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Synthesis of Six Common Amino Acid Sequence Fragments of Thymosins β_4, β_8 and β_9 and Determination of Their Effects on the Low E-Rosette Forming Cells of Lupus Nephritis Patients[1)]

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Six common amino acid sequence fragments of thymosins β_4, β_8 and β_9, H-Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-OH (positions 16—26), H-Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-OH (positions 31—39), H-Asp-Lys-Pro-Asp-OH (positions 2—5), H-Phe-Asp-Lys-OH (positions 12—14), H-Leu-Pro-OH (positions 28—29) and H-Glu-Ile-OH (positions 8—9), were synthesized by the solution method, and were tested to determine their effects on the low E-rosette forming cells of lupus nephritis patients.

Two of the fragments, H-Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-OH and H-Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-OH, increased E-rosette forming capacity when incubated *in vitro* with patient's blood though they were less effective than thymosin β_9, but the other four peptide fragments, H-Asp-Lys-Pro-Asp-OH, H-Phe-Asp-Lys-OH, H-Leu-Pro-OH and H-Glu-Ile-OH, had no effect.

Keywords——thymosin β_4; thymosin β_8; thymosin β_9; lupus nephritis patient; low E-rosette forming cell; six fragments of thymosin β_9; *N*-hydroxybenzotriazole-dicyclohexylcarbodiimide procedure; -Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-; -Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-

Thymosin fraction 5 contains at least 30 different polypeptides and is active in several different *in vitro* bioassays which assess the induction of mature T-cell surface characteristics and T-cell functional properties.[2)] One of the thymosin polypeptides isolated from bovine fraction 5 has been termed thymosin β_4.[3)] This peptide exhibits several biological activities

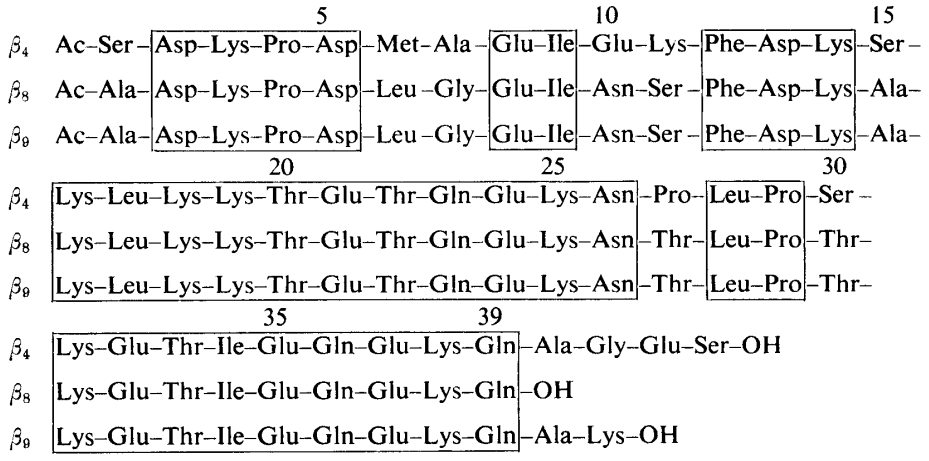

Fig. 1. Comparison of Amino Acid Sequences of Thymosin β_4, Thymosin β_8 and Thymosin β_9

Identical sequences are shown in boxes.

that are important for maturation and functioning of the immune system in man and animals.[3,4] Recently Hannappel *et al.*[5] have isolated two peptides, thymosins β_8 and β_9, from thymosin fraction 5, and their amino acid sequences were established. Thymosin β_9 is identical to thymosin β_8 except for the presence of an additional dipeptide, –Ala–Lys–OH at the C-terminus, and 32 of its 41 amino acid residues are identical to those of thymosin β_4.[5] The similarity in structures of thymosins β_4 and β_9 suggests that they may have related functions.

Our previous studies[6,7] demonstrated that synthetic thymosins β_8 and β_9 could increase the peripheral T-cells when incubated *in vitro* with lupus nephritis patient's blood. In this paper we subdivided the amino acid sequence of thymosin β_9 into six peptide fragments in common with those of thymosins β_4 and β_8 to collect further information on the essential residues for restoration of E-rosette forming cells of the lupus nephritis patients by thymosin β_9.

Results and Discussion

In the previous papers,[6,7] we reported that, in contrast to normal persons, patients with lupus nephritis have reduced percentages of T-cells and we showed that synthetic thymosin β_9 could increase the E-rosette forming capacity in patients with lupus nephritis.

The six peptide fragments of thymosin β_9 investigated here had the following compositions, H–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–OH (positions 16—26), H–Lys–Glu–Thr–Ile–Glu–Gln–Glu–Lys–Gln–OH (positions 31—39), H–Asp–Lys–Pro–Asp–OH (positions 2—5), H–Phe–Asp–Lys–OH (positions 12—14), H–Leu–Pro–OH (positions 28—29) and H–Glu–Ile–OH (positions 8—9).

In the present syntheses, as illustrated in Figs. 2 and 3, amino acid derivatives bearing protecting groups, *i.e.*, Asn–ONb, Lys(Z)–ONb, Ile–OBzl, Pro–OBzl, Gln–OBzl, Asp(OBzl)–OBzl, Lys(Z), Glu(OBzl), Asp(OBzl) and Z–Asp(OBzl), which could be removed by catalytic hydrogenation were used. Hydroxyl groups of threonine residues were not protected. These

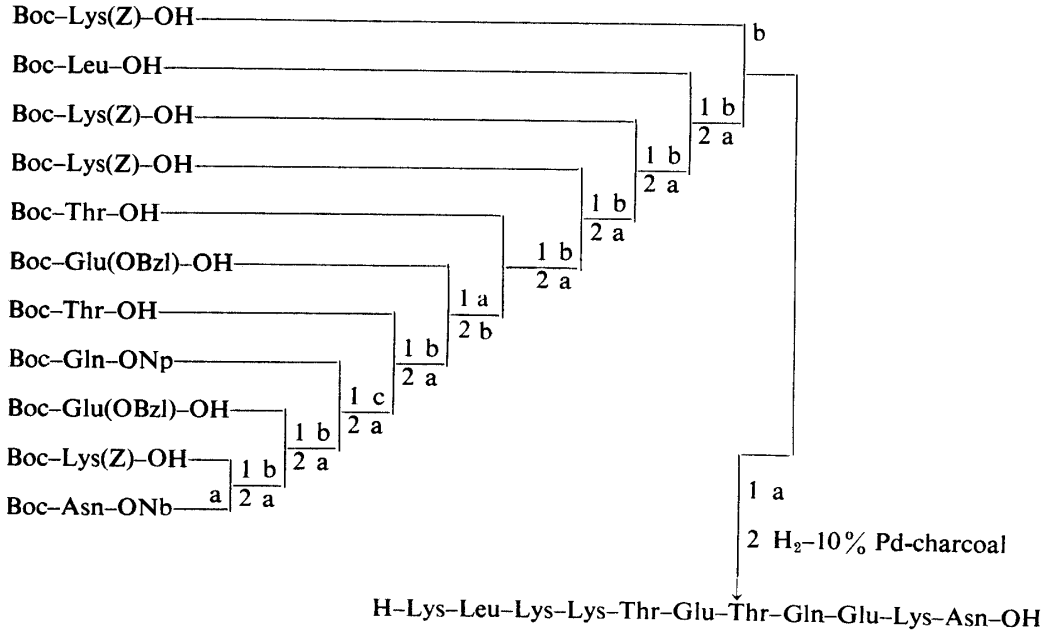

Fig. 2. Synthetic Scheme for H–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–OH (Positions 16—26)

a, TFA–anisole; b, HOBT–WSCI; c, active ester.

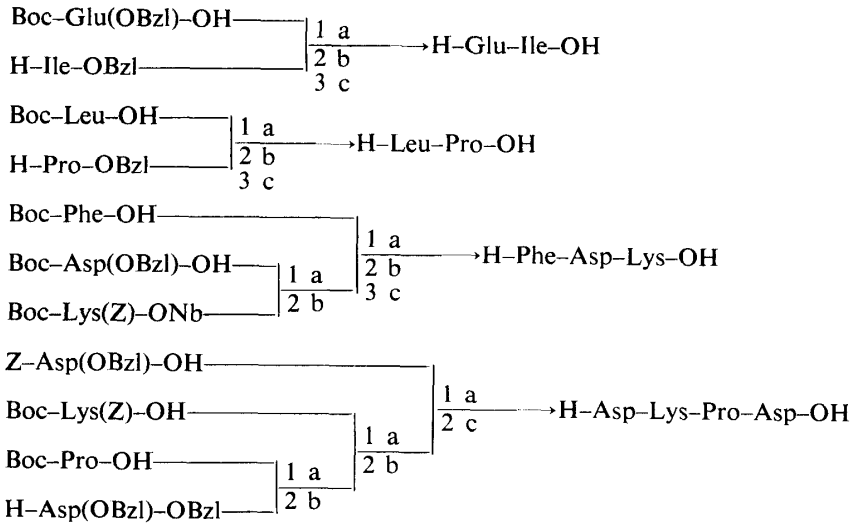

Fig. 3. Synthetic Scheme for H–Asp–Lys–Pro–Asp–OH (Positions 2—5), H–Phe–Asp–Lys–OH (Positions 12—14), H–Leu–Pro–OH (Positions 28—29) and H–Glu–Ile–OH (Positions 8—9)

a, HOBT–WSCI; b, TFA-anisole; c, H_2–10% Pd-charcoal.

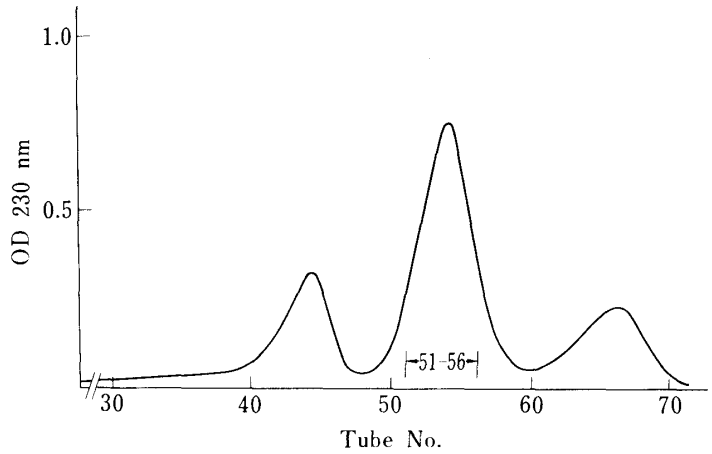

Fig. 4. Purification of Synthetic Undecapeptide (H–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–OH) by Partition Column Chromatography on Sephadex G-25

Column: 2.8 × 75 cm, fraction: 5 ml, solvent: BuOH–AcOH–H_2O (4 : 1 : 5) upper phase, flow rate: 5 ml/17 min.

protecting groups survive mostly intact during careful treatment with trifluoroacetic acid for the removal of the Boc group, employed as a temporary α-amino protecting group. After removal of the α-protecting Boc group with trifluoroacetic acid (TFA)–anisole, the peptide chain was elongated from the C-terminus to the N-terminus by the *N*-hydroxybenzotriazole–dicyclohexylcarbodiimide (HOBT–DCC) procedure,[8] except for Gln residues, for which the NP active ester procedure[9] was employed. Each protected product was purified by batchwise washing followed by precipitation from appropriate solvents.

The homogeneity of these protected intermediates was assessed by paper chromatography and elemental analysis. Following the final condensation, the Boc group was removed by TFA–anisole treatment and then the other protecting groups were removed by catalytic hydrogenation over 10% Pd-charcoal catalyst. The deblocked undecapeptide XI was purified by gel filtration through Sephadex G-15 using 1% AcOH, followed by partition column

chromatography (Fig. 4) on Sephadex G-25 according to Yamashiro.[10] The undecapeptide, H–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–OH (XI), thus obtained was found to be homogeneous by paper chromatographies in two different solvent systems. The purity of XI was further assessed by amino acid analyses of the 6 N HCl hydrolysate and the aminopeptidase (AP-M) digest.[11]

The protected nonapeptide, Boc–Lys(Z)–Glu(OBzl)–Thr–Ile–Glu(OBzl)–Gln–Glu–(OBzl)–Lys(Z)–Gln–OBzl, which was synthesized in our laboratory,[7] was treated with TFA–anisole and the resulting product was hydrogenated over 10% Pd-charcoal. The deblocked nonapeptide XII was purified by gel filtration on Sephadex G-15 using 1% AcOH, followed by preparative TLC. The nonapeptide, H–Lys–Glu–Thr–Ile–Glu–Gln–Glu–Lys–Gln–OH (XII), thus obtained was found to be homogeneous by paper chromatography in two different solvent systems. The amino acid compositions in the acid hydrolysate and the AP-M digest of XII gave molar ratios in good agreement with the expected values.

The hydrogenated product of Z–Asp(OBzl)–Lys(Z)–Pro–Asp(OBzl)–OBzl (XV) was purified by column chromatography on Sephadex G-10. The tetrapeptide, H–Asp–Lys–Pro–Asp–OH (XVI), thus obtained was found to be homogeneous on paper chromatography using two different solvent systems. Ratios of amino acids in the acid hydrolysate of the tetrapeptide were in good agreement with the theoretical values.

After TFA–anisole treatment of Boc–Phe–Asp(OBzl)–Lys(Z)–ONb (XVIII), the resulting tripeptide ester was hydrogenated over 10% Pd-charcoal in aqueous AcOH. The hydrogenated product was purified by column chromatography on Sephadex G-10. The tripeptide, H–Phe–Asp–Lys–OH (XIX), thus obtained was found to be homogeneous by paper chromatography in two different solvent systems. The amino acid analyses of the acid hydrolysate and the AP-M digest of XIX gave values in good agreement with the theoretical values.

TABLE I. Effects of the Six Synthetic Fragment Peptides on the Low E-Rosette-Forming Cells from Lupus Nephritis Patients

Peptide	Dose (Molar concentration)	E-Rosette-forming cells[d] (%)
—[a]	—	74±6
—[b]	—	45±7
Thymosin β_9[b,c]	1.0×10^{-9}	43±6
	2.2×10^{-8}	67±7
H–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–OH[b,c]	1.4×10^{-8}	45±7
	4.5×10^{-7}	43±6
	1.0×10^{-6}	63±5
H–Lys–Glu–Thr–Ile–Glu–Gln–Glu–Lys–Gln–OH[b,c]	1.9×10^{-8}	42±6
	6.0×10^{-7}	45±5
	1.6×10^{-6}	61±7
H–Asp–Lys–Pro–Asp–OH[b,c]	1.3×10^{-6}	46±6
	7.4×10^{-6}	43±7
H–Phe–Asp–Lys–OH[b,c]	1.5×10^{-6}	42±5
	8.7×10^{-6}	44±6
H–Glu–Ile–OH[b,c]	2.3×10^{-6}	46±5
	1.4×10^{-5}	43±7
H–Leu–Pro–OH[b,c]	2.6×10^{-6}	43±7
	1.5×10^{-5}	41±7

a) Normal venous blood.
b) Patient's venous blood.
c) Incubation was carried out for 60 min at 37 °C.
d) Each value represents the mean ± S.D. of triplicate measurements.

As shown in Fig. 3, the other two dipeptide fragments were obtained by condensation by the HOBT–DCC procedure. After the condensations, the Boc groups were removed by TFA–anisole treatment and then the C-terminal benzyl ester groups were removed by catalytic hydrogenation and the products were purified by recrystallization from appropriate solvents. The two dipeptide fragments, H–Leu–Pro–OH (XXI) and H–Glu–Ile–OH (XXIII), thus obtained were found to be homogeneous by paper chromatography in two different solvent systems. The amino acid analyses of these two dipeptide fragments gave values in good agreement with the theoretical values.

Incubation of blood from patients in the presence of synthetic peptides (1.0×10^{-9}—1.5×10^{-5} M) was carried out to investigate the recovery of E-rosette formation. The *in vitro* effects of the six fragments on low E-rosette forming cells of lupus nephritis patients are shown in Table I. The activities of H–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–OH and H–Lys–Glu–Thr–Ule–Glu–Gln–Glu–Lys–Gln–OH were lower than that of synthetic thymosin β_9.[7] The observed activities of these peptides were in order thymosin β_9 > H–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–OH > H–Lys–Glu–Thr–Ile–Glu–Gln–Lys–Gln–OH. However, the other four fragments, H–Asp–Lys–Pro–Asp–OH, H–Phe–Asp–Lys–OH, H–Leu–Pro–OH and H–Glu–Ile–OH, had no effect on the low E-rosette forming cells in patients. E-Rosette formation with 10^{-5}—10^{-6} M of these synthetic peptides (Table I) was considered negative; at higher concentrations these peptides reacted with normal lymphocytes and gave inconsistent results. These results indicate that two portions of amino acid sequence, –Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn– (positions 16—26) and –Lys–Glu–Thr–Ile–Glu–Gln–Lys–Gln– (positions 31—39), are important structures in thymosin β_9 for the restoration of activity on low E-rosette forming cells from lupus nephritis. Thymosin β_9 was effective at lower concentrations than the synthetic peptides, so other portions of thymosin β_9 may also be necessary for the full activity.

Experimental

Melting points are uncorrected. Rotations were measured with an Atago Polax machine (cell length: 10 cm). Amino acid compositions of acid hydrolysates and AP-M digests were determined with a JEOL JLC-8AH amino acid analyzer (one-column system). Solutions were concentrated in a rotary evaporator under reduced pressure at a temperature of 30—40 °C. Boc groups of the protected peptides were removed by TFA–anisole treatment. The resulting amino components were chromatographed on filter paper, Toyo Roshi No. 51, at room temperature. Rf^a values refer to the Partridge system[12] and Rf^b values refer to BuOH–pyridine–AcOH–H_2O (30:20:6:24).[13] Aminopeptidase (3501, Aminopeptidase 210520) was purchased from the Protein Research Foundation, Osaka, Japan. Venous blood samples from three lupus nephritis patients and three normal volunteers were drawn into heparinized syringes and sedimented at room temperature.

Boc–Lys(Z)–Asn–ONb (I)——Boc–Asn–ONb (1.8 g) was treated with TFA–anisole (10 ml–2 ml) in an ice-bath for 40 min, then TFA was removed by evaporation. The residue was washed with dry ether, dried over KOH pellets *in vacuo* for 2 h and then dissolved in DMF (18 ml). To this ice-chilled solution, Boc–Lys(Z)–OH DCHA (3 g), HOBT (744 mg) and WSCI (1.1 g) were successively added. After being stirred at 0 °C for 3 h and at room temperature for 12 h, the mixture was extracted with EtOAc, and the extract was washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O, then dried over $MgSO_4$ and concentrated *in vacuo*. The residue was recrystallized from MeOH and ether; yield 3.1 g (94%), mp 119—121 °C, $[\alpha]_D^{22}$ −7.5° (c=1.0, DMF), Rf^a 0.66, Rf^b 0.71, single ninhydrin-positive spot. *Anal.* Calcd for $C_{30}H_{39}N_5O_{10} \cdot H_2O$: C, 55.63; H, 6.38; N, 10.81. Found: C, 55.99; H, 6.62; N, 10.66.

Boc–Glu(OBzl)–Lys(Z)–Asn–ONb (II)——I (2.1 g) was treated with TFA–anisole (10 ml–2 ml) as described above and the resulting powder was dissolved in DMF (20 ml) together with NMM (0.37 ml). To this ice-chilled solution, Boc–Glu(OBzl)–OH (1.2 g), HOBT (495 mg) and WSCI (725 mg) were successively added. After being stirred at 0 °C for 3 h and at room temperature for 12 h, the mixture was extracted with EtOAc and the extract was washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O, dried over $MgSO_4$ and then concentrated *in vacuo*. The residue was precipitated from EtOAc and *n*-hexane. The powder was reprecipitated from EtOAc and ether; yield 2.3 g (82%), mp 70—75 °C, $[\alpha]_D^{23}$ −8.9° (c=1.0, DMF), Rf^a 0.70, Rf^b 0.74, single ninhydrin-positive spot. *Anal.* Calcd for $C_{42}H_{52}N_6O_{13} \cdot H_2O$: C, 58.91; H, 6.28; N, 9.69. Found: C, 58.55; H, 6.45; N, 9.23.

Boc–Gln–Glu(OBzl)–Lys(Z)–Asn–ONb (III)——II (2 g) was treated with TFA–anisole (10 ml–2 ml) as described

above and the resulting powder was dissolved in DMF (20 ml) together with NMM (0.26 ml). Boc–Gln–ONp (1.1 g) was added and the reaction mixture was stirred at room temperature for 18 h, then diluted with 1 N NH_4OH (3 ml) with stirring to saponify the unchanged *p*-nitrophenyl ester. After 1 h, the mixture was poured into ice-chilled 1 N NH_4OH with vigorous stirring. The precipitate formed was washed successively with 1 N NH_4OH, H_2O, 1 N citric acid and H_2O. The dried product was precipitated from AcOH and H_2O; yield 1.4 g (60%), mp 142—148 °C, $[\alpha]_D^{23}$ −14.3° (*c* = 1.0, DMF), Rf^a 0.75, Rf^b 0.74, single ninhydrin-positive spot. *Anal.* Calcd for $C_{47}H_{60}N_8O_{15}$: C, 57.78; H, 6.19; N, 11.47. Found: C, 57.92; H, 6.21; N, 11.79.

Boc–Thr–Gln–Glu(OBzl)–Lys(Z)–Asn–ONb (IV)——III (651 mg) was treated with TFA–anisole (3 ml–0.6 ml) as described above and the resulting powder was dissolved in DMF (6 ml) together with NMM (0.1 ml). To this ice-chilled solution, Boc–Thr–OH (161 mg), HOBT (99 mg) and WSCI (145 mg) were successively added. After 3 h at 0 °C and 18 h at room temperature, the mixture was poured into ice-chilled 1 N $NaHCO_3$ with stirring. The precipitate thus formed was washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O. The dried product was recrystallized from EtOAc and ether; yield 519 mg (72%), mp 121—124 °C, $[\alpha]_D^{23}$ −42.3° (*c* = 1.0, DMF), Rf^a 0.83, Rf^b 0.87, single ninhydrin-positive spot. *Anal.* Calcd for $C_{51}H_{67}N_9O_{17}$: C, 56.82; H, 6.26; N, 11.69. Found: C, 56.43; H, 6.53; N, 11.89.

Boc–Glu(OBzl)–Thr–Gln–Glu(OBzl)–Lys(Z)–Asn–ONb (V)——This compound was prepared from IV (431 mg), Boc–Glu(OBzl)–OH (149 mg), HOBT (60 mg) and WSCI (88 mg) essentially as described for the preparation of IV. The product was recrystallized from EtOAc and ether; yield 389 mg (73%), mp 136—140 °C, $[\alpha]_D^{23}$ −2.8° (*c* = 1.0, DMF), Rf^a 0.79, Rf^b 0.85, single ninhydrin-positive spot. *Anal.* Calcd for $C_{63}H_{80}N_{10}O_{20}\cdot 2H_2O$: C, 56.75; H, 6.35; N, 10.51. Found: C, 56.33; H, 6.67; N, 10.21.

Boc–Thr–Glu(OBzl)–Thr–Gln–Glu(OBzl)–Lys(Z)–Asn–ONb (VI)——This compound was prepared from V (324 mg), Boc–Thr–OH (61 mg), HOBT (37 mg) and WSCI (55 mg) essentially as described for the preparation of IV. The product was recrystallized from MeOH and ether; yield 312 mg (89%), mp 114—119 °C, $[\alpha]_D^{23}$ −16.8° (*c* = 1.0, DMF), Rf^a 0.80, Rf^b 0.81, single ninhydrin-positive spot. *Anal.* Calcd for $C_{67}H_{87}N_{11}O_{22}\cdot 8H_2O$: C, 52.17; H, 6.73; N, 9.99. Found: C, 52.30; H, 6.73; N, 9.74.

Boc–Lys(Z)–Thr–Glu(OBzl)–Thr–Gln–Glu(OBzl)–Lys(Z)–Asn–ONb (VII)——This compound was prepared from VI (254 mg), Boc–Lys(Z)–OH DCHA (112 mg), HOBT (28 mg) and WSCI (40 mg) essentially as described for the preparation of IV. The product was recrystallized from EtOAc and ether; yield 239 mg (79%), mp 121—127 °C, $[\alpha]_D^{23}$ −15.0° (*c* = 1.0, DMF), Rf^a 0.71, Rf^b 0.79, single ninhydrin-positive spot. *Anal.* Calcd for $C_{81}H_{105}N_{13}O_{25}$: C, 58.58; H, 6.37; N, 10.96. Found: C, 58.20; H, 6.18; N, 11.15.

Boc–Lys(Z)–Lys(Z)–Thr–Glu(OBzl)–Thr–Gln–Glu(OBzl)–Lys(Z)–Asn–ONb (VIII)——This compound was prepared in essentially the same manner as described for the preparation of IV by using VII (208 mg), Boc–Lys(Z)–OH DCHA (77 mg), HOBT (19 mg) and WSCI (28 mg). The product was reprecipitated from AcOH and H_2O; yield 189 mg (76%), mp 130—135 °C, $[\alpha]_D^{24}$ −18.3° (*c* = 1.0, DMF), Rf^a 0.85, Rf^b 0.84, single ninhydrin-positive spot. *Anal.* Calcd for $C_{95}H_{123}N_{15}O_{28}$: C, 59.33; H, 6.45; N, 10.93. Found: C, 59.25; H, 6.28; N, 10.86.

Boc–Leu–Lys(Z)–Lys(Z)–Thr–Glu(OBzl)–Thr–Gln–Glu(OBzl)–Lys(Z)–Asn–ONb (IX)——This compound was prepared in essentially the same manner as described for the preparation of IV by using VIII (148 mg), Boc–Leu–OH (21 mg), HOBT (12 mg) and WSCI (17 mg). The product was recrystallized from MeOH and ether; yield 137 mg (87%), mp 136—140 °C, $[\alpha]_D^{24}$ −10.1° (*c* = 1.0, DMF), Rf^a 0.81, Rf^b 0.88, single ninhydrin-positive spot. *Anal.* Calcd for $C_{101}H_{134}N_{16}O_{29}\cdot 5H_2O$: C, 56.57; H, 6.77; N, 10.45. Found: C, 56.58; H, 6.72; N, 10.26.

Boc–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr–Glu(OBzl)–Thr–Gln–Glu(OBzl)–Lys(Z)–Asn–ONb (X)——This compound was prepared in essentially the same manner as described for the preparation of IV by using IX (101 mg), Boc–Lys(Z)–OH DCHA (32 mg), HOBT (10 mg) and WSCI (13 mg). The product was reprecipitated from AcOH and H_2O; yield 97 mg (85%), mp 148—153 °C, $[\alpha]_D^{23}$ −18.1° (*c* = 1.0, DMF), Rf^a 0.88, Rf^b 0.90, single ninhydrin-positive spot. *Anal.* Calcd for $C_{115}H_{152}N_{18}O_{32}\cdot 3H_2O$: C, 58.71; H, 6.77; N, 10.72. Found: C, 58.61; H, 6.70; N, 10.38.

H–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–OH (XI)——X (60 mg) was treated with TFA–anisole (2 ml–0.4 ml) in an ice-bath for 40 min, then dry ether was added. The resulting powder was collected by filtration, washed with ether, and dried over KOH pellets *in vacuo* for 2 h. The N^{α}-deprotected peptide ester was hydrogenated in 50% AcOH (12 ml) over 10% Pd-charcoal for 24 h. The catalyst was removed with the aid of celite. The solution was evaporated to dryness and the residue was dried over KOH pellets *in vacuo*. A solution of the deprotected undecapeptide in 1% AcOH (2 ml) was applied to a Sephadex G-15 column (2.8 × 97 cm) and eluted with 1% AcOH. Fractions of 5 ml each were collected at a flow rate of 5 ml per 18 min with an automatic fraction collector, and the absorbancy of each fraction was determined at 230 nm. The eluates in tube Nos. 59 to 65 were pooled and lyophilized. Analysis by paper chromatography revealed the presence of two ninhydrin-positive spots with Rf^b 0.11 (major) and 0.29 (minor). The product was dissolved in a small amount of the upper phase of a solvent system consisting of BuOH–AcOH–H_2O (4 : 1 : 5). The solution was applied to a column of Sephadex G-25 (2.8 × 75 cm) previously equilibrated with the lower phase of the above solvent system. The column was developed with the same upper phase and individual fractions (5 ml each) were collected. The absorbancy at 230 nm was determined. The fractions corresponding to the main peak (tube Nos. 51—56) were combined. The solvent was removed by evaporation and the residue was lyophilized from 1% AcOH to give fluffy powder; yield 12 mg (34%), mp 201—211 °C (dec.), $[\alpha]_D^{24}$ −55.3°

($c=0.3$, 2% AcOH), Rf^a 0.03, Rf^b 0.10, single ninhydrin-positive spot. Amino acid ratios in 6 N HCl hydrolysate: Glu 2.83, Asp 1.12, Leu 1.00, Thr 1.69, Lys 3.82 (recovery of Leu 82%). Amino acid ratios in an AP-M digest: Glu 2.06, Leu 1.00, Gln + Thr 2.76, Asn 0.84, Lys 3.78 (recovery of Leu 79%). (Gln emerged at the same position as Thr, and was calculated as Thr).

H–Lys–Glu–Thr–Ile–Glu–Gln–Glu–Lys–Gln–OH (XII)——Boc–Lys(Z)–Glu(OBzl)–Thr–Ile–Glu(OBzl)–Gln–Glu(OBzl)–Lys(Z)–Gln–OBzl[7] (80 mg) was treated with TFA–anisole (2 ml–0.4 ml) as described above and the resulting powder was hydrogenated in AcOH–H_2O (6 ml–6 ml) in the presence of 10% Pd-charcoal for 24 h. After removal of the catalyst by filtration with the aid of celite, the filtrate was evaporated to dryness. The deblocked peptide thus obtained was dissolved in 1% AcOH (2 ml), applied to a column of Sephadex G-15 (2.8 × 101 cm), and eluted with the same solvent. Fractions of 5 ml were collected per 18 min, and the absorption at 230 nm was determined. Fractions corresponding to the front main peak (tube Nos. 60—66) were collected and lyophilized. The fluffy powder thus obtained was dissolved in H_2O (1 ml) and subjected to preparative TLC (Whatman PLK-5, 20 × 20 cm) using the Partridge system as a developing solvent. The zone corresponding to Rf^a 0.09 was separated and extracted with 1% AcOH. The extracts were concentrated to a small volume and subjected to Sephadex G-15 column chromatography as described above; yield 14 mg (33%), mp 238—246 °C (dec.), $[\alpha]_D^{26}$ −18.6° ($c=0.3$, 2% AcOH), Rf^a 0.08, Rf^b 0.13, single ninhydrin-positive spot. Amino acid ratios in 6 N HCl hydrolysate: Glu 4.72, Ile 1.00, Thr 0.73, Lys 2.03 (recovery of Ile 80%). Amino acid ratios in an AP-M digest: Glu 2.80, Ile 1.00, Thr + Gln 2.74, Lys 1.85 (recovery of Ile 81%) (Gln emerged at the same position as Thr, and was calculated as Thr).

Boc–Pro–Asp(OBzl)–OBzl (XIII)——HOBT (149 mg) and WSCI (218 mg) were added to a solution of Boc–Pro–OH (237 mg) and H–Asp(OBzl)–OBzl Tos (486 mg) in DMF (5 ml) containing NMM (to keep the solution slightly alkaline) with stirring at 4 °C. The mixture was stirred for 3 h at 0 °C and for 16 h at room temperature, then the mixture was extracted with EtOAc. The extract was washed as described above, then evaporated to dryness. The residue was precipitated from EtOAc and petroleum ether; yield 426 mg (83%) (oily material), $[\alpha]_D^{23}$ −6.5° ($c=1.0$, DMF), Rf^a 0.67, Rf^b 0.75, single ninhydrin-positive spot. *Anal.* Calcd for $C_{28}H_{34}N_2O_7$: C, 65.87; H, 6.71; N, 5.49. Found: C, 56.89; H, 6.45; N, 5.36.

Boc–Lys(Z)–Pro–Asp(OBzl)–OBzl (XIV)——This compound was prepared from XIII (340 mg), Boc–Lys(Z)–OH DCHA (413 mg), HOBT (100 mg) and WSCI (145 mg) essentially as described for the preparation of I. The product was reprecipitated from EtOAc and *n*-hexane; yield 318 mg (62%), mp 68—71 °C, $[\alpha]_D^{24}$ −9.7° ($c=1.0$, DMF), Rf^a 0.68, Rf^b 0.72, single ninhydrin-positive spot. *Anal.* Calcd for $C_{42}H_{52}N_4O_{10}\cdot 2H_2O$: C, 62.36; H, 6.98; N, 6.93. Found: C, 62.30; H, 7.04; N, 6.76.

Z–Asp(OBzl)–Lys(Z)–Pro–Asp(OBzl)–OBzl (XV)——XIV (258 mg) was treated with TFA–anisole (3 ml–0.6 ml) as described above and the resulting powder was dissolved in DMF (4 ml) together with NMM (0.04 ml). To this ice-chilled solution, Z–Asp(OBzl)–OH (131 mg), HOBT (50 mg) and WSCI (73 mg) were successively added. After being stirred at 0 °C for 3 h and at room temperature for 16 h, the mixture was extracted with EtOAc and the extract was washed successively with 1 N $NaHCO_3$, H_2O, 1 N HCl and H_2O, dried over $MgSO_4$ and then concentrated *in vacuo*. The residue was reprecipitated from EtOAc and petroleum ether; yield 229 mg (68%), mp 82—86 °C, $[\alpha]_D^{23}$ −12.1° ($c=1.0$, DMF). *Anal.* Calcd for $C_{56}H_{61}N_5O_{13}$: C, 66.46; H, 6.08; N, 6.92. Found: C, 66.62; H, 6.29; N, 6.70.

H–Asp–Lys–Pro–Asp–OH (XVI)——The protected tetrapeptide ester XV (80 mg) was hydrogenated in AcOH–H_2O (6 ml–6 ml) in the presence of 10% Pd-charcoal for 12 h. After removal of the catalyst by filtration with the aid of celite, the filtrate was evaporated to dryness. The protected peptide thus obtained was dissolved in 1% AcOH (2 ml), applied to a column of Sephadex G-10 (2.8 × 103 cm), and eluted with the same solvent. Fractions of 5 ml were collected per 16 min, and the absorption at 230 nm was determined. Fractions corresponding to the front main peak (tube Nos. 66—77) were collected and lyophilized; yield 16 mg (43%), mp 238—244 °C (dec.), $[\alpha]_D^{23}$ −10.8° ($c=0.5$, 1 N AcOH), Rf^a 0.02, Rf^b 0.12, single ninhydrin-positive spot. Amino acid ratios in 6 N HCl hydrolysate: Asp 2.12, Lys 1.00, Pro 0.84 (recovery of Lys 82%).

Boc–Asp(OBzl)–Lys(Z)–ONb (XVII)——This compound was prepared from Boc–Lys(Z)–ONb (515 mg), Boc–Asp(OBzl)–OH (355 mg), HOBT (149 mg) and WSCI (218 mg) essentially as described for the preparation of I. The product was recrystallized from EtOAc and *n*-hexane; yield 606 mg (82%), mp 98—101 °C, $[\alpha]_D^{23}$ −19.3° ($c=1.0$, DMF), Rf^a 0.74, Rf^b 0.77, single ninhydrin-positive spot. *Anal.* Calcd for $C_{37}H_{44}N_4O_{11}\cdot H_2O$: C, 60.15; H, 6.28; N, 7.58. Found: C, 60.01; H, 6.34; N, 7.36.

Boc–Phe–Asp(OBzl)–Lys(Z)–ONb (XVIII)——This compound was prepared from XVII (360 mg), Boc–Phe–OH (146 mg), HOBT (75 mg) and WSCI (109 mg) essentially as described for the preparation of I. The product was reprecipitated from MeOH and ether; yield 387 mg (89%), mp 117—120 °C, $[\alpha]_D^{23}$ −9.7° ($c=1.0$, DMF), Rf^a 0.77, Rf^b 0.73, single ninhydrin-positive spot. *Anal.* Calcd for $C_{46}H_{53}N_5O_{12}$: C, 63.66; H, 6.16; N, 8.07. Found: C, 63.88; H, 6.11; N, 8.20.

H–Phe–Asp–Lys–OH (XIX)——XVIII (100 mg) was treated with TFA–anisole (2 ml–0.4 ml) as described above and the resulting powder was hydrogenated in 50% AcOH (12 ml) over 10% Pd-charcoal for 20 h. The catalyst was removed with the aid of celite. The filtrate was evaporated to dryness and the hydrogenated product thus obtained was dissolved in 1% AcOH (2 ml) and applied to a column of Sephadex G-10 (2.8 × 101 cm), eluting with the same solvent. Individual fractions (4 ml each) were collected and the absorbancy at 260 nm was determined. Fractions

corresponding to the main peak (tube Nos. 79—87) were combined and the solvent was removed by lyophilization; yield 27 mg (47%), mp 174—182 °C (dec.), $[\alpha]_D^{23}$ −29.3° (c=1.0, 1 N AcOH), Rf^a 0.09, Rf^b 0.41, single ninhydrin-positive spot. Amino acid ratios in 6 N HCl hydrolysate: Asl 1.06, Phe 1.00, Lys 0.98 (recovery of Phe 78%). Amino acid ratios in an AP-M digest: Asp 1.02, Phe 1.00, Lys 0.87 (recovery of Phe 81%).

Boc–Leu–Pro–OBzl (XX)——This compound was prepared from H–Pro–OBzl HCl (242 mg), Boc–Leu–OH (274 mg), HOBT (149 mg) and WSCI (218 mg) essentially as described for the preparation of XIII; yield 284 mg (68%) (oily material), $[\alpha]_D^{23}$ −36.2° (c=1.0, DMF), Rf^a 0.89, Rf^b 0.95, single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{34}N_2O_5$: C, 66.01; H, 8.19; N, 6.69. Found: C, 56.79; H, 8.30; N, 6.50.

H–Leu–Pro–OH (XXI)——XX (200 mg) was treated with TFA–anisole (3 ml–0.6 ml) as described above. The deblocked peptide ester was hydrogenated in AcOH–H_2O (6 ml–6 ml) over Pd-charcoal for 12 h. The catalyst was removed with the aid of celite. The filtrate was evaporated to dryness and dried over KOH pellets *in vacuo*. The dried product was recrystallized from EtOH and ether; yield 94 mg (86%), mp 131—136 °C, $[\alpha]_D^{23}$ −45.3° (c=1.0, 1 N AcOH), Rf^a 0.76, Rf^b 0.84, single ninhydrin-positive spot. Amino acid ratios in 6 N HCl hydrolysate: Leu 1.00, Pro 0.87 (recovery of Leu 83%).

Boc–Glu(OBzl)–Ile–OBzl (XXII)——This compound was prepared from H–Ile–OBzl Tos (197 mg), Boc–Glu(OBzl)–OH (186 mg), HOBT (75 mg) and WSCI (109 mg) essentially as described for the preparation of XIII. The product was precipitated from EtOAc and *n*-hexane; yield 181 mg (67%), mp 110—113 °C, $[\alpha]_D^{23}$ −15.2° (c=1.0, DMF), Rf^a 0.81, Rf^b 0.87, single ninhydrin-positive spot. *Anal.* Calcd for $C_{30}H_{40}N_2O_7$: C, 66.65; H, 7.46; N, 5.18. Found: C, 66.37; H, 7.70; N, 4.82.

H–Glu–Ile–OH (XXIII)——XXII (100 mg) was treated with TFA–anisole (2 ml–0.4 ml) as described above. The deblocked peptide ester was hydrogenated in 50% AcOH (12 ml) over 10% Pd-charcoal for 12 h. The catalyst was removed with the aid of celite. The filtrate was evaporated to dryness and dried over KOH pellets *in vacuo*. The dried product was recrystallized from MeOH and ether; yield 32 mg (67%), mp 159—167 °C (dec.), $[\alpha]_D^{23}$ −20.4° (c=1.0, 1 N AcOH), Rf^a 0.22, Rf^b 0.38, single ninhydrin-positive spot. Amino acid ratios in 6 N HCl hydrolysate: Ile 1.00, Glu 1.13 (recovery of Ile 80%). Amino acid ratios in an AP-M digest: Ile 1.00, Glu 1.02 (recovery of Ile 83%).

E-Rosette Formation——A patient's blood was incubated with a synthetic peptide for 1 h at 37 °C and then lymphocytes were isolated in a Hypaque–Ficoll gradient[14] for the testing of E-rosette formation. Isolated lymphocytes were adjusted to 5×10^5 cells/ml with PBS. Contamination by monocytes and polymorphonuclear cells amounted to less than 7%.[15] Sheep erythrocytes (Kyokutō Pharmaceutical Co.) were washed with PBS, and a suspension (8×10^6/ml) was prepared. The lymphocytes were washed with GVB^{2+} and centrifuged for 10 min at 1500 rpm, then suspended in FCS (Dainippon Pharmaceutical Co.) (1 ml). The suspension was mixed with the suspension of sheep erythrocytes (0.5 ml) and incubated for 12 h at 4 °C. The mixture was then centrifuged for 5 min at 900 rpm. Triplicate wet-cell preparations were checked by phase contrast microscopy. For each preparation, 200 lymphocytes were counted, and the proportion binding more than three erythrocytes was determined.

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References and Notes

1) The amino acid residues mentioned in this paper are of the L-configuration. The abbreviations used to denote amino acid derivatives and peptides are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **11**, 1726 (1972). Other abbreviations: DMF, dimethylformamide; WSCI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; TFA, trifluoroacetic acid; HOBT, *N*-hydroxybenzotriazole; AcOH, acetic acid; EtOAc, ethyl acetate; DCC, dicyclohexylcarbodiimide; NMM, *N*-methylmorpholine; Tos, *p*-toluenesulfonic acid; Boc, *tert*-butoxycarbonyl; ONb, *p*-nitrobenzyl ester; DCHA, dicyclohexylamine; OBzl, benzyl ester; Z, benzyloxycarbonyl; ONp, *p*-nitrophenyl ester; TLC, thin-layer chromatography; NP, *p*-nitrophenyl; E-rosette, a rosette with sheep erythrocytes; FCS, fetal calf serum; GVB^{2+}, gelatin veronal buffer; PBS, phosphate-buffered saline.
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