Electronic Supplementary Information

for

The DS2-specific flavonoid-based probe with unique dual-emissive response to human serum albumin

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1. Experimental Section

1.1 Materials and instruments

All biological components including human serum albumin, glutathione (GSH), cysteine (Cys), homocysteine (Hcy), trypsin, thymidine, ribonucleic acid and lipase were purchased from Sigma-Aldrich and used as received. All chemicals and solvents were purchased from Energy Chemical China without further purification. ^{1}H NMR and ^{13}C NMR spectra were measured using a Bruker 600-MHz NMR spectrometer. UV-Vis spectra were obtained on a Thermo spectrophotometer. Fluorescence spectra were recorded on a Thermo Lumina fluorometer. Fluorescence lifetimes were obtained by Horiba DeltaFlex with 392 nm Laser Nano LED. Fluorescence quantum yields were measured by HAMAMATCU Quantaurus-QY.

1.2 General testing method

Stock solution of the probes **1HHF**, **2HHF**, **3HHF** and **HF** was diluted to 0.2 mmol/L in DMSO and restored in the refrigerator for further usage. For the test of spectroscopic properties, a volume of 10 μL of test solution was injected into 2 mL deionized water to yield detection solution with probe concentration of 10^{-6} mol/L. All spectroscopic measurements were performed at room temperature after the mixtures were shaken for 1 min.

1.3 Molecular docking

The 3D geometry of the molecules was constructed using the Gaussian viewer, then optimized at the level of B3LYP/6-31G* with PCM implicit water solvent model. The structure of HSA (PDB ID: 4K2C) was obtained from the Brookhaven Protein Data Bank (http://www.rcsb.org/pdb). The R-value and the resolution of the file were 0.213 and 3.23Å, respectively. The Flexible ligand docking was performed by AutoDock 4.2 molecular docking program using the implemented empirical free energy function

and the Lamarckian Genetic Algorithm. The first step is to set the docking box of the probes and the HSA ligand. Then used Autogrid to calculate grids. Set the parameters as follows: 60 grid points per dimension, grid point interval: 0.375 Å, the number of runs: 20 times, the maximum energy evaluation: 2.5 million times.

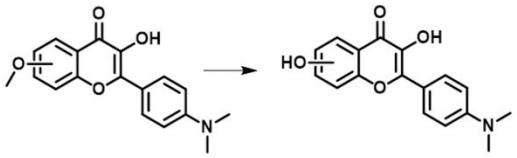
1.4 Site-specific competition experiments

The Site-specific competition experiments were carried out using albumin-binding drugs warfarin (DS1 inhibitor) and ibuprofen (DS2 inhibitor) were prepared in distilled water. The stock solutions of drugs with different concentration (1-5 μmol/L) were added proportionally into the HSA (1 μmol/L) and probe (1 μmol/L) mixture solution at room temperature, followed by shaking for 2 min. Then, fluorescence spectra were measured.

1.5 The fluorescence lifetime

The fluorescence decay curves were performed on a Horiba instrument using a tunable pulse laser radiation (nano-light emitting diode (nano-LED): Peak wavelength: 392nm; Pulse duration:< 1.4ns) as the excitation. Generally, the radiation decays of fluorophores were fitted to a multi exponential decay function, $I(t) = I_0 \sum \alpha_i \exp(-t/\tau_i)$, where Iis the luminescence intensity at time t and I_0 are the luminescence intensity initially, the α_i values are pre-exponential factors, which represent the fractional amount of fluorophore in each environment, the τ_i are the lifetimes for the exponential components. Furthermore, the effective lifetime constant (τ^*) can be calculated as: $\tau^* = \sum \alpha_i \tau_i$.

2. Synthesis and characterizations



General procedure. The methoxyl-substituted flavonoids could be firstly synthesized according to our previous work (*Chem. Lett.*, 2019, **48**, 1383-1386). Boron tribromide (4.8 mmol) was added into a solution of methoxyl-substituted flavonoids (1.6 mmol) in dry dichloromethane (150 mL) under a nitrogen atmosphere at 78 °C. The mixture was stirred at room temperature for overnight. Then, the resulting mixture was poured into cool water, and sodium bicarbonate was added dropwise until no gas was liberated. The mixture was extracted with ethyl acetate for 3 times, dried over anhydrous Na_2SO_4. The products were purified by recrystallization from ethanol/ tetrahydrofuran mixture.

1HHF (yield ~ 78%).^{1}H-NMR (400 MHz, DMSO-d_6): δ= 9.888 (d, *J* =10.8 Hz, 1H), 8.992 (d, *J* = 9.6 Hz, 1H), 8.123 (t, 2H), 7.587 (t, 1H), 7.356 (d, *J* = 8.0 Hz, 1H), 7.225 (t, 1H), 6.858 (t, 2H), 3.025 (d, *J* = 7.2Hz, 6H). ^{13}C-NMR (150 MHz, DMSO-d_6): δ= 172.09, 154.39, 151.40, 148.70, 146.96, 137.17, 129.31, 122.95, 122.63, 119.90, 118.64, 111.85, 107.28, 41.3.

2HHF (yield ~ 80%). ^{1}H-NMR (600 MHz, DMSO-d_6): δ=10.670 (s, 1H), 8.906 (s, 1H), 8.069 (d, *J* = 9.0 Hz, 2H), 7.914 (d, *J* = 8.4 Hz, 1H), 6.925 (d, *J* = 1.8 Hz, 1H), 6.897 (m, 1H), 6.839 (d, *J* = 9.0 Hz, 2H), 3.003 (s, 6H). ^{13}C-NMR (150 MHz, DMSO-d_6): δ=172.04, 162.50, 156.65, 151.25, 146.18, 137.02, 129.07, 126.80, 118.72, 114.93, 114.77, 111.88, 102.38, 67.87.

3HHF. (yield ~ 74%). ^{1}H-NMR (400 MHz, DMSO-d_6): δ= 10.369 (s, 1H), 9.085 (s, 1H), 8.170 (d, *J* = 8.4 Hz, 2H), 7.489 (s, 1H), 7.201 (s, 2H), 6.864 (d, *J* = 8.4 Hz, 2H), 3.011 (s, 6H). ^{13}C-NMR (150 MHz, DMSO-d_6): δ= 172.54, 151.44, 147.16, 146.79,

144.67, 137.60, 129.43, 124.65, 123.18, 118.70, 118.20, 114.52, 111.86, 56.50.

3. Additional Figures, Schemes and Tables

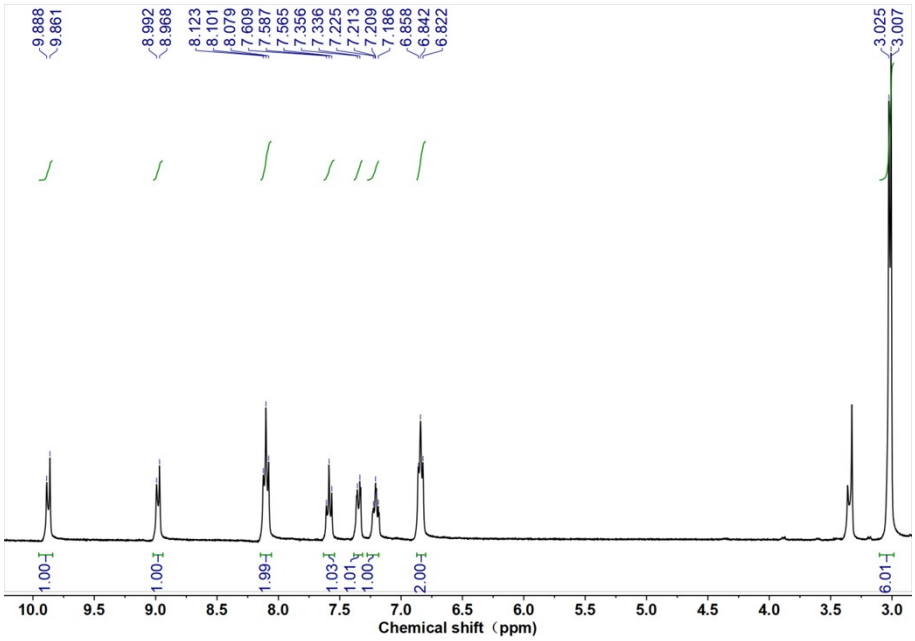


Fig. S1. ^{1}H NMR spectrum of **1HHF**

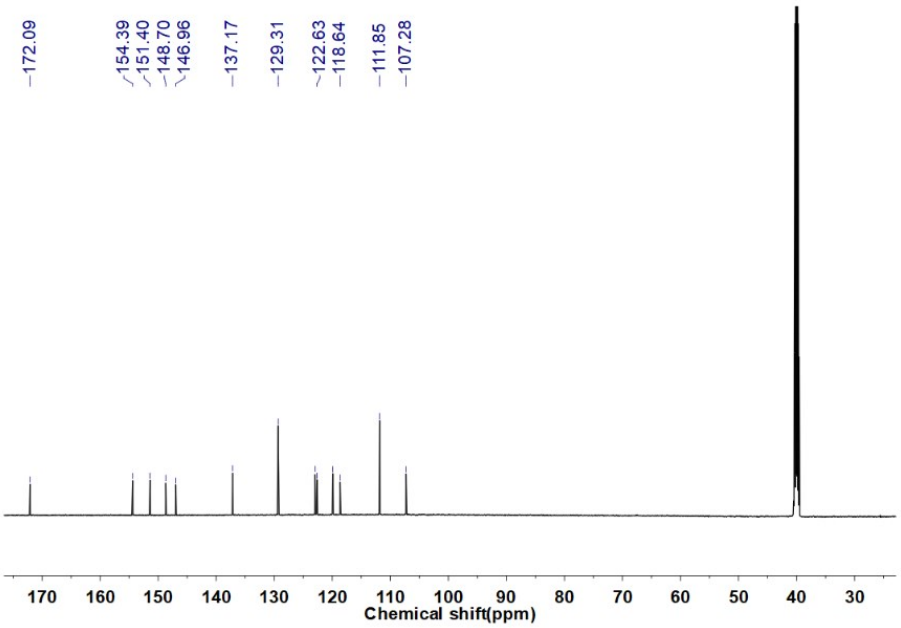


Fig. S2. ^{13}C NMR spectrum of **1HHF**

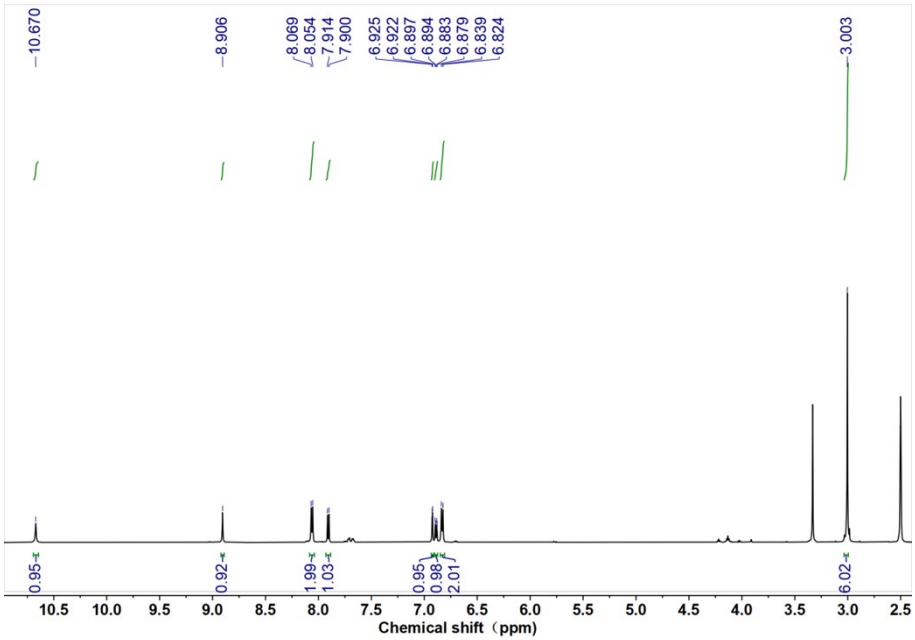


Fig. S3. ^{1}H NMR spectrum of **2HHF**

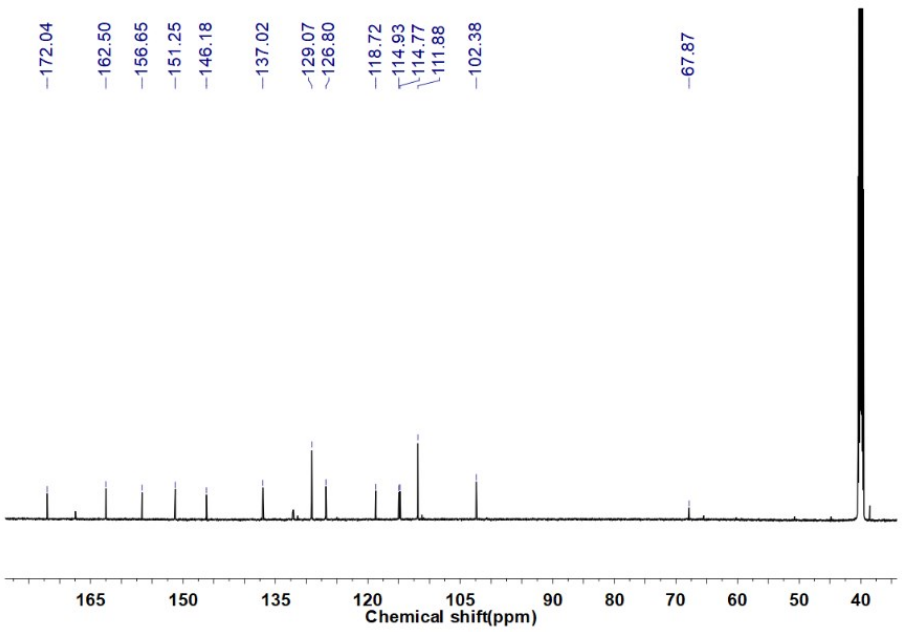


Fig. S4. ^{13}C NMR spectrum of **2HHF**

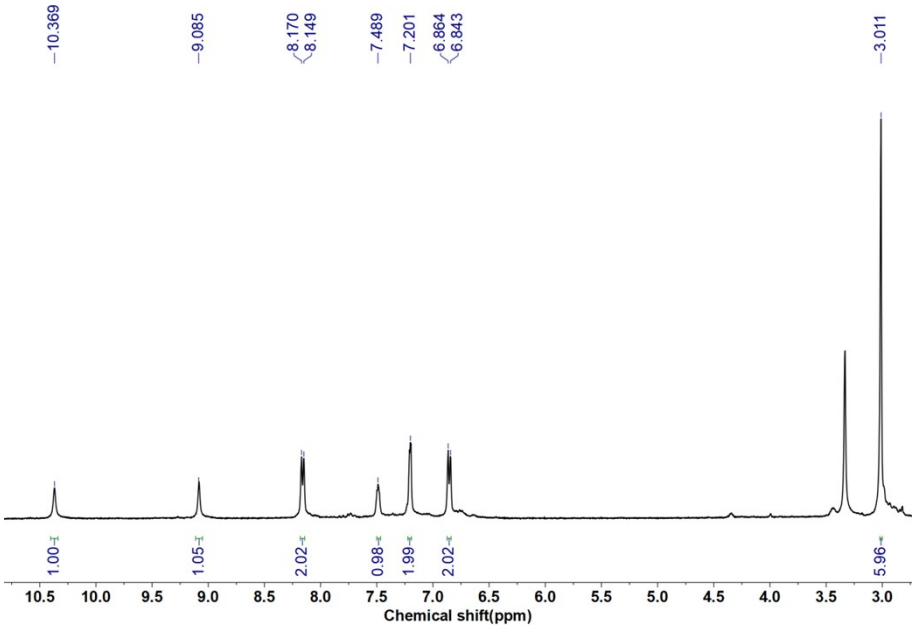


Fig. S5. ^{1}H NMR spectrum of **3HHF**

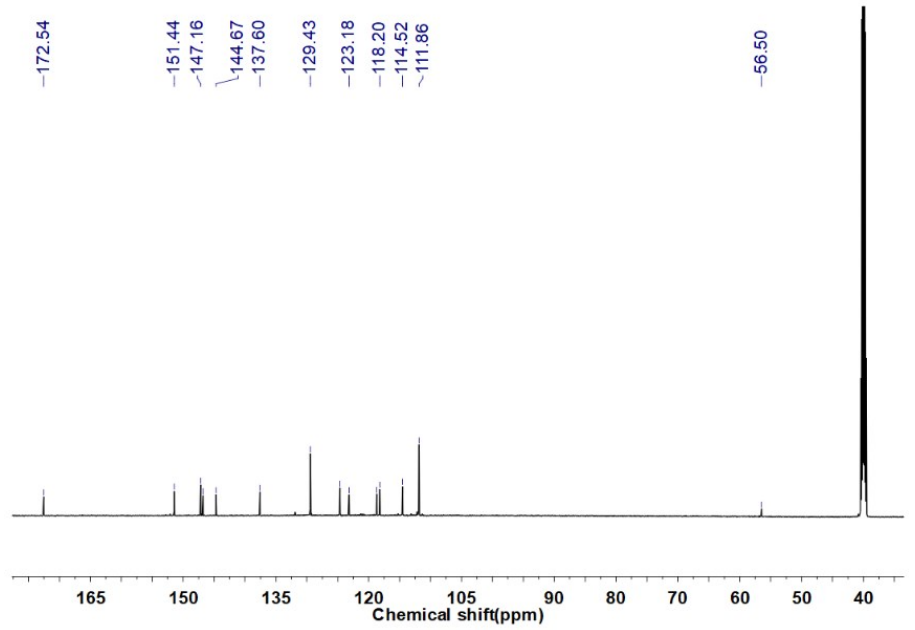


Fig. S6. ^{13}C NMR spectrum of **3HHF**

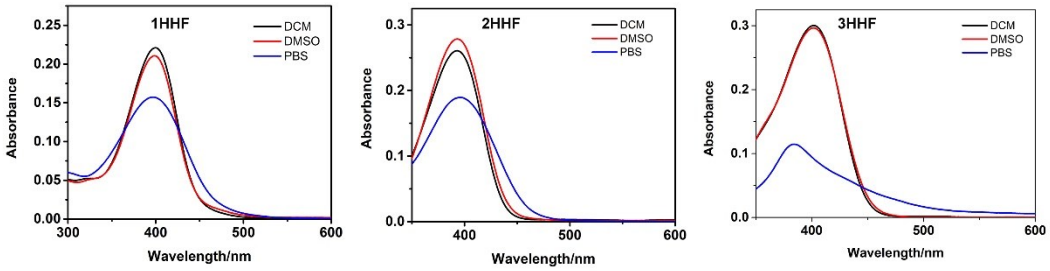


Fig. S7. UV-vis absorption spectra of **1HHF-3HHF** (10 μM) in DCM, DMSO, and PBS buffer (pH~7.4, 1 mM).

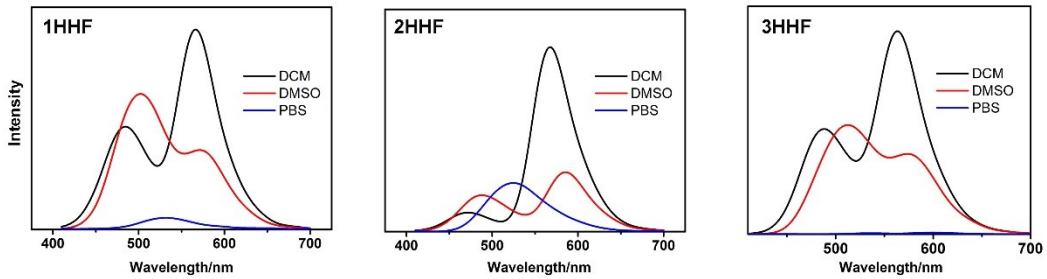


Fig. S8. Fluorescent spectra of **1HHF-3HHF** (10 μM) in DCM, DMSO, and PBS buffer (pH~7.4, 1 mM). λ_{ex} = 400 nm.

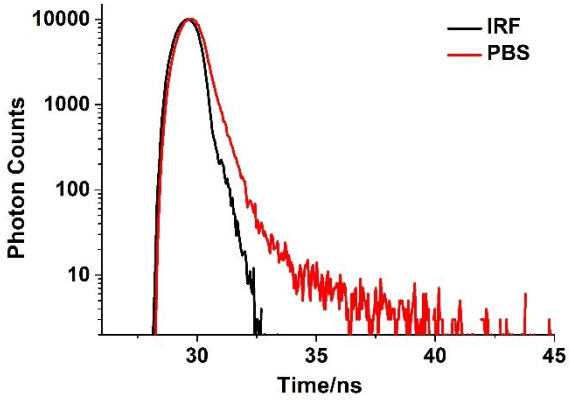


Fig. S9. The fluorescence decay curves of **2HHF** in PBS buffer. The data were recorded at 525 nm. IRF: instrument response function (prompt). Laser resource: 392 nm nanoLED. [**2HHF**] = 1 μM.

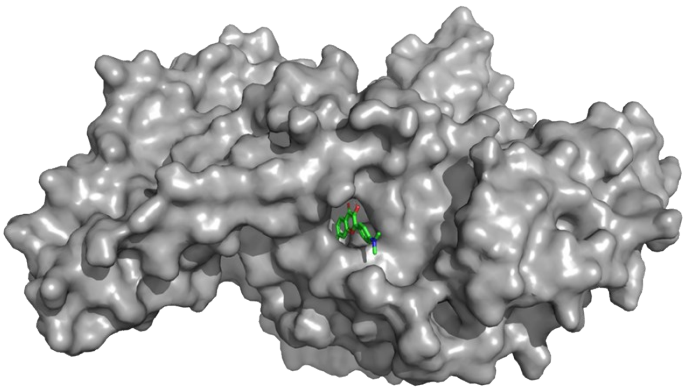


Fig. S10. The overview of **2HHF** bound into the DS2 of HSA. The surface of HSA was colored in gray.

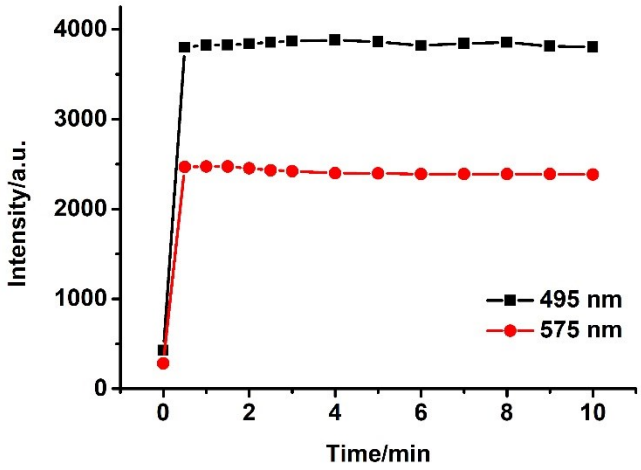


Fig. S11. The time-dependent fluorescent intensities (495 nm and 575 nm) of **2HHC** upon addition of 5 equiv. of HSA. [**2HHF**] = 1 μM. λ_{ex} = 400 nm.

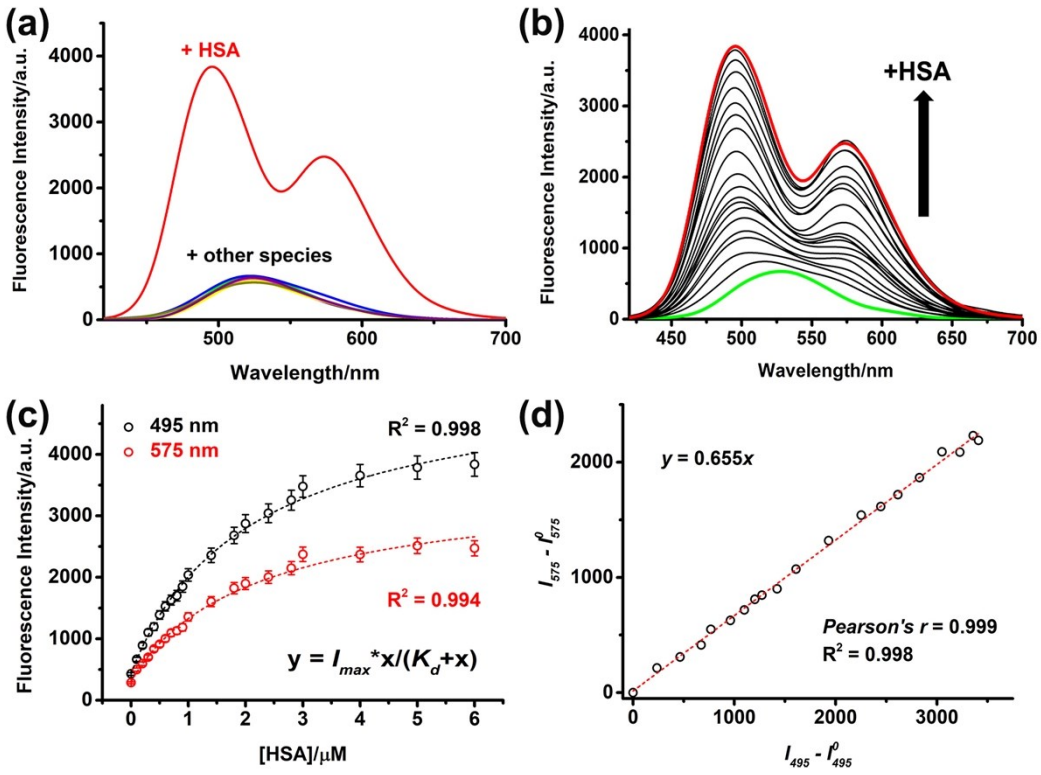


Fig. S12. (a) The selectivity experiments of **2HHF** in the presence of different biological species (6 equiv.). (b) The fluorescent spectra and (c) intensities of **2HHF** upon addition of HSA (up to 6 equiv.). [**2HHF**] = 1 μM. λ_{ex} = 400 nm, λ_{em} = 495 nm and 575 nm, error bars = ±SD, n = 3. (d) The correlation between intensity enhancements ($\boldsymbol{I}$-$\boldsymbol{I^0}$) at 495 nm and 575 nm. $\boldsymbol{I^0}$ and $\boldsymbol{I}$ represent the fluorescent intensities in the absence and presence of HSA, respectively.

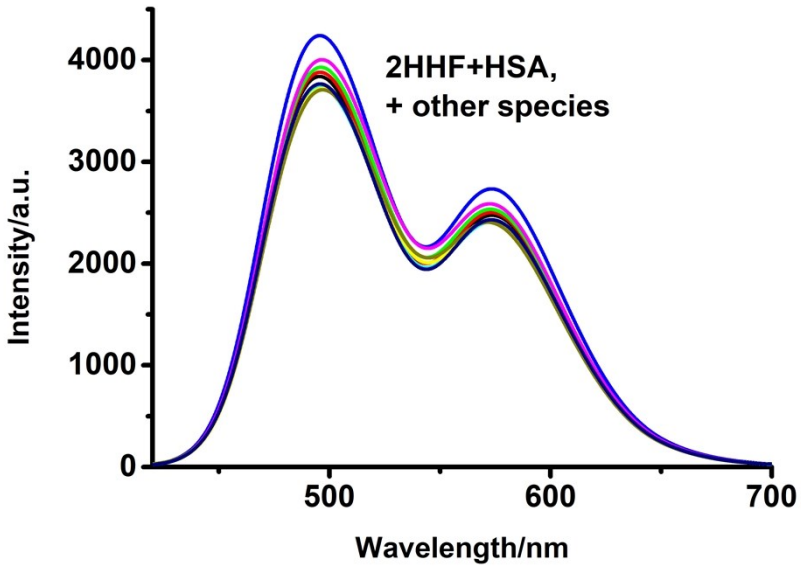


Fig. S13. The fluorescent spectra of **2HHF** pretreating with biological species (6 equiv.), including cysteine, lysozyme, globulin, ribonuclease, trypsin, fibrinogen, and lipase, then adding with HSA (6 equiv.). [**2HHF**] = 1 μM. λ_{ex} = 400 nm.

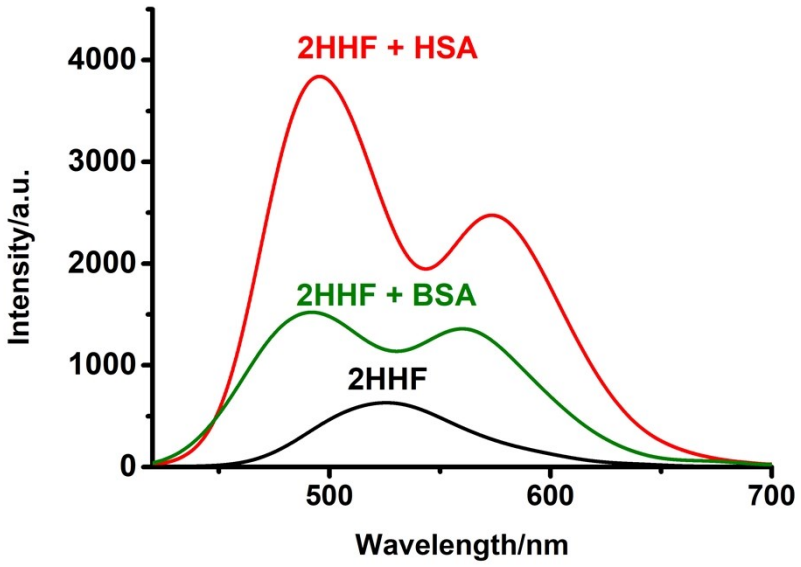
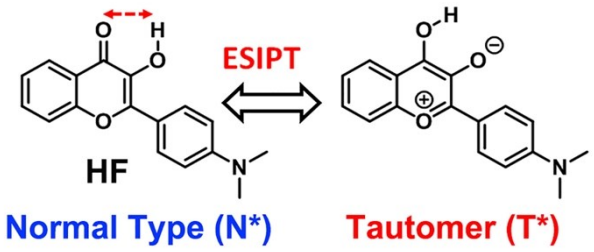
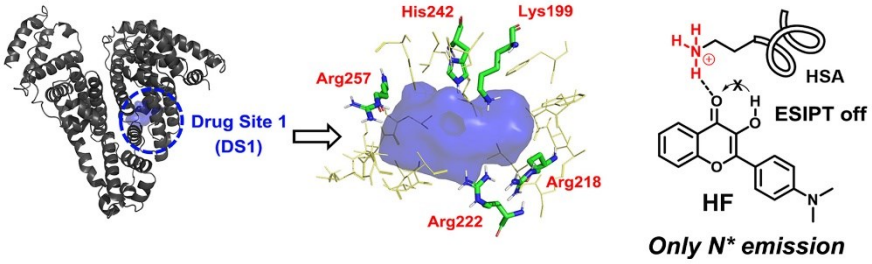


Fig. S14. The fluorescence responce of **2HHF** towards BSA and HSA (6 equiv.). [**2HHF**] = 1 μM. λ_{ex} = 400 nm.



Scheme S1 The ESIPT process of representative flavonoid



Scheme S2 The ESIPT process may be interrupted by the amino acids in DS1 of HSA

Table S1. The fluorescence lifetime of **2HHF** in PBS buffer, DCM and HSA

	λ/nm	$\tau_1{}^a$/ns	$\alpha_1{}^b$	$\tau_2{}^a$/ns	$\alpha_2{}^b$	τ^c/ns
In DCM	460 (N*)	0.089	0.95	1.26	0.05	0.15
	570 (T*)	/	/	1.11	1	1.11
In PBS	525	0.38	1	/	/	0.38
In HSA	495 (N*)	0.76	0.85	3.45	0.15	1.16
	575 (T*)	/	/	3.93	1	3.93

NOTE: [a] τ_1 and τ_2 are relative short- (<1 ns) and long-lived (>1 ns) fluorescence lifetimes. [b] α_1 and α_2 are pre-exponential coefficients. [c] The average lifetime $\tau = \tau_1 \times \alpha_1 + \tau_2 \times \alpha_2$. λ_{ex} = 400 nm.