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Supporting Information

A ratiometric fluorescent probe based on two-isophorone fluorophore for detecting cysteine

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Contents

1. Reagent.

All reagents and solvents are commercially available without further refinement.

Drug: acryloyl chloride (Energy Chemical), malonitrile (Kelong Chemical), Isophorone (Energy Chemical), 4-Hydroxy-2,6-dimethoxybenzaldehyde (Energy Chemical), sodium sulfate anhydrous (Kelong Chemical), disodium hydrogen phosphate (Kelong Chemical), potassium dihydrogen phosphate (Kelong Chemical), Column chromatographic silica gel (Qingdao Marine chemical plant, 200-300 mesh), glycine (Guangfu Chemical), alanine (Kelong Chemical), valine (Kelong Chemical), leucine (Kelong Chemical), isoleucine (Guangfu Chemical), proline (Guangfu Chemical), phenylalanine (Kelong Chemical), tryptophan (Guangfu Chemical), methionine (Kelong Chemical), serine (Guangfu Chemical), threonine (Kelong Chemical), tyrosine (Kelong Chemical), asparagine (Guangfu Chemical), glutamine (Guangfu Chemical), aspartic acid (Kelong Chemical), glutamate (Kelong Chemical), arginine (Guangfu Chemical), histidine (Kelong Chemical), cysteine (Kelong Chemical), homocysteine (TCI), glutathione (Kelong Chemical).

Solvent: anhydrous dichloride (analytically pure), absolute ethanol (analytically pure), tetrahydrofuran (analytical pure), petroleum ether (Chemically pure), ethyl acetate (Chemically pure).

2. Instrument.

Large-scale instruments: The UV-Visible spectrum was measured by Varian CARY 5000 Conc (U.S.) UV-Vis spectrometer; Hitachi F-7000 (JPN) fluorescence spectrometer was used to measure fluorescence spectra; JEOL ECS 400 M (JPN) nuclear magnetic resonance spectrometer (The internal standard is TMS and the solvent is Chloroform-*d*) was used to measure ^{1}H NMR and ^{13}C NMR spectra; Finnigan MAT LCQ Mass spectrometer System (USA) was used to measure Mass Spectrometry; Cell images were obtained from EVOS FLoid Cell Imaging Station (Thermo Fisher Scientifi, America); Cytotoxicity test was carried out on Multiskan Ascent enzyme-labeled instrument (Thermo Fisher Scientifi, USA).

Other commonly used instruments: ENF-260C/FBE Spectro-line handheld ultraviolet lamp; FB-10 SARTOSIUS desktop pH meter; ZNCL-GS magnetic stirrer; AL-204 electronic balance (Mettler-Toledo); ZNHW-II electronic temperature controller (Yuhua instrument); SHB-III circulating vacuum water pump (Great Wall Science, Industry and Trade); Heidolph Laborota 4000 high-efficiency (GER) rotary evaporator; KQ2200B ultrasonic cleaning instrument (Kunshan ultrasonic instrument); Thermo Scientific F1-ClipTip microinjector; Electric thermostatic blast drying box (Jinghong experimental equipment).

3. Synthesis of compound 1 and TIFC-OH.

Synthesis of Compound 1. Isophorone (6.9 g, 50 mmol) and malononitrile (4 g, 60.6 mmol) were weighed into a flask and absolute ethanol (100 mL) was added to dissolve, and then piperidine (5 drops) was added. Obtained solution was stirred at 110 ℃ for a period of time until the original material disappeared (TLC plate detection). When the temperature of the solution was cooled to about room temperature, a reduced pressure operation was performed to remove the solvent. Then water (180ml) was added to it, and after refrigerating for about half an hour, solid precipitation was observed, and the precipitated solid was filtered and dissolved in dichloromethane, then dried, and finally filtered to obtain the filtrate, which was purified to obtain an orange solid (2.5 g, yield: 27%). MS (m/z) for [$C_{12}H_{14}N_2$]: 186.1157, Found [M + H^+]: 187.1259 (Fig. S9); ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.55 (s, 1H), 2.53 (s, 2H), 2.23 (s, 2H), 2.04 (s, 3H), 0.95 (s, 6H) (Fig.S10).

Synthesis of TIFC-OH. 4-Hydroxybenzene-1,3-dicarbaldehyde (0.3 g, 2 mmol) and compound 1 (0.74 g, 4 mmol) were weighed in a round-bottomed flask, then added absolute ethanol (20 mL) to dissolve, and then piperidine (2 drops) was added. The obtained solution was refluxed at 110°C for about 12 hours. When the temperature of the solution cooled, the mixed solution was placed in a refrigerator at 4°C for a period of time, and then ethanol was added to concentrate the solution. Finally, a dark red solid (0.58 g, yield: 59.8%) was obtained by filtration. MS (m/z) for [$C_{32}H_{30}ON_4$]: 486.2420, Found [M – H^+]: 485.3007 (Fig. S11); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.69 (s, 1H), 8.13 (d, J = 1.8 Hz, 1H), 7.54 (dd, J = 8.5, 1.9 Hz, 1H), 7.45 (d, J = 3.2 Hz, 2H), 7.27 (q, J = 16.1 Hz, 2H), 6.94 (d, J = 8.5 Hz, 1H), 6.85 (d, J = 11.1 Hz, 2H), 2.61 (d, J = 2.1 Hz, 4H), 2.54 (s, 4H), 1.03 (d, J = 3.8 Hz, 12H) (Fig.S12); ^{13}C NMR (101 MHz, DMSO-D_6) δ 170.73, 170.66, 157.03, 156.71, 138.59, 132.28, 132.11, 129.34, 127.64, 127.15, 123.75, 122.90, 121.99, 117.55, 114.68, 114.49, 113.97, 113.78, 76.28, 75.32, 56.55, 42.78, 38.59, 32.21, 32.19, 27.98, 27.92, 19.10 (Fig.S13).

4. The time-varying fluorescence spectrum of GSH and Hcy.

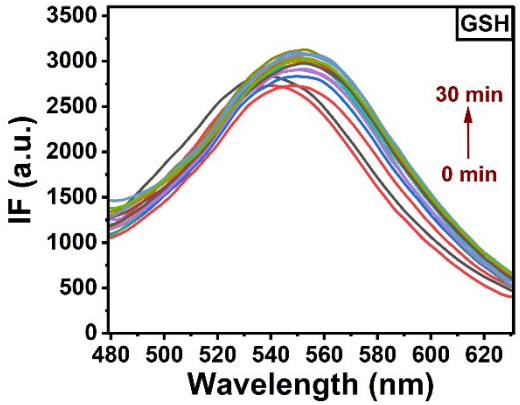


Fig. S1 Time-dependent fluorescence changes of target probe **TIFC** (10μM) towards GSH (50μM) in PBS-THF solution (20mM, 1/1, v/v, PBS/THF, pH=7.40). (λ_{ex} = 457 nm).

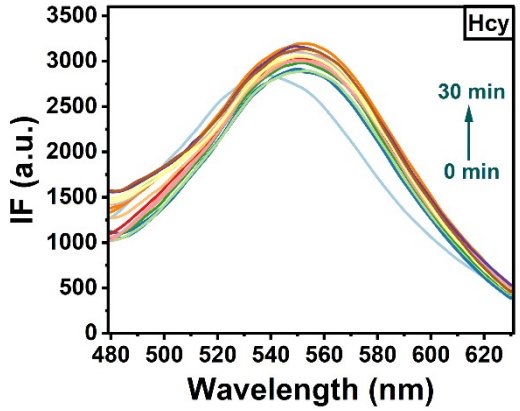


Fig. S2 Time-dependent fluorescence changes of target probe **TIFC** (10μM) towards Hcy (50μM) in PBS-THF solution (20mM, 1/1, v/v, PBS/THF, pH=7.40). (λ_{ex} = 457 nm).

5. Influence of pH.

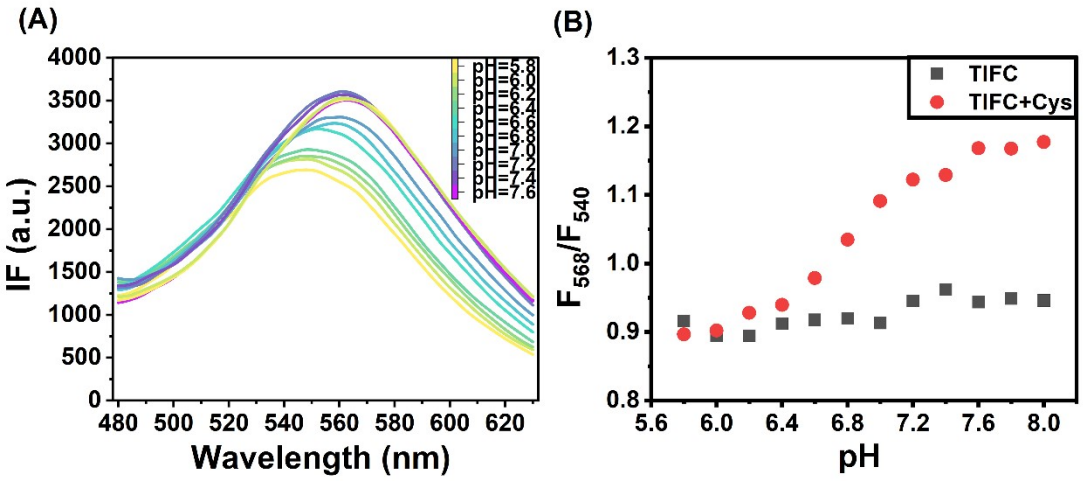


Fig. S3 Fluorescence spectra of **TIFC** of different pH reacted with Cys in PBS-THF solution (20mM, 1/1, v/v, PBS/THF, pH=7.40) and the change of fluorescence intensity. **(A)** Emission spectra of probe (10μM) at different pH in PBS-THF solution. (λ_{ex}=457 nm). **(B)** pH effect on the fluorescence intensity ratio (F_{568}/F_{540}) of the target probe (in black) and on the fluorescence intensity ratio (F_{568}/F_{540}) of the target probe with Cys (in red). The resulting solution was shaken well and incubated for 20 min at room temperature before recording the spectra.

6. Linear relationship between the fluorescence intensity and the Cys concentration.

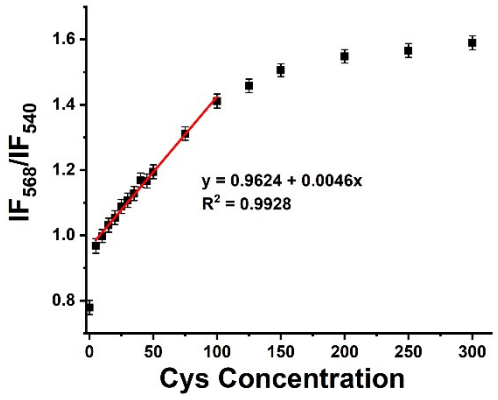


Fig. S4 linear relationship between the F_{568}/F_{540} and the Cys concentration (0 to 300μM) added to **TIFC** (10μM).

7. pK_a of the fluorophore TIFC-OH.

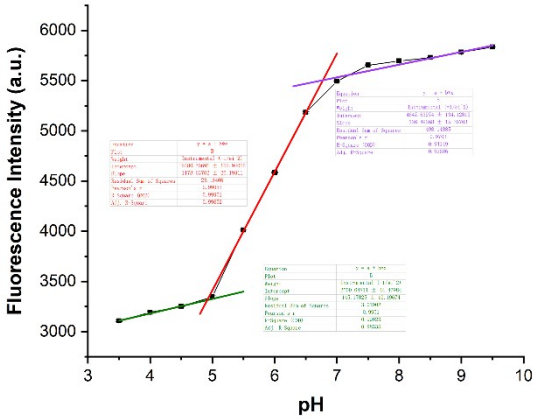


Fig. S5 pK_a of the fluorophore **TIFC-OH**.

8. Supplemental Selective Studies.

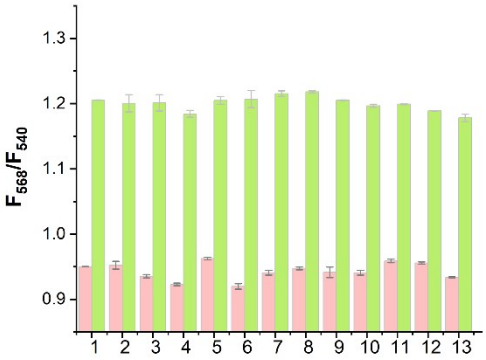


Fig. S6 Fluorescence responses of **TIFC** (10 μM) toward various relevant species (50 μM) without (pink bar) and with (green bar) the presence of 50 μM Cys in PBS-THF solution (0.01 M, pH 7.4, v/v,1/1) at 25 ℃. 1. Blank, 2. Na^+, 3. K^+, 4. Ca^{2+}, 5. NH^{4+}, 6. Cu^{2+}, 7. Fe^{3+}, 8. Zn^{2+}, 9. F^-, 10. Br^-, 11. $ONOO^-$, 12. HClO, 13. SCN^-. The resulting solution was shaken well and incubated for 20 min at room temperature before recording the spectra.

9. Detection limit.

First, the concentration of 10μM **TIFC** was taken to complete the fluorescence titration experiment of Cys, taking the concentration of the Cys (0-300μM) as the x-axis and the ratio fluorescence intensity (F_{568}/F_{540}) as the y-axis, the slope k was obtained by linearly fitting. Keep the same conditions of the **TIFC** concentration and repeat the experiment 20 times in succession, calculate the population standard deviation σ of the many times with the following formula, and substitute it into the formula (A.1):

$$\sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(Xi - \mu)^2} \qquad LOD = \frac{3\sigma}{k}$$

Eq. (A.1)

In this formula, 3 represents confidence level, k is the slope obtained above, and σ is the standard deviation of blank samples. Where N is the total number of samples, μ is the average of all samples, and X_i represents each sample. In this experiment, k was 0.0047 (as shown in Fig. S4), N was 20, and the calculated σ was 0.000165. The experimental results show that the detection limit of **TIFC** for Cys is 105.6nM.

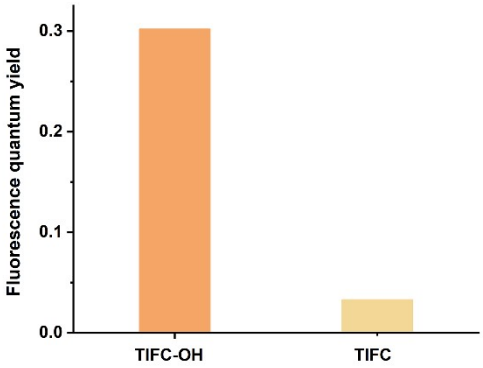
10. Fluorescence quantum yield.

Fluorescence quantum yield is the report of the number of photons emitted by a fluorescent substance after absorption of light to the number of absorbed photons. It is normally measured by the relative method, and the value is between 0.01 and 1. The higher the ratio is, the higher the utilization rate of photons will be. Fluorescence quantum yield of **TIFC-OH** was calculated according to the following equation (A.2).

$$\Phi = \Phi_{ref} \frac{I}{I_{ref}} \frac{A_{ref}}{A} \frac{n^2}{n_{ref}^2}$$

Eq. (A.2)

Where Φ_{ref} is the quantum yield of the reference (standard, Rhodamine), I is the integrated area of the analyte. A is the absorbance at the excitation wavelength of the analyte. And n is the refractive index of the solvent used. Subscript ref means reference. The experimental results show that the fluorescence quantum yield of **TIFC-OH** is 0.302. Incidentally, the fluorescence quantum yield of **TIFC** was measured to be 0.033 according to the above formula.



Fig, S7 Fluorescence quantum yield (Φ) of **TIFC-OH** and **TIFC** in PBS-THF buffer (20mM, 1/1, v/v, PBS/THF, pH=7.40).

11. Cytotoxicity test results.

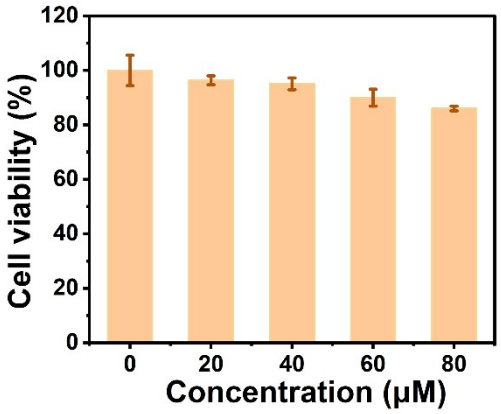
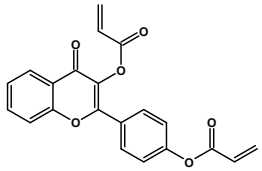
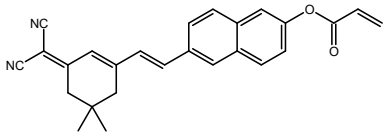
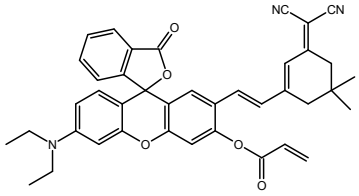
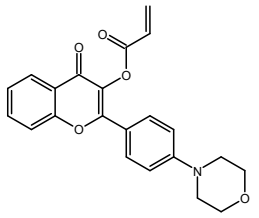
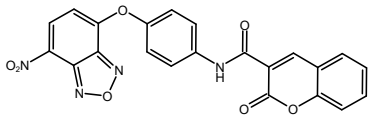
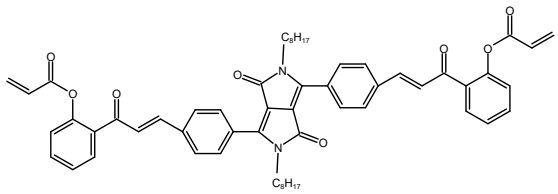
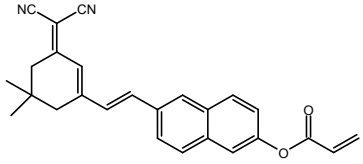
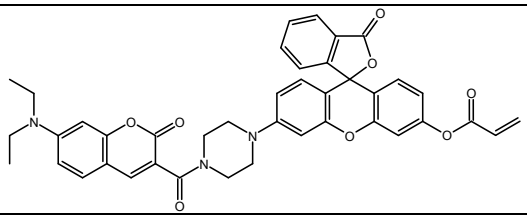
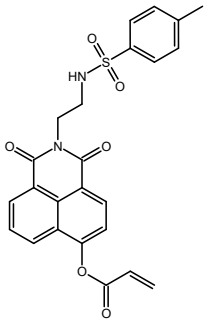
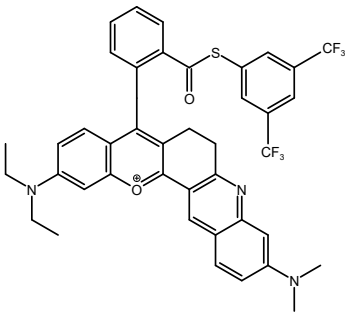
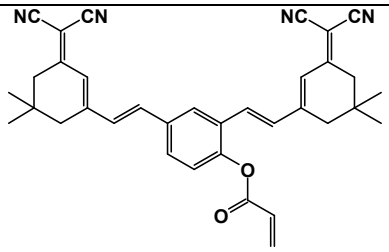


Fig. S8 Cell viability (%) estimated by MTT assay under incubation with different concentrations of **TIFC**. Osteoblasts were incubated with **TIFC** (0, 20, 40, 60, 80μM) for 24h at 37°C in a humidified incubator atmosphere with 5% CO_2. These data obtained are the mean ± standard deviation. (n = 2).

12. Research comparison.

Table S1. Comparison of detection limits of **TIFC** with other reported cysteine probes

Probe	emission type	Detection Limit	Literature
	OFF- ON	9.7×10^{-8}M	[1] Anal Chim Acta, 2021, 1176, 338763.
	OFF- ON	0.89μM	[2] Microchemical Journal, 2022, 174.
	OFF- ON	0.168μM	[3] Anal Methods, 2021, 13, 5369- 5376
	OFF- ON	0.16μM	[4] Spectrochim Acta A Mol Biomol Spectrosc, 2022, 280, 121552.
	OFF- ON	0.43μM	[5] Dyes and Pigments, 2021, 184.
	Ratiometric	0.18μM, 2.9μM	[6] Dyes and Pigments, 2018, 157, 284- 289.
	Ratiometric	0.48μM	[7] Molecules, 2018, 23.
	Ratiometric	2.0×10^{-7}M	[8] New Journal of Chemistry, 2019, 43, 14763- 14771.

	Ratiometric	1.8μM	[9] Anal Chem, 2019, 91, 5513-5516.
	Ratiometric	0.25μM	[10] Dyes and Pigments, 2020, 183.
	Ratiometric	105.6nM	This work

13. NMR Data and Mass spectra.

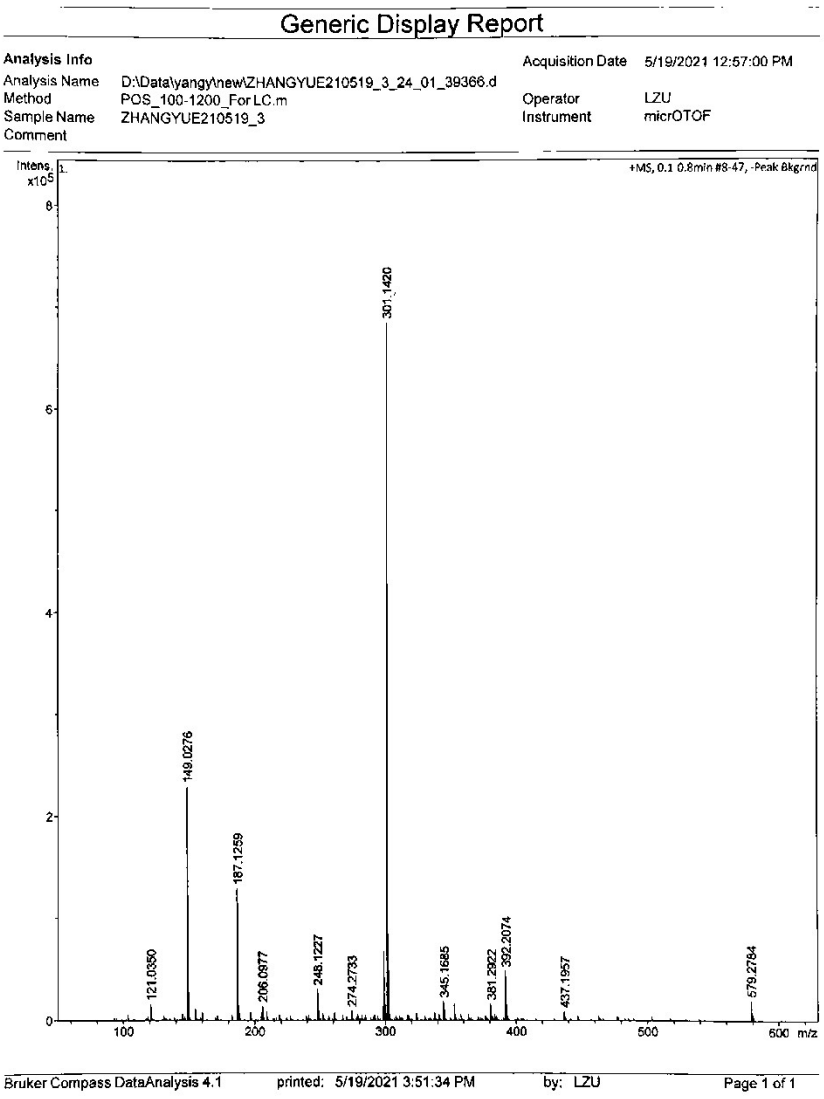


Fig. S9. The Mass spectra of Compound **1**.

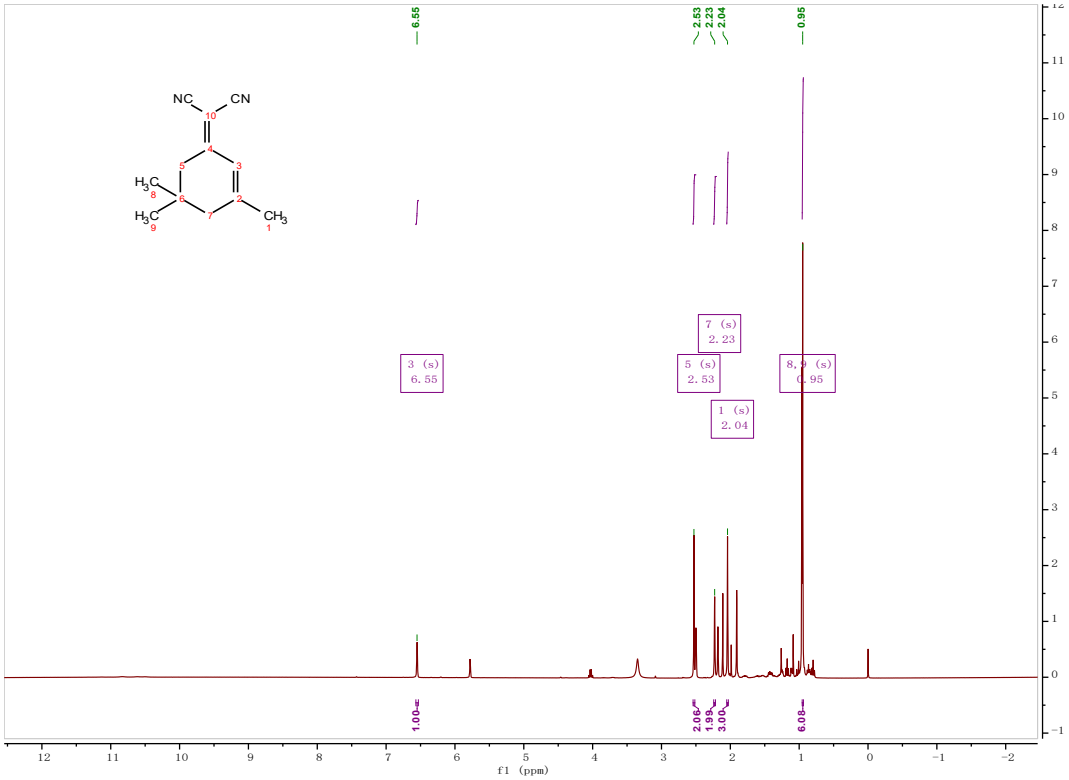


Fig. 10. The ^{1}H NMR spectra of the Compound **1** in DMSO-d_6.

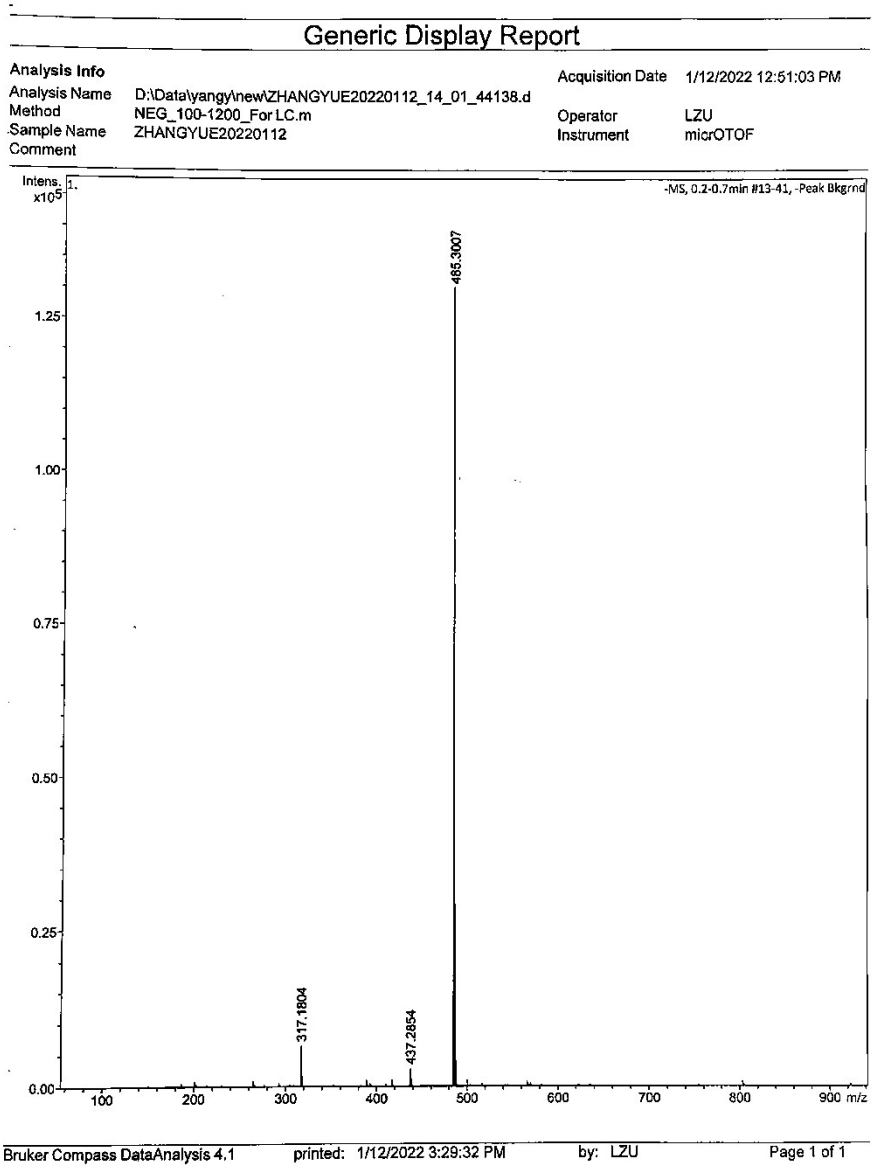


Fig. S11. The Mass spectra of **TIFC-OH**.

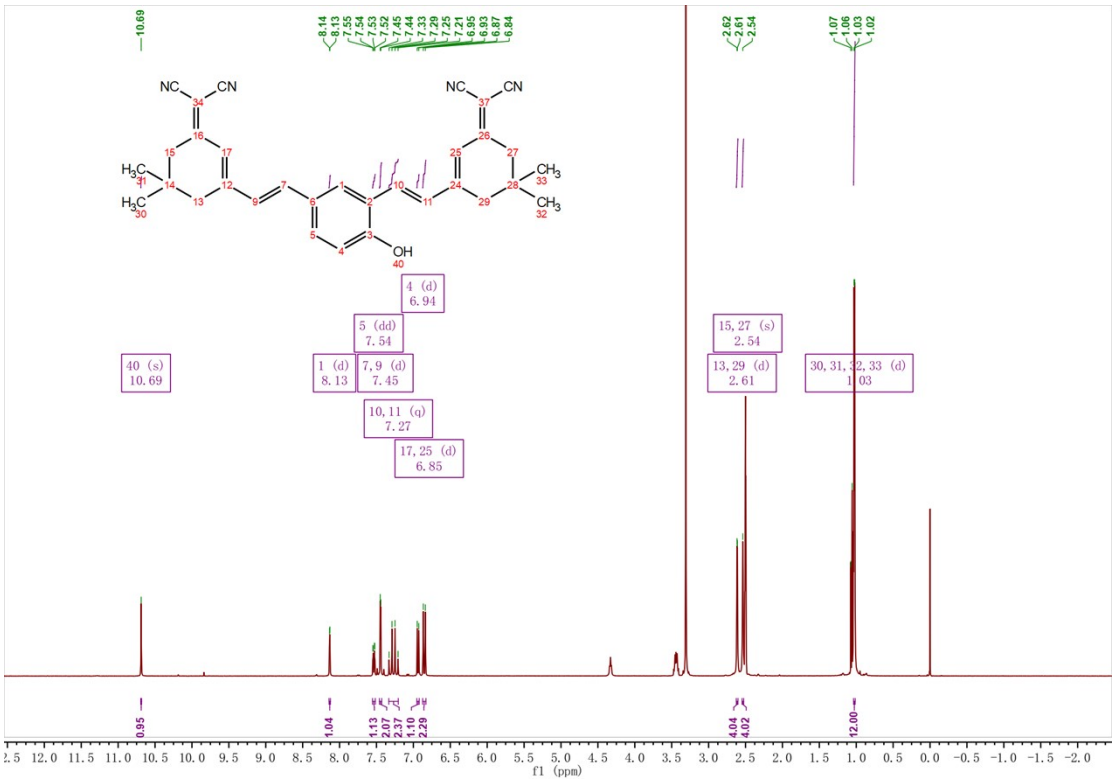


Fig. 12. The ^{1}H NMR spectra of the **THFC-OH** in DMSO-d_6.

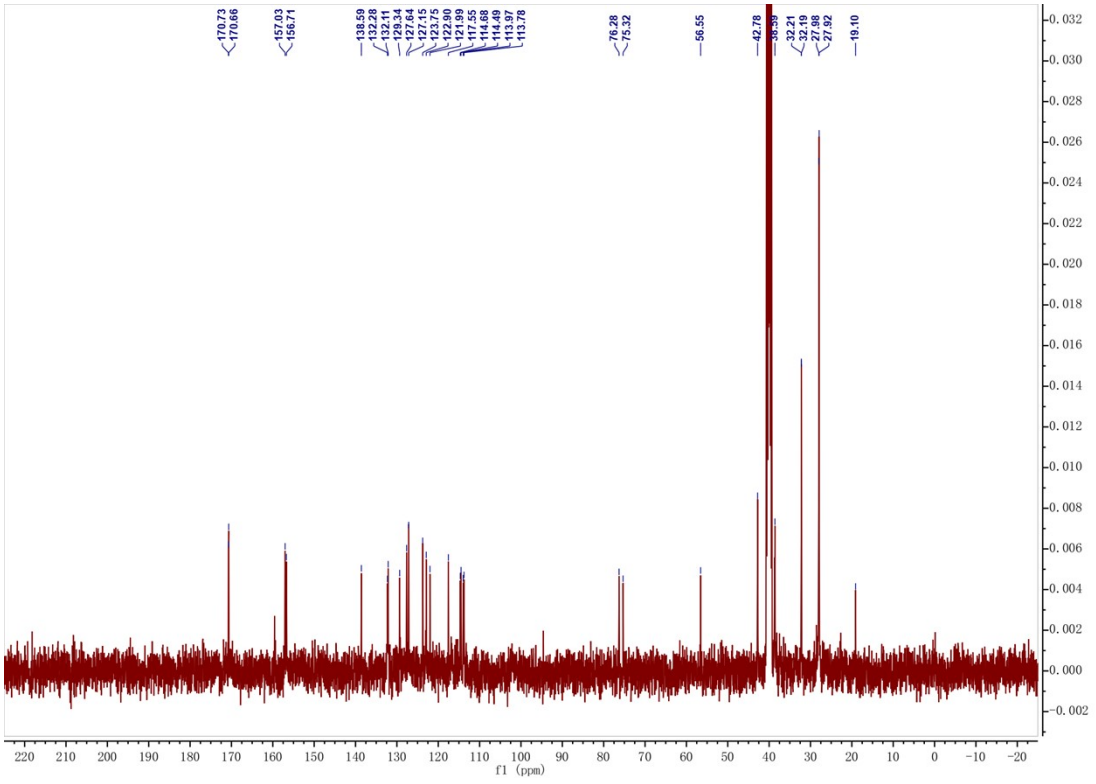


Fig. S13. The ^{13}C NMR spectra of the **TIFC-OH** in DMSO-d_6.

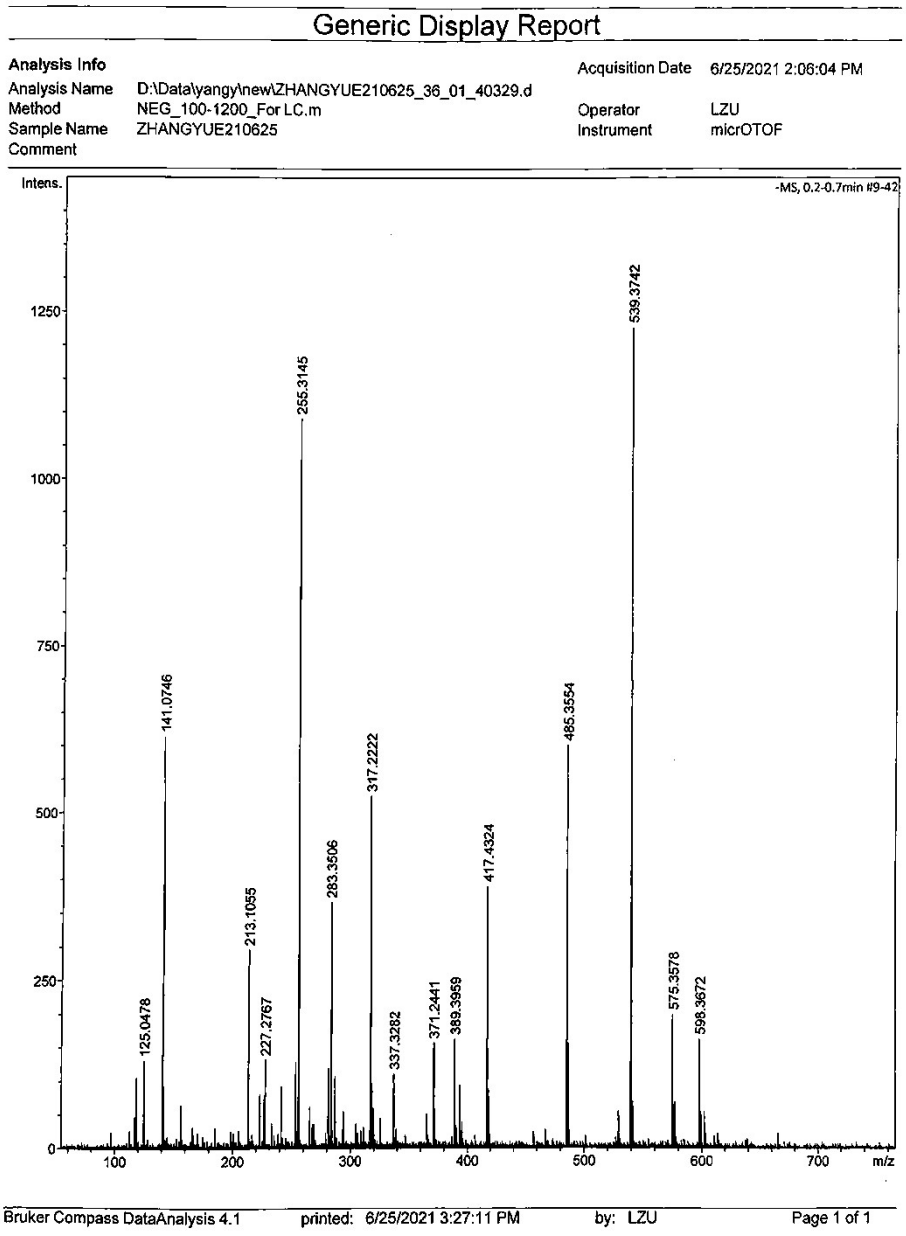


Fig. S14. The Mass spectra of **TIFC**.

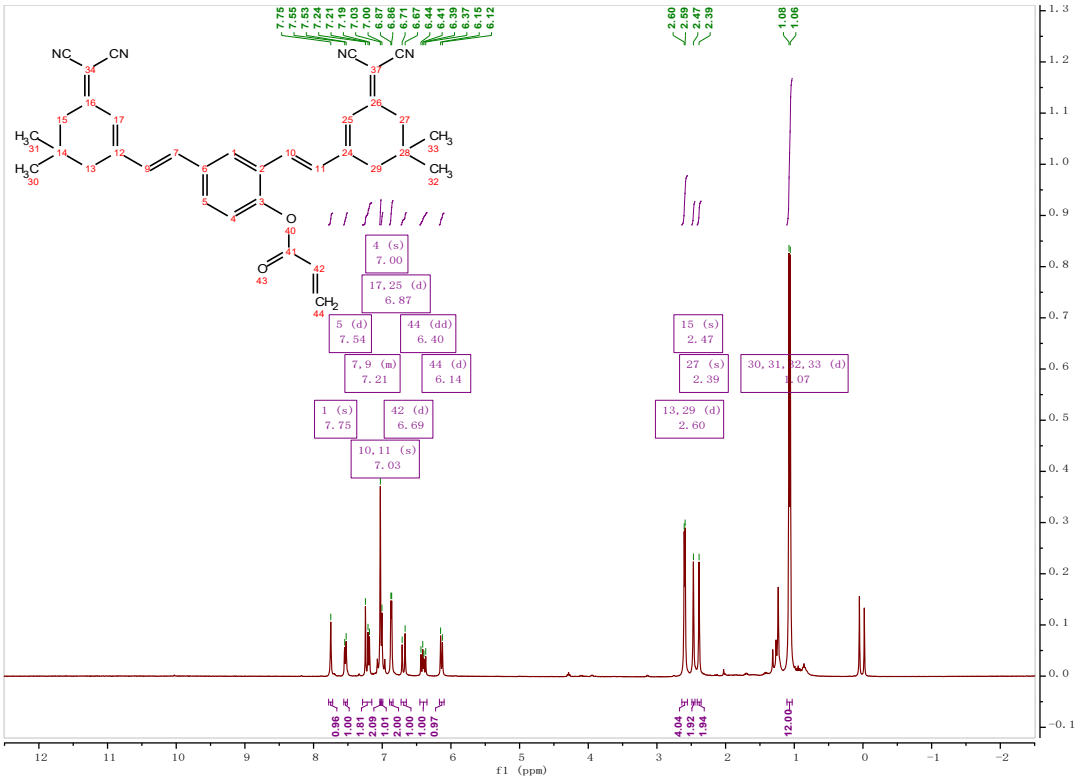


Fig. S15. The ^{1}H NMR spectra of **TIFC** in Chloroform-*d*.

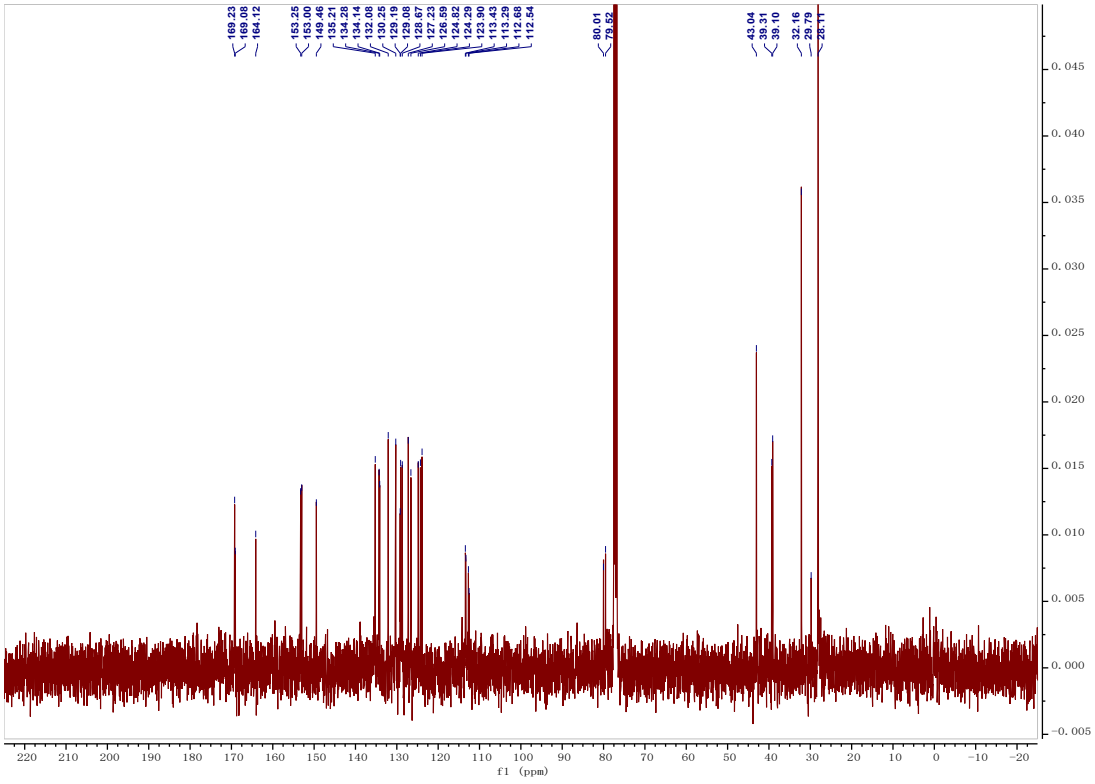


Fig. S16. The ^{13}C NMR spectra of **TIFC** in Chloroform-*d*.