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ESI of

A Novel FA1-Targeting Fluorescent Probe for Specific Discrimination and Identification of human serum albumin from bovine serum albumin

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1. Experimental section

1.1 materials and instruments

5-Bromo-2,4-dimethoxybenzaldehyde, Boron tribromide, salicylaldehyde, 2-aminobenzenethiol, palladium diacetate, 4-bromo-2-hydroxy-5-methoxybenzaldehyde, 1,3-Diphenylisobenzofuran and triphenylphosphine were purchased from Energy Chemical (China). Benzothiazole and N-(3-chlorophenyl)carbonohydrazonoyl dicyanide were purchased from Tokyo Chemical Industry (Japan). All organic solvents were obtained from Chron Chemicals. Human Serum Albumin (HSA) and bovine serum albumin (BSA) purchased from Sigma-Aldrich (America).

1.2 Spectroscopic measurements

Fluorescence spectra and absorption spectra were recorded with a RF-6000 (Shimadzu, Japan) and UV-1750 (Shimadzu, Japan), respectively. The ^{1}H NMR and ^{13}C NMR spectra were recorded using Bruker AVANCE III 500 (Bruker, Germany). The TOF mass spectrum were recorded using LC-30A TripleTOF5600+ (AB SCIEX, Singapore). Stock solutions of 10 mM in DMSO were diluted to a concentration of 10 μM in the corresponding solvent. The diluted solutions were then transferred to a 1 cm × 1 cm quartz cuvette for testing.

1.3 Preparation of buffer solution

Phosphate buffered saline (PBS) buffer solution: Sodium dihydrogen phosphate (NaH_2PO_4, 10 mm) and disodium hydrogen phosphate (Na_2HPO_4, 10 mm) were mixed at different ratio, and the pH value of the mixed solution was examined with PHS-3C (Leici, China).

1.4 Molecular docking

The 3D geometry of flavonoids was constructed using the Chem3D. The structure of HSA (PDB ID: 1AO6) and BSA (PDB ID: 4F5S) was obtained from the Brookhaven protein data bank (http://www.rcsb.org/pdb). The resolutions of the files were 2.5 Å (HSA) and 2.47 Å (BSA). Semi flexible docking was performed by AutoDock 4.2 molecular docking program using the Lamarckian Genetic Algorithm. The output from AutoDock was rendered with PyMol and the ligand site analysis was assisted with LigPlus.

1.5 Selective experiments

1. KCl (2.5 mM), 2. NaCl (2.5 mM), 3. $MgCl_2$ (2.5 mM), 4. $CaCl_2$ (2.5 mM), 5. $ZnCl_2$ (2.5 mM), 6. $FeCl_3$ (2.5 mM), 7. Ovalbumin (200 μg/mL), 8. Myoglobin (200 μg/mL), 9. Casein (200 μg/mL), 10. γ-Globins (200 μg/mL), 11. Concanavalin (200 μg/mL), 12. Glucose (2.5 mM), 13. Urea (2.5 mM), 14. Formaldehyde (0.5 mM), 15. H_2O_2 (0.5 mM), 16. NaClO (0.025 mM), 17. Glutathione (0.5 mM), 18. Cysteine(0.5 mM), 19. Adenosine triphosphate (ATP, 0.5 mM), 20. Adenosine Diphosphate (ADP, 0.5 mM), 21. L-Proline (0.5 mM), 22. L-Threonine (0.5 mM), 23. L-Methionine (0.5 mM), 24. L-Histidine (0.5 mM), 25. 3-phenyl-L-alanine (0.5 mM), 26. L-Tryptophan (0.5 mM), 27. L-Lysine (0.5 mM), 27. L-Alanine (0.5 mM), 29.BSA (50 μg/mL), 30. HSA (20 μg/mL)

1.6 Competition experiments

C1 was mixed with biological species, and then measure the fluorescence signal change of **C1** at 446nm after adding HSA (100 μg/mL).

1. KCl (1 mM), 2. NaCl (1 mM), 3. $MgCl_2$ (1 mM), 4. $CaCl_2$ (1 mM), 5. $ZnCl_2$ (0.5 mM), 6. $FeCl_3$ (0.5 mM), 7. Ovalbumin (100 μg/mL), 8. Myoglobin (100 μg/mL), 9. Casein (100 μg/mL), 10. γ-Globins (100 μg/mL), 11. Concanavalin (100 μg/mL), 12. Glucose (1 mM), 13. Urea (1 mM), 14. Formaldehyde (0.25 mM), 15. H_2O_2 (0.25 mM), 16. NaClO (0.025 mM), 17. Glutathione (0.25 mM), 18. Cysteine(0.25 mM), 19. Adenosine triphosphate (ATP, 0.25 mM), 20. Adenosine Diphosphate (ADP, 0.25 mM), 21. L-Proline (0.25 mM), 22. L-Threonine (0.25 mM), 23. L-Methionine (0.25 mM), 24. L-Histidine (0.25 mM), 25. 3-phenyl-L-alanine (0.25 mM), 26. L-Tryptophan (0.25 mM), 27. L-Lysine (0.25 mM), 27. L-Alanine (0.25 mM)

1.7 Cells culture and Cytotoxicity assay

Streptomycin (100 μg per mL), penicillin (100 units per mL), and 10% fetal bovine serum were added to all culture media unless otherwise stated. HepG2 cell were incubated with RPMI-1640 complete medium. Cells were incubated at 37 ^{o}C in a 5% CO_2 humidified incubator.

HepG2 cells were seeded in 96-well microplates (Nest, China). After 24 h, when the cells had attached. The culture medium was removed. The cells were then cultured in RPMI-1640 medium with 0, 2.5, 5, 10, 15 and 20 μM of **C1** for 24 h. Cells in culture medium without **C1** were used as the control. A test concentration of 10 μL of MTT (5 mg/mL), prepared in RPMI-1640 medium, was added to each well, and the plates were incubated for another 4 h. The medium was then carefully removed, and the purple crystals were lysed in 200 μL DMSO. Optical density (OD) was determined with Spark (Tecan Austria GmbH, Austria) at 490 nm. Cell survival was calculated from the ratio of OD values of experimental and control groups.

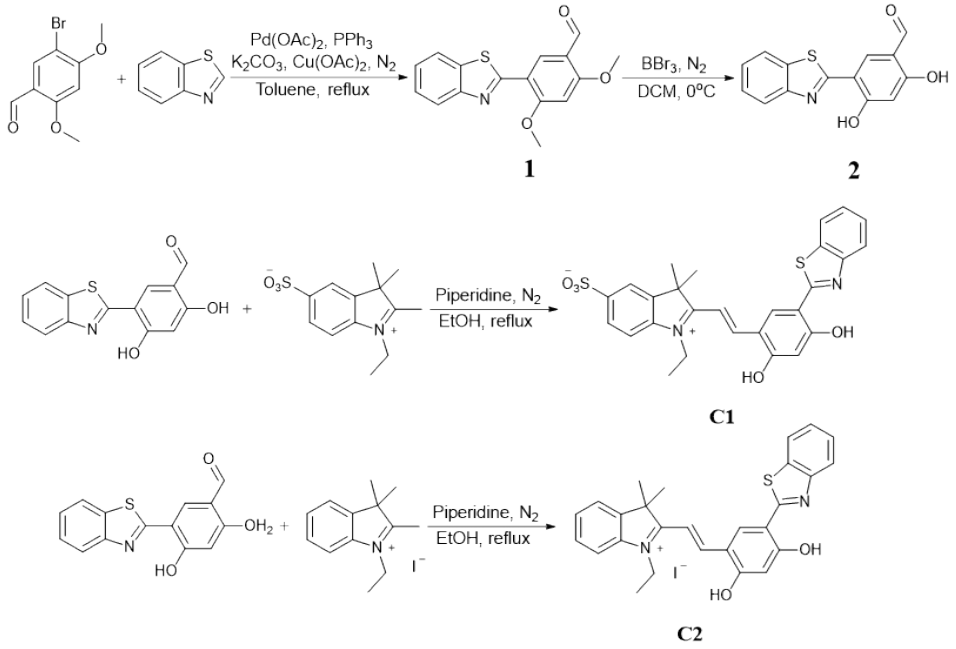
1.8 Preparation of test strips

The filter paper was cut into uniformly sized circles, which were then immersed in a **C1** ethanol solution (150 μM) for a duration of 10 min. Subsequently, the soaked filter paper pieces were carefully retrieved and subjected to drying in an oven.

1.9 Clinical samples

Serum samples, generously provided by the University Hospital of Northwest A&F University, had their HSA concentrations determined using the Bromocresol Green (BCG) assay.

2. Synthesis and characterizations



Scheme S1 Synthetic routes of **C1** and **C2**.

Synthesis of compound **1**. In a 250 mL single-necked round-bottom flask, 80 mL of toluene, 5-bromo-2,4-dimethoxybenzaldehyde (1 g, 4.1 mmol), benzothiazole (1.34 mL, 12.24 mmol), palladium acetate (92 mg, 0.41 mmol), copper acetate (163.2 mg, 0.82 mmol), potassium carbonate (1.13 g, 8.2 mmol) and triphenylphosphine (430.2 mg, 1.64 mmol) were added. The mixed solution was refluxed for 24 h under nitrogen atmosphere and the system gradually turned black. After the reaction was brought to room temperature without post-treatment, the white powder (920 mg, 75%) was purified by silica gel column. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.31 (s, 1H), 8.83 (s, 1H), 8.14 (s, 1H), 8.10 (s, 1H), 7.57 (s, 1H), 7.47 (s, 1H), 7.02 (s, 1H), 4.25 (s, 3H), 4.10 (s, 3H).

Synthesis of compound **2**. In a 50 mL double bottomed flask, compound **1** (300 mg, 1 mmol) was dissolved in 8 mL of dry dichloromethane and boron tribromide (950 μL, 10 mmol) was added dropwise while stirring in an ice bath under nitrogen atmosphere. At the end of the reaction, the reaction was quenched by slow addition of water at 0 °C. A green solid was observed in the round bottom flask. After completion of the reaction, the reaction was filtered under reduced pressure, washed with ice water (20 mL×3) and the solid was collected and dried under vacuum to obtain a light green powder. The powder was purified on a silica gel column to give a white solid (171 mg，63%）.^{1}H NMR (500 MHz, DMSO-d_6) δ 12.49 (s, 1H), 11.33 (s, 1H), 10.15 (s, 1H), 8.56 (s, 1H), 8.08 (d, *J*=7.9 Hz, 1H), 8.02 (d, *J*=8.1 Hz, 1H), 7.51 (t, *J*=7.6 Hz, 1H), 7.40 (t, *J*=7.5 Hz, 1H), 6.63 (s, 1H).

Synthesis of **C1**. In a 25 mL double-bottomed flask, 8 mL of anhydrous ethanol, compound 2 (80 mg, 0.295 mmol), 1-ethyl-2,3,3-trimethyl-3H-indol-1-ium-5-sulfonate (160 mg, 0.6 mmol) and hexahydropyridine (55 μL, 0.59 mmol) were added and refluxed for 8 h. After the reaction

was completed, the solid was collected by cooling and filtering, washed with ice-ethanol (10 mL × 3). A red solid (71 mg, 43%) was obtained after drying. ^{1}H NMR (500 MHz, DMSO) δ 8.91 (s, 1H), 8.58 (d, *J* = 16.0 Hz, 1H), 8.15 (d, *J* = 7.8 Hz, 1H), 8.06 (d, *J* = 9.1 Hz, 2H), 7.83 (d, *J* = 11.2 Hz, 3H), 7.56 (t, *J* = 7.6 Hz, 1H), 7.45 (t, *J* = 7.5 Hz, 1H), 6.76 (s, 1H), 4.57 (q, *J* = 6.9 Hz, 2H), 1.83 (s, 6H), 1.47 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 182.28, 164.20, 163.87, 163.17, 151.82, 151.22, 149.44, 143.55, 140.90, 135.20, 134.75, 126.94, 125.37, 122.34, 120.75, 116.00, 114.32, 113.95, 110.67, 103.63, 56.50, 52.21, 42.28, 26.63, 19.03, 13.51. TOF-MS: m/z calcd for $[M+H]^+$: 535.1356, found: 535.1354.

Synthesis of **C2**. In a 25 mL double-bottomed flask, 8 mL of anhydrous ethanol, compound 2 (80 mg, 0.295 mmol), 1-ethyl-2,3,3,5-tetramethyl-3H-indolium (160 mg, 0.59 mmol) and hexahydropyridine (55 µL, 0.59 mmol) were added and refluxed for 4 h. After the reaction was completed, the solid was collected by cooling and filtering, washed with ice-ethanol (10 mL × 3). A dark red solid (70 mg, 42%) was obtained after drying. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.92 (s, 1H), 8.59 (d, *J*=16.0 Hz, 1H), 8.18 (d, *J*=7.4 Hz, 1H), 8.09 (d, *J*=7.6 Hz, 1H), 7.98–7.83 (m, 3H), 7.69–7.54 (m, 3H), 7.47 (t, *J*=6.7 Hz, 1H), 6.79 (s, 1H), 4.61 (q, *J*=6.3 Hz, 2H), 1.85 (s, 6H), 1.51 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.82, 164.12, 163.81, 163.07, 151.84, 151.18, 143.95, 141.03, 135.27, 134.81, 129.54, 129.21, 126.95, 125.37, 123.54, 122.47, 122.35, 115.98, 114.99, 113.99, 110.77, 103.66, 52.16, 42.23, 26.71, 13.59. TOF-MS: m/z calcd for $[M+H]^+$: 441.1631, found: 441.1624.

3. Supporting Figures and Table

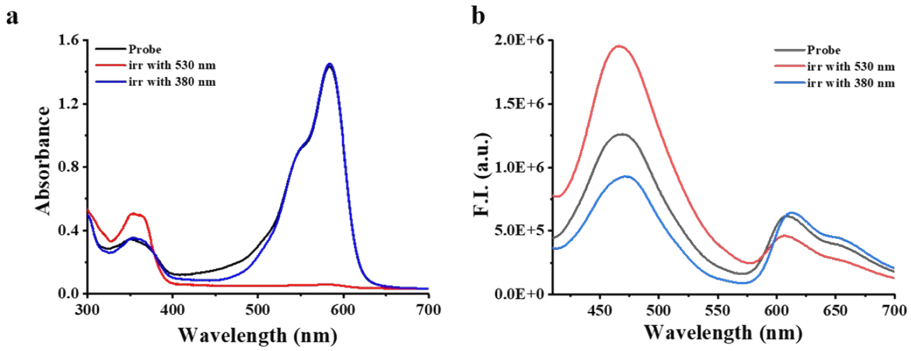


Fig. S1 The absorption spectra (a) and fluorescence spectra (b) of **C1** (10 µM) in EtOH (5% water) after treatment with 380 or 530 nm illumination. The excitation wavelength (λ_{ex}) for fluorescence spectra is at 380 nm.

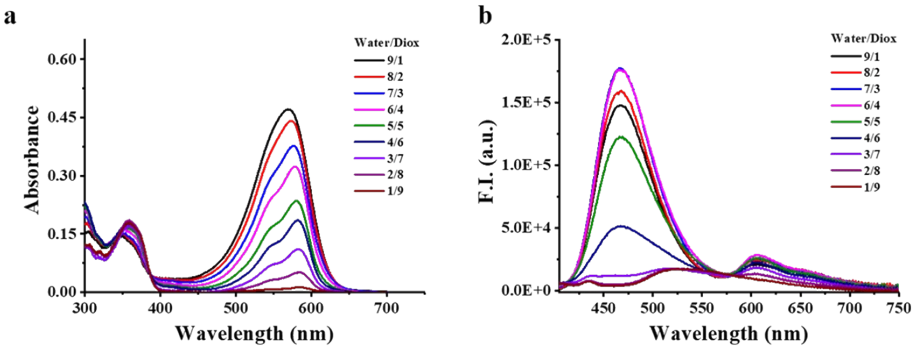


Fig. S2 The absorption spectra (a) and fluorescence spectra (b) of **C1** (10 μM) in water-dioxane mixtures. λ_{ex} = 380 nm.

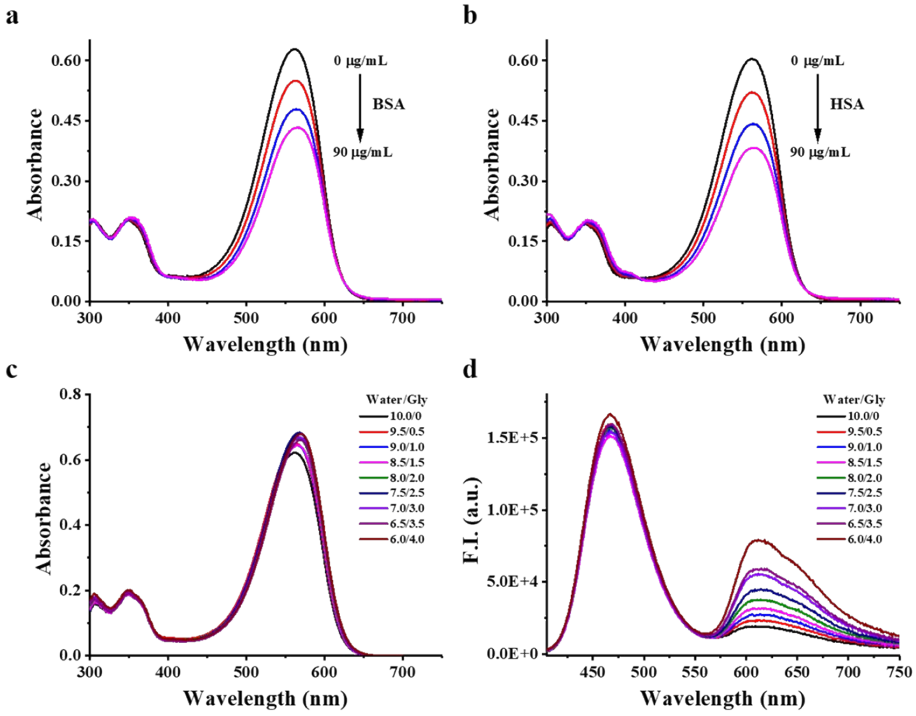


Fig. S3 The absorption spectra of **C1** (10 μM) in the presence of various concentrations of HSA (b) or BSA (c) from 0 to 90 μg/mL in PBS buffer (10 mM, pH7.4). The absorption spectra (c) and fluorescence spectra (d) of **C1** (10 μM) in water-glycerol mixtures. λ_{ex} = 380 nm.

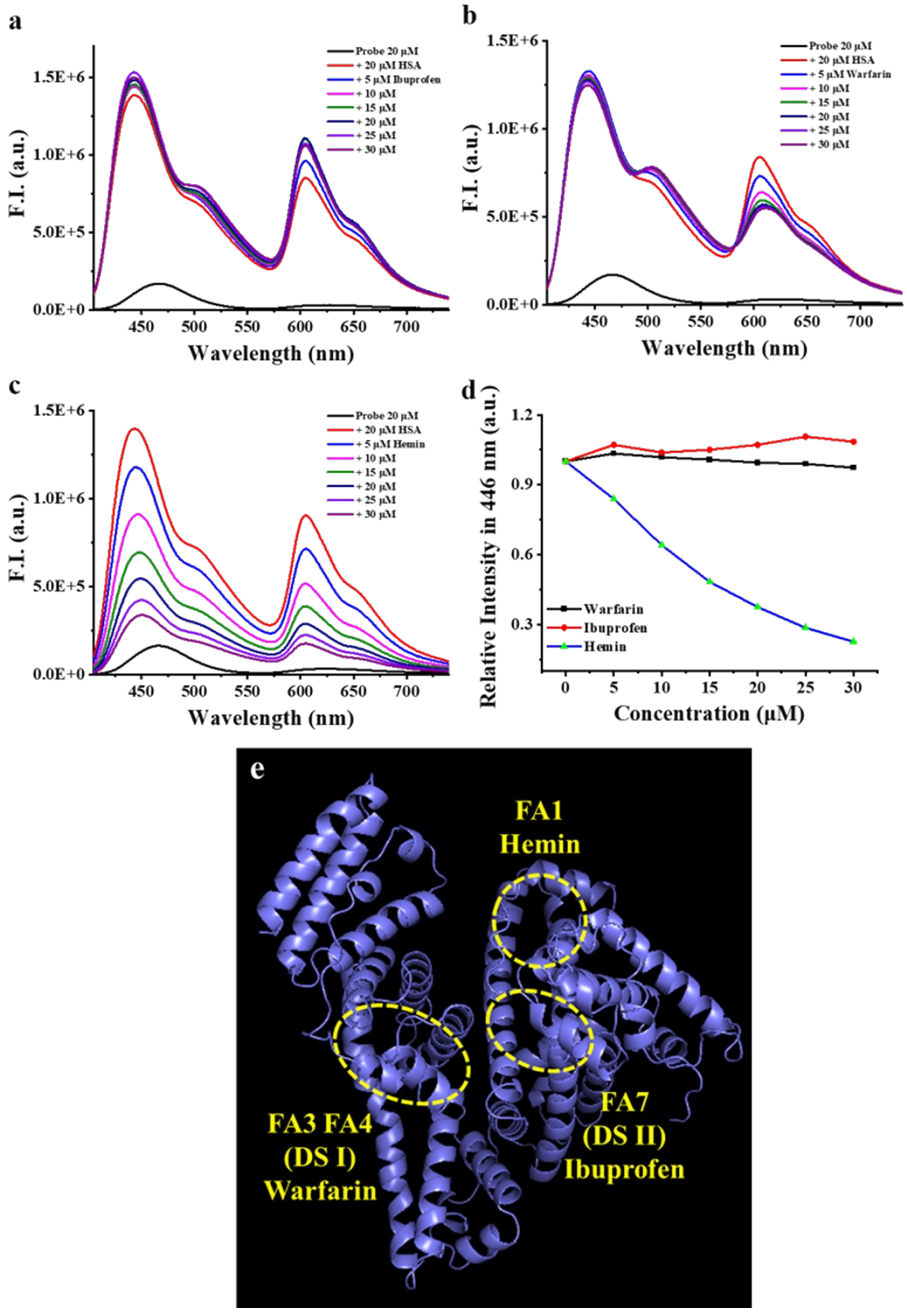


Fig. S4 The fluorescence spectra of **C1**@HSA in the presence of various concentrations of ibuprofen (a), warfarin (d) or hemin (c) in PBS buffer (10 mM, pH7.4). (d) Relative intensity of **C1**@HSA in the presence of ibuprofen, warfarin and hemin. (e) Schematic representation of the drug binding site of HSA. λ_{ex} = 380 nm.

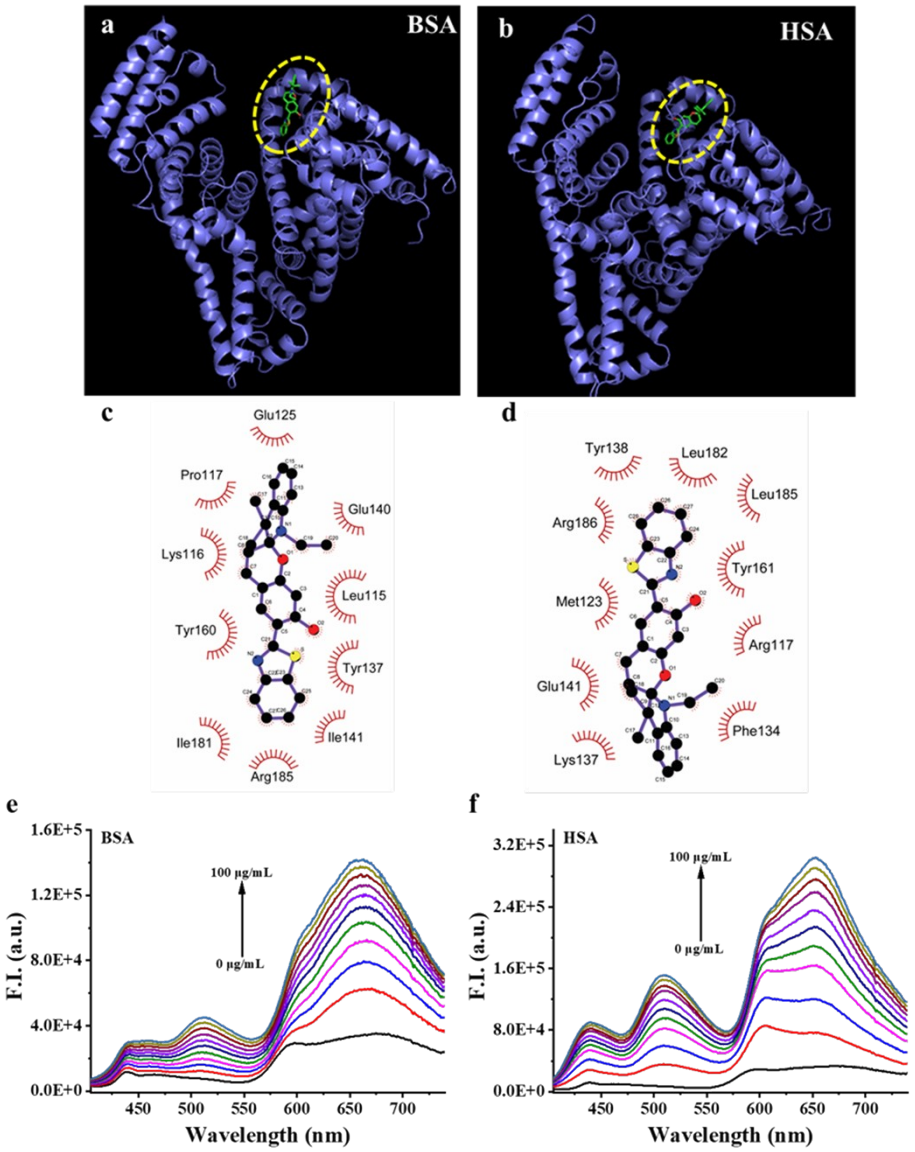


Fig. S5 Calculated binding modes of **C2** ring-closing isomer with BSA (a) or HSA (b) by using the molecular docking (Autodock 4.2). Possible formed hydrogen bonds between **C2** ring-closing isomer and amino acid residues in BSA (c) or HSA (d) pocket. The fluorescence spectra of **C2** (10 μM) in the presence of various concentrations of BSA (e) or HSA (f) from 0 to 0.1 mg/mL. λ_{ex} = 380 nm.

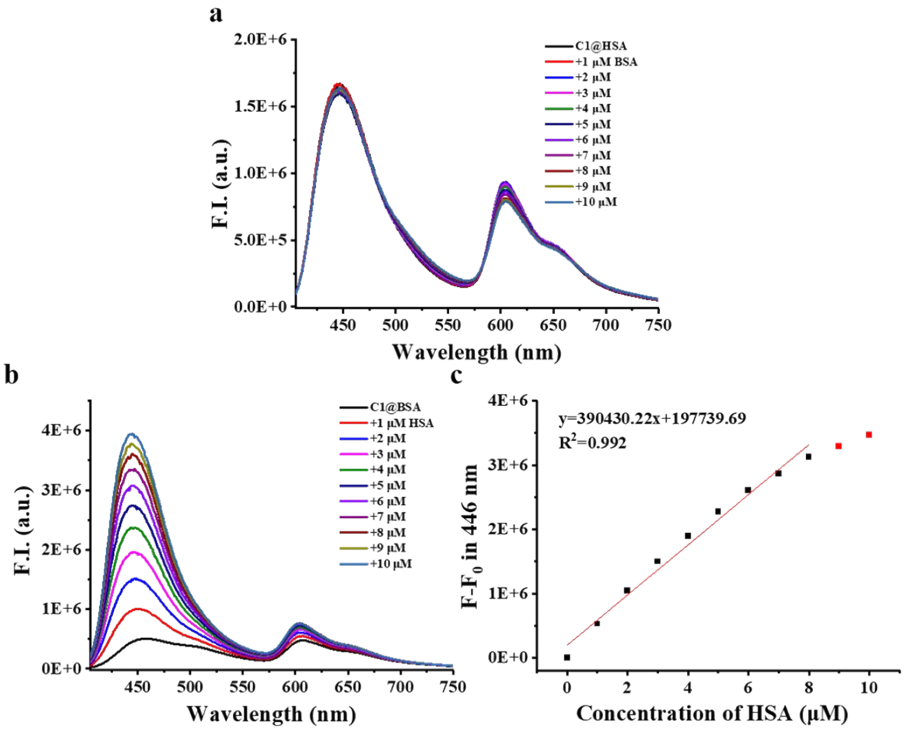
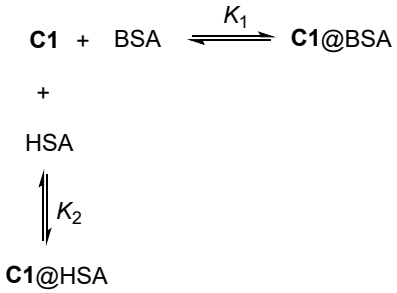


Fig. S6 (a) The fluorescence spectra of **C1**@HSA (**C1** 10 μM, HSA 10 μM) in the presence of various concentrations of BSA. (b) The fluorescence spectra of **C1**@BSA (**C1** 10 μM, BSA 10 μM) in the presence of various concentrations of HSA. (c) Relationship between fluorescence intensity in 446 nm of **C1**@BSA (**C1** 10 μM, BSA 10 μM) and the concentration of HSA. λ_{ex} = 380 nm. Error bars stand for the mean value of three experiments.



Scheme S2 Schematic diagram of competitive binding equilibrium of **C1** between BSA and HSA.

Compared to BSA, HSA exhibits a lower binding energy to **C1** and more easily bind to **C1** to form **C1**@HSA (Here K_2>K_1, K_1 and K_2 are the binding constant of **C1**@BSA and **C1**@HSA, respectively). In the solution of **C1**@BSA (**C1**=10 μM, BSA=10 μM), due to the binding equilibrium there exists free **C1** and BSA. With the adding of HSA to the solution of **C1**@BSA, the free **C1** was bound to HSA and **C1**@HSA continuously forms (the fluorescence intensity correspondingly increases), and meanwhile more free BSA was released. When HSA concentrations exceed 9 μM, it means that more than 9 μM free BSA was released. In the presence of high concentration of free BSA, **C1**@BSA is hard to further release free **C1** that was used to bind with HSA to form **C1**@HSA, which is the plausible reason that there are apparent outliers in Figure S6C when HSA concentrations exceed 9 μM.

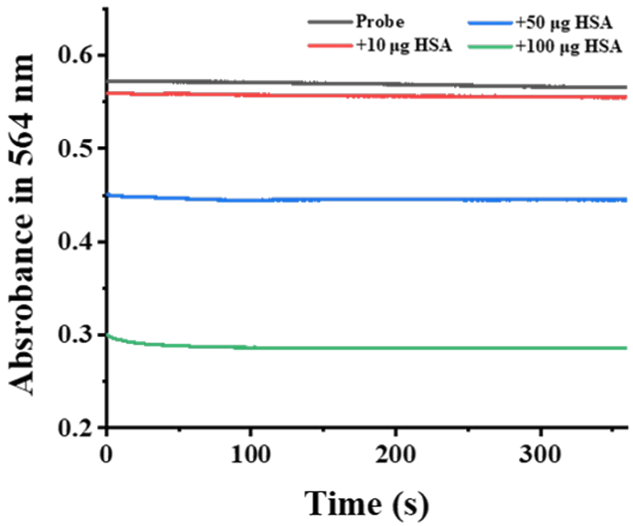


Fig. S7 Time-dependent absorbance change at 563 nm of **C1** (10 μM) in the presence and the absence of HSA in PBS buffer (10 mM, pH 7.4).

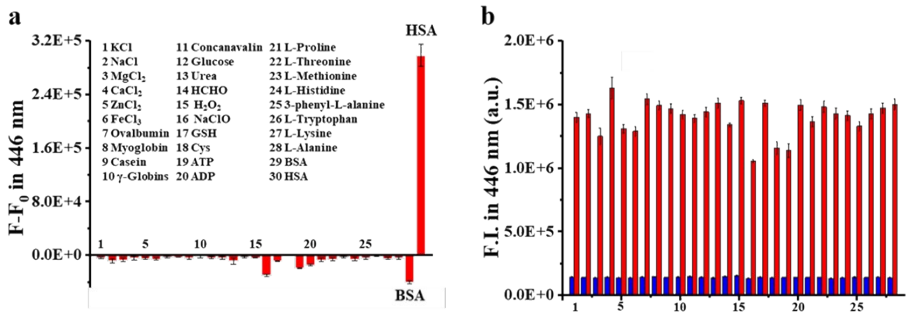


Fig. S8 (a) F-F_0 at 446 nm of **C1** (10 μM) in the presence of different biological species. (b) The fluorescence intensity at 446 nm of **C1** (10 μM) in the presence of different biological species (blue bars), red bars represent the relative intensity with subsequent addition of HSA (100 μg/mL). Error bars stand for the mean value of three experiments.

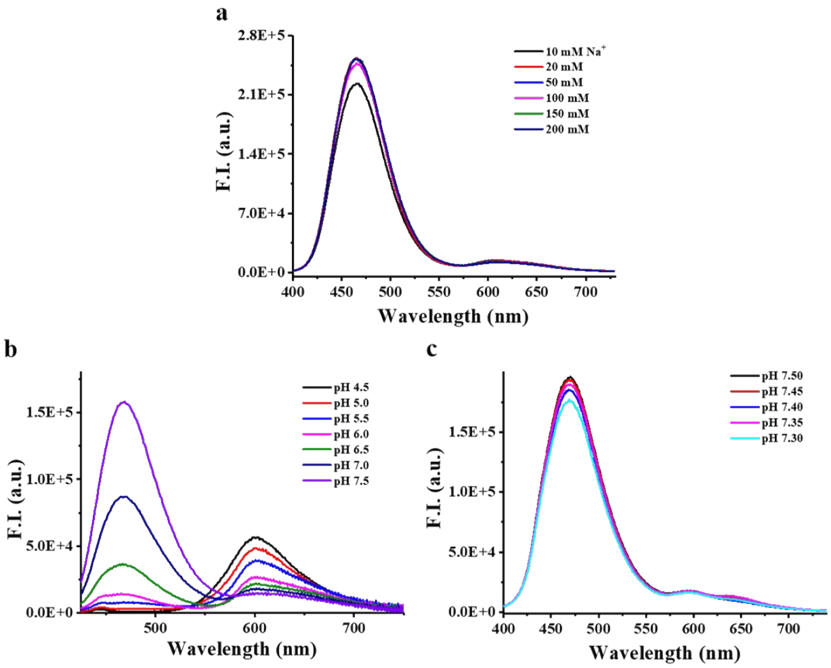


Fig. S9 (a) The fluorescence spectra of **C1** (10 μM) in PBS buffer (pH7.4) with different ionic strengths. (b) The fluorescence spectra of **C1** (10 μM) in BR buffer (10 mM) with different pH value (4.5-7.5). (c) The fluorescence spectra of **C1** (10 μM) in BR buffer (10 mM) with different pH value (7.3-7.5). λ_{ex} = 380 nm.

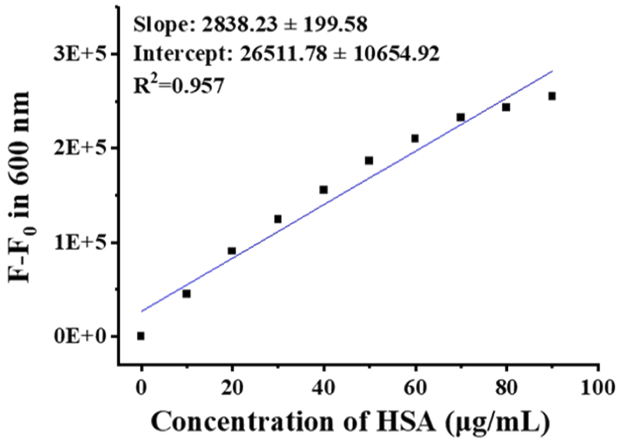


Fig. S10 Relationship between fluorescence intensity of **C1** (10 μM) at 600 nm and the concentration of HSA. λ_{ex} = 380 nm.

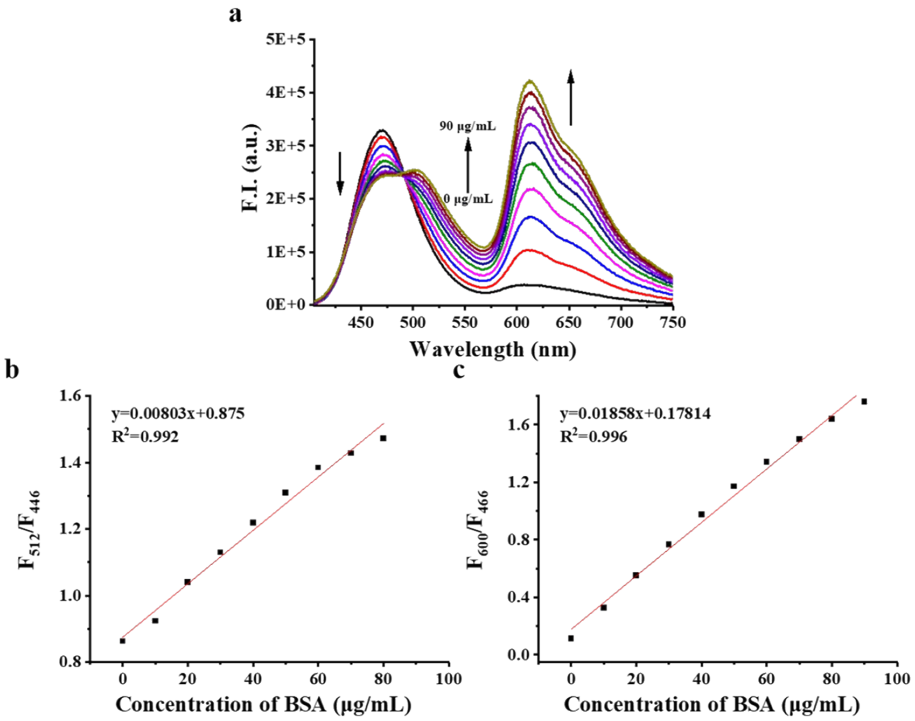


Fig. S11 (a) The fluorescence spectra of **C1** (10 μM) in the presence of various concentrations of BSA from 0 to 90 μg/mL. (b) Relationship between F_{512}/F_{446} of **C1** (10 μM) and the concentration of BSA. (c) Relationship between F_{600}/F_{466} of **C1** (10 μM) and the concentration of HSA. λ_{ex} = 380 nm.

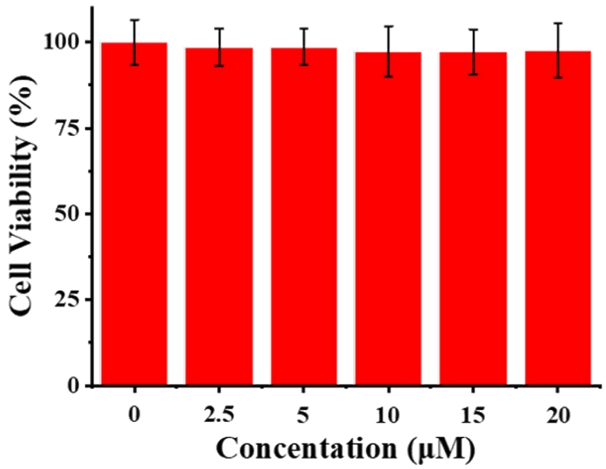


Fig. S12 The cell viability of HepG2 cells treated with different concentration of **C1**. Error bars stand for the mean value of five experiments.

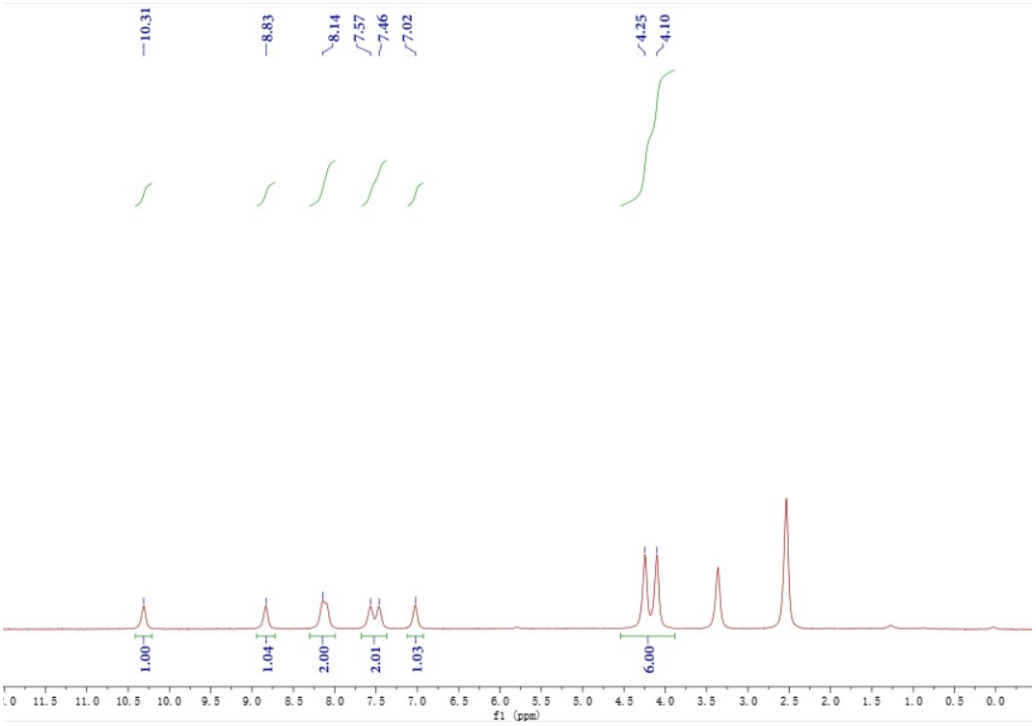


Fig. S13 ^{1}H NMR spectrum of compound **1** in DMSO-d_6 solvent.

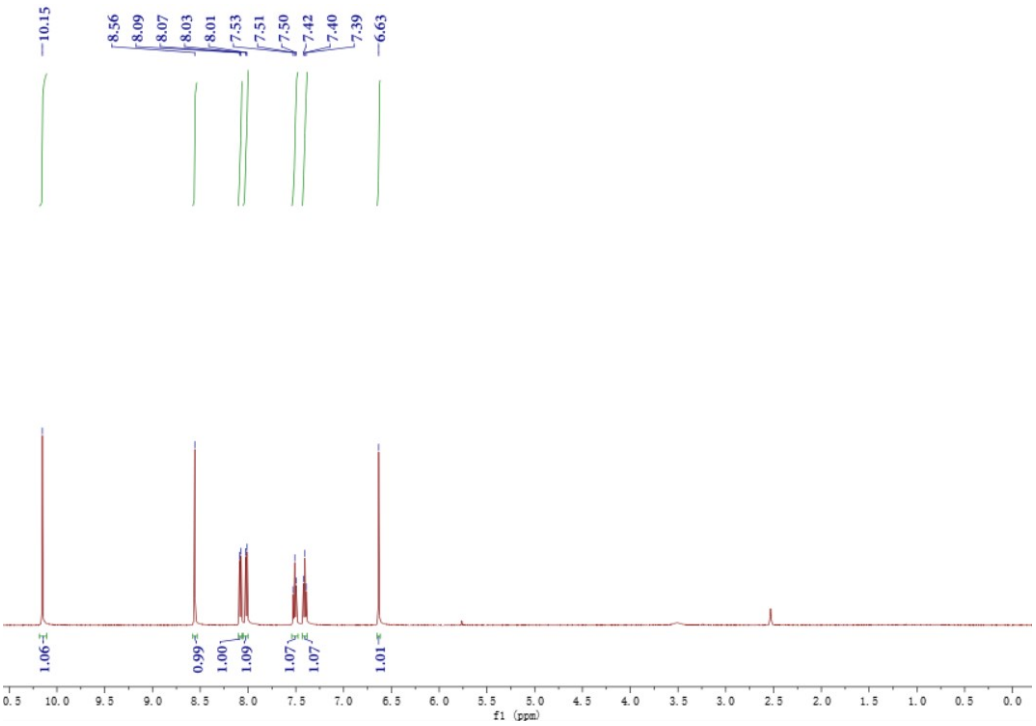


Fig. S14 ^{1}H NMR spectrum of compound **2** in DMSO-d_6 solvent.

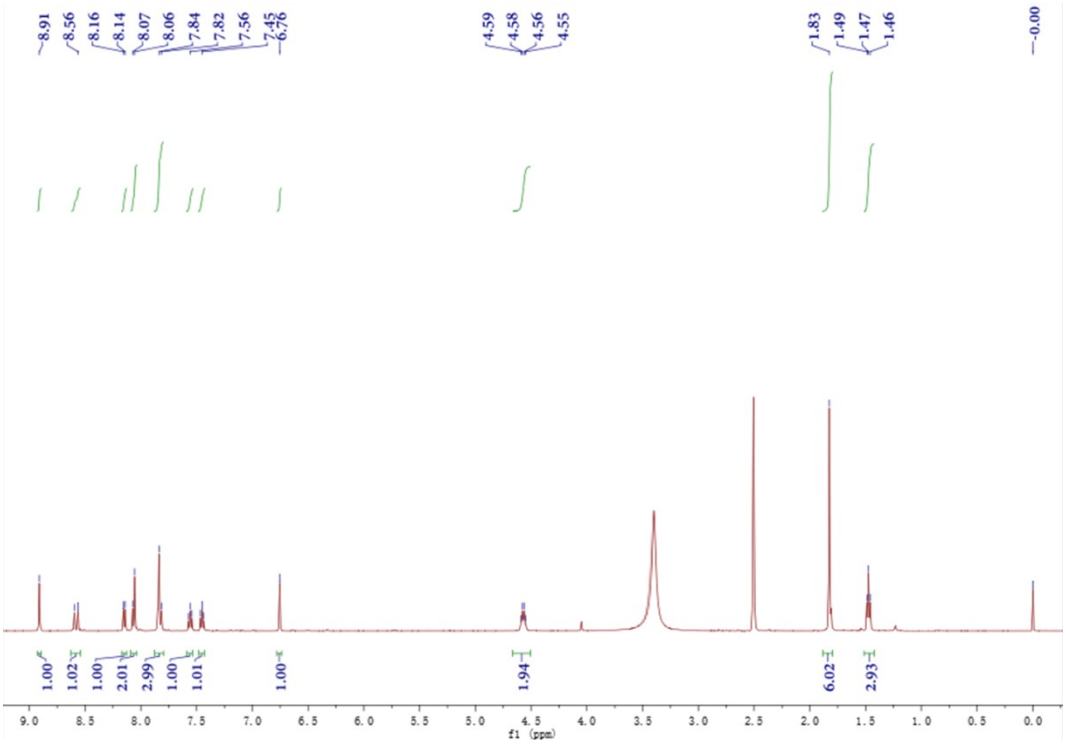


Fig. S15 ^{1}H NMR spectrum of **C1** in DMSO-d_6 solvent.

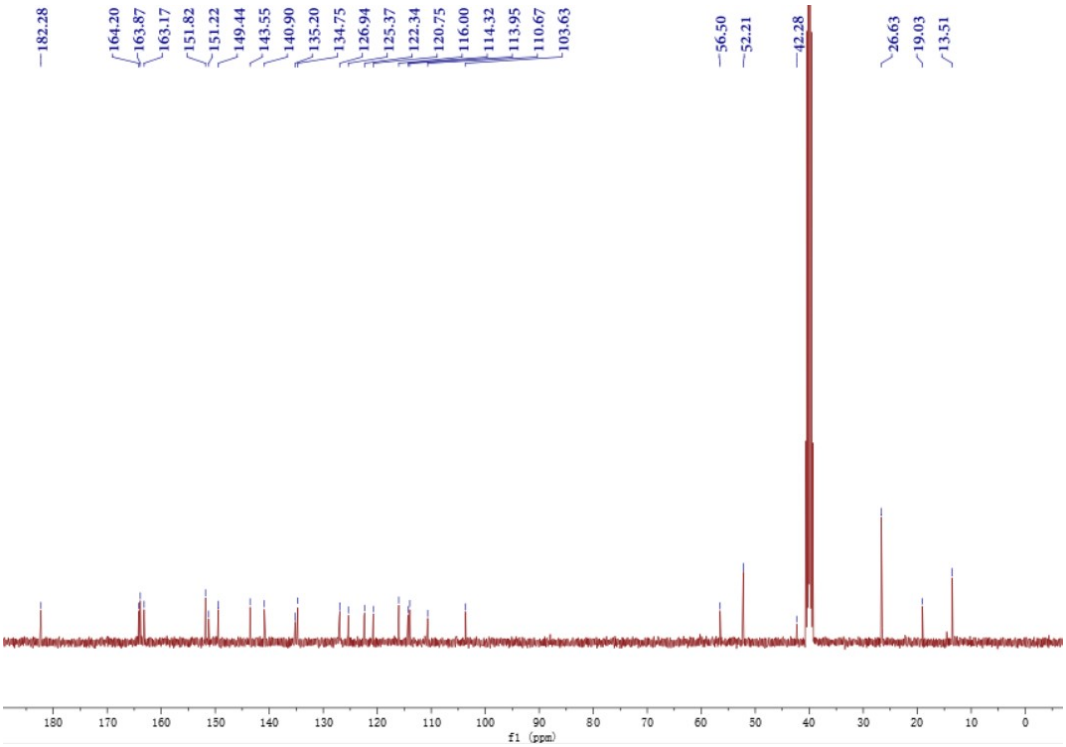


Fig. S16 ^{13}C NMR spectrum of **C1** in DMSO-d_6 solvent.

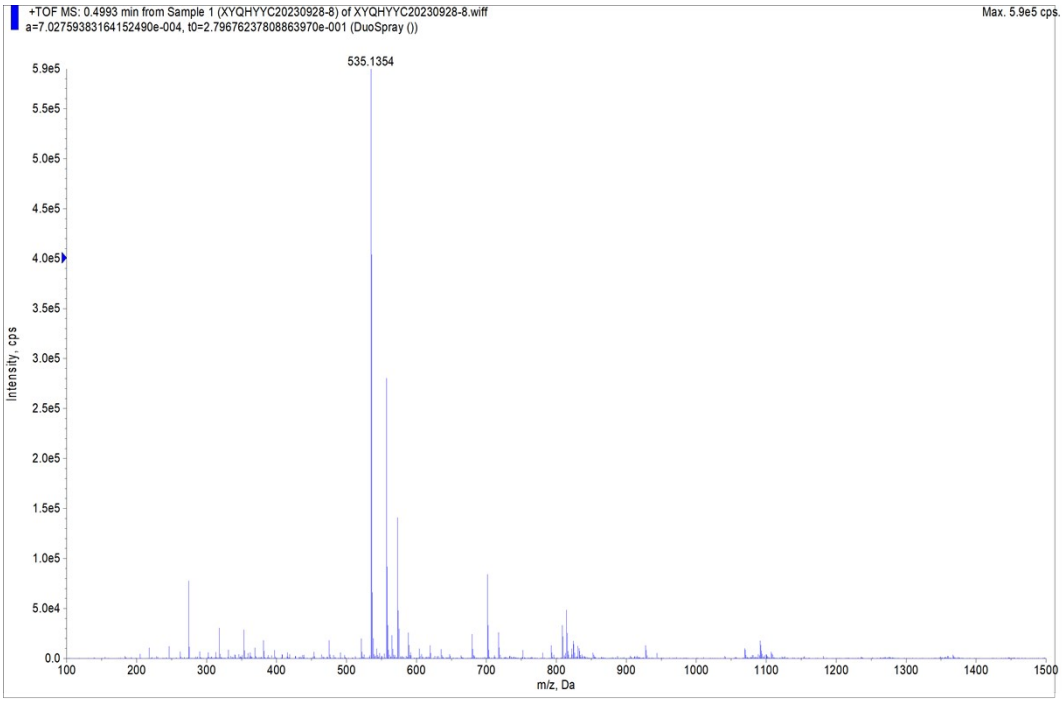


Fig. S17 TOF mass spectrum of **C1.**

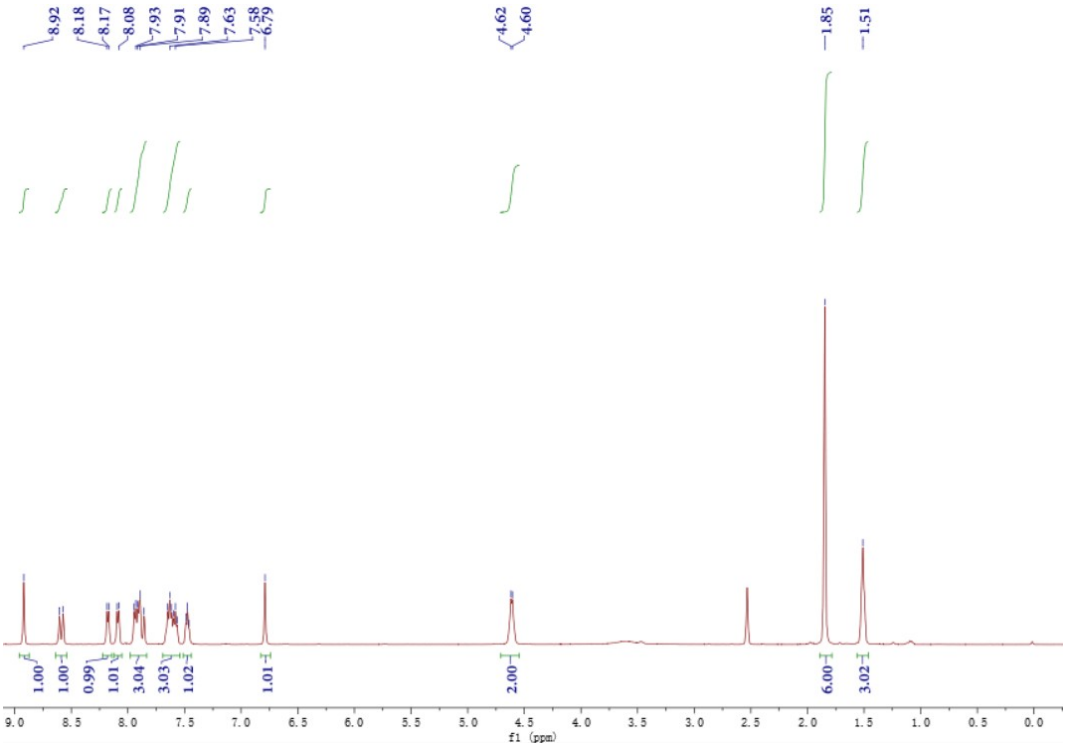


Fig. S18 ^{1}H NMR spectrum of **C2** in DMSO-d_6 solvent.

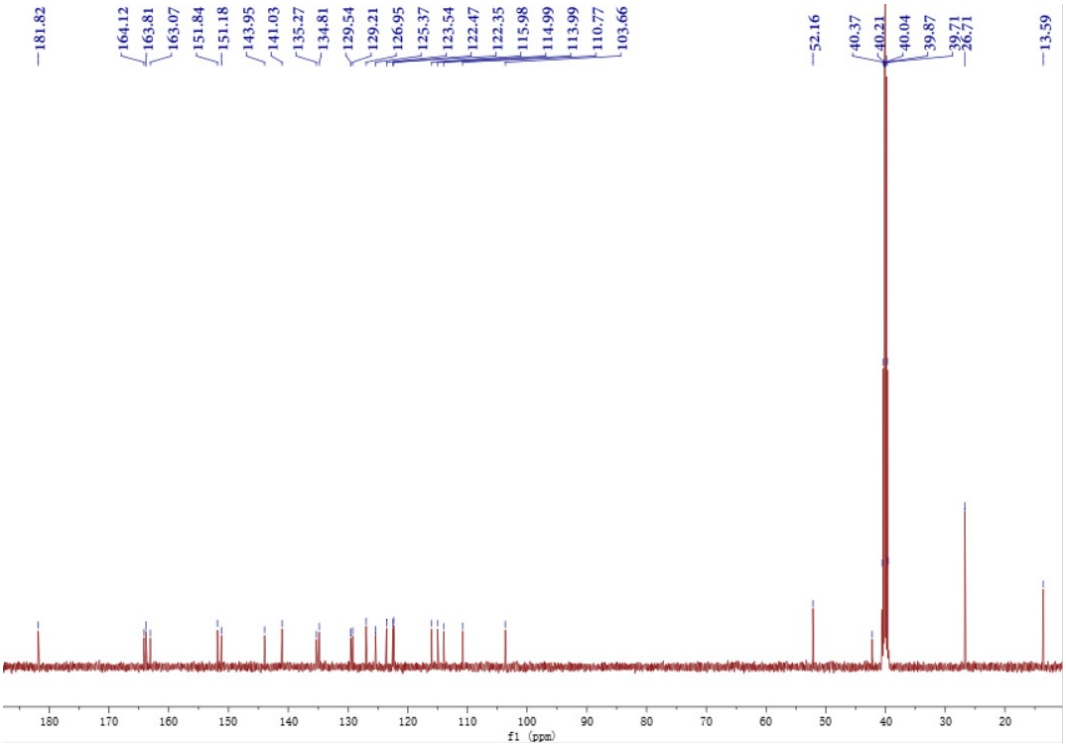


Fig. S19 ^{13}C NMR spectrum of **C2** in DMSO-d_6 solvent.

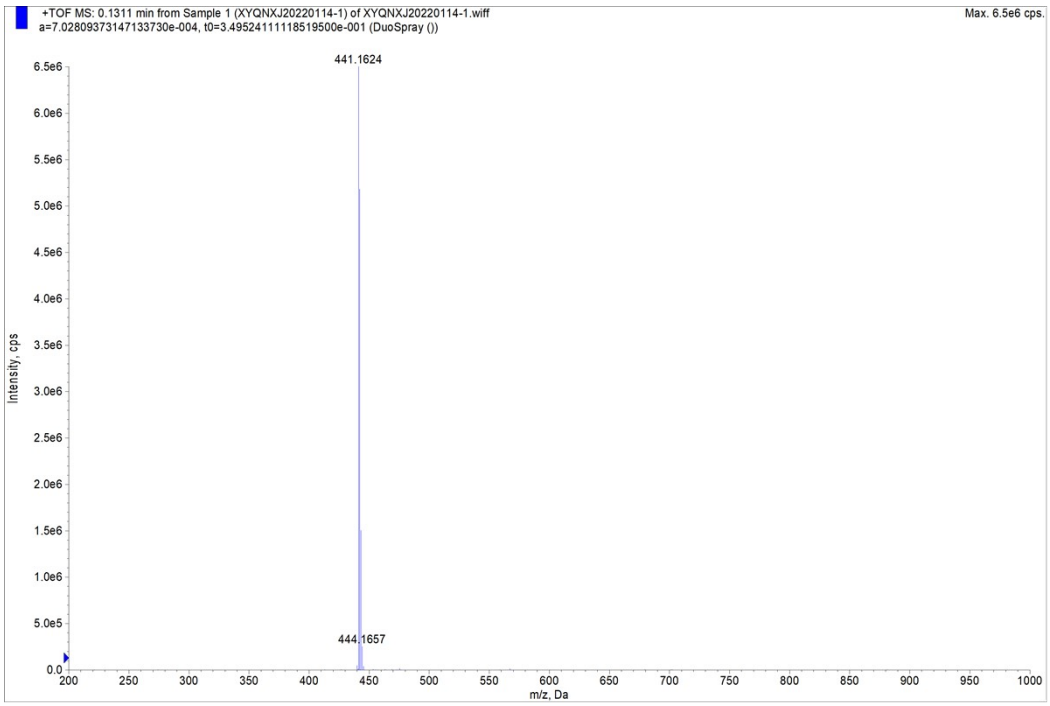


Fig. S20 TOF mass spectrum of **C2.**

Table S1 The Binding Energy of **C1** with HSA or BSA

Binding Energy/Kcal*mol^{-1}	Ring-opening	Ring-closing
BSA	-8.47	-8.98
HSA	-9.14	-10.4

Table S2 The Binding Energy and ΔE of **C1** and **C1** ring-closing isomer with HSA or BSA.

Binding Energy/Kcal*mol^{-1}	HSA	BSA	ΔE (E_{BSA}-E_{HSA})
C1	-10.4	-8.98	1.42
C2	-7.81	-7.36	0.45

Table S3 The concentration of HSA in serum samples measured by BCG assay and Test strips

Sample number	1	2	3	4	5	6	7	8
BCG assay (mg/mL)	44	45	45	45	46	42	45	46
Test strips (mg/mL)	49	49	48	42	45	47	47	48