A TP II analogue, [l-Nal³] TP II, was synthesized by a conventional solution method, followed by deprotection with 1M TFMSA-thioanisole (molar ratio 1:1) in TFA in the presence of Me₂Se and m-cresol as scavengers. The synthetic [l-Nal³] TP II, TP II and [Phe (4 F)³] TP II were tested for comparative effect on the impaired T-lymphocyte transformation by PHA in uremic patients suffering from recurrent infectious diseases. The synthetic analogue was found to have stronger restorative activity than those of our synthetic TP II and [Phe (4 F)³] TP II.

Synthesis of [l-Nal³] thymopoietin II and examination of its immunological effect on the uremic T-lymphocytes

T. Abiko[CA] and S. Nakatsubo

Research Laboratory, Global Shinwa Pharmaceutical Co. Ltd, 2–546–1, Yoriki, Matsuomura, Iwate-gun, Iwate-ken 028–7302, Japan

[CA] Corresponding Author
   Fax: 0195–78–3357
   E-mail: nakatubo@mx2.alpha-web.ne

Introduction

Thymopoietins are polypeptide hormones of the thymus, which induce T-lymphocyte differentiation and perform a restorative activity on impaired T-lymphocyte transformation by PHA in uremic patients.[1-6]

On the other hand, evidence of impaired immune function in patients with chronic uremia has been elucidated. This impairment is reflected in depressed cell-mediated immune function both *in vitro* and *in vivo* and thymic atrophy with degenerative changes has been observed in uremic patients, though the cause of thymic atrophy in uremia is as yet unknown.

In 1995, we reported that an analogue of TP II. [Phe (4 F)³] TP II, exhibited stronger restorative activity than that of TP II on the impaired PHA stimulation of T-lymphocytes *in vitro* in uremic patients.[7]

In 1998, we reported that an analogue of THF-g2, [l-Nal⁷]-THF-g2, which belongs to the naphtyl group rather than the phenyl group, exhibited a stronger restorative effect than that of THF-g2 and [Phe (4 F)⁷]-THF-g2 on the impaired PHA response of T-lymphocytes from uremic patients.[8]

These results seem to suggest an important role in regulating immunological activities[7,8] and prompted us to synthesize a TP II analogue containing l-naphthylalanine residue instead of 3-position of phenylalanine by the conventional solution method, to examine the activity of our synthetic [l-Nal³] TP II on the impaired T-lymphocytes of uremic patients and to compare the relative activity between our synthetic TP II [Phe (4 F)³] TP II and [l-Nal³] TP II.

The synthetic route we employed is almost the same as those employed for our previous synthesis of [Phe (4 F)³] TP II.[7]

As illustrated in Fig. 1, the TFA-labile Boc group was employed for N^a-protection and amino-acid derivatives bearing protective groups removable by the thioanisole-mediated TFMSA deprotection procedure were employed, i.e. Lys (Z), Glu (OBzl), Thr (Bzl), Asp (OcHex) and Arg (Mts).

N^a-deprotection with TFA was performed in the presence of anisole, prior to each condensation reaction in the usual fashion. Asp (OcHex) was employed to minimize aspartimide formation during the synthesis of Asp-containing peptides. The C-terminal protected dotetracontapeptide ester[5] was available from our previous synthesis of TP II. The N-terminal protected heptapeptide, Boc-Pro-Glu (OBzl)-l-Nal-Leu-Glu (OBzl)-Asp (OcHex)-Pro-NHNH-Troc (III), was prepared stepwise starting from Boc-Leu-Glu (OBzl)-Asp (OcHex)-Pro-NHNH-Troc[5] by the HOBT-WSCI procedure to minimize racemization.

For the preparation of the N-terminal protected hepatapeptide hydrazide containing Glu (OBzl) and Asp (OcHex), Boc-Pro-Glu (OBzl)-l-Nal-Leu-Glu (OBzl)-Asp (O cHex)-Pro-NHNH₂ (IV), we employed a substituted hydrazide, Troc-NHNH₂, 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)-l-Nal-Leu-Glu (OBzl)-Asp (OoHex)-Pro-NHNH₂ (IV), in analytically pure form. The hydrazine test on the thin-layer chromatograms and elemental
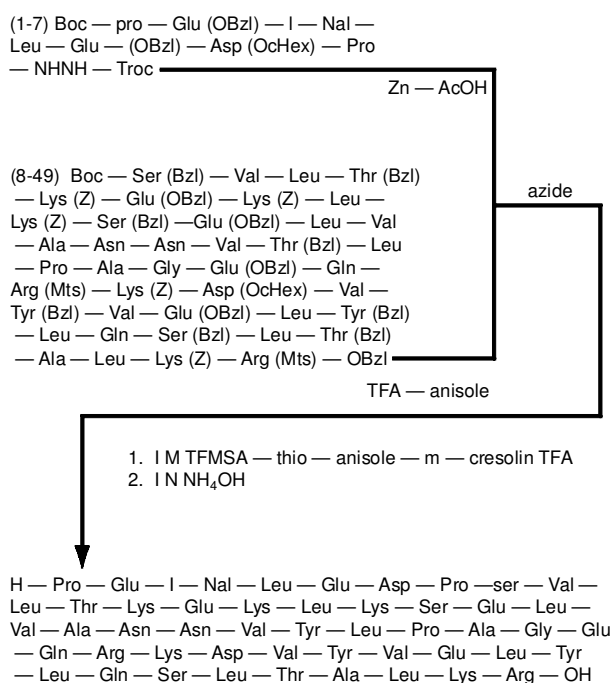
(1-7) Boc — pro — Glu (OBzl) — I — Nal —
Leu — Glu — (OBzl) — Asp (OcHex) — Pro
— NHNH — Troc

Zn — AcOH

(8-49) Boc — 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

azide

TFA — anisole

1. 1 M TFMSA — thio — anisole — m — cresolin TFA
2. 1 N NH$_4$OH

H — Pro — Glu — I — Nal — Leu — Glu — Asp — Pro —ser — Val —
Leu — Thr — Lys — Glu — Lys — Leu — Lys — Ser — Glu — Leu —
Val — Ala — Asn — Asn — Val — Tyr — 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

FIG. 1. Synthetic route to [l-Nal^3]-TP-II.

analysis data were consistent with homogeneity of the desired product.

The two fragments were assembled by the azide procedure, according to the route illustrated in Fig. 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 new incorporation of the N-terminal fragment in condensation, the reaction was confirmed. The homogeneity of the purified protected nonatetracontapeptide corresponding to the entire amino-acid sequence of [l-Nal^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 protective groups. The deprotected crude peptide was purified by Sephadex G-50 then by ion-exchange column chromatography on a CM-Biogel A column, followed by preparative TLC.

The immunological effect of the synthetic TP II [Phe (4F)^3] TP II and [l-Nal^3] TP II was examined by the JIMRO (Japan immunoresearch Laboratories Co. Ltd) fluorometric blast-formation test.[8]

Materials and methods

General experimental procedures used in this paper are essentially the same as described in our previous papers.[6,7] 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 Rf values refer to the following solvent systems: Rf^9, BuOH-AcOH-H$_2$O (4:1:5); Rf^2: BuOH-pyridine-AcOH-H$_2$O. The final product corresponding to the entire amino-acid sequence of [l-Nal^3] TP II was chromatographed on cellulose plates (Merck). Rf^3 value refers to BuOH-AcOH-H$_2$O (4:1:1) and Rf^4 value refers to BuOH-pyridine-AcOH-H$_2$O (30:20:6:24). Troc-NHNH$_2$ was purchased from Kokusan Chemical Works Ltd, Japan. Kite 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 m Bondapak C18 column with a gradient of acetonitrile (15→38%) in TFA at a flow rate of 1.0 ml/min and the eluate was monitored at 230 nm. The FAB-MS spectrum was obtained on an Auto Spec Q (UQ Analytical Co, UK) mass spectrometer equipped with an OPUS data processor.

Patient selection

Three uremic patients 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 11,283, 10,994 and 11,342 cpm respectively (normal values 40,993–41,835 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 a UVLOG-FLOUSPEC-llA fluorometer.

Boc-l-Nal-Leu-Glu (OBzl)-Asp (OcHex)-Pro-NHNH-Troc (I)

Boc-Leu-Glu (OBzl)-Asp (OcHex)-Pro-NHNH-Troc^4 (2.4 g) was treated with TFA-anisole (12–2.4 ml) in an ice-bath for 40 min, then dry Et$_2$O was added, 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-l-Nal-OH (694 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 an ice-chilled 5% citric acid solution with stirring. The precipitate thereby formed was reprecipitated from AcOH with H$_2$O. Yield: 2.1 g (64%), mp 141–147°C, [a]$^{21}_D$ – 18.9° (*c* = 1.0, DMF), Rf^1

0.59, Rf² 0.64, single ninhydrin-positive spot. Anal. Calcd. for $C_{54}H_{70}Cl_3N_7O_{13} \cdot 3 H_2O$: C, 54.71; H, 6.46; N, 8.72. Found: C, 54.63: H, 6.50; N, 8.39.

Boc-Glu (OBzl)-l-Nal-Leu-Glu (OBzl)-Asp (OcHex)-Pro-NHNH-Troc (II)

This compound was prepared from I (1.6 g), Boc-Glu (OBzl)-OH (500 mg), HOBT (237 mg) and WSCI (328 mg) essentially in the same manner as described for the preparation of I. The product was re-precipitated from hot MeOH with H_2O. Yield: 1.5 g (83%), mp 129–135°C, $[a]^{21}$ D - 12.3° (c = 1.0, DMF), Rf¹ 0.60, Rf² 0.64, single ninhydrin-positive spot. Anal. Calcd. for $C_{66}H_{83}Cl_3N_8O_{16}$: C, 58.68; H, 6.19; N, 8.30. Found: C, 58.49; H, 6.32; N, 8.47.

Boc-Pro-Glu (OBzl)-l-Nal-Leu-Glu (OBzl)-Asp (OcHex)-Pro-NHNH-Troc (III)

This compound was prepared essentially in the same manner as described for the preparation of I by using II (1.4 g), Boc-Pro-OH (250 mg), HOBT (160 mg) and WSCI (232 mg). The product was re-precipitated from AcOH with H_2O. Yield: 1.3 g (81%), mp 127–134°C, $[a]^{21}$ D - 11.5° (c = 1.0, DMF), Rf¹ 0.65, Rf² 0.68, single ninhydrin-positive spot. Anal. Calcd. for $C_{71}H_{90}Cl_3N_9O_{17} \cdot 4 H_2O$: C, 56.11; H, 6.50; N, 8.29. Found: C, 55.86; H, 6.83; N, 8.47.

Boc-Pro-Glu (OBzl)-l-Nal-Leu-Glu (OBzl)-Asp (OcHex)-Pro-NHNH₂ (IV)

The compound III (1.1 g) in a mixture of AcOH (5 ml) and DMF (5 ml) was treated with Zn dust (480 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 re-precipitated from DMF with H_2O. Yield: 805 mg (82%), mp 164–168°C, $[a]^{21}$ D - 10.1° (c = 1.0, DMF), Rf¹ 0.60, Rf² 0.63, single hydrazine-positive spot. Anal. Calcd. for $C_{66}H_{89}N_9O_{15}$: C, 64.18; H, 7.05; N, 9.91. Found: C, 63.85; H, 7.26; N, 9.47.

Boc-Pro-Glu (OBzl)-l-Nal-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-(8–49)-OBzl[5] (187 mg) was treated with TFA-anisole (3–0.6 ml) as described above and N^a-deprotected peptide was dissolved in DMF–DMSO

(1:1, 4 ml) containing NMM (0.003 ml). The azide (prepared from 112 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 40 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 AcOH, the mixture was poured into ice-chilled 5% citric acid and stirred. 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 × 90 cm), which was eluted with the same solvent. The fractions with Rf¹ 0.54 were combined and the solvent was removed by evaporation. Treatment of the residue with EtOH afforded a powder. Yield: 132 mg (62%), mp 170–177°C, $[a]^{21}$D - 31.4°C (c = 1.0, DMSO), Rf¹ 0.54, Rf² 0.62, single ninhydrin-positive spot. Anal. Calcd. for $C_{435}H_{580}N_{64}O_{94}S_2 \cdot 16 H_2O$: C, 61.43; H, 7.27; N, 10.45. Found: C, 61.10; H, 7.53; N. 10.40. Amino acid ratios in a 6 N HCl hydrolysate: Leu 9.02, Val 5.04, Ala 3.03, Gly 1.00, Pro 2.89, l-Nal 0.96, Tyr 1.93, Ser 2.88, Thr 2.95, Glu 8.02, Asp 3.99, Lys 4.98, Arg 1.92 (recovery of Gly 86%).

H-Pro-Glu-l-Nal-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 [l-Nal³]-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 ml) and Me_2Se (60 ml) in an ice-bath for 120 min, then the 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% AcOH (5 ml). The solution, after being stirred with Amberlite IRA-400 (acetate form, approximately, 1g) 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 the solution was lyophilized to give a fluffy powder. The powder was dissolved in 2% AcOH (2 ml), applied to a column of Sephadex G-50 (3.0 × 95 cm) and eluted with 2% AcOH. Individual fractions (5 ml each) were collected and absorbancy at 260 nm was determined. The front main peak (tube Nos 52–58) was collected and the solvent was 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
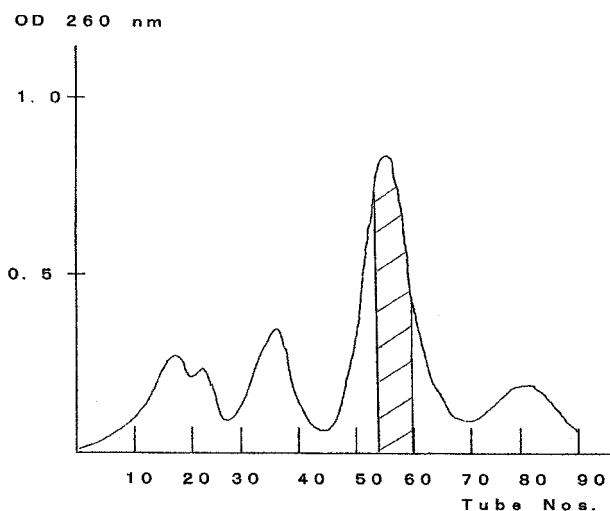
OD 260 nm

FIG. 2. Purification of synthetic [l-Nal³]-TP-II by ion-exchange chromatography on a CM-Biogel A column.

(tube NOs 55–60) was collected and the solvent was removed by lyophilization. Analysis by TLC revealed the presence of two ninhydrin-positive spots with Rf^4 0.56 (main) and 0.79 (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-AcOH-H_2O (30:20:6:24) as a developing solvent. The zone corresponding to Rf^4 0.56 was separated and extracted with 2% AcOH. The single main peak fractions were combined and the solvent was removed by lyophilization to give a fluffy white powder. Yield: 8.2 mg (15%), $[a]^{21}_D$ - 80.6° (c = 0.3, 1 N AcOH), Rf^3 0.59, Rf^4 0.56, single ninhydrin- and Sakaguchi-positive spot. The synthetic peptide exhibited a single spot on a paper electrophoresis: Toyo Roshi No. 51 (2 × 40 cm), acetate buffer at pH 2.79: mobility 8.3 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 mBondapak C18 column (0.39 × 30 cm) at a retention time of 14.20 min, when eluted with a linear gradient of acetonitrile (15→38% in 20 min) in 0.1% TFA at a flow rate of 1.0 ml/min (Fig. 3).

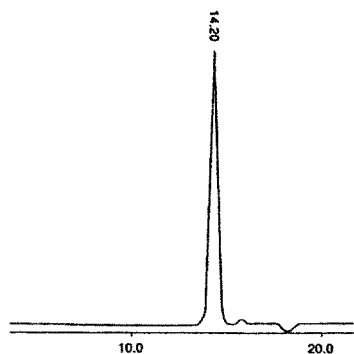FAB-MS m/z: calculated $(M + H)^+$ 6524, observed $(M + H)^+$ 5625. Amino acid ratios in 6 N HCl

FIG. 3. HPLC profile of synthetic [l-Nal³]-TP-II.

hydrolysate: Leu 9.04, Val 5.02, Ala 2.94, Pro 2.90, Thr 2.91, Ser 2.87, l-Nal 0.93, Glu 7.96, Asp 3.88, Lys 5.03, Arg 1.95, Tyr 2.01, Gly 1.00 (recovery of Gly 87%).

Fluorometric blast-formation test

A 3-ml aliquot of venous blood was drawn into a syringe containing 25 U/ml of heparin and then mixed with 3 ml of PBS. Lymphocytes were isolated in a Hypaque–Ficoll gradient. 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 one of our synthetic peptides in a humidified atmosphere of 5% CO_2 in air for 12 h and then PHA (0.125%, 0.5 ml) was added to each well and incubation was continued under the same conditions for 60 h. Lymphocytes in each well were transfered into a test tube and centrifuged for 10 min at $240 × g$, then the supernatant was added to the residue and stirred for 20 min at room temperature; the 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 essentially in the same manner as described in our previous papers.[5-8]

Results and discussion

Our synthetic route to [l-Nal³] TP II is illustrated in Fig. 1, which shows two fragments selected as building blocks to construct the entire amino-acid sequence of [l-Nal³] TP II. Protected C-terminal dotetracontapeptide ester was identical with that employed in our previous synthesis of TP II.[5] Thus, one fragment, Boc-Pro-Glu (OBzl)-l-Nal-Leu-Glu (OBzl)-Asp (OcHex)-Pro-NHNH₂ (IV), which covers the area of sequence variation between TP II,[5] [Phe (4F)³] TP II and [l-Nal³] TP II, was newly synthesized.

The Boc group of Boc-(8–49)-OBzl[5] was removed by the usual TFA-anisole treatment and the corresponding free amine was condensed with the protected N-terminal heptapeptide hydrazide IV by the azide procedure to yield the protected [l-Nal³] TP II. The protected nonatetracontapeptide ester was treated with 1 M TFMSA-thioanisole in TFA in the presence of m-cresol and Me2Se. m-Cresol was used as additional cation scavenger to suppress a side reaction, i.e. O-sulfation of Tyr residues. Me2Se was employed to facilitate acidolytic cleavage of protecting groups.

The deprotected peptide was next precipitated with dry Et_2O, converted to the corresponding acetate with Amberlite IRA-400 (acetate form) and then treated with 1M NH_4OH to reverse a possible N →O shift at the Ser and Thr residues.

Table 1. Effect of synthetic TP-II, [Phe (4 F)³] – and [l-Nal³]-TP-II on impaired PHA-stimulation of T-lymphocytes of uremic patients

Peptide	Dose (mg/ml)	SI[a,b]
(1) ■[c]	■	310.6 ± 51.6
(2) ■[d]	■	119.3 ± 54.9[f]
(3) TP-II[d,e]	0.1	117.5 ± 53.4
(4) TP-II[d,e]	1.0	196.6 ± 52.2[g]
(5) TP-II[d,e]	2.0	268.3 ± 55.3[g]
(6) [Phe (4 F)³]-[d,e]	0.1	202.1 ± 53.6[g]
(7) [Phe (4 F)³]-[d,e]	1.0	270.0 ± 51.8[g]
(8) [l-Nal³]-[d,e]	0.01	115.9 ± 53.2
(9) [l-Nal³]-[d,e]	0.05	198.3 ± 52.6[g]
(10) [l-Nal³]-[d,e]	0.5	269.4 ± 54.6[g]
(11) [l-Nal³]-[d,e]	1.0	267.9 ± 52.7[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 ref.[2]

The crude peptide was purified by gelfiltration on Sephadex G-50 and then by ion-exchange column chromatography on a CM-Biogel A column with linear gradient elution using pH 6.50 ammunium acetate buffer (0 → 0.25 M) (Fig. 2), followed by preparative TLC. Desalting on Sephadex G-25 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 2.79 acetate buffer). The peptide also exhibited a single peak on HPLC (Fig. 3). The molecular weight of the synthetic peptide was ascertained by FAB-MS spectrometry. Homogeneity of the synthetic [l-Nal³] TP II was further ascertained by amino-acid analysis after 6 N HCl hydrolysis.

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

The *in vitro* effect of the synthetic TP II,[5] [Phe (4 F)³] TP II and [l-Nal³] TP II on the impaired PHA stimulation of T-lymphocytes from uremic patients is shown in Table 1.

When peripheral T-lymphocytes isolated from uremic patients were incubated with various amounts of the synthetic TP II from 0.1 to 2.0 mg/ml, restoration of the impaired PHA stimulation of T-lymphocytes was observed at a concentration of 1 mg/ml and above. One of the synthetic two analogues in which Phe (3 position of TP II) was replaced by Phe (4 F) exhibited more potent restorative activity than that of the synthetic TP II (Table 2). The other synthetic peptide [l-Nal3] TP II, exhibited the most potent restorative activity beyond that of our synthetic TP II and [Phe (4 F)³] TP II.

The relative potency of the synthetic [l-Nal³] TP II was four times stronger than that of the synthetic TP II (Table 2). In normal subjects, no effects of TP II [Phe (4 F)³] TP II and [l-Nal³] TP II were observed (data not shown).

In our previous paper,[8] we reported that one of our synthetic THF-g2 analogues, [l-Nal⁷]-THF-g2, exhibited the most potent restorative effect on the impaired blastogenic response of PHA-stimulated T-lymphocytes of uremic patients and similar results were reported here on TP II analogues. Between the two analogues, [l-Nal³] TP II showed a stronger restorative effect on the impaired blastogenic response of uremic T-lymphocytes than that of [Phe (4 F)³] TP II. This result exhibited that not only a 4-fluorophenyl ring but also a more bulky naphthyl ring could bind with the receptor more strongly than that of TP II. These results seem to suggest that aromaticity of the side chain of 3 position on TP II is important for immunological activities.

Table 2. Relative potencies of synthetic TP II, [Phe (4 F)³] TP II and [l-Nal³] TP II

Peptide	Relative potency (molar basis)
TP II	1.00
[Phe (4 F)³] TP II	2.98
[l-Nal³] TP II	4.03

Abbreviations

TP, thymopoietin; l-Nal, l-naphythylalanine; Phe (4 F), 4-fluoro-acetic acid; Z, benzyloxycarbonyl; OBzl, benzyl ester; Boc, tert-butoxycarbonyl; Troc, b, b, b-tri-chloroethoxycarbonyl; Bzl, benzyl; Mts, mesitylene-2-sulfonyl; OcHex, cyclohexyl ester; HOBT, l-hy-droxybenzotriazole; WSCI, l-ethyl-3-(3-dimethyla-minopropyl) carbodimide; NMM, N-methyl-morpholine; EDTA, ethylenedi-aminetetraacetic acid; DMF, dimethyformamide; SMSO, dimethylsufoxide; TFMSA, trifluoromethanesulfonic acid; PHA, phytohe-magglutinin; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; RPMI, Rosewell Park Memorial Institute; FCS, fetal calf serum; AcOH, acetic acid; Et$_2$O, ethyl ether; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; THF, thymic humoral factor.

References

1. Basch RS, Goldstein G. Induction of T cell differentiation *in vitro* by thymine, a purified polypeptide hormone of the thymus. *Proc Natl Acad Sci* USA 1974; **71**:1474–8
2. Scheid MP, Goldstein G, Boyse EA. The generation and regulation of lymphocyte populations. Evidence from differentiative induction systems *in vitro*. *J Exp Med* 1978; **147**:1727–43
3. Sunshine GJ, Basch RS, Coffey RS, Cohen KW, Goldstein G, Hadden JW. Thymopoietin enhances the allogeneic response and cyclic GMP levels of mouse peripheral thymus-derived lymphocytes. *J Immunol* 1978; **120**:1594–9
4. Abiko T, Shishido H, Sekino H. Synthesis of nonatetracontapeptide corresponding to the entire amino acid sequence of thympoietin III and its effect on the impaired T-lymphocyte transformation of a patient with common variable immunodefieciency. *Chem Pharm Bull* 1986; **34**:2133–43
5. Abiko T, Sekino H. Synthesis of the revised amino acid sequence of thymopoietin II and examination of its immunological effect on the impaired T-lymphocyte transformation of a uremic patient with pneumonia. *Chem Pharm Bull* 1987; **35**:2016–24
6. Abiko T, Sekino H. Synthesis of human thymopoietin (hTP) and examination of its immunological effect on the impaired blastogenic response of T-lymphocytes of uremic patients. *Chem Pharm Bull* 1988; **36**:2506–16
7. Abiko T, Sekino H. Synthesis of [Phe (4 F)[3]] thymopoietin II and examination of its immunological effect on the impaired blastogenic response of T-lymphocytes of uremic patients. *Bioorganic & Medicinal Chemistry* 1995; **3**:1369–75
8. Abiko T, Sekino H. Functional roles of phenylalanine[7] of thymic humoral factor-g2 in the impaired blastogenic response of uremic T-lymphocytes. *Drug Development and Industrial Pharmacy* 1998; **24**:569–72