Synthesis of Rat Parathymosin α Fragment 1—28 and Examination of Its Inhibitory Activity towards the Restoring Activity of Thymosin α_1 on the Impaired T-Lymphocytes of Uremic Patients[1)]

Takashi ABIKO* and Hiroshi SEKINO

Kidney Research Laboratory, Kojinkai, 1-6 Tsutsujigaoka 2-chome, Miyagino-ku, Sendai 980, Japan. *Received May 8, 1991*

A fragment corresponding to *N*-terminal octaeicosapeptide of rat parathymosin α was synthesized by assembling 5 peptide fragments, followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole (molar ratio 1 : 1) in trifluoroacetic acid in the presence of dimethylselenium. Incubation of impaired T-lymphocytes isolated from uremic patients with the synthetic parathymosin α fragment 1—28 showed no immunological restoring effect, but when it was administered together with thymosin α_1, it appeared to suppress the restoring effect of the thymosin α_1 on the impaired T-lymphocytes of uremic patients.

Keywords parathymosin α fragment 1—28 synthesis; trifluoromethanesulfonic acid deprotection; impaired T-lymphocyte blastogenic response; restoring effect; restoring effect suppression

Cellular immunity is known to be impaired in uremic patients. This impairment has been implicated in a high susceptibility to infections and an increased incidence of malignancy. The thymus plays an essential role in the development and maintenance of cellular immune competence.

Thymosin α_1, a component of the thymus-derived polypeptide mixture thymosin fraction 5, has been shown to exhibit various activity *in vitro* immunological assays.[2,3)] *In vivo*, thymosin α_1 seems to play an important regulatory role in the late stages of T-lymphocyte differentiation.[2,4)]

In previous papers,[5-8)] we reported the syntheses of deacetylthymosin α_1, thymosin α_1 and its fragments, and showed that synthetic thymosin α_1 and some of its C-terminal fragments could have a restoring effect on impaired cell-mediated immunological functions.

	1				5					10
parathymosin α	Ac–	Ser–	Lys–	Ser–	Glu–	Val–	Glu–	Ala–	Ala–	Ala–	Glu–
prothymosin α	Ac–	Ser–	Asp–	Ala–	Ala–	Val–	Asp–	Thr–	Ser–	Ser–	Glu–
	11				15					20	
parathymosin α	Leu–	Ser–	Ala–	Lys–	Asp–	Leu–	Lys–	Glu–	Lys–	Lys–	
prothymosin α	Ile–	Thr–	Thr–	Lys–	Asp–	Leu–	Lys–	Glu–	Lys–	Lys–	
	21				25						
parathymosin α	Asp–	Lys–	Val–	Glu–	Glu–	Lys–	Ala–	Gly–			
prothymosin α	Glu–	Val–	Val–	Glu–	Glu–	Ala–	Glu–	Asn–			

Fig. 1. Comparison of the N-Terminal Amino Acid Sequences of Prothymosin α and Parathymosin α

The positions of identity are underlined.

In 1985, Haritos *et al.*[9)] reported that a peptide parathymosin α, containing about 105 amino acid residues, had been isolated from the rat thymus, and the sequence of the first 30 residues at the N-terminus had been determined. In this region, a 43% structural identity is shown between thymosin α_1 and parathymosin α.

Preliminary assay results suggest that parathymosin α may act to modulate the immunoenhancing activity exhibited by prothymosin α.

In our preceding paper,[6)] we reported evidence of impaired immune function in patients with chronic uremia. We also reported[5-8)] that the synthetic thymosin α_1 and some of its fragments show a restoring effect on impaired cell-mediated immunological functions.

These results prompted us to synthesize the N-terminal peptide fragment corresponding to amino acids 1—28 of parathymosin α. This paper deals with the synthesis of parathymosin α fragment 1—28, with an examination of the immunological effect of this peptide and our synthetic thymosin α_1[7)] on the impaired blastogenic response of T-lymphocytes of uremic patients. From a synthetic viewpoint, compared with our previous syntheses of deacetylthymosin α_1,[8)] thymosin α_1[7)] and its fragments,[5-7)] the thioanisole-mediated trifluoromethanesulfonic acid (TFMSA) deprotection procedure[10,11)] was applied in the final step of the present synthesis instead of catalytic hydrogenation or hydrogen fluoride.

According to the scheme shown in Fig. 2, the oc-

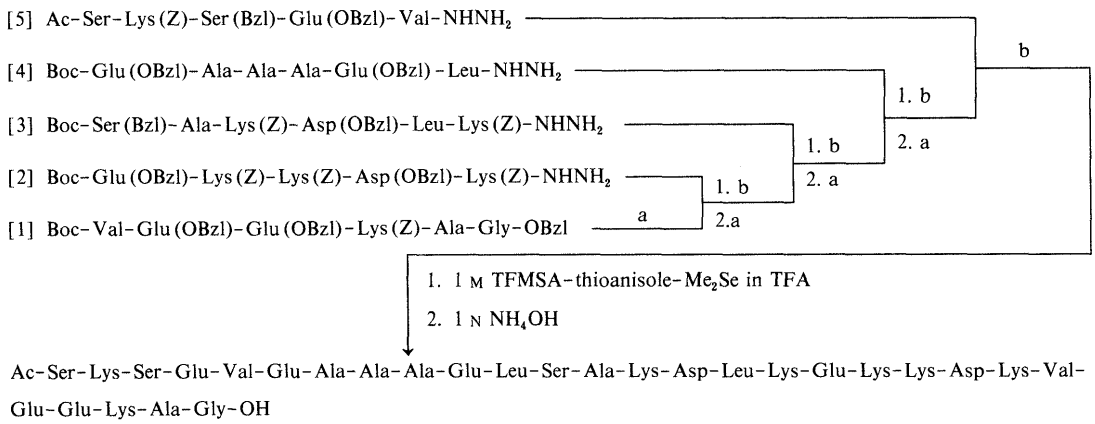

Fig. 2. Synthetic Route to Parathymosin α Fragment 1—28

a, TFA–anisole; b, azide.

taeicosapeptide corresponding to sequence 1—28 of parathymosin α was synthesized. Amino acid derivatives bearing protecting groups removable by 1 M TFMSA–thioanisole in TFA[10] were employed, *i.e.*, Lys(Z), Ser(Bzl), Asp(OBzl), Glu(OBzl) and Gly–OBzl. These protecting groups survive mostly intact under careful TFA treatment for removal of Boc or Z(OMe) group, employed as a temporary α-amino protecting group. Five protected intermediate peptide fragments, Ac–(1—5)–NHNH–Troc [XI], Boc–(6—11)–NHNH–Troc [XV], Boc–(12—17)–NHNH–Troc [X], Boc–(18—22)–NHNH–Troc [VIII] and Boc–(23—28)–OBzl [1], were chosen to construct the full sequence. In order to prepare the peptide hydrazides containing Asp(OBzl) or Glu(OBzl), these four fragments were synthesized with Troc–$NHNH_2$.[11] This Troc group is known to be cleaved by Zn[12] in AcOH without affecting other functional groups.

Throughout the syntheses of these intermediates and fragments, the purity of every intermediate was checked by thin-layer chromatography (TLC), elemental analysis and amino acid analysis. The analytical results were within ±0.44% of theoretical values in all cases.

The five fragments, [5], [4], [3], [2] and [1], were prepared in a stepwise manner starting from a protected C-terminal amino acid by the Su active ester procedure,[13] except for introduction of Ac–Ser, for which the azide procedure[14] was employed. The resulting protected peptide fragments, [XI], [XV], [X] and [VIII], were treated with Zn[11,12] in AcOH to remove the Troc group, and the zinc acetate was removed by treatment with EDTA to give the required hydrazides in analytical pure forms. The hydrazine test on the thin-layer chromatograms and the elemental analysis data were consistent with the homogeneity of the desired products. The five fragments were assembled successively from the C-terminal fragment to the N-terminal fragment using the azide procedure[14] according to the routes illustrated in Fig. 2.

The amount of acyl component in each fragment condensation was increased from 1.5 to 4 eq as the chain elongation progressed in order to secure complete condensation. The solubility of the protected intermediates in DMF decreased remarkably with chain elongation. Consequently, a mixture of DMF–DMSO had to be employed for the subsequent condensation reactions. Some of the intermediates were purified by repeated precipitation from DMF with MeOH, and others were purified by gel-filtration on Sephadex LH-20 using DMF as an eluent.

Throughout the synthesis, Gly was taken as the diagnostic amino acid in acid hydrolysis. By comparing of the recovery of Gly with that of newly incorporated amino acids, satisfactory incorporation of each fragment in each condensation was confirmed.

Starting with the C-terminal hexapeptide ester corresponding to positions 23 to 28 of parathymosin α [1], four fragments, Boc–(18—22)–$NHNH_2$ [2], Boc–(12—17)–$NHNH_2$ [3], Boc–(6—11)–$NHNH_2$ [4] and Ac–(1—5)–$NHNH_2$ [5], were successively condensed by the azide procedure[14] to give the protected octaeicosapeptide ester corresponding to the parathymosin α fragment 1—28.

In the final step of the synthesis, the protected octaeicosapeptide ester was treated with 1 M TFMSA–thioanisole in TFA in the presence of Me_2Se. Me_2Se was employed to facilitate acidic cleavage of the protecting groups.[15] The deprotected peptide was precipitated with dry ether, converted to the corresponding acetate with Amberlite IRA-400 (acetate form) and then treated with 1 N NH_4OH to reverse a possible N → O shift at the Ser residues.[16] The product was purified by gel-filtration on Sephadex G-25, followed by partition column chromatography on Sephadex G-25 according to Yamashiro.[17] Analysis of the main fractions by TLC using Partridge's solvent system[18] revealed the presence of one major chlorine-tolidine-positive spot and one minor spot. The crude octaeicosapeptide was further purified by preparative TLC developed with Partridge's solvent system. The purified peptide so obtained was found to be a single component from the results of TLC using two different solvent systems. The purified product was then subjected to Sephadex G-25 column chromatography as described above. The amino acid ratios in the acid hydrolysate of XXIV agreed well with theoretical values. The peptide also gave a single spot on paper electrophoresis and exhibited a single peak on HPLC. The molecular weight of the synthetic peptide was ascertained by FAB-MS spectrometry.

The immunological effect of the synthetic parathymosin α fragment 1—28 and thymosin α_1[7] was examined by the JIMRO (Japan Immunoresearch Laboratories Co., Ltd.) fluorometric blast-formation test according to Itoh and Kawai.[19] Responses of T-lymphocytes to mitogenic stimulation were lower in uremic patients than those of normal persons. The *in vitro* effect of the synthetic peptides on the impaired PHA response of T-lymphocytes from uremic patients is shown in Table I.

Comparison of the stimulation index (SI) values of the blastogenic transformation of T-lymphocytes into lymphoblasts with mitotic activity upon PHA stimulation shows that, in the case of the uremic patients investigated, the synthetic thymosin α_1 exhibited a restoring effect at a dose of 1 μg/ml, but the synthetic parathymosin α fragment 1—28 had no restoring effect on the impaired mitotic activity induced by PHA stimulation under the same conditions. Apparently, the synthetic parathymosin α fragment 1—28 is considerably far less active than thymosin α_1 in our *in*

TABLE I. Effect of the Synthetic Parathymosin α Fragment 1—28 and Thymosin α_1 on the Impaired PHA-Stimulation of T-Lymphocytes of Uremic Patients

Peptide	Dose (μg/ml)	SI[a,b]
—[c]	—	284.3±51.4
—[d]	—	106.5±50.7[g]
Thymosin α_1[d,e]	1.0	231.6±48.4[h]
Parathymosin α fragment 1—28[d,e]	1.0	111.2±49.3
Thymosin α_1 + parathymosin α fragment 1—28[d,e]	1.0+1.0	126.3±51.6
Thymosin α_1 + H–Gly–Gly–His–OH[d,e,f]	1.0+1.0	227.9±52.1[h]

a) Each value represents the mean±S.D. of triplicate measurements. *b*) SI (Stimulation index) was calculated according to the following formula: $SI = (I_2 - I_0/I_1 - I_0) \times 100$, where I_2 = mean fluorescence intensity of PHA-activated lymphocytes, I_1 = fluorescence intensity of PHA-nonactivated lymphocytes and I_0 = fluorescence intensity of ethidium bromide. *c*) Normal venous lymphocytes. *d*) Uremic patiens' lymphocytes. *e*) Incubation was carried out at 37°C in a humidified atmosphere of 5% CO_2 in air at 37°C for 12 h in the presence of each synthetic peptide or both. *f*) Control: purchased from the Peptide Institute Inc., Osaka. *g*) $p<0.05$, when compared to the normal persons using Student's *t* test. *h*) $p<0.01$, when compared to the uremic patients using Student's *t* test.

vitro assay test. Interestingly, when the synthetic parathymosin α fragment 1—28 was administered together with thymosin α_1, it appeared to neutralize the immunoenhancing effect of the latter at a dose of 1 μg/ml.

These results suggest that parathymosin α fragment 1—28 may act to modulate the immunoenhancing activity exhibited by thymosin α_1. In the case of normal subjects, *in vitro* addition of these peptides had no effect on the mitotic activity induced by PHA stimulation under the same conditions (data not shown).

Experimental

General experimental procedures used in this paper are essentially the same as described in the previous papers.[20,21] Azides were prepared according to Honzl and Rudinger[14] with isoamyl nitrite. Melting points are uncorrected. Rotations were measured with an Atago Polax machine (cell length: 10 cm). The amino acid compositions of the hydrolysates were determined with a Hitachi type 835-50 amino acid analyzer. HPLC was conducted with a Shimadzu LC-3A apparatus coupled to a YMC AM-312 column (6.0 × 150 mm). FAB-MS spectra was obtained on a Auto spec Q instrument (UQ Analytical Co., England) mass spectrometer equipped with an OPUS data processor. Solutions were concentrated in a rotary evaporator under reduced pressure at a temperature of 30—45 °C. Z(OMe) or Boc groups of the protected peptides were removed by TFA–anisole treatment. The resulting amino components were chromatographed on silica gel plates (Kieselgel G, Merck) and *Rf* values refer to the following solvent system: Rf^1 $CHCl_3$–MeOH–H_2O (8 : 3 : 1). The final product corresponding to the N-terminal octaeicosapeptide fragment of parathymosin α was chromatographed on cellulose plates (Merck). Rf^2 value refers to the Partridge system,[18] and Rf^3 value refers to BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24).[22] Patient selection: two uremic patients who were suffering from recurrent infections diseases were selected. Examination of the cellular immunocompentence of these patients revealed a significant decrease in blast-formation by PHA. ^{3}H-Thymidine incorporation values of these patients were 12160 and 11641 cpm respectively (normal values: 41694—42319 cpm). Venous blood was obtained from these uremic patients for the fluorometric blast-formation test. Venous blood samples from three healthy donors were used as a control. The fluorescence excitation spectrum was measured with an Oyo-Bunko ulog-flouspec 11 A fluorometer. Kits for the fluorometric blast-formation test were purchased from Japan Immunoresearch Laboratories Co., Ltd., Japan. After each coupling reaction, each product was purified by one of two procedures. Procedure A: For purification of protected peptides soluble in EtOAc, the extract was washed with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O, then dried over $MgSO_4$ and concentrated. The residue was reprecipitated or recrystallized from appropriate solvents. Procedure B: For purification of protected peptides almost insoluble in EtOAc, the reaction mixture was poured into ice-chilled 5% citric acid with stirring. The powder thereby formed was washed with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O. The dried product was recrystallized or reprecipitated from appropriate solvents. Preparations of protected intermediates were repeated several times in order to obtain sufficient quantities for the next step.

Boc–Ala–Gly–OBzl [I] H–Gly–OBzl·Tos (3.4 g) was dissolved in DMF (20 ml) containing NMM (1.2 ml). To this solution, Boc–Ala–OSu (3.2 g) was added, and the mixture was stirred at room temperature for 6 h. The reaction mixture was purified by procedure A, followed by reprecipitation from EtOAc with petroleum ether: Yield 3.1 g (86%), mp 80—85 °C, $[\alpha]_D^{21}$ −21.3° (c = 1.0, DMF), Rf^1 0.52, single ninhydrin-positive spot. *Anal.* Calcd for $C_{17}H_{24}N_2O_5 \cdot H_2O$: C, 57.62; H, 7.40; N, 7.90. Found: C, 57.59; H. 7.58; N, 8.02.

Boc–Lys(Z)–Ala–Gly–OBzl [II] I (3 g) was treated with TFA–anisole (20 ml–4 ml) in an ice-bath for 40 min, and TFA was removed by evaporation. The residue was washed with *n*-hexane, dried over KOH pellets *in vacuo* for 2 h, and then dissolved in DMF (15 ml) containing NMM (1 ml). To this solution, Boc–Lys(Z)–OSu (4.4 g) was added, and the mixture was stirred at room temperature for 6 h. The product was pufified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane: Yield 4.1 g (76%), mp 79—84 °C, $[\alpha]_D^{21}$ −9.7° (c = 1.0, DMF), Rf^1 0.54, single ninhydrin-positive spot. *Anal.* Calcd for $C_{31}H_{42}N_4O_8 \cdot 2H_2O$: C, 58.66; H, 7.31; N, 8.83. Found: C, 58.47; H, 7.56; N, 8.95.

Boc–Glu(OBzl)–Lys(Z)–Ala–Gly–OBzl [III] This compound was prepared essentially in the same manner as described for the preparation of II by using II (3.2 g) and Boc–Glu(OBzl)–OSu (2.4 g). The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane: Yield 3 g (71%), mp 92—98 °C, $[\alpha]_D^{21}$ −11.6° (c = 1.0, DMF), Rf^1 0.64, single ninhydrin-positive spot. *Anal.* Calcd for $C_{43}H_{55}N_5O_{11} \cdot H_2O$: C, 61.78; H, 6.87; N, 8.38. Found: C, 61.81; H, 7.13; N, 8.06.

Boc–Glu(OBzl)–Glu(OBzl)–Lys(Z)–Ala–Gly–OBzl [IV] This compound was prepared essentially in the same manner as described for the preparation of II using III (2.8 g) and Boc–Glu(OBzl)–OSu (1.6 g). The product was purified by procedure A, followed by reprecipitation from EtOAc with ether: Yield 2.6 g (74%), mp 114—123 °C, $[\alpha]_D^{21}$ −8.2° (c = 1.0, DMF), Rf^1 0.67, single ninhydrin-positive spot. *Anal.* Calcd for $C_{55}H_{68}N_6O_{14}$: C, 63.69; H, 6.61; N, 8.10. Found: C, 63.40; H, 6.86; N, 7.82.

Boc–Val–Glu(OBzl)–Glu(OBzl)–Lys(Z)–Ala–Gly–OBzl [I] This compound was prepared essentially in the same manner as described for the preparation of II by using IV (2.1 g) and Boc–Val–OSu (692 mg). The product was purified by procedure B, followed by reprecipitation from MeOH with ether: Yield 2 g (83%), mp 123—129 °C, $[\alpha]_D^{21}$ −13.4° (c = 1.0, DMF), Rf^1 0.69, single ninhydrin-positive spot. *Anal.* Calcd for $C_{60}H_{77}N_7O_{15} \cdot 2H_2O$: C, 61.47; H, 6.97; N, 8.36. Found: C, 61.28; H, 7.16; N, 8.54.

Boc–Asp(OBzl)–Lys(Z)–NHNH–Troc [V] This compound was prepared essentially in the same manner as described for the preparation of II by using Boc–Lys(Z)–NHNH–Troc (2.3 g) and Boc–Asp(OBzl)–OSu (1.9 g). The product was purified by procedure A, followed by reprecipitation from EtOAc with petroleum ether: Yield 2.1 g (70%), mp 74—82 °C, $[\alpha]_D^{21}$ −7.4° (c = 1.0, DMF), Rf^1 0.53, single ninhydrin-positive spot. *Anal.* Calcd for $C_{33}H_{42}Cl_3N_5O_{10}$: C, 49.38; H, 5.66; N, 9.36. Found: C, 49.03; H, 5.38; N, 9.71.

Boc–Lys(Z)–Asp(OBzl)–Lys(Z)–NHNH–Troc [VI] This compound was prepared from V (2 g) and Boc–Lys(Z)–OSu (1.4 g) essentially as described for the preparation of II. The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane: Yield 2.1 g (75%), mp 91—97 °C, $[\alpha]_D^{21}$ −10.1° (c = 1.0, DMF), Rf^1 0.59, single ninhydrin-positive spot. *Anal.* Calcd for $C_{47}H_{60}Cl_3N_7O_{13} \cdot H_2O$: C, 53.49; H, 5.92; N, 9.29. Found: C, 53.48; H, 6.17; N, 9.46.

Boc–Lys(Z)–Lys(Z)–Asp(OBzl)–Lys(Z)–NHNH–Troc [VII] This compound was prepared from VI (2 g) and Boc–Lys(Z)–OSu (1 g) essentially as described for the preparation of II. The product was purified by procedure A, followed by reprecipitation from MeOH with ether: Yield 1.8 g (72%), mp 110—116 °C, $[\alpha]_D^{21}$ −12.6° (c = 1.0, DMF), Rf^1 0.64, single ninhydrin-positive spot. *Anal.* Calcd for $C_{61}H_{78}Cl_3N_9O_{16} \cdot 2H_2O$: C, 54.85; H, 6.19; N, 9.44. Found: C, 54.64; H, 6.42; N, 9.58.

Boc–Glu(OBzl)–Lys(Z)–Lys(Z)–Asp(OBzl)–Lys(Z)–NHNH–Troc [VIII] This compound was prepared from VII (1.7 g) and Boc–Glu(OBzl)–OSu (597 mg) essentially as described for the preparation of II. The product was purified by procedure B, followed by recrystallization from hot EtOAc: Yield 1.4 g (70%), mp 121—128 °C, $[\alpha]_D^{21}$ −7.5° (c = 1.0, DMF), Rf^1 0.69, single ninhydrin-positive spot. *Anal.* Calcd for $C_{73}H_{91}Cl_3N_{10}O_{19} \cdot H_2O$: C, 57.05; H, 6.10; N, 9.11. Found: C, 56.87; H, 6.38; N, 9.46.

Boc–Glu(OBzl)–Lys(Z)–Lys(Z)–Asp(OBzl)–Lys(Z)–NHNH$_2$ [2] VIII (1.3 g) in a mixture of AcOH (6 ml) and DMF (6 ml) was treated with Zn dust (545 mg) at 4 °C for 12 h. The solution was filtered, and the residue was treated with 3% EDTA and then with $NaHCO_3$ to adjust the pH to neutral. The resulting gelatinous mass was washed with H_2O and reprecipitated from DMF with H_2O: Yield 1 g (83%), mp 146—153 °C, $[\alpha]_D^{21}$ −13.2° (c = 1.0, DMF), Rf^1 0.57, single hydrazin-test-positive spot. *Anal.* Calcd for $C_{70}H_{90}N_{10}O_{17} \cdot 3H_2O$: C, 60.16; H, 6.92; N, 10.02. Found: C, 59.86; H, 7.19; N, 10.27.

Boc–Ala–Lys(Z)–Asp(OBzl)–Leu–Lys(Z)–NHNH–Troc [IX] This compound was prepared essentially in the same manner as described for the preparation of II by using Z(OMe)–Lys(Z)–Asp(OBzl)–Leu–Lys(Z)–NHNH–Troc[8] (1.5 g) and Boc–Ala–OSu (394 mg). The product was purified by procedure A, followed by reprecipitation from EtOAc with ether; Yield 1.2 g (80%), mp 102—110 °C, $[\alpha]_D^{21}$ −13.7° (c = 1.0, DMF), Rf^1 0.63, single ninhydrin-positive spot. *Anal.* Calcd for $C_{56}H_{76}Cl_3N_9O_{15} \cdot H_2O$: C, 55.61; H, 6.50; N, 10.42. Found: C, 55.38; H, 6.89; N, 10.27.

Boc–Ser(Bzl)–Ala–Lys(Z)–Asp(OBzl)–Leu–Lys(Z)–NHNH–Troc [X] This compound was prepared from IX (1.2 g) and Boc–Ser(Bzl)–OSu (432 mg) essentially as described for the preparation of II. The product was purified by procedure B, followed by reprecipitation from MeOH with ether: Yield 1.1 g (79%), mp 118—125 °C, $[\alpha]_D^{21}$ −12.7° (c = 1.0, DMF), Rf^1 0.66, single ninhydrin-positive spot. *Anal.* Calcd for

$C_{66}H_{87}Cl_3N_{10}O_{17}\cdot 2H_2O$: C, 55.25; H, 6.39; N. 9.76. Found: C, 55.02, H, 6.64; N, 9.48.

Boc–Ser(Bzl)–Ala–Lys(Z)–Asp(OBzl)–Leu–Lys(Z)–NHNH$_2$ [3] This compound was prepared from X (1 g) and Zn dust (467 mg) essentially as described for the preparation of [2]. The product was recrystallized from MeOH: Yield 801 mg (90%), mp 129—137 °C, $[\alpha]_D^{21}$ −16.5° (c=1.0, DMF), Rf^1 0.49, single hydrazin-test-positive spot. *Anal.* Calcd for $C_{63}H_{86}N_{10}O_{15}\cdot 3H_2O$: C, 59.23; H, 7.26; N, 10.96. Found: C, 59.01; H, 7.49; N, 10.58.

Boc–Glu(OBzl)–Leu–NHNH–Troc [XI] This compound was prepared from Boc–Leu–NHNH–Troc (2.2 g) and Boc–Glu(OBzl)–OSu (2.4 g) essentially as described for the preparation of II. The product was purified by procedure A, followed by reprecipitation from EtOAc with petroleum ether: Yield 2.6 g (79%), mp 90—94 °C, $[\alpha]_D^{21}$ −28.9 °C (c=1.0, DMF), Rf^1 0.55, single ninhydrin-positive spot. *Anal.* Calcd for $C_{26}H_{37}Cl_3N_4O_8\cdot H_2O$: C, 47.46; H, 5.98; N, 8.52. Found: C, 47.59; H, 6.26; N, 8.21.

Boc–Ala–Glu(OBzl)–Leu–NHNH–Troc [XII] This compound was prepared assentially in the same manner as described for the preparation of II by using XI (2.2 g) and Boc–Ala–OSu (1.1 g). The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane: Yield 2 g (83%), mp 86—95 °C, $[\alpha]_D^{21}$ −17.3° (c=1.0, DMF), Rf^1 0.62, single ninhydrin-positive spot. *Anal.* Calcd for $C_{29}H_{42}Cl_3N_5O_9$: C, 48.99; H, 5.95; N, 9.85. Found: C, 48.72; H, 6.28; N, 9.90.

Boc–Ala–Ala–Glu(OBzl)–Leu–NHNH–Troc [XIII] This compound was prepared essentially in the same manner as described for the preparation of II by using XII (1.8 g) and Boc–Ala–OSu (787 mg). The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane: Yield 1.6 g (80%), mp 96—102 °C, $[\alpha]_D^{21}$ −14.2° (c=1.0, DMF), Rf^1 0.59, single ninhydrin-positive spot. *Anal.* Calcd for $C_{32}H_{47}Cl_3N_6O_{10}\cdot H_2O$: C, 48.04; H, 6.17; N, 10.50. Found; C, 47.93; H, 6.43; N, 10.24.

Boc–Ala–Ala–Ala–Glu(OBzl)–Leu–NHNH–Troc [XIV] This compound was prepared from XIII (1.3 g) and Boc–Ala–OSu (525 mg) essentially as described for the preparation of II. The product was purified by procedure A, followed by reprecipitation from EtOAc with ether: Yield 1.2 g (86%), mp 108—116 °C, $[\alpha]_D^{21}$ −19.3° (c=1.0, DMF), Rf^1 0.63, single ninhydrin-positive spot. *Anal.* Calcd for $C_{35}H_{52}Cl_3N_7O_{11}\cdot H_2O$: C, 48.25; H, 6.25; N, 11.25. Found: C, 48.23; H, 6.49; N, 11.02.

Boc–Glu(OBzl)–Ala–Ala–Ala–Glu(OBzl)–Leu–NHNH–Troc [XV] This compound was prepared from XIV (1.1 g) and Boc–Glu(OBzl)–OSu (597 mg) essentially as described for the preparation of II. The product was purified by procedure B, followed by reprecipitation from DMF with H_2O: Yield 1.2 g (86%), mp 126—133 °C, $[\alpha]_D^{21}$ −12.8° (c=1.0, DMF), Rf^1 0.68, single ninhydrin-positive spot. *Anal.* Calcd for $C_{47}H_{65}Cl_3N_8O_{14}\cdot 2H_2O$: C, 52.35; H, 6.45; N, 10.39. Found: C, 52.18; H, 6.77; N, 10.53.

Boc–Glu(OBzl)–Ala–Ala–Ala–Glu(OBzl)–Leu–NHNH$_2$ [4] This compound was prepared from XV (1.1 g) and Zn dust (654 mg) essentially as described for the preparation of [2]. The product was reprecipitated from DMF with ether: Yield 864 mg (89%), mp 149—157 °C, $[\alpha]_D^{21}$ −19.7° (c=1.0, DMF), Rf^1 0.54, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{44}H_{64}N_8O_{12}\cdot 3H_2O$: C,55.61; H, 7.42; N, 11.78. Found: C, 55.61; H, 7.86; N, 11.39.

Boc–Glu(OBzl)–Val–NHNH–Troc [XVI] This compound was prepared from Boc–Val–NHNH–Troc (2 g) and Boc–Glu(OBzl)–OSu (2.4 g) essentially as described for the preparation of II. The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane: Yield 2.6 g (81%), mp 72—76 °C, $[\alpha]_D^{21}$ −10.2° (c=1.0, DMF), Rf^1 0.65, single ninhydrin-positive spot. *Anal.* Calcd for $C_{25}H_{35}Cl_3N_4O_8\cdot H_2O$: C, 46.63; H, 5.79; N, 8.70. Found: C, 46.60; H, 5.96; N, 8.82.

Boc–Ser(Bzl)–Glu(OBzl)–Val–NHNH–Troc [XVII] This compound was prepared essentially in the same manner as described for the preparation of II by using XVI (2.2 g) and Boc–Ser(Bzl)–OSu (1.4 g). The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane: Yield 2.1 g (75%), mp 90—98 °C, $[\alpha]_D^{21}$ −8.3° (c=1.0, DMF), Rf^1 0.72, single ninhydrin-positive spot. *Anal.* Calcd for $C_{35}H_{46}Cl_3N_5O_{10}\cdot H_2O$: C, 51.20; H, 5.89; N, 8.53. Found: C, 51.33; H, 6.12; N, 8.87.

Boc–Lys(Z)–Ser(Bzl)–Glu(OBzl)–Val–NHNH–Troc [XVIII] This compound was prepared from XVII (2 g) and Boc–Lys(Z)–OSu (1.3 g) essentially as described for the preparation of II. The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane: Yield 2 g (74%), mp 94—99 °C, $[\alpha]_D^{21}$ −14.1° (c=1.0, DMF), Rf^1 0.64, (single ninhydrin-positive spot. *Anal.* Calcd for $C_{49}H_{64}Cl_3N_7O_{13}\cdot 2H_2O$: C, 53.43; H, 6.22; N, 8.90. Found: C, 53.09; H, 6.51; N, 8.66.

Ac–Ser–Lys(Z)–Ser(Bzl)–Glu(OBzl)–Val–NHNH–Troc [XIX] XVIII (1.4 g) was treated with TFA–anisole (14 ml–2.8 ml) as usual and the resulting powder was dissolved in DMF (10 ml) together with NMM (0.28 ml). A solution of Ac–Ser–NHNH$_2$ (214 mg) in DMF (3 ml) was chilled in dry ice-80% EtOH bath to −60 °C. To this solution, 4 N HCl in dioxane (0.52 ml) was added, followed by isoamyl nitrite (0.3 ml). The mixture was stirred for 20 min until the hydrazine-test was negative. The mixture was neutralized with NMM (0.28 ml) at −60 °C. To a cold solution of the tetrapeptide, a cold solution of Ac–Ser–N$_3$ was added and stirred at −10 °C for 36 h. The mixture was poured into cold 5% citric acid. To the suspension thereby formed, 50% NH_4OAc was added dropwise with stirring to form a precipitate. The precipitate was collected and washed successively with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O. The dried product was reprecipitated from MeOH with ether: 1.1 g (73%), mp 118—127 °C, $[\alpha]_D^{21}$ −23.6° (c=1.0, DMF), Rf^1 0.66, single chlorine–tolidine-positive spot. *Anal.* Calcd for $C_{49}H_{63}Cl_3N_8O_{14}\cdot 3H_2O$: C, 51.25; H, 6.06; N, 9.76. Found: C, 51.43; H, 6.41; N, 9.72.

Ac–Ser–Lys(Z)–Ser(Bzl)–Glu(OBzl)–Val–NHNH$_2$ [5] This compound was prepared from XIX (1 g) and Zn dust (568 mg) essentially as described for the preparation of [2]. The product was recrystallized from hot MeOH: Yield 721 mg (89%), mp 140—147 °C, $[\alpha]_D^{21}$ −71.6° (c=1.0, DMF), Rf^1 0.52, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{46}H_{61}N_8O_{12}\cdot H_2O$: C, 59.03; H, 6.78; N, 11.97. Found: C, 58.79; H, 7.10; N, 11.65.

Synthesis of Protected Parathymosin α Fragment 1—28 Successive azide condensations of five fragments were carried out according to Fig. 2. Prior to condensation, the Boc group was removed from the respective amino component (1 ml per 0.1 g of the peptide) in the presence of anisole (10 eq) in an ice-bath for 40 min. The TFA-treated sample was precipitated with dry ether, dried over KOH pellets *in vacuo* for 2 h, and dissolved in DMF or DMF–DMSO (1 : 1) containing NMM (1 : 1 eq). The corresponding azide (the amount was increased from 1.5 to 4 eq as chain elongation progressed) in DMF or DMF–DMSO (1 : 1) and NMM (1 : 1 eq) was added to the above ice-chilled solution and the mixture was stirred at −10 °C until the solution became negative to the ninhydrin-test. The mixture was neutralized by adding a few drops of AcOH and poured into ice-chilled 5% citric acid with stirring. The precipitate thereby formed was successively washed with 5% citric acid, H_2O and MeOH. The dried product was

TABLE II. Characterization of the Protected Parathymosin α Fragment 1—28 and Its Intermediates

	Puri. proc. (Yield %)	mp (°C)	Rf^1	$[\alpha]_D^{21}$ (c=1.0, DMSO)	Formula	Analysis (%) Calcd (Found)		
						C	H	N
Boc–(18—28)–OBzl	B (71)	140—148	0.62	−17.6	$C_{125}H_{155}N_{15}O_{30}\cdot 6H_2O$	61.14 (60.87	6.86 6.97	8.56 8.42)
Boc–(12—28)–OBzl	B (63)	152—161	0.69	−12.4	$C_{183}H_{229}N_{23}O_{43}\cdot 9H_2O$	61.10 (61.22	6.91 7.18	7.18 8.59)
Boc–(6—28)–OBzl	A (55)	164—173	0.59	−15.7	$C_{222}H_{281}N_{29}O_{53}\cdot 11H_2O$	60.57 (60.48	6.94 7.24	9.23 9.50)
Boc–(1—28)–OBzl	A (65)	159—167	0.67	−26.4	$C_{263}H_{330}N_{35}O_{63}\cdot 13H_2O$	60.47 (60.16	6.87 7.13	9.39 9.42)

A, precipitation from DMF or DMSO with MeOH; B, gel-filtration on Sephadex LH-20.

purified by one of the following procedures. A: Precipitation from DMF with MeOH. B: Gel-filtration on Sephadex LH-20 using DMF containing 3% H_2O as an eluent. In procedure B, eluates (4 ml each fractions) were examined by measuring the ultraviolet (UV) absorption at 260 nm and fractions corresponding to the front main peak were combined. The solvent was removed by evaporation and the residue was treated with ether to afford a powder. The purification procedure, yield, physical constants and analytical data of protected parathymosin α fragment 1—28 and its intermediates are listed in Tables II and III.

Ac–Ser–Lys–Ser–Glu–Val–Glu–Ala–Ala–Glu–Leu–Ser–Ala–Lys–Asp–Leu–Lys–Glu–Lys–Lys–Asp–Lys–Val–Glu–Glu–Lys–Ala–Gly–OH (Corresponding to Parathymosin α Fragment 1—28) [XXIV] The protected octaeicosapeptide ester (100 mg) was treated with 1 M TFMSA–thioanisole in TFA (4 ml) in the presence of Me_2Se (100 μl) in an ice-bath for 110 min, then dry ether was added. The resulting powder was collected by centrifugation, dried over KOH pellets for 2 h and dissolved in 1 N AcOH (5 ml). The solution, after being stirred with Amberlite IRA-400 (acetate form, approximately 1 g) for 30 min, was filtered. The pH of the filtrate was adjusted to pH 8.0 with 1 N NH_4OH, and after 30 min to pH 6.0 with 1 N AcOH, and the solution was lyophilized. The residue was dissolved in 2% AcOH (2 ml), applied to a column of Sephadex G-25 (3.0 × 96 cm), and eluted with the same solvent. Fractions of 4 ml each were collected per 20 min, and the absorption at 230 nm was determined. Fractions corresponding to the front main peak (tube Nos. 68—79) were combined and the solvent was removed by lyophilization. The Sephadex-purified sample 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 (3.0 × 94 cm) previously equilibrated with the lower phase of the above solvent system. The column was developed with the same upper phase. Individual fractions (4 ml each) were collected and the absorbancy at 230 nm was determined. The fractions corresponding to the main peak (tube Nos. 60—68) were combined. The solvent was removed by evaporation. Analysis by TLC revealed the presence of two chlorine-tolidine-positive spots with Rf^2 0.05 (main) and 0.31 (minor). The crude peptide was subscribed to preparative TLC (cellulose phase, 20 × 40 cm) using the solvent system of BuOH–AcOH–H_2O (4 : 1 : 5, upper phase). The zone corresponding to Rf^2 0.05 was separated and extracted with 2% AcOH. The extract was concentrated to a small volume, applied to a Sephadex G-25 column (3.0 × 96 cm), and eluted with 2% AcOH. The main fractions containing a single component were combined and the solvent was removed by lyophilization: Yield 8.3 mg (14%), $[\alpha]_D^{21}$ −79.5° (c = 0.3, 2% AcOH), Rf^2 0.05, Rf^3 0.08, single chlorine–tolidine-positive spot. The synthetic peptide exhibited a single spot on paper electrophoresis: Toyo Roshi No. 51 (2 × 40 cm), acetate buffer at pH 2.86, mobility 2.1 cm from the origin toward the cathode after running at 1.5 mA, 600 V for

TABLE III. Amino Acid Ratios in 6 *N* HCl Hydrolysates of the Protected Parathymosin α Fragment 1—28 and Its Intermediates[a)]

	Protected peptides				Residue
	18—28	12—28	6—28	1—28	
Gly	1.00	1.00	1.00	1.00	1
Ala	1.04	2.04	5.06	5.07	5
Leu		0.97	1.96	2.02	2
Val	0.95	0.95	0.91	1.91	2
Ser		0.94	0.93	2.93	3
Asp	0.93	1.90	1.99	1.97	2
Glu	2.99	3.03	4.92	5.89	6
Lys	4.06	5.91	5.92	7.04	7

a) The results are expressed as ratios to the value for Gly, which was taken as the diagnostic amino acid in acid hydrolysates.

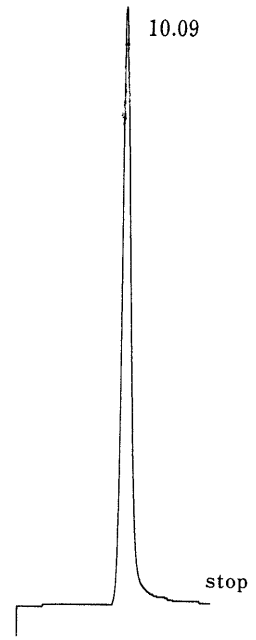

Fig. 3. HPLC of the Synthetic Parathymosin α Fragment 1—28

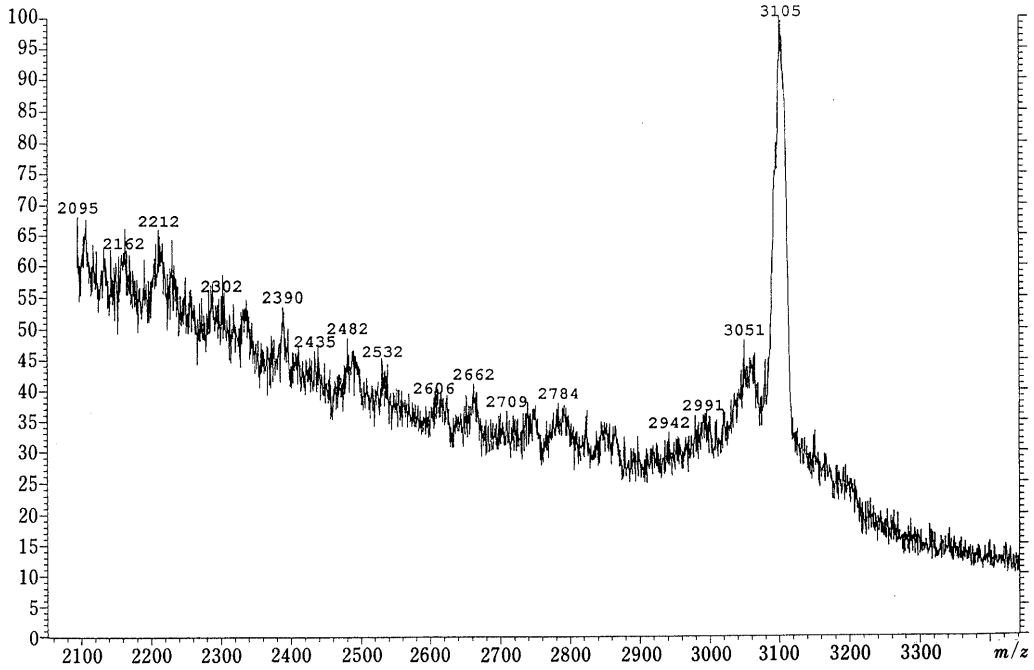

Fig. 4. FAB-MS of the Synthetic Parathymosin α Fragment 1—28

75 min. Amino acid ratios in 6 N HCl hydrolysate: Gly 1.00, Ala 5.07, Leu 1.95, Val 1.98, Ser 2.90, Asp 2.03, Glu 5.90, Lys 7.04 (recovery of Gly 83%). The synthetic peptide exhibited a single peak on HPLC using an analytical YMC AM-312 column (6.0 × 150 mm) at a retention time of 10.09 min, when eluted with a gradient of acetonitrile 32 to 45% in 0.1% TFA at a flow rate of 1 ml per min (Fig. 3). FAB-MS *m/z*: 3105 $(M+H)^+$.

Fluorometric Blast-Formation Test A 3-ml aliquot of venous blood from uremic patients was drawn into a syringe containing 25U/ml of heparin, and the mixed with 3 ml of PBS. Lymphocytes were isolated in a Hypaque-Ficoll gradient.[23] Isolated lymphocytes were adjusted to 1.0×10^6/ml with PBS. The lymphocytes were cultured in 0.5 ml of RPMI 1640 (Gibco) with FCS (Dainippon Pharmaceutical Co.) in microplates. Cultures of each combination were incubated at 37 °C in the presence of the peptide in a humidified atmosphere of 5% CO_2 in air for 12 h and PHA (0.125%, 0.5 ml) was added to each well. Incubation was continued under the same conditions for 60 h. T-lymphocytes in each well were transfered to a test tube and centrifuged for 10 min at 240 *g*, and an aliquot of 0.125% SDS was added to the residue and stirred for 20 min at room temperature; lymphocytes were completely destroyed and solubilized by this procedure. An ethidium bromide solution was added to the above solution and the mixture was stirred for 15 min at room temperature. The fluorescence excitation spectrum was measured according to the method of Itoh and Kawai.[19]

References and Notes

1) Abbreviations used: TFA, trifluoroacetic acid; Z, benzyloxycarbonyl; OBzl, benzyl ester; Z(OMe), *p*-methoxybenzyloxycarbonyl; Ac, acetyl; Boc, *tert*-butoxycarbonyl; Troc, β,β,β-trichloroethoxycarbonyl; Bzl, benzyl; NMM, *N*-methylmorpholine; OSu, *N*-hydroxysuccinimide ester; Su, *N*-hydroxysuccinimide; DMF, dimethylformamide, DMSO, dimethylsulfoxide; AcOH, acetic acid; MeOH, methanol; EtOAc, ethyl acetate; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; PHA, phytohemagglutinin; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; PRMI, Rosewell Park Memorial Institute; FCS, fetal calf serum; FAB-MS, fast atom bombardment mass spectrometry; Tos, *p*-toluenesulfonic acid; EtOH, ethanol; NH_4OAc, ammonium acetate.

2) T. L. Low, G. B. Thurman, C. Chincarini, J. E. McClure, G. D. Marshall, K. Hu and A. L. Goldstein, *Ann. N. Y. Acad. Sci.*, **253**, 33 (1979).
3) A. L. Goldstein, T. L. K. Low, M. McAdoo, J. McClure, G. B. Thurman, J. Rossio, C.-Y. Lai, D. Chang, S.-S. Wang, C. Harvey, A. H. Remel and J. Meinhofer, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 725 (1977).
4) T. L. K. Low, G. B. Thurman, M. McAdoo, J. McClure, J. L. Rossio, P. H. Nayler and A. L. Goldstein, *Biol. Chem.*, **254**, 981 (1979).
5) T. Abiko, I. Onodera and H. Sekino, *Chem. Pharm. Bull.*, **27**, 3171 (1979).
6) T. Abiko, H. Sekino and H. Higuchi, *Chem. Pharm. Bull.*, **28**, 3411 (1980).
7) T. Abiko, I. Onodera and H. Sekino, *Chem. Pharm. Bull.*, **28**, 3542 (1980).
8) T. Abiko and H. Sekino, *Chem. Pharm. Bull.*, **30**, 1776 (1982).
9) A. A. Haritos, S. B. Salvin, R. Blacher, S. Stein and B. L. Horecker, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 1050 (1985).
10) H. Yajima, N. Fijii, H. Ogawa and H. Kawatani, *J. Chem. Soc., Chem. Commun.*, **1974**, 107; H. Yajima and N. Fujii, *J. Am. Chem. Soc.*, **103**, 5867 (1981); Y. Kiso, S. Nakamura, K. Ito, K. Ukawa, K. Katagawa, T. Akito and H. Moritoki, *J. Chem. Soc., Chem. Commun.*, **1979**, 971.
11) H. Yajima and Y. Kiso, *Chem. Pharm. Bull.*, **19**, 420 (1971).
12) R. B. Woodward, K. Heusler, J. Gosteri, P. Naegeli, W. Oppolzer, R. Ramage, S. Rangnathan and H. Vorbrüggen, *J. Am. Chem. Soc.*, **88**, 852 (1966).
13) G. W. Anderson, J. E. Zimmerman and F. Callahan, *J. Am. Chem. Soc.*, **85**, 3039 (1963); *idem*, *ibid.*, **86**, 1839 (1964).
14) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).
15) M. Shimokura, Y. Kiso, A. Nagata, M. Tsuda, H. Seki, Y. Kai, N. Fujii and H. Yajima, *Chem. Pharm. Bull.*, **34**, 1814 (1986).
16) S. Sakakibara, "Chemistry and Biochemistry of Amino Acids, Peptides and Proteins," Vol. 1, ed. by B. Weinstein, Academic Press Inc., New York, 1971, p. 51.
17) D. Yamashiro, Nature (London), **201**, 76 (1964).
18) S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).
19) Y. Itoh and T. Kawai, *Rinsho Kensa*, **27**, 928 (1983).
20) T. Abiko and H. Sekino, *Chem. Pharm. Bull.*, **37**, 2467 (1989).
21) T. Abiko and H. Sekino, *Chem. Pharm. Bull.*, **39**, 752 (1991).
22) S. G. Walley and G. Watson, *Biochem. J.*, **55**, 328 (1953).
23) R. Harris and E. U. Ukaejiofo, *Br. J. Haematol.*, **18**, 229 (1970).