Supporting Information

A novel internal standard ratio fluorescent probe for nitroreductase detection in cells

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1. Fluorescent probes for NTR

Probe	ratio	λ_{em} (nm)	DL (ng/mL)	Response time (min)	Reference
T-TPE-NO_2	NO	626	46.3	40	1
Pro-CN	NO	560	4.4	25	2
PC-NTR	NO	635	13.8	30	3
NORP	Yes	455 520	30	/	4
ETH1-NO_2	NO	603	562	/	5
Ox-NTR	NO	683	90	/	6
CS-CN-NO	Yes	530 700	70	16	**This Work**

2.Supplementary methods

Cell image

The cells were plated at 1×10^5 cells/mL suspension in a Glass bottomed petri dish and allowed to culture overnight with (normoxia) or without (hypoxia) $CoCl_2$ (100μM). The cells were incubated with CS-CN-NO for 30 min before imaging and then washed with PBS three times. Fluorescent cell images were obtained by confocal imaging microscopy. Unless otherwise specified, the probe concentration is always 20 μM.

Instruments

UV−Vis absorption spectra were recorded using a PerkinElmer Lambda 650S UV/Vis spectrometer. Fluorescence spectra were recorded using a PerkinElmer LS55 fluorescence spectrometer. ^{1}H NMR and ^{13}C NMR spectra were acquired using a Bruker AvanceAVII-500 MHz spectrometer. High-resolution mass spectra were acquired with a Waters Xevo G2-XS QTOF mass spectrometer. All live cell images were obtained using a Nikon A1 confocal microscope.

General procedure for fluorescence detection

Stock solutions of interference ions were prepared in ultra-filter deionized water with the concentration of 1.0×10^{-2} M and then diluted to a desired concentration. The concentrations of interfering ions and enzymes were 10 mM and 5 U/mL, respectively. Probes stock solution was prepared at the concentration of 1.0×10^{-3} M in DMSO and

then diluted to 2.0 × 10^{-5} M for titration experiments. NTR was prepared at the concentration of 200 μg/mL in PBS. NADH was prepared at the concentration of 1.0 × 10^{-2} M in PBS and then diluted to 1.0 × 10^{-4} M for titration experiments. UV-vis and fluorescence titration experiments were operated in PBS/CH_3CN = 3:7 (10 mM pH 7.4), (λ_{ex} = 480 nm, excitation slit =15.0 nm, emission slit = 15.0 nm). Because the enzyme requires a milder and more stable environment in the organism, in order to better detect the activity of NTR, all experiments were carried out at 37°C.

Determination of detecting limits

The detecting limits (DL) were calculated according to Eq.

$$DL = 3.29\sigma/k$$

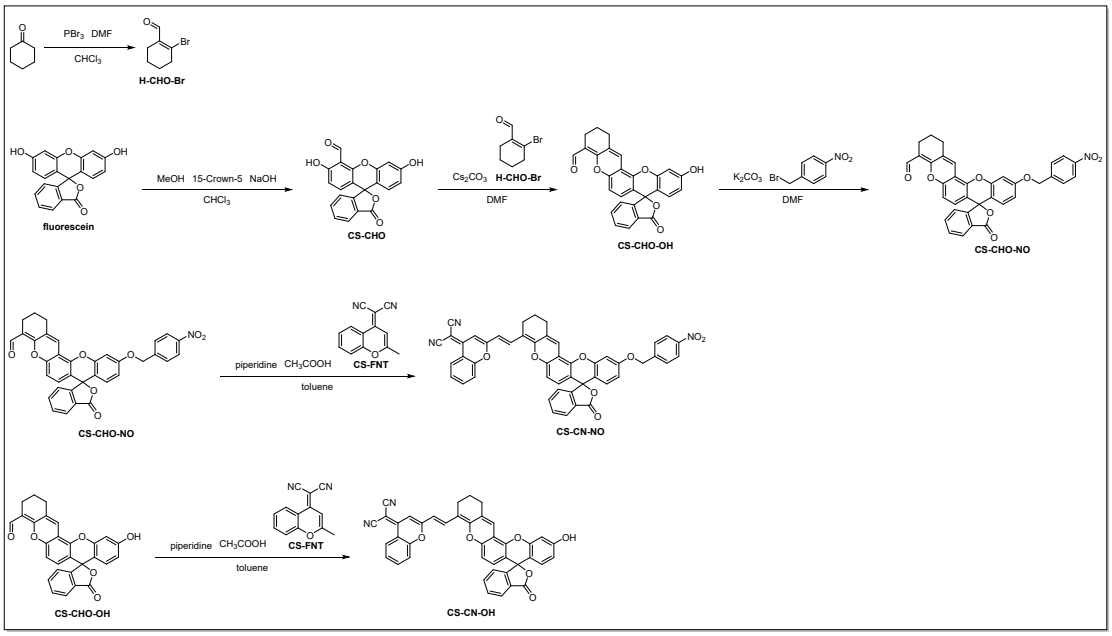
Where σ is the standard derivation of blank solution and k is the slope of calibration curve.

MTT assay for the cell cytotoxicity.

Cell cytotoxicity was evaluated by MTT assay. Cells were cultivated in a 96-well plate until 70% confluence, and incubated with different concentrations of CS-CN-NO(0-30 μM) or $CoCl_2$ (0-100 μM) for 24 h. Then 20 μL 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL) was added for 4 h at 37°C. After MTT was washed, 110 μL DMSO was added. Absorbance was measured at 570 nm with a multi-function microplate reader. All experiments were repeated 3 times, and the data were presented as the percentage of control cells.

3.Supplementary Schemes

Syntheses of CS-CN-NO



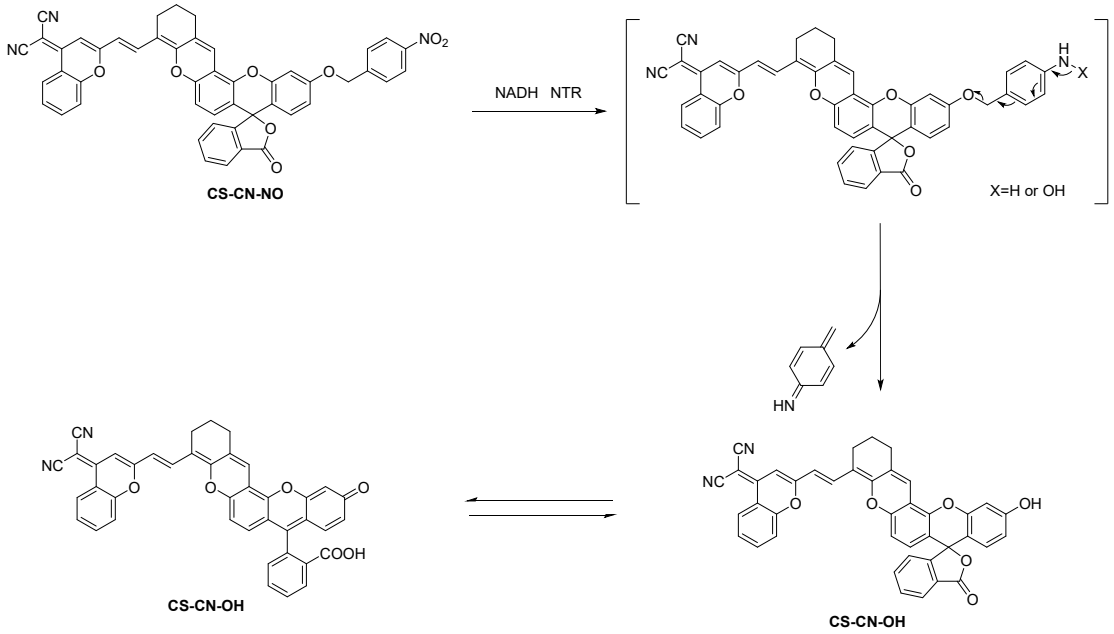
Scheme. S1. Reaction scheme for probe and fluorophore synthesis.

Compound H-CHO-Br, CS-CHO, CS-CHO-OH, CS-CN-OH, CS-FNT was synthesized by literature method[7].

CS-CHO-NO: Compound CS-CHO-OH (301 mg, 0.66 mmol), K_2CO_3(276 mg, 2mmol) and 4-nitrobenzyl bromide (173 mg, 0.80mmol) were dissolved in DMF (8 mL). After stirring overnight at 40 ℃ and dark environment. After the reaction was completed, it was extracted by DCM. After extraction, dried on Na_2SO_4 and solvent was removed by reducing pressure. The product was purified by silica gel column chromatography (DCM/MeOH = 250:1, v/v) to give the pure compound as a light yellow solid (106 mg, 27%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.32 (s, 1H), 8.31 – 8.24 (m, 3H), 8.07 – 8.00 (m, 1H), 7.73 – 7.59 (m, 5H), 7.18 – 7.13 (m, 1H), 6.91 (d, J = 2.3 Hz, 1H), 6.83 – 6.66 (m, 4H), 5.23 (s, 2H), 2.70 (dd, J = 8.0, 5.0 Hz, 2H), 2.48 (t, J = 6.1 Hz, 2H), 1.78 (h, J = 5.8 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 187.85, 169.08, 159.90, 159.42, 152.96, 152.66, 151.87, 147.80, 146.41, 143.48, 135.28, 130.31, 130.10, 129.51, 128.75, 127.60, 126.60, 125.33, 123.99, 123.80, 119.80, 113.91, 113.84, 112.59, 112.05, 111.55, 110.44, 102.10, 82.17, 68.98, 30.41, 21.50, 20.22.

CS-CN-NO: Compound CS-CHO-NO (80 mg, 0.14 mmol), Acetic acid (0.5 mL), piperidine (1mL) and Compound CS-FNT (44 mg, 0.21mmol) were dissolved in toluene (40 mL). After stirring overnight at 80 ℃ and dark environment. After the

reaction was completed, removed solvent by reducing pressure. The product was purified by silica gel column chromatography (DCM/MeOH = 250:1, v/v) to give the pure compound as a purple solid (11 mg, 10%). ^{1}H NMR (500 MHz, CDCl3) δ 8.83 (d, *J* = 8.2 Hz, 1H), 8.21 (d, *J* = 8.5 Hz, 2H), 8.04 – 7.95 (m, 2H), 7.67 – 7.50 (m, 5H), 7.45 (d, *J* = 8.4 Hz, 1H), 7.34 (t, *J* = 7.6 Hz, 1H), 7.10 (d, *J* = 7.5 Hz, 1H), 7.00 (s, 1H), 6.84 (s, 1H), 6.77 – 6.64 (m, 4H), 6.57 (d, *J* = 8.7 Hz, 1H), 6.10 (d, *J* = 15.4 Hz, 1H), 5.16 (s, 2H), 2.62 (t, *J* = 5.9 Hz, 2H), 2.46 (t, *J* = 5.7 Hz, 2H), 1.83 (p, *J* = 2.8 Hz, 2H). ^{13}C NMR (126 MHz, Pyr) δ 175.82, 169.20, 160.38, 159.24, 153.70, 152.98, 152.48, 152.35, 151.49, 147.70, 147.58, 146.06, 144.29, 139.38, 139.19, 134.39, 133.38, 131.79, 131.12, 130.27, 129.57, 128.46, 127.97, 126.87, 125.55, 125.28, 125.19, 124.84, 123.85,119.35, 118.73, 117.80, 116.45, 115.34, 112.36, 112.26, 111.63, 111.31, 105.94, 102.43, 82.37, 69.05, 64.40, 30.21, 24.52, 20.50.HRMS Calc for $C_{48}H_{29}N_3O_8$ $[M+H]^+$ Calcd for 776.2028, found 776.2032.



Scheme. S2. The proposed recognition mechanism of CS-CN-NO toward NTR.

4. Study on Spectral Properties

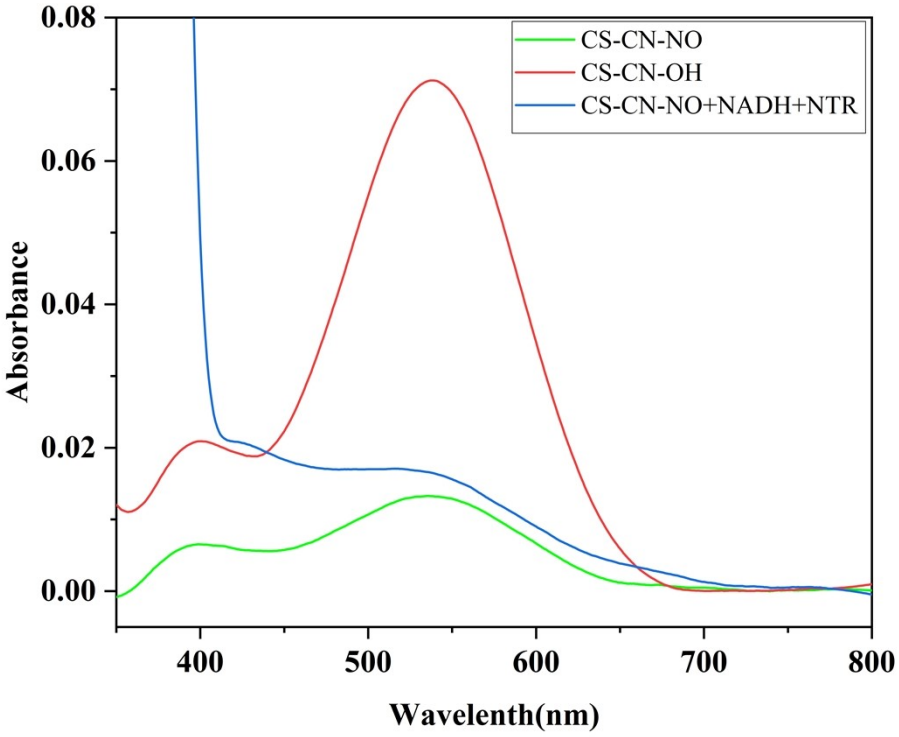


Fig. S1. UV-vis absorbance of CS-CN-NO(20 μM) , CS-CN-OH (20 μM) and CS-CN-NO (20 μM) with NADH (100 μM) and NTR (5 μg/mL).

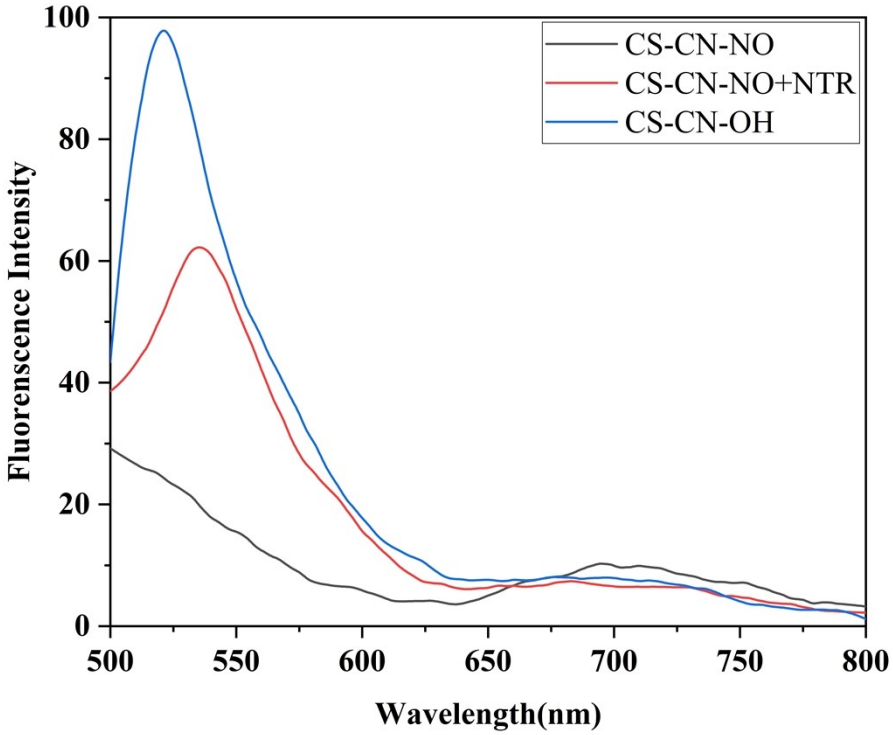


Fig. S2. Fluorescent intensity of CS-CN-NO (20 μM), CS-CN-OH (20 μM) and CS-CN-NO (20 μM)with NADH (100μM) and NTR (5μg/mL).

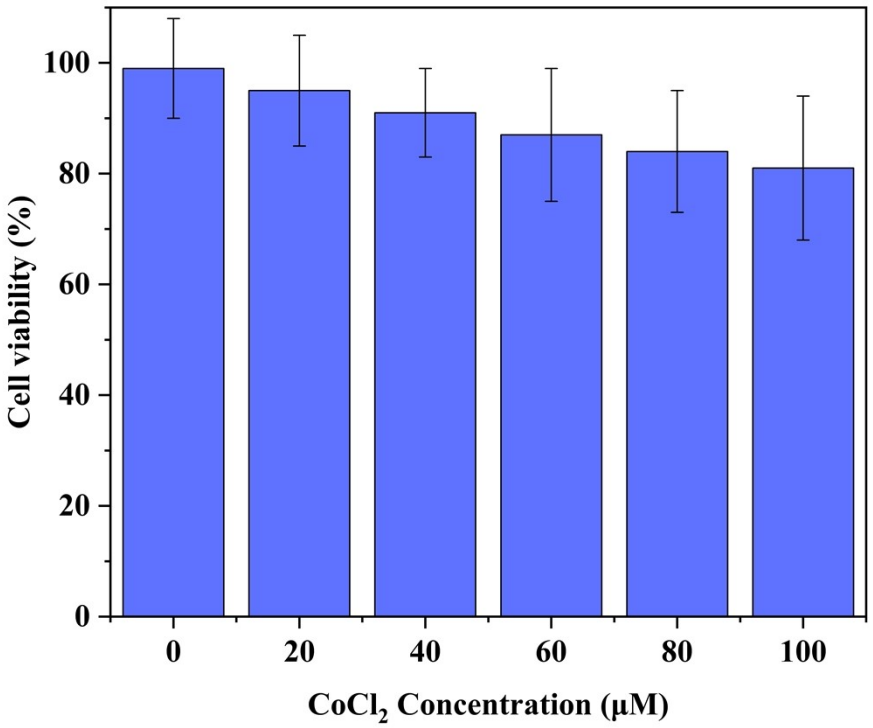


Fig. S3. MTT assay for the survival rate of HeLa cells treated with various concentrations of $CoCl_2$ (from 0 to 100 μM) for 24 h. Error bars represent the standard deviation (n = 3).

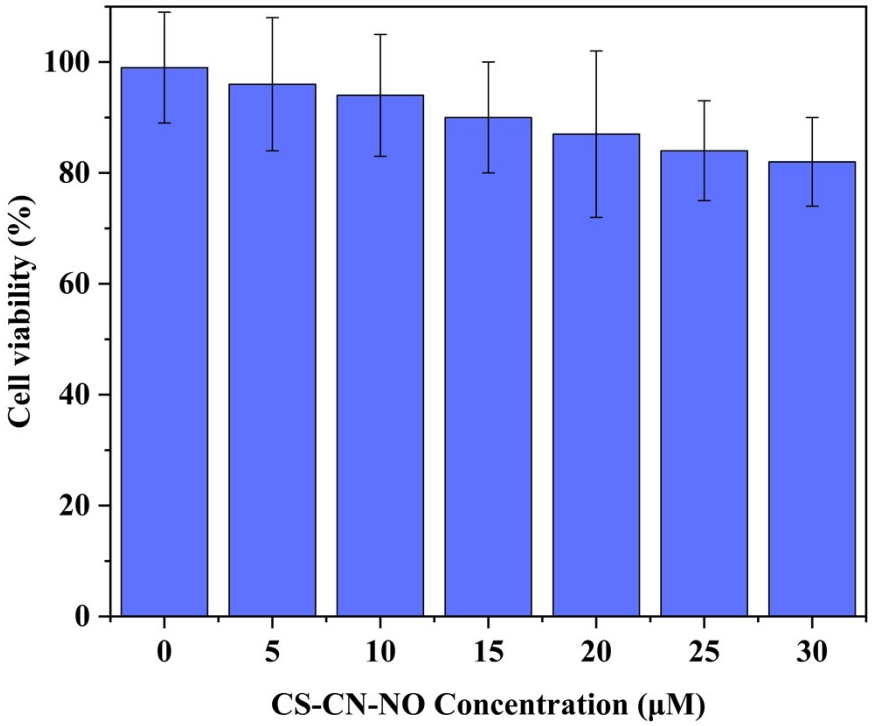


Fig. S4. MTT assay for the survival rate of HeLa cells treated with various concentrations of CS-CN-NO (from 0 to 30 μM) for 24 h. Error bars represent the standard deviation (n = 3).

5. HRMS,^{1}H-NMR and ^{13}C-NMR

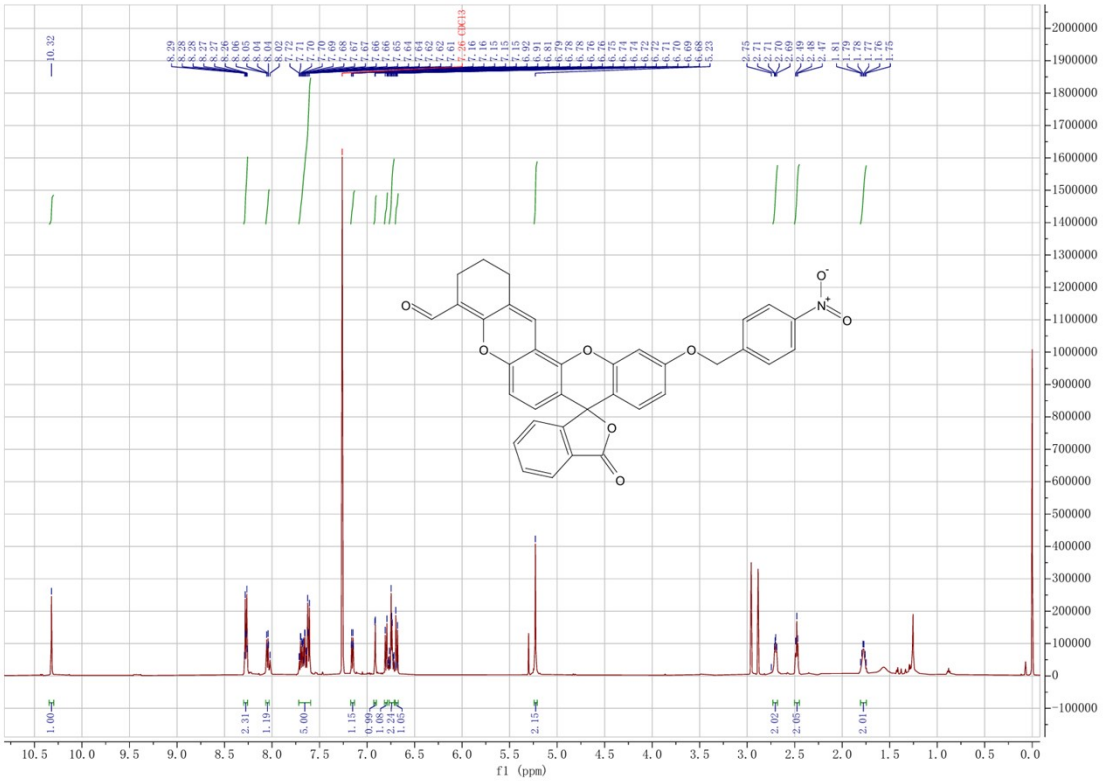


Fig. S5. ^{1}H-NMR spectrum of CS-CHO-NO in $CDCl_3$.

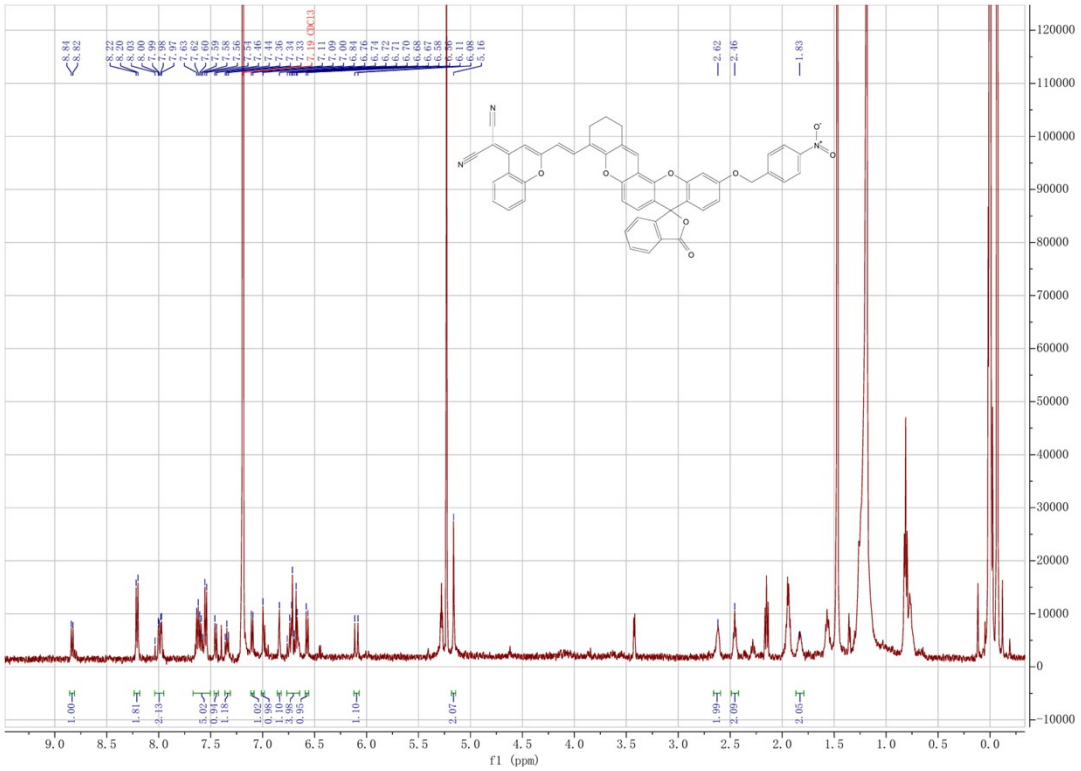


Fig. S6. ^{1}H NMR spectrum of CS-CN-NO in $CDCl_3$.

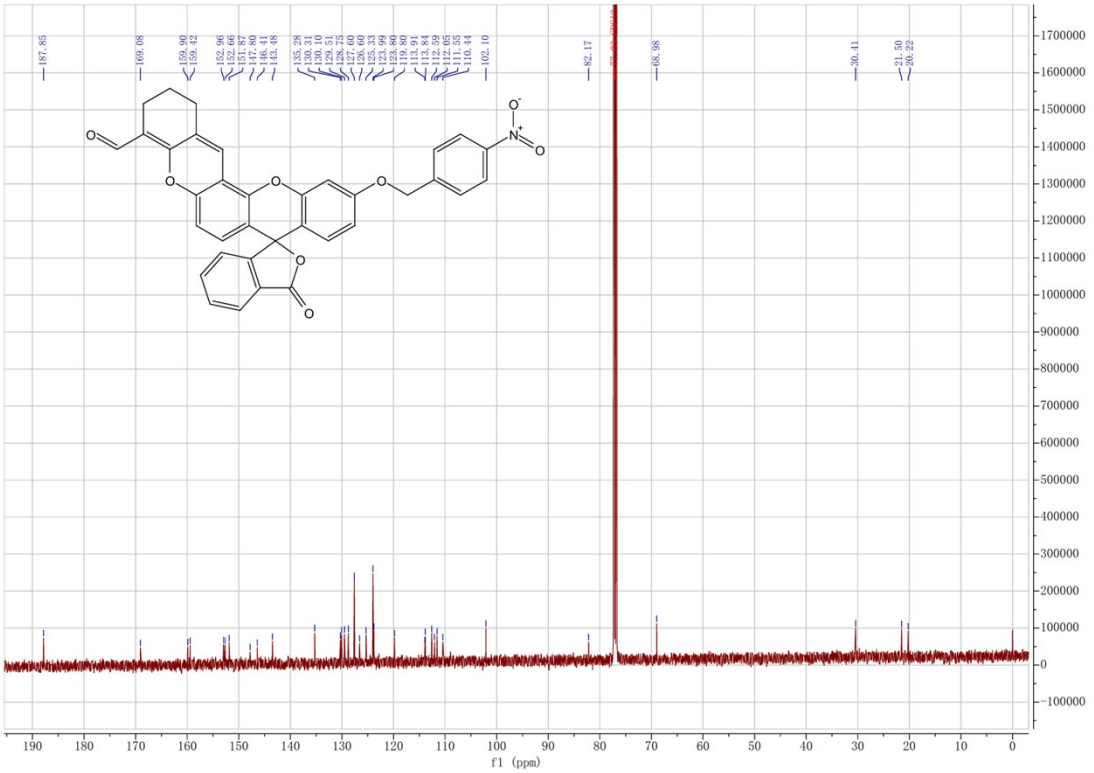


Fig. S7. ^{13}C-NMR spectrum of CS-CHO-NO in $CDCl_3$.

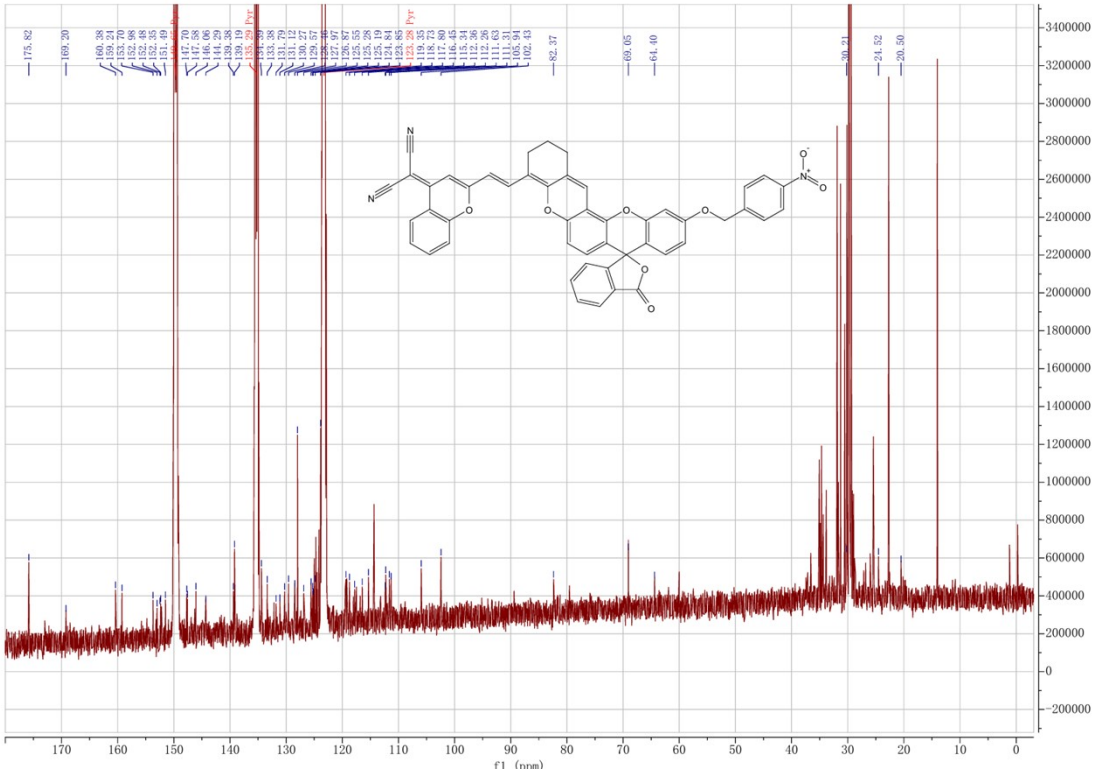


Fig. S8. ^{13}C NMR spectrum of CS-CN-NO in pyridine-d5.

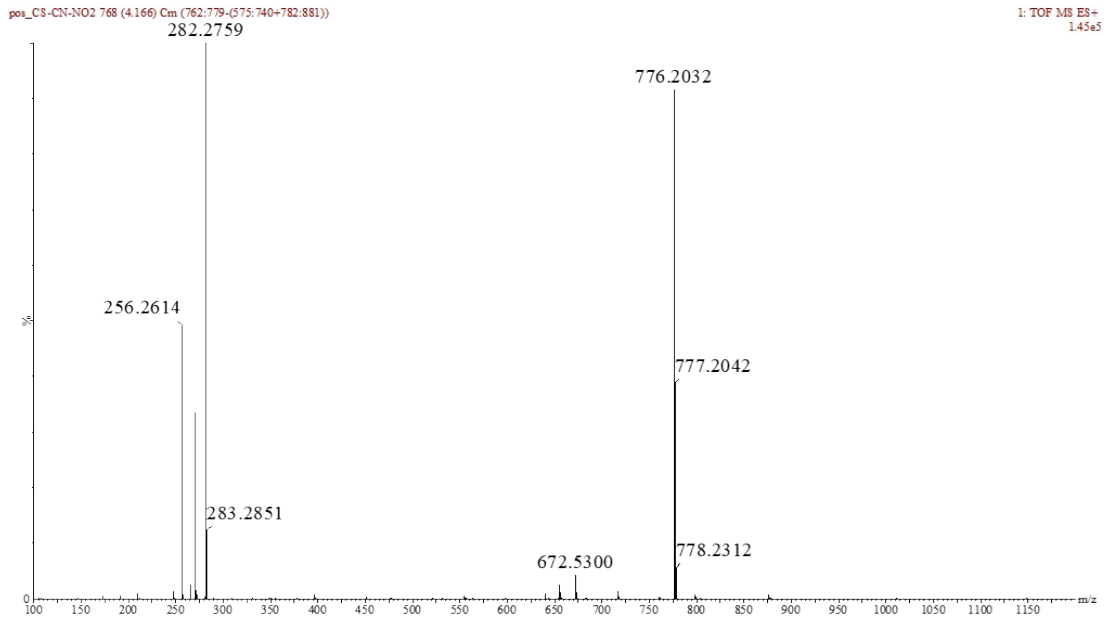


Fig. S9. HRMS spectrum of CS-CN-NO.

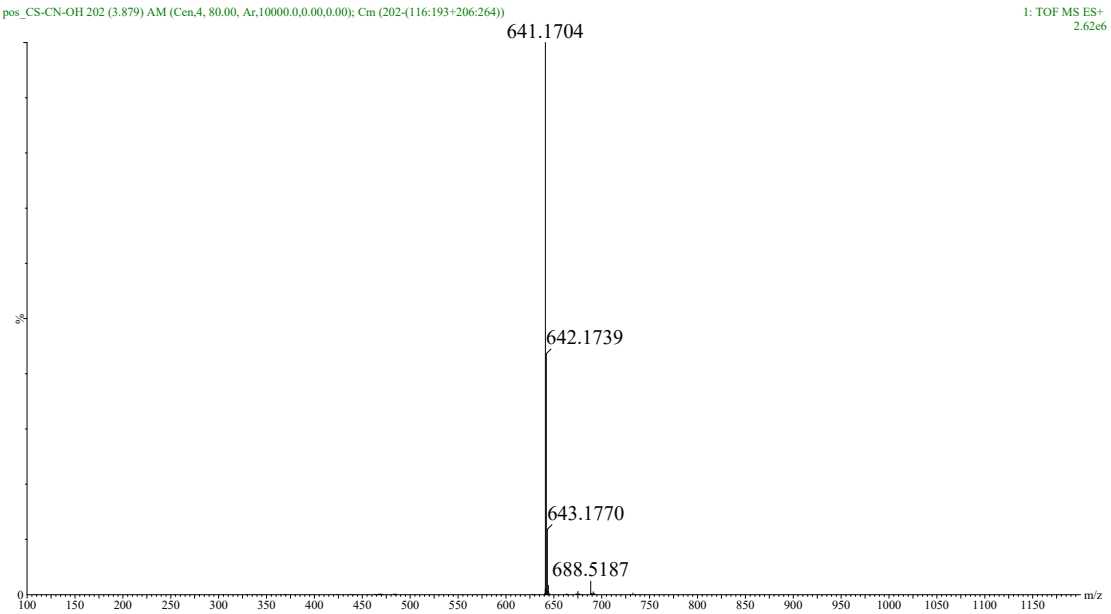


Fig. S10. HRMS spectrum of CS-CN-OH.

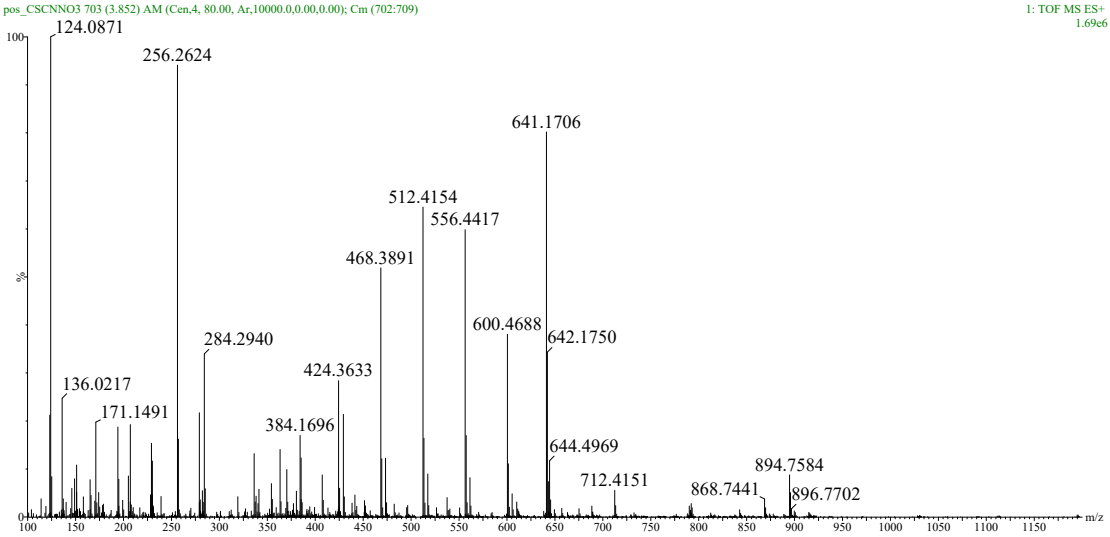


Fig. S11. HRMS spectrum of CS-CN-NO + NTR +NADH.

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