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Synthesis of the Nonatetracontapeptide Corresponding to the Entire Amino Acid Sequence of Thymopoietin III and Its Effect on the Impaired T-Lymphocyte Transformation of a Patient with Common Variable Immunodeficiency[1)]

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The nonatetracontapeptide corresponding to the entire amino acid sequence of thymopoietin III 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 *m*-cresol. The synthetic nonatetracontapeptide was tested for effect on impaired T-lymphocyte transformation by phytohemagglutinin (PHA) in common variable immunodeficiency. The synthetic nonatetracontapeptide was found to have restoring activity on the T-lymphocyte transformation by PHA. However, the restoring activity of the synthetic nonatetracontapeptide was lower than that of the synthetic thymopoietin I at a dose of 2 μg/ml.

Keywords——thymopoietin III; trifluoromethanesulfonic acid deprotection; impaired T-lymphocyte transformation; common variable immunodeficiency; β,β,β-trichloroethyloxycarbonyl-hydrazine; fluorometric blast-formation test

Thymopoietin, a polypeptide hormone of the thymus discovered by its effect on neuromusclar transmission,[2)] was later shown to induce T-lymphocyte differentiation[3,4)] and to affect immunoregulatory balance.[5,6)] Two biochemically homogeneous thymic polypeptides, thymopoietins I and II, were isolated, and both were active in this system.[2)] These purified polypeptides subsequently proved to be active in induction of early T-lymphocyte differentiation and modulation of mature lymphocytes.[3,4,7)] The complete amino acid sequence of thymopoietin II was determined,[8)] and the biological activity was shown to residue in fragment (29—41) of thymopoietin II by chemical synthesis.[9)] Subsequently the pentapeptide, corresponding to residues 32—36 of thymopoietin II, was shown to retain the biological activity of thymopoietin II and thus probably corresponds to a biological active site of the parent peptide.[10)]

Fujino and coworkers[11)] reported the first synthetic peptide to exhibit the full activity of thymopoietin II in 1977. Then we reported that the decapeptide (32—41)[12)] of thymopoietin II and the pentapeptide (32—36)[13)] induce the same recovery of E-rosette formation in the uremic state. In 1982, we also reported that the octadecapeptide (32—49)[14)] 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.*[15)]

In 1982, we reported[16)] 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 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).

In 1981, Goldstein *et al.*[15)] elucidated the primary structure of thymopoietin III, which

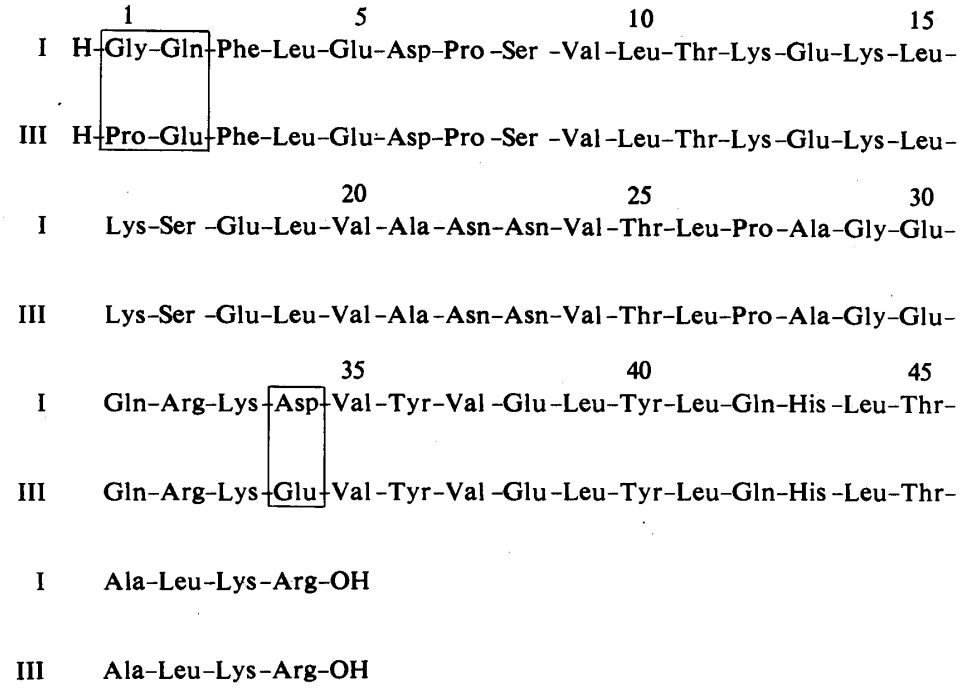

Fig. 1. Comparison of Amino Acid Sequences of Thymopoietin I and Thymopoietin III; Differences in the Sequences are Enclosed in Boxes

was isolated from bovine spleen. The three peptides, thymopoietins I, II and III, have identical sequences except for the amino acid residues at positions 1, 2, 34 and 43. As shown in Fig. 1, the structure of thymopoietin III differs from that of thymopoietin I in only three positions, 1 (Pro), 2 (Glu) and 34 (Glu).

In 1985, we also reported[17] the synthesis of the nonatetracontapeptide corresponding to the entire amino acid sequence of thymopoietin I and showed that this peptide could restore the E-rosette-forming capacity in a uremic patient.

It is generally accepted that a high percentage of patients with common variable immunodeficiency have a defect of cell-mediated immunity.[18] In contrast to normal persons, the patients with common variable immunodeficiency were reported to have depressed T-lymphocyte response to PHA.

We now wish to report the first solution synthesis of a nonatetracontapeptide corresponding to the entire amino acid sequence of thymopoietin III.[15] Further, we have compared the *in vitro* effects of this synthetic nonatetracontapeptide and the synthetic thymopoietin I[17] on the impaired T-lymphocyte transformation by PHA in common variable immunodeficiency.

We synthesized thymopoietin III by a method different from that employed for the previous synthesis of thymopoietin I.[17] For the present synthesis, the thioanisole–mediated TFMSA deprotecting procedure[19] was employed instead of the hydrogen fluoride deprotecting procedure.[20] Ten relatively small peptide fragments were selected as building blocks to construct the entire amino acid sequence by means of the azide procedure[21] and HOSu–WSCI procedure,[22] because of the low risk of racemization involved in these procedures, as compared to other amido-forming reactions.

As shown in Fig. 2, the TFA-labile Boc group was employed for N^{α}-protection and amino acid derivatives bearing protecting groups removable by 1 M TFMSA/TFA[19] were employed, *i.e.*, Lys(Z), Glu(OBzl), Thr(Bzl), Ser(Bzl), Tyr(Bzl), His(Tos), Asp(OcHex)[23] and Arg(Mts).[24] This peptide contains one residue of Asp, which from the synthetic viewpoint

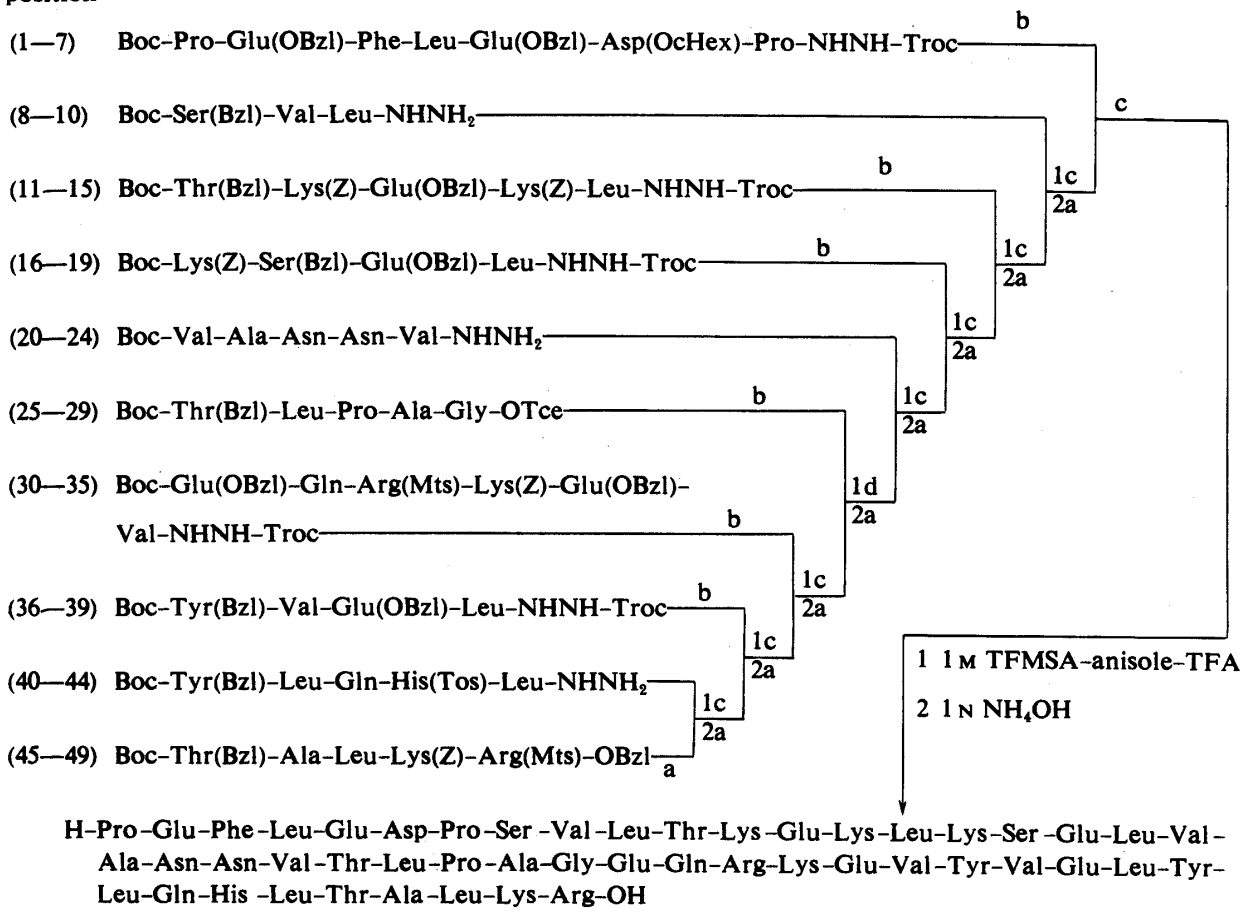

Fig. 2. Synthetic Route to Thymopoietin III

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

involves some difficulty regarding its functional group. In order to minimize the base-catalyzed ring closure of Asp(OBzl),[25] Asp(OcHex) was employed[23] instead of Asp(OBzl).

Hydrazides containing the Asp(OcHex) or Glu(OBzl) residue were synthesized with the aid of substituted hydrazine, Troc–$NHNH_2$,[26] the protecting group of which can be removed by Zn[27] in AcOH without affecting other functional groups.

Throughout the syntheses of these intermediates and fragments, the purity of every fragment and intermediate was checked by thin-layer chromatography (TLC), elemental analysis and amino acid analysis. The analytical results were within $\pm 0.4\%$ of theoretical values in all cases. Boc groups of intermediates were removed by treatment with TFA–anisole prior to the next coupling reaction.

First, the C-terminal fragment, Boc–(45—49)–OBzl, was prepared stepwise starting from Boc–Arg(Mts)–OBzl by the HOBT–WSCI procedure.[28] Next, Boc–(40—44)–OMe, Boc–(20—24)–OMe[17] and Boc–(8—10)–OMe, were prepared stepwise by the HOBT–WSCI procedure[28] except for the introduction of Gln or Asn residues, which were introduced by the NP active ester procedure.[29] The three fragment esters thus obtained were smoothly converted to the corresponding hydrazides, Boc–(40—44)–$NHNH_2$, Boc–(20—24)–$NHNH_2$[17] and Boc–(8—10)–$NHNH_2$ in the usual manner. The hydrazine test on the thin-layer chromatograms and elemental analysis data of these three peptide fragments were consistent with homogeneity of the desired products.

Next, for the preparation of the five fragments containing Glu(OBzl) and/or Asp(OcHex), Boc–(36—39)–NHNH–Troc, Boc–(30—35)–NHNH–Troc, Boc–(16—19)–

NHNH–Troc, Boc–(11—15)–NHNH–Troc[17] and Boc–(1—7)–NHNH–Troc, we employed a substituted hydrazide, Troc–$NHNH_2$,[26] the protecting group which is known to be removed by Zn[27] without affecting side chain protecting groups such as Boc, Mts, Z, Bzl, OBzl and OcHex. Thus, these fragments were prepared without exposing the corresponding methyl or ethyl esters to hydrazide. The five fragments, Boc–(36—39)–NHNH–Troc, Boc–(30—35)–NHNH–Troc, Boc–(16—19)–NHNH–Troc, Boc–(11—15)–NHNH–Troc[17] and Boc–(1—7)–NHNH–Troc, were prepared stepwise by the HOBT–WSCI procedure[28] except for the introduction of Gln residue, which was introduced by the NP active ester procedure.[29] The five fragments thus obtained were treated with Zn[27] in AcOH to remove Troc groups, and the zinc acetate was removed by treatment with EDTA to give the required hydrazide in analytical pure form. The hydrazine test on the thin-layer chromatograms and elemental analysis data were consistent with homogeneity of the desired products.

Then, Boc–(25—29)–OTce was prepared stepwise starting from Z–Gly–OTce by the HOBT–WSCI procedure.[28] The Z group of Z–Gly–OTce was removed by treatment with 25% HBr in AcOH in the presence of anisole prior to the next coupling reaction. The resulting pentapeptide ester was treated with Zn[30] in AcOH–H_2O (4 : 1) to remove the Tce, and the zinc acetate was removed by treatment with EDTA to give Boc–(25—29)–OH in analytical pure form.

The ten fragments thus obtained were then 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 a 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 III, 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)–$NHNH_2$,[17] 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 the entire amino acid sequence of thymopoietin III. 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 benzyl ester was treated with 1 M TFMSA–thioanisole in TFA in the presence of *m*-cresol. *m*-Cresol was used as an additional cation-scavenger to suppress a side reaction, *i.e.*, *O*-sulfonation of Tyr residue.[24] The treatment was repeated again to ensure complete deprotection. The deprotected peptide was next precipitated with dry ether, converted to the corresponding acetate with Amberlite IR-400 (acetate form) and then treated with 1 N NH_4OH to reverse a possible $N \rightarrow O$ shift at the Ser and Thr residues.[31] The crude peptide was purified by gel-filtration on Sephadex G-50 and then by ion-exchange column chromatography on a CM-cellulose column with linear gradient elution using pH 6.50 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 electrophoresis (pH 2.80 acetate buffer). The peptide also exhibited a single peak on HPLC (Fig. 4). Its purity was further confirmed by amino acid analysis after acid hydrolysis and after

TABLE I. Yields and Physical Constants of Protected Intermediates

Peptides	Yield (%)	mp (°C)	$[\alpha]_D^{21}$ (c=1.0, DMF)	TLC[a]	
				Rf^1	Rf^2
Boc–(48—49)–OBzl	73	81—84	−10.4	0.31	0.34
Boc–(47—49)–OBzl	83	128—134	−15.2	0.42	0.59
Boc–(46—49)–OBzl	81	128—130	−25.6	0.41	0.76
Boc–(45—49)–OBzl	71	124—130	−14.2	0.34	0.75
Boc–(43—44)–OMe	78	82—86	−9.3	0.71	0.84
Boc–(42—44)–OMe	65	132—137	−14.6	0.49	0.80
Boc–(41—44)–OMe	88	121—126	−15.8	0.56	0.82
Boc–(40—44)–OMe	71	126—130	−18.3	0.52	0.81
Boc–(40—44)–$NHNH_2$	90	167—176	−23.6	0.73[b]	0.70[b]
Boc–(38—39)–NHNH–Troc	88	82—84	−19.3	0.56	0.84
Boc–(37—39)–NHNH–Troc	85	132—137	−41.4	0.80	0.79
Boc–(36—39)–NHNH–Troc	87	146—151	−13.9	0.82	0.81
Boc–(36—39)–$NHNH_2$	87	183—191	−7.5	0.70[b]	0.72[b]
Boc–(34—35)–NHNH–Troc	80	76—80	−9.6	0.79	0.80
Boc–(33—35)–NHNH–Troc	79	85—89	−9.3	0.80	0.83
Boc–(32—35)–NHNH–Troc	86	96—101	−20.7	0.81	0.79
Boc–(31—35)–NHNH–Troc	88	141—146	−34.9	0.72	0.80
Boc–(30—35)–NHNH–Troc	80	126—133	−20.1	0.82	0.81
Boc–(30—35)–$NHNH_2$	88	174—183	−15.6	0.74[b]	0.70[b]
Boc–(28—29)–OTce	84	72—76	−10.2	0.61	0.70
Boc–(27—29)–OTce	85	79—83	−11.3	0.56[c]	0.67[c]
Boc–(26—29)–OTce	88	80—83	−15.2	0.74	0.74
Boc–(25—29)–OTce	87	110—113	−13.4	0.82	0.84
Boc–(25—29)–OH	86	131—135	−30.2	0.32	0.20
Boc–(17—19)–NHNH–Troc	85	92—95	−5.9	0.75	0.71
Boc–(16—19)–NHNH–Troc	77	104—109	−6.8	0.80	0.74
Boc–(16—19)–$NHNH_2$	95	179—188	−11.8	0.72[b]	0.69[b]
Boc–(11—15)–$NHNH_2$	82	189—197	−10.5	0.59[b]	0.62[b]
Boc–(9—10)–OMe	85	121—124	−29.5	0.79	0.76
Boc–(8—10)–OMe	81	96—101	−7.2	0.83	0.82
Boc–(8—10)–$NHNH_2$	86	201—209	−12.1	0.49[b]	0.50[b]
Boc–(6—7)–NHNH–Troc	77	84—87	−10.8	0.58	0.65
Boc–(5—7)–NHNH–Troc	78	104—110	−21.4	0.77	0.81
Boc–(4—7)–NHNH–Troc	84	121—124	−13.8	0.80	0.83
Boc–(3—7)–NHNH–Troc	81	141—147	−27.1	0.83	0.86
Boc–(2—7)–NHNH–Troc	69	120—126	−18.3	0.81	0.84
Boc–(1—7)–NHNH–Troc	81	135—139	−15.2	0.79	0.84
Boc–(1—7)–$NHNH_2$	86	156—164	−9.6	0.74[b]	0.77[b]

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

enzymic digestion. Despite the presence of the Pro residues,[32] complete digestion of this synthetic peptide with commercial aminopeptidase (AP-M)[33] was achieved and the presence of Asn and Gln residues in the product was thus confirmed.

The immunological effects of the synthetic thymopoietins I[17] and III, were examined by the JIMRO (Japan Immunoresearch Laboratories Co., Ltd.) fluorometric blast-formation test according to Itoh and Kawai.[34]

In contrast to normal persons, the transformation of the T-lymphocytes into lymphoblasts with mitotic activity after PHA stimulation is depressed in patients with common variable immunodeficiency. The *in vitro* effects of the synthetic peptides on the impaired T-

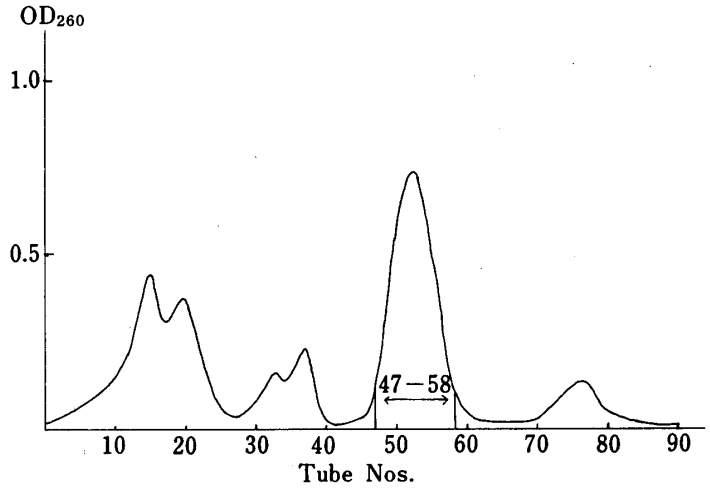

Fig. 3. Purification of Synthetic Thymopoietin III by Ion-Exchange Chromatography on a CM-Cellulose Column

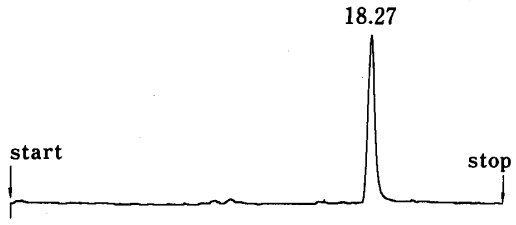

Fig. 4. HPLC of Synthetic Thympoietin III

TABLE II. Effects of the Synthetic Thymopoietins I and III on the Impaired PHA Stimulation of T-Lymphocytes of a Patient with Common Variable Immunodeficiency

Peptides	Dose (μg/ml)	SI[a,b]
—[c]	—	301.5±65.4
—[d]	—	142.3±61.3
Synthetic thymopoietin I[d,e]		
	1	189.4±64.7
	2	288.3±60.9
	3	284.5±67.3
	4	280.6±59.6
Synthetic thymopoietin III[d,e]		
	1	138.2±65.4
	2	191.8±62.4
	3	233.4±59.7
	4	284.1±60.3

a) Each value represents the mean±S.D. of triplicate measurements. *b*) SI (stimulation index) was calculated according to the following formula: $SI = \frac{I_2 - I_0}{I_1 - I_0} \times 100$ where I_2 = mean fluorescence intensity of PHA-P activated lymphocytes, I_1 = fluorescence intensity of PHA-P nonactivated lymphocytes and I_0 = fluorescence intensity of ethidium bromide. *c*) Normal venous lymphocytes. *d*) Patient's venous lymphocytes. *e*) Incubation was carried out for 90 min at 37 °C.

lymphocytes in a patient with common variable immunodeficiency are shown in Table II.

Incubation of peripheral venous blood from a patient in the presence of various amounts of the synthetic peptides from 1 to 4 μg/ml resulted in restoration of activity of T-lymphocyte transformation was observed with the synthetic thymopoietin III at a concentration as low as 2 μg/ml. However, the synthetic thymopoietin III exhibited lower activity than synthetic thymopoietin I. We also know that a pentapeptide, Arg–Lys–Asp–Val–Tyr, corresponding to amino acids 32—36 of thymopoietin, shows the biological activities of the parent molecule and was considered to represent the active site.[10] The increased activity of E-rosette-forming cells from uremic patients induced by Arg–Lys–Glu–Val–Tyr,[13] which corresponds to

residues 32 to 36 of thymopoietin III, was lower than that of Arg–Lys–Asp–Val–Tyr, which corresponds to residues 32—36 of thymopoietin I. These results seem to suggest that replacing Asp^{34} of thymopoietin I by Glu gave an analog with lower potency for restoring cell-mediated immunological activities.

Experimental

General experimental procedures used in this paper are essentially the same as described in the previous paper.[35] An azide was prepared according to Honzl and Rudinger[21] with isoamyl nitrite. Unless otherwise mentioned, products were purified by one of the following two procedures. Procedure 1: For purification of protected peptides soluble in EtOAc, the extract was washed with 1 N citric acid, H_2O, 5% $NaHCO_3$ and H_2O, then dried over $MgSO_4$ and concentrated. The residue was precipitated or recrystallized from appropriate solvents. Procedure 2: For purification of protected peptides almost insoluble in EtOAc, the reaction mixture was poured into ice-chilled 1 N citric acid with stirring. The powder thereby formed was washed with 1 N citric acid, H_2O, 5% $NaHCO_3$ and H_2O. The dried product was recrystallized or precipitated from appropriate solvents. General procedure for obtaining protected peptide hydrazides from Troc-derivatives: Troc-derivatives in DMF or MeOH were treated with Zn dust in the presence of AcOH for 6 h. The solution was filtered, the filtrate was concentrated and the residue was treated with 3% EDTA. The resulting powder was washed with 5% $NaHCO_3$ and H_2O and precipitated from appropriate solvents. The procedure for obtaining Boc–Thr(Bzl)–Leu–Pro–Ala–Gly–OH from Boc–Thr(Bzl)–Leu–Pro–Ala–Gly–OTce: The Tce ester derivative in MeOH was treated with Zn dust in the presence of AcOH according to Olsen *et al.*[30]

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—40 °C. Boc groups of the protected peptides were removed by TFA–anisole treatment and Z group of Z–Gly–OTce was removed with 25% HBr in AcOH 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, $CHCl_3$–MeOH–H_2O (8 : 3 : 1, lower phase); Rf^2, the Partridge system.[36] The final product corresponding to the entire amino acid sequence of thymopoietin III was chromatographed on filter paper, Toyo Roshi No. 51, at room temperature. Rf^3 value refers to the Partridge system[36] and Rf^4 value refers to BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24).[37] Troc–$NHNH_2$ was purchased from the Kokusan Chemical Works Ltd., Japan. Kits for the fluorometric blast-formation test were purchased from the Japan Immunoresearch Laboratories Co., Ltd., Japan. Preparations of protected intermediates were repeated several times in order to obtain sufficient quantities for the next step. Aminopeptidase (3501, Aminopeptidase 210520) was purchased from the Peptide Institute Inc., Minoh, Osaka 562, Japan. Venous blood was obtained from a patient suffering from common variable immunodeficiency. Venous blood samples from three healthy donors were 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 Cosmosil $5C_{18}$ column.

Boc–Tyr(Bzl)–Leu–Gln–His(Tos)–Leu–Thr(Bzl)–Ala–Leu–Lys(Z)–Arg(Mts)–OBzl (I)——Boc–Thr(Bzl)–Ala–Leu–Lys(Z)–Arg(Mts)–OBzl (1.2 g) was treated with TFA–anisole (10 ml–2 ml) in an ice-bath for 40 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 (10 ml) containing NMM (0.11 ml). The azide [prepared from 2 g of Boc–Tyr(Bzl)–Leu–Gln–His(Tos)–Leu–$NHNH_2$ (2 eq)] in DMF (10 ml) and NMM (0.35 ml) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C for 36 h, was concentrated. The residue was poured into ice-chilled 1 N citric acid with stirring. The precipitate thereby formed was successively washed with 1 N citric acid and H_2O. The dried product was recrystallized twice from DMF with MeOH; yield 1.5 g (71%), mp 203—211 °C, $[\alpha]_D^{21}$ −19.8° (c = 1.0, DMF), Rf^1 0.80, Rf^2 0.79, single fluorescamine-positive spot. *Anal.* Calcd for $C_{107}H_{143}N_{17}O_{22}S_2$: C, 61.68; H, 6.92; N, 11.43. Found: C, 61.52; H, 7.04; N, 11.29. Amino acid ratios in a 6 N HCl hydrolysate: Leu 3.06, Ala 1.00, Tyr 0.92, His 0.88, Thr 0.87, Glu 0.89, Lys 1.04, Arg 0.85 (recovery of Ala 80%).

Boc–Tyr(Bzl)–Val–Glu(OBzl)–Leu–Tyr(Bzl)–Leu–Gln–His(Tos)–Leu–Thr(Bzl)–Ala–Leu–Lys(Z)–Arg(Mts)–OBzl (II)——The above Boc–(40—49)–OBzl (I) (1.4 g) was treated with TFA–anisole (10 ml–2 ml) as described above and the N^{α}-deprotected peptide was dissolved in DMF (12 ml) containing NMM (0.074 ml). The azide [prepared from 1.1 g of Boc–Tyr(Bzl)–Val–Glu(OBzl)–Leu–$NHNH_2$ (2 eq)] in DMF (10 ml) and NMM (0.24 ml) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C for 36 h, was concentrated. The residue was treated with 1 N citric acid and H_2O. The dried product was recrystallized twice from DMF with MeOH; yield 1.3 g (65%), mp 211—219 °C, $[\alpha]_D^{21}$ −15.6° (c = 1.0, DMF), Rf^1 0.74, Rf^2 0.70, single fluorescamine-positive spot. *Anal.* Calcd for $C_{146}H_{191}N_{21}O_{29}S_2 \cdot 11H_2O$: C, 59.11; H, 7.24; N, 9.92. Found: C, 59.10; H, 7.42; N, 9.76. Amino acid ratios in a 6 N HCl hydrolysate: Leu 4.07, Ala 1.00, Tyr 2.02, Val 1.08, His 0.89, Thr 0.86, Glu 1.87, Lys 0.97, Arg 0.90 (recovery of Ala 81%).

Boc–Glu(OBzl)–Gln–Arg(Mts)–Lys(Z)–Glu(OBzl)–Val–Tyr(Bzl)–Val–Glu(OBzl)–Leu–Tyr(Bzl)–Leu–Gln–His(Tos)–Leu–Thr(Bzl)–Ala–Leu–Lys(Z)–Arg(Mts)–OBzl (III)——The above Boc–(36—49)–OBzl (II) (1.1 g) was treated with TFA–anisole (10 ml–2 ml) and the N^{α}-deprotected peptide ester, isolated as usual, was dissolved in DMF–DMSO (1 : 1, 10 ml) containing NMM (0.04 ml). The azide [prepared from 1.5 g of Boc–Glu(OBzl)–Gln–Arg(Mts)–Lys(Z)–Glu(OBzl)–Val–$NHNH_2$ (3 eq)] in DMF–DMSO (1 : 1, 10 ml) and NMM (0.22 ml) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C for 36 h, was neutralized with AcOH and concentrated. The residue was treated with 1 N citric acid and the precipitate thereby formed was washed with 1 N citric acid and H_2O. The resulting powder was purified by gel-filtration on Sephadex LH-60 (3 × 98 cm) with DMF containing 5% H_2O. The ultraviolet (UV) absorption at 260 nm was determined, and fractions containing a UV-absorbing substance of Rf^1 0.69 were combined. The solvent was removed by evaporation and the residue was treated with ether to afford a powder; yield 1.1 g (69%), mp 219—226 °C, $[\alpha]_D^{21}$ −21.6° (c = 1.0, DMSO), Rf^1 0.69, Rf^2 0.64, single fluorescamine-positive spot. *Anal.* Calcd for $C_{209}H_{274}N_{32}O_{44}S_3 \cdot 15H_2O$: C, 58.31; H, 7.12; N, 10.41. Found: C, 58.42; H, 7.28; N, 10.38. Amino acid ratios in a 6 N HCl hydrolysate: Leu 4.08, Val 2.10, Ala 1.00, Tyr 1.92, His 0.89, Thr 0.87, Glu 4.94, Lys 2.03, Arg 1.85 (recovery of Ala 80%).

Boc–Thr(Bzl)–Leu–Pro–Ala–Gly–Glu(OBzl)–Gln–Arg(Mts)–Lys(Z)–Glu(OBzl)–Val–Tyr(Bzl)–Val–Glu(OBzl)–Leu–Tyr(Bzl)–Leu–Gln–His(Tos)–Leu–Thr(Bzl)–Ala–Leu–Lys(Z)–Arg(Mts)–OBzl (IV)——Boc–(30—49)–OBzl (III) (1 g) was treated with TFA–anisole (10 ml–2 ml) and the N^{α}-deprotected peptide, isolated as usual, was dissolved in DMF–DMSO (1 : 1, 10 ml) together with NMM (0.026 ml). To this were added Boc–Thr(Bzl)–Leu–Pro–Ala–Gly–OH (441 mg, 3 eq), HOSu (88 mg) and WSCI (174 mg) at 0 °C. After 24 h, the reaction mixture was evaporated and then triturated with 1 N citric acid. The powder obtained was washed successively with 1 N citric acid, H_2O, 5% $NaHCO_3$ and H_2O. The powder was further purified by reprecipitation three times from DMSO with MeOH; yield 894 mg (69%), mp 236—245 °C, $[\alpha]_D^{21}$ −14.8° (c = 1.0, DMSO), Rf^1 0.57, Rf^2 0.54, single fluorescamine-positive spot. *Anal.* Calcd for $C_{236}H_{313}N_{37}O_{50}S_3$: C, 62.10; H, 6.91; N, 11.35. Found: C, 61.84; H, 7.16; N, 11.08. Amino acid ratios in a 6 N HCl hydrolysate: Leu 5.11, Val 2.07, Ala 2.12, Gly 1.00, Pro 0.94, Tyr 1.95, His 0.89, Thr 1.87, Glu 5.02, Lys 1.92, Arg 1.84 (recovery of Gly 79%).

Boc–Val–Ala–Asn–Asn–Val–Thr(Bzl)–Leu–Pro–Ala–Gly–Glu(OBzl)–Gln–Arg(Mts)–Lys(Z)–Glu(OBzl)–Val–Tyr(Bzl)–Val–Glu(OBzl)–Leu–Tyr(Bzl)–Leu–Gln–His(Tos)–Leu–Thr(Bzl)–Ala–Leu–Lys(Z)–Arg(Mts)–OBzl (V)——The above Boc–(25—49)–OBzl (IV) (761 mg) was treated with TFA–anisole (8 ml–1.6 ml) and the N^{α}-deprotected peptide, isolated as usual, was dissolved in DMF–DMSO (1 : 1, 6 ml) containing NMM (0.019 ml). The azide [prepared from 324 mg of Boc–Val–Ala–Asn–Asn–Val–$NHNH_2$[17] (3 eq)] in DMF–DMSO (1 : 1, 6 ml) and NMM (0.067 ml) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 36 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 DMSO with MeOH; yield 612 mg (69%), mp 231—238 °C, $[\alpha]_D^{21}$ −17.3 °C (c = 1.0, DMSO), Rf^1 0.43, Rf^2 0.49, single fluorescamine-positive spot. *Anal.* Calcd for $C_{257}H_{348}N_{44}O_{57}S_3 \cdot 14H_2O$: C, 58.09; H, 7.13; N, 11.60. Found: C, 58.14; H, 7.16; N, 11.48. Amino acid ratios in a 6 N HCl hydrolysate: Leu 5.10, Val 4.03, Ala 3.11, Gly 1.00, Pro 0.92, Tyr 1.98, His 0.87, Thr 1.85, Glu 4.84, Asp 1.86, Lys 2.09, Arg 1.87 (recovery of Gly 80%).

Boc–Lys(Z)–Ser(Bzl)–Glu(OBzl)–Leu–Val–Ala–Asn–Asn–Val–Thr(Bzl)–Leu–Pro–Ala–Gly–Glu(OBzl)–Gln–Arg(Mts)–Lys(Z)–Glu(OBzl)–Val–Tyr(Bzl)–Val–Glu(OBzl)–Leu–Tyr(Bzl)–Leu–Gln–His(Tos)–Leu–Thr(Bzl)–Ala–Leu–Lys(Z)–Arg(Mts)–OBzl (VI)——The above Boc–(20—49)–OBzl (V) (531 mg) was treated with TFA–anisole (6 ml–1.2 ml) and the N^{α}-deprotected peptide obtained as described above was dissolved in DMF–DMSO (1 : 2, 8 ml) containing NMM (0.011 ml). The azide [prepared from 383 mg of Boc–Lys(Z)–Ser(Bzl)–Glu(OBzl)–Leu–$NHNH_2$ (4 eq)] in DMF–DMSO (1 : 1, 4 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. The ninhydrin-negative solution was neutralized with AcOH and poured into ice-chilled MeOH with stirring. The precipitate formed was washed with MeOH, H_2O, 1 N citric acid and H_2O. The dried product was reprecipitated from DMSO with MeOH; yield 518 mg (86%), mp 198—207 °C, $[\alpha]_D^{21}$ −25.3° (c = 1.0, DMSO), Rf^1 0.44, Rf^2 0.42, single fluorescamine-positive spot. *Anal.* Calcd for $C_{299}H_{401}N_{49}O_{66}S_3 \cdot 11H_2O$: C, 59.45; H, 7.21; N, 11.36. Found: C, 59.41; H, 7.39; N, 11.20. Amino acid ratios in a 6 N HCl hydrolysate: Leu 6.13, Val 4.07, Ala 3.01, Gly 1.00, Pro 0.89, Tyr 2.04, His 0.88, Thr 1.89, Ser 0.87, Glu 5.91, Asp 1.86, Lys 3.09, Arg 1.90 (recovery of Gly 82%).

Boc–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(Mts)–Lys(Z)–Glu(OBzl)–Val–Tyr(Bzl)–Val–Glu(OBzl)–Leu–Tyr(Bzl)–Leu–Gln–His(Tos)–Leu–Thr(Bzl)–Ala–Leu–Lys(Z)–Arg(Mts)–OBzl (VII)——The above Boc–(16—49)–OBzl (VI) (465 mg) was treated with TFA–anisole (5 ml–1 ml) and the N^{α}-deprotected peptide obtained as described above was dissolved in DMF–DMSO (1 : 2, 6 ml) containing NMM (0.009 ml). The azide [prepared from 293 mg of Boc–Thr(Bzl)–Lys(Z)–Glu(OBzl)–Lys(Z)–Leu–$NHNH_2$ (3 eq)] in DMF–DMSO (1 : 2, 4 ml) and NMM (0.03 ml) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 36 h. Additional azide [prepared from 1 eq of hydrazide in DMF–DMSO (1 : 2, 3 ml) and NMM (0.013 ml) were added and stirring was continued for an additional 24 h. After neutralization with a few drops of AcOH, the solution was concentrated and the residue was treated with 1 N citric acid. The resulting powder was washed with 1 N citric acid and H_2O. The dried product was

precipitated from DMSO and MeOH. The crude product was dissolved in DMSO containing 5% H_2O (3 ml) and the solution was applied to a column of Sephadex LH-60 (3 × 93 cm), which was eluted with the same solvent. The fractions with Rf^1 0.51 were combined and the solvent was removed by evaporation. Treatment of the residue with ether afforded a powder; yield 403 mg (73%), mp 184—192 °C, $[\alpha]_D^{21}$ −17.2° (c=1.0, DMSO), Rf^1 0.51, Rf^2 0.49, single fluorescamine-positive spot. *Anal.* Calcd for $C_{356}H_{474}N_{56}O_{78}S_3 \cdot 14H_2O$: C, 59.93; H, 7.09; N, 11.00. Found: C, 59.85; H, 7.21; N, 10.87. Amino acid ratios in a 6 N HCl hydrolysate: Leu 7.12, Val 4.06, Ala 3.10, Gly 1.00, Pro 0.99, Tyr 1.87, His 0.86, Thr 2.87, Ser 0.85, Asp 2.03, Glu 7.03, Lys 4.89, Arg 1.89 (recovery of Gly 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(Mts)–Lys(Z)–Glu(OBzl)–Val–Tyr(Bzl)–Val–Glu(OBzl)–Leu–Tyr(Bzl)–Leu–Gln–His(Tos)–Leu–Thr(Bzl)–Ala–Leu–Lys(Z)–Arg(Mts)–OBzl (VIII)——The above Boc–(11—49)–OBzl (356 mg) was treated with TFA–anisole (4 ml–0.8 ml) and the N^{α}-deprotected peptide as described above was dissolved in DMF–DMSO (1 : 2, 4 ml) containing NMM (0.006 ml). The azide [prepared from 81 mg of Boc–Ser(Bzl)–Val–Leu–$NHNH_2$ (3 eq)] in DMF–DMSO (1 : 2, 3 ml) and NMM (0.02 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 hydrazide] in DMF–DMSO (1 : 2, 3 ml) and NMM (0.009 ml) were added and stirring was continued at 4 °C for an additional 24 h. After being neutralized with a few drops of AcOH, the ninhydrin-negative solution was concentrated and the residue was treated with MeOH, 1 N citric acid and H_2O. The product was reprecipitated twice from DMSO with MeOH; yield 218 mg (62%), mp 178—185 °C, $[\alpha]_D^{21}$ −18.2° (c=1.0, DMSO), Rf^1 0.46, Rf^2 0.41, single fluorescamine-positive spot. *Anal.* Calcd for $C_{356}H_{505}N_{59}O_{82}S_3$: C, 60.92; H, 7.25; N, 11.77. Found: 60.70; H, 7.24; N, 11.86. Amino acid ratios in a 6 N HCl hydrolysate: Leu 8.05, Val 5.08, Ala 3.01, Gly 1.00, Pro 0.91, Tyr 1.87, His 0.89, Thr 2.90, Ser 1.80, Glu 6.87, Asp 1.90, Lys 4.92, Arg 1.87 (recovery of Gly 79%).

Boc–Pro–Glu(OBzl)–Phe–Leu–Glu(OBzl)–Asp(OcHex)–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(Mts)–Lys(Z)–Glu(OBzl)–Val–Tyr(Bzl)–Val–Glu(OBzl)–Leu–Tyr(Bzl)–Leu–Gln–His(Tos)–Leu–Thr(Bzl)–Ala–Leu–Lys(Z)–Arg(Mts)–OBzl (IX)——Boc–(8—49)–OBzl (VIII) (176 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 : 3, 4 ml) containing NMM (0.003 ml). The azide [prepared from 122 mg of Boc–Pro–Glu(OBzl)–Phe–Leu–Glu(OBzl)–Asp(OcHex)–Pro–$NHNH_2$ (4 eq)] in DMF–DMSO (1 : 3, 3 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 hydrazide] in DMF–DMSO (1 : 3, 3 ml) and NMM (0.003 ml) were added and stirring was continued for an additional 32 h. After being neutralized with a few drops of AcOH, the solution was concentrated and 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 DMSO with MeOH. The crude product was dissolved in DMSO containing 5% H_2O (3 ml) and the solution was applied to a column of Sephadex LH-60 (3 × 94 cm), which was eluted with the same solvent. The fractions with Rf^1 0.48 were combined and the solvent was removed by evaporation. Treatment of the residue with EtOAc afforded a powder; yield 108 mg (51%), mp 169—177 °C, $[\alpha]_D^{21}$ −26.3° (c=1.0, DMSO), Rf^1 0.48, Rf^2 0.34, single ninhydrin-positive spot. *Anal.* Calcd for $C_{415}H_{580}N_{66}O_{95}S_3 \cdot 18H_2O$: C, 59.10; H, 7.36; N, 10.96. Found: C, 59.09; H, 7.53; N, 10.84. Amino acid ratios in a 6 N HCl hydrolysate: Leu 9.12, Val 5.04, Ala 3.09, Gly 1.00, Pro 2.87, Phe 1.06, Tyr 1.91, His 0.85, Thr 2.89, Ser 1.84, Glu 8.97, Asp 2.88, Lys 5.08, Arg 1.91 (recovery of Gly 81%).

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–Glu–Val–Tyr–Val–Glu–Leu–Tyr–Leu–Gln–His–Leu–Thr–Ala–Leu–Lys–Arg–OH (Corresponding to Thymopoietin III) (X)——The above protected nonatetracontapeptide (70 mg) was treated with 1 M TFMSA–thioanisole in TFA (3 ml) in the presence of *m*-cresol (0.1 ml) in an ice-bath for 60 min and dry ether was added. The resulting powder was collected by centrifugation and dried over KOH pellets *in vacuo* for 60 min. This treatment was repeated again to ensure complete deprotection. 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 1 N NH_4OH, then after 30 min, to 6.0 with 1 N 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 (3.0 × 96 cm) and eluted with the same solvent. Individual fractions (5 ml each) were collected and the absorbancy at 260 nm was determined. The front peak (tube Nos. 77—84) 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 × 15 cm, Serva). The 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 6.50). Individual fractions (4 ml each) were collected and the absorbancy at 260 nm was determined. The main peak (tube Nos. 47—58) was collected and then the solvent was removed by lyophilization. The product was dissolved in 2% AcOH (2 ml) and the solution was then applied to a Sephadex G-25 column (3.0 × 96 cm), and eluted with the same solvent. The single main peak fractions were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 6.4 mg (14%), $[\alpha]_D^{21}$ −81.2° (c=0.3, 1 N AcOH), Rf^3 0.10, Rf^4 0.18, 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, 8.9 cm from the origin toward the anode, after running at 1 mA, 600 V for 60 min. The synthetic

peptide exhibited a single peak on HPLC using a Cosmosil $5C_{18}$ column (4.6 × 150 mm) at a retention time of 18.27 min, when eluted with a gradient of acetonitrile (30 to 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 5.10, Ala 3.12, Gly 1.00, Pro 2.88, Phe 1.01, Tyr 1.89, His 0.88, Thr 2.85, Ser 1.90, Glu 9.03, Asp 2.86, Lys 5.03, Arg 1.86 (recovery of Gly 83%). Amino acid ratios in an AP-M digest: Leu 9.06, Val 5.01, Ala 3.10, Gly 1.00, Pro 3.04, Phe 1.02, Tyr 2.10, His 0.90, Thr + Gln 4.83 (Calcd. as Thr), Asn + Ser 3.86 (Calcd. as Ser), Glu 6.94, Asp 0.92, Lys 5.06, Arg 1.92 (recovery of Gly 82%).

Fluorometric Blast-Formation Test——A 3 ml aliquot of venous blood was drawn into a syringe containing 25 U/ml heparin and incubated with the synthetic peptide for 90 min at 37 °C, then mixed with 3 ml of PBS. Lymphocytes were then isolated in a Hypaque–Ficoll gradient.[38] Isolated lymphocytes were adjusted to 1.0×10^6/ml with PBS. The lymphocytes were cultered 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 a humidified atmosphere of 5% CO_2 in air for 72 h. Lymphocytes in each well were transferred into a test tube and centrifuged for 10 min at 240 *g*, then the supernatant was removed. Next, 2 ml of 0.125% SDS was added to the residue and the mixture was stirred for 20 min at room temperature; lymphocytes were completely destroyed and solubilized by this procedure. Ethidium bromide solution (2 ml) was 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.[34]

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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 peptides 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; Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl; ONp, *p*-nitrophenyl ester; NP, *p*-nitrophenyl; Mts, mesitylene-2-sulfonyl; Tos, *p*-tolylsufonyl; OBzl, benzyl ester; Bzl, benzyl; Troc, β,β,β-trichloroethyloxycarbonyl; OcHex, cyclohexyl ester; OTce, β,β,β-trichloroethyl 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.
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