Supporting Information

Dynamic Visualization of Endoplasmic Reticulum Viscosity in Living Cells with Multirotor-Based Fluorescent Probe

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1. General Chemicals and Instruments

All chemicals and solvents used for synthesis were purchased from commercial suppliers and applied directly in the experiments without further purification. The reaction progress was monitored via thin-layer chromatography (TLC) on precoated silica gel plates, with spots visualized under UV light at 254 nm. Silica gel (100-200 mesh) was employed for column chromatography purification. ^{1}H and ^{13}C NMR Nuclear magnetic resonance (NMR) spectra (^{1}H and ^{13}C) were recorded on a Q.ONE AS 400 spectrometer (400 MHz for ^{1}H NMR, 101 MHz for ^{13}C NMR) at room temperature. Chemical shifts are reported in parts per million (ppm) relative to residual solvent peaks ($CDCl_3$ = 7.26 ppm; DMSO-d_6 = 2.50 ppm). Coupling constants (*J*) are reported in Hertz (Hz), and multiplicity is denoted as follows: s (singlet), d (doublet), dd (doublet doublet), t (triplet), m (multiplet). High-Resolution Mass Spectrum (HRMS) were obtained using a Q-Exactive Orbitrap HRMS in positive ion mode. UV-visible spectra were recorded on a UV-3600 UV-VIS-NIR spectrophotometer (Shimadzu, Japan). Fluorescence spectra were using an F-380 pectrophotometer (Tianjin Gangdong Sci & Tech., Development. Co., Ltd). Bioimaging data were collected using a confocal microscope (Olympus FV1000).

2. Spectroscopic Studies

Probe 1-3 were dissolved in DMSO to a final concentration of 5 mM to prepare the stock solution. Seven solvents, including toluene (Tol), dioxane (Dio), ethyl acetate (EtOAc), tetrahydrofuran (THF), dichloromethane (DCM), dimethyl formamide (DMF), methanol (MeOH), PBS, and glycerol (Gly), were selected to investigate the solvent-dependent absorption and fluorescence spectra of probe 1-3. The viscosity sensitivity of probe 1 was tested in a mixture of methanol and glycerol. The chemical stability of probe 1 was evaluated in the presence of multiple substances within a PBS-Gly mixture (1/1, v/v). These substances include NaCl, $MgCl_2$, $CuCl_2$, $FeCl_3$, $CaCl_2$, $ZnCl_2$, Na_2SO_4, Na_2CO_3, $NaNO_2$, $NaHSO_3$, H_2O_2, HClO, H_2S, Cys, Hcy, and GSH. The concentration of sulfhydryl compounds (Cys, Hcy, and GSH) is 1 mM, while that of the other compounds is 100 μM. The pH stability of probe 1 was evaluated in a PBS-Gly mixture (1:1, v/v) across a range of pH values. Excitation and emission slit widths were set at 5 nm and 10 nm, respectively.

3. Cell Imaging

HeLa cells were cultured at 37 ^{o}C and 5% CO2 in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, and 4 mM L-glutamine.

For cytotoxicity test, cells were seeded into 96-well microplates and cultured in DMEM for 24 h. After overnight culture, cells were incubated with each concentration of probe 1 for 3 h. After that, the medium was removed, and cells were washed twice with 100 μL PBS buffer. Subsequently, 100 μL fresh medium and 10 μL of CCK-8 was added, followed

by incubation for 1 h. Finally, the absorbance value was recorded at 450 nm on a Spectramax microwell plate reader.

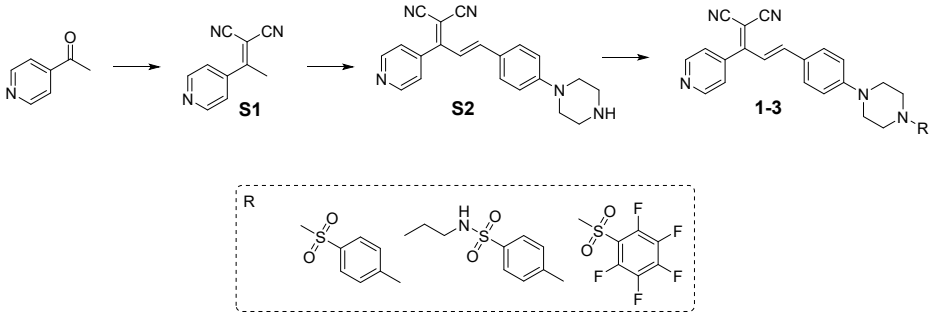
For organelle colocalization experiment, HeLa cells were co-incubated with commercial organelle-specific dyes and probe 1 (5 μM) for 30 min. After washing with PBS three times, the cells were imaged with green channel (500-550 nm, excitation at 488 nm) and red channel (600-650 nm, excitation at 488 nm). The concentrations of ER-tracker Green, Lyso-tracker Green and DODIPY 493/503 were 1 μM, 500 nM, and 500 nM, respectively.

For monensin induced cell viscosity imaging, HeLa cells were first treated with monensin for different times (0, 1, 2, 4 h) or different concentrations (0, 5, 10, 20 μM), then incubated with probe 1 (5 μM) for 30 min. After washing with PBS three times, the above cells were used for imaging immediately.

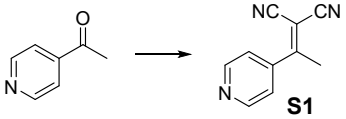
For drug induced cell viscosity imaging, HeLa cells were first treated with LPS (10 μg/mL) or CPT (10 μM) for 30 min, then incubated with probe 1 (5 μM) for 30 min. After washing with PBS three times, the above cells were used for imaging immediately.

For imaging of starvation-induced ER-phagy, HeLa cells were cultured in high-glucose DMEM without FBS supplementation for different times (0, 0.5, 1, 2 h). The cells were then incubated with probe 1 (5 μM) and Lyso-tracker Green (500 nM) for 30 min. After washing with PBS three times, the above cells were used for imaging immediately.

4. Synthesis

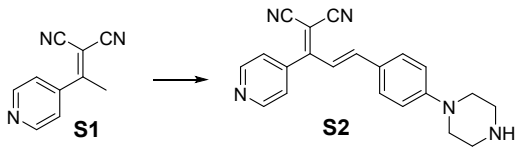


Scheme S1 Synthetic route of probe **1-3**



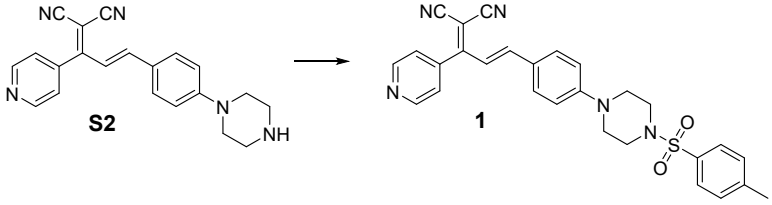
Synthesis of compound S1

4-acetylpyridine (0.450 mL, 4.12 mmol), malononitrile (545 mg, 8.25 mmol) and HMDS (0.86 mL, 4.12 mmol) were dissolved in 5 mL of acetic acid and stirred at 75 °C for 45 min. The cooling to room temperature, the pH was adjusted to 7.0 using a saturated sodium bicarbonate solution. The resulting solution was extracted with dichloromethane, washed with a saturated sodium chloride aqueous solution, and concentrated under reduced pressure to afford the crude compounds S1, which was used without further purification.



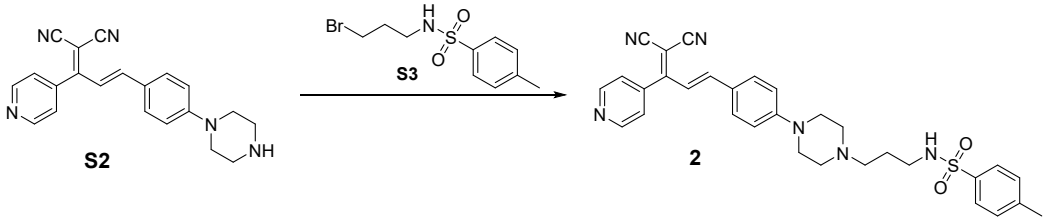
Synthesis of compound S2

Compound S1 (1.69 g, 10.0 mmol) and 1-Boc-4-(4-formylphenyl)piperazine (3.48 g, 12.0 mmol) were dissolved in dry THF (35 mL). The mixture was refluxed for 10 hours, after which the solvent was removed under reduced pressure. The crude product was purified by column chromatography ($V_{petroleum\ ether}/V_{ethyl\ acetate}$ = 4 / 1) to give reddish brown solid. The solid was dissolved in a TFA/DCM solution (v/v = 1:1, 25 mL). After stirring at room temperature for 4 hours, the reaction mixture was neutralized to pH 7.0 with a saturated $NaHCO_3$ aqueous solution. The resulting solution was extracted with DCM, washed with saturated NaCl aqueous solution, and concentrated to afford compound S2 as brown solid (1.71 g, yield 50%). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.80 (d, J = 6.0 Hz, 2H), 7.55 (d, J = 4.8 Hz, 2H), 7.48 (d, J = 6.0 Hz, 2H), 7.27 (d, J = 15.6 Hz, 1H), 6.94 (d, J = 8.0 Hz, 2H), 6.80 (d, J = 15.2 Hz, 1H), 3.32-3.29 (m, 4H), 2.80 - 2.78 (m, 4H), 1.23 (s, 1H).



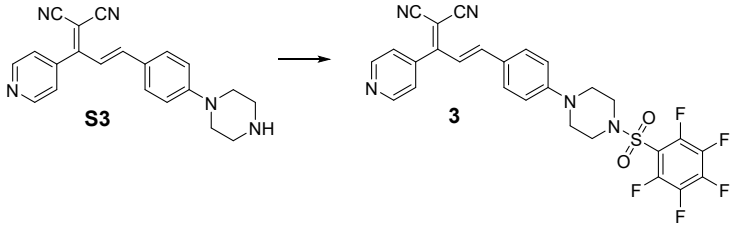
Synthesis of probe 1

Compound S2 (341 mg, 1.00 mmol), tosyl chloride (248 mg, 1.30 mmol) and triethylamine (202 mg, 2.00 mmol) were sequentially addedto dry acetonitrile (10 mL). The reaction mixture was stirred for 8 hours at room temperature. Subsequently, the solvent was removed under reduced pressure. The crude product was purified by column chromatography (V_{DCM}/V_{MeOH} = 10 / 1) to give probe 1 as brown solid (352 mg, 71 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.82 (d, J = 4.8 Hz, 2H), 7.65 (d, J = 8.4 Hz, 2H), 7.41 - 7.33 (m, 5H), 7.26 (s, 1H), 7.25 (s, 1H), 6.78 (d, J = 8.8 Hz, 2H), 6.65 (d, J = 15.2 Hz, 1H), 3.46 - 3.43 (m, 4H), 3.14 - 3.12 (m, 4H), 2.43 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 168.14, 152.94, 150.70, 149.35, 144.28, 141.70, 132.23, 131.37, 129.97, 127.97, 124.88, 123.13, 119.98, 114.94, 113.37, 112.90, 79.80, 47.03, 45.73, 27.03, 21.69. HRMS: $C_{28}H_{26}N_5O_4S$ $[M+H]^+$ calcd. for 496.1802, found 496.1802.



Synthesis of probe 2

Compound S2 (341 mg, 1.00 mmol), S3 (380 mg, 1.30 mmol) and potassium carbonate (276 mg, 2.00 mmol) were sequentially addedto dry acetonitrile (10 mL). The reaction mixture was stirred over night at room temperature. Subsequently, the solvent was removed under reduced pressure. The crude product was purified by column chromatography (V_{DCM}/V_{MeOH} = 10 / 1) to give probe 2 as brown solid (138 mg, 25 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.82 (d, J = 6.0 Hz, 2H), 7.72 (d, J = 8.4 Hz, 2H), 7.42 (d, J = 8.8 Hz, 2H), 7.38 (d, J = 15.2 Hz, 2H), 7.30 (d, J = 8.0 Hz, 2H), 7.27 (d, J = 6.0 Hz, 2H), 6.84 (d, J = 9.2 Hz, 2H), 6.68 (d, J = 15.6 Hz, 1H), 3.41 - 3.38 (m, 4H), 3.08 (t, J = 5.6 Hz, 2H), 2.55 (t, J = 4.8 Hz, 4H), 2.49 - 2.45 (m, 2H), 2.42 (s, 3H), 1.72 - 1.67 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 13C NMR (101 MHz, CDCl3) δ 168.24, 153.57, 150.70, 149.78, 143.36, 141.87, 137.21, 131.51, 129.78, 127.12, 124.21, 123.18, 119.37, 114.44, 113.62, 113.12, 78.93, 57.86, 52.77, 47.13, 43.94, 24.52, 21.66. HRMS: $C_{31}H_{33}N_6O_2S$ $[M+H]^+$ calcd. for 553.2380, found 553.2380.



Synthesis of probe 3

Compound S2 (341 mg, 1.00 mmol), pentafluorobenzenesulfonyl chloride (346 mg, 1.30 mmol) and triethylamine (202 mg, 2.00 mmol) were sequentially addedto dry acetonitrile (10 mL). The reaction mixture was stirred for 8 hours at room temperature. Subsequently, the solvent was removed under reduced pressure. The crude product was purified by column chromatography (V_{DCM}/V_{MeOH} = 10 / 1) to give probe 1 as brown solid (242 mg, 49 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.85 - 8.83 (m, 2H), 7.47 - 7.38 (m, 3H), 7.29 - 7.26 (m, 2H), 6.89 (d, J = 8.8 Hz, 1H), 6.84 (d, J = 8.8 Hz, 1H), 6.69 (dd, J = 15.6, 8.0 Hz, 1H), 3.54 - 3.50 (m, 6H), 3.40 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 13C NMR (101 MHz, CDCl3) δ 168.13, 153.35, 152.81, 150.76, 149.41, 149.17, 141.71, 141.61, 131.43, 131.36, 125.23, 124.89, 123.13, 123.10, 120.29, 119.97, 115.20, 114.85, 113.40, 113.28, 112.95, 112.84, 80.20, 79.76, 50.19, 47.89, 47.31, 45.34.

5. Spectroscopic analysis of the probes

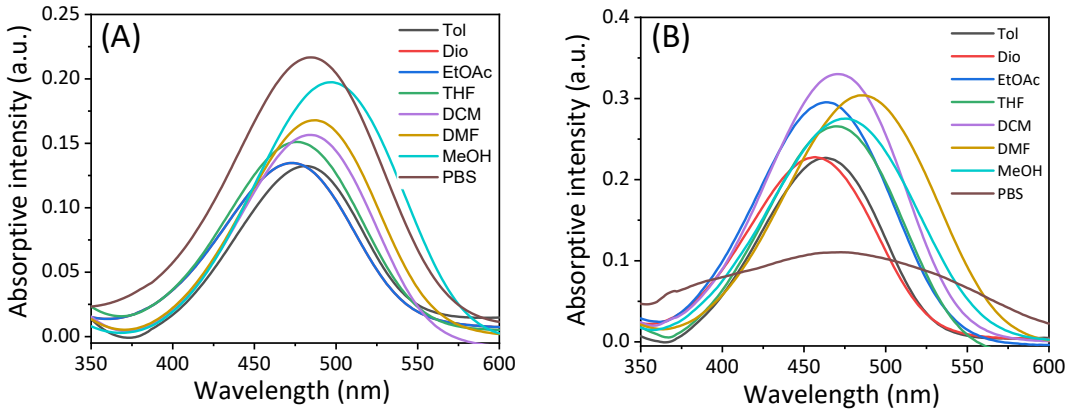


Fig. S1 The absorptive spectra of probe 2 (A, 5 μM) and probe 3 (B, 5 μM)in different solutions.

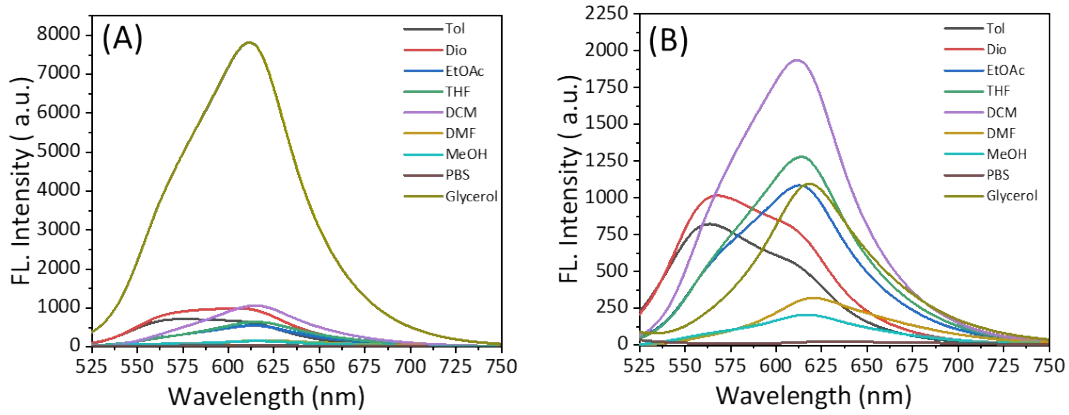


Fig. S2 The fluorescence spectra of probe 2 (A, 5 μM) and probe 3 (B, 5 μM) in different solutions.

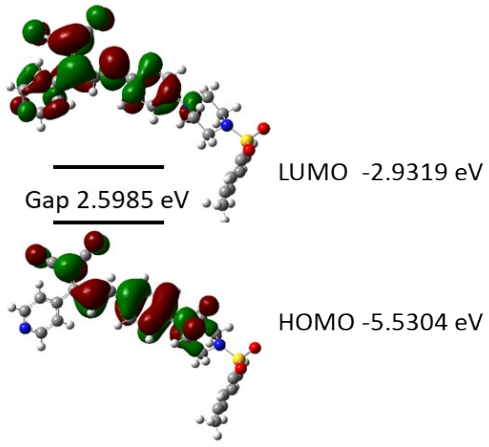


Fig. S3 Computational results of probe 1 in dichloromethane. The calculated distributions, energy levels (E_{HOMO} and E_{LUMO}) of HOMO and LUMO, energy gaps ($\Delta E = E_{LUMO} - E_{HOMO}$) of probe 1 at b3lyp/6-31G(d, p) level.

Table S1 Photophysical properties of probe 1 in different solutions

Solvent	Abs. (nm)	FL. (nm)	ε ($M^{-1}cm^{-1}$)	ϕ (%)	Stokes shift (cm)
Toluene	468	563	16492	0.048	95
Dioxene	461	567	23238	0.059	106
EtOAc	464	611	22898	0.04	147
THF	470	612	23852	0.049	142
DCM	474	610	26004	0.058	136
DMF	482	619	32402	0.012	137
MeOH	472	615	37478	0.007	143
PBS	472	619	11948	0.005	147
Glycerol	470	615	40308	0.570	145

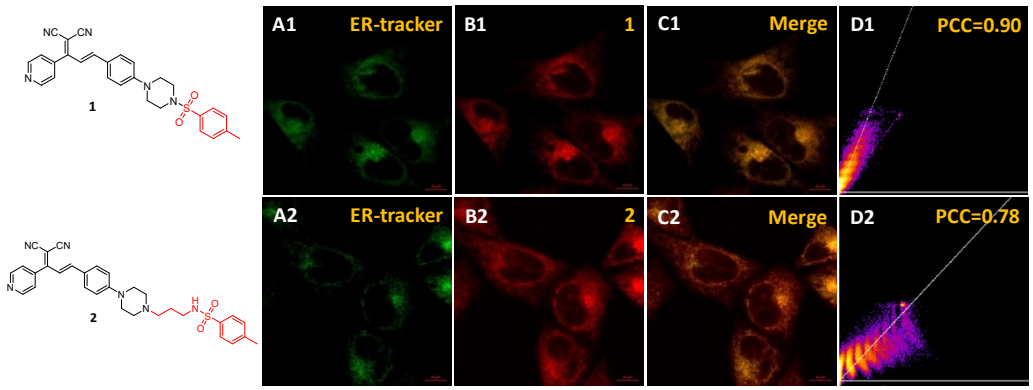


Fig. S4 The ER colocalization imaging in HeLa cells. (A) Green channel of ER-tracter (1 μm, λ_{ex} = 488 nm, λ_{em} = 500-550 nm); (B) Probe 1 and 2 (5 μm, λ_{ex} = 488 nm, λ_{em} = 600-650nm); (C) merged filed, (D) Scatter plot with PCC. Scale bar, 10 μm.

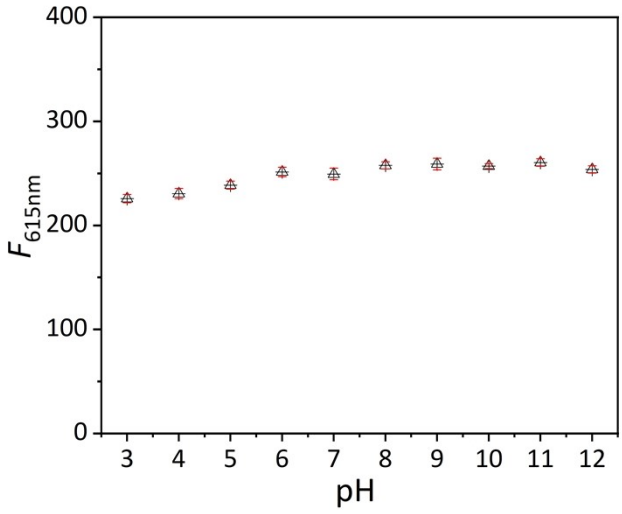


Fig. S5 The pH stability of probe 1 in a PBS-Gly mixture (1:1, v/v) across a range of pH values

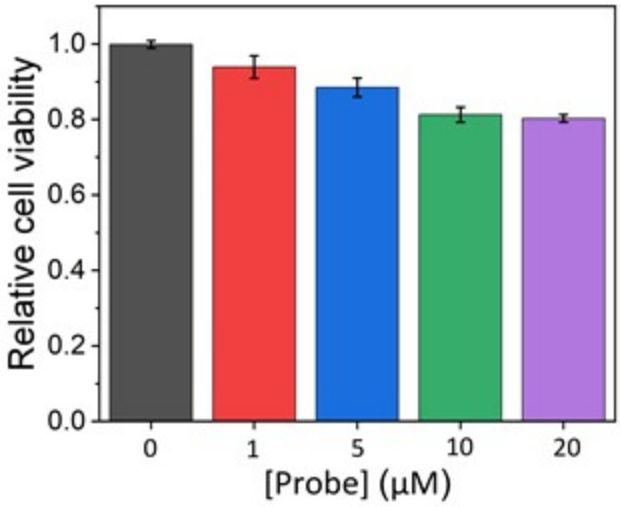


Fig. S6 The cytotoxicity of the probe 1 evaluated by MTT assay. Error bars are ± SEM.

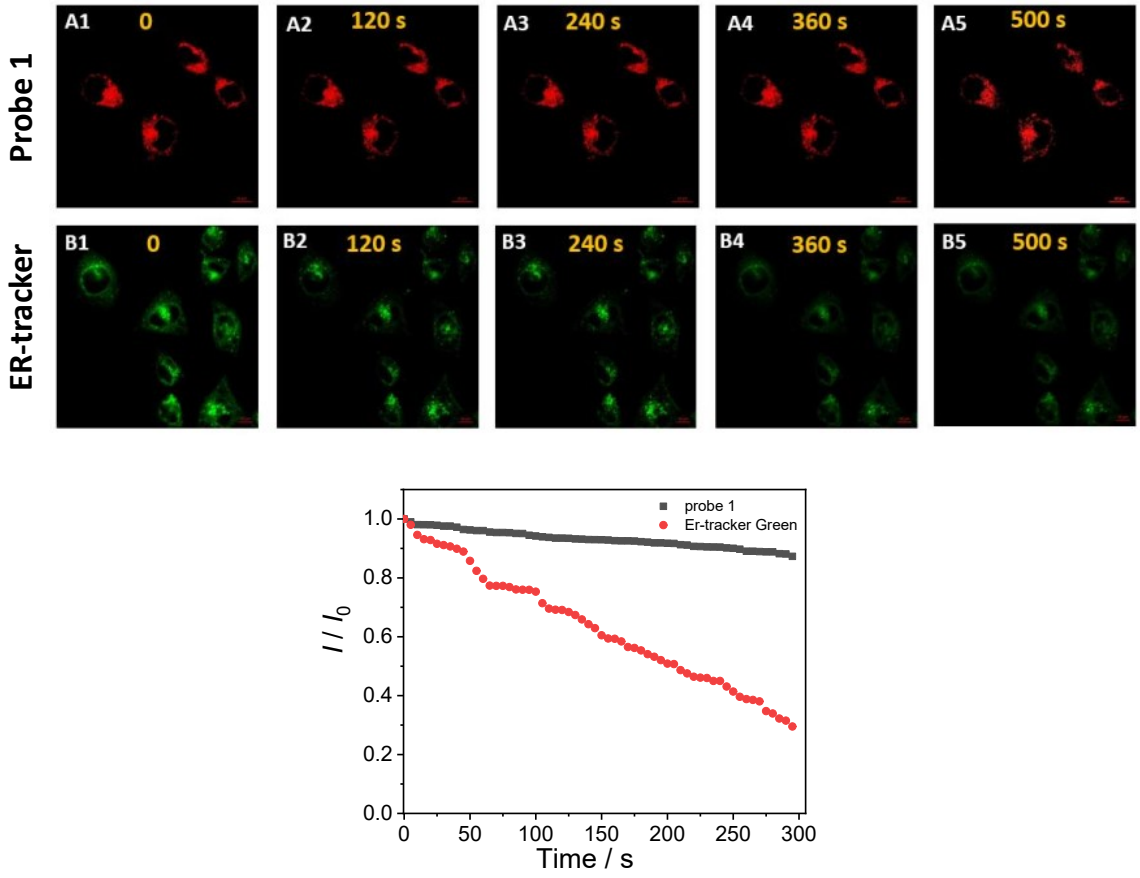


Fig. S7 The photostability of probe 1 (A, 5 μm, λ_{ex} = 488 nm, λ_{em} = 600-650 nm) and Er-tracker Green in HeLa cells during continuous laser irradiation (B, 5 μm, λ_{ex} = 488 nm, λ_{em} = 500-550 nm)

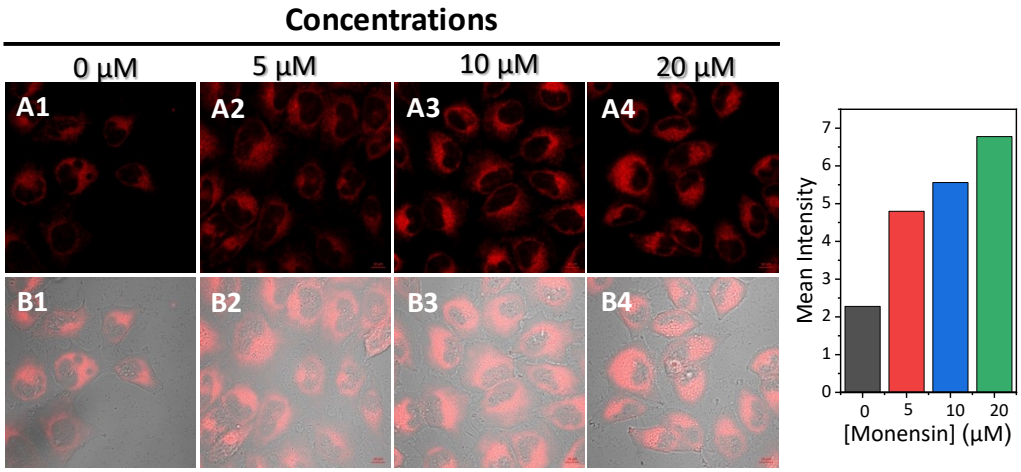


Fig. S8 Confocal fluorescence imaging of HeLa cells after treatment of Monensin (5 μm) for different concentrations and then stain with probe 1 (5 μm) for 30 min. Scale bar, 10 μm.

6. Supplementary spectra of NMR and HRMS

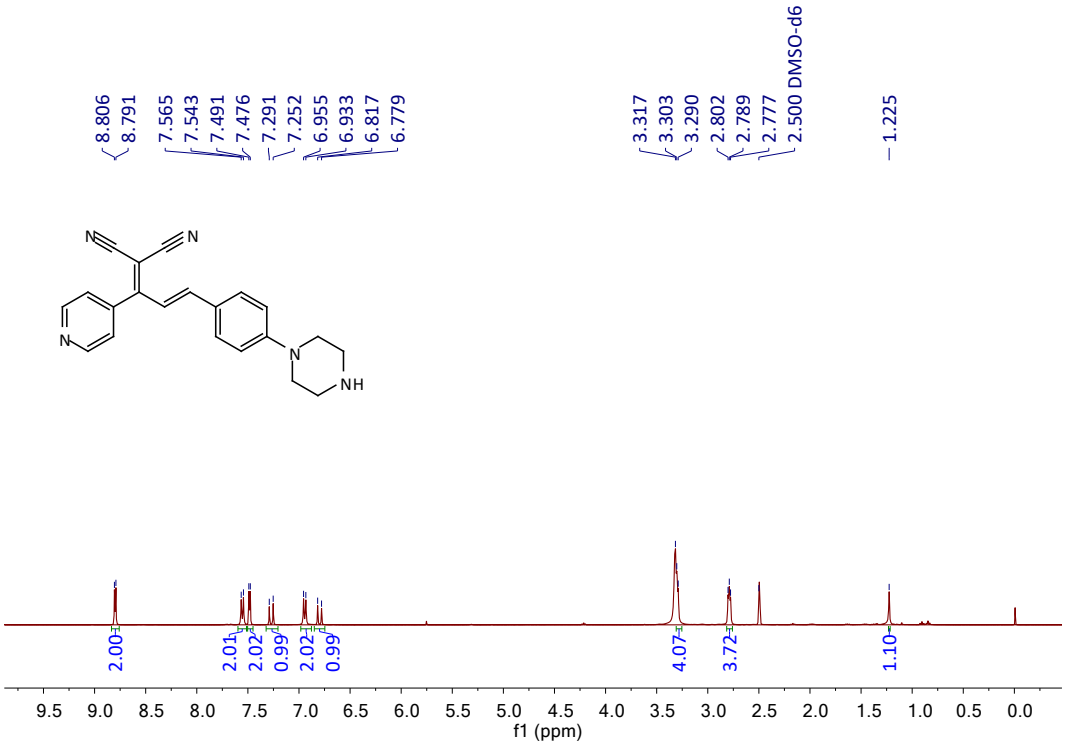


Fig. S9 ^{1}H NMR of compound S2

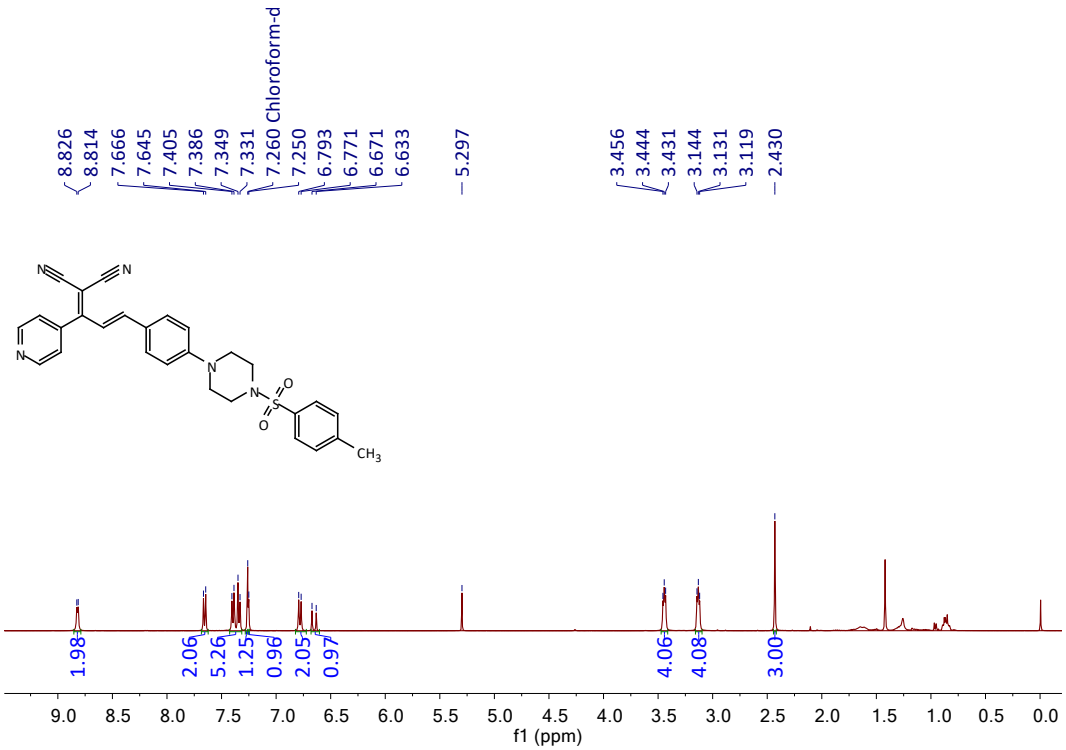


Fig. S10 ^{1}H NMR of probe 1

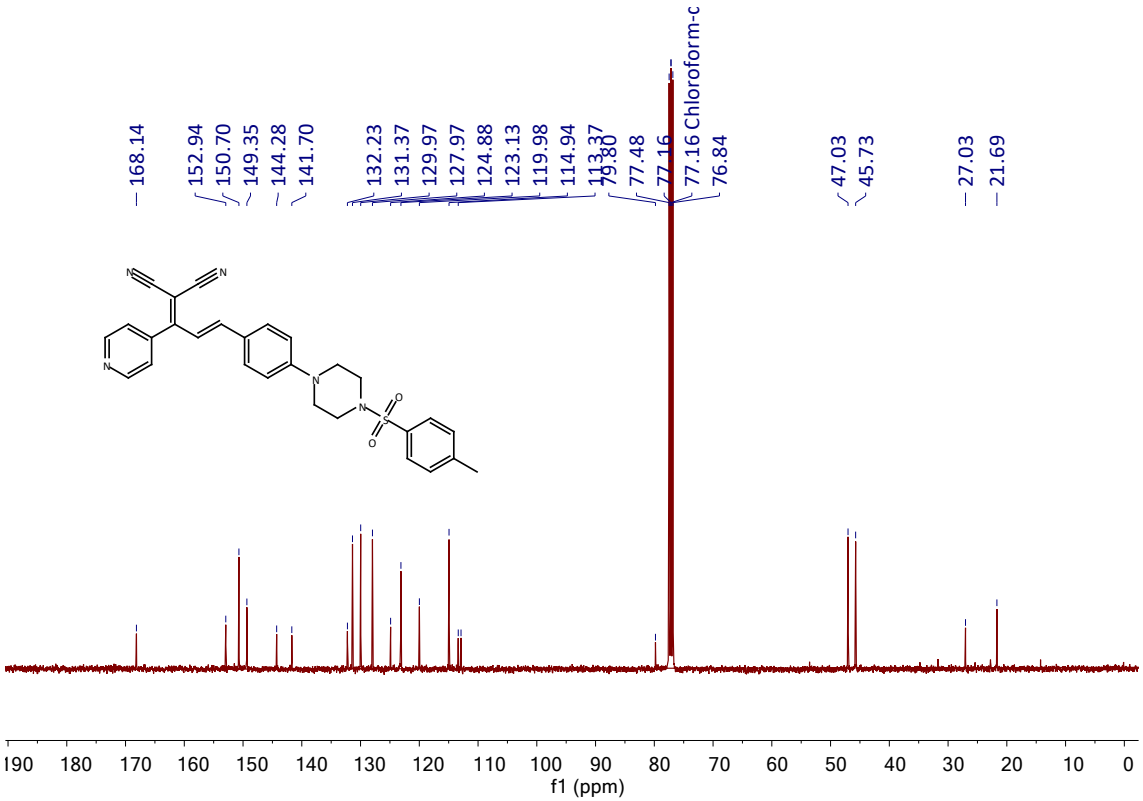


Fig. S11 ^{13}C NMR of probe 1

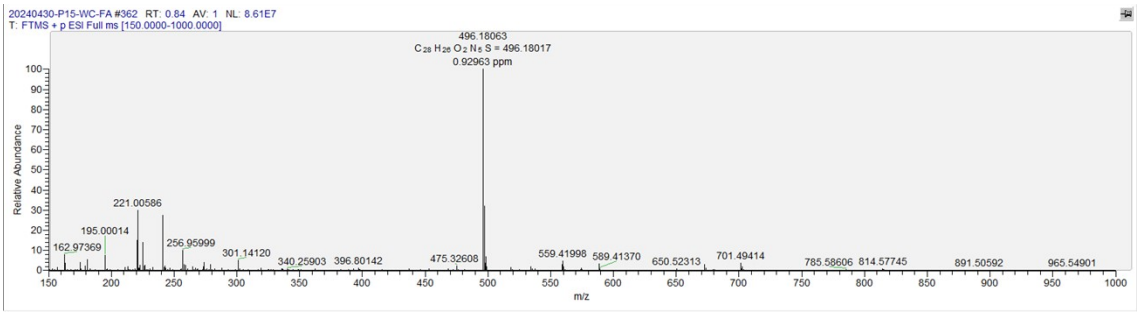


Fig. S12 HRMS of probe 1

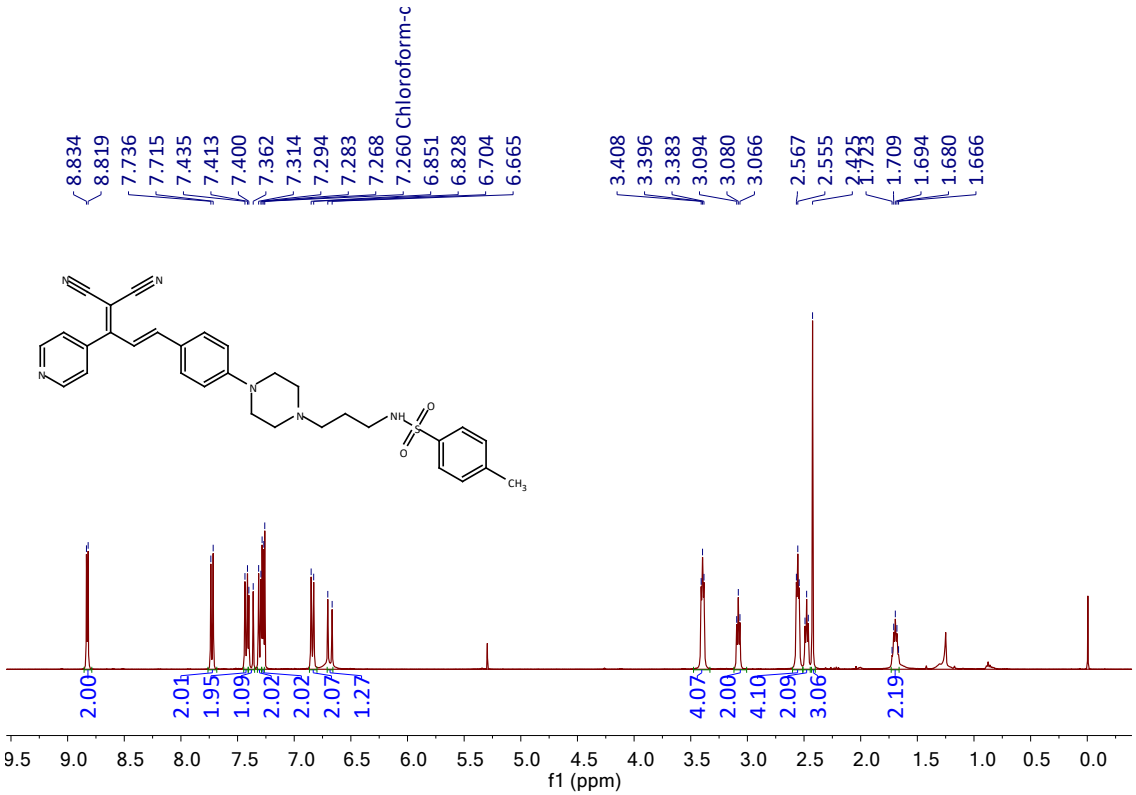


Fig. S13 ^{1}H NMR of probe 2

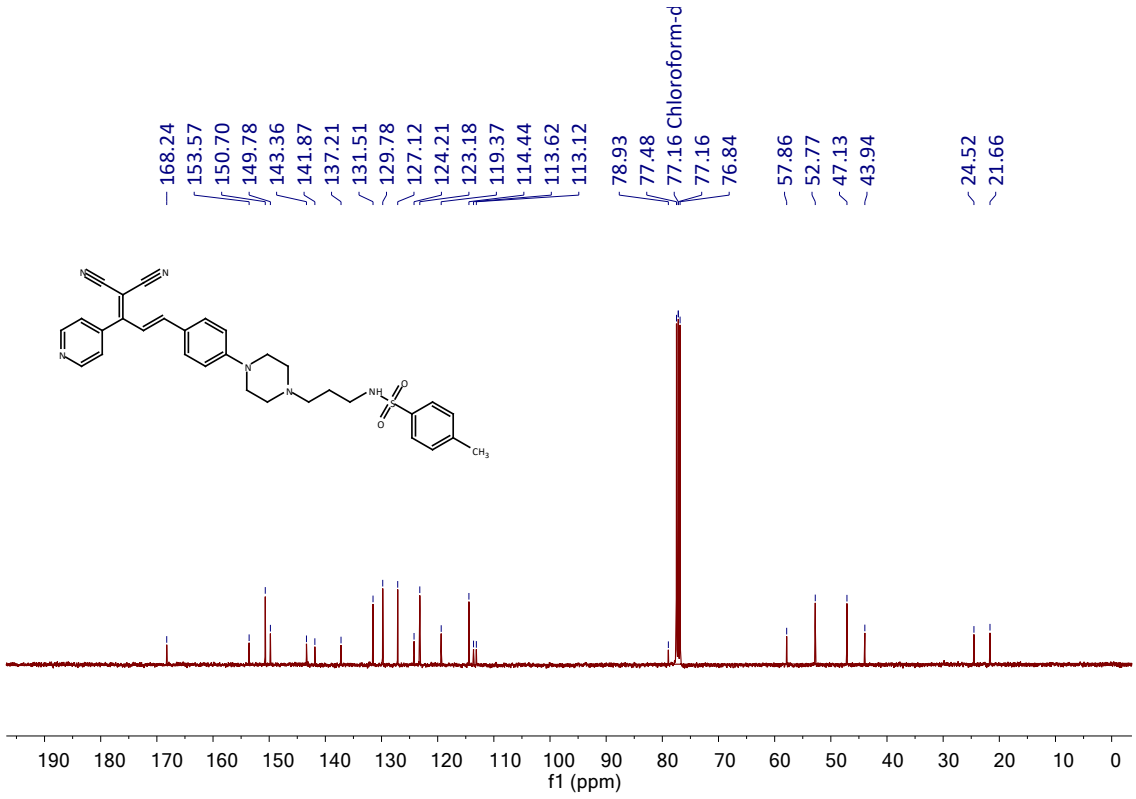


Fig. S14 ^{13}C NMR of probe 2

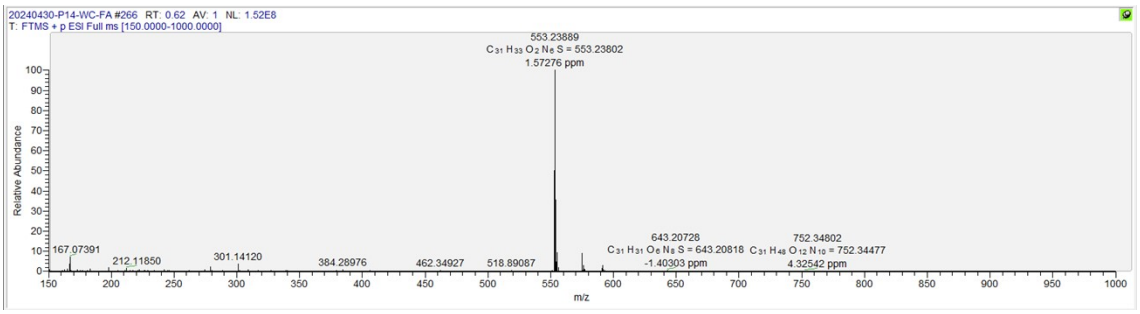


Fig. S15 HRMS of probe 2

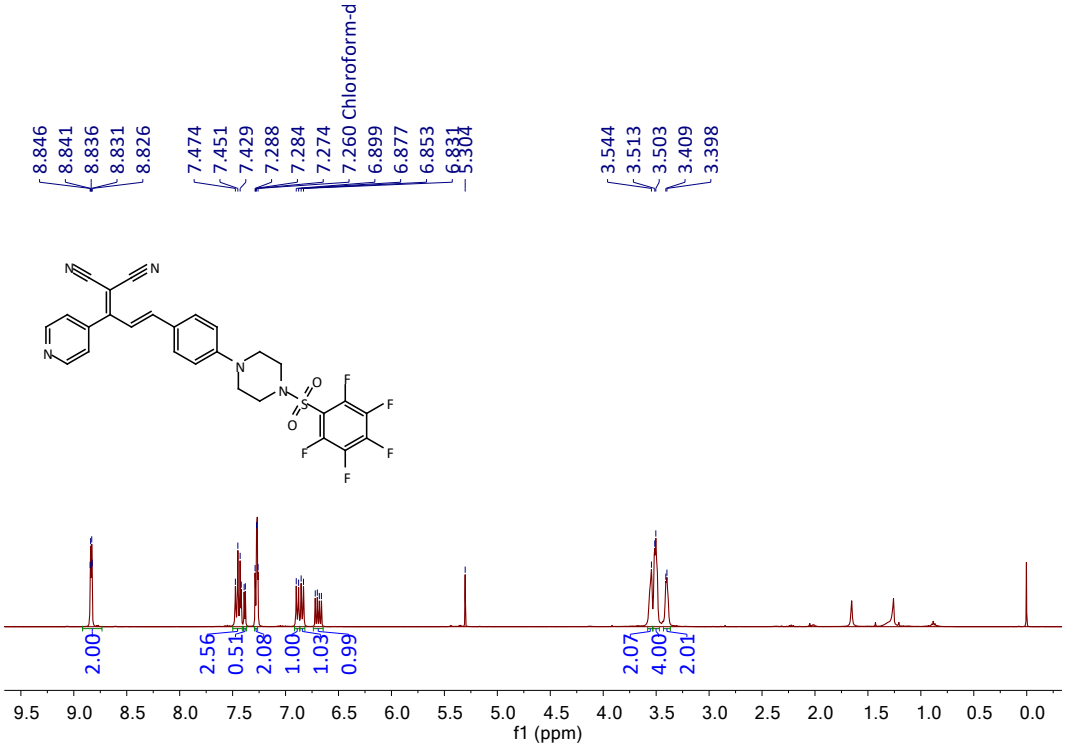


Fig. S16 ^{1}H NMR of probe 3

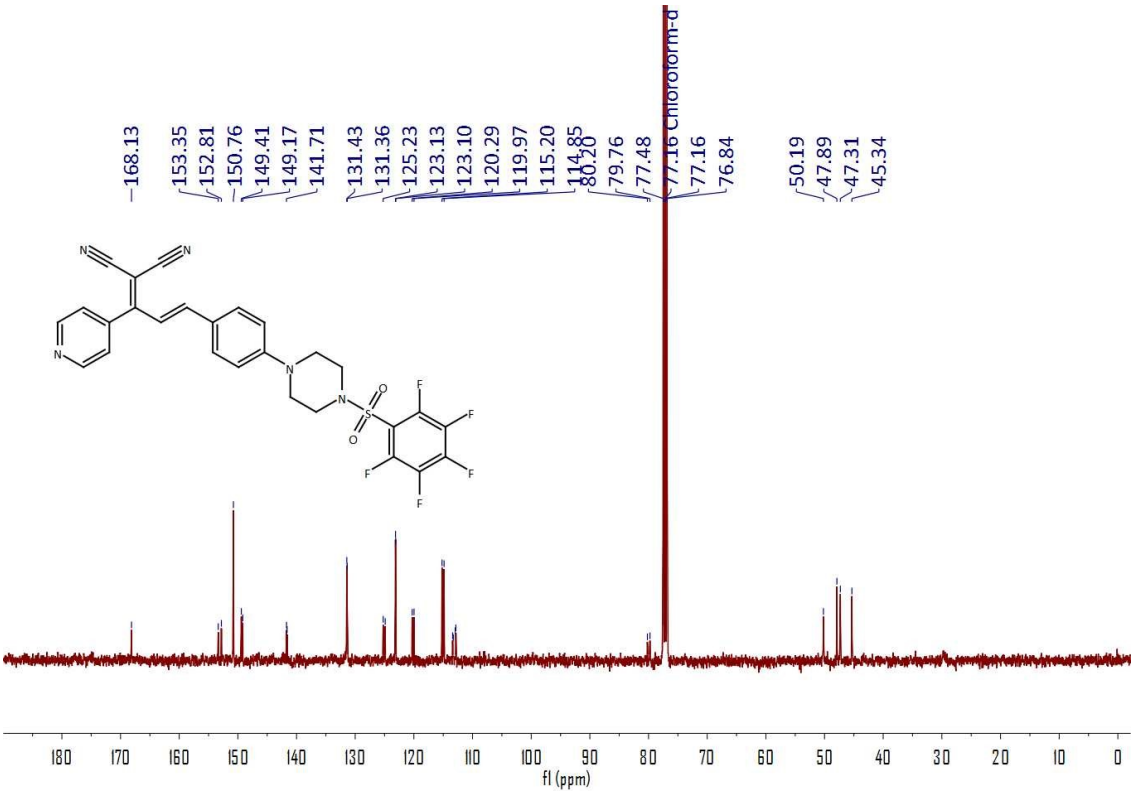


Fig. S17 ^{13}C NMR of probe 3