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# The Effect of Thymopoietin II Fragments and Their Analogs on E-Rosette Forming Cells in the Uremic State[1]

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The synthesis of four fragments from dipeptide to pentapeptide, corresponding to amino acids 32 to 36 of thymopoietin II, and four analogs is described, together with their effects on E-rosette forming cells in the uremic state. After incubation with amounts of H–Arg–Lys–Asp–Val–Tyr–OH (residues 32—36) varying from 100 to 200 μg/ml of cell suspension, maximum T-cell rosette formation ranged from 65 to 73% compared with 50 to 52% without the peptide. The activity of H–Arg–Lys–Glu–Val–Tyr–OH was lower than that of H–Arg–Lys–Asp–Val–Tyr–OH. The other fragments and analogs had no effect on the E-rosette formation-inhibiting activity of uremic serum at a dose of 200 μg/ml

**Keywords**——thymopoietin II fragments; E-rosette forming cells; uremic serum; HOBT-DCC method; uremic state

Thymopoietin II is a polypeptide hormone of the thymus that consists of a 49-amino acid single polypeptide chain.[3] Goldstein *et al.* described the chemical synthesis of a tridecapeptide with an amino acid sequence corresponding to positions 29—41 of thymopoietin II, and showed it to have the same selective T cell differentiating capacity as thymopoietin II.[4] Recently, they reported the synthesis of pentapeptide corresponding to amino acids 32 to 36 thymopoietin II, and showed that it also has biological properties characteristic of thymopoietin II.[5] Human peripheral blood lymphocytes are surrounded *in vitro* by E in a rosette formation,[6] and these rosette-forming cells are thymus-derived cells (T cells).[7] Altered cellular immunity in the uremic state is a well established phenomenon. There are studies showing that renal failure serum depresses the number of E-rosette forming cells.[8] A decapeptide of thymopoietin II, consisting of amino acids 32 to 41, had been synthesized by us[9] and shown to increase E-rosette forming capacity in the uremic state.[9]

We describe here the synthesis of four thymopoietin II fragments corresponding to residues 32—36 and four analogs, as well as the *in vitro* response to E-rosette forming cells[8] of the uremic state in the presence of the synthetic peptides. The structure-activity relationship of the pentapeptide, and in particular, the contribution of each amino acid in this peptide to the E-rosette forming capacity in the uremic state, is also described. The following fragments and analogs were synthesized by a solution method: H–Val–Tyr–OH, H–Asp–Val–

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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; HOBT, N-hydroxybenzotriazole; FCS, fetal calf serum; $Et_3N$, triethylamine; PBS, phosphate-buffered saline; E, sheep erythrocytes; $GVB^{2+}$, gelatin veronal buffer; Tos, *p*-toluenesulfonic acid.
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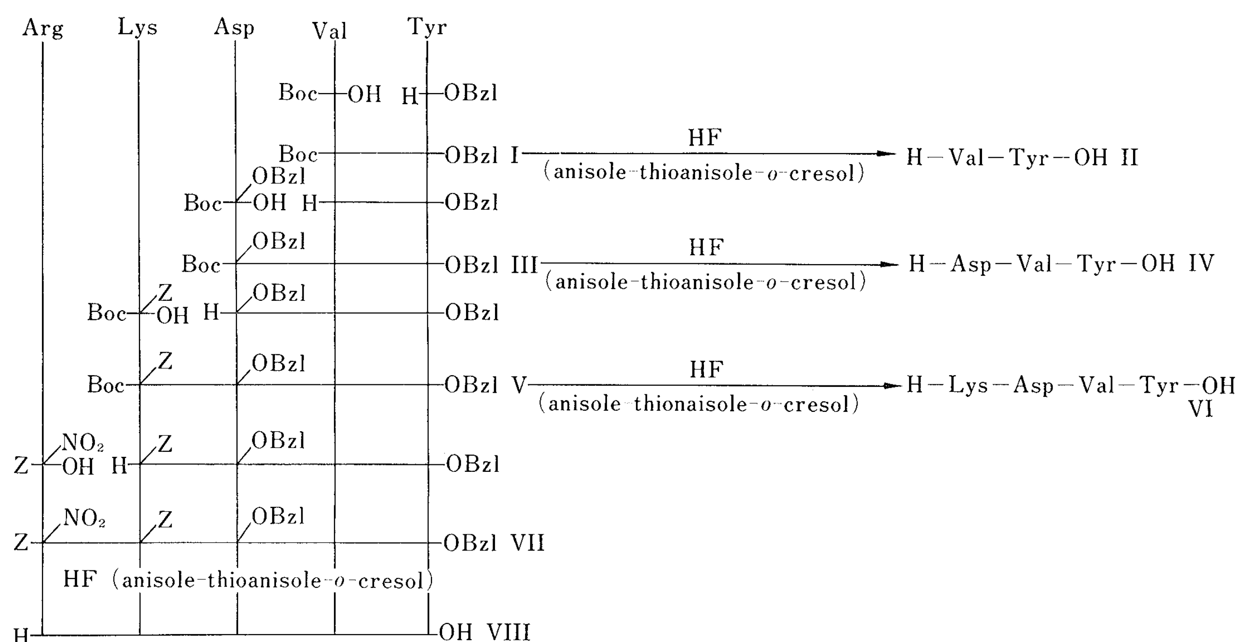


Fig. 1. Synthetic Scheme for H–Arg–Lys–Asp–Val–Tyr–OH and Its Fragments

Tyr–OH, H–Lys–Asp–Val–Tyr–OH, H–Arg–Lys–Asp–Val–Tyr–OH, H–Val–Lys–Asp–Val–Tyr–OH, H–Lys–Lys–Asp–Val–Tyr–OH, H–Arg–Lys–Glu–Val–Tyr–OH and H–Arg–Lys–Asp–Val–Tyr–OMe. The synthetic routes to the pentapeptide (H–Arg–Lys–Asp–Val–Tyr–OH) and their three fragments are illustrated in Fig. 1 as examples.

H–Tyr–OBzl Tos was condensed with Boc–Val–OH by the HOBT–DCC (dicyclohexylcarbodiimide) method[10] giving Boc–Val–Tyr–OBzl (I). The protected dipeptide I was then treated for 30 min with anhydrous HF[11] in the presence of anisole–thioanisole–*o*-cresol (1:1:1, v/v)[12] to suppress side reaction of H–Tyr–OH[13] 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-10. The dipeptide (II) thus obtained was found to be homogeneous on paper chromatography using two different solvent systems. The protected dipeptide I was treated with TFA-anisole to remove the Boc group and the product was coupled with Boc–Asp(OBzl)–OH by the HOBT–DCC method to give Boc–Asp(OBzl)–Val–Tyr–OBzl (III). Deblockings of III as described for preparation of the dipeptide II yielded the desired H–Asp–Val–Tyr–OH (IV). The Boc group of III was similarly removed and the free base was condensed with Boc–Lys(Z)–OH by the HOBT–DCC method to afford Boc–Lys(Z)–Asp(OBzl)–Val–Tyr–OBzl (V). Deblockings of V as described for preparation of the dipeptide II yielded the desired H–Lys–Asp–Val–Tyr–OH (VI). The Boc group of V was similarly removed and the free base was condensed with Z–Arg($NO_2$)–OH by the HOBT–DCC method to afford Z–Arg($NO_2$)–Lys(Z)–Asp(OBzl)–Val–Tyr–OBzl (VII). Deblockings of VII as described for preparation of the dipeptide II yielded the desired H–Arg–Lys–Asp–Val–Tyr–OH (VIII). The other four analogs, H–Val–Lys–Asp–Val–Tyr–OH, H–Lys–Lys–Asp–Val–Tyr–OH, H–Arg–Lys–Glu–Val–Tyr–OH and H–Arg–Lys–Asp–Val–Tyr–OMe were prepared by essentially the same approach that had been used to obtain VIII.

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Serum from an uremic patient with chronic renal failure had low proportions of T cell rosettes, ranging from 50 to 52% (Table IV). After incubation with amounts of H–Arg–Lys–Asp–Val–Tyr–OH varying from 100 to 200 μg/ml of cell suspension, maximum T cell rosette formation ranged from 65 to 73%. Table IV shows the effects of thymopoietin II fragments and analogs on the inhibition of E-rosette formation by uremic serum in comparison with H–Arg–Lys–Asp–Val–Tyr–OH. The activity of H–Arg–Lys–Glu–Val–Tyr–OH was lower

Table I. Physical Constants and Analytical Data for the Protected Peptides

| Compound | Recryst. solv. | Yield (%) | $[\alpha]_D^{26}$ ($c$=1.0, DMF) | Formula | Analysis (%) Calcd (Found) C | H | N | $Rf^1$ | $Rf^2$ | mp (°C) |
|---|---|---|---|---|---|---|---|---|---|---|
| Boc–Val–Tyr–OBzl | A[b] | 72 | −5.0 | $C_{26}H_{34}N_2O_6$ | 66.36 (65.97 | 7.82 7.46 | 5.95 5.47) | 0.87[a] | 0.91[a] | 146—157 |
| Boc–Asp(OBzl)–Val–Tyr–OBzl | B[b] | 80 | −10.0 | $C_{37}H_{45}N_3O_9$ | 65.76 (65.48 | 6.71 7.04 | 6.22 5.89) | 0.80[a] | 0.70[a] | 125—130 |
| Boc–Lys(Z)–Asp(OBzl)–Val–Tyr–OBzl | B[b] | 92 | −15.0 | $C_{51}H_{63}N_5O_{12}$ | 65.30 (65.16 | 6.77 6.35 | 7.47 7.60) | 0.70[a] | 0.75[a] | 75— 81 |
| Z–Arg(NO$_2$)–Lys(Z)–Asp(OBzl)–Val–Tyr–OBzl | B[b] | 85 | −11.1 | $C_{60}H_{72}N_{10}O_{15}$ | 61.42 (61.22 | 6.19 6.21 | 11.94 12.30) | | | 87— 91 |
| Z–Val–Lys(Z)–Asp(OBzl)–Val–Tyr–OBzl | B[b] | 63 | −9.0 | $C_{59}H_{70}N_6O_{11}$ | 68.19 (68.35 | 6.79 6.63 | 8.09 8.21) | | | 96—105 |
| Boc–Lys(Z)–Lys(Z)–Asp(OBzl)–Val–Tyr–OBzl | B[b] | 77 | −14.0 | $C_{60}H_{72}N_6O_{14}$ | 67.14 (67.45 | 6.76 6.84 | 7.83 7.45) | 0.71[a] | 0.91[a] | 75— 82 |
| Boc–Glu(OBzl)–Val–Tyr–OBzl | B[b] | 60 | −7.5 | $C_{38}H_{47}N_3O_9$ | 66.16 (65.86 | 6.87 6.46 | 6.09 5.70) | 0.84[a] | 0.76[a] | 120—125 |
| Boc–Lys(Z)–Glu(OBzl)–Val–Tyr–OBzl | B[b] | 74 | −12.0 | $C_{52}H_{65}N_5O_{12}$ | 65.60 (65.57 | 6.88 6.50 | 7.36 7.54) | 0.69[a] | 0.74[a] | 88— 97 |
| Z–Arg(NO$_2$)–Lys(Z)–Glu(OBzl)–Val–Tyr–OBzl | B[b] | 70 | −8.0 | $C_{61}H_{74}N_{10}O_{15}$ | 61.71 (61.86 | 6.28 6.09 | 11.80 11.36) | | | 100—108 |
| Z–Val–Tyr–OMe | A[b] | 82 | −17.0 | $C_{23}H_{28}N_2O_6$ | 64.47 (64.08 | 6.59 7.01 | 6.55 6.49) | 0.63[a] | 0.83[a] | 134—137 |
| Boc–Asp(OBzl)–Val–Tyr–OMe | B[b] | 53 | −12.6 | $C_{31}H_{41}N_3O_9$ | 62.09 (61.63 | 6.89 7.06 | 7.01 6.92) | 0.70[a] | 0.87[a] | 63— 65 |
| Boc–Lys(Z)–Asp(OBzl)–Val–Tyr–OMe | B[b] | 98 | −25.6 | $C_{45}H_{59}N_5O_{12}$ | 62.07 (62.41 | 6.90 7.31 | 8.31 8.29) | 0.89[a] | 0.95[a] | 86— 95 |
| Z–Arg(NO$_2$)–Lys(Z)–Asp(OBzl)–Val–Tyr–OMe | C[b] | 92 | −20.0 | $C_{54}H_{68}N_{10}O_{15}$ | 59.11 (58.76 | 6.25 6.66 | 12.77 12.78) | | | 136—147 |

a) Ninhydrin-positive spot.
b) Abbreviations: A=EtOAc–*n*-hexane; B=EtOAc–ether; C=EtOAc.

Table II. Physical Constants and Analytical Data for the Peptides

| | Compound No. | mp (°C) | Yield (%) | $[\alpha]_D^{26}$ (10% AcOH) | $Rf^1$ | $Rf^2$ |
|---|---|---|---|---|---|---|
| I | H–Val–Tyr–OH | 180—185 | 86 | +5.0($c$ =1.0) | 0.60[a] | 0.61[a] |
| IV | H–Asp–Val–Tyr–OH | 141—147 | 80 | −12.1($c$ =1.0) | 0.07[a] | 0.18[a] |
| VI | H–Lys–Asp–Val–Tyr–OH | 108—116 | 83 | −21.0($c$ =1.0) | 0.03[a] | 0.17[a] |
| VIII | H–Arg–Lys–Asp–Val–Tyr–OH | 143—147 | 93 | −26.4($c$ =1.0) | 0.08[a] | 0.17[a] |
| X | H–Val–Lys–Asp–Val–Tyr–OH | 151—159 | 85 | −30.4($c$ =0.4) | 0.27[a] | 0.40[a] |
| XII | H–Lys–Lys–Asp–Val–Tyr–OH | 142—154 | 85 | −19.1($c$ =0.3) | 0.07[a] | 0.18[a] |
| XVI | H–Arg–Lys–Glu–Val–Tyr–OH | 148—155 | 68 | −32.1($c$ =0.4) | 0.05[b] | 0.14[b] |
| XXI | H–Arg–Lys–Asp–Val–Tyr–OMe | 120—131 | 84 | −29.0($c$ =1.0) | 0.11[b] | 0.30[b] |

a) Ninhydrin-positive spot.
b) Ninhdrin- and Sakaguch -positive spot.

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than that of H–Arg–Lys–Asp–Val–Tyr–OH. The other fragments and analogs had no effect on the E-rosette formation inhibiting activity of uremic serum at a dose of 200 μg/ml. These results strongly suggest that the COOH–terminal amino acid (Tyr) must have a free carboxyl group, that the Arg residue is important for the receptor affinity and that the positive charge of the guanidinium side chain is required for agonistic activity towards E-rosette inhibition by uremic serum.

Table III. Results of Amino Acid Analysis of the Peptides

| | Compound No. | Acid hydrolysate (average recovery %) | AP–M digest average recovery %) |
|---|---|---|---|
| II | H–Val–Tyr–OH | Tyr 0.99, Val 1.00 (85) | Tyr 0.94, Val 1.00 (81) |
| IV | H–Asp–Val–Tyr–OH | Tyr 0.94, Val 0.89, Asp 1.00 (81) | Tyr 0.91, Val 0.94, Asp 0.89 (84) |
| VI | H–Lys–Asp–Val–Tyr–OH | Asp 0.87, Val 0.91, Tyr 0.88, Lys 1.02 (81) | Asp 0.85, Val 0.90, Tyr 0.84, Lys 0.99 (82) |
| VIII | H–Arg–Lys–Asp–Val–Tyr–OH | Asp 0.84, Val 0.99, Tyr 0.82, Arg 0.81, Lys 1.00 (84) | Asp 0.88, Val 0.79, Tyr 0.91, Arg 0.94, Lys 0.85 (80) |
| X | H–Val–Lys–Asp–Val–Tyr–OH | Asp 0.79, Val 1.63, Tyr 0.92, Lys 1.02 (86) | Asp 0.81, Val 1.63, Tyr 0.89, Lys 0.88 (85) |
| XII | H–Lys–Lys–Asp–Val–Tyr–OH | Asp 0.79, Val 0.80, Tyr 0.91, Lys 1.74 (88) | Asp 0.83, Val 1.01, Tyr 0.99, Lys 1.65 (83) |
| XVI | H–Arg–Lys–Glu–Val–Tyr–OH | Glu 0.97, Val 1.00, Tyr 0.79, Arg 0.78, Lys 0.89 (81) | Glu 0.99, Val 0.91, Tyr 0.84, Arg 0.81, Lys 0.92 (85) |
| XXI | H–Arg–Lys–Asp–Val–Tyr–OMe | Asp 1.01, Val 0.87, Typ 0.91, Arg. 0.79, Lys 0.99 (81) | |

Table IV. Effects of H–Arg–Lys–Asp–Val–Tyr–OH (Thymopoietin II 32—36), Its Fragments and Analogs on the Inhibition of E-Rosette Formation by Uremic Serum

| Material | Dose (μg/ml) | E-Rosettes (%) |
|---|---|---|
| —[a] | | 80±4 |
| —[b] | | 81±4 |
| —[c] | | 51+4 |
| H–Arg–Lys–Asp–Val–Tyr–OH[c,d] | 100 | 65±3 |
| | 200 | 73±2 |
| H–Lys–Asp–Val–Tyr–OH[c,d] | 100 | 53±4 |
| | 200 | 52±3 |
| H–Asp–Val–Tyr–OH[c,d] | 100 | 53±4 |
| | 200 | 51±4 |
| H–Val–Tyr–OH[c,d] | 100 | 51±3 |
| | 200 | 50±5 |
| H–Val–Lys–Asp–Val–Tyr–OH[c,d] | 100 | 52±5 |
| | 200 | 53±4 |
| H–Lys–Lys–Asp–Val–Tyr–OH[c,d] | 100 | 53±4 |
| | 200 | 52±5 |
| H–Arg–Lys–Glu–Val–Tyr–OH[c,d] | 100 | 60±4 |
| | 200 | 66±3 |
| H–Arg–Lys–Asp–Val–Tyr–OMe[c,d] | 100 | 53±5 |
| | 200 | 51±6 |

a) Normal lymphocytes.
b) Lymphocytes were incubated for 3 hr at 37° with normal serum.
c) Lympocytes were incubated for 3 hr at 37° with uremic serum.
d) Lympocytes were incubated for 1 hr at 37° with synthetic peptide.

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## Experimental

Melting points are uncorrected. Optical rotations were measured with an Atago Polax machine. The amino acid hydrolysates and aminopeptidase-M (AP-M) digests[14] were analyzed with a JEOL JLC-8AH amino acid analyzer according to the directions give by Moore *et al.*[15] The Boc groups of the protected peptides were deblocked with TFA. The resulting amino components were chromatographed on filter paper (Toyo Roshi No. 51) at room temperature. $Rf^1$ values refer to the Partridge system[16] and $Rf^2$ values refer to BuOH–pyridine–AcOH–$H_2O$ (30: 20: 6: 24).[17] Concentration was carried out in a rotary evaporator under reduced pressure at a temperature of 35—38°. 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 −80° until use.

**General Procedure for the Synthesis of the Peptide starting with Amino Acid Benzyl Ester or Amino Acid Methyl Ester**——Amino acid benzyl ester Tos (0.01 mol) or amino acid methyl ester HCl (0.01 mol) was dissolved in DMF (20 ml) together with $Et_3N$ (0.01 mol), N-*tert*-butoxycarbonyl amino acid (0.011 mol) or N-benzyloxycarbonyl amino acid (0.011 mol) and HOBT (0.011 mol). After addition of WSCI (0.011 mol), the solution was stirred at 0° for 18 hr and then diluted with EtOAc. The solution was washed successively with 1 N $NaHCO_3$, $H_2O$, 1 N citric acid and $H_2O$. The EtOAc layer was dried over anhydrous $MgSO_4$, concentrated to small volume, and *n*-hexane was added to give the product, which was reprecipitated or recrystallized from an appropriate solvent as listed in Table I. The Boc groups of the protected peptides were deblocked with TFA in the presence of anisole except that deblocking of the Z group of Z–Val–Tyr–OMe was done by catalytic hydrogenation. The coupling of the next amino acid to the peptide benzyl ester TFA salt or the peptide methyl ester AcOH salt was carried out in the same manner. The fully protected peptides (0.0005 mol) thus obtained were deblocked with HF (approximately 5 ml) in the presence of anisole–thioanisole–*o*-cresol (1: 1: 1 v/v, 0.5 ml) in an ice-bath for 30 min. The excess HF was removed by evaporation *in vacuo* at 0° and the residue was washed with ether and then dissolved in $H_2O$ (10 ml). The solution was treated with Amberlite CG-4B (acetate form, approximately 5 g) for 30 min. The resin was removed by filtration and the filtrate was concentrated *in vacuo*. The residue was applied to a column of Sephadex G-10 (2.6×94 cm). Individual fractions (4 ml each) eluted with 3% AcOH were collected and the absorbancy at 260 nm was determined to locate the final peptide.

**E-Rosette Formation Procedure**——Peripheral blood lymphocytes were isolated in a Hypaque-Ficoll gradient[18] for T cell rosette formation. Isolated lymphocytes were adjusted to $5 \times 10^5$ cells/ml with PBS. Contamination by monocytes and polymorphonuclear cells amounted to less than 5%. Sheep erythrocytes were washed with PBS and a suspension ($1 \times 10^6$/ml) was prepared. Lymphocytes were suspended in $GVB^{2+}$ or FCS (1.0 ml) and incubated for 3 hr at 37° with uremic patient's serum (0.22 ml). Next, lymphocytes were washed with $GVB^{2+}$ and centrifuged for 10 min at 1500 rpm, then suspended in $GVB^{2+}$ (1.0 ml) and incubated for 1 hr at 37° with a synthetic peptide (100—200 μg/ml). Lymphocytes were washed with $GVB^{2+}$ and centrifuged for 10 min at 1500 rpm, then suspended in $GVB^{2+}$ (1.0 ml). The suspension was mixed with sheep erythrocytes (0.5 ml) and incubated for 16 hr at 4°. Triplicate wet-cell preparations were checked by phase contrast microscopy. For each preparation, 200 lymphocytes were counted, and the proportion binding more than three sheep erythrocytes was determined (Table IV).

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