Supporting Information

Sequential Activation Fluorescent Probes Based on Purine Scaffold: Enabling Precise Spatiotemporal Monitoring of H_2O_2/TH in Brainstem NTS for Spontaneous Hypertension

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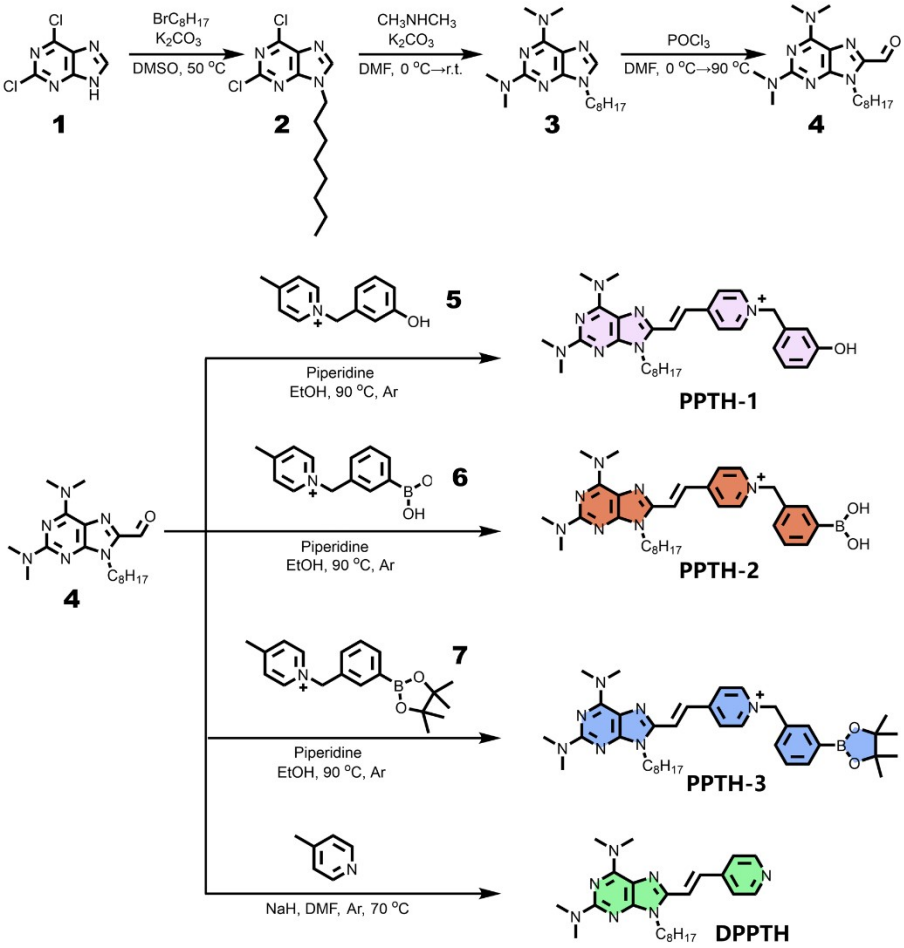
1. Synthesis and Characterization

1.1 Materials and instruments

^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker AM400 NMR spectrometer. Proton chemical shifts of the NMR spectra are given in ppm relative to an internal reference, TMS (1H, 0.00 ppm). ESI-MS and HRMS spectral data were recorded on a Finnigan LCQDECA and a Bruker Daltonics Bio TOF mass spectrometer, respectively. Fluorescence emission spectra were tested using a Horiba Duetta at 298 K. The imaging experiments of living cells were performed on a ZEISS LSM 780 confocal laser scanning microscope (CLSM).

1.2 Synthesis of Compounds



Scheme S1. Synthetic routes of **PPTHs** and Product **DPPTH**.

Compound 2: Commercially available 2,6-dichloropurine **1** (3.78 g, 20.0 mmol, 1.0 equiv) and potassium carbonate (4.14 g, 30.0 mmol, 1.5 equiv) were weighed and added to a round-bottom flask. DMSO (50 mL) was then introduced as the reaction solvent, and the mixture was stirred thoroughly until homogeneous. The reaction system was heated and stirred at 50 °C, followed by the slow addition of 1-bromooctane (11.60 g, 60.0 mmol, 3.0 equiv). Stirring was continued for an additional 6 hours. The reaction progress was monitored by thin-layer chromatography (TLC). Upon completion, the mixture was cooled to room temperature (RT), quenched with water, and extracted with ethyl acetate. The organic phase was collected, dried over anhydrous sodium sulfate, and concentrated under reduced pressure to remove the organic solvent. The crude product was purified by column chromatography using petroleum ether/ethyl acetate (1:2, v/v) as the eluent, affording compound **2** as a white solid (3.92 g, 65% yield).

Compound 3: Compound **2** (2.62 g, 8.7 mmol, 1.0 equiv) and potassium carbonate (3.63 g, 26.3 mmol, 3.0 equiv) were weighed and added to a round-bottom flask. DMF (10 mL) was then added as the reaction solvent, and the mixture was stirred thoroughly until homogeneous in an ice-water bath. Subsequently, dimethylamine (40% aqueous solution, 4 mL) was introduced into the system. The mixture was continuously stirred in the ice-water bath for 1 hour, followed by heating and stirring at 100 °C for 48 hours.

The reaction progress was monitored continuously by TLC. Upon confirmed completion, the reaction system was allowed to cool to RT and then slowly poured into ice water. Thereafter, the mixture was subjected to suction filtration to separate the liquid and solid phases, and the resulting solid product was collected. The crude solid was further purified by column chromatography using petroleum ether/ethyl acetate (1:1, v/v) as the eluent, affording compound **3** as a white solid (1.88 g, 68% yield).

Compound 4: DMF (4 mL) was added to a round-bottom flask as the solvent, and the flask was placed in an ice-water bath for cooling. After the solvent was completely cooled down, phosphorus oxychloride (935 μL) was added to the flask, with continuous stirring maintained. Thirty minutes later, compound **3** (318.47 mg, 1 mmol, 1.0 equiv) dissolved in 2 mL of DMF was introduced into the flask. Subsequently, the reaction system was heated to 90 °C and stirred for 24 hours. The reaction progress was monitored by TLC. Upon confirmed completion, the cooled reaction mixture was poured into ice water. The resulting mixture was extracted with dichloromethane, and the organic phase was collected, dried over anhydrous sodium sulfate, and concentrated under reduced pressure to remove the solvent. The crude product was purified by column chromatography using petroleum ether/ethyl acetate (10:1, v/v) as the eluent, affording compound **4** as a yellow solid (138.59 mg, 40% yield).

Compound **5**, **6**, **7**, and **DPPTH** were synthesized according to previously report studies.[1-3]

PPTH-1: Compound **4** (173.24 mg, 0.5 mmol, 1.0 equiv) and compound **5** (100 mg, 0.5 mmol, 1.0 equiv) were added to a flask, which was then evacuated and backfilled with argon three times. Piperidine (42.82 mg, 0.5 mmol, 1.0 equiv) and ethanol (2.5 mL) were subsequently introduced into the flask. The reaction system was stirred at 79 °C for 12 hours. Upon completion of the reaction, the solvent was immediately removed under reduced pressure. The crude product was recrystallized from boiling ethanol and finally purified by silica gel chromatography using dichloromethane/methanol (15:1, v/v) as the eluent, yielding the corresponding target product **PPTH-1** as a reddish-brown solid (180.92 mg, 70% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.75 (s, 1H), 9.03 (d, J = 7.8 Hz, 2H), 8.41 (d, J = 6.5 Hz, 2H), 7.99 (d, J = 14.7 Hz, 1H), 7.66 (d, J = 17.1 Hz, 1H), 7.27 – 7.21 (m, 1H), 7.15 (d, J = 7.0 Hz, 1H), 6.80 (d, J = 7.3 Hz, 1H), 6.73 (d, J = 6.0 Hz, 1H), 5.63 (s, 2H), 4.33 – 4.19 (m, 2H), 3.09 (d, J = 24.8 Hz, 12H), 1.69 (dd, J = 15.2, 6.5 Hz, 2H), 1.26 – 1.09 (m, 10H), 0.82 – 0.75 (t, J = 16.16, 8.4 Hz, 3H). ESI-HRMS (m/z): calculated for $C_{31}H_{42}N_7O^+$, $[M+H]^+$, 529.3518; found 529.3532.

PPTH-2: Compound **4** (178.38 mg, 0.5 mmol, 1.0 equiv) and compound **6** (114.06 mg, 0.5 mmol, 1.0 equiv) were added to a flask, which was then evacuated and backfilled with argon three times. Piperidine (42.82 mg, 0.5 mmol, 1.0 equiv) and ethanol (2.5 mL) were subsequently added to the system. The reaction mixture was stirred at 79 °C for 12 hours. Upon completion of the reaction, the solvent was immediately removed under reduced pressure. The crude product was recrystallized from boiling ethanol and finally purified by silica gel chromatography using dichloromethane/methanol (15:1, v/v) as the eluent, yielding the corresponding product **PPTH-2** as a reddish-brown solid (200.29 mg, 72% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.03 (d, J = 9.9 Hz, 2H), 8.42 (s, 1H), 8.00 (s, 1H), 7.65 (d, J = 14.4 Hz, 1H), 7.19 (m, 2H), 6.87 – 6.70 (m, 3H), 5.63 (s, 2H), 4.28 (d, J = 6.5 Hz, 2H), 3.08 (d, J = 24.9 Hz, 12H), 1.68 (dd, J = 21.4, 10.7 Hz, 2H), 1.17 (dd, J = 21.1, 10.3 Hz, 10H), 0.82 – 0.72 (t, J = 17.9, 9.3 Hz, 3H). ESI-HRMS (m/z): calculated for $C_{31}H_{43}BN_7O^{2+}$, [M], 556.3566; found 556.3565.

PPTH-3: Compound **4** (178.38 mg, 0.5 mmol, 1.0 equiv) and compound **7** (155.10 mg, 0.5 mmol, 1.0 equiv) were added to a flask, which was then evacuated and backfilled with argon three times. Piperidine (42.82 mg, 0.5 mmol, 1.0 equiv) and ethanol (2.5 mL) were subsequently added to the system. The reaction mixture was stirred at 79 °C for 12 hours. Upon completion of the reaction, the solvent was immediately removed under reduced pressure. The crude product was recrystallized from boiling ethanol and finally purified by silica gel chromatography using dichloromethane/methanol (15:1, v/v) as the eluent, yielding the corresponding product **PPTH-3** as a reddish-brown solid (207.57 mg, 65% yield).

DPPTH: DMF (10 mL) and sodium hydride (NaH, 42.72 mg, 1.78 mmol, 0.44 equiv) were added to a two-necked flask, which was then evacuated and backfilled with argon three times. Subsequently, 4-methylpyridine (800 μL, 8.1 mmol, 2.01 equiv) was introduced, and the mixture was stirred at room temperature (RT) for 30 minutes. Compound **4** (1.39 g, 4.02 mmol, 1.0 equiv), synthesized in the previous step, was then added to the mixture. The reaction system was stirred at RT until the reaction was confirmed complete by TLC. Thereafter, the reaction was quenched with 1.5 mL of brine and extracted with ethyl acetate. The collected organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure. Upon standing for a period of time, a yellow solid crude product of **DPPTH** was formed. The crude product was then collected by suction filtration and rinsed repeatedly with ethyl acetate, affording the pure product **DPPTH** (931.48 mg, 55% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.58 (d, J = 5.7 Hz, 2H), 7.56 (d, J = 15.8 Hz, 1H), 7.38 (d, J = 6.1 Hz, 2H), 7.15 (d, J = 15.8 Hz, 1H), 4.16 (t, J = 7.1 Hz,

2H), 3.51 (s, 6H), 3.18 (s, 6H), 1.82 – 1.75 (m, 2H), 1.35 – 1.23 (m, 10H), 0.85 (dd, J = 8.9, 4.8 Hz, 3H). ESI-HRMS (m/z): calculated for $C_{24}H_{35}N_7$, $[M+H]^+$, 422.3027; found 422.3026.

2. General methods

2.1 Absorption Spectra and Molar Extinction Coefficients

Preparation of Probe Stock Solutions: Taking **PPTH-1** as an example, the product **PPTH-1** (52.87 mg, 0.1 mmol) was accurately weighed and dissolved in 1.0 mL of HPLC-grade DMSO. The prepared solution was transferred to a 5.0 mL volumetric flask, and additional DMSO was added to dilute the solution to the mark. The mixture was thoroughly shaken to obtain Stock Solution I with a concentration of 20 mM. Subsequently, 500 μL of Stock Solution I was mixed with 500 μL of HPLC-grade DMSO, and the resultant solution was vortexed to homogeneity to yield Stock Solution II at a concentration of 10 mM. Stock Solution II was aliquoted into 200 μL Eppendorf (EP) tubes and stored in a refrigerator at -20 °C for later use. The preparation procedures for the stock solutions of **PPTH-2**, **PPTH-3** and product **DPPTH** were identical to that of **PPTH-1**.

Determination of Absorption Spectra: The 10 mM stock solutions of **PPTHs** and **DPPTH** were separately diluted with PBS buffer solution to a series of test solutions with concentrations of 1 μM, 2 μM, 4 μM, 6 μM, 8 μM, and 10 μM. Using a Duetta instrument, the absorption spectra of these test solutions were measured over the wavelength range of 300–800 nm, thus acquiring the UV–vis absorption spectra of the corresponding compounds at the aforementioned concentrations.

Calculation of Molar Extinction Coefficients: According to the Lambert–Beer Law ($A=\varepsilon bc$), absorbance (A) is proportional to the optical path length (b) and analyte concentration (c) within a relatively low concentration range, where ε denotes the molar extinction coefficient of the substance. The experimentally measured absorbance changes (ΔA) were plotted against the corresponding concentration changes (Δc) to generate a linear fitting plot. The slope of the fitted line was calculated via linear regression analysis, which corresponds to the molar extinction coefficient (ε) of the probe in PBS buffer.

2.2 Cytotoxicity Assay

To evaluate the cytotoxicity of the probes, the Cell Counting Kit-8 (CCK-8) assay was employed to determine cell viability. First, SH-SY5Y cells in the logarithmic growth phase were selected, and the density of the cell suspension was accurately quantified using a hemocytometer. The cells were then digested and prepared into a homogeneous cell suspension, which was finally seeded uniformly into a 96-well plate for subsequent cytotoxicity analysis. Each well was filled with 100 μL of complete DMEM containing 1.0×10^4 cells, followed by incubation in a cell culture incubator supplied with 5% CO^2. After 24 hours of incubation, the original medium was discarded, and 100 μL of complete DMEM medium containing the probes at various concentrations (0, 1.25, 2.5, 5.0, 10.0, and 20.0 μM) was added to each well. The 96-well plate was then placed back into the incubator for another 24 hours of incubation. Subsequently, the medium was removed, and 100 μL of phosphate-buffered saline (PBS) containing 10% CCK-8 reagent was added to each well. The plate was incubated again for approximately 1 hour. After incubation, the absorbance of each well at a wavelength of 450 nm was measured using a microplate reader. The cell viability (CV, %) was calculated as the ratio of the OD_{450} value of the sample group to that of the control group. Five independent replicate samples were tested for each concentration, and the final results were expressed as mean ± standard deviation (SD).

2.3 Living Cell imaging

SH-SY5Y and HeLa cells in the logarithmic growth phase were digested with trypsin, and the resulting cell suspensions were transferred into glass-bottom confocal dishes. The cells were incubated in a cell culture incubator for 24–36 hours. Subsequent imaging experiments were performed only after microscopic observation confirmed that the cells were fully adherent to the dish bottom and exhibited a well-spread morphology. Prior to conducting confocal imaging experiments, the original medium was first discarded, and the cells were gently rinsed once with pre-warmed PBS (37 °C). The cells were then incubated with DMEM medium containing probes at specific concentrations or commercially available dyes for a predetermined duration. After incubation, the cells were gently rinsed with PBS, and confocal imaging was immediately performed.

For the photostability imaging assay, SH-SY5Y cells were incubated with DMEM medium containing 10 μM **PPTH-1**, **PPTH-2**, and **PPTH-3** at 37 °C for 50 min. Subsequently, the cells were scanned continuously for 50 cycles using 488 nm and 405 nm lasers at 2% confocal laser power. A laser scanning confocal

microscope (LSM 780) was employed to record the changes in intracellular fluorescence intensity after each scanning cycle.
PPTH-1/2/3: Excitation at 543 nm, emission wavelength range of 670–758 nm;
Product **DPPTH**: Excitation at 405 nm, emission wavelength range of 480–550 nm.

3. Additional Data

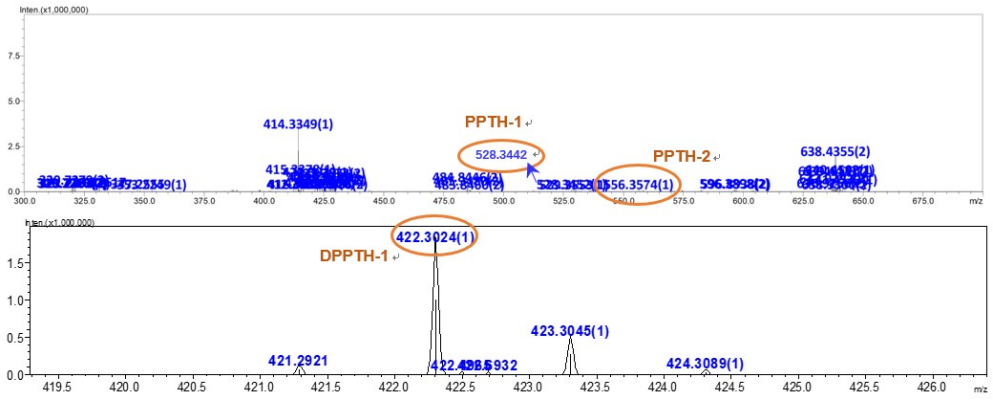


Figure S1. high-resolution mass spectrometry (HRMS) for the reaction of probe **PPTH-2** (10 μM) in SH-SY5Y cell lysate with H_2O_2 (1 μM).

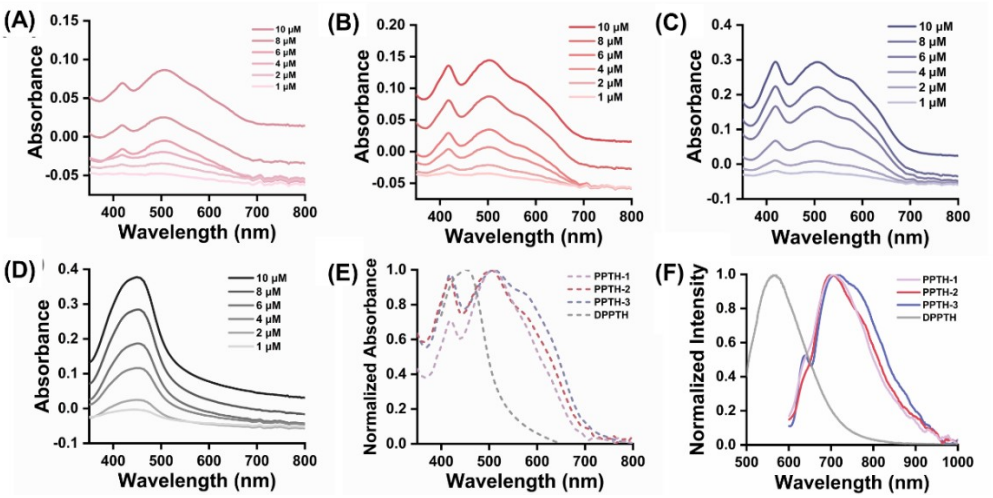


Figure S2. Absorption spectra of (A) **PPTH-1**, (B) **PPTH-2**, (C) **PPTH-3**, and (D) **DPPTH** at different concentrations in PBS; (E) Normalized UV–vis absorption spectra of **PPTHs** and **DPPTH**; (F) Normalized fluorescence spectra of **PPTHs** and **DPPTH**.

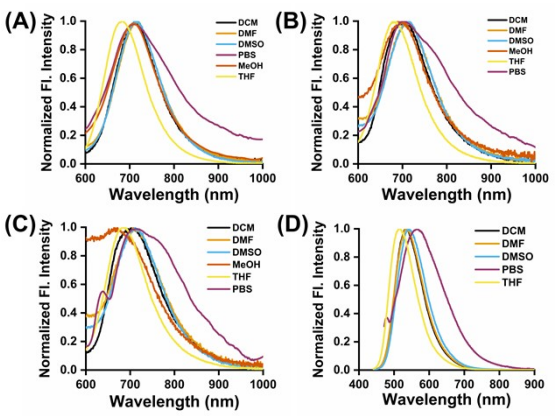


Figure S3. Normalized fluorescence spectra (A) **PPTH-1**, (B) **PPTH-2**, (C) **PPTH-3**, and (D) **DPPTH** in different solvent.

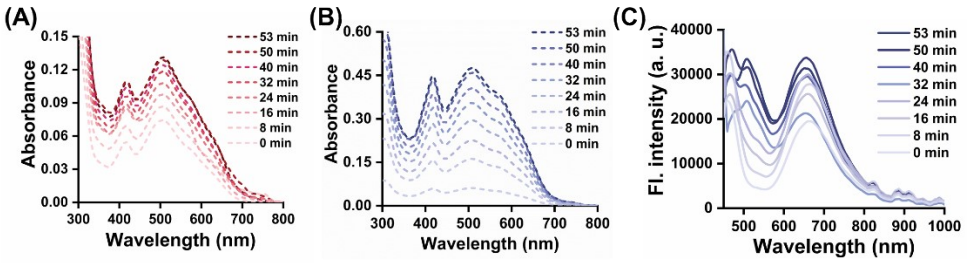


Figure S4. Time-dependent (A) absorption spectra of **PPTH-2** (10 μM), (B) absorption spectra of **PPTH-3** (10 μM), and (C) fluorescence spectra of **PPTH-3** (10 μM) in SH-SY5Y cell lysate (3.0×10^5 cells/mL).

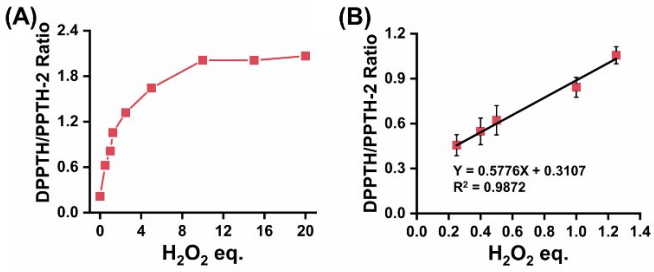


Figure S5. (A) Variation in the ratio of the maximum fluorescence intensity of **DPPTH** to that of **PPTH-2** as a Function of H_2O_2 concentration in SH-SY5Y cell lysate (3.0×10^5 cells/mL); (B) Linear correlation between the ratio of the maximum fluorescence intensity of **DPPTH** to that of **PPTH-2** and H_2O_2 concentration in SH-SY5Y cell lysate (3.0×10^5 cells/mL).

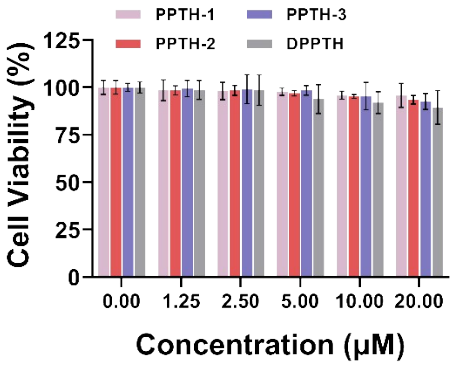


Figure S6. Cytotoxicity assay results of **PPTHs** and **DPPTH** against SH-SY5Y cells.

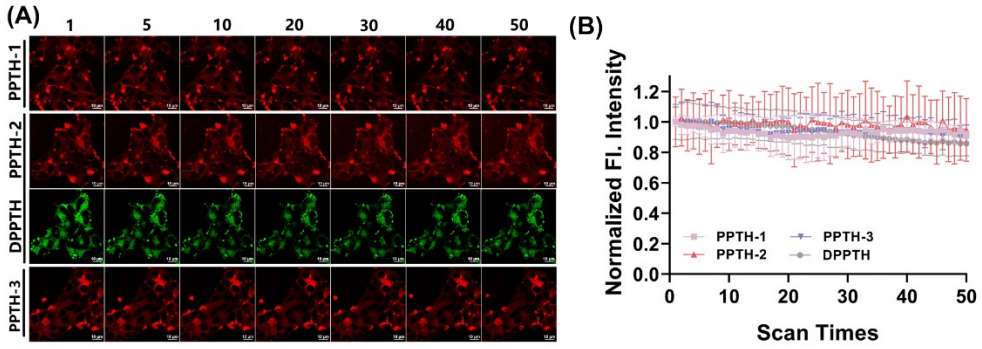


Figure S7. (A) Photostability study of **PPTHs** and **DPPTH** in SH-SY5Y cells; confocal images captured at specific time points during 50 consecutive scans with 2% confocal laser intensity; scale bar: 10 μm; (B) Normalized fluorescence intensity of the probes and product per scan in SH-SY5Y cells. **PPTH-1**: λ_{ex}=543 nm, λ_{em}=670−758 nm; **PPTH-2**: λ_{ex}=543 nm, λ_{em}=670−758 nm; **PPTH-3**: λ_{ex}=543 nm, λ_{em}=670−758 nm; **DPPTH**: λ_{ex}=405 nm, λ_{em}=480−550 nm.

Table S1. Optical Properties of Probes.

	Abs (nm)	Em (nm)	ε (M^{-1} cm^{-1})	Stokes shifts (nm)	Φ_{fl} (%) PBS	Φ_{fl} (%) DCM
PPTH-1	506	705	1.45×10^4	199	1.28	9.58
PPTH-2	502	702	1.95×10^4	200	0.84	8.33
PPTH-3	510	712	3.48×10^4	202	1.45	10.35
PTH-1	352	730	6.88×10^3	378	0.99	3.83
PTH-2	358	750	8.52×10^3	392	0.64	2.16

Table S2. Blood pressure-related data of normal and spontaneously hypertensive rats

	No.	HR	SBP	MBP	DBP		No.	HR	SBP	MBP	DBP
	A1	391	104	91	84		A7	369	168	137	121
Control	A2	354	114	97	88	**SHR**	A8	305	136	114	103
	A3	389	130	105	92		A9	390	213	176	158

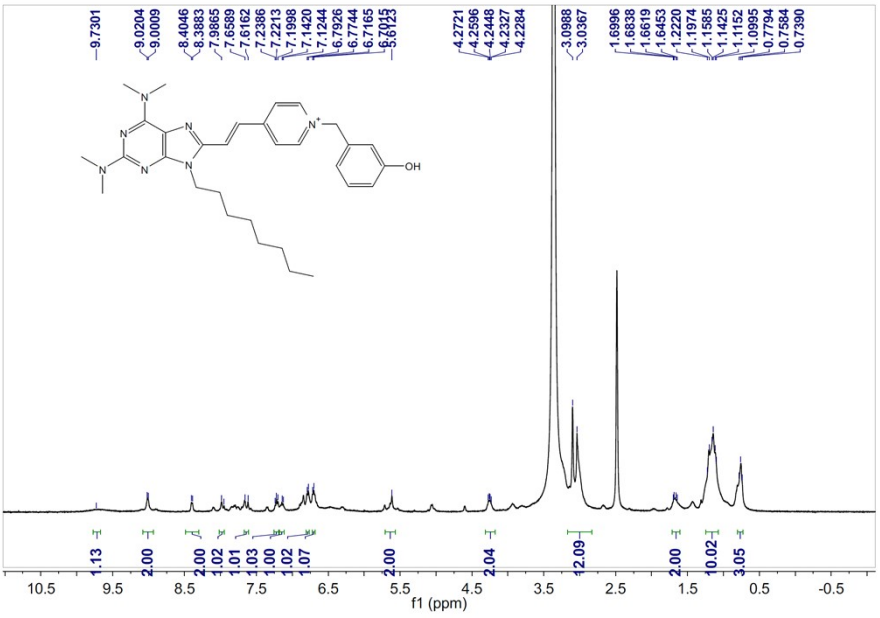


Figure S8. ^{1}H NMR Spectrum of **PPTH-1** (400 MHz, DMSO-d_6).

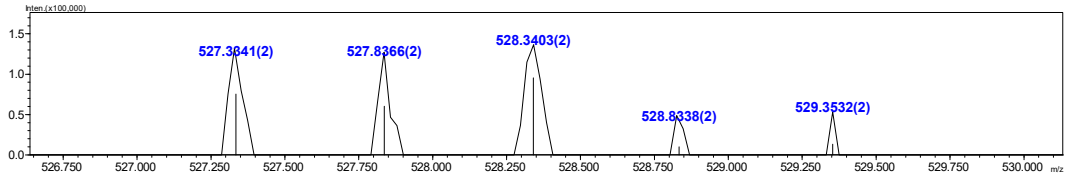


Figure S9. HRMS Spectrum of **PPTH-1**.

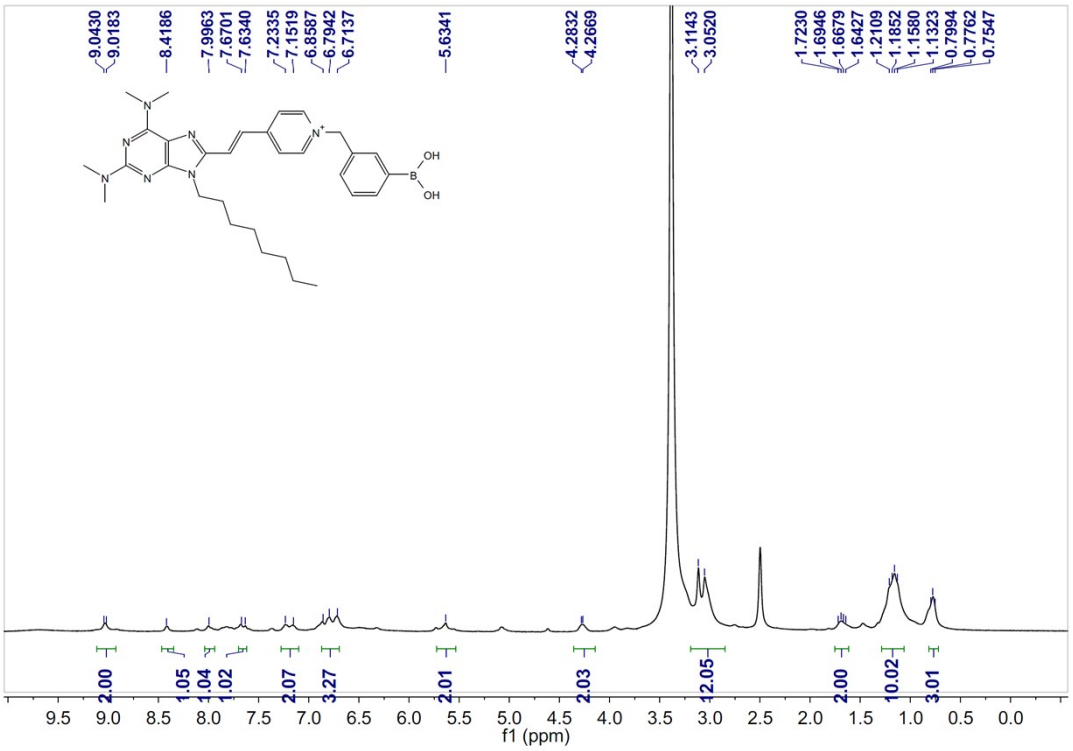


Figure S10. ^{1}H NMR Spectrum of **PPTH-2** (400 MHz, DMSO-d_6).

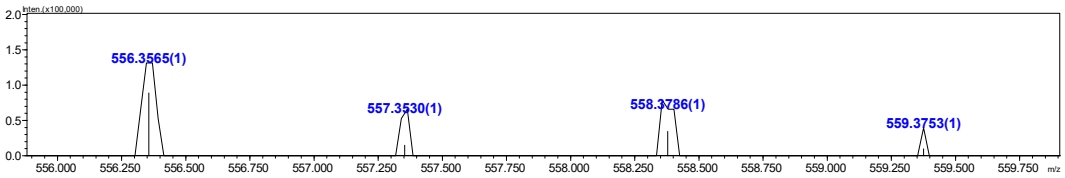


Figure S11. HRMS Spectrum of **PPTH-2**

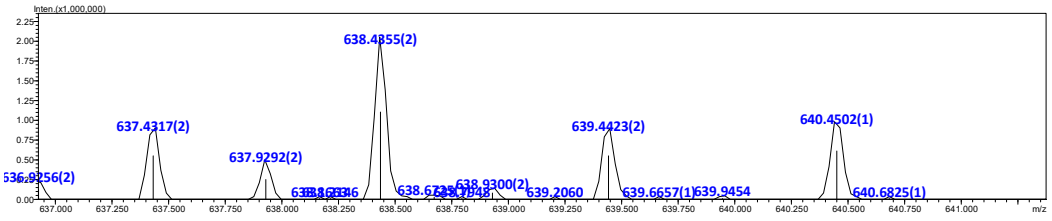


Figure S12. HRMS Spectrum of **PPTH-3**

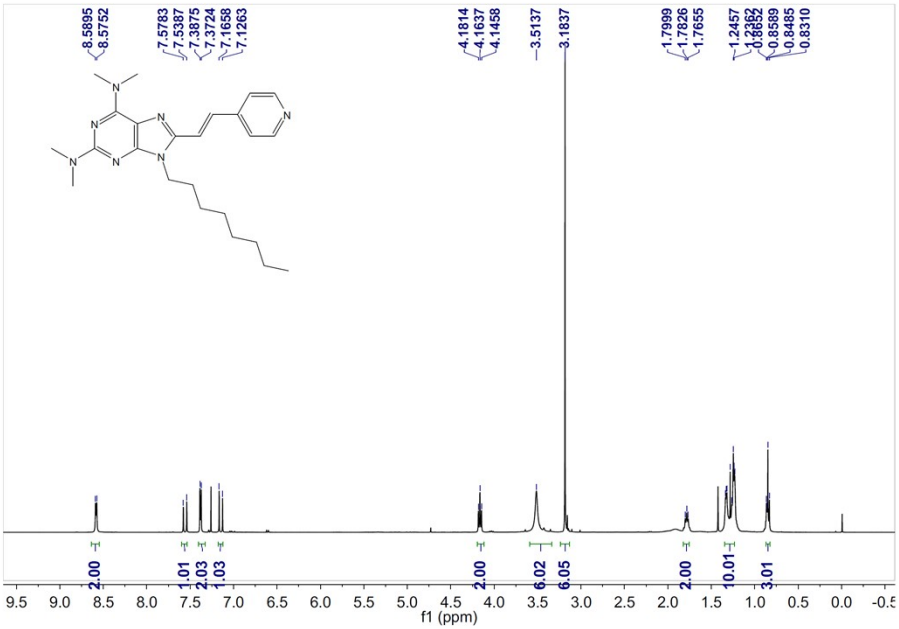


Figure S13. ^{1}H NMR Spectrum of **DPPTH** (400 MHz, $CDCl_3$).

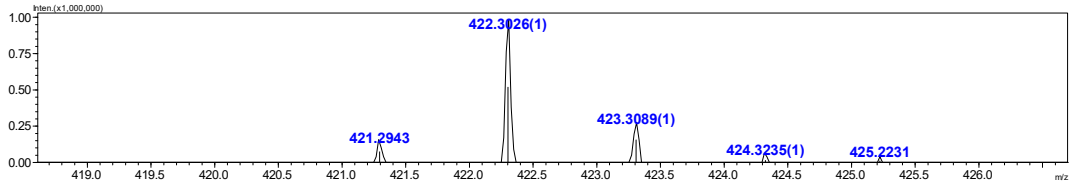


Figure S14. HRMS Spectrum of **DPPTH**.

4. References

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