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Synthesis of the Nonatetracontapeptide Corresponding to the Entire Amino Acid Sequence of Thymopoietin I and Its Effect on the Low E-Rosette-Forming Cells of a Uremic Patient[1)]

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The nonatetracontapeptide corresponding to the entire amino acid sequence of thymopoietin I was synthesized by successive azide condensation of seven fragments: Boc-(36—41)-$NHNH_2$, Boc-(30—35)-$NHNH_2$, Boc-(25—29)-$NHNH_2$, Boc-(20—24)-$NHNH_2$, Boc-(16—19)-$NHNH_2$, Boc-(8—15)-$NHNH_2$ and Boc-(1—7)-$NHNH_2$ with H-(42—49)-OBzl. The deprotection of the protected nonatetracontapeptide was achieved by treatment with hydrogen fluoride in the presence of anisole-thioanisole-*m*-cresol. An increase of E-rosette-forming cells was obtained after incubation of peripheral blood from a uremic patient with the synthetic nonatetracontapeptide at the dose of 1 μg/ml.

Keywords——thymopoietin I; uremic patient; low E-rosette-forming cell; HOBT-WSCI procedure; azide procedure; NP active ester procedure

Two peptides, thymopoietins I and II, which are T-cell differentiating hormones of thymus, were purified and characterized by Goldstein, using an *in vitro* neuromuscular assay.[2)] Each peptide contains 49 amino acid residues and both peptides were shown to be related and to differ by only two amino acid residues.[3)] Fujino and coworkers[4)] reported the first synthetic peptide to exhibit the full activity of thymopoietin II in 1977.

In 1975, a chemical synthesis by Schlesinger *et al.*[5)] revealed that biological activity was exhibited by fragment (29—41) of thymopoietin II. Subsequently the pentapeptide corresponding to residues 32—36 of thymopoietin II was shown to retain the biological activity of thymopoietin II.[6)] Then we reported that the decapeptide (32—41)[7)] of thymopoietin II and the pentapeptide (32—36)[8)] induce some recovery of E-rosette formation in the uremic state. In 1981, we also reported that the octadecapeptide (32—49)[9)] of thymopoietin II induces some recovery of E-rosette formation in the blood of patients with rheumatoid arthritis.

In 1981, the proposed structures of thymopoietins I and II were revised by Goldstein *et al.*[10)] In 1982, we reported[11)] 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). As shown in Fig. 1, the revised structure of thymopoietin I[10)] differs from that of thymopoietin II in only three positions, positions 1 (Gly), 2 (Gln) and 43 (His).

On the other hand, Harris *et al.* have reported evidence of impaired immune function in patients with chronic uremia.[12)] This impairment is reflected in depressed cell-mediated immune function both *in vitro* and *in vivo*. Further, many patients with chronic uremia have thymic atrophy.[13)]

We now wish to report the first solution synthesis of a nonatetracontapeptide corresponding to the entire revised amino acid sequence of thymopoietin I[10)] and to describe the

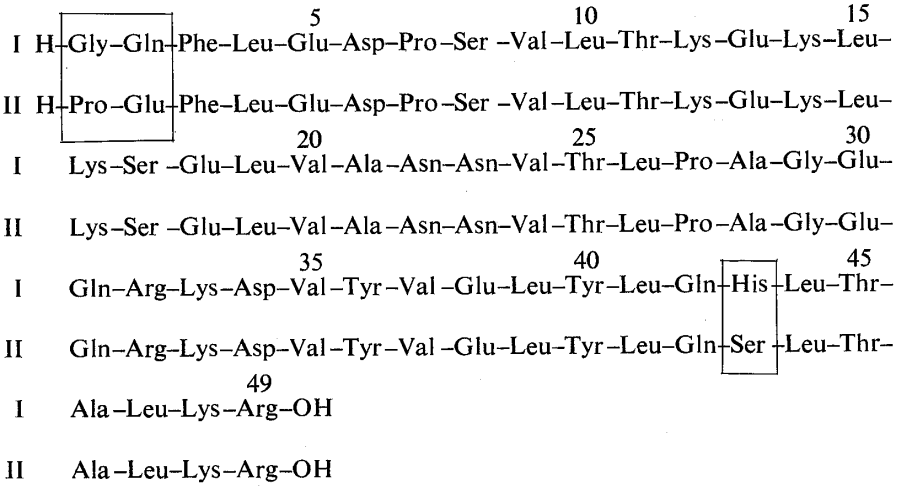

Fig. 1. Comparison of Amino Acid Sequences of Thymopoietin I and Thymopoietin II

Differences in the sequences are enclosed in boxes.

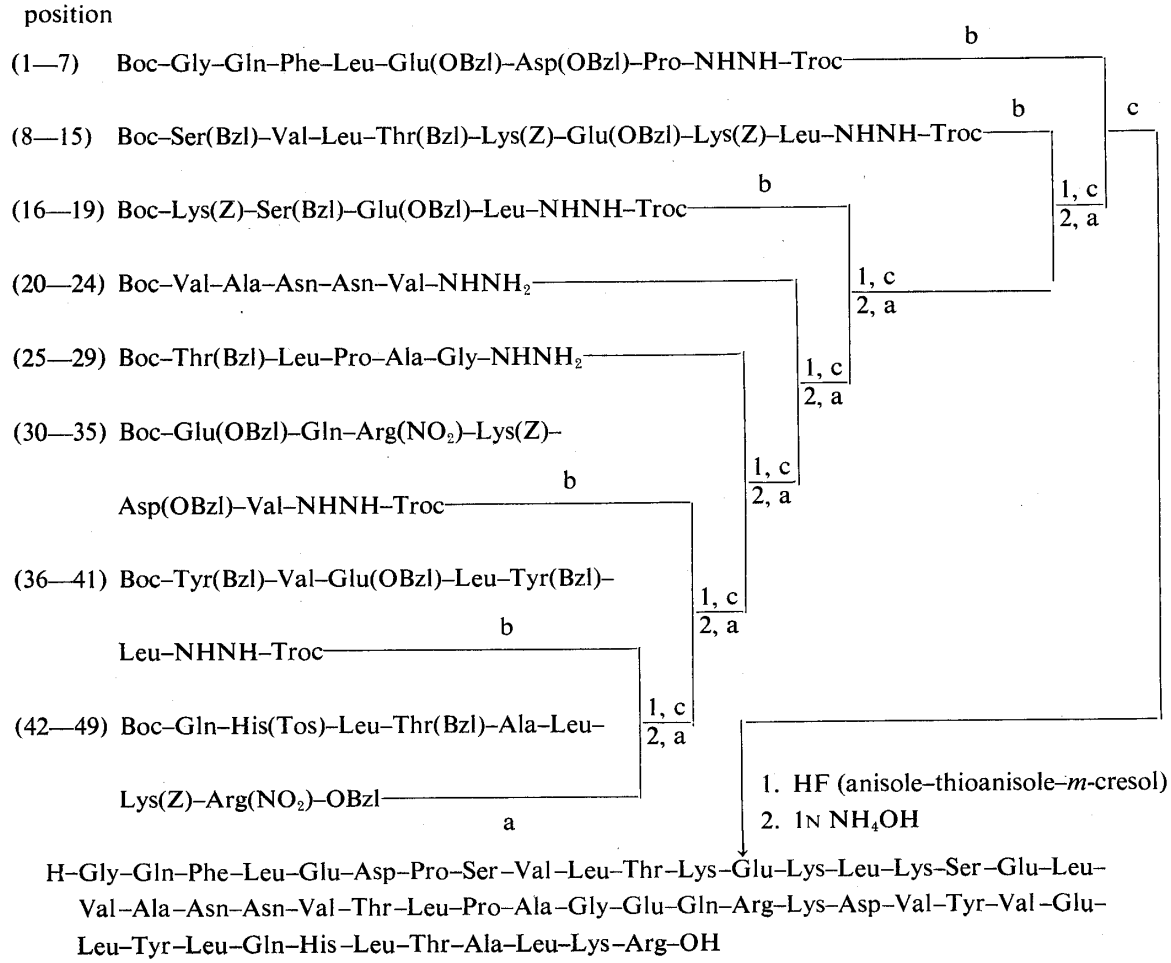

Fig. 2. Synthetic Routes to Thymopoietin I

a, TFA–anisole; b, Zn–AcOH; c, azide.

in vitro effect of this peptide on the low E-rosette-forming cells of a uremic patient.

The nonatetracontapeptide was synthesized in a manner similar to that described for our synthesis of calf thymosin β_8.[14] In the present synthesis, as illustrated in Fig. 2, amino acid

derivatives bearing protecting groups, *i.e.*, Tyr(Bzl), Thr(Bzl), Ser(Bzl), Arg(NO_2), His(Tos), Lys(Z), Glu(OBzl), Asp(OBzl) and Arg(NO_2)-OBzl, which could be removed by treatment with hydrogen fluoride,[15] were used. The protecting groups survive mostly intact during TFA treatment for the removal of the Boc group, employed as a temporary α-amino protecting group.

As shown in Fig. 2, eight peptides, Boc–(42—49)–OBzl, Boc–(36—41)–$NHNH_2$, Boc–(30—35)–$NHNH_2$, Boc–(25—29)–$NHNH_2$, Boc–(20—24)–$NHNH_2$, Boc–(16—19)–$NHNH_2$, Boc–(8—15)–$NHNH_2$ and Boc–(1—7)–$NHNH_2$, were chosen as building blocks for the construction of the full sequence of thymopoietin I. The procedure using azide[16] was applied to condense these fragments successively, because of the low risk of racemization involved in this procedure, as compared to other amido-forming reactions. Throughout the syntheses of these intermediates and fragments, the purity of every fragment and intermediate was confirmed by paper chromatography, TLC, acid hydrolysate and elemental analysis. The analytical results were within $\pm 0.4\%$ of theoretical values in all cases.

First, the C-terminal fragment, Boc–(42—49)–OBzl, was prepared stepwise starting from Boc–Ala–Leu–Lys(Z)–Arg(NO_2)–OBzl[11] by the HOBT–WSCI procedure,[17] except for the introduction of Gln residue, which was introduced by the NP active ester procedure.[18] Next, for the preparation of the five fragments containing Glu(OBzl) and/or Asp(OBzl), Boc–(36—41)–NHNH–Troc, Boc–(30—35)–NHNH–Troc, Boc–(16—19)–NHNH–Troc, Boc–(8—15)–NHNH–Troc and Boc–(1—7)–NHNH–Troc, we employed a substituted hydrazide, Troc–$NHNH_2$,[19] the protecting group of which is known to be removed by Zn[20] without affecting side chain protecting groups such as Boc, NO_2, Z, Tos and Bzl. Thus, these fragments were prepared without exposing the corresponding methyl or ethyl esters to hydrazide. The five fragments, Boc–(36—41)–NHNH–Troc, Boc–(30—35)–NHNH–Troc, Boc–(16—19)–NHNH–Troc, Boc–(8—15)–NHNH–Troc and Boc–(1—7)–NHNH–Troc, were prepared stepwise by the HOBT–WSCI procedure[17] except for the introduction of Gln residues. Gln residues were introduced by the NP active ester procedure,[18] and the Boc groups of intermediates were removed by treatment with TFA–anisole prior to the next coupling reaction. The five fragments thus obtained were treated with Zn[20] in AcOH and DMF to remove the Troc groups, and the zinc acetate was removed by treatment with EDTA to give the required hydrazide in analytically pure form. The hydrazine test on the paper chromatograms and elemental analysis data were consistent with homogeneity of the desired products. Next, Boc–(25—29)–OEt was prepared stepwise starting from H–Gly–OEt·HCl by the HOBT–WSCI procedure.[17] The resulting pentapeptide ester was smoothly converted to the corresponding hydrazide, Boc–(25—29)–$NHNH_2$, in the usual manner. Fragment Boc–(20—24)–OMe was also prepared stepwise starting from H–Val–OMe·HCl by the HOBT–WSCI procedure,[17] except for the introduction of Asn residues, which were introduced by the NP active ester procedure.[18] The resulting pentapeptide ester was also smoothly converted to the corresponding hydrazide, Boc–(20—24)–$NHNH_2$, in the usual manner. The hydrazine test on the paper chromatograms and elemental analysis data of these two peptide fragments, Boc–(25—29)–$NHNH_2$ and Boc–(20—24)–$NHNH_2$, were also consistent with homogeneity of the desired products.

The eight fragments thus obtained were then assembled successively by the azide procedure[16] according to the route illustrated in Fig. 2. The amount of the acyl component in each fragment condensation was increased from 1.8 to 4 eq as the chain was elongated. The solubility of protected intermediates in DMF decreased remarkably with chain elongation. Consequently, mixtures of DMF–DMSO had to be employed for subsequent fragment condensation reactions. Throughout this synthesis, Ala was selected as the diagnostic amino acid in acid hydrolysis.

Starting with the side-chain-protected octapeptide ester corresponding to positions 42 to

TABLE I. Yields and Physical Constants of Protected Intermediates

Peptides	Yield (%)	mp (°C)	$[\alpha]_D^{21}$ (c=1.0, DMF)	Paper chromato.[a] Rf^a	Rf^b
Boc-(45—49)-OBzl	71	92—98	−14.1	0.78	0.89
Boc-(44—49)-OBzl	88	121—128	−24.5	0.80	0.91
Boc-(43—49)-OBzl	78	124—129	−9.6	0.79	0.84
Boc-(42—49)-OBzl	93	132—137	−19.4	0.73	0.78
Boc-(40—41)-NHNH-Troc	77	80—83	−12.6	0.81	0.87
Boc-(39—41)-NHNH-Troc	67	133—137	−13.9	0.85	0.90
Boc-(38—41)-NHNH-Troc	86	121—126	−2.5	0.81	0.88
Boc-(37—41)-NHNH-Troc	91	130—135	−8.2	0.77	0.80
Boc-(36—41)-NHNH-Troc	66	136—140	−7.9	0.76	0.79
Boc-(36—41)-$NHNH_2$	87	176—183	−8.9	0.70[b]	0.64[b]
Boc-(34—35)-NHNH-Troc	70	70—73	−6.4	0.77	0.80
Boc-(33—35)-NHNH-Troc	90	72—74	−4.3	0.72	0.78
Boc-(32—35)-NHNH-Troc	67	78—81	−12.4	0.76	0.79
Boc-(31—35)-NHNH-Troc	75	119—126	−16.7	0.78	0.83
Boc-(30—35)-NHNH-Troc	78	108—113	−13.0	0.81	0.86
Boc-(30—35)-$NHNH_2$	91	173—181	−18.6	0.71[b]	0.68[b]
Boc-(28—29)-OEt	79	Oil	−23.6	0.70	0.75
Boc-(27—29)-OEt	67	Oil	−7.3	0.80	0.84
Boc-(26—29)-OEt	68	76—80	−14.1	0.83	0.87
Boc-(25—29)-OEt	69	112—114	−9.1	0.84	0.88
Boc-(25—29)-$NHNH_2$	74	148—155	−13.5	0.71[b]	0.70[b]
Boc-(23—24)-OMe	88	136—137	−15.8	0.65	0.78
Boc-(22—24)-OMe	62	228—230	−12.3	0.24	0.62
Boc-(21—24)-OMe	78	243—245	−10.1	0.23	0.64
Boc-(20—24)-OMe	77	128—134	−10.9	0.43	0.70
Boc-(20—24)-$NHNH_2$	77	230—237	−9.5	0.44[b]	0.68[b]
Boc-(18—19)-NHNH-Troc	75	70—74	−8.1	0.74	0.87
Boc-(17—19)-NHNH-Troc	85	73—76	−13.6	0.76	0.85
Boc-(16—19)-NHNH-Troc	85	74—83	−9.3	0.73	0.86
Boc-(16—19)-$NHNH_2$	85	167—173	−13.1	0.65[b]	0.70[b]
Boc-(14—15)-NHNH-Troc	91	71—73	−8.7	0.76	0.81
Boc-(13—15)-NHNH-Troc	65	76—80	−26.7	0.73	0.77
Boc-(12—15)-NHNH-Troc	84	77—83	−5.8	0.75	0.82
Boc-(11—15)-NHNH-Troc	67	131—139	−9.8	0.75	0.79
Boc-(10—15)-NHNH-Troc	76	124—129	−15.3	0.83	0.87
Boc-(9—15)-NHNH-Troc	90	78—83	−6.7	0.77	0.81
Boc-(8—15)-NHNH-Troc	77	101—107	−10.1	0.84	0.87
Boc-(8—15)-$NHNH_2$	79	136—143	−29.8	0.75[b]	0.79[b]
Boc-(6—7)-NHNH-Troc	83	Oil	−8.4	0.70	0.73
Boc-(5—7)-NHNH-Troc	78	68—70	−10.8	0.74	0.76
Boc-(4—7)-NHNH-Troc	78	73—77	−14.6	0.80	0.83
Boc-(3—7)-NHNH-Troc	74	81—85	−4.8	0.81	0.86
Boc-(2—7)-NHNH-Troc	92	96—104	−19.7	0.73	0.75
Boc-(1—7)-NHNH-Troc	86	103—107	−3.1	0.75	0.74
Boc-(1—7)-$NHNH_2$	83	151—158	−34.6	0.77[b]	0.84[b]

a) The protected peptides were deblocked with TFA unless otherwise mentioned. Ninhydrin-positive spot. *b*) Hydrazine-positive spot.

49 of thymopoietin I, H–(42—49)–OBzl, seven fragments, Boc–(36—41)–$NHNH_2$, Boc–(30—35)–$NHNH_2$, Boc–(25—29)–$NHNH_2$, Boc–(20—24)–$NHNH_2$, Boc–(16—19)–$NHNH_2$, Boc–(8—15)–$NHNH_2$ and Boc–(1—7)–$NHNH_2$, were successively condensed by the azide procedure[16] as shown in Fig. 2 to give the protected nonatetracontapeptide corresponding to

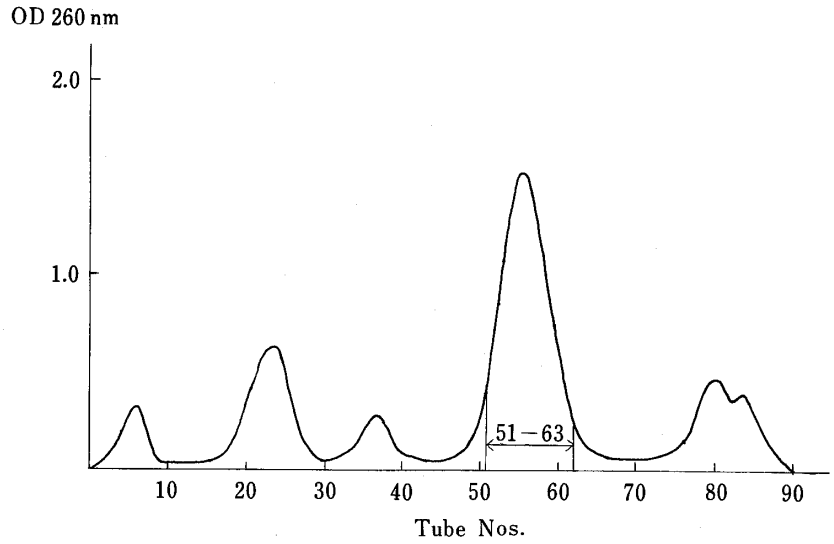

Fig. 3. Purification of Synthetic Thymopoietin I by Ion-Exchange Chromatography on CM-Cellulose

TABLE II. Effects of the Synthetic Thymopoietin I on the Low E-Rosette-Forming Capacity of Cells of a Uremic Patient

Peptide	Dose (μg/ml)	E-Rosette-forming cells[d] (%)
—[a]		68±5
—[b]		33±6
Synthetic thymopoietin I[b,c]	0.1	32±7
	1.0	46±5
	10.0	57±6
	100.0	58±6

a) Normal venous blood. *b*) Patient's venous blood. *c*) Incubation was carried out at 37 °C for 1 h. *d*) Each value represents the mean±S.D. of triplicate measurements by a single observer.

the entire amino acid sequence of thymopoietin I. The homogeneities of the peptides were assessed by elemental analysis, TLC and amino acid analyses of the acid hydrolysates. In the final step of the synthesis, all protecting groups, Boc, Z, OBzl, Tos and NO_2, were removed from the protected thymopoietin I by treatment with hydrogen fluoride,[15] in the presence of anisole–thioanisole–*m*-cresol (1 : 1 : 1, v/v) to suppress possible reaction of Asp(OBzl) residue[21] and Tyr residue.[22] The deblocked peptide was precipitated by dry ether, converted to the corresponding acetate with Amberlite CG-4B (acetate form) and then treated with 1 N NH_4OH for 30 min. The latter treatment was performed because of the reversible N→O shift at Ser and Thr residues during the hydrogen fluoride treatment.[23,24] The crude peptide was purified by gel-filtration on Sephadex G-50 and then by ion-exchange chromatography on a CM-cellulose column with linear gradient elution using pH 6.53 ammonium acetate buffer (0→0.25 M). Desalting on Sephadex G-25 gave a fluffy powder, which exhibited a single spot (ninhydrin- and Sakaguchi-positive) on paper chromatography in two different solvent systems and on paper electrophoresis (pH 2.81 acetate buffer). Its purity was further assessed by amino acid analysis of the acid hydrolysate.

The *in vitro* effect of the synthetic nonatetracontapeptide on low E-rosette-forming cells of a uremic patient is shown in Table II. Incubation of peripheral venous blood from a uremic patient in the presence of various amounts of the synthetic peptide from 0.1 to 100 μg/ml

resulted in recovery of E-rosette formation (Table II). Increased activity for E-rosette formation was observed with the synthetic nonatetracontapeptide up to a concentration of 1 μg/ml. The synthetic nonatetracontapeptide, however, had no effect on E-rosette-forming cells of this patient at a dose of 0.1 μg/ml (Table II). These results suggest that the synthetic peptide is active to restore the low E-rosette-forming cells *in vitro* in cases of uremic immunodeficiency.

Experimental

Melting points are uncorrected. Rotations were measured with an Atago Polax machine (cell length: 10 cm). Amino acid compositions of acid hydrolysates 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—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,[25] Rf^b value refer to BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24)[26] and Rf^d value refer to BuOH–EtOAc–AcOH–H_2O (1 : 1 : 1 : 1). TLC was performed on silica gel (Kieselgel G, Merck) plates and Rf^c values refer to $CHCl_3$–MeOH–H_2O (8 : 3 : 1, lower phase). Troc–$NHNH_2$ was purchased from the Kokusan Chemical Works, Ltd., Japan. Azide was prepared according to Honzl and Rudinger[16] with isoamyl nitrite. All eight fragments for the construction of thymopoietin I were newly synthesized by a method similar to that employed previously.[14,27] Preparations of protected intermediates were repeated several times in order to obtain sufficient quantities for the next step. Venous blood samples were obtained from a patient suffering from chronic uremia. Venous blood samples from three healthy donors were used as controls.

Boc–Tyr(Bzl)–Val–Glu(OBzl)–Leu–Tyr(Bzl)–Leu–Gln–His(Tos)–Leu–Thr(Bzl)–Ala–Leu–Lys(Z)–Arg(NO_2)–OBzl, Boc–(36—49)–OBzl(I)——Boc–Gln–His(Tos)–Leu–Thr(Bzl)–Ala–Leu–Lys(Z)–Arg(NO_2)–OBzl (395 mg) was treated with TFA–anisole (4 ml–0.8 ml) in an ice-bath for 50 min, then dry ether was added. The resulting powder was washed with dry ether, dried over KOH pellets *in vacuo* for 2 h and then dissolved in DMF (3 ml) containing NMM (0.033 ml). The azide [prepared from 571 mg of Boc–(36—41)–$NHNH_2$] in DMF (3 ml) and NMM (0.11 ml) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C for 48 h, was concentrated. The residue was treated with 1 N citric acid and H_2O and precipitated twice from DMF with H_2O. The dried product was dissolved in DMF containing 5% H_2O (3 ml) and the solution was applied to a column of Sephadex LH-20 (2.8 × 100 cm), which was eluted with the same solvent. Each fraction (5 ml) was examined for ultraviolet (UV) absorption at 260 nm; fractions corresponding to the main peak (tube Nos. 59—71) were combined and the solvent was removed by evaporation. Treatment of the residue with EtOAc afforded a powder; yield 416 mg (63%), mp 143—150 °C, $[\alpha]_D^{21}$ −3.7° (c = 1.0, DMF), Rf^c 0.51, single fluorescamine-positive spot. *Anal.* Calcd for $C_{137}H_{180}N_{22}O_{29}S$: C, 62.54; H, 6.90; N, 11.71. Found: C, 62.74; H, 6.89; N, 11.31. Amino acid ratios in a 6 N HCl hydrolysate: Leu 4.12, Val 1.07, Tyr 1.95, Ala 1.00, Thr 0.92, Glu 1.87, Lys 1.04, His 0.84, Arg 0.90 (recovery of Ala 89%).

Boc–Glu(OBzl)–Gln–Arg(NO_2)–Lys(Z)–Asp(OBzl)–Val–Tyr(Bzl)–Val–Glu(OBzl)–Leu–Tyr(Bzl)–Leu–Gln–His(Tos)–Leu–Thr(Bzl)–Ala–Leu–Lys(Z)–Arg(NO_2)–OBzl, Boc–(30—49)–OBzl (II)——Boc–(36—49)–OBzl (I) (329 mg) was treated with TFA–anisole (4 ml–0.8 ml) as describe above and the N^{α}-deprotected peptide was dissolved in DMF–DMSO (1 : 2, 4 ml) containing NMM (0.01 ml). The azide [prepared from 568 mg of Boc–(30—35)–$NHNH_2$] in DMF (4 ml) and NMM (0.06 ml) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C for 48 h, was concentrated. The residue was treated with 1 N citric acid. The resulting powder was washed with 1 N citric acid and H_2O, and precipitated from DMF with EtOAc to form a powder. The powder was dissolved in DMF (4 ml) containing 5% H_2O and the solution was applied to a column of Sephadex LH-60 (2.8 × 102 cm), which was eluted with the same solvent. Each fraction (5 ml) was examined for UV absorption at 260 nm; fractions corresponding to the main peak (tube Nos. 56—69) were combined and the solvent was removed by evaporation. Treatment of the residue with ether afforded a powder; yield 398 mg (85%), mp 181—190 °C, $[\alpha]_D^{21}$ −7.5° (c = 1.0, DMSO), Rf^c 0.54, single fluorescamine-positive spot. *Anal.* Calcd for $C_{190}H_{250}N_{34}O_{44}S \cdot 13H_2O$: C, 57.33; H, 6.99; N, 11.96. Found: C, 57.21; H, 7.20; N, 11.87. Amino acid ratios in a 6 N HCl hydrolysate: Leu 4.13, Val 2.08, Tyr 1.84, Ala 1.00, Thr 0.87, Glu 3.96, Asp 0.90, Lys 2.12, His 0.84, Arg 1.84 (recovery of Ala 91%).

Boc–Thr(Bzl)–Leu–Pro–Ala–Gly–Glu(OBzl)–Gln–Arg(NO_2)–Lys(Z)–Asp(OBzl)–Val–Tyr(Bzl)–Val–Glu(OBzl)–Leu–Tyr(Bzl)–Leu–Gln–His(Tos)–Leu–Thr(Bzl)–Ala–Leu–Lys(Z)–Arg(NO_2)–OBzl, Boc–(25—49)–OBzl (III)——Boc–(30—49)–OBzl (II) (312 mg) was treated with TFA–anisole (3 ml–0.6 ml) and the N^{α}-deprotected peptide ester, isolated as usual, was dissolved in DMF–DMSO (1 : 2, 4 ml) containing NMM (0.009 ml). The azide [prepared from 110 mg of Boc–(25—29)–$NHNH_2$] in DMF (2 ml) and NMM (0.05 ml) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 48 h. After addition of a few drops of AcOH to the mixture, the solvent was removed by evaporation. Trituration of the residue with 1 N citric acid and ether afforded a powder, which was purified by the washing procedure as described above, followed by precipitation twice from DMF with EtOAc; yield 328 mg (92%), mp 168—177 °C, $[\alpha]_D^{21}$ −38.4° (c = 1.0, DMSO), Rf^c 0.55, single fluorescamine-positive

spot. *Anal.* Calcd for $C_{217}H_{289}N_{39}O_{50}S \cdot 17H_2O$: C, 56.88; H, 7.11; N, 11.92. Found: C, 56.94; H, 7.20; N, 11.75. Amino acid ratios in a 6 N HCl hydrolysate: Leu 5.18, Val 2.13, Gly 1.06, Tyr 1.98, Ala 2.00, Pro 0.87, Thr 1.87, Glu 3.84, Asp 0.92, Lys 2.04, His 0.82, Arg 1.83 (recovery of Ala 88%).

Boc–Val–Ala–Asn–Asn–Val–Thr(Bzl)–Leu–Pro–Ala–Gly–Glu(OBzl)–Gln–Arg(NO_2)–Lys(Z)–Asp(OBzl)–Val–Tyr(Bzl)–Val–Glu(OBzl)–Leu–Tyr(Bzl)–Leu–Gln–His(Tos)–Leu–Thr(Bzl)–Ala–Leu–Lys(Z)–Arg(NO_2)–OBzl, Boc–(20—49)–OBzl (IV)——The above Boc–(25—49)–OBzl (III) (305 mg) was treated with TFA–anisole (3 ml–0.6 ml) and the N^{α}-deprotected peptide, isolated as usual, was dissolved in DMF–DMSO (1 : 2, 4 ml) containing NMM (0.0079 ml). The azide [prepared from 90 mg of Boc–(20—24)–$NHNH_2$] in DMF (2 ml) and NMM (0.007 ml) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 48 h. After addition of a few drops of AcOH, the solvent was removed by evaporation and the residue was treated with 1 N citric acid to form a powder, which was washed with H_2O and precipitated three times from DMF with EtOAc; yield 287 mg (76%), mp 146—155 °C, $[\alpha]_D^{21}$ −34.2° (c=1.0, DMSO), Rf^c 0.52, single fluorescamine-positive spot. *Anal.* Calcd for $C_{238}H_{324}N_{46}O_{57}S \cdot 18H_2O$: C, 56.08; H, 7.12; N, 12.46. Found: C, 56.38; H, 7.40; N, 12.79. Amino acid ratios in a 6 N HCl hydrolysate: Leu 5.15, Val 4.11, Gly 1.09, Tyr 1.87, Ala 3.00, Pro 0.86, Thr 1.80, Glu 3.92, Asp 2.86, Lys 2.03, His 0.85, Arg 1.84 (recovery of Ala 90%).

Boc–Lys(Z)–Ser(Bzl)–Glu(OBzl)–Leu–Val–Ala–Asn–Val–Thr(Bzl)–Leu–Pro–Ala–Gly–Glu(OBzl)–Gln–Arg(NO_2)–Lys(Z)–Asp(OBzl)–Val–Tyr(Bzl)–Val–Glu(OBzl)–Leu–Tyr(Bzl)–Leu–Gln–His(Tos)–Leu–Thr(Bzl)–Ala–Leu–Lys–(Z)–Arg(NO_2)–OBzl, Boc–(16—49)–OBzl (V)——The above Boc–(20—49)–OBzl (213 mg) was treated with TFA–anisole (3 ml–0.6 ml) and the N^{α}-deprotected peptide obtained as described above was dissolved in DMF–DMSO (1 : 2, 3 ml) containing NMM (0.005 ml). The azide [prepared from 72 mg of Boc–(16—19)–$NHNH_2$] in DMF (2 ml) and NMM (0.005 ml) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 48 h. The ninhydrin-negative solution was diluted with 1 N citric acid and the resulting powder was purified by gel-filtration on Sephadex LH-60 (2.8 × 100 cm) using DMF–DMSO (1 : 1) containing 5% H_2O as an eluent as described above. The desired fractions (each fraction 5 ml, tube Nos. 49—60) were combined, the solvent was evaporated off and the residue was treated with MeOH to afford a powder; yield 210 mg (95%), mp 148—160 °C, $[\alpha]_D^{21}$ −24.3° (c=0.4, DMSO), Rf^c 0.51, single fluorescamine-positive spot. *Anal.* Calcd for $C_{280}H_{377}N_{51}O_{66}S \cdot 16H_2O$: C, 57.65; H, 7.07; N, 12.25. Found: C, 57.43; H, 7.39; N, 12.04. Amino acid ratios in a 6 N HCl hydrolysate: Leu 6.15, Val 4.08, Gly 1.09, Tyr 1.93, Ala 3.00, Pro 0.85, Thr 1.83, Ser 0.82, Glu 4.87, Asp 2.92, Lys 3.10, His 0.84, Arg 1.85 (recovery of Ala 81%).

Boc–Ser(Bzl)–Val–Leu–Thr(Bzl)–Lys(Z)–Glu(OBzl)–Lys(Z)–Leu–Lys(Z)–Ser(Bzl)–Glu(OBzl)–Leu–Val–Ala–Asn–Asn–Val–Thr(Bzl)–Leu–Pro–Ala–Gly–Glu(OBzl)–Gln–Arg(NO_2)–Lys(Z)–Asp(OBzl)–Val–Tyr(Bzl)–Val–Glu–(OBzl)–Leu–Tyr(Bzl)–Leu–Gln–His(Tos)–Leu–Thr(Bzl)–Ala–Leu–Lys(Z)–Arg(NO_2)–OBzl, Boc(8—49)–OBzl (VI)——Boc–(16—49)–OBzl (V) (185 mg) was treated with TFA–anisole (3 ml–0.6 ml) in an ice-bath for 50 min, then dry ether was added. The resulting powder was collected by filtration, washed with ether, dried over KOH pellets *in vacuo* for 2 h and then dissolved in DMF–DMSO (1 : 2, 3 ml) containing NMM (0.004 ml). The azide [prepared from 157 mg (3 eq) of Boc–(8—15)–$NHNH_2$] in DMF–DMSO (1 : 1, 2 ml) and NMM (0.015 ml) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 48 h. Additional azide (prepared from 1 eq of the hydrazide) in DMF–DMSO (1 : 1, 2 ml) and NMM (0.008 ml) were added and stirring was continued for an additional 24 h until the solution became ninhydrin-negative. The solution was poured into 1 N citric acid with stirring. The resulting powder was washed with 1 N citric acid and H_2O and purified by gel-filtration on Sephadex LH-60 (2.8 × 104 cm) using DMF–DMSO (1 : 1) containing 5% H_2O as an eluent. The desired fractions (each 5 ml, tube Nos. 57—69) were combined, and the residue was treated with EtOAc to afford a powder; yield 171 mg (77%), mp 169—175 °C, $[\alpha]_D^{21}$ −25.7° (c=0.5, DMSO), Rf^c 0.55, single fluorescamine-positive spot. *Anal.* Calcd for $C_{358}H_{481}N_{61}O_{82}S \cdot 20H_2O$: C, 58.55; H, 7.15; N, 11.64. Found: C, 58.71; H, 7.48; N, 11.49. Amino acid ratios in a 6 N HCl hydrolysate: Leu 8.15, Val 5.07, Gly 1.11, Tyr 1.88, Ala 3.00, Pro 0.87, Thr 2.80, Ser 1.82, Glu 5.98, Asp 3.09, Lys 4.96, His 0.81, Arg 1.87 (recovery of Ala 83%).

Boc–Gly–Gln–Phe–Leu–Glu(OBzl)–Asp(OBzl)–Pro–Ser(Bzl)–Val–Leu–Thr(Bzl)–Lys(Z)–Glu(OBzl)–Lys(Z)–Leu–Lys(Z)–Ser(Bzl)–Glu(OBzl)–Leu–Val–Ala–Asn–Asn–Val–Thr(Bzl)–Leu–Pro–Ala–Gly–Glu(OBzl)–Gln–Arg–(NO_2)–Lys(Z)–Asp(OBzl)–Val–Tyr(Bzl)–Val–Glu(OBzl)–Leu–Tyr(Bzl)–Leu–Gln–His(Tos)–Leu–Thr(Bzl)–Ala–Leu–Lys(Z)–Arg(NO_2)–OBzl, Boc–(1—49)–OBzl (VII)——Boc–(8—49)–OBzl (VI) (133 mg) was treated with TFA–anisole (2 ml–0.4 ml) and the N^{α}-deprotected peptide obtained as described above was dissolved in DMF–DMSO (1 : 1, 3 ml) containing NMM (0.002 ml). The azide [prepared from 66 mg (3 eq) of Boc–(1—7)–$NHNH_2$] in DMF–DMSO (1 : 1, 2 ml) and NMM (0.01 ml) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 48 h. Additional azide (prepared from 1 eq of the hydrazide) in DMF–DMSO (2 ml) and NMM (0.003 ml) were then added and stirring was continued for an additional 24 h. The ninhydrin-negative solution was diluted with 1 N citric acid and the resulting powder was purified by gel-filtration on Sephadex LH-60 as described above, followed by precipitation twice from DMSO with EtOAc; yield 102 mg (64%), mp 179—186 °C, $[\alpha]_D^{21}$ −24.2° (c=0.3, DMSO), Rf^c 0.58, single fluorescamine-positive spot. *Anal.* Calcd for $C_{408}H_{543}N_{69}O_{94}S \cdot 18H_2O$: C, 59.22; H, 7.03; N, 11.68. Found: C, 59.17; H, 7.35; N, 11.42. Amino acid ratios in a 6 N HCl hydrolysate: Leu 9.11, Val 5.17, Phe 0.93, Gly 2.09, Tyr 1.84, Ala 3.00, Thr 2.81, Ser 1.84, Glu 8.05, Asp 3.92, Lys 4.87, His 0.81, Arg 1.86 (recovery of Ala 80%).

H–Gly–Gln–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–His–Leu–Thr–Ala–Leu–Lys–Arg–OH (Corresponding to Thymopoietin I) (VIII)——The protected nonatetracontapeptide VII (60 mg) was treated with HF (approximately 3 ml) in the presence of anisole–thioanisole–*m*-cresol (1 : 1 : 1, 0.9 ml) at −5 °C for 60 min. After removal of the HF, dry ether was added to the residue and the resulting powder was dissolved in H_2O (6 ml). The solution was treated with Amberlite CG-4B (acetate form, approximately 3 g) for 30 min, and filtered by suction. The filtrate was adjusted to pH 9 with 1 N NH_4OH and stirred in an ice-bath for 30 min to reverse a possible N→O shift at the Ser and Thr residues. The pH of the solution was adjusted to pH 6 with a few drops of AcOH and the solution was lyophilized to give a fluffy powder. The powder was dissolved in 1% AcOH (2 ml), applied to a column of Sephadex G-50 (2.8 × 101 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. 71—80) 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-cellulose (2.3 × 18 cm, Serva). The CM-cellulose column was eluted first with H_2O (50 ml) and then with a linear gradient from H_2O (250 ml) to 0.25 M ammonium acetate buffer (250 ml, pH 6.53). Individual fractions (4 ml each) were collected and the absorbancy at 260 nm was determined. The main peak (tube Nos. 51—63) was collected and then the solvent was removed by lyophilization. The product was dissolved in 2% AcOH (2 ml) and the solution was then subjected to Sephadex G-25 column chromatography (2.8 × 98 cm), and eluted with the same solvent. The main peak fractions were combined and the solvent was removed by lyophilization to give a white fluffy powder; yield 8.6 mg (21%), $[\alpha]_D^{21}$ −78.4° (c=0.3, 1 N AcOH), Rf^b 0.17, Rf^d 0.38, single ninhydrin- and Sakaguchi-positive spot and the peptide stayed at the origin on paper chromatography in Partridge system. The synthetic peptide exhibited a single spot on paper electrophoresis: Toyo Roshi No. 51 (2 × 40 cm), acetate buffer at pH 2.81. Mobility, 7.8 cm from the origin toward the anode, after running at 1 mA, 600 V for 60 min. Amino acid ratios in a 6 N HCl hydrolysate: Leu 9.06, Val 5.10, Phe 0.90, Gly 2.06, Tyr 1.87, Ala 3.00, Thr 2.83, Ser 1.82, Glu 7.92, Asp 4.08, Lys 5.14, His 0.84, Arg 1.82 (recovery of Ala 83%).

E-Rosette Formation Test——The test involves the *in vitro* incubation of patient's blood with the synthetic peptide and then the *in vitro* incubation of isolated lymphocytes from the peripheral blood with sheep red blood cells. The results of incubations with and without the synthetic peptide are compared. In this case, lymphocytes were incubated with sheep red blood cells at 4 °C for 2 h rather than for 16 h,[11] because in our laboratory, counts obtained at 2 h were similar to those obtained at 16 h. To study the effects of the synthetic peptide, we performed experiments in which all cells binding even one sheep red blood cell were counted as T-cells, because the effect of the synthetic thymopoietin I might be to increase the number of sheep red blood cell binding sites on the surface of maturing T-cells.

A 5 ml aliquot of venous blood was drawn into a syringe containing 1000 U of heparin and was incubated with the synthetic peptide for 1 h at 37 °C, then lymphocytes were isolated in a Hypaque-Ficoll gradient[28] for testing of E-rosette formation. The isolated lymphocytes were adjusted to 5×10^5 cells/ml with PBS. Contamination by monocytes and polymorphonuclear cells amounted to less than 7%.[29] Sheep erythrocytes (Kyokutō Pharmaceutical Co.) were washed with GVB^{2+}, centrifuged for 10 min at 1500 rpm, and then suspended in PBS (1 ml). The suspension was mixed with the suspension of sheep erythrocytes (0.5 ml) and the mixture was incubated for 2 h at 4 °C, 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 one erythrocyte was determined.

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References and Notes

1) The amino acid residues mentioned in this paper are of the L-configuration except for glycine. 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 used: DMF, dimethylformamide; DMSO, dimethylsulfoxide; WSCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; TFA, trifluoroacetic acid; MeOH, methanol; HOBT, 1-hydroxybenzotriazole; AcOH, acetic acid; EtOAc, ethyl acetate; NMM, *N*-methylmorpholine; Boc, *tert*-butoxycarbonyl; OBzl, benzyl ester; Bzl, benzyl; Z, benzyloxycarbonyl; HF, hydrogen fluoride; Troc, β,β,β-trichloroethyloxycarbonyl; TLC, thin-layer chromatography; Tos, *p*-tolylsulfonyl; NP, *p*-nitrophenyl; E-rosette, a rosette with sheep erythrocytes; PBS, phosphate-buffered saline; GVB^{2+}, gelatin veronal buffer; EDTA, ethylenediaminetetraacetic acid; OMe, methyl ester; OEt, ethyl ester; CM, carboxymethyl.
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