

Synthesis, aggregation, and stimuli-responsive disaggregation of an amphiphilic cyclophane having an alkyl disulfide moiety

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Abstract

An amphiphilic cyclophane (**1**) having an alkyl chain with a cleavable disulfide linkage was developed as a reduction-responsive host aiming at guest release. Host **1** was synthesized by stepwise condensation of a tetraaza[6.1.6.1]paracyclophane skeleton having a carboxylic acid succinimidyl ester (**3**) with a cystamine dihydrochloride and a stearic acid derivative in this sequence, followed by removal of the protecting groups in a fairly good yield. Host **1** formed vesicle-like aggregates with an average diameter of *ca.* 260 nm, which were confirmed by dynamic light scattering (DLS) measurements and transmission electron microscopy (TEM) observation. The self-aggregates formed with **1** was found to bind fluorescent guests such as TNS strongly with a binding constant of $3.4 \times 10^4 \text{ M}^{-1}$. Disassembly of the supramolecular aggregates composed of **1** was successfully performed upon addition of dithiothreitol (DTT), to give the corresponding thiol of cyclophane (**2**) having poor guest-binding affinity that led to release of the entrapped guest molecules to the bulk aqueous phase.

Key words: Cyclophane; Aggregation; Stimuli-responsive guest release

1. Introduction

Cyclophanes are a macrocyclic host having an internal cavity that form host-guest complexes with various organic molecules [1-3]. Even though cyclophanes have been frequently used as a molecular framework for host-guest chemistry, the guest-binding affinity of monocyclic cyclophanes was relatively weak [4]. On the other hand, biological ligand-receptor interactions are often amplified by multivalent receptors. That is, naturally occurring multivalent clusters of receptors are known to exhibit extremely strong binding capability toward substrates, although the affinities of monovalent receptors are weak [5-8]. On these grounds, we have recently developed covalently-linked cyclophane oligomers, on the basis of molecular design to connect several cyclophanes in order to enhance the guest-binding ability [9-12]. In the preceding paper, we reported a synthesis of pendent-type water-soluble cyclophane dimer, trimer, tetramer, and pentamer by aminolysis of succinimidyl ester derivative of tetraaza-[6.1.6.1]paracyclophane with a lysine and the corresponding lysine peptide as a scaffold [13]. The guest-binding ability of the cyclophane oligomers enhanced

toward fluorescence guests, reflecting the multivalency effects in macrocycles [13]. However, the synthetic yield of the cyclophane pentamer was moderate under the reaction conditions of amide formation, which was not satisfied to get further insights into a variety of multivalent host systems. In the course of our ongoing research on multivalent cyclophanes, we became interested in developing non-covalent aggregates based on cyclophanes, which open a new way for investigation of multivalent hosts. On these grounds, we designed and synthesized an amphiphilic cyclophane having an alkyl disulfide moiety (**1**). The cyclophane has both hydrophilic and hydrophobic moieties. Therefore, when **1** is dissolved in water, molecular aggregates composed of **1** are expected to form through hydrophobic interaction (Figure 1). In addition, disassembly of the aggregates can be expected by a cleavage of the disulfide bond of the host through external stimuli such as reducing reagents, with releasing the alkyl chain (Figure 1). In this context, we now report synthesis, self-aggregation, and stimuli-responsive disaggregation of an amphiphilic cyclophane having an alkyl chain with a cleavable disulfide linkage.

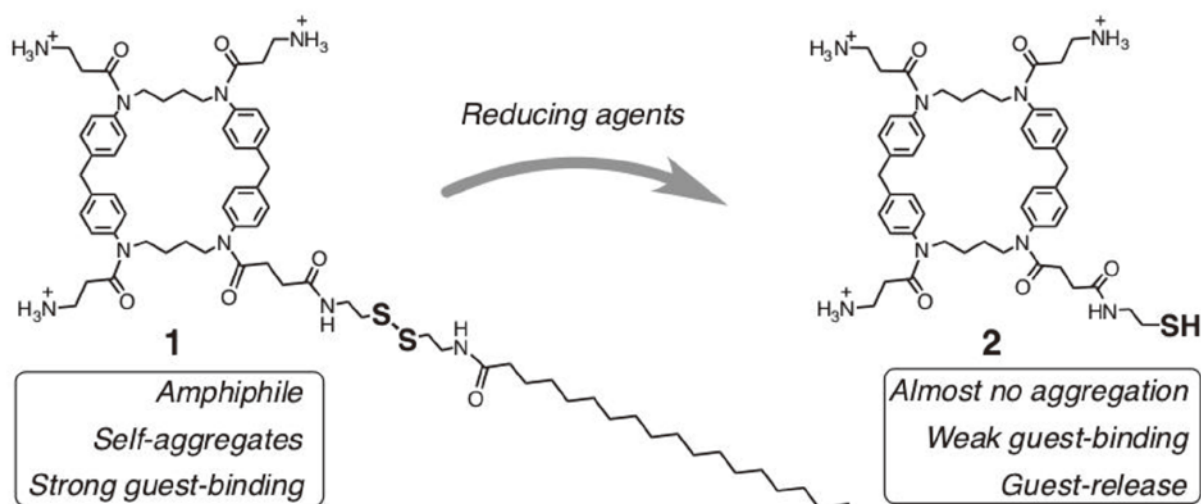


Figure 1. An amphiphilic cyclophane having an alkyl chain with a cleavable disulfide linkage (**1**) and water-soluble cyclophane (**2**).

2. Results and discussion

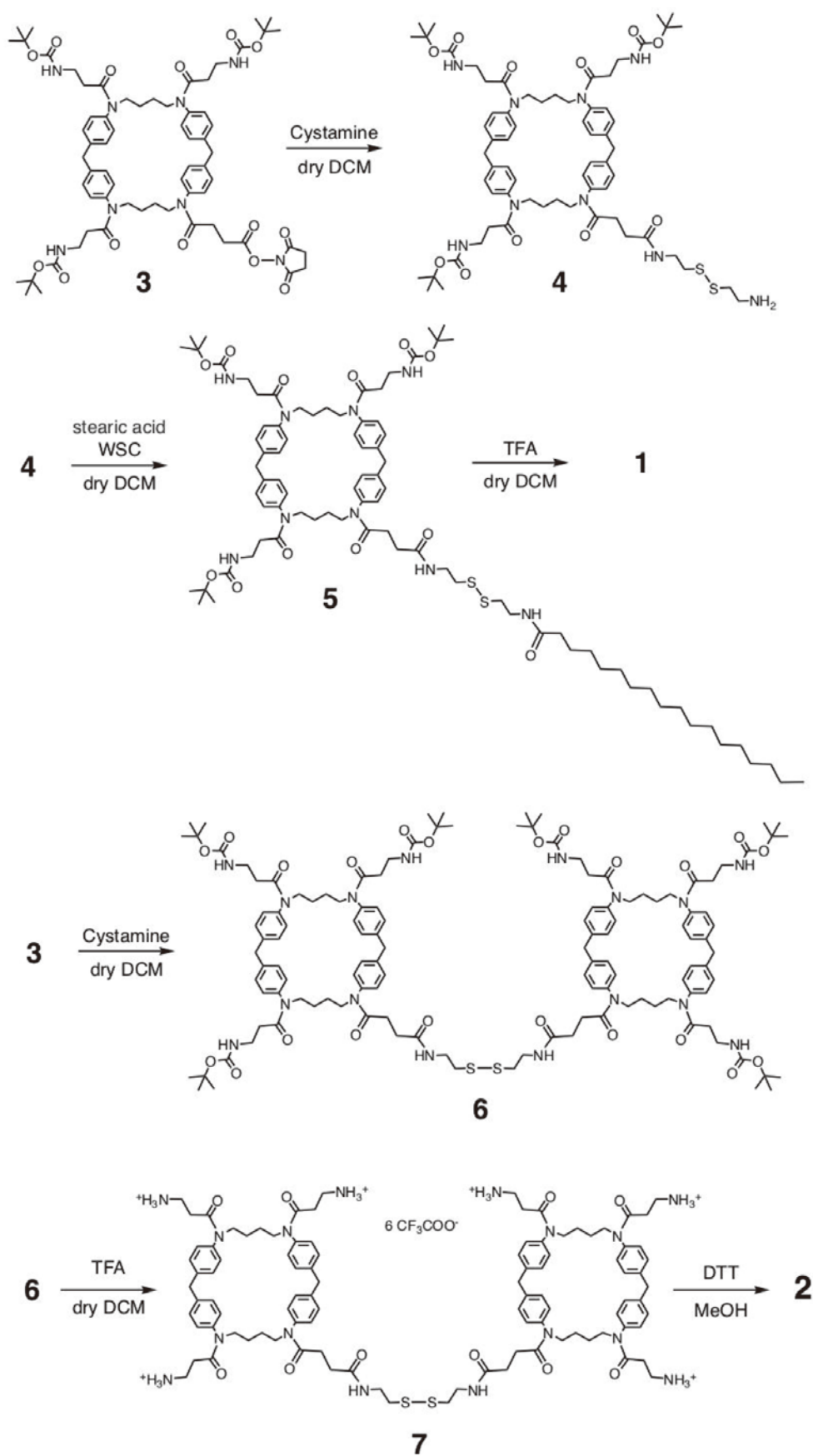
2-1. Design and synthesis of amphiphilic cyclophane having an alkyl disulfide moiety

We designed an amphiphilic cyclophane having an alkyl chain with a cleavable disulfide linkage **1**. Cyclophane **1** was constructed with a tetraaza[6.1.6.1]paracyclophane, three hydrophilic side chains having a terminal ammonium group, and a hydrophobic alkyl chain connected with a disulfide bond. The former side chains afford advantage of water-solubility to the host. Cyclophane **1** was synthesized by following the reaction sequence shown in Scheme 1. In the previous paper, we have reported a synthesis of a cyclophane derivative having a carboxylic acid succinimidyl ester and three Boc- β -alanine residues (**3**) [13]. A cyclophane having a terminal amino group with a disulfide linkage (**4**) was synthesized by condensation of **3** with cystamine dihydrochloride in a fairly good yield. Then, a precursor (**5**) of **1** was synthesized by condensation of **4** with steric acid in the presence of water-soluble carbodiimide (WSC). Cyclophane **1** was derived from **5** by removal of the protecting groups with trifluoroacetic acid (TFA). In addition, an cyclophane having three hydrophilic side chains having a terminal ammonium group and a terminal thiol one (**2**) was also prepared as a control water-soluble host, as shown in Scheme 1. All the

synthesized compounds were identified by ^1H and ^{13}C NMR spectroscopy, matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS), and elemental analysis.

2-2. Aggregation behavior of amphiphilic cyclophane

Compound **1** showed good solubility in common organic solvents such as methanol, ethanol, ethyl acetate, and chloroform. In addition, despite of the presence of hydrophobic cyclophane skeleton and an alkyl chain, **1** was fairly soluble in water at neutral pH ranges owing to the three peripheral polar side chains, indicating that **1** has amphiphilic property to form self-assemblies. The aqueous dispersion of **1**, which was prepared and filtered by membrane filter (pore size, 0.8 μm) was investigated by dynamic light scattering (DLS) was used to measure the size of the aggregates. DLS studies for **1** in a dispersion state showed the presence of hydrodynamic particle with an average diameter of *ca.* 260 nm (Figure 2a). Aggregation behavior of **1** was also investigated by negative staining transmission electron microscopy (TEM), while phosphotungstic acid was used as a stain. Vesicle-like aggregates ranging from *ca.* 100 to 500 nm were found by TEM observation for **1** (Figure 2b), which was almost comparable in size to that indicated by DLS measurements.


 Scheme 1. Preparation of cyclophanes **1** and **2**.

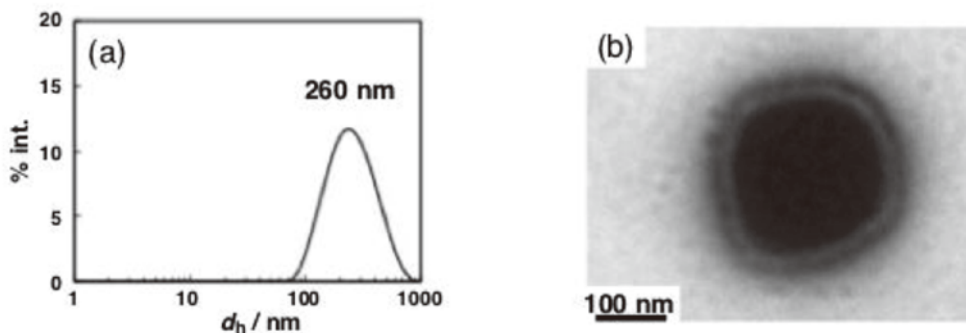


Figure 2. DLS histogram (a) and TEM image (b, the scale bar is 100 nm) for self-aggregates composed of **1**. Phosphotungstic acid was used as a stain.

2-3. Enhanced guest-binding ability of amphiphilic cyclophane

As mentioned above, we have previously prepared pendent-type cyclophane dimer, trimer, tetramer, and pentamer [13]. We have clarified that the guest-binding affinity of pendent-type cyclophane oligomers with fluorescence guests such as 6-*p*-toluidino-naphthalene-2-sulfonate (TNS) [14] was much enhanced relative to that of the corresponding monocyclic cyclophane, as evaluated by fluorescence spectroscopy. In order to evaluate the multivalent effect on the non-covalent aggregates based on cyclophanes and compare the guest-binding affinity of pendent-type cyclophane pentamers, we investigated in this work the guest-binding behavior of **1** toward fluorescence guests by the identical methods. The guest-binding behavior of **1** toward TNS and 6-anilino-naphthalene-2-sulfonate (2,6-ANS) was examined by fluorescence spectroscopy in aqueous 2-[4-(2-hydroxy-ethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer (0.01 M, pH 7.4, 0.15 M with NaCl) at 298K. The fluorescence intensity originated from each guest increased along with

a concomitant blue shift of the fluorescence maximum upon addition of the hosts as shown in Figure 3. Binding constants (*K*) for the formation of inclusion complexes of **1** with TNS and 2,6-ANS in a 1:1 molar ratio were evaluated on the basis of the Benesi-Hildebrand relationship [15] under the conditions at a large excess amount of the host. The evaluated *K* values of **1** with TNS and 2,6-ANS were 3.4×10^4 and $4.6 \times 10^4 \text{ M}^{-1}$, respectively, and were about 10 times larger than that of the corresponding monocyclic cyclophane. Therefore, multivalent effect of non-covalent aggregates of **1** was somewhat equivalent to that of the cyclophane trimer (11-fold) [13].

2-4. Reduction-responsive disaggregation of amphiphilic cyclophane

Stimuli-responsive control of aggregation/disaggregation of supramolecules is an attractive research issue. As regards the reduction-responsive functions, the present cyclophanes have a disulfide linkage that is cleavable to thiols by a treatment with reducing reagents. In fact, cyclophane **1** was easily transformed to the

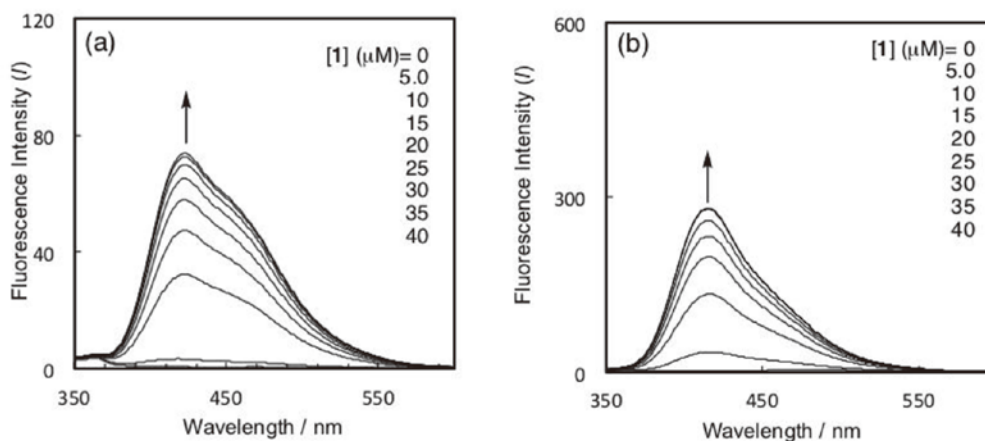


Figure 3. Fluorescence spectra for aqueous solutions of TNS (a) and 2,6-ANS (b) upon addition of **1** in HEPES buffer at 298K: Ex, 326 nm.

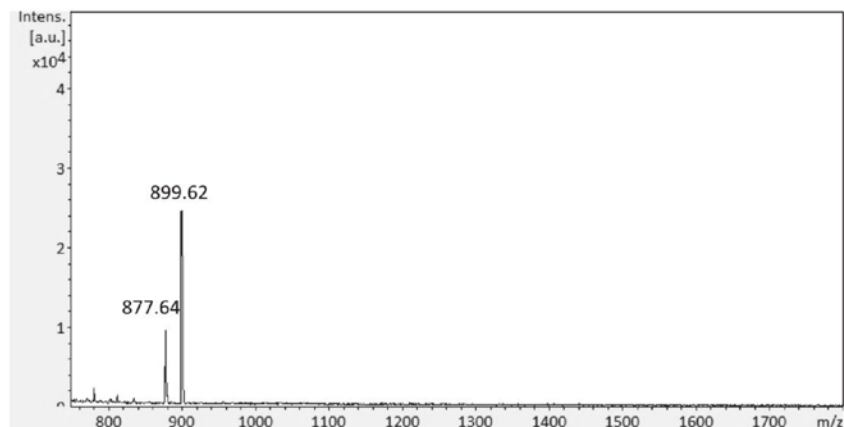


Figure 4. MALDI-TOF MS spectrum of a solution of **1** and DTT in methanol.

corresponding thiol derivatives by reducing reagents such as dithiothreitol (DTT), which was confirmed by MALDI-TOF MS spectrometry (Figure 4). That is, upon addition DTT to aqueous dispersion of **1**, a peak originating from reduced form, thiol **2**, was also observed; m/z , 877.6 and 899.6 was assigned to $[M + H]^+$ and $[M + Na]^+$, respectively (Figure 4). Similar reduction of **1** was also observed by using glutathione (GSH) in place of DTT. Disaggregation of the aggregates composed of **1** was confirmed by DLS measurements. The dispersion of **1** in the presence of DTT did not give any meaningful DLS signals, indicating the absence of large particles such as aggregates. In addition, such disaggregation of the aggregates composed of **1** was confirmed by TEM observation. Therefore, **1** was found to compose aggregates of which disaggregation was controlled by reducing reagents.

Reduction-responsive guest-releasing behavior of **1** was investigated in a similar manner by fluorescence spectroscopy. Upon addition of DTT to a HEPES buffer solution containing host-guest complexes of **1** with TNS, the fluorescence intensity originated from the guest molecules was subjected to decrease, as shown in Figure 5. These results indicate that **2** having weak guest-binding affinity generated by the reduction of disulfide bond of **1** by DTT. Accordingly, quite a few entrapped guest molecules by **1** were released to the bulk aqueous phase after 50 min incubation, reflecting reduction-responsive cleavage of the macrocycle. Similarly, upon addition of GSH (10mM) to a HEPES buffer solution containing host-guest complexes of **1** with TNS, decrease in fluorescence intensity was also observed, reflecting the release of the entrapped guest molecules.

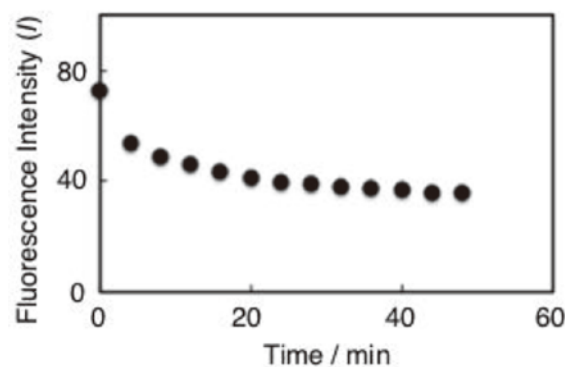


Figure 5. Time course for changes of fluorescence intensity originating TNS ($1 \mu\text{M}$) in HEPES buffer in the presence of **1** ($40 \mu\text{M}$) upon addition of DTT (1mM) at 298K.

3. Conclusions

We have synthesized amphiphilic cyclophane having an alkyl chain with a cleavable disulfide linkage **1** by introducing alkyl disulfide moiety into a tetraaza[6.1.6.1] paracyclophane having three Boc- β -alanine residues, followed by removal of the external Boc-protecting groups. Host **1** formed vesicle-like aggregates with an average diameter of *ca.* 260 nm, which were confirmed by DLS measurements and TEM observation. The self-aggregates formed with **1** was found to bind fluorescent guest such as TNS and 2,6-ANS strongly with binding constant of 3.4×10^4 and $4.6 \times 10^4 \text{M}^{-1}$, respectively. Disassembly of the supramolecular aggregates composed of **1** was successfully performed upon addition of DTT, to give the corresponding thiol of cyclophane **2** having poor guest-binding affinity that led to release of the entrapped guest molecules to the bulk aqueous phase. It is known that intracellular concentration of reductive GSH is about 2-10 mM and is often elevated in some cancer cells, being higher than the level outside

the cells [16,17]. Combined these characteristics suggest that **1** is expected to act as a reduction-responsive vehicles capable of being degradable and releasing the entrapped drugs (guest) under the reducing condition such as cancer cells. These subjects of interest will be explored further in the future.

4. Experimental section

4-1. A cyclophane having a terminal amino group with a disulfide linkage **4**

A solution of succinimidyl ester derivative of tetraaza[6.1.6.1]paracyclophane having Boc-protected β -alanine residues (**3**) [13] (163 mg, 0.13 mmol) in dry dichloromethane (DCM, 6 mL) was added dropwise to a solution of cystamine dihydrochloride (304 mg, 1.4 mmol) in dry DCM (10 mL), and the mixture was stirred for 1 h at ambient temperature. The solvent was distilled on a rotatory evaporator to give white solid. The crude product was chromatographed on a column of Sephadex LH-20 with methanol as an eluent for purification. Evaporation of the main fraction on a rotatory evaporator gave a white solid (121 mg, 72%): $^1\text{H NMR}$ (400 MHz, CDCl_3 , 298 K) δ 1.44 (m, 35H), 2.13 (m, 6H), 2.36 (t, 2H), 2.55 (t, 2H), 3.01 (t, 2H), 3.12 (t, 2H), 3.29 (m, 6H), 3.45 (m, 2H), 3.64 (m, 10H), 3.97 (s, 4H), 5.35 (s, 3H), 6.99 (m, 8H), 7.22 (m, 8H), 7.75 (m, 2H). $^{13}\text{C NMR}$ (100 MHz, CDCl_3 , 298 K): δ 24.8, 25.4, 26.1, 28.4, 29.5, 30.5, 30.6, 32.3, 33.2, 34.8, 36.4, 41.1, 48.8, 48.9, 70.0, 128.3, 130.2, 140.3, 140.5, 154.4, 155.9, 170.6, and 171.9. IR: 1635, 1701 cm^{-1} (C=O). MALDI-TOF MS m/z : 1275 [M + Na] $^+$, where M shows $\text{C}_{68}\text{H}_{97}\text{N}_9\text{O}_{11}\text{S}_2$ (monoamine derivative of cyclophane **4**).

4-2. A precursor (**5**) of **1**

Dicyclohexylcarbodiimide (DCC, 39 mg, 0.19 mmol) was added to a solution of stearic acid (45 mg, 0.16 mmol) in dry DCM (2 mL) at 0 $^\circ\text{C}$, and the mixture was allowed to stand at the same temperature while being stirred for 20 min. The mixture was added to a solution of **4** (100 mg, 0.08 mmol) in dry DCM (2 mL), and the resulting mixture was stirred for 2 days at room temperature. Precipitates that formed (*N,N'*-dicyclohexylurea) were removed by filtration, the solvent was eliminated under reduced pressure, and the residue was dissolved in ethyl acetate (EtOAc, 10 mL). Insoluble materials were removed by filtration, and the filtrate was evaporated to dryness under reduced pressure. The crude product was chromatographed on a column of Sephadex LH-20 with methanol as an eluent for

purification. Evaporation of the main fraction on a rotatory evaporator gave a white solid (107 mg, 89%): $^1\text{H NMR}$ (400 MHz, CDCl_3 , 298 K) δ 0.88 (t, 3H), 1.25 (s, 28H), 1.42 (s, 35H), 2.08 (m, 6H), 2.19 (t, 2H), 2.27 (t, 2H), 2.42 (t, 2H), 2.80 (m, 4H), 3.26 (m, 6H), 3.61 (m, 12H), 3.95 (s, 4H), 5.31 (s, 3H), 6.41 (s, 1H), 6.73 (s, 1H), 6.94 (m, 8H), 7.20 (m, 8H). $^{13}\text{C NMR}$ (100 MHz, CDCl_3 , 298K) δ 14.1, 22.7, 25.0, 25.8, 28.4, 29.3, 30.3, 31.5, 31.9, 34.8, 36.4, 36.6, 37.7, 38.0, 38.3, 38.4, 41.1, 48.7, 49.0, 79.0, 128.3, 130.31, 140.2, 155.9, 171.4, 171.6, 172.8, and 173.7. IR 1645, 1709 cm^{-1} (C=O). Found: C, 64.08; H, 8.38; N, 7.96; Calcd for $\text{C}_{84}\text{H}_{127}\text{N}_9\text{O}_{12}\text{S}_2 \cdot 3\text{H}_2\text{O}$: C, 64.13; H, 8.52; N, 8.01. MALDI TOF MS m/z , 1541.0 [M + Na] $^+$, where M shows $\text{C}_{84}\text{H}_{127}\text{N}_9\text{O}_{12}\text{S}_2$.

4-3. Amphiphilic cyclophane having an alkyl disulfide moiety **1**

Trifluoroacetic acid (1 mL) was added to a solution of **5** (114 mg, 0.08 mmol) in dry DCM (2 mL), and the mixture was stirred for 2 hours at room temperature. Evaporation of the solvent under reduced pressure and the residue was purified by gel filtration chromatography on a column of Sephadex LH-20 with methanol as an eluent. Evaporation of the product fraction under reduced pressure gave a red solid (108 mg, 93 %): $^1\text{H NMR}$ (400 MHz, CD_3OD , 298 K) δ 0.90 (t, 3H), 1.26 (m, 28H), 1.46 (t, 8H), 1.61 (t, 2H), 2.11 (t, 2H), 2.21 (m, 4H), 2.37 (m, 2H), 2.44 (m, 4H), 2.83 (t, 4H), 3.05 (t, 2H), 3.13 (m, 2H), 3.49 (t, 8H), 3.69 (s, 8H), 4.04 (s, 4H), 6.99 (m, 4H), 7.19 (m, 4H), and 7.39 (m, 8H). $^{13}\text{C NMR}$ (100 MHz, CD_3OD , 298K) δ 13.0, 22.3, 24.1, 25.6, 28.9, 29.5, 30.4, 31.1, 31.6, 35.7, 37.2, 38.17, 40.4, 118.3, 128.3, 130.2, 140.1, 141.6, 161.8, 173.9, 175.9, 177.3, and 178.99. IR 1635 cm^{-1} (C=O). Found: C, 56.83; H, 7.04; N, 7.97; Calcd for $\text{C}_{84}\text{H}_{127}\text{N}_9\text{O}_{12}\text{S}_2 \cdot 3\text{H}_2\text{O}$: C, 57.06; H, 6.90; N, 7.98. MALDI-TOF MS: m/z 1218.9 [M + H] $^+$, 1240.9 [M + Na] $^+$, where M shows $\text{C}_{84}\text{H}_{127}\text{N}_9\text{O}_{12}\text{S}_2$.

4-4. Boc-protected cyclophane dimer having an alkyl disulfide spacer **6**

A solution of **3** (175 mg, 0.14 mmol) in dry DCM (5 mL) was added dropwise to a solution of cystamine dihydrochloride (16 mg, 0.07 mmol) in dry DCM (5 mL), and the mixture was stirred for 1 h at ambient temperature. The solvent was distilled on a rotatory evaporator to give white solid. The crude product was chromatographed on a column of silica gel (Wako Gel C-100) with methanol-chloroform (1 : 1 v/v) as eluant. Evaporation of the main fraction on a rotatory evaporator gave a white solid (137

mg, 81%): ^1H NMR (400 MHz, CDCl_3 , 298 K) δ 1.44 (m, 70H), 2.10 (m, 12H), 2.30 (t, 4H), 2.46 (t, 4H), 2.83 (t, 4H), 3.28 (s, 12H), 3.55 (m, 4H), 3.64 (s, 16H), 3.97 (s, 8H), 5.34 (s, 6H), 6.97 (m, 17H), 7.22 (m, 16H). ^{13}C NMR (100 MHz, CDCl_3 , 298 K): δ 24.9, 28.4, 30.2, 31.4, 34.8, 36.4, 37.8, 38.4, 41.0, 48.7, 48.9, 78.9, 128.3, 130.2, 140.2, 155.9, 171.4, and 172.7. IR: 1644, 1708 cm^{-1} (C=O). Found: C, 62.69; H, 7.36; N, 9.03; Calcd for $\text{C}_{128}\text{H}_{174}\text{N}_{16}\text{O}_{22}\text{S}_2 \cdot 5\text{H}_2\text{O}$: C, 62.93; H, 7.59; N, 9.17. MALDI-TOF MS m/z : 1218.9 $[\text{M} + \text{H}]^+$, 1240.9 $[\text{M} + \text{Na}]^+$, where M shows $\text{C}_{128}\text{H}_{174}\text{N}_{16}\text{O}_{22}\text{S}_2$.

4-5. Water-soluble cyclophane dimer having an alkyl disulfide spacer 7

Trifluoroacetic acid (2 mL) was added to a solution of **6** (138 mg, 0.06 mmol) in dry DCM (5 mL), and the mixture was stirred for 2 hours at room temperature. Evaporation of the solvent under reduced pressure and the residue was purified by gel filtration chromatography on a column of Sephadex LH-20 with methanol as an eluent. Evaporation of the product fraction under reduced pressure gave a red solid (139 mg, 97 %): ^1H NMR (400 MHz, CD_3OD , 298 K) δ 1.46 (s, 16H), 2.11 (m, 4H), 2.23 (m, 4H), 2.37 (t, 4H), 2.44 (t, 8H), 2.79 (t, 4H), 3.04 (t, 4H), 3.12 (t, 8H), 3.45 (t, 4H), 3.69 (t, 16H), 4.04 (s, 8H), 6.99 (s, 8H), 7.18 (d, 8H), 7.38 (m, 16H). ^{13}C NMR (100 MHz, CD_3OD , 298K) δ 24.1, 29.6, 30.5, 31.1, 35.6, 37.1, 38.2, 40.4, 118.0, 128.2, 130.2, 139.3, 140.0, 141.1, 141.6, 170.1, 172.1, and 173.4. IR 1637 cm^{-1} (C=O). Found: 53.25; H, 5.49; N, 8.93; Calcd for $\text{C}_{110}\text{H}_{132}\text{F}_{18}\text{N}_{16}\text{O}_{22}\text{S}_2 \cdot 2\text{H}_2\text{O}$: C, 53.43; H, 5.54; N, 9.06. MALDI-TOF MS: m/z 1752.2 $[\text{M} + \text{H}]^+$, 1774.1 $[\text{M} + \text{Na}]^+$, where M shows $\text{C}_{96}\text{H}_{126}\text{N}_{16}\text{O}_{10}\text{S}_2$.

4-6. Water-soluble cyclophane 2

Dithiothreitol (65 mg, 0.42 mmol) was added to solution of **7** (110 mg, 0.005 mmol) in methanol- H_2O (1 : 1 v/v, 5 ml), the resulting mixture was stirred for 1 day at room temperature. The solution was evaporated to dryness under reduced pressure. The crude product was purified by gel filtration chromatography on a column of Sephadex LH-20 with methanol as an eluent. Evaporation of the product fraction under reduced pressure gave a white solid. (91 mg, 84 %): ^1H NMR (400 MHz, CD_3OD , 298 K) δ 1.46 (s, 8H), 2.09 (t, 2H), 2.21 (t, 2H), 2.36 (t, 2H), 2.44 (s, 4H), 2.58 (t, 2H), 3.04 (t, 2H), 3.13 (s, 4H), 3.70 (s, 8H), 4.04 (s, 4H), 6.97 (d, 4H), 7.19 (d, 4H), and 7.39 (m, 8H). ^{13}C NMR (100 MHz, CD_3OD , 298K) δ 23.1, 24.1, 30.4, 31.1, 35.6, 40.4, 42.5, 118.3, 128.3, 130.2, 139.4, 140.1, 141.1, 141.6,

169.9, 172.1, and 173.4. IR 1638 cm^{-1} (C=O). Found: 52.57; H, 5.60; N, 8.87; Calcd for $\text{C}_{55}\text{H}_{67}\text{F}_9\text{N}_8\text{O}_{11}\text{S} \cdot 2\text{H}_2\text{O}$: C, 52.62; H, 5.70; N, 8.93. MALDI-TOF MS: m/z 877.6 $[\text{M} + \text{H}]^+$, 899.6 $[\text{M} + \text{Na}]^+$, where M shows $\text{C}_{48}\text{H}_{63}\text{N}_8\text{O}_5\text{S}$.

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