Synthesis of a Thymosin β_4-Like Peptide, Deacetyl-thymosin β_4^{Xen}, and Its Restorative Effect on Depressed Lymphocyte Blastogenic Response to Phytohemagglutinin (PHA) in Uremic Patients[1)]

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An analog of thymosin β_4^{Xen} isolated from oocytes of *Xenopus laevis*, deacetyl-thymosin β_4^{Xen}, was synthesized by assembling 6 peptide fragments, followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole (molar ratio, 1 : 1) in trifluoroacetic acid in the presence of dimethylselenium. Finally, the deprotected peptide was incubated with dithiothreitol to reduce sulfoxide on the methionine side chain. The synthetic tritetracontapeptide was found to have a restoring effect on the impaired blastogenic response of T-lymphocytes isolated from uremic patients.

Keywords deacetyl-thymosin β_4^{Xen} synthesis; trifluoromethanesulfonic acid deprotection; dithiothreitol reduction; uremic patient; impaired T-lymphocyte blastogenic response; restoring effect; fluorometric blast-formation test

The uremic state causes T-cell immune impairment in patients with chronic renal failure,[2,3)] reflected by a decreased response to the T-cell mitogen phytohemagglutinin (PHA). We and others have reported evidence of impaired immune function in patients with uremia.[4–8)] This impairment is reflected in both *in vitro* and *in vivo* depressed cell-mediated immune function.

On the other hand, the amino acid sequence of thymosin β_4, a polypeptide isolated from calf thymus, was determined.[9)] Thymosin β_4 is one of several components present in thymosin fraction 5 that participate in the regulation, differentiation and function of thymus-dependent thymocytes. The amino acid sequences of thymosins β_8 and β_9, which were also isolated from calf thymus by Hannappel *et al.*, were found to be homologous to thymosin β_4.[10)]

In the previous papers,[4,11,12)] we reported syntheses of deacetyl-thymosin β_4, thymosin β_8 and thymosin β_9, and showed that these synthetic thymus peptides could have restoring effects on the impaired cell-mediated immunological functions.

In 1988, a new thymosin β_4-like peptide, thymosin β_4^{Xen}, was isolated from oocytes of *Xenopus laevis*.[13)] Thymosin β_4 and thymosin β_4^{Xen} differ in the amino acid residues at positions 15, 40 and 41. At position 15 Ser is replaced by Ala and at 41—42 the sequence is Thr–Ser instead of Ala–Gly.

Following our solution syntheses of deacetyl-thymosin β_4,[4)] and thymosins β_8[11)] and β_9,[12)] we wish to report the solution synthesis of deacetyl-thymosin β_4^{Xen} and the *in vitro* effect of this peptide on the impaired blastogenic response of T-lymphocytes of uremic patients. Since the acetyl group at the N-terminal Ser residue of thymosin β_4 is not required for the expression of immunological activity in cases of chronic renal failure,[4)] we chose to synthesize deacetyl-thymosin β_4^{Xen}.

From the synthetic viewpoint, compared with our previous syntheses of deacetyl-thymosin β_4,[4)] thymosins β_8[11)] and β_9,[12)] the thioanisole-mediated trifluoromethanesulfonic acid (TFMSA) deprotecting procedure[14,15)] was applied in the final step of the present synthesis instead of hydrogen fluoride.

Our synthetic route to deacetyl-thymosin β_4^{Xen} is illustrated in Fig. 1, which shows six fragments selected as building blocks to construct the entire amino acid sequence of deacetyl-thymosin β_4^{Xen}. Of these, four fragments, [2], [3], [5] and [6], are those employed for our previous synthesis of deacetyl-thymosin β_4.[4)] Thus, two fragments, [1] and [4], which cover the areas of sequence variation between thymosin β_4 and thymosin β_4^{Xen}, were newly synthesized. The Boc group, removable by trifluoroacetic acid (TFA), was adopted as a temporary N^{α}-protecting group for every intermediate. Amino acid derivatives bearing protecting groups removable by 1 M TFMSA–thioanisole in TFA[14,15)] were employed, *i.e.*, Ser(Bzl), Thr(Bzl), Lys(Z), Glu(OBzl), Asp(OBzl) and Ser(Bzl)–OBzl. The Met residue was reversibly protected as its sulfoxide[16)] in order to prevent partial S-alkylation during the N^{α}-TFA deprotection as well as partial air oxidation during the synthesis. The substituted hydrazine, Troc–$NHNH_2$,[17)] was employed for the preparation of five fragments, [2], [3], [4], [5] and [6], containing the Glu(OBzl) or the Asp(OBzl) residue. This Troc group is known to be cleaved by Zn[18)] in AcOH without affecting other functional groups.

Throughout the synthesis of these fragments and intermediates, 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 decapeptide, Boc–(34—43)–OBzl [1], was prepared stepwise starting from Boc–Glu(OBzl)–Ser(Bzl)–OBzl[4)] by the Su active ester procedure[19)] except for the introduction of the Gln residue, which was introduced by the NP active ester procedure.[20)] Next, the protected octapeptide hydrazide, Boc–(13—20)–$NHNH_2$ [4], was prepared stepwise starting from Boc–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–NHNH–Troc[4)] by the Su active ester procedure to yield Boc–Asp(OBzl)–Lys(Z)–Ala–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–NHNH–Troc [X]. The protected octapeptide thus obtained was treated with Zn[17,18)] in AcOH to remove the Troc group, and the zinc acetate was removed by treatment with EDTA to give the required hydrazide, Boc–(13—20)–$NHNH_2$ [4], in analytically pure form. The hydrazine test on the thin-layer chromatogram and the elemental analysis data were consistent with homogeneity of the desired product.

The six fragments were successively condensed by the azide procedure[21)] according to the route shown in Fig. 1. Every reaction was carried out in a mixture of DMF and DMSO and the amount of the acyl component was in-

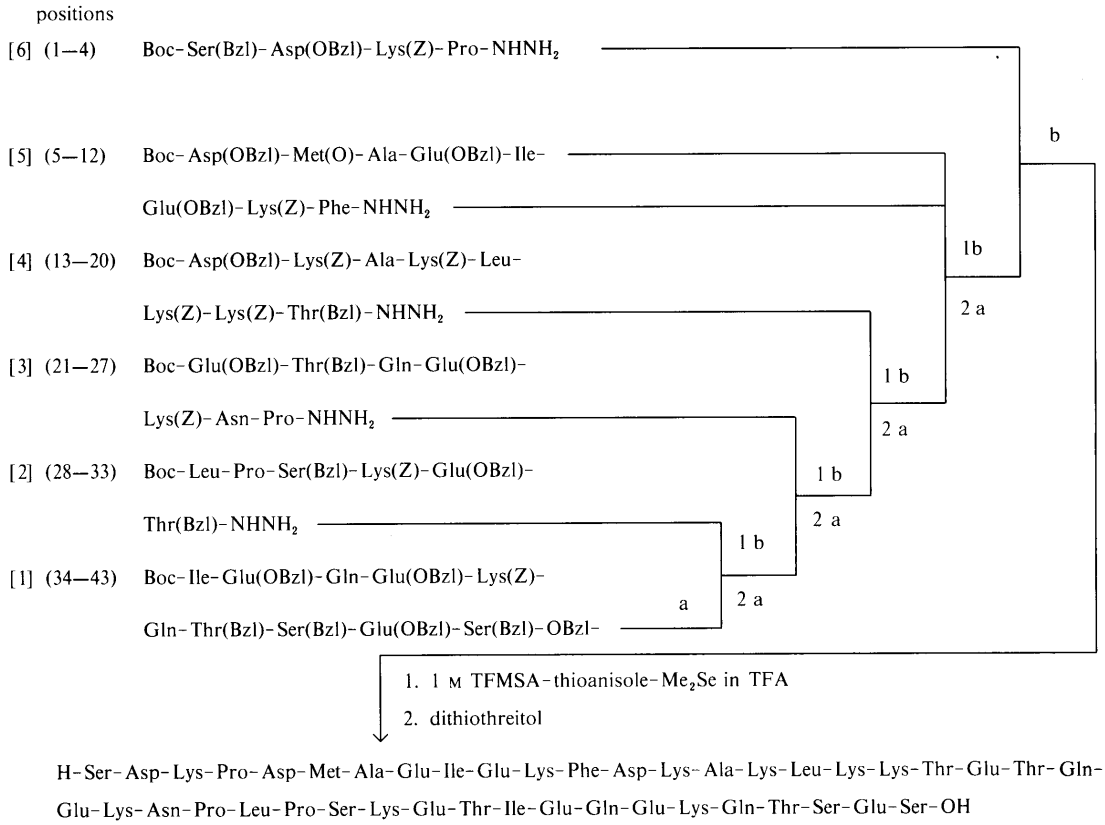

Fig. 1. Synthetic Route to Deacetyl-thymosin β_4^{Xen}
a, TFA-anisole; b, azide.

creased from 2 to 4eq as the chain elongation progressed. Every product was purified either by precipitation from DMF or DMSO with MeOH or by gel-filtration on Sephadex LH-60. Throughout the synthesis, Ile was used as a diagnostic amino acid in acid hydrolysis (Table III). By comparison of recovery of Ile with those of newly incorporated amino acids, satisfactory incorporation of each fragment was asscertained.

Starting with the C-terminal decapeptide ester corresponding to positions 34 to 43 of deacetyl-thymosin β_4^{Xen}, Boc-(34—43)-OBzl, five fragments, Boc-(28—33)-$NHNH_2$, Boc-(21—27)-$NHNH_2$, Boc-(13—20)-$NHNH_2$, Boc-(5—12)-$NHNH_2$ and Boc-(1—4)-$NHNH_2$, were successively condensed by the azide procedure[21] as shown in Fig. 1 to give the protected tritetracontapeptide corresponding to the entire amino acid sequence of deacetyl-thymosin β_4^{Xen}. The homogeneity of the peptide was checked by elemental analysis, TLC and amino acid analysis of the acid hydrolysate.

In the final step of the synthesis, the protected tritetracontapeptide ester was treated with 1 M TFMSA–thioanisole in TFA in the presence of Me_2Se. Me_2Se was employed to facilitate acidic cleavage of protecting groups.[22] 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 at pH 8.0 to reverse a possible N→O shift[23] at the Ser and Thr residues. The Met(O) residue was reduced back to Met in two steps, firstly with thioanisole and Me_2Se[22] during the above acid treatment,

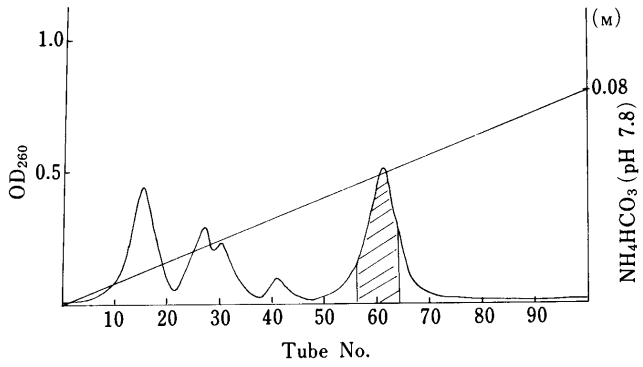

Fig. 2. Purification of Synthetic Deacetyl-thymosin β_4^{Xen} by Ion-Exchange Chromatography on a DEAE-Cellulose Column

and secondly with dithiothreitol during incubation of the deprotected peptide.

The reduced product was purified by gel-filtration on Sephadex G-25, followed by ion-exchange column chromatography on a diethylaminoethyl (DEAE)-cellulose column with linear gradient elution using pH 7.8 NH_4HCO_3 buffer, followed by preparative TLC. Desalting on a Sephadex G-25 column gave a fluffy powder, which exhibited a single spot (ninhydrin- and chlorine-tolidine-positive) on TLC in two different solvent systems and on paper electrophoresis (pH 7.2 pyridinium–acetate buffer). Its purity was further confirmed by amino acid analysis after acid hydrolysis and enzymatic digestion.

This peptide is slightly sensitive to air-oxidation. The

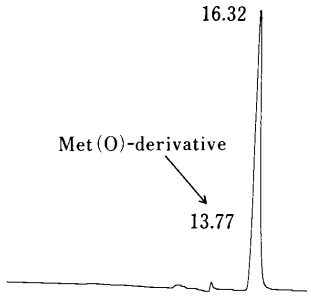

Fig. 3. HPLC of Synthetic Deacetyl-thymosin β_4^{Xen}

TABLE I. Effect of the Synthetic Deacetyl-thymosin β_4^{Xen} on the Impaired PHA-Stimulation of T-Lymphocytes of Uremic Patients

Peptides	Dose (μg/ml)	$SI^{a,b)}$
—[c)]	—	278.1±51.6
—[d)]	—	107.2±53.2[g)]
H-Leu-Gly-Gly-OH[d-f)]	10.0	104.3±54.3
Deacetyl-thymosin β_4^{Xen} [d,f)]	1.0	181.3±52.1[h)]
Deacetyl-thymosin β_4^{Xen} [d,f)]	10.0	228.4±56.5[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 ethidium 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 the normal persons by using Student's *t* test. *h*) $p<0.02$, when compared to the uremic patients by using Student's *t* test.

purity of the product as estimated by HPLC was about 99%. The minor impurity (approximately 1%) seemed to be the Met(O)-derivative, since the main product was converted to this minor component by excess H_2O_2 treatment.

The immunological effect of the synthetic deacetyl-thymosin β_4^{Xen} was examined by means of the JIMRO (Japan Immunoresearch Laboratories Co., Ltd.) fluorometric blast-formation test according to Itoh and Kawai.[24] Responses of T-lymphocytes to mitogenic stimulation were lower in uremic patients than those of normal persons. 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 stimulation index (*SI*) values of the blastogenic transformation of T-lymphocytes into lymphoblasts with mitotic activity upon PHA stimulation shows that, in the case of the uremic patients investigated, the synthetic deacetyl-thymosin β_4^{Xen} exhibited a restoring effect at a dose of 1 μg/ml. In the case of normal subjects, *in vitro* addition of this peptide had no effect on the mitotic activity induced by PHA stimulation under the same conditions (data not shown).

In the preceding papers,[6,25] we reported that the tricosapeptide fragment corresponding to amino acids 16 to 38 of calf thymosin β_4, which contains two immunologically active sites, -Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-(16—26) and -Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys- (31—38), within its molecule, has restoring activity on the impaired blastogenic response of PHA-stimulated T-lymphocytes of uremic patients. The tricosapeptide moiety corresponding to amino acids 16—38 of calf thymosin β_4, which was found to be an important moiety in calf thymosin β_4 for restorative activity on impaired immunological deficiency, is conserved in the molecule of thymosin β_4^{Xen}. These results seem to suggest that deacetyl-thymosin β_4^{Xen} treatment *in vitro* restores immune competence of T-lymphocytes immunosuppressed as a result of uremia.

Experimental

General experimental procedures used were essentially the same as described in the previous papers.[4,6] Azides were prepared according to Honzl and Rudinger[21] 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 type 835-50 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^1 values refer to the following solvent systems: $CHCl_3$-MeOH-H_2O (8:3:1). The final product corresponding to the entire amino acid sequence of deacetyl-thymosin β_4^{Xen} was chromatographed on a cellulose plate (Merck). Rf^2 value refers to BuOH-AcOH-H_2O (4:1:1) and Rf^3 value refers to BuOH-pyridine-AcOH-H_2O (30:20:6:24).[26] 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: Two uremic patients who were suffering from recurrent infectious diseases were selected. Examination of the cellular immunocompetence of these patients revealed a significant decrease in blast-formation by PHA. [^{3}H]Thymidine incorporation values of these patients were 11692 and 10503 cpm, respectively (normal values: 37934—39458 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 11A fluorometer. HPLC was conducted with a Shimadzu LC-3A apparatus equipped with a Nucleosil $5C_{18}$ column.

Boc-Ser(Bzl)-Glu(OBzl)-Ser(Bzl)-OBzl [I] Boc-Glu(OBzl)-Ser(Bzl)-OBzl[4] (3 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 (15 ml) containing NMM (0.6 ml). To this solution, Boc-Ser(Bzl)-OSu (2 g) was added, and the mixture was stirred at room temperature for 7 h. The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane. Yield 3.3 g (81%), mp 89—94 °C, $[\alpha]_D^{21}$ −12.1° (c=1.0, DMF), Rf^1 0.64, single ninhydrin-positive spot. *Anal.* Calcd for $C_{44}H_{51}N_3O_{10}\cdot 2H_2O$: C, 64.61; H, 6.78; N, 5.14. Found: C, 64.38; H, 6.92; N, 5.33.

Boc-Thr(Bzl)-Ser(Bzl)-Glu(OBzl)-Ser(Bzl)-OBzl [II] This compound was prepared essentially in the same manner as described for the preparation of I by using I (2.7 g) and Boc-Thr(Bzl)-OSu (1.4 g). The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane. Yield 2.5 g (78%), mp 92—100 °C, $[\alpha]_D^{21}$ −16.5° (c=1.0, DMF), Rf^1 0.69, single ninhydrin-positive spot. *Anal.* Calcd for

TABLE II. Characterization of the Protected Deacetyl-thymosin β_4^{Xen} and Its Intermediates

	Puri. proc. (Yield %)	mp (°C)	Rf^1	$[\alpha]_D^{21}$ ($c=1.0$, DMSO)	Formula	Analysis (%) Calcd C	H	N	Found C	H	N
Boc–(28—43)–OBzl	A (84)	172—183	0.71	−15.4	$C_{166}H_{208}N_{20}O_{38}\cdot 10H_2O$	60.94	7.03	8.56	60.69	7.36	8.67
Boc–(21—43)–OBzl	B (71)	160—171	0.63	−33.8	$C_{229}H_{286}N_{30}O_{54}\cdot 11H_2O$	60.84	6.87	9.29	60.59	7.05	9.43
Boc–(13—43)–OBzl	B (68)	190—199	0.58	−23.7	$C_{316}H_{398}N_{42}O_{73}\cdot 13H_2O$	61.35	6.91	9.51	61.24	7.18	9.28
Boc–(5—43)–OBzl	B (55)	179—188	0.68	−20.3	$C_{388}H_{487}N_{51}O_{90}S\cdot 15H_2O$	61.26	6.59	9.39	60.94	6.83	9.44
Boc–(1—43)–OBzl	A (64)	192—201	0.62	−26.6	$C_{428}H_{534}N_{56}O_{99}S\cdot 18H_2O$	61.17	6.84	9.33	60.89	7.09	9.46

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

TABLE III. Amino Acid Ratios in 6 N HCl Hydrolysates of the Protected Deacetyl-thymosin β_4^{Xen} and Its Intermediates[a]

	Protected peptides 28—43	21—43	13—43	5—43	1—43	Residue
Ile	1.00	1.00	2.00	2.00	2.00	2
Leu	1.03	1.02	1.98	2.05	2.01	2
Ala			1.02	2.01	2.03	2
Met				0.85	0.84	1[b]
Phe				0.94	0.95	1
Pro	0.91	1.87	1.90	1.88	2.89	3
Ser	2.84	2.86	2.84	2.85	3.84	4
Thr	1.86	2.89	3.88	3.87	3.87	4
Glu	5.93	8.81	9.07	10.91	10.94	11
Asp		0.95	1.87	3.02	3.89	4
Lys	2.02	2.91	6.92	7.93	8.92	9

a) The results are expressed as ratios to the value for Ile, which was taken as the diagnostic amino acid in acid hydrolysates. *b*) Met + Met(O).

$C_{55}H_{64}N_4O_{12}$: C, 67.88; H, 6.63; N, 5.76. Found: C, 67.52; H, 6.89; N, 5.89.

Boc–Gln–Thr(Bzl)–Ser(Bzl)–Glu(OBzl)–Ser(Bzl)–OBzl [III] II (2.2 g) was treated with TFA–anisole (12 ml–2.4 ml) as usual and the resulting powder was dissolved in DMF (15 ml) together with NMM (0.27 ml). Boc–Gln–ONp (1 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 2 g (80%), mp 143—149 °C, $[\alpha]_D^{21}$ −7.4° ($c=1.0$, DMF), Rf^1 0.52, single ninhydrin-positive spot. *Anal.* Calcd for $C_{60}H_{72}N_6O_{14}\cdot H_2O$: C, 64.39; H, 6.66; N, 7.51. Found: C, 64.20; H, 6.74; N, 7.33.

Boc–Lys(Z)–Gln–Thr(Bzl)–Ser(Bzl)–Glu(OBzl)–Ser(Bzl)–OBzl [IV] This compound was prepared essentially in the same manner as described for the preparation of I by using III (1.1 g) and Boc–Lys(Z)–OSu (526 mg). The product was purified by procedure A, followed by reprecipitation from EtOAc with ether. Yield 1.1 g (79%), mp 129—137 °C, $[\alpha]_D^{21}$ −17.2° ($c=1.0$, DMF), Rf^1 0.66, single ninhydrin-positive spot. *Anal.* Calcd for $C_{74}H_{90}N_8O_{17}\cdot 3H_2O$: C, 62.70; H, 6.83; N, 7.90. Found: C, 62.47; H, 6.91; N, 10.04.

Boc–Glu(OBzl)–Lys(Z)–Gln–Thr(Bzl)–Ser(Bzl)–Glu(OBzl)–Ser(Bzl)–OBzl [V] This compound was prepared essentially in the same manner as described for the preparation of I by using IV (1 g) and Boc–Glu(OBzl)–OSu (341 mg). The product was purified by procedure B, followed by reprecipitation from MeOH with ether. Yield 1 g (88%), mp 149—156 °C, $[\alpha]_D^{21}$ −12.1° ($c=1.0$, DMF), Rf^1 0.68, single ninhydrin-positive spot. *Anal.* Calcd for $C_{86}H_{103}N_9O_{20}\cdot 2H_2O$: C, 63.81; H, 6.66; N, 7.79. Found: C, 63.72; H, 6.85; N, 7.48.

Boc–Gln–Glu(OBzl)–Lys(Z)–Gln–Thr(Bzl)–Ser(Bzl)–Glu(OBzl)–Ser(Bzl)–OBzl [VI] This compound was prepared from V (900 mg) and Boc–Gln–ONp (261 mg) essentially as described for the preparation of III. The product was purified by procedure C, followed by reprecipitation from AcOH with H_2O. Yield 829 mg (84%), mp 142—151 °C, $[\alpha]_D^{21}$ −10.9° ($c=1.0$, DMF), Rf^1 0.53, single ninhydrin-positive spot. *Anal.* Calcd for $C_{91}H_{111}N_{11}O_{22}\cdot 4H_2O$: C, 61.30; H, 6.73; N, 8.64. Found: C, 61.24; H, 6.95; N, 8.42.

Boc–Glu(OBzl)–Gln–Glu(OBzl)–Lys(Z)–Gln–Thr(Bzl)–Ser(Bzl)–Glu(OBzl)–Ser(Bzl)–OBzl [VII] This compound was prepared from VI (713 mg) and Boc–Glu(OBzl)–OSu (191 mg) essentially as described for the preparation of I. The product was purified by procedure B, followed by recrystallization from MeOH with ether. Yield 706 mg (87%), mp 146—153 °C, $[\alpha]_D^{21}$ −13.6° ($c=1.0$, DMF), Rf^1 0.69, single ninhydrin-positive spot. *Anal.* Calcd for $C_{103}H_{124}N_{12}O_{25}\cdot 6H_2O$: C, 60.70; H, 6.73; N, 8.25. Found: C, 60.38; H, 6.84; N, 8.49.

Boc–Ile–Glu(OBzl)–Gln–Glu(OBzl)–Lys(Z)–Gln–Thr(Bzl)–Ser(Bzl)–Glu(OBzl)–Ser(Bzl)–OBzl [1] This compound was prepared from VII (679 mg) and Boc–Ile–OSu (121 mg) essentially as described for the preparation of I. The product was purified by procedure B, followed by recrystallization from hot EtOAc. Yield 605 mg (85%), mp 147—156 °C, $[\alpha]_D^{21}$ −7.3° ($c=1.0$, DMF), Rf^1 0.65, single ninhydrin-positive spot. *Anal.* Calcd for $C_{109}H_{135}N_{13}O_{26}\cdot 5H_2O$: C, 61.37; H, 6.85; N, 8.54. Found: C, 61.08; H, 7.11; N, 8.48.

Boc–Ala–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–NHNH–Troc [VIII] This compound was prepared from Boc–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–NHNH–Troc[4] (1.4 g) and Boc–Ala–OSu (315 mg) essentially as described for the preparation of I. The product was purified by procedure A, followed by reprecipitation from EtOAc with ether. Yield 1.2 g (80%), mp 94—100 °C, $[\alpha]_D^{21}$ −5.9° ($c=1.0$, DMF), Rf^1 0.66, single ninhydrin-positive spot. *Anal.* Calcd for $C_{70}H_{96}Cl_3N_{11}O_{17}$: C, 57.20; H, 6.58; N, 10.48. Found: C, 56.84; H, 6.76; N, 10.71.

Boc–Lys(Z)–Ala–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–NHNH–Troc [IX] This compound was prepared essentially in the same manner as described for the preparation of I by using VIII (1.1 g) and Boc–Lys(Z)–OSu (375 mg). The product was purified by procedure A, followed by reprecipitation from MeOH with ether. Yield 1.1 g (85%), mp 108—115 °C, $[\alpha]_D^{21}$ −11.8° ($c=1.0$, DMF), Rf^1 0.70, single ninhydrin-positive spot. *Anal.* Calcd for $C_{84}H_{114}Cl_3N_{13}O_{20}\cdot 2H_2O$: C, 57.06; H, 6.73; N, 10.30. Found: C, 56.78; H, 6.95; N, 10.14.

Boc–Asp(OBzl)–Lys(Z)–Ala–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–NHNH–Troc [X] This compound was prepared from IX (1 g) and Boc–Asp(OBzl)–OSu (272 mg) essentially as described for the preparation of I. The product was purified by procedure A, followed by reprecipitation from EtOAc with ether. Yield 930 mg (83%), mp 113—119 °C, $[\alpha]_D^{21}$ −7.9° ($c=1.0$, DMF), Rf^1 0.61, single ninhydrin-positive spot. *Anal.* Calcd for $C_{95}H_{125}Cl_3N_{14}O_{23}\cdot 3H_2O$: C, 57.30; H, 6.63; N, 9.85. Found: C, 57.16; H, 6.90; N, 9.82.

Boc–Asp(OBzl)–Lys(Z)–Ala–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–$NHNH_2$ [4] X (797 mg) in a mixture of AcOH (4 ml) and DMF (4 ml) was treated with Zn dust (261 mg) at 4 °C for 12 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 631 mg (86%), mp 169—177 °C, $[\alpha]_D^{21}$ −18.3° ($c=1.0$, DMF), Rf^1 0.56, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{92}H_{124}N_{14}O_{21}\cdot 4H_2O$: C, 60.25; H, 7.25; N, 10.69. Found: C, 60.04; H, 7.46; N, 10.33.

Synthesis of Protected Deacetyl-thymosin β_4^{Xen} Successive azide condensations of six fragments were carried out according to Fig. 1. 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–DMSO (1 : 1) or DMF–DMSO (1 : 2) containing NMM (1.1 eq). The corresponding azide (the amount was increased from 2 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 become negative to the ninhydrin test. 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 MeOH. The dried product was purified by one of the following 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_4^{Xen} and its intermediates are listed in Tables II and III.

H-Ser-Asp-Lys-Pro-Asp-Met-Ala-Glu-Ile-Glu-Lys-Phe-Asp-Lys-Ala-Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Pro-Leu-Pro-Ser-Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-Thr-Ser-Glu-Ser-OH (Corresponding to Deacetyl-thymosin β_4^{Xen}) The protected tritetracontapeptide (50 mg) was treated with 1 M TFMSA–thioanisole in TFA (2 ml) in the presence of Me_2Se (60 μl) in an ice-bath for 110 min, then peroxide-free dry 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 (30 mg) at 40 °C for 12 h and then lyophilized. The product was purified by gel-filtration on Sephadex G-25 (3.6 × 93 cm) using 2% AcOH as an eluant. The fractions (5 ml each) corresponding to the front main peak (tube Nos. 54—66, determined by UV absorption measurement at 260 nm) were combined and the solvent was removed by lyophilization to give a fluffy powder. The product was dissolved in H_2O (3 ml) and the solution was applied to a column of DEAE-celulose (Brown, 2.3 × 10.3 cm), which was eluted with a linear gradient of 300 ml each of H_2O–0.08 M NH_4HCO_3 buffer at pH 7.8. Individual fractions (5 ml each) were collected and the absorbancy at 260 nm was determined. Main peak fractions of the gradient eluates (tube Nos. 56—65) were combined and the solvent was evaporated *in vacuo*. Analysis by TLC revealed the presence of two ninhydrin-positive spots with Rf^3 0.08 (main) and 0.26 (minor). The crude product was dissolved in a small amount of water and subjected to preparative TLC (cellulose pate, 20 × 40 cm) using BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24) as a developing solvent. The zone corresponding to Rf^3 0.08 was separated and extracted with 2% AcOH. The extracts were concentrated to a small volume, applied to a Sephadex G-25 column (3.6 × 93 cm), and eluted with 2% AcOH as described above and the solvent was removed by lyophilization. Yield 7.4 mg (25%), $[\alpha]_D^{21}$ −72.8° (c = 0.3, 2% AcOH), Rf^2 0.02, Rf^3 0.08, single ninhydrin- and chlorine–tolidine-positive spot. The synthetic peptide exhibited a single spot on paper electrophoresis: Toyo Roshi No. 51 (2 × 40 cm), pyridinium–acetate buffer at pH 7.2, mobility 1.5 cm from the origin toward the anode after running at 2 mA, 600 V for 75 min. Amino acid ratios in a 6 N HCl hydrolysate: Ile 2.00, Leu 2.03, Ala 2.04, Met 0.89, Phe 0.98, Pro 2.87, Ser 3.84, Thr 3.86, Glu 10.89, Asp 3.97, Lys 8.91 (recovery of Ile 83%). Amino acid ratios in papain plus leucine aminopeptidase digest: Ile 2.00, Leu 2.01, Ala 1.98, Met 0.92, Phe 0.96, Pro 2.84, Ser 3.90, Thr 3.91, Glu 7.83, Asp 1.86, Lys 8.98; Gln and Asn were not determined (recovery of Ile 84%). The synthetic peptide exhibited two peaks on HPLC using an analytical Nucleosil $5C_{18}$ column (4 × 150 mm) at retention times of 13.77 min (1%) and 16.32 min (99%), when eluted with a gradient of acetonitrile (20 to 45%) in 0.1% TFA at a flow rate of 1 ml per min (Fig. 3).

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.[27] 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 PHA (0.125%, 0.5 ml) was added to each well. Incubation was continued under the same conditions for 60 h. T-Lymphocytes in each well were transferred into a test tube and centrifuged for 10 min at 240 *g*, then the supernatant was removed. A 2 ml aliquot of 0.125% SDS was added to the residue and stirred for 20 min at room temperature; 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.[24]

References and Notes

1) Amino acids and their derivatives used in this investigation were of the L-configuration. The following abbreviations are used: DMF, dimethylformamide; DMSO, dimethyl sulfoxide; Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl; NP, *p*-nitrophenyl; ONp, *p*-nitrophenyl ester; OBzl, benzyl ester; Bzl, benzyl; Troc, β,β,β-trichloroethoxycarbonyl; Su, *N*-hydroxysuccinimide; NMM, *N*-methylmorpholine; OSu, *N*-hydroxysuccinimide ester; EDTA, ethylenediaminetetraacetic acid; AcOH, acetic acid; EtOAc, ethyl acetate; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; FCS, fetal calf serum; MeOH, methanol; RPMI, Rosewell Park Memorial Institute; SDS, sodium dodecyl sulfate.
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