Amino Acids and Peptides. XXX. Synthesis of Eglin c (41—49) and Eglin c (60—63) and Examination of Their Inhibitory Activity towards Human Leukocyte Elastase, Cathepsin G, Porcine Pancreatic Elastase and α-Chymotrypsin[1,2]

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H–Ser–Pro–Val–Thr–Leu–Asp–Leu–Arg–Tyr–OH and H–Thr–Asn–Val–Val–OH, which correspond to the sequences 41—49 and 60—63 of eglin c, respectively, were synthesized by a conventional solution approach using the newly developed 6-chloro-2-pyridyl ester method. The inhibitory activities of the above two peptides against human leukocyte elastase, cathepsin G, porcine pancreatic elastase and α-chymotrypsin were examined in comparison with those of the corresponding methyl esters.

Keywords eglin c-related peptide; chemical synthesis; inhibitory activity; human leukocyte elastase; cathepsin G; porcine pancreatic elastase; α-chymotrypsin

Eglin c, isolated from the leech *Hirudo medicinalis*,[3] consists of 70 amino acid residues[4] (Fig. 1) and effectively inhibits chymotrypsin and subtilisin as well as leukocyte elastase and cathepsin G. The latter two enzymes have attracted our interest due to their possible involvement in connective tissue turnover and diseases such as emphysema, rheumatoid arthritis and inflammation.[5,6]

Previously, we reported that a small peptide, H–Ser–Pro–Val–Thr–Leu–Asp–Leu–Arg–Tyr–OMe, which corresponds to the sequence 41—49 of eglin c, exhibited significant inhibitory activity against cathepsin G and α-chymotrypsin but not against leukocyte elastase, while the peptide H–Thr–Asn–Val–Val–OMe, which corresponds to the sequence 60—63 of eglin c, inhibited leukocyte elastase but not cathepsin G or α-chymotrypsin.[7]

This paper deals with the synthesis of H–Ser–Pro–Val–Thr–Leu–Asp–Leu–Arg–Tyr–OH and H–Thr–Asn–Val–Val–OH by using the newly developed 6-chloro-2-pyridyl active ester method and with an examination of their inhibitory activity against human leukocyte elastase, cathepsin G, α-chymotrypsin and porcine pancreatic elastase in order to study the roles of the C-terminal methyl ester groups of the above two peptides in the manifestation of the inhibitory activity.

According to the scheme shown in Fig. 2, the nonapeptide corresponding to the sequence 41—49 of eglin c was synthesized. Boc–Arg(Mts)–OH and H–Tyr–OBzl were coupled by the DPPA method[8] to avoid lactam formation,[9] to give Boc–Arg(Mts)–Tyr–OBzl. After removal of the Boc group by TFA treatment, Boc–Leu–OPyCl, Boc–Asp(OBzl)–OPyCl, Boc–Leu–OPyCl, Boc–Thr–ONSu, Boc–Val–OPyCl and Boc–Pro–OPyCl were added successively to afford Boc–Pro–Val–Thr–Leu–Asp(OBzl)–Leu–Arg(Mts)–Tyr–OBzl. After removal of the Boc group, Boc–Ser–$NHNH_2$ was coupled by the azide method[10] to give a protected nonapeptide. Boc-amino acid 6-chloro-2-pyridyl

H–Thr–Glu–Phe–Gly–Ser–Glu–Leu–Lys–Ser–Phe–Pro–Glu–Val–Val–Gly–Lys–Thr–Val–Asp–Gln–Ala–Arg–Glu–Tyr–Phe–Thr–Leu–His–Tyr–Pro–Gln–Tyr–Asp–Val–Tyr–Phe–Leu–Pro–Glu–Gly–Ser–Pro–Val–Thr–Leu–Asp–Leu–Arg–Tyr–Asn–Arg–Val–Arg–Val–Phe–Tyr–Asn–Pro–Gly–Thr–Asn–Val–Val–Asn–His–Val–Pro–His–Val–Gly–OH

Fig. 1. Structure of Eglin c

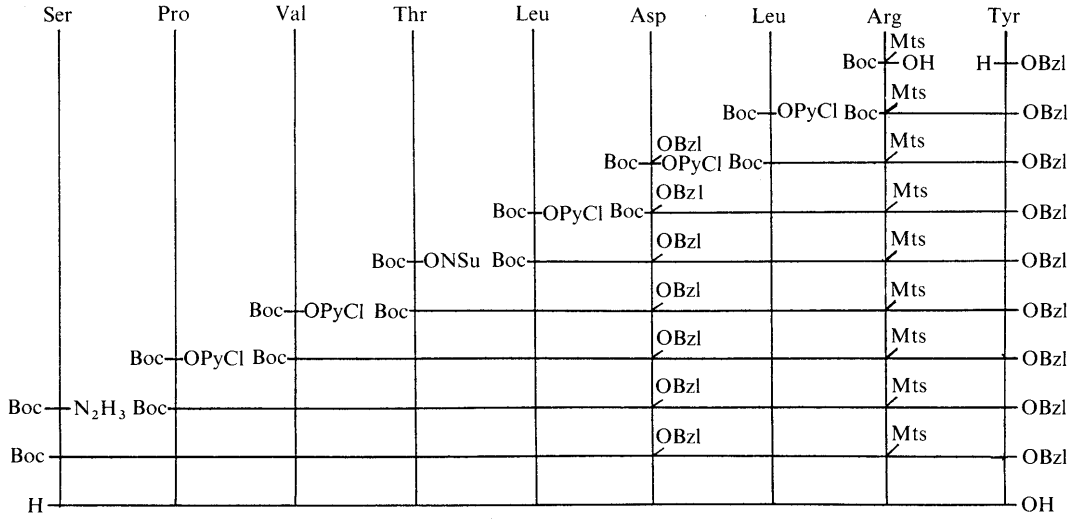

Fig. 2. Synthetic Scheme for H-(Eglin c 41—49)-OH

TABLE I. K_i Values of Eglin c (41—49) and Eglin c (60—63)

	K_i (M)			
	LE[a]	Cathepsin G[b]	α-Chymotrypsin[b]	PE[c]
H–(41—49)–OH·AcOH	ND	3.7×10^{-5}	7.2×10^{-5}	2.7×10^{-4}
H–(41—49)–OMe·2AcOH	ND	4.0×10^{-5}	2.0×10^{-5}	3.0×10^{-4}
H–(60—63)–OH	2.3×10^{-3}	ND	ND	4.8×10^{-3}
H–(60—63)–OMe·HCl	1.6×10^{-4}	ND	ND	ND

a) The substrate for LE (leukocyte elastase) was Suc–Ala–Tyr–Leu–Val–*p*NA. *b*) The substrate for cathepsin G and α-chymotrypsin was Suc–Ile–Pro–Phe–*p*NA. *c*) The substrate for PE (pancreatic elastase) was Suc–Ala–Ala–Ala–*p*NA. ND: not detectable ($K_i > 5.0\times10^{-3}$ M).

esters[11] reacted with amino groups very rapidly and the liberated pyridinol derivative was easily removed. Homogeneity of protected peptide intermediates was ascertained by thin layer chromatography (TLC), elemental analysis and amino acid analysis. The protected nonapeptide was treated with HF at 0 °C in the presence of thioanisole and *m*-cresol to give the desired peptide H–Ser–Pro–Val–Thr–Leu–Asp–Leu–Arg–Tyr–OH.

H–Thr–Asn–Val–Val–OH was synthesized as follows: Boc–Val–OPyCl and H–Val–OBzl were coupled to give Boc–Val–Val–OBzl. After removal of the Boc group, Boc–Asn–ONp and Z–Thr–OH were coupled successively by the active ester method and the DPPA method, respectively, to give Z–Thr–Asn–Val–Val–OBzl. This protected peptide was hydrogenated over a palladium catalyst to give H–Thr–Asn–Val–Val–OH. Both deprotected peptides were purified by gel-filtration on Sephadex G-25 using 3% AcOH as an eluant. The homogeneity of the peptides was ascertained by TLC, high performance liquid chromatography (HPLC) and amino acid analysis.

Next, the inhibitory activities of the above two peptides towards human leukocyte elastase, cathepsin G, α-chymotrypsin and porcine pancreatic elastase were examined and the results are summarized in Table I in comparison with those for the corresponding methyl esters.[7] Leukocyte elastase and pancreatic elastase preferentially split valyl and alanyl bonds, respectively, and therefore they have quite different substrate specificities from those of cathepsin G and α-chymotrypsin.

H–Ser–Pro–Val–Thr–Leu–Asp–Leu–Arg–Tyr–OH exhibited similar inhibitory activity to the corresponding methyl ester towards cathepsin G and α-chymotrypsin, supporting the previous conclusion that the Leu^{45}–Asp^{46} sequence of eglin c is a reactive site and important for the manifestation of the inhibitory activity.[12] It is of interest that although the above two peptides did not show inhibitory activity against leukocyte elastase, they inhibited porcine pancreatic elastase with K_i values of 2.7×10^{-4} and 3.0×10^{-4} M, respectively, suggesting differences in the structure of the active center between leukocyte elastase and pancreatic elastase.

The inhibitory activity of H–Thr–Asn–Val–Val–OH towards leukocyte elastase was decreased compared with that of the corresponding methyl ester. Both peptides exhibited weaker inhibitory activity towards pancreatic elastase than towards leukocyte elastase.

These results cast light on the enzymatic differences between leukocyte elastase and pancreatic elastase and suggest the feasibility of developing small-molecular inhibitors of leukocyte elastase for practical therapeutic use by modifying the methyl group.

Experimental

The melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-360 (Japan Spectroscopic Co.). Amino acid compositions of an acid hydrolysate (6 N HCl, 110 °C, 20 h) were determined with an amino acid analyzer, K-101 AS (Kyowa Seimitsu Co.). HPLC was conducted with a Waters M 600 instrument [columns: YMC-PACK A-312 ODS (6×150 mm) and YMC-PACK D-ODS-5 (20×250 mm)]. On TLC (Kieselgel G, Merck), Rf^1, Rf^2, Rf^3, Rf^4 and Rf^5 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), *n*-BuOH, pyridine, AcOH and H_2O (4:1:1:2), *n*-BuOH, pyridine, AcOH and H_2O (1:1:1:1) and $CHCl_3$, respectively.

General Procedure for Synthesis of Boc-Amino Acid 6-Chloro-2-Pyridyl Esters An N^{α}-protected amino acid (8.5 mmol) and 6-chloro-2-hydroxypyridine (1.10 g, 8.5 mmol) were dissolved in DMF (30 ml) and the solution was cooled with ice-salt. DCC (2.10 g, 10.2 mmol) was added, and the reaction mixture was stirred at 4 °C overnight. After removal of dicyclohexylurea and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated down *in vacuo* to give the title compound. Boc–Asp(OBzl)–OPyCl: yield 75.4%, oil, $[\alpha]_D^{27}$ −3.4° ($c=1.0$, benzene), Rf^1 0.81, Rf^5 0.17. Boc–Pro–OPyCl: yield 78.5%, oil, $[\alpha]_D^{27}$ −1.0° ($c=1.0$, benzene), Rf^5 0.38.

Boc–Arg(Mts)–Tyr–OBzl DPPA (1.4 ml, 5.2 mmol) and Et_3N (1.3 ml, 9.5 mmol) were added to a solution of Boc–Arg(Mts)–OH (1.98 g, 4.3 mmol) and H–Tyr–OBzl·Tos–OH (1.91 g, 4.3 mmol) in DMF (20 ml) under cooling with ice. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and H_2O, dried over Na_2SO_4 and evaporated down. The crude material was purified by silica gel column (2.3×44 cm) chromatography to give crystals, yield 1.90 g (63.4%), mp 80—90 °C, $[\alpha]_D^{27}$ −8.5° ($c=1.0$, MeOH), Rf^1 0.6, Rf^2 0.91. *Anal.* Calcd for $C_{36}H_{47}N_5O_8S\cdot3/2H_2O$: C, 58.7; H, 6.85; N, 9.50. Found: C, 58.7; H, 6.67; N, 9.46.

Boc–Leu–Arg(Mts)–Tyr–OBzl Boc–Leu–OPyCl[11] (1.11 g, 3.2 mmol) and H–Arg(Mts)–Tyr–OBzl [prepared from Boc–Arg(Mts)–Tyr–OBzl (1.8 g, 2.5 mmol), TFA (2.0 ml, 27 mmol), anisole (0.59 ml, 5.4 mmol) and *m*-cresol (0.57 ml, 5.4 mmol) as usual] were dissolved in DMF (30 ml) containing Et_3N (0.38 ml, 2.5 mmol). 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, 10% citric acid and H_2O, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to afford crystals, which were collected by filtration, yield 2.03 g (99.9%), mp 51.5—65 °C, $[\alpha]_D^{27}$ −19.7° ($c=1.0$, MeOH), Rf^1 0.47. *Anal.* Calcd for $C_{42}H_{58}N_6O_9S\cdot1/2H_2O$: C, 60.0; H, 7.26; N, 10.2. Found: C, 60.1; H, 7.38; N, 10.3.

Boc–Asp(OBzl)–Leu–Arg(Mts)–Tyr–OBzl The title compound was prepared from Boc–Asp(OBzl)–OPyCl (0.95 g, 2.8 mmol) and H–Leu–Arg(Mts)–Tyr–OBzl [prepared from Boc–Leu–Arg(Mts)–Tyr–OBzl (1.81 g, 2.3 mmol) and TFA (1.8 ml, 23.0 mmol)]. The crude product was purified by silica gel column (2.5×28.7 cm) chromatography, yield 1.34 g (57.5%), mp 88.5—91 °C, $[\alpha]_D^{27}$ −26.4° ($c=1.0$, MeOH), Rf^1 0.67. *Anal.* Calcd for $C_{53}H_{69}N_7O_{12}S$: C, 61.4; H, 6.86; N, 9.64. Found: C, 61.7; H, 6.96; N, 9.44.

Boc–Leu–Asp(OBzl)–Leu–Arg(Mts)–Tyr–OBzl The title compound was prepared from Boc–Leu–OPyCl[11] (0.45 g, 1.3 mmol) and H–Asp(OBzl)–Leu–Arg(Mts)–Tyr–OBzl [prepared from Boc–Asp(OBzl)–Leu–Arg(Mts)–Tyr–OBzl (1.1 g, 1.1 mmol) and TFA (1.3 ml, 16.5 mmol)], yield

1.18 g (95.2%), mp 94.5—102.5 °C, $[\alpha]_D^{27}$ −29.0° (c=0.3, MeOH), Rf^1 0.61. *Anal.* Calcd for $C_{59}H_{80}N_8O_{13}S$: C, 61.7; H, 7.15; N, 9.92. Found: C, 61.4; H, 7.21; N, 9.74.

Boc–Thr–Leu–Asp(OBzl)–Leu–Arg(Mts)–Tyr–OBzl The title compound was prepared from Boc–Thr–ONSu (185 mg, 0.58 mmol) and H–Leu–Asp(OBzl)–Leu–Arg(Mts)–Tyr–OBzl [prepared from Boc–Leu–Asp(OBzl)–Leu–Arg(Mts)–Tyr–OBzl (549 mg, 0.486 mmol) and TFA (0.4 ml, 4.860 mmol)], yield 394 mg (65.9%), mp 114—121 °C, $[\alpha]_D^{27}$ −29.5° (c=0.5, MeOH), Rf^1 0.57, Rf^2 0.67. *Anal.* Calcd for $C_{63}H_{87}N_9O_{15}S$: C, 60.5; H, 7.14; N, 10.2. Found: C, 60.3; H, 7.07; N, 10.1.

Boc–Val–Thr–Leu–Asp(OBzl)–Leu–Arg(Mts)–Tyr–OBzl The title compound was prepared from Boc–Val–OPyCl[11] (136 mg, 0.465 mmol) and H–Thr–Leu–Asp(OBzl)–Leu–Arg(Mts)–Tyr–OBzl [prepared from Boc–Thr–Leu–Asp(OBzl)–Leu–Arg(Mts)–Tyr–OBzl (382 mg, 0.310 mmol) and TFA (0.3 ml, 3.1 mmol)], yield 213 mg (51.7%), mp 205—209 °C, $[\alpha]_D^{27}$ −21.5° (c=1.0, DMF), Rf^1 0.54. *Anal.* Calcd for $C_{68}H_{96}N_{10}O_{16}S \cdot 3/2H_2O$: C, 59.6; H, 7.44; N, 10.2. Found: C, 59.3; H, 7.22; N, 10.4.

Boc–Pro–Val–Thr–Leu–Asp(OBzl)–Leu–Arg(Mts)–Tyr–OBzl The title compound was prepared from Boc–Pro–OPyCl (55 mg, 0.16 mmol) and H–Val–Thr–Leu–Asp(OBzl)–Leu–Arg(Mts)–Tyr–OBzl [prepared from Boc–Val–Thr–Leu–Asp(OBzl)–Leu–Arg(Mts)–Tyr–OBzl (150 mg, 0.113 mmol) and TFA (0.17 ml, 2.26 mmol)], yield 27 mg (16.9%), mp 183—190 °C, $[\alpha]_D^{27}$ −24.0° (c=0.5, DMF), Rf^1 0.58, Rf^2 0.68. *Anal.* Calcd for $C_{73}H_{103}N_{11}O_{17}S$: C, 60.9; H, 7.23; N, 10.7. Found: C, 60.8; H, 7.32; N, 10.5.

Boc–Ser–Pro–Val–Thr–Leu–Asp(OBzl)–Leu–Arg(Mts)–Tyr–OBzl The title compound was prepared from Boc–Ser–N_3 [prepared from Boc–Ser–$NHNH_2$ (30 mg, 0.137 mmol) and isopentyl nitrite (0.019 ml, 0.137 mmol)] and H–Pro–Val–Thr–Leu–Asp(OBzl)–Leu–Arg(Mts)–Tyr–OBzl [prepared from Boc–Pro–Val–Thr–Leu–Asp(OBzl)–Leu–Arg(Mts)–Tyr–OBzl (163 mg, 0.114 mmol) and TFA (0.17 ml, 2.28 mmol)], yield 115 mg (54.0%), mp 130—139 °C, $[\alpha]_D^{27}$ −25.3° (c=1.0, DMF), Rf^1 0.45, Rf^2 0.67. *Anal.* Calcd for $C_{76}H_{110}N_{12}O_{19}S \cdot H_2O$: C, 59.0; H, 7.32; N, 10.9. Found: C, 58.8; H, 7.27; N, 10.8.

H–Ser–Pro–Val–Thr–Leu–Asp–Leu–Arg–Tyr–OH The protected nonapeptide (50 mg, 0.026 mmol) was treated with HF (10 ml) in the presence of thioanisole (0.30 ml) and *m*-cresol (0.1 ml) in an ice-bath for 90 min. After removal of HF, dry ether was added to the residue to afford crystals, which were collected by filtration and dissolved in H_2O. The pH of the solution was adjusted to 8 with 1 M NH_4OH and the solution was kept at 4 °C for 30 min and applied to a column of Sephadex G-25 (2.2 × 58.7 cm), which was equilibrated and eluted with 3% AcOH. The fractions (3 g each, tube Nos. 41—49) were combined and the solvent was removed by lyophilization to give a fluffy powder. The crude product was purified by preparative HPLC, yield 23 mg (83%), $[\alpha]_D^{27}$ −88.9° (c=0.1, 5% AcOH). Amino acid ratios in an acid hydrolysate: Ser (1) 0.88; Pro (1) 1.20; Val (1) 0.97; Thr (1) 0.89; Leu (2) 2.00; Asp (1) 0.84; Arg (1) 0.70; Tyr (1) 0.92 (average recovery 74.3%).

Boc–Val–Val–OBzl The title compound was prepared from Boc–Val–OPyCl[11] (1.50 g, 4.6 mmol) and H–Val–OBzl·Tos–OH (1.16 g, 3.0 mmol), yield 1.80 g (97.0%), mp 51—55 °C, $[\alpha]_D^{27}$ −46.5° (c=1.2, MeOH), Rf^1 0.77. *Anal.* Calcd for $C_{22}H_{34}N_2O_5$: C, 65.0; H, 8.45; N, 6.89. Found: C, 65.2; H, 8.67; N, 6.78. [*p*-Nitrophenyl ester method: yield 76%, oily material, $[\alpha]_D^{27}$ −44.0° (c=1.0, MeOH), Rf^1 0.77].

Boc–Asn–Val–Val–OBzl The title compound was prepared from Boc–Asn–ONp and H–Val–Val–OBzl [prepared from Boc–Val–Val–OBzl (1.1 g, 2.7 mmol) and 7.2 N HCl–dioxane (2.0 ml, 13.7 mmol)], yield 0.70 g (48.9%), mp 193—197 °C, $[\alpha]_D^{27}$ −56.3° (c=1.0, MeOH), Rf^1 0.69. *Anal.* Calcd for $C_{26}H_{40}N_4O_7$: C, 60.0; H, 7.76; N, 10.8. Found: C, 59.9; H, 7.69; N, 10.7.

Z–Thr–Asn–Val–Val–OBzl DPPA (0.48 ml, 1.7 mmol) and Et_3N (0.45 ml, 3.2 mmol) were added to a solution of Z–Thr–OH (0.36 g, 1.4 mmol) and H–Asn–Val–Val–OBzl [prepared from Boc–Asn–Val–Val–OBzl (0.50 g, 1.0 mmol) and 7.2 N HCl–dioxane (0.67 ml, 4.8 mmol)] in DMF (7 ml) under cooling with ice. 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, yield 0.47 g (75.4%), mp 230—232 °C, $[\alpha]_D^{27}$ −26.0° (c=1.0, DMF), Rf^1 0.78. *Anal.* Calcd for $C_{33}H_{45}N_5O_9 \cdot 1/2H_2O$: C, 59.6; H, 6.99; N, 10.5. Found: C, 59.7; H, 6.95; N, 10.8.

H–Thr–Asn–Val–Val–OH Z–Thr–Asn–Val–Val–OBzl (0.10 g, 0.15 mmol) in DMF (3 ml) was hydrogenated over Pd catalyst. After removal of Pd and the solvent, the residue was dissolved in H_2O and lyophilized to give a fluffy powder, yield 56 mg (85.7%), $[\alpha]_D^{27}$ −50.0° (c=0.1, MeOH), Rf^3 0.47, Rf^4 0.87. Amino acid ratios in an acid hydrolysate: Thr (1) 0.90; Asp (1) 1.00; Val (2) 1.78; NH_3 (1) 1.20 (average recovery 75.3%).

Assay Procedure Human leukocyte elastase[13] and cathepsin G[14] were prepared in our laboratory according to the procedure described previously. α-Chymotrypsin was purchased from Miles Co., Ltd., Elkhart. Porcine pancreatic elastase was purchased from Sigma Chemical Co., St. Louis. Enzymatic activities of leukocyte elastase, porcine pancreatic elastase, cathepsin G and α-chymotrypsin were assayed by the method described previously using Suc–Ala–Tyr–Leu–Val–*p*NA[15] for leukocyte elastase, Suc–Ala–Ala–Ala–*p*NA for porcine pancreatic elastase and Suc–Ile–Pro–Phe–*p*NA[16] for cathepsin G and α-chymotrypsin. For determination of the effects of synthetic peptides on the enzymes, the enzymatic activity towards each substrate was assayed in the presence and in the absence of the peptide to be examined.

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References and Note

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