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ORIGINAL ARTICLE

Synthesis of coumarin-appended cyclophanes and evaluation of their complexation with myoglobin

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Abstract Coumarin-appended cyclophanes bearing positively or negatively charged side chains were synthesized as a water-soluble host (**1a** or **1b**, respectively). Host **1a** and **1b** showed fluorescence bands with fluorescence maxima at 404 nm originated from coumarin moiety. As a host for guest molecules by using macrocyclic cavity, cationic host **1a** binds anionic guests such as 6-*p*-toluidinonaphthalene-2-sulfonate (TNS), 6-anilinonaphthalene-2-sulfonate (2,6-ANS), and 8-anilinonaphthalene-1-sulfonate (1,8-ANS) more strongly than anionic host **1b**, reflecting intermolecular electrostatic interactions. In addition, both host **1a** and **1b** showed protein surface recognition and fluorescence response toward myoglobin, a small and globular protein. The fluorescence intensity originating from the hosts decreased upon the addition of myoglobin, reflecting the formation of **1a-** and **1b**-myoglobin complexes. On the other hand, such fluorescence response of **1a** and **1b** was almost negligible for other proteins such as egg white albumin, bovine serum albumin, human albumin, concanavaline A, fibrinogen, γ-globlin, peanut agglutinin, trypsin, and lysozyme.

Keywords Cyclophane · Host–guest chemistry · Coumarin · Fluorescence sensing

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Introduction

Macrocyclic cyclophanes bearing polar side chains furnish hydrophobic cavities in aqueous media [1–3]. The cyclophanes act as a water-soluble host for incorporation of organic guest molecules [4]. Guest recognition can be exercised by the water-soluble cyclophanes, because size and shape of the cavities are easily designed for binding of target guest molecules [5, 6]. More sophisticated capabilities such as specific binding to proteins can be achieved by macrocyclic hosts having functional groups such as peptides [7–10], saccharides [11–13], and charged moieties [14–17]. We have previously reported that water-soluble cyclophane having four anionic resorcinarene moieties tightly bound to histone, a basic protein in eukaryotic chromosomes, as confirmed by surface plasmon resonance (SPR) measurements [14]. In addition, host analogue bearing three anionic resorcinarene moieties and a dansyl moiety as a fluorophore was also developed for fluorescence sensing of histone [15]. The dansyl-appended anionic cyclophane was able to perform discrimination and fluorescence sensing toward histone surfaces, as confirmed by fluorescence spectroscopy [15]. Besides the dansyl derivatives, many other fluorophores such as fluoresceins [18], rhodamines [19], pyrene derivatives [20, 21], and coumarin derivatives [22, 23] have been used to investigate interactions of biomolecular complexes and assemblies. Among them, coumarin and its derivatives take advantages in a viewpoint of their high quantum yield, thermal stability, and less toxicity [24]. In particular, a certain coumarin derivative was known to exhibit binding affinity toward myoglobin [25]. In the series of our research on fluorophore-appended cyclophanes, we began to develop coumarin-appended cyclophanes with the aim of sensing for specific proteins. We designed and synthesized

coumarin-appended hosts by introducing the 7-methoxycoumarin moiety and three polar side chains into the cyclophane skeleton. In this context, we report the preparation of coumarin-appended cyclophanes bearing cationic and anionic polar side chains **1a** and **1b**, respectively (Fig. 1), and their protein surface recognition in aqueous medium by fluorescence spectroscopy, with an emphasis on the selectivity.

Results and discussion

Design and synthesis of coumarin-appended cyclophanes

We have now designed new cationic and anionic fluorescent cyclophanes (**1a** and **1b**, respectively), which are composed of a tetraaza[6.1.6.1]paracyclophane skeleton, three polar side chains, and a coumarin moiety as a fluorophore. Actually, we used a strategy to prepare coumarin-appended cyclophanes by introducing a coumarin moiety into tetraaza[6.1.6.1]paracyclophane [26] through a 3-aminopropanoic acid (β-alanine) spacer. Coumarin-appended cyclophanes bearing cationic and anionic polar side chains **1a** and **1b**, respectively, were synthesized as shown in Scheme 1. In the preceding paper, we have prepared a tetraaza[6.1.6.1]paracyclophane derivative having Fmoc- and Boc- protected β-alanine residues **2** as a key intermediate [27]. A precursor (**3**) of **1a** was synthesized by aminolysis of succinimidyl ester derivative of coumarin [28] with a monoamine of cyclophane, which was easily obtained from **2** by a treatment with piperidine to remove the Fmoc protecting group. Cationic cyclophane bearing a coumarin moiety **1a** was derived from **3** by a reaction with trifluoroacetic acid (TFA). Then, anionic cyclophane having carboxylic acid residues **1b** was obtained from **1a** by a reaction with succinic anhydride. All the new compounds were purified by gel permeation chromatography and identified by ^{1}H and ^{13}C NMR and MS spectroscopy as well as by elemental analyses. Although compounds **1a** and **1b** contain a hydrophobic core structure, both compounds were soluble in aqueous neutral media owing to three polar side chains. From a practical point of view, cyclophanes **1a** and **1b** were water-soluble and had good solubility of 0.36 and 0.16 g/mL, respectively.

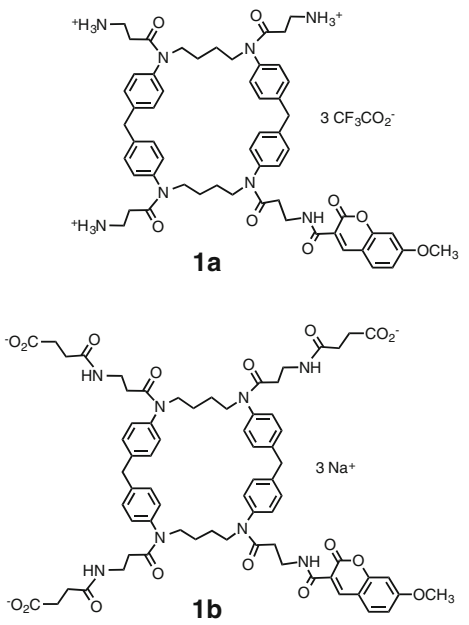


Fig. 1 Cationic and anionic cyclophanes having a coumarin moiety (**1a** and **1b**)

Guest-binding behavior of coumarin-appended cyclophanes

Coumarin-appended water-soluble cyclophanes **1a** and **1b** showed fluorescence spectra originated from a coumarin moiety in aqueous media with a fluorescence maximum of 404 nm. Compounds **1a** and **1b** have subtle amphiphilic characteristics due to a hydrophobic cyclophane and charged side-chains. First, the concentration dependency of the fluorescence spectra was investigated for an aqueous 2-[4-(2-hydroxy-ethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) buffer (0.01 M, pH 7.4, with 0.15 M NaCl) of **1a** and **1b** at various concentrations at 298 K. At least in the concentrations below 8.0×10^{-6} M, a linear increase in fluorescence intensity originating from the coumarin groups were observed for **1a** and **1b** with increasing the concentrations without any changes in its maximum (see the Electronic supplementary material). These results indicate that **1a** and **1b** are in a monomeric state under these conditions.

In order to evaluate guest-binding ability of **1a** and **1b** as a host, we adopted environmentally-responsive fluorescent probes such as 6-*p*-toluidinonaphthalene-2-sulfonate (TNS), 6-anilinonaphthalene-2-sulfonate (2,6-ANS), and 8-anilinonaphthalene-1-sulfonate (1,8-ANS) as a guest, because their fluorescence intensity is extremely sensitive to the surrounding microenvironmental polarity [29]. First, the guest-binding behavior of cyclophane **1a** and **1b** toward TNS, 2,6-ANS, and 1,8-ANS was examined by fluorescence spectroscopy at 298 K. Upon addition of TNS to an aqueous HEPES buffer containing **1a**, a fluorescence intensity originated from coumarin moiety of **1a** at 404 nm decreased accompanying a slightly increase of the fluorescence intensity of entrapped TNS molecules at around 530 nm, as shown in Fig. 2, indicating the formation of host–guest complexes. Such fluorescence quenching of **1a**

Scheme 1 Preparation of coumarin-appended cyclophanes **1a** and **1b**

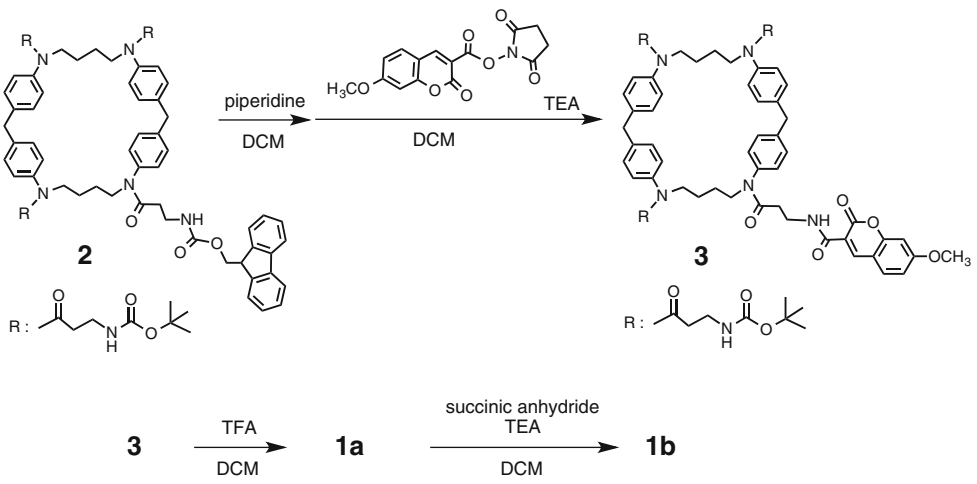
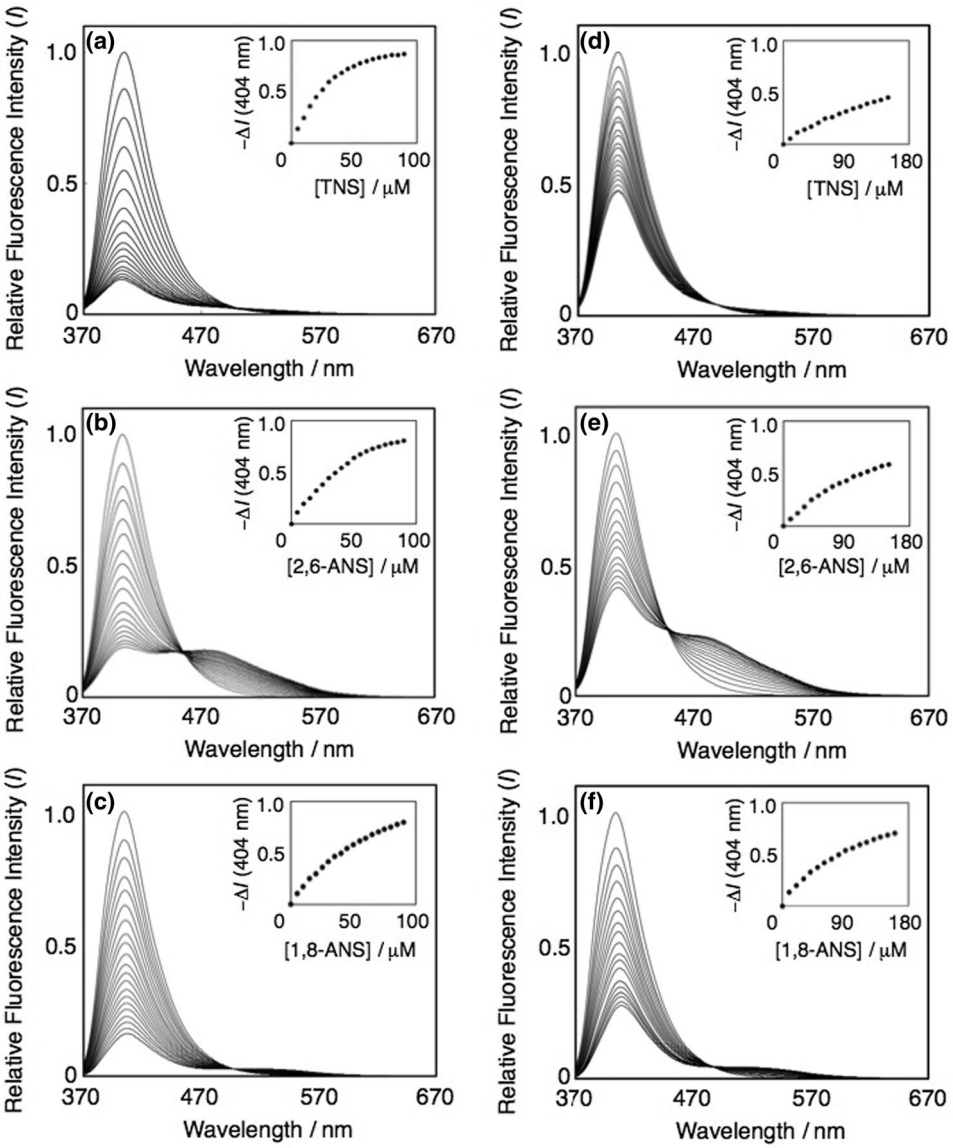


Fig. 2 Fluorescence spectral changes for aqueous solutions of **1a** and **1b** upon addition of TNS, 2,6-ANS, and 1,8-ANS: **1a** with TNS (**a**), **1a** with 2,6-ANS (**b**), **1a** with 1,8-ANS (**c**), **1b** with TNS (**d**), **1b** with 2,6-ANS (**e**), and **1b** with 1,8-ANS (**f**) in HEPES buffer at 298 K; [**1a**] = [**1b**] = 1.0 μM, [guest] (for **1a**) = 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, and 90 μM (from *top* to *bottom* at 404 nm), [guest] (for **1b**) = 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, and 160 μM (from *top* to *bottom* at 404 nm). Ex. 353 nm. *Inset* the corresponding titration curves



at 404 nm seems to be caused by the interactions between coumarin group of **1a** and entrapped 2,6-ANS molecules. A similar trend of guest-binding behavior was also observed for complexation of **1a** and **1b** with the other guests (Fig. 2). The stoichiometry for the complexes were 1:1 host:guest as revealed by Job's plots (see ESM). Binding constants (K) for the 1:1 host–guest complexes were evaluated by means of Benesi–Hildebrand plots [30] on the basis of spectroscopic data obtained at various concentrations of the guests. The K values of cationic host **1a** toward TNS, 2,6-ANS, and 1,8-ANS were calculated to be 4.0×10^4, 2.0×10^4, and 1.4×10^4 M^{-1}, respectively. On the other hand, anionic host **1b** moderately bound TNS, 2,6-ANS, and 1,8-ANS with the K values of 4.3×10^3, 5.9×10^3, and 8.1×10^3 M^{-1}, respectively, indicating that intermolecular electrostatic interactions play important roles in the formation of complexes. In addition, the enhancement in the binding constants for TNS from **1a** to **1b** ($K_{\mathbf{1a}\bullet\text{TNS}}/K_{\mathbf{1b}\bullet\text{TNS}} = 9.3$) is much larger than those for 2,6-ANS and 1,8-ANS ($K_{\mathbf{1a}\bullet\text{2,6-ANS}}/K_{\mathbf{1b}\bullet\text{2,6-ANS}} = 3.4$ and $K_{\mathbf{1a}\bullet\text{1,8-ANS}}/K_{\mathbf{1b}\bullet\text{1,8-ANS}} = 1.7$, respectively). These differences in the enhancement seem to be caused by geometrical arrangements of host and guest molecules in complexes. The electrostatic interactions between cationic side-chains of **1a** and sulfonate moiety of TNS are most effective among those of host–guest complexes.

Recognition and fluorescence sensing of myoglobin by coumarin-appended cyclophanes

Coumarin and its derivatives have been frequently used as a fluorescent probe for sensing and labeling biological molecules in the field of biology, analytical science, and medicine. As mentioned above, some species of coumarin derivative interact with myoglobin, according to the literature reported previously [25]. On these grounds, we investigated myoglobin recognition abilities of **1a** and **1b** by fluorescence spectroscopy. Upon the addition of myoglobin to each aqueous HEPES buffer solutions containing **1a** and **1b**, the fluorescence intensity originating from the coumarin moiety of **1a** and **1b** decreased with simple saturation behavior while the fluorescence maximum were splitting into two peaks (from 404 to 394 and 426 nm), as shown in Fig. 3. Such fluorescence quenching of **1a** and **1b** seems to be caused by the energy transfer in the complexes upon the addition of the heme protein, in a manner similar to those for TNS, 2,6-ANS, and 1,8-ANS. These results showed that **1a** and **1b** interacted with myoglobin. The 1:1 binding constant (K) of **1a** toward myoglobin was evaluated based on the Benesi-Hildebrand method applied to the fluorescence titration data (K; 1.5×10^5 M^{-1}). Electrostatic interactions between the hosts and myoglobin were not effectively performed, because the K value of anionic host **1b** with myoglobin was almost the same with that of cationic host **1a** with myoglobin: K, 1.5×10^5 M^{-1} for **1b** with myoglobin. According to the literature [31], isoelectric point of myoglobin is 7.2. Under the experimental

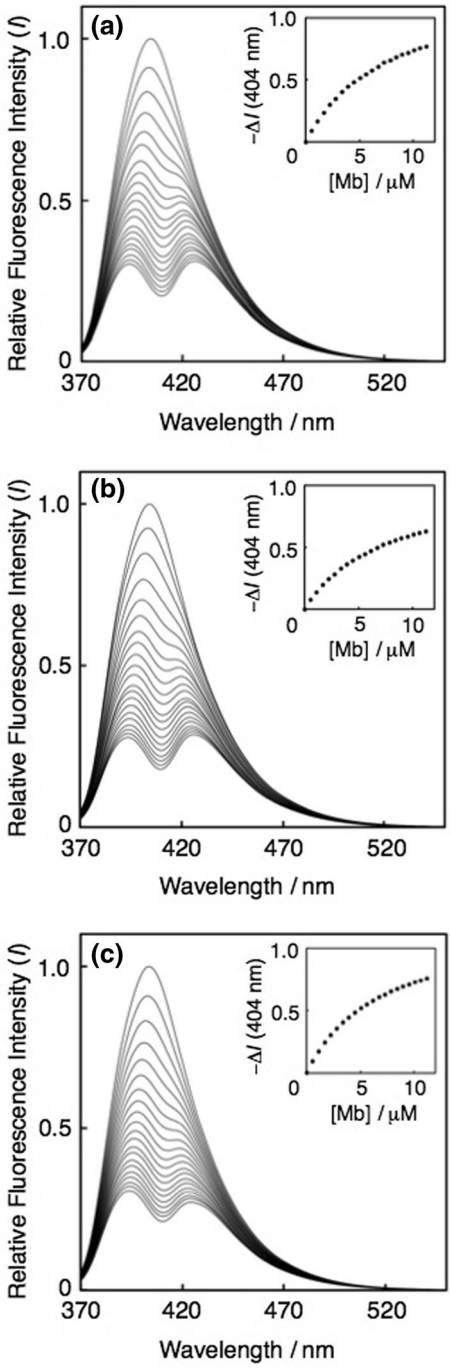


Fig. 3 Fluorescence spectral changes for aqueous solutions of hosts upon addition of myoglobin (Mb): **1a** (**a**), **1b** (**b**), and **4** (**c**) in HEPES buffer at 298 K; [**1a**] = [**1b**] = [4] = 0.5 μM, [myoglobin] = 0, 0.6, 1.1, 1.7, 2.2, 2.8, 3.4, 3.9, 4.5, 5.0, 5.6, 6.2, 6.7, 7.3, 7.8, 8.4, 9.0, 9.5, 10.1, 10.6, and 11.2 μM (from *top* to *bottom* at 404 nm). *Inset* the corresponding titration curves

condition of pH 7.4, myoglobin maintains almost balance of positive and negative charges on its surface. Therefore, there seems to be no significant difference on the electrostatic interactions with myoglobin between **1a** and **1b**. The binding constants of the hosts with myoglobin happen to be the same value. In addition, as a control experiment, a similar spectral change in fluorescence spectroscopy was obtained for 7-methoxycoumarin derivative **4** (Fig. 4) as shown in Fig. 3: K, 1.5×10^5 M^{-1}. These results indicate that the binding affinity of **1a** and **1b** was retained, even when coumarin moiety was covalently bound to the cyclophane. As regards the protein surface selectivity by the hosts, the fluorescence spectral changes of **1a** were almost negligible upon the addition of other proteins such as egg white albumin, bovine serum albumin, human albumin, concanavalin A, fibrinogen, γ-globulin, peanut agglutinin, trypsin, and lysozyme to an aqueous HEPES buffer containing **1a**, as shown in Fig. 5. A similar binding trend was also observed for **1b**. Therefore, these results indicated that **1a** and **1b** are the potent hosts showing fluorescence response to myoglobin (See ESM).

Conclusions

In the paper, water-soluble and coumarin-appended cyclophanes **1a** and **1b** were designed and synthesized by introduction of a coumarin moiety into an appropriate site of the cyclophane skeleton through a spacer. Both cyclophanes showed fluorescence spectra originated from a coumarin moiety in aqueous media. Cationic host **1a** bound anionic guests such as TNS, 2,6-ANS, and 1,8-ANS with the K values of 4.3×10^3, 5.9×10^3, and 8.1×10^3 M^{-1}, respectively. These binding affinities were much larger than those of anionic host **1b,** reflecting the intermolecular electrostatic interactions. In addition, a response in the emission spectra was observed for the aqueous solutions of **1a** and **1b** upon complexation with myoglobin. The binding affinity of **1a** and **1b** toward myoglobin was evaluated to be 1.5×10^5 M^{-1}.

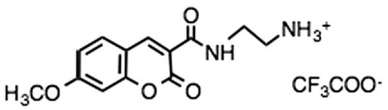


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Fig. 4 7-Methoxycoumarin derivative **4**

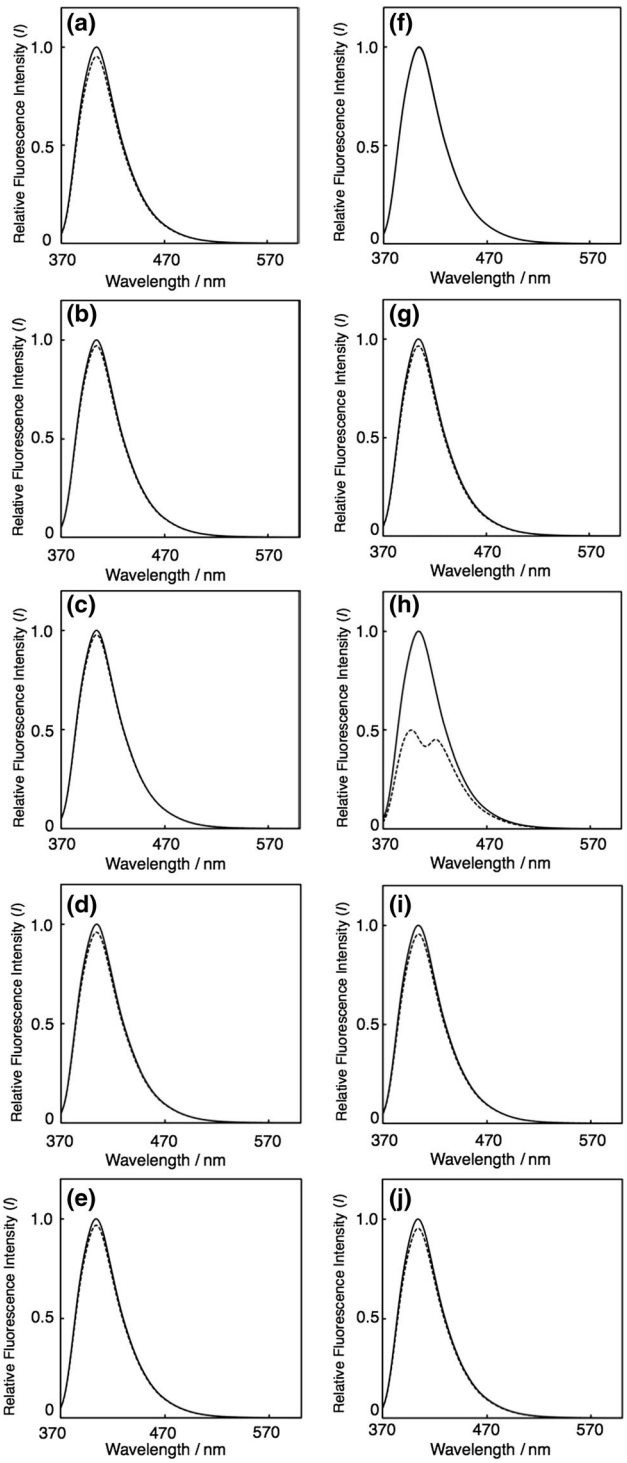

Fig. 5 Fluorescence spectral changes for aqueous solutions of **1a** (1.0 μM) upon addition of proteins in HEPES buffer at 298 K; without any proteins (*solid lines*) and in the presence of proteins (50 μg/ml) (*broken lines*). Egg white albumin (**a**), bovine serum albumin (**b**), human albumin (**c**), concanavaline A (**d**), fibrinogen (**e**), γ-globlin (**f**), peanut agglutinin (**g**), myoglobin (**h**), trypsin (**i**), and lysozyme (**j**)

Experimental section

Precursor of 1a (3)

Piperidine (1.0 mL) was added to a solution of tetra-aza[6.1.6.1]-paracyclophane derivative having Boc- and Fmoc- protected β-alanine residues (**2**) (300 mg, 0.23 mmol) in dry dichloromethane (DCM, 1 mL), and the mixture was stirred for 12 h at room temperature. Evaporation of the solvent under reduced pressure gave a pale yellow solid. The monoamine of cyclophane was purified by gel filtration chromatography on a column (Sephadex LH-20) with methanol (MeOH) as an eluant. The precursor fraction was evaporated to dryness under reduced pressure to give a pale yellow solid (cyclophane monoamine, 293 mg). Triethylamine (0.1 mL) was added to a solution of the monoamine of cyclophane (119 mg, 0.11 mmol) in dry DCM (5 mL), and the mixture was stirred at room temperature. The mixture was added to a solution of *N*-succinimidyl-7-methoxycoumarin-3-carboxylate (38 mg, 0.12 mmol) in dry DCM (1 mL), and the resulting mixture was stirred for 12 h at room temperature. After being dried (Na_2SO_4), the solution was evaporated to dryness under reduced pressure to give a pale yellow solid. The crude product was then purified by gel filtration chromatography on a column of Sephadex LH-20 with MeOH as an eluant. Evaporation of the product fraction under reduced pressure gave a pale yellow solid (133 mg, 94 %): mp 138–140 °C. ^{1}H NMR (400 MHz, $CDCl_3$, 298 K) δ 1.44 (s, 35H), 2.10 (m, 6H), 2.26 (m, 2H), 3.28 (m, 6H), 3.64 (m, 10H), 3.94–3.96 (m, 7H), 5.34 (m, 3H), 6.89 (m, 1H), 6.96 (m, 9H), 7.20 (m, 8H), 7.57 (m, 1H), 8.79 (m, 1H), and 9.03 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K) δ 24.9, 28.4, 34.4, 34.8, 35.8, 36.4, 41.0, 48.6, 56.0, 79.0, 100, 112, 114, 115, 128, 130, 131, 140, 148, 156, 157, 161, 165, and 171. IR 1646, 1705 cm^{-1} (C=O). Found: C, 66.16; H, 6.94; N, 8.55. Calcd for $C_{72}H_{90}N_8O_{14}\cdot H_2O$: C, 66.04; H, 7.08; N, 8.56. MALDI-TOF MS (positive mode): m/z 1314 $[M + Na]^+$, where M denotes $C_{72}H_{90}N_8O_{14}$.

Cationic cyclophane bearing a coumarin moiety (1a)

Trifluoroacetic acid (1.0 mL) was added to a solution of **3** (72 mg, 0.055 mmol) in dry DCM (3 mL), and the mixture was stirred for 12 h at room temperature. Evaporation of the solvent under reduced pressure gave a pale yellow solid. The crude product was purified by gel filtration chromatography on a column of Sephadex LH-20 with methanol as an eluant. Evaporation of the product fraction under reduced pressure gave a pale yellow solid (71 mg, 96 %): mp 149–151 °C (decomp.). ^{1}H NMR (400 MHz, CD_3OD, 298 K) δ 1.46 (m, 8H), 2.13–2.44 (m, 8H), 3.02–2 3.11 (m, 6H), 3.51 (m, 2H), 3.71 (m, 8H), 3.97–4.-3(m, 7H), 6.89–7.19 (m, 10H), 7.26–7.38 (m, 8H), 7.75 (d, 1H), and 8.57 (s, 1H). ^{13}C NMR (100 MHz, CD_3OD, 298 K) δ 23.7, 31.1, 33.4, 35.6, 40.3, 46.5, 55.4, 100, 112, 114, 115, 18, 128, 130, 131, 139, 141, 148, 157, 161, 163, 166, 170, and 171. IR 1645 cm^{-1} (C=O). Found: C, 54.54; H, 5.68; N, 7.82. Calcd for $C_{63}H_{69}F_9N_8O_{14}\cdot 3H_2O$: C, 54.54; H, 5.45; N, 8.08. MALDI-TOF MS (positive mode): m/z 1014 $[M + Na]^+$, where M denotes triamine derivative of cyclophane as a free base (M, $C_{57}H_{66}N_8O_8$).

Anionic cyclophane bearing a coumarin moiety (1b)

Succinic anhydride (41 mg, 0.41 mmol) was added to a solution of cyclophane **1a** (61 mg, 0.05 mmol) and triethylamine (0.5 mL) in dry DCM (4 mL) at room temperature, and the mixture was stirred for 12 h. Ethylenediamine (0.1 mL, 1.5 mmol) was added to the mixture to quench the reaction. After being dried (Na_2SO_4), the solution was evaporated to dryness under reduced pressure to give a pale yellow solid. The crude product was purified by gel filtration chromatography on a column of Sephadex LH-20 with methanol as an eluant. Evaporation of the product fraction under reduced pressure gave a pale yellow solid (54 mg, 73 %): Then added 0.1 M NaOH aq. (2 ml) and stirred 20 min at room temperature. After purification by Sephadex LH-20 chromatography with methanol as an eluant, solvent of the product fraction was evaporated off under reduced pressure to give a pale yellow solid (54 mg, 95 %): mp 136–138 °C (decomp.). ^{1}H NMR (400 MHz, CD_3OD, 293 K) δ 1.42 (m, 8H) 2.05 (m, 2H), 2.19 (m, 6H), 2.41–2.46 (m, 12H), 3.27 (m, 6H), 3.53 (m, 2H), 3.71 (m, 8H), 3.96–4.03 (m, 7H), 6.91–7.07 (m, 10H), 7.26 (m, 8H), 7.76 (d, 1H), and 8.78 (m, 1H). ^{13}C NMR (100 MHz, CD_3OD, 293 K) δ 7.85, 23.9, 31.4, 33.8, 35.4, 40.5, 46.4, 52.5, 55.4, 99.9, 112, 114, 128, 130, 131, 140–141, 148, 156, 161, 163, 165, 171, and 173. IR 1635 cm^{-1} (C=O). Found: C, 63.27; H, 6.37; N, 8.54. Calcd for $C_{69}H_{78}N_8O_{17}\cdot H_2O$: C, 63.29; H, 6.16; N, 8.56. MALDI-TOF MS (positive mode): m/z 1314 $[M + Na]^+$, where M denotes carboxylic acid of cyclophane (M, $C_{69}H_{78}N_8O_{17}$).

Binding constants of cyclophane with fluorescence guests

To each solution of host (1.0 μM) in HEPES buffer were added increasing amounts of the guests such as TNS, 2,6-ANS, and 1,8-ANS, and the fluorescence intensity was monitored after each addition of the guests by excitation at 353 nm. The binding constants were calculated on the basis of the Benesi–Hildebrand method for titration data.

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