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Synthesis of the Nonatriacontapeptide corresponding to the Entire Amino Acid Sequence of Calf Thymosin β_8 and Its Effect on the Impaired T-Cell Subsets in Patients with Lupus Nephritis[1)]

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The nonatriacontapeptide corresponding to the entire amino acid sequence of calf thymosin β_8 was synthesized by the azide condensation of four fragments, (1—7), (8—20), (21—29) and (30—39), followed by deprotection with hydrogen fluoride in the presence of anisole–thioanisole. The synthetic nonatriacontapeptide increased almost the entire peripheral T-cell population and a suppressor T-cell subset when incubated *in vitro* with a lupus nephritis patient's peripheral blood, but the percentage of a helper T-cell subset did not change under these conditions.

Keywords——calf thymosin β_8; patients with lupus nephritis; monoclonal antibody; β,β,β-trichloroethyloxycarbonylhydrazide; azide condensation

A partially purified thymosin preparation termed "thymosin fraction 5"[2)] has been used most extensively for studies of biological activity,[3-6)] as well as in clinical trials.[7-9)] Thymosin fraction 5 is a potent immunopotentiating preparation and can act instead of the thymus gland to reconstitute some immune functions in thymus-deprived or immuno-deprived individuals.

Analytical polyacrylamide gel electrophoresis and isoelectric focusing have demonstrated that fraction 5 consists of 10—15 major components and 20 or more minor components with molecular weights ranging between 1000 and 15000.[10)]

In 1982, Hannappel *et al.*[11)] elucidated the primary structure of thymosin β_8, which was isolated from calf thymus fraction 5. As shown in Fig. 1, thymosin β_8 is a polypeptide with a molecular weight of 4517, consisting of 39 amino acid residues. The amino terminus of thymosin β_8 is blocked by an acetyl group. Comparison of the sequence of thymosin β_8 with the published sequences of other thymus hormones, such as thymosin β_4 and thymosin β_9 reveals clear homology.[11,12)] It is generally recognized that thymosin β_4 acts on lymphoid stem cells and controls the early stages of the maturation process of thymus-dependent lymphocytes.[12,13)]

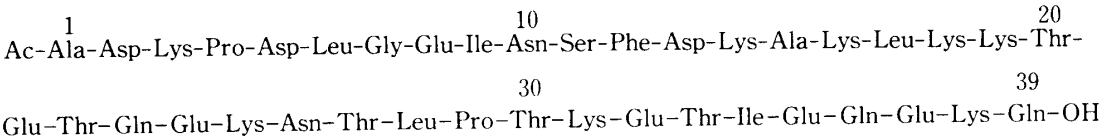

Fig. 1. Structure of Calf Thymosin β_8 (Hannappel *et al.* 1982)

On the other hand, it is generally accepted that a high percentage of lupus nephritis patients have a defect of cell-mediated immunity.[14,15)] In contrast to normal persons, the patients with lupus nephritis were reported to have reduced percentages of all T-cells and suppressor T-cells, while helper T-cells remained normal.[16,17)]

Thymosin β_4 has been chemically synthesized by Wang *et al.*,[13)] but thymosin β_8 has not been synthesized yet. Now we wish to report the synthesis of the nonatriacontapeptide

corresponding to the entire amino acid sequence of calf thymosin β_8. Further, we have tested the *in vitro* effect of the synthetic nonatriacontapeptide on the impaired T-cell subsets of lupus nephritis patients. In the previous papers,[18,19] we reported the syntheses of thymosin α_1 and deacetyl-thymosin α_1 by the solution method and showed that these peptides could increase the E-rosette forming capacity in patients with cell-mediated immunodeficiency diseases. In the present synthesis, as illustrated in Fig. 2, amino acid derivatives bearing protecting groups, *i.e.*, Gln–OBzl, Lys(Z), Glu(OBzl) and Asp(OBzl), which could be removed by treatment with hydrogen fluoride[20] were used. Hydroxy groups of Ser and Thr residues

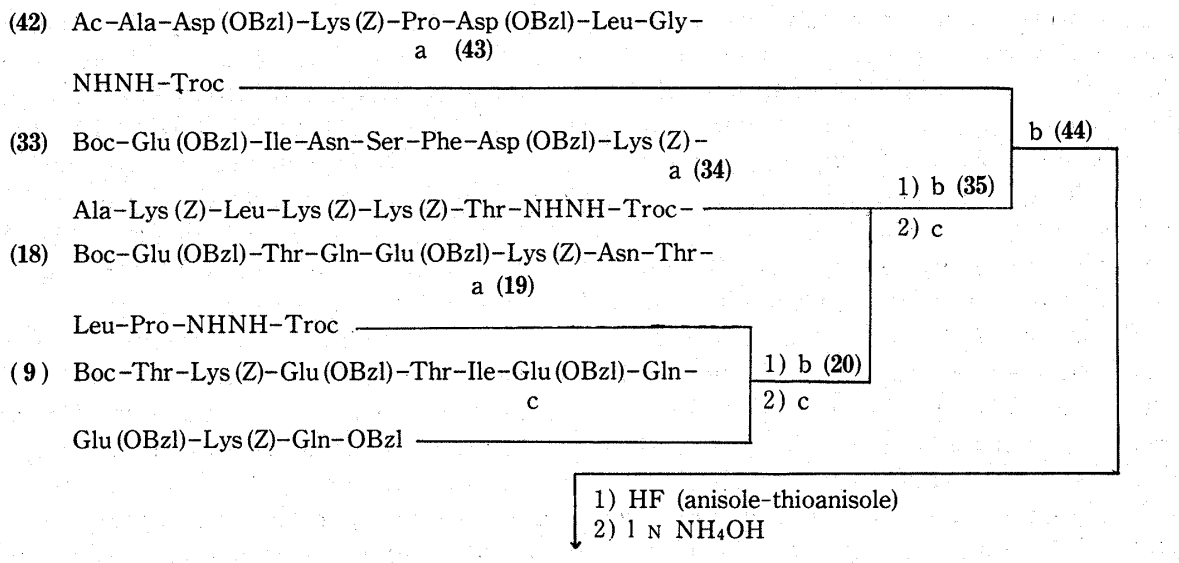

Fig. 2. Synthetic Route to Nonatriacontapeptide corresponding to Calf Thymosin β_8
a, Zn-AcOH; b, azide; c, TFA-anisole.

were not protected. The above 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, four peptide subunits, Boc–(30—39)–OBzl (**9**), Boc–(21—29)–$NHNH_2$ (**19**), Boc–(8—20)–$NHNH_2$ (**34**) and Ac–(1—7)–$NHNH_2$ (**43**) served as building blocks for the construction of the nonatriacontapeptide corresponding to the entire amino acid sequence of calf thymosin β_8.[11] The azide procedure[21] was applied as a main tool to condense these fragments successively, since much less risk of racemization is involved in this procedure, compared to other amido-forming reactions.

First, the C-terminal decapeptide fragment, Boc–(30—39)–OBzl (**9**), was prepared stepwise starting from H–Gln–OBzl Tos by the HOBT–DCC procedure,[22] except for the introduction of a Gln residue, which was introduced by the NP active ester procedure.[23] Next, for the preparation of the three fragments containing Glu(OBzl) and Asp(OBzl), (**18**), (**33**) and (**42**), we employed a substituted hydrazide, Troc–$NHNH_2$,[24] the protecting group of which is known to be removed by Zn[25] without affecting side chain protecting groups such as Z, Ac and Bzl. Thus, these fragments were prepared without exposing the corresponding methyl or ethyl esters to hydrazide. First, Boc–Amino acid was condensed with Troc–$NHNH_2$ by the HOBT–DCC procedure.[22] Then, the three fragments, Boc–(21—29)–NHNH–Troc (**18**), Boc–(8—20)–NHNH–Troc (**33**) and Ac–(1—7)–NHNH–Troc (**42**) were prepared stepwise by the HOBT–DCC procedure[22] except for the introduction of Asn and Gln residues. These residues were also

introduced by the NP active ester procedure,[23] and the Boc groups of intermediates were removed by treatment with TFA–anisole prior to the next coupling reaction.

The four fragments thus obtained were assembled successively according to Fig. 2 by Rudinger's azide procedure.[21] Boc–(21—29)–NHNH–Troc (**18**) was treated with Zn[25] to remove the Troc group, and the last trace of contaminant zinc acetate was removed by treatment with EDTA to give the decapeptide hydrazide (**19**) 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–(30—39)–OBzl (**9**) was removed by the usual TFA–anisole treatment and the corresponding free amine was condensed with Boc–(21—29)–$NHNH_2$ (**19**) by the azide procedure[21] to yield Boc–(21—39)–OBzl (**20**), which was purified by column chromatography on Sephadex LH-20 with DMSO. The homogeneity of the peptide was assessed by elemental analysis, TLC and amino acid analysis of the 6 N HCl hydrolysate. The solubility of protected intermediates in DMF decreased remarkably with chain elongation. Consequently, mixture solvents of DMSO–DMF or NMP–DMF had to be employed for subsequent fragment condensation reactions. Next, after removal of the Troc group of Boc–(8—20)–NHNH–Troc (**33**) by treatment with Zn in AcOH and DMF, the resulting tridecapeptide hydrazide, Boc–(8—20)–$NHNH_2$ (**34**), was condensed with H–(21—39)–OBzl by the azide procedure to yield Boc–(8—39)–OBzl (**35**), which was purified by Sephadex LH-20 column chromatography with DMSO. The homogeneity of the peptide was assessed by elemental analysis, TLC and amino acid analysis of the 6 N HCl hydrolysate. Treatment of Ac–(1—7)–NHNH–Troc (**42**), with Zn in AcOH and DMF afforded the fragment, Ac–(1—7)–$NHNH_2$ (**43**). The final condensation was performed using 3 eq of acyl component (**43**) and after 48 h, additional azide (3 eq) was added. The heptapeptide hydrazide, Ac–(1—7)–$NHNH_2$ (**43**), was condensed with H–(8—39)–OBzl by the azide procedure to yield Ac–(1—39)–OBzl (**44**), which was purified by silica gel column chromatography with BuOH–AcOH–H_2O (4: 1: 5, upper phase). The homogeneity of the peptide was assessed by elemental analysis, TLC and amino acid analysis of the 6 N HCl hydrolysate. The protected nonatriacontapeptide ester thus obtained was treated with hydrogen fluoride in the presence of anisole–thioanisole (1: 1, v/v) to suppress side reaction of the Asp(OBzl) residue.[26] The deprotected nonatriacontapeptide (**45**) was precipitated with ether, converted to the corresponding acetate by treatment with Amberlite CG-4B and then treated with 1 N NH_4OH to reverse a possible N→O shift at the Thr and Ser residues.[27,28] Finally, the product was purified by gel -filtration on Sephadex G-25 using 1% AcOH, followed by partition column chromatography on Sephadex G-25 according to Yamashiro.[29] The nonatriacontapeptide (**45**) thus obtained was found to be homogeneous by paper chromatographies in two different solvent systems.

Its purity was further assessed by amino acid analysis of the 6 N HCl hydrolysate. Amino acid analysis of the 6 N HCl hydrolysate gave molar ratios in good agreement with the expected values. These data indicate clearly that the synthetic nonatriacontapeptide has a high degree of purity.

The *in vitro* effects of the synthetic nonatriacontapeptide on the impaired T-cell subsets in lupus nephritis patients are shown in Table I.

For this analysis, we used monoclonal antibodies to the cell-surface antigens of helper (T 4) and suppressor (T 8) T-cell subsets and to a common T-cell antigen (T 3) defining all peripheral T-cells.[30,31] In contrast to normal persons, we found that the patients with lupus nephritis had reduced percentages of suppressor T-cells and all

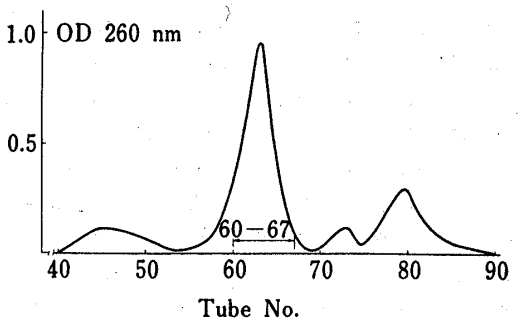

Fig. 3. Purification of Synthetic Nonatriacontapeptide corresponding to Calf Thymosin β_8 by Partition Column Chromatography on Sephadex G-25

Column: 2.8×94 cm. Fraction: 4 ml.

TABLE I. Effects of the Symthetic Nonatriacontapeptide on the Impaired T-Cell Subsets in Patients with Lupus Nephritis

Peptide	Dose (μg/ml)	No. of samples	Reactivity with monoclonal antibodies[d] (%)		
			Anti-T3	Anti-T4	Anti-T8
—[a]		6	64 ± 5	41 ± 5	19 ± 4
Synthetic peptide[a,c]	100	6	67 ± 4	40 ± 5	21 ± 3
—[b]		6	43 ± 4	37 ± 3	8 ± 4
Synthetic peptide[b,c]	100	6	59 ± 3	35 ± 4	17 ± 4

a) Normal venous blood. b) Patient's blood. c) Incubation was carried out for 1 h at 37 °C. d) Each value is the mean ± S. D. for 6 samples.

peripheral T-cells, but the percentage of helper T-cells was at a normal level (Table I). Reduced percentages of suppressor T-cells and all peripheral T-cells have been demonstrated by several investigators[16,17] and also in our laboratory, as described above.

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 nonatriacontapeptide (Table I) shows that, in the group of patients investigated, the synthetic nonatriacontapeptide restored to nearly normal values the percentages of suppressor T-cells and all peripheral T-cells at a dose of 100 μg/ml, but this peptide did not change the percentage of helper T-cells under the same conditions. In normal subjects *in vitro* additions of the synthetic nonatriacontapeptide slightly increased the percentages of suppressor T-cells and all peripheral T-cells (Table I).

These results indicate that the synthetic nonatriacontapeptide has activity to restore the defect of suppressor T-cells *in vitro* in cases of lupus nephritis.

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[32] and Rf^b values refer to BuOH–pyridine–AcOH–H_2O (30: 20: 6: 24).[33] TLC was performed on silica gel (Kieselgel G, Merck) plates and Rf^c values refer to $CHCl_3$–MeOH–H_2O (8: 3: 1). Ac–Ala–OH and Troc-$NHNH_2$ were purchased from the Kokusan Chemical Works, Ltd., Japan. Azide was prepared according to Honzl and Rudinger with isoamylnitrite. 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 lupus nephritis. 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-Lys(Z)-Gln-OBzl (1)——HOBT (1.5 g) and WSCI (2.2 g) were added to a mixture of Boc–Lys(Z)–OH DCHA (6 g) and H–Gln–OBzl Tos (4.1 g) in DMF (25 ml). After being stirred at 0°C for 3 h and at room temperature for 12 h, the mixture was extracted with EtOAc and the extract was washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O, dried over $MgSO_4$ and then concentrated *in vacuo*. The residue was recrystallized from MeOH and ether; yield 4.8 g (80%), mp 135—139°C, $[\alpha]_D^{26}$ −24.3° (c=1.0, DMF), Rf^a 0.88, Rf^b 0.90, single ninhydrin-positive spot. *Anal.* Calcd for $C_{31}H_{42}N_4O_8$: C, 62.19; H, 7.07; N, 9.36. Found: C, 61.90; H, 7.35; N, 9.32.

Boc-Glu(OBzl)-Lys(Z)-Gln-OBzl (2)——1 (3 g) was treated with TFA–anisole (10 ml–2 ml) as usual, then TFA was removed by evaporation. The residue was washed with dry ether, dried over KOH pellets *in vacuo* and dissolved in DMF (20 ml) containing NMM (0.56 ml). To this ice-chilled solution, Boc–Glu(OBzl)–OH (1.9 g), HOBT (744 mg) and WSCI (1.1 g) were successively added. After being stirred at 0°C for 3 h and at room temperature for 12 h, the mixture was extracted with EtOAc and the extract was washed successively

with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O, dried over $MgSO_4$ and then concentrated *in vacuo*. The residue was recrystallized from EtOAc and *n*-hexane; yield 3.8 g (93%), mp 80—84°C, $[\alpha]_D^{26}$ −3.1° (c=1.0, DMF), Rf^a 0.88, Rf^b 0.92, single ninhydrin-positive spot. *Anal.* Calcd for $C_{43}H_{55}N_5O_{11}$: C, 63.14; H, 6.78; N, 8.56. Found: C, 63.05; H, 6.92; N, 8.75.

Boc-Gln-Glu(OBzl)-Lys(Z)-Gln-OBzl (3)——**2** (3 g) was treated with TFA–anisole (10 ml–2 ml) as usual and the resulting powder was dissolved in DMF (20 ml) together with NMM (0.38 ml). Boc–Gln–ONp (1.8 g) was added and the solution was stirred at room temperature for 16 h. The reaction mixture was diluted with 1 N NH_4OH (4 ml) with stirring to saponify the unchanged *p*-nitrophenyl ester. After 1 h, the mixture was extracted with EtOAc and the extract was washed successively with 1 N NH_4OH, H_2O, 1 N citric acid and H_2O, dried over $MgSO_4$ and evaporated *in vacuo*. The residue was recrystallized from MeOH and ether; yield 2.4 g (69%), mp 118—123°C, $[\alpha]_D^{26}$ −21.4° (c=1.0, DMF), Rf^a 0.73, Rf^b 0.89, single ninhydrin-positive spot. *Anal.* Calcd for $C_{48}H_{63}N_7O_{13}$: C, 60.94; H, 6.71; N, 10.36. Found: C, 60.68; H, 6.70; N, 10.54.

Boc-Glu(OBzl)-Gln-Glu(OBzl)-Lys(Z)-Gln-OBzl (4)——This compound was prepared from **3** (1.4 g), Boc–Glu(OBzl)–OH (530 mg), HOBT (212 mg) and WSCI (310 mg) essentially as described for the preparation of **2**. The product was reprecipitated from MeOH and ether; yield 1.3 g (76%), mp 86—91°C, $[\alpha]_D^{26}$ −16.9° (c=1.0, DMF), Rf^a 0.76, Rf^b 0.82, single ninhydrin-positive spot. *Anal.* Calcd for $C_{60}H_{76}N_8O_{16}$: C, 61.84; H, 6.57; N, 9.62. Found: C, 62.12; H, 6.49; N, 9.38.

Boc-Ile-Glu(OBzl)-Gln-Glu(OBzl)-Lys(Z)-Gln-OBzl (5)——This compound was prepared from **4** (1.2 g), Boc–Ile–OH (254 mg), HOBT (149 mg) and WSCI (218 mg) essentially as described for the preparation of **2**. The product was reprecipitated from EtOAc and ether; yield 1.1 g (85%), mp 70—73°C, $[\alpha]_D^{26}$ −4.9° (c=1.0, DMF), Rf^a 0.78, Rf^b 0.83, single ninhydrin-positive spot. *Anal.* Calcd for $C_{66}H_{87}N_9O_{17}\cdot 2H_2O$: C, 60.31; H, 6.98; N, 9.59. Found: C, 60.16; H, 6.99; N, 9.37.

Boc-Thr-Ile-Glu(OBzl)-Gln-Glu(OBzl)-Lys(Z)-Gln-OBzl (6)——This compound was prepared from **5** (984 mg), Boc–Thr–OH (186 mg), HOBT (114 mg) and WSCI (167 mg) as described for the preparation of **2**. The product was recrystallized from MeOH and ether; yield 756 mg (69%), mp 116—121°C, $[\alpha]_D^{26}$ −20.7° (c=1.0, DMF), Rf^a 0.78, Rf^b 0.84, single ninhydrin-positive spot. *Anal.* Calcd for $C_{70}H_{94}N_{10}O_{19}$: C, 60.94; H, 6.87; N, 10.15. Found: C, 61.19; H, 7.25; N, 9.78.

Boc-Glu(OBzl)-Thr-Ile-Glu(OBzl)-Gln-Glu(OBzl)-Lys(Z)-Gln-OBzl (7)——This material was prepared from **6** (690 mg), Boc–Glu(OBzl)–OH (186 mg), HOBT (75 mg) and WSCI (109 mg) as described for the preparation of **2**. The product was recrystallized from EtOAc and ether; yield 587 mg (73%), mp 106—113°C, $[\alpha]_D^{26}$ −4.5° (c=1.0, DMF), Rf^a 0.76, Rf^b 0.89, single ninhydrin-positive spot. *Anal.* Calcd for $C_{82}H_{107}N_{11}O_{22}$: C, 61.60; H, 6.75; N, 9.64. Found: C, 61.29; H, 6.59; N, 9.93.

Boc-Lys(Z)-Glu(OBzl)-Thr-Ile-Glu(OBzl)-Gln-Glu(OBzl)-Lys(Z)-Gln-OBzl (8)——**7** (400 mg) was treated with TFA–anisole (3 ml–0.6 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–DMSO (3 ml–3 ml). To this ice-chilled solution, Boc–Lys(Z)–OH DCHA (154 mg), HOBT (38 mg) and WSCI (54 mg) were successively added. After being stirred at 0°C for 3 h and at room temperature for 18 h, 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 dried product was recrystallized from hot EtOAc; yield 357 mg (76%), mp 139—145°C, $[\alpha]_D^{26}$ −37.5° (c=1.0, DMF), Rf^a 0.80, Rf^b 0.84, single ninhydrin-positive spot. *Anal.* Calcd for $C_{96}H_{125}N_{13}O_{25}$: C, 61.96; H, 6.77; N, 9.78. Found: C, 62.30; H, 7.01; N, 9.49.

Boc-Thr-Lys(Z)-Glu(OBzl)-Thr-Ile-Glu(OBzl)-Gln-Glu(OBzl)-Lys(Z)-Gln-OBzl (9)——This compound was prepared from **8** (233 mg), Boc–Thr–OH (32 mg), HOBT (20 mg) and WSCI (30 mg) essentially as described for the preparation of **8**. The product was recrystallized from hot MeOH; yield 217 mg (88%), mp 140—146°C, $[\alpha]_D^{26}$ −32.6° (c=1.0, DMF), Rf^a 0.83, Rf^b 0.88, single ninhydrin-positive spot. *Anal.* Calcd for $C_{100}H_{132}N_{14}O_{27}$: C, 61.21; H, 6.78; N, 9.99. Found: C, 60.84; H, 7.13; N, 10.05.

Boc-Pro-NHNH-Troc (10)——HOBT (5 g) and WSCI (7.3 g) were added to a solution of Boc–Pro–OH (7.2 g) and Troc–$NHNH_2$ (7.6 g) in tetrahydrofuran (30 ml) with stirring at 0°C. The reaction mixture was stirred for 16 h at 4°C. Then, the mixture was extracted with EtOAc and the extract was washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O, dried over $MgSO_4$, then concentrated *in vacuo*. The residue was reprecipitated from EtOAc and petroleum ether. Attempts to crystallize the oily residue were unsuccessful; yield 12.4 g (oily material) (92%), $[\alpha]_D^{26}$ −13.6° (c=1.0, DMF), Rf^a 0.64, Rf^b 0.69, single ninhydrin-positive spot. *Anal.* Calcd for $C_{13}H_{20}Cl_3N_3O_5\cdot H_2O$: C, 36.94; H, 5.25; N, 9.94. Found: C, 36.85; H, 5.41; N, 9.62.

Boc-Leu-Pro-NHNH-Troc (11)——This compound was prepared from **10** (4.1 g), Boc–Leu–OH (2.8 g), HOBT (1.5 g) and WSCI (2.2 g) essentially as described for the preparation of **2**. The product was reprecipitated from EtOAc and petroleum ether; yield 4.4 g (83%), mp 70—72°C, $[\alpha]_D^{26}$ −12.4° (c=1.0, DMF), Rf^a 0.72, Rf^b 0.65, single ninhydrin-positive spot. *Anal.* Calcd for $C_{19}H_{31}Cl_3N_4O_6$: C, 44.07; H, 6.03; N, 10.82. Found: C, 43.81; H, 6.35; N, 10.76.

Boc-Thr-Leu-Pro-NHNH-Troc (12)——This material was prepared from **11** (2.6 g), Boc–Thr–OH (1.2 g), HOBT (744 mg) and WSCI (1.1 g) as described for the preparation of **2**; yield 3 g (97%), mp 64—66°C, $[\alpha]_D^{26}$ −19.1° (c=1.0, DMF), Rf^a 0.14, Rf^b 0.40, single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{38}Cl_3N_5O_8$: C, 44.63; H, 6.19; N, 11.32. Found: C, 44.27; H, 0.48; N, 11.26.

Boc-Asn-Thr-Leu-Pro-NHNH-Troc (13)——This compound was prepared from **12** (3.1 g) and Boc–Asn–ONp (2 g) as described for the preparation of **3**. The product was reprecipitated from EtOAc and *n*-hexane; yield 2.9 g (78%), mp 73—76°C, $[\alpha]_D^{26}$ −13.2° (c=1.0, DMF), Rf^a 0.64, Rf^b 0.71, single ninhydrin-positive spot. *Anal.* Calcd for $C_{27}H_{44}Cl_3N_7O_{10}$: C, 44.24; H, 6.05; N, 13.38. Found: C, 43.87; H, 6.11; N, 13.40.

Boc-Lys(Z)-Asn-Thr-Leu-Pro-NHNH-Troc (14)——This compound was prepared from **13** (2 g), Boc–Lys(Z)–OH DCHA (1.7 g), HOBT (393 mg) and WSCI (587 mg) essentially as described for the preparation of **2**; yield 1.7 g (63%), mp 71—74°C, $[\alpha]_D^{26}$ −11.4° (c=1.0, DMF), Rf^a 0.72, Rf^b 0.79, single ninhydrin-positive spot. *Anal.* Calcd for $C_{41}H_{62}Cl_3N_9O_{13}$: C, 49.48; H, 6.28; N, 12.67. Found: C, 49.20; H, 6.52; N, 12.39.

Boc-Glu(OBzl)-Lys(Z)-Asn-Thr-Leu-Pro-NHNH-Troc (15)——This compound was prepared from **14** (1 g), Boc–Glu(OBzl)–OH (371 mg), HOBT (149 mg) and WSCI (218 mg) as described for the preparation of **2**; yield 916 mg (76%), mp 72—75°C, $[\alpha]_D^{26}$ −10.5° (c=1.0, DMF), Rf^a 0.52, Rf^b 0.63, single ninhydrin-positive spot. *Anal.* Calcd for $C_{53}H_{75}Cl_3N_{10}O_{16}$: C, 52.41; H, 6.22; N, 11.53. Found: C, 52.67; H, 6.56; N, 11.22.

Boc-Gln-Glu(OBzl)-Lys(Z)-Asn-Thr-Leu-Pro-NHNH-Troc (16)——This compound was prepared from **15** (810 mg) and Boc–Gln–ONp (314 mg) as described for the preparation of **3**. The product was recrystallized from EtOAc and ether; yield 731 mg (82%), mp 84—87°C, $[\alpha]_D^{26}$ −12.3° (c=1.0, DMF), Rf^a 0.70, Rf^b 0.74, single ninhydrin-positive spot. *Anal.* Calcd for $C_{58}H_{83}Cl_3N_{12}O_{18}$: C, 51.88; H, 6.23; N, 12.52. Found: C, 51.50; H, 6.04; N, 12.89.

Boc-Thr-Gln-Glu(OBzl)-Lys(Z)-Asn-Thr-Leu-Pro-NHNH-Troc (17)——This compound was prepared from **16** (671 mg), Boc–Thr–OH (120 mg), HOBT (75 mg) and WSCI (109 mg) essentially as described for the preparation of **2**. The product was reprecipitated from EtOAc and ether; yield 558 mg (77%), mp 107—113°C, $[\alpha]_D^{26}$ −16.5° (c=1.0, DMF), Rf^a 0.61, Rf^b 0.73, single ninhydrin-positive spot. *Anal.* Calcd for $C_{62}H_{90}Cl_3N_{13}O_{20}$: C, 51.58; H, 6.28; N, 12.61. Found: C, 51.67; H, 5.96; N, 12.40.

Boc-Glu(OBzl)-Thr-Gln-Glu(OBzl)-Lys(Z)-Asn-Thr-Leu-Pro-NHNH-Troc (18)——This material was prepared from **17** (481 mg), Boc–Glu(OBzl)–OH (124 mg), HOBT (50 mg) and WSCI (73 mg) as described for the preparation of **2**. The product was reprecipitated from EtOAc and ether; yield 432 mg (78%), mp 97—101°C, $[\alpha]_D^{26}$ −18.7° (c=1.0, DMF), Rf^a 0.79, Rf^b 0.84, single ninhydrin-positive spot. *Anal.* Calcd for $C_{74}H_{103}Cl_3N_{14}O_{23}\cdot 3H_2O$: C, 51.76; H, 6.40; N, 11.42. Found: C, 51.50; H, 6.38; N, 11.21.

Boc-Glu(OBzl)-Thr-Gln-Glu(OBzl)-Lys(Z)-Asn-Thr-Leu-Pro-NHNH$_2$ (19)——**18** (333 mg) in a mixture of AcOH (2.5 ml) and DMF (2.5 ml) was treated with Zn dust (130 mg) at room temperature for 12 h. The solution was filtered, the filtrate was concentrated *in vacuo* and the residue was treated with a saturated solution of EDTA, then with $NaHCO_3$ to adjust the pH to neutral. The resulting powder was washed with H_2O and recrystallized from MeOH; yield 251 mg (84%), mp 151—159°C (dec.), $[\alpha]_D^{26}$ −23.4° (c=1.0, DMF), Rf^a 0.64, Rf^b 0.65, sngle hydrazine-test-positive spot. *Anal.* Calcd for $C_{71}H_{102}N_{14}O_{21}$: C, 57.32; H, 6.91; N, 13.18. Found: C, 57.46; H, 7.20; N, 13.06.

Boc-Glu(OBzl)-Thr-Gln-Glu(OBzl)-Lys(Z)-Asn-Thr-Leu-Pro-Thr-Lys(Z)-Glu(OBzl)-Thr-Ile-Glu(OBzl)-Gln-Glu(OBzl)-Lys(Z)-Gln-OBzl (20)——**9** (200 mg) was treated with TFA–anisole (2 ml–0.4 ml) as usual and the N^a-deprotected peptide isolated as described above was dissolved in DMSO–DMF (1: 1, 2 ml) containing NMM (0.01 ml). The azide (prepared from 224 mg of **19**) in DMF (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 poured into ice-chilled 1 N $NaHCO_3$ with stirring and the resulting powder thus formed was washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O and precipitated twice from DMF with H_2O. For further purification, the product was dissolved in DMSO (3 ml) and the solution was applied to a column of Sephadex LH-20 (3×97 cm), which was eluted with the same solvent with a flow rate of 4 ml/12 min. The ultraviolet spectrum (UV) absorption at 260 nm was determined in each fraction. The fractions corresponding to the main peak (tube Nos. 42—51) were combined and the solvent was removed by evaporation. The residue was treated with ether to afford a powder; yield 251 mg (72%), mp 153—159°C, $[\alpha]_D^{26}$ −27.9° (c=1.0, DMSO), Rf^c 0.52, single ninhydrin-positive spot. *Anal.* Calcd for $C_{166}H_{222}N_{26}O_{46}\cdot 5H_2O$: C, 58.51; H, 6.86; N, 10.69. Found: C, 58.23; H, 7.16; N, 10.37. Amino acid ratios in a 6 N HCl hydrolysate: Asp 0.89, Glu 8.01, Thr 3.72, Ile 1.16, Leu 1.00, Pro 1.06, Lys 3.15 (recovery of Leu 78%).

Boc-Thr-NHNH-Troc (21)——This compound was prepared from Boc–Thr–OH (5.5 g), Troc–$NHNH_2$ (5.4 g), HOBT (3.7 g) and WSCI (5.4 g) essentially as described for the preparation of **10**; yield 7.9 g (77%), mp 78—80°C, $[\alpha]_D^{26}$ −15.1° (c=1.0, DMF), Rf^a 0.88, Rf^b 0.91, single ninhydrin-positive spot. *Anal.* Calcd for $C_{12}H_{20}Cl_3N_3O_6$: C, 35.27; H, 4.93; N, 10.28. Found: C, 35.61; H, 5.30; N, 10.73.

Boc-Lys(Z)-Thr-NHNH-Troc (22)——This compound was prepared from **21** (4.1 g), Boc–Lys(Z)–OH DCHA (6.2 g), HOBT (1.5 g) and WSCI (2.2 g) as described for the preparation of **2**. The product was reprecipitated from EtOAc and petroleum ether; yield 5.8 g (87%), mp 61—63°C, $[\alpha]_D^{26}$ −19.0° (c=1.0, DMF), Rf^a 0.79, Rf^b 0.78, single ninhydrin-positive spot. *Anal.* Calcd for $C_{26}H_{38}Cl_3N_5O_9\cdot H_2O$: C, 45.33; H, 5.85; N, 10.17. Found: C, 45.20; H, 5.94; N, 10.26.

Boc-Lys(Z)-Lys(Z)-Thr-NHNH-Troc (23)——This material was prepared from **22** (3.4 g), Boc–Lys(Z)–OH DCHA (3.1 g), HOBT (744 mg) and WSCI (1.1 g) as described for the preparation of **2**; yield 4.2 g (89%), mp 64—66°C, $[\alpha]_D^{26}$ −15.2° (c=1.0, DMF), Rf^a 0.72, Rf^b 0.79, single ninhydrin-positive spot. *Anal.* Calcd for $C_{40}H_{56}Cl_3N_7O_{12}\cdot H_2O$: C, 50.50; H, 6.15; N, 10.31. Found: C, 50.35; H, 6.42; N, 10.07.

Boc-Leu-Lys(Z)-Lys(Z)-Thr-NHNH-Troc (24)——This compound was prepared from **23** (3.1 g), Boc-Leu-OH (914 mg), HOBT (495 mg) and WSCI (725 mg) essentially as described for the preparation of **2**. The product was reprecipitated from MeOH and ether; yield 2.3 g (66%), mp 80—86°C, $[\alpha]_D^{26}$ −2.9° (c=1.0, DMF), Rf^a 0.83, Rf^b 0.93, single ninhydrin-positive spot. *Anal.* Calcd for $C_{46}H_{67}Cl_3N_8O_{13}$: C, 52.80; H, 6.45; N, 10.71. Found: C, 52.98; H, 6.70; N, 10.47.

Boc-Lys(Z)-Leu-Lys(Z)-Lys(Z)-Thr-NHNH-Troc (25)——This compound was prepared from **24** (2.1 g), Boc-Lys(Z)-OH DCHA (1.2 g), HOBT (297 mg) and WSCI (435 mg) as described for the preparation of **2**. The product was reprecipitated fom EtOAc and ether; yield 2 g (80%), mp 83—86°C, $[\alpha]_D^{26}$ −3.6° (c=1.0, DMF), Rf^a 0.75, Rf^b 0.93, single ninhydrin-positive spot. *Anal.* Calcd for $C_{60}H_{85}Cl_3N_8O_{13}\cdot 2H_2O$: C, 56.80; H, 7.07; N, 8.83. Found: C, 56.49; H, 7.08; N, 8.56.

Boc-Ala-Lys(Z)-Leu-Lys(Z)-Lys(Z)-Thr-NHNH-Troc (26)——This material was prepared from **25** (19 g), Boc-Ala-OH (297 mg), HOBT (212 mg) and WSCI (310 mg) as described for the preparation of **2**. The product was reprecipitated from MeOH and ether; yield 1.7 g (85%), mp 102—106°C, $[\alpha]_D^{26}$ −11.4° (c=1.0, DMF), Rf^a 0.84, Rf^b 0.94, single ninhydrin-positive spot. *Anal.* Calcd for $C_{63}H_{90}Cl_3N_9O_{14}$: C, 58.04; H, 6.96; N, 9.67. Found: C, 58.23; H, 6.91; N, 10.03.

Boc-Lys(Z)-Ala-Lys(Z)-Leu-Lys(Z)-Lys(Z)-Thr-NHNH-Troc (27)——This compound was prepared from **26** (1.5 g), Boc-Lys(Z)-OH DCHA (686 mg), HOBT (165 mg) and WSCI (242 mg) as described for the preparation of **2**. The product was reprecipitated from MeOH and ether; yield 1.3 g (72%), mp 84—88°C, $[\alpha]_D^{26}$ −14.1° (c=1.0, DMF), Rf^a 0.72, Rf^b 0.84, single ninhydrin-positive spot. *Anal.* Calcd for $C_{77}H_{108}Cl_3N_{11}O_{17}\cdot 3H_2O$: C, 57.08; H, 7.09; N, 9.51. Found: C, 57.01; H, 6.98; N, 9.30.

Boc-Asp(OBzl)-Lys(Z)-Ala-Lys(Z)-Leu-Lys(Z)-Lys(Z)-Thr-NHNH-Troc (28)——This compound was prepared from **27** (1.1 g), Boc-Asp(OBzl)-OH (254 mg), HOBT (107 mg) and WSCI (155) gm as described for the preparation of **2**. The product was reprecipitated from acetone and ether; yield 930 mg (75%), mp 102—106°C, $[\alpha]_D^{26}$ −25.6° (c=1.0, DMF), Rf^a 0.81, Rf^b 0.89, single ninhydrin-positive spot. *Anal.* Calcd for $C_{88}H_{119}Cl_3N_{12}O_{20}$: C, 59.67; H, 6.77; N, 9.49. Found: C, 59.40; H, 6.83; N, 9.62.

Boc-Phe-Asp(OBzl)-Lys(Z)-Ala-Lys(Z)-Leu-Lys(Z)-Lys(Z)-Thr-NHNH-Troc (29)——**28** (886 mg was) treated with TFA–anisole (4 ml–0.5 ml) as usual, then dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* and dissolved in DMF (10 ml) containing NMM (0.06 ml). To this ice-chilled solution, Boc-Phe-OH (146 mg), HOBT (75 mg) and WSCI (109 mg) were added. After being stirred at 0°C for 3 h and at room temperature for 18 h, the mixture was poured into ice-chilled 1 N $NaHCO_3$ with stirring. The precipitate formed was washed as described above. The precipitate was reprecipitated from AcOH and H_2O; yield 746 mg (78%), mp 96—103°C, $[\alpha]_D^{26}$ −36.2° (c=1.0, DMF), Rf^a 0.83, Rf^b 0.87, single ninhydrin-positive spot. *Anal.* Calcd for $C_{97}H_{128}Cl_3N_{13}O_{21}$: C, 60.73; H, 6.73; N, 9.49. Found: C, 60.90; H, 7.06; N, 9.24.

Boc-Ser-Phe-Asp(OBzl)-Lys(Z)-Ala-Lys(Z)-Leu-Lys(Z)-Lys(Z)-Thr-NHNH-Troc (30)——This compound was prepared from **29** (640 mg), Boc-Ser-OH (75 mg), HOBT (50 mg) and WSCI (73 mg) essentially as described for the preparation of **29**. The product was reprecipitated from MeOH and ether; yield 532 mg (79%), mp 124—129°C, $[\alpha]_D^{26}$ −18.3° (c=1.0, DMF), Rf^a 0.78, Rf^b 0.89, single ninhydrin-positive spot. *Anal.* Calcd for $C_{100}H_{133}Cl_3N_{14}O_{23}$: C, 59.89; H, 6.69; N, 9.78. Found: C, 60.24; H, 6.65; N, 10.10.

Boc-Asn-Ser-Phe-Asp(OBzl)-Lys(Z)-Ala-Lys(Z)-Leu-Lys(Z)-Lys(Z)-Thr-NHNH-Troc (31)——**30** (401 mg) was treated with TFA–anisole (3 ml–0.6 ml) as described above and the resulting powder was dissolved in DMF (5 ml) together with NMM (0.02 ml). Boc-Asn-ONp (80 mg) was added and the solution was stirred at room temperature for 24 h. The reaction mixture was diluted with 1 N NH_4OH (1 ml) with stirring to saponify the unchanged *p*-nitrophenyl ester. After 1 h, the mixture was poured into ice-chilled 1 N NH_4OH with stirring. The precipitate thereby formed was purified by washing as described above, followed by precipitation from MeOH and ether; yield 378 mg (89%), mp 146—151°C, $[\alpha]_D^{24}$ −18.3° (c=1.0, DMF), Rf^a 0.74, Rf^b 0.82, single ninhydrin-positive spot. *Anal.* Calcd for $C_{104}H_{139}Cl_3N_{16}O_{25}$: C, 58.93; H, 6.61; N, 10.57. Found: C, 59.20; H, 6.54; N, 10.21.

Boc-Ile-Asn-Ser-Phe-Asp(OBzl)-Lys(Z)-Ala-Lys(Z)-Leu-Lys(Z)-Lys(Z)-Thr-NHNH-Troc (32)——This compound was prepared from **31** (303 mg), Boc-Ile-OH (40 mg), HOBT (24 mg) and WSCI (34 mg) as described for the preparation of **29**. The product was recrystallized from hot EtOAc; yield 301 mg (95%), mp 163—169°C, $[\alpha]_D^{26}$ −9.8° (c=1.0, DMF), Rf^a 260.80, Rf^b 0.89, single ninhydrin-positive spot. *Anal.* Calcd for $C_{110}H_{150}Cl_3N_{17}O_{26}\cdot 5H_2O$: C, 56.88; H, 6.94; N, 10.25. Found: C, 56.92; H, 6.77; N, 9.86.

Boc-Glu(OBzl)-Ile-Asn-Ser-Phe-Asp(OBzl)-Lys(Z)-Ala-Lys(Z)-Leu-Lys(Z)-Lys(Z)-Thr-NHNH-Troc (33)——This compound was prepared from **32** (280 mg), Boc-Glu(OBzl)-OH (50 mg), HOBT (20 mg) and WSCI (30 mg) as described for the preparation of **29**. The product was reprecipitated from DMF and H_2O; yield 258 mg (84%), mp 189—192°C, $[\alpha]_D^{24}$ −22.7° (c=1.0, DMF), Rf^a 0.84, Rf^b 0.88, single ninhydrin-positive spot. *Anal.* Calcd for $C_{122}H_{163}Cl_3N_{18}O_{20}\cdot 4H_2O$: C, 58.05; H, 6.83; N, 9.99. Found: C, 58.01; H, 6.76; N, 9.68.

Boc-Glu(OBzl)-Ile-Asn-Ser-Phe-Asp(OBzl)-Lys(Z)-Ala-Lys(Z)-Leu-Lys(Z)-Lys(Z)-Thr-NHNH$_2$ (34)——This compound was prepared from **33** (223 mg) and Zn dust (59 mg) essentially as described for the preparation of **19**. The product was reprecipitated from DMF and ether; yield 191 mg (92%), mp 171—176°C (dec.), $[\alpha]_D^{26}$ −32.7° (c=1.0, DMF), Rf^a 0.77, Rf^b 0.86, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{119}H_{162}N_{18}O_{27}\cdot 5H_2O$: C, 60.39; H, 7.33; N, 10.65. Found: C, 60.45; H, 7.21; N, 10.53.

Boc-Glu (OBzl)-Ile-Asn-Ser-Phe-Asp (OBzl)-Lys(Z)-Ala-Lys (Z)-Leu-Lys (Z)-Lys (Z)-Thr-Glu (OBzl)-Thr-Gln-Glu(OBzl)-Lys(Z)-Asn-Thr-Leu-Pro-Thr-Lys(Z)-Glu(OBzl)-Thr-Ile-Glu (OBzl)-Gln-Glu (OBzl)-Lys (Z)-Gln-OBzl (35)——Boc-(21—39)-OBzl (**20**) (166 mg) was treated with TFA–anisole (2 ml–0.4 ml) in an ice-bath for 60 min, then dry ether was added. The resulting powder was dissolved in DMF–NMP (1.5 ml–1.5 ml) containing NMM (0.005 ml). The azide (prepared from 171 mg of **34**) in DMF (2 ml) and NMM (0.01 ml) were added and the mixture was stirred at 4°C for 30 h. Additional azide (prepared from the same amount of the hydrazide) in DMF (2 ml) and NMM (0.01 ml) were added and stirring was continued for an additional 34 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 crystallized from hot EtOAc. The product was further purified by column chromatography on Sephadex LH-20 (3×96 cm), equilibrated and eluted with DMSO. The UV absorption at 260 nm was determined. The fractions corresponding to the main peak (tube Nos. 40—47) were combined and the solvent was removed by evaporation. The residue was treated with ether to afford a powder; yield 147 mg (53%), mp 162—169°C (dec.), $[\alpha]_D^{24}$ −18.7° (c=0.3, DMSO), Rf^c 0.54, single chlorine–tolidine-positive spot. *Anal.* Calcd for $C_{280}H_{372}N_{44}O_{74}\cdot 6H_2O$: C, 59.56; H, 6.86; N, 10.92. Found: C, 59.70; H, 6.65; N, 10.51. Amino acid ratios in a 6 N HCl hydrolysate: Asp 2.80, Ser 0.74; Glu 9.19, Thr 4.70, Ala 1.00, Ile 2.26, Leu 2.21, Phe 0.84, Pro 0.80, Lys 7.03 (recovery of Ala 81%).

Boc-Gly-NHNH-Troc (36)——This compound was prepared from Boc–Gly–OH (1.8 g), Troc–$NHNH_2$ (2.3 g), HOBT (1.5 g) and WSCI (2.2 g) essentially as described for the preparation of **10**; yield 3.2 g (82%), mp 124—125°C, Rf^a 0.80, Rf^b 0.92, single ninhydrin-positive spot. *Anal.* Calcd for $C_{10}H_{18}Cl_3N_3O_6$: C, 31.39; H, 4.74; N, 10.98. Found: C, 31.38; H, 4.37; N, 10.64.

Boc-Leu-Gly-NHNH-Troc (37)——This compound was prepared from **36** (2.6 g), Boc–Leu–OH (1.9 g), HOBT (1 g) and WSCI (1.4 g) as described for the preparation of **2**. The product was reprecipitated from EtOAc and petroleum ether; yield 2.2 g (65%), mp 93—98°C, $[\alpha]_D^{26}$ −8.1° (c=1.0, DMF), Rf^a 0.79, Rf^b 0.88, single ninhydrin-positive spot. *Anal.* Calcd for $C_{16}H_{29}Cl_3N_4O_7$: C, 38.76; H, 5.90; N, 11.30. Found: C, 38.93; H, 5.81; N, 11.63.

Boc-Asp(OBzl)-Leu-Gly-NHNH-Troc (38)——This compound was prepared from **37** (1.7 g), Boc–Asp-(OBzl)–OH (1.2 g), HOBT (496 mg) and WSCI (725 mg) as described for the preparation of **2**. The product was reprecipitated from EtOAc and petroleum ether; yield 1.7 g (71%), mp 75—79°C, $[\alpha]_D^{26}$ −8.9° (c=1.0, DMF), Rf^a 0.87, Rf^b 0.96, single ninhydrin-positive spot. *Anal.* Calcd for $C_{27}H_{40}Cl_3N_5O_{10}$: C, 46.26; H, 5.75; N, 9.99. Found: C, 46.38; H, 5.83; N, 9.98.

Boc-Pro-Asp(OBzl)-Leu-Gly-NHNH-Troc (39)——This compound was prepared from **38** (1.2 g), Boc–Pro–OH (395 mg), HOBT (248 mg) and WSCI (363 mg) as described for the preparation of **2**; yield 1 g (71%), mp 80—83°C, $[\alpha]_D^{26}$ −21.4° (c=1.0, DMF), Rf^a 0.71, Rf^b 0.84, single ninhydrin-positive spot. *Anal.* Calcd for $C_{32}H_{47}Cl_3N_6O_{11}$: C, 48.16; H, 5.94; N, 10.53. Found: C, 47.80; H, 6.17; N, 10.19.

Boc-Lys(Z)-Pro-Asp(OBzl)-Leu-Gly-NHNH-Troc (40)——This compound was prepared from **39** (1 g), Boc–Lys(Z)–OH DCHA (772 mg), HOBT (186 mg) and WSCI (272 mg) as described for the preparation of **2**. The product was reprecipitated from EtOAc and petroleum ether; yield 1.1 g (85%), mp 80—84°C, $[\alpha]_D^{24}$ −40.1° (c=1.0, DMF), Rf^a 0.74, Rf^b 0.80, single ninhydrin-positive spot. *Anal.* Calcd for $C_{46}H_{65}Cl_3N_8O_{14}$: C, 52.10; H, 6.18; N, 10.57. Found: C, 52.24; H, 6.19; N, 10.40.

Boc-Asp(OBzl)-Lys(Z)-Pro-Asp(OBzl)-Leu-Gly-NHNH-Troc (41)——This compound was prepared from **40** (884 mg), Boc–Asp(OBzl)–OH (296 mg), HOBT (124 mg) and WSCI (182 mg) as described for the preparation of **2**. The product was reprecipitated from EtOAc and ether; yield 910 mg (83%), mp 84—89°C, $[\alpha]_D^{24}$ −34.6° (c=1.0, DMF), Rf^a 0.70, Rf^b 0.83, single ninhydrin-positive spot. *Anal.* Calcd for $C_{57}H_{76}Cl_3N_9O_{17}\cdot 2H_2O$: C, 52.60; H, 6.20; N, 9.69. Found: C, 52.55; H, 6.33; N, 9.23.

Ac-Ala-Asp(OBzl)-Lys(Z)-Pro-Asp(OBzl)-Leu-Gly-NHNH-Troc (42)——The above protected hexapeptide (422 mg) was treated with TFA–anisole (3 ml–0.6 ml) as usual, then dry ether was added and the resulting powder was collected by filtration, washed with ether, dried over KOH pellets *in vacuo* for 2 h and then dissolved in DMF (4 ml) together with NMM (0.035 ml), Ac–Ala–OH (48 mg), HOBT (50 mg) and WSCI (73 mg) at 0°C. After being stirred at 0°C for 3 h and at room temperature for 16 h, the mixture was extracted with EtOAc and the extract was washed successively with 1 N $NaHCO_3$, H_2O, 1 N HCl and H_2O, dried over $MgSO_4$ and then concentrated *in vacuo*. The residue was crystallized from MeOH and ether and then recrystallized from hot EtOAc; yield 329 mg (77%), mp 106—107°C, $[\alpha]_D^{26}$ −5.4° (c=1.0, DMF), Rf^a 0.76, Rf^b 0.87, single chlorine–tolidine-positive spot. *Anal.* Calcd for $C_{57}H_{75}Cl_3N_{10}O_{17}$: C, 53.54; H, 5.91; N, 10.95. Found: C, 53.41; H, 6.19; N, 10.83.

Ac-Ala-Asp(OBzl)-Lys(Z)-Pro-Asp(OBzl)-Leu-Gly-$NHNH_2$ (43)——This compound was prepared from **42** (256 mg) and Zn dust (131 mg) essentially as described for the preparation of **19**. The product was recrystallized from MeOH and ether; yield 201 mg (91%), mp 154—160°C, $[\alpha]_D^{26}$ −17.3° (c=1.0, DMF), Rf^a 0.73, Rf^b 0.89, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{54}H_{74}N_{10}O_{15}$: C, 58.79; H, 6.76; N, 12.70. Found: C, 58.54; H, 6.47; N, 12.93.

Ac-Ala-Asp (OBzl)-Lys (Z)-Pro-Asp (OBzl)-Leu-Gly-Glu (OBzl)-Ile-Asn-Ser-Phe-Asp (OBzl)-Lys (Z)-Ala-Lys(Z)-Leu-Lys(Z)-Lys(Z)-Thr-Glu(OBzl)-Thr-Gln-Glu(OBzl)-Lys(Z)-Asn-Thr-Leu-Pro-Thr-Lys (Z)-Glu-(OBzl)-Thr-Ile-Glu(OBzl)-Gln-Glu(OBzl)-Lys(Z)-Gln-OBzl (44)——Boc-(8—39)-OBzl (**35**) (111 mg) was

treated with TFA–anisole (3 ml–0.6 ml) in an ice-bath for 60 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–NMP (1 ml–2 ml) containing NMM (0.002 ml). The azide (prepared from 66 mg of **43**) in DMF (1 ml) and NMM (0.006 ml) were added to the above ice-chilled solution and the mixture was stirred at 4°C for 36 h. Additional azide (prepared from the same amount of the hydrazide) in DMF (1 ml) and NMM (0.006 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 HCl and H_2O and precipitated from DMSO and H_2O. The crude product was applied to a column of Sephadex LH-20 (2.3 × 105 cm), which was eluted with DMSO containing 3% H_2O. Individual fractions (4 ml each) were collected and the absorbancy at 260 nm was determined. The fractions corresponding to the main peak (tube Nos. 39—46) were combined and evaporated to dryness. Ether was added to the residue to give a precipitate, which was collected by centrifugation. It was further purified by silica gel (Kieselgel 60 Merck, 2.1 × 48 cm) column chromatography using BuOH, AcOH and H_2O (4: 1: 5, upper phase) as an eluent. The desired fractions (4 ml each, tube Nos. 28—31) were collected and the solvent was removed by evaporation. The product was recrystallized from hot DMF and EtOAc; yield 90 mg (68%), mp 171—177°C (dec.), $[\alpha]_D^{24}$ −25.9° (c=0.3, DMSO), Rf^c 0.50, single chlorine–tolidine-positive spot. *Anal.* Calcd for $C_{335}H_{444}N_{52}O_{88}\cdot 8H_2O$: C, 59.60; H, 6.87; N, 10.79. Found: C, 59.62; H, 6.82; N, 10.72. Amino acid ratios in a 6 N HCl hydrolysate: Asp 5.04, Ser 0.71, Glu 8.69, Thr 4.67, Gly 1.00, Ala 2.20, Ile 2.06, Leu 3.24, Phe 0.96, Pro 1.84, Lys 7.76 (recovery of Gly 79%).

Ac-Ala-Asp-Lys-Pro-Asp-Leu-Gly-Glu-Ile-Asn-Ser-Phe-Asp-Lys-Ala-Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Thr-Leu-Pro-The-Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-OH (corresponding to calf thymosin β_8) (45)——The protected nonatriacontapeptide, Ac-(1—39)-OBzl (66 mg), was treated with HF (approximately 4 ml) in the presence of anisole-thioanisole (1 ml) in an ice-chilled 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 2 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. The pH of the solution was adjusted to pH 5 with a few drops of AcOH and the solution was lyophilized. The crude peptide thus obtained was dissolved in 1% AcOH (2 ml), applied to a column of Sephadex G-25 (2.8 × 98 cm), and eluted with the same solvent. Fractions of 5 m per 18 min were collected, and the absorption at 260 nm was determined. Fractions corresponding to the main peak (tube Nos. 59—74) were collected and the solvent was removed by lyophilization. The product was next dissolved in a small amount of the upper phase of a solvent system consisting of BuOH–AcOH–H_2O (4: 1: 5, by volume). The solution was subjected to partition column chromatography on Sephadex G-25 (2.8 × 94 cm) previously equilibrated with the lower phase of the above solvent system. The main peak fractions (tube Nos. 60—67) were combined and evaporated *in vacuo*. The residue was subjected to Sephadex G-25 column chromatography as described above; yield 9.9 mg (23%), mp 246—257°C (dec.), $[\alpha]_D^{24}$ −69.3° (c=0.2, 1 N AcOH), Rf^a 0.02, Rf^b 0.10, single chlorine–tolidine-positive spot. Amino acid ratios in a 6 N HCl hydrolysate: Asp 5.18, Ser 0.74, Glu 8.72, Thr 4.70, Gly 1.00, Ala 2.22, Ile 1.83, Leu 3.15, Phe 0.85, Pro 1.93, Lys 8.31 (recovery of Gly 80%).

Distribution of T-Cell Subsets in Patients with Lupus Nephritis and Effect of Synthetic Nonatriacontapeptide: Analysis with Monoclonal Antibodies——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 T-cells were isolated in a Hypaque–Ficoll gradient.[34] T-cells obtained on the Hypaque–Ficoll gradient were stained for membrane antigens by indirect immunofluorescence with murine monoclonal antibodies (OKT3, OKT4 OKT8) according to Hoffman[35] and Janossy *et al.*[36] Anti-T3 (OKT3) reacted with 100% of peripheral T-cells. In contrast, anti-T4 (OKT4) defined the helper T-cell subset and anti-T8 (OKT8) defined the suppressor T-cell subset. Briefly, 10^6 cells in 50 μl phosphate-buffered saline were incubated for 15 min at 20°C with conventio al antisera (1: 20 dilution) and monoclonal antibodies (peritoneal exudate 1: 200 to 1: 500 dilution), then washed twice and reincubated at 4°C for 30 min with goat anti-mouse-immunoglobulin–TRITC and goat anti-rabbit or horse-immunoglobulin–FITC (1: 20 dilution), followed by washing and counting of the labelled cells under a Nikon VFD-TR fluorescence microscope. The reactivity of antisera was evaluated concomitally 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: DMF, dimethylformamide; WSCI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; TFA, trifluoro-

acetic acid; HOBT, *N*-hydroxybenzotriazole; AcOH, acetic acid; EtOAc, ethyl acetate; FITC, fluorescein-isothiocyanate; TRITC, tetramethylrhodamine-isothiocyanate; Ac, acetyl; NMM, *N*-methylmorpholine; DMSO, dimethylsulfoxide; NMP, *N*-methyl-2-pyrrolidone; Boc, *t*-butyloxycarbonyl; ONp, *p*-nitrophenyl ester; OBzl, benzyl ester; Z, benzyloxycarbonyl; HF, hydrogen fluoride; DCHA, dicyclohexylamine; Tos, *p*-toluenesulfonic acid; NHNH-Troc, β,β,β-trichloroethyloxycarbonylhydrazide; TLC, thin-layer chromatography; DCC, dicyclohexylcarbodiimide; NP, *p*-nitrophenyl; E-rosette, a rosette with sheep erythrocytes; EDTA, ethylenediamine-tetraacetic acid.

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