Vertebrate Collagenase Inhibitor. II.[1] Tetrapeptidyl Hydroxamic Acids

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To develop a potent and specific collagenase inhibitor, a series of tetrapeptidyl hydroxamic acids were synthesized, based on the previous findings with tripeptidyl derivatives (*Chem. Pharm. Bull.*, 38, 1007—1011, 1990). Among the series of tetrapeptidyl derivatives synthesized, R-Gly-Pro-Leu-Ala-NHOH and R-Gly-Pro-D-Leu-D-Ala-NHOH were found to be highly specific and potent inhibitors against vertebrate collagenase with an IC_{50} of 10^{-6} M order, where R stands for Boc or acyl group. Analysis of their structure-activity relationships showed a characteristic feature of the substrate-binding site of collagenase as follows: 1) the S_1 subsite forms a shallow hydrophobic pocket, although glycine residue corresponds to the subsite of the natural collagen substrate: 2) the S_2 subsite constitutes a bulky pocket with less requirement for hydrophobicity: 3) the S_3 subsite preferentially accommodates Pro residue: and 4) the accommodation of the P_4–P_1 subsites of peptidyl collagenase inhibitor to the S_4–S_1 subsites is required to form a tight binding of its hydroxamic acid moiety to the zinc ion at the catalytic site of the enzyme. The introduction of an enantiometric dipeptide unit, D-Leu-D-Ala, to the P_2–P_1 subsites demonstrated an increased binding capacity to the extended S_4–S_1 subsites of collagenase, thus providing proteinase-resistant inhibitor.

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

Introduction

The involvement of matrix metalloproteinases, that is, collagenase, gelatinase and stromelysin, in tissue degrading diseases such as rheumatoid arthritis and tumor metastasis has become evident recently.[2] The level of these proteinase activities is regulated in at least three different ways: 1) modulation of transcriptional levels of metalloproteinase genes by cytokines and glucocorticoids, 2) activation of the precursor forms of these enzymes which are latent and 3) inhibition of activated enzymes by tissue inhibitor of metalloproteinases or by synthetic inhibitors.[1,3]

In our previous paper,[1] we reported that tripeptidyl hydroxamic acid with the sequence of Z–Pro–Leu–Ala–NHOH showed a higher inhibitory activity against human skin fibroblast collagenase (IC_{50} value: 10^{-6} M order) than Z–Pro–Leu–Gly–NHOH (IC_{50} value: 10^{-5} M order), which was designed from the amino acid sequence of the collagenase cleavage site of type I collagen molecule and synthetic substrate (Fig. 1).[4,5]

These results strongly suggest that a potent peptidyl inhibitor for collagenase can be designed to recognize only Sn site of the enzyme molecule. This is quite a contrast to previous studies on collagenase inhibitors, which were mostly designed based on P'_n site of collagen cleavage site, –Gly–Ile(Leu)–, where collagenase was supposed to recognize Ile(Leu)residue (P'_1–S'_1 interaction)[6] (Fig. 1).

In this paper, we describe the synthesis of a series of tetrapeptidyl hydroxamic acids and assays of their inhibitory activity against vertebrate collagenase. The relationship between structure and activity of the inhibitors in terms of P*n*–S*n* interaction is also discussed.

Results and Discussion

Tetrapeptidyl hydroxamic acids were synthesized as shown in Chart 1. A Boc-derivative of amino acid was coupled with *O*-benzylhydroxylamine (NH_2-OBzl) by the dicyclohexylcarbodiimide (DCC) –*N*-hydroxybenzotriazole (HOBt) method[7] to obtain its fully protected hydroxamic

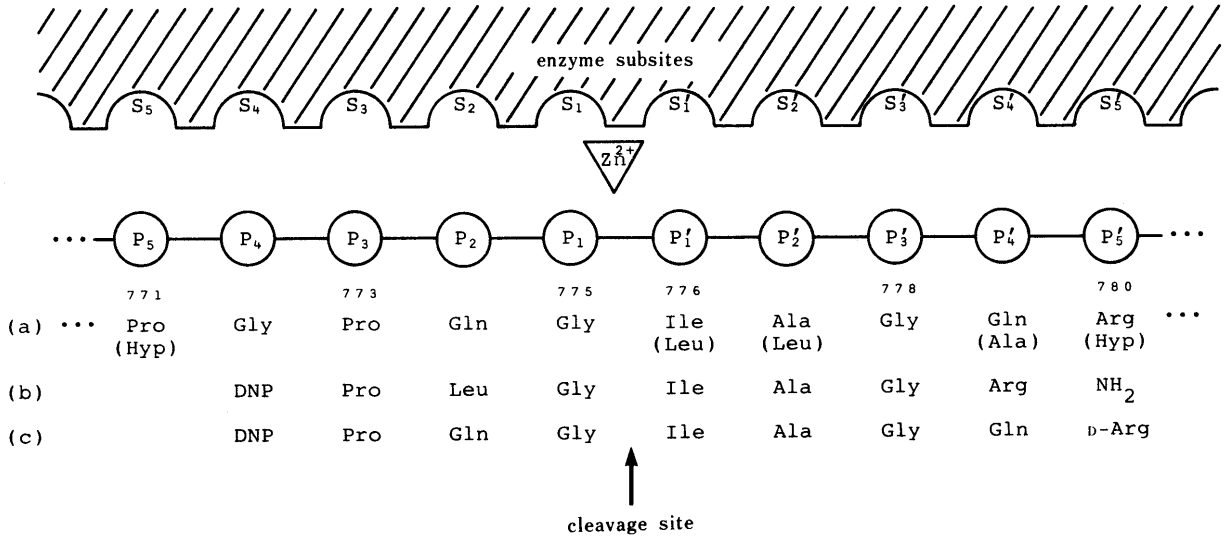

Fig. 1. Schematic Drawing Showing an Expected Interaction between Vertebrate Collagenase and the Cleavage Region of α1 and α2 (in Parentheses) Chains of Bovine Type I Collagen (a) and Synthetic Substrates (b and c) for Vertebrate Collagenase[5]

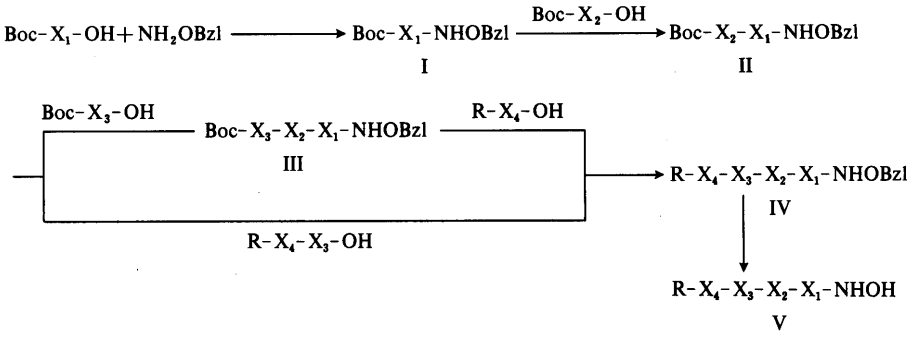

Chart 1. The Synthetic Route to Tetrapeptidyl Hydroxamic Acids

TABLE I. Inhibition (IC_{50}) of Vertebrate Collagenase and Other Enzymes by Hydroxamic Acid Derivatives of a Series of Oligopeptides

Compd. No.	Structure	Tadpole collagenase (M)	Bacterial collagenase (M)	Urease (M)	Thermolysin (M)
1	Boc–Leu–Gly–NHOH	6.8×10^{-4}	9.2×10^{-3}	1.0×10^{-4}	1.3×10^{-2}
2	Boc–Pro–Leu–Gly–NHOH	9.6×10^{-5}	2.0×10^{-2}	3.0×10^{-2}	3.2×10^{-2}
3	Boc–Gly–Pro–Leu–Gly–NHOH	3.3×10^{-5}	3.0×10^{-2}	5.5×10^{-2}	3.4×10^{-2}
4	Boc–Pro–Gly–Pro–Leu–Gly–NHOH	3.9×10^{-5}	1.6×10^{-2}	1.1×10^{-2}	1.6×10^{-2}
5	Boc–Gln–Gly–NHOH	4.1×10^{-4}	1.9×10^{-2}	9.6×10^{-3}	2.0×10^{-2}
6	Boc–Pro–Gln–Gly–NHOH	2.0×10^{-4}	7.0×10^{-2}	8.6×10^{-2}	3.3×10^{-2}
7	Boc–Gly–Pro–Gln–Gly–NHOH	5.6×10^{-5}	5.7×10^{-2}	7.5×10^{-2}	3.9×10^{-2}

acid derivative (I). Then, dipeptide derivative (II) was prepared from (I) by eliminating its Boc group, and coupling with a desired Boc–amino acid (Boc–X_2–OH; functional group(s) in its side chain was protected with Bzl or Z by the DCC–HOBt method). After synthesizing fully protected tri-(III) and then tetrapeptidyl hydroxamic acid (IV) as shown in Chart 1, its Bzl group was eliminated by treatment with hydrogen on Pd–C to give rise to tetrapeptidyl hydroxamic acid (V).

Enzyme inhibitory activities of a series of di- to tetrapeptidyl hydroxamic acids thus synthesized were assayed against tadpole collagenase[8] as reported,[9] and compared with those against clostridial collagenase (Sigma Chem. Co., St. Louis, MO), urease (jack bean, Toyobo Co., Osaka)[10] and thermolysin (Wako Pure Chemical Industries, Osaka)[11] to design vertebrate collagenase-specific inhibitors.

The relationships between primary structure and enzyme inhibitory activity as well as enzyme specificity of the peptidyl hydroxamic acids assayed are summarized as follows:

Peptide Chain Length and Enzyme Inhibitory Activity To obtain the size of peptide giving the maximum inhibitory activity against collagenase with high specificity, peptide length was elongated along the sequence of the P*n* subsite of the collagenase cleavage region of type I collagen and synthetic substrates (see Fig. 1). As shown in Table I, the extension to tetrapeptide covering P_4–P_1 subsites reached a maximum inhibitory activity against collagenase with high specificity, indicating that both P_3–S_3 and P_4–S_4 interactions are indispensable to give a tight binding of collagenase and inhibitor. Based on these findings, Boc–Gly–Pro–Leu–Gly–NHOH was selected as a leading compound in the following experiments. The kinetic analysis of this leading compound against tadpole collagenase by Lineweaver–Burk plot showed the mode of inhibition to be competitive with a K_i value of 7.7 μM ($K_m = 1.6\,\mu$M).

TABLE II. Inhibition (IC_{50}) of Vertebrate Collagenase and Other Enzymes by R–Gly–Pro–Leu–Gly–NHOH

Compd. No.	R	Tadpole collagenase (M)	Bacterial collagenase (M)	Urease (M)	Thermolysin (M)
3	Boc	3.3×10^{-5}	3.0×10^{-2}	5.5×10^{-2}	3.4×10^{-2}
8	H	1.1×10^{-4}	5.0×10^{-2}	1.8×10^{-3}	9.8×10^{-2}
9	CH_3	2.3×10^{-4}	5.4×10^{-2}	9.6×10^{-3}	1.5×10^{-1}
10	Ac	8.6×10^{-5}	3.2×10^{-2}	1.7×10^{-2}	2.9×10^{-1}
11	Bz	2.8×10^{-5}	7.0×10^{-3}	1.4×10^{-3}	3.9×10^{-2}
12	Boc(CH_3)	4.1×10^{-5}	3.1×10^{-2}	3.9×10^{-2}	3.6×10^{-2}

TABLE III. Inhibition (IC_{50}) of Vertebrate Collagenase and Other Enzymes by Boc–Gly–Pro–Leu–X_1–NHOH

Compd. No.	X_1	Tadpole collagenase (M)	Bacterial collagenase (M)	Urease (M)	Thermolysin (M)
3	Gly	3.3×10^{-5}	3.0×10^{-2}	5.5×10^{-2}	3.4×10^{-2}
25	β-Ala	5.2×10^{-3}	2.9×10^{-2}	1.3×10^{-3}	2.0×10^{-3}
26	GABA[a]	1.3×10^{-2}	7.8×10^{-3}	1.3×10^{-3}	1.3×10^{-2}
27	Ala	8.9×10^{-6}	4.9×10^{-2}	$>1.0\times10^{-1}$	3.6×10^{-2}
28	D-Ala	3.6×10^{-4}	7.2×10^{-3}	6.0×10^{-2}	3.7×10^{-2}
29	Val	9.8×10^{-6}	3.4×10^{-2}	$>1.0\times10^{-2}$	1.0×10^{-2}
30	Sar[b]	2.7×10^{-5}	2.1×10^{-3}	7.0×10^{-3}	1.5×10^{-2}

a) γ-Aminobutyric acid. *b*) Sarcosine.

Since the removal of Boc group from tetrapeptidyl derivative showed a marked decrease in collagenase inhibitory activity (compound **8**), and benzoylation of the unmasked amino group resulted in a full return of its inhibitory activity (compound **11**), S_5 subsite of the enzyme is apparently a bulky hydrophobic pocket (Table II). The finding that protection of the amino group with methyl- or

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

Compd. No.	X_2	X_1	Tadpole collagenase (M)	Bacterial collagenase (M)	Urease (M)	Thermolysin (M)
31	Leu	Gly	2.8×10^{-5}	7.0×10^{-3}	1.4×10^{-3}	3.9×10^{-2}
32	Leu	Ala	7.7×10^{-6}	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$
33	D-Leu	D-Ala	3.1×10^{-6}	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$
34	Leu	Leu	2.3×10^{-5}	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$
35	D-Leu	D-Leu	5.3×10^{-6}	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$
36	Leu	Sar	1.1×10^{-5}	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$
37	D-Leu	Sar	1.2×10^{-5}	$>1.0\times10^{-3}$	$>1.0\times10^{-2}$	$>1.0\times10^{-2}$

acetyl-group showed only a partial recovery of collagenase inhibitory activity (compounds **9** and **10**) supports this hypothesis.

P_1–S_1 Interaction To detect the optimum P_1 subsite for tight interaction with enzyme S_1 subsite, X_1 residue in the sequence of Boc–Gly–Pro–Leu–X_1–NHOH was substituted with a variety of amino acids. As shown in Table III, substitution with sarcosine for Gly did not affect its collagenase inhibitory activity (compare compounds **3** and **30**). This suggests that the NH-moiety of Gly at P_1 subsite may not be significantly involved in the interaction with S_1 subsite. However, substitution with β-Ala (compound **25**) or GABA (compound **26**) showed a marked decrease in collagenase inhibitory activity, indicating that the presence of α-amino acid at P_1 subsite is essential for its hydroxamic acid moiety to access Zn ion at the catalytic site of the enzyme.

On the other hand, substitution with Ala (compound **27**) or Val (compound **29**) for Gly at X_1 residue caused an increase in the inhibitory activity against collagenase without impairing enzyme specificity. This strongly suggests that S_1 subsite constitutes a shallow hydrophobic pocket, since introduction of Leu to P_1 subsite resulted in the reduction of collagenase inhibitory activity (Table IV).

P_2–S_2 and P_3–S_3 Interactions Table V shows that S_2 subsite seems to be a bulky pocket but is less strict in the requirement for hydrophobicity, since X_2 residue corresponding to P_2 subsite can be substituted with D-Leu, Gln, Glu, Ile or even with Phe for Leu without impairing enzyme specificity. However, some charge effect seems to be involved in the interaction between P_2 subsite and S_2 subsite, since the reduction of collagenase inhibitory activity was observed with compound **23** containing Lys at P_2 subsite. The findings that substitution of X_2 residue with Gly or Ser resulted in a marked decrease in their collagenase inhibitory activity strongly suggest that tight interaction of P_2 subsite with bulky S_2 subsite is essential for the hydroxamic acid moiety to access Zn ion in the enzyme.

Of greatest importance in this study was the finding that S_3 subsite preferentially accommodates Pro residue as P_3 subsite. Neither Hyp nor D-Pro residue can be employed as a substitute for Pro (Table VI). Substitution with Ala residue showed a comparable inhibitory activity to that of the leading compound, but became less specific to collagenase. A marked decrease in collagenase inhibitory activity of Boc–Gly–Pro–Pro–Gly–NHOH (compound **24** in Table V) indicates that S_3–S_1 subsites require an extended structure of P_3–P_1 subsites to accommodate.

Configuration Requirement for P_2–P_1 Subsite Since collagenase inhibitors designed from the sequence of P*n*

TABLE V. Inhibition (IC_{50}) of Vertebrate Collagenase and Other Enzymes by Boc–Gly–Pro–X_2–Gly–NHOH

Compd. No.	X_2	Tadpole collagenase (M)	Bacterial collagenase (M)	Urease (M)	Thermolysin (M)
3	Leu	3.3×10^{-5}	3.0×10^{-2}	5.5×10^{-2}	3.4×10^{-2}
17	D-Leu	1.2×10^{-5}	5.7×10^{-2}	$>1.0\times10^{-2}$	2.4×10^{-3}
7	Gln	5.6×10^{-5}	5.7×10^{-2}	7.5×10^{-2}	3.9×10^{-2}
18	Glu	5.8×10^{-5}	8.0×10^{-3}	7.6×10^{-2}	1.5×10^{-2}
19	Gly	$>1.0\times10^{-3}$	$>1.0\times10^{-2}$	3.4×10^{-2}	4.8×10^{-3}
20	Ile	2.4×10^{-5}	6.9×10^{-3}	5.2×10^{-4}	6.8×10^{-3}
21	Phe	9.7×10^{-5}	1.1×10^{-2}	1.4×10^{-3}	7.3×10^{-3}
22	Ser	2.4×10^{-4}	5.2×10^{-2}	7.8×10^{-3}	2.7×10^{-2}
23	Lys	5.1×10^{-4}	2.6×10^{-2}	N.D.	2.5×10^{-2}
24	Pro	$>1.0\times10^{-3}$	1.4×10^{-2}	1.0×10^{-2}	1.7×10^{-2}

N.D.: not determined.

TABLE VI. Inhibition (IC_{50}) of Vertebrate Collagenase and Other Enzymes by Boc–Gly–X_3–Leu–Gly–NHOH

Compd. No.	X_3	Tadpole collagenase (M)	Bacterial collagenase (M)	Urease (M)	Thermolysin (M)
3	Pro	3.3×10^{-5}	3.0×10^{-2}	5.5×10^{-2}	3.4×10^{-2}
13	Hyp	3.8×10^{-4}	6.2×10^{-3}	4.4×10^{-4}	1.2×10^{-2}
14	Gly	3.4×10^{-4}	5.3×10^{-3}	1.8×10^{-4}	8.3×10^{-3}
15	Ala	5.0×10^{-5}	5.6×10^{-3}	6.3×10^{-4}	8.5×10^{-3}
16	D-Pro	5.8×10^{-4}	8.2×10^{-3}	6.5×10^{-3}	2.4×10^{-2}

subsites at the cleavage site of type I collagen require at least tetrapeptidyl derivative to give maximum inhibitory activity, introduction of D-form of amino acids to P_2–P_1 subsites was attempted to develop a proteinase-resistant collagenase inhibitor. To our surprise, introduction of D-Leu–D-Ala (compound **33** in Table IV), an enantiomeric dipeptide unit against L-Leu–L-Ala, to P_2–P_1 subsites showed collagenase inhibitory activity as high as that of its parent inhibitor carrying L-Leu–L-Ala (compound **32** in Table IV), although substitution with D-Leu–L-Ala[1] or L-Leu–D-Ala, a diastereomeric dipeptide unit (compound **28** in Table III), to the same subsites resulted in a marked decrease in the inhibitory activity. This again indicates that S_2–S_1 subsites of vertebrate collagenase constitute an extended bulky pocket to accommodate enantiomeric Leu–Ala dipeptide unit. Bz–Gly–Pro–D-Leu–D-Ala–NHOH thus prepared showed little reduction in collagenase inhibitory activity even after overnight incubation with pronase, trypsin or leukocyte elastase at pH 7.5 and 37 °C, while the inhibitory activity of its parent compound containing L-Leu–L-Ala decreased with incubation time

under the same conditions (data not shown).

Experimental

General experimental procedures used in this paper are the same as described in the previous paper.[1] The coupling reactions using DCC–HOBt and their post-treatments were performed as follows. DCC solution in CH_2Cl_2 was added at −15 °C to a dimethylformamide (DMF) solution containing both the components to be coupled and HOBt and the reaction mixture was stirred at 5 °C overnight. After removal of DC urea by filtration, the filtrate was evaporated *in vacuo*. The residue was dissolved in AcOEt and washed with 1 N HCl, H_2O, 10% Na_2CO_3 and finally with H_2O, then dried over $MgSO_4$ and evaporated *in vacuo*. The resulting residue was purified by chromatography on silica gel and/or by recrystallization from appropriate solvents.

All catalytic hydrogenations of *O*-benzyl ethers were performed in MeOH in the presence of 5% Pd–C at atmospheric pressure.

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 (80:10:5, v/v); Rf^2, *n*-BuOH–AcOH–H_2O (4:1:1, v/v); Rf^3, $CHCl_3$–MeOH (14:1, v/v); Rf^4, $CHCl_3$–MeOH–AcOH (95:5:3, v/v) and Rf^5, *n*-BuOH–AcOH–H_2O (4:2:1, v/v).

FITC-labeled collagen (bovine type I collagen, K-21) was obtained from Cosmo Bio (Tokyo). Bacterial collagenase (collagenase-Sterile, type IA-S, *Clostridium histolyticum*), and furylacryloyl–Gly–Leu–NH_2 was purchased from Sigma Chemical Co. (St. Louis, MO). Urease (jack bean, 100 U/mg grade) was from Toyobo Co. (Osaka). Thermolysin (lyophilized, 7000 U/mg, *Bacillus thermoproteolyticus* ROKKO) from Wako Pure Chemical Industries (Osaka) and urea from Nakarai Chemicals (Kyoto).

Boc–Leu–Gly–NHOH (**1**), Boc–Pro–Leu–Gly–NHOH (**2**), Bod–Gln–Gly–NHOH (**5**) and Boc–Pro–Gln–Gly–NHOH (**6**) were prepared as described in the previous paper.[1]

Boc–Gly–Pro–Leu–Gly–NHOH (3) As a typical example of tetrapeptidyl hydroxamic acid synthesis, Boc–Pro–Leu–Gly–NHOBzl[1] (9.87 g, 20.1 mmol) was treated with 4.2 N HCl/AcOEt (50 ml) at room temperature for 1 h and evaporated *in vacuo*, and further dried in a vacuum desiccator with solid NaOH. The residue was coupled with Boc–Gly–OH (3.36 g, 19.2 mmol) by triethylamine (TEA, 4.22 ml, 30.2 mmol), DCC (5.16 g, 25.0 mmol) and HOBt (2.60 g, 19.2 mmol). The product was purified by chromatography on a silica gel with AcOEt to give Boc–Gly–Pro–Leu–Gly–NHOBzl (5.30 g, 50%) as colorless oil. $[\alpha]_D^{25}$ −68.6° (c=1.0, EtOH), Rf^1, 0.72; Rf^3, 0.37; single ninhydrin-positive spot.

Catalytic hydrogenation of Boc–Gly–Pro–Leu–Gly–NHOBzl (5.30 g, 9.68 mmol) by the conventional procedure gave compound **3** (3.71 g, 84%) as a white powder from MeOH–Et_2O. mp 90—104 °C, $[\alpha]_D^{25}$ −84.3° (c=1.0, EtOH), Rf^1, 0.34; Rf^2, 0.67; single ninhydrin-positive spot. *Anal.* Calcd for $C_{20}H_{35}N_5O_7$: C, 52.5; H, 7.71; N, 15.3. Found: C, 52.45; H, 7.53; N, 15.11. Compounds **7** and **13** were prepared in the same manner from Boc–Pro–Gln–Gly–NHOBzl[1] and Boc–Pro–Leu–Gly–NHOBzl[1] respectively.

7: A hygroscopic white power. $[\alpha]_D^{25}$ −67.1° (c=1.0, EtOH), Rf^1, 0.08; $Rf^2$0.43; single ninhydrin-positive spot. *Anal.* Calcd for $C_{19}H_{32}N_6O_8$: C, 48.29; H, 6.82; N, 17.78. Found: C, 48.55; H, 7.01; N, 17.5.

12: A white powder from MeOH–Et_2O. mp 94—99 °C, $[\alpha]_D^{25}$ −80.6° (c=1.0, EtOH), Rf^1, 0.42; Rf^2, 0.66; single ninhydrin-positive spot. *Anal.* Calcd for $C_{21}H_{37}N_5O_7 \cdot 1/2H_2O$: C, 52.48; H, 7.96; N, 14.57. Found: C, 52.63; H, 7.81; N, 14.55.

Boc–Pro–Gly–Pro–Leu–Gly–NHOH (4) Boc–Pro–Gly–Pro–Leu–Gly–NHOBzl was prepared from Boc–Gly–Pro–Leu–Gly–NHOBzl (3.0 g, 5.48 mmol) was Boc–Pro–OH (1.10 g, 5.11 mmol) as described for the preparation of Boc–Gly–Pro–Leu–Gly–NHOBzl. The product was purified by chromatography on a silica gel with $CHCl_3$–MeOH (15:1, v/v) to give Boc–Pro–Gly–Pro–Leu–Gly–NHOBzl (2.70 g, 82%) as colorless oil, $[\alpha]_D^{25}$ −106° (c=1.0, EtOH), Rf^1, 0.70; Rf^3, 0.29; single ninhydrin-positive spot.

Catalytic hydrogenation of Boc–Pro–Gly–Pro–Leu–Gly–NHOBzl (0.30 g, 0.46 mmol) by the conventional procedure gave compound **4** (0.19 g, 74%) as a white powder from MeOH–Et_2O. mp 115—120 °C, $[\alpha]_D^{25}$ −120.8° (c=0.5, EtOH), Rf^1, 0.31; Rf^2, 0.52; single ninhydrin-positive spot. *Anal.* Calcd for $C_{25}H_{42}N_6O_8 \cdot H_2O$: C, 52.43, H, 7.74, N, 14.67. Found: C, 52.23; H, 7.48; N, 14.67.

H–Gly–Pro–Leu–Gly–NHOH AcOH (8) As a typical example of tetrapeptide synthesis, compound **3** (100 mg, 0.22 mmol) was treated with 4.2 N HCl/AcOEt at room temperature for 1 h and concentrated *in vacuo*. The residue was passed through a PA-308 anion exchange column (AcO^- form) and purified by partition chromatography (Sephadex G-25, *n*-BuOH : AcOH : H_2O = 4 : 1 : 5). The product thus obtained was lyophilized to give compound **8** (50 mg, 55%) as a hygroscopic white powder. $[\alpha]_D^{25}$ −87.2° (c=0.5, EtOH), Rf^5, 0.24; single ninhydrin-positive spot. *Anal.* Calcd for $C_{17}H_{31}N_5O_7 \cdot 3/2H_2O$: C, 45.93; H, 7.70; N, 15.75. Found: C, 45.89; H, 7.62; N, 15.70. Compound **9** was prepared in the same manner from compound **12**.

9: A hygroscopic white powder. $[\alpha]_D^{25}$ −79.1° (c=1.0, EtOH), Rf^2, 0.19; Rf^5, 0.24; single ninhydrin-positive spot. *Anal.* Calcd for $C_{18}H_{33}N_5O_7 \cdot H_2O$: C, 48.09; H, 7.84; N, 15.58. Found: C, 48.01; H, 7.85; N, 15.28.

Ac–Gly–Pro–Leu–Gly–NHOH (10) The Boc- group of Boc–Gly–Pro–Leu–Gly–NHOBzl (0.55 g, 1.0 mmol) was removed by treatment with 4.2 N HCl/AcOEt. TEA (0.20 ml, 1.43 mmol), HOBt (0.14 g, 1.04 mmol) and AcOSu (0.19 g, 1.2 mmol) were added to 0 °C to a DMF solution of the HCl salt thus obtained and the reaction mixture was stirred for 1 h at room temperature and concentrated *in vacuo*. The residue was dissolved in AcOEt, and washed with 1 N HCl, H_2O, 10% Na_2CO_3 and finally with H_2O. The organic layer was dried over $MgSO_4$ and evaporated *in vacuo*. The residue was purified by chromatography on a silica gel with $CHCl_3$–MeOH (20:1, v/v), followed by recrystallization from AcOEt–*n*-Hexane to give Ac–Gly–Pro–Leu–Gly–NHOBzl (0.32 g, 65%) as a colorless crystal. mp 105—109 °C, $[\alpha]_D^{25}$ −76.1° (c=1.0, EtOH), Rf^1, 0.48; Rf^3, 0.13; single ninhydrin-positive spot. *Anal.* Calcd for $C_{24}H_{35}N_5O_6$: C, 58.88; H, 7.20; N, 14.30. Found: C, 58.82; H, 7.34; N, 14.11.

Catalytic hydrogenation of Ac–Gly–Pro–Leu–Gly–NHOBzl (0.28 g, 0.57 mmol) by the conventional procedure gave compound **10** (0.21 g, 92%) as a white powder from MeOH–Et_2O. mp 90—115 °C, $[\alpha]_D^{25}$ −91.3° (c=1.0, EtOH), Rf^1, 0.13; Rf^2, 0.43; single ninhydrin-positive spot. *Anal.* Calcd for $C_{17}H_{29}N_5O_6 \cdot 1/2H_2O$: C, 49.99; H, 7.40; N, 17.14. Found: C, 49.98; H, 7.34; N, 16.87. Compound **11** was prepared in the same manner from Boc–Gly–Pro–Leu–Gly–NHOBzl using Bz–Cl instead of AcOSu.

11: A white powder from MeOH–Et_2O. mp 110—123 °C, $[\alpha]_D^{25}$ −77.4° (c=1.0, EtOH), Rf^1, 0.23; Rf^2, 0.60; single ninhydrin-positive spot. *Anal.* Calcd for $C_{22}H_{31}N_5O_6$: C, 57.25; H, 6.77; N, 15.17. Found: C, 57.25; H, 6.79; N, 15.24.

Boc–Gly–Hyp–Leu–Gly–NHOH (13) Boc–Hyp–Leu–Gly–NHOBzl was prepared from Boc–Leu–Gly–NHOBzl[1] (3.89 g, 9.89 mmol) and Boc–Hyp–OH (1.99 g, 8.60 mmol) as described for the preparation of Boc–Pro–Leu–Gly–NHOBzl.[1] The product was purified by recrystallization from AcOEt to give Boc–Hyp–Leu–Gly–NHOBzl (2.45 g, 56%) as colorless crystals. mp 168—173 °C, $[\alpha]_D^{25}$ −50.8° (c=1.0, EtOH), Rf^3, 0.40; Rf^4, 0.19; single ninhydrin-positive spot. *Anal.* Calcd for $C_{25}H_{38}N_4O_7$: C, 59.27; H, 7.56; N, 11.05. Found: C, 59.16, H; 7.66; N, 10.93.

Boc–Gly–Hyp–Leu–Gly–NHOBzl was prepared from Boc–Hyp–Leu–Gly–NHOBzl (2.0 g, 3.95 mmol) and Boc–Gly–OSu (1.27 g, 4.35 mmol) in the same manner as described for the preparation of Ac–Gly–Pro–Leu–Gly–NHOBzl. The product was purified by chromatography on a silica gel with $CHCl_3$–MeOH (30:1, v/v) to give Boc–Gly–Hyp–Leu–Gly–NHOBzl (1.50 g, 67%) as colorless oil. $[\alpha]_D^{25}$ −55.5° (c=1.0, EtOH), Rf^3, 0.37; Rf^4, 0.13; single ninhydrin-positive spot.

Catalytic hydrogenation of Boc–Gly–Hyp–Leu–Gly–NHOBzl (0.75 g, 1.33 mmol) by the conventional procedure gave compound **13** (0.60 g, 95%) as a white powder from MeOH–Et_2O. mp 178—183 °C, $[\alpha]_D^{25}$ −73.8° (c=1.0, EtOH), Rf^1, 0.10; Rf^2, 0.51; single ninhydrin-positive spot. *Anal.* Calcd for $C_{20}H_{35}N_5O_8 \cdot H_2O$: C, 48.87; H, 7.58; N, 14.24. Found: C, 48.82; H, 7.63; N, 14.28. Compounds **14**, **15** and **16** were prepared in the same manner from Boc–Gly–Leu–Gly–NHOBzl, Boc–Ala–Leu–Gly–NHOBzl and Boc–D-Pro–Leu–Gly–NHOBzl, respectively.

14: A white powder, mp 120—129 °C, $[\alpha]_D^{25}$ −12.5° (c=1.0, EtOH), Rf^1, 0.18; Rf^2, 0.65; single ninhydrin-positive spot. *Anal.* Calcd for $C_{17}H_{31}N_5O_7 \cdot 1/2H_2O$: C, 47.87; H, 7.56; N, 16.42. Found: C, 47.72; H, 7.57; N, 16.45.

15: A white powder, mp 148—152 °C, $[\alpha]_D^{25}$ −42.2° (c=1.0, EtOH), Rf^1, 0.24; Rf^2, 0.67; single ninhydrin-positive spot. *Anal.* Calcd for $C_{18}H_{33}N_5O_7$: C, 50.10; H, 7.70; N, 16.23. Found: C, 50.20; H, 7.65; N, 16.11.

16: A white powder, mp 98—105 °C, $[\alpha]_D^{25}$ +18.4° (c=1.0, EtOH), Rf^1, 0.55; Rf^2, 0.70; single ninhydrin-positive spot. *Anal.* Calcd for $C_{20}H_{35}N_5O_7$: C, 52.50; H, 7.71; N, 15.30. Found: C, 52.55; H, 7.63; N, 15.28.

Boc–Gly–Pro–D-Leu–Gly–NHOH (17) As a typical example of tetrapeptide synthesis containing D-amino acid, Boc–D-Leu–Gly–NHOBzl

was prepared from Boc-D-Leu-OH·H_2O (2.53 g, 10.1 mmol) and Boc-Gly-NHOBzl (3.36 g, 12.0 mmol) as described for the preparation of Boc-Leu-Gly-NHOBzl.[1] The product was purified by chromatography on a silica gel with $CHCl_3$–MeOH (50:1, v/v) to give Boc-D-Leu-Gly-NHOBzl (3.56 g, 90%) as colorless oil. $[\alpha]_D^{25}$ +7.8° (c=1.0, EtOH), Rf^4, 0.56, single ninhydrin-positive spot.

Boc-Gly-Pro-D-Leu-Gly-NHOBzl was prepared from Boc-D-Leu-Gly-NHOBzl (2.26 g, 5.74 mmol) and Boc-Gly-Pro-OH (1.42 g, 5.22 mmol) as described for the preparation of Boc-Gly-Pro-Leu-Gly-NHOBzl. The product was purified by chromatography on a silica gel with $CHCl_3$–MeOH (50:1, v/v), followed by reprecipitation from AcOEt to give Boc-Gly-Pro-D-Leu-Gly-NHOBzl (1.1 g, 39%) as a hydroscopic white powder. $[\alpha]_D^{25}$ −6.2° (c=1.0, EtOH), Rf^3, 0.18; Rf^4, 0.25; single ninhydrin-positive spot. *Anal.* Calcd for $C_{27}H_{41}N_5O_7$: C, 59.21: H, 7.54; N, 12.78. Found: C, 59.05; H, 7.46; N, 12.84.

Catalytic hydrogenation of Boc-Gly-Pro-D-Leu-Gly-NHOBzl (0.56 g, 1.02 mmol) by the conventional procedure gave compound **17** (0.30 g, 64%) as a white powder from AcOEt–*n*-Hexane. mp 90—100 °C, $[\alpha]_D^{25}$ −20.3° (c=1.0, EtOH), Rf^1, 0.50; Rf^2, 0.73; single ninhydrin-positive spot. *Anal.* Calcd for $C_{20}H_{35}N_5O_7 \cdot 1/3H_2O$: C, 51.82; H, 7.75; N, 15.11. Found: C, 52.01; H, 7.64; N, 14.78. Compounds **18**—**24** were prepared in the same manner from the corresponding dipeptide derivatives and Boc-Gly-Pro-OH, respectively.

18: A white powder. mp 90—100 °C, $[\alpha]_D^{25}$ −64.9° (c=1.0, EtOH), Rf^1, 0.12; Rf^2, 0.49; single ninhydrin-positive spot. *Anal.* Calcd for $C_{19}H_{31}N_5O_9 \cdot 1/2H_2O$: C, 47.29; H, 6.65; N, 14.51. Found: C, 47.10, H, 6.62; N, 14.48.

19: A white powder. mp 178—180 °C, $[\alpha]_D^{25}$ −52.3° (c=1.0, DMF), Rf^1, 0.16; Rf^2, 0.44; single ninhydrin-positive spot. *Anal.* Calcd for $C_{16}H_{27}N_5O_7$: C, 47.87; H, 6.77; N, 17.44. Found: C, 47.79; H, 6.71; N, 17.54.

20: A white powder. mp 123—129 °C, $[\alpha]_D^{25}$ −78.3° (c=1.0, EtOH), Rf^1, 0.37; Rf^2, 0.64; single ninhydrin-positive spot. *Anal.* Calcd for $C_{20}H_{35}N_5O_7 \cdot 1/3H_2O$: C, 51.82; H, 7.75; N, 15.11. Found: C, 51.70; H, 7.57; N, 15.18.

21: A white powder. mp 166—171 °C, $[\alpha]_D^{25}$ −89.7° (c=1.0, MeOH), Rf^1, 0.44; Rf^2, 0.71; single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{33}N_5O_7$: C, 56.20; H, 6.76; N, 14.24. Found: C, 56.19; H, 6.71; N, 14.31.

22: A white powder. mp 92—105 °C, $[\alpha]_D^{25}$ −72.1° (c=1.0, EtOH), Rf^1, 0.12; Rf^2, 0.45; single ninhydrin-positive spot. *Anal.* Calcd for $C_{17}H_{29}N_5O_8 \cdot 1/3H_2O$: C, 46.68; H, 6.83; N, 16.01. Found: C, 46.61; H, 6.91; N, 15.78.

23: A hygroscopic white powder (purified by partition chromatography as described for compound **8**. $[\alpha]_D^{25}$ −56.1° (c=1.0, EtOH), Rf^2, 0.25; single ninhydrin-positive spot. *Anal.* Calcd for $C_{20}H_{36}N_6O_6 \cdot CH_3CO_2H \cdot 1/2H_2O$: C, 50.27; H, 7.86; N, 15.98. Found: C, 50.11; H, 7.86; N, 15.78.

24: A hygroscopic white powder. $[\alpha]_D^{25}$ −98.1° (c=1.0, EtOH), Rf^1, 0.31; Rf^2, 0.46; single ninhydrin-positive spot. *Anal.* Calcd for $C_{19}H_{31}N_5O_7$: C, 51.69; H, 7.07; N, 15.86. Found: C, 51.51; H, 7.26; N, 15.65.

Boc-Gly-Pro-Leu-β-Ala-NHON (25) Boc-β-Ala-NHOBzl was prepared from Boc-β-Ala-OH (9.46 g, 50 mmol) and HCl·NH_2OBzl (12 g, 75 mmol) as described for the preparation of Boc-Gly-NHOBzl.[1] The product was purified by recrystallization from AcOEt to give Boc-β-Ala-NHOBzl (12.5 g, 85%) as a colorless crystal. mp 102—103 °C, Rf^3, 0.57; Rf^4, 0.80; single ninhydrin-positive spot. *Anal.* Calcd for $C_{15}H_{22}N_2O_4$: C, 61.20; H, 7.53; N, 9.51. Found: C, 61.01; H, 7.55; N, 9.49.

Boc-Leu-β-Ala-NHOBzl was prepared from Boc-β-Ala-NHOBzl (4.42 g, 15.0 mmol) and Boc-Leu-OH (3.15 g, 13.6 mmol) as described for the preparation of Boc-D-Leu-Gly-NHOBzl. The product was purified by chromatography on a silica gel with AcOEt–*n*-Hexane (5:1, v/v) to give Boc-Leu-β-Ala-NHOBzl (5.49 g, 99%) as colorless oil. $[\alpha]_D^{25}$ −17.8° (c=1.0, EtOH), Rf^1, 0.80; Rf^3, 0.53; single ninhydrin-positive spot.

Boc-Gly-Pro-Leu-β-Ala-NHOBzl was prepared from Boc-Leu-β-Ala-NHOBzl (4.07 g, 10 mmol) and Boc-Gly-Pro-OH (2.46 g, 10 mmol) as described for the preparation of Boc-Gly-Pro-D-Leu-Gly-NHOBzl. The product was purified by chromatography on a silica gel with $CHCl_3$–MeOH (25:1, v/v) to give Boc-Gly-Pro-Leu-β-Ala-NHOBzl (3.98 g, 71%) as colorless oil. $[\alpha]_D^{25}$ −60.1° (c=1.0, EtOH), Rf^1, 0.73; Rf^3, 0.33; single ninhydrin-positive spot.

Catalytic hydrogenation of Boc-Gly-Pro-Leu-β-Ala-NHOBzl (0.75 g, 1.34 mmol) by the conventional procedure gave compound **25** (0.49 g, 78%) as a white powder from MeOH–Et_2O. mp 100–112 °C, $[\alpha]_D^{25}$ −70.2° (c=1.0, EtOH), Rf^1, 0.38; Rf^2, 0.64; single ninhydrin-positive spot. *Anal.* Calcd for $C_{21}H_{37}N_5O_7$: C, 53.48; H, 7.90; N, 14.85. Found: C, 53.78; H, 8.08; N, 14.56. Compounds **26**—**29** were prepared in the same manner from the corresponding dipeptide derivatives, respectively.

26: A white powder. mp 80—93 °C, $[\alpha]_D^{25}$ −56.9° (c=1.0, EtOH), Rf^1, 0.38; Rf^2, 0.64; single ninhydrin-positive spot. *Anal.* Calcd for $C_{22}H_{39}N_5O_7 \cdot 1/2H_2O$: C, 53.42; H, 8.15; N, 14.16. Found: C, 53.38; H, 8.31; N, 13.87.

27: A white powder. mp 110—118 °C, $[\alpha]_D^{25}$ −85.0° (c=1.0, EtOH), Rf^1, 0.39; Rf^2, 0.67; single ninhydrin-positive spot. *Anal.* Calcd for $C_{21}H_{37}N_5O_7 \cdot 1/2H_2O$: C, 52.48; H, 7.96; N, 14.57. Found: C, 52.77; H, 7.72; N, 14.71.

28: A white powder. mp 110—120 °C, $[\alpha]_D^{25}$ −89.3° (c=1.0, EtOH), Rf^1, 0.55; Rf^2, 0.70; single ninhydrin-positive spot. *Anal.* Calcd for $C_{21}H_{37}N_5O_7 \cdot 2/5H_2O$: C, 52.68; H, 7.95; N, 14.62. Found: C, 52.80; H, 7.83; N, 14.54.

29: A white powder. mp 116—123 °C, $[\alpha]_D^{25}$ −113.6° (c=0.5, EtOH), Rf^1, 0.53; Rf^2, 0.73; single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{41}N_5O_7 \cdot 1/2H_2O$: C, 54.31; H, 8.32; N, 13.76. Found: C, 54.31, H, 8.04; N, 13.76.

Bz-Gly-Pro-Leu-Ala-NHOH (32) As a typical example of Bz-tetrapeptide derivative synthesis, Bz-Gly-Pro-Leu-Ala-NHOBzl was prepared from Boc-Leu-Ala-NHOBzl[1] (4.89 g, 12.0 mmol) and Bz-Gly-Pro-OH (2.76 g, 10.0 mmol) as described for the preparation of Boc-Gly-Pro-Leu-Gly-NHOBzl. The product was purified by chromatography on a silica gel with $CHCl_3$–MeOH (50:1, v/v) to give Bz-Gly-Pro-Leu-Ala-NHOBzl (4.63 g, 82%) as colorless oil. $[\alpha]_D^{25}$ −73.2° (c=1.0, EtOH), Rf^1, 0.72; Rf^3, 0.22; single ninhydrin-positive spot.

Catalytic hydrogenation of Bz-Gly-Pro-Leu-Ala-NHOBzl (0.57 g, 1.0 mmol) by the conventional procedure gave compound **32** (0.36 g, 75%) as a white powder from MeOH–Et_2O. mp 202—204 °C, $[\alpha]_D^{25}$ −71.7° (c=1.0, DMF), Rf^1, 0.78; Rf^2, 0.75; single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{33}N_5O_6 \cdot 1/2H_2O$: C, 57.01; H, 7.07; N, 14.45. Found: C, 57.14; H, 6.99; N, 14.72. Compounds **33**—**35** were prepared in the same manner from the corresponding dipeptide derivatives and Bz-Gly-Pro-OH, respectively.

33: Colorless needles from MeOH. mp 172—174 °C, $[\alpha]_D^{25}$ +10.8 (c=1.0, DMF), Rf^1, 0.63; Rf^2, 0.70; single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{33}N_5O_6 \cdot 2/3H_2O$: C, 54.96; H, 7.21; N, 13.93. Found: C, 55.04; H, 7.07; N, 13.86.

34: A white powder. mp 130—135 °C, $[\alpha]_D^{25}$ −74.0° (c=0.5, EtOH), Rf^1, 0.35; Rf^2, 0.66; single ninhydrin-positive spot. *Anal.* Calcd for $C_{26}H_{39}N_5O_6$: C, 60.33; H, 7.59; N, 13.52. Found: C, 60.38; H, 7.42; N, 13.28.

35: A white powder (from MeOH–Et_2O). mp 187—190 °C, $[\alpha]_D^{25}$ +25.6° (c=0.5, EtOH), Rf^1, 0.69; Rf^2, 0.74; single ninhydrin-positive spot. *Anal.* Calcd for $C_{26}H_{39}N_5O_6$: C, 60.33; H, 7.59; N, 13.52. Found: C, 60.21; H, 7.65; N, 13.34.

Boc-Gly-Pro-Leu-Sar-NHOH (30) As a typical example of Sar-containing tetrapeptide synthesis, Boc-Sar-NHOBzl was prepared from Boc-Sar-OH (4.73 g, 25 mmol) and HCl·NH_2OBzl (6.0 g, 37.5 mmol) as described for the preparation of Boc-Gly-NHOBzl.[1] The product was purified by recrystallization from AcOEt–*n*-Hexane to give Boc-Sar-NHOBzl (6.62 g, 90%) as a colorless crystal. mp 102—103 °C, Rf^3, 0.85; Rf^4, 0.69; single ninhydrin-positive spot. *Anal.* Calcd for $C_{15}H_{22}N_2O_4$: C, 61.2; H, 7.53; N, 9.51. Found: C, 61.11; H, 7.55; N, 9.36.

Boc-Gly-Pro-Leu-Sar-NHOBzl was prepared from Boc-Sar-NHOBzl (0.60 g, 2.04 mmol) and Boc-Gly-Pro-Leu-OH (0.70 g, 1.8 mmol) as described for the preparation of Boc-Gly-Pro-Leu-Gly-NHOBzl. The product was purified by chromatography on a silica gel with $CHCl_3$–MeOH (20:1, v/v) to give Boc-Gly-Pro-Leu-Sar-NHOBzl (0.75 g, 77%) as a hygroscopic white powder. $[\alpha]_D^{25}$ −67.0° (c=1.0, EtOH), Rf^3, 0.44; Rf^4, 0.49; single ninhydrin-positive spot.

Catalytic hydrogenation of Boc-Gly-Pro-Leu-Sar-NHOBzl (0.36 g, 0.64 mmol) was performed by the conventional procedure. The product was purified by chromatography on a silica gel with $CHCl_3$–MeOH (30:1, v/v), followed by reprecipitation from MeOH–Et_2O to give compound **30** (75 mg, 25%) as a hygroscopic white powder. $[\alpha]_D^{25}$ −79.8° (c=1.0, EtOH), Rf^1, 0.35; Rf^2, 0.54; single ninhydrin-positive spot. *Anal.* Calcd for $C_{21}H_{37}N_5O_7 \cdot 1/2H_2O$: C, 52.48; H, 7.96; N, 14.57. Found: C, 52.55; H, 7.96; N, 14.30. Compounds **36** and **37** were prepared in the same manner from the corresponding Sar-containing dipeptide derivatives, respectively.

36: A pale yellow powder. mp 102—110 °C, $[\alpha]_D^{25}$ −94.5° (c=1.0,

EtOH), Rf^1, 0.44; Rf^2, 0.58; single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{33}N_5O_6 \cdot 1/2H_2O$: C, 57.01; H, 7.07; N, 14.45. Found: C, 57.20; H, 7.04; N, 14.49.

37: A colorless powder. mp 110—119 °C, $[\alpha]_D^{25}$ −36.9° ($c=1.0$, DMF), Rf^1, 0.49; Rf^2, 0.60; single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{33}N_5O_6 \cdot 1/2H_2O$: C, 57.01; H, 7.07; N, 14.45. Found: C, 57.23; H, 7.10; N, 14.31.

Enzyme Inhibition Assay Inhibitory activities of tetrapeptidyl hydroxamic acids against collagenase (tadpole and bacterial) were assayed using FITC-labeled collagen as substrate, as reported previously.[9] 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.*[10] Inhibitory activity against thermolysin was assayed using furylacryloyl–Gly–Leu–NH_2 as substrate, as previously reported by Feder.[11]

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