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Synthesis of the Octadecapeptide corresponding to Positions 32 to 49 of the Revised Amino Acid Sequence of Thymopoietin II and Its Effect on Low E-Rosette Forming Cells of an Aged Patient with Chronic Renal Failure[1)]

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The octadecapeptide, H–Arg–Lys–Asp–Val–Tyr–Val–Glu–Leu–Tyr–Leu–Gln–Ser–Leu–Thr–Ala–Leu–Lys–Arg–OH, corresponding to positions 32 to 49 of the revised amino acid sequence of bovine thymopoietin II was synthesized by the azide condensation of three fragments, (32—36), (37—41) and (42—49), followed by deprotection with hydrogen fluoride in the presence of anisole–thioanisole–*o*-cresol. The *in vitro* addition of the synthetic thymopoietin II (32—49) significantly restored the low E-rosette forming capacity of cells from an aged patient with chronic renal failure to normal levels. The *in vitro* effect of [Gln^{38}, Thr^{43}, Val^{47}]-thymopoietin II (32—49) on the low E-rosette forming capacity of cells from the aged patient with chronic renal failure was also compared with that of the synthetic thymopoietin II (32—49). The [Gln^{38}, Thr^{43}, Val^{47}]-thymopoietin II (32—49) was approximately equipotent with the synthetic thymopoietin II (32—49) at a concentration of 100 μg/ml.

Keywords——thymopoietin II (32—49); [Gln^{38}, Thr^{43}, Val^{47}]-thymopoietin II (32—49); low rosette forming cells with sheep erythrocytes; aged patient with chronic renal failure; azide condensation

Thymopoietin II is a polypeptide hormone of the thymus, and consists of a 49-amino acid single polypeptide chain.[2)] In 1977, Fujino *et al.* reported the first total synthesis of thymopoietin II using the HONB–DCC procedure.[3)] The synthetic thymopoietin II was active in the incubation of thymocyte differentiation from prothymocytes *in vitro* and the potency of this peptide was more than 10 times that of the synthetic tridecapeptide corresponding to the sequence 29—41 of the hormone.[4)] In 1979, Goldstein *et al.*[5)] reported the synthesis of a pentapeptide corresponding to amino acids 32 to 36 of thymopoietin, and showed that it also has the biological properties of the parent molecule. Then, we reported that the octadecapeptide (positions 32—49)[6)] induces some recovery of E-rosette formation in rheumatoid arthritis. On the other hand, in aged animals and humans, thymus-dependent immunity is impaired as manifested by depression of delayed hypersensitivity and graft *versus* host reactions[7,8)] and a depressed response of lymphocyte stimulation with T-cell mitogens.[9)]

The immune deficiencies that develop during aging may be related to the involution of the thymus and the decline in the serum concentration of thymus hormone,[10)] and may be associated with the high incidence of infections and cancer in aged people.

Moreover, cell-mediated immunity is impaired in chronic renal failure.[11–13)] For example, skin graft rejection is usually delayed and the cutaneous hypersensitivity response to various antigens is depressed.[11,13)] A decrease of E-rosette forming cells in these patients has been demonstrated by several investigators.[14)]

Recently, several investigators[15)] reported that the *in vitro* addition of the thymopoietin pentapeptide (positions 32—36) could restore to normal values the low activity of E-rosette forming cells in aged people with minor pathology.

Later, the proposed structure of bovine thymopoietin II was revised by Goldstein *et al.*[16)] in 1981. Glu residue instead of Gln at positions 38, Ser residue instead of Thr at position 43 and Leu residue instead of Val at position 47 were placed in the octadecapeptide corresponding

to positions 32 to 49 of the revised structure of bovine thymopoietin II.

We describe here the synthesis of the octadecapeptide corresponding to positions 32 to 49 (H–Arg–Lys–Asp–Val–Tyr–Val–Glu–Leu–Tyr–Leu–Gln–Ser–Leu–Thr–Ala–Leu–Lys–Arg–OH) of the revised amino acid sequence of bovine thymopoietin II. Further we compared the *in vitro* effects of this octadecapeptide and [Gln[38], Thr[43], Vla[47]]-thymopoietin II (32—49)[6] on low E-rosette forming cells of an aged patient with chronic renal failure. In the previous paper,[6] we reported the synthesis of [Gln[38], Thr[43], Val[47]]-thymopoietin II (32—49) by the solution method and showed that this peptide could increase the E-rosette forming capacity in patient with rheumatoid arthritis.

In the present synthesis, as illustrated in Fig. 1, amino acid derivatives bearing protecting groups, *i.e.*, Arg(NO_2)–OBzl, Lys(Z), Z–Arg(NO_2), Glu(OBzl) and Asp(OBzl), which could be removed by treatment with hydrogen fluoride[17] were used. Hydroxy groups of Ser, Thr and Tyr residues were not protected. The above protecting groups survive mostly intact under careful TFA treatment for removal of the Boc group, employed as a temporary α-amino protecting group. As shown in Fig. 1, three peptide subunits, Boc–Gln–Ser–Leu–Thr–Ala–Leu–Lys(Z)–Arg(NO_2)–OBzl (VI), Boc–Val–Glu(OBzl)–Leu–Tyr–Leu–NHNH–Troc (XI) and Z–Arg(NO_2)–Lys(Z)–Asp(OBzl)–Val–Tyr–NHNH–Troc (XVI) served as building blocks for the construction of the full sequence corresponding to positions 32 to 49 of bovine thymopoietin II.[16] First, the C-terminal octapeptide, Boc–Gln–Ser–Leu–Thr–Ala–Leu–Lys(Z)–Arg(NO_2)–OBzl (VI), was synthesized by the stepwise elongation method. The protected heptapeptide ester, Boc–Ser–Leu–Thr–Ala–Leu–Lys(Z)–Arg(NO_2)–OBzl (V), was prepared stepwise by the HOBT–DCC procedure.[18] starting from Boc–Lys(Z)–Arg(NO_2)–OBzl.[6] After the TFA–anisole treatment of V, the resulting heptapeptide ester was condensed with Boc–Gln–ONp to give the protected octapeptide, Boc–Gln–Ser–Leu–Thr–Ala–Leu–Lys(Z)–Arg(NO_2)–OBzl (VI). Next, in order to prepare the peptide hydrazides containing Asp(OBzl) and Glu(OBzl), these two fragments were synthesized starting with Troc–$NHNH_2$.[19]

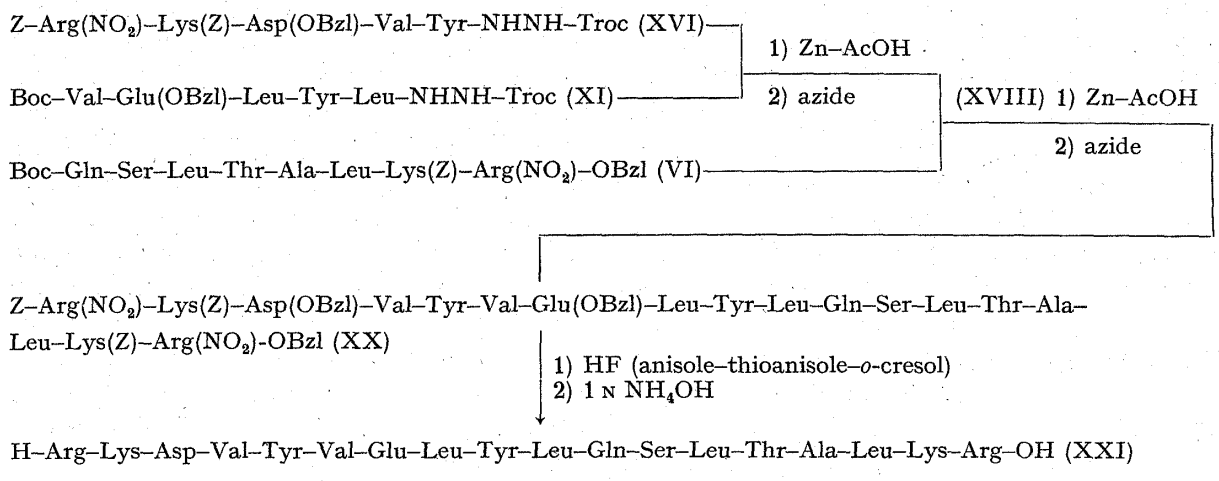

Fig. 1. Synthetic Scheme for the Revised Sequence of Thymopoietin II (32—49)

First, Boc-amino acid was condensed with Troc–$NHNH_2$ by the HOBT–DCC procedure.[18] Then, the two fragments, Boc–Val–Glu(OBzl)–Leu–Tyr–Leu–NHNH–Troc (XI) and Z–Arg(NO_2)–Lys(Z)–Asp(OBzl)–Val–Tyr–NHNH–Troc (XVI), were synthesized stepwise by the HOBT–DCC procedure and the Boc groups of intermediates were removed by treatment with TFA–anisole prior to the next coupling reaction. The three fragments thus obtained were assembled successively according to Fig. 1 by Rudinger's azide procedure.[20] The Troc group of Z–Arg(NO_2)–Lys(Z)–Asp(OBzl)–Val–Tyr–NHNH–Troc (XVI) was removed by treatment

with Zn dust[21] in AcOH and DMF to give Z–Arg(NO_2)–Lys(Z)–Asp(OBzl)–Val–Tyr–$NHNH_2$ (XVII). The last trace of metal contamination was removed by treatment with EDTA. The Boc group of Boc–Val–Glu(OBzl)–Leu–Tyr–Leu–NHNH–Troc (XI) was removed by usual TFA–anisole treatment and the corresponding free base was condensed with Z–Arg(NO_2)–Lys(Z)–Asp(OBzl)–Val–Tyr–$NHNH_2$ (XVII) by the azide procedure[20] to yield Z–Arg(NO_2)–Lys(Z)–Asp(OBzl)–Val–Tyr–Val–Glu(OBzl)–Leu–Tyr–Leu–NHNH–Troc (XVIII), which was purified by column chromatography on silica gel with DMF and MeOH (1 : 3). Its homogeneity was confirmed by elemental analysis and paper chromatographies in two different solvent systems. Next, after removal of the Troc group of Z–Arg(NO_2)–Lys(Z)–Asp(OBzl)–Val–Tyr–Val–Glu(OBzl)–Leu–Tyr–Leu–NHNH–Troc (XVIII) by treatment with Zn dust in AcOH and DMF, the resulting decapeptide hydrazide, Z–Arg(NO_2)–Lys(Z)–Asp(OBzl)–Val–Tyr–Val–Glu(OBzl)–Leu–Tyr–Leu–$NHNH_2$ (XIX), was condensed with H–Gln–Ser–Leu–Thr–Ala–Leu–Lys(Z)–Arg(NO_2)–OBzl by the azide procedure to yield Z–Arg(NO_2)–Lys(Z)–Asp(OBzl)–Val–Tyr–Val–Glu(OBzl)–Leu–Tyr–Leu–Gln–Ser–Leu–Thr–Ala–Leu–Lys(Z)–Arg(NO_2)–OBzl (XX), which was purified by silica gel column chromatography with BuOH and DMF (3 : 1). The homogeneity of the peptide was assessed by elemental analysis and paper chromatography in two different solvent systems. The protected octadecapeptide ester thus obtained was treated with hydrogen fluoride, in the presence of anisole–thioanisole–*o*-cresol (1 : 1 : 1, v/v)[22] to suppress side reaction of H–Tyr–OH,[23] to remove all protecting groups. The deblocked peptide was precipitated by adding dry ether, and converted to the corresponding acetate on Amberlite CG-4B (acetate form), then treated with 1 N NH_4OH at pH 10 for 30 min. The latter treatment was performed because of the reversible N→O shift at the Thr and Ser residues during the hydrogen fluoride treatment.[24,25] Finally, the product was purified by gel-filtration on Sephadex G-25 using 2% AcOH, followed by partition column chromatography on Sephadex G-25 according to Yamashiro.[26] The octadecapeptide (XXI) thus obtained was found to be homogeneous by paper chromatographies in two different solvent systems. Its purity was further assessed by amino acid analysis of the 6 N HCl hydrolysates and the aminopeptidase (AP-M) digest.[27] Amino acid analyses of both hydrolysates gave molar ratios in good agreement with the expected values.

The *in vitro* effects of the synthetic thymopoietin II (32—49) and [Gln^{38}, Thr^{43}, Val^{47}]-thymopoietin II (32—49) on low E-rosette forming cells in an aged patient with chronic renal failure are shown in Table I. Incubation of blood from the aged patient with chronic renal failure in the presence of synthetic peptides at a concentration of 100 μg/ml resulted in recovery of E-rosette formation (Table I). The biological activity of the synthetic [Gln^{38}, Thr^{43}, Val^{47}]-thymopoietin II (32—49) was equal to that of the revised thymopoietin II (32—49) at a concentration of 100 μg/ml (Table I). The results presented in this paper indicate that the three amino acid residues Glu (position 38), Ser (position 43) and Leu (position 47) of bovine

Table I. Effects of the Synthetic Thymopoietin II (32—49) and [Gln^{38}, Thr^{43}, Val^{47}]-Thymopoietin II (32—49) on the E-Rosette Forming Cells of an Aged Patient with Chronic Renal Failure

Peptide	Dose (μg/ml)	E-Rosette forming cells (%)
—[a]		78±6[d]
—[b]		47±7[d]
Thymopoietin II (32—49)[b,c]	100	72±6[d]
[Gln,[38] Thr^{43}, Val^{47}]-thymopoietin II (32—49)[b,c]	100	71±7[d]

a) Normal venous blood.
b) Aged patient's venous blood.
c) Incubation was carried out for 30 min at 37°C.
d) Each value represents the mean±S.D. of triplicate measurements.

thymopoietin II, are not essential for increasing the activity of E-rosette forming cells in aged patients with chronic renal failure.

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). Evaporation of solvents was carried out in a rotary evaporator under reduced pressure at 35 to 45°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[28] and Rf^b values refer to BuOH–pyridine–AcOH–H_2O (30: 20: 6: 24).[29] For paper chromatography, the α-Z groups of protected peptides were not deblocked.

Venous blood was obtained from an aged patient (male, 76 years old) suffering from chronic renal failure. Venous blood samples from three healthy donors (23—28 years old) were used as a control. Aminopeptidase (hog kidney, EC 3.4.11.2, Lot. No. 20214) was purchased from Pierce Chemical Company, Rockford, Illinois, USA. Troc-$NHNH_2$ was purchased from the Kokusan Chemical Works, Ltd., Japan.

Boc-Leu-Lys(Z)-Arg(NO_2)-OBzl (I)——Boc–Lys(Z)–Arg(NO_2)–OBzl[6] (2.7 g) was dissolved in TFA (10 ml)–anisole (2 ml) and the solution was allowed to stand at room temperature for 20 min. The TFA was evaporated off and the residue was treated with ether, and collected by filtration. The powder obtained was dried over KOH pellets *in vacuo* and then dissolved in DMF (15 ml) and the solution was neutralized with NMM (0.44 ml). To this ice-chilled solution, Boc–Leu–OH (1.1 g), HOBT (594 mg) and WSCI (683 mg) were successively added. After being stirred overnight at 0°C, 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 reprecipitated from EtOAc and *n*-hexane; yield 2.6 g (81%), mp 108—114°C, $[\alpha]_D^{25}$ −21.3° (c=1.0, DMF), Rf^a 0.79, Rf^b 0.87, single ninhydrin-positive spot. *Anal.* Calcd for $C_{38}H_{56}N_8O_{16}$: C, 58.15; H, 7.19; N, 14.28. Found: C, 57.86; H, 7.16; N, 14.17.

Boc-Ala-Leu-Lys(Z)-Arg(NO_2)-OBzl (II)——This compound was prepared from I (2 g), Boc–Ala–OH (520 mg), HOBT (372 mg) and WSCI (427 mg) essentially as described for the preparation of I. The product was reprecipitated from MeOH and ether; yield 1.4 g (64%), mp 87—91°C, $[\alpha]_D^{25}$ −10.8° (c=1.0, DMF), Rf^a 0.76, Rf^b 0.85, single ninhydrin-positive spot. *Anal.* Calcd for $C_{41}H_{61}N_9O_{11}$: C, 57.53; H, 7.18; N, 14.73. Found: C, 57.30; H, 7.30; N, 14.60.

Boc-Thr-Ala-Leu-Lys(Z)-Arg(NO_2)-OBzl (III)——This compound was prepared from II (1.1 g), Boc–Thr–OH (301 mg), HOBT (186 mg) and WSCI (213 mg) essentially as described for the preparation of I. The product was reprecipitated from EtOAc and ether; yield 891 mg (74%), mp 78—82°C, $[\alpha]_D^{26}$ −15.1° (c=1.0, DMF), Rf^a 0.73, Rf^b 0.87, single ninhydrin-positive spot. *Anal.* Calcd for $C_{45}H_{68}N_{10}O_{13}$: C, 56.47; H, 7.16; N, 14.64. Found: C, 56.71; H, 7.01; N, 14.32.

Boc-Leu-Thr-Ala-Leu-Lys(Z)-Arg(NO_2)-OBzl (IV)——This compound was prepared from III (957 mg), Boc–Leu–OH (172 mg), HOBT (92 mg) and WSCI (107 mg) essentially as described for the preparation of I. The product was reprecipitated from acetone and ether; yield 449 mg (67%), mp 118—126°C, $[\alpha]_D^{25}$ −93.0° (c=1.0, DMF), Rf^a 0.82, Rf^b 0.94, single ninhydrin-positive spot. *Anal.* Calcd for $C_{51}H_{79}N_{11}O_{14}$: C, 57.24; H, 7.44; N, 14.40. Found: C, 57.52; H, 7.76; N, 14.56.

Boc-Ser-Leu-Thr-Ala-Leu-Lys(Z)-Arg(NO_2)-OBzl (V)——This compound was prepared from IV (214 mg), Boc–Ser–OH (50 mg), HOBT (30 mg) and WSCI (34 mg) essentially as described for the preparation of I. The product was reprecipitated from MeOH and ether; yield 211 mg (91%), mp 103—107°C, $[\alpha]_D^{26}$ −22.4° (c=1.0, DMF), Rf^a 0.84, Rf^b 0.92, single ninhydrin-positive spot. *Anal.* Calcd for $C_{54}H_{84}N_{12}O_{16}$: C, 56.04; H, 7.32; N, 14.52. Found: C, 56.28; H, 7.55; N, 14.30.

Boc-Gln-Ser-Leu-Thr-Ala-Leu-Lys(Z)-Arg(NO_2)-OBzl (VI)——V (193 mg) was treated with TFA (2 ml)–anisole (0.4 ml) as described above and the resulting powder was dissolved in DMF (3 ml) together with NMM (0.02 ml). Boc–Gln–ONp (78 mg) was added and the solution was stirred at room temperature for 18 h. The reaction mixture was diluted with 1 N NH_4OH (1 ml) with stirring to saponify the unchanged *p*-nitrophenyl ester. After 1 h, the mixture was extracted with EtOAc and the extract was washed successively with 1 N NH_4OH, H_2O, 1 N citric acid and H_2O, dried over $MgSO_4$ and evaporated *in vacuo*. The residue was recrystallized from EtOAc; yield 159 mg (74%), mp 158—164°C, $[\alpha]_D^{26}$ −15.5° (c=1.0, DMF), Rf^a 0.72, Rf^b 0.86, single ninhydrin-positive spot. *Anal.* Calcd for $C_{59}H_{92}N_{14}O_{18}$: C, 55.13; H, 7.21; N, 15.26. Found: C, 55.36; H, 7.42; N, 14.95.

Boc-Leu-NHNH-Troc (VII)——HOBT (1.6 g) and WSCI (1.8 g) were added to a solution of Boc–Leu–OH (2.5 g) and Troc–$NHNH_2$ (2.3 g) in THF (20 ml) with stirring at 0°C. The reaction mixture was stirred for 16 h at 4°C. Then, 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 $MgSO_4$, then concentrated *in vacuo*. The residue was reprecipitated from EtOAc and petroleum ether; yield 3.9 g (oily material) (89%), $[\alpha]_D^{26}$ −21.6° (c=1.0,

DMF), Rf^a 0.64, Rf^b 0.83, single ninhydrin-positive spot. *Anal.* Calcd for $C_{14}H_{24}Cl_3N_3O_5 \cdot H_2O$: C, 38.33; H, 5.97; N, 9.58. Found: C, 38.42; H, 6.21; N, 9.87.

Boc-Tyr-Leu-NHNH-Troc (VIII)——This compound was prepared from VII (3.5 g), Boc–Tyr–OH (2.5 g), HOBT (1.3 g) and WSCI (1.4 g) essentially as described for the preparation of I. The product was reprecipitated from EtOAc and petroleum ether; yield 3.2 g (65%), mp 60—63°C, $[\alpha]_D^{25}$ −50.4° (c=1.0, DMF), Rf^a 0.71, Rf^b 0.87, single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{33}Cl_3N_4O_7$: C, 47.31; H, 5.70; N, 9.60. Found: C, 47.57; H, 5.57; N, 9.68.

Boc-Leu-Tyr-Leu-NHNH-Troc (IX)——This compound was prepared from VIII (2.9 g), Boc–Leu–OH (1.4 g), HOBT (764 mg) and WSCI (854 mg) essentially as described for the preparation of I; yield 2.3 g (66%), mp 86—90°C, $[\alpha]_D^{25}$ −43.2° (c=1.0, DMF), Rf^a 0.86, Rf^b 0.91, single ninhydrin-positive spot. *Anal.* Calcd for $C_{29}H_{44}Cl_3N_5O_8$: C, 54.35; H, 6.36; N, 10.05. Found: C, 54.47; H, 6.16; N, 10.42.

Boc-Glu(OBzl)-Leu-Tyr-Leu-NHNH-Troc (X)——This compound was prepared from IX (1.5 g), Boc–Glu(OBzl)–OH (1.2 g), HOBT (317 mg) and WSCI (363 mg) essentially as described for the preparation of I; yield 1.3 g (68%), mp 80—83°C, $[\alpha]_D^{25}$ −17.2° (c=1.0, DMF), Rf^a0.78, Rf^b0.89, single ninhydrin-positive spot. *Anal.* Calcd for $C_{41}H_{57}Cl_3N_6O_{11}$: C, 53.74; H, 6.27; N, 9.17. Found: C, 53.39; H, 6.01; N, 8.43.

Boc-Val-Glu(OBzl)-Leu-Tyr-Leu-NHNH-Troc (XI)——This compound was prepared from X (916 mg), Boc–Val–OH (239 mg), HOBT (149 mg) and WSCI (171 mg) essentially as described for the preparation of I. The product was reprecipitated from EtOH and ether; yield 734 mg (72%), mp 124—129°C, $[\alpha]_D^{26}$ −15.8° (c=1.0, DMF), Rf^a 0.78, Rf^b 0.87, single ninhydrin-positive spot. *Anal.* Calcd for $C_{46}H_{66}Cl_3N_7O_{12} \cdot H_2O$: C, 53.46; H, 6.63; N, 9.49. Found: C, 53.48; H, 6.85; N, 9.27.

Boc-Tyr-NHNH-Troc (XII)——This compound was prepared from Boc–Tyr–OH (2 g), Troc–$NHNH_2$ (1.7 g), HOBT (1.1 g) and WSCI (1.2 g) essentially as described for the preparation of VII. The product was reprecipitated from EtOAc and *n*-hexane; yield 2.9 g (oily material) (85%), $[\alpha]_D^{26}$ +12.1° (c=1.0, DMF), Rf^a 0.73, Rf^b 0.76, single ninhydrin-positive spot. *Anal.* Calcd for $C_{17}H_{22}Cl_3N_3O_6 \cdot H_2O$: C, 41.78; H, 4.95; N, 8.60. Found: C, 41.85; H, 5.06; N, 8.42.

Boc-Val-Tyr-NHNH-Troc (XIII)——This compound was prepared from XII (2.4 g), Boc–Val–OH (1.2 g), HOBT (744 mg) and WSCI (854 mg) essentially as described for the preparation of I; yield 2.1 g (70%), mp 60—63°C, $[\alpha]_D^{26}$ −18.9° (c=1.0, DMF), Rf^a 0.76, Rf^b 0.81, single ninhydrin-positive spot. *Anal.* Calcd for $C_{22}H_{31}Cl_3N_4O_7 \cdot H_2O$: C, 44.95; H, 5.66; N, 9.53. Found: C, 45.01; H, 5.52; N, 9.27.

Boc-Asp(OBzl)-Val-Tyr-NHNH-Troc (XIV)——This compound was prepared from XIII (1.4 g), Boc–Asp(OBzl)–OH (890 mg), HOBT (372 mg) and WSCI (427 mg) essentially as described for the preparation of I; yield 1.4 g (74%), mp 74—81°C, $[\alpha]_D^{26}$ −19.5° (c=1.0, DMF), Rf^a 0.71, Rf^b 0.82, single ninhydrin-positive spot. *Anal.* Calcd for $C_{33}H_{42}Cl_3N_5O_{10}$: C, 51.14; H, 5.46; N, 9.04. Found: C, 50.82; H, 5.79; N, 8.75.

Boc-Lys(Z)-Asp(OBzl)-Val-Tyr-NHNH-Troc (XV)——This compound was prepared from XIV (553 mg), Boc–Lys(Z)–OH dicyclohexylammonium salt (441 mg), HOBT (107 mg) and WSCI (122 mg) essentially as described for the preparation of I. The product was reprecipitated from MeOH and ether; yield 521 mg (70%), mp 121—124°C, $[\alpha]_D^{26}$ −36.5° (c=1.0, DMF), Rf^a 0.74, Rf^b 0.89, single ninhydrin-positive spot. *Anal.* Calcd for $C_{47}H_{60}Cl_3N_7O_{13}$: C, 54.42; H, 5.83; N, 9.45. Found: C, 54.29; H, 6.09; N, 9.57.

Z-Arg(NO_2)-Lys(Z)-Asp(OBzl)-Val-Tyr-NHNH-Troc (XVI)——XV (346 mg) was treated with TFA (2 ml)–anisole (0.4 ml) as described above. The resulting powder was dissolved in DMF (3 ml). Z–Arg–(NO_2)–OH (130 mg), HOBT (50 mg) and WSCI (57 mg) were added to the above ice-chilled solution, followed by NMM (0.04 ml) to keep the solution slightly alkaline. After 16 h at 4°C, the reaction 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 $MgSO_4$, then concentrated *in vacuo*. The residue was reprecipitated from MeOH and ether; yield 318 mg (75%), mp 131—136°C, $[\alpha]_D^{26}$ −26.3° (c=1.0, DMF), Rf^a 0.86, Rf^b 0.92, single chlorine–tolidine-positive spot. *Anal.* Calcd for $C_{56}H_{69}Cl_3N_{12}O_6$: C, 52.85; H, 5.47; N, 13.21. Found: C, 53.11; H, 5.48; N, 12.93.

Z-Arg(NO_2)-Lys(Z)-Asp(OBzl)-Val-Tyr-$NHNH_2$ (XVII)——A solution of XVI (259 mg) in a mixture of AcOH (2 ml) and DMF (2 ml) was treated with Zn dust (80 mg) at room temperature for 3 h. The mixture was filtered, the filtrate was concentrated *in vacuo* and the residue was treated with 1% EDTA. The resulting gelatinous mass was washed batchwisely with 1 N $NaHCO_3$ and H_2O and then recrystallized from MeOH and H_2O; yield 196 mg (79%), mp 148—154°C, $[\alpha]_D^{26}$ −40.9° (c=1.0, DMF). *Anal.* Calcd for $C_{53}H_{68}N_{12}O_{14}$: C, 53.81; H, 6.87; N, 16.86. Found: C, 53.54; H, 6.45; N, 16.66.

Z-Arg(NO_2)-Lys(Z)-Asp(OBzl)-Val-Tyr-Val-Glu(OBzl)-Leu-Tyr-Leu-NHNH-Troc (XVIII)——XI (127 mg) was treated with TFA (2 ml)–anisole (0.4 ml) as usual and dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and dissolved in DMF (2 ml) containing NMM (0.01 ml). The azide[20] (prepared from 150 mg of XVII with 0.1 ml of 6 N HCl in dioxane and 0.06 ml of isoamylnitrite at −60°C) in DMF (2 ml)–DMSO (1 ml) and NMM (0.15 ml) were added to the above ice-chilled solution and the mixture was stirred for 48 h at 4°C. Then the mixture was poured into ice-chilled 1 N $NaHCO_3$ with stirring. Next, 50% NH_4OAc was added dropwise with stirring to form a precipitate. The precipitate was collected and washed successively with 1 N $NaHCO_3$, H_2O, 1 N HCl and H_2O. The product was precipitated from MeOH and ether. The dried powder was further purified by column chromatography on silica gel (2.1×46 cm), equilibrated and eluted with DMF–MeOH (1: 3). The desired fractions (4 ml each, tube Nos. 26—31) were combined and the solvent was removed by evaporation. Ether was added

to the residue to give a precipitate; yield 231 mg (81%), mp 186—192°C, $[\alpha]_D^{26}$ −37.4° (c=1.0, DMF), Rf^a 0.88, Rf^b 0.93, single chlorine–tolidine-positive spot. *Anal.* Calcd for $C_{94}H_{122}Cl_3N_{17}O_{24}\cdot 2H_2O$: C, 55.99; H, 6.29; N, 11.81. Found: C, 55.54; H, 6.45; N, 12.01.

Z-Arg(NO_2)-Lys(Z)-Asp(OBzl)-Val-Tyr-Val-Glu(OBzl)-Leu-Tyr-Leu-NHNH$_2$ (XIX)——This compound was prepared from XVIII (192 mg) and Zn dust (65 mg) essentially as described for the preparation of XVII. The product was reprecipitated from DMF and H_2O; yield 154 mg (83%), mp 146—153°C, $[\alpha]_D^{26}$ −50.1° (c=1.0, DMF). *Anal.* Calcd for $C_{91}H_{121}N_{17}O_{22}\cdot 3H_2O$: C, 58.80; H, 6.88; N, 12.81. Found: C, 58.59; H, 6.93; N, 12.72.

Z-Arg(NO_2)-Lys(Z)-Asp(OBzl)-Val-Tyr-Val-Glu(OBzl)-Leu-Tyr-Leu-Gln-Ser-Leu-Thr-Ala-Leu-Lys(Z)-Arg(NO_2)-OBzl (XX)——VI (64 mg) was treated with TFA (1 ml)–anisole (0.2 ml) as described above. The resulting octapeptide ester trifluoroacetate was dissolved in DMF (2 ml) containing NMM (0.01 ml). The azide (prepared from 140 mg of XIX with 0.013 ml of 6 N HCl in dioxane and 0.012 ml of isoamylnitrite at −60°C) in DMF (1 ml)–DMSO (1 ml) and NMM (0.06 ml) were added to the above ice-chilled solution and the mixture was stirred for 60 h at 4°C. After that, 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 HCl and H_2O. The product was further purified by column chromatography on silica gel (2.1×46 cm), equilibrated and eluted with DMF–BuOH (1: 3). The desired fractions (4 ml each, tube Nos. 26—29) were collected and the solvent was removed by evaporation. Ether was added to the residue to give a precipitate. The product was recrystallized from hot MeOH; yield 85 mg (58%), mp 210—221°C, $[\alpha]_D^{26}$ −21.4° (c=1.0, DMF), Rf^a 0.89, Rf^b 0.96, single chlorine–tolidine-positive spot. *Anal.* Calcd for $C_{145}H_{201}N_{29}O_{38}$: C, 58.87; H, 6.84; N, 13.73. Found: C, 59.06; H, 6.50; N, 13.99.

H-Arg-Lys-Asp-Val-Tyr-Val-Glu-Leu-Tyr-Leu-Gln-Ser-Leu-Thr-Ala-Leu-Lys-Arg-OH (XXI)——The protected octadecapeptide XX (50 mg) was treated with HF (approximately 4 ml) in the presence of anisole–thioanisole–*o*-cresol (1 ml) in an ice-chilled bath for 1 h. After removal of the HF, dry ether was added to the residue and the resulting powder was dissolved in H_2O (5 ml). The solution was treated with Amberlite CG-4B (acetate form, approximately 2 g) for 30 min, and filtered by suction. The filtrate was adjusted to pH 10 with 1 N NH_4OH and stirred in an ice-chilled bath for 30 min. The pH of the solution was adjusted to pH 5 with a few drops of AcOH and the solution was lyophilized. The crude peptide thus obtained was dissolved in 2% AcOH (2 ml), applied to a column of Sephadex G-25 (2.8×92 cm), and eluted with the same solvent. Fractions of 4 ml per 15 min were collected, and the absorption at 260 nm was determined. Fractions corresponding to the front main peak (tube Nos. 62—69) were combined and lyophilized. The resulting powder was dissolved in a small amount of the upper phase of a solvent system consisting of BuOH–AcOH–H_2O (4: 1: 5, by volume). The solution was subjected to partition column chromatography on Sephadex G-25 (2.8×65 cm) previously equilibrated with the lower phase of the above solvent system. The column was developed with the same upper phase. Fractions of 4 ml each were collected (one fraction per 28 min) and the absorbance at 260 nm was determined. The fractions corresponding to the main peak (tube Nos. 35—38) were combined and evaporated to dryness *in vacuo*, and then the residue was lyophilized; yield 13 mg (35%), mp 231—243°C (dec.), $[\alpha]_D^{26}$ −53.7° (c=0.3, 1 N AcOH), Rf^a 0.08, Rf^b 0.17, single ninhydrin- and Sakaguchi-positive spot. Amino acid ratios in an acid hydrolysate: Leu 4.11, Val 2.09, Ala 1.00, Arg 1.74, Lys 1.96, Asp 0.83, Tyr 1.79, Ser 0.79, Thr 0.82, Glu 1.72 (recovery of Ala 80%); amino acid ratios in an AP-M digest: Leu 4.04, Val 2.10, Ala 1.00, Arg 1.83, Lys 2.01, Asp 0.92, Tyr 1.96, Ser 0.88, Thr+Gln 1.48, Glu 1.49 (recovery of Ala 83%). (This enzyme partially digested glutamine to glutamic acid, but the data are not corrected; Gln emerged at the same position as Thr, and was calculated as Thr).

E-Rosette Formation——A 10 ml aliquot of venous blood was drawn into a syringe containing 1000 U of heparin and was incubated with the synthetic peptide for 30 min at 37°C, then lymphocytes were isolated in a Hypaque-ficoll gradient.[30] Isolated lymphocytes were adjusted to 5×10^5 cells/ml with PBS. Contamination by monocytes and polymorphonuclear cells amounted to less than 7%.[31] Sheep erythrocytes (Kyokuto Pharmaceutical Co.) were washed with PBS, and a suspension (1×10^8/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 16 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. Two hundred lymphocyte-like cells were counted and cells associtaed with three or more sheep erythrocytes were considered positive.

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References and Notes

1) The amino acid residues 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; DCC, dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; HOBT, *N*-hydroxybenzotriazole; AcOH, acetic acid; EtOAc, ethyl acetate; HF, hydrogen fluoride; NMM, *N*-methylmorpholine; Boc, *t*-butyloxycarbonyl; ONp, *p*-nitrophenyl ester; OBzl, benzyl ester; Z, benzyloxycarbonyl; NHNH-Troc, trichloroethyloxycarbonylhydrazide; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline; GVB^{2+}, gelatin veronal buffer; E-rosette, a rosette with sheep erythrocytes; HONB, *N*-hydroxy-5-norbornene-2,3-dicarboximide; THF, tetrahydrofuran.

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