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Supporting Information

Pyrene appended Thymine derivative for selective turn-on fluorescence sensing of Uric Acid in live cell

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Experimental Section:

Materials and Methods:

1-pyrenemethylamine hydrochloride, thymine, bromoacetyl chloride and uric acid were purchased from Sigma-Aldrich Pvt.Ltd. (India). Unless otherwise mentioned, materials were obtained from commercial suppliers and were used without further purification. Solvents were dried according to standard procedures. Elix Millipore water was used throughout all experiments. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz instrument. For NMR spectra, DMSO-d_6 and for NMR titration D_2O was used as solvent using TMS as an internal standard. Chemical shifts are expressed in δ ppm units and ^{1}H–^{1}H and ^{1}H–C coupling constants in Hz. The mass spectrum (HRMS) was carried out using a micromass Q-TOF Micro™ instrument by using Methanol as a solvent. Fluorescence spectra were recorded on a Perkin Elmer Model LS 55 spectrophotometer. UV spectra were recorded on a SHIMADZU UV-3101PC spectrophotometer. FTIR spectra was recorded as KBr pellets using a SHIMADZU FTIR-8400S spectrophotometer. Elemental analysis of the compounds was carried out on Perkin-Elmer 2400 series CHNS/O Analyzer. The following abbreviations are used to describe spin multiplicities in ^{1}H NMR spectra: s = singlet; d = doublet; t = triplet; m = multiplet.

Synthetic Procedure:

2-Bromo-N-(pyren-1-ylmethyl)acetamide (1): 1-pyrenemethylamine hydrochloride (0.804 g, 3 mmol) mixed with potassium carbonate (1.68 g,12 mmol) is suspended into a mixture of ethyl acetate (120 mL) and water(120 mL). Then, bromoacetyl chloride (7.07 g, 4.5 mmol) in ethyl acetate (10 mL) is added dropwise into the solution. After 2 h stirring at room temperature, the organic layer is isolated and dried by MgSO4. The ethyl acetate solvent is removed by rotary evaporation to give the crude product that is purified by column chromatography (silica, 220–400 mesh, hexane/ EtOAc = 1:3 v/v). The product is isolated as a white powder 1 (0.76 g, 72%). ^{1}H-NMR (DMSO-d6, 400 MHz): δ (ppm) 4.41 (s, 2H), 5.05 (d, J = 8 Hz, 2H), 8.07–8.33 (m, 9H), 8.92 (t, J = 8 Hz, 1H); ^{13}C-NMR (DMSO-d6, 400 MHz): δ (ppm) 30.1, 123.7, 124.3, 124.5, 125.2,125.7, 125.8, 126.8, 127.3, 127.6, 127.8, 128.3, 128.6, 130.7, 131.2, 132.7, 167.0. ESI/MS: m/z calcd for $C_{19}H_{14}BrNO$: 352.02, found $[M+H]^{+}$: 353.04. Anal.Calcd for $C_{19}H_{14}BrNO$: C, 64.79; H, 4.01; N, 3.89. Found: C, 64.88; H, 4.07; N, 3.81.

PTA: To a solution of anhydrous K_2CO_3 (2.5g, 18 mmol) in dry DMF was added thymine (0.2 g, 1.50 mmol). The mixture was stirred for 0.5 h. Then compound **1** (0.85 g, 2.5 mmol) was added to the solution and stirred for 48 h. Then, the reaction mixture was poured into water. The

solution was extracted with EtOAc (3×50 mL), and the combined organic layer was washed with 5% aqueous HCl (50 mL), 10% aqueous Na_2CO_3 (50 mL) and finally with water and then was dried over by anhydrous $MgSO_4$. After removing the solvents, the residue was chromatographed on silica gel with Ethyl acetate/methanol=8:1 v/v as eluent to give 0.05g (23%) of compound **1** as brown solid.1H NMR (DMSO-d6, 400 MHz): δ (ppm) 1.78 (s, 3H), 4.41 (s,2H), 5.05 (d, J = 8 Hz, 2H), 7.51 (s, 1H), 8.07-8.38 (m, 10H), 8.92 (t, J = 8 Hz, 1H). ^{13}C-NMR (DMSO-d6, 400 MHz): δ(ppm) 12.87,41.45,50.50,63.75,108.93,124.08,124.84,124.94,125.65,126.12,126.22, 127.21,127.56,128.01,128.32,128.55,131.09,131.25,131.73,133.35,143.25,152.05,165.63,167.94. FTIR (cm^{-1}) : 3160.98 (N-H str.), 2370.35 (C=C), 1695.31 (C=O str.), 2910.68 (sp^3-CH_3). HRMS (TOF MS): (m/z, %): **420.2314** [($M+Na^+$), 100 %]; Calculated for $C_{24}H_{19}N_3O_3$: 397.4271. Anal.Calcd for $C_{24}H_{19}N_3O_3$: C, 72.55; H, 4.79; N, 10.57; O, 12.09; Found: C, 72.58; H, 4.77; N, 10.59; O, 12.06.

^{1}H NMR of compound 1 in DMSO-d_6:

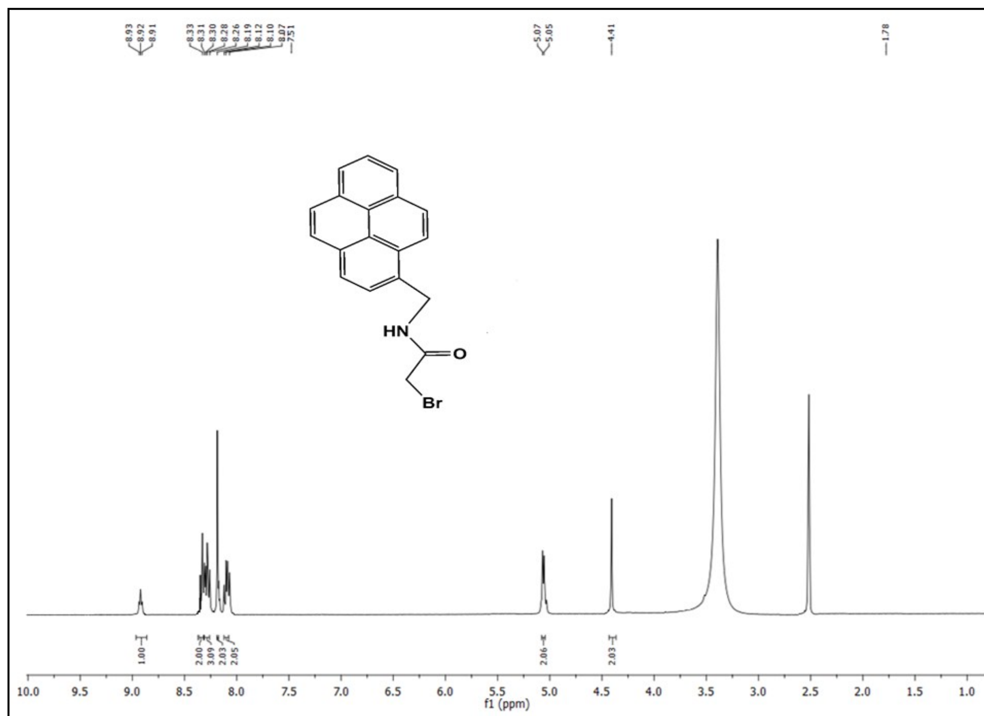

Figure S1. ^{1}H NMR of compound **1** in d_6-DMSO (400 MHz).

^{13}C NMR of compound 1 in DMSO-d_6:

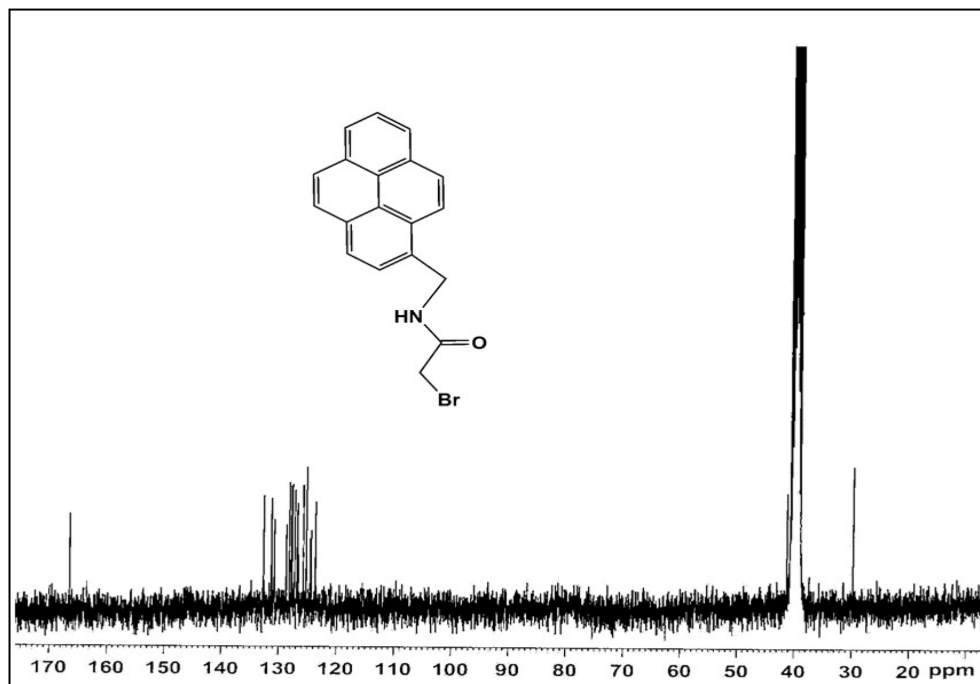

Figure S2. ^{13}C NMR of compound **1** in d_6-DMSO (400 MHz).

^{1}H NMR of PTA in DMSO-d_6:

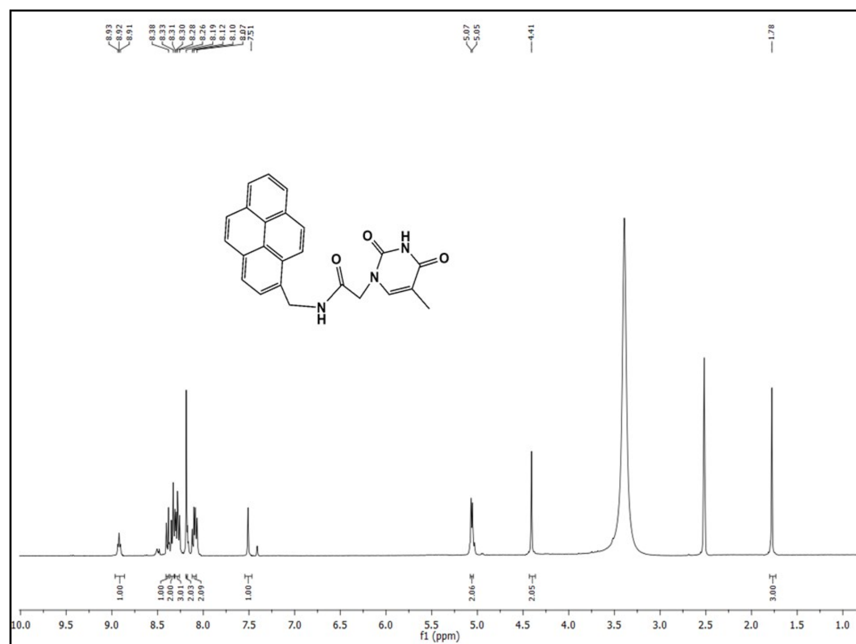

Figure S3. ^{1}H NMR of P**TA** in d_6-DMSO (400 MHz).

^{13}C NMR of compound PTA in DMSO-d_6:

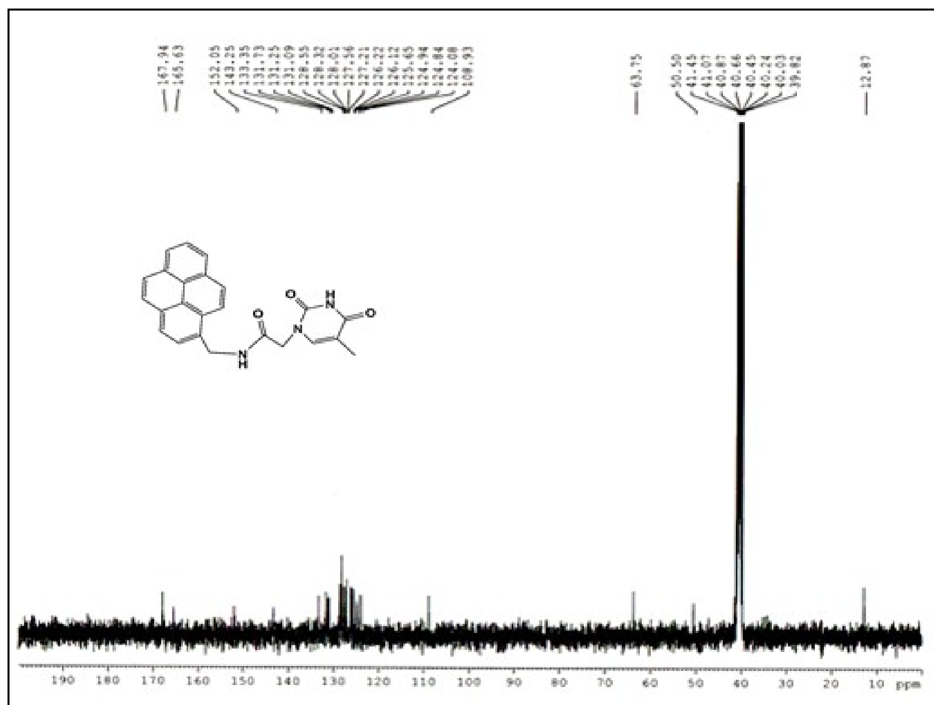

Figure S4. ^{13}C NMR of P**TA** in d_6-DMSO (400 MHz)..

FTIR spectrum of PTA:

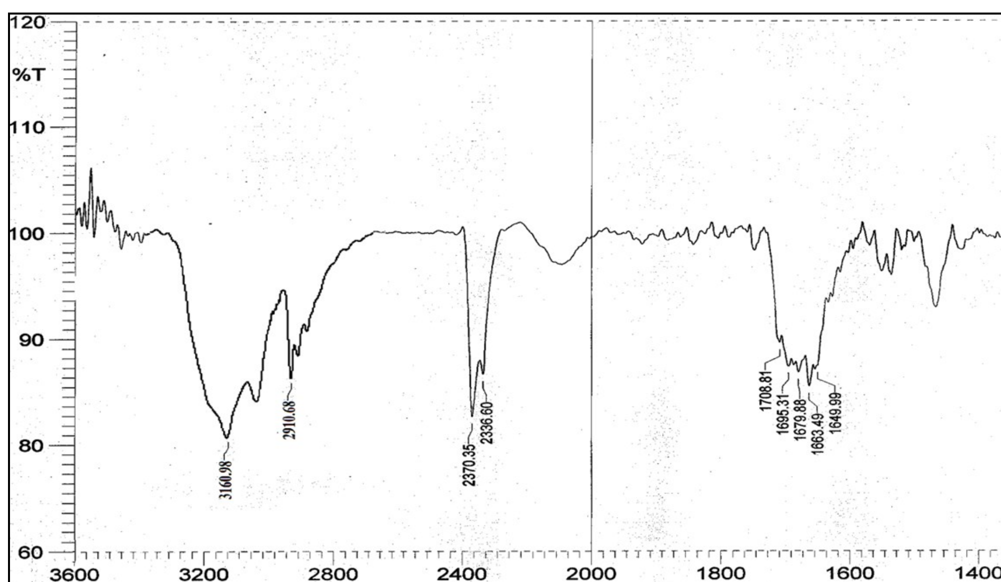

Figure S5. IR-Spectrum of **PTA**.

Mass spectrum of PTA :

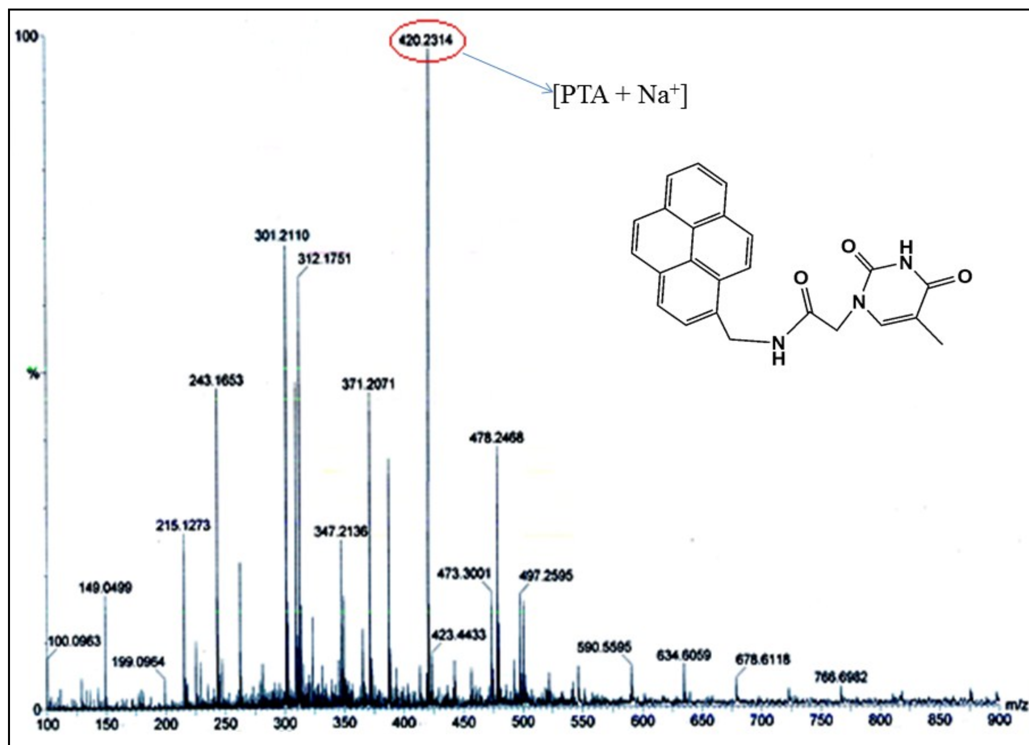

Figure S6. HRMS of **PTA**

^{1}H NMR titration spectrum of PTA with UA:

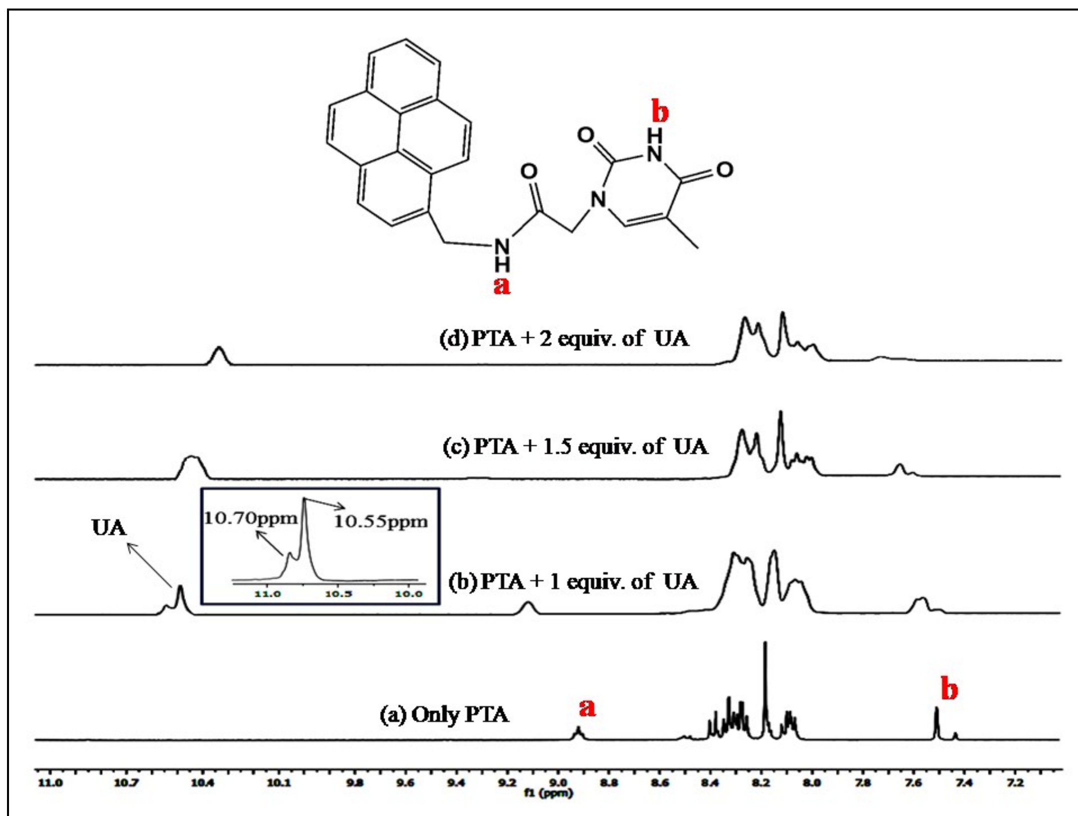

Figure S7. Partial ^{1}H NMR titration [400MHz] of **PTA** in D_2O at 25^0C and the corresponding changes after the gradual addition of different equiv. of **UA** in D2O from a) only **PTA**, b) **PTA**+ 1 equiv. of **UA**, c) **PTA**+1.5 equiv. of **UA**, d) **PTA**+ 2ev. of **PTA**; [inset] **UA**.

8. Evaluation of the Association constants for the formation of PTA-UA:

By UV-Vis Method:

The substrate binding interaction was calculated according to the Benesi-Hildebrand equation.

$$\frac{A_0}{A - A_0} = \left(\frac{\varepsilon_0}{\varepsilon_0 - \varepsilon}\right)^2 \left(\frac{1}{K_B [Substrate]^2} + 1\right) \quad \text{............(i)}$$

Here A_o is the absorbance of receptor in the absence of guest, A is the absorbance recorded in the presence of added guest, ε_0 and ε are the corresponding molar absorption co-efficient and K_B represents the substrate binding interaction with guest.

Binding constant calculation graph (Absorption method):

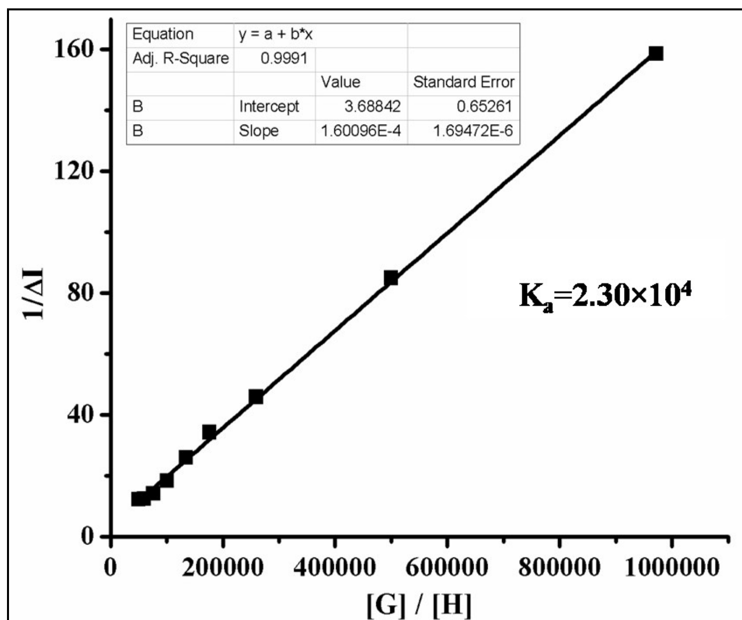

Figure S8. Linear regression analysis (1/[G] vs 1/ΔI) for the calculation of association constant values by UV- titration method.

The association const.(K_a) of **PTA** for sensing **UA** was determined from the equation: K_a = intercept/slope. From the linear fit graph we get intercept= 3.66842, slope = 1.60096×10^{-4}. Thus we get, $K_a = 3.66842/(1.60096 \times 10^{-4}) = 2.30 \times 10^{4}$.

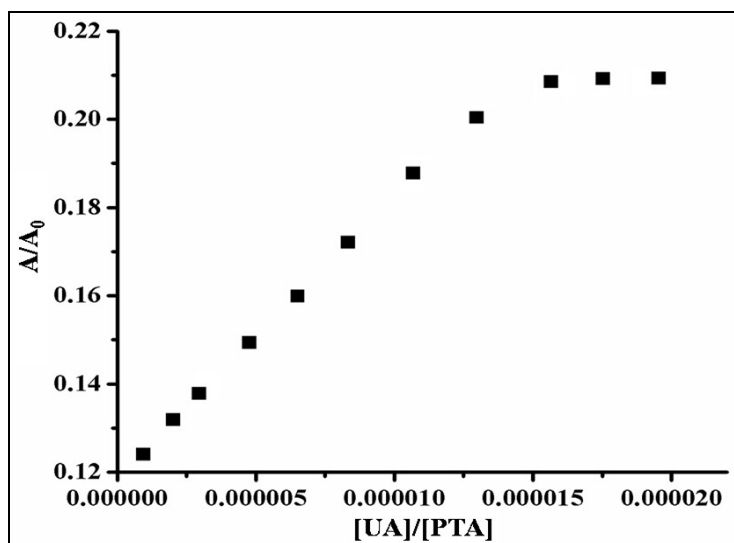

Figure S9. Changes of relative absorption intensity (A/A_0) of **PTA** (0.1 μM) as a function of [**UA**]/[**PTA**].

By Fluorescence Method:

Binding constant of the chemosensor **PTA** also be calculated through emission method by using the following equation.

$$1/(I - I_0) = 1/K(I_{max} - I_0)[G] + 1/(I_{max} - I_0) \quad \text{..............(ii)}$$

where I_0, I_{max}, and I represent the emission intensity of free **PTA**, the maximum emission intensity observed in the presence of added **UA** at 377 nm (λ_{ex} = 315 nm), [G] is the concentration of the guest **UA** and the emission intensity at a certain concentration of the **UA**, respectively.

8B. Binding constant calculation graph (Fluorescence method):

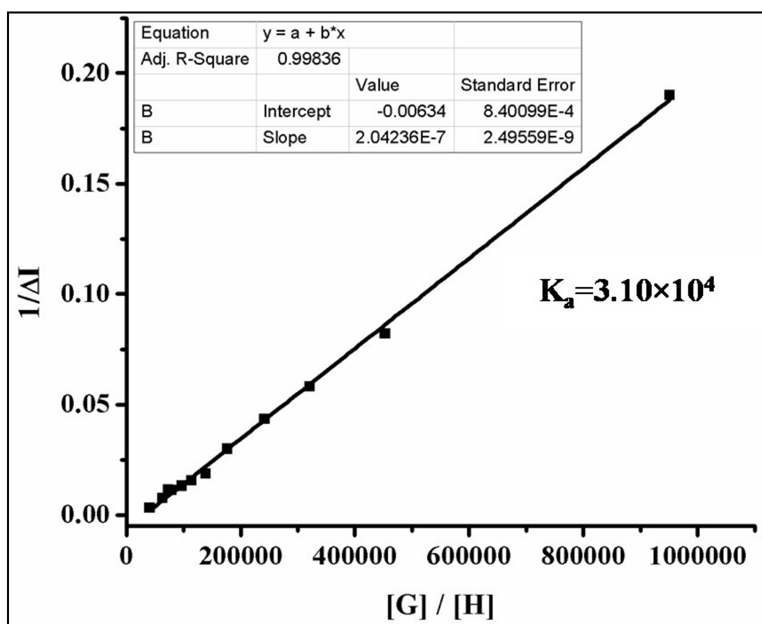

Figure S10. Linear regression analysis (1/[G] vs 1/ΔI) for the calculation of association constant values by Fluorescence titration method.

The association const.(K_a) of **PTA** for sensing uric acid was determined from equation: K_a = intercept/slope. From the linear fit graph we get intercept= -0.00634, slope = 2.04236×10^{-7}. Thus we get, K_a= 0.00634/2.04236 = 3.10×10^4.

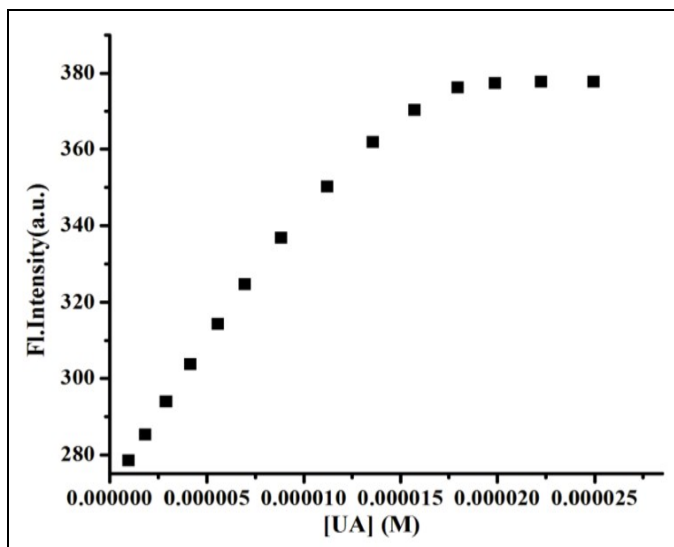

Figure S11. Fluorescence intensity changes of **PTA** (0.1 μM) upon addition of various concentration of **UA**.

Calculation of limit of detection (LOD):

The detection limit of the receptors for **UA** was calculated on the basis of fluorescence titration. To determine the standard deviation for the fluorescence intensity, the emission intensity of four individual receptors without **UA** was measured by 10 times and the standard deviation of blank measurements was calculated.
The limit of detection (LOD) of PTA for sensing **UA** was determined from the following equation:

$$LOD = K \times SD/S$$

Where K = 2 or 3 (we take 2 in this case); SD is the standard deviation of the blank receptor solution; S is the slope of the calibration curve.

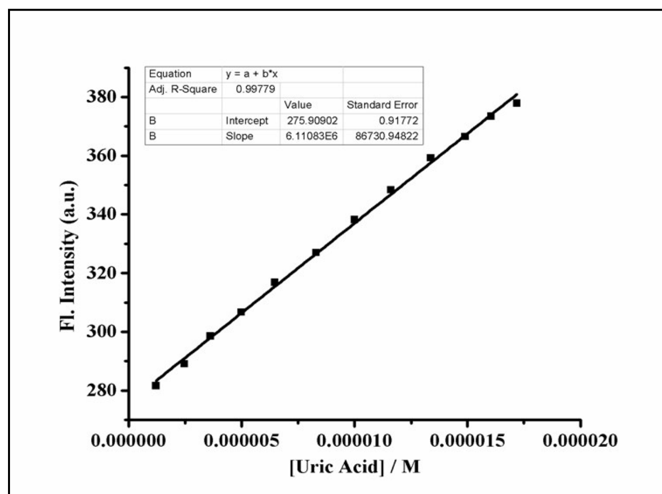

Figure S12. Linear fit curve of **PTA** at 377 nm with respect to **UA** concentration.

For **PTA** with **UA**:
From the linear fit graph we get slope = 6.11083×10^{6}, and SD value is 1.58283
Thus using the above formula we get the Limit of Detection = 5.1804×10^{-7} M**.** i.e 0.518 µM.
Therefore **PTA** can detect **UA** up to this very lower concentration by fluorescence techniques.

Job's plot for determining the stoichiometry of PTA and UA by fluorescence method:

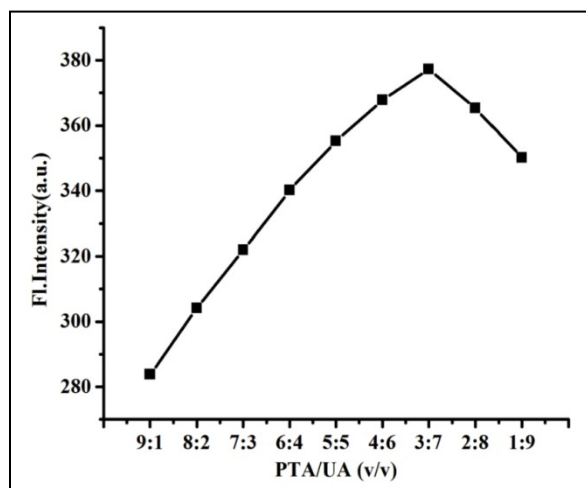

Figure S13. Job's plot of **PTA** with **UA** in water, 10 mM HEPES buffer, pH 7.4, ([**PTA**] = [**UA**] = 1×10^{-4} M) by fluorescence method, which indicates 1:2 stoichiometry.

pH titration study of PTA:

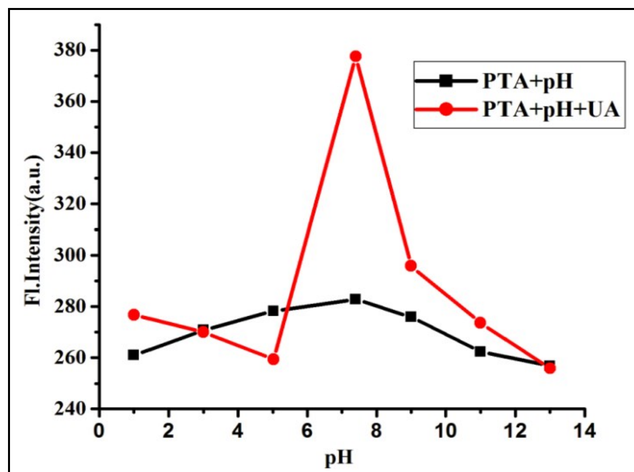

Figure S14. Change in fluorescence intensity of free chemosensor **PTA** (black) and in presence of 2 equiv. of **UA** in water ($1x10^{-4}$M) (red) at λ_{ex} 315 nm (10 mM HEPES buffer, pH 7.4) with different pH conditions.

UV-vis spectral studies:

A stock solution of probe **PTA** (1×10^{-5} M) was prepared in water. An **UA** solution of concentration 1×10^{-4} M was prepared in Millipore water. All experiments were carried out in water (10 mM HEPES buffer, pH 7.4). During titration, each time a 1×10^{-5} M solution of **PTA** was filled in a quartz optical cell of 1 cm optical path length and **UA** stock solution was added into the quartz optical cell gradually by using a micropipette. Spectral data were recorded at 1 min after the addition of **UA**.

Fluorescence spectral studies:

A stock solution of probe **PTA** (1×10^{-5} M) was prepared in water. An **UA** solution of concentration 1×10^{-4} M was prepared in Millipore water. All experiments were carried out in water (10 mM HEPES buffer, pH 7.4). During titration, each time a 1×10^{-5} M solution of **PTA** was filled in a quartz optical cell of 1 cm optical path length and **UA** stock solution was added into the quartz optical cell gradually by using a micropipette. Spectral data were recorded at 1 min after the addition of **UA**. For fluorescence measurements of **PTA**, excitation was provided at 315 nm, and emission was collected from 360 to 460 nm.

Competitive absorbance study:

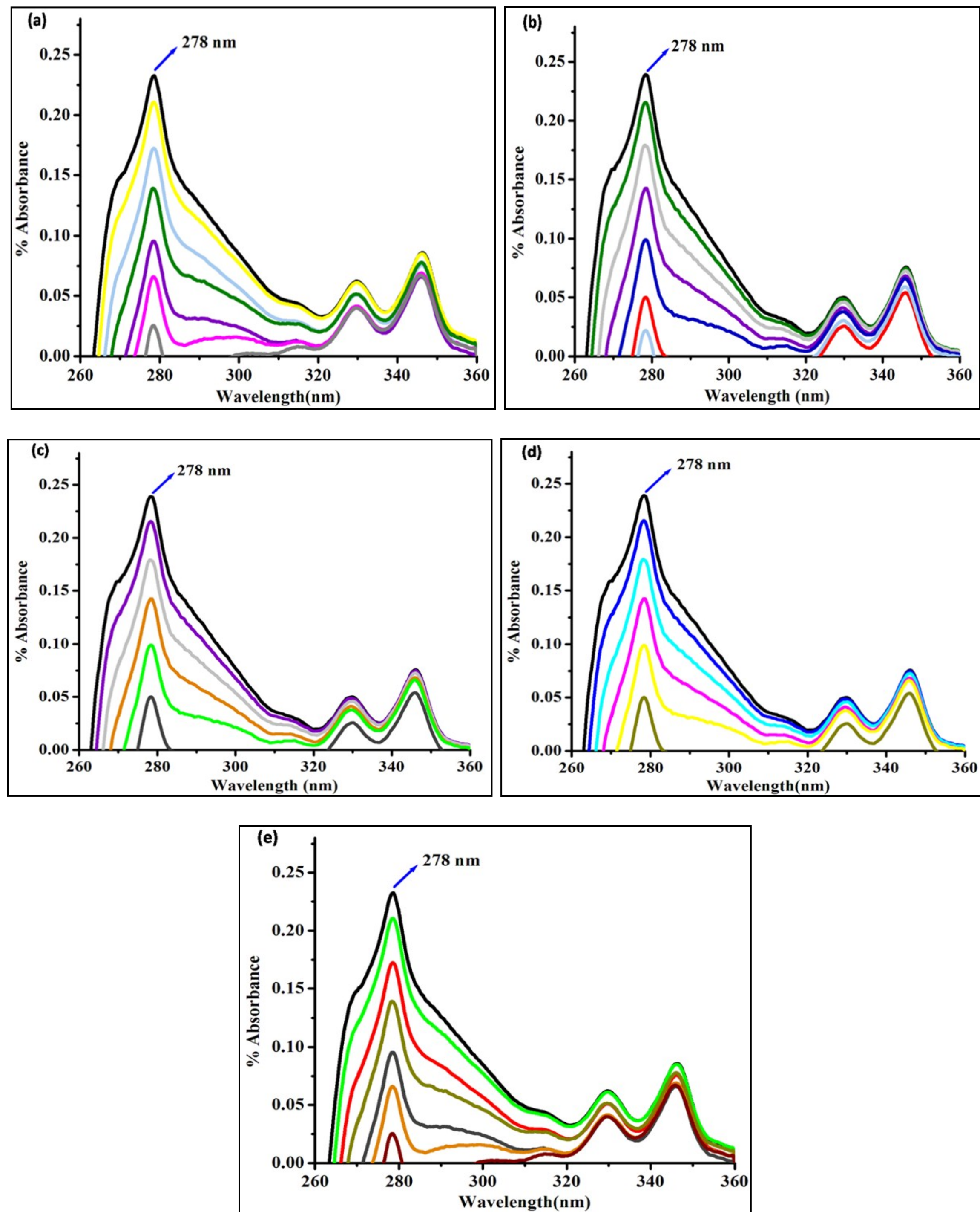

Figure S15. UV-vis absorption spectra of **PTA** ($c = 1\times 10^{-5}$ M) upon addition of (a) Adenine ($c = 1\times 10^{-4}$ M), (b) Guanine ($c = 1\times 10^{-4}$ M), (c) Caffeine ($c = 1\times 10^{-4}$ M), (d) Theophylline ($c = 1\times 10^{-4}$ M), (e) Hypoxanthine ($c = 1\times 10^{-4}$ M). (10 mM HEPES buffer, pH 7.4).

Competitive fluorescence study:

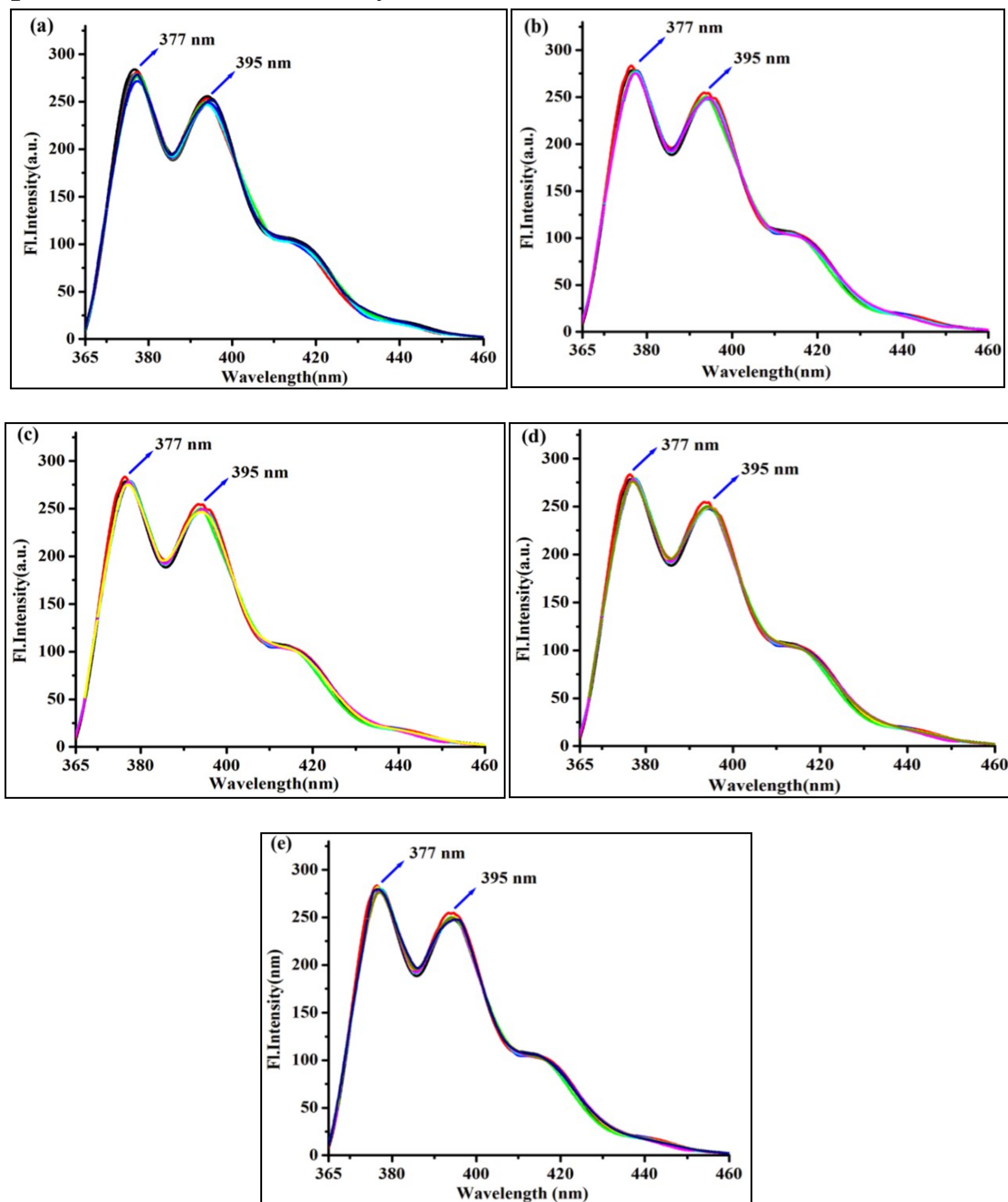

Figure S16. Fluorescence emission spectra (λ_{ex}= 315 nm) of **PTA** (c = 1× 10^{-5} M) upon addition of (a) Adenine (c = 1× 10^{-4} M), (b) Guanine (c = 1× 10^{-4} M), (c) Caffeine (c = 1× 10^{-4} M), (d) Theophylline (c = 1× 10^{-4} M), (e) Hypoxanthine (c = 1× 10^{-4} M). (10 mM HEPES buffer, pH 7.4).

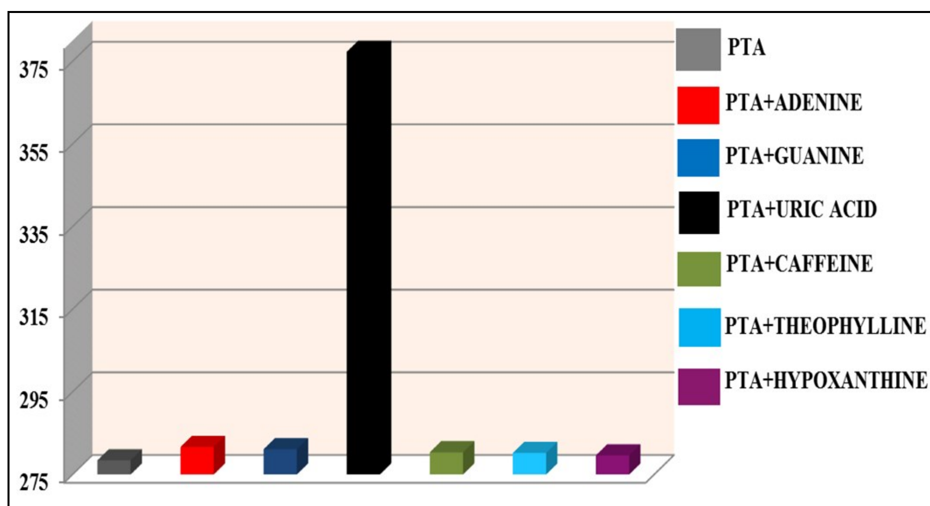

Figure S17. Bar diagram represents competitive fluorescence spectra of **PTA** with different purine bases at 377 nm (10 mM HEPES buffer, pH 7.4).

DFT Study:

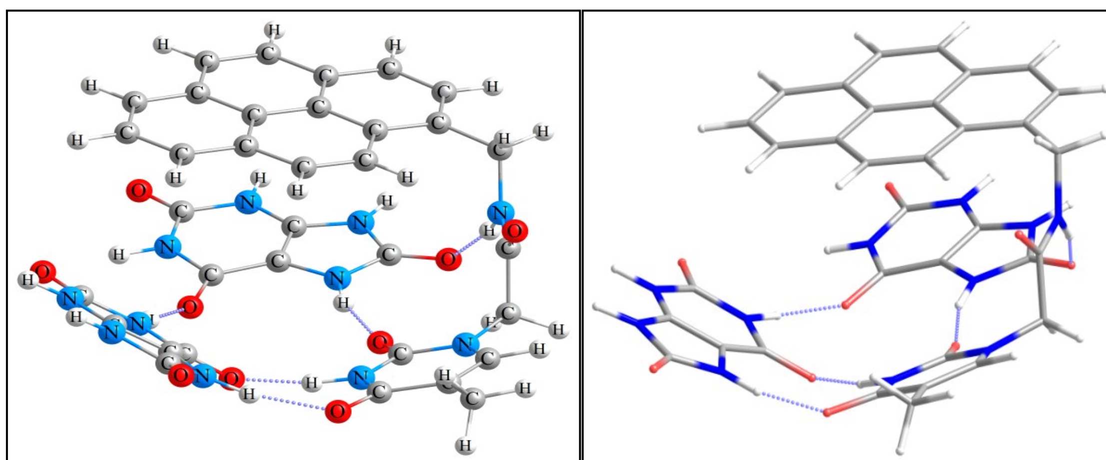

Figure S18. The energy Optimized structure of **PTA-UA** by TDDFT//B3LYP/6-31+G(*d,p*) + solv(COSMO) method.

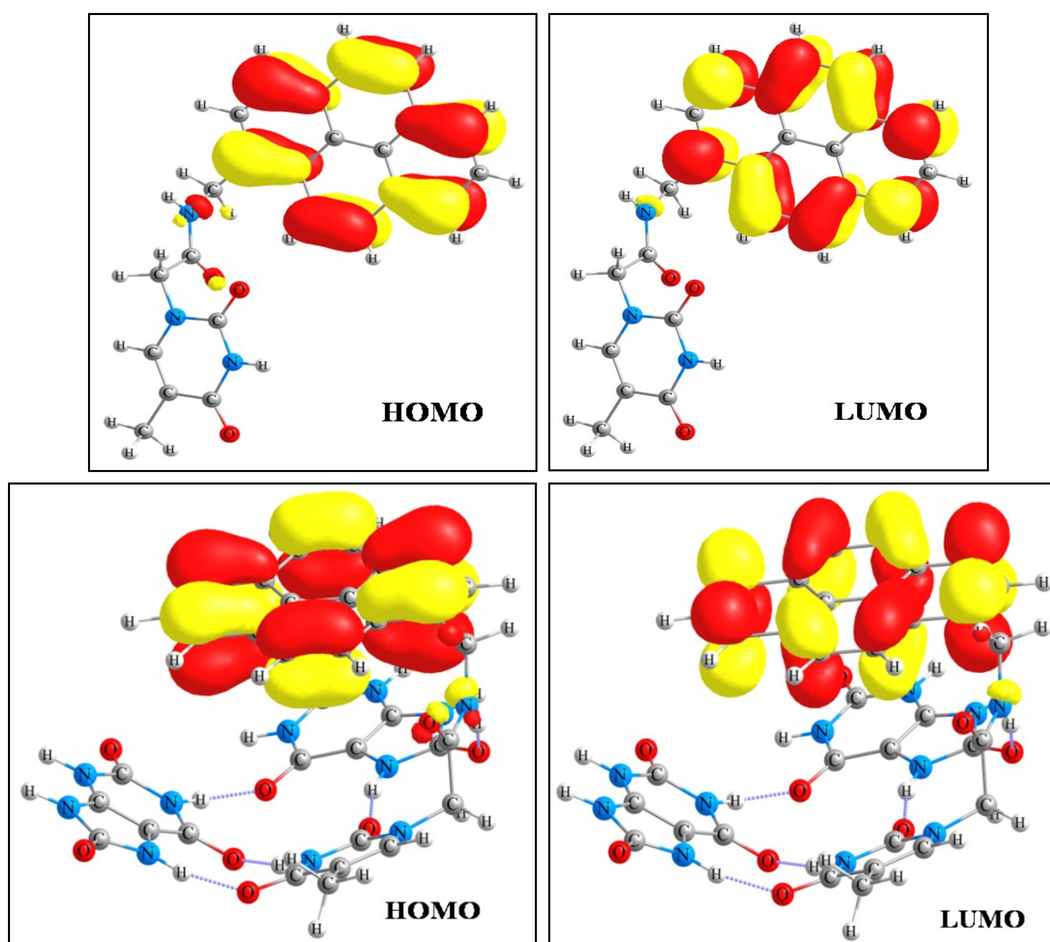

Figure S19. Molecular orbitals and electronic contribution of the relevant excitations of **PTA** (above) and **PTA-UA** complex (below).

Table S1. Selected electronic excitation energies (eV), oscillator strengths (f), main configurations of the low-lying excited states of all the molecules and complexes. The data were calculated by TDDFT//B3LYP/6-31+G(*d,p*) + solv(COSMO) based on the optimized ground state geometries.

Molecules	Electronic Transition	Excitation Energy[a]	f[b]	Composition[c]
UA	$S_0 \rightarrow S_1$	4.861 eV 255.1 nm	0.1905	H → L
	$S_0 \rightarrow S_3$	5.373 eV 230.8 nm	0.2038	H → L +1
	$S_0 \rightarrow S_{12}$	7.161 eV 173.1 nm	0.3764	H-4 → L
PTA	$S_0 \rightarrow S_2$	3.793 eV 326.9 nm	0.3485	H → L
	$S_0 \rightarrow S_8$	4.788 eV 259.0 nm	0.4216	H-2 → L
PTA-UA	$S_0 \rightarrow S_4$	3.758 eV 329.9 nm	0.2459	H → L
	$S_0 \rightarrow S_{18}$	4.750 eV 261.0 nm	0.2808	H → L + 2 H - 4 → L

[a] Only selected excited states were considered. The numbers in parentheses are the excitation energy in wavelength. [b] Oscillator strength. [c] H stands for HOMO and L stands for LUMO.

Table S2. Energies of the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO):

Species	E_{HOMO} (a.u)	E_{LUMO}(a.u)	ΔE(a.u)	ΔE(eV)	ΔE(kcal/mol)
UA	-0.209224	-0.024793	0.184431	5.018625713	115.7322046
PTA	-0.20384	-0.066943	0.136897	3.725159026	85.90416802
PTA-UA	-0.201718	-0.09682	0.104898	2. 85442017	65.82446244

Computational details: Geometries have been optimized using the B3LYP/Def2SVP level of theory in presence of solvent water. Solvent effects were incorporated using COSMO solvent model[1] implemented in ORCA.[2] The geometries are verified as proper minima by frequency calculations. Time-dependent density functional theory calculation has also been performed at the same level of theory. All the calculations have been carried out using ORCA software suite. [2]

(1) Sinnecker, S.; Rajendran, A.; Klamt, A.; Diedenhofen, M.; Neese, F. (2006) Calculation of Solvent Shifts on Electronic G-Tensors with the Conductor-Like Screening Model (COSMO) and its Self-Consistent Generalization to Real Solvents (COSMO-RS), J. Phys. Chem. A, 110, 2235-2245.

(2) Neese, F. (2012) The ORCA program system, Wiley Interdiscip. Rev.: Comput. Mol. Sci., 2, 73-78.

Live Cell Imaging:

Cell line and cell culture

Vero cell (Vero 76, ATCC No CRL-1587) lines were prepared from continuous culture in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (100 μg/mL), and streptomycin (100 μg/mL). The Vero 76 were obtained from the American Type Culture Collection (Rockville, MD) and maintained in DMEM containing 10% (v/v) fetal bovine serum and antibiotics in a CO_2 incubator. Cells were initially propagated in 75 cm^2 polystyrene, filter-capped tissue culture flask in an atmosphere of 5% CO_2 and 95% air at 37°C in CO_2 incubator. When the cells reached the logarithmic phase, the cell density was adjusted to 1.0 x 10^5 per/well in culture media. The cells were then used to inoculate in a glass bottom dish, with 1.0 mL (1.0 x 10^4 cells) of cell suspension in each dish. After cell adhesion, culture medium was removed. The cell layer was rinsed twice with phosphate buffered saline (PBS), and then treated according to the experimental need.

Cell imaging study

For confocal imaging studies Vero cells, 1 x 10^4 cells in 1000 μL of medium, were seeded on sterile 35 mm μ-Dish, glass bottom culture dish (ibidi GmbH, Germany), and incubated at 37°C in a CO_2 incubator for 10 hrs. Then cells were washed with 500 μL DMEM followed by incubation with 1.0 x 10^{-4} M **UA** dissolved in 500 μL DMEM at 37°C for 1 hr in a CO_2 incubator and observed under an Olympus IX81 microscope equipped with a FV1000 confocal system using 1003 oil immersion Plan Apo (N.A. 1.45) objectives. Images obtained through section scanning were analyzed by Olympus Fluoview (version 3.1a; Tokyo, Japan) with excitation at 285nm and 312 nm monochromatic laser beam, and emission spectra were integrated at over the range 300-450 nm (single channel). The cells were again washed thrice with phosphate buffered saline PBS (pH 7.4) to remove any free **UA** and incubated in PBS containing probes **PTA** to a final concentrations of 10^{-5} M, incubated for 10 min followed by washing with PBS three times to remove excess probe outside the cells and images were captured. According to the need of the experiment we follow similar procedures to label the cell nuclei by treatment with DAPI (1 μg/mL) followed by three times wash with PBS and subsequently image was captured with excitation wavelength of laser was 350 nm, and emission was 470 nm. For all images, the confocal microscope settings, such as transmission density, and scan speed, were held constant to compare the relative intensity of intracellular fluorescence.

Cytotoxicity Assay

The cytotoxic effects of probe **PTA** and **PTA-UA** complex were determined by an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay following the manufacturer's instruction (MTT 2003, Sigma-Aldrich, MO). Vero cells were cultured into 96-well plates (10^4 cells per well) for 24 h. After overnight incubation, the medium was removed and various concentrations of **PTA** and **PTA-UA** complex (0, 5, 25, 50, 75 and 100 μM) made in DMEM were added to the cells and incubated for 24 h. Control experiments were set with DMSO, cells without any treatment and cell-free medium were also included in the study. Following incubation, the growth medium was removed and fresh DMEM containing MTT solution was added. The plate was incubated for 3−4 h at 37°C. Subsequently, the supernatant was removed, the insoluble colored formazan product was solubilized in DMSO, and its absorbance was measured in a microplate reader (Perkin-Elmer) at 570 nm. The assay was performed in triplicate for each concentration of **PTA** and **PTA-UA**. The OD value of wells containing only DMEM medium was subtracted from all readings to get rid of the background influence. The cell viability was calculated by the following formula: (mean OD in treated wells / mean OD in control wells) X 100.

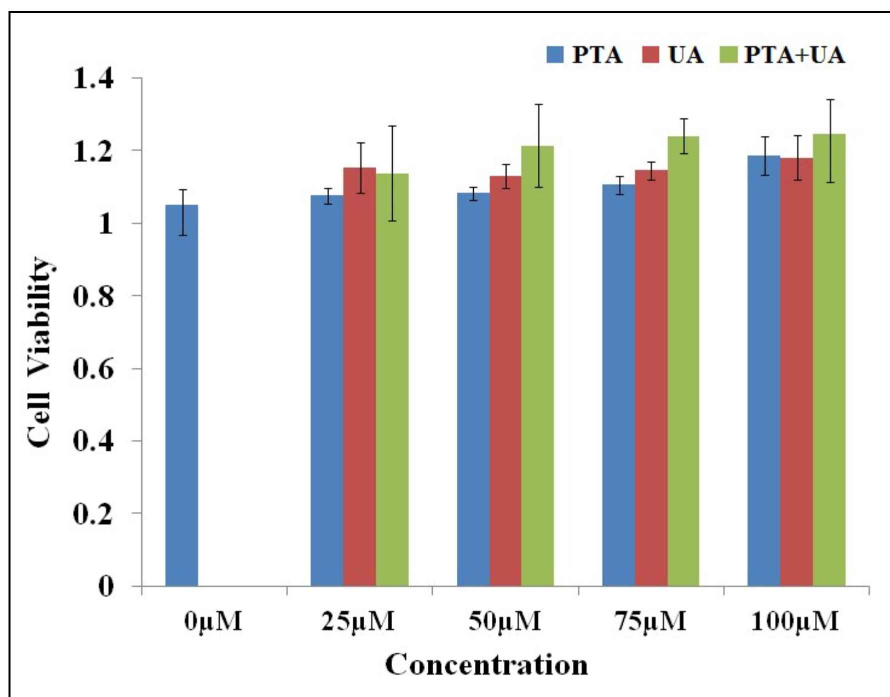

Figure SI20: MTT assay to determine the cytotoxic effect of **PTA** and **PTA-UA** complex on Vero 76 cells.