Supramolecular Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713649759

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First published on: 13 April 2007

To cite this Article Kemp, Sharon , Wheate, Nial J. , Stootman, Frank H. and Aldrich-Wright, Janice R.(2007) 'The Host-Guest Chemistry of Proflavine with Cucurbit[6,7,8]urils', Supramolecular Chemistry, 19: 7, 475 — 484, First published on: 13 April 2007 (iFirst)
To link to this Article: DOI: 10.1080/10610270601124019
URL: http://dx.doi.org/10.1080/10610270601124019

Supramolecular Chemistry, October–November 2007 Vol. 19(7), pp. 475–484

The Host-Guest Chemistry of Proflavine with Cucurbit[6,7,8]urils

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(Received 17 August 2006; Accepted 15 November 2006)

The binding of the polyaromatic guest, 3,6-diaminoacridine (Proflavine) to cucurbit[*n*]uril (CB[*n*]) where $n = 6, 7$ and 8 has been studied by fluorescence spectrophotometry and binding constants determined using a least squares fitting method. Titration of CB[8] into a solution of Proflavine results in a 95% decrease in fluorescence up to a CB[8] to Proflavine ratio of 2:1. From the induced fluorescence spectra a binding constant of $1.9 \times 10^7\,M^{-1}$ was determined. When Proflavine is titrated into a solution of CB[8] a similar binding constant is calculated ($1.3 \times 10^7\,M^{-1}$). Titration of CB[6] into a solution of Proflavine yields a decrease in fluorescence of 18–20%, but no binding is observed beyond what is seen within experimental error. Finally, titration of CB[7] into a solution of Proflavine results in an increase in fluorescence (32%) and a blue-shift of the emission wavelength from 509 nm to 485 nm. From the induced fluorescence spectra a binding constant of $1.65 \times 10^7\,M^{-1}$ was determined. From ^{1}H NMR it appears that the decrease in fluorescence for Proflavine with CB[6] and CB[8] is due to collisional quenching, whereas the increase in fluorescence with CB[7] may be due to rotational restriction.

Keywords: Proflavine; DNA intercalator; Cucurbituril; Flourescence spectroscopy; Binding constant; 1H NMR

INTRODUCTION

Cucurbit[*n*]uril (CB[*n*]) is a small barrel-shaped organic molecule made from the condensation of glycoluril and formaldehyde in concentrated acid solutions [1]. In recent years, the CB[*n*] family has been expanded to include CB[*n*] of different sizes (where $n = 5–8, 10$) [1–4], substituted and partially-substituted CB[*n*] [5,6], partially-inverted CB[*n*] [7], and hemicucurbit[*n*]urils [8,9].

All cucurbit[*n*]urils have hydrophilic oxygen-lined portals and a hydrophobic cavity within which these molecules are able to bind a variety of guests [10]. Encapsulation within the cavity is stabilized by hydrogen bonding and/or electrostatic interactions between guests and the CB[*n*] portals and/or hydrophobic interactions between the guest and the inner walls of CB[*n*] [10]. The CB[n] family can form host-guest complexes with a wide range of compounds, thus giving it a large number of applications [11], including: entrapment of gases and pollutants [12,13], as a stationary phase in column chromatography [14], in drug delivery [15–17], and as nanomachine components [18,19].

The encapsulation of guests by CB[*n*] can be monitored via a number of spectroscopic techniques including: X-ray diffraction, nuclear magnetic resonance spectrometry, UV-visible spectrophotometry, mass spectrometry and fluorescence spectrophotometry. In the latter case, the guest molecule is inherently fluorescent [19–23], or a modified CB[*n*] containing fluorescent chromophores is used [24,25]. The change in the fluorescence of either the guest or the modified CB[*n*] is highly dependent on both the nature of the guest and the CB[*n*] size. Fluorescence intensity may either increase or decrease upon encapsulation of the guest within CB[*n*] [20,21,23–26], and there may also be a concurrent blue or red shift of the emission peak [19,20,23,26].

In this study the encapsulation of 3,6-diaminoacridine (Proflavine) in different sized cucurbit[6,7 and 8]urils (Fig. 1) using fluorescence spectrophotometry is reported. The binding constants were simultaneously determined at all wavelengths on each emission peak from the induced fluorescence spectra using a least squares fitting method. ^{1}H NMR was then used to explain the differences in fluorescence spectra between CB[7] and CB[6 and 8].

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ISSN 1061-0278 print/ISSN 1029-0478 online © 2007 Taylor & Francis
DOI: 10.1080/10610270601124019

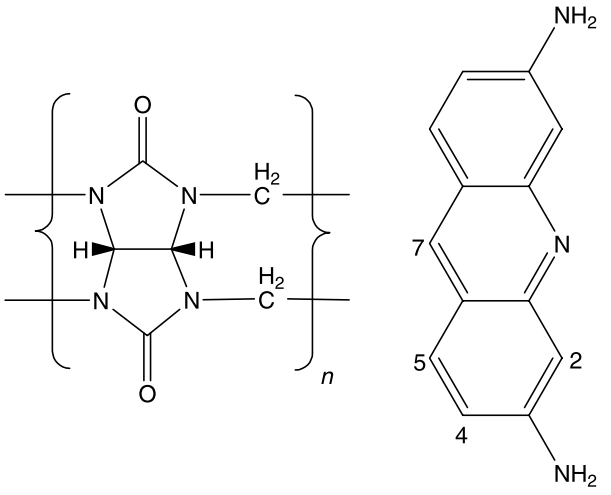

FIGURE 1 The chemical structures of cucurbit[*n*]uril (where $n = 6$, 7 or 8) and 3,6-diaminoacridine (Proflavine), showing the proton numbering scheme used for nuclear magnetic resonance spectrometry.

RESULTS AND DISCUSSION

Initially, ethidium bromide was used to study the host-guest interactions of a DNA intercalator with CB[*n*], but upon addition of an equimolar amount of CB[7] there was no change in either the wavelength (617 nm) nor the intensity of the ethidium fluorescence spectrum. It is unclear why there was no change, but the curved shape of the ethidium may prevent it from properly encapsulating. Instead 3,6-diaminoacridine (Proflavine), a synthetic acridine dye used as a powerful antiseptic during World War II, was used [27]. In recent years it has been studied extensively in novel RNA-targeted antiviral drugs and as an intercalator in cancer treatments [28,29]. Proflavine is a straight, three chromophore compound with terminal amino groups capable of stabilising encapsulation within CB[*n*] by hydrogen bonding to portal-oxygens [30]. Native Proflavine fluoresces at 510 nm from an excitation wavelength of 400 nm.

The addition of an excess amount of CB[8] to Proflavine (2:1) results in a 95% decrease in fluorescence intensity, but no significant change (<1 nm) in fluorescence wavelength (Fig. 2a). Subtraction of the spectrum of Proflavine in the absence of CB[8] from the spectra gives the induced fluorescence spectra (IFS; Fig. 2b). Whilst a binding curve can be generated directly from the fluorescence spectrum for CB[8], the method utilized here calculates the binding constant at a set wavelength [31]. Since any change in the emission wavelength will affect the binding constant, the use of IFS negates any possible change in wavelength, allowing the calculation of K_b at any given point on an emission peak. From the IFS a binding curve and corrected binding curve for Proflavine and CB[8] were plotted (Fig. 3). The fact that the slope of the binding curve never approaches zero is of interest.

The need for a corrected binding curve is better illustrated when the CB[8]-Proflavine titration is reversed, with Proflavine being added incrementally into a fixed concentration of CB[8]. As can be seen in Fig. 4a, fluorescence due to Raman scattering, at an excitation wavelength of 400 nm, is clearly observed. The use of IFS negates the fluorescence attributable to Raman scattering (Fig. 4b). With increasing Proflavine concentration the fluorescence at 509 nm increases. Beyond the saturation point, the fluorescence

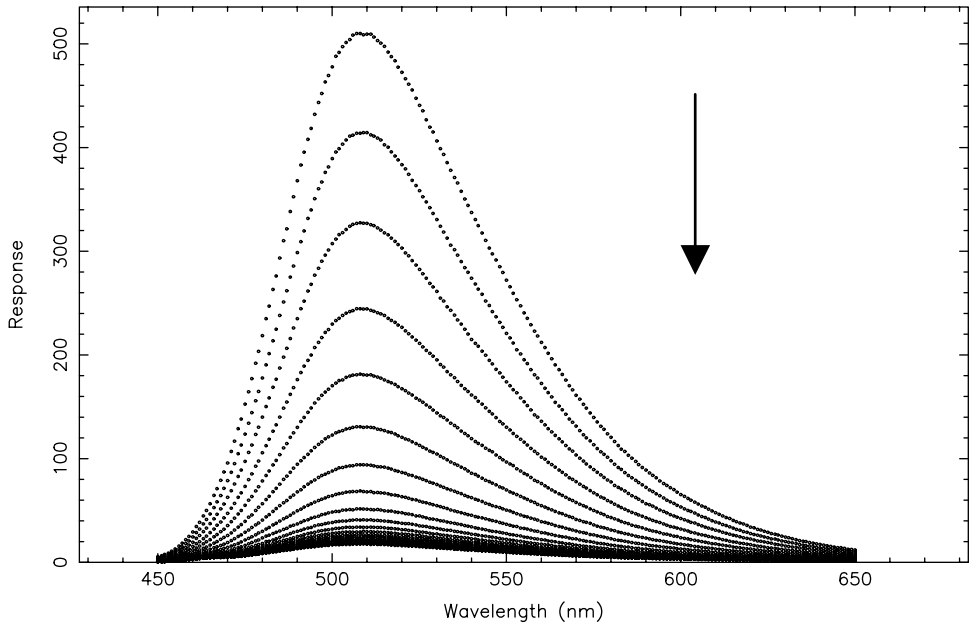

FIGURE 2A The fluorescence spectra of Proflavine (2.7 μM) showing the decrease in fluorescence intensity with increasing CB[8] concentration (up to 5.4 μM).

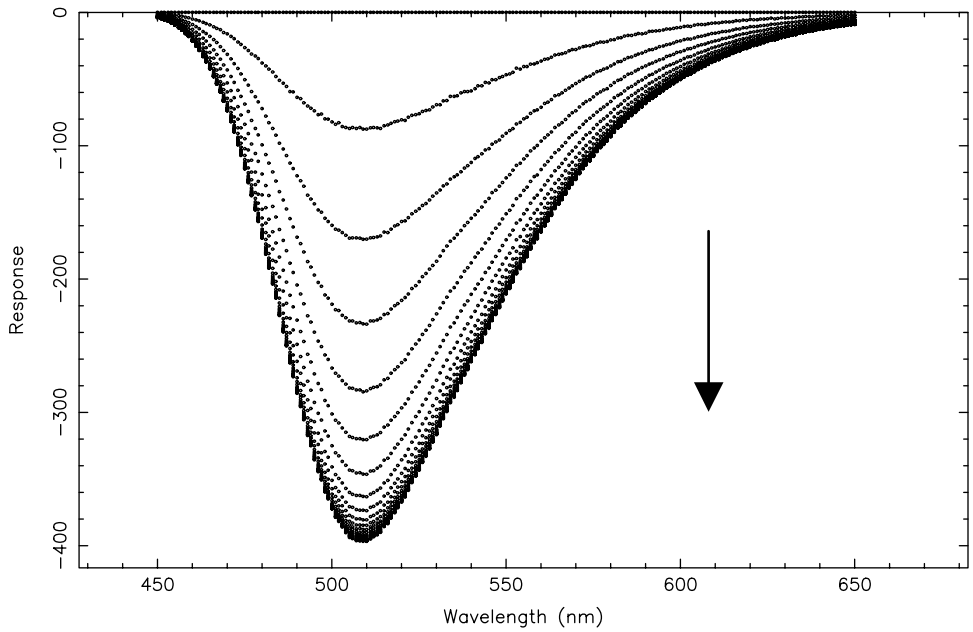

FIGURE 2B The induced fluorescence spectra (IFS), obtained by subtracting the fluorescence spectrum of Proflavine in the absence of CB[8] from the spectrum at varying concentrations of CB[8].

continues to increase linearly. Subtraction of the linear increase from the graph produces an ideal binding curve and removes the component of free Proflavine from the graph.

From the titrations of CB[8] and Proflavine, binding constants of $1.9 \times 10^7\,M^{-1}$ and $1.3 \times 10^7\,M^{-1}$ were calculated. This level of binding is consistent with the binding of a variety of guests within cucurbit[*n*]uril. Nile red is encapsulated by CB[6] with a K_b of $8.2 \times 10^6\,M^{-1}$ [25]; biologically relevant molecules such as amino acids and nucleobases by a CB[6] analogue with values between $10^3\,M^{-1}$ to $10^6\,M^{-1}$ [24]; 2-aminoanthracene by CB[7] $8 \times 10^5\,M^{-1}$ [19]; and 2,3-diazabicyclo[2.2.2]oct-2-ene by CB[7] with a K_b of $4 \times 10^5\,M^{-1}$ [22]. Analysis of the non-linear double reciprocal plot indicates 2:1 Proflavine to CB[8] binding at low concentrations of CB[8], but 1:1 at higher concentrations (supplementary information) [20]. Therefore, the binding constants calculated here represent only

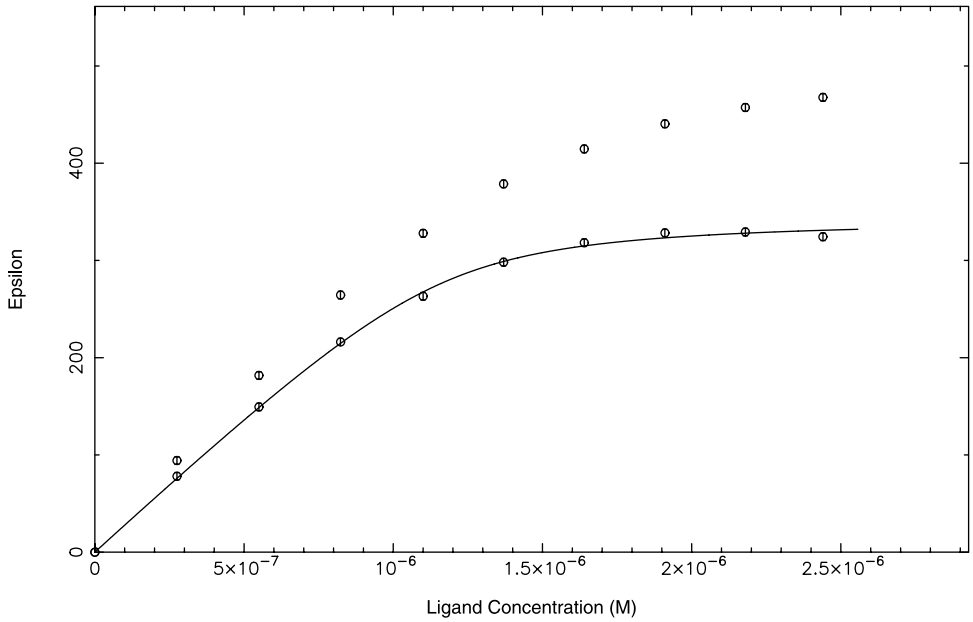

FIGURE 3 The binding curve (dots and filled line) and corrected binding curve (dots and filled line) of the titration of CB[8] (up to 5.4 μM) into a solution of Proflavine (2.7 μM) calculated at a wavelength of 509 nm. The corrected binding curve is obtained by subtracting the slope of the line beyond the saturation point from the overall graph.

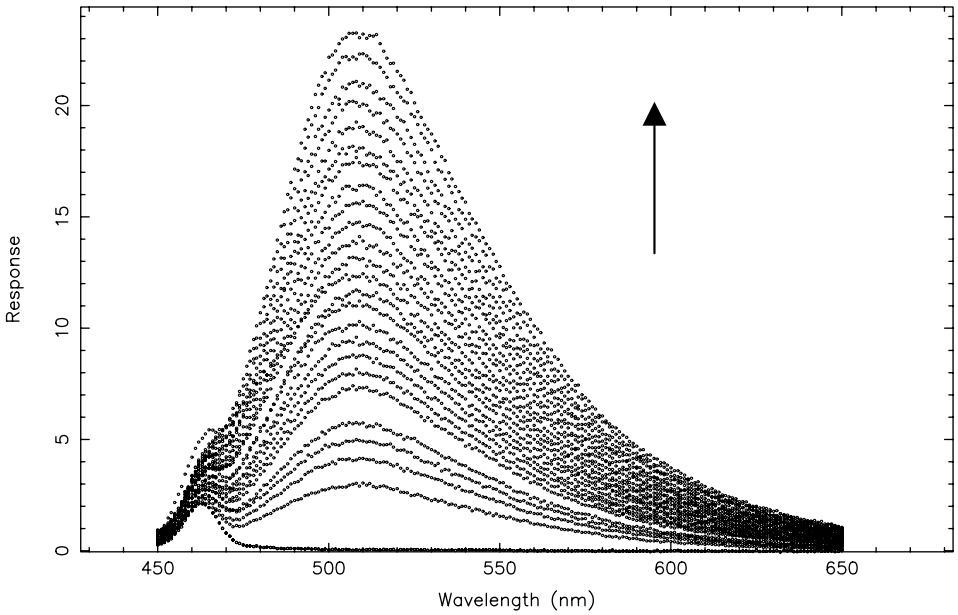

FIGURE 4A The fluorescence spectra of CB[8] and Proflavine showing the increase in intensity with the titration of Proflavine (up to 5.4 μM) into a fixed concentration of CB[8] (2.7 μM). The inherent fluorescence due to Raman scattering by water is observed through the small peak at 463 nm, in the absence, or at low concentrations, of Proflavine.

the overall binding strength of Proflavine to CB[8] and not the binding constants for separate 2:1 and 1:1 binding events.

The addition of CB[6] results in a smaller decrease in fluorescence intensity compared to CB[8], and is in the order of 18–20%. The K_b value for CB[6] is very low ($2.0\,M^{-1}$) which, within experimental error, indicates no interactions between Proflavine and CB[6] at all. This is in contrast to curcumin and CB[6] where portal binding results in K_b values of $72\,M^{-1}$ and $260\,M^{-1}$ for individual binding events [23].

The addition of an equimolar concentration of CB[7] to Proflavine results in both an increase in fluorescence intensity (32%) and a blue-shift of the emission maximum from 509 nm to 485 nm (Fig. 5a). By subtracting the fluorescence spectrum of Proflavine in an absence of CB[7] from the spectra with increasing CB[7] concentration, IFS are obtained (Fig. 5b). This gives two peaks, a positive peak at 475 nm and a negative peak 543 nm, at which a binding curve, and from these, a K_b can be calculated. From the binding curve of fluorescence

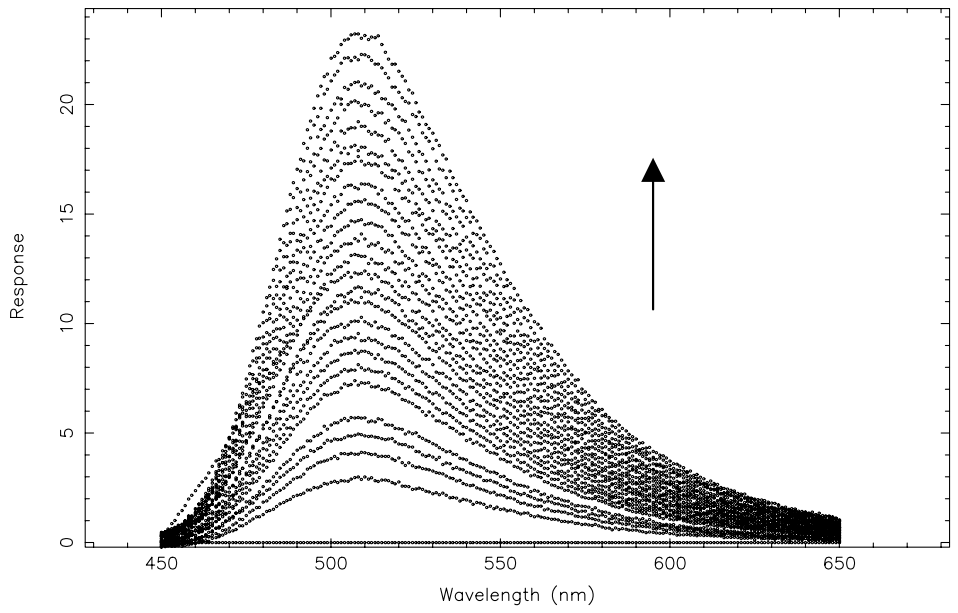

FIGURE 4B The induced fluorescence spectra of CB[8] and Proflavine which negates the fluorescence effect attributable to Raman scattering and allows the generation of a corrected binding curve from the increase in fluorescence intensity from the Proflavine only.

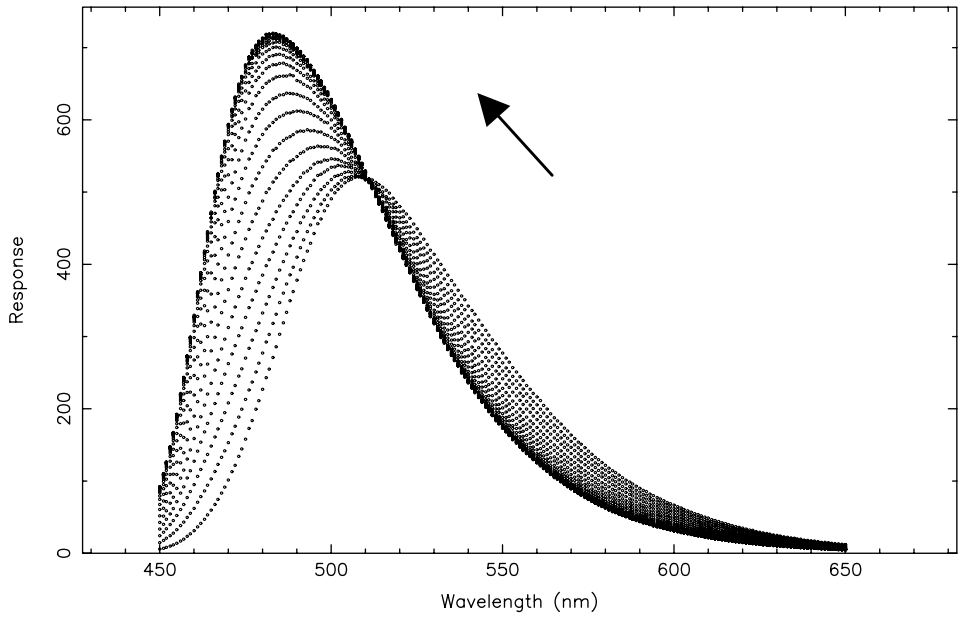

FIGURE 5A The fluorescence spectra of Proflavine (2.7 μM) and CB[7] showing the increase in fluorescence intensity and the wavelength blue-shift from 509 nm to 485 nm with increasing CB[7] concentration (up to 5.4 μM).

at 475 nm the K_b of Proflavine and CB[7] is $2.0 \times 10^7\,M^{-1}$ (Fig. 6). A similar value is obtained when calculated at 543 nm ($1.3 \times 10^7\,M^{-1}$). As stated previously, K_b can be calculated at any point on an emission peak, not necessarily the maximum [31]. Analysis of the linear double reciprocal plot, which is linear, indicates 1:1 Proflavine to CB[7] binding at all concentrations (supplementary information) [20].

By plotting the K_b values calculated at every wavelength (1 nm increments) on the emission peaks the precision of the K_b values were determined. Figures 7a and 7b show a plot of K_b versus wavelength for CB[7] binding of Proflavine between the wavelengths of 520 nm to 577 nm and 462 nm to 492 nm, respectively. The small relative scatter (all within the same order of magnitude) therefore confirms the precision of the K_b values determined.

As the results show, the fluorescence spectrum of Proflavine changes differently with each different sized CB[*n*]. Addition of CB[6] and CB[8] decrease the fluorescence intensity, but to much different levels (18–20 and 95% respectively), whilst CB[7]

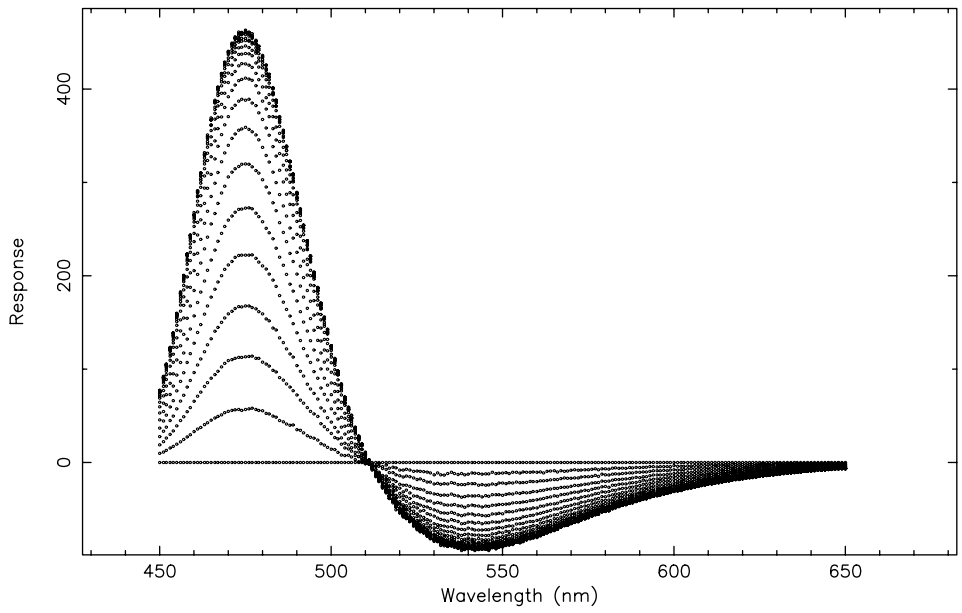

FIGURE 5B The induced fluorescence spectra of Proflavine, obtained by subtracting the fluorescence spectrum of Proflavine in the absence of CB[7] from the spectrum at varying concentrations of CB[7]. The induced fluorescence spectra gave one maxima (475 nm) and one minima (543 nm) at which K_b was calculated.

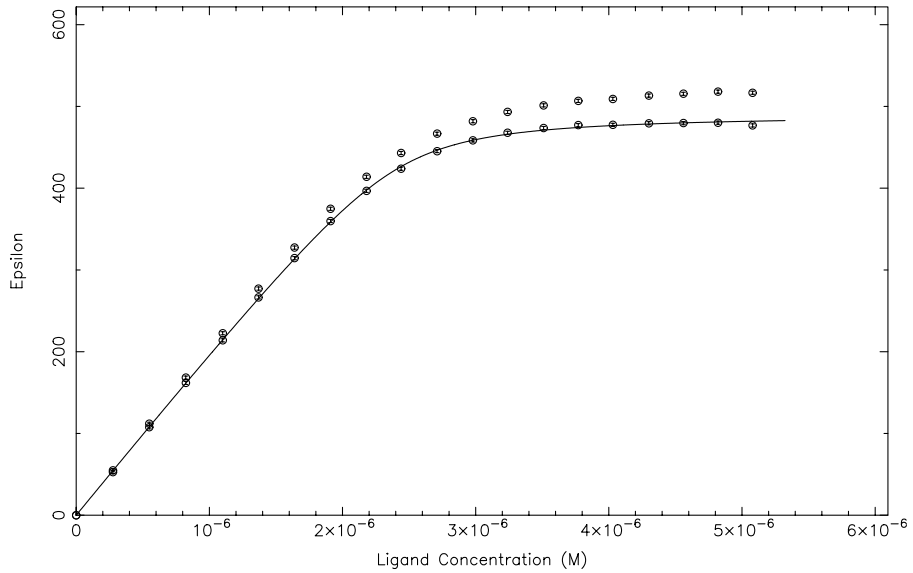

FIGURE 6 The binding curve (dotted line) and corrected binding curve (dotted and filled line) of the titration of CB[7] (up to 5.4 μM) into a solution of Proflavine (2.7 μM) calculated at a wavelength of 475 nm.

increases the intensity and causes a blue-shift in the fluorescence maxima. Since Proflavine has three basic functional groups and cucurbituril samples are known to contain acidic residues from their synthesis, it is possible that these results reflect differing proton transfer processes on Proflavine. As such, the fluorescence titration experiments were also completed in buffered solutions; however, in phosphate buffer, no difference in the fluorescence of Proflavine with CB[6], CB[7] or CB[8] compared to the fluorescence of the same host-guest complexes in water was observed. Therefore proton transfer processes are unlikely to account for differences in the fluorescence spectra.

The fluorescence of Proflavine in the presence and absence of CB[7,8] was also determined in ethanol solutions. Proflavine at equimolar concentrations is less fluorescent in ethanol than in water (12% decrease in intensity) and changes the emission wavelength from 509 nm to 492 nm, the latter being consistent with the fluorescence wavelength of Proflavine and CB[7] in water. Upon addition of an equimolar concentration of either CB[7] or CB[8] to Proflavine in ethanol there is a small increase

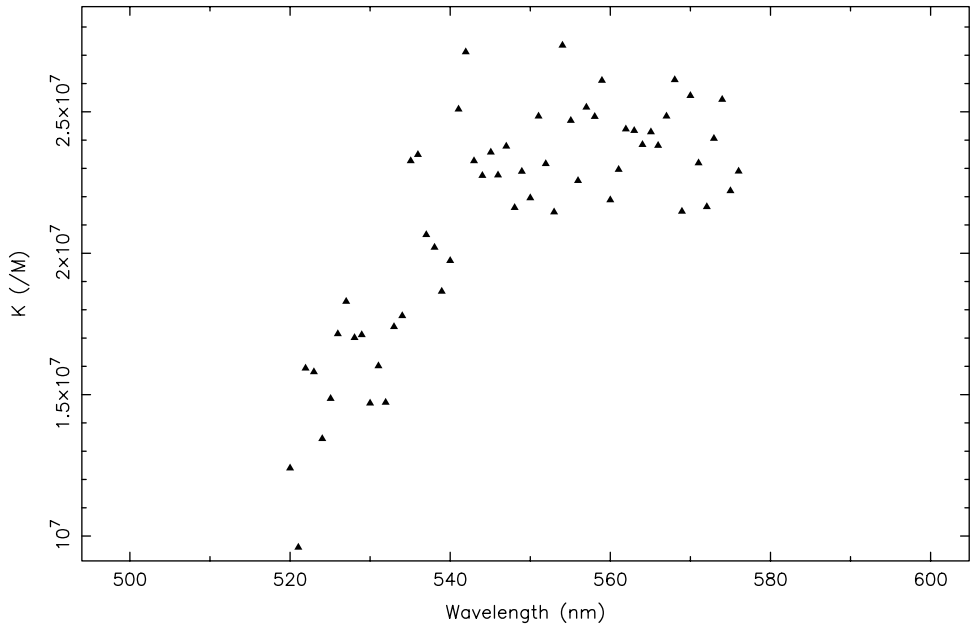

FIGURE 7A The variation of K_b from calculations made between the wavelengths of 520 nm and 577 nm of the induced fluorescence spectra of Proflavine (2.7 μM) and CB[7] (up to 5.4 μM).

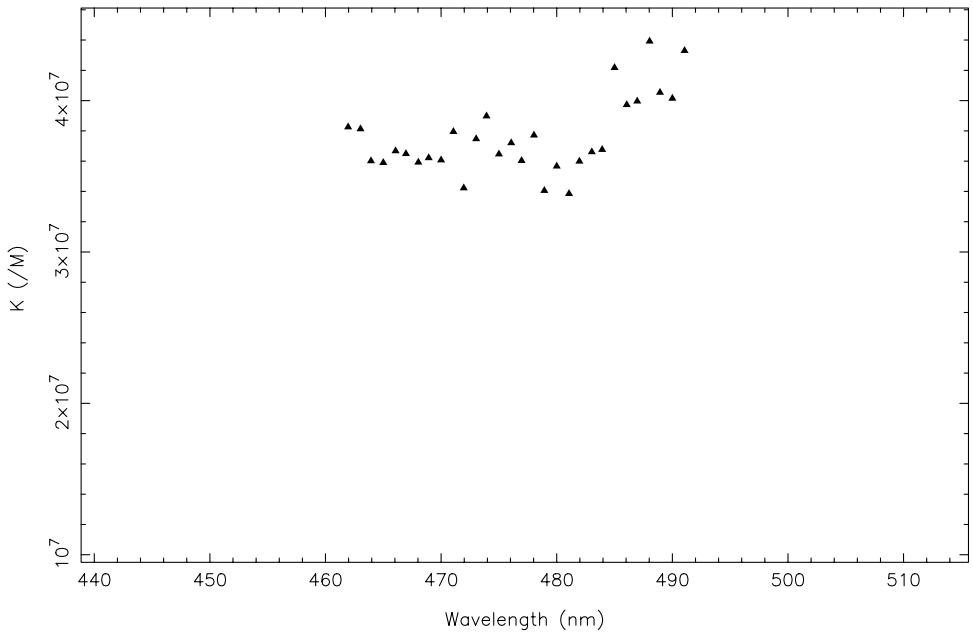

FIGURE 7B The variation of K_b from calculations made between the wavelengths of 462 nm and 492 nm of the induced fluorescence spectra of Proflavine and CB[7].

in fluorescence intensity (ca. 5%) but the emission intensity remains less than that of Proflavine in water and the emission wavelength remains at 492 nm.

The differences observed between CB[6], CB[7] and CB[8] can also be explained, in part, through ^{1}H NMR. The ^{1}H NMR proton resonances for Proflavine in CB[6,7 and 8] were assigned via NOESY and DQCOSY experiments. The addition of CB[6] to Proflavine produces no significant shifts in the proton resonances of Proflavine (see Table I), indicating that it is not encapsulated within the CB[6] cavity. Proflavine has a terminal nitrogen–nitrogen length of approximately 9.5 Å (a perfect length for cucurbit[*n*]uril which is 9.1 Å) but Proflavine is significantly wider (5.0 Å) than the width of the CB[6] portal (3.9 Å). Therefore, Proflavine can only portal bind with CB[6]. The linear decrease in the fluorescence intensity is most probably then a simple collisional quenching mechanism [32] through the oxygen-lined CB[6] portals. Because the amount of quenching is directly related to the CB[6] concentration, then the fluorescence intensity decreases linearly with increasing CB[6]. Simple collisional quenching, as is the case here, does not generally change the fluorescence wavelength [32].

From the ^{1}H NMR spectrum of Proflavine and CB[7] at a ratio of 1:1, the Proflavine proton resonances move downfield, a most unexpected result (see Table I). The H7 resonance moves 0.66 ppm, the H2 and H5 by 0.39 ppm and 0.23 ppm respectively, and H4 by 0.13 ppm. Importantly, only one set of aromatic resonances is observed, indicating symmetrical encapsulation of Proflavine. The pKa of the central nitrogen atom of Proflavine is 9.4 [33], whilst the pKa of the terminal amino groups is around 2.5. From pH-NMR titration experiments, increasing the pH of a solution of Proflavine to around 9.4 in the absence

TABLE I The ^{1}H NMR chemical shifts of free Proflavine and Proflavine added to CB[6,7 and 8] at various ratios. All values are reported as ppm, referenced to the solvent peak. The change in chemical shift relative to free Proflavine is given in brackets. A negative sign indicates a downfield shift.

			Proflavine encapsulated within CB[n] at different added ratios				
			1:1				2:1
Proton	Proflavine (pH ~ 5)	Proflavine (pH ~ 10)	CB[6]	CB[7]	CB[8] - sharp	CB[8] - broad	CB[8]
H7	7.83	8.63 (−0.80)	7.83 (0.00)	8.49 (−0.66)	8.01 (−0.18)	7.48 (+0.35)	8.08 (−0.25)
H5/H9	7.21	7.85 (−0.64)	7.21 (0.00)	7.44 (−0.23)	7.09 (+0.12)	6.81 (+0.40)	7.15 (+0.06)
H4/H10	6.60	7.07 (−0.47)	6.62 (−0.02)	6.74 (−0.13)	6.56 (+0.04)	6.23 (+0.38)	6.64 (−0.04)
H2/H12	5.87	6.97 (−1.1)	5.90 (−0.03)	6.27 (−0.39)	5.55 (+0.32)	5.86 (+0.32)	5.91 (−0.04)

of CB[*n*], induces large downfield shifts of the Proflavine resonances.

From this result it is proposed that encapsulation within the CB[7] cavity promotes deprotonation of the central nitrogen atom; lowering its pKa. This would explain the large downfield movement of the Proflavine resonances, even though upfield movements are expected upon encapsulation. The deshielding effect of Proflavine deprotonation appears to have a larger effect on proton resonance chemical shift than the shielding effect from encapsulation within CB[7]. Therefore, the net effect is a downfield shift of the Proflavine resonances. Unfortunately, this does not explain why CB[7] induces a change in the fluorescence wavelength and an increase in fluorescence intensity. When the pH of Proflavine is either increased to pH 12, or lowered to a pH of 2 in the absence of CB[*n*], the effect in both cases is to decrease the fluorescence intensity, with no significant change in fluorescence wavelength (<2 nm). The difference in the fluorescence spectra of CB[7] compared to CB[6] and CB[8] is therefore most likely due to rotational restriction; either of the whole molecule within the CB[7] cavity or just the terminal amino groups at the CB[7] portals. With a width of 5 Å, Proflavine is a near perfect fit through the CB[7] portals (5.4 Å) [10].

The ^{1}H NMR spectra of CB[8] and Proflavine further support this model. At a Proflavine to CB[8] ratio of 1:1, there are two sets of peaks in the spectrum; a broad set of resonances representing encapsulation of two Proflavine molecules per CB[8], and a set of sharp resonances that represent 1:1 binding. Upon the addition of excess CB[8] up to a CB[8] to Proflavine of 2:1 the broad resonances disappear and the sharp resonances move downfield and approach the chemical shifts of free Proflavine. The chemical shifts of the Proflavine resonances at added Proflavine to CB[8] of 2:1 are much further downfield from where encapsulated resonances would be expected and indicates that CB[8] also promotes deprotonation of the central nitrogen atom. Therefore it is proposed that the differences in the fluorescence spectra of Proflavine in CB[7] and CB[8] are not due to this charge effect.

The mechanism by which Proflavine is quenched is charge transfer [34]. This can occur from Proflavine forming non-fluorescent dimers or removal from an aqueous solvent into a low-polarisable guest (typically DNA) [34–37]. The decrease in Proflavine fluorescence when bound to CB[8] is certainly consistent with these results particularly given the low polarisability of cucurbiturils [38], and the large cavity of CB[8] which can stabilise and hold Proflavine dimers. In contrast, while a decrease in Proflavine fluorescence would be expected when bound to CB[7], it is proposed that the observed increase in fluorescence and change in emission wavelength is dominated by rotation restriction of the terminal amino groups, giving rise to twisted intramolecular charge transfer (TICT). Whilst TICT has not previously been observed for Proflavine, nor discounted, TICT has been observed for another amino-containing aromatic compound, *p*-dimethylaminobenzoic acid, inside a molecular container (β-cyclodextrin) [39]. Similarly, a blue-shift and increase in fluorescence were observed upon encapsulation, consistent with the results obtained for Proflavine and CB[7].

CONCLUSIONS

In this study the encapsulation of 3,6-diaminoacridine (Proflavine) in different sized cucurbit[6,7 and 8]urils has been reported. Binding constants were determined using a least squares fitting method from the induced fluorescence spectra. The binding constant of Proflavine in CB[7] and CB[8] is similar ($\sim 10^7\,M^{-1}$) whilst the small portal size of CB[6] effectively prevents Proflavine encapsulation. Interestingly, encapsulation of Proflavine within CB[7] results in an increase in fluorscence intensity and a blue-shift of the fluorescence peak, whereas encapsulation within CB[8] results in a decrease in fluorescence intensity. From the ^{1}H NMR spectra it was observed that encapsulation within CB[7] or CB[8] promotes deprotonation of the central nitrogen atom of Proflavine leading to unexpected downfield shifts of the Proflavine proton resonances. The differences in the fluorescence spectra of Proflavine in CB[7] and CB[8], however, are not due to this charge effect, but due to ease of rotation of Proflavine within CB[*n*] or rotation of the Proflavine terminal amino groups at the CB[*n*] portals.

EXPERIMENTAL SECTION

Materials

NaOD, DCl and Proflavine were purchased from Aldrich, while cucurbit[6,7 and 8]urils were a gift from Dr Anthony Day (UNSW), which were made as previously described [1].

NMR

One- and two-dimensional NMR spectra were obtained on a 300 MHz Oxford NMR spectrometer in D_2O, referenced internally to the solvent. NMR were run at 25°C unless otherwise stated. For one-dimensional spectra, a spectral width of 7,000 Hz was used with 50,000 data points and a relaxation delay of 3.7 s. Two-dimensional nuclear overhauser effect (NOESY) spectra were obtained using

a 3700 Hz spectral width with 256 increments in the t1 dimension, 2048 points in the t2 dimension and a mixing time of 0.3 s, by the method of States *et al.* [40]. Two-dimensional double quantum correlated spectroscopy (DQCOSY) experiments were recorded over a spectral width of 3700 Hz using 256 increments in the t1 dimension, 2048 points in the t2 dimension and a pulse repetition delay of 3.0 s. Cucurbit[*n*]uril to Proflavine ratios were determined by integration.

Fluorescence Spectrophotometry

Spectra were recorded on a Varian Cary Eclipse Fluorescence spectrophotometer, in 1 cm quartz cuvettes operating at room temperature. The excitation wavelength was 400 nm, and emissions were recorded from 450 nm to 650 nm, using slit widths of 5 nm. The binding constant (K_b) of Proflavine with CB[*n*] was determined using Eq. 1 [31]:

$$y = 0.5R\left\{A + B + x - \sqrt{(A + B + x)^2 - 4Bx}\right\} \quad (1)$$

where Proflavine is titrated into CB[*n*]: $A = 1/K_b$, B is the concentration of CB[*n*], x is the variable concentration of Proflavine, y is the fluorescence response, and R is a scaling factor. Where CB[*n*] is titrated into Proflavine: B is the concentration of Proflavine and x is the variable concentration of CB[*n*].

Fluorescence Titration Experiments

Stock solutions of Proflavine (0.16 mM) and cucurbit[6,7,8]uril (0.16 mM) were prepared in Milli-Q water, ethanol or phosphate buffer (1 mM) containing NaCl (13.2 mM). In the case of CB[6], 20 mM NaCl was used to aid dissolution. 50 μL of the Proflavine solution was pipetted into a 1 cm quartz cuvette and 2900 μL of water added. CB[6,7,8] was then titrated into the Proflavine solution up to a CB[*n*]:Proflavine ratio of 2:1. Alternatively, CB[6,7,8] (50 μL) was pipetted into a cuvette and 2900 μL of water added before Proflavine was titrated into the solution, up to a Proflavine:CB[*n*] ratio of 2:1. The final concentrations of Proflavine and CB[6,7,8] used in the fluorescence experiments were between 2.7 μM and 5.4 μM.

pH NMR Titrations

Stock solutions of Proflavine (600 μL, 39 mM) in D_2O were prepared in NMR sample tubes. Deuterium chloride, DCl, (35 wt. %) in D_2O or sodium deuteroxide, NaOD, (40 wt. %) was then titrated in 1 μL increments into the Proflavine solution until pH 2 or pH 12 was reached respectively.

pH Fluorescence Titrations

To a stock solution of Proflavine (2900 μL, 2.7 mM) HCl (1 M) or NaOH (1 M) was added in (1–5) μL increments until pH 2 or pH 12 was reached.

SUPPLEMENTARY INFORMATION

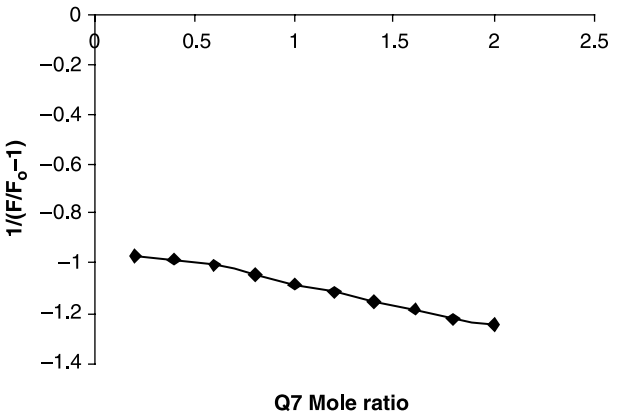

FIGURE S1 The double reciprocal plot of Proflavine and CB[7] which indicates 1:1 binding at all concentrations.

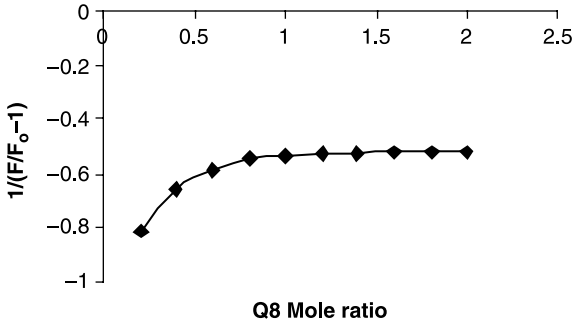

FIGURE S2 The double reciprocal plot of Proflavine and CB[8]. The non-linear nature of the graph indicates 2:1 Proflavine to CB[8] at low concentrations but 1:1 binding at higher concentration of CB[8].

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