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Synthesis of Deacetyl-thymosin β_7 and Examination of Its Immunological Effect on the Blastogenic Response of T-Lymphocytes of a Uremic Patient with Cell-Mediated Immunodeficiency[1)]

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The untetracontapeptide corresponding to the entire amino acid sequence of deacetyl-thymosin β_7 was synthesized by assembling seven peptide fragments *via* the azide followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole in trifluoroacetic acid in the presence of dimethylselenide. The synthetic peptide was tested for effect on the impaired blastogenic response of phytohemagglutinin-stimulated T-lymphocytes of a uremic patient. The synthetic peptide was found to have restoring activity on the impaired blastogenic response of T-lymphocytes.

Keywords——deacetyl-thymosin β_7; deacetyl-thymosin β_9; trifluoromethanesulfonic acid deprotection; uremic patient; T-lymphocyte impaired blastogenic response; fluorometric blast-formation test

Hannappel *et al.*[2)] reported the isolation and sequencing of thymosins β_8 and β_9, homologous to thymosin β_4. Of the 41 amino acid residues in thymosin β_9, 32 are identical to corresponding residues in thymosin β_4. Several recent studies have demonstrated that thymosin β_4 is important for maturation and functioning of the immune system in man and animals.[3-5)] Previous to their report[2)] on thymosins β_8 and β_9, Low and Goldstein[6)] reported the isolation of thymosin β_7 from calf thymus. Their preliminary structural studies indicate that thymosin β_7 has an identical amino acid sequence to calf thymosin β_9.

Following our solution syntheses of calf thymosin β_9[7)] and deacetyl-thymosin β_4,[8)] we wish to report the solution synthesis of calf deacetyl-thymosin β_7, the structure of which possesses the same sequence as deacetyl-thymosin β_9.

In the preceding paper,[8)] we concluded that the acetyl group at the N-terminal Ser of thymosin β_4 is not required for increasing the activity of helper T-cells in cases of chronic renal failure. In the light of this result, we decided to synthesize deacetyl-thymosin β_7 by a solution method. We then examined its ability to restore *in vitro* the impaired response to PHA stimulation of lymphocytes from a uremic patient with cell-mediated immunodeficiency.

As compared with our previous syntheses of thymosins,[7-9)] several improvements have been made in the present synthesis. The thioanisole–mediated TFMSA deprotecting procedure[10,11)] was employed. Besides Lys(Z), Thr(Bzl), Ser(Bzl) and Lys(Z)–OBzl, two new amino acid derivatives bearing protecting groups removable by 1 M TFMSA–thioanisole in TFA were employed, *i.e.*, Glu(OcHex) and Asp(OcHex).[12)] Asp(OcHex) was employed to minimize aspartimide formation during the synthesis of Asp-containing peptide.[12)] Glu(OcHex) was also employed to suppress base-catalyzed pyrrolidone formation.[13)] These protecting groups survive mostly intact under careful TFA treatment for removal of the Boc group, employed as a temporary α-amino protecting group. As shown in Fig. 1, seven fragments were selected as building blocks to construct the entire peptide backbone of deacetyl-thymosin β_7 by the azide procedure.[14)] Of these, fragments, [2], [3], [4], and [7], are

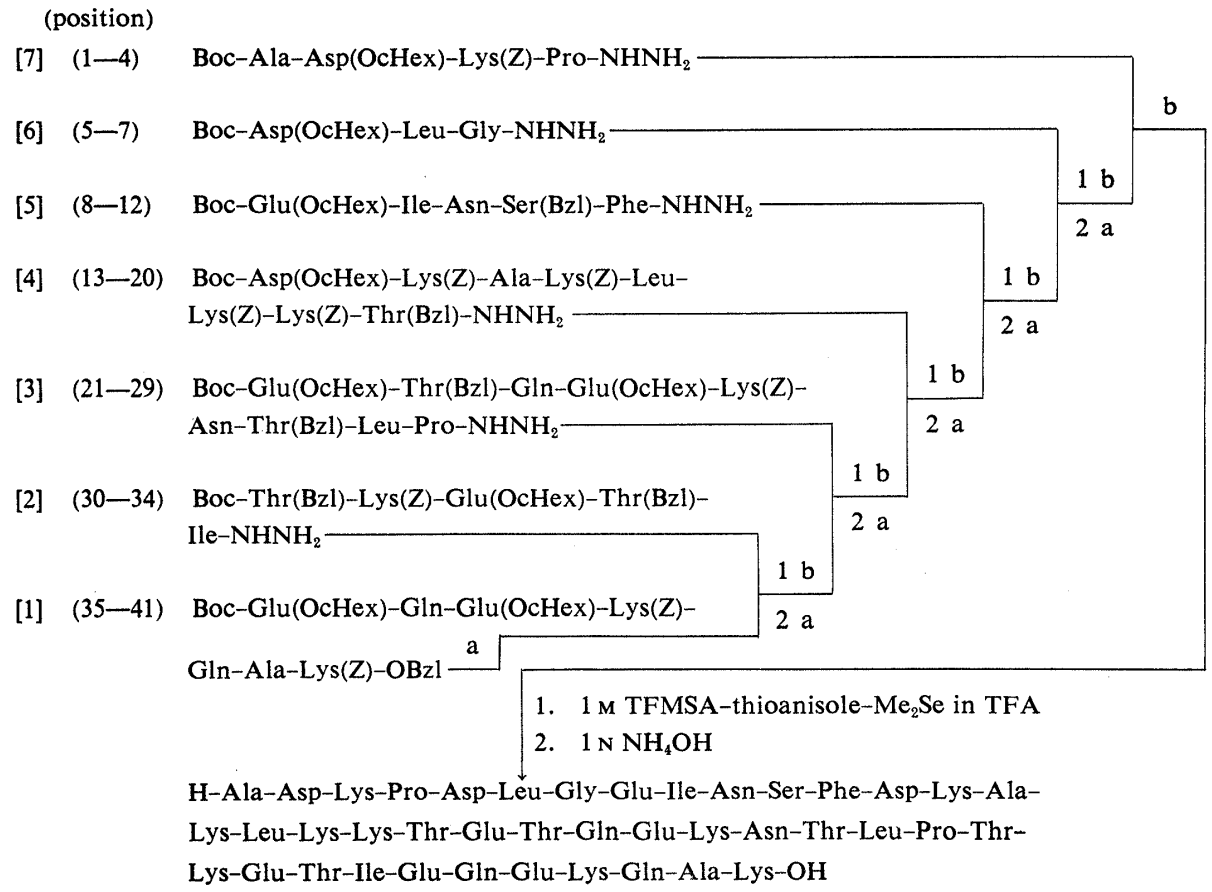

Fig. 1. Synthetic Route to Deacetyl-thymosin β_7

a, TFA–anisole; b, azide.

those employed for our previous synthesis of deacetyl-thymosin β_{10}.[15]

In the present synthesis, three fragments, [1], [5] and [6], which cover the areas of different amino acid residues in deacetyl-thymosin β_7, were newly synthesized. The procedure using azide[14] 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 TLC, amino acid analysis of acid hydrolysates and elemental 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–(35—41)–OBzl [1], was prepared in a stepwise manner starting with Boc–Lys(Z)–OBzl. The respective amino acid residues were introduced by an active ester procedure, *i.e.*, the Su method for Ala and Lys(Z) and the NP method for Gln. Glu (OcHex) residues were introduced by the HOBT–WSCI method.[16] For introduction of Gln and Asn residues, HOBT was employed as an accelerator.[16] Next, two fragments, Boc–(8—12)–NHNH–Troc and Boc–(5—7)–NHNH–Troc, were prepared stepwise from Boc–Ser(Bzl)-Phe–NHNH–Troc[15] and Boc–Leu–Gly–NHNH–Troc[17] by the HOBT–WSCI method[16] except for the introduction of the Asn residue. The Asn residue was introduced by the NP method. The two protected peptides, Boc–(8—12)–NHNH–Troc and Boc–(5—7)–NHNH–Troc, thus obtained were treated with Zn[18,19] in AcOH to remove the Troc groups, and the zinc acetate was removed by treatment with EDTA to give the desired hydrazides, Boc–(8—12)–$NHNH_2$ [5] and Boc–(5—7)–$NHNH_2$ [6], in analytically pure

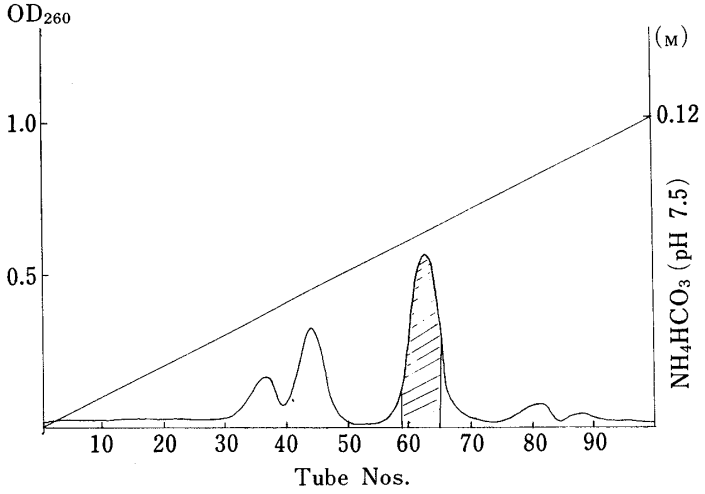

Fig. 2. Purification of Synthetic Deacetyl-thymosin β_7 by Ion-Exchange Chromatography on a DEAE-Sephadex A-25 Column

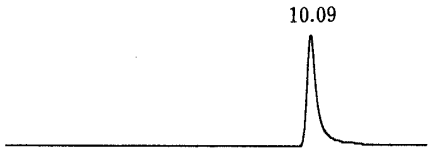

Fig. 3. HPLC of Synthetic Deacetyl-thymosin β_7

forms. The hydrazine test on the thin-layer chromatograms and elemental analysis data were consistent with homogeneity of the desired products.

Seven fragments thus obtained were assembled by the azide procedure according to the route 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.

The protected peptides obtained after condensation reactions of fragments [1] to [4] were purified by gel-filtration on Sephadex LH-60 using DMF or DMSO as an eluant and the rest of the products, including the protected deacetyl-thymosin β_7, by precipitation from DMF or DMSO with MeOH. Throughout this synthesis, Ile was selected as the diagnostic amino acid in acid hydrolysates. By comparison of the recovery of Ile with those of newly incorporated amino acids, satisfactory incorporation of each fragment in each condensation reaction was confirmed. The homogeneity of every product was further ascertained by elemental analysis and TLC.

In the final step of the synthesis, the protected untetracontapeptide benzyl ester was treated with 1 M TFMSA–thioanisole in TFA in the presence of Me_2Se in an ice-bath for 90 min to remove all the protecting groups employed. Me_2Se was used as an accelerator for the cleavage of protecting groups.[20] The deprotected peptide was next precipitated with dry 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.[21] The crude peptide was purified by gel-filtration on Sephadex G-25 and then ion-exchange column chromatography on a DEAE-Sephadex A-25 column with a linear gradient of pH 7.5 ammonium bicarbonate buffer (0→0.12 M). The main product was rechromatographed on the DEAE-Sephadex A-25 column as described above. After being desalted by repeated lyophilization, the product was further purified by column chromatography on cellulose powder using Walley's solvent system[22] as an eluant. The product thus obtained was then subjected to Sephadex G-25 column chromatography as described above. The product thus obtained gave a single spot on TLC in two different solvent systems and behaved as a single band on disk isoelectrofocusing (Pharmalyte, pH 3—10). Homogeneity of the synthetic deacetyl-thymosin β_7 was further ascertained by amino acid analysis, after 6 N HCl hydrolysis

TABLE I. Effect of the Synthetic Deacetyl-thymosin β_7 on the Impaired PHA-Stimulation of T-Lymphocytes of a Uremic Patient with Immunodeficiency

Peptides	Dose (μg/ml)	SI [a,b]
—[c]	—	266.1±47.2
—[d]	—	119.3±46.7[f]
Thymosin β_9[7,d,e]	10.0	234.6±42.5[g]
Deacetyl-thymosin β_7[d,e]	0.1	114.1±47.3[g]
Deacetyl-thymosin β_7[d,e]	1.0	189.5±41.5[g]
Deacetyl-thymosin β_7[d,e]	10.0	237.8±45.9[g]

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 peripheral lymphocytes. *d*) Patient's peripheral lymphocytes. *e*) Incubation was carried out for 12 h at 37 °C. *f*) Significantly different from normal persons at a *p* value of 0.05 or less. *g*) Significantly different from the uremic patient at a *p* value of 0.01 or less.

and enzymatic digestion. The peptide exhibited a single peak in analytical HPLC.

The immunological effect of the synthetic deacetyl-thymosin β_7 was examined by means of the JIMRO (Japan Immunoresearch Laboratories Co., Ltd.) fluorometric blast-formation test according to Itoh and Kawai.[23] In contrast to normal persons, the blastogenesis of the T-lymphocytes into lympholasts with mitotic activity after PHA stimulation is depressed in a uremic patient with symptoms of upper respiratory tract infection. The *in vitro* effect of the synthetic peptide on the impaired PHA response of T-lymphocytes from the uremic patient 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 patient investigated, the synthetic deacetyl-thymosin β_7 restored the blastogenesis to nearly normal values at a dose of 10 μg/ml. In the 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 deacetyl-thymosin β_7 has activity to restore the blastogenic transformation of T-lymphocytes into lymphoblasts with mitotic activity upon PHA stimulation in a uremic patient with cell-mediated immunodeficiency, and the acetyl group at the N-terminal Ala residue of thymosin β_7 is not required for the restorative activity on the impaired blastogenic response of PHA-stimulated T-lymphocytes of uremic patients with symptoms of upper respiratory tract infection.

Experimental

General experimental procedures used in this paper are essentially the same as described in the previous papers.[15,24] Azides were prepared according to Honzl and Rudinger[14] with isoamyl nitrite. Unless otherwise mentioned, products were 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.

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 $CHCl_3$–MeOH–H_2O (8:3:1). Rf^2 BuOH–pyridine–AcOH–H_2O (30:20:6:24).[22] Troc-$NHNH_2$ was purchased from Kokusan Chemical Works Ltd., Japan. Papain (No. P-3125) and leucine aminopeptidase (No. L-9876) were purchased from Sigma Chemical Co. Patient selection: A 69-year-old uremic patient (male) had suffered from recurrent episodes of fever due to symptoms of upper respiratory tract infection. Examination of cellular immunocompetence revealed a significant decrease in blast-formation by PHA. The PHA blast-formation was 9281 cpm (normal values: 33451—36924 cpm). Venous blood was obtained from the uremic patient 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 Ōyō-Bunko ULOG-FLOUSPEC 11A fluorometer. HPLC was conducted with a Shimadzu LC-3A apparatus coupled to a μBondapak C_{18} column.

Boc–Ala–Lys(Z)–OBzl (I)——Boc–Lys(Z)–OBzl (2.4 g) was treated with TFA–anisole (20 ml–4 ml) in an ice-bath for 40 min, 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 (1.4 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 ether. Yield 2.7 g (96%), mp 134—141 °C, $[\alpha]_D^{21}$ −16.3° (c=1.0, DMF), Rf^1 0.64, Rf^2 0.70, single ninhydrin-positive spot. *Anal.* Calcd for $C_{29}H_{39}N_3O_7$: C, 64.31; H, 7.26; N, 7.76. Found: C, 64.09; H, 7.51; N, 7.53.

Boc–Gln–Ala–Lys(Z)–OBzl (II)——I (2.2 g) was treated with TFA-anisole (15 ml–3 ml) as usual and the resulting powder was dissolved in DMF (12 ml) together with NMM (0.45 ml). HOBT (550 mg) and Boc–Gln–ONp (1.9 g) were added and the solution was stirred at room temperature for 7 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 DMF with H_2O. Yield 2.3 g (85%), mp 141—149 °C, $[\alpha]_D^{21}$ −10.3° (c=1.0, DMF), Rf^1 0.44, Rf^2 0.50, single ninhydrin-positive spot. *Anal.* Calcd for $C_{34}H_{47}N_5O_9 \cdot 2H_2O$: C, 57.86; H, 7.28; N, 9.92. Found: C, 57.73; H, 7.41; N, 9.57.

Boc–Lys(Z)–Gln–Ala–Lys(Z)–OBzl (III)——This compound was prepared essentially in the same manner as described for the preparation of I by using II (1.9 g) and Boc–Lys(Z)–OSu (1.3 g). The product was purified by procedure A, followed by recrystallization from EtOAc. Yield 2.2 g (82%), mp 146—157 °C, $[\alpha]_D^{21}$ −18.4° (c=1.0, DMF), Rf^1 0.59, Rf^2 0.68, single ninhydrin-positive spot. *Anal.* Calcd for $C_{48}H_{65}N_7O_{12} \cdot H_2O$: C, 60.68; H, 7.11; N, 10.32. Found: C, 60.47; H, 7.38; N, 10.20.

Boc–Glu(OcHex)–Lys(Z)–Gln–Ala–Lys(Z)–OBzl (IV)——III (1.9 g) was treated with TFA–anisole (15 ml–3 ml) as usual and the resulting powder was dissolved in DMF (16 ml) together with NMM (0.22 ml). To this ice-chilled solution, Boc–Glu(OcHex)–OH (723 mg), HOBT (297 mg) and WSCI (422 mg) were successively added. After having been stirred at 4 °C for 8 h, the product was purified by procedure B, followed by recrystallized from EtOAc. Yield 2.1 g (91%), mp 132—141 °C, $[\alpha]_D^{21}$ −7.9° (c=1.0, DMF), Rf^1 0.67, Rf^2 0.68, single ninhydrin-positive spot. *Anal.* Calcd for $C_{59}H_{82}N_8O_{15}$: C, 61.98; H, 7.23; N, 9.80. Found: C, 61.74; H, 7.50; N, 9.93.

Boc–Gln–Glu(OcHex)–Lys(Z)–Gln–Ala–Lys(Z)–OBzl (V)——This compound was prepared from IV (1.1 g), Boc–Gln–ONp (470 mg) and HOBT (140 mg) essentially as described for the preparation of II. The product was purified by procedure C, followed by recrystallization from EtOAc. Yield 983 mg (80%), mp 167—175 °C, $[\alpha]_D^{21}$ −7.2° (c=1.0, DMF), Rf^1 0.53, Rf^2 0.61, single ninhydrin-positive spot. *Anal.* Calcd for $C_{64}H_{90}N_{10}O_{17} \cdot 3H_2O$: C, 57.99; H, 7.30; N, 10.57. Found: C, 57.83; H, 7.49; N, 10.39.

Boc–Glu(OcHex)–Gln–Glu(OcHex)–Lys(Z)–Gln–Ala–Lys(Z)–OBzl [1]——This compound was prepared from V (636 mg), Boc–Glu(OcHex)–OH(181 mg), HOBT (74 mg) and WSCI (106 mg) as described for the preparation of IV. The product was purified by procedure B, followed by recrystallization from EtOAc. Yield 709 mg (96%), mp 172—185 °C, $[\alpha]_D^{21}$ −5.6° (c=1.0, DMF), Rf^1 0.64, Rf^2 0.59, single ninhydrin-positive spot. *Anal.* Calcd for $C_{75}H_{107}N_{11}O_{20}$: C, 60.75; H, 7.27; N, 10.39. Found: C, 60.44; H, 7.56; N, 10.45.

Boc–Asn–Ser(Bzl)–Phe–NHNH–Troc (VI)——This compound was prepared from Boc–Ser(Bzl)–Phe–NHNH–Troc[15] (1.1 g), Boc–Asn–ONp (660 mg) and HOBT (248 mg) essentially as described for the preparation of II. The product was purified by procedure C, followed by reprecipitation from DMF with H_2O. Yield 937 mg (72%), mp 100—105 °C, $[\alpha]_D^{21}$ −7.2° (c=1.0, DMF), Rf^1 0.60, Rf^2 0.62, single ninhydrin-positive spot. *Anal.* Calcd for $C_{31}H_{39}Cl_3N_6O_9$: C, 49.91; H, 5.27; N, 11.27. Found: C, 49.76; H, 5.48; N, 11.09.

Boc–Ile–Asn–Ser(Bzl)–Phe–NHNH–Troc (VII)——This compound was prepared from VI (746 mg), Boc–Ile–OH (260 mg), HOBT (149 mg) and WSCI (210 mg) as described for the preparation of IV. The product was purified by procedure B, followed by reprecipitation from MeOH with ether. Yield 713 mg (83%), mp 116—120 °C, $[\alpha]_D^{21}$ −8.6° (c=1.0, DMF), *Anal.* Calcd for $C_{37}H_{50}Cl_3N_7O_{10}$: C, 51.72; H, 5.87; N, 11.41. Found: C, 51.36; H, 5.99; N, 11.56.

Boc–Glu(OcHex)–Ile–Asn–Ser(Bzl)–Phe–NHNH–Troc (VIII)——This compound was prepared from VII

(614 mg), Boc–Glu (OcHex)–OH (258 mg), HOBT (106 mg) and WSCI (151 mg) as described for the preparation of IV. The product was purified by procedure B, followed by recrystallization from EtOAc. Yield 603 mg (78%), mp 124—131 °C, $[\alpha]_D^{21}$ −10.5° ($c=1.0$, DMF), Rf^1 0.58, Rf^2 0.59, single ninhydrin-positive spot. *Anal.* Calcd for $C_{48}H_{67}Cl_3N_8O_{13}\cdot H_2O$: C, 52.97; H, 6.39; N, 10.29. Found: C, 52.78; H, 6.54; N, 9.92.

Boc–Glu(OcHex)–Ile–Asn–Ser(Bzl)–Phe–NHNH$_2$ [5]——VIII (544 mg) in a mixture of AcOH (4 ml) and DMF (2 ml) was treated with Zn dust (300 mg) at 4 °C for 2 h and then at room temperature for 10 h. The solution was filtered, the filtrate was concentrated *in vacuo*, and the residue was treated with 3% EDTA to form a powder, which was washed with 5% $NaHCO_3$, H_2O and MeOH. The product was recrystallized from MeOH. Yield 431 mg (96%), mp 141—150 °C, $[\alpha]_D^{21}$ −16.4° ($C=1.0$ DMF), Rf^1 0.48, Rf^2 0.45, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{45}H_{66}N_8O_{11}$: C, 60.39; H, 7.43; N, 12.52. found: C, 60.28; H, 7.70; N, 12.65.

Boc–Asp(OcHex)–Leu–Gly–NHNH–Troc (IX)——This compound was prepared from Boc–Leu–Gly–NHNH–Troc[17] (1 g), Boc–Asp(OcHex)–OH (656 mg), HOBT (297 mg) and WSCI (422 mg) as described for the preparation of IV. The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane. Yield 948 mg (68%), mp 80—83 °C, $[\alpha]_D^{21}$ −6.2° ($c=1.0$, DMF), Rf^1 0.74, Rf^2 0.76, single ninhydrin-positive spot. *Anal.* Calcd for $C_{26}H_{44}Cl_3N_5O_{10}$: C, 45.06; H, 6.40; N, 10.11. Found: C, 45.01; H, 6.73; N, 9.86.

Boc–Asp(OcHex)–Leu–Gly–NHNH$_2$ [6]——IX (866 mg) in a mixture of AcOH (6 ml) and DMF (3 ml) was treated with Zn dust (409 mg) at 4 °C for 2 h and then at room temperature for 10 h. The solution was filtered, the filtrate was concentrated *in vacuo*, and the residue was treated with 3% EDTA to form a powder, which was washed with 5% $NaHCO_3$ and H_2O. The product was recrystallized from MeOH. Yield 512 mg (79%), mp 140—149 °C, $[\alpha]_D^{21}$ −14.9° ($c=1.0$, DMF), Rf^1 0.44, Rf^2 0.46, single hydrazine-test positive spot. *Anal.* Calcd for $C_{23}H_{43}N_5O_8$: C, 53.37; H, 8.37; N, 13.53. Found: C, 53.46; H, 8.69; N, 13.24.

Synthesis of the Protected Deacetyl-thymosin β_7——Successive azide condensations of the seven fragments were carried out according to the scheme in Fig. 2. Prior to condensation, the Boc group was removed from the amino component by TFA–anisole treatment as described in the general experimental procedure. A TFA-treated sample was precipitated with ether, dried over KOH pellets *in vacuo* for 2 h and dissolved in DMF–DMSO (1 : 1) except for the condensation reaction of fragment [2]. In this case, DMF alone was an adequate solvent. The solution was neutralized with NMM. The azide (1.5 to 4 eq according to the degree of chain elongation) in DMF or DMF–DMSO (1 : 1) and NMM (1 eq) were added to the above ice-chilled solution and the mixture was stirred at −10 °C for 32 h to 46 h until the solution became ninhydrin-negative. The mixture was neutralized by adding a few drops of AcOH and poured into ice-chilled 5% citric acid with stirring. The precipitate thereby formed was successively washed with 5% citric acid, H_2O and ether. The dried product was purified by one of the following two procedures. A: Precipitation from DMF or DMSO with MeOH. B: Gel-filtration on Sephadex LH-60 using DMF or DMSO as an eluant. In procedure B, eluates (5 ml fractions) were examined by measuring the ultraviolet (UV) absorption at 260 nm and the fractions corresponding to the front main peak were combined. The solvent was removed by evaporation and the residue was treated with ether to afford a powder. The purification procedure, yield, physical constants and analytical data of protected deacetyl-thymosin β_7 and its intermediates are listed in Tables II and III.

H–Ala–Asp–Lys–Pro–Asp–Leu–Gly–Glu–Ile–Asn–Ser–Phe–Asp–Lys–Ala–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–Thr–Leu–Pro–Thr–Lys–Glu–Thr–Ile–Glu–Gln–Glu–Lys–Gln–Ala–Lys–OH (Corresponding to Deacetyl-thymosin β_7)——The protected untetracontapeptide (50 mg) was treated with 1 M TFMSA–thioanisole in TFA (2 ml) in the presence of Me_2Se (50 μl) in an ice-bath for 90 min, then dry ether was added. The powder thus formed was dissolved in H_2O (5 ml). The solution was treated with Amberlite IRA-400 (acetate form, approximately 1.5 g) for 30 min, and filtered by suction. The filtrate was adjusted to pH 8.0 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 6.2 with a few drops of 1 N AcOH and the solution was lyophilized. The residue was dissolved in a small amount of 2% AcOH and then applied to a column of Sephadex G-25 (3 × 97 cm), which was eluted with the same solvent. Individual fractions (4 ml each) were collected and the absorbancy at 260 nm was determined for each fraction. The fractions corresponding to the front peak (tube Nos. 54—63) were combined and the solvent was removed by lyophilization. The residue was dissolved in H_2O (2 ml) and the solvent was applied to a column of DEAE-Sephadex A-25 (2.3 × 65 cm), and eluted with a linear gradient formed from 250 ml each of H_2O and 0.12 M NH_4HCO_3 buffer at pH 7.5. Individual fractions (5 ml each) were collected and the absorbancy at 260 nm was determined. A main peak present in the gradient eluates (tube Nos. 59—65) was collected. The solvent was evaporated off and the residue was rechromatographed on a DEAE-Sephadex A-25 column as described above. Next, the residue was dissolved in Walley's solvent system (1 ml) and the solution was applied to a column of cellulose (2.3 × 98 cm), which was eluted with the same solvent. Each fraction was examined by use of the ninhydrin test. The fractions which exhibited a ninhydrin-positive single spot (Rf^2 0.11) on TLC were combined and the solvent was evaporated off. The residue was dissolved in 2% AcOH. The solution was subjected to Sephadex G-25 column chromatography as described above. Yield 6.3 mg (22%), $[\alpha]_D^{21}$ −81.4 °C ($c=0.3$, 1% AcOH), Rf^1 0.04, Rf^2 0.11, single ninhydrin-positive spot. The synthetic peptide exhibited a single band in disk electrofocusing on 12.5% polyacrylamide gel (0.5 × 6.3 cm) containing Pharmalyte (pH 3—10): mobility, 1.9 cm from the origin toward the cathodic end of the gel, after running at 200 V for 4 h (stained with Coomassie Brilliant Blue G-250, Sigma). The synthetic peptide exhibited a single peak

TABLE II. Characterization of the Protected Deacetyl-thymosin β_7 and Its Intermediates

	Puri. proc. (Yield %)	mp (°C)	$[\alpha]_D^{21}$ ($c=1.0$, DMSO)	Formula	Analysis Calcd (Found) C	H	N	Rf[1]
Boc–(30—41)–OBzl	A (76)	174—187	−19.1	$C_{128}H_{179}N_{17}O_{31}\cdot 8H_2O$	59.22 (59.14)	7.57 (7.91)	9.17 (8.87)	0.64
Boc–(21—41)–OBzl	A (71)	184—193	−14.3	$C_{206}H_{289}N_{29}O_{50}\cdot 11H_2O$	59.34 (59.21)	7.52 (7.77)	9.74 (9.54)	0.66
Boc–(13—41)–OBzl	A (62)	190—201	−20.4	$C_{292}H_{405}N_{41}O_{69}\cdot 13H_2O$	60.18 (59.96)	7.46 (7.54)	9.85 (9.51)	0.61
Boc–(8—41)–OBzl	B (59)	201—213	−24.8	$C_{332}H_{459}N_{47}O_{78}\cdot 14H_2O$	60.34 (60.01)	7.43 (7.43)	9.96 (9.74)	0.50
Boc–(5—41)–OBzl	B (51)	234—248	−15.3	$C_{350}H_{488}N_{50}O_{83}\cdot 12H_2O$	60.57 (60.58)	7.44 (7.49)	10.09 (9.87)	0.65
Boc–(1—41)–OBzl	B (73)	231—246	−18.5	$C_{382}H_{533}N_{55}O_{91}\cdot 18H_2O$	59.77 (59.48)	7.47 (7.62)	10.04 (9.84)	0.59

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

TABLE III. Amino Acid Ratios in 6 N HCl Hydrolysates of the Protected Deacetyl-thymosin β_7 and Its Intermediates[a]

	Protected peptides 30—41	21—41	13—41	8—41	5—41	1—41	Residue
Ile	1.00	1.00	1.00	2.00	2.00	2.00	2
Leu		1.03	2.08	2.09	3.07	3.06	3
Gly					1.04	0.98	1
Pro		0.91	0.84	0.84	0.89	1.88	2
Ala	1.06	1.05	2.02	2.00	1.95	3.07	3
Phe				0.92	0.99	1.02	1
Thr	1.84	3.80	4.86	4.82	4.90	4.78	5
Ser				0.84	0.91	0.82	1
Glu	4.88	7.82	7.85	8.91	8.80	8.99	9
Asp		0.92	2.01	2.93	4.02	4.87	5
Lys	3.02	4.01	7.80	8.04	7.84	9.02	9

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

on HPLC using a μBondapak C_{18} column (0.39 × 30 cm) at a retention time of 10.09 min, when eluted with a gradient of acetonitrile (33 to 38% within 16 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: Ile 2.00, Leu 3.02, Gly 1.03, Pro 1.82, Ala 3.01, Phe 0.94, Thr 4.80, Ser 0.85, Glu 8.87, Asp 4.91, Lys 8.94 (recovery of Ile 81%). Amino acid ratios in papain plus leucine aminopeptidase digest: Ile 2.00, Leu 2.98, Gly 0.99, Pro 1.94, Ala 3.03, Phe 0.89, Thr 4.90, Ser 0.87, Glu 5.87, Asp 2.81, Lys 8.81; Asn and Gln were not determined (recovery of Ile 79%).

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.[25] Isolated lymphocytes were adjusted to 1.0×10^6/ml with PBS. The lymphocytes were cultured in 0.5 ml of RPMI 1640 (Gibco) with 10% FCS (Dainippon Pharmaceutical Co.) in microplates. Cultures of each combination were incubated at 37 °C in the presence of 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 removed. A 2 ml aliquot of 0.125% SDS was added to the residue and 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.[23)]

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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; OSu, *N*-hydroxysuccinimide ester; NMM, *N*-methylmorpholine; EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; AcOH, acetic acid; EtOAc, ethyl acetate; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; ONp, *p*-nitrophenyl ester; PBS, phosphate-buffered saline; RPMI, Rosewell Park Memorial Institute; FCS, fetal calf serum; PHA, phytohemagglutinin; SDS, sodium dodecyl sulfate.
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