[Chem. Pharm. Bull. 36(7)2506—2516(1988)]

Synthesis of Human Thymopoietin (hTP) and Examination of Its Immunological Effect on the Impaired Blastogenic Response of T-Lymphocytes of Uremic Patients[1)]

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(Received December 3, 1987)

The octatetracontapeptide corresponding to the entire amino acid sequence of human thymopoietin (hTP) 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. Finally, the deprotected peptide was incubated with dithiothreitol to reduce sulfoxide on the methionine side chain. The synthetic peptide was found to have a restoring effect on the impaired blastogenic response of T-lymphocytes isolated from uremic patients.

Keywords——human thymopoietin; trifluoromethanesulfonic acid deprotection; uremic patient; impaired T-lymphocyte blastogenic response; fluorometric blast-formation test

Two peptides isolated from bovine thymus, bovine thymopoietin I (bTP-I) and bovine thymopoietin II (bTP-II), were purified and characterized by Goldstein.[2)] Each peptide contains 49 amino acid residues and bTP-II has a molecular weight of 5562. The peptides were shown to be related and to differ by only two amino acid residues.[3)]

A tridecapeptide, corresponding to bTP-II residues 29—41, has been synthesized and demonstrated to have biological activity similar to that of the native molecule.[4)]

Within this sequence, a pentapeptide called thymopentin (32—36), was shown to be the smallest active fragment of thympoietin. We then reported that the pentapeptide (32—36)[5)] induces some recovery of E-rosette formation in the uremic state. In 1982, we also reported that octadecapeptide (32—49)[6)] of bTP-II induces some recovery of E-rosette formation in the blood of patients with rheumatoid arthritis.

In 1981, the amino acid sequences of bTP-I and bTP-II were revised and bovine thymopoietin III (bTP-III) was also isolated from bovine spleen.[7)] The three peptides have largely identical sequences, except for the amino acid residues at positions 1, 2, 34 and 43.

In 1982, we reported the synthesis of the octadecapeptide (32—49),[8)] which corresponds to a part of the revised structure of bTP-II, and showed that the biological acitivity 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 bTP-II fragment (32—49).[6)] In 1977, Fujino and coworkers[9)] reported the first total synthesis of bTP-II, and the synthetic peptide exhibited full activity of bTP-II.

Recently, human thymopoietin[10)] (hTP) was isolated from human thymus and the complete amino acid sequence of this 48-amino-acid polypeptide was determined. hTP possesses the same sequence as bTP-II, except for replacement of eleven residues at positions, 1, 2, 3, 4, 6, 8, 13, 23, 31, 43 and 48, *i.e.*, Pro, Glu, Phe, Leu, Asp, Ser, Glu, Asn, Gln, Ser and Lys with Gly, Leu, Pro, Lys, Val, Ala, Gln, Gly, Met, His and His, respectively.

On the other hand, we and others have reported evidence of impaired immune function in

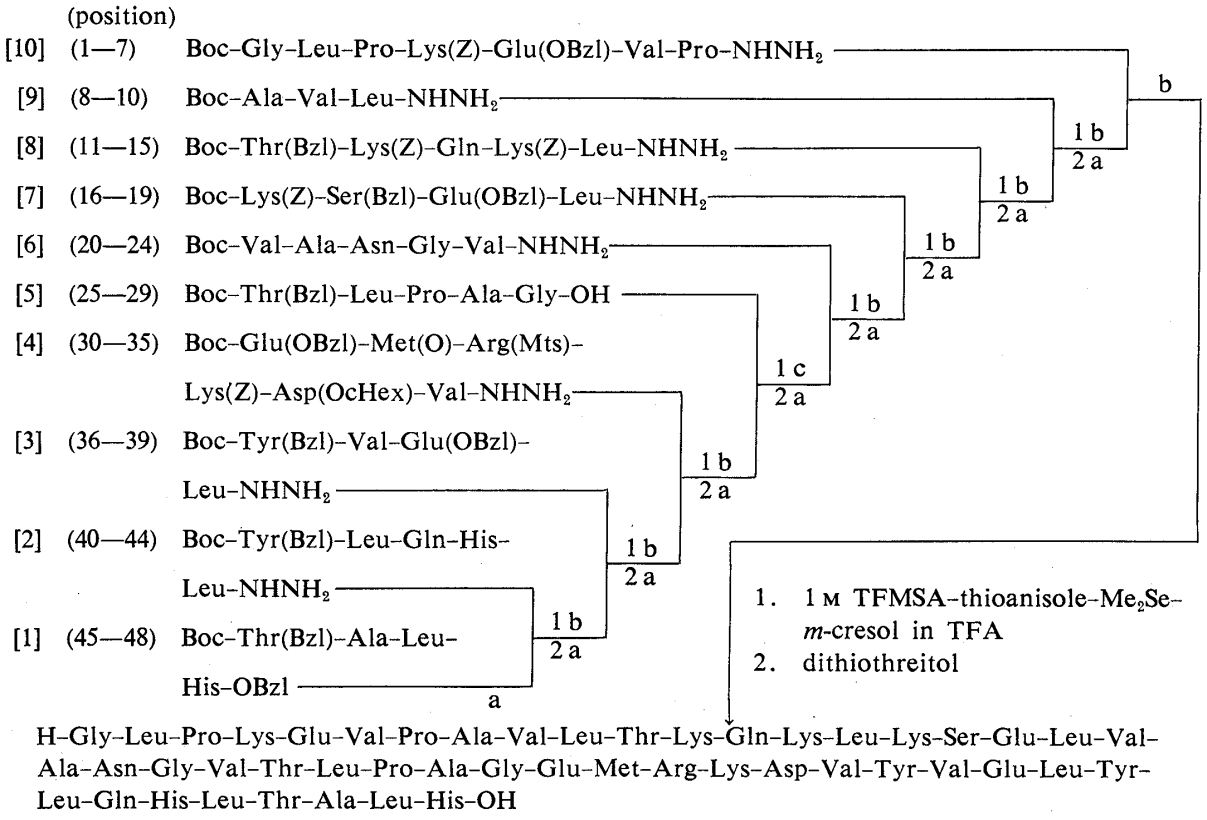

Fig. 1. Synthetic Route to hTP

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

patients with chronic uremia.[5,8,11,12] This impairment is reflected in depressed cell-mediated immune function both *in vitro* and *in vivo*.

Patients with chronic uremia may have thymic atrophy.[12] Thymic atrophy with degenerative changes has been observed in uremic patients,[12] though the cause of thymic atrophy in uremia is currently unknown. These observations suggested to us that the cell-mediated immune abnormalities seen in chronic uremia might be attributable to thymic hormone deficiency.

Following our solution syntheses of bTP-I,[13] bTP-II[14] and bTP-III,[15] we wish to report the solution synthesis of hTP. We then examined its ability to restore *in vitro* the impaired response to PHA stimulation of T-lymphocytes isolated from uremic patients.

Our synthetic route to hTP is illustrated in Fig. 1, which shows the ten fragments selected as building blocks to construct the entire amino acid sequence of hTP. Of these, fragments Boc–(36—39)–$NHNH_2$ [3], Boc–(32—35)–NHNH–Troc, Boc–(25—29)–OH [5], Boc–(16—19)–$NHNH_2$ [7] and Boc–(9—10)–OMe, are available from our previous syntheses of bTP-II[14] and bTP-III.[15]

The Boc group, removable by trifluoroacetic acid (TFA), was adopted as a temporary N^{α}-protecting group for every intermediate. In the present synthesis, the thioanisole–mediated trifluoromethanesulfonic acid (TFMSA) deprotecting procedure[16,17] was employed, *i.e.*, Lys(Z), Glu(OBzl), Thr(Bzl), Ser(Bzl), Tye(Bzl), Asp(OcHex)[18] and Arg(Mts).[19] The Met residue was reversibly protected as its sulfoxide[20] in order to prevent partial S-alkylation during the N^{α}-TFA deprotection as well as partial air oxidation during the synthesis. Asp(OcHex) was employed to minimize aspartimide formation during the synthesis of Asp-containing peptide.[18] N^{α}-Deprotection with TFA was performed in the presence of anisole, prior to each condensation reaction as usual. The substituted hydrazine, Troc-$NHNH_2$,[21]

was employed for preparation of fragments containing the Asp(OcHex) or Glu(OBzl) residue. This Troc group is known to be cleaved by Zn[22] in AcOH without affecting other functional groups. In the present synthesis, seven fragments, [1], [2], [4], [6], [8], [9] and [10], which cover the areas of different amino acid residues in hTP, were newly synthesized. 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.

The protected C-terminal tetrapeptide, Boc–(45—48)–OBzl [1], was prepared stepwise starting from H-His-OBzl 2 Tos by the Su active ester procedure.[23] Next, protected peptide esters, Boc–(40—44)–OMe, Boc–(20—24)–OMe, Boc–(11—15)–OMe and Boc–(8—10)–OMe, were prepared stepwise starting from C-terminal amino acid esters by the Su active ester procedure[23] except for the introduction of Gln and Asn, which were introduced by the NP active ester procedure.[24] The protected peptide esters thus obtained were smoothly converted to the corresponding hydrazides, Boc–(40—44)–$NHNH_2$ [2], Boc–(20—24)–$NHNH_2$ [6], Boc–(11—15)–$NHNH_2$ [8] and Boc–(8—10)–$NHNH_2$ [9], in the usual manner. The hydrazine test on the thin-layer chromatograms and the elemental analysis data of these fragments were consistent with homogeneity of the desired products. The two fragments, Boc–(30—35)–NHNH–Troc and Boc–(1—7)–NHNH–Troc which contain Glu(OBzl) or Asp(OcHex), were prepared stepwise by the Su active ester procedure.[23] The two fragments thus obtained were treated with Zn[21,22] in AcOH to remove Troc groups, and the zinc acetate was removed by treatment with EDTA to give the required hydrazides in analytically pure form. The hydrazine test on the thin-layer chromatograms and the elemental analysis data were consistent with homogeneity of the desired products.

The ten fragments were assembled successively by the azide procedure[25] and the HOSu–WSCI procedure[26] according to the routes illustrated in Fig. 2. The amount of the acyl component in each fragment condensation was increased from 1.5 to 4 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 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 C-terminal pentapeptide ester corresponding to positions 45 to 48 of hTP, Boc–(45—48)–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[25] and the HOSu–WSCI procedure[26] as shown in Fig. 2 to give the protected octatetracontapeptide corresponding to the entire amino acid sequence of hTP. 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 octatetracontapeptide 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.[19] Me_2Se was employed to facilitate acidic cleavage of protecting groups.[27] The deprotected peptide was next precipitated with peroxide-free ether, converted to the corresponding acetate with Amberlite IRA-400 (acetate form) and then treated with 1 N NH_4OH to reverse a possible N→O shift at the Ser and Thr residues.[28] The Met(O) residue

was reduced back to Met in two steps, firstly with thioanisole and Me_2Se[27] during the above acid treatment, and secondly with dithiothreitol during incubation of the deprotected peptide. The reduced product was purified by gel-filtration on Sephadex G-50, followed by ion-exchange column chromatography on a CM-Biogel A column with linear gradient elution using pH 6.50 ammonium acetate buffer, 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 2.84 acetate buffer). The peptide also exhibited a single peak on HPLC. Homogeneity of the synthetic hTP was further ascertained by amino acid analysis, after 6 N

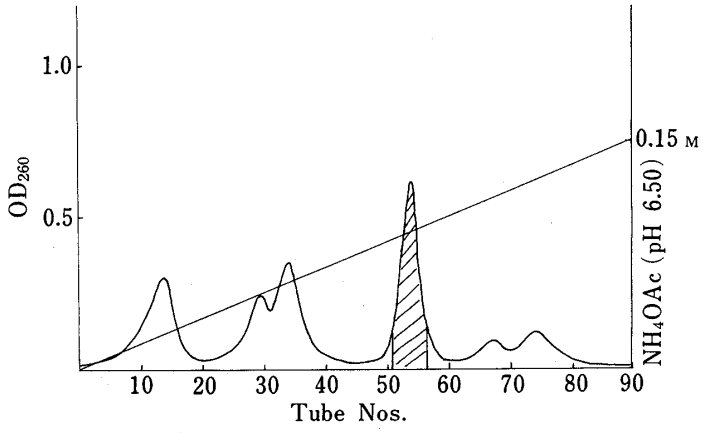

Fig. 2. Purification of Synthetic hTP by Ion-Exchange Chromatography on a CM-Biogel A Column

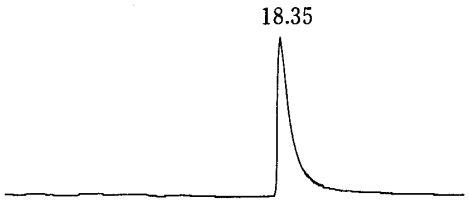

Fig. 3. HPLC of Synthetic hTP

TABLE I. Effect of the Synthetic hTP on the Impaired PHA Stimulation of T-Lymphocytes of Uremic Patients

Peptides	Dose (μg/ml)	$SI^{a,b)}$
—[c)]	—	289±55.4
—[d)]	—	116±57.2[g)]
H–Leu–Gly–Gly–OH[d–f)]	1.0	118±60.7
	2.0	109±59.6
Synthetic hTP[d,f)]	1.0	191±62.6[h)]
	2.0	256±61.5[h)]

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-activated lymphocytes, I_1 = fluorescence intensity of PHA-nonactivated lymphocytes and I_0 = fluorescence intensity of ethydium bromide. *c*) Normal venous lymphocytes. *d*) Patient's venous lymphocytes. *e*) Control: This peptide was purchased from the Peptide Institute, Inc., Osaka, Japan. *f*) Incubation was carried out at 37 °C in a humidified atmosphere of 5% CO_2 in air for 12 h. *g*) $p < 0.05$, when compared to normal persons by using Student's *t*-test. *h*) $p < 0.01$, when compared to uremic patients by using Student's *t*-test.

HCl hydrolysis and enzymatic digestion.

The immunological effect of the synthetic hTP was examined by means of the JIMRO (Japan Immunoresearch Laboratories Co., Ltd.) fluorometric blast-formation test according to Itoh and Kawai.[29] In contrast to normal persons, the blastogenesis of the T-lymphocytes into lymphoblasts with mitotic activity after PHA stimulation is depressed in uremic patients with recurrent pneumonia. The *in vitro* effect of the synthetic peptide on the impaired PHA response of T-lymphocytes from the uremic patients is shown in Table I.

Comparison of the *SI* values of the blastogenic transformation of T-lymphocytes into lymphoblasts with mitotic activity upon PHA stimulation shows that, in the case of the patients investigated, the synthetic hTP exhibited a restoring effect at a dose of 1 μg/ml. In the case of normal subjects, *in vitro* addition of this peptide did not have any effect on the mitotic activity induced by PHA stimulation under the same conditions (data not shown). These results indicate that the synthetic hTP has activity to restore the impaired blastogenic transformation of T-lymphocytes into lymphoblasts with mitotic activity upon PHA stimulation in uremic patients with cell-mediated immunodeficiency. This preliminary communication reports evidence to suggest that the cell-mediated immunodeficiency seen in uremic patients with infectious diseases may be attributable to a deficiency of the thymic hormone hTP.

Experimental

General experimental procedures used in this paper are essentially the same as described in the previous papers.[14,15] Azides were prepared according to Honzl and Rudinger[25] with isoamyl nitrite. After each coupling reaction, each product was purified by one of the following three procedures. Procedure A: For purification of protected peptides soluble in EtOAc, the extract was washed with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O, then dried over $MgSO_4$ and concentrated. The residue was reprecipitated or recrystallized from appropriate solvents. Procedure B: For purification of protected peptides almost insoluble in EtOAc, the reaction mixture was poured into ice-chilled 5% citric acid with stirring. The powder thereby formed was washed with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O. The dried product was recrystallized or reprecipitated from appropriate solvents. Procedure C: For purification of protected peptides almost insoluble in EtOAc, the reaction mixture was poured into ice-chilled 1 N NH_4OH with stirring. The powder thereby formed was washed with 1 N NH_4OH until the yellow color disappeared, and then washed with H_2O, 5% citric acid and H_2O. The dried product was recrystallized or reprecipitated from appropriate solvents. Preparations of protected intermediates were repeated several times in order to obtain sufficient quantities for the next step.

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 the protected peptides were removed by TFA–anisole treatment. 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[30]; Rf^2 BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24).[31] The final product corresponding to the entire amino acid sequence of hTP 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).[31] Troc–$NHNH_2$ was purchased from Kokusan Chemical Works Ltd., Japan. Papain (No. P-3125) and leucine aminopeptidease (No. L-9876) were purchased from Sigma Chemical Co. Patient selection: Two uremic patients who were suffering from recurrent pneumonia were selected. Examination of cellular immunocompetence of these patients revealed a significant decrease in blast-formation by PHA. ^{3}H-Thymidine incorporation values of these patients were 10900 and 9126 cpm, respectively (normal values: 35426—37694 cpm). Venous blood was obtained from these uremic patients for the fluorometric blast-formation test. Venous blood samples from three healthy donors were used as a control. The fluorescence excitation spectrum was measured with an Oyo-Bunko ULOG-FLOUSPEC 11 A fluorometer. HPLC was conducted with a Shimadzu LC-3A apparatus coupled to a Vydac $5C_{18}$ column.

Boc–Leu–His–OBzl (I)——Boc–Leu–OSu (3.5 g) was added to a mixture of H–His–OBzl 2 Tos (59 g) and NMM (2.2 ml) in DMF (30 ml) and the solution, after being stirred at room temperature for 5 h, was concentrated. The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane. Yield 4 g (87%), mp 108—113 °C, $[\alpha]_D^{21}$ −14.6° (c = 1.0, DMF), Rf^1 0.42, Rf^2 0.49, single ninhydrin-positive spot. *Anal.* Calcd for $C_{24}H_{34}N_4O_5$: C, 62.86; H, 7.47; N, 12.22. Found: C, 62.61; H, 7.73; N, 11.90.

Boc–Ala–Leu–His–OBzl (II)——I (2.3 g) was treated with TFA–anisole (20 ml–4 ml) in an ice-bath for 40 min,

TABLE II. Physical Constants and Analytical Data of Protected hTP and Its Intermediates

	Puri. proc. (Yield %)	Rf^1	mp (°C)	$[\alpha]_D^{21}$ ($c=1.0$, DMSO)	Formula	Analysis (%) Calcd (Found)		
						C	H	N
Boc–(40—48)–OBzl	A (78)	0.62	192—203	−14.3	$C_{77}H_{104}N_{14}O_{15}\cdot 5H_2O$	59.44 (59.35	7.39 7.40	12.60 12.46)
Boc–(36—48)–OBzl	A (73)	0.71	184—196	−18.9	$C_{116}H_{152}N_{18}O_{23}\cdot 8H_2O$	60.30 (60.41	7.33 7.56	10.91 10.82)
Boc–(30—48)–OBzl	B (69)	0.71	213—224	−20.4	$C_{177}H_{238}N_{28}O_{38}S_2\cdot 11H_2O$	58.59 (58.44	7.23 7.60	10.81 10.94)
Boc–(25—48)–OBzl	A (57)	0.70	224—237	−17.6	$C_{204}H_{277}N_{33}O_{44}S_2\cdot 13H_2O$	58.42 (58.36	7.28 7.34	11.02 10.84)
Boc–(20—48)–OBzl	A (66)	0.76	231—244	−21.7	$C_{223}H_{309}N_{39}O_{50}S_2\cdot 10H_2O$	58.48 (58.32	7.24 7.22	11.93 12.17)
Boc–(16—48)–OBzl	A (62)	0.59	236—248	−16.3	$C_{265}H_{362}N_{44}O_{59}S_2\cdot 15H_2O$	58.48 (58.20	7.26 7.39	11.32 11.35)
Boc–(11—48)–OBzl	B (68)	0.71	239—250	−19.2	$C_{315}H_{430}N_{52}O_{70}S_2\cdot 18H_2O$	58.63 (58.59	7.28 7.42	11.29 11.07)
Boc–(8—48)–OBzl	A (52)	0.60	240—253	−21.3	$C_{329}H_{455}N_{55}O_{73}S_2\cdot 14H_2O$	59.29 (59.37	7.31 7.58	11.56 11.42)
Boc–(1—48)–OBzl	B (59)	0.53	256—268	−29.6	$C_{378}H_{523}N_{63}O_{84}S_2\cdot 16H_2O$	59.38 (59.40	7.73 7.49	11.54 11.68)

A, Precipitation from DMF with MeOH. B, Gel filtration on Sephadex LH-60.

TABLE III. Amino Acid Ratios in 6 N HCl Hydrolysates of Protected hTP and Its Intermediates[a]

	Protected peptides									Residue
	40—48	36—48	30—48	25—48	20—48	16—48	11—48	8—48	1—48	
Gly				1.00	2.00	2.00	2.00	2.00	3.00	3
Ala	1.00	1.00	1.00	2.04	3.05	3.02	3.06	3.91	4.02	4
Val		0.98	2.01	2.06	3.94	4.07	4.03	5.01	5.93	6
Leu	3.04	4.06	4.03	4.92	5.03	6.04	6.94	7.88	9.01	9
Tyr	0.91	2.02	1.92	2.01	1.93	2.06	1.91	2.04	1.92	2
Met			0.92	0.90	0.91	0.90	0.89	0.92	0.85	1[b]
Ser						0.86	0.89	0.87	0.88	1
Thr	0.90	0.89	0.88	1.84	1.87	1.81	2.82	2.88	2.86	3
Pro				0.90	0.92	0.89	0.87	0.86	2.84	3
Glu	0.95	1.89	2.84	2.86	3.02	3.90	4.91	4.86	5.92	6
Asp			1.03	0.93	1.99	2.04	1.95	2.04	1.99	2
His	1.88	1.92	1.85	1.86	1.89	1.84	1.87	1.84	1.86	2
Lys			0.94	0.91	0.93	1.99	3.90	3.94	4.86	5
Arg			0.90	0.89	0.88	0.87	0.92	0.87	0.86	1

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. *b*) Met + Met(O).

and TFA was then removed by evaporation. The residue was washed with *n*-hexane, dried over KOH pellets *in vacuo* for 2 h, and then dissolved in DMF (20 ml) containing NMM (0.6 ml). To this solution, Boc–Ala–OSu (1.6 g) was added, and the mixture was stirred at room temperature for 5 h. The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane. Yield 2.4 g (86%), mp 113—121 °C, $[\alpha]_D^{21}$ −10.2° ($c=1.0$, DMF), Rf^1 0.49, Rf^2 0.55, single ninhydrin-positive spot. *Anal.* Calcd for $C_{27}H_{39}N_5O_9\cdot 2H_2O$: C, 57.33; H, 7.60; N, 12.38. Found: C, 57.25; H, 7.59; N, 12.24.

Boc–Thr(Bzl)–Ala–Leu–His–OBzl [1]——This compound was prepared essentially in the same manner as

described for the preparation of II by using II (2.3 g) and Boc–Thr(Bzl)–OSu (1.8 g). The product was purified by procedure A, followed by reprecipitation from EtOAc with ether. Yield 2.1 g (70%), mp 124—132 °C, $[\alpha]_D^{21}$ −8.9° (c = 1.0, DMF), Rf^1 0.50, Rf^2 0.55, single ninhydrin-positive spot. *Anal.* Calcd for $C_{38}H_{52}N_6O_8 \cdot H_2O$: C, 61.77; H, 7.37; N, 11.37. Found: C, 61.59; H, 7.63; N, 11.18.

Boc–His–Leu–OMe (III)——This compound was prepared essentially in the same manner as described for the preparation of I using H–Leu–OMe HCl (1.8 g) and Boc–His–OSu (3.7 g). The product was purified by procedure A, followed by reprepcipitation from EtOAc with *n*-hexane. Yield 2.9 g (73%), mp 77—81 °C, $[\alpha]_D^{21}$ −12.9° (c = 1.0, DMF), Rf^1 0.44, Rf^2 0.50, single ninhydrin-positive spot. *Anal.* Calcd for $C_{18}H_{30}N_4O_5 \cdot H_2O$: C, 53.99; H, 8.05; N, 13.99. Found: C, 53.78; H, 8.14; N, 13.72.

Boc–Gln–His–Leu–OMe (IV)——III (1.6 g) was treated with TFA-anisole (12 ml–2 ml) as usual and the resulting powder was dissolved in DMF (16 ml) together with NMM (0.4 ml). Boc–Gln–ONp (1.8 g) was added and the solution was stirred at room temperature for 8 h. The reaction mixture was diluted with 1 N NH_4OH (4 ml) with stirring to saponify the unchanged *p*-nitrophenyl ester. After 1 h, the product was purified by procedure C, followed by reprecipitation from MeOH with ether. Yield 1.4 g (70%), mp 142—149 °C, $[\alpha]_D^{21}$ −8.9° (c = 1.0, DMF), Rf^1 0.42, Rf^2 0.48, single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{38}N_6O_7$: C, 54.11; H, 7.50; N, 16.46. Found: C, 53.38; H, 7.81; N, 16.49.

Boc–Leu–Gln–His–Leu–OMe (V)——This compound was prepared essentially in the same manner as described for the preparation of II by using IV (1.1 g) and Boc–Leu–OSu (803 mg). The product was purified by procedure A, followed by reprecipitation from EtOAc with ether. Yield 1.2 g (86%), mp 107—115 °C, $[\alpha]_D^{21}$ −11.2° (c = 1.0, DMF), Rf^1 0.51, Rf^2 0.55, single ninhydrin-positive spot. *Anal.* Calcd for $C_{29}H_{49}N_7O_8 \cdot 2H_2O$: C, 52.29; H, 8.10; N, 14.86. Found: C, 52.58; H, 8.06; N, 14.50.

Boc–Tyr(Bzl)–Leu–Gln–His–Leu–OMe (VI)——This compound was prepared essentially in the same manner as described for the preparation of II using V (1.1 g) and Boc–Tyr(Bzl)–OSu (681 mg). The product was purified by procedure B, followed by reprecipitation from MeOH with ether. Yield 1.2 g (80%), mp 141—150 °C, $[\alpha]_D^{21}$ −16.9° (c = 1.0, DMF), Rf^1 0.60, Rf^2 0.64, single ninhydrin-positive spot. *Anal.* Calcd for $C_{45}H_{64}N_8O_{10}$: C, 61.63; H, 7.36; N, 12.78. Found: C, 61.74; H, 7.61; N, 12.93.

Boc–Tyr(Bzl)–Leu–Gln–His–Leu–NHNH$_2$ [2]——VI (1.1 g) was dissolved in DMF–MeOH (1 : 1, 12 ml). Hydrazine hydrate (0.6 ml) was added, and the solution was kept standing at room temperature for 24 h. After evaporation of the MeOH, the residue was poured into ice-chilled H_2O with vigorous stirring. The precipitate thereby formed was collected by filtration and washed with H_2O. The dried product was recrystallized from MeOH. Yield 1 g (91%), mp 157—165 °C, $[\alpha]_D^{21}$ −15.4° (c = 1.0, DMF), Rf^1 0.51, Rf^2 0.49, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{44}H_{64}N_{10}O_9 \cdot H_2O$: C, 59.04; H, 7.43; N, 15.65. Found: C, 59.11; H, 7.64; N, 15.37.

Boc–Met(O)–Arg(Mts)–Lys(Z)–Asp(OcHex)–Val–NHNH–Troc (VII)——This compound was prepared from Boc–Arg(Mts)–Lys(Z)–Asp(OcHex)–Val–NHNH–Troc[14] (1.2 g) and Met(O)–OSu (398 mg) essentially as described for the preparation of II. The product was purified by procedure B, followed by reprecipitation from AcOH with H_2O. Yield 1.1 g (79%), mp 146—153 °C, $[\alpha]_D^{21}$ −14.2° (c = 1.0, DMF), Rf^1 0.60, Rf^2 0.64, single ninhydrin-positive spot. *Anal.* Calcd for $C_{57}H_{86}Cl_3N_{11}O_{16}S_2$: C, 50.64; H, 6.41; N, 11.40. Found: C, 50.29; H, 7.67; N, 11.09.

Boc–Glu(OBzl)–Met(O)–Arg(Mts)–Lys(Z)–Asp(OcHex)–Val–NHNH–Troc (VIII)——This compound was prepared from VII (1 g) and Boc–Glu(OBzl)–OSu (368 mg) essentially as described for the preparation of II. The product was purified by procedure B, followed by reprecipitation from MeOH with ether. Yield 967 mg (81%), mp 110—117 °C, $[\alpha]_D^{21}$ −19.1° (c = 1.0, DMF), Rf^1 0.56, Rf^2 0.61, single ninhydrin-positive spot. *Anal.* Calcd for $C_{69}H_{99}Cl_3N_{12}O_{19}S_2 \cdot 2H_2O$: C, 51.57; H, 6.46; N, 10.46. Found: C, 51.38; H, 6.72; N, 10.25.

Boc–Glu(OBzl)–Met(O)–Arg(Mts)–Lys(Z)–Asp(OcHex)–Val–NHNH$_2$ [4]——VIII (536 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 501 mg (91%), mp 164—172 °C, $[\alpha]_D^{21}$ −15.9° (c = 1.0, DMF), Rf^1 0.46, Rf^2 0.49, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{66}H_{98}Cl_3N_{12}O_{17}S_2$: C, 63.65; H, 5.96; N, 10.13. Found: C, 63.54; H, 6.08; N, 9.84.

Boc–Gly–Val–OMe (IX)——This compound was prepared from H–Val–OMe HCl (1.7 g) and Boc–Gly–OSu (2.9 g) essentially as described for the preparation of I. The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane. Yield 3 g (91%) (oily material), $[\alpha]_D^{21}$ −13.4° (c = 1.0, DMF), *Anal.* Calcd for $C_{13}H_{26}N_2O_6 \cdot H_2O$: C, 48.14; H, 8.70; N, 8.64. Found: C, 48.21; H, 8.62; N, 8.39.

Boc–Asn–Gly–Val–OMe (X)——This compound was prepared essentially in the same manner as described for the preparation of IV by using IX (1.6 g) and Boc–Asn–ONp (2 g). The product was purified by procedure C, followed by reprecipitation from EtOAc with ether. Yield 1.2 g (59%), mp 148—154 °C, $[\alpha]_D^{21}$ −11.2° (c = 1.0, DMF), Rf^1 0.45, Rf^2 0.48, single ninhydrin-positive spot. *Anal.* Calcd for $C_{17}H_{32}N_4O_8$: C, 48.56; H, 7.62; N, 13.33. Found: C, 48.51; H, 7.89; N, 13.52.

Boc–Ala–Asn–Gly–Val–OMe (XI)——This compound was prepared from X (842 mg) and Boc–Ala–OSu (600 mg) essentially as described for the preparation of II. The product was purified by procedure A, followed by reprecipitation from EtOAc with ether. Yield 903 mg (82%), mp 121—128 °C, $[\alpha]_D^{21}$ −18.3° (c = 1.0, DMF), Rf^1 0.50,

Rf^2 0.54, single ninhydrin-positive spot. *Anal.* Calcd for $C_{22}H_{37}N_5O_9 \cdot 2H_2O$: C, 48.08; H, 7.52; N, 12.74. Found: C, 48.14; H, 7.79; N, 12.47.

Boc–Val–Ala–Asn–Gly–Val–OMe (XII)——This compound was prepared from XI (550 mg) and Boc–Val–OSu (345 mg) essentially as described for the preparation of II. The product was purified by procedure A, followed by reprecipitation from MeOH with ether. Yield 518 mg (84%), mp 103—108 °C, $[\alpha]_D^{21}$ −12.8° (c=1.0, DMF), Rf^1 0.54, Rf^2 0.59, single ninhydrin-positive spot. *Anal.* Calcd for $C_{27}H_{46}N_6O_{10}$: C, 52.76; H, 7.54; N, 13.67. Found: C, 52.48; H, 7.81; N, 13.54.

Boc–Val–Ala–Asn–Gly–Val–NHNH$_2$ [6]——This compound was prepared from XII (410 mg) and hydrazine hydrate (0.3 ml) essentially as described for the preparation of [2]. The product was recrystallized from MeOH. Yield 407 mg (97%), mp 167—175 °C, $[\alpha]_D^{21}$ −17.6° (c=1.0, DMF), Rf^1 0.41, Rf^2 0.44, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{26}H_{46}N_8O_9 \cdot H_2O$: C, 49.36; H, 7.65; N, 17.71. Found: C, 49.51; H, 7.80; N, 17.59.

Boc–Lys(Z)–Leu–OMe (XIII)——This compound was prepared essentially in the same manner as described for the preparation of I by using H–Leu–OMe HCl (1.8 g) and Boc–Lys(Z)–OSu (5.2 g). The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane. Yield 4.3 g (83%), mp 98—101 °C, $[\alpha]_D^{21}$ 9.1° (c=1.0, DMF), Rf^1 0.64, Rf^2 0.62, single ninhydrin-positive spot. *Anal.* Calcd for $C_{26}H_{41}N_3O_7 \cdot H_2O$: C, 59.41; H, 8.25; N, 7.99. Found: C, 59.18; H, 8.46; N, 7.68.

Boc–Gln–Lys(Z)–Leu–OMe (XIV)——This compound was prepared from XIII (2.1 g) and Boc–Gln–ONp (1.9 g) essentially as described for the preparation of IV. The product was purified by procedure C, followed by reprecipitation from MeOH with ether. Yield 1.9 g (70%), mp 124—132 °C, $[\alpha]_D^{21}$ −11.6° (c=1.0, DMF), Rf^1 0.51, Rf^2 0.53, single ninhydrin-positive spot. *Anal.* Calcd for $C_{31}H_{49}N_5O_9$: C, 54.35; H, 7.21; N, 10.22. Found: C, 54.48; H, 7.46; N, 9.84.

Boc–Lys(Z)–Gln–Lys(Z)–Leu–OMe (XV)——This compound was prepared from XIV (1.1 g) and Boc–Lys(Z)–OSu (876 mg) essentially as described for the preparation of II. The product was purified by procedure A, followed by recrystallization from EtOAc with ether. Yield 1.2 g (80%), mp 123—130 °C, $[\alpha]_D^{21}$ −10.4° (c=1.0, DMF), Rf^1 0.52, Rf^2 0.63, single ninhydrin-positive spot. *Anal.* Calcd for $C_{45}H_{67}N_7O_{12} \cdot 2H_2O$: C, 57.86; H, 7.66; N, 10.50. Found: C, 57.71; H, 7.83; N, 10.29.

Boc–Thr(Bzl)–Lys(Z)–Gln–Lys(Z)–Leu–OMe (XVI)——This compound was prepared essentially in the same manner as described for the preparation of II by using XV (934 mg) and Boc–Thr(Bzl)–OSu (450 mg). The product was purified by procedure A, followed by reprecipitation from MeOH with ether. Yield 920 mg (85%), mp 130—137 °C, $[\alpha]_D^{21}$ −15.1° (c=1.0, DMF), Rf^1 0.55, Rf^2 0.59, single ninhydrin-positive spot. *Anal.* Calcd for $C_{56}H_{80}N_8O_{14}$: C, 61.75; H, 7.40; N, 10.92. Found: C, 61.48; H, 7.69; N, 10.03.

Boc–Thr(Bzl)–Lys(Z)–Gln–Lys(Z)–Leu–NHNH$_2$ [8]——This compound was prepared from XVI (726 mg) and hydrazine hydrate (0.4 ml) essentially as described for the preparation of [2]. The product was recrystallized from MeOH. Yield 665 mg (90%), mp 153—161 °C, $[\alpha]_D^{21}$ −21.3° (c=1.0, DMF), Rf^1 0.47, Rf^2 0.55, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{55}H_{80}N_{10}O_{13} \cdot H_2O$: C, 59.66; H, 7.47; N, 12.65. Found: C, 59.46; H, 7.60; N, 12.39.

Boc–Ala–Val–Leu–OMe (XVII)——This compound was prepared from Boc–Val–Leu–OMe[15] (725 mg) and Boc–Ala–OSu (630 mg) essentially as described for the preparation of II. The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane. Yield 711 mg (86%), mp 119—126 °C, $[\alpha]_D^{21}$ −20.1° (c=1.0, DMF), Rf^1 0.61, Rf^2 0.66, single ninhydrin-positive spot. *Anal.* Calcd for $C_{20}H_{37}N_3O_6$: C, 57.75; H, 8.98; N, 10.11. Found: C, 57.49; H, 9.12; N, 9.80.

Boc–Ala–Val–Leu–NHNH$_2$ [9]——This compound was prepared from XVII (519 mg) and hydrazine hydrate (0.6 ml) essentially as described for the preparation of [2]. The product was recrystallized from MeOH. Yield 506 mg (93%), mp 149—161 °C, $[\alpha]_D^{21}$ −16.3° (c=1.0, DMF), Rf^1 0.46, Rf^2 0.48, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{19}H_{37}N_5O_5 \cdot H_2O$: C, 52.64; H, 9.07; N, 16.15. Found: C, 52.47; H, 9.18; N, 16.22.

Boc–Val–Pro–NHNH–Troc (XVIII)——This compound was prepared essentially in the same manner as described for the preparation of II by using Boc–Pro–NHNH–Troc (2 g) and Boc–Val–OSu (1.5 g). The product was purified by procedure A, followed by reprecipitation from EtOAc with petroleum ether. Yield 2.1 g (84%) (oily material), $[\alpha]_D^{21}$ −8.3° (c=1.0, DMF), Rf^1 0.59, Rf^2 0.61, single ninhydrin-positive spot. *Anal.* Calcd for $C_{18}H_{29}Cl_3N_4O_6$: C, 42.91; H, 5.80; N, 11.12. Found: C, 42.63; H, 6.12; N, 11.37.

Boc–Glu(OBzl)–Val–Pro–NHNH–Troc (XIX)——This compound was prepared essentially in the same manner as described for the preparation of II by using XVIII (1.7 g) and Boc–Glu(OBzl)–OSu (1.6 g). The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane. Yield 2.1 g (84%), mp 80—84 °C, $[\alpha]_D^{21}$ −8.6° (c=1.0, DMF), Rf^1 0.56, Rf^2 0.61, single ninhydrin-positive spot. *Anal.* Calcd for $C_{30}H_{42}Cl_3N_5O_9 \cdot H_2O$: C, 48.62; H, 5.99; N, 9.45. Found: C, 48.46; H, 6.23; N, 9.56.

Boc–Lys(Z)–Glu(OBzl)–Val–Pro–NHNH–Troc (XX)——This compound was prepared from XIX (1.9 g) and Boc–Lys(Z)–OSu (1.3 g) essentially as described for the preparation of II. The product was purified by procedure A, followed by reprecipitation from EtOAc with ether. Yield 2 g (80%), mp 116—123 °C, $[\alpha]_D^{21}$ −13.4° (c=1.0, DMF), Rf^1 0.62, Rf^2 0.64, single ninhydrin-positive spot. *Anal.* Calcd for $C_{44}H_{60}Cl_3N_7O_{12}$: C, 53.63; H, 6.14; N, 9.95. Found: C, 53.41; H, 6.38; N, 10.14.

Boc–Pro–Lys(Z)–Glu(OBzl)–Val–Pro–NHNH–Troc (XXI)——This compound was prepared from XXI (1.6 g)

and Boc–Pro–OSu (573 mg) essentially as described for the preparation of II. The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane. Yield 1.5 g (83%), mp 113—121 °C, $[\alpha]_D^{21}$ −9.5° ($c=1.0$, DMF), Rf^1 0.59, Rf^2 0.64, single ninhydrin-positive spot. *Anal.* Calcd for $C_{49}H_{67}Cl_3N_8O_{13}\cdot H_2O$: C, 53.48; H, 6.32; N, 10.18. Found: C, 53.41; H, 6.49; N, 10.35.

Boc–Leu–Pro–Lys(Z)–Glu(OBzl)–Val–Pro–NHNH–Troc (XXII)——This compound was prepared essentially in the same manner as described for the preparation of II by using XXI (1.1 g) and Boc–Leu–OSu (361 mg). The product was purified by procedure B, followed by recrystallization from MeOH with ether. Yield 932 mg (78%), mp 120—128 °C, $[\alpha]_D^{21}$ −15.6° ($c=1.0$, DMF), Rf^1 0.70, Rf^2 0.68, single ninhydrin-positive spot. *Anal.* Calcd for $C_{55}H_{78}Cl_3N_9O_{14}$: C, 55.25; H, 6.58; N, 10.54. Found: C, 55.11; H, 6.80; N, 10.67.

Boc–Gly–Leu–Pro–Lys(Z)–Glu(OBzl)–Val–Pro–NHNH–Troc (XXIII)——This compound was prepared essentially in the same manner as described for the preparation of II by using XXII (797 mg) and Boc–Gly–OSu (200 mg). The product was purified by procedure B, followed by reprecipitation from MeOH with ether. Yield 614 mg (72%), mp 130—141 °C, $[\alpha]_D^{21}$ −4.2° ($c=1.0$, DMF), Rf^1 0.59, Rf^2 0.65, single ninhydrin-positive spot. *Anal.* Calcd for $C_{57}H_{81}Cl_3N_{10}O_{15}\cdot 2H_2O$: C, 53.13; H, 6.65; N, 10.87. Found: C, 53.09; H, 6.74; N, 10.64.

Boc–Gly–Leu–Pro–Lys(Z)–Glu(OBzl)–Val–Pro–NHNH$_2$ [10]——This compound was prepared from XXIII (516 mg) and Zn dust (261 mg) essentially as described for the preparation of **[4]**. The product was reprecipitated from DMF with H_2O. Yield 423 mg (93%), mp 142—151 °C, $[\alpha]_D^{21}$ −10.6° ($c=1.0$, DMF), Rf^1 0.40, Rf^2 0.45, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{54}H_{80}N_{10}O_{13}\cdot 3H_2O$: C, 57.33; H, 7.66; N, 12.38. Found: C, 57.47; H, 7.75; N, 12.11.

Synthesis of Protected hTP——1. Successive azide condensation of nine fragments except for Boc–(25—29)–OH [5], which was condensed by the HOSu–WSCI procedure, were carried out according to 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 1.5 to 4 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, H_2O and MeOH. The dried product was purified by one of the following two procedures. A: Precipitation from DMF with MeOH. B: Gel-filtration on Sephadex LH-60 using DMF 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—48)–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 (2.5 eq), HOSu (2.5 eq) and WSCI (2.5 eq) at 0 °C. After 24 h, the reaction mixture was evaporated *in vacuo* and then triturated with 5% $NaHCO_3$. The powder thus obtained was washed successively with 5% $NaHCO_3$, H_2O, 5% citric acid and H_2O. The powder was further purified by reprecipitation three times from DMF with MeOH. The purification procedure, yield, physical constants and analytical data of protected hPT and its intermediates are listed in Tables II and III.

H–Gly–Leu–Pro–Lys–Glu–Val–Pro–Ala–Val–Leu–Thr–Lys–Gln–Lys–Leu–Lys–Ser–Glu–Leu–Val–Ala–Asn–Gly–Val–Thr–Leu–Pro–Ala–Gly–Glu–Met–Arg–Lys–Asp–Val–Tyr–Val–Glu–Leu–Tyr–Leu–Gln–His–Leu–Thr–Ala–Leu–His–OH (Corresponding to hTP)——The protected octatetracontapeptide ester (50 mg) was treated with 1 M TFMSA–thioanisole in TFA (3 ml) in the presence of *m*-cresol (100 μl) and Me_2Se (50 μl) in an ice-bath for 110 min, then peroxide-free ether was added. The resulting powder was collected by centrifugation, dried over KOH pellets for 2 h and dissolved in 1 N AcOH (5 ml). The solution, after being stirred with Amberlite IRA-400 (acetate form, approximately 1 g) for 30 min, was filtered. The pH of the filtrate was adjusted to pH 8.0 with 1 N NH_4OH and after 30 min to pH 6.0 with 1 N AcOH. The solution was incubated with dithiothreitol (60 mg) at 40°C for 10 h and then lyophilized. The product was purified by gel-filtration on Sephadex G-50 (3.6 × 90 cm) using 2% AcOH as an eluant. The fractions (4 ml each) corresponding to the front main peak (tube Nos. 71—78, determined by UV absorption measurement at 260 nm) were combined and the solvent was removed by lyophilization to give a fluffy powder. 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 × 14 cm), which was eluted first with H_2O (100 ml) and then with a linear gradient from H_2O (250 ml) to 0.15 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. Nos. 51—57) was collected and the solvent was removed by lyophilization. Analysis by TLC revealed the presence of two ninhydrin-positive spots with Rf^4 0.49 (main) and Rf^4 0.68 (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^4 0.49 was separated and extracted with 2% AcOH. The extract was concentrated to a small volume, applied to a Sephadex G-25 column (3.6 × 92 cm), and eluted with 3% AcOH. The single main fractions were combined and the solvent was removed by lyophilization to give a white fluffy powder. Yield 4.6 mg (13%), $[\alpha]_D^{21}$ −87.6° ($c=0.3$, 3%

AcOH), Rf^3 0.38, Rf^4 0.49, 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 2.84; mobility, 8.6 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 Vydac $5C_{18}$ column (4.6 × 250 mm) at a retention time of 18.35 min, when eluted with a gradient of acetonitrile (20—45% in 30 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: Gly 3.00, Ala 4.04, Leu 8.97, Val 5.89, Pro 2.85, Met 0.84, Tyr 2.03, Ser 0.87, Thr 2.90, Asp 2.08, Glu 5.87, His 1.86, Lys 4.98, Arg 0.88 (recovery of Gly 84%). Amino acid ratios in papain plus leucine aminopeptidase digest: Gly 3.00, Ala 4.02, Leu 9.03, Val 6.10, Pro 2.87, Met 0.86, Tyr 1.94, Ser 0.90, Thr 2.85, Asp 0.87, Glu 3.89, His 1.89, Lys 4.97, Arg 0.91; Asn and Gln were not determined (recovery of Gly 81%).

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 Hypaque-Ficoll gradient.[32] 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 the peptide in a humidified atmosphere of 5% CO_2 in air for 12 h and then PHA (0.125%) was added to each well and incubation was continued under the same conditions for 60 h. Lymphocytes in each well were transferred into a test tube and centrifuged for 10 min at 240 g, then the supernatant 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) 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.[29]

References and Notes

1) Amino acids and their derivatives used in this investigation were of the L-configuration except for glycine. The following abbreviations are used: DMF, dimethylformamide; DMSO, dimethylsulfoxide; WSCI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; HOBT, 1-hydroxybenzotriazole; Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl; NP, *p*-nitrophenyl; OBzl, benzyl ester; Bzl, benzyl; Troc, β,β,β-trichloroethoxycarbonyl; OcHex, cyclohexyl ester; Su, *N*-hydroxysuccinimide; NMM, *N*-methylmorpholine; EDTA, ethylenediaminetetraacetic acid; AcOH, acetic acid; EtOAc, ethyl acetate; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; Tos, *p*-toluenesulfonic acid; FCS, fetal calf serum; PHA, phytohemagglutinin; MeOH, methanol; CM, carboxymethyl; Mts, mesitylene-2-sulfonyl; E-rosette, a rosette with sheep erythrocytes; OSu, *N*-hydroxysuccinimide ester; RPMI, Rosewell Park Memorial Institute.
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