Synthesis and Circular Dichroism Spectra of Sperm Whale Myoglobin-(57—96)-Tetracontapeptide[1)]

Chikao HASHIMOTO* and Ichiro MURAMATSU†
Department of Chemistry, The Jikei University School of Medicine, 8-3-1, Kokuryo-cho, Chofu-shi, Tokyo 182
† Department of Chemistry, College of Science, Rikkyo University, Toshima-ku, Nishi-ikebukuro, Tokyo 171
(Received June 12, 1992)

A protected sperm whale myoglobin-(57—96)-tetracontapeptide (**29**) was synthesized by successive condensations of three fragments, Boc-(70—76)-OH, Boc-(62—69)-OH, and Boc-(57—61)-OH, to a partially protected eicosapeptide ester, H-(77—96)-OBzl. After removal of the protecting groups of **29**, the crude product was purified with reversed-phase high-performance liquid chromatography to yield sperm whale myoglobin-(57—96)-tetracontapeptide (**30**). The circular dichroism spectra showed that this peptide was in a random conformation in 0.10 M phosphate buffer (pH 6.50) and in a 69% α-helix conformation in 60% 2,2,2-trifluoroethanol–0.10 M phosphate buffer (pH 6.50).

Myoglobin is a globular heme protein which plays a role in the storage of oxygen in the cells of muscle. A three-dimensional structure of sperm whale myoglobin was elucidated by means of an X-ray analysis by Kendrew et al.[2,3)] The myoglobin molecule is folded with eight α-helical and seven random coil segments. The E and F helical regions[3)] of sperm whale myoglobin include distal (E7) and proximal (F8) histidine residues which coordinate a heme iron atom and contribute to the physiological functions of myoglobin. It is not yet known whether a fragment peptide consisting of only an E–EF–F segment[3)] forms a three-dimensional structure similar to that of the corresponding region in the Mb molecule, and exhibits a physiological function similar to Mb.

Bayer et al.[4,5)] tried to prepare a protected Mb-(58—73)-hexadecapeptide of the E region and a protected Mb-(81—94)-tetradecapeptide of the F region. They succeeded in obtaining the desired peptide of the E region, but not the peptide of the F region. Several research groups[6—9)] investigated the three-dimensional structure of the fragment peptides obtained from native Mb by chemical and enzymatic degradations. However, it is difficult to obtain arbitrary fragment peptides necessary for systematic investigation by those methods. In previous papers,[10,11)] we reported on the syntheses of protected and free sperm whale Mb-(77—96)-eicosapeptides consisting mainly of the EF–F region and the CD spectra of these peptides and the related fragment peptides. Among the protecting group-free peptides, sperm whale Mb-(85—96)-dodecapeptide and sperm whale Mb-(77—96)-eicosapeptide in 60% TFE–0.10 M (1 M=1 mol dm^{-3}) phosphate buffer (pH 6.50) showed CD spectra patterns characteristic of α-helix, although their helicities were less than 22%.

In the present study, we synthesized a peptide consisting mainly of the E–EF–F region, sperm whale myoglobin-(57—96)-tetracontapeptide (**30**), and investigated the CD spectra of this peptide.

Results and Discussion

Synthesis. A protected tetracontapeptide (**29**), Boc-(57—96)-OBzl, was synthesized from four fragment peptides according to the synthetic route shown in Fig. 1. The synthesis of a protected eicosapeptide (**26**), Boc-(77—96)-OBzl, was reported in the previous paper.[10)] The other three fragment peptides were prepared by the synthetic schemes illustrated in Figs. 2, 3, 4, and 5.

Protected Heptapeptide (70—76). The synthesis of fragment (**8**), Boc-Thr-Ala-Leu-Gly-Ala-Ile-Leu-OH, is illustrated in Fig. 2. Boc-Ala-Ile-Leu-OMe (**2**) and Boc-Thr-Ala-Leu-Gly-OEt (**5**) were constructed stepwise by the MA method.[12)] Compound **5** was converted to the corresponding acid (**6**), Boc-Thr-Ala-Leu-Gly-OH, by saponification. After removal of the Boc group of **2**, the tripeptide ester was coupled with **6** by EDC[13)] in the presence of HOBt[14)] to afford fragment (**7**), Boc-Thr-Ala-Leu-Gly-Ala-Ile-Leu-OMe. The methyl ester of **7** was hydrolyzed to give **8**. All of the fragment peptides in this sequence were obtained in high yields.

Protected Octapeptide (62—69). The synthesis of fragment (**16**), Boc-Lys(Z)-Lys(Z)-His-Gly-Val-Thr-Val-Leu-OPac, was carried out by the two routes illustrated in Figs. 3 and 4.

In the first route (Fig. 3), the starting material, H-Leu-OPac, was successively elongated by the MA method to produce fragment (**11**), Boc-Val-Thr-Val-Leu-OPac. Compound **9** was purified by chromatography on a silica-gel column using a chloroform-methanol solvent system as eluent.

In the preliminary synthesis of dipeptide **12a**, Boc-His(Boc)-OH and Boc-His(Boc)-N_3 were condensed with H-Gly-OPac[15)] by MA and azide[16)] methods, respectively. The MA method gave many by-products, and the desired peptide could not be isolated. The azide method also gave many by-products. Although a major product was isolated, it was not the expected one and was identified as **12b**[17)] by ^{1}H NMR, IR, FAB-MS, and

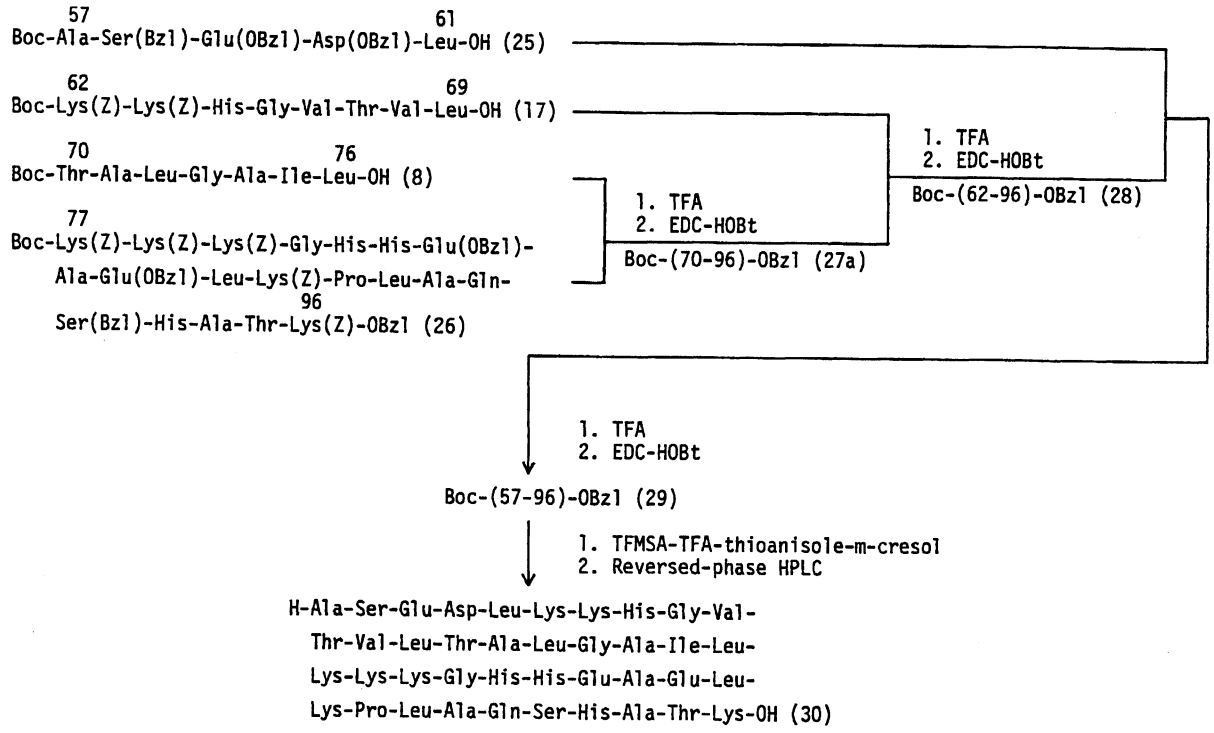


Fig. 1. Synthetic scheme of sperm whale Mb-(57—96)-tetracontapeptide (**30**).

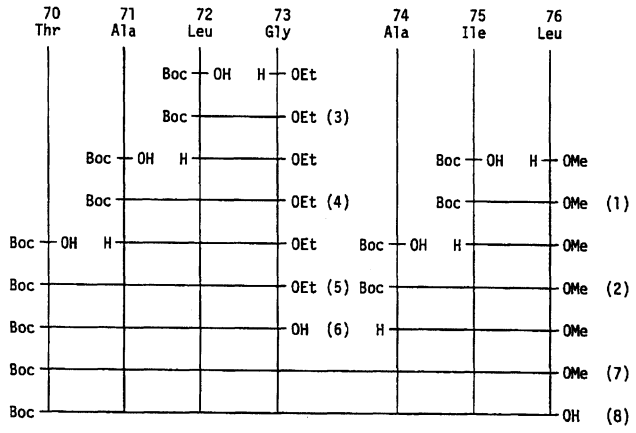
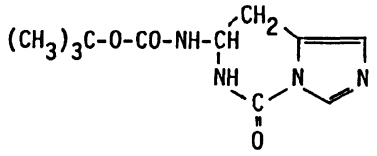


Fig. 2. Synthetic scheme of protected heptapeptide (70—76).



12b

Chart 1.

amino acid analyses (Chart 1).

Finally, the coupling of H–Gly–OEt with Boc–His(Boc)–OH by the EDC–HOBt method successfully gave the desired peptide (**12a**), Boc–His(Boc)–Gly–OEt. It was purified by silica-gel column chromatography. After treatment of **12a** with TFA, the dipeptide ester was elongated to compound **14** by the EDC–HOBt procedure. Fragment peptides **13** and **14** were also purified on a silica-gel column. After saponification of fragment **14**, the product **15** was similarly purified. After the removal of the Boc group of **11** with TFA, the tetrapeptide ester was coupled with **15** by the EDC–HOBt procedure. Fragment **16** was purified by recrystallization, and then reduced with zinc powder in acetic acid to yield fragment (**17**), Boc–Lys(Z)–Lys(Z)–His–Gly–Val–Thr–Val–Leu–OH.

In order to avoid the complicated purification of compound **16**, it was also synthesized by the second route shown in Fig. 4. Compounds **18**—**20** and **16** were successively synthesized by the EDC–HOBt method. However, compound **16** obtained by this method also contained a similar by-product. The N^{im}-Boc groups of peptides **12a** and **19** were completely removed by TFA without the side reaction mentioned in the previous paper.[10]

Prior to the preparation of peptides **8** and **17**, the synthesis of a fragment, Boc–Val^{66}–Thr(Bzl)–Val–Leu–Thr(Bzl)–Ala–Leu–Gly^{73}–OEt, was carried out. However, this fragment was scarcely soluble in several solvents (DMF, DMSO, *N*-methyl-2-pyrrolidone, and HMPT). Also, both fragment peptides, Boc–Val–Thr(Bzl)–Val–Leu–OEt [or OH] and Boc–Thr(Bzl)–Ala–Leu–Gly–OEt, were sparingly soluble. These problems were solved by employing unprotected threonine as in

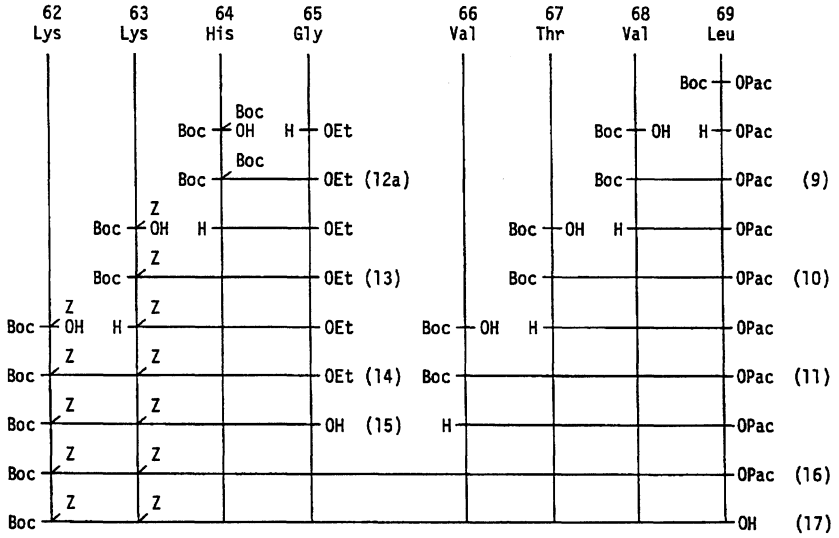


Fig. 3. Synthetic scheme of protected octapeptide (62—69).

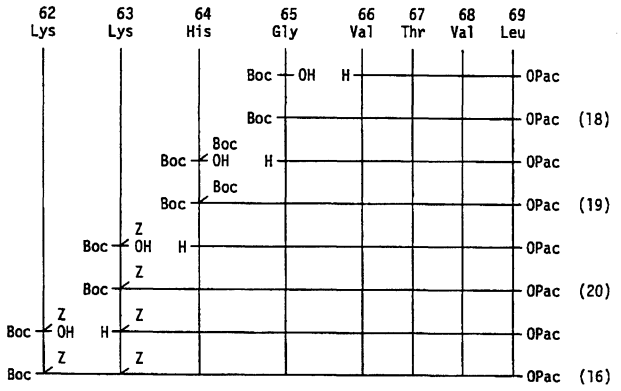


Fig. 4. Alternative synthetic scheme of protected octapeptide (62—69).

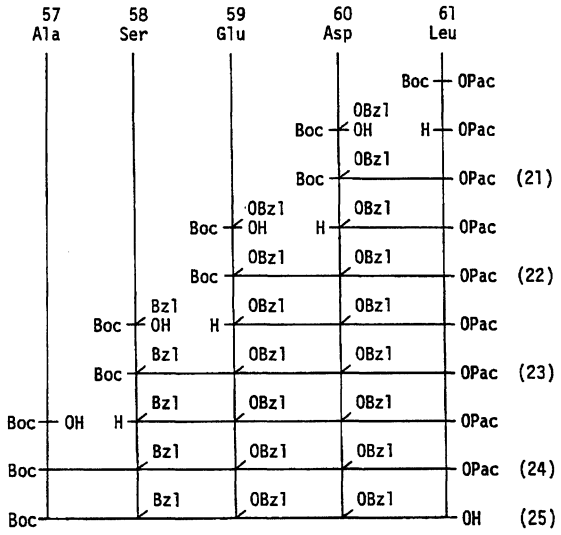


Fig. 5. Synthetic scheme of protected pentapeptide (57—61).

peptides **5** and **11**.

Protected Pentapeptide (57—61). Figure 5 shows the synthesis of fragment (**25**), Boc–Ala–Ser-(Bzl)–Glu(OBzl)–Asp(OBzl)–Leu–OH. Each peptide was synthesized by the MA method. Compounds **22** and **23** were purified by silica-gel chromatography employing a chloroform–methanol solvent system. Fragment **25** was obtained by the reduction of **24** with zinc powder in acetic acid.

Protected Tetracontapeptide (57—96). The synthesis of a protected tetracontapeptide **29** is illustrated in Fig. 1. After removal of the Boc group of a protected eicosapeptide (**26**), Boc–(77—96)–OBzl,[10] the resulting ester was coupled with **8** by using EDC in the presence of HOBt to afford a protected heptaeicosapeptide (**27a**), Boc–(70—96)–OBzl. The crude product was purified by silica-gel chromatography using 1-butanol–acetic acid–water (4 : 1 : 1) as eluent (repeated three times). Two components were isolated and their structures were confirmed by amino acid, elemental, and FAB-MS analyses: one component was the desired peptide **27a** (Fig. 6), the other was a by-product, **27b**. The FAB-MS analysis indicated that the $(M+H)^+$ ion of **27b** had a mass 74 units less than that of **27a** (Fig. 7). From this result, it was presumed that the by-product, **27b**,[18] had an oxazolidone structure resulting from the cyclization of threonine at the N-terminal (Chart 2). As mentioned in the previous paper,[10] silica-gel chromatography was also a very efficient tool for purification of protected long-chain peptides such as **27a**. After treatment of compound **27a** with TFA, the resulting ester was coupled with **17** by the EDC–HOBt procedure. The crude product was applied to a Sephadex LH-60 column using 5% acetic acid–DMF as eluent to give a protected pentatriacontapeptide (**28**), Boc–(62—96)–OBzl. After removal of the Boc group of **28**, the resulting ester was condensed with **25** by using EDC in the

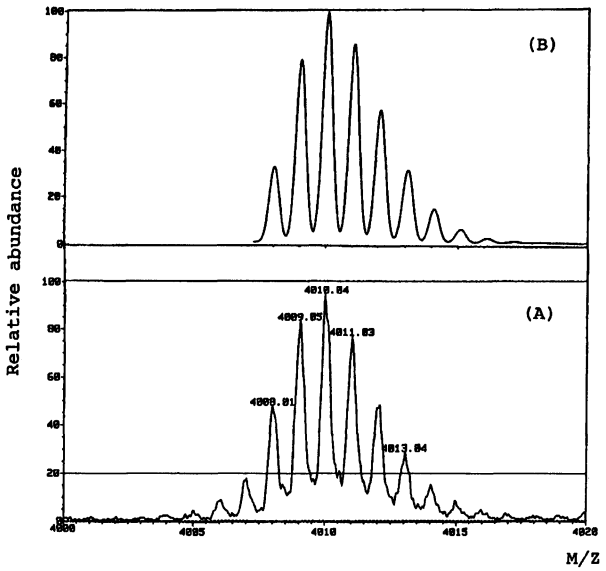


Fig. 6. FAB-MS spectra of Boc-(70—96)-OBzl (**27a**). (A) Found, (B) Theoretical ion distribution for $C_{201}H_{280}O_{48}N_{39}$.

presence of HOBt to afford a protected tetracontapeptide (**29**), Boc-(57—96)-OBzl.

Free Tetracontapeptide (57—96). The protecting groups of compound **29** were removed with TFMSA-TFA-thioanisole-*m*-cresol (Fig. 1).[19,20] It has been pointed out that in such an acid treatment of Ser- or Thr-containing peptides, an acyl rearrangement from a nitrogen atom to an oxygen atom of the hydroxy amino acid residues may take place.[21] In order to reverse this possible rearrangement, the crude peptide was treated with 0.5 M aq NH_3 according to the usual method. The peptide was converted to the acetate form by Dowex 1×4 ion exchanger, and then purified by reversed-phase HPLC by using gradient and isocratic elution methods to give sperm whale Mb-(57—96)-tetracontanepeptide (**30**). The HPLC and FAB-MS profiles of peptide **30** are shown in Figs. 8 and 9, respectively. The amino acid sequence of this peptide was also confirmed by a sequence analysis.

The analytical data of compounds **1**, **3**—**5**, **9**, **10**, **13**, **14**, and **18**—**23** are given in Table 1.

Circular Dichroism Spectra. The CD spectra of peptide **30** were measured in the 190—270 nm region. For the CD measurements, peptide **30** was dissolved in 0.10 M phosphate buffer (pH 6.50), and 30% and 60% TFE-0.10 M phosphate buffer (pH 6.50). 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.[7] 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

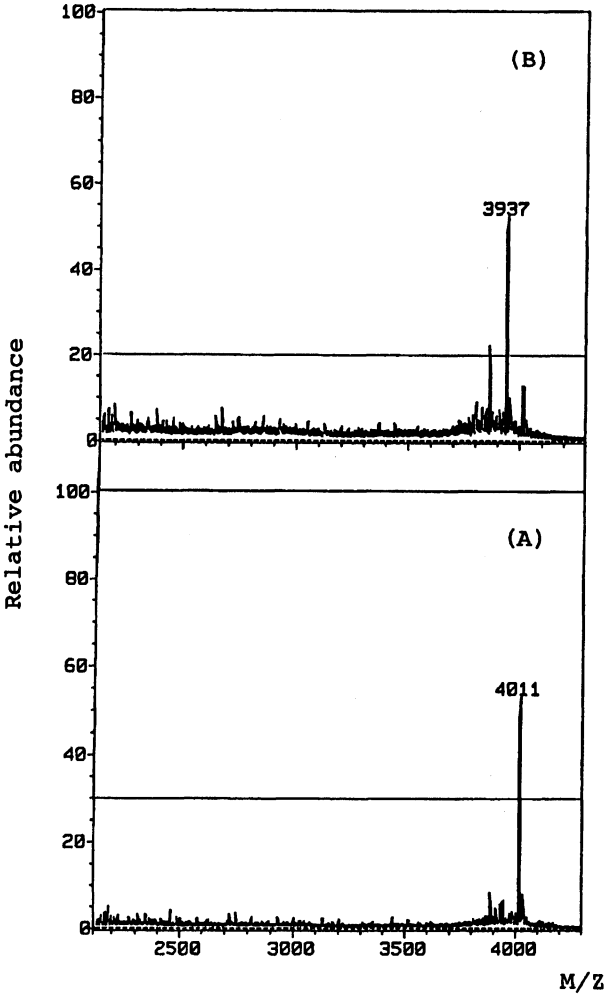
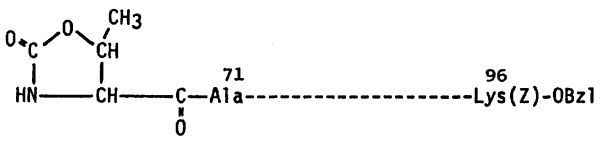


Fig. 7. FAB-MS spectra. (A) Boc-(70—96)-OBzl (**27a**), (B) Compound **27b**.



27b

Chart 2.

known three-dimensional structure of Mb.

In the amino acid sequence of sperm whale myoglobin-(57—96)-tetracontapeptide (**30**), -Ala-Ser-Glu-Asp-Leu-Lys-Lys-His-Gly-Val-Thr-Val-Leu-Thr-Ala-Leu-Gly-Ala-Ile-Leu-Lys-Lys-Lys-Gly-His-His-Glu-Ala-Glu-Leu-Lys-Pro-Leu-Ala-Gln-Ser-His-Ala-Thr-Lys-, the underlined amino acid residues take part in the helical structure according to the results of X-ray analysis.[3] The theoretical helicity of peptide **30** is 75%.

In 0.10 M phosphate buffer (pH 6.50), the spectrum of peptide **30** (Fig. 10) had a shoulder at 215 nm and a trough at 198 nm, and did not show unequivocal ex-

Table 1. Yields, Physical Properties, and Analytical Data of Intermediate Protected Peptides [a]

Compound	Yield/%	Mp θ_m/°C	$[\alpha]_D^{24}$/° (c 1.0, MeOH)	Molecular formula	Elemental analysis/% Found (F:), Calcd (C:) C	H	N	R_f [b] R_f^1	R_f^2	R_f^3	R_f^4	R_f^5
1 [c]	96	149.5—150.5	−55.0	$C_{18}H_{34}O_5N_2$	F: 60.35	9.60	7.71	0.86	0.92			
					C: 60.31	9.56	7.81					
3 [d]	92	83.5—84	−42.8	$C_{15}H_{28}O_5N_2$	F: 56.81	8.99	8.79	0.79	0.87		0.86	
					C: 56.94	8.92	8.85					
4	95	154—155	−74.2	$C_{18}H_{33}O_6N_3$	F: 55.50	8.74	10.59	0.52	0.75			0.93
					C: 55.80	8.58	10.84					
5	74	155—157	−61.8	$C_{22}H_{40}O_8N_4$	F: 53.77	8.23	11.37	0.32	0.57		0.56	
					C: 54.08	8.25	11.47					
9	53	127—128	−72.4	$C_{24}H_{36}O_6N_2$	F: 64.29	8.07	6.49	0.83	0.91		0.77	
					C: 64.26	8.09	6.35					
10	75	137.5—138.5	−78.4	$C_{28}H_{43}O_8N_3 \cdot 0.5H_2O$	F: 60.66	7.91	7.68	0.47	0.74		0.64	
					C: 60.20	7.94	7.52					
13 [e]	79	119—121	−38.6	$C_{29}H_{42}O_8N_6$	F: 57.45	7.02	13.86	0.11	0.39		0.10	
					C: 57.79	7.02	13.94					
14	81	107—110.5	−37.0	$C_{43}H_{60}O_{11}N_8 \cdot 0.5H_2O$	F: 58.91	7.06	12.88		0.39		0.08	
					C: 59.09	7.03	12.82					
18	84	198.5—200.5	−11.0 (c 1.0, DMF)	$C_{35}H_{55}O_{10}N_5 \cdot H_2O$	F: 58.27	7.96	9.72	0.25	0.59		0.73	
					C: 58.08	7.94	9.67					
19	57	197—199	−21.6 (c 1.0, DMF)	$C_{46}H_{70}O_{13}N_8 \cdot H_2O$	F: 57.50	7.55	11.76	0.28	0.49		0.49	
					C: 57.49	7.55	11.66					
20 [f]	43	199—202 (decomp)	−24.2 (c 1.0, DMF)	$C_{55}H_{80}O_{14}N_{10} \cdot 4.5H_2O$	F: 55.65	6.93	11.74			0.49		0.22
					C: 55.68	7.56	11.81					
21	91	Oil						0.93				
22	79	Oil						0.86	0.92			
23	53	127—128	−30.0	$C_{52}H_{62}O_{13}N_4 \cdot 0.5H_2O$	F: 65.09	6.58	5.87	0.83	0.93			
					C: 65.05	6.61	5.84					

a) The amino acid composition of the acid hydrolyzate of each compound agreed well with the theoretical value. b) The TLC solvent systems are shown in the experimental section. c) Mp 144—146°C in Ref. 22. d) Mp 79—80°C in Ref. 23. e) FAB-MS, m/z 603 $(M+H)^+$. Calcd for $C_{29}H_{43}O_8N_6$: (M+H), 603. f) FAB-MS, m/z 1105 $(M+H)^+$. Calcd for $C_{55}H_{81}O_{14}N_{10}$: (M+H), 1105.

istence of helical structure. The value of $[\theta]_{222}$ was −2350. Even if it is assumed that this value results entirely from the helical structure, the helicity is estimated to be only 7.4%. Compared with the theoretical helicity of peptide **30** described above, the helicity of the peptide in the aqueous solution was extremely low. In 30% and 60% TFE–0.10 M phosphate buffer (pH 6.50), the spectra of peptide **30** (Fig. 10) showed troughs at 208 and 222 nm, and a peak at 193 nm, and those profiles are characteristic of a helical structure. The values of $[\theta]_{222}$ in 30% and 60% TFE–0.10 M phosphate buffer were −17640 and −21950, respectively. From these data, the helicities were calculated as 55% and 69%, respectively. Thus, the helicity of the peptide increased with an increase in the concentration of TFE, and these results indicate that the addition of TFE (a noninteracting solvent)[24] to the phosphate buffer enhanced the hydrophobicity surrounding the peptide, and brought about the stabilization of the helical structure. The helicity of peptide **30** in 60% TFE–0.10 M phosphate buffer was approximately the same as the theoretical one. This result suggests that peptide **30** has a three-dimensional structure similar to that of the corresponding region in Mb.

A helical ratio has been used for comparing the helicities of fragment peptides of different sizes.[8] The helical ratio of peptide **30** in 60% TFE–0.10 M phosphate buffer (pH 6.50) was 0.92. This value indicates that peptide **30** possesses a helical content similar to that of the corresponding parts of Mb. The helical ratios of free sperm whale Mb-(85—96)-dodecapeptide and sperm whale Mb-(77—96)-eicosapeptide were 0.26 and 0.48, respectively,[11] that is, the elongation of the peptide chain induced the increase of the helical ratio. These results suggest that various interactions among amino acid residues located separately from each other in the primary structure contribute to stabilization of the helical structure, as was noted in the previous papers.[10,11]

In conclusion, the small helical content of peptide **30** in 0.10 M phosphate buffer (pH 6.50) strongly indicates the lack of a hydrophobic environment around this molecule. The increase in the helical content brought about by the addition of TFE suggests that a noninteracting (inert) solvent,[24] such as TFE, stabilizes the helical structure by enhancing the hydrophobicity around the backbone of the peptide. It is well known that meto- and apo-Mb exhibit CD spectra characteristic of α-helix structure even in aqueous solution.[25—29] The

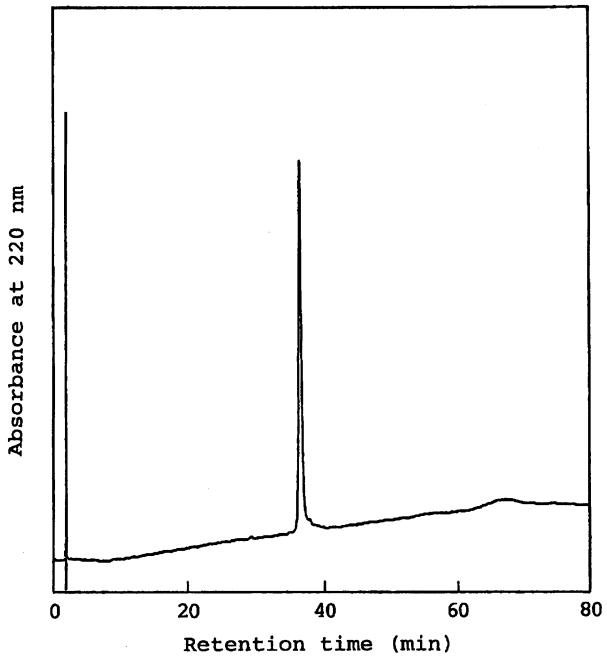


Fig. 8. HPLC profile of sperm whale Mb-(57—96)-tetracontapeptide (**30**). Eluent: 20—30% CH_3CN/H_2O–0.1% TFA (60 min), 30% CH_3CN/H_2O–0.1% TFA (20 min); Column: Waters μ-Bondasphere 5μ C18-300 Å (3.9×150 mm); Flow rate: 1.0 ml min^{-1}; Column temperature: 40°C.

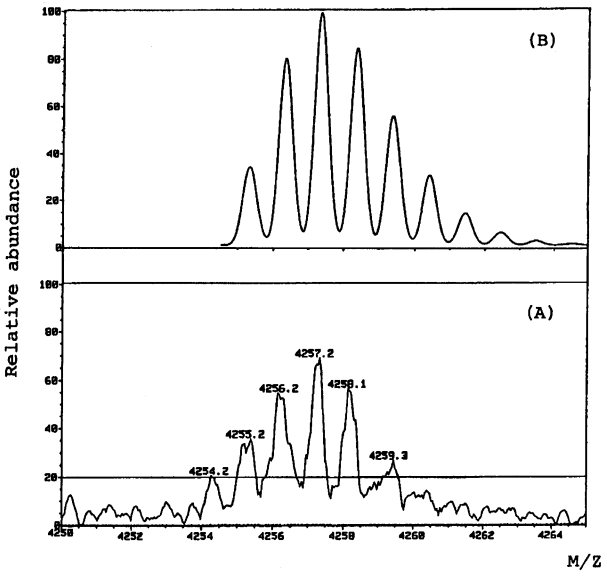


Fig. 9. FAB-MS spectra of sperm whale Mb-(57—96)-tetracontapeptide (**30**). (A) Found, (B) Theoretical ion distribution for $C_{189}H_{321}O_{55}N_{56}$.

present results afford strong evidence that hydrophobic surroundings brought from the regions other than the E–EF–F region play a very important role in helix formation of this region in the entire Mb molecule.

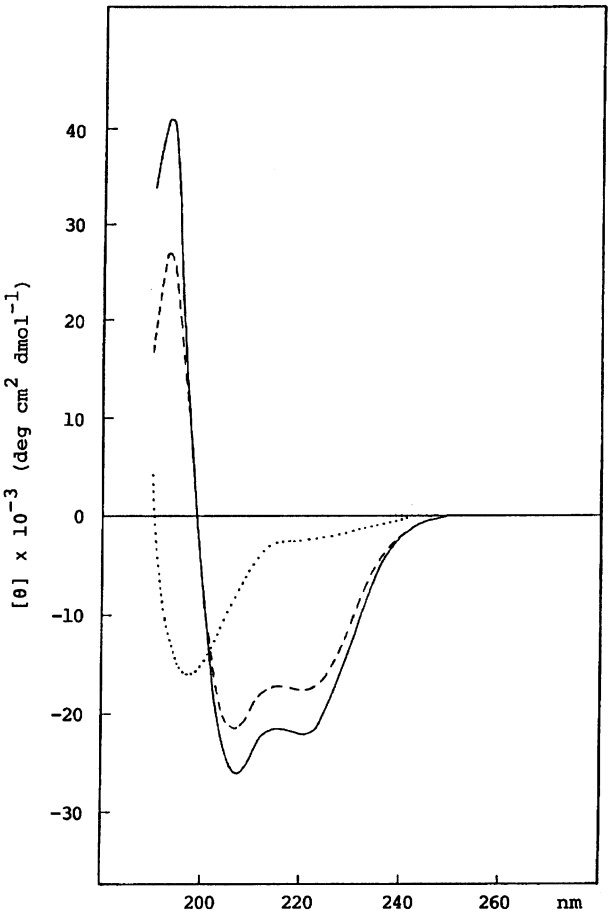


Fig. 10. CD spectra of sperm whale Mb-(57—96)-tetracontapeptide (**30**) in 0.10 M phosphate buffer (pH 6.50) (····), 30% TFE–0.10 M phosphate buffer (pH 6.50) (---), and 60% TFE–0.10 M phosphate buffer (pH 6.50) (—).

Experimental

Melting points were measured by the capillary method using a Mel-Temp (Mitamura Riken Kogyo) and are given as uncorrected values. Optical rotations were determined with a DIP-370 digital type (JASCO) polarimeter. Amino acid analyses were performed on a JLC-6AS, a JLC-300 (JEOL), or an L-8500 amino acid analyzer (Hitachi) after acid hydrolysis with 6 M HCl in a sealed tube at 110°C for 24 h or AP-M (Pierce Co.) digestion at 37°C for 48 h. The theoretical values of amino acid ratios are shown in parentheses after each result. FAB-MS spectra were measured on a JMS-SX mass spectrometer (JEOL). ^1H NMR spectra were taken with a JEOL GX-400 (400 MHz), using tetramethylsilane as an internal standard. IR spectra were obtained on a model IR-G spectrophotometer (JASCO).

In the synthetic procedures, the imidazolyl group of histidine was temporarily protected by a Boc group on the occasion of incorporation of a histidine residue into a peptide chain. The functional groups of side chains, other than the hydroxyl group of threonine, were protected by the following protecting groups: *t*-butoxycarbonyl group for α-amino groups; benzyloxycarbonyl group for lysine ε-ami-

no groups; methyl, ethyl, benzyl, or phenacyl ester for α-carboxyl groups; benzyl ester for β- and γ-carboxyl groups of asparatic and glutamic acids, respectively; benzyl group for serine hydroxyl groups.

The N^{α}- and N^{im}-Boc protecting groups were removed by treatment with TFA in the presence of anisole, or TFA, at room temperature or in an ice-bath for 40—95 min. The Boc groups of compounds **3**, **4**, **9**, **12a**, **13**, and **18**—**22** were similarly removed with TFA. After evaporation of the solvents, the residue was triturated with diethyl ether to give a product.

Compounds **1**, **3**—**5**, **9**, **10**, and **21**—**23** were synthesized by the MA method, and compounds **13**, **14**, **16** in Fig. 3, and **18**—**20** by the EDC–HOBt method. Unless otherwise described, the reaction mixtures were stirred for 2—3 h at 0°C, and then for 13—42 h at room temperature. After coupling, products dissolved in organic solvent were washed successively with water, 5% citric acid, water, 5% sodium hydrogencarbonate, and water, and then dried over anhydrous sodium sulfate except when noted otherwise. The evaporation of solvents was carried out under reduced pressure. Unless stated otherwise, residues and crystals were dried over NaOH pellets and P_2O_5 in vacuo at room temperature.

The silica gel used in column chromatography was Kieselgel 60 (230—400 mesh; Merck and Co.) for compounds **9**, **12**—**14**, **16** in Fig. 3, **18**, **19**, **22**, and **23**; and Kieselgel 100 (70—230 mesh; Merck and Co.) for compounds **15**, **25**, and **27a**. Thin-layer chromatography was performed on a TLC plate of silica gel (Kieselgel 60 F_{254}) using the following solvent systems (volume ratios): chloroform–methanol, R_f^1 (95:5), R_f^2 (9:1), R_f^3 (6:1); chloroform–methanol–acetic acid, R_f^4 (95:5:5), R_f^5 (9:1:1); 1-butanol–acetic acid–water, R_f^6 (10:1:1), R_f^7 (8:1:1), R_f^8 (6:1:1), R_f^9 (4:1:1), R_f^{10} (3:1:1); 1-butanol–pyridine–acetic acid–water, R_f^{11} (4:1:1:2). Development of compounds on TLC was conducted by spraying with ninhydrin, HBr–ninhydrin, and/or Rydon[30] reagents.

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 at 21°C using a J-500 A spectropolarimeter (JASCO) with a DP-500 N Data Processor. A cell with a light path of 0.5 mm was used. The peptide concentration during the measurements was 6.90×10^{-5} M (calculated on the basis of the result of amino acid analyses). A phosphate buffer (pH 6.50) was prepared with 0.10 M NaH_2PO_4 and 0.10 M Na_2HPO_4.

Boc–Ala–Ile–Leu–OMe (2). Compound **1** (8.930 g) was treated with TFA (25 ml) in the usual manner to yield an oily residue. Boc–Ala–OH (5.656 g) was dissolved in THF (40 ml) and the solution was cooled to below −20°C. To this solution were added NMM (3.29 ml) and ECC (2.87 ml) with stirring, and the mixture was stirred for 5 min at −15—−16°C, and then cooled to below −20°C. To this solution was then added the ice-chilled solution of the above oily residue and Et_3N (11.0 ml) in THF (50 ml). The mixture was stirred in an ice-bath for 3 h and then at room temperature for 63 h. After the solvent had been evaporated, the residue was dissolved in ethyl acetate (200 ml), and the solution was washed in the routine manner. After concentration, the residue was recrystallized from ethyl acetate–hexane: Yield of **2**, 10.439 g (98%); mp 114—115°C; $[\alpha]_D^{24}$ −89.8° (*c* 1.0, MeOH); and R_f^1 0.62, R_f^2 0.85, R_f^4 0.85. Amino acid ratios in acid hydrolyzate: Ala 0.97 (1), Ile 0.97 (1), Leu 1.06 (1). Found: C, 57.88; H, 9.23; N, 9.53%. Calcd for $C_{21}H_{39}O_6N_3\cdot0.5H_2O$: C, 57.51; H, 9.19; N, 9.58%.

Boc–Thr–Ala–Leu–Gly–OH (6). To an ice-chilled solution of **5** (8.143 g) in ethanol (35 ml) was added 1.98 M NaOH (9.3 ml). After being stirred for 1.6 h at room temperature, the solution was neutralized by adding 1.0 M HCl (19.3 ml) in an ice-bath. After evaporation of the ethanol, the residual solution was extracted with ethyl acetate (250 ml). The organic layer was washed with saturated NaCl aq solution (10 ml×5) and water (10 ml×1). After removal of the solvent, the residue was recrystallized from ethyl acetate–diethyl ether: Yield of **6**, 7.329 g (95%); mp 150—152.5°C; $[\alpha]_D^{24}$ −14.6° (*c* 1.0, DMF); and R_f^4 0.09, R_f^5 0.36, R_f^9 0.76. Amino acid ratios in acid hydrolyzate: Thr 0.96 (1), Gly 1.01 (1), Ala 1.04 (1), Leu 0.99 (1). Found: C, 51.44; H, 7.95; N, 11.70%. Calcd for $C_{20}H_{36}O_8N_4\cdot0.5\ H_2O$: C, 51.16; H, 7.94; N, 11.93%.

Boc–Thr–Ala–Leu–Gly–Ala–Ile–Leu–OMe (7). Compound **2** (3.780 g) was treated with TFA (20 ml) to afford a solid. Then to an ice-chilled solution of **6** (3.684 g) in DMF (15 ml) were added HOBt (1.622 g), EDC·HCl (1.687 g), and an ice-chilled solution of the above solid dissolved in DMF (20 ml) to which had been added Et_3N (1.22 ml). The mixture was stirred in an ice-bath for 3 h, then at room temperature for 16 h. It was then treated in a similar manner to that described for the preparation of **2**. 1-Butanol (200 ml) was used as the solvent for extraction of the product. The product was recrystallized from methanol–ethyl acetate: Yield of **7**, 5.907 g (96%); mp 226.5—228°C (decomp); $[\alpha]_D^{24}$ −24.4° (*c* 1.0, DMF); and R_f^1 0.15, R_f^2 0.49, R_f^5 0.75. Amino acid ratios in acid hydrolyzate: Thr 0.92 (1), Gly 1.01 (1), Ala 2.00 (2), Ile 0.99 (1), Leu 2.00 (2). Found: C, 55.52; H, 8.53; N, 12.67%. Calcd for $C_{36}H_{65}O_{11}N_7\cdot0.5H_2O$: C, 55.37; H, 8.52; N, 12.55%.

Boc–Thr–Ala–Leu–Gly–Ala–Ile–Leu–OH (8). Compound **7** (5.707 g) was suspended in a mixture of methanol (35 ml) and water (10 ml). To this mixture was added 1.98 M NaOH (6.2 ml) at 0°C, and after stirring for 4.8 h at room temperature, 1.0 M HCl (12.8 ml) was added to the solution in an ice-bath. After evaporation of the methanol, the residual solution was extracted with a solvent mixture of ethyl acetate (200 ml) and 1-butanol (30 ml), and the organic layer was washed with water (10 ml×5). After the solvent had been evaporated, the residue was recrystallized from methanol–ethyl acetate: Yield of **8**, 5.241 g (94%); mp 203°C (decomp); $[\alpha]_D^{24}$ −22.6° (*c* 1.0, DMF); and R_f^5 0.46, R_f^9 0.88. Amino acid ratios in acid hydrolyzate: Thr 0.95 (1), Gly 1.01 (1), Ala 2.00 (2), Ile 0.98 (1), Leu 2.01 (2). Found: C, 54.22; H, 8.41; N, 12.62%. Calcd for $C_{35}H_{63}O_{11}N_7\cdot H_2O$: C, 54.18; H, 8.44; N, 12.64%.

Boc–Val–Thr–Val–Leu–OPac (11). Compound **10** (3.979 g) was treated with TFA (20 ml) containing anisole (0.865 ml) to produce a solid. A solution of Boc–Val–OH (1.887 g) in DMF (20 ml) was cooled to below −25°C. To this solution were added NMM (0.956 ml) and ECC (0.834

ml) with stirring below −20°C. The mixture was stirred for 5 min at −15 — −16°C and mixed with an ice-chilled solution of the above solid dissolved in DMF (30 ml) to which had been added Et_3N (1.11 ml). The mixture was treated in a similar way to that for the preparation of **7**. By the addition of water (100 ml) to the DMF solution (30 ml) of the crude product, **11** was obtained as a precipitate, and was recrystallized from methanol: Yield of **11**, 3.208 g (68%); mp 185—186°C; $[\alpha]_D^{24}$ −20.0° (*c* 1.0, DMF); and R_f^1 0.38, R_f^2 0.69, R_f^4 0.63. Amino acid ratios in acid hydrolyzate: Thr 0.91 (1), Val 2.01 (2), Leu 0.99 (1). Found: C, 59.05; H, 8.13; N, 8.44%. Calcd for $C_{33}H_{52}O_9N_4 \cdot H_2O$: C, 59.44; H, 8.16; N, 8.40%.

Boc–His(Boc)–Gly–OEt (12a).[31] To an ice-chilled solution of Boc–His(Boc)–OH (8.885 g) in DMF (30 ml) were added HOBt (5.067 g), EDC·HCl (5.272 g), and an ice-cooled solution of HCl·H–Gly–OEt (4.188 g) dissolved in DMF (30 ml) to which had been added Et_3N (4.17 ml). The mixture was worked up according to the procedure for the preparation of **7**. Ethyl acetate (150 ml) was used as a solvent in extraction and washing of the crude product. After removal of the solvent, the residue was dissolved in chloroform (20 ml). This solution was applied to a silica-gel column (2.2×37.4 cm), which was then eluted with a chloroform-methanol solvent system (stepwise gradient method). The fractions containing the desired peptide were combined and the solvent was evaporated. The residue was recrystallized from diethyl ether–petroleum ether: Yield of **12a**, 4.406 g (40%); mp 106—107°C; $[\alpha]_D^{24}$ −8.6° (*c* 1.0, MeOH); and R_f^1 0.49, R_f^2 0.82, R_f^4 0.65. Amino acid ratios in acid hydrolyzate: Gly 1.00 (1), His 0.99 (1). Found: C, 54.15; H, 7.43; N, 12.73%. Calcd for $C_{20}H_{32}O_7N_4$: C, 54.53; H, 7.32; N, 12.72%. FAB-MS, *m/z* 441 $(M+H)^+$. Calcd for $C_{20}H_{33}O_7N_4$: (M+H), 441.

7-(*t*-Butoxycarbonylamino)-7,8-dihydroimidazo[1,5-*c*]pyrimidin-5(6*H*)-one (12b). Boc–His–$NHNH_2$ was treated with isopentyl nitrite according to the usual azide method. The reaction mixture was added to an H–Gly–OPac solution. However, the product obtained after purification by silica-gel chromatography and recrystallization was **12b**: Mp 169—170°C (decomp) and R_f^1 0.30, R_f^2 0.52, R_f^4 0.51. FAB-MS, *m/z* 253 $(M+H)^+$. Calcd for $C_{11}H_{17}O_3N_4$: (M+H), 253. ^{1}H NMR (DMSO-d_6) δ=1.40 (9H, s, $(CH_3)_3C$), 2.92 (1H, dd, *J*=15.9 and 4.4 Hz, 8-CH_2), 3.10 (1H, dd, *J*=15.9 and 3.9 Hz, 8-CH_2), 5.15 (1H, m, 7-CH), 6.83 (1H, s, 1-CH), 7.80 (1H, d, *J*=6.3 Hz, –O–CO–NH–), 8.07 (1H, s, 3-CH), and 8.63 (1H, d, *J*=3.3 Hz, 6-NH); IR (KBr) 3300, 1740, 1710, 1680, 1515, 1410, and 1250 cm^{-1}.

Boc–Lys(Z)–Lys(Z)–His–Gly–OH (15). Compound **14** (1.118 g) was suspended in ethanol (16 ml), and then 1.98 M NaOH (0.78 ml) was added to the suspension in an ice-bath. The mixture became clear with saponification. After stirring for 2 h at room temperature, 1.0 M HCl (1.55 ml) was added to the solution in an ice-bath. Addition of water to the solution gave a precipitate, which was collected and recrystallized from ethanol. The crude product (0.830 g) was dissolved in 1-butanol–acetic acid–water (4:1:1) (6.0 ml) and the solution was applied to a silica-gel column (2.2×40.9 cm) equilibrated with the same solvent, and then eluted with the same solvent. Fractions 28—46 (5.0 g each) were combined and the solvent was evaporated. The residue was recrystallized from methanol: Yield of **15**, 0.484 g (58%); mp 176—179°C (decomp); $[\alpha]_D^{24}$ −14.0° (*c* 1.0, DMF); and R_f^7 0.43, R_f^9 0.66, R_f^{10} 0.70. Amino acid ratios in acid hydrolyzate: Gly 1.03 (1), His 0.98 (1), Lys 1.99 (2). Found: C, 57.79; H, 6.92; N, 12.94%. Calcd for $C_{41}H_{56}O_{11}N_8 \cdot H_2O$: C, 57.60; H, 6.84; N, 13.11%.

Boc–Lys(Z)–Lys(Z)–His–Gly–Val–Thr–Val–Leu–OPac (16) in Fig. 2. Compound **11** (0.763 g) was treated with TFA (15.0 ml) containing anisole (0.153 ml) to yield a solid. To an ice-chilled solution of **15** (0.895 g) in DMF (8 ml) were added HOBt (0.217 g), EDC·HCl (0.225 g), and an ice-cooled solution of the above solid dissolved in DMF (22 ml) to which had been added Et_3N (0.163 ml). The mixture was treated in a similar way to that for **7**; 1-butanol was used as the solvent in the extraction. After evaporation of the solvent, the residue was triturated with water to give a crude product (1.390 g). The product (0.695 g) was recrystallized several times each from methanol, ethanol–chloroform–ethyl acetate, methanol–ethanol–chloroform-ethyl acetate, and methanol–ethanol–chloroform solvent systems to show a single spot on TLC. Yield of **16**, 0.222 g (30%) and R_f^2 0.23, R_f^3 0.48, R_f^9 0.85. Amino acid ratios in acid hydrolyzate: Thr 0.96 (1), Gly 1.01 (1), Val 2.00 (2), Leu 0.98 (1), His 0.99 (1), Lys 2.02 (2).

Boc–Lys(Z)–Lys(Z)–His–Gly–Val–Thr–Val–Leu–OH (17). Compound **16** (0.284 g) was dissolved in acetic acid (8.0 ml), and zinc powder (0.70 g) was added to the solution. The mixture was stirred for 3.5 h at 40°C, and after removal of the precipitate, the solvent was evaporated. The residue was triturated with water and twice with hot methanol: Yield of **17**, 0.209 g (80%); mp 213.5—215.5°C (decomp); $[\alpha]_D^{24}$ −18.2° (*c* 1.0, DMF); and R_f^6 0.57, R_f^9 0.79. Amino acid ratios in acid hydrolyzate: Thr 0.99 (1), Gly 1.03 (1), Val 2.00 (1), Leu 0.97 (1), His 1.00 (1), Lys 2.01 (2). Found: C, 54.89; H, 7.11; N, 12.62%. Calcd for $C_{61}H_{92}O_{16}N_{12} \cdot 4.5H_2O$: C, 55.07; H, 7.65; N, 12.63%.

Boc–Ala–Ser(Bzl)–Glu(OBzl)–Asp(OBzl)–Leu–OPac (24). Compound **23** (10.219 g) was treated with TFA (25.0 ml) – anisole (1.28 ml) to afford a solid. Boc–Ala–OH (2.440 g) was dissolved in DMF (25.0 ml), and to this solution were added NMM (1.42 ml) and ECC (1.24 ml) at below −25°C. The mixture was stirred for 5 min at −14—−16°C, and to it was then added an ice-cooled solution of the above solid dissolved in DMF (25.0 ml) to which had been added Et_3N (1.49 ml). This mixture was stirred in an ice-bath for 3 h and then at room temperature for 43.3 h. After concentration of the solution, the residue was recrystallized twice from DMF–water. The product was further triturated with hot methanol: Yield of **24**, 9.539 g (87%); mp 156—158°C; $[\alpha]_D^{24}$ −17.6° (*c* 1.0, DMF); and R_f^1 0.79, R_f^2 0.90, R_f^4 0.73. Amino acid ratios in acid hydrolyzate: Asp 1.03 (1), Ser 0.90 (1), Glu 1.09 (1), Ala 0.98 (1), Leu 1.00 (1). Found: C, 63.19; H, 6.49; N, 6.72%. Calcd for $C_{55}H_{67}O_{14}N_5 \cdot 1.5H_2O$: C, 62.96; H, 6.72; N, 6.68%.

Boc–Ala–Ser(Bzl)–Glu(OBzl)–Asp(OBzl)–Leu–OH (25). The OPac group of **24** (2.500 g) was removed in a similar way to that of **16**. Yield of **25**, 1.805 g (82%); mp 179—180°C; $[\alpha]_D^{24}$ −13.2° (*c* 1.0, DMF); and R_f^2 0.22, R_f^4 0.53, R_f^5 0.92. Amino acid ratios in acid hydrolyzate: Asp 1.01 (1), Ser 0.90 (1), Glu 1.07 (1), Ala 0.99 (1), Leu 1.04 (1). Found: C, 61.72; H, 6.77; N, 7.60%. Calcd for $C_{47}H_{61}O_{13}N_5 \cdot 0.5H_2O$: C, 61.83; H, 6.84; N, 7.67%.

Protected Heptaeicosapeptide (27a); Boc–(70—96)–OBzl. Compound **26** (1.300 g) synthesized previously[10] was treated with TFA (20.0 ml) containing anisole (0.209 ml) to give a solid. To an ice-cooled solution of **8** (1.170 g) in DMF (8.0 ml) were added HOBt (0.417 g), EDC·HCl (0.325 g), and an ice-chilled solution of the above solid dissolved in DMF (11.0 ml) to which had been added Et_3N (0.450 ml). This mixture was stirred in an ice-bath for 4 h and then at room temperature for 62.5 h. After the solvent had been evaporated, the residue was dissolved in 1-butanol saturated with water (ca. 150 ml), and the organic layer was washed by the routine method. After evaporation of the solvent, the residue was crystallized from methanol-water. The crude material obtained was dissolved in 1-butanol–acetic acid–water (4 : 1 : 1) (5.0 ml), and the solution was subjected to a silica-gel column (2.2×29.2 cm) which had been equilibrated with the same solvent, and then eluted with the same solvent (600 ml) and with further 1-butanol–pyridine–acetic acid–water (4 : 1 : 1 : 1). Fractions of 10 g each were collected.

Fractions 11—17 containing the desired peptide were combined and the solution was concentrated. The crude product was further purified twice by the same chromatography as described above and the oily residue obtained was crystallized from a 5% $NaHCO_3$ solution (10 ml). The product was dissolved in 1-propanol, and the insoluble materials removed. After the solution had been concentrated, the residue was crystallized by adding diethyl ether. This product was ascertained to be the desired peptide from the following analytical data: Yield of **27a**, 0.374 g (24%); mp 190°C (decomp); $[\alpha]_D^{19}$ −3.1° (*c* 1.0, DMF); and R_f^8 0.65, R_f^9 0.79. Amino acid ratios in acid hydrolyzate: Thr 1.99 (2), Ser 0.99 (1), Glu 3.02 (3), Pro 0.94 (1), Gly 2.04 (2), Ala 5.26 (5), Ile 1.05 (1), Leu 4.24 (4), His 2.73 (3), Lys 4.75 (5). Found: C, 58.05; H, 6.92; N, 13.31%. Calcd for $C_{201}H_{279}O_{48}N_{39}\cdot 8H_2O$: C, 58.12; H, 7.16; N, 13.15%. FAB-MS, *m/z* 4010.04 $(M+H)^+$. Calcd for $C_{201}H_{280}O_{48}N_{39}$: (M+H), 4010.072.

On the other hand, fractions 31—68 were combined and the solvent was evaporated. The residue was purified by the same treatment as that described above: Yield 0.408 g. However, this compound was presumed to be a by-product, **27b**, from the following analytical data: Mp 184°C (decomp); $[\alpha]_D^{19}$ −2.7° (*c* 1.0, DMF); and R_f^8 0.55 (trace 0.22, 0.65), R_f^9 0.73 (trace 0.79). Amino acid ratios in acid hydrolyzate: Thr 1.86 (2), Ser 0.87 (1), Glu 3.09 (3), Pro 0.95 (1), Gly 2.23 (2), Ala 5.09 (5), Ile 1.10 (1), Leu 4.01 (4), His 2.91 (3), Lys 4.87 (5), NH_3 1.17 (1). Found: C, 57.02; H, 7.03; N, 13.44%. Calcd for $C_{197}H_{269}O_{47}N_{39}\cdot 12H_2O$: C, 56.99; H, 7.11; N, 13.16%. FAB-MS, *m/z* 3937 $(M+H)^+$. Calcd for $C_{197}H_{270}O_{47}N_{39}$: (M+H), 3937.

Protected Pentatriacontapeptide (28); Boc–(62—96)–OBzl. Compound **27a** (0.266 g) was treated with TFA (10.0 ml)–anisole (0.083 ml) to yield a solid. To an ice-chilled solution of **17** (0.160 g) in DMF (2.0 ml) were added HOBt (0.026 g), EDC·HCl (0.030 g), and an ice-cooled solution of the above solid dissolved in DMF (6.0 ml) to which had been added Et_3N (0.036 ml). The mixture was stirred in an ice-bath for 4 h and then at room temperature for 15.3 h. After the solution had been concentrated, the residue was triturated with water. The resulting precipitate was dissolved in 5% acetic acid–DMF (3.0 ml) and this solution was applied to a Sephadex LH-60 column (1.70×122.3 cm) by the use of the same solvent as the eluate. Fractions 25—30 (5.0 g each) were combined, and after evaporation of the solvent, the residue was crystallized from hot methanol. An additional crop from fractions 19—24 was purified by rechromatography on the same column: Yield of **28**, 0.156 g (47%) and R_f^8 0.55 (trace 0.23, 0.39, 0.64), R_f^9 0.75 (trace 0.56, 0.81), R_f^{11} 0.83 (trace 0.89).

Protected Tetracontapeptide (29); Boc–(57—96)–OBzl. Compound **28** (120 mg) was treated with TFA (6.0 ml) containing anisole (0.038 ml) to give a solid. To an ice-cooled solution of **25** (63.1 mg) in DMF (0.50 ml) were added HOBt (14.17 mg), EDC·HCl (15.99 mg), and an ice-chilled solution of the above solid dissolved in a mixture of DMF (2.0 ml) and DMSO (1.0 ml) to which had been added Et_3N (0.0162 ml). The mixture was stirred in an ice-bath for 4 h and then at room temperature for 15 h, and after removal of the DMF, water was added to the residue. The precipitate was successively triturated with hot methanol, chloroform, and methanol-water to give product **29**: Yield 88.67 mg (64%) and R_f^8 0.72 (trace 0.82), R_f^9 0.86 (trace 0.89, 0.95), R_f^{11} 0.96.

Sperm Whale Mb-(57—96)-Tetracontapeptide (30). Compound **29** (70.94 mg) was treated with TFMSA (0.215 ml)–TFA (2.0 ml)–thioanisole (0.284 ml)–*m*-cresol (0.251 ml) in an ice-bath for 2.0 h. After evaporation of the TFA, diethyl ether was added to the remaining solution. The solid obtained was dissolved in 0.5 M aq NH_3 (10.0 ml), and the solution was stirred for 30 min in an ice-bath. After the addition of acetic acid (4.0 ml), the solution was subjected to an ion exchange column (1.7×19.3 cm; Dowex 1×4; acetate form). The product was eluted with 10% acetic acid (100 ml), and the eluates were collected, concentrated, and the residue lyophylized from water (70.85 mg). The product dissolved in water (1.0 ml) was eluted from a Waters μ-Bondasphere 5μ C18-300 Å column (19×150 mm) by the use of a gradient elution device from 2% CH_3CN/H_2O–0.1% TFA to 50% CH_3CN/H_2O–0.1% TFA as eluent (injection: 10 times). The eluates obtained in retention time 77—80 min (flow rate, 5.0 ml min^{-1}) were combined and concentrated, and the residue lyophylized from water (8.96 mg). The crude product dissolved in water (0.50 ml) was further purified in the same column by an isocratic elution device with 28% CH_3CN/H_2O–0.1% TFA as eluent (injection: 9 times). The eluates obtained in retention time 19—21 min (flow rate, 5.0 ml min^{-1}) were collected and concentrated, and the residue lyophylized from water to give product **30**: Yield 3.67 mg (4.2%).[32] Amino acid ratios in acid hydrolyzate: Asp 1.12 (1), Thr 2.82 (3), Ser 1.97 (2), Glu 3.95 (4), Pro 0.84 (1), Gly 3.11 (3), Ala 6.00 (6), Val 2.06 (2), Ile 0.95 (1), Leu 6.02 (6), His 3.91 (4), Lys 6.80 (7). Amino acid ratios in AP-M digest: Asp 1.25 (1), Thr+Gln 4.09 (4), Ser 2.65 (2), Glu 3.44 (3), Pro not calculated (1), Gly 2.98 (3), Ala 6.05 (6), Val 2.11 (2), Ile 1.08 (1), Leu 6.00 (6), His 3.94 (4), Lys 6.62 (7). FAB-MS, *m/z* 4257.2 $(M+H)^+$. Calcd for $C_{189}H_{321}O_{55}N_{56}$: (M+H), 4257.41. CD: (*c* 0.050, 0.10 M phosphate buffer; pH 6.50, 21°C) $[\theta]_{222}$ −2350, $[\theta]_{199}$ −15680, and $[\theta]_{190.5}$ 0; (*c* 0.050, 60% TFE-0.10 M phosphate buffer; pH 6.50, 21°C) $[\theta]_{222}$ −21950, $[\theta]_{208}$ −25970, $[\theta]_{199}$ 0, and $[\theta]_{193}$ 41160. The sequence analysis by an automatic sequencer (Shimadzu PSQ-1) showed the proper sequence in accordance with the synthetic design.

We wish to thank Professor Machiko Tozawa, Associate Professor Tomoyoshi Takahashi, and emeritus Professor Tsunetaka Kushimoto of The Jikei University School of Medicine, and Professor Shosuke Sofuku of Rikkyo University for their continuing interest and encouragement. We also thank the members of the Radiochemical Laboratory of Rikkyo University for CD measurements, the Research Laboratory of Toyo Jozo Co. Ltd. for elemental, amino acid, peptide sequence, and FAB mass analyses, Dr. Yoshiaki Motoki of POLA Pharmaceutical Research Laboratory for optical rotation measurements and amino acid analyses, and Lecturer Toshio Takido of Nihon University and Professor Mitsuo Kodomari of Shibaura Institute of Technology for NMR measurements. The present work was partially supported by a Grant-in-Aid of the Kaneko from Protein Research Foundation.

References

1) The amino acids used were of L-configuration except glycine. The abbreviations used are those recommended by the IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN): *Eur. J. Biochem.*, **138**, 9 (1984). The following additional abbreviations are used: CD, circular dichroism; TLC, thin-layer chromatography; FAB-MS, fast atom bombardment mass spectroscopy; HPLC, high-performance liquid chromatography; MA, mixed anhydride; Mb, sperm whale myoglobin; HOBt, 1-hydroxybenzotriazole; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; Et_3N, triethylamine; NMM, *N*-methylmorpholine; ECC, ethyl chloroformate; TFA, 2,2,2-trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; DMF, *N,N*-dimethylformamide; TFE, 2,2,2-trifluoroethanol; DMSO, dimethyl sulfoxide; HMPT, hexamethylphosphoric triamide; THF, tetrahydrofuran; OPac, phenacyl ester; OBu^t, *t*-butyl ester; AP-M, aminopeptidase M.

2) J. C. Kendrew, R. E. Dickerson, B. E. Strandberg, R. G. Hart, D. R. Davies, D. C. Phillips, and V. C. Shore, *Nature (London)*, **185**, 422 (1960).

3) J. C. Kendrew, H. C. Watson, B. E. Strandberg, R. E. Dickerson, D. C. Phillips, and V. C. Shore, *Nature (London)*, **190**, 666 (1961).

4) R. Uhmann and E. Bayer, *Justus Liebigs Ann. Chem.*, **1974**, 1955.

5) H. Eckstein and E. Bayer, *Justus Liebigs Ann. Chem.*, **1978**, 1607.

6) M. J. Crumpton and P. A. Small, Jr., *J. Mol. Biol.*, **26**, 143 (1967).

7) R. M. Epand and H. A. Scheraga, *Biochemistry*, **7**, 2864 (1968).

8) R. P. Singhal and M. Z. Atassi, *Biochemistry*, **9**, 4252 (1970).

9) M. Z. Atassi and R. P. Singhal, *J. Biol. Chem.*, **245**, 5122 (1970).

10) C. Hashimoto and I. Muramatsu, *Bull. Chem. Soc. Jpn.*, **62**, 1900 (1989).

11) C. Hashimoto, *Bull. Chem. Soc. Jpn.*, **65**, 1268 (1992).

12) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Am. Chem. Soc.*, **88**, 1338 (1966).

13) J. C. Sheehan, J. Preston, and P. A. Cruickshank, *J. Am. Chem. Soc.*, **87**, 2492 (1965).

14) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).

15) J. B. Hendrickson and C. Kandall, *Tetrahedron Lett.*, **1970**, 343.

16) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).

17) The formation of a similar product was reported in the investigation of the side reaction of Z–His–N_3: M. Shin and K. Inouye, "Peptide Chemistry 1977," ed by T. Shiba, Protein Research Foundation, Osaka (1978), p. 67.

18) The formation of similar products was found in the preparation of Z–Ser–OH and Z–Thr–OH: T. Kaneko and T. Inui, *Nippon Kagaku Zasshi*, **82**, 1075 (1961).

19) H. Yajima and N. Fujii, *J. Am. Chem. Soc.*, **103**, 5867 (1981).

20) N. Fujii, M. Shimokura, H. Yajima, F. Shono, M. Tsuda, and A. Yoshitake, *Chem. Pharm. Bull.*, **32**, 1193 (1984).

21) In the present study, this acyl migration was not checked: M. Fujino, M. Wakimasu, S. Shinagawa, C. Kitada, and H. Yajima, *Chem. Pharm. Bull.*, **26**, 539 (1978).

22) S. S. Wang, B. F. Gisin, D. P. Winter, R. Makofske, I. D. Kulesha, C. Tzougraki, and J. Meienhofer, *J. Org. Chem.*, **42**, 1286 (1977).

23) D. J. Woodman, L. C. Butler, and J. Stewart, *Tetrahedron Lett.*, **1973**, 1557.

24) N. Lotan, A. Berger, and E. Katchalski, *Ann. Rev. Biochem.*, **41**, 869 (1972).

25) S. C. Harrison and E. R. Blout, *J. Biol. Chem.*, **240**, 299 (1965).

26) S. Beychok and E. R. Blout, *J. Mol. Biol.*, **3**, 769 (1961).

27) G. Holzwarth and P. Doty, *J. Am. Chem. Soc.*, **87**, 218 (1965).

28) N. Greenfield and G. D. Fasman, *Biochemistry*, **8**, 4108 (1969).

29) E. Breslow, S. Beychok, K. D. Hardman, and F. R. N. Gurd, *J. Biol. Chem.*, **240**, 304 (1965).

30) H. N. Rydon and P. W. G. Smith, *Nature (London)*, **169**, 922 (1952).

31) Mp 104—106°C: E. Schnabel, H. Herzog, P. Hoffmann, E. Klauke, and I. Ugi, *Justus Liebigs Ann. Chem.*, **716**, 175 (1968).

32) The yield is based on the molar quantity calculated from the result of amino acid analyses.