Synthesis of Some Pseudo-Peptide Analogs of Thiol Proteinase Inhibitors[1)]

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Some pseudo-peptide analogs of thiol proteinase inhibitors were synthesized by a conventional solution method. Among them, Suc-Ala–Val–Val–Ala-ψ-(CH_2-NH)-Ala-*p*NA (peptide 1) and Suc-Ala–Val–Val-ψ-(CH_2-NH)-Ala–Ala-*p*NA (peptide 2) showed a stronger inhibitory activity compared with parent peptide such as Suc-Ala–Val–Val–Ala–Ala-*p*NA. In particular, peptide 2 was about 10-fold as active as the parent peptide (IC_{50}=8 μM). Inserting ψ-(CH_2-NH) possibly makes the inhibitor less susceptible to papain and, as a result, produces more potent inhibition.

Key words pseudo-peptide analog; synthetic thiol proteinase inhibitor (TPI); endogenous TPI; HPLC analysis; synthetic TPI degradation; papain

The lysosomal thiol proteinases all belong to the papain superfamily, and their activity in the body is controlled by several endogenous proteinase inhibitors. Recent studies have shown that abnormal activity of thiol proteinase is involved in several inflammatory diseases, suggesting an important role for thiol proteinase inhibitors in the pathophysiology of disease.[2—4)] Although low-molecular-weight proteinase inhibitors have been found in microbial products and widely used in biochemical and medical studies,[5)] potent synthetic inhibitors of thiol proteinases have also been developed.

Thiol proteinase inhibitors, including kininogens isolated from various tissues, have a fairly conservative common amino acid sequence, Gln–Val–Val–Ala–Gly. Conservation of this sequence strongly suggests that this site may be one of the reaction sites of thiol proteinase inhibitors.[6)] Previously, we synthesized various kinds of Gln–Val–Val–Ala–Gly derivatives.[7,8)] Suc-Ala–Val–Val–Ala–Ala-*p*NA was an analog peptide with the common sequence. The *N*-terminal Gln and *C*-terminal Gly residues were replaced by Ala residues in this peptide, which strongly inhibited the amidolytic activity of papain toward Bz-Arg-β NA.[8)]

In order to obtain more potent inhibitors for clinical use, we prepared pseudo-peptide analogs of Suc-Ala-Val-Val-Ala-Ala-*p*NA with a reduced peptide bond (Fig. 1). The use of peptide bond surrogates is an established approach to overcome one of the major drawbacks in the use of peptides as therapeutic agents, namely, their rapid degradation by peptidases. Among these peptide bond replacements, one of the simplest is the reduced peptide bond (CH_2NH), which has been widely and successfully used in the design of metaboli-

Suc-Ala-Val-Val-Ala-Ψ-(CH_2-NH)-Ala-pNA (peptide 1)
mp >230°C (dec.), $[\alpha]_D^{18}$ 168.3°(c=0.2, H_2O); $C_{28}H_{45}O_9N_7$(635.7) MS(MALD/I-TOF): m/z=636(M^+)

Suc-Ala-Val-Val-Ψ-(CH_2-NH)-Ala-Ala-pNA (peptide 2)
mp 103-107°C; $[\alpha]_D^{18}$ −79.4°(c=0.4, H_2O); $C_{28}H_{45}O_9N_7$(635.7) MS(MALD/I-TOF): m/z=636(M^+)

Suc-Ala-Val-Ψ-(CH_2-NH)-Val-Ala-Ala-pNA (peptide 3)
mp 133-135°C; $[\alpha]_D^{18}$ −124.0°(c=0.3, H_2O); $C_{29}H_{45}O_9N_7$(635.7) MS(MALD/I-TOF): m/z=636(M^+)

Suc-Ala-Ψ-(CH_2-NH)-Val-Val-Ala-Ala-pNA (peptide 4)
mp 152-163°C; $[\alpha]_D^{18}$ 83.2°(c=0.4, H_2O); $C_{29}H_{45}O_9N_7$(635.7) MS(MALD/I-TOF): m/z=636(M^+)

Fig. 1. Synthetic Pseudo-Peptides

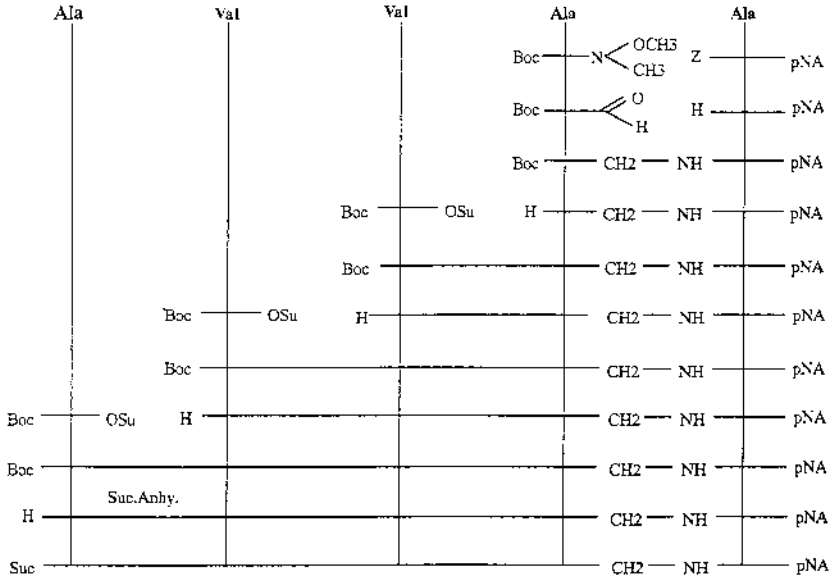

Fig. 2. Synthetic Scheme for the Pseudo-Peptide Analogs

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cally stable agonists or antagonists of natural peptides, or enzyme inhibitors.[9—15)]

We now report the synthesis of pseudo-peptide analogs of Suc-Ala–Val–Val–Ala–Ala-*p*NA, in which each of the peptide bonds was consecutively reduced, and describe the relationship between structure and effect on thiol proteinase (papain); in addition, we examined the enzymatic breakdown of the peptides by papain using HPLC.

Cleavage of peptide bonds by papain reduces the inhibitory activity of the peptide toward papain. The reduced peptides, however, resist enzymatic degradation.

The synthetic route to the pseudo-peptide analog is shown in Fig. 2. The pentapeptide was prepared stepwise, starting from the *C*-terminal residue, by the *N*-hydroxysuccinimide active-ester method, except for the pseudo-peptide bond. Boc-Ala-OH and Boc-Val-OH were converted into the corresponding aldehyde,[16)] which could be stored for 6 months in a refrigerator after purification. Then, the aldehyde was reacted with the HBr salt of H-Ala-*p*NA according to the procedure of Martinez *et al*.[9)] with a minor modification to obtain the desired pseudo-peptide. Purification of the peptide by Sephadex LH-20 column chromatography was very efficient after each, stepwise, elongation procedure. All peptides obtained here were homogeneous upon silica gel thin-layer chromatography and reversed-phase HPLC. The result of the elemental analysis for intermediates and the result of TOF-MS for 4 kinds of desired peptides and one of the intermediates were in good agreement with theoretically expected values (Fig.1, Table1). The inhibitory activity of these synthetic peptides against papain was determined with a synthetic substrate, Bz-Arg-β NA, using techniques previously described[17)] and the results are summarized in Figs. 3 and 4. Peptide 1 and 2 showed a strong inhibitory activity compared with their parent peptides [Suc-Gln–Val–Val–Ala–Ala-*p*NA (IC_{50}=59

Table 1. Yield, Melting Point, $[\alpha]_D^{20}$ and Elemental Analysis of the Synthetic Intermediates

Compound	Yield (%)	mp (°C)	$[\alpha]_D^{20}$	Formula	Elemental analysis Calcd (Found)
Boc-A–V–V–A-ψ-A-*p*NA	85.4	235—238	−13.5 (*c*=0.2, DMF)	$C_{30}H_{49}O_8N_7 \cdot H_2O$	55.1 7.86 15.0 (55.3 7.73 15.0)
Boc-V–V–A-ψ-A-*p*NA	68.7	130—131	−50.6 (*c*=0.2, MeOH)	$C_{27}H_{44}O_7N_6$	57.4 7.85 14.9 (57.4 7.85 14.9)
Boc-V–A-ψ-A-*p*NA	62.3	175—177	−31.3 (*c*=0.2, MeOH)	$C_{22}H_{35}O_6N_5$	57.1 7.95 14.9 (56.5 7.69 14.9)
Boc-A-ψ-A-*p*NA	7.4	134—135	−40.1 (*c*=0.2, MeOH)	$C_{17}H_{26}O_5N_4$	54.3 7.43 14.1 (54.0 7.43 14.1)
Boc-A–V–V-ψ-A–A-*p*NA	42.5	223—226	−68.4 (*c*=0.2, MeOH)	$C_{30}H_{49}O_8N_7$	56.7 7.77 15.4 (56.3 7.86 15.5)
Boc-V–V-ψ-A–A-*p*NA	21.2	148—149	−53.3 (*c*=0.2, MeOH)	$C_{27}H_{44}O_7N_6$	57.4 7.85 14.9 (57.1 7.86 14.9)
Boc-V-ψ-A–A-*p*NA	48.6	178—180	−50.4 (*c*=0.2, MeOH)	$C_{22}H_{35}O_6N_5$	56.8 7.58 14.9 (57.1 7.79 14.9)
Boc-A–V-ψ-V–A–A-*p*NA	72.7	167—171	−89.9 (*c*=0.2, MeOH)	$C_{30}H_{49}O_8N_7$	M^+ 636[a)]
Boc-V-ψ-V–A–A-*p*NA	24.4	178—180	−90.2 (*c*=0.2, MeOH)	$C_{27}H_{44}O_7N_6 \cdot H_2O$	55.7 7.96 14.4 (55.7 7.88 14.5)
Boc-A-ψ-V–V–A–A-*p*NA	16.4	192—196	−111.6 (*c*=0.2, MeOH)	$C_{30}H_{49}O_8N_7 \cdot 3H_2O$	52.3 8.04 14.2 (52.3 7.64 14.4)

a) Mesured by MALD/I TOF-MS.

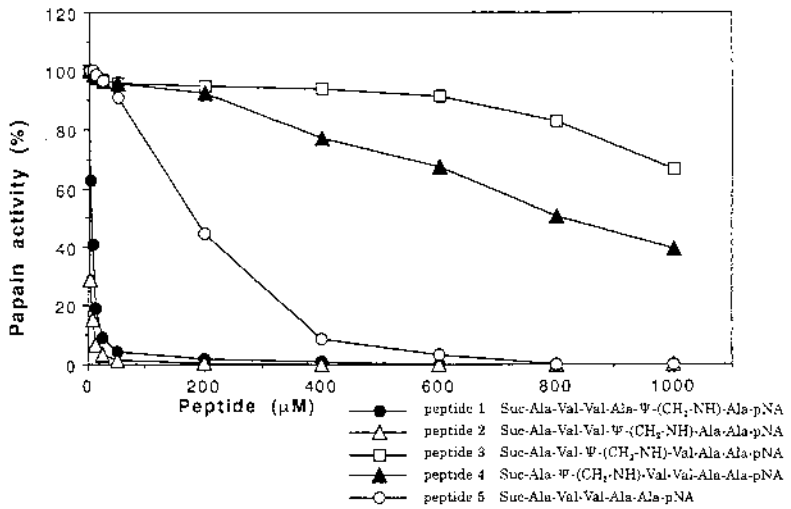

Fig. 3. Inhibition of Papain Activity by Suc-Ala–Val–Val–Ala–Ala-*p*NA Analogs

Inhibition of papain activity by Suc-Ala–Val–Val–Ala–Ala-*p*NA and its analogs. Papain activity was measured using Bz-Arg-β NA as the substrate in the presence of Suc-Ala–Val–Val–Ala–Ala-*p*NA and its analogs at concentrations shown in the figure. Each point represents the mean of 2 assays.

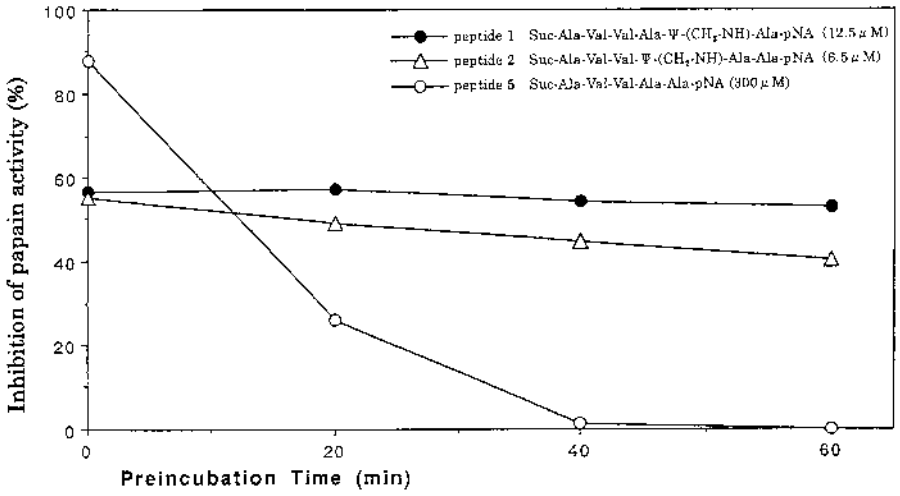

Fig. 4. Inactivation of the Papain-Inhibitory Activity of Suc-Ala–Val–Val–Ala–Ala-*p*NA analogs Preincubated with Papain

Abolition of the papain-inhibitory activity of Suc-Ala–Val–Val–Ala–Ala-*p*NA and its analogs by pretreatment with papain. Papain was preincubated in the presence or absence of Suc-Ala–Val–Val–Ala–Ala-*p*NA and its analogs for the period shown in the figure. After preincubation, Bz-Arg-β NA was added to measure the enzyme activity. Each point represents the mean of 2 assays.

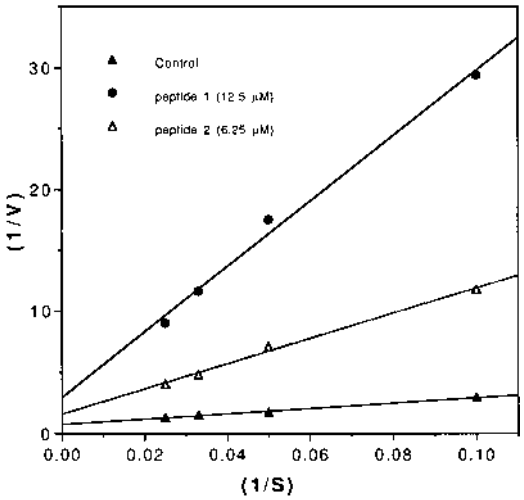

Fig. 5. Lineweaver–Burk Plot of Papain Activity

1/S: 1/Bz-Arg-NA (mg/ml), 1/V: 1/absorbance at 520 nm for 20 min.

μM), Suc-Ala–Val–Val–Ala–Ala-*p*NA (as potent as the former)].[7] In particular peptide 2 is about 10-fold as active as the parent peptide (IC_{50}=8 μM). When the results were plotted according to the method of Lineweaver and Burk, peptide 1 and 2 inhibited papain non-competitively (Fig.5), like Suc-Gln–Val–Val–Ala–Ala-*p*NA reported previously.[7]

Thus, it is clear that substitution of the peptide bond is responsible for the strong activity which may result from increased flexibility of the structure or increased resistance to proteinase (papain). Therefore, the stability of the parent peptide (Suc-Ala–Val–Val–Ala–Ala-*p*NA) to papain was tested by measuring the degradation products by HPLC. The expected products by papain (H-Ala–*p*NA, H-Ala–Ala-*p*NA, H-Val–Ala–Ala-*p*NA and H-Val–Val–Ala–Ala-*p*NA) were prepared by deblocking the N^{α}-protecting groups of the corresponding protected peptides in the usual manner. Incubation of Suc-Ala–Val–Val–Ala–Ala-*p*NA with papain in phosphate buffer at 37 °C caused a rapid decline of the parent peptide peak. Moreover, the appearance of peaks for H-Ala–Ala–*p*NA and H-Ala-*p*NA appeared at the same time as

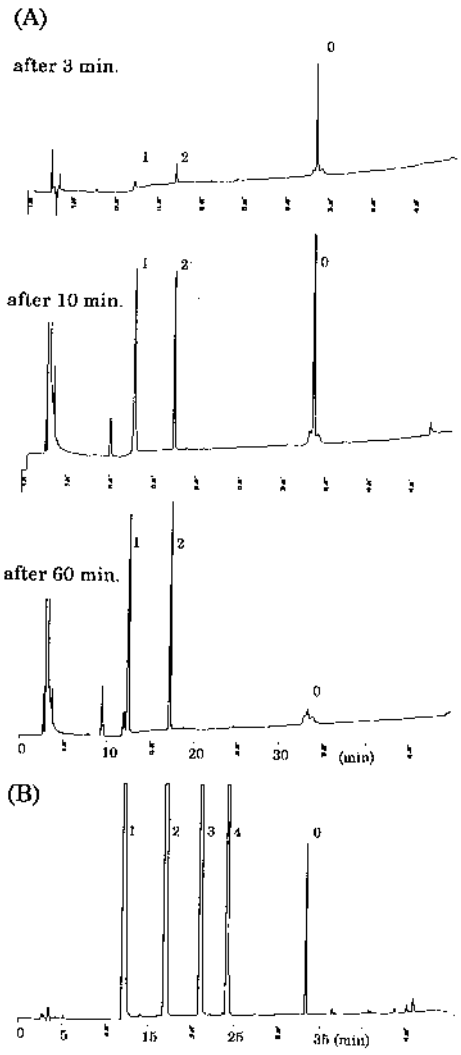

Fig. 6. HPLC Analyses of Suc-Ala–Val–Val–Ala–Ala-*p*NA Digestion by Papain

(A), Digestion of parent peptide by papain. (B), Synthetic fragments. Retention time of the peptides, (0) Suc-Ala–Val–Val–Ala–Ala-*p*NA (33.6 min), (1) H-Ala-pNA (12.3 min), (2) H-Ala-Ala-*p*NA (17.2 min), (3) H-Val-Ala-Ala-pNA (21.2 min), (4) H-Val–Val–Ala–Ala-*p*NA (24.2 min).

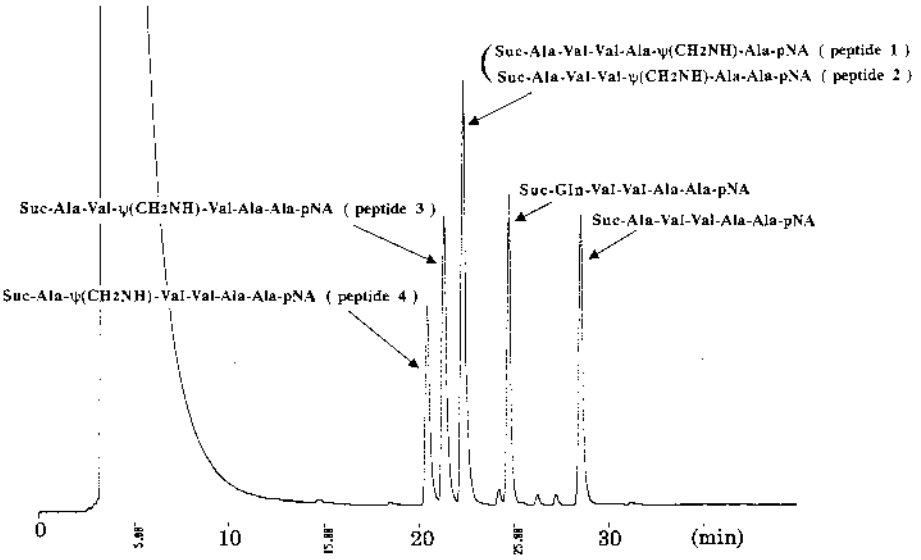

Fig. 7. HPLC Pattern of Suc-Ala–Val–Val–Ala–Ala-*p*NA Analogs

Column: Cosmosil 5C_{18}-AR (4.6×250 mm); Detection: 220 nm; Flow: 1 ml/min; Solvent: A, H_2O (0.05% TFA), B, CH_3CN (0.05% TFA); Gradient A/B: 80/20→(40 min)→40/60.

the disappearance of the Suc-Ala–Val–Val–Ala–Ala-*p*NA peak, which strongly suggests enzymatic breakdown of the peptide inhibitor (Fig. 6). The strong inhibitory activity of peptide 1 and peptide 2, therefore, could be attributed to their resistance to enzymatic degradation by papain. The solubility of these 4 kinds of pseudo-peptides in water (over 1 mg/1 ml) as greater than for lead compounds such as Suc-Ala–Val–Val–Ala–Ala-*p*NA. The HPLC characteristics of these peptides also indicated their hydrophilicity (Fig. 7), which seems to favor solubility in buffer.

Experimental

The melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-360 (Japan Spectroscopic Co., Ltd.). Amino acid compositions of acid hydrolysates (6 N HCl, 110 °C, 18 h; for peptides containing a Val–Val bond, 6 N HCl, 110 °C, 72 h) were determined with an amino acid analyzer, K-101 AS (Kyowa Seimitsu Co., Ltd.). TLC was carried out on silica gel plates (Merck, Silica gel 60 F254), Rf^1, Rf^2,Rf^3 and Rf^4 values refer to the systems $CHCl_3$, MeOH and AcOH (90 : 8 : 2), $CHCl_3$, MeOH and H_2O (8 : 3 : 1, lower phase), *n*-BuOH, AcOH and H_2O (4 : 1 : 5,upper phase) and $CHCl_3$, MeOH and H_2O (17 : 2 : 1), respectively.

Analytical reversed-phase HPLC was performed on a Waters M-600 model using a YMC-Pack Pro C_{18} AS-303 (0.46×25 cm) column and a linear gradient of water (A) and acetonitrile (B), both containing 0.05%TFA, at a flow rate of 1 ml/min. The following gradient systems were used: B: 15 to 50% (20 min). TOF-MS was conducted using a Kratos Kompact MALD/I 3 mass spectrometer. The samples were dissolved in MeOH and matrix was used. Papain was purchased from Nacalai Tesque (code 260-36, 17 U/mg protein)

N-Methoxy-*N*-methyl-α-(*tert*-butoxycarbonylamino)-carboxamides and α-(*tert*-butoxycarbonylamino)-aldehydes were prepared according to the procedure of Martinez *et al*.[9] and others.[16,18,19]

(*tert*-Butoxycarbonyl)-L-valine *N*,*O*-Dimethylhydroxamate Boc-L-Val-OH (8.45 g, 38.9 mmol) was dissolved in DMF (50 ml) containing the hydrochloride salt of *N*,*O*-dimethylhydroxylamine (3.9 g, 40 mmol). Bop-reagent (17.4 g, 38.9 mmol) was added, followed by Et_3N (5.5 ml, 50 mmol). The reaction mixture was stirred at 4 °C overnight. The solvent was removed *in vacuo* and the residue was dissolved in AcOEt (250 ml) and washed with a 5% citric acid solution (3×50 ml), saline (3×50 ml), saturated sodium bicarbonate solution (3×50 ml) and saline (3×50 ml). The organic layer was dried over $MgSO_4$ and then concentrated *in vacuo*, to give an oily compound. Yield 10.1 g (100%), Rf^1 0.44, Rf^2 0.49.

(*tert*-Butoxycarbonyl)-L-valinal[16] Lithium aluminum hydride (1.5 g,) was added to a stirred solution of (*tert*-butoxycarbonyl)-L-valine *N*,*O*-dimethylhydroxamate (10 g, 38.9 mmol) in dry ether (100 ml), at −20 °C. Reduction was complete in 1 h. The mixture was hydrolyzed with a solution of 10% citric acid (ca. 50 ml) instead of potassium hydrogen sulfate. The aqueous phase was separated and extracted with ether (200 ml×3), which was then washed with 10% citric acid, saline, 5% sodium bicarbonate solution and saline. The organic layer was dried over sodium sulfate and concentrated *in vacuo*. The oily residue was left standing overnight in a refrigerator. The crystalline compound produced was triturated and filtered with hexane. Yield 6.8 g (80.9%), mp 48—49 °C, Rf^4 0.65.

(*tert*-Butoxycarbonyl)-L-alaninal[16] Yield 843 mg (48.7%), mp 88—89 °C, Rf^1 0.63, Rf^4 0.47 (same procedure as described above).

Boc-Val-ψ-(CH_2-NH)-Ala–Ala-*p*NA (Incorporation of the Pseudo-peptide Bond, General Procedure) Boc-L-valinal (1.19 g, 5.5 mmol) was dissolved in methanol (20 ml) containing the TFA salt of H-Ala–Ala-*p*NA [Boc-Ala–Ala-*p*NA (1.04 g, 2.74 mmol) was treated with TFA (2 ml) for 1 h and the addition of ether (100 ml) yielded a white powder which was collected by filtration and dried *in vacuo* over KOH]. Sodium cyanoborohydride (207 mg, 3.3 mmol) was added in small portions over 30 min. After 1 h, when no more TFA salt could be detected by TLC, the reaction mixture was cooled in an ice-water bath, and saturated sodium bicarbonate solution (30 ml) was added under stirring. AcOEt (100 ml) was added to the reaction mixture. The organic layer was collected, then washed with saturated sodium bicarbonate solution, saline, 5% citric acid solution and saline, and dried over $MgSO_4$. The solution was concentrated *in vacuo* to yield an oil that was triturated with ether to give a white powder. Yield 620 mg (48.6%), mp 178—180 °C. Physical constants and analytical data are shown in Table 1.

Boc-Ala–Val-ψ-(CH_2-NH)-Val–Ala–Ala-*p*NA (One of the Typical Active Ester Condensation Procedures) Boc-Ala-ONSu (0.152 g, 0.531 mmol) and H-Val-ψ-(CH_2-NH)-Val–Ala–Ala-*p*NA. TFA [prepared from 0.3 g (0.531 mmol) Boc-Val-ψ-(CH_2-NH)-Val–Ala–Ala-*p*NA and 1 ml TFA] were dissolved in DMF (15 ml) containing Et_3N (0.112 ml, 0.797 mmol). The reaction mixture was stirred at room temperature overnight. Boc-Ala-ONSu (50 mg, 0.175 mmol) was added to the reaction mixture which was stirred for several hours. After removal of the solvent, EtOH (2 ml) was added to the residue which was applied to a Sephadex LH-20 column (2.2×125 cm), equilibrated and eluted with EtOH directly. Fractions (5 g each) were collected. After removal of the solvent of the appropriate eluent (fraction Nos. 26—30), ether was added to the residue to give an amorphous powder which included a small amount of impurity [Rf^1 0.24 (main spot), 0.27 (impurity)]. This powder was purified by silica gel column chromatography (2.7×30 cm) using 3% MeOH in $CHCl_3$ as eluent. The pure fraction (Rf^1 0.24) was pooled and the solvent was removed *in vacuo*. The residue was crystallized spontaneously, and was triturated with hexane to yield crystalline compound. The crystalline compound was collected by filtration, rinsed with a small amount of ether, and dried *in vacuo*. Yield 245 mg (72.7%). Physical constants and analytical data are shown in Table 1.

Suc-Ala–Val-ψ-(CH_2-NH)-Val–Ala–Ala-*p*NA (General Procedure for the Preparation of Suc-Peptides) H-Ala–Val-ψ-(CH_2-NH)-Val–Ala–Ala-*p*NA [prepared from 100 mg (0.157 mmol) Boc-Ala–Val-(CH_2-NH)-Val–Ala–Ala-*p*NA and 1 ml TFA] was dissolved in pyridine (2 ml) and Et_3N (22 μl, 0.157 mmol). Succinic anhydride (47.2 mg, 0.472 mmol) was added to the above solution at 0 °C and the reaction mixture was stirred at room temperature overnight. After removal of solvent, EtOH was added to the residue which was then subjected to Sephadex LH-20 column (1.3×135 cm) chromatography, using EtOH as an eluent. Fractions (6 g each) were collected. After removal of the solvent of the appropriate eluent (fraction Nos. 13—14), ether was added to the residue to afford crystals, which were collected by filtration and dried *in vacuo*. Yield 53.5 mg (52.5%), Rf^2 0.4. Physical constants and analytical data are shown in Table 1.

HPLC Analyses of Suc-Ala–Val–Val–Ala–Ala-*p*NA Degradation by Papain Analytical reversed-phase HPLC was performed on a Waters M-600 model using a YMC-Pack Pro C_{18} AS-303 (0.46×25 cm) column and a linear gradient of water (A) and acetonitrile (B), both containing 0.05%TFA, at a flow rate of 1 ml/min. The following gradient system was used: B: 15 to 50% (20 min). Absorbance was monitored at 220 nm.

Preparation of the samples: Papain (0.3 mg) was dissolved in 500 μl 0.1 M phosphate buffer (pH 6.0). Peptide (1.3 mg) was dissolved in a mixture of phosphate buffer (100 μl) and DMSO (100 μl). Both solutions (50 μl each) were mixed, then incubated in a water-bath at 37 °C.

After incubation of the combined samples of the parent peptide (Suc-Ala–Val–Val–Ala–Ala-*p*NA) and papain, in 0.1 M phosphate buffer (pH 6.0) containing DMSO at 37 °C for 3 min, over 90% of the parent peptide remained intact. After incubation for 20 min less than 5% of the parent peptide remained intact, with the formation of almost an equal amount of metabolite [retention time (RT) 12.3 min], H-Ala-*p*NA and metabolite (RT 17.2 min,H-Ala–Ala-*p*NA). When the incubation period was extended to 1 h, the HPLC profile was similar to that after a 20 min incubation. The appearance of the metabolites mirrored the disappearance of the parent peptide, which strongly suggested enzymatic breakdown of the synthetic inhibitors.

N^{α}-Deprotection: Free peptide analogs were prepared by the following procedure. The N^{α}-protecting group, Boc was cleaved by TFA (ca. 1 ml per 100 mg peptide) at ice-bath temperature for 60 min. After removal of the TFA *in vacuo* at 15—20 °C, the residue was treated with dry ether. If a powder was obtained, it was collected by filtration, dried over KOH pellets *in vacuo* for 3 h and then used as the standard sample.

Z-Ala-*p*NA was treated with 25%HBr/AcOH as described in our previous report.[7]

The TFA salt of H-Ala–Ala-*p*NA (3 mg), H-Val–Ala–Ala-*p*NA (4 mg), H-Val–Val–Ala–Ala-*p*NA (5 mg) and the HBr salt of H-Ala-*p*NA (2 mg) were dissolved in water or DMSO (0.5—1.0 ml) for injection.

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References and Notes

1) The customary L indication for amino acid residues is omitted. Standard abbreviations for amino acids and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: Biochemistry, **5**, 3485 (1966); *ibid*., **6**, 362 (1967); *ibid*., **11**, 1726 (1972). Other abbreviations used are: Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl; Suc, succinyl; *p*NA, *p*-nitroanilide; OSu (ONSu), succimido-oxy (*N*-hydroxysuccinimide ester), DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; Et_3N, triethylamine; AcOH, acetic acid; TFA, trifluoroacetic acid; Bz-Arg-βNA, N^{α}-benzoyl-D,L-Arg-2-naphthylamide; NMM,N-methylmorpholine; MALD/I TOF-MS, matrix-assisted laser desorption ionization time of flight mass spectrometry.
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