Supplementary Materials

Dual-functional fluorescent molecular rotor for endoplasmic reticulum microviscosity imaging during reticulophagy

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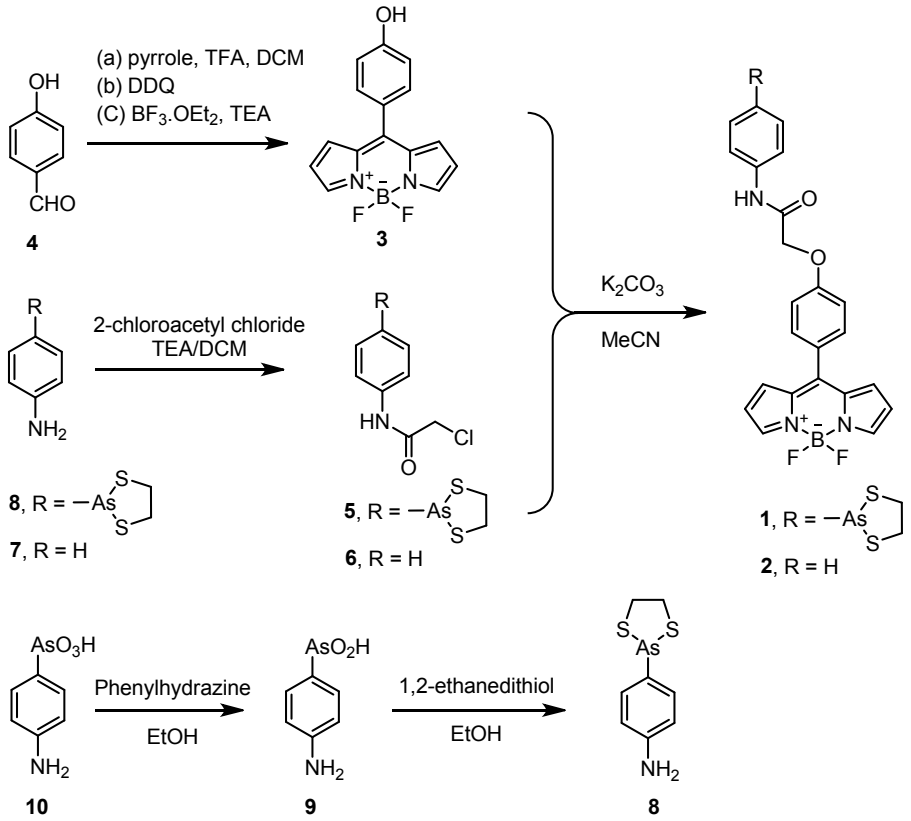
1. Materials and Methods

1.1 Synthesis and preparation of target compounds (1, 2)

In brief, initially, arsanilic acid was reduced with phenylhydrazine in refluxing ethanol to provide intermediate **10**, which was subsequently reacted with ethanedithiol to generate compound **9**. Compound **9** was further modified by treating it with chloroacetyl chloride and was then subjected to reaction with BODIPY to generate probe **1**. The reference probe **2** was made according to a similar procedure (See Scheme S1).

All reactions were performed in an oven-dried apparatus, and the solvent was freshly distilled. All reagents were purchased from commercial suppliers and used without further purification. A rotary evaporator was used to concentrate the reaction mixtures. Thin layer chromatography was performed using glass-backed sheets of silica gel and visualized under a UV lamp (254 nm and 365 nm). Column chromatography was performed to purify the compounds using silica gel 60 (200–400 mesh).



Scheme S1 Synthetic protocol of target compound and reference (**1, 2**)

Synthesis of 9

Arsanilic acid **10** (10.85 g, 50 mmol) was dissolved in EtOH (60 mL) and the solution was heated under reflux, phenylhydrazine (10.3 mL, 100 mmol) was added drop wise for 10 minutes. The solution was then heated under reflux for 1 h. After completion of the reaction, the mixture was concentrated, treated with water (85 mL), NaOH (0.1 M, 60 mL) and then washed with Ether (150 mL). The aqueous layer was treated with aqueous NH_4Cl solution (5 M, 40 mL) at 0 °C. Precipitate was then filtered and washed with ice-water (25 mL × 2) which gave **9** as white solid (3.86 g, 35% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.33 (d, J = 8.5 Hz, 2H, Ar-H), 6.61 (d, J = 8.4 Hz, 2H, Ar-H), 5.44 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 151.30, 135.90, 131.07, 113.76.

Synthesis of 8

To a solution of **9** (1.29 g, 6.42 mmol) in absolute ethanol (10 mL), 1,2-ethanedithiol (0.65 mL, 8.46 mmol) was added slowly, and then the solution was stirred for 10 min under reflux. The solution was cooled down to room temperature and concentrated to get a white solid. Recrystallization of the white solid from ethanol yielded compound **8** as white crystals (0.92 g, 56% yield). ^{1}H NMR (400 MHz, CD_3OD): δ 7.33 (d, J = 7.9 Hz, 2H, Ar-H), 6.65 (d, J = 7.9 Hz, 2H, Ar-H), 3.27 (m, 4H, S-CH_2). ^{13}C NMR (100MHz, CD_3OD): δ 130.85, 113.29, 40.2

Synthesis of 5

To a solution of **8** (4.5 g, 50 mmol) and TEA (9.8 mL, 70 mmol) in DCM (100 mL) was slowly added chloroacetyl chloride (6.1 g, 55 mmol) at 0 °C. The mixture was stirred at r.t. for another 2 h. After the completion it was quenched with water (70 mL) and extracted with DCM (40 mL × 3). The combined organic layer was washed with hydrochloric acid (2 M, 100 mL) and saturated solution of $NaHCO_3$ (100 mL) respectively, dried over Na_2SO_4 and concentrated to afford **5** as white solid (6.6 g, 78% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.29 (s, 1H), 7.57 (d, J = 7.4 Hz, 2H, N-H), 7.38 (t, J = 7.9 Hz, 2H, Ar-H), 7.19 (t, J = 7.4 Hz, 1H, Ar-H), 4.21 (s, 2H, CH_2). ^{13}C NMR

(100 MHz, $CDCl_3$) δ 163.84, 136.69, 129.14, 125.26, 120.16, 42.91.

Synthesis of 6

The synthesis of **6** is similar to **5.** To a solution of aniline **(7)** (1.3 g, 5 mmol) and TEA (1.0 mL, 7 mmol) in DCM (20 mL) was slowly added chloroacetyl chloride (0.6 g, 5.5 mmol) at 0 °C. The reaction mixture was then stirred at r.t. for 2 h and quenched with water (20 mL) and extracted with DCM (10 mL × 3). The combined organic layer was washed with hydrochloric acid (2 M, 10 mL) and saturated solution of $NaHCO_3$ (10 mL). The organic phase was then dried over Na_2SO_4 and concentrated to afford **6** (1.3 g, 82% yield). ^{1}H NMR (400MHz, $CDCl_3$): δ 8.27 (Br, 1H, N-H), 7.63 (d, J = 8.55 Hz, 2H, Ar-H), 7.55 (d, J = 8.55 Hz, 2H, Ar-H), 4.18 (s, 2H, Cl-CH_2), 3.35 (m, 2H, S-CH_2), 3.14 (bp, 2H, S-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.10, 140.27, 137.68, 131.87, 120.04, 43.09, 42.08.

Synthesis of 3

To a solution of 4-hydroxybenzaldehyde (**4)** (1.0 g, 8.2 mmol) in dry DCM (20 mL) was added and pyrrole (1.2 g, 18 mmol) under nitrogen atmosphere and a drop of trifluoroacetic acid (TFA) was added. The reaction mixture was then stirred at r.t. for 4 h for the complete consumption of 4-hydroxybenzaldehyde. At this point, a solution of 2, 3-dichloro-5, 6-dicyano-1, 4-benzoquinone (DDQ) (1.1 g, 8.2 mmol) in 20 mL dry DCM was added drop wise and stirring was continued for another 1 h followed by the addition of 5 mL Et_3N and 5 mL of $BF_3{\cdot}Et_2O$ respectively. After stirring for another 2 h the reaction mixture was washed with water, dried over Na_2SO_4 and evaporated to dryness to give a residue which on column chromatographic purification (silica gel; Hexane/Ethyl acetate, 1:2 v/v) provided **3** as a dark red solid (252 mg, 11% yield).
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.90 (s, 2H, Ar-H), 7.45 (d, J = 8.6 Hz, 2H, Ar-H), 6.97 (m, 4H, Ar-H), 6.55 (m, 2H, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.18, 147.86, 143.39, 135.00, 132.93, 131.78, 126.18, 118.53, 115.88.

Synthesis of 2

To a solution of **3** (284 mg, 1 mmol) in dry MeCN (50 mL) was added K_2CO_3 (495 mg,

5 mmol) and **6** (169 mg, 1 mmol). Then the reaction mixture was heated under reflux for 6 h. After completion, the reaction mixture was cooled down to room temperature, solid excess $K_2CO_3$was removed by filtration and the solution was evaporated to dryness. The crude product was purified with column chromatography (silica gel; CH_2Cl_2/Hexane 8:2 v/v) to give product **2** (133 mg, 32% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.26 (s, 1H, N-H), 7.97 (s, 2H, Ar-H), 7.65 (d, *J* = 2.6 Hz, 2H, Ar-H), 7.62 (d, *J* = 3.1 Hz, 2H, Ar-H), 7.41 (t, *J* = 7.8 Hz, 2H, Ar-H), 7.20 (m, 3H, Ar-H), 6.97 (d, *J* = 4.2 Hz, 2H, Ar-H), 6.58 (d, *J* = 2.6 Hz, 2H, Ar-H), 4.75 (s, 2H, CH_2). ^{13}C NMR (120 MHz, $CDCl_3$): δ 144.02, 132.57, 131.31, 129.21, 125.16, 120.22, 118.54, 114.95, 67.76.

Synthesis of 1

The synthesis procedure of compound **1** is very similar to **2**. To a solution of **3** (284 mg, 1 mmol) in dry MeCN (50 mL) was added K_2CO_3 (495 mg, 5 mmol) and **5** (334 mg, 1 mmol). Then the reaction mixture was heated under reflux for 6 hours. After cooling down to room temperature, excess solid K_2CO_3 was removed by filtration and the solution was evaporated to give a residue which was purified with column chromatography (silica gel; CH_2Cl_2/Hexane 8:2 v/v) to give product **1** (209 mg, 36% yield) ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.29 (s, 1H, N-H), 7.93 (s, 2H, Ar-H), 7.83 – 7.47 (m, 6H, Ar-H), 7.15 (d, *J* = 8.5 Hz, 2H, Ar-H), 6.93 (d, *J* = 4.3 Hz, 2H), 6.55 (d, *J* = 4.3, 2H, Ar-H), 4.70 (s, 2H, CH_2), 3.36 (m, 2H, S-CH_2), 3.17 (m, 2H, S-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.75, 159.23, 146.63, 144.19, 140.10, 137.69, 135.03, 132.77, 131.91, 131.54, 128.29, 120.13, 118.80, 115.11, 67.79, 42.08.

1.2 UV/Vis and fluorescence spectroscopy

Stock solutions of probe **1** (1 mM) were prepared in dimethyl sulfoxide. The absorption spectrum was recorded using the V-560 UV/VIS spectrophotometer (JASCO, Japan), and fluorescence spectra were recorded on the RF-5301 PC spectrofluorometer (Shimadzu) equipped with a xenon lamp. Other details regarding the excitation

wavelength and solution are given in the figure captions.

1.3 Formation of reduced and oxidized bovine serum albumin (rBSA and oBSA)

rBSA was obtained by treating a BSA solution with 1 mM dithiothreitol (DTT; Sigma–Aldrich) overnight at 4 °C, and oBSA was obtained by treating BSA with 100 μM H_2O_2 for 10 min at 25 °C. These samples were diluted 50 times with distilled water and the proteins were recovered by precipitation in 50% acetone for 2 h at –20 °C.

1.4 SDS-PAGE and fluorescence imaging of gels

The selectivity of compound **1** towards proteins or cells was verified by 10% SDS-PAGE. Samples were treated with probe **1** in de-ionized water or Dulbecco's modified Eagle medium (DMEM), and then incubated at 5% CO_2 with 95% humidity at 37°C for 1 h. After labeling, the samples were precipitated with 50% (v/v) acetone for 2 h at –20 °C and then mixed with SDS-PAGE loading buffer with Tris(2-carboxyethyl) phosphine hydrochloride (Sigma-Aldrich), and electrophoresis was started immediately. The gel was imaged by a fluorescent scanner (Typhoon FLA 9400, GE) at an excitation wavelength of 488 nm, with a band pass filter ranging from 500 nm to 540 nm. The same gel was stained by Coomassie brilliant blue (CBB) G250 after the fluorescent image was obtained. Other details regarding the excitation wavelength and emission filter are given in the figure captions.

1.5 Cell culture

Raw 264.7 cells (American Type Culture Collection, Manassas, VA, USA) and HeLa cells (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (WelGene), penicillin (100 units/mL), and streptomycin (100 mg/mL) under 5% CO_2 and 95% humidity at 37 °C.

1.6 Confocal microscopy

Co-localization of probe **1** with 1 μM ER-Tracker Red (BODIPY TR Glibenclamide),

Mito-Tracker Deep Red FM, and Lyso-Tracker Red DND-99 (all from Invitrogen, Oregon, USA) was detected using confocal microscopy. Probe **1** was excited at 488 nm, and fluorescence collected at 500–550 nm (peak at 516 nm); Lyso-tracker was excited at 577 nm, and fluorescence was collected at 586–659 nm (peak at 590 nm); ER-tracker was excited at 587 nm, and fluorescence was collected at 595–681 nm (peak at 615 nm); and Mito-Tracker was excited at 581 nm, and fluorescence was collected at 619–701 nm (peak at 644 nm). One day before imaging, the cells were seeded on a cover glass bottom dish (SPL Lifesciences Co., Ltd.), which was incubated in a humidified atmosphere containing 5% (v/v) CO_2 at 37 °C. Cell images were obtained using confocal laser-scanning microscopy (Leica, Wetzlar, Germany).

1.7 FLIM

Time-domain FLIM experiments were performed on a time-correlated single-photon counting (TCSPC) system (PicoQuant) attached to a Leica TCS SP8 microscope (Leica, Wetzlar, Germany) with a 100× 1.40 NA oil immersion objective. Excitation was achieved using a pulsed white light laser (Leica; 80 MHz pulsing) operating at 488 nm. Emission data from 500 nm to 530 nm were collected with a photomultiplier tube and processed by a PicoHarp 300 TCSPC system (PicoQuant, Berlin, Germany). Lifetime analysis was carried out using SymPhoTime 200 software. Bi-exponential fittings were applied.

1.8 Immunofluorescence imaging

HeLa cells were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.5% Triton X-100 for 15 min, and incubated with 5% BSA for 30 min at 37 °C. The cells were then incubated overnight with rabbit polyclonal anti-microtubule-associated protein light chain 3B (LC3B) antibody (1:1000, Abcam, Shanghai, China; catalog no. ab51520) at 4°C, followed by incubation with goat anti-rabbit IgG H&L (1:1000, Abcam, Alexa Fluor® 647, cat. No. ab150079) antibodies in a dark room at 37 °C for 120 min and then rinsed in phosphate-buffered saline (PBS).

2. Experiment results:

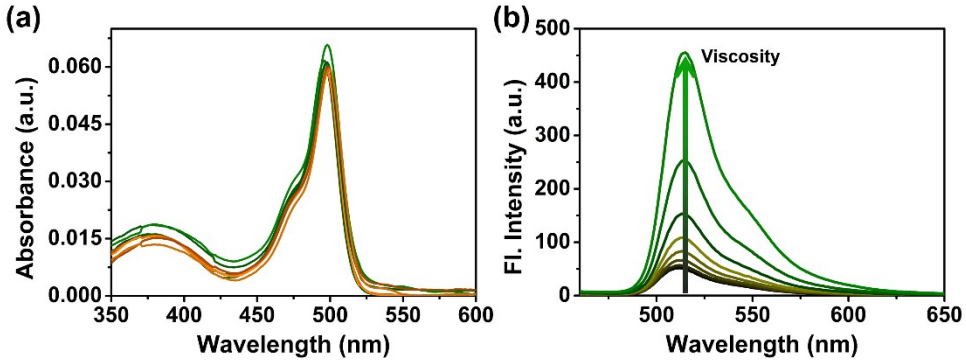


Fig. S1 (a) Absorbance spectra and (b) Fluorescence spectra of **1** (both spectra were conducted in different viscosity of solution in mixture of ethanol-glycerol, excited at 488 nm).

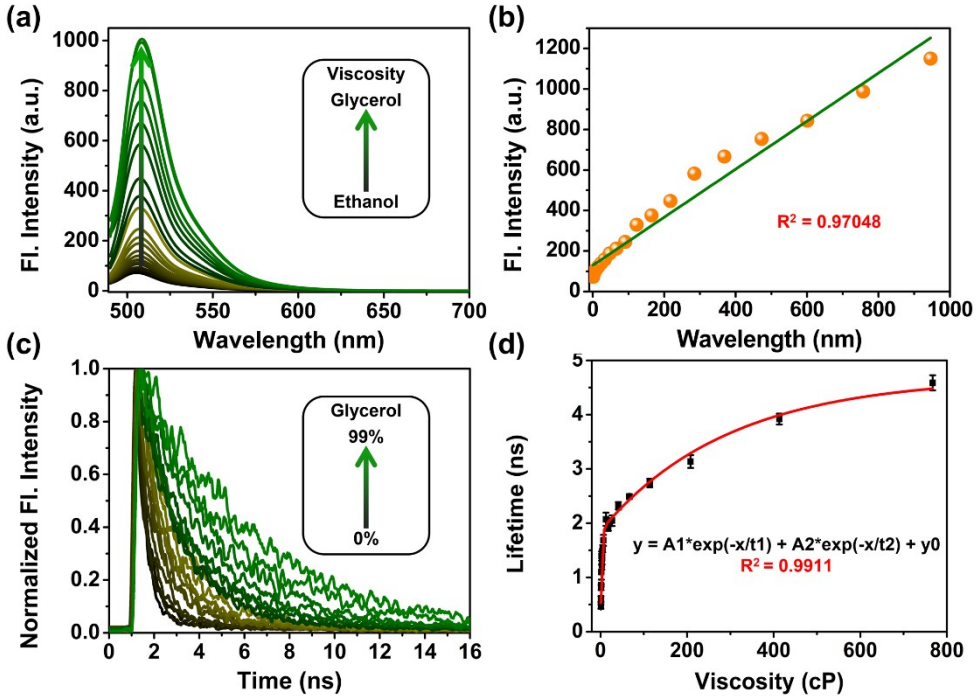


Fig. S2 (a) Fluorescence changes of probe **1** (2 μM) with the variations of solution viscosity in Ethanol-Glycerol. (b) Linear relationship between fluorescence intensity and viscosity of probe **1** (2 μM) in mixed solvents, excited at 488 nm. (c) Fluorescence decay of probe **1** (2 μM) in Ethanol-Glycerol and (d) Exponential relationship between fluorescence lifetime and solution viscosity of probe **1** (2 μM) in Ethanol-Glycerol respectively (Excited at 488 nm, detected at *ca.* 515 nm, using a time correlated single photon counting equipment (TCSPC) commercial DCS-120 system(Becker & Hickl, Germany)).

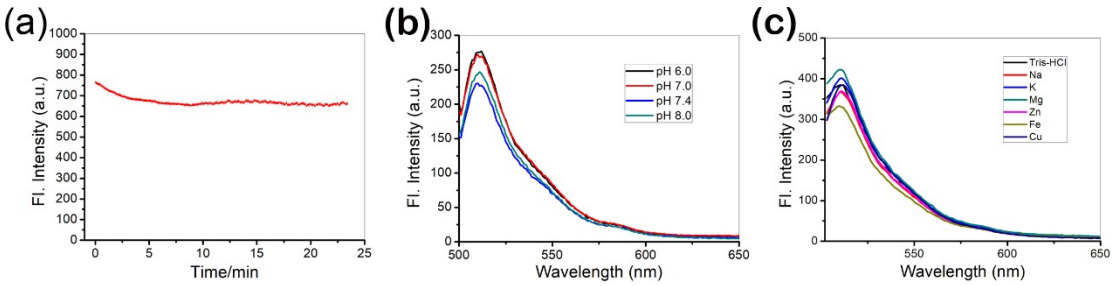


Fig. S3 (a) fluorescence stability assay of probe 1 under continuous light exposure (excited at 488 nm, detected at *ca.* 515 nm). (b) fluorescence intensity assay of probe 1 in the various pHs. (c) fluorescence intensity assay of probe 1 in the presence of biologically abundant metal ions (all the metal ions were diluted in 1M Tris-HCl solution separately, final concentration was 1 mM).

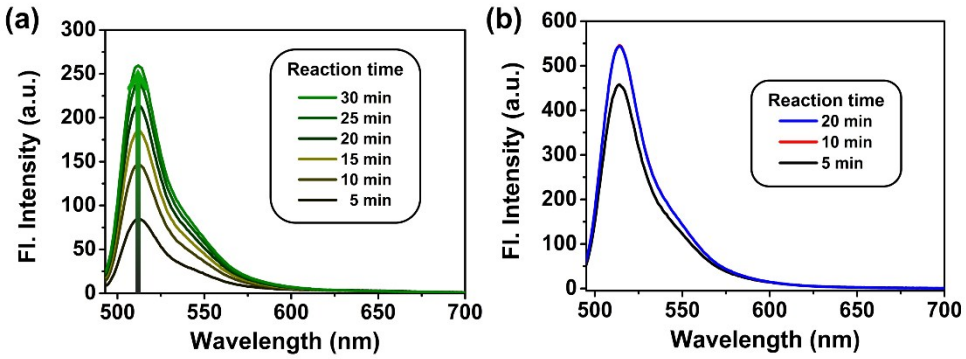


Fig. S4 (a) Fluorescence spectra of probe **1** (1 μM) and (b) probe **2** (1 μM) changing reaction time in the presence of rBSA.

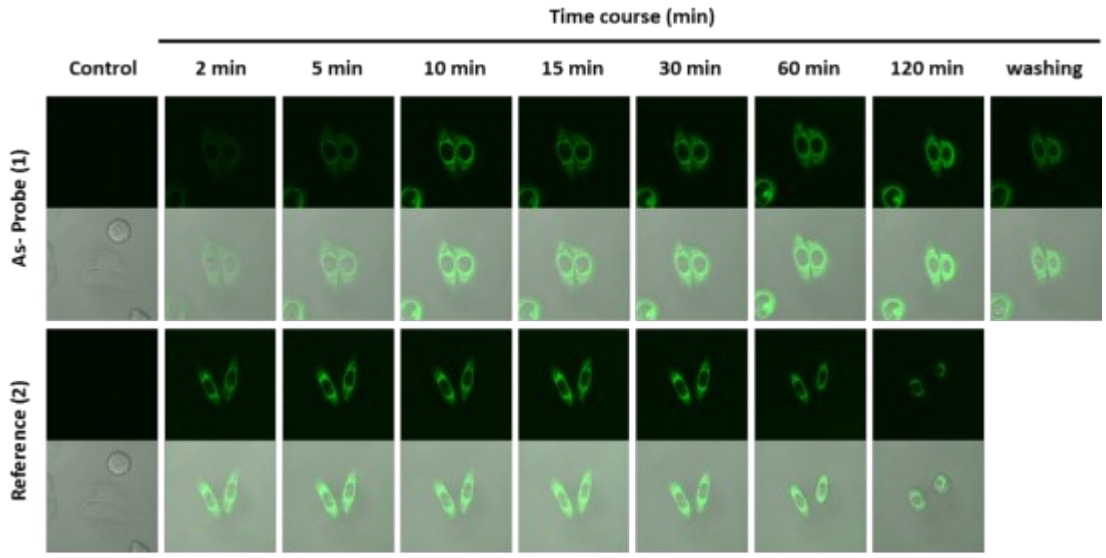


Fig. S5 Fluorescence imaging of probe **1** and **2** in HeLa cells with different incubating time and the cells were washed with PBS×1. Both probes incubated in DMEM with NCS for 24 hours.

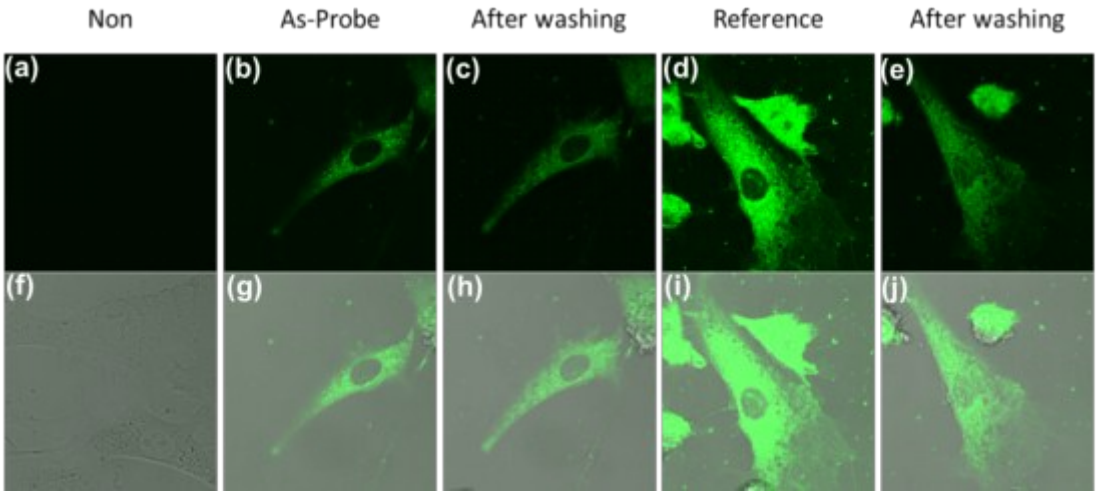
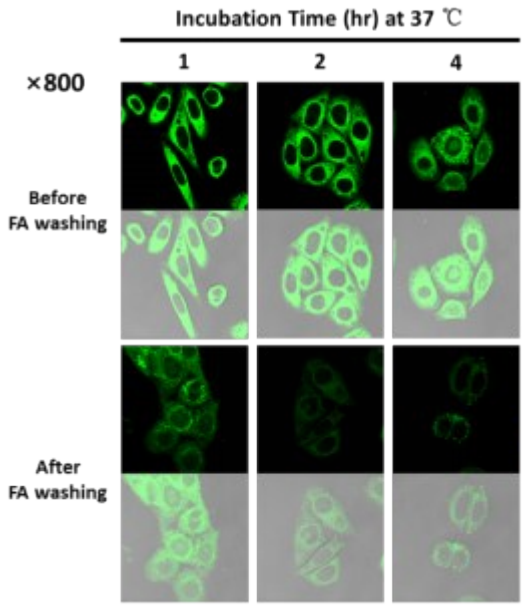
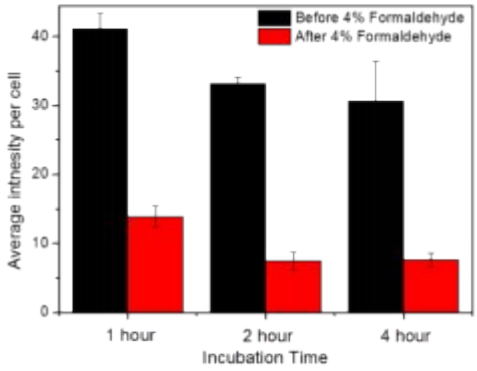


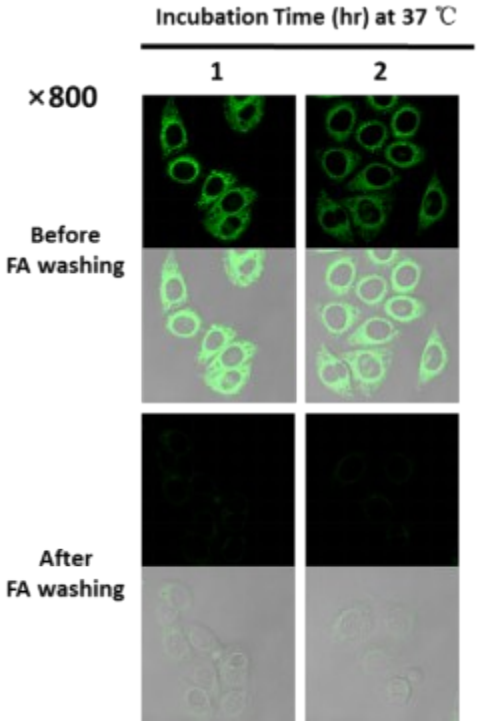
Fig. S6 Pre-adipocytes were treated with (a) blank, (b) probe **1** (1 μM) and (d) reference probe **2** (1 μM). After treated with (c) As-probe and (e) reference probe, washed with PBS×1. Panel f-j was the merged images by fluorescent images and DIC figures. Both probes incubated in DMEM with NCS for 24 hours; at 18 °C circumstances, time course confocal images were obtained following the excitation at 488 nm and 505 nm long pass emission filters.

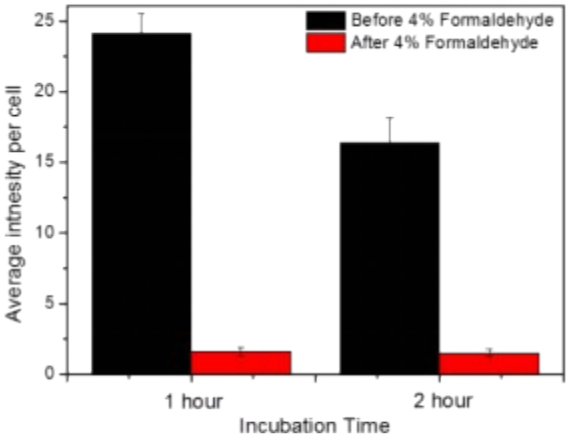




Incubation Time (hr)	Decreasing rate
1	66.14
2	77.33
4	75.07

Fig. S7 Fluorescence changes of probe **1** (1 μM) in HeLa cells before and after washed by 4% formaldehyde solutions.





Incubation Time (hr)	Decreasing rate
1	93.26 %
2	90.64 %

Fig. S8 Fluorescence changes of probe **2** (1 μM) in HeLa cells before and after washed by 4% formaldehyde solutions.

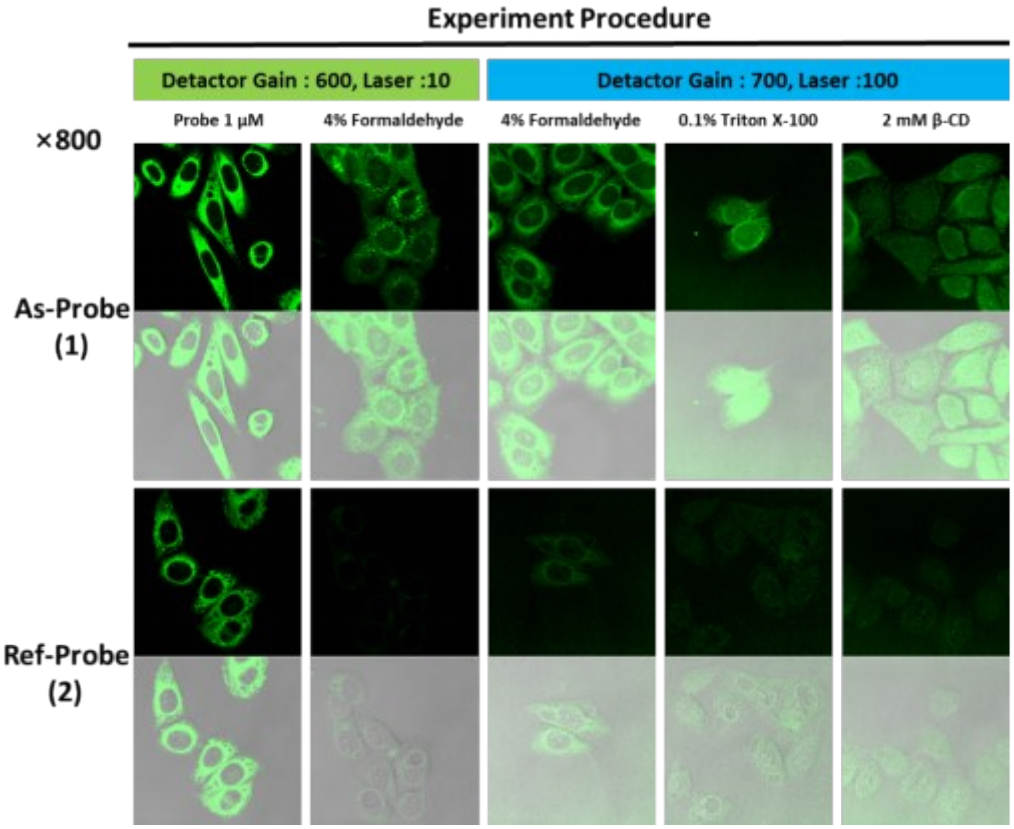


Fig. S9 Fluorescence changes of probe **1** and **2** (1 μM) in HeLa cells treated by different reagents.

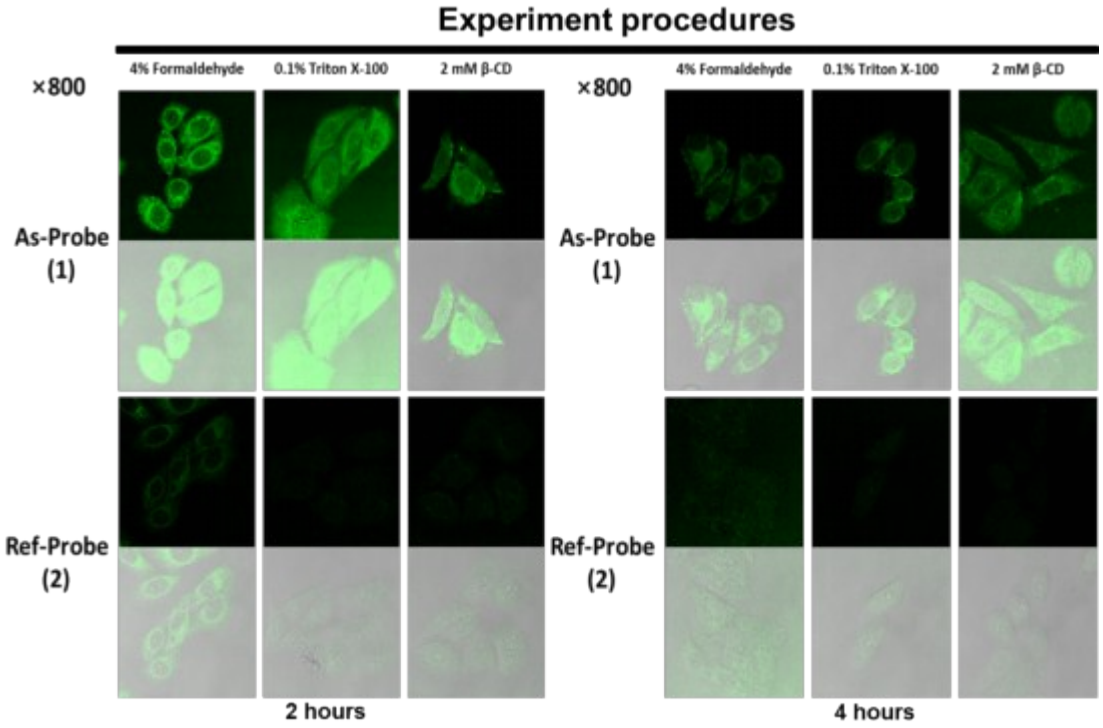


Fig. S10 Fluorescent changes of probe **1** and **2** (1 μM) in HeLa cells washed by different reagents under 2 hours' treatment (Left), 4 hours' treatment (Right), respectively.

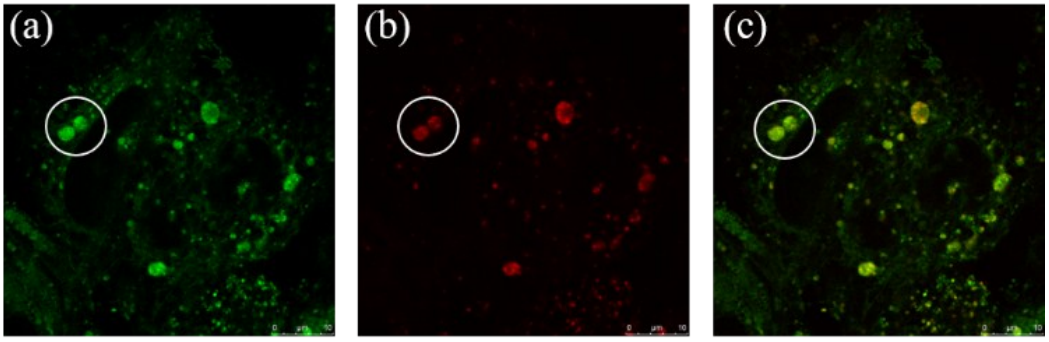


Fig. S11 Colocalization of probe **1** and lyso-tracker. After 1 hour, part of probe **1** was located in lysosomes, Pearson's coefficient r = 0.82. (a) Fluorescence imaging of probe **1**; (b) Fluorescence imaging of lyso-tracker; (c) Merged picture of (a) and (b). (a) excited at 488 nm, fluorescence collected at 500-550 nm (peak at 516 nm); (b) excited at 577 nm, fluorescence collected at 586-659 nm (peak at 590 nm); (a)-(c), scale bar = 10 μm.

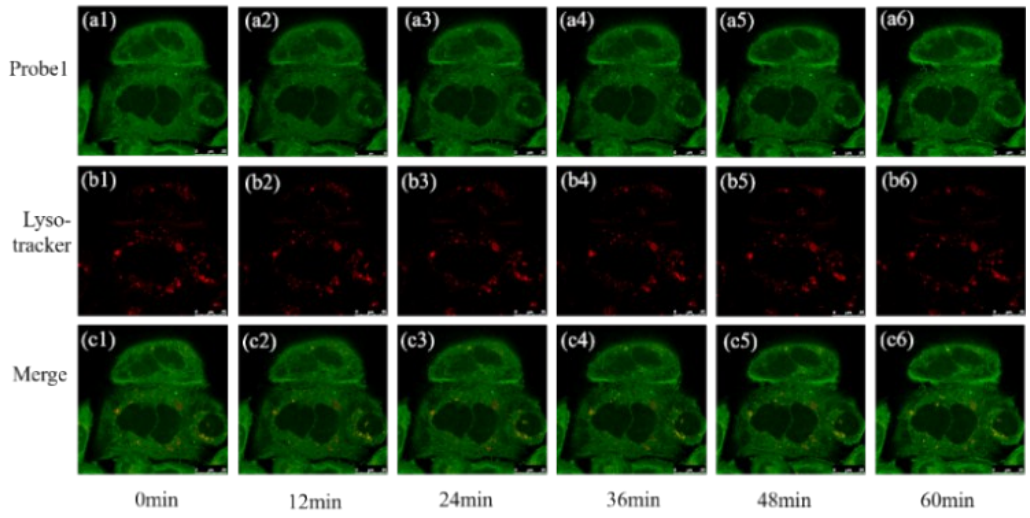


Fig. S12 Time-lapse imaging of probe **1** (1 μM) and Lyso-Tracker in HeLa cells. (a1)–(a6) Probe **1** signal changes in HeLa cells, excited at 488 nm; fluorescence collected at 500–550 nm. (b1)-(b6) Lyso-Tracker signal changes in HeLa cells, excited at 577 nm; fluorescence collected at 586–659 nm. (c1)– (c6) Merged images.

3. Structural Identifications of Target compounds

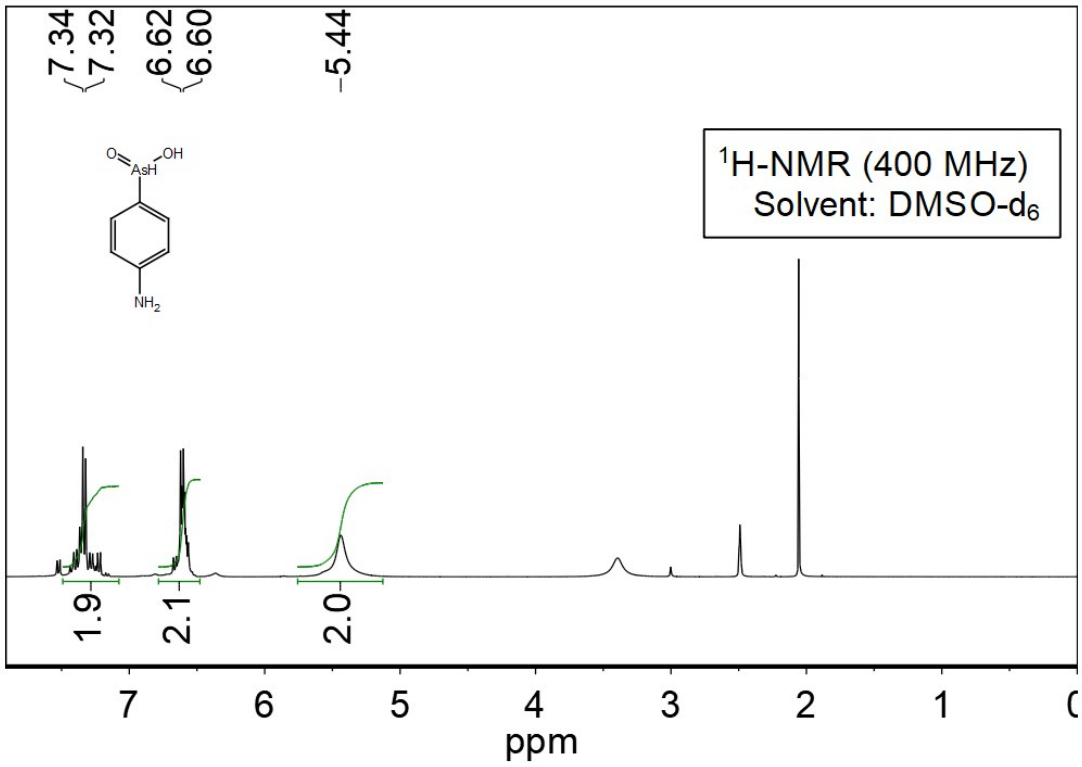


Fig. S13 ^{1}H NMR (298 K, 400 MHz, DMSO-d_6) spectrum for **10.**

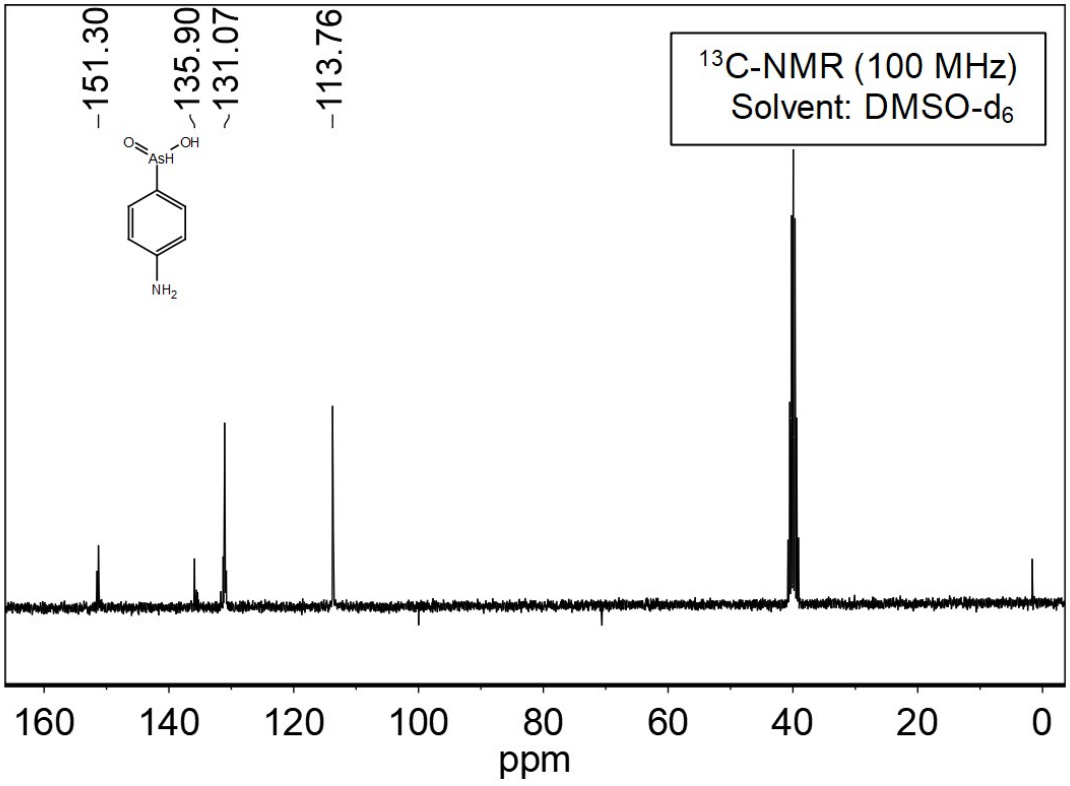


Fig.S14 ^{13}C NMR (298 K, 400 MHz, DMSO-d_6) spectrum for **10.**

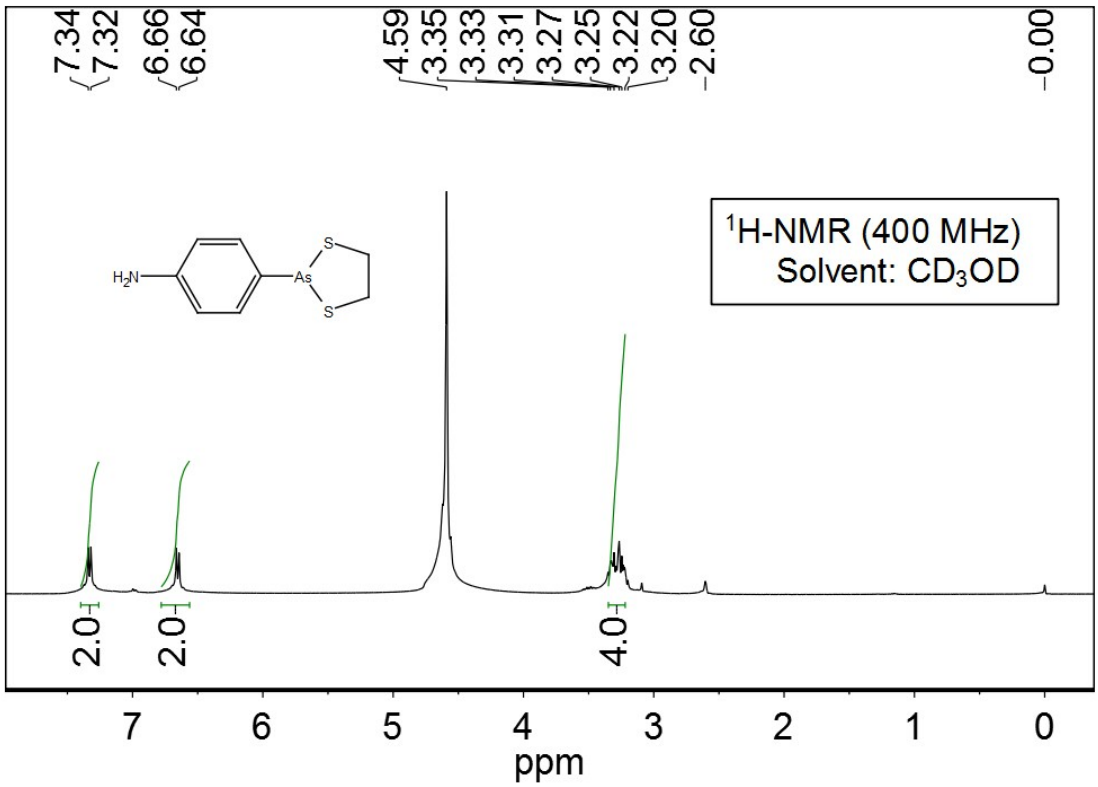


Fig. S15 ^{1}H NMR (298 K, 400 MHz, Methanol-d_4) spectrum for **9.**

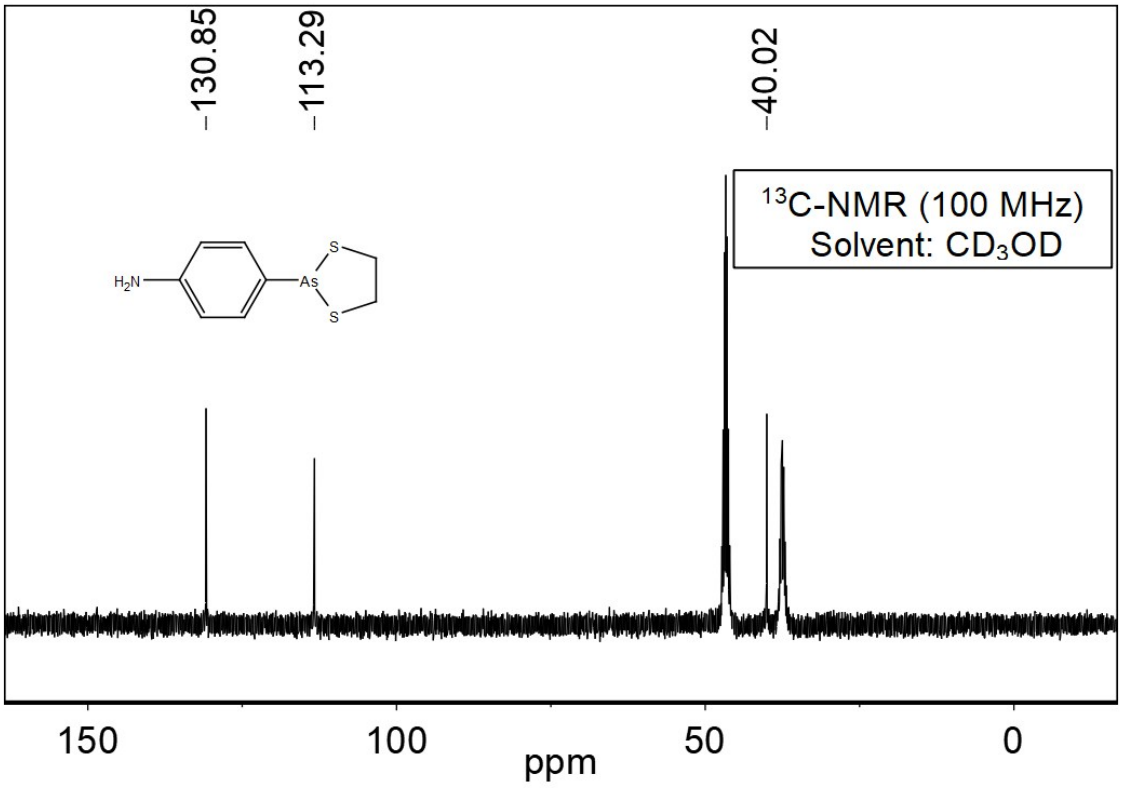


Fig. S16 ^{13}C NMR (298 K, 100 MHz, Methanol-d_4) spectrum for **9.**

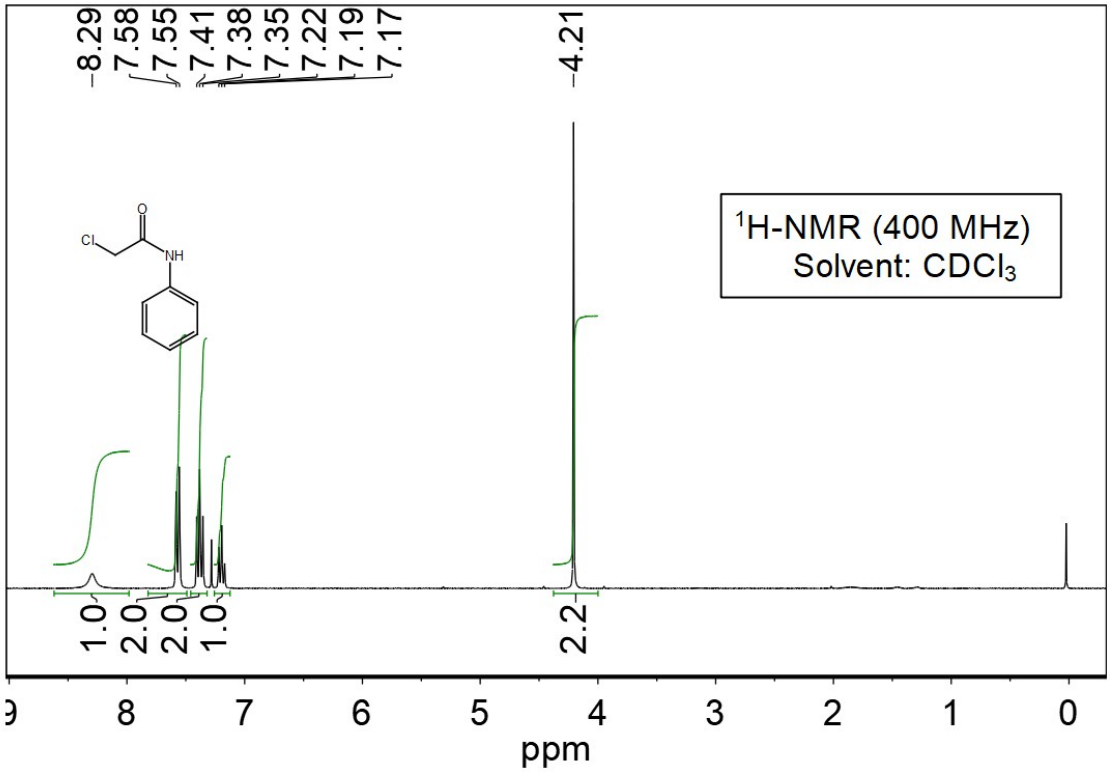


Fig. S17 ^{1}H NMR (298 K, 400 MHz, $CDCl_3$) spectrum for **6.**

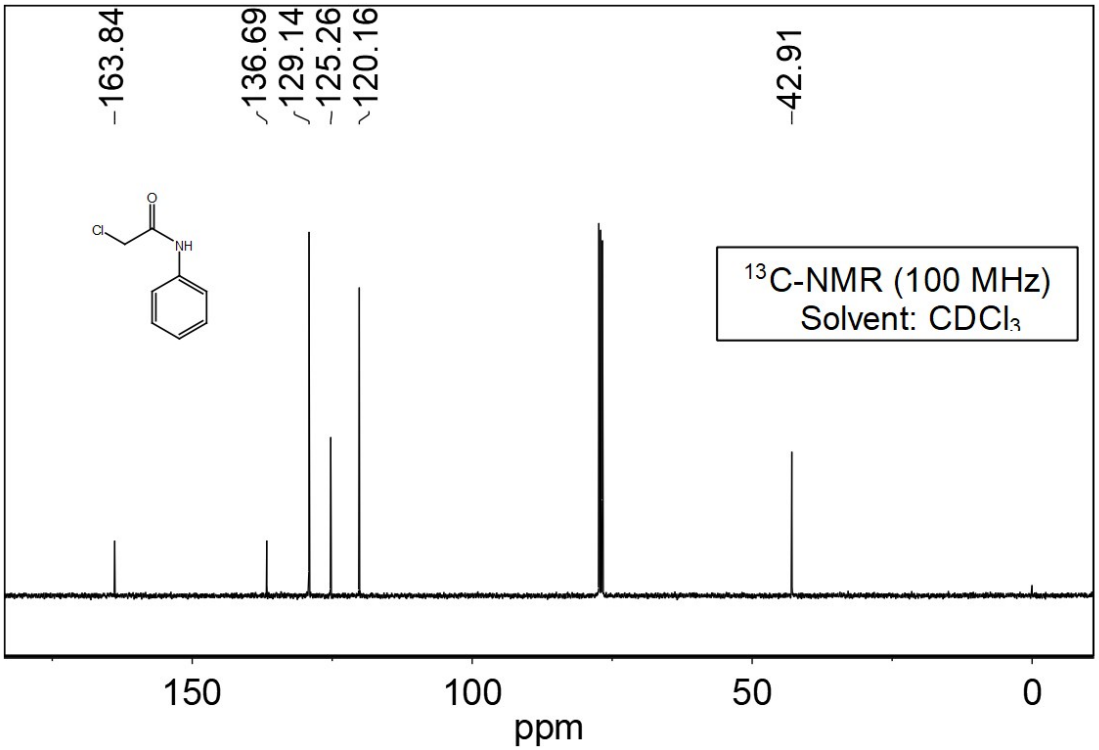


Fig. S18 ^{13}C NMR (298 K, 400 MHz, $CDCl_3$) spectrum for **6.**

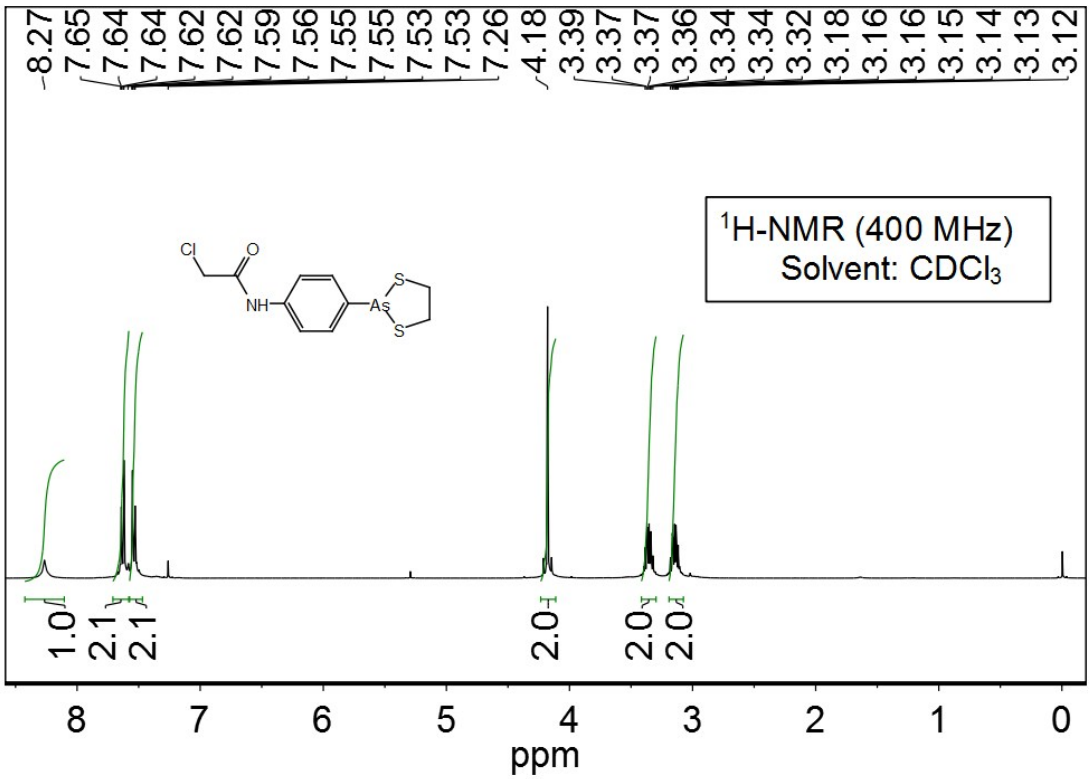


Fig. S19 ^{1}H NMR (298 K, 400 MHz, $CDCl_3$) spectrum for **5.**

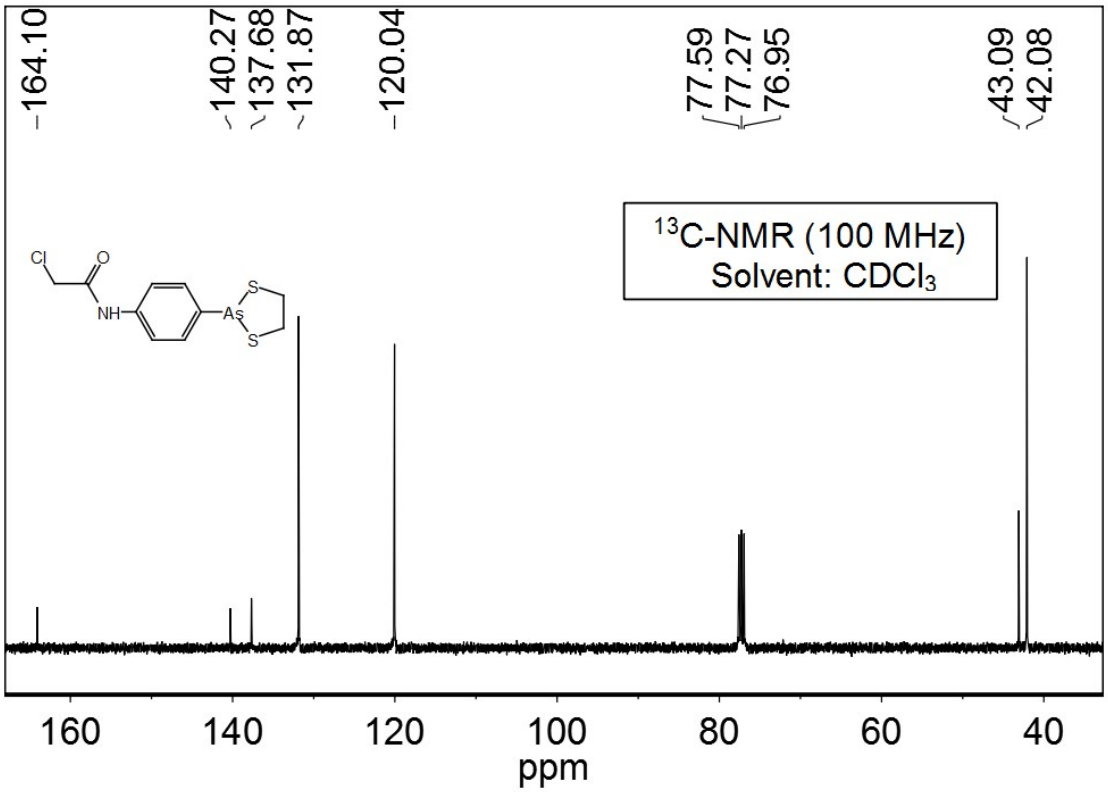


Fig. S20 ^{13}C NMR (298 K, 100 MHz, $CDCl_3$) spectrum for **5.**

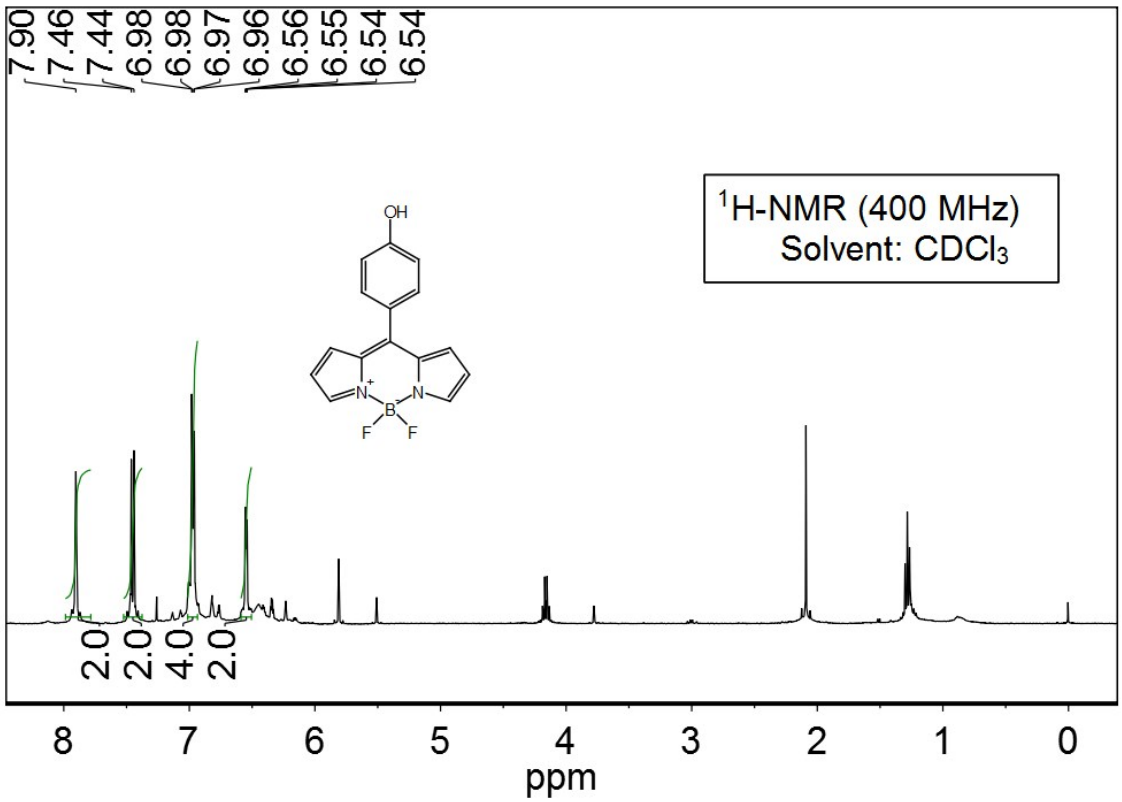


Fig. S21 ^{1}H NMR (298 K, 400 MHz, $CDCl_3$) spectrum for **3.**

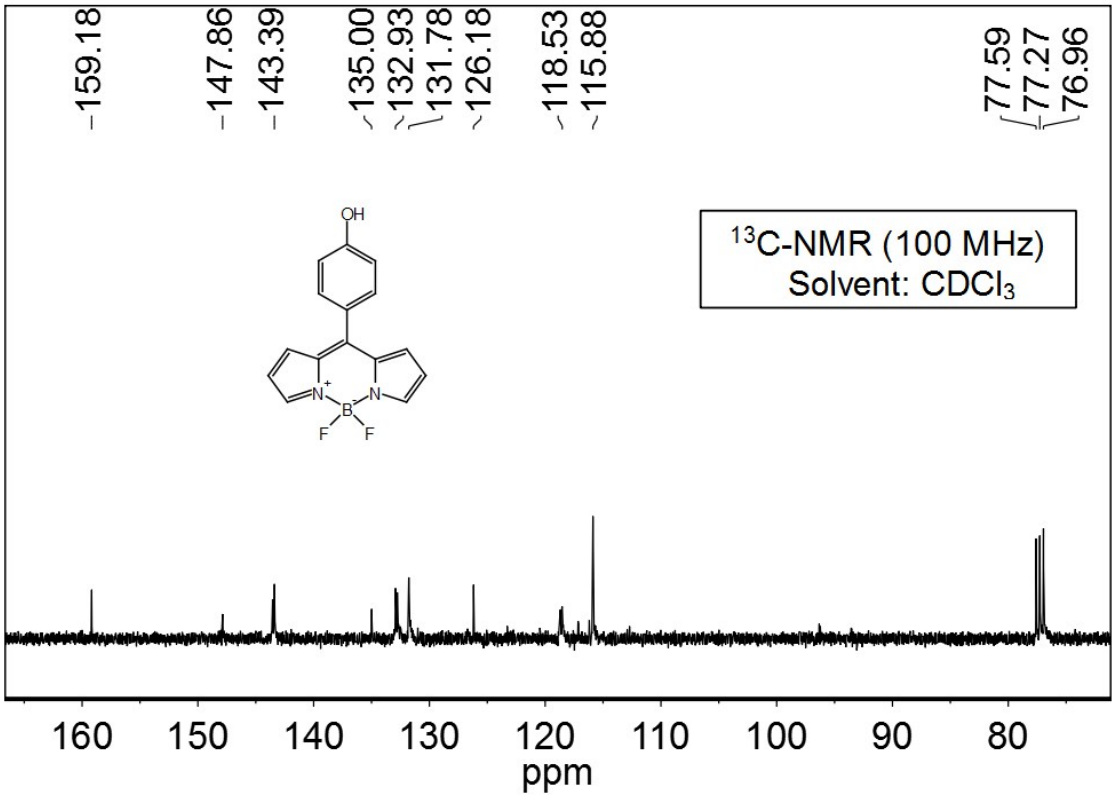


Fig. S22 ^{13}C NMR (298 K, 100 MHz, $CDCl_3$) spectrum for **3.**

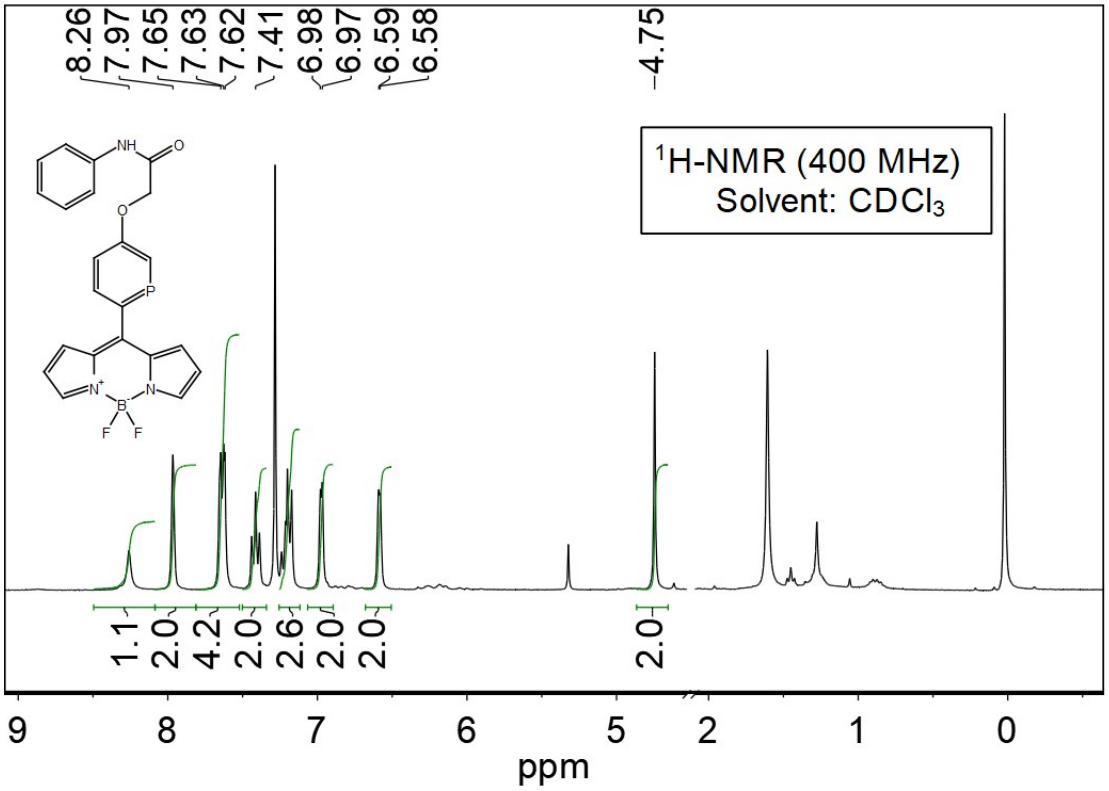


Fig. S23 ^{1}H NMR (298 K, 400 MHz, $CDCl_3$) spectrum for **2.**

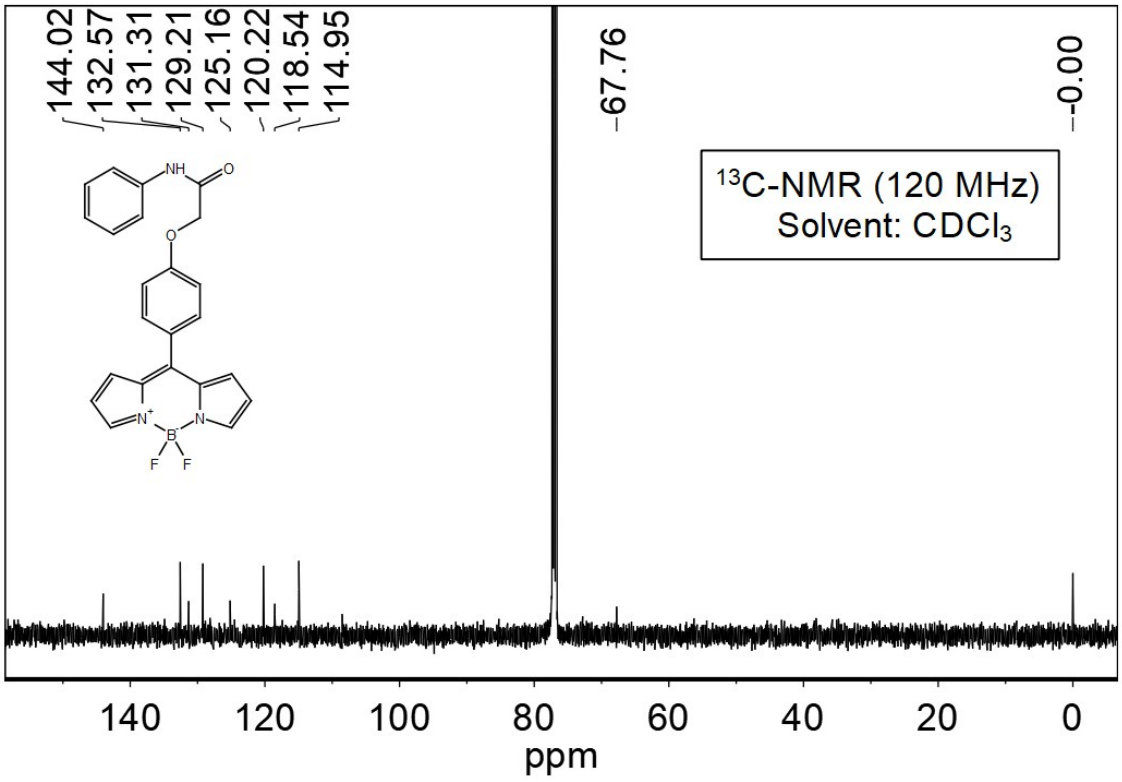


Fig. S24 ^{13}C NMR (298 K, 100 MHz, $CDCl_3$) spectrum for **2.**

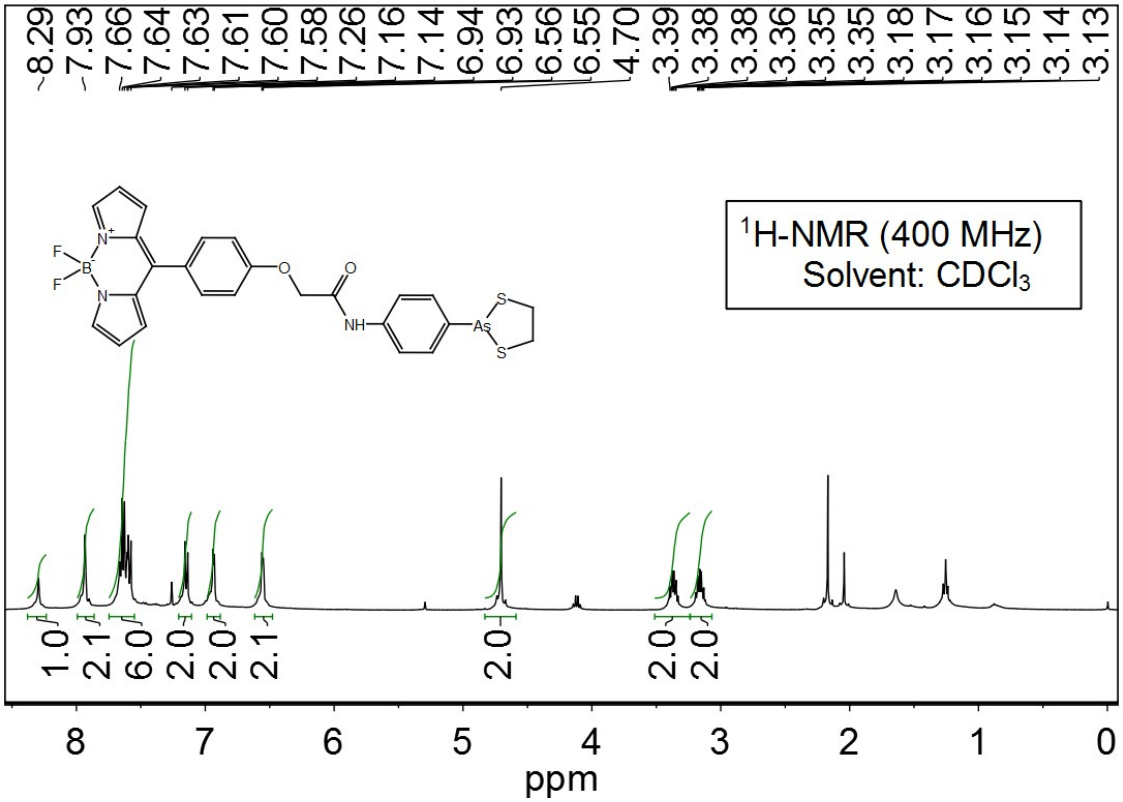


Fig. S25 ^{1}H NMR (298 K, 400 MHz, $CDCl_3$) spectrum for **1.**

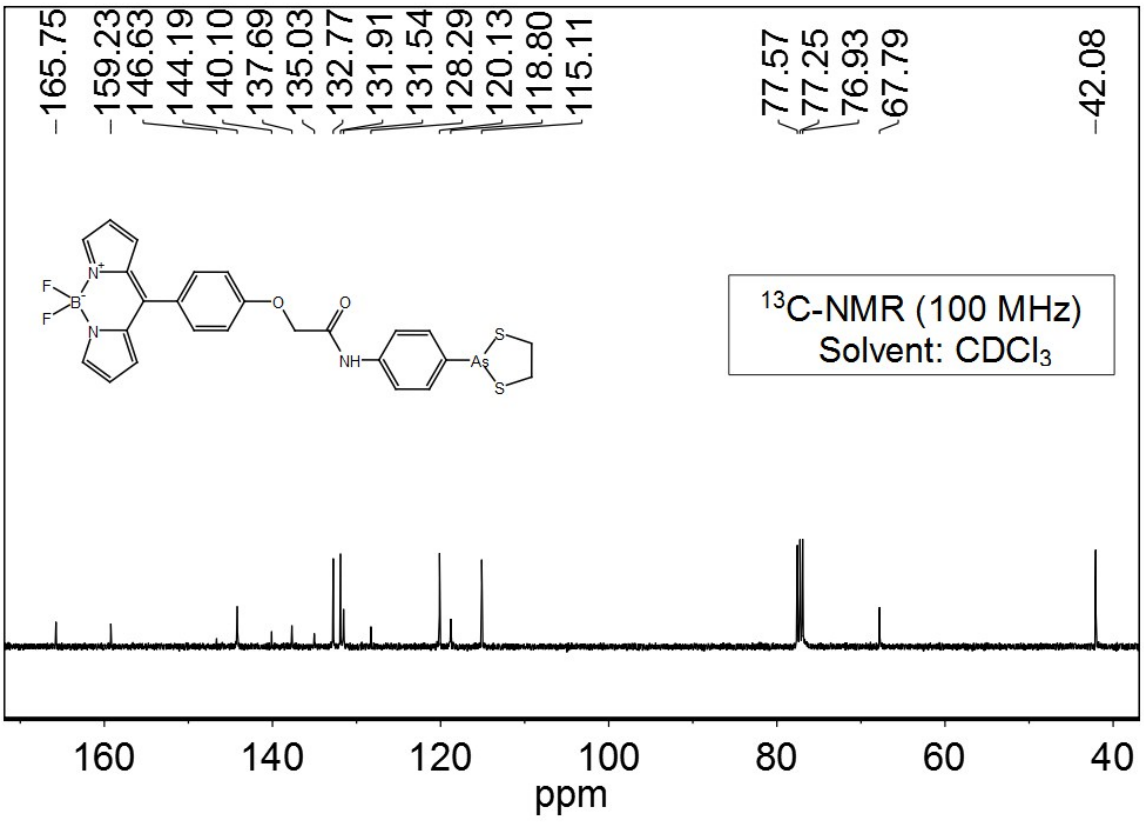


Fig. S26 ^{13}C NMR (298 K, 100 MHz, $CDCl_3$) spectrum for **1.**