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Electronic Supporting Information (ESI)

Colorimetric and fluorescence recognition of tryptophan and histidine using phthalaldehyde based probe: Experimental, computational, cell imaging and fish tissue analysis

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Materials and methods

High-purity *o*-phthalaldehyde and 2-aminobenzimidazole were purchased from Sigma-Aldrich (India). L-tryptophan (Trp) and L-histidine (His) were purchased from SRL (Mumbai, India). Solvents used were of spectroscopic grade. The other chemicals used are of analytical reagent grade and used without further purification except when specified. Milli-Q Millipore® 18.2 M**Ω** cm^{-1} water was used throughout all of the experiments. Absorption studies were carried out using a Shimadzu (model no. 1800) UV−Vis spectrophotometer. Fourier transform infrared (FTIR) spectra were recorded on a Shimadzu FTIR spectrophotometer (model no. IR Prestige 21). Mass spectral analyses were performed in a QTOF Micro YA 263 mass spectrometer in electron spray ionization (ESI) positive mode. ^{1}H NMR spectra were recorded using a Bruker Advance 600 (600 MHz) while ^{13}C NMR spectra were performed using a Bruker Advance 300 (300 MHz) instrument. Steady-state fluorescence Measurements were performed with a Perkin Elmer Precisely LS55 spectrofluorimeter. The fluorescence imaging system is comprised of an

inverted fluorescence microscope (Leica DM 1000 LED), digital compact camera (Leica DFC 420C), and an image processor (Leica Application Suite v3.3.0). The microscope is equipped with a 50 W mercury arc lamp. The measurement of pH was performed with a Systronics digital pH meter (model 335). All the spectra were recorded at room temperature.

Determination of detection limit

Fluorescence titration of BENPH with Trp and His are carried out by the addition of aliquot of micromolar concentration of Trp and His respectively. The minimum concentration of Trp or His which result a sharp change in the fluorescence intensity multiplied with the concentration of BENPH give the detection limit (DL).[1]

DL = C (BENPH) × C (Q)

Where C (BENPH) = Concentration of BENPH; C (Q) = Concentration of Trp/His at which fluorescence enhanced.

Thus; DL = $2.0 \times 10^{-5} \times 0.5 \times 10^{-6} = 0.5 \times 10^{-6} = 5.0 \times 10^{-7}$

Estimation of binding constants

The binding ability of BENPH in 0.1 M HEPES buffered ethanol−water (v/v, 1/1, pH 7.4) towards Trp and His separately has been estimated following the modified Benesi−Hildebrand equation[2] as written below:

$$1/\Delta F = 1/\Delta F_{max} + (1/ K[Q]^n)(1/\Delta F_{max})$$

Here $\Delta F = F_x - F_0$ and $\Delta F_{max} = F_{max} - F_0$, where Q is either Trp or His, n is the number of Trp or His moiety bound per BENPH (presently, n = 1). F_0, F_x, and F_{max} are the emission intensities of

BENPH in the absence of Trp or His, at an intermediate Trp or His concentration, and at a concentration of complete interaction, respectively. K is the binding constant, the values of which are 1.5×10^4 M^{-1} for Trp and 1.7×10^4 M^{-1} for His (Fig.S25).

Calculation of quantum yield

Fluorescence quantum yields (Φ) were estimated by integrating the area under the fluorescence curves using the equation,

$$\Phi_{sample} = \frac{OD_{standard} \times A_{sample}}{OD_{sample} \times A_{standard}} \times \Phi_{standard} \times \frac{\eta_{sample}^2}{\eta_{standard}^2}$$

where A indicate the area under the fluorescence spectral curve, OD, optical density of the probe at excitation wavelength[3] and η is the refractive indices of the solvent. Anthracene was used as quantum yield standard (quantum yield, 0.27 in ethanol)[4] for measuring the quantum yields of BENPH and [BENPH + His] adduct (λ_{ex} = 330 nm) while Tris(2,2'-bi pyridyl)ruthenium(II) was used as quantum yield standard (quantum yield, 0.042 in water)[5] for measuring the quantum yields of BENPH and [BENPH + Trp] (λ_{ex} = 410 nm).

Preparation of cells

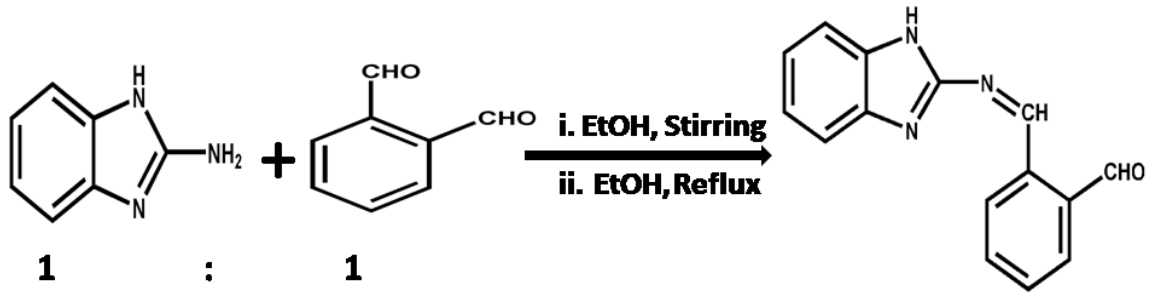
The pollen pellet was prepared following published procedure[6] and incubated in a solution of (1) Trp and (2) His (0.1 mg mL^{-1}) respectively for one hour at ambient temperature. After incubation, they were again washed in normal saline and observed under fluorescence microscope using UV filter in presence and absence of BENPH. Both Trp/ His treated and untreated cells were stained with BENPH and observed under fluorescence microscope.

Synthesis of BENPH

The probe BENPH, as shown in the scheme (Scheme **S1**), have been synthesized by a simple step. A dry 10 mL ethanol solution of *o*-phthalaldehyde (0.500 g, 3.731 mmol) is added dropwise to a 10 mL solution of 2-amino benzimidazole (0.4969 g, 3.731 mmol) in dry ethanol under stirring condition. The process of stirring is continued for further half an hour followed by reflux for 8 h. A white precipitate which appeared was filtered and washed with ethanol thrice. Crude product was purified by recrystallization from ethanol as white solid with 83% yield. The purity and structure were confirmed by ^{1}H NMR, ^{13}C NMR, QTOF – MS ES^{+} and FTIR spectroscopic studies. ^{1}H NMR (600 MHz, DMSO-d_6) (Fig. **S2**): 6.3 (2H, m, J = 6.6), 6.9 (2H, m, J = 6), 7.3 (2H, q, J = 6), 7.5 (2H, m, J = 4.8), 7.8/8.0 (1H, m, J = 6.6), 10.48 (1H, s) and 11.26 (1H, s).^{13}C NMR (300 MHz, DMSO-d_6) (Fig. **S3**): δ193.63, 152.85, 141.63, 141.31, 140.40, 136.81, 134.33, 130.58, 129.56, 129.41, 129.28, 124.06, 123.93, 123.15, 123.02. QTOF – MS ES^{+} (Fig. **S1**) [M + H]$^{+}$: 250.03. FTIR (cm^{-1}) (Fig. **S4**): υ(NH)3433.85, υ(C=O)1642, υ(C=N)1577.79.

Synthesis of adducts of BENPH with Trp and His

To 4 mL solution of Trp (0.030 g, 0.1468 mmol) in water, 6 mL ethanol solution of BENPH (0.0365 g, 0.1468 mmol) was added dropwise and stirred for 10 min. Similarly, a solution of His (0.030 g, 0.1933 mmol) in 4 mL water was added to an ethanol solution of BENPH (0.0481 g, 0.1933mmol, 6 mL) dropwise and stirred for 10 min. A grey and chocolate colour precipitates were obtained for Trp and His respectively. QTOF – MS ES^{+} (Fig. **S19** and **S20**) and FTIR (**S21**) spectral analysis confirmed the formation of adducts. Job's plots (Fig. **S24**) reveal 1:1 stoichiometry (mole ratio) of the adducts.



Scheme S1 Synthesis of BENPH

Method for estimation of free Trp and His in muscle of *Labeo rohita* (Hamilton)

Fish, a major source of animal protein and *Labeo rohita* (Hamilton) is the most available carp species in India. Selective estimation of Trp and His in fish muscle is important to have idea on the nutritive value of fish samples. The developed method has been very useful for quick and easy estimation of free Trp and His in fish muscle.

Three healthy *Labeo rohita* (average weight 264.85 ± 7.25 g) were collected from a local fish market at Burdwan and acclimatized in standard laboratory conditions for two days. During this time they were fed with commercial fish feed with 32% crude protein. On the day of the experiment fish samples were anesthetized with MS 222 and dissected on ice trays. The muscle was collected and washed several times with phosphate buffer (0.1 M, pH 7.4) with 0.89% of NaCl to remove blood and other debris. Samples were homogenized separately at 4° C in a tissue homogenizer and centrifuged at 10,000 rpm for 30 minutes at 4° C in 80% ethanol. The extraction process was repeated twice, the clear supernatant was collected and the pH was adjusted to pH 7.4 by HEPES buffer (0.1 M) and used for the quantitative estimation of free His and free Trp. For flourimetric analysis 2 mL of muscle extract was mixed with 2 mL (160 μM) of BENPH and emission intensity was noted against reagent blank (2 mL 80% ethyl alcohol

mixed with 2 mL BENPH, 160 µM) after 36 minutes and 34 minutes of mixing of BENPH for Trp and His respectively. Standard curves were prepared with L-His and L-Trp and results were expressed as µmoles of free His or free Trp g^{-1} of wet weight of muscle. Standard methods for biochemical estimation of free Trp (Gaitonde and Dovey, 1970) and free His (Newman and Turnbull, 1960) were also carried out with the same fish muscle samples for comparison.

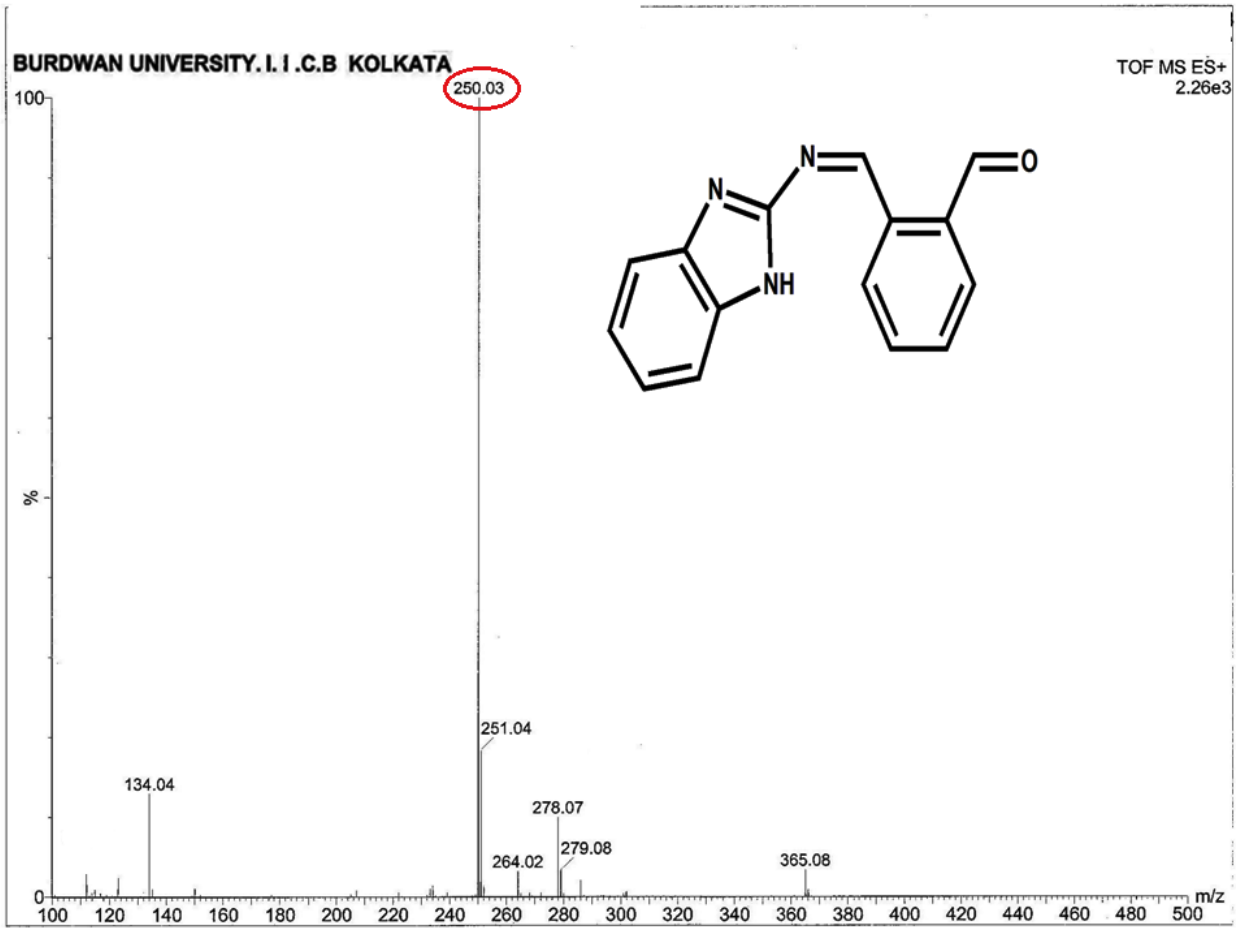


Fig.S1 QTOF-MS spectrum of BENPH.

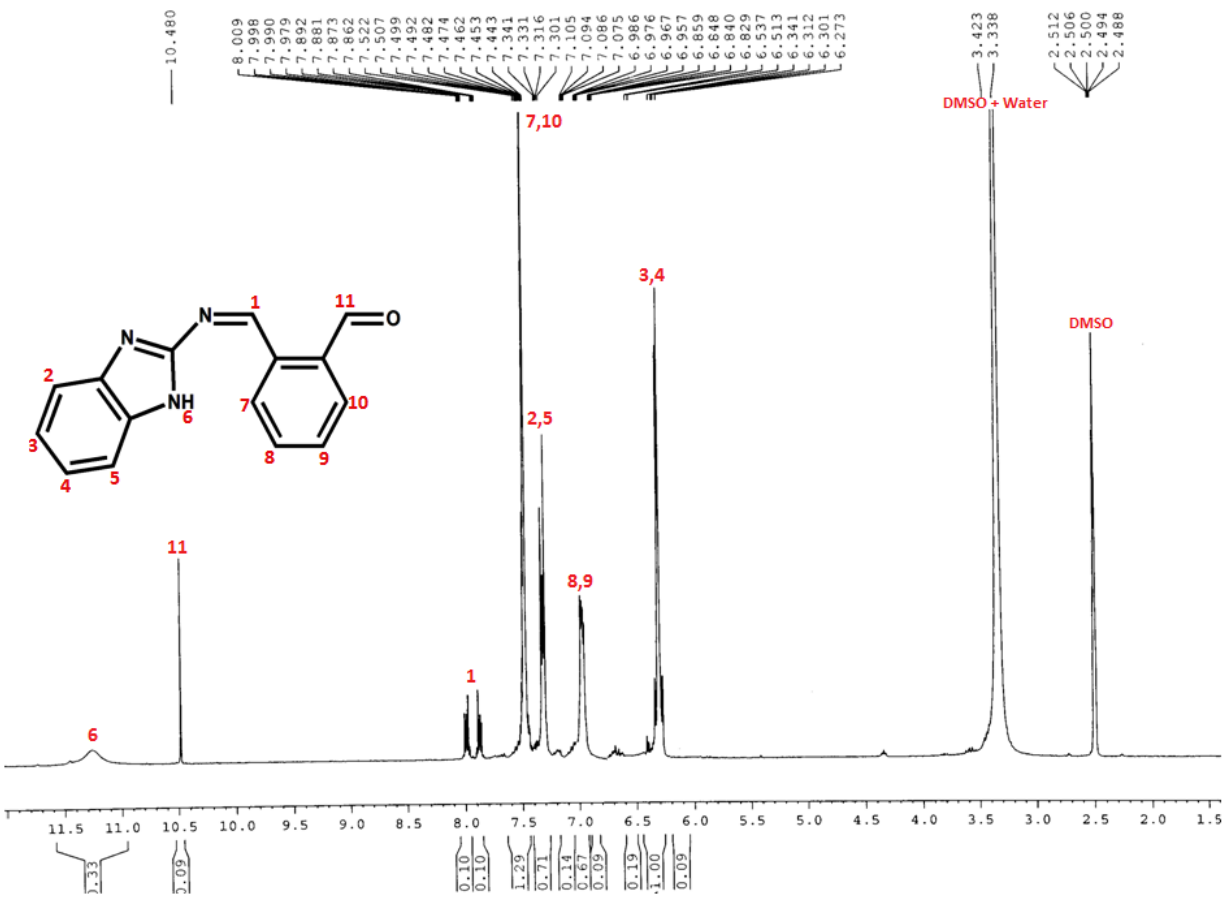


Fig.S2 ^{1}H NMR spectrum of BENPH in DMSO-d_6.

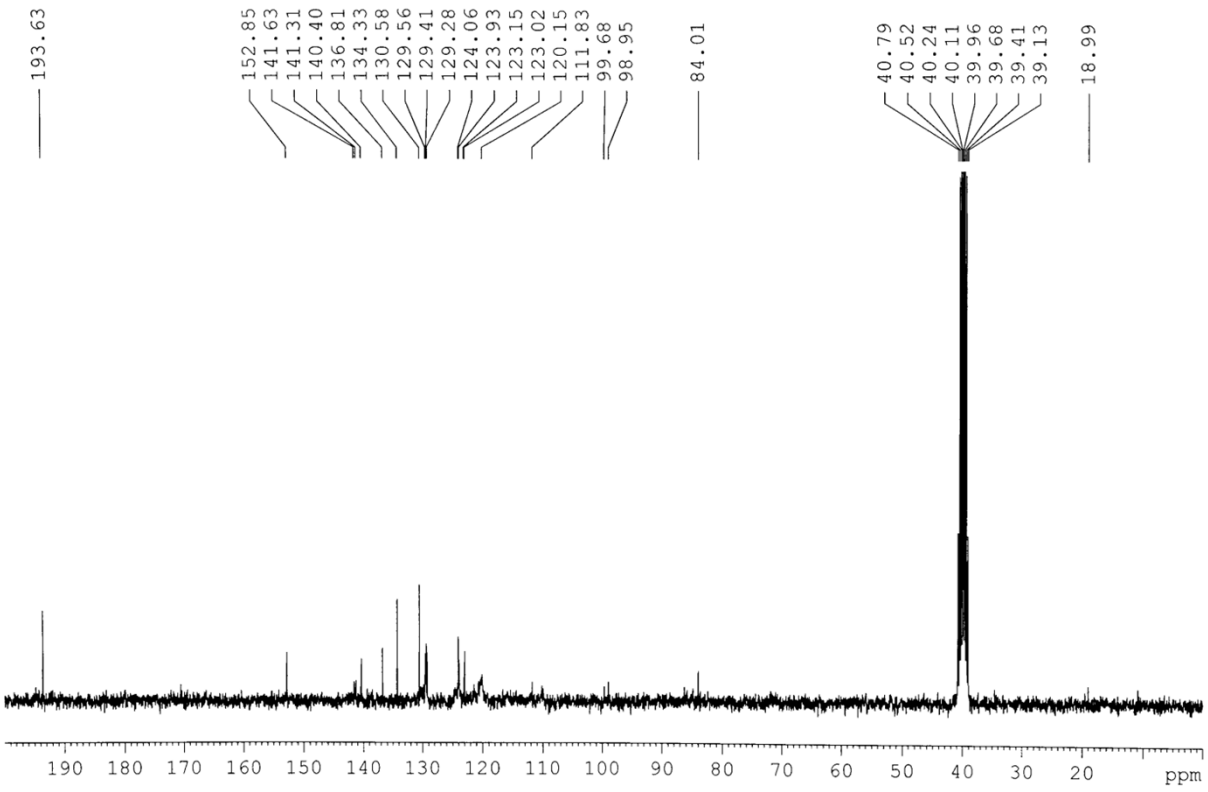


Fig.S3 ^{13}C NMR spectrum of BENPH in DMSO-d_6.

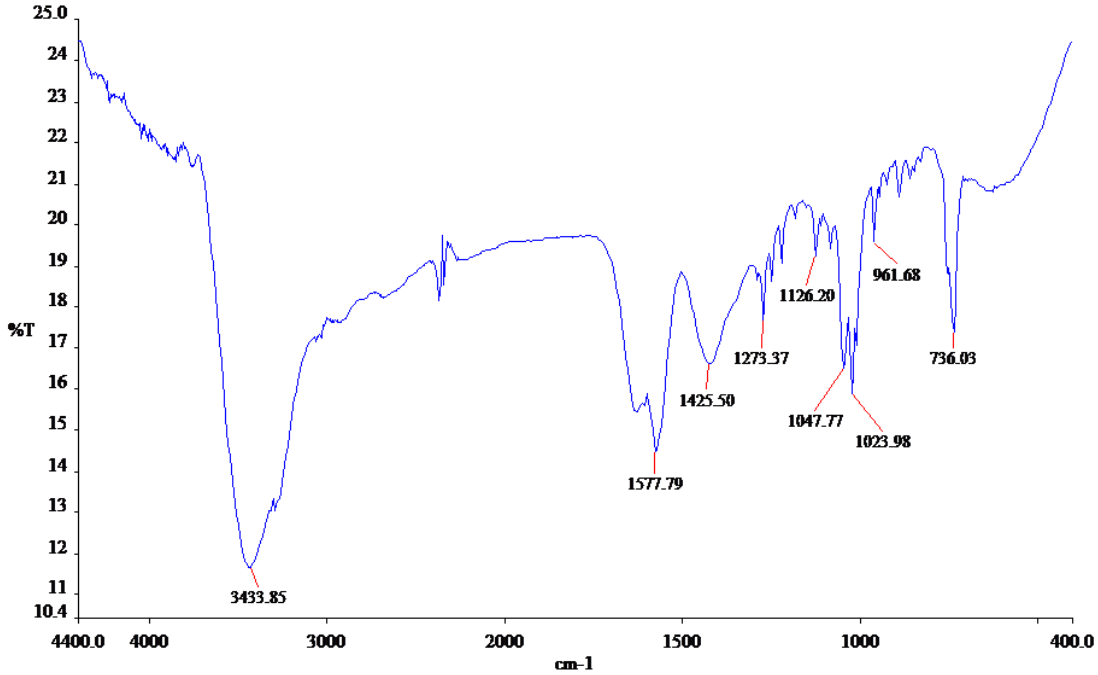


Fig.S4 FTIR spectrum of BENPH.

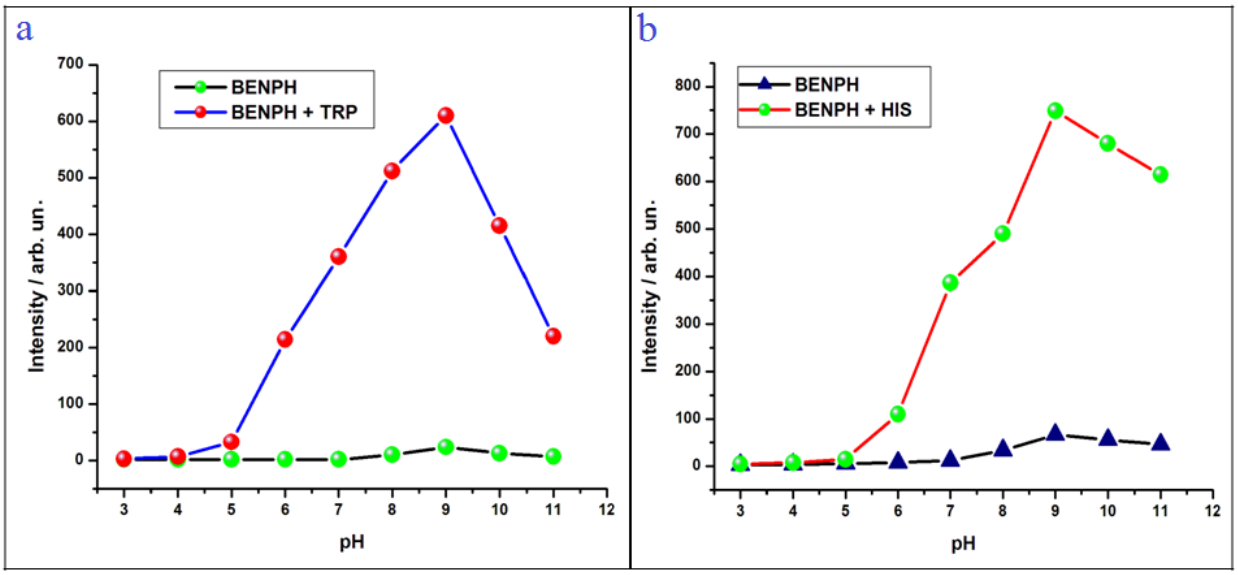


Fig.S5 Changes in the emission intensity of BENPH (20 μM, ethanol/water, 1/1, v/v) and its adducts with 300 μM of (a) Trp (λ_{ex}, 410 nm; λ_{em}, 495 nm) and (b) His (λ_{ex}, 330 nm; λ_{em}, 430 nm) *vs.* pH of the media.

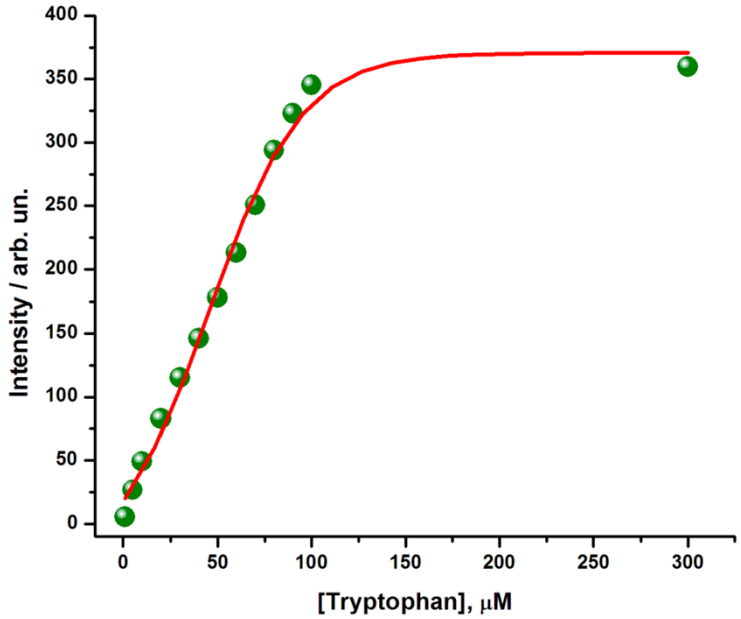


Fig.S6 Emission intensities of BENPH (20 μM, λ_{ex} = 410 nm, λ_{em} = 495 nm) as a function of externally added Trp (1-300 μM).

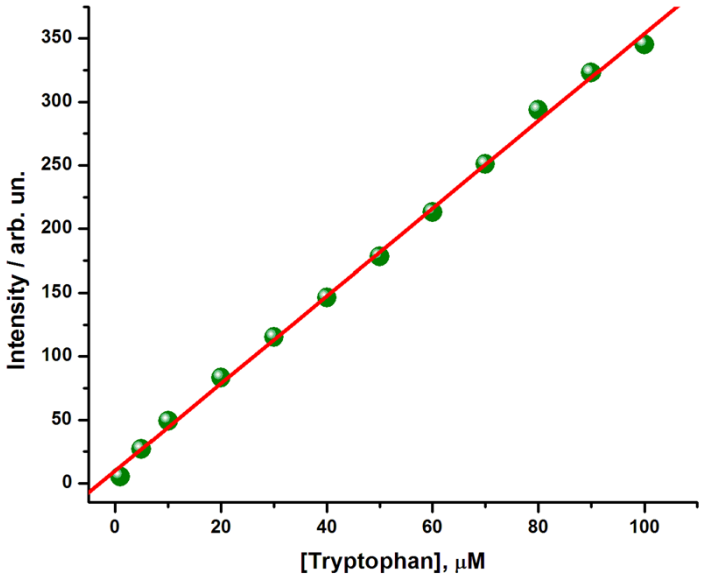


Fig.S7 Emission intensities of BENPH (20 μM, λ_{ex} = 410 nm, λ_{em} = 495 nm) as a function of externally added Trp (up to 100 μM).

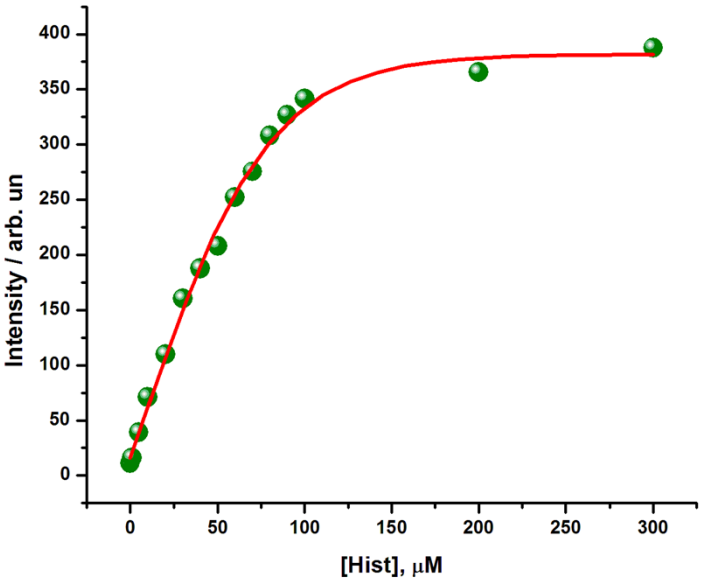


Fig.S8 Emission intensities of BENPH (20 μM, λ_{ex} = 330 nm, λ_{em} = 430 nm) as a function of externally added His (1-300 μM).

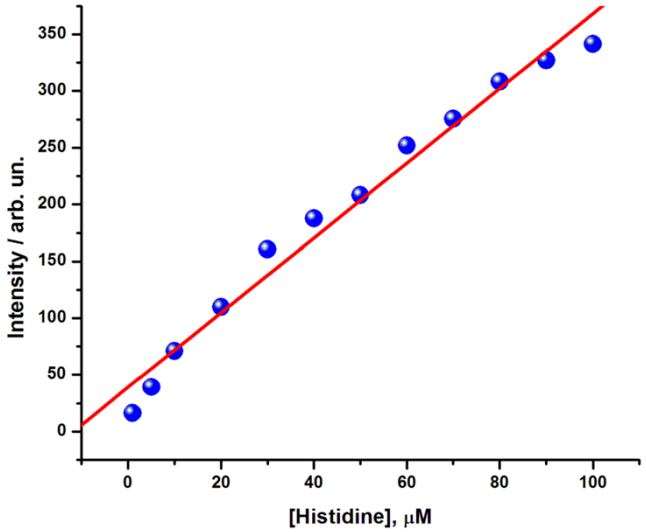


Fig.S9 Emission intensities of BENPH (20 μM, λ_{ex} = 330 nm, λ_{em} = 430 nm) as a function of externally added His (up to 100 μM).

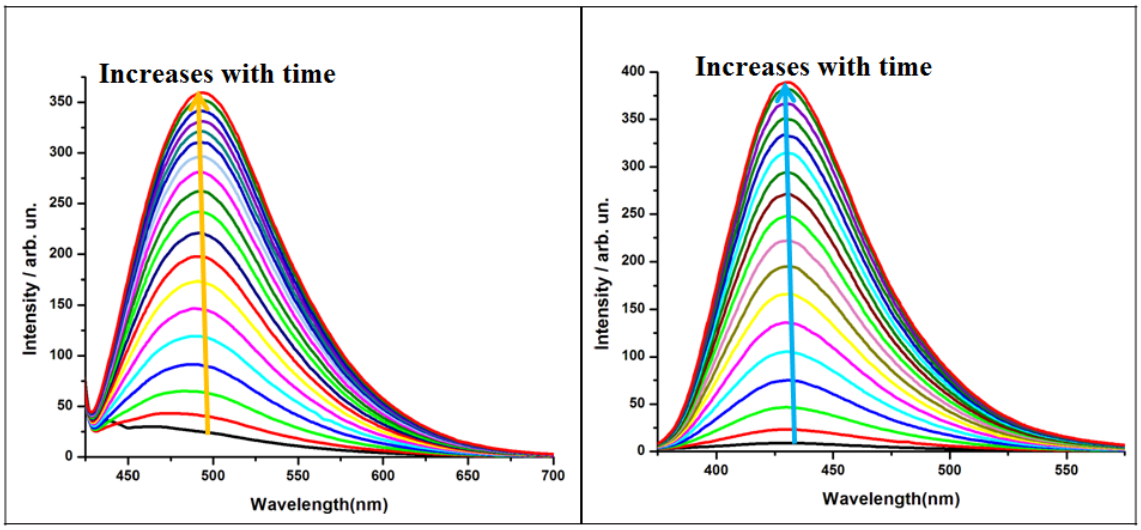


Fig.S10 Changes in the fluorescence spectra with time (2 min interval) of BENPH (20 μM, 0.1 M HEPES buffered ethanol/ water = 1/1, v/v, pH 7.4) upon addition of 300 μM (a) Trp (increases up to 36 min) and (b) His (increases up to 34 min).

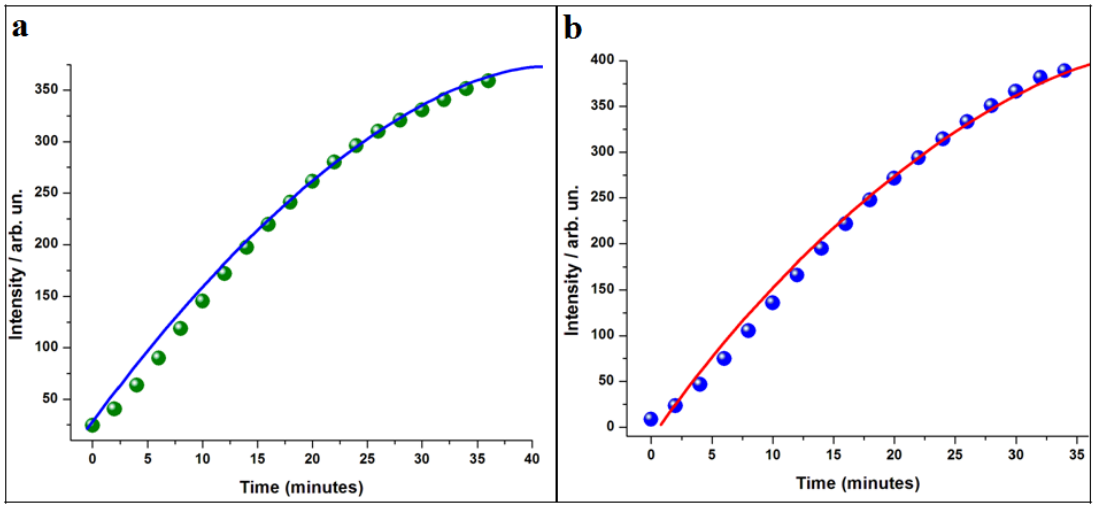


Fig.S11 Changes in the emission intensity of BENPH (20 µM, 0.1 M HEPES buffered ethanol/ water = 1/1, v/v, pH 7.4) with time upon addition of 300 µM (a) Trp (increases up to 36 min, λ_{ex}, 410 nm, λ_{em}, 495 nm) and (b) His (increases up to 34 min, λ_{ex}, 330 nm, λ_{em}, 430 nm).

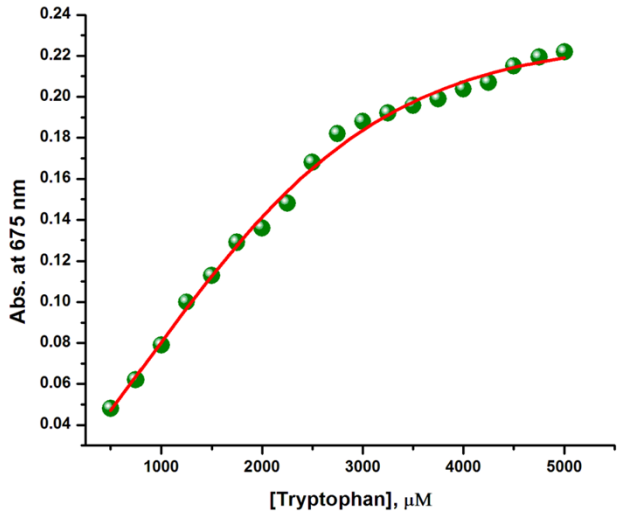


Fig.S12 Changes in the absorbance of BENPH (80 µM, at 675 nm) as a function of externally added Trp (1-5000 µM).

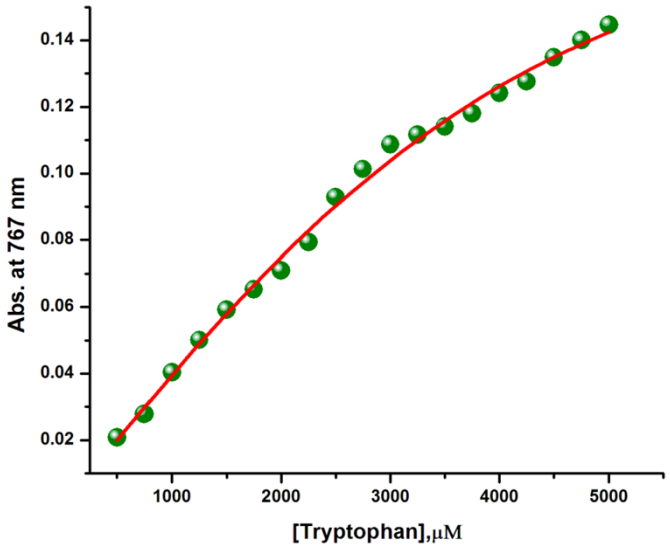


Fig.S13 Changes in the absorbance of BENPH (80 μM, at 767 nm) as a function of externally added Trp (1-5000 μM).

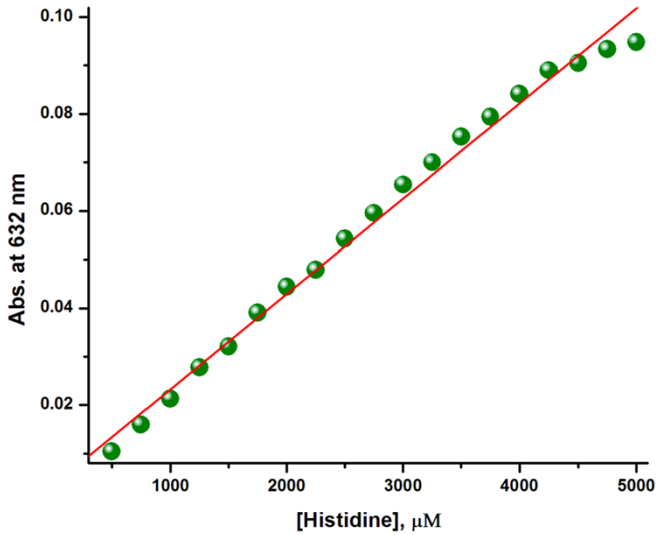


Fig.S14 Absorbance of BENPH (80 μM) at 632 nm as a function of externally added His (1-5000 μM).

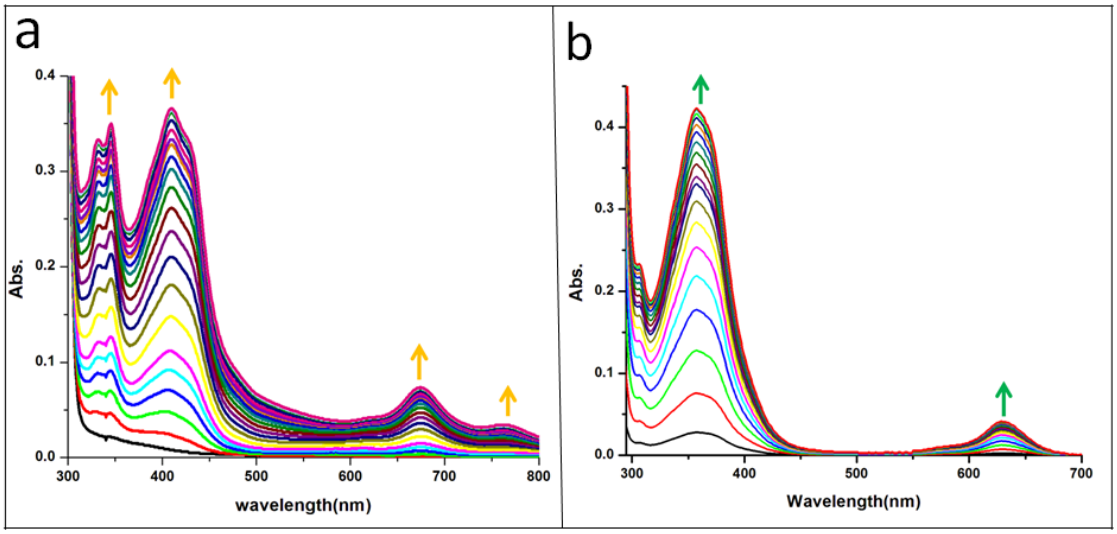


Fig.S15 Changes in the UV-Vis spectra of BENPH (20 μM, 0.1 M HEPES buffered ethanol/water = 1/1, v/v, pH 7.4) with time (2 min interval) upon addition of 2000 μM (a) Trp and (b) His.

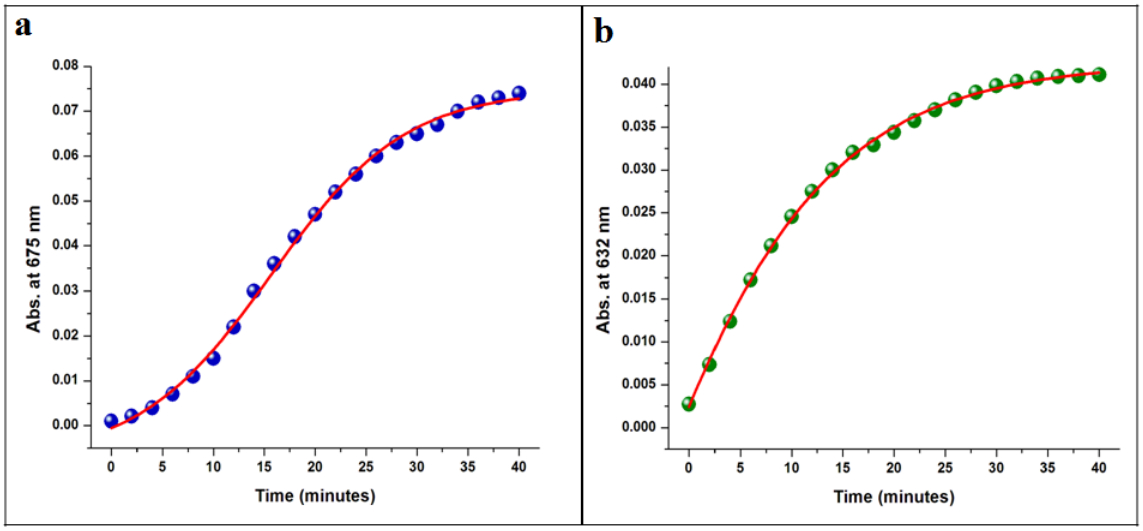


Fig.S16 Changes in the absorbance of BENPH (80 μM, 0.1 M HEPES buffered ethanol/ water = 1/1, v/v, pH 7.4) at 675 nm and 632 nm with time upon addition of 5000 μM of (a) Trp and (b) His respectively.

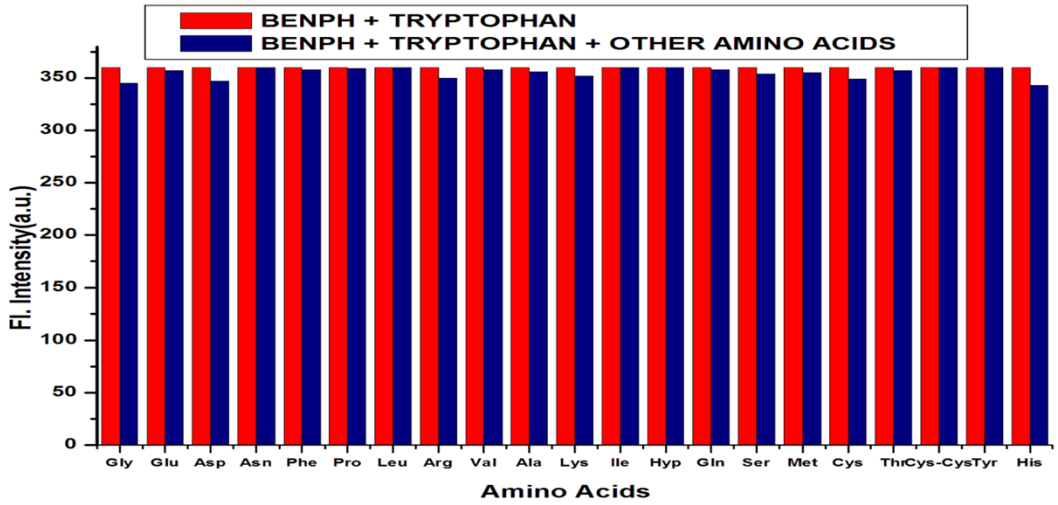


Fig.S17 Amino acids selectivity of BENPH (20 μM) in HEPES buffer (0.1 M; EtOH/H_2O, 1/1, v/v; pH 7.4) Red bars represent the emission intensity of [BENPH + Trp (300 μM)] system and blue bars show emission intensity of [BENPH + Trp] system in presence of 300 μM different amino acids ($\lambda_{em,}$ 495 nm).

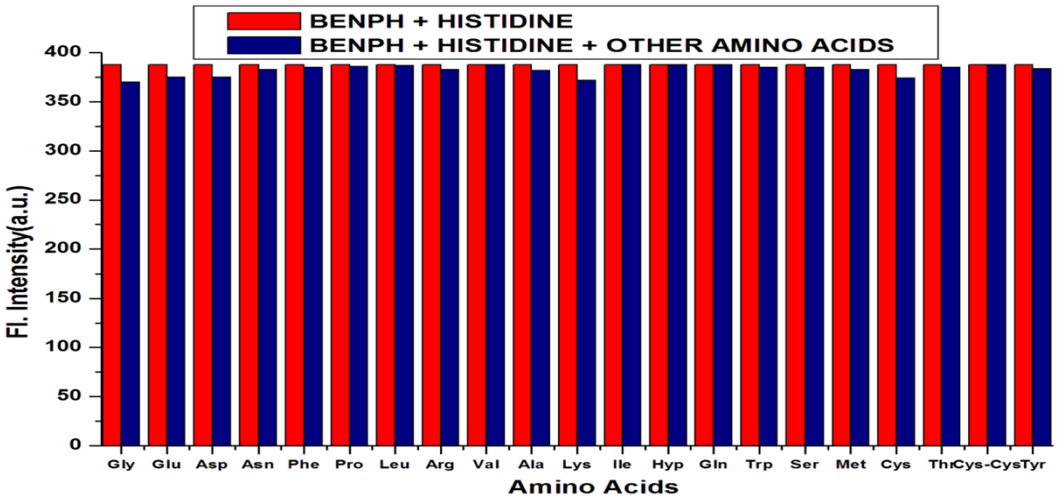


Fig.S18 Amino acids selectivity of BENPH (20 μM) in HEPES buffer (0.1 M; EtOH/H_2O, 1/1, v/v; pH 7.4). Red bars represent the emission intensity of [BENPH + His (300 μM)] system and blue bars show the emission intensity of [BENPH + His] system in presence of 300 μM different amino acids ($\lambda_{em,}$ 430 nm).

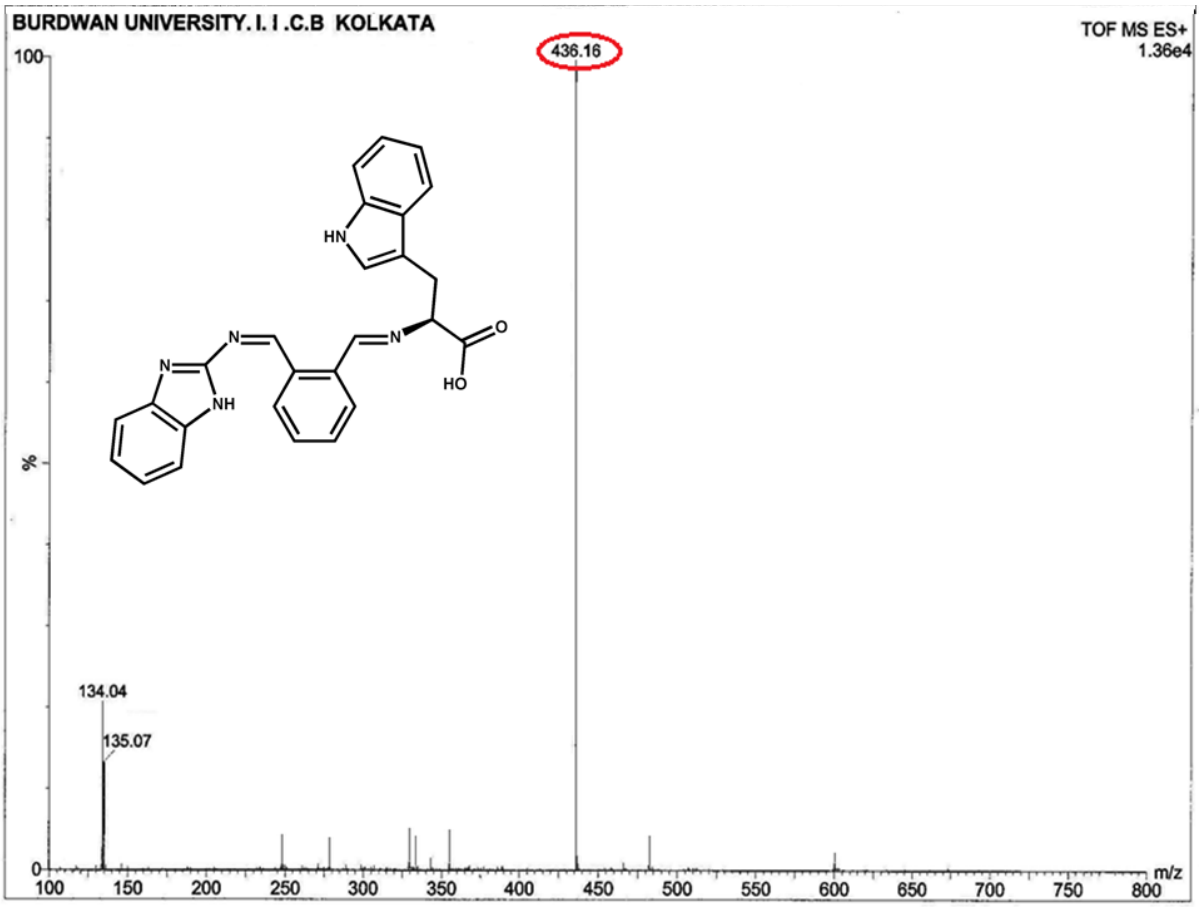


Fig.S19 QTOF-MS spectrum of [BENPH + Trp] adduct.

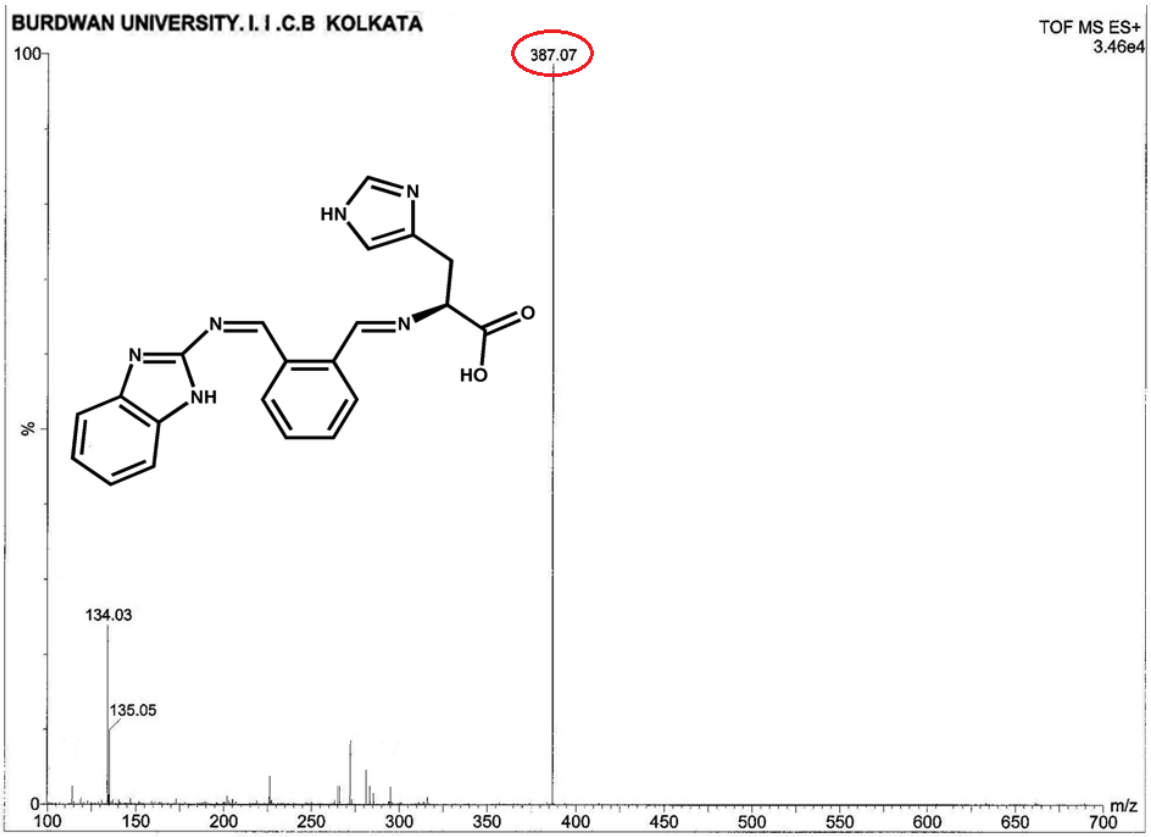


Fig.S20 QTOF-MS spectrum of [BENPH + His] adduct.

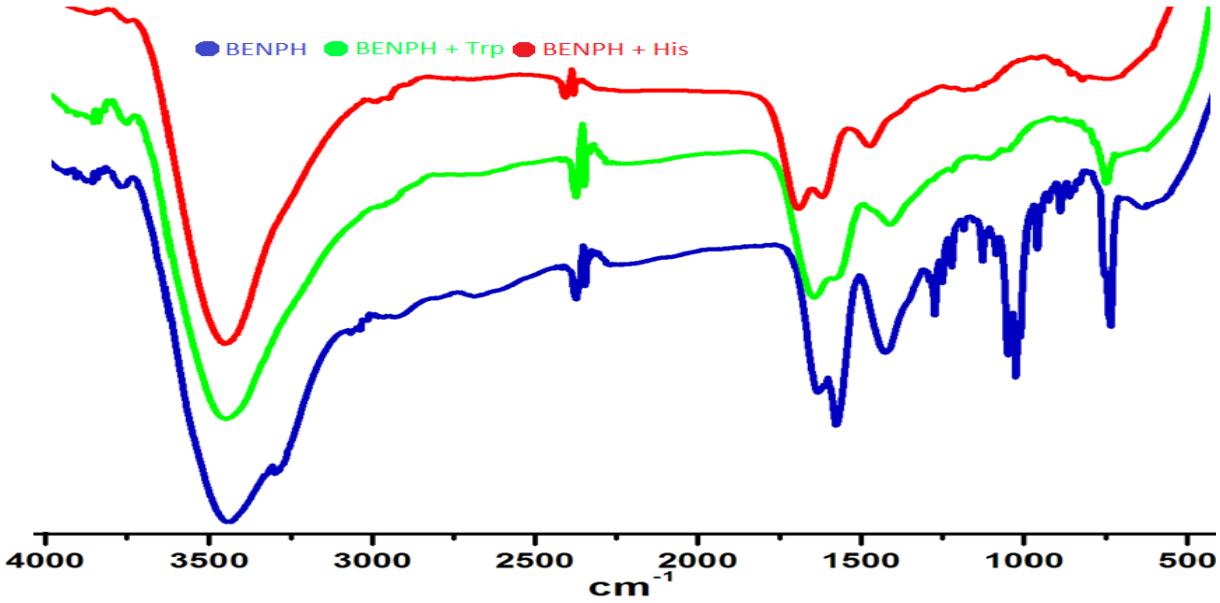


Fig.S21 FTIR spectral changes of BENPH upon interaction with Trp and His

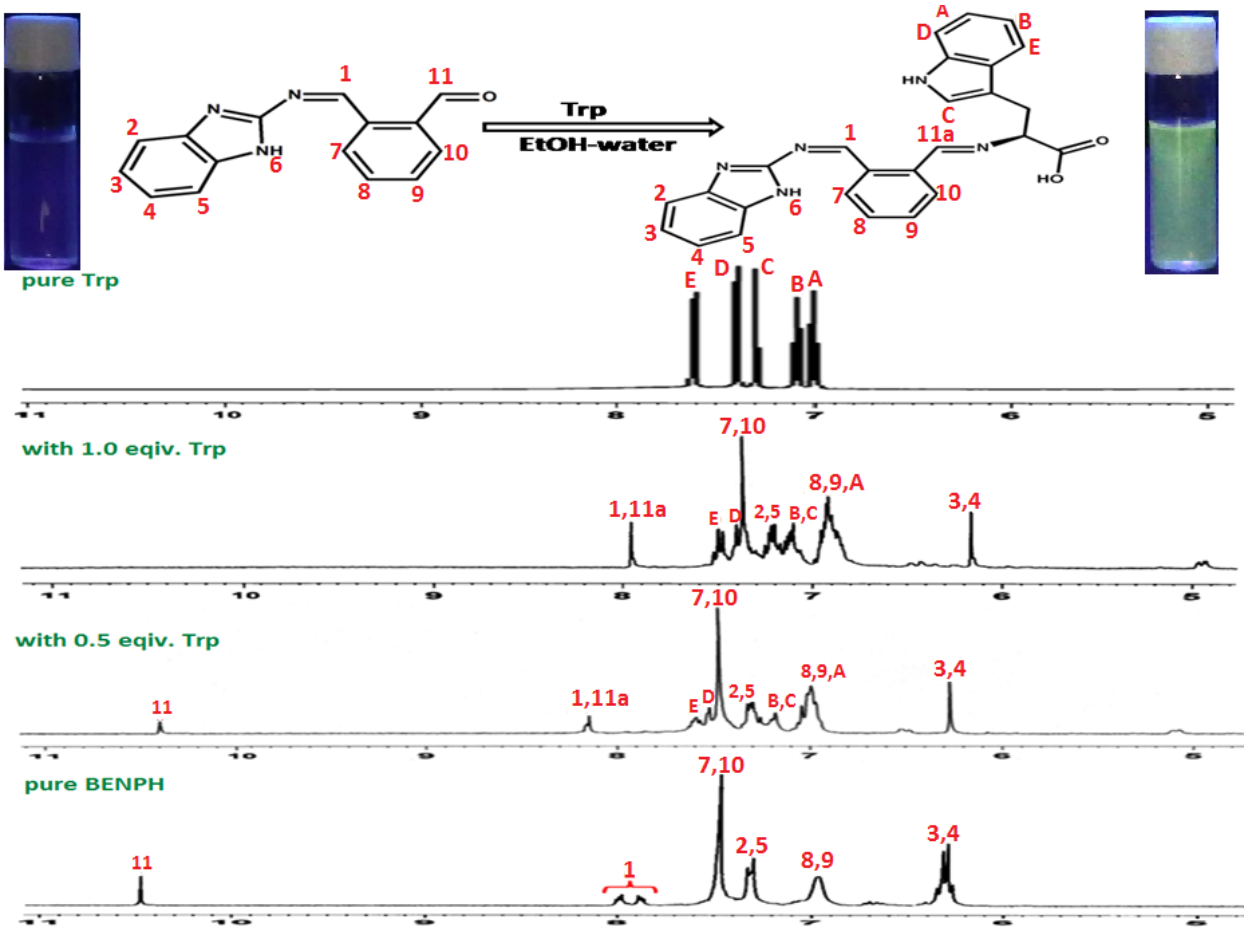


Fig.S22 Changes in the ^{1}H NMR spectra of BENPH upon interaction with Trp (in $DMSOd_6$ - D_2O).

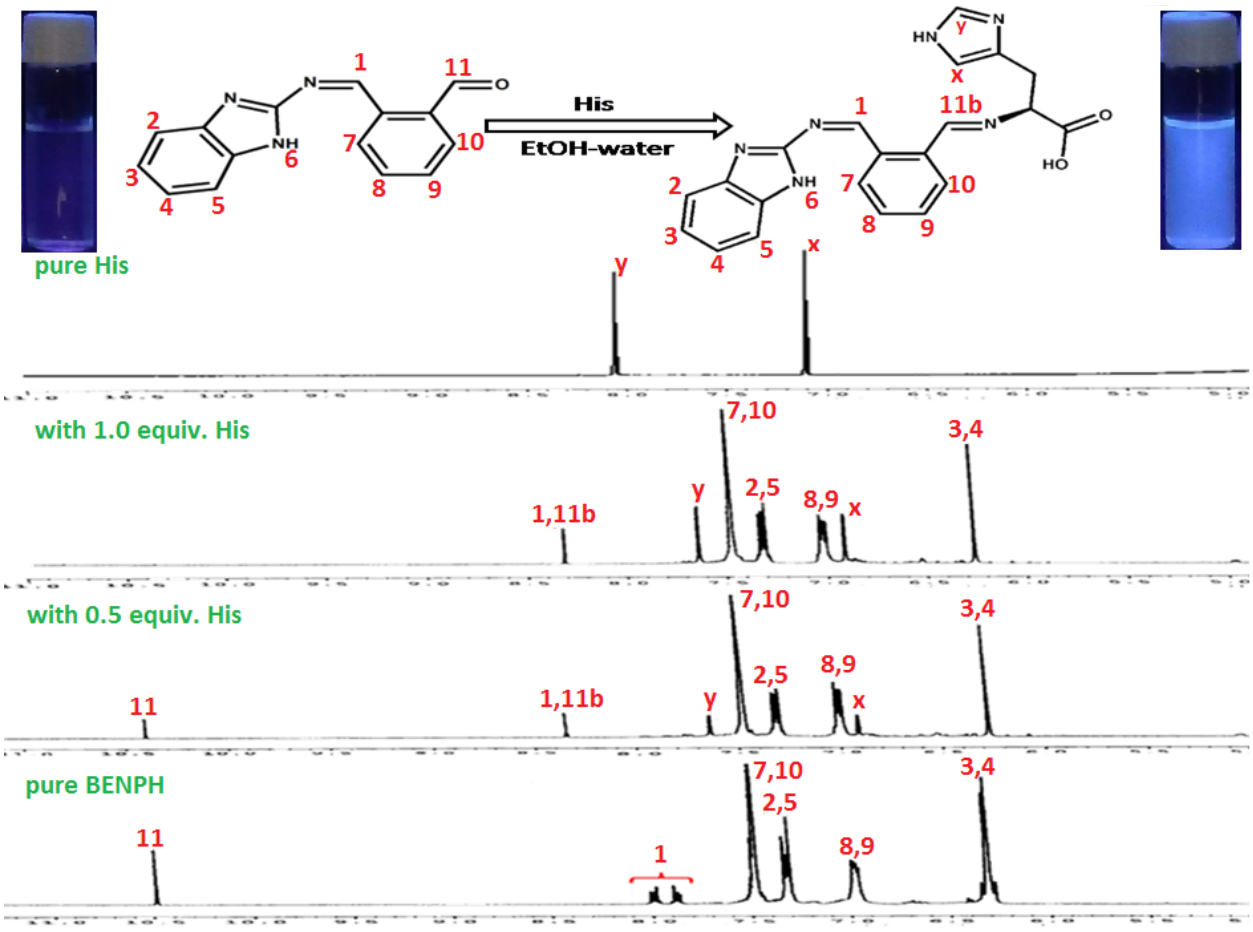


Fig.S23 Changes in the ^{1}H NMR spectra of BENPH upon interaction with His (in $DMSOd_6$ - D_2O).

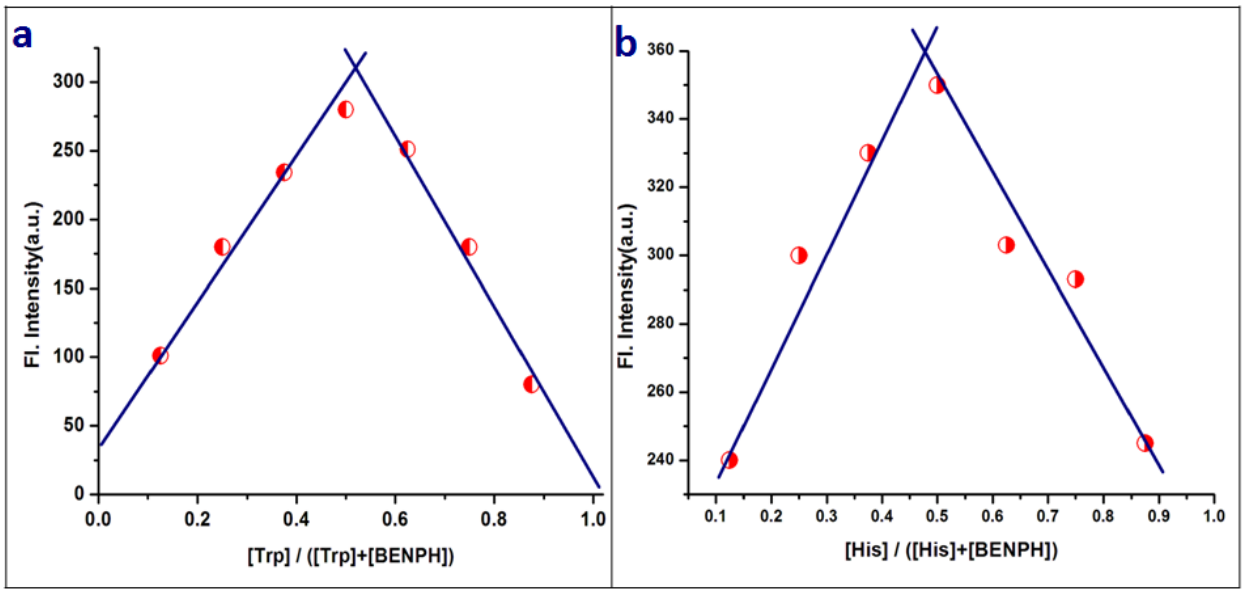


Fig.S24 Job's plot (stoichiometry determination) of the adducts between BENPH and (a) Trp and (b) His (HEPES buffer, 0.1 M, ethanol/water = 1/1, v/v, pH 7.4).

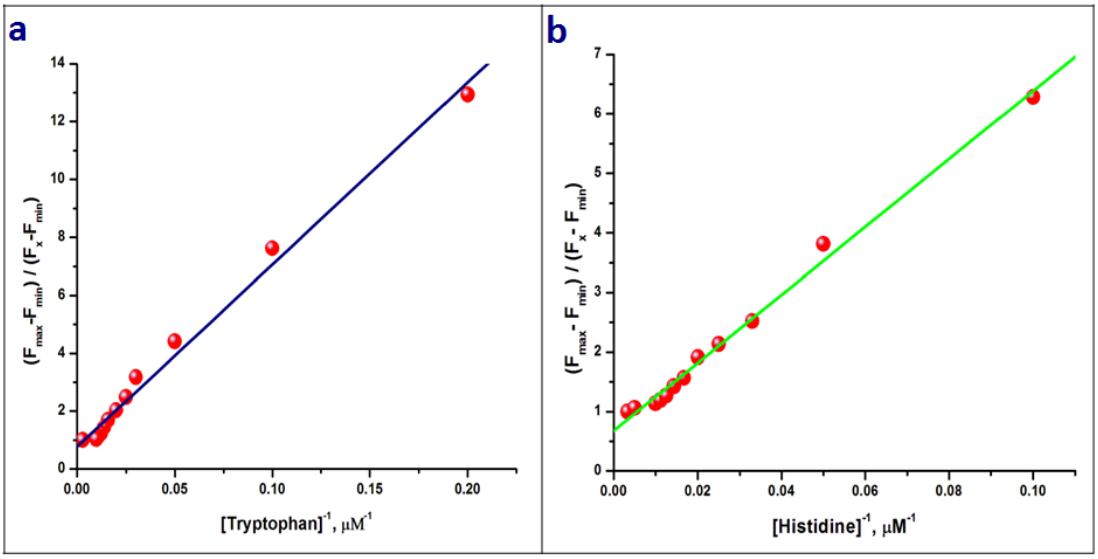


Fig.S25 Determination of binding constant of BENPH with (a) Trp (λ_{ex}, 410 nm; λ_{em}, 495 nm) and (b) His (λ_{ex}, 330 nm; λ_{em}, 430 nm) in HEPES buffered (0.1 M, ethanol/water = 1/1, v/v, pH 7.4).

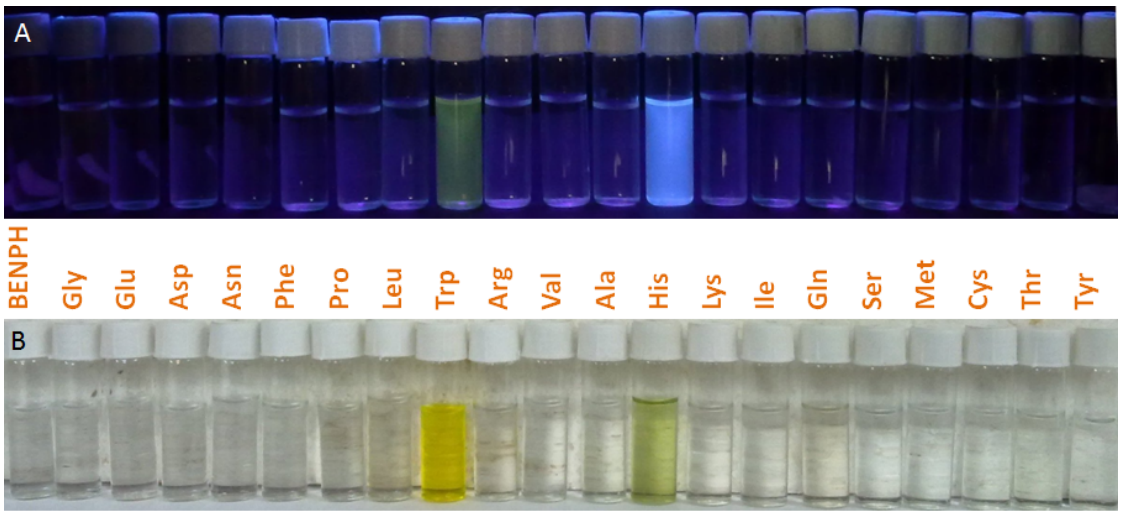


Fig.S26 Visual color changes of BENPH (80 μM) upon addition of equimolar (1500 μM) various amino acids under UV light (A) and through naked eye (B)

Table S1 Changes in chemical shifts (δ ppm) values of BENPH protons during ^{1}H NMR titration with Trp and His.

Proton number	δ (ppm)				
	Free BENPH	BENPH + 0.5 eq.		BENPH + 1.0 eq.	
		Trp	His	Trp	His
3,4	6.32	6.26	6.27	6.16	6.26
8,9	6.97	7.04	7.00	6.96	7.02
2,5	7.38	7.30	7.33	7.20	7.33
7,10	7.54	7.47	7.47	7.35	7.46
1	7.8, 8.0	8.18	8.34	8.00	8.34
11	10.48	10.48	----	10.48	----
6	----	----	----	----	----

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