Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Screening of dicyanoisophorone-based probes for highly sensitive detection of viscosity changes in living cells and zebrafish

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Contents

1. General information

2. Synthesis of probes

3. Determination of the fluorescence quantum yield

4. Calculation of pKa values of DCO-5

5. Supplemental figures

6. ^{1}H NMR, ^{13}C NMR and HR MS spectra

1. General information

The chemicals used in this study were commercially purchased and were not purified unless otherwise specified. Bruker 400NMR spectrometers and a Bruker maxis UHR-TOF instrument were used for NMR and high-resolution mass spectra collection, respectively. A Shimadzu UV-1700 vis spectrophotometer and a HITACHI F-4600 fluorescence spectrophotometer were used to record absorption and fluorescence spectra. Cell imaging and in vivo imaging experiments were measured on a Leica SP8 confocal fluorescence spectrophotometer.

Absorbtion and Fluorescence Spectroscopic Measurements. **DCO-5** was dissolved in DMSO to prepare the stock solution with a concentration of 2 mM. A mixed solution which was prepared by mixing different proportions of PBS (pH=7.4) and glycerol was used as an experimental system. Probe **DCO-5** was added to a mixture of different ratios of PBS and glycerol, which were treated by ultrasound for 20 min to eliminate bubbles. The final concentration of **DCO-5** was 10 μM. Different ratios of mixed solutions were measured by Shimadzu UV-1700 or F-4600 at room temperature.

Polarity and pH Effects. DCO-5 solutions with different pH values were prepared by adding 5 μL probe **DCO-5** stock solution to 2 mL of mixed solution with different pH values, and the final concentration of probe was 10 μM. Add 5μL **DCO-5** stock solution to 2 mL of N, N-dimethylformamide, dimethylsulfoxide, methanol, ethanol, acetonitrile, ethyl acetate, dichloromethane and PBS buffer, respectively. Every solution was measured in a F-4600 fluorometer at room temperature.

Selectivity. This experiment explored the stability of the probe in 19 substances such as metal ions, amino acids, and active components. **DCO-5** was added to a mixture of different ratios of PBS and glycerol, then add a certain volume of interfering substances respectively. The solution was treated by ultrasound for 20 min to eliminate bubbles. Solution was measured in a F-4600 fluorometer at room temperature.

Cytotoxicity Assay. The cytotoxicity of probe **DCO-5** was investigated by standard MTT method. Add **DCO-5** with a concentration of 0-30 μM to a 96-well culture plate, the number of cells in each well was kept the same, and each concentration will be in three parallel groups. After 12 hours of incubation, use a multifunctional microplate reader to measure the absorbance at a wavelength of 490 nm.

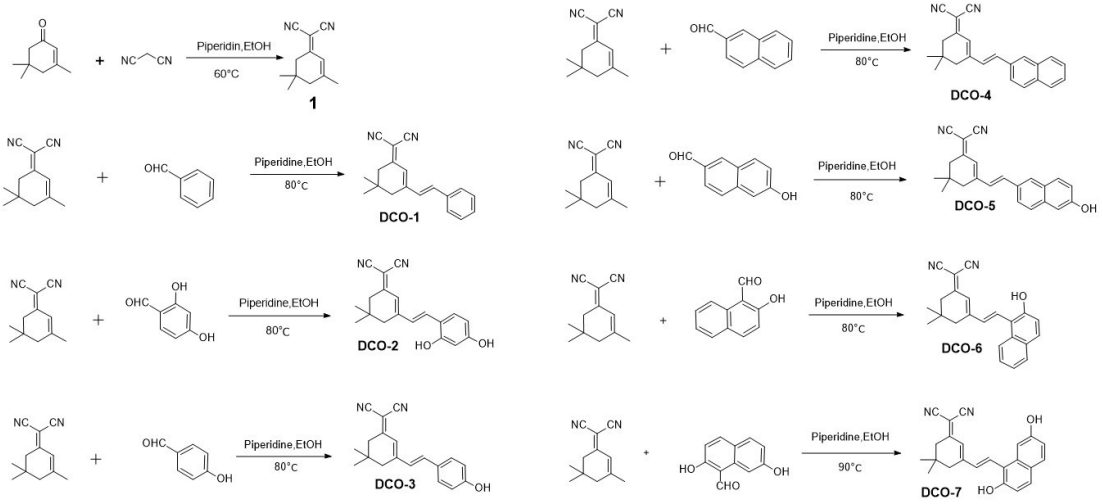
Cell Incubation and Fluorescence Imaging in Living Cells. There were four cell lines were used, including L02 cells, HepG2 cells, HeLa cells and PC12 cells. L02 cells were cultured in Roswell Park Memorial Institute (RPMI-1640) supplemented with penicillin/streptomycin and 10% fetal bovine serum in a 5% CO_2 incubator at 37 ℃. HepG2 cells, HeLa cells and PC12 cells were cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin/streptomycin, and 10% fetal bovine serum in a 5% CO_2 incubator at 37 °C. Cells were plated on confocal dish and allowed to adhere for 24 h. The cell experiment was divided into three groups. The first group was pretreated with nystatin (10 μM) for 40 min and then incubated with **DCO-5** (10 μM) for another 20 min. The second group was pretreated with rotenone (10 μM) for 15 h and subsequently incubated with **DCO-5** (10 μM) for another

20 min. The third group was cultured under starvation conditions including **DCO-5** (10 μM) for 40 min. Cells were washed three times by PBS buffer before imaging experiments.

Imaging of Viscosity in PD Models. Highly differentiated PC12 cells with neuron-like characteristics were selected, which have been widely used in the model construction of nervous system diseases due to their easier cultivation and enhanced expression of neuronal characteristics. AD model was obtained by incubating highly differentiated PC12 cells with 1 mM glutamate (Glu) and 100 μM H_2O_2 for 4 h, the AD model was incubated with **DCO-5** (10 μM) for another 20 min and the change of viscosity in AD model was measured.

Imaging of Viscosity in Zebrafish. The zebrafish (3 days post-fertilization) were provided by Nanjing EzeRinka Biotechnology Co., Ltd. Larval zebrafish were kept under the best breeding conditions. Before the fluorescence imaging experiment, the three-day-old zebrafish were transferred into three glass petri dishes with a disposable dropper. Among three glass petri dishes, one of them was used as the blank group without any stimulation, the second group was incubated with **DCO-5** (10 μM) for 30 minutes, the other petri dish was pretreated with nystatin (10 μM) for 50 min and subsequently incubated with **DCO-5** (10 μM) for another 30 min. After the probe was incubated, the zebrafish were gently washed with PBS, a small amount of medium was used to place the zebrafish on the fixative for confocal fluorescence imaging. Using a 10 X objective lens.

2. Synthesis of Probes



Scheme S1. Synthesis of probes DCO **1** -**7**

Synthesis of Compound 1.

To a solution of malononitrile (1.3 g, 20 mmol) and piperidine (0.2 mL) in ethanol (40 mL) was added isoflurone (3 mL, 20 mmol). The mixture was stirred at 60 °C for 8 hours. The reaction mixture is poured into ice water (100 mL), then the solid formed was filtered. After drying, 3.10 g (84%) of product was afforded. ^{1}H NMR (400 MHz, DMSO-d_6)：δ 6.56 (s, 1H), 2.53 (s, 2H), 2.23 (s, 2H), 2.05 (s, 3H), 0.96 (s, 6H); ^{13}C NMR (101 MHz, DMSO-d_6)：δ 171.90, 162.97, 119.88, 113.92, 113.12, 76.49, 45.29,

42.45, 32.42, 27.74, 25.43. HRMS:(ESI, m/z) Calculated for $C_{12}H_{14}N_2$ [M-H]⁻：185.1073，found：185.1056.

Synthesis of Compound DCO-1.

To a solution of compound 1 (300 mg, 1.6 mmol) and piperidine (0.2 mL) in ethanol (8 mL) was added benzaldehyde (170 mg, 1.6 mmol), the mixture was stirred at 80 °C. After complete reaction, as judged by TLC, the reaction solution was cooled to room temperature, and the solid produced was filtered, washed with ice ethanol, dried under vacuum to obtain 0.31g of the product. Yield: 71%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.52 (dd, *J* = 8.0, 1.2 Hz, 2H), 7.39 (dt, *J* = 8.4, 6.8 Hz, 3H), 7.03 (d, *J* = 13.9 Hz, 2H), 6.85 (s, 1H), 2.61 (s, 2H), 2.48 (s, 2H), 1.09 (s, 6H)；^{13}C NMR (101 MHz, $CDCl_3$) δ 169.37, 153.85, 137.05, 135.57, 129.76, 129.09, 129.03, 127.55, 123.59, 113.50, 112.72, 78.66, 58.47, 42.97, 39.14, 32.04, 28.02, 18.43. HR MS:(ESI, m/z) Calculated for $C_{19}H_{18}N_2$ [M-H]⁻：273.1386，found：273.1379.

Synthesis of Compound DCO-2.

To a solution of compound 1 (300 mg, 1.6 mmol) and piperidine (0.2 mL) in ethanol (8 mL) was added 2,4-dihydroxybenzaldehyde (200 mg, 1.4 mmol), the mixture was stirred at 80 °C for 5 hours. The reaction solution was cooled to room temperature, and the solvent was removed by rotary evaporation, then the water and methylene chloride were used for extraction. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. Purification by flash chromatography on silica gel (methanol: dichloromethane = 1:20) afforded 90 mg (21% yield) of **DCO-2**.^{1}H NMR (400 MHz, DMSO-d_6)：δ 10.17 (s, 1H), 9.98 (s, 1H), 7.53 (d, *J* = 8.6 Hz, 1H), 7.42 (d, *J* = 16.1 Hz, 1H), 7.13 (d, *J* = 16.1 Hz, 1H), 6.70 (s, 1H), 6.35 (d, *J* = 2.1 Hz, 1H), 6.30 (d, *J* = 8.6 Hz, 1H), 2.50 (d, *J* = 6.0 Hz, 4H), 1.00 (s, 6H)；^{13}C NMR (101 MHz, DMSO-d_6) δ 170.72, 161.24, 158.57, 158.01, 134.08, 129.48, 125.49, 120.98, 114.98, 114.87, 114.05, 108.64, 102.86, 73.99, 55.33, 42.78, 38.58, 32.13, 27.86. HRMS: (ESI, m/z) Calculated for $C_{19}H_{18}N_2O_2$ [M-H]⁻：305.1284，found：305.1281.

Synthesis of Compound DCO-3.

To a solution of compound 1 (300 mg, 1.6 mmol) and piperidine (0.2 mL) in ethanol (8 mL) was added p-hydroxybenzaldehyde (200 mg, 1.6 mmol), the mixture was stirred at 80 °C for 5 hours. The reaction solution was cooled to room temperature, and the solvent was removed by rotary evaporation, then the water and methylene chloride were used for extraction. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. Purification by flash chromatography on silica gel (petroleum ether: ethyl acetate = 10: 1) afforded 153 mg (33% yield) of **DCO-3**. ^{1}H NMR (400 MHz, DMSO-d_6)：δ 10.00 (s, 1H), 7.56 (d, *J* = 8.7 Hz, 2H), 7.21 (d, *J* = 5.0 Hz, 2H), 6.83 – 6.76 (m, 3H), 2.60 (s, 2H), 2.53 (s, 2H), 1.01 (s, 6H)；^{13}C NMR (101 MHz, DMSO-d_6) δ 191.46, 170.79, 159.81, 157.23, 138.79, 132.60, 130.37, 127.58, 126.72, 121.85, 116.33, 114.64, 113.81, 75.22, 42.75, 38.60, 32.15, 27.91. HRMS：(ESI, m/z) Calculated for $C_{19}H_{18}N_2O$ [M-H]⁻：289.1335，found：289.1330.

Synthesis of Compound DCO-4.

To a solution of compound 1 (300 mg, 1.6 mmol) and piperidine (0.2 mL) in ethanol (8

mL) was added 2-naphthaldehyde (200 mg, 1.3 mmol), the mixture was stirred at 80 °C. After complete reaction, as judged by TLC, the reaction solution was cooled to room temperature, and the solid produced was filtered, washed with ice ethanol, dried under vacuum to obtain 270 mg of the product. Yield: 65%. ^{1}H NMR (400 MHz, DMSO-d_6)：δ 8.16 (s, 1H), 7.92 (d, *J* = 10.3 Hz, 4H), 7.55 (d, *J* = 9.3 Hz,3H), 7.44 (d, *J* = 16.1 Hz, 1H), 6.93 (s, 1H), 2.59 (d, *J* = 11.0 Hz, 4H), 1.03 (s, 6H)；^{13}C NMR (101 MHz, DMSO-d_6)：δ 170.76, 156.23, 137.98, 134.15, 133.87, 133.53, 130.50, 129.34, 128.94, 128.81, 128.17, 127.52, 127.22, 124.47, 123.45, 114.33, 113.53, 76.93, 42.75, 38.64, 32.15, 27.92. HRMS: (ESI, m/z) Calculated for $C_{23}H_{20}N_2$ [M-H]$^-$：323.1542，found：323.1538.

Synthesis of Compound DCO-5.

To a solution of compound 1 (300 mg, 1.6 mmol) and piperidine (0.2 mL) in ethanol (10 mL) was added 6-hydroxy-2-naphthaldehyde (150 mg, 0.87 mmol), the mixture was stirred at 80 °C. After complete reaction, as judged by TLC, the reaction solution was cooled to room temperature, and the solid produced was filtered, washed with ice ethanol, dried under vacuum to obtain 210 mg of the product. Yield: 69%. ^{1}H NMR (400 MHz, DMSO-d_6)：δ 10.01 (s, 1H), 8.02 (s, 1H), 7.89 – 7.59 (m, 3H), 7.42 (d, *J* = 8.2 Hz, 2H), 7.13 (d, *J* = 2.7 Hz, 2H), 6.88 (s, 1H), 2.59 (d, *J* = 14.0 Hz, 4H), 1.03 (s, 6H)；^{13}C NMR (101 MHz, DMSO-d_6)：δ 170.76, 157.14, 156.70, 138.71, 135.79, 131.05, 130.68, 129.65, 128.83, 128.05, 127.24, 124.73, 122.70, 119.73, 114.49, 113.68, 109.54, 76.06, 55.37, 42.78, 38.66, 32.15, 27.93. HRMS: (ESI, m/z) Calculated for $C_{23}H_{20}N_2O$ [M-H]$^-$：339.1491，found：339.1505.

Synthesis of Compound DCO-6.

To a solution of compound 1 (300 mg, 1.6 mmol) and piperidine (0.2 mL) in ethanol (10 mL) was added 2-hydroxy-1-naphthaldehyde (150 mg, 0.87 mmol), the mixture was stirred at 80 °C. After complete reaction, as judged by TLC, the reaction solution was cooled to room temperature, and the solid produced was filtered, washed with ice ethanol, dried under vacuum to obtain 180 mg of the product. Yield: 61%. ^{1}H NMR (400 MHz, DMSO-d_6)：δ 10.74 (d, *J* = 61.1 Hz, 1H), 8.21 (d, *J* = 8.6 Hz, 1H), 7.87 – 7.76 (m, 3H), 7.54 (ddd, *J* = 6.6, 5.1, 2.7 Hz, 2H), 7.36 (t, *J* = 7.5 Hz, 1H), 7.25 (t, *J* = 9.2 Hz, 1H), 6.75 (s, 1H), 2.65 (d, *J* = 37.7 Hz, 4H), 1.06 (s, 6H)；^{13}C NMR (101 MHz, DMSO-d_6) δ 170.80, 157.64, 156.12, 133.88, 132.99, 131.95, 131.76, 129.17, 128.61, 127.73, 123.67, 123.30, 122.38, 118.85, 114.72, 114.55, 113.73, 75.82, 56.49, 42.84, 38.22, 32.16, 27.94. HRMS: (ESI, m/z) Calculated for $C_{23}H_{20}N_2O$ [M-H]$^-$：339.1491，found：339.1488.

Synthesis of Compound DCO-7.

To a solution of compound 1 (300 mg, 1.6 mmol) and piperidine (0.2 mL) in ethanol (8 mL) was added 2,7-dihydroxy-1-naphthaldehyde (150 mg, 0.8 mmol), the mixture was stirred at 80 °C for 5 hours. The reaction solution was cooled to room temperature, and the solvent was removed by rotary evaporation, then the water and methylene chloride were used for extraction. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. Purification by flash chromatography on silica gel (methanol: dichloromethane = 1:60) afforded 53 mg (31% yield) of **DCO-7**. ^{1}H NMR (400 MHz, DMSO-d_6)：δ 10.47 (s, 1H), 9.77 (s, 1H), 7.67 (t, *J* =

12.4 Hz, 3H), 7.54-7.38 (m, 2H), 7.00 (d, *J* = 8.8 Hz, 1H), 6.90 (d, *J* = 8.7 Hz, 1H), 6.73 (s, 1H), 2.66 (d, *J* = 17.2 Hz, 4H), 1.08 (s, 6H)；^{13}C NMR (101 MHz, DMSO-d_6)：δ 170.75, 157.72, 157.33, 156.69, 134.90, 132.89, 132.51, 131.83, 130.94, 123.25, 121.99, 115.68, 115.29, 114.59, 113.79, 113.35, 105.48, 75.48, 49.07, 42.90, 38.43, 32.21, 27.99. HRMS: (ESI, m/z) Calculated for $C_{23}H_{20}N_2O_2$ $[M-H]^-$：355.1441，found：355.1414.

3. Determination of the fluorescence quantum yield

Fluorescence quantum yield (Φ) was determined by using rhodamine B (Φ = 0.89, in ethanol) as the fluorescence standard. The quantum yield was calculated using the following equation.

$$\Phi_x = \Phi_{st}\left(D_x/D_{st}\right)\left(A_{st}/A_x\right)\left(\eta_x^2/\eta_{st}^2\right)$$

Where Φ_{st}, D and A refer to the reported quantum yield of the standard, the area under the emission spectra and the absorbance at the excitation wavelength. η stands for the refractive index of the solvent used. Subscripts x and st represents sample and standard, respectively. Absorbance of sample and reference at their respective excitation wavelengths was controlled to be lower than 0.05. (Ref. Anal. Chem. 2021, 93, 3241-3249).

4. Calculation of pKa Value of DCO-5

Absorbance or fluorescence emission intensity of indicators was measured in buffer solution at different pH values. The pH profiles of absorbance or fluorescence intensity were fitted to the Henderson-Hasselbach equation for determination of pKa values. (Ref. Dyes Pigments 2012, 95, 112-115.)

$$\mathrm{p}K_\mathrm{a} = \mathrm{pH}\text{-}\log\frac{I_\mathrm{max} - I}{I - I_\mathrm{min}}$$

Where I is the absorbance or fluorescence intensity of the testing compound at a given wavelength, I_{max} and I_{min} are the maximum and minimum limiting values of I, respectively.

5. Supplemental figures

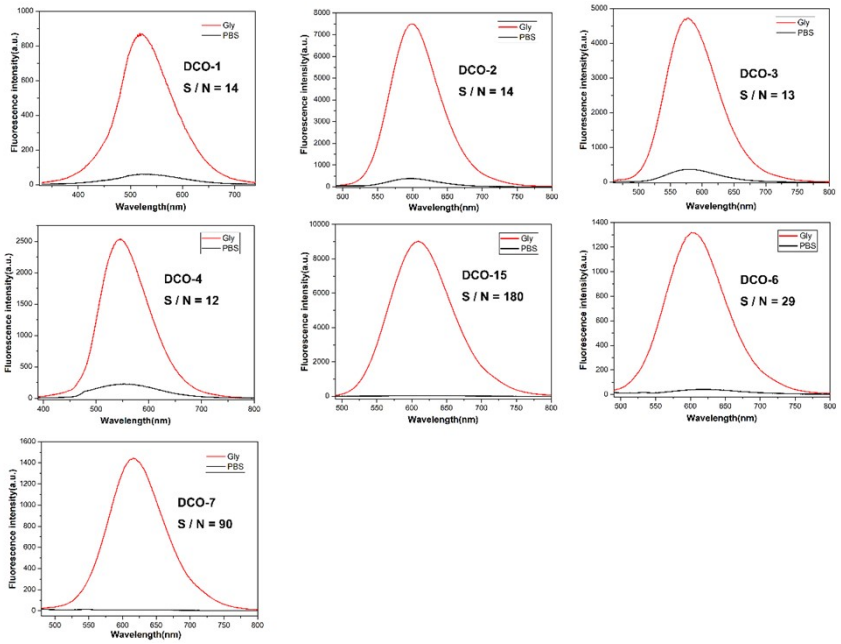


Figure S1. Fluorescence intensity of DCO**1-7** (10 μM) in PBS and the 99% v/v glycerol solution. Excitation was at the maximum absorption wavelength of each.

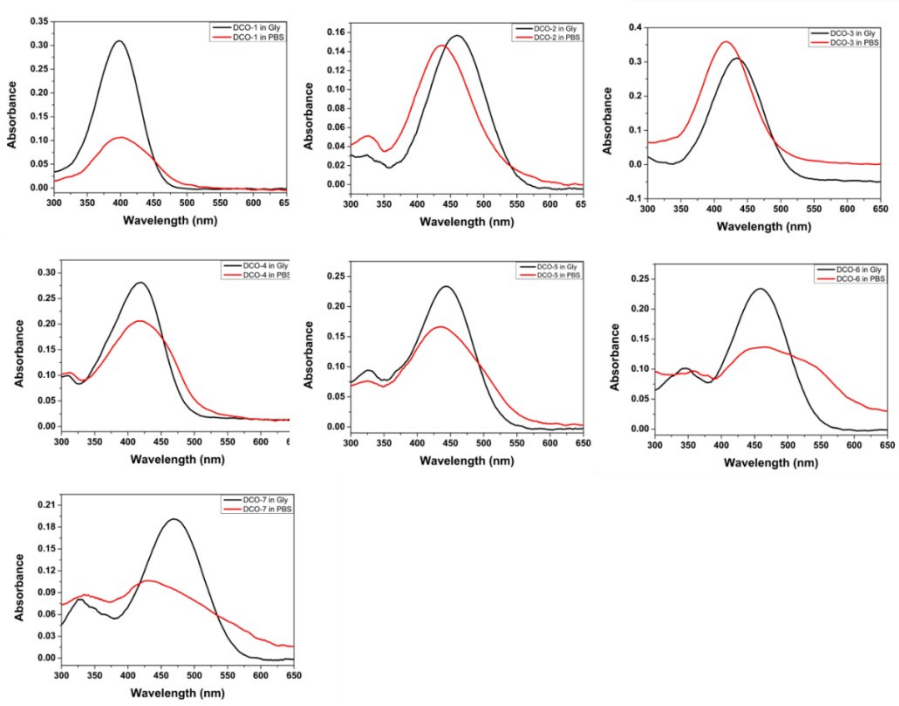


Figure S2. Absorption spectrum of DCO **1-7** (10 μM) in PBS and the 99% v/v glycerol solution.

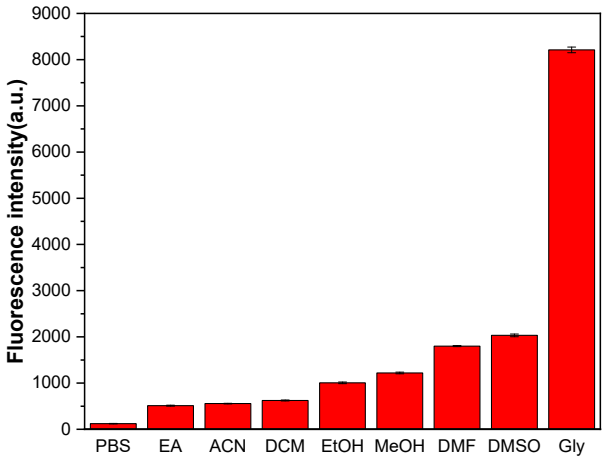


Figure S3. Fluorescence intensity of **DCO-5** (10 μM) in different solvents. Excitation was at 430 nm.

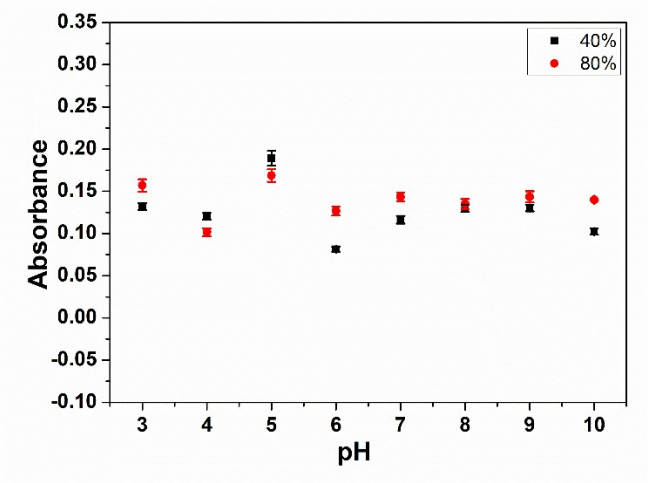


Figure S4. Absorption spectrum of DCO-5 (10 μM) in 40% glycerol solution (gray) and in 80% v/v glycerol solution (red) at different pH values.

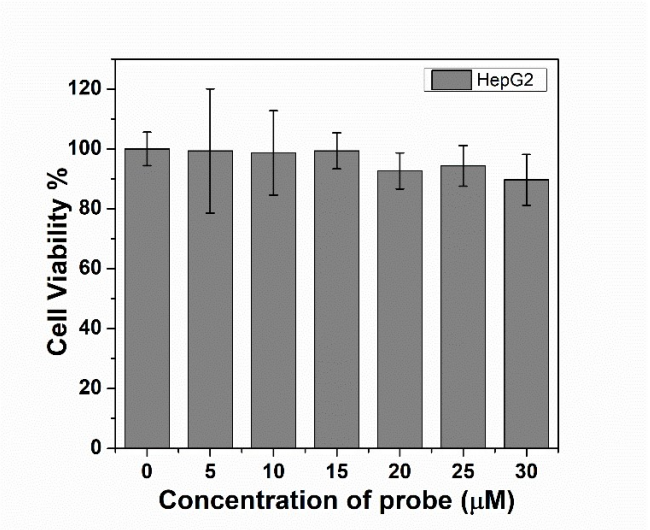


Figure S5. Cell cytotoxicity of **DCO-5** against HepG2 cells evaluated by MTT assay.

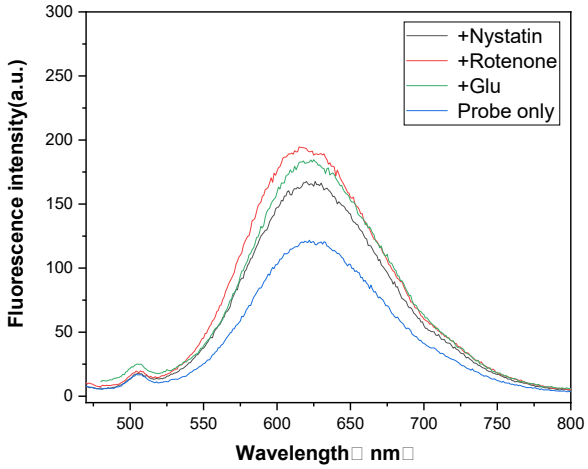


Figure S6. Fluorescence spectra of **DCO-5** (10 μM) in present of rotenone (10 μM), nystatin (10 μM) and glutamate (1mM) in PBS. Excitation was at 430 nm.

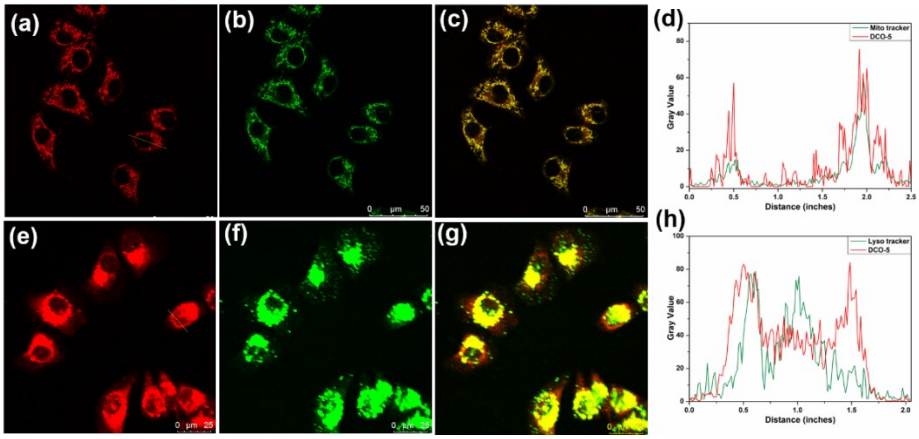


Figure S7. Fluorescence images of HepG2 cells co-incubated with (a, e) **DCO-5** (10 μM, 30 min) and (b) Mito-Tracker Green (0.1 μM, 30 min) or (f) Lyso-Tracker Red (1 μM, 30 min). (c, g) Merged images. (d) Pearson's colocalization correlation of **DCO-5** with Mito-Tracker intensities (PC = 0.69). (h) Pearson's colocalization correlation of **DCO-5** with Lyso-Tracker intensities (PC = 0.63). **DCO-5**: λ_{ex} = 488 nm, λ_{em} = 560-700 nm; Mito-Tracker Green : λ_{ex} =490 nm, λ_{em} = 495-560 nm. Lyso-Tracke : λ_{ex} = 575 nm, λ_{em} = 590-700 nm.

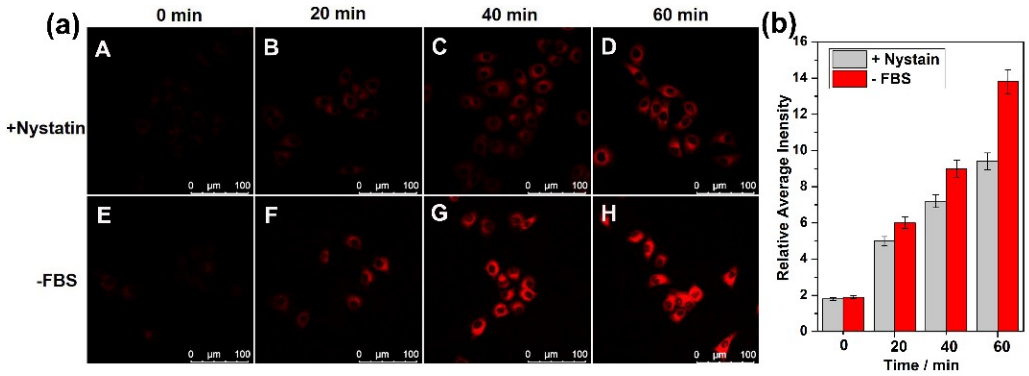


Figure S8. (a) Time-dependent fluorescence images of HepG2 cells incubated with nystatin. (A-D) or in fetal bovine serum-free medium (E-H) with **DCO-5** (10 μM). The cells were pre-treated with nystatin (10 μM) or in serum-free medium and then incubated with **DCO-5** (10 μM) followed by imaging at different time points (0, 20, 40, and 60 min). The images were recorded with 488 nm excitation and 560-700 nm collection. (b) The relative fluorescence intensities of the images A-H.

6. ^{1}H NMR, ^{13}C NMR and HR MS spectra

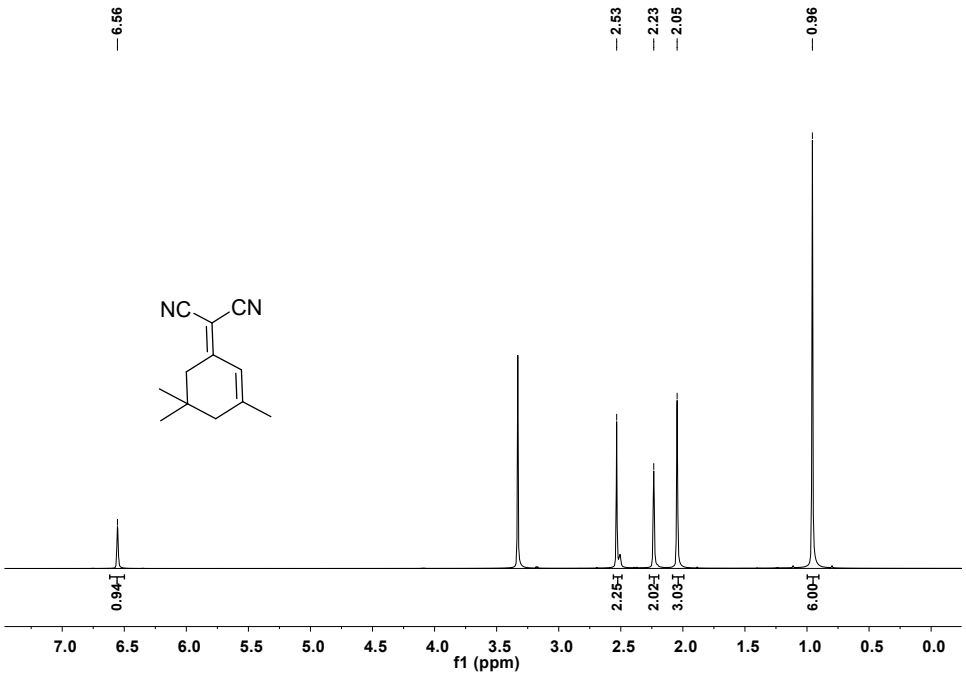


Fig. S6 ^{1}H-NMR spectrum of compound **1** in DMSO-d_6 (400 MHz).

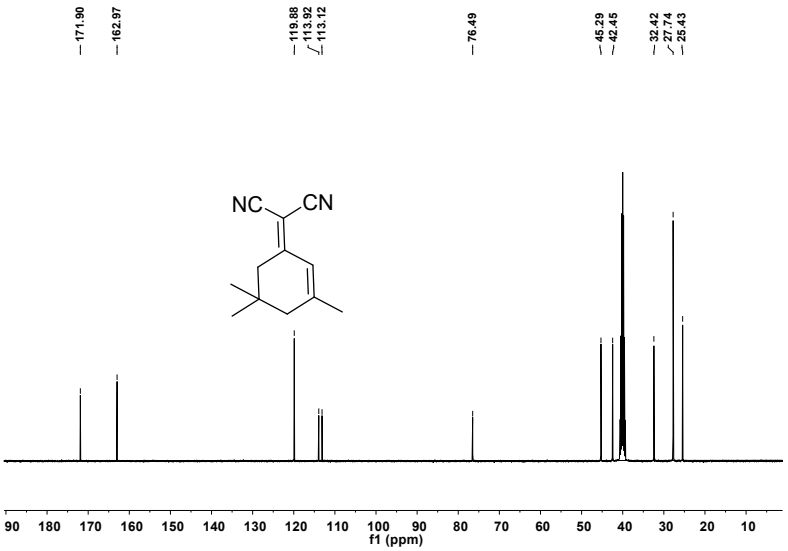


Fig. S7 ^{13}C-NMR spectrum of compound **1** in DMSO-d_6 (400 MHz).

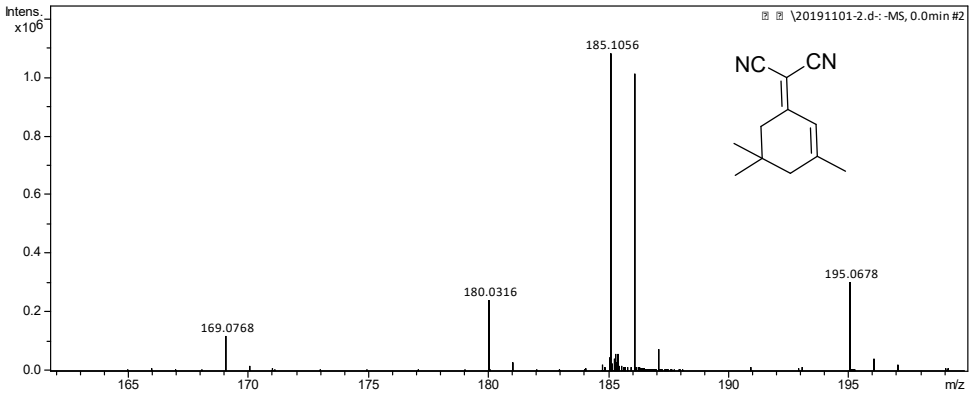


Fig. S8 HR MS spectrum of compound **1**

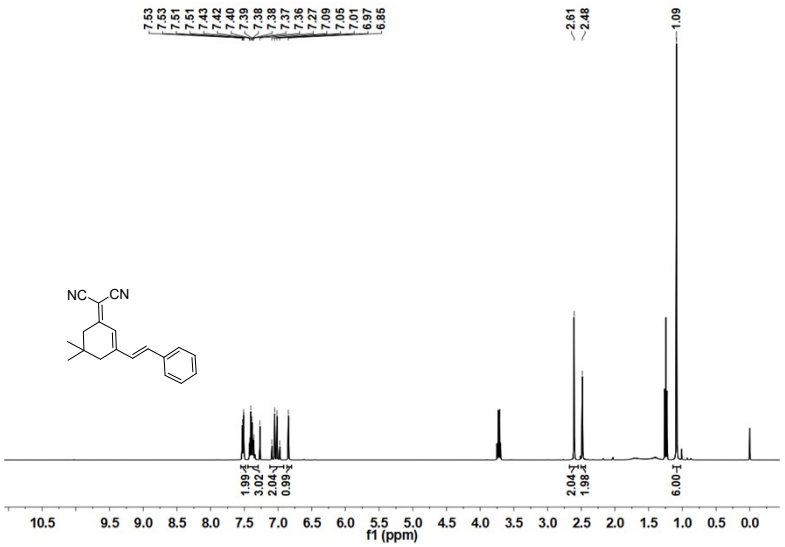


Fig. S9 ^{1}H-NMR spectrum of DCO **1** in $CDCl_3$ (400 MHz)

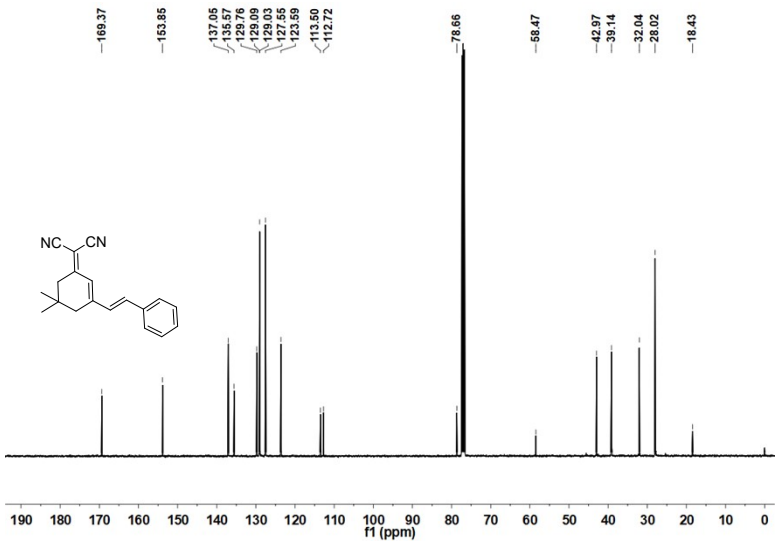


Fig. S10 ^{13}C-NMR spectrum of DCO **1** in $CDCl_3$ (400 MHz)

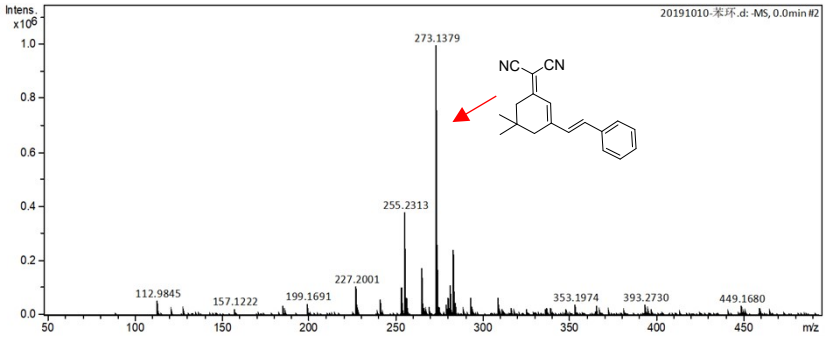


Fig. S11 HR MS spectrum of compound **1**

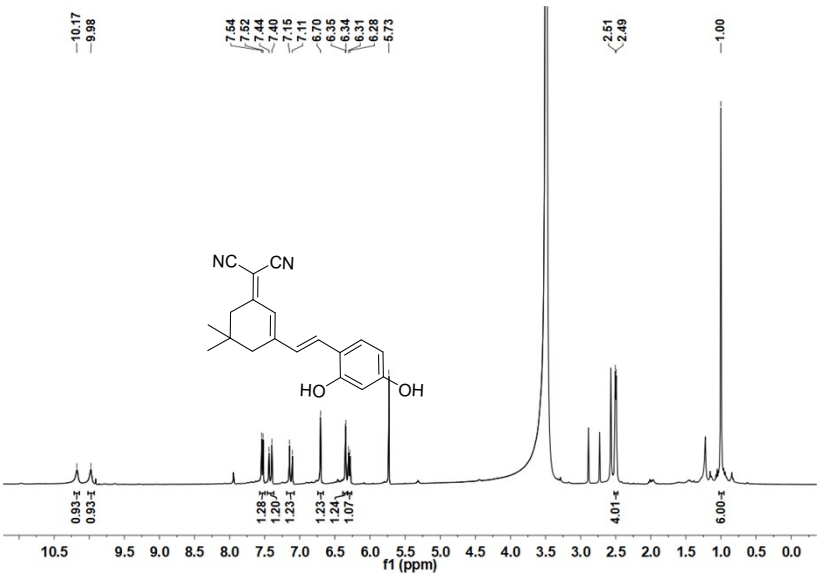


Fig. S12 ^{1}H-NMR spectrum of **DCO 2** in DMSO-d_6 (400 MHz).

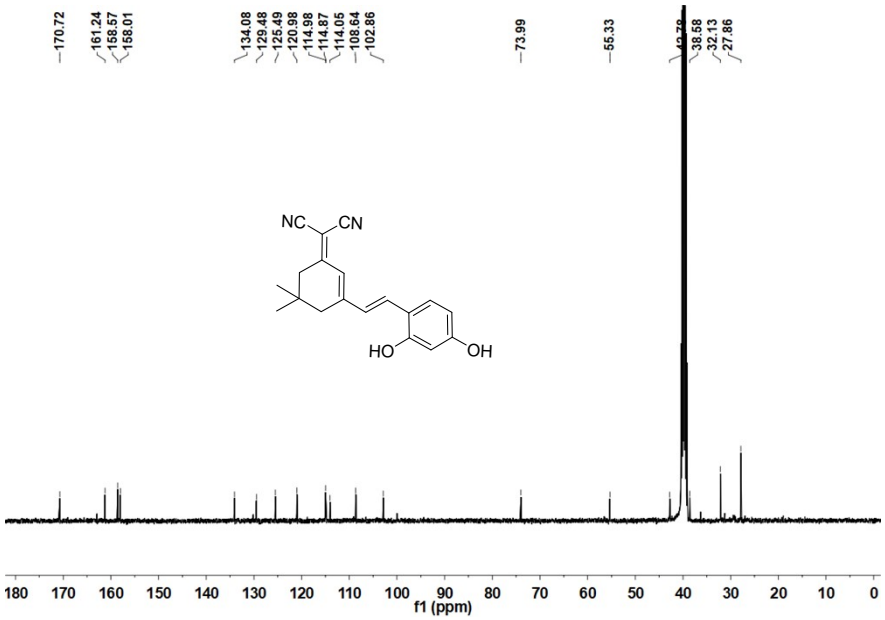


Fig. S13 ^{13}C-NMR spectrum of **DCO 2** in DMSO-d_6 (400 MHz)

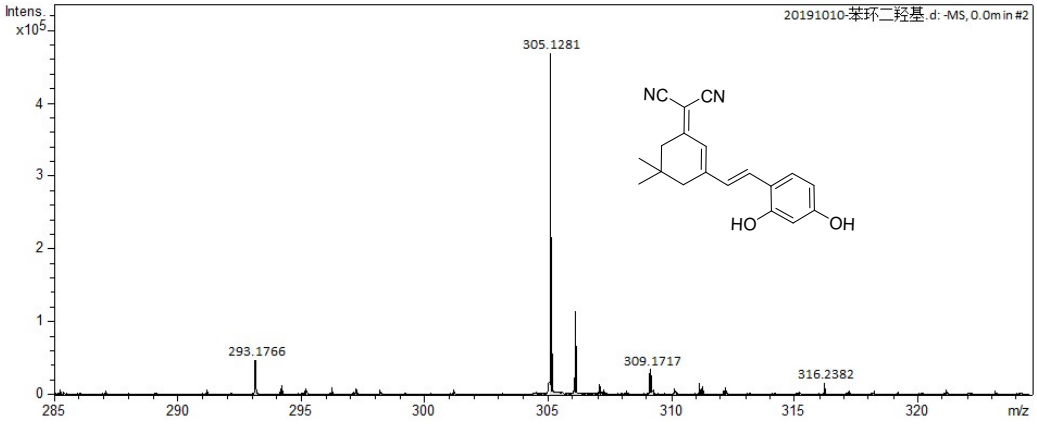


Fig. S14 HR MS spectrum of **DCO 2**

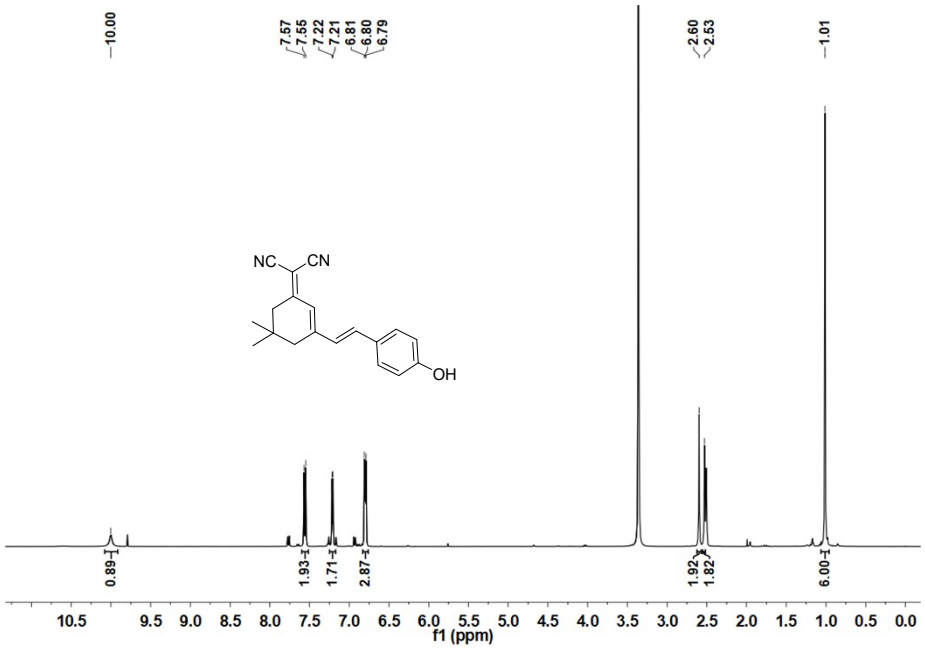


Fig. S15 ^{1}H-NMR spectrum of **DCO 3** in DMSO-d_6 (400 MHz).

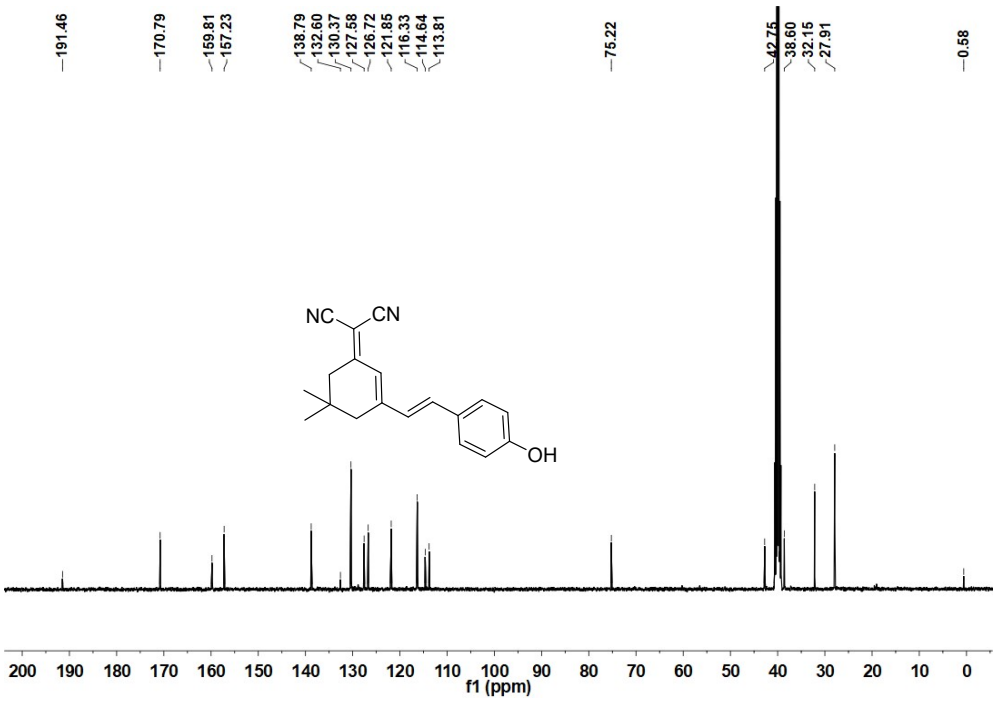


Fig. S16 ^{13}C-NMR spectrum of **DCO 3** in DMSO-d_6 (400 MHz)

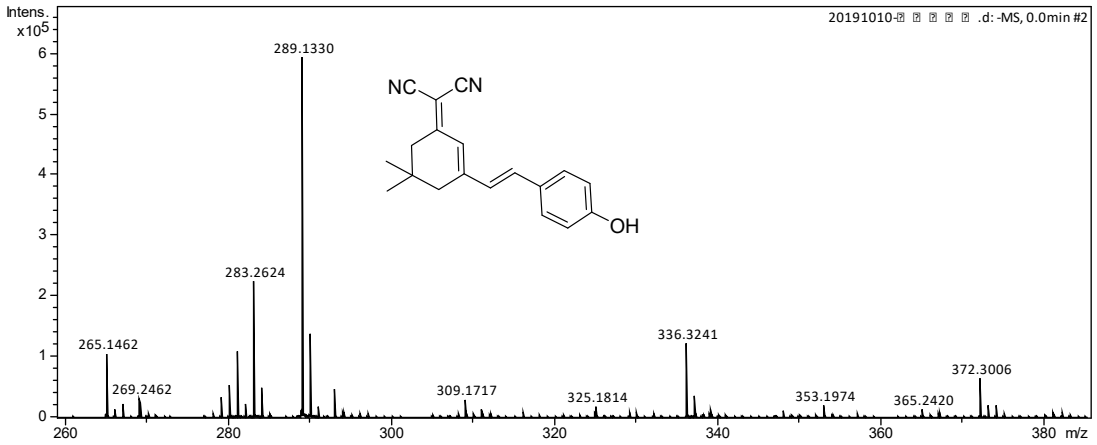


Fig. S17 HR MS spectrum of **DCO 3**

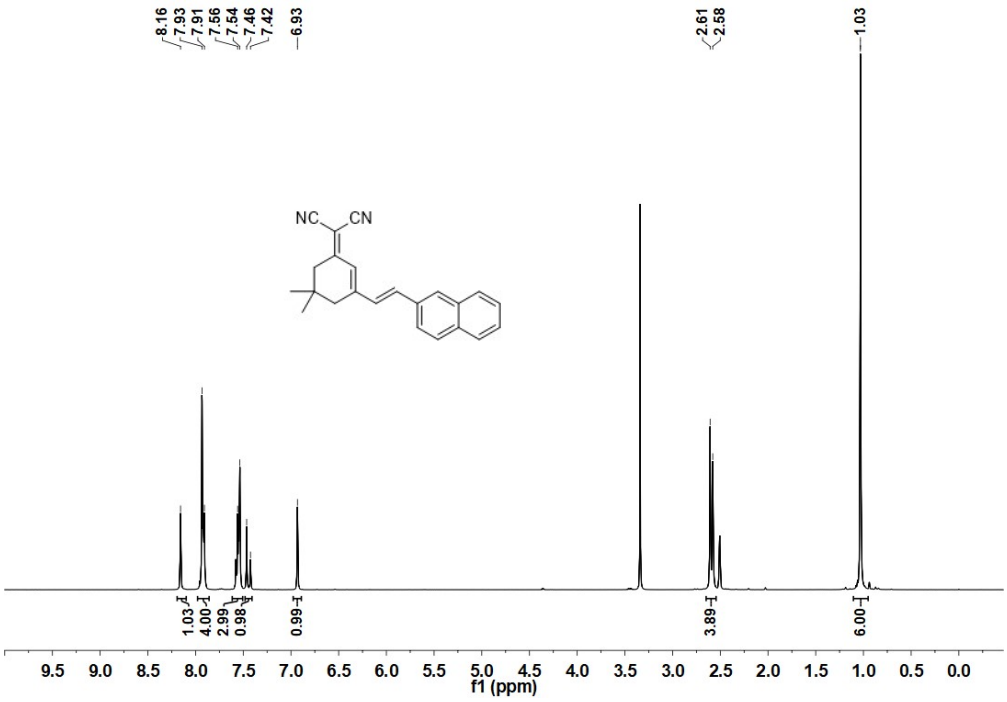


Fig. S18 ^{1}H-NMR spectrum of **DCO 4** in DMSO-d_6 (400 MHz).

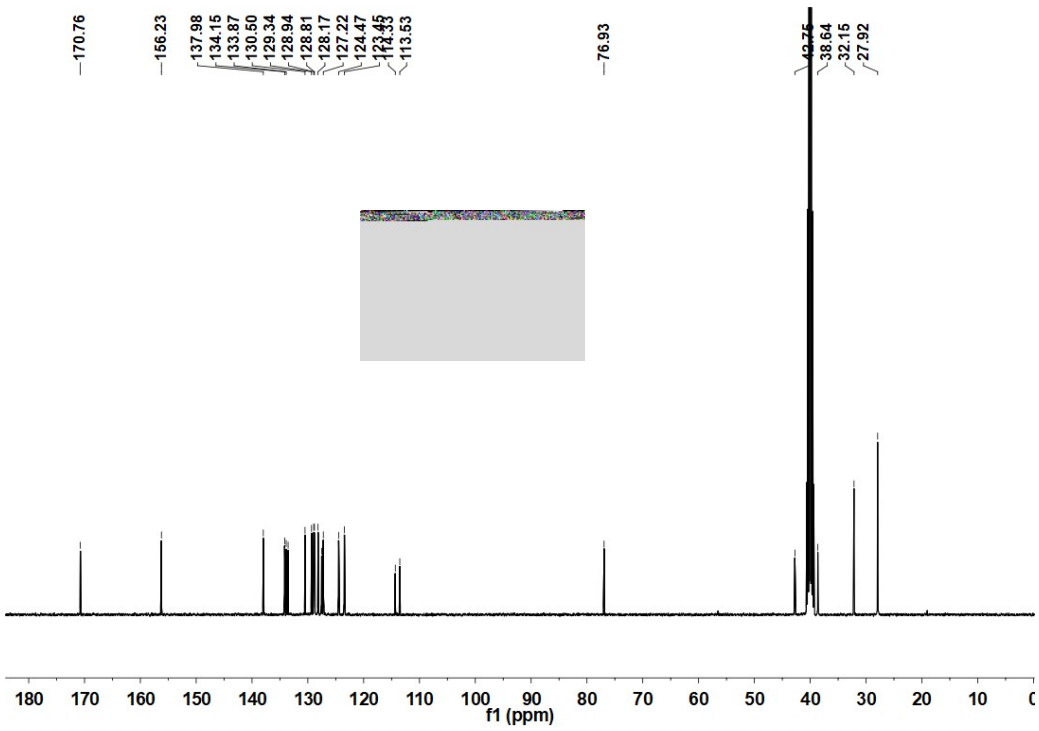


Fig. S19 ^{13}C-NMR spectrum of **DCO 4** in DMSO-d_6 (400 MHz)

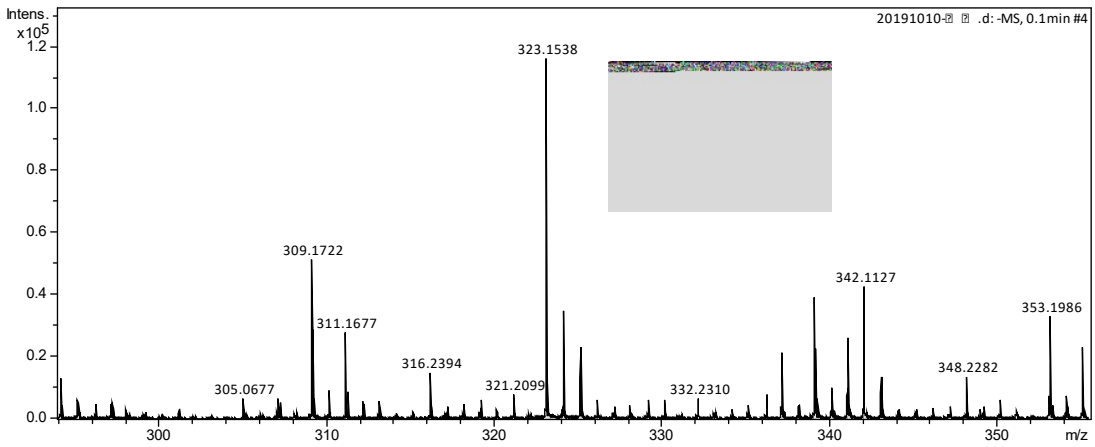


Fig. S20 HR MS spectrum of **DCO 4**

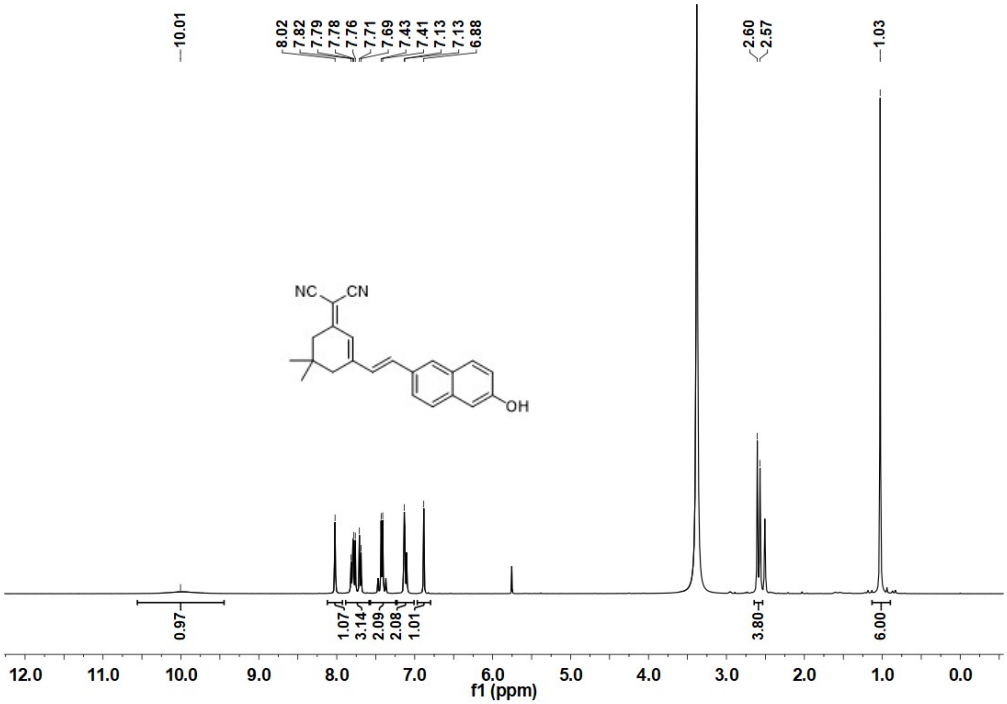


Fig. S21 ^{1}H-NMR spectrum of **DCO 5** in DMSO-d_6 (400 MHz).

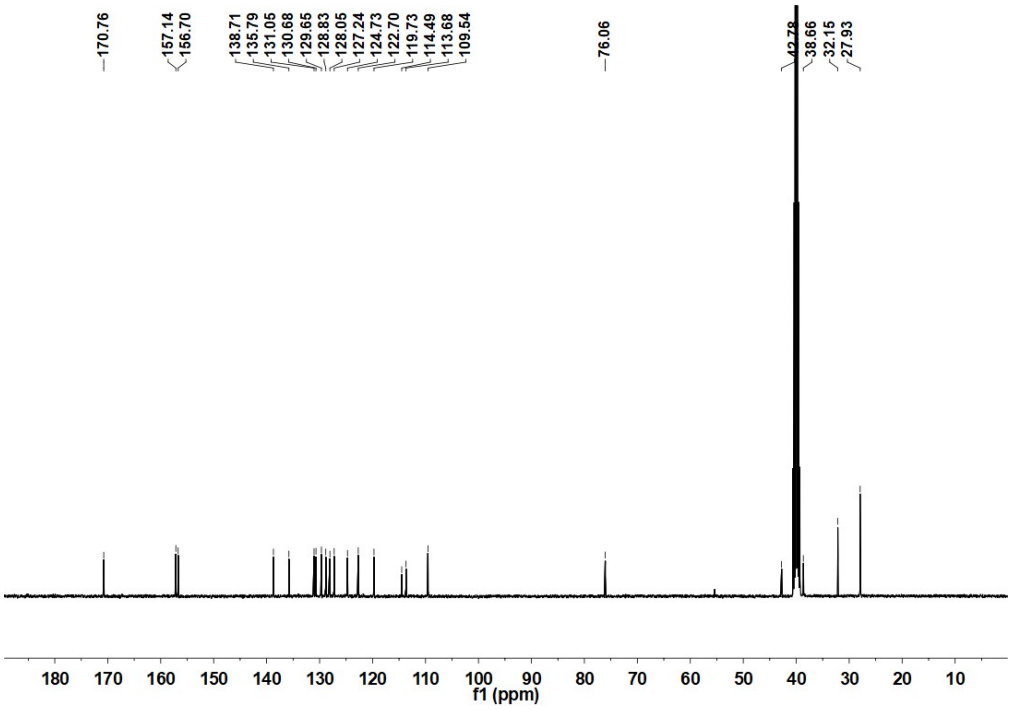


Fig. S22 ^{13}C-NMR spectrum of **DCO 5** in DMSO-d_6 (400 MHz)

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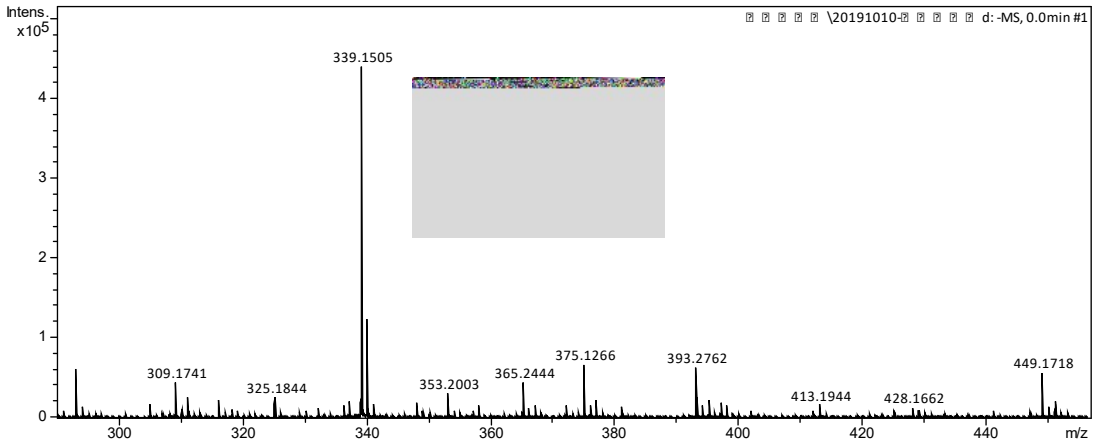

Fig. S23 HR MS spectrum of **DCO 5**

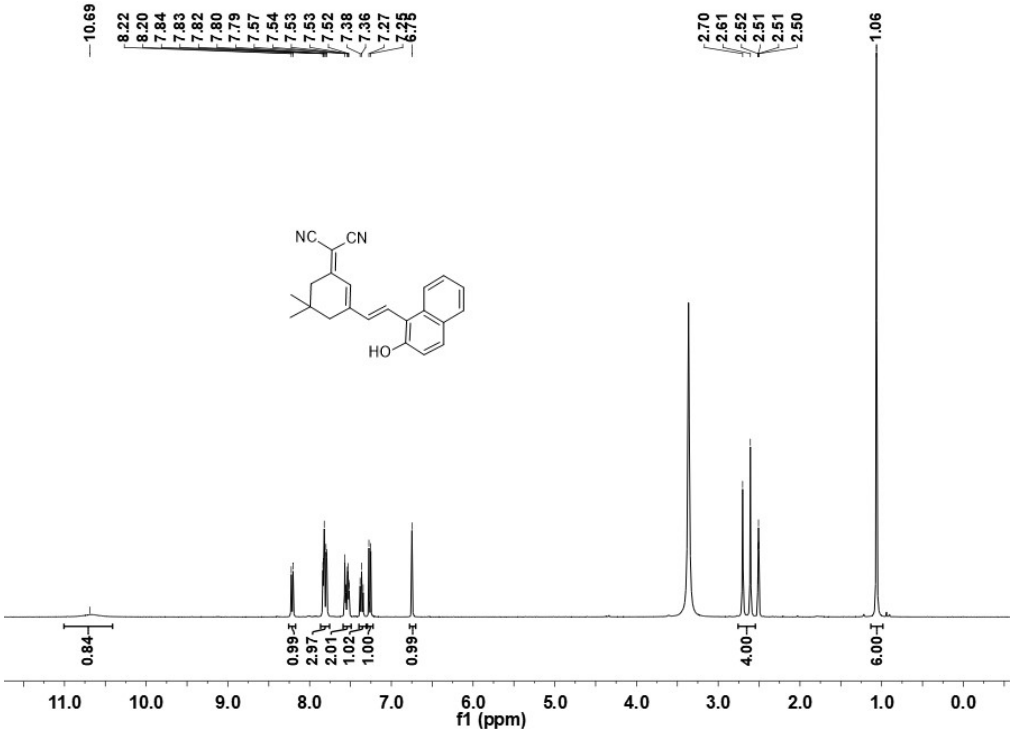

Fig. S24 ^{1}H-NMR spectrum of **DCO 6** in DMSO-d_6 (400 MHz).

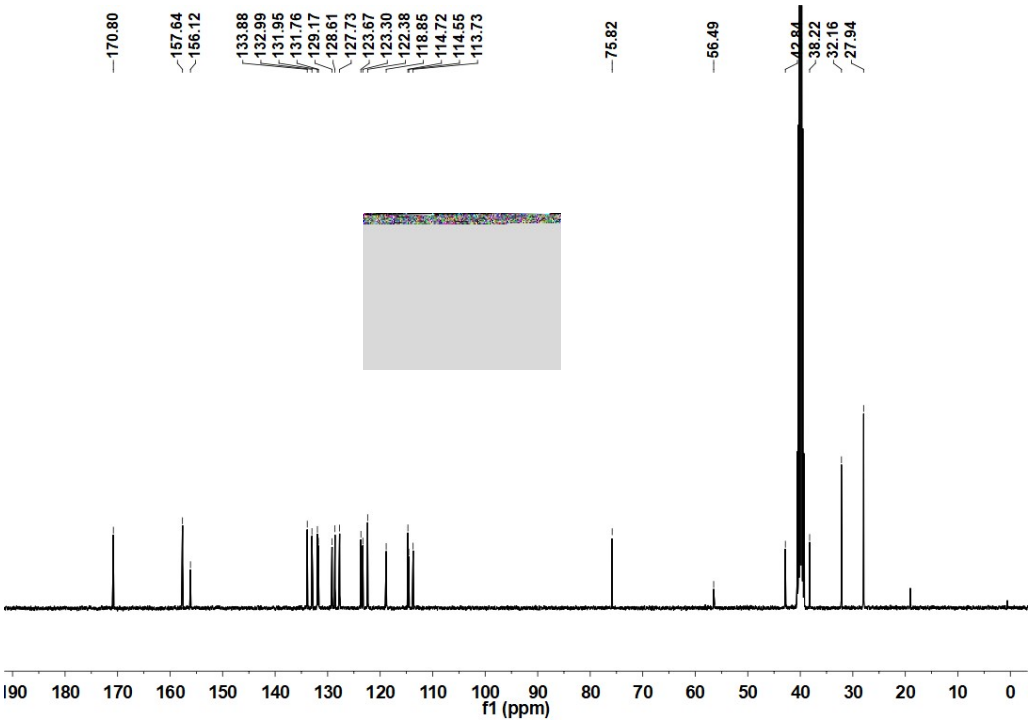

Fig. S25 ^{13}C-NMR spectrum of **DCO 6** in DMSO-d_6 (400 MHz)

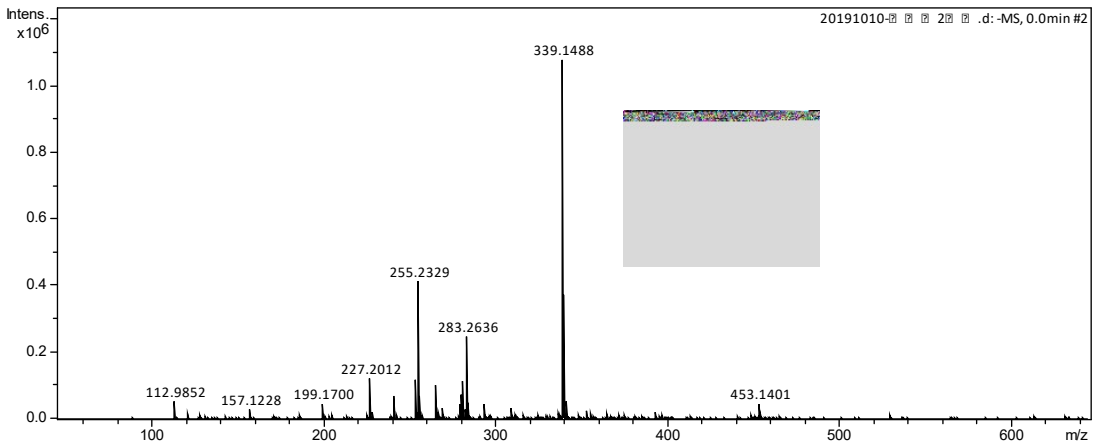

Fig. S26 HR MS spectrum of **DCO 6**

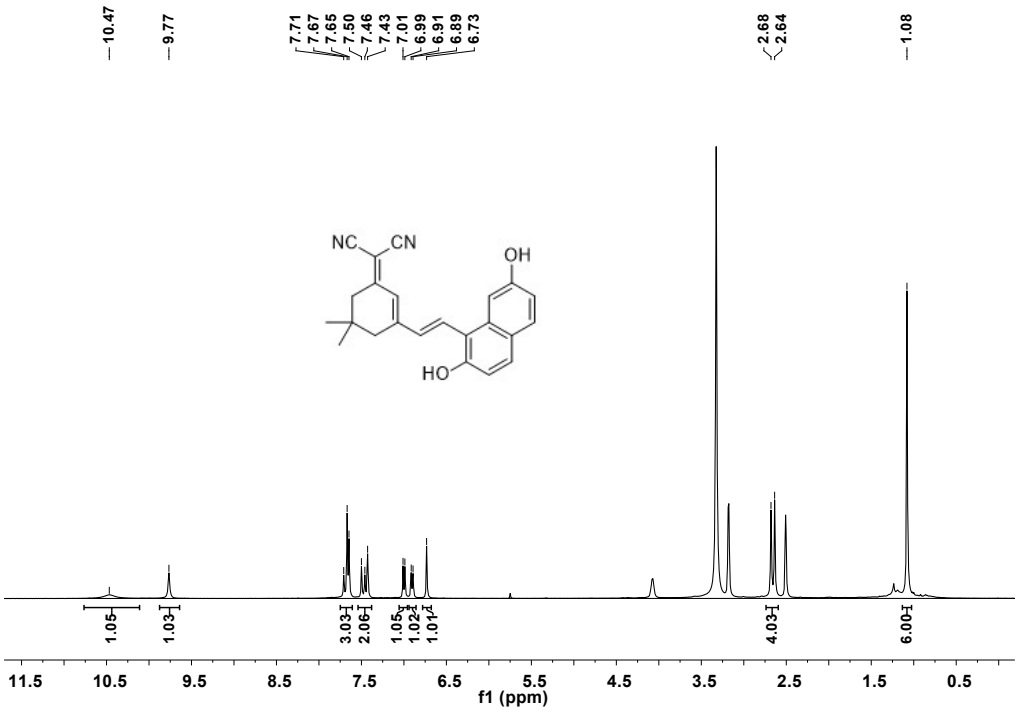

Fig. S27 ^{1}H-NMR spectrum of **DCO 7** in DMSO-d_6 (400 MHz).

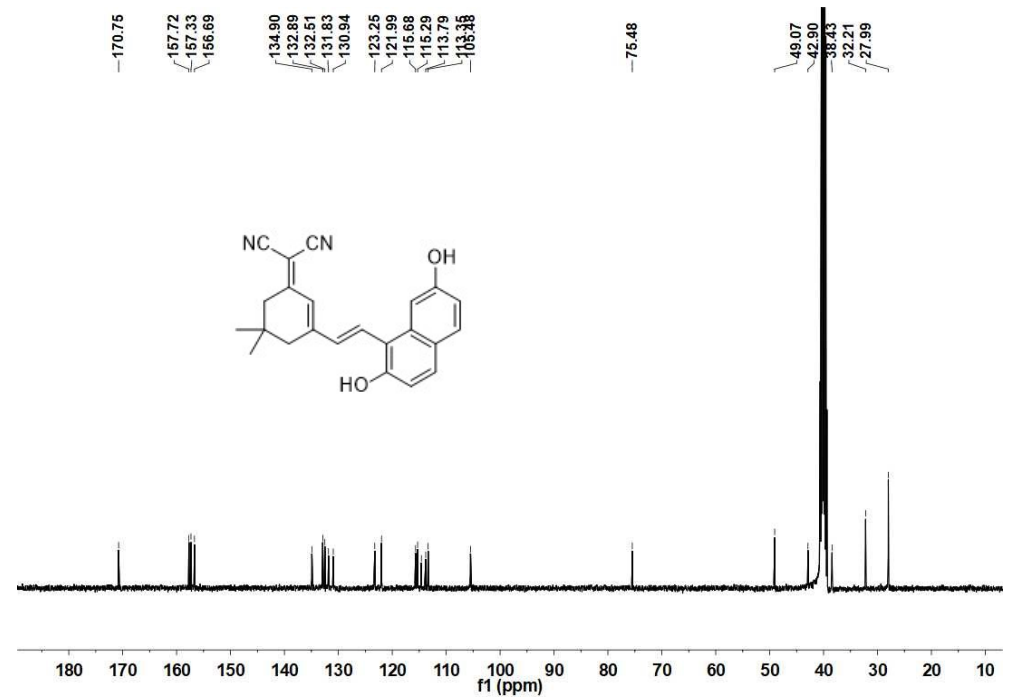

Fig. S28 ^{13}C-NMR spectrum of **DCO 7** in DMSO-d_6 (400 MHz)

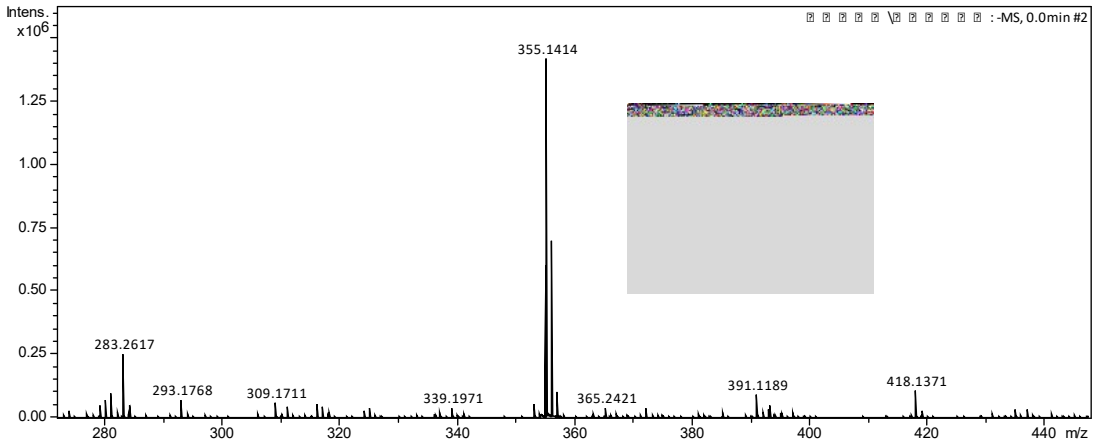

Fig. S29 HR MS spectrum of **DCO 7**