Amino Acids and Peptides. XXXIX. Synthesis of iNoc–Gln–Val–Val–Ala–Ala–*p*NA and Its Action on Thiol Proteinases[1,2]

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Based on the results of X-ray analysis of the complex between Suc–Gln–Val–Val–Ala–Ala–*p*NA, a fairly potent thiol proteinase inhibitor, and papain, iNoc–Gln–Val–Val–Ala–Ala–*p*NA was designed and prepared and its inhibitory activity against thiol proteinases was examined. iNoc–Gln–Val–Val–Ala–Ala–*p*NA inhibited cathepsin L fairly specifically, although its potency is not high.

Keywords Gln–Val–Val–Ala–Gly sequence; isonicotinyloxycarbonyl derivative; non-competitive inhibition; papain; cathepsin L

Thiol proteinases and endogenous thiol proteinase inhibitors function cooperatively to maintain homeostasis. However, an imbalance between thiol proteinases and natural inhibitors causes serious disorders such as muscular dystrophy,[3] osteoporosis,[4] tumor invasion[5] and so on. Therefore, low-molecular inhibitors which can specifically control the proteolytic activities of thiol proteinases could be powerful tools for investigating the roles of enzymes and could have clinical applications. Under these circumstances, our studies were directed to the development of potent and selective thiol proteinase inhibitors with small molecular size.

Thiol proteinase inhibitors, including kininogens isolated from various species, have a fairly conservative common amino acid sequence, Gln–Val–Val–Ala–Gly, as shown in Fig. 1. This site may be one of the reactive sites of thiol proteinase inhibitors.[6]

Therefore, we decided to mimick the Gln–Val–Val–Ala–Gly sequence. Although numerous attempts have been made to design synthetic inhibitory peptides which mimic the reactive sites of protein inhibitors of serine proteinases, our approach to the design of synthetic thiol proteinase inhibitors by mimicking the structure of natural thiol proteinase inhibitors is new. Previously, we prepared various kinds of Gln–Val–Val–Ala–Gly derivatives and studied the relationship between the structure and inhibitory activity.[7–10] Based on these studies, we prepared Suc–Gln–Val–Val–Ala–Ala–*p*NA,[11] which inhibited papain activity toward Bz–Arg–βNA with a K_i value of 1.0×10^{-5} M, and we studied its inhibitory mechanism by measuring CD spectra. In order to obtain further information about the interaction mode of the inhibitor and papain, we analyzed the crystal structure of the papain–Suc–Gln–Val–Val–Ala–Ala–*p*NA complex.[12]

As shown in Fig. 2, the peptide (a) is positioned at the R-domain side of the cleft created by the R- and L-domains. The Cys-25 of the active center of papain is located at the left side (L-domain side), where the Gln–Val–Val–Ala–Gly region of stefin B, a natural thiol proteinase inhibitor (b) is positioned.[13] Oxygen atoms of the *p*NA nitro group of the peptide are hydrogen-bonded to the amide NH_2 group of Gln-142 of papain. One of them further participates in an electrostatic interaction with the Ala-137 NH group. The NH group of Ala-5 of the peptide is linked with the Asp-158 C=O group by a hydrogen bond. Thus, the Ala–Ala–*p*NA moiety of the peptide is tightly bound with papain. In the Suc–Gln–Val–Val moiety of the peptide, hydrogen bond formation is possible between C=O of Gln-1 of the peptide and ε-NH_2 of Lys-156. Furthermore, this moiety is bound with pa-

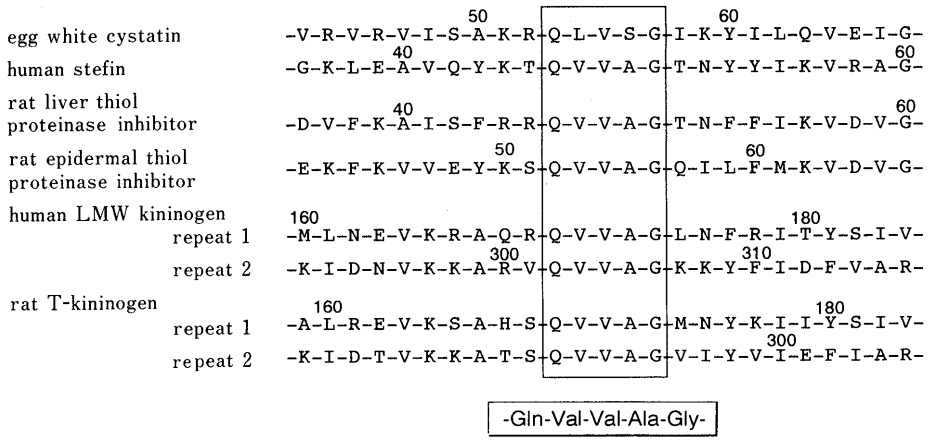

Fig. 1. Partial Structure of Thiol Proteinase Inhibitors

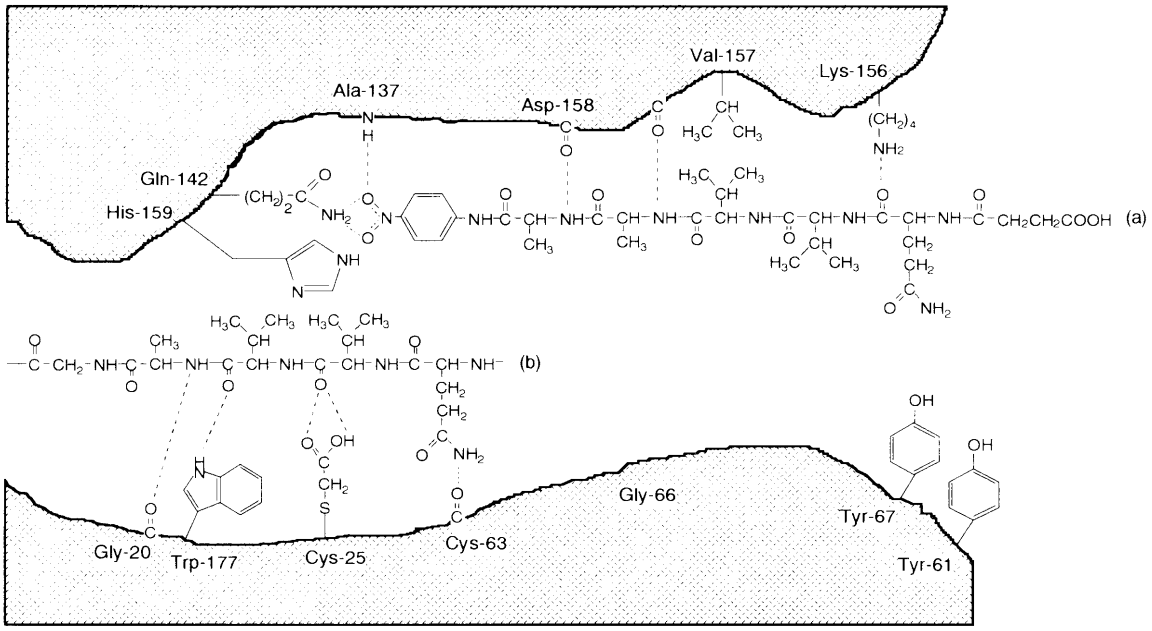

Fig. 2. Schematic Presentation of the Interaction Modes of Suc–Gln–Val–Val–Ala–Ala–*p*NA (a) and the Gln–Val–Val–Ala–Gly Region of Stefin B (b) with the Papain Active Site

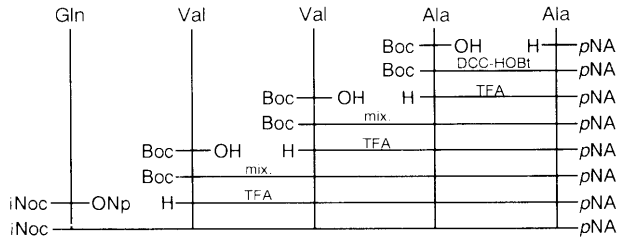

Fig. 3. Synthetic Scheme for iNoc Gln Val Val Ala Ala *p*NA

pain by hydrophobic interaction with Lys-156 and Val-157. Based on the results of X-ray analysis of the Suc–Gln–Val–Val–Ala–Ala–*p*NA–papain complex, we designed isonicotinyl (iNoc)–Gln–Val–Val–Ala–Ala–*p*NA, because we concidered that the aromatic ring of the iNoc group might stack with the phenyl ring of Tyr-61 or Tyr-67 of papain (Fig. 2). Previously, we prepared benzyloxycarbonyl (Z), benzoyl and naphthoyl derivatives and examined their inhibitory activity against papain.[7-11] Although these derivatives inhibited papain fairly potently, they showed insufficient solubility. Therefore, the iNoc group was selected in order to increase the solubility of the peptide.

According to the synthetic scheme shown in Fig. 3, iNoc–Gln–Val–Val–Ala–Ala–*p*NA was prepared.

As shown in Fig. 4a and 4b, the CD spectra of iNoc–Gln–Val–Val–Ala–Ala–*p*NA–papain complex were measured as in the case of the Suc derivative. Previously, we reported that the interaction of the *p*NA moiety of Suc–Gln–Val–Val–Ala–Ala–*p*NA and the R-domain of papain induced a CD spectral change at around 300 nm (see Fig. 4a).[11,12] From Fig. 4b, it can be deduced that C-terminal Ala–*p*NA moiety of the iNoc derivative interacts with papain in a similar manner to the Suc derivative. It can be seen that at the wavelength region around 270 nm, the spectrum measured for the iNoc–Gln–Val–Val–Ala–Ala–*p*NA and papain complex is slightly different from that calculated for an equimolar mixture of the inhibitor and papain (Fig. 4b). Lindahl *et al.* reported that the formation of chicken cystatin–papain complex caused a pronounced change in the region of 260—290 nm in the CD spectra.[14] Furthermore, it was demonstrated that the chicken cystatin exhibited inhibitory activity against papain by interacting with the R- and L-domains of papain.[15] Therefore, the decrease of the CD spectrum in the 270 nm region observed in the iNoc–Gln–Val–Val–Ala–Ala–*p*NA–papain complex might be due to the interaction of iNoc group with some part of papain.

Finally, the inhibitory activities of iNoc–Gln–Val–Val–Ala–Ala–*p*NA against various thiol proteinases were examined. This peptide inhibited papain non-competitively with a K_i value of 1.5×10^{-5} M, as shown in Fig. 5. Although Suc–Gln–Val–Val–Ala–Ala–*p*NA did not exhibit any specific inhibitory activity against cathepsin L, cathepsin B or cathepsin H (IC_{50} values 1.2, 0.72 mM and >0.5 mM, respectively),[16] iNoc–Gln–Val–Val–Ala–Ala–*p*NA inhibited cathepsin L specifically as summarized in Table I. These results suggest that cathepsin L has some region which can interact with the aromatic moiety of the iNoc group. Therefore, modification of this compound might generate a specific inhibitor of cathepsin L and a candidate drug for treatment of osteoporosis.

Experimental

The melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-360 (Japan Spectroscopic Co., Ltd.). CD spectra were measured with a JASCO J-20 spectropolarimeter. On TLC (Kieselgel G Merck), Rf^1, Rf^2, Rf^3 and Rf^4 values refer to the systems of $CHCl_3$, MeOH and AcOH (90:8:2), $CHCl_3$, MeOH and H_2O (89:10:1), $CHCl_3$, MeOH and H_2O (8:3:1, lower phase) and *n*-BuOH, pyridine, AcOH and H_2O (1:1:1:1), respectively.

Boc–Ala–Ala–*p*NA DCC (1.7 g, 8.4 mmol) was added to a solution

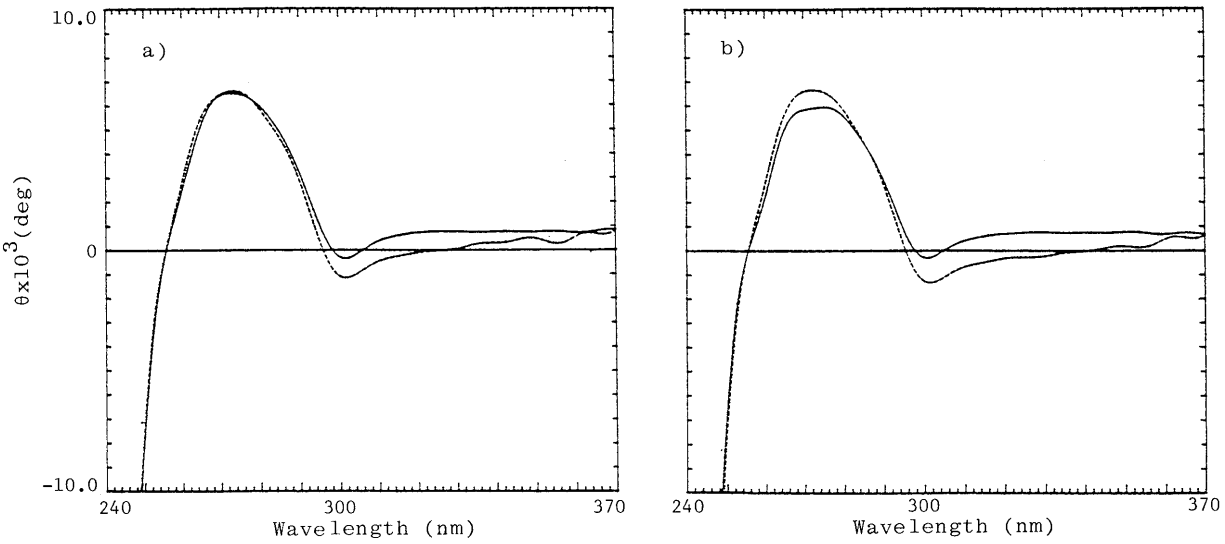

Fig. 4a. Comparison of the Observed and Calculated CD Spectra for a Mixture of Suc–Gln–Val–Val–Ala–Ala–*p*NA and Papain

——, measured curve; ------, calculated curve from CD spectra of Suc–Gln–Val–Val–Ala–Ala–*p*NA and papain.

Fig. 4b. Comparison of the Observed and Calculated CD Spectra for a Mixture of iNoc–Gln–Val–Val–Ala–Ala–*p*NA and Papain

——, measured curve; ------, calculated curve from CD spectra of iNoc–Gln–Val–Val–Ala–Ala–*p*NA and papain.

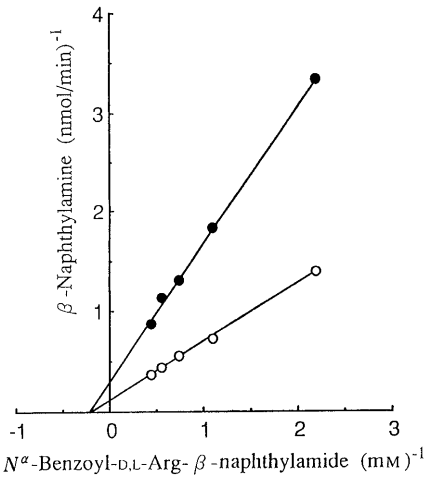

Fig. 5. Kinetic Plots of the Inhibition of Papain by iNoc–Gln–Val–Val–Ala–Ala–*p*NA

●: with inhibitor (iNoc–Gln–Val–Val–Ala–Ala–*p*NA, 40 μM). ○: without inhibitor.

TABLE I. Inhibition of Thiol Proteinases by iNoc–Gln–Val–Val–Ala–Ala–*p*NA

Concentration (M)	Inhibition (%)			
	Cathepsin L (rat liver)	Cathepsin L (human recombinant)	Cathepsin B (human liver)	Cathepsin H (rat liver)
10^{-6}	6.4	0	0	0
10^{-5}	20.1	8.6	0	0
10^{-4}	75.0	86.2	0	0

Each value indicates the mean of 3 observations.

of Boc–Ala–OH (1.3 g, 6.9 mmol), H–Ala–*p*NA · HBr [prepared from Z–Ala–*p*NA (2.38 g, 6.9 mmol) and 25% HBr/AcOH (6.5 ml, 20 mmol) as usual] and HOBt (0.93 g, 6.9 mmol) in DMF (50 ml) containing Et_3N (0.97 ml, 6.9 mmol) under cooling with ice-salt. The reaction mixture was stirred at room temperature overnight. After removal of DCC–urea and the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and concentrated to a small volume to afford crystals, which were collected by filtration, yield 2.1 g (80%), mp 197—200 °C, $[\alpha]_D^{25}$ −17.5° (c=2.0, DMF), Rf^1 0.88. *Anal.* Calcd for $C_{17}H_{24}N_4O_6$: C, 53.7; H, 6.36; N, 14.7. Found: C, 53.8; H, 6.41; N, 14.7.

Boc–Val–Ala–Ala–*p*NA 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 (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 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to give crystals, which were collected by filtration and recrystallized from AcOEt and ether, yield 1.1 g (88.3%), mp 105—112 °C, $[\alpha]_D^{25}$ −16.6° (c=1.2, DMF), Rf^1 0.67, Rf^2 0.62. *Anal.* Calcd for $C_{22}H_{33}N_5O_7$: C, 55.1; H, 6.94; N, 14.6. Found: C, 54.8; H, 6.99; N, 14.5.

Boc–Val–Val–Ala–Ala–*p*NA A solution of mixed anhydride [prepared from Boc–Val–OH (522 mg, 2.08 mmol) and isobutyl chloroformate (0.27 ml, 2.08 mmol) as usual] in THF (10 ml) was added to a solution of H–Val–Ala–Ala–*p*NA · TFA [prepared from Boc–Val–Ala–Ala–*p*NA (1.0 g, 2.08 mmol) and TFA (1.55 ml, 21 mmol) as usual] in DMF (10 ml) containing Et_3N (0.29 ml, 2.08 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 5% Na_2CO_3 and water and concentrated to a small volume. Ether was added to the residue to give crystals, which were collected by filtration, yield 950 mg (79.0%), mp 230—233 °C, $[\alpha]_D^{25}$ −28.3° (c=0.8, DMF), Rf^1 0.67. *Anal.* Calcd for $C_{27}H_{42}N_6O_8 \cdot H_2O$: C, 54.3; H, 7.43; N, 14.1. Found: C, 54.0; H, 7.11; N, 14.1.

iNoc–Gln–OH A solution of isonicotinyl *p*-nitrophenyl carbonate[17] (4.3 g, 15 mmol) in DMF (30 ml) was added to a solution of Gln (1.7 g, 12 mmol) in H_2O (30 ml) containing Et_3N (1.68 ml, 12 mmol) and the reaction mixture was stirred at room temperature for 48 h. After removal of the solvent, AcOEt and water were added to the residue. The water layer was evaporated down and MeOH was added to the residue to give a crude material. This material was applied to a silica gel column (3.0 × 31 cm), equilibrated and eluted with $CHCl_3$, MeOH and H_2O (16 : 3 : 1, lower phase 1.2 l), followed by 10% MeOH in $CHCl_3$. After removal of the solvent of the effluent (10% MeOH in $CHCl_3$ 2.4—4.0 l), petroleum ether was added to the residue to afford a precipitate, yield 1.29 g (40%), mp 154—159 °C, $[\alpha]_D^{25}$ −17.6° (c=0.6, DMF), Rf^3 0.10, Rf^4 0.62. *Anal.* Calcd for $C_{12}H_{15}N_3O_5 \cdot 2/3H_2O$: C, 49.1; H, 5.20; N, 14.3. Found: C, 49.2; H, 5.44; N, 14.2.

iNoc–Gln–ONp DCC (0.52 g, 2.5 mmol) was added to a solution of iNoc–Gln–OH (0.56 g, 2.0 mmol) and *p*-nitrophenol (0.29 g, 2.1 mmol) in DMF (50 ml) under cooling with ice-salt. The reaction mixture was stirred at 4 °C overnight. After removal of DCC–urea and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to give an amorphous powder, which was collected and washed with AcOEt, yield 500 mg (62%), mp 133—135 °C, $[\alpha]_D^{25}$ −24.3° (c=0.8, DMF), Rf^1 0.20, Rf^3 0.65. *Anal.* Calcd for $C_{18}H_{18}N_4O_7$: C, 53.7; H, 4.51; N, 13.9. Found: C, 54.1; H, 4.85; N, 13.8.

iNoc–Gln–Val–Val–Ala–Ala–*p*NA iNoc–Gln–ONp (100 mg, 0.25 mmol) and H–Val–Val–Ala–Ala–*p*NA · TFA [prepared from Boc–Val–Val–Ala–Ala–*p*NA (145 mg, 0.25 mmol) and TFA (0.185 ml, 2.5 mmol) as usual] were dissolved in DMF (20 ml) containing Et_3N (0.035 ml, 0.25 mmol). The reaction mixture was stirred at room temperature for 48 h. After removal of the solvent, AcOEt and water were added to the residue to afford crystals, which were collected by filtration, yield 160 mg (86.3%), mp 286 °C (dec.), $[\alpha]_D^{25}$ −26.0° (c=0.5, DMF), Rf^3 0.68. *Anal.* Calcd for $C_{34}H_{47}N_9O_{10} \cdot H_2O$: C, 53.7; H, 6.50; N, 16.6. Found: C, 54.0; H, 6.40; N, 16.8.

Assay Procedure Rat liver cathepsins B, H and L were purified according to the published methods.[18-20] Cathepsin activities were measured with Z–Arg–Arg–MCA at pH 6.0 for cathepsin B, Arg–MCA at pH 7.0 for cathepsin H and Z–Phe–Arg–MCA at pH 5.5 for cathepsin L, by the method of Barrett and Kirschke.[21] An inhibitor was pre-incubated with the enzyme and 8 mM cysteine hydrochloride for 5 min and then the reaction was started by addition of the substrate. The activity of each enzyme was adjusted to 0.5 units (one unit corresponds to the production of 1 μmol of 7-amino-4-methylcoumarin per min from 20 μM substrate at 37 °C). The fluorescence of 7-amino-4-methylcoumarin liberated from the substrate was monitored by a Hitachi fluorescence spectrometer.

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; iNoc, isonicotinyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Suc, succinyl; *p*NA, *p*-nitroanilide; ONp, *p*-nitrophenyl ester; DCC, *N,N'*-dicyclohexylcarbodiimide; HOBt, *N*-hydroxybenzotriazole; DMF, *N,N*-dimethylformamide; AcOH, acetic acid; Bz–Arg–βNA, N^{α}-benzoyl-D,L-Arg-2-naphthylamide; MCA, 7-amino-4-methyl coumarin; CD, circular dichroism; THF, tetrahydrofuran; TFA, trifluoroacetic acid.
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