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Synthesis of the Revised Amino Acid Sequence of Thymopoietin II and Examination of Its Immunological Effect on the Impaired T-Lymphocyte Transformation of a Uremic Patient with Pneumonia[1)]

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The nonatetracontapeptide corresponding to the revised amino acid sequence of thymopoietin II was synthesized by assembling ten peptide fragments in solution followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole (molar ratio, 1 : 1) in trifluoroacetic acid in the presence of dimethylselenide and *m*-cresol. The synthetic nonatetracontapeptide was tested for effect on impaired T-lymphocyte transformation by phytohemagglutinin (PHA) in a uremic patient with pneumonia. The synthetic peptide was found to have restoring activity on the impaired PHA stimulation of T-lymphocytes; the minimum effective concentration was 1 μg/ml.

Keywords——thymopoietin II; trifluoromethanesulfonic acid deprotection; uremic patient; pneumonia; impaired T-lymphocyte transformation; fluorometric blast-formation test

Two T-cell-differentiating hormones of thymus, thymopoietins I and II, were isolated from bovine thymus by Goldstein.[2)] Each peptide contains 49 amino acid residues, and the two peptides are closely related, differing by only two amino acid residues.[3)] Fujino and coworkers[4)] reported the first total synthesis of thymopoietin II using the HONB–DCC procedure in 1977. In 1981, the proposed structures of thymopoietins I and II were revised by Goldstein *et al.*[5)] They also reported the isolation of thymopoietin III from bovine spleen and the complete amino acid sequence of thymopoietin III.[5)] The revised structure of thymopoietin II differs from the previously proposed structure of thymopoietin II in the replacement of five amino acid residues: Ser (position 1), Gln (position 2), Gln (position 38), Thr (position 43) and Val (position 47) in the unrevised thymopoietin II are replaced by Pro, Glu, Glu, Ser

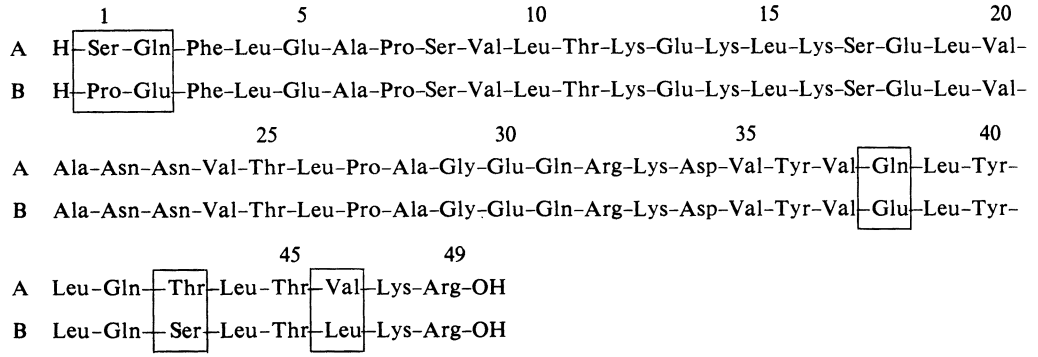

Fig. 1. Comparison of the Formerly Proposed Amino Acid Sequence of the Thymopoietin II (A) and the Revised Sequence of Thymopoietin II (B); Differences in the Sequences are Enclosed in Boxes[5)]

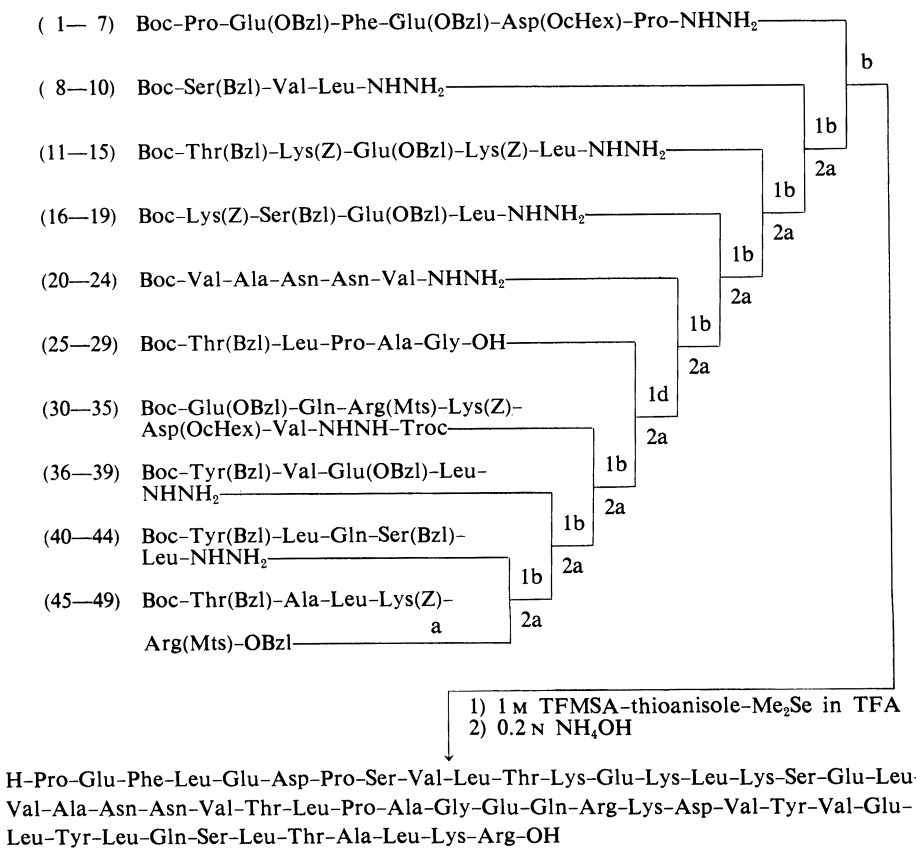

Fig. 2. Synthetic Route to Thymopoietin II

a, TFA–anisole; b, azide; c, Zn–AcOH; d, HOSu–WSCI.

and Leu, respectively.

In 1982, we reported[6] the synthesis of the octadecapeptide (32—49), which corresponds to a part of the revised structure of thymopoietin II, and showed that the biological activity of the revised thymopoietin II fragment (32—49) on low E-rosette-forming cells of an aged patient with chronic renal failure was equal to that of the unrevised thymopoietin II fragment (32—49).[7]

Following the syntheses of thymopoietins I and III[8,9] we wish to report the solution synthesis of the revised structure of thymopoietin II, *via* the route illustrated in Fig. 2 and to describe the *in vitro* effect of this peptide on the impaired PHA stimulation of T-lymphocytes from a uremic patient with pneumonia.

The life of patients with chronic renal failure has been prolonged by recent advances in hemodialysis therapy. However, the complication of infectious diseases in chronic hemodialysis patients still poses a grave problem in term of their prognosis. It is an accepted fact that there is a decrease in immunity, particularly cell-mediated immunity, in uremic patients.[10] This impairement is reflected in both *in vitro* and *in vivo* depressed cell-mediated immune function. Patients with chronic uremia may also have thymic atrophy. The thymus may show a marked reduction in lymphoid elements and extensive replacement with fat. These observations[11] suggested to us that one of the cell-mediated immune abnormalities seen chronic uremia might be attributable to thymic hormone deficiency. Thus, we were interested in the immunological effect of the revised amino acid sequence of thymopoietin II

on the impaired immune function in uremic patients.

The synthetic routes that we employed here are almost the same as those employed for our previous syntheses of thymopoietins I and III.[8,9] As illustrated in Fig. 2, the TFA-labile Boc group was employed for N^{α}-protection and amino acid derivatives bearing protecting groups removable by the thioanisole-mediated TFMSA deprotecting procedure[12,13] were employed, *i.e.*, Lys(Z), Glu(OBzl), Thr(Bzl), Ser(Bzl), Tyr(Bzl), Asp(OcHex)[14] and Arg(Mts).[15] N^{α}-Deprotection with TFA was performed in the presence of anisole, prior to each condensation reaction as usual. Asp(OcHex) was employed to minimize aspartimide formation during the synthesis of Asp-containing peptides.[14] Eight protected peptide hydrazides, one protected pentapeptide and the C-terminal protected pentapeptide ester were selected as building blocks to construct the revised amino acid sequence of thymopoietin II (Fig. 2). Of these, eight fragments, Boc–(1—7)–$NHNH_2$ [10], Boc–(8—10)–$NHNH_2$ [9], Boc–(11—15)–$NHNH_2$ [8], Boc–(16—19)–$NHNH_2$ [7], Boc–(20—24)–$NHNH_2$ [6], Boc–(25—29)–OH [5], Boc–(36—39)–$NHNH_2$ and Boc–(45—49)–OBzl [1], are available from our previous syntheses of thymopoietins I and III.[8,9] The protected pentapeptide ester, Boc–(40—44)–OMe, was prepared stepwise starting from H–Leu–OMe·HCl by the HOBT–WSCI procedure[16] except for the introduction of Gln, which was introduction by the NP active ester procedure.[17] The protected pentapeptide ester thus obtained was smoothly converted to the corresponding hydrazide, Boc–(40—44)–$NHNH_2$ [2], in the usual manner. The hydrazine test on the thin-layer chromatograms and elemental analysis data of this peptide fragment were consistent with homogeneity of the desired product. Next, for the preparation of the protected hexapeptide fragment containing Glu(OBzl) and Asp(OcHex), Boc–(30—35)–NHNH–Troc, we employed a substituted hydrazide, Troc–$NHNH_2$,[18] a protecting group which is known to be removed by Zn[19] in AcOH without affecting side chain protecting groups such as Boc, Mts, Z, OBzl and OcHex. Boc–(30—35)–NHNH–Troc was prepared stepwise starting from Boc–Val–NHNH–Troc by the HOBT–WSCI procedure[16] except for the introduction of the Gln residue, which was introduced by the NP active ester procedure,[17] and the introduction of the Arg(Mts) residue, which was introduced by the MA procedure.[20] The protected hexapeptide, Boc–(30—35)–NHNH–Troc, was treated with Zn[19] in AcOH to remove the Troc group, and the zinc acetate was removed by treatment with EDTA to give the required hydrazide, Boc–(30—35)–$NHNH_2$ [2], in analytically pure form. The hydrazine test on the thin-layer chromatograms and elemental analysis data were consistent with homogeneity of the desired product.

The ten fragments were assembled successively by the azide procedure[21] and the HOSu–WSCI procedure[22] according to the routes illustrated in Fig. 2. The amount of the acyl component in each fragment condensation was increased from 2 to 5 eq as the chain elongation proceeded. The solubility of protected intermediates in DMF decreased remarkably with chain elongation. Consequently, mixtures of DMF–DMSO had to be employed for the subsequent condensation reactions. Some of the intermediates were purified by repeated precipitation from DMF or DMSO with MeOH and others were purified by gel-filtration on Sephadex LH-60 using DMF or DMSO as the eluent. Throughout this synthesis, Ala or Gly was taken as the diagnostic amino acid in acid hydrolysis. By comparison of the recovery of Ala or Gly with those of newly incorporated amino acids, satisfactory incorporation of each fragment in each condensation reaction was confirmed.

Starting with the side-chain-protected pentapeptide ester corresponding to positions 45 to 49 of thymopoietin II, Boc–(45—49)–OBzl, nine fragments, Boc–(40—44)–$NHNH_2$, Boc–(36—39)–$NHNH_2$, Boc–(30—35)–$NHNH_2$, Boc–(25—29)–OH, Boc–(20—24)–$NHNH_2$, Boc–(16—19)–$NHNH_2$, Boc–(11—15)–$NHNH_2$, Boc–(8—10)–$NHNH_2$ and Boc–(1—7)–$NHNH_2$, were successively condensed by the azide procedure[21] and the HOSu–WSCI procedure[22] as shown in Fig. 2 to give the protected nonatetracontapeptide corresponding to

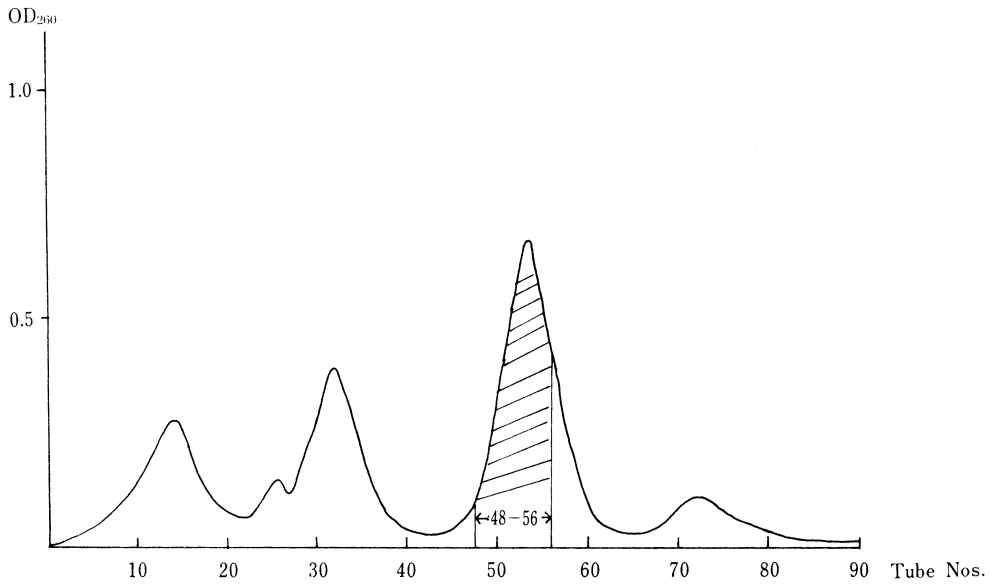

Fig. 3. Purification of Synthetic Thymopoietin II by Ion-Exchange Chromatography on a CM-Biogel A Column

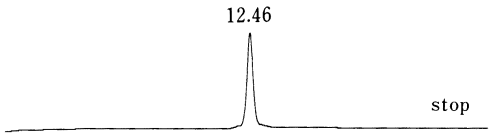

Fig. 4. HPLC of Synthetic Thymopoietin II

the entire amino acid sequence of thymopoietin II. The homogeneities of the peptides were checked by elemental analysis, TLC and amino acid analyses of the acid hydrolysates.

In the final step of the synthesis, the protected nonatetracontapeptide ester was treated with 1 M TFMSA–thioanisole in TFA in the presence of *m*-cresol and Me_2Se. *m*-Cresol was used as an additional cation scavenger to suppress a side reaction, *i.e.*, *O*-sulfonation of Tyr residues.[15] Me_2Se was employed to facilitate acidolytic cleavage of protecting groups.[23] The deprotected peptide was next precipitated with dry ether, converted to the corresponding acetate with Amberlite IR-400 (acetate form) and then treated with 0.2 N NH_4OH to reverse a possible N→O shift at the Ser and Thr residues.[24] The crude peptide was purified by gel-filtration on Sephadex G-50 and then by ion-exchange column chromatography on a CM-Biogel A column with linear gradient elution using pH 6.5 ammonium acetate buffer (0→0.25 M), followed by preparative TLC.

Desalting on Sephadex G-25 gave a fluffy powder, which exhibited a single spot (ninhydrin- and Sakaguchi-positive) on TLC in two different solvent systems and on paper electrophoresis (pH 2.80 acetate buffer). The peptide also exhibited a single peak on HPLC (Fig. 4). Homogeneity of the synthetic thymopoietin II was further ascertained by amino acid analyses, after 6 N HCl hydrolysis and enzymatic digestion.

The immunological effect of the synthetic thymopoietin II was examined by the JIMRO (Japan Immunoresearch Laboratories Co., Ltd.) fluorometric blast-formation test according to Itoh and Kawai.[25] In contrast to normal persons, the transformation of the T-lymphocytes into lymphoblasts with mitotic activity after PHA stimulation is depressed in severely uremic patients with pneumonia. The *in vitro* effect of the synthetic peptide on the impaired PHA

TABLE I. Effect of the Synthetic Thymopoietin II on the Impaired PHA Stimulation of T-Lymphocytes of a Uremic Patient with Pneumonia

Peptide	Dose (μg/ml)	$SI^{a,b)}$
—[c)]	—	311.4±60.8
—[d)]	—	129.7±64.7
Synthetic thymopoietin II[d,e)]	0.1	118.6±63.1
	1.0	192.6±66.3
	2.0	283.2±59.4
	3.0	280.3±62.4

a) Each value represents the mean±S.D. of triplicate measurements. *b*) *SI* (stimulation index) was calculated according to the following formula: $SI=\{(I_2-I_o)/(I_1-I_o)\}\times 100$, where I_2 = mean fluorescence intensity of PHA-P activated lymphocytes, I_1 = fluorescence intensity of PHA-P nonactivated lymphocytes and I_o = fluorescence intensity of ethidium bromide. *c*) Normal venous lymphocytes. *d*) Patient's venous lymphocytes. *e*) Incubation was carried out at 37 °C in a humidified atmosphere of 5% CO_2 in air for 12 h.

stimulation of T-lymphocytes from a uremic patient with pneumonia is shown in Table I. When peripheral T-lymphocytes isolated from the patient were incubated with various amounts of the synthetic thymopoietin II from 0.1 to 3 μg/ml, restoration of the impaired PHA stimulation of T-lymphocytes was observed at concentrations of 1 μg/ml and above. In normal subjects, no effects of thymopoietin II were observed (data not shown). The results suggest that the synthetic thymopoietin II is able to restore the impaired T-lymphocyte transformation *in vitro* in severely uremic patients with infectious diseases such as pneumonia.

Experimental

General experimental procedures used in this paper are essentially the same as described in the previous papers.[8,9] An azide was prepared according to Honzl and Rudinger[21] with isoamyl nitrite. Melting points are uncorrected. Rotations were measured with an Atago Polax machine (cell length: 10 cm). The amino acid compositions of the acid and enzymatic hydrolysates were determined with a Hitachi 835-50 type amino acid analyzer. Solutions were concentrated in a rotary evaporator under reduced pressure at a temperature of 30—45 °C. Boc groups of protected peptides were removed by TFA–anisole treatment in the presence of anisole. The resulting amino components were chromatographed on silica gel plates (Kieselgel G, Merck) and *Rf* values refer to the following solvent systems: Rf^1, the Partridge system[26]; Rf^2, BuOH–pyridine–AcOH–H_2O (30:20:6:24).[27] The final product corresponding to the entire amino acid sequence of thymopoietin II was chromatographed on a cellulose plate (Merck). Rf^3 value refers to BuOH–AcOH–H_2O (4:1:1) and Rf^4 value refers to BuOH–pyridine–AcOH–H_2O (30:20:6:24).[27] Troc–$NHNH_2$ was purchased from Kokusan Chemical Works Ltd., Japan. Kits for the fluorometric blast-formation test were purchased from Japan Immunoresearch Laboratories Co., Ltd., Japan. Preparations of protected intermediates were repeated several times in order to obtain sufficient quantities for the next step. Papain (No. p-3125) and leucine aminopeptidase (No. L-9876) were purchased from Sigma Chemical Co. Venous blood was obtained from a severely uremic patient with pneumonia. Venous blood from three healthy doners was used as a control. The fluorescence excitation spectra was measured with a UVLOG-FlOUSPEC-11A fluorometer. HPLC was conducted with a Shimadzu LC-3A apparatus coupled to a μBondapak C_{18} column.

Boc–Ser(Bzl)–Leu–OMe (I)——H–Leu–OMe·HCl (1.8 g) was dissolved in THF (10 ml) containing NMM (1.1 ml). To this ice-chilled solution, Boc–Ser(Bzl)–OH (3 g), HOBT (1.5 g) and WSCI (2.1 g) were successively added. After being stirred at 4 °C for 12 h, the mixture was extracted with EtOAc, and the extract was washed successively with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O, dried over $MgSO_4$, and then concentrated *in vacuo*. The residue was reprecipitated from EtOAc with petroleum ether: Yield 4 g (95%), mp 71—74 °C, $[\alpha]_D^{21}-6.8°$ ($c=1.0$, DMF), Rf^1 0.52, Rf^2 0.60, single ninhydrin-positive spot. *Anal.* Calcd for $C_{22}H_{34}N_2O_6\cdot H_2O$: C, 59.98; H, 8.24; N, 6.36. Found: C, 59.86; H, 8.32; N, 6.27.

Boc–Gln–Ser(Bzl)–Leu–OMe (II)——I (3.5 g) was treated with TFA–anisole (10 ml–2 ml) in an ice-bath for 40 min, and TFA was then removed by evaporation. The residue was washed with *n*-hexane, and dried over KOH pellets *in vacuo* for 2 h. The dried residue was dissolved in DMF (20 ml) together with NMM (0.93 ml). Boc–Gln–ONp (3.6 g) was added and the solution was stirred at room temperature for 12 h. The reaction mixture was 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 stirring, and the precipitate thereby formed was washed successively with 1 N NH_4OH, H_2O, 5% citric acid and H_2O. The product was reprecipitated from DMF with 1 N NH_4OH: Yield 4.1 g (87%), mp 139—144 °C, $[\alpha]_D^{21} - 16.2°$ ($c = 1.0$, DMF), Rf^1 0.46, Rf^2 0.47, single ninhydrin-positive spot. *Anal.* Calcd for $C_{27}H_{52}N_4O_8$: C, 57.84; H, 9.35; N, 9.99. Found: C, 57.49; H, 9.40; N, 9.81.

Boc–Leu–Gln–Ser(Bzl)–Leu–OMe (III)——II (2.2 g) was treated with TFA–anisole (10 ml–2 ml) as described above and the resulting powder was dissolved in DMF (15 ml) containing NMM (0.44 ml). To this ice-chilled solution, Boc–Leu–OH (1.1 g), HOBT (594 mg) and WSCI (844 mg) were successively added. After 12 h at 4 °C, the mixture was extracted with EtOAc, and the extract was washed successively with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O, dried over $MgSO_4$ and then concentrated *in vacuo*. The residue was reprecipitated from EtOH with ether: Yield 1.9 g (73%), mp 136—143 °C, $[\alpha]_D^{21} - 24.6°$ ($c = 1.0$, DMF), Rf^1 0.48, Rf^2 0.52, single ninhydrin-positive spot. *Anal.* Calcd for $C_{33}H_{63}N_5O_9 \cdot 2\,H_2O$: C, 55.38; H, 9.51; N, 9.87. Found: C, 55.74; H, 9.46; N, 9.74.

Boc–Tyr(Bzl)–Leu–Gln–Ser(Bzl)–Leu–OMe (IV)——This compound was prepared essentially in the same manner as described for the preparation of III by using III (1.4 g), Boc–Tyr(Bzl)–OH (819 mg), HOBT (298 mg) and WSCI (422 mg). The product was reprecipitated from acetone with ether: Yield 1.3 g (68%), mp 124—132 °C, $[\alpha]_D^{21} - 11.4$ ($c = 1.0$, DMF), Rf^1 0.68, Rf^2 0.69, single ninhydrin-positive spot. *Anal.* Calcd for $C_{49}H_{78}N_6O_{11}$: C, 63.48; H, 8.48; N, 9.06. Found: C, 63.17; H, 8.69; N, 8.85.

Boc–Tyr(Bzl)–Leu–Gln–Ser(Bzl)–Leu–NHNH$_2$ (V)——IV (927 mg) was dissolved in DMF–MeOH (1 : 1, 10 ml). Hydrazine hydrate (0.48 ml) was added, and the solution was kept standing at room temperature for 36 h. After evaporation of the MeOH, the residue was poured into ice-chilled H_2O with vigorous stirring. The precipitate thereby formed was filtered off and washed with H_2O. The dried product was recrystallized from hot MeOH: Yield 742 mg (80%), mp 136—143 °C, $[\alpha]_D^{21} - 15.6°$ ($c = 1.0$, DMF), Rf^1 0.44, Rf^2 0.47, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{48}H_{78}N_8O_{10}$: C, 62.18; H, 8.48; N, 12.09. Found: C, 62.03; H, 8.76; N, 11.87.

Boc–Asp(OcHex)–Val–NHNH–Troc (VI)——This compound was prepared essentially in the same manner as described for the preparation of III by using Boc–Val–NHNH–Troc (1 g), Boc–Asp(OcHex)–OH (821 mg), HOBT (327 mg) and WSCI (528 mg). The product was reprecipitated from EtOAc with petroleum ether: Yield 1.1 g (85%), mp 70—73 °C, $[\alpha]_D^{21} - 8.4°$ ($c = 1.0$, DMF), Rf^1 0.50, Rf^2 0.51, single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{37}Cl_3N_4O_8$: C, 45.74; H, 6.18; N, 9.28. Found: C, 45.62; H, 6.32; N, 9.45.

Boc–Lys(Z)–Asp(OcHex)–Val–NHNH–Troc (VII)——This compound was prepared essentially in the same manner as described for the preparation of III by using VI (1 g), Boc–Lys(Z)–OH · DCHA (1.1 g), HOBT (248 mg) and WSCI (352 mg). The product was reprecipitated from EtOAc with *n*-hexane: Yield 1.3 g (93%), mp 78—84 °C, $[\alpha]_D^{21} - 21.3$ ($c = 1.0$, DMF), Rf^1 0.54, Rf^2 0.57, single ninhydrin-positive spot. *Anal.* Calcd for $C_{37}H_{55}Cl_3N_6O_{11} \cdot 2\,H_2O$: C, 49.26; H, 6.59; N, 9.31. Found: C, 49.52; H, 6.73; N, 9.18.

Boc–Arg(Mts)–Lys(Z)–Asp(OcHex)–Val–NHNH–Troc (VIII)——VII (1.1 g) was treated with TFA–anisole (10 ml–2 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–THF (1 : 1, 10 ml) containing NMM (0.14 ml). To this ice-chilled solution, a solution of the mixed anhydride [prepared from 741 mg of Boc–Arg(Mts)–OH · DCHA with 0.16 ml of ethylchlorocarbonate at −10 °C] in THF (4 ml) and acetonitrile (4 ml) was added. The mixture was stirred at 4 °C for 2 h and then at room temperature for 6 h. The mixture was evaporated *in vacuo*, the residue was extracted with EtOAc, and the extract was washed successively with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O, dried over $MgSO_4$, and then concentrated *in vacuo*. The residue was reprecipitated from EtOAc with *n*-hexane: Yield 1 g (67%), mp 74—77 °C, $[\alpha]_D^{21} - 15.7°$ ($c = 1.0$, DMF), Rf^1 0.60, Rf^2 0.54, single ninhydrin-positive spot. *Anal.* Calcd for $C_{52}H_{77}Cl_3N_{10}O_{14}S$: C, 51.85; H, 6.44; N, 11.63. Found: C, 51.73; H, 6.70; N, 11.59.

Boc–Gln–Arg(Mts)–Lys(Z)–Asp(OcHex)–Val–NHNH–Troc (IX)——This compound was prepared from VIII (803 mg) and Boc–Gln–ONp (313 mg) essentially as described for the preparation of IV. The dried product was reprecipitated from EtOAc with ether: Yield 759 mg (86%), mp 123—129 °C, $[\alpha]_D^{21} - 30.8°$ ($c = 1.0$, DMF), Rf^1 0.49, Rf^2 0.52, single ninhydrin-positive spot. *Anal.* Calcd for $C_{57}H_{85}Cl_3N_{12}O_{16}S$: C, 51.37; H, 6.43; N, 12.61. Found: C, 51.23; H, 6.74; N, 12.24.

Boc–Glu(OBzl)–Gln–Arg(Mts)–Lys(Z)–Asp(OcHex)–Val–NHNH–Troc (X)——This compound was prepared from IX (666 mg), Boc–Glu(OBzl)–OH (186 mg), HOBT (74 mg) and WSCI (106 mg) essentially as described for the preparation of III. The reaction mixture was poured into ice-chilled 5% citric acid with stirring. The precipitate thereby formed was washed successively with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O. The dried product was reprecipitated from MeOH with ether: Yield 592 mg (76%), mp 132—137 °C, $[\alpha]_D^{21} - 16.2°$ ($c = 1.0$, DMF), Rf^1 0.56, Rf^2 0.55, single ninhydrin-positive spot. *Anal.* Calcd for $C_{69}H_{98}Cl_3N_{13}O_{19}S \cdot 3\,H_2O$: C, 51.60; H, 6.53; N, 11.34. Found: C, 51.62; H, 6.48; N, 11.73.

Boc–Glu(OBzl)–Gln–Arg(Mts)–Lys(Z)–Asp(OcHex)–Val–NHNH$_2$ (XI)——X (517 mg) in a mixture of AcOH (4 ml) and DMF (4 ml) was treated with Zn dust (218 mg) at 4 °C for 2 h and then at room temperature for 8 h. The solution was filtered, the filtrate was concentrated *in vacuo*, and the residue was treated with 3% EDTA and then with $NaHCO_3$ to adjust the pH to neutral. The resulting powder was washed with H_2O and reprecipitated from DMF with H_2O: Yield 419 mg (89%), mp 164—173 °C, $[\alpha]_D^{21} - 16.2°$ ($c = 1.0$, DMF), Rf^1 0.50, Rf^2 0.54, single hydrazine-

test-positive spot. *Anal.* Calcd for $C_{66}H_{97}N_{13}O_{17}S \cdot 2\,H_2O$: C, 56.12; H, 7.21; N, 12.89. Found: C, 56.09; H, 7.41; N, 12.91.

Synthesis of the Protected Thymopoietin II——1. Successive azide condensations of nine fragments except for Boc–(25—29)–OH [5], which was condensed by the HOSu–WSCI procedure, were carried out according to the Fig. 2. Prior to condensation, the Boc group was removed from the respective amino component (1 ml per 0.1 g of the peptide) in the presence of anisole (10 eq) in an ice-bath for 40 min. The TFA-treated sample was precipitated with dry ether, dried over KOH pellets *in vacuo* for 2 h and dissolved in DMF or DMF–DMSO (1 : 1) containing NMM (1.1 eq). The corresponding azide (the amount was increased from 2 to 5 eq as chain elongation progressed) in DMF or DMF–DMSO (1 : 1) and NMM (1.1 eq) were added to the above ice-chilled solution and the mixture was stirred at −10 °C until the solution became negative to the ninhydrin test. The mixture was poured into ice-chilled 5% citric acid with stirring. The precipitate thereby formed was successively washed with 5% citric acid and H_2O. The dried product was purified by one of the following two procedures. A: Precipitation from DMF or DMSO with MeOH. B: Gel-filtration on Sephadex LH-60 using DMF or DMSO as an eluant. In procedure B, eluates (5 ml fractions) were examined by measuring the ultraviolet (UV) absorption at 260 nm and the fractions corresponding to the front main peak were combined. The solvent was removed by evaporation and the residue was treated with ether to afford a powder.

2. The HOSu–WSCI condensation procedure: Boc–(30—49)–OBzl was treated with TFA–anisole and the N^{α}-deprotected peptide, isolated as usual, was dissolved in DMF–DMSO (1 : 1) together with NMM (1.1 eq). To this were added Boc–Thr(Bzl)–Leu–Pro–Ala–Gly–OH (3 eq), HOSu (3 eq) and WSCI (3 eq) at 0 °C. After 24 h, the reaction mixture was evaporated *in vacuo* and then triturated with 5% citric acid. The powder obtained was washed successively with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O. The powder was further purified by reprecipitaion three times from DMSO with MeOH. The purification procedure, yield, physical constants and analytical data of protected thymopoietin II and its intermediates are listed in Tables II and III.

H–Pro–Glu–Phe–Leu–Glu–Asp–Pro–Ser–Val–Leu–Thr–Lys–Glu–Lys–Leu–Lys–Ser–Glu–Leu–Val–Ala–Asn–Asn–Val–Thr–Leu–Pro–Ala–Gly–Glu–Gln–Arg–Lys–Asp–Val–Tyr–Val–Glu–Leu–Tyr–Leu–Gln–Ser–Leu–Thr–Ala–Leu–Lys–Arg–OH (Corresponding to Thymopoietin II)——The protected nonatetracontapeptide (85 mg) was treated with 1M TFMSA–thioanisole in TFA (3 ml) in the presence of *m*-cresol (70 μl) and Me_2Se (60 μl) in an ice-bath for 110 min, then dry ether was added. The resulting powder was collected by centrifugation, and dried over KOH pellets *in vacuo* for 60 min. The deprotected peptide thus obtained was dissolved in H_2O (5 ml) and treated with Amberlite IR-400 (acetate form, approximately 2 g) for 30 min with stirring. The pH of the filtrate was adjusted to 8.0 with ice-chilled 0.2 N NH_4OH, then after 30 min, to 6.0 with 0.2 N AcOH, and the solution was lyophilized to give a

TABLE II. Characterization of the Protected Thymopoietin II (Revised Amino Acid Sequence) and Its Intermediates

	Puri. proc. (Yield %)	Rf^{1}	mp (°C)	$[\alpha]_D^{21}$ (c = 1.0, DMF)	Formula	Analysis (%) Calcd (Found)		
						C	H	N
Boc–(40—49)–OBzl	A (76)	0.72	186—195	−17.4	$C_{104}H_{131}N_{15}O_{21}S \cdot 8H_2O$	59.39	7.04	9.99
						(59.57	7.10	9.87)
Boc–(36—49)–OBzl	A (79)	0.79	207—215	−26.3	$C_{143}H_{189}N_{19}O_{28}S \cdot 10H_2O$	60.60	7.34	9.39
						(60.48	7.52	9.22)
Boc–(30—49)–OBzl	B (73)	0.72	224—234	−25.4	$C_{204}H_{274}N_{30}O_{43}S_2 \cdot 13H_2O$	59.29	7.32	10.17
						(59.31	7.59	9.85)
Boc–(25—49)–OBzl	A (75)	0.61	218—227	−16.3	$C_{231}H_{313}N_{35}O_{49}S_2 \cdot 15H_2O$	59.74	7.31	10.56
						(59.51	7.42	10.29)
Boc–(20—49)–OBzl	A (71)	0.51	241—259	−26.4	$C_{242}H_{348}N_{42}O_{56}S_2 \cdot 11H_2O$	58.09	7.45	11.76
						(58.01	7.67	11.52)
Boc–(16—49)–OBzl	A (80)	0.48	218—229	−14.9	$C_{294}H_{401}N_{47}O_{65}S_2 \cdot 16H_2O$	58.99	7.29	11.00
						(58.82	7.45	10.87)
Boc–(11—49)–OBzl	B (74)	0.52	231—242	−21.6	$C_{351}H_{474}N_{54}O_{77}S_2 \cdot 14H_2O$	60.24	7.23	10.18
						(60.13	7.41	10.56)
Boc–(8—49)–OBzl	B (71)	0.50	211—224	−17.6	$C_{372}H_{505}N_{57}O_{81}S_2 \cdot 16H_2O$	59.90	7.31	10.70
						(59.99	7.52	10.57)
Boc–(1—49)–OBzl	B (52)	0.44	180—197	−31.2	$C_{431}H_{578}N_{64}O_{94}S_2 \cdot 17H_2O$	60.69	7.23	10.51
						(60.47	7.38	10.29)

A, precipitation from DMF or DMSO with MeOH. B, gel-filtration on Sephadex LH-60.

TABLE III. Amino Acid Ratios in 6 N HCl Hydrolysates of the Protected Thymopoietin II (Revised Amino Acid Sequence) and Its Intermediates[a]

	Protected peptides									Residue
	40—49	36—49	30—49	25—49	20—49	16—49	11—49	8—49	1—49	
Leu	3.02	4.03	4.01	5.02	4.99	6.10	7.08	8.04	9.06	9
Val		1.01	1.92	2.03	4.02	4.02	4.00	5.02	5.01	5
Ala	1.00	1.00	2.10	3.04	3.05	2.94	3.01	3.10	3.08	3
Gly				1.00	1.00	1.00	1.00	1.00	1.00	1
Pro				0.91	0.93	0.91	0.86	0.87	2.84	3
Phe									0.92	1
Tyr	0.81	1.94	1.92	1.97	1.96	1.92	1.83	1.90	1.87	2
Ser	0.89	0.87	0.88	0.87	0.86	1.89	1.80	2.80	2.84	3
Thr	0.91	0.89	0.90	1.84	1.88	1.87	2.85	2.88	2.83	3
Glu	0.94	1.95	4.02	4.01	3.92	4.87	5.89	5.86	7.91	8
Asp			0.99	0.92	2.90	2.86	2.94	2.91	3.90	4
Lys	1.01	0.97	2.01	1.98	2.04	3.02	4.92	4.90	4.91	5
Arg	0.89	0.88	1.87	1.85	1.84	1.82	1.85	1.83	1.84	2

a) The results are expressed as ratios to the value for Ala or Gly, which was taken as the diagnostic amino acid in acid hydrolysates.

fluffy powder. The powder was dissolved in 1% AcOH (2 ml), applied to a column of Sephadex G-50 (3.6 × 97 cm) and eluted with 1% AcOH. Individual fractions (5 ml each) were collected and the absorbancy at 260 nm was determined. The front peak (tube Nos. 79—86) was collected and the solvent was removed by lyophilization. Next, the Sephadex-purified sample was dissolved in H_2O (2 ml) and the solution was applied to a column of CM-Biogel A (2.3 × 12 cm), which was eluted first with H_2O (100 ml) and then with a linear gradient from H_2O (250 ml) to 0.25 M NH_4OAc buffer (250 ml, pH 6.50). Individual fractions (4 ml each) were collected and the absorbancy at 260 nm was determined. The main peak (tube Nos. 48—56) was collected and the solvent was removed by lyophilization. Analysis by TLC revealed the presence of two ninhydrin-positive spots with Rf 0.57 (main) and Rf 0.74 (minor). The crude peptide was dissolved in a small amount of H_2O and subjected to preparative TLC (cellulose plate, 20 × 40 cm) using BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24) as a developing solvent. The zone corresponding to Rf 0.57 was separated and extracted with 2% AcOH. The extracts were concentrated to a small volume, applied to a Sephadex G-25 column (3.6 × 90 cm), and eluted with 2% AcOH. The single main peak fractions were combined and the solvent was removed by lyophization to give a fluffy powder: Yield 6.1 mg (11%), $[\alpha]_D^{21}$ −73.2° (c=0.3, 1 N AcOH), Rf^3 0.58, Rf^4 0.57, single ninhydrin- and Sakaguchi-positive spot. The synthetic peptide exhibited a single spot on paper electrophoresis: Toyo Roshi No. 51 (2 × 40 cm), acetate buffer at pH 2.80, Mobility, 8.0 cm from the origin toward the anode, after running at 1.5 mA, 600 V for 60 min. The synthetic peptide exhibited a single peak on HPLC using a μBondapak C_{18} column (0.39 × 30 cm) at a retention time of 12.46 min, when eluted with a gradient of acetonitrile (30—35% in 20 min) in 0.1% TFA at a flow rate of 1.0 ml per min (Fig. 4). Amino acid ratios in a 6 N HCl hydrolysate: Leu 9.04, Val 4.92, Ala 3.09, Gly 1.00, Pro 2.82, Phe 0.94, Tyr 1.89, Ser 2.85, Thr 2.90, Glu 7.95, Asp 3.89, Lys 4.92, Arg 1.83 (recovery of Gly 83%). Amino acid ratios in papain plus leucine aminopeptidase digest: Leu 9.01, Val 5.04, Ala 2.98, Gly 1.00, Pro 2.91, Phe 0.99, Tyr 2.03, Ser 2.91, Thr 2.94, Glu 5.92, Asp 1.87, Lys 5.01, Arg 1.93; Asn and Gln were not determined (recovery of Gly 80%).

Fluorometric Blast-Formation Test——A 3 ml aliquot of venous blood was drawn into a syringe containing 25 U/ml of heparin and then mixed with 3 ml of PBS. Lymphocytes were isolated in a Hypague-Ficoll gradient.[28] Isolated lymphocytes were adjusted to 1.0 × 10^6/ml with PBS. The lymphocytes were cultured in 0.5 ml of RPMI 1640 (Gibco) with 10% FCS (Dainippon Pharmaceutical Co.) in microplates. Cultures of each combination were incubated at 37 °C in the presence of peptide (0.1—3 μg/ml) in a humidified atmosphere of 5% CO_2 in air for 12 h and then PHA-P was added to each well and incubation was continued under the same conditions for 60 h. T-Lymphocytes in each well were transferred into a test tube and centrifuged for 10 min at 240 g, then the supernatant was removed. A 2 ml aliquot of 0.125% SDS was added to the residue and stirred for 20 min at room temperature; the lymphocytes were completely destroyed and solubilized by this procedure. Ethidium bromide solution (2 ml) added to the above solution and the mixture was stirred for 15 min at room temperature. The fluorescence excitation spectrum was measured according to Itoh and Kawai.[25]

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References and Notes

1) The amino acid residues mentioned in this paper are of L-configuration except for glycine. The abbreviations used to denote amino acid derivatives and peptide are those recommended by the IUPAC-IUB Joint Commission on Biochemical Nomenclature: *Eur. J. Biochem.*, **138**, 9 (1984); *Int. J. Peptide Protein Res.*, **24**, No. 1 (1984). Other abbreviations used: DMF, dimethylformamide; DMSO, dimethylsulfoxide; WSCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBT, 1-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; AcOH, acetic acid; EtOAc, ethyl acetate; NMM, *N*-methylmorpholine; MeOH, methanol; THF, tetrahydrofuran; TFA, trifluoroacetic acid; Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl; ONp, *p*-nitrophenyl ester; NP, *p*-nitrophenyl; Mts, mesitylene-2-sulfonyl; OBzl, benzyl ester; Troc, β,β,β-trichloroethyloxycarbonyl; Bzl, benzyl; OcHex, cyclohexyl ester; EDTA, ethylenediaminetetraacetic acid; OMe, methyl ester; CM, carboxymethyl; FCS, fetal calf serum; RPMI, Rosewell Park Memorial Institute; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; E-rosette, a rosette with sheep erythrocytes; TFMSA, trifluoromethanesulfonic acid; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; DCC, *N,N'*-dicyclohexylcarbodiimide; HONB, *N*-hydroxy-5-norbornene-2,3-dicarboximide; PHA, phytohemagglutinin; MA, mixed anhydride; DCHA, dicyclohexylamine.
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