Supporting Information

Excited state intramolecular proton transfer induced phosphate ion targeted ratiometric fluorescent switch to monitor phosphate ion in human peripheral blood mononuclear cells

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1. *Experimental*

General:

Chemicals and solvents were purchased from Sigma-Aldrich and used without further purification. Silica gel (100-200 mesh, Merck) was used for column chromatography. NMR spectra were recorded on a Varian VXR-400 spectrometer (^{1}H at 300 MHz, ^{13}C at 75 MHz) at 298 K in commercially available d^6 DMSO, with TMS as an internal standard. Chemical shifts are expressed in δ units and coupling constants in Hz. UV-Vis spectra were recorded using a Cary 5000 high performance UV-Vis-NIR spectrophotometer, controlled by Cary WinUV software. Fluorescence was recorded using a Horiba Fluorolog-3 spectrometer using FluorEssence software. IR spectra were recorded on a JASCO FT/IR-460 plus spectrometer, using KBr discs. Melting points were determined on a hot-plate melting point apparatus in an open-mouth capillary and are uncorrected.

General method of UV-Vis absorption and fluorescence emission titrations:

For both UV-Vis and fluorescence titrations, a stock solution of **BTP** was prepared (10 μM) in CH_3OH-H_2O (1/4, v/v) in the presence of HEPES buffer (10 mM) solution at pH = 7.2. The solution of the guest anions using their sodium salts at 10 μM were prepared in buffered deionised water at pH 7.2. The absorption spectra of these solutions were recorded by means of UV-Vis methods using a 10 mm path length quartz cuvette. Fluorescence emission was measured in a 10 mm path length quartz cuvette with the excitation wavelength 380 nm. Fluorescence lifetimes were measured using a time-resolved spectrofluorometer from IBH, UK. The instrument uses a picoseconds diode laser (NanoLed-07, 380 nm) as the excitation source and works on the principle of time-correlated single photon counting. The goodness of fit was evaluated by χ^2 criterion and visual inspection of the residuals of the fitted function to the data.

Details of bio-imaging

Materials Methods

We have conducted an experiment to validate the ability of **BTP** to detect intracellular phosphate. For this purpose, we have isolated peripheral blood mononuclear cells (PBMCs) from venous blood. Approximately 10 ml venous blood was obtained from a healthy, male volunteer donor (age - 32 years) with his informed consent. PBMCs were isolated by density gradient centrifugation utilizing histopaque-1077 gradient [SIGMA]. PBMCs were washed two times with HEPES buffer (SIGMA) and suspended serum-free DMEM supplemented with 2 mmol/l l-glutamine and 50 μg/ml gentamicin having

approximately 3 x 10^6 cells. The cells were incubated with 500 µM Pi or 5mM ATP at 37°C for 2 hours at 5% CO_2 and 95% air. Then the cells treated with ATP were incubated with 1U, 1.5U or 2U of apyrase for 1 hour respectively. After that, the cells were incubated with 10 µM of BTP and incubated for 1 hour at 37°C. Cells were washed twice with 1ml HEPES buffer. Intracellular fluorescence intensity was detected under a fluorescence microscope (Carl Zeiss HBO 100) under 40X magnification with fluorescence emissions at 560 nm (540 nm – 570 nm, Yellow channel) and 480 nm (480 nm – 530 nm, Green channel), respectively.

MTT assay

To determine cell viability against **BTP**, PBMCs were treated with different concentrations of **BTP** solution (5-50 µM) with or without Pi (500 µM) for 1 hour at 37^0C against control cell suspension without **BTP**. Cell density remains 0.05 x 10^6 cells per well in a 96- well plate. 100 µl of MTT solution (5 mg/ml) was added to each well including control and incubated for 4 hours at 37^0C. The purple-colored formazan crystals were dissolved with 100 µl DMSO and the absorbance were measured at 570 nm. Cell viability was calculated using the following calculation:

$$\% \text{ of Cell Viability} = \frac{(\text{Absorbance of treatment group - blank})}{(\text{Absorbance of control group - blank})} \text{ X } 100$$

2. Determination of detection limit:

The detection limit was calculated based on the fluorescence titration. To determine the S/N ratio, the emission intensity of **BTP** without PO_4^{3-} was measured by 10 times and the standard deviation of blank measurements was determined. The detection limit (DL) of **BTP** for PO_4^{3-} was determined from the following equation: $DL = K \times Sb_1/S$ where K = 2 or 3 (we take 3 in this case); Sb_1 is the standard deviation of the blank solution; S is the slope of the calibration curve. For PO_4^{3-}: From the graph we get slope = 81061.3736, and Sb_1 value is 0.00225. Thus using the formula we get the Detection Limit = 8.33×10^{-8} M i.e. **BTP** can detect PO_4^{3-} in this minimum concentration by fluorescence techniques.

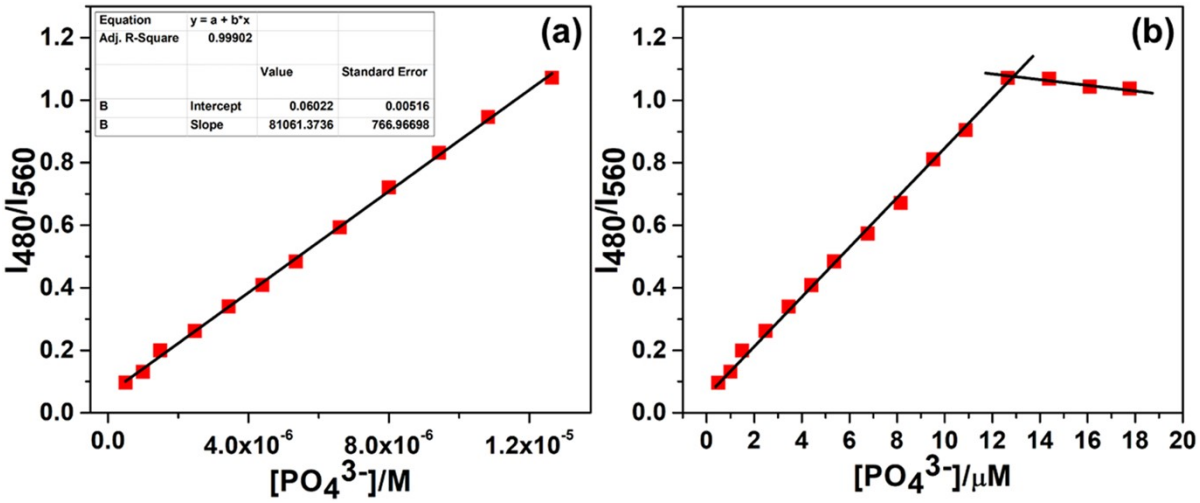

Figure S1: Emission intensity ratio I_{480}/I_{560} of **BTP** depending on the concentration of PO_4^{3-}

3. ***Linear responsive curve BTP depending on PO_4^{3-} concentration:***

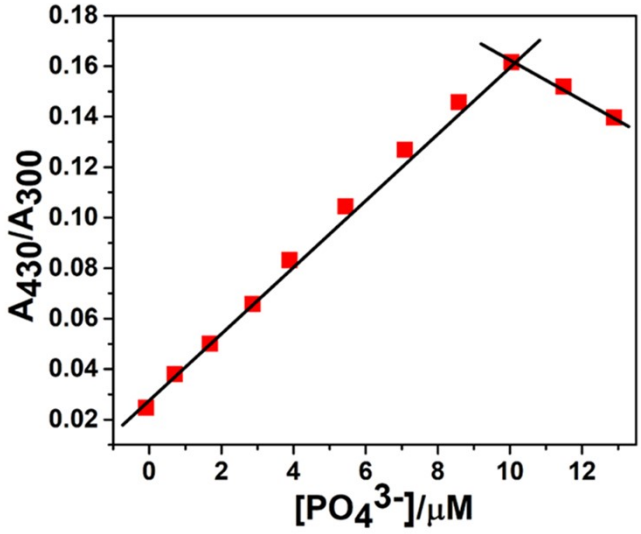

Figure S2: UV-Vis intensity ratio A_{430}/A_{300} of **BTP** respectively depending on the concentration of PO_4^3

4. ***Determination of binding constant***

By Fluorescence method:

Association constant was calculated according to the Benesi-Hildebrand equation. K_a was calculated following the equation stated below.

$$1/(I-I_o) = 1/\{K(I_{max}–I_o)\,[M^{x+}]^n\} + 1/[I_{max}-I_o]$$

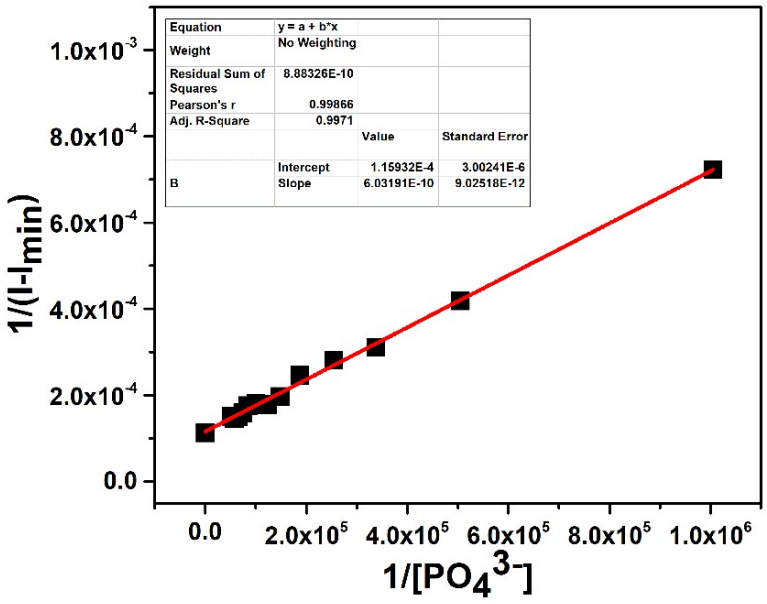

Figure S3: Benesi–Hildebrand plot from UV-Vis titration data of receptor **BTP** (10 μM) with PO_4^{3-}.

Here I_o is the emission of receptor in the absence of guest, I is the emission recorded in the presence of added guest, I_{max} is emission in presence of added $[M^{x+}]_{max}$ and K_a is the association constant, where $[M^{X+}]$ is $[PO_4^{3-}]$. The association constant (K_a) could be determined from the slope of the straight line of the plot of $1/(I-I_o)$ against $1/[PO_4^{3-}]$ and is found to be 1.92×10^5 M^{-1}.

5. **Job's Plot**

Stock solution of same concentration of the receptors and the guest were prepared in the order of 2.0 x 10^{-5} ML^{-1} CH_3OH-H_2O (1:1, *v/v*). The emission in each case with different *host–guest* ratio but equal in volume was recorded. Job plots were drawn by plotting ΔI. X_{host} *vs* X_{host} (ΔI = change of intensity of the emission spectrum during titration and X_{host} is the mole fraction of the host).

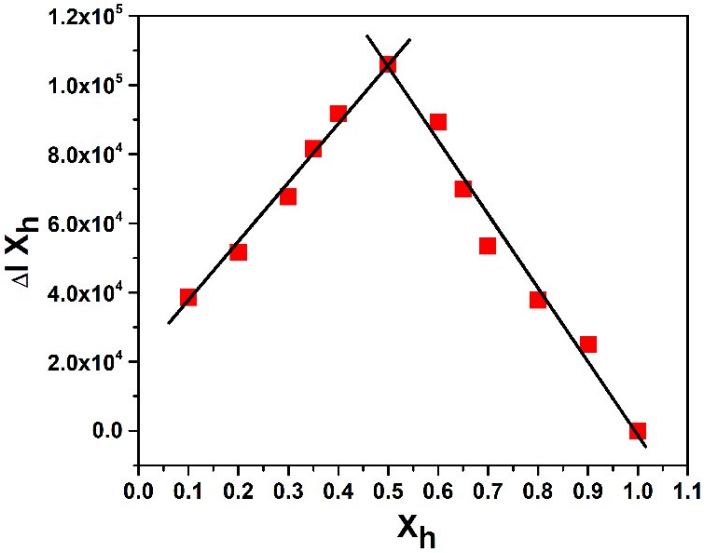

Figure S4: Job plot diagram of **BTP** with PO_4^{3-} (where X_h is the mole fraction of **BTP** and ΔI indicates the change of emission at 480 nm).

6. Determination of fluorescence Quantum Yield (Φ) of BTP and its complex with PO_4^{3-}

To measure the quantum yields of **BTP** and its complex with PO_4^{3-}, the absorbance of the compounds in methanol solution were recorded. The emission spectra were then recorded using the maximum excitation wavelengths, and the integrated areas of the fluorescence-corrected spectra were measured. The quantum yield of **BTP** was then calculated by comparison with fluorescein (Φs = 0.97 in basic ethanol) as a reference. The quantum yield of **BTP** - PO_4^{3-} was calculated by comparison with rhodamine B (Φs = 0.66 in ethanol) as a reference. In each case the following equation was used:

$$\Phi_x = \Phi_s \times \left(\frac{Ix}{Is}\right) \times \left(\frac{As}{Ax}\right) \times \left(\frac{nx}{ns}\right)^2$$

where, *x* and *s* indicate the unknown and standard solution, respectively, *Φ* is the quantum yield, *I* is the integrated area under the fluorescence spectra, *A* is the absorbance and *n* is the refractive index of the solvent. The quantum yield calculated for BTP using the above equation was 0.26. The quantum yield calculated for BTP-PO_4^{3-} using the above equation was 0.44.

7. **pH Study:**

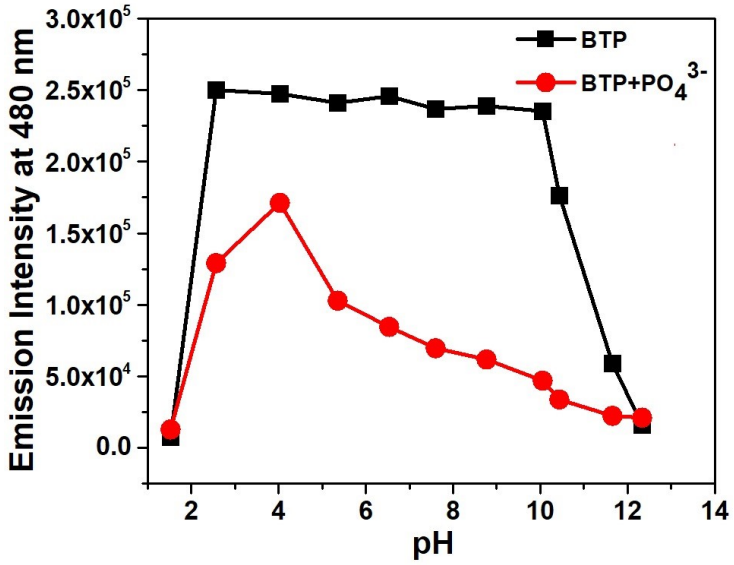

Figure S5: Fluorescence response of **BTP** and BTP-PO_4^{3-} as a function of pH in CH_3OH/H_2O (1/4, v/v), pH is adjusted by using aqueous solutions of 1 M HCl or 1 M NaOH.

8. UV-Vis and Fluorescence study of BTP in presence of other guest analytes.

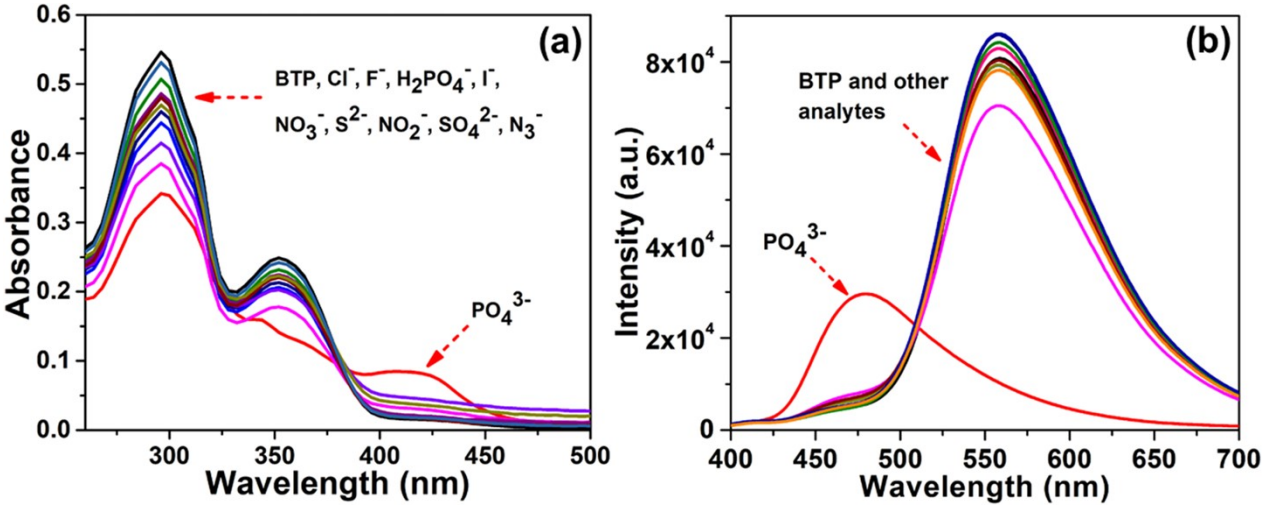

Figure S6: (a) UV-vis spectra and (b) Fluorescence spectra of **BTP** upon gradual addition of 5 equivalents of stated guest anions. BTP (10 µM) in $MeOH/H_2O$ (1/4, v/v), HEPES buffer (10 mM), pH 7.2, 25 °C**.**

Comparison studies with cations:

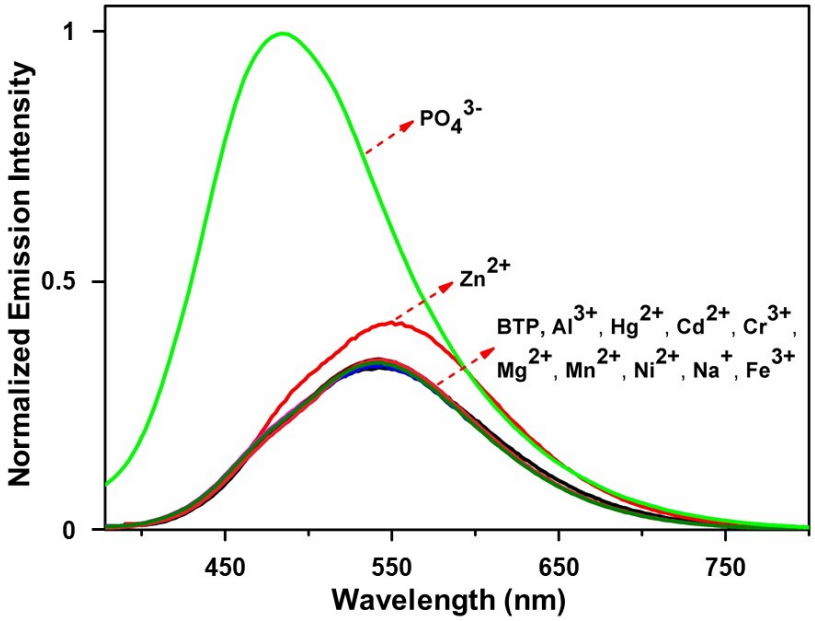

Figure S6 C: Fluorescence spectra of **BTP** upon gradual addition of 3 equivalents of stated guest cations. BTP (10 μM) in MeOH/H_2O (1/4, v/v), HEPES buffer (10 mM), pH 7.2, 25 °C**.**

9. ***Comparison Study of Reversibility Experiment***

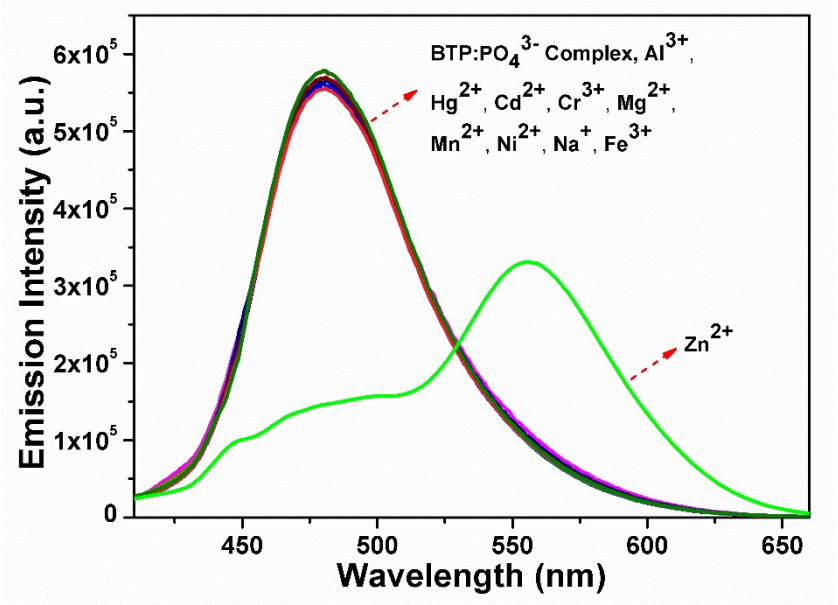

Figure S7: Fluorescence spectra of **BTP-PO_4^{3-}** upon gradual addition of 5 equivalents of stated guest cations. **BTP-PO_4^{3-}** (10 μM) in MeOH/H_2O (1/4, v/v), HEPES buffer (10 mM), pH 7.2, 25 °C**.**

10. IR spectra of BTP

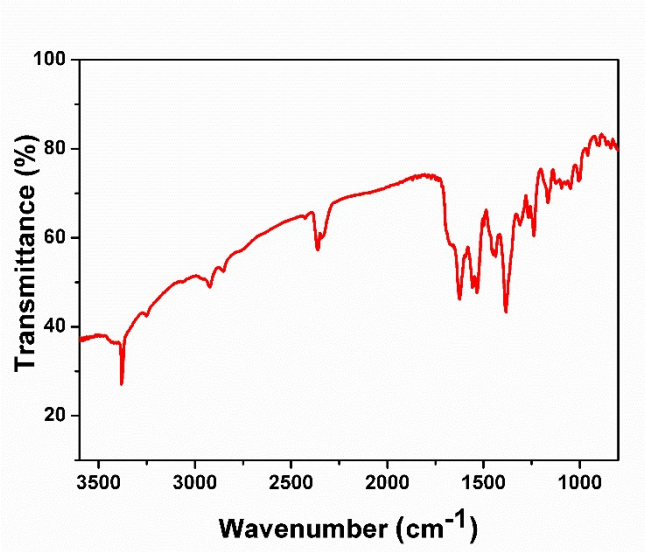

Figure S8: FT-IR spectrum of BTP

11. Fluorescence life time of BTP

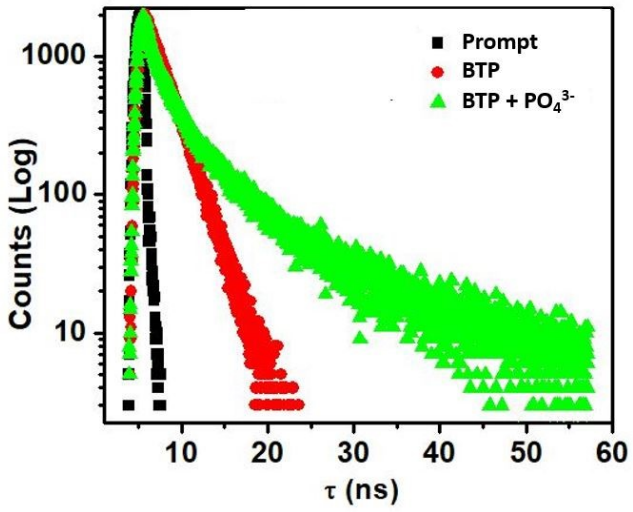

Figure S9: Time-resolved fluorescence decay of **BTP** (Red) and **BTP** + PO_4^{3-} (Green).

Table S1 Fluorescence lifetime data of **BTP**

Entry	Φ	τ (ns)	k_r ($10^8 \times s^{-1}$)	k_{nr} ($10^8 \times s^{-1}$)
BTP	0.26	2.31	1.16	3.16
BTP-PO_4^{3-}	0.44	4.66	0.94	1.19

12. Truth table for INHIBIT Logic Gate

Table S2

Input 1 (PO_4^{3-})	Input 2 (Zn^{2+})	Output (Emission intensity at 480 nm)
0	0	0
1	0	1
0	1	0
1	1	0

13. [1]H NMR spectrum of BTP:

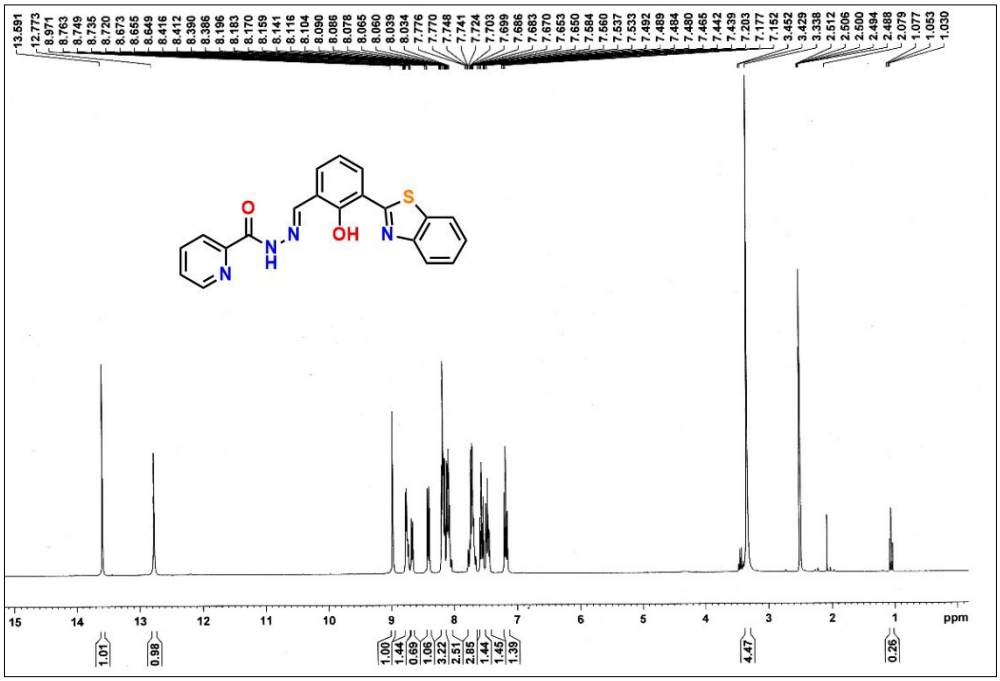

Figure S10: [1]H NMR (300 MHz) spectrum of **BTP** in d_6-DMSO (298 K).

14. [13]C NMR spectrum of BTP

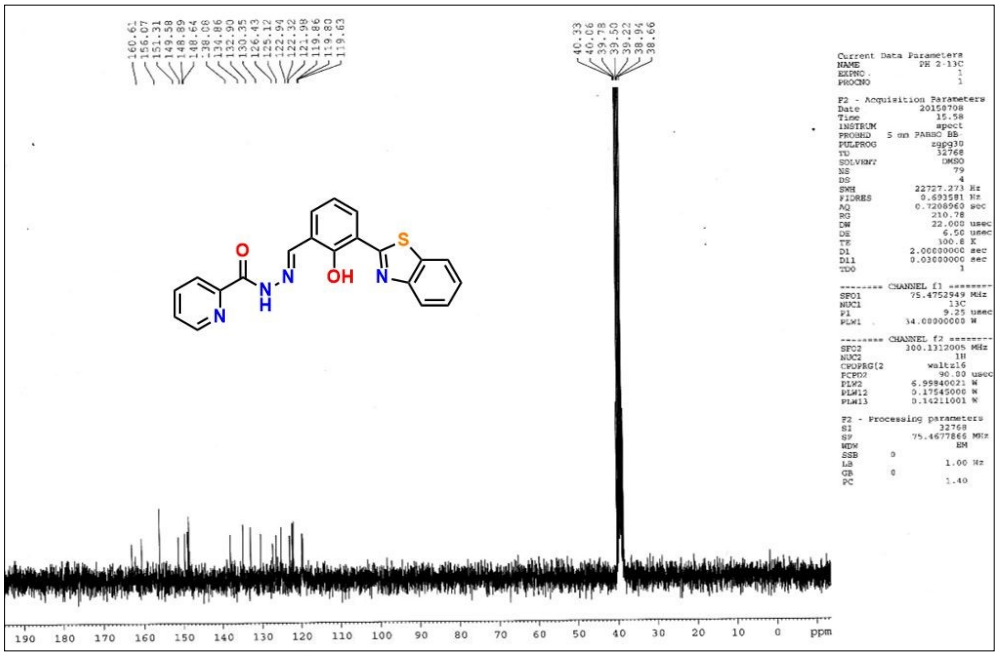

Figure S11: [13]C NMR (75 MHz) spectrum of **BTP** in d_6-DMSO (298 K).

15. HRMS of BTP:

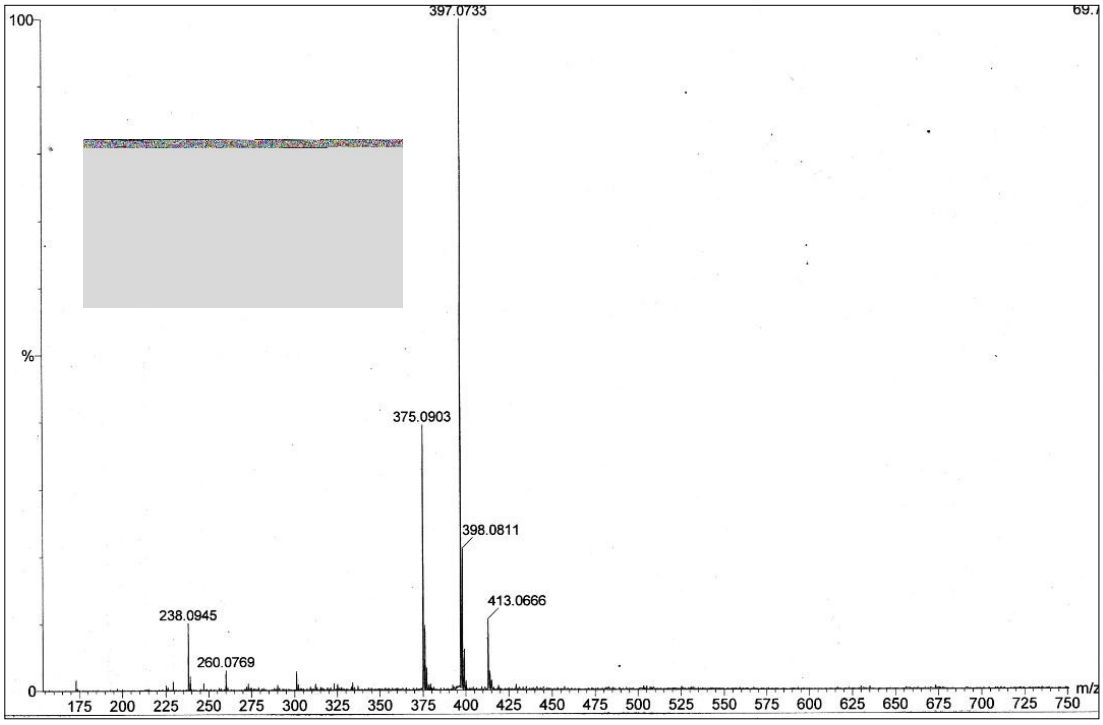

Figure S12: HRMS spectrum of the **BTP** (positive ESI mode)

16. ***^{1}H NMR titration of BTP with PO_4^{3-}***

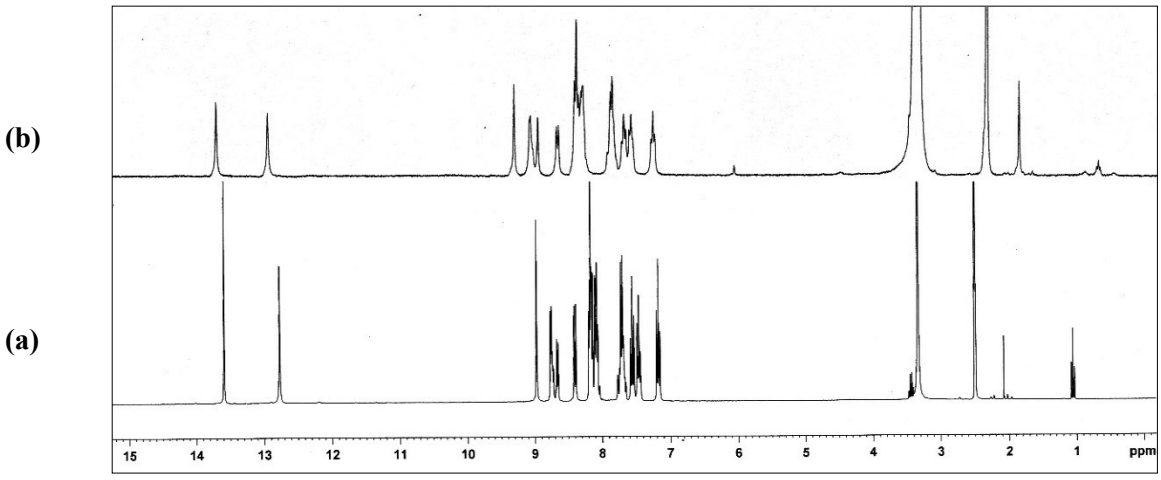

Figure S13: Partial ^{1}H NMR (300 MHz) spectra of (a) **PBT** and (b) **PBT +PO_4^{3-}** in d_6-DMSO. [BTP] = 2.12×10^{-3} M; [PO_4^{3-}] = 4.24×10^{-3} M (298 K).

17. ***Comparison Study:***

Sr. No	**Fluorophore Used**	**Ratiometric Fluorescence Change**	**Detection Limit**	**Bioimaging Studies (endogenous phosphate detection)**	**References**
1.	Dimethylcarbamodithioate -calix[4]arene	No	7.9 nM	No	Tetrahedron Letters, 2021, 71, 153046
2.	Coumarin	No	8.11×10^{-7} M	Yes	Anal. Chem. 2015, 87, 2, 1196–1201
3.	Pyridine−Biquinoline	No	0.85 μM	Yes (But not endogenous phosphate detection)	10.1021/ac504536q
4.	methylphenol with 4-(1Hbenzo[d]imidazol-2-yl)aniline	No	1.67 nM	Yes (But not endogenous phosphate detection)	Anal. Chem. 2015, 87, 13, 6974–6979.
5.	BINOL	No	95 nM	Yes (But not endogenous phosphate detection)	10.1039/c4an01615g
6.	Dihydroxyaniline bisimidazolium derivative	No	5.4 ppb	Yes (But not endogenous phosphate detection)	10.1039/C4CC00752B.
7.	2-hydroxybenzohydrazide	No	2.7 nM	No	10.1039/c4dt01799d
8.	Cyanine dye	Yes	9.37×10^{-7} M	Yes	10.1016/j.dyepig.2016.04.032
9.	Benzo[d]thiazol-2-yl)-2-picolinohydrazide	Yes	8.33×10^{-8} M	Yes	Present Work