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Syntheses and Effects of Human Splenin (hSP) Fragment 32—48 and an Analog on the Reduced B-Lymphocytes of Uremic Patients[1)]

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A heptadecapeptide, H–Arg–Lys–Ala–Val–Tyr–Val–Glu–Leu–Tyr–Leu–Gln–Ser–Leu–Thr–Ala–Glu–His–OH, corresponding to amino acids 32 to 48 of human splenin (hSP) and an analog in which the amino acid residue at position 34 is changed from Ala to Glu, were synthesized. These peptides were synthesized using conventional solution synthesis and were tested for their effect on reduced B-lymphocytes of uremic patients. Incubation of peripheral lymphocytes isolated from uremic patients with these two synthetic heptadecapeptides, hSP fragment 32—48 and [Glu^{34}]hSP fragment 32—48, had an enhancing effect on the reduced B-lymphocytes, but synthetic bovine thymopoietin II (bTP-II) fragment 32—49 had no effect under the same conditions.

Keywords human splenin (hSP) fragment synthesis; trifluoromethanesulfonic acid deprotection; preparative thin-layer chromatography; uremic patient; reduced B-lymphocyte; enhancing effect

Differentiation of lymphoid precursor cells in a variety of species is induced by polypeptide hormones such as bovine thymopoietin (bTP) for T-lymphocytes and bovine splenin (bSP) for B-lymphocytes. bTP and bSP are 49-amino acid polypeptides that differ in only 2 amino acids at positions 34 and 43[2)]; synthetic pentapeptides corresponding to residues 32—36, called thymopentin (TP-5) and splenopentin (SP-5) reproduce the biological activities of bTP and bSP, respectively.[3,4)] TP-5 affects neuromuscular transmission, whereas SP-5 does not.[5)] Also, whereas TP-5 is a selective inducer of prothymocytes, SP-5 induces T- and B-lymphocyte precursors indiscriminately.[4)] All these differences must be ascribed to the single amino acid substitution that distinguishes TP-5 from SP-5.

In 1987, Audhya *et al.*[5)] 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 it was shown that these 48-amino acid polypeptides differed at four positions.[5)] As shown in Fig. 1, position 34 in the active site of hSP has changed from Glu in bSP to Ala in hSP.

In our preceding paper,[6)] we reported that a synthetic analog of hSP, [Glu^{34}]hSP, has enhancing activity on the reduced percentage of B-lymphocytes of uremic patients.

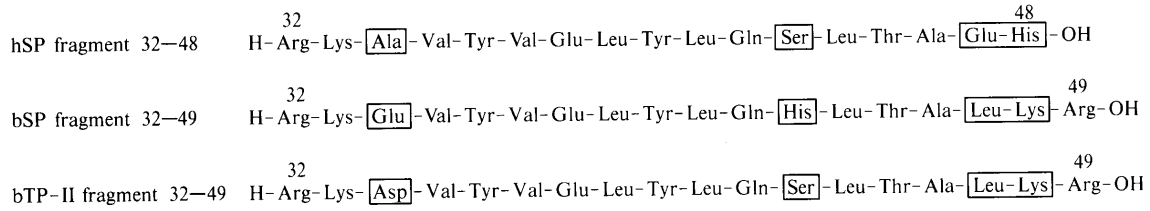

Fig. 1. Sequences of hSP Fragment 32—48, bSP Fragment 32—49 and bTP-II Fragment 32—49

Differences between the sequences are enclosed in boxes.

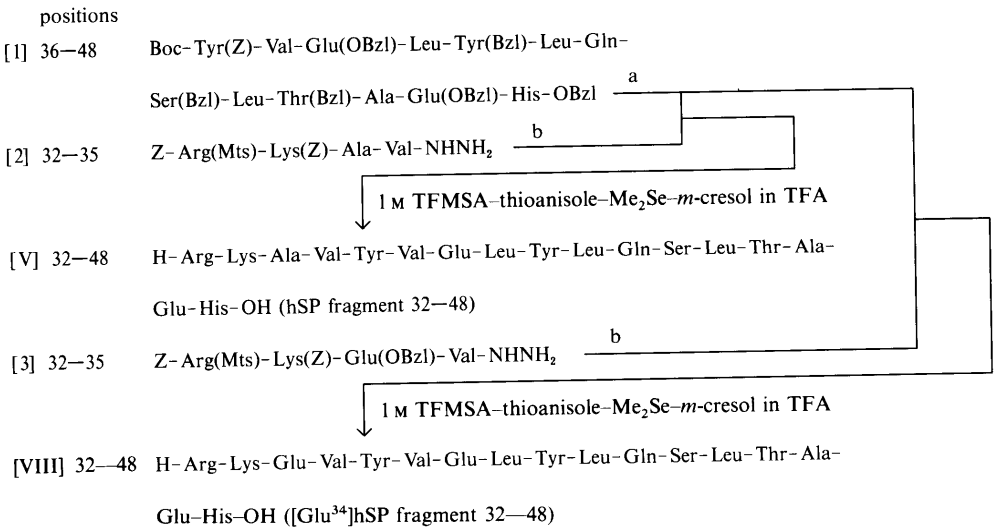

Fig. 2. Synthetic Routes to hSP Fragment 32—48 and [Glu^{34}]hSP Fragment 32—48

a, TFA-anisole; b, azide.

This result prompted us to synthesize the C-terminal peptide fragment corresponding to amino acids 32 to 48 of hSP, which has Ala instead of Glu at position 43. We described here the syntheses of a fragment corresponding to positions 32 to 48 of hSP and its analog in which Ala^{34} is replaced by Glu. Further we compared the *in vitro* effects of these peptides on the reduced percentage of B-lymphocytes of uremic patients.

It is generally accepted that uremia is associated with depression of total T- and B-lymphocyte numbers when compared with normal subjects.[7,8] The cause of depression of the immune system in uremia is still unknown.

The synthetic routes to hSP fragment 32—48 and [Glu^{34}]hSP fragment 32—48 that we have employed are shown in Fig. 2.

In the present synthesis, as illustrated in Fig. 2, amino acid derivatives bearing protecting groups, *i.e.*, Thr(Bzl), Ser(Bzl), Glu(OBzl), Lys(Z), Z–Arg(Mts), Tyr(Bzl) and His–OBzl, that could be removed by 1 M TFMSA–thioanisole in TFA[9] were employed. Those protecting groups survive mostly intact during careful TFA treatment for removal of the Boc group, employed as a temporary α-amino protecting group.

Throughout the syntheses of hSP fragment 32—48 and [Glu^{34}]hSP fragment 32—48, the homogeneity of each fragment and intermediate was checked by TLC and elemental analysis. The purity of the final deprotected peptides was also checked by TLC using two different solvent systems and amino acid analyses after acid hydrolysis and enzymatic digestion. Prior to every condensation reaction, the Boc group was removed by TFA in the presence of anisole as usual.

The two protected peptides, Boc–(36—48)–OBzl [1] and Boc–(33—35)–NHNH–Troc, are identical with those employed for the [Glu^{34}]hSP synthesis[6] and bSP synthesis[10] respectively and two, [2] and [3], were newly synthesized. Fragment [2], Z–Arg(Mts)–Lys(Z)–Ala–Val–$NHNH_2$, was prepared in a stepwise manner starting with H–Val–OMe·HCl. The respective amino acids were introduced by the Su procedure[11] and the MA method[12] followed by the usual hydrazine treatment. Fragment [3], Z–Arg(Mts)–Lys(Z)–Glu(OBzl)–Val–$NHNH_2$, was easily prepared by condensation of Boc–Lys(Z)–Glu(OBzl)–Val–NHNH–Troc[10] with Z–Arg(Mts)–OH·DCHA by the MA procedure and from the resulting tetrapeptide derivative, Z–Arg(Mts)–Lys(Z)–Glu(OBzl)–Val–NHNH–Troc, the Troc group[13] was removed by treatment with Zn/AcOH.[14] These hydrazides and their intermediates were characterized by TLC and elemental analysis. The Boc group of Boc–(36—48)–OBzl[6] [1] was removed by the usual TFA–anisole treatment and the corresponding free amine was condensed with [2] by the azide procedure[15] to yield Z–(32—48)–OBzl [IV]. The homogeneity of the peptide was assessed by elemental analysis, TLC and amino acid analysis of the 6 N HCl hydrolysate. The protected Glu^{34}-substituted heptadecapeptide ester VII was synthesized in a manner similar to that described for the preparation of IV. These two protected heptadecapeptides, IV and VII, obtained after fragment condensation were purified by gel-filtration on Sephadex LH-20 followed by precipitation from DMF with ether. Throughout this synthesis, Ala was taken as a diagnostic amino acid in acid hydrolysates. By comparison of the recovery of Ala with that of newly incorporated amino acids, satisfactory condensation of N-terminal tetrapeptide fragments was confirmed.

The protected heptadecapeptide ester IV thus obtained was treated with 1 M TFMSA–thioanisole in TFA[9] in the presence of *m*-cresol and Me_2Se in an ice-bath for 90 min to remove all protecting groups. *m*-Cresol was used as an additional scavenger to suppress a side reaction, *i.e.*, O-sulfation of the Tyr residues.[16] Me_2Se was employed to facillitate acidic cleavage of protecting groups.[17] The deprotected peptide was converted to the corresponding acetate by treatment with Amberlite IRA-400, and treated with 1 N NH_4OH to reverse the N→O shift.[18]

The deprotected peptide was purified by gel-filtration on a Sephadex G-25 column using 2% AcOH, followed by partition column chromatography on Sephadex G-25 according to Yamashiro.[19] Analysis of the main fractions by TLC using Partridge's solvent system[20] revealed the presence of one major ninhydrin- and Sakaguchi-positive spot and one minor spot. The crude heptadecapeptide was further purified by preparative TLC developed with Partridge's solvent system. The heptadecapeptide V so obtained was found to be a single compound from the result of TLC using two different solvent systems. The purified product thus obtained was then subjected to Sephadex G-25 column chromatography as described above. The amino acid ratios in the acid hydrolysate of V and aminopeptidase (AP)-M digest agreed with theoretical values. The [Glu^{34}]hSP fragment 32—48 VIII was also prepared essentially in the same manner as described for the preparation of V. The heptadecapeptide, [Glu^{34}]hSP fragment 32—48, obtained was also found to be a single compound from the result of TLC using two different solvent systems. The amino acid ratios in the acid hydrolysate of VIII and AP-M digest agreed with theoretical values.

These two peptides each gave a single spot on paper electrophoresis and exhibited a single peak on HPLC.

The *in vitro* effects of these two synthetic heptadecapeptides and the synthetic octadecapeptide[21] corresponding to amino acids 32 to 49 of bTP-II on reduced B-lymphocytes of uremic patients are shown in Table I.

In contrast to normal persons, the percentage of the B-lymphocytes of uremic patients is reduced. Incubation of lymphocytes from uremic patients in the presence of syn-

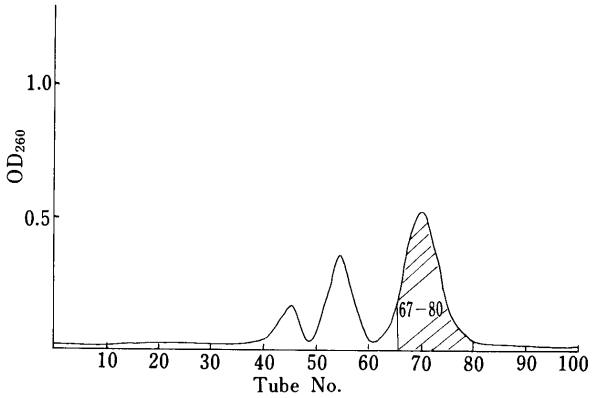

Fig. 3. Purification of Synthetic [Glu^{34}]hSP Fragment 32—48 by Partition Column Chromatography on Sephadex G-25

Column: 3.0 × 90 cm. Eluent: BuOH–AcOH–H_2O (4 : 1 : 5, upper phase).

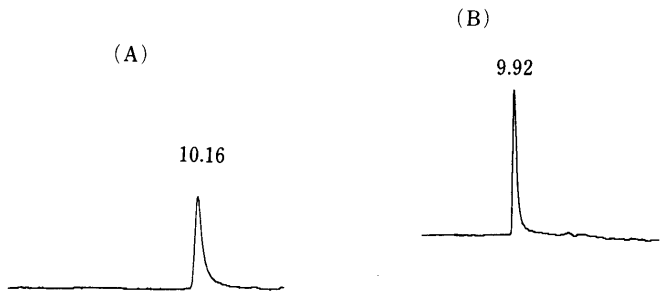

Fig. 4. HPLC of Synthetic hSP Fragment 32—48 (A) and [Glu^{34}]hSP Fragment 32—48 (B)

TABLE I. Effects of the Synthetic hSP Fragment 32—48 and an Analog [Glu^{34}]hSP Fragment 32—48 on the Reduced B-Lymphocytes of Uremic Patients

Peptides	No.	Dose (μg/ml)	B-Lymphocytes[a] (%)
(1) —[b]	3	—	18.2±5.5
(2) —[c]	3	—	7.4±3.4[e]
(3) hSP fragment 32—48[c,d]	3	0.1	7.6±3.6
(4) hSP fragment 32—48[c,d]	3	2.0	11.3±4.8[f]
(5) hSP fragment 32—48[c,d]	3	20.0	15.2±5.2[f]
(6) [Glu^{34}]hSP fragment 32—48[c,d]	3	0.1	7.3±3.7
(7) [Glu^{34}]hSP fragment 32—48[c,d]	3	2.0	10.8±4.9[f]
(8) [Glu^{34}]hSP fragment 32—48[c,d]	3	20.0	15.6±5.3[f]
(9) bTP-II fragment 32—49[c,d]	3	2.0	7.1±3.6
(10) bTP-II fragment 32—49[c,d]	3	20.0	6.3±3.5

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 15 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).

thetic peptides (0.1—20 μg/ml) was carried out to investigate the recovery of B-lymphocytes. The synthetic hSP fragment 32—48 and [Glu^{34}]hSP fragment 32—48 both induced some recovery of the reduced percentage of B-lymphocytes although bTP-II fragment 32—49 did not show any such effect under the same conditions.

In the case of normal subjects, *in vitro* addition of these synthetic peptides did not have any enhancing effect on the percentage of B-lymphocytes under the same conditions (data not shown). These results seem to suggest that not only bSP or SP-5 but also the heptadecapeptide corresponding to the C-terminal region of hSP acts on differentiation of B-lymphocytes, whereas the octadecapeptide corresponding to the C-terminal region of bTP-II does not. These results indicate that the C-terminal heptadecapeptide sequence of hSP contains an active region on B-lymphocyte differentiation, as does that of bSP.

Experimental

General experimental procedures used were essentially the same as described in the previous papers.[6,22] Azides were prepared according to Honzl and Rudinger[15] 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 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 products corresponding to the C-terminal heptadecapeptide fragment of hSP and its analog, [Glu^{34}]hSP fragment 32—48, were chromatographed on cellulose plates (Merck). *Rf*^{2} values refer to the Partridge system[20] and *Rf*^{3} values refer to BuOH–pyridine–AcOH–H_2O (30:20:6:24).[23] AP-M (EC 3.4.11.2, Aminopeptidase 20214) was purchased from the Peptide Institute Inc., Osaka. Labelled B-lymphocyte counting was done under a Nikon UFD-TR fluorescence microscope. Patient selection: Peripheral lymphocytes were obtained from three uremic patients suffering from chronic renal failure. In these patients, the percentage 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–Ala–Val–OMe [I] H–Val–OMe·HCl (1.7 g) was dissolved in DMF (15 ml) containing NMM (1.2 ml). To this solution, Boc–Ala–OSu (3 g) was added, and the mixture was stirred at room temperature for 6 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 petroleum ether: Yield 2.6 g (79%) (oily material), $[\alpha]_D^{21}$ −21.3° (c=1.0, DMF), *Rf*^{1} 0.70, single ninhydrin-positive spot. *Anal.* Calcd for $C_{14}H_{26}N_2O_5\cdot H_2O$: C, 52.49; H, 8.81; N, 8.74. Found: C, 52.36; H, 8.70; N, 8.91.

Boc–Lys(Z)–Ala–Val–OMe [II] I (1.6 g) was treated with TFA–anisole (16 ml–3.6 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 (16 ml) containing NMM (0.6 ml). To this solution, Boc–Lys(Z)–OSu (2.5 g) was added, and the solution was stirred at room temperature for 6 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 *n*-hexane: Yield 2.1 g (70%), mp 91—94 °C, $[\alpha]_D^{21}$ −11.6° (c=1.0, DMF), *Rf*^{1} 0.64, single ninhydrin-positive spot. *Anal.* Calcd for $C_{28}H_{44}N_4O_8\cdot 2H_2O$: C, 55.99, H, 8.05; N, 9.33. Found: C, 59.56; H, 7.85; N, 9.92.

Z–Arg(Mts)–Lys(Z)–Ala–Val–OMe [III] II (2 g) was treated with TFA–anisole (20 ml–4 ml) as described above and the resulting powder was dissolved in THF (10 ml) containing NMM (0.4 ml). To this ice-chilled solution, a solution of the mixed anhydride [prepared from 2 g of Z–Arg(Mts)–OH·DCHA with 0.46 ml of ethylchlorocarbonate and 0.4 ml of NMM at −10 °C] in THF (10 ml) and acetonitrile (5 ml) was added. The mixture was stirred at 4 °C for 2 h and then at room temperature for 8 h. The mixture was evaporated *in vacuo*. The residue was extracted with EtOAc, and the extract was washed successively with 1 N HCl, H_2O, 5% $NaHCO_3$ and H_2O, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was reprecipitated from EtOAc with ether: Yield 2 g (65%), mp 116—121 °C, $[\alpha]_D^{21}$ −8.6° (c=1.0, DMF), *Rf*^{1} 0.59, single chlorine–tolidine-positive spot. *Anal.* Calcd for $C_{46}H_{64}N_8O_{11}$ S: C, 58.96; H, 6.88; N, 11.96. Found: C, 58.54; H, 7.14; N, 12.30.

Z–Arg(Mts)–Lys(Z)–Ala–Val–NHNH$_2$ [2] III (1.9 g) was dissolved in DMF–MeOH (1:1, 12 ml). To this solution, hydrazine hydrate (1 ml) was added and the solution was kept standing at room temperature for 24 h. After evaporation of MeOH, the residue was poured into ether. The precipitate formed was washed with MeOH and ether. The product was recrystallized from MeOH: Yield 1.4 g (74%), mp 171—176 °C, $[\alpha]_D^{21}$ −13.4° (c=1.0, DMF), *Rf*^{1} 0.47, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{45}H_{64}N_{10}S\cdot H_2O$: C, 56.59; H, 6.97; N, 14.66. Found: C, 56.47; H, 6.98; N, 14.34.

Z–Arg(Mts)–Lys(Z)–Ala–Val–Tyr(Bzl)–Val–Glu(OBzl)–Leu–Tyr(Bzl)–Leu–Gln–Ser(Bzl)–Leu–Thr(Bzl)–Ala–Glu(OBzl)–His–OBzl [IV] Boc–Tyr(Bzl)–Val–Glu(OBzl)–Leu–Tyr(Bzl)–Leu–Gln–Ser(Bzl)–Leu–Thr(Bzl)–Ala–Glu(OBzl)–His–OBzl[6] [1] (125 mg) was treated with TFA–anisole (2 ml–0.4 ml) as usual and dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo*, and dissolved in DMF–DMSO (1:1, 3 ml) containing NMM (0.008 ml). The azide [prepared from 96 mg of [2] (2 eq)] in DMF–DMSO (1:1, 2 ml) and NMM (0.028 ml) were added to the above ice-chilled solution. The mixture, after being stirred at −10 °C for 36 h, was neutralized with AcOH and concentrated *in vacuo*. The residue was treated with 5% citric acid and the precipitate thereby formed was washed with 5% citric acid and H_2O. The resulting powder was purified by gel-filtration on Sephadex LH-20 (3×94 cm) with DMF containing 3% H_2O. The UV absorption at

280 nm was determined in each fraction (4 ml) and the desired fractions containing a substance of Rf^1 0.59 (chlorine–tolidine-positive spot) were combined. The solvent was removed by evaporation and the residue was treated with ether to afford a powder: Yield 98 mg (59%), mp 170—183 °C, $[\alpha]_D^{21}$ −19.2° (c=1.0, DMSO), Rf^1 0.59, single chlorine–tolidine-positive spot. *Anal.* Calcd for $C_{166}H_{210}N_{24}O_{33}S \cdot 11\ H_2O$: C, 60.42; H, 7.09; N, 10.19. Found: C, 60.20; H, 7.31; N, 10.43. Amino acid ratios in a 6 N HCl hydrolysate: Ala 2.00, Leu 3.06, Val 1.94, Tyr 1.88, Ser 0.91, Thr 0.92, Glu 2.93, His 0.92, Lys 1.02, Arg 0.94 (recovery of Ala 83%).

H–Arg–Lys–Ala–Val–Tyr–Val–Glu–Leu–Tyr–Leu–Gln–Ser–Leu–Thr–Ala–Glu–His–OH (Human Splenin Fragment 32—48) [V] The protected heptadecapeptide IV (80 mg) was treated with 1 M TFMSA–thioanisole in TFA (3 ml) in the presence of *m*-cresol (150 μl) and Me_2Se (80 μl) in an ice-bath for 90 min, then dry 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 2 g) for 30 min, was filtered. 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 and lyophilized. The product was purified by gel-filtration on Sephadex G-25 (3.2 × 94 cm) using 2% AcOH as an eluant. The fractions (4 ml each) corresponding to the front main peak (tube Nos. 52—61, determined by UV absorption measurement at 260 nm) were combined and the solvent was removed by lyophilization to give a fluffy powder. The crude product thus obtained was purified by partition column chromatography on Sephadex G-25 according to Yamashiro.[19] The product was dissolved in a small amount of the upper phase of BuOH–AcOH–H_2O (4 : 1 : 5). The solution was applied to a column of Sephadex G-25 (2.7 × 93 cm) previously equilibrated with the lower phase of the above solvent system. The column was developed with the same upper phase. Individual fractions (4 ml each) were collected and the absorbancy at 260 nm was determined. The fractions corresponding to the main peak (tube Nos. 61—76) were combined. The solvent was removed by evaporation. Analysis by TLC revealed the presence of two ninhydrin- and Sakaguchi-positive spots with Rf^2 0.09 (major) and 0.31 (minor). The crude product was subjected to preparative TLC (cellulose plate, 20 × 40 cm) using the Partridge system as a developing solvent. The zone corresponding to Rf^2 0.09 was separated and extracted with 2% AcOH. The extracts were concentrated to a small volume and subjected to Sephadex G-25 column chromatography as described above: Yield 9.1 mg (19%), $[\alpha]_D^{21}$ −56.3° (c=0.3, 2% AcOH), Rf^2 0.09, Rf^3 0.27, 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 2.80; 5.3 cm from the origin toward the anode after running at 1.5 mA, 550 V for 70 min. The synthetic peptide exhibited a single peak on HPLC using a Nucleosil $5C_{18}$ column (4.0 × 150 mm) at a retention time of 10.16 min, when eluted with a gradient of acetonitrile (25—45% in 30 min) in 0.1% TFA at a flow rate of 1.0 ml per min (Fig. 4). Amino acid ratios in a 6 N HCl hydrolysate: Ala 2.00, Leu 2.93, Val 1.91, Tyr 2.04, Ser 0.91, Thr 0.93, Glu 2.95, His 0.94, Lys 0.97, Arg 0.93 (recovery of Ala 84%). Amino acid ratios in an AP-M digest: Ala 2.00, Leu 3.03, Val 2.04, Tyr 1.99, Ser 0.92, Glu 1.89, His 0.95, Lys 0.97, Arg 0.93, Thr + Gln 1.84 (recovery of Ala 84%) (Gln emerged at the same position as Thr, and was calculated as Thr).

Z–Arg(Mts)–Lys(Z)–Glu(OBzl)–Val–NHNH–Troc [VI] This compound was prepared essentially in the same manner as described for the preparation of III by using Boc–Lys(Z)–Glu(OBzl)–Val–NHNH–Troc[10] (1.8 g), Z–Arg(Mts)–OH·DCHA (1.2 g) and ethylchlorocarbonate (0.26 ml). The product was reprecipitated from EtOAc with ether: Yield 2.2 g (85%), mp 121—127 °C, $[\alpha]_D^{21}$ −17.3° (c=1.0m DMF), Rf^1 0.66, single chlorine–tolidine-positive spot. *Anal.* Calcd for $C_{57}H_{37}Cl_3N_{10}O_{14}S$: C, 54.31; H, 5.84; N, 11.11. Found: C, 54.04; H, 6.10; N, 11.37.

Z–Arg(Mts)–Lys(Z)–Glu(OBzl)–Val–$NHNH_2$ [3] A solution of VI (2.1 g) in a mixture of AcOH (7 ml) and DMF (7 ml) was treated with Zn dust (1.1 g) at 0 °C for 2 h and then for 10 h at room temperature. The mixture was filtered, the filtrate was concentrated *in vacuo*, and the residue was treated with 3% EDTA. The resulting gelatinous mass was washed in batches with 3% EDTA, 5% $NaHCO_3$ and H_2O, and then reprecipitated from DMF with H_2O: Yield 1.6 g (84%), mp 164—176 °C, $[\alpha]_D^{21}$ −18.2° (c=1.0, DMF), Rf^1 0.53, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{54}H_{72}N_{10}O_{12}S \cdot 2H_2O$: C, 57.84; H, 6.83; N, 12.49. Found: C, 57.69; H, 6.78; N, 12.25.

Z–Arg(Mts)–Lys(Z)–Glu(OBzl)–Val–Tyr(Bzl)–Val–Glu(OBzl)–Leu–Tyr(Bzl)–Leu–Gln–Ser(Bzl)–Leu–Thr(Bzl)–Ala–Glu(OBzl)–His–OBzl [VII] Boc–Tyr(Bzl)–Val–Glu(OBzl)–Leu–Tyr(Bzl)–Leu–Gln–Ser(Bzl)–Leu–Thr(Bzl)–Ala–Glu(OBzl)–His–OBzl [1] (125 mg) was treated with TFA–anisole (3 ml–0.6 ml) as described above and the resulting powder was dissolved in DMF–DMSO (1 : 1, 3 ml) containing NMM (0.008 ml). The azide [prepared from 112 mg of 3 (2 eq)] in DMF–DMSO (1 : 1, 3 ml) and NMM (0.028 ml) were added to the above ice-chilled solution and the mixture was stirred at −10 °C for 36 h. After addition of a few drops of AcOH, the ninhydrin-negative solution was poured into ice-chilled 5% citric acid with stirring. The precipitate thereby formed was washed with 5% citric acid and H_2O. The resulting powder was purified by gel-filtration on Sephadex LH-20 (3 × 94 cm) with DMF containing 3% H_2O. The UV absorption at 280 nm was determined in each fraction (4 ml) and the desired fractions containing a substance of Rf^1 0.68 (chlorine–tolidine-positive spot) were combined. The solvent was removed by evaporation and the residue was treated with ether to afford a powder: Yield 121 mg (69%), mp 181—190 °C, $[\alpha]_D^{21}$ −30.2° (c=1.0, DMSO). *Anal.* Calcd for $C_{180}H_{227}N_{24}O_{37}S \cdot 8H_2O$: C, 61.86; H, 7.01; N, 9.62. Found: C, 61.59; H, 7.24; N, 10.03. Amino acid ratios in a 6 N HCl hydrolysate: Ala 1.00, Leu 3.03, Val 2.01, Tyr 1.94, Ser 0.91, Thr 0.93, Glu 3.90, His 0.93, Lys 1.02, Arg 0.94 (recovery of Ala 85%).

H–Arg–Lys–Glu–Val–Tyr–Val–Glu–Leu–Tyr–Leu–Gln–Ser–Leu–Thr–Ala–Glu–His–OH ([Glu^{34}]hSP Fragment 32—48) [VIII] The protected heptadecapeptide VII (87 mg) was treated with 1 M TFMSA–thioanisole in TFA (3 ml) in the presence of *m*-cresol (150 μl) and Me_2Se (80 μl) in an ice-bath for 90 min, then dry 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 2 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 and lyophilized. The product was purified by gel-filtration on Sephadex G-25 (3.2 × 93 cm) using 2% AcOH as an eluant. The fractions (4 ml each) corresponding to the front main peak (tube Nos. 56—64; 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 a small amount of the upper phase of BuOH–AcOH–H_2O (4 : 1 : 5). The solvent was applied to a column of Sephadex G-25 (3.0 × 90 cm) previously equilibrated with the lower phase of the above solvent system. The column was developed with the same upper phase. Individual fractions (4 ml each) were collected and the absorbancy at 260 nm was determined. The fractions corresponding to the main peak (tube Nos. 67—80) were combined. The solvent was removed by evaporation. Analysis by TLC revealed the presence of three Sakaguchi-positive spots with Rf^2 0.12 (main), 0.39 (minor) and 0.69 (minor). The crude peptide was subjected to preparative TLC (cellulose plate, 20 × 40 cm) using the Partridge system (upper phase) as a developing solvent. The zone corresponding to Rf^2 0.12 was separated and extracted with 2% AcOH. The extract was concentrated to a small volume, applied to a Sephadex G-25 column (3.2 × 94 cm), and eluted with 2% AcOH. The main fractions containing a single component were combined and the solvent was removed by lyophilization: Yield 14 mg (27%), $[\alpha]_D^{21}$ −62.1° (c=0.6, 2% AcOH), Rf^2 0.12, Rf^3 0.31, 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 4.9 cm from origin toward the anode after running at 1.5 mA, 550 V for 70 min. The synthetic peptide exhibited a single peak on HPLC using a Nucleosil $5C_{18}$ column (4.0 × 150 mm) at a retention time of 9.92 min, when eluted with a gradient of acetonitrile (25—45% in 30 min) in 0.1% TFA at a flow rate of 1.0 ml per min (Fig. 4). Amino acid ratios in a 6 N HCl hydrolysate: Ala 1.00, Leu 2.98, Val 2.03, Tyr 1.94, Ser 0.92, Thr 0.94, Glu 3.96, His 0.93, Lys 1.02, Arg 0.96 (recovery of Ala 84%). AP-M digest: Ala 1.00, Leu 3.02, Val 1.97, Tyr 1.98, Ser 0.93, Glu 2.96, His 0.96, Lys 0.99, Arg 0.94, Thr + Gln 1.84 (recovery of Ala 82%) (Gln emerged at the same position as Thr, and was calculated as Thr).

B-Lymphocyte Assay Blood samples for B-lymphocyte assay were collected in heparinized tubes. Venous blood (20 ml) was obtained from patients. A lymphocyte suspension relatively free of monocytes and granulocytes was harvested by the method of Loos and Roos[24] 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 15 h. Then B-lymphocytes were detected by the method described by Shevach *et al.*[25] 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 theen 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.

References and Notes

1) Abbreviations used: DMF, dimethylformamide; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; Boc, *tert*-butoxycarbonyl; OSu, *N*-hydroxysuccinimide ester; Z, benzyloxycarbonyl; OBzl, benzyl ester; Bzl, benzyl; Troc, β,β,β-trichloroethoxycarbonyl; Su, *N*-hydroxysuccinimide; NMM, *N*-methylmorpholine; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; MA, mixed anhydride; EDTA, ethylenediaminetetraacetic acid; AcOH, acetic acid; EtOAc, ethyl acetate; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; OMe, methyl ester; Mts, mesitylene-2-sulfonyl; BSS, balanced salt solution; DCHA, dicyclohexylamine; UV, ultraviolet.
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