Electronic Supplementary Information

A Hydrogen Sulfide and Tyrosinase Responsive Dual-locked Fluorophore for Selective Imaging of Melanoma Cells

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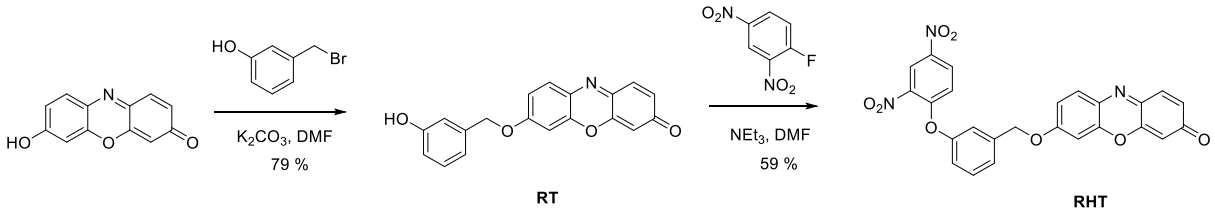
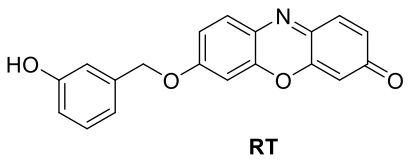
1. Materials and Instruments

All chemicals and reagents used in the experiment were commercially available and used as received, unless otherwise stated. $Na_2S.9H_2O$, Kojic acid, $ZnCl_2$, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and Dimethyl sulfoxide (DMSO) Hybri-MaxTM were purchased from Sigma-Aldrich (MO, USA). Roswell Park Memorial Institute 1640 (RPMI 1640), Dulbecco's Modified Eagle Medium (DMEM), McCoy's 5A medium were used for cell culture. Phosphate-buffered saline (PBS) tablets were obtained from Biomatik Corp. (ON, Canada). The Mbraun MBSPS5 solvent drying machine was used to produce dry solvents for reactions. Reactions performed under inert atmosphere were carried out using a schlenk line and argon gas. Column chromatography was performed by using thick-walled glass columns and silica Gel 60 (Merck 230-400 mesh). Thin-layer chromatography (TLC) was performed using commercially prepared silica gel plates (0.25 mm thickness, Merck Silica Gel 60 F254), and visualization was done using a UV lamp. Electronic absorption spectra and emission spectra in solutions were acquired using Biotek Synergy H1 Hybrid Microplate Reader and Agilent Cary Eclipse fluorescence spectrophotometer. Mass spectrum was recorded on Waters Synapt G1 High-Definition mass spectrometer. HPLC analyses were conducted on an Agilent 1260 series.

2. Synthetic Details

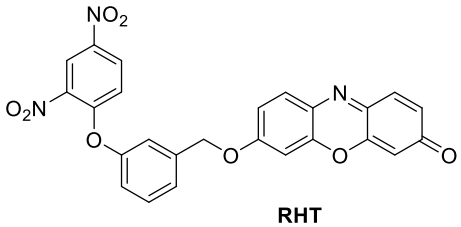


Scheme S1. Synthesis of **RHT** and **RT**.



Synthesis of **RT**: Commercially available resorufin (100 mg, 0.469 mmol) and K_2CO_3 (130 mg, 0.928 mmol) were dissolved in 5 mL dry DMF and stirred at room temperature for half an hour. 3-hydroxybenzyl bromide (175 mg, 0.928 mmol) was added portion-wise to the reaction flask and it was stirred overnight at 65 °C. The reaction mixture was then diluted with 70 mL ethyl acetate, washed with 2x20 mL brine, and then 2x30 mL saturated $NaHCO_3$. Organic layers were collected, dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (Hex:EtOAc 1:1). The product was obtained as red crystal (118 mg, 79 %). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 9.60 (s, 1H), 7.84 (d, *J* = 8.9 Hz, 1H), 7.59 (d, *J* = 9.8 Hz, 1H), 7.28 – 7.22 (m, 2H), 7.18 (dd, *J* = 8.9, 2.7 Hz, 1H), 6.97 – 6.90 (m, 2H), 6.85 (dd, *J* = 9.8, 2.1 Hz, 1H), 6.80 (dd, *J* = 8.0, 2.4 Hz, 1H), 6.33 (d, *J* = 2.0 Hz, 1H), 5.27 (s, 2H). ^{13}C NMR (126 MHz, DMSO-*d*6) δ 185.81, 162.80, 158.01, 150.20, 145.72, 145.68, 137.88, 135.39, 134.21, 131.81, 130.10, 128.45, 118.71, 115.57,

114.94, 114.84, 106.13, 101.66, 70.71.HR-MS spectrum of **RT** calculated: 320.0923 and found: 320.0921 for $[M+H]^+$.



RHT

Synthesis of **RHT**: 40 mg (0.125 mmol) of **RT** was dissolved in 3 mL dry DMF and stirred at room temperature. Next, triethyl ammine (35 µL, 0.25 mmol) was added to the flask and stirred for 20 minutes. Finally, 2,4-dinitrofluorobenzene (46 mg, 0.25 mmol) was added portion-wise, and the reaction was stirred overnight at 50 °C. Reaction was cooled down to room temperature, and solvent was evaporated under reduced pressure. Then, reaction mixture was diluted with 50 mL of ethyl acetate and washed with brine. Organic layer was separated, dried under Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (Hex:EtOAc 1:1), and obtained as bright yellow crystal (36 mg, 59 %).^{1}H NMR (500 MHz, $CDCl_3$) δ 8.86 (d, *J* = 2.7 Hz, 1H), 8.33 (dd, *J* = 9.3, 2.8 Hz, 1H), 7.73 (d, *J* = 8.9 Hz, 1H), 7.54 (t, *J* = 7.9 Hz, 1H), 7.44 – 7.37 (m, 2H), 7.27 (s, 1H), 7.16 – 7.12 (m, 1H), 7.07 (d, *J* = 9.2 Hz, 1H), 7.00 (dd, *J* = 8.9, 2.7 Hz, 1H), 6.87 (d, *J* = 2.7 Hz, 1H), 6.84 (dd, *J* = 9.8, 2.0 Hz, 1H), 6.32 (d, *J* = 2.1 Hz, 1H), 5.21 (s, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 186.43, 162.12, 155.86, 154.26, 149.83, 146.25, 145.72, 141.86, 139.91, 139.02, 134.88, 134.57, 131.89, 131.29, 128.95, 128.84, 125.31, 122.32, 120.43, 119.37, 118.86, 114.08, 107.05, 101.27, 69.89. HR-MS spectrum of **RHT** calculated: 486.0937 and found: 486.0935 for $[M+H]^+$.

3. Photophysical Characterization

General Assay for Photophysical Characterization: **RHT** was firstly treated with fixed or varying concentrations of $Na_2S.9H_2O$ (0-50 µM) in DMSO and then incubated with fixed or varying concentrations of Tyr (0-90 U/mL) in PBS (pH, 7.4, 10% DMSO).

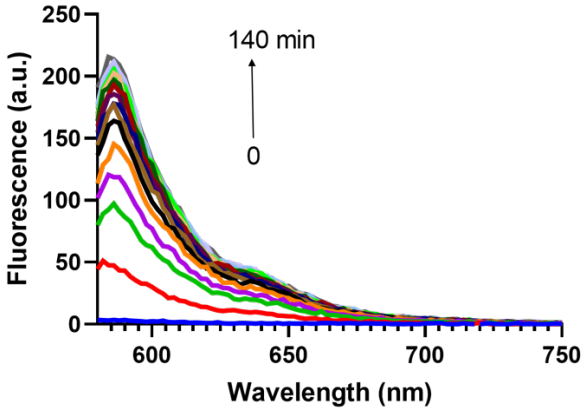


Figure S1. Fluorescence spectra of **RHT** (10 µM) co- treated with Na_2S (50 µM) and Tyr (90 U/mL) in PBS (pH 7.4, 10% DMSO). Spectra were recorded for 140 minutes at 10-minute intervals. λ_{ex} = 570 nm, Slit widths: 5-5 nm.

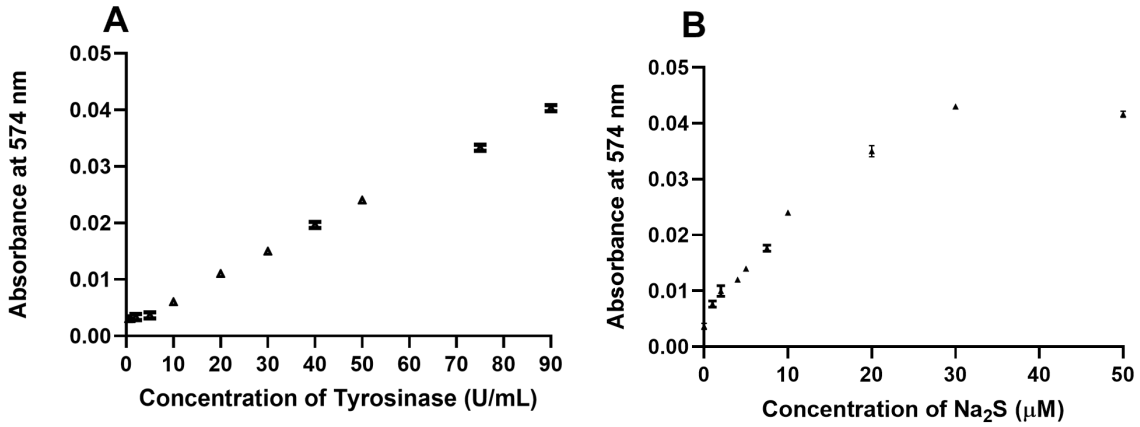


Figure S2. The absorbance intensity of **RHT** (10 μM) **(A)** treated with Na_2S (50 μM) and then various Tyr concentrations (0-90 U/mL) or **RHT** (10 μM) **(B)** treated with varying Na_2S concentration (0-50 μM) and then Tyr (90 U/mL) in PBS (pH 7.4, 10% DMSO). Signals were recorded at 574 nm, 3 hours after the treatment. R^2 was calculated as 0.998 for **(A)** and 0.989 for **(B)** within the concentration regions of **(A)** (0.5-90 U/mL Tyr) and **(B)** (1-20 μm Na_2S). λ_{ex} = 570 nm.

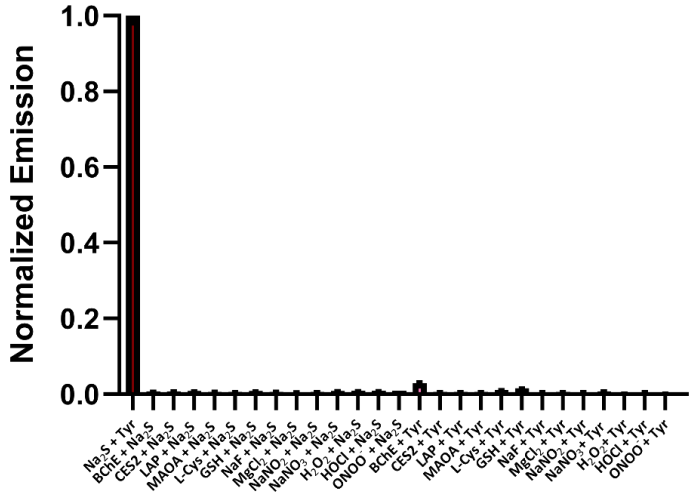


Figure S3. Normalized emission of **RHT** (10 μM) recorded at 600 nm in the co-presence of biologically relevant analytes (Na_2S: 50 μM, GSH: 5 mM, L-Cys: 5 mM, AChE and BChE: 20 U/mL, LAP: 10 ng/mL, MAOA: 10 μg/mL, CES2: 50 nM and other: 1mM). (λ_{ex} = 570 nm) n=3.

Fluorescence Quantum Yield

The fluorescence quantum yield (Φ_f) of **RHT** was calculated by following similar method. Formula as given below were used:

$$\Phi_f\,(PS) = \Phi f\,(\mathrm{ref}) x\, \frac{F(PS)}{F\,(Ref)}\; x\; \frac{A(Ref)}{F\,(PS)}$$

F is the area under the fluorescence spectra, A is the absorption signal recorded at 570 nm. As reference compound resorufin salt (Φ_f = 0.74) was used and PS stands for **RHT** (10 μM) treated with Na_2S (50 μM) and tyrosinase (90 U/mL).

4. HPLC Analysis

HLPC analyses were conducted using an Agilent 1260 series equipped with an analytical column (ACE 5C18-300, 250 × 4.6 mm). As mobile phase gradients of acetonitrile in water (0.01% TFA, v/v) were used. Similar to photophysical characterization, **RHT**, **RT** or resorufin injected to HPLC instrument and their retention times were observed. Then, **RHT** treated with Na_2S, tyrosinase or both were injected into the instrument. All the corresponding peaks were evaluated and depicted accordingly. The gradient program of acetonitrile (Phase A) and water with 0.1 % TFA (Phase B) is given below:

Time/min	Phase A/%	Phase B/%
0	50.0	50.0
0.2	95.0	5.0
3.00	85.0	15.0
13.0	25.0	75.0
16.0	100.0	0.0

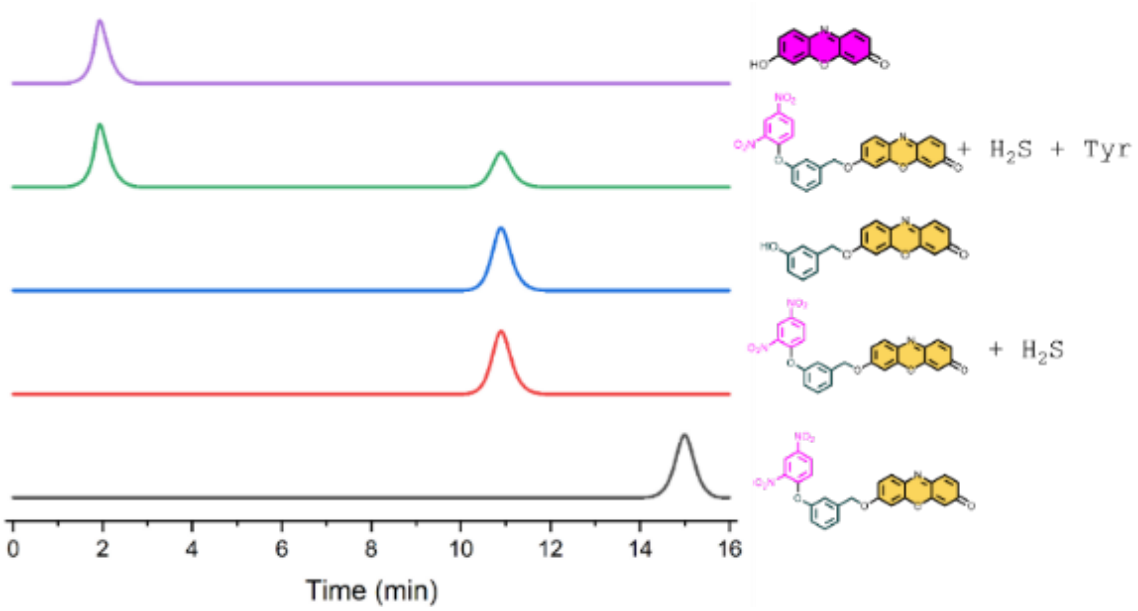


Figure S4. HPLC chromatograms for **RHT**, **RHT** + Na_2S, **RT**, **RHT** + Na_2S + Tyr, and resorufin.

5. Mass Analysis

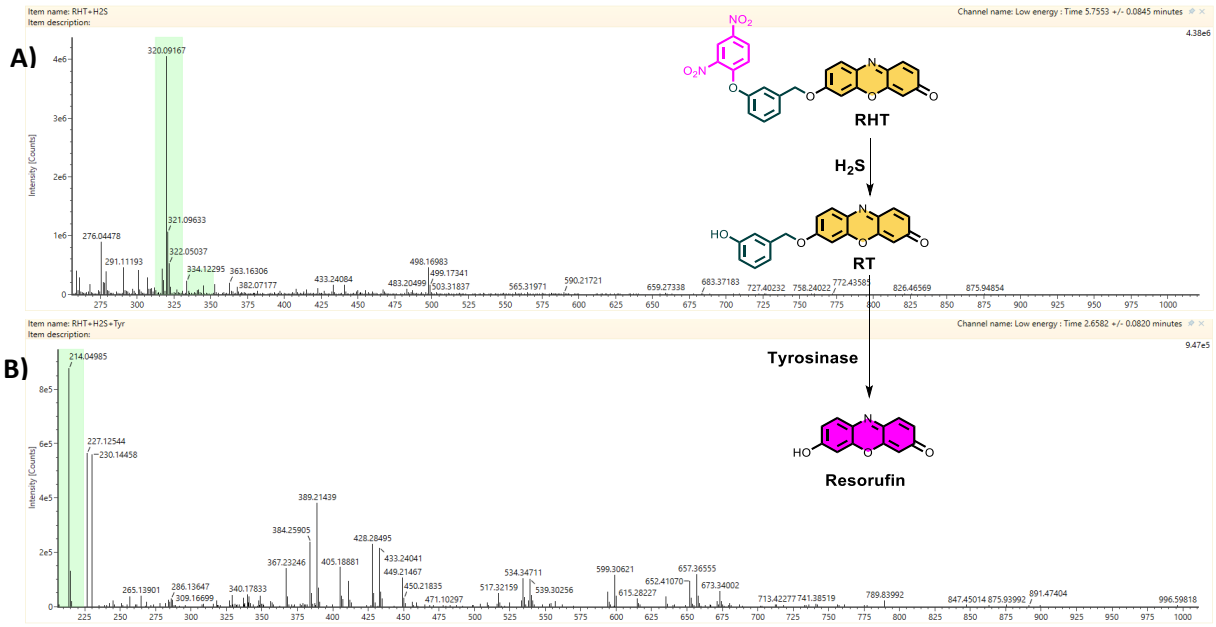


Figure S5. (A) HR-MS spectrum of **RHT** treated with Na_2S, Calculated: 320.09228, Found: 320.09167 for $[M+H]^+$. **(B)** HR-MS spectrum of **RHT** treated with Na_2S and Tyr, Calculated: 214.05042, Found: 214.04985 for $[M+H]^+$.

6. *In Vitro* Experiments

The cells were grown in a culture medium consisting of 1% penicillin-streptomycin, 10% Fetal Bovine Serum (FBS), and High Glucose Dulbecco's Modified Eagle Medium (DMEM). When the cells reached 70% confluency, they were passaged using Dulbecco's phosphate-buffered saline (DPBS) and Trypsin-EDTA (0.05% with phenol red). For kinetic measurements and viability assays, the cells were seeded at a density of 10,000 cells per well in 96-well plates with clear bottoms and black sides. For live cell confocal studies, the cells were seeded at a density of 20,000 cells per dish in 35 mm glass bottom cell culture dishes. The cells were then incubated in an Eppendorf Galaxy 170S incubator at a temperature of 37°C with 5% CO_2. Kinetic luminescence measurements were performed using a Biotek Synergy H1 MF microplate reader. Confocal studies were carried out using a Leica DMI8 SP8 Inverted Confocal Microscope. **RHT** was applied in 1% DMSO containing DMEM solutions. Inhibitions were performed by 10 minutes incubation with 300 µM $ZnCl_2$ and an hour incubation with 1 mM Kojic Acid. The inhibitors were removed and RHT incubation was started later.

Detection of Fluorescence Signal in Cells

A poly-L-lysine coated 96-well plate was seeded with 1×10^4 per well and incubated overnight at 37 °C, with 5% CO_2 in an Eppendorf Galaxy 170S incubator. The media was removed, washed with DPBS and the live cells were treated with varying concentrations of RHT (0-20 µM). The fluorescence restored at 600 nm after the treatment was recorded for 9 hours. (λ_{ex} = 560 nm) Corresponding fluorescence signal at the end of the incubation period was noted and depicted.

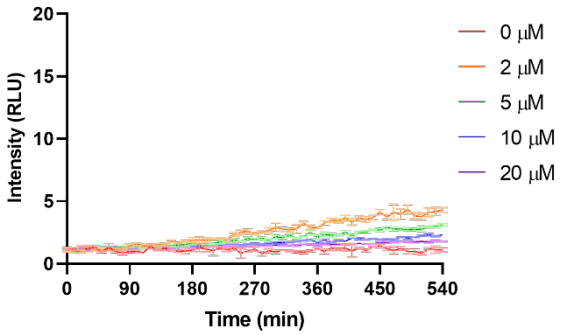


Figure S6. Time and concentration dependent fluorescence of **RHT** (0-20 μM) treated BJ cells, (n=3, 1% DMSO).

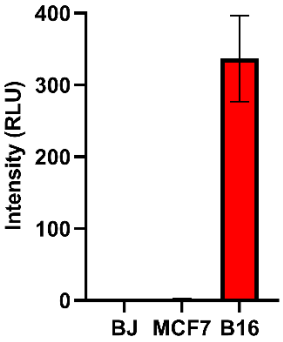


Figure S7. Fluorescence intensity of 20 μM **RHT** in B16-F10, MCF-7 and BJ at 9 hours. (n=3, 1% DMSO).

MTT Assay

Cells were treated with 0-20 μM RHT for an hour. Then, RHT bearing media was replaced with fresh DMEM. After 23 h of incubation, the medium was removed and the 5 mg/ml MTT working solution was added to each well. The plate was incubated for 4 hours. The solution was removed, and DMSO was added to dissolve the formazan crystals. The absorbance was measured at 570 nm to quantify cell viability.

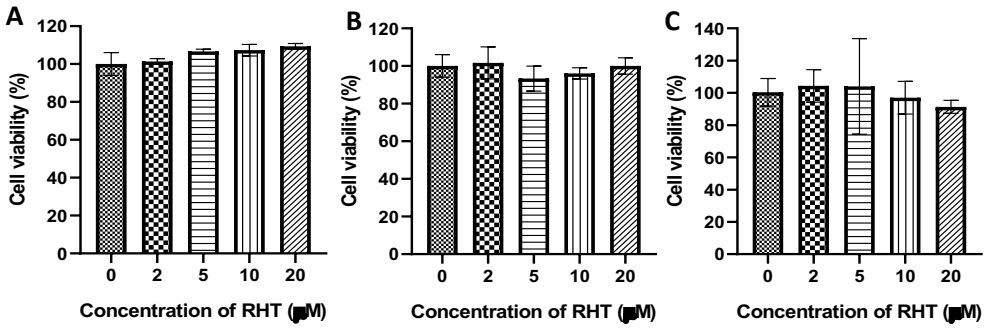


Figure S8. Cell viability results based on MTT viability assay. **(A)** BJ, **(B)** MCF-7, **(C)** B16-F10. (n=3, 1% DMSO).

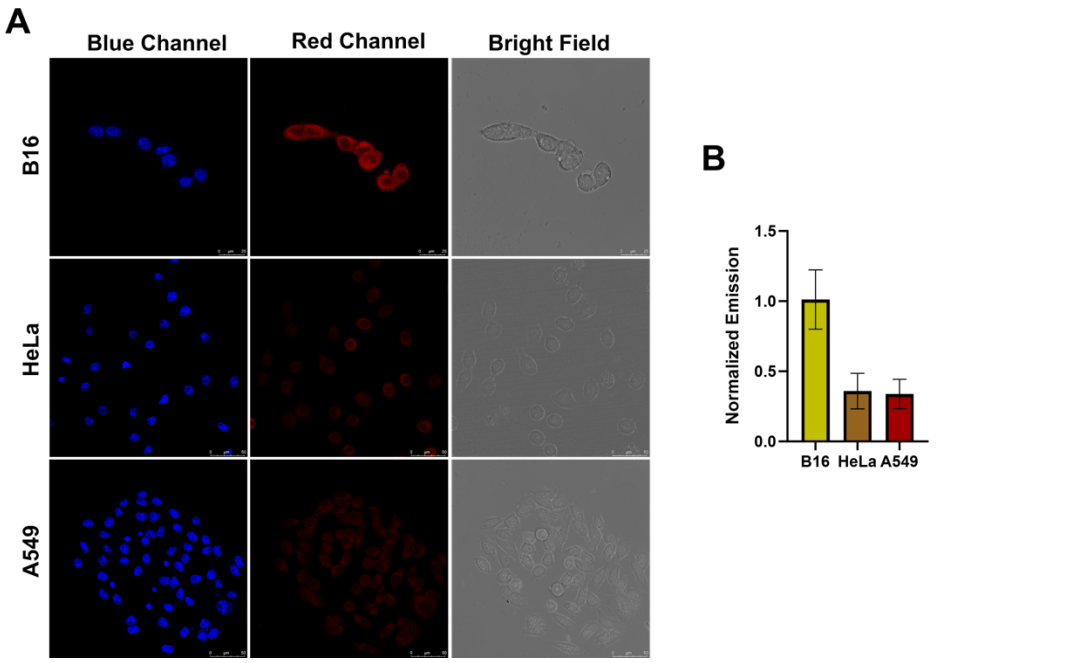


Figure S9. (A) Fluorescence imaging of B16, HeLa and A549 cells upon treating with **RHT** (2 μM). **(B)** Quantification of the fluorescence signal. Blue channel: Hoechst; Red channel: **RHT**.

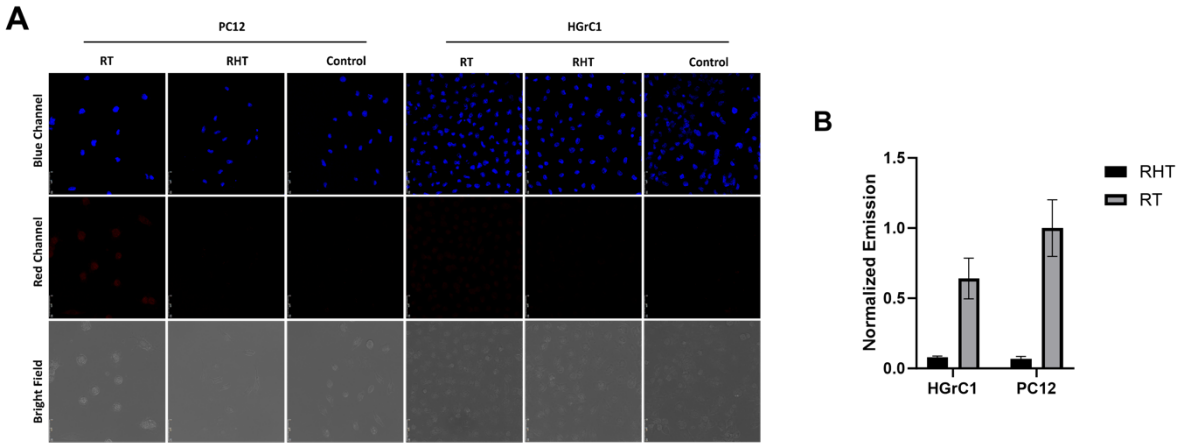


Figure S10. (A) Fluorescence imaging of PC12 and HGrC1 cells upon treating with either **RHT** (2 μM) or **RT** (2 μM). **(B)** Quantification of the fluorescence signal. Blue channel: Hoechst; Red channel: **RHT** or **RT**.

7. NMR Spectra

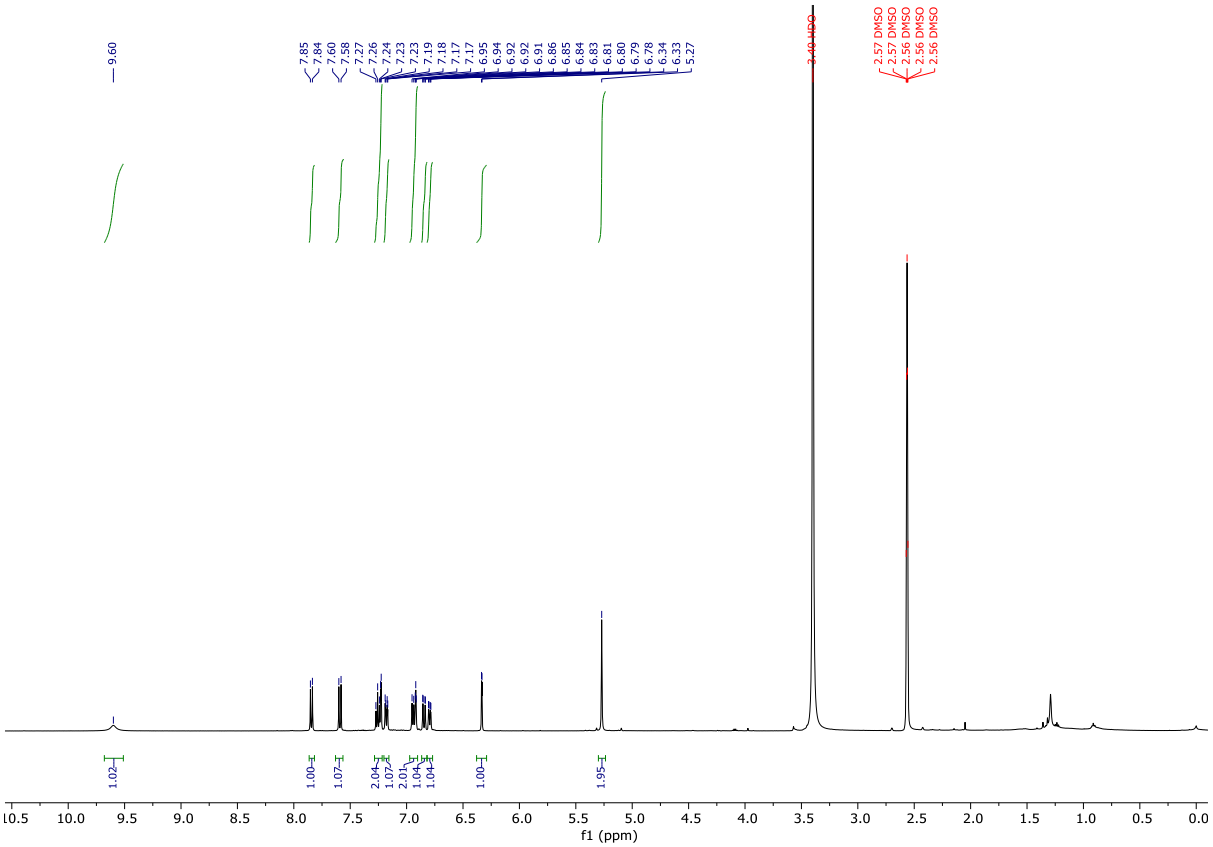


Figure S11. ^{1}H NMR of **RT** in DMSO-*d*6.

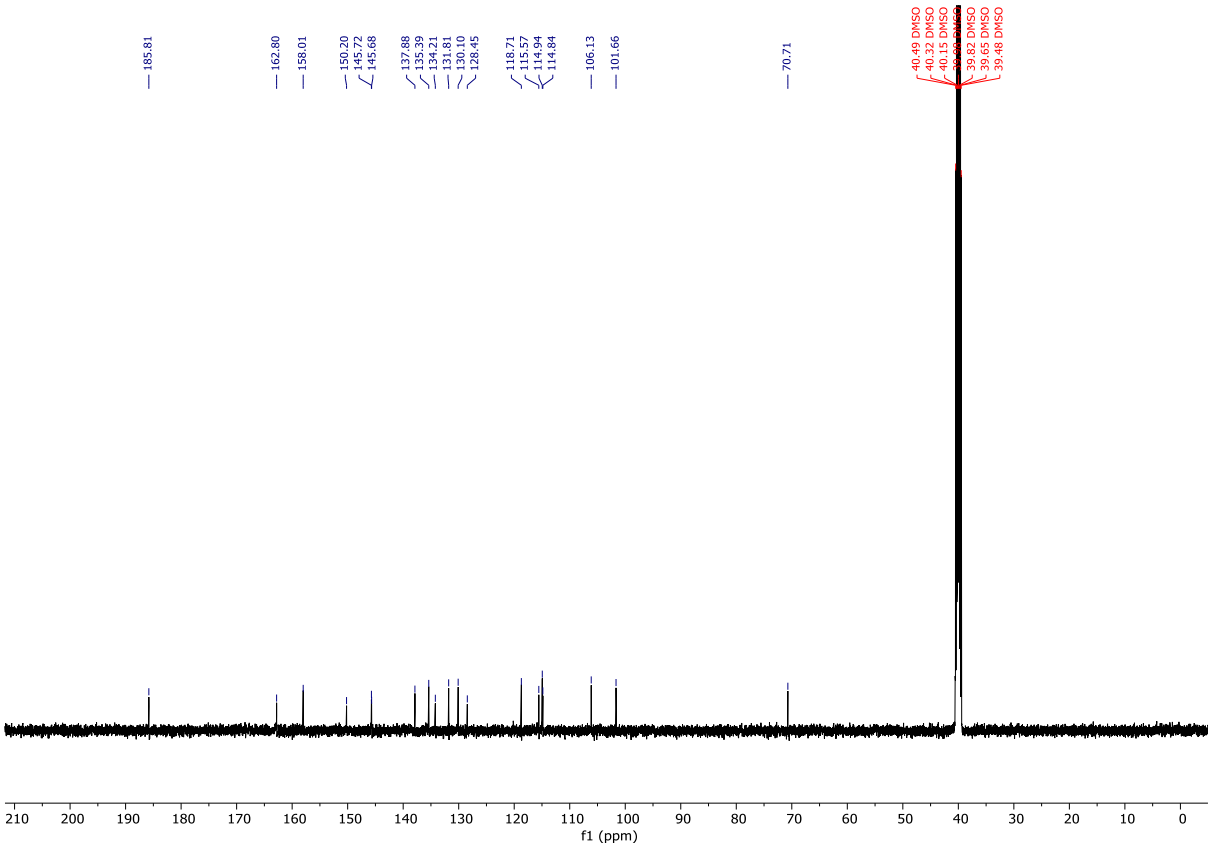


Figure S12. ^{13}C NMR of of **RT** in DMSO-*d*6.

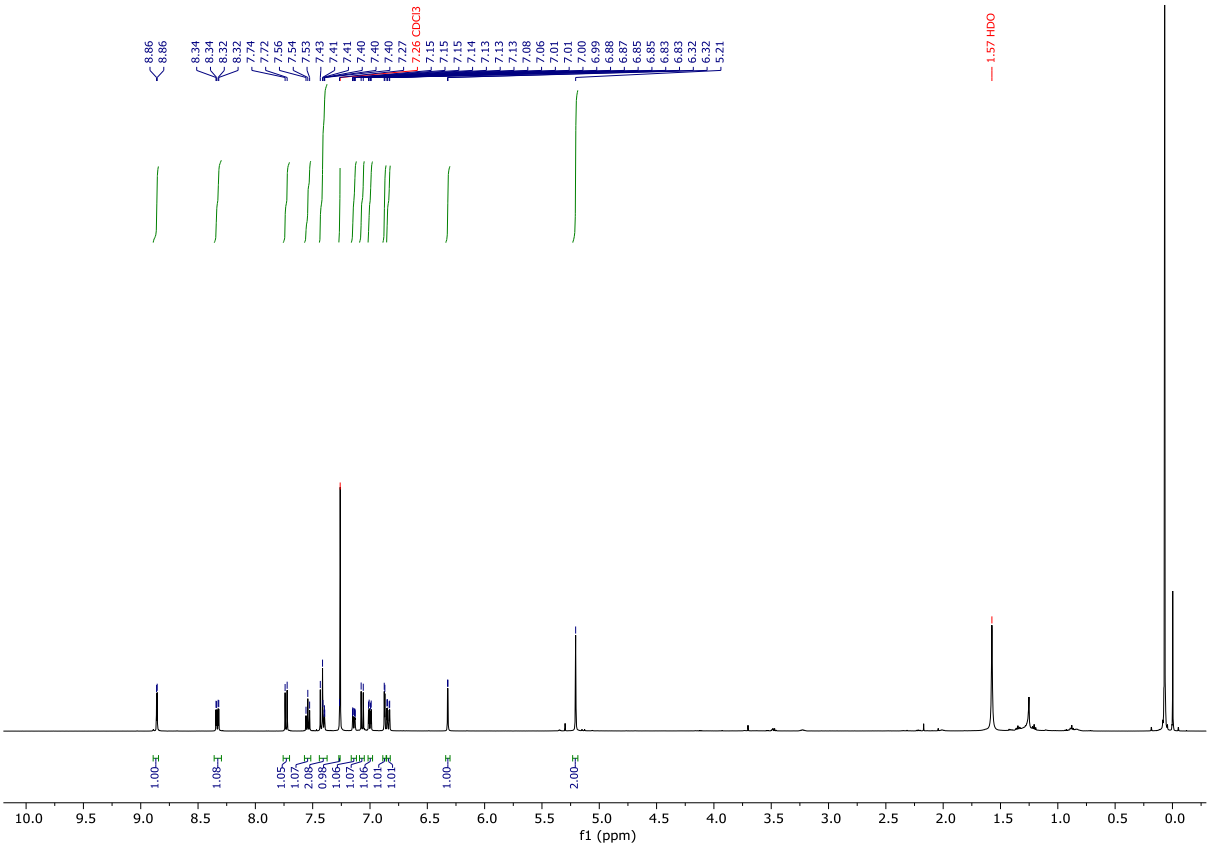


Figure S13. ^{1}H NMR of **RHT** in $CDCl_3$.

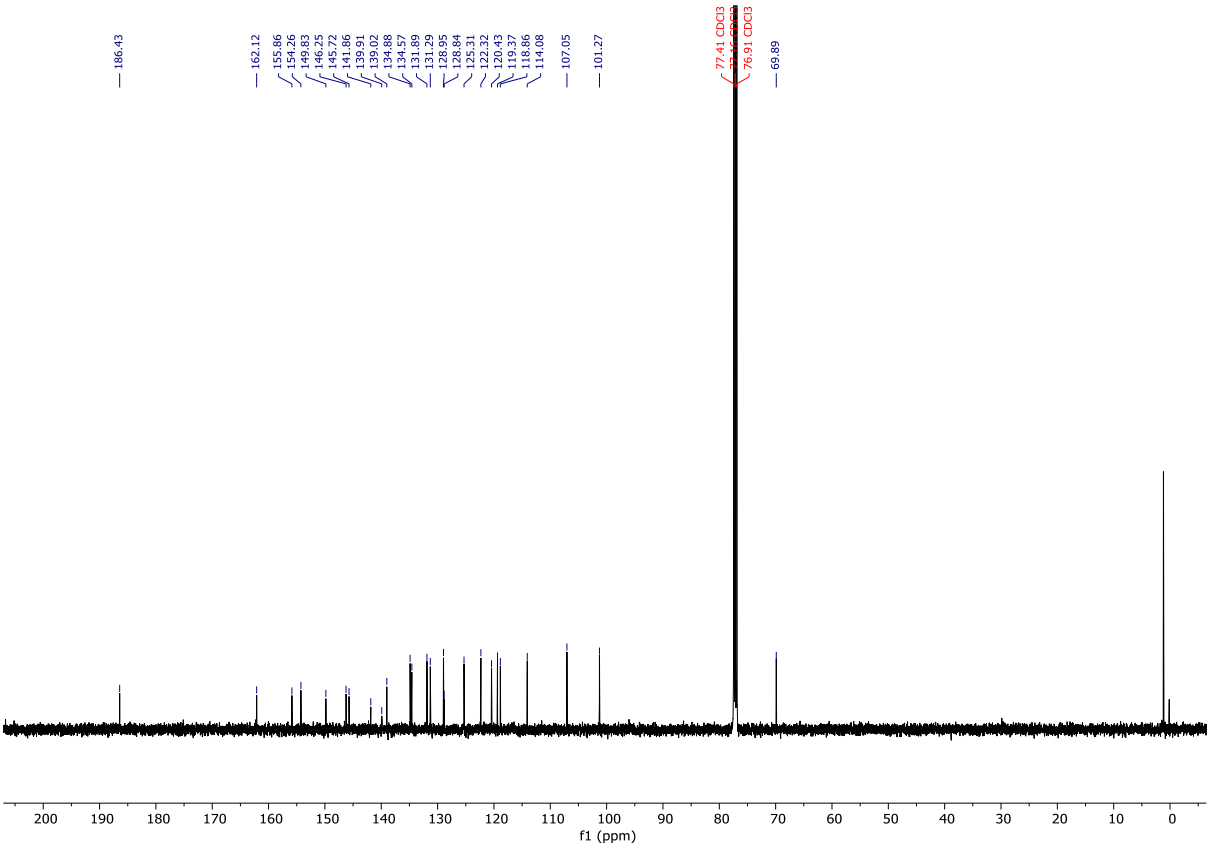


Figure S14. ^{13}C NMR of of **RHT** in $CDCl_3$.

HRMS Spectra

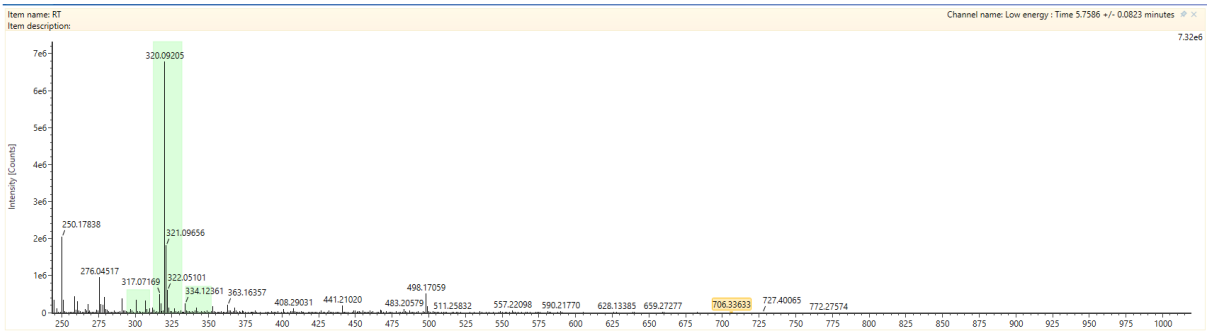


Figure S15. HRMS spectrum of **RT**.

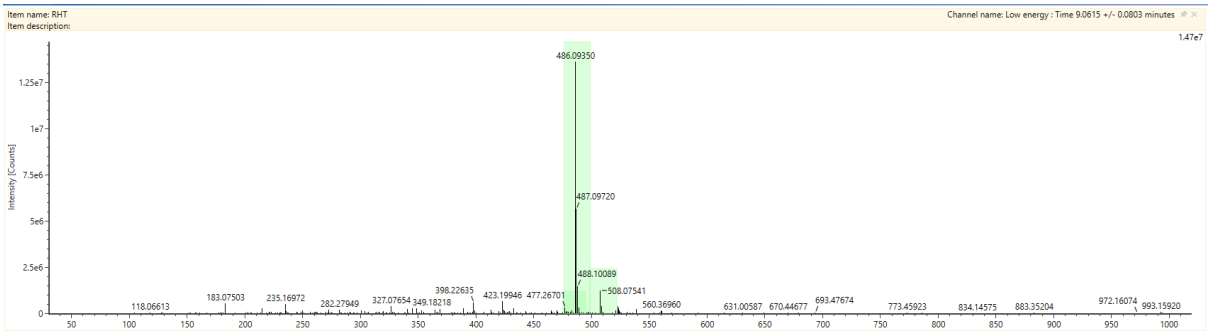


Figure S16. HRMS spectrum of **RHT**.