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Deacetyl-Thymosin α_1: Synthesis and Immunological Effect on Lipoid Nephrosis Lymphocytes[1]

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We have replaced the Ac-Ser residue of bovine thymosin α_1 (position 1) with Ser in order to examine the resulting change in immunological effect on the low rosette-forming capacity with sheep erythrocytes of cells from a lipoid nephrosis patient. The deacetyl-thymosin α_1 was synthesized by the solution method. For the synthesis of the protected octaeicosapeptide, five peptide fragments were prepared by the stepwise elongation method with *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline as a coupling reagent. The condensations of the fragments were achieved by Rudinger's azide procedure. Finally, all protecting groups were removed by HF treatment followed by catalytic hydrogenation.

The *in vitro* addition of the synthetic deacetyl-thymosin α_1 at a dose of 100 μg/ml was able to restore the rosette-forming capacity with sheep erythrocytes of lipoid nephrosis cells to normal levels. Our preparations of deacetyl-thymosin α_1 and thymosin α_1 were found to be equally active in cases of lipoid nephrosis.

Keywords——deacetyl-thymosin α_1; lipoid nephrosis; *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; rosette-forming cells with sheep erythrocytes; defect of cell-mediated immunity

Thymosin α_1 was isolated from bovine thymus and sequenced by Goldstein *et al.*[2] This acidic peptide is composed of 28 amino acid residues with acetylserine as the *N*-terminus.[2] This immunologically active octaeicosapeptide was shown to be 10—1000 times as active as thymosin fraction 5, from which the α_1 component was isolated, *in vitro* and *in vivo*.[3] Thymosin α_1 induces the expression of T-lymphocyte markers and potentiates immunologic reactions mediated through or regulated by T-cells.[3,4] In certain immunopathological states, incubation of lymphocytes with thymosin α_1 has been shown to cause an increase in fraction of T-lymphocytes.[5,6]

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 the solid-phase method and tested for biological activity in the rosette inhibition assay.[8]

On the other hand, it is well known that many lipoid nephrosis have a defect of cell-mediated immunity.[9] A decrease of E-rosette-forming cells in these patients has been demonstrated.[9]

In this paper, we wish to describe the synthesis of deacetyl-thymosin α_1 by the solution method. Furthermore, we compared the *in vitro* effects of this deacetyl-thymosin α_1 and thymosin α_1[6] on the E-rosette-forming capacity of cells of a lipoid nephrosis patient.

In the present synthesis, as illustrated in Fig. 1, amino acid derivatives bearing protecting groups removable by hydrogen fluoride treatment[10] were employed; *i.p.*, Lys(Z), Asp(OBzl) and Glu(OBzl). The *p*-nitrobenzyl ester group of C-terminal Asn was removed by catalytic hydrogenation. These protecting groups survive mostly intact under careful TFA treatment for removal of the Z(OMe) group,[11] employed as a temporary α-amino protecting group. Five intermediate peptides, Z(OMe)–Val–Glu(OBzl)–Glu(OBzl)–Ala–Glu(OBzl)–Asn–ONb (23—28) (V), Z(OMe)–Glu(OBzl)–Lys(Z)–Lys(Z)–Glu(OBzl)–Val–NHNH–Troc (18—22) (X), Z(OMe)–Thr–Thr–Lys(Z)–Asp(OBzl)–Leu–Lys(Z)–NHNH–Troc (12—17) (XVIII), Z(OMe)–Asp(OBzl)–Thr–Ser–Ser–Glu(OBzl)–Ile–NHNH–Troc (6—11) (XXVI) and Z(OMe)–Ser–Asp-(OBzl)–Ala–Ala–Val–NHNH–Troc (1—5) (XXX), were chosen to construct the full sequence.

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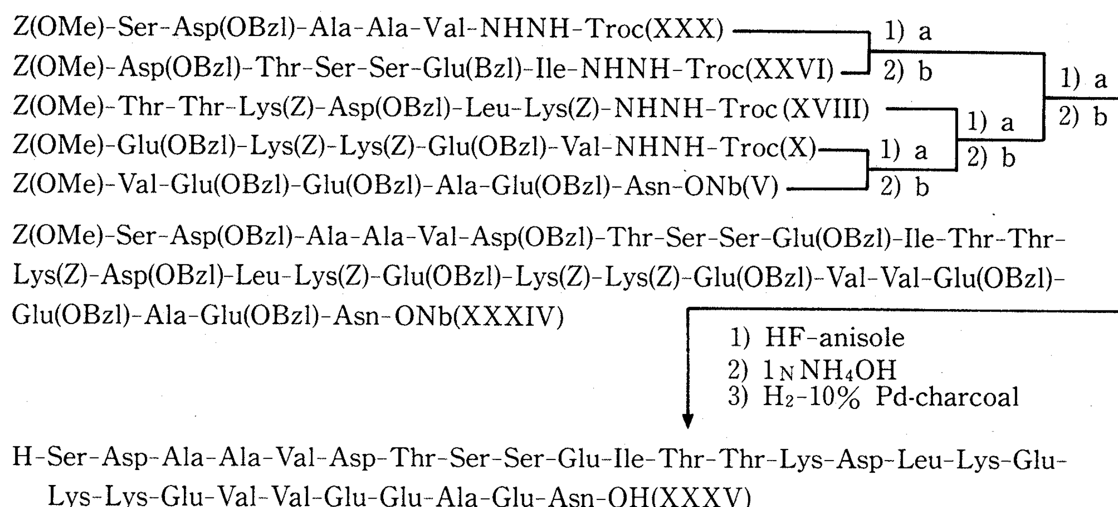

Fig. 1. Synthetic Scheme for Deacetyl-Thymosin α_1
a: Zn–AcOH. b: azide.

In order to prepared the peptide hydrazides containing Asp(OBzl) and Glu(OBzl), these four fragments were synthesized starting with Troc–$NHNH_2$.[12]

First, Z(OMe)-amino acid was condensed with Troc–$NHNH_2$ by the HOBT–DCC method.[13] These five fragments were prepared by the stepwise elongation method with EEDQ as a coupling reagent[14] to minimize undesirable racemization[15] and Z(OMe) groups of intermediates were removed by treatment with TFA-anisole prior to the next coupling reaction. The five fragments thus obtained were assembled successively according to Fig. 1 by Rudinger's azide procedure.[16] The Troc group of Z(OMe)–Glu(OBzl)–Lys(Z)–Lys(Z)–Glu(OBzl)–Val–NHNH–Troc (X) was removed by treatment with Zn dust[17] in AcOH and DMF to give Z(OMe)–Glu(OBzl)–Lys(Z)–Lys(Z)–Glu(OBzl)–Val–$NHNH_2$ (XI). The last trace of metal contamination was removed by treatment with EDTA. The Z(OMe) group of the hexapeptide V was removed by usual TFA-anisole treatment and the corresponding free base was condensed with Z(OMe)–Glu(OBzl)–Lys(Z)–Lys(Z)–Glu(OBzl)–Val–$NHNH_2$ XI by the azide procedure[16] to yield Z(OMe)–Glu(OBzl)–Lys(Z)–Lys(Z)–Glu(OBzl)–Val–Val–Glu(OBzl)–Glu(OBzl)–Ala–Glu(OBzl)–Asn–ONb (XII). After removal of Troc group of Z(OMe)–Thr–Thr–Lys(Z)–Asp(OBzl)–Leu–Lys(Z)–NHNH–Troc (XVIII) by treatment with Zn dust in AcOH and DMF, the resulting hexapeptide hydrazide, Z(OMe)–Thr–Thr–Lys(Z)–Asp(OBzl)–Leu–Lys(Z)–$NHNH_2$ (XIX), was condensed with H–Glu(OBzl)–Lys(Z)–Lys(Z)–Glu(OBzl)–Val–Val–Glu(OBzl)–Glu(OBzl)–Ala–Glu(OBzl)–Asn–ONb by the azide procedure to yield Z(OMe)–Thr–Thr–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–ONb (XX). Next, after removal of Troc group of Z(OMe)–Ser–Asp(OBzl)–Ala–Ala–Val–NHNH–Troc (XXX), the resulting pentapeptide hydrazide (XXXI) was condensed with H–Asp(OBzl)–Thr–Ser–Ser–Glu(OBzl)–Ile–NHNH–Troc by the azide procedure to yield Z(OMe)–Ser–Asp(OBzl)–Ala–Ala–Val–Asp-(OBzl)–Thr–Ser–Ser–Glu(OBzl)–Ile–NHNH–Troc (XXXII). The final azide coupling reaction of the TFA-anisole-treated sample of the protected heptadecapeptide, Z(OMe)–Thr–Thr–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–ONb (XX), with the undecapeptide, Z(OMe)–Ser–Asp(OBzl)–Ala–Ala–Val–Asp(OBzl)–Thr–Ser–Ser–Glu(OBzl)–Ile-$NHNH_2$ XXXIII, was performed using *N*-methyl-2-pyrrolidone as a solvent because of the poor solubility of the amino component in DMF. The protected octaeicosapeptide, Z(OMe)–Ser–Asp(OBzl)–Ala–Ala–Val–Asp(OBzl)–Thr–Ser–Ser–Glu(OBzl)–Ile–Thr–Thr–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–ONb (XXXIV), thus

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obtained was purified by silica gel column chromatography with BuOH and DMF (1:1). The homogeneity of the peptide was assessed by paper chromatography using two different solvent systems, amino acid analysis of the acid hydrolysate, and elemental analysis.

The protected octaeicosapeptide XXXIV was treated with hydrogen fluoride in the presence of anisole.[10] The free peptide ester, precipitated by adding dry ether, was converted to the corresponding acetate by treatment with Amberlite CG-4B (acetate form) and then treated with 1 N NH_4OH for 30 min. The latter treatment was performed because of the reversible N→O shift at the Ser and Thr residues during the hydrogen fluoride treatment.[18,19] Then the deblocked peptide ester was hydrogenated over 10% Pd-charcoal in aqueous AcOH for 20 h. Finally, the product was purified by gel-filtration on Sephadex G-25 using 2% AcOH, followed by preparative TLC chromatography. The octaeicosapeptide (XXXV) thus obtained was found to be homogeneous by paper chromatography in two different solvent systems. The amino acid compositions in the acid hydrolysate and aminopeptidase (AP-M)[20] digest of XXXV agreed well with the theoretical values.

The *in vitro* effects of the synthetic deacetyl-thymosin α_1 and thymosin α_1 on E-rosette-forming cells of a lipoid nephrosis patient are shown in Table I.

Table I. Effects of the Synthetic Thymosin α_1 and Deacetyl-thymosin α_1 on the Low E-Rosette-forming Capacity of Cells of a Lipoid Nephrosis Patient

Peptide	Dose (μg/ml)	E-Rosette-forming cells (%)
—[a]		81±6
—[b]		49±7
Thymosin α_1[b,c]	1	57±5
	10	65±6
	100	77±6
Deaetyl-thymosin α_1[b,c]	1	58±6
	10	67±7
	100	78±5

a) Normal lymphocytes.
b) Patient's lymphocytes.
c) Incubation was carried out for 30 min at 37°C with synthetic peptide.

Incubation of lymphocytes from a lipoid nephrosis patient in the presence of various amounts of synthetic peptides from 1 μg/ml to 100 μg/ml resulted in recovery of E-rosette formation (Table I). Our preparations of thymosin α_1 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_1 is not required for increasing the activity of E-rosette-forming cells in cases of lipoid nephrosis.

Experimental

Melting points are uncorrected. Rotations were measured with an Atago Polax machine (cell length: 10 cm). Amino acid compositions of acid hydrolysate and AP-M digest were determined with a JEOL JLC-8AH amino acid analyzer (one-column system). Evaporation of solvents was carried out in a rotary evaporator under reduced pressure at 35 to 45°C. Z(OMe) 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^1 values refer to the Partridge system[21] and Rf^2 values refer to BuOH-pyridine-AcOH-H_2O (30:20:6:24).[22] Preparations of protected intermediates were repeated several times in order to obtain sufficient quantities for the next step.

Venous blood samples from a lipoid nephrosis patient and normal subjects were drawn into heparinized syringes and sedimented at room temperature. Thymosin α_1 was synthesized in our laboratory.[6] Amino-

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peptidase (3501, Aminopeptidase 210520) was purchased from the Protein Research Foundation, Osaka, Japan. Troc-$NHNH_2$ was purchased from the Kokusan Chemical Works, Ltd., Japan.

Z(OMe)-Glu(OBzl)-Asn-ONb (I)——Z(OMe)–Asn–ONb (4.3 g) was treated with TFA (7 ml)–anisole (1.5 ml) at room temperature for 30 min, then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in THF (8 ml)–DMF (8 ml). Z(OMe)–Glu-(OBzl)–OH (4.4 g) and EEDQ[14] (2.8 g) were added to the above solution, followed by NMM[23] to keep the solution slightly alkaline. After 16 h at 4°C, the reaction mixture was concentrated *in vacuo*. The residue was extracted with EtOAc and the extract was washed successively with 1 N citric acid, H_2O, 1 N $NaHCO_3$ and H_2O, dried over $MgSO_4$ and then concentrated *in vacuo*. The residue was precipitated from EtOAc and *n*-hexane. The precipitate was recrystallized from MeOH and ether; yield 4.4 g (68%), mp 168—169°C, $[\alpha]_D^{32}$ −10.4° (c=1.0, DMF), Rf^1 0.73, Rf^2 0.88, single ninhydrin-positive spot. *Anal.* Calcd for $C_{32}H_{34}N_4O_{11}$: C, 59.07; H, 5.27; N, 8.61. Found: C, 58.80; H, 5.30; N, 8.39.

Z(OMe)-Ala-Glu(OBzl)-Asn-ONb (II)——This compound was prepared from I (5.4 g), Z(OMe)–Ala–OH (2.2 g) and EEDQ (2.3 g) essentially as described for the preparation of I. The product was recrystallized from MeOH; yield 4.3 g (72%), mp 124—127°C, $[\alpha]_D^{30}$ −25.3° (c=1.0, DMF), Rf^1 0.65, Rf^2 0.91, single ninhydrin-positive spot. *Anal.* Calcd for $C_{35}H_{39}N_5O_{12}$: C, 58.25; H, 5.45; N, 9.70. Found: C, 57.83; H, 5.32; N, 9.70.

Z(OMe)-Glu(OBz)-Ala-Glu(OBz)-Asn-ONb (III)——This compound was prepared from II (2.4 g), Z(OMe)-Glu(OBzl)–OH (1.4 g) and EEDQ (907 mg) essentially as described for the preparation of I. The product was recrystallized from THF and ether; yield 2.1 g (68%), mp 147—149°C, $[\alpha]_D^{29}$ −10.9° (c=1.0, DMF), Rf^1 0.82, Rf^2 0.96, single ninhydrin-positive spot. *Anal.* Calcd for $C_{47}H_{52}N_6O_{15}$: C, 59.99; H, 5.57; N, 8.93. Found: C, 59.65; H, 5.54; N, 8.98.

Z(OMe)-Glu(OBzl)-Glu(OBzl)-Ala-Glu(OBzl)-Asn-ONb (IV)——This compound was prepared from III (1.9 g), Z(OMe)–Glu(OBzl)–OH (883 mg) and EEDQ (545 mg) essentially as described for the preparation of I. The product was reprecipitated from MeOH and ether; yield 1.6 g (70%), mp 90—96°C, $[\alpha]_D^{30}$ −12.3° (c=1.0, DMF), Rf^1 0.89, Rf^2 0.94, single ninhydrin-positive spot. *Anal.* Calcd for $C_{59}H_{65}N_7O_{18}$: C, 61.08; H, 5.65; N, 8.45. Found: C, 61.44; H, 5.69; N, 8.88.

Z(OMe)-Val-Glu(OBzl)-Glu(OBzl)-Ala-Glu(OBzl)-Asn-ONb (V)——This compound was prepared from IV (1.1 g), Z(OMe)–Val–OH (282 mg) and EEDQ (248 mg) essentially as described for the preparation of I. The product was recrystallized from acetone and ether; yield 814 mg (68%), mp 85—89°C, $[\alpha]_D^{29}$ −5.4° (c=1.0, DMF), Rf^1 0.87, Rf^2 0.95, single ninhydrin-positive spot. *Anal.* Calcd for $C_{64}H_{74}N_8O_{19}$: C, 61.04; H, 5.92; N, 8.90. Found: C, 61.60; H, 5.76; N, 9.25.

Z(OMe)-Val-NHNH-Troc (VI)——HOBT (1.6 g) and WSCI (1.8 g) were added to a solution of Z(OMe)–Val–OH (2.8 g) and Troc–$NHNH_2$ (2.3 g) in THF (20 ml) with stirring at 0°. The reaction mixtur ewas stirred for 16 h at 4°C. Then, the mixture was extracted with EtOAc and the extract was washed successively with 1 N citric acid, H_2O, 1 N $NaHCO_3$ and H_2O, dried over $MgSO_4$ and then concentrated *in vacuo*. The residue was reprecipitated from EtOAc and *n*-hexane; yield 3.1 g (oily material) (66%), $[\alpha]_D^{29}$ −61.3° (c=1.0, DMF), Rf^1 0.75, Rf^2 0.95, single ninhydrin-positive spot. *Anal.* Calcd for $C_{17}H_{35}Cl_3N_3O_6$: C, 43.38; H, 4.71; N, 8.93. Found: C, 43.28; H, 4.72; N, 8.69.

Z(OMe)-Glu(OBzl)-Val-NHNH-Troc (VII)——This compound was prepared from VI (2.4 g), Z(OMe)–Glu(OBzl)–OH (2.2 g) and EEDQ (1.3 g) essentially as described for the preparation of I. The product was recrystallized from EtOH; yield 3.3 g (94%), mp 84—91°C, $[\alpha]_D^{30}$ −19.1° (c=1.0, DMF), Rf^1 0.79, Rf^2 0.91, single ninhydrin-positive spot. *Anal.* Calcd for $C_{29}H_{35}Cl_3N_4O_9$: C, 50.48; H, 5.11; N, 8.12. Found: C, 50.12; H, 4.96; N, 7.86.

Z(OMe)-Lys(Z)-Glu(OBzl)-Val-NHNH-Troc (VIII)——This compound was prepared from VII (1.7 g), Z(OMe)–Lys(Z)–OH DCHA (1.7 g) and EEDQ (680 mg) essentially as described for the preparation ofI. The product was precipitated from EtOAc and *n*-hexane. Then the dried powder was recrystallized from MeOH; yield 2.1 g (88%), mp 98—104°C, $[\alpha]_D^{29}$ −24.7° (c=1.0, DMF), Rf^1 0.89, Rf^2 0.95, single ninhydrin-positive spot. *Anal.* Calcd for $C_{43}H_{53}Cl_3N_6O_{12}$: C, 54.24; H, 5.61; N, 8.83. Found: C, 54.45; H, 5.84; N, 8.65.

Z(OMe)-Lys(Z)-Lys(Z)-Glu(OBzl)-Val-NHNH-Troc (IX)——This compound was prepared from VIII (2.4 g), Z(OMe)–Lys(Z)–OH DCHA (1.6 g) and EEDQ (680 mg) essentially as described for the preparation of I. The product was recrystallized from EtOH; yield 2.5 g (81%), mp 134—141°C, $[\alpha]_D^{29}$ −50.2° (c=1.0, DMF), Rf^1 0.86, Rf^2 0.92, single ninhydrin-positive spot. *Anal.* Calcd for $C_{57}H_{71}Cl_3N_8O_{15}$: C, 56.37; H, 5.89; N, 9.23. Found: C, 56.49; H, 5.47; N, 9.02.

Z(OMe)-Glu(OBzl)-Lys(Z)-Lys(Z)-Glu(OBzl)-Val-NHNH-Troc (X)——This compound was prepared from IX (1.2 g), Z(OMe)–Glu(OBzl)–OH (441 mg) and EEDQ (272 mg) essentially as described for the preparation of I. The product was recrystallized from MeOH and H_2O; yield 1.3 g (93%), mp 114—121°C, $[\alpha]_D^{29}$ −36.3° (c=1.0, DMF), Rf^1 0.81, Rf^2 0.94, single ninhydrin-positive spot. *Anal.* Calcd for $C_{69}H_{84}Cl_3N_9O_{18}$: C, 57.80; H, 5.91; N, 8.79. Found: C, 58.13; H, 5.84; N, 8.46.

Z(OMe)-Glu(OBzl)-Lys(Z)-Lys(Z)-Glu(OBzl)-Val-$NHNH_2$ (XI)——A solution of X (1.4 g) in a mixture of AcOH (5 ml) and DMF (5 ml) was treated with Zn dust (1 g) at room temperature for 3 h. The mixture was filtered, the filtrate was concentrated *in vacuo* and the residue was treated with 1% EDTA. The resulting

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gelatinous mass was washed batchwisely with 1 N $NaHCO_3$ and H_2O and then recrystallized from MeOH; yield 816 mg (68%), mp 124—136°C, $[\alpha]_D^{29}$ −18.9° (c=1.0, DMF), *Anal.* Calcd for $C_{66}H_{83}N_9O_{16}$: C, 62.99; H, 6.65; N, 10.02. Found: C, 63.44; H, 6.63; N, 10.31.

Z(OMe)-Glu(OBzl)-Lys(Z)-Lys(Z)-Glu(OBzl)-Val-Val-Glu(OBzl)-Glu(OBzl)-Ala-Glu(OBzl)-Asn-ONb (XII)——V (315 mg) was treated with TFA (2 ml)-anisole (0.4 ml) as usual and dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and dissolved in DMF (3 ml) containing NMM (0.06 ml). The azide[16] (prepared from 379 mg of XI with 0.4 ml of 6 N HCl in dioxane and 0.1 ml of isoamylnitrite at −60°C) in DMF (2 ml)–DMSO (1 ml) and NMM (0.9 ml) were added to the above ice-chilled solution and the mixture was stirred for 48 h at 4°C. Then the mixture was poured into ice-chilled 1 N $NaHCO_3$ with stirring. Next, 50% NH_4OAc was added dropwise with stirring to form a precipitate. The precipitate was collected and washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O. The product was further purified by column chromatography on silica gel (2.1×40 cm), equilibrated and eluted with $CHCl_3$–water-saturated BuOH–DMF (2: 1: 1). The desired fractions (4 ml each, tube Nos. 26—29) were combined and the solvent was removed by evaporation. Ether was added to the residue to give a precipitate. The product was recrystallized from EtOAc; yield 514 mg (89%), mp 164—172°C, $[\alpha]_D^{29}$ −17.4° (c=1.0, DMF), Rf^1 0.86, Rf^2 0.91, single ninhydrin-positive spot. *Anal.* Calcd for $C_{121}H_{145}N_{15}O_{32}$: C, 62.60; H, 6.30; N, 9.05. Found: C, 62.49; H, 6.04; N, 8.85. Amino acid ratios in acid hydrolysate: Val 2.16, Ala 1.00, Lys 1.84, Glu 4.72, Asp 0.82 (average recovery 84%).

Z(OMe)-Lys(Z)-NHNH-Troc (XIII)——This compound was prepared from Z(OMe)–Lys(Z)–OH (2.1 g), Troc-$NHNH_2$ (1.1 g), HOBT (744 mg) and WSCI (854 mg) essentially as described for the preparation of VI; yield 3.0 g (oily material) (97%), $[\alpha]_D^{29}$ −40.1° (c=1.0, DMF), Rf^1 0.80, Rf^2 0.93, single ninhydrin-positive spot. *Anal.* Calcd for $C_{26}H_{31}Cl_3N_4O_8$: C, 49.26; H, 4.93; N, 8.84. Found: C, 49.08; H, 4.67; N, 8.52.

Z(OMe)-Leu-Lys(Z)-NHNH-Troc (XIV)——This compound was prepared from XIII (2.1 g), Z(OMe)–Leu–OH (1.8 g) and EEDQ (906 mg) essentially as described for the preparation of I; yield 1.8 g (72%), mp 61—64°C, $[\alpha]_D^{30}$ −16.3° (c=1.0, DMF), Rf^1 0.81, Rf^2 0.90, single ninhydrin-positive spot. *Anal.* Calcd for $C_{32}H_{42}Cl_3N_5O_9$: C, 51.15; H, 5.67; N, 9.37. Found: C, 50.87; H, 5.42; N, 9.13.

Z(OMe)-Asp(OBzl)-Leu-Lys(Z)-NHNH-Troc (XV)——This compound was prepared from XIV (1.5 g), Z(OMe)–Asp(OBzl)–OH (853 mg) and EEDQ (545 mg) essentially as described for the preparation of I. The product was recrystallized from MeOH; yield 1.6 g (84%), mp 106—115°C, $[\alpha]_D^{28}$−41.9° (c=1.0, DMF), Rf^1 0.84, Rf^2 0.94, single ninhydrin-positive spot. *Anal.* Calcd for $C_{43}H_{53}Cl_3N_6O_{12}$: C, 54.24; H, 5.61; N, 8.83. Found: C, 53.85; H, 5.39; N, 8.59.

Z(OMe)-Lys(Z)-Asp(OBzl)-Leu-Lys(Z)-NHNH-Troc (XVI)——This compound was prepared from XV (1.4 g), Z(OMe)–Lys(Z)–OH DCHA (1.1 g) and EEDQ (453 mg) essentially as described for the preparation of I. The product was recrystallized from EtOH; yield 1.4 g (78%), mp 91—97°C, $[\alpha]_D^{29}$ −32.8° (c=1.0, DMF), Rf^1 0.87, Rf^2 0.94, single ninhydrin-positive spot. *Anal.* Calcd for $C_{57}H_{71}Cl_3N_8O_{15}$: C, 56.37; H, 5.89; N, 9.23. Found: C, 56.54; H, 5.53; N, 8.98.

Z(OMe)-Thr-Lys(Z)-Asp(OBzl)-Leu-Lys(Z)-NHNH-Troc (XVII)——This compound was prepared from XVI (1.2 g), Z(OMe)–Thr–OH (311 mg) and EEDQ (272 mg) essentially as described for the preparation of I. The product was recrystallized from MeOH and H_2O; yield 923 mg (71%), mp 104—109°C, $[\alpha]_D^{29}$ −18.6° (c=1.0, DMF), Rf^1 0.81, Rf^2 0.90, single ninhydrin-positive spot. *Anal.* Calcd for $C_{61}H_{78}Cl_3N_9O_{17}\cdot 2H_2O$: C, 54.20; H, 6.12; N, 9.33. Found: C, 53.93; H, 6.45; N, 8.98.

Z(OMe)-Thr-Thr-Lys(Z)-Asp(OBzl)-Leu-Lys(Z)-NHNH-Troc (XVIII)——This compound was prepared from XVII (658 mg), Z(OMe)–Thr–OH (156 mg) and EEDQ (136 mg) essentially as described for the preparation of I. The product was recrystallized from EtOH and H_2O; yield 539 mg (76%), mp 138—144°C, $[\alpha]_D^{29}$ −4.3° (c=1.0, DMF), Rf^1 0.87, Rf^2 0.94, single ninhydrin-positive spot. *Anal.* Calcd for $C_{65}H_{85}Cl_3N_{10}O_{19}$: C, 55.10; H, 6.05; N, 9.89. Found: C, 54.82; H, 5.79; N, 9.81.

Z(OMe)-Thr-Thr-Lys(Z)-Asp(OBzl)-Leu-Lys(Z)-$NHNH_2$ (XIX)——This compound was prepared from XVIII (354 mg) and Zn dust (370 mg) essentially as described for the preparation of XI. The product was reprecipitated from MeOH and H_2O; yield 270 mg (87%), mp 118—124°C, $[\alpha]_D^{29}$ −61.3° (c=1.0, DMF). *Anal.* Calcd for $C_{62}H_{84}N_{10}O_7$: C, 59.99; H, 6.82; N, 11.28. Found: C, 59.72; H, 6.54; N, 10.90.

Z(OMe)-Thr-Thr-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-ONb (XX)——XII (464 mg) was treated with TFA (3 ml)–anisole (0.6 ml) as described above. The resulting undecapeptide ester trifluoroacetate was dissolved in DMF (2 ml) containing NMM (0.03 ml). The azide (prepared from 298 mg of XIX with 0.26 ml of 6 N HCl in dioxane and 0.07 ml of isoamylnitrite at −60°C) in DMF (2 ml)–DMSO (1 ml) and NMM (0.04 ml) were added to the above ice-chilled solution and the mixture was stirred for 48 h at 4°C. 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. The product was further purified by column chromatography on silica gel (2.1×48 cm), equilibrated and eluted with water-saturated BuOH–DMF (2: 1). The desired fractions (4 ml each, tube Nos. 31—36) were combined and the solvent was removed by evaporation. Ether was added to the residue to give a precipitate. The product was recrystallized from EtOAc; yield 600 mg (89%), mp 144—156°C, $[\alpha]_D^{29}$ −40.8° (c=1.0, DMF), Rf^1 0.81, Rf^2 0.94, single ninhydrin-positive spot. *Anal.* Calcd for $C_{174}H_{217}N_{23}O_{46}$: C, 62.08; H, 6.50; N, 9.57. Found: C, 62.51; H, 6.16; N, 9.82. Amino acid ratios in acid

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hydrolysate: Val 2.09, Leu 1.12, Ala 1.00, Thr 1.80, Lys 3.73, Glu 4.71, Asp 1.89 (average recovery 84%).

Z(OMe)-Ile-NHNH-Troc (XXI)——This compound was prepared from Z(OMe)–Ile–OH (9.9 g), Troc–$NHNH_2$ (7.6 g), HOBT (5 g) and WSCI (5.7 g) essentially as described for the preparation of VI; yield 13 g (oily material) (81%), $[\alpha]_D^{29}$ 0° (c=1.0, DMF), Rf^1 0.88, Rf^2 0.92, single ninhydrin-positive spot. *Anal.* Calcd for $C_{18}H_{24}Cl_3N_3O_6$: C, 44.60; H, 4.99; N, 8.67. Found: C, 44.29; H, 5.26; N, 8.84.

Z(OMe)-Glu(OBzl)-Ile-NHNH-Troc (XXII)——This compound was prepared from XXI (2.4 g), Z(OMe)–Glu(OBzl)–OH (2.3 g) and EEDQ (1.3 g) essentially as described for the preparation of I. The product was recrystallized from MeOH; yield 2.6 g (74%), mp 129—134°C, $[\alpha]_D^{29}$ −15.0° (c=1.0, DMF), Rf^1 0.71, Rf^2 0.73, single ninhydrin-positive spot. *Anal.* Calcd for $C_{30}H_{37}Cl_3N_4O_9 \cdot 3H_2O$: C, 47.53; H, 5.72; N, 7.39. Found: C, 47.66; H, 5.81; N, 7.15.

Z(OMe)-Ser-Glu(OBzl)-Ile-NHNH-Troc (XXIII)——This compound was prepared from XXII (2.4 g), Z(OMe)–Ser–OH (1 g) and EEDQ (836 mg) essentially as described for the preparation of I. The product was recrystallized from EtOH; yield 2 g (77%), mp 111—116°C, $[\alpha]_D^{29}$ −8.5° (c=1.0, DMF), Rf^1 0.69, Rf^2 0.75, single ninhydrin-positive spot. *Anal.* Calcd for $C_{33}H_{42}Cl_3N_5O_{11}$: C, 48.34; H, 5.54; N, 9.17. Found: C, 48.29; H, 5.38; N, 9.18.

Z(OMe)-Ser-Ser-Glu(OBzl)-Ile-NHNH-Troc (XXIV)——This compound was prepared from XXIII (1.8 g), Z(OMe)–Ser–OH (740 mg) and EEDQ (680 mg) essentially as described for the preparation of I. The product was recrystallized from MeOH; yield 1.7 g (81%), mp 98—104°C, $[\alpha]_D^{29}$ −15.6° (c=1.0, DMF), Rf^1 0.75, Rf^2 0.88, single ninhydrin-positive spot. *Anal.* Calcd for $C_{36}H_{47}Cl_3N_6O_{13}$: C, 49.24; H, 5.40; N, 9.57. Found: C, 49.43; H, 5.56; N, 9.49.

Z(OMe)-Thr-Ser-Ser-Glu(OBzl)-Ile-NHNH-Troc (XXV)——This compound was prepared from XXIV (1.5 g), Z(OMe)–Thr–OH (519 mg) and EEDQ (453 mg) essentially as described for the preparation of I. This compound was recrystallized from MeOH; yield 1.6 g (94%), mp 136—139°C, $[\alpha]_D^{29}$ −48.3° (c=1.0, DMF), Rf^1 0.76, Rf^2 0.84, single ninhydrin-positive spot. *Anal.* Calcd for $C_{40}H_{54}Cl_3N_7O_{15}$: C, 49.06; H, 5.56; N, 10.01. Found: C, 49.02; H, 5.37; N, 9.86.

Z(OMe)-Asp(OBzl)-Thr-Ser-Ser-Glu(OBzl)-Ile-NHNH-Troc (XXVI)——This compound was prepared from XXV (1.4 g), Z(OMe)–Asp(OBzl)–OH (608 mg) and EEDQ (388 mg) essentially as described for the preparation of I. The product was recrystallized from MeOH and H_2O; yield 1.2 g (71%), mp 107—109°, $[\alpha]_D^{29}$ −75.4° (c=1.0, DMF), Rf^1 0.74, Rf^2 0.81, single ninhydrin-positive spot. *Anal.* Calcd for $C_{51}H_{65}Cl_3N_8O_{18}$: C, 51.74; H, 5.54; N, 9.47. Found: C, 51.56; H, 5.68; N, 9.23.

Z(OMe)-Ala-Val-NHNH-Troc (XXVII)——This compound was prepared from VI (1.2 g), Z(OMe)–Ala–OH (647 mg) and EEDQ (680 mg) essentially as described for the preparation of I; yield 1.3 g (oily material) (93%), $[\alpha]_D^{29}$ −11.7° (c=1.0, DMF), Rf^1 0.76, Rf^2 0.92, single ninhydrin-positive spot. *Anal.* Calcd for $C_{20}H_{27}Cl_3N_4O_7 \cdot H_2O$: C, 42.91; H, 5.22; N, 10.01. Found: C, 42.73; H, 5.18; N, 9.85.

Z(OMe)-Ala-Ala-Val-NHNH-Troc (XXVIII)——This compound was prepared from XXVII (1.1,)g Z(OMe)–Ala–OH (517 mg) and EEDQ (545 mg) essentially as described for the preparation of I; yield 1 g (83%), mp 54—57°, $[\alpha]_D^{29}$ −24.8° (c=1.0, DMF), Rf^1 0.73, Rf^2 0.84, single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{32}Cl_3N_5O_8 \cdot H_2O$: C, 43.79; H, 5.43; N, 11.10. Found: C, 43.81; H, 5.14; N, 11.69.

Z(OMe)-Asp(OBzl)-Ala-Ala-Val-NHNH-Troc (XXIX)——This compound was prepared from XXVIII (1 g), Z(OMe)–Asp(OBzl)–OH (711 mg) and EEDQ (453 mg) essentially as described for the preparation of I. The product was recrystallized from MeOH; yield 1.2 g (92%), mp 89—94°C, $[\alpha]_D^{29}$ −30.9° (c=1.0, DMF), Rf^1 0.78, Rf^2 0.89, single ninhydrin-positive spot. *Anal.* Calcd for $C_{34}H_{43}Cl_3N_6O_{11} \cdot 2H_2O$: C, 47.81; H, 5.55; N, 9.84. Found: C, 47.52; H, 5.68; N, 9.61.

Z(OMe)-Ser-Asp(OBzl)-Ala-Ala-Val-NHNH-Troc (XXX)——This compound was prepared from XXIX (1 g), Z(OMe)–Ser–OH (371 mg) and EEDQ (340 mg) essentially as described for the preparation of I. The product was reprecipitated from THF and *n*-hexane; yield 928 mg (84%), mp 102—105°C, $[\alpha]_D^{29}$ −62.1° (c=1.0, DMF), Rf^1 0.71, Rf^2 0.83, single ninhydrin-positive spot. *Anal.* Calcd for $C_{37}H_{48}Cl_3N_7O_{13} \cdot H_2O$: C, 48.14; H, 5.46; N, 10.62. Found: C, 48.32; H, 5.67; N, 10.31.

Z(OMe)-Ser-Asp(OBzl)-Ala-Ala-Val-NHNH$_2$ (XXXI)——This compound was prepared from XXX (905 mg) and Zn dust (1 g) essentially as described for the preparation of XI; yield 816 mg (68%), mp 124—136°C, $[\alpha]_D^{29}$ −18.9° (c=1.0, DMF). *Anal.* Calcd for $C_{66}H_{83}N_9O_{16}$: C, 62.99; H, 6.65; N, 10.02. Found: C, 63.44; H, 6.63; N, 10.31.

Z(OMe)-Ser-Asp(OBzl)-Ala-Ala-Val-Asp(OBzl)-Thr-Ser-Ser-Glu(OBzl)-Ile-NHNH-Troc (XXXII)——XXVI (237 mg) was treated with TFA (2 ml)-anisole (0.4 ml) as described above. The resulting hexapeptide ester trifluoroacetate was dissolved in DMF (2 ml) containing NMM (0.03 ml). The azide (prepared from 193 mg of XXXI with 0.3 ml of 6 N HCl in dioxane and 0.1 ml of isoamylnitrite at −60°C) in DMF (2 ml)–DMSO (1 ml) and NMM (0.6 ml) were added to the above ice-chilled solution and the mixture was stirred for 48 h at 4°C. The mixture was poured into ice-chilled 1 N $NaHCO_3$ with stirring. The precipitate thereby formed was washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O. The product, was purified by column chromatography on silica gel (2.1×48 cm), equilibrated and eluted with $CHCl_3$–water-saturated BuOH (1:2). The desired fractions (4 ml each, tube Nos. 21—25) were combined and the solvent was removed by evaporation. Then, ether was added to the residue to obtain a precipitate; yield 333 mg (97%), mp 103—109°C, $[\alpha]_D^{29}$ −12.4°, (c=1.0, DMF), Rf^1 0.79, Rf^2 0.86, single ninhydrin-positive spot. *Anal.* Calcd for

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$C_{76}H_{100}Cl_3N_{13}O_{26}\cdot 3H_2O$: C, 51.51; H, 6.03; N, 10.28. Found: C, 51.38; H, 6.01; N, 10.14. Amino acid ratios in acid hydrolysate: Val 1.00, Ala 2.21, Ile 1.14, Ser 2.75, Thr 0.84, Glu 0.94, Asp 1.78 (average recovery 81%).

Z(OMe)-Ser-Asp(OBzl)-Ala-Ala-Val-Asp(OBzl)-Thr-Ser-Ser-Glu(OBzl)-Ile-$NHNH_2$ (XXXIII)——This compound was prepared from XXXII (286 mg) and Zn dust (316 mg) essentially as described for the preparation of XI; yield 238 mg (93%), mp 105—110°C, $[\alpha]_D^{29}$ +20.6° (c=1.0, DMF). *Anal.* Calcd for $C_{73}H_{99}N_{13}O_{24}$: C, 56.84; H, 6.47; N, 11.80. Found: C, 56.94; H, 6.69; N, 11.76.

Z(OMe)-Ser-Asp(OBzl)-Ala-Ala-Val-Asp(OBzl)-Thr-Ser-Ser-Glu(OBzl)-Ile-Thr-Thr-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-ONb (XXXIV)——XX (337 mg) was treated with TFA (2 ml)–anisole (0.4 ml) as described above. The resulting heptadecapeptide ester trifluoroacetate was dissolved in N-methyl-2-pyrrolidone (3 ml) containing NMM (0.01 ml). The azide (prepared from 308 mg of XXXIII with 0.26 ml of 6 N HCl in dioxane and 0.07 ml of isoamylnitrite at −60°C) in N-methyl-2-pyrrolidone (2 ml) and NMM (0.2 ml) were added to the above ice-chilled solution and the mixture was stirred for 60 h at 4°C. 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. The product was further purified by column chromatography on silica gel (2.1×48 cm), equilibrated and eluted with BuOH–DMF (1 : 1). The desired fractions (4 ml each, tube Nos. 39—43) were combined and the solvent was removed by evaporation. Ether was added to the residue to give a precipitate. The product was recrystallized from MeOH; yield 331 mg (70%), mp 143—152°C, $[\alpha]_D^{29}$ −21.3° (c=1.0, DMF), Rf^1 0.83, Rf^2 0.90, single ninhydrin-positive spot. *Anal.* Calcd for $C_{238}H_{304}N_{34}O_{67}\cdot 4H_2O$: C, 59.74; H, 6.57; N, 9.95. Found: C, 59.48; H, 6.64; N, 9.80. Amino acid ratios in acid hydrolysate: Val 3.08, Ile 1.12, Leu 1.00, Ala 3.06, Ser 2.72, Thr 2.71, Glu 5.73, Asp 3.81, Lys 3.75 (average recovery 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-Gul-Glu-Ala-Glu-Asn-OH (XXXV)——The protected octaeicosapeptide XXXIV (157 mg) was treated with HF (approximately 4 ml) in the presence of anisole (0.8 ml) in an ice-bath for 1 h. 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 filtrate was adjusted to pH 10 with 1 N NH_4OH and stirred in an ice-chilled bath for 30 min. The pH of the solution was adjusted to pH 5 with a few drops of AcOH and the solution was lyophilized. The crude octaeicosapeptide ester was hydrogenated in a 1 : 1 mixture of AcOH and H_2O (15 ml) for 20 h over Pd-charcoal (200 mg). The catalyst was removed with the aid of cellite. The solution was evaporated to dryness *in vacuo*. The residue was dissolved in 2% AcOH (2 ml), applied to a column of Sephadex G-25 (2.8×96 cm), and eluted with the same solvent. Fractions of 4 ml were collected per 20 min, and the absorption at 230 nm was determined. Fractions corresponding to the front main peak (tube Nos. 81—92) were combined and the solvent was removed by lyophilization. The fluffy powder thus obtained was dissolved in H_2O (1 ml) and subjected to preparative TLC (Whatman PLK-5, 20×20 cm) using the Partridge system as a developing solvent. The zone corresponding to Rf 0.11 was separated and extracted with 2% AcOH. The extracts were concentrated to a small volume and subjected to Sephadex G-25 chromatography as described above; yield 37 mg (36%), mp 181—186°C (dec.), $[\alpha]_D^{29}$ −84.9° (c=1.0, 2 N AcOH), Rf^1 0.06, Rf^2 0.12, single ninhydrin-positive spot. Amino acid ratios in acid hydrolysate: Val 3.12; Ile 1.09, Leu 1.00, Ala 2.83, Ser 2.69, Thr 2.74, Asp 3.79, Glu 5.81, Lys 3.82 (average recovery 81%). Amino acid ratios in AP-M digest: Val 3.09, Ile 0.94, Leu 1.00, Ala 3.02, Ser 2.86, Thr 2.93, Asp 3.04, Glu 5.79, Asn 0.77, Lys 3.90 (average recovery 83%).

E-Rosette Formation——Peripheral blood lymphocytes were isolated in a Hypaque-Ficoll gradient[24] for T-cell rosette formation. Isolated lymphocytes were adjusted to 5×10^5 cell/ml with PBS. Contamination by monocytes and polymorphonuclear cells was assessed according to the method of Tachibana *et al.*[25] Sheep erythrocytes were washed with PBS, and a suspension (6×10^8/ml) was prepared. Lymphocytes were suspended in GVB^{2+} (1 ml) and incubated for 30 min at 37°C with deacetyl-thymosin α_1. Next, they were washed with GVB^{2+} and centrifuged for 10 min at 1500 rpm, then suspended in FCS (1 ml). The suspension was mixed with the suspension of sheep erythrocytes (0.5 ml) and incubated for 16 h at 4°C. The mixture was 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 three erythrocytes was determined.

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References and Notes

1) The amino acid residues are of the L-configuration. 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: EEDQ, N-ethoxycarbony-2-ethoxy-1,2-

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dihydroquinoline; THF, tetrahydrofuran; DCHA, dicyclohexylamine; EDTA, ethylenediaminetetraacetic acid; HOBT, *N*-hydroxybenzotriazole; WSCI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; DCC, dicyclohexylcarbodiimide; Z(OMe), *p*-methoxybenzyloxycarbonyl; Z, benzyloxycarbonyl; OBzl, benzyl ester; ONb, *p*-nitrobenzyl ester; NHNH-Troc, trichloroethyloxycarbonylhydrazide; NMM, *N*-methylmorpholine; DMF, dimethylformamide; TFA, trifluoroacetic acid; HF, hydrogen fluoride; DMSO, dimethylsulfoxide; TLC, thin-layer chromatography; E-rosette, a rosette with sheep erythrocytes; PBS, phosphate-buffered saline; GVB^{2+}, gelatin veronal buffer; FCS, fetal calf serum; AcOH, acetic acid; EtOAc, ethyl acetate.

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