Vertebrate Collagenase Inhibitor. I. Tripeptidyl Hydroxamic Acids

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A series of tripeptidyl analogues carrying hydroxamic acid residue at the C-terminus of the molecule were synthesized, and their inhibitory activities against vertebrate collagenase and other metalloenzymes including bacterial collagenase were examined. Both Z–Pro–Leu–Ala–NHOH and Z–Pro–D-Leu–D-Ala–NHOH showed highly specific and potent inhibitory activity against tadpole and human skin collagenases with an IC_{50} of 10^{-6} M order.

Keywords collagenase; hydroxamic acid; inhibitor; peptide synthesis; tripeptide

Introduction

It is well known that collagenase is a highly specific tissue proteinase which selectively cleaves collagen molecule at a 3/4 position from the N-terminus, and is involved in the initial step of collagen metabolism in tissues. Collagenase is, however, also involved in tissue-degrading diseases such as rheumatoid arthritis, periodontitis, skin ulcer and tumor invasion.[1] Therefore, intensive efforts have been made to develop collagenase inhibitors with high specificity as a therapeutic means to modulate the enzyme activity.

In this study, we prepared a series of tripeptidyl hydroxamic acids to establish specific collagenase inhibitors based on our previous studies on peptidyl inhibitors of urease.[2]

Results and Discussion

Because of the pathophysiological importance of collagenase in tissue-degrading diseases, numerous works exploring and developing vertebrate collagenase inhibitors have been reported recently. They include peptides[3] isolated from tissues as well as nonpeptidyl compounds.[4] Since collagenase is a member of the Zn-metalloproteinase family, most of these compounds are small peptides composed of a sequence similar to collagenase-cleavage site of collagen molecule and metallochelating groups, such as thiol (–SH),[5] carboxylic acid (–COOH),[6] hydroxamic acid (–CONHOH),[7] ketone (–CO–)[8] and phosphonate ($-PO(OH)_2$)[9] which are commonly introduced into many metalloproteinase inhibitors.

(1) –Pro–Gly–Pro–Gln–Gly—Ile(Leu)–Ala–Gly–Gln–Arg–Gly–
(2) DNP–Pro–Gln–Gly—Ile–Ala–Gly–Gln–D-Arg
DNP–Pro–Leu–Gly—Ile–Ala–Gly–Arg–NH_2
↑
cleavage site

Chart 1. Amino Acid Sequence of the Collagenase-Cleavage Site of Type I Collagen Molecule (1) and Synthetic Substrates for Vertebrate Collagenase (2)[10]

DNP = 2,4-dinitrophenyl.

Previously, we reported the synthesis of dipeptidyl hydroxamic acids, X–Gly–NHOH (X: protected or unprotected amino acid residues), as a potent inhibitor of urease (IC_{50} value: 10^{-6} M order). Since, among the peptidyl hydroxamic acids synthesized, Boc–Gln–Gly–NHOH and Boc–Leu–Gly–NHOH include the sequence of the cleavage site of either type I collagen or synthetic peptide substrates for vertebrate collagenase[10] (see Chart 1), these two compounds were tested for inhibitory activity against tadpole collagenase, and it was found that their IC_{50} values were 10^{-4} M order.

Based on these findings, attempts to develop a novel class of vertebrate collagenase inhibitors with high specificity has been undertaken. Since peptidyl hydroxamic acids are known to be an effective inhibitor of metalloenzymes such as thermolysin,[11] 5-lipoxygenase[12] and bacterial collagenase,[13] as well as urease,[14] hydroxamic acid residue can also be effectively introduced to the vertebrate collagenase inhibitor. Furthermore, it is essential for the inhibitor to include a peptide sequence corresponding to the collagenase cleavage site of collagen molecule to provide high affinity with the enzyme. Based on this molecular design for collagenase inhibitor, we first introduced Pro residue to the N-terminus of Boc–Gln–Gly–NHON and Boc–Leu–Gly–NHOH to give rise to tripeptidyl hydroxamic acids as leading compounds, then modified the second and third amino acid residues serially to obtain a potent and specific inhibitor against vertebrate collagenase.

Tripeptidyl hydroxamic acids were synthesized by the following two methods as shown in Chart 2.

Firstly, tripeptides (III, R = NHOBzl) prepared from Boc–X_1–NHOBzl (I) by the conventional procedure for peptide synthesis were hydrogenated. Secondly, tripeptidyl hydroxamic acid (IV) was prepared by the reaction of tripeptides (III, R = OMe or OEt) with hydroxylamine in alkaline methanolic solution.

The peptidyl hydroxamic acids prepared were assayed for enzyme inhibitory activities against collagenases purified

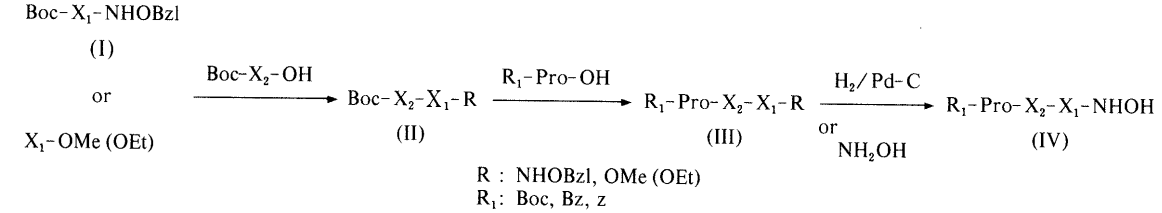

Chart 2. The Synthetic Route to Tripeptidyl Hydroxamic Acids

TABLE I. Inhibition (IC_{50}) of Vertebrate Collagenase and Other Enzymes by R_1–Pro–X_2–X_1–NHOH (IV)

Compd. No.	Residue			Human skin fibroblast collagenase	Tadpole collagenase	Bacterial collagenase	Urease	Thermolysin
	R_1	X_2	X_1					
IV-1	Boc	Leu	Gly		9.6×10^{-5}	2.0×10^{-2}	3.0×10^{-2}	3.2×10^{-2}
IV-2	Boc	Gln	Gly		2.0×10^{-4}	7.0×10^{-2}	8.6×10^{-2}	3.3×10^{-2}
IV-3	H	Leu	Gly		3.1×10^{-4}	4.1×10^{-2}	1.2×10^{-3}	6.6×10^{-2}
IV-4	Boc	Leu	Ala		1.7×10^{-5}	17%[a]	0%[a]	19%[b]
IV-5	Z	Leu	Ala	2.6×10^{-6}	7.3×10^{-6}	21%[a]	5%[a]	1.3×10^{-3}
IV-6	Bz	Leu	Ala		1.4×10^{-5}	13%[a]	1%[a]	1.1×10^{-2}
IV-7	Boc	D-Leu	Ala		2.5×10^{-3}	14%[a]	5%[a]	6.3×10^{-3}
IV-8	Z	D-Leu	Ala		1.5×10^{-3}	5%[a]	2%[a]	6.1×10^{-3}
IV-9	Bz	D-Leu	Ala		1.8×10^{-3}	8%[a]	0%[a]	4.9×10^{-3}
IV-10	Boc	Phe	Ala		4.3×10^{-5}	18%[a]	3%[a]	4.6×10^{-3}
IV-11	Z	Phe	Ala		2.3×10^{-5}	39%[a]	0%[a]	1.6×10^{-3}
IV-12	Bz	Phe	Ala		3.0×10^{-5}	29%[a]	1%[a]	2.5×10^{-3}
IV-13	Z	Leu	Leu	2.7×10^{-5}	3.5×10^{-5}	35%[a]	10%[a]	23%[a]
IV-14	Z	D-Leu	D-Ala	4.1×10^{-6}	1.3×10^{-6}	32%[a]	29%[a]	10%[a]
A	Boc–Leu–Gly–NHOH				6.8×10^{-4}	9.2×10^{-3}	1.0×10^{-4}	1.3×10^{-2}
B	Boc–Gln–Gly–NHOH				4.1×10^{-4}	1.9×10^{-2}	9.6×10^{-3}	2.0×10^{-2}
C	Z–Pro–Leu–Gly–NHOH			4.0×10^{-5} [c]	1.9×10^{-5}	39%[a]	42%[a]	1.1×10^{-3}

a) Inhibition % (2.0×10^{-3} M). *b*) Inhibition % (4.0×10^{-3} M). *c*) From ref. 7*a* (similar results were obtained in this study).

from tadpole skin explants[15] and human skin fibroblasts in culture (Nagai *et al.*, to be published elsewhere) by comparing them with those against bacterial collagenase (Sigma Chem. Co., St. Louis, MO), urease (Toyobo Co., Osaka) and thermolysin (Wako Pure Chemical Industries, Osaka) to deduce peptide sequence requirement(s) for potent and specific peptidyl inhibitors against vertebrate collagenase.

As shown in Table I, elongation of dipeptidyl analogues by introducing Pro as N-terminal amino acid residue showed an increase in inhibitory activity against vertebrate (tadpole) collagenase and a decrease in that against urease (compare compounds IV-1 and IV-2 with compounds A and B in Table I), indicating that the introduction of Pro residue into leading compounds provides a higher affinity with vertebrate collagenase by reducing their inhibitory activities against bacterial collagenase and thermolysin.

Further interesting findings obtained through this study were as follows (see Table I):

Firstly, the replacement of Gly residue at the C-terminus (X_1) with Ala residue resulted in several fold increase in its inhibitory activity against vertebrate collagenase, although the corresponding amino acid residue at the cleavage site of type I collagen is Gly (compare compound IV-1 with IV-4). However, replacement with more bulky amino acid such as Leu (IV-13) failed to improve its inhibitory activity. Secondly, the conversion of N-protecting group (R_1) from Boc (IV-4) to Z (IV-5) indicated ten fold increase in collagenase inhibitory activity.

Thirdly, of most interest was the introduction of D-amino acid residues to X_1 and X_2 positions without affecting collagenase inhibitory capacity. The replacement of L-Leu at X_2 with D-Leu and L-Ala at X_1 with D-Ala (IV-14) showed favorable inhibitory activity (IC_{50} value: 10^{-6} M order) as high as L-form compound, although replacement of L-Leu with D-Leu alone (IV-7—9) resulted in marked decreases in their inhibitory activity, indicating that the conformational structure extended over X_1 and X_2 residues in the compound is a key factor in its tight binding to the affinity site of vertebrate collagenase. Both compounds (IV-5) and (IV-14) also showed potent inhibitory activity against human skin fibroblast collagenase (IC_{50} value: 10^{-6} M order) as observed with tadpole collagenase, which is ten fold higher than Z-Pro-Leu-Gly-NHOH (compound C in Table I).[7a]

In summarizing, tripeptidyl hydroxamic acids carrying potent inhibitory activity against vertebrate collagenase were prepared. These compounds were highly specific to the enzyme with an IC_{50} value of 10^{-6} M order, and showed few inhibitory activities against thermolysin and bacterial collagenase which also belong to the metalloenzyme family. The replacement of constituting amino acids at both X_1 and X_2 positions with D-form moiety did not affect the inhibitory capacity against vertebrate collagenase. This may serve for *in vivo* stability of the compound resisting to the action of peptidases and proteases present in body fluids and tissues. Further studies along this line to develop a novel class of collagenase inhibitors are now in progress.

Experimental

Coupling reactions by using dicyclohexylcarbodiimide (DCC)–*N*-hydroxybenzotriazole (HOBt) were performed in dimethylformamide (DMF). DCC solution in CH_2Cl_2 was added dropwise at -15 °C to a mixture of paired amino acid (or peptide) derivatives to be coupled and the reaction mixture was stirred ovenight at 5 °C. After removal of DCUrea by filtration, the fitrate was evaporated *in vacuo* at 30—40 °C. The residue was purified by one of the following two procedures. Procedure A: For a case in which the product was soluble in AcOEt, the organic layer was washed successively with 1 M HCl, H_2O, 10% Na_2CO_3 and finally with H_2O, then dried over $MgSO_4$ and evaporated *in vacuo*. The residue was chromatographed on silica gel and/or recrystallized from appropriate solvents. Procedure B: For a case in which the product was insoluble in AcOEt, the crude product was triturated with AcOEt. The powder thus obtained was washed with 1 M HCl, H_2O, 10% Na_2CO_3 and finally with H_2O. The dried powder was recrystallized from appropriate solvents.

Melting points were determined on a Yanagimoto melting apparatus (Kyoto) without correction. Specific rotations were measured with a Jasco DIP-140 apparatus (Tokyo). The purity of all new compounds was monitored by analytical thin-layer chromatography (TLC) on Merck silica gel plates in the following solvent systems: Rf^1, $CHCl_3$–MeOH–AcOH (95:5:3, v/v); Rf^2, $CHCl_3$–MeOH (20:1, v/v); Rf^3, $CHCl_3$–MeOH–AcOH (80:10:5, v/v); Rf^4, *n*-BuOH–AcOH–H_2O (4:1:1, v/v); Rf^5, *n*-BuOH–AcOH–H_2O (4:2:1, v/v).

FITC-labeled collagen (bovine type I collagen, K-21), bacterial collagenase (collagenase-Sterile, Type IA-S, *Clostridium histolyticum*), urease (jack bean, 100 U/mg, Grade II), thermolysin (lyophilized, 7000 U/mg, *Bacillus thermoproteolyticus*), and furylacryloyl-Gly-Leu-NH_2 were purchased from Cosmo Bio (Tokyo), Sigma Chem. Co. (St. Louis, MO), Toyobo Co. (Osaka), Wako Pure Chemical Industries (Osaka), and Sigma Chem. Co., respectively.

Boc–Gly–NHOBzl (I-1) Triethylamine (TEA, 9.8 ml, 70 mmol), HOBt (7.43 g, 55 mmol), and Boc–Gly–OH (8.76 g, 50 mmol) was added to a solution of HCl·NHOBzl (11.2 g, 70 mmol) in DMF (100 ml) at −15 °C. Then, DCC (14.5 g, 70 mmol) in CH_2Cl_2 (30 ml) was added with stirring and the reaction mixture was stirred overnight at 5 °C. The crude product was purified by procedure A, followed by chromatography on silica gel with AcOEt–hexane (1 : 1, v/v). Light yellow oil (13 g, 93%). Rf^1, 0.58; Rf^2, 0.51.

Boc–Ala–NHOBzl (I-2) This compound was prepared from Boc–Ala–OH and HCl·NHOBzl as described for the preparation of I-1. The product was purified by procedure A, followed by recrystallization from AcOEt–hexane. Colorless needles (91%), mp 98—99 °C, $[\alpha]_D^{25}$ −42.1 ° (c = 1.0, EtOH). Rf^1, 0.58; Rf^2, 0.60. *Anal.* Calcd for $C_{15}H_{22}N_2O_4$: C, 61.2; H, 7.53; N, 9.51. Found: C, 61.38; H, 7.51; N, 9.62.

Boc–Leu–Gly–NHOBzl (II-1) As a typical example of dipeptide (II) synthesis, I-1 (11.8 g, 42.1 mmol) was dissolved in 4.2 M HCl/AcOEt (100 ml) at room temperature for 1 h and concentrated *in vacuo*. The residue was precipitated with ether, collected by filtration, and dried in a vacuum desiccator with solid NaOH. The white powder (HCl·Gly–NHOBzl, 8.3 g, 38.3 mmol) and Boc–Leu–OH (8.05 g, 34.8 mmol) were coupled as described for the preparation of I-1. The crude product was purified by procedure A, followed by recrystallization from AcOEt–hexane. Colorless crystals (12.6 g, 93%), mp 109—113 °C, $[\alpha]_D^{25}$ −8.3 ° (c = 1.0, EtOH). Rf^1, 0.56; Rf^2, 0.45. *Anal.* Calcd for $C_{20}H_{31}N_3O_5$: C, 61.04; H, 7.94; N, 10.67. Found: C, 60.8; H, 8.01; N, 10.62. Other dipeptides, II-2—7 were prepared as described for II-1 and the results are shown in Table II.

Boc–Pro–Leu–Gly–NHOBzl (III-1) As a typical example of tripeptide (III) synthesis, this compound was prepared as described for the preparation of II-1 by using II-1 (12.7 g, 32.3 mmol) and Boc–Pro–OH (6.30 g, 29.3 mmol). The product was purified by procedure B, followed by recrystallization from AcOEt. Colorless crystals (11.6 g, 81%), mp 181—184 °C, $[\alpha]_D^{25}$ −54.4 ° (c = 1.0, EtOH). Rf^1, 0.52; Rf^2, 0.40. *Anal.* Calcd for $C_{25}H_{38}N_4O_6$: C, 61.2; H, 7.80; N, 11.42. Found; C, 61.42; H, 7.65; N, 11.22. Compounds III-2—5, 9 and 10 were prepared from II-2—7 respectively in the same manner and are listed in Table III.

Bz–Pro–Leu–Ala–NHOBzl (III-6) Boc group of Boc–Pro–Leu–Ala–NHOBzl (II-4) (0.98 g, 1.94 mmol) was eliminated by the procedure described for II-1. HCl·Pro–Leu–Ala–NHOBzl thus obtained was dissolved in DMF and Bz-Cl (0.27 ml, 2.32 mmol) was added at −10 °C. The solution was adjusted to pH 8.0 and kept its pH constant with TEA for 3 h. The reaction mixture was concentrated *in vacuo* and purified by procedure A, followed by recrystallization from MeOH–ether. Colorless needles (0.5 g, 50%), mp 199—202 °C, $[\alpha]_D^{25}$ −116 ° (c = 1.0, EtOH). Rf^1, 0.50; Rf^2, 0.31. *Anal.* Calcd for $C_{28}H_{36}N_4O_5$: C, 59.75; H, 7.25; N, 13.27. Found: C, 59.79; H, 7.17; N, 13.18. Compounds III-7 and 8 were prepared by the

TABLE II. Physicochemical and Analytical Data for Boc–X_2–X_1–R (II)

Compd. No.	Residue X_2	Residue X_1	Residue R	Puri. proc. (Yield %)	mp (°C) Recryst. solv.	$[\alpha]_D^{25}$ (°) (c, solv.)	Rf^1 Rf^2	Formula	Analysis Calcd (Found) C	H	N
II-2	Gln	Gly	NHOBzl	B (80)	159—163 EtOH–Et_2O	−2.4 (0.5, DMF)	0.09 0.04	$C_{19}H_{28}N_4O_6$	55.87 (55.92	6.9 6.93	13.71 13.52)
II-3	Leu	Ala	NHOBzl	A (91)	164—166 AcOEt–hexane	−46.0 (1.0, EtOH)	0.60 0.52	$C_{21}H_{33}N_3O_5$	61.89 (61.71	8.16 8.17	10.31 10.22)
II-4	D-Leu	Ala	NHOBzl	A (89)	—[a]	−13.2 (1.0, EtOH)	0.61 0.60	$C_{21}H_{33}N_3O_5$			
II-5	Phe	Ala	NHOBzl	A (84)	126—127 MeOH–Et_2O	−17.6 (1.0, EtOH)	0.59 0.55	$C_{24}H_{31}N_3O_5$	65.28 (65.15	7.07 7.09	9.51 9.48)
II-6	Leu	Leu	OEt	A (88)	133—134 Et_2O	−49.6 (1.0, EtOH)	0.81 0.78	$C_{19}H_{36}N_2O_5$	61.26 (61.03	9.74 9.80	7.52 7.49)
II-7	D-Leu	D-Ala	OMe	A (95)	105—109 Et_2O–hexane	+43.6 (1.0, EtOH)	0.69 0.72	$C_{15}H_{28}N_2O_5$	56.94 (56.81	8.91 8.95	8.85 8.82)

a) Oily compound: chromatographed on silica gel with $CHCl_3$–MeOH (50 : 1).

TABLE III. Physicochemical and Analytical Data for R_1–Pro–X_2–X_1–R (III)

Compd. No.	Residue R_1	Residue X_2	Residue X_1	Residue R	Puri. proc. (Yield %)	mp (°C) Recryst. solv.	$[\alpha]_D^{25}$ (°) (c, solv.)	Rf^1 Rf^2	Formula	Analysis Calcd (Found) C	H	N
III-2	Boc	Gln	Gly	NHOBzl	B (74)	183—187 MeOH–Et_2O	−31.6 (0.5, DMF)	0.10 0.04	$C_{24}H_{35}N_5O_7$	57.01 (57.23	6.97 6.99	13.85 13.58)
III-3	Boc	Leu	Ala	NHOBzl	B (90)	233—235 AcOEt–hexane	−84.0 (1.0, EtOH)	0.57 0.43	$C_{26}H_{40}N_4O_6$	61.88 (61.85	7.98 7.99	11.1 11.08)
III-4	Boc	D-Leu	Ala	NHOBzl	A (95)	—[a]	−11.1 (1.0, EtOH)	0.59 0.49	$C_{26}H_{40}N_4O_6$			
III-5	Boc	Phe	Ala	NHOBzl	B (66)	218—220 MeOH–Et_2O–hexane	−58.5 (1.0, EtOH)	0.56 0.44	$C_{29}H_{38}N_4O_6$	64.66 (64.69	7.11 7.10	10.4 10.43)
III-7	Bz	D-Leu	Ala	NHOBzl	A (89)	—[b]	+7.2 (1.0, EtOH)	0.58 0.41	$C_{28}H_{36}N_4O_5$			
III-8	Bz	Phe	Ala	NHOBzl	B (72)	200—206 AcOEt	−22.0 (0.5, EtOH)	0.53 0.33	$C_{31}H_{34}N_4O_5$	68.61 (68.69	6.31 6.41	10.32 10.25)
III-9	Z	Leu	Leu	OEt	A (88)	137—139 AcOEt–hexane	−89.6 (0.5, EtOH)	0.67 0.74	$C_{27}H_{41}N_3O_6$	64.39 (64.12	8.2 8.05	8.34 8.33)
III-10	Z	D-Leu	D-Ala	OMe	A (70)	—[b]	+20.6 (0.5, EtOH)	0.69 0.59	$C_{23}H_{33}N_3O_6$			

Oily compound: chromatographed on silica gel with *a*) $CHCl_3$: MeOH (100 : 1) or *b*) $CHCl_3$: MeOH (50 : 1).

TABLE IV. Physicochemical and Analytical Data for R_1–Pro–X_2–X_1–NHOH (IV)

Compd. No.	Residue			mp (°C) Recryst. or reprecipit. solv.	Yield (%)	$[\alpha]_D^{25}$ (c, solv.)	Rf^3 Rf^4	Formula	Analysis Calcd (Found)		
	R_1	X_2	X_1						C	H	N
IV-2	Boc	Gln	Gly	—	68	−50.6	0.12	$C_{17}H_{29}N_5O_7$	49.14	7.03	16.85
				MeOH–Et_2O		(1.0, EtOH)	0.52		(48.80	6.91	16.73)
IV-4	Boc	Leu	Ala	190—205	81	−59.0	0.63	$C_{19}H_{34}N_4O_6$	55.05	8.26	13.51
				MeOH–Et_2O		(1.0, DMF)	0.81		(54.90	8.57	13.43)
IV-6	Bz	Leu	Ala	78—84	87	−134	0.51	$C_{21}H_{30}N_4O_5 \cdot 1/5\,H_2O$	59.75	7.25	13.27
				MeOH–Et_2O		(1.0, EtOH)	0.74		(59.79	7.17	13.18)
IV-7	Boc	D-Leu	Ala	105—111	84	−7.4	0.69	$C_{19}H_{34}N_4O_6 \cdot 1/5\,H_2O$	54.58	8.29	13.40
				MeOH–Et_2O		(1.0, EtOH)	0.83		(54.53	8.30	13.29)
IV-8	Z	D-Leu	Ala	174—179	35	−1.6	0.75	$C_{22}H_{32}N_4O_6 \cdot 1/10\,H_2O$	58.67	7.20	12.44
				AcOEt–Et_2O		(1.0, EtOH)	0.84		(58.58	7.10	12.50)
IV-9	Bz	D-Leu	Ala	133—136	67	+9.2	0.63	$C_{21}H_{30}N_4O_5 \cdot 1/5\,H_2O$	59.75	7.25	13.27
				MeOH–Et_2O		(1.0, EtOH)	0.79		(59.70	7.14	13.22)
IV-10	Boc	Phe	Ala	176—185	90	−46.9	0.67	$C_{22}H_{32}N_4O_6$	58.91	7.19	12.49
				MeOH–Et_2O		(1.0, DMF)	0.74		(58.80	7.23	12.46)
IV-11	Z	Phe	Ala	170—179	31	−48.6	0.71	$C_{25}H_{30}N_4O_6$	62.22	6.26	11.61
				MeOH–Et_2O		(1.0, DMF)	0.81		(62.15	6.39	11.70)
IV-12	Bz	Phe	Ala	120—130	79	−77.7	0.58	$C_{24}H_{28}N_4O_5$	63.70	6.23	12.38
				MeOH–Et_2O		(1.0, EtOH)	0.75		(63.39	6.19	12.27)
IV-14	Z	D-Leu	D-Ala	154—157	73	+4.6	0.69	$C_{22}H_{32}N_4O_6$	58.91	7.19	12.49
				MeOH–AcOEt		(0.5, EtOH)	0.79		(58.85	7.25	12.20)

same manner from (II-4) and (II-5) respectively and the results are shown in Table III.

Boc–Pro–Leu–Gly–NHOH (IV-1) As a typical example of hydroxamic acid synthesis from O-benzylhydroxamic acid, a mixture of Boc–Pro–Leu–Gly–NHOBzl (III-1) (0.50 g, 1.02 mmol) and 5% Pd–C (0.2 g) in MeOH was vigorously stirred for 1 h at room temperature under a hydrogen flow. The catalyst was filtered off and the filtrate was concentrated *in vacuo*. The residue was reprecipitated from MeOH–AcOEt. A white powder (0.33 g, 81%), mp 160—167 °C, $[\alpha]_D^{25}$ −67.1° (c=1.0, EtOH). Rf^3, 0.45; Rf^4, 0.75. *Anal.* Calcd for $C_{18}H_{32}N_4O_6$: C, 53.98; H, 8.05; N, 13.99. Found: C, 53.93; H, 7.90; N, 14.02. Compounds IV-2, 4, 6, 7, 9, 10 and 12 were prepared from III-2, 3, 6, 4, 7, 5 and 8 respectively in the same manner and the listed in Table IV.

Z–Pro–Leu–Leu–NHOH 1 M NH_2OH/MeOH (2 ml) [a solution of HCl·NH_2OH (2.8 g, 40 mmol) in MeOH (25 ml) was added to a solution of 85% KOH (3.69 g, 55.9 mmol) in MeOH (15 ml), and KCl formed were filtered off] was added to Z–Pro–Leu–Leu–OEt (III-9) (0.35 g, 0.69 mmol) at 4 °C. The reaction mixture was stirred for 5 h, and pH of the solution was adjusted to 2 with 1 M HCl, and H_2O (10 ml) was added. The white precipitates produced were collected by filtration (washed with H_2O) and dried over *in vacuo*, followed by reprecipitation from MeOH–ether. A white powder (0.32 g, 94%), mp 162—167 °C, $[\alpha]_D^{25}$ −95.4° (c=0.5, EtOH). Rf^3, 0.76; Rf^4, 0.82. *Anal.* Calcd for $C_{25}H_{38}N_4O_6$: C, 61.2; H, 7.80; N, 11.42. Found: C, 61.05; H, 7.90; N, 11.22. (IV-14) was prepared from (III-10) in the same manner except for reaction time (2 h), and is listed in Table IV.

HCl·Pro–Leu–Gly–NHOH (IV-3) (IV-1) (0.15 g, 0.37 mmol) was treated with 4.2 M HCl/AcOEt for 1 h. The precipitates formed were collected by filtration (washed with AcOEt) and dried in a vacuum desiccator with solid NaOH. A hygroscopic white powder (0.10 g, 79%), $[\alpha]_D^{25}$ −40.1° (c=1.0, EtOH). Rf^5, 0.28. *Anal.* Calcd for $C_{13}H_{25}ClN_4O_4 \cdot H_2O$: C, 44.0; H, 7.66; N, 15.78. Found: C, 43.91; H, 7.67; N, 15.75.

Z–Pro–Leu–Ala–NHOH (IV-5) Boc group elimination of Boc–Pro–Leu–Ala–NHOH was carried out in the same manner as described for the preparation of IV-3. The HCl·Pro–Leu–Ala–NHOH thus formed (0.45 g, 1.28 mmol) was dissolved in DMF, and TEA (0.18 ml, 1.3 mmol) was added at 0 °C. The suspension was added to a solution of Z–N_3 (1.67 mmol, prepared from $ZNHNH_2$[16] according to the method of Honzl and Rudinger[17] with isoamyl nitrite) in DMF at −70 °C, and the reaction mixture was allowed to warm to −10 °C slowly (kept pH at 8) and was stirred overnight at 4 °C. After filtration, the filtrate was concentrated *in vacuo* and the residue was purified by procedure A, followed by reprecipitation from MeOH–ether. A white powder (0.4 g, 70%), mp 156—161 °C, $[\alpha]_D^{25}$ −51.4° (c=1.0, DMF). Rf^3, 0.65; Rf^4, 0.79. *Anal.* Calcd for $C_{22}H_{32}N_4O_6 \cdot 1/3H_2O$: C, 57.37; H, 7.29; N, 12.16. Found: C, 57.24; H, 7.18; N, 12.41. Compounds IV-8 and 11 were prepared from IV-7 and 10, respectively, as described for IV-5 and listed in Table IV.

Enzyme Inhibition Assay Inhibitory activities of tripeptidyl hydroxamic acids against collagenase (tadpole, human skin fibroblast, and bacterial) were assayed by using FITC-labeled collagen as substrate, as reported previously.[18] Inhibitory activity against urease was assayed by measuring pH changes with phenol red due to hydrolysis of urea, as described by K. Kobashi *et al.*[14d] Inhibitory activity against thermolysin was assayed using furylacryloyl–Gly–Leu–NH_2 as substrate, as previously reported by J. Feder.[19]

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