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Sulfamide derivatives as transition state analogue inhibitors for carboxypeptidase A

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Abstract—3-Phenyl-2-sulfamoyloxypropionic acid (**2**), 2-benzyl-3-sulfamoylpropionic acid (**3**), and *N*-(*N*-hydroxysulfamoyl)phenylalanine (**5**) have been synthesized and evaluated as inhibitors for carboxypeptidase A (CPA) to find that they inhibit the enzyme competitively with the K_i values in the μM range, suggesting that their binding modes to CPA are analogous to each other, and resemble the binding mode of *N*-sulfamoylphenylalanine (**1**) that has been established by the X-ray crystallographic method to form a complex with CPA in a manner reminiscent of the binding of a transition state in the catalytic pathway. It was concluded thus that they are a new type of transition state analogue inhibitors for CPA. (*R*)-*N*-Hydroxy-*N*-sulfamoyl-β-phenylalanine (**8**) was shown to be also a potent CPA inhibitor ($K_i = 39$ μM), the high potency of which may be ascribed to the involvement of the hydroxyl in the binding of CPA, most likely forming bidentate coordinative bonds to the zinc ion in CPA together with the sulfamoyl oxygen atom.

1. Introduction

Carboxypeptidase A (CPA) catalyzes the hydrolysis of peptides, removing the carboxy-terminal amino acid residue having a hydrophobic side chain such as phenylalanine.[1] CPA is a most extensively investigated zinc protease and represents the large family of zinc proteolytic enzymes, and has served as a model target enzyme for developing design protocols of inhibitors that are effective against pathologically important zinc proteases such as angiotensin converting enzyme (ACE)[2] and matrix metalloproteinases (MMPs).[3]

The catalytically essential zinc ion at the active site of CPA is coordinated by the carboxylate of Glu-72 and two imidazoles of His-69 and His-196. A water molecule is loosely bound to the zinc ion as the fourth ligand. The positively charged guanidinium of Arg-145 forms bifurcated hydrogen bonds with the C-terminal carboxylate of substrate and the carboxylate of Glu-270 is intimately involved in the peptide bond hydrolysis reaction, serving as a general base that activates the zinc-bound water molecule. There is present at the active site a hydrophobic pocket, which recognizes substrate by accommodating the aromatic side chain of P_1' residue of substrate.[4] It has been known that the CPA-catalyzed hydrolysis of peptides is initiated by the attack of the zinc-bound water molecule on the scissile peptide bond to generate a tetrahedral transition state, which is stabilized by the active site zinc ion and the guanidinium moiety of Arg-127.[1,5]

Recently, we have reported that *N*-sulfamoylphenylalanine (**1**) is a potent inhibitor for CPA.[6] The inhibitor was designed as a transition state analogue taking advantage of the physicochemical properties of sulfamides. Sulfamide has been reported to have the shape of tetrahedron with its sulfur atom occupying the center.[7] Furthermore, its nitrogen atoms are sufficiently basic thus to allow metal ion coordination. Indeed, the X-ray crystal structure of the CPA·(*S*)-**1** complex revealed that the inhibitor molecule interacts with functional groups including the zinc ion at the active site of CPA in a fashion reminiscent of the postulated stabilization mode of a tetrahedral transition state generated in the CPA-catalyzed hydrolysis of a peptide substrate.[6] Thus, the terminal nitrogen atom in the sulfamoyl moiety of (*S*)-**1** is coordinated to the zinc ion with the distance of 1.92 Å, one of the oxygens of the sulfamide moiety is involved in the hydrogen bonding with one of guanidininium

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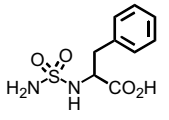
nitrogens of Arg-127, and the Glu-270 carboxylate forms a hydrogen bond with the internal amino group in the inhibitor.

It was thought to be of interest to evaluate as CPA inhibitors compounds **2** and **3**, in which the internal amino group of **1** is replaced with an oxygen atom and methylene unit, respectively. We have also synthesized and evaluated compound **5** in which a hydroxyl group is introduced on the terminal amino group of the sulfamide moiety in **1**.



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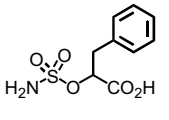

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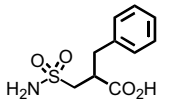

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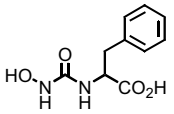

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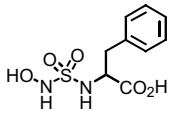

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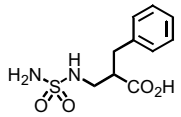
In the previous study,[6] we have observed that the CPA inhibitory activity of sulfamoylated derivative of β-Phe (**6**) is drastically reduced compared with **1**. In comparison, the hydroxamate derivative of β-Phe, that is, **7** was shown to be a potent inhibitor for CPA.[8] In light of the fact that the high inhibitory activity shown by **7** may be attributed to the formation of a bidentate coordinative bond between the hydroxamate moiety and the active site zinc ion, it was expected that an introduction of a hydroxyl group on the internal nitrogen in **6** might improve the binding affinity of **6**. Accordingly, we have synthesized **8** and evaluated its CPA inhibitory activity.



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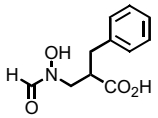

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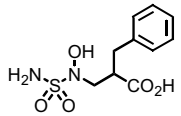

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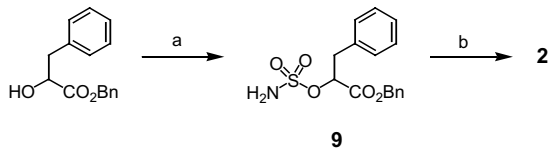
2. Results and discussion

2.1. Chemistry

Compound **2** was synthesized by the route outlined in Scheme 1. Addition of formic acid to neat chlorosulfonyl isocyanate generated sulfamoyl chloride, which was then allowed to react with (*S*)-phenyllactic acid in the presence of 1.5 equiv amount of pyridine to obtain **9** in 57% yield. Hydrogenolysis of **9** using 10% Pd–C afforded **2** in 99% yield. Compound **10** required for the synthesis of **3** was prepared according to the method reported by Mazdiyasni et al.[9] Treatment of **10** using PCl_5 in toluene gave the corresponding sulfonyl chloride, which was then treated with *tert*-butylamine to give **11** in 72% yield. Alkaline hydrolysis of the ester moiety in **11** using 2 N sodium hydroxide solution provided **12**, and subsequent treatment of **12** with trifluoroacetic acid gave **3** (Scheme 2).



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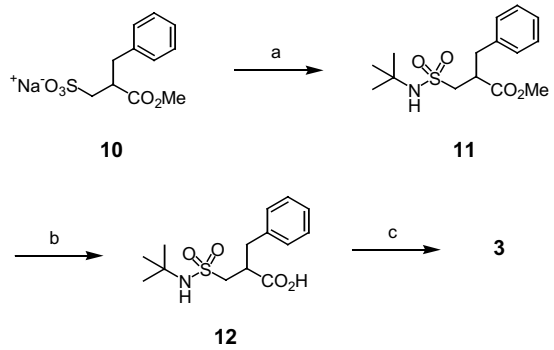
Scheme 1. Reagents, conditions, and yield: (a) (i) formic acid (1.5 equiv), chlorosulfonyl isocyanate (1.5 equiv), 0 °C, 4 h; (ii) (*S*)-phenyllactic acid, pyridine (1.5 equiv), 0 °C, 12 h; 57%; (b) H_2, Pd–C, MeOH, rt, 2 h, 99%.

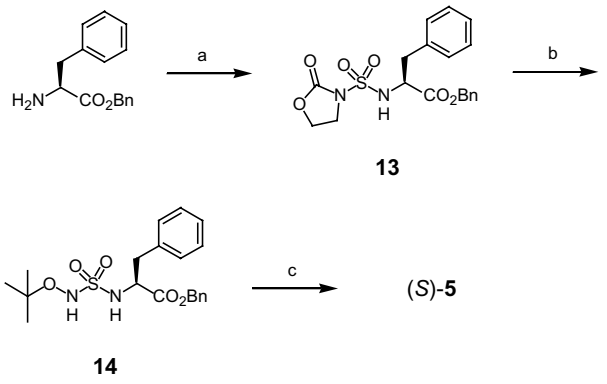
Compound (*S*)-**5** was synthesized starting with L-phenylalanine benzyl ester in three steps (Scheme 3). Chlorosulfonyl isocyanate was treated with 2-chloroethanol to generate *N*-(chloroethyl)sulfamoyl chloride, which was then allowed to react with phenylalanine benzyl ester to afford **13** in 95% yield. Transsulfamoylation of *N*-sulfonyloxazolidinone moiety in **13** with *tert*-butyloxyamine hydrochloride was effected under the conditions reported by Montero and co-workers,[10] providing **14** in 49% yield. Compound **14** was treated with titanium chloride in dry dichloromethane[11] to give the *N*-hydroxysulfamide compound, which was then subjected to hydrolgenolysis in the presence of 10% Pd–C to obtain (*S*)-**5** (Scheme 3). Compound (*R*)-**5** was prepared starting with D-phenylalanine benzyl ester in a manner analogous to that used for the preparation of (*S*)-**5**.

(3*R*)-*N*-Benzyloxy-3-benzyl-2-azetidine (**15**) that was obtained from 3-phenylpropanoic acid in six steps according to the method reported by Jin and Kim[12] was



10 **11** **12**

Scheme 2. Reagents, conditions, and yield: (a) (i) PCl_5, toluene, rt, 2 h; (ii) $^tBuNH_2$ (3 equiv), toluene, 0 °C, 1 h, 71.5%; (b) 2 N NaOH, MeOH, rt, 12 h, 52.3%; (c) TFA, rt, 12 h, 95%.



Scheme 3. Reagents, conditions, and yield: (a) *N*-(chloroethyl)sulfamoyl chloride, Et_3N, CH_2Cl_2, rt, 12 h, 95%; (b) $^tBuONH_2{\cdot}HCl$, Et_3N, MeCN, reflux, 12 h, 49%; (c) (i) $TiCl_4$ (1.5 equiv), CH_2Cl_2, rt, 30 min; (ii) H_2, Pd–C, MeOH, rt, 2 h, 37%.

treated with methanol in the presence of tetramethylsilyl chloride (TMSCl) to give **16**. 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 **17**. The subsequent alkaline hydrolysis of **17** with LiOH solution in MeOH/H_2O followed by hydrolgenolysis in the presence of 10% Pd–C afforded (*R*)-**8** (Scheme 4). In an analogous fashion, (*S*)-**8** was prepared.

2.2. Kinetic studies

The compounds thus synthesized were assayed for CPA inhibitory activity at pH 7.5 using *O*-(*trans*-*p*-chlorocinnamoyl)-L-phenyllactic acid (ClCPL) as substrate. They are shown to be competitive inhibitors for CPA from the Dixon plots.[13] The inhibitory constants (K_i values) estimated from the respective Dixon plot are collected in Tables 1 and 2. Figure 1 exemplifies the Dixon plots.

As can be seen from Table 1, the high binding affinity of **1** was essentially unchanged by the replacement of the internal amino group with an oxygen atom or methylene unit, suggesting strongly that **2** and **3** bind CPA in a mode that is closely analogous to the binding of **1** and a transition state in the enzymic reaction. It may thus be inferred that **2** and **3** serve as analogues of a transition state in the CPA catalyzed proteolytic reaction, binding tightly to CPA. The present results also suggest that the hydrogen bond that is formed between the carboxylate of Glu-270 and the internal amino group of **1** in the binding of **1** to CPA does not contribute to any great extent in binding the inhibitor to CPA. In fact, it has been proposed that the amino group in the tetrahedral transition state in the CPA-catalyzed hydrolysis of peptide substrate undergoes protonation to result in to

Table 1. Comparison of the K_i values of sulfamide type inhibitors

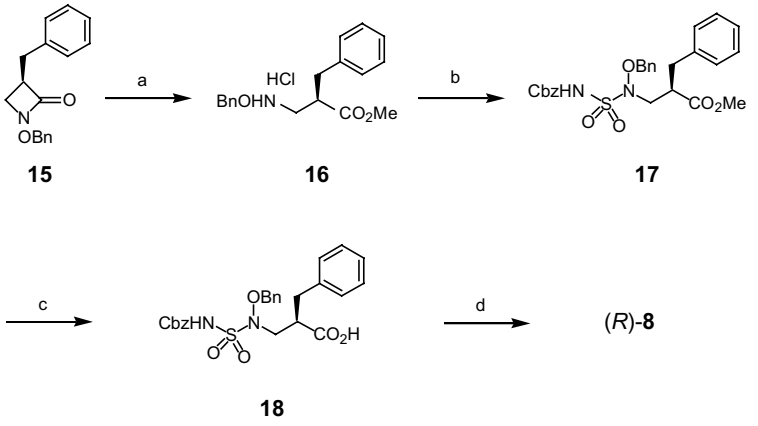
Inhibitor	K_i (μM)
(*S*)-**1**	0.65 ± 0.05[a]
(*R*)-**1**	470 ± 110[a]
(*RS*)-**1**	1.42 ± 0.22[b]
(*RS*)-**2**	1.98 ± 0.32
(*RS*)-**3**	3.53 ± 0.51
(*RS*)-**4**	2.09 ± 0.16[c]
(*S*)-**5**	3.2 ± 0.3
(*R*)-**5**	72 ± 16

[a] Ref. 6.
[b] Unpublished result.
[c] Ref. 14.

Table 2. Inhibitory potencies of *N*-hydroxysulfamoylPhe for CPA inhibition

Inhibitor	K_i (μM)
(*S*)-**6**	2900 ± 930[a]
(*R*)-**6**	1400 ± 190[a]
(*RS*)-**7**	67 ± 5.3
(*S*)-**7**	4.95[b]
(*R*)-**7**	0.56[b]
(*S*)-**8**	1900 ± 270
(*R*)-**8**	39 ± 2.2

[a] Ref. 6.
[b] Ref. 8.



Scheme 4. 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) LiOH (2 equiv), MeOH/H_2O (3:1), rt, 2 h, 93%; (d) H_2, Pd–C, MeOH, rt, 2 h, 95.5%.

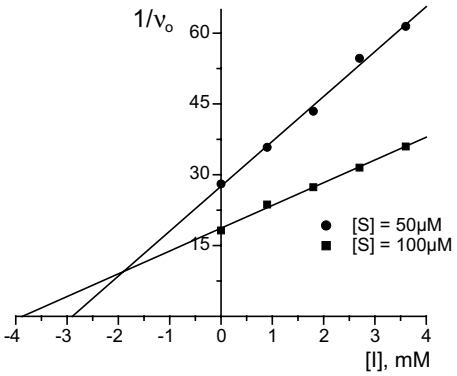


Figure 1. The Dixon plot for the inhibition of carboxypeptidase A by (*RS*)-**2**.

facilitate the cleavage of the C–N bond in the transition state.[1]

It has been reported that the introduction of a hydroxyl group on the terminal nitrogen of *N*-(aminocarbonyl)phenylalanine to give *N*-(hyroxyaminocarbonyl)-phenylalanine (**4**) improves the binding affinity by 28-fold.[14] It was thus thought to be of interest to evaluate compound **5** that is obtained by the introduction of a hydroxyl group on the terminal amino group of **1**. We have obtained the K_i value of 3.2 μM for (*S*)-**5**. Thus, the binding affinity of **1** to CPA is attenuated by fivefold by the hydroxyl introduction. The marginal change in the binding affinity caused by the hydroxylation tends to indicate that **5** binds CPA in a fashion analogous to that of **1**, mimicking a transition state in the enzymic reaction rather than the hydroxyl being involved in the formation of a coordinative bond to the active site zinc ion. The slightly lowered binding affinity of **5** compared with **1** may be ascribed to the somewhat reduced $N \rightarrow Zn^{2+}$ bond strength resulted by the electron withdrawing effect of the hydroxyl oxygen atom. Recently, Scozzafava and Supuran[15] employed the *N*-hydroxysulfonamide moiety in designing inhibitors for matrix metalloproteases,[3] zinc proteases involved in the degradation of extracellular matrix.

As described in Introduction, *N*-sulfamoylated β-phenylalanine (**6**) is only marginally active inhibitor for CPA,[6] although *N*-sulfamoylated α-phenylalanine (**1**) is a remarkably potent CPA inhibitor. *N*-Formyl-*N*-hydroxyphenylalanine (**7**) that carries a N–OH group at the γ-position to the carboxylate was reported to be a potent CPA inhibitor having the K_i value of 0.98 μM.[8] We were interested in knowing CPA inhibitory activity of **8**, which bears a N–OH group also at the γ-position to the carboxylate. (*R*)-**8** was shown to have a potent CPA inhibitory activity with the K_i value of 39 μM, which corresponds to 36-fold enhancement in binding affinity of **6** by the introduction of a hydroxyl group. Apparently, the hydroxyl group in **8** functions differently from that in **5**, contributing much for the binding of **8** to CPA. This remarkable increase in the binding affinity may be reconciled by the proposition that the hydroxyl group in **8** together with the sulfamoyl oxygen atom, may form a bidentate coordinative bond to the active site zinc ion like inhibitor **7** does. Inhibitor (*S*)-**8** is only marginally active.

Although it is highly unlikely that the present inhibitors would render inhibitory activity toward other zinc-containing proteases, we nevertheless assayed the most potent CPA inhibitor in the present study, that is, (*RS*)-**2** for thermolysin,[16] a zinc containing endopeptidase whose active site and mode of catalytic action are closely analogous to CPA. The compound was indeed found to be a very poor inhibitor for thermolysin with the K_i value of 11.67 ± 0.05 mM, revealing that the inhibitor, (*RS*)-**2** is highly selective for CPA.

3. Conclusion

Compounds **2**, **3**, and **5** whose structures resemble closely to **1** are shown to be potent competitive inhibitors for CPA having the K_i values in the same order of magnitude as that of **1**. Previously, the binding manner of **1** to CPA has been established to be reminiscent of the binding mode of a postulated transition state in the catalytic pathway by the X-ray diffraction study of the CPA·(*S*)-**1** complex. It is thus inferred from the structure-activity consideration of the series of the inhibitors that inhibitors **2**, **3**, and **5** may also be transition state analogue inhibitors of CPA. Although the hydroxyl group in **5** may not be directly involved in the interactions with the enzyme, the hydroxyl in **8** appears to participate in the binding interactions with CPA, possibly coordinating to the zinc ion in a bidentate fashion together with a sulfamoyl oxygen atom. The novel inhibitors reported in the present study may serve as prototypes useful for designing inhibitors that are effective against zinc proteases of medicinal interest.

4. Experimental

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. Low resolution mass spectra were obtained with a KRATOS MS 25 RFA instrument. 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). Optical rotations were measured on a RUDOLPH RESEARCH AUTOPOL III digital polarimeter. 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 chemicals were of reagent grade obtained from Aldrich Chemical Co. Dichloromethane, toluene, and dimethylformamide were distilled over calcium hydride and stored under nitrogen. Tetrahydrofuran was distilled over sodium-benzophenone before use.

4.1. 3-Phenyl-2-sulfamoyloxypropaonic acid benzyl ester (9)

Formic acid (0.67 mL, 17.2 mmol) was added dropwise to neat chlorosulfonyl isocyanate (1.5 mL, 17.2 mmol) at 0 °C with rapid stirring. Vigorous gas evolution was observed during the addition. The resulting viscous suspension was stirred for 5 min at 0 °C. methylene chloride was added and the solution was stirred for 1 h at 0 °C, and then for 4 h at 25 °C. To the solution was slowly added a mixture of (*S*)-phenyllactic acid benzyl ester (2.95 g, 11.5 mmol) and pyridine (1.4 mL, 17.3 mmol) at 0 °C. The resulting solution was stirred for 12 h. After evaporation of the solvent, the residue was dissolved in ethyl acetate (50 mL), washed successively with 1 N HCl solution (30 mL × 3) and brine (30 mL × 3), and dried over anhydrous $MgSO_4$. The dried solution was concentrated in vacuo to give the product (2.2 g, 57%) as a white solid. Mp 86.5–87 °C; $[\alpha]_D^{20}$ −25.7 (*c* 1.04, MeOH); IR (neat) 1186, 1385, 1745 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 3.14–3.24 (m, 2H), 5.02 (s, 2H), 5.13–5.18 (m, 3H), 7.13–7.35 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 38.4, 68.3, 79.8, 127.9, 129.0, 129.1, 129.2, 129.3, 130.0, 134.9, 135.0, 169.7. Anal. Calcd for $C_{16}H_{17}NO_5S$: C, 57.30; H, 5.11; N, 4.18. Found: C, 57.24; H, 4.78; N, 4.20.

4.2. 3-Phenyl-2-sulfamoyloxypropanoic acid (2)

A solution of **9** (1.0 g, 3 mmol) in anhydrous MeOH (25 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 the product (0.73 g, 99%) as a white product. Mp 109–111 °C; $[\alpha]_D^{20}$ −10.1 (*c* 1.61, MeOH); IR (neat) 1183, 1368, 1734 cm^{-1}; ^1H NMR 300 MHz (CD_3OD) δ 3.15 (d, 2H), 4.99 (m, 1H), 7.25 (m, 5H); ^{13}C NMR 300 MHz (CD_3OD) δ 38.4, 79.5, 127.0, 128.4, 129.7, 135.8, 170.2. Anal. Calcd for $C_9H_{11}NO_5S$: C, 44.08; H, 4.52; N, 5.71. Found: C, 43.72; H, 4.31; N, 5.61.

4.3. 2-Benzyl-3-*tert*-butylsulfamoylpropnonic acid methyl ester (11)

To a suspension of **10**[9] (7 g, 25 mmol) in toluene was added PCl_5 (5.2 g, 25 mmol) and the resulting suspension was stirred for 2 h. The suspension was filtered off and the filtrate was cooled to 0 °C. ^{t}*tert*-Butylamine (5.7 mL, 75 mmol) was added slowly to the filtrate at 0 °C and the resulting clear solution was stirred for 1 h. The solution was evaporated under reduced pressure and the residue was dissolved in ethyl acetate, washed with 1 N HCl solution (30 mL × 3) and brine (30 mL × 3), and dried over anhydrous $MgSO_4$. The residue that was obtained by evaporation of the dried solution was purified by column chromatography (EtOAc/*n*-hexane = 1:4) to give the product (5.6 g, 71.5%) as a white solid. Mp 75–76 °C; IR ($CHCl_3$) 1133, 1324, 1734 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.23 (s, 9H), 2.81 (m, 1H), 3.05 (m, 2H), 3.21 (m, 1H), 3.53 (dd, 1H), 3.65 (s, 3H), 4.06 (s, 1H), 7.14–7.31 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 30.4, 38.4, 43.8, 52.6, 55.3, 56.4, 127.5, 129.2, 129.5, 137.4, 174.2. Anal. Calcd for $C_{15}H_{23}NO_4S$: C, 57.48; H, 7.40; N, 4.47. Found: C, 57.20; H, 7.56; N, 4.59.

4.4. 2-Benzyl-3-*tert*-butylsulfamoylpropaonic acid (12)

To a solution of **11** (1.5 g, 4.8 mmol) in methanol (15 mL), was added 2 N NaOH solution (2.4 mL) and the resulting solution was stirred for 12 h. After evaporation of the solvent, the residue was dissolved in 1 N HCl solution and the solution was extracted with ethyl acetate (30 mL × 3). The extract was dried over anhydrous $MgSO_4$ and evaporated under reduced pressure to give the product (0.75 g, 52.3%) as an oil, which solidified on standing. Mp 119–120 °C; IR ($CHCl_3$) 1125, 1311, 1717 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.22 (s, 9H), 2.82 (dd, 1H), 3.04 (dd, 2H), 3.13–3.50 (m, 2H), 3.53 (dd, 1H), 4.43 (s, 1H), 7.20–7.44 (m, 5H); ^{13}C NMR 300 MHz (CD_3OD) δ 29.3, 37.9, 43.7, 54.0, 55.8, 127.0, 128.7, 129.4, 138.1, 175.8. Anal. Calcd for $C_{15}H_{23}NO_4S$: C, 56.16; H, 7.07; N, 4.68. Found: C, 56.17; H, 6.97; N, 4.70.

4.5. 2-Benzyl-3-sulfamoylpropaonic acid (3)

Compound **12** (0.75 g, 2.51 mmol) was dissolved in trifluoroacetic acid (5 mL) and the resulting solution was stirred for 12 h, then evaporated under reduced pressure, and the residue was recrystallized from ethyl acetate and *n*-hexane to give the product (0.58 g, 95%) as a white solid. Mp 98–100 °C; IR ($CHCl_3$) 1121, 1325, 1717, 3446 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 2.83 (dd, 1H), 3.06–3.19 (m, 2H), 3.27 (dd, 1H), 3.59 (dd, 1H), 5.30 (br, 2H), 7.16–7.61 (m, 5H), 9.02 (s, 1H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 37.8, 43.3, 55.4, 126.9, 128.6, 129.3, 138.0, 175.6. Anal. Calcd for $C_{15}H_{23}NO_4S \cdot 1/2\ H_2O$: C, 48.47; H, 5.49; N, 5.65. Found: C, 48.74; H, 5.21; N, 5.73.

4.6. (*S*)-2-(2-Oxo-oxazolidine-3-sulfonylamino)-3-phenylpropaonic acid benzyl ester ((*S*)-13)

To an ice-chilled solution of chlorosulfonyl isocyanate (2 mL, 23 mmol) in methylene chloride (25 mL) was added 2-chloroethanol (1.54 mL, 23 mmol) and stirred at 0 °C for 1.5 h. To the resulting mixture was added a solution of phenylalanine benzyl ester *p*-toluenesulfonate (9.83 g, 23 mmol) and triethylamine (9.62 mL, 69 mmol) obtained by dissolving in CH_2Cl_2 (100 mL). The temperature was remained under 5 °C during the addition. The resulting solution was allowed to warm to room temperature and stirred for 12 h. The reaction was

quenched with 1 N HCl solution, the organic phase was separated, dried over anhydrous $MgSO_4$, and evaporated in vacuo to give a white solid (8.8 g, 95%). Mp 147–147.5 °C; $[\alpha]_D^{20}$ −20.9 (*c* 1.24, $CHCl_3$); IR ($CHCl_3$) 1636, 1764 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 3.03–3.16 (m, 2H), 3.72–3.83 (m, 2H), 4.10–4.21 (m, 2H), 4.63 (t, 1H), 5.14 (s, 2H), 7.06–7.37 (m, 10H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 39.2, 45.5, 58.5, 62.9, 68.4, 127.8, 129.2, 129.9, 135.1, 135.2, 153.4, 171.1. Anal. Calcd for $C_{19}H_{20}N_2O_6S{\cdot}1/4H_2O$: C, 55.80; H, 5.05; N, 6.85. Found: C, 55.91; H, 4.98; N, 6.98. Anal. Calcd for $C_{19}H_{20}N_2O_6S{\cdot}1/4H_2O$: C, 55.80; H, 5.05; N, 6.85. Found: C, 55.90; H, 4.90; N, 6.93.

4.7. (*R*)-2-(2-Oxo-oxazolidine-3-sulfonylamino)-3-phenylpropaonic acid benzyl ester ((*R*)-13)

Compound (*R*)-**13** was prepared from (*R*)-phenylalanine benzyl ester in a manner analogous to that used for the preparation of (*S*)-**13**. $[\alpha]_D^{20}$ +21.6 (*c* 0.43, $CHCl_3$). Mp and spectral data are identical with those of (*S*)-**13**. Anal. Calcd for $C_{19}H_{20}N_2O_6S{\cdot}1/4H_2O$: C, 55.80; H, 5.05; N, 6.85. Found: C, 55.91; H, 4.98; N, 6.98.

4.8. (*S*)-*N*-(*O*-*tert*-Butyloxy)sulfamoylphenylalanine benzyl ester ((*S*)-14)

The solution of (*S*)-**13** (5.0 g, 12.4 mmol) and benzyloxyamine hydrochloride (1.97 g, 12.4 mmol) in acetonitrile (50 mL) was heated under reflux for 12 h. The resulting solution was evaporated under reduced pressure to give an oily residue, which upon purification by flash column chromatography (*n*-hexane/EtOAc = 4:1) gave a solid product, which was recrystallized from ethyl acetate and *n*-hexane (12.47 g, 49%). Mp 102–103 °C; $[\alpha]_D^{20}$ −21.4 (*c* 0.55, $CHCl_3$); IR ($CHCl_3$) 1736 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.17 (s, 9H), 3.09 (dd, 2H), 4.53 (m, 1H), 5.05–5.18 (q, 2H), 5.23 (d, 1H), 6.60 (s, 1H), 7.07–7.36 (m, 10H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 26.9, 39.9, 58.8, 68.1, 82.1, 127.7, 129.1, 129.9, 135.1, 135.4, 171.6. Anal. Calcd for $C_{20}H_{26}N_2O_5S$: C, 59.09; H, 6.45; N, 6.89. Found: C, 59.11; H, 6.52; N, 6.86. Anal. Calcd for $C_{20}H_{26}N_2O_5S$: C, 59.09; H, 6.45; N, 6.89. Found: C, 59.09; H, 6.45; N, 6.85.

4.9. (*R*)-*N*-(*O*-*tert*-Butyloxy)sulfamoylphenylalanine benzyl ester ((*R*)-14)

Compound (*R*)-**14** was prepared from (*R*)-**13** in a manner analogous to that used for the preparation of (*S*)-**14**. $[\alpha]_D^{20}$ +23.4 (*c* 0.59, $CHCl_3$). The mp and spectral data are identical with those of (*S*)-**14**. Anal. Calcd for $C_{20}H_{26}N_2O_5S$: C, 59.09; H, 6.45; N, 6.89. Found: C, 59.11; H, 6.52; N, 6.86.

4.10. (*S*)-*N*-(*N*-Hydroxysulfamoyl)phenylalanine ((*S*)-5)

To an ice-chilled solution of (*S*)-**14** (2.0 g, 4.92 mmol) in dry methylene chloride was added titanium tetrachloride (1.4 g, 7.38 mmol) slowly. The resulting solution was stirred at room temperature for 30 min. The solution was quenched with saturated aqueous NH_4Cl solution and evaporated in vacuo. The residue was dissolved in anhydrous MeOH (30 mL) and 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*)-**5** as an oil (0.47 g, 37%). $[\alpha]_D^{20}$ +13.6 (*c* 0.23, MeOH); ^1H NMR 300 MHz (DMSO-d_6) δ 2.94–2.99 (m, 2H), 4.07 (q, 1H), 7.18–7.30 (m, 5H), 7.52 (d, 1H), 8.86 (s, 1H); ^{13}C NMR 300 MHz (DMSO-d_6) δ 49.4, 57.8, 127.3, 128.9, 130.2, 137.7, 173.6. HRMS (FAB+) $(M+H)^+$: calcd for $C_8H_{13}N_2O_5S$, 261.0545; found 261.0547.

4.11. (*R*)-*N*-(*N*-Hydroxysulfamoyl)phenylalanine ((*R*)-5)

Compound (*R*)-**5** was prepared from (*R*)-**14** in a manner analogous to that used for the preparation of (*S*)-**5**. $[\alpha]_D^{20}$ −17.3 (*c* 0.1, MeOH). Mp and spectral data are identical to those of (*S*)-**5**. HRMS (FAB+) $(M+H)^+$: calcd for $C_8H_{13}N_2O_5S$, 261.0545; found 261.0545.

4.12. (*R*)-2-Benzyl-3-(*N*-benzyloxy)aminopropanoic acid methyl ester hydrochloride ((*R*)-16)

To the solution of (*R*)-**15**[12] (2.2 g, 8.2 mmol) in anhydrous MeOH (20 mL) was added slowly tetramethylsilyl chloride (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 crystalline product (2.7 g, 98%). Mp 152.5–153 °C; $[\alpha]_D^{20}$ +25.4 (*c* 1.1, EtOH); IR (KBr) 1726 cm^{-1}; ^1H 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. Calcd for $C_{18}H_{22}NClO_3$: C, 64.38; H, 6.60; N, 4.17. Found: C, 64.30; H, 6.67; N, 4.25.

4.13. (*S*)-2-Benzyl-3-(*N*-benzyloxy)aminopropanoic acid methyl ester hydrochloride ((*S*)-16)

Compound (*S*)-**16** was prepared from (*S*)-**15** in a manner analogous to that used for the preparation of (*R*)-**16**. $[\alpha]_D^{20}$ −25.7 (*c* 0.8, EtOH). Mp and spectral data are identical with those of (*R*)-**15**. Anal. Calcd for $C_{18}H_{22}NClO_3$: C, 64.38; H, 6.60; N, 4.17. Found: C, 64.28; H, 6.57; N, 4.26.

4.14. (*R*)-2-Benzyl-3-(*N*-benzyloxy-*N*-(*N*-benzyloxycarbonyl)sulfamoyl)aminopropanoic acid methyl ester ((*R*)-17)

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)

and stirred for 30 min. The solution thus obtained and triethylamine (0.5 mL) in anhydrous dichloromethane were concurrently added dropwise to an ice-chilled solution of (*R*)-**16** (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 dissolved 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%): $[\alpha]_D^{25}$ −18.9 (*c* 1.1, $CHCl_3$); IR (neat) 1386, 1738 cm^{-1}; ^1H 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.

4.15. (*S*)-2-Benzyl-3-(*N*-benzyloxy-*N*-(*N*-benzyloxycarbonyl)sulfamoyl)aminopropanoic acid methyl ester ((*S*)-17)

Compound (*S*)-**17** was prepared from (*S*)-**16** in a manner analogous to that used for the preparation of (*R*)-**17**. $[\alpha]_D^{20}$ −19.6 (*c* 0.94, $CHCl_3$). The mp and spectral data are identical with those of (*R*)-**17**.

4.16. (*R*)-*N*-(*N*-Benzyloxycarbonyl)sulfamoyl)-*N*-(*O*-benzylhydroxy)-β-phenylalanine ((*R*)-18)

A mixture of (*R*)-**17** (1.0 g, 2 mmol) and lithium hydroxide (0.164 g, 4 mmol), in MeOH/H_2O (1:1, 5 mL) was stirred for 2 h at room temperature, then acidified with 1 N HCl solution and extracted with ethyl acetate. The combined extract was evaporated under reduced pressure to afford the product as an oil (0.2 g, 93%). $[\alpha]_D^{20}$ −19.9 (*c* 0.5, $CHCl_3$); IR (neat) 1739, 3392 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 2.77–3.07 (m, 3H), 3.43 (m, 1H) 3.73 (m, 1H), 5.01 (d, 2H), 5.14 (s, 2H), 7.12–7.40 (m, 15H).

4.17. (*S*)-*N*-(*N*-Benzyloxycarbonyl)sulfamoyl)-*N*-(*O*-benzylhydroxy)-β-phenylalanine ((*S*)-18)

Compound (*S*)-**18** was prepared from (*S*)-**17** in a manner analogous to that used for the preparation of (*R*)-**18**. $[\alpha]_D^{20}$ +20.1 (*c* 1.42, $CHCl_3$). The mp and spectral data are identical with those of (*R*)-**18**.

4.18. (*R*)-*N*-Hydroxy-*N*-sulfamoyl-β-phenylalanine ((*R*)-8)

A solution of (*R*)-**18** (0.9 g, 1.8 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 the product (0.47 g, 95.5%) as a white solid. Mp 109–111 °C; $[\alpha]_D^{20}$ +6.4 (*c* 0.55, MeOH); IR (KBr) 1355, 1716, 3372 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 2.92 (m, 3H), 3.17 (dd, 2H), 7.15–7.24 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 35.8, 44.2, 47.8, 126.5, 128.4, 129.1, 139.1, 176.5. Anal. Calcd for $C_{10}H_{14}N_2O_5S{\cdot}1/3H_2O$: C, 42.85; H, 5.27; N, 9.99. Found: C, 42.96; H, 4.93; N, 10.08.

4.19. (*R*)-*N*-hydroxy-*N*-sulfamoyl-β-phenylalanine ((*S*)-8)

Compound (*S*)-**8** was prepared from (*S*)-**18** in a manner analogous to that used for the preparation of (*R*)-**8**. $[\alpha]_D^{20}$ −5.7 (*c* 0.48, MeOH). Mp and spectral data are identical to those of (*R*)-**8**. Anal. Calcd for $C_{10}H_{14}N_2O_5S{\cdot}1/3H_2O$: C, 42.85; H, 5.27; N, 9.99. Found: C, 43.10; H, 5.13; N, 10.11.

4.20. Inhibition assay for CPA

Typically, the enzyme stock solution was added to a solution containing *O*-(*trans*-*p*-chlorocinnamoyl)-L-phenyllactic acid (final concentrations: 50 and 100 μM) and inhibitor (five different final concentrations in the range of $0.5K_i$–$2K_i$ 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.[13] The correlation coefficients for the Dixon plots were above 0.990.

Inhibition assay for thermolysin was performed as described in the literature.[17]

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