Supplementary Information

Long-wavelength fluorescent boronate probes for the detection and intracellular cell imaging of peroxynitrite

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Content

1. General information

Cell culture. Hep-G2, HeLa and RAW264.7 cells were maintained in a Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Gland Island, NY, USA), and A549 cells were cultured in Ham's F-12 nutrient mixture supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air at 37 ºC and split when the cells reached 90% confluency.

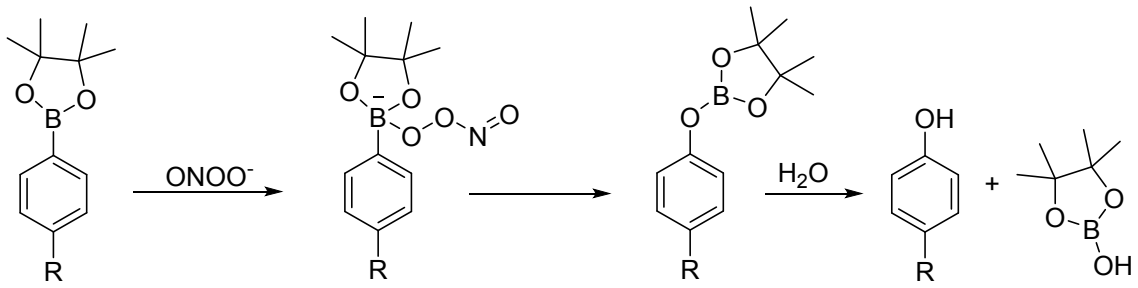
Fluorescence imaging of cells. Cells were seeded on a black 96-well microplate with optically clear bottom (Greiner bio-one, Germany) overnight. To produce $ONOO^-$ exogenously, the cells were first incubated with **TCFB2** (10 μM, 1% DMSO in PBS, pH 7.4) for 30 min, followed by incubation with Sin-1 (500 μM) for 30 min. To quench the exogenously produced $ONOO^-$, the cells were incubated with **TCFB2** (10 μM) for 30 min, then uric acid (100 μM) for 1 h, and finally Sin-1 (500 μM) for 30 min. To simulate endogenous peroxynitrite, lipopolysaccharide (1 μg mL^{-1}) was first added to RAW264.7 cells. After 24 hours, the cells were incubated with **TCFB2** (10 μM) for 30 min. The cell nuclei were stained with Hoechst 33342 (5 μg mL^{-1}) at 37 °C in a humidified atmosphere of 5% CO_2 in air for 5 min. Then, cells were washed with PBS (phosphate buffered saline) three times. The fluorescence images were recorded using an Operetta high-content imaging system (Perkinelmer, US) and quantified and plotted by columbus analysis system (Perkinelmer, US).

Confocal laser scanning microscopy. Cells were incubated sequentially with Mito-Tracker Green (1 μM, 1% DMSO in PBS, pH 7.4) and **TCFB2** (10 μM, 1% DMSO in PBS, pH 7.4) in an atmosphere of 5% CO_2 and 95% air for 40 min at 37 °C. Then the cells on the microplate were rinsed by PBS and fixed by 4% paraformaldehyde for 20 min at room temperature. After rinsing twice with PBS, the cells were stained with Hoechst 33342 (5 μg mL^{-1}) at 37 °C in a humidified atmosphere of 5% CO_2 in air for 5 min. After three rinses with PBS, the fluorescence was detected and photographed with confocal laser scanning microscopy.

Cell viability assay. Cells were plated overnight on 96-well plates in growth medium. After seeding, cells were treated with **TCFB2** of different concentrations for 48 hours. Then, 10 μL per well of MTS/PMS (20:1, Promega Corp) solution was added to each well containing 100 μL of growth medium. After incubation at 37 °C under 5% CO_2 for 2 h, the absorbance of the solutions was measured at 490 nm, using an M5 microplate reader (Molecular Device, USA). The optical density of the result in MTS assay was directly proportional to the number of viable cells. The optical density of the result in MTS assay was directly proportional to the number of viable cells.

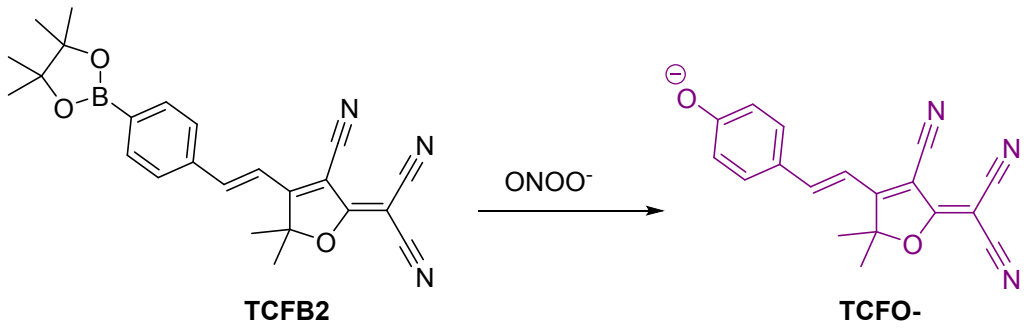
2. Mechanism

The mechanism for the oxidation of aryl boronates by $ONOO^-$ is shown in **Scheme S1**.[1]

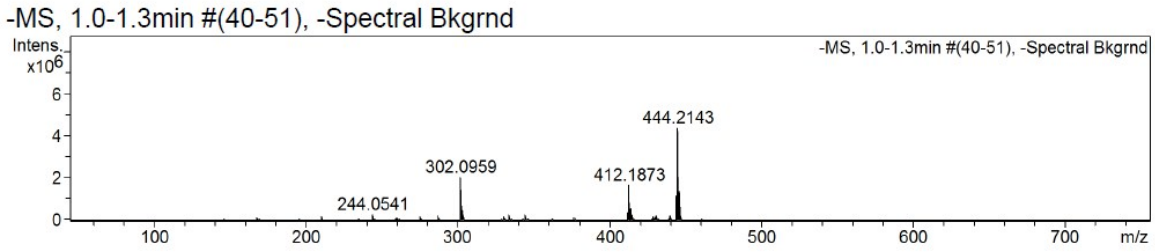


Scheme S1 – Mechanism of the $ONOO^-$ mediated oxidation of an aryl boronate

We confirmed that the addition of $ONOO^-$ to **TCFB2** results in the formation of phenoxide **TCFO-** (**Scheme S2**). The reaction to produce **TCFO-** was confirmed using mass spectrometry. Addition of $ONOO^-$ to a solution of **TCFB2** resulted in the formation of **TCFO-** m/z = 302.0959. Unreacted **TCFB2** was also observed m/z = 413.1910 (**Fig. S1**)



Scheme S2 – $ONOO^-$ mediated oxidation of **TCFB2**



#	m/z	I	I %	Area	S/N
1	244.0541	287202	6.6	12382	1368.0
2	302.0959	2037178	46.7	112064	4689.9
3	303.0982	454010	10.4	24411	1035.0
4	334.1207	264719	6.1	15836	712.2
5	411.1899	380905	8.7	27400	578.3
6	412.1873	1653878	37.9	123283	2426.8
7	413.1910	575728	13.2	40453	816.7
8	443.2164	1151813	26.4	92481	822.3
9	444.2143	4359249	100.0	382102	3062.3
10	445.2157	1357035	31.1	104916	937.8

Generate Molecular Formula Parameters

Charge	Tolerance	SearchRadius	H/C Ratio min.	H/C Ratio max.	Electron Conf.	Nitrogen Rule	sigma limit
negative	20 ppm	0.05 m/z	0	3	both	true	0.05

Expected Formula C18 H13 N3 O2 **Adduct(s):** H, Na

#	meas. m/z	theo. m/z	Err[ppm]	Sigma	Formula
1	302.0959	302.0935	7.90	0.0439	C 18 H 12 N 3 O 2

Fig. S1 – Mass spec of a sample of TCFB2 after addition of $ONOO^-$

3. Fluorescence analysis

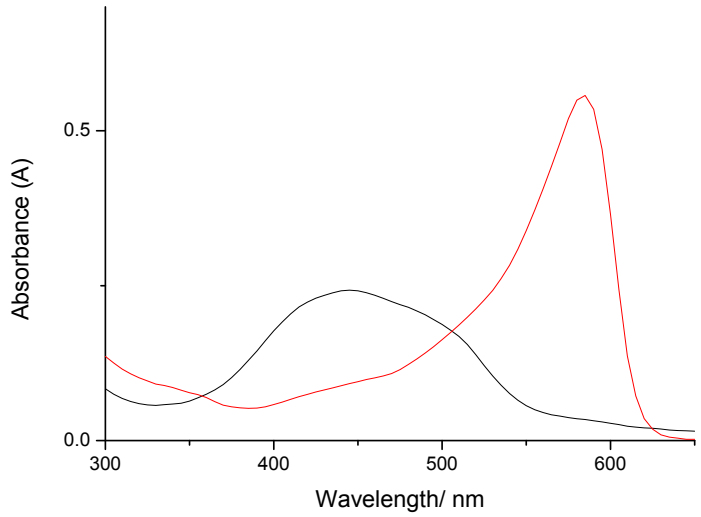


Fig. S2 – UV-Vis spectra of TCF B1 (10 µM) with (red line) and without $ONOO^-$ (black line) in PBS buffer 20 % DMSO pH = 8.00

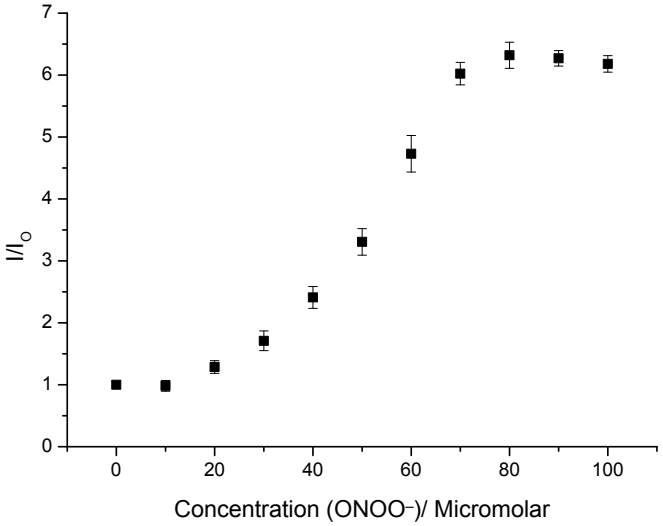


Fig. S3 - Fluorescence intensity changes (I/I_O) for **TCFB1** (10 μM) with addition of $ONOO^-$ (0 – 100 μM) in PBS buffer 20 % DMSO, pH = 8.00. λex = 560 nm/ λem = 606. Slit widths ex = 10 nm and em = 15 nm

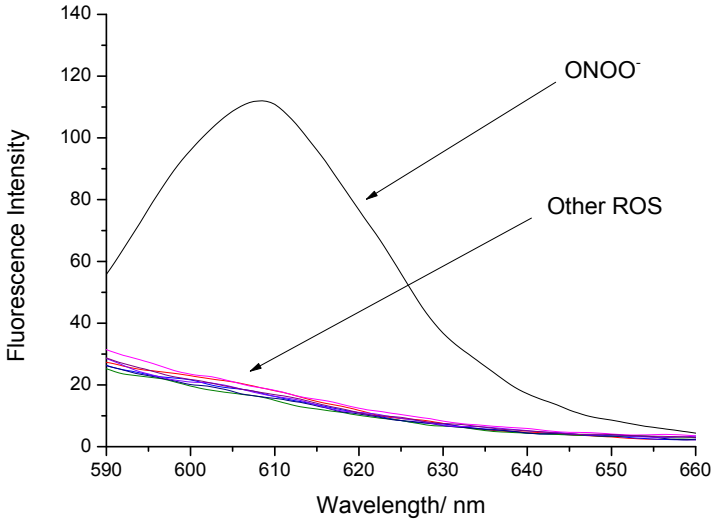


Fig. S4 – Fluorescence spectra of **TCFB1** (10 μM) in the presence of various ROS/RNS: $ONOO^-$ (100 μM, 1 min), ^-OCl (100 μM, 30 min), H_2O_2 (100 μM, 30 min), $ROO\cdot$ (100 μM, 30 min), $^-O_2$ (100 μM, 1 min), $\cdot OH$ (100 μM, 1 min) λex 560 nm in PBS buffer 20 % DMSO, pH = 8.00, Slit widths ex = 10 nm and em = 15 nm

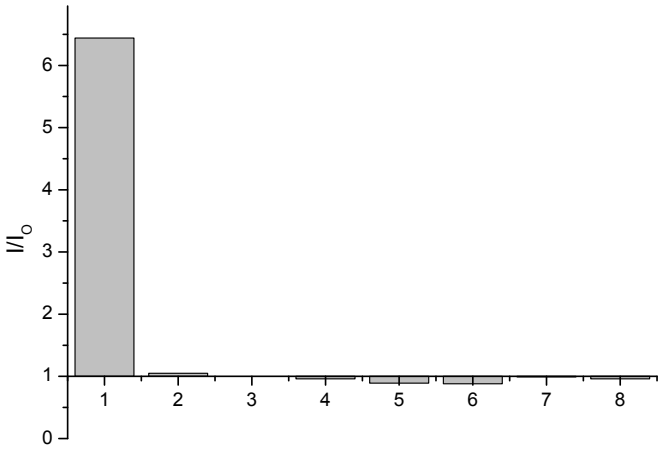


Fig. S5 - Selectivity bar chart of **TCFB1** (10 µM), 100 µM for ROS/RNS – (1 – $ONOO^-$, 2 – H_2O_2, 3 – ClO^-, 4 – KO_2, 5 – $^1O_2$, 6 – $ROO^\cdot$, 7 – $HO^\cdot$). Concentration of each ROS/RNS: $ONOO^-$ (10 µM, 1 min), ^-OCl (100 µM, 30 min), H_2O_2 (100 µM, 30 min), $ROO^\cdot$ (100 µM, 30 min), $^-O_2$ (100 µM, 1 min), $^\cdot OH$ (100 µM, 1 min). λ_{ex} 560 nm/ λem 606 nm in PBS buffer solution, 20 % DMSO at pH = 8.00. Slit widths ex = 10 nm and em = 15 nm

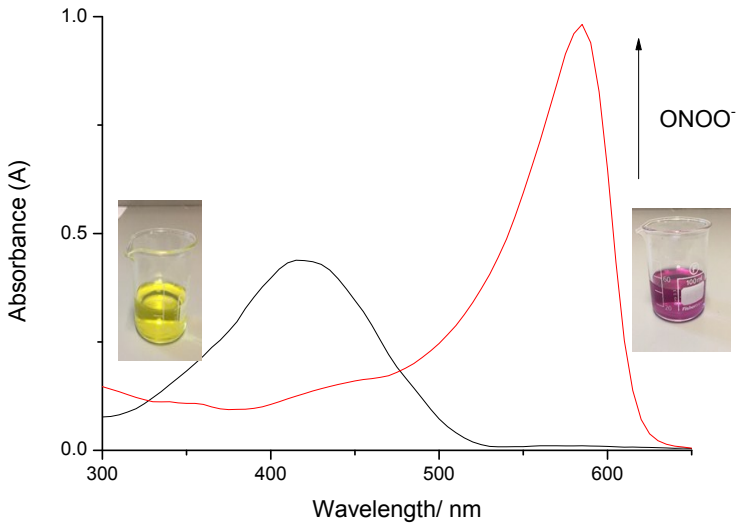


Fig. S6 - UV-Vis spectra of **TCF B2** (10 µM) with (red line) and without $ONOO^-$ (Black line) in PBS buffer solution, 20 % DMSO at pH = 8.00

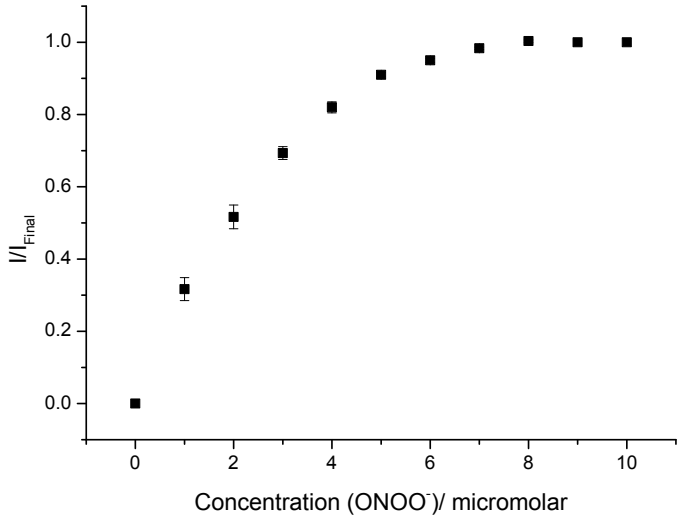


Fig. S7 - Fluorescence intensity changes (I/I_{Final}) for **TCFB2** (10 μM) with addition of $ONOO^-$ (0 – 10 μM) in PBS buffer solution, 20 % DMSO, pH = 8.00. λ_{ex} = 560 nm/ λ_{em} = 606. Slit widths ex = 10 nm and em = 15 nm

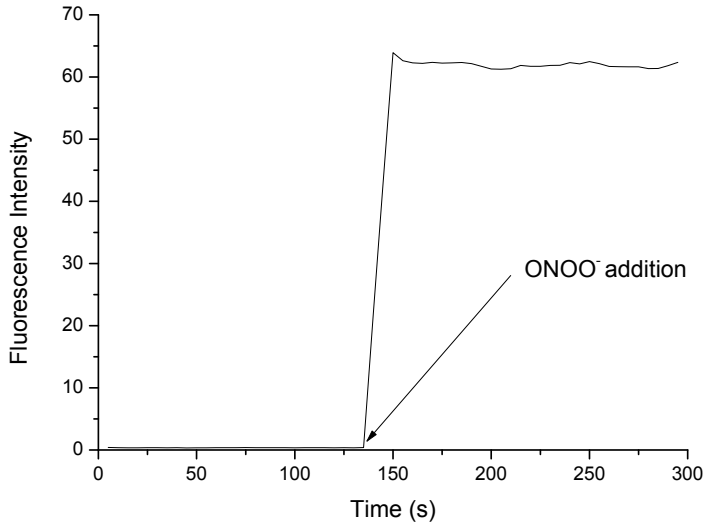


Fig. S8 - Fluorescence intensity versus time for **TCFB2** (10 μM) with addition of $ONOO^-$ (5 μM) in PBS buffer solution, 20 % DMSO, pH = 8.00. λ_{ex} = 560 nm/ λ_{em} = 606. Slit widths ex = 10 nm and em = 15 nm.

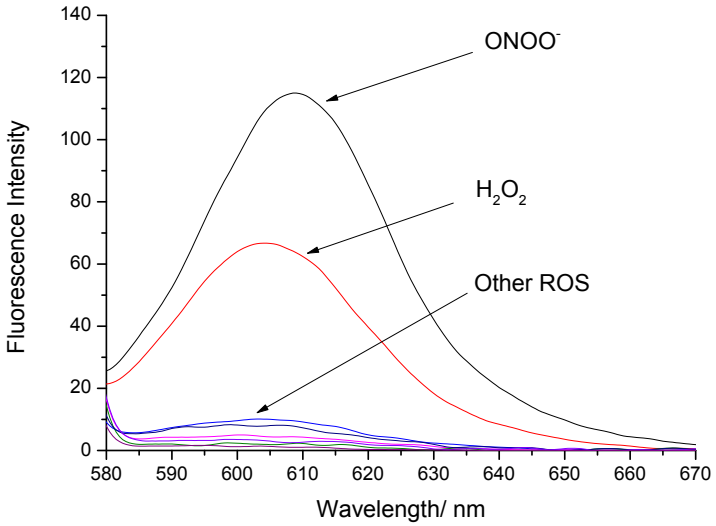


Fig. S9 - Fluorescence spectra of **TCFB2** (10 µM) in the presence of various ROS/RNS: $ONOO^-$ (10 µM, 1 min), ^-OCl (100 µM, 30 min), H_2O_2 (100 µM, 30 min), $ROO^\cdot$ (100 µM, 30 min), $^-O_2$ (100 µM, 1 min), $^\cdot OH$ (100 µM, 1 min) λ_{ex} 560 nm in PBS buffer 20 % DMSO, pH = 8.00, Slit widths ex = 10 nm and em = 15 nm

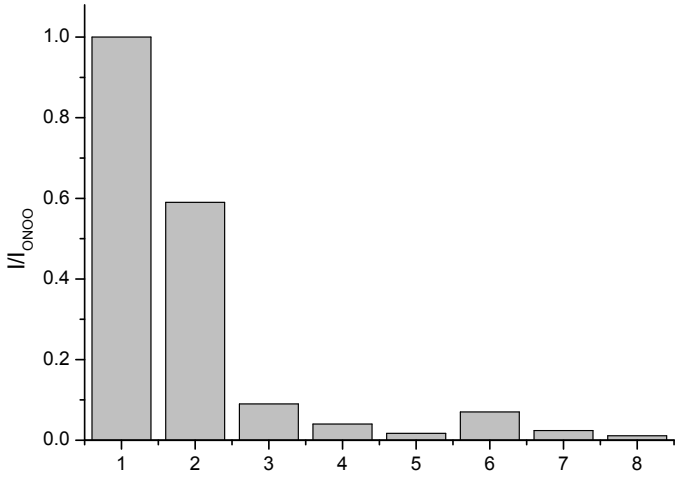


Fig. S10 - Selectivity bar chart of **TCFB2** (10 µM) – (1 – $ONOO^-$, 2 – H_2O_2, 3 – ClO^-, 4 – KO_2, 5 – $^1O_2$, 6 – $ROO^\cdot$, 7 – $HO^\cdot$). Concentration of each ROS/ RNS: $ONOO^-$ (10 µM, 1 min), ^-OCl (100 µM, 30 min), H_2O_2 (100 µM, 30 min), $ROO^\cdot$ (100 µM, 30 min), $^-O_2$ (100 µM, 1 min), $^\cdot OH$ (100 µM, 1 min). λex =560 nm/ λem 606 nm in PBS buffer 20 % DMSO pH = 8.00. Slit widths ex = 10 nm and em = 15 nm

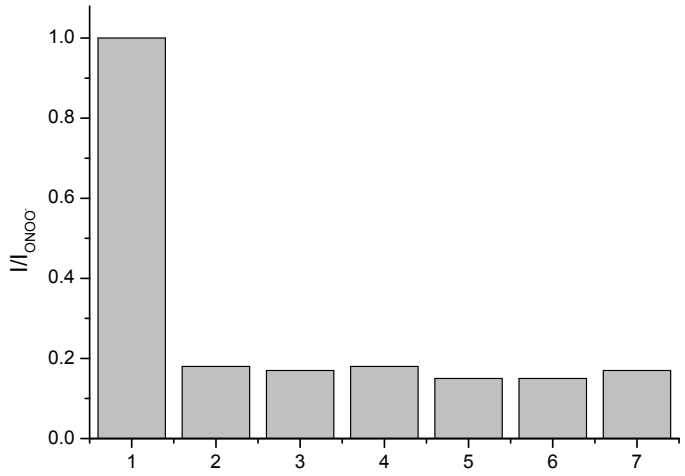


Fig. S11 - Selectivity bar chart of **TCFB1** (10 μM) – (1 – $ONOO^-$, 2 – H_2O_2, 3 – ClO^-, 4 – KO_2, 5 – $^1O_2$, 6 – $ROO^\cdot$, 7 – $HO^\cdot$) Concentration of each ROS/ RNS: $ONOO^-$ (100 μM, 1 min), ^-OCl (100 μM, 30 min), H_2O_2 (100 μM, 30 min), $ROO^\cdot$ (100 μM, 30 min), $^-O_2$ (100 μM, 1 min), $^\cdot OH$ (100 μM, 1 min). λex =560 nm/ λem 606 nm in PBS buffer 20 % DMSO pH = 8.00. Slit widths ex = 10 nm and em = 15 nm

Surprisingly, no fluorescence change was observed for the addition of hypochlorite (ClO^-) to **TCFB1** and **TCFB2** in PBS buffer with 20 % DMSO at pH = 8.00 (Fig. S8 and S9), this observation can be ascribed to the deactivation of hypochlorite (ClO^-) by DMSO in the buffer solution. Therefore, we carried out the titration of **TCFB2** with hypochlorite ClO^- (100 μM) in 100 % PBS buffer solution at pH 7.4. Reassuringly, under these conditions a fluorescence response with **TCFB2** was observed, clearly demonstrating that the reaction between **TCFB2** and ClO^- is affected by DMSO in the buffer.

[NB Titration of **TCFB1** in 100 % PBS buffer solution at pH 7.4 was not carried out due to the low solubility]

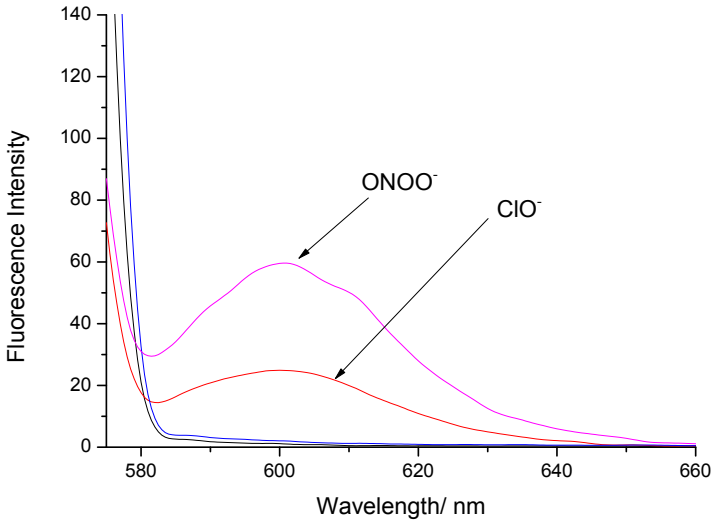


Fig. S12 - Fluorescence spectra of **TCFB2** (10 μM) with addition of ClO^- (100 μM) and $ONOO^-$ (10 μM) in PBS buffer solution pH = 7.4. λ_{ex} = 560 nm. Slit widths ex = 10 nm and em = 15 nm

4. Quantum yield Φ

Table 1 – Quantum Yields for TCB1, TCB2 and TCFO-

	Φ
TCB1[a]	3.02×10^{-4}
TCFB2[b]	1.83×10^{-5}
TCFO-[c]	2×10^{-3}

(a) The quantum yield of **TCFB1** was estimated by dividing **ΦTCFO-** by the 6.62-fold intensity increase for **TCFB1** at 614 nm for the addition of $ONOO^-$ (100 μM). (b) The quantum yield of **TCFB2** was estimated by dividing **ΦTCFO$^-$** by the 109-fold intensity increase for **TCFB2** at 614 nm for the addition of $ONOO^-$ (10 μM). (c) The quantum yield of **TCFO-** (Φ**TCFO-)** was previously reported.[2]

5. Cell viability and lysosome colocalisation

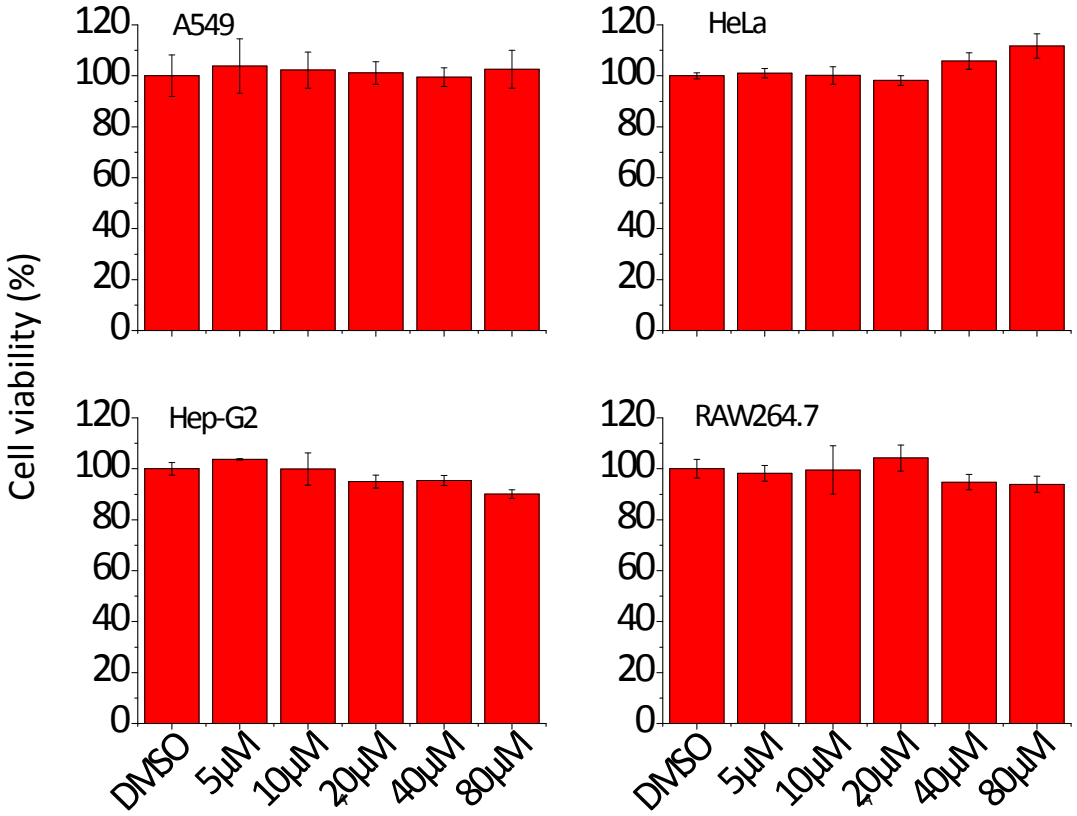


Figure S13. Viability of different cells upon incubation with different concentrations of **TCFB2**, as determined by MTS cell proliferation assay.

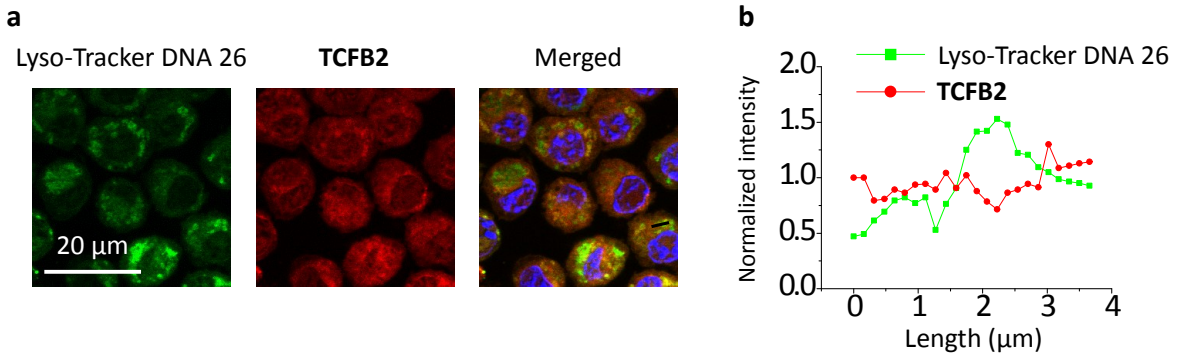
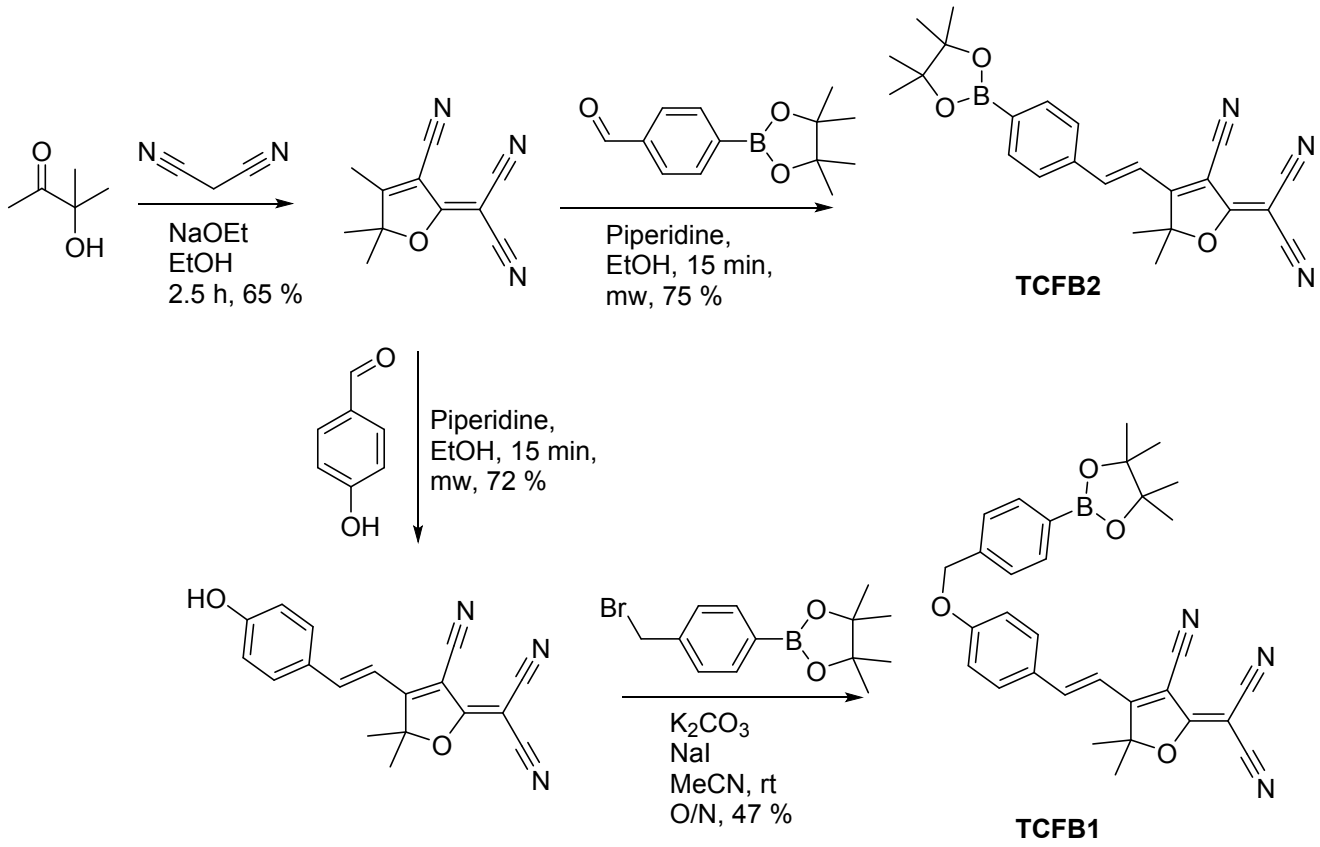
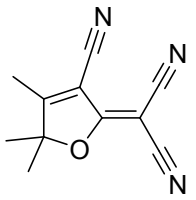


Figure S14. (a) Fluorescence co-localisation of **TCFB2** (10 μM) with Lyso-tracker DNA 26 (1 μM) in RAW264.7 cells (scale bar = 20 μm). (b) Fluorescence quantification of **TCFB2** and Lyso-tracker DNA 26 of a selected section (the black line in "Merged" panel) of a RAW264.7 cell. Excitation wavelength for Lyso-tracker DNA 26 and **TCFB2** is 489 and 579 nm, respectively. Emission wavelength for Lyso-tracker DNA 26 and **TCFB2** is 506 and 603 nm, respectively. The cell nuclei were stained by Hoechst 33342.

6. Experimental

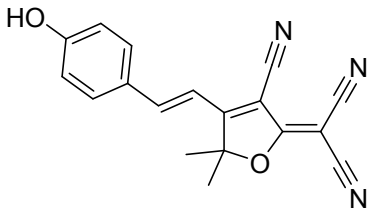


2-(3-cyano-4,5,5-trimethylfuran-2(5H)-ylidene)malononitrile



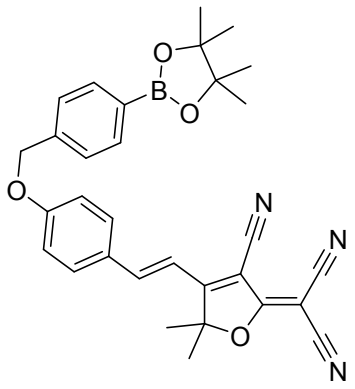
NaOEt (0.391 g, 5.75 mmol) was added to a solution of 3-hydroxy-3-methyl-2-butanone (4 mL, 38 mmol) and malonitrile (4.9 g, 74 mmol) in EtOH (10 mL) and stirred for 1.5 h. The reaction mixture was then refluxed for 1 h, which was then cooled to rt. The mixture was cooled and the solid precipitate was filtered to afford the title compound as a pale grey solid (4.92 g, 24.70 mmol, 65 %); M.p. 204 – 208 ^{o}C (decomp). ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.37 (s, 3 H), 1.64 (s, 6 H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 182.6, 175.2, 111.1, 110.4, 109.0, 104.8, 99.8, 58.5, 24.4, 14.2; I.R (thinfilm) ν max (cm^{-1}): 2232.78, 2222.00 (CN); HRMS (FTMS-NSI): m/z calculated for $C_{11}H_9N_3O$: requires 200.0108 for $[M+H]^+$, found 200.0108.

(E)-2-(3-cyano-4-(4-hydroxystyryl)-5,5-dimethylfuran-2(5H)-ylidene)malononitrile



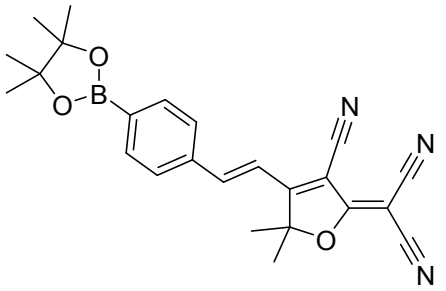
Two drops of Piperidine were added to a mixture of 4-hydroxybenzaldehyde (0.122 g, 1 mmol) and TCF (0.228 g, 1.15 mmol) in EtOH (10 mL). The reaction mixture was heated in the microwave for 15 min at 100 ^{o}C, which was then cooled to rt. The solid precipitate was filtered off to afford the title compound as an orange solid (0.218 g, 0.72 mmol, 72 %) M.p. 202 – 206 ^{o}C (decomp). ^{1}H NMR (300MHz, DMSO-d_6) 7.95 - 7.73 (m, 3 H), 7.01 (d, J = 16.2 Hz, 1 H), 6.89 (d, J = 8.7 Hz, 2 H), 1.77 (s, 6 H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ 177.6, 176.2, 162.7, 148.7, 132.7, 126.0, 116.8, 113.3, 112.5, 112.0, 111.6, 99.4, 96.9, 53.5, 25.7; I.R (thinfilm) ν max (cm^{-1}): 3361.61 (O-H), 2224.73 (CN); HRMS (FTMS-NSI): m/z calculateed for $C_{18}H_{13}N_3O_2$: requires 304.1081 for $[M+H]^+$, found 304.1084.

(E)-2-(3-cyano-5,5-dimethyl-4-(4-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)oxy)styryl)furan-2(5H)-ylidene)malononitrile



(E)-2-(3-cyano-4-(4-hydroxystyryl)-5,5-dimethylfuran-2(5H)-ylidene)malononitrile (0.090 g, 0.297 mmol), 2-(4-(bromomethyl)phenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (0.088 g, 0.297 mmol), K_2CO_3 (0.123 g, 0.89 mmol) and NaI (0.044 g, 0.297 mmol) in MeCN (5 mL) was stirred overnight at rt. The reaction mixture was partitioned with EtOAc (50 mL) and H_2O (50 mL). The organic layer was washed with H_2O (2 x 50 mL), Brine (50 mL) and dried ($MgSO_4$). The solvent was removed *in-vacuo* and the crude material was triturated (MeOH) to afford the title compound as a red solid (0.073 g, 0.14 mmol, 47 %) M.p. 267 – 269 ^oC. ^1H NMR (500MHz, $CDCl_3$) δ 7.88 - 7.82 (d, *J* = 8.3 Hz, 2 H), 7.67 - 7.57 (m, 3 H), 7.48 - 7.40 (d, *J* = 8.3 Hz, 2 H), 7.05 (d, *J* = 8.8 Hz, 2 H), 6.90 (d, *J* = 16.1 Hz, 1 H), 5.19 (s, 2 H), 1.79 (s, 6 H), 1.36 (s, 12 H); ^{13}C NMR (125.5 MHz, $CDCl_3$) δ 175.5, 174.0, 162.8, 147.2, 138.8, 135.2, 131.3, 126.9, 126.5, 116.0, 112.5, 111.8, 111.0, 110.4, 97.4, 83.9, 70.3, 26.5, 24.8; I.R (thinfilm) ν max (cm^{-1}): 2227.39 (CN); HRMS (FTMS-NSI): m/z calculated for $C_{31}H_{30}BN_3O_4$: requires 536.2704 for $[M+NH_4]^+$, found 536.2699

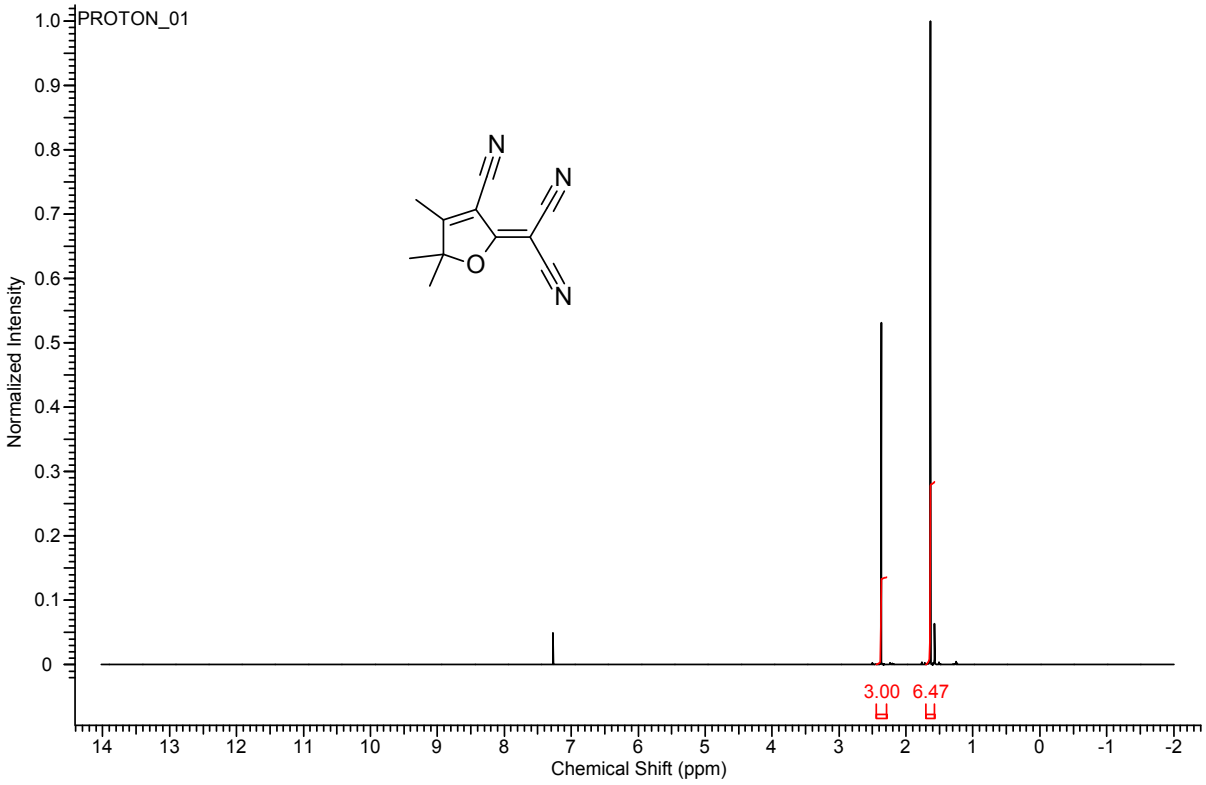
(E)-2-(3-cyano-5,5-dimethyl-4-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)styryl)furan-2(5H)-ylidene)malononitrile



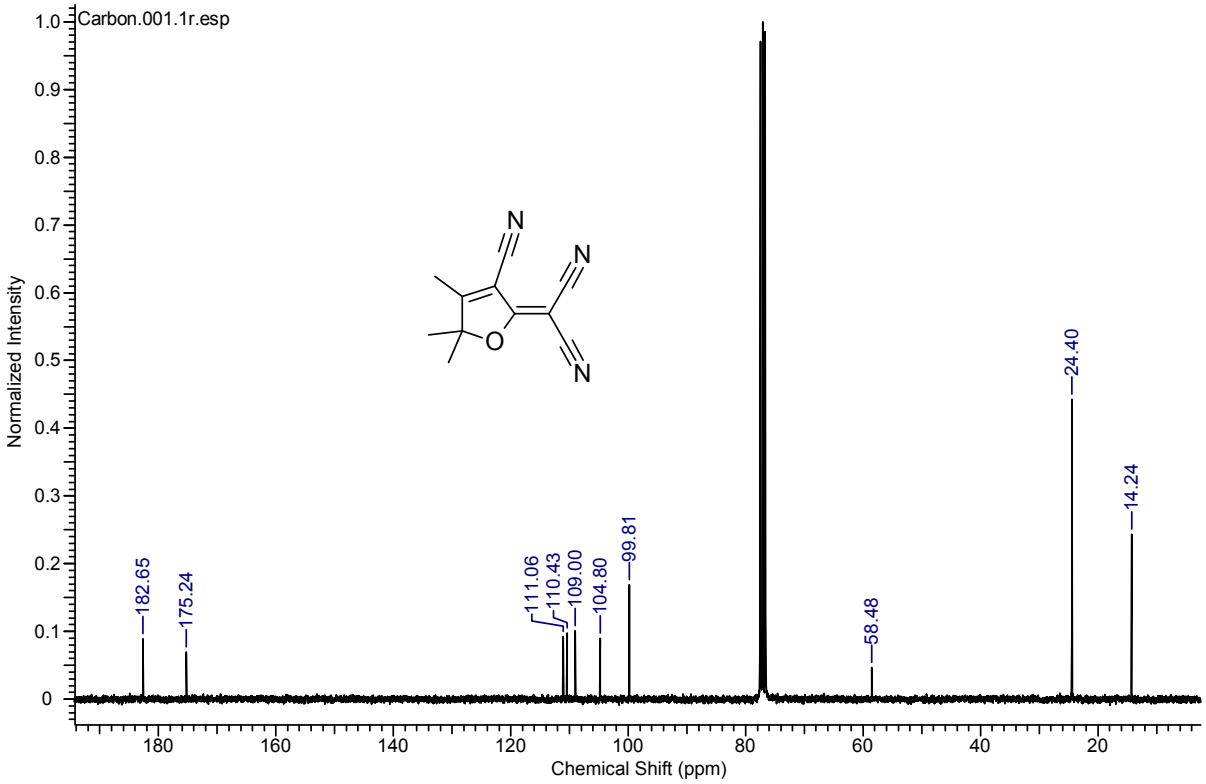
Two drops of Piperidine was added to a mixture of 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzaldehyde (0.232 g, 1 mmol) and TCF (0.288 g, 1.15 mmol) in EtOH (10 mL). The reaction mixture was heated in the microwave at 100 ^{o}C for 15 min. The reaction mixture was cooled and the solid was filtered off to afford the title compound as an orange solid (0.310 g, 0.75 mmol, 75 %) M.p. 275 – 278 ^{o}C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.90 (d, *J* = 8.1 Hz, 2 H), 7.71 - 7.59 (m, 3 H), 7.11 (d, *J* = 16.4 Hz, 1 H), 1.82 (s, 6 H), 1.37 (s, 12 H); ^{13}C NMR (75.5 MHz, $CDCl_3$-d) δ 175.2, 173.7, 147.3, 135.9, 135.7, 128.1, 115.6, 111.6, 110.8, 110.1, 100.6, 97.9, 84.4, 58.2, 26.5, 24.9; I.R (thinfilm) ν max (cm^{-1}): 2231.63 (CN); HRMS (FTMS-NSI): m/z calculated for $C_{24}H_{24}BN_3O_3$: requires 430.2285 for $[M+NH_4]^+$, found 430.2287.

7. NMR

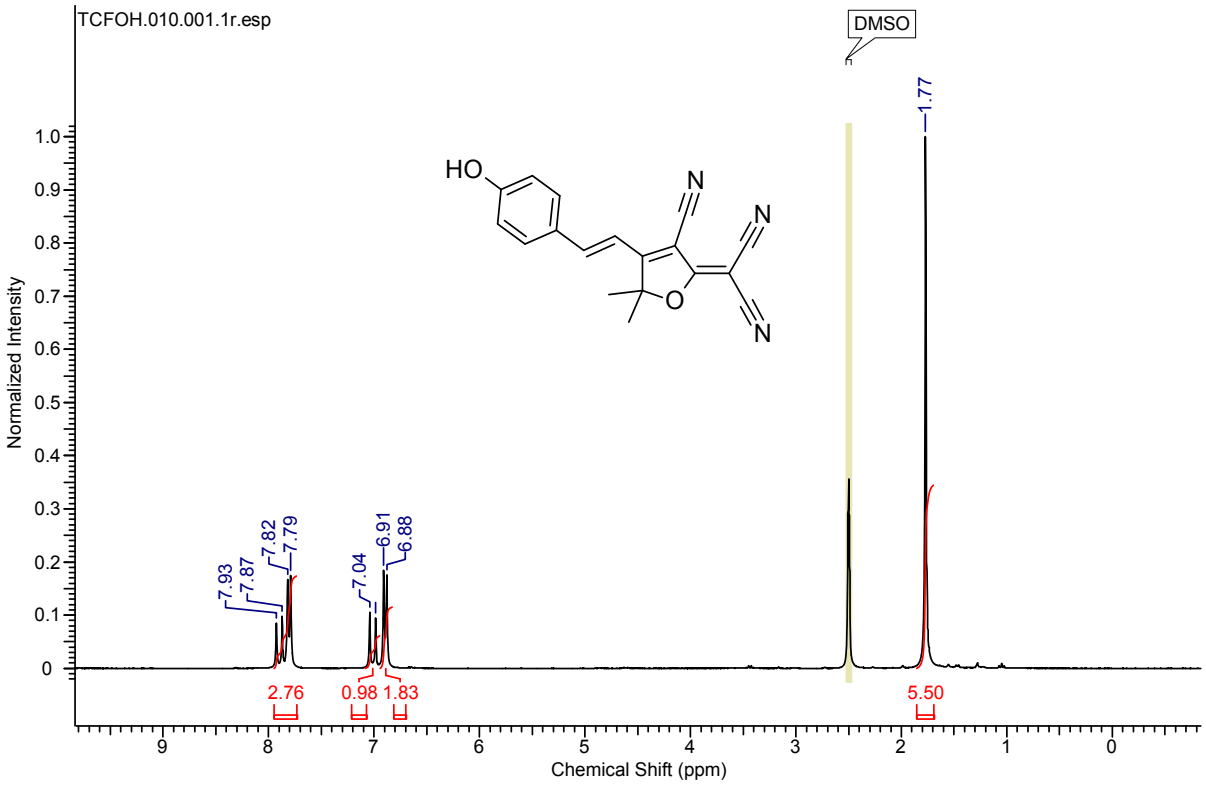
2-(3-cyano-4,5,5-trimethylfuran-2(5H)-ylidene)malononitrile (500 MHz, $CDCl_3$)



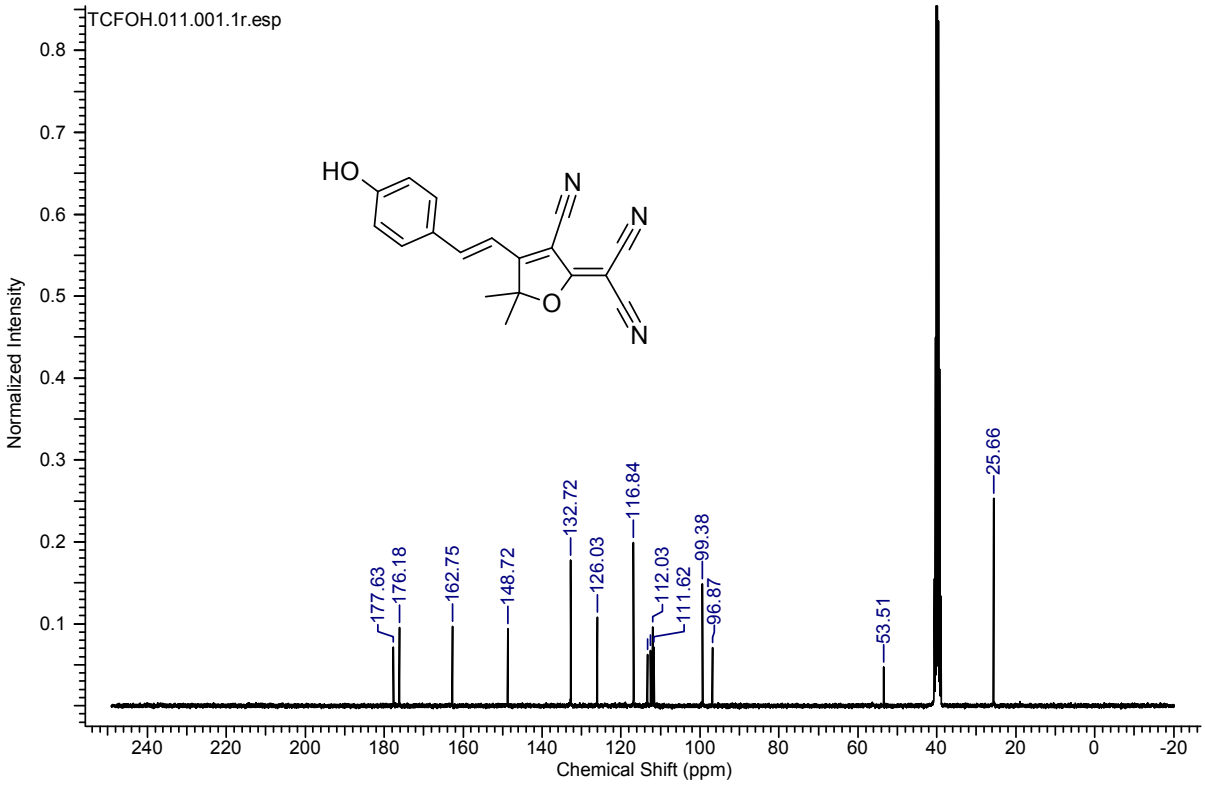
2-(3-cyano-4,5,5-trimethylfuran-2(5H)-ylidene)malononitrile (75.5 MHz, $CDCl_3$)



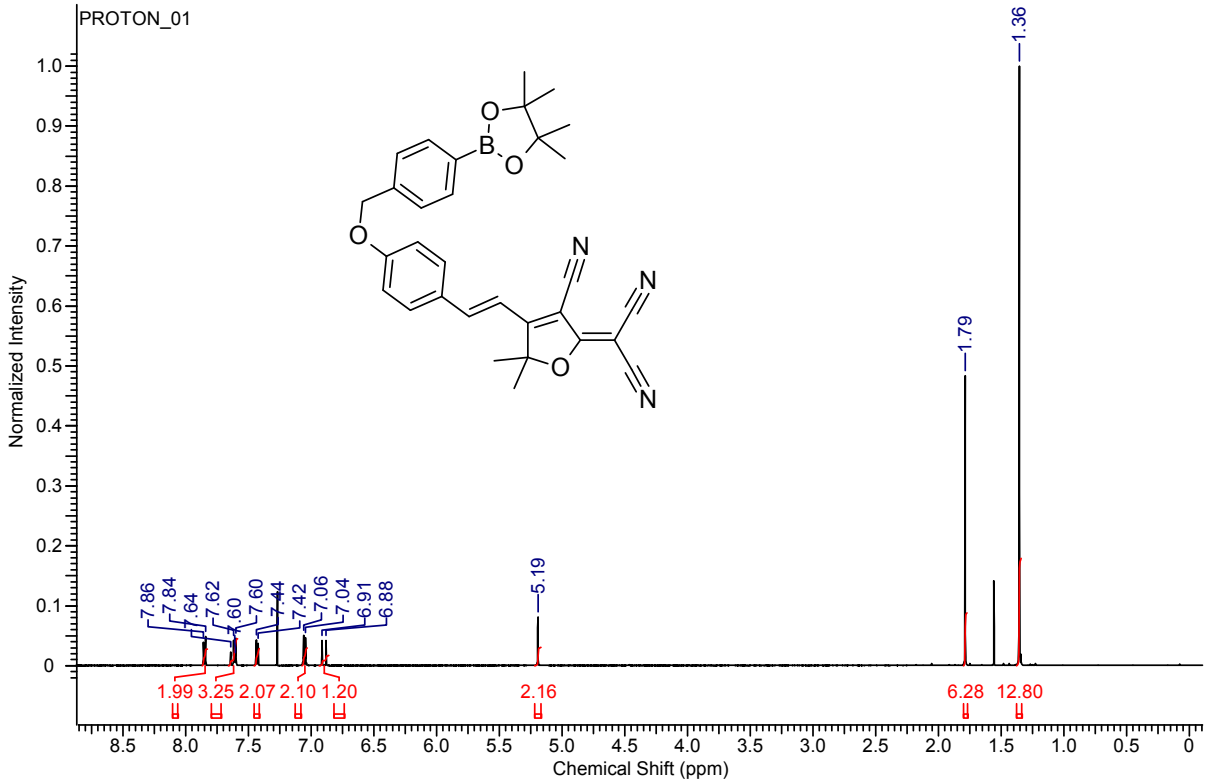
(E)-2-(3-cyano-4-(4-hydroxystyryl)-5,5-dimethylfuran-2(5H)-ylidene)malononitrile (300 MHz, $CDCl_3$)



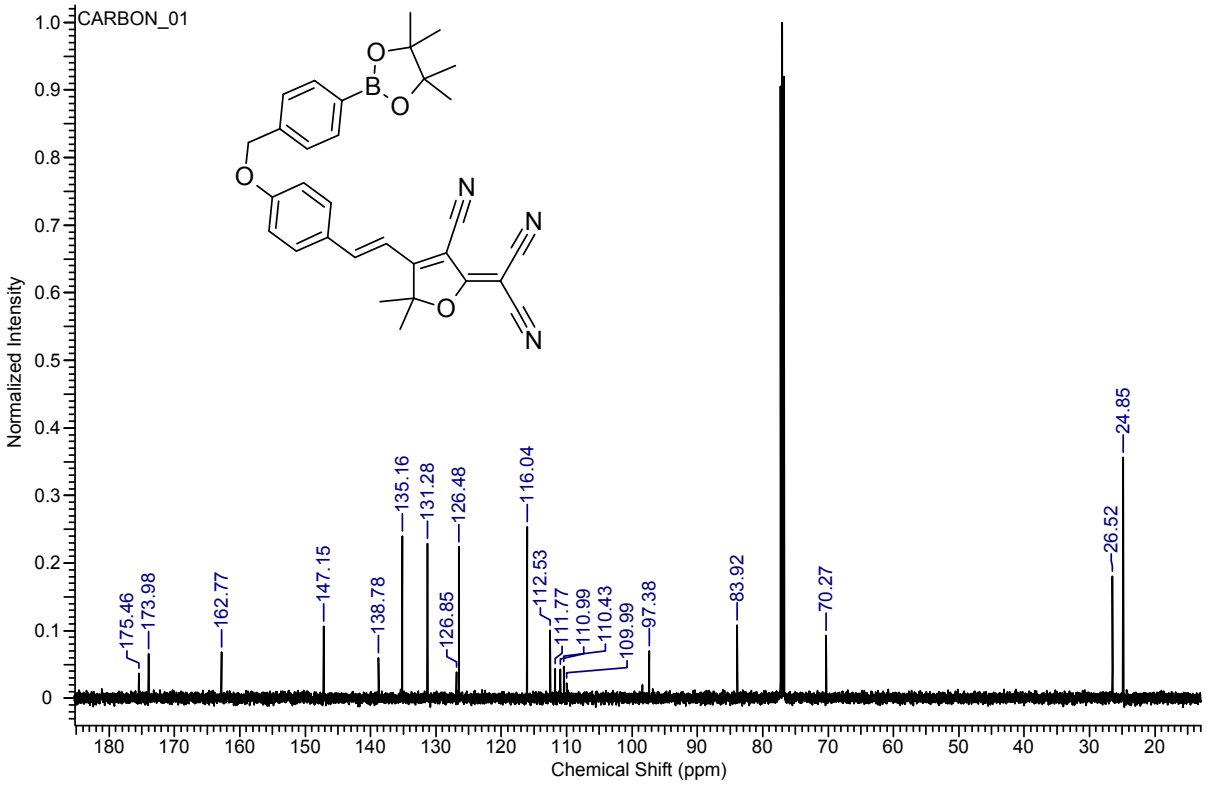
(E)-2-(3-cyano-4-(4-hydroxystyryl)-5,5-dimethylfuran-2(5H)-ylidene)malononitrile (75.5 MHz, $CDCl_3$)



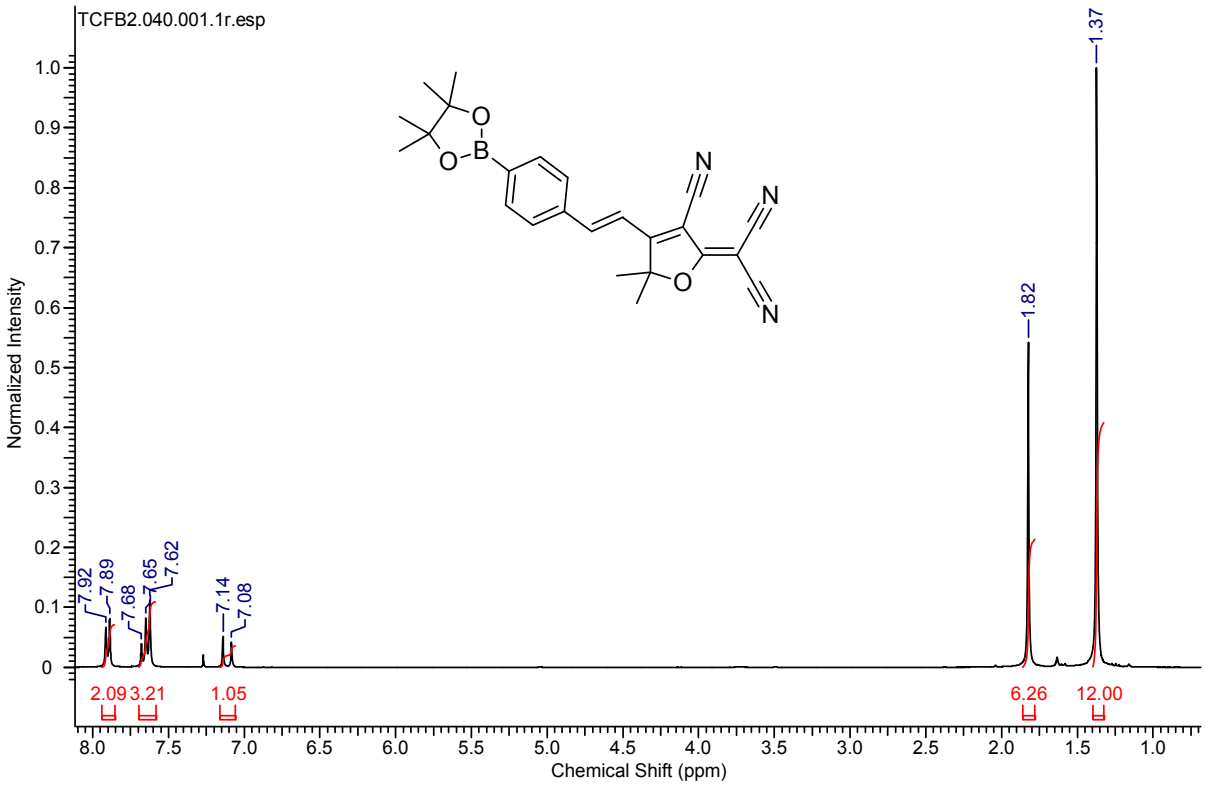
(E)-2-(3-cyano-5,5-dimethyl-4-(4-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)oxy)styryl)furan-2(5H)-ylidene)malononitrile (500 MHz, $CDCl_3$)



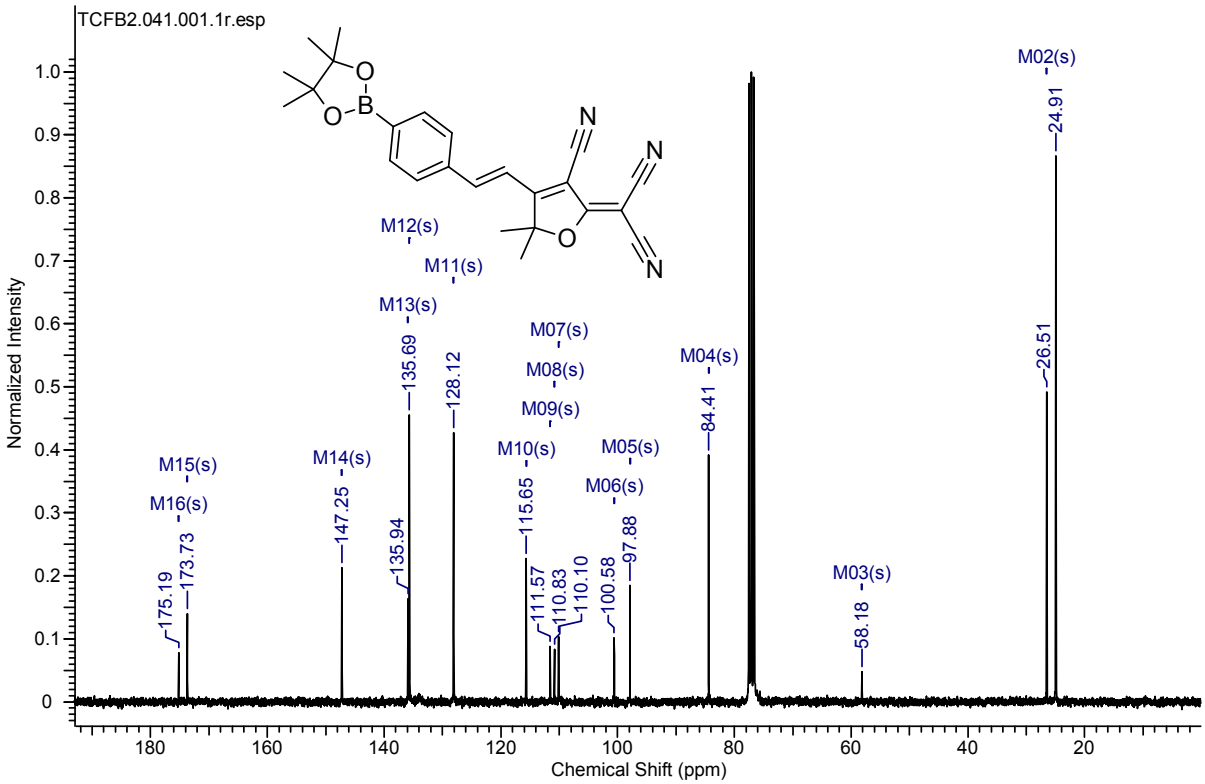
(E)-2-(3-cyano-5,5-dimethyl-4-(4-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)oxy)styryl)furan-2(5H)-ylidene)malononitrile (125.5 MHz, $CDCl_3$)



(E)-2-(3-cyano-5,5-dimethyl-4-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)styryl)furan-2(5H)-ylidene)malononitrile (300 MHz, $CDCl_3$)



(E)-2-(3-cyano-5,5-dimethyl-4-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)styryl)furan-2(5H)-ylidene)malononitrile (75.5 MHz, $CDCl_3$)



8. References

(1) Sikora, A.; Zielonka, J.; Lopez, M.; Joseph, J.; Kalyanaraman, B. Direct oxidation of boronates by peroxynitrite: Mechanism and implications in fluorescence imaging of peroxynitrite. *Free Radic. Biol. Medic.,* **2009,** *47* (10), 1401.

(2) Ipuy, M.; Billon, C.; Micouin, G.; Samarut, J.; Andraud, C.; Bretonniere, Y. Fluorescent push-pull pH-responsive probes for ratiometric detection of intracellular pH. *Org. Biomol. Chem.,* **2014,** *12* (22), 3641.