Electronic Supplementary Information (ESI)

A Fluorescent Probe for the Site I Binding and Sensitive Discrimination of HSA from BSA

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CONTENTS:

1. Materials and instruments

All solvents used were of analytical grade without further purification. Solutions of cations were prepared from NaCl, KCl, $MgCl_2$, $FeCl_3$, $Cu(NO_3)_2$, $AlCl_3$, $ZnCl_2$, $Ni(NO_3)_2$, $Hg(NO_3)_2$, $MnCl_2$, by separately dissolving, 10.0 mM of each cation in distilled water, corresponding to Na^+, K^+, Mg^{2+}, Fe^{3+}, Cu^{2+}, Al^{3+}, Zn^{2+}, Ni^{2+}, Hg^{2+}, and Mn^{2+} respectively. Sodium salts, such as NaCl, $NaNO_3$, Na_2SO_4, Na_2CO_3, NaClO, $NaClO_4$, NaOAc were separately dissolved in distilled water to prepare the stock solutions of different anions (10.0 mM for each). All proteins including human serum albumin, bovine serum albumin, chymotrysin, collagen, haemoglobin, lysozyme, chymotrypsinogen A, histones as well as other biomolecules like cysteine, homocysteine and glutathione were dissolved in distilled water to prepare stock solutions with concentrations of 5.0 mg/mL. The solutions (1.0 mM) of **DH1** and **DH2** was prepared in DMSO (5 mL) and stored in a refrigerator before use. Measurements were done just after the addition of different analytes to the solutions of **DH1** or **DH2**. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a VARIAN INOVA-400 spectrometer. The following abbreviations are used to indicate the multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad.

2. UV-visible and fluorescence spectroscopy

UV-visible spectra were collected on a Perkin Elmer Lambda 35 UV-Vis spectrophotometer under the control of a Windows-based PC running the manufacturers' supplied software. Fluorescence measurements were performed on a VAEIAN CARY Eclipse Fluorescence Spectrophotometer (Serial No. FL1109-M018) with slit widths were set at 10 nm and 10 nm for excitation and emission, respectively. The samples were excited at 520 nm and the emission intensities were collected. All spectra were corrected for emission intensity using the manufacturer supplied photomultiplier curves.

3. Photostability experiment

DH1 was dissolved in PBS buffer (0.2 M, pH 7.4, 50% DMSO) at a concentration of 5 μM. The solutions were irradiated under a *500W* iodine-tungsten lamp for 4 h at a distance of 300 mm away. A saturated sodium nitrite aqueous solution was placed between the samples and the lamp as a light filter (to cut off the light shorter than 400 nm) and heat filter.

4. Determination of the detection limit

The detection limit was calculated based on the method reported in the previous literature [S1]. The fluorescence emission spectrum of **DH1** was measured by three times and the standard deviation of blank measurement was achieved. The fluorescence ratio of 670 nm was plotted as a concentration of HSA. The detection limit was calculated by using detection limit = 3σ/k: Where σ is the standard deviation of blank measurement, k is the slope between the fluorescence intensity versus HSA concentration.

5. HSA detection in human urine

Urine was obtained form a normal adult without addition of any detergents. The test system was adjusted to be PBS buffer (0.2 M, pH 7.4)–urine (1/1, v/v) with a probe concentration of 5 μM. The changes of the fluorescene spectrum were monitored through fluorescence spectrophotometer when different concentrations of HSA were added to the test solution.

6. Theoretical calculations

Molecular modeling systems. The X-ray structures of HSA and BSA were obtained from the PDB database (http://www.rcsb.org/pdb). The 2D structures of the probes were drawn by the program MDL ISIS Draw 2.5 standalone and transferred to 3D structures by Accelrys Discovery Studio 2.5 (DS, http://www.accelrys.com/). Superimpose serum albumins by sequence alignment and LigandFit were used for molecular modelling on a Windows XP workstation. The resultant structures were analyzed and the visualization of complexes was employed by DS too.

Comparison of binding pockets. The X-ray crystal structures of HSA compounded with phenylbutazone (PDB code 2BXP, resolution 2.30Å), HSA compounded with ibuprofen (PDB code 2BXG, resolution 2.70Å) and BSA (PDB code 4F5S, resolution 2.47Å) were collected from the PDB database. Using basic sequence alignment of DS, the multiple sequence alignment of 4F5S with 2BXG and 2BXP was performed, respectively. And then the structure superimpose was done calling the result of basic sequence alignment.

Binding investigation of HSA with fluorescent probes by molecular docking simulation. The Prepare Protein protocol prepared HSA structure for input into LigandFit protocol, performing tasks such as inserting missing atoms in incomplete residues, modeling missing loop regions, deleting alternate conformations, removing waters, standardizing atom names, and protonating titratable residues using predicted pKs. Since the arginine residues in the protein couldn't be prepared correctly in DS CHARMm field, we did it manually. LigandFit, a docking tool, was used to dock ligands in the protein binding site, calculate scores, and rank them accordingly. The docking site was defined using the LigandFit site search utility. The binding site definition was offered by LigandFit: cavities can be detected from a bound ligand. In our case, the binding site I was generated from the X-ray pose of the bound ibuprofen. The binding site cavity was expanded with the program expansion tool to a distance of 4 Å in all directions. After characterization of the binding pockets, a Monte Carlo method was employed for the conformational search of the probe. Dreiding force field with Gasteiger charges was used for calculating ligand-receptor interaction energies during docking. Steepest Descent Algorithm was set to perform the minimization. Default settings were kept for the other parameters. The probes were docked within the identified site with possible conformations, with each ranked according to the dock score provided in LigandFit. Dock scoring was performed with LigScore1 scoring function. Three descriptors are used to calculate LigScore1 (a program-internal scoring function), a softened Lennard-Jones potential (vdW descriptor), a count of the buried polar surface area between a receptor and ligand which involves

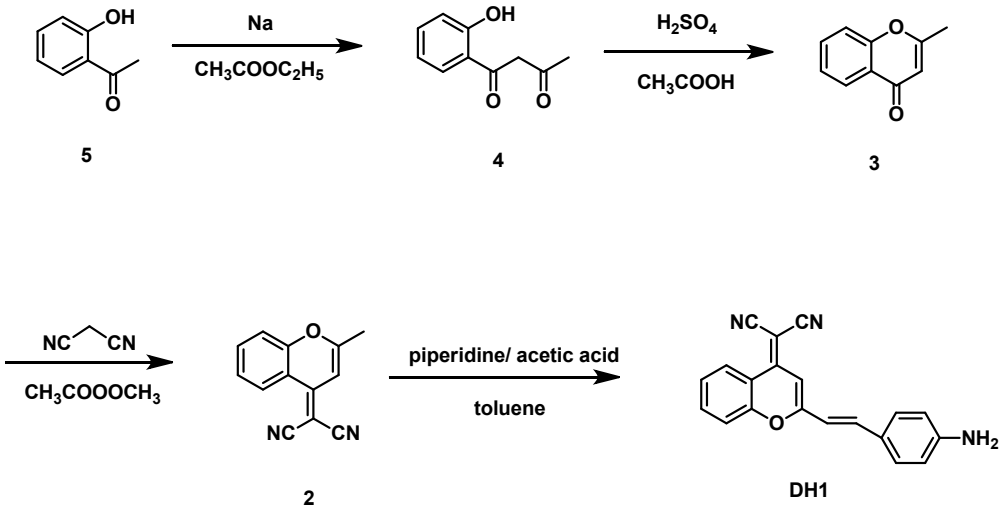
attractive receptor-ligand interactions (C+pol descriptor), and the squared sum of the total polar surface area of the receptor and the ligand (TotPol2).(Krammer et al., 2005) The 3-descriptor system that could be found is equation (1):

LigScore1_Dreiding = -0.3498 - 0.04673*vdW + 0.1653*C+pol -0.001132*TotPol2

where vdW indicates the van der Waals energy of protein-ligand interactions, which is computed via a Lennard-Jones potential using van der Waals radii and energy parameters of either the consistent force field (CFF) or the Dreiding force field. So in the case of the solvent sensitive probes, the contributions of three key descriptors (vdW and two surface descriptors) may accurately embody the interactions between the probes and serum albumins.

7. Synthetic procedures

Scheme S1. Synthesis of the compounds



Synthesis

1-(2-hydroxyphenyl)butane-1,3-dione (4):

1-(2-hydroxyphenyl)ethanone (10.0 g, 73.5 mmol) was dissolved in 200 mL ethyl acetate, and then sodium (8.00 g, 0.34 mmol) was added into the solution. The grayish-green solid was filtered after violently stirring for 4h at room temperature. The solid was dissolved in 100 mL deionized water, followed by the adjustment of pH

of the solution to neutral. The aqueous solution was extracted with 200 mL EtOAc and the organic layers were dried over Na_2SO_4, filtered, and concentrated to yield the final crude product as a brown solid (6.95 g, 53%) which was directly used in the next reaction without further purification.

2-methyl-4H-chromen-4-one (3):

Sulfuric acid (4.6 mL) was slowly added to a AcOH solution (70 mL) containing 1-(2-hydroxy phenyl)butane-1,3-dione (6.95 g, 38.9 mmol). The mixture was refluxed at 120 ^oC for about 30 min and then was poured into 800 mL ice water, followed by the adjustment of pH of the solution to neutral with Na_2CO_3. The aqueous solution was extracted with methylene dichloride twice and the organic layers were dried over Na_2SO_4, filtered, and concentrated to yield the final crude product as an acicular gray solid (4.78 g, 76.9%). The crude product was directly used in the next reaction without further purification.

2-(2-methyl-4H-chromen-4-ylidene)malononitrile (2):

2-methyl-4H-chromen-4-one (4.78 g, 29.9 mmol) and malononitrile (2.40 g, 36.2 mmol) were dissolved in 25 mL acetic anhydride. The solution was refluxed at 140 ^oC for 14 h and then the solvent was evaporated in vacuo. Deionized water (80 mL) was added to the residue and the mixture was refluxed for anther 0.5 h, followed by extraction with methylene dichloride. The organic layers were dried over Na_2SO_4, filtered, and concentrated. The obtained crude product was purified by silica column chromatography to yield compound **2** as an orange solid (2.02 g, 32.5 %).^{1}H NMR (400 MHz, $CDCl_3$) , δ (ppm): 8.92 (d, 1H, J = 8.0 Hz), 7.72 (t, 1H, J = 8.0 Hz), 7.46 (d, 1H, J = 8.0 Hz), 7.45 (t, 2H, J = 8.0 Hz), 6.72 (s, 1H), 2.44 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$), δ 161.68, 153.24, 152.91, 134.58, 126.04, 125.82, 118.64, 117.57, 116.57, 115.45, 105.48, 62.40, 20.47; HPLC-MS (API-ES): $[M+H]^+$ 209.1, found 209.1; $[M+Na]^+$ 231.1, found 231.1.

(E)-2-(2-(4-azidostyryl)-4H-chromen-4-ylidene)malononitrile (DH1):

2-(2-methyl-4H-chromen-4-ylidene)malononitrile (63.0 mg, 0.30 mmol), and N-(4-formyl-phenyl)acetamide (45.0 mg, 0.28 mmol) were dissolved in toluene (10 mL) with piperidine (0.15 mL) and acetic acid (0.15 mL) under argon protection at room temperature. Then the mixture was refluxed at 115 ^{o}C for 3 h to give an orange precipitate. After filtration, the orange solid was refluxed in a solution of conc. HCl and ethanol (2:1, 30 mL) for another 2 h before the pH of the solution was adjusted to neutral. The aqueous solution was extracted with ethyl acetate and then the organic layers were dried over Na_2SO_4, filtered, and concentrated to obtain the crude product which was purified by silica column chromatography to yield **DH1** as a crimson solid (40.0 mg, 42.9%). ^{1}H NMR (400 MHz, DMSO), δ (ppm): 8.73 (d, 1H, J = 8.0 Hz), 7.89 (t, 1H, J = 8.0 Hz), 7.76 (d, 1H, J = 8.0 Hz), 7.66 (d, 1H, J = 16.0 Hz), 7.56 (t, 1H, J = 8.0 Hz), 7.49 (d, 1H, J = 8.0 Hz), 7.10 (d, 1H, J = 16.0 Hz), 6.87 (s, 1H), 6.61 (d, 2H, J = 8.0 Hz), 5.99 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 160.30, 153.05, 152.56, 152.53, 141.13, 135.47, 131.15, 126.35, 125.01, 122.79, 119.35, 118.25, 117.68, 116.89, 114. 28, 112.88, 105.16, 57.95; HPLC-MS (API-ES): $[M+H]^+$ 312.1, found 312.0; $[M+Na]^+$ 334.1, found 334.0.

Reference [S1]: B. Zhu, C. Gao, Y. Zhao, C. Liu, Y. Li, Q. Wei, Z. Ma, B. Du, X. Zhang. *Chem. Commun.* **2011**, 47, 8656-8658.

8. Fluorescence studies of DH1

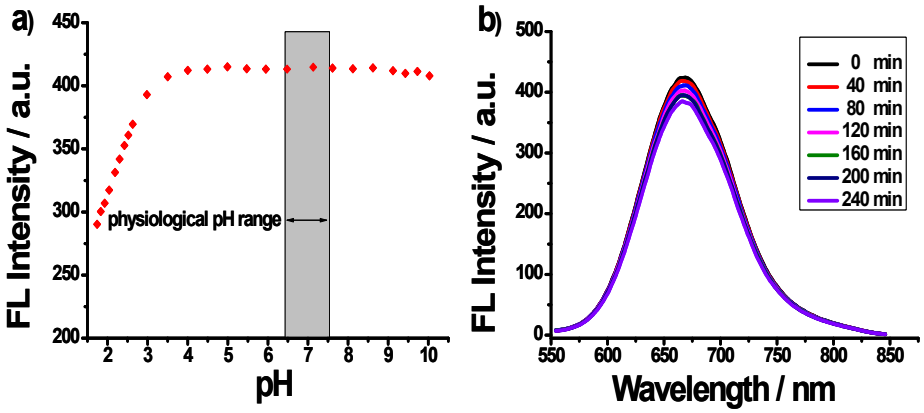


Figure S1. a) Fluorescence responses of **DH1** (5 μM) to various pH. b) Fluorescence changes of **DH1** were measured during light irradiation for 4 h using a 500 W *I–W* lamp as the light source. Conditions: λex = 520 nm, PBS buffer (0.2 M, pH 7.4, 50% DMSO), points showed the fluorescence intensity at 670 nm.

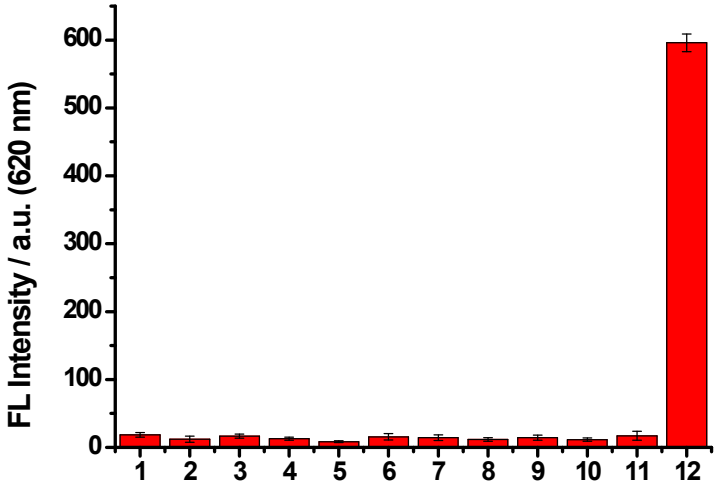


Figure S2. Fluorescence responses of **DH1** (5 μM) to HSA (0.17 mg/mL) and various cations (500 μM) at 620 nm. Conditions: PBS buffer (0.2 M, pH 7.4), λ_{ex} = 520 nm. Red bars represent the addition of HSA and cations to the solution of **DH1**. 1. blank, 2. Na^{+}, 3. Mg^{2+}, 4. Fe^{3+}, 5. Cu^{2+}, 6. Al^{3+}, 7. Zn^{2+}, 8. Ni^{2+}, 9. Hg^{2+}, 10 Mn^{2+}, 11. K^{+}, 12. HSA. (Three times for each experiment).

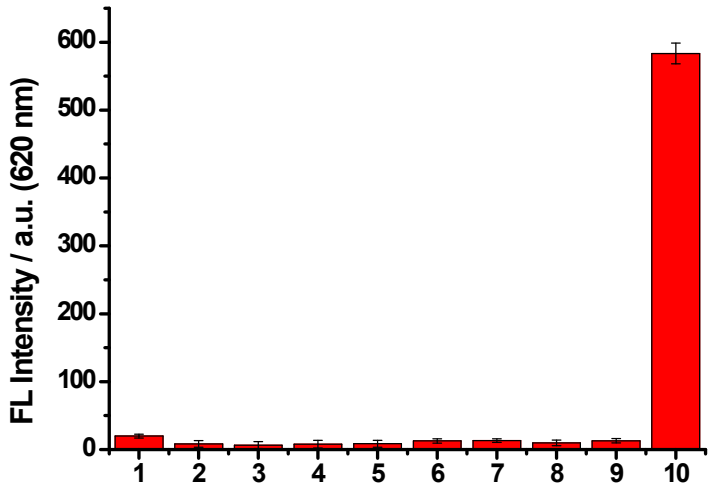


Figure S3. Fluorescence responses of **DH1** (5 μM) to HSA (0.17 mg/mL) and various anions (500 μM) at 620 nm. Conditions: PBS buffer (0.2 M, pH 7.4), λ_{ex} = 520 nm. Red bars represent the addition of HSA and aninos to the solution of **DH1**. 1. blank, 2. Cl^-, 3. NO_3^-, 4. SO_4^{2-}, 5. CO_3^{2-}, 6. ClO^-, 7. ClO_4^-, 8. Ac^-, 9. I^-, 10. HSA. (Three times for each experiment).

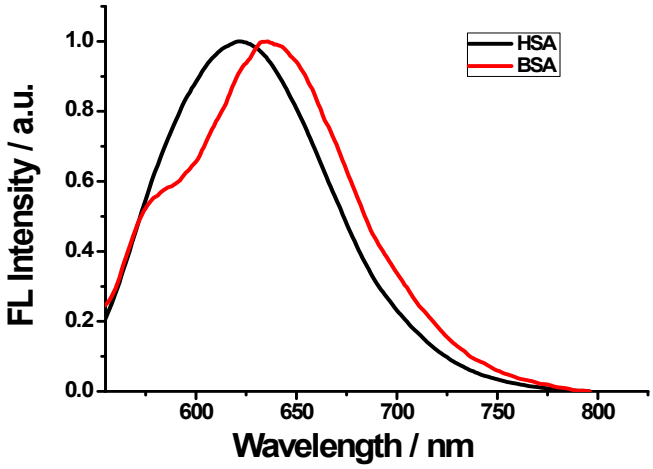


Figure S4. The normalized fluorescence emission spectra of **DH1** (5 μM) after upon addition of HSA (0.17 mg/mL) and BSA (0.17 mg/mL) in PBS buffer (pH 7.4, 0.2 M). λ_{ex} = 520 nm.

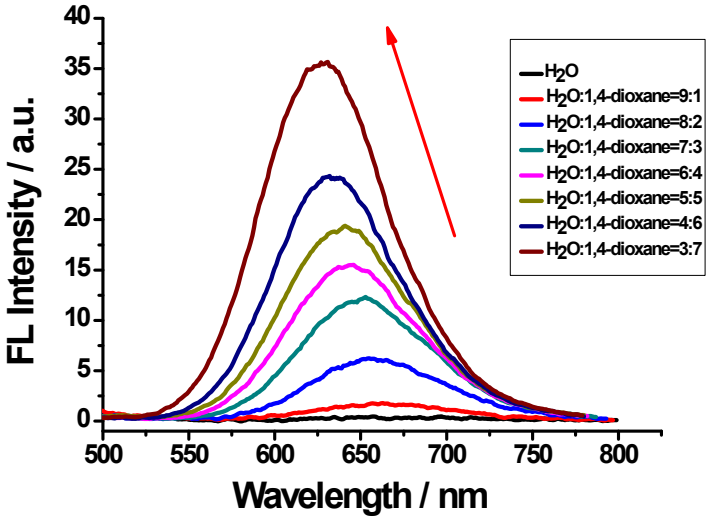


Figure S5. The fluorescence intensity spectra of **DH1** (5.0 μM) in water with different proportion of 1,4-dioxane (v/v, from 0-70%).

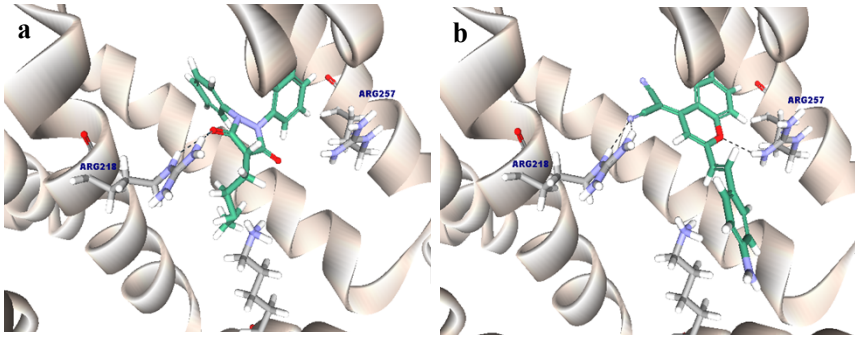


Figure S6. Docking conformation of the probes in the binding site I of HSA. The carbon atoms of the probes are green shown in stick mode. Hydrogen bonds are displayed as black dotted lines. (a) The pose of Phenylbutazone in 2BXP. (b) A docked pose of **DH1** in 2BXP.

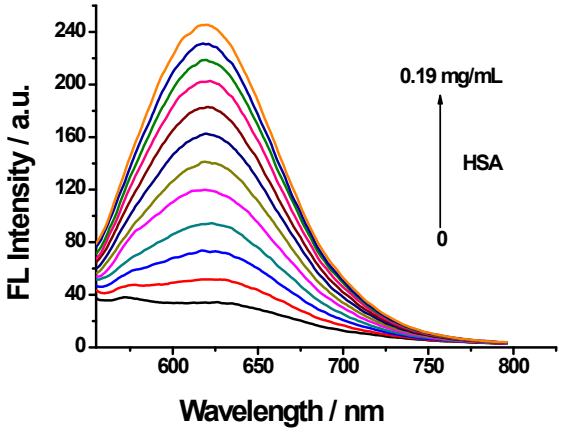


Figure S7. Fluorescence emission spectra of **DH1** (5 μM) upon the addition of increasing concentrations of HSA (0–0.19 mg/mL). The arrows indicate the change in the emission intensity with the increased HSA.

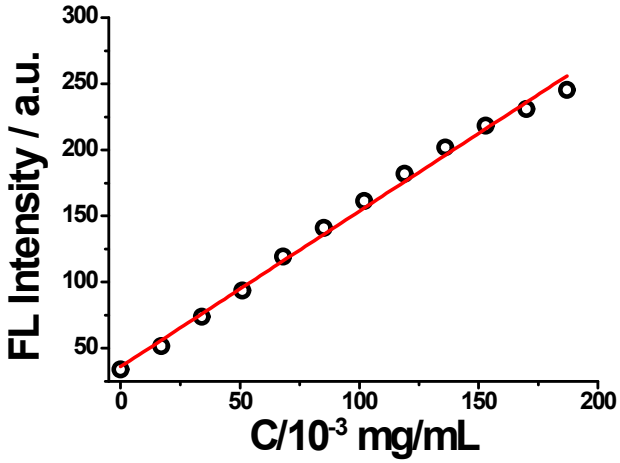


Fig. S8 The fluorescence intensity at 620 nm of probe **DH1** (5 μM) was linearly related to the concentrations of HSA (0–0.19 mg/mL), Y = 36.0410 + 1.17638 * X, R= 0.995. Conditions: PBS buffer (0.2 M, pH 7.4) and human urine (1:1, v/v), λex = 520 nm..

Table S1. Determination of HSA concentration in healthy human urine.

Samples	HSA Added (10^{-3} mg mL^{-1})	Proposed method	
		Found (10^{-3} mg mL^{-1})	Recovery (%)
1	30.0	31.3	104.3
2	45.0	44.8	99.5
3	85.0	86.0	101.2
4	140.0	136.3	97.4
5	180.0	176.8	98.2

9. NMR and MS data for compounds

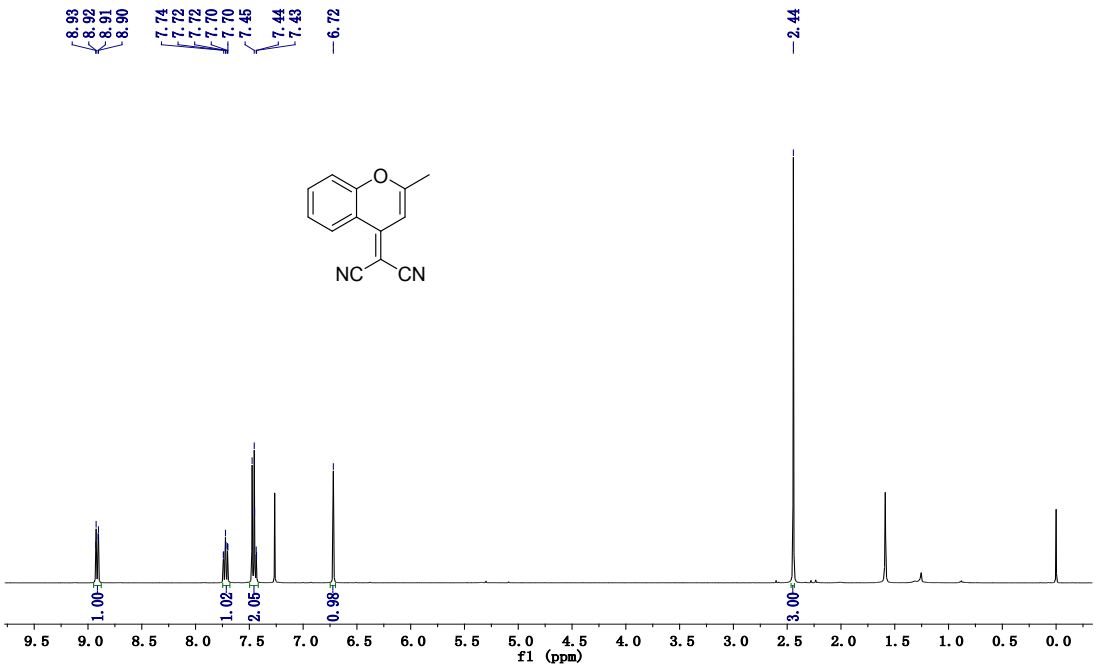


Figure S9. ^{1}H NMR spectrum of compound **2** in $CDCl_3$

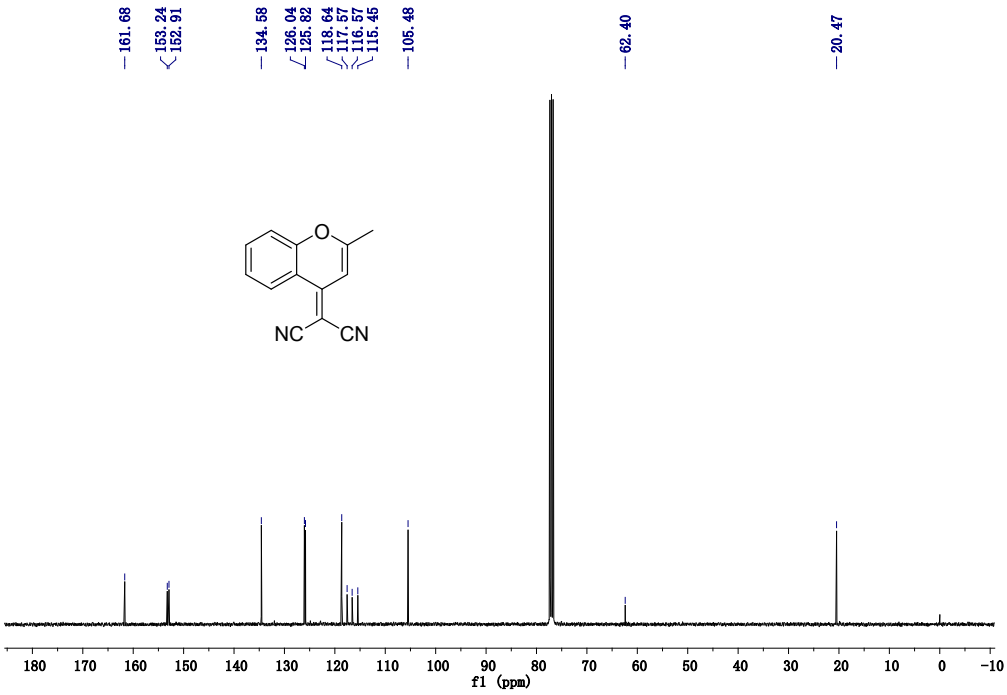
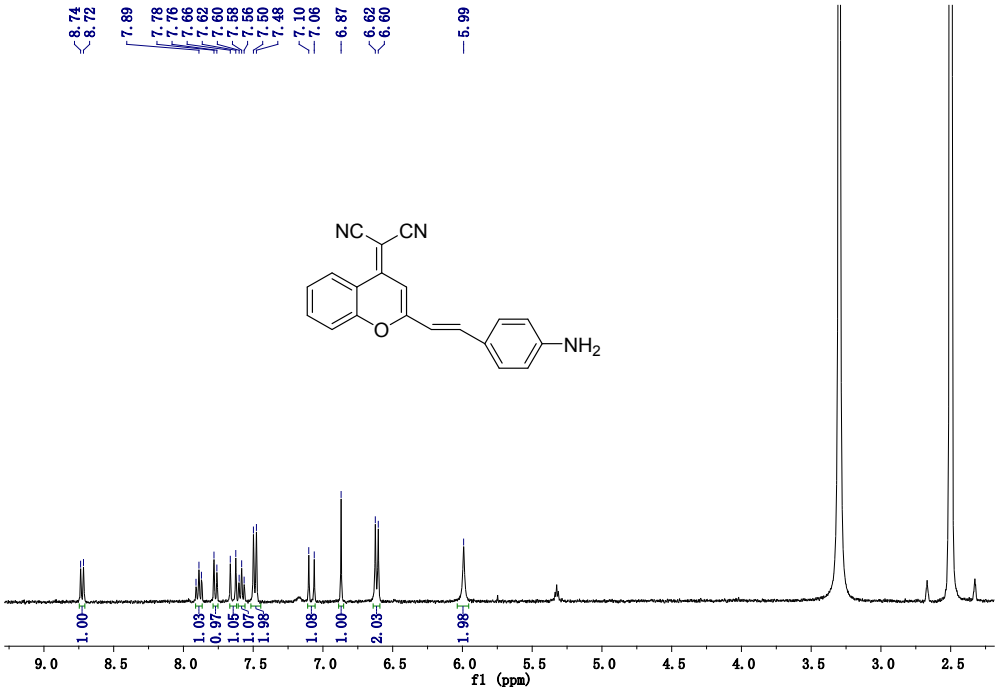


Figure S10. ^{13}C NMR spectrum of compound **2** in $CDCl_3$



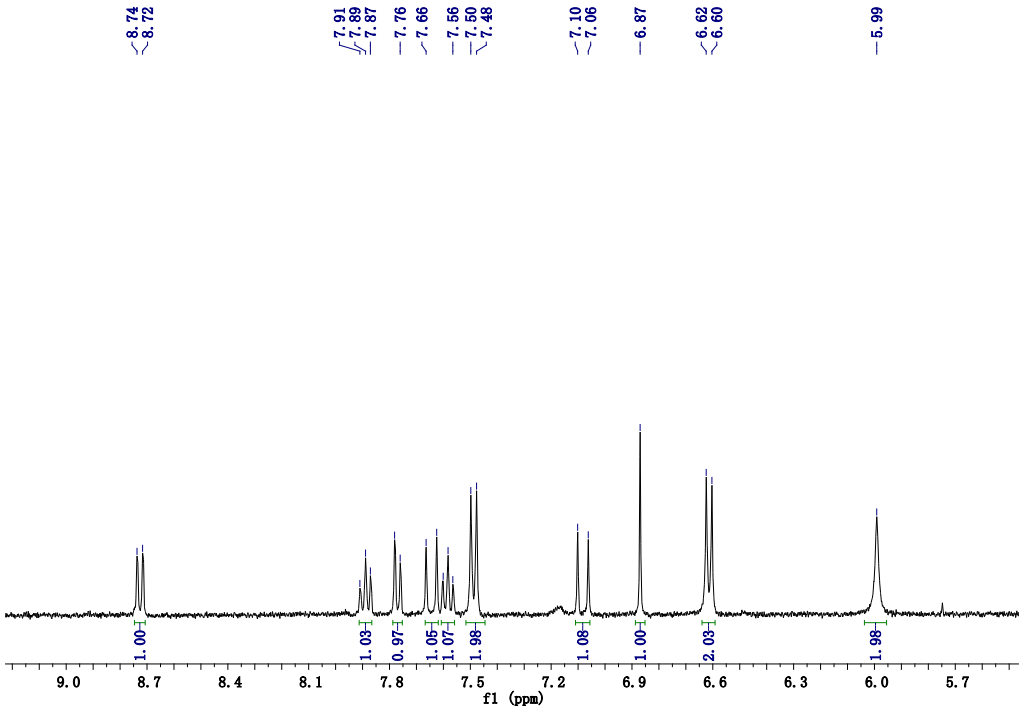


Figure S11. ^{1}H NMR spectrum of compound **DH1** in DMSO-d_6

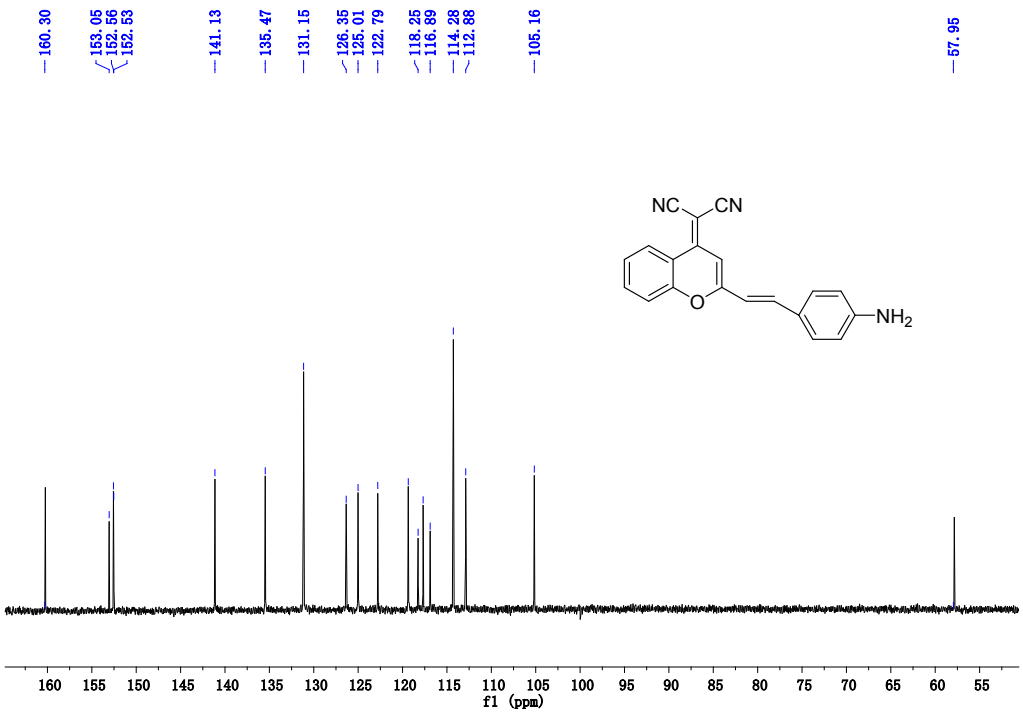


Figure S12. ^{13}C NMR spectrum of compound **DH1** in DMSO-d_6.