Electronic Supplementary Material (ESI) for ChemComm.

Thiophene bridged hydrocyanine − a new fluorogenic ROS probe

Santanu Maity [a], Subhamoy Das [a], Corinne M. Sadlowski [a], Jingtuo Zhang,[a] Giri K. Vegesna [b], Niren Murthy[a]

[a]Department of Bioengineering, UC Berkeley, CA 94720
[b]Profusa Inc., 345 Allerton Ave, South San Francisco, CA 94080

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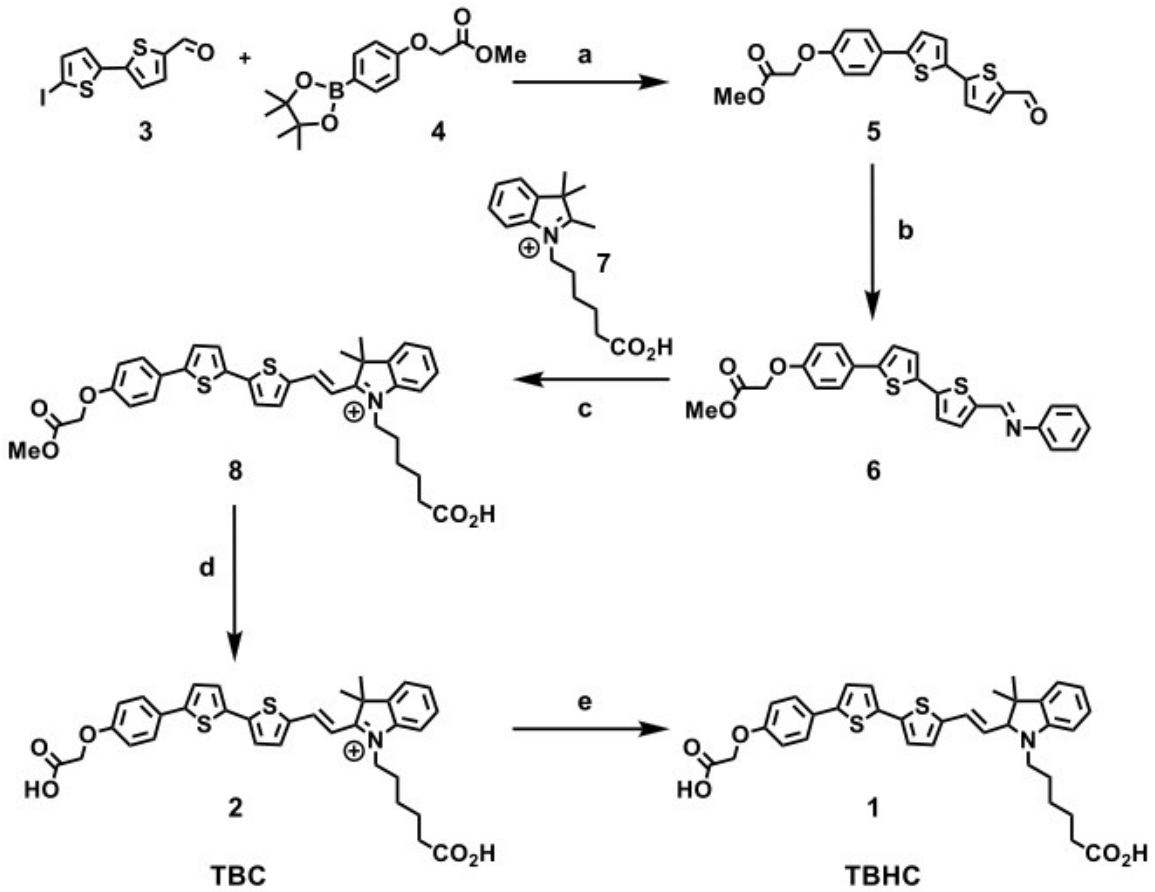
Materials: All the chemicals used in the synthesis of TBC and TBHC were purchased from Sigma-Aldrich. 0.1 M phosphate buffered saline (PBS), syringes, needles, pipette tips, microcentrifuge tubes, and nuclear magnetic resonance (NMR) tubes were purchased from VWR. ^{1}H-NMR spectra were recorded in $CDCl_3$, Acetone-d_6 and DMSO-d_6 in a Bruker 300 MHz and 400 MHz spectrometers at 300K. TMS (δ (ppm) H = 0.00) was used as the internal reference. ^{13}C-NMR spectra were recorded in $CDCl_3$, Acetone-d_6 and DMSO-d_6 at a 100MHz on a Bruker 900 MHz spectrometer, using the central resonances of $CDCl_3$ (δ (ppm) C = 77.23). Chemical shifts are reported in ppm and multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), and m (multiplet). Coupling constants, J, are reported in hertz (Hz). High-resolution mass spectra (HRMS) were obtained on an AB SCIEX TOF/TOF 5800 system and are reported as m/z (relative intensity). Accurate masses are reported for the molecular ion (M^+) or a suitable fragment ion. Chemicals were purchased from Aldrich or VWR and used without further purification. All solvents were purified using standard methods. Flash chromatography was carried out using silica gel (230-400 mesh). All reactions were performed under anhydrous conditions under N_2 or Argon and monitored by TLC on Kieselgel 60 F254 plates (Merck). Detection was accomplished by examination under UV light (254 nm). Macrophage RAW 264.7 cells were obtained from the UCB Cell Culture Facility, which is supported by The University of California Berkeley. A laser scanning confocal microscope (LSM 710) was used for imaging RAW 264.7 macrophage cells. Fluorescent images were analyzed and quantified using Fiji (NIH).

A. Synthesis of TBC and TBHC

A1. Synthesis of compound 5

In an oven dried two-neck round bottom flask was added **3** (100 mg, 0.312 mmol), **4** (96 mg, 0.327 mmol) and triphenylphosphine (62 mg, 0.125 mmol). The resulting reaction mixture was dissolved into degassed and dry DMF (20 mL). Pd_2dba_3 (16 mg, 5 mol%) and cesium fluoride (141 mg, 0.936 mmol) were sequentially added to the reaction mixture. The reaction mixture was degassed and refilled with nitrogen five times and heated to 85 °C for 16h. The reaction mixture was poured into a large excess of water (120 mL) and extracted with ethyl acetate (3 x 35 mL). The organic layers were collected together and washed with brine (3 x 20 mL) and dried over anhydrous Na_2SO_4. The ethyl acetate was removed under reduced pressure to afford a yellow oil. The crude product was purified by silica gel chromatography to afford a



Scheme S1: Synthesis of TBHC and TBC: (a) $Pd(PPh_3)_4$, CsF, DMF, 80°C, 82%; (b) Aniline, Toluene, reflux, quantitative; c) 2,2,3-trimethyl-N-hexyl-indeloic acid, acetic anhydride, 80°C, 20%; d) porcine liver esterase (1000 unit), PBS (pH = 7.83), 82.5%; (e) $NaBH_4$, methanol, 63%.

solid product. **Compound 5** was purified by silica gel column chromatography (Toluene/DCM (8/2) to afford **compound 5** (92 mg, 82%) as yellow solid. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 9.85 (s, 1H), 7.95 (d, 1H, *J* = 3.0 Hz), 7.62-7.54 (m, 5H), 6.99 (d, 2H, *J* = 6.0 Hz), 4.83 (s, 2H), 3.69 (s, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 183.4, 168.8, 157.7, 145.4, 144.8, 140.9, 138.8, 133.2,

127.9, 126.7, 126.1, 124.6, 124.0, 115.2, 64.6, 51.6; EI-HR (*m/z*): $(M+H)^+$ Calculated for ($C_{18}H_{15}O_4S_2$), 359.0334, found 359.0351.

A2. Synthesis of compound 6

In an oven dried round bottom flask were added **compound 5** (50 mg, 0.14 mmol) and aniline (1.27 mL, 14 mmol). The resulting reaction mixture was suspended in toluene (50 mL). The reaction was heated to reflux for 48 h. The reaction mixture was cooled down to room temperature and filtered. Product was washed with toluene. The crude **compound 6** was used in the next step.

A3. Synthesis of compound 7

Compound 7 was synthesized following the procedure detailed in the paper by Dueckert *et. al*.[1]

A4. Synthesis of compound 8

To the crude reaction mixture that we obtained after the synthesis of **compound 6** were added **compound 7** (70 mg, 0.2 mmol) and acetic anhydride (40 mL). The reaction mixture was heated to 80°C for 1.5 hours. The reaction mixture was poured into water and extracted with ethyl acetate (3 × 50 mL). The cumulative organic phases were dried over anhy. Na_2SO_4 and the ethyl acetate were removed under reduced pressure. The crude product was purified by silica gel chromatography (acetonitrile/water = 9/1) to yield 17 mg (20% in overall two steps) of **compound 8** as a purple solid.

^{1}H NMR (MeOD-d_4, 400 MHz): δ □□□□ (d, *J* = 16.0 Hz, 1H), 7.94 (d, *J* = 4.0 Hz, 1H), 7.75 (dd, *J* = 8 Hz, 2H), 7.65-7.57 (m, 5H), 7.51 (d, *J* = 4.0 Hz, 1H), 7.39 (d, *J* = 4.0 Hz, 1H), 7.16 (d, *J* = 16.0 Hz, 1H), 7.00 (d, *J* = 8 Hz, 2H), 4.80 (s, 2H), 4.57 (t, *J* = 4.0 Hz), 3.80 (s, 3H), 2.27 (t, *J* = 4.0 Hz, 2H), 1.98 (m, 2H), 1.84 (s, 6H), 1.73 (m, 2H), 1.57 (m, 2H).

^{13}C NMR (MeOD-d_4, 125 MHz): δ 182.1, 171.3, 160.0, 150.3, 148.6, 147.7, 147.6, 145.0, 142.5, 140.2, 135.5, 130.7, 130.6, 129.9, 128.4, 128.3, 127.2, 125.4, 124.2, 116.5, 115.8, 66.2, 57.5 (m), 53.6 (d), 52.8, 50.0, 49.8, 49.7, 49.6, 49.5, 47.6, 37.0, 29.5, 27.6, 26.9, 26.6, 17.5 (m).

HRMS (*m/z*): $[M+H]^+$, calculated for ($C_{35}H_{36}NO_5S_2$+), 614.2035, found 614.2214.

A5. Synthesis of TBC

In an oven dried round bottom flask was added **compound 8** (3 mg, 0.005 mmol) and the compound was suspended into PBS (pH = 7.83) (700 μL). A 500 unit of porcine liver esterase suspended into 500 μL of PBS (pH = 7.83) was added into the reaction mixture at RT and the resulting reaction mixture was stirred at RT for 20 hours. Additionally another 500 unit of porcine liver esterase in 500 μL of PBS (pH = 7.83) was added and the stirring continues for another 20 hour. The reaction mixture was diluted with methanol (15 mL) and the solid suspension was purified by centrifuge at 4000xg for 10 min at 25 °C. The solid was further washed with 15 mL of methanol. Filtrates were combined and evaporated to achieve a crude TBC. The crude TBC was redissolved into copious amount of methanol (2 mL) and precipitated into a large volume of diethyl ether (50 mL). The solid product was collected via centrifuge at 4000xg for 10 min at 25 °C. Further, the solid product was washed with DCM

several times to achieve 86% pure TBC. Further, TBC was purified by HPLC using mobile phase A: deionized distilled water with 0.1% TFA (v/v) and mobile phase B: HPLC grade acetonitrile with 0.1% TFA (v/v) to achieve **TBC** as a dark purple solid (2.1 mg, 82%).

^{1}H NMR (MeOD-D_4, 900 MHz): δ 8.64 (d, *J* = 18 Hz, 1H), 7.98 (d, *J* = 9 Hz, 1H), 7.78 (d, *J* = 9 Hz, 1H), 7.76 (d, *J* = 9 Hz, 1H), 7.68-7.60 (m, 5H), 7.54 (d, *J* = 9 Hz, 1H), 7.41 (d, *J* = 9 Hz, 1H), 7.17 (d, *J* = 18 Hz, 1H), 7.04 (d, *J* = 9 Hz, 2H), 4.75 (s, 2H), 4.61 (t, *J* = 9 Hz, 2H), 2.37 (t, *J* = 9 Hz, 2H), 2.02 (m, 2H), 1.86 (s, 6H), 1.76 (m, 2H), 1.59 (m, 2H).
^{13}C NMR (MeOD-D_4, 125 MHz): 182.3, 177.3, 172.6, 160.2, 150.4, 148.8, 147.8, 147.7, 145.0, 142.5, 140.2, 135.5, 130.8, 130.6, 129.9, 128.4, 128.3, 127.2, 125.4, 124.2, 116.6, 115.7, 110.1, 66.0, 57.6, 53.6, 49.8, 47.5, 34.6, 29.4, 27.3, 26.9, 25.7.
HRMS (*m/z*): $[M]^+$ calculated for ($C_{34}H_{34}NO_5S_2$+), 600.1878, found 600.1904.

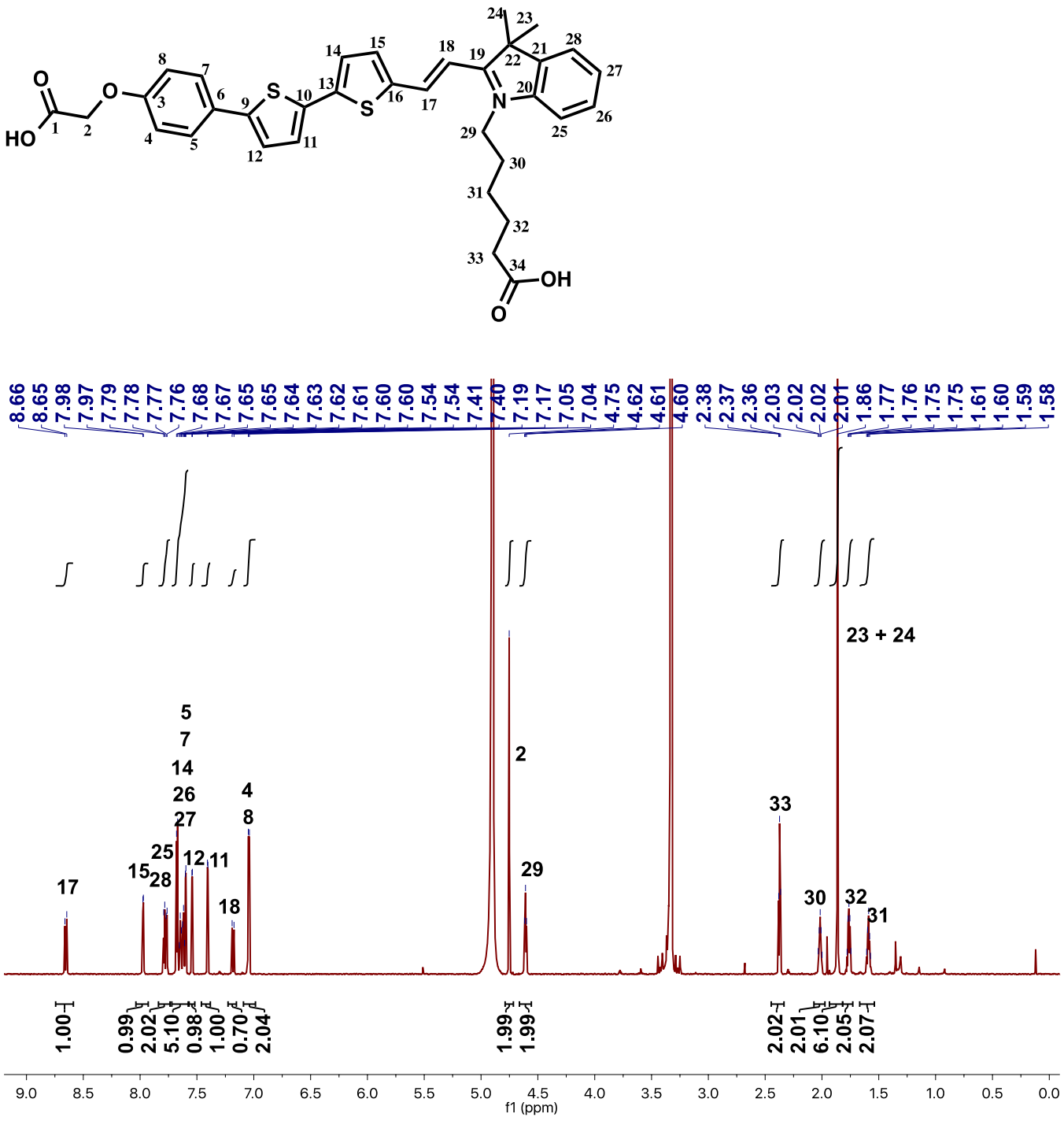
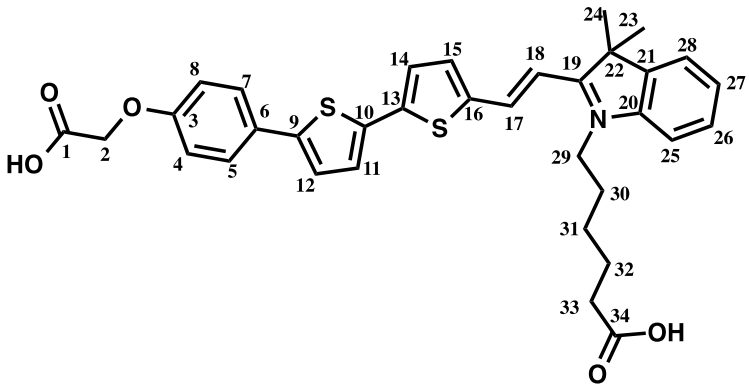


Figure S1: 1H NMR of TBC



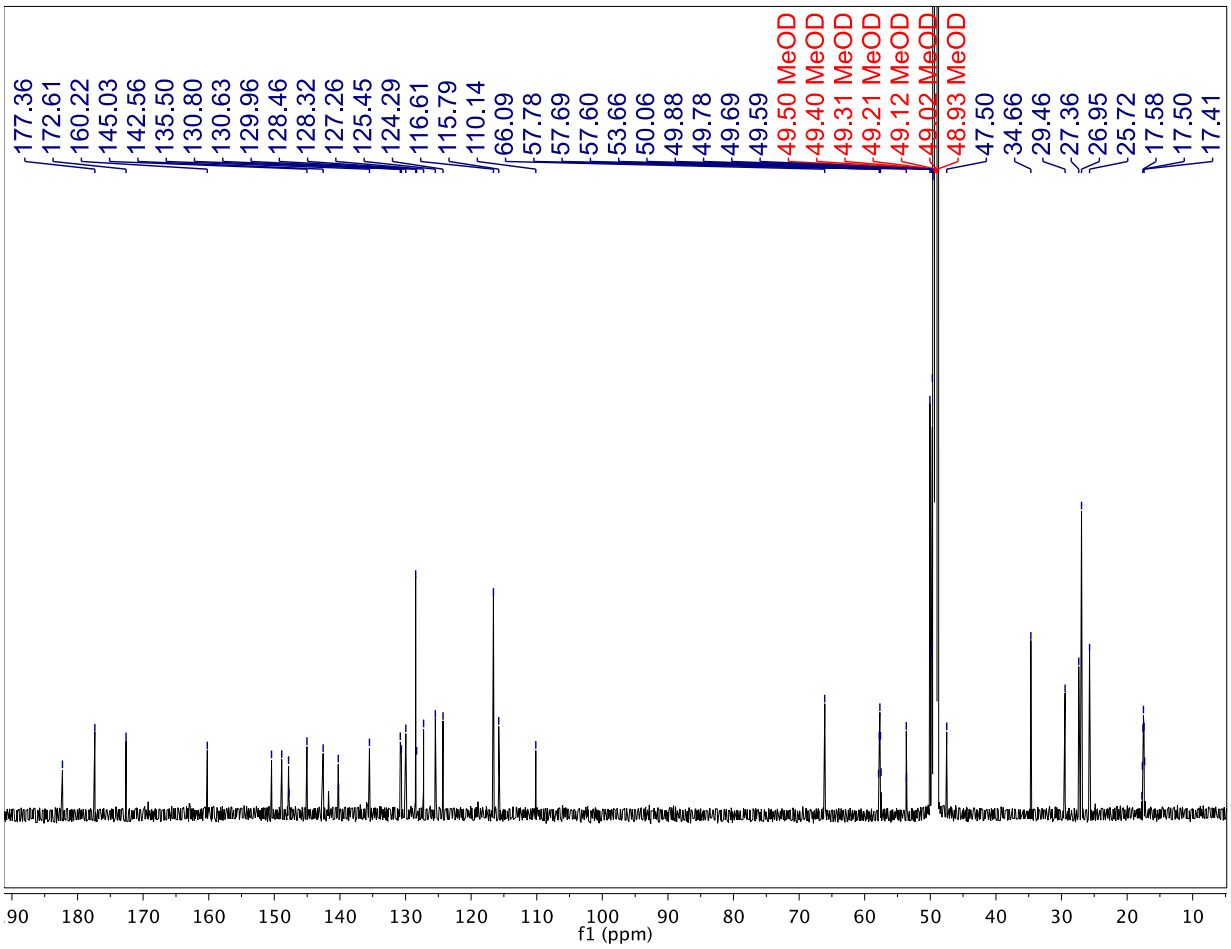


Figure S2: 13C NMR of TBC

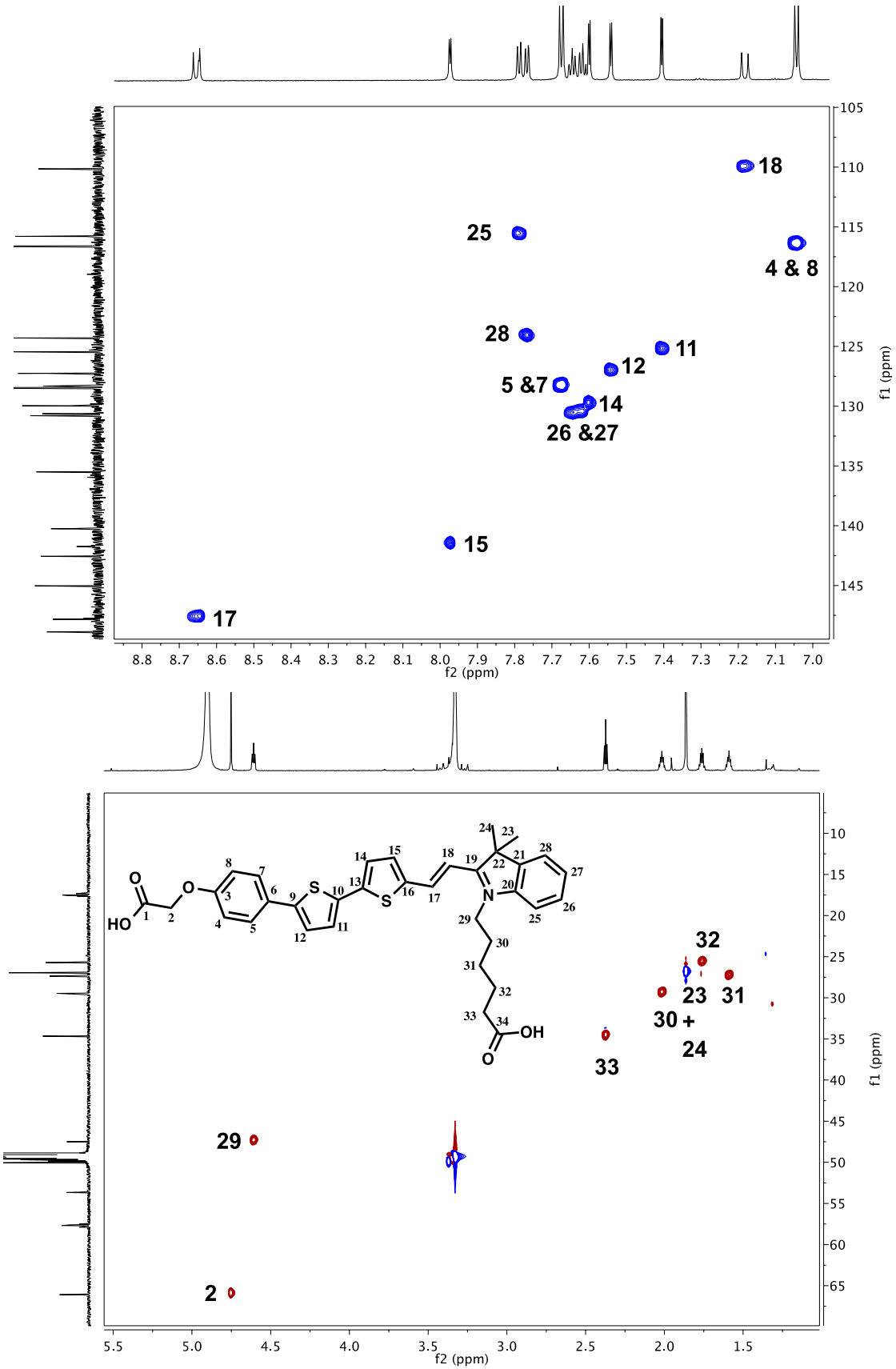


Figure S3: HSQC spectra of TBC. CHs and CH3s are annotated as blue and CH2s annotated as red

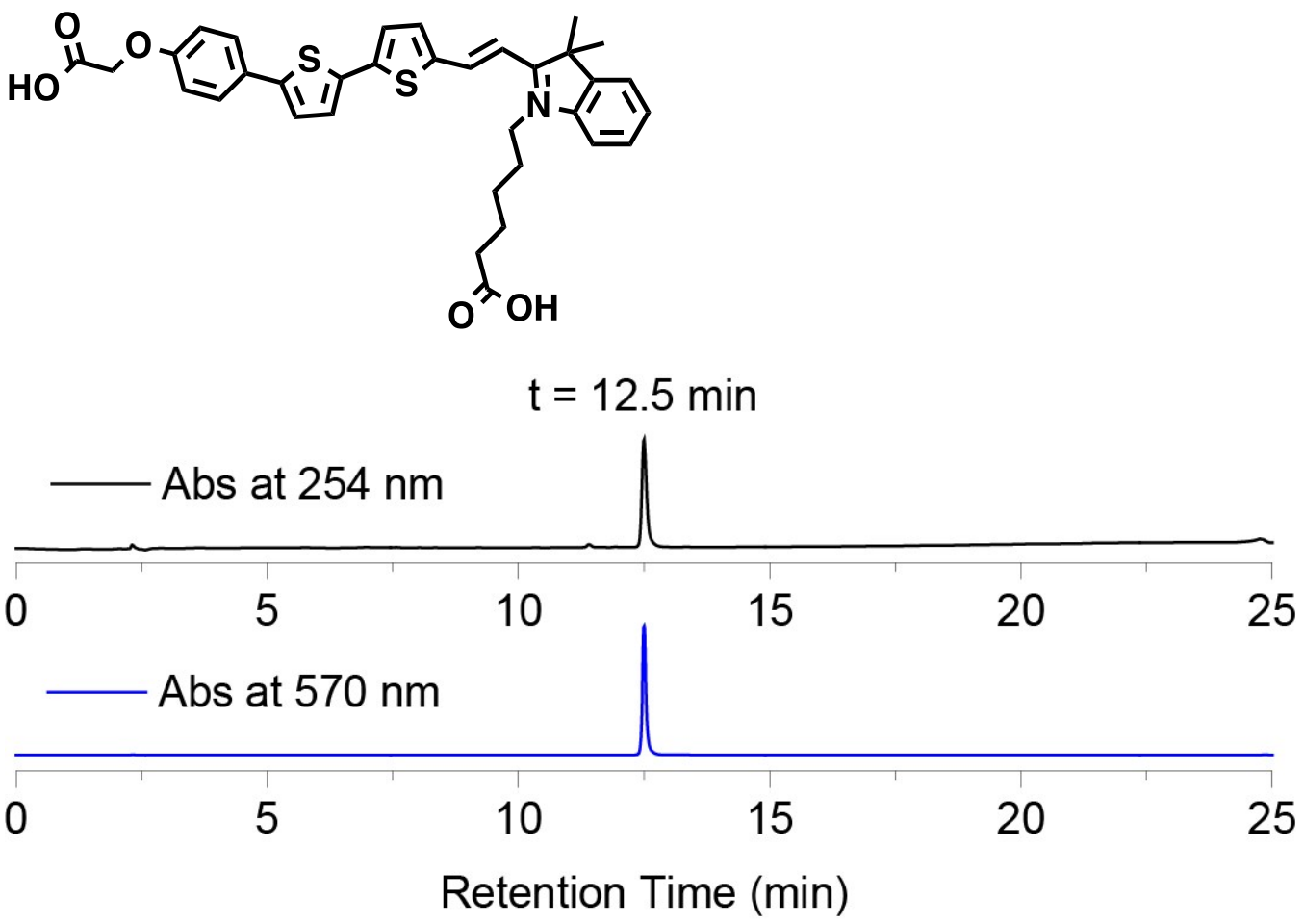


Figure S4: HPLC trace of TBC. Using Mobile phase A : Deionized Distilled water with 0.1% TFA (v/v). Mobile phase B : HPLC grade acetonitrile with 0.1% TFA (v/v).

A6. Synthesis of compound TBHC

In an oven dried round bottom flask was added **compound 2 (TBC)** (3 mg, 4.9 μmol). The compound was dissolved in 2 mL of methanol. Then 3 μL of a methanolic solution of $NaBH_4$ (34 mg of $NaBH_4$ in 500 μL of methanol) was added to the reaction mixture. After the addition of methanolic $NaBH_4$, the solution turns light yellow and the reaction mixture was further stirred for 10 min at rt. Solvent was removed under vacuum and washed with diethyl ether (5 x 10 mL). The crude product was purified by HPLC using Mobile phase A : Deionized Distilled water with 0.1% TFA (v/v). Mobile phase B : HPLC grade acetonitrile with 0.1% TFA (v/v). Using flow rate 1.0 mL/min; 0-1 min: 1% B ; 1 – 18 min 1% – 100% B; 18 – 21min: 100% B; 21 – 24 min: 100% – 1% B; 24 – 25 min: 1% B. To afford **compound 1** (**TBHC**) 1.8 mg (63%) as a yellow powder (Caution: TBHC is prone to auto-oxidation in solid form, therefore it is recommended to store in a solution)

1H NMR (MeOD-D_4, 900 MHz): δ 7.56 (d, *J* = 9.0 Hz, 2H), 7.21 (d, *J* = 9 Hz, 1H), 7.19 (d, *J* = 9 Hz, 1H), 7.12 (d, *J* = 9 Hz, 1H), 7.04 (t, *J* = 9 Hz, 1H), 7.00-6.98 (m, 4H), 6.85 (d, *J* = 9 Hz, 1H), 6.65 (t, *J* = 9 Hz, 1H), 6.51 (d, *J* = 9 Hz, 1H), 6.06 (dd, *J* = 9.0 Hz, 1H), 4.43 (s, 2H), 3.70 (d, *J* = 9.0 Hz, 1H), 3.19 (m, 1H), 3.07 (m, 1H), 2.17 (m, 2H), 1.91-1.60 (m, 4H), 1.42-1.10 (m, 8H).
13C NMR (MeOD-D_4, 125 Hz): δ 183.1, 180.5, 176.5, 170.5, 161.7, 160.2, 151.6, 144.9, 142.1, 140.0, 137.7, 136.8, 128.8, 128.7, 128.4, 128.3, 127.8, 126.0, 124.8, 124.1, 122.8, 119.0, 116.4, 108.6, 79.2, 68.6, 57.8, 57.7, 57.6, 57.5, 57.4, 50.0, 49.8, 49.7, 49.6, 49.5, 47.6, 45.7, 39.5, 39.4, 33.2, 31.1 (q), 28.7, 28.0, 27.9, 27.4, 26.8, 24.8, 24.7, 24.4, 23.9, 17.4 (q).
HRMS (*m/z*): $[M]^{-1}$ Calculated for ($C_{34}H_{34}NO_5S_2$-), 600.1957, found 600.1878.

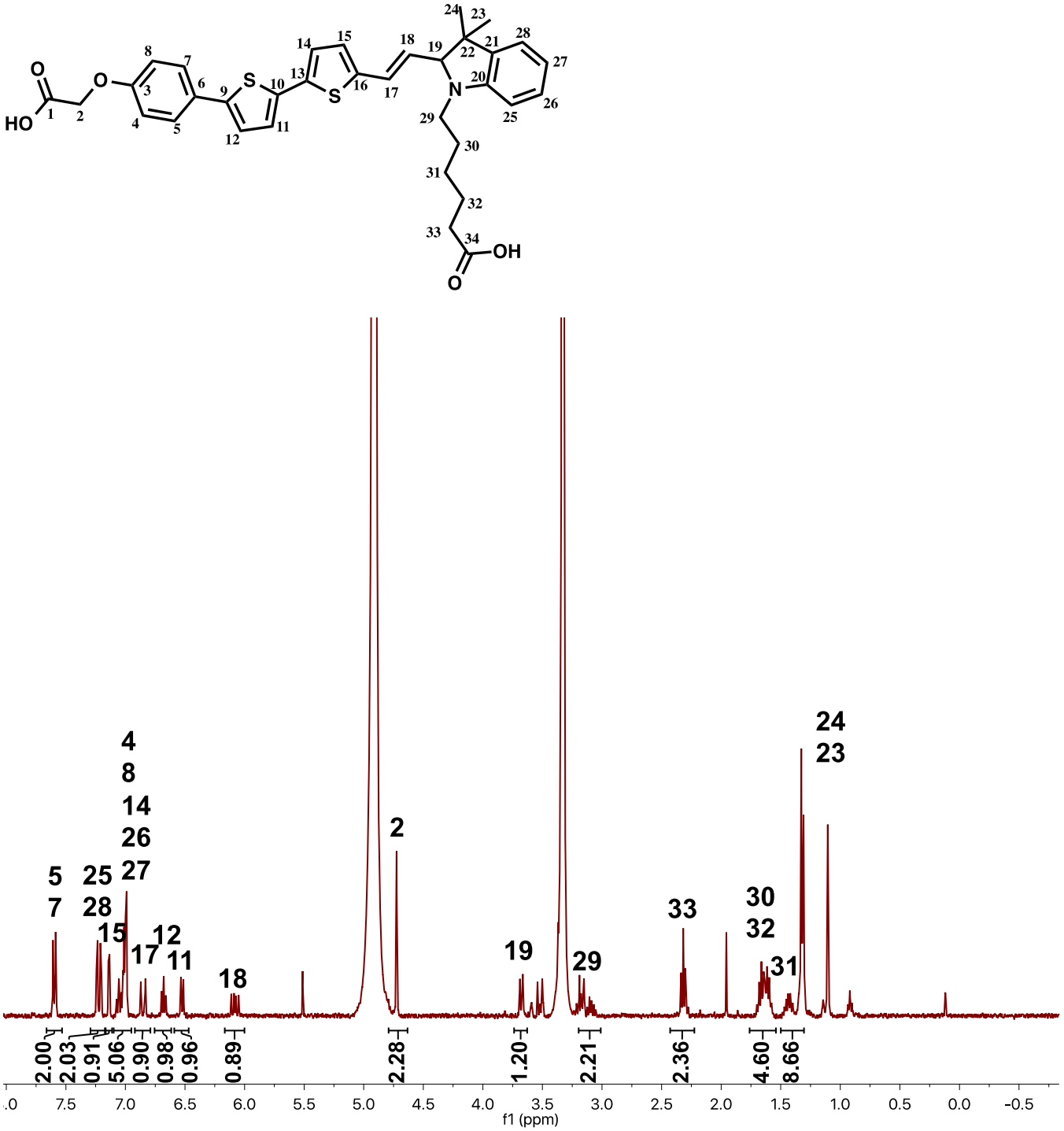


Figure S5: 1H NMR of TBHC

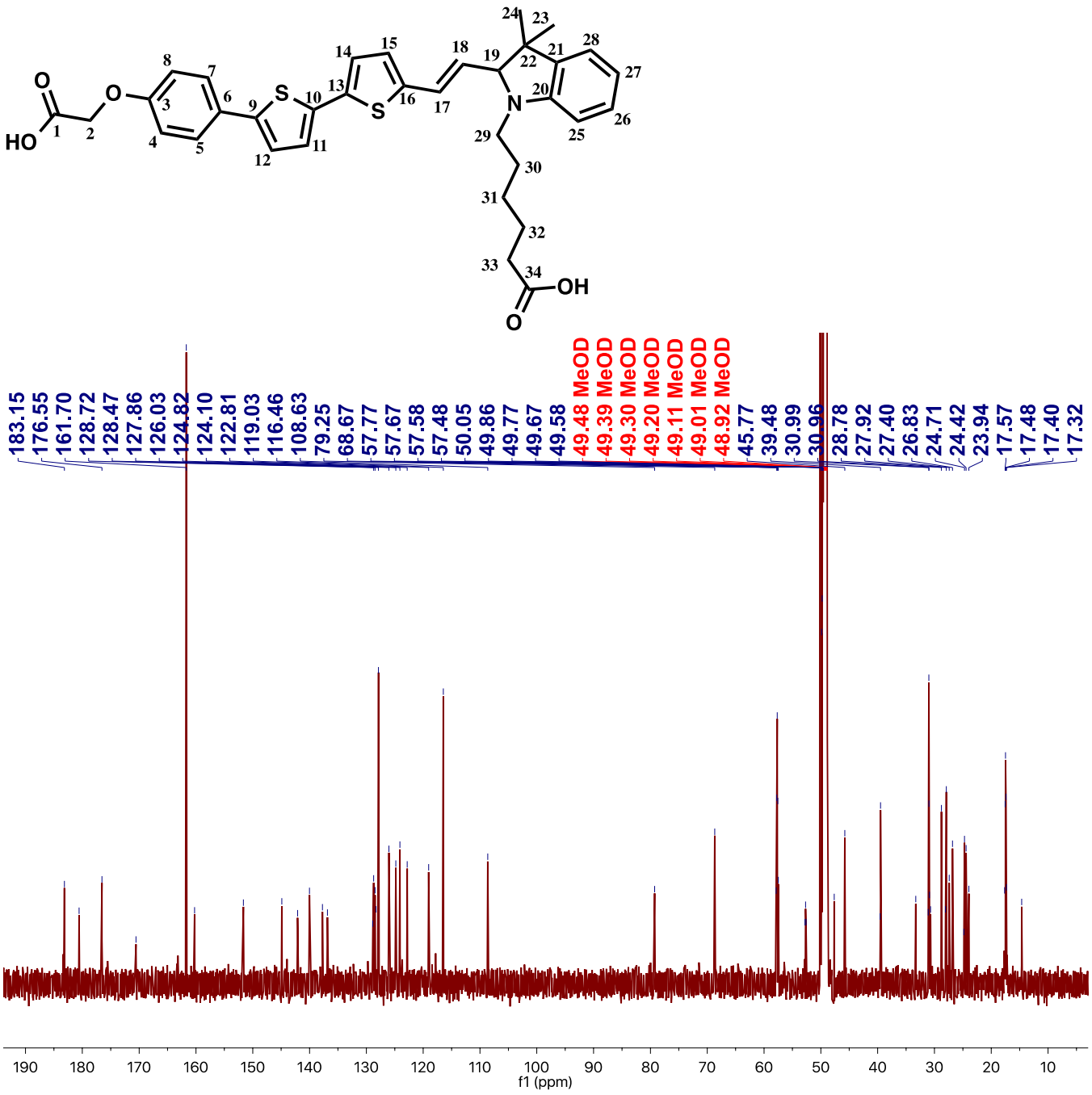


Figure S6: 13C NMR spectra of TBHC

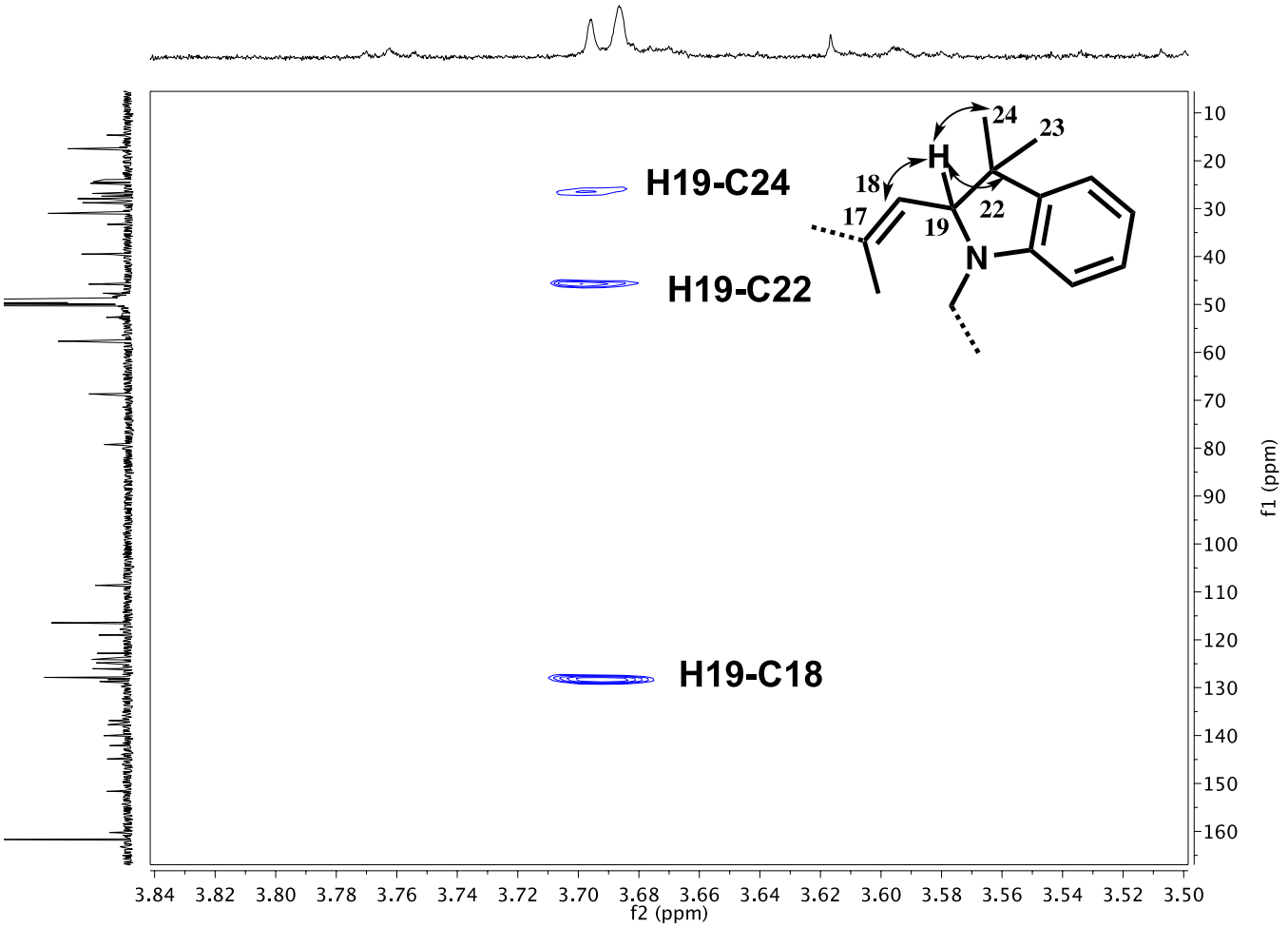


Figure S7: HMBC spectra of TBHC. H19 of TBHC showed 2-3 bonds coupling with C18, C22 and C24 respectively.

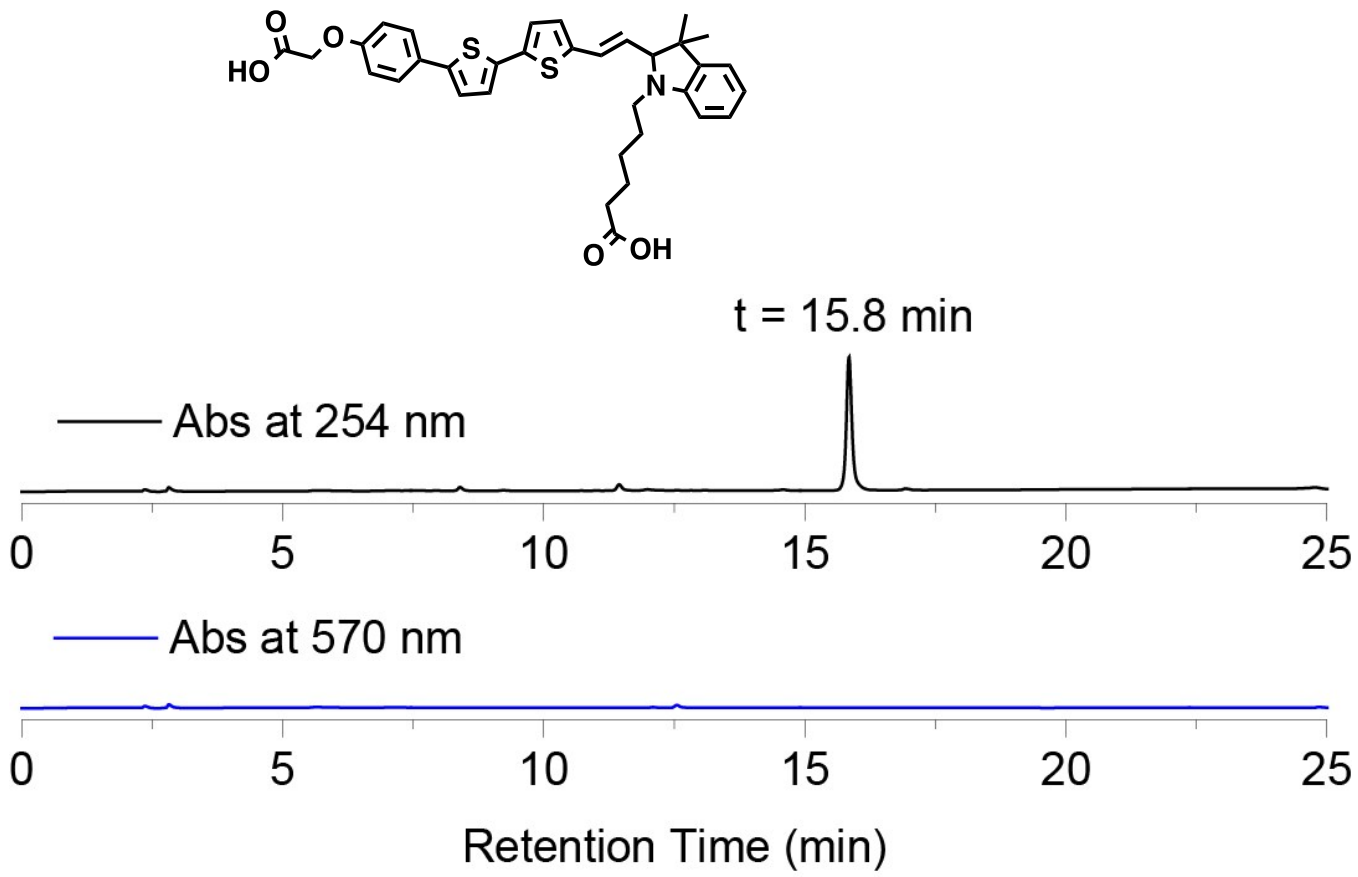


Figure S8: HPLC trace of TBHC. Using Mobile phase A : Deionized Distilled water with 0.1% TFA (v/v). Mobile phase B : HPLC grade acetonitrile with 0.1% TFA (v/v).

B. Measurement of absorbance and molar absorption coefficient of TBC and TBHC

Absorbance spectra of 25 μM, 30 μM, 50 μM, 75 μM and 100 μM solution in PBS of either TBHC or TBC were measured by a Tecan plate reader. Subsequently, molar absorption coefficients were measured using Lambert-Beer law.

Molar absorption coefficient of TBC (**2**): 0.28×10^4 M^{-1} cm^{-1}

Molar absorption coefficient of TBHC (**1**): 0.38×10^3 M^{-1} cm^{-1}

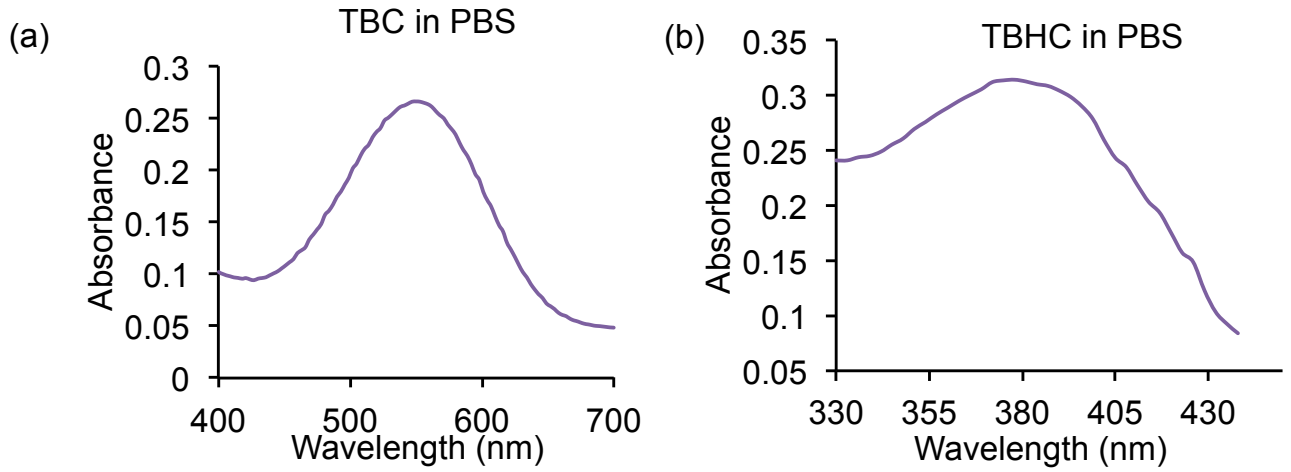


Figure S9: Absorbance spectra of 75 μM solution of TBC (a) and TBHC (b) in PBS.

C. Measurement of fluorescence spectra and quantum yield of TBC and TBHC

TBC and TBHC were dissolved in DMSO (2 mM) and were diluted to a 50 μM concentration in PBS. The fluorescence spectra of TBC and TBHC were measured using a Tecan plate reader. The fluorescence quantum yield was measured following this equation and using cyanine 5.5 as standard:[1]

$$\varphi_{TBC} = \varphi_{STD} (I_{TBC}/I_{STD})(OD_{STD}/OD_{TBC})(\eta^2_{TBC}/\eta^2_{STD})$$

Where φ_{TBC} = quantum yield of TBC; φ_{STD} = quantum yield of cyanine 5.5 in PBS; I_{TBC} = Integrated intensity of TBC; I_{STD} = Integrated intensity of cyanine 5.5 in PBS, OD_{STD} = Optical density of cyanine 5.5 in PBS, OD_{TBC} = Optical density of TBC in PBS, η = Refractive index of PBS

Quantum yield of TBC in PBS: 0.11%
Quantum yield of TBC in DMSO: 23%
Quantum yield of TBC in 10% (w/v) albumin: 2.4%
Quantum yield of TBHC in 10% (w/v) albumin: 0.005%

D. Preparation of reactive oxygen species

1. **$OONO^-$**: 3-morpholinosydnonimine hydrochloride was placed (SIN-1: purchased from Abcam) (1.3 mg, 6.4 μM) in a sterilized Eppendorf tube and a 50 μL of 0.1N NaOH was added to it. The resultant solution was incubated at room temperature for 15 min. The generation of peroxynitrite (^-ONOO) was quantified by measuring the absorbance at 302 nm (ε = 1670 $M^{-1}cm^{-1}$). The efficiency of peroxynitrite production was 1.8%. The solution was stored at 4 °C during the experiments.

2. **ClO^-**: In an Eppendorf tube a 5% solution of sodium hypochlorite (purchased from Sigma) was diluted with PBS to make an approximate 450 μM of final concentration. The concentration of hypochlorite was quantified by measuring UV absorbance at 292 nm (ε = 350 $M^{-1}cm^{-1}$). The newly calculated concentration of hypochlorite was 250 μM.

3. **H_2O_2**: The stock H_2O_2 solution was purchased from Sigma-Aldrich. The stock H_2O_2 solution was diluted with PBS to make an approximate concentration of 1 mM. The effective concentration was determined by measuring the absorbance at 240 nm with a molar extinction coefficient of 43.6 M^{-1} cm^{-1}. The newly calculated concentration of H_2O_2 was 877 μM.

4. **•OH**: To an Eppendorf tube were added hydrogen peroxide (20 μM) and Horseradish Peroxidase (4 unit). Generation of hydroxyl radical was quantified by 2,7-dichlorodihydrofluorescein (H_2DCF).[3] The newly calculated hydroxyl radical concentration was 25 μM.

5. **•O_2^-**: KO_2 (2 mg, 0.028 mmol) was dissolved in PBS (pH = 6.5) and added to a glass vial. 5 μL of the stock solution was added into 100 μL of PBS. The concentration of superoxide was quantified by hydro-Cy3.[4]

6. **$^1O_2$**: 3,3'-(naphthalene-1,4-diyl)dipropionic acid endoperoxide (2 mg) was dissolved into a PBS and generation of singlet oxygen was quantified by the generation of 3,3'-

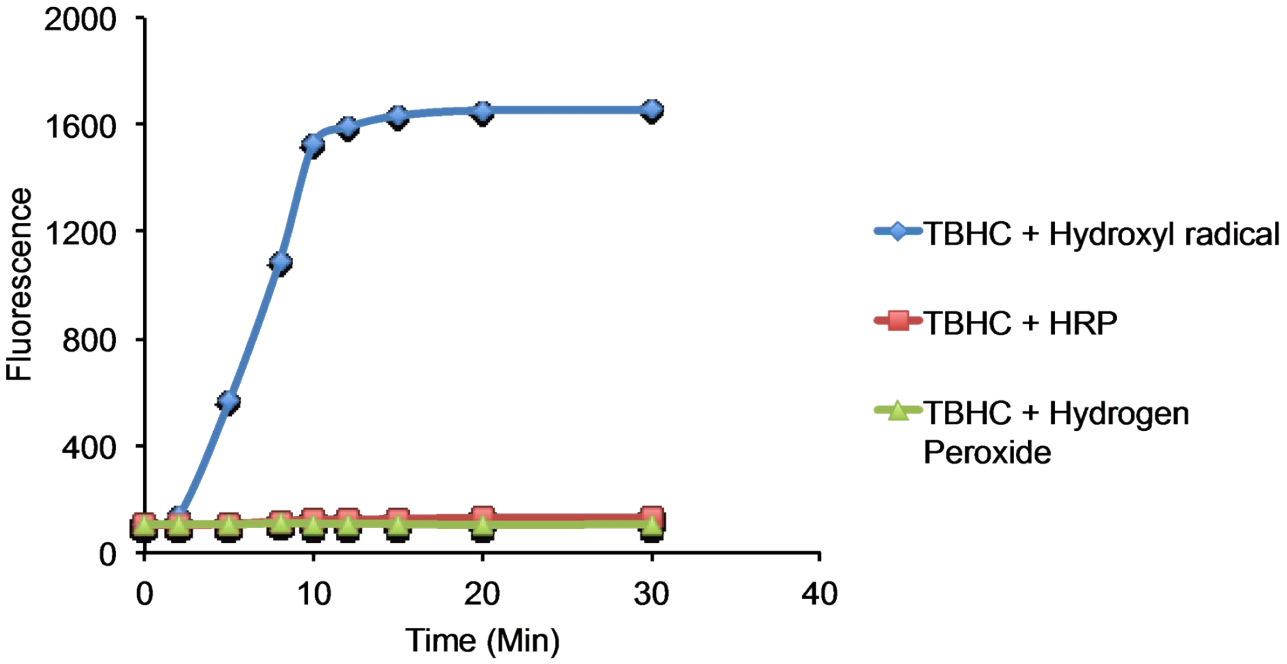


Figure S10: **Time course measurement of TBHC to TBC oxidation in response to hydroxyl radicals.** TBHC were either treated with hydroxyl radicals (HRP + H_2O_2) (blue) or HRP (red) or H_2O_2 (green). In presence of HRP + H_2O_2, fluorescence at 700 nm was linearly increasing for 10 min.

(naphthalene-1,4-diyl)dipropionic acid at 290 nm.

7. **•NO**: NOC 5 (2 mg, 0.011 mmol) was dissolved in 100 mL of PBS to achieve nitric oxide in the solution.

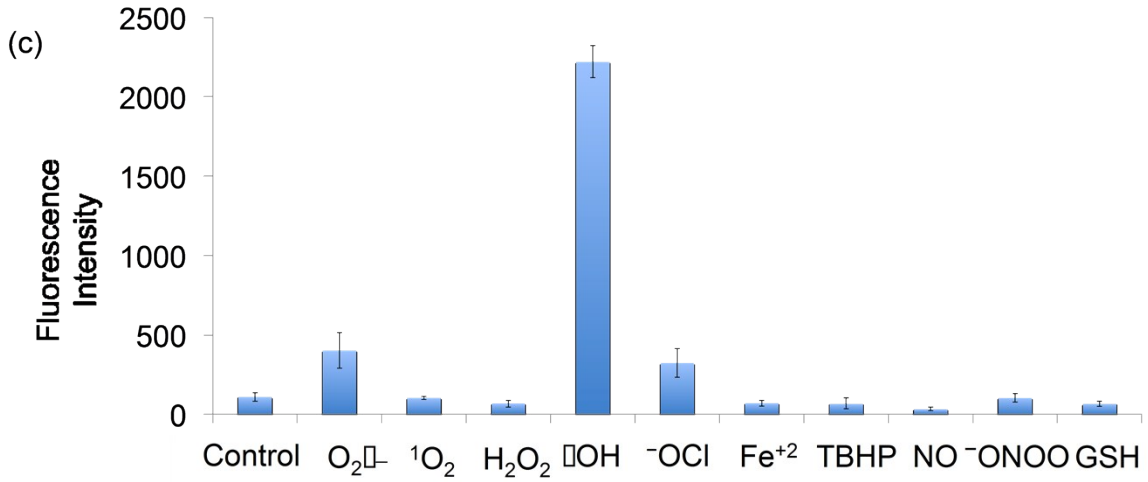


Figure S11: **TBHC can detect hydroxyl radical selectively**. TBHC was treated with various reactive oxygen species and it is selective for radical oxidants, such as superoxide and hydroxyl radical.

E. Time Course of fluorescence change in response to hydroxyl radicals

To a 300 μM solution of TBHC in PBS were added 75 μM of H_2O_2 and 8 units of Horseradish Peroxidase. The reaction mixture was incubated at 37 °C and the increase in fluorescence at 573/700 was recorded for 30 min.

F. Detection of Hydroxyl Radicals

The hydroxyl radical was generated *in situ* by reacting hydrogen peroxide with horseradish peroxidase in the following experiments. To a 100 μL solution of TBHC in PBS (300 μM) various quantities of a hydrogen peroxide stock solution were added, to generate hydroxyl radical concentrations within the 1-100 nM range. 4 units of horseradish peroxidase were then added to the TBHC/H_2O_2 solution to generate a 1-100 nM range of concentration. The resulting solution was kept at ambient temperature for 15 min and the fluorescence intensity was measured ($\lambda_{ex}/\lambda_{em}$ = 573/700 nm). Similarly, as a control 300 μM solution of TBHC was either incubated with H_2O_2 (1-100 nM) or with 4 units of horseradish peroxidase or in the PBS for 15 min and the increase in fluorescence was measured ($\lambda_{ex}/\lambda_{em}$ = 573/700 nm) at room temperature.

Table S1: A summary of hydroxyl radical imaging organic fluorescent probes

	$\lambda_{ex}/\lambda_{em}$[a]	Stoke's Shift (nm)[b]	Response type[c]	Detection Limit[d]	Detection Media[e]	Reference
1.	560/588	28	Off/on	670 nM	PBS with 0.1% DMSO at pH = 4	*Org. Lett*. 2012, **14**, 50–53

2.	371 and 461/418 and 552	47 and 91	Ratiometric	200 nM	Water/DMF (98:2)	*Chem.Comm.* 2014, **50**, 4843–4845
3.	410/450 or 528	40 and 118	Ratiometric (FRET)	10 □M	PBS	*Anal. Chem*. 2011, **83**, 2576–2581
4.	488/520 and 635	32 and 147	Ratiometric (FRET)	680 nM	PBS	*Anal. Chem*. 2014, **86**, 1829–1836
5.	500/550	50	Off-on	2 □M	TRIS buffer (pH = 7.4) with 1% DMF	*Chem.Comm.* 2013, **49**, 7959–7961
6.	573/700	127	Off-on	35 nM	PBS (pH = 7.4)	This work

a: excitation and emission maximum of probe; b: difference between excitation and emission maxima; c: mode of detection of ROS; d: detection sensitivity denotes lowest concentration of ROS a probe can detect; e: reaction medium of ROS mediated oxidation.

G. Stability of TBHC and hydro-C5 to auto-oxidation

A solution of hydro-Cy5 or TBHC (80 μM) was made in PBS buffer using DMSO as co-solvent (1%) and incubated at room temperature for 48 hours. The stability profile of hydro-Cy5 and TBHC was obtained by monitoring the formation of their oxidized products Cy5 and TBC, by recording the absorbance at either 640 nm or 573 nm. The half-life of hydro-Cy5 and TBHC was determined by plotting the data and fitting it to the best equation, we obtained zero–order kinetics for this process for both hydro-Cy5 and TBHC..

This suggests the following reaction scheme, where the $^1O_2$, is being continuously generated and has a very low concentration in comparison to the concentration of TBHC or hydro-Cy5. From the experimental data, we deduce the integrated zero order rate law as:

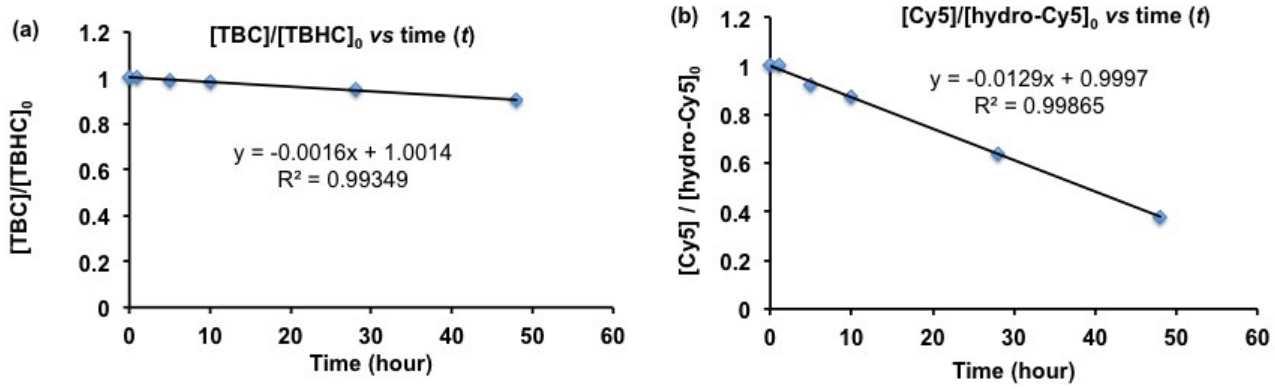


Figure S12: A plot of [TBC]/[TBHC]$_0$ *vs t* (a) and [Cy5]/[hydro-Cy5]$_0$ *vs t* (b) can be fitted with the equation y= -mx + c, which represents zero order kinetics.

Half-life of TBHC = 250 hours
Half-life of hydro-Cy5 = 31 hours

From this TBHC has an auto-oxidation half-life that 8.06 fold greater than hydro-Cy5.

H. Stability of TBC to ROS

A 25 μM solution of either TBC or Cy5 were incubated with 250 μM of •OH, $^{-}$ONOO and $^{-}$OCl. Hydroxyl radical was generated in-situ via a reaction between H_2O_2 (300 μM) and 4 units of horseradish peroxidase. $^{-}$ONOO and $^{-}$OCl were generated by the addition of 3-morpholinosydnonimine (SIN-1) (250 μM final concentration of peroxynitrite) and a solution of hypochlorite (250 μM final concentration of hypochlorite) respectively.

I. Fluorescence recovery of TBHC in response to hydroxyl radical

A 25 μM solution of either TBHC or hydro-Cy5 were mixed with 300 μM of H_2O_2 in the presence of 8 units of horseradish peroxidase at room temperature. The reaction mixture was incubated for 15 min and the absorbance was either measured at 573 nm (for oxidized product T BC) or 640 nm (for oxidized product Cy5).

J. pH dependent fluorescence response of TBHC

A 300 μM solution of TBHC at various pHs was treated with H_2O_2 (75 μm final concentration) and Horseradish Peroxidase (8 units in 100 μL). The reaction mixture was incubated for 15 min and the increase in fluorescence was measured at 700 nm. Acidic pH solutions were

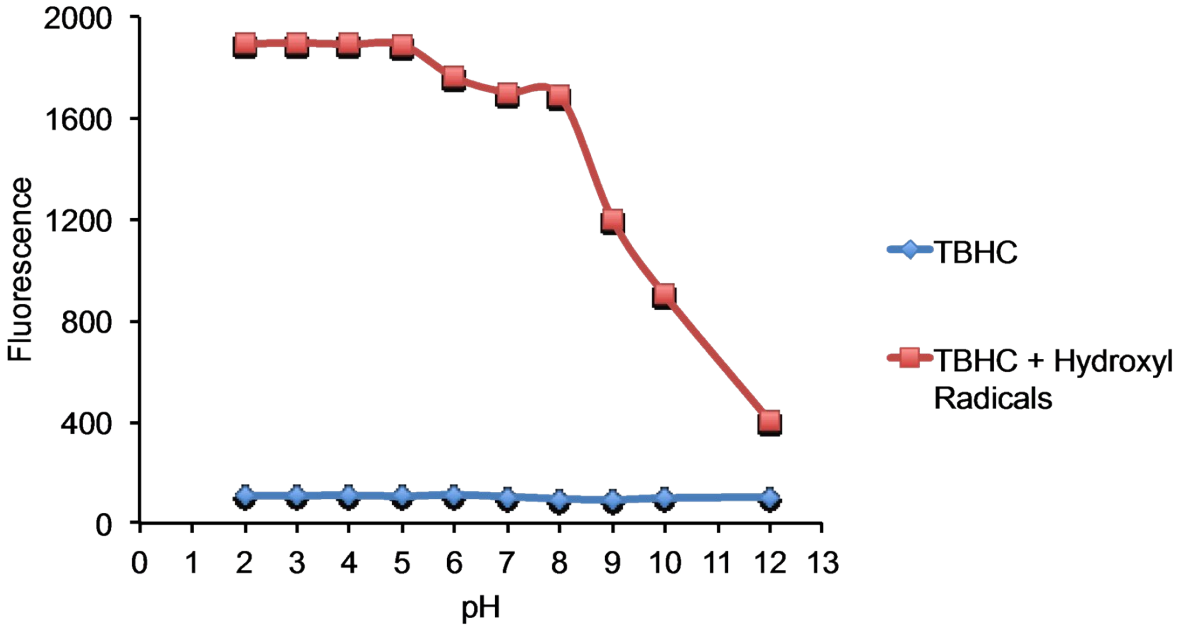


Figure S13. pH dependent fluorescence response of TBHC in the presence of hydroxyl radicals. TBHC has no response in presence/absence of acidic/basic pHs (blue line). However, hydroxyl radical mediated oxidation of tertiary amine to iminium is partially inhibited at basic pHs due to instability of hydroxyl radical (red line).

prepared by addition of HCl into PBS and basic pH solutions were prepared by addition of 1M NaOH into PBS.

K. Cell culture

RAW 264.7 murine macrophages were acquired from the UC Berkeley Cell Facility (Biosciences Divisional Services). The cells were cultured in high glucose DMEM with 10% fetal bovine serum (GIBCO), 1% Penicillin-Streptomycin (GIBCO), 1% L-Glutamine (GIBCO) and 1% Non-Essential Amino acids (GIBCO). The cells were grown up to 90% confluence before passaging into new plates. For all the *in vitro* experiments, 35mm dishes (Corning) were used. Briefly, 10^5 cells were plated per well of the EZ SLIDE with media containing Phenol Red. The day before treatment, the previous media was replaced with media without Phenol Red, to reduce background. The cells were then either activated with 1 µg/ml of Lipopolysaccharide (Santa Cruz Bio – sc221855A) or 1X PBS (GIBCO) for overnight (16 hours). The wells were washed three times with 1X PBS for 5 minutes each. Media without Phenol Red is added with the either TBHC or hydro-Cy5 dye at 100µM final concentration for 45 minutes. The cells were again washed three times with 1X PBS for 5 minutes each. Finally, media without phenol red is added and the cells are imaged live.

L. Cell viability (MTT) assay

RAW 264.7 macrophages were plated in a 96 well plate at the density of 10^4 cells per well. The cells were allowed to attach overnight in the media with phenol red. The following day, the old media was changed to media without phenol red after a quick 1X PBS wash. TBHC dye was added to the wells at increasing concentrations from 0-500 µM and incubated for 45 minutes. The cells were then allowed to recover from the treatment for 48 hours. The wells were washed 3 times with 1X PBS and kept in 100µL PBS. The MTT reagent (12mM) was added to each well (10µL) and incubated for 2 hours at 37 °C. SDS-HCl solution was added later to permeabilize the cells and incubated for 2 hours at 37 °C. The 96-well plate was finally inserted into a plate reader to read absorbance at 570 nm. Absorbance values were recorded and analyzed in Microsoft Excel.

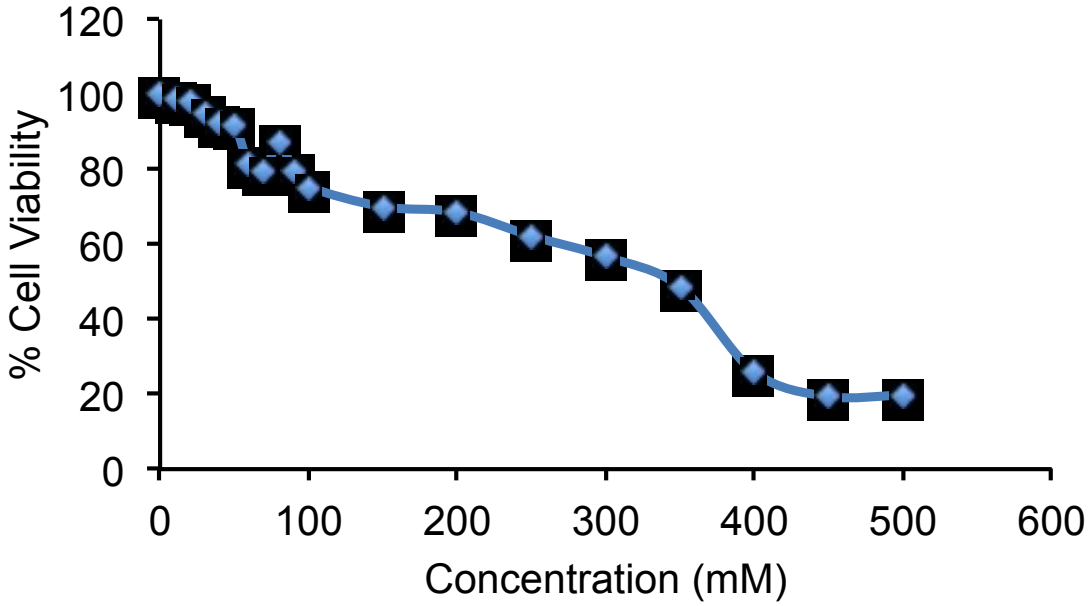


Figure S14: RAW 264.7 viability study with various concentrations of TBHC.

M. Microscopy and quantification

All the imaging studies were done using the Prarie Technologies Swept Field Confocal microscope (CIRM QB3 Shared Stem Cell Facility) with a 63X water immersion lens. The imaging conditions maintained across treatment conditions were: 200-millisecond exposure, 30 µm pinhole, EM Gain of 300, readout frequency of 10 MHz and field of view of 134.7 µm X 134.7 µm. The 561 nm laser was used to excite the TBHC dye and the 643 laser was used for the hydro-Cy5 dye. Images were acquired at various points of the cell culture dish. The images were quantified using Fiji (NIH) as described before.[3] In brief, we take 12 slices of image in the z-stack and subsequently performed maximum projection of 12 slices image. The bar charts in Figures 6f and 6g were generated by drawing a region of interest (ROI) around the cells and determining the average fluorescent intensity in that region. The average intensity from 30 randomly chosen cells from 3 pictures (10 from each picture) was averaged, and is presented in Figures 6f and 6g. Fiji software from the NIH was used to analyze the cells

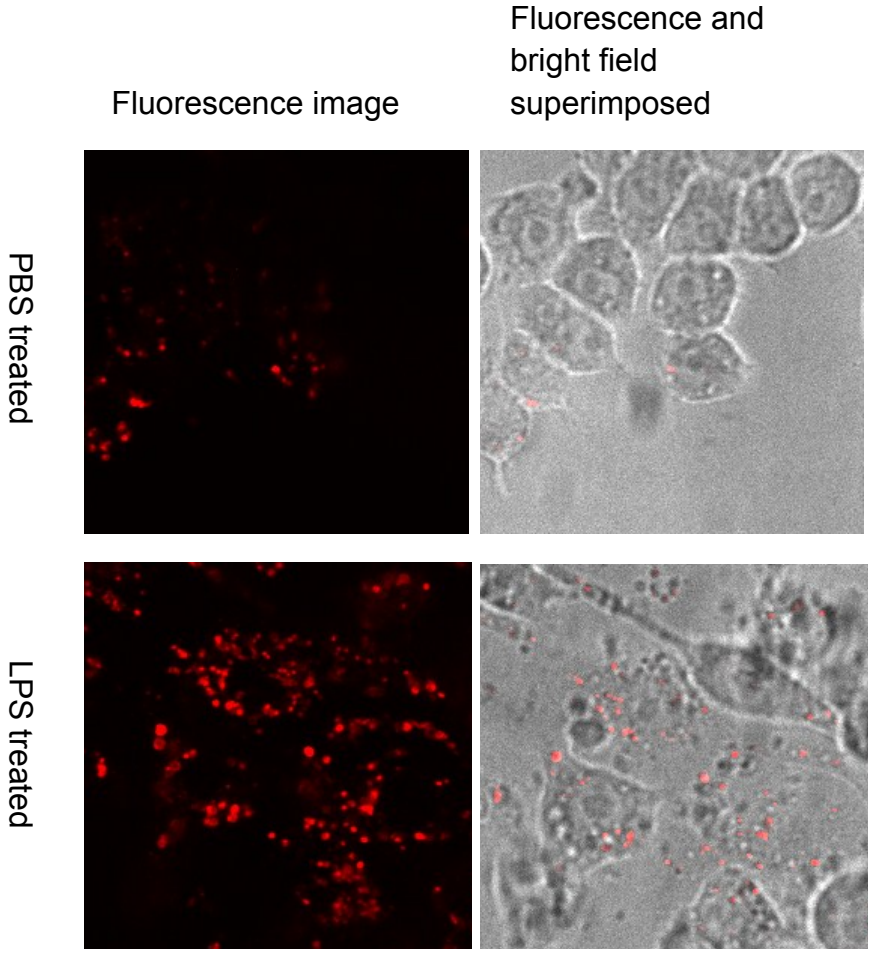


Figure S15. **TBHC can image ROS that produced from LPS stimulated macrophages**. Macrophages were either treated with PBS or LPS and after that it was treated with TBHC (100 µM). A superimposed image of bright field and fluorescence image clearly shows that majority of fluorescence signal is cellular

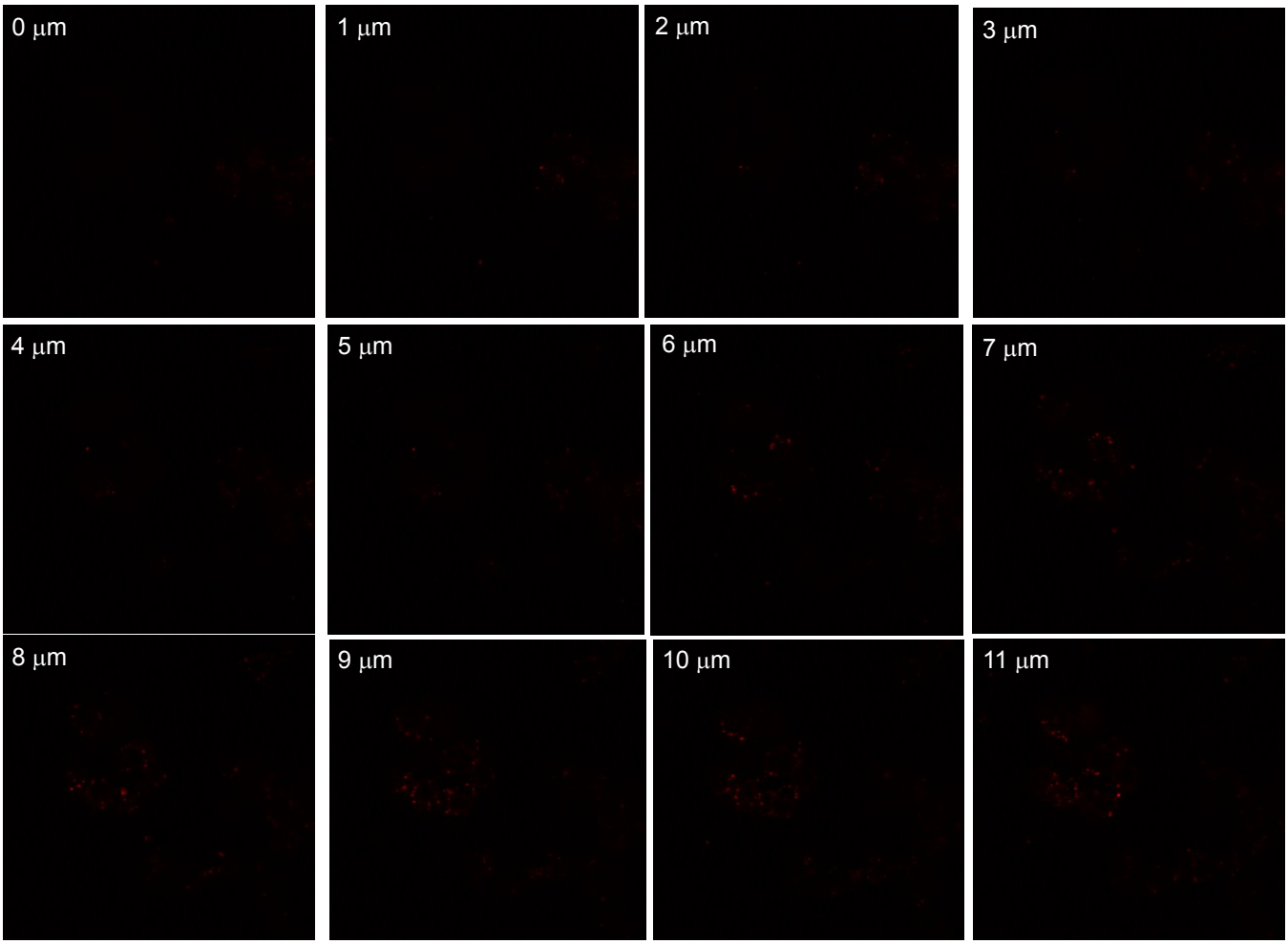


Figure S16: Z-stack of RAW 264.7 cells treated with TBHC (100 μM) + LPS. Raw 264.7 cells were activated by LPS and subsequently treated with TBHC for 45 min. 12 images were taken at different depths within the cells, in the z-stack with a 1 μm interval between images. Fluorescence is observed at multiple depths within the cells, demonstrating that the fluorescence is intracellular.

N. References

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