Synthesis of Human Splenin (hSP) and Examination of Its Immunological Effects on the Impaired T- and B-Lymphocytes in Uremic Patients[1)]

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Human splenin (hSP) was synthesized by assembling eight peptide fragments followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole (molar ratios, 1:1) in trifluoroacetic acid in the presence of *m*-cresol and dimethylselenium. Finally, the deprotected peptide was incubated with dithiothreitol to reduce sulfoxide on the methionine side chain. Incubation of peripheral lymphocytes isolated from uremic patients with the synthetic hSP showed an enhancing effect on the reduced B-lymphocytes, but had no restoring effect on the impaired blastogenic response of T-lymphocytes.

Keywords human splenin (hSP) synthesis; trifluoromethanesulfonic acid deprotection; dithiothreitol reduction; uremic patient; reduced B-lymphocyte; impaired T-lymphocyte blastogenic response; enhancing effect

Evidence of impaired immune function is well recognized in uremic patients.[2,3)] Uremia is therefore associated with impaired response to PHA stimulation of T-lymphocytes and depression of B-lymphocyte numbers.

In our previous paper,[4)] we reported that a synthetic analog of human splenin (hSP) [Glu^{34}]hSP, has enhancing activity on the reduced percentage of B-lymphocytes of uremic patients. Recently, we also reported the syntheses of hSP fragment 32—48 and its analog in which Ala^{34} is replaced by Glu, [Glu^{34}]hSP fragment 32—48, and showed that not only the synthetic [Glu^{34}]fragment 32—48 but also the synthetic hSP fragment 32—48 has enhancing activity on the reduced percentage of B-lymphocytes of uremic patients.[5)] These results prompted us to synthesize the octatetracontapeptide corresponding to the entire amino acid sequence of hSP.

We describe here the solution synthesis of hSP in order to examine whether our synthetic hSP has an enhancing effect on the marked reduction of B-lymphocytes and a restoring effect on the impaired blastogenic response of PHA-stimulated T-lymphocytes of uremic patients.

Our synthetic route to hSP is illustrated in Fig. 1. The methods we employed here are essentially the same as employed for our previous synthesis of [Glu^{34}]hSP.[4)] Amino acid derivatives bearing protecting groups removable by 1 M TFMSA–thioanisole in TFA[6)] were employed, *i.e.*, Arg (Mts), Glu (OBzl), Lys (Z), Thr (Bzl), Ser (Bzl), Tyr (Bzl) and His–OBzl. The Met was reversibly protected as its sulfoxide[7)] in order to prevent partial S-alkylation during the N$^{\alpha}$-TFA-deprotection as well as partial air

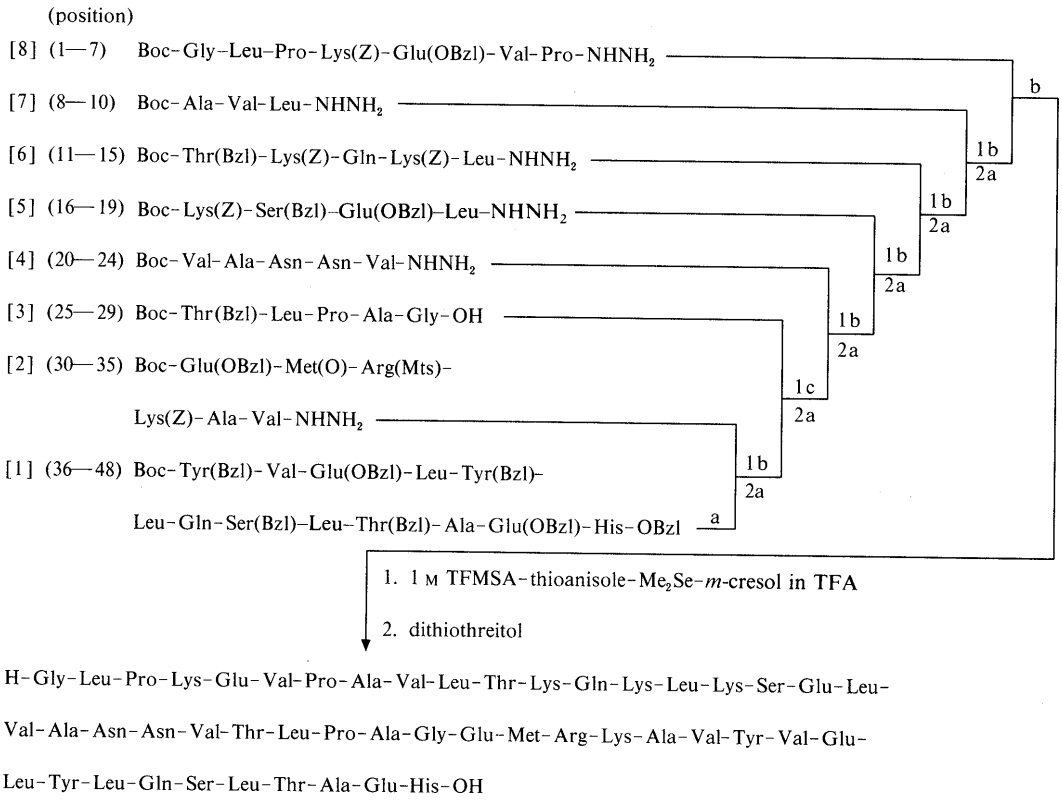

Fig. 1. Synthetic Route to hSP

a, TFA–anisole; b, azide; c, HOSu-WSCI.

oxidation during the synthesis.

Of eight fragments used in our present synthesis, seven fragments, Boc–(36—48)–OBzl [1], Boc–(25—29)–OH [3], Boc–(20—24)–$NHNH_2$ [4], Boc–(16—19)–$NHNH_2$ [5], Boc–(11—15)–$NHNH_2$ [6], Boc–(8—10)–$NHNH_2$ [7] and Boc–(1—7)–$NHNH_2$ [8], are identical with those employed for our previous syntheses of human thymopoietin,[8] bovine thymopoietin III[9] and [Glu^{34}]hSP.[4] The remaining fragment, Boc–(30—35)–$NHNH_2$ [2], was newly synthesized. The Boc group, removable by TFA, was adopted as a temporary N^{α}-protecting group for every intermediate. N^{α}-Deprotection was performed in the presence of anisole prior to each condensation reaction as usual.

The substituted hydrazine, Troc–$NHNH_2$,[10] was employed for the preparation of fragment [2] containing the Glu(OBzl) residue. This Troc group is known to be cleaved by Zn[11] in AcOH without affecting other functional groups.

Throughout the syntheses of these intermediates and the fragment, the purity of every intermediate was checked by thin-layer chromatography (TLC), elemental analysis and amino acid analysis. The analytical results were within $\pm 0.4\%$ of theoretical values in all cases.

The fragment, Boc-Glu(OBzl)-Met(O)-Arg(Mts)-Lys(Z)-Ala-Val-$NHNH_2$ [2], was prepared in a stepwise manner starting from Boc-Val-NHNH-Troc by the Su active ester procedure[12] except for introduction of Arg(Mts), for which the MA procedure[13] was employed, and the resulting hexapeptide was treated with Zn[10,11] in AcOH to remove the Troc group, and the zinc acetate was removed by treatment with EDTA to give the required hydrazide [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 eight fragments were assembled successively from the C-terminal fragment to the N-terminal fragment by the azide procedure[14] and the HOSu–WSCI procedure[15] according to the routes illustrated in Fig. 1. The amount of the acyl component in each fragment condensation was increased from 2 to 4 eq as the chain elongation progressed in order to secure complete condensation. The solubility of protected intermediates in DMF decreased remarkably with chain elongation. Consequently, mixtures of DMF–DMSO or DMF–HMPA had to be employed for the subsequent condensation reactions. Some of the intermediates were purified by repeated precipitation from DMF or DMSO or HMPA with MeOH, and others were purified by gel-filtration on Sephadex LH-60 using DMF or DMSO as an eluant.

Throughout the synthesis, Ala or Gly was taken as the diagnostic amino acid in acid hydrolysis. By comparison of the recovery of Ala or Gly with those of newly incorporated amino acids, satisfactory incorporation of each fragment in each condensation was confirmed.

Starting with the C-terminal tridecapeptide ester corresponding to positions 36 to 48 of hSP, Boc–(36—48)–OBzl [1], seven fragments, Boc–(30—35)–$NHNH_2$ [2], Boc–(25—29)–OH [3], Boc–(20—24)–$NHNH_2$ [4], Boc–(16—19)–$NHNH_2$ [5], Boc–(11—15)–$NHNH_2$ [6], Boc–(8—10)–$NHNH_2$ [7], and Boc–(1—7)–$NHNH_2$ [8], were successively condensed by the azide procedure[14] and the HOSu–WSCI procedure[15] to give the protected octatetracontapeptide ester corresponding to the entire amino acid sequence of hSP.

In the final step of the synthesis, the protectd octatetracontapeptide ester was treated with 1 M TFMSA–thioanisole in TFA in the presence of *m*-cresol and Me_2Se. *m*-Cresol was used as an additional cation scavenger to suppress a side reaction *i.e.*, O-sulfation of Tyr residues.[16] Me_2Se was employed to facilitate acidic cleavage of protecting groups.[17] 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.[18] The Met(O) residue was reduced back to Met in two steps, firstly with thioanisole and Me_2Se[17] 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 followed by ion-exchange column chromatography on a CM-Sephadex C-25 column with linear gradient elution using pH 6.50 ammonium acetate buffer. The main product was rechromatographed on the CM-Sephadex C-25 column as described above. After being desalted by repeated lyophilization, the product was further purified by column chromatography on cellulose powder using the Partridge solvent system[19] as an eluant. The product thus obtained was then applied to a Sephadex G-50 column as described above. The product thus obtained gave a single spot (ninhydrin- and Sakaguchi-positive) on TLC in two different solvent systems and on paper electrophoresis (pH 2.90 acetate buffer). The peptide also exhibited a single peak on HPLC. Homogeneity of the synthetic hSP was further ascertained by amino acid analysis after 6 N HCl hydrolysis and enzymatic digestion.

The *in vitro* effects of the synthetic hSP on reduced B-

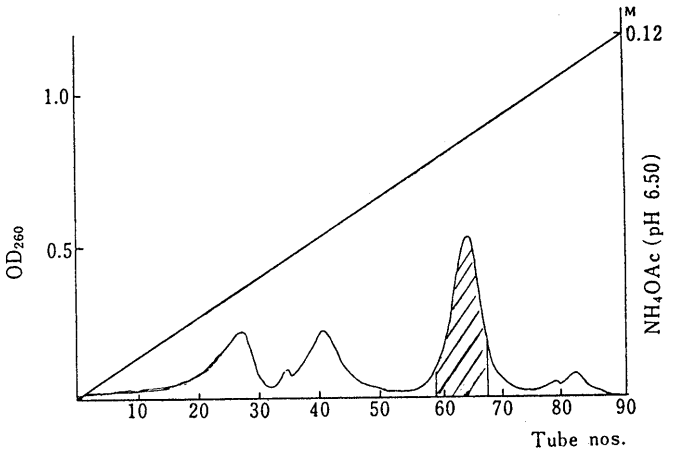

Fig. 2. Purification of Synthetic hSP by Ion-Exchange Chromatography on a CM-Sephadex C-25 Column

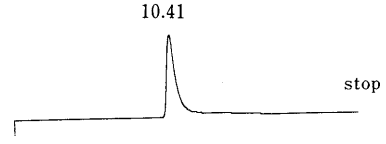

Fig. 3. HPLC of the Synthetic hSP

TABLE I. Effect of the Synthetic hSP on the Reduced B-Lymphocytes of Uremic Patients

Peptide	Dose (μg/ml)	B-Lymphocytes[a] (%)
(1) —[b] ($n=3$)	—	17.9±5.4
(2) —[c] ($n=2$)	—	7.8±4.0[e]
(3) hSP[c,d] ($n=2$)	1.0	12.1±3.7[f]
(4) hSP[c,d] ($n=2$)	10.0	14.9±4.4[f]
(5) hTP[c,d] ($n=2$)	10.0	7.2±4.3

a) Each value represents the mean±S.D. of triplicate measurements (based on counts of 200 cells each on 1 d by a single observer). *b*) Normal peripheral lymphocytes. *c*) Patient's peripheral lymphocytes. *d*) Incubation was carried out at 37 °C in a humidified atmosphere of 5% CO_2 in air for 13 h. *e*) The significance of differences of mean values was analyzed by means of Student's *t* test. $p<0.001$ as compared with (1). *f*) The significance of differences of mean values was analyzed by means of Student's *t* test. $p<0.001$ as compared with (2).

TABLE II. Effect of the Synthetic hSP on the Impaired PHA Stimulation of T-Lymphocytes of Uremic Patients

Peptide	Dose (μg/ml)	*SI*[a,b]
(1) —[c] ($n=3$)	—	281.4±47.2
(2) —[d] ($n=2$)	—	113.2±49.6[f]
(3) hSP[d,e] ($n=2$)	1.0	117.2±42.9
(4) hSP[d,e] ($n=2$)	10.0	108.6±45.3
(5) hTP[d,e] ($n=2$)	10.0	226.4±46.8[g]

a) Each value represents the mean±S.D. of triplicate measurements. *b*) *SI* (stimulation index) was calculated according to the following formala: $SI=\frac{I_2-I_0}{I_1-I_0}\times 100$, where I_2 = mean fluorescence intensity of PHA-activated lymphocytes, I_1 = fluorescence intensity of PHA-nonactivated lymphocytes and I_0 = fluorescence intensity of ethidium bromide. *c*) Normal peripheral lymphocytes. *d*) Patient's lymphocytes. *e*) Incubation was carried out at 37 °C in a humidified atmosphere of 5% CO_2 in air for 12 h. *f*) Significantly different from normal persons (1) at a *p* value of 0.03 or less. *g*) Significantly different from uremic patients (2) at a *p* value of 0.01 or less.

lymphocytes of uremic patients and on the impaired PHA response of T-lymphocytes of uremic patients are shown in Tables I and II.

B-Lymphocytes were detected by the method described by Shevach *et al.*[20] and the blastogenic response of T-lymphocytes was examined by means of the JMRO (Japan Immunoresearch Laboratories Co., Ltd.) fluorometric blast-formation test according to Itoh and Kawai.[21]

Incubation of peripheral venous lymphocytes isolated from uremic patients in the presence of various amounts of the synthetic peptide from 0.1 to 10 μg/ml resulted in recovery of a reduced percentage of B-lymphocytes. Some enhancing activity was observed with synthetic hSP up to a concentration of 1 μg/ml. However, no enhancement was observed after incubation with 10 μg/ml of the synthetic hTP[8] under the same conditions (Table I).

On the contrary, the synthetic hTP had some restoring activity on the impaired blastogenic response of T-lymphocytes isolated from the uremic patients, whereas the synthetic hSP had no effect under the same conditions (Table II). In the case of normal subjects, *in vitro* additions of the synthetic hSP and hTP did not have any effect on the percentages of B-lymphocytes and the blastogenic response of T-lymphocytes (data not shown).

In our previous paper,[4] we reported that the [Glu^{34}]hSP has enhancing activity on the reduced percentage of B-lymphocytes of uremic patients. In addition to this fact, we recently reported that not only the synthetic [Glu^{34}]hSP fragment 32—48 but also the synthetic hSP fragment 32—48 has the same enhancing activity on the reduced percentage of B-lymphocytes of uremic patients. These results seem to suggest that hSP has enhancing activity on the reduced percentage of B-lymphocytes isolated from uremic patients while hTP is ineffective.

Experimental

General experimental procedures used in this paper are essentially the same as described in the previous papers.[4,8,9] Azides were prepared according to Honzl and Rudinger[14] with isoamyl nitrite. Preparations of protected intermediates were repeated several times in order to obtain sufficient quantities for the next step.

Melting points are uncorrected. Rotations were measured with an Atago Polax machine (cell length: 10 cm). The amino acid compositions of the acid and enzymatic hydrolysates were determined with a Hitachi type 835-50 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* values refer to the following solvent system: *Rf*[1] $CHCl_3$–MeOH–H_2O (8:3:1). The final product corresponding to the entire amino acid sequence of hSP was chromatographed on cellulose plates (Merck). *Rf*[2] value refers to the Partridge system[19] and *Rf*[3] value refers to BuOH–pyridine–AcOH–H_2O (30:20:6:24).[22] Troc–$NHNH_2$ was purchased from Kokusan Chemical Works Ltd., Japan. Papain (No. P-3125) and leucine aminopeptidase (No. L-9876) were purchased from Sigma Chemical Co. Labelled B-lymphocyte counting was done under a Nikon UFD-TR fluorescence microscope. Kits for the fluorometric blast-formation test were purchased from Japan Immunoresearch Laboratories Co., Ltd., Japan. Patient selection: Peripheral lymphocytes were obtained from two uremic patients suffering from chronic renal failure. In these uremic patients, the percentages of B-lymphocytes were significantly reduced and examination of cellular immunocompetence revealed a significant decrease in blast formation by PHA when compared with controls (Tables I and II). Venous blood samples from three healthy donors were used as a control. The fluorescence excitation spectrum was measured with an Oyo-Bunko ULOG-FLOUSPEC 11A fluorometer. HPLC was conducted with a Shimadzu LC-3A apparatus equipped with an Asahipak ODP 50 column.

Boc-Ala-Val-NHNH-Troc (I) Boc-Val-NHNH-Troc (2 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 (20 ml) containing NMM (0.6 ml). To this solution, Boc-Ala-OSu (1.5 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 2.1 g (88%), mp 81—84 °C, $[\alpha]_D^{21}$ −6.2° ($c=1.0$, DMF), *Rf*[1] 0.69, single ninhydrin-positive spot. *Anal.* Calcd for $C_{16}H_{27}Cl_3N_4O_6$: C, 40.22: H, 5.70; N, 11.73. Found: C, 40.06; H, 5.92; N, 11.49.

Boc-Lys(Z)-Ala-Val-NHNH-Troc (II) I (2 g) was treated with TFA–anisole (20 ml–4 ml) as described above and the resulting powder was dissolved in DMF (20 ml) containing NMM (0.3 ml). To this solution, Boc-Lys(Z)-OSu (1.1 g) was added, and the solution was stirred at room temperature for 7 h. The mixture 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 petroleum ether: Yield 1.7 g (81%), mp 98—105 °C, $[\alpha]_D^{21}$ −12.1° ($c=1.0$, DMF), *Rf*[1] 0.71, single ninhydrin-positive spot. *Anal.* Calcd for $C_{30}H_{45}Cl_3N_6O_9\cdot 2H_2O$: C, 46.43; H, 6.36; N, 10.83. Found: C, 46.51; H, 6.47; N, 10.78.

Boc-Arg(Mts)-Lys(Z)-Ala-Val-NHNH-Troc (III) II (1.6 g) was treated with TFA–anisole (16 ml–3.2 ml) as described above and the resulting powder was dissolved in THF–DMF (1:1, 6 ml) containing NMM (0.23 ml). To this ice-chilled solution, a solution of the mixed anhydride [prepared from 1.1 g of Boc–Arg(Mts) OH CHA with 0.26 ml of ethylchlorocarbonate and 0.23 ml of NMM at −10 °C] in THF–AcCN (1:1, 10 ml) was added. The mixture was stirred at 4 °C for 2 h and then at room temperature for 8 h, and evaporated *in vacuo*. The residue 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 evaporated *in vacuo*. The

TABLE III. Physical Constants and Analytical Data of Protected hSP and Its Intermediates

	Puri. Proc. (Yield %)	Rf^1	mp (°C)	$[\alpha]_D^{21}$ ($c=1.0$, DMSO)	Formula	Analysis (%) Calcd (Found)		
						C	H	N
Boc-(30—48)-OBzl	B (65)	0.69	214—223	−13.9	$C_{180}H_{244}N_{26}O_{38}S_2 \cdot 9H_2O$	59.94 (59.72)	7.33 7.59	10.10 9.87)
Boc-(25—48)-OBzl	A (71)	0.72	219—227	−14.1	$C_{207}H_{283}N_{31}O_{44}S_2 \cdot 8H_2O$	60.38 (60.45)	7.32 7.61	10.55 10.28)
Boc-(20—48)-OBzl	A (66)	0.79	238—250	−16.4	$C_{228}H_{318}N_{38}O_{51}S_2 \cdot 10H_2O$	58.87 (58.62)	7.32 7.63	11.44 11.49)
Boc-(16—48)-OBzl	A (64)	0.74	251—263	−8.6	$C_{270}H_{371}N_{43}O_{60}S_2 \cdot 12H_2O$	59.40 (59.29)	7.29 7.50	11.03 10.85)
Boc-(11—48)-OBzl	B (71)	0.81	261—270	−17.9	$C_{320}H_{439}N_{51}O_{71}S_2 \cdot 14H_2O$	59.59 (59.68)	7.30 7.48	11.07 11.36)
Boc-(8—48)-OBzl	A (54)	0.75	256—271	−20.5	$C_{334}H_{464}N_{54}O_{74}S_2 \cdot 16H_2O$	59.24 (59.40)	7.38 7.61	11.17 11.29)
Boc-(1—48)-OBzl	B (53)	0.77	268—279	−16.7	$C_{387}H_{532}N_{62}O_{85}S_2 \cdot 16H_2O$	59.61 (59.46)	7.37 7.62	11.25 11.29)

A, precipitation from DMF or DMSO or HMPA with MeOH. B, gel-filtration on Sephadex LH-60.

residue was reprecipitated from EtOAc with ether: Yield 1.7 g (74%), mp 116—174 °C, $[\alpha]_D^{21}$ −13.4° ($c=1.0$, DMF), Rf^1 0.78, single ninhydrin-positive spot. *Anal.* Calcd for $C_{45}H_{67}Cl_3N_{10}O_{12}S \cdot H_2O$: C, 49.29; H, 6.34; N, 12.77. Found: C, 49.36; H, 6.50; N, 12.94.

Boc-Met(O)-Arg(Mts)-Lys(Z)-Ala-Val-NHNH-Troc (IV) III (1.4 g) was treated with TFA–anisole (14 ml–2.8 ml) as described above and the resulting powder was dissolved in DMF (14 ml) containing NMM (0.16 ml). To this solution, Boc-Met(O)-OSu (476 mg) was added, and the mixture was stirred at room temperature for 8 h. The mixture was poured into an ice-chilled 5% citric acid solution with stirring. The precipitate thereby formed was washed successively with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O. The dried product was reprecipitated from MeOH with ether: Yield 1.3 g (81%), mp 141—148 °C, $[\alpha]_D^{21}$ −9.8° ($c=1.0$, DMF), Rf^1 0.74, single ninhydrin-positive spot. *Anal.* Calcd for $C_{50}H_{76}Cl_3N_{11}O_{14}S_2 \cdot 2H_2O$: C, 47.60; H, 6.39; N, 12.21. Found: C, 47.53; H, 6.62; N, 11.95.

Boc-Glu(OBzl)-Met(O)-Arg(Mts)-Lys(Z)-Ala-Val-NHNH-Troc (V) This compound was prepared essentially in the same manner as described for the preparation of IV using IV (1.3 g) and Boc-Glu(OBzl)-OSu (478 mg). The product was reprecipitated from acetone with ether: Yield 1.1 g (73%), mp 132—140 °C, $[\alpha]_D^{21}$ −14.2° ($c=1.0$, DMF), Rf^1 0.75, single ninhydrin-positive spot. *Anal.* Calcd for $C_{62}H_{89}Cl_3N_{12}S_2 \cdot 3H_2O$: C, 49.68; H, 6.39; N, 11.21. Found: C, 49.57; H, 6.51; N, 11.56.

Boc-Glu(OBzl)-Met(O)-Arg(Mts)-Lys(Z)-Ala-Val-NHNH$_2$ [2] V (1 g) in a mixture of AcOH (5 ml) and DMF (5 ml) was treated with Zn powder (327 mg) at room temperature for 10 h. Fresh Zn powder (100 mg) was added and the solution, after being stirred for an additional 3 h, was filtered. The filtrate was concentrated and the residue was treated with 3% EDTA to form a powder, which was washed with 5% $NaHCO_3$ and H_2O. The powder was reprecipitated from DMF with H_2O: Yield 726 mg (83%), mp 170—181 °C, $[\alpha]_D^{21}$ −17.9° ($c=1.0$, DMF), Rf^1 0.52, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{59}H_{88}N_{12}O_{15}S_2 \cdot 2H_2O$: C, 54.28; H, 7.10; N, 12.88. Found: C, 53.89; H, 7.32; N, 12.86.

Synthesis of hSP 1. Successive azide condensations of the six fragments except for Boc-(25—29)-OH [3], which was condensed by the HOSu–WSCI procedure, were carried out according to Fig. 1. Prior to condensation, the Boc group was removed from the respective amino component (1 ml per 0.1 g of the peptide) in the presence of anisole (10 eq) in an ice-bath for 40 min. The TFA-treated sample was precipitated with dry ether, dried over KOH pellets *in vacuo* for 2 h, and dissolved in DMF–DMSO (1 : 1) or DMF–HMPA (1 : 1) containing NMM (1 : 1 eq). The corresponding azide (the amount was increased from 2 to 4 eq as chain elongation pregressed) in DMF–DMSO (1 : 1) and NMM (1.1 eq) were added to the above ice-chilled solution and the mixture was stirred at −10 °C until the solution become negative to the ninhydrin test. The mixture was poured into ice-chilled 5% citric acid with stirring. The precipitate thereby formed was successively washed with 5% citric acid, H_2O and MeOH. The dried product was purified by one of the following two procedures. A: Precipitation from DMF or DMSO with MeOH. B: Gel-filtration on Sephadex LH-60 using DMF or DMSO as an eluant. In procedure B, eluates (5 ml fractions) were examined by measuring the ultraviolet (UV) absorption at 260 nm and the fractions corresponding to the front main peak were combined. The solvent was rmoved by evaporation and the residue was treated with ether to afford a powder.

TABLE IV. Amino Acid Ratios in 6 N HCl Hydrolysates of Protected hSP and Its Intermediates[a]

	Protected peptides							Residues
	30—48	25—48	20—48	16—48	11—48	8—48	1—48	
Gly		1.00	1.00	1.00	1.00	1.00	2.00	2
Ala	2.00	3.02	3.89	4.03	4.01	5.02	5.06	5
Val	2.06	1.99	3.93	3.90	3.98	5.06	6.00	6
Leu	3.07	4.01	4.05	4.98	6.07	7.03	8.01	8
Tyr	1.92	2.03	1.89	1.95	1.87	1.96	1.95	2
Met	0.89	0.92	0.91	0.90	0.87	0.93	0.89	1[b]
Ser	0.88	0.91	0.90	1.87	1.88	1.86	1.88	2
Thr	0.90	1.85	1.93	1.90	2.91	2.94	2.91	3
Pro		0.89	0.93	0.89	0.92	0.93	2.90	3
Glu	3.95	3.94	3.96	4.93	5.94	5.98	6.94	7
Asp			1.87	1.99	1.92	1.97	2.02	2
His	0.92	0.90	0.91	0.89	0.93	0.92	0.90	1
Lys	0.98	0.96	0.97	1.94	3.84	3.92	4.95	5
Arg	0.87	0.90	0.88	0.94	0.95	0.91	0.92	1

a) The results are expressed as ratios to the value for Ala or Gly, which was taken as the diagnostic amino acid in acid hydrolysates. *b*) Met + Met(O).

2. The HOSu–WSCI condensation procedure: Boc-(30—48)-OBzl was treated with TFA–anisole and the N^{α}-deprotected peptide, isolated as usual, was dissolved in DMF–DMSO (1 : 1) together with NMM (1 : 1 eq). To this, Boc-Thr(Bzl)-Leu-Pro-Ala-Gly-OH (2.5 eq), HOSu (2.5 eq) and WSCI (2.5 eq) were added at 0 °C. After 24 h, the reaction mixture was evaporated *in vacuo* and the residue was triturated with 5% $NaHCO_3$. The powder thus obtained was washed successively with 5% $NaHCO_3$, H_2O, 5% citric acid and H_2O. The product was further purified by reprecipitation three times from DMF with MeOH. The purification procedure, yield, physical constants and analytical data of the protected hSP and its intermediates are listed in Tables III and IV.

H-Gly-Leu-Pro-Lys-Glu-Val-Pro-Ala-Val-Leu-Thr-Lys-Gln-Lys-Leu-Lys-Ser-Glu-Leu-Val-Ala-Asn-Asn-Val-Thr-Leu-Pro-Ala-Gly-Glu-Met-Arg-Lys-Ala-Val-Tyr-Val-Glu-Leu-Tyr-Leu-Gln-Ser-Leu-Thr-Ala-Glu-His-OH (hSP) The protected hSP (50 mg) was treated with 1 M TFMSA–thioanisole in TFA (2 ml) in the presence of *m*-cresol (100 μl) and 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 1 N 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 (3.6 × 96 cm) using 2% AcOH as an eluant. The fractions (5 ml each) corresponding to the main peak (tube Nos. 55—69, determined by UV absorption measurement at 260 nm), were combined and the solvent was removed by lyophilization to give a fluffy powder. The Sephadex-purified sample was dissolved in H_2O (2 ml) and the solution was applied to a column of CM-Sephadex C-25 (2.3 × 60 cm), which was eluted first with H_2O (100 ml) and then with a linear gradient from H_2O (260 ml) to 0.12 M NH_4OAc buffer (260 ml, pH 6.50). Individual fractions (4 ml each) were collected and absorbancy at 260 nm was determined. The main peak (tube Nos. 59—67) was collected and the solvent was removed by lyophilization. Analysis by TLC revealed the presence of three Sakaguchi-positive spots with Rf^2 0.29 (main), 0.46 (minor) and 0.58 (minor). The crude peptide was dissolved in a small amount of the upper phase of Partridge's solvent system. The solution was applied to a column of cellulose (2.3 × 91 cm), which had previous been equilibrated with the same solvent. Each fraction was examined by means of the Sakaguchi test and the fractions that exhibited a single Sakaguchi-positive spot (Rf^2 0.29) were collected and evaporated to dryness. The residue was dissolved in 2% AcOH, and the solution was then subjected to Sephadex G-50 column chromatography as described above: Yield 5.9 mg (15%), $[\alpha]_D^{21}$ −81.3° (c=0.3, 2% AcOH), Rf^2 0.29, Rf^3 0.48, single ninhydrin- and Sakaguchi-positive spot. The synthetic peptide exhibited a single spot on paper electrophoresis: Toyo Roshi No. 51 (2 × 40 cm), acetate buffer at pH 2.90; mobility, 7.8 cm from the origin toward the cathode after running at 1.5 mA, 600 V for 70 min. The retention time was 10.41 min in HPLC on an analytical Asahipak ODP 50 column (4.6 × 150 mm) on gradient elution with AcCN (30 to 45%, 30 min) in 0.1% TFA at a flow rate of 1 ml per min. Amino acid ratios in a 6 N HCl hydrolysate: Gly 2.00, Ala 5.01, Leu 8.06, Ser 1.87, Thr 2.88, Asp 1.94, Glu 6.87, Val 5.99, Pro 2.89, Met 0.90, Tyr 1.96, His 0.89, Lys 4.92, Arg 0.89 (recovery of Gly 84%). Amino acid ratios in papain plus leucine aminopeptidase digest: Gly 2.00, Ala 5.02, Leu 8.04, Val 6.02, Pro 2.86, Met 0.91, Tyr 1.95, Ser 1.86, Thr 2.98, Glu 4.92, His 0.96, Lys 4.97, Arg 0.91; Asn and Gln were not determined (recovery of Gly 83%).

B-Lymphocyte Assay Blood samples for B-lymphocyte assay were collected in heparinized tubes (20 ml of venous blood was collected from patients). A lymphocyte suspension relatively free of monocytes and granulocytes was harvested by the method of Loos and Roos,[23] by density centrifugation of the blood on Lymphoprep (Nyegaad and Co., A/S, Oslo). Washed lymphocytes were then separated into aliquots each containing 2×10^6 cells. Cultures of each combination were incubated at 37 °C in the presence of the peptide in a humidified atmosphere of 5% CO_2 in air for 13 h. Then B-lymphocytes were detected by the method described by Shevach *et al.*[20] One aliquot of lymphocytes resuspended in 0.1 cm^3 BSS was incubated with 0.1 cm^3 polyspecific fluorescein-conjugated goat anti-human-γ-globulin (Gibco, Grand Island, N.Y.) for 30 min at 4 °C. The lymphocytes were then washed three times and the preparation was examined by bright field and fluorescence microscopy. Two hundred lymphocytes were counted and those with surface immunofluorescence were classified as B-lymphocytes.

Fluorometric Blast-Formation Test on T-Lymphocytes A 3-ml aliquot of venous blood was drawn into a syringe containing 25 U/ml of heparin and mixed with 3 ml of PBS. Lymphocytes were isolated in a Hypaque-Ficoll gradient.[24] Isolated lymphocytes were adjusted to 1.0×10^6/ml with PBS. The lymphocytes were cultured in 0.5 ml of RPMI 1640 (Gibco) with 10% FCS (Dainippon Pharmaceutical Co.) in microplates. Cultures of each combination were incubated at 37 °C in the presence of the peptide in a humidified atmosphere of 5% CO_2 in air for 12 h, then PHA (0.125%) was added to each well, and incubation was continued under the same conditions for 60 h. Lymphocytes in each well were transferred into a test tube and centrifuged for 10 min at 240 g, then the supernatant was removed. A 2-ml aliquot of 0.125% SDS was added to the residue and the mixture was stirred for 20 min at room temperature; lymphocytes were completely destroyed and solubilized by this procedure. Ethidium bromide solution (2 ml) was added to the above solution and the mixture was stirred for 15 min at room temperature. The fluorescence excitation spectrum was measured according to Itoh and Kawai.[21]

References and Notes

1) Amino acids and their derivatives used in this investigation were of the L-configuration except for glycine. The following abbreviations are used: DMF, dimethylformamide; DMSO, dimethylsulfoxide; HMPA, hexamethylphosphoramide; THF, tetrahydrofuran; AcCN, acetonitrile; WSCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOSu, *N*-hydroxysuccinimide; Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl; OBzl, benzyl ester; Bzl, benzyl; Troc, β,β,β-trichloroethoxylcarbonyl; OSu, *N*-hydroxysuccinimide ester; NMM, *N*-methylmorpholine; EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; AcOH, acetic acid; EtOAc, ethyl acetate; MeOH, methanol; EtOH, ethanol; HPLC, high-performance liquid chromatography; Mts, mesityl-2-sulfonyl; CM, carboxymethyl; BSS, balanced salt solution; Su, succinimide; PHA, phytohemagglutinin; SDS, sodium dodecyl sulfate; FCS, fetal calf serum; PBS, phosphate-buffered saline; hTP, human thymopoietin; RPMI, Rosewell Park Memorial Institute.
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