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Effects of Synthetic Thymopoietin II Fragments on E-Rosette Forming Cells of a Rheumatoid Arthritis Patient[1]

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An octadecapeptide, H–Arg–Lys–Asp–Val–Tyr–Val–Gln–Leu–Tyr–Leu–Gln–Thr–Leu–Thr–Ala–Val–Lys–Arg–OH, corresponding to the C-terminal portion of thymopoietin II was synthesized using protecting groups removable by hydrogen fluoride treatment. The *in vitro* addition of the synthetic octadecapeptide was able to restore the low E-rosette forming capacity of cells in a rheumatoid arthritis patient to normal levels. The *in vitro* effects of pentapeptide (positions 32—36), nonapeptide (positions 33—41) and decapeptide (positions 32—41) fragments of thymopoietin on the low E-rosette forming capacity of cells of a rheumatoid arthritis patient were also compared with that of the synthetic octadecapeptide. The relative potency of the pentapeptide was 10.56 and that of the decapeptide was 25.43 based on the octadecapeptide (100.00) as a standard, but the nonapeptide was ineffective.

Keywords——rheumatoid arthritis patient; E-rosette forming cells; HOBT-DCC procedure; HONB-DCC procedure; thymopoietin II

Thymopoietin II is a polypeptide of the thymus, and the 49-amino acid sequence of this T-cell differentiating hormone has been determined.[2] In addition to the induction of early T-cell differentiation, thymopoietin also appears to regulate more mature populations.[3] Two synthetic fragments were shown to be biologically active–first a tridecapeptide corresponding to positions 29—41[4] and, subsequently, a pentapeptide corresponding to 32—36.[5] Thus, the synthetic pentapeptide, H–Arg–Lys–Asp–Val–Tyr–OH, appears to correspond to a biologically active site of the parent thymopoietin molecule. On the other hand, it is generally accepted that a high percentage of rheumatoid arthritis patients have a defect of cell-mediated immunity.[6] A decrease of E-rosette forming cells in these patients has been demonstrated by several investigators.[6,7] Recently, Auteri *et al.*[8] reported that the *in vitro* addition of the thymopoietin pentapeptide (positions 32—36) could restore to normal values the low activity of E-rosette forming cells in rheumatoid arthritis patients. We describe here the synthesis of an octadecapeptide with an amino acid sequence corresponding to positions 32—49 (H–Arg–Lys–Asp–Val–Tyr–Val–Gln–Leu–Tyr–Leu–Gln–Thr–Leu–Thr–Ala–Val–Lys–Arg–OH) of thymopoietin II. Furthermore, we compared the *in vitro* effects of this octadecapeptide, thymopoietin pentapeptide[9] (positions 32—36), its nonapeptide[10] (positions 33—41) and its decapeptide[10] (positions 32—41) on low E-rosette forming cells of a rheumatoid arthritis patient. In the previous papers,[9,10] we reported syntheses of a pentapeptide (positions 32—36), nonapeptide (positions 33—41) and decapeptide (positions 32—41) by the solution method and we showed that the pentapeptide and decapeptide could increase the E-rosette forming capacity in the uremic state.

In the present synthesis, protecting groups of amino acid derivatives, Z–Arg(NO_2), Asp(OBzl), Arg(NO_2)–OBzl and Lys(Z) were removed by hydrogen fluoride treatment.[11] These protecting groups survive mostly intact under TFA treatment for removal of the Boc group, employed as a temporary α-amino protecting group. First, the C-terminal octapeptide, Boc–Gln–Thr–Leu–Thr–Ala–Val–Lys(Z)–Arg(NO_2)–OBzl (VII) was synthesized stepwise according to Chart 1. The protected heptapeptide ester, Boc–Thr–Leu–Thr–Ala–Val–Lys(Z)–Arg(NO_2)–OBzl (VI) was synthesized stepwise by the HOBT–DCC procedure[12] starting from H–Arg(NO_2)–OBzl 2 Tos. After the TFA-anisole treatment of VI, the resulting hepta-

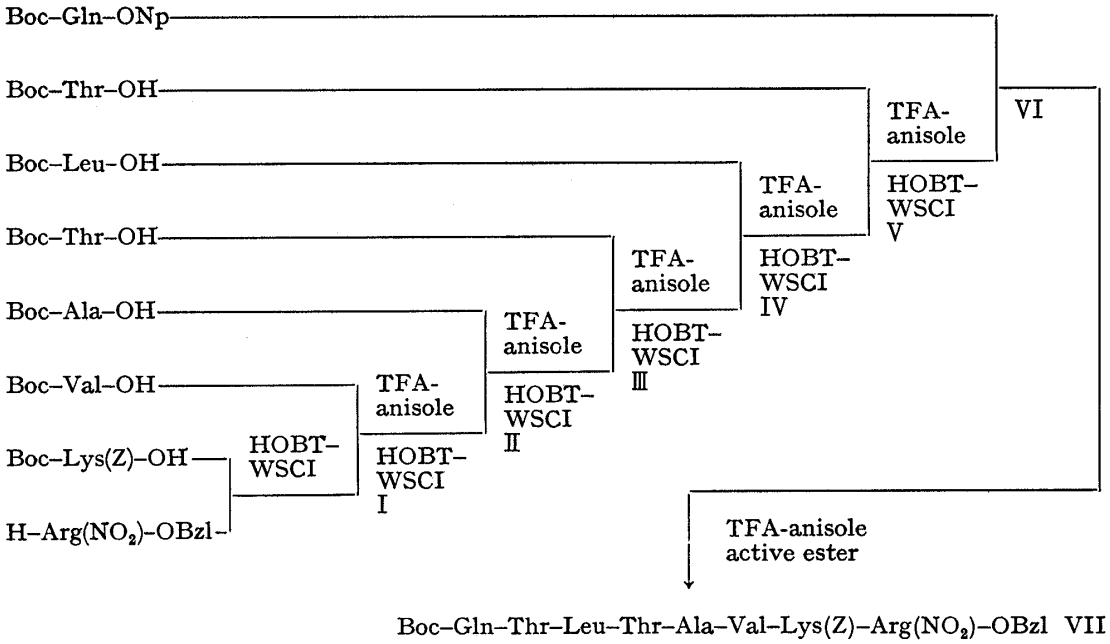

Chart 1. Synthetic Route for the Protected Octapeptide (Positions 42—49)

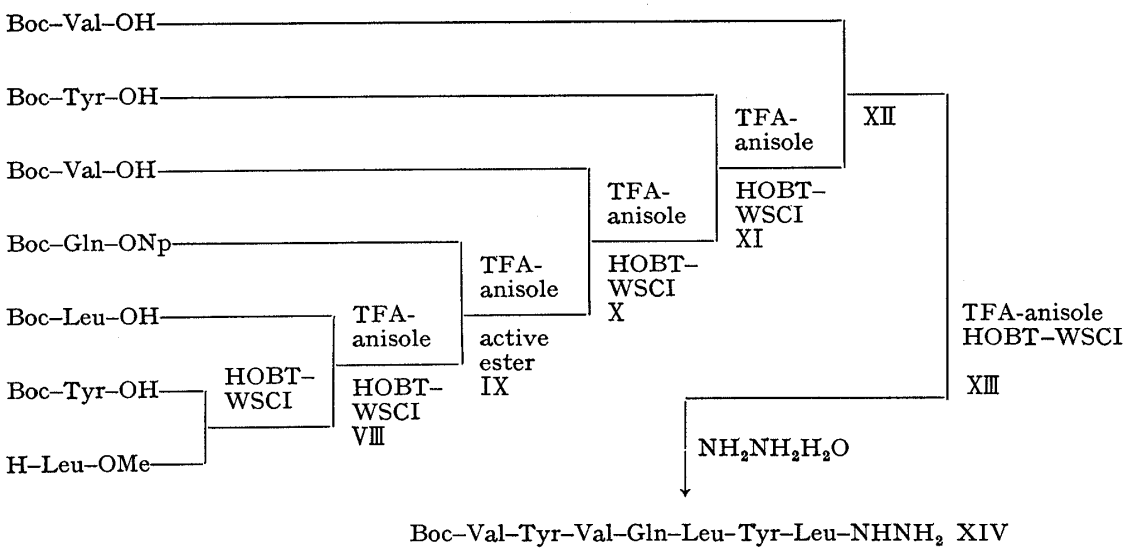

Chart 2. Synthetic Route for the Protected Heptapeptide Hydrazide (Positions 35—41)

peptide ester was condensed with Boc–Gln–ONp to give the protected octapeptide, Boc–Gln–Thr–Leu–Thr–Ala–Val–Lys(Z)–Arg(NO_2)–OBzl (VII). The synthetic scheme for the protected heptapeptide hydrazide, Boc–Val–Tyr–Val–Gln–Leu–Tyr–Leu–$NHNH_2$ (XIV), is illustrated in Chart 2. The protected heptapeptide ester, Boc–Val–Tyr–Val–Gln–Leu–Tyr–Leu–OMe (XIII) was also synthesized stepwise by the HOBT–DCC procedure and the *p*-nitrophenyl ester method starting from H–Leu–OMe HCl. This protected heptapeptide ester (XIII) was therefore converted to the corresponding hydrazide, Boc–Val–Tyr–Val–Gln–Leu–Tyr–Leu–$NHNH_2$ (XIV), by the usual hydrazine treatment, which permitted us to check its homogeneity by elemental analysis. The protected octapeptide ester VII was converted to the corresponding amine by TFA-anisole treatment and was subjected to coupling with the azide derived from XIV by treatment with HCl, followed by isoamylnitrile, to afford the protected pentadecapeptide ester, Boc–Val–Tyr–Val–Gln–Leu–Tyr–Leu–Gln–Thr–Leu–Thr–Ala–Val–Lys(Z)–Arg(NO_2)–OBzl (XV). XV was purified by silica gel column chromato-

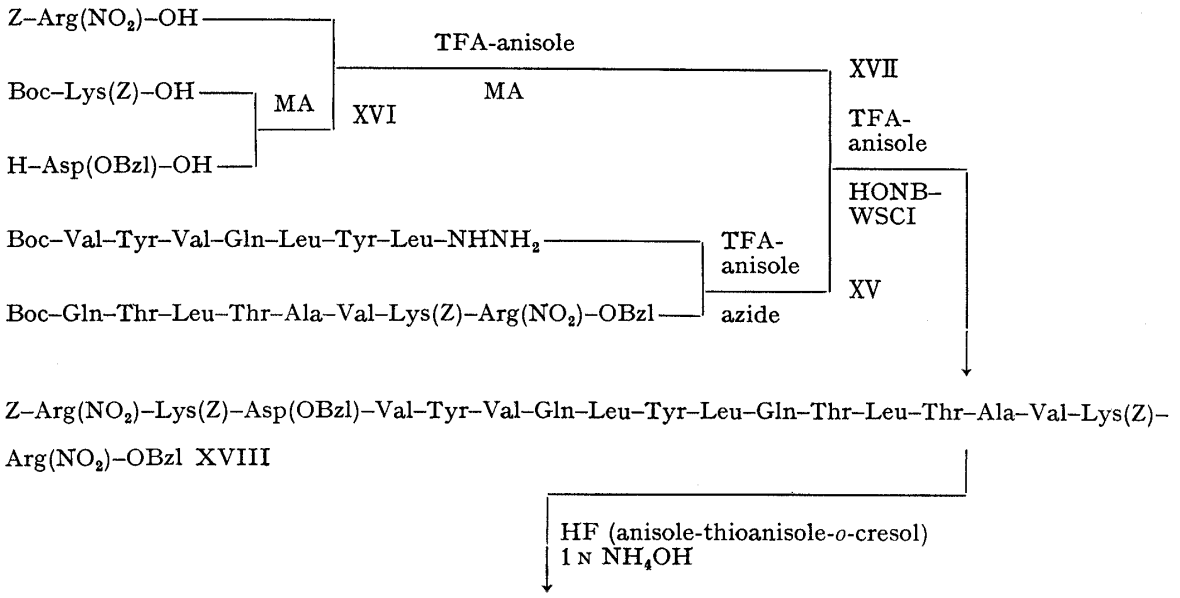

Chart 3. Synthetic Route for the Thymopoietin II Fragment (Positions 32—49)

graphy. The synthetic route to the thymopoietin II octadecapeptide fragment, H–Arg–Lys–Asp–Val–Tyr–Val–Gln–Leu–Tyr–Leu–Gln–Thr–Leu–Thr–Ala–Val–Lys–Arg–OH (XIX), is illustrated in Chart 3. The protected tripeptide, Z–Arg(NO_2)–Lys(Z)–Asp(OBzl)–OH (XVII), was synthesized in a stepwise manner by the MA procedure[13] starting from H–Asp(OBzl)–OH. The pentadecapeptide XV was treated with TFA-anisole and the product was condensed with the tripeptide XVII by the HONB–DCC procedure to minimize undesirable racemization[14] to afford Z–Arg(NO_2)–Lys(Z)–Asp(OBzl)–Val–Tyr–Val–Gln–Leu–Tyr–Leu–Gln–Thr–Leu–Thr–Ala–Val–Lys(Z)–Arg(NO_2)–OBzl (XVIII). The protected octadecapeptide was then treated with hydrogen fluoride, in the presence of anisole–thioanisole–*o*-cresol (1:1:1, v/v)[15] to suppress side reaction of H–Tyr–OH,[16] to remove all protecting groups. The deblocked peptide was precipitated by dry ether, converted to the corresponding acetate by Amberlite CG-4B (acetate form) and then treated with 1 N NH_4OH for 30 min. The latter treatment was performed because of the reversible N→O shift at the Thr residues during the hydrogen fluoride treatment.[17,18] Finally, the product was purified by gel-filtration on Sephadex G-25 using 2% AcOH, followed by partition column chromatography on Sephadex G-25 according to Yamashiro.[19] The octadecapeptide (XIX) thus obtained was found to be homogeneous by paper chromatography in two different solvent systems. The amino acid compositions in the acid hydrolysate and aminopeptidase (AP-M)[20] digest of XIX agreed well with the theoretical values. These results indicate that the synthetic octadecapeptide possesses a high degree of homogeneity and the L-configuration of constituent amino acid residues. As shown in schemes 1—3, our synthetic route to the thymopoietin II fragment is different from those employed for the synthesis of thymopoietin II by Fujino *et al.*[21] The *in vitro* effects and relative potencies of the synthetic thymopoeitin II fragments on E-rosette forming cells in a rheumatoid arthritis patient are shown in Tables I and II. Incubation of blood from a rheumatoid arthritis patient in the presence of various amounts of synthetic fragment peptides from 10 μg/ml to 300 μg/ml resulted in recovery of E-rosette formation (Table I). The potency of the synthetic octadecapeptide (positions 32—49) was more than 9 times the potency of the synthetic pentapeptide (positions 32—36) (Table II). The synthetic decapeptide (positions 32—41) showed 1/4 of the activity of the synthetic octadecapeptide. However, the synthetic nonapeptide (positions 33—41) had no effect on E-rosette forming cells

Table I. Effects of the Synthetic Thymopoietin II Fragments on the Low E-Rosette Forming Cells of a Rheumatoid Arthritis Patient

Peptide	Dose (μg/ml)	E-Rosette forming cell (%)
——[a]		78±4
——[b]		51±3
Octadecapeptide[b,c] (positions 32—49)	10	73±4
Nonapeptide[b,c] (positions 33—41)	300	52±4
Decapeptide[b,c] (positions 32—41)	39	74±3
Pentapeptide[b,c] (positions 32—36)	95	75±4

a) Normal lymphocytes.
b) Patient's lymphocytes.
c) Incubation was carried out for 1 h at 37 °C with synthetic peptide.

Table II. Relative Potencies of the Synthetic Thymopoietin II Fragments on the Low E-Rosette Forming Cells of a Rheumatoid Arthritis Patient

Peptides	Relative potency (molar basis)
Octapeptide (positions 32—49)	100.00
Nonapeptide[a] (positions 33—41)	—
Decapeptide (positions 32—41)	25.43±3
Pentapeptide (positions 32—36)	10.56±4

a) This peptide had no effect on the low E-rosette forming cells in a rheumatoid arthritis patient at a dose of 300 μg/ml.

of this patient at a dose of 300 μg/ml (Table I). These results strongly suggest that the Arg residue at the 32 position of thymopoietin II is required for increasing the activity of E-rosette forming cells in cases of rheumatoid arthritis.

Experimental

Melting points are uncorrected. Rotations were determined in a Atago Polax machine (cell length: 10 cm). The amino acid compositions of the acid and enzymatic hydrolysates were determined with a JEOL JLC-8AH amino acid analyzer (one-column system). Solvents were removed by evaporation *in vacuo* at 35 to 40°C in a rotary evaporator. Boc groups of the protected peptides were removed by TFA-anisole treatment. The resulting amino components were chromatographed on filter paper, Toyo Roshi No. 51, at room temperature. Rf^1 values refer to the Partridge system[22] and Rf^2 values refer to BuOH–pyridine–AcOH–H_2O (30: 20: 6: 24).[23] Venous blood from a rheumatoid arthritis patient and normal subjects was draun into heparinized syringes and sedimented at room temperature. Aminopeptidase (3501, Aminopeptidase 210520) was purchased from the Protein Research Foundation, Osaka, Japan.

Boc-Lys(Z)-Arg(NO_2)-OBzl (I)——HOBT (744 mg) and WSCI (854 mg) were added to a solution of Boc–Lys(Z)–OH DCHA (3.1 g), H–Arg(NO_2)–OBzl 2 Tos (3.3 g) and Et_3N (0.77 ml) in DMF (20 ml) with stirring at 0°C. The mixture was stirred for 12 h at 4°C. The reaction mixture was extracted with EtOAc and the extract was washed successively with 1 N citric acid, H_2O, 1 N $NaHCO_3$ and H_2O, dried over $MgSO_4$ and then concentrated *in vacuo*. The residue was reprecipitated from EtOAc and *n*-hexane: 3.1 g (91%), mp 63—64°C, $[\alpha]_D^{26}$ −24.3° (c=1.0, AcOH), Rf^1 0.86, Rf^2 0.93, single ninhydrin–positive spot. *Anal.* Calcd for $C_{32}H_{45}N_7O_9$: C, 57.21; H, 6.75; N, 14.60. Found: C, 56.93; H, 6.86; N, 14.79.

Boc-Val-Lys(Z)-Arg(NO_2)-OBzl (II)——I (2.2 g) was treated with TFA (5 ml)–anisole (0.5 ml) at room temperature for 30 min, then excess TFA was removed by evaporation. The residue was washed with dry ether and then dried over KOH pellets *in vacuo* and the deprotected peptide was dissolved in DMF (15 ml). To this ice-chilled solution, Et_3N (0.51 ml), Boc–Val–OH (796 mg), HOBT (495 mg) and WSCI (570 mg) were successively added. After stirring overnight at 0°C, the mixture was extracted with EtOAc and the extract was washed successively with 1 N citric acid, H_2O, 1 N $NaHCO_3$ and H_2O, dried over $MgSO_4$ and concentrated *in vacuo*. *n*-Hexane was added to the residue and the precipitate formed was filtered off *in vacuo*. The

product was reprecipitated from EtOAc and ether: 1.5 g (60%), mp 81—83°C, $[\alpha]_D^{27}$ −28.9° (c=1.0, DMF), Rf^1 0.84, Rf^2 0.92, single ninhydrin-positive spot. *Anal.* Calcd for $C_{37}H_{54}N_8O_{10}$: C, 57.65; H, 7.06; N, 14.54. Found: C, 57.66; H, 7.18; N, 14.59.

Boc-Ala-Val-Lys(Z)-Arg(NO_2)-OBzl (III)——This compound was prepared from II (1.1 g), HOBT (212 mg), Boc–Ala–OH (297 mg) and WSCI (244 mg) essentially as described for the preparation of II. The product was reprecipitated from MeOH and ether: 948 mg (82%), mp 93—96°C, $[\alpha]_D^{27}$ −48.3° (c=1.0, DMF), Rf^1 0.89, Rf^2 0.95, single ninhydrin–positive spot. *Anal.* Calcd for $C_{40}H_{59}N_9O_{11}$: C, 57.06; H, 7.06; N, 14.97. Found: C, 56.82; H, 7.32; N, 15.01.

Boc-Thr-Ala-Val-Lys(Z)-Arg(NO_2)-OBzl (IV)——This compound was prepared from III (843 mg), Boc–Thr–OH (241 mg), HOBT (149 mg) and WSCI (171 mg) essentially as described for the preparation of II. The product was reprecipitated from EtOAc and ether: 568 mg (60%), mp 99—101°C, $[\alpha]_D^{26}$ −21.0° (c=1.0, DMF), Rf^1 0.81, Rf^2 0.91, single ninhydrin–positive spot. *Anal.* Calcd for $C_{44}H_{66}N_{10}O_{13}$: C, 56.04; H, 7.06; N, 14.85. Found: C, 55.87; H, 7.24; N, 15.09.

Boc-Leu-Thr-Ala-Val-Lys(Z)-Arg(NO_2)-OBzl (V)——This compound was prepared from IV (370 mg), Boc–Leu–OH (110 mg), HOBT (60 mg) and WSCI (68 mg) essentially as described for the preparation of II. After 16 h at 0°C, the reaction mixture was extracted with water-saturated BuOH. The extract was washed successively with 1 N citric acid, H_2O, 1 N $NaHCO_3$ and H_2O, and concentrated *in vacuo*. *n*-Hexane was added to the residue and the precipitate formed was filtered off *in vacuo*. The product was recrystallized from hot EtOAc: 283 mg (68%), mp 175—183°C, $[\alpha]_D^{24}$ −23.7° (c=1.0, DMF), Rf^1 0.81, Rf^2 0.92, single ninhydrin-positive spot. *Anal.* Calcd for $C_{50}H_{77}N_{11}O_{14}$: C, 56.86; H, 7.35; N, 14.59. Found: C, 56.55; H, 7.43; N, 14.64.

Boc-Thr-Leu-Thr-Ala-Val-Lys(Z)-Arg(NO_2)-OBzl (VI)——V (264 mg) was treated with TFA (2 ml)–anisole (0.2 ml) as described above. To an ice-chilled solution of the resulting hexapeptide ester trifluoroacetate in DMF (3 ml), Boc–Thr–OH (61 mg), HOBT (37 mg) and WSCI (43 mg) were added, followed by addition of Et_3N (0.05 ml) to keep the solution slightly alkaline. After 16 h at 0°C, the reaction mixture was poured into 1 N $NaHCO_3$ with stirring. The precipitate thus formed was washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O. The product was recrystallized from hot EtOAc: 154 mg (53%), mp 167—176°C, $[\alpha]_D^{26}$ −29.8° (c=1.0, DMF), Rf^1 0.80, Rf^2 0.93, single ninhydrin–positive spot. *Anal.* Calcd for $C_{54}H_{84}N_{12}O_{16}\cdot 2H_2O$: C, 54.35; H, 7.43; N, 14.09. Found: C, 54.64; H, 7.71; N, 13.86.

Boc-Gln-Thr-Leu-Thr-Ala-Val-Lys(Z)-Arg(NO_2)-OBzl (VII)——VI (116 mg) was treated with TFA (1 ml)–anisole (0.1 ml) as described above. Boc–Gln–ONp (47 mg) was added to a solution of this product in DMF (2 ml), followed by Et_3N to keep the solution slightly alkaline. After 24 h at room temperature, the reaction mixture was diluted with 1 N NH_4OH (1 ml) with stirring to saponify the unchanged *p*-nitrophenyl ester. After 1 h, the mixture was extracted with water-saturated BuOH and the extract was washed successively with 1 N NH_4OH, H_2O, 1 N citric acid and H_2O. The solution was evaporated to dryness *in vacuo* and the residue was recrystallized from hot EtOAc: 103 mg (80%), mp 151—163°C, $[\alpha]_D^{26}$ −33.7° (c=1.0, DMF), Rf^1 0.83, Rf^2 0.95, single ninhydrin–positive spot. *Anal.* Calcd for $C_{59}H_{92}N_{14}O_{18}$: C, 55.12; H, 7.21; N, 15.26. Found: C, 55.21; H, 7.41; N, 15.48.

Boc-Tyr-Leu-OMe (VIII)——This compound was prepared from H–Leu–OMe·HCl (1.8 g), Boc–Tyr–OH (3.1 g), HOBT (1.6 g) and WSCI (1.8 g) essentially as described for the preparation of I: 3.4 g (83%), mp 64—66°C, $[\alpha]_D^{26}$ −18.1° (c=1.0, AcOH), Rf^1 0.81, Rf^2 0.90, single ninhydrin–positive spot. *Anal.* Calcd for $C_{21}H_{32}N_2O_6\cdot H_2O$: C, 59.14; H, 8.06; N, 6.58. Found: C, 58.89; H, 8.32; N, 6.25.

Boc-Leu-Thr-Leu-OMe (IX)——This compound was prepared from VIII (2 g), Boc–Leu–OH (1.5 g), HOBT (744 mg) and WSCI (854 mg) essrntially as described for the preparation of II: 1.8 g (69%), mp 76—79°C, $[\alpha]_D^{26}$ −38.5° (c=1.0, DMF), Rf^1 0.84, Rf^2 0.89, single ninhydrin–positive spot. *Anal. Calcd* for $C_{27}H_{43}N_3O_7$: C, 62.16; H, 8.31; N, 8.02. Found: C, 61.87; H, 8.67; N, 7.92.

Boc-Gln-Leu-Tyr-Leu-OMe (X)——This compound was prepared from IX (1 g), Boc–Gln–ONp (941 mg) essentially as described for the preparation of VII. The reaction mixture was extracted with EtOAc and the extract was washed successively with 1 N NH_4OH, H_2O, 1 N citric acid and H_2O, dried over $MgSO_4$ and evaporated *in vacuo*. The residue was recrystallized from hot EtOAc: 1.1 g (85%), mp 203—209°C (dec.), $[\alpha]_D^{26}$ −27.2° (c=1.0, DMF), Rf^1 0.81, Rf^2 0.89, single ninhydrin–positive spot. *Anal.* Calcd for $C_{32}H_{51}N_5O_9\cdot H_2O$: C, 57.55; H, 8.00; N, 10.49. Found: C, 57.64; H, 8.30; N, 10.25.

Boc-Val-Gln-Leu-Tyr-Leu-OMe (XI)——This compound was prepared from X (650 mg), Boc–Val–OH (240 mg), HOBT (149 mg) and WSCI (171 mg) essentially as described for the preparation of II. The reaction mixture was poured into ice-chilled 1 N $NaHCO_3$ with stirring. The precipitate thus obtained was washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O. The product was recrystallized from hot EtOAc: 730 mg (98%), mp 203—211°C (dec.), $[\alpha]_D^{24}$ −26.1° (c=1.0, DMF), Rf^1 0.86, Rf^2 0.91, single ninhydrin–positive spot. *Anal.* Calcd for $C_{37}H_{60}N_6O_{10}$: C, 59.34; H, 8.08; N, 11.22. Found: C, 59.25; H, 8.29; N, 11.45.

Boc-Tyr-Val-Gln-Leu-Tyr-Leu-OMe (XII)——This compound was prepared from XI (500 mg), Boc–Tyr–OH (207 mg), HOBT (99 mg) and WSCI (114 mg) essentially as described for the preparation of II. The reaction mixture was then treated essentially in the same manner as described for XI. The product was recrystallized from hot MeOH: 401 mg (66%), mp 176—185°C, $[\alpha]_D^{24}$ −16.3° (c=1.0, AcOH), Rf^1 0.80,

Rf^2 0.89, single ninhydrin–positive spot. *Anal. Calcd* for $C_{46}H_{69}N_7O_{12}$: C, 57.22; H, 7.63; N, 10.75. Found: C, 57.46; H, 7.79; N, 10.42.

Boc-Val-Tyr-Val-Gln-Leu-Leu-Tyr-Leu-OMe (XIII)——This compound was prepared from XII (365 mg), Boc–Val–OH (96 mg), HOBT (60 mg) and WSCI (68 mg) essentially as described for the preparation of II. The product was recrystallized from MeOH and ether: 298 mg (74%), mp 236—248°C (dec.), $[\alpha]_D^{26}$ −35.8° (c=1.0, DMF), Rf^1 0.88, Rf^2 0.96, single ninhydrin–positive spot. *Anal.* Calcd for $C_{51}H_{78}N_8O_{13}$: C, 60.57; H, 7.78; N, 11.08. Found: C, 60.29; H, 7.89; N, 10.79.

Boc-Val-Tyr-Gln-Leu-Tyr-Leu-NHNH$_2$ (XIV)——XIII (182 mg) was dissolved in MeOH (2 ml)–DMF (1 ml). To this solution, hydrazine hydrate (0.11 ml) was added and the whole was left to stand at room temperature for 48 h. After the removal of MeOH by evaporation, the residue was recrystallized from MeOH and ether: 123 mg (61%), mp 158—164°C, $[\alpha]_D^{26}$ −22.4° (c=1.0, AcOH), *Anal.* Calcd for $C_{50}H_{78}N_{10}O_{12}$: C, 59.39; H, 7.78; N, 13.85. Found: C, 59.16; H, 7.81; N, 13.96.

Boc-Val-Tyr-Val-Gln-Leu-Tyr-Leu-Gln-Thr-Leu-Thr-Ala-Val-Lys(Z)-Arg(NO$_2$)-OBzl (XV)——VII (100 mg) was treated with TFA (1.5 ml)–anisole (0.2 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 (2 ml) containing N-methylmorpholine[24] (0.01 ml). The azide[25] (prepared from 87 mg of Boc–Val–Tyr–Val–Gln–Leu–Tyr–Leu–NHNH$_2$ with 0.023 ml of 6 N HCl in dioxane and 0.012 ml of isoamylnitrite at −60°C) in DMF (2 ml) and N-methylmorpholine (0.01 ml) were added to the above ice-chilled solution and the mixture was stirred for 48 h at 4°C. Then the mixture was poured into 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 $NaHCO_3$, H_2O, 1 N citric acid and H_2O. The product was further purified by column chromatography on silica gel (2.3×31 cm), equilibrated and eluted with $CHCl_3$, MeOH and H_2O (8:3:1). The eluate (4 ml fractions, tube Nos. 14—18) was collected and the solvent was removed by evaporation. Ether was added to the residue to give a precipitate. The product was recrystallized from hot EtOAc: 129 mg (76%), mp 243—248°C (dec.), $[\alpha]_D^{26}$ −26.8° (c=0.3, DMF), Rf^1 0.90, Rf^2 0.97, single ninhydrin–positive spot. *Anal.* Calcd for $C_{106}H_{158}N_{21}O_{28}$: C, 58.55; H, 7.32; N, 13.53. Found: C, 58.84; H, 7.61; N, 13.80.

Boc-Lys(Z)-Asp(OBzl)-OH (XVI)——A mixed anhydride prepared from Boc–Lys(Z)–OH (1.0 g) with N-methylmorpholine[24] (0.22 ml) and ethyl chlorocarbonate[13] (0.21 ml) at −10°C in THF (3 ml) and acetonitrile (3 ml) was added to a cold solution of H-Asp(OBzl)–OH- (446 mg) with N-methylmorpholine (0.22 ml) in DMF (3 ml). The solution was stirred in an ice-bath for 6 h then the mixture was extracted with EtOAc, and washed with 1 N $NaHCO_3$. The aqueous layer was acidified with 1 M citric acid to give a precipitate. The product (a colorless oil) was extracted with EtOAc and washed successively with 1 N citric acid and H_2O. The product was precipitated from EtOAc and *n*-hexane: 689 mg (59%), mp 60—64°C, $[\alpha]_D^{26}$ −10.2° (c=1.0, DMF), Rf^1 0.71, Rf^2 0.76, 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, 61.27; H, 6.98; N, 7.02.

Z-Arg(NO$_2$)-Lys(Z)-Asp(OBzl)-OH (XVII)——XVI (293 mg) was treated with TFA (2 ml)–anisole (0.2 ml) as usual and *n*-hexane was added. The resulting oil was dried over KOH pellets *in vacuo*, and dissolved in DMF (3 ml) containing N-methylmorpholine (0.06 ml). To this ice-chilled solution, the mixed anhydride (prepared from 195 mg of Z–Arg(NO$_2$)–OH with 0.1 ml of ethyl chlorocarbonate and 0.06 ml of N-methylmorpholine at −10°C) in THF (2 ml)–acetonitrile (2 ml) was added. The solution was stirred at 4°C for 6 h, then concentrated, and the residue was diluted with EtOAc. The solution was washed with 1 N citric acid and H_2O, dried over $MgSO_4$ and evaporated to dryness. The residue was reprecipitated from EtOAc and ether: 321 mg (78%), mp 61—63°C, $[\alpha]_D^{26}$ −28.9° (c=1.0, MeOH), *Anal.* Calcd for $C_{39}H_{49}N_8O_{12}$: C, 56.99; H, 6.10; N, 13.64. Found: C, 56.68; H, 6.45; N, 13.82.

Z-Arg(NO$_2$)-Lys(Z)-Asp(OBzl)-Val-Tyr-Val-Gln-Leu-Tyr-Leu-Gln-Thr-Leu-Thr-Ala-Val-Lys(Z)-Arg(NO$_2$)-OBzl (XVIII)——XV (109 mg) was treated with TFA (1 ml)–anisole (0.1 ml) as described above. To an ice-chilled solution of this product in DMF (3 ml), XVII (50 mg), HONB[14] (10 mg) and WSCI (9 mg) were added, followed by N-methylmorpholine to keep the solution slightly alkaline. After 48 h at −10°C. the reaction mixture was poured into 1 N $NaHCO_3$ with stirring. The precipitate thus formed was washed successively with 1 N $NaHCO_3$, H_2O, 1 N HCl and H_2O. The dried product was recrystallized from hot MeOH: 81 mg (56%), mp 255—269°C (dec.), $]\alpha]_D^{26}$ −14.8° (c=0.3, DMF), *Anal.* Calcd for $C_{140}H_{196}N_{29}O_{37}$: C, 58.44; H, 6.87; N, 14.12. Found: C, 58.72; H, 6.73; N, 14.41.

H-Arg-Lys-Asp-Val-Tyr-Val-Gln-Leu-Tyr-Leu-Gln-Thr-Leu-Thr-Ala-Val-Lys-Arg-OH (XIX)——The protected octadecapeptide XVIII (48 mg) was treated with HF (approximately 3 ml) in the presence of anisole–thioanisole–*o*-cresol (1:1:1 v/v, 0.5 ml) in an ice-bath for 1 h. After removal of the HF, dry ether was added and the resulting powder was dissolved in H_2O (5 ml). The solution was treated with Amberlite CG-4B (acetate form, approximately 1 g) for 30 min, filtered by suction, and evaporated to dryness *in vacuo*, then 1 N NH_4OH (2 ml) was added to the residue. The solution was left to stand at 0°C for 30 min, and lyophilized. The crude peptide thus obtained was dissolved in 2% AcOH (2 ml), applied to a column of Sephadex G-25 (2.8×90 cm), and eluted with the same solvent. Fractions of 5 ml were collected per 15 min, and the absorption at 260 nm was determined. Fractions corresponding to the front main peak (tube Nos. 67—72) were combined and lyophilized. The resulting powder was dissolved in a small amount of the upper phase of a solvent system consisting of BuOH–AcOH–H_2O (4:1:5, by volume). The solution was

subjected to partition column chromatography on Sephadex G-25 (2.8×60 cm) previously equilibrated with the lower phase of the above solvent system. The column was developed with the same upper phase, fractions of 4 ml were collected (one fraction per 24 min) and the absorbancy at 260 nm was determined. The fractions corresponding to the main peak (tube Nos. 37—41) were combined and evaporated to dryness *in vacuo*, and then the residue was lyophilized: 18 mg (49%), mp 244—258°C (dec.), $[\alpha]_D^{26}$ −59.1° (c=0.4, 10% AcOH), Rf^1 0.07, Rf^2 0.21, single ninhydrin- and Sakaguchi-positive spot. Amino acid ratios in an acid hydrolysate: Arg 1.86, Lys 1.91, Asp 0.89, Thr 1.80, Glu 1.92, Ala 1.00, Val 3.11, Leu 3.07, Tyr 1.69 (average recovery 81%). Amino acid ratios in an AP-M digest: Arg 1.96, Lys 2.03, Asp 1.11, (Thr+Gln) 3.86, Ala 1.00, Val 3.13, Leu 2.98, Tyr 1.84 (average recovery 80%) (Gln emerged at the same position as Thr and was calculated as Thr).

E-Rosette Formation——A patient's blood was incubated with the synthetic peptide for 1 h at 37°C and then lymphocytes were isolated in a Hypaque-Ficoll gradient[26] for testing of E-rosette formation. Isolated lymphocytes were adjusted to 5×10^5 cells/ml with PBS. Contamination by monocytes and polymorphonuclear cells amount to less than 5%.[27] Sheep erythrocytes (Kyokutō Pharmaceutical Co.) were washed with PBS, and a suspension (1×10^8/ml) was prepared. The lymphocytes were washed with GVB^{2+} and centrifuged for 10 min at 1500 rpm, then suspended in FCS (Dainippon-Pharmaceutical Co.) (1 ml). The suspension was mixed with the suspension of sheep erythrocytes (0.5 ml) and incubated for 16 h at 4°C. The mixture was then centrifuged for 5 min at 900 rpm. Triplicate wet-cell preparations were checked by phase contrast microscopy. For each preparation, 200 lymphocytes were counted, and the proportion binding more than three erythrocytes was determined.

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References and Notes

1) The amino acid residues are of the L-configuration. The abbreviations used to denote amino acid derivatives and peptides are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **11**, 1726 (1972). Other abbreviations: DMF, dimethylformamide; WSCI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; DCC, dicylohexylcarbodiimide; TFA, trifluoroacetic acid; HOBT, N-hydroxybenzotriazole; HONB, N-hydroxy-5-norbornene-2,3-dicarboximide; MA, mixed anhydride; THF, tetrahydrofuran; AcOH, acetic acid; Et_3N, triethylamine; DCHA, dicyclohexylamine; EtOAc, ethyl acetate; FCS, fetal calf serum; E, sheep erythrocytes; HF, hydrogen fluoride; Z, benzyloxycarbonyl; OBzl, benzyloxy; Boc, *t*-butoxycarbonyl; ONp, *p*-nitrophenoxy; Tos, *p*-toluenesulfonic acid; OMe, methoxy; PBS, phosphate-buffered saline; GVB^{2+}, gelatin veronal buffer.
2) D.H. Schlesinger, and G. Goldstein, *Cell*, **5**, 361 (1975).
3) M.E. Weksler, J.B. Innes, and G. Goldstein, *J. Exp. Med.*, **148**, 996 (1978).
4) D.H. Schlesinger, G. Goldstein, M.P. Scheid, and E.A. Boyse, *Cell*, **5**, 367 (1975).
5) G. Goldstein, M.P. Scheid, E.A. Boyse, D.H. Schlesinger, and J.V. Wauwe, *Science*, **204**, 1309 (1979).
6) B.L. Gordon, and J.P. Keeman, *Annals of Allergy*, **35**, 342 (1975).
7) Y. Schuermans, *Lancet*, **1**, 111 (1975).
8) A. Auteri, F. Laghi, A.L. Pasqui, R. Bilenchi, and T. Di Perri, *Boll. Soc. Ital. Biol. Sper.*, **56**, 308 (1980).
9) T. Abiko, M. Kumikawa, and H. Sekino, *Chem. Pharm. Bull.*, **27**, 2233 (1979).
10) T. Abiko, I. Onodera, and H. Sekino, *Chem. Pharm. Bull.*, **28**, 2507 (1980).
11) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).
12) W. König, and R. Geiger, *Chem. Ber.*, **106**, 3626 (1973).
13) R.A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951); T. Wieland, and H. Bernhand, *Ann. Chem.*, **572**, 190 (1951).
14) M. Fujino, S. Kobayashi, M. Obayashi, T. Fukuda, S. Shinagawa, and O. Nishimura, *Chem. Pharm. Bull.*, **22**, 1857 (1974).
15) H. Yajima, K. Akaji, H. Saito, H. Adachi, M. Oishi, and Y. Akazawa, *Chem. Pharm. Bull.*, **27**, 2283 (1979).
16) M. Engelhard, and R.B. Merrifield, *J. Am. Chem. Soc.*, **100**, 3559 (1978).
17) S. Sakakibara, in "*Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*" ed. by B. Weinstein, Marcel Dekker Inc., New York, 1971, Vol. 1, P. 51.
18) M. Fujino, M. Wakimasu, S. Shinagawa, C. Kitada, and H. Yajima, *Chem. Pharm. Bull.*, **26**, 539 (1978).
19) D. Yamashiro, *Nature* (London), **201**, 76 (1964).
20) K. Hofman, F.M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966).
21) M. Fujino, S. Shinagawa, T. Fukuda, M. Takaoki, H. Kawaji, and Y. Sugino, *Chem. Pharm. Bull.*, **25**, 1486 (1977).
22) S.M. Partridge, *Biochem. J.*, **42**, 238 (1948).

23) S.G. Waley, and G. Watson, *Biochem., J.* **55**, 328 (1953).
24) G.W. Anderson, F.M. Callahan, and J.E. Zimmerman, *J. Am. Chem. Soc.*, **89**, 178 (1967).
25) J. Honzl, and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).
26) R. Harris, and E.O. Ukaejiofo, *Brit. J. Haematol.*, **18**, 229 (1970).
27) T. Tachibana, A. Yoshida, and H. Takada, *Igakunoayumi*, **90**, 434 (1974).