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ARTICLE

Inhibition of the Prototropic Tautomerism in Chrysazine by *p*-Sulfonatocalixarene Hosts

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This study explores the interesting effect of *p*-sulfonatocalix[n]arene hosts (SCXn) on the excited-state tautomeric equilibrium of Chrysazine (CZ), a model antitumour drug molecule. Detailed photophysical investigations reveal that conversion of CZ from its more dipolar, excited normal form (N*) to the less dipolar, tautomeric form (T*) is hindered in SCXn-CZ host-guest complexes, which is quite unexpected considering the nonpolar cavity of the hosts. The atypical effect of SCXn is proposed to arise due to the partial inclusion or external binding of CZ with the hosts, which facilitates H-bonding interactions between CZ and the sulfonate groups present at the portals of the host. The intermolecular H-bonding subsequently leads to weakening of the pre-existing intramolecular H-bond network within CZ, and thus hinders the tautomerizaion process. Our results suggest that rather than the binding affinity, it is the orientation of CZ in the SCXn-CZ complexes, and its proximity to the portals of the host that plays a predominant role in influencing the tautomeric equilibrium. These observations are supported by quantum chemical calculations. Thermodynamic studies validate that SCXn-CZ interaction is essentially enthalpy driven and accompanied by small entropy loss, which is consistent with the binding mechanisms.

Introduction

Prototropic tautomerism refers to the dynamic equilibrium between two stable forms (tautomers) of a molecule by the repositioning or movement of proton(s) from one molecular site to another.[1-5] This phenomenon plays important roles in numerous biological processes like, enzymatic catalysis, drug activity and nucleic acid chemistry.[6-9] The ability of DNA bases to tautomerize is well-recognized to be the origin of spontaneous point mutations.[9-11] Since tautomerization is often accompanied by changes in optical properties, this process also finds applications in various fields of science and technology such as, optical switches, laser technology, sensing and molecular data processing.[3,12-15]

The necessary criterion for a molecule to depict prototropic tautomerism is that it should possess both proton donating and proton accepting substituents. The proton-donating and accepting groups may be vicinal and intramolecularly H-bonded, in which case the relocation of the proton occurs directly through the pre-existing H-bond.[3,4,16-18] Alternatively, the two substituents may be distal, in which case the proton relocation is mediated through solvent H-bonded bridges[5,19-21] or through doubly H-bonded dimers of the molecules.[2,22] In many instances, proton transfer is found to be more favourable in the excited state than in the ground state, due to modulation in the acid-base characteristics of molecules upon electronic excitation.[2-5,13,16-25]

Being an essential process in various biochemical systems, prototropic tautomerism of many different kinds of molecules are extensively studied in both homogeneous[5,16,19] and microheterogeneous solvent media[20,21,26-30]. The effects of solvent,[5,31,32] temperature,[33,34] pH,[35,36] and additives like, metal ions[23,37,38] and macrocycle cavitands,[18,39-41] are investigated to determine the factors that can influence tautomeric equilibria, for their better applications. The studies with macrocyclic cavitands are especially significant, because host molecules can trap suitable prototropic guest molecules within their cavity and thus mimic the confined pockets existing in biomolecular assemblies.[42,43] Understanding the effect of host-guest interactions on tautomeric equilibria provides a means to visualize how biological environments are able to selectively transform one tautomer form of a molecule to another or choose the desired tautomeric form, by inducing or inhibiting the tautomerization process.

This paper describes the effect of two *p*-sulfonatocalix[n]arene hosts on the prototropic tautomerism of a model anthracycline antitumour drug, 1-8-dihydroxy-9-10-anthraquinone or Chrysazine (CZ), based on ground state absorption, steady-state fluorescence and time-resolved fluorescence measurements. The photophysics of CZ is quite interesting because it has two equivalent proton donating hydroxyl groups that are intramolecularly H-bonded from either side with the central proton accepting carbonyl group

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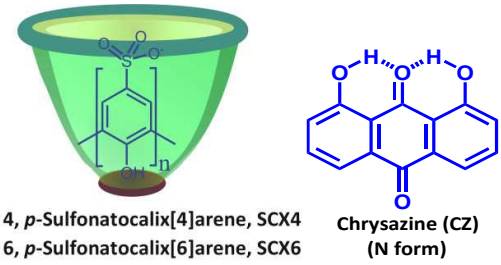
(Scheme 1).[4,44-47] In the ground state, CZ exists exclusively in a single tautomeric form that is designated as the normal form (N). However, upon photoexcitation in the first excited singlet (S_1) state, the dye undergoes rapid proton transfer from either of the two hydroxyl groups to the carbonyl group, to yield the excited tautomer form (T*).[4,44-47] In accordance with this mechanism, the absorption spectrum of CZ shows the signature of a single species (the normal or N form), whereas the steady-state emission spectrum shows two distinct emission bands. The lower wavelength emission band (LWEB) represents the excited state of the normal form (N*) and the higher wavelength band (HWEB) represents the excited tautomeric form (T*). Due to the strong pre-existing intramolecular H-bonding in CZ, its excited state tautomerization is reported to be very facile and efficient. The prototropic tautomerism remains operative in a variety of solvents and under different temperature conditions.[4,44-47]



Scheme 1 Molecular structures of the hosts (SCX4 and SCX6) and the guest, Chrysazine (CZ).

The two host molecules used in the present work, namely *p*-sulfonatocalix[4]arene (SCX4) and *p*-sulfonatocalix[6]arene (SCX6), are homologues of the *p*-sulfonatocalix[n]arene series of macrocycles. These cup shaped container molecules are comprised of *p*-hydroxybenzenesulfonate units linked with methylene groups (Scheme 1).[48-50] They have an interior hydrophobic cavity with two asymmetrical hydrophilic portals. Their high water solubility, flexible π-electron rich cavities and ability to provide anchoring points of sulfonate groups at one of the portals, endows them with versatile complexation properties for different guest molecules.[42,48-53] The host-guest binding in these systems occur primarily due to favorable electrostatic, hydrophobic, H-bonding, π-stacking and CH-π interactions.[48,51] Since SCX4 and SCX6 have similar chemical constitutions but differ in their cavity dimensions,[51,54,55] these two hosts were particularly chosen to determine whether cavity size has any influence on the binding interaction and consequently on the prototropic tautomerism of the guest, CZ.

Our results show that both SCX4 and SCX6 form complexes with CZ and also inhibit the otherwise facile excited state tautomerization of CZ. Surprisingly, it is observed that although SCX6 (which is the macrocycle with the larger cavity size) has a relatively weaker binding affinity with CZ as compared to SCX4, the former host is capable of inhibiting the prototropic tautomerism of CZ to a much larger extent than the latter host. All of these results are rationalized by considering the partial inclusion or external binding of CZ with the hosts, as a result of which there is a strong tendency for intermolecular H-bonding interactions between the hydroxyl groups of CZ and the sulfonate groups present at the portals of the host, and a subsequent weakening of the pre-existing intramolecular H-bond network of CZ. These propositions are well supported by FTIR and quantum chemical calculations. Our study further reveals that the SCXn-CZ host-guest interaction is in essence an enthalpy driven process and accompanied by a small overall loss in entropy.

Experimental

The dye, CZ, was procured from Sigma-Aldrich and was recrystallised from acetonitrile. The hosts, SCX4 and SCX6, were purchased from TCI Mark, Tokyo and were used as received. A concentrated stock solution of CZ was prepared in ethanol and small aliquots of this stock solution were added to water for preparing aqueous solutions of CZ with a concentration around 3 μM. The interaction of CZ with SCX4 and SCX6 was studied by adding different weighed amounts of the corresponding hosts to the aqueous solution of CZ. Nanopure water with a conductivity of less than 0.1 μS cm^{-1} was obtained from a Millipore Elix-3/A10 water purification system. The solutions were freshly prepared prior to the experiments. The slight decrease in the pH of the solutions on addition of the SCXn hosts was disregarded in the present study, because pH change within the range of 3-8 does not have any effect on the absorption and emission spectral characteristics of the dye (Fig. S1, ESI†). Hence, any spectral changes of CZ in the presence of the hosts are attributed to specific host-guest interactions.

Absorption spectra were recorded with a Jasco UV-vis spectrophotometer (model V-650). Steady-state fluorescence spectra were obtained with a spectrofluorimeter (model FS5, Edinburgh Instruments). Time-resolved fluorescence measurements were carried out using a time-correlated single photon counting (TCSPC) instrument (Horiba Jobin Yvon, UK), where samples were excited by the light pulses from a nano-LED source (406 nm; repetition rate: 1 MHz) and the fluorescence was detected using a PMT based detection module (model TBX4). The instrument response function (IRF) of the present setup is about 185 ps. Measurements were carried out at magic angle configuration to eliminate the contribution of the rotational depolarization of CZ on the observed fluorescence decays. The fluorescence decay traces were analyzed by the reconvolution method, considering mono-exponential function. The quality of the fit was judged by the reduced chi-square (χ^2) value and the distribution of the weighted residuals among the data channels. For a good fit, the χ^2 value was close to unity and the weighted residuals were distributed randomly among the data channels.[56] FTIR spectra were recorded with an IR-Affinity-1 Spectrometer (Shimadzu), using diamond ATR setup.

Unless otherwise stated, all measurements were carried out at ambient temperature, 25 ± 1°C. For the binding studies at different temperatures, a temperature controller (Quantum

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Northwest TC125) was used to adjust the temperature of the solutions (within ± 1°C).

Quantum chemical calculations were carried out using the general *ab initio* quantum package, Gaussian 16.[57] The minimum energy ground-state structures of the hosts, SCX4 and SCX6, the guest, CZ, as well as those of the 1:1 host-guest complexes were determined after considering several initial guess geometries, for optimization under isolated gas phase condition. The optimized gas phase structures were allowed to relax further in water medium applying a macroscopic solvent model. The model considers a self-consistent reaction field (SCRF) method based on solute electron density (SMD). All electronic structure calculations were carried out applying B97D density functional and adopting Dunning correlated atomic basis functions, namely, cc-pVDZ as well as Pople style basis set, 6-311++G(d,p). The DFT functional considered in the present study is known to take into account the long range dispersion correction, which plays a crucial role in shaping the structures of host-guest complexes.

Results and discussion

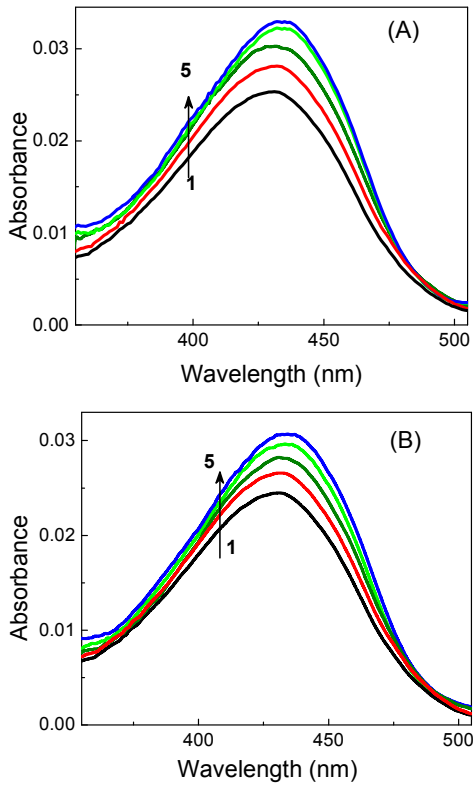

Fig. 1 Absorption spectra of CZ (3 μM) in water with increasing host concentrations; (A) SCX4, (1-5)/mM: 0, 5.5, 10.5, 21, 26 and (B) SCX6, (1-5)/mM: 0, 4.6, 7.6, 15.2, 18.

Fig. 1 shows the absorption spectra of CZ in water with increasing concentrations of the hosts, SCX4 and SCX6. The absorption spectrum of CZ in aqueous solutions has a peak at 430 nm. According to previous literature reports, this absorption band is ascribed to the S_0 to S_1 transition of the normal form (N) of CZ.[4,44,46] With increase in the host concentration, there is marginal increase in the absorbance along with a slight red shift in the absorption maximum, and gradual saturation in the absorption changes, for both SCX4-CZ and SCX6-CZ systems. The changes in the absorption features are indicative of interaction between the dye and the hosts. However, as the changes in the absorption characteristics are not that large, these were not used further for any quantitative estimation of binding affinities.

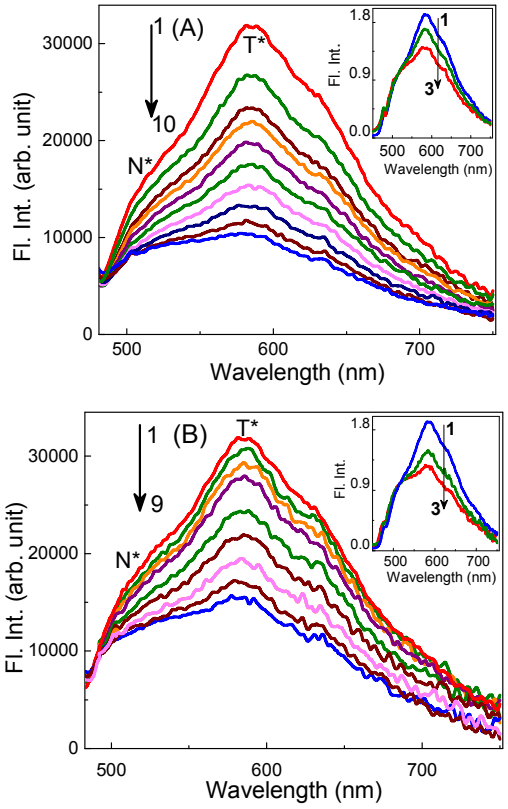

Fig. 2 (A) Emission spectra of CZ (3 μM) in water with 0, 2.0, 3.4, 4.4, 6.7, 9.4, 12.5, 17.2, 23.3 and 31.0 mM SCX4 (1-10). Inset shows the fluorescence intensity of CZ normalized at 515 nm in the presence of 0, 17.2 and 31.0 mM SCX4. (B) Emission spectra of CZ (3 μM) in water with 0, 1.0, 2.2, 3.2, 5.3, 8.1, 11.2, 15.3 and 20.5 mM SCX6 (1-9). Inset shows the fluorescence intensity of CZ normalized at 515 nm in the presence of 0, 11.2 and 20.5 mM SCX6. Excitation wavelength was 415 nm.

Considering that fluorescence is a more sensitive parameter than absorbance, we next investigated the fluorescence characteristics of CZ in the presence of the hosts. It may be mentioned that in many previous studies on host-guest interactions, considerable changes have been noticed in the emission characteristics of the dyes, although under similar conditions, the changes in the absorption characteristics may not be that significant.[49,51,58-60] Based on the changes in the fluorescence characteristics, especially that in the intensities, binding affinities over a wide range (~10^2-10^7 M^{-1}) have been reliably estimated for a variety of host-guest systems.[49,59-63]

The steady-state fluorescence spectra of CZ in aqueous solution, with increasing concentration of SCX4 and SCX6, are presented in Figs. 2A and 2B, respectively. In pure water, CZ

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shows a weak lower wavelength emission band (LWEB) at around 515 nm and a strong higher wavelength emission band (HWEB) with maximum at 585 nm. As mentioned before, the LWEB is due to the emission from excited-state normal form (N^*) while, the HWEB is attributed to the excited-state tautomeric form (T^*) of the dye.[4,44,46]

On addition of the SCXn hosts, two kinds of changes are observed in the emission spectra of CZ. First, the emission intensity is gradually quenched with increasing concentration of the hosts. Second, the nature of the fluorescence spectra of CZ systematically changes such that at the highest concentration of the hosts, the emission contribution of HWEB (T^* emission), which is initially much stronger than the LWEB (N^* emission) becomes almost at par with LWEB.

The decrease in the fluorescence intensity of CZ with increasing concentration of SCXn is justified, because the quinoid moiety of CZ is a good electron acceptor, while phenoxy moieties of the hosts are known to be good electron donors. Consequently, the host can quench the fluorescence intensity of the dye due to electron transfer or charge transfer type of interactions.[49,51]

In accordance with the decrease in the steady-state fluorescence intensity, we also observed a gradual decrease in the fluorescence decay time of CZ with increasing concentration of SCXn. Representative decay traces for the SCX4-CZ system monitored at both LWEB (515 nm, N^* emission) and HWEB (585 nm, T^* emission) are shown in Fig. S2, ESI†. The corresponding fluorescence decay times are presented in Table S1, ESI†. It may be mentioned that the de-excitation rates for both N* and T* forms of the dye are quite similar (τ_f=0.29 ns for the free dye), indicating a kinetic equilibrium between the two forms of the excited dye at the time scales probed by TCSPC measurements (Note S1, ESI†).[4,44,46]

Apart from the fluorescence quenching, the change in the relative intensities of the LWEB and HWEB in the emission spectrum of CZ (cf. Fig. 2), is quite an interesting observation. It suggests that the existing equilibrium between the N* and T* forms of CZ is disturbed when the dye is bound to the SCXn hosts. More specifically, the emission contribution of the T* form (HWEB) is observed to be lower for the SCXn-CZ systems as compared to CZ alone. This is illustrated more clearly in the insets of Fig. 2, which depict the representative emission spectra of the SCXn-CZ systems with their intensities normalized at the LWEB (N* form). It can be seen that with increasing SCXn concentration, as the population of SCXn-CZ complexes increases in the solution, there is a noticeable decrease in the relative contribution of the T* form (HWEB) over the N* form. It is thus evident that the binding interaction of SCXn with CZ hinders the formation of T* from N*.

According to literature reports, the N* form of CZ has a higher dipole moment than the T* form.[4,44,64] This is also evident from the emission spectra of CZ recorded in solvents of different polarities (Fig. S3, ESI†). It is clear that while the position of the HWEB (T* form) remains unaffected by the polarity of the solvent medium, the LWEB (N* form) shows a systematic redshift along with marginal increase in the relative intensity with increasing polarity, which is in accordance with the greater dipolar character of the N* form of CZ. Therefore, based on a comparison of the dipolar natures of the two tautomer forms, it was anticipated that the encapsulation of CZ within the hydrophobic cavity of SCXn hosts would facilitate its tautomerization from the more polar N* form to the relatively less polar T* form. That the SCXn-CZ systems behave in an opposite manner and hinder the prototropic tautomerism of the bound dye from the N* to T* form, is an interesting deviation from the normal expectation.

Excitation spectra were recorded to understand the origin of the two emission bands, LWEB (515 nm) and HWEB (585 nm) for the bound CZ dye. As in the case of free CZ, for the SCXn-CZ systems as well, a single excitation band is observed, which closely resembles the absorption spectrum (Fig. S4, ESI†). This indicates that for both CZ and SCXn-CZ, the tautomeric forms (N* and T*) arise from the same initially excited normal form of the dye.

To decipher the mechanism by which the SCXn hosts affect the prototropic tautomerism of CZ in such a unique manner, so as to inhibit the transformation of N* to T*, we looked into the binding affinities of CZ with SCX4 and SCX6. The binding constants for the SCXn-CZ systems were calculated by considering the changes in the fluorescence intensity of CZ at both the LWEB (515 nm) and the HWEB (585 nm), with varying host concentrations. The host-guest binding stoichiometry was considered to be 1:1, as indicated from the inflection observed at 0.5 mole fraction of the host in the Job plots for the SCX4-CZ and SCX6-CZ systems (Fig. S5, ESI†), and from the geometry optimization studies (discussed later). The formation of 1:1 SCXn-CZ complexes is also reasonably supported by ESI-MS (Fig. S6, ESI†).

The host-guest complex formation of SCXn with CZ can be expressed as,

$$N + H \underset{}{\overset{K_{eq}}{\rightleftharpoons}} C_N \qquad (1)$$

where H represents the SCXn host, N is the normal form of CZ (which is the only form that exists in the ground state) and C_N is the normal form of the complexed dye in the ground state. If K_{eq} is not very large and the host concentration, $[H]_0$, is much higher than the dye concentration, $[N]_0$, then one can assume that the equilibrium host concentration in the solution is effectively the same as the actually added host concentration, i.e., $[H] \approx [H]_0$. In this situation, the equilibrium constant can be expressed as,

$$K_{eq} = \frac{[C_N]}{[N][H]_0} \qquad (2)$$

Further, considering that excited-state lifetimes of guest dyes are generally much shorter (sub-ns for CZ, cf. Table S1, ESI†) than the exchange rate between the free and host-bound forms of the dye (occurs in microseconds),[65] the fluorescence intensity at any host concentration is a composite of the fluorescence intensity contributions from the complexed and uncomplexed forms of the dye. Accordingly, the fluorescence intensity of CZ at 515 nm can be expressed as,

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$$I_{N(obs)} = I_N^0 \frac{[N]}{[N_0]} + I_N^\infty \frac{[C_N]}{[N_0]} \quad (3)$$

Where I_N^0 is the initial fluorescence intensity of the neutral form of CZ in the absence of the host and I_N^∞ is the extrapolated fluorescence intensity when the neutral form is completely complexed with SCXn (Note S2, ESI†). The change in the fluorescence intensity at 515 nm, ΔI_N, can thus be correlated with the equilibrium constant as,

$$\Delta I_N = \Delta I_N^\infty \frac{K_{eq}[H]_0}{1+K_{eq}[H]_0} \quad (4)$$

Where, ΔI_N^∞ is the extrapolated difference in the emission intensity at 515 nm in the presence of zero and infinite host concentrations (Note S2, ESI†).

Similarly, the fluorescence intensity changes at 585 nm corresponding to the tautomer form, ΔI_T, can be correlated as, $\Delta I_T = \Delta I_T^\infty \frac{K_{eq}[H]_0}{1+K_{eq}[H]_0}$ (5)

where, ΔI_T^∞ is the extrapolated difference in the emission intensity at 585 nm in the presence of zero and infinite host concentrations (Note S2, ESI†).

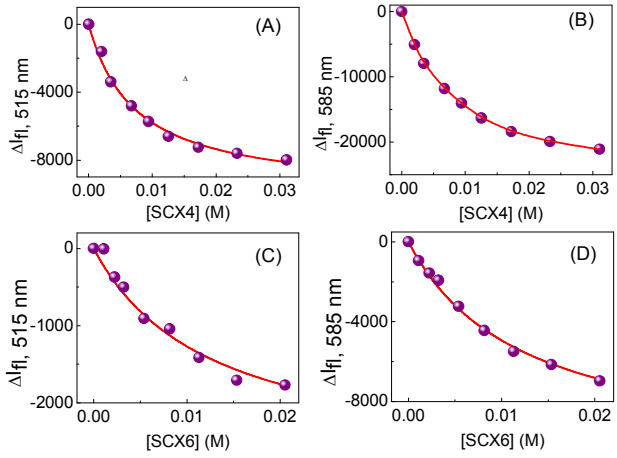

Fig. 3 The binding isotherms for SCX4-CZ system following the variation in the fluorescence intensity at, (A) 515 nm (B) 585 nm, and for SCX6-CZ system following the variation in the fluorescence intensity at, (C) 515 nm (D) 585 nm.

Figs. 3A and B show the variations in the fluorescence intensity of SCX4-CZ complex at the LWEB (515 nm) and HWEB (585 nm) with increasing SCX4 concentration, along with the fitted curves according to equation (4) and equation (5), respectively. Similar plots for the SCX6-CZ system are shown in Figs. 3C and D. Reasonably good fits are obtained in all the cases. The fitted parameters for both the systems are presented in Table 1.

It may be noted that the values of the binding constants (K_{eq}) estimated by equations 4 and 5, (by monitoring the fluorescence intensity changes of either the normal or the tautomer forms of CZ), are quite similar within experimental error. This result is expected because only the normal form of the dye exists in the ground state, and therefore the binding of SCXn occurs exclusively with the normal form. It is only in the excited state that the free and the bound forms of CZ convert to the corresponding tautomeric forms.

Table 1 Binding parameters for the interaction of CZ with the SCXn hosts.

system	parameters obtained by fitting to eq. 4	parameters obtained by fitting to eq. 5	I_T^0/I_N^0 [a]	I_T^∞/I_N^∞ [b]
SCX4-CZ	K_{eq} = 135 M^{-1} ΔI_N^∞ = -10038 (arb. unit)	K_{eq} = 118 M^{-1} ΔI_T^∞ = -27117 (arb. unit)	2.1	0.6
SCX6-CZ	K_{eq} = 79 M^{-1} ΔI_N^∞ = -2878 (arb. unit)	K_{eq} = 81 M^{-1} ΔI_T^∞ = -12038 (arb. unit)	2.1	0.3

[a] Calculated from the experimental data for the free dye [b] Calculated from fitted parameters for the SCXn-CZ complexes.

In the present host-guest systems, in addition to the binding equilibrium, the two other equilibria that exist due to the prototropic tautomerism of the free and bound forms of excited CZ are expressed as follows.

$$N \underset{}{\overset{K_T}{\rightleftharpoons}} T \quad (6)$$

$$C_N \overset{K'_T}{\rightleftharpoons} C_T \quad (7)$$

Here K_T and K'_T are the equilibrium constants for the transformation of the excited normal form, N^*, to the excited tautomer form, T^*, of free CZ and SCXn-CZ complex, respectively.

Considering that the absorption spectrum of CZ does not change to a very large extent in the presence of the hosts (cf. Fig. 1) and assuming that both N* and T* forms of CZ are equally quenched by SCXn, it can be derived that $K_T \propto I_T^0/I_N^0$ and $K'_T \propto I_T^\infty/I_N^\infty$ (Note S2, ESI†). From the experimentally observed intensity data and the fitted parameters (Table 1), the I_T^0/I_N^0 value for free dye is determined to be about 2.1, while the I_T^∞/I_N^∞ values for SCX4-CZ and SCX6-CZ systems are determined to be about 0.6 and 0.3, respectively. These values provide approximate estimates for the tautomerization equilibria of the free and bound dye, and confirm that when CZ is bound to the SCXn hosts, the transformation of the N* form of the dye to the T* form is indeed hindered very significantly. Surprisingly, it is found that although the binding constant of SCX4-CZ system is much higher (K_{eq}~127±9 M^{-1}; average of the values obtained from equations 4 and 5, cf. Table 1) than that of SCX6-CZ (K_{eq}=80±2 M^{-1}; average of the values obtained from equations 4 and 5, cf. Table 1), the prototropic

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tautomerization from N* to T* is inhibited to a much larger extent by the SCX6 host than SCX4.

The fact that the binding of CZ with the SCXn hosts inhibits the conversion of N* to T* rather than promoting it, together with the result that the SCX6 host, which has a lower binding affinity for CZ than SCX4, is able to arrest the conversion of N* to T* more efficiently compared to SCX4, suggests that it is not the inclusion of the guest into the hydrophobic host cavity per se, that affects the prototropic equilibrium. Therefore, it is necessary to consider other factors to explain these interesting and unusual observations made with the SCXn-CZ systems.

Taking into account the relative dimensions of the guest, and the hosts (Table S2, ESI†),[51,54,55] it may be anticipated that CZ cannot penetrate deep within the cavity of either of the SCXn hosts. The guest dye possibly remains close to the periphery of the cavitands, where the sulfonate groups of SCXn can provide suitable anchoring points for stabilization of the host-guest complexes through H-bonding interactions. However, since the upper rim diameter of SCX4 cavity is smaller than that of SCX6, a tight host-guest binding is more likely for SCX4-CZ system compared to SCX6-CZ, leading to a higher binding constant value for the former.

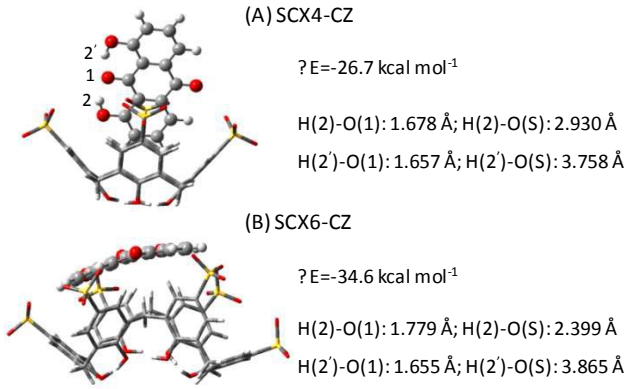
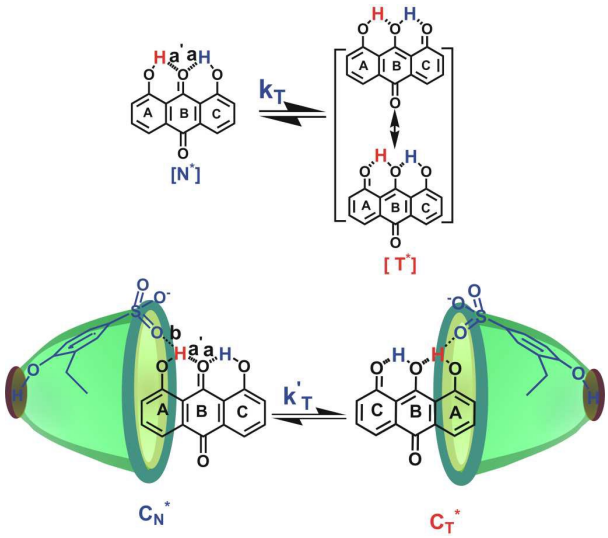


Fig. 4 Geometry optimized structures of the SCX4-CZ and SCX6-CZ host-guest complexes along with their corresponding stabilization energies and H-bond distances for the H atoms in the two hydroxyl groups of CZ. O(S) represents the oxygen atom of the host sulfonate group that is nearest to each of the indicated H atoms in CZ.

Since the low solubility of CZ in aqueous medium precluded us from obtaining any NMR signals, we resorted to quantum chemical calculations to understand the binding modes of CZ with the hosts and substantiate our propositions regarding the structures of the SCXn-CZ complexes. Such calculations are widely used to understand and complement host-guest interaction studies in many cases.[51,66-69] The most stable geometry optimized structures obtained for the 1:1 SCXn-CZ complexes are shown in Fig. 4. It is clearly seen that CZ is partly accommodated within the cavity of SCX4, but remains externally bound at the portals of SCX6, suggesting that the dye cannot experience much of the hydrophobic environments in either of the host-guest complexes.

Based on the aforementioned results that CZ resides in the vicinity of the portals instead of being embedded deeply within the host cavities, it is rationalised that rather than simple hydrophobic effects, it is the competition between intramolecular H-bonding within CZ and intermolecular H-bonding of CZ with the sulfonate groups of the hosts, that is mainly responsible for inhibition of the prototropic tautomerism in CZ by SCXn. As depicted in Scheme 2, in the free dye, the hydroxyl groups of either ring A or ring C can take part in the tautomerization reaction along the pre-existing intramolecular H-bonds with the carbonyl group (a and a'; both H-bonds being equivalent), thereby converting the N* form to the T* form in a facile manner. On binding to the hosts, it can be envisaged that there will be a strong tendency for CZ to form intermolecular H-bonds with the sulfonate groups present at the portals of the host. The formation of new H-bonds by the hydroxyl group of CZ that is suitably located in the vicinity of a sulfonate group of the host (depicted as b for the SCXn-CZ complex in Scheme 2), will quite expectedly lead to a weakening of the pre-existing intramolecular H-bonding of CZ. Consequently, the tautomerization of CZ from the N* form to the T* form may be anticipated to reduce considerably in SCXn-CZ systems (cf. Scheme 2) compared to that of free CZ, as observed in the present study.



Scheme 2 Schematic of intramolecular (a, a') and intermolecular (b) H-bonding in CZ and SCXn-CZ, respectively, and the tautomerization equilibria in each case.

These propositions are indeed corroborated by the quantum chemical calculations, where a clear trend is observed in the bond lengths for the intramolecular H-bonds of CZ vis a vis the intermolecular H-bonding in SCXn-CZ. From the geometry optimized structures of the SCXn-CZ complexes, it is revealed that the intramolecular H-bond distances between the central carbonyl group of CZ ($O(1)_{CZ}$) and the H-atoms of the two hydroxyl groups on either side ($H(2)_{CZ}$ and $H(2')_{CZ}$), are not of equal length in the bound dye, although these H-bonds are equivalent in free CZ. Due to the availability of another H-bond accepting oxygen atom (belonging to the sufonate group of the host) in the vicinity of the complexed

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dye, the H-atom of one of its hydroxyl groups forms a new alternate H-bond with the host and this leads to the elongation of the corresponding intramolecular H-bond. Thus, for both host-guest complexes (cf. Fig. 4) it is noticed that the $H(2)_{CZ}$-$O(S)_{SCXn}$ distance is less than $H(2')_{CZ}$-$O(S)_{SCXn}$ and accordingly, $H(2)_{CZ}$-$O(1)_{CZ}$ bond length is greater than $H(2')_{CZ}$-$O(1)_{CZ}$ bond length. Interestingly, the calculations also reveal that the elongation in the intramolecular H-bond of CZ occurs to a much larger extent in SCX6-CZ (1.655 Å to 1.779 Å) than in SCX4-CZ (1.657 Å to 1.678 Å), as the H-atom of the corresponding hydroxyl group of CZ and the O-atom of the sulfonate group of the host are more closely placed in the former than in the latter (intermolecular H-bond distances are 2.399 Å and 2.939 Å, respectively). This result is in accordance with the experimental observation that tautomerization of CZ is hindered to a larger extent in SCX6-CZ than in SCX4-CZ despite the binding constant value being higher for the latter. Therefore, the specific orientations of the host-guest systems and the relative propensities of intramolecular H-bonding within CZ and the intermolecular H-bonding between CZ and SCXn, essentially determines the prototropic tautomerism of CZ in the studied host-guest complexes.

The formation of SCXn-CZ intermolecular H-bonds is further substantiated by FTIR studies. The OH bending mode of CZ which appears around 1400 cm^{-1} for the free dye is shifted toward lower frequency (~1370 cm^{-1}) in the presence of both SCX4 and SCX6 (Fig. S7, ESI†), which is a signature of intermolecular H-bonding in the host-guest systems.[70,71]

To have an idea about the thermodynamics of the host-guest interaction, we estimated the binding constants at various temperatures ranging from 30°C to 60°C. The temperature dependent binding isotherms obtained by monitoring the emission intensity changes at the LWEB (515 nm) for SCX4-CZ and SCX6-CZ, are shown in the Figs. 5A and B. The binding isotherms obtained by monitoring the emission intensity changes at the HWEB (585 nm) for both the systems are shown in Fig. S8, ESI†. Since the ratio between the intensities of the normal and tautomeric forms of free CZ remains invariant with temperature, it was assumed that the same would be true for the bound dye as well. Accordingly, the binding constants estimated from the fitted curves using equations 4 and 5, are listed in Table S3, ESI†. It is observed that the K_{eq} values decrease systematically with increase in the temperature. The entropy and enthalpy changes for the binding of CZ to SCX6 and SCX4 were determined following the van't Hoff equation (equation 8),[72] and the corresponding plots are shown in the insets of Fig. 5 (and insets of Fig. S8, ESI†).

$$\ln K_{eq} = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} \qquad (8)$$

The calculated thermodynamic parameters presented in Table 2 (obtained from the temperature dependent changes in K_{eq}), indicate that interactions between SCXn and CZ are mainly driven by favorable enthalpy changes accompanied by small entropy losses.

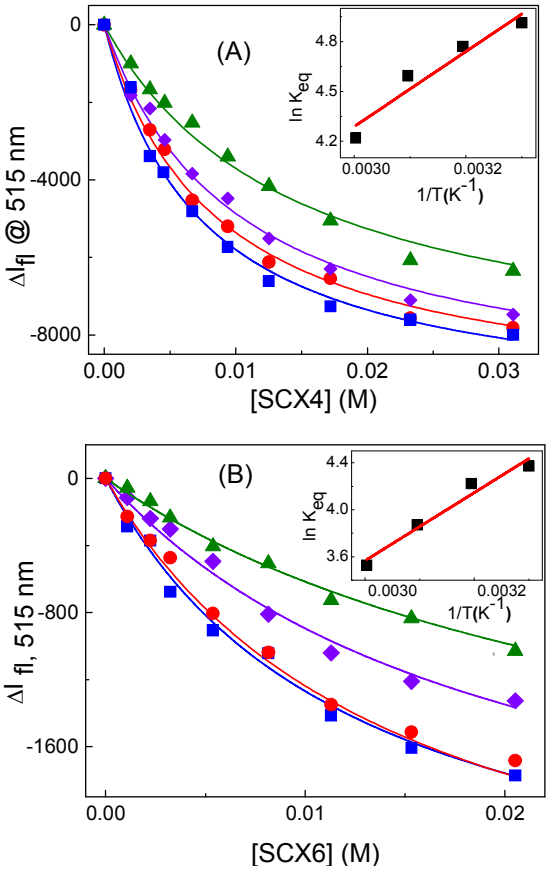

Fig. 5 Binding isotherms for (A) SCX4-CZ and (B) SCX6-CZ systems at 30°C (■), 40°C (●), 50°C (▼) and 60°C (▲). Insets show the corresponding van't Hoff plots.

The negative enthalpy change originates from H-bonding, electrostatic and van der Waals interactions,[73-80] and suggests that a combination of these favourable noncovalent forces drives the complexation between CZ and the hosts. The entropy change, on the other hand, originates from the interplay between entropic gain due to release of well-ordered water molecules initially surrounding the individual host and guest molecules (desolvation effect), and the entropic loss due to decrease in the motional freedoms upon mutual complexation (configurational effect).[73-78] In the present systems, the latter effect seems to have a predominant contribution, resulting in an overall entropy loss. Since, SCXn are known to be flexible molecules that can adopt different conformations,[78] the loss in motional freedom on binding with the guest is quite understandable. The larger entropy loss for SCX6-CZ system than for SCX4-CZ (cf. Table 2) is in accordance with the greater flexibility of the higher homologue of the host. The enthalpy change is also observed to be higher for the SCX6-CZ system than for SCX4-CZ, which correlates well with the internal energy changes obtained from quantum chemical calculations (cf. Fig. 4). Overall, however, the Gibbs free energy change, $\Delta G^0=\Delta H^0-T\Delta S^0$, is estimated to be higher for the SCX4-

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CZ complex (ΔG^0 ~-14 kJ mol^{-1}) than the SCX6-CZ complex (ΔG^0 ~-11.8 kJ mol^{-1}), which is in agreement with the higher binding constant for the former complex than the latter.

Table 2 Thermodynamic parameters for the SCXn-CZ systems (T=298 K).

system	parameters obtained from van't Hoff analysis	
	K_{eq} determined by fitting to eq. 4	K_{eq} determined by fitting to eq. 5
SCX4-CZ	ΔH^0 = -18.8 kJ mol^{-1}	ΔH^0 = -21.7 kJ mol^{-1}
	$T\Delta S^0$ = -6.1 kJ mol^{-1}	$T\Delta S^0$ = -6.4 kJ mol^{-1}
	ΔG^0 = -12.7 kJ mol^{-1}	ΔG^0 = -15.3 kJ mol^{-1}
SCX6-CZ	ΔH^0 = -24.4 kJ mol^{-1}	ΔH^0 = -23.8 kJ mol^{-1}
	$T\Delta S^0$ = -12.5 kJ mol^{-1}	$T\Delta S^0$ = -12.2 kJ mol^{-1}
	ΔG^0 = -11.9 kJ mol^{-1}	ΔG^0 = -11.6 kJ mol^{-1}

Conclusions

Our study reveals that the facile prototropic transformation of CZ is considerably inhibited when the dye is bound to SCXn hosts. This result is contradictory to usual expectations because binding of CZ within the relatively hydrophobic host cavity should have facilitated its tautomerization from N* form to the T* form instead of inhibiting the process, as T^* is less dipolar in character than the N^* form. Furthermore, it is observed that the tautomerization equilibrium of CZ is more severely hindered in the SCX6-CZ system than in SCX4-CZ, although the binding affinity of the former host-guest pair is lower. All of these unusual effects are proposed to arise due to the specific intermolecular H-bonding interactions of CZ with the portals of SCXn hosts, rather than due to the inclusion of the dye within the host cavities. The formation of new intermolecular H-bonds between the hydroxyl groups of CZ and the sulfonate groups present at the portals of the hosts, effectively disrupts the pre-existing intramolecular H-bond network in CZ, and consequently hinders the tautomerization process. Moreover, the proximity of CZ near the portals of the larger host SCX6, allows the intermolecular H-bonding to be more efficient in the SCX6-CZ system than in SCX4-CZ. As a result, the tautomerism from N* to T* form of CZ is reduced to a larger extent in the former case, despite its lower binding affinity. The present results emphasize that it is not only the localized binding pockets provided by biological environments, but also the orientations and specific interactions, that must be considered in interpreting the course of any tautomeric equilibria occuring in natural systems. These additional parameters influence the tautomerization of vital biomolecules and thereby impart selectivity to various biochemical processes.

Conflicts of interest

There are no conflicts of interest to declare.

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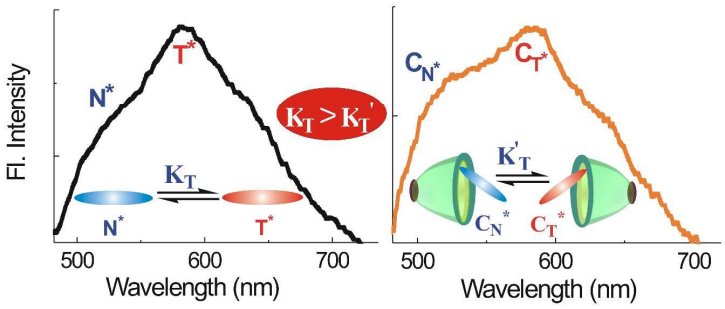
Graphical Abstract



This study reveals the unusual inhibition of excited-state prototropic tautomerism of Chrysazine by *p*-sulfonatocalix[4,6]arene hosts.