SUPPORTING INFORMATION

Benzimidazole-Piperazine-Coumarin/Cucurbit[7]uril Supramolecular PET Fluorochrome for Detection of Carnosol by Stimuli-Responsive Dye Displacement and $pK_a$ Tuning

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**Part I: Interaction of the indicator dye with CB7 and pKₐ determination**

**Figure S1.** Evolution of the UV–visible absorption spectra of (a) 4PBZC (35 µM) and (b) 4PBZC/CB7 (35 µM 4PBZC + 500 µM CB7) upon changing the pH in the range 3–9 in aqueous solution at 298 K.

The formation of host–guest inclusion complexes between 4PBZC and 4PBZH⁺C and CB7 was confirmed by UV–visible and NMR titrations at different pH (pD) values. The appearance of isosbestic points at 300 nm (Figure S2a) and the fitting analysis confirmed the 2:1 binding stoichiometry for the 4PBZH⁺C/CB7 complex, see Experimental Section. The di-protonated dye enters the CB7 cavity through its BZ and coumarin rings (Figure S4). Inclusion was also confirmed because two proton resonances, H-1, and H-2, shifted upfield by approximately 0.4–0.9 ppm with the addition of 1 equivalent of CB7, while other proton resonances H-8, H-9, H-3, and H-4 slightly shifted to higher ppm upon addition of one equivalent of CB7 whereas H-6, H-7 and the methyl protons exhibited insignificant shifts. Furthermore, consequently to the addition of 2.0 molar equivqlent of CB7, two resonance proton H-6, H7 and the methyl proton shifted upfield, indicating the encalpsulation of the coumarin unit (Figure S4).
Figure S2. Variations in the UV–visible absorption spectra of 5 μM of 4PBZH⁺C at pH 3 (a) and 4PBZC at pH 9 (b) with the addition of up to 300 μM of CB7.

Figure S3. Binding curve resulted from the measurements the UV–visible absorption spectra of 4PBZH⁺C (5 µM) with different concentrations of CB7 at pH 3 in aqueous solution at 298 K with the nonlinear fit to a 2:1 host–guest binding model (solid line and Experimental Section). The change in optical density (ΔOD) is the difference between the absorbance in the absence and presence of CB7.
**Figure S4.** $^1$H NMR spectra of 1 mM 4PBZH•C with CB7 (0–4 molar equivalents) in D$_2$O of pD 3 (400 MHz) with CB7 peaks indicated.

The extracted 2:1 host–guest stability constants (Figure S3) based on the UV–visible absorption data was $5.5 \times 10^6$ M$^{-2}$ (Figure S2a) at approximately pH 3 (pD 3). In contrast, very low binding affinity of the mono-protonated form with CB7 was noticed from the corresponding binding UV-titration data at pH 9 (Figure S2b). The NMR titration data (Figure S4), however, allowed us to extract a 1:1 host–guest stability constant (Figure S5) of $2.4 \times 10^4$ M$^{-1}$ at approximately pH (pD) of 3 by monitoring the shifts in the NMR peak position of H-1 proton.

Although the $^1$H NMR results clearly indicated 2:1 binding stoichiometry (Figure S4) in agreement with the optical data, the data in Figure S5 were fitted according to a 1:1 binding model, due to the fact that $^1$H NMR shifts of the BZ due to complexation with CB7 is independent on the binding of the fluorophore (C), albeit both have experienced complexation induced down-field shifts.
Figure S5. Binding curve resulted from the $^1$H NMR spectral measurements of 4PBZH$^+\text{C}$ (1 mM) with different concentrations of CB7 at pH (pD) 3 in aqueous solution with the nonlinear fit to a 1:1 host–guest binding model (solid line and Experimental Section). The peak at ~7.18 ppm (H-1) was monitored to construct the binding titration plot. $\Delta \delta$ represents the difference between the NMR peak for H-1 in the absence and presence of CB7.

Part II: Supramolecular effects on fluorescence and apparent host-induced pK$_a^*$ shifts

Figure S6. Fluorescence spectra recorded at different pH values for 35 µM of (a) free and (b) CB7-complexed dye (500 µM for CB7) at different pH values in aqueous solution and at room temperature upon the excitation of coumarin group at 375 nm.

The spectra in Figure S6 reveal a significant fluorescence enhancement upon the addition of HCl in the absence or presence of CB7 with less amounts of acid needed in the latter case. The di-
protonated and mono-protonated forms emit a fluorescence signal in water at 410 nm and 460 nm, respectively, in the absence (Figure S6a) and the presence of CB7 (Figure S6b). The changes which give the plot in Figure 5 agree with the host-induced pKₐ shift in Figure 2, that, both the free or CB7-complexed BZ unit are either protonated at pH< 4 or neutral at pH> 6. Addition of CB7 (Figure S7) slightly enhanced the emission from the mono-protonated form, possibly because of some restriction imposed by the cavity of the host to their free rotation (confinement effects).¹

**Figure S7.** Variations in the fluorescence spectra of 5 μM of 4PBZH⁺C (a) and 4PBZC (b) with the addition of up to 300 μM of CB7, at pH 3 and pH 9, respectively.

The extracted excited-state 2:1 host–guest binding constant (Figure S8) from photoluminescence data was 3.6 ± 0.3 × 10⁶ M⁻² at approximately pH 3.
Figure S8. Binding curve resulted from the measurements the fluorescence spectra of 5 μM 4PBZH⁺C with different concentrations of CB7 at pH 3 in aqueous solution at 298 K with the nonlinear fit to a 2:1 host–guest binding model (solid line and Experimental Section). The change in fluorescence intensity (Relative Int.) as the difference between the emission in the absence and presence of CB7.
Figure S9. DFT-optimized structures of CB7/4PBZC (a), CB7/4PBZH+C (b), and CB7/CAR (c), considering the different complexation modes. The corresponding binding energies are given in kcal/mol.

Part III: The host-retarded PET: Mechanism of fluorescence sensing
**Figure S10.** Frontier-orbital contour plots for the optimized geometries (at the M062X/6-31++G** level of theory) of the mono-protonated (a) and di-protonated (b) 4PBZC.

**Figure S11.** The changes in the fluorescence (a) and UV–visible absorption (b) spectra of the indicator dye (30 µM) upon the addition of CB7 up to 2 mM in aqueous solution at pH 6 and 298 K. Excitation wavelength was at 375 nm.

**Part IV: Interaction of CAR with CB7.**
Encapsulation of CAR into the cavity of CB7 was confirmed by the NMR experiment (Figure S12) as result of three proton resonances, H-e, H-f and H-g, shifted upfield by approximately 0.2−0.5 ppm with the addition of 1 equivalent of CB7, and two proton resonance, H-c and H-d are completely hidden under the CB7’s peak, while three more proton resonance H-a, H-b, H-h and the hydroxyl protons exhibited no or slight downfield shifts. This chemical shift explains the encapsulation of CAR into the cavity of CB7 with resonance proton H-a and H-b positioned outside the cavity.

Figure S12. $^1$H NMR spectra of Carnosol with CB7 (0−1 molar equivalents) in 50% DMSO-$d_6$ and 50% D$_2$O (400 MHz) at pD 7 with peaks indicated as labelled in the embedded structure (a−h). Solvents (HOD and DMSO) and CB7 peaks are also indicated.

Part V: Synthesis of the indicator dye 4PBZC

Preparation of tert-butyl 4-(1H-benzo[d]imidazol-2-yl)piperazine-1-carboxylate
A blend of 19.6 mmol of 2-chlorobenzimidazole amounting to 3.0 g and 3.7 g N-tert-butoxycarbonyl piperazine, which equivalent to 20 mmol, were added to 40 mL of 1-butanol and heated to reflux overnight. The resulted precipitate was collected, washed with ether and dried over high vacuum to get a white powder, corresponding to tert-butyl 4-(1H-benzimidazol-2-yl)piperazine-1-carboxylate compound with 95% yield. The obtained solid showed a melting point ranging between 317 to 319 °C, Rf = 0.8 (1:1 Ethyl acetic acid derivation/Hexane). The IR spectrum (KBr, cm⁻¹) confirms the functional groups present (carbonyl at 1650 and amines at 3407), while the ¹H NMR (400 MHz) signifies the present protons and location in deuterated chloroform: δ 7.49 (dd, J = 5.9, 3.2 Hz, 2H), 7.15 (dd, J = 5.9, 3.2 Hz, 2H), 4.04 – 3.83 (m, 4H), 3.74 – 3.53 (m, 4H), 1.45 (s, 9H).

**Preparation of 2-(piperazin-1-yl)-1H-benzo[d]imidazole**
One equivalent of N-Boc ensured amine (~ 5.3 g) in 10 mL of dioxin were combined with 4 M hydrochloric acid in 106 mL of dioxane and mixed at room temperature for 3 h. The resulted mixture in precipitate form was sifted and dried under high vacuum to obtain a white solid 2-(piperazin-1-yl)-1H-benzimidazole with a total mass of 4.85 g corresponding to 96% yield. The melting point was estimated to range between 317-319 °C, Ethyl acetate Rf = 0.25, IR spectroscopy was carried out in KBr with wavenumber unit cm\(^{-1}\) and NH peaks appeared at 3380, while \(^1\)H NMR in deuterated chloroform solvent indicated chemical shifts as follow: 7.50 - 7.41 (m, 2H), 7.38 - 7.32 (m, 2H), 4.01 - 3.91 (m, 4H, 4x Pip-H), 3.55 - 3.46 (m, 4H, 4x Pip-H), and finally \(^{13}\)C NMR in CD\(_3\)OD signifies chemical shifts as follows: 149.91, 129.72, 124.11, 111.38, 43.25, 41.92 ppm.

**Figure S13.** \(^1\)H NMR spectra for 4PBZC in D\(_2\)O (400 MHz, pH 7) with peaks indicated as labelled in the embedded structure (a–h).
Figure S14. $^{13}$C NMR spectra for 4PBZC in DMSO (400 MHz).

Figure S15. ESI-MS (positive) spectrum of 4PBZH$^+$ C. Calc. 391.1770, found 391.1722 m/z.
Part VI: Procedures and equations for determination of binding affinities and p$K_a$ values

**pH and binding titration studies**

Titration experiment of 4PBZC (~ 3.0 mL) with respect to the change in pH was carried out by UV-Visible assimilation (absorption) and fluorescence spectroscopy utilizing a 1-cm optical way length, rectangular quartz cuvette as sample holders. Solutions of NaOH and HCl were added in drops (microliters) to modify the pH as desired. The p$K_a$ was resolved from the fitted titration information at a chosen wavelength to a sigmoidal equation got from Henderson-Hasselbalch and Lager Lambert laws. The fitting calculations were given by SigmaPlot programming (rendition 14.1; SPSS, Inc., Chicago, IL, USA).

In the titration test, the complete centralization of the 4PBZC was kept consistent and that of the host, while contender/competitor were slowly expanded at steady pH. The pH of known volume of H$_2$O was first acclimated to bring down pH, somewhere in the range of 2 and 3, to
which the stock arrangement of 30 μM of 4PBZC was set up in a specific volume of the acidified H₂O. A calculated weight of CB7 amounting to 2 mM was added to a required volume of 30 μM of 4PBZC stock to prepare the complex of 4PBZC/CB7. The pH of both stocks’ solutions was raised to desired pH (6).

The solution with the final concentration of CB7 was prepared by gradually adding the increment volume of the complex’s stock solution to 2.6 mL of the free 4PBZC directly in the quartz cuvette. The absorption or fluorescence spectra were measured for each solution. The signal at certain wavelength was plotted as a function of host concentrations.²

The intramolecular interaction between CB7 and 4PBZC and 4PBZC/CB7 and carnosol was quantified by the affinity constant known as the association equilibrium constant or binding constant (K). This is associated with the binding/unbinding interaction of receptor/guest (4PBZC) and ligand/host (CB7), as describe below:

\[
\begin{array}{c}
\text{4PBZC + CB7} \quad 4PBZC/CB7, \\
\end{array}
\]

\[
k_f \quad k_r
\]

For an equilibrium reaction, the forward rate of reaction (k_f) should be equal to the backward rate of reaction (k_b) provided the equation of reaction is balanced.

Therefore, \( k_f = [4BZPC][CB7] = k_b[4BZPC/CB7] \) (2)

Binding constant, \( K = \frac{k_f}{k_b} = \frac{[4BZPC/CB7]}{[4BZPC][CB7]} \) (3)

The average concentration of 4PBZC (C₄PBZC) and CB7 (C_CB7) in the new solution can be estimated with the equation below.
\[ K = \frac{[4PBZC/CB7]}{[4PBZC][CB7]}, \]  

(4)

\[ C_{4PBZC} = [4PBZC] + [4PBZC/CB7], \text{ and} \]  

(5)

\[ C_{CB7} = [CB7] + [4PBZC/CB7], \]  

(6)

Wherein \( C_{4PBZC} \) and \( C_{CB7} \) are the total concentrations of 4PBZC and CB7, respectively. It can be written as:

\[ Y \text{ (Reading at certain } \lambda \text{) } = \text{constant 1 } \times [4PBZC] + \text{constant 2 } \times [4PBZC/CB7] \]  

(7)

Subtracting equation (7) from (5), we obtain:

\[ \Delta Y = \frac{\Delta(\text{constant})C_{CB7}}{KC_{4PBZC}^{-1} - K_{CB7} + \sqrt{(1 - KC_{4PBZC} + K_{CB7})^2 + 4KC_{4PBZC}}} \]  

(8)

The titration by using a UV-Visible spectrophotometer of 4PBZC with CB7 was also conducted to obtain the binding constant \( K' \) between two CB7 macrocycles and one 4PBZC molecule. The expression for the changes of absorbance signals \( Y \) as a function of the total concentrations of CB7 \( ([CB7]_0) \) can be derived by considering the two equilibria below and the subsequent mass balance equations:

\[ \text{CB7} + 4\text{PBZC} \rightleftharpoons 4\text{PBZC/CB7} \]  

(9)

\[ K = \frac{[CB7/4PBZC]}{[CB7][4PBZC]} \]  

(10)

\[ \text{CB7/4PBZC} + \text{CB7} \rightleftharpoons (\text{CB7})_24\text{PBZC} \]  

\[ K' = \frac{[(\text{CB7})_24\text{PBZC}]}{[\text{CB7/4PBZC}][\text{CB7}]} \]  

(10)

The absorbance of the free 4PBZC \( (Y_{4PBZC}) \) is:

\[ Y_{4PBZC} = \varepsilon_{4PBZC}[4PBZC]_0 \]  

(11)
[4PBZC]₀ is the total concentration of 4PBZC. Upon the addition of CB7 at a concentration of [CB7]₀, the absorbance (Y) becomes:

\[ Y = \varepsilon_{4PBZC} \cdot [4PBZC] + \varepsilon_{4PBZC/CB7} \cdot [4PBZC/CB7] + \varepsilon_{(CB7)_2 \cdot 4PBZC} \cdot [(CB7)_2\cdot 4PBZC] \]  

(12)

\( \varepsilon_{4PBZC}, \varepsilon_{4PBZC/CB7}, \) and \( \varepsilon_{(CB7)_2 \cdot 4PBZC} \) are the molar absorptivity of 4PBZC, 4PBZC/CB7, and \((CB7)_2 \cdot 4PBZC\), respectively.

When the signal reaches a steady value, it is assumed that only \((CB7)_2 \cdot 4PBZC\) is present. The absorbance becomes:

\[ Y_{(CB7)_2 \cdot 4PBZC} = \varepsilon_{(CB7)_2 \cdot 4PBZC} \cdot [4PBZC]₀ \]  

(13)

Mass balance equations for CB7 and 4PBZC are:

\[ [4PBZC]₀ = [4PBZC] + [4PBZC/CB7] + [(CB7)_2 \cdot 4PBZC] \]  

(14)

\[ [CB7]₀ = [CB7] + [4PBZC/CB7] + 2[(CB7)_2 \cdot 4PBZC] \]  

(15)

From Eqs. 9–15 we get:

\[ Y = \frac{Y_{4PBZC} + [CB7]₀^2 \cdot \varepsilon_{4PBZC/CB7} \cdot K + Y_{(CB7)_2 \cdot 4PBZC} \cdot KK' [CB7]₀^2}{1 + K [CB7]₀ + KK' [CB7]₀^2} \]  

(16)

For the titration try, the all-out focus \([4PBZC]₀\) was kept steady and that of the host \([CB7]₀\) was bit by bit expanded. Thus, we expected the estimation \([CB7]₀ = [CB7]\) is legitimate. The coupling consistent \((K')\) was then controlled by utilizing the nonlinear least-squares recipe (Eq. 15) for the plot of Y versus \([CB7]₀\). All boundaries \((K, Y_{(CB7)_2 \cdot 4PBZC}, \varepsilon_{4PBZC/CB7})\) except \(Y_{4PBZC}\) were left
unconstrained. The examination by Levenberg-Marquardt calculation was given by SigmaPlot's product adaptation 6.1, USA).

References

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