Supporting Information

Two-photon excitable and ratiometric fluorogenic nitric oxide photoreleaser and its biological applications

Xilei Xie,‡ Jilin Fan,‡ Muwen Liang, Yong Li, Xiaoyun Jiao, Xu Wang* and Bo Tang*

College of Chemistry, Chemical Engineering and Materials Science, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Institute of Molecular and Nano Science, Shandong Normal University, Jinan 250014, P. R. China.

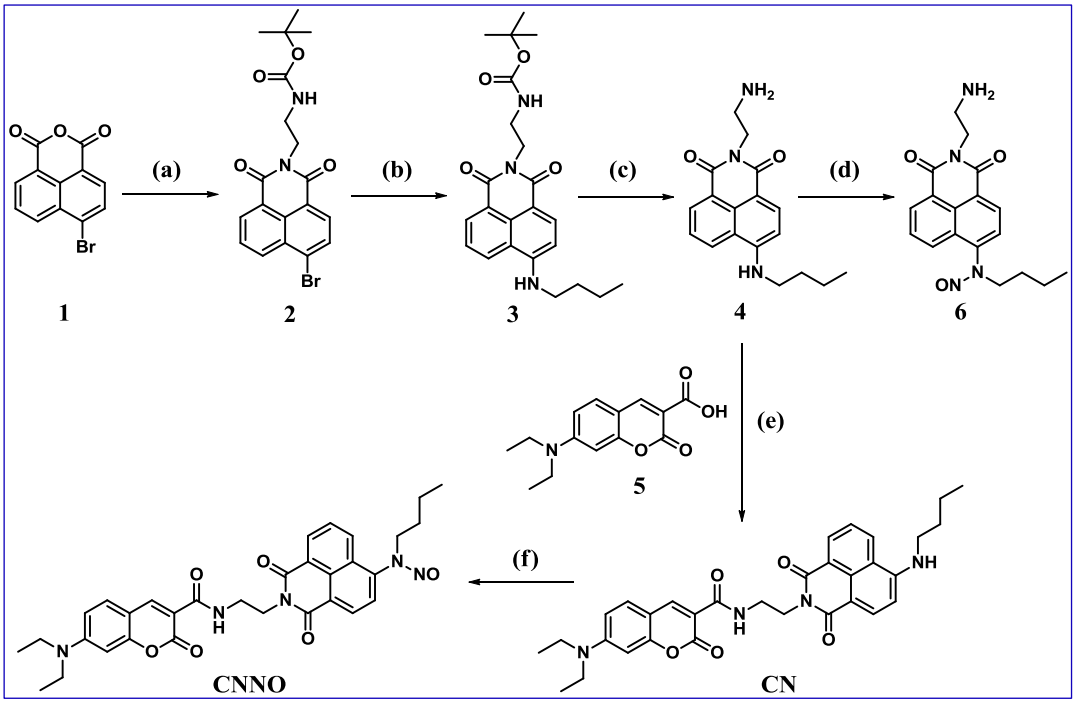
E-mail: tangb@sdnu.edu.cn; wangxu@sdnu.edu.cn

‡ These authors contributed equally to this work.

1. Materials and instruments

Reagents and solvents were acquired from commercial sources, and the solvents were distilled or purified before use if necessary. NMR spectroscopic characterization was taken on a Bruker Advance 400 MHz spectrometer. HRMS spectra were obtained by a Bruker MaXis UHR-TOF instrument. Fourier transform infrared (FT-IR) spectra were obtained using a Bruker Alpha spectrometer equipped with a Bruker platinum ATR accessory. The photoreleaser was dissolved in dimethyl sulfoxide (DMSO) to produce 1.0 mM stock solution and was diluted to 10 μM by phosphate buffer (pH=7.4, 50 mM, 50% ethanol) before use. Absorption spectra were recorded on a UV-1700 spectrophotometer, and fluorescence measurements were performed using a FLS-980 fluorospectrometer from Edinburgh Instruments. One-photon or two-photon fluorescence imaging of live cells was performed on a Zeiss LSM 880 confocal laser scanning microscope with an objective lens (×20), and the one-photon excitation wavelength and two-photon excitation wavelength were 405 nm and 840 nm, respectively. EPR spectra were recorded on an A300 electron paramagnetic resonance spectrometer. The solutions or cells were irradiated with a CEL-HXF300 Xenon lamp with power of 15 mW/cm^2. The vasodilation of mouse aorta was monitored with a 620M Multi Myograph System (DMT).

2. Synthetic procedure



Reagents and conditions: (a) *N*-Boc-ethylenediamine, ethanol, 80 ℃, 4 h, 88%; (b) *n*-butylamine, 2-methoxyethanol, 125 ℃, 12 h, 85%; (c) TFA, CH_2Cl_2, rt, 2h, 95%; (d) concd HCl, AcOH, CH_2Cl_2, $NaNO_2$, 0 ℃; then, rt, 2 h, 75%; (e) HOBt, EDC, DMF, 50 ℃, 12 h, 76%; (f) concd HCl, AcOH, CH_2Cl_2, $NaNO_2$, 0 ℃; then, rt, 2 h, 79%.

Scheme S1 Synthetic pathway of **CNNO**

*Compound **2**.* Compound **1** (12.5 mmol, 3.46 g) was dissolved in ethanol (15 mL). After addition of *N*-Boc-ethylenediamine (12.5 mmol, 2.0 mL), the mixture was stirred at 80 ℃ for 4 h. The solvent was evaporated, and the residue was purified by column chromatography on silica gel (dichloromethane/methanol, 200:1 v/v) to afford a white solid (4.6 g, 88% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.62 (d, J = 7.1 Hz, 1H), 8.51 (d, J = 8.4 Hz, 1H), 8.37 (d, J = 7.7 Hz, 1H), 8.00 (d, J = 7.7 Hz, 1H), 7.81 (t, J = 7.8 Hz, 1H), 4.33 (br s, 2H), 3.52 (br s, 2H), 1.24 (s, 9H). HRMS (ESI): calculated for $C_{19}H_{20}BrN_2O_4^+$ ($M+H^+$) 419.0601, found 419.0610.

*Compound **3**.* Compound **2** (4.8 mmol, 2.0 g) was dissolved in 2-methoxyethanol (20 mL). After addition of *n*-butylamine (72 mmol, 7.0 mL), the mixture was stirred at 125 ℃ for 12 h. The solvent was evaporated, and the residue was purified by column chromatography on silica gel (dichloromethane/methanol, 160:1 v/v) to afford a

yellow solid (1.68 g, 85% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.49 (d, J = 6.7 Hz, 1H), 8.41 (d, J = 8.4 Hz, 1H), 8.07 (d, J = 8.2 Hz, 1H), 7.53 (t, J = 7.2 Hz, 1H), 6.71 (d, J = 7.4 Hz, 1H), 4.32 (t, J = 5.1 Hz, 2H), 3.59−3.45 (m, 2H), 3.39 (t, J = 7.2 Hz, 2H), 1.86−1.71 (m, 2H), 1.61−1.44 (m, 2H), 1.33 (s, 9H), 1.01 (t, J = 7.3 Hz, 3H). HRMS (ESI): calculated for $C_{23}H_{29}N_3NaO_4^+$ ($M+Na^+$) 434.2050, found 434.2050.

*Compound **4***. Compound **3** (4.0 mmol, 1.65 g) was dissolved in dichloromethane (10 mL). After addition of trifluoroacetic acid (3 mL), the mixture was stirred at room temperature for 2 h. The solvent was evaporated, and the residue was obtained (1.2 g, 95% yield) and directly used without further purification.

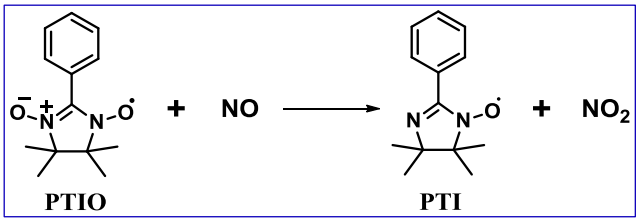
*Compound **6***. Compound **4** (0.3 mmol, 93 mg) was dissolved in a combined solvent of HCl (0.3 mL), AcOH (3.0 mL), and CH_2Cl_2 (0.6 mL). After cooled to 0 ℃, sodium nitrite (0.6 mmol, 41 mg) was added slowly while the mixture was constantly stirred. The mixture was then stirred for additional 2 h at room temperature. A concentrated $NaHCO_3$ solution was added to adjust the solution pH to neutral before the mixture was extracted with CH_2Cl_2. The organic layer was dried with Na_2SO_4, filtered, and evaporated under reduced pressure to afford the crude product, which was purified by column chromatography on silica gel (dichloromethane/ methanol, 10:1 v/v) to obtain a light yellow solid (77 mg, 75% yield). HRMS (ESI): calculated for $C_{18}H_{21}N_4O_3^+$ ($M+H^+$) 341.1608, found 341.1584.

*Compound **CN***. Compound **4** (3.8 mmol, 1.2 g), compound **5** (3.8 mmol, 0.99 g), HOBt (3.8 mmol, 0.51 g), and EDC (3.8 mmol, 0.73 g) were dissolved in 5 mL of DMF, and the mixture was stirred at 50 ℃ for 12 h. After solvent was evaporated, the crude product was purified by column chromatography on silica gel (dichloromethane/ethyl acetate, 2:1 v/v) to obtain a yellow solid (1.6 g, 76% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.01 (s, 1H), 8.64 (s, 1H), 8.47 (d, J = 7.0 Hz, 1H), 8.41 (d, J = 8.1 Hz, 1H), 8.02 (d, J = 8.1 Hz, 1H), 7.39 (t, J = 7.4 Hz, 1H), 7.33 (d, J = 8.8 Hz, 1H), 6.69−6.53 (m, 2H), 6.43 (s, 1H), 5.57 (s, 1H), 4.48 (t, J = 4.8 Hz, 2H), 3.86 (dd, J = 5.3 Hz, 2H), 3.43 (dd, J = 14.1, 7.2 Hz, 4H), 3.39−3.32 (m, 2H), 1.86−1.73 (m, 2H), 1.59−1.45 (m, 2H), 1.22 (t, J = 6.7 Hz, 6H), 1.01 (t, J = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.89, 164.21, 163.57, 162.57, 157.55, 152.40,

149.86, 148.02, 134.63, 131.12, 131.06, 129.86, 126.27, 124.18, 122.50, 120.01, 110.27, 109.82, 109.32, 108.30, 104.03, 96.45, 45.09, 43.48, 39.33, 38.61, 30.86, 20.43, 13.94, 12.45. HRMS (ESI): calculated for $C_{32}H_{35}N_4O_5^+$ ($M+H^+$) 555.2602, found 555.2605.

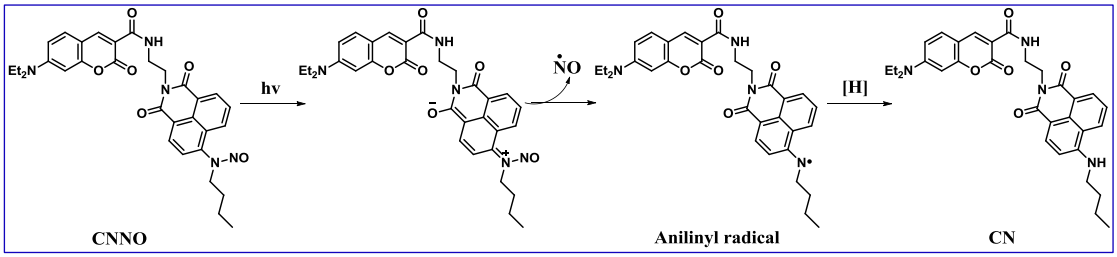
*Compound **CNNO***. Compound **CN** (0.5 mmol, 277 mg) was dissolved in a combined solvent of HCl (0.4 mL), AcOH (4.0 mL), and CH_2Cl_2 (0.8 mL). After cooled to 0 ℃, sodium nitrite (1.0 mmol, 69 mg) was added slowly while the mixture was constantly stirred. The mixture was then stirred for additional 2 h at room temperature. A concentrated $NaHCO_3$ solution was added to adjust the solution pH to neutral before the mixture was extracted with CH_2Cl_2. The organic layer was dried with Na_2SO_4, filtered, and evaporated under reduced pressure to afford the crude product, which was purified by column chromatography on silica gel (dichloromethane/ethyl acetate, 3:1 v/v) to obtain a yellow solid (230 mg, 79% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.98 (dd, J = 12.2, 6.0 Hz, 1H), 8.75−8.69 (m, 1H), 8.69−8.62 (m, 2H), 8.06 (d, J = 8.5 Hz, 1H), 7.86−7.74 (m, 1H), 7.75−7.63 (m, 1H), 7.42 (t, J = 8.9 Hz, 1H), 6.69 (d, J = 8.8 Hz, 1H), 6.53 (s, 1H), 4.59−4.47 (m, 2H), 4.21−4.05 (m, 2H), 3.92−3.80 (m, 2H), 3.45 (q, J = 7.0 Hz, 4H), 1.57−1.46 (m, 2H), 1.33−1.27 (m, 2H), 1.23 (t, J = 7.0 Hz, 6H), 0.87 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.88, 163.63, 163.42, 162.53, 157.53, 152.31, 148.06, 142.96, 141.55, 132.11, 131.12, 129.46, 129.12, 128.19, 127.98, 125.48, 123.91, 123.13, 122.96, 109.97, 108.42, 96.42, 47.60, 45.16, 39.69, 38.20, 28.49, 20.31, 13.53, 12.38. HRMS (ESI): calculated for $C_{32}H_{34}N_5O_6^+$ ($M+H^+$) 584.2504, found 584.2507.

3. EPR analysis using PTIO



Scheme S2 The reaction between PTIO and NO

4. Photolysis mechanism



Scheme S3 Proposed mechanism of photolysis of **CNNO**

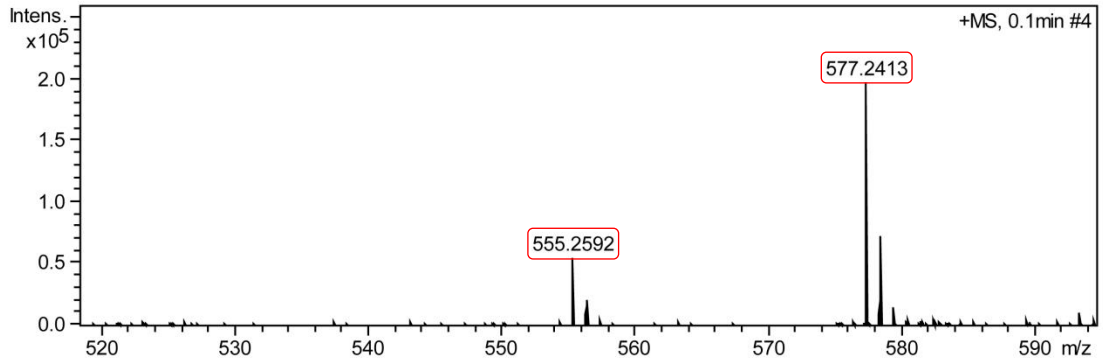
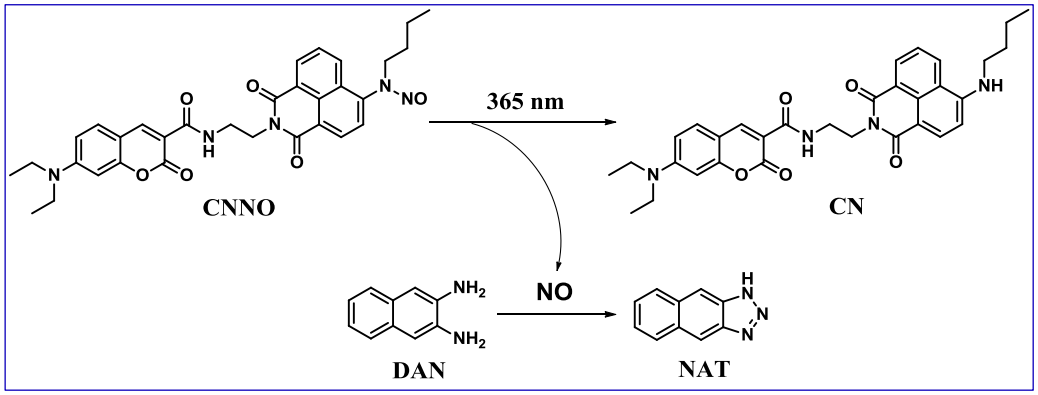


Fig. S1 HRMS spectral analysis of photolysis of **CNNO**. **CNNO** (10 μM) was irradiated with 365 nm light (15 mW/cm^2) for 30 min, and then the mixture was characterized by HRMS spectrometry.

5. Fluorescence measurement during NO release



Scheme S4 Detection of NO release from **CNNO** upon irradiation

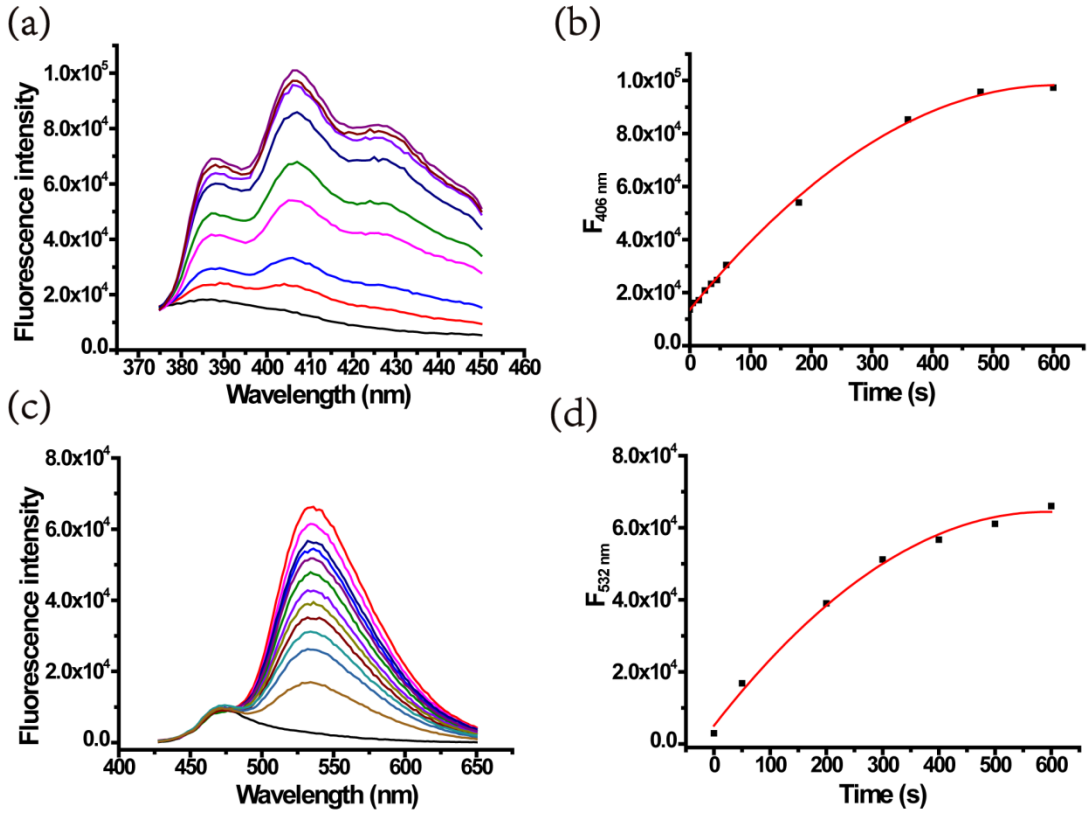


Fig. S2 Spectroscopic monitoring (a) and fluorescence enhancement at 406 nm (b) of the solution containing DAN (10 μM) and **CNNO** (10 μM) in phosphate buffer (50 mM, pH = 7.4, 50% ethanol) during irradiation with 365 nm light (15 mW/cm^2), and detected with excitation at 360 nm. Spectroscopic monitoring (c) and fluorescence enhancement at 532 nm (d) of the above solution during irradiation with 365 nm light (15 mW/cm^2), upon excitation at 408 nm.

6. Quantification of NO release

Different concentrations (2.0, 4.0, 6.0, 8.0, 10.0, 12.0, and 14.0 μM) of **CN** in phosphate buffer (50 mM, pH = 7.4, 50% ethanol) was prepared, and the fluorescence intensity at 532 nm was determined with excitation at 408 nm. Thus, we obtained the regression equation: $F = 1.27\times10^4 + 7.13\times10^3$ [**CN**] μM with a linear coefficient of 0.996. Then, 10.0 μM **CNNO** in phosphate buffer (50 mM, pH = 7.4, 50% ethanol) was irradiated at 365 nm light for 20 min, and the fluorescence intensity at 532 nm was determined. The concentration of released NO was equal to that of the byproduct

CN which was calculated according to the above fluorescence calibration curve.

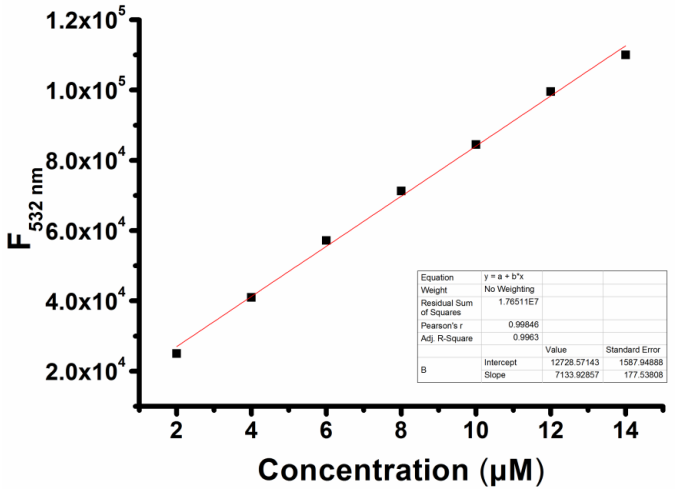


Fig. S3 Fluorescence calibration curve was established based on the relationship between fluorescence intensity at 532 nm and concentration of **CN**, against which dose of NO photoreleased from **CNNO** is easily estimated.

7. Absorption and emission spectra

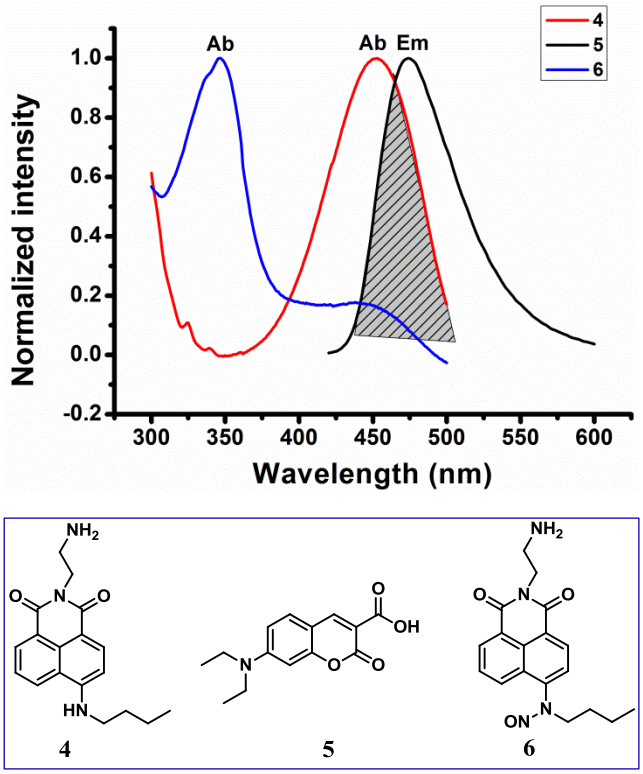


Fig. S4 Absorption spectra of naphthalimide derivatives **4** (red) and **6** (blue)**,** fluorescence emission spectrum of coumarin derivative **5** (black)**,** and their chemical structures.

8. Quantum yield

Fluorescence quantum yields of **CNNO** and **CN** were determined by using quinine sulphate (Φ_f = 0.54 in 0.1 M H_2SO_4) as a fluorescence standard. The quantum yield was calculated using the following equation:

$$\Phi_{F(X)} = \Phi_{F(S)} (A_S F_X / A_X F_S) (n_X / n_S)^2$$

Where Φ_F is the fluorescence quantum yield, A is the absorbance at the excitation wavelength, F is the area under the corrected emission curve, and n is the refractive index of the solvents used. Subscripts $_S$ and $_X$ refer to the standard and to the unknown, respectively. For the synthesized dyes, the excitation wavelength was at 408 nm while keeping the absorption below 0.05.

9. Test of photostability

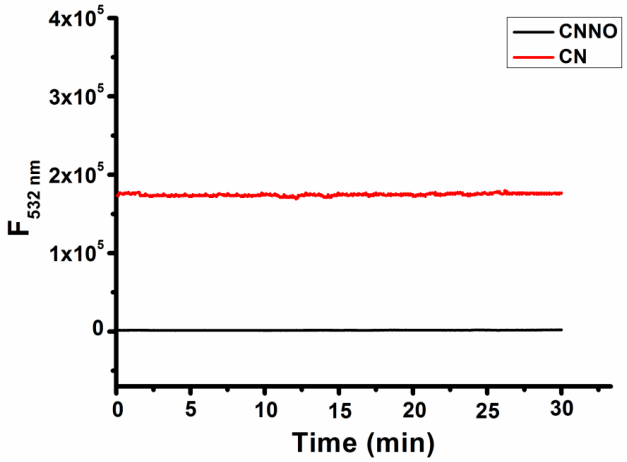


Fig. S5 Photostability of **CNNO** (10 μM, black) and **CN** (10 μM, red) in phosphate buffer (50 mM, pH = 7.4, 50% ethanol) during irradiation by 408 nm light from a xenon lamp of the fluorospectrometer.

10. MTT assay

Hela cells were seeded into a 96-well microtiter plate at 37 ℃ in a 5% CO_2/95% air incubator for 24 h. The cells were incubated for an additional 24 h with different concentrations of tested compounds (0, 10, 20, 50, 100, and 200 μM), respectively.

Then the cells were washed with PBS three times. Subsequently, MTT solution (200 μL, 0.5 mg/mL) was added to each well and the cells were incubated at 37 ℃. After 4 h, the remaining MTT was removed, and the formazan crystals were dissolved in 200 μL of DMSO with gentle agitation for 5 min. The absorbance at 490 nm was measured using a TRITURUS microplate reader.

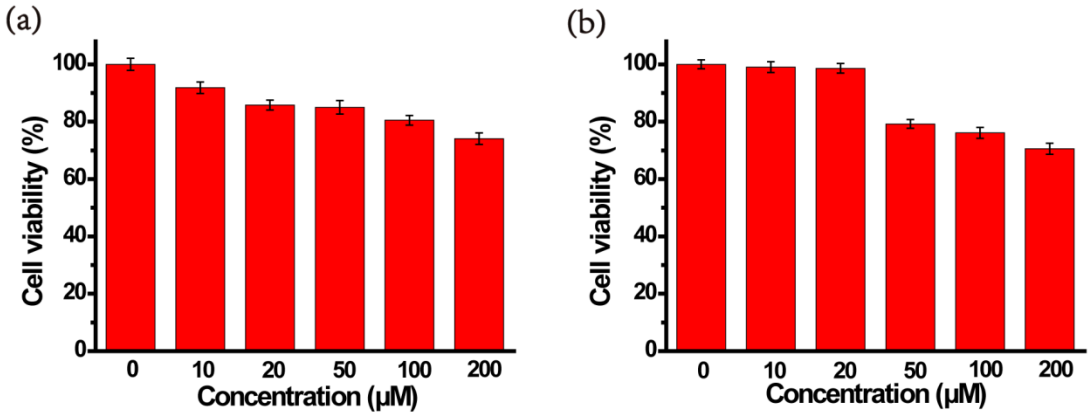


Fig. S6 MTT assay of Hela cells with different concentrations of **CNNO** (a) and its photodecomposed product **CN** (b).

11. Spatial controlling NO release in live cells

Hela cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin at 37 ℃ in a 5% CO_2/95% air incubator MCO-5AC (SANYO, Tokyo, Japan). One day before imaging, the cells were detached and were replanted on glass-bottomed dishes. For NO release, the cells were incubated with 10 μM **CNNO** for 15 min before being washed three times with PBS. Then for one-photon irradiation, the cells were subjected to a CEL-HXF300 Xenon lamp with power of 15 mW/cm^2 for 5 min. For two-photon irradiation, a region of interest of the cells were subjected to the confocal fluorescence microscopy (Zeiss LSM 880) and bleached with an 800 nm laser (20 mW) for 2 min. After irradiation, the images were captured with 405 nm excitation or 840 nm excitation.

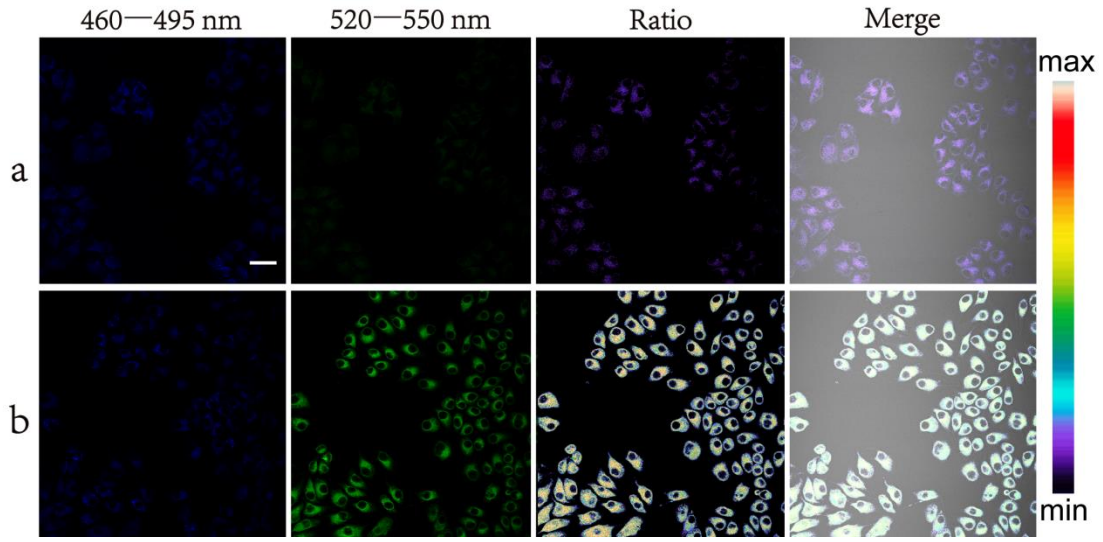


Fig. S7 Two-photon fluorescence images of NO release from **CNNO** in live Hela cells. Cells were treated with 10 μM **CNNO** without (a) or with (b) irradiation by 365 nm light (15 mW/cm^2) for 5 min. The ratiometric images ($F_{520-550}/F_{460-495}$, the third column) were obtained from channel 1 (460−495 nm, the first column) and channel 2 (520−550 nm, the second column) with the two-photon excitation at 840 nm. The fourth column represents the merged images of the ratio channel and the corresponding bright field images. Scale bar represents 50 μm.

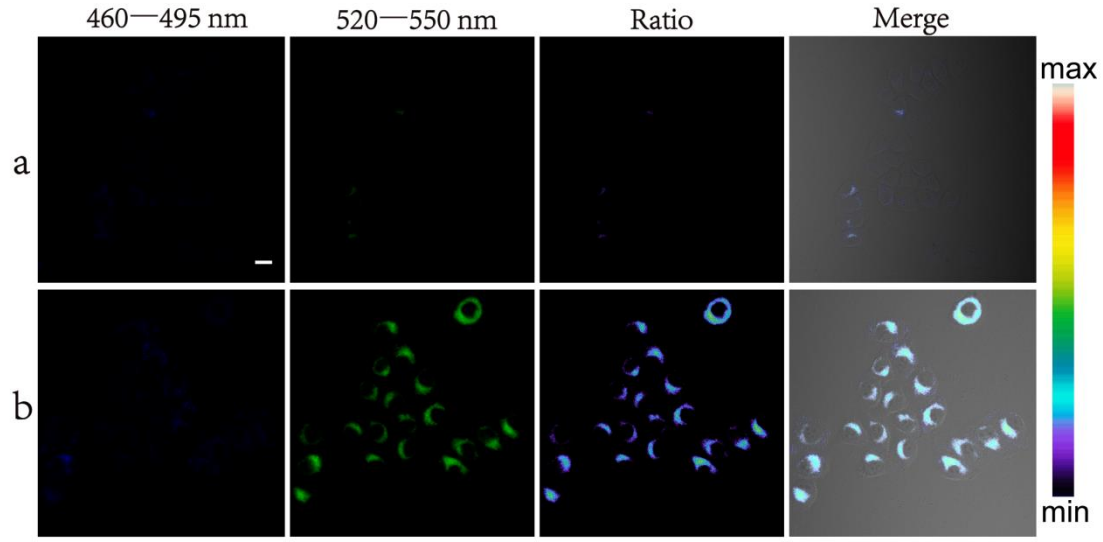


Fig. S8 One-photon fluorescence images of NO release from **CNNO** in live Hela cells. Cells were treated with 10 μM **CNNO** without (a) or with (b) irradiation by 365 nm light (15 mW/cm^2) for 5 min. The ratiometric images ($F_{520-550}/F_{460-495}$, the third column) were obtained from channel 1 (460−495 nm, the first column) and channel 2 (520−550 nm, the second column) with the one-photon excitation at 405 nm. The

fourth column represents the merged images of the ratio channel and the corresponding bright field images. Scale bar represents 20 μm.

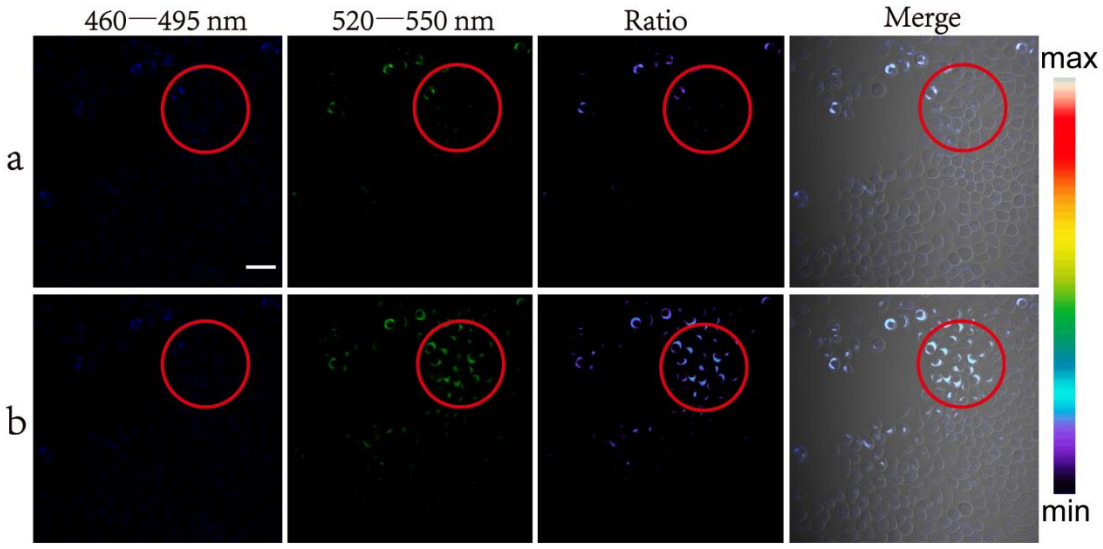


Fig. S9 One-photon fluorescence images of NO release from **CNNO** in live Hela cells. Cells were treated with 10 μM **CNNO.** (a) The dish was imaged without photoirradiation. (b) The dish was photoirradiated inside the selected circle using a two-photon laser at 800 nm (20 mW) for 2 min before imaged. The ratiometric images ($F_{520-550}/F_{460-495}$, the third column) were obtained from channel 1 (460−495 nm, the first column) and channel 2 (520−550 nm, the second column) with the one-photon excitation at 405 nm. The fourth column represents the merged images of the ratio channel and the corresponding bright field images. Scale bar represents 50 μm.

12. Temporal modulation of vasodilation by CNNO

All animal experiments were in strict accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. A strip of mouse thoracic aorta was placed and fixed in one chamber of 620M Multi Myograph System, which was filled with PSS buffer (4.7 mM KCl, 119 mM NaCl, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 5.5 mM glucose, and 0.03 mM EDTA, pH 7.4) at 37 ℃. Then the strip was pretreated with 10 μM L-NAME, and then incubated in KPSS buffer (123.7 mM KCl, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$,

1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 5.5 mM glucose, and 0.03 mM EDTA, pH 7.4). After equilibration, 10 μM **CNNO** was added and the chamber was irradiated with 365 nm light (15 mW/cm^2) for 60 s and 30 s in turn. As the negative control, another strip of aorta was irradiated for 90 s without treatment of **CNNO**.

13. NMR and HRMS spectra

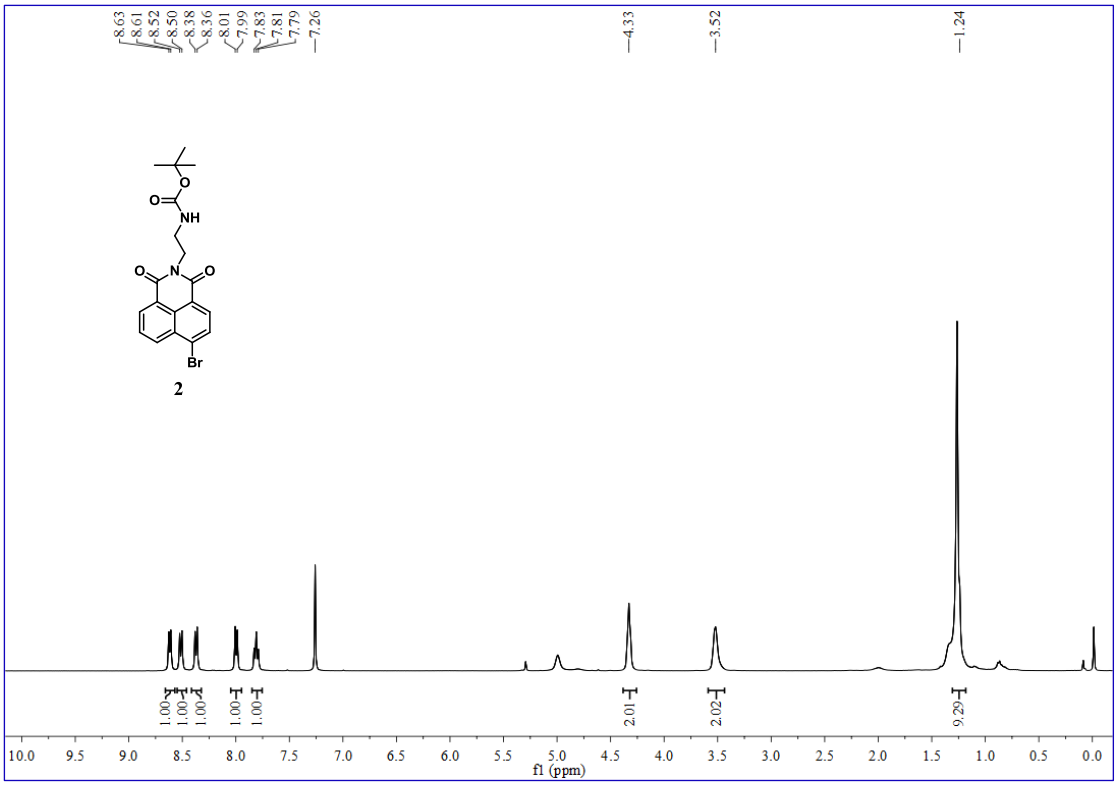


Fig. S10 ^{1}H NMR spectrum of compound **2**

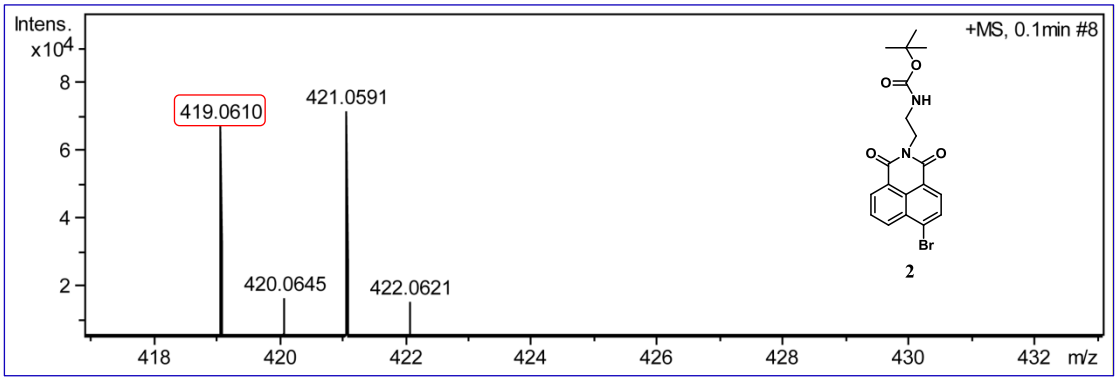


Fig. S11 HRMS spectrum of compound **2**

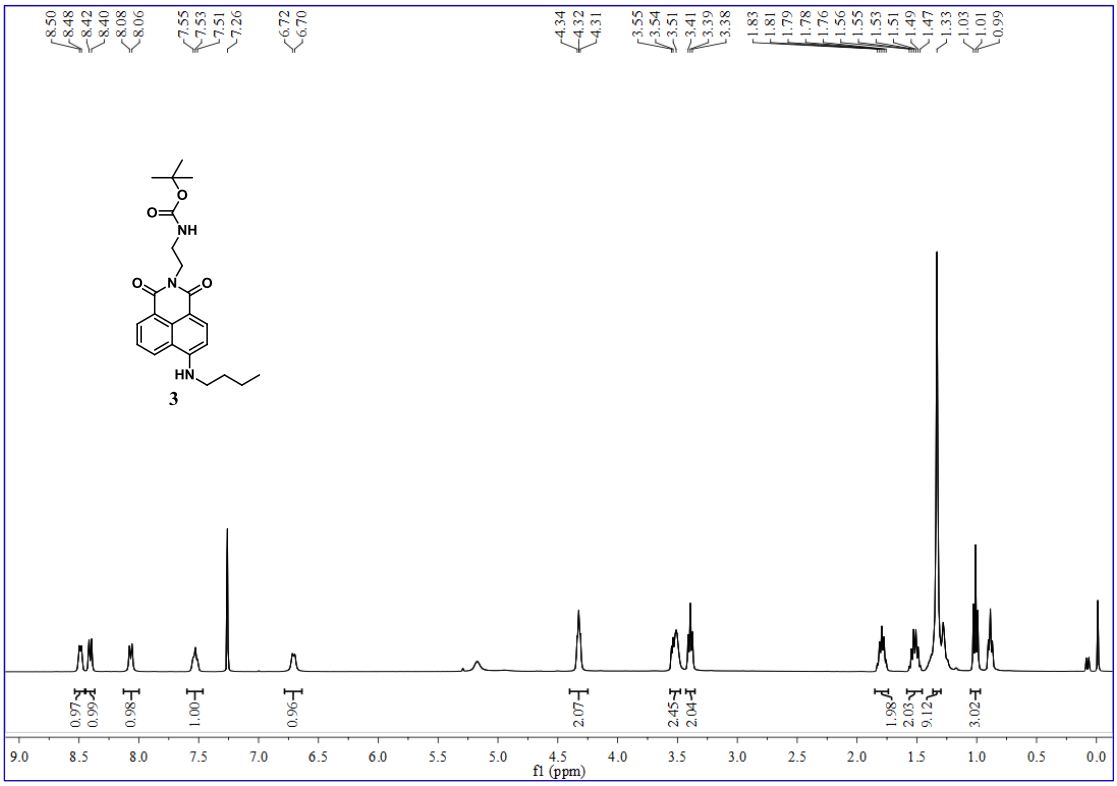


Fig. S12 ^{1}H NMR spectrum of compound **3**

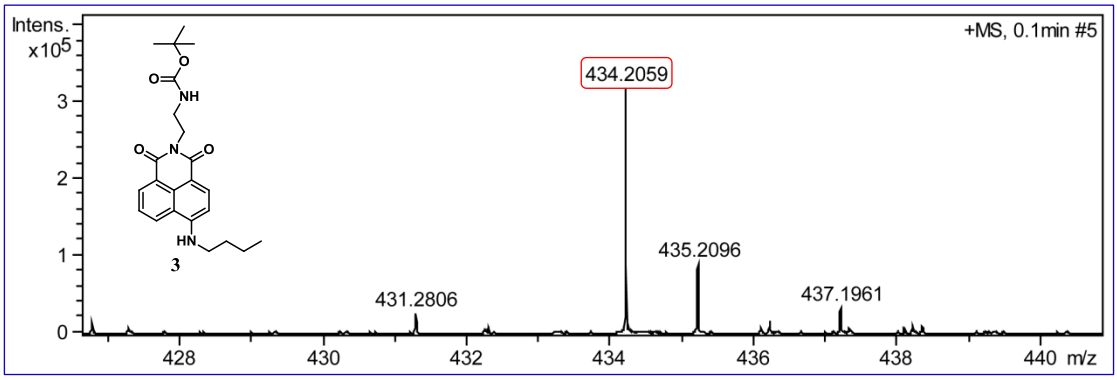


Fig. S13 HRMS spectrum of compound **3**

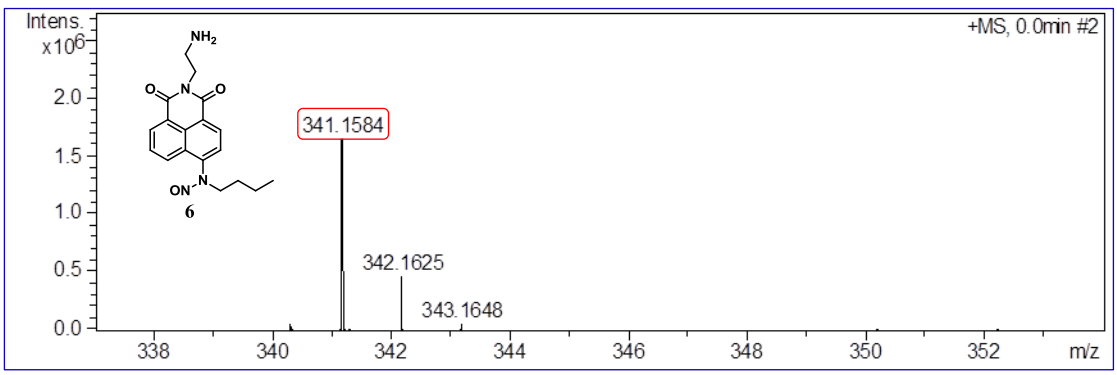


Fig. S14 HRMS spectrum of compound **6**

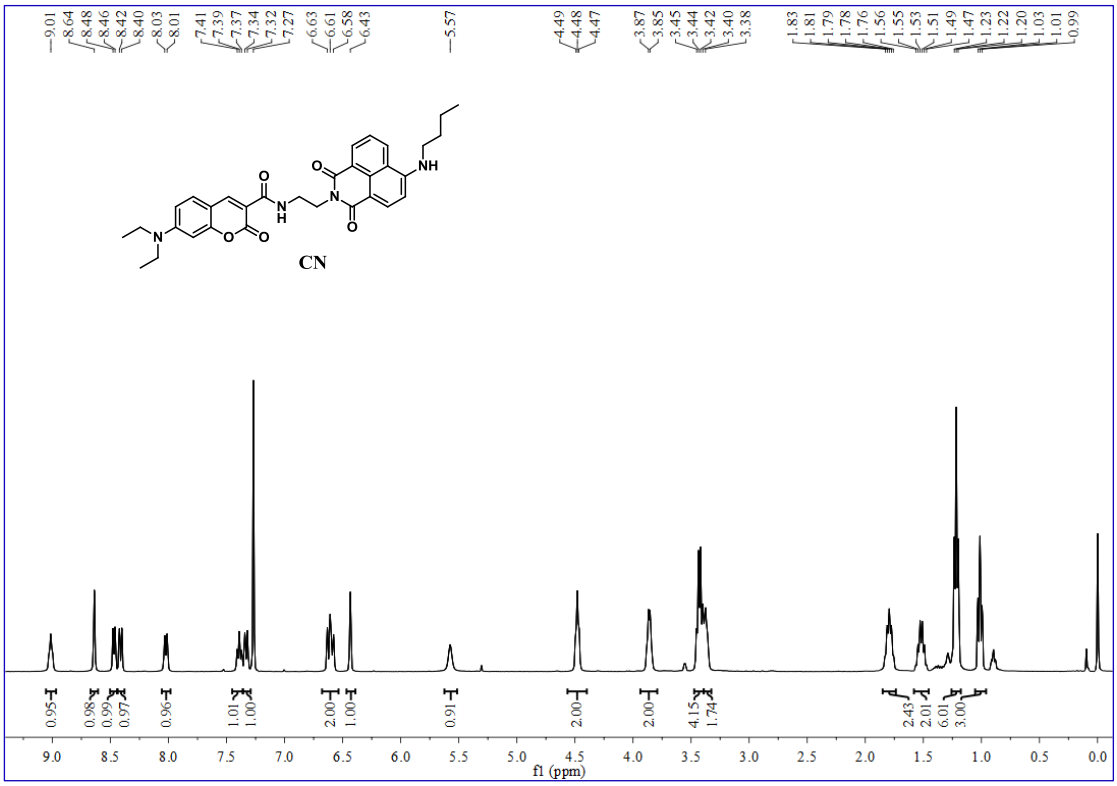


Fig. S15 ^{1}H NMR spectrum of compound **CN**

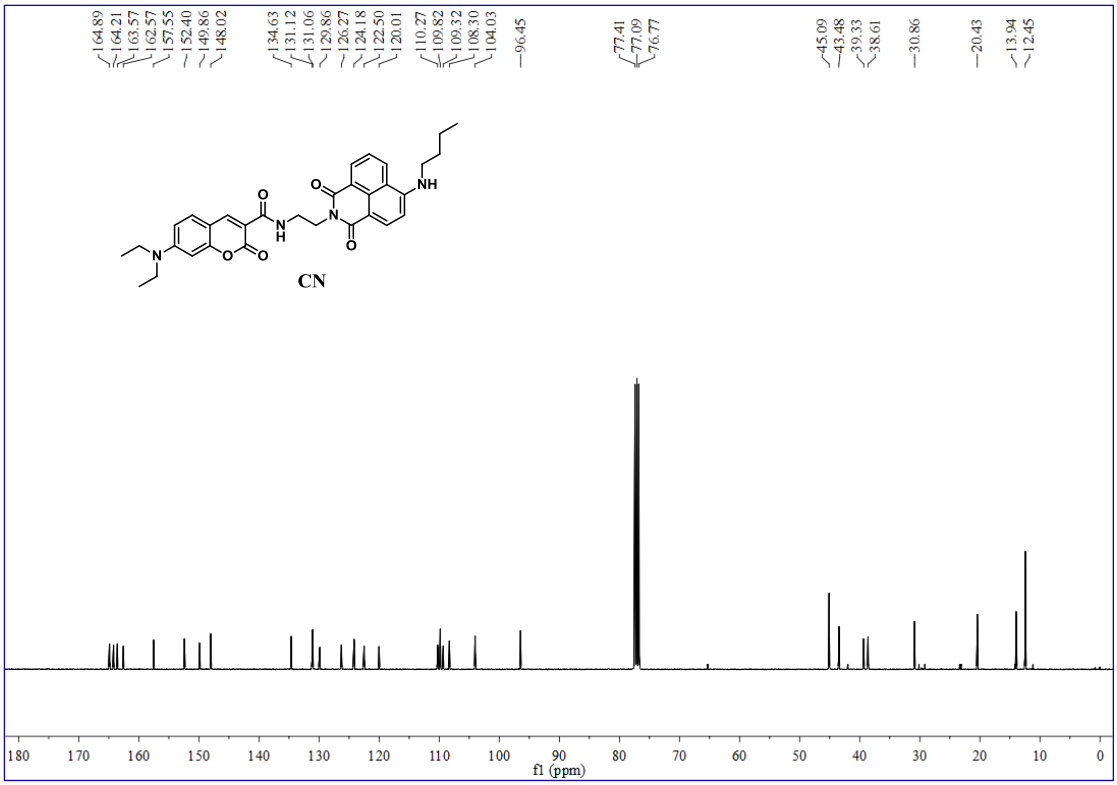


Fig. S16 ^{13}C NMR spectrum of compound **CN**

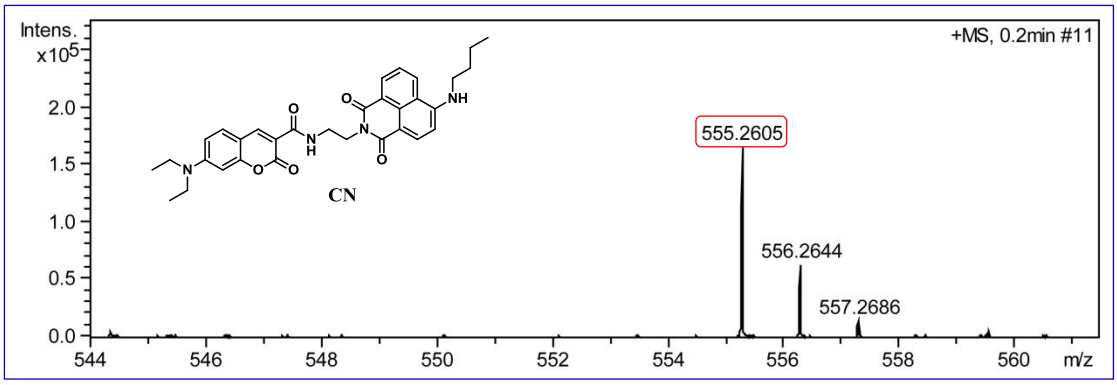


Fig. S17 HRMS spectrum of compound **CN**

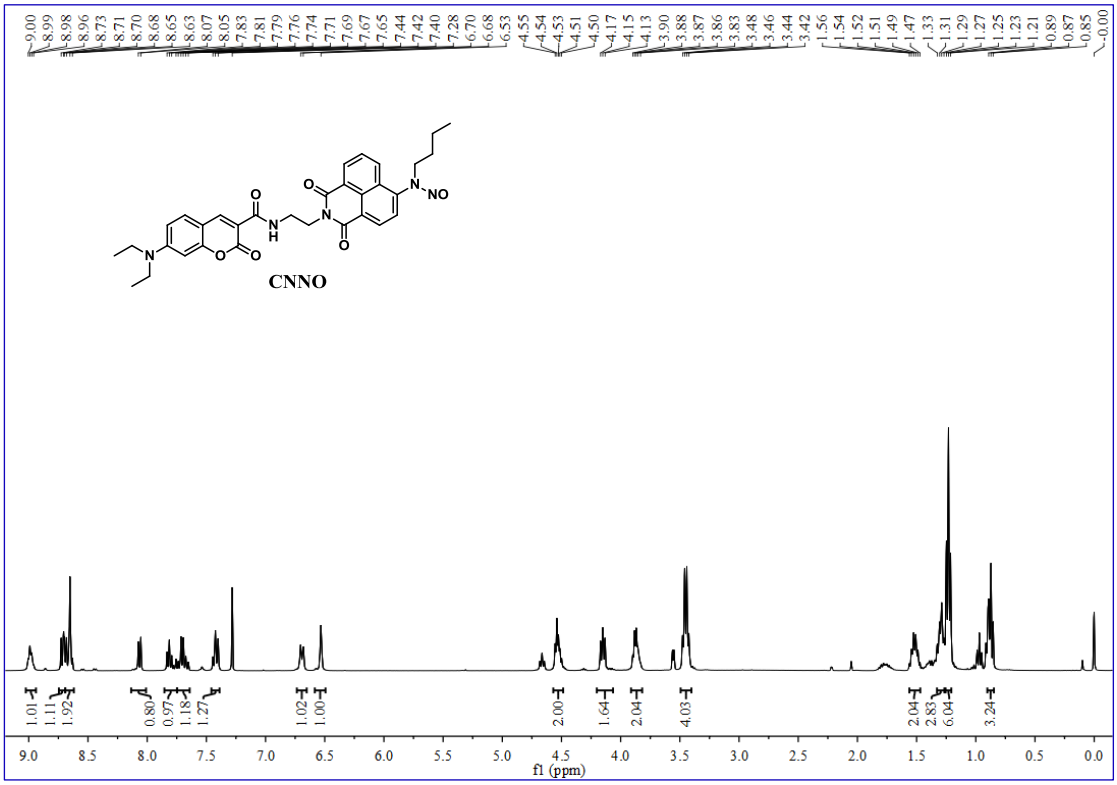


Fig. S18 ^{1}H NMR spectrum of compound **CNNO**

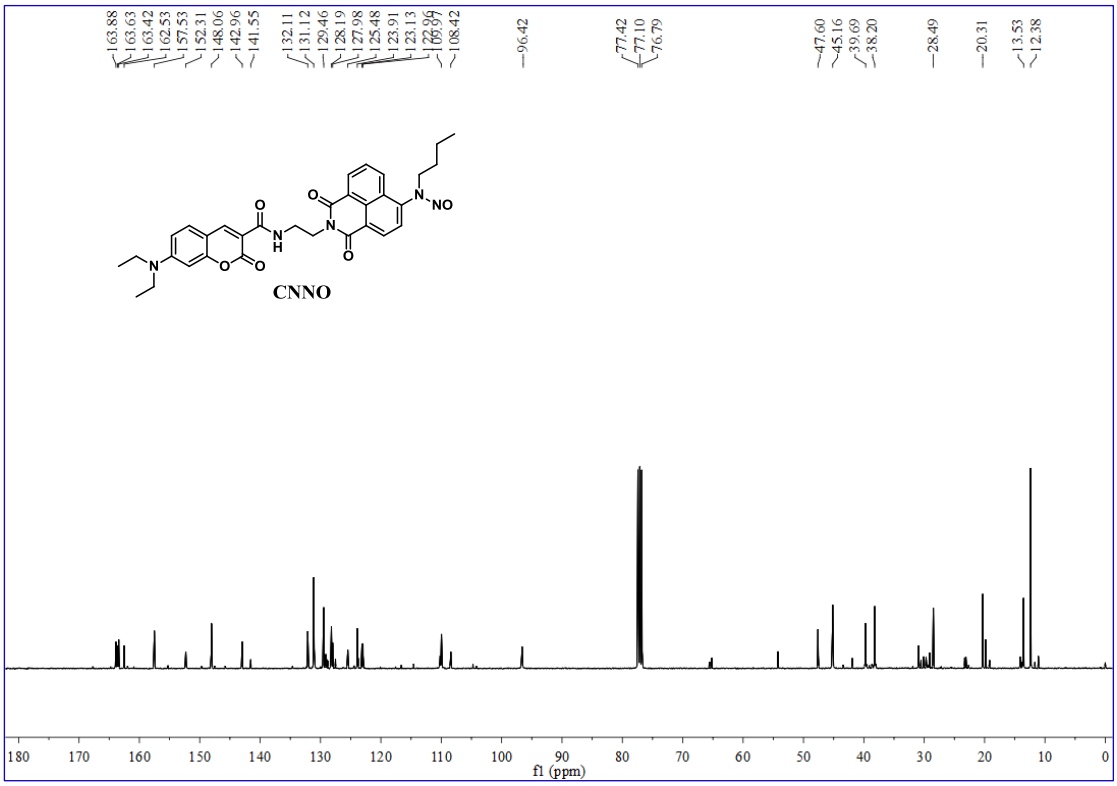


Fig. S19 ^{13}C NMR spectrum of compound **CNNO**

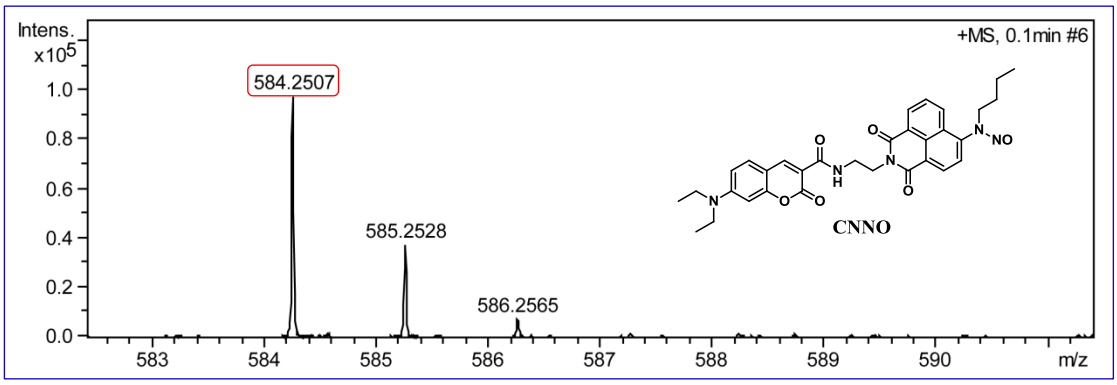
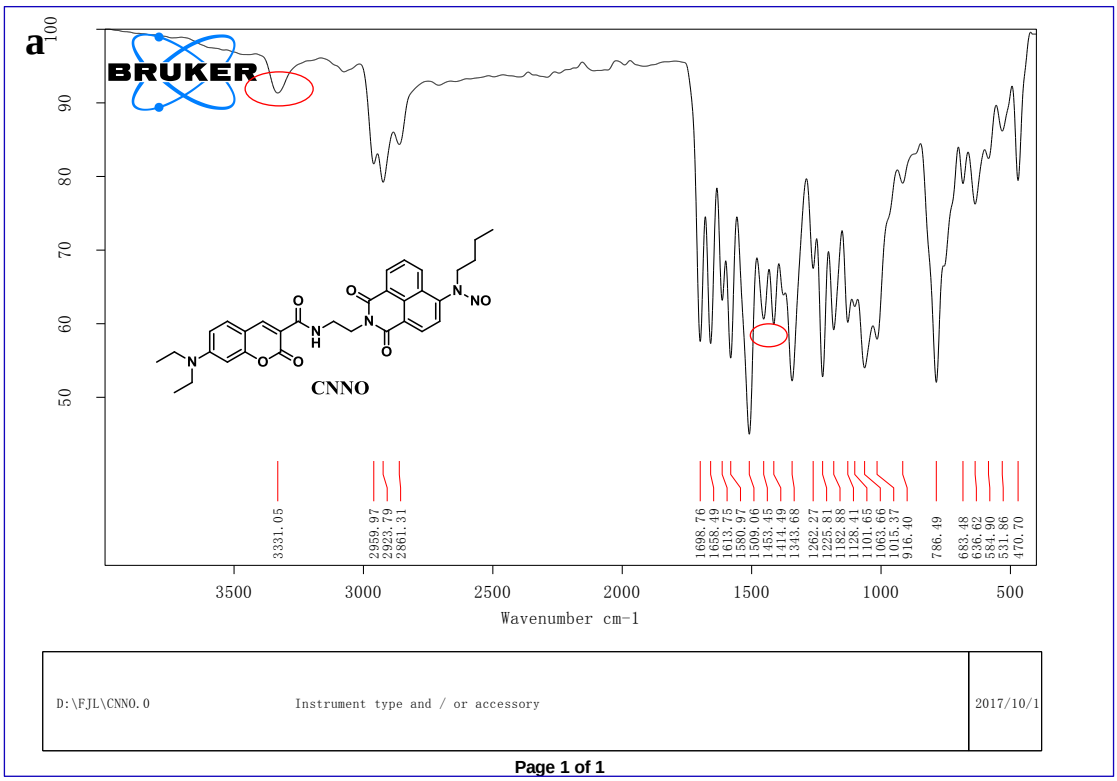


Fig. S20 HRMS spectrum of compound **CNNO**

14. IR spectra of CNNO and CN



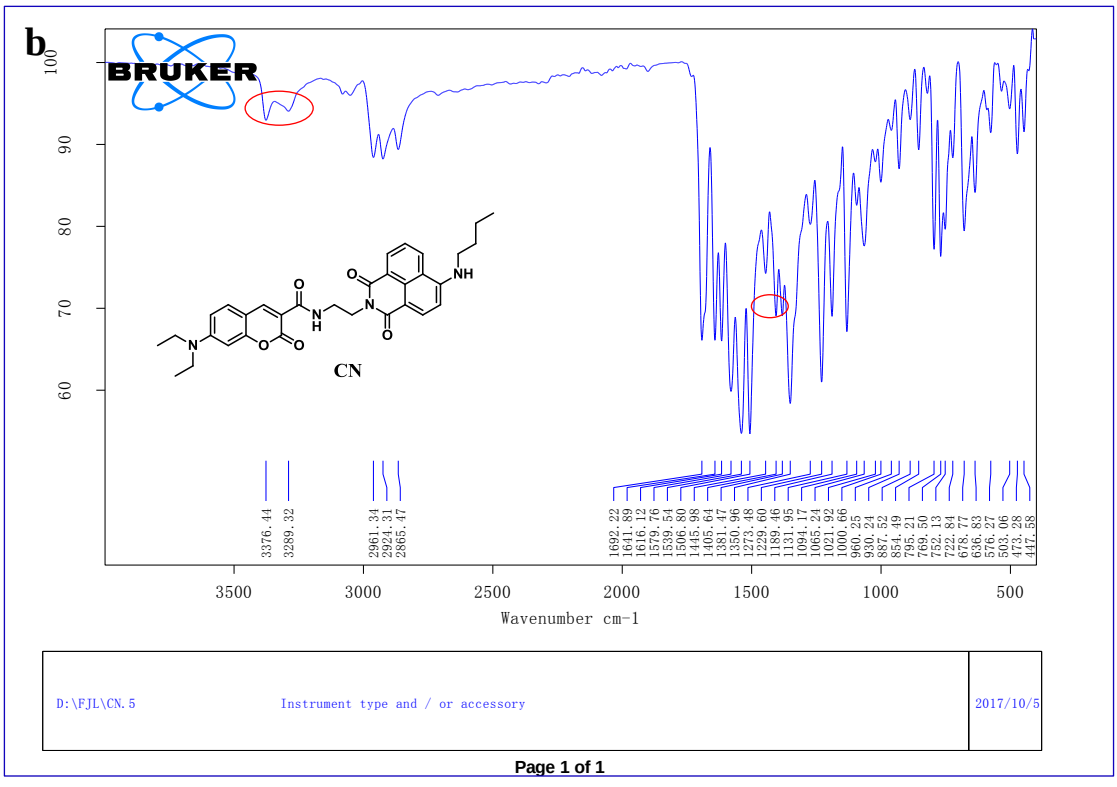


Fig. S21 IR spectra of **CNNO** (a) and **CN** (b). Spectrum a shows only one –NH– stretching frequency at 3331 cm^{-1}, while spectrum b shows two –NH– stretching frequencies at 3376 cm^{-1} and 3289 cm^{-1}. Additionally, the two spectra present quite

different absorption in the range of 1500−1400 cm^{-1} where the stretching frequency of –N=O emerges. All the above spectrum features are in accord with the characteristic structures of **CNNO** and **CN**.

15. HPLC analysis

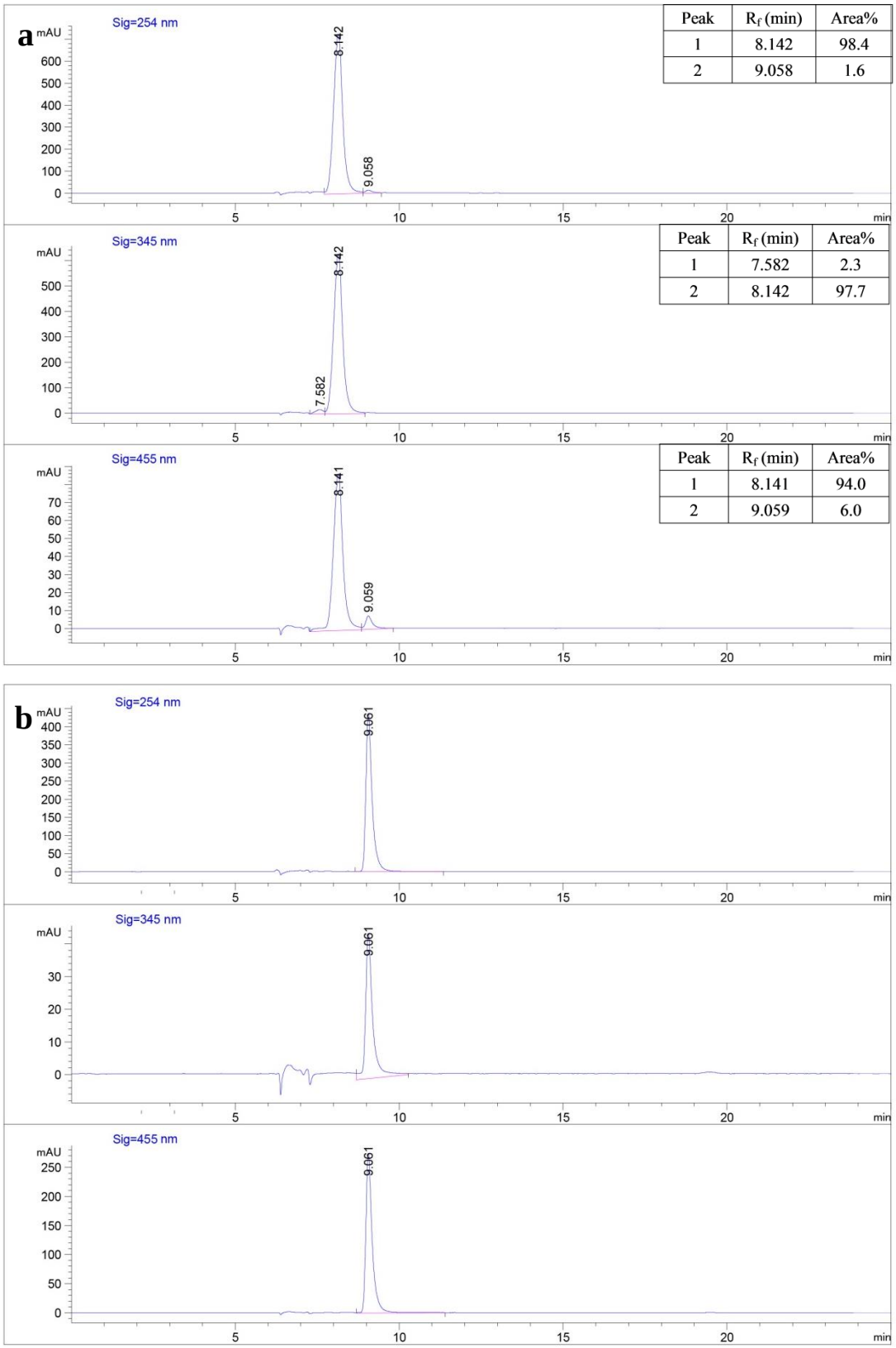


Fig. S22 HPLC spectra of **CNNO** (a) and **CN** (b). The HPLC analysis was operated

on an Agilent 1260 Infinity instrument equipped with an Agilent 1260 Infinity Diode array detector VL (G1315D). Separation was performed on a Shim-pack CLC-ODS column (250 × 4.6 mm, 5 μm). The mobile phase was water/acetonitrile (0.1% formic acid) 5:95, and the flow rate was 0.5 mL/min. The detection wavelengths were set at 254, 345, and 455 nm, respectively. The retention times of **CNNO** and **CN** were 8.14 and 9.06 min respectively, indicating the huge difficulty to completely remove **CN** from **CNNO**. According to the HPLC results, we estimate that the purity of **CNNO** is ca. 95%, and the main impurity in it is the raw material **CN** which is also the photolytic product of **CNNO**.