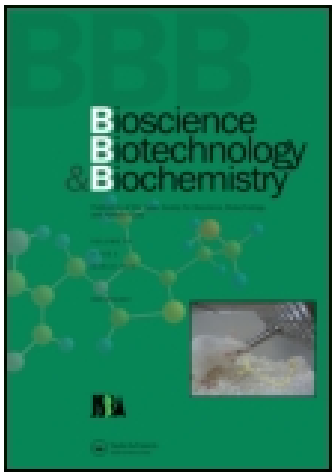

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Inhibition of *Achromobacter* Protease I by Lysinal Derivatives

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Z-Val-, Z-Pro-, Z-Leu-Leu-, and Z-Leu-Pro-lysinals and Bz-DL-lysinal were chemically synthesized and tested as novel inhibitors for *Achromobacter* protease I (API), a lysine-specific serine protease. Among the lysinal derivatives tested, Z-Val-lysinal was the most potent competitive inhibitor, its K_i being estimated as 6.5 nM in an esterolytic assay with Tos-Lys-OMe. In an amidolytic assay, Z-Leu-Leu-lysinal was the most potent inhibitor and the apparent mode of inhibition was non-competitive. The K_is of the other lysinal derivatives in both esterolytic and amidolytic assays were more than 10^3 times lower than that of leupeptin. Z-Val-lysinol, lacking the aldehyde group, was a poor competitive inhibitor. These results suggest that acyl-, acylaminoacyl-, and acylpeptidyllysinals function as a transition-state inhibitor for *Achromobacter* protease I.

Achromobacter protease I (EC 3.4.21.50., API), isolated from a culture filtrate of *Achromobacter lyticus* M497-1, is a lysine-specific serine protease that specifically hydrolyzes peptide bonds at the carboxyl side of lysine residues.[1] The narrow specificity to lysine residues, the high catalytic activity relative to bovine trypsin, the wide pH optimum and the resistance against denaturation with urea and SDS are all distinct from trypsin. API has recently become a useful tool for the fragmentation of the peptide chain in protein sequence analysis.[2,3] It has been shown that the enzyme is inhibited by diisopropylfluorophosphate and phenylmethylsulfonylfluoride.[4] However, the rate of inactivation with these reagents of API is much slower than that of bovine trypsin and a *Streptomyces erythraeus* trypsin-like protease (T. Masaki *et al.*, unpublished results). In contrast, API is rapidly inactivated by *N*-tosyl-lysine chloromethyl ketone but not inactivated by *N*-tosyl-arginine chloromethyl ketone.[4] Recently, the amino acid sequence of API has been determined[5] and that of prepro-API (653 amino acids) has been deduced from the nucleotide sequence of the cloned API gene.[6] Mature API consists of a single peptide chain of 268 residues with three disulfide bonds. Comparison of amino acid sequences between this protease and other serine proteases of bacterial and mammalian origins has found that API is mammalian-type. It has been suggested that the catalytic triad is composed of His^{57}, Asp^{113}, and Ser^{194}, although the degree of identity in amino acid sequence between API and trypsin is as low as 20%.

The conspicuous structural difference of API, specific for lysine, from trypsin, specific for both arginine and lysine, prompted us to examine the inhibitory potency of a transition-state analog of the lysine substrate, aiming at synthesizing a novel, potent, and specific inhibitor for API. Then, we chemically synthesized several lysinal derivatives with the C-terminal aldehyde group and tested their inhibition of API. This paper describes the synthesis of Bz-DL-lysinal and several other lysinal derivatives and their inhibition of the hydrolytic activity of API and related serine proteases involving in blood coagulation.

Materials and Methods

Materials. Achromobacter protease I was purified as described previously.[4] The following materials were purchased from the indicated sources: *N*-tosylphenylalanine chloromethylketone-treated bovine trypsin from Worthington Biochemicals; Z-Lys(Boc)-OH, Z-Val-OH, Z-Pro-OH, Z-Leu-OH, Z-Leu-Pro-OH, Bz-DL-Lys(Z)-OH, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and dicyclohexylcarbodiimide (DCC) from Wako Pure Chemical Industries; Tos-Lys-OMe and leupeptin from the Peptide Institute; Ac-Lys-pNA from Bachem Feinchemikalien AG; and human plasmin, human thrombin, porcine pancreas kallikrein, human factor Xa, Tos-Gly-Pro-Lys(or Arg)-pNA, D-Val-cyclohexyl-Ala-Arg-pNA, and Bz-Ile-Glu-Gly-Arg-pNA from Boehringer Mannheim GmbH. All amino acid derivatives used were of the L-configuration, unless otherwise stated.

Synthesis of lysinal-containing peptide derivatives. All NMR spectra of lysinal derivatives synthesized as described below were obtained on a JEOL JNM-PMX60SI NMR spectrometer. All melting points were uncorrected.

Z-Lys(Boc)-ol (**1**). Z-Lys(Boc)-ol was prepared by reduction with $NaBH_4$ of Z-Lys-OSu by the method of Kubota *et al.*[7] Yield, 82.6%. mp 49—51° (hexane). $[\alpha]_D^{20}$ −12.4° (c=1.2, MeOH). *Anal.* Found: C 62.05, H 8.23, N 7.86. Calcd. for $C_{19}H_{30}N_2O_5$: C 62.77, H 8.25, N 7.64%.

Z-Lys(Boc)(OMe)$_2$ (**2**). Compound **1** was oxidized by the method of Pfitzner and Moffatt.[8] To a solution of compound **1** (9.0 g, 24.6 mmol) in DMSO was added EDC (15.5 g, 81.1 mmol) and dichloroacetic acid (1.22 ml, 14.8 mmol), and the solution was stirred for 30 hr at room temperature. The reaction mixture was diluted with ethyl acetate, washed with water, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The oily aldehyde was treated with trimethylorthoformate (5.8 g, 54.6 mmol) and *p*-toluenesulfonic acid monohydrate (100 g) in methanol. Crude dimethylacetal was purified by silica gel chromatography using chloroform–ethyl acetate (1 : 1, v/v) and obtained as crystals from hexane. Yield, 7.08 g (70.2%). mp 54—56°C. $[\alpha]_D^{20}$ −17.3° (c=1.1, MeOH). *Anal.* Found: C 60.83, H 8.68, N 6.93. Calcd. for $C_{21}H_{34}N_2O_6$: C 61.44, H 8.35, N 6.82%.

Abbrevations: Ac, *N*-acetyl; Bz, *N*-benzoyl; boc, *t*-butyloxycarbonyl; DMSO, dimethylsulfoxide; OMe, methylester; OSu, hydroxysuccinimide; pNA, *p*-nitroanilide; Tos, *p*-toluenesulfonyl; Z, benzyloxycarbonyl.

Table I. Analytical Data for Lysinal Derivatives

Lysinal derivatives	Yields (%)	Mp. (°C)	$[\alpha]_D^{20}$ ($c=1$, MeOH)	Formula	*Anal.* (%) Calcd. (Found)		
					C	H	N
Z-Pro-lysinal	84.7	[a]	−47.9	$C_{19}H_{27}N_3O_4 \cdot HCl \cdot H_2O$	54.87 (54.73)	7.27 (7.08)	10.10 (9.88)
Z-Leu-Leu-lysinal[b]	74.9	141—143	−47.0	$C_{26}H_{42}N_4O_5 \cdot HCl \cdot H_2O$	57.28 (57.35)	8.32 (8.13)	10.28 (10.65)
Z-Leu-Pro-lysinal[b]	61.8	[c]	−67.5	$C_{25}H_{38}N_4O_5 \cdot HCl \cdot H_2O$	56.75 (56.11)	7.81 (7.55)	10.59 (10.58)

[a] Hygroscopic. [b] Recrystallized from ether. [c] Amorphous.

Z-Val-Lys(Boc)(OMe)$_2$ (**3**). Compound **2** (812 mg, 2 mmol) was hydrogenated over palladium in methanol and the free base thus obtained was coupled with Z-Val-OH (503 mg, 2 mmol) in CH_2Cl_2 (30 ml) containing DCC (412 mg, 2 mmol) for 20 hr at room temperature. After filtration, the filtrate was washed with 10% citric acid, 5% sodium hydrogencarbonate, and water successively, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was crystallized from ethyl acetate–hexane. Yield, 612 mg (60.0%). mp 104—106°C. $[\alpha]_D^{20}$ −21.7° ($c=1.1$, MeOH). *Anal.* Found: C 60.90, H 8.47, N 8.15. Calcd. for $C_{26}H_{43}N_3O_7 \cdot 1/2H_2O$: C 60.20, H 8.55, N 8.10%.

Z-Val-Lys-al (**4**). Compound **3** (473 mg, 0.93 mmol) was treated with TFA (5 ml) for 1 hr. After removal of TFA, the residue was dissolved in 6 N HCl, concentrated *in vacuo*, and crystallized with ether. Recrystallization from ether gave pure compound **4**. Yield, 161 mg (43.3%). mp 180—182°C. $[\alpha]_D^{20}$ −29.8° ($c=1.0$, MeOH). *Anal.* Found: C 57.16, H 7.41, N 10.89. Calcd. for $C_{19}H_{29}N_3O_4 \cdot HCl$: C 57.06, H 7.56, N 10.51%.

Similarly, Z-Pro-lysinal, Z-Leu-Pro-lysinal, and Z-Leu-Leu-lysinal were synthesized. The analytical data of these three lysinal derivatives are listed in Table I.

Z-Val-Lys(Boc)-ol (**5**). Compound **1** (2.20 g, 6 mmol) was hydrogenated and coupled with Z-Val-OH (1.51 g, 6 mmol) using the same method in the preparation of compound **3** and crystallized from ethyl acetate. Yield, 2.08 g (74.6%). mp 120—123°C. $[\alpha]_D^{20}$ −16.7° ($c=1.0$, MeOH). *Anal.* Found: C 61.64, H 8.38, N 9.16. Calcd. for $C_{24}H_{39}N_3O_6$: C 61.91, H 8.44, N 9.03%.

Z-Val-Lys-ol (**6**). Compound **5** (466 mg, 1 mmol) was treated with TFA as described for the preparation of compound **4** and crystallized from ether. Yield, 301 mg (74.9%). mp 132—134°C. $[\alpha]_D^{20}$ −20.6° ($c=1.0$, MeOH). *Anal.* Found: C 56.30, H 7.83, N 10.21. Calcd. for $C_{19}H_{31}N_3O_4 \cdot HCl$: C 56.77, H 8.03, N 10.46%.

Bz-DL-Lys-al (**7**). Bz-DL-Lys(Z)-OSu (1.2 g, 2.5 mmol) was reduced by $NaBH_4$ (473 mg, 12.5 mmol) by the method of Kubota *et al.*[7] Crude Bz-DL-Lys(Z)-ol was purified by silica gel chromatography using chloroform–methanol (9 : 1, v/v) and the oily alcohol (811 mg, 87.7%) was obtained. The alcohol (680 mg, 1.83 mmol) was oxidized with DMSO-DCC-dichloroacetic acid[8] to Bz-DL-Lys(Z)-al (340 mg, 55.3%). The benzyloxycarbonyl group was removed with 25% HBr in acetic acid and Bz-DL-Lys-al was crystallized from ether. Yield, 270 mg (73.9%). mp 136—139°C. *Anal.* Found: C 39.68, H 4.99, N 7.57. Calcd. for $C_{13}H_{18}N_2O_2 \cdot 2HBr$: C 39.42, H 5.09, N 7.07%.

Measurement of enzyme activity. Esterolytic activities of API and bovine trypsin were measured using Tos-Lys-OMe as a substrate.[9] The reaction mixture (3 ml) contained 40 mM Tris–HCl buffer (pH 8.0), the enzyme (2.7 nM for API and 18 nM for trypsin) and various concentrations of substrates (600—90 μM for both enzymes) and different concentrations of inhibitors (180—7 nM for API and 30—0.05 μM for trypsin). The initial rate was estimated by measuring the absorbance at 247 nm at 30°C for 5 min.

The amidolytic activities of API and trypsin were measured using Ac-Lys-pNA as a substrate.[10] The reaction mixture (0.75 ml) contained 0.1 M Tris–HCl buffer (pH 8.0), the enzyme (0.048 μM for API and 0.48 μM for trypsin) and various concentrations of substrates (330—17 μM for API and 830—34 μM for trypsin) and different concentrations of inhibitors (90—0.02 μM for API and 110—28 μM for trypsin). After incubation for 10 min at 30°C, the reaction was terminated by adding 0.25 ml of 45% acetic acid, and the absorbance at 405 nm was measured. The amidolytic activities of plasmin, thrombin, factor X_a, and kallikrein were assayed in 0.1 M Tris–HCl buffer (pH 8.0) using Tos-Gly-Pro-Lys-pNA, Tos-Gly-Pro-Arg-pNA, Bz-Ile-Glu-Gly-Arg-pNA, and D-Val-cyclohexyl-Ala-Arg-pNA, respectively.[11,12]

Results and Discussion

The synthesis of lysinal peptides is for the first time described in this paper. Briefly, the aldehyde precursor of C-terminal lysine, N^{α}-Z,N^{ε}-Boc-lysinal dimethylacetal, was synthesized by the oxidation of N^{α}-Z,N^{ε}-Boc-lysinol with the Pfitzner and Moffatt reagent[8] and subsequent derivatization to dimethylacetal. N^{α}-Z,N^{ε}-Boc-lysinal dimethylacetal was stable enough to be useful both for purification by silica gel chromatography and the synthesis of lysinal peptides. After the synthesis of the rest of a peptide, the C-terminal aldehyde group was regenerated by TFA treatment, which caused removal of the Boc groups simultaneously.

The elemental analyses of the four lysinal peptides were satisfactory. In the ^{1}H-NMR spectrum of Z-Val-lysinal, the signal of an aldehyde proton appeared at 9.65 ppm. For N^{α}-Z,N^{ε}-Boc-lysinal and the three other lysinal peptides, single aldehyde proton signals were consistently detected at 9—10 ppm (data not shown).

The inhibitory effects of Bz-DL-lysinal and four related peptide derivatives on API were examined for ester and amide substrates. The inhibition constants (K_i) of lysinal derivatives were calculated by a Dixon plot[13] using Tos-Lys-OMe and Ac-Lys-pNA as the specific substrate.[14] Bovine trypsin was used for comparison. As shown in Table II, all lysinal derivatives inhibited API as well as leupeptin inhibits trypsin. Interestingly, the inhibition of Z-Val-lysinal was extremely strong among the seven inhibitors tested, a very low K_i (6.5 nM) being measured for this dipeptide-like inhibitor when assayed with Tos-Lys-OMe. Z-Pro-, Z-Leu-Leu-, and Z-Leu-Pro-lysinals were also better inhibitors for API than for trypsin. The K_is of these inhibitors were estimated to be one to three order of magnitude lower than those obtained with trypsin. The K_is of Z-Pro-, Z-Leu-Leu-, and Z-Leu-Pro-lysinals for API were one order of magnitude lower than that of Bz-DL-lysinal. This suggests that the interaction between subsites P_2 of the inhibitor and S_2 of the enzyme (the notation of substrate binding subsites are made as described by Schechter and Berger[15]) in the inhibitor-enzyme complex is critical in the inhibitory potency of lysinal derivatives. On the other hand, the inhibitory potency of lysinal derivatives toward trypsin was increased about 10-fold as the sites of the interaction were extended from P_2–S_2 to P_3–S_3 (Table II), suggesting that the interaction at subsites P_3–S_3 and subsites P_2–S_2 contributes to the inhibition to the same extent. However, the reason why the P_3–S_3 interaction of Z-Leu-Leu/Pro-lysinal with API has little effect on the inhibitory potency remains to be clarified.

All acyl-, aminoacyl-, and peptidyllysinals tested are

Table II. Inhibition Constants of *Achromobacter* Protease I and Bovine Trypsin by Lysinal-Containing Peptide Derivatives

Details of experimental conditions are given in Materials and Methods.

Inhibitors	K_i (μM)			
	Achromobacter protease I		Bovine trypsin	
	Tos-Lys-OMe	Ac-Lys-pNA	Tos-Lys-OMe	Ac-Lys-pNA
Bz-DL-lysinal	0.3[a]	0.7[a]	30.0[a]	85.0[a]
Z-Val-lysinal	0.0065[a]	0.12[b]	3.0[a]	5.0[a]
Z-Pro-lysinal	0.04[a]	0.08[b]	3.10[a]	2.50[a]
Z-Leu-Leu-lysinal	0.04[a]	0.04[b]	0.15[a]	0.43[a]
Z-Leu-Pro-lysinal	0.04[a]	0.08[b]	0.10[a]	0.20[a]
Z-Val-lysinol	30.0[a]	43.0[a]		
Leupeptin	110.0[a]	180.8[b]	0.04[a]	0.1[a]

[a] Competitive inhibition. [b] Non-competitive inhibition.

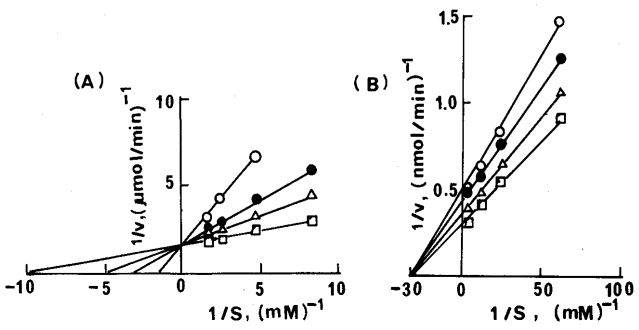

Fig. 1. Double Reciprocal Plots of *Achromobacter* Protease I with and without Z-Val-Lysinal.

(A) Competitive inhibition of the esterolytic activity by Z-Val-lysinal.
The reactions were started by adding 0.1 ml of the enzyme solution (2.7 nM) to 2.9 ml of a solution containing 0.04 M Tris–HCl buffer (pH 8.0), various concentrations of Tos–Lys-OMe and different concentrations of Z-Val-lysinal, and the enzyme activities were measured as described in Materials and Methods. The concentrations of Z-Val-lysinal were: (—□—) 0 nM; (—△—) 6.8 nM; (—●—) 13.7 nM; (—○—) 27.3 nM.
(B) Noncompetitive inhibition of the amidolytic activity by Z-Val-lysinal.
The reactions were started by adding 0.05 ml of the enzyme solution (48 nM) to 0.70 ml of a solution containing 0.1 M Tris–HCl buffer (pH 8.0), various concentrations of Ac-Lys-pNA, and different concentrations of Z-Val-lysinal. After 10 min of incubation at 30°C, the reactions were stopped by adding 0.25 ml of 45% acetic acid. The enzyme activities were measured as described in Materials and Methods. The concentrations of Z-Val-lysinals were: (—□—) 0 nM; (—△—) 44 nM; (—●—) 66 nM; (—○) 83 nM.

analogs of typical substrates having lysine at subsite P_1. So it was expected that the mode of inhibition would be competitive. However, two types of inhibition, competitive and non-competitive, were observed depending on the type of substrate used for the inhibition assay. For instance, the inhibition of Z-Val-lysinal was competitive when assayed with Tos-Lys-OMe, but it was non-competitive when assayed with Ac-Lys-pNA (Fig. 1). However, the inhibition of Bz-DL-lysinal, a less potent inhibitor than others, was competitive for both ester and amide substrates. Since no evidence has been reported so far for the presence of a possible second binding site to which only an amide substrate can bind, the non-competitive mode of inhibition observed for strong inhibitors of the lysinal type must be explained on the basis of more reasonable reasons. Interestingly, it has been reported that the mode of inhibition with an essentially competitive inhibitor changes to the non-competitive mode in the inhibition of trypsin with leupeptin[16] and of prolylendopeptidase with Z-Pro-prolinal.[17,18] In these cases, amide substrates have been exclusively used and the mode of inhibition changed. Accordingly, it has been proposed that tight binding of a competitive inhibitor to the active site is responsible for the change in the mode of inhibition. Similarly, the change from the competitive to non-competitive mode of inhibition was observed when inhibition of API was assayed with an amide substrate. However, when assayed with an ester substrate, the mode of inhibition with those strong inhibitors remained competitive. If the rate of hemiacetal formation between API and the C-terminal lysinal is comparable with the rate of acyl-enzyme formation, the mode of inhibition would be competitive. The inhibition observed with ester substrate may be the case. On the other hand, if hemiacetal formation is rapid and acyl-enzyme formation are slow, the apparent mode of inhibition would be non-competitive. The inhibition observed with the amide substrate may be the case.

Z-Val-lysinol was a very weak competitive inhibitor, suggesting that the aldehyde portion of the inhibitor is essential for strong inhibitory activity. As in the case of leupeptin[19,20] and other aldehyde-type inhibitors for serine proteases,[21,22] it is thought that Ser^{194} in API and the C-terminal lysinal residue of our inhibitors form hemiacetal, a transition-state analog.

Table III. Competitive Inhibition Constants of Several Coagulation Serine Proteases by Lysinal-Containing Peptide Derivatives

The activity with each substrate was measured at 30°C in 0.1 M Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl or 0.005 M $CaCl_2$ as described in refs. 11 and 12. The following substrates were used: Tos-Gly-Pro-Lys-pNA for plasmin; Tos-Gly-Pro-Arg-pNA for thrombin; Bz-Ile-Glu-Gly-Arg-pNA for factor X_a; D-Val-cyclohexyl-Ala-Arg-pNA for kallikrein.

Inhibitors	K_i (μM)			
	Plasmin	Thrombin	Factor X_a	Kallikrein
Bz-DL-lysinal	120.0	>1700.0		>1700.0
Z-Val-lysinal	47.0	50.0		>50.0
Z-Pro-lysinal	45.0	40.0		>200.0
Z-Leu-Leu-lysinal	0.30	75.0	250.0	>290.0
Z-Leu-Pro-lysinal	0.5	25.0	250.0	

Coagulation proteases such as plasmin, thrombin, factor Xa, and kallikrein are trypsin-type serine proteases. In the hydrolysis of peptide bonds, plasmin prefers lysyl bonds and the three other proteases prefer arginyl bonds.[23,24] To confirm the substrate preference, the inhibitory power of the lysinal derivatives were tested with these four proteases (Table III). The five lysinal derivatives, which all have K_is of 0.04—0.12 μM on API, were weak inhibitors, showing that the affinity of API toward lysinal derivatives was extremely strong compared with that of the four proteases involved in blood coagulation. Nevertheless, there is the clear difference in inhibitory power between plasmin and the three other proteases. Among the five lysinal derivatives tested, both Z-Leu-Leu-lysinal and Z-Leu-Pro-lysinal were potent inhibitors for plasmin, their K_is being as low as those estimated for trypsin. However, the inhibition of plasmin by Z-Val-lysinal and Z-Pro-lysinal was two orders of magnitude weaker than the inhibition by the two peptidyllysinals mentioned above.

This is suggestive of the importance of the interaction at subsite P_3 in plasmin inhibition by lysinal derivatives. As a result, Z-Leu-Leu-lysinal and Z-Leu-Pro-lysinal will be used to preferentially inhibit plasmin among four typical serine proteases involved in blood coagulation.

Since lysinal derivative can selectively bind to the Ser^{194} hydroxyl group in the active site of API, lysinal derivatives may be used as the ligand for affinity chromatography of API as in the case of a set of trypsin and leupeptin.[25] We have already prepared an immobilized lysinal adsorbent and tested the capability of a column for affinity chromatography of API. The results of these experiments will be published elsewhere.

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