Considerable Change of Fluorescence Properties upon Multiple Binding of Coralyne to 4-Sulfonatocalixarenes

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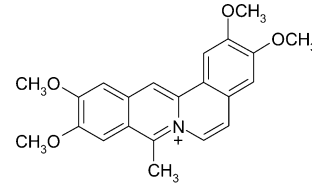
The interaction of coralyne, an analogue of natural protoberberine alkaloids, with 4-sulfonatocalixarenes (SCXn) was studied in aqueous solution at pH 2 to reveal the major factors determining the stability, stoichiometry, and fluorescent properties of the species formed. Addition of SCXn to coralyne solution brought about remarkable fluorescence intensity diminution and hypochromism in the 300−440 nm absorption domain. SCXn hosts were capable of binding as many coralyne molecules as the number of their hydroxybenzenesulfonate units. The SCXn-promoted interaction among coralyne molecules was evidenced by the appearance of a long-lived fluorescence component. In dilute alkaloid solution, 1:1 and 1:2 coralyne/4-sulfonatocalix[4]arene complexes were formed, but only 1:1 association occurred with 4-sulfonatocalix[8]arene. Time-resolved fluorescence measurements demonstrated that photoinduced electron transfer from a hydroxybenzenesulfonate moiety to the singlet-excited coralyne can compete efficiently with the other deactivation processes.

1. Introduction

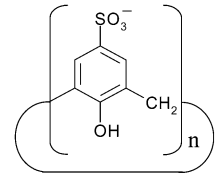
The highly water-soluble 4-sulfonatocalixarenes (SCXn), synthesized first in 1984 by Shinkai and co-workers,[1] represent a particularly important, versatile family of building blocks in supramolecular chemistry. This class of cavitands is composed of *n* 4-hydroxy-benzenesulfonate units linked by methylene groups (Scheme 1), forming π-electron rich, flexible macrocycles, which can encapsulate a wide variety of organic compounds, dyes, and cations.[2,3] The fluorescence intensity change upon the extrusion of a probe from the host cavity was exploited to signal and quantify the confinement of nonfluorescent guests.[4,5] 4-Sulfonatocalixarenes have many analytical[6] and crystal-engineering[7] applications and can be used as metalloenzyme models.[8] The biological activity of these macrocyclic compounds has also received considerable attention. Recent reviews provide a good overview of their antiviral, antibacterial, antithrombotic, and ion channel blocking behavior, as well as of the studies of their toxicity.[9,10] They are capable of solubilizing drugs[11] and complexing amino acids, peptides, proteins, or other compounds of pharmaceutical interest.[9,10] Despite the importance of supramolecular complexation in drug delivery, very little information is available on the interaction of alkaloids with macrocyclic compounds.

We have previously studied the photophysical characteristics of berberine, a clinically important natural isoquinoline alkaloid, in confined microenvironments.[12−14] Remarkably strong binding to 4-sulfonatocalix[8]arene was found in aqueous solution,[14] which led to a fluorescence quantum yield increase of a factor of about 40 at pH 2. The size of the macrocycle proved to be the dominant factor determining the binding constant, whereas pH exerted the largest effect upon the fluorescence quantum yield of the berberine−SCXn inclusion complexes. Extrusion of berberine from SCXn cavity by the competitive binding of ionic liquids revealed that the lengthening of the aliphatic side chain of the imidazolium moiety of ionic liquids diminishes the equilibrium constant of complexation with SCX4, but enhances the stability of SCX6 complexes.[5]

SCHEME 1



Coralyne cation



n = 4, 6, 8

4-Sulfonatocalix[n]arene (SCXn)

In the present study, we focus on coralyne (Scheme 1), a synthetic analogue of natural protoberberine alkaloids. It has received extensive attention because of its anticancer acivity[15] and DNA- and RNA-targeting properties.[16] Owing to its fully conjugated aromatic system, coralyne has planar conformation, which facilitates aggregation in aqueous solution.[17,18] Dimerization of fluorescent dyes on cancer-targeting proteins can be used to generate activatable optical probes for in vivo molecular imaging.[19] Binding to DNA induces the formation of coralyne aggregates stacked along the deoxyribose phosphate backbone.[20] We have shown that the remarkably strong interaction with negatively charged polysaccharides, such as chondroitin-6-sulfate or dextran sulfate, promotes the coralyne dimer formation.[21] As an extension of this work, now, we reveal how the complex stoichiometry and the size of macrocycle affect the fluorescence of coralyne-SCXn complexes. It is demonstrated that the binding characteristics of coralyne is entirely different from that reported for the structurally similar berberine alkaloid.

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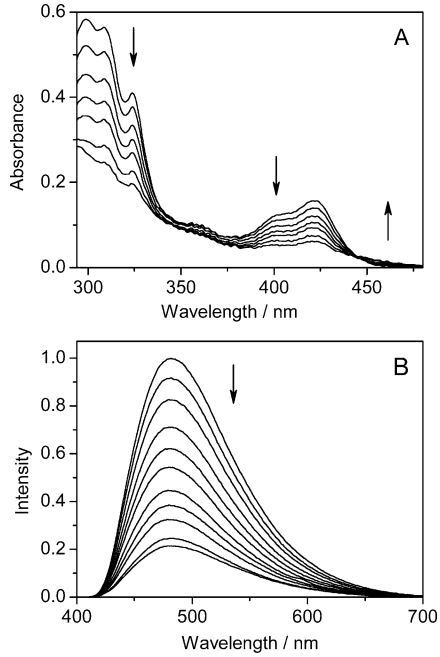

Figure 1. Spectra of 3.18 μM coralyne in water at pH 2; (A) absorption in the presence of 0, 0.56, 1.30, 1.74, 2.55, 6.49, and 27.1 μM SCX4 using 5 cm optical path; (B) fluorescence in the presence of 0, 0.19, 0.71, 1.71, 2.75, 4.31, 8.59, 12.5, 29.8, 133, and 371 μM SCX4.

2. Experimental Section

Coralyne chloride (Acros Organics) was recrystallized from ethanol, and 4-hydroxybenzenesulfonate sodium salt (Aldrich) was used as received. 4-Sulfonatocalix[4]arene (SCX4) (Fluka), 4-sulfonatocalix[6]arene (SCX6), and 4-sulfonatocalix[8]arene (SCX8) (Acros Organics) held 1:21, 1:13, and 1:9 stoichiometric amounts of water in their crystal structures.[22] All experiments were carried out at pH 2 and 296 K. The pH of the solutions was measured with a Consort C832 apparatus. The UV−vis absorption spectra were recorded on a Unicam UV 500 spectrophotometer. Corrected fluorescence spectra were obtained on a Jobin-Yvon Fluoromax-P photon-counting spectrofluorometer. Fluorescence decays were measured with the time-correlated single-photon counting technique. A Picoquant diode laser (pulse duration ~70 ps, wavelength 372 nm) excited the samples, and the fluorescence decays were detected with a Hamamatsu R3809U-51 microchannel plate photomultiplier, which was connected to a Picoquant Timeharp 100 electronics (36 ps/channel time resolution). Fluorescence decays were fitted by a nonlinear least-squares deconvolution method using Picoquant FluoFit software. The experimental data were analyzed by the ORIGINPRO8 software or with a homemade program written in MATLAB 7.9.

3. Results and Discussion

The sulfonic acid moieties of SCXn are completely dissociated[23] at pH 0.4, and the pK_a value of the first dissociation step of the phenolic OH groups is in the 3.26−3.73 range showing slight growth with the ring size.[23−25] To avoid phenolate formation, the interaction with coralyne was studied at pH 2 by adding an appropriate amount of HCl to the solutions. As a representative example, Figure 1 shows the changes in the absorption and fluorescence spectra upon addition of gradually increasing amounts of SCX4 to 3.18 μM coralyne aqueous solution. Because the equilibrium constant of coralyne dimerization[17,21] is 1.1×10^5 M^{-1}, about 68% of the alkaloid molecules

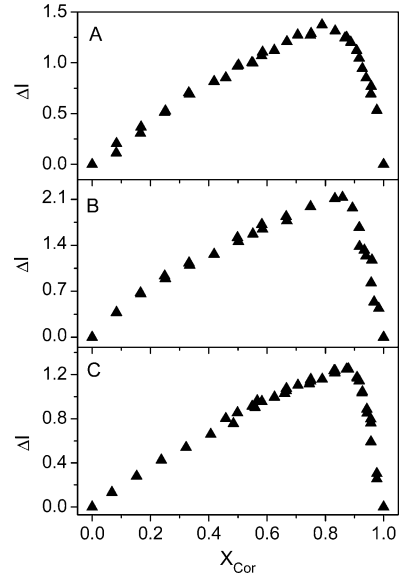

Figure 2. Job plot of the fluorescence intensity change at 490 nm vs the mole fraction of coralyne for the binding to SCX4 (A), SCX6 (B), and SCX8 (C) at pH 2. The total concentration [coralyne] + [SCXn] is constant (10 μM).

are present as monomers at this concentration. The marked hypochromicity in the absorption spectra below 445 nm and the slight rise of the absorbance in the 445−475 nm range evidence coralyne's binding to SCX4. As seen in Figure 1B, complex formation leads to significant fluorescence intensity diminution. SCX6 and SCX8 induce similar spectral alterations and bring about not only fluorescence quenching but also a bathochromic displacement of the fluorescence maximum.

The complexation stoichiometry is studied by Job's continuous variation method.[26] The difference in fluorescence intensity measured at 490 nm in the absence and presence of SCXn (ΔI) is plotted as a function of the mole fraction of coralyne (X_{Cor}) holding the total concentration of reactants (10 μM) fixed (Figure 2). The maxima appear at 0.8, 0.86, and 0.88 coralyne mole fractions, which correspond to 4:1, 6:1, and 8:1 stoichiometry for coralyne binding to SCX4, SCX6, and SCX8, respectively. These results suggest that as many coralyne as the number of 4-hydroxybenzenesulfonate units of the macrocycle can be complexed. Of course, complexes with other stoichiometries also coexist in the solution in dynamic equilibrium. As Gil and Oliveira pointed out,[27] the sensitivity of Job's method is poor above 3:1 complexation stoichiometry. Nevertheless, the data in Figure 2 show unambiguously the shift of the peak toward larger coralyne mole fraction with growing macrocycle size. In principle, the dimerization of coralyne could affect the Job plot,[27] but the strong 1:1 binding (vide infra) ensures the practically complete dissociation of the dimer, and therefore, it has negligible influence on the location of the peaks in Figure 2.

Figure 3 presents the alteration of the absorbance at the maximum of the first absorption band (422 nm) as a function of the ratio of coralyne and SCXn concentrations. In these experiments, the coralyne concentration (3.2 μM) is kept constant. All 4-sulfonatocalixarenes induce significant absorbance diminution, but the concentration needed to reach the effect varied to a large extent with the size of the macrocycle. In the case of SCX8, the largest and least rigid host, the absorbance change levels off at about 1:8 [SCX8]/[coralyne] concentration ratio, indicating very strong binding (Figure 3A). As the size and flexibility of the calixarene ring diminish, multiple association of coralyne with SCXn becomes more

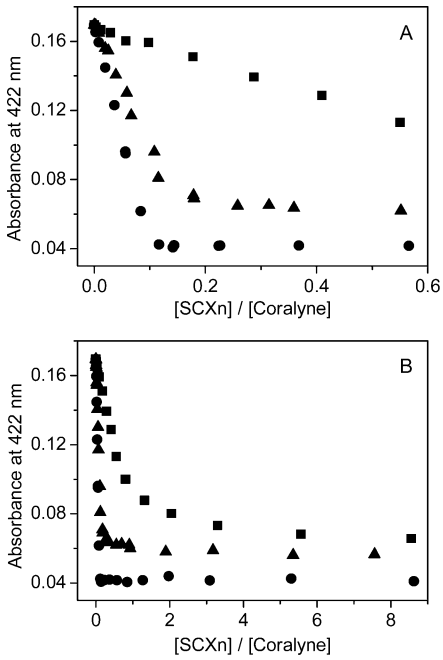

Figure 3. Variation of the absorbance at 422 nm with the concentration of SCXn relative to that of coralyne (3.2 μM) at pH 2. Upper plot is the zoomed view of the data presented in the lower figure. Squares, triangles, and circles show the results obtained in the presence of SCX4, SCX6, and SCX8, respectively.

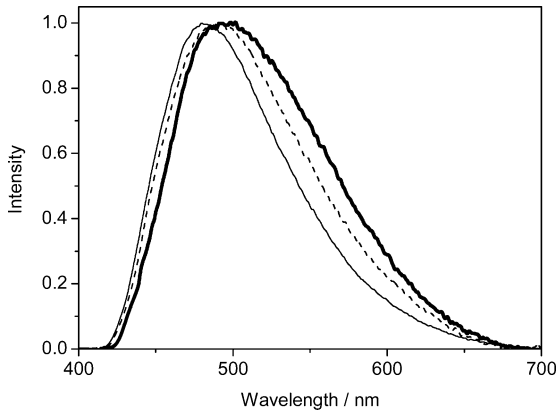

Figure 4. Normalized fluorescence spectra of 3.2 μM coralyne in the presence of 30 μM SCX4 (thin line), SCX6 (dashed line), or SCX8 (thick line) at pH 2. Excitation is at 372 nm.

hindered sterically, leading to reduced complexation affinity. Figure 3B demonstrates that the absorbance where the changes level off rises in the order SCX8 < SCX6 < SCX4. This trend reflects the lessening probability of the $\pi-\pi$ interaction among bound coralyne molecules.

The SCXn concentration dependence of the coralyne fluorescence intensity exhibits parallel diminution with the absorbance. The shape of the fluorescence spectra of the various coralyne−SCXn complexes in the presence of 30 μM host concentration are compared in Figure 4. A broadening of the band and a gradual shift of the maximum to lower energy are observed when the number of 4-hydroxybenzenesulfonate moieties of the macrocycle is raised from 4 to 8. Analogous spectral alterations were found upon dimerization of coralyne.[21] Despite the ~3 diminution factor of the fluorescence intensity upon addition of 30 μM SCX4 (inset to Figure 5), the spectrum of the coralyne−SCX4 complex barely differs from that of the free alkaloid, indicating that the interaction among the bound

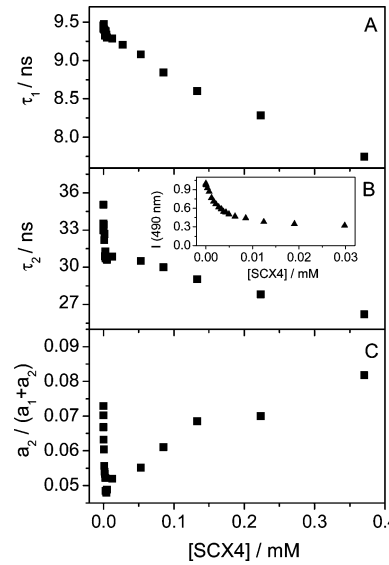

Figure 5. Effects of SCX4 concentration variation on the fluorescence decay parameters (A, B) and the fractional amplitude of the long-lived component (C) detected at 520 nm in 3.2 μM coralyne solution at pH 2. Inset presents the variation of the fluorescence intensity at 490 nm (excitation at 372 nm).

coralyne molecules is sterically restricted by the small and relative inflexible macrocycle.

The fluorescence intensity decays at 520 nm follow dual exponential kinetics, irrespective of the size of the host. In the absence of SCXn, the fluorescence component with a lifetime of $\tau_1 = 9.5$ ns is assigned to free coralyne monomer, whereas the longer-lived component with a lifetime of $\tau_2 = 35$ ns is due to the excited dimer.[21] In the 0−4 μM SCX4 concentration range, the fractional amplitude of the dimer emission ($a_2/(a_1 + a_2)$) diminishes because the binding of coralyne to SCX4 competes with alkaloid dimerization. A substantial fluorescence intensity decrease also takes place in this concentration domain (inset to Figure 5), which arises mainly from the lessening of the absorbance at the 372 nm excitation wavelength upon complexation (Figure 1). At higher concentrations, $a_2/(a_1 + a_2)$ somewhat grows but remains a fairly low value. This trend implies that the diminution of the number of coralyne molecules bound to a macrocycle facilitates the structural changes within the complex, increasing thereby the probability of the interaction between the alkaloid molecules.

When SCX8 serves as a host, the decrease of $a_2/(a_1 + a_2)$ from 0.075 to 0.063 appeared at much lower (0−0.2 μM) concentration domain and further addition of SCX8 leads to a considerable rise of the fractional amplitude of the long-lived fluorescence, reaching a maximum at 2:1 coralyne/SCX8 molar ratio (Figure 6C). As SCX8 concentration is raised further, $a_2/(a_1 + a_2)$ becomes smaller because of the increasing probability of 1:1 complex formation.

Depending on the molar ratio of the constituents, the stoichiometry of the coralyne−SCXn species varies, and the complexes can have many conformations with different fluorescence lifetimes. However, the complexes, in which coralyne components are able to interact, can be distinguished from others easily because of their much longer excited-state lifetime. As shown below, intracomplex electron transfer from the 4-hydroxybenzenesulfonate moieties of the macrocycle to the excited coralyne may contribute to the shortening of the fluorescence lifetime of the complexes presented in Figure 6A and B. The

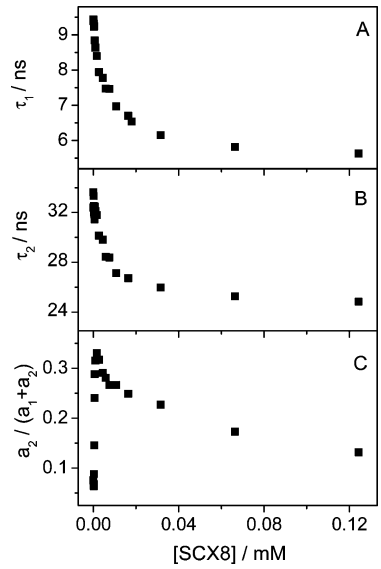

Figure 6. SCX8 concentration dependence of the fluorescence decay parameters (A, B) and the fractional amplitude of the long-lived component (C) detected at 520 nm in 3.2 μM coralyne solution at pH 2.

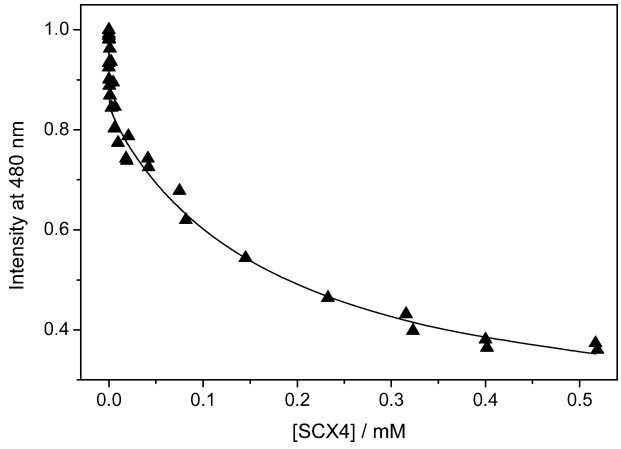

Figure 7. Fluorescence intensity of 0.11 μM coralyne solution at pH 2 vs SCX4 concentration (excitation at 372 nm, detection at 480 nm). The line represents the result of nonlinear least-squares analysis.

characteristics of the time-resolved fluorescence of coralyne−SCX6 complexes are analogous to those observed using SCX8 as a host.

SCXn Complex Formation in 0.11 μM Coralyne Solution. Multiple binding to a host is less probable at low coralyne concentration. To decrease the types of the complexes coexisting in the solution, the association with SCXn is studied at 0.11 μM coralyne concentration. Under this condition, only about 2% of coralyne is expected to be dimerized on the basis of the equilibrium constant of coralyne dimer formation[17,21] (1.1×10^5 M^{-1}). The exponential coralyne fluorescence decay kinetics confirmed that alkaloid association plays a negligible role. Figure 7 presents the effect of SCX4 addition on the fluorescence intensity of coralyne recorded at 480 nm. After a steep initial decline, a much slower intensity diminution appears when SCX4 concentration is raised above 6 μM. The two distinct domains suggest that not only one but also two SCX4 can form a complex with a coralyne molecule. The equilibrium constants are defined as

$$K_1 = \frac{[\text{Complex1}]}{[\text{Cor}][\text{SCX4}]} \quad (1)$$

$$K_2 = \frac{[\text{Complex2}]}{[\text{Complex1}][\text{SCX4}]} \quad (2)$$

and the mass balance equations for the total coralyne and SCX4 concentrations are

$$[\text{Cor}]_T = [\text{Cor}] + [\text{Complex1}] + [\text{Complex2}] \quad (3)$$

$$[\text{SCX4}]_T = [\text{SCX4}] + [\text{Complex1}] + 2[\text{Complex2}] \quad (4)$$

On the basis of eqs 1−4, the concentration of unbound SCX4 is given by the following third-order equation:

$$K_1K_2[\text{SCX4}]^3 + (K_1 - K_1K_2[\text{SCX4}]_T + 2K_1K_2[\text{Cor}]_T)[\text{SCX4}]^2 + (1 - K_1[\text{SCX}]_T + K_1[\text{Cor}]_T)[\text{SCX4}] - [\text{SCX4}]_T = 0 \quad (5)$$

whereas the concentrations for the other species are

$$[\text{Cor}] = [\text{Cor}]_T/(1 + K_1[\text{SCX4}] + K_1K_2[\text{SCX4}]^2) \quad (6)$$

$$[\text{Complex1}] = K_1[\text{Cor}][\text{SCX4}] \quad (7)$$

$$[\text{Complex2}] = K_1K_2[\text{Cor}][\text{SCX4}]^2 \quad (8)$$

The normalized fluorescence intensity (I) is expressed by

$$I = [\text{Cor}]/[\text{Cor}]_T + f_1[\text{Complex1}] + f_2[\text{Complex2}] \quad (9)$$

where the f_1 and f_2 constants are proportional to the fluorescence quantum yields of complexes 1 and 2, respectively.

The experimental data presented in Figure 7 were analyzed by a homemade MATLAB 7.9 program. Starting with the initial estimates of K_1 and K_2, eq 5 was solved numerically. Then, the fluorescence intensity (I) was calculated on the basis of eqs 6−9, and the iterations were repeated until the best fit was achieved. The line in Figure 7 corresponds to $K_1 = 1.8 \times 10^7$ M^{-1}, $K_2 = 6100$ M^{-1}, whereas $f_1 = \Phi_F(\text{Complex1})/\Phi_F(\text{Coralyne}) = 0.86$ and $f_2 = \Phi_F(\text{Complex2})/\Phi_F(\text{Coralyne}) = 0.55$ were obtained for the optimized fluorescence quantum yield ratios. The much lower stability of the 1:2 complex is probably due to the electrostatic repulsion between the two SCX4 ligands.

On the basis of the equilibrium constant of 1:1 binding obtained from the analysis of the steady-state fluorescence intensity data ($K_1 = 1.8 \times 10^7$ M^{-1}), we can conclude that coralyne is almost fully converted to 1:1 complex in the presence of 6 μM SCX4. Nevertheless, time-resolved fluorescence measurements give unchanged, single-exponential decay kinetics up to this SCX4 concentration, indicating that 1:1 binding to SCX4 barely affects the fluorescence lifetime of coralyne ($\tau_1 = 9.5$ ns). Above 6 μM SCX4 concentration, a new emission appears with a 2.0 ns lifetime and growing amplitude (Figure

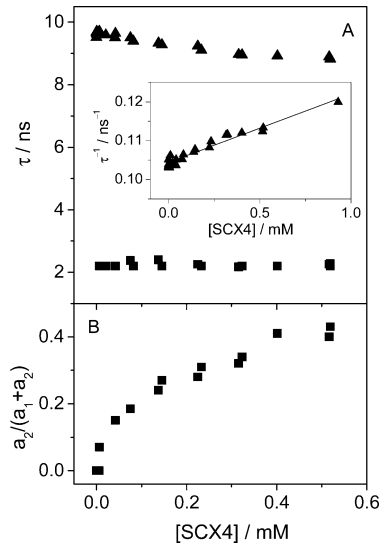

Figure 8. Change of the fluorescence decay parameters and the fractional amplitude of the shorter-lived species detected at 520 nm with SCX4 concentration in 0.11 μM coralyne solution at pH 2. Inset displays the reciprocal fluorescence lifetime of the longer-lived component as a function of the SCX4 concentration.

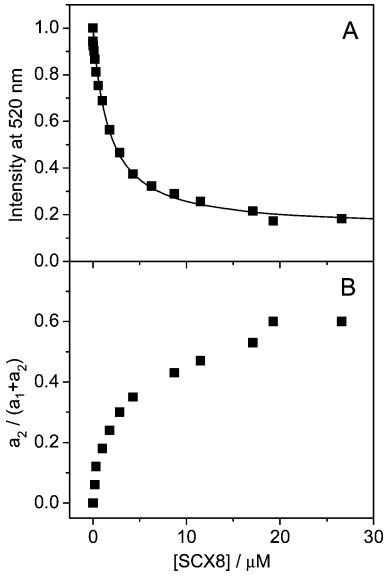

Figure 9. SCX8 concentration dependence of the fluorescence intensity (A) and the fractional amplitude of the shorter-lived species (B) detected at 520 nm in 0.11 μM coralyne solution at pH 2. Excitation is at 370 nm.

8), which is assigned to the 1:2 coralyne/SCX4 complex. The small size and relatively inflexible character of SCX4 macrocycle does not allow the inclusion of coralyne. The marked difference in the fluorescence lifetime of 1:1 and 1:2 complexes implies dissimilar structure. The orientation of the macrocycle does not facilitate electron transfer from the phenolic units to the excited coralyne within the 1:1 complex. Therefore, the fluorescence decay kinetics does not change upon the formation of this species. The drop of the fluorescence intensity upon 1:1 binding probably originates from the lower molar absorption coefficient of the coralyne/SCX4 ion pair compared to that of the free coralyne. Figures 1 and 3 demonstrate the absorbance diminution upon binding to SCX4. A considerable decrease in the molar absorption coefficient upon ion pairing has also been reported for berberine, an alkaloid possessing molecular structure similar to coralyne.[28] The 1:2 complex may have a sandwichlike structure, in which an aromatic ring of both SCX4 molecules interacts with coralyne, and the sulfonato groups of the two macrocycles point toward opposite directions. Such a molecular assembly permits photoinduced intracomplex electron transfer, leading to a shortening of the fluorescence lifetime of coralyne to 2.0 ns in the 1:2 complex.

Figure 8 demonstrates that the lifetime of the excited 1:2 complex is constant within the limits of experimental errors, whereas the increase of SCX4 concentration slightly accelerates the fluorescence decay of 1:1 complex. The plot of the reciprocal fluorescence lifetime as a function of the SCX4 concentration (inset to Figure 8) gives a linear correlation with a slope of 1.8 $\times$ 10^{10} M^{-1} s^{-1}. This suggests that the excited 1:1 complex is quenched in a practically diffusion controlled bimolecular reaction with SCX4.

When SCX8 is used as a complexing agent, two distinct domains do not appear in the ligand concentration dependence of the coralyne fluorescence intensity (I_F) (Figure 9). This implies that only the 1:1 complex is produced with SCX8 in 0.11 μM coralyne solution. The experimental data were fitted by the following function:[29]

$$I_F = I_F^0 + \frac{I_F^\infty - I_F^0}{2}\left\{1 + \frac{[SCX8]_0}{[Cor]_0} + \frac{1}{K[Cor]_0} - \left[\left(1 + \frac{[SCX8]_0}{[Cor]_0} + \frac{1}{K[Cor]_0}\right)^2 - 4\frac{[SCX8]_0}{[Cor]_0}\right]^{1/2}\right\} \quad (10)$$

where K represents the equilibrium constant of 1:1 binding, $[Cor]_0$ stands for the initial guest concentration, and I_F^∞ and I_F^0 denote the fluorescence intensity of the fully complexed and free coralyne. As seen in Figure 9A, the calculated function matches the experimental I_F values, confirming 1:1 complexation stoichiometry. The nonlinear least-squares analysis provides K = 6.4 $\times$ 10^5 M^{-1} and I_F^∞/I_F^0= 0.138. Fluorescence intensity decays follow dual exponential kinetics from 1 μM SCX8 concentration with practically constant 9.5 and 2.1 ns lifetimes. The shorter-lived emission is assigned to the 1:1 complex, since the fractional amplitude of this component ($a_2/(a_1 + a_2)$) grows with SCX8 concentration (Figure 9B). The accelerated deactivation of the excited 1:1 complex compared to unbound coralyne probably arises from intracomplex electron transfer. This process cannot occur in 1:1 coralyne/SCX4 complex due to the unfavorable orientation of the constituents. The augmentation of the conformational mobility with the number of phenolic units renders the macrocycle more adaptable to the geometrical features of coralyne and thereby promotes the electron transfer. The importance of the guest binding induced calixarene conformation change, the so-called "template effect", has been emphasized in previous papers.[30]

Coralyne Interaction with 4-Hydroxybenzenesulfonate. To confirm that electron transfer is possible from SCXn to excited coralyne, the effect of 4-hydroxybenzenesulfonate (HBS), which is a building block of SCXn macrocycles, was also studied. Fluorescence lifetime measurements were performed at 0.11 μM coralyne concentration to avoid complications originating from coralyne dimerization. The interaction with HBS expedites the relaxation of the singlet-excited coralyne, but the fluorescence decay kinetics remains exponential. Figure 10 presents the reciprocal fluorescence lifetime as a function of HBS concentration. The slope gives k_q = 4.3 $\times$ 10^9 M^{-1} s^{-1} for the rate constant of the reaction between singlet-excited coralyne and HBS at pH 2. Singlet-excited coralyne is expected to be a good

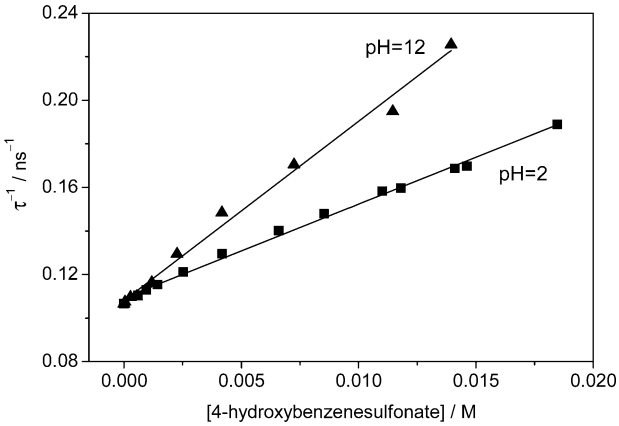

Figure 10. Linear correlations between the reciprocal fluorescence lifetime of coralyne and 4-hydroxybenzenesulfonate concentration at pH 2 (■) and 12 (▲).

electron acceptor because of its positive charge. Thus, the dynamic quenching takes place probably via electron transfer mechanism. This is corroborated by the acceleration of the process at pH 12, where $k_q = 8.2 \times 10^9$ M^{-1} s^{-1} is obtained. Under strongly alkaline conditions, the HO moiety of HBS is deprotonated. Because phenolates can be oxidized more easily than the corresponding phenols, the driving force for photoinduced electron transfer is enhanced, leading to the more rapid quenching at pH 12. We found $k_q = 7.8 \times 10^9$ M^{-1} s^{-1} for the rate constant of singlet-excited coralyne reaction with unsubstituted phenol at pH 2. This k_q value is larger than that obtained for HBS because the removal of an electron withdrawing substituent, such as a sulfonate group, from phenols decreases the oxidation potential.[31]

4. Conclusions

We have demonstrated that the characteristics of coralyne binding to SCXn is totally different from those found previously for the encapsulation of a structurally related natural alkaloid, berberine. The latter compound produced only a 1:1 complex with SCXn and showed significant fluorescence intensity enhancement upon confinement in the macrocycle. In contrast, coralyne complexation leads to fluorescence quenching, whereas the stoichiometry of its binding to SCXn strongly depends on the size of the macrocycle and the coralyne concentration. SCXn hosts are able to associate with as many coralyne molecules as the number of their 4-hydroxybenzenesulfonate moieties. The different behavior of the two alkaloids arises from their dissimilar molecular shape. Berberine has a nonaromatic ring and consequently, a nonplanar molecular structure, whereas the conjugated aromatic rings of coralyne ensure planar conformation. The flat coralyne molecules can readily participate in $\pi-\pi$ stacking interactions, resulting in multiple binding to SCXn. Moreover, the conjugated aromatic system also facilitates the reduction of coralyne. Hence, SCXn is able to quench singlet-excited coralyne via electron transfer. Analogous reaction from SCXn to berberine is not possible in acidic solution because of the unfavorable driving force.

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