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Biotinylated Cyclophane: Synthesis, Cyclophane-Avidin Conjugates, and Their Enhanced Guest-Binding Affinity

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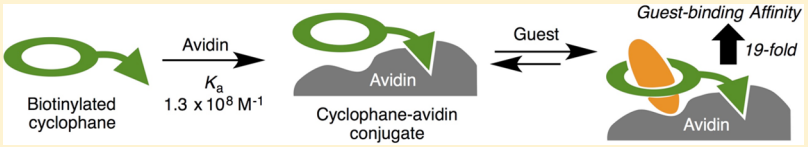
Supporting Information



ABSTRACT: Cationic and anionic cyclophanes bearing a biotin moiety were synthesized as a water-soluble host (**1a** and **1b**, respectively). Both hosts **1a** and **1b** were found to strongly bind avidin with binding constants of 1.3×10^8 M^{-1}, as confirmed by surface plasmon resonance measurements. The present conjugate of **1a** with avidin (**1a**-avidin) showed an enhanced guest binding affinity toward fluorescence guests such as TNS and 2,6-ANS. The *K* values of **1a**-avidin conjugate with TNS and 2,6-ANS were ~19-fold larger than those of monocyclic cyclophane **1a** with the identical guests. Favorable hydrophobic and electrostatic interactions between **1a**-avidin and TNS were suggested by computer-aided molecular modeling calculations. Moreover, addition of excess biotin to the complexes of **1a**-avidin with the guests resulted in dissociation of **1a**-avidin to avidin and **1a** having less guest-binding affinity. Conversely, such enhancements in the guest-binding affinity were not obviously observed for the conjugate of anionic **1b** with avidin (**1b**-avidin) due to electrostatic repulsion between anionic **1b** and anionic guests.

INTRODUCTION

Macrocyclic hosts, such as cyclophanes and related compounds, exhibit potent molecular recognition capabilities toward molecular guests.[1−3] In particular, water-soluble hosts based on tetraaza-[6.1.6.1]paracyclophane provide a hydrophobic cavity suitable for guest-binding in aqueous media.[4] The major driving force for the guest binding is considered to be hydrophobic interaction, whereas other additional noncovalent effects, such as electrostatic and hydrogen bonding interactions, become effective recognition factors.[5] On these grounds, various functionalized cyclophanes have been synthesized by introducing side-chains into the macrocyclic skeleton.[6] For instance, water-soluble cyclophanes bearing cationic or anionic charged side-chains recognize and bind hydrophobic guests in aqueous media through hydrophobic and electrostatic interactions.[7] Considering the importance of multivalency on the interactions,[8] we also developed functionalized cyclophanes with multiple carboxylate groups, which exhibited binding capability toward histones, basic nuclear proteins in eukaryotic chromatins, in aqueous media.[9] In addition, saccharide cyclophanes having carbohydrate residues have been synthesized as functionalized hosts.[10] These saccharide cyclophanes having carbohydrate residues not only incorporate guest molecules but also bind a carbohydrate-binding protein (lectin) through lectin-ligand interactions.[11] In our ongoing research on functionalized cyclophanes, we began to develop cyclophane−protein conjugates as functionalized hosts for binding molecular guests in aqueous media.

Avidin, a basic glycoprotein found in egg white, is known to show strong and specific binding affinity for biotin with dissociation constants in the range of 10^{-15} M, according to literature reported previously.[12] The high affinity and specificity of avidin−biotin interactions have been exploited for diverse applications in immunology,[13] affinity chromatography,[14] and many other areas.[15] On these grounds, we decided to develop conjugates composed of biotin-functionalized cyclophanes and avidin to create a new-class of cyclophane-avidin conjugates as a semiartificial host. We designed biotin-appended cyclophanes by introducing a biotin moiety and three cationic or anionic charged side-chains into the macrocyclic skeletons **1a** and **1b**, respectively (Figure 1). In this context, we report the synthesis of **1a** and **1b** and the formation of cyclophane-avidin conjugates. Moreover, the guest binding behavior of the present cyclophane-avidin conjugates was also examined by fluorescent spectroscopy with emphasis on enhancements of the guest-binding affinity.

RESULTS AND DISCUSSION

Synthesis of Biotinylated Water-Soluble Cyclophanes.

We designed water-soluble and biotinylated cyclophane (**1a**), which is composed of a tetraaza[6.1.6.1]paracyclophane skeleton, three cationic side chains, and a biotin moiety as a ligand to avidin. Actually, we adopted a synthetic strategy to prepare biotinylated cyclophane **1a** by connecting a biotin moiety into the macrocyclic skeleton through a hydrophilic spacer having ethylene glycol. An analogous biotinylated cyclophane having three anionic side chains (**1b**) was also

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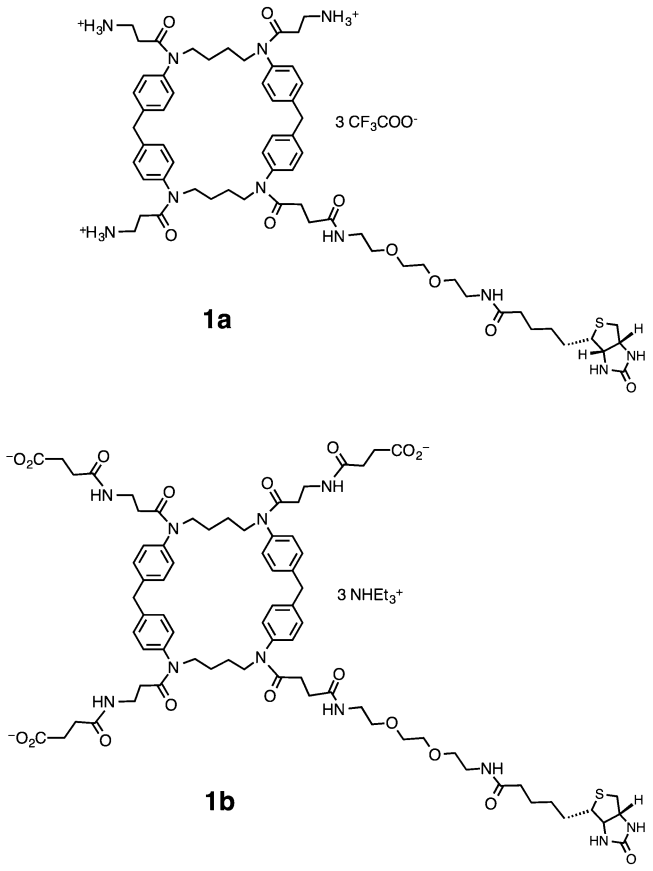


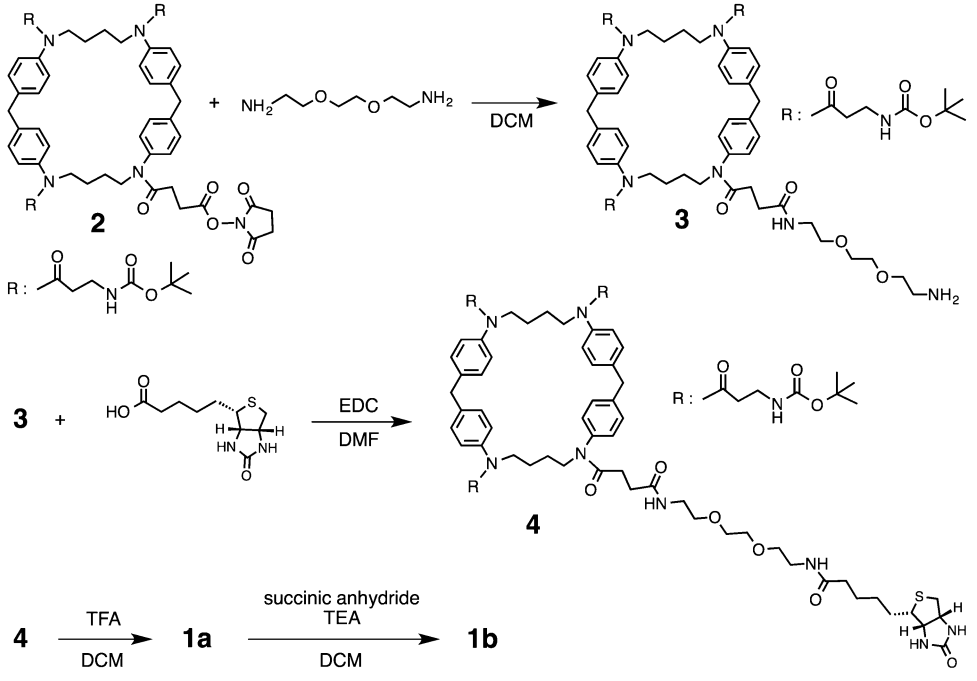
Figure 1. Cationic and anionic cyclophanes bearing a biotin moiety (**1a** and **1b**).

designed to investigate electrostatic effects on the host−guest molecular recognition. Biotinylated cyclophanes bearing cationic and anionic polar side chains, **1a** and **1b**, respectively, were synthesized as shown in Scheme 1. In the previous paper, we reported the synthesis of a succinimidyl ester derivative of tetraaza-[6.1.6.1]paracyclophane having Boc-protected *β*-alanine residues (**2**).[16] A monoamine derivative of cyclophane **3** was synthesized by aminolysis of **2** with 1,2-bis(2-aminoethoxy)ethane. A precursor (**4**) of **1a** was synthesized by condensation of **3** with a biotin in the presence of 1-ethyl-3(3-(dimethylamino)propyl)carbodiimide (EDC). Cationic cyclophane bearing a biotin moiety **1a** was derived from **4** by a reaction with trifluoroacetic acid (TFA). Then, anionic cyclophane having three carboxylate groups (**1b**) was obtained from **1a** by a reaction with succinic anhydride. All of the new compounds were purified by size exclusion chromatography and identified by ^{1}H and ^{13}C NMR and MALDI-TOF MS spectroscopy as well as by elemental analysis.

Computer-aided molecular CPK modeling studies of **1a** and **1b** show that these cyclophanes provide relatively large internal cavities for guest inclusion (see Supporting Information). In addition, terminal hydrophilic side chains on the periphery play an important role for the water-solubility of **1a** and **1b**. Actually, compounds **1a** and **1b** exhibit good solubility in ultrapure water (0.20 and 0.03 g/mL, respectively).

Conjugation of Biotinylated Water-Soluble Cyclophanes with Avidin. Each of the water-soluble cyclophanes **1a** and **1b** has a biotin ligand to avidin. Interactions of **1a** and **1b** with avidin were conveniently investigated by surface plasmon resonance (SPR) measurements. First, avidin protein was covalently immobilized on an SPR sensor chip with a carboxymethyl dextran-coated surface through an amine-carboxyl coupling method using *N*-hydroxysuccinimide (NHS)-EDC reagents in a manner similar to that reported previously (see Supporting Information).[17] Then, when an aqueous HEPES solution of **1a** was injected over the surface-immobilized avidin, strong association between **1a** and avidin was observed in an SPR sensorgram, as shown in Figure 2. After finishing the injection of **1a**, very slow dissociation of **1a** from

Scheme 1. Preparation of Cationic and Anionic Cyclophanes (1a and 1b, Respectively) Bearing a Biotin Moiety



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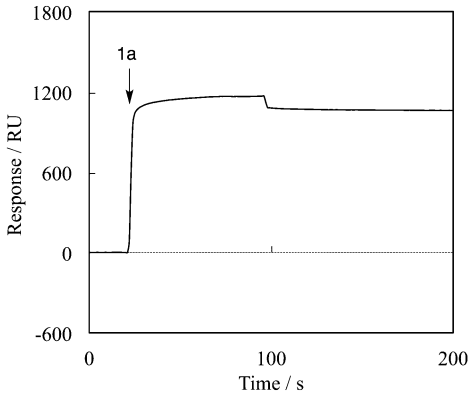

Figure 2. SPR sensorgram with an injection of **1a** onto surface-immobilized avidin. Flow rate of 40 μL min^{-1} in HEPES buffer (0.01 M, pH 7.4, 0.15 with NaCl). Arrow represents the injection of **1a**.

avidin was observed (Figure 2). The association and dissociation rate constants (k_a and k_d) were evaluated to be 1.6×10^4 M^{-1} s^{-1} and 1.2×10^{-4} s^{-1}, respectively, on the basis of kinetic analysis by using curve-fitting methods applied to the SPR sensorgram. Accordingly, association constant (K_a) between **1a** with surface-immobilized avidin was calculated to be 1.3×10^8 M^{-1}. In addition, a similar SPR sensorgram was observed when anionic cyclophane **1b** was applied onto the avidin. Obtained kinetic and binding parameters of **1b** with the avidin were almost equal to those of **1a** (k_a, 1.7×10^4 M^{-1} s^{-1}; k_d, 1.3×10^{-4} s^{-1}; and K_a, 1.3×10^8 M^{-1}; see Supporting Information).

According to the literature reported by Green,[12a] the K_a value between biotin and avidin was determined to be 10^{15} M^{-1} by using ^{14}C isotope-labeled biotin in an aqueous solution. This K_a value is larger than those of **1a** and **1b**. As mentioned above, we evaluated the K_a values of biotinylated cyclophanes **1a** and **1b** with surface-immobilized avidin by SPR measurements. Accordingly, some factors, such as the differences in analytical methods and the steric hindrance of the cyclophane moieties, are considered to affect the binding affinity of the biotinylated cyclophanes with avidin. Nevertheless, these results showed that the avidin-binding capabilities of **1a** and **1b** were preserved to some extent, even though a biotin moiety was covalently connected to the cyclophane skeleton. Considering the K_a values on an order of 10^8 M^{-1}, it is strong enough to maintain the formation of cyclophane-avidin conjugates under the fluorescence titration experimental conditions.

Enhanced Guest-Binding Affinity by Cyclophane-Avidin Conjugates. We have previously reported that monocyclic cyclophanes showed relatively weak guest-binding affinity toward fluorescence guests such as TNS and 2,6-ANS (Figure 3),[18] whose emission is in response to microenvironments exerted by the surrounding medium in both intensity and wavelength.[16] Accordingly, the first thing we examined was the guest-binding behavior of **1a** and **1b** with employing TNS and 2,6-ANS as a guest. Upon the addition of **1a** to an aqueous HEPES buffer containing TNS, the fluorescence intensity originating from TNS slightly increased, indicating a weak formation of host–guest complexes, as shown in Figure 4a.

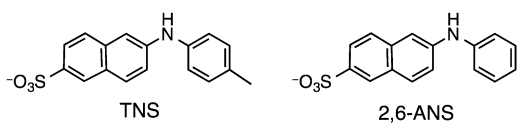

Figure 3. TNS and 2,6-ANS as hydrophobic fluorescent guests.

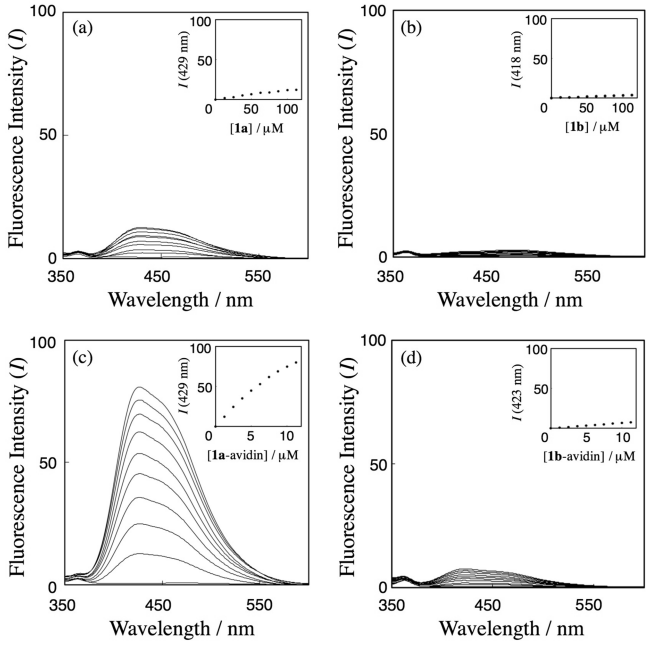

Figure 4. Fluorescence titration and spectral changes showing the enhanced guest-binding affinity of **1a**-avidin. Fluorescence spectra of TNS (0.5 μM) by adding incremental amounts of **1a** (a), **1b** (b), **1a**-avidin (c), and **1b**-avidin (d) in HEPES buffer at 298 K. [**1a**] = [**1b**] = 0, 12.5, 25,0, 37.5, 50.0, 62.5, 75.0, 87.5, 100.0, and 112.5 μM. [**1a**-avidin] = [**1b**-avidin] = 0, 1.25, 2.50, 3.75, 5.0, 6.25, 7.50, 8.75, 10.0, and 11.25 μM. Each inset shows the corresponding titration curve. Ex. = 326 nm.

Regarding the stoichiometry for the complexes formed with **1a** and TNS, Job's continuous variation plot revealed that **1a** bound TNS in a 1:1 molar ratio (see Supporting Information). Binding constant (K) for 1:1 host:guest complex formation was calculated by the Benesi–Hildebrand method[19] in a manner described previously.[18] The results reveal that **1a** moderately binds TNS with a K value of 3.2×10^3 M^{-1}. Similar fluorescence spectral changes were also observed when 2,6-ANS was used as a guest for titration experiments of **1a** (K, 3.2×10^3 M^{-1}, see Supporting Information). These K values of **1a** with TNS and 2,6-ANS are comparable to those by the other water-soluble monocyclic cyclophanes toward the identical guests.[16,20] Conversely, upon the addition of anionic **1b** to an aqueous HEPES buffer containing TNS or 2,6-ANS, the increases in fluorescence intensity originating from each guest were too small to be accurately determine K values owing to an electrostatic repulsion between anionic **1b** and anionic guest molecules (Figure 4b for TNS, see Supporting Information for 2,6-ANS). These results are consistent with previously reported findings.[7b,c]

Interestingly, when **1a**-avidin conjugate was added to an aqueous HEPES buffer containing TNS, the fluorescence intensity originating from TNS was significantly increased with the saturation behavior, as shown in Figure 4c. The K value of the **1a**-avidin conjugate with TNS was evaluated to be 6.1×10^4 M^{-1}, which is ~19-fold larger than that of **1a**. A similar guest-binding enhancement in complex formation of **1a** with 2,6-ANS was also observed by fluorescence titration experiments (see

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Supporting Information). That is, the K value of **1a**-avidin conjugate with 2,6-ANS was calculated to be 6.2×10^4 M^{-1}, which is ~19-fold larger than that of **1a**.

Conversely, upon the addition of avidin to an aqueous HEPES buffer of the guest, such as TNS and 2,6-ANS, meaningful changes in the fluorescence spectra were not observed (see Supporting Information). These results indicate that the binding occurs at the cyclophane site of the **1a**-avidin conjugate.

In addition, we carried out fluorescence polarization measurements (P) to prove the existence of **1a**-avidin-TNS. A relatively large P value was observed for TNS in the presence of the **1a**-avidin conjugate (0.23). This P value was larger than that of TNS molecules bound to **1a** (0.15), although the P value of TNS alone was not determined due to its weak fluorescence. These results indicate that TNS molecules with a marked motional repression bind to the **1a**-avidin conjugate, which is also reflected in the change in the fluorescence intensity.

When **1b**-avidin conjugate was added to an aqueous HEPES buffer containing TNS, the fluorescence intensity originating from TNS increased to some extent. The K value of the **1b**-avidin conjugate with TNS was calculated to be 4.1×10^3 M^{-1}, which was smaller by one order magnitude than that for the **1a**-avidin conjugate with the identical guest. A similar guest-binding ability in the complex formation of **1b**-avidin conjugate with 2,6-ANS was also observed by fluorescence titration experiments (see Supporting Information, K, 3.1×10^3 M^{-1}). These differences in K values reflect the electrostatic interactions between hosts and the protein surfaces as well as the guests.

Biotin acts as a competitive inhibitor as a result of strong but unproductive binding as a host. Upon the addition of biotin to aqueous solutions containing complexes of **1a**-avidin with TNS or 2,6-ANS, the fluorescence intensity originating from entrapped guest molecules decreased, reflecting the dissociation of **1a**-avidin conjugate into avidin and **1a** having less guest-binding affinity (Figure 5).

To study intermolecular interactions of the **1a**-avidin conjugate with TNS, we examined conformations of the complex by means of computer-aided molecular modeling techniques.[21] In an optimized conformation for the complex, the biotin moiety of **1a** gets into a concavity site of avidin while cationic side chains of **1a** interact with anionic residues (aspartic acids) of the avidin surface through electrostatic interactions, as shown in Figure 6. In addition, a hydrophobic moiety of the TNS molecule is partly incorporating into the macrocyclic skeleton of **1a** while one of the cationic side chains of **1a** interacts electrostatically with a sulfonate group of TNS. These favorable electrostatic and hydrophobic interactions between the **1a**-avidin conjugate and TNS molecule seem to be responsible for stabilization of the complexes and enhancements of the guest-binding affinity.

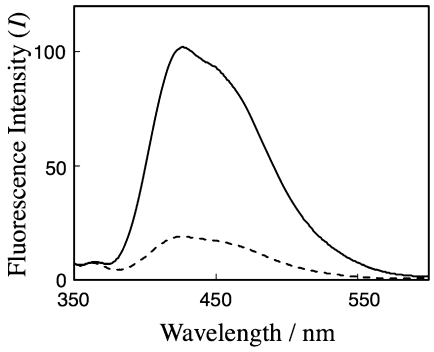

Figure 5. Fluorescence experiment showing the dissociation of the **1a**-avidin conjugate to **1a** and avidin upon the addition of biotin as a competitive inhibitor. Fluorescence spectra of TNS (0.5 μM) in the presence of **1a**-avidin (17.5 μM) in a HEPES buffer at 298 K without biotin (solid line) and in the presence of biotin (1.5 mM) (dotted line). Ex. = 326 nm.

CONCLUSIONS

We synthesized cationic and anionic cyclophanes bearing a biotin moiety as a water-soluble host (**1a** and **1b**, respectively). Strong binding of **1a** with avidin was confirmed by SPR measurements to give a supramolecular **1a**-avidin conjugate. The resulting conjugate of **1a** with avidin showed enhanced guest binding ability toward fluorescence guests, such as TNS and 2,6-ANS. The K values of **1a**-avidin conjugate with TNS and 2,6-ANS were ~19-fold larger than those by monocyclic cyclophane **1a** toward the identical guests. Such enhancements in K values came from hydrophobic and electrostatic effects by the conjugates as suggested by computer-aided molecular modeling calculations. Conjugation of cyclophanes with naturally occurring avidin is a feasible strategy to enhance their recognition abilities.

EXPERIMENTAL SECTION

Monoamine Derivative of Cyclophane 3. A solution of succinimidyl ester derivative of tetraaza-[6.1.6.1]paracyclophane having Boc-protected β-alanine residues (**2**) (381 mg, 0.31 mmol) in dry dichloromethane (DCM, 10 mL) was added dropwise to a solution of 1,2-bis(2-aminoethoxy)ethane (464 mg, 3.1 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 pale yellow oil. The oil 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 (366 mg, 94%). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ 1.44 (m, 35H), 1.60 (m, 2H), 2.00 (m, 6H), 2.30 (t, 2H), 2.45 (t, 2H), 3.01 (t, 2H), 3.29 (m, 6H), 3.45 (m, 2H), 3.64 (m, 16H), 3.97 (s 4H), 5.35 (s, 3H), 6.97 (m, 8H), 7.21 (m, 8H). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K): δ 24.9, 28.4, 29.9, 31.1, 34.8, 36.4, 39.3, 41.0, 48.8, 70.1, 71.8, 79.0, 128, 130, 141, 156, 171, 172. IR: 1645, 1704 cm^{-1} (C═O). Found: C, 64.55; H, 7.89; N, 9.92; Calcd for $C_{68}H_{97}N_9O_{13}\cdot H_2O$: C, 64.48; H, 7.88; N, 9.95. MALDI-TOF MS m/z: 1271 $[M + Na]^+$, where M shows $C_{68}H_{97}N_9O_{13}$ (monoamine derivative of cyclophane).

Precursor of Biotinylated Water-Soluble Cyclophane 4. Biotin (142 mg, 0.58 mmol) was added to a solution of **3** (361 mg, 0.29 mmol) and EDC (111 mg, 0.58 mmol) in dry *N*,*N*-dimethylformamide (DMF, 2.5 mL), and the mixture was stirred for 2 days at room temperature. The solvent was distilled on a rotatory evaporator to give a white solid. The solid 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 (397 mg, 92%). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ 1.42 (m, 37H), 1.74 (m, 4H), 2.12 (m, 6H), 2.28 (m, 4H), 2.44 (m, 2H), 2.78 (m, 1H), 2.95 (m, 1H), 3.28 (m, 7H), 3.44 (m, 4H), 3.65 (m, 16H), 3.96 (s, 4H), 4.38 (m, 1H), 4.55 (m, 1H), 5.35 (m, 3H), 6.97 (m, 8H), 7.20 (m, 8H). ^{13}C NMR (100 MHz, CD_3OD, 298 K): δ 25.4, 26.7, 28.8, 29.4, 29.7, 30.9, 31.8, 35.7, 37.7, 40.2, 40.8, 41.7, 48.8, 56.9, 61.5, 63.2, 70.5, 71.2, 79.9, 129, 131, 141, 142, 158, 166, 173, 176. IR: 1635, 1696 cm^{-1} (C═O). Found: C, 62.19; H, 7.46; N, 10.22; Calcd for $C_{78}H_{111}N_{11}O_{15}S\cdot 2H_2O$: C, 62.01; H, 7.67; N, 10.20.

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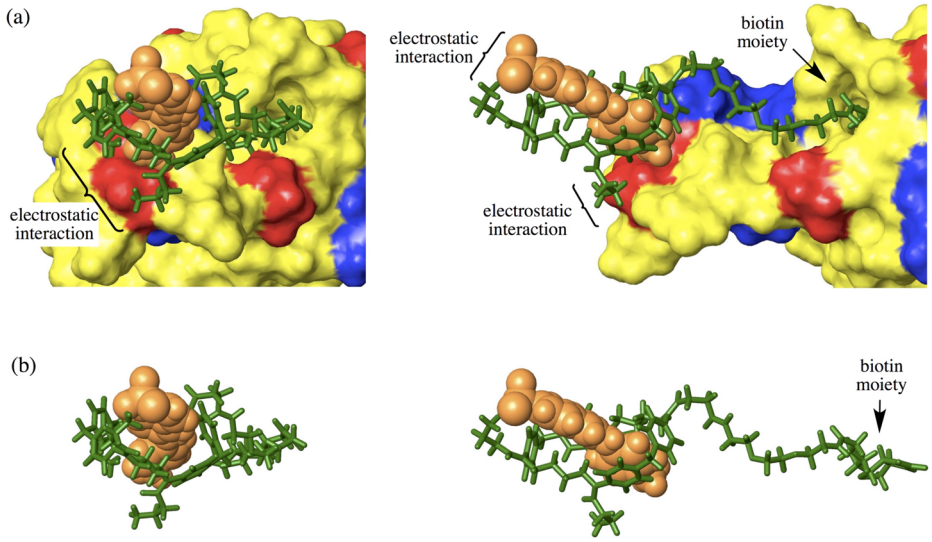

Figure 6. Front and side views of plausible computer-generated molecular models for the complex of the **1a**-avidin conjugate with TNS (a). Avidin was omitted for simplicity (b). **1a**, green stick model; TNS, orange CPK model; and avidin, surface model with acidic (red), basic (blue), and the other (yellow) amino acid residues.

MALDI-TOF MS *m*/*z*: 1497 [M + Na]$^+$, where M shows $C_{78}H_{111}N_{11}O_{15}S$.

Cationic Cyclophane Bearing a Biotin Moiety 1a. To a solution of **4** (222 mg, 0.15 mmol) in dry DCM (3 mL) was added trifluoroacetic acid (TFA, 1 mL). The mixture was stirred for 5 h at ambient temperature. After the solvent was distilled on a rotatory evaporator, methanol (10 mL) was added to the residue, and this procedure was repeated 3 times to remove remaining TFA. Evaporation of the solvent on a rotatory evaporator gave a pale yellow solid. The solid 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 (triamine derivative of cyclophane as the trifluoroacetic acid salt, 211 mg, 92%). ^{1}H NMR (400 MHz, CD_3OD, 298 K): δ 1.28 (t, 2H), 1.49 (m, 8H), 1.70 (m, 4H), 2.10 (m, 6H), 2.28 (m, 4H), 2.43 (t, 2H), 2.79 (d, 1H), 2.88 (m, 6H), 2.96 (m, 1H), 3.21 (m, 1H), 3.50 (m, 4H), 3.66 (m, 16H), 3.98 (s, 4H), 4.28 (m, 1H), 4.55 (m, 1H), 7.02 (m, 8H), 7.23 (m, 8H). ^{13}C NMR (100 MHz, CD_3OD, 298 K): δ 28, 29.4, 32.3, 34.3, 35.0, 39.6, 42.9, 43.6, 44.3, 52.3, 59.5, 64.2, 65.9, 70.5, 73.2, 73.8, 132, 134, 145, 165, 168, 174, 178. IR: 1634 cm^{-1} (C═O). Found: C, 54.49; H, 6.20; N, 10.15; Calcd for $C_{69}H_{90}F_9N_{11}O_{15}S$: C, 54.65; H, 5.98; N, 10.16. MALDI-TOF MS *m*/*z*: 1197 [M + Na]$^+$, where M shows $C_{63}H_{87}N_{11}O_9S$ (triamine derivative of cyclophane).

Anionic Cyclophane Bearing a Biotin Moiety 1b. To a solution of cyclophane **1a** (190 mg, 0.13 mmol) and triethylamine (TEA, 1 mL) in dry DCM (5 mL) was added succinic anhydride (113 mg, 1.13 mmol). The mixture was stirred for 24 h at ambient temperature. The solution was distilled off under reduced pressure to give a white solid. The solid 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 (137 mg, 62%). The resulting salt was converted into the free acid by ion-exchange chromatography on a column of Amberlite IR-120B with methanol as eluent. The solvent was distilled under reduced pressure, and the crude product was chromatographed on a column of Sephadex LH-20 with methanol as eluant. Evaporation of the main fraction on a rotatory evaporator gave a white solid. ^{1}H NMR (400 MHz, CD_3OD, 298 K): δ 1.34 (m, 10H), 1.55 (m, 4H), 2.04 (m, 10H), 2.29−2.40 (m, 14H), 2.62 (d, 1H), 2.81 (m, 1H), 3.15 (m, 1H), 3.21 (m, 10H), 3.44 (m, 4H), 3.50 (m, 12H), 3.92 (s, 4H), 4.21 (m, 1H), 4.39 (m, 1H), 6.89 (m, 8H), 7.19 (m, 8H). ^{13}C NMR (100 MHz, CD_3OD, 298 K): δ 24.1, 25.4, 28.1, 28.3, 29.5, 29.9, 30.4, 30.7, 33.7, 35.3, 38.9, 39.6, 40.3, 48.2, 55.5, 60.2, 61.9, 69.1, 69.9, 128, 130, 140, 141, 165, 171, 172, 173, 175, 176. IR: 1632, 1728 cm^{-1} (C═O). Found: C, 59.58; H, 6.81; N, 10.00; Calcd for $C_{75}H_{99}N_{11}O_{18}S{\cdot}2H_2O$: C, 59.63; H, 6.87; N,10.20. MALDI-TOF MS *m*/*z*: 1497 [M + Na]$^+$, where M shows $C_{75}H_{99}N_{11}O_{18}S$ (carboxylic acid of cyclophane).

SPR Measurements. Preparation of sensor surface: the carboxyl groups on the sensor surfaces of CM5 were activated with an injection of a solution containing 0.4 M EDC and 0.1 M *N*-hydroxy-succinimide (NHS) at a flow rate of 5 μL min^{-1}. The specific surface was obtained by injecting avidin (1.0 mg mL^{-1}) in 10 mM acetate buffer at pH 4. The immobilization procedure was completed by a 7 min injection of 1 M ethanolamine hydrochloride to block the remaining ester groups.

Interaction of **1a** and **1b** with immobilized avidin: HEPES buffer (0.01 M, pH 7.4, 0.15 with NaCl) solutions containing **1a** (10 μM) or **1b** (10 μM) were injected for 1.25 min at a flow rate of 40 μL min^{-1}.

Preparation of Cyclophane-Avidin Conjugates as a Stock Solution. Avidin was dissolved in ultrapure water at 1.0 mM (as a tetramer). By combining the avidin (25 μL), **1a** (10 mM, 10 μL), and ultrapure water (65 μL), a stock solution of **1a**-avidin conjugate was prepared. The concentration of **1a**-avidin is considered to be 1.0 mM (as a cyclophane unit) because of a high K_a value (1.3×10^8 M^{-1}). The use of **1b** in place of **1a** afforded the corresponding stock solutions (1.0 mM) of **1b**-avidin.

Fluorescence Titration Experiments. By adding incremental amounts of hosts, such as **1a**, **1b**, **1a**-avidin, and **1b**-avidin, to a HEPES buffer (0.01 M, pH 7.4, 0.15 with NaCl) containing TNS (0.5 μM) at 298 K, the fluorescence spectra were recorded after each addition with excitation at 326 nm. When these hosts were added to an aqueous HEPES buffer of 2,6-ANS, excitation at 318 nm was employed.

Computations. The initial structure of avidin is taken from the Protein Data Bank (PDB ID: 3FDC). Hydrogen atoms are added using MacroModel 7.5 software. Conversely, an optimized structure of **1a** is obtained by molecular mechanics calculations (MacroModel 7.5, OPLS 2005, water). For the initial structure of the **1a**-avidin conjugate, the biotin moiety of **1a** is set to the binding site of avidin. Optimization is done by MM calculations (OPLS 2005, water, constant dielectric) while freezing the structure of avidin.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.5b01809.

^{1}H and ^{13}C NMR spectra for **3**, **4**, **1a**, and **1b**. Computer-generated CPK models for **1a** and **1b**. Job's plots and additional fluorescence titration experiments (PDF)

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Tables of computational data and total energies (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) (a) Rebek, J., Jr. *Chem. Commun.* **2000**, 637−643. (b) Biros, S. M.; Rebek, J., Jr. *Chem. Soc. Rev.* **2007**, *36*, 93−104. (c) Berryman, O. B.; Sather, A. C.; Lledó, A.; Rebek, J., Jr. *Angew. Chem., Int. Ed.* **2011**, *50*, 9400−9403.

(2) Vögtle, F.; Seel, C.; Windscheif, P.-M. In *Comprehensive Supramolecular Chemistry*; Vögtle, F., Ed.; Pergamon: Oxford, 1996; Vol. 2, Chapter 7, pp 211−265.

(3) (a) Seward, E.; Diederich, F. *Tetrahedron Lett.* **1987**, *28*, 5111−5114. (b) Seward, E.; Hopkins, R. B.; Sauerer, W.; Tam, S.-W.; Diederich, F. *J. Am. Chem. Soc.* **1990**, *112*, 1783−1790.

(4) (a) Odashima, K.; Itai, A.; Iitaka, Y.; Arata, Y.; Koga, K. *Tetrahedron Lett.* **1980**, *21*, 4347−4350. (b) Diederich, F.; Schuermann, G.; Chao, I. *J. Org. Chem.* **1988**, *53*, 2744−2757. (c) Koga, K.; Odashima, K. *J. Inclusion Phenom. Mol. Recognit. Chem.* **1989**, *7*, 53−60. (d) Odashima, K.; Itai, A.; Iitaka, Y.; Koga, K. *J. Org. Chem.* **1985**, *50*, 4478−4484. (e) Ariga, K.; Terasaka, Y.; Sakai, D.; Tsuji, H.; Kikuchi, J. *J. Am. Chem. Soc.* **2000**, *122*, 7835−7836. (f) Ariga, K.; Nakanishi, T.; Terasaka, Y.; Tsuji, H.; Sakai, D.; Kikuchi, J. *Langmuir* **2005**, *21*, 976−981.

(5) (a) Fujita, M.; Umemoto, K.; Yoshizawa, M.; Fujita, N.; Kuzukawa, T.; Biradha, K. *Chem. Commun.* **2001**, 509−518. (b) Biros, S. M.; Rebek, J., Jr. *Chem. Soc. Rev.* **2007**, *36*, 93−104. (c) Akine, S.; Kusama, D.; Nabeshima, T. *Tetrahedron Lett.* **2013**, *54*, 205−209. (d) Akine, S.; Piao, S.; Miyashita, M.; Nabeshima, T. *Tetrahedron Lett.* **2013**, *54*, 6541−6544.

(6) (a) Hamuro, Y.; Calama, M. C.; Park, H. S.; Hamilton, A. D. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2680−2683. (b) Wei, Y.; Mclendon, G. L.; Hamilton, A. D.; Case, M. A.; Purring, C. B.; Lin, Q.; Park, H. S.; Lee, C. S.; Yu, T. *Chem. Commun.* **2001**, 1580−1581. (c) Peczuh, M. W.; Hamilton, A. D. *Chem. Rev.* **2000**, *100*, 2479−2494. (d) Hayashida, O.; Nakamura, Y. *Bull. Chem. Soc. Jpn.* **2013**, *86*, 223−229.

(7) (a) Hayashida, O.; Nakashima, T. *Chem. Lett.* **2011**, *40*, 134−135. (b) Hayashida, O.; Ichimura, K.; Sato, D.; Yasunaga, T. *J. Org. Chem.* **2013**, *78*, 5463−5469. (c) Hayashida, O.; Kaku, Y. *J. Org. Chem.* **2013**, *78*, 10437−10442.

(8) (a) Lee, Y. C.; Lee, R. T. *Acc. Chem. Res.* **1995**, *28*, 321−327. (b) Gestwicki, J. E.; Strong, L. E.; Cairo, C. W.; Boehm, F. J.; Keesling, L. L. *Chem. Biol.* **2002**, *9*, 163−169. (c) Huang, Y. F.; Liu, H.; Xiong, X.; Chen, Y.; Tan, W. *J. Am. Chem. Soc.* **2009**, *131*, 17328−17334. (d) Tomas, S.; Milanesi, L. *Nat. Chem.* **2010**, *2*, 1077−1083.

(9) (a) Hayashida, O.; Uchiyama, M. *J. Org. Chem.* **2007**, *72*, 610−616. (b) Hayashida, O.; Ogawa, N.; Uchiyama, M. *J. Am. Chem. Soc.* **2007**, *129*, 13698−13705.

(10) (a) Bukownik, R. R.; Wilcox, C. S. *J. Org. Chem.* **1988**, *53*, 463−467. (b) Coteron, J. M.; Vicent, C.; Bosso, C.; Penades, S. *J. Am. Chem. Soc.* **1993**, *115*, 10066−10076. (c) Hayashida, O.; Kato, M.; Akagi, K.; Aoyama, Y. *J. Am. Chem. Soc.* **1999**, *121*, 11597−11599. (d) Hayashida, O.; Takaoka, Y.; Hamachi, I. *Tetrahedron Lett.* **2005**, *46*, 6589−6592. (e) Murphy, P. V. *Eur. J. Org. Chem.* **2007**, *2007*, 4177−4187. (f) Hayashida, O.; Sato, D. *J. Org. Chem.* **2008**, *73*, 3205−3211.

(11) (a) Hayashida, O.; Hamachi, I. *Chem. Lett.* **2003**, *32*, 632−633. (b) Hayashida, O.; Hamachi, I. *J. Inclusion Phenom. Mol. Recognit. Chem.* **2005**, *53*, 57−61.

(12) (a) Green, N. M. *Biochem. J.* **1963**, *89*, 585−591. (b) Ahn, D. J.; Kim, J.-M. *Acc. Chem. Res.* **2008**, *41*, 805−816. (c) Ward, T. R. *Acc. Chem. Res.* **2011**, *44*, 47−57. (d) Ren, W. X.; Han, J.; Uhm, S.; Jang, Y. J.; Kang, C.; Kim, J.-H.; Kim, J. S. *Chem. Commun.* **2015**, *51*, 10403−10418.

(13) (a) Struthers, L.; Patel, R.; Clark, J.; Thomas, S. *Anal. Biochem.* **1998**, *255*, 20−31. (b) Nordlund, H. R.; Hytoenen, V. P.; Laitinen, O. H.; Kulomaa, M. S. *J. Biol. Chem.* **2005**, *280*, 13250−13255. (c) Verdoliva, A.; Bellofiore, P.; Rivieccio, V.; Catello, S.; Colombo, M.; Albertoni, C.; Rosi, A.; Leoni, B.; Anastasi, A. M.; De Santis, R. *J. Biol. Chem.* **2010**, *285*, 9090−9099. (d) Pei, H.; Wan, Y.; Li, J.; Hu, H.; Su, Y.; Huan, Q.; Fan, C. *Chem. Commun.* **2011**, *47*, 6254−6256.

(14) (a) Moroianu, J.; Fett, J. W.; Riordan, J. F.; Vallee, B. L. *Proc. Natl. Acad. Sci. U. S. A.* **1993**, *90*, 3815−3819. (b) Mirzaei, H.; Regnier, F. *Anal. Chem.* **2005**, *77*, 2386−2392. (c) Pan, Y.; Ye, M.; Zheng, H.; Cheng, K.; Sun, Z.; Liu, F.; Liu, J.; Wang, K.; Qin, H.; Zou, H. *Anal. Chem.* **2014**, *86*, 1170−1177.

(15) (a) Liljestrom, V.; Mikkila, J.; Kostiainen, M. A. *Nat. Commun.* **2014**, *5*, 4445−4453. (b) Sirianni, R. W.; Zheng, M.-Q.; Patel, T. R.; Shafbauer, T.; Zhou, J.; Saltzman, W. M.; Carson, R. E.; Huang, Y. *Bioconjugate Chem.* **2014**, *25*, 2157−2165. (c) Lepoitevin, M.; Nguyen, G.; Bechelany, M.; Balanzat, E.; Janot, J.-M.; Balme, S. *Chem. Commun.* **2015**, *51*, 5994−5997.

(16) Hayashida, O.; Nakashima, T. *Bull. Chem. Soc. Jpn.* **2012**, *85*, 715−723.

(17) Osmond, R. I. W.; Kett, W. C.; Skett, S. E.; Coombe, D. R. *Anal. Biochem.* **2002**, *310*, 199−207.

(18) Slavik, J. *Biochim. Biophys. Acta, Rev. Biomembr.* **1982**, *694*, 1−25.

(19) Benesi, H. A.; Hildebrand, J. H. *J. Am. Chem. Soc.* **1949**, *71*, 2703−2707.

(20) Hayashida, O.; Hamachi, I. *J. Org. Chem.* **2004**, *69*, 3509−3516.

(21) The calculations were carried out using Macromodel 7.5 software. (a) Tong, Y.; Mei, Y.; Li, Y. L.; Ji, C. G.; Zhang, Z. H. *J. Am. Chem. Soc.* **2010**, *132*, 5137−5142. (b) General, I. J.; Dragomirova, R.; Meirovitch, H. *J. Phys. Chem. B* **2011**, *115*, 168−175.

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