Synthesis of [Glu^{34}]Human Splenin (hSP) and Examination of Its Immunological Effect on the Reduced B-Lymphocytes of Uremic Patients[1)]

Takashi ABIKO* and Hiroshi SEKINO

Kidney Research Laboratory, Kojinkai, 1-6, Tsutsujigaoka 2-chome, Sendai 980, Japan. *Received July 20, 1988*

[Glu^{34}]human splenin (hSP) was synthesized in a conventional manner by assembling ten peptide fragments followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole (molar ratio, 1:1) in trifluoroacetic acid in the presence of *m*-cresol and dimethylselenide. 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 [Glu^{34}]hSP showed an enhancing effect on the reduced B-lymphocytes, but synthetic human thymopoietin (hTP) had no effect under the same conditions.

Keywords [Glu^{34}]human splenin synthesis; trifluoromethanesulfonic acid deprotection; dithiothreitol reduction; uremic patient; reduced B-lymphocyte; enhancing effect

Bovine thymopoietin I (bTP-I) and bovine thymopoietin II (bTP-II) share a common pentapeptide at residues 32—36, –Arg–Lys–Asp–Val–Tyr–,[2)] named thymopentin or TP-5, which reproduces the biological activity of thymopoietin[3-5)] and thus was considered to represent the active site. In 1981, Audhya *et al.*[6)] reported revised structures of bTP-I and bTP-II, and a newly identified polypeptide, bovine splenin (bSP), isolated from bovine spleen. The only structural difference between splenin and thymopoietins I and II, both of bovine origin, is the substitution of Glu for Asp at position 34, and splenin was first named thymopoietin III (bTP-III).

bTP and thymopentin affect neuromuscular transmission and induce the phenotype differentiation of T-precursor cells *in vitro* while inhibiting phenotype differentiation of B-lymphocytes. However, bSP and splenopentin corresponding to amino acids 32 to 36, –Arg–Lys–Glu–Val–Tyr–, of bSP, in contrast, do not affect neuromuscular transmission, and they induce both T- and B-precursors.[7)]

In 1987, Audhya *et al.*[8)] reported the isolation of human thymopoietin (hTP) and splenin (hSP) from human thymus and spleen respectively. The complete amino acid sequences of purified hTP and hSP were determined and the two molecules were shown to be 48-amino-acid polypeptides differing at four positions.[8)] The pentapeptide active site of thymopoietin (positions 32—36) is the same in hTP and bTP, but position 34 in the active site of hSP has changed from Glu in bSP to Ala in hSP.

Uremia is associated with depression of total T- and B-lymphocyte numbers. In the uremic condition, total T- and B-lymphocytes were reduced when compared with controls.[9,10)] In the preceding paper,[11)] we reported the synthesis of hTP, and showed that the synthetic hTP exhibited restoring activity on the impaired blastogenic response of T-lymphocytes of uremic patients.

Following our solution syntheses of bTP-I,[12)] bTP-II,[13)] bTP-III[14)] and hTP,[11)] we now wish to report the solution synthesis of [Glu^{34}]hSP in order to examine whether our synthetic [Glu^{34}]hSP has a restoring effect on the marked reduction of B-lymphocytes in uremic patients, since replacement of Ala with Glu in hSP gives a common pentapeptide with that at residues 32—36 of bSP, which induces B-precursors.[7)]

Our synthetic route to [Glu^{34}]hSP is illustrated in Fig. 1.

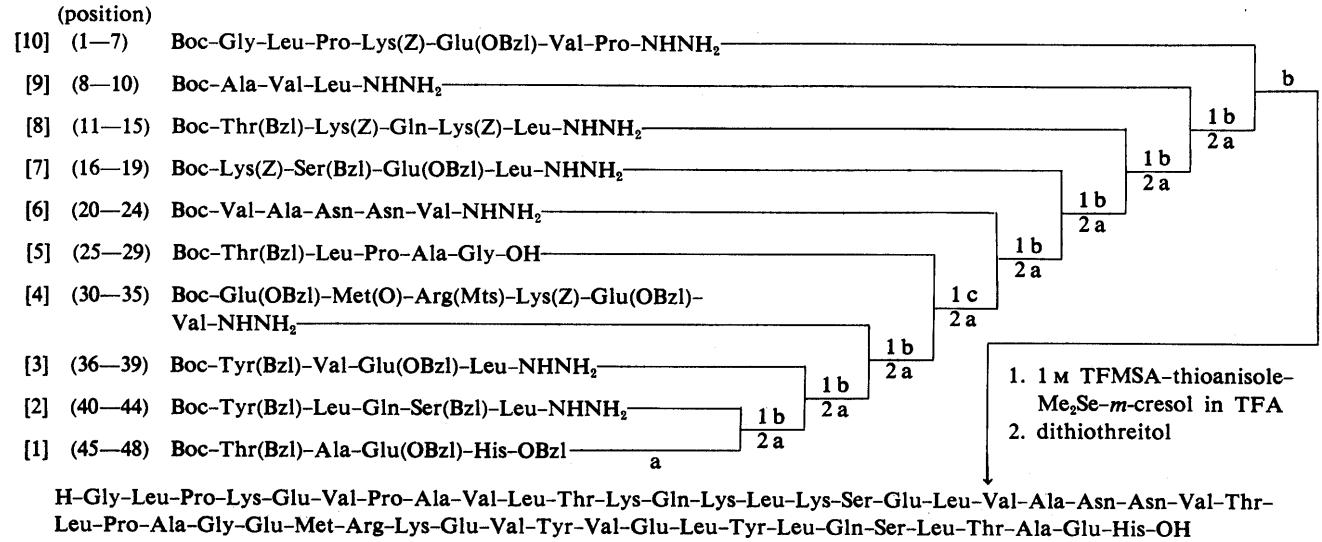

Fig. 1. Synthetic Route to [Glu^{34}]hSP

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

The methods we employed here are essentially the same as employed for our previous synthesis of hTP.[11] Amino acid derivatives bearing protecting groups removable by 1 M TFMSA–thioanisole in TFA[15] 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[16] in order to prevent partial S-alkylation during the N^{α}-TFA deprotection as well as partial air oxidation during the synthesis.

Of ten fragments used in our present synthesis, six fragments, Boc–(36—39)–$NHNH_2$ [3], Boc–(25—29)–OH [5], Boc–(16—19)–$NHNH_2$ [7], Boc–(11—15)–$NHNH_2$ [8], Boc–(8—10)–$NHNH_2$ [9] and Boc–(1—7)–$NHNH_2$ [10], are identical with those employed for our previous syntheses of hTP[11] and bTP-III.[14] The other four fragments, Boc–(45—48)–OBzl [1], Boc–(40—44)–$NHNH_2$ [2], Boc–(30—35)–$NHNH_2$ [4] and Boc–(20—24)–$NHNH_2$ [6], were 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–$NHNH_2$[17] was employed for preparation of fragment [4] containing the Glu (OBzl) residue. This Troc group is known to be cleaved by Zn[18] in AcOH without affecting other functional groups.

Throughout the syntheses of these intermediates and fragments, the purity of every fragment and 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 C-terminal fragment, Boc–Thr(Bzl)–Ala–Glu(OBzl)–His–OBzl [1], was prepared in a stepwise manner starting from H–His–OBzl 2 Tos by the Su active ester procedure.[19] Next, protected peptide esters, Boc–Tyr(Bzl)–Leu–Gln–Ser(Bzl)–Leu–OMe and Boc–Val–Ala–Asn–Asn–Val–OMe, were prepared in a stepwise manner starting from C-terminal amino acid esters by the Su active ester procedure[19] except for introduction of Gln and Asn, for which the NP active ester procedure was employed.[20] The protected peptide esters thus obtained were smoothly converted to the corresponding hydrazides, Boc–Tyr(Bzl)–Leu–Gln–Ser(Bzl)–Leu–$NHNH_2$ [2] and Boc–Val–Ala–Asn–Asn–Val–$NHNH_2$ [6], in the usual manner. The hydrazine test on the thin-layer chromatograms and the elemental analysis data of these fragments were consistent with homogeneity of the desired products. The fragment, Boc–Glu(OBzl)–Met(O)–Arg(Mts)–Lys(Z)–Glu(OBzl)–Val–$NHNH_2$ [4], was prepared starting with Boc–Arg(Mts)–Lys(Z)–Glu(OBzl)–Val–NHNH–Troc, an intermediate of our previous synthesis of bTP-III.[14] The tetrapeptide, after TFA treatment, was condensed with Boc–Met(O)–OSu and Boc–Glu(OBzl)–OSu, respectively, and the resulting hexapeptide was treated with Zn[17,18] in AcOH to remove the Troc group, and the zinc acetate was removed by treatment with EDTA to give the required hydrazide [4] in analytically pure form. The hydrazine test on the thin-layer chromatograms and the elemental analysis data were consistent with homogeneity of the desired product.

The ten fragments were assembled successively by the azide procedure[21] and the HOSu–WSCI procedure[22] according to the routes illustrated in Fig. 1. The amount of the acyl component in each fragment condensation was increased from 1.5 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 this 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 tetrapeptide ester corresponding to positions 45 to 48 of hSP, Boc–(45—48)–OBzl [1], nine fragments, Boc–(40—44)–$NHNH_2$ [2], Boc–(36—39)–$NHNH_2$ [3], Boc–(30—35)–$NHNH_2$ [4], Boc–(25—29)–OH [5], Boc–(20—24)–$NHNH_2$ [6], Boc–(16—19)–$NHNH_2$ [7], Boc–(11—15)–$NHNH_2$ [8], Boc–(8—10)–$NHNH_2$ [9] and Boc–(1—7)–$NHNH_2$ [10], were successively condensed by the azide procedure[21] and the HOSu–WSCI procedure[22] as shown in Fig. 1 to give the protected octatetracontapeptide corresponding to the entire amino acid sequence of [Glu^{34}]hSP.

The homogeneities of the peptides were checked by elemental analysis, TLC and amino acid analyses of the acid hydrolysates.

In the final step of the synthesis, the protected 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.[23] Me_2Se was employed to facilitate acidic cleavage of protecting groups.[24] The deprotected peptide was next 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.[25] The Met(O) residue was reduced back to Met in two steps, firstly with thioanisole and Me_2Se[24] 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 6.45 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[26] 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 2.88 acetate buffer). The peptide also exhibited a single peak on HPLC. Homogeneity of the synthetic [Glu^{34}]hSP was further ascertained by amino acid analysis, after 6 N

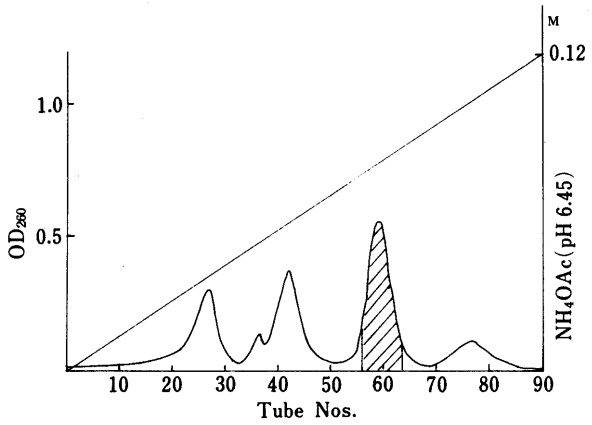

Fig. 2. Purification of Synthetic [Glu^{34}]hSP by Ion-Exchange Chromatography on a CM-Sephadex C-25 Column

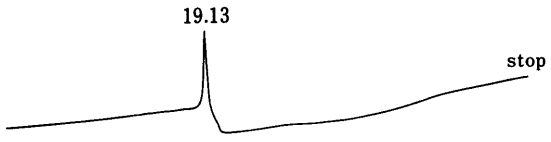

Fig. 3. HPLC of Synthetic [Glu^{34}]hSP

TABLE I. Effect of the Synthetic [Glu^{34}]hSP on the Reduced B-Lymphocytes of Uremic Patients

	Peptide		Dose (μg/ml)	B-Lymphocytes[a] (%)
(1)	—[b]	($n=3$)		17.6±5.6
(2)	—[c]	($n=2$)		7.6±3.5[e]
(3)	[Glu^{34}]hSP[c,d]	($n=2$)	0.1	7.5±3.3
(4)	[Glu^{34}]hSP[c,d]	($n=2$)	1.0	11.3±5.0[f]
(5)	[Glu^{34}]hSP[c,d]	($n=2$)	10.0	14.6±5.3[f]
(6)	hTP[c,d,11]	($n=2$)	10.0	6.9±3.4

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*) Patients' peripheral lymphocytes. *d*) Incubation was carried out at 37 °C in a humidified atmosphere of 5% CO_2 in air for 16 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).

HCl hydrolysis and enzymatic digestion.

The *in vitro* effect of the synthetic octatetracontapeptide on reduced B-lymphocytes of uremic patients is shown in Table I. Incubation of peripheral venous lymphocytes isolated from uremic patients in the presence of various amount of the synthetic peptide from 0.1 to 10 μg/ml resulted in recovery of the reduced percentage of B-lymphocytes. Some enhancing activity was also observed with synthetic [Glu^{34}]hSP up to a concentration of 1 μg/ml. However, our synthetic hTP[11] had no effect on the reduced percentage of B-lymphocytes of these patients at a dose of 10 μg/ml (Table I). In the case of normal subjects, *in vitro* addition of the synthetic [Glu^{34}]hSP did not have any enhancing effect on the percentage of B-lymphocytes under the same conditions (data not shown).

It is well known[7] that splenopentin, Arg–Lys–Glu–Val–Tyr, is a selective inducer of B-cell precursors, but thymopentin, Arg–Lys–Asp–Val–Tyr, is not. These results seem to suggest that replacing Ala^{34} of hSP by Glu gives an analog with activity to enhance the reduced percentage of B-lymphocytes in uremic patients.

Experimental

General experimental procedures used in this paper are essentially the same as described in the previous papers.[11,13,14] Azides were prepared according to Honzl and Rudinger[21] with isoamyl nitrite. After each coupling reaction, each product was purified by one of the following three procedures. Procedure A: For purification of protected peptides soluble in EtOAc, the extract was washed with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O, then dried over $MgSO_4$ and concentrated. The residue was reprecipitated or recrystallized from appropriate solvents. Procedure B: For purification of protected peptides almost insoluble in EtOAc, the reaction mixture was poured into ice-chilled 5% citric acid with stirring. The powder thereby formed was washed with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O. The dried product was recrystallized or reprecipitated from appropriate solvents. Procedure C: For purification of protected peptides almost insoluble in EtOAc, the reaction mixture was poured into ice-chilled 1 N NH_4OH with stirring. The powder thereby formed was washed with 1 N NH_4OH until the yellow color disappeared, and then washed with H_2O, 5% citric acid and H_2O. The dried product was recrystallized or reprecipitated from appropriate solvents. 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 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* 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 [Glu^{34}]hSP was chromatographed on a cellulose plate (Merck). Rf^2 value refers to the Partridge system[26] and Rf^3 value refers to BuOH–pyridine–AcOH–H_2O (30:20:6:24).[27] 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. 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 when compared with controls (Table I). Control peripheral lymphocytes were obtained from three healthy persons. HPLC was conducted with a Shimadzu LC-3A apparatus coupled to a Nucleosil $5C_{18}$ column (4×150 mm).

Boc–Glu(OBzl)–His–OBzl (I) Boc–Glu(OBzl)–OSu (2.4 g) was added to a mixture of H–His–OBzl·2 Tos (3 g) and NMM (1 ml) in DMF (15 ml) and the solution, after being stirred at room temperature for 6 h, was concentrated. The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane. Yield 2.7 g (90%), mp 89—94 °C, $[\alpha]_D^{21}$ −8.3° (*c*=1.0, DMF), Rf^1 0.44, single ninhydrin-positive spot. *Anal.* Calcd for $C_{30}H_{36}N_4O_7 \cdot H_2O$: C, 61.84; H, 6.57; N, 9.62. Found: C, 61.72; H, 6.81; N, 9.46.

Boc–Ala–Glu(OBzl)–His–OBzl (II) I (2.3 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 (0.4 ml). To this solution, Boc–Ala–OSu (1.3 g) was added, and the mixture was stirred at room temperature for 6 h. The product was purified by procedure A followed by reprecipitation from EtOAc with *n*-hexane. Yield 2.6 g (81%), mp 106—115 °C, $[\alpha]_D^{21}$ −10.9° (*c*=1.0, DMF), Rf^1 0.50, single ninhydrin-positive spot. *Anal.* Calcd for $C_{40}H_{47}N_5O_8 \cdot H_2O$: C, 64.59; H, 6.64; N, 9.41. Found: C, 64.35; H, 6.87; N, 9.29.

Boc–Thr(Bzl)–Ala–Glu(OBzl)–His–OBzl [1] This compound was prepared essentially in the same manner as described for the preparation of II by using II (2.2 g) and Boc–Thr(Bzl)–OSu (1.3 g). The product was purified by procedure A, followed by reprecipitation from EtOH with ether. Yield 2 g (87%), mp 124—131 °C, $[\alpha]_D^{21}$ −6.5° (*c*=1.0, DMF), Rf^1 0.55, single ninhydrin-positive spot. *Anal.* Calcd for $C_{44}H_{54}N_6O_{10}$: C, 63.91; H, 6.58; N, 10.16. Found: C, 63.84; H, 6.80; N, 10.03.

Boc–Ser(Bzl)–Leu–OMe (III) This compound was prepared essen-

TABLE II. Physical Constants and Analytical Data of Protected [Glu[34]]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–(40—48)–OBzl	A (77)	0.58	149—160	−16.3	$C_{87}H_{110}N_{12}O_{18}\cdot 4H_2O$	62.05 (61.84)	7.06 7.24	9.98 9.69)
Boc–(36—48)–OBzl	A (87)	0.73	177—189	−14.4	$C_{126}H_{168}N_{16}O_{25}\cdot 8H_2O$	60.63 (60.35)	7.43 7.59	8.98 9.11)
Boc–(30—48)–OBzl	B (69)	0.72	206—217	−12.5	$C_{194}H_{261}N_{26}O_{40}S_2\cdot 7H_2O$	61.52 (61.39)	7.32 7.50	9.62 9.63)
Boc–(25—48)–OBzl	A (64)	0.65	231—243	−13.8	$C_{227}H_{300}N_{31}O_{46}S_2\cdot 9H_2O$	60.98 (60.74)	7.36 7.59	9.98 9.85)
Boc–(20—48)–OBzl	A (68)	0.75	240—254	−18.5	$C_{242}H_{338}N_{38}O_{53}S_2\cdot 11H_2O$	59.44 (59.26)	7.42 7.61	10.89 10.74)
Boc–(16—48)–OBzl	A (61)	0.62	248—260	−9.2	$C_{284}H_{391}N_{43}O_{62}S_2\cdot 8H_2O$	60.83 (60.52)	7.32 7.48	10.24 10.31)
Boc–(11—48)–OBzl	B (72)	0.73	255—268	−21.3	$C_{334}H_{459}N_{51}O_{73}S_2\cdot 13H_2O$	60.28 (59.97)	7.35 7.39	10.73 10.59)
Boc–(8—48)–OBzl	A (54)	0.71	264—277	−23.6	$C_{348}H_{484}N_{54}O_{76}S_2\cdot 13H_2O$	60.24 (60.21)	7.41 7.68	10.90 10.79)
Boc–(1—48)–OBzl	B (58)	0.65	259—269	−18.4	$C_{397}H_{552}N_{62}O_{87}S_2\cdot 15H_2O$	60.21 (59.88)	7.41 7.64	10.97 10.23)

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

TABLE III. Amino Acid Ratios in 6 N HCl Hydrolysates of Protected [Glu[34]]hSP and Its Intermediates[a]

	Protected peptides									Residues
	40—48	36—48	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	1.00	1.00	1.00	2.03	2.93	3.02	3.06	4.03	3.94	4
Val		0.98	2.04	1.93	3.90	4.05	4.03	4.92	6.05	6
Leu	2.03	3.04	3.06	4.05	4.02	4.94	5.90	7.01	7.99	8
Tyr	0.93	1.93	1.89	1.99	1.87	1.89	1.95	1.94	1.92	2
Met			0.94	0.90	0.95	0.93	0.94	0.91	0.92	1[b]
Ser	0.88	0.89	0.90	0.93	0.88	1.86	1.84	1.85	1.89	2
Thr	0.91	0.87	0.92	1.88	1.84	1.87	2.90	2.83	2.84	3
Pro				0.95	0.91	0.96	0.89	2.86	2.87	3
Glu	1.92	2.89	4.88	4.87	5.02	5.85	7.06	6.94	7.88	8
Asp					1.92	1.90	2.02	1.98	1.94	2
His	0.90	0.90	0.91	0.97	0.88	0.94	0.92	0.91	0.97	1
Lys			1.02	1.02	0.93	1.97	3.86	3.95	4.89	5
Arg			0.92	0.91	0.87	0.86	0.88	0.87	0.90	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).

tially in the same manner as described for the preparation of I using H–Leu–OMe·HCl (1.8 g) and Boc–Ser(Bzl)–OSu (4 g). The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane. Yield 3.4 g (8.3%), mp 80—84 °C $[\alpha]_D^{21}$ −14.2° ($c=1.0$, DMF), Rf^1 0.59, single nihydrin-positive spot. *Anal.* Calcd for $C_{22}H_{34}N_2O_6$: C, 62.54; H, 8.11; N, 6.63. Found: C, 62.30; H, 8.36; N, 6.51.

Boc–Gln–Ser(Bzl)–Leu–OMe (IV) III (2.8 g) was treated with TFA–anisole (20 ml–4 ml) as usual and the resulting powder was dissolved in DMF (20 ml) together with NMM (0.7 ml). Boc–Gln–ONp (3 g) was added and the solution was stirred at room temperature for 8 h. The reaction mixture was diluted with 1 N NH_4OH (5 ml) with stirring to saponify the unchanged *p*-nitrophenyl ester. After 1 h, the product was purified by procedure C, followed by reprecipitation from MeOH with ether. Yield 3.3 g (87%), mp 141—150 °C, $[\alpha]_D^{21}$ −12.6° ($c=1.0$, DMF), Rf^1 0.48, single ninhydrin-positive spot. *Anal.* Calcd for $C_{27}H_{42}N_4O_8\cdot H_2O$: C, 57.03; H, 7.80; N, 9.85. Found: C, 56.91; H, 7.92; N, 9.72.

Boc–Leu–Gln–Ser(Bzl)–Leu–OMe (V) This compound was prepared essentially in the same manner as described for the preparation of II by using IV (2.8 g) and Boc–Leu–OSu (1.8 g). The product was purified by procedure A, followed by reprecipitation from EtOAc with ether. Yield 2.4 g (75%), mp 121—129 °C, $[\alpha]_D^{21}$ −15.4° ($c=1.0$, DMF), Rf^1 0.61, single ninhydrin-positive spot. *Anal.* Calcd for $C_{33}H_{53}N_5O_9$: C, 61.57; H, 8.30; N, 10.88. Found: C, 61.48; H, 8.46; N, 10.97.

Boc–Tyr(Bzl)–Leu–Gln–Ser(Bzl)–Leu–OMe (VI) This compound was prepared essentially in the same manner as described for the preparation of II by using V (2.2 g) and Boc–Tyr(Bzl)–OSu (1.3 g). The product was purified by procedure B, followed by reprecipitation from MeOH with ether. Yield 2.4 g (73%), mp 142—150 °C, $[\alpha]_D^{21}$ −10.8° ($c=1.0$ DMF), Rf^1 0.62, single ninhydrin-positive spot. *Anal.* Calcd for $C_{49}H_{68}N_6O_{11}\cdot H_2O$: C, 60.98; H, 7.31; N, 11.81. Found: C, 60.72; H, 7.54; N, 11.63.

Boc–Tyr(Bzl)–Leu–Gln–Ser(Bzl)–Leu–NHNH$_2$ [2] VI (1.9 g) was dissolved in DMF–MeOH (1:1, 20 ml). Hydrazine hydrate (0.97 ml) was added, and the solution was kept standing at room temperature for 26 h. After evaporation of the MeOH, the residue was poured into ice-chilled H_2O with vigorous stirring. The precipitate thereby formed was collected by filtration and washed with H_2O. The dried product was recrystallized from hot MeOH. Yield 1.5 g (79%), mp 172—183 °C, $[\alpha]_D^{21}$ −23.7° ($c=1.0$, DMF), Rf^1 0.52, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{48}H_{68}N_8O_{10}\cdot 2H_2O$: C, 61.52; H, 7.74; H, 11.96. Found: C, 61.43; H, 7.91; N, 11.70.

Boc–Met(O)–Arg(Mts)–Lys(Z)–Glu(OBzl)–Val–NHNH–Troc (VII) This compound was prepared from Boc–Arg(Mts)–Lys(Z)–Glu(OBzl)–Val–NHNH–Troc[14] (1.2 g) and Boc–Met(O)–OSu (398 mg) essentially as described for the preparation of II. The product was purified

by procedure B, followed by recrystallization from hot EtOAc. Yield 1.1 g (73%), mp 131—143 °C, $[\alpha]_D^{21}$ −18.4° (c=1.0, DMF), Rf^1 0.63, single ninhydrin-positive spot. *Anal.* Calcd for $C_{64}H_{93}Cl_3N_{11}O_{16}S_2 \cdot 2H_2O$: C, 52.10; H, 6.62; N, 10.43. Found: C, 52.13; H, 6.85; N, 10.25.

Boc–Glu(OBzl)–Met(O)–Arg(Mts)–Lys(Z)–Glu(OBzl)–Val–NHNH–Troc (VIII) This compound was prepared from VII (1 g) and Boc–Glu(OBzl)–OSu (319 mg) essentially as described for the preparation of II. The product was purified by procedure B, followed by reprecipitation from EtOH with ether. Yield 1 g (91%), mp 139—148 °C, $[\alpha]_D^{21}$ −9.2° (c=1.0, DMF), Rf^1 0.67, single ninhydrin-positive spot. *Anal.* Calcd for $C_{76}H_{106}Cl_3N_{12}O_{19}S_2$: C, 54.92; H, 6.43; N, 10.11. Found: C, 54.68; H, 6.56; N, 9.79.

Boc–Glu(OBzl)–Met(O)–Arg(Mts)–Lys(Z)–Glu(OBzl)–Val–NHNH$_2$ [4] VIII (941 mg) in a mixture of AcOH (5 ml) and DMF (5 ml) was treated with Zn dust (363 mg) at 4 °C for 8 h. The solution was filterd, the filtrate was concentrated *in vacuo*, and the residue was treated with 3% EDTA and then with $NaHCO_3$ to adjust the pH to neutral. The resulting powder was washed with H_2O and reprecipitated from DMF with H_2O. Yield 728 mg (85%), mp 174—185 °C, $[\alpha]_D^{21}$ −16.9° (c=1.0, DMF), Rf^1 0.59, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{73}H_{105}N_{12}S_2 \cdot 3H_2O$: C, 56.90; H, 7.26; N, 10.91. Found: C, 56.65; H, 7.47; N, 10.73.

Boc–Asn–Val–OMe (IX) This compound was prepared from H–Val–OMe·HCl (1.7 g) and Boc–Asn–ONp (1.5 g) essentially as described for the preparation of IV. The product was purified by procedure C, followed by recrystallization from hot EtOAc with ether. Yield 3.1 g (89%), mp 141—150 °C, $[\alpha]_D^{21}$ −11.4° (c=1.0, DMF), Rf^1 0.46, single ninhydrin-positive spot. *Anal.* Calcd for $C_{15}H_{30}N_3O_6$: C, 51.71; H, 8.68; N, 12.06. Found: C, 51.42; H, 8.77; N, 11.89.

Boc–Asn–Asn–Val–OMe (X) This compound was prepared essentially in the same manner as described for the preparation of IV by using IX (1.9 g) and Boc–Asn–ONp (827 mg). The product was purified by procedure C, followed by reprecipitation from MeOH with H_2O. Yield 1.8 g (75%), mp 146—155 °C, $[\alpha]_D^{21}$ −8.3° (c=1.0, DMF), Rf^1 0.51 single ninhydrin-positive spot. *Anal.* Calcd for $C_{19}H_{36}N_5O_8 \cdot H_2O$: C, 47.49; H, 7.97; N, 14.57. Found: C, 47.38; H, 7.86; N, 14.30.

Boc–Ala–Asn–Asn–Val–OMe (XI) This compound was prepared from X (1.6 g) and Boc–Ala–OSu (1.1 g) essentially as described for the preparation of II. The product was purified by procedure A, followed by reprecipitation from EtOAc with ether. Yield 1.5 g (83%), mp 136—145 °C, $[\alpha]_D^{21}$ −7.2° (c=1.0, DMF), Rf^1 0.51, single ninhydrin-positive spot. *Anal.* Calcd for $C_{22}H_{41}N_6O_9$: C, 49.52; H, 7.75; N, 15.75. Found: C, 49.36; H, 7.91; N, 15.48.

Boc–Val–Ala–Asn–Asn–Val–OMe (XII) This compound was prepared from XI (1.1 g) and Boc–Val–OSu (692 mg) essentially as described for the preparation of II. The product was purified by procedure B, followed by reprecipitation from MeOH with ether. Yield 1 g (77%), mp 132—140 °C, $[\alpha]_D^{21}$ −8.3° (c=1.0, DMF), Rf^1 0.49, single ninhydrin-positive spot. *Anal.* Calcd for $C_{27}H_{50}N_7O_{10} \cdot H_2O$: C, 49.84; H, 8.05; N, 15.07. Found: C, 49.68; H, 8.21; N, 15.37.

Boc–Val–Ala–Asn–Asn–Val–NHNH$_2$ [6] This compound was prepared from XII (814 mg) and hydrazine hydrate (0.59 ml) essentially as described for the preparation of **2**. The product was recrystallized from hot MeOH. Yield 618 mg (78%), mp 180—193 °C, $[\alpha]_D^{21}$ −12.1° (c=1.0, DMF), Rf^1 0.47, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{26}H_{50}N_9O_9$: C, 49.36; H, 7.97; N, 19.92. Found: C, 49.08; H, 8.20; N, 19.64.

Synthesis of Protected [Glu^{34}]hSP 1. Successive azide condensations of nine fragments except for Boc–(25—29)–OH **[5]**, 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 or DMF–DMSO (1 : 1) or DMF–HMPA (1 : 1) containing NMM (1.1 eq). The corresponding azide (the amount was increased from 1.5 to 4 eq as chain elongation progressed) in DMF or 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-fitration 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 removed by evaporation and the residue was treated with ether to afford a powder.

2. The HOSu–WSCI condensation procedure: Boc–(30—48)–OBzl was treated with TFA–anisole and the N^a-deprotected peptide, isolated as usual, was dissolved in DMF–DMSO (1 : 1) together with NMM (1.1 eq). To this were added Boc–Thr(Bzl)–Leu–Pro–Ala–Gly–OH (2.5 eq), HOSu (2.5 eq) and WSCI (2.5 eq) 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 protected [Glu^{34}]hSP and its intermediates are listed in Tables II and III.

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–Glu–Val–Tyr–Val–Glu–Leu–Tyr–Leu–Gln–Ser–Leu–Thr–Ala–Glu–His–OH ([Glu^{34}]hSP) The protected [Glu^{34}]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 × 92 cm) using 2% AcOH as an eluant. The fractions (5 ml each) corresponding to the front main peak (tube Nos. 52—68, 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 × 68 cm), which was eluted first with H_2O (100 ml) and then with a linear gradient from H_2O (250 ml) to 0.12 M NH_4OAc buffer (250 ml, pH 6.45). Individual fractions (5 ml each) were collected and absorbancy at 260 nm was determined. The main peak (tube Nos. 56—64) was collected and the solvent was removed by lyophilization and the residue was rechromatographed under the same conditions. Analysis by TLC revealed the presence of two Sakaguchi-positive spots with Rf^2 0.31 (main) and 0.54 (minor). The crude peptide was dissolved in a small amount of the upper phase of Partridge's solvent system.[26] The solution was applied to a column of cellulose (2.3 × 84 cm), which was previously equilibrated with the same upper phase and eluted with the same solvent. Each fraction was examined by means of the Sakaguchi test, and the fractions that exhibited a Sakaguchi-positive single spot (Rf^2 0.31) were evaporated off. The residue was dissolved in 2% AcOH, and the solution was then subjected to Sephadex G-50 column chromatography as described above. Yield 5.1 mg (15%), $[\alpha]_D^{21}$ −81.9° (c=0.3, 2% AcOH), Rf^2 0.31, Rf^3 0.47, 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 2.88, mobility 7.6 cm from the origin toward the anode, after running at 1.5 mA, 600 V for 70 min. The retention time was 19.13 min in HPLC on an analytical Nucleosil $5C_{18}$ column (4 × 150 mm) on gradient elution with acetonitrile (25 to 45%) 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 3.96, Leu 8.03, Val 5.92, Pro 2.89, Met 0.91, Tyr 1.93, Ser 1.85, Thr 2.87, Asp 1.89, Glu 7.86, His 0.90, Lys 4.91, Arg 0.87 (recovery of Gly 83%). Amino acid ratios in papain plus leucine aminopeptidase digest: Gly 2.00, Ala 4.03, Leu 7.89, Val 5.94, Pro 2.84, Met 0.89, Tyr 1.96, Ser 1.87, Thr 2.84, Glu 5.87, His 0.92, Lys 4.88, Arg 0.92; Asn and Gln were not determined (recovery of Gly 82%).

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,[28] by density centrifugation of the blood on Lymphoprep (Nyegaard 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 16 h. Then B-lymphocytes were detected by the method described by Shevach *et al.*[29] One aliquat 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 fluorescent microscopy. Two hundred

lymphocytes were counted and those with surface immunofluorescence were classified as B-lymphocytes.

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; WSCI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; HOSu, *N*-hydroxysuccinimide; Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl; NP, *p*-nitrophenyl; OBzl, benzyl ester; Bzl, benzyl; Troc, β,β,β-trichloroethoxycarbonyl; 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; Tos, *p*-toluenesulfonic acid; OMe, methyl ester; Mts, mesitylene-2-sulfonyl; ONp, *p*-nitrophenyl ester; CM, carboxymethyl; BSS, balanced salt solution; Su; succinimide; hTP, human thymopoietin.
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