Real-time detection and imaging for exogenous and endogenous Zn^{2+} in PC12 cells model of depression with NIR fluorescence probe

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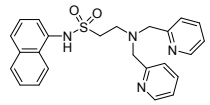
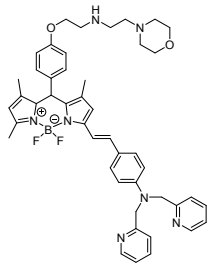
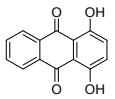
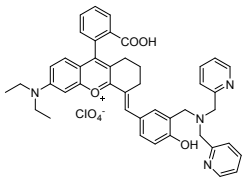
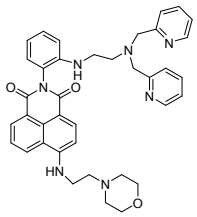
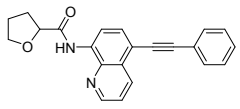
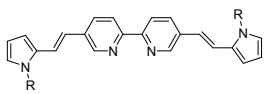
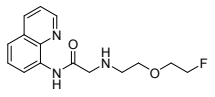
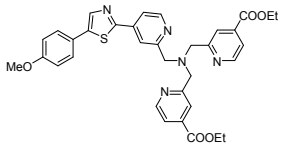
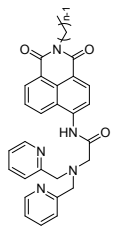
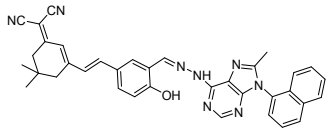
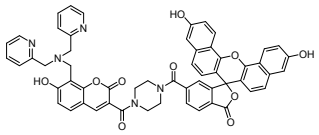
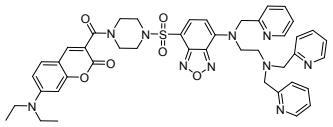
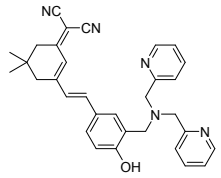
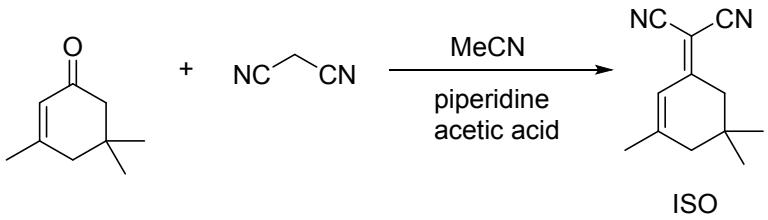
1. Comparison of fluorescence probes for sensing Zn^{2+}

Table S1 Comparison of fluorescence probes for sensing Zn^{2+}:

Probe	$\lambda_{ex}/\lambda_{em}$ (nm)	Stokes shift (nm)	Detection limit (nM)	Quantum yield	Application	References
	360/532	172	42	1.5%-5.5%	HeLa cells	Inorg. Chem. Commun. 2011, 14, 304-307.
	568/578	10	480	NR-23%	MCF-7 cells, NSCs cells and HeLa cells	Biomater. Sci. 2014, 2, 89-97.
	499/600	101	NR	NR	Techoma stans pollen grains	J. Photochem. Photobiol. B. 2015, 148, 181-187.
	576/701	125	190	NR	HUVEC-C cells and MDA-MB231 cells	ACS Sens. 2016, 1, 1408-1415.
	444/536 (two-photon)	/	180	3%-23%	NIH 3T3 cells, mouse brain tissues	Chem. Commun. 2016, 52, 124-127.
	390/525	135	41	45%-27%	HepG-2 cells	RSC Adv. 2017, 7, 40615-40620.
	475/600	125	NR	NR	C6 glioma cells and hippocampal slices	Sci.Rep. 2018, 8, 9069.

	320/500	180	NR	4.2%-35%	RWPE-1 and PC-3 cells	Chem. Commun. 2018, 54, 3227-3230.
	366/520	154	NR	32%-71%	NIH 3T3 mouse fibroblasts	ACS Sens. 2018, 3, 458-467.
	405/519	114	NR	NR	HT-19 cells	Chem. Commun. 2019, 55, 15045-15048.
	502/670	168	12	NR	HeLa cells	Anal. Methods. 2019, 11, 2396-2403.
	390/460	70	320	32%-52%	PC12 cells, brains of mice	Anal. Chem. 2020, 92, 4101-4107.
	415/480	65	82	NR	MCF-7 cells, SKOV3 cells, zebrafish larva	Chem. Sci. 2020, 11, 11037-11041.
	475/660	185	106	8.3%-44%	PC12 cells	This work

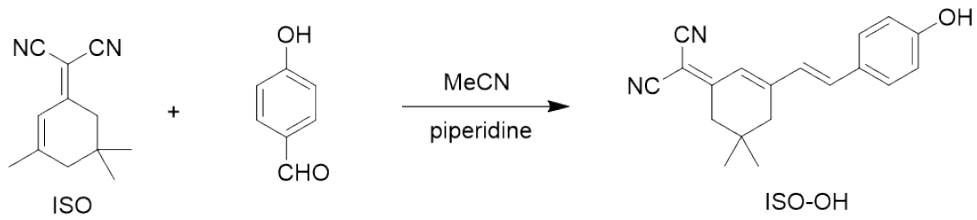
2. Synthesis of intermediate compounds



Scheme S1 Synthesis of compound **ISO**.

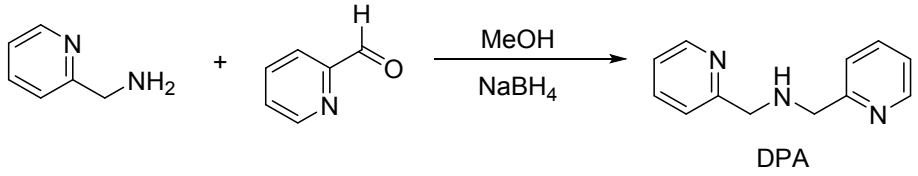
Isophorone (6.91 g, 50 mmol) and malononitrile (3.96 g, 60 mmol) were dissolved in 50 mL

acetonitrile. Piperidine (0.5 mL, 5 mmol) and anhydrous acetic acid (0.30 g, 5 mmol) were added into the solution. The reaction mixture was refluxed at 80 ℃ for six hours and monitored by TLC. The solvent was removed under reduced pressure. Dichloromethane (50 mL) was added and the solution was washed with water (50 mL×2). The organic phase was collected and the solvent was removed under reduced pressure. The residue was purified by silica column using petroleum and dichloromethane (2:1) as eluent to give **ISO** as a pale-yellow solid (7.26 g,78% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ(ppm): 6.62 (1H, s), 2.51 (2H, s), 2.18 (2H, s), 2.03 (3H, s), 1.01 (6H, s).



Scheme S2 Synthesis of compound **ISO-OH**.

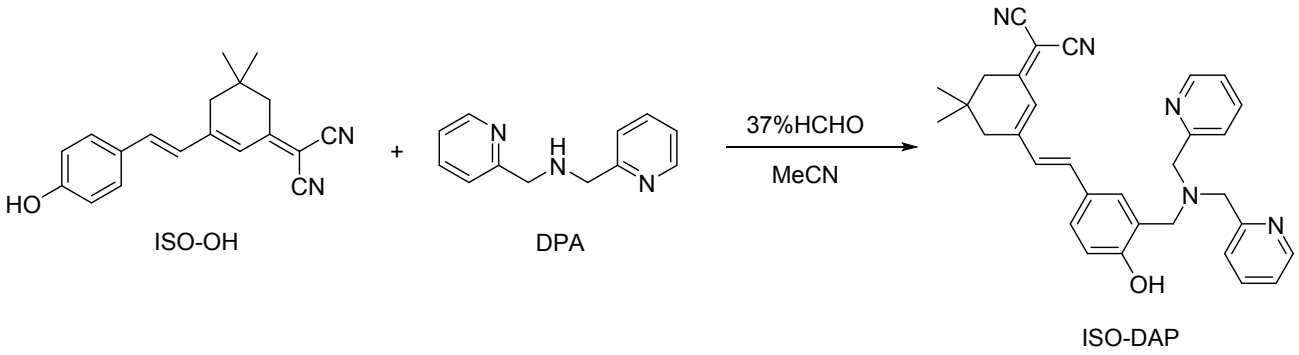
The similar synthesis route of **ISO-OH** can refer to ref 1. **ISO** (0.75 g, 4 mmol) and p-hydroxybenzaldehyde (0.49 g, 4 mmol) were dissolved in 50 mL acetonitrile. Eight drops of piperidine was added into the solution. The reaction mixture was refluxed at 80 ℃ for six hours and monitored by TLC. The solvent was removed under reduced pressure. Dichloromethane (60 mL) was added and then washed with water (60 mL×2). The organic phase was collected and the solvent was removed under reduced pressure, and the residue was purified by silica column using petroleum and dichloromethane (1:1) as eluent to give **ISO-OH** as an orange solid (0.98 g, 85% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 10.00 (1H, s), 7.55 (2H, d, J = 8.4 Hz), 7.21 (2H, dd, J = 16.1 Hz, J = 16.2 Hz), 6.79 (2H, d, J = 8.5 Hz), 6.79 (1H, s), 2.60 (2H, s), 2.53 (2H, s), 1.01(6H, s).



Scheme S3 Synthesis of compound **DPA**.

The similar synthesis route of **DPA** can refer to ref 2. To a solution of 12.00 g (110.8 mmol) of 2-(aminomethyl) pyridine in 30 mL methanol, 12.00 g (112.0 mmol) of pyridine-2-carboxaldehyde in 30 mL methanol were slowly added at 0 °C. A dark yellow solution was obtained and stirred for 1

h at room temperature. Then cooled to 0 °C, 1.56 g (110.8 mmol) of $NaBH_4$ was added slowly to the solution and the reaction mixture was stirred for 12 h. After the reaction completed, the solution color changed into light yellow, and methanol was removed under reduced pressure. The solid product was dissolved in dilute hydrochloric and adjusted the pH to 3. The clear yellow solution was washed with dichloromethane (3 × 50 mL) to remove the unreacted substrates. A saturated solution of Na_2CO_3 was added to the aqueous layer to adjust the pH to 10. The solution was then extracted with dichloromethane (3 × 100 mL) and dried over magnesium sulfate. The solvent was removed under vacuum to obtain **DPA** as a yellow oil (14 g,70% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ(ppm):8.55(2H, d, *J* = 4.0 Hz), 7.66 (2H, t, *J* = 8.0 Hz), 7.36 (2H, d, *J* = 8.0 Hz), 7.17 (2H, t, *J* = 4.0 Hz), 4.0 (4H, s).



Scheme S4 Synthesis of compound **ISO-DPA**.

To a solution of **DPA** (0.19 g, 1 mmol) in 10 mL of acetonitrile, aqueous formaldehyde (37%) (0.11 mL, 1.2 mmol) was added. The mixture was heated at 60 ℃ for two hours. Then a solution of **ISO-OH** (0.29 g, 1 mmol) in 10 mL of acetonitrile was slowly added. The reaction mixture was refluxed at 60 ℃ for twelve hours monitored by TLC. The solvent was removed under reduced pressure. The residue was purified by silica column using petroleum and ether-ethyl acetate (1:2) as eluent to give **ISO-DPA** as an orange solid (0.27 g, 54% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 11.36 (1H, s), 8.54 (2H, d, *J* = 4.0 Hz), 7.78 (2H, td, *J* = 8.0 Hz, *J* = 1.7 Hz), 7.66 (1H, d, *J* = 1.7 Hz), 7.48 (1H, dd, *J* = 8.0 Hz, *J* = 2.0 Hz), 7.44 (2H, d, *J* = 7.8 Hz), 7.30-7.15(4H, m), 6.82(1H, d, *J* = 8.4 Hz), 6.78 (1H, s), 3.83 (4H, s), 3.74 (2H, s), 2.60 (2H, s), 2.53 (2H, s),1.01 (6H, s). ^{13}C NMR (100 MHz, $CDCl_3$) δ(ppm): 169.36, 159.96, 158.03, 154.73, 148.70, 137.47, 137.11, 130.12, 129.20, 126.70, 126.13, 123.79, 123.19, 122.42, 117.66, 113.90,113.11, 77.12, 58.77, 56.79, 43.05, 39.24, 32.04, 28.06. HRMS (ESI), m/z calculated for $C_{32}H_{30}N_5O$ $[M - H]^-$:500.2450, found: 500.2451.

3. Preparation of ROS / RNS

The solutions of H_2O_2, ^-OCl, ·OH, $ONOO^-$ were prepared according to ref 3.

H_2O_2: The concentration of H_2O_2 was determined from the absorption at 240 nm (ε = 43.6 M^{-1} cm^{-1}).

^-OCl: The concentration of ^-OCl was determined from the absorption at 292 nm (ε = 350 M^{-1} cm^{-1}).

·OH: Hydrogen peroxide (H_2O_2, 10 eq) was added to $FeSO_4 \cdot 7H_2O$ in ultrapure water, hydroxyl radical (·OH) was achieved by the Fenton reaction. The concentration of ·OH was determined by the concentration of $FeSO_4 \cdot 7H_2O$.

$ONOO^-$: KNO_2 (0.6 M, 10 mL), H_2O_2 (0.7 M, 10 mL) and HCl (0.6 M, 10 mL) were successively added to a NaOH solution (3 M, 10 mL) at 0 ºC. The concentration of $ONOO^-$ was determined by the absorption at 302 nm (ε =1670 cm^{-1} M^{-1}) in 0.1 M NaOH solution.

4. Cytotoxicity of ISO-DPA

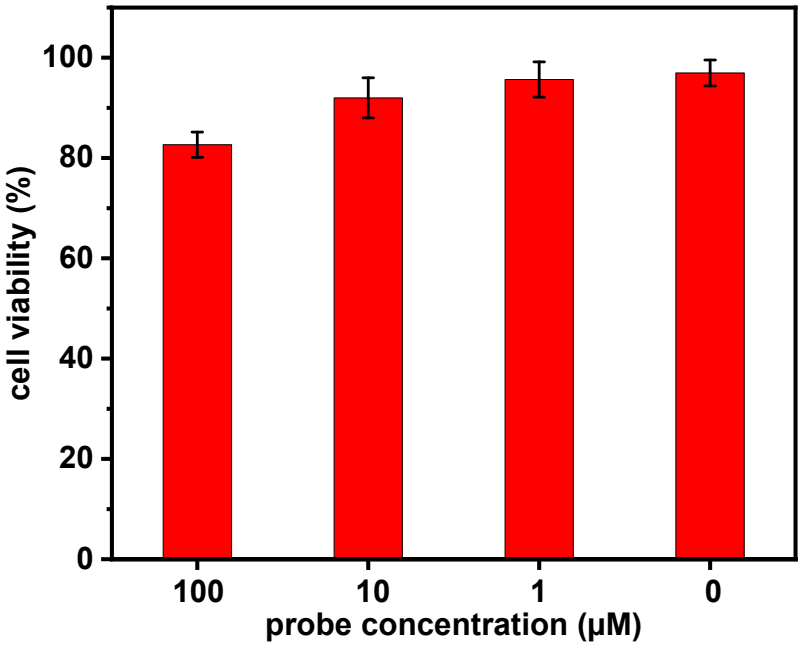


Fig. S1 Cell viability of PC12 cells incubated with probe **ISO-DPA** (100, 10, 1, 0 μM) at 37 ℃ for 24 h. Error bars represent the standard deviation (±S.D.) with n ≥3.

5. Determination of excitation wavelength of confocal laser scanning microscope via fluorescence spectra

The maximum absorption wavelength and maximum emission wavelength of **ISO-DPA** (10 μM) after adding Zn^{2+} (10 μM) is 475 nm and 660 nm. And confocal laser scanning microscope has three

suitable excitation wavelengths including 488 nm, 541 nm and 561 nm. So we carried out fluorescence spectra measurement of **ISO-DPA** with three different excitation wavelengths. The following spectra show that **ISO-DPA** exhibits weakest fluorescence intensity at 561 nm excitation. We chose 561 nm as excitation wavelength of confocal laser scanning microscope to avoid the fluorescence of the probe itself in PC12 cells.

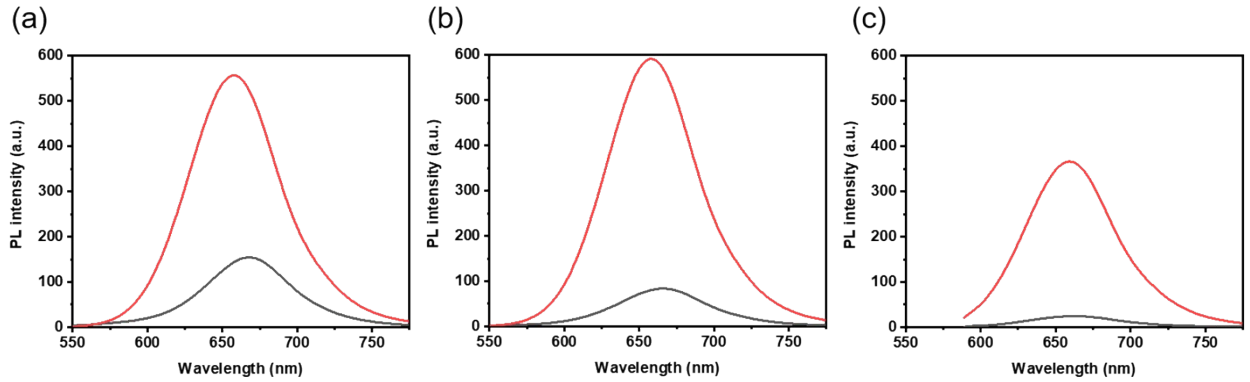


Fig. S2 Fluorescence intensities of **ISO-DPA** (10 μM) before (black) and after (red) adding Zn^{2+} (10 μM) in a mixture solution of HEPES buffer solution (10 mM, pH = 7.46) and DMSO (V/V = 1:1) (a): λ_{ex} = 488 nm, (b): λ_{ex} = 514 nm, (c): λ_{ex} = 561 nm.

6. NMR and MS data for compounds

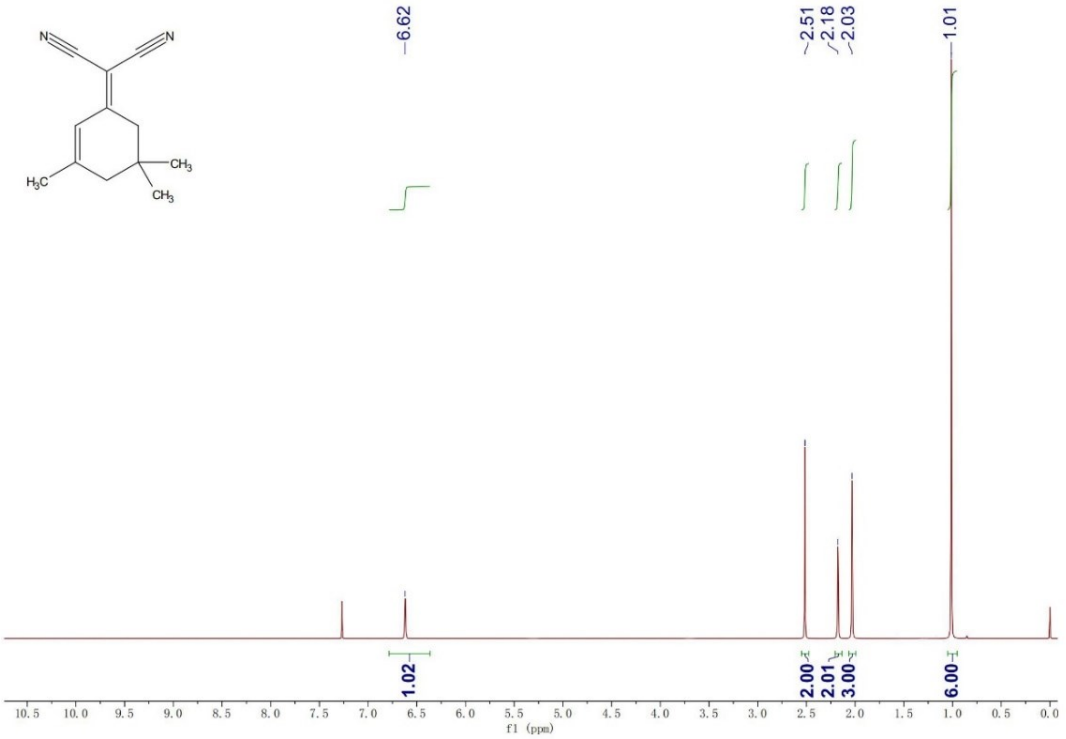


Fig. S3 The ^1H NMR spectrum of compound **ISO** in $CDCl_3$.

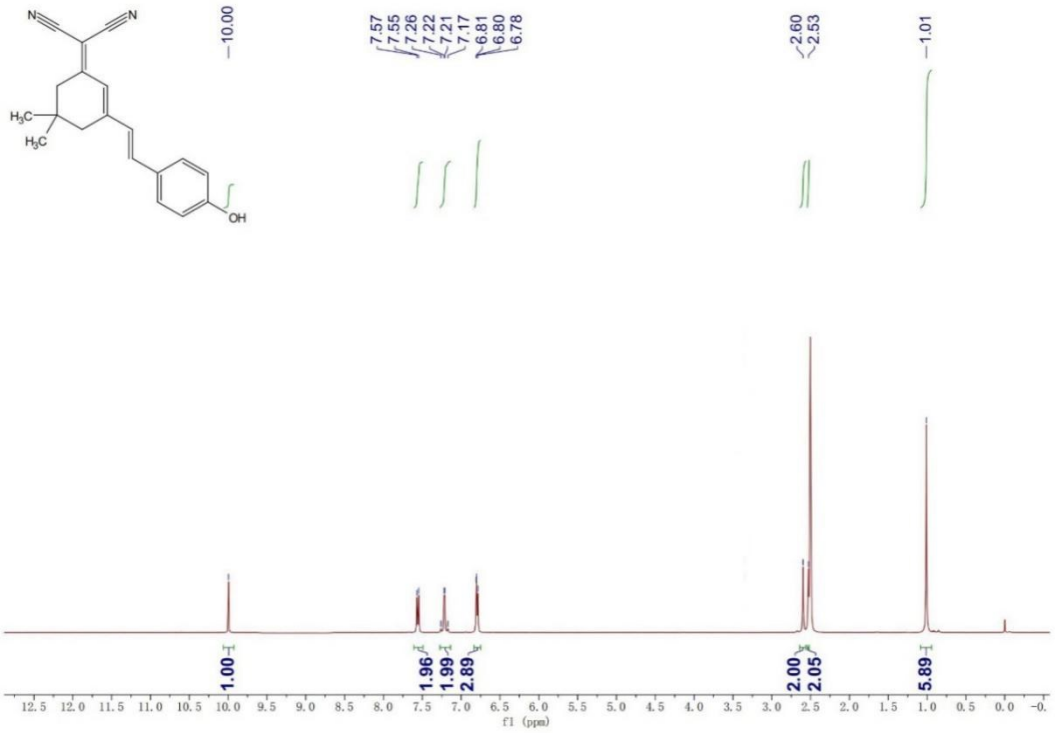


Fig. S4 The ^{1}H NMR spectrum of compound **ISO-OH** in DMSO.

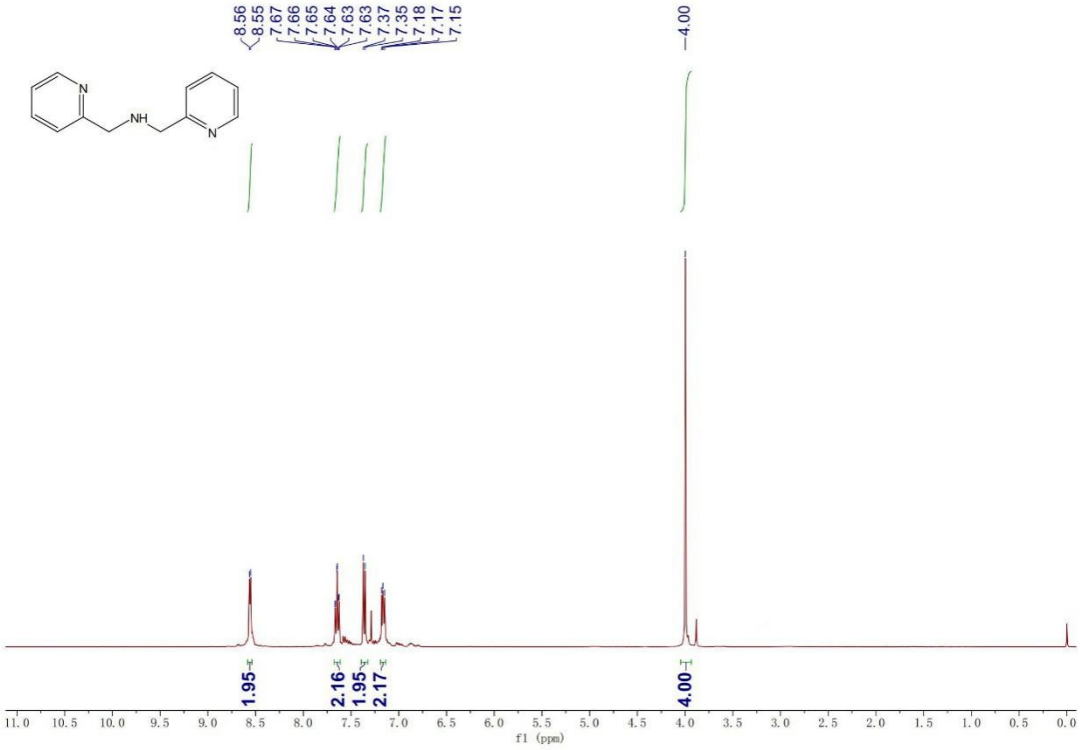


Fig. S5 The ^{1}H NMR spectrum of compound **DPA** in $CDCl_3$.

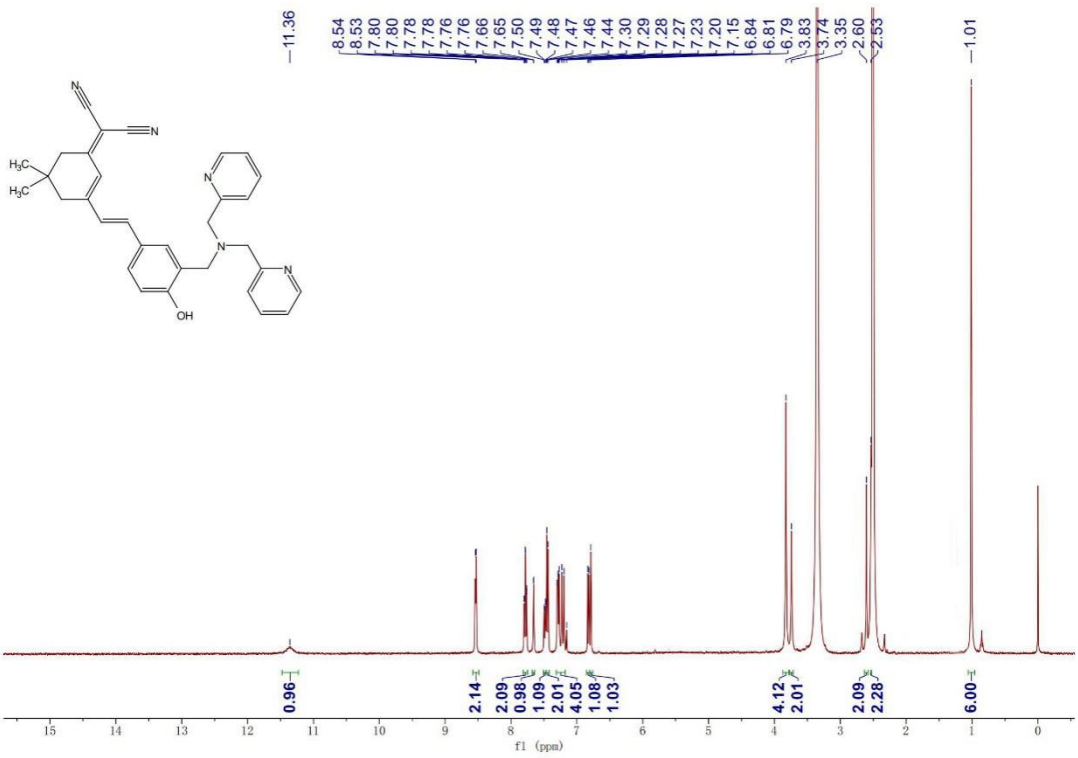


Fig. S6 The ^{1}H NMR spectrum of compound **ISO-DPA** in DMSO.

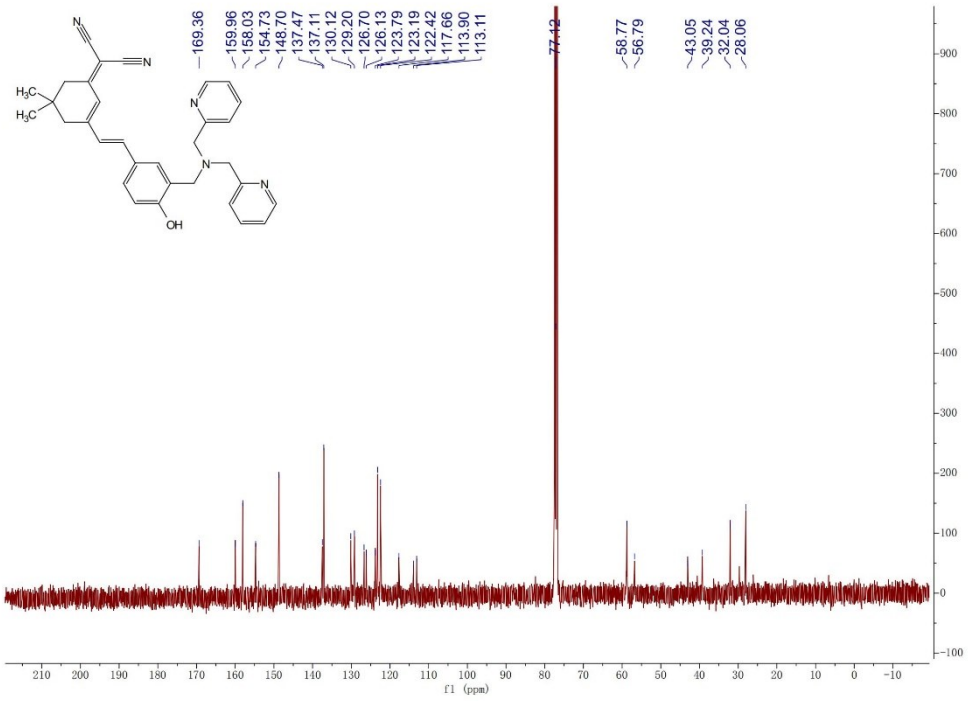


Fig. S7 The ^{13}C NMR spectrum of compound **ISO-DPA** in $CDCl_3$.

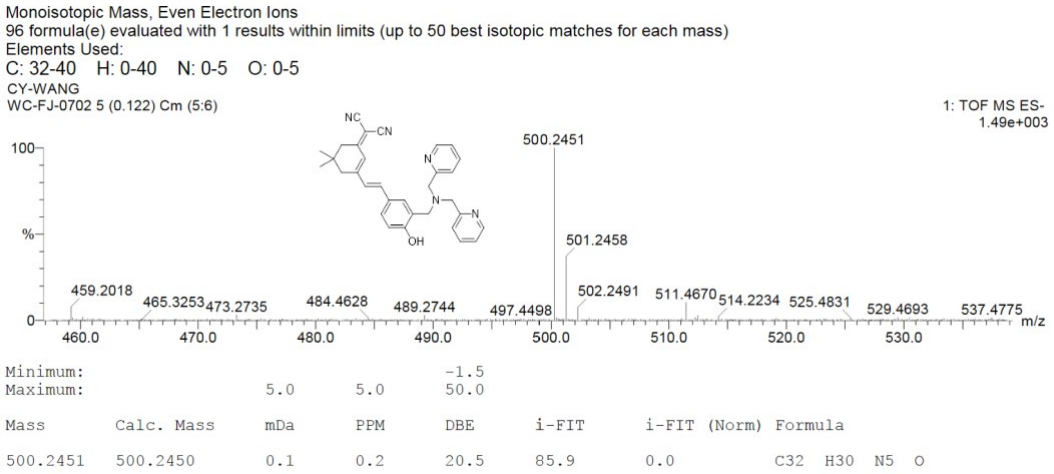


Fig. S8 The HR-MS (ESI) spectrum of the probe **ISO-DPA**.

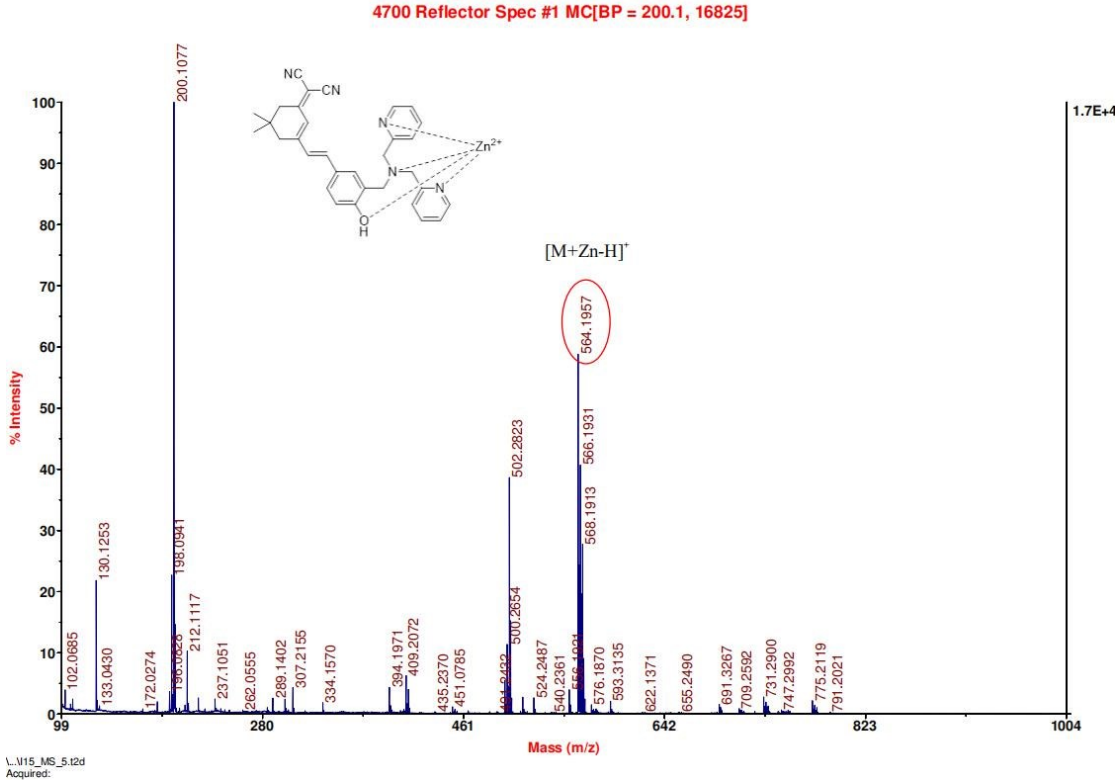


Fig. S9 The MALDI-TOF-MS spectrum of probe **ISO-DPA**+Zn^{2+}.

7. Materials and instruments

All reactants and solvents (analysis level) used were get from commercial suppliers. The products were purified by column chromatography. The Ultraviolet-visible light (UV-vis) absorption spectra were performed on Varian Cary 500 spectrophotometer and fluorescence spectra were performed on Agilent Cary Eclipse spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AM-400 spectrometer. High resolution mass spectra were recorded on a Waters LCT Permier XE spectrometer. Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry was performed on AB SCIEX 4800 Plus MALDI TOF/TOF^{TM}. The fluorescence

quantum yields were determined with rhodamine B (Φ_f = 0.89 in ethanol) as reference. The measurements of pH were done using a pH-10C digital pH meter.

8. Measurement of relative fluorescence quantum yield

The fluorescence quantum yield (Φ_f) of the related compounds in ethanol was determined by using rhodamine B as a reference with a known Φ_f value of 0.89 in ethanol. The calculation formula is listed as follow:

$$\Phi_f^{sample} = \Phi_f^{rhb} \times \left(\frac{F^{sample}}{F^{rhb}}\right) \times \left(\frac{Abs^{rhb}}{Abs^{sample}}\right)$$

Where Φ_f^{sample} is fluorescence quantum yield of the samples to be tested; Φ_f^{rhb} is fluorescence quantum yield of rhodamine B; Abs^{rhb} is absorbance of rhodamine B; F^{rhb} is the area under the fluorescence spectra of rhodamine B; Abs^{sample} is absorbance of the test samples; F^{sample} is the area under the fluorescence spectra of the test sample.

Table S2 Photophysical properties of the **rhb**, **ISO-DPA**, **ISO-DPA**+Zn^{2+} in ethanol:

	F	Abs	Φ_f
rhb	29281.3600	0.0346	0.89
ISO-DPA	3773.2577	0.0474	0.083
ISO-DPA+Zn^{2+}	14025.1053	0.0330	0.44

9. Job's plot

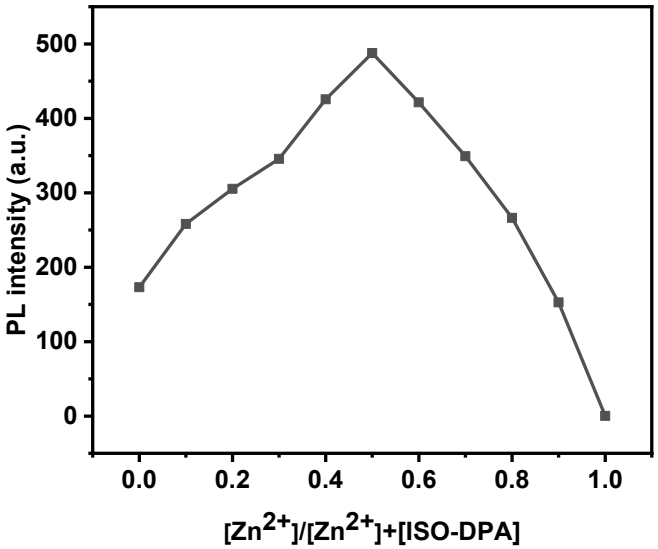


Fig. S10 Job's plot of the complexation of fluorescence probe **ISO-DPA** with Zn^{2+} was plotted as function of the different molar ratio [Zn^{2+}]/([Zn^{2+}] + [**ISO-DPA**]) by fluorescence method, indicating the 1:1 stoichiometry. The total concentration of **ISO-DPA** and Zn^{2+} was constantly maintained in 20 μM in a mixture solution of HEPES

buffer solution (10 mM, pH = 7.46) and DMSO (V/V = 1:1). λ_{ex} = 475 nm, λ_{em} = 660 nm.

10. Determination of binding constant

Binding constant (*K*a) was calculated from the fluorescence titration experiment based on the modified Benesi-Hildbrand plot. Fluorescence intensities were obtained from the titration curves of 10 μM **ISO-DPA** in the presence of different concentration of mental ions at 660 nm in a mixture solution of HEPES buffer solution (10 mM, pH = 7.46) and DMSO (V/V = 1:1). *K*a was calculated according to the following equation:

$$\frac{1}{K_a[M](I_t - I_0)} + \frac{1}{I_t - I_0} = \frac{1}{I - I_0}$$

"I" stands for the fluorescence intensity of **ISO-DPA**. I_t refers to the fluorescence intensity at saturated metal ion concentration. I_0 means the fluorescence intensity in the absence of metal ion. K_a is the binding constant and [M] is the concentration of metal ion.

According to the plot of fluorescence intensity verses Zn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} concentration, K_a is calculated as 1.3×10^5 M^{-1}, 3.9×10^5 M^{-1}, 6.3×10^4 M^{-1}, 4.9×10^5 M^{-1}, 1.3×10^5 M^{-1} respectively.

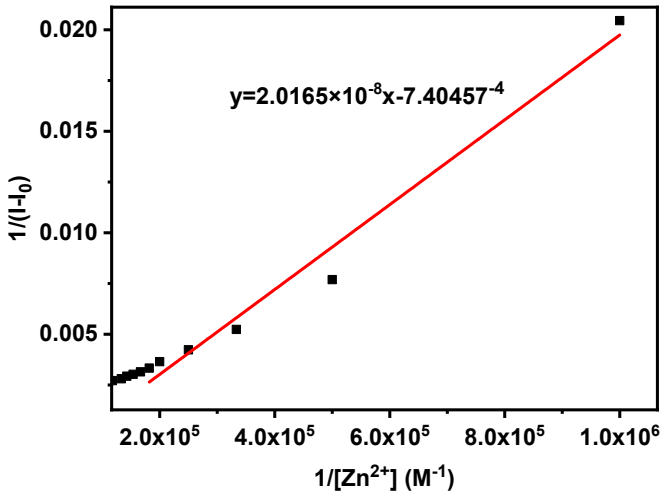


Fig. S11 Benesi-Hildbrand equation plot of 10 μM of **ISO-DPA** with Zn^{2+} ions obtained from fluorescence titration data. The binding constant was determined to be 1.3×10^5 M^{-1}. λ_{ex} = 475 nm, λ_{em} = 660 nm.

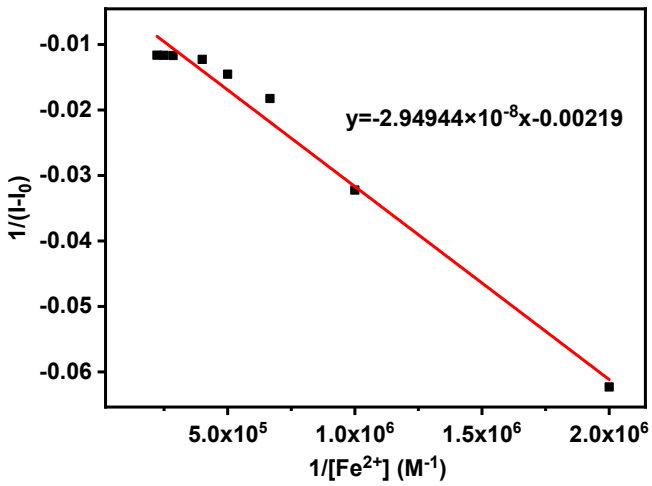


Fig. S12 Benesi-Hildbrand equation plot of 10 μM of **ISO-DPA** with Fe^{2+} ions obtained from fluorescence titration data. The binding constant was determined to be 3.9×10^5 M^{-1}. λ_{ex} = 475 nm, λ_{em} = 660 nm.

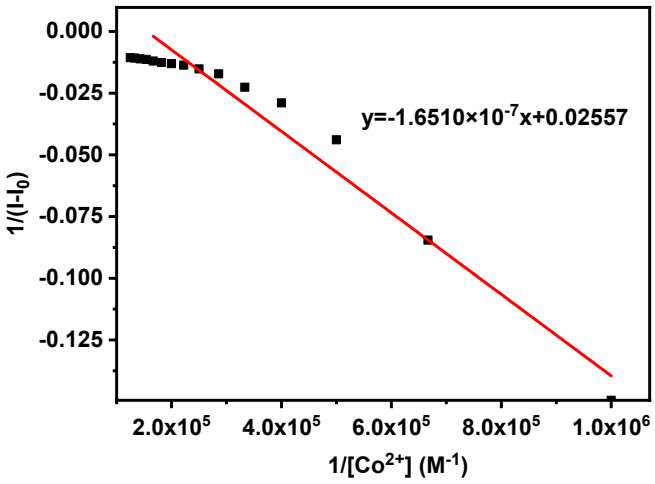


Fig. S13 Benesi-Hildbrand equation plot of 10 μM of **ISO-DPA** with Co^{2+} ions obtained from fluorescence titration data. The binding constant was determined to be 6.3×10^4 M^{-1}. λ_{ex} = 475 nm, λ_{em} = 660 nm.

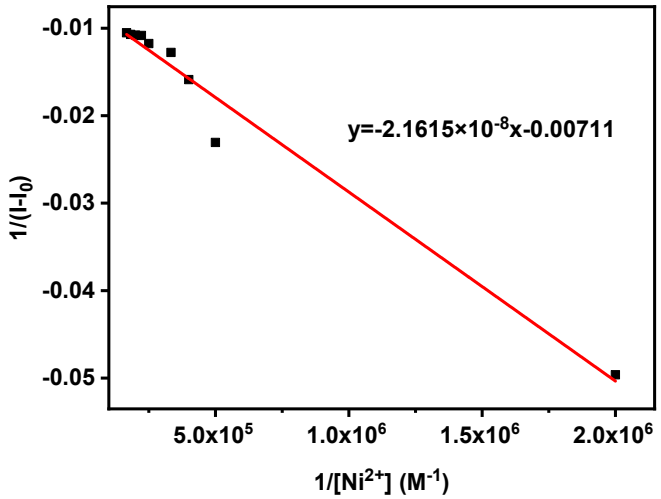


Fig. S14 Benesi-Hildbrand equation plot of 10 μM of **ISO-DPA** with Ni^{2+} ions obtained from fluorescence titration data. The binding constant was determined to be 4.9×10^5 M^{-1}. λ_{ex} = 475 nm, λ_{em} = 660 nm.

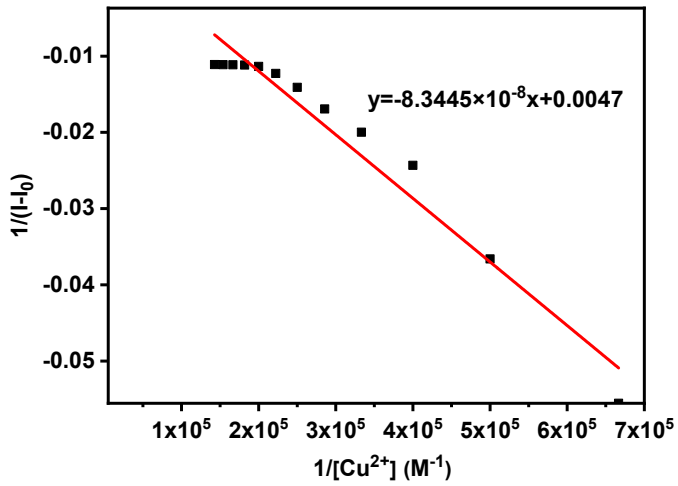


Fig. S15 Benesi-Hildbrand equation plot of 10 μM of **ISO-DPA** with Cu^{2+} ions obtained from fluorescence titration data. The binding constant was determined to be 1.3×10^5 M^{-1}. λ_{ex} = 475 nm, λ_{em} = 660 nm.

11. Time courses of the fluorescence responses

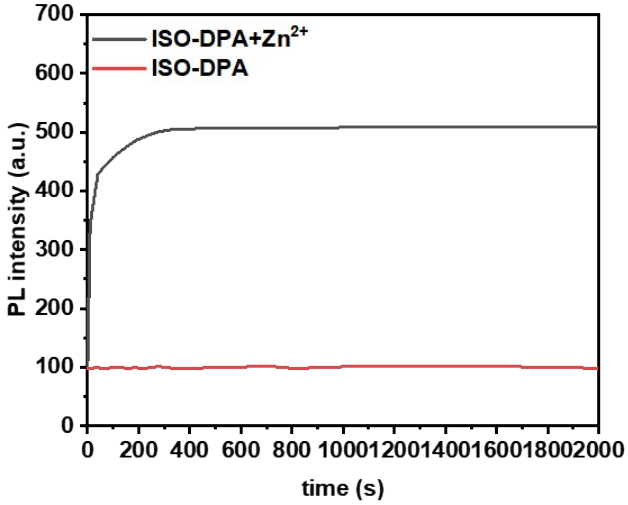


Fig. S16 Photostability of **ISO-DPA** (10 μM) in the absence and presence of Zn^{2+} (10 μM) in a mixture solution of HEPES buffer solution (10 mM, pH = 7.46) and DMSO (V/V = 1:1). λ_{ex} = 475 nm, λ_{em} = 660 nm.

12. Selectivity of ISO-DPA

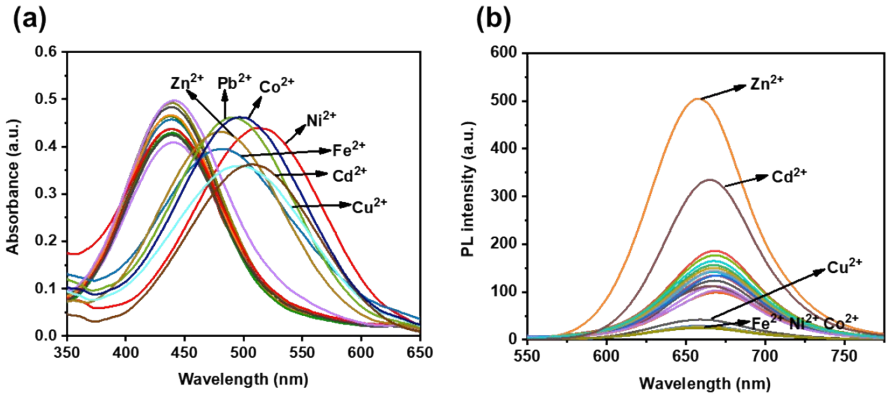


Fig. S17 Absorption spectra and fluorescence spectra of **ISO-DPA** (10 μM) in the presence of 100 μM of various ions including (Na^{+}, Mg^{2+}, Al^{3+}, K^{+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Cd^{2+} Pb^{2+}, F^{-}, Cl^{-}, Br^{-}, I^{-}, AcO^{-}, CO_3^{2-}) and 10 μM of Zn^{2+} in a mixture solution of HEPES buffer solution (10 mM, pH = 7.46) and (V/V = 1:1). λ_{ex} = 475 nm.

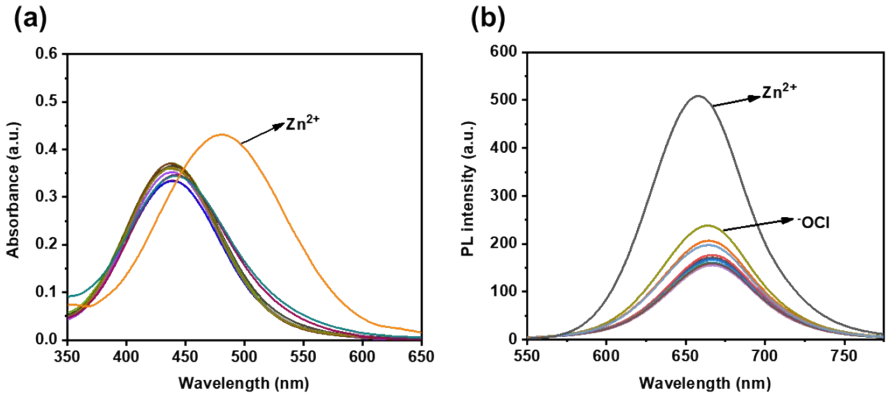


Fig. S18 Absorption spectra and fluorescence spectra of **ISO-DPA** (10 μM) in the presence of 100 μM of ROS,

RNS and RSS species including (H_2O_2, ^-OCl, $\cdot OH$, $ONOO^-$, HS^-, HSO_3^-, $S_2O_3^{2-}$, $S_2O_5^{2-}$, GSH, Cys, Hcy) and 10 μM of Zn^{2+} in a mixture solution of HEPES buffer solution (10 mM, pH = 7.46) and (V/V = 1:1). λ_{ex} = 475 nm.

13. Demetalation of ISO-DPA-Zn^{2+} using TPEN

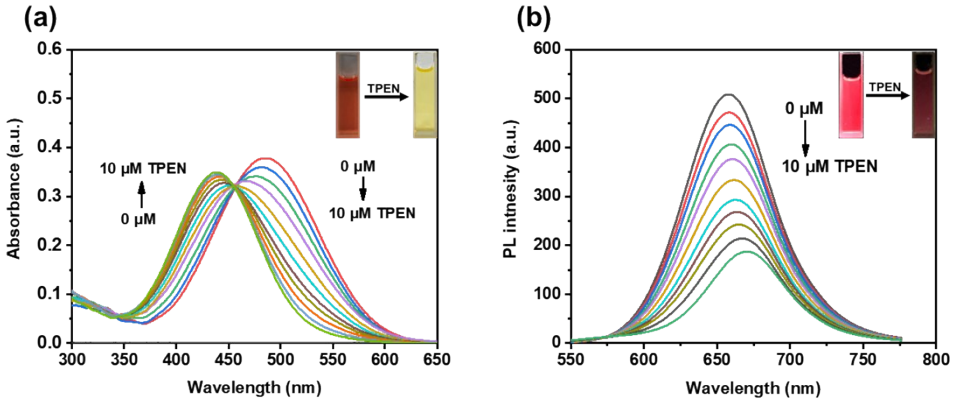


Fig. S19 (a) UV-vis spectra of **ISO-DPA** (10 μM) upon adding different concentration of TPEN (0-10 μM). (b) Fluorescence spectra changes of **ISO-DPA** after adding TPEN (0-10 μM). All tests were performed in a mixture solution of HEPES buffer solution (10 mM, pH = 7.46) and DMSO (V/V = 1:1). λ_{ex} = 475 nm.

References

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2. S. Goswami, A. K. Das, B. Pakhira, S. B. Roy, A. K. Maity, P. Saha and S. Sarkar, *Dalton Trans.*， 2014， **43**， 12689-12697.

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