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Inhibition of α-chymotrypsin with thiol-bearing substrate analogues in the presence of zinc ion

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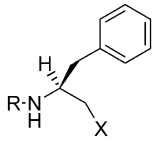
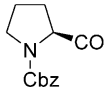
Abstract—We have demonstrated that thiol-bearing analogues of α-chymotrysin (α-CT) substrates such as (*S*)-(1-benzyl-2-thiol-ethyl)-carbamic acid, benzyl ester (**3**) inhibits α-CT, a prototypical serine protease, in the presence of Zn(II) ion. They constitute a novel class of small molecule inhibitors for α-CT believed to inhibit the enzyme by forming a ternary complex consisting of α-CT, Zn(II) ion, and the inhibitor.

1. Introduction

Serine proteases have received increasing attention in recent years because of their roles in the pathology of numerous diseases such as rheumatoid arthritis, thrombosis, and pulmonary emphysema.[1] These enzymes are characterized by having a catalytic triad consisting of Ser, His, and Asp as the essential catalytic machinery. The serine hydroxyl group in the catalytic triad in collaboration with the His and Asp residues attacks the scissile peptide or ester bond of the enzyme bound substrates, generating an acyl-enzyme intermediate which, in turn, is hydrolyzed by the active site water molecule to yield the second product with regeneration of the free enzyme. α-Chymorypsin (α-CT) is a much studied serine protease whose structure and catalytic mechanism have been well established.[2] There is present at the active site of α-CT a large hydrophobic pocket (S1 subsite), the primary function of which involves accommodation of the P_1 residue having a bulky hydrophobic side chain such as Phe. As a prototypical enzyme for a large family of proteases, α-CT has served as a model target enzyme for the development of design strategies that can be utilized to inhibit serine proteases of medicinal interest. As a consequence, numerous design protocols for α-CT inhibitors are known and a vast number of α-CT inhibitors have been reported.[3] We wish to report herein a new class of inhibitors that bind strongly to α-CT in the presence of Zn(II) ion. These inhibitors are substrate analogues that bear a methylmercapto moiety in place of hydrolysable peptide bond in the substrate of α-CT.



1; R = CH_3CO, X = SH
2; R = C_6H_5CO, X = SH
3; R = $C_6H_5CH_2OCO$, X = SH
4; R =  , X = SH
5; R = $C_6H_5CH_2OCO$, X = OH

Recently, Katz et al. have developed a novel strategy for designing inhibitors that are highly effective against trypsin and other serine proteases.[4] The design protocol takes advantage of the high propensity of Zn(II) ion to form a tetrahedrally coordinated complex. The binding affinity of bis-(5-amidino-2-benzimidazolyl)-methane (BABIM) toward trypsin was enhanced by as much as 1.7×10^4-fold in the presence of Zn(II) ion. The X-ray crystallographic structural analysis of the complex of trypsin formed with BABIM revealed that the Zn(II) ion is coordinated by one nitrogen from each of the two benzimidazoles in BABIM, the hydroxyl of Ser-195, and the imidazole of His-57 of the catalytic

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triad of the enzyme, thus to bridge the inhibitor and trypsin.

It has been well established that a mercapto group has a high tendency to form a coordinative bond to Zn(II) ion.[5] In fact, this property of mercapto group has been extensively utilized in the design of inhibitors for zinc-containing metalloenzymes.[6] We have envisioned that appropriately modified mercapto-bearing analogues of α-CT substrate would bind strongly to α-CT in the presence of Zn(II) ion, forming a ternary complex, as depicted in Figure 1. The phenyl ring in such inhibitors is expected to anchor in the primary substrate recognition pocket (S1 pocket) and the thiol group would ligate the zinc ion that is held by the side chains of Ser-195 and His-57 present at the active site of α-CT. A water molecule is thought to bind the metal ion as the fourth ligand. It is known that when sulfur is bound, the Zn(II) ion prefers to have the coordination number of 4.[7,8] The water molecule may be coordinated to the Zn(II) in a deprotonated form. It has been known that the pK_a value of the water molecule coordinated to Zn(II) can be lowered as much as 9 units.[9]

2. Results and discussion

2.1. Synthesis

Compound **6** that was prepared by the literature[10] method was treated with hydrochloric acid, then the product was allowed to react with acetic anhydride to give **7** which was treated with potassium carbonate in an aqueous methanol solution to obtain inhibitor **1**[11] (Scheme 1). In a similar fashion, inhibitor **2** was synthesized.[11] In the synthesis of **3**, the hydroxyl group in **5** that was obtained by the reduction of **8** was converted into the corresponding bromide using tetrabromomethane and triphenylphophine,[12] then the product was subjected to thioacetylation using potassium thioacetate to give **10**. Hydrolysis of the latter compound afforded **3**[13] (Scheme 2). Inhibitor **4** was synthesized starting with 2-(1-benzyl-2-hydroxy-ethylcarbamoyl)-pyrrolidine-1-carboxylic acid, benzyl ester,[14] whose hydroxyl group was converted directly into the thioacetate by the modified Mitsunobu reaction,[15] and the product thus obtained was hydrolyzed to yield **4**.[16]

The α-CT inhibition potencies are expressed by the IC_{50} value which is estimated from the plot of the percent enzymic activity remaining in the presence of an inhibitor and Zn(II) ion versus concentration of the inhibitor, as exemplified by Figure 2. The assay was performed using Suc-Ala-Ala-Pro-Phe-*p*-NO_2-anilide[17] as substrate in a Tris buffer solution of pH 7.5. The IC_{50} values[18] thus obtained are listed in Table 1. None of the inhibitors exhibited inhibitory activity against α-CT up to the

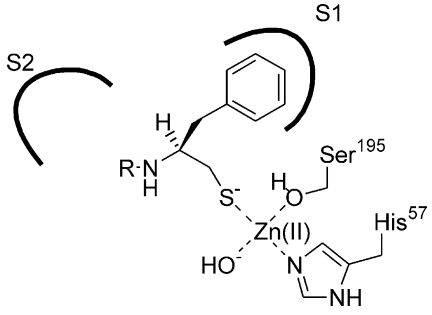
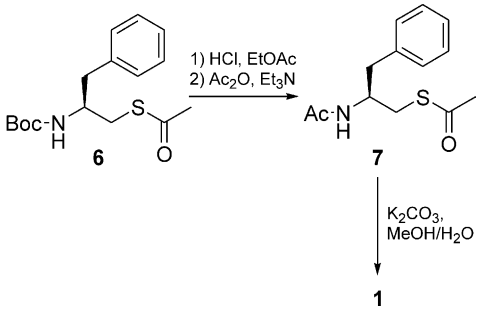
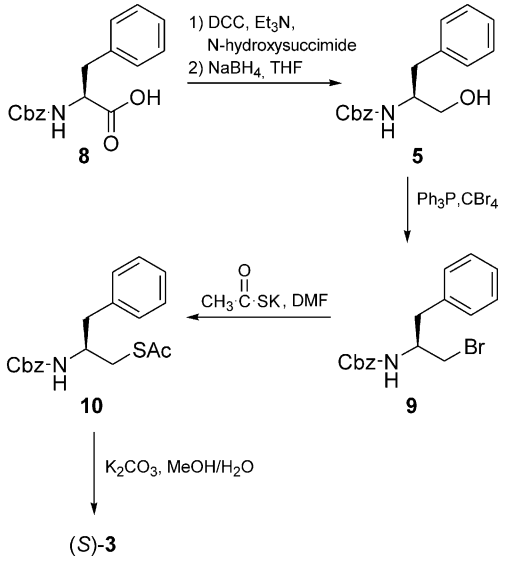


Figure 1. Schematic representation of inhibitor design rationale: mercapto-bearing substrate analogues inhibit α-chymotrypsin in the presence of Zn(II) ion by forming ternary complexes.



Scheme 1.



Scheme 2.

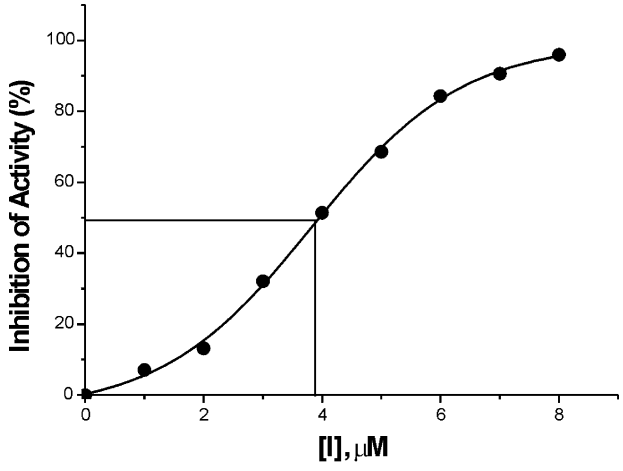


Figure 2. Plot of the percent enzymic activity remaining in the presence of an inhibitor and Zn(II) ion versus concentration of the inhibitor (*S*)-**3**.

Table 1. Kinetic parameters for the inhibition of α-CT in the presence of Zn(II) (500 μM)

Inhibitor	IC_{50} (μM)
1	>800
2	23
(*S*)-**3**	3.8
(*R*)-**3**	6.0
4	8.0
(*S*)-**5**	>3000
(*R*)-**5**	>3000

concentration of 2 mM when tested in the absence of Zn(II) ion, suggesting that the metal ion plays a critical role in the inhibition, most likely by bridging the inhibitor to the enzyme with the formation of a ternary complex as depicted in Figure 1. Since some proteases such as carboxypeptidase A are known to be inactivated by Zn(II) ion,[19] we have investigated the effect of Zn(II) ion alone on α-CT to find that the enzymic activity is essentially unchanged by Zn(II) ion up to the concentration of 2 mM. These results suggest strongly that in the inhibitions of α-CT both the thiol and Zn(II) ion are involved, which is in line with the proposed design rationale depicted schematically in Figure 1.

As can be seen in Figure 3, the extent of Zn(II) mediated inhibition of α-CT by (*S*)-**3** is dependent on the concentration of Zn(II) ion. The enzymic activity is essentially abolished when the concentration of Zn(II) ion reaches 150 μM. We were interested in knowing the effect of other metal ions than Zn(II) on the α-CT inhibitory property of the thiol-bearing inhibitors, and evaluated four different metal ions including Zn(II) ion to find that Cu(II) ion is as effective as Zn(II) ion in mediating (*S*)-**3** to inhibit the α-CT. Although the extent of the inhibition caused by the two metal ions is comparable, Cu(II) ion is much more efficient than Zn(II) ion. That is, the maximum inhibition is achieved by a much smaller amount of Cu(II) ion than Zn(II) ion (Fig. 3).[20]

The limited structure–activity relationships study on the Zn(II) ion mediated inhibition of α-CT by thiol derivatives

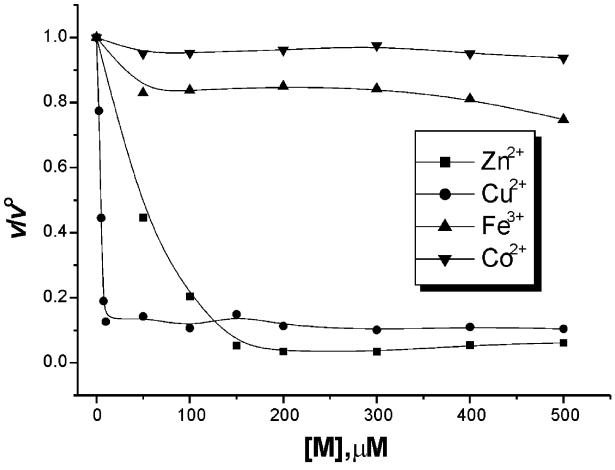


Figure 3. Inhibition of α-CT by (*S*)-**3** (5.0 μM) in the presence of different metal ion (0–500 μM) in Tris buffer 7.5 (substrate, Suc-Ala-Ala-Pro-Phe-*p*-NO_2-anilide (300 μM); [E] = 0.4 μg/mL).

revealed that (*S*)-**3** that carries a Cbz moiety on the amino group is most potent, suggesting that the phenyl ring in the Cbz may play a role in binding of the inhibitor to the enzyme, possibly the phenyl group being accommodated in the S2 pocket. It has been known that the S2 pocket of α-CT prefers a large residue.[21] Segel and others showed that substrates of α-CT having a proline at the P2 position bind the enzyme with high affinity by virtue of its hydrophobic interactions possibly with the side chain of Ile-99 as well as a smaller entropy decrease upon binding to the enzyme due to its restricted rotational freedom, in addition to hydrogen bondings between the substrate and active site backbone residues of the enzyme.[22] Accordingly, we have synthesized **4** and evaluated it as an α-CT inhibitor in the presence of Zn(II) ion. Contrary to the expectation, **4** was found to be less effective than **3**. The replacement of the thiol group in (*S*)-**3** with a hydroxyl essentially abolishes the α-CT inhibitory activity in accordance with the proposition that the enzyme inhibition is effected via the formation of the metal-centered ternary complex, and indicates that the thiol group coordinates the metal ion much more strongly than a hydroxyl. Recently, Vahrenkamp and associates have demonstrated that a thiol has a stronger coordination power toward Zn(II) than a hydroxyl in forming the tetrahedral Zn(II) complex.[23]

Zn(II) ion has a filled d-shell and thus is redox-inert which is a desirable feature for the metal ion being used as a cofactor of proteolytic enzymes. In fact, Zn(II) ion is the most abundantly and widely distributed transition metal ion next to Fe(II) and Fe(III) ions in the biological systems: The concentrations of Zn(II) ion in human tissue and blood amount to 60–120 μM.[24] Comparative trace metal analyses of cancerous and noncancerous human tissue have revealed that the concentration of Zn(II) ion in cancerous cell such as breast carcinoma is much higher by 700% compared with that in normal breast cells.[25] Hence, the design protocol reported in this communication may be applied for developing inhibitors that selectively inhibit serine proteases in cells of high Zn(II) ion concentrations. In employing the present protocol in designing inhibitors that are effective in the living system, there is a strong possibility of the inhibitors may undergo dimerization with loss of the inhibitory activity. Use of the inhibitors in a form of prodrug such as *S*-acylated derivatives may circumvent the problem.

3. Conclusion

We have demonstrated that thiol-bearing analogues of α-CT substrate functions as inhibitors for α-CT in the presence of Zn(II) ion. In the inhibitions, the Zn(II) plays a mediating role by bridging the inhibitors to the enzyme, forming the ternary complexes: The Zn(II) ion is coordinated with the thiol of the inhibitors, the hydroxyl of Ser-195 and the imidazole of His-57 in α-CT, and a water molecule. In addition to the high selectivity towards serine proteases in cells of high Zn(II) ion concentrations, this type of inhibitors are thought to manifest high inhibition power in light of the fact that coordinative

bonds to a metal ion are in general much stronger than hydrogen bonds or hydrophobic interactions that are involved in the binding of inhibitors to enzymes. Furthermore, these compounds are structurally simple having an amino acid structural frame, and hence there is an ample room for structural modifications for the activity improvements.

Acknowledgements

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7. In general, Zn(II) ion complexes may have coordination numbers of 4, 5, and 6, although in the biological systems, Zn(II) complexes having coordination number 4 are most common (Dudev, T.; Lim, C. *J. Am. Chem. Soc.* **2000**, *122*, 11146).
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11. **Thioactic acid, (*S*)-(2-acethylamino-3-phenyl-propyl) ester (7)**. Thioacetic acid, (*S*)-(2-*tert*-butoxycarbonylamino-3-phenylpropyl) ester (Fournib-Zaluski, M.-C.; Coric, P.; Turcaud, S.; Bruetschy, L.; Lucas, E.; Noble, F.; Roques, B. P. *J. Med. Chem.* **1992**, *35*, 1259) (4.0 g, 12.93 mmol) was added to a solution of saturated hydrochloric acid in ethyl acetate (80 mL). The reaction mixture was stirred overnight, evaporated under reduced pressure, and the residue was dissolved in methylene chloride (50 mL). To the solution was added triethylamine (0.64 g, 6.23 mmol) and acetic anhydride (0.35 g, 3.39 mmol) at 0 °C, then stirred for 1 h at room temperature. The reaction mixture was washed with 0.1 N hydrochloric acid solution, 1 N sodium bicarbonate solution, dried over magnesium sulfate, and concentrated under reduced pressure. The crude product was purified by recrystallization (toluene/hexane) to afford the product (0.58 g, 82%). Mp 121–122 °C; $[\alpha]^{20}$ −18.7 (*c* 1, $CHCl_3$); IR (KBr) 3299, 1731, 1678, 1605 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.27 (m, 5H), 5.90 (b, 1H), 4.28 (m, 1H), 2.99 (m, 3H), 2.81 (m, 1H), 2.34 (s, 3H), 1.89 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 196.99, 170.29, 137.60, 129.70, 128.97, 127.12, 51.35, 40.48, 32.78, 30.98, 23.68. Anal. calcd for $C_{13}H_{17}NO_2S$: C, 62.12; H, 6.82; N, 5.57. Found: C, 62.41; H, 6.95; N, 5.60. **Thioacetic acid, (*S*)-(2-benzoylamino-3-phenyl-propyl) ester (11)** was prepared similarly starting with thioacetic acid, (*S*)-(2-*tert*-butoxycarbonylamino-3-phenyl-propyl) ester. Benzoyl chloride was used in lieu of acetic anhydride. Yield 71%. Mp 137–138 °C; $[\alpha]^{20}$ +1.3 (*c* 1, $CHCl_3$); IR (KBr) 3350, 1703, 1654, 1505 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.71 (d, 2H), 7.41 (m, 3H), 7.26 (m, 5H), 6.65 (b, 1H), 4.45 (m, 1H), 3.12 (m, 3H), 2.85 (m, 1H), 2.30 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 197.97, 167.40, 137.64, 134.59, 131.89, 129.84, 129.06, 128.97, 127.31, 127.19, 52.48, 40.70, 32.63, 31.00. Anal. calcd for $C_{18}H_{19}NO_2S$: C, 68.98; H, 6.11; N, 4.47. Found: C, 68.84; H, 6.14; N, 4.58. **(*S*)-*N*-(1-Mercaptomethyl-2-phenyl-ethyl)-acetamide (1).** Compound **7** (0.25 g, 1.0 mmol) was added to a solution of potassium carbonate (0.027 g, 0.2 mmol) in $MeOH/H_2O$ (10 mL, 95/5). The reaction mixture was stirred for 3 h at room temperature. The reaction mixture was diluted with methylene chloride and washed with 0.1 N hydrochloric acid solution, dried over magnesium sulfate, and concentrated under reduced pressure. The crude product was purified by column chromatography (EtOAc–hexane = 1/1) to afford the product (0.18 g, 85%) as a white solid. Mp 70–71 °C; $[\alpha]^{20}$ −18.8 (*c* 1, $CHCl_3$); IR (KBr) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.28 (m, 5H), 5.74 (d, 1H), 4.38 (m, 1H), 2.94 (m, 2H), 2.64 (m, 2H), 1.95 (s, 3H), 1.32 (t, 1H); ^{13}C NMR ($CDCl_3$) δ 170.32, 137.95, 129.95, 129.34, 127.43, 51.65, 38.96, 28.46, 24.05. HRMS-EI *m*/*z*: calcd for $C_{11}H_{15}NOS$, 209.0874, found: 209.0872. **(*S*)-*N*-(1-Mercaptomethyl-2-phenyl-ethyl)-benzamide (2)** was prepared in a similar fashion from **11**. Yield 59%. Mp 135–136 °C; $[\alpha]^{20}$ −19.7 (*c* 1, $CHCl_3$); IR (KBr) 3299, 1632, 1530 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.73–7.24 (m, 10H), 6.34 (d, 1H), 4.56 (m, 1H), 3.03 (m, 2H), 2.76 (m, 2H), 1.38 (t, 1H); ^{13}C NMR ($CDCl_3$) δ 167.66, 137.86, 135.11, 132.30, 129.44, 129.31, 127.55, 52.00, 39.02, 28.55. HRMS-EI *m*/*z*: calcd for $C_{16}H_{17}NOS$, 271.1031, found: 271.1029.
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13. **(*S*)-2-(Benzyloxycarbonylamino)-3-phenyl-1-propanol [(*S*)-5]**. To an ice-chilled stirred solution of **8** (13.6 g, 46 mmol) and *N*-hydroxysuccinimide (5.52 g, 48 mmol) in ethylene glycol dimethyl ether (80 mL) was added 1,3-dicyclohexylcarbodiimide (9.88 g, 48 mmol). The mixture was stirred overnight and then filtered through a Celite pad, the filtrate was evaporated under reduced pressure, and the residue was dissolved in THF (80 mL). To the ice-chilled stirred solution of THF was added sodium borohydride (4.53 g, 120 mmol), and stirred for 18 h, then poured into vigorously stirred ice-water. The product was extracted with ethyl acetate, and the extract was washed with water and brine, dried over magnesium sulfate, and concentrated to give (*S*)-**5** (7.3 g, 92%) mp 89–91 °C. lit (Loeffler, L. J.; Sajadi, Z.; Hall, I. H. *J. Med. Chem.* **1977**, *20*, 1578) mp 89–90 °C. **(*S*)-(1-Benzyl-2-bromoethyl)-carbamic-acid, benzyl ester [(*S*)-9]**. To an ice-chilled stirred solution of (*S*)-**5** (13.6 g, 47.7 mmol) and triphenylphospine (13.75 g, 52.4 mmol) in methylene chloride (80 mL) was added tetrabromomethane (17.4 g, 52.4 mmol). The resulting mixture was stirred overnight and concentrated under reduced pressure. The crude product was purified by column chromatography (EtOAc–hexane = 1/4) to

afford **9** (11.95 g, 72%). Mp 69–70 °C; $[\alpha]^{20}$ −5.3 (*c* 1, $CHCl_3$); IR (KBr) 3321, 1698 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.30 (m, 10H), 5.09 (s, 2H), 5.05 (s, 1H), 4.12 (m, 1H), 3.40 (dd, 2H), 2.91 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 155.89, 137.19, 136.65, 129.66, 129.18, 128.99, 128.65, 128.52, 127.37, 67.34, 52.43, 39.17, 37.43. Anal. calcd for $C_{17}H_{18}BrNO_2$: C, 58.63; H, 5.21; N, 4.02. Found: C, 58.51; H, 5.20; N, 4.08. **(*S*)-2-[(benzyloxycarbonyl)amino]-3-phenylpropyl ethanethioate [(*S*)-10]**. Potassium thioacetate (3.07 g, 27 mmol) was added to a solution of (*S*)-**9** (7.8 g, 22 mmol) in DMF and the reaction mixture was stirred for 1 h at room temperature. The reaction mixture was diluted with ethyl acetate and washed with 5% aqueous solution of sodium thiosulfate to remove DMF. The organic layer was washed with 0.1 N hydrochloric acid solution, dried over magnesium sulfate, and concentrated under reduced pressure to afford the product (7.5 g, 98%). Mp 89–90 °C, Lit. (Higashiura, K.; Ienga, K. *J. Org. Chem.* **1992**, *57*, 764) mp 89–90 °C; $[\alpha]^{20}$ −6.2 (*c* 1, $CHCl_3$); IR (KBr) 3333, 1692 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.29 (m, 10H), 5.07 (s, 2H), 4.88 (m, 1H), 4.05 (m, 1H), 2.94 (m, 4H), 2.32 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 196.31, 156.27, 137.55, 136.99, 129.77, 129.02, 128.92, 128.48, 128.41, 127.16, 67.01, 52.94, 40.82, 33.15, 30.99. **(*S*)-(1-Benzyl-2-thiolethyl)-carbamic acid, benzyl ester [(*S*)-3]**. Compound (*S*)-**10** (0.4 g, 1.14 mmol) was added to a solution of potassium carbonate (0.03 g, 0.23 mmol) in $MeOH/H_2O$ (50 mL, 95/5). The reaction mixture was stirred for 3 h at room temperature, then diluted with methylene chloride, and washed with 0.1 N hydrochloric acid solution, dried over magnesium sulfate, and concentrated under reduced pressure. The crude product was purified by column chromatography (EtOAc–hexane = 1/4) to afford (*S*)-**3** (0.23 g, 68%) Mp 56–57 °C; $[\alpha]^{20}$ −13.0 (*c* 0.67, $CHCl_3$); IR (KBr) 3323, 2550, 1697 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.27 (m, 10H), 5.08 (s, 2H), 4.95 (d, 1H), 4.07 (m, 1H), 2.87 (m, 2H), 2.65 (m, 2H), 1.33 (t, 1H); ^{13}C NMR ($CDCl_3$) δ 156.07, 137.64, 136.90, 129.61, 129.04, 128.89, 128.49, 128.39, 127.13, 67.17, 53.75, 39.24, 28.56; HRMS-EI *m*/*z*: calcd for $C_{17}H_{19}NO_2S$, 301.1137, Found: 301.1135.

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16. **(2*R*,3*S*)-2-(2-Acetylsulfanyl-1-benzyl-ethylcarbamoyl)-pyrrolidine-1-carboxylic acid benzyl ester (12)**. To the mixture of 2-(1-benzyl-2-hydroxy-ethylcarbamoyl)-pyrrolidine-1-carboxylic acid, benzyl ester (Pfeiffer, F. R.; Chambers, P. A.; Hilbert, E. E.; Woodward, P. W.; Ackerman, D. M. *J. Med. Chem.* **1984**, *27*, 325) (0.3 g, 0.73 mmol) and triphenylphospine (0.23 g, 0.86 mmol) in THF (10 mL) were added dropwise dimethyl azodicarboxylate (0.32 mL of 40% solution in toluene, 0.86 mmol) and thioacetic acid (0.06 mL, 0.86 mmol), and stirred for 1 h. The reaction mixture was evaporated under reduced pressure to give a reddish oil which was purified by column chromatography (EtOAc–hexane = 1/2) to yield **12** (0.26 g, 76%). Mp 75–77 °C; $[\alpha]^{20}$ −77 (*c* 0.8, $CHCl_3$); IR (KBr) 3309, 1691 cm^{-1}; ^1H NMR (DMSO, 100 °C) δ?7.28 (m, 10H), 5.07 (m, 2H), 4.17 (m, 1H), 4.15 (m, 1H), 3.41 (m, 2H), 3.10 (m, 1H), 2.87 (m, 1H), 2.79 (m, 2H), 2.28 (m, 3H), 2.03 (m, 1H), 1.76 (m, 3H); ^{13}C NMR (DMSO, 100 °C) δ 193.87, 170.76, 153.62, 137.51, 137.51, 136.51, 128.33, 127.51, 127.39, 126.85, 126.59, 125.42, 65.38, 59.54, 49.15, 46.17, 32.30, 29.59, 22.54; HRMS-EI *m*/*z*: calcd for $C_{24}H_{28}O_4N_2S$, 440.1770, found: 440.1765. **(2*R*,3*S*)-2-(1-Benzyl-2-mercapto-ethylcarbamoyl)-pyrrolidine-1-carboxylic acid, benzyl ester (4)**. Compound **12** (1.2 g, 2.72 mmol) was added to a solution of potassium carbonate (0.07 g, 0.5 mmol) in $MeOH/H_2O$ (50 mL, 95/5). The reaction mixture was stirred for 3 h at room temperature, then diluted with methylene chloride, washed with 0.1 N hydrochloric acid solution, dried over magnesium sulfate, and concentrated under reduced pressure. The crude product was purified by column chromatography (EtOAc–hexane = 1/1) to afford 4 (0.68 g, 63%). Mp 87–89 °C; $[\alpha]^{20}$ −88 (*c* 0.8, $CHCl_3$); IR (KBr) 2551, 1691 cm^{-1}; ^1H NMR (DMSO, 100 °C) δ 7.28 (m, 10H), 5.06 (m, 2H), 4.20 (m, 1H), 3.41 (t, 1H), 2.49 (m, 2H), 2.18 (m, 2H), 2.12 (m, 1H), 1.93 (m, 1H), 1.78 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 170.88, 153.69, 137.83, 136.49, 128.34, 127.54, 127.41, 126.86, 126.62 125.37, 65.43, 59.62, 51.88, 46.22, 42.64, 37.97, 27.12, 22.60; HRMS-EI *m*/*z*: calcd for $C_{22}H_{26}N_2O_3S$, 398.1664, Found: 398.1664.
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18. Determination of IC_{50} values: Assay mixtures were prepared by dissolving Suc-AAPF-pNA (300 μM), zinc chloride (500 μM), and various concentrations of the inhibitor in pH 7.5 buffer (50 mM $CaCl_2$, 50 mM TRIZMA). Enzyme stock solution was added to the assay mixture to afford a final concentration of 0.4 μg/mL. Residual enzyme activities were determined by using initial rates monitored at 405 nm at 25 °C. IC_{50} of inhibitors were determined by plotting the percentage of residual enzyme activity versus the concentration of inhibitors.
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