Supplementary Information

for

A ratiometric two-photon fluorescent probe for quantification of nitroreductase in hypoxic neurons

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Experimental section

Reagents and Chemicals

All chemicals were purchased from commercial suppliers and without further purification. 4-Nitro-1,8-naphthalicanhydride, 4-amino-1,8-naphthalicanhydride, 3-amino-1-propanol, triethylamine, 7-(diethylamino)-2-oxo-2H-chromene-3-carbaldehyde, 4-methylpyridine, tribromophosphine, NaI were purchased from Adamas-Beta Co., Ltd. (Shanghai, China). Petroleum ether (PE), ethyl acetate (EA), dichloromethane (DCM), methanol (MeOH) and N, N-dimethylformamide (DMF) were bought from General Reagents Co., Ltd. (Shanghai, China). Nitro reductase (NTR), monoamine oxidase-A (MAO-A), monoamine oxidase-B (MAO-B), protein tyrosine phosphatase (PTP), gamma-glutamyl transferase (GCT), bovine serum albumin (BSA), human serum albumin (HSA), tyrosinase (TYR) and alkaline phosphatase (ALP) were purchased from Aladdin Chemistry Co. Ltd. (China). Phosphate buffer solution (PBS, pH 7.4) with concentration of 0.1 M was prepared from KH_2PO_4, $K_2HPO_4 \cdot 3H_2O$ and KCl. All aqueous solutions were prepared with Milli-Q water (18.2 M Ω cm, Millipore) and all chemicals were used as purchase without further purification.

Instruments

The ^{1}H NMR and ^{13}C NMR spectra were obtained from a 500 MHz Bruker NMR spectrometer (Bruker, Germany). The mass spectra were detected by a Bruker ESI time-of-flight MS system (Bruker, Germany). The fluorescence spectrum and the UV-vis absorption spectrum were recorded by using a Hitachi F-4500 fluorescence

spectrometer (Hitachi, Japan) and a Hitachi UH-5300 spectrometer (Hitachi, Japan), respectively. The fluorescence imaging was obtained from a Leica TCS SP8 confocal laser scanning microscope (Leica, Germany) equipped with two-photon laser (Chameleon Ultra II, Coherent, UK). The cytotoxicity assays were measured by Varioskan LUX multimode microplate reader (Thermo Fisher scientific, USA). The apoptosis assay was carried out by a FACS Calibur flow cytometry (Becton, Dickinson and Company, USA).

Cell culture

The acquisition and cultivation of neurons were conducted as a previous reported procedure. Newborn within 24 hours C57BL/6 wild-type mice were anesthetized by halothane, and then their brains were removed quickly and put in Hanks' balanced salt solution (HBSS, free Mg^{2+} and Ca^{2+}) at 0 °C. Tissues of the cortex were stripped out and then incubated with papain at 37 °C for 12 min, after that they were dispersed into poly-d-lysine-coated 35 mm Petri dishes at a density of 1×10^6 cells/dish. The neurons were cultured with neurobasal medium containing L-glutamine and B27 and the medium was changed three times a week. After maintained at 37 °C in a humidified atmosphere with 5% CO_2 for a week, the neurons could be used for imaging. All experiments involving mice were carried out in accordance with the principles and guidelines approved by the Animal Care and Use Committee of Jiaxing University.

Cytotoxicity and apoptosis assay

The cytotoxicity assays were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Neurons in 96-well plates were incubated with

different concentrations of the **IFRP** probe (0, 10, 20, 30 and 40 μM) and cultured for 12 and 24 h. Then, the neurons in each well were treated with 20 μL, 5 mg/mL MTT solution and further continuously incubated for 4 h at 37 °C. After that, MTT solution was removed and 100 μL Formazan solvent was added to each well until the crystalline formazan products were dissolved. Absorbance was next measured at 490 nm in a Varioskan LUX multimode microplate reader (Thermo Fisher scientific, USA). Cell viability was defined as the ratio of absorbance in the experimental groups to that in the blank control groups. For apoptosis assays, the Annexin V-FITC Apoptosis Detection Kit was used to determine the degree of cell apoptosis. Neurons were incubated with the **IFRP** probe (0, 10, 30 and 50 μM) for 24 h, then they were collected with the help of EDTA-free trypsin and washed by 5 mL PBS for three times. Moreover, PBS was removed by centrifugation of 1000 rpm for 5 min and neurons were incubated with 195 μL binding buffer of Annexin V-FITC, 5 μL Annexin V-FITC and 10 μL Propidium Iodide (PI) at room temperature in the dark for 30 min. After these procedures, neurons were used for the flow cytometry and detected at an excitation wavelength of 488 nm.

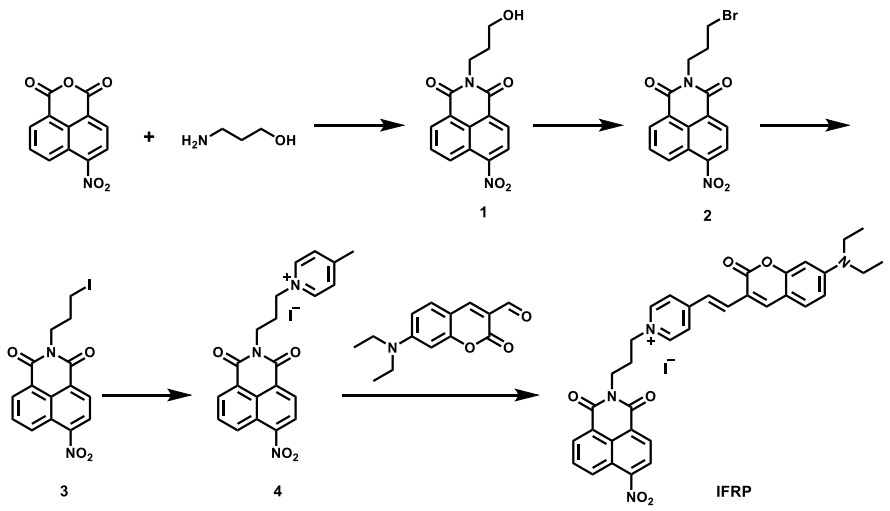
NTR analysis in cell lysates

The cultured neuronal cells were subjected to centrifugal treatment to remove the nutrient solutions and then dispersed in normal saline. Pulsed sonication, with a cycle of 2 seconds on and 2 seconds off, was performed at a power out of 600 W for 2 min to break the cells (Scientz-IID, Ningbo Scientz Biotechnology Co., Ltd., China). Subsequently, the neuronal cell lysates were clarified through a centrifugation process at 10,000 rpm at 4 ℃ for 5 min and stored at -20 ℃ for further use. The procedure for

NTR analysis in neuronal cell lysates was identical to that in PBS solution.

Subcellular imaging

The subcellular localization of **IFRP** was studied through colocalization imaging experiments, in which neurons labelled with **IFRP** (5.0 μM, labeling time: 0.5 h) were further labelled with MitoLite blue FX490 (5.0 μM) for additional 0.5 h. Following staining, neurons were gently washed thrice with PBS before conducting confocal fluorescence imaging experiments. MitoLite blue FX490 was excited using a 405 nm laser, and emission signals were collected within the 420-490 nm wavelength range. As for **IFRP**, fluorescence was excited at 800 nm, and images were collected within 470-550 nm (F_{515} channel) and 600-700 nm (F_{640} channel) wavelength range.



Scheme S1. The synthesis procedures of **IFRP**.

Synthesis of Compound **1**: 4-Nitro-1,8-naphthalicanhydride (486 mg, 2.0 mmol) and 3-amino-1-propanol (300 mg, 4.0 mmol) were dissolved in ethanol (20 mL). The reaction mixture was stirred and refluxed for 3 h under a nitrogen atmosphere, the resulting mixture was evaporated to dryness. After the solvent was evaporated under reduced pressure, the crude product was purified by silica gel column chromatography using PE/EA (20:1, v/v) as eluent to get Compound **1** as brown solid. ^{1}H NMR (500 MHz, 298 K, DMSO-d_6) δ 8.65-8.63 (d, J = 10.0 Hz, 1H), 8.57-8.49 (m, 3H), 8.06-8.03 (m, 1H), 4.53-4.51 (m, 1H), 4.11-4.08 (m, 2H), 3.53-3.50 (m, 2H), 1.83-1.77 (m, 2H). ^{13}C NMR (125 MHz, 298 K, DMSO-d_6) δ 163.33, 162.53, 149.44, 132.04, 130.51, 129.96, 129.09, 128.68, 127.06, 124.68, 123.17, 123.11, 59.43, 40.49, 40.32, 40.16, 39.99, 39.82, 39.65, 39.49, 38.44, 31.21. HR-MS (ESI): m/z calcd for $C_{15}H_{12}N_2NaO_5$ $[M+Na]^+$: 323.0638; found: 323.0646.

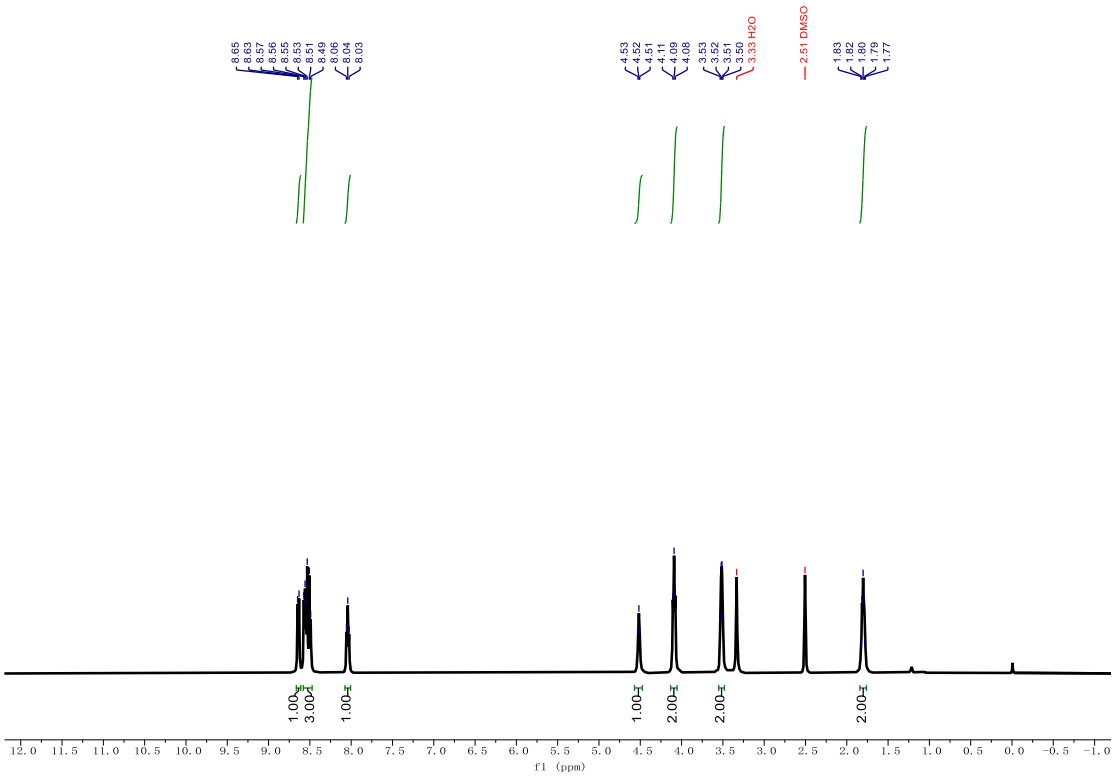


Fig. S1. ^{1}H NMR spectrum of Compound **1**.

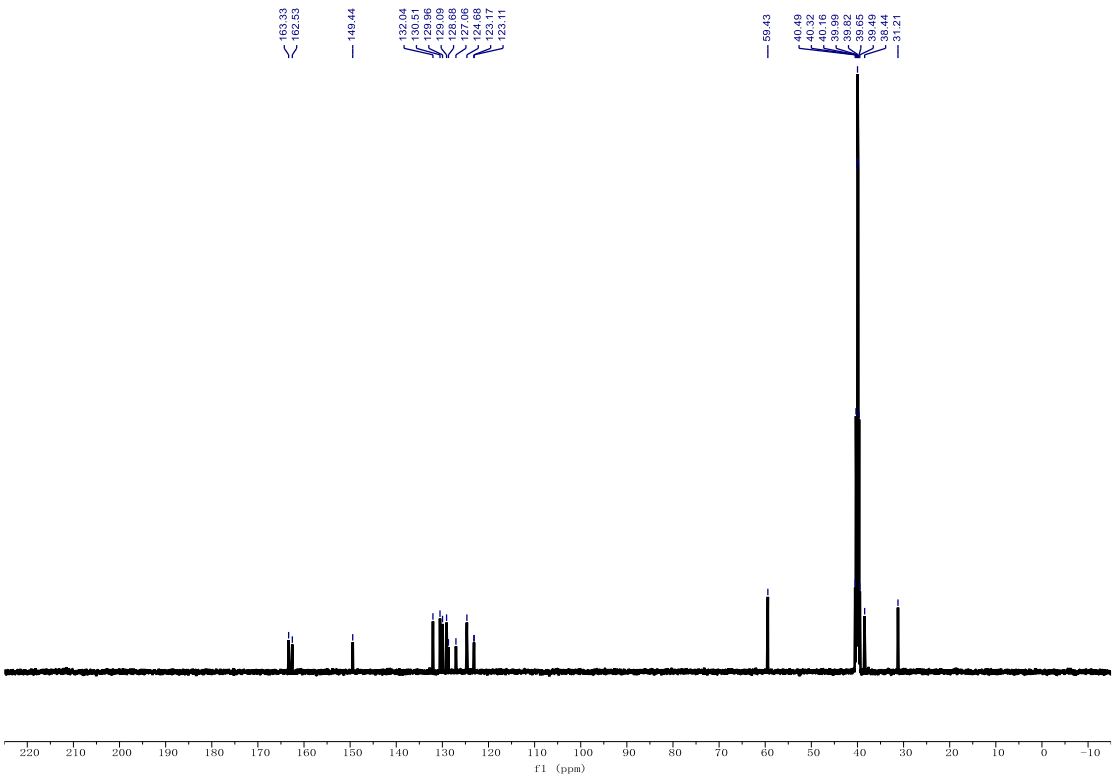
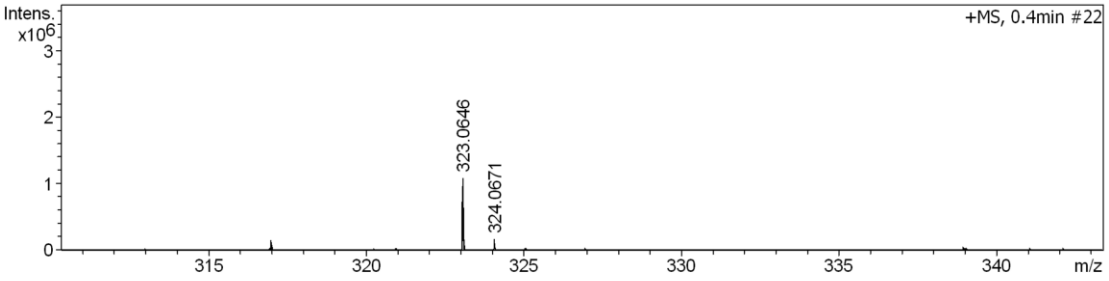


Fig. S2. ^{13}C NMR spectrum of Compound **1**.



#	m/z	Res.	S/N	I	I %	FWHM
1	323.0646	26044	1513.9	1091136	100.0	0.0124
2	324.0671	17710	235.3	169736	15.6	0.0183
3	325.0689	15152	31.8	22928	2.1	0.0215

Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma		Score	rdb	e¯ Conf	N-Rule
323.0646	1	C15H12N2NaO5	323.0638	-2.5	10.2	1	100.00	10.5	even	ok

Fig. S3. HR-MS spectrum of Compound **1**.

Synthesis of Compound **CMP**: 4-amino-1,8-naphthalicanhydride (426 mg, 2.0 mmol) and 3-amino-1-propanol (300 mg, 4.0 mmol) were dissolved in ethanol (20 mL). The reaction mixture was stirred and refluxed for 3 h under a nitrogen atmosphere, the resulting mixture was evaporated to dryness. After the solvent was evaporated under reduced pressure, the crude product was purified by silica gel column chromatography using PE/EA (5:1, v/v) as eluent to get Compound1 as yellow solid. ^{1}H NMR (500 MHz, 298 K, DMSO-d_6) δ 8.62-8.60 (d, J = 8.5 Hz, 1H), 8.43-8.42 (d, J = 7.0 Hz, 1H), 8.20-8.18 (d, J = 8.5 Hz, 1H), 7.66-7.63 (m, 1H), 7.43 (s, 2H), 6.86-8.84 (d, J = 8.0 Hz, 1H), 4.96-4.47 (m, 1H), 4.08-4.05 (m, 2H), 3.50-3.47 (m, 2H), 1.78-1.75 (m, 2H). ^{13}C NMR (125 MHz, 298 K, DMSO-d_6) δ 164.25, 163.41, 153.12, 134.37, 131.41, 130.11, 129.70, 124.40, 122.24, 119.80, 108.60, 108.04, 59.48, 37.45, 31.69. HR-MS (ESI): m/z calcd for $C_{15}H_{14}N_2NaO_3$ $[M+Na]^+$: 293.0897; found: 293.0900.

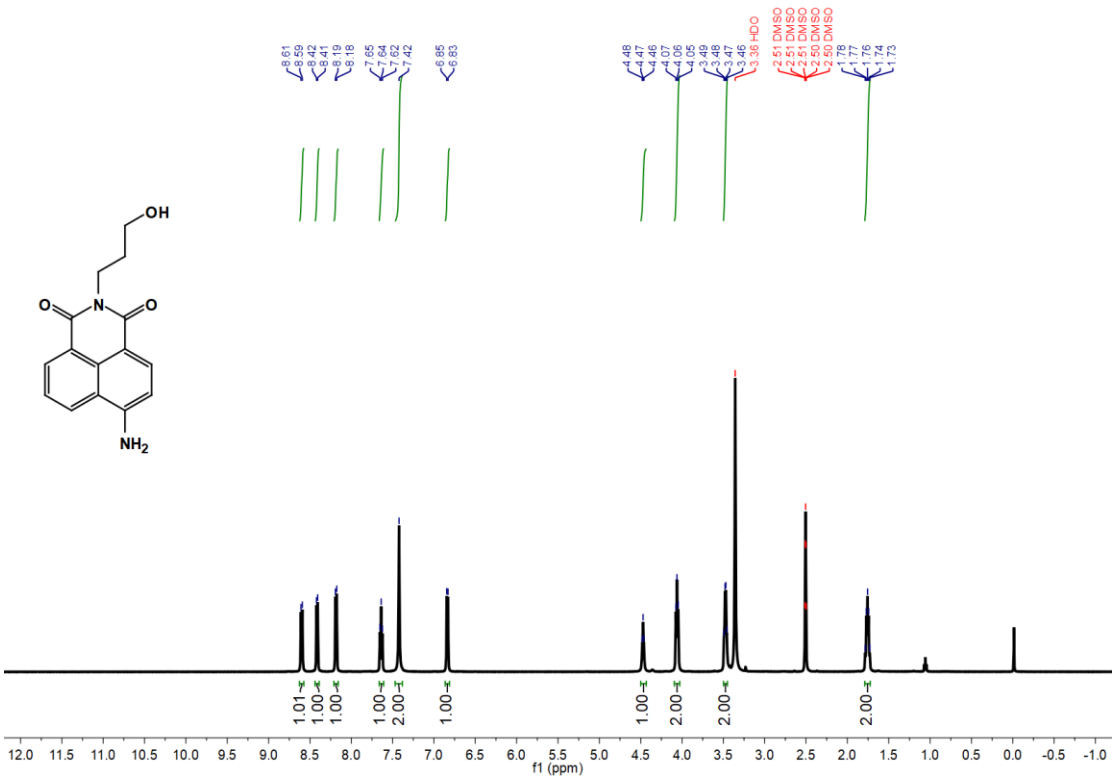


Fig. S4. ^{1}H NMR spectrum of Compound **CMP**.

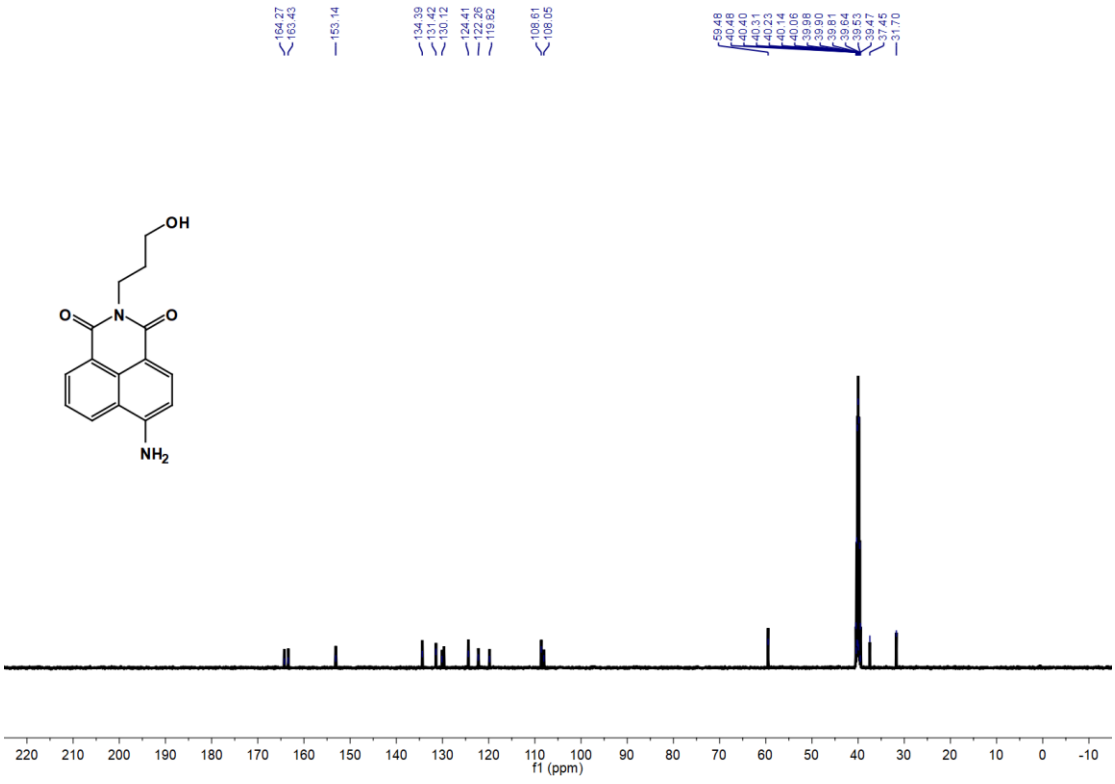
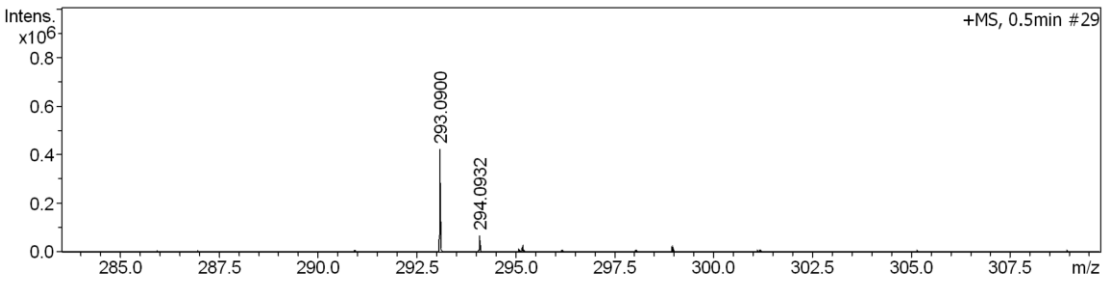


Fig. S5. ^{13}C NMR spectrum of Compound **CMP**.



#	m/z	Res.	S/N	I	I %	FWHM
1	293.0900	17243	481.6	422992	100.0	0.0170
2	294.0932	15361	78.9	69296	16.4	0.0191
3	295.0956	13548	9.2	8056	1.9	0.0218

Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma		Score	rdb	e¯ Conf	N-Rule
293.0900	1	C15H14N2NaO3	293.0897	-1.0	4.9	1	100.00	9.5	even	ok

Fig. S6. HR-MS spectrum of Compound **CMP**.

Synthesis of Compound **DMP**: 7-(diethylamino)-2-oxo-2H-chromene-3-carbaldehyde (490 mg, 2 mmol) and 4-methylpyridine (279 mg, 3.0 mmol) were dissolved in acetonitrile (20 mL). The reaction mixture was stirred and refluxed for 8 h under nitrogen atmosphere, the resulting mixture was evaporated under reduced pressure, the crude product was purified by silica gel column chromatography using DCM/MeOH (200:1, v/v) as eluent to get **DMP** as red solid. ^{1}H NMR (500 MHz, 298 K, DMSO-d_6) δ 8.79-8.78 (d, J = 5.5 Hz, 2H), 8.24 (s, 1H), 8.16-8.14 (m, 2H), 7.83-7.81 (d, J = 13.5 Hz, 1H), 7.68-7.66 (d, J = 13.0 Hz, 1H), 7.55-7.54 (d, J = 8.0 Hz, 1H), 6.81-6.79 (m, 1H), 6.60-6.59 (d, J = 2.0 Hz, 1H), 4.23 (s, 3H), 3.51-3.47 (m, 4H) , 1.16-1.14 (m, 6H). ^{13}C NMR (125 MHz, 298 K, DMSO-d_6) δ 13C NMR (151 MHz, DMSO) δ 160.07, 156.76, 153.36, 152.40, 145.78, 145.25, 137.12, 131.20, 123.46, 123.08, 114.11, 110.49, 108.82, 96.68, 47.18, 44.84, 40.42, 40.28, 40.14, 40.00, 39.86, 39.72, 39.58, 12.86. HR-MS (ESI): m/z calcd for $C_{21}H_{23}N_2O_2$ [M-I]$^+$: 335.1754; found: 335.1760.

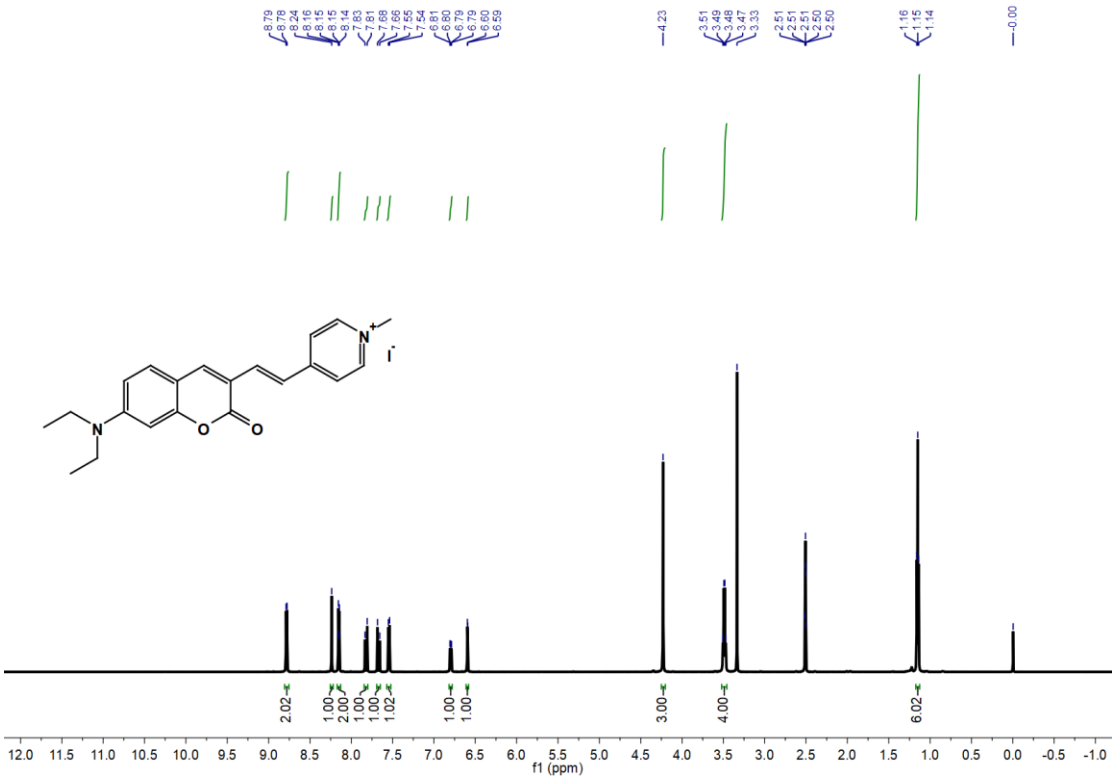


Fig. S7. ^{1}H NMR spectrum of Compound **DMP**.

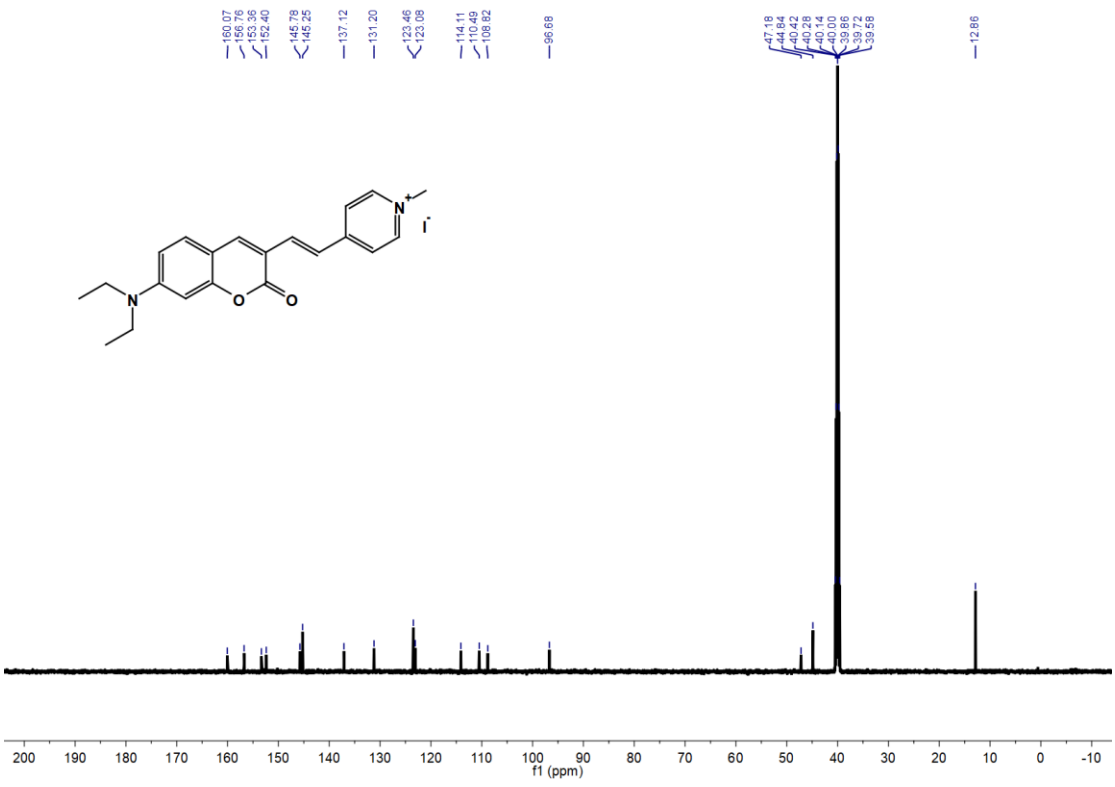
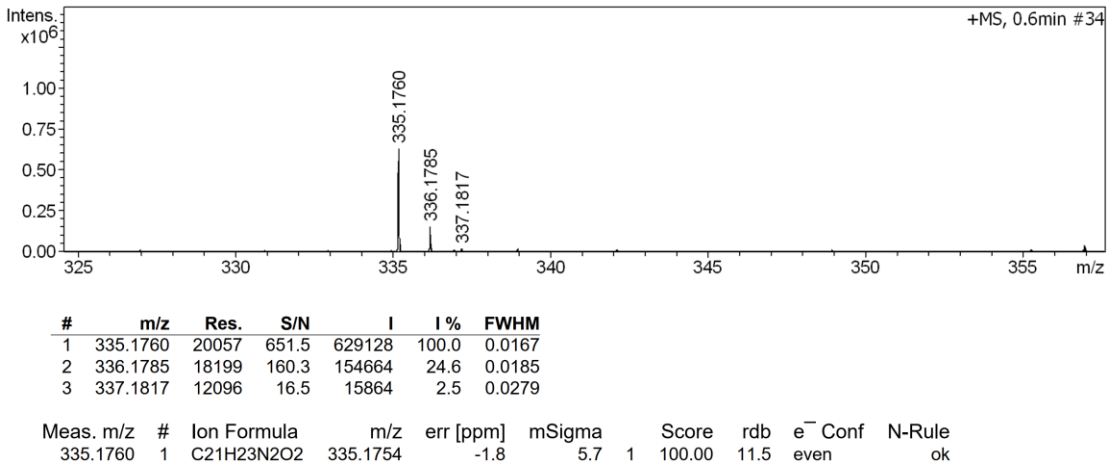


Fig. S8. ^{13}C NMR spectrum of Compound **DMP**.



#	m/z	Res.	S/N	I	I %	FWHM
1	335.1760	20057	651.5	629128	100.0	0.0167
2	336.1785	18199	160.3	154664	24.6	0.0185
3	337.1817	12096	16.5	15864	2.5	0.0279

Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma		Score	rdb	e¯ Conf	N-Rule
335.1760	1	C21H23N2O2	335.1754	-1.8	5.7	1	100.00	11.5	even	ok

Fig. S9. HR-MS spectrum of Compound **DMP**.

Synthesis of Compound **2**: To a solution of compound 1 (300 mg, 1.0 mmol) in dichloromethane (30 mL) at 0 ℃, tribromophosphine (0.19 mL, 2.0 mmol) was added dropwise. The resulting reaction mixture was warmed up to room temperature and stirred for 6 h. Then, the mixture was diluted with dichloromethane (20 ml), and washed three times with water (20 mL). The organic layer was separated and dried over anhydrous Na_2SO_4. The solvent was removed by evaporation under reduced pressure, and the purified by silica-gel column chromatography PE/EA (20:1, v/v) to afford compound **2** as yellow solid. ^{1}H NMR (500 MHz, 298 K, DMSO-d_6) δ 8.64-8.62 (d, J = 10.0 Hz, 1H), 8.57-8.47 (m, 3H), 8.05-8.02 (m, 1H), 4.12-4.07 (m, 4H), 2.01-1.94 (m, 2H). ^{13}C NMR (125 MHz, 298 K, DMSO-d_6) δ 170.76, 163.41, 162.61, 149.46, 132.07, 130.51, 129.99, 129.14, 128.69, 126.98, 124.68, 123.10, 62.58, 40.49, 40.32, 40.24, 40.16, 40.08, 39.99, 39.91, 39.82, 39.66, 39.49, 37.76, 27.12, 21.07. HR-MS (ESI): m/z calcd for $C_{15}H_{11}BrN_2NaO_4$ $[M+Na]^+$: 384.9794; found: 384.9777.

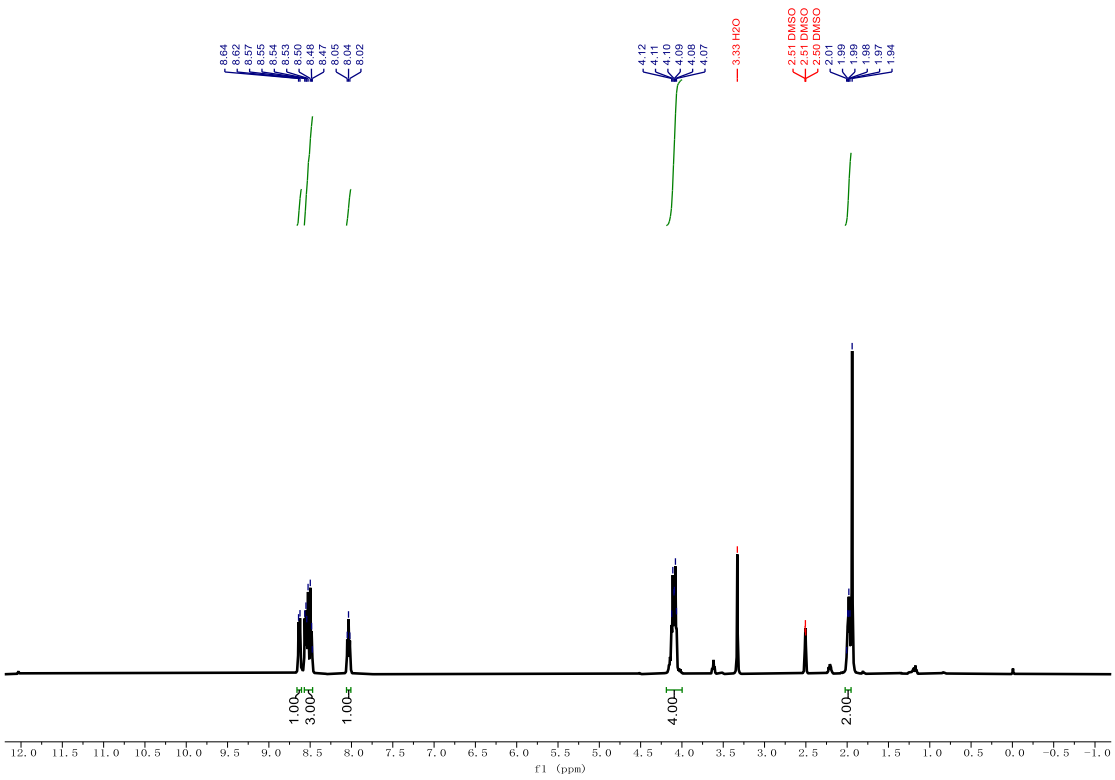


Fig. S10. ^{1}H NMR spectrum of Compound **2**.

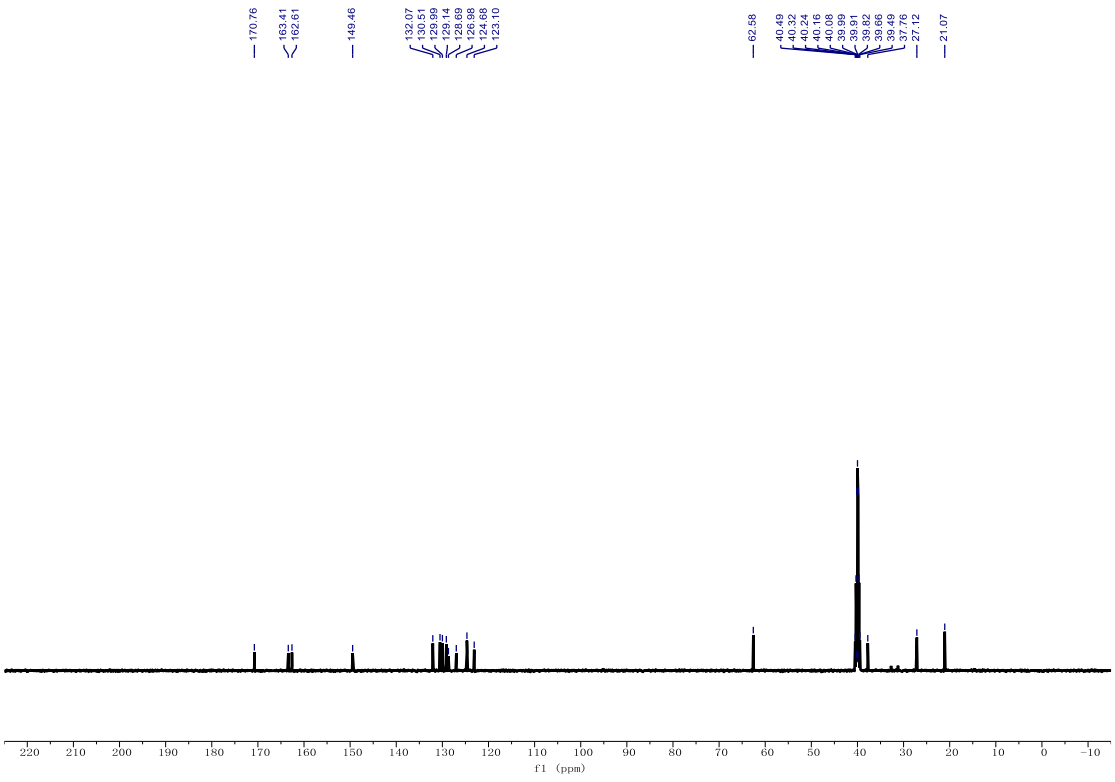
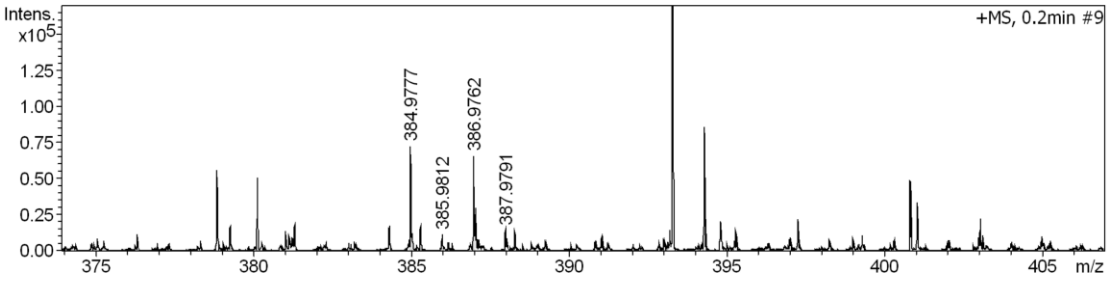


Fig. S11. ^{13}C NMR spectrum of Compound **2**.



#	m/z	Res.	S/N	I	I %	FWHM
1	384.9777	17520	75.2	72016	100.0	0.0220
2	385.9812	14370	11.6	11184	15.5	0.0269
3	386.9762	17159	67.8	65408	90.8	0.0226
4	387.9791	20845	14.5	14028	19.5	0.0186

Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma		Score	rdb	e¯ Conf	N-Rule
384.9777	1	C15H11BrN2NaO4	384.9794	4.6	42.2	2	58.24	10.5	even	ok

Fig. S12. HR-MS spectrum of Compound **2**.

Synthesis of Compound **3**: Compound **2** (363 mg, 1.0 mmol) and NaI (450 mg, 4 mmol) were dissolved in tetrahydrofuran (50 mL). The reaction mixture was stirred and refluxed for 12 h under a nitrogen atmosphere, the resulting mixture was evaporated to dryness. The solvent was evaporated under reduced pressure to get Compound **3** as yellow solid. ^{1}H NMR (500 MHz, 298 K, DMSO-d_6) δ 8.70-8.67 (m, 1H), 8.63- 8.58 (m, 2H), 8.56-8.54 (m, 1H), 8.11-8.07 (m, 1H), 4.15-4.06 (m, 4H), 2.01-1.96 (m, 2H). ^{3}C NMR (125 MHz, 298 K, DMSO-d_6) δ 163.27, 162.76, 146.08, 136.61, 134.44, 134.24, 134.12, 131.15, 130.15, 129.97, 129.88, 129.61, 124.43, 123.13, 123.03, 122.99, 41.36, 40.88, 40.48, 40.31, 40.24, 40.14, 39.98, 39.90, 39.81, 39.72, 39.64, 39.47, 31.96, 5.01. R-MS (ESI): m/z calcd for $C_{15}H_{11}IN_2NaO_4$ $[M+Na]^+$: 432.9656; found: 432.9650.

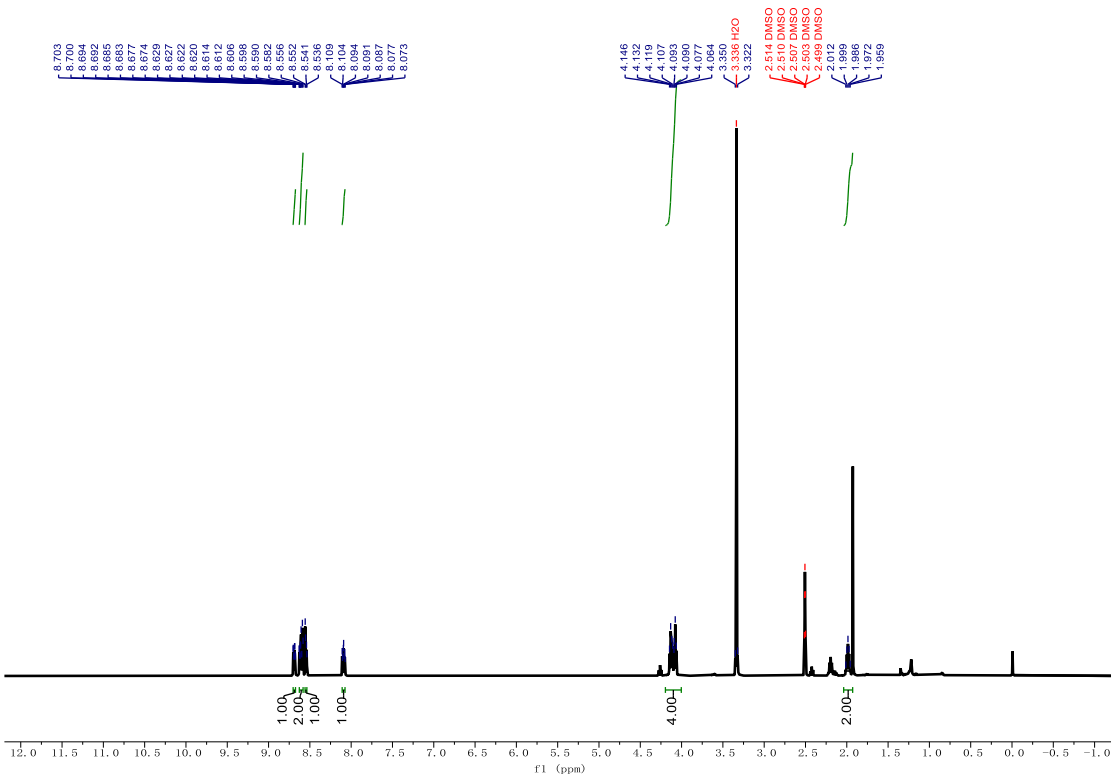


Fig. S13. ^{1}H NMR spectrum of Compound **3**.

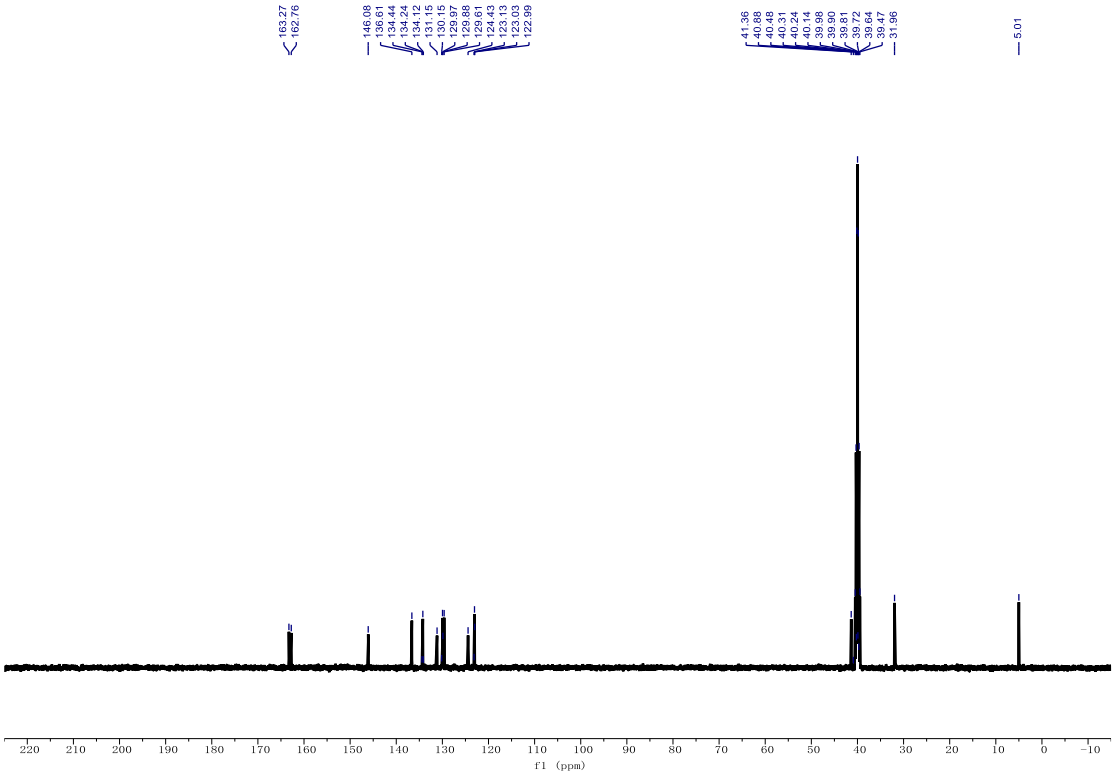
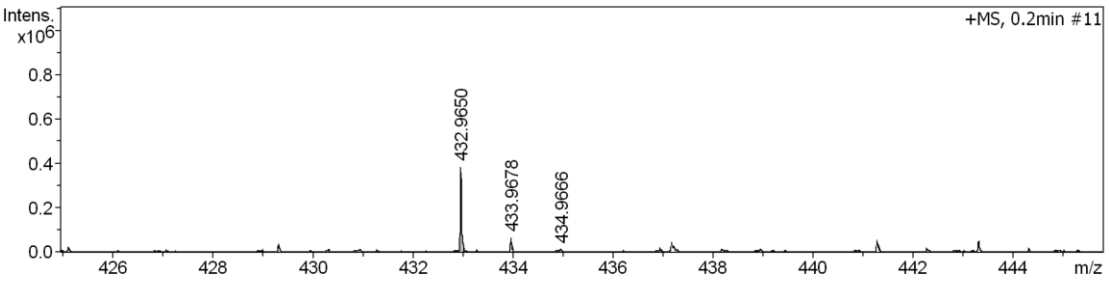


Fig. S14. ^{13}C NMR spectrum of Compound **3**.



#	m/z	Res.	S/N	I	I %	FWHM
1	432.9650	21043	265.0	384300	100.0	0.0206
2	433.9678	15381	45.0	65292	17.0	0.0282
3	434.9666	12806	8.5	12360	3.2	0.0340

Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma		Score	rdb	e⁻ Conf	N-Rule
432.9650	1	C15H11IN2NaO4	432.9656	1.3	5.9	1	100.00	10.5	even	ok

Fig. S15. HR-MS spectrum of Compound **3**.

Synthesis of Compound **4**: Compound 3 (1.0 g, 2.5 mmol) and 4-methylpyridine (279 mg, 3.0 mmol) were dissolved in acetonitrile (20 mL). The reaction mixture was stirred and refluxed for 8 h under a nitrogen atmosphere, the resulting mixture was evaporated to dryness. After the solvent was evaporated under reduced pressure, the crude product was purified by silica gel column chromatography using DCM/MeOH (200:1, v/v) as eluent to get Compound **4** as brown solid. ^{1}H NMR (500 MHz, 298 K, DMSO-d_6) δ 9.50 (s, 1H), 8.95-8.94 (m, 3H), 8.81-8.79 (d, J = 10.0 Hz, 1H), 8.69-8.67 (d, J = 10.0 Hz, 1H), 8.10-8.07 (m, 1H), 8.00-7.99 (d, J = 5.0 Hz, 2H), 4.67-4.63 (m, 2H), 4.16-4.14 (m, 2H), 2.60 (s, 1H), 2.37-2.31 (m, 2H). ^{13}C NMR (125 MHz, 298 K, DMSO-d_6) δ 163.58, 163.10, 159.32, 146.25, 144.26, 136.81, 134.38, 131.31, 130.18, 130.07, 129.78, 128.93, 128.79, 124.63, 123.20, 58.43, 40.49, 40.33, 40.25, 40.16, 39.99, 39.92, 39.83, 39.73, 39.66, 39.49, 37.41, 30.07, 21.86. HR-MS (ESI): m/z calcd for $C_{21}H_{18}N_3O_4$ $[M]^+$: 376.1292; found: 376.1294.

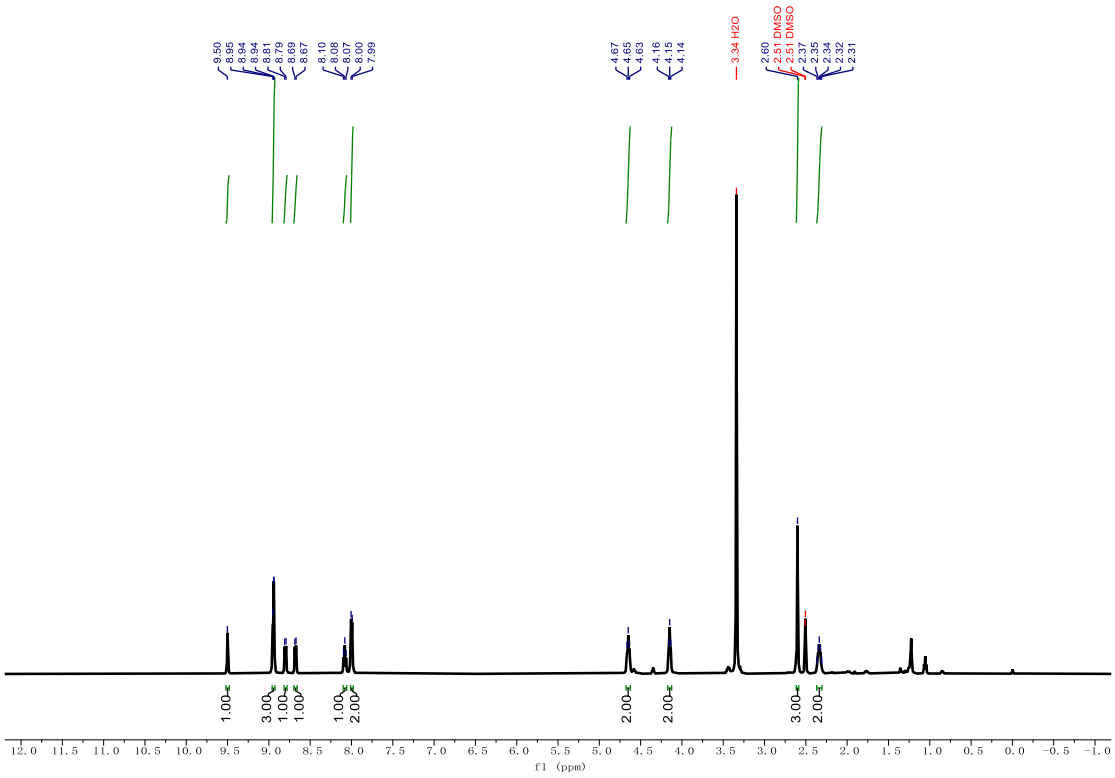


Fig. S16. ^{1}H NMR spectrum of Compound **4**.

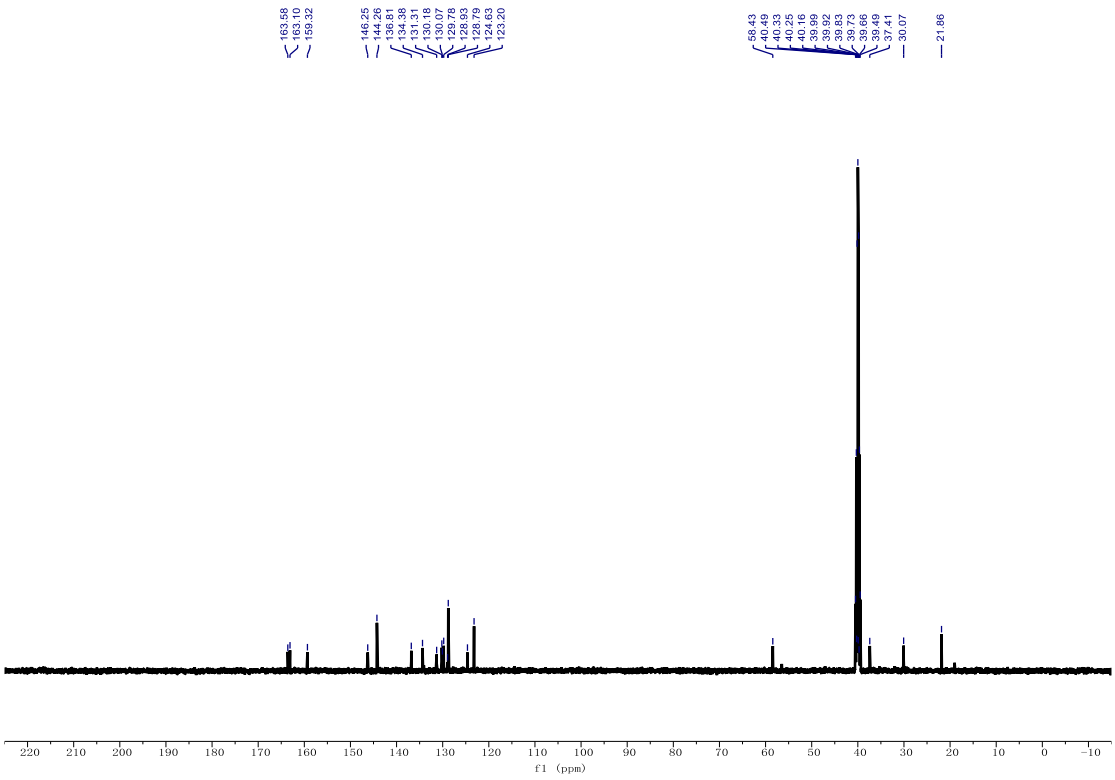
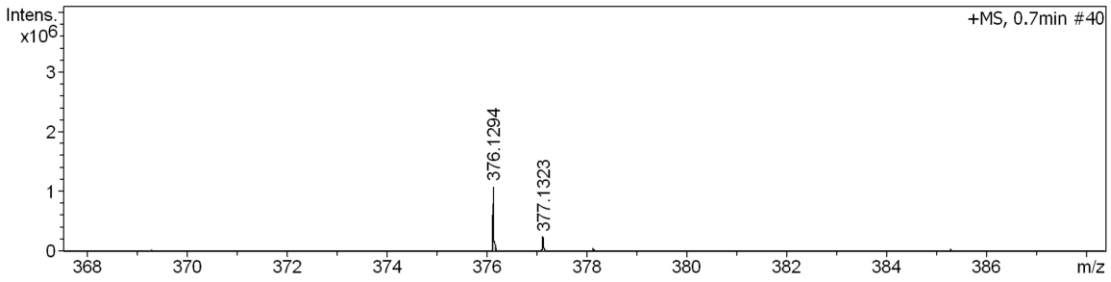


Fig. S17. ^{13}C NMR spectrum of Compound **4**.



#	m/z	Res.	S/N	I	I %	FWHM
1	376.1294	24327	1362.4	1082584	100.0	0.0155
2	377.1323	16876	293.4	233356	21.6	0.0223
3	378.1346	17917	49.9	39688	3.7	0.0211

Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma		Score	rdb	e¯ Conf	N-Rule
376.1294	1	C21H18N3O4	376.1292	-0.5	13.1	1	100.00	14.5	even	ok

Fig. S18. HR-MS spectrum of Compound **4**.

Synthesis of Compound **IFRP**: Compound 4 (753 mg, 2 mmol) and 7-(diethylamino)-2-oxochromene-3-carbaldehyde (589 mg, 2.4 mmol) were dissolved in ethanol (50 mL). The reaction mixture was stirred and refluxed for 8 h under a nitrogen atmosphere, the resulting mixture was evaporated to dryness. After the solvent was evaporated under reduced pressure, the crude product was purified by silica gel column chromatography using DCM/MeOH (100:1, v/v) as eluent to get compound **IFRP** as purple solid. ^{1}H NMR (500 MHz, 298 K, DMSO-d_6) δ 8.87 (s, 2H), 8.67-8.55 (m, 3H), 8.33 (s, 1H), 8.17-8.08 (m, 4H), 7.77-7.58 (m, 2H), 7.50-7.49 (d, J = 9.0 Hz, 1H), 6.77-6.75 (d, J = 9.0 Hz, 1H), 6.54 (s, 1H), 4.58-4.57 (d, J = 7.5 Hz, 2H), 4.14 (s, 2H), 3.47-3.46 (d, J = 6.5 Hz, 4H), 2.34 (s, 2H), 1.15-1.14 (d, J = 7.0 Hz, 6H). ^{13}C NMR (125 MHz, 298 K, DMSO-d_6)δ 163.48, 162.99, 159.95, 159.32, 156.71, 156.36, 153.82, 152.41, 146.15, 146.12, 145.76, 144.45, 144.25, 137.38, 136.78, 134.35, 131.25, 131.16, 130.13, 129.95, 129.74, 128.79, 124.47, 124.18, 123.70, 123.13, 123.05, 122.91, 114.00, 110.48,

108.77, 96.60, 44.85, 40.50, 40.34, 40.17, 40.00, 39.84, 39.67, 39.50, 37.48, 37.39, 30.07, 29.93, 21.87, 12.86. HR-MS (ESI): m/z calcd for $C_{35}H_{31}N_4O_6$ $[M]^+$: 603.2238; found: 603.2241.

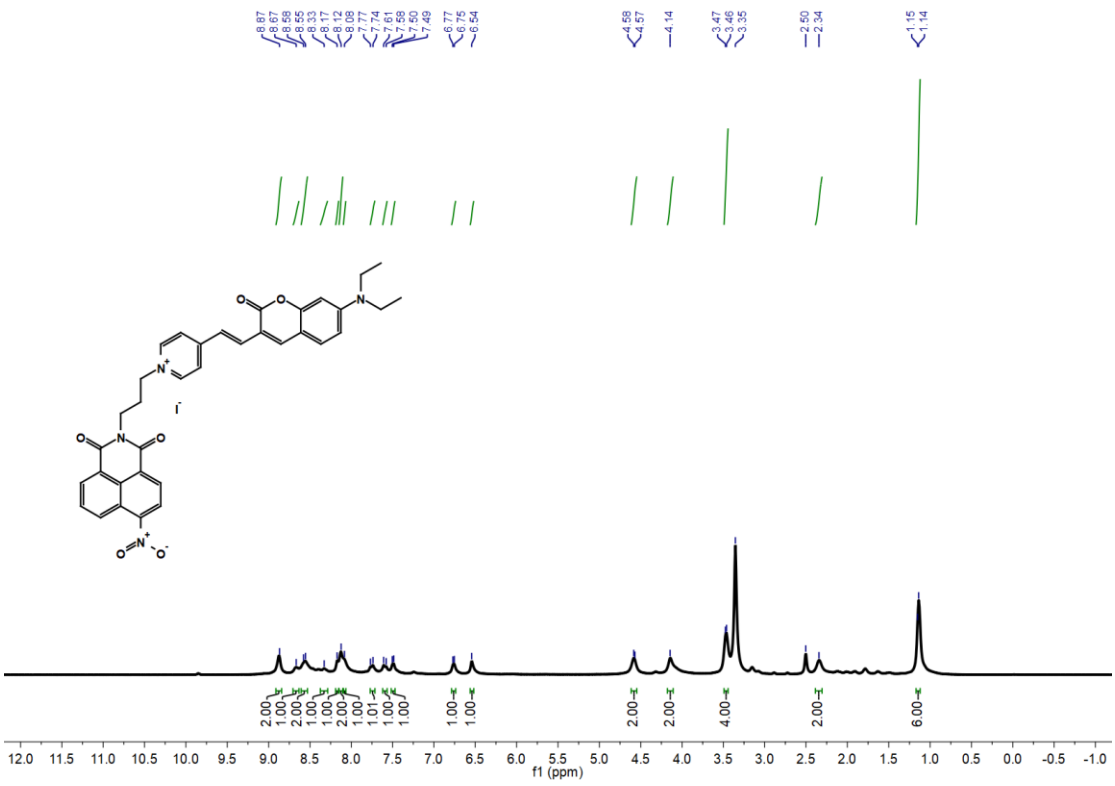


Fig. S19. ^{1}H NMR spectrum of **IFRP**.

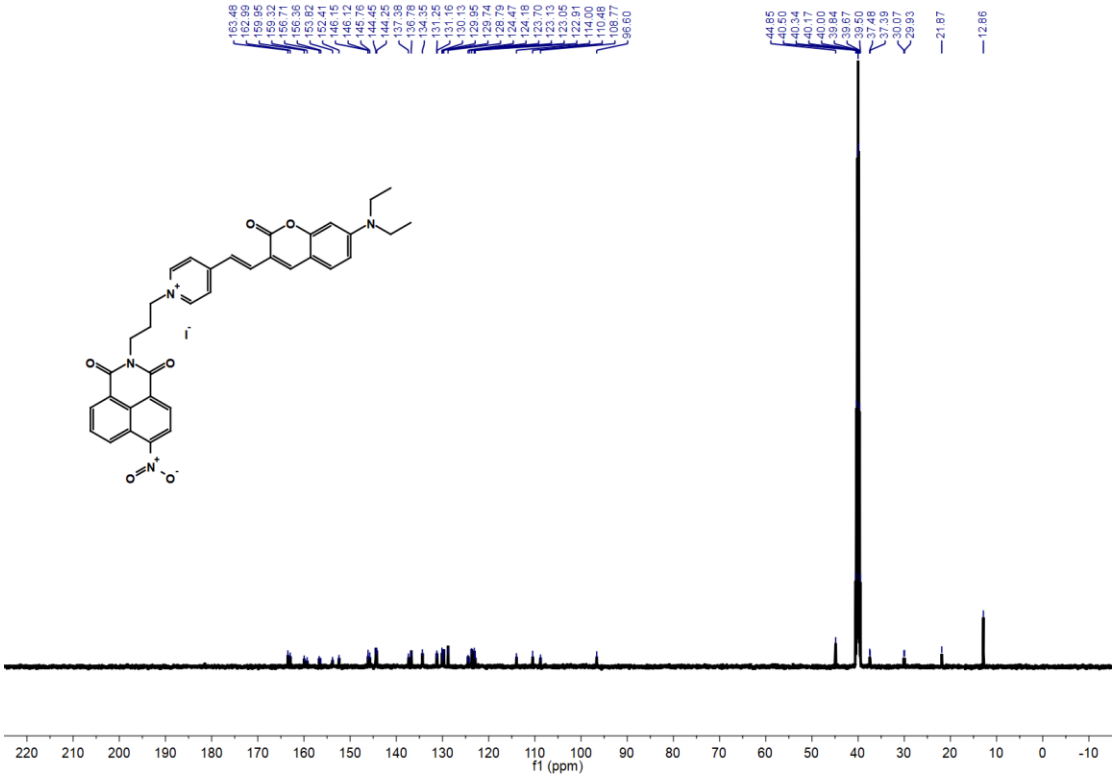
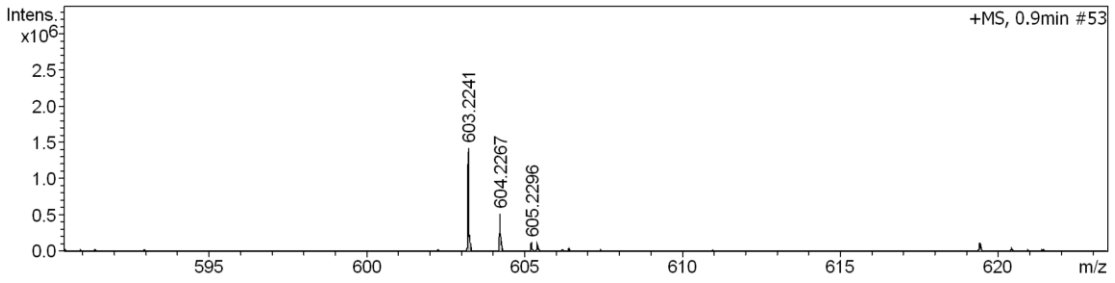


Fig. S20. ^{13}C NMR spectrum of **IFRP**.



#	m/z	Res.	S/N	I	I %	FWHM
1	603.2241	24702	1623.2	1419200	100.0	0.0244
2	604.2267	19920	587.8	514180	36.2	0.0303
3	605.2296	16440	132.0	115508	8.1	0.0368

Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma		Score	rdb	e¯ Conf	N-Rule
603.2241	1	C35H31N4O6	603.2238	-0.4	18.8	2	100.00	22.5	even	ok

Fig. S21. HR-MS spectrum of **IFRP**.

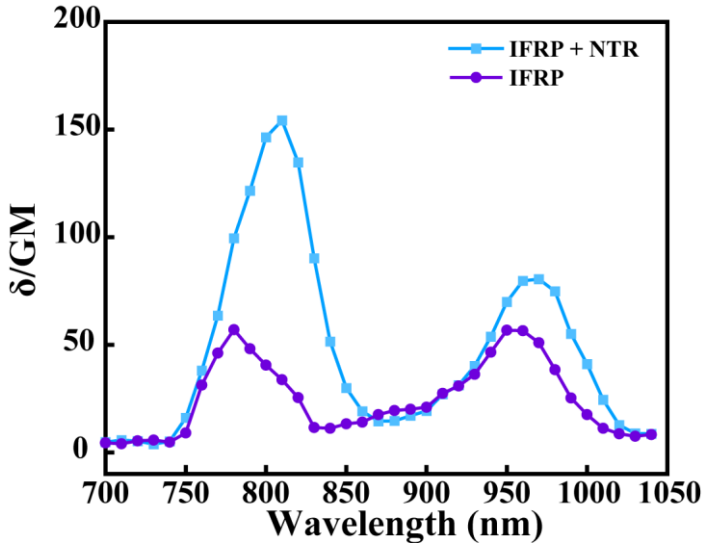


Fig. S22. Two-photon fluorescence spectra of probe **IFRP** before and after addition of NTR.

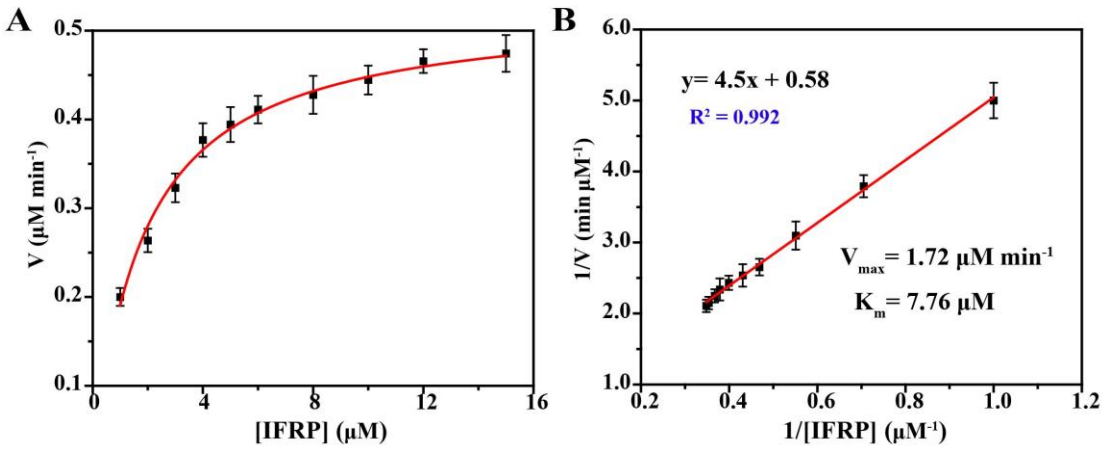
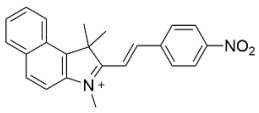
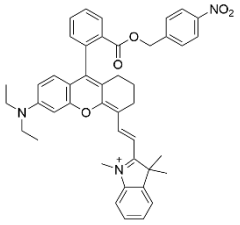
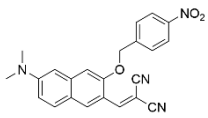
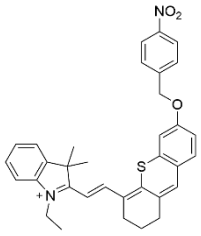
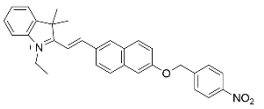
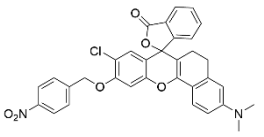
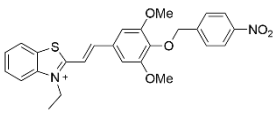
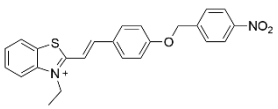
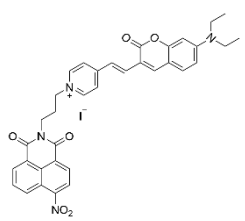


Fig. S23. (A) Michaelis-Menten plot for enzymatic reaction of 10 mg/mL NTR with **IFPR** at various concentrations (1, 2, 3, 4, 5, 6, 8, 10, 15 μM). (B) Lineweaver-Burk plot of the enzyme-catalyzed reaction.

Table S1. Comparison of the sensing performances of our probe to the reported fluorescent probes for NTR.

Structure of probes	λ_{ex} / λ_{em} (nm)	Linear Range (μg mL^{-1})	LOD (ng mL^{-1})	Km (μM)	Ref.
	495 / 595	0 - 10	2.2	12.67	S1
	700 / 740	0 - 2	43	25.9	S2
	760 / 595	0 - 10	0.142	71.77	S3
	670 / 730	0 - 5	2.5	72.6	S4
	450 / 580	0 - 20	26	46.82	S5
	561 / 624	0.05 – 0.3	0.79	35.07	S6
	548 / 603	0 - 50	562	-	S7
	490 / 555	0 - 20	153	-	S7
	800 / 640, 515	1.0 – 9.0	250	7.76	This work

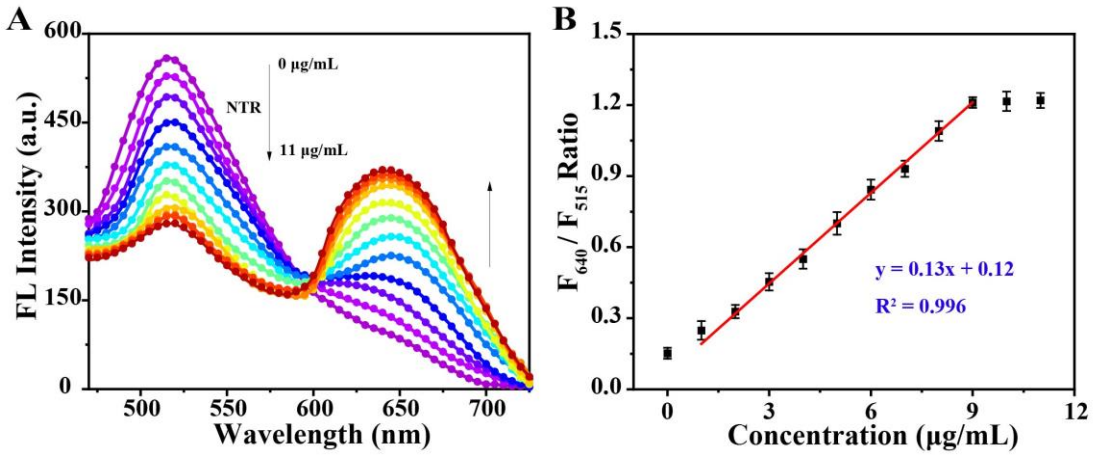


Fig. S24. (A) Two-photon fluorescence responses of **IFRP** to different concentrations of NTR (0-11 μg mL^{-1}) in cell lysates. (B) Corresponding linear relationship of the fluorescence intensity ratio F_{640}/F_{515} (F_{515}: 470-550 nm, F_{640}: 600-700 nm) versus the concentration of NTR.

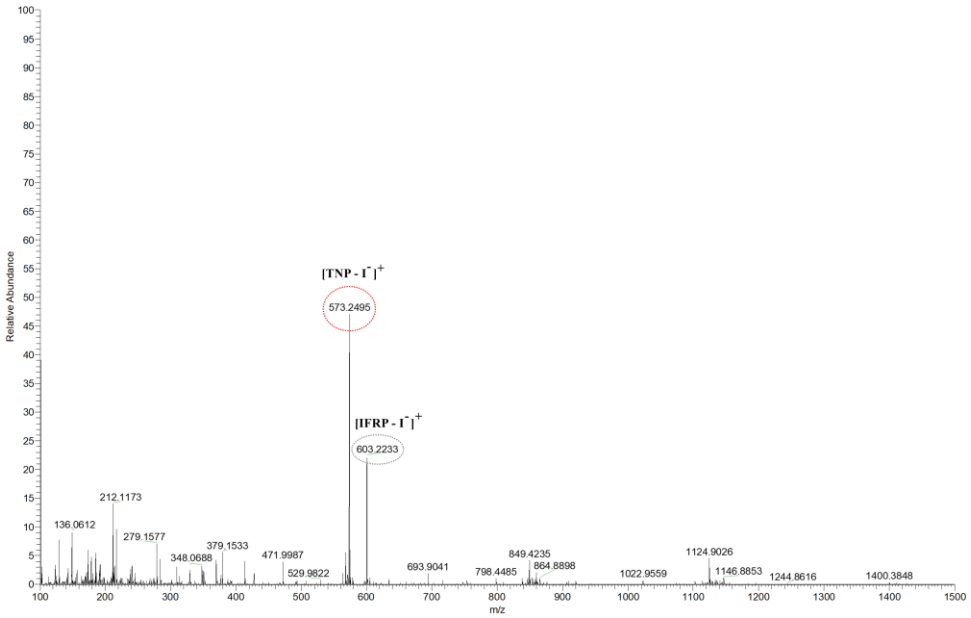


Fig. S25. HR-MS of probe **IFRP** after interacted with NTR.

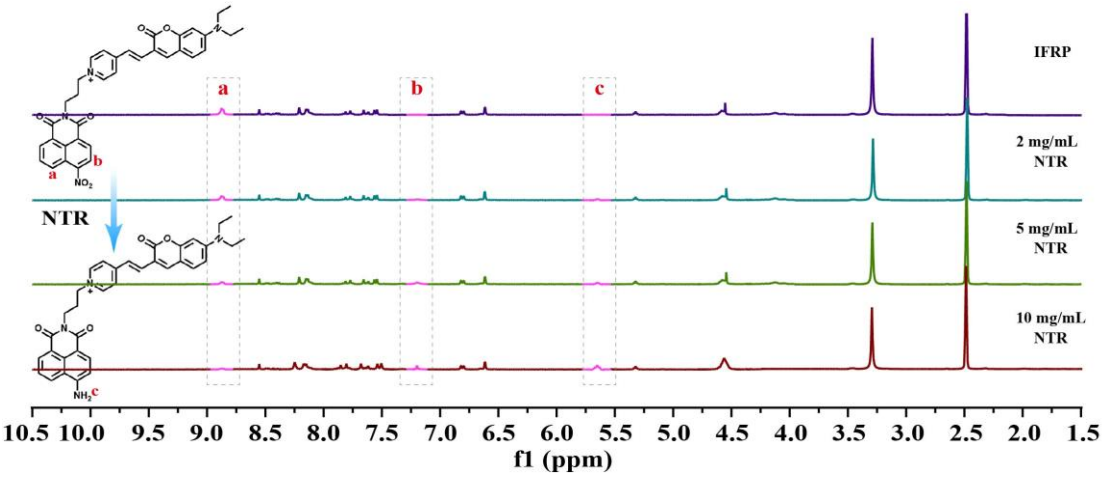


Fig. S26. ^{1}H NMR spectrum of **IFRP** (5 mM) upon addition of NTR (0-10 mg/mL).

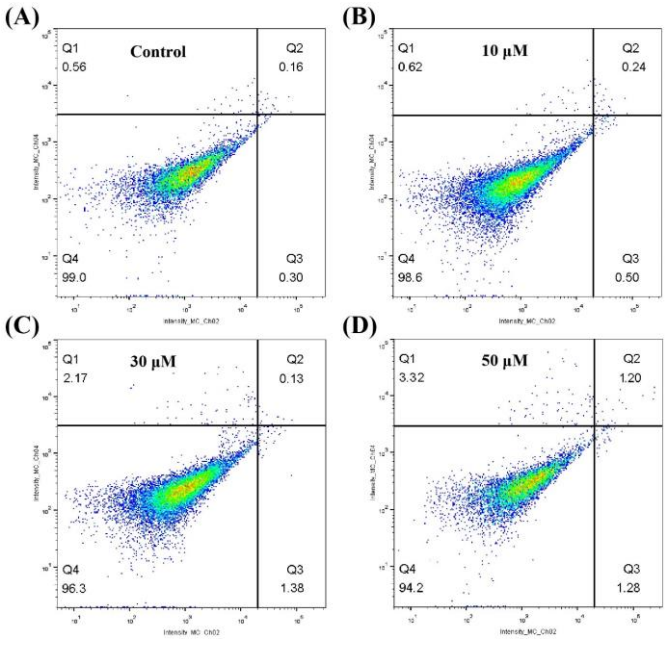


Fig. S27. The apoptosis assay of neurons incubated with probe **IFRP** under different concentrations (A) 0 μM, (B) 10 μM, (C) 30 μM, and (D) 50 μM for 24 h. Q1, Q2, Q3, and Q4 represent the regions of dead neurons, late apoptotic neurons, early apoptotic neurons, and normal neurons, respectively.

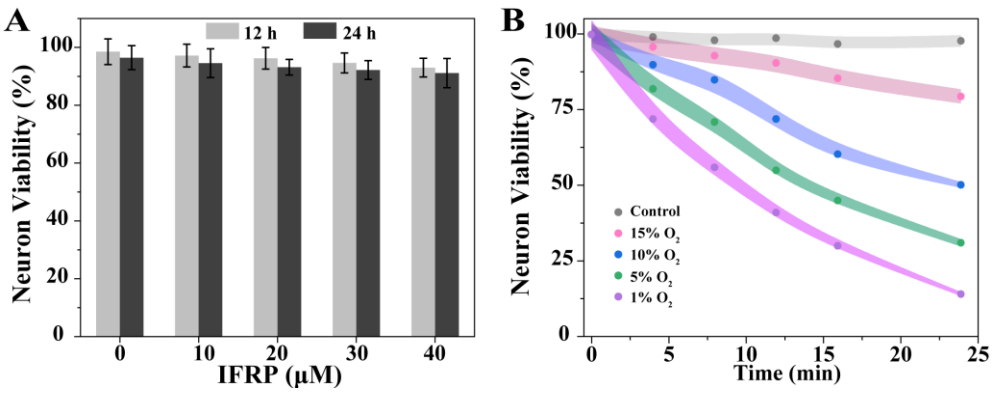


Fig. S28. (A) The MTT assay for neurons upon incubation with **IFRP** at different concentrations (0, 10, 20, 30, and 40 μM) after 12 h and 24 h, respectively. (B) Summarized data of neuron viability stimulated by various concentrations of O_2 (20% O_2, 15% O_2, 10% O_2, 5% O_2 and 1% O_2) stimulation for different times. Error bars, n = 10, S.D.

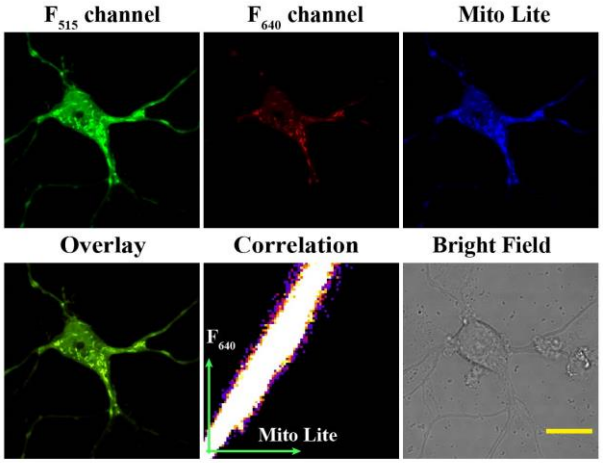


Fig. S29. Fluorescence images of neurons co-incubated with **IFRP** and MitoLite blue FX490. The images of **IFRP** were obtained under 800 nm excitation and 470-550 nm collection for the F_{515} channel and 600-700 nm for the F_{640} channel. MitoLite blue FX490 was excited using a 405 nm laser, and emission signals were collected within the 420-490 nm wavelength range.

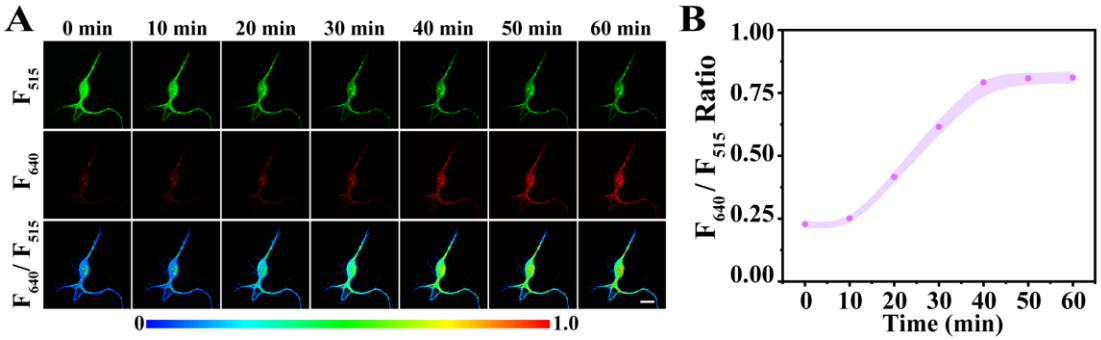


Fig. S30. (A) Confocal microscopy imaging of neurons after hypoxic stimulation at different time (0, 10, 20, 30, 40, 50 and 60 min). (B) Quantification of the intensity ratio (F_{640}/F_{515}) after hypoxic stimulation at different times. Scale bar: 30 μm.

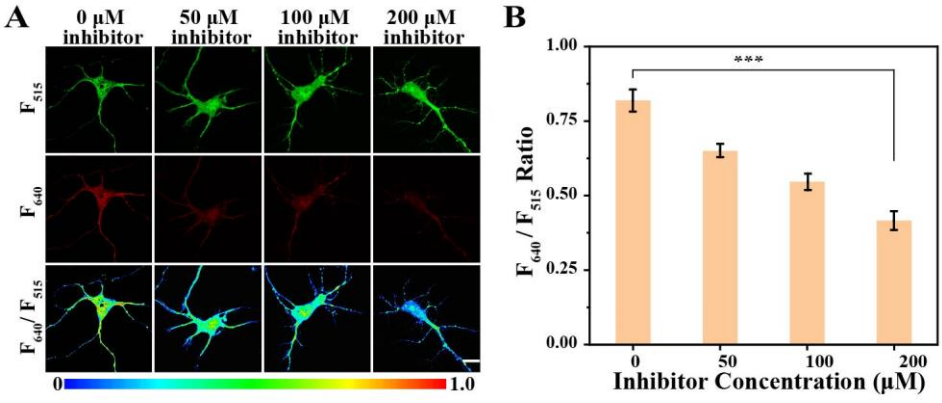


Fig. S31. Confocal microscopy imaging of **IFRP** (5 μM) in neurons under hypoxia (1% O_2) stimulation with the presence of different concentrations dicoumarin (0, 50, 100, 200 μM). Scale bar: 30 μm.

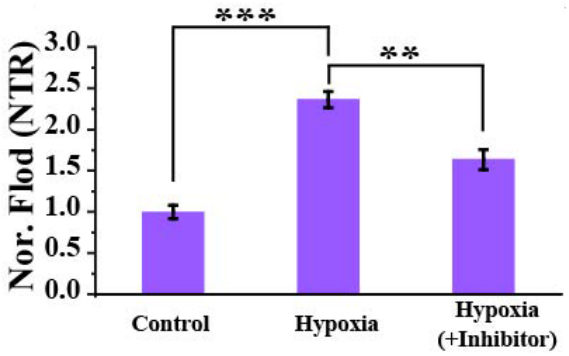


Fig. S32. The relative mRNA of NTR in neurons under normoxia or hypoxic condition with or without the presence of dicoumarin (n = 10; **: $p < 0.01$; ***: $p < 0.001$).

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