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Amino Acids and Peptides. XX. Inhibition of Papain by Succinyl–Gln–Val–Val–Ala–Ala–*p*-Nitroanilide, a Common Sequence of Endogenous Thiol Proteinase Inhibitors[1,2]

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Thiol proteinase inhibitors isolated from various tissues have a fairly conservative common amino acid sequence, Gln–Val–Val–Ala–Gly. We synthesized various kinds of Gln–Val–Val–Ala–Gly derivatives by the solution method. Z–Gln–Val–Val–Ala–Ala–*p*NA, Z–Gln–Val–Val–Ala–*p*NA and Z–Gln–Val–Val–*p*NA weakly inhibited the amidolytic activity of papain toward Bz–Arg–βNA and exhibited a stronger protective activity than corresponding Z-peptide methyl ester against thiol proteinase inhibitor (such as T-kininogen)-induced inhibition of papain. Suc–Gln–Val–Val–Ala–Ala–*p*NA exhibited more potent inhibitory activity toward papain than Z–Gln–Val–Val–Ala–Ala–*p*NA, but it did not show any protective effect. The circular dichroism spectra of the *p*NA derivatives and papain suggested that the *p*NA moiety of the peptide participated in binding with some part of the enzyme other than the catalytic site.

Keywords——Gln–Val–Val–Ala–Gly sequence; non-competitive inhibition; protection activity; papain; T-kininogen; *p*-nitroanilide moiety; CD spectra

Thiol proteinase inhibitors, including kininogens isolated from various tissues, have a fairly conservative common amino acid sequence, Gln–Val–Val–Ala–Gly. This conservation of the sequence strongly suggests that this site may be one of the reactive sites of thiol proteinase inhibitors.[3] Previously, we synthesized Z–Gln–Val–Val–Ala–Gly–OMe and found that this peptide weakly inhibited the amidolytic activity of papain toward Bz–Arg–βNA and exhibited a remarkable protective activity against thiol proteinase inhibitor-induced inhibition of papain.[4]

This report deals with the synthesis of Gln–Val–Val–Ala–Gly derivatives modified at the N and/or C-terminus by the solution method, the relationship between structure and effect on thiol proteinase (papain) and an examination of the inhibitory and protective mechanisms by measuring circular dichroism (CD) spectra.

In order to obtain compounds which exhibit more potent inhibitory and protective effects on thiol proteinases and are suitable for examination of the interaction between synthetic peptides and thiol proteinases by measuring CD spectra, we prepared *p*-nitroanilide derivatives according to our previous report.[5] A Gly residue was replaced by an Ala residue to introduce chirality at the *p*NA part. As an example, the synthetic route to Z–Gln–Val–Val–Ala–Ala–*p*NA (**1**) is shown in Fig. 1. Z–Ala–*p*NA was prepared by the phosphazo method.[6] N^{α}-Protected peptide and amino acids were coupled by the azide method, the mixed anhydride method and the *p*-nitrophenyl active ester method. All peptides obtained here were homogeneous upon silica gel thin-layer chromatography (TLC). Amino acid ratios in acid hydrolysates and the results of elemental analysis were in good agreement with theoretically

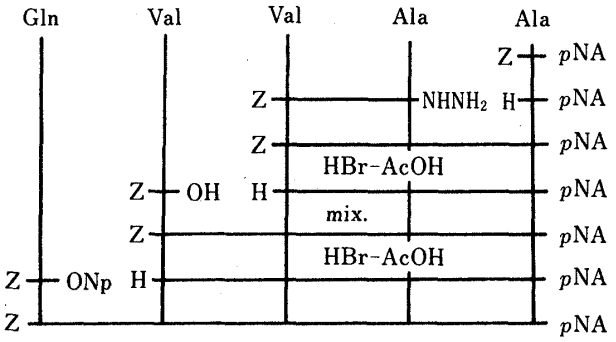

Fig. 1. Synthetic Scheme for Z–Gln–Val–Val–Ala–Ala–*p*NA (**1**)

TABLE I. Effects of Z–Gln–Val–Val–Ala–Ala–*p*NA (**1**) and Its Derivatives on the Amidolytic Activity of Papain and on the Inhibitory Activity of T-Kininogen toward Papain

Compound		Inhibition of papain (%)[a]	Protection of papain from T-kininogen-induced inhibition[b]
Z–Gln–Val–Val–Ala–Ala–*p*NA	(**1**)	55.0	14.3
Z–Gln–Val–Val–Ala–Ala–OMe	(**2**)	50.0	2.7
Z–Gln–Val–Ala–Ala–Ala–*p*NA	(**3**)	68.2	15.9
Z–Gln–Val–Ala–Ala–Gly–OMe	(**4**)[c]	9.1	0.9
Z–Gln–Val–Val–Ala–*p*NA	(**5**)	9.6	10.6
Z–Gln–Val–Val–Ala–OMe	(**6**)[c]	9.3	2.7
Z–Gln–Val–Val–*p*NA	(**7**)	8.2	15.7
Z–Gln–Val–Val–OMe	(**8**)[c]	10.5	1.9
Z–Gln–Val–*p*NA	(**9**)	0	1.0
Z–Gln–Val–OMe	(**10**)[c]	10.5	1.0

a) The inhibitory effects of the peptides were determined by adding the peptides (0.18 mM) to the assay medium. The values represent the means of 4 experiments. *b*) The protective effects of peptides on the inhibitory activity of T-kininogen toward papain were calculated as follows:

$$\text{factor} = \frac{IC_{50} \text{ of T-kininogen in the presence of peptide}}{IC_{50} \text{ of T-kininogen in the absence of peptide}}$$

c) See reference 9.

expected values. The inhibitory activities of these synthetic peptides against papain and protective activity against T-kininogen-induced inhibition of papain were determined with a synthetic substrate, Bz–Arg–βNA[7] by means of the techniques previously described[8] and are summarized in comparison with those of the corresponding methyl ester derivatives[9] in Table I.

The *p*NA derivatives and the corresponding methyl ester derivatives exhibited roughly similar inhibitory activities except for **3** and **4**. The amino acid sequences of these peptides (**3** and **4**) are different at the C-terminus. Although we have reported that in order to manifest some effect on papain, the Z–Gln–Val–Val sequence is very important,[8,9] the Z–Gln–Val–Ala in **3** seems not prevent the inhibitory activity; the Ala–*p*NA moiety might increase the affinity between **3** and papain to manifest more potent inhibitory activity than that of the peptide (**4**). On the other hand, regarding the protective activity, the *p*NA derivatives (**1**, **3**, **5** and **7**) protected papain from T-kininogen-induced inhibition much more strongly than the corresponding Z-peptide methyl ester derivatives (**2**, **4**, **6** and **8**), demonstrating that the *p*NA moiety of the derivatives plays a very important role in binding with some part of papain, other than the catalytic site, in competition with T-kininogen.[9]

TABLE II. Effects of Suc–Gln–Val–Val–Ala–Ala–*p*NA (**11**) and Its Derivatives on the Amidolytic Activity of Papain and on the Inhibitory Activity of T-Kininogen toward Papain

Compound	Inhibition of papain (%)[a]	Protection of papain from T-kininogen-induced inhibition[b]
Suc–Gln–Val–Val–Ala–Ala–*p*NA (**11**)	90.0	1.0
Suc–Gln–Val–Val–Ala–Ala–OMe (**12**)	58.0	1.0
Suc–Ala–Val–Val–Ala–Ala–*p*NA (**13**)	87.0	1.0
Suc–Gln–Val–Val–Ala–*p*NA (**14**)	18.2	1.0
Suc–Gln–Val–Val–*p*NA (**15**)	8.6	1.4
Suc–Gln–Val–*p*NA (**16**)	0	1.0

a) The inhibitory effects of the peptides were determined by adding the peptides (0.18 mM) to the assay medium. The values represent the means of 4 experiments. *b*) The protective effects of peptides on the inhibitory activity of T-kininogen toward papain were calculated as follows:

$$\text{factor} = \frac{IC_{50} \text{ of T-kininogen in the presence of peptide}}{IC_{50} \text{ of T-kininogen in the absence of peptide}}$$

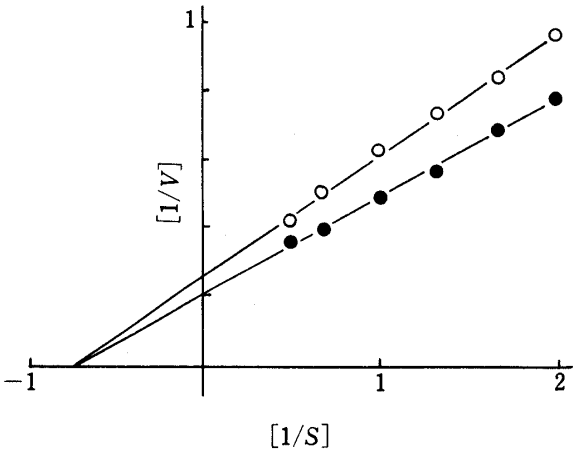

Fig. 2. Kinetic Plots of the Inhibition of Papain by Suc–Gln–Val–Val–Ala–Ala–*p*NA (**11**)

○, in the presence of 36 μM **11**; ●, in the absence of **11**. Substrate, Bz–Arg–βNA.

Next, in order to increase the solubility of *p*NA derivatives in the buffer used for measurement of CD spectra, a succinyl (Suc) group was substituted for the Z group by succinylation of the corresponding Z-deblocked *p*-nitroanilide derivatives with succinic anhydride. Their inhibitory and protective activities were examined and the results are summarized in Table II. Suc–Gln–Val–Val–Ala–Ala–*p*NA (**11**) exhibited fairly potent inhibition of papain activity toward Bz–Arg–βNA in a dose-dependent manner (IC_{50}: 59 μM), although Suc–Gln–Val–Val–Ala–Ala–OMe (**12**) inhibited papain with an IC_{50} of 150 μM. However, the peptide (**11**) did not show any protective effect against T-kininogen-induced inhibition of papain. We expected that Suc–Ala–Val–Val–Ala–Ala–*p*NA (**13**) would not show any effect on papain, since Z–Ala–Val–Val–Ala–Gly–OMe was ineffective.[9] However, the peptide (**13**) inhibited papain as potently as **11**. The *p*NA moiety in **13** clearly plays an important role in the inhibitory effect, and the distance between the *p*NA group and the Suc group seems to be critical, because the inhibitory activities of **14**, **15** and **16** are very weak. These peptide anilides (**11**—**16**) did not show any protective effect against T-kininogen-induced inhibition of papain. This phenomenon is compatible with our previous report that in order to protect papain from thiol proteinase inhibitor-induced inhibition, an aromatic ring at the N-terminus of a pentapeptide was required.[10]

It is possible that the inhibitory mechanism of Suc–Gln–Val–Val–Ala–Ala–*p*NA (**11**)

against papain is different from that of Z–Gln–Val–Val–Ala–Ala–*p*NA (**1**) or Z–Gln–Val–Val–Ala–Ala–OMe (**2**), because the former peptide (**11**) fairly potently inhibited papain activity toward Bz–Arg–βNA but could not protect papain from T-kininogen-induced inhibition, while **1** or **2** inhibited papain less potently than **11** but remarkably protected papain from T-kininogen-induced inhibition. In order to examine the above possibility, amidolytic activity of papain was assayed using Bz–Arg–βNA as a substrate in the absence and presence of Suc–Gln–Val–Val–Ala–Ala–*p*NA (**11**) at six concentrations of substrate, and the results were plotted according to the method of Lineweaver and Burk[11] (Fig. 2). As can be seen in Fig. 2, this peptide (**11**) inhibits papain non-competitively, demonstrating that it does not bind with the active site of papain. From our experimental result that Z–Gln–Val–Val–*p*NA (**7**, inhibition percent 8.2 and protection factor 15.7) exhibited a weak but definite protective effect against Suc–Gln–Val–Val–Ala–Ala–*p*NA-induced inhibition of papain, it was clear that Suc-derivatives and Z-derivatives bind with the same part of papain (not the active site). In order to compete with the Gln–Val–Val–Ala–Gly part of T-kininogen, an aromatic group at the N-terminus of peptides seems to be more favorable than a Suc group.

In order to study the interaction between peptide anilides and papain, the CD spectra of the enzyme–Suc–Gln–Val–Val–Ala–Ala–*p*NA or Suc–Ala–Val–Val–Ala–Ala–*p*NA mixture were measured. The molar ratio of peptide to enzyme was 1 : 1. The ellipticity (θ) was obtained from

$$\theta = H \times S$$

where H is the measured value (cm) and S is the scale (deg/cm). The CD spectra of Suc–Gln–Val–Val–Ala–Ala–*p*NA (**11**), papain and their 1 : 1 mixture are shown in Fig. 3a. Figure 3b illustrates the CD spectrum of the mixture of the peptide and papain in comparison with the curve calculated from the CD spectra of **11** and papain. From Fig. 3b, it can be seen that the peak due to the *p*NA moiety appeared at around 330 nm, although no peak was observed in the calculated curve. It can be deduced that the *p*NA moiety of **11** interacts with some part of papain, resulting in the appearance of the peak at around 330 nm, as well as causing the manifestation of inhibitory activity against papain. We previously reported that the degree of the discrepancy between the calculated curve and the measured curve at around 330 nm was proportional to the potency of the inhibitory activity in peptide-*p*-nitroanilide-α-chymo-

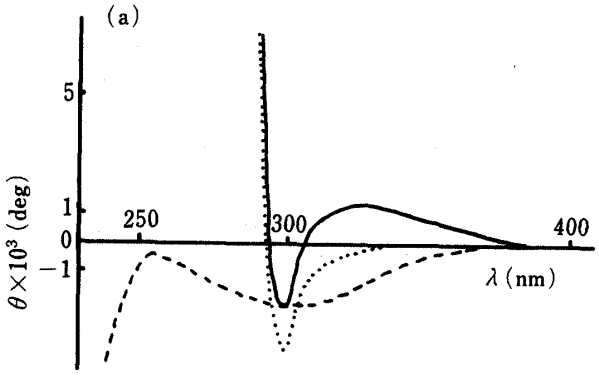

Fig. 3a. CD Spectra of Suc–Gln–Val–Val–Ala–Ala–*p*NA (**11**), Papain and Their Mixture

——, Suc–Gln–Val–Val–Ala–Ala–*p*NA (**11**) (0.045 mg/ml); ------, papain (1.11 mg/ml); ——, **11** (0.045 mg/ml) + papain (1.11 mg/ml). Buffer, 0.1 M Na, K-phosphate buffer (pH 6.0) in the presence of 18% DMSO.

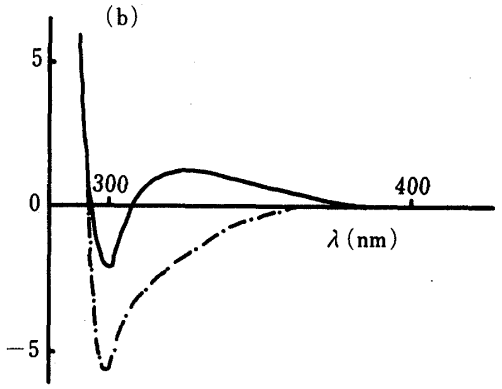

Fig. 3b. Comparison of the Observed and Calculated CD Spectra for the Same Mixture as in Fig. 3a

——, measured curve; — · —, calculated from CD spectra of **11** and papain.

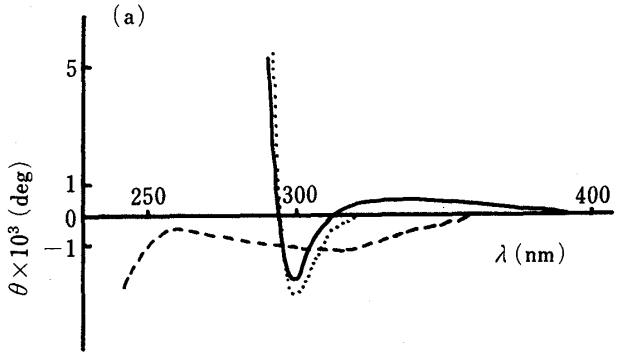

Fig. 4a. CD Spectra of Suc–Ala–Val–Val–Ala–Ala–*p*NA (**13**), Papain and Their Mixture

——, Suc–Ala–Val–Val–Ala–Ala–*p*NA (**13**) (0.032 mg/ml); ------, papain (0.96 mg/ml); ——, **13** (0.032 mg/ml) + papain (0.96 mg/ml). Buffer, 0.1 M Na, K-phosphate buffer (pH 6.0) in the presence of 15% DMSO.

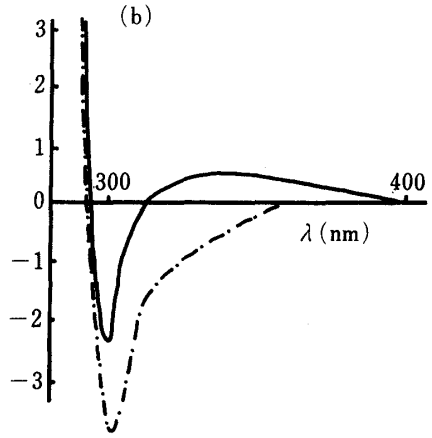

Fig. 4b. Comparison of the Observed and Calculated CD Spectra for the Same Mixture as in Fig. 4a

——, measured curve; — · —, calculated from CD spectra of **13** and papain.

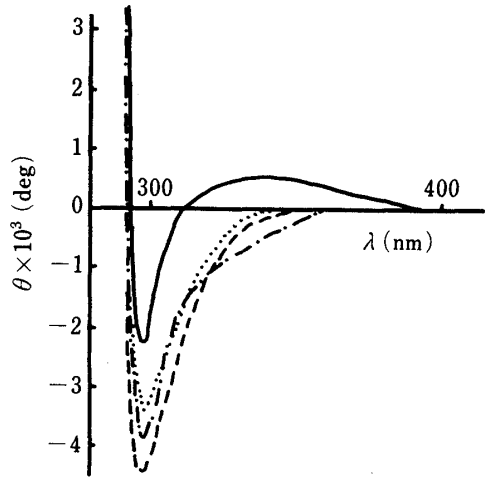

Fig. 5. Change of CD Spectra of the Mixture of Suc–Ala–Val–Val–Ala–Ala–*p*NA (**13**) and Papain on Adding T-Kininogen

——, measured curve of the mixture of **13** (0.032 mg/ml) and papain (0.96 mg/ml); — · —, calculated from the CD spectra of **13** and papain; ------, **13**-papain mixture containing T-kininogen (1.25 mg/ml); – – – –, **13**-papain mixture containing T-kininogen (2.50 mg/ml). Buffer, 0.1 M Na, K-phosphate buffer (pH 6.0) in the presence of 15% DMSO.

trypsin systems.[5,12]

The interaction of **13** and papain was also studied by measuring the CD spectra in the same manner as described above. Similar results to those in the case of **11** and papain were obtained, as shown in Figs. 4a and 4b.

Next, the effect of T-kininogen on the interaction between the peptide (**13**) and papain was studied by measuring the CD spectra. T-Kininogen was added to a mixture of **13** and papain. The broad peak at around 330 nm due to the interaction of the *p*NA moiety of **13** and papain disappeared, as shown in Fig. 5. From this result, it can be deduced that the peptide (**13**) bound with papain is easily replaced by T-kininogen. This might be a reason why **11** and **13**, which exhibit fairly potent inhibitory effects on papain, can not protect papain from T-kininogen-induced inhibition.

From the above experimental results, it was concluded that Suc–Gln–Val–Val–Ala–Ala–*p*NA (**11**) inhibited papain activity by binding with some part of papain other than the catalytic site and *p*NA moiety of this peptide (**11**) had an important role in increasing the affinity between the peptide and papain, resulting in the manifestation of potent inhibitory activity against papain. Consequently, substitution of the *p*NA group at the C-terminus of the

pentapeptide with various kinds of aromatic moieties is under way in our laboratory with the objective of obtaining more potent inhibitors of thiol proteinases.

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.). CD spectra were measured with JASCO J-20 spectropolarimeter. On TLC (Kieselgel G, Merck), Rf^1, Rf^2 and Rf^3 values refer to the systems of $CHCl_3$, MeOH and AcOH (90 : 8 : 2), $CHCl_3$, MeOH and H_2O (89 : 10 : 1) and $CHCl_3$, MeOH and H_2O (8 : 3 : 1, lower phase).

Z–Val–Ala–Ala–*p*NA——H–Ala–*p*NA · HBr (prepared from 0.8 g of Z–Ala–*p*NA[13] and 2.2 ml of HBr/AcOH in the usual manner) was dissolved in DMF (25 ml) containing Et_3N (0.32 ml). Z–Val–Ala–$NHNH_2$ (0.9 g)[14] was dissolved in DMF (20 ml) and cooled to −30 °C. To this cold solution, 7.0 N HCl/dioxane (0.77 ml) was added, followed by isopentyl nitrite (0.38 ml). The reaction mixture was stirred at the same temperature for 15 min, and the pH of the solution was adjusted to 8 with Et_3N. This azide was combined with the above solution. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, AcOEt and H_2O were added to the residue to afford crystals, which were collected by filtration and recrystallized from MeOH, yield 0.88 g (80.2%), mp 228—230 °C, $[\alpha]_D^{30}$ −17.5° (c = 1.0, DMF), Rf^1 0.42. *Anal.* Calcd for $C_{25}H_{31}N_5O_7$: C, 58.5; H, 6.10; N, 13.6. Found: C, 58.5; H, 6.11; N, 13.6.

Z–Val–Val–Ala–Ala–*p*NA——H–Val–Ala–Ala–*p*NA · HBr (prepared from 0.8 g of Z–Val–Ala–Ala–*p*NA and 1.6 ml of 25% HBr/AcOH) was dissolved in DMF (5.0 ml) containing Et_3N (0.23 ml). A mixed anhydride (prepared from 0.6 g of Z–Val–OH, 0.33 ml of isobutylchloroformate and 0.34 ml of Et_3N) in THF (10 ml) was combined with the above solution. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, AcOEt and H_2O were added to the residue to give crystals, which were collected by filtration and recrystallized from MeOH, yield 0.54 g (55.1%), mp 285—288 °C, $[\alpha]_D^{30}$ −17.2° (c = 1.0, DMF), Rf^1 0.50, Rf^2 0.52. *Anal.* Calcd for $C_{30}H_{40}N_6O_8$: C, 58.8; H, 6.59; N, 13.7. Found: C, 58.6; H, 6.67; N, 13.8.

Z–Gln–Val–Val–Ala–Ala–*p*NA (1)——Z–Gln–ONp (0.4 g) and H–Val–Val–Ala–Ala–*p*NA · HBr (prepared from 0.4 g of Z–Val–Val–Ala–Ala–*p*NA and 0.6 ml of 25% HBr/AcOH) were dissolved in DMF (5 ml) containing Et_3N (0.1 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, MeOH was added to the residue to give crystals, which were collected by filtration and recrystallized from MeOH, yield 0.38 g (78.7%), mp 288 °C (dec.), $[\alpha]_D^{30}$ −10.9° (c = 0.1, DMF), Rf^1 0.24, Rf^3 0.78. Amino acid ratios in an acid hydrolysate: Glu 1.03; Val 1.33; Ala 2.00 (average recovery 78.0%). *Anal.* Calcd for $C_{35}H_{48}N_8O_{10} \cdot 1/2H_2O$: C, 56.0; H, 6.60; N, 14.9. Found: C, 56.0; H, 6.56; N, 14.9.

Z–Val–Ala–Ala–OMe——Z–Val–Ala–N_3 (prepared from 3.1 g of Z–Val–Ala–$NHNH_2$, 2.6 ml of 7.6 N HCl/dioxane and 1.3 ml of isopentyl nitrite) in DMF (50 ml) was added to a solution of H–Ala–OMe (prepared from 2.9 g of H–Ala–OMe · HCl and 1.0 ml of Et_3N) in DMF (50 ml). This reaction mixture was stirred at 4 °C overnight. After removal of the solvent, AcOEt and H_2O were added to the residue to give crystals, which were collected and recrystallized from MeOH, yield 2.0 g (72.2%), mp 183—185 °C, $[\alpha]_D^{30}$ −15.1° (c = 1.0, DMF), Rf^1 0.80, Rf^2 0.58. *Anal.* Calcd for $C_{20}H_{29}N_3O_6$: C, 59.0; H, 7.17; N, 10.3. Found: C, 58.8; H, 7.14; N, 10.3.

Z–Val–Val–Ala–Ala–OMe——The title compound was prepared from Z–Val–OH (0.75 g) and H–Val–Ala–Ala–OMe · HBr (prepared from 1.4 g of Z–Val–Ala–Ala–OMe and 3.0 ml of 25% HBr/AcOH) by a mixed anhydride method in the same manner as described for the synthesis of Z–Val–Val–Ala–Ala–*p*NA and recrystallized from MeOH, yield 1.4 g (94.5%), mp 230—233 °C, $[\alpha]_D^{23}$ −16.2° (c = 1.0, DMF), Rf^1 0.77. *Anal.* Calcd for $C_{25}H_{38}N_4O_7 \cdot 2H_2O$: C, 55.3; H, 7.80; N, 10.3. Found: C, 55.6; H, 7.88; N, 10.8.

Z–Gln–Val–Val–Ala–Ala–OMe (2)——The title compound was prepared from Z–Gln–ONp (1.4 g) and H–Val–Val–Ala–Ala–OMe · HBr (prepared from 1.3 g of Z–Val–Val–Ala–Ala–OMe and 2.3 ml of 25% HBr/AcOH) in the same manner as described for the synthesis of **1** and recrystallized from MeOH, yield 0.8 g (53.2%), mp 274 °C (decomp.), $[\alpha]_D^{23}$ −35.3° (c = 0.5, DMSO), Rf^1 0.80. Amino acid ratios in an acid hydrolysate: Glu 1.08; Val 1.45; Ala 2.00 (average recovery 85.0%). *Anal.* Calcd for $C_{30}H_{46}N_6O_9 \cdot H_2O$: C, 55.2; H, 7.10; N, 12.9. Found: C, 54.9; H, 7.24; N, 13.1.

Z–Val–Ala–Ala–Ala–*p*NA——The title compound was prepared from Z–Val–OH (1.0 g) and H–Ala–Ala–Ala–*p*NA · HBr (prepared from 1.2 g of Z–Ala–Ala–Ala–*p*NA[15] and 2.4 ml of 25% HBr/AcOH) by a mixed anhydride method as described above and recrystallized from MeOH, yield 1.1 g (78.3%), mp 279—282 °C, $[\alpha]_D^{25}$ −13.0° (c = 0.3, DMSO), Rf^1 0.43, Rf^3 0.87. *Anal.* Calcd for $C_{28}H_{36}N_6O_8 \cdot 1/2H_2O$: C, 56.7; H, 6.39; N, 14.2. Found: C, 56.7; H, 6.26; N, 14.1.

Z–Gln–Val–Ala–Ala–Ala–*p*NA (3)——The title compound was prepared from Z–Gln–ONp (1.0 g) and H–Val–Ala–Ala–Ala–*p*NA · HBr (prepared from 1.0 g of Z–Val–Ala–Ala–Ala–*p*NA and 1.7 ml of 25% HBr/AcOH) in the same manner as described for the synthesis of **1** and recrystallized from MeOH, yield 0.34 g (28.3%), mp 252—253 °C,

$[\alpha]_D^{24}$ −37.9° (c=1.0, DMSO), Rf^1 0.57, Rf^2 0.58. Amino acid ratios in an acid hydrolysate: Glu 1.02; Val 1.14; Ala 3.00 (average recovery 100%). *Anal.* Calcd for $C_{33}H_{44}N_8O_{10}\cdot 5/4H_2O$: C, 53.9; H, 6.39; N, 15.2. Found: C, 54.1; H, 6.30; N, 14.9.

Z–Val–Ala–*p*NA——A mixed anhydride (prepared from 0.80 g of Z–Val–OH, 0.45 ml of Et_3N and 0.4 ml of isobutylchloroformate) in THF (30 ml) was added to a solution of H–Ala–*p*NA·HBr (prepared from 0.9 g of Z–Ala–*p*NA and 2.5 ml of 25% HBr/AcOH) in DMF (10 ml) containing Et_3N (0.36 ml) cooled to 0 °C. The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 1 N HCl and H_2O, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to give crystals, which were collected by filtration, yield 0.66 g (57.1%), mp 90—95 °C, $[\alpha]_D^{25}$ −10.6° (c=1.0, DMF), Rf^1 0.45, Rf^3 0.54. *Anal.* Calcd for $C_{22}H_{26}N_4O_6\cdot 1/4H_2O$: C, 59.1; H, 6.99; N, 12.5. Found: C, 59.2; H, 6.67; N, 12.4.

Z–Val–Val–Ala–*p*NA——The title compound was prepared from Z–Val–OH (0.4 g) and H–Val–Ala–*p*NA·HBr (prepared from 0.6 g of Z–Val–Ala–*p*NA and 1.4 ml of 25% HBr/AcOH) by a mixed anhydride method as described for the synthesis of Z–Val–Ala–*p*NA and recrystallized from AcOEt and ether, yield 0.33 g (44.0%), mp 255—257 °C, $[\alpha]_D^{25}$ −16.0° (c=1.0, DMF), Rf^1 0.73, Rf^3 0.74. *Anal.* Calcd for $C_{27}H_{35}N_5O_7$: C, 59.9; H, 6.53; N, 12.9. Found: C, 59.6; H, 6.51; N, 12.7.

Z–Gln–Val–Val–Ala–*p*NA (5)——The title compound was prepared from Z–Gln–ONp (0.27 g) and H–Val–Val–Ala–*p*NA (prepared from 0.3 g of Z–Val–Val–Ala–*p*NA and 0.54 ml of 25% HBr/AcOH) in the same manner as described for the synthesis of **1** and recrystallized from MeOH, yield 0.34 g (91.9%), mp 270 °C (dec.), $[\alpha]_D^{24}$ −26.9° (c=1.0, DMSO), Rf^1 0.24, Rf^2 0.41. Amino acid ratios in an acid hydrolysate: Glu 1.05; Val 1.45; Ala 1.00 (average recovery 88.5%). *Anal.* Calcd for $C_{32}H_{43}N_7O_9\cdot 1/2H_2O$: C, 56.6; H, 6.55; N, 14.4. Found: C, 56.6; H, 6.44; N, 14.5.

Z–Val–Val–*p*NA——The title compound was prepared from Z–Val–OH (1.6 g) and H–Val–*p*NA·HBr (prepared from 2.0 g of Z–Val–*p*NA[12] and 5.3 ml of 25% HBr/AcOH) in the same way as described for the synthesis of Z–Val–Ala–*p*NA and recrystallized from AcOEt and ether, yield 1.8 g (73.6%), mp 204—209 °C, $[\alpha]_D^{24}$ +21.0° (c=1.0, DMF), Rf^1 0.55, Rf^3 0.91. *Anal.* Calcd for $C_{24}H_{30}N_4O_6$: C, 61.3; H, 6.44; N, 11.8. Found: C, 61.3; H, 6.43; N, 11.8.

Z–Gln–Val–Val–*p*NA (7)——The title compound was prepared from Z–Gln–ONp (1.3 g) and H–Val–Val–*p*NA·HBr (prepared from 1.0 g of Z–Val–Val–*p*NA and 2.0 ml of 25% HBr/AcOH) in the same way as described for the synthesis of **1** and recrystallized from MeOH, yield 1.1 g (89.6%), mp 275 °C (dec.), $[\alpha]_D^{24}$ +2.7° (c=1.0, DMSO), Rf^1 0.33, Rf^3 0.69. Amino acid ratios in an acid hydrolysate: Glu 1.00; Val 1.45 (average recovery 85.6%). *Anal.* Calcd for $C_{29}H_{28}N_6O_8$: C, 57.3; H, 6.48; N, 13.8. Found: C, 57.6; H, 6.50; N, 13.6.

Z–Gln–Val–*p*NA (9)——The title compound was prepared from Z–Gln–ONp (1.5 g) and H–Val–*p*NA·HBr (prepared from 0.9 g of Z–Val–*p*NA and 2.5 ml of 25% HBr/AcOH) in the same manner as described for the synthesis of **1** and recrystallized from MeOH, yield 0.5 g (38.0%), mp 249—252 °C, $[\alpha]_D^{30}$ +5.2° (c=1.1, DMSO), Rf^1 0.40. Amino acid ratios in an acid hydrolysate: Glu 1.01; Val 1.00 (average recovery 85.6%). *Anal.* Calcd for $C_{24}H_{29}N_5O_7$: C, 57.7; H, 5.78; N, 14.0. Found: C, 57.8; H, 5.72; N, 14.0.

Suc–Gln–Val–Val–Ala–Ala–*p*NA (11)——H–Gln–Val–Val–Ala–Ala–*p*NA·HBr (prepared from 100 mg of **1** and 0.14 ml of 25% HBr/AcOH) was dissolved in pyridine (3 ml) containing Et_3N (0.02 ml). Succinic anhydride (40.5 mg) was added to the above solution in three equal portions over a period of 30 min at 0 °C. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, AcOEt (30 ml) and 3% AcOH (30 ml) were added to the residue. Crystals formed in the AcOEt layer were collected and washed with H_2O and MeOH, yield 63.4 mg (66.5%), mp >300 °C, $[\alpha]_D^{30}$ −1.5° (c=1.1, DMSO). Amino acid ratios in an acid hydrolysate: Glu 0.98; Val 1.52; Ala 2.00 (average recovery 100%). *Anal.* Calcd for $C_{31}H_{46}N_8O_{11}\cdot 5/4H_2O$: C, 51.0; H, 6.54; N, 15.3. Found: C, 51.2; H, 6.39; N, 15.0.

Suc–Gln–Val–Val–Ala–Ala–OMe (12)——The title compound was prepared from H–Gln–Val–Val–Ala–Ala–OMe·HBr (prepared from 0.77 g of **2** and 1.1 ml of 25% HBr/AcOH) and succinic anhydride (0.34 g) in the same manner as described for the synthesis of **11**, yield 0.28 g (38.9%), mp 275 °C (dec.), $[\alpha]_D^{24}$ +9.2° (c=1.1, DMSO), Rf^1 0.88. Amino acid ratios in an acid hydrolysate: Glu 1.10; Val 1.26; Ala 2.00 (average recovery 66.9%). *Anal.* Calcd for $C_{26}H_{44}N_6O_{10}\cdot 9/4H_2O$: C, 48.7; H, 7.62; N, 13.1. Found: C, 48.8; H, 7.34; N, 12.7.

Z–Ala–Val–Val–Ala–Ala–*p*NA——The title compound was prepared from Z–Ala–OH (0.1 g) and H–Val–Val–Ala–Ala–*p*NA·HBr (prepared from 0.23 g of Z–Val–Val–Ala–Ala–*p*NA and 0.4 ml of 25% HBr/AcOH) by the mixed anhydride method as described for the synthesis of Z–Val–Val–Ala–Ala–*p*NA and recrystallized from MeOH, yield 0.14 g (55.7%), mp 289—292 °C, $[\alpha]_D^{24}$ −20.0° (c=0.1, DMSO), Rf^1 0.60. *Anal.* Calcd for $C_{33}H_{45}N_7O_9\cdot 3/4H_2O$: C, 55.4; H, 6.46; N, 13.7. Found: C, 55.4; H, 6.43; N, 14.0.

Suc–Ala–Val–Val–Ala–Ala–*p*NA (13)——The title compound was prepared from H–Ala–Val–Val–Ala–Ala–*p*NA·HBr (prepared from 100 mg of Z–Ala–Val–Val–Ala–Ala–*p*NA and 0.14 ml of 25% HBr/AcOH) and succinic anhydride (43.2 mg) in the same manner as described for the synthesis of **11**, yield 40.5 mg (42.5%), mp 280 °C (dec.), $[\alpha]_D^{24}$ −44.0° (c=0.3, DMSO). Amino acid ratios in an acid hydrolysate: Ala 3.00; Val 1.88 (average recovery 85.9%). *Anal.* Calcd for $C_{29}H_{43}N_7O_{10}\cdot 7/4H_2O$: C, 49.8; H, 6.46; N, 14.0. Found: C, 50.0; H, 6.19; N, 13.8.

Suc–Gln–Val–Val–Ala–*p*NA (14)——The title compound was prepared from H–Gln–Val–Val–Ala–*p*NA·HBr (prepared from 100 mg of **5** and 0.15 ml of 25% HBr/AcOH) and succinic anhydride (45 mg) in the same manner as

described for the synthesis of **11**, yield 59.0 mg (61.9%), mp 247—251 °C, $[\alpha]_D^{24}$ −41.2° (c=0.5, DMSO), Rf^3 0.89. Amino acid ratios in an acid hydrolysate: Glu 1.11; Val 2.00; Ala 1.00 (average recovery 82.5%). *Anal.* Calcd for $C_{28}H_{41}N_7O_{10}\cdot 1/2H_2O$: C, 52.2; H, 6.58; N, 15.2. Found: C, 52.3; H, 6.43; N, 15.0.

Suc–Gln–Val–Val–*p*NA (15)——The title compound was prepared from H–Gln–Val–Val–*p*NA·HBr (prepared from 100 mg of **7** and 0.17 ml of 25% HBr/AcOH) and succinic anhydride (51 mg) in the same manner as described for the synthesis of **11**, yield 43.0 mg (44.8%),mp 234—237 °C, $[\alpha]_D^{24}$ −8.8° (c=0.5, DMSO), Rf^3 0.70. Amino acid ratios in an acid hydrolysate: Glu 1.00; Val 1.69 (average recovery 87.5%). *Anal.* Calcd for $C_{25}H_{36}N_6O_9\cdot H_2O$: C, 51.5; H, 6.33; N, 14.4. Found: C, 51.9; H, 6.58; N, 14.1.

Suc–Gln–Val–*p*NA (16)——The title compound was prepared from H–Gln–Val–*p*NA·HBr (prepared from 0.30 g of **9** and 0.6 ml of 25% HBr/AcOH) and succinic anhydride (0.17 g) in the same manner as described for the synthesis of **11**, yield 0.11 g (40.9%), mp 187—189 °C, $[\alpha]_D^{30}$ −32.3° (c=1.0, DMSO), Rf^3 0.27. Amino acid ratios in an acid hydrolysate: Glu 1.05; Val 1.00 (average recovery 99.4%). *Anal.* Calcd for $C_{20}H_{27}N_5O_8\cdot 1/2H_2O$: C, 50.6; H, 5.94; N, 14.8. Found: C, 50.6; H, 5.81; N, 14.7.

References and Notes

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2) Amino acids, peptides and their derivatives mentioned in this paper are of the L-configuration. 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: Z, benzyloxycarbonyl; Suc, succinyl; *p*NA, *p*-nitroanilide; OMe, methyl ester; ONp, *p*-nitrophenyl ester, DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide, THF, tetrahydrofuran; AcOH, acetic acid; Bz–Arg–βNA, N^{α}-benzoyl-D,L-Arg-2-naphthylamide.
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