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Intermolecular proton transfer from flavonol to human serum albumin triggers a red-shifted ratiometric fluorescence response

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1. Experimental section

1.1 materials and instruments

All biological analytes including human serum albumin (HSA), tyrosinase, EC3.1.1.1, globulin, lysozyme (Lyso), trypsin, lipase, RNA, DNA, glucose, lactose, creatinine, carbamide, cysteine (Cys), homocysteine (Hcy), glutathione (GSH) lysine (Lys), tryptophan (Trp), histidine (His), methionine (Met), glutamine (Glu), and leucine (Leu) were purchased from Sigma-Aldrich without further purification. All chemicals and solvents were purchased from Energy Chemical China without further purification. Phosphate buffered saline (PBS, 10×, pH 7.4) was purchased J&K scientific. The experiments on serum samples were permitted by the department of clinical laboratory in Shenzhen Longhua Central Hospital (No. 0273178, 0221043, and 1561417). ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker AVANCE III 500 MHz or 600-MHz spectrometer using Chloroform-*d* or DMSO-d_6 as solvent and tetramethylsilane (TMS, δ = 0) as internal standard. Fluorescence spectra were recorded by a Thermo Lumina Fluorescent spectrometer.

1.2 General testing method

The stock solution of HFH, HFM, and HF was prepared in DMSO with a concentration of 10 mM and restored in the refrigerator for further usage. For the test of spectroscopic properties, a volume of 10 μL of 10 mM flavonoids stock solution was

injected into 2 mL solution to yield a detection solution with a concentration of 5 μM. The mixture was shaken for 30 sec and then measured by spectroscopic measurements at room temperature. The excitation wavelength is 360 nm. For Job's Plot experiments, the emission spectra were measured in the different ratios of HFH and HSA with the total concentration at 10 μM. For drug-displacement experiments, two site-specific drugs Warfarin (DS1) and Ibuprofen (DS2) were added proportionally into the HFH@HSA complex solution, and the emission spectra were measured after shaking for 1 min. For fluorescent response time experiments, the intensity decay curves were measured in the solution of fluorescent probe HFH with or without HSA. For fluorescence titration experiments, small aliquots of HSA solution were successively added into the solution of HFH and allowed to shake for at least 30 sec every time before measurement. Absorption spectra and fluorescence spectra data were plotted using Origin graphing software (OriginLab Origin 2022B).

1.3 Molecular docking

The 3D geometry of flavonoids 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 3.23 Å, 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 molecules and the HSA ligands. 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: 2500000 times. The output from AutoDock was rendered with PyMol and the ligand site analysis was assisted with LigPlus.

1.4 Procedure for sensing HSA in real serum samples

The standard serum sample (from 1μL to 10 μL) was dropped in the 2 mL PBS solution with the concentration of HFH at 5 μM. After sharking for 30 sec, the mixture was incubated for 1 min at room temperature and then was measured by fluorometer at room temperature. For real serum samples, 2 μL real serum samples were dropped in the 1998 μL PBS buffer with the concentration of HFH at 5 μM. After sharking for 30 sec, the mixture was incubated for 1 min at room temperature and then was measured by fluorometer at room temperature.

1.5 BCG method

The human serum was added in BCG solution and transported into 96-well plate. The signals were collected by a Microplate Reader using ultraviolet-visible

spectrometry at 630 nm. The concentrations of HSA were calculated by following equation (1):

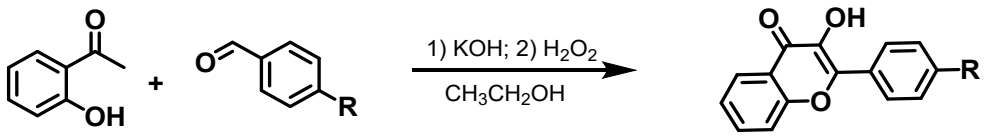
$$\frac{A}{A_0} = \frac{C}{C_0} \qquad (1)$$

Where A and A_0 are the absorption of sample and standard sample, respectively. C and C_0 are HSA concentration in serum and standard serum, respectively.

1.6 RGB analysis

The testing solutions were put into a dark box equipped with a 365 nm UV lamp (10 w). The image of solution was captured by the camera of iPhone 13 (Wide Angle Camera, ISO 3200, 26 mm, f 1.6). For RGB analysis, photos were imported into the RGB software (ImageJ software). The plot of the RGB value versus the concentration of HSA was obtained by the Origin software.

2. Synthesis and characterizations



General procedure: the flavonoids were prepared following the previously reported method. 2-Hydroxy-acetonphenone (10 mmol) and benzaldehyde (10 mmol) were stirred in ethanol with the addition of an aqueous potassium hydroxide solution (1

g/mL). The mixture was reacted for 12~14 h at room temperature, and 30% H_2O_2 solution was slowly added to the reaction solution, which was placed in an ice-water bath. After being stirred at room temperature for 10~12 h, the mixture was poured into an ice-water bath, and the precipitate was collected via filtration and washed with ethanol. The product was purified by recrystallization from ethanol,

HFH (yield: 68%) ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.14-8.06 (m, 3H), 7.81-7.75 (m, 1H), 7.73 (d, J = 8.1 Hz, 1H), 7.48-7.41 (m, 1H), 6.95 (d, J = 8.9 Hz, 2H).

3-hydroxy-2-(4-methoxyphenyl)-4H-chromen-4-one, HFM (yield: 66%) ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.48 (s, 1H), 8.22 (d, *J* = 8.8 Hz, 2H), 8.12 (d, *J* = 7.9 Hz, 1H), 7.85 – 7.74 (m, 2H), 7.48 (t, *J* = 7.4 Hz, 1H), 7.15 (d, *J* = 8.8 Hz, 2H), 3.87 (s, 3H).

2-(4-(dimethylamino)phenyl)-3-hydroxy-4H-chromen-4-one, HF (yield: 79%) ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.23 (dd, *J* = 8.0, 1.6 Hz, 1H), 8.20 (d, *J* = 9.0 Hz, 2H), 7.66 (ddd, *J* = 8.6, 7.0, 1.7 Hz, 1H), 7.56 (dd, *J* = 8.5, 1.0 Hz, 1H), 7.39 (ddd, *J* = 8.0, 7.0, 1.0 Hz, 1H), 6.82 (d, *J* = 8.9 Hz, 2H), 3.08 (s, 6H).

3. Supporting Figures and Table

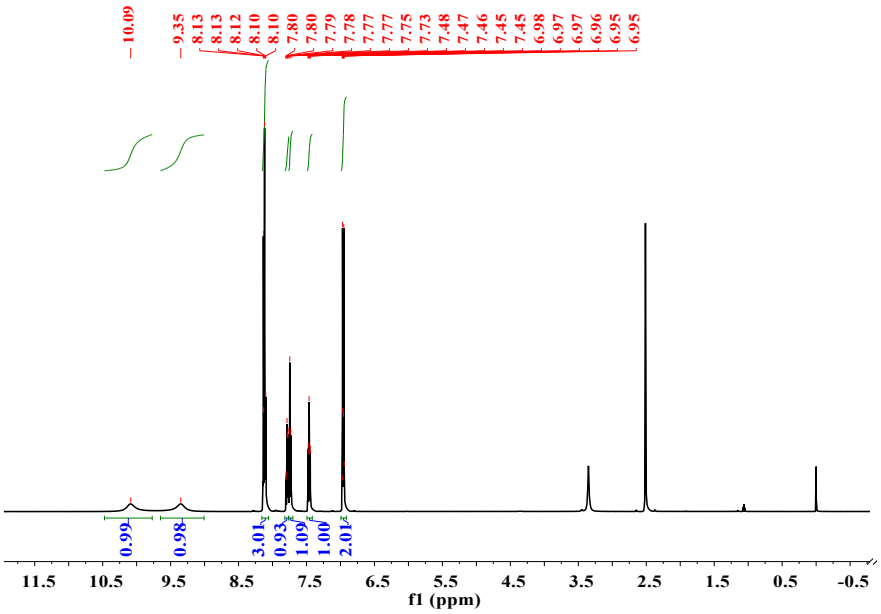


Fig. S1 ^{1}H NMR spectrum of HFH in DMSO-d_6

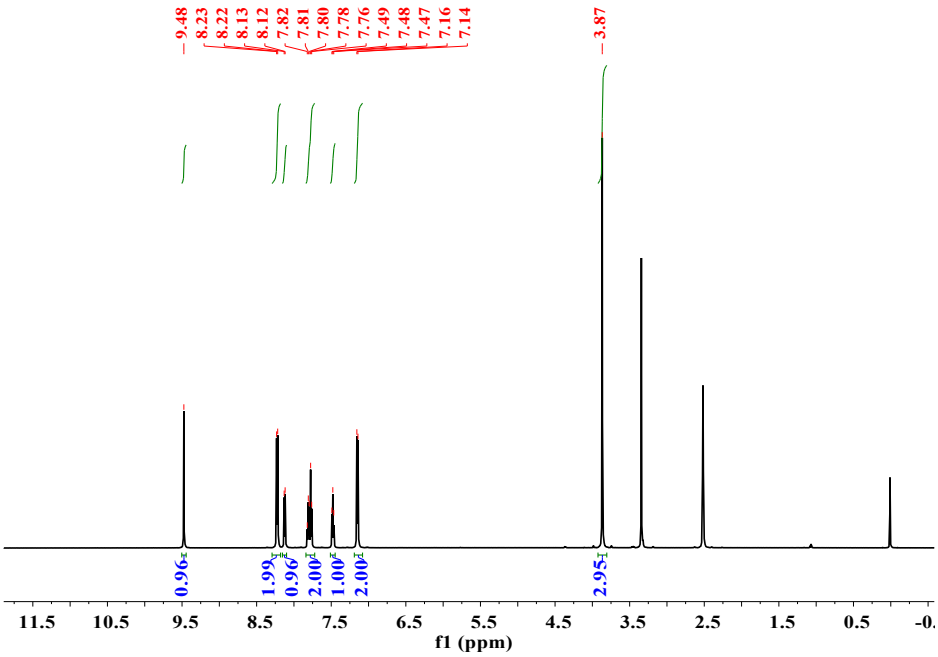


Fig. S2 ^{1}H NMR spectrum of HFM in DMSO-d_6

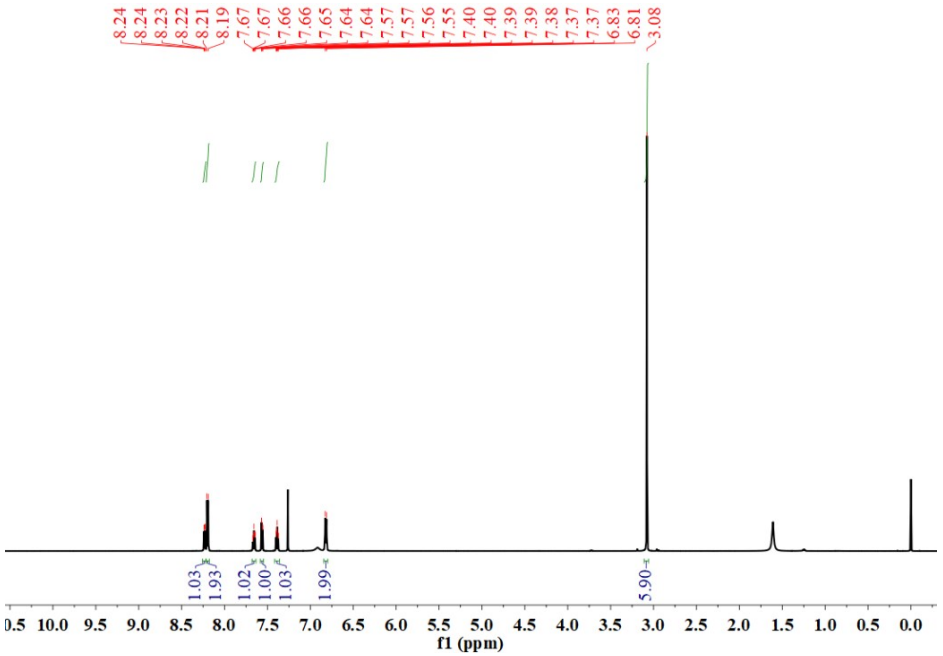


Fig. S3 ^{1}H NMR spectrum of HF in chloroform-*d*

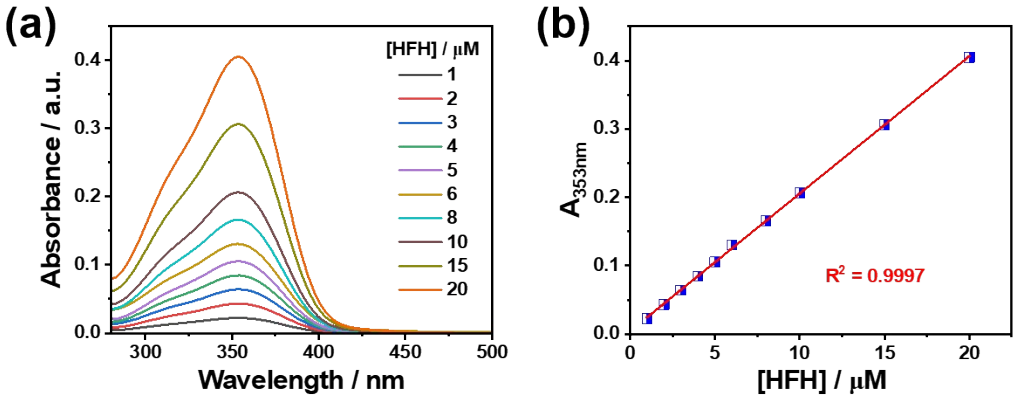


Fig. S4. (a) The absorption spectra of HFH with different concentration in aqueous solution. (b) The linear relationship between absorption at 353 nm and the concentration of HFH.

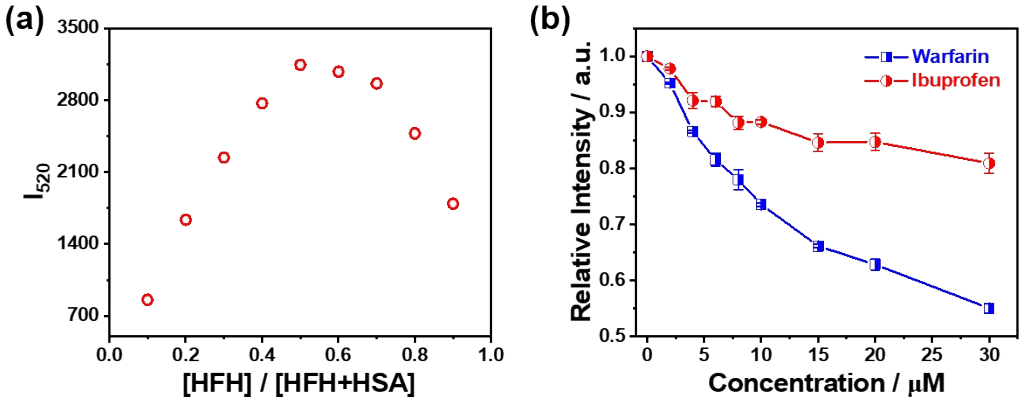


Fig. S5 (a) Job's Plot analysis of HFH with HSA in PBS buffer. [HFH + HSA] = 10 μM. (b) Relative intensity of HFH@HSA in the presence of two site-specific drugs (DS1: Warfarin, DS2: Ibuprofen). HFH@HSA was prepared by mixing same concentration of HFH and HSA. [HFH@HSA] = 10 μM. λ_{ex} = 360 nm.

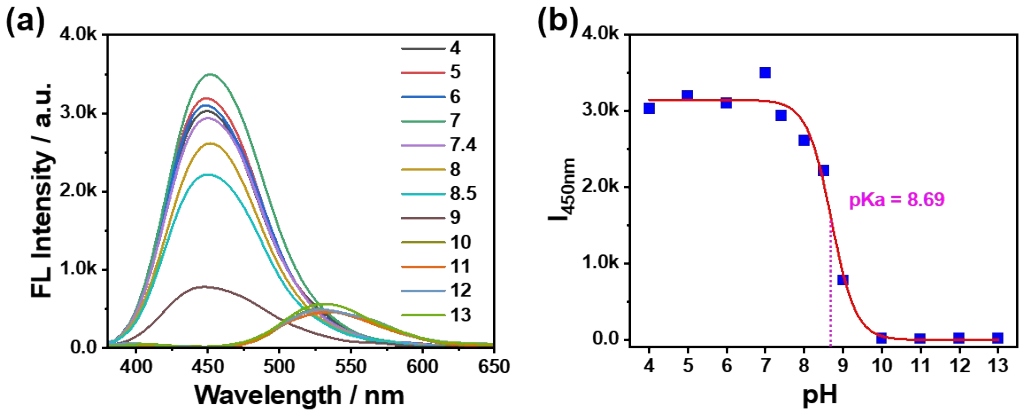


Fig. S6 (a) The pH-dependent fluorescence spectra of HFH (10 μM). (b) Plot of fluorescence intensity at 450 nm of HFH with different pH. [HFH] = 5 μM. λ_{ex} = 360 nm.

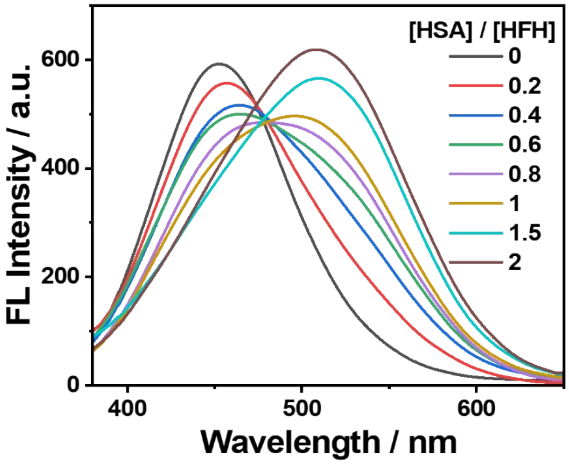


Fig. S7 Fluorescence spectra of HFH (1 μM) in the presence of different concentrations of HSA (0-2 μM) in PBS buffer. λ_{ex} = 360 nm.

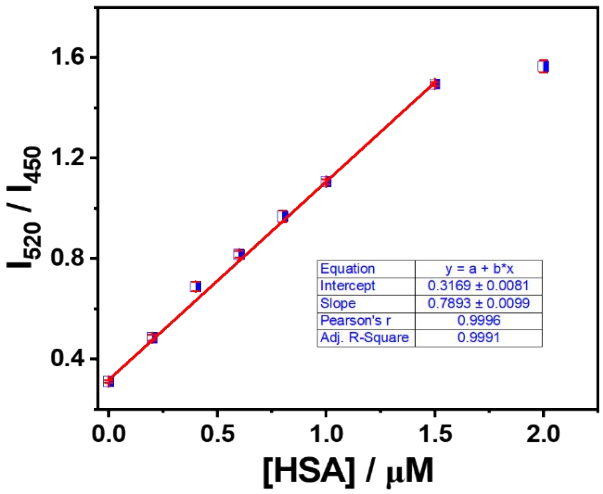


Fig. S8 Intensity ratio (I_{520}/I_{450}) of HFH (1 μM) with the titration of HSA (0-1.5 μM). LOD = 3σ/k = 3 × 0.0051/0.7893 = 0.0194 μM. λ_{ex} = 360 nm.

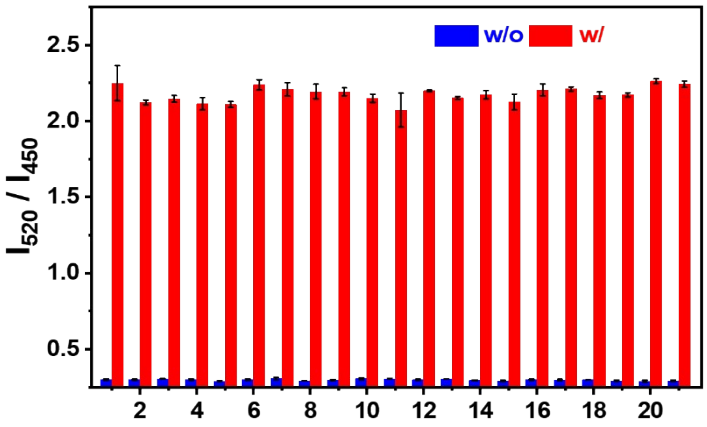


Fig. S9 Intensity ratios of HFH (blue bar, 5 μM) and HFH@HSA (red bar, 5 μM) for common ions and biomolecules. (1 Control, 2 K^+, 3 Na^+, 4 Mg^{2+}, 5 Ca^{2+}, 6 NH_4^+, 7 Cl^-, 8 CO_3^-, 9 SO_4^{2-}, 10 HSO_3^-, 11 SO_3^{2-}, 12 NO_3^-, 13 GSH, 14 Cys, 15 Hcy, 16 Lys, 17 Trp, 18 His, 19 Met, 20 Glu, 21 Leu). [ions] = [biomolecules] = 10 μM, λ_{ex} = 360 nm. Error bars = ±SD, n = 3.

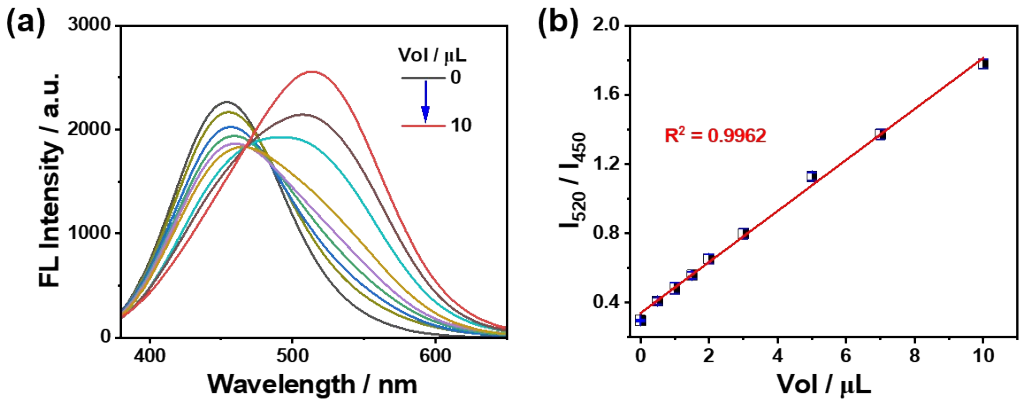
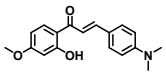
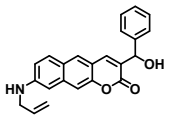
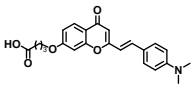
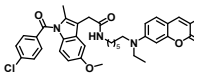
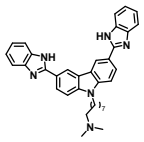
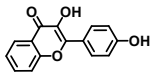


Fig. S10 (a) Fluorescence spectra and (b) intensity ratio (I_{520}/I_{450}) of HFH (5 μM) in PBS buffer upon titration of different volumes of standard serum (0-10 μL). λ_{ex} = 360

nm. Error bars = ±SD, n = 3.

Table S1. Reported ratiometric fluorescent probes for HSA detection.

Probe	Synthetic route	Signal change(nm)	Line μg/mL	LOD μg/mL	Response time	Mechanism	Ref.
	simple	620/520	0-590	1.08	< 5 min	DSE	**1**
	complicated	580/490	0-14	0.2	< 1 min	EC	**2**
	complicated	614/562	0-33	3.38	< 1 min	EC	**3**
	complicated	575/475	0-500	4.65	> 5 min	EC	**4**
	complicated	400/454	0-80	0.27	120 min	EC	**5**
	simple	450/520	0-99	1.28	< 5 sec.	IPT	This work

Note: DSE: dual-state-emission; EC: environmental change (polarity or viscosity); IPT: intermolecular proton transfer.

4. References

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