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# Synthesis of [Phe(4F)$^3$]Thymopoietin II and Examination of its Immunological Effect on the Impaired Blastogenic Response of T-Lymphocytes of Uremic Patients

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**Abstract**—[Phe(4F)$^3$]thymopoietin II was synthesized using a conventional solution method. The deprotection of the protected [Phe(4F)$^3$]thymopoietin II was achieved by treatment with 1 M trifluoromethanesulfonic acid:thioanisole (molar ratio 1:1) in trifluoroacetic acid in the presence of dimethylselenide and *m*-cresol. The synthetic [Phe(4F)$^3$]thymopoietin II and thymopoietin II were tested for effect on impaired T-lymphocyte transformation by phytohemagglutinin in uremic patients suffering from recurrent infectious diseases. The restoring activity on the impaired phytohemagglutinin stimulation of T-lymphocytes was obtained after incubation of peripheral lymphocytes isolated from uremic patients with the synthetic [Phe(4F)$^3$]thymopoietin II. This peptide exhibited far stronger restoring effect than that of our synthetic thymopoietin II.

## Introduction

Bovin thymopoietin II (TP-II) is a 49-amino acid polypeptide hormone of the thymus, which proves to be active in induction of early T-lymphocyte differentiation and modulation of mature lymphocytes.[1–3]

The complete amino acid sequence of TP-II was determined,[4] and the biological activity was shown by chemical synthesis to reside in a fragment (positions 29–41) of TP-II.[5] Subsequently, the pentapeptide corresponding to residues 32–36 of TP-II, was shown to retain the biologically active site of the parent peptide.[7] However, the potency of the synthetic TP-II was more than 10 times the potency of the synthetic tridecapeptide corresponding to the sequence 29–41 of TP-II.[7]

Thymus peptides are an object of intensive studies in our laboratories.[8–17] The life of patients with chronic renal failure has been prolonged by recent advances in hemodialysis therapy. However, the complication of infectious diseases in chronic hemodialysis patients still poses a grave problem in terms of their prognosis. It is an accepted fact that there is a decrease in immunity, particularly cell-mediated immunity, in uremic patients.[18] This impairement is reflected in both *in vitro* and *in vivo* depressed cell-mediated immune function. Patients with chronic uremia may also have thymic atrophy. The thymus may show a marked reduction in lymphoid elements and extensive replacement with fat.

These observations[19] suggested to us that one of the cell-mediated immune abnormalities seen in chronic uremia might be attributable to thymic hormone deficiency.

In our preceding papers,[8–17] we synthesized TP-I, TP-II, TP-III, human TP and their fragments by a conventional method to study the immunological effect on impaired lymphocytes from uremic patients with cell-mediated immunodeficiency. Among them, we reported that our synthetic TP-II exhibited restoring activity on the impaired PHA stimulation of T-lymphocytes *in vitro* in uremic patients with infectious diseases such as pneumonia.

In 1994, we reported[20] that an analog of THF-γ2, [Phe(4F)$^7$]THF-γ2, exhibited far stronger restoring activity on the impaired PHA stimulation of T-lymphocytes from uremic patients than that of THF-γ2. These results prompted us to synthesize a TP-II analog containing *p*-fluorinated Phe$^3$ residue by a conventional solution method and to examine the activity of our synthetic [Phe(4F)$^3$]TP-II on the impaired T-lymphocytes of uremic patients and to compare the relative activity between our synthetic TP-II and [Phe(4F)$^3$]TP-II.

*Abbreviations*: TP, thymopoietin; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl; OBzl, benzyl ester; Boc, tert-butoxycarbonyl; Troc, β,β,β-trichloroethoxycarbonyl; Bzl, benzyl; Mts, mesitylene-2-sulfonyl; OcHex, cyclohexyl ester; HOBT, 1-hydroxybenzotriazole; WSCI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; NMM, *N*-methylmorpholine; EDTA, ethylenediaminetetraacetic acid; DMF, dimethylformamide; DMSO, dimethylsulfoxide; TFMSA, trifluoromethanesulfonic acid; PHA, phytohemagglutinin; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; PRMI, Rosewell Park Memorial Institute; FCS, fetal calf serum; THF, thymic humoral factor.

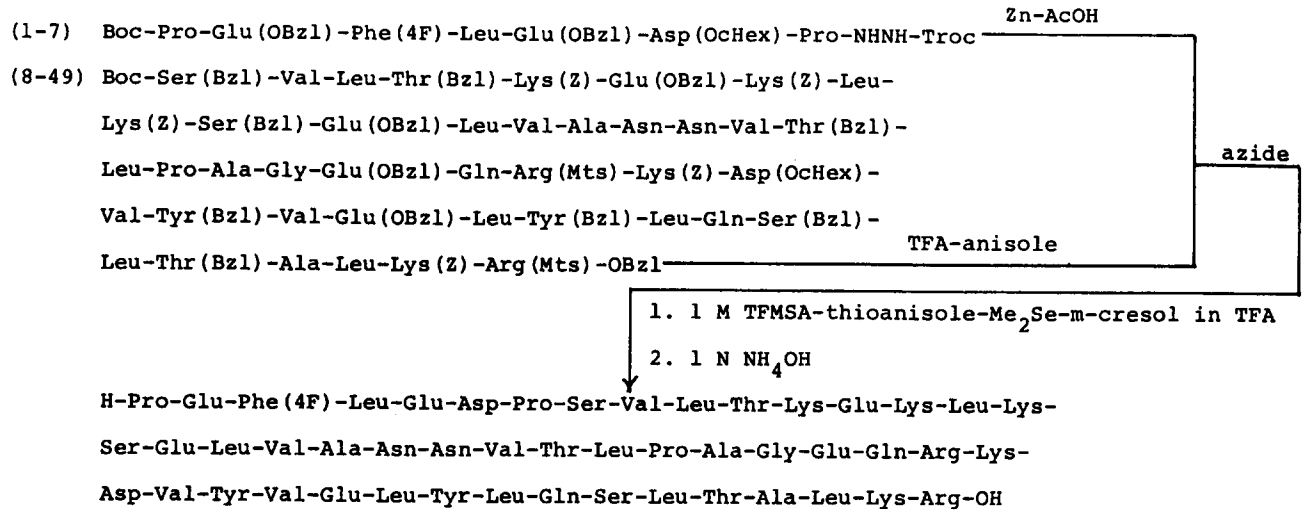


**Figure 1.** Synthetic route to [Phe(4F)$^3$]TP-II.

The synthetic route that we employed is almost the same as those employed for our previous syntheses of TP-II and TP-III.[14,15] As illustrated in Figure 1, the TFA-labile Boc group was employed for $N^{\alpha}$-protection and amino acid derivatives bearing protecting groups removable by the thioanisole-mediated TFMSA deprotecting procedure[21,22] were employed, i.e. Lys(Z), Glu(OBzl), Thr(Bzl), Ser(Bzl), Tyr(Bzl), Asp(OcHex) and Arg(Mts).

$N^{\alpha}$-Deprotection with TFA was performed in the presence of anisole, prior to each condensation reaction as usual. Asp(OcHex) was employed to minimize aspartimide formation during the synthesis of Asp-containing peptides.[23] The C-terminal protected dotetracontapeptide ester[15] was available from our previous synthesis of TP-II. The N-terminal protected heptapeptide, Boc-Pro-Glu(OBzl)-Phe(4F)-Leu-Glu(OBzl)-Asp(OcHex)-Pro-NHNH-Troc (**III**), was prepared stepwise starting from Boc-Leu-Glu(OBzl)-Asp(OcHex)-Pro-NHNH-Troc[15] by the HOBT-WSCI procedure to minimize racemization.

For the preparation of the N-terminal protected heptapeptide hydrazide containing Glu(OBzl) and Asp(OcHex), Boc-Pro-Glu(OBzl)-Phe(4F)-Leu-Glu(O-Bzl)-Asp(OcHex)-Pro-$NHNH_2$ (**IV**), we employed a substituted hydrazide, Troc-$NHNH_2$,[24] a protecting group which is known to be removed by Zn in acetic acid without affecting side chain protecting groups, such as Boc, OBzl and OcHex, and zinc acetate was removed by treatment with EDTA to give the required hydrazide, Boc-Pro-Glu(OBzl)-Phe(4F)-Leu-Glu(OBzl)-Asp(OcHex)-Pro-$NHNH_2$ (**IV**), in analytically pure form. The hydrazine test on the thin-layer chromatograms and elemental analysis data were consistent with homogeneity of the desired product.

The two fragments were assembled by the azide procedure,[25] according to the route illustrated in Figure 1. The procedure for the coupling reaction was the use of mixture of DMF–DMSO instead of DMF, which could dissolve both N- and C-terminal protected peptides. After coupling, Gly was taken as the diagnostic amino acid in acid hydrolysis. By comparison of the recovery of Gly with newly incorporated amino acids, satisfactory incorporation of the N-terminal fragment in condensation reaction was confirmed. The homogeneity of the purified protected nonatetracontapeptide corresponding to the entire amino acid sequence of [Phe(4F)$^3$]TP-II was checked by elemental analysis, TLC and amino acid analysis of the acid hydrolysate.

In the final step of the synthesis, the protected nonatetracontapeptide ester was treated with 1 M TFMSA–thioanisole in TFA to remove all protecting groups. The deprotected crude peptide was purified by Sephadex G-50 and then by ion-exchange column chromatography on a CM-Biogel A column, followed by preparative TLC. The immunological effect of the synthetic TP-II[15] and [Phe(4F)$^3$]TP-II was examined by the JIMRO (Japan Immunoresearch Laboratories Co. Ltd) fluorometric blast-formation test according to Itoh and Kawai.[26]

## Results and Discussion

Our synthetic route to [Phe(4F)$^3$]TP-II is illustrated in Figure 1, which shows two fragments selected as building blocks to construct the entire amino acid sequence of [Phe(4F)$^3$]TP-II. Protected C-terminal dotetracontapeptide ester was identical with that employed in our previous synthesis of TP-II.[15] Thus, one fragment, Boc-Pro-Glu(OBzl)-Phe(4F)-Leu-Glu(OBzl)-Asp(OcHex)-Pro-$NHNH_2$ (**IV**), which covers the area of sequence variation between TP-II and [Phe(4F)$^3$]TP-II, was newly synthesized.

The Boc group of Boc-(8–49)-OBzl[15] was removed by the usual TFA–anisole treatment and the corresponding

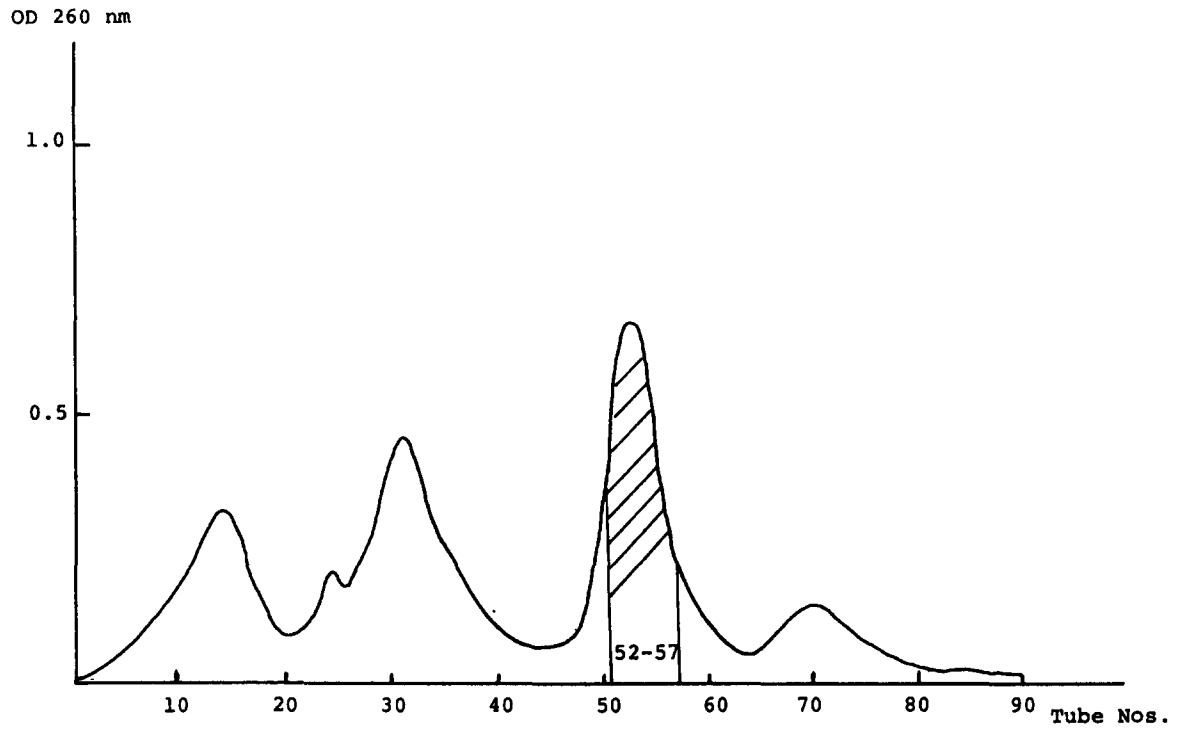


**Figure 2.** Purification of synthetic [Phe(4F)$^3$]TP-II by ion exchange chromatography on a CM-Biogel A column.

free amine was condensed with the protected N-terminal heptapeptide hydrazide **IV** by the azide procedure[25] to yield the protected [Phe(4F)$^3$]TP-II. The protected nonatetracontapeptide 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.[27] $Me_2Se$ was employed to facilitate acidolytic cleavage of protecting groups.[28] The deprotected peptide was next precipitated with dry ether, converted to the corresponding acetate with Amberlite IRA-400 (acetate form) and then treated with 1 M $NH_4OH$ to reverse a possible N → O shift at the Ser and Thr residues.[29]

The crude peptide was purified by gel-filtration on Sephadex G-50 and then by ion-exchange column chromatography on a CM-Biogel A column with linear gradient elution using pH 6.5 ammonium acetate buffer (O → 0.25 M) (Fig. 2), followed by preparative TLC. Desalting on Sephadex G-50, gave a fluffy powder, which exhibited a single spot (ninhydrin- and Sakaguchi-positive) on TLC in two different solvent systems and on paper electrophoresis (pH 2.80 acetate buffer). The peptide also exhibited a single peak on HPLC (Fig. 3).

The molecular weight of the synthetic peptide was ascertained by FAB-mass spectrometry. Homogeneity of the synthetic [Phe(4F)$^3$]TP-II was further ascertained by amino acid analysis after 6 M HCl hydrolysis.

In contrast to normal persons, the transformation of T-lymphocytes into lymphoblasts with mitotic activity after PHA stimulation is depressed in severe uremic patients with infectious diseases.

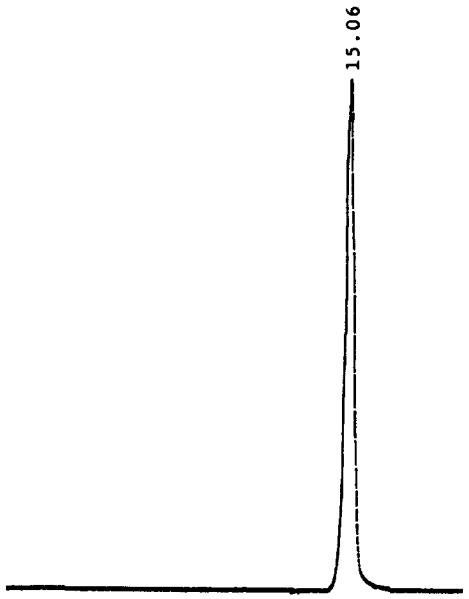


**Figure 3.** HPLC of synthetic [Phe(4F)$^3$]TP-II.

The *in vitro* effect of the synthetic TP-II[15] and [Phe(4F)$^3$]TP-II on the impaired PHA stimulation of T-lymphocytes from uremic patients is shown in Table 1. When peripheral T-lymphocytes isolated from patients were incubated with various amounts of the synthetic TP-II from 0.1 to 2.0 μg mL$^{-1}$ restoration of the impaired PHA stimulation of T-lymphocytes was observed at a concentration of 1 μg mL$^{-1}$ and above. The synthetic analog in which Phe$^3$ was replaced by Phe(4F)

**Table 1.** Effect of the synthetic TP-II and [Phe(4F)$^3$]TP-II on the impaired PHA-stimulation of T-lymphocytes of uremic patients

| | Peptide | Dose (μg/ml) | SI[a,b] |
|---|---|---|---|
| (1) | —[c] | — | 312.1 ± 58.4 |
| (2) | —[d] | — | 124.5 ± 60.2[f] |
| (3) | Tp-II[d,e] | 0.1 | 121.3 ± 58.9 |
| (4) | TP-II[d,e] | 1.0 | 197.7 ± 59.7[g] |
| (5) | TP-II[d,e] | 2.0 | 279.6 ± 61.3[g] |
| (6) | [Phe(4F)$^3$]TP-II[d,e] | 0.1 | 203.9 ± 62.4[g] |
| (7) | [Phe(4F)$^3$]TP-II[d,e] | 1.0 | 282.4 ± 61.7[g] |
| (8) | [Phe(4F)$^3$]TP-II[d,e] | 2.0 | 280.4 ± 60.3[g] |

[a]Each value represents the mean ± SD of triplicate measurements.

[b]SI (stimulation index) was calculated according to the following formula: $SI = \frac{I_2 - I_o}{I_1 - I_o} \times 100$,

where $I_2$ = mean fluorescence intensity of PHA-activated lymphocytes, $I_1$ = fluorescence intensity of PHA-nonactivated lymphocytes and $I_o$ = 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]The significance of differences of mean values was analyzed by means of Student's $t$ test. $p < 0.03$ as compared with (1).

[g]The significance of differences of mean values was analyzed by means of Student's $t$ test. $p < 0.01$ as compared with (2).

exhibited more potent restoring activity than that of the synthetic TP-II. The relative potency of the synthetic TP-II[15] was one-half of that of the synthetic [Phe(4F)$^3$]TP-II. In normal subjects, no effects of TP-II and [Phe(4F)$^3$]TP-II were observed (data not shown).

In our previous paper,[20] we reported that one of our synthetic THF-γ2 analogs, [Phe(4F)$^7$]THF-γ2 exhibited the most potent restoring effect on the impaired blastogenic response of PHA-stimulated T-lymphocytes of uremic patients. Similar results were reported by Maeda *et al.*[30] that [Phe(4F)$^4$]Leu-enkephalin showed much stronger activity in the guinea-pig ileum and mouse vas deferens assays as compared with Leu-enkephalin.

Regarding TP-II, Fujino *et al.*[7] reported that the relative potency of their synthetic TP-II was more than 10 times as potent as the synthetic tridecapeptide corresponding to the sequence 29–41 of TP-II. Schlesinger *et al.*[4] also reported that the fragment 29–41 of TP-II exhibited approximately 10% activity by weight when compared with natural TP-II. A pentapeptide corresponding to residue 32–36 of TP-II is found to be the minimal sequence (active site) that reproduces the immunological effects of TP-II, but these effects are weaker than those of TP-II. These results seem to suggest that the pentapeptide (32–36) requires other regions of the TP-II sequence to exhibit full activity.

Interestingly, our synthetic analog modified at Phe$^3$ with Phe(4F), which has an electron-withdrawing atom, [Phe(4F)$^3$]TP-II, enhances the immunological potency of TP-II. Phe$^3$ of TP-II is an immunologically silent residue, but the modification of Phe$^3$ with Phe(4F) seems to affect immunological activity of TP-II and enhances its biological potency.

## Experimental

General experimental procedures used in this paper are essentially the same as described in previous papers.[14–16] An azide was prepared according to Honzl and Rudinger[25] with isoamyl nitrite. Melting points are uncorrected. Rotations were measured with an Atago Polax machine (cell length: 10 cm). The amino acid compositions of acid hydrolysates were determined with a Hitachi 835-50 type amino acid analyzer. Solvents were concentrated in a rotary evaporator under reduced pressure at a temperature of 30–45 °C. Boc groups of protected peptides were removed by TFA–anisole treatment. The resulting amino components were chromatographed on silica gel plates (Kieselgel G, Merck) and $R_f$ values refer to the following solvent systems: $R_f^1$, BuOH:HOAc:$H_2O$ (4:1:5); $R_f^2$, BuOH:pyridine:HOAc:$H_2O$ (30:20:6:24). The final product corresponding to the entire amino acid sequence of [Phe(4F)$^3$]TP-II was chromatographed on cellulose plates (Merck). $R_f^3$ value refers to BuOH:HOAc:$H_2O$ (4:1:1) and $R_f^4$ value refers to BuOH:pyridine:HOAc:$H_2O$ (30:20:6:24). Troc-$NHNH_2$ was purchased from Kokusan Chemical Works Ltd, Japan.

Kits for the fluorometric blast-formation test were purchased from Japan Immunoresearch Laboratories Ltd, Japan. HPLC was conducted with a Shimadzu LC-5A apparatus coupled to a μBondapak C18 column with a gradient of acetonitrile (15–35%) in 0.1% TFA at 1.0 mL min$^{-1}$ and the elution was monitored at 230 nm. The FAB-mass spectrum was obtained on an Auto spec Q with a OPUS data processor.

### *Patient selection*

Two uremic patients who were suffering from recurrent

infectious diseases were selected. Examination of the cellular immunocompetence of these patients revealed a significant decrease in blast-formation by PHA. [$^3$H]Thymidine incorporation values of these patients were 10,918 and 11,467 cpm, respectively, (normal values 41,745–42,392 cpm). Venous blood was obtained from these uremic patients for the fluorometric blast-formation test. 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.

*Boc-Phe(4F)-Leu-Glu(OBzl)-Asp(OcHex)-Pro-NHNH-Troc (I).* Boc-Leu-Glu(OBzl)-Asp(OcHex)-Pro-NHNH-Troc[14] (2.4 g) was treated with TFA–anisole (12–2.4 mL) in an ice-bath for 40 min, then dry $Et_2O$ was added. The resulting powder was washed with dry $Et_2O$, dried over KOH pellets *in vacuo* for 2 h and then dissolved in DMF (15 mL) containing NMM (0.31 mL). To this ice-chilled solution, Boc-Phe(4F)-OH (779 mg), HOBT (372 mg) and WSCI (527 mg) were successively added. After being stirred at 4 °C for 12 h, the mixture was poured into ice-chilled 5% citric acid with stirring. The precipitate thereby formed was washed successively with 5% citric acid, $H_2O$, 5% $NaHCO_3$ and $H_2O$. The product was reprecipitated from HOAc with $H_2O$. Yield 2.0 g (74%), mp 130–137 °C, $[\alpha]_D^{21}$ –22.9° (*c* 1.0; DMF), $R_f^1$ 0.56, $R_f^2$ 0.61, single ninhydrin-positive spot. Anal. Calcd for $C_{50}H_{67}Cl_3FN_7O_{13}$: C, 54.46; H, 6.14; N, 8.92. Found: C, 54.41; H, 6.38; N, 9.17.

*Boc-Glu(OBzl)-Phe(4F)-Leu-Glu(OBzl)-Asp(OcHex)-Pro-NHNH-Troc (II).* This compound was prepared essentially in the same manner as described for the preparation of **I** by using **I** (1.6 g), Boc-Glu(OBzl)-OH (530 mg), HOBT (212 mg) and WSCI (301 mg). The product was reprecipitated from hot MeOH with $H_2O$. Yield 1.4 g (70%), mp 137–144 °C, $[\alpha]_D^{21}$ –14.3° (*c* 1.0; DMF), $R_f^1$ 0.63, $R_f^2$ 0.65, single ninhydrin-positive spot. Anal. Calcd for $C_{62}H_{80}Cl_3FN_8O_{16}\cdot 2H_2O$: C, 55.00; H, 6.25; N, 8.27. Found: C, 54.76; H, 6.46; N, 8.39.

*Boc-Pro-Glu(OBzl)-Phe(4F)-Leu-Glu(OBzl)-Asp(OcHex)-Pro-NHNH-Troc (III).* This compound was prepared from **II** (1.4 g), Boc-Pro-OH (237 mg), HOBT (149 mg) and WSCI (211 mg) essentially in the same manner as described for the preparation of **I**. The product was reprecipitated from HOAc with $H_2O$. Yield 12 g (80%), mp 137–143 °C, $[\alpha]_D^{21}$ –13.7° (*c* 1.0; DMF), $R_f^1$ 0.64, $R_f^2$ 0.70, single ninhydrin-positive spot. Anal. Calcd for $C_{67}H_{87}Cl_3FN_9O_{17}\cdot 3H_2O$: C, 54.75; H, 6.25; N, 8.58. Found: C, 54.41; H, 6.56; N, 8.29.

*Boc-Pro-Glu(OBzl)-Phe(4F)-Leu-Glu(OBzl)-Asp(OcHex)-Pro-NHNH$_2$ (IV).* Compound **III** (1.1 g) in a mixture of HOAc (5 mL) and DMF (5 mL) was treated with Zn dust (467 mg) at 4 °C for 2 h and then at room temperature for 8 h. The solution was filtered, 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 738 mg (75%), mp 161–167 °C, $[\alpha]_D^{21}$ –11.3° (*c* 1.0; DMF), $R_f^1$ 0.62, $R_f^2$ 0.66, single hydrazine-test-positive spot. Anal. Calcd for $C_{64}H_{86}FN_9O_{15}\cdot 4H_2O$: C, 58.57; H, 7.22; N, 9.61. Found: C, 58.72; H, 7.51 N, 9.28.

*Boc-Pro-Glu(OBzl)-Phe(4F)-Leu-Glu(OBzl)-Asp(OcHex)-Pro-Ser(Bzl)-Val-Leu-Thr(Bzl)-Lys(Z)-Glu(OBzl)-Lys(Z)-Leu-Lys(Z)-Ser(Bzl)-Glu(OBzl)-Leu-Val-Ala-Asn-Asn-Val-Thr(Bzl)-Leu-Pro-Ala-Gly-Glu(OBzl)-Gln-Arg(Mts)-Lys(Z)-Asp(OcHex)-Val-Tyr(Bzl)-Val-Glu(OBzl)-Leu-Tyr(Bzl)-Leu-Gln-Ser(Bzl)-Leu-Thr(Bzl)-Ala-Leu-Lys(Z)-Arg(Mts)-OBzl (V).* Boc-(42–49)-OBzl[15] (187 mg) was treated with TFA–anisole (3–0.6 mL) as described above and $N^{\alpha}$-deprotected peptide was dissolved in DMF:DMSO (1:1, 4 mL) containing NMM (0.003 mL). The azide (prepared from 98 mg of **IV**) in DMF:DMSO (1:1, 2 mL) and NMM (0.008 mL) were added and the mixture was stirred at –10 °C for 48 h. Additional azide (prepared from 33 mg of **IV**) in DMF:DMSO (1:1, 2 mL) and NMM (0.003 mL) were added and stirring was continued for an additional 18 h until the solution became ninhydrin-negative. After being neutralized with a few drops of HOAc, the mixture was poured into ice-chilled 5% citric acid with stirring. The resulting powder was washed successively with 5% citric acid, $H_2O$ and MeOH. The crude product was dissolved in DMSO containing 5% $H_2O$ (3 mL) and the solution was applied to a column of Sephadex LH-60 (3 × 94 cm), which was eluted with the same solvent. The fractions with $R_f^1$ 0.52 were combined and the solvent was removed by evaporation. Treatment of the residue with EtOAc afforded a powder. Yield 121 mg (57%), mp 174–186 °C, $[\alpha]_D^{21}$ –29.6° (*c* 1.0; DMSO), $R_f^1$ 0.52, $R_f^2$ 0.53, single ninhydrin-positive spot. Anal. Calcd for $C_{431}H_{577}FN_{64}O_{94}S_2\cdot 15H_2O$: C, 60.82; H, 7.12; N, 10.53. Found: C, 60.47; H, 7.39; N, 10.88. Amino acid ratios in a 6 M HCl hydrolysate: Leu 9.06, Val 5.03, Ala 3.05, Gly 1.00, Pro 2.91, Phe(4F) 0.95, Tyr 1.90, Ser 2.87, Thr 2.91, Glu 7.98, Asp 3.97, Lys 4.89, Arg 1.91 (recovery of Gly 84%).

*H-Pro-Glu-Phe(4F)-Leu-Glu-Asp-Pro-Ser-Val-Leu-Thr-Lys-Glu-Lys-Leu-Lys-Ser-Glu-Leu-Val-Ala-Asn-Asn-Val-Thr-Leu-Pro-Ala-Gly-Glu-Gln-Arg-Lys-Asp-Val-Tyr-Val-Glu-Leu-Tyr-Leu-Gln-Ser-Leu-Thr-Ala-Leu-Lys-Arg-OH (corresponding to [Phe(4F)$^3$]TP-II) (VI).* Compound **V** (85 mg) was treated with 1 M TFMSA–thioanisole in TFA (3 mL) in the presence of *m*-cresol (70 μL) and $Me_2Se$ (60 μL) in an ice-bath for 120 min, then dry $Et_2O$ was added. The resulting powder was collected by centrifugation, dried over KOH pellets *in vacuo* for 2 h and dissolved in 2% HOAc (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 M HOAc and the solution was lyophilized to give a fluffy powder. The powder was dissolved in 2% HOAc (2 mL), applied to a column of Sephadex G-50 (3.0 × 96 cm) and eluted with 2% HOAc. Individual fractions (5 mL each) were collected and absorbancy at 260 nm was determined.

The front peak (tube nos 49–57) was collected and the solvent removed by lyophilization. Next, the residue was dissolved in $H_2O$ (2 mL) and was applied to a column of CM-Biogel A (2.3 × 12 cm), which was eluted first with $H_2O$ (100 mL) and then with a linear gradient from $H_2O$ (250 mL) to 0.25 M $NH_4OAc$ buffer (250 mL, pH 6.50). Individual fractions (4 mL each) were collected and absorbancy at 260 nm was determined. The main peak (tube nos 52–57) was collected and the solvent removed by lyophilization. Analysis by TLC revealed the presence of two ninhydrin-positive spots with $R_f^4$ 0.54 (main) and 0.76 (minor). The crude peptide was dissolved in a small amount of $H_2O$ and subjected to preparative TLC (cellulose plate, 20 × 40 cm) using BuOH:pyridine: HOAc:$H_2O$ (30:20:6:24) as a developing solvent. The zone corresponding to $R_f^4$ 0.54 was separated and extracted with 2% HOAc. The extracts were concentrated to a small volume and subjected to Sephadex G-50 column chromatography (3.0 × 94 cm) and eluted with 2% HOAc. The single main peak fractions were combined and the solvent was removed by lyophilization to give a white fluffy powder. Yield 7.3 mg (13%), $[\alpha]_D^{21}$ −79.8° (*c* 0.3; 1 N HOAc), $R_f^3$ 0.56, $R_f^4$ 0.54, 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; mobility 8.1 cm from the origin toward the anode after running at 2 mA, 600 V for 80 min. The synthetic peptide exhibited a single peak on HPLC using a μBondapak C18 column (0.39 × 30 cm) at a retention time of 15.06 min, when eluted with a gradient of acetonitrile (15–35% in 20 min) in 0.1% TFA at a flow rate of 1.0 mL $min^{-1}$ (Fig. 3). FAB-MS m/z: calculated $(M + H)^+$ 5593, observed $(M + H)^+$ 5592. Amino acid ratios in a 6 M HCl hydrolysate: Leu 9.03, Val 4.94, Ala 3.05, Gly 1.00, Pro 2.92, Phe(4F) 0.97, Glu 7.96, Asp 3.94, Lys 5.01, Arg 1.92 (recovery of Gly 85%).

*Fluorometric blast-formation test*

A 3-mL aliquot of venous blood from uremic patients was drawn into a syringe containing 25 U $mL^{-1}$ heparin and then mixed with 3 mL PBS. Lymphocytes were isolated in a Hypaque–Ficoll gradient.[31] Isolated lymphocytes were adjusted to $1.0 \times 10^6$ $mL^{-1}$ with PBS. The lymphocytes were cultured in 0.5 mL of RPMI1640 (Gibco) with 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 and PHA (0.125%, 0.5 mL) was added to each well. Incubation was continued under the same conditions for 60 h. T-Lymphocytes in each well were transferred to a test tube and centrifuged for 10 min at 240 *g*, and an aliquot of 0.125% SDS was added to the residue and stirred for 20 min at room temperature; lymphocytes were completely destroyed and solubilized by this procedure. An ethidium bromide solution 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 the method of Itoh and Kawai.[26]

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