Amino Acids and Peptides. XLIV. Synthesis of Stereoisomeric Pentapeptides of Thiol Proteinase Inhibitor [1,2]

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Received February 29, 1996; accepted April 8, 1996

Stereoisomers of thiol proteinase inhibitor (TPI) were synthesized by a conventional solution method. Among them, iNoc–D-Gln–Val–Val–Ala–Ala–*p*NA weakly inhibited the amidolytic activity of papain, although iNoc–Gln–Val–Val–Ala–Ala–*p*NA inhibited papain activity fairly potently. However, the other five D-amino acid-containing peptides did not show any inhibitory effects on papain. The circular dichroism (CD) spectra of the enzyme–peptides mixtures were measured in order to study the relationship between the peptide anilides-papain interaction and the inhibitory activity.

Key words thiol proteinase inhibitor; TPI common sequence; stereoisomeric pentapeptide; structure–activity relationship; circular dichroism spectra

We have previously synthesized various derivatives of Gln–Val–Val–Ala–Gly, which is a common sequence of endogenous thiol protease inhibitors (TPI).[3] Suc–Gln–Val–Val–Ala–Ala–*p*NA exhibited fairly potent inhibition of papain activity toward Bz–Arg–βNA in a dose-dependent manner (IC_{50}: 59 μM)[3] and iNoc–Gln–Val–Val–Ala–Ala–*p*NA showed similar inhibitory activity.[4] The circular dichroism (CD) spectra of these *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.[3,4] Furthermore, X-ray analysis of a crystal of the above Suc-pentapeptide-*p*NA-papain complex showed that the inhibitor is located at the R-domain site, not in the center of the binding site created by the R- and L-domains of papain.[5] It was also pointed out that the inhibitory activity of pentapeptide derivatives (**1** and **5**, see Table 1) decreased as a function of the time of interaction with the enzyme, presumably due to their enzymatic degradation. In an attempt to prevent such degradation, we decided to substitute constituent amino acids of the pentapeptide with D-amino acids to afford stereoisomers.

In this paper, we wish to report the synthesis of eight kinds of pentapeptides, *i.e.*, two parents and six stereoisomers (Table 1), by a conventional solution method, as well as studies on the relationship between the inhibitory activity and the interacting mode with thiol proteinase (papain) based on analysis of the CD spectra. Synthetic routes to the stereoisomeric pentapeptides are illustrated in Fig. 1. In contrast to our previous syntheses,[3,4,6] we employed predominantly the mixed anhydride method for stepwise elongation. For D-Gln coupling, we chose the new coupling reagent, HATU,[7] instead of the active ester method, because Boc–D-Gln–ON*p* was difficult to prepare and couple, and gave the desired product in only a low yield, although in the case of Z–L-Gln–ON*p*, it was possible to get the desired pentapeptide in a fairly good yield.[3] Possibly there is steric hindrance between Boc–D-Gln–ON*p* and tetrapeptide-*p*NA. All peptides obtained here were homogeneous upon silica gel thin-layer chromatography and reversed-phase HPLC. Amino acid ratios in acid hydrolysates (6 N HCl, 60 h) (Table 2) and the results of elemental analysis were in good agreement with theoretically expected values.

The inhibitory activities of these synthetic peptides against papain were determined with a synthetic substrate, Bz–Arg–βNA by means of the techniques previously described,[6] and are summarized in Fig. 2 and Fig. 3.

Those D-amino acid-containing derivatives did not show inhibitory activity except for iNoc–D-Gln–Val–Val–Ala–Ala–*p*NA (**8**), which showed a weak inhibitory activity compared with that of the parent compound (**5**). The duration of the inhibition activity of this compound (**8**) was tested, because within 60 min, the inhibitory activity of the parent compound (**5**) fell to 15% due to degradation by papain. Compound **8** exhibited a similar tendency to **5** in this regard (data not shown).

From Fig. 4, it can be seen that the CD spectra of **2** and **6**, which had no inhibitory activity against papain, showed similar differences between the calculated curve and the measured curve at around 330 nm, although **8**, which had a weak inhibitory activity showed a different measured curve from those of **2** and **6**, especially at around 330 nm. These observations suggested that the degree of the discrepancy between the calculated curve and the measured curve at around 330 nm is proportional to the potency of the inhibitory activity. Thus, manifestation of inhibitory activity might be due to interaction of the nitro group with some part of the enzyme.

Similarly, Fig. 5 illustrates the CD spectra of the mixtures of these stereoisomeric peptides (including cystatin) and papain. Compounds **1** and **5**, which fairly

Table 1. Synthetic Stereoisomeric Pentapeptides

Suc–Gln–Val–Val–Ala–Ala–*p*NA (**1**)	iNoc–Gln–Val–Val–Ala–Ala–*p*NA (**5**)
Suc–Gln–Val–Val–Ala–Ala–*p*NA (**2**)	iNoc–Gln–Val–Val–Ala–Ala–*p*NA (**6**)
Suc–Gln–Val–Val–Ala–Ala–*p*NA (**3**)	iNoc–Gln–Val–Val–Ala–Ala–*p*NA (**7**)
Suc–Gln–Val–Val–Ala–Ala–*p*NA (**4**)	iNoc–Gln–Val–Val–Ala–Ala–*p*NA (**8**)

—, D-Configuration.

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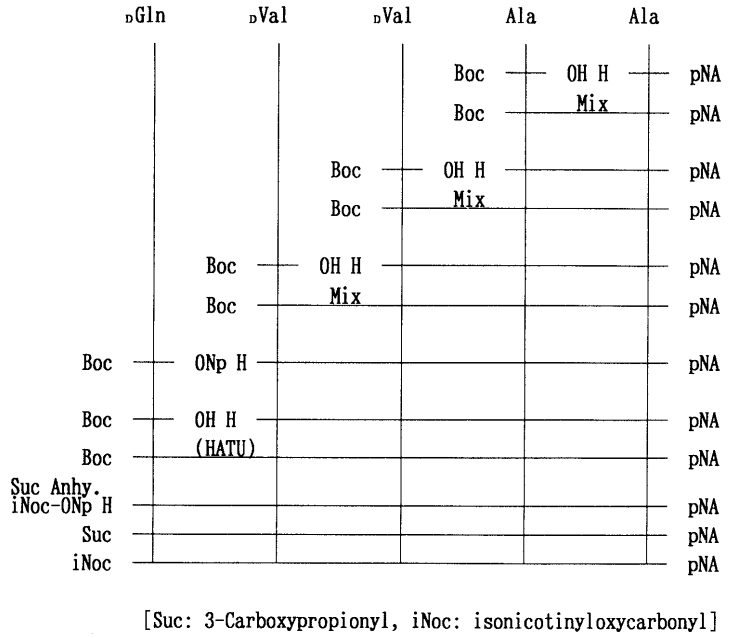


Fig. 1. Synthetic Scheme for the Pentapeptide *p*-Nitroanilide

Table 2. Amino Acid Analysis Data for Stereoisomeric Pentapeptides

	1	2	3	4	5	6	7	8
Glu	0.98	0.91	0.93	0.97	0.96	0.94	0.97	0.96
Val	1.52	2.04	2.05	1.91	1.92	1.91	1.92	1.94
Ala	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Recovery (%)	100	96.2	100	82.4	87.9	99.0	100	80.7

6 N HCl, 110 °C, 60 h.

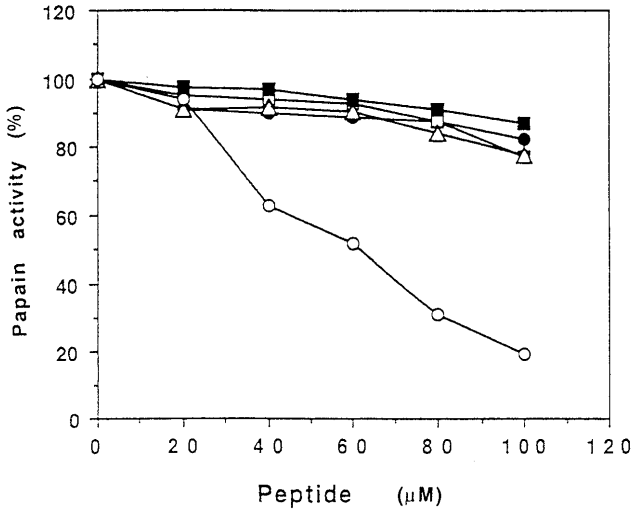


Fig. 2. Inhibitory Effects of Stereoisomeric Suc–Gln–Val–Val–Ala–Ala–*p*NA on Papain Activity

Suc–^{1}Gln–^{2}Val–^{3}Val–^{4}Ala–^{5}Ala–*p*NA

—●—, D-Ala-4; —□—, D-Val-3 (**2**); —■—, D-Val-2 (**3**); —△—, D-Gln-1 (**4**); —○—, All-L (**1**).

potently inhibited papain activity toward Bz–Arg–βNA, and compounds **2** and **6**, which were ineffective, showed different CD features at around 330 nm. On the other hand, compound **8**, which had a weak inhibitory activity, showed an intermediate pattern. Cystatin–papain interaction changed the spectral shape of cystatin appreciably at around 270—300 nm. It can be deduced that the cystatin

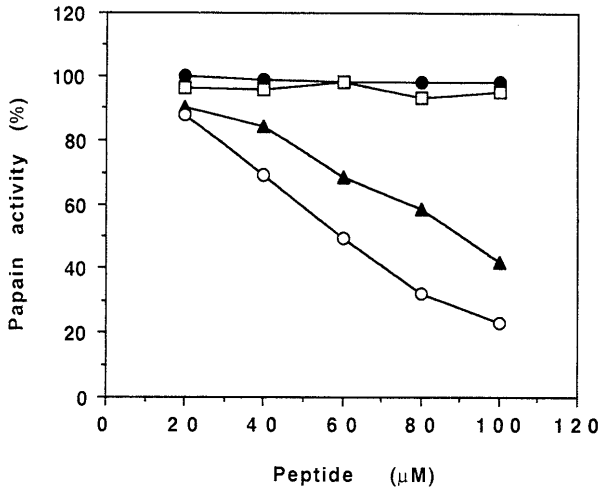


Fig. 3. Inhibitory Effects of Stereoisomeric iNoc–Gln–Val–Val–Ala–Ala–*p*NA on Papain Activity

iNoc–^{1}Gln–^{2}Val–^{3}Val–^{4}Ala–^{5}Ala–*p*NA

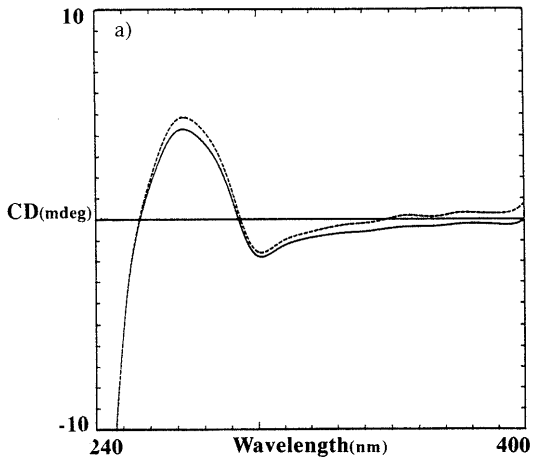
—●—, D-Val-3 (**6**); —□—, D-Val-2 (**7**); —▲—, D-Gln-1 (**8**); —○—, All-L (**5**).

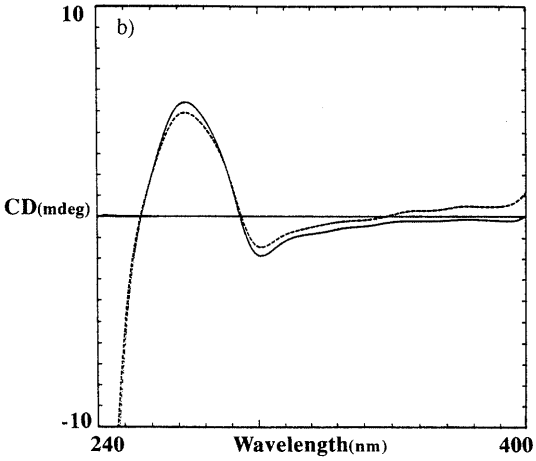
moiety strongly interacted with papain at a different region of papain from that at which pentapeptide anilides interact. These results are compatible with our previous findings.[3,4]

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, 60 h) were determined with an amino acid analyzer, K-101 AS (Kyowa Seimitsu Co., Ltd.). CD spectra were measured with a 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 (8:3:1, lower phase) and *n*-BuOH, AcOH, H_2O (4:1:5, upper phase). General experimental methods which were employed here are essentially the same as those described in Part XX[3] of this series.

N^{α}-Deprotection The N^{α}-protecting group, Boc, was cleaved by trifluoroacetic acid (TFA) (*ca.* 1 ml per 100 mg of peptide) at ice-bath





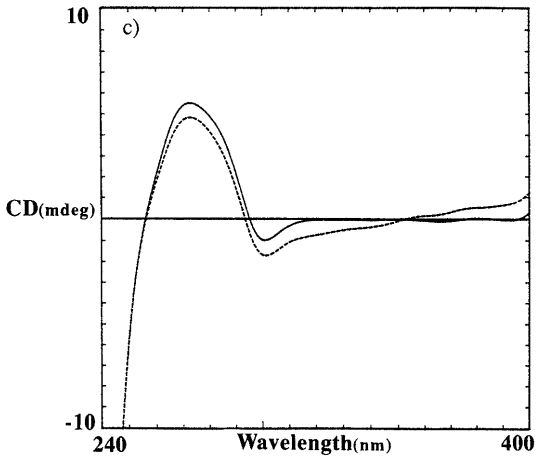


Fig. 4. Comparison of Observed and Calculated CD Spectra

a) —— measured curve, ------ calculated curve from the CD spectra of peptide **2** (19.5 μM) and papain (15 μM). b) —— measured curve, ------ calculated curve from the CD spectra of peptide **6** (19.5 μM) and papain (15 μM). c) —— measured curve, ------ calculated curve from the CD spectra of peptide **8** (19.5 μM) and papain (15 μM).

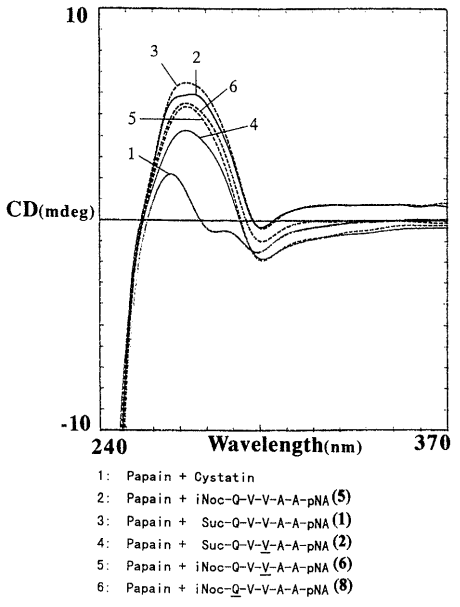


Fig. 5. CD Spectra of Papain Inhibitor Mixtures

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 for the condensation reaction. If an oily precipitate was obtained, it was washed with *n*-hexane, dried over KOH pellets *in vacuo* for 3 h and then used for the condensation reaction.

Condensation Reactions A mixed anhydride was prepared using isobutyl chloroformate and allowed to react with an amino component in an ice-bath overnight. $(Suc)_2O$ or iNoc-active ester condensation was performed at room temperature.

Purification Products were purified by the following procedures. After removal of the solvent, the residue was extracted with AcOEt, then the extract was washed with 10% citric acid, 5% Na_2CO_3 and saline, dried over $MgSO_4$ and concentrated. The residue was crystallized or precipitated from appropriate solvents (ether or hexane). HPLC was conducted with a Waters M-600 instrument equipped with a YMC $5C_{18}$ (4.6 × 250 mm) column using linear gradient elution with acetonitrile (10 to 40%, 20 min) in 0.5% TFA at a flow rate of 1.0 ml/min.

Boc–Val–Ala–Ala–*p*NA (Typical Mixed Anhydride Condensation Procedure) A solution of mixed anhydride [prepared from Boc–Val–OH (650 mg, 2.6 mmol) and isobutyl chloroformate (0.34 ml, 2.6 mmol) as usual] in THF (10 ml) was added to a solution of H–Ala–Ala–*p*NA. TFA [prepared from Boc–Ala–Ala–*p*NA[4] (1.0 g, 2.6 mmol) and TFA (1.9 ml, 26 mmol) as usual] in DMF (10 ml) containing Et_3N (0.36 ml, 2.6 mmol) under cooling with ice-salt. 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 10% citric acid, 5% Na_2CO_3 and saline, dried over $MgSO_4$ and evaporated down. Ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from AcOEt and ether. Yield 1.1 g (88.3%). Physical constants and analytical data are shown in Table 3.

Boc–D-Gln–Val–Val–Ala–Ala–*p*NA Three coupling methods were tested. The HATU method gave the desired peptide.

a) Active Ester Method: The reaction was poor; only the starting material was recovered. Boc–D-Gln–ON*p* and H–Val–Val–Ala–Ala–*p*NA. TFA [prepared from 1.24 g (2.14 mmol) of Boc–Val–Val–Ala–Ala–*p*NA and 4 ml of TFA] were dissolved in DMF (15 ml) containing NMM (0.4 ml, 3.64 mmol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, AcOEt was added to the residue to give crystals, which were collected by filtration. This material in MeOH (50 ml) was applied to a Sephadex LH-20 column (2.2 × 125 cm), equilibrated and eluted with MeOH. Individual fractions (7 g each) were collected. After removal of the solvent of the appropriate effluent (fraction Nos. 59—68), ether was added to the residue to give an amorphous powder. Yield 1.01 g (98.6%, starting material). Amino acid ratios in an acid hydrolysate (60 h): Ala 2.00; Val 1.83 (average

Table 3. Yield, Melting Point, $[\alpha]_D$ and Elemental Analysis of the Synthetic Peptides

Compound	Yield (%)	mp (°C)	$[\alpha]_D^{29}$	Formula	Elemental Analysis Calcd (Found)		
Boc–V–A–A–*p*NA	84.3	238—241	9.46 (c=0.4, DMF)	$C_{22}H_{33}N_5O_7 \cdot 2/5H_2O$	54.3	7.00	14.4
					(54.5	6.81	14.4)
Boc–V–A–A–*p*NA	88.3	105—112	−16.6 (c=1.0, DMF)	$C_{22}H_{33}N_5O_7$	55.1	6.94	14.6
					(54.8	6.99	14.5)
Boc–V–V–A–A–*p*NA	76.0	256—258	−9.61 (c=0.4, DMF)	$C_{27}H_{42}N_6O_8 \cdot 1/2H_2O$	55.2	7.38	14.3
					(55.5	7.26	14.5)
Boc–V–V–A–A–*p*NA	88.2	218—226	−29.1 (c=0.6, DMF)	$C_{27}H_{42}N_6O_8$	54.3	7.43	14.1
					(54.0	7.11	14.1)
Boc–V–V–A–A–*p*NA	69.8	203—205	−3.21 (c=0.4, DMF)	$C_{27}H_{42}N_6O_8$	56.0	7.32	14.5
					(55.8	7.20	14.6)
Boc–Q–V–V–A–A–*p*NA	26.8	215—222	−28.8 (c=0.4, DMSO)	$C_{32}H_{50}N_8O_{10} \cdot 3/2H_2O$	52.4	7.28	15.3
					(52.4	6.92	15.2)
Boc–Q–V–V–A–A–*p*NA	31.3	175—235	−27.0 (c=0.4, DMSO)	$C_{32}H_{50}N_8O_{10} \cdot 3/5H_2O$	53.5	7.18	15.6
					(53.7	7.27	15.0)
Boc–Q–V–V–A–A–*p*NA	68.2	Amorph.	−24.7 (c=0.4, DMSO)	$C_{32}H_{50}N_8O_{10} \cdot 3/2H_2O$	52.4	7.28	15.3
					(51.9	6.81	15.8)
Suc–Q–V–V–A–A–*p*NA (**1**)	66.5	>300	−1.5 (c=1.1, DMSO)	$C_{31}H_{46}N_8O_{11} \cdot 5/4H_2O$	51.0	6.54	15.3
					(51.2	6.39	15.0)
Suc–Q–V–V–A–A–*p*NA (**2**)	81.3	238—241	−30.4 (c=0.4, DMSO)	$C_{31}H_{46}N_8O_{11} \cdot 3/2H_2O$	50.7	6.73	15.3
					(50.4	6.36	15.2)
Suc–Q–V–V–A–A–*p*NA (**3**)	78.7	238—241	−29.9 (c=0.5, DMSO)	$C_{31}H_{46}N_8O_{11} \cdot H_2O$	51.4	6.68	15.5
					(51.2	6.64	15.4)
Suc–Q–V–V–A–A–*p*NA (**4**)	78.6	Amorph.	−29.2 (c=0.4, DMSO)	$C_{31}H_{46}N_8O_{11}$	52.7	6.56	15.9
					(52.5	6.52	15.9)
iNoc–Q–V–V–A–A–*p*NA (**5**)	86.3	286 dec.	−26.0 (c=0.5, DMF)	$C_{34}H_{47}N_9O_{10} \cdot H_2O$	53.7	6.50	16.6
					(54.0	6.40	16.8)
iNoc–Q–V–V–A–A–*p*NA (**6**)	79.3	266—271	−2.62 (c=0.4, DMSO)	$C_{34}H_{47}N_9O_{10} \cdot 5/2H_2O$	51.9	6.66	16.0
					(51.8	6.56	16.6)
iNoc–Q–V–V–A–A–*p*NA (**7**)	52.1	249—271	−27.8 (c=0.3, DMSO)	$C_{34}H_{47}N_9O_{10} \cdot 5/2H_2O$	51.9	6.66	16.0
					(51.6	6.48	16.7)
iNoc–Q–V–V–A–A–*p*NA (**8**)	67.5	255—262	−33.9 (c=0.4, DMSO)	$C_{34}H_{47}N_9O_{10} \cdot 5/2H_2O$	51.9	6.66	16.0
					(51.3	6.47	16.5)

—, D-Configuration.

recovery 83.1%).

b) DCC/HOBt Method: The above recovered material (1.01 g, 2.11 mmol), Boc-D-Gln–OH (571 mg, 2.53 mmol), DCC (522 mg, 2.53 mmol) and HOBt (285 mg, 2.11 mmol) were dissolved in DMF (10 ml), and the reaction mixture was stirred at room temperature overnight. After removal of the dicyclohexylurea and the solvent, MeOH was added to the residue to afford a gelatinous compound, which was collected by filtration. Yield 205 mg (13.8%). Rf^1 0.23, Rf^2 0.50, Amino acid ratios in an acid hydrolysate (60 h): Glu 0.90; Ala 2.00; Val 2.06 (average recovery 95.0%)

c) HATU Method: Boc-D-Gln–OH (296 mg, 1.2 mmol) and H–Val–Val–Ala–Ala–*p*NA. TFA (606 mg, 1 mmol) were dissolved in DMF (6 ml) containing NMM (0.165 ml, 1.5 mmol) and the mixture was cooled with an ice-bath. HATU (456 mg, 1.2 mmol) was added to the solution and the reaction mixture was stirred at room temperature overnight. After removal of the solvent, MeOH was added to the residue to afford crystals, which were collected by filtration and washed with hexane. Yield 482 mg (68.2%), Rf^1 0.23, Rf^2 0.50. Physical constants and analytical data are shown in Table 3.

Suc–Gln–Val–D-Val–Ala–Ala–*p*NA (General Procedure for the Preparation of Stereo-Isomeric Suc-Peptides) H–Gln–Val–D-Val–Ala–Ala–*p*NA. TFA [prepared from 250 mg (0.35 mmol) of Boc–Gln–Val–D-Val–Ala–Ala–*p*NA and 1.5 ml of TFA as usual] was dissolved in pyridine (3 ml) and DMF (2 ml) containing Et_3N (0.1 ml, 0.71 mmol). Succinic anhydride (88 mg, 0.88 mmol) was added to the above solution at 0 °C. The reaction mixture was stirred at room temperature overnight. After removal of the solvent, MeOH was added to the residue. Crystals formed were collected and washed with hexane. Yield 203 mg (81.3%), Rf^1 0.09, Rf^2 0.32. Physical constants and analytical data are shown in Table 3.

iNoc–Gln–D-Val–Val–Ala–Ala–*p*NA (General Procedure for the Preparation of Stereo-Isomeric iNoc-Peptides) H–Gln–D-Val–Val–Ala–Ala–*p*NA. TFA [prepared from 200 mg (0.28 mmol) of Boc–Gln–D-Val–Val–Ala–Ala–*p*NA and 1.0 ml of TFA as usual] was dissolved in DMF (5 ml) containing NMM (62 μl, 0.57 mmol)). iNoc–ON*p* (233 mg, 0.88 mmol) was added to the above solution and the reaction mixture was stirred at room temperature overnight. After removal of the solvent, AcOEt was added to the residue. Crystals formed were collected by filtration. The product was triturated with EtOH and then collected by filtration. Yield 109 mg (52.1%), Rf^2 0.53, Rf^3 0.45. Physical constants and analytical data are shown in Table 3.

References and Notes

1) Part XLIII: Okada Y., Mu Y., *J. Chem. Soc., Perkin Trans. 1*, "submitted."
2) The customary L indication for amino acid residues is omitted; only D isomers are indicated. 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; iNoc, isonicotinyl; *p*NA, *p*-nitroanilide; OMe, methyl ester; ON*p*, *p*-nitrophenyl ester; DMF, *N*,*N*-dimethylformamide; THF, tetrahydrofuran; AcOH, acetic acid; Bz–Arg–βNA, N^{α}-benzoyl-D,L-Arg-2-naphthylamide; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; NMM, *N*-methylmorpholine.
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