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Supplementary Information for

A Tandem Activity-Based Sensing and Labeling Strategy Enables Imaging of Transcellular Hydrogen Peroxide Signaling

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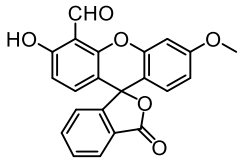
This PDF includes:

Synthesis

PF2. Synthesis of **PF2** was synthesized according to literature procedures[1].

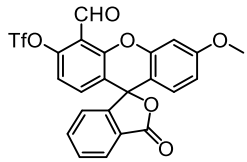
Compound 1. Compound **1** was synthesized according to literature procedures[2].

Compound 2



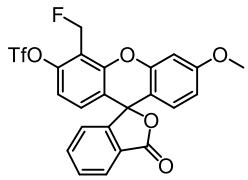
To a stirred solution of compound **1** (2.4 g, 6.9 mmol) in trifluoroacetic acid (70 mL) was added hexamethylenetetramine (1.0 g, 7.6 mmol, 1.1 equiv). The mixture was stirred at 90 °C for 16 h and then H_2O (70 mL) was added to the mixture. The resulting mixture was stirred at 95 °C for another 1 h, and then cooled to room temperature. The mixture was extracted with CH_2Cl_2 three times, dried over Na_2SO_4, and evaporated. The crude product was purified by silica-gel column chromatography (eluent: CH_2Cl_2/MeOH = 100/0 to 100/1) to yield 900 mg (34%) of **2** as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.17 (s, 1H), 10.68 (s, 1H), 8.10 – 7.97 (m, 1H), 7.67 (ddd, *J* = 8.8, 7.2, 1.3 Hz, 2H), 7.21 – 7.12 (m, 1H), 6.91 (d, *J* = 8.9 Hz, 1H), 6.86 – 6.80 (m, 1H), 6.80 – 6.49 (m, 3H), 3.89 – 3.80 (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 193.38, 169.20, 164.56, 161.65, 153.26, 152.66, 151.40, 137.15, 135.42, 130.20, 129.27, 126.80, 125.36, 123.93, 113.92, 112.78, 111.12, 109.81, 109.01, 101.05, 82.09, 55.81. LRMS: calcd for $[M+H]^+$, 375.0; found 375.0.

Compound 3



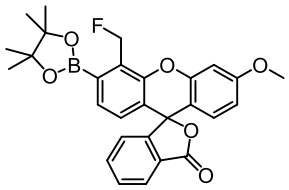
A mixture of compound **2** (900 mg, 2.4 mmol), *N*-phenyl-bis(trifluoromethanesulfonimide) (1.28 g, 3.6 mmol, 1.5 equiv) and Cs_2CO_3 (1.56 g, 4.8 mmol, 2.0 equiv) in MeCN (30 mL) was stirred at ambient temperature for 1 h. After the filtration, the filtrate was evaporated under the reduced pressure. The resulting residue was purified by silica-gel column chromatography with CH_2Cl_2 eluent to yield 1.2 g (98%) of **3** as a pale yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.79 (d, *J* = 0.7 Hz, 1H), 8.11 – 8.02 (m, 1H), 7.80 – 7.63 (m, 2H), 7.20 (dt, *J* = 7.6, 0.9 Hz, 1H), 7.13 (d, *J* = 8.8 Hz, 1H), 7.00 (d, *J* = 8.8 Hz, 1H), 6.88 (dd, *J* = 2.2, 0.7 Hz, 1H), 6.76 – 6.72 (m, 2H), 3.87 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 185.57, 168.87, 162.08, 153.68, 152.27, 151.27, 148.34, 135.80, 134.87, 130.65, 129.73, 129.05, 127.52, 126.22, 125.70, 123.95, 123.64, 121.41, 117.78, 117.32, 113.54, 110.32, 101.19, 80.97, 77.48, 77.36, 77.16, 76.84, 55.93; ^{19}F NMR (376 MHz, $CDCl_3$) δ -72.38. LRMS: calcd for $[M]^+$, 506.0; found 506.9.

Compound 4



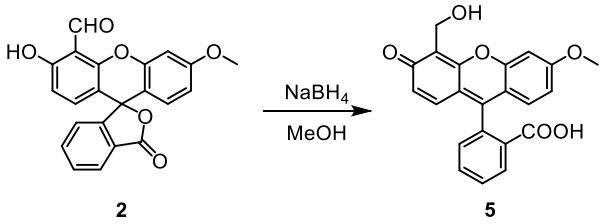
To a solution of compound **3** (50 mg, 0.098 mmol) in THF-MeOH (2 mL/2 mL) was added AcOH (6 mL, 0.098 mmol, 1.0 equiv) and $NaBH(OAc)_3$ (25 mg, 0.118 mmol, 1.2 equiv). The mixture was stirred for 1 h at ambient temperature under N_2 atmosphere, diluted with sat. NH_4Cl aq., and then extracted with EtOAc three times. The combined organic layer was dried over Na_2SO_4, and concentrated. The crude product was dissolved in CH_2Cl_2 (10 mL) and cooled to -20 °C. (Dimethylamino)sulfur trifluoride (DAST, 15 mL, 0.118 mmol, 1.2 equiv) was added to the mixture in one portion and stirred at ambient temperature for 1 h. After addition of MeOH to quench excess of DAST, the mixture was evaporated. The crude product was purified by silica-gel column chromatography with CH_2Cl_2 eluent to yield 40 mg (80%) of **4** as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05 (dq, *J* = 7.4, 0.9 Hz, 1H), 7.76 – 7.62 (m, 2H), 7.19 (dt, *J* = 7.5, 0.9 Hz, 1H), 7.07 (d, *J* = 8.9 Hz, 1H), 6.97 (ddd, *J* = 8.9, 2.3, 0.7 Hz, 1H), 6.88 (d, *J* = 2.4 Hz, 1H), 6.76 – 6.65 (m, 2H), 5.95 – 5.61 (m, 2H), 3.87 (d, *J* = 0.7 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.00, 161.93, 152.58, 151.75, 151.26, 149.13, 135.61, 131.20, 130.43, 129.04, 126.40, 125.54, 124.03, 120.35, 117.21, 116.64, 113.14, 110.49, 101.13, 81.66, 73.90, 72.23, 55.87, 30.45, 29.84.; ^{19}F NMR (376 MHz, $CDCl_3$) δ -72.43, -210.36. LRMS: calcd for $[M+H]^+$, 511.0; found 511.0.

Peroxy Green 1- Fluoromethyl (PG1-FM)



A mixture of **4** (160 mg, 0.31 mmol), bis(pinacolato)diboron (102 mg, 0.40 mmol, 1.3 equiv), $Pd(dppf)Cl_2$ (22 mg, 0.031 mmol, 0.1 equiv), and potassium acetate (91 mg, 0.93 mmol, 3.0 equiv) in dioxane (3 mL) was stirred at 90 °C for 3 h. After being cooled, the reaction mixture was diluted with CH_2Cl_2, filtered over Celite pad and concentrated. The crude product was purified by silica-gel column chromatography with CH_2Cl_2 eluent and washing with MeOH to yield 90 mg (59%) of **PG1-FM** as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.03 (dd, *J* = 6.1, 2.1 Hz, 1H), 7.63 (ddd, *J* = 6.8, 4.7, 1.4 Hz, 2H), 7.46 (d, *J* = 7.9 Hz, 1H), 7.11 (dd, *J* = 6.4, 2.1 Hz, 1H), 6.91 – 6.79 (m, 2H), 6.72 (d, *J* = 8.8 Hz, 1H), 6.63 (dd, *J* = 8.8, 2.5 Hz, 1H), 6.03 (d, $J_{H\text{-}F}$= 47.1 Hz, 2H), 3.86 (s, 3H), 1.35 (d, *J* = 1.3 Hz, 13H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.57, 161.65, 153.55, 152.16, 149.98, 135.24, 130.19, 130.17, 129.94, 129.35, 129.20, 128.91, 128.48, 126.29, 125.28, 123.92, 121.74, 112.40, 110.82, 101.07, 84.42, 82.53, 78.62, 55.77, 24.91; ^{19}F NMR (376 MHz, $CDCl_3$) δ -204.00. HRMS: calcd for $[M+H]^+$, 489.1879; found, 489.1883 (+0.0004 mmu).

Compound 5



2 **5**

To a solution of compound **2** (40 mg, 0.106 mmol) in THF-MeOH (2 mL/2 mL) was added $NaBH_4$ (5 mg, 0.128 mmol, 1.2 equiv). The mixture was stirred for 16 h at ambient temperature under N_2 atmosphere, diluted with 2N HCl, and then extracted with EtOAc three times. The combined organic layer was dried over Na_2SO_4, and concentrated. The crude product was purified by silica-gel column chromatography (eluent: CH_2Cl_2/MeOH = 100/2.5) and washing with MeOH to yield 20 mg (50%) of compound **5** as a yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.04 – 7.95 (m, 1H), 7.72 – 7.54 (m, 2H), 7.21 – 7.09 (m, 1H), 6.72 (d, *J* = 2.4 Hz, 1H), 6.69 – 6.51 (m, 4H), 5.28 – 5.14 (m, 2H), 3.79 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.02, 161.54, 158.72, 152.80, 152.41, 149.11, 135.25, 129.91, 129.07, 128.24, 126.92, 125.23, 124.20, 113.29, 112.00, 111.75, 111.13, 110.50, 100.94, 58.24, 55.73. LRMS: calcd for $[M+H]^+$, 377.0; found 377.0.

ROS Selectivity Analysis

PG1-FM was dissolved in DMSO to obtain a 1 mM stock solution. The fluorescence spectra of **PG1-FM** (1 μM) were measured in PBS (-) containing 25 μM of H_2O_2, OCl^-, TBHP, $O_2^{-\cdot}$, $ONOO^-$, or NO. OCl^-, $O_2^{-\cdot}$, $ONOO^-$, or NO were generated by using NaOCl (Fluka), potassium superoxide (SIGMA), Peroxynitrite (Cayman), and PROLI NONOate (Cayman), respectively. The fluorescence spectra of **PG1-FM** were collected an emission window between 500 nm to 650 nm with excitation at 488 nm.

HPLC Analyses of the Reaction Between PG1-FM and H_2O_2

PG1-FM (10 μM) and compound **5** (10 μM) were diluted with eluent (A/B = 50/50(v/v)) or PBS (-) to prepare 100 μM solutions containing 1% DMSO. The reaction mixture of **PG1-FM** (100 μM) and H_2O_2 (10 μM) in PBS (-) was incubated at 37 °C for 30 min, and then analyzed by HPLC. LC analyses were performed on an Agilent Technologies 1200 system using eluent A (H_2O containing 0.05% formic acid) and eluent B (acetonitrile containing 0.05% formic acid), and monitored at 254 nm and 470 nm.

SDS-PAGE Experiments in the Presence of BSA

PG1-FM (10 μM) and BSA (0.5 mg/mL) were incubated at 25 °C for 1 h with or without H_2O_2 (100 μM). Afterward, Ebselen (100 μM) was added to the mixture to quench excess H_2O_2. The obtained samples were separated with Novex™ Mini Gel (Thermo). The gel was first analyzed by ChemiDoc™ MP (Bio-Rad) to obtain fluorescence images (488 nm excitation) and stained with Coomassie brilliant blue (CBB) solution.

SDS-PAGE Experiment in the Presence of Cell Lysate

PG1-FM (10 μM) or **Ctrl-PG1** (10 μM) were gently mixed with MFP 231 cell lysate (1 mg/mL) and treated with varying concentrations of H_2O_2 (200-1000 μM). Samples were incubated at 25 °C for 1 h. The obtained samples were separated with Novex™ Mini Gel (Thermo). The gel was first analyzed by ChemiDoc™ MP (Bio-Rad) to obtain fluorescence images (488 nm excitation) and stained with Coomassie brilliant blue (CBB) solution.

Supplementary figures and legends

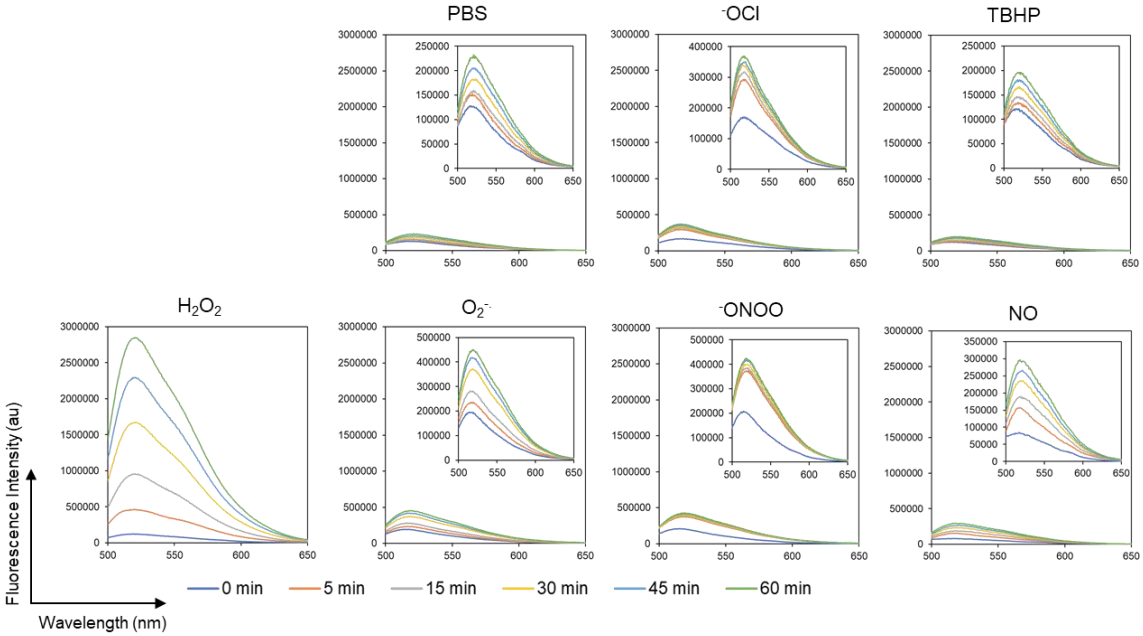


Figure S1. Fluorescence spectra of **PG1-FM** reacted with H_2O_2, OCl^-, TBHP, $O_2^{\cdot-}$, $ONOO^-$, NO or PBS (control). **PG1-FM** (1 μM) was incubated with 25 μM of H_2O_2, OCl^-, TBHP, $O_2^{\cdot-}$, $ONOO^-$, or NO at 37 ^oC in PBS (-). The mixtures were measured using a fluorimeter at 0, 5, 15, 30, 45, and 60 min.

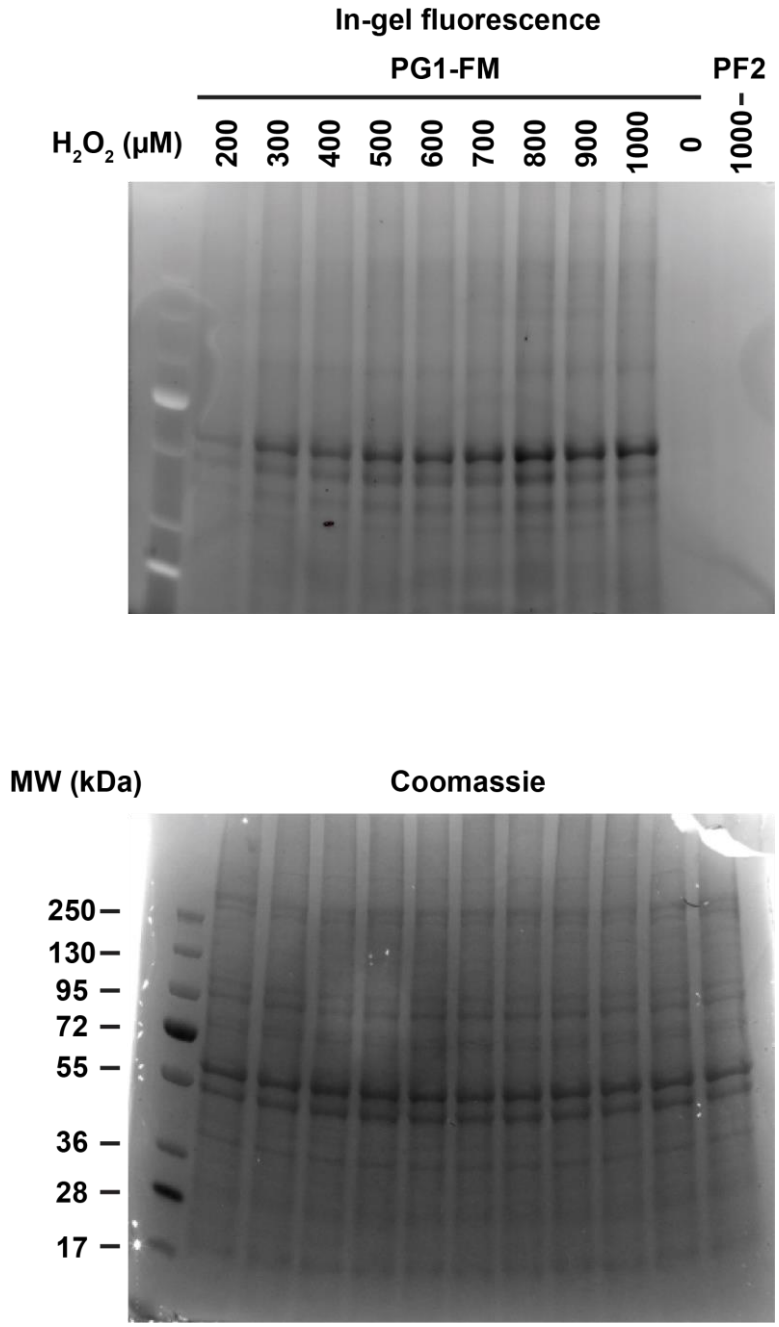


Figure S2. H_2O_2-dependent activity-based labeling of **PG1-FM** or **PF2** in an MFP 231 cell lysate solution.

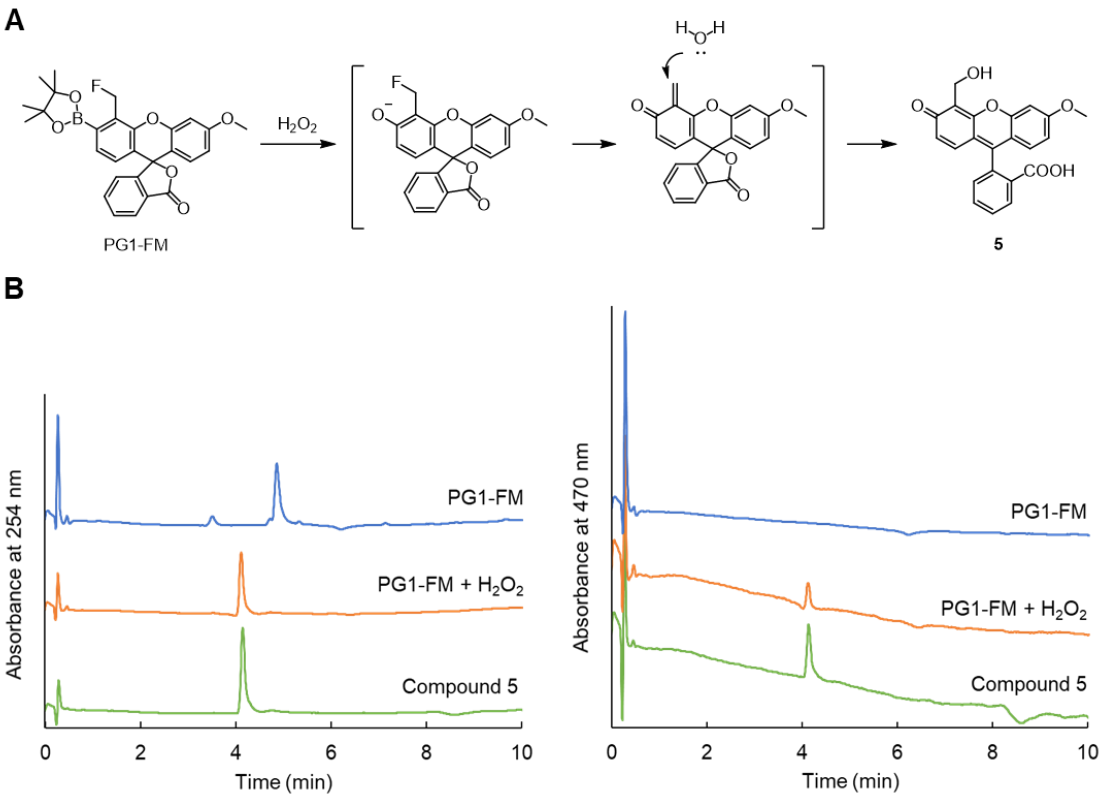


Figure S3. The reaction mechanism (A) and HPLC analyses (B) of the reaction product using **PG1-FM** and H_2O_2. 10 μM **PG1-FM** was treated with 10 μM H_2O_2 in PBS (-), containing 1% DMSO, and the mixture was incubated at 37 °C for 30 min. HPLC analysis was performed under a gradient condition (A: H_2O containing 0.05% formic acid; B: acetonitrile containing 0.05% formic acid; A/B = 75%/25% to 25%/75% in 15 min). Absorbance at 254 nm and 470 nm were monitored. The spectra show before (blue) and after (orange) reaction with H_2O_2, and its putative product, hydroxymethyl compound **5** (green), which is obtained by boronate deprotection and quenching of the quinone methide by water.

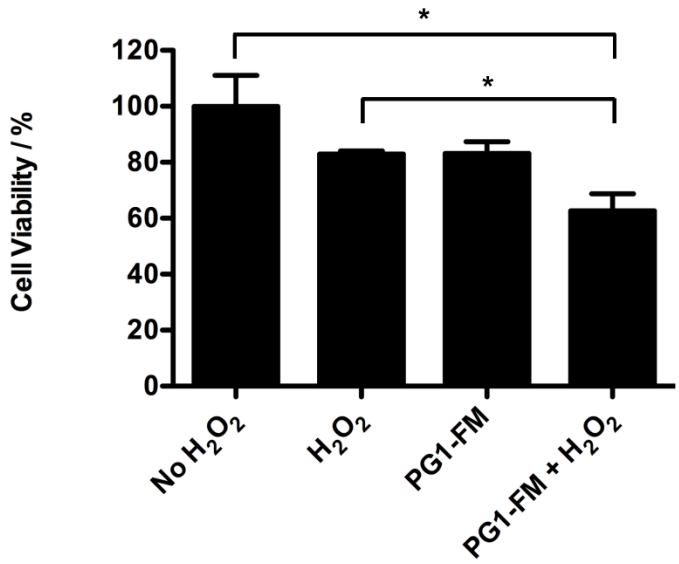


Figure S4. Cell viability assay of **PG1-FM** treated HeLa cells in the presence of H_2O_2. HeLa cells (96-well plate, 20,000 cells/well) were treated with **PG1-FM** (10 µM, 100 µL in HBSS) and placed in a humidified 5% CO_2 incubator at 37 °C for 30 minutes. The solution in wells were replaced with a 100 µM solution of H_2O_2 in HBSS (100 µL) and placed in a humidified 5% CO_2 incubator at 37 °C for one hour. The cells were washed once with HBSS (100 µL) at which point 100 µL of a 1:2000 dilution of 1 mM HOECHST 33342 stain in MilliQ water was added to each sample. The cells were placed in a humidified 5% CO_2 incubator at 37 °C for 10 minutes. Measurements λ_{ex}=350 nm/ λ_{em}= 460 nm were performed using a microplate reader. Mean ± SEM, normalized to the untreated control condition. N = 3. *P < 0.05.

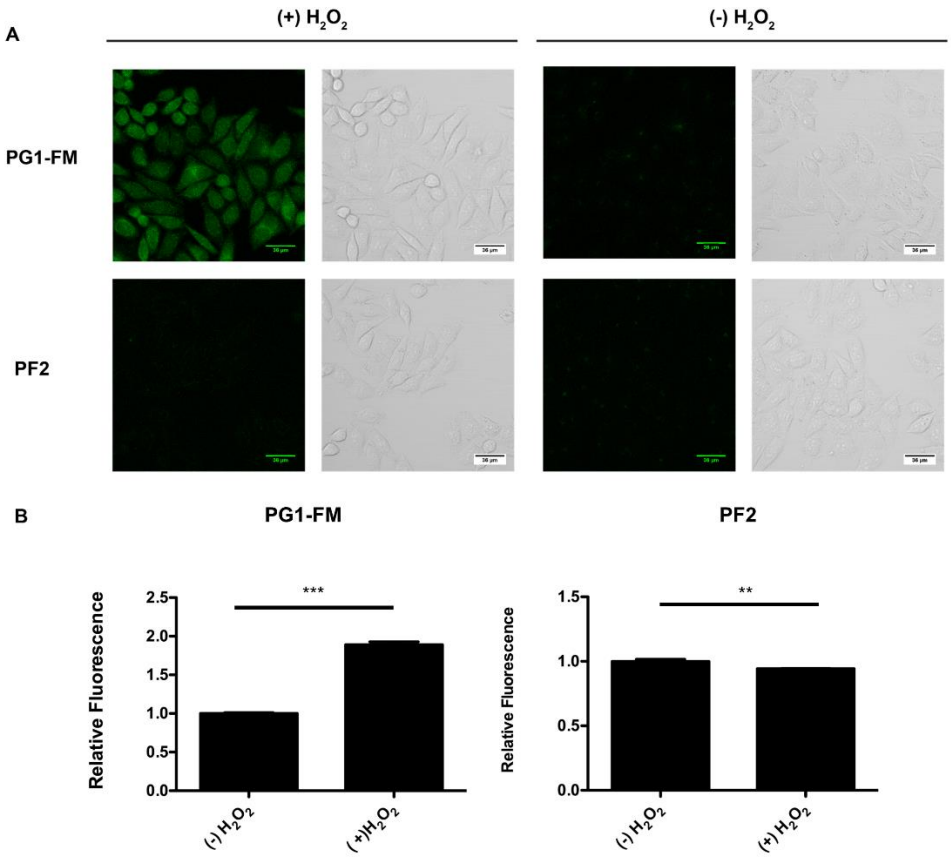


Figure S5. (A) CLSM images of HeLa cells stained with **PG1-FM** or **PF2** in the presence or absence of H_2O_2 (100 μM). Scale bar: 36 μm. (B) Quantification of **PG1-FM** and **PF2** fluorescence intensity. Mean ± SEM, normalized to the untreated control condition. N = 3, with 4 fields examined per repeat. ***P* < 0.01, ****P* < 0.001.

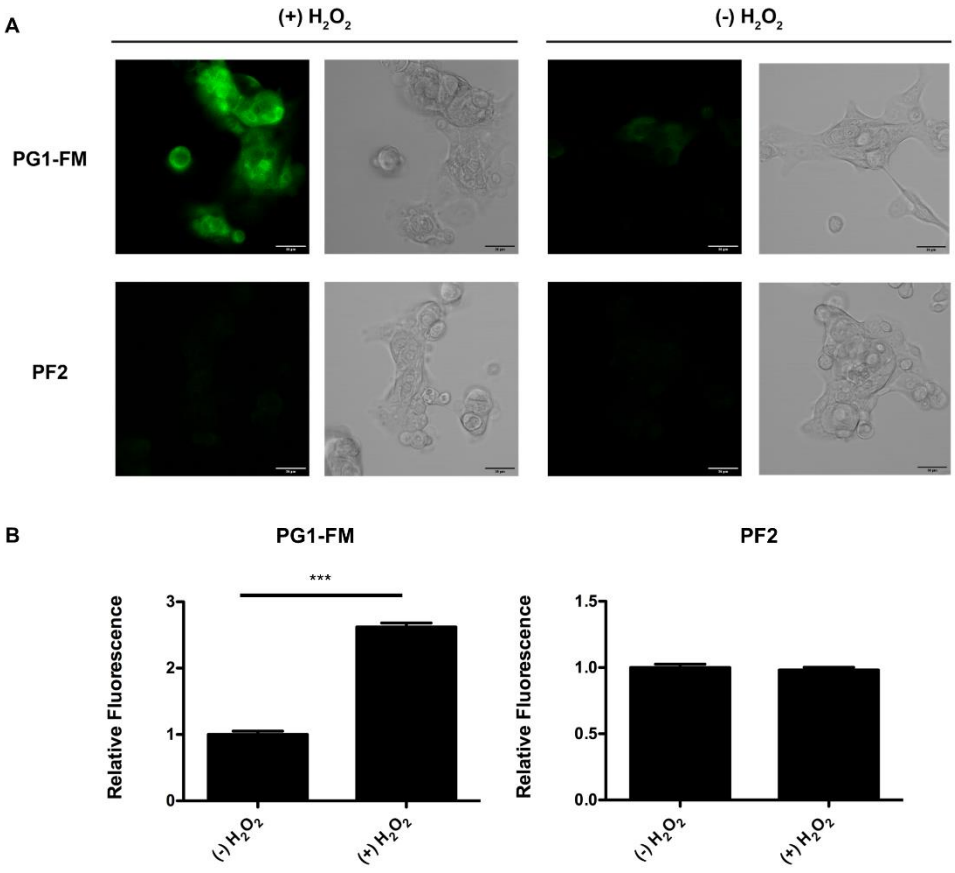


Figure S6. (A) CLSM images of MCF10A cells stained with **PG1-FM** or **PF2** in the presence or absence of H_2O_2 (100 μM). Scale bar: 36 μm. (B) Quantification of **PG1-FM** and **PF2** fluorescence intensity. Mean ± SEM, normalized to the untreated control condition. N = 3, with 4 fields examined per repeat. ****P* <0.001.

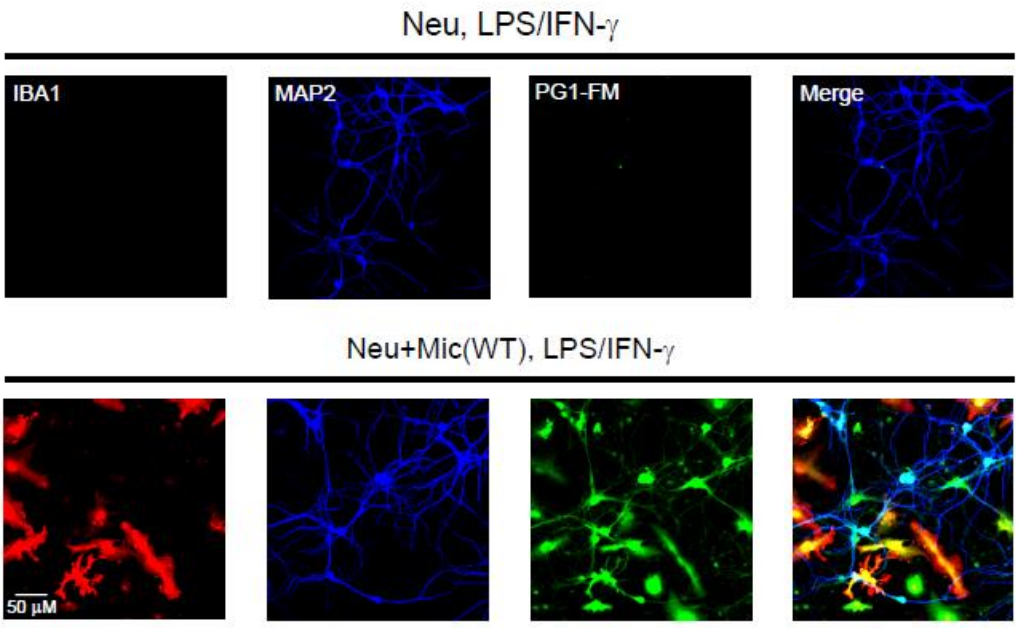


Figure S7. H_2O_2 signal in neurons in triple-labeled cultures. Representative images of cultures treated overnight with 500 ng/mL LPS + 50 ng/mL IFN-γ and incubated with 10 μM **PG1-FM** (green) for 1 h. Cultures were immunostained for the neuronal marker MAP2 (blue) and the microglia marker Iba-1 (red). Neu: neurons, Mic: microglia. Scale bar = 50 μm.

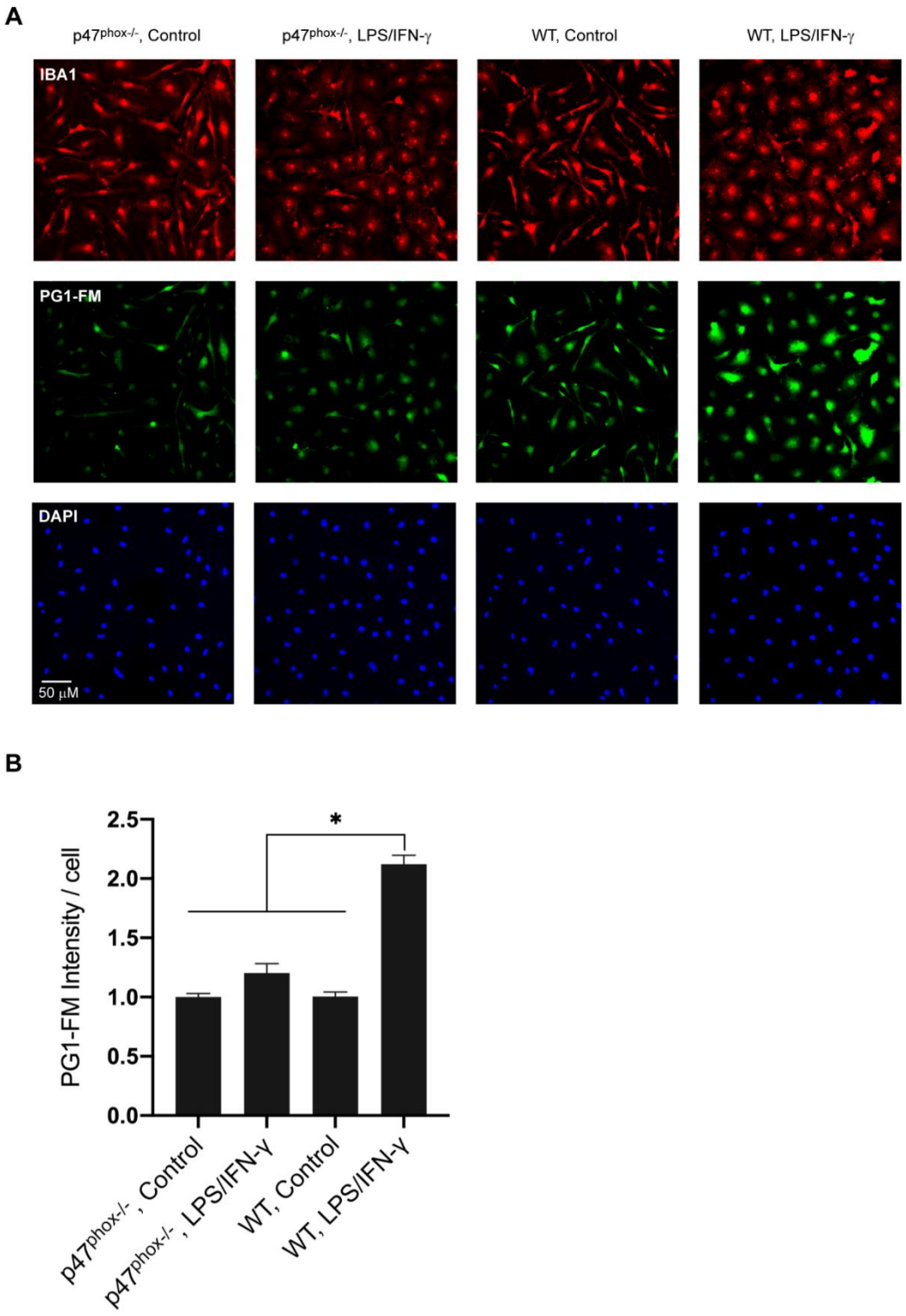


Figure S8. H_2O_2 production in WT and p47$^{phox-/-}$ microglia in response to LPS and INFγ. (A) Representative images of microglia treated overnight with 500 ng/mL LPS + 50 ng/mL IFN-γ. The cultures were incubated with 10 μM **PG1-FM** (green) for 1 h and immunostained for the microglial marker IBA1 (red), with nuclei labeled with DAPI (blue). Scale bar = 50 μm. (B) Values were

plotted as **PG1-FM** fluorescence intensity ± SEM, normalized to p47$^{phox-/-}$ control condition. N = 3, with > 200 cells evaluated per repetition. * P < 0.05.

Small Molecule Characterization

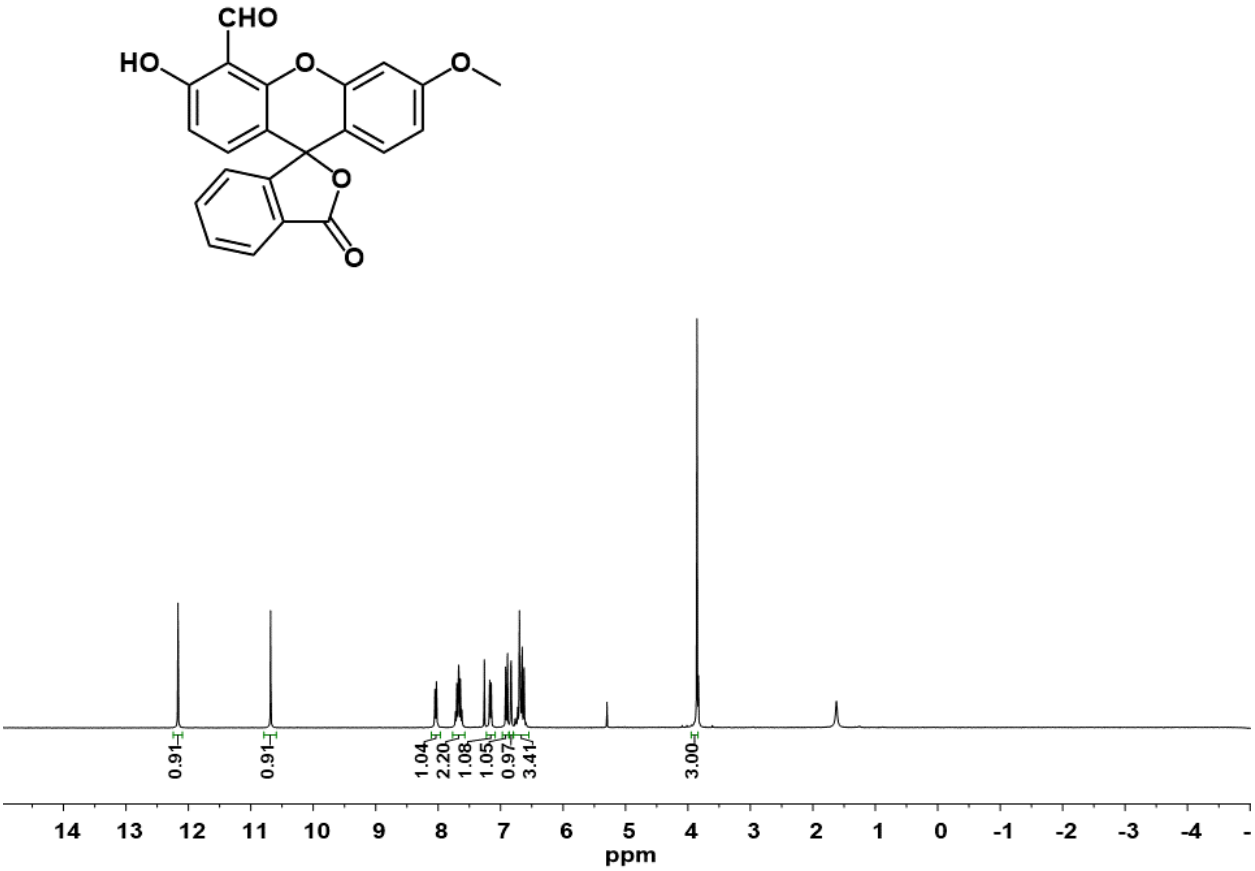


Figure S9. ^{1}H NMR spectrum of **2**.

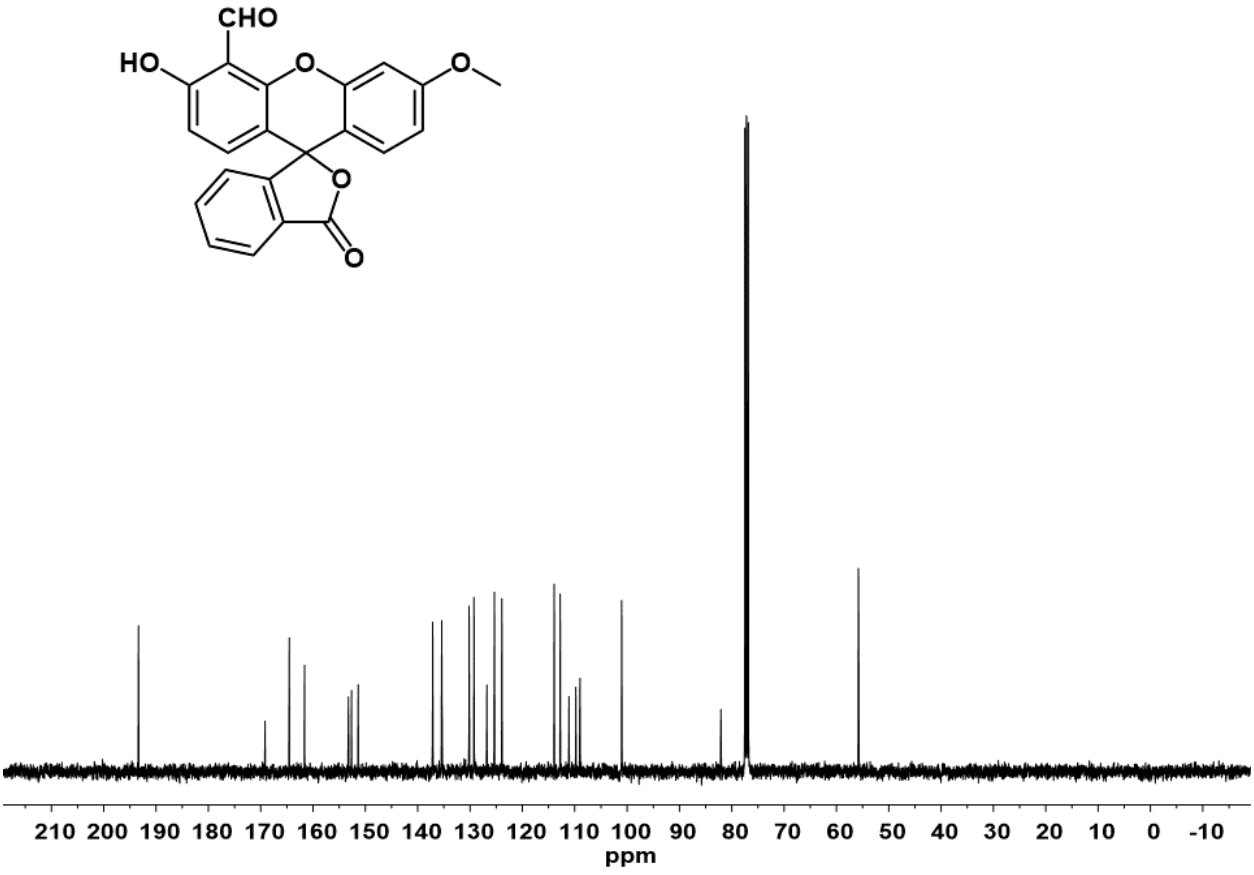


Figure S10. ^{13}C NMR spectrum of **2**.

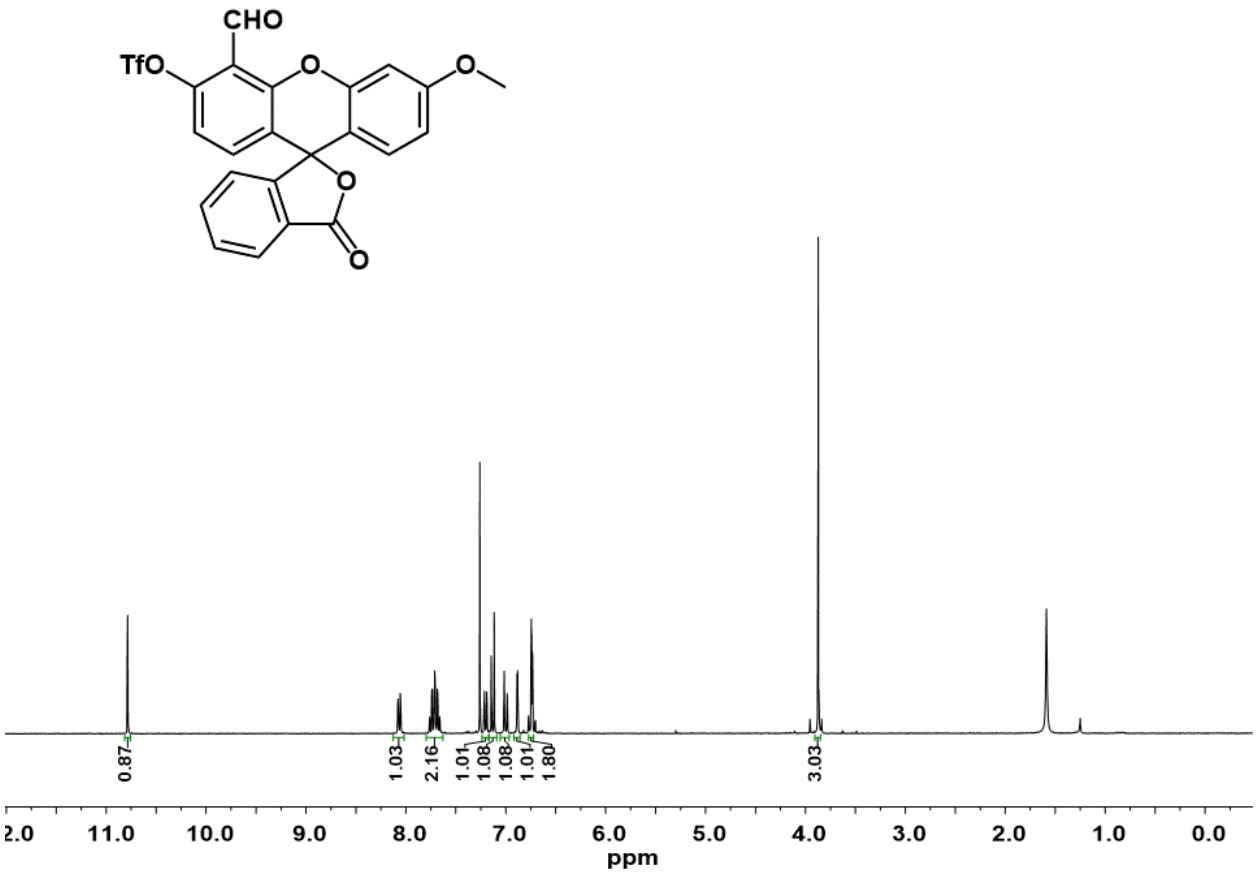


Figure S11. ^{1}H NMR spectrum of **3**.

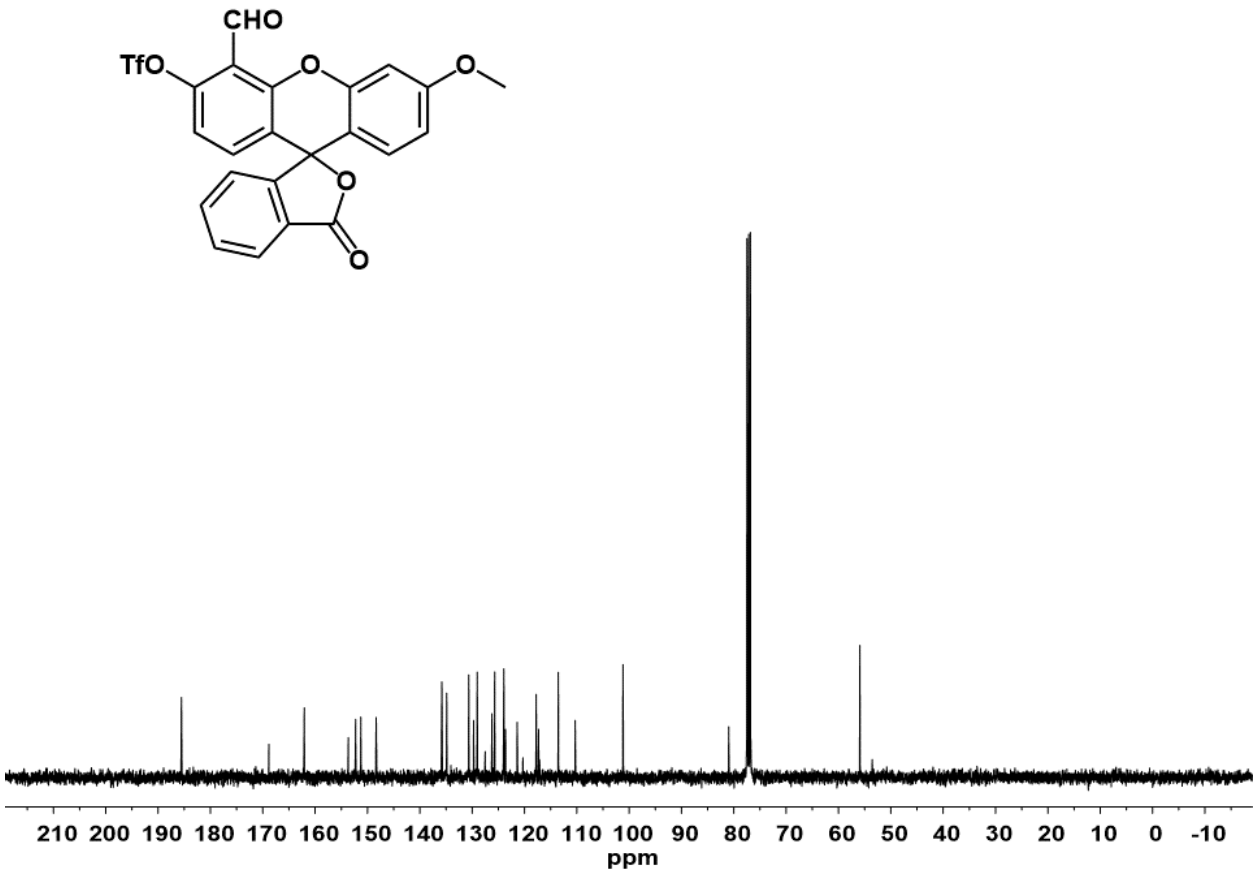


Figure S12. ^{13}C NMR spectrum of **3**.

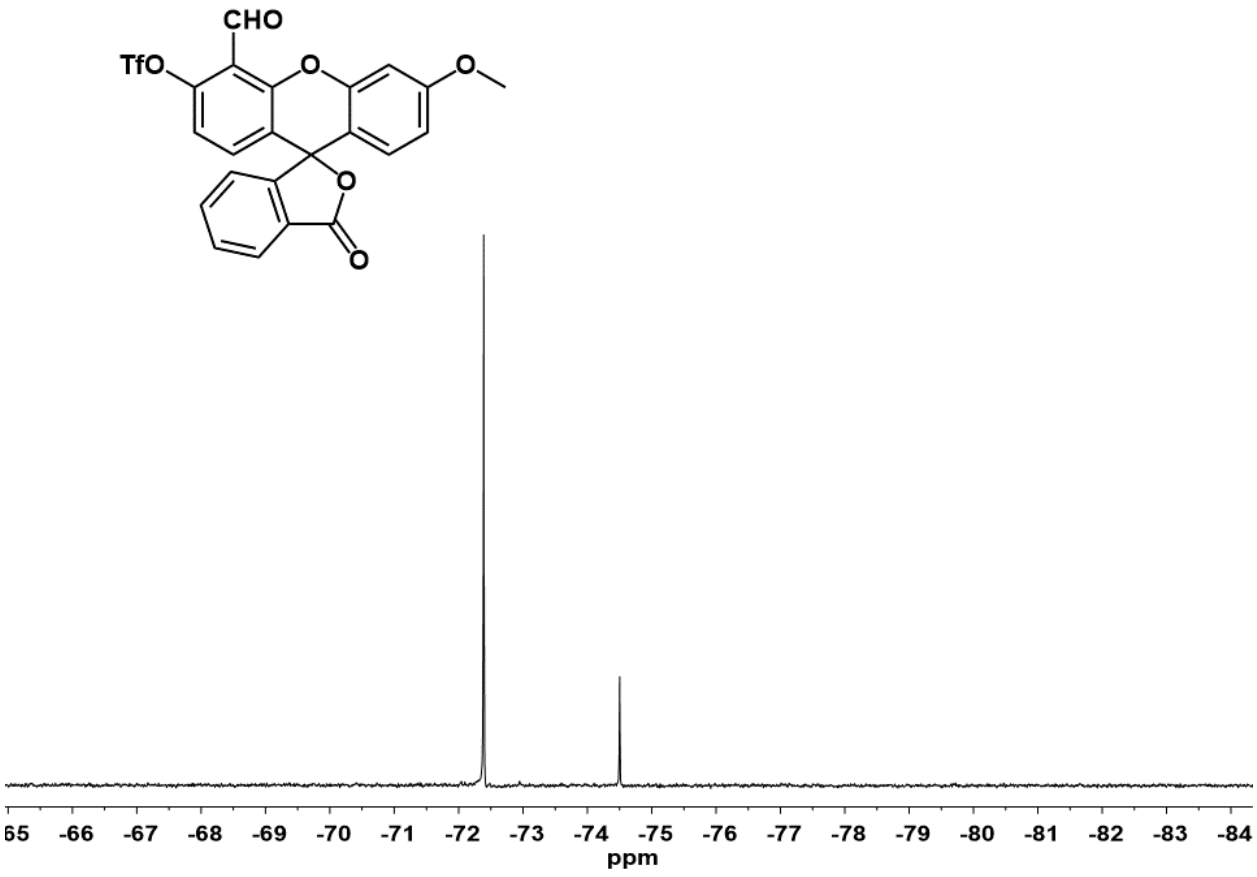


Figure S13. ^{19}F NMR spectrum of **3**.

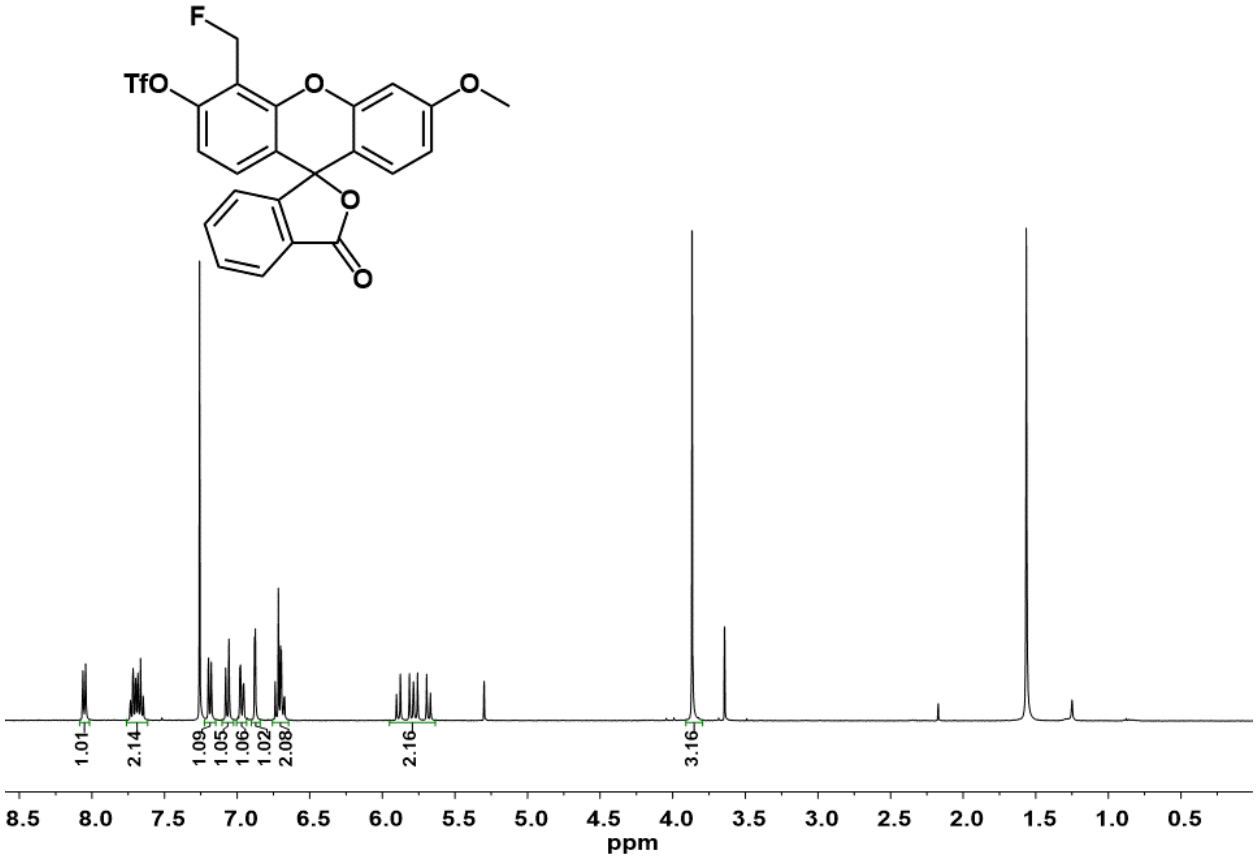


Figure S14. ^{1}H NMR spectrum of **4**.

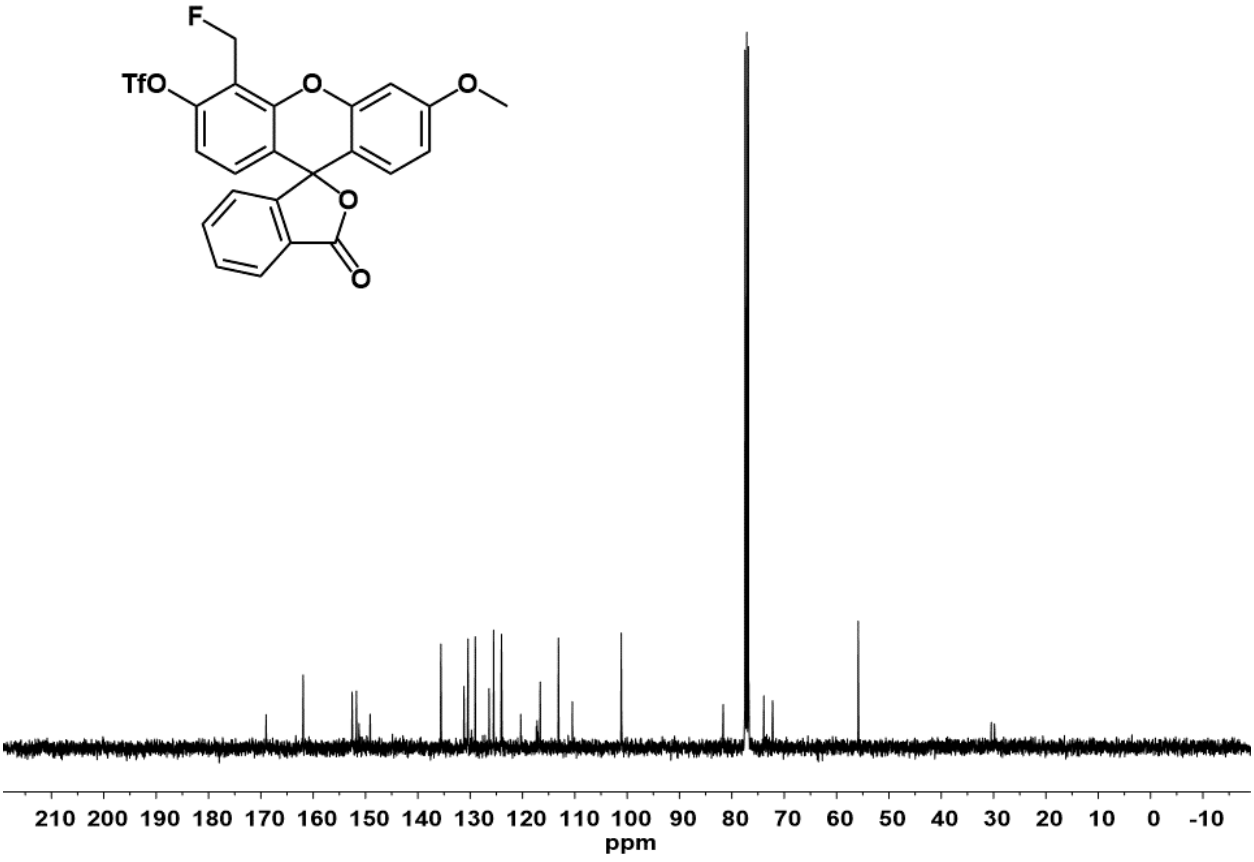


Figure S15. ^{13}C NMR spectrum of **4**.

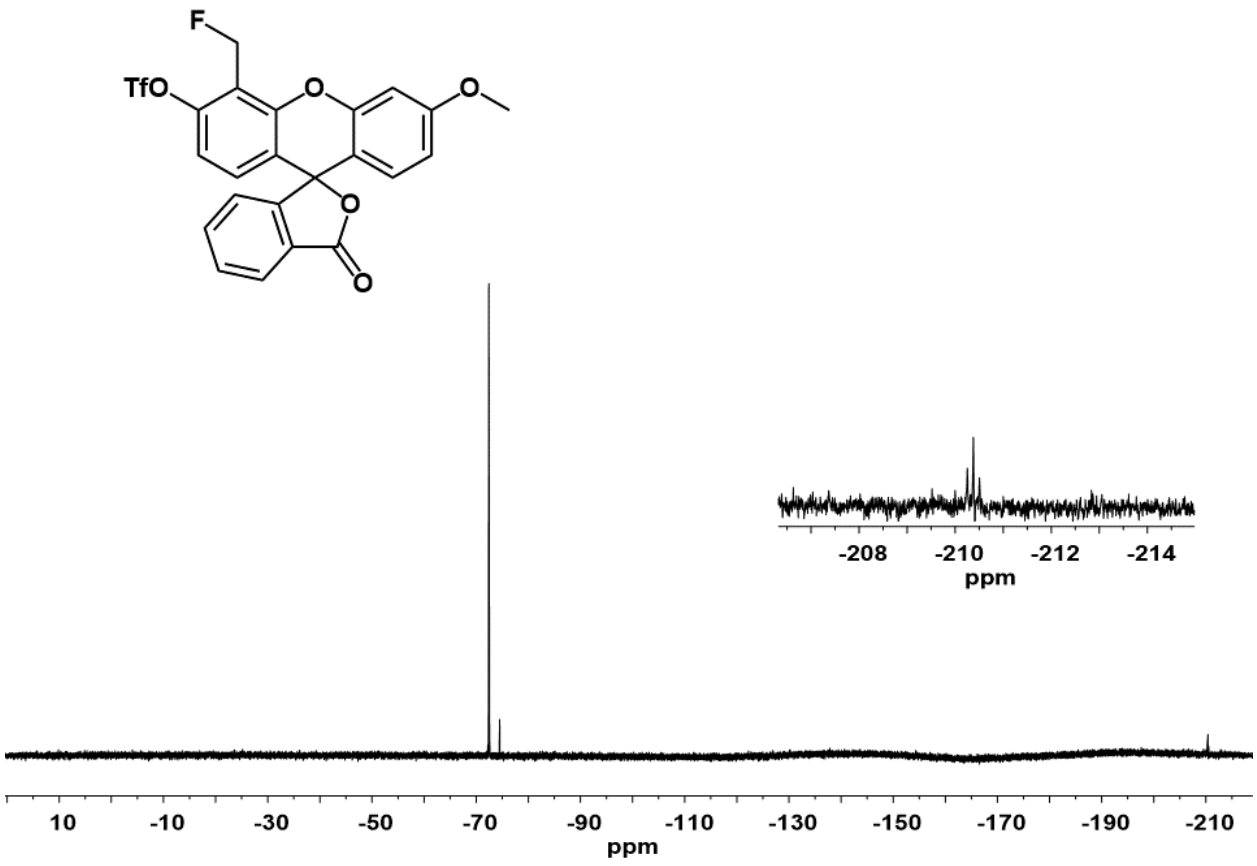


Figure S16. ^{19}F NMR spectrum of **4**.

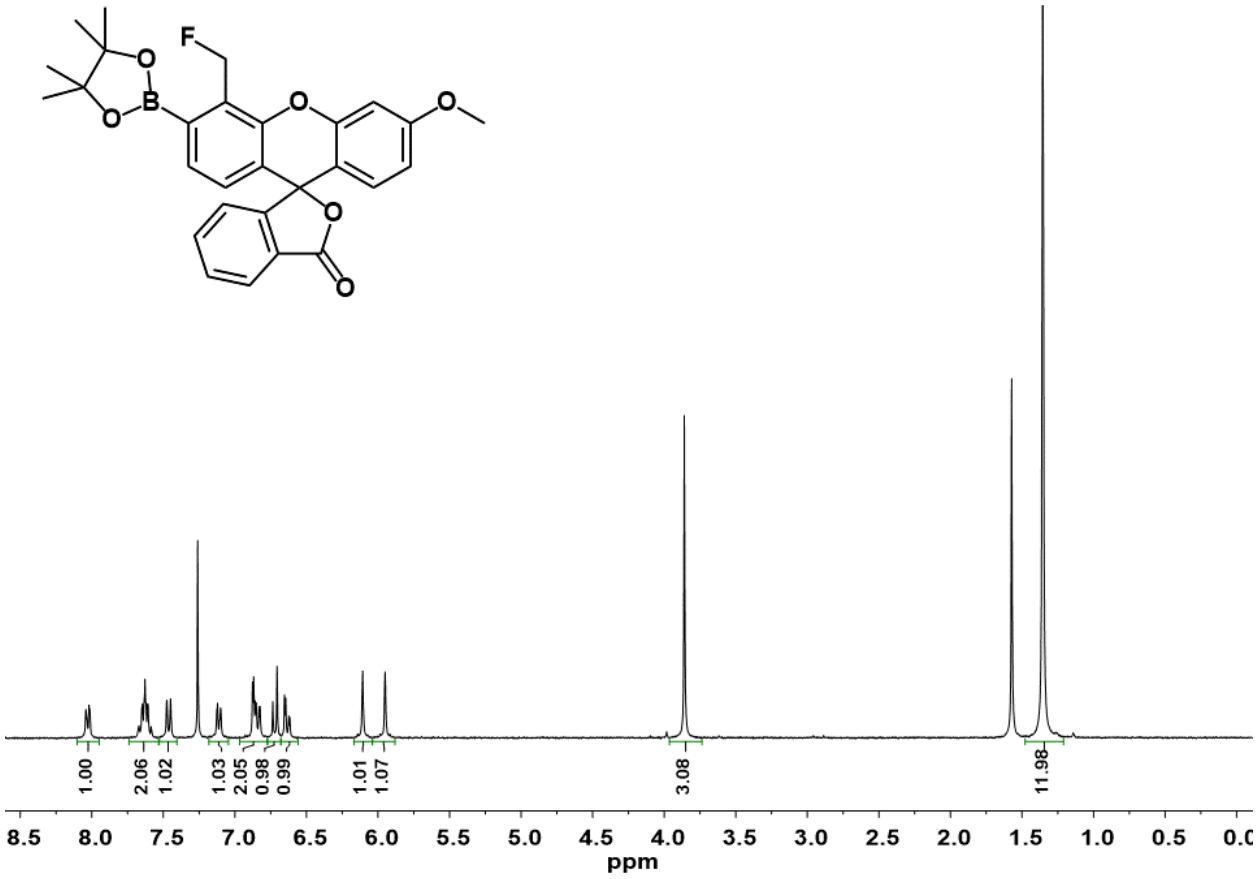


Figure S17. ^{1}H NMR spectrum of **PG1-FM**.

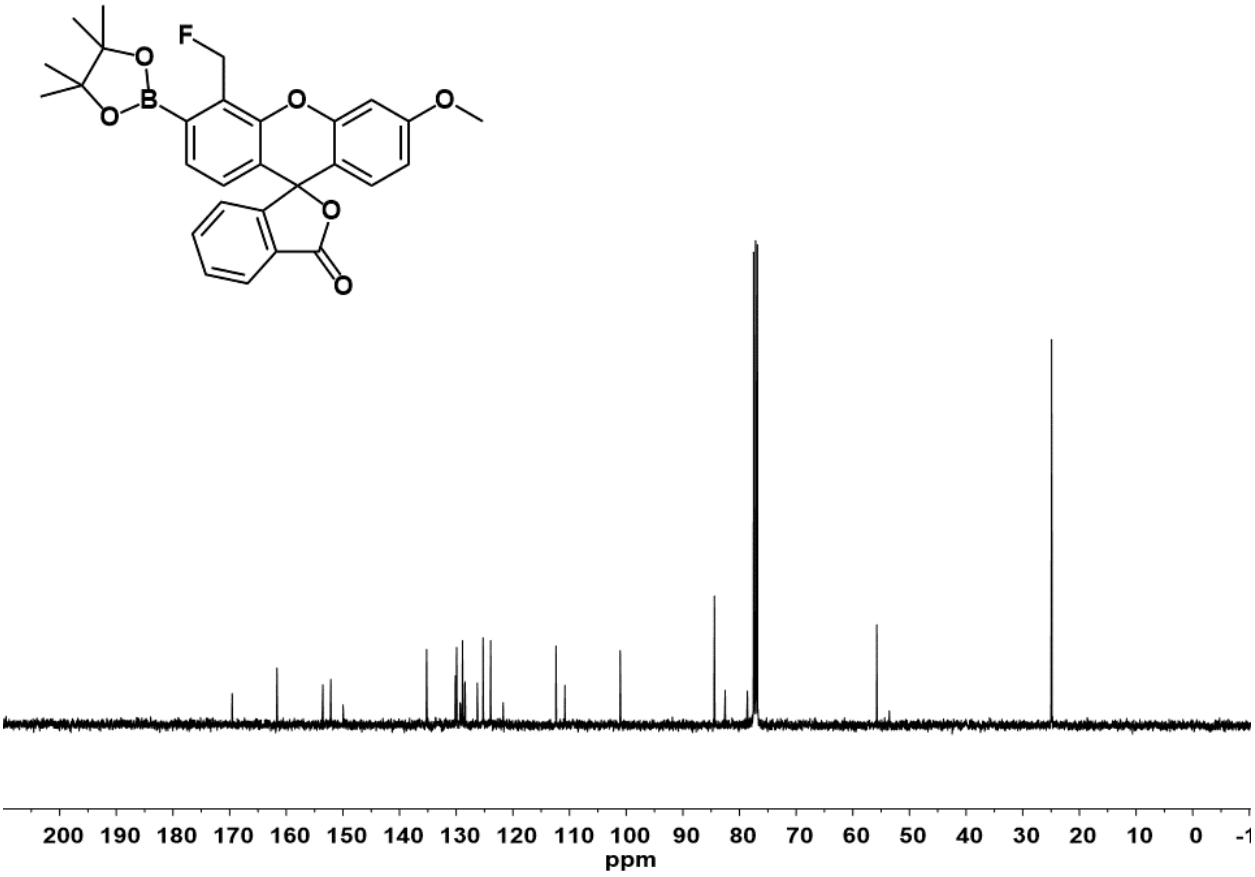


Figure S18. ^{13}C NMR spectrum of **PG1-FM**.

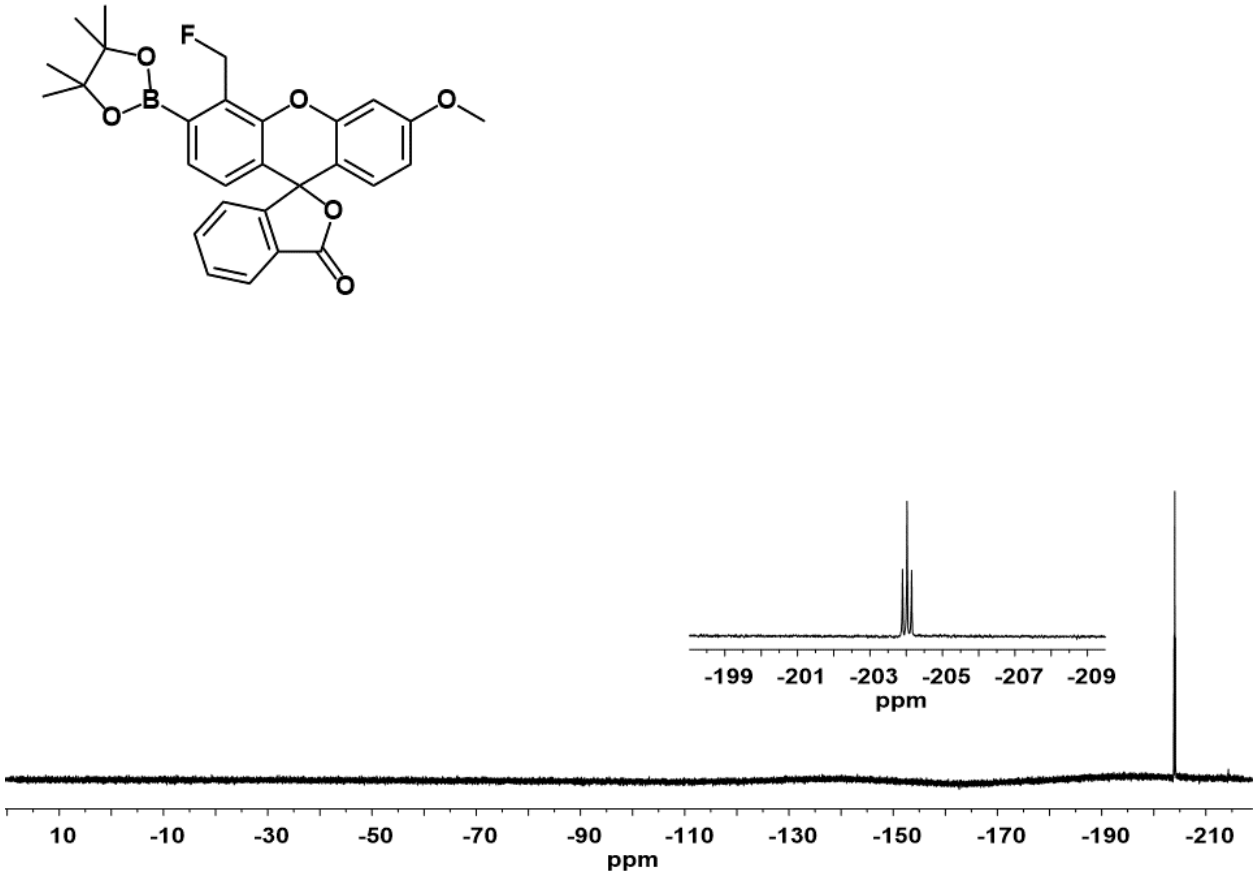


Figure S19. ^{19}F NMR spectrum of **PG1-FM**.

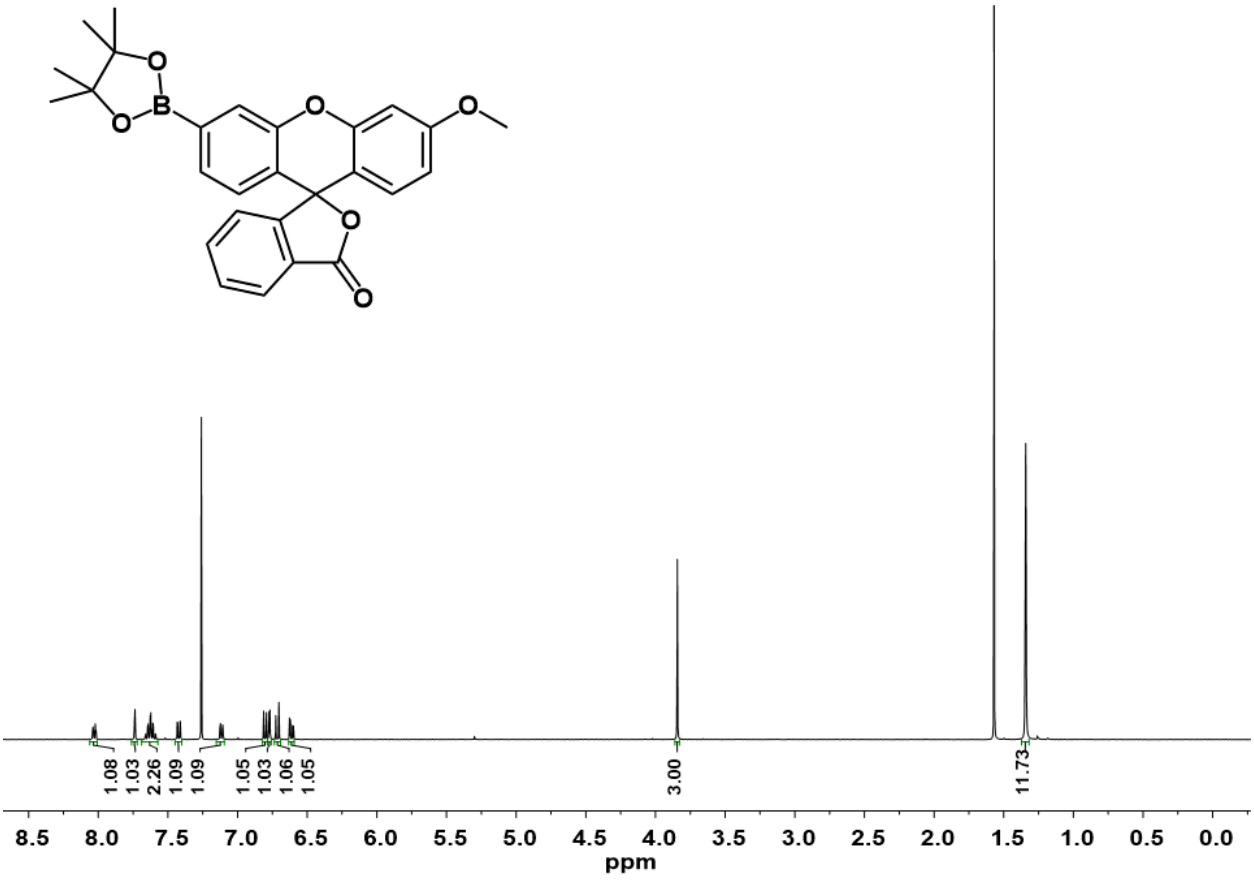


Figure S20. ^{1}H NMR spectrum of **PF2**.

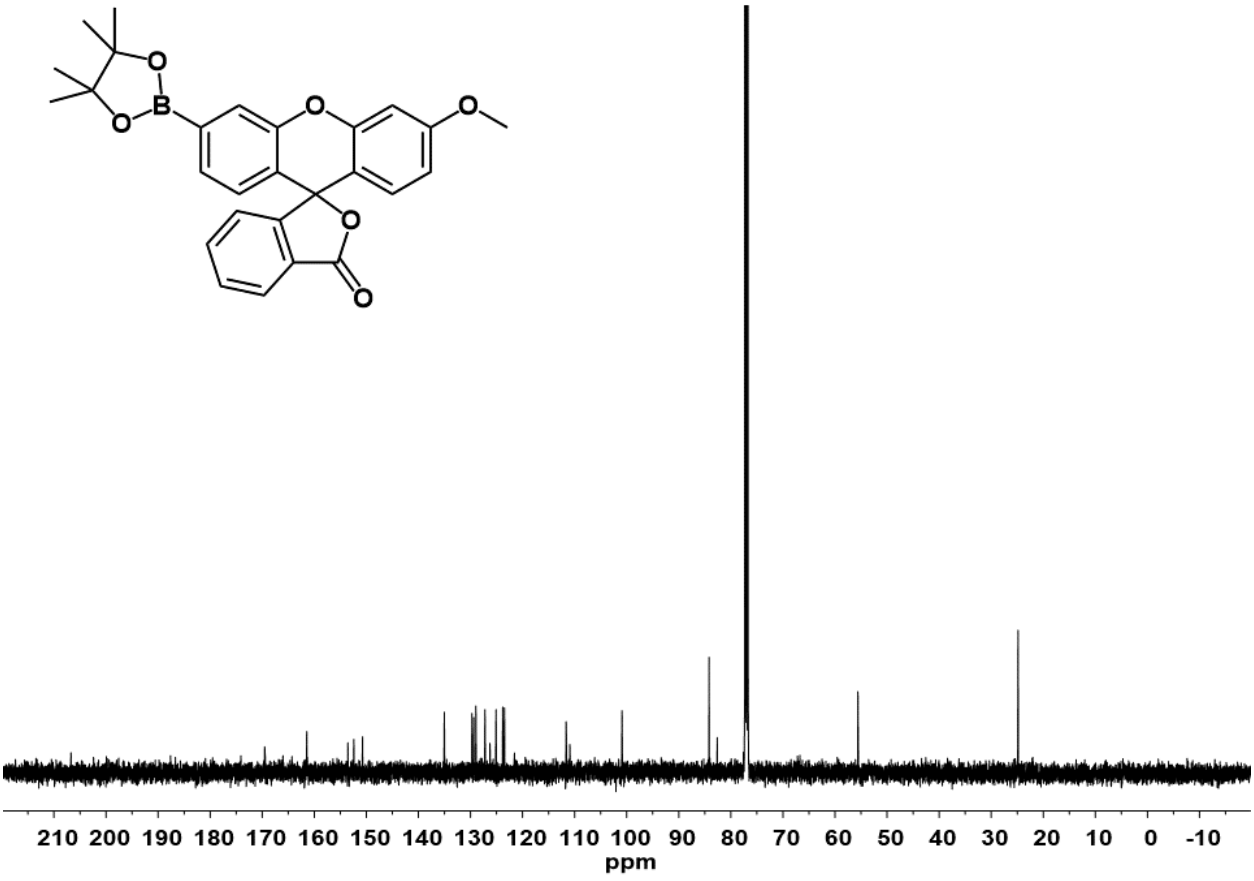


Figure S21. ^{13}C NMR spectrum of **PF2**.

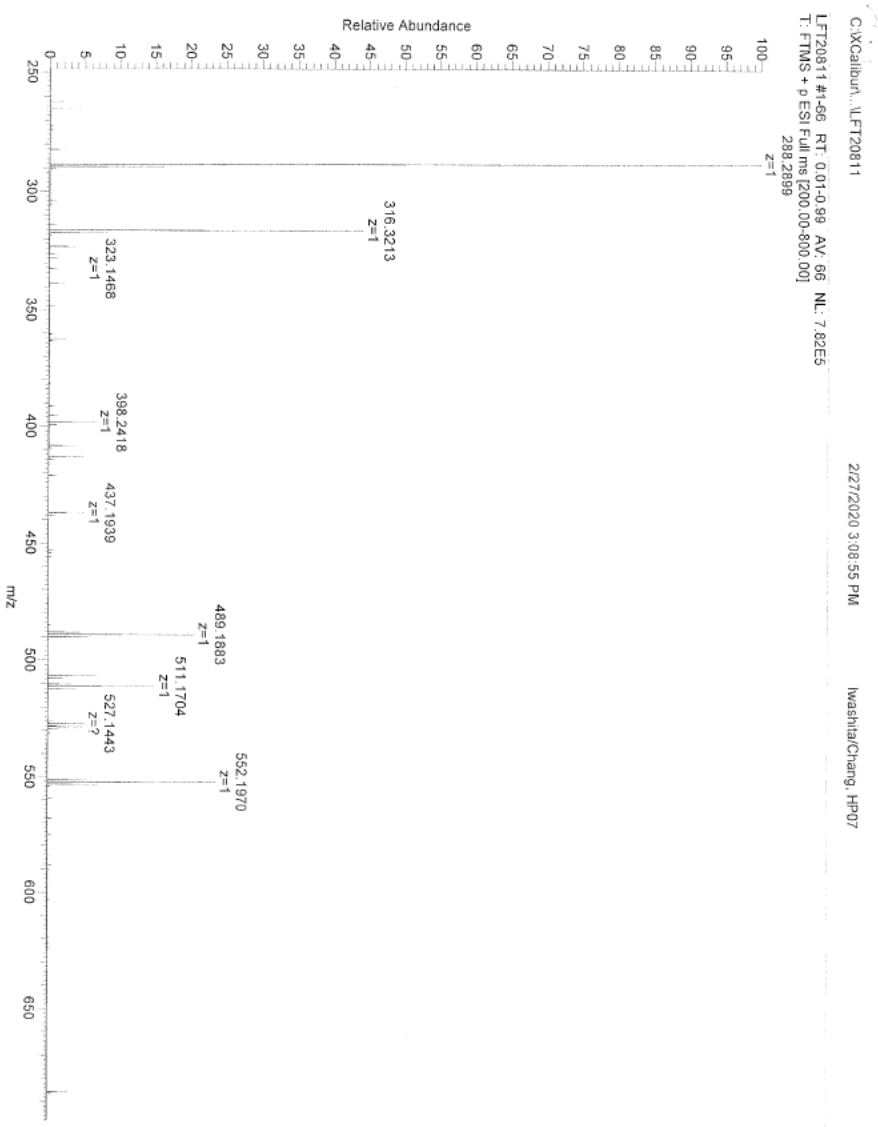


Figure S22. HRMS spectrum of **PG1-FM**.

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