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Development of Benzothiazole-Functionalized Red-Emission Pyronin Dye and Its Dihydro Derivative for Imaging Lysosomal Viscosity and Tracking Endogenous Peroxynitrite

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1. General Information

All reagents and solvents in this study were purchased from commercial sources and were of the highest grade. Solvents were dried according to standard methods. Absorption spectra were performed on a Varian Carry 4000 spectrophotometer. Fluorescence spectra were measured on Hitachi F-7000 fluorescence spectrometer. The ^{1}H NMR and ^{13}C NMR spectra were acquired on 600 and 150 MHz, respectively. High resolution mass spectra were collected on a Varian QFT-ESI mass spectrometer. Live cell fluorescence images were acquired by Zeiss LSM 880+ Airyscan Laser Scanning Confocal Microscope with a 60×oil–immersion objective lens. Stock solutions of both **BTP** and **HBTP** were prepared in CH_3CN, and the concentration is 2 mM.

2. Experimental Section

*2.1 Synthesis of **BTP** and **HBTP***

Xanthone 1 was synthesized by the procedures reported previously.[1]

Compound BTP. To a flame-dried flask flushed with argon, benzothiazole (405 mg, 3.0 mmol) and dry THF (30 mL) were added. The solution was cooled to -78 °C, 2.4 M *n*-BuLi (1.25 mL, 3.0 mmol) was added, and the mixture was stirred for 90 min at the same temperature. Xanthone **1** (338mg, 1.0 mmol) in anhydrous THF (20 mL) was slowly added, and then the mixture was slowly warmed up to room temperature and

stirred for 5 h. The reaction was quenched by addition of 2 N HCl and then stirred at room temperature for 10 min. The resulting mixture was extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4 and removed out under reduced pressure. The crude product was purified by flash column chromatography dichloromethane/methanol (20/1) to afford pure product as a dark blue solid (354 mg, 78% yield). ^{1}H NMR (600 Hz, $CDCl_3$) δ 8.25 (d, J = 8.4 Hz, 1H), 8.11 (d, J = 7.8 Hz, 1H), 7.68 (t, J = 8.4 Hz, 3H), 7.61 (t, J = 7.2 Hz, 1H), 7.03 (d, J = 7.2 Hz, 2H), 6.92 (s, 2H), 3.72 (s, 8H), 1.37 (s, 12H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 177.8, 157.4, 155.7, 153.4, 145.9, 136.1, 131.4, 127.1, 124.4, 122.0, 115.1, 113.0, 97.2, 46.7, 12.9; ESI-MS $[M]^+$: Calcd for 456.2110, Found 456.2101.

Compound HBTP. Compound **BTP** (200 mg, 0.4 mmol) was dissolved in methanol (20 mL) and $NaBH_4$ (16.6 mg，0.44 mmol) was added portion wise to the solution for 1 min. After stirred for further 5 min at the same temperature, the reaction was quenched by addition saturated $NaHCO_3$ aqueous solution. The resulting mixture was extracted with CH_2Cl_2. After dried over Na_2SO_4, the organic layer was removed under reduced pressure and the mixture was purified by flash column chromatography (PE/EA = 5/1) to afford pure product as yellow solid (160 mg, 88% yield). ^{1}H NMR (600 Hz, $CDCl_3$) 8.02 (d, J = 7.8 Hz, 1H), 7.71 (d, J = 7.8 Hz, 1H), 7.44 (t, J = 7.2 Hz, 1H), 7.30 (d, J = 7.8 Hz, 1 H), 7.16 (d, J = 8.4 Hz, 2 H), 6.42 (d, J = 2.4 Hz，2 H), 6.40 (d, J = 3.0 Hz，1 H), 6.39 (d, J = 2.4 Hz, 1 H), 5.66 (s, 1 H), 3.36 (m, J = 7.2 Hz，8 H), 1.28 (s, 1 H), 1.18 (t, J = 7.2 Hz, 12 H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 179.8, 152.9, 151.8, 148.5, 136.4, 130.5, 125.6, 124.5, 122.9, 121.7, 108.0, 107.5, 98.6, 44.4, 42.0, 12.6; ESI-MS $[M]^+$: Calcd for 458.2266, Found 458.2256.

2.2 Quantum yield determination

Fluorescence quantum yields of **BTP** in various solvents were determined with cresyl violet as standard (Φ = 0.53 in MeOH), respectively. The quantum yield was calculated by Eq.1.

$$\Phi_u = [(A_s F A_u \eta^2)/ (A_u F A_s \eta_0^2)]\ \Phi_s. \qquad \text{(Eq.1)}$$

A_s and A_u are the absorbance of the reference and sample solution at the reference excitation wavelength, respectively; FA_s and FA_u are the corresponding integrated

fluorescence intensity, and η and η_0 are the solvent refractive indexes of sample and reference, respectively. Absorbance of sample and reference at their respective excitation wavelengths was controlled to be lower than 0.05.

2.3 Cytoxicity assays

The CCK-8 cell proliferation assay was applied to determine the viabilities of the cells treated with **BTP** and **HBTP**. The Hela cells were seeded into a 96-well plate with a density of 5.0×10^3 cells/well in 100 μL cell culture medium. After cell attachment, the cells were treated by **BTP** or **HBTP** with different concentrations (0 – 10 μM). After cells were incubated 24 h and washed there times with PBS, CCK-8 (10 μL) in fresh medium was added. Then the plate was shaken for 30 min, and absorbance at 450 nm was measured by Synergy™ Mx Multi-Mode Microplate Reader. Cell viability (%) = $(A_{\text{with probe}}-A_{\text{blank}}/\ A_{\text{control}}-A_{\text{blank}}) \times 100\%$.

2.4 Cell culture and fluorescence imaging

HeLa and Raw 264.7 cell lines were kindly provided by Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education (China). HeLa cells were cultured in DMEM medium, and Raw 264.7 macrophage Cells were grown in RPMI 1640 medium. Cells were supplemented with 10 % FBS (Fetal Bovine Serum) and 1% antibiotics at 37 ºC in humidified environment of 5% CO_2. Cells were seeded in glass bottom cell culture dish (30 mm) and allowed to adhere for 12 hours. Before we started experiments, cells were washed with PBS 3 times.

To evaluate the imaging ability of **BTP** in living cells, HeLa cells were treated with 2 μM **BTP** in PBS for 30 min, and after washed with PBS 3 times, the fluorescence imaging assays were performed. To evaluate the subcellular localization, HeLa cells were co-stained with **BTP** (2 μM, 20 min) and MitoTracker Green FM (0.2 μM, 10 min), or LysoTracker Green DND-26 (0.1 μM, 10 min) in PBS at 37 ºC, and after washed with PBS 3 times, the fluorescence imaging assays were performed. To test pH effect on the lysosome-targeted ability of **BTP**, HeLa cells were pre-treated with **BTP** (2 μM, 30 min) or LysoTracker Green DND-26 (0.1 μM, 10 min) in PBS and then treated with 20 μM chloroquine, a cell permeable base that can increase lysosomal pH, for 12 min. Images were obtained at the indicated time point.

*2.5 Preparation of the test solution for evaluating the sensing performances of **HBTP** for $ONOO^-$*

Deionized water and spectroscopic grade MeCN were used for spectroscopic studies. The solutions of Cys, GSH, Hcy, NADH and various amino acids were prepared in deionized water. Superoxide solution (O_2^-) was prepared by adding KO_2 (1 mg) to dry dimethyl sulfoxide (1 mL) and stirring vigorously for 10 min. Hydroxyl radical ($OH^\bullet$) was generated in situ by the Fenton reaction (to generate $OH^\bullet$, Fe^{2+} was added in the presence of 10 eq of H_2O_2). Singlet oxygen ($^1O_2$) was generated in situ by addition of the HClO stock into a solution containing 10 eq of H_2O_2. Hypochlorite and hydrogen peroxide solution were prepared by the dilution of commercial NaClO solution and H_2O_2 solution in deionized water. NO was generated from a commercially available NO donor NOC-9 (dissolved in 0.1 M NaOH solution). Peroxynitrite solution was synthesized as reported method.[2] The peroxynitrite concentration was estimated by using an extinction coefficient of 1670 $M^{-1}cm^{-1}$ at 302 nm. C_{ONOO^-} = Abs $_{302\ nm}$/1.67 (mM).

*2.6 Imaging $ONOO^-$ in living cells and mouse models using **HBTP***

Before the experiments, Raw 264.7 macrophage cells were washed with PBS 3 times. Then, the cells were pretreated with 5 μM **HBTP** for 30 min, and then incubated with the $ONOO^-$ donor SIN-1 for 30 min in PBS at 37 °C. To check whether **HBTP** is affected by HClO and H_2O_2, Raw 264.7 macrophage cells were loaded with 5 μM **HBTP** for 30 min, washed, and treated with 50 μM NaClO and H_2O_2, respectively, in PBS at 37 °C. After washed with PBS three times, imaging experiments were performed. Emission was collected at 570–670 nm (λ_{ex} = 561 nm).

The cells were pretreated with 1 μg/mL LPS and 50 ng/mL INF-γ for 4 h and then incubated with **HBTP** (5 μM) for 30 min at 37 °C. After washed with PBS three times, imaging experiments were performed. For inhibition assays, the cells were activated with LPS (1 μg/mL) and INF-γ (50 ng/mL) in the presence of AG (5 mM) or TEMPO (300 μM) for 4 h and then loaded with 5 μM **HBTP** for 30 min. After washed with PBS three times, imaging experiments were performed. Emission was collected at 570–670 nm (λ_{ex} = 561 nm).

A Bruker In-Vivo FX Pro small animal optical imaging system with an excitation filter 550 nm and an emission filter 600 nm was used for the living animal imaging assays. For imaging endogenous $ONOO^-$, the mouse was first i.p. injected with LPS (1 mg/mL, 100 μL) for 12 h, and then i.p. injected with **HBTP** (10 μM, 100 μL) for 30 min. Then, the mouse was anesthetized and applied to the imaging system. The untreated mouse was used as a control. The experiments were performed in compliance with the relevant laws and institutional guidelines, and animal care and handing procedures were reviewed and approved by Animal Care and Use Committee of Shanxi University.

3. Supplementary Spectra and Imaging data

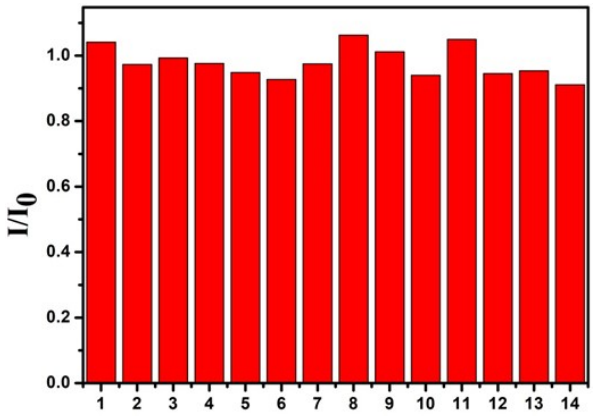

Fig. S1 Fluorescence responses of **BTP** (5 μM) in PBS (10 mM, pH = 7.4) toward various amino acids, ions, and H_2O_2. (1) Ca^{2+}, (2) Cu^{2+}, (3) Fe^{2+}, (4) Fe^{3+}, (5) Hg^{2+}, (6) K^+, (7) Mg^{2+}, (8) Na^+, (9) Zn^{2+}, (10) Cys, (11) Hcy, (12) GSH, (13) NADH, (14) H_2O_2. Concentrations for (1-11, 13), 500 μM; for (12), 10 mM; for (14), 100 μm. $\mathbf{I_0}$ refers to fluorescence intensity of **BTP** at 648 nm, and **I** refers to fluorescence intensity of **BTP** at 648 nm treated with amino acids, ions, and H_2O_2. Slits: 5/5 nm; $\lambda_{ex}/\lambda_{em}$ = 590 nm/648 nm; Voltage: 600 V.

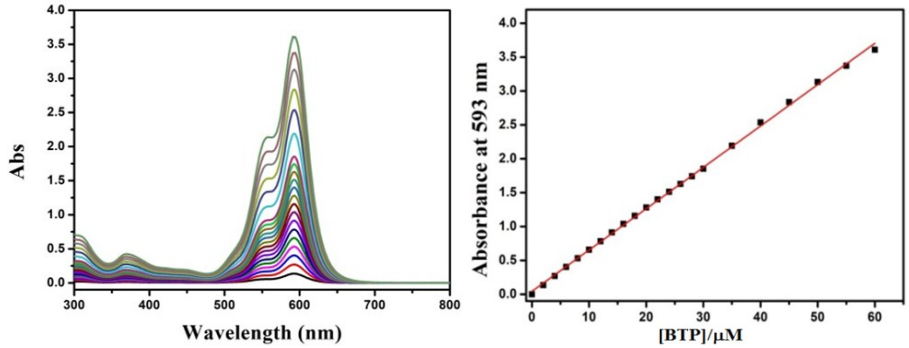

Fig. S2 (A) Solubility evaluation of **BTP** in PBS (10 mM, pH = 7.4) by UV-vis spectra. (B) Plots of absorption intensity *vs* concentrations of **BTP**.

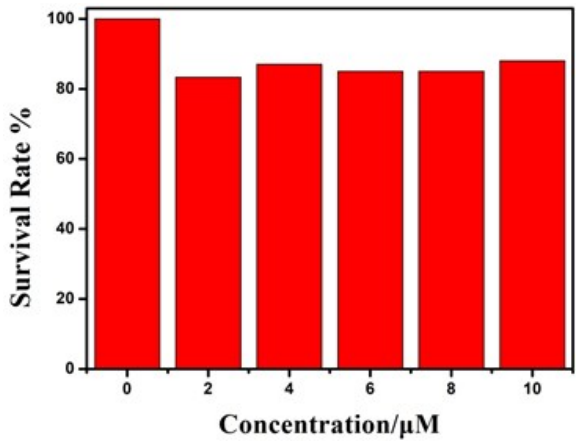

Fig. S3 Survival rate of HeLa cells treated with **BTP** (2–10 μM) for 24 hr, determined by CCK8 assays.

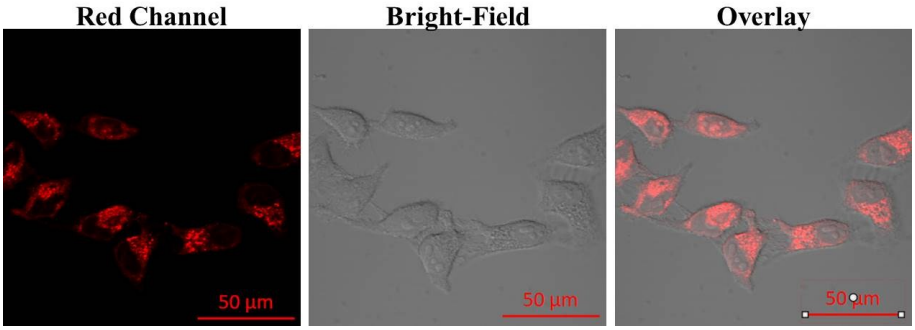

Fig. S4 Fluorescence image of HeLa cells treated with **BTP** (2 μM) for 30 min in PBS. Emission was collected at 570–670 nm (λ_{ex} = 561 nm).

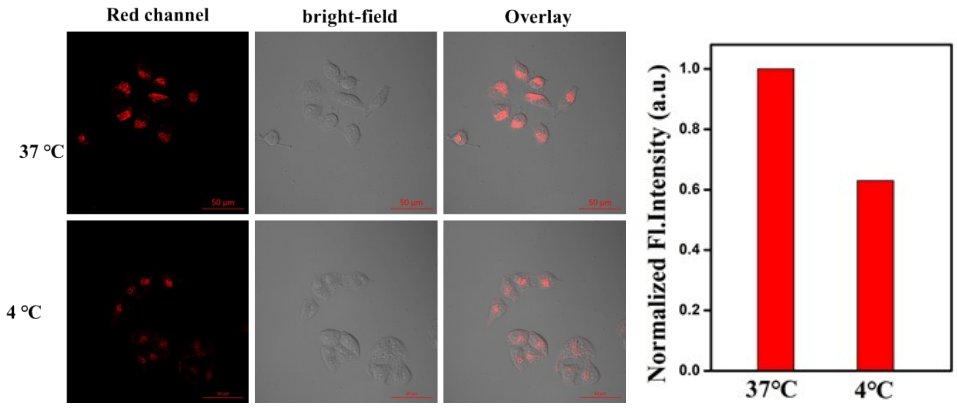

Fig. S5 Fluorescence images and corresponding normalized fluorescence intensities of HeLa cells incubated with **BTP** (2 μM) at 37 °C and at 4 °C, respectively, for 30 min. Emission was collected at 570–670 nm (λ_{ex} = 561 nm). Scale bar: 50 μm

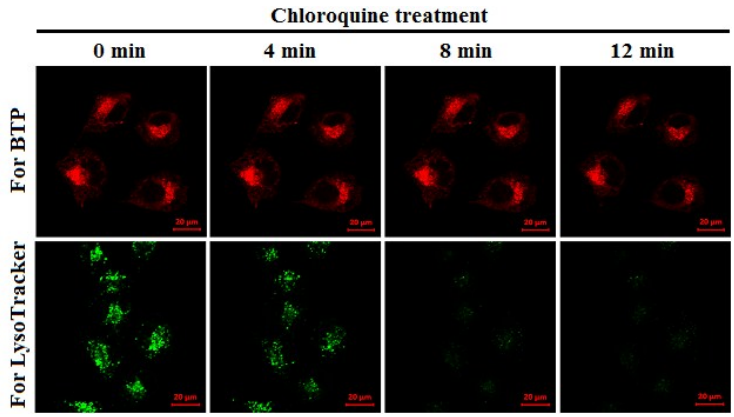

Fig. S6 Fluorescence images of **BTP** (2 μM)- or LysoTracker (0.1 μM)-loaded HeLa cells after treated with chloroquine (20 μM). HeLa cells were irradiated in indicated time point by a semiconductor laser. For **BTP**, emission was collected at 570–670 nm (λ_{ex} = 561 nm), and for LysoTtracker, emission was collected at 500–550 nm (λ_{ex} = 488 nm). Scale bar = 20 μm.

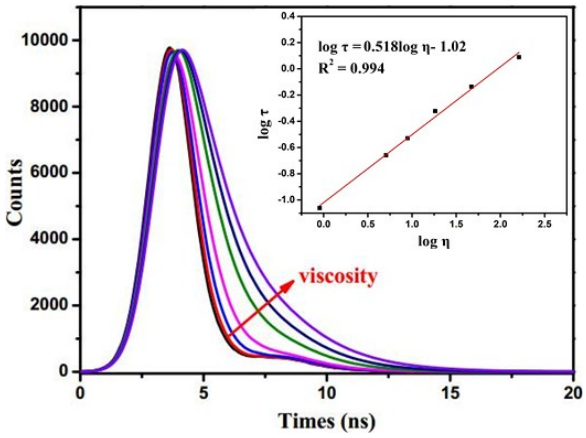

Fig. S7 Fluorescence lifetime spectra of **BTP** (5 μM) under different solution viscosities in the water/glycerol system. Inset: the linear relationship between log τ and log η (R2 = 0.994).

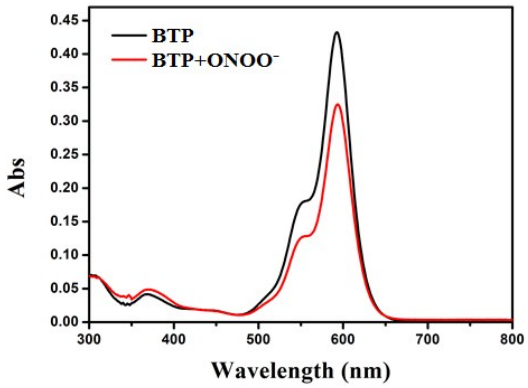

Fig. S8 Comparison of the absorption spectra of **BTP** (5 μM) and **HBTP** (5 μM) treated with 5 equiv of $ONOO^-$ in PBS (10 mM, pH 7.4, 20% CH_3CN).

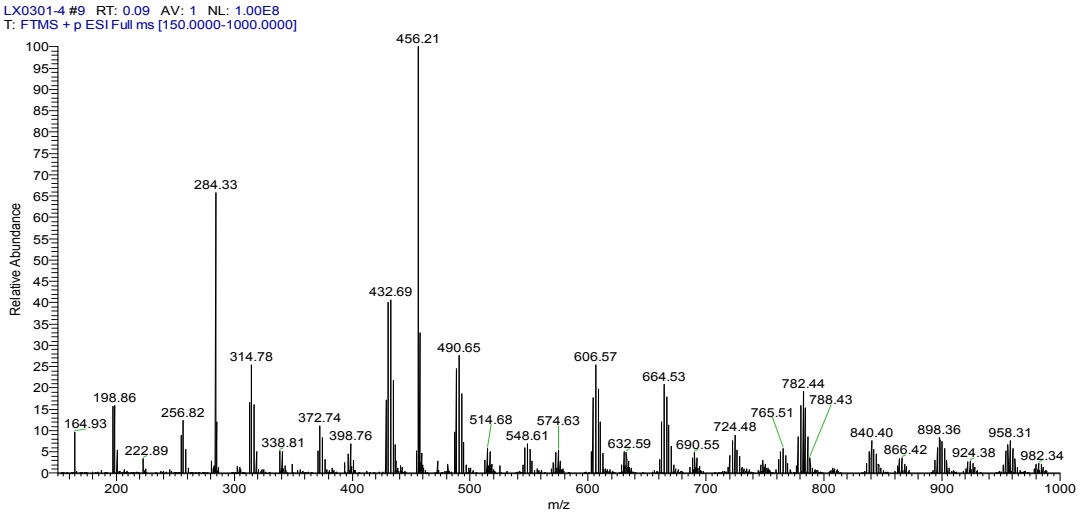

Fig. S9 HRMS chart of **HBTP** in the presence of $ONOO^-$.

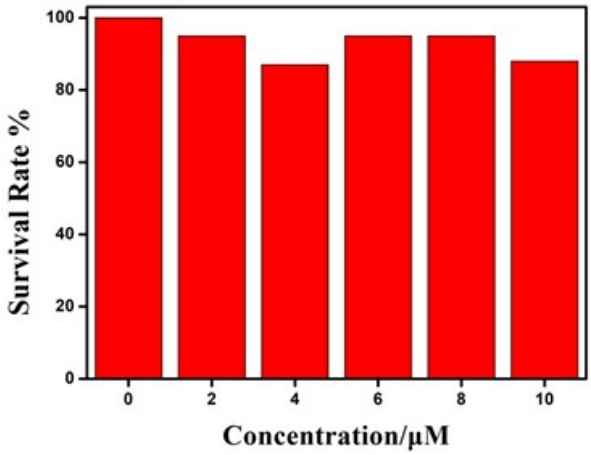

Fig. S10 Survival rate of HeLa cells treated with **HBTP** (0–10 μM) for 24 hr, determined by CCK8 assays.

4. ^{1}H NMR, ^{13}C NMR, and HRMS charts of BTP and HBTP

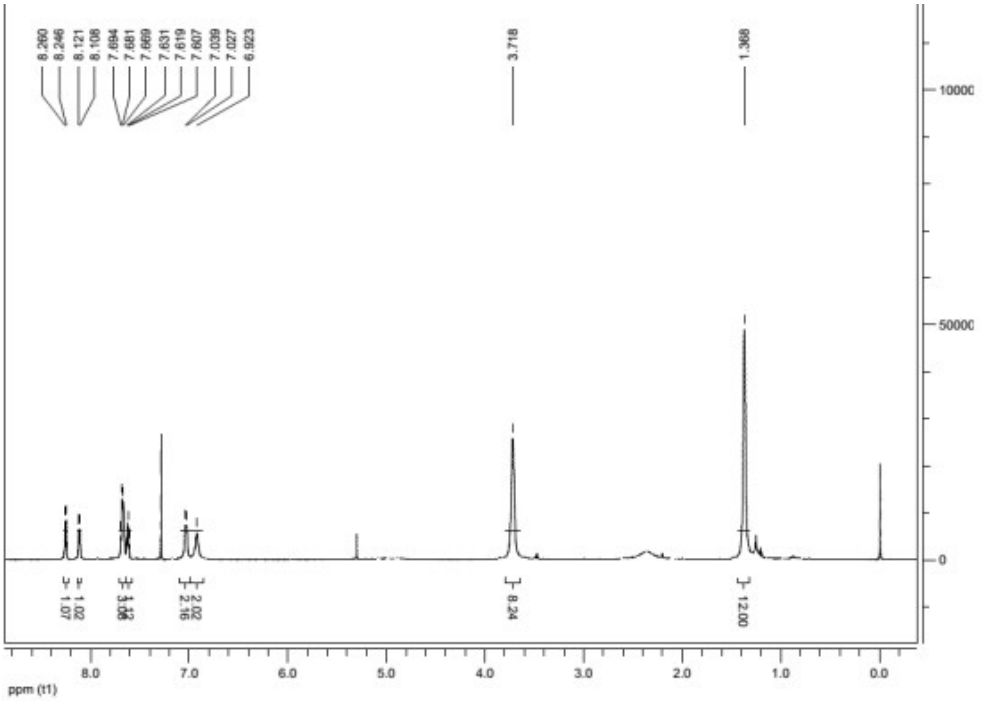

Fig. S11 ^{1}H NMR chart of compound **BTP** (600 MHz, $CDCl_3$).

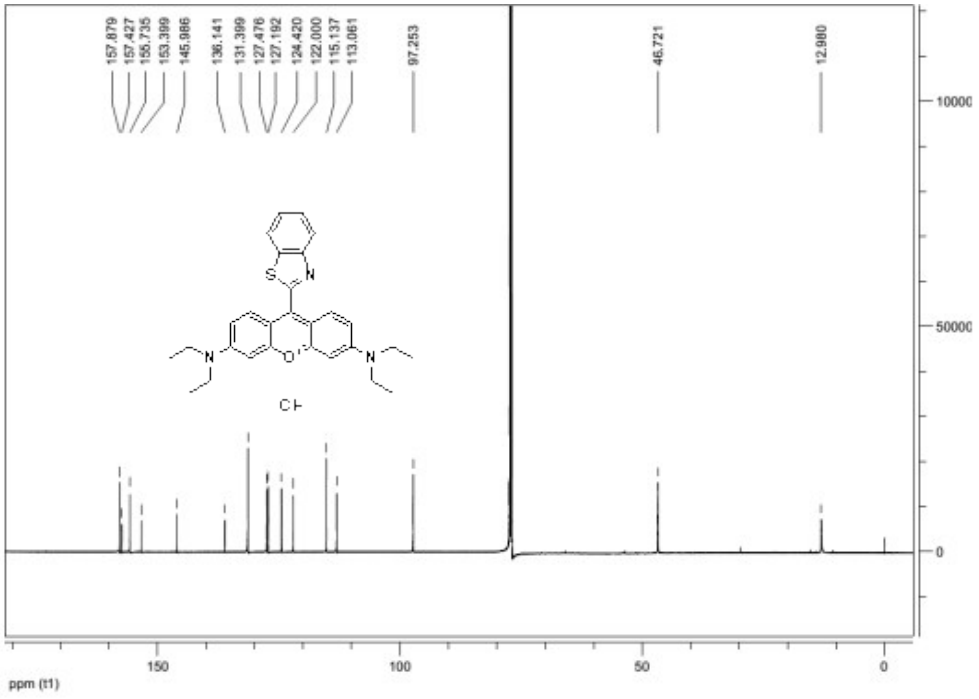

Fig. S12 ^{13}C NMR chart of compound **BTP** (150MHz, $CDCl_3$).

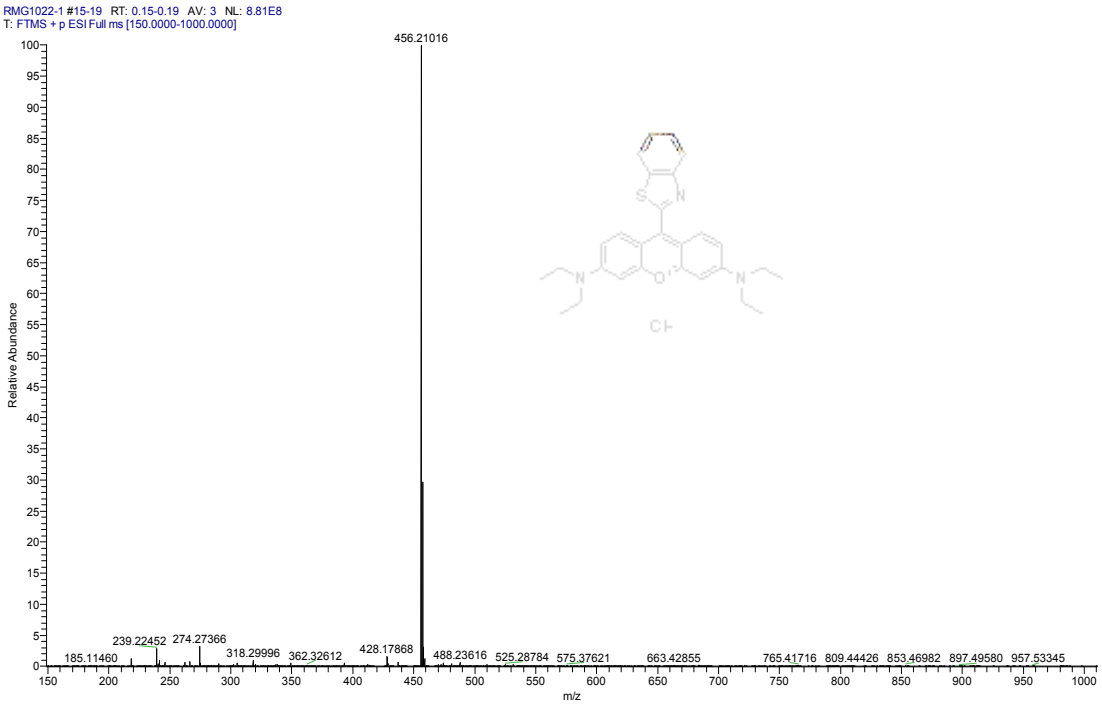

Fig. S13 HRMS chart of compound **BTP**.

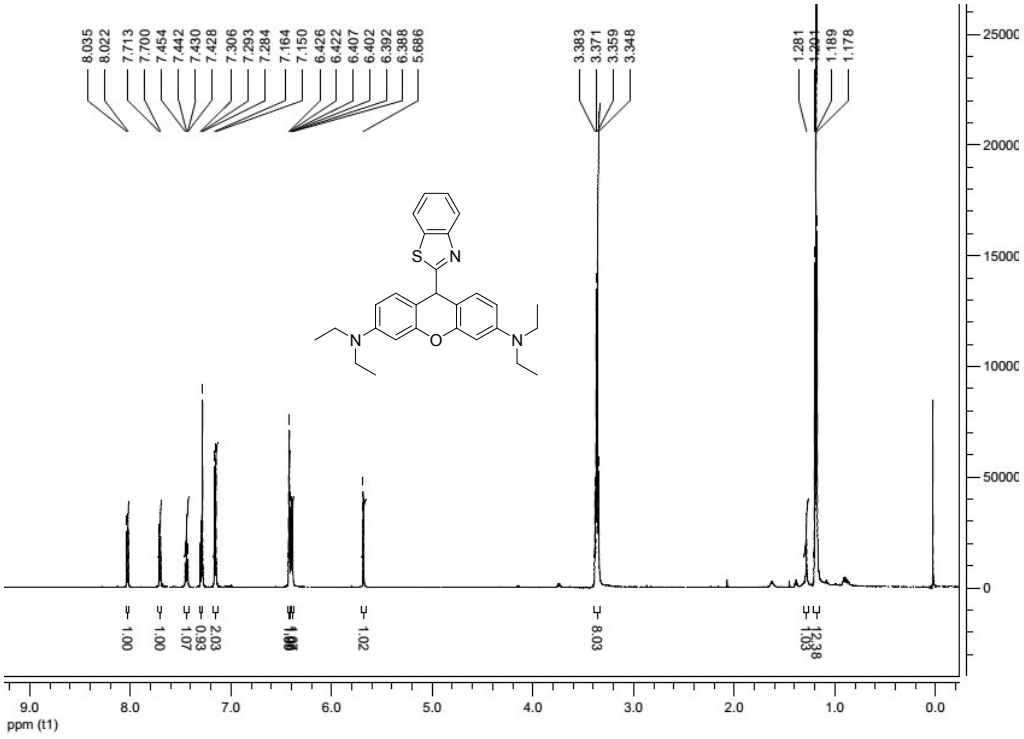

Fig. S14 ^{1}H NMR chart of compound **HBTP** (600 MHz, $CDCl_3$).

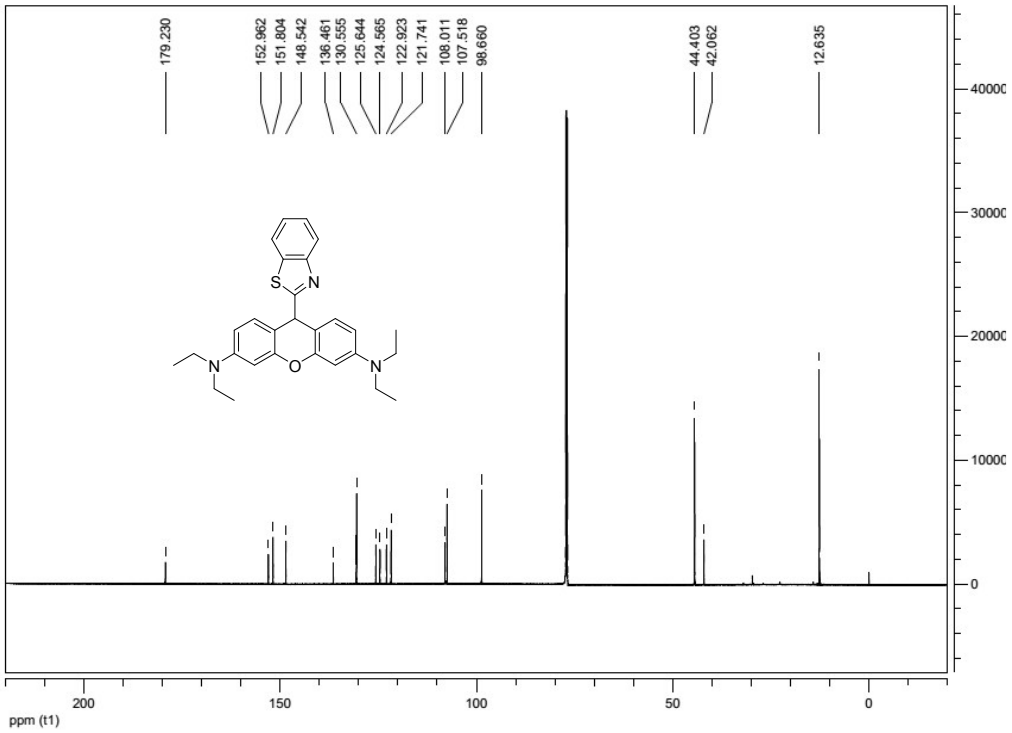

Fig. S15 ^{13}C NMR chart of compound **HBTP** (150MHz, $CDCl_3$).

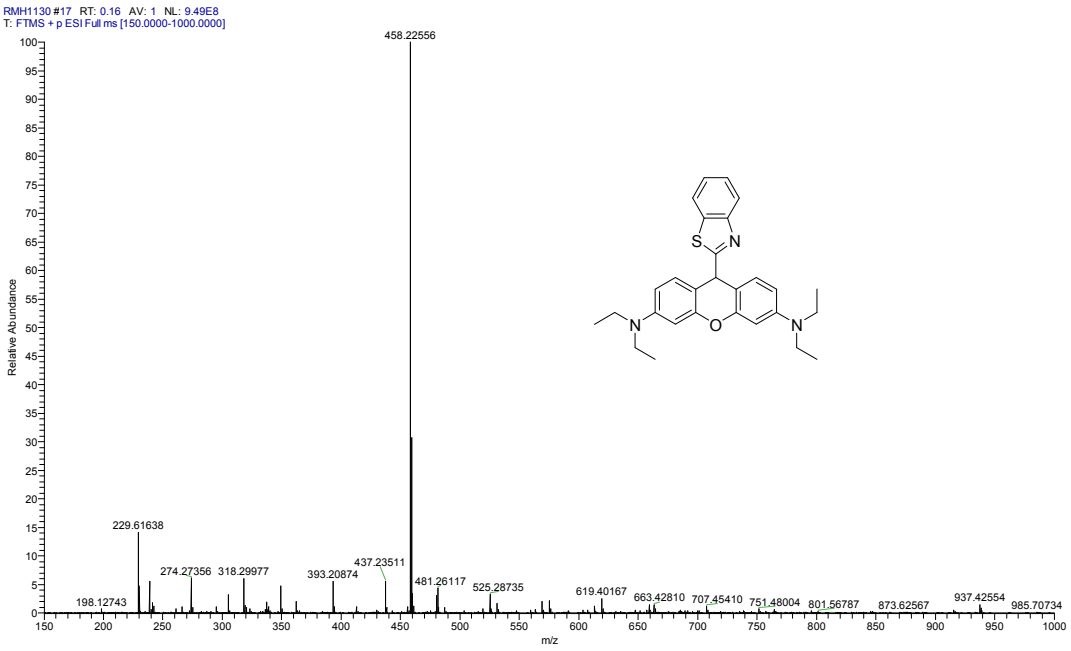

Fig. S16 HRMS chart of compound **HBTP**.

5. References

[1] H. Zhang, J. Liu, B. Hu, L. Wang, Z. Yang, X. Han, J. Wang, W. Bai, W. Guo, *Chem. Sci.*, 2018, **9**, 3209–3214.

[2] R.M. Uppu, W.A. Pryor, *Anal. Biochem.*, 1996, **236**, 242-249.