

Synthesis and Effects of Two Peptide Fragments of Thymopoietin II on E-Rosette Forming Cells in the Uremic State¹⁾

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Two peptides, H-Lys-Asp-Val-Tyr-Val-Gln-Leu-Tyr-Leu-OH and H-Arg-Lys-Asp-Val-Tyr-Val-Gln-Leu-Tyr-Leu-OH (fragment of thymopoietin II), were synthesized in a conventional manner utilizing the hydrogen fluoride procedure, and were tested to determine their effects on E-rosette forming cells in the uremic state. The synthetic decapeptide increased E-rosette forming capacity in the presence of uremic serum. The synthetic nonapeptide had no effect on the E-rosette formation inhibiting activity of uremic serum.

Keywords—uremic state; thymopoietin II; uremic serum; E-rosette; terminal chronic renal failure

The amino acid sequence of bovine thymopoietin II was elucidated by Goldstein *et al.*³⁾ This T cell differentiating hormone of thymus is a single 49-amino acid polypeptide chain.³⁾ Chemical synthesis of a tridecapeptide with an amino acid sequence corresponding to positions 29—41 of thymopoietin II (H-Gly-Glu-Gln-Arg-Lys-Asp-Val-Tyr-Val-Gln-Leu-Tyr-Leu-OH) was reported Goldstein *et al.*⁴⁾ and they showed it to have selective T cell differentiating capacity like that of thymopoietin II. Their data established that the key residues involved in the active site of thymopoietin II are present within this synthetic tridecapeptide, which constitutes only a minor portion of the amino acid sequence of thymopoietin II.⁴⁾ Various fragment peptides of biologically active peptides have been shown to possess *in vitro* biological activity much higher than that of the parent peptides.⁵⁾

On the other hand, cell mediated immunity is impaired in the uremic state.⁶⁻⁸⁾ For example, skin graft rejection is usually delayed⁶⁾ and E-rosette inhibiting activity of uremic serum is also observed.⁹⁾ Lymphocytes that bind *in vitro* to sheep erythrocytes in a rosette formation are thymus-derived. In the present communication, the synthesis of two fragment peptides of the thymopoietin II fragment⁴⁾ was achieved and the *in vitro* response to uremic serum⁹⁾ of E-rosette forming cells in the presence of the synthetic peptides was investigated. The solution method for peptide synthesis is used in this investigation. The synthetic routes to the decapeptide (H-Arg-Lys-Asp-Val-Tyr-Val-Gln-Leu-Tyr-Leu-OH) and the nonapeptide (H-Lys-Asp-Val-Tyr-Val-Gln-Leu-Tyr-Leu-OH) are illustrated in Fig. 1.

- 1) Symbols for amino acid derivatives and peptides are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochem. J.*, **126**, 773 (1972). Other abbreviations: WSCI, water-soluble carbodiimide; DMF, dimethylformamide; TFA, trifluoroacetic acid; GVB²⁺, gelatin veronal buffer; FCS, fetal calf serum; Tos, *p*-toluenesulfonic acid; E, sheep erythrocytes; HONB, N-hydroxy-5-norbornene-2,3-dicarboximide; PBS, phosphate-buffered saline; Et₃N, triethylamine.
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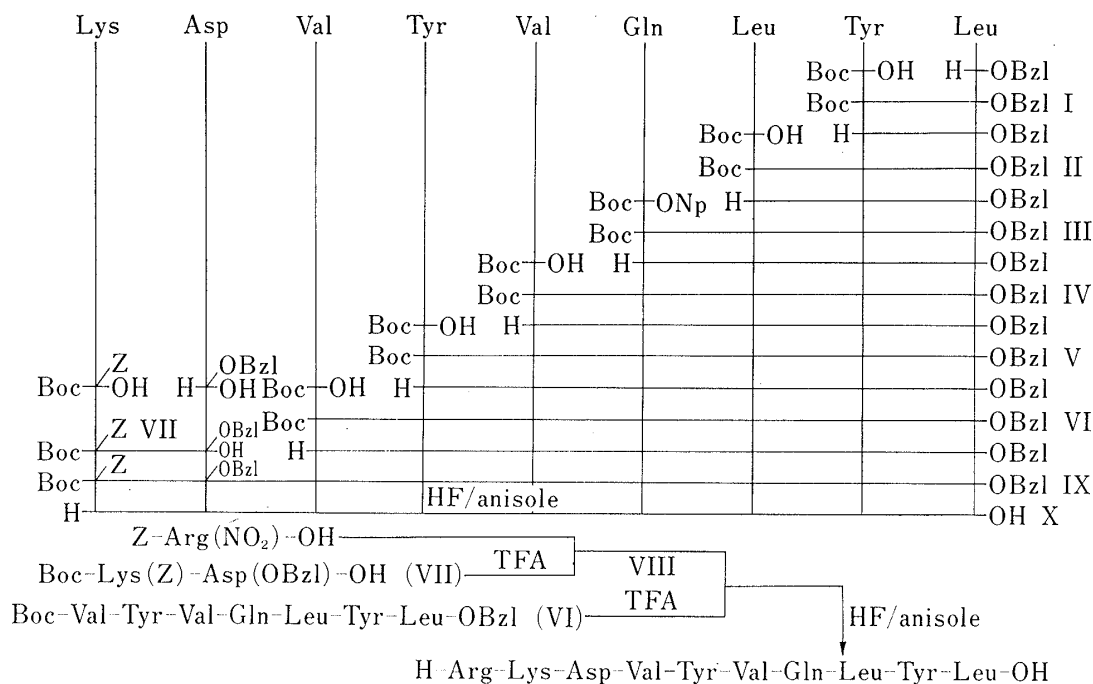


Fig. 1. Synthetic Scheme for the Thymopoietin II Nona- and Decapeptides

H-Leu-OBzl Tos was condensed with Boc-Tyr-OH by the HONB-DCC method¹⁰⁾ giving Boc-Tyr-Leu-OBzl (I). The protected dipeptide I was treated with TFA to remove the Boc group and the product was coupled with Boc-Leu-OH by the HONB-DCC method to give Boc-Leu-Tyr-Leu-OBzl (II). The Boc group of the tripeptide II was similarly removed and the free base was condensed with Boc-Gln-ONp to afford Boc-Gln-Leu-Tyr-Leu-OBzl (III). The Boc group of the tetrapeptide III was similarly removed and the free base was condensed with Boc-Val-OH by the HONB-DCC method to afford Boc-Val-Gln-Leu-Tyr-Leu-OBzl (IV). The Boc group of IV was again similarly removed and the free base was condensed with Boc-Tyr-OH by the HONB-DCC method to afford Boc-Tyr-Val-Gln-Leu-Tyr-Leu-OBzl (V). As before, the Boc group of V was removed and the free base was condensed with Boc-Val-OH by the HONB-DCC method to afford Boc-Val-Tyr-Val-Gln-Leu-Tyr-Leu-OBzl (VI). Boc-Lys(Z)-OH was condensed with H-Asp(OBzl)-OH by the mixed anhydride procedure¹¹⁾ to give Boc-Lys(Z)-Asp(OBzl)-OH (VII), which after conversion to the de-Boc peptide, was further condensed with Z-Arg(NO₂)-OH by the mixed anhydride procedure to give Z-Arg(NO₂)-Lys(Z)-Asp(OBzl)-OH (VIII). The heptapeptide VI was treated with TFA and the product was condensed with VII by the HONB-DCC method to give Boc-Lys(Z)-Asp(OBzl)-Val-Tyr-Val-Gln-Leu-Tyr-Leu-OBzl (IX). The protected nonapeptide IX was then treated with anhydrous hydrogen fluoride¹²⁾ in the presence of anisole at 0° for 30 min to remove all protecting groups and the deblocked peptide was converted into the corresponding acetate with Amberlite CG-4B (acetate form). The product was purified by column chromatography on Sephadex G-15. The nonapeptide (X) thus obtained was found to be homogeneous on paper chromatography using two different solvent systems. The Boc group of the heptapeptide VI was removed as described above and the free base was condensed with VIII by the HONB-DCC method to give Z-Arg(NO₂)-Lys(Z)-Asp(OBzl)-Val-Tyr-Val-Gln-Leu-Tyr-Leu-OBzl (XI). Deblockings of XI as describ-

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TABLE I. Effects of Thymopoietin II Fragments on the Inhibition of E-Rosette Formation by Uremic Serum

Material	Dose ($\mu\text{g/ml}$)	E-Rosettes (%)
— a)		77
— b)		42
H-Lys-Asp-Val-Tyr-Val-Gln-Leu-Tyr-Leu-OH ^{c)}	10	44
	50	45
	100	42
H-Arg-Lys-Asp-Val-Tyr-Val-Gln-Leu-Tyr-Leu-OH ^{c)}	10	53
	50	59
	100	66

a) Normal lymphocytes.

b) Lymphocytes were incubated for 30 min at 37° with uremic serum.

c) Lymphocytes were incubated for 30 min at 37° with uremic serum and thymopoietin II fragments.

ed for the preparation of the nonapeptide X yielded the desired H-Arg-Lys-Asp-Val-Tyr-Val-Gln-Leu-Tyr-Leu-OH (XII).

Serum from an uremic patient with chronic renal failure had low proportions of T-cell rosettes, ranging from 39 to 42%. After incubation with amounts of the decapeptide varying from 10 to 100 $\mu\text{g/ml}$ of cell suspension, maximum T-cell rosette formation ranged from 53 to 66% (Table I). The nonapeptide has no effect on the E-rosette formation inhibiting activity of uremic serum at a dose of 100 $\mu\text{g/ml}$. These results strongly suggest that the lower limit of peptide chain length for agonistic activity towards E-rosette formation inhibition by uremic serum is the decapeptide (XII). The results also suggest strongly that the N-terminal arginine residue of the decapeptide plays an important role in eliciting full agonistic activity towards E-rosette inhibition by uremic serum.

Experimental

All melting points are uncorrected. Rotations were determined with an Atago Polax machine. The amino acid hydrolysates and aminopeptidase M (AP-M) digests¹³⁾ were analyzed with a JEOL JLC-8AH amino acid analyzer according to the directions given by Moore *et al.*¹⁴⁾ The Z-group of the protected peptide was deblocked by catalytic hydrogenation in the presence of 10% Pd-C, and the Boc group with TFA. The deblocked peptides were chromatographed on filter paper, Toyo Roshi No. 51, at room temperature. Rf^1 values refer to the Partridge system¹⁵⁾ and Rf^2 values refer to a system of BuOH-pyridine-AcOH-H₂O (30:20:6:24).¹⁶⁾ Evaporations were carried out in a rotary evaporator under reduced pressure at a temperature of 30–35°. Uremic blood was obtained from an uremic patient suffering from terminal chronic renal failure. The blood was centrifuged and the separated serum was stored at –20° until use.

Boc-Tyr-Leu-OBzl (I)—H-Leu-OBzl Tos (3.9 g) was condensed with Boc-Tyr-OH (3.1 g) in DMF (30.0 ml) in the presence of Et₃N (1.5 ml), HONB (1.8 g) and WSCI (2.0 g) at 0° for 18 hr. The mixture was diluted with EtOAc and the solution was washed with 1 N NaHCO₃, H₂O, 1 N citric acid and H₂O, then dried over anhydr. MgSO₄, and the solvent was evaporated off. The residue was precipitated from EtOAc and petroleum ether: 4.5 g (94%), mp 52–53°, $[\alpha]_D^{25} -16.0^\circ$ ($c=1.0$, DMF), Rf^1 0.68, Rf^2 0.76, single ninhydrin-positive spot. *Anal.* Calcd. for C₂₇H₃₆N₂O₆: C, 66.92; H, 7.49; N, 5.78. Found: C, 66.51; H, 7.87; N, 5.66.

Boc-Leu-Tyr-Leu-OBzl (II)—I (4.9 g) was dissolved in TFA (5.0 ml) and the solution was allowed to stand at room temperature for 15 min, then evaporated down. The residue was dried over KOH pellets in a vacuum and then dissolved in DMF (30.0 ml) together with Et₃N (1.6 ml). HONB (2.0 g), Boc-Leu-OH (2.6 g) and WSCI (1.7 g) were added to the above solution at –10°. The mixture was stirred at 0° for 18 hr then extracted with EtOAc, and the solution was washed successively with 1 N NaHCO₃, H₂O, 1 N citric acid and H₂O. The solution was dried over anhydr. MgSO₄ and concentrated to a small volume, then petro-

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leum ether was added to the residue. The precipitate thereby formed was reprecipitated from EtOAc and hexane: 4.3 g (69%), mp 62—68°, $[\alpha]_D^{25} - 39.4^\circ$ ($c=1.0$, DMF), Rf^1 0.78, Rf^2 0.88, single ninhydrin-positive spot. *Anal.* Calcd. for $C_{23}H_{47}N_3O_7 \cdot H_2O$: C, 64.37; H, 8.02; N, 6.83. Found: C, 64.83; H, 8.41; N, 7.21.

Boc-Gln-Leu-Tyr-Leu-OBzl (III)—II (1.5 g) was treated with TFA (2.0 ml) and the resulting residue was dissolved in DMF (15.0 ml) together with Et_3N (0.4 ml). Boc-Gln-ONp (1.2 g) was added and the solution was stirred at room temperature for 18 hr. The reaction mixture was poured into cold 1 N NH_4OH with stirring. Next, 50% NH_4OAc was added dropwise with stirring to form a precipitate. The precipitate was collected and washed successively with 1 N NH_4OH , H_2O , 1 N citric acid and H_2O . The product was reprecipitated from DMF and 1 N citric acid: 1.7 g (94%), mp 164—168°, $[\alpha]_D^{25} - 33.2^\circ$ ($c=1.0$, DMF), Rf^1 0.71, Rf^2 0.78, single ninhydrin-positive spot. *Anal.* Calcd. for $C_{35}H_{55}N_5O_9$: C, 62.87; H, 7.64; N, 9.65. Found: C, 63.15; H, 7.49; N, 10.09.

Boc-Val-Gln-Leu-Tyr-Leu-OBzl (IV)—III (1.500 g) was treated with TFA (2.0 ml) as described above and the resulting residue was dissolved in DMF (15.0 ml) with Et_3N (0.3 ml). HONB (394 mg), Boc-Val-OH (479 mg) and WSCI (341 mg) were added to this solution at -10° . The mixture was stirred at 0° for 18 hr, then poured into cold 1 N $NaHCO_3$ with stirring. Next, 50% NH_4OAc was added dropwise with stirring to form a precipitate. The precipitate was collected and washed successively with 1 N NH_4OH , H_2O , 1 N citric acid and H_2O . The product was reprecipitated from DMF and 1 N citric acid: 1.2 g (71%), mp 207—212°, $[\alpha]_D^{25} - 45.8^\circ$ ($c=1.0$, DMF), Rf^1 0.87, Rf^2 0.91, single ninhydrin-positive spot. *Anal.* Calcd. for $C_{43}H_{64}N_6O_{10}$: C, 62.60; H, 7.82; N, 10.19. Found: C, 62.19; H, 7.88; N, 9.91.

Boc-Tyr-Val-Gln-Leu-Tyr-Leu-OBzl (V)—This compound was prepared from IV (825 mg), HONB (198 mg), Boc-Tyr-OH (309 mg) and WSCI (171 mg) essentially as described for the preparation of IV: 800 mg (81%), mp 170—190°, $[\alpha]_D^{25} - 46.3^\circ$ ($c=1.0$, DMF), Rf^1 0.88, Rf^2 0.91, single ninhydrin-positive spot. *Anal.* Calcd. for $C_{52}H_{73}N_7O_{12}$: C, 63.20; H, 7.45; N, 9.92. Found: C, 62.86; H, 7.68; N, 9.45.

Boc-Val-Tyr-Val-Gln-Leu-Tyr-Leu-OBzl (VI)—This compound was prepared from V (494 mg), HONB (99 mg), Boc-Val-OH (155 mg) and WSCI (86 mg) essentially as described for the preparation of IV: 430 mg (79%), mp 189—199°, $[\alpha]_D^{25} - 45.8^\circ$ ($c=1.0$, DMF), Rf^1 0.91, Rf^2 0.94, single ninhydrin-positive spot. *Anal.* Calcd. for $C_{57}H_{82}N_8O_{13}$: C, 62.96; H, 7.60; N, 10.13. Found: C, 63.01; H, 7.66; N, 9.98.

Boc-Lys(Z)-Asp(OBzl)-OH (VII)—A solution of Boc-Lys(Z)-OH (3.8 g) and N-methylmorpholine (1.1 ml) in a mixture of tetrahydrofuran (5.0 ml) and acetonitrile (5.0 ml) was cooled to -10° and ethylchloroacetate (1.0 ml) added thereto. After 15 min, a precooled solution of H-Asp(OBzl)-OH (2.3 g) and N-methylmorpholine (1.0 ml) in DMF (5.0 ml) was added and the reaction mixture was left overnight at 4° . The mixture was extracted with EtOAc and washed successively with 1 N citric acid and H_2O . The solvent was dried over anhydr. $MgSO_4$ and evaporated off in a vacuum. The residue was precipitated from EtOAc and petroleum ether: 3.6 g (62%), mp 50—51°, $[\alpha]_D^{25} - 17.0^\circ$ ($c=1.0$, DMF), Rf^1 0.42, Rf^2 0.52, single ninhydrin-positive spot. *Anal.* Calcd. for $C_{30}H_{39}N_3O_9$: C, 61.52; H, 6.71; N, 7.18. Found: C, 61.16; H, 7.02; N, 6.91.

Z-Arg(NO₂)-Lys(Z)-Asp(OBzl)-OH (VIII)—VII (1.200 g) was treated with TFA (2.0 ml) as described above and the resulting residue was dissolved in DMF (5.0 ml). A solution of Z-Arg(NO₂)-OH (0.780 g) and N-methylmorpholine (0.2 ml) in a mixture of tetrahydrofuran (5.0 ml) and acetonitrile (5.0 ml) was cooled to -10° and treated with ethylchloroacetate (0.3 ml). After standing for some 25 min, a cold DMF solution of H-Lys(Z)-Asp(OBzl)-OH trifluoroacetate and N-methylmorpholine (0.22 ml) was added, and the mixture was allowed to stand overnight at 4° . The mixture was evaporated down in a vacuum, extracted with EtOAc, and washed with 1 N HCl and H_2O . The solvent was dried over anhydr. $MgSO_4$ and evaporated down in a vacuum. The residue was precipitated from EtOAc and ether: 1.2 g (71%), mp 58—59°, $[\alpha]_D^{25} - 42.0^\circ$ ($c=1.0$, DMF). *Anal.* Calcd. for $C_{39}H_{49}N_8O_{12}$: C, 57.09; H, 6.01; N, 13.64. Found: C, 56.80; H, 5.88; N, 13.65.

Boc-Lys(Z)-Asp(OBzl)-Val-Tyr-Val-Gln-Leu-Tyr-Leu-OBzl (IX)—This compound was prepared from VI (155 mg), HONB (29 mg), VII (95 mg) and WSCI (25 mg) essentially as described for the preparation of IV. The dried product was reprecipitated from EtOH and H_2O : 203 mg (91%), mp 166—174°, $[\alpha]_D^{25} - 35.5^\circ$ ($c=1.0$, DMF), Rf^1 0.88, Rf^2 0.93, single ninhydrin-positive spot. *Anal.* Calcd. for $C_{82}H_{111}N_{11}O_{19}$: C, 63.34; H, 7.20; N, 9.90. Found: C, 62.91; H, 7.45; N, 10.11.

H-Lys-Asp-Val-Tyr-Val-Gln-Leu-Tyr-Leu-OH (X)—The above protected nonapeptide (180 mg) was treated with HF (approximately 10.0 ml) in the presence of anisole (0.2 ml) in an ice bath for 30 min. After removal of HF by evaporation, was stored over KOH pellets in a vacuum and the residue was dissolved in a small amount of H_2O , which was washed with EtOAc then treated with Amberlite CG-4B (type 1, acetate form, 5 g) for 30 min. The resin was removed by filtration, the filtrate was lyophilized and the residue was dissolved in a small amount of 1% AcOH. The solution was applied to a column of Sephadex G-15 (2.8×60.0 cm), and eluted with the same solvent. Individual fractions (4 ml each) were collected and the absorbancy at 260 nm was determined. Fractions corresponding to the main peak (tube Nos. 38—52) were combined. The solution was concentrated *in vacuo* and the product lyophilized: 60 mg (46%), mp 140—150°, $[\alpha]_D^{25} - 55.0^\circ$ ($c=1.0$, H_2O), Rf^1 0.03, Rf^2 0.11, single ninhydrin-positive spot. Amino acid ratios in the acid hydrolysate: Leu 1.90, Tyr 1.81, Val 1.89, Glu 0.91, Asp 1.00, Lys 0.98 (average recovery 84%). Amino acid ratios in the AP-M digest: Leu 1.94, Tyr 1.90, Val 2.00, Gln 0.90, Asp 0.91, Lys 0.94 (average recovery 81%).

Z-Arg(NO₂)-Lys(Z)-Asp(OBzl)-Val-Tyr-Val-Gln-Leu-Tyr-Leu-OBzl (XI)—This compound was prepared from VI (218 mg), HONB (39 mg), VIII (180 mg) and WSCI (34 mg) essentially as described for the preparation of IV. The product was recrystallized from EtOH and H₂O: 310 mg (85%), mp 141—150°, $[\alpha]_D^{25} -39.5^\circ$ ($c=1.0$, DMF). *Anal.* Calcd. for C₉₁H₁₂₂N₁₆O₂₃H₂O: C, 60.45; H, 6.80; N, 12.40. Found: C, 59.51; H, 7.31; N, 11.80.

H-Arg-Lys-Asp-Val-Tyr-Val-Gln-Leu-Tyr-Leu-OH (XII)—The decapeptide was prepared from XI (200 mg) essentially as described for the preparation of X. XI was treated with HF at 0° for 30 min. The deblocked peptide was applied to a column (2.0 × 60.0 cm) of Sephadex G-15 and eluted with 10% AcOH. The eluates in the Nos. 31 to 50 containing the decapeptide were pooled and treated in the usual way: 68 mg (48%), mp 184—198°, $[\alpha]_D^{25} -64.5^\circ$ ($c=1.0$, H₂O), Rf^1 0.03, Rf^2 0.10, single ninhydrin- and Sakaguchi-positive spot. Amino acid ratios in the acid hydrolysate: Leu 1.82, Tyr 1.78, Val 1.91, Glu 0.89, Asp 0.91, Arg 0.98, Lys 1.00 (average recovery 81%). Amino acid ratios in the AP-M digest: Leu 1.91, Tyr 1.88, Val 1.99, Gln 0.91, Asp 0.98, Arg 0.99, Lys 0.91 (average recovery 81%).

E-Rosette Formation Procedure¹⁷⁾—Peripheral lymphocytes were isolated by Hypaque-Ficoll gradient centrifugation. For T cell rosette formation, isolated lymphocytes were adjusted to a concentration of 5×10^5 ml with PBS. Contamination with monocytes and polymorphonuclear cells was less than 5%. Sheep erythrocytes were washed with PBS and a suspension (1×10^6 /ml) was prepared. Lymphocytes were suspended in GVB²⁺ or FCS (0.7 ml) and incubated for 30 min at 37° with uremic serum (0.3 ml) and the synthetic peptides. Lymphocytes were washed with GVB²⁺ and centrifuged for 10 min at 1500 rpm, then suspended in GVB²⁺ or FCS (1.0 ml). The suspension was mixed with sheep erythrocytes (0.5 ml) and then incubated for 18 hr at 4°. The mixture was centrifuged for 5 min at 900 rpm. Triplicate wet-cell preparations were studied by phase microscopy. In each preparation, 200 lymphocytes were counted, and the proportion binding three or more sheep erythrocytes (rosettes) was determined (normal, 77%). Monocytes or polymorphonuclear cells forming rosettes were excluded (Table I).

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