Synthesis of a Thymosin β_4-Like Peptide, Thymosin β_9^{Met}, and Its Effect on Low E-Rosette-Forming Lymphocytes of Lupus Nephritis Patients[1)]

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A thymosin β_4-like peptide, thymosin β_9^{Met} isolated from pork spleen, was synthesized using a conventional solution method. The deprotection of the protected thymosin β_9^{Met} was achieved by treatment with Zn–90% AcOH to remove C-terminal *p*-nitrobenzyl ester and then with 1 M trifluoromethanesulfonic acid–thioanisole (molar ratio, 1:1) in trifluoroacetic acid in the presence of dimethylselenium. Finally, the deprotected peptide was incubated with dithiothreitol to reduce sulfoxide on the methionine side chain.

The increase of the E-rosette-forming lymphocytes was obtained after incubation of peripheral blood from lupus nephritis patients with the synthetic thymosin β_9^{Met}. The synthetic thymosin β_9^{Met} was approximately equal in potency to that of our synthetic calf thymosin β_9.

Keywords thymosin β_9^{Met} synthesis; trifluoromethanesulfonic acid deprotection; dithiothreitol reduction; lupus nephritis patient; low E-rosette-forming lymphocyte; restorative effect

Thymosin β_4 exhibits several biological activities that are important for maturation and functioning of the immune system.[2,3)] The amino acid sequences of thymosin β_8 and β_9, which were isolated from calf thymus by Hannappel *et al.*, were found to be homologous to thymosin β_4.[4)]

A new thymosin β_4-like peptide, thymosin β_9^{Met}, was recently isolated from pork spleen by Hannappel *et al.*[5)] The only difference between it and thymosin β_9 is substitution of leucine by methionine at position 6.

In previous papers,[6–8)] we reported syntheses of deacetyl-thymosin β_4, thymosins β_8 and β_9, and showed that these synthetic peptides could have restorative effects on impaired cell-mediated immunological functions.

On the other hand, it is well recognized that a high percentage of lupus nephritis patients has a defect cell-mediated immunity.[9,10)] A decrease of T-lymphocytes in these patients has also been demonstrated by several investigators[11)] and in our laboratory.[7,8)]

We reported earlier[8)] that synthetic thymosin β_9 could increase the peripheral E-rosette-forming lymphocytes when incubated *in vitro* with blood from lupus nephritis patients. These results prompted us to synthesize thymosin β_9^{Met}.

Following our solution syntheses of deacetyl-thymosin β_4,[6)] and thymosins β_8[7)] and β_9,[8)] we describe here the solution synthesis of thymosin β_9^{Met} and the *in vitro* effect of this peptide on the low E-rosette-forming lymphocytes of lupus nephritis patients, since many biologically active peptides contain Met in their active regions.

In contrast to our previous syntheses of deacetyl-thymosin β_4,[6)] and thymosins β_8[7)] and β_9,[8)] the thioanisole-mediated trifluoromethanesulfonic acid (TFMSA) deprotecting procedure[12,13)] was applied in the final step of this synthesis instead of hydrogen fluoride.

Our synthetic route to thymosin β_9^{Met} is illustrated in Fig. 1, which shows two fragments selected as building blocks to construct the entire amino acid sequence of thymosin β_9^{Met}. Protected C-terminal tetratriacontapeptide ester [1], was identical with that employed in our previous synthesis of thymosin β_9.[8)] Thus, one fragment, Ac–Ala–Asp(OBzl)–Lys(Z)–Pro–Asp(OBzl)–Met(O)–Gly–$NHNH_2$ [2], which covers the area of sequence variation between thymosin β_9 and thymosin β_9^{Met}, was newly synthesized. The Boc group, removable by trifluoroacetic acid (TFA), was adopted as a temporary N^{α}-protecting group for each intermediate.

Amino acid derivatives bearing protective groups removable by 1 M TFMSA–thioanisole in TFA[12,13)] were employed except for C-terminal *p*-nitrobenzyl ester, *i.e.*, Lys(Z), Glu(OBzl) and Asp(OBzl). The *p*-nitrobenzyl group was cleaved by Zn in 90% AcOH[14)] before the 1 M TFMSA–thioanisole in the TFA deprotecting procedure,[12,13)] since it is not easy to remove *p*-nitrobenzyl group completely by the thioanisole-mediated TFMSA deprotecting procedure. The Met was reversibly protected as its sulfoxide[15)] in order to prevent partial *S*-alkylation during

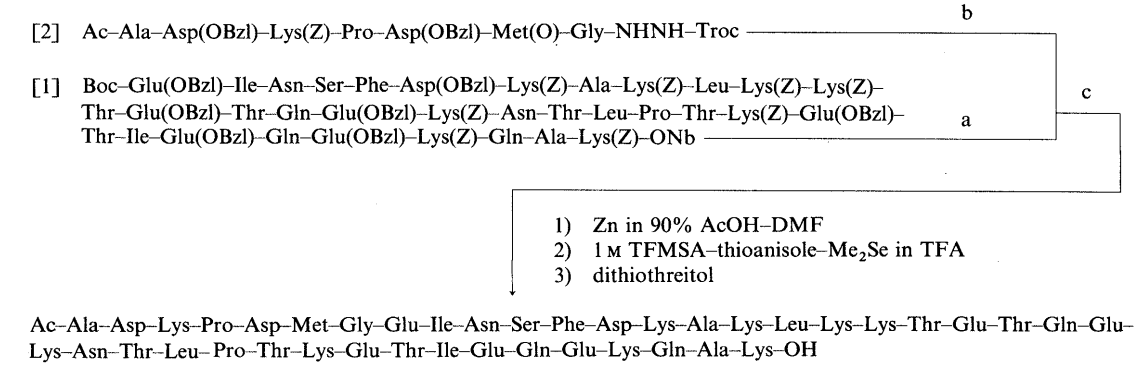

Fig. 1. Synthetic Route to Thymosin β_9^{Met}

a, TFA–anisole; b, Zn–AcOH; c, azide.

the N^{α}-deprotection as well as partial air oxidation during the synthesis.

The substituted hydrazide, Troc-$NHNH_2$,[16] was employed for the preparation of the N-terminal fragment, Ac–Ala–Asp(OBzl)–Lys(Z)–Pro–Asp(OBzl)–Met(O)–Gly–$NHNH_2$ [2], containing the Asp(OBzl) residues. This Troc group is known to be cleaved by Zn[17] in AcOH without affecting other functional groups. Throughout the syntheses of the fragment [2] and its intermediates, the purity of the fragment [2] and its intermediates was checked by thin-layer chromatography (TLC) and elemental analysis. The analytical results were within ±0.4% of theoretical values in all cases.

The protected N-terminal heptapeptide, Ac–Ala–Asp(OBzl)–Lys(Z)–Pro–Asp(OBzl)–Met(O)–Gly–NHNH–Troc, was prepared stepwise starting from Boc–Gly–NHNH–Troc by the Su active ester procedure.[18] Prior to each condensation reaction, the Boc group was removed by TFA in the presence of anisole as usual. The protected heptapeptide thus obtained was treated with Zn[16,17] in AcOH to remove the Troc group, and zinc acetate was removed by treatment with EDTA to give the required hydrazide, Ac–Ala–Asp(OBzl)–Lys(Z)–Pro–Asp(OBzl)–Met(O)–Gly–$NHNH_2$ [2], in analytically pure form. The hydrazine test on the thin-layer chromatogram and the elemental analysis data were consistent with homogeneity of the desired product.

The Boc group of Boc–(8—41)–ONb[8] [1] was removed by the usual TFA–anisole treatment and the corresponding free amine was condensed with the protected N-terminal heptapeptide hydrazide [2] by the azide procedure[19] to yield Ac–(1-41)–ONb [VII] corresponding to the protected entire amino acid sequence of thymosin β_9^{Met}. The homogeneity of the protected untetracontapeptide ester [VII] was checked by elemental analysis, TLC and amino acid analysis of the acid hydrolysate.

In the final step of the synthesis, the protected untetracontapeptide was treated with Zn in 90% AcOH–DMF[14] to remove the *p*-nitrobenzyl group. The last traces of metal contamination were removed by treatment with 5% EDTA. The partially deprotected untetracontapeptide was next treated with 1 M TFMSA–thioanisole in TFA in the presence of Me_2Se. Me_2Se was employed to facilitate acidic cleavage of protecting groups.[20] The deprotected peptide was precipitated with peroxide-free ether, converted to the corresponding acetate with Amberlite IRA-400 (acetate form) and then treated with 1 N NH_4OH to reverse a possible N→O shift at the Ser and Thr residues.[21] The Met(O) residue was reduced back to Met in two steps, firstly with thioanisole and Me_2Se[20] during the above acid treatment, and secondly with dithiothreitol during incubation of the deprotected peptide. The reduced product was purified by gel-filtration on Sephadex G-50 using 2% AcOH, followed by partition column chromatography on Sephadex G-25 according to Yamashiro[22] as shown in Fig. 2.

The main product was rechromatographed on the Sephadex G-25 column as described above, and the highly purified product thus obtained was then applied to a Sephadex G-50 column as described above. The product thus obtained gave a single spot (chlorine–tolidine-positive) on TLC in two different solvent systems and on paper electrophoresis (pH 7.3, pyridinium–acetate buffer). The peptide also exhibited a single peak on HPLC. Homogeneity of the synthetic thymosin β_9^{Met} was further ascertained by amino acid analysis after 6 N HCl hydrolysis.

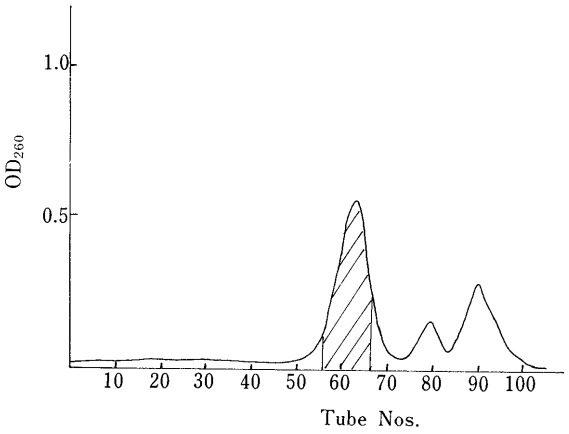

Fig. 2. Purification of Synthetic Untetracontapeptide Corresponding to Thymosin β_9^{Met} by Partition Column Chromatography on Sephadex G-25

Column, 3.2 × 94 cm; fraction, 4 ml; solvent, BuOH–AcOH–H_2O (4 : 1 : 5) upper phase.

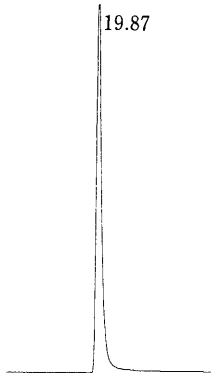

Fig. 3. HPLC of Synthetic Thymosin β_9^{Met}

TABLE I. Effects of the Synthetic Thymosins β_9 and β_9^{Met} on the Low E-Rosette-Forming Capacity of Lymphocytes of Lupus Nephritis Patients

Peptides	Dose (μg/ml)	No.of determinations	E-rosette-forming lymphocytes[d] (%)
(1) —[a]	—	3	73±5
(2) —[b]	—	3	42±6[e]
(3) Thymosin β_9[b,c]	1	3	40±7
(4) Thymosin β_9[b,c]	10	3	55±5[f]
(5) Thymosin β_9[b,c]	100	3	67±7[f]
(6) Thymosin β_9^{Met}[b,c]	1	3	43±6
(7) Thymosin β_9^{Met}[b,c])	10	3	56±5[f]
(8) Thymosin β_9^{Met}[b,c]	100	3	65±6[f]

a) Normal venous blood. *b*) Patient's venous blood. *c*) Incubation was carried out at 37 °C for 70 min. *d*) Each value represents the mean±S.D. of triplicate measurements. *e*) The significance of differences of mean values was analyzed by means of Student's *t* test. $p<0.02$ as compared with (1). *f*) The significancce of differences of mean values was analyzed by means of Student's *t* test. $p<0.01$ as compared with (2).

The *in vitro* effects of the synthetic thymosins β_9[8] and β_9^{Met} on low E-rosette-forming lymphocytes of lupus nephritis patients are shown in Table I.

Incubation of peripheral venous blood from lupus nephritis patients in the presence of various amounts of the

synthetic peptides from 1 to 100 μg/ml resulted in recovery of E-rosette formation (Table I). Increased activity for E-rosette formation was observed with the synthetic thymosin β_9^{Met} up to a concentration of 10 μg/ml. This peptide was approximately equal in potency to that of the synthetic calf thymosin β_9.

In normal subjects, *in vitro* additions of the synthetic thymosins β_9 and β_9^{Met} did not have any effect on the percentages of E-rosette-forming lymphocytes (data not shown).

In an earlier paper,[23] we reported that undecapeptide fragment corresponding to amino acids 16 to 26 of calf thymosin β_9 has increasing activity on low E-rosette-forming lymphocytes of lupus nephritis patients. The undecapeptide moiety corresponding to amino acids 16—26 of calf thymosin β_9, which was found to contain an important moiety in calf thymosin β_9 for restorative activity on impaired immunological deficiency, is conserved in the molecule of thymosin β_9^{Met}.

These results seem to suggest that not only calf thymosin β_9 but also thymosin β_9^{Met} elicits a restorative effect in the cases of lupus nephritis.

Experimental

General experimental procedures used were essentially the same as previously described.[6-8] Ac–(1—7)–N_3 was prepared according to Honzl and Rudinger[19] with isoamyl nitrite.

Melting points are uncorrected. Rotations were measured with an Atago Polax machine (cell length: 10 cm). The amino acid compositions of the acid hydrolysates were determined with a Hitachi 835-50 type amino acid analyzer. Solutions were concentrated in a rotary evaporator under reduced pressure at a temperature of 30—45 °C. Boc groups of the protected peptides were removed by TFA–anisole treatment. The resulting amino components were chromatographed on silica gel plates (Kieselgel G, Merck) and Rf^1 values are based on the following solvent system: $CHCl_3$–MeOH–H_2O (8:3:1). The final product corresponding to the entire amino acid sequence of thymosin β_9^{Met} was chromatographed on cellulose plates (Merck). Rf^2 value refers to BuOH–AcOH–H_2O (4:1:2) and Rf^3 value refers to BuOH–pyridine–AcOH–H_2O (30:20:6:24).[24] Venous blood samples were obtained from two patients suffering from lupus nephritis. Venous blood samples from two healthy donors were used as a control. HPLC was conducted with a Shimadzu LC-3A apparatus equipped with a Nucleosil $5C_{18}$ column.

Boc–Met(O)–Gly–NHNH–Troc [I] Boc–Gly–NHNH–Troc (2.6 g) was treated with TFA–anisole (20 ml–4 ml) in an ice-bath for 40 min, and TFA was then removed by evaporation. The residue was washed with *n*-hexane, dried over KOH pellets *in vacuo* for 2 h, and then dissolved in DMF (15 ml) containing NMM (0.8 ml). To this solution, Boc–Met(O)–OSu (2.6 g) was added, and the mixture was stirred at room temperature for 7 h. The product was extracted with EtOAc and the extract was washed successively with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O, dried over $MgSO_4$ and then concentrated *in vacuo*. The residue was reprecipitated from EtOAc with *n*-hexane: Yield 2.4 g (69%), mp 82—86 °C, $[\alpha]_D^{21}$ +12.1° (c=1.0, DMF), Rf^1 0.63, single ninhydrin-positive spot. *Anal.* Calcd for $C_{15}H_{25}Cl_3N_4O_7S$: C, 35.20; H, 4.92; N, 10.95. Found: C, 34.89; H, 5.23; N, 10.97.

Boc–Asp(OBzl)–Met(O)–Gly–NHNH–Troc [II] This compound was prepared essentially in the same manner as described for the preparation of I using I (1.7 g) and Boc–Asp(OBzl)–OSu (1.5 g). The product was reprecipitated from EtOAc with petroleum ether: Yield 1.6 g (67%), mp 79—83 °C, $[\alpha]_D^{21}$ −5.9° (c=1.0, DMF), Rf^1 0.74, single ninhydrin-positive spot. *Anal.* Calcd for $C_{26}H_{36}Cl_3N_5O_{10}S \cdot H_2O$: C, 42.49; H, 5.21; N, 9.35. Found: C, 42.36; H, 5.50; N, 9.46.

Boc–Pro–Asp(OBzl)–Met(O)–Gly–NHNH–Troc [III] This compound was prepared essentially in the same manner as desdribed for the preparation of I using II (1.2 g) and Boc–Pro–OSu (546 mg). The product was reprecipitated from EtOAc with *n*-hexane: Yield 1 g (77%), mp 76—81 °C, $[\alpha]_D^{21}$ −14.3° (c=1.0, DMF), Rf^1 0.69, single ninhydrin-positive spot. *Anal.* Calcd for $C_{31}H_{43}Cl_3N_6O_{11}S$: C, 45.74; H, 5.32; N, 10.32. Found: C, 45.48; H, 5.61; N, 10.58.

Boc–Lys(Z)–Pro–Asp(OBzl)–Met(O)–Gly–NHNH–Troc [IV] This compound was prepared from III (1 g) and Boc–Lys(Z)–OSu (657 mg) essentially as described for the preparation of I. The product was reprecipitated from EtOAc with ether: Yield 1.1 g (79%), mp 96—101 °C, $[\alpha]_D^{21}$ −16.4° (c=1.0, DMF), Rf^1 0.76, single ninhydrin-positive spot. *Anal.* Calcd for $C_{45}H_{61}Cl_3N_8O_{14}S \cdot 2H_2O$: C, 48.59; H, 5.89; N, 10.07. Found: C, 48.42; H, 6.12; N, 10.28.

Boc–Asp(OBzl)–Lys(Z)–Pro–Asp(OBzl)–Met(O)–Gly–NHNH–Troc [V] This compound was prepared from IV (927 mg) and Boc–Asp(OBzl)–OSu (385 mg) essentially as described for the preparation of I. The product was reprecipitated from MeOH with ether: Yield 914 mg (82%), mp 98—105 °C, $[\alpha]_D^{21}$ −16.3° (c=1.0, DMF), Rf^1 0.79, single ninhydrin-positive spot. *Anal.* Calcd for $C_{56}H_{72}Cl_3N_9O_{17}S \cdot 3H_2O$: C, 50.36; H, 5.89; N, 9.44. Found: C, 50.19; H, 6.14; N, 9.51.

Ac–Ala–Asp(OBzl)–Lys(Z)–Pro–Asp(OBzl)–Met(O)–Gly–NHNH–Troc [VI] This compound was prepared essentially in the same manner as described for the preparation of I using V (668 mg) and Ac–Ala–OSu (126 mg). The product was recrystallized from hot EtOAc: Yield 512 mg (79%), mp 121—129 °C, $[\alpha]_D^{21}$ −7.8° (c=1.0, DMF), Rf^1 0.77, single chlorine–tolidine-positive spot. *Anal.* Calcd for $C_{56}H_{71}Cl_3N_{10}O_{17}S$: C, 51.95; H, 5.53; N, 10.82. Found: C, 51.68; H, 5.86; N, 10.95.

Ac–Ala–Asp(OBzl)–Lys(Z)–Pro–Asp(OBzl)–Met(O)–Gly–NHNH$_2$ [2] VI (432 mg) in AcOH (4 ml) was treated with Zn dust (218 mg) at room temperature for 12 h. The solution was filtered, the filtrate was concentrated *in vacuo*, and then with 5% $NaHCO_3$ to adjust the pH to neutral. The resulting powder was washed with H_2O and reprecipitated from DMF with ether: Yield 312 mg (80%), mp 168—176 °C, $[\alpha]_D^{21}$ −26.8° (c=1.0, DMF), Rf^1 0.59, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{53}H_{70}N_{10}O_{15}S \cdot 3H_2O$: C, 54.27; H, 6.53; N, 11.94. Found: C, 53.97; H, 6.85; N, 11.67.

Ac–Ala–Asp(OBzl)–Lys(Z)–Pro–Asp(OBzl)–Met(O)–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–Ala–Lys(Z)–ONb [VII] Boc–(8—41)–ONb[8] (100 mg) was treated with TFA–anisole (2 ml–0.4 ml) as described above and the N^{α}-deprotected peptide was dissolved in DMF–DMSO (1:1, 3 ml) containing NMM (0.002 ml). The azide [prepared from 60 mg of 2] in DMF–DMSO (1:1, 2 ml) and NMM (0.01 ml) were added and the mixture was stirred at −10 °C for 48 h. Additional azide [prepared from 20 mg of 2] in DMF–DMSO (1:1, 2 ml) and NMM (0.003 ml) were added and stirring was continued for an additional 20 h until the solution became ninhydrin-negative. The mixture was poured into ice-chilled 5% citric acid with stirring. The resulting powder was washed successively with 5% citric acid, H_2O and MeOH. The crude product was then dissolved in DMSO (2 ml) and the solution was applied to a column of Sephadex LH-60 (3 × 96 cm), which was eluted with DMSO containing 3% H_2O. Individual fractions (5 ml each) were collected and the absorbancy at 260 nm was determined. The fractions corresponding to the main peak (tube Nos. 48—57) were combined and concentrated *in vacuo*. The residue was treated with EtOAc to afford a powder: Yield 70 mg (57%), mp 183—196 °C, $[\alpha]_D^{21}$ −10.4° (c=0.4, DMSO), Rf^1 0.72, single chlorine–tolidine-positive spot. *Anal.* Calcd for $C_{345}H_{452}N_{56}O_{93}S \cdot 16H_2O$: C, 57.62; H, 6.78; N, 10.91. Found: C, 57.46; H, 6.90; N, 11.04. Amino acid ratios in a 6 N HCl hydrolysate: Gly 1.00, Phe 0.96, Ala 3.04, Leu 2.01, Ile 1.96, Pro 1.89, Met + Met(O) 0.88, Ser 0.91, Thr 4.87, Asp 4.93, Glu 8.90, Lys 9.05 (recovery of Gly 84%).

Ac–Ala–Asp–Lys–Pro–Asp–Met–Gly–Glu–Ile–Asn–Ser–Phe–Asp–Lys–Ala–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–Thr–Leu–Pro–Thr–Lys–Glu–Thr–Ile–Glu–Gln–Glu–Lys–Gln–Ala–Lys–OH (Corresponding to Thymosin β_9^{Met}) [VIII] Zn dust (10 mg) was added to a solution of VII (55 mg) in 90% AcOH–DMF (1:1, 3 ml). The mixture was stirred at 0 °C for 30 min, then at room temperature for 1 h. Fresh Zn dust (5 mg) was added and the solution, after being stirred for an additional 1 h, was filtered. The filtrate was concentrated and the residue was treated with 5% EDTA to form a powder, which was washed with 1 N HCl and H_2O. The dried product was treated with 1 M TFMSA–thioanisole in TFA (2 ml) in the presence of Me_2Se (50 μl) in an ice-bath for 110 min, then peroxide-free ether was added. The resulting powder was collected by centrifugation, dried over KOH pellets for 2 h and dissolved in 2% AcOH (5 ml). The solution after being stirred with Amberlite IRA-400 (acetate form, approximately 1 g) for 30 min, was filtered. The pH of the filtrate was adjusted to pH 8.0 with 1 N NH_4OH and after 30 min to pH 6.0 with 1 N AcOH. The solution was incubated with dithiothreitol (50 mg) at 40 °C for 12 h and then lyophilized. The product was purified by gel-filtration

on Sephadex G-50 (3.2 × 96 cm) using 2% AcOH as an eluant. The fractions (5 ml each) corresponding to the front main peak (tube Nos. 54—69, determined by UV absorption measurement at 260 nm) were combined and the solvent was removed by lyophilization to give a fluffy powder. The product was next dissolved in a small amount of the upper phase of BuOH–AcOH–H_2O (4 : 1 : 5, v/v). The solution was subjected to partition column chromatography on Sephadex G-25 column (3.2 × 94 cm) previously equilibrated with the lower phase of the above solvent system. The column was developed with the same upper phase. The main peak fractions (tube Nos. 57—67) were collected and the solvent was removed by evaporation. The residue was rechromatographed on a column of Sephadex G-25 as described above. The residue was subjected to Sephadex G-50 column chromatography as described above: Yield 7.4 mg (20%), $[\alpha]_D^{21}$ −74.6° (c=0.2, 2% AcOH), Rf^2 0.03, Rf^3 0.10, single chlorine–tolidine-positive spot. The synthetic peptide exhibited a single spot on paper electrophoresis: Toyo Roshi No. 51 (2 × 40 cm), pyridinium–acetate buffer at pH 7.3; mobility 1.2 cm from the origin toward the anode after running at 2 mA, 600 V for 70 min. The synthetic peptide exhibited a single peak on HPLC using an analytical Nucleosil $5C_{18}$ column (4 × 150 cm, 5 μm) at a retention time of 19.87 min, when eluted with a gradient of acetonitrile (15 to 40% in 30 min) in 0.1% TFA at a flow rate of 1.0 ml per min at room temperature, detection at 1.0 a.u.f.s. (absorbance unit full scale) at 260 nm (Fig. 3). Amino acid ratios in a 6 N HCl hydrolysate: Gly 1.00, Phe 1.02, Ala 3.06, Leu 2.04, Ile 1.98, Pro 1.88, Met 0.89, Ser 0.88, Thr 4.90, Asp 4.95, Glu 8.94, Lys 9.01 (recovery of Gly 84%).

E-Rosette Formation Test A 5-ml aliquot of venous blood was drawn into a syringe containing 300 U of heparin and incubated with the synthetic peptide for 70 min at 37 °C. Lymphocytes were then isolated in a Hypaque–Ficoll gradient.[25] Isolated lymphocytes were adjusted to 5×10^5 cell/ml with PBS. Contamination by monocytes and polymorphonuclear cells amounted to less than 7%.[26] Sheep erythrocytes (Kyokuto Pharmaceutical Co.) were washed with PBS, and a suspension (1×10^7 ml) was prepared. The lymphocytes were washed with GVB^{2+}, centrifuged for 10 min at 1500 rpm, and then suspended in FCS (Dainippon Pharmaceutical Co.) (1 ml). The suspension was mixed with the suspension of sheep erythrocytes (0.5 ml) and incubated for 12 h at 4 °C. The mixture was then contrifuged 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.

References and Notes

1) Abbreviations used: DMF, dimethylformamide; DMSO, dimethylsulfoxide; Boc, *tert*-butoxycarbonyl; OSu, *N*-hydroxysuccinimide ester; Z, benzyloxycarbonyl; OBzl, benzyl ester; Troc, β,β,β-trichloroethoxycarbonyl; Su, *N*-hydroxysuccinimide; ONb, *p*-nitrobenzyl ester; NMM, *N*-methylmorpholine; EDTA, ethylenediaminetetraacetic acid; AcOH, acetic acid; EtOAc, ethyl acetate; MeOH, methanol; HPLC, high-performance liquid chromatography; UV, ultraviolet; Ac, acetyl; E-rosette, a rosette with sheep erythrocytes; FCS, fetal calf serum; GVB^{2+}, gelatin veronal buffer; PBS, phosphate-buffered saline.

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