Photoresponsive Melittin Having a Spyropyran Residue in the Hydrophobic Region of the Sequence

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A melittin derivative carrying a spiropyran group at the third residue (MSp) was prepared by a semisynthetic method. MSp showed reversible photochromism in a phospholipid bilayer membrane. The kinetics of thermal decolouration and the maximum absorption of MSp in the merocyanine form indicated that the merocyanine moiety is located at the membrane surface. The disturbance of the membrane structure by MSp, as studied by CF leakage, was enhanced by UV-light irradiation. The kinetics of CF leakage indicate that MSp in the merocyanine form disturbs the membrane structure more significantly than that in the spiropyran form.

Many biologically active peptides are amphiphilic in order to help distribution to the phospholipid bilayer membrane.[1] Melittin is composed of 26 amino acid residues. Among them, six residues (21–26) of the C-terminal part are hydrophilic and most of the other residues involved in the N-terminal part are hydrophobic.[2] Melittin disturbs the membrane structure due to the primary amphiphilic α-helical structure.[3,4] It has been proposed that the hydrophobic helical part (residues 1–20) penetrates into the lipid bilayer membrane and the cationic C-terminal part (residues 21–26) is anchored to the surface of the bilayer membrane.[5] Consequently, more than four melittin molecules associate together to form a pore in the membrane, through which water and charged molecules are transported, leading to osmotic cell lysis. Therefore, it is believed that the pore formation due to the hydrophobicity of the N-terminal part is the key step in the melittin activity.

We designed a melittin derivative in which a spiropyran group is introduced into the hydrophobic part of the peptide chain. The spiropyran group reversibly isomerizes from a non-polar spiropyran form to a polar merocyanine form by UV-light irradiation and in the opposite direction thermally or by visible-light irradiation. Several compounds having a spiropyran group have been synthesized for a photoresponsive transport system[6] and photosensitive enzymes.[7–9] These activity changes are principally based on the polarity change of the spiropyran group. It is expected that the melittin derivative carrying the spiropyran group is photoresponsive in the interaction with and in the lysis of the phospholipid bilayer membrane.

The spiropyran group was connected to the third residue of melittin. Upon UV-light irradiation, the highly polar zwitterionic merocyanine group is produced and forms a hydrophilic cluster with Lys-7, Thr-10 and Thr-11 residues. This situation is illustrated by the helical wheel as shown in Fig. 1. The secondary amphiphilic helix tends to stay at the membrane surface in contrast to the primary amphiphilic helix before UV-light irradiation.[10] The different orientations of the peptide in the membrane with and without photoirradiation should lead to different activities.

We report here photocontrol of the membrane-perturbation activity of the melittin derivative having a spiropyran group. The spiropyran group was introduced through an amide linkage at the δ-amino group of the Orn-3 residue. [Orn-3(Sp), ε[7,21,23]-amidinated]melittin (MSp) was prepared by a semisynthetic method. The photoisomerization kinetics and irradiation effects on the membrane-disturbing activity are investigated.

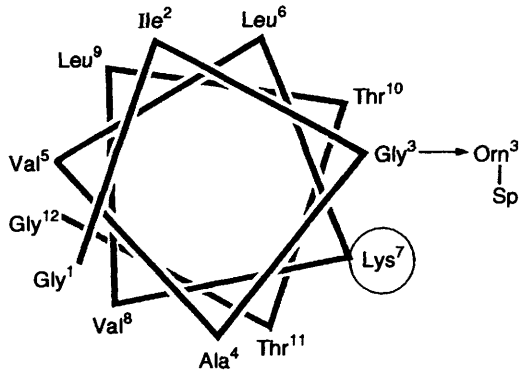

Fig. 1 Helical wheel presentation of the N-terminal part of modified melittin; Gly-3 is replaced by (N^{δ}-spiropyranyl)Orn-3. A charged amino acid residue is indicated with a circle.

Experimental

Materials.—Melittin extracted from a bee venom was obtained from Sigma Chemical Co., USA. N^{α}-*tert*-butoxycarbonylglycine succinimide ester (Boc-Gly-OSu), N^{α}-*tert*-butoxycarbonylisoleucine succinimide ester (Boc-Ile-OSu) and N^{α}-*tert*-butoxycarbonylornithine (Boc-Orn-OH) were obtained from Kokusan Chemical Works, Ltd., Japan. Sephadex G-25 and G-50 gels were purchased from Pharmacia Co., USA. Other chemicals used were of the highest purity available.

Synthesis.—The melittin analogue was synthesized by a semisynthetic method. The semisynthesis is a useful and proper way to prepare proteins and peptides replaced by a specific amino acid.[11,12] Fig. 2 summarizes the synthetic route of the melittin derivative. The purity of synthetic peptides was checked by TLC with a solvent system of chloroform–methanol–acetic acid (95 : 5 : 3 v/v/v). All the intermediate peptides were identified by ^{1}H NMR spectroscopy.

Boc-Gly-Ile-Orn(Sp)-OH. A photochromic compound, 1-hydroxycarbonylmethyl-3,3-dimethyl-6′-nitrospiro[indoline-2,2′-2*H*-benzopyran] (HO_2C-Sp), was prepared by the reaction of 1-ethoxycarbonylmethyl-2,3,3-trimethylindolinium iodide and 5-nitrosalicylaldehyde in the presence of piperidine followed by hydrolysis of the ethyl ester with NaOH in dioxane–methanol.[13,14] The spiropyran derivative was coupled with *N*-hydroxysuccinimide by using dicyclohexylcarbodiimide (DCC) and condensed *in situ* with N^{α}-*tert*-butoxycarbonylornithine (N^{α}-Boc-Orn-OH) to obtain Boc-Orn(Sp)-OH. HO_2C-Sp (475 mg, 1.30 mmol) was dissolved in dimethylformamide (DMF)

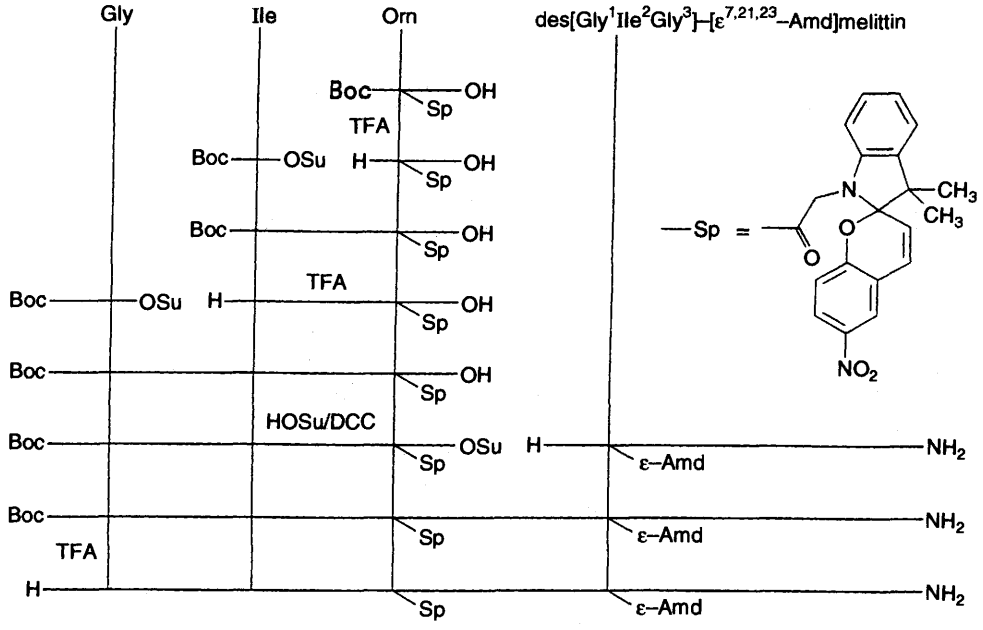

Fig. 2 Semisynthetic method for the preparation of MSp

(15 cm^3), and *N*-hydroxysuccinimide (180 mg) and DCC (322 mg) were added at 0 °C. The reaction mixture was stirred for 24 h. The precipitated solid was removed by filtration and N^{α}-Boc-Orn-OH in DMF and triethylamine (236 mm^3) (1 mm^3 = 1 μl) were added to the filtrate at 0 °C. After stirring for 17 h, the solution was condensed under reduced pressure and the residue was dissolved in ethyl acetate. The organic solution was washed with 10% citric acid and NaCl aqueous solutions and was dried ($MgSO_4$). The solution was concentrated and purified through a Sephadex LH-20 column using methanol as eluent to obtain Boc-Orn(Sp)-OH; $\delta_H(CD_3OD)$ 1.44 (s, 6 H, 3-CH_3), 1.60 [s, 9 H, C(CH_3)$_3$], 1.76 (dt, 2 H, NH[CH$_2$]$_2$CH_2), 1.84 (tt, 2 H, NHCH$_2$CH_2CH$_2$), 3.36 (br, 2 H, CONHCH_2CH$_2$), 3.92 [q, 1 H, NHCH(CO$_2$H)CH$_2$], 4.24 (s, 2 H, NCH_2CO), 6.16 (d, *J* 11.52, 1 H, 3′-CH=), 6.64 (d, *J* 8.64, 1 H, 4′-CH=), 7.12 (m, 5 H, PhH) and 8.16 (m, 2 H, PhH). TLC, R_f = 0.41. By treatment with trifluoroacetic acid (TFA, 1.0 cm^3) in the presence of anisole (100 mm^3) for 30 min, ornithine N^{δ}-spiropyran derivative [H-Orn(Sp)-OH] was obtained (60.2 mg) as a solid.

A DMF solution of Boc-Ile-OSu (0.15 mmol), TFA H-Orn(Sp)-OH (0.10 mmol) and triethylamine (0.20 mmol) was stirred for 15 h at room temperature. The product was purified through a Sephadex LH20 column using methanol as eluent. Yield: 48%. $\delta_H(CD_3OD)$ 1.09 [d, 3 H, CH-$^{\gamma'}$CH_3 (Ile)], 1.09 [s, 3 H, $^{\delta}$CH$_3$ (Ile)], 1.48 (s, 6 H, 3-CH$_3$), 1.63 [s, 9 H, C(CH$_3$)$_3$], 1.84 [m, 8 H, NHCH$_2$CH_2CH_2, $^{\beta}$CH$_2$ (Ile), $^{\gamma}$CH$_2$ (Ile)], 3.44 (br, 2 H, CONHCH_2CH$_2$), 3.84 (s, 2 H, NCH_2CO), 3.92 [q, 1 H, $^{\alpha}$CH (Ile)], 4.48 [q, 1 H, $^{\alpha}$CH (Orn)], 6.16 (d, *J* 11.52, 1 H, 3′-CH=), 6.64 (d, *J* 8.64, 1 H, 4′-CH=), 7.12 (m, 5 H, PhH) and 8.16 (m, 2 H, PhH). TLC, R_f = 0.28. The dipeptide was treated with TFA and precipitated by the addition of diethyl ether. TFA H-Ile-Orn(Sp)-OH was obtained as a solid. Yield, 22.0 mg.

The dipeptide was coupled with Boc-Gly-Osu (0.048 mmol) in the same way as for the coupling of Boc-Ile-OSu with H-Orn(Sp)-OH. Boc-Gly-Ile-Orn(Sp)-OH was obtained as a solid. Yield: 71%. $\delta_H(CD_3OD)$ 1.09 [d, 3 H, CH$^{\gamma'}$CH_3 (Ile)], 1.09 [s, 3 H, $^{\delta}$CH$_3$ (Ile)], 1.48 (s, 6 H, 3-CH$_3$), 1.63 [s, 9 H, -C(CH$_3$)$_3$], 1.84 [m, 8 H, NHCH$_2$CH_2CH_2, $^{\beta}$CH$_2$ (Ile), $^{\gamma}$CH$_2$ (Ile)], 3.44 (br, 2 H, CONHCH_2CH$_2$), 3.84 (s, 2 H, NCH_2CO), 3.92 [q, 1 H, $^{\alpha}$CH (Ile)], 4.00 [q, 2 H, $^{\alpha}$CH$_2$ (Gly)], 4.48 [q, 1 H, $^{\alpha}$CH (Orn)], 6.16 (d, *J* 11.52, 1 H, 3′-CH=), 6.64 (d, *J* 8.64, 1 H, 4′-CH), 7.12 (m, 5 H, PhH) and 8.16 (m, 2 H, PhH). TLC, R_f = 0.12.

Des[Gly^1*Ile*^{2}*Gly*^{3}*]-[ε*^{7,21,23}*-Amd]melittin.* (Amd represents the acetimidyl group.) ε-Amino groups of melittin were blocked by the acetimidyl group according to the previously reported method.[15] Melittin was treated with excess methyl acetimidate hydrochloride at pH 10.1–10.5. The reaction mixture was eluted through a Sephadex G-25 column (2.6 × 43 cm) with a Tris HCl buffer (0.1 mol dm^{-3}, pH 9.4) to remove excess reagents. The pH of the concentrated solution was adjusted to 6.9 with 1 mol dm^{-3} hydrochloric acid and the solution was desalted by a Sephadex G-25 column (2.6 × 35 cm). Acetimidylmelittin, [ε^{7,21,23}-Amd]melittin, was obtained as a white powder (8.5 mg) after lyophilization. The free amino group of acetimidylmelittin was determined by labelling with 2,4,6-trinitrobenzenesulfonic acid (TNBS).[16] One amino group remained unblocked, which is assigned to the N-terminal α-amino group.

Acetimidylmelittin was subjected to three cycles of Edman degradation to remove the N-terminal residues Gly-1, Ile-2 and Gly-3 from the peptide chain. The stepwise degradation was checked by the TLC analysis. The degraded peptide was purified with a Sephadex G-50 column. Des[Gly-1-Ile-2-Gly-3]-[ε^{7,21,23}-Amd]melittin was obtained as a white powder (5.0 mg) after lyophilization.

[Orn^3*(Sp)]-[ε*^{7,21,23}*-Amd]melittin (MSp).* The tripeptide, Boc-Gly-Ile-Orn(Sp)-OH, was reacted with des[Gly-1-Ile-2-Gly-3]-[ε^{7,21,23}-Amd]melittin by using DCC (1.5 mol equiv.) and HOBt (1.5 mol equiv.) as coupling reagents. The product was eluted through a Sephadex LH-20 column with DMF (5 × 100 cm). The Boc group was removed by treatment with TFA and the product was precipitated with diethyl ether. The solid product was further purified by a reverse-phase HPLC (COSMOSIL 5C$_{18}$ column, acetonitrile–water). After lyophilization, [Orn^3(Sp)]-[ε^{7,12,23}-Amd]melittin was obtained as a red powder (1.2 mg).

Spectroscopic Measurements.—UV- and visible-absorption spectra were recorded on a JASCO Ubest-50 UV–VIS spectrophotometer with a cell of 1 cm optical path length. Circular dichroism spectra were measured on a JASCO J-600 CD spectropolarimeter with a cell of 0.1 cm optical path length.

^{1}H NMR spectra were recorded on a JEOL FX90Q spectrometer.

Thermal Isomerization Kinetics.—Thermal decolouration rates were determined by monitoring the decrease of absorbance at the absorption maximum (λ_{max}) of the merocyanine group. UV ($310 < \lambda < 360$ nm) and visible ($500 \text{ nm} < \lambda$) light irradiations were performed by using 100 W high-pressure mercury lamp and glass filters.

The decay curves were analysed using eqn. (1), where A_t,A_0

$$(A_t\text{-}A_\infty)/(A_0\text{-}A_\infty) = F\exp(-k_{fast}t) + (1 - F)\exp(-k_{slow}t) \quad (1)$$

and A_∞ are the absorbance at λ_{max} at time t, zero and infinity, respectively and F and $1 - F$ are the fractions of k_{fast} and k_{slow} components, respectively.

Vesicle Preparation.—DPPC vesicles encapsulating 5/6-carboxyfluorescein (CF) were prepared by a sonication method.[16] HEPES buffer (10 mmol dm^{-3}, pH 7.4, with 100 mmol dm^{-3} NaCl, 0.67 cm^3) and aqueous CF solution (130 mmol dm^{-3}; 2.0 cm^3) were added to dry dipalmitoylphosphatidylcholine (DPPC, 25 mg) and the mixture was sonicated using a bath-type sonicator (40 W) at 50 °C under an N_2 atmosphere. The suspension was centrifuged at 100 000 g for 30 min at 25 °C to obtain small unilamellar vesicles encapsulating CF in the supernatant. This suspension was subjected to gel filtration on a Sephadex G-50 superfine column (1 × 25 cm) using HEPES buffer (10 mmol dm^{-3}, pH 7.4, with 100 mmol dm^{-3} NaCl) as an eluent. The DPPC vesicles without CF were prepared in otherwise the same way as that for the CF-containing DPPC vesicles using a HEPES buffer without CF.

CF Leakage.—When CF is entrapped in vesicles at more than 100 mmol dm^{-3}, the dye is subject to concentration quenching. However, the dye recovers the fluorescence upon leakage from vesicles due to dilution.[16] MSp was added to the CF-encapsulated DPPC vesicle at 25 °C and the fluorescence intensity at 515 nm (F) was measured. The excitation wavelength was 470 nm. The fluorescence intensity without MSp (F_0) was determined by the addition of water. Maximal leakage was attained by addition of Triton X-100 (F_{Triton}), which disrupts vesicular structure. The percentage of CF leakage was calculated using eqn. (2). The leakage rates were calculated by $1/(100 - L)\,dL/dt$ from the time course of the leakage.

$$\text{Leakage } (L\%) = [(F - F_0) \times 100]/(F_{Triton} - F_0) \quad (2)$$

When MSp was irradiated by UV light for 10 min and was added to the CF-containing DPPC vesicles, the curve of the leakage rate was fitted to a two-component exponential function (3), with $G_1 < 0$ and $G_2 > 0$.

$$1/(100 - L)dL/dt = G_1\exp(-k_{acc}t) + G_2\exp(-k_{dec}t) \quad (3)$$

Results and Discussion

Thermal Decolouration of MSp.—MSp exhibited photochromism either in an organic solution or in a lipid membrane. The colourless solution turned blue or violet upon UV-light irradiation and the coloured solution was faded thermally or by visible-light irradiation. The thermal decay of the merocyanine form of MSp was monitored by the absorbance at λ_{max} ($\lambda > 500$ nm) and analysed in terms of the two-exponential process. The rate constants and the fractions of two components are summarized in Table 1. The thermal isomerization rate from merocyanine to spiropyran is strongly dependent on the medium polarity.[17] Two components in the decay were ascribed to two conformers of merocyanine, in which the slow component is stabilized in a polar environment and the fast one in an apolar environment.[18] The higher fraction of the slow component than the fast component suggests that the merocyanine group is located at the polar surface of lipid membrane. This belief is supported by the fact that the merocyanine group absorbs at a shorter wavelength (533 nm) in the lipid membrane.[15]

Table 1 Kinetic constants and pre-exponential factors of the thermal decay of the merocyanine group of MSp in the presence of DPPC vesicle

	Slow component		Fast component	
T/°C	Factor	k_{slow}	Factor	k_{fast}
15	0.15 (65%)	6.6×10^{-4}	0.08 (35%)	5.4×10^{-3}
25	0.28 (82%)	3.2×10^{-3}	0.06 (18%)	2.5×10^{-2}
35	0.61 (97%)	4.1×10^{-3}	0.02 (3%)	5.9×10^{-2}

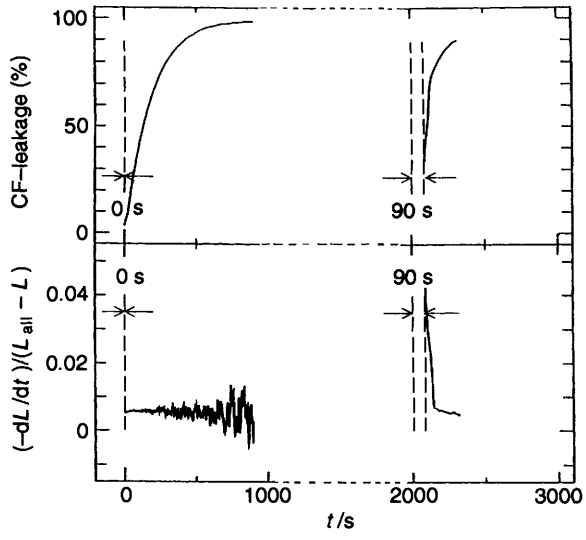

Fig. 3 CF leakage from DPPC vesicles induced by MSp at 15 °C. Upper: the percentage of CF leaked from the vesicles. Lower: the rate constant of CF leakage. dL/dt was calculated by numerical differentiation of the experimental data. Left: without UV-light irradiation. Right: UV-light irradiation for 90 s.

CF Leakage Induced by MSp.—The activity of MSp in a membrane was studied by CF leakage from DPPC vesicles. A simple exponential leakage of entrapped CF was observed when MSp was added to the vesicles (Fig. 3). In this case, the kinetic constant, $k_{obs} = 1/(L_\infty - L)\,dL/dt$ (L and L_∞ represent CF leakage at time t and infinity), was nearly constant until 80% CF-leakage (Fig. 3). When UV light was irradiated for 90 s after addition of the melittin derivative, a *ca.* eight-fold increase of k_{obs} was observed immediately after the irradiation (Fig. 3).

To estimate precisely the effect of UV irradiation on CF leakage, CF leakage was measured at 15, 25 and 35 °C in the presence of pre-irradiated (10 min) MSp (Fig. 4). If CF leakage is accelerated by MSp in the spiropyran form, accumulation of MSp in the spiropyran form in the lipid membrane should cause a simple exponential acceleration of k_{obs} because the amount of MSp in the membrane increases in an exponential way. On the other hand, if CF leakage is accelerated by the merocyanine form of MSp, k_{obs} should show a two component exponential change consisting of acceleration and deceleration, representing binding of the merocyanine-type MSp and its thermal isomerization, respectively. As shown in Fig. 4, k_{obs} was initially

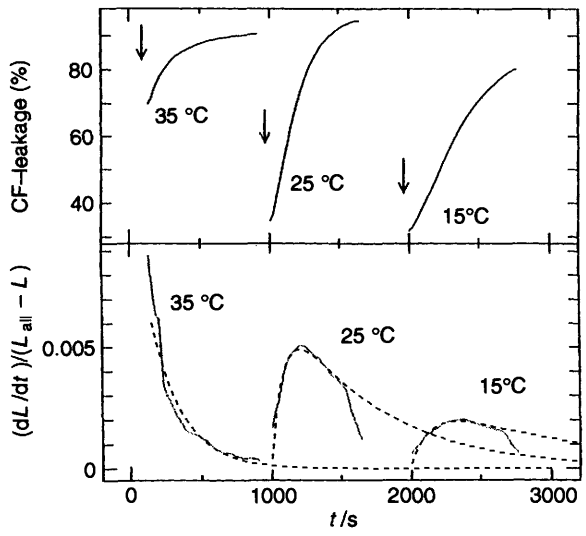

Fig. 4 CF leakage from DPPC vesicles induced by adding pre-irradiated (10 min) MSp; broken lines were obtained by the bicomponent exponential fitting

Table 2 Kinetic constants and pre-exponential factors of CF leakage induced by the addition of MSp

	Deceleration process		Acceleration process	
T/°C	Factor	k_{slow}	Factor	k_{fast}
15	0.029 (52%)	8.4×10^{-4}	−0.027 (48%)	7.1×10^{-3}
25	0.076 (51%)	1.4×10^{-3}	−0.072 (49%)	1.1×10^{-2}
35	0.119 (55%)	4.6×10^{-3}	−0.097 (45%)	4.5×10^{-2}

accelerated and followed by deceleration, indicating that MSp in the merocyanine form disturbs the membrane structure more significantly than MSp in the spiropyran form.

The curve of the CF-leakage rate was fitted by a two-component exponential function. The fractions of the acceleration and deceleration processes are nearly the same as shown in Table 2. This result is explained in terms of the kinetic scheme shown in Fig. 5. The kinetic scheme predicts that the pre-exponential factor of the acceleration process (binding of M_w to the membrane resulted in an increase of M_L) becomes the same as that of the deceleration process (thermal isomerization of M_L to S_L resulted in a decrease of M_L) under the condition that MSp, taking a merocyanine form in the lipid membrane, is the predominant species responsible for the CF leakage. The validity of this belief is confirmed by the agreement of the kinetic constants between the slow component of the thermal decay (Table 1) and the deceleration process of the CF leakage

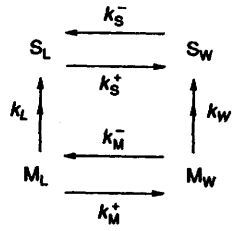

Fig. 5 Thermal decolouration pathways of MSp in the presence of vesicles. The subscripts L and W represent the lipid membrane phase and the aqueous phase, respectively. S and M represent MSp in the form of spiropyran and merocyanine, respectively.

(Table 2) at all temperatures. The result is consistent with the molecular design described in the introduction. The merocyanine-type MSp takes the secondary amphiphilic α-helical structure due to alignment of the hydrophilic residues, Orn(Sp)-3, Lys-7, Thr-10 and Thr-11 localized at one surface of the helix peptide, which should disturb the membrane structure severely.

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Paper 4/03883E
Received 27*th June* 1994
Accepted 27*th September* 1994