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Deacetyl-thymosin β_4: Synthesis and Effect on the Impaired Peripheral T-Cell Subsets in Patients with Chronic Renal Failure[1)]

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Deacetyl-thymosin β_4 was synthesized by successive azide condensations of five peptide fragments, Boc-(1—4)-$NHNH_2$, Boc-(5—12)-$NHNH_2$, Boc-(13—20)-$NHNH_2$, Boc-(21—27)-$NHNH_2$ and Boc-(28—33)-$NHNH_2$, with H-(34—43)-OBzl, followed by deprotection with hydrogen fluoride in the presence of anisole and thioanisole. Finally, the deprotected peptide was incubated with dithiothreitol to reduce sulfoxide on the methionine side chain. The synthetic deacetyl-thymosin β_4 increased almost the entire peripheral T-cell population and a helper T-cell subset when incubated *in vitro* with uremic patient's blood but a suppressor T-cell subset was unaffected under these conditions.

Keywords——deacetyl-thymosin β_4; chronic renal failure; impaired peripheral T-cell subset; azide condensation; monoclonal antibody

Introduction

Low *et al.* have reported[2,3] the isolation and complete amino acid sequence of thymosin β_4 purified from thymosin fraction 5. Thymosin β_4 is composed of 43 amino acid residues with acetyl-serine at the N-terminus, and has an isoelectric point of 5.1.

As shown in Fig. 1, thymosin β_4 was found to be highly homologous to thymosin β_8, differing only in eight amino acid substitutions in the presence of four residues at the C-terminus.[4)] Several recent studies have also demonstrated that thymosin β_4 is important for maturation and functioning of the immune system in man and animals.[2-5)] Thymosin β_4 has been chemically synthesized by solid phase procedure[6,7)] and the proposed structure of thymosin β_4 was confirmed.

In 1983, we reported[8)] the synthesis of calf thymosin β_8 and showed that the synthetic nonatriacontapeptide could increase the entire peripheral T-cell population and a suppressor

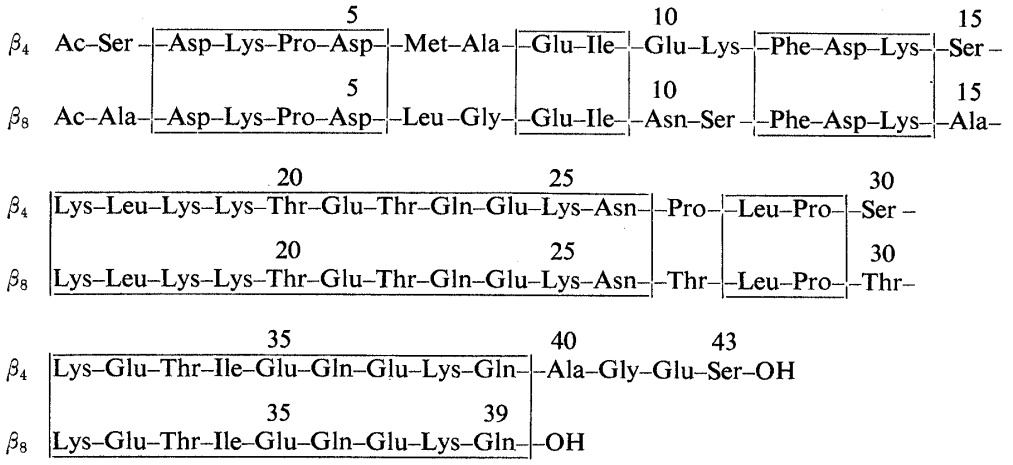

Fig. 1. Comparison of Amino Acid Sequences of Thymosin β_4 and Thymosin β_8

Identical sequences are in boxes.

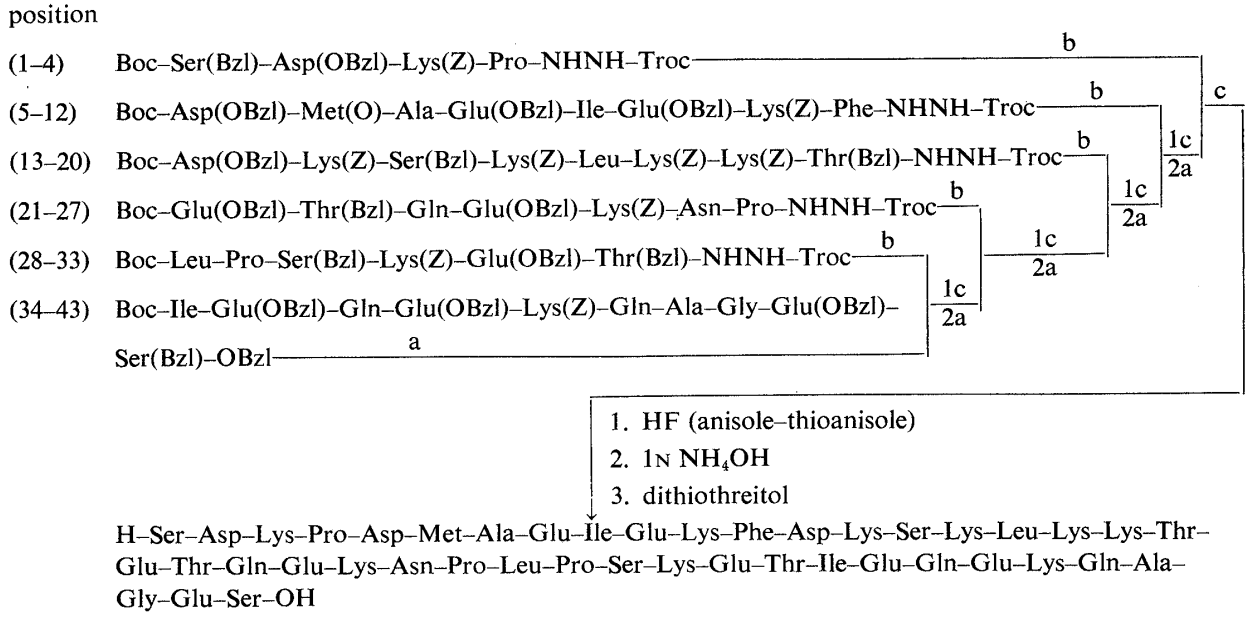

Fig. 2. Synthetic Routes to Deacetyl-thymosin β_4 (1—43)

a, TFA–anisole; b, Zn–AcOH; c, azide.

T-cell subset when incubated *in vitro* with lupus nephritis patient's blood. In 1982, we also reported[9] the synthesis of deacetyl-thymosin α_1 and showed that the synthetic octaeicosapeptide could increase population of the peripheral E-rosette-forming cells when incubated *in vitro* with lipoid nephrosis patient's blood. Therefore, we concluded that the acetyl group at the N-terminal Ser residue of thymosin α_1 is not required for increasing the activity of E-rosette-forming cells in lipoid nephrosis.

On the other hand, it is well known that cell-mediated immunity is impaired in chronic renal failure.[10-12] In 1975, Harris *et al.* reported[13] that thymosin fraction 5 has therapeutic potential for the treatment of uremic patients with impaired immunity.

In this paper, we describe the synthesis of deacetyl-thymosin β_4 by the liuqid phase procedure and the *in vitro* effect of this peptide on the impaired T-cell subsets in patients with chronic renal failure. In the present synthesis, as illustrated in Fig. 2, amino acid derivatives bearing protecting groups, *i.e.*, Ser(Bzl), Thr(Bzl), Lys(Z), Asp(OBzl), Glu(OBzl) and Ser(Bzl)–OBzl, which could be removed by treatment with hydrogen fluoride,[14] were used. These protecting groups survive mostly intact under careful TFA treatment for removal of the Boc group, employed as a temporary α-amino protecting group. Met residue was protected as the corresponding sulfoxide in order to prevent partial oxidation and S-alkylation during the synthesis.[15]

As shown in Fig. 2, six peptides, Boc–(34—43)–OBzl, Boc–(28—33)–$NHNH_2$, Boc–(21—27)–$NHNH_2$, Boc–(13—20)–$NHNH_2$, Boc–(5—12)–$NHNH_2$ and Boc–(1—4)–$NHNH_2$ served as building blocks for the construction of the full sequence of deacetyl-thymosin β_4. The procedure using azide[16] was applied to condense these fragments successively, because of the low risk of racemization involved in this procedure, as compared to other amido-forming reactions. Throughout the syntheses of these intermediates and fragments, the purity of every fragment and intermediate was confirmed by paper chromatography, TLC, acid hydrolysis and elemental analysis. The analytical results were within $\pm 0.4\%$ of theoretical values in all cases.

First, the C-terminal fragment, Boc–(34—43)–OBzl, was prepared stepwise starting from H–Ser(Bzl)–OBzl Tos by the HOBT–WSCI procedure,[17] except for the introduction of Gln residues, which were introduced by the NP active ester procedure.[18] Next, for the preparation

of the five fragments containing Glu(OBzl) and Asp(OBzl), Boc–(28—33)–NHNH–Troc, Boc–(21—27)–NHNH–Troc, Boc–(13—20)–NHNH–Troc, Boc–(5—12)–NHNH–Troc and Boc–(1—4)–NHNH–Troc, we employed a substituted hydrazide, Troc–$NHNH_2$,[19] the protecting group of which is known to be removed by Zn[20] without affecting side chain protecting groups such as Boc, Z and Bzl. Thus, these fragments were prepared without exposing the corresponding methyl or ethyl esters to hydrazide. Then the five fragments, Boc–(28—33)–NHNH–Troc, Boc–(21—27)–NHNH–Troc, Boc–(13—20)–NHNH–Troc, Boc–(5—12)–NHNH–Troc and Boc–(1—4)–NHNH–Troc, were prepared stepwise by the HOBT–WSCI procedure[17] except for the introduction of Asn and Gln residues. These residues were also introduced by the NP active ester procedure,[18] and the Boc groups of intermediates were removed by treatment with TFA–anisole prior to the next coupling reaction. The six fragments thus obtained were assembled successively according to Fig. 2 by the procedure of Rudinger using azide.[16] By comparison of the recovery of Gly with those of newly incorporated amino acids, satisfactory incorporation of each fragment in condensation reactions was confirmed. Boc–(28—33)–NHNH–Troc was treated with Zn[20] in AcOH and DMF to remove the Troc group, and the last trace of zinc acetate was removed by treatment with EDTA to give the hexapeptide hydrazide in analytically pure form. The hydrazine test on the paper chromatograms and elemental analysis data were consistent with homogeneity of the desired product. The Boc group of Boc–(34—43)–OBzl was removed by the usual TFA–anisole treatment and the corresponding free amine was condensed with Boc–(28—33)–$NHNH_2$ (2 eq) by the azide procedure[16] to give Boc–(28—43)–OBzl (I), which was purified by column chromatography on Sephadex LH-20 using DMF containing 5% H_2O as an eluent. The homogeneity of the peptide was assessed by elemental analysis, TLC and amino acid analysis of the hydrolysate with 6 N HCl. The solubility of protected intermediates in DMF decreased remarkably with chain elongation. Consequently, mixtures of DMF–DMSO had to be employed for subsequent fragment condensation reactions. Next, after removal of the Troc group of Boc–(21—27)–NHNH–Troc by treatment with Zn in AcOH and DMF, the resulting heptapeptide hydrazide, Boc–(21—27)–$NHNH_2$ (2 eq), was condensed with H–(28—43)–OBzl by the azide procedure to yield Boc–(21—43)–OBzl (II), which was purified by column chromatography on Sephadex LH-60 with DMSO containing 5% H_2O. The homogeneity of the peptide was assessed by elemental analysis, TLC and amino acid analysis of the acid hydrolysate. The Boc group of the trieicosapeptide II was removed and the corresponding free base was condensed with the N-terminal peptide Boc–(13—20)–$NHNH_2$ (3 eq), by the procedure using azide in the same manner as described above to give Boc–(12—43)–OBzl (III), which was also purified by column chromatography on Sephadex LH-60 with DMSO containing 5% H_2O. The homogeneity of the peptide was also assessed by elemental analysis, TLC, and amino acid analysis of the acid hydrolysate. The Boc group of the untriacontapeptide III was removed and the corresponding free base was condensed with Boc–(5—12)–$NHNH_2$ by the azide procedure. This condensation reaction was performed using 3 eq of acyl component, Boc–(5—12)–$NHNH_2$, followed by addition of further azide (2 eq) after 48 h to yield the protected nonatriacontapeptide, Boc–(5—43)–OBzl (IV), which was also purified by column chromatography on Sephadex LH-60 with DMSO containing 5% H_2O. The homogeneity of the peptide was also assessed by elemental analysis, TLC, and amino acid analysis of the acid hydrolysate. The Boc group of the nonatriacontapeptide IV was removed and the corresponding free base was condensed with the N-terminal subunit, Boc–(1—4)–$NHNH_2$, by the azide procedure. This condensation reaction was performed using 3 eq of acyl component, Boc–(1—4)–$NHNH_2$, followed by addition of further azide (2 eq) after 48 h to yield the protected tritetracontapeptide V corresponding to the entire amino acid sequence of calf thymosin β_4, which was purified by repeated precipitation from DMSO with MeOH. The homogeneity of the peptide was assessed by elemental analysis, TLC, and

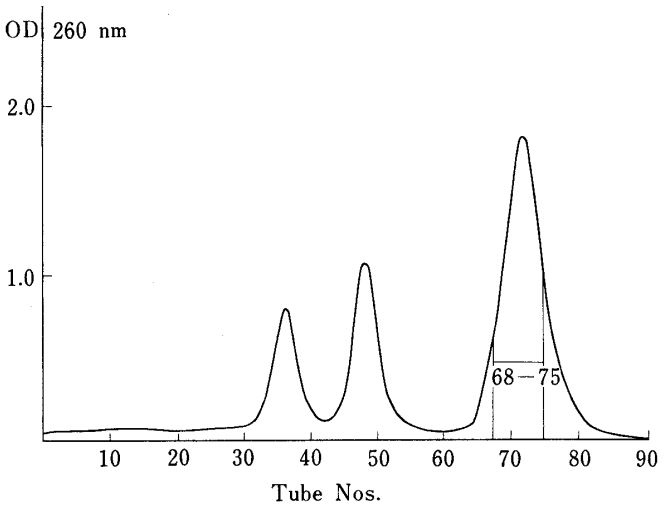

Fig. 3. Purification of Synthetic Deacetyl-thymosin β_4 by Ion-Exchange Chromatography on DEAE-Cellulose

amino acid analysis of the acid hydrolysate. The protected tritetracontapeptide ester V was then treated with hydrogen fluoride[14] in the presence of anisole–thioanisole (1 : 1, v/v) to suppress side reaction of the Asp(OBzl) residue,[21] and the deblocked peptide hydrogen fluoride was converted into the corresponding acetate with Amberlite CG-4B and then treated with 1 N NH_4OH for 30 min. The latter treatment was performed because of the reversible N→O shift at Ser and Thr residues during the hydrogen fluoride treatment.[22,23] The treated product was incubated with dithiothreitol to reduce Met(O) residue[24] in H_2O at 60 °C for 36 h. After removal of the reducing reagent by gel-filtration on Sephadex G-25, the product was purified by ion-exchange chromatography on a DEAE-cellulose column. The product was eluted with a gradient up to 0.08 M ammonium bicarbonate buffer (pH 7.8). As shown in Fig. 3, two side peaks were detected in front of the main peak. These two peaks seem to be due to incomplete deprotection of products, since these materials were less soluble in water than the main product. These two side peaks also seem to be due to the unreduced Met(O)-derivatives, since Met recoveries in these hydrolysates were lower than that of the main product. The main product was rechromatographed on a DEAE-cellulose column. The product thus obtained was desalted completely by gel-filtration on Sephadex G-25. The product thus purified was found to be homogeneous by paper chromatography in two different solvent systems and behaved as a single component upon paper electrophoresis. Its purity was further assessed by amino acid analysis. Amino acid analysis of the acid hydrolysate gave molar ratios in good agreement with expected values. These data indicate clearly that the synthetic tritetracontapeptide (deacetyl-thymosin β_4) has a high degree of purity.

Results

The *in vitro* effects of the synthetic deacetyl-thymosin β_4 on the impaired T-cell subsets in patients with chronic renal failure are shown in Table I.

For this immunological analysis, we used monoclonal antibodies against the cell-surface antigens of helper (T4) and suppressor (T8) T-cell subsets and to a common T-cell antigen (T3) defining all peripheral T-cells.[25,26] In contrast to normal persons, we found that the patients with chronic renal failure had reduced percentages of helper T-cells and all peripheral T-cells, but the percentage of suppressor T-cells was at a normal level (Table I). Comparison of the results of statistical analysis of the data for percentages of T-cell subsets in peripheral blood incubated with or without the synthetic deacetyl-thymosin β_4 shows that, in the group

TABLE I. Effect of the Synthetic Deacetyl-thymosin β_4 on the Impaired T-Cell Subsets of Patients with Chronic Renal Failure

Peptide	Dose (μg/ml)	No. of samples	Reactivity with monoclonal antibodies[d] (%)		
			Anti-T3	Anti-T4	Anti-T8
—[a]		3	68±5	45±6	24±4
—[b]		3	29±5	16±5	26±4
Deacetyl-thymosin β_4[b,c]	0.1	3	43±4	27±5	24±5
Deacetyl-thymosin β_4[b,c]	1.0	3	55±5	33±5	27±5
Deacetyl-thymosin β_4[b,c]	10.0	3	63±4	39±4	25±4

a) Normal venous blood.
b) Patient's venous blood.
c) Incubation was carried out for 60 min at 37 °C.
d) Each value is the mean ± S.D. for three samples.

of patients investigated, the synthetic deacetyl-thymosin β_4 restored to nearly normal values the percentages of helper T-cells and all peripheral T-cells at a dose of 10 μg/ml, but this peptide did not change the percentage of suppressor T-cells under the same conditions. In normal subjects, *in vitro* addition of this peptide did not increase the percentages of helper T-cells and all peripheral T-cells under the same conditions (data not shown). These results indicate that the synthetic deacetyl-thymosin β_4 has activity to restore the defect of helper T-cells *in vitro* and the acetyl group at the N-terminal Ser residue of thymosin β_4 is not required for increasing the activity of helper T-cells in cases of chronic renal failure.

Experimental

Melting points are uncorrected. Rotations were measured with an Atago Polax machine (cell length: 10 cm). Amino acid compositions of acid hydrolysates were determined with a JEOL JLC-8AH amino acid analyzer (one-column system). Solutions were concentrated in a rotary evaporator under reduced pressure at a temperature of 30—40 °C. Boc groups of the protected peptides were removed by TFA–anisole treatment. The resulting amino components were chromatographed on filter paper, Toyo Roshi No. 51, at room temperature. Rf^a values refer to the Partridge system[27] and Rf^b values refer to BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24).[28] TLC was performed on silica gel (Kieselgel 60 F 254, Merck) plates and Rf^c values refer to $CHCl_3$–MeOH–H_2O (8 : 3 : 1, lower phase). Troc–$NHNH_2$ was purchased from the Kokusan Chemical Works, Ltd., Japan. Azide was prepared according to Honzl and Rudinger[16] with isoamylnitrite. Boc–Thr(Bzl)–NHNH–Troc and Boc–Phe–NHNH–Troc were prepared according to the HOBT–WSCI procedure[17] with Boc–Thr(Bzl)–OH or Boc–Phe–OH and Troc–$NHNH_2$. All six fragments for the construction of deacetyl-thymosin β_4 were newly synthesized by a method similar to that employed previously.[8,9] Preparations of protected intermediates were repeated several times in order to obtain sufficient quantities for the next step. Venous blood samples were obtained from three patients suffering from chronic renal failure. Venous blood samples from three healthy donors were used as a control. Monoclonal antibodies (Ortho Diagnostic Systems K. K., New Jersey, USA) used were OKT 3 (all peripheral T-cells), OKT 4 (helper T-cells) and OKT 8 (suppressor T-cells).

Boc–Leu–Pro–Ser(Bzl)–Lys(Z)–Glu(OBzl)–Thr(Bzl)–Ile–Glu(OBzl)–Gln–Glu(OBzl)–Lys(Z)–Gln–Ala–Gly–Glu(OBzl)–Ser(Bzl)–OBzl (I)——Boc–Ile–Glu(OBzl)–Gln–Glu(OBzl)–Lys(Z)–Gln–Ala–Gly–Glu(OBzl)–Ser(Bzl)–OBzl (301 mg) was treated with TFA–anisole (3 ml–0.6 ml) in an ice-bath for 50 min, then dry ether was added. The resulting powder was washed with dry ether, dried over KOH pellets *in vacuo* for 2 h and then dissolved in DMF (3 ml) containing NMM (0.018 ml). The azide (prepared from 394 mg of Boc–(28—33)–$NHNH_2$) in DMF (3 ml) and NMM (0.17 ml) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C for 48 h, was concentrated. The residue was poured into ice-chilled 1 N $NaHCO_3$ with stirring and the resulting powder thus obtained was washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O and precipitated twice from DMF and H_2O. The crude product thus obtained was purified by column chromatography on Sephadex LH-20 using DMF containing 5% H_2O as an eluent and further precipitated from AcOH and H_2O; yield 403 mg (78%), mp 143—147 °C, $[\alpha]_D^{20}$ −12.1° (c=1.0, DMF), Rf^c 0.51, single fluorescamine-positive spot. *Anal.* Calcd for $C_{151}H_{192}N_{20}O_{36} \cdot 12H_2O$: C, 58.90; H, 7.07; N, 9.10. Found: C, 59.03; H, 7.21; N, 8.92. Amino acid ratios

in a 6 N HCl hydrolysate: Leu 1.12, Ile 1.09, Ala 1.06, Gly 1.00, Pro 0.90, Ser 1.79, Thr 0.89, Glu 6.02, Lys 2.17 (recovery of Gly 82%).

Boc–Glu(OBzl)–Thr(Bzl)–Gln–Glu(OBzl)–Lys(Z)–Asn–Pro–Leu–Pro–Ser(Bzl)–Lys(Z)–Glu(OBzl)–Thr(Bzl)–Ile–Glu(OBzl)–Gln–Glu(OBzl)–Lys(Z)–Gln–Ala–Gly–Glu(OBzl)–Ser(Bzl)–OBzl (II)——Boc–(28—43)–OBzl (I) (308 mg) was treated with TFA–anisole (3 ml–0.6 ml) as described above and N^{α}-deprotected peptide was dissolved in DMF–DMSO (1 : 1, 3 ml) containing NMM (0.01 ml). The azide (prepared from 272 mg of Boc–(21—27)–$NHNH_2$) in DMF–DMSO (1 : 1, 2 ml) and NMM (0.05 ml) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C for 48 h, was concentrated. The residue was treated with EtOAc and 1 N citric acid. The resulting powder thus formed was washed successively with 1 N citric acid, H_2O, 1 N $NaHCO_3$ and H_2O and precipitated twice from DMSO with H_2O. For further purification, the product was dissolved in DMSO containing 5% H_2O (3 ml) and the solution was applied to a column of Sephadex LH-60 (2.3 × 96 cm), which was eluted with the same solvent at a flow rate of 4 ml/30 min. The ultraviolet (UV) absorption at 260 nm was determined in each fraction. The fractions corresponding to the main peak (tube Nos. 43—49) were combined and evaporated to dryness. The residue was treated with ether to afford a powder; yield 318 mg (74%), mp 141—149 °C, $[\alpha]_D^{20}$ −40.1° (c = 1.0, DMSO), Rf^c 0.54, single fluorescamine-positive spot. *Anal.* Calcd for $C_{214}H_{270}N_{30}O_{52} \cdot 10H_2O$: C, 60.13; H, 6.84; N, 9.83. Found: C, 60.28; H, 6.84; N, 9.49. Amino acid ratios in a 6 N HCl hydrolysate: Leu 1.10, Ile 1.04, Ala 1.07, Gly 1.00, Pro 1.86, Ser 1.80, Thr 1.82, Glu 9.02, Asp 0.90, Lys 3.06 (recovery of Gly 84%).

Boc–Asp(OBzl)–Lys(Z)–Ser(Bzl)–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–Glu(OBzl)–Thr(Bzl)–Gln–Glu(OBzl)–Lys(Z)–Asn–Pro–Leu–Pro–Ser(Bzl)–Lys(Z)–Glu(OBzl)–Thr(Bzl)–Ile–Glu(OBzl)–Gln–Glu(OBzl)–Lys(Z)–Gln–Ala–Gly–Glu(OBzl)–Ser(Bzl)–OBzl (III)——Boc–(21—43)–OBzl (II) (216 mg) was treated with TFA–anisole (3 ml–0.6 ml) in an ice-bath for 50 min, then dry ether was added. The resulting powder was collected by filtration, washed with ether, dried over KOH pellets *in vacuo* for 2 h and then dissolved in DMF–DMSO (1 : 1, 3 ml) containing NMM (0.006 ml). The azide (prepared from 272 mg of Boc–(13—20)–$NHNH_2$) in DMF–DMSO (1 : 1, 2 ml) and NMM (0.06 ml) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 72 h. The solution was concentrated and the residue was precipitated twice from DMSO and EtOAc. The dried product was applied to a column of Sephadex LH-60 (2.3 × 91 cm), equilibrated and eluted with DMSO containing 5% H_2O. Individual fractions (4 ml each) were collected and the desired fractions (tube Nos. 45—51) were combined and evaporated to dryness. Ether was added to the residue to afford a precipitate; yield 256 mg (85%), mp 171—178 °C (dec.), $[\alpha]_D^{20}$ −34.6° (c = 1.0, DMSO), Rf^c 0.56, single fluorescamine-positive spot. *Anal.* Calcd for $C_{308}H_{388}N_{42}O_{72} \cdot 11H_2O$: C, 61.36; H, 6.86; N, 9.76. Found: C, 61.46; H, 6.89; N, 9.49. Amino acid ratios in a 6 N HCl hydrolysate: Leu 2.03, Ile 1.04, Ala 1.07, Gly 1.00, Pro 1.90, Ser 2.79, Thr 2.85, Glu 8.87, Asp 1.93, Lys 7.06 (recovery of Gly 84%).

Boc–Asp(OBzl)–Met(O)–Ala–Glu(OBzl)–Ile–Glu(OBzl)–Lys(Z)–Phe–Asp(OBzl)–Lys(Z)–Ser(Bzl)–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–Glu(OBzl)–Thr(Bzl)–Gln–Glu(OBzl)–Lys(Z)–Asn–Pro–Leu–Pro–Ser(Bzl)–Lys(Z)–Glu(OBzl)–Thr(Bzl)–Ile–Glu(OBzl)–Gln–Glu(OBzl)–Lys(Z)–Gln–Ala–Gly–Glu(OBzl)–Ser(Bzl)–OBzl (IV)——Boc–(13—43)–OBzl (III) (151 mg) was treated with TFA–anisole (2 ml–0.4 ml) in an ice-bath for 50 min, then dry ether was added. The resulting powder was collected by filtration, washed with ether, dried over KOH pellets *in vacuo* for 2 h and then dissolved in DMF–DMSO (1 : 2, 3 ml) containing NMM (0.003 ml). The azide (prepared from 114 mg of Boc–(5—12)–$NHNH_2$) in DMF–DMSO (1 : 2, 2 ml) and NMM (0.04 ml) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 48 h. Additional azide (prepared from 2 eq of the hydrazide) in DMF–DMSO (2 ml) and NMM (0.027 ml) were added and stirring was continued for an additional 26 h. After that, the mixture was poured into ice-chilled 1 N $NaHCO_3$ with stirring. The precipitate thus formed was washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O and precipitated twice from DMSO and MeOH. The dried product was applied to a column of Sephadex LH-60 (2.3 × 93 cm), equilibrated and eluted with DMSO containing 5% H_2O. Individual fractions (4 ml each) were collected and the desired fractions (tube Nos. 42—48) were combined and evaporated to dryness. The residue was precipitated from DMSO with H_2O; yield 123 mg (65%), mp 158—164 °C, $[\alpha]_D^{20}$ −18.7° (c = 1.0, DMSO), Rf^c 0.58, single fluorescamine-positive spot. *Anal.* Calcd for $C_{380}H_{477}N_{51}O_{89}S \cdot 14H_2O$: C, 61.12; H, 6.82; N, 9.75. Found: C, 61.22; H, 6.90; N, 9.49. Amino acid ratios in a 6 N HCl hydrolysate: Leu 2.01, Ile 2.07, Met(O) 0.92, Ala 2.14, Gly 1.00, Phe 0.92, Pro 1.87, Ser 2.79, Thr 2.84, Glu 10.83, Asp 3.02, Lys 7.94 (recovery of Gly 85%).

Boc–Ser(Bzl)–Asp(OBzl)–Lys(Z)–Pro–Asp(OBzl)–Met(O)–Ala–Glu(OBzl)–Ile–Glu(OBzl)–Lys(Z)–Phe–Asp(OBzl)–Lys(Z)–Ser(Bzl)–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–Glu(OBzl)–Thr(Bzl)–Gln–Glu(OBzl)–Lys(Z)–Asn–Pro–Leu–Pro–Ser(Bzl)–Lys(Z)–Glu(OBzl)–Thr(Bzl)–Ile–Glu(OBzl)–Gln–Glu(OBzl)–Lys(Z)–Gln–Ala–Gly–Glu(OBzl)–Ser(Bzl)–OBzl (V)——Boc–(5—43)–OBzl (IV) (94 mg) was treated with TFA–anisole (2 ml–0.4 ml) in an ice-bath for 50 min, then dry ether was added. The resulting powder was collected by filtration, washed with ether, dried over KOH pellets *in vacuo* for 2 h and then dissolved in DMSO (2 ml) containing NMM (0.001 ml). The azide (prepared from 33 mg of Boc–(1—4)–$NHNH_2$) in DMF (1 ml) and NMM (0.013 ml) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 48 h. Additional azide (prepared from 2 eq of the hydrazide) in DMF (1 ml) and NMM (0.009 ml) were added and stirring was continued for a further 26 h. After that, the mixture was concentrated and the residue was treated with 1 N citric acid and EtOAc. The re-

sulting powder was purified by washing as described above followed by precipitation three times from DMSO with MeOH; yield 68 mg (67%), mp 179—187 °C (dec.), $[\alpha]_D^{20}$ −21.3° (c=0.4, DMSO), Rf^c 0.59, single fluorescamine-positive spot. *Anal.* Calcd for $C_{420}H_{524}N_{56}O_{98}S \cdot 15H_2O$: C, 61.32; H, 6.79; N, 9.53. Found: C, 61.28; H, 6.70; N, 9.17. Amino acid ratios in a 6 N HCl hydrolysate: Leu 2.09, Ile 2.07, Met(O) 0.89, Ala 2.06, Gly 1.00, Pro 2.84, Phe 0.93, Ser 3.80, Thr 2.83, Glu 11.02, Asp 4.06, Lys 9.10 (recovery of Gly 82%).

H–Ser–Asp–Lys–Pro–Asp–Met–Ala–Glu–Ile–Glu–Lys–Phe–Asp–Lys–Ser–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–Pro–Leu–Pro–Ser–Lys–Glu–Thr–Ile–Glu–Gln–Glu–Lys–Gln–Ala–Gly–Glu–Ser–OH (Corresponding to Deacetyl-Thymosin β_4) (VI)——The protected tritetracontapeptide (48 mg) was treated with HF (ap-

TABLE II. Yields and Physical Constants of Protected Intermediates

Peptides	Yield (%)	mp (°C)	$[\alpha]_D^{20}$ (c=1.0, DMF)	Paper chromato.[a] Rf^a	Rf^b
Boc-(42—43)-OBzl	62	92—96	−13.1	0.76	0.81
Boc-(41—43)-OBzl	85	74—77	−8.7	0.78	0.84
Boc-(40—43)-OBzl	77	76—78	−20.4	0.75	0.86
Boc-(39—43)-OBzl	74	83—89	−24.8	0.71	0.80
Boc-(38—43)-OBzl	91	90—94	−26.8	0.88	0.89
Boc-(37—43)-OBzl	74	119—124	−13.1	0.80	0.88
Boc-(36—43)-OBzl	78	113—118	−9.8	0.88	0.89
Boc-(35—43)-OBzl	79	131—135	−12.5	0.83	0.88
Boc-(34—43)-OBzl	85	134—139	−9.6	0.85	0.87
Boc-(32—33)-NHNH-Troc	75	76—79	−10.2	0.80	0.85
Boc-(31—33)-NHNH-Troc	93	83—87	−13.6	0.71	0.79
Boc-(30—33)-NHNH-Troc	75	92—97	−19.7	0.80	0.81
Boc-(29—33)-NHNH-Troc	82	110—115	−4.2	0.84	0.89
Boc-(28—33)-NHNH-Troc	89	96—99	−9.2	0.86	0.87
Boc-(28—33)-$NHNH_2$	83	171—177	−2.6	0.70[b]	0.68[b]
Boc-(26—27)-NHNH-Troc	69	110—114	−23.6	0.72	0.83
Boc-(25—27)-NHNH-Troc	70	80—84	−25.7	0.81	0.83
Boc-(24—27)-NHNH-Troc	71	88—93	−11.3	0.77	0.89
Boc-(23—27)-NHNH-Troc	72	91—94	−17.3	0.80	0.82
Boc-(22—27)-NHNH-Troc	72	121—126	−7.0	0.87	0.89
Boc-(21—27)-NHNH-Troc	81	132—137	−13.1	0.86	0.90
Boc-(21—27)-$NHNH_2$	94	172—177	−17.2	0.80[b]	0.71[b]
Boc-(19—20)-NHNH-Troc	97	88—90	−1.3	0.84	0.97
Boc-(18—20)-NHNH-Troc	95	93—97	−4.6	0.80	0.89
Boc-(17—20)-NHNH-Troc	66	86—89	−8.5	0.84	0.87
Boc-(16—20)-NHNH-Troc	82	82—85	−3.7	0.79	0.85
Boc-(15—20)-NHNH-Troc	73	89—93	−6.7	0.79	0.86
Boc-(14—20)-NHNH-Troc	82	96—100	−14.2	0.89	0.87
Boc-(13—20)-NHNH-Troc	70	101—107	−11.6	0.80	0.91
Boc-(13—20)-$NHNH_2$	83	160—167	−15.6	0.82[b]	0.83[b]
Boc-(11—12)-NHNH-Troc	93	74—80	−19.1	0.68	0.76
Boc-(10—12)-NHNH-Troc	77	70—73	−13.0	0.79	0.83
Boc-(9—12)-NHNH-Troc	73	126—129	−6.9	0.78	0.89
Boc-(8—12)-NHNH-Troc	72	98—104	−15.4	0.81	0.88
Boc-(7—12)-NHNH-Troc	75	126—129	−9.3	0.85	0.86
Boc-(6—12)-NHNH-Troc	89	110—116	−8.9	0.82	0.87
Boc-(5—12)-NHNH-Troc	90	143—148	−12.8	0.85	0.86
Boc-(5—12)-$NHNH_2$	70	180—187	−15.4	0.76[b]	0.79[b]
Boc-(3—4)-NHNH-Troc	94	72—74	−6.4	0.73	0.79
Boc-(2—4)-NHNH-Troc	72	82—85	−14.7	0.78	0.80
Boc-(1—4)-NHNH-Troc	67	80—82	−10.8	0.86	0.90
Boc-(1—4)-$NHNH_2$	82	112—115	−19.2	0.64[b]	0.65[b]

a) Ninhydrin-positive spot unless otherwise noted.
b) Hydrazine-positive spot.

proximately 3 ml) in the presence of anisole–thioanisole (1 : 1, 1 ml) at −5 °C for 60 min. After removal of the excess HF, dry ether was added to the residue and the resulting powder was dissolved in H_2O (6 ml). The solution was treated with Amberlite CG-4B (acetate form, approximately 3 g) for 30 min, and filtered by suction. The filtrate was adjusted to pH 10 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 6.5 with a few drops of AcOH and the solution was lyophilized. The residue was dissolved in H_2O (10 ml). The solution, after addition of dithiothreitol (20 mg), was incubated at 60 °C under N_2 gas for 36 h. The solvent was evaporated off *in vacuo* and the residue was dissolved in a small amount of 1% AcOH and then applied to a column of Sephadex G-25 (2.3 × 96 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. 55—62) were combined and the solvent was removed by lyophilization. The residue was dissolved in H_2O (3 ml) and the solution was applied to a column of DEAE-cellulose (Brown, 2.3 × 60 cm), eluted with a linear gradient formed from 300 ml each of H_2O and 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. A main peak present in the gradient eluates (tube Nos. 68—75) was collected. The solvent was evaporated off and the residue was rechromatographed on a DEAE-cellulose column as described above. The residue obtained from the peak fractions was dissolved in 1% AcOH (2 ml). This solution was then subjected to Sephadex G-25 column chromatography as described above; yield 6.4 mg (22%), $[\alpha]_D^{20}$ −74.8° (c=0.3, 1 N AcOH), Rf^a 0.01, Rf^b 0.07, single ninhydrin-positive spot. The synthetic peptide exhibited a single spot on paper electrophoresis: Toyo Roshi No. 51 (2 × 40 cm), pyridinium-acetate buffer at pH 7.1. Mobility, 2.3 cm from the origin toward the anode, after running at 2 mA, 600 V for 60 min. Amino acid ratios in a 6 N HCl hydrolysate: Leu 2.03, Ile 2.10, Met 0.87, Ala 2.11, Gly 1.00, Pro 2.85, Phe 0.93, Ser 3.82, Thr 2.81, Glu 11.06, Asp 3.98, Lys 8.89 (recovery of Gly 85%).

Distribution of T-Cell Subsets in Patients with Chronic Renal Failure and Effect of Synthetic Deacetyl-thymosin β_4: Analysis with Monoclonal Antibodies——A 10 ml of aliquot of venous blood was drawn into a syringe containing 1000 U of heparin and was incubated with the synthetic peptide for 60 min at 37 °C, then the T-cells were isolated from venous blood by Ficoll–Hypaque density centrifugation as described elsewhere.[29] T-cells obtained on the Ficoll–Hypaque density centrifugation were stained for membrane antigens by indirect immunofluorescence with murine monoclonal antibodies (OKT3, OKT4 and OKT8) according to Hoffman[30] and Janossy *et al.*[31] Briefly, the isolated T-cells were incubated with OKT3, OKT4 or OKT8 monoclonal antibodies (Ortho Diagnostic Systems, Roritan, N. J.) (1×10^6 mononuclear cells in 0.2 ml of minimum essential medium with 5% fetal calf serum in 10 μl of antibody) at 4 °C for 30 min, then washed twice, and mixed and incubated for 30 min with 0.1 ml of an appropriate dilution of fluorescein-labelled goat antimouse immunoglobulins. The cells were then washed three times and resuspended in 1.0 ml of minimum essential medium supplemented with 5% fetal calf serum and 3 mM EDTA. Labelled-cell counting was done under a Nikon UFD-TR fluorescence microscope. The reactivity of antisera was evaluated concomitantly with determination of the phase contrast morphology of cells. More than 200 cells were counted.

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References and Notes

1) The amino acid residues mentioned in this paper are of the L-configuration except for glycine. The abbreviations used to denote amino acid derivatives and peptides are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **11**, 1726 (1972). Other abbreviations used: DMF, dimethylformamide; DMSO, dimethylsulfoxide; WSCI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; TFA, trifluoroacetic acid; MeOH, methanol; HOBT, 1-hydroxybenzotriazole; AcOH, acetic acid; EtOAc, ethyl acetate, Ac, acetyl; NMM *N*-methylmorpholine; Boc, *tert*-butoxycarbonyl; OBzl, benzyl ester; Bzl, benzyl; Z, benzyloxycarbonyl; Troc, β,β,β-trichloroethyloxycarbonyl; HF, hydrogen fluoride; TLC, thin-layer chromatography; NP, *p*-nitrophenyl; E-rosette, a rosette with sheep erythrocytes; EDTA, ethylenediamine tetraacetic acid; DEAE, diethylaminoethyl.
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