Synthesis of Sperm Whale Myoglobin-(77—96)-Eicosapeptide and Circular Dichroism Spectra of the Related Peptides[1)]

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Sperm whale myoglobin-(91—96)- (**6a**), -(85—90)- (**7a**), -(77—84)- (**8**), -(85—96)- (**9a**), and -(77—96)- (**10**) peptides were prepared. The products, derived from protected myoglobin-(91—96)-, -(85—90)-, and -(85—96)-peptides, partly changed into peptides containing a pyroglutamic acid residue in the course of purification by various forms of column chromatography. The circular dichroism (CD) spectra of free peptides **6a, 7a, 8, 9a,** and **10** in 0.10 M phosphate buffers at pH 4.00, 6.50, and 8.50 were not typical of the helical structure. However, the CD spectra of peptides **7a, 9a,** and **10** in 60% 2,2,2-trifluoroethanol–0.10 M phosphate buffers at the same pHs showed profiles characteristic of a helical structure.

In the previous paper,[2)] we reported the synthesis of a protected Mb-(77—96)-eicosapeptide (**5**) and the CD spectra of the related protected peptides. The CD spectra of a protected Mb-(85—96)-peptide (**4**) and **5** in TFE suggested that the helical structure of these peptides is stabilized by contributions not only from the solvent (TFE), but also from hydrophobic interactions among the hydrophobic protecting groups and side chains arranged spatially near to each other.

Several studies have been reported concerning the conformation of sperm whale Mb-fragments in water and/or in water–methanol by means of CD and/or ORD.[3-6)] The results showed that short-range and long-range interactions play an important role in stabilizing the α-helical structure of the fragment peptides and Mb in solutions.

This paper reports on the preparation of an Mb-(77—96)-eicosapeptide (**10**) and the intermediate protecting group-free peptides together with their CD spectra. The factors regarding helix formation are discussed on the basis of those spectra.

Results and Discussion

Final Deprotection of Protected Peptides. Five protecting group-free sperm whale Mb-(91—96)- (**6a**), -(85—90)- (**7a**), -(77—84)- (**8**), -(85—96)- (**9a**) peptides, and **10** were prepared from the corresponding protected sperm whale Mb-(91—96)- (**1**), -(85—90)- (**2**), -(77—84)- (**3**) peptides, **4**, and **5** (Table 1).[2)] All of the protecting groups of protected peptides **1**, **2**, and **4** were removed by a treatment with methanesulfonic acid.[7)] The products obtained from **1** and **4** were treated with 0.50 M (1 M=1 mol dm^{-3}) aq NH_3 in order to reverse possible acyl migration from the nitrogen atom of the amide to oxygen atoms in the side chains of Ser and Thr residues.[8)] Compound **3** was treated with TFA, and then hydrogenated over palladium black catalyst under a hydrogen atmosphere. All of the protecting groups of **5** were removed by treating with 10% pyridine–HF[9-11)] containing anisole. All of the deprotected peptides were converted to the corresponding acetate form by passing through an ion-exchange column (Dowex 1×4); the

Table 1. The Structures of Protected and Free Fragment Peptides

Compound
Protected fragment peptides[a)]
Boc–Gln–Ser(Bzl)–His–Ala–Thr–Lys(Z)–OBzl (**1**)
Boc–Glu(OBzl)–Leu–Lys(Z)–Pro–Leu–Ala–OH (**2**)
Boc–Lys(Z)–Lys(Z)–Lys(Z)–Gly–His–His–Glu(OBzl)–Ala–OH (**3**)
Boc–Glu(OBzl)–Leu–Lys(Z)–Pro–Leu–Ala–Gln–Ser(Bzl)–His–Ala–Thr–Lys(Z)–OBzl (**4**)
Boc–Lys(Z)–Lys(Z)–Lys(Z)–Gly–His–His–Glu(OBzl)–Ala–Glu(OBzl)–Leu–Lys(Z)–Pro–Leu–Ala–Gln–Ser(Bzl)–His–Ala–Thr–Lys(Z)–OBzl (**5**)
Free fragment peptides
H–Gln–Ser–His–Ala–Thr–Lys–OH (**6a**)
pGlu–Ser–His–Ala–Thr–Lys–OH (**6b**)
H–Glu–Leu–Lys–Pro–Leu–Ala–OH (**7a**)
pGlu–Leu–Lys–Pro–Leu–Ala–OH (**7b**)
H–Lys–Lys–Lys–Gly–His–His–Glu–Ala–OH (**8**)
H–Glu–Leu–Lys–Pro–Leu–Ala–Gln–Ser–His–Ala–Thr–Lys–OH (**9a**)
pGlu–Leu–Lys–Pro–Leu–Ala–Gln–Ser–His–Ala–Thr–Lys–OH (**9b**)
H–Lys–Lys–Lys–Gly–His–His–Glu–Ala–Glu–Leu–Lys–Pro–Leu–Ala–Gln–Ser–His–Ala–Thr–Lys–OH (**10**)

a) Ref. 2.

acetates were purified by various chromatographic methods.

A side reaction occurred while purifying the free peptide derived from **1**. Immediately after a treatment of **1** with methanesulfonic acid, the product gave two spots of a major component (A) and a minor one (B) on TLC. During the course of purification by the above-mentioned column chromatography, the amount of component A decreased, whereas that of the component B increased. Components A and B were separately isolated as a homogeneous material. However, component A was obtained in only minute quantity. Amino acid analysis of an acid hydrolyzate of component B exhibited the presence of glutamic acid, but not ammonia. Moreover, an amino acid analysis of an AP-M digest of component B did not show the presence of glutamine or serine. These results suggest that component B contains a cyclic imide produced by a ring closure between the α-amino group and the γ-amide group of the glutamine residue at the N-terminal position. An FAB mass analysis of component B showed an MH^+ ion corresponding to **6b**. Component A was confirmed as being the desired peptide **6a** which possesses a glutamine residue at the N-terminal position, based on the results of the same analyses mentioned above. However, small amounts (4%) of **6a** changed to **6b**, even during the course of concentration of the eluate after purification by reversed-phase HPLC. It has been reported that acetic acid acts as a catalyst in the deamination-cyclization reaction of glutamine,[12,13] and that the formation of pyroglutamic acid is catalyzed by the imidazole ring of a neighboring histidine residue.[13-15] In the present study, this side reaction occurred easily during the course of purification by column chromatography at room temperature; that is, it may be attributed not only to the influence of acetic acid in the eluent, but also to the effect by the imidazole ring.

Other examples of a similar side reaction were observed in the preparations of **7a** and **9a**. By-products, **7b** and **9b**, respectively contain pyroglutamic acid residue which was formed by dehydration of glutamic acid residue at the N-terminal positions of **7a** and **9a**.[13,16,17] The structures of these compounds were also confirmed by FAB mass and amino acid analysis after acid hydrolysis and AP-M digestion.

Peptides **6b**, **7b**, and **9b**, containing pyroglutamic acid residue at the N-terminal position, increase their retention time on a reversed-phase HPLC column relative to the corresponding uncyclized peptides: **6a**, **7a**, and **9a** (Fig. 1A—C). The longer retention times may be due to an increase in the hydrophobicity of the cyclized peptides.[18] The purity of all the protecting group-free fragment peptides was assured by reversed-phase HPLC. The HPLC profile of the final peptide **10** is indicated in Fig. 1D.

The present study shows that the formation of pyroglutamic acid in **6a**, **7a**, and **9a** containing glutamine or glutamic acid residue at the N-terminal position is brought about by the use of a solvent system which includes acetic acid in the purification process. The yield of **9a** was increased by using a solvent system containing a strong acid, such as TFA, instead of acetic acid.

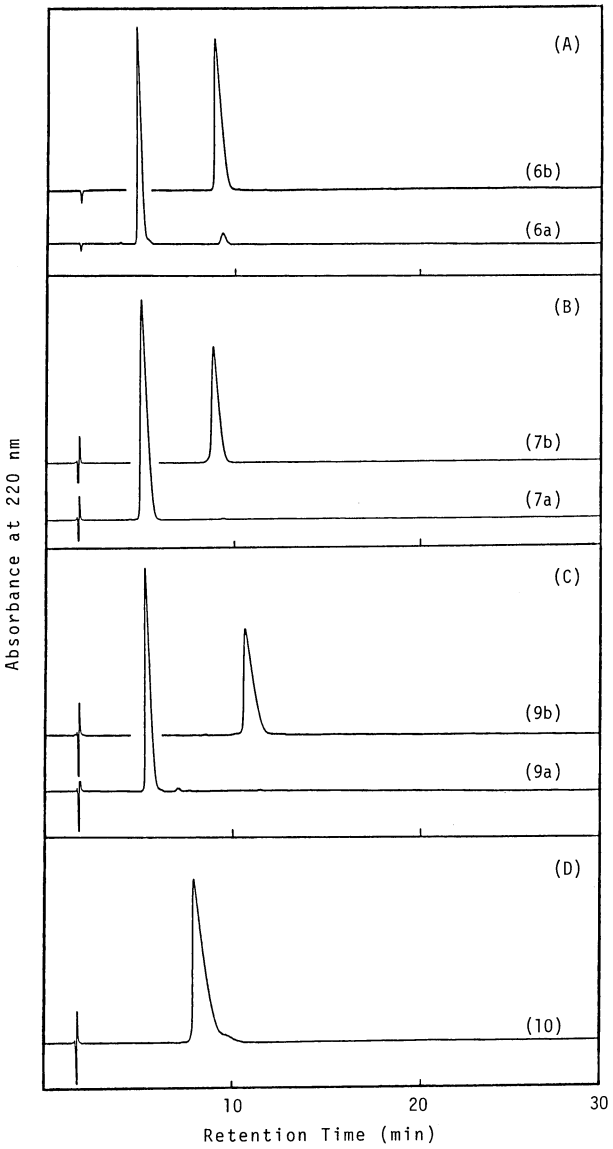

Fig. 1. HPLC profiles. (A) Compounds **6a** and **6b**: Eluent, 0.5% CH_3CN/H_2O–0.1% TFA; (B) Compounds **7a** and **7b**: Eluent, 15% CH_3CN/H_2O–0.1% TFA; (C) Compounds **9a** and **9b**: Eluent, 13% CH_3CN/H_2O–0.1% TFA; (D) Compound **10**: Eluent, 13% CH_3CN/H_2O–0.1% TFA. Column: Waters μ-Bondasphere 5μ C18-300 Å (3.9×150 mm); column temperature: 40°C; flow rate: 1.0 ml min^{-1}; detection: absorbance at 220 nm.

It is worthwhile mentioning that the "AP-M" digestion of peptides **6b**, **7b**, and **9b** resulted in a cleavage of the amide bond between the second and the third residue from the N-terminus in these peptides.

Circular Dichroism Spectra. The CD spectra were

measured in the 190—270 nm region. The free peptides were dissolved in 0.10 M phosphate buffers and 60% TFE–0.10 M phosphate buffers at pH 4.00, 6.50, and 8.50. The CD data are indicated as mean residue ellipticity ($[\theta]$) which are expressed in degrees square centimeters per decimole. For calculating the helical content, the value of $[\theta]_{222}$ for a completely helical structure, −32000, was used.[4] The theoretical helicity of a fragment peptide is expressed by the proportion of the number of amino acid residues located in the helical part to the total number of residues in the peptide, on the basis of the known three-dimensional structure of Mb. In the amino acid sequence of positions 77—96 of Mb, -Lys-Lys-Lys-Gly-His-His-Glu-Ala-Glu-Leu-Lys-Pro-Leu-Ala-Gln-Ser-His-Ala-Thr-Lys-, the underlined residues take part in a helical structure, according to the result of an X-ray analysis.[19,20]

As for the CD spectra in a 0.10 M phosphate buffer (pH 6.50), peptides **6a**, **7a**, and **8** exhibit a small peak, or a shoulder, near 220 nm and a trough near 195 nm (Fig. 2). These spectra are not of helical structure. The spectra of peptides **9a** and **10** in a 0.10 M phosphate buffer (pH 6.50) show a small trough near 230 nm, a small peak near 222 nm, and a trough near 200 nm (Figs. 3 and 4). Even if a calculation is made on the basis of the assumption that all of the $[\theta]_{222}$ values of those five peptides result from a helical structure, the helical contents of those peptides are estimated only as a few percent. These results show that those small peptides can hardly form a helical structure in an aqueous solution.

Regarding the CD spectra in a 60% TFE–0.10 M

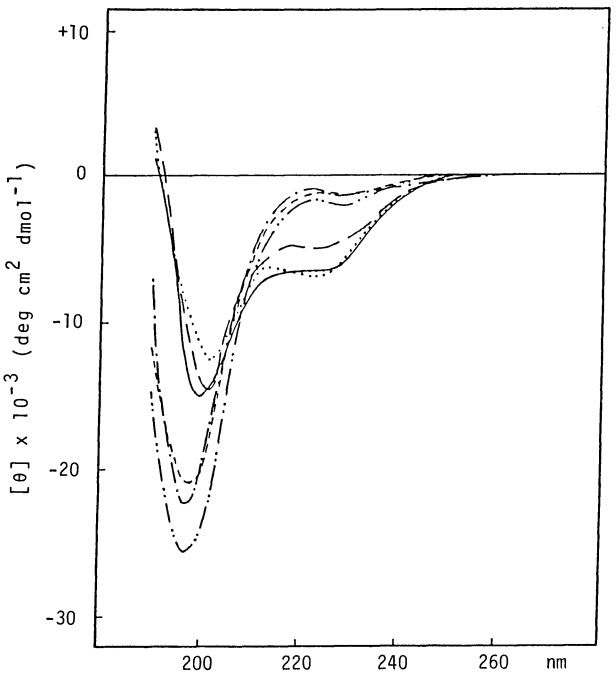

Fig. 3. CD spectra of **9a**; —·—· at pH 4.00, ------ at pH 6.50, and —··—·· at pH 8.50 in 0.10 M phosphate buffers; – – – at pH 4.00, —— at pH 6.50, and ····· at pH 8.50 in 60% TFE–0.10 M phosphate buffers.

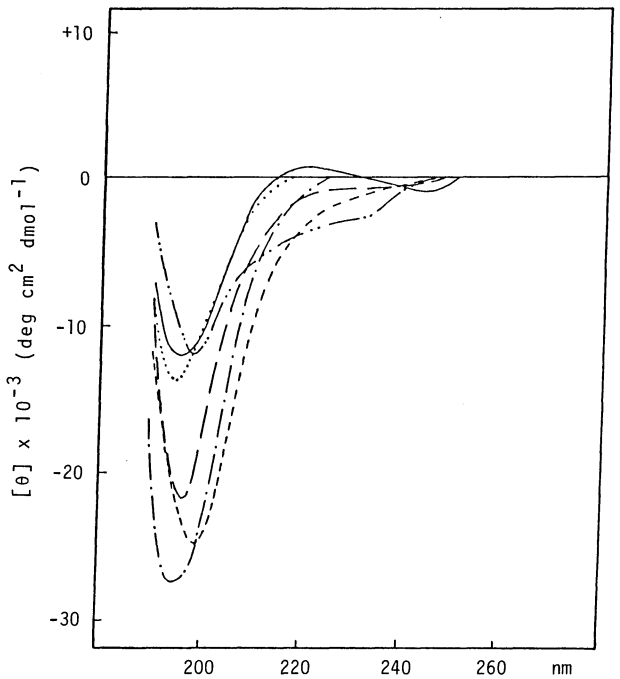

Fig. 2. CD spectra of peptides **6a** (—·—·), **7a** (------), and **8** (——) in 0.10 M phosphate buffer (pH 6.50) and those of peptides **6a** (– – –), **7a** (—··—··), and **8** (·····) in 60% TFE–0.10 M phosphate buffer (pH 6.50).

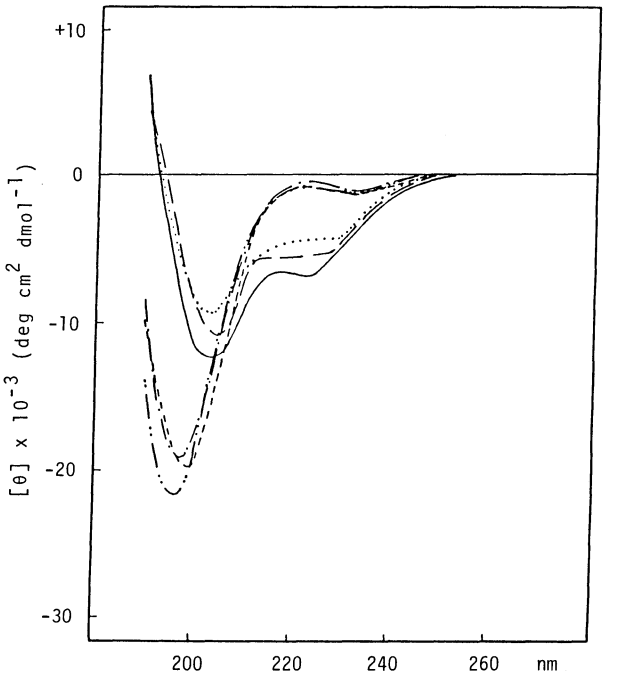

Fig. 4. CD spectra of **10**; —·—· at pH 4.00, ------ at pH 6.50, and —··—·· at pH 8.50 in 0.10 M phosphate buffers; – – – at pH 4.00, —— at pH 6.50, and ····· at pH 8.50 in 60% TFE- 0.10 M phosphate buffers.

phosphate buffer (pH 6.50), peptides **6a** and **8** do not indicate any characteristic band other than a trough near 195 nm (Fig. 2). It is reasonable that peptides **6a** and **8** do not take the helical structure, since both of these peptides are small and **8** also belongs a nonhelical part of Mb. Although **7a** is also a short peptide, the spectrum suggests the existence of a helical structure (Fig. 2). The spectra of peptides **9a** and **10** in a 60% TFE–0.10 M phosphate buffer (pH 6.50) indicate a trough or a shoulder near 222 nm and a trough near 200 nm (Figs. 3 and 4). These spectra show an increase in the negative ellipticity near 222 nm, and a decrease in negative ellipticity near 200 nm. The results clearly indicate the presence of a helical structure. The $[\theta]_{222}$ values of peptides **7a**, **9a**, and **10** in a 60% TFE–0.10 M phosphate buffer (pH 6.50) are −3500, −6333, and −6900, respectively. From these data, the helicities of peptides **7a**, **9a**, and **10** were calculated to be 10.9% (theoretical helicity; 83%), 19.8% (theoretical helicity; 75%), and 21.6% (theoretical helicity except Lys^{77}; 45%), respectively, although the helicities of the three peptides are much lower than values expected from the three-dimensional structure proposed by X-ray analysis.[19,20] These results show that those peptides including one helical part (Leu^{86}—Ala^{94}) of Mb can form helical structures by adding TFE to 0.10 M phosphate buffers.

The increase in the helical content accompanied with an elongation of the peptide chain was not observed for peptides **6a**, **9a**, and **10** in a 0.10 M phosphate buffer (pH 6.50). A helical ratio has been used for comparing the helicities of the fragment peptides of different size.[5] The helical ratios of peptides **9a** and **10** in a 60% TFE–0.10 M phosphate buffer (pH 6.50) are estimated to be 0.26 and 0.48, respectively. Although peptide **10** was prepared by a combination of peptide **8**, including a nonhelical part, with peptide **9a** containing the F helical region (Leu^{86}—Ala^{94}), the helical content of **10** was larger than that of **9a**. As described in a previous paper,[2] this result also suggests the importance of the interaction among amino acid residues located remotely from each other in the primary structure.

An α-helical wheel[21] of the F region is shown in Fig. 5. As it indicates, the hydrophobic amino acid residues are mostly located on one side onto the plane perpendicular to the helix axis. The addition of TFE (a noninteracting solvent)[22] to 0.10 M phosphate buffers resulted in a stabilization of the helical structure of the F region by enhancing the interaction among these hydrophobic amino acid residues.

The helical content (22%) of free peptide **10** in 60% TFE–0.10 M phosphate buffer (pH 6.50) is less than that (26%)[2] of the corresponding protected peptide **5**. A comparison of the CD profiles between peptides, **5** and **10**, suggests that the former peptide has more helical property than does the latter. These results may be elucidated as follows: in peptide **5**, the interaction among the hydrophobic protecting groups at the side chains of

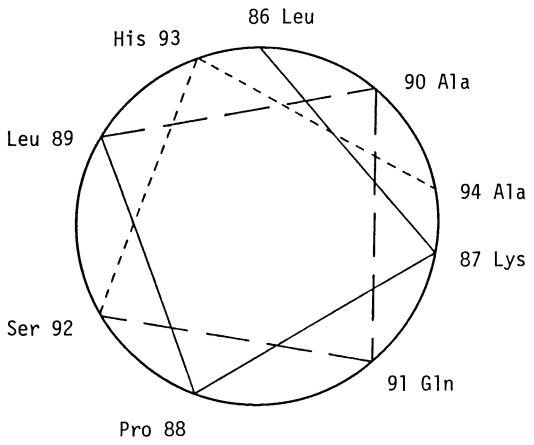

Fig. 5. The α-helical wheel of F region (Leu^{86}—Ala^{94}).

Lys^{87} and Ser^{96} and the neighboring hydrophobic side chains of other residues results in a stabilization of helical structure.

The pH dependency of the helical content of peptides **9a** and **10** was also measured at pH 4.00 and 8.50. The spectra of peptides **9a** and **10** in 0.10 M phosphate buffers (pH 4.00 and 8.50) (Figs. 3 and 4) are similar to those spectra in a 0.10 M phosphate buffer (pH 6.50). Both of the peptides have a nearly disordered structure under these conditions. In 60% TFE–0.10 M phosphate buffers, the $[\theta]_{222}$ values of peptide **9a** increased with increasing pH (Fig. 3). Under the same conditions, the $[\theta]_{222}$ value of peptide **10** at pH 6.50 was larger than those at pH 4.00 and 8.50 (Fig. 4). These results suggested that the differences in ionization-state at the side chains of the Glu and Lys residues in those molecules affect the stability of the helical structure. This consideration may be supported by the suggestion that the stability of the helical structure depends on the charged groups in a peptide.[23,24]

It has been reported regarding helical sequences that acidic amino acid residues occur in high frequency near the N-terminus, whereas basic amino acid ones occur near the C-terminus.[23] Peptides **9a** and **10** possess acidic and basic amino acid residues near both ends of the helical segment, that is, sequences of these peptides seem to be preferable for the formation of a helical structure.

Experimental

The optical rotation was determined with a DIP-140 type or a DIP-370 digital (JASCO) polarimeter. Amino acid analysis was performed by a JLC-6AS automatic analyzer (JEOL) or an L-8500 amino acid analyzer (Hitachi) after acid hydrolysis with 6 M HCl in a sealed tube at 110 °C for 20 h or AP-M (Pierce or Sigma Co.) digestion at 37 °C for 24 h. The theoretical values of the amino acid ratios are shown in parentheses after each results.

Thin-layer chromatography was performed on TLC plates of silica gel (Kieselgel 60 F_{254}; Merck and Co.) and cellulose (Avicel SF) using the following solvent systems (volume ratios):

R_f^1, 1-butanol–acetic acid–water (4 : 1 : 1); R_f^2, 1-butanol–pyridine–acetic acid–water (4 : 1 : 1 : 2); R_f^3, 1-butanol–pyridine–acetic acid–water (16 : 3 : 6 : 12); R_f^4, 1-butanol–pyridine–acetic acid–water (8 : 3 : 3 : 6); R_f^5, 1-butanol–pyridine–acetic acid–water (16 : 10 : 3 : 12).

Evaporation of the solvent was carried out under reduced pressure at below 40°C.

Reversed-phase HPLC was carried out with a Tri Rotar VI HPLC system (JASCO) on an analytical column (3.9×150 mm; flow rate, 1.0 ml min^{-1}) and a preparative column (19×150 mm; flow rate, 5.0 ml min^{-1}) using a Waters μ-Bondasphere 5μ C18-300 Å resin. The column temperature was kept at 40°C. The elution of peptides was monitored at 220 nm with a Sic 7000 B recorder.

CD measurements were performed in a J-500 A spectropolarimeter with a DP-500 N Data Processor at 21—23°C. A cell with 0.1-mm light path was used. Measurements of each peptide were carried out in solutions at concentration of 5.0×10^{-4} M. Phosphate buffers of 0.10 M NaH_2PO_4–0.10 M H_3PO_4 (pH 4.00), 0.10 M NaH_2PO_4–0.10 M Na_2HPO_4 (pH 6.50), and 0.10 M NaH_2PO_4–0.10 M Na_2HPO_4 (pH 8.50) were employed as a solvent.

H-Gln-Ser-His-Ala-Thr-Lys-OH (6a). Compound **1** (150 mg) was treated with methanesulfonic acid (1.5 ml) in the presence of anisole (0.045 ml) for 30 min at room temperature. After the addition of diethyl ether to the solution, the precipitate was dried over NaOH pellets in vacuo. It was then dissolved in 0.50 M aq NH_3 (6.0 ml), and the solution pH was adjusted to 9.0. After being stirred for 15 min in an ice-bath, the solution was applied to an ion-exchange column (1.7×16.7 cm; Dowex 1×4; acetate form) with 20% acetic acid (100 ml). The eluate was pooled and concentrated. The TLC of the residue showed the presence of a major component (A) and a minor component (B). The residue was dissolved in 20% acetic acid (2.0 ml). The solution was applied to a Sephadex G-10 column (1.7×105.8 cm) with the same solvent. Fractions 18—28 (5.0 g each) were combined and the solvent was evaporated. TLC of the residue showed an increase in the amount of component B, and a decrease in component A. After the residue was dissolved in 1-butanol–pyridine–acetic acid–water (8 : 3 : 3 : 6) (2.0 ml), it was subjected to a Sephadex G-25 column (1.7×31.1 cm) using the same solvent system. Fractions of 5.0 g each were collected. The eluates in fractions 26—34 contained only component B, but those in fractions 38—55 contained components A and B.

The former fractions containing component B were combined and the solvent was evaporated. The product was dissolved in water and lyophilized; 82.12 mg (78%). Component B was identified as being pGlu–Ser–His–Ala–Thr–Lys–OH (**6b**) from the following analytical data. Yield 86%; $[\alpha]_D^{24}$ −50.81° (*c* 1.0, H_2O); R_f^2 0.12, R_f^3 0.32, R_f^4 0.29 (silica); R_f^2 0.26, R_f^3 0.56, R_f^4 0.54 (cellulose). Amino acid ratios in acid hydrolyzate: Thr 0.95 (1), Ser 0.96 (1), Glu 1.10 (1), Ala 0.97 (1), His 1.00 (1), Lys 1.02 (1). Amino acid ratios in AP-M digest: Thr 1.02 (1), Ala 0.99 (1), His 0.99 (1), Lys 1.00 (1). Found: C, 43.46; H, 7.11; N, 15.58%. Calcd for $C_{27}H_{43}O_{10}N_9\cdot CH_3COOH\cdot5H_2O$: C, 43.33; H, 7.15; N, 15.68%. FAB mass *m/z* 654 (MH^+, $C_{27}H_{44}O_{10}N_9$).

The latter fractions containing the two components were combined and concentrated. The crude material (48.13 mg) was further purified on a preparative reversed-phase column using 2% CH_3CN/H_2O–0.1% TFA as an eluent. Eluates (retention time 18.2—23.4 min) containing **6b** were collected and lyophilized; 9.77 mg (8%). On the other hand, eluates (retention time 13.3—16.1 min) including component A were collected and the solvent evaporated. The residue was then dissolved in water and lyophilized. However, 4% of this purified peptide changed to **6b** during solvent evaporation. Component A was identified as being a desired peptide **6a** from the analytical data described below. Yield 10.08 mg (7%); $[\alpha]_D^{24}$ −32.2° (*c* 1.34, H_2O); R_f^2 0.07, R_f^3 0.27, R_f^4 0.19 (silica); R_f^2 0.18, R_f^3 0.45, R_f^4 0.35 (cellulose). Amino acid ratios in acid hydrolyzate: Thr 0.97 (1), Ser 0.92 (1), Glu 1.05 (1), Ala 1.02 (1), His 1.02 (1), Lys 1.02 (1). Amino acid ratios[25] in AP-M digest: Thr 1.02 (1), Ser 0.95 (1), Gln 0.23 (1), Ala 1.01 (1), His 0.99 (1), Lys 1.02 (1). Found: C, 35.87; H, 4.66; N, 12.52%. Calcd for $C_{27}H_{46}O_{10}N_{10}\cdot4CF_3COOH\cdot2H_2O$: C, 36.15; H, 4.68; N, 12.05%. FAB mass: *m/z* 671 (MH^+, $C_{27}H_{47}O_{10}N_{10}$).

H-Glu-Leu-Lys-Pro-Leu-Ala-OH (7a). Compound **2** (150 mg) was treated with methanesulfonic acid (5.0 ml)–anisole (0.328 ml) for 30 min at room temperature. To the solution was added diethyl ether. The residue was changed to the acetate form by using an ion-exchange column (11.7×10.5 cm; Dowex 1×4) with 10% acetic acid (70 ml). After the solution was concentrated, the residue was purified by repeated chromatography: 1st, a silica-gel column (1.7×24.8 cm; Kieselgel 60; 230—400 mesh) with 1-butanol–pyridine–acetic acid–water (16 : 10 : 3 : 12); 2nd, a Sephadex G-25 column (1.6×34.5 cm) with the same solvent system. The weight of the product was 65.53 mg. A part of the product (51.91 mg) was further purified on a preparative reversed-phase column using 15% CH_3CN/H_2O–0.1% TFA as an eluent.

The eluates (retention time 19.9—29.4 min) were collected and concentrated. The residue was dissolved in water and lyophilized; 44.51 mg. This compound was confirmed as being **7a** from the following analytical data. Yield 37%; $[\alpha]_D^{24}$ −74.8° (*c* 1.35, H_2O); R_f^1 0.20, R_f^2 0.40, R_f^5 0.43 (silica); R_f^2 0.69, R_f^5 0.67 (cellulose). Amino acid ratios in acid hydrolyzate: Glu 1.04 (1), Pro 0.98 (1), Ala 0.99 (1), Leu 2.00 (2), Lys 0.99 (1). Amino acid ratios in AP-M digest: Glu 1.01 (1), Pro 0.94 (1), Ala 1.00 (1), Leu 2.04 (2), Lys 1.01 (1). Found: C, 43.58; H, 6.14; N, 10.06%. Calcd for $C_{31}H_{55}O_9N_7\cdot3CF_3COOH\cdot1/2H_2O$: C, 43.53; H, 5.82; N, 9.60%. FAB mass: *m/z* 670 (MH^+, $C_{31}H_{56}O_9N_7$).

The eluates (retention time 43.2—49.8 min) were collected and lyophilized; 9.39 mg. It was ascertained as pGlu–Leu–Lys–Pro–Leu–Ala–OH (**7b**) from the analytical data described below. Yield 8%; $[\alpha]_D^{24}$ −96.1° (*c* 1.27, H_2O); R_f^1 0.38, R_f^2 0.51, R_f^5 0.56 (silica); R_f^2 0.85, R_f^5 0.83 (cellulose). Amino acid ratios in acid hydrolyzate: Glu 1.02 (1), Pro 1.01 (1), Ala 0.97 (1), Leu 2.02 (2), Lys 0.98 (1). Amino acid ratios in AP-M digest: Pro 0.96 (1), Ala 1.01 (1), Leu 1.08 (2), Lys 1.03 (1). Found: C, 45.93; H, 6.41; N, 11.11%. Calcd for $C_{31}H_{53}O_8N_7\cdot2CF_3COOH\cdot2H_2O$: C, 45.90; H, 6.49; N, 10.71%. FAB mass *m/z* 652 (MH^+, $C_{31}H_{54}O_8N_7$).

H-Lys-Lys-Lys-Gly-His-His-Glu-Ala-OH (8). Compound **3** (200 mg) was treated with TFA (10 ml)–anisole (0.071 ml) at room temperature for 1.0 h. After solvent evaporation, the residue was triturated with diethyl ether. The precipitate dissolved in 50% acetic acid (15 ml) was hydrogenated over palladium black (300 mg) under a hydrogen atmosphere for 3 d at room temperature. After removing the catalyst, the solvent was evaporated. The residue was changed to the acetate form using an ion-exchange column

(1.7×10.5 cm; Dowex 1×4) with 20% acetic acid (100 ml). The acetate was further purified by a Sephadex G-25 column (1.7×36.3 cm) with 1-butanol–pyridine–acetic acid–water (16 : 3 : 6 : 12). Fractions 49—64 (5.0 g each) were combined and the solvent was evaporated. The residue was dissolved in water and lyophilized. Yield 75.42 mg (44%); $[\alpha]_D^{24}$ −28.61° (*c* 1.0, H_2O); R_f^3 0.11 (silica); R_f^3 0.41, R_f^5 0.08 (cellulose). Amino acid ratios in acid hydrolyzate: Glu 1.07 (1), Gly 0.99 (1), Ala 0.98 (1), His 2.01 (2), Lys 2.95 (3). Amino acid ratios in AP-M digest: Glu 1.15 (1), Gly 0.95 (1), Ala 0.95 (1), His 1.96 (2), Lys 2.99 (3). Found: C, 43.14; H, 7.48; N, 15.48%. Calcd for $C_{40}H_{67}O_{11}N_{15}$·4CH_3COOH·9H_2O: C, 43.14; H, 7.62; N, 15.72%.

H-Glu-Leu-Lys-Pro-Leu-Ala-Gln-Ser-His-Ala-Thr-Lys-OH (9a). Method (A). Compound **4** (150 mg) was treated with methanesulfonic acid (1.5 ml)–anisole (0.05 ml) at room temperature. After stirring for 50 min, the mixture was triturated with diethyl ether. The precipitate was dissolved in 0.50 M aq NH_3 (5.0 ml) and the solution (pH 9.0) was stirred for 15 min in an ice-bath. The crude product was changed to the acetate form using an ion-exchange column (1.7×17.5 cm; Dowex 1×4). The acetate was purified by column chromatographies: 1st, a silica-gel column (1.5×37.0 cm; Kieselgel 100; 70—230 mesh; repeated twice) with 1-butanol–pyridine–acetic acid–water (16 : 3 : 6 : 12); 2nd, a Sephadex G-10 column (1.7×104.2 cm) with 20% acetic acid; 3rd, a Sephadex G-25 column (1.7×36.3 cm; collected, 5.0 g each) with 1-butanol–pyridine–acetic acid–water (16 : 10 : 3 : 12).

Fractions 18—30 in the 3rd chromatography were combined and the solvent removed. The residue was dissolved in water and lyophilized; 7.40 mg. This material was further purified on a preparative reversed-phase column using 15% CH_3CN/H_2O–0.1% TFA as an eluent. The eluates (retention time 25.3—30.5 min) were combined and the solvent was evaporated. The residue was dissolved in water and lyophilized; 2.56 mg. This compound was confirmed as being pGlu–Leu–Lys–Pro–Leu–Ala–Gln–Ser–His–Ala–Thr–Lys–OH (**9b**) from the analytical data described below. R_f^2 0.28, R_f^3 0.52 (silica); R_f^2 0.55, R_f^3 0.75, R_f^5 0.59 (cellulose). Amino acid ratios in acid hydrolyzate: Thr 0.95 (1), Ser 0.92 (1), Glu 2.15 (2), Pro 1.00 (1), Ala 2.04 (2), Leu 1.99 (2), His 0.97 (1), Lys 1.99 (2). Amino acid ratios[25] in AP-M digest: Thr 1.01 (1), Ser 1.00 (1), Gln 0.24 (1), Pro 0.98 (1), Ala 2.02 (2), Leu 1.05 (2), His 0.96 (1), Lys 2.03 (2). FAB mass: *m*/*z* 1304 (MH^+, $C_{58}H_{98}O_{17}N_{17}$).

Fractions 46—60 in the 3rd chromatography were combined and the solvent removed. The residue dissolved in 20% acetic acid (2.0 ml) was subjected to a Sephadex G-10 column (1.7×104.2 cm) using the same solvent. Fractions 19—25 (5.0 g each) were combined and the solvent evaporated. The residue was dissolved in water and lyophilized; 39.75 mg. A part of the product (30.0 mg) was purified on a preparative reversed-phase column using 15% CH_3CN/H_2O–0.1% TFA as an eluent. The eluates (retention time 15.1—17.9 min) were collected and the solution concentrated. The residue was dissolved in water and lyophilized; 20.11 mg. This compound was ascertained as desired peptide **9a** from the following analytical data. Yield 15%; $[\alpha]_D^{24}$ −69.4° (*c* 1.30, H_2O); R_f^2 0.17, R_f^3 0.50 (silica); R_f^2 0.43, R_f^3 0.70, R_f^5 0.45 (cellulose). Amino acid ratios in acid hydrolyzate: Thr 0.94 (1), Ser 0.90 (1), Glu 2.12 (2), Pro 1.05 (1), Ala 2.01 (2), Leu 1.98 (2), His 0.99 (1), Lys 2.00 (2). Amino acid ratios[25] in AP-M digest: Thr 1.00 (1), Ser 1.01 (1), Glu 1.03 (1), Gln 0.24 (1), Pro 0.90 (1), Ala 1.99 (2), Leu 2.08 (2), His 0.99 (1), Lys 2.03 (2). Found: C, 38.91; H, 4.81; N, 9.87%. Calcd for $C_{58}H_{99}O_{18}N_{17}$·8CF_3COOH·4H_2O: C, 38.53; H, 5.02; N, 10.32%. FAB mass: *m*/*z* 1322 (MH^+, $C_{58}H_{100}O_{18}N_{17}$).

Method (B). Compound **4** (80 mg) was treated with methanesulfonic acid (1.5 ml) and anisole (0.040 ml) for 60 min at room temperature. The mixture was triturated with diethyl ether. The precipitate was dissolved in 0.50 M aq NH_3 (3.0 ml) and the solution (pH 9.0) was stirred for 25 min in an ice-bath. The mixture was applied to an ion-exchange column (1.7×21.0 cm; Dowex 1×4) with 10% acetic acid (100 ml). The eluate was pooled and concentrated, and the residue then lyophilized from water. The crude material was purified on a preparative reversed-phase column using a gradient method (2% CH_3CN/H_2O–0.1% TFA—50% CH_3CN/H_2O–0.1% TFA, 0—100 min; combined, retention time 31.0—33.0 min) and then an isocratic method (14% CH_3CN/H_2O–0.1% TFA: combined, retention time 16.2—20.1 min). Compound **9a** was obtained in a yield 35.43 mg (38%).

H-Lys-Lys-Lys-Gly-His-His-Glu-Ala-Glu-Leu-Lys-Pro-Leu-Ala-Gln-Ser-His-Ala-Thr-Lys-OH (10). Compound **5** (181.5 mg) was treated with 10% pyridine–HF (20 ml) in the presence of anisole (0.117 ml) for 1.0 h at ice-bath temperature. After removing HF, the residue was dried over CaO and NaOH pellets in vacuo. The residue was converted to the acetate form using an ion-exchange column (1.7×15.5 cm; Dowex 1×4; repeated twice) with 20% acetic acid (100 ml). The acetate was purified by column chromatographies: 1st a Sephadex G-10 column (1.7×105.7 cm) with 20% acetic acid; 2nd, a Sephadex G-25 column (1.6×94.8 cm; repeated twice) with 1-butanol–pyridine–acetic acid–water (16 : 3 : 6 : 12). The residue was dissolved in water and the solution lyophilized. Yield 39.62 mg (25%); $[\alpha]_D^{24}$ −76.5° (*c* 1.0, H_2O); R_f^3 0.10 (silica); R_f^2 0.11, R_f^3 0.57, R_f^5 0.13 (cellulose). Amino acid ratios in acid hydrolyzate: Thr 0.93 (1), Ser 0.90 (1), Glu 3.06 (3), Pro 1.17 (1), Gly 1.01 (1), Ala 2.92 (3), Leu 2.01 (2), His 2.97 (3), Lys 5.02 (5), NH_3 1.26 (1). Amino acid ratios[26] in AP-M digest: Thr+Gln 1.93 (2), Ser 1.01 (1), Glu 2.16 (2), Pro 0.42 (1), Gly 1.02 (1), Ala 2.98 (3), Leu 2.15 (2), His 3.02 (3), Lys 4.65 (5). Found: C, 44.46; H, 7.62; N, 15.27%. Calcd for $C_{98}H_{164}O_{28}N_{32}$·6$CH_3COOH$·20$H_2O$: C, 44.65; H, 7.77; N, 15.15%.

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nol; ORD, optical rotatory dispersion; TFA, 2,2,2-trifluoroacetic acid; TLC, thin-layer chromatography; AP-M, aminopeptidase M; FAB, fast atom bombardment; HPLC, high-performance liquid chromatography; pGlu, pyroglutamic acid.

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