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The Effect of Ubiquitin Hexadecapeptide Fragment on E-Rosette Forming Cells of a Uremic Patient[1)]

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A hexadecapeptide, H–Tyr–Asn–Ile–Gln–Lys–Glu–Ser–Thr–Leu–His–Leu–Val–Leu–Arg–Leu–Arg–OH, corresponding to the C-terminal sequence 59—74 of bovine ubiquitin, was synthesized in a conventional manner. The synthetic hexadecapeptide was tested for effect on E-rosette forming cells of a patient in the uremic state. After incubation with amounts of the synthetic hexadecapeptide in the range of 200 to 300 μg/ml of uremic blood, recovery of E-rosette forming ability was observed.

Keywords——uremic patient; ubiquitin; E-rosette forming cells; HOBT-DCC procedure; azide procedure; peripheral blood lymphopenia

Ubiquitin was first isolated from extracts of bovine thymus during the isolation of thymopoietin.[2)] Ubiquitin is a polypeptide hormone of the thymus that consists of a 74 amino acid polypeptide chain of 8451 daltons.[2)] The key role of the thymus in the development and maintenance of cellular immune competence in animals and man is well established. The consequences of thymectomy in animals or the results of failure of the thymus to develop normally in man have shown that this organ has an important influence on immunodeficiency states.[3)] However, ubiquitin was also found in extracts of other mammalian tissues and, after the development of a radioimmunoassay, was shown to be present in extracts of various organisms including yeasts, bacteria and higher plants.[4)] The complete amino acid sequence of bovine ubiquitin was determined by Schlesinger *et al.*[5)] This peptide induces early T- and B-cell differentiation *in vivo* in chicken systems.[4,6)] On the other hand, Goldstein *et al.* reported that ubiquitin was not involved in any thymic hormone activities.[7,8)] However, the synthesis of ubiquitin fragments and the immunological activities of synthetic ubiquitin fragments have not been reported yet. It is known that cell-mediated immunity is impaired in the uremic state.[9–11)] Peripheral blood lymphopenia is well recognized in uremic subjects,[12)] with a reduction in the total numbers both T- and B-cells. The lack of acquired cellular resistance to some infectious organisms is also very likely. Lymphocytes from uremic patients exihibit decreased *in vitro* reactivity to mitogens, and uremic plasma also has an inhibitory influence on the transformation of lymphocytes *in vitro*.[13)]

We describe here the synthesis of a hexadecapeptide, H–Tyr–Asn–Ile–Gln–Lys–Glu–Ser–Thr–Leu–His–Leu–Val–Leu–Arg–Leu–Arg–OH, corresponding to the C-terminal sequence 59—74 of bovine ubiquitin proposed by Schlesinger *et al.*[5)] and report the *in vitro* effects of this hexadecapeptide on the impaired E-rosette forming cells of a uremic patient.

In the present synthesis, protecting groups of amino acid derivatives, Arg(NO_2)–OBzl, Glu(OBzl), Lys(Z) and His(Tos) were removed by hydrogen fluoride treatment.[14)] These protecting groups survive mostly intact during TFA treatment for the removal of the Boc group, employed as a temporary α-amino protecting group. First, the C-terminal undecapeptide, Boc–Glu(OBzl)–Ser–Thr–Leu–His(Tos)–Leu–Val–Leu–Arg(NO_2)–Leu–Arg(NO_2)–OBzl (XI), was synthesized according to Chart 1. The protected pentapeptide ester, Boc–Val–Leu–Arg(NO_2)–Leu–Arg(NO_2)–OBzl (IV), was synthesized stepwise by the HOBT–DCC procedure[15)] starting from H–Arg(NO_2)–OBzl 2 Tos. The protected pentapeptide hydrazide, Boc–Ser–Thr–Leu–His(Tos)–Leu–$NHNH_2$ (IX), was synthesized in a stepwise manner by the HOBT–DCC procedure starting from H–Leu–OBzl Tos. The protected pentapeptide

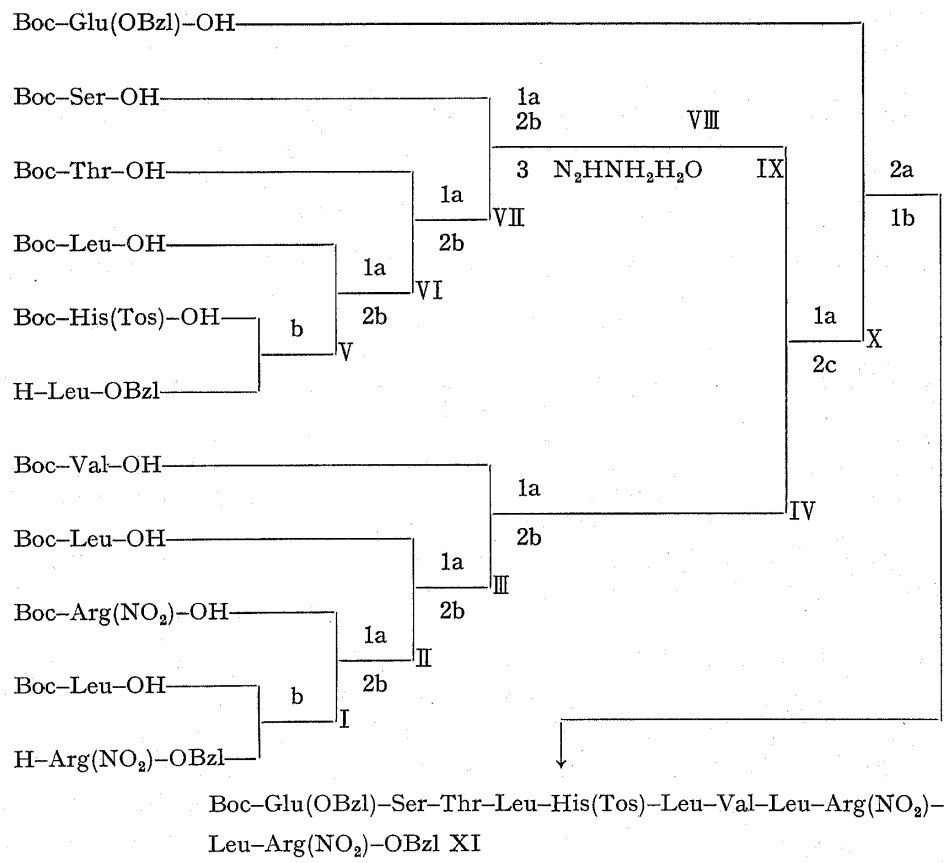

Chart 1. Synthetic Route to the Protected Undecapeptide (positions 64—74)

a: TFA–anisole. b: HOBT–WSCI. c: azide.

ester, Boc–Ser–Thr–Leu–His(Tos)–Leu–OBzl (VII), was therefore converted to the corresponding hydrazide, Boc–Ser–Thr–Leu–His(Tos)–Leu–$NHNH_2$ (IX), which permitted a check on its homogeneity by elemental analysis. After the TFA–anisole treatment of IV, the resulting pentapeptide amine was coupled with IX by the azide procedure[16] to give the protected decapeptide ester, Boc–Ser–Thr–Leu–His(Tos)–Leu–Val–Leu–Arg(NO_2)–Leu–Arg(NO_2)–OBzl (X). X was further purified by silica gel column chromatography using CH_3Cl–MeOH (2: 1) as an eluent. The decapeptide X was treated with TFA–anisole and the product was condensed with Boc–Glu(OBzl)–OH by the HOBT–DCC procedure to give the undecapeptide, Boc–Glu(OBzl)–Ser–Thr–Leu–His(Tos)–Leu–Val–Leu–Arg(NO_2)–Leu–Arg(NO_2)–OBzl (XI). Next, the protected pentapeptide hydrazide, Boc–Tyr–Asn–Ile–Gln–Lys(Z)–$NHNH_2$ (XVI) was synthesized according to Chart 2 in a stepwise manner starting from H–Lys(Z)–OMe HCl. First, Boc–Gln–ONp was incorporated by the active ester procedure[17] and subsequently Boc–Ile–OH was incorporated by the HOBT–DCC procedure to give the protected tripeptide ester, Boc–Ile–Gln–Lys(Z)–OMe (XIII). After the TFA-anisole treatment of XIII, the resulting tripeptide ester was condensed with Boc–Asn–ONp to give the protected tetrapeptide ester, Boc–Asn–Ile–Gln–Lys(Z)–OMe (XIV). Then, the tetrapeptide XIV was treated with TFA-anisole to remove the Boc group of XIV and the resulting free base of XIV was condensed with Boc–Tyr–OH by the HOBT–DCC procedure to give the protected pentapeptide ester, Boc–Tyr–Asn–Ile–Gln–Lys(Z)–OMe (XV). This peptide ester was converted to the corresponding hydrazide, Boc–Tyr–Asn–Ile–Gln–Lys(Z)–$NHNH_2$ (XVI), which permitted us to check its homogeneity by elemental analysis. After the TFA-anisole treatment of XI, the resulting undecapeptide amine was coupled with XVI by the azide procedure[16] to give the protected hexadecapeptide ester XVII, Boc–Tyr–Asn–Ile–Gln–Lys(Z)–Glu(OBzl)–Ser–Thr–Leu–His(Tos)–Leu–Val–Leu–Arg(NO_2)–Leu–Arg(NO_2)–OBzl. XVII was purified by

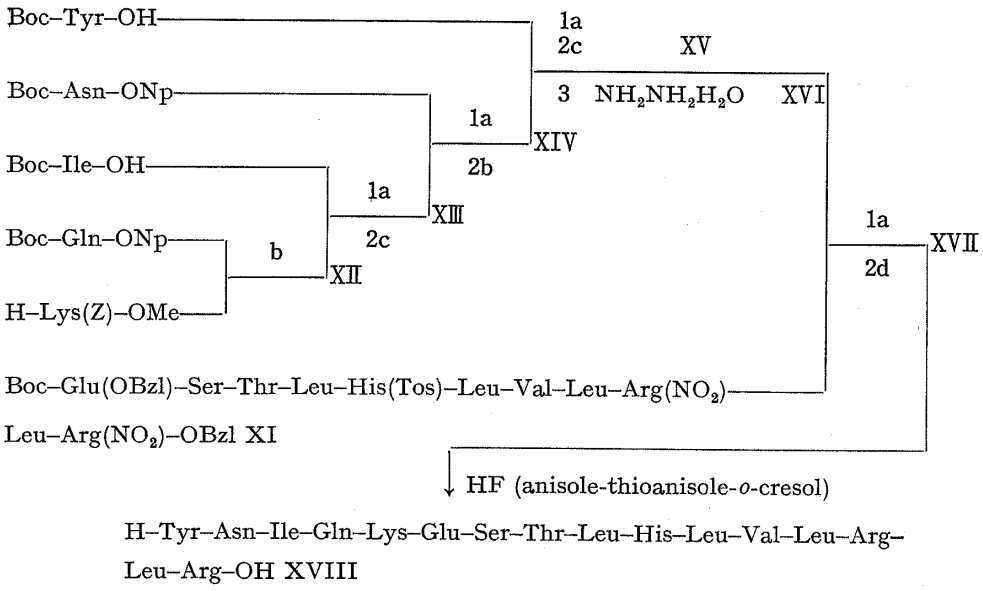

Chart 2. Synthetic Route to the Ubiquitin Fragment (positions 59—74)
a: TFA–anisole. b: active ester. c: HOBT–WSCI. d: azide.

silica gel column chromatography using CH_3Cl–BuOH–DMF (2:1:1) as an eluent. The protected hexadecapeptide ester was then treated with hydrogen fluoride in the presence of anisole–thioanisole–*o*-cresol (1:1:1, V/V),[18] which suppresses side reaction of H–Tyr–OH,[19] to remove all protecting groups. The deblocked peptide, precipitated by dry ether, was converted to the corresponding acetate by Amberlite CG-4B (acetate form) treatment and then treated with 0.5 N NH_4OH for 30 min. The latter treatment was performed because of the reversible N→O shift at the Thr residue during the hydrogen fluoride treatment.[20,21] After that, the product was passed through a carboxy (CM-) celulose column. Analysis of the main fraction by paper chromatography using Partridge's solvent system[22] revealed the presence of one major ninhydrin-, Sakaguchi- and Pauly-positive spot and one minor spot. The crude hexadecapeptide was further purified by gel-filtration on Sephadex G-25 according to Yamashiro.[23] The hexadecapeptide (XVIII) thus obtained was found to be homogeneous by paper chromatography in two different solvent systems. The amino acid hydrolysate and aminopeptidase (AP-M)[24] digest of XVIII gave values in good agreement with the theoretical values. The *in vitro* effect of the synthetic ubiquitin fragment on E-rosette forming cells of a uremic patient are shown in Table I.

Incubation of heparinized blood from a uremic patient in the presence of various amounts of the peptide fragment (200 to 300 μg/ml) resulted in recovery of E-rosette forming ability (Table I). Our data establish that the key residues involved in the active site for agonistic activity against E-rosette forming inhibition by uremic toxin are present within our synthetic hexadecapeptide.

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 hydrolysate were determined with a JEOL JLC-8AH amino acid analyzer (two-column system). Solvents were removed by evaporation *in vacuo* at 35 to 40° 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).[25] Venous blood from a uremic patient and normal subjects was drawn into heparinized syringes and sedimented at room temperature. Aminopeptidase (3501, Aminopeptidase 210520) and angiotensin II (human) were purchased from the Protein Research Foundation, Osaka, Japan.

Boc-Leu-Arg(NO_2)-OBzl (I)——HOBT (743 mg) and WSCI (854 mg) were added to a solution of Boc–Leu–OH (1.3 g), and Et_3N (0.77 ml) in DMF (15 ml) with stirring at 0°C. Then, the mixture was stirred for 12 h at 4°C. The reaction mixture was extracted with EtOAc and 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: 2 g (80%), mp 68—72°C, $[\alpha]_D^{27}$ −30.1° (c=1.0, DMF), Rf^1 0.88, Rf^2 0.93, single ninhydrin–positive spot. *Anal.* Calcd for $C_{24}H_{39}N_5O_5$: C, 60.35; H, 8.23; N, 14.67. Found: C, 60.24; H, 8.56; N, 14.74.

Boc-Arg(NO_2)-Leu-Arg(NO_2)-OBzl (II)——I (1.9 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 *n*-hexane and then dried over KOH pellets *in vacuo*. The product was dissolved in DMF (10 ml) and the solution was neutralized with Et_3N (0.66 ml). To this ice–chilled solution of Boc–Arg(NO_2)–OH (1.4 g) in DMF (10 ml), HOBT (594 mg) and WSCI (683 mg) were added, and the mixture was stirred at 0°C for 16 h. The reaction mixture was extracted with EtOAc and then washed successively with 1 N citric acid, H_2O, 1 N $NaHCO_3$ and H_2O, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was reprecipitated from THF and *n*-hexane: 1.8 g (62%), mp 86—92°C, $[\alpha]_D^{27}$ −25.4° (c=1.0, DMF), Rf^1 0.84, Rf^2 0.91, single ninhydrin-positive spot. *Anal.* Calcd for $C_{30}H_{49}N_{11}O_{10}\cdot H_2O$: C, 48.57; H, 6.93; N, 20.77. Found: C, 48.43; H, 7.23; N, 20.48.

Boc-Leu-Arg(NO_2)-Leu-Arg(NO_2)-OBzl (III)——This compound was prepared from II (2.4 g), HOBT (495 mg), Boc–Leu–OH (880 mg) and WSCI (570 mg) essentially as described for the preparation of II: oily material, 1.9 g (68%), $[\alpha]_D^{27}$ −28.3° (c=1.0, DMF), Rf^1 0.84, Rf^2 0.92, single ninhydrin–positive spot. *Anal.* Calcd for $C_{36}H_{60}N_{12}O_{11}\cdot 2H_2O$: C, 49.53; H, 7.39; N, 19.26. Found: C, 49.36; H, 7.65; N, 19.07.

Boc-Val-Leu-Arg(NO_2)-Leu-Arg(NO_2)-OBzl (IV)——This compound was prepared from III (1.7 g), HOBT (297 mg), Boc–Val–OH (479 mg) and WSCI (342 mg) essentially as described for the preparation of II. The product was reprecipitated from MeOH and ether: 1.2 g (63%), mp 132—137°C, $[\alpha]_D^{27}$ −25.6° (c=1.0, DMF), Rf^1 0.84, Rf^2 0.91, single ninhydrin–positive spot. *Anal.* Calcd for $C_{41}H_{69}N_{13}O_{12}$: C, 52.60; H, 7.43; N, 19.45. Found: C, 52.64; H, 7.49; N, 19.67.

Boc-His(Tos)-Leu-OBzl (V)——This compound was prepared from H–Leu–OBzl Tos (2 g), Boc–His(Tos)–OH (2.2 g), HOBT (743 mg) and WSCI (854 mg) essentially as described for the preparation of I: 2.2 g (71%), mp 61—63°C, $[\alpha]_D^{27}$ −6.4° (c=1.0, DMF), Rf^1 0.87, Rf^2 0.92, single ninhydrin–positive spot. *Anal.* Calcd for $C_{31}H_{40}N_4O_7S$: C, 60.76; H, 6.58; N, 9.15. Found: C, 60.53; H, 6.81; N, 8.89.

Boc-Leu-His(Tos)-Leu-OBzl (VI)——This compound was prepared from V (2 g), Boc–Leu–OH (900 mg), HOBT (495 mg) and WSCI (570 mg) essentially as described for the preparation of II. The product was reprecipitated from EtOAc and *n*-hexane: 2 g (83%), mp 62—64°C, $[\alpha]_D^{27}$ −25.3° (c=1.0, DMF), Rf^1 0.84, Rf^2 0.95, single ninhydrin-positive spot. *Anal.* Calcd for $C_{37}H_{51}N_5O_8S$: C, 61.21; H, 7.08; N, 9.65. Found: C, 61.07; H, 7.24; N, 9.82.

Boc-Thr-Leu-His(Tos)-Leu-OBzl (VII)——This compound was prepared from VI (2.1 g), Boc–Thr–OH (689 mg), HOBT (425 mg) and WSCI (488 mg) essentially as described for the preparation of II. The product was reprecipitated from EtOAc and *n*-hexane: 1.5 g (63%), mp 66—69°C, $[\alpha]_D^{27}$ −19.8° (c=1.0, DMF), Rf^1 0.79, Rf^2 0.82, single ninhydrin-positive spot. *Anal.* Calcd for $C_{41}H_{58}N_6O_{10}S$: C, 59.54; H, 7.07; N, 10.16. Found: C, 59.68; H, 7.16; N, 9.87.

Boc-Ser-Thr-Leu-His(Tos)-Leu-OBzl (VIII)——This compound was prepared from VII (414 mg), Boc–Ser–OH (113 mg), HOBT (75 mg) and WSCI (86 mg) essentially as described for the preparation of II. The product was reprecipitated from EtOAc and *n*-hexane: 332 mg (73%), mp 85—92°C, $[\alpha]_D^{27}$ −70.6° (c=1.0, DMF), Rf^1 0.84, Rf^2 0.86, single ninhydrin-positive spot. *Anal.* Calcd for $C_{44}H_{63}N_7O_{12}S$: C, 57.81; H, 6.95; N, 10.73. Found: C, 56.83; H, 7.24; N, 10.46.

Boc-Ser-Thr-Leu-His(Tos)-Leu-$NHNH_2$ (IX)——VIII (203 mg) in MeOH (3 ml) was treated with 80% hydrazine hydrate (0.2 ml) at 50° overnight. The resulting mass was collected by filtration, washed with MeOH and recrystallized from MeOH and ether: 154 mg (83%), mp 139—146°C, $[\alpha]_D^{27}$ −23.4° (c=1.0, DMF). *Anal.* Calcd for $C_{37}H_{59}N_9O_{11}S$: C, 53.03; H, 7.10; N, 15.05. Found: C, 52.68; H, 7.40; N, 14.81.

Boc-Ser-Thr-Leu-His(Tos)-Leu-Val-Leu-Arg(NO_2)-Leu-Arg(NO_2)-OBzl (X)——IV (234 mg) was treated with TFA (2 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 NMM[26] (0.02 ml). The azide[16] (prepared from 230 mg of IX with 0.02 ml of 6 N HCl in dioxane and 0.02 ml of isoamylnitrite at −60°) in DMF (2 ml) and NMM (0.02 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_4-OAc 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 with $CHCl_3$ and MeOH (2: 1). The desired eluate fractions (4 ml each, tube Nos. 17—21) were collected and the solvent was removed by evaporation. Ether was added to the residue to give a precipitate: 323 mg (79%), mp 211—219°C (dec.), $[\alpha]_D^{27}$ −36.8° (c=1.0, DMF), Rf^1 0.91, Rf^2 0.94, single ninhydrin–positive spot. *Anal.* Calcd for $C_{73}H_{116}N_{20}O_{21}S$: C, 53.40; H, 7.21; N, 17.06. Found: C, 53.10; H, 7.45; N, 16.83.

Boc-Glu(OBzl)-Ser-Thr-Leu-His(Tos)-Leu-Val-Leu-Arg(NO_2)-Leu-Arg(NO_2)-OBzl (XI)——X (164 mg) was treated with TFA (1 ml)–anisole (0.1 ml) as described above. To an ice-chilled solution of this product in DMF (2 ml), Boc–Glu(OBzl)–OH (57 mg), HOBT (16 mg), and WSCI (18 mg) were added, followed by Et_3N to keep the solution slightly alkaline. After 16 h at 0°C, the reaction mixture was poured into ice-chilled 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 dried product was recrystallized from EtOAc: 121 mg (65%), mp 241—250°C (dec.), $[\alpha]_D^{27}$ −46.8° (c=1.0, DMF), *Anal.* Calcd for $C_{85}H_{129}N_{21}O_{24}S$: C, 54.81; H, 6.99; N, 15.81. Found: C, 55.60; H, 7.25; N, 15.48.

Boc-Gln-Lys(Z)-OMe (XII)——Boc–Gln–ONp (941 mg) was added to a solution of H–Lys(Z)–OMe HCl (662 mg) in DMF (5 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 (1.5 ml) with stirring to saponify the unchanged *p*-nitrophenyl ester. After 1 h, the mixture was extracted with EtOAc and washed successively with 1 N NH_4OH, H_2O, 1 N citric acid and H_2O. The solution was dried over $MgSO_4$ and concentrated to a small volume, then petroleum ether was added. The precipitate thereby formed was reprecipitated from THF and *n*-hexane: 685 mg (62%), mp 131—142°C, $[\alpha]_D^{26}$ −14.6° (c=1.0, DMF), Rf^1 0.78, Rf^2 0.81, single ninhydrin-positive spot. *Anal.* Calcd for $C_{25}H_{38}N_4O_8$: C, 57.46; H, 7.33; N, 10.72. Found: C, 57.59; H, 7.46; N, 10.98.

Boc-Ile-Gln-Lys(Z)-OMe (XIII)——This compound was prepared from XII (261 mg), Boc–Ile–OH (128 mg), HOBT (75 mg) and WSCI (86 mg) essentially as described for the preparation of II: 281 mg (91%), mp 66—71°C, $[\alpha]_D^{27}$ −36.4° (c=1.0, DMF), Rf^1 0.75, Rf^2 0.83, single ninhydrin-positive spot. *Anal.* Calcd for $C_{31}H_{49}N_5O_9 \cdot H_2O$: C, 58.38; H, 8.06; N, 10.98. Found: C, 58.09; H, 8.23; N, 10.71.

Boc-Asn-Ile-Gln-Lys(Z)-OMe (XIV)——XIII (318 mg) was treated with TFA (1 ml)–anisole (0.1 ml) as described above. Boc–Asn–ONp (200 mg) was added to a solution of this product in DMF (3 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. After 1 h, the mixture was poured into ice-chilled 1 N NH_4OH with stirring. The precipitate thus formed was washed successively with 1 N NH_4OH, H_2O, 1 N citric acid and H_2O. The precipitate was reprecipitated from MeOH and ether: 245 mg (65%), mp 146—156°C, $[\alpha]_D^{27}$ −41.2° (c=1.0, DMF), Rf^1 0.81, Rf^2 0.89, single ninhydrin-positive spot. *Anal.* Calcd for $C_{35}H_{55}N_7O_{11}$: C, 56.06; H, 7.39; N, 13.08. Found: C, 55.89; H, 7.52; N, 12.86.

Boc-Tyr-Asn-Ile-Gln-Lys(Z)-OMe (XV)——XIV (107 mg) was treated with TFA (1 ml)–anisole (0.1 ml) as described above. To an ice-chilled solution of this product in DMF (2 ml), Boc–Tyr–OH (44 mg), HOBT (22 mg) and WSCI (25 mg) were added, followed by Et_3N to keep the solution slightly alkaline. After 16 h at 4°C, the reaction mixture was poured into 1 N $NaHCO_3$ with stirring. The precipitate thus formed was washed successively with 1N $NaHCO_3$, H_2O, 1 N citric acid and H_2O. The dried product was reprecipitated from MeOH and ether: 123 mg (95%), mp 184—195°C (dec.), $[\alpha]_D^{27}$ −32.8° (c=1.0, DMF), Rf^1 0.82, Rf^2 0.91, single ninhydrin-positive spot. *Anal.* Calcd for $C_{44}H_{64}N_8O_{13}$: C, 57.88; H, 7.07; N, 12.27. Found: C, 58.09; H, 7.14; N, 11.98.

Boc-Tyr-Asn-Ile-Gln-Lys(Z)-$NHNH_2$ (XVI)——(100 mg) was dissolved in MeOH (1 ml)–DMF (3 ml). To this solution, 80% hydrazine hydrate (0.1 ml) was added and the mixture was kept standing at room temperature for 48 h. The MeOH was evaporated off and the residue was poured into ether with stirring. The precipitate thus obtained was filtered off under suction. The product was recrystallized from MeOH: 821 mg (82%), mp 208—210°C (dec.), $[\alpha]_D^{27}$ −30.5° (c=1.0, DMF). *Anal.* Calcd for $C_{43}H_{64}N_{10}O_{12}$: C, 56.56; H, 7.07; N, 15.34. Found: C, 56.92; H, 7.34; N, 15.45.

Boc-Tyr-Asn-Ile-Gln-Lys(Z)-Glu(OBzl)-Ser-Thr-Leu-His(Tos)-Leu-Val-Leu-Arg(NO_2)-Leu-Arg(NO_2)-OBzl (XVII)——XI (93 mg) was treated with TFA (1 ml)-anisole (0.1 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 (1 ml) containing NMM[26] (0.02 ml). The azide[16] (prepared from 69 mg of XVI with 0.01 ml of 6 N HCl in dioxane and 0.01 ml of isoamylnitrite at −60°) in DMF (1 ml) and NMM (0.03 ml) were added to the above ice-chilled solution and the mixture was stirred for 48 h at 4°C then poured into ice-chilled 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 (2.1×36 cm), equilibrated and eluted with a mixture of $CHCl_3$, BuOH and DMF (2:1:1). The desired eluate fractions (4 ml each, tube Nos. 21—25) were collected and the solvent was removed by evaporation. Ether was added to the residue to give a precipitate. The product was recrystallized from EtOAc: 72 mg (55%), mp 238—249°C (dec.), $[\alpha]_D^{27}$ −32.1° (c=1.0, DMF), Rf^1 0.90, Rf^2 0.91, single ninhydrin-positive spot. *Anal.* Calcd for $C_{123}H_{181}N_{29}O_{34}S$: C, 55.91; H, 6.91; N, 15.38. Found: C, 56.23; H, 7.28; N, 15.47.

H-Tyr-Asn-Ile-Gln-Lys-Glu-Ser-Thr-Leu-His-Leu-Val-Leu-Arg-Leu-Arg-OH (XVIII)——The protected hexadecapeptide (45 mg) was treated with HF (approximately 3 ml) in the presence of anisole–thioanisole–*o*-cresole (1:1:1 v/v, 0.5 ml) in an ice-bath for 1 h. After removal of the HF, dry ether was added to the residue 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, evaporated to dryness *in vacuo*, and then mixed with 0.5 N NH_4OH (2 ml). The solution was left to stand at 0°C for 30 min, then lyophilized. The aqueous solution (2 ml) of this product was applied to a CM-cellulose column (2.1×12 cm) which was

eluted with a linear gradient from H_2O (200 ml) in the mixing chamber to 0.15 M NH_4OAc (pH 6.51, 200 ml) in the reservoir. Fractions of 5 ml each were collected at a flow rate of 1 ml/min with an automatic fraction collector, and the absorbancy of each fraction was determined at 260 nm. The eluates in tube Nos. 48 to 54 containing the hexadecapeptide were pooled, evaporated to dryness *in vacuo* and lyophilized. Analysis by paper chromatography using Partridge's solvent system revealed the presence of two ninhydrin-, Sakaguchi- and Pauly-positive spots with Rf^1 0.09 (major) and 0.21 (minor). The crude peptide thus obtained was dissolved in a small amount of the upper phase of the solvent system consisting of BuOH–AcOH–H_2O (4: 1: 5, volume). The solution was subjected to partition coumn chromatography on Sephadex G-25 (2.8 × 60 cm) previously equilibrated with the lower phase of the above solvent system. Elution was carried out with the upper phase. Individual fractions (5 ml each) were collected and the absorption at 260 nm was determined. The fractions corresponding to the main peak (tube Nos. 35—40) were combined and evaporated to dryness *in vacuo*, then the residue was lyophilized: 12 mg (35%), mp 248—255°C (dec.), $[\alpha]_D^{27}$ −79.4° (c=0.3, 1 N AcOH), Rf^1 0.10, Rf^2 0.21, single ninhydrin-positive spot. Amino acid composition of the acid hydrolysate: Asp 0.84, Glu 1.80, Leu 3.62, Ile 1.13, Val 1.00, Tyr 0.81, Thr 0.80, Ser 0.79, His 0.84, Lys 0.86, Arg 1.78 (average recovery 80%). Amino acid composition of the AP–M digest: Glu 0.85, Leu 4.01, Ile 1.06, Val 1.00, Tyr 0.91, Thr 0.87, Ser 0.83, Asn 0.91, Gln 0.81, His 0.90, Lys 1.11, Arg 1.82 (average recovery 83%).

E-Rosette Formation——A 10 ml aliquot of venous blood was drawn into a suringe containing 1000 U of heparin and incubated with the synthetic peptide for 1 h at 37°C, then lymphocytes were isolated in a Hypaque-Ficoll gradient[27] for E-rosette formation. The isolated lymphocytes were adjusted to 5×10^5 cells/ml with PBS. Contamination by monocytes and polymorphonuclear cells amounted to less than 6%.[28] Sheep erythrocytes (Kyokutō Pharmaceutical Co.) were washed with PBS, and a suspension (6×10^6/ml) was prepared. Next, lymphocytes were washed with GVB^{2+} and centrifuged for 10 min at 1500 rpm, then suspended in GVB^{2+} (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. An E-rosette forming cell was defined as one surrounded by at least three sheep erythrocytes. For each preparation, 200 lymphocytes were counted (Table I).

Table I. Effect of the Synthetic Ubiquitin Fragment on the Impaired E-Rosette Forming Cells from a Uremic Patient

Peptides	Dose (μg/ml)	E-Rosette forming cells (%)
—[a]		68±4
—[b]		42±3
Angiotensin II (human)[b,c,d]	200	41±5
	300	43±3
	500	42±4
H–Tyr–Asn–Ile–Gln–Lys–Glu–Ser–Thr–Leu–His–Leu–Val–Leu–Arg–Leu–Arg–OH (positions 59—74)[b,d]	200	54±4
	300	63±3

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

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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, dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; HOBT, N-hydroxybenzotriazole; AcOH, acetic acid; Et_3N, triethylamine; EtOAc, ethyl acetate; HF, hydrogen fluoride; Boc, *t*-butoxycarbonyl; ONp, *p*-nitrophenyl ester; Tos, *p*-toluenesulfonic acid; OMe, methyl ester; OBzl, benzyl ester; Z, benzyloxycarbonyl; PBS, phosphate-buffered saline; GVB^{2+}, gelatin veronal buffer; NMM, N-methylmorpholine; THF, tetrahydrofuran; E-rosette, a rosette with sheep erythrocytes.

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