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A Membrane Protein Model: Polypeptides with Four α-Helix Bundle Structure on 5,10,15,20-Tetrakis[2-(carboxymethoxy)phenyl]porphyrin

Toru Arai, Keisuke Kobata, Hisakazu Mihara,# Tsutomu Fujimoto, and Norikazu Nishino*

Department of Applied Chemistry, Faculty of Engineering, Kyushu Institute of Technology,
Tobata-ku, Kitakyushu 804

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A novel template for the polypeptide–porphyrin hybrid, 5,10,15,20-tetrakis[2-(ethoxycarbonylmethoxy)phenyl]porphyrin (**2**) was synthesized in 17% yield. Four protected carboxyl groups are installed in **2** at the *ortho*-phenyl groups as the anchoring points, to which the N-termini of the polypeptides can be linked after the hydrolysis. Thus, **2** is a new analog of the porphyrin template tetrakis[2-(methoxycarbonyl)phenyl]porphyrin. The atropisomerization of **2** was much more rapid than that of tetrakis[2-(methoxycarbonyl)phenyl]porphyrin: At 353 K, **2** reached their equilibrium within 15 min. Such atropisomeric flexibility of **2** allows the self-organization of the α-helical peptide segments which are connected to **2**. After the hydrolyses of the ethyl ester groups of **2**, four α-helical 21-peptides, H–(–Gln–Leu–Leu–Gln–Ala–Leu–Ala–)$_3$–$NHCH_2CH_2OH$ were combined through the amide bonds to yield a new polypeptide–porphyrin hybrid (**12**). The polypeptide moieties of **12** showed typical α-helix profiles on circular dichroism measurements in methanol–water (3/1, v/v) and in Tris·HCl buffer solution. Thus, the four α-helix polypeptides are considered to fold into a bundle structure on the porphyrin template through the hydrophobic interaction. The circular dichroism spectrum of **12** in methanol showed an induced Cotton effect at the porphyrin region, and the CD band was symmetrically split. The hybrid (**12**) was also successfully incorporated into the egg yolk lecithin membrane almost quantitatively. When the molar ratio of egg yolk lecithin/**12** was 500/1, the α-helix structure of the peptide segments in **12** was completely retained in the vesicles.

The porphyrin-containing proteins are of biological importance and show a wide variety of functions: for instance, catalysis, photochemical property, and redox activity. Several researchers have tried to construct model systems of the proteins containing porphyrins.[1)] In the biological systems, porphyrins are fixed to the polypeptides and are often embedded into the lipid membrane. Such porphyrin-containing proteins in the membrane may exhibit different properties from the artificial models built in the solution. Therefore, one goal of the artificial models at present is to construct a polypeptide–porphyrin hybrid that can be stably embedded into the membrane.[1c,1d)] On the other hand, a polypeptide with a desired three-dimensional structure can nowadays be built up by using the *de novo* design strategy.[2)] One of the features of the peptides compared with the other organic materials, is their capability of self-organization. The four α-helix bundle structure is the representative motif of the peptide of well-defined structure, which is made up by amphiphilic α-helix peptide segments.[3)] The porphyrins bearing four functional groups that can anchor polypeptide segments thus seem to be suitable for the template for the polypeptide assembly.[1a,1b)] Moreover, a novel type of artificial functional polypeptides can be constructed in combination with the porphyrin function.

So far, the four α-helix bundle structures have been constructed through the intramolecular aggregation on the porphyrin rings by Groves et al. using tetrakis(3-carboxyphenyl)porphyrin and by Sasaki and Kaiser using coproporphyrin I.[1a,1b,1d)] We have reported the synthesis of a polypeptide–porphyrin hybrid using the αααα-atropisomer of 5,10,15,20-tetrakis(2-carboxyphenyl)porphyrin as a template (Fig. 1).[1c)] Our template, tetrakis(2-carboxyphenyl)porphyrin (**3**), seemed to be most efficient in folding the bundle structure, because the peptide segments are bound to the tetraphenylporphyrin framework at the *ortho*-phenyl positions and are located close to each other. In this polypeptide–tetrakis(2-carboxyphenyl)porphyrin hybrid, four α-helix polypeptide segments are oriented on one face of the porphyrin plane. Therefore, the polypeptides filled

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#Present address: Department of Bioengineering, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta, Yokohama 226.

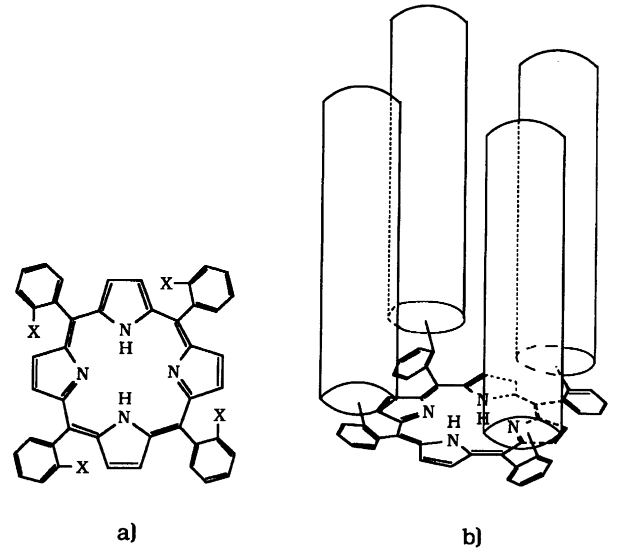


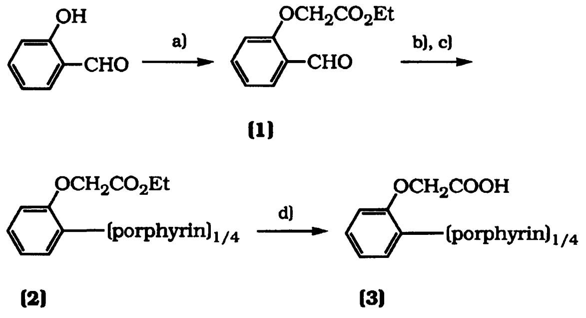
Fig. 1. a) Structures of polypeptide–porphyrin hybrid and its precursors. 5,10,15,20-Tetrakis(2-carboxyphenyl)porphyrin, –X = –CO_2H; 5,10,15,20-tetrakis-[2- (ethoxycarbonylmethoxy)phenyl]porphyrin (**2**), –X=–OCH_2CO_2Et; **3**, –X=–OCH_2CO_2H; **12**, –X= –OCH_2CO_2–(–Gln–Leu–Leu–Gln–Ala–Leu–Ala–$)_3$–$NHCH_2CH_2OH$. b) Illustration of the four α-helix peptide segments of **12** folded into a bundle structure. The piles indicate the α-helix peptide segments.

the porphyrin plane and the pillar-like structure of the hybrid enabled the stable embedding into the lipid bilayers. However, the isolation of the αααα-isomer of the tetrakis(2-carboxyphenyl)porphyrin was tedious and resulted in low yield (3.8% after repeating conversion of other isomers).[4]

In the present paper, we demonstrate the use of tetrakis(2-carboxylmethoxyphenyl)porphyrin (**3**) as a novel template for the polypeptide–porphyrin hybrid. In this template, four carboxyl groups are linked to the *ortho*-phenyl positions of the tetraphenylporphyrin framework like tetrakis[2-(carboxymethoxy)-phenyl]porphyrin (Fig. 1). In the atropisomerization, the rotation of the phenyl groups is not strictly hindered in the protected precursor of the template, tetrakis[2-(ethoxycarbonylmethoxy)phenyl]porphyrin (**2**). Therefore, the intramolecular aggregation of the four peptide segments bound to the porphyrin may prefer the formation of the αααα-atropisomer through the rotation of the phenyl groups, due to the low energy barrier.

Results and Discussion

Synthesis and Atropisomerism of 5,10,15,20-Tetrakis[2-(ethoxycarbonylmethoxy)phenyl]-porphyrin (2). 5,10,15,20-Tetrakis[2-(ethoxycarbonylmethoxy)phenyl]porphyrin (**2**) (Fig. 1a) was synthesized from 2-(ethoxycarbonylmethoxy)benzaldehyde (**1**) and pyrrole by Lindsey's method in 17% yield (Fig. 2).[5] As is well known, the *ortho*-substituted porphyrin is obtained as an atropisomeric mixture because of the restricted rotation of the C–C bonds between the porphyrin ring and the phenyl groups. In fact, the four atropisomers of **2** were detected both by TLC (silica gel) and reversed-phase HPLC analyses (Table 1, Fig. 3). In the TLC analysis of the atropisomeric mixture of **2** (chloroform/methanol=49/1, v/v), the fraction with largest R_f value (0.53) was determined to be the αβαβ-isomer. This was the least polar of the isomers. Similarly, the fraction with the smallest R_f value (0.33) was determined to be the most polar isomer, the αααα- one. The other two fractions with R_f values of 0.47 and 0.38 were presumed to be ααββ- and αααβ-isomers, respectively. In the reversed-phase HPLC, the four atropisomers of **2** were eluted in the order of αβαβ-, ααββ-, αααβ-, and αααα-, the same in the case of tetrakis[2-(methoxycarbonyl)phenyl]porphyrin.[4] The most polar



a) ethyl bromoacetate, K_2CO_3 b) pyrrole, $BF_3 \cdot OEt_2$
c) chloranil d) NaOH aq.

Fig. 2. Scheme for the synthesis of the porphyrin template, tetrakis[2-(carboxymethoxy)phenyl]porphyrin (**3**).

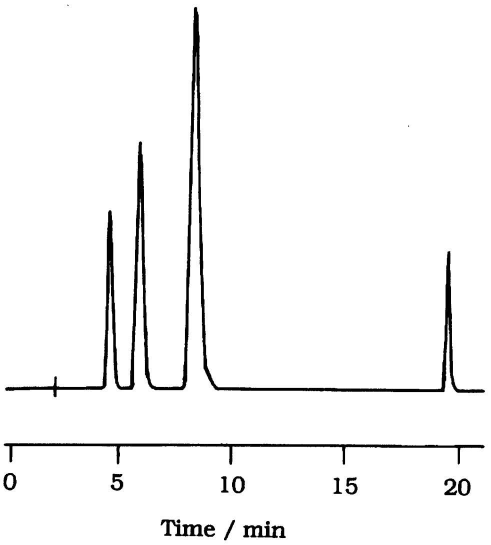


Fig. 3. Reversed-phase HPLC analysis of the atropisomers of **2**. Column, Waters μBondasphere C18 (3.9×150 mm); eluent, a linear gradient of acetonitrile/water=88/12 to 100/0 (v/v) over 10 min, and 100% acetonitrile (10—30 min); flow rate, 1.0 cm^3 min^{-1}; detected at 420 nm.

Table 1. Atropisomers of **2**

Fraction	R_f value[a)]	Retention time/min[b)]	Assignment	Content at equilibrium/%	Statistic ratio/%
1	0.53	4.46	$\alpha\beta\alpha\beta$-	16	12.5
2	0.47	5.76	$\alpha\alpha\beta\beta$-	26	25.0
3	0.38	8.11	$\alpha\alpha\alpha\beta$-	49	50.0
4	0.33	19.22	$\alpha\alpha\alpha\alpha$-	9	12.5

a) Silica gel, chloroform/methanol=(49/1, v/v). b) See Fig. 3 for the HPLC conditions.

$\alpha\alpha\alpha\alpha$-isomer was eluted from the octadecyl-silica gel column with 100% acetonitrile. Because of the fast rotation of the phenyl groups of **2** (see below), the ^{1}H NMR analyses of the isolated isomers were not performed. However, the assignments of the atropisomers of **2** were determined unambiguously from the comparison of the TLC and HPLC profiles with those of the known atropisomers of tetrakis[2-(methoxycarbonyl)phenyl]porphyrin. These assignments of the isomers are also confirmed by the thermal atropisomerization experiments (see below).

The $\alpha\beta\alpha\beta$-isomer of **2** was quickly separated from the equilibrium mixture of the isomers by silica gel chromatography eluted with chloroform. The thermal atropisomerism of **2** was studied in toluene, immediately after the isolation of the $\alpha\beta\alpha\beta$-isomer. At 353 K in toluene, the thermal atropisomerization of $\alpha\beta\alpha\beta$-**2** occurred rapidly. The amount of $\alpha\beta\alpha\beta$-isomer diminished and the corresponding amounts of the other isomers were observed. The mixture reached equilibrium within 15 min (Fig. 4a). The HPLC analysis gave the molar ratio of the isomers at equilibrium as $\alpha\beta\alpha\beta$- : $\alpha\alpha\beta\beta$- : $\alpha\alpha\alpha\beta$- : $\alpha\alpha\alpha\alpha$-$=16:26:49:9$, which was close to the statistic ratio of $12.5:25.0:50.0:12.5$ $(1:2:4:1)$. This atropisomer ratio at equilibrium is also very close to that of tetrakis[2-(methoxycarbonyl)phenyl]porphyrin, where the atropisomer ratio was $18:27:47:8$.[4)] These results indicated that the intramolecular interaction did not exist between methoxycarbonyl substituent groups of **2**, like in the case of methoxycarbonyl groups in tetrakis[2-(methoxycarbonyl)phenyl]porphyrin.

However, the atropisomeric conversion of **2** is much more rapid than that of tetrakis[2-(methoxycarbonyl)phenyl]porphyrin. At 353 K in toluene, the first-order rate constant k_1 for the isomerization of $\alpha\beta\alpha\beta$-**2** was 1.77×10^{-3} s^{-1}, and the activation free energy $\Delta G^{\neq}$ was calculated as 105 kJ mol^{-1} (these values were calculated from the first stages of the reactions). In contrast, k_1 and $\Delta G^{\neq}$ for the isomerization of $\alpha\beta\alpha\beta$-isomer of tetrakis[2-(methoxycarbonyl)phenyl]porphyrin was 8.33×10^{-5} s^{-1} and 115 kJ mol^{-1}, respectively (353 K in toluene).[4)] Even at 313 K, the thermal atropisomerization of **2** was observed. The $\alpha\beta\alpha\beta$-isomer decreased and $\alpha\alpha\alpha\beta$-isomer was detected after 5 min. The $\alpha\alpha\beta\beta$- and $\alpha\alpha\alpha\alpha$-isomers then appeared and the equilibrium was reached within 10 h (Fig. 4b). At 313 K, the first-order rate constant k_1 for the isomerization of $\alpha\beta\alpha\beta$-**2** was 9.18×10^{-5} s^{-1} and the activation free energy $\Delta G^{\neq}$ was 118 kJ mol^{-1}, respectively. In contrast, the isomers of tetrakis[2-(methoxycarbonyl)phenyl]porphyrin were thermally stable and no isomerization was observed at 313 K. The *ortho*-substituent group of the tetraphenylporphyrin derivatives strongly effected the rotation barrier of the C–C bonds between the porphyrin ring and the phenyl groups. The ether-oxygen atom in the *ortho*-substituent group of **2**, $-OCH_2CO_2Et$, does not restrict the rotation of the phenyl ring. On the other hand, the *ortho*-substituent

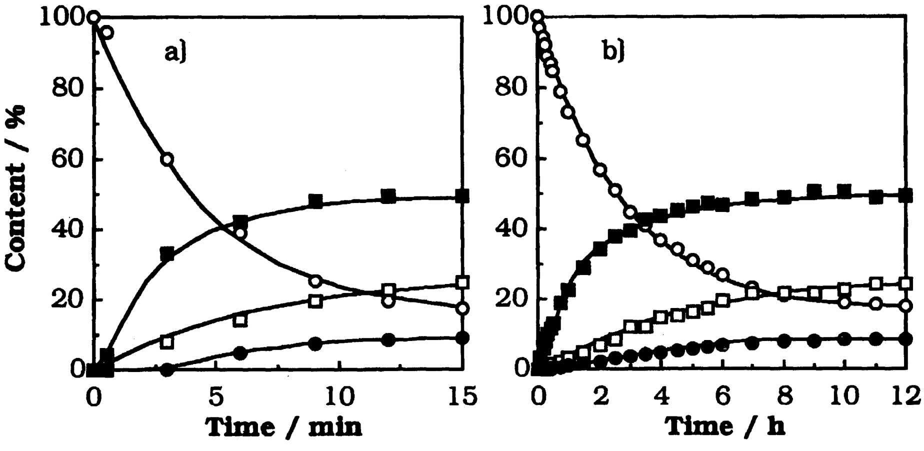


Fig. 4. Time courses of the thermal isomerizations of $\alpha\beta\alpha\beta$-**2** in toluene at a) 353 K and b) 313 K. (○), $\alpha\beta\alpha\beta$-; (□), $\alpha\alpha\beta\beta$-; (■), $\alpha\alpha\alpha\beta$-; (●), $\alpha\alpha\alpha\alpha$-isomer.

of the tetrakis[2-(methoxycarbonyl)phenyl]porphyrin is $-CO_2Me$, and the carbonyl group restricts the rotation of the phenyl ring due to the branched structure. In this connection, it is noteworthy that the rapid rotations of the phenyl groups are reported for tetrakis(2-methoxyphenyl)porphyrin and tetrakis(2-hydroxyphenyl)porphyrin in nitrobenzene.[6]

Thus, the atropisomerization of **2** took place very much rapidly because of the less sterically hindering *ortho*-substituent $-OCH_2CO_2Et$ group. This characteristic property in the rapid atropisomerism of **2** motivated us to use **2** as a new template for the construction of the artificial proteins with porphyrin function. The four carboxyl groups of **2** can link the N-termini of the peptide segments like tetrakis(2-carboxyphenyl)porphyrin. However, the rapid atropisomerization of **2** suggests the rotation of the phenyl groups even after hybridization with polypeptides, in contrast to the hybrid based on tetrakis(2-carboxyphenyl)porphyrin.

Synthesis and Properties of 5,10,15,20-Tetrakis[2-(polypeptidylcarbonylmethoxy)phenyl]porphyrin (12). The 21-peptide Boc–(–Gln–Leu–Leu–Gln–Ala–Leu–Ala–)$_3$–$NHCH_2CH_2OH$ (**10**) was designed to take an amphiphilic α-helix conformation with polar Gln (2/7), hydrophobic Leu (3/7), and neutral Ala (2/7) residues.[7] Figure 5 depicts the wheel diagram of the α-helix peptide **10**, where the polar Gln residues and hydrophobic Leu residues are on each side of the helix. The C-terminus of polypeptide was protected with 2-aminoethanol, which may improve the hydrophilicity of the C-terminus and would make the polypeptide–porphyrin hybrid stably embedded into the lipid membrane. The Boc–21-peptide–$NHCH_2CH_2OH$ (**10**) was synthesized by the solid-phase-syntheses of the heptapeptide fragments on Kaiser's *p*-nitrobenzophenone oxime resin and fragment condensations in the solution phase (Fig. 6).[8] The CD spectrum of **10** in methanol (Fig. 7a) gave a typical α-helix profile, showing double minima at 208 and 222 nm. The θ value at 222 nm per 21 amino acid residue ($[\theta]^{\mathrm{MRW}}_{222}=-25300$ deg cm^2 dmol^{-1}) corresponded to 77% α-helicity (α-he-

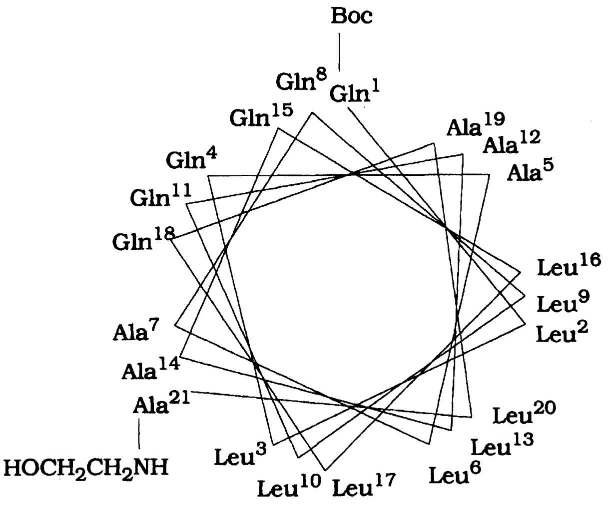
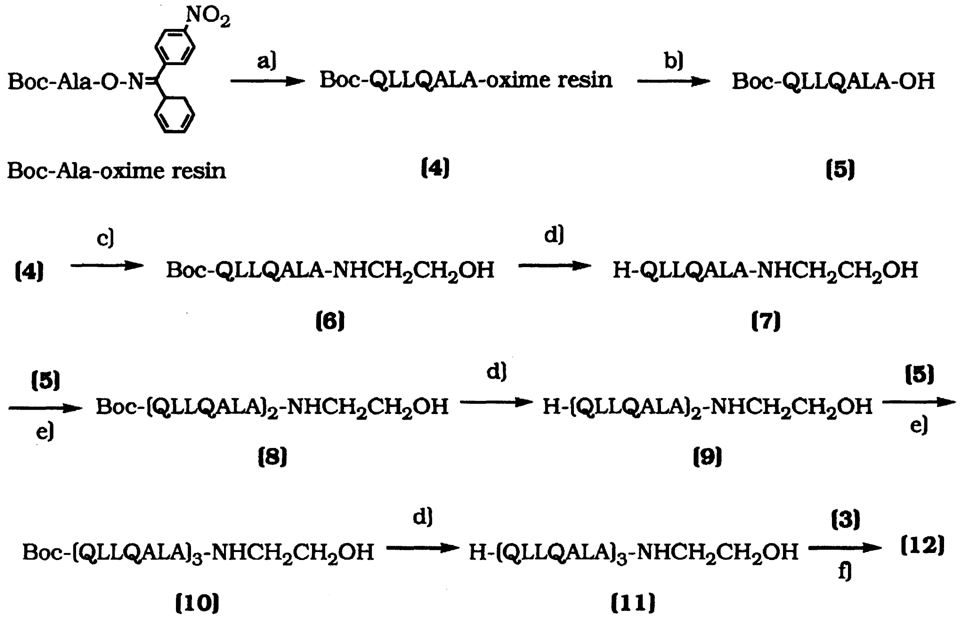


Fig. 5. The amphiphilic α-helix character of the protected 21-peptide (**10**) represented by the wheel drawing.



a) stepwise solid-phase-syntheses b) i) 1-hydroxypiperidine, ii) $Na_2S_2O_4$ aq.
c) 2-aminoethanol, acetic acid d) TFA e) EDC/HOBt f) BOP/HOBt$^+$

Fig. 6. Scheme for the synthesis of the polypeptide-porphyrin hybrid, (**12**). Q; Gln, L; Leu, A; Ala.

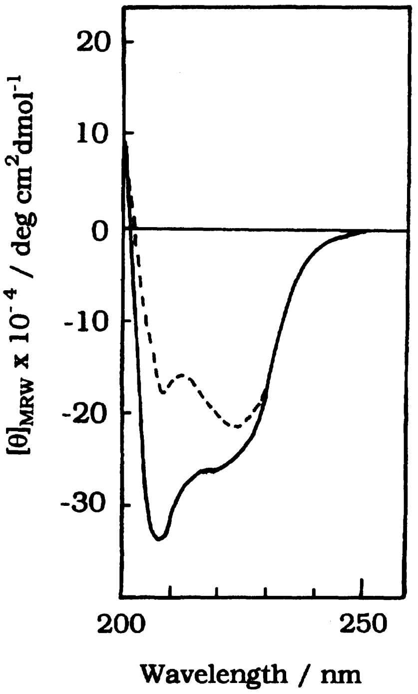


Fig. 7. CD spectra of the protected 21-peptide (**10**) in (a, —) methanol and (b, - - -) methanol–water (1/3, v/v). [**10**]=50 μmol dm^{-3}.

lix content).[9] Such a high α-helicity of **10** is reasonably explained by the α-helical character of Gln, Len, and Ala residues. The addition of a large amount of water to the solvent decreased the α-helicity of **10**. The α-helicity was 70% in methanol-water (3/1, v/v). This lower α-helicity in the water-containing solvent is probably because water weakened the hydrogen bondings of the amide groups (Fig. 7b, Table 2).

The ethyl ester moieties of **2** were hydrolyzed by aqueous NaOH solution and the N-terminus of the Boc–21-peptide (**10**) was deprotected by TFA. The desired polypeptide-porphyrin hybrid (**12**) was synthesized by the coupling of tetrakis[2-(carboxymethoxy)-phenyl]porphyrin (**3**) and deprotected polypeptide (**11**) with BOP/HOBt reagents, using excess amounts of **11**. The obtained polypeptide–porphyrin hybrid (**12**) was purified by size exclusion chromatography (two times with Sephadex LH-60 column with DMF as eluent). Figure 8 shows the profiles of the chromatography in the purification of **12**. The sharp symmetrical peak eluting around fraction numbers 48-53 (Fig. 8b) suggests the successful synthesis of the polypeptide-porphyrin hybrid (**12**). The sharp peak eluting around fraction numbers 72—76, observed only in the first purification, corresponds to the elution of **11**. The peak volume corresponded to the molecular weight of **12** (molecular weight=10067). The UV-vis spectrum of **12** (Fig. 9) is similar to that of **2** in methanol. Furthermore, the UV-vis spectrum of **12** in methanol differed slightly from that in methanol–water (1/3, v/v) (see Experimental section). The CD spectra of **12** in methanol (Fig. 10a) gave a typical α-helix CD profile with double minima at 208 and 222 nm. The θ value at 222 nm ($[\theta]_{222}^{MRW}$) was −23800 deg cm^2 dmol^{-1}, which corresponds to 78% α-helicity (Table 2). Such a high α-helicity unambiguously indicates that four peptide segments are introduced to the porphyrin template. If only three segments

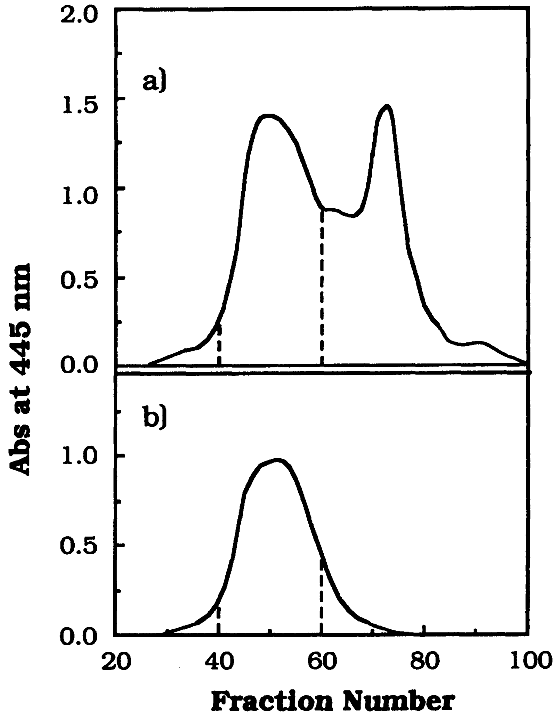


Fig. 8. Elution profile of **12** in Sephadex LH-60 size exclusion chromatography (DMF). a) The first purification of the reaction mixture. The fractions of Nos. 40 to 60 was collected and submitted to the re-chromatography. b) The second purification. The fractions of Nos. 40 to 60 were collected.

Table 2. α-Helicity of 21-Peptide (**10**) and Polypeptide–Porphyrin Hybrid (**12**)[a]

Substrate	α-Helicity/%				
	MeOH	MeOH–H$_2$O (3/1, v/v)	MeOH–H$_2$O (1/1, v/v)	MeOH–H$_2$O (1/3, v/v)	Tris•HCl Buffer[b]
10	83	81	81	70	—
12	78	70	91	90	83

a) α-Helicity is determined from the molar ellipticity at 222 nm ($[\theta]_{222}^{MRW}$). b) Tris•HCl 20 mM in the presence of 2.5 mM SDS.

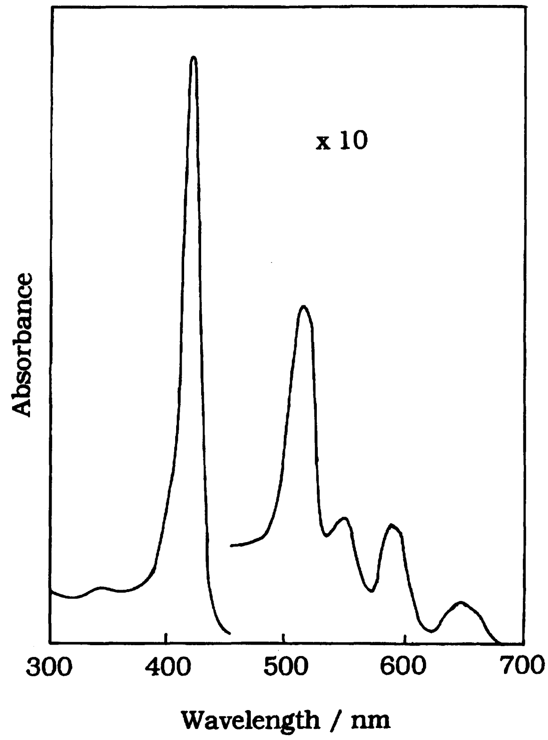


Fig. 9. UV-vis spectra of **12** in methanol. [**12**]= 10 μmol dm^{-3}.

were connected to the porphyrin, the $[\theta]_{222}^{\mathrm{MRW}}$ value becomes improbable. For example, the α-helicity of **12** in methanol–water (1/3, v/v) is 90%, assuming that four peptide segments are connected to the porphyrin template. This value becomes over 100% if three peptide segments are connected, which is unlikely.

The addition of water to the solvent did not diminish the α-helicity of the peptide segments in **12**, which is a contrast to the simple 21-peptide (**10**) (Table 2). Moreover, the α-helicity of **12** was rather increased in the water-containing solvents. For instance, the α-helicity of **12** was 90% in methanol–water (1/3, v/v). This fact indicates that the four 21-peptide segments in **12** aggregate intramolecularly through hydrophobic helix–helix interactions in the water-containing solvent. The hydrophobic face of the amphiphilic α-helix (Leu residues) may be placed inside and the hydrophilic face (Gln residues) outside. Thus, the peptide chains fold into a bundle structure (see Fig. 1b). In this connection, we and others have already reported the folding of intramolecular peptide chains into a parallel bundle structure connected on the porphyrin templates.$^{1)}$ Furthermore, the hydrophobic interaction of Leu residues is known to stabilize the α-helix structure in such solvents.$^{10)}$ Such a construction of a bundle structure strongly suggests that the peptide segments of **12** may be on one face of the porphyrin plane, that is, *αααα*-isomer of **12** is formed preferentially through the intramolecular aggregation of the polypeptides. The rotations of the phenyl rings of the porphyrin template take place with slight restriction in **12**, and the attractive interaction between the peptide segments would stabilize the *αααα*-isomer of **12**. In the water-containing solvent, the α-helix structure of the peptide segment in *αβαβ*-, *ααββ*-, or *αααβ*-isomers has less or no chance to be stabilized. However, only the α-helix structure of the peptide segment in *αααα*-isomer is stabilized by the intramolecular self-aggregation by the hydrophobic interaction. As a result, the ratio of $[\theta]_{208}/[\theta]_{222}$ of **12** almost equals 1 (see Fig. 10), which suggests the tight interaction of α-helices like the coiled coil structure.$^{11)}$

In the CD spectrum of **12** in methanol, a symmetrically split CD band was observed at 420 nm, which corresponds to the absorption of the porphyrin ring (Fig. 10a).$^{12)}$ The split CD profile indicates that the porphyrin chromophore is under the symmetrical arrangement by the attachment of the chiral polypeptide moiety in methanol. However, this induced Cotton effect disappeared in the water-containing solvent (Fig. 10b). The arrangement of the polypeptide segments could be considered unsymmetrical in such a solvent, although the detailed orientation of the peptide segments is not yet clear. It is very interesting that the polypeptide-porphyrin hybrid **12** is soluble in Tris•HCl buffer solution (pH 7.4) in the presence of 2.5 mmol dm^{-3} of sodium dodecyl sulfate. The CD spectra of **12** in Tris•HCl buffer solution with sodium dodecyl sulfate indicated that the α-helicity of the peptide is 83% (Fig. 10c). However, the induced CD band was completely unsymmetrical. Sodium dodecyl sulfate below its critical micellar content is known to loosen the bundle structure of the α-helix peptides, without destabilizating the α-helix structures of each peptide unit.$^{3e)}$ Sodium dodecyl sulfate also loosened the four α-helix bundle structure of the polypeptide–porphyrin hybrid (**12**) on the porphyrin template.

Membrane-Formation Containing 12 and Construction of the Artificial Membrane Protein Model. The polypeptide–porphyrin hybrid (**12**) was successfully dispersed in the egg yolk lecithin membrane at room temperature. However, **12** was not doped into the dipalmitoyl phosphatidylcholine membrane either at room temperature or at 323 K. This was probably because dipalmitoyl phosphatidylcholine formed somewhat rigid vesicles. The formation of the porphyrin-containing vesicle was performed by the sonication of the mixture of egg yolk lecithin and **12** (molar ratio, 50/1) in 20 mM Tris•HCl buffer solution. The mixture was directly subjected to the size exclusion chromatography (Sephadex G-75) eluted by the same buffer solution (Fig. 11). The porphyrin-containing vesicle was collected at the elution volume of 11 to 15 ml. The amount of **12** embedded into the vesicle was estimated by the UV-vis spectra. About 92% amount of **12** was revealed to be successfully introduced into the membrane. Thus, construction of a membrane-penetrating polypeptide–porphyrin hybrid was achieved. Figure 12

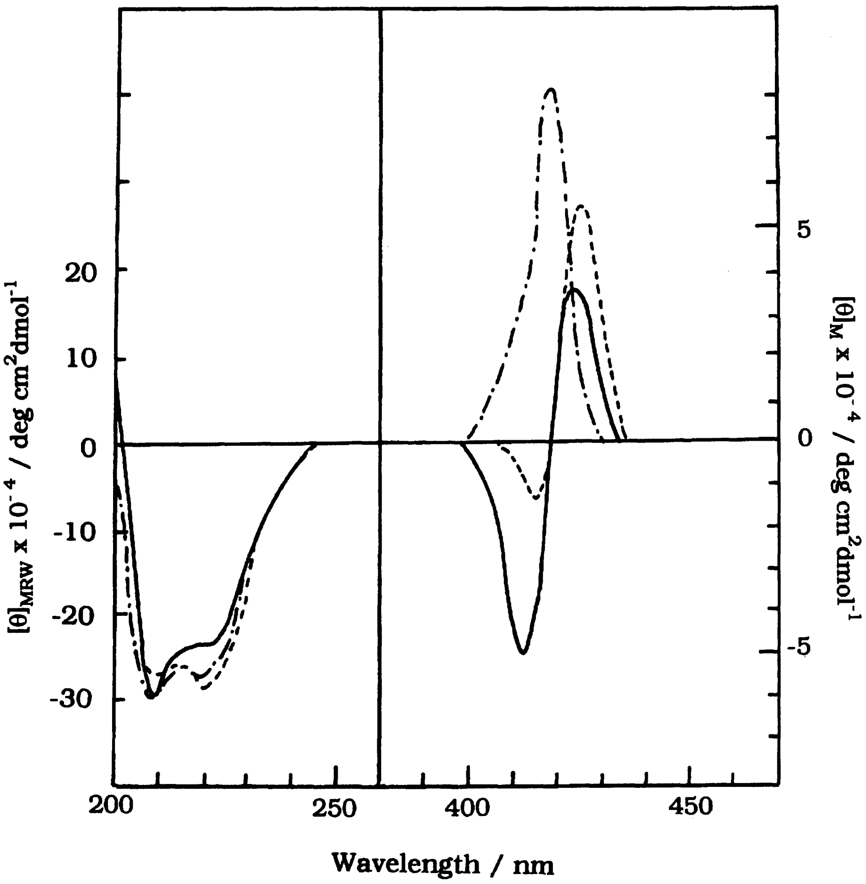


Fig. 10. CD spectra of **12** in (a —) methanol and (b - - -) methanol-water (1/3, v/v). [**12**]=12.5 μmol dm^{-3}. (c —-—) **12** in 20 mmol dm^{-3} Tris•HCl buffer solution in the presence of 2.5 mmol dm^{-3} sodium dodecyl sulfate. [**12**]=5.0 μmol dm^{-3}.

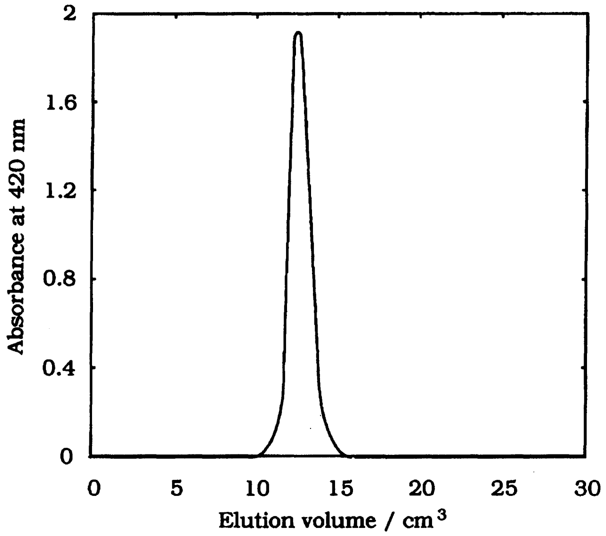


Fig. 11. Elution profile in Sephadex G-75 (10 mm×250 mm) size exclusion chromatography of the porphyrin-containing vesicles. The vesicles were prepared by the sonication of egg yolk lecithin and **12** (molar ration is 50/1) in 20 mmol dm^{-3} Tris•HCl buffer solution, which was eluted with the same solvent.

shows the CD spectra of the **12**-containing egg yolk lecithin vesicle. In the lower molar ratio of the egg yolk lecithin/**12** (50/1 to 125/1), the membrane solution in Tris•HCl buffer solution became turbid. In such cases, the CD spectra showed smaller θ values for the shorter wavelength region, probably because of the light scattering (Figs. 12a and 12b). Thus, polypeptide–porphyrin hybrid (**12**) seems to fuse the lipid membrane in such a low molar ration of egg yolk lecithin/**12**. In the higher molar ratio of the egg yolk lecithin/**12** (250/1 and 500/1, Figs. 12c and 12d), the membrane solution was clear and the typical CD spectra for the α-helix peptides were observed. The α-helicities were 77% in these conditions. Thus, successful incorporation of **12** into a membrane while retaining the α-helix structure was observed. Unfortunately, the induced Cotton effect for the porphyrin region (420 nm) was not detected because of the low concentration of **12**. Therefore, the orientation of the peptide segments on the porphyrin plane in the membrane environment was not revealed. However, in the hydrophilic environment of the membrane, the hydrophilic faces of the α-helix peptide chains (Gln residue) may aggregate with each other, probably constructing a water-in-oil reversed micellar structure.

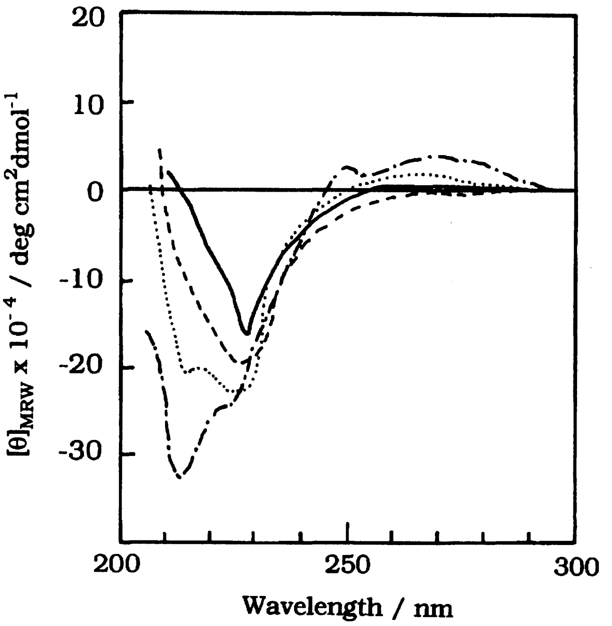


Fig. 12. CD spectra of **12**-containing egg yolk lecithin vesicle in Tris•HCl buffer solution (20 mmol dm^{-3}, pH 7.4). [Egg yolk lecithin] = 0.50 mmol dm^{-3}. (a —) [**12**] = 10 μmol dm^{-3} (egg yolk lecithin/**12** = 50/1), (b ---)) [**12**] = 4.0 μmol dm^{-3}, (125/1), (c ····) [**12**] = 2.5 μmol dm^{-3} (250/1), (d ---) [**12**] = 1.0 μmol dm^{-3} (500/1).

Experimental

Analytical Methods. The HPLC analysis was carried out on a Hitachi L-6200 intelligent pump equipped with a Hitachi L-4200 UV-vis detector and a Hitachi D-2500 chromato-integrator. The analysis for the atropisomers of **2** was performed on a Waters μBondasphere C18 (3.9×150 mm) column eluting with a linear gradient of acetonitrile/water=88/12 to 100/0 (v/v) over 10 min, and 100% acetonitrile (10—30 min), flow rate, 1.0 $cm^3 min^{-1}$, and detection at 420 nm. The analysis for the polypeptide was performed on a Wakosil 5C4 (4.6×150 mm) column eluting with a linear gradient of acetonitrile/water/TFA=33/67/0.1 to 100/0/0.1 (v/v) over 30 min, and 100% acetonitrile containing 0.1% TFA (30—60 min), flow rate, 1.0 $cm^3 min^{-1}$, and detection at 220 nm. UV-vis spectra were recorded on a Hitachi 150-20 spectrometer using quartz cells of 1 and 10 mm pathlength. CD spectra were recorded on a JASCO J-500A spectropolarimeter using a quartz cell of 1 mm pathlength at 298 K. In the CD measurement of the polypeptide, the concentration of the polypeptide was 50 μmol dm^{-3}. In the CD measurement of the polypeptide-porphyrin hybrid, the concentration of the hybrid was 12.5 μmol dm^{-3} unless otherwise noted. EI and FAB-MS were obtained by JEOL DX-300 mass spectrometer.

2-(Ethoxycarbonylmethoxy)benzaldehyde (1). Ethyl bromoacetate (3.3 g, 20 mmol), salicylaldehyde (2.4 g, 20 mmol), and potassium carbonate (4.1 g, 30 mmol) were refluxed in dry acetone (50 cm^3) for 1 h. After evaporation of the solvent, the residue was dissolved in dichloromethane and washed with aqueous NaCl solution. The organic phase was dried by $MgSO_4$ and evaporated. Then the residual material was chromatographed over silica gel (Wakogel C-300, chloroform). **1** was yielded as a pale yellow oil. Yield 3.1 g (15 mmol, 75%). MS (EI), *m/z* (rel intensity) 208 (M^+; 14), 162 (21), 135 (57), 121 (100).

5,10,15,20-Tetrakis[2-(ethoxycarbonylmethoxy)phenyl]porphyrin (2). To the dichloromethane (1.5 dm^3) prepurged by N_2, **1** (3.1 g, 15 mmol), pyrrole (1.0 g, 15 mmol), and BF_3–Et_2O (0.21 g, 1.5 mmol) were added successively. The mixture was stirred at room temperature for 15 h. Then tetrachloro-1,4-benzoquinone (2.7 g, 11 mmol) was added, and the resultant mixture was stirred for a further 24 h. The solvent was evaporated, and the residue was chromatographed over silica gel (Wakogel C-300, dichloromethane). The crude **1** was yielded as a purple powder, this was recrystallized from chloroform–hexane. Yield 660 mg (0.65 mmol, 17%). MS (FAB, 2,2′-dithiodiethanol) *m/z* 1023 (M^+). UV-vis (dichloromethane) 416 (ε 444000), 512 (19300), 544 (4900), 587 (5800), 642 (2200) nm. R_f value (Merck silica gel Art. 5715, chloroform/methanol=49/1, v/v), 0.53 (αβαβ), 0.47 (ααββ), 0.38 (αααβ), 0.33 (αααα). Found: C, 69.94; H, 5.43; N, 5.30%. Calcd for $C_{60}H_{54}N_4O_{12}$•1/2H_2O: C, 69.82; H, 5.37; N, 5.43%.

5,10,15,20-Tetrakis[2-(carboxymethoxy)phenyl]porphyrin (3). Aqueous NaOH (1 M, 1 M=1 mol dm^{-3}) (1.2 ml, 1.2 mmol) was added to the methanol (5 cm^3) solution of **2** (30 mg, 0.03 mmol), and the mixture was stirred overnight at room temperature. The solvent was evaporated to dryness, and 0.7 ml of 10% (v/v) acetic acid in water (1.2 mmol for acetic acid) was added with ice-cooling. The solid porphyrin (**3**) was obtained by filtration and dried under vacuum. Yield, 25 mg (0.03 mmol, 100%). The porphyrin (**3**) thus obtained was used for the further synthesis without purification.

Boc–Gln–Leu–Leu–Gln–Ala–Leu–Ala–OH (5). The Boc–heptapeptide–*p*-nitrobenzophenone oxime resin, Boc–Gln–Leu–Leu–Gln–Ala–Leu–Ala-oxime resin (**4**) (0.4 mmol) was synthesized by the solid-phase-synthesis on *p*-nitrobenzophenone oxime resin according to the literature method.[8] To **4** suspended in DMF (15 cm^3), 1-hydroxypiperidine (0.16 g, 1.6 mmol) was added, and the mixture was stirred for 24 h. After the resin was filtered off, water was added to the solution to solidify the Boc–heptapeptide–O–piperidino ester, (Boc–Gln–Leu–Leu–Gln–Ala–Leu–Ala–O–piperidino). After this Boc–heptapeptide–O–piperidino ester was obtained by filtration, the peptide was washed and dried under vacuum. HPLC, retention time 13.18 min. This solid Boc–heptapeptide–O–piperidino ester was treated by sodium dithionite (0.28 g, 1.6 mmol) in acetic acid–water (10/1, v/v) for 1 h. Then the solvent was evaporated and the product was solidified by the addition of water to yield the Boc–heptapeptide–OH (**5**). Reprecipitation from ethyl ether/petroleum ether gave 190 mg (0.21 mmol, 48%) of pure **5**. MS (FAB, glycerol) *m/z* 856 (M^+). HPLC, retention time 6.51 min.

Boc–Gln–Leu–Leu–Gln–Ala–Leu–Ala–$NHCH_2CH_2OH$ (6). To the Boc–heptapeptide–oxime resin (**4**) (4 mmol) suspended in DMF (20 cm^3), 2-aminoethanol (1.2 g, 20 mmol) and acetic acid (1.2 g, 20 mmol) were added. The resulting mixture was stirred for 24 h. Then the resin was filtered off and the filtrate was evaporated to dryness. The solid was washed with aqueous citric acid solution to yield the Boc–heptapeptide–$NHCH_2CH_2OH$ (**6**). Reprecipitation from ethyl ether/petroleum ether gave 280 mg (0.31 mmol, 66%) of pure **6**. MS (FAB, glycerol) *m/z* 899 (M^+). HPLC, retention time, 6.73 min.

Boc– (–Gln– Leu– Leu– Gln– Ala– Leu– Ala–)$_2$–NHCH$_2$CH$_2$OH (8). The Boc–heptapeptide–NHCH$_2$CH$_2$OH (**6**) (180 mg, 0.2 mmol) was dissolved in TFA (5 cm^3) at ice-cooled temperature and stirred at that temperature for 0.5 h. The solvent was evaporated, and ether was added to the residues. The TFA salt of deprotected H–heptapeptide–NHCH$_2$CH$_2$OH, TFA·H–Gln–Leu–Leu–Gln–Ala–Leu–Ala–NHCH$_2$CH$_2$OH (**7**) was obtained as a white precipitate and dried under vacuum. The deprotected H–heptapeptide–NHCH$_2$CH$_2$OH, (**7**) (160 mg, 0.2 mmol), Boc–heptapeptide–OH, (**5**) (150 mg, 0.2 mmol), and HOBt (40 mg, 0.3 mmol) were dissolved in 2,2,2-trifluoroethanol/dichloromethane (1/2, v/v, 10 cm^3), which was known as a well-dissolving solvent system.[13)] To this solution were added triethylamine (24 mg, 0.24 mmol) and EDC (77 mg, 0.4 mmol) with ice-cooling. After 24 h, water was added to the solution to solidify the Boc–14-peptide–NHCH$_2$CH$_2$OH (**8**) as a white powder. The crude **8** was reprecipitated from ethyl ether/petroleum ether. Yield, 250 mg (0.15 mmol. 77%). MS (FAB, glycerol) *m/z* 1637 ($M+H^+$). HPLC, retention time 28.01 min.

Boc– (–Gln– Leu– Leu– Gln– Ala– Leu– Ala–)$_3$–NHCH$_2$CH$_2$OH (10). The Boc–21-peptide–NHCH$_2$CH$_2$OH (**10**) was synthesized in almost the same way as **8** was, although longer reaction time was required because of the lower reactivity of the 14-peptide. The Boc–14-peptide–NHCH$_2$CH$_2$OH (**8**) (230 mg, 0.14 mmol) was treated by TFA to yield the TFA salt of deprotected H–14-peptide–NHCH$_2$CH$_2$OH, TFA·H–(–Gln–Leu–Leu–Gln–Ala–Leu–Ala–)$_2$–NHCH$_2$CH$_2$OH (**9**). The deprotected H–14-peptide–NHCH$_2$CH$_2$OH, (**9**) (220 mg, 0.14 mmol), Boc–heptapeptide–OH, (**5**) (180 mg, 0.21 mmol), and HOBt (57 mg, 0.42 mmol) were dissolved in 2,2,2-trifluoroethanol/dichloromethane (1/2, v/v, 10 cm^3). To this solution were added triethylamine (17 mg, 0.17 mmol) and EDC (110 mg, 0.56 mmol) successively with ice-cooling. After 2 d, HPLC analysis indicated the disappearance of **9** and the decrease of the theoretical amount of **5**. Water was added to the solution, then the crude Boc–21-peptide–NHCH$_2$CH$_2$OH (**10**) was obtained as a white powder. Since this polypeptide was soluble in methanol, the insoluble materials were filtered off. The reprecipitation from ethyl ether/petroleum ether gave **10**. The mass spectrometry analysis of **10** failed because of its high molecular weight. Yield, 260 mg (0.11 mmol. 81%). HPLC, 30.69 min.

5,10,15,20-Tetrakis[2-{[(Gln–Leu–Leu–Gln–Ala–Leu– Ala–)$_3$–NHCH$_2$CH$_2$OH]- carbonylmethoxy}-phenyl]porphyrin (5,10,15,20-Tetrakis[2-(polypeptidylcarbonylmethoxy)phenyl]porphyrin) (12). The Boc–21-peptide–NHCH$_2$CH$_2$OH (**10**) (260 mg, 0.11 mmol) treated with TFA (5 cm^3) as described for the preparation of **7** to give TFA·H–(–Gln–Leu–Leu–Gln–Ala–Leu–Ala–)$_3$–NHCH$_2$CH$_2$OH (**11**) as white precipitate (yield, 250 mg, 0.11 mmol, 100%). The DMF solution (0.7 cm^3) of the deprotected H–21-peptide–NHCH$_2$CH$_2$OH (**11**) (230 mg, 0.10 mmol) was added to the DMF–triethylamine solution (1.2 cm^3 24/1, v/v) containing the porphyrin template (**3**) (13 mg, 16 μmol), BOP (44 mg, 0.10 mmol), and HOBt (14 mg, 0.10 mmol) with ice-cooling After 18 h, the mixture was directly subjected to the size exclusion chromatography over Sephadex LH-60 with DMF as eluent. The porphyrin-containing fragments were rechromatographed to give 56 mg of **12** (5.6 μmol, yield 35%). UV-vis (methanol) 416 (ε 437000), 513 (21800), 545 (6400), 586 (6400), 642 (2600) nm. UV-vis (methanol–water 1/3, v/v) 419 (ε 428000), 515 (26200), 548 (9600), 588 (8300), 642 (3800) nm.

Thermal Isomerization of 2. The $\alpha\beta\alpha\beta$-isomer of **2** was isolated from the atropisomeric mixture by silica gel chromatography eluted with chloroform. The fast moving band was collected, and evaporated to dryness. HPLC analysis indicated that the fraction contains only the desired isomer. The $\alpha\beta\alpha\beta$-isomer (1.0 mg, 1.0 μmol) was dissolved in toluene (10 cm^3), which had been thermostatted at an appropriate temperature. Small aliquots were withdrawn at appropriate intervals, and analyzed by the reversed-phase HPLC. Retention times of the isomers were: 4.46 min ($\alpha\beta\alpha\beta$), 5.76 min ($\alpha\alpha\beta\beta$), 8.12 min ($\alpha\alpha\alpha\beta$), and 19.47 min ($\alpha\alpha\alpha\alpha$). The peak intensities in the chromatograms were not corrected.

Incorporation of the Polypeptide–Porphyrin Hybrid (12) into the Egg Yolk Lecithin Membrane. The solution of **12** (1.0 mg, 0.1 μmol) in methanol–chloroform (1/3, v/v) (0.5 cm^3, 0.2 mmol dm^{-3}) and the solution of 4.0 mg (5.0 μmol) of egg yolk lecithin (Wako Pure Chemical, the average molecular weight is 800) in methanol–chloroform (1/1, v/v, 0.5 cm^3) were mixed in a pear-like flask. The solvent was evaporated by a stream of N_2 and Tris·HCl buffer solution (20 mM, pH 7.4, 8.0 ml) was added. After the sonication for 10 min at room temperature, a clear solution was obtained. The vesicles thus obtained were kept for 2 h without movement. The chromatography over Sephadex G-75 columns (10 mm×250 mm) was carried out with the same buffer solution as the eluent. The eluates were collected by every 1.0 cm^3, and the content of porphyrin was determined by the absorption at 420 nm.

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References

1) a) T. Sasaki and E. T. Kaiser, *J. Am. Chem. Soc.*, **111**, 380 (1989); b) T. Sasaki and E. T. Kaiser, *Biopolymers*, **29**, 79 (1990); c) H. Mihara, N. Nishino, R. Hasegawa, and T. Fujimoto, *Chem. Lett.*, **1992**, 1805; d) K. S. Åkerfeldt, R. M. Kim, D. Camac, J. T. Groves, J. D. Lear, and W. F. DeGrado, *J. Am. Chem. Soc.*, **114**, 9656 (1992); e) C. T. Choma, J. D. Lear, M. J. Nelson, P. L. Dutton, D. E. Robertson, and W. F. DeGrado, *J. Am. Chem. Soc.*, **116**, 856 (1994).

2) S. F. Bets, D. P. Raleigh, and W. F. DeGrado, *Curr. Opin. Struct. Biol.*, **3**, 501 (1993). References sited therein.

3) a) J. S. Richardson and D. C. Richardson, *Trends Biochem. Sci.* (*Pers. Ed.*), **14**, 304 (1989); b) W. F. DeGrado, Z. R. Wasserman, and J. D. Lear, *Science*, **243**, 622 (1989); c) N. Nishino, M. Mihara, T. Tanaka, and T. Fujimoto, *Tetrahedron Lett.*, **33**, 5767 (1992); d) N. Nishino, H. Mihara, T. Uchida, and T. Fujimoto, *Chem. Lett.*, **1993**, 53; e) H. Mihara, K. Tomizaki, N. Nishino, and T. Fujimoto, *Chem. Lett.*, **1993**, 1533.

4) T. Fujimoto, H. Umekawa, and N. Nishino, *Chem. Lett.*, **1992**, 37.

5) J. S. Lindsey, I. C. Schreiman, H. C. Hsu, P. C. Kearney, and A. M. Marguerettaz, *J. Org. Chem.*, **52**, 827 (1987); J. S. Lindsey and R. W. Wagner, *J. Org. Chem.*, **54**, 828 (1989).

6) J. W. Dirks, G. Underwood, J. C. Matheson, and D. Gust, *J. Org. Chem.*, **44**, 2551 (1979).

7) Abbreviations used are according to IUPAC-IUB Commissions, *Eur. J. Biochem.*, **138**, 9 (1984). Other abbreviations: Boc, *t*-butoxycarbonyl; BOP, benzotriazol-1-yloxytris(diethylamino)phosphonium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; DMF, *N*,*N*-dimethylformamide; SDS; sodium dodecyl sulfate, Tris, tris(hydroxylmethyl)aminomethane; TFA, trifluoroacetic acid; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; CD, circular dichroism.

8) N. Nishino, M. Xu, H. Mihara, and T. Fujimoto, *Bull. Chem. Soc. Jpn.*, **65**, 991 (1992). References sited therein.

9) J. M. Scholtz, H. Qian, E. J. York, J. M. Stewart, and R. L. Baldwin, *Biopolymers*, **31**, 1463 (1991).

10) E. K. O'Shea, J. D. Klemm, P. S. Kim, and T. Alber, *Science*, **254**, 539 (1991); H. Mihara, N. Nishino, and T. Fujimoto, *Chem. Lett.*, **1991**, 1809.

11) C. García-Echeverría, *J. Am. Chem. Soc.*, **116**, 6031 (1994); N. E. Zhou, C. M. Kay, and R. S. Hodges, *Biochemistry*, **31**, 5739 (1992).

12) N. Nishino, H. Mihara, and T. Fujimoto, *J. Chem. Soc., Chem. Commun.*, **1992**, 692.

13) H. Kuroda, Y.-N. Chen, T. Kimura, and S. Sakakibara, *Int. J. Peptide Protein Res.*, **40**, 294 (1992).