Amphiphilic α-helical structure in water stabilized by dioctadecyl chain

Jinbao Zhao, Shunsaku Kimura and Yukio Imanishi*
Department of Polymer Chemistry, Kyoto University, Yoshida Honmachi, Sakyo-Ku, Kyoto, Japan 606-01

The peptides H-(Leu-Aib-Lys-Aib-Aib-Lys-Aib)$_3$-X [X = OMe, P21OMe; X = -Ala-N($C_{18}H_{37}$)$_2$, P21D], have been synthesized as models for biologically active peptides having long alkyl chains, and the effect of the alkyl chain on the peptide conformation has been studied. CD measurements revealed that the α-helix content of P21D in water was higher than in P21OMe, while their conformations were nearly the same in methanol. Guanidine hydrochloride (GdnHCl), which is a denaturant of proteins, increased the helix content of P21D, but decreased the helix content of P21OMe. The stabilization of the helical structure of P21D at high concentrations of GdnHCl is ascribed to a reduction in the electrostatic repulsion between the ammonium groups of P21D at high ionic strength. It is notable that in water the major fraction of P21D exists as a monomer, but the number of P21D molecules involved in higher aggregates is significant, as revealed by dynamic light scattering measurement. A short-chain peptide, H-Leu-Aib-Lys-Aib-Aib-Lys-Aib-Ala-N($C_{18}H_{37}$)$_2$, formed much larger aggregates in water than P21D. It probably formed micelles without taking on a helical conformation. Therefore, the connection of two long alkyl chains at the C terminal of a long-chain amphiphilic helical peptide is a suitable method for stabilizing the helix conformation without being accompanied by severe aggregation.

Introduction

A number of proteins are covalently linked to lipids, *e.g.* myristic acid and palmitic acid, and are termed 'acylproteins'.[1] Since acylproteins have various functions, it is difficult to deduce a common role for protein-bound fatty acids.[2] One function of the long-chain fatty acid is to anchor the protein to cellular membranes. For example, the myristyl moiety of $p60^{src}$ and $Pr65^{gag}$ is essential for these proteins to be bound to the inner face of a plasma membrane.[3,4] The myristyl group is thought to be inserted into the lipid membrane simply due to its hydrophobicity. However, another interpretation has been proposed for the myristyl group that it acts as a signal to the receptor protein to locate the acyl protein at a specific site in the cell.[5] In addition, there are several myristylated proteins that are not bound to cellular membranes such as the catalytic subunit of the cAMP-dependent protein kinase.[6] Therefore, it is best to consider that the fatty acid group changes the physical properties of the protein so as to influence interactions with receptor proteins, lipid membranes or nucleic acids. This consideration prompted us to investigate the effects of connecting long alkyl chains to the terminal position of a model peptide on its physical properties, especially the secondary structure of the peptide in a buffer solution.

Amphiphilic helical structures are frequently found in proteins and peptide hormones.[7,8] Amphiphilicity is considered to be important for stabilization of the tertiary structure of proteins as exemplified by 4 α-helix bundle structure.[9,10] The amphiphilic structure also plays an important role in the partition of a peptide to a phospholipid bilayer membrane and in binding to a receptor protein.[11] It is, therefore, useful to design peptide molecules which take a stable amphiphilic structure in a buffer solution.

We have reported that the amphiphilic polypeptide, poly(Lys-Aib-Leu-Aib), took an α-helical conformation in water, and the amphiphilic helical structure is considered to be the reason that the peptide can induce fusion of dipalmitoylphosphatidylcholine (DPPC) vesicles in an alkaline solution.[12] However, the sequential peptide possesses winding hydrophobic and hydrophilic surfaces around the helix axis. In the present paper, -(Leu-Aib-Lys-Aib-Aib-Lys-Aib)$_3$-, was designed and synthesized as an amphiphilic model peptide, which has hydrophobic and hydrophilic surfaces straight along the helix axis. The molecular structures of the synthesized peptides are shown in Fig. 1 with the helical wheel of the peptide part. Two octadecyl chains were attached to the peptide, which may stabilize the helical structure by intramolecular association of the alkyl chains with the hydrophobic surface of the peptide. A peptide with a stable amphiphilic helical conformation should manifest enhanced activities. In the present study, dioctadecylamine was chosen for the acylation reaction with the C terminal carboxylic acid group of the peptide, because a single alkyl chain such as myristyl and palmityl has a smaller cross section than the peptide, and may favour micelle formation.[13] Micelle formation is unfavourable in the construction of a specified structure because micelles do not have a defined structure. However, the

H-[Leu-Aib-Lys-Aib-Aib-Lys-Aib]$_n$-X

n = 1, X: -OBz(P7Bz), -Ala-N($C_{18}H_{37}$)$_2$(P7D)
n = 3, X: -OMe(P21OMe), -Ala-N($C_{18}H_{37}$)$_2$(P21D)

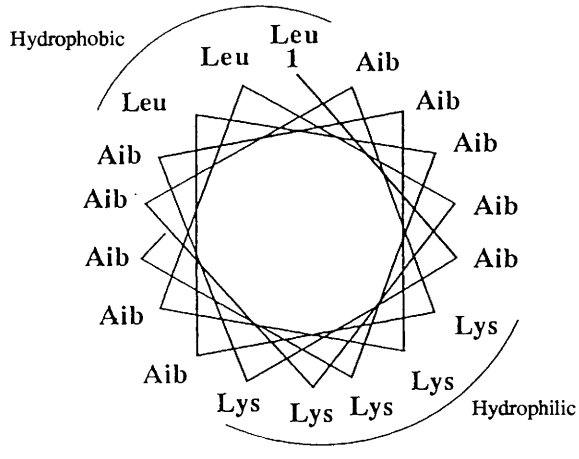

Fig. 1 Molecular structure of the peptides synthesized. The henicosapeptide is designed to take an amphiphilic α-helical structure as shown by the helical wheel presentation.

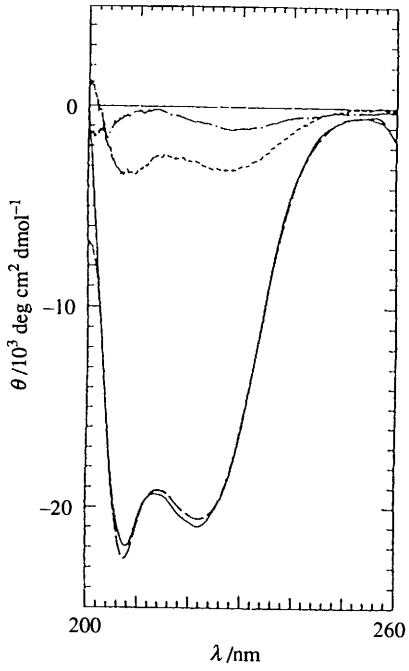

Fig. 2 CD spectra of P21D (—), P21OMe (– – –), P7D (- - -) and P7Bz (– · –) in methanol at 30 °C. [P21D] = [P21OMe] = 5.0 μmol dm^{-3}. [P7D] = [P7Bz] = 40 μmol dm^{-3}.

cross section of the dioctadecyl group in H-(Leu-Aib-Lys-Aib-Aib-Lys-Aib)$_3$-Ala-N($C_{18}H_{37}$)$_2$ (P21D) is comparable to that of the peptide part in a helical conformation, and will sterically hinder micelle formation.[13] The peptide part is composed of α-aminoisobutyric acid (Aib) residues to promote helix formation.[14] Our aim is not to form large aggregates or micelles of the peptide, but to investigate the effects of the long alkyl chain on the peptide structure.

Results and discussion

Conformation

CD spectra of P21D, P21OMe, P7D and P7Bz in methanol are shown in Fig. 2.† The double-minimum pattern of P21D and P21OMe indicates an α-helical conformation, which agrees with previous reports that Aib-containing peptides favour the helical conformation.[15] The helix content of both peptides is *ca.* 60%. However, the low molar ellipticities of P7D and P7Bz suggest an irregular conformation, which is probably due to the peptide chains not being long enough.

The helix content of P21OMe decreased to 26% in water. However, the helix content of P21D was not influenced by the change of solvent, indicating that the terminal dioctadecyl group of P21D stabilizes the helical conformation of the peptide part. Despite the presence of dioctadecyl groups, P7D did not take a specific conformation in water, suggesting that a heptapeptide chain is not long enough to take a helical conformation in solution.

CD spectra of P21D and P21OMe were measured at various NaCl concentrations, and the intensity at 222 nm or the helix

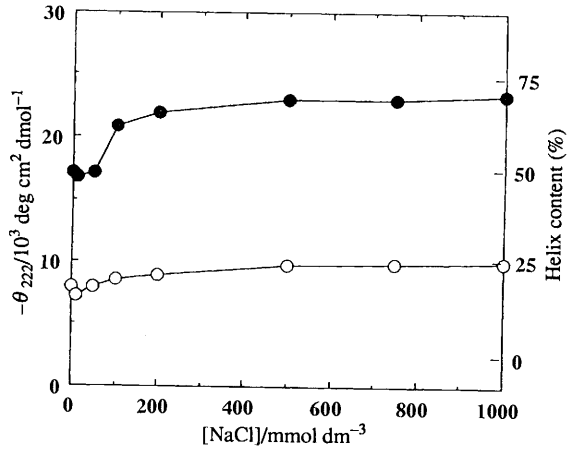

Fig. 3 Change in the ellipticity at 222 nm of P21D (●) and P21OMe (○) with NaCl concentration in water at 30 °C; [peptide] = 5.0 μmol dm^{-3}

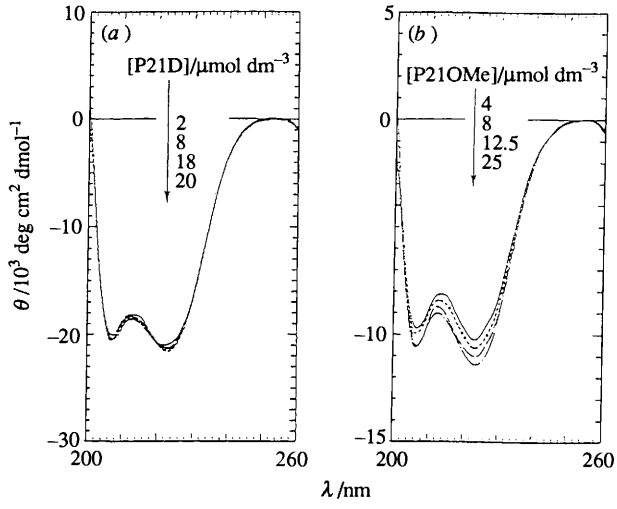

Fig. 4 Change of CD spectra of (*a*) P21D and (*b*) P21OMe with peptide concentration in a buffer solution (10 mmol dm^{-3} Tris, pH 7.4) at 30 °C. The numbers in the figures represent the peptide concentrations.

content was plotted against the concentration (Fig 3). The helix content of P21D increased significantly with the addition of 100 mmol dm^{-3} NaCl. This observation can be explained by a reduction in the electrostatic repulsion between ammonium groups of Lys side chains with increasing ionic strength of the solution.

Aggregation

CD spectra of P21D and P21OMe in a buffer solution were measured at various peptide concentration (Fig. 4). The Cotton effect at 222 nm of P21OMe increased as the peptide concentration increased, while the spectral pattern was independent of peptide concentration. Therefore, P21OMe might be aggregated in a buffer solution at higher concentrations than 4 μmol dm^{-3}. On the other hand, CD spectra of P21D were not influenced by the peptide concentration in the range of 2 to 20 μmol dm^{-3}, suggesting that no aggregation of P21D is occuring although the connection of long alkyl groups at the C terminal was considered to promote the association of peptides due to hydrophobic interactions.

P21D and P7D were subjected to a dynamic light scattering measurement to examine the size distribution of the peptides in water (Fig. 5). The number-average diameter of the aggregates was 2.3 and 15 nm for P21D and P7D, respectively, assuming

† *Abbreviations:* Aib, 2-aminoisobutyric acid; ANS, 8-anilinonaphthalene-1-sulfonic acid; Boc-, *tert*-butoxycarbonyl; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; GdnHCl, guanidine hydrochloride; HOBt, *N*-hydroxybenzotriazole; -OBzl, benzyl ester; -OMe, methyl ester; P7D, H-Leu-Aib-Lys-Aib-Aib-Lys-Aib-Ala-N($C_{18}H_{37}$)$_2$; P7Bz, H-Leu-Aib-Lys-Aib-Aib-Lys-Aib-OBzl; P21D, H-(Leu-Aib-Lys-Aib-Aib-Lys-Aib)$_3$-Ala-N($C_{18}H_{37}$)$_2$, P21OMe, H-(Leu-Aib-Lys-Aib-Aib-Lys-Aib)$_3$-OMe; THF, tetrahydrofuran; TFA, trifluoroacetic acid.

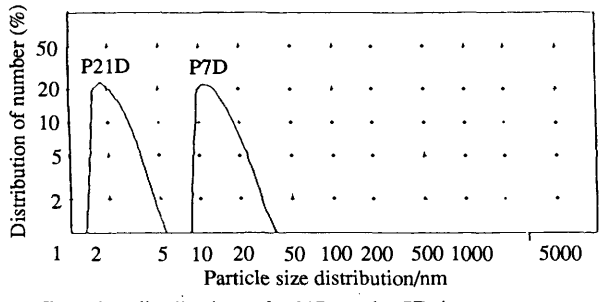

Fig. 5 The size distribution of P21D and P7D in water at room temperature as measured by dynamic light scattering. The size is expressed by the diameter of the solvated sphere.

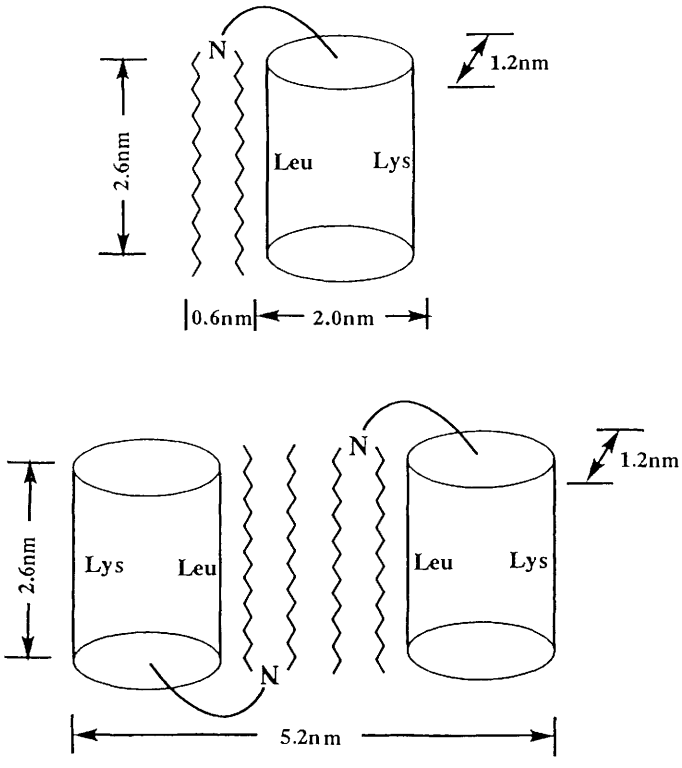

Fig. 6 Schematic presentation of the molecular shape of P21D in a monomer and a dimer state

that the peptides are in hydrated spheres. P7D is clearly shown to form larger aggregates than P21D. Since the peptide part of P7D does not take on a regular structure, the molecular shape of P7D should be wedge-shaped. In the wedged structure of P7D, the hydrophilic peptide part has a larger cross section than the hydrophobic dialkyl chain. This shape is suitable for micelle formation.[13]

The translational diffusion coefficient depends on the shape of a particle.[16] We assumed that P21D takes the molecular shape in water as shown in Fig. 6, where the dioctadecyl group is associated with the hydrophobic surface of the helix cylinder of P21D. The shape of the cross section of the helix cylinder is an ellipse, because the side chains of Leu and Lys extend to the outside along the long axis (as shown by the helical wheel presentation in Fig. 1). The length of the long axis is about 2.6 nm.[17] The side chains of Aib are directed along the short axis, which is about 1.2 nm. The length of the long axis, 2.6 nm, is nearly the same as that of the molecular length which is the sum of the length of the extended octadecyl chain and the length of the C terminal turn of the peptide chain. Therefore, the structure can be roughly regarded as tetragonal column with a = 1.2, b = 2.6 and c = 2.6 nm. The radius of gyration, R_g, is

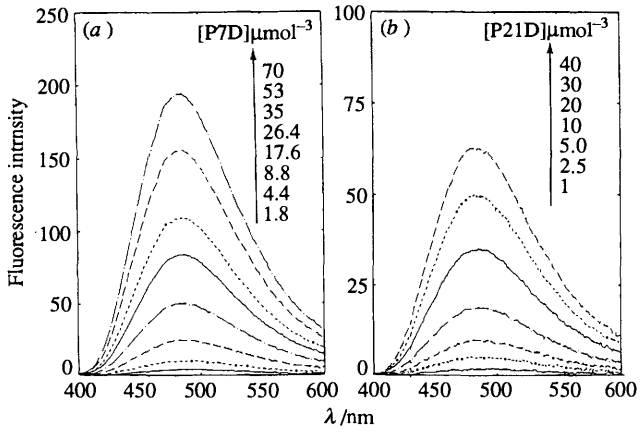

Fig. 7 Fluorescence spectra of ANS in the presence of different concentrations of (*a*) P21D and (*b*) P7D in a buffer solution (10 mmol dm^{-3} Tris, pH 7.4) at 30 °C; [ANS] = 50 μmol dm^{-3}. The numbers in the figures represent the peptide concentrations.

calculated to be 1.1 nm. When two peptides form a dimer with the dioctadecyl groups facing each other, the shape is a tetragonal column with a = 1.2, b = 5.2 and c = 2.6 nm, and R_g is 1.7 nm. The experimental value of R_g is 0.9 nm, indicating that the monomeric state predominates in water. The same conclusion is reached by assuming an ellipsoidal shape.

However, the formation of dimer, tetramer and higher aggregates cannot be excluded because the distribution was not so narrow (Fig. 5), and the amount of aggregates with diameter larger than 2.6 nm was 22.4%. Therefore, more than half of P21D molecules are present in aggregates, while the monomer forms the largest single species.

ANS is a fluorescent probe for hydrophobic environments. Fluorescence of ANS was measured in the presence of different concentrations of P21D, P7D and P21OMe (Fig. 7). A significant increase of fluorescence was observed in the presence of P7D or P21D, but the increase in the presence of P21OMe was slight (less than 1/20-fold of the effect of P21D). These experimental results lead to the conclusion that P7D and P21D form a hydrophobic domain for ANS binding. P7D might form micelles by the association of dioctadecyl chains, providing a hydrophobic environment suitable for ANS binding. This consideration is consistent with the result of dynamic light scattering measurements. The hydrophobic domain of P21D should be provided by the hydrophobic surface of the secondary amphiphilic structure and the octadecyl chain. On the other hand, P21OMe was shown to aggregate at concentrations above 4 μmol dm^{-3} by CD measurement (Fig. 4), and is considered to form a hydrophobic domain by association of the hydrophobic surface of the amphiphilic helical peptides. However, this domain is not so hydrophobic as to enhance ANS binding. Binding of negatively charged ANS is influenced by electrostatic interactions as well as hydrophobic interactions. P7D possesses three positive charges and P21D seven positive charges. Nevertheless, the extent of fluorescence enhancement of ANS by P7D was higher than that by P21D. It is, therefore, considered that the micelle formation of P7D is very suitable for ANS-binding due to association of octadecyl chains. On the other hand, ANS should be located at the hydrophobic surface of the bent conformation of P21D with the formation of an ion pair between ANS and Lys. This configuration should provide a hydrophobic environment in which ANS can increase the fluorescence intensity. The hydrophobicity of the ANS-binding site of the peptides increases in the order of P21OMe < P21D < P7D.

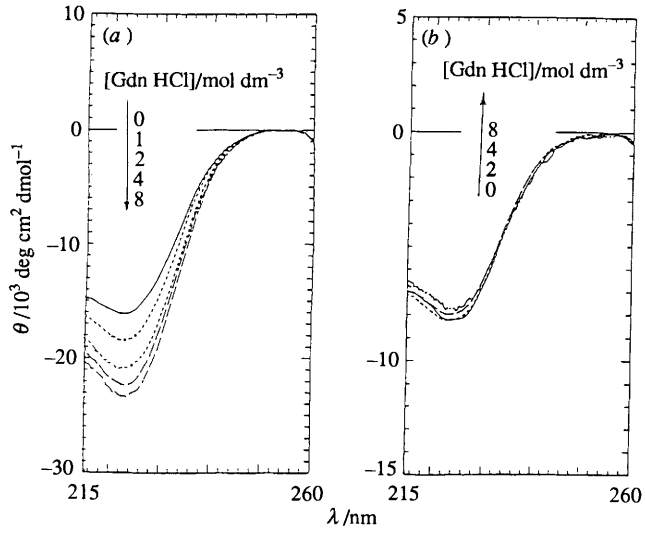

Fig. 8 Change in the CD spectra of (*a*) P21D and (*b*) P21OMe on addition of guanidine hydrochloride in water at 30 °C; [peptide] = 5.0 μmol dm^{-3}. The numbers in the figures represent the concentrations of guanidine hydrochloride.

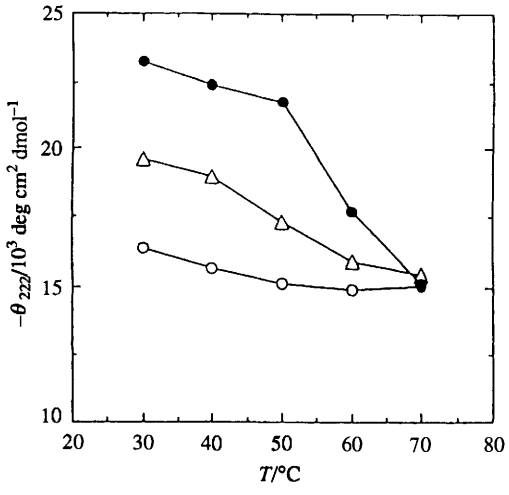

Fig. 9 Temperature dependence of the ellipticity at 222 nm of P21D and in the presence of 4 mol dm^{-3} (△) and 8 mol dm^{-3} (●) guanidine hydrochloride and in the absence of guanidine hydrochloride (○) in water; [peptide] = 5.0 μmol dm^{-3}

Helix stability

The stability of the α-helical conformation of P21D and P21OMe was evaluated by the addition of guanidine hydrochloride. The Cotton effect at 222 nm of P21OMe was slightly weakened by the addition of 8 mol dm^{-3} guanidine hydrochloride. On the other hand, that of P21D was strengthened by more than 40% by the presence of guanidine hydrochloride (Fig. 8). This result is interpreted as the denaturation effect of guanidine hydrochloride being overwhelmed by the increasing ionic strength of the medium, resulting in relaxation of the electrostatic interactions of the Lys side chains of P21D. In addition, conformational stability of P21D against guanidine hydrochloride should be gained from the two dioctadecyl chains shielding the guanidine hydrochloride from access to the peptide bonds, which are buried in the domain formed by association of the octadecyl group and the hydrophobic surface of the helical peptide.

Thermal denaturation of P21D was examined by CD spectroscopy in the presence of various concentrations of guanidine hydrochloride (Fig. 9). The helical conformation of P21D was destroyed as the temperature was raised. It is notable that the temperature at which the half of P21D is denatured in the presence of 8 mol dm^{-3} guanidine hydrochloride is higher than that without guanidine hydrochloride, showing that the helical structure of P21D is more stable in the presence of guanidine hydrochloride.

Conclusions

The amphiphilic peptide, P21OMe, which is composed of helix-forming amino acids, does not take a stable helical structure in water, where the helix content was half of that in methanol. The α-helical structure of P21OMe is destabilized in water due to hydration of peptide bonds, which prevents formation of intramolecular hydrogen bonds. When the amphiphilic peptide is connected to a dioctadecyl group at the C terminal (P21D), the helical structure in water became as stable as in methanol. This stabilization is due to intramolecular association of the dioctadecyl group with the hydrophobic surface of the amphiphilic α-helical structure. The bent structure of P21D, which allows association between the peptide chain and the dioctadecyl group, is in contrast to the aggregation (micelle formation) of P7D. The peptide chain of P7D is too short to be hydrophobic enough to associate with the dioctadecyl group, so that the molecular shape becomes wedge-like. These situations should lead to micelle formation of P7D. It is notable that the helix content of P21D is 70% at 50 °C in the presence of 8 mol dm^{-3} guanidine hydrochloride. The remarkable stability of the amphiphilic α-helical peptide is useful for the *de novo* design of biologically active peptides.

Experimental

Materials

N^{α}-Boc-N^{ε}-formyl-L-lysine [Boc-Lys(F)] and *N*-Boc-2-amino-isobutyric acid (Boc-Aib) were prepared by the methods reported previously.[18,19] Other N^{α}-Boc amino acids were purchased from Kokusan Chem. Inc., Japan. 8-Anilinonaphthalene-1-sulfonic acid (ANS) was purchased from Molecular Probes Inc., USA. Dioctadecylamine [$HN(C_{18}H_{37})_2$] was obtained from Fluka, Switzerland.

Synthesis

The peptides were synthesized by a conventional liquid-phase method according to the synthetic route exemplified by the synthesis of P21OMe in Fig. 10. The products were identified by NMR measurements, and their purity was checked by TLC using Silica Gel 60 F_{254} aluminum plates (Merck, USA). The peptides were visualized by UV light, iodine, ninhydrin and/or chlorine–tolidine methods. Solvent systems for TLC were (*A*) chloroform–methanol–acetic acid (95:5:3), (*B*) chloroform–methanol–aqueous ammonia (65:25:5) and (*C*) butanol–acetic acid–water (100:10:30).

Boc-Leu-Aib-Lys(F)-Aib-Aib-Lys(F)-Aib-OBzl (Boc7OBzl). Coupling reactions were carried out using dicyclohexylcarbodiimide (DCC) and *N*-hydroxybenzotriazole (HOBt) as shown in Fig. 10. The product was purified twice with a Sephadex LH-20 column using *N*,*N*-dimethylformamide (DMF) and methanol as eluent. The major fraction was collected and precipitated with light petroleum to give the title compound, $R_f(A)$ = 0.45, $R_f(B)$ = 0.80, $R_f(C)$ = 0.64 (Found: C, 57.9; H, 8.4; N, 12.7. Calc. for $C_{48}H_{79}N_9O_{12}\cdot H_2O$: C, 58.11; H, 8.23; N, 12.70%).

Boc-[Leu-Aib-Lys(F)-Aib-Aib-Lys(F)-Aib]$_3$-OBzl (Boc-21-OBzl). The Boc group and benzyl ester of Boc7OBzl were removed by treatment with trifluoroacetic acid (TFA) and catalytic hydrogenation, respectively. The heptapeptides were coupled, and further fragment condensation gave Boc-21-OBzl, $R_f(B)$ = 0.85, $R_f(C)$ = 0.39 (Found: C, 56.4; H, 8.1; N, 14.7.

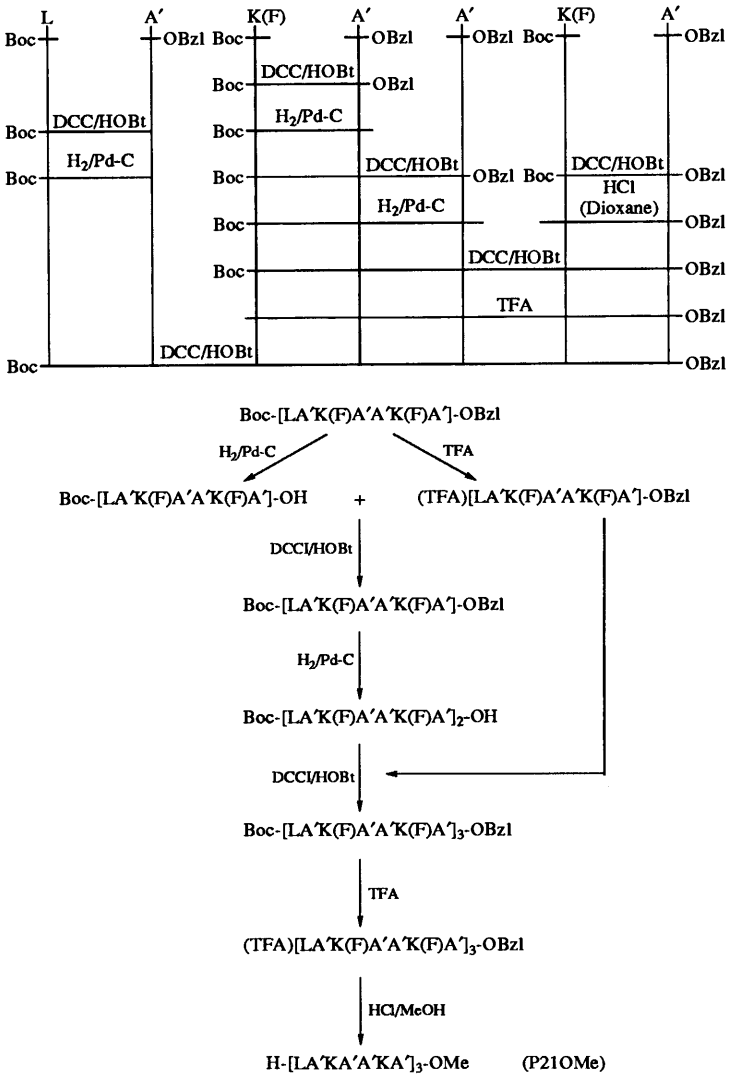

Fig. 10 Synthetic route to the peptides

Calc. for $C_{120}H_{205}N_{27}O_{30}$·2.5 H_2O: C, 56.49; H, 8.29; N, 14.82%).

Boc-L-Ala-2C18. $HN(C_{18}H_{37})_2$ (522 mg, 1.0 mmol) and Boc-L-Ala (227 mg, 1.2 mmol) in a mixture of DMF–chloroform (1:4 v/v) were coupled by DCC (339 mg, 1.5 mmol) and HOBt (203 mg, 1.5 mmol) at 0 °C for 4 h and then at room temperature for 24 h. The solution was centrifuged to remove dicyclohexylurea and then evaporated. The residue was dissolved in chloroform and the solution was washed three times with aqueous $KHSO_4$, NaCl, $NaHCO_3$ and NaCl, and then dried with Na_2SO_4. The solvent was evaporated and the residue was purified by a silica gel column using ethyl acetate as eluent to give the title compound, $R_f(A)$ = 0.70, R_f(ethyl acetate) = 0.90.

Boc-7-Ala-2C18. To a DMF–chloroform (1:2 v/v) solution containing Boc7-OH (45 mg, 0.051 mmol) and HCl H_2N-Ala-2C18 (48 mg, 0.077 mmol, prepared by deprotection of Boc-L-Ala-2C18 by 4 mol dm^{-3} HCl–dioxane), DCC (17 mg, 0.077 mmol), HOBt (10 mg, 0.077 mmol) and triethylamine (10.7 mm^3, 0.077 mmol) were added at 0 °C. The solution was left to stand at room temperature, and then condensed under reduced pressure. The residue was dissolved in tetrahydrofuran (THF), and dicyclohexylurea was removed by centrifugation. The solution was purified with a Sephadex LH-20 column using THF as eluent to give the title compound (80%), $R_f(A)$ = 0.23, $R_f(B)$ = 0.72, $R_f(C)$ = 0.40 (Found: C, 66.1; H, 10.5; N, 10.8. Calc. for $C_{80}H_{151}N_{11}O_{12}$: C, 65.85; H, 10.43; N, 10.56%).

Boc-21-Ala-2C18. To a DMF–chloroform (2:3 v/v) solution containing Boc-14-OH (35 mg, 0.021 mmol) and HCl H_2N-7-Ala-2C18 (32 mg, 0.023 mmol), DCC (7 mg, 0.032 mmol), HOBt (4.3 mg, 0.032 mmol) and triethylamine (3.2 mm^3, 0.023 mmol) were added. The solution was stirred at 0 °C for 4 h and then at room temperature for 1 day. Further DCC (2.4 mg, 0.01 mmol) was added and the solution was stirred for a further 2 days. The product was separated, dissolved in THF, and washed and purified as described above to give the title compound, $R_f(B)$ = 0.91, $R_f(C)$ = 0.38 (Found: C, 60.65; H, 9.4; N, 13.6. Calc. for $C_{152}H_{277}N_{29}O_{30}$·1.5$H_2O$: C, 60.49; H, 9.35; N, 13.46%).

Deprotection. The four kinds of peptides, Boc-7-OBzl, Boc-7-Ala-2C18, Boc-21-OBzl and Boc-21-Ala-2C18, were treated with trifluoroacetic acid at 0 °C for 1 h. The resulting solution was condensed and diethyl ether was added to precipitate the product. The precipitate was dissolved in 0.5 mol dm^{-3} HCl–methanol, and the solution was left to stand for 3 days at room temperature. The reaction mixture was concentrated, and diethyl ether was added to precipitate the product. The precipitate was purified twice by partition chromatography using a Sephadex G-25 column, which was equilibrated with an upper layer of butanol–acetic acid–water (4:1:5 v/v/v) and eluted by the lower layer. The major fraction was collected, and

the aqueous solution was freeze-dried to give the deprotected product, P21D: $R_f(B) = 0.14$. P21OMe: $R_f(B) = 0.22$. P7D: $R_f(B) = 0.56$. P7Bz: $R_f(B) = 0.33$.

Measurements

Circular dichroism (CD) spectra were recorded on a JASCO-J600 spectropolarimeter using a cell with 1 cm optical path length. All the samples were dissolved in Tris buffer (10 mmol dm^{-3}, pH 7.4), and incubated for 10 min at 30 °C before the measurement unless otherwise specified. The helix content, f_H, was calculated from the strength of the Cotton effect at 222 nm according to the eqn. (1) reported by Chen *et al.*[20] This method

$$f_H = -(\theta_{222} + 2340)/30300 \quad (1)$$

was chosen because the peptides take on a mixed conformation of α-helix and random coil as shown by analysis using a secondary structure analysis program.

Fluorescence measurements were performed on a Hitachi MPF-4 spectrophotometer. The peptides were dissolved in a buffer solution of ANS (50 μmol dm^{-3}). The solution was incubated at 30 °C for 10 min, and the fluorescence spectra were recorded. The excitation wavelength was 345 nm.

The dynamic light scattering of the peptide in water was measured on a Malvern 7032 multicorrelator, and the data were analysed by the SYSTEM 4700C. The peptide concentration was 4.0 mg cm^{-3}. Water purified by double distillation after ion-exchange was used, and the sample solutions were filtered before the measurement was taken. The data were analysed by Malvern's software to obtain size distribution. For solvated spheres, the translational diffusion coefficient is given by the Stokes–Einstein relations as given in eqn. (2),[16] where η_0 and

$$D = kT/6\pi\eta_0 R \quad (2)$$

R represent the viscosity of the solvent and the radius, respectively. The Gyration radius, R_g, for spheres is calculated using eqn. (3). However, R_g for a tetragonal column of $2a$ long,

$$R_g = (3/5)^{\frac{1}{2}} R \quad (3)$$

$2b$ broad and $2c$ high is calculated using eqn. (4). For ellipsoids, a

$$R_g = [(a^2 + b^2 + c^2)/3]^{\frac{1}{2}} \quad (4)$$

shape-dependent factor, $G(\rho)$, as shown in eqn. (5) is taken into

$$G(\rho) = (1 - \rho^2)^{-\frac{1}{2}} \ln\{[1 + (1 - \rho^2)^{\frac{1}{2}}]/\rho\}, \quad \rho < 1 \quad (5)$$

consideration, where ρ is the axial ratio (the ratio of the lengths of the minor and the major semi-axes).

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