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Synthesis and Immunological Effect of Deacetyl-thymosin α_{11} on Low E-Rosette-Forming Cells of a Rheumatoid Arthritis Patient[1)]

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Deacetyl-thymosin α_{11} was synthesized by successive azide condensations of seven peptide fragments, Boc-(1—3)-$NHNH_2$, Boc-(4—7)-$NHNH_2$, Boc-(8—11)-$NHNH_2$, Boc-(12—16)-$NHNH_2$, Boc-(17—22)-$NHNH_2$, Boc-(23—26)-$NHNH_2$ and Boc-(27—29)-$NHNH_2$, with H-(30—35)-OBzl, followed by deprotection with hydrogen fluoride in the presence of anisole and thioanisole. An increase of E-rosette-forming cells was obtained after incubation of peripheral blood from a rheumatoid arthritis patient with the synthetic deacetyl-thymosin α_{11}. This synthetic deacetyl-thymosin α_{11} was approximately equal in potency to our synthetic deacetyl-thymosin α_1 in cases of rheumatoid arthritis.

Keywords——deacetyl-thymosin α_{11}; rheumatoid arthritis; E-rosette-forming cell; azide condensation; β,β,β-trichloroethoxycarbonylhydrazide

In 1983, Caldarella *et al.*[2)] reported that thymosin α_{11}, which was isolated from a preparation of calf thymosin fraction 5, contains seven additional amino acid residues at the C-terminus of thymosin α_1. Thymosin α_{11} is 30 times as potent as thymosin fraction 5 and approximately equal in potency to thymosin α_1.

On the other hand, thymosin α_1 was isolated from bovine thymus and sequenced by Goldstein *et al.*[3)] This acidic peptide is composed of 28 amino acid residues with acetylserine as the N-terminus.[2)] Thymosin α_1 induces the expression of T-lymphocyte markers and potentiates immunological reactions mediated through or regulated by T-cells.[4,5)] The chemical synthesis of thymosin α_1 has been achieved by both solution and solid phase methods.[6-8)] Deacetyl-thymosin α_1 was also synthesized by both solid phase and solution methods and tested for biological activities.[8,9)]

Immune abnormalities have long been known to exist in partients with rheumatoid arthritis. It is generally accepted that a high percentage of rheumatoid arthritis patients have a defect of cell-mediated immunity.[10)] A decrease of E-rosette-forming cells in these patients has been demonstrated by several investigators.[10,11)]

In the preceding papers,[6,9)] we reported the syntheses of thymosin α_1 and deacetyl-thymosin α_1 by the solution method and we showed that the synthetic thymosin α_1 was able to increase the E-rosette-forming capacity in cases of minimal change nephrotic syndrome[6)] and the synthetic deacetyl-thymosin α_1 was also able to restore the E-rosette-forming capacity in cases of lipoid nephrosis.[9)] Our preparations of deacetyl-thymosin α_1 and thymosin α_1 were found to be equally active in cases of lipoid nephrosis.[9)] This result suggests that the acetyl

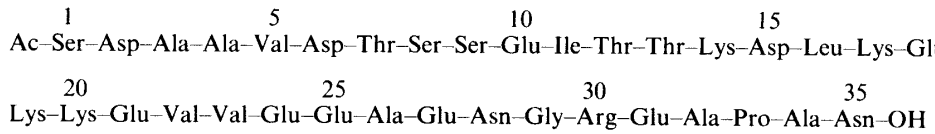

Fig. 1. The Amino Acid Sequence of Thymosin α_{11}

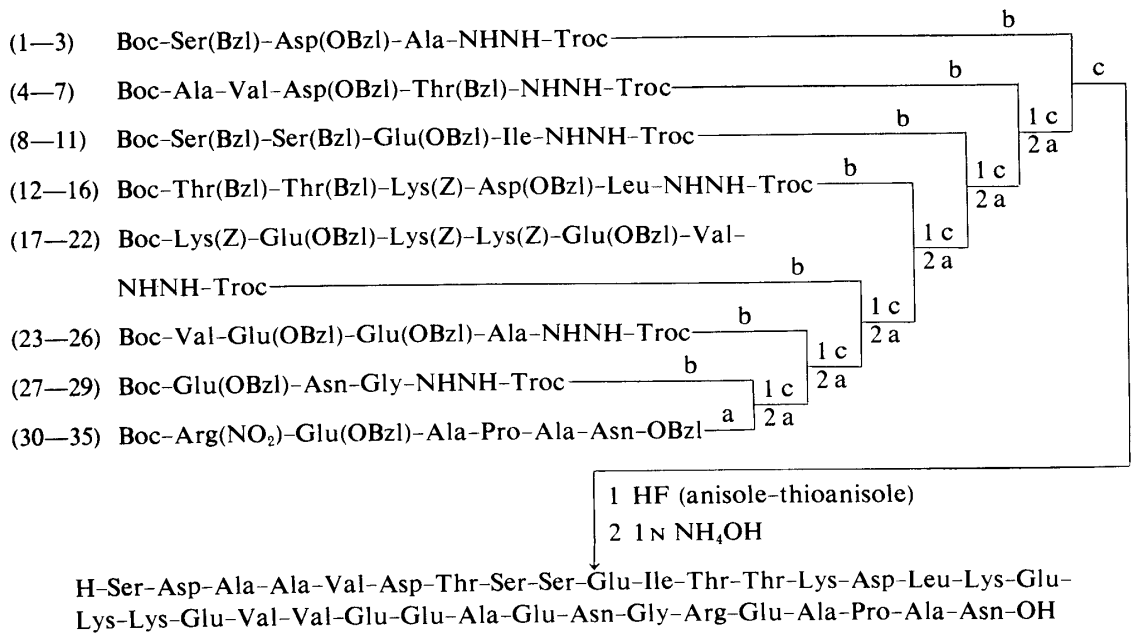

Fig. 2. Synthetic Route to Deacetyl-thymosin α_{11}

a, TFA–anisole; b, Zn–AcOH; c, azide.

group at the N-terminal Ser residue of thymosin α_1 is not required for increasing the activity of E-rosette-forming cells in cases of lipoid nephrosis.[9]

In this paper, we describe the synthesis of deacetyl-thymosin α_{11} by the solution method and a comparison of the *in vitro* effects of this deacetyl-thymosin α_1[9] on the E-rosette-forming capacity of cells of a rheumatoid arthritis patients.

In the present synthesis, as illustrated in Fig. 2, amino acid derivatives bearing protecting groups removable by hydrogen fluoride treatment[12] were employed, *i.e.*, Lys(Z), Glu(OBzl), Asp(OBzl), Arg(NO_2), Ser(Bzl), Thr(Bzl) and Asn–OBzl. 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. 2, eight peptides, Boc–(30—35)–OBzl, Boc–(27—29)–$NHNH_2$, Boc–(23—26)–$NHNH_2$, Boc–(17—22)–$NHNH_2$, Boc–(12—16)–$NHNH_2$, Boc–(8—11)–$NHNH_2$, Boc–(4—7)–$NHNH_2$ and Boc–(1—3)–$NHNH_2$, served as building blocks for the construction of the full sequence of deacetyl-thymosin α_{11}. The procedure using azide[13] 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–(30—35)–OBzl, was prepared stepwise starting from H–Asn–OBzl Tos by the HOBT–WSCI procedure.[14] Next, for the preparation of the seven fragments containing Glu(OBzl) or Asp(OBzl), Boc–(27—29)–NHNH–Troc, Boc–(23—26)–NHNH–Troc, Boc–(17—22)–NHNH–Troc, Boc–(12—16)–NHNH–Troc, Boc–(8—11)–NHNH–Troc, Boc–(4—7)–NHNH–Troc and Boc–(1—3)–NHNH–Troc, we employed a substituted hydrazide, Troc–$NHNH_2$,[15] the protecting group of which is known to be cleaved by Zn[16] or Cd[17] 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. The seven fragments, Boc–(27—29)–NHNH–Troc, Boc–(23—26)–NHNH–Troc, Boc–(17—22)–NHNH–Troc, Boc–(12—16)–NHNH–Troc, Boc–(12—16)–NHNH–Troc,

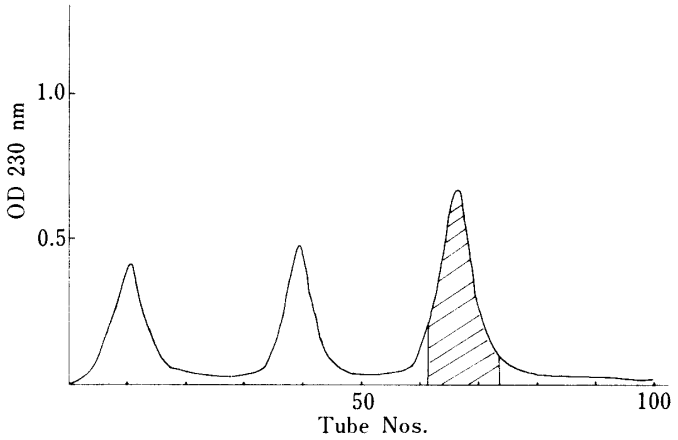

Fig. 3. Purification of Synthetic Deacetyl-thymosin α_{11} by Ion-Exchange Chromatography on a DEAE-cellulose Column

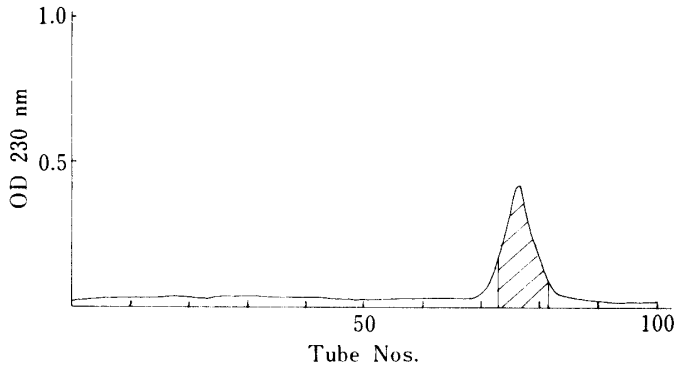

Fig. 4. Gel-Filtration of the Synthetic Deacetyl-thymosin α_{11} on Sephadex G-25

Boc–(8—11)–NHNH–Troc, Boc–(4—7)–NHNH–Troc and Boc–(1—3)–NHNH–Troc, were prepared stepwise by the HOBT–WSCI procedure[14] except for the introduction of Asn and Arg(NO_2) residues. Asn residue was introduced by the NP active ester procedure.[18] Arg(NO_2) residue was introduced by the MA procedure.[19] Boc groups of intermediates were removed by treatment with TFA–anisole prior to the next coupling reaction. The eight fragments thus obtained were assembled successively according to Fig. 2 by the procedure of Rudinger and Honzl using azide.[13] 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–(27—29)–NHNH–Troc was treated with Zn[16] in AcOH and MeOH to remove the Troc group, and the last trace of zinc acetate was removed by treatment with EDTA to give the tripeptide hydrazide in analytically pure form. The hydrazine test on the paper chromatograms and elemental analysis data were consistent with homogeneity of the desired product. Then, every fragment condensation was performed in DMF or DMF–DMSO and some of the products were purified by crystallization from EtOAc or precipitation from DMF or DMSO with MeOH. The Boc group of Boc–(30—35)–OBzl was removed by the usual TFA–anisole treatment and the corresponding free amine was condensed with Boc–(27—29)–$NHNH_2$ (1.5 eq) by the azide procedure[13] to give Boc–(27—35)–OBzl (I), which was purified by repeated crystallization from hot EtOAc. 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–(23—26)–NHNH–Troc by treatment with Zn in AcOH and DMF, the resulting tetrapeptide hydrazide (1.5 eq) was condensed with H–(27—35)–OBzl by the azide procedure to yield Boc–(23—35)–OBzl (II), which was purified by repeated precipitation from DMF with MeOH. The homogeneity of the peptide was assessed by elemental analysis, TLC

TABLE I. Yields and Physical Constants of Protected Intermediates

Peptides	Yield (%)	mp (°C)	$[\alpha]_D^{21}$ (c=1.0, DMF)	Paper chromato.[a] Rf^a	Rf^b
Boc–(43—35)–OBzl	65	72—75	−12.5	0.68	0.74
Boc–(33—35)–OBzl	79	75—78	−8.7	0.71	0.76
Boc–(32—35)–OBzl	65	98—104	−5.4	0.78	0.85
Boc–(31—35)–OBzl	62	106—110	−12.7	0.81	0.86
Boc–(30—35)–OBzl	73	113—118	−7.6	0.84	0.89
Boc–(28—29)–NHNH–Troc	81	118—124	−4.8	0.68	0.65
Boc–(27—29)–NHNH–Troc	94	120—127	−10.6	0.84	0.89
Boc–(27—29)–$NHNH_2$	88	156—164	−16.4	0.48[b]	0.42[b]
Boc–(25—26)–NHNH–Troc	69	Oil	−13.5	0.79	0.74
Boc–(24—26)–NHNH–Troc	80	81—90	−7.4	0.85	0.88
Boc–(23—26)–NHNH–Troc	78	130—135	−5.8	0.87	0.86
Boc–(23—26)–$NHNH_2$	88	171—178	−10.2	0.78[b]	0.83[b]
Boc–(21—22)–NHNH–Troc	86	Oil	−8.2	0.71	0.76
Boc–(20—22)–NHNH–Troc	83	76—80	−14.1	0.78	0.85
Boc–(19—22)–NHNH–Troc	93	131—137	−12.3	0.81	0.87
Boc–(18—22)–NHNH–Troc	70	84—91	−6.7	0.86	0.88
Boc–(17—22)–NHNH–Troc	81	92—95	−6.3	0.88	0.86
Boc–(17—22)–$NHNH_2$	82	162—174	−14.7	0.77[b]	0.74[b]
Boc–(15—16)–NHNH–Troc	81	116—120	−4.2	0.79	0.84
Boc–(14—16)–NHNH–Troc	74	91—94	−11.8	0.80	0.88
Boc–(13—16)–NHNH–Troc	65	90—93	−7.2	0.82	0.90
Boc–(12—16)–NHNH–Troc	79	127—130	−3.4	0.87	0.90
Boc–(12—16)–$NHNH_2$	86	146—153	−18.4	0.72[b]	0.76[b]
Boc–(10—11)–NHNH–Troc	84	123—126	−6.9	0.70	0.74
Boc–(9—11)–NHNH–Troc	80	118—123	−2.4	0.83	0.86
Boc–(8—11)–NHNH–Troc	74	105—113	−5.5	0.85	0.89
Boc–(8—11)–$NHNH_2$	89	134—139	−13.8	0.80[b]	0.84[b]
Boc–(6—7)–NHNH–Troc	79	75—79	+4.2	0.75	0.79
Boc–(5—7)–NHNH–Troc	74	101—106	−3.1	0.84	0.83
Boc–(4—7)–NHNH–Troc	78	121—126	−10.6	0.88	0.89
Boc–(4—7)–$NHNH_2$	87	171—177	−19.8	0.79[b]	0.74[b]
Boc–(2—3)–NHNH–Troc	77	71—74	−8.4	0.72	0.74
Boc–(1—3)–NHNH–Troc	72	74—76	−6.7	0.86	0.88
Boc–(1—3)–$NHNH_2$	83	128—134	−8.3	0.69[b]	0.71[b]

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

and amino acid analysis of the acid hydrolysate. The Boc group of Boc–(23—35)–OBzl (II) was removed and the corresponding free base was condensed with Boc–(17—22)–$NHNH_2$ (1.5 eq), by the procedure using azide in the same manner as described above to give Boc–(17—35)–OBzl (III), which was purified by column chromatography on Sephadex LH-60 with DMF 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 Boc–(17—35)–OBzl (III) was removed and the corresponding free base was condensed with Boc–(12—16)–$NHNH_2$ (2.5 eq) by the azide procedure to yield Boc–(12—35)–OBzl (IV), which was also purified by repeated precipitation from DMSO with MeOH. The homogeneity of the peptide was also assessed by elemental analysis, TLC, and amino acid analysis of the acid hydrolysate. The Boc group of Boc–(12—35)–OBzl (IV) was removed and the corresponding free base was condensed with Boc–(8—11)–$NHNH_2$ by the azide procedure. This condensation reaction was performed using 2 eq of acyl component, Boc–(8—11)–$NHNH_2$, followed by addition of further azide (1 eq) after 48 h to yield Boc–(8—35)–OBzl (V), which was purified by column

TABLE II. Effects of the Synthetic Deacetyl-thymosin α_{11} and Deacetyl-thymosin α_1 on the Low E-Rosette-Forming Cells of a Rheumatoid Arthritis Patient

Peptide	Dose (μg/5 ml)	E-Rosette-forming cells (%)
—[a]		66 ± 7[d]
—[b]		32 ± 6
Deacetyl-thymosin α_{11}[b,c]	1	30 ± 6
	10	44 ± 6
	100	52 ± 6
Deacetyl-thymosin α_1[b,c]	1	31 ± 7
	10	42 ± 6
	100	54 ± 6

a) Normal venous blood. *b*) Patient's venous blood. *c*) Incubation was carried out for 1 h at 37 °C. *d*) Each value represents the mean ± S.D. of triplicate measurements.

chromatography on Sephadex LH-60 with DMF–DMSO (1 : 2) 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 Boc–(8—35)–OBzl (V) was removed and the corresponding free base was condensed with Boc–(4—7)–$NHNH_2$ by the azide procedure. This condensation reaction was performed using 3 eq of acyl component, Boc–(4—7)–$NHNH_2$, followed by addition of further azide (1 eq) after 48 h to yield Boc–(4—35)–OBzl (VI), which was also purified by column chromatography on Sephadex LH-60 with DMF–DMSO (1 : 2) containing 1% 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 Boc–(4—35)–OBzl was removed and the corresponding free base was condensed with the N-terminal subunit, Boc–(1—3)–$NHNH_2$, by the azide procedure. This condensation reaction was performed using 3 eq of acyl component, Boc–(1—3)–$NHNH_2$, followed by addition of further azide (1 eq) after 48 h to yield the protected pentatriacontapeptide VII corresponding to the entire amino acid sequence of calf thymosin α_{11}, which was purified by repeated precipitation from DMSO with MeOH. The homogeneity of the peptide was assessed by elemental analysis, TLC and amino acid analysis of the acid hydrolysate. The protected pentatriacontapeptide ester VII was then treated with hydrogen fluoride[12] in the presence of anisole–thioanisole (1 : 1, v/v) to suppress side reaction of the Asp(OBzl) residue,[20] and the deblocked peptide hydrogen fluoride was converted into the corresponding acetate with Amberlite CG-4B and then treated with 1 N NH_4OH at pH 8.5 in order to reverse the possible N→O shift[21,22] at the Ser and Thr residues during the hydrogen fluoride treatment. The product was purified by gel-filtration on Sephadex G-25 using 2% AcOH, followed by ion-exchange chromatography on a DEAE-cellulose column. The product was eluted with a gradient up to 0.12 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. The main product was rechromatographed on a DEAE-cellulose column as described above. 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 acid hydrolysis gave the amino acid ratios predicted by theory. Its purity was further confirmed by enzymic digestion. Despite the presence of the Pro residue,[23] complete digestion of this synthetic peptide with commercial AP-M[24,25] was achieved and the presence of Asn residues in the product was thus confirmed. We assumed that this

phenomenon was due to the presence of prolidase in our commercial AP-M.

The *in vitro* effects of the synthetic deacetyl-thymosin α_{11} and deacetyl-thymosin α_1 on low E-rosette-forming cells of a rheumatoid arthritis patient are shown in Table II. Incubation of peripheral venous blood from the rheumatoid arthritis patient in the presence of various amounts of the synthetic peptide from 1 to 100 μg/5 ml resulted in recovery of E-rosette formation (Table II). Our preparations of deacetyl-thymosin α_{11} and deacetyl-thymosin α_1 were found to be equally active in this assay system, suggesting that the acetyl group at the N-terminal Ser residue of thymosin α_{11} and the seven amino acid residues at the C-terminus of trymosin α_{11} are not required for increasing the activity of E-rosette-forming cells in cases of rheumatoid arthritis.

Experimental

General experimental procedures used in this paper are essentially the same as described in the previous paper.[26] An azide was prepared according to Honzl and Rudinger[13] with isoamyl nitrite. Unless otherwise mentioned, products were purified by one of the following two procedures. Procedure 1: For purification of protected peptides soluble in EtOAc, the extract was washed with 1 N citric acid, H_2O, 1 N $NaHCO_3$ and H_2O, then dried over $MgSO_4$ and concentrated. The residue was precipitated or recrystallized from appropriate solvents. Procedure 2: For purification of protected peptides almost insoluble in EtOAc, the reaction mixture was poured into ice-chilled 1 N citric acid with stirring. The powder thereby formed was washed with 1 N citric acid, H_2O, 1 N $NaHCO_3$ and H_2O. The dried product was recrystallized or precipitated from appropriate solvents. General procedure for obtaining protected peptide hydrazides from Troc-derivatives: Troc-derivatives in DMF or MeOH were treated with Zn dust in the presence of AcOH for 6 h. The solution was filtered, the filtrate was concentrated and the residue was treated with 3% EDTA. The resulting powder was washed with 1 N $NaHCO_3$ and H_2O, and precipitated 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 JEOL JLC-8AH amino acid analyzer (one-column system). Solvents were removed by evaporation *in vacuo* at 30 to 40 °C in a rotary evaporator. 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 G, Merck) plates and Rf^c values refer to $CHCl_3$–MeOH–H_2O (8 : 3 : 1). Troc–$NHNH_2$ was purchased from the Kokusan Chemical Works, Ltd., Japan. Aminopeptidase (3501, Aminopeptidase 210520) was purchased from the Protein Research Foundation, Osaka, Japan. Venous blood from a rheumatoid arthritis patient and normal subjects was drawn into heparinized syringes and sedimented at room temperature.

Boc–Glu(OBzl)–Asn–Gly–Arg(NO_2)–Glu(OBzl)–Ala–Pro–Ala–Asn–OBzl (I)——Boc–Arg(NO_2)–Glu(OBzl)–Ala–Pro–Ala–Asn–OBzl (327 mg) was treated with TFA–anisole (3 ml–0.6 ml) in an ice-bath for 40 min, then dry ether was added. The resulting powder was washed with dry ether, dried over KOH pellets *in vacuo* for 2 h and then dissolved in DMF (3 ml) containing NMM (0.037 ml). The azide [prepared from 261 mg of Boc–(27—29)–$NHNH_2$] in DMF (2 ml) and NMM (0.06 ml) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C for 48 h, was acidified with a few drops of AcOH. The mixture was poured into ice-chilled 1 N citric acid with stirring and the powder thus obtained was washed successively with 1 N citric acid, H_2O, 1 N $NaHCO_3$ and H_2O and precipitated twice from DMF with H_2O. The dried product was recrystallized twice from hot EtOAc; yield 381 mg (72%), mp 136—145 °C, $[\alpha]_D^{21}$ −7.1° (c = 1.0, DMF), Rf^a 0.88, Rf^b 0.90, single ninhydrin-positive spot. *Anal.* Calcd for $C_{58}H_{85}N_{15}O_{20}$: C, 53.08; H, 6.53; N, 16.01. Found: C, 52.82; H, 6.49; N, 16.40. Amino acid ratios in a 6 N HCl hydrolysate: Ala 2.04, Gly 1.00, Pro 0.87, Glu 2.06, Asp 1.83, Arg 0.81 (recovery of Gly 80%).

Boc–Val–Glu(OBzl)–Glu(OBzl)–Ala–Glu(OBzl)–Asn–Gly–Arg(NO_2)–Glu(OBzl)–Ala–Pro–Ala–Asn–OBzl (II)——Boc–(27—35)–OBzl (I) (328 mg) was treated with TFA–anisole (3 ml–0.6 ml) as described above and the N^{α}-deblocked peptide was dissolved in DMF (3 ml) containing NMM (0.027 ml). The azide [prepared from 291 mg of Boc–(23—26)–$NHNH_2$] in DMF (2 ml) and NMM (0.042 ml) were added to the above ice-chilled solution and the mixture, after being stirred for 48 h, was acidified with a few drops of AcOH. The mixture was poured into an ice-chilled 1 N citric acid. The powder thus formed was washed successively with 1 N citric acid, H_2O, 1 N $NaHCO_3$ and H_2O. The dried product was prepipitated twice from DMF with MeOH; yield 426 mg (86%), mp 162—171 °C (dec.), $[\alpha]_D^{21}$ −4.8° (c = 1.0, DMF), Rf^c 0.03, single fluorescamine-positive spot. *Anal.* Calcd for $C_{90}H_{125}N_{19}O_{28}\cdot H_2O$: C, 55.75; H, 6.60; N, 13.73. Found: C, 55.75; H, 6.48; N, 13.51. Amino acid ratios in a 6 N HCl hydrolysate: Ala 3.12, Gly 1.00, Pro 0.85, Val 1.06, Glu 3.87, Asp 1.84, Arg 0.84 (recovery of Gly 87%).

Boc–Lys(Z)–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Val–Val–Glu(OBzl)–Glu(OBzl)–Ala–Glu(OBzl)–Asn–Gly–Arg(NO_2)–Glu(OBzl)–Ala–Pro–Ala–Asn–OBzl (III)——Boc–(23—35)–OBzl (II) (329 mg) was treated with TFA–anisole (3 ml–0.6 ml) in an ice-bath for 40 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 (3 ml) containing NMM (0.019 ml). The azide [prepared from 374 mg of Boc–(17—22)–$NHNH_2$] in DMF (3 ml) and NMM (0.028 ml) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 62 h. After addition of a few drops of AcOH, the mixture was treated with 1 N citric acid to form a powder, which was washed with 1 N citric acid, H_2O and MeOH. The product was precipitated from DMF with MeOH. The dried product was dissolved in DMF containing 5% H_2O (3 ml) and the solution was applied to a column of Sephadex LH 60 (2.8 × 90 cm), which was eluted with the same solvent. Each fraction (5 ml) was examined for ultraviolet (UV) absorption at 260 nm; fractions corresponding to the main peak (tube Nos. 54—67) were combined and the solvent was removed by evaporation. Treatment of the residue with EtOAc afforded a powder; yield 361 mg (67%), mp 180—187 °C (dec.), $[\alpha]_D^{21}$ −9.4° (c = 1.0, DMF), Rf^c origin, fluorescamine-positive spot. *Anal.* Calcd for $C_{161}H_{214}N_{28}O_{44} \cdot 10H_2O$: C, 56.45; H, 6.89; N, 11.45. Found: C, 56.59; H, 6.72; N, 11.27. Amino acid ratios in a 6 N HCl hydrolysate: Ala 3.14, Gly 1.00, Val 2.10, Pro 0.91, Glu 5.78, Asp 1.81, Lys 2.04, Arg 0.88 (recovery of Gly 84%).

Boc–Thr(Bzl)–Thr(Bzl)–Lys(Z)–Asp(OBzl)–Leu–Lys(Z)–Glu(OBzl)–Lys(Z)–Lys(Z)–Glu(OBzl)–Val–Val–Glu(OBzl)–Glu(OBzl)–Ala–Glu(OBzl)–Asn–Gly–Arg(NO_2)–Glu(OBzl)–Ala–Pro–Ala–Asn–OBzl (IV)——Boc–(17—35)–OBzl (III) (286 mg) was treated with TFA–anisole (3 ml–0.6 ml) in an ice-bath for 40 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 (0.01 ml). The azide [prepared from 228 mg of Boc–(12—16)–$NHNH_2$] in DMF–DMSO (1 : 1, 2 ml) and NMM (0.023 ml) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 62 h. After addition of a few drops of AcOH, the mixture was treated with 1 N citric acid, H_2O and MeOH. The product was precipitated twice from DMSO with MeOH; yield 269 mg (74%), mp 188—196 °C (dec.), $[\alpha]_D^{21}$ −14.6° (c = 1.0, DMSO), Rf^c origin, fluorescamine-positive spot. *Anal.* Calcd for $C_{214}H_{280}N_{34}O_{55} \cdot 7H_2O$: C, 59.30; H, 6.84; N, 10.99. Found: C, 59.23; H, 6.80; N, 10.67. Amino acid ratios in a 6 N HCl hydrolysate: Ala 3.13, Gly 1.00, Val 2.04, Pro 0.86, Leu 1.02, Thr 1.82, Glu 5.87, Asp 2.80, Lys 4.01, Arg 0.85 (recovery of Gly 88%).

Boc–Ser(Bzl)–Ser(Bzl)–Glu(OBzl)–Ile–Thr(Bzl)–Thr(Bzl)–Lys(Z)–Asp(OBzl)–Leu–Lys(Z)–Glu(OBzl)–Lys(Z)–Lys(Z)–Glu(OBzl)–Val–Val–Glu(OBzl)–Glu(OBzl)–Ala–Glu–(OBzl)–Asn–Gly–Arg(NO_2)–Glu(OBzl)–Ala–Pro–Ala–Asn–OBzl (V)——Boc–(12—35)–OBzl (IV) (217 mg) was treated with TFA–anisole (3 ml–0.6 ml) as usual and dissolved in DMF–DMSO (1 : 2, 3 ml) containing NMM (0.0052 ml). The azide [prepared from 84 mg of Boc–(8—11)–$NHNH_2$] in DMF–DMSO (2 ml) and NMM (0.011 ml) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 48 h. Additional azide (1 eq) in DMF–DMSO (2 ml) and NMM (0.0055 ml) were then added and stirring was continued for an additional 24 h, until the solution became ninhydrin-negative. The solution was poured into 1 N citric acid. The resulting powder was washed with 1 N citric acid and H_2O and purified by gel-filtration on Sephadex LH-60 (2.8 × 92 cm) using DMF–DMSO (1 : 2) containing 5% H_2O as an eluent. The desired fractions (each 5 ml, tube Nos. 51—62) were combined, the solvent was evaporated off, and the residue was treated with EtOAc to afford a powder; yield 174 mg (71%), mp 194—209 °C (dec.), $[\alpha]_D^{21}$ −6.3° (c = 1.0, DMSO), Rf^c origin, fluorescamine-positive spot. *Anal.* Calcd for $C_{252}H_{326}N_{38}O_{63} \cdot 14H_2O$: 58.80; H, 6.93; N, 10.34. Found: 58.76; H, 6.69; N, 10.42. Amino acid ratios in a 6 N HCl hydrolysate: Ala 3.11, Gly 1.00, Ile 1.09, Val 2.12, Pro 0.90, Leu 1.06, Ser 1.79, Thr 1.83, Glu 6.79, Asp 2.84, Lys 4.07, Arg 0.82 (recovery of Gly 89%).

Boc–Ala–Val–Asp(OBzl)–Thr(Bzl)–Ser(Bzl)–Ser(Bzl)–Glu(OBzl)–Ile–Thr(Bzl)–Lys(Z)–Asp(OBzl)–Leu–Lys(Z)–Glu(OBzl)–Lys(Z)–Lys(Z)–Glu(OBzl)–Val–Val–Glu(OBzl)–Glu(OBzl)–Ala–Glu(OBzl)–Asn–Gly–Arg–(NO_2)–Glu(OBzl)–Ala–Pro–Ala–Asn–OBzl (VI)——Boc–(8—35)–OBzl (V) (122 mg) was treated with TFA–anisole (2 ml–0.4 ml) as usual and dissolved in DMF–DMSO (1 : 2, 3 ml) containing NMM (0.0028 ml). The azide [prepared from 57 mg of Boc–(4—7)–$NHNH_2$] in DMF–DMSO (2 ml) and NMM (0.01 ml) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 48 h. Additional azide (1 eq) in DMF–DMSO (2 ml) and NMM (0.003 ml) were then added and stirring was continued for an additional 24 h until the solution became ninhydrin-negative. The solution was poured into 1 N citric acid. The resulting powder was washed with 1 N citric acid, H_2O and MeOH and purified by gel-filtration on Sephadex LH-60 (2.8 × 98 cm) using DMF–DMSO (1 : 2) containing 1% H_2O as an eluent. The desired fractions (each 5 ml, tube Nos. 56—69) were combined, the solvent was evaporated off, and the residue was treated with MeOH to afford a powder; yield 101 mg (71%), mp 198—209 °C (dec.), $[\alpha]_D^{21}$ −6.5° (c = 1.0, DMSO), Rf^c origin, fluorescamine-positive spot. *Anal.* Calcd for $C_{282}H_{361}N_{42}O_{70} \cdot 16H_2O$: C, 58.93; H, 6.89; N, 10.24. Found: C, 58.72; H, 6.90; N, 10.06. Amino acid ratios in a 6 N HCl hydrolysate: Ala 4.11, Gly 1.00, Ile 0.94, Val 3.14, Leu 1.01, Pro 0.82, Ser 1.80, Thr 2.86, Glu 7.03, Asp 3.96, Lys 4.13, Arg 0.90 (recovery of Gly 90%).

Boc–Ser(Bzl)–Asp(OBzl)–Ala–Ala–Val–Asp(OBzl)–Thr(Bzl)–Ser(Bzl)–Ser(Bzl)–Glu(OBzl)–Ile–Thr(Bzl)–Thr(Bzl)–Lys(Z)–Asp(OBzl)–Leu–Lys(Z)–Glu(OBzl)–Lys(Z)–Lys(Z)–Glu(OBzl)–Val–Val–Glu(OBzl)–Glu(OBzl)–Ala–Glu(OBzl)–Asn–Gly–Arg(NO_2)–Glu(OBzl)–Ala–Pro–Ala–Asn–OBzl (VII)——Boc–(4—35)–OBzl (VI) (82 mg) was treated with TFA–anisole (2 ml–0.4 ml) as usual and dissolved in DMF–DMSO (1 : 2, 3 ml) containing NMM (0.0016 ml). The azide [prepared from 24 mg of Boc–(1—3)–$NHNH_2$] and NMM (0.005 ml) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 48 h. Additional azide (1 eq) in DMF–DMSO (2 ml) and NMM (0.0018 ml) were added and stirring was continued for a further 24 h. The solution was poured into 1 N citric acid. The resulting powder was washed with 1 N citric acid, H_2O and MeOH. The product was precipitated three

times from DMSO with MeOH; yield 59 mg (68%), mp 211—226 °C (dec.), $[\alpha]_D^{21}$ −8.0° (c=0.4, DMSO), Rf^c origin, fluorescamine-positive spot. *Anal.* Calcd for $C_{306}H_{388}N_{45}O_{76} \cdot 12H_2O$: C, 59.97; H, 6.78; N, 10.28. Found: C, 59.82; H, 6.86; N, 10.03. Amino acid ratios in a 6 N HCl hydrolysate: Ala 5.05, Gly 1.00, Ile 1.02, Val 3.06, Leu 0.98, Pro 0.81, Ser 2.76, Thr 2.80, Glu 6.87, Asp 4.86, Arg 0.83 (recovery of Gly 82%).

H–Ser–Asp–Ala–Ala–Val–Asp–Thr–Ser–Ser–Glu–Ile–Thr–Thr–Lys–Asp–Leu–Lys–Glu–Lys–Lys–Glu–Val–Val–Glu–Glu–Ala–Glu–Asn–Gly–Arg–Glu–Ala–Pro–Ala–Asn–OH (Corresponding to Deacetyl-thymosin α_{11}) (VIII)——The protected pentatriacontapeptide (VII) (40 mg) was treated with HF (approximately 4 ml) in the presence of anisole–thioanisole (1 : 1, 1 ml) in an ice-bath for 60 min. After removal of the HF, dry ether was added to the residue and the resulting powder was dissolved in H_2O (5 ml). The solution was treated with Amberlite CG-4B (acetate form, approximately 3 g) for 30 min, and filtered by suction. The pH of the filtrate was adjusted to pH 8.5 with 1 N NH_4OH and, after being stirred for 30 min in an ice-bath, the filtrate was readjusted to pH 6.5 with 1 N AcOH and was lyophilized. The residue was dissolved in 2% AcOH (2 ml), applied to a column of Sephadex G-25 (2.8 × 101 cm), and eluted with the same solvent. Fractions of 4 ml per 16 min were collected, and the absorption at 230 nm was determined. Fractions corresponding to the front main peak (tube Nos. 73—82) were combined and the solvent was removed by lyophilization. The residue was dissolved in H_2O (2 ml) and the solution was applied to a column of DEAE-cellulose (Brown, 2.3 × 12 cm), which was eluted with a linear gradient formed from 300 ml each of H_2O and 0.12 M ammonium bicarbonate buffer at pH 7.8. Individual fractions (5 ml each) were collected and the absorbancy at 230 nm was determined. A main peak present in the gradient eluates (tube Nos. 62—74) was collected. The solvent was evaporated off and the residue was rechromatographed on a Sephadex G-25 column as described above. The peptide purified by DEAE-cellulose ion-exchange column chromatography exhibited a single peak on Sephadex G-25 column chromatography; yield 6.7 mg (27%), $[\alpha]_D^{21}$ −71.3° (c=0.3, 1 N AcOH), Rf^a 0.02, Rf^b 0.10, single ninhydrin- and Sakaguchi-positive spot. The synthetic peptide exhibited a single spot on paper electrophoresis: Toyo Roshi No. 51 (2 × 40 cm), pyridinium–acetate buffer at pH 6.4. Mobility, 3.4 cm from the origin toward the anode, after running at 2 mA, 600 V for 70 min. Amino acid ratios in a 6 N HCl hydrolysate: Ala 5.13, Gly 1.00, Val 2.88, Ile 1.14, Leu 1.06, Pro 0.87, Ser 2.76, Thr 2.82, Asp 5.04, Glu 6.81, Lys 4.07, Arg 0.86 (recovery of Gly 83%). Amino acid ratios in an AP-M digest: Ala 5.08, Gly 1.00, Val 3.02, Ile 0.96, Leu 0.99, Pro 0.83, Ser 2.83, Asp 2.85, Glu 7.04, (Asn+Thr) 4.89, Lys 4.03, Arg 0.80 (recovery of Gly 79%) (Asn emerged at the same position as Thr and was calculated as Thr).

E-Rosette Formation Test——The test involves the *in vitro* incubation of patient's blood with the synthetic peptide and then the *in vitro* incubation of isolated lymphocytes from the peripheral blood with sheep erythrocytes. The results of incubation with and without the synthetic peptide are compared. In this case, lymphocytes were incubated with sheep erythrocytes at 4 °C for 2 h rather than for 12 h,[29] because in our laboratory, counts obtained at 2 h were similar to those obtained at 12 h. To study the effects of the synthetic peptide, we performed experiments in which all cells binding even one sheep erythrocyte were counted as T-cells, because the effect of the synthetic deacetyl-thymosin α_{11} might be to increase the number of sheep erythrocyte binding sites on the surface of maturing T-cells. A 5 ml aliquot of venous blood was drawn into a syringe containing 1000 U of heparin and was incubated with the synthetic peptide for 1 h at 37 °C, then lymphocytes were isolated in a Hypaque–Ficoll gradient[30] for testing of E-rosette formation. The isolated lymphocytes were adjusted to 5×10^5 cells/ml with PBS. Contamination by monocytes and polymorphonuclear cells amounted to less than 7%.[31] Sheep erythrocytes (Kyokutō Pharmaceutical Co.) were washed with GVB^{2+}, centrifuged for 10 min at 1500 rpm, and then suspended in PBS (1 ml). The suspension was mixed with the suspension of sheep erythrocytes (0.5 ml) and the mixture was incubated for 2 h at 4 °C, then centrifuged for 5 min at 900 rpm. Triplicate wet-cell preparations were checked by phase-contrast microscopy. For each preparation, 200 lymphocytes were counted, and the proportion binding more than one erythrocyte was determined.

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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, dimethyl sulfoxide; WSCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; TFA, trifluoroacetic acid; MeOH, methanol; HOBT, 1-hydroxybenzotriazole; AcOH, acetic acid; EtOAc, ethyl acetate; NMM, *N*-methylmorpholine; Boc, *tert*-butoxycarbonyl; OBzl, benzyl ester; Bzl, benzyl; Z, benzyloxycarbonyl; HF, hydrogen fluoride; Troc, β,β,β-trichloroethoxycarbonyl; TLC, thin-layer chromatography; Tos, *p*-tolylsulfonyl; NP, *p*-nitrophenyl; E-rosette, a rosette with sheep erythrocytes; PBS, phosphate-buffered saline; GVB^{2+}, gelatin veronal buffer; AP-M, aminopeptidase M; EDTA, ethylenediaminetetraacetic acid; MA, mixed anhydride.

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