[Chem. Pharm. Bull. 23(10)2301—2305(1975)]

UCD 547.466.1.057.09.615.31.076.9

Studies on Peptides. LIII.[1,2] Synthesis of the Docosapeptide Corresponding to the Entire Amino Acid Sequence of Porcine ACTH-Like Intermediate Lobe Peptide (CLIP)

KANAME KOYAMA, YOSHIRO MORI, YOSHIAKI KISO, SATOSHI HIRABAYASHI, and HARUAKI YAJIMA

Faculty of Pharmaceutical Sciences, Kyoto University[3]

(Received March 6, 1975)

The docosapeptide corresponding to positions 18 to 39 of the revised amino acid sequence of porcine ACTH (porcine ACTH-like intermediate lobe peptide, CLIP) was synthesized and its radioimmunoassay was conducted. It was shown that its mode of the cross reaction with the ACTH antiserum (prepared from porcine ACTH) was parallel, like synthetic human and bovine type CLIPs, to that of porcine ACTH, when ^{125}I-synthetic human CLIP was used as a tracer.

In the preceding papers, we described the syntheses of two docosapeptides which correspond to positions 18 to 39 of ACTHs of bovine and human origins respectively.[4,5] These peptides should be defined as respective ACTH-like intermediate lobe peptide (CLIP) of these mammalian species. In this paper we wish to record the synthesis of CLIP which corresponds to position 18 to 39 of porcine ACTH. This peptide was first isolated from pars intermediate lobe of porcine pituitary and characterized by Scott, *et al.*[6] in 1973.

As shown in Fig. 1, synthetic scheme we employed here is essentially the same as described in the synthesis of human type CLIP, since the revised structure of porcine ACTH[7,8] is identical with that of human ACTH,[8] except one amino acid residue at position 31. Porcine ACTH possesses the Leu residue at this position, while human ACTH has Ser. Available fragments for the synthesis of human CLIP,[5] Z–Arg(NO_2)–Pro–OH (I), Z(OMe)–Val–Lys(Z)–Val–Tyr–Pro–Asn–Gly–OH (II), H–Glu(OBzl)–Ala–Phe–Pro–OH (III) and H–Leu–Glu–(OBzl)–Phe–OBzl (IV) served to construct the entire sequence of porcine CLIP.

A middle sequence was newly synthesized according to the route illustrated in Fig. 1. Z(OMe)–Leu–Ala–$NHNH_2$ was coupled with H–Glu(OBzl)–Ala–Phe–Pro–OH (III)[5] by the azide procedure of Honzl and Rudinger[9] to give Z(OMe)–Leu–Ala–Glu(OBzl)–Ala–Phe–Pro–OH, which was then condensed with H–Leu–Glu(OBzl)–Phe–OBzl (IV)[4] by N-isobutoxy-

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[1] Part LII: N. Fujii and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), 23, 1596 (1975).

[2] Peptides and peptide derivatives mentioned in this communication are of the L-configuration. Abbreviations used are those recommended by IUPAC-IUB Commission of Biochemistry Nomenclature: *Biochemistry*, **11**, 1726 (1972), *ibid.*, **6**, 362 (1967), *ibid.*, **5**, 2485 (1966). Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Bzl=benzyl, ONP=*p*-nitrophenyl ester, OPCP=pentachlorophenyl ester.

[3] Location: *Sakyo-ku, Kyoto.*

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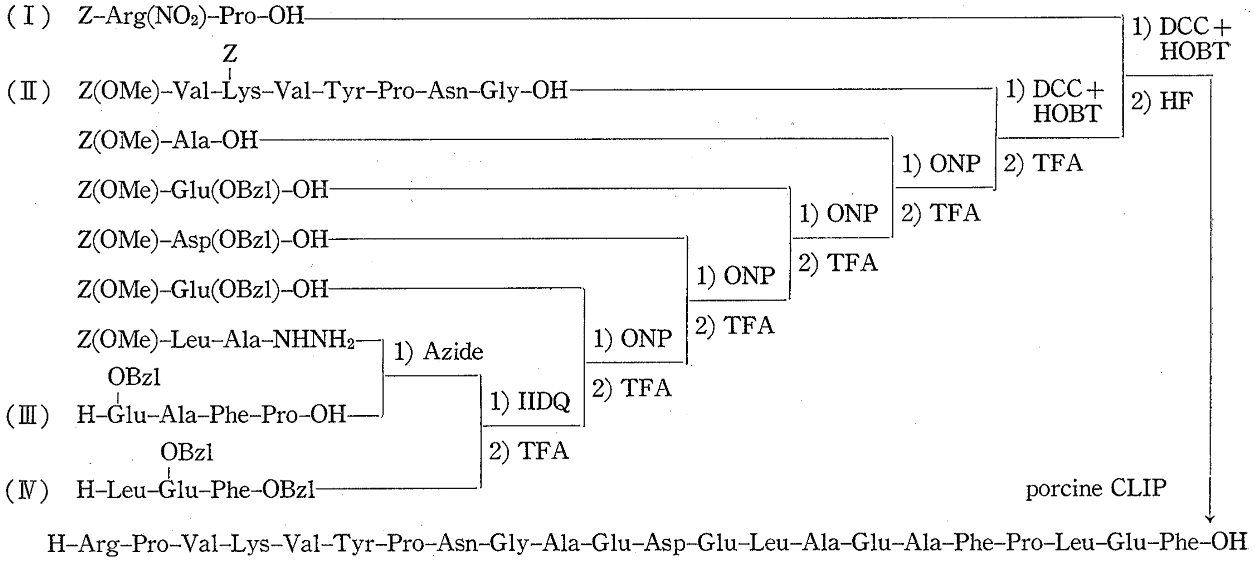

IIDQ=N-isobutoxycarbonyl-2-isobutoxy-1,2-dihydroquinoline

Fig. 1. Synthetic Route to Porcine CLIP

carbonyl-2-isobutoxy-1,2-dihydroquinoline (IIDQ).[10] The result was comparable to those performed by the dicyclohexylcarbodiimide (DCC) plus N-hydroxybenzotriazole (HOBT) procedure[11] for the syntheses of analogous CLIPs.[4,5] Usefulness of this newly introduced coupling reagent for the fragment condensation reaction was further confirmed. Thus the typical C-terminal portion of porcine CLIP was prepared in the protected form, *i.e.*, Z(OMe)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl.

Next, combination of the TFA treatment[12] for the deprotection of the Z(OMe) group and the *p*-nitrophenyl ester procedure[13] was applied to elongate the above nonapeptide chain stepwisely to the protected tridecapeptide ester, Z(OMe)-Ala-Glu(OBzl)-Asp(OBzl)-Glu-(OBzl)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl. Such stepwise elongation procedure gave nearly 90% or better yield in each step.

Fragment condensation of Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-OH (II)[5] with the TFA treated sample of the above protected tridecapeptide ester and subsequent coupling of Z-Arg(NO_2)-Pro-OH were performed in essentially the same manner as described previously[5] by the DCC plus HOBT procedure.

The protected docosapeptide ester, Z-Arg(NO_2)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, thus obtained was subjected to the hydrogen fluoride treatment,[14] rather catalytic hydrogenation, to remove all protecting groups since reductive removal of the NO_2 group from such a relatively large peptide is known to require a long period of time. The resulting free peptide was purified by partition column chromatography on Sephadex G-25 according to Yamashiro.[15] The solvent system consisting of *n*-butanol, acetic acid and water (4: 1: 5) was applied to elute the desired compound. The absorbancy due to the Tyr residue was the guide of this chromatographic purification. Homogeneity of the synthetic porcine CLIP

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was confirmed by thin-layer chromatography, acid hydrolysis and elemental analysis. Despite of the presence of the Pro residue,[16] complete digestion of this synthetic peptide was achieved by aminopeptidase (AP-M),[17] when the enzyme-substrate ratio was slightly increased. The result was parallel to that of the acid hydrolysis except for Asp. In the enzymatic hydrolysate, nearly one equivalent amount of Asn was detected.

Radioimmunoassay of bovine and human CLIPs[4,5] and newly synthesized porcine CLIP has been conducted by Professor H. Imura and Dr. T. Hirata of the Internal Medicine, Kobe University, School of Medicine. When ^{125}I-synthetic human CLIP was used as a tracer, the mode of cross reaction of these peptides with the ACTH-antiserum (prepared by natural porcine ACTH) was nearly parallel to that of porcine ACTH. Detailed account of these investigations will be published by the above authors.

Experimental

Thin-layer chromatography was performed on silica gel (Kieselgel G, Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8: 3: 1), Rf_2 $CHCl_3$–MeOH (9: 1), Rf_3 $CHCl_3$–MeOH–AcOH (9: 1: 0.5), Rf_4 *n*-BuOH–pyridine–AcOH–H_2O: (4: 1: 1: 2).

Z(OMe)-Leu-Ala-OMe——Z(OMe)–Leu–OH (16.81 g) and DCC (12.91 g) were added to a solution of H–Ala–OMe (prepared from 7.98 g of the hydrochloride and 8.3 ml of Et_3N) in DMF (170 ml) and the mixture was stirred at room temperature for 48 hr. The solvent was evaporated and the residue was dissolved in AcOEt, which was washed with 5% Na_2CO_3, 5% citric acid and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was triturated with ether and recrystallized from AcOEt and ether; yield 12.80 g (58%), mp 120—121°, $[\alpha]_D^{20}$ $-11.3°$ (c=1.1, DMF). Rf_1 0.80. *Anal.* Calcd. for $C_{19}H_{28}O_6N_2$: C, 59.98; H, 7.42; N, 7.36. Found: C, 60.11; H, 7.57; N, 7.66.

Z(OMe)-Leu-Ala-NHNH₂——To a solution of Z(OMe)–Leu–Ala–OMe (12.50 g) in MeOH (120 ml), 80% hydrazine hydrate (6.5 ml) was added and the mixture was kept on standing overnight. The resulting solid was recrystallized from MeOH and dioxane to give fine needles; yield 11.72 g (94%), mp 176—178°. *Anal.* Calcd. for $C_{18}H_{28}O_5N_4$: C, 56.82; H, 7.42; N, 14.73. Found: C, 56.96; H, 7.44; N, 14.78.

Z(OMe)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-OH——In the usual manner, Z(OMe)–Glu(OBzl)–Ala–Phe–Pro–OH (20.06 g)[4] was treated with TFA (20 ml) in the presence of anisole (10 ml) at room temperature for 45 min, when dry ether was added. The resulting precipitate was washed with ether, dried over KOH pellets *in vacuo* overnight and then dissolved in DMF (20 ml). To this ice-chilled solution, Et_3N (7.8 ml) and the azide (prepared from 11.50 g of Z(OMe)–Leu–Ala–$NHNH_2$ with 33.3 ml of 1.8N HCl–DMF, 4.0 ml of isoamylnitrite and 12.4 ml of Et_3N) in DMF (30 ml) were added and the mixture was stirred at 4° for 48 hr. The solvent was evaporated *in vacuo* and the residue was dissolved in H_2O, which after washing with AcOEt, was acidified with citric acid. The resulting oily precipitate turned to solid on standing in a refrigerator, which was washed with 5% citric acid and H_2O and then recrystallized from AcOEt and ether; yield 14.0 g (55%), mp 177—180°, $[\alpha]_D^{20}$ $-33.5°$ (c=0.7 DMF). Rf_1 0.32. *Anal.* Calcd. for $C_{47}H_{60}O_{12}N_6$: C, 62.65; H, 6.71; N, 9.33. Found: C, 62.70; H, 6.68; N, 9.13.

Z(OMe)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl——As reported previously,[4,5] Z(OMe)–Leu–Glu(OBzl)–Phe–OBzl (7.50 g) was treated with TFA (10 ml) in the presence of anisole (3 ml) at 0° for 60 min. The excess TFA was evaporated *in vacuo* and the residue was dissolved in AcOEt, which was washed with 5% K_2CO_3 and H_2O–NaCl, dried over Na_2SO_4 and then evaporated *in vacuo* (bath temperature 30°). The residue turned to solid with ether; yield 5.40 g (92%), Rf_1 0.74. This tripeptide ester (4.34 g) and Z(OMe)–Leu–Ala–Glu(OBzl)–Ala–Phe–Pro–OH (4.50 g) were dissolved in DMF (60 ml), to which IIDQ (2.10 g) was added and the mixture was stirred at room temperature for 48 hr. After evaporation of the solvent, the residue was treated with ether and the resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and then recrystallized from MeOH and ether; yield 5.58 g (76%), mp 148—153°, $[\alpha]_D^{20}$ $-31.5°$ (c=0.6, DMF). Rf_1 0.95, Rf_2 0.45. *Anal.* Calcd. for $C_{81}H_{99}O_{17}N_9$: C, 66.15; H, 6.79; N, 8.57. Found: C, 65.89; H, 6.86; N, 8.36.

Z(OMe)-Glu(OBzl)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl——The above protected nonapeptide ester (4.40 g) was treated with TFA (6 ml) in the presence of anisole (2 ml) at 0° for 60 min, when dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (40 ml), to which Et_3N (0.4 ml), Z(OMe)–Glu(OBzl)–ONP (3.13 g) and HOBT

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17) G. Pfleiderer and P.G. Celliers, *Biochem. Z.*, 339, 186 (1963); K. Hofmann, F.M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966). Rohm & Haas (Darmstadt), P.R.F. (191226) was employed.

(20 mg) were combined. The mixture was stirred at room temperature for 48 hr and then the solvent was evaporated. The residue was treated with ether to form the solid, which after washing with 5% citric acid and H_2O, was recrystallized twice from THF and ether; yield 4.70 g (95%), mp 187—191°, $[\alpha]^{22}_{D}$ −28.6° (c=0.5, DMF). Rf_2 0.50. *Anal.* Calcd. for $C_{93}H_{112}O_{20}N_{10}\cdot H_2O$: C, 65.40; H, 6.70; N, 8.20. Found: C, 65.42; H, 6.63; N, 8.30.

Z(OMe)-Asp(OBzl)-Glu(OBzl)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl——The above protected decapeptide ester (5.60 g) was treated as usual. with TFA (9 ml) in the presence of anisole (3 ml) at 0° for 60 min, when dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (60 ml), to which Et_3N (0.5 ml), Z(OMe)–Asp(OBzl)–ONP (3.36 g) and HOBT (20 mg) were combined. After the solution was stirred at room temperature overnight, the solvent was evaporated and the residue was treated with ether. The resulting powder was washed with 5% citric acid and H_2O and then recrystallized twice from THF and ether; yield 5.95 g (95%), mp 215—218°, $[\alpha]^{22}_{D}$ −30.9° (c=0.5, DMF). Rf_2 0.58. *Anal.* Calcd. for $C_{104}H_{123}O_{23}N_{11}$: C, 65.91; H, 6.54; N, 8.13. Found: C, 65.90; H, 6.46; N, 8.20.

Z(OMe)-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl—— In the usual manner, the above protected undecapeptide ester (5.90 g) was treated with TFA (9 ml) in the presence of anisole (3 ml) at 0° for 60 min, when dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (50 ml), to which Et_3N (0.4 ml), Z(OMe)–Glu(OBzl)–ONP (3.24 g), HOBT (20 mg) were combined. The solution, after stirring at room temperature for 24 hr, was condensed *in vacuo*. Addition of ether to the residue afforded powder, which was washed with 5% citric acid and H_2O and then precipitated twice from THF with ether; yield 6.30 g (95%), mp 245—248°, $[\alpha]^{22}_{D}$ −27.3° (c=0.4, DMF). Rf_2 0.58. *Anal.* Calcd. for $C_{116}H_{136}O_{26}N_{12}$: C, 65.89; H, 6.48; N, 7.95. Found: C, 65.70; H, 6.46; N, 8.18.

Z(OMe)-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl ——As usual, the above protected dodecapeptide ester (6.20 g) was treated with TFA (10 ml) in the presence of anisole (3 ml) at 0° for 60 min, when dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (40 ml), to which Et_3N (0.4 ml), Z(OMe)–Ala–ONP (2.25 g), HOBT (20 mg) were combined. The solution, after stirring at room temperature for 48 hr, was condensed *in vacuo* and ether was added to the residue to form the solid which was washed with 5% citric acid and H_2O and then precipitated twice from THF with ether; yield 5.89 g (93%), mp 255—260°, $[\alpha]^{22}_{D}$ −26.8° (c=0.7, DMF), Rf_2 0.59. Amino acid ratios in an acid hydrolysate: Ala 3.00, Glu 4.42, Asp 1.03, Leu 1.92, Phe 1.96, Pro 1.07 (average recovery 92%). *Anal.* Calcd. for $C_{119}H_{141}O_{27}N_{13}$: C, 65.40; H, 6.50; N, 8.33. Found: C, 65.52; H, 6.40; N, 8.60.

Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl——The above protected tridecapeptide ester (4.20 g) was treated with TFA (8 ml) in the presence of anisole (2 ml) as stated above. The powder formed by addition of dry ether was dissolved in 1N HCl-dioxane (3 ml) and the solution was lyophilized. The hydrochloride thus obtained was dissolved in DMF (40 ml). To this solution, Et_3N (0.3 ml), Z(OMe)–Val–Lys(Z)–Val–Tyr–Pro–Asn–Gly–OH (3.22 g), HOBT (0.81 g) and DCC (0.83 g) were added and the mixture was stirred at room temperature for 72 hr. The solution was filtered and the filtrate was condensed *in vacuo*. The residue was treated with ether. The resulting powder was washed with 5% citric acid and H_2O and precipitated twice from THF with MeOH; yield 4.60 g (75%), mp 245—250°, $[\alpha]^{22}_{D}$ −14.0° (c=0.5, DMF). Rf_2 0.10, Rf_3 0.24. Amino acid ratios in an acid hydrolysate: Val 1.72, Lys 0.86, Pro 1.74, Asp 2.05, Gly 0.84, Ala 3.00, Glu 4.46, Leu 1.82, Phe 1.93 (average recovery 91%). *Anal.* Calcd. for $C_{163}H_{202}H_{38}N_{22}$: C, 63.62; H, 6.62; N, 10.01. Found: C, 63.45; H, 6.58; N, 9.78.

Z-Arg(NO$_2$)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Leu-Ala-Glu(OBzl)-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl——The above protected eicosapeptide ester (0.62 g) was treated as usual with TFA (1.2 ml) in the presence of anisole (0.3 ml) in an ice-bath for 90 min. The excess TFA was evaporated *in vacuo* and dry ether was added. The TFA salt isolated as a fine powder was converted to the free base as stated above by treatment with 4.2N HCl-dioxane (0.2 ml) and subsequently with Et_3N (0.2 ml). To a solution of this free base in DMF (6 ml), Z–Arg(NO$_2$)–Pro–OH (0.18 g), HOBT (54 mg) and DCC (84 mg) were added and the mixture was stirred at room temperature for 48 hr. The solution was filtered, the filtrate was condensed *in vacuo* and the residue was treated with ether. The resulting powder was washed batchwisely with 5% Na_2CO_3, 10% citric acid and H_2O and applied to a column of silica (2.5 × 15 cm), which was eluted with the solvent system of $CHCl_3$–MeOH–H_2O (8:3:1). Fractions containing the substance of Rf_1 0.60 were combined and the solvent was evaporated. Treatment of the residue with H_2O gave the fine powder which was precipitated from DMF with AcOEt. Yield 0.34 g (51%), mp 236—238°, $[\alpha]^{26}_{D}$ −17.3° (c=0.6, DMF). Rf_1 0.60. Amino acid ratios in an acid hydrolysate: Arg+Orn 0.94, Pro 2.78, Val 1.76, Lys 0.99, Tyr 0.27, Asp 1.99, Gly 1.00, Glu 4.45, Leu 1.90, Ala 3.05, Phe 1.97 (average recovery 95%).[17] *Anal.* Calcd. for $C_{173}H_{218}O_{41}N_{28}$: C, 62.10; H, 6.57; N, 11.72. Found: C, 61.88; H, 6.43; N, 11.58.

H-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Leu-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH, Porcine CLIP——The above protected docosapeptide ester (205 mg) was treated with HF (approximately

10 ml) in the presence of anisole (1 ml) in an ice-bath for 60 min. The excess HF was removed by evaporation under reduced pressure. The residue was dissolved in H_2O (10 ml), which was treated with Amberlite IR-4B (acetate form, approximately 5 g) for 30 min with stirring. The solution , after stirring for 30 min, was filtered, the filtrate was lyophilized to give fluffy powder (125 mg). This was dissolved in 3% AcOH and the solution was applied to a column of Sephadex G-25 (2.6×55 cm), which was eluted with 3% AcOH. Individual fractions collected (6 ml each) were examined by UV absorbancy at 275 mμ. Fractions corresponding to the front main peak (tube No. 18—32) were collected and the solvent was removed by lyophilization. The resulting powder was dissolved in a small amount of the upper phase of the solvent system consisting of *n*-BuOH–AcOH–H_2O (4:1:5). The solution was applied to a column of Sephadex G-25 (2.6×55 cm) equilibrated previously with the lower phase of the above solvent system. The column was developed with the same upper phase and individual fractions (10 ml each) were collected. Examination of the ultraviolet spectrum (UV) absorbancy at 275 mμ revealed the presence of a main peak (tube No. 9—18) and two minor peaks. Fractions corresponding to the main peak were collected and the solvent was removed by evaporation and the residue was lyophilized from 3% AcOH to give fluffy white powder; yield 81 mg (51%), $[\alpha]_D^{26}$ $-69.1°$ ($c=0.4$, 3% AcOH). Rf_4 0.54. Amino acid ratios in an acid hydrolysate: Arg 1.19, Pro 2.85, Val 1.83, Lys 0.99, Tyr 0.79, Asp 2.07, Gly 1.00, Ala 2.94, Glu 4.15, Leu 1.85, Phe 1.95 (average recovery 94%). Amino acid ratios in a AP-M digest (enzyme/substrate=2 IU/1 μmol at pH 7.73): Arg 0.94, Pro 3.20, Val 2.19, Lys 0.94, Tyr 1.10, Asn 1.09, Gly 1.00, Ala 2.74, Asp 0.85, Glu 4.12, Leu 2.09, Phe 2.16 (average recovery 81%). *Anal.* Calcd. for $C_{115}H_{171}O_{35}N_{27}\cdot 2CH_3COOH\ 5H_2O$: C, 52.90; H, 7.05; N, 14.00. Found: C, 52.99; H, 6.95; N, 14.20.

Acknowledgement The authors express their sincere appreciation to Prof. H. Imura and Dr. Y. Hirata of the Internal Medicine, Kobe University, School of Medicine, for the radioimmunoassay of our synthetic peptides.