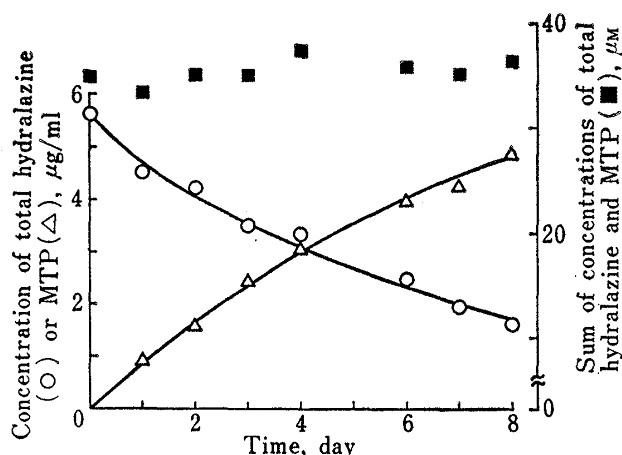

Fig. 3. Time Course of Concentrations of Total Hydralazine (Hydralazine plus its Pyruvate Hydrazone) and MTP in the Presence of a Physiological Concentration Ratio of Pyruvic Acid to Hydralazine (22: 1) at pH 7.4 and 37°

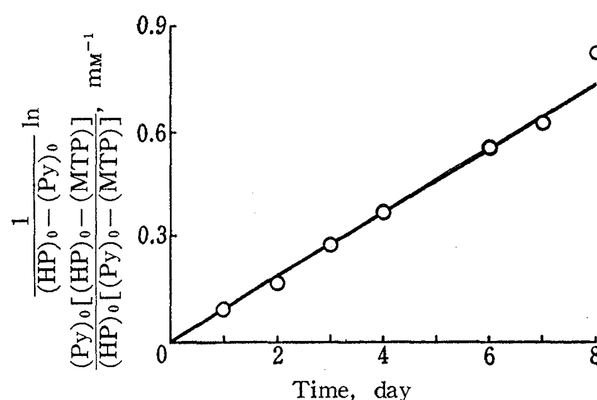

Fig. 4. Second-Order Plot for the Formation of 3-Methyl-*s*-triazolo[3,4-*a*]phthalazine (MTP) from Hydralazine (HP) in the Presence of a Physiological Concentration Ratio of Pyruvic Acid (Py) at pH 7.4 and 37°

tion in the blood. Because the formation of MTP is a second-order reaction, the apparent reaction rate is affected by the concentrations of both hydralazine and pyruvic acid. Thus, it is necessary to study the reaction with drug levels of the same order as that in blood.

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The Effect of Thymosin α_1 Fragments on T-Lymphocyte Transformation in the Uremic State[1]

TAKASHI ABIKO, HIROSHI SEKINO,[2a] and HIROKI HIGUCHI[2b]

Kidney Center, Sendai Insurance Hospital[2a] *and Kidney Research Laboratory, Kōjinkai*[2b]

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The thymosin α_1 C-terminal fragment H-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-OH (positions 14—28) was synthesized by a conventional method. Two thymosin α_1 fragments, H-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-OH (positions 19—28) and the pentadecapeptide fragment synthesized in this study, were tested for effect on lymphocyte transformation in the uremic state. The synthetic pentadecapeptide increased ^{3}H-thymidine incorporation into DNA in the uremic state, but the synthetic decapeptide had no effect on the ^{3}H-thymidine incorporation.

Keywords——thymosin α_1 fragment; uremic serum; chronic renal failure; HONB-DCC; lymphocytes transformation

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1) Abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **11**, 1726 (1972). Other abbreviations: DMF, dimethylformamide; PHA, phytohemagglutinin; WSCI, water-soluble carbodiimide; MA, mixed anhydride; TFA, trifluoroacetic acid; HONB, N-hydroxy-5-norbornene-2,3-dicarboximide; HOBT, N-hydroxybenzotriazole; DCC, dicycloxylcarbodiimide.

2) Location: *a*) *Tsutsumimachi 3-16-1, Sendai, 980, Japan*; *b*) *Higashi-shichibanchō 84, Sendai, 980, Japan.*

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Goldstein *et al.*[3] prepared a biologically active heat-stable polypeptide, named thymosin, from bavine thymus. Thymosin α_1, an immunologically active polypeptide, highly acidic with an isoelectric point of 4.2. This molecule is composed of 28 amino acid residues with acetylserine as the NH_2 terminus.[4] Thymosin α_1 is a potent immunopotentiating agent. It is from 10 to 1000 times as active as the parent thymosin fraction 5 preparation in a number of bioassay systems designed to measure the maturation and function of T-lymphocytes.[4] Partially purified bovine thymosin preparations, when administered to neonatally thymectomized mice, have been shown to slow the development of wasting disease,[5] and to increase the incidence of cell-mediated immune responses.

On the other hand, the transformation of the T-lymphocytes into lymphoblasts with mitotic activity after antigen stimulation is known to be depressed in chronic renal failure.[6] The resulting loss of cellular immunity is demonstrable by measurement of ^{3}H-thymidine uptake in lymphocyte cultures after PHA stimulation. Addition of uremic serum to the cultures decreases metabolic DNA synthesis as well as impairing cell viability.[7] The authors have synthesized a C-terminal thymosin α_1 fragment (positions 14—28) by a conventional method in order to examine its effect on lymphocyte transformation in the uremic state. The synthesis of the C-terminal decapeptide (positions 19—28) was described in the preceding paper,[8] and the synthetic route to the pentadecapeptide (positions 14—28) is illustrated in Fig. 1.

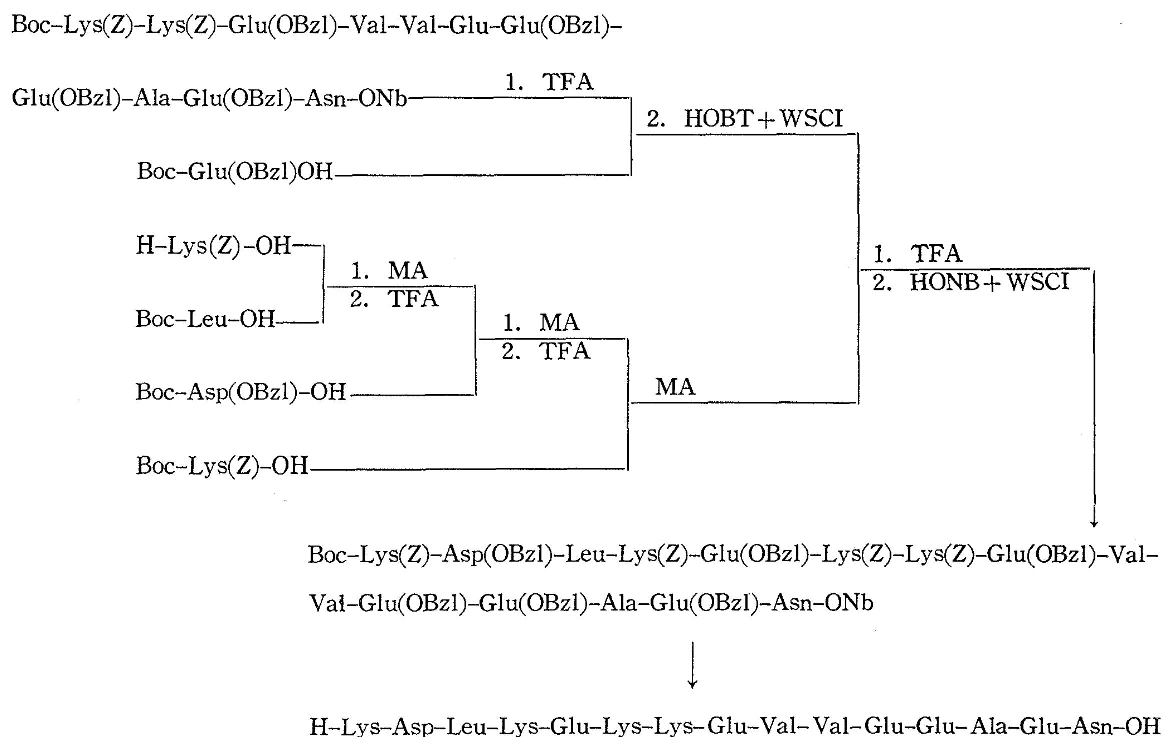

Fig. 1. Synthetic Scheme for the Thymosin α_1 Fragment (Residues 14—18)

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Boc–Lys(Z)–Lys(Z)–Glu(OBzl)–Val–Val–Glu(OBzl)–Glu(OBzl)–Ala–Glu(OBzl)–Asn–ONb[8] was treated with TFA in the presence of anisole to remove the Boc group and the resulting product was condensed with Boc–Glu(OBzl)–OH by the HOBT–DCC procedure[9] to give Boc–Glu(OBzl)–Lys(Z)–Lys(Z)–Glu(OBzl)–Val–Val–Glu(OBzl)–Glu(OBzl)–Ala–Glu(OBzl)–Asn–ONb (I). H–Lys(Z)–OH was condensed with Boc–Leu–OH by the MA procedure[10] to give Boc–Leu–Lys(Z)–OH (II), which, after conversion to the de-Boc peptide, was further condensed with Boc–Asp(OBzl)–OH by the MA procedure to give Boc–Asp(OBzl)–Leu–Lys(Z)–OH (III). The tripeptide III was treated with TFA and the product was also condensed with Boc–Lys(Z)–OH by the MA procedure to give Boc–Lys(Z)–Asp(OBzl)–Leu–Lys-(Z)–OH (IV). The undecapeptide I was treated with TFA and the product was condensed with IV by the HONB–DCC procedure to minimize undesirable racemization[11] to provide Boc–Lys(Z)–Asp(OBzl)–Leu–Lys(Z)–Glu(OBzl)–Lys(Z)–Lys(Z)–Glu(OBzl)–Val–Val–Glu-(OBzl)–Ala–Glu(OBzl)–Asn–ONb (V). N–Methyl-2-pyrrolidone had to be used as a solvent, because of the poor solubility of the amino component in DMF. After removal of the Boc group of V with TFA, the resulting pentadecaptide ester was hydrogenated over 10% Pd–C in aqueous AcOH for 18 hr. The hydrogenated product was purified by partition column chromatography on Sephadex G-25 according to Yamashiro.[12] A solvent system consisting of BuOH, AcOH, and H_2O (4: 1: 5) was used to elute the desired compound. The absorbancy (230 nm) due to the peptide bond was used as a guide in this chromatogrophic purification. Homogeneity of the synthetic pentadecapeptide thus obtained was confirmed by paper chromatography and amino acid analysis. The 5.5 *N* HCl hydrolysate contained the constituent amino acids in the ratios predicted by theory. In addition, complete digestion of the synthetic pentadecapeptide was achieved by aminopeptidase M (AP-M).[13] Serum from a uremic patient with chronic renal failure was found to inhibit markedly lymphocyte transformation by PHA (Table I). After incubation with amounts of the pentadecapeptide ranging from 100 to 200 μg/ml of cell culture, the amount of ^{3}H-thymidine incorporation into DNA was increased (Table I). The decapeptide has no effect on the lymphocyte transformation-inhibiting activity of uremic serum at a dose of 200 μg/ml. These results suggest that the key residues involved

Table I. Effect of Thymosin α_1 Fragments on the Inhibition of T-Lymphocyte Transformation by Uremic Serum

Peptide	Dose (μg/ml)	^{3}H-Thymidine incorporation (cpm)
—[a,b]		310±30
—[b,c]		34981±3461
—[c,d]		12658±3645
H–Lys–Lys–Glu–Val–Val–Glu–Glu–Ala–	100	12489±4136
Glu–Asn–OH[c,d]	200	12045±2948
H–Lys–Asp–Leu–Lys–Glu–Lys–Lys–Glu–	100	24664±4009
Val–Val–Glu–Glu–Ala–Glu–Asn–OH[c,d]	200	29982±3627

a) PHA (−).
b) Lymphocytes were incubated with normal serum (0.02 ml) for three days at 37°.
c) PHA (+).
d) Lymphocytes were incubated with uremic serum (0.02 ml) for three days at 37°.

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in the active site of thymosin α_1 for agonistic activity towards lymphocyte transformation inhibition by uremic serum are present within our synthetic pentadecapeptide.

Experimental

Melting points are uncorrected. Rotations were determined in a Atago Polax (cell length: 10 cm). The amino acid compositions of the acid and enzymatic hydrolysates were determined with a JEOL JLC-8AH amino acid analyzer. Solvents were removed by evaporation *in vacuo* at a bath temperature of 40 to 50° in a rotary evaporator. 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[14] and Rf^2 values refer to BuOH–pyridine–AcOH–H_2O (30: 20: 6: 24).[15] Uremic blood and T-lymphocytes were obtained from a uremic patient suffering from terminal chronic renal failure. The blood was centrifuged and the separated serum was stored at −20° until use. Control serum was obtained from a healthy person. AP-M was purchased from the Protein Research Foundation (Osaka).

Boc-Glu(OBzl)-Lys(Z)-Lys(Z)-Glu(OBzl)-Val-Val-Glu(OBzl)-Glu(OBzl)-Ala-Glu(OBzl)-Asn-ONb (I)——Boc–Lys(Z)–Lys(Z)–Glu(OBzl)–Val–Val–Glu(OBzl)–Glu(OBzl)–Ala–Glu(OBzl)–Asn–ONb[8] (204 mg) was treated with TFA (1.0 ml) in the presence of anisole (0.1 ml) at room temperature for 30 min, and the TFA salt precipitated by addition of dry ether was collected by filtration and dried over KOH pellets *in vacuo*. The powder was dissolved in ice-chilled DMF (3.0 ml) together with triethylamine (0.02 ml), HOBT (15 mg) and Boc–Glu(OBzl)–OH (37 mg). After addition of WSCI (18 mg), the mixture was stirred at 0° for 18 hr and poured into 1 N citric acid with vigorous stirring. The resulting precipitate was washed batchwise with 1 N citric acid, H_2O, 1 N $NaHCO_3$ and H_2O and then recrystallized from hot MeOH; yield 152 mg (71%), mp 198—205°, $[\alpha]_D^{26}$ −21.0° (c=1.0, DMF), Rf^1 0.83, Rf^2 0.91, single ninhydrin-positive spot. *Anal.* Calcd for $C_{117}H_{145}N_{15}O_{31}$: C, 62.25; H, 6.48; N, 9.31. Found: C, 62.14; H, 6.66; N, 9.48.

Boc-Leu-Lys(Z)-OH (II)——A mixed anhydride prepared from Boc–Leu–OH (1.3 g) with N-methylmorpholine (0.54 ml) and ethylchlorocarbonate (0.51 ml) at −10° in tetrahydrofuran (THF) (5 ml) and acetonitrile (5 ml) was added to a cold solution of H–Lys(Z)–OH (1.3 g) in DMF (5 ml). The solution was stirred in an ice-bath for 6 hr, then the solvent was removed and the residue was dissolved in EtOAc. The solution was washed successively with 1 N citric acid and H_2O, dried over $MgSO_4$ and concentrated. The residue was reprecipitated from EtOAc and n-hexane; yield 1.3 g (52%), mp 51—53°, $[\alpha]_D^{26}$ −50.0° (c=1.0, DMF), Rf^1 0.68, Rf^2 0.85, single ninhydrin-positive spot. *Anal.* Calcd for $C_{25}H_{39}N_3O_7H_2O$: C, 60.58; H, 8.34; N, 8.49. Found: C, 60.21; H, 8.65; N, 8.10.

Boc-Asp(OBzl)-Leu-Lys(Z)-OH (III)——The above protected dipeptide (993 mg) was treated with TFA (4 ml) in the presence of anisole (0.5 ml) as usual and n-hexane was added. The resulting oil was dried over KOH pellets *in vacuo*, and dissolved in DMF (5 ml) containing N-methylmorphiline (0.2 ml). To this ice-chilled solution, the mixed anhydride (prepared from 647 mg of Boc–Asp(OBzl)–OH with 0.21 ml of ethylchlorocarbonate and 0.2 ml of N-methylmorpholine at −10°) in THF (5 ml) and acetonitrile (5 ml) was added. The solution was stirred at 4° for 6 hr, then concentrated, and the residue was diluted with EtOAc. The solution was washed as described above and then precipitated from EtOAc and n-hexane; yield 1.0 g (71%), mp 48—56°, $[\alpha]_D^{26}$ −17.0° (c=1.0, DMF), Rf^1 0.74, Rf^2 0.84, single ninhydrin-positive spot. *Anal.* Calcd for $C_{36}H_{50}N_4O_{10}$: C, 61.88; H, 7.21; N, 8.02. Found: C, 62.21; H, 7.52; N, 7.93.

Boc-Lys(Z)-Asp(OBzl)-Leu-Lys(Z)-OH (IV)——Compound III (349 mg) was treated with TFA (1 ml)-anisole (0.1 ml) in the usual manner and the deprotected peptide was dissolved in DMF (1 ml) containing N-methylmorpholine (0.05 ml). To this ice-chilled solution, the mixed anhydride (prepared from 281 mg of Boc–Lys(Z)–OH with 0.1 ml of ethylchlorocarbonate and 0.05 ml of N-methylmorpholine at −10°) in THF (2 ml) and acetonitrile (2 ml) was added. The solution was stirred at 4° for 6 hr, then concentrated, and the residue was diluted with EtOAc. The solution was washed as described above and then precipitated from EtOAc and n-hexane; yield 291 mg (61%), mp 47—51°, $[\alpha]_D^{26}$ −24.5° (c=1.0, DMF), Rf^1 0.84, Rf^2 0.89, single ninhydrin-positive spot. *Anal.* Calcd for $C_{50}H_{68}N_6O_{13}H_2O$: C, 61.33; H, 7.21; N, 8.58. Found: C, 61.54; H, 7.52; N, 9.01.

Boc-Lys(Z)-Asp(OBzl)-Leu-Lys(Z)-Glu(OBzl)-Lys(Z)-Lys(Z)-Glu(OBzl)-Val-Val-Glu(OBzl)-Glu(OBzl)-Ala-Glu(OBzl)-Asn-ONb (V)——I (90 mg) was treated with TFA (1 ml)-anisole (0.1 ml) as described above. IV (45 mg), HONB (8 mg)[11] and WSCI (7 ml) were added to an ice-chilled solution of the resulting undecapeptide ester trifluoroacetate in N-methyl-2-pyrrolidone (2 ml), followed by addition of N-methyl-morpholine[16] to keep the solution slightly alkaline. After 36 hr at 0°, 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 precipitate was recrystallized from hot MeOH; yield 76 mg (61%), mp 181—195°, $[\alpha]_D^{26}$ −37.3°

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(c=0.2, DMF), Rf^1 0.88, Rf^2 0.92, single ninhydrin-positive spot. *Anal.* Calcd for $C_{162}H_{203}N_{21}O_{41}$: C, 62.75; H, 6.60; N, 9.49. Found: C, 62.89; H, 6.69; N, 9.21.

H-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-OH (VI)——The protected pentadecapeptide V (50 mg) was treated with TFA (1 ml)-anisole (0.1 ml) at room temperature for 40 min, then dry ether was added. The resulting powder was washed with ether and dried over KOH pellets *in vacuo*. The de-Boc peptide ester was hydrogenated in 60% AcOH (15 ml) over 10% Pd-C for 20 hr. The catalyst was removed with the aid of cellite. The filtrate was evaporated to dryness and the residue was dried over KOH pellets *in vacuo*. The hydrogenated product was dissolved in a small amount of the upper phase of BuOH–AcOH–H_2O (4:1:5). The solution was applied to a column of Sephadex G-25 (2.6×96 cm) previously equilibrated with lower phase of the above solvent system. The column was developed with the same upper phase. Individual fractions (5 ml each) were collected and the absorbancy at 230 nm was determined. The fractions corresponding to the main peak (tube No. 78—91) were combined. The solvent was removed by evaporation and the residue was lyophilized from H_2O to give a fluffy white powder; yield 13 mg (59%), mp 248—255° (dec.), $[\alpha]_D^{26}$ −42.1° (c=0.1, 10% AcOH), Rf^1 0.06, Rf^2 0.15, single ninhydrin-positive spot. Amino acid ratios in the acid hydrolysate: Glu 4.51, Asp 1.69, Val 2.02, Ala 1.03, Leu 0.94, Lys 3.54 (average recovery 84%). Amino acid ratios in the AP-M digest: Glu 4.58, Asp 0.93, Val 2.01, Ala 1.01, Leu 0.89, Asn 0.88, Lys 3.56 (average recovery 86%).

Effect of Thymosin α_1 Fragments on the Inhibition of T-Lymphocyte Transformation by Uremic Serum——Peripheral blood lymphocytes were isolated in a Hypaque-Ficoll gradient[17] for T-cell transformation. The cells were cultured in 0.2 ml of RPMI-1640 containing 0.02 ml of uremic serum in microtiter plates (Falcon—3040) and 0.02 ml (final 1 μg/ml) of PHA was added, with 0.02 ml (100—200 μg/ml) of thymosin α_1 fragment. Triplicate cultures of each combination of 5×10^5 cells per well were incubated at 37° in a humidified atmosphere of 5% CO_2 in air for three days. Twenty-four hr before harvest, 1 μCi/ml of ^{3}H-thymidine was added per culture. The amount of thymidine incorporated into DNA was measured in a scintillator.

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Modification of Cytosine Moieties of Nucleic Acids with Hydrogen Sulfide (Nucleosides and Nucleotides. XXXIV)[1]

Kazunobu Miura and Tohru Ueda

Faculty of Pharmaceutical Sciences, Hokkaido University[2]

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The chemical introduction of 4-thiouridine(S^4U) residues into nucleic acids was carried out by the reaction of nucleic acids with liquid hydrogen sulfide in aqueous pyridine, which caused the conversion of cytosine moieties to 4-thiouracil moieties.

Keywords——chemical modification; sulfhydrolysis; hydrogen sulfide; 4-thiouridine; nucleic acids; yeast RNA; yeast tRNA; calf thymus DNA; ultraviolet absorption spectrum

It is important to investigate the chemical and physical properties of oligo- and polynucleotides containing 4-thiouridine (S^4U) in order to obtain information on the function of the S^4U residue in tRNA. Therefore, it seems desirable to be able to synthesize oligonucleotides containing S^4U by a simple procedure.

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2) Location: *Kita-12, Nishi-6, Kita-ku, Sapporo, 060, Japan.*

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