Articles

Inactivation of Thermolysin with *N*-Chloroacetyl-*N*-hydroxy-β-L-phenylalanine *N*-methylamide

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N-Chloroacetyl-*N*-hydroxy-β-Phe-NHMe and *N*-chloroacetyl-*N*-hydroxy-α-Phe-OMe were designed, synthesized and evaluated as irreversible inhibitors of thermolysin, a representative zinc protease. Analysis of kinetic data of the enzymic activity of thermolysin in the presence of these inhibitors revealed that they are indeed potent inactivators of thermolysin having the k_{inact}/K_I values of 3.06 and 0.05 M^{-1} s^{-1}, respectively. We have established that the inhibitory activity of *N*-chloroacetyl-*N*-hydroxy-β-Phe-NHMe stems mainly from the (*R*)-enantiomer that belongs to the "L" series. The (*R*)-enantiomer is also responsible for the inactivation in the case of *N*-chloroacetyl-*N*-hydroxy-α-Phe-OMe, but this enantiomer belongs to the "D"-series.

Introduction

Thermolysin is a much studied thermostable proteolytic enzyme isolated from *Bacillus thermoproteolyticus*.[1] The enzyme is a neutral endopeptidase having specificity for peptide bond on the imino side of hydrophobic amino acid residue. At the active site of the enzyme, there is present a zinc ion which plays an essential role in the catalytic bond cleavage by activating the carbonyl group of the scissile peptide bond for a nucleophilic attack.[1] Thermolysin has received much attention because the enzyme shares many critical mechanistic features with mammalian metalloproteases such as angiotensin converting enzyme and matrix metalloproteases.[1,2] The latter enzymes participate in metabolic processes of physiological importance and inhibitors of these enzymes are potentially useful as therapeutic agents.[3,4] Accordingly, the inhibitor design strategy developed with thermolysin is of considerable practical value in connection with drug discovery.

There have been developed a large number of thermolysin inhibitors of a wide structural variety, but all of them except one[5] inhibit the enzyme in a reversible competitive manner.[1] We wish to report herein new irreversible thermolysin inhibitors of improved potency, the rationale employed designing them, and the stereochemistry associated with the inactivation.

Results and Discussion

Hydroxamic acid is known to form a stable complex with zinc ion,[6] and this property of hydroxamate has been extensively utilized in the design of reversible inhibitors of zinc proteases.[7] The X-ray crystallographic study of the enzyme that is inhibited by hydroxamates reveals that the C=O and OH groups simultaneously coordinate to the active site zinc ion to form thermodynamically favorable five-membered chelate complexes.[8]

Recently, we have reported that *N*-formyl-*N*-hydroxy-β-Phe-NHMe is a potent reversible inhibitor for thermolysin.[9] The aromatic ring of the benzyl group at the α-position of the inhibitor is thought to be accommodated in the primary substrate recognition pocket (S_1' pocket) in the enzyme and the hydroxamate forms bidentate coordinative bonds to the active site zinc ion.[9] We envisioned that the replacement of the formyl group in the reversible inhibitor with a chloroacetyl group would generate a potential affinity label for the enzyme by allowing the activated carbon bearing the chloride to undergo a substitution reaction with a nucleophile at the active site. In light of the X-ray crystallographic report[10] that the carboxylate of Glu-143 becomes covalently modified upon treatment of thermolysin with *N*-chloroacetyl-*N*-hydroxy-Leu-OMe, it is not unreasonable to expect that the same nucleophile would displace the activated chloride of the potential inhibitor, resulting in the inhibitor to tether the enzyme (Figure 1). The covalently modified thermolysin thus generated will no longer perform its enzymic function. On the basis of the foregoing design rationale, we have synthesized *N*-chloroacetyl-*N*-hydroxy-β-Phe-NHMe (**1**) and *N*-chloroacetyl-*N*-hydroxy-α-Phe-OMe (**2**) as inactivators for thermolysin.

The target compound (**1**) was prepared readily by treatment of 2-benzyl-3-benzyloxyamino-*N*-methylpropanamide[9] with chloroacetyl chloride followed by removal of the benzyl group according to the method reported by Fujii *et al.* with a modification[11] (Scheme 1). Inhibitor **2** was synthesized by the method described in the literature.[12]

Kinetic study for the enzyme inhibition was carried out as described in the literature using *N*-[3-(2-furyl)acryloyl]-Gly-Leu-NH_2 as substrate,[12] and the kinetic data are listed in Table 1. The values of K_I and k_{inact} were obtained from the

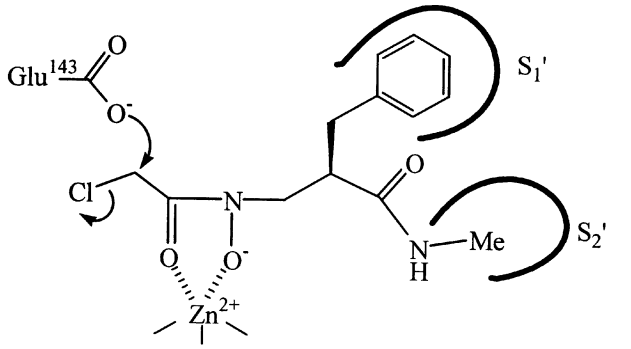
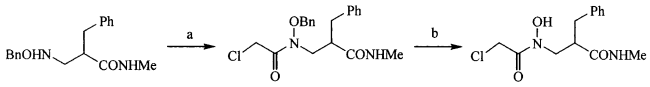


Figure 1. Schematic representation of the active site of thermolysin, which is occupied by *N*-chloroacetyl-*N*-hydroxyamino-*β*-L-phenylalanine *N*-methylamide. In the enzyme-inhibitor complex, the nucleophilic displacement of chloro group in the inhibitor by the carboxylate of Glu-143 would result in tethering of the inhibitor to the enzyme.



Scheme 1. Reagents, conditions, and (yields): (a) Ac_2O (8.0 eq), HCO_2H, 0 °C (100%); (b) chloroacetyl chloride (1.0 eq), TEA (1.1 eq), 0 °C, CH_2Cl_2 (93%); (c) *o*-cresol (3.0 eq), thioanisole (5.0 eq), TMS-trifluoromethanesulfonate (5.0 eq), TFA (70%).

Table 1. Structures of *N*-chloroacetyl-*N*-hydroxy-amino acid derivatives and their kinetic parameters determined for the inhibition of thermolysin

Compd No.	Structure	K_I (mM)	k_{inact} ($\times 10^3$ s^{-1})	k_{inact}/K_I ($M^{-1}s^{-1}$)
(*R,S*)-**1**	(*R,S*)-ClCH$_2$C(=O)-N(OH)-CH(CH$_2$Ph)-C(=O)-NHMe	3.5	10.7	3.06
(*R*)-**1**	(*R*)-ClCH$_2$C(=O)-N(OH)-CH(CH$_2$Ph)-C(=O)-NHMe	1.7	11.0	6.47
(*S*)-**1**	(*S*)-ClCH$_2$C(=O)-N(OH)-CH(CH$_2$Ph)-C(=O)-NHMe	NI[a]		
(*R*)-**2**	(*R*)-ClCH$_2$C(=O)-N(OH)-CH(CH$_2$Ph)-C(=O)-OMe	10.0	5.0	0.50
(*S*)-**2**	(*S*)-ClCH$_2$C(=O)-N(OH)-CH(CH$_2$Ph)-C(=O)-OMe	NI[a]		

[a] No inhibitory activity was observed at the concentration up to 10 mM.

double reciprocal plot of k_{obs} *vs* concentrations of inhibitors as shown in Figure 2. The k_{obs} (first-order rate constant) values were obtained from the progress curves for the enzymic hydrolysis of the substrate in the presence of respective inhibitor at different concentrations. The inhibition of thermolysin by (*R*)-**1** was slowed at higher concentration of the substrate as can be seen from Figure 3, indicating that (*R*)-**1** competes with the substrate for the active site. This observation supports that the inhibition of thermolysin by (*R*)-**1** takes place at the active site of the enzyme. (*R,S*)- and (*R*)-**1** showed a time-dependant loss of the enzymic activity (Figure 4) to suggest that the enzyme is inhibited in an irreversible manner. On the other hand, (*S*)-**1** failed to inhibit the enzyme at concentrations up to 10 mM. The irreversible nature of the thermolysin inhibitions by (*R,S*)- and (*R*)-**1** was

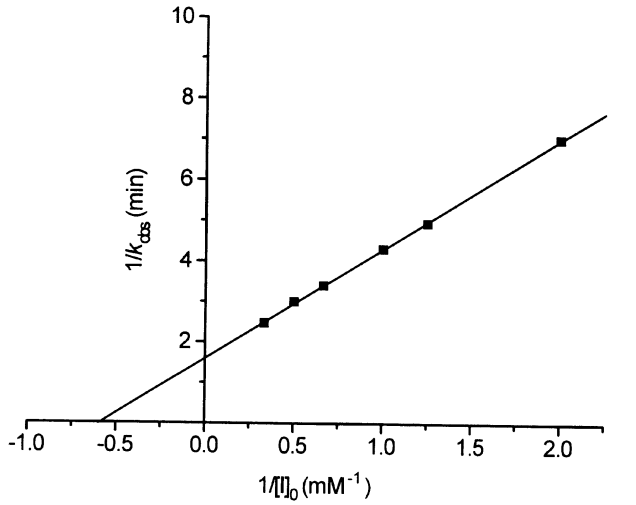


Figure 2. Double receprocal plot of k_{obs} *vs* $[I]_0$ in the inactivation of thermolysin with (*R*)-**1**. The y-intercept of the straight corresponds to $1/k_{inact}$ and the x-intercept shows $1/K_I$.

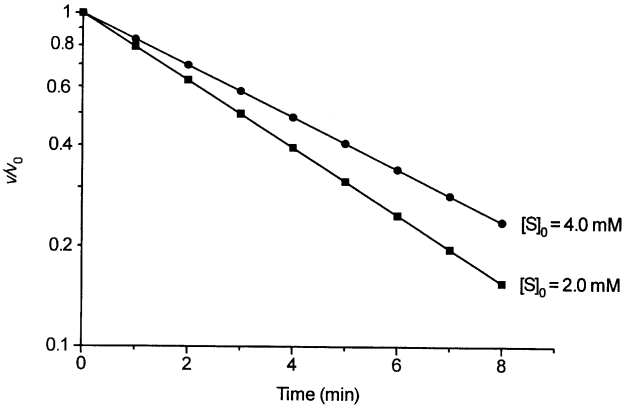


Figure 3. Loss of enzymic activity of thermolysin as a function of time during competitive substrate assay at the constant concentration (1.0 mM) of (*R*)-**1** with varying concentration of substrate. $[S]_0$ = concentration of substrate.

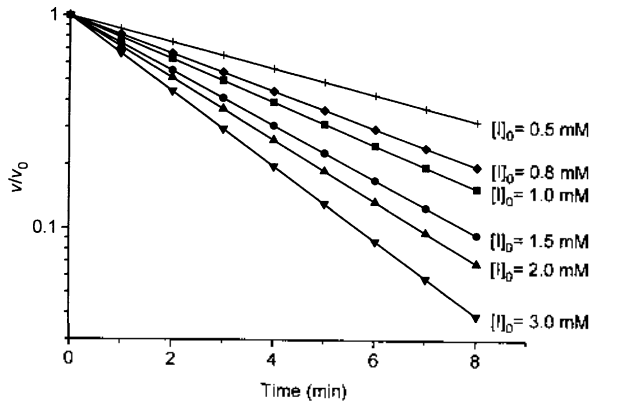


Figure 4. Loss of enzymic activity of thermolysin as a function of time during incubation with (*R*)-**1** of different concentrations. $[I]_0$ = concentration of (*R*)-**1**.

substantiated by the dialysis experiment, in which the inactivated thermolysin by incubating the enzyme with the inhibitors failed to regain the enzymic activity. The second-order rate constant (k_{inact}/K_I) reflects inactivation potency. As can be seen from the Table, the most potent inactivator for thermolysin is found to be (*R*)-**1** with the k_{inact}/K_I value of 6.47 $M^{-1}s^{-1}$. It is interesting to note that the *N*-chloroacetyl-*N*-hydroxy derivative prepared from β-Phe is considerably more potent than that obtained from α-Phe. A similar trend was noted previously for reversible competitive inhibitors of hydroxamate type,[9] suggesting that the inactivating chemical reaction becomes facilitated as the inactivator forms a tighter enzyme-inactivator complex. Furthermore, it is remarkable that the stereochemistry of the inhibitors plays a critical role in effecting the inactivation: in the case of **1**, the active form corresponds to the stereochemsitry of the L-series, but in the case of **2**, the compound that belongs to the D-series inactivates the enzyme. Previously, we have reported that the inactivation property of *N*-chloroacetyl-*N*-hydroxyleucine methyl ester is vested in the D-enantiomer,[12] which is in accord with the present observation. The reversal of stereochemistsry observed with the inactivator prepared from β-Phe in the present study is also parallel to the stereochemistry observed with *N*-formyl-*N*-hydroxy-β-L-Phe-NHMe which is competitive inhibitor for the enzyme.[9] In light of these observations, it may be concluded that the stereochemistry in the inactivation of thermolysin by the present inhibitors is established in the process of enzyme-inhibitor complex formation.

In summary, *N*-chloroacetyl-*N*-hydroxy-β-L-Phe-NHMe (**1**) is a potent competitive inactivator of thermolysin and its inactivating property rests mainly on the (*R*)-enantiomer which belongs to the L-series. In the case of *N*-chloroacetyl-*N*-hydroxy-α-Phe-OMe (**2**) the inactivating activity also stems from the (*R*)-enantiomer but this enantiomer belongs to the stereochemistry of unnatural amino acids, *i.e.*, the D-series.

Experimental Section

Melting points were taken on a Thomas-Hoover capillary melting point apparatus and were uncorrected. ^{1}H NMR spectra were recorded on a Bruker AM 300 (300 MHz) using tetramethylsilane as the internal standard. IR spectra were recorded on a Bruker EQUINOX 55 FTIR spectrometer. Mass spectra and elemental analyses were performed at the Center for Biofunctional Molecules, Pohang University of Science and Technology, Pohang, Korea. Optical rotations were measured on a Rudolph Research Autopol III digital polarimeter. Flash chromatography was performed on silica gel-60 (200-400 mesh) and thin-layer chromatography (TLC) was carried out on silica-coated glass sheet (Merck silica gel 60F-254).

3-[(*N*-Benzyloxy-*N*-chloroacetyl)amino]-2-benzyl-*N*-methylpropanamide. To a stirred cooled (0 °C) solution of 2-benzyl-3-benzyloxyamino-*N*-methylpropanamide (1.49 g, 5 mmol) and triethylamine (0.56 g, 5.5 mmol) in CH_2Cl_2 (20 mL) was added chloroacetyl chloride (5 mmol) dropwise. The resulting solution was stirred at 0 °C for 2 h. The solution was washed with 1 N HCl (2 × 5 mL), saturated $NaHCO_3$ (2 × 5 mL), brine (5 mL), and dried over $MgSO_4$. After evaporation, the residue was purified by column chromatography to give the product as a colorless oil (1.75 g, 93%). (*R*)-enantiomer : $[\alpha]_D^{18}$ = +20.0 (c 0.62, MeOH); (*S*)-enantiomer : $[\alpha]_D^{18}$ = −20.5 (c 0.80, MeOH); IR (neat) 1658,1605, 1550, 1505 cm^{-1}; EI MS 374 (M^+); ^{1}H NMR ($CDCl_3$) δ 7.41-7.16 (m, 10H), 5.35 (s, 1H), 4.90-4.77 (q, 2H), 4.07 (s, 2H), 3.96 (d, 2H), 3.00-2.70 (m, 3H), 2.61 (d, 3H).

3-[(*N*-Chloroacetyl-*N*-hydroxy)amino]-2-benzyl-*N*-methylpropanamide (1). To a stirred solution of 3-[(*N*-benzyloxy-*N*-chloroacetyl)amino]-2-benzyl-*N*-methylpropionamide (374 mg, 1 mmol), *o*-cresol (324 mg, 3 mmol) and thioanisole (0.6 mL, 5 mmol) in trifluoroacetic acid (3 mL) was added trimethylsilyl trifluoromethanesulfonate (0.9 mL, 5 mmol) dropwise. The resulting solution was stirred for 1 h at room temperature. After evaporation of trifluoroacetic acid, the residue was purified by column chromatography to give the product as a white solid (200 mg, 70%). (*R, S*)-**1**: 124-125 °C; (*R*)-**1**: mp 133-134 °C, $[\alpha]_D^{16}$ = + 37.4 (c 1.10, $CHCl_3$); (*S*)-**1**: mp 133-134 °C, $[\alpha]_D^{16}$ = −37.2 (c 1.10, $CHCl_3$); IR (KBr) 3310, 1650, 1560, 1505 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 9.73 (s, 1H); 7.20-7.00 (m, 5H), 6.50 (s, 1H), 4.30-4.18 (q, 2H), 4.10-3.90 (m, 2H), 2.90-2.65 (m, 3H), 2.60-2.50 (d, 3H); Anal. Cacld for $C_{18}H_{22}N_2O_2$: C, 54.84; H, 6.02; N, 9.84. Found: C, 54.53; H, 5.96; N, 9.61.

***N*-Chloroacetyl-*N*-hydroxyphenylalanine methyl ester (2).** To a solution of *N*-hydroxyphenylalanine methyl ester (0.976 g, 5 mmol) and 2,6-lutidine (1.2 mL, 10 mmol) in THF (20 mL) was added chlorotrimethylsilane (1.3 mL, 10 mmol) dropwise. The resulting solution was stirred at room temperature for 3 h. The solution was cooled to 0 °C and chloroacetyl chloride (0.4 mL, 5 mmol) was added dropwise. After stirring for 3 h at 0 °C, ethyl acetate (50 mL) was added and the solution was washed with 1 N HCl (2 × 5 mL), saturated $NaHCO_3$ (2 × 5 mL), brine (5 mL), and dried over $MgSO_4$. After evaporation, the residue was purified by column chromatography to give the product as a solid (1.21 g, 89%). (*R*)-**2**: mp 93-94 °C, $[\alpha]_D^{28}$ = +51.2 (c 1.20, MeOH); (*S*)-**2**: mp 93-94 °C, $[\alpha]_D^{28}$ = −54.0 (c 1.20, MeOH); IR (KBr) 3180, 1740, 1630 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.30-7.21 (m, 5H), 7.00 (s, 1H), 5.50-5.40 (dd, 1H), 4.15 (d, 1H), 4.02 (d, 3H), 3.80 (s, 3H), 3.30-3.20 (m, 2H). Anal. Cacld for $C_{12}H_{14}ClNO_4$: C, 53.05; H, 5.18; N, 5.16. Found: C, 53.32; H, 5.36; N, 4.80.

Kinetic Studies. Thermolysin was purchased from the Sigma Chemical Co. and used without further purification. The enzyme stock solution was prepared by dissolving the enzyme in 0.1 M Tris/0.01 M $CaCl_2$, pH 7.2 buffer solution and the enzyme concentration was determined from the absorbance at 278 nm (ε_{278} = 6.64 × 104 $M^{-1}cm^{-1}$). TLN substrate *N*-[3-(2-furyl)acryloyl]-Gly-L-Leu-NH_2 was purchased from the Sigma Chemical Co. The stock solutions of substrate and inhibitors were prepared in DMF. The rate of

substrate hydrolysis at 25 °C was monitored at 345 nm using computer-assisted Perkin-Elmer HP8435 UV/VIS spectrometer.

Determination of K_I and k_{inact}. Into the 1.0 mL cuvette containing the buffer solutions of substrate and inhibitor, the thermolysin stock solution was added to start the enzymic hydrolysis. The final concentrations in the assay mixture were 0.5-3.0 mM, 2.0 mM and 0.3-0.4 μM for inhibitors, substrate and the enzyme, respectively. The change in absorbance at 345 nm was recorded over such a time interval that the control curve in the absence of inhibitor was linear. The pseudo-first-order rate constant k_{obs} were obtained from computer-assisted UV spectrometer. A replot of $1/k_{obs}$ *vs* $1/[I]_0$ yielded the inactivation parameters K_I and k_{inact}.

Dialysis. The stock solution of thermolysin (500 μL) was incubated with racemic or (*R*)-**1** (100 μL) for 6 hours to ensure complete inactivation of the enzyme. After dialysis for 24 h using a dialysis kit against buffer (500 mL × 2) at 4 °C, aliquots (60 μL) were added to the buffer solution of substrate and the remaining enzyme activity was determined immediately to find no enzyme activity being regained. The control experiment carried out in the absence of inactivator showed that 95% of the enzymic activity remains.

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