Supporting information for

Bis-Reaction-Trigger as a Strategy to Improve the Selectivity of

Fluorescent Probes

1. Materials and methods

Reagents and apparatus

All chemicals were from commercial supplies and used without further purification except otherwise indicated. Dry DMF and dry Et$_3$N were distilled from CaH$_2$. Dry toluene was distilled from Na. $^1$H NMR spectra were recorded on a Bruker 500 Fourier transform spectrometer (500 MHz). $^{13}$C NMR spectra were obtained on a Bruker 500 Fourier transform spectrometer (125 MHz) spectrometer. All NMR spectra were calibrated using the residual solvent (CDCl$_3$) as internal reference ($^1$H NMR = 7.26, $^{13}$C NMR = 77.16). All chemical shifts were reported in parts per million (ppm) and coupling constants ($J$) in Hz. The following abbreviations were used to explain the multiplicities: d = doublet, t = triplet, m = multiplet. Mass spectra (MS) were measured on a SHIMADZU LCMS-2020 spectrometer. UV-vis spectra were taken on a HITACHI U-3010 Spectrophotometer. Fluorescence spectra were measured on an Agilent Cary Eclipse Fluorescence Spectrophotometer.

Probe synthesis and characterization

![Chemical structures](image)

**Synthesis of intermediate 2**

Compound 1 (1.0 g, 3.9 mmol) and N,N-Bis (trifluoromethylsulfonyl)aniline (2.1 g, 5.9 mmol) was dissolved in dry DMF (10 mL) and the mixture was cooled to 0 °C with an ice bath. Dry triethylamine (1.1 mL, 7.9 mmol) was added to the mixture dropwise under a nitrogen atmosphere. The reaction was stirred at ambient temperature overnight. After completion of the reaction as monitored by TLC analysis, H$_2$O (10 mL) was added to quench the reaction. The mixture was then diluted with ethyl acetate (20 mL) and then transferred to a separatory funnel. The organic phase was
washed subsequently with H$_2$O (4×10 mL) and brine (1×10 mL), dried over anhydrous Na$_2$SO$_4$, and concentrated under reduced pressure to give the crude product which was purified by flash column chromatography (SiO$_2$, petroleum ether/EtOAc, 25:1) to yield the product as a white solid (1.2 g, 79% yield).

M.p.: 79.6-80.7 °C.

R$_f$ = 0.44 (1:25, EtOAc:PE).

IR (cm$^{-1}$): 3074, 2923, 2842, 1710, 1581, 1491, 1427, 1217, 1135, 868

$^1$H NMR (500 MHz, CDCl$_3$): δ 10.12 (s, 1H), 8.70 (d, J = 2.1 Hz, 1H), 8.17 (d, J = 8.2 Hz, 1H), 8.07 (dd, J = 8.5, 2.1 Hz, 1H), 7.97 (d, J = 8.0 Hz, 1H), 7.67 (d, J = 8.5 Hz, 1H), 7.61 - 7.54 (m, 1H), 7.53 -7.44 (m, 1H).

$^{13}$C NMR (126 MHz, CDCl$_3$): δ 189.70, 159.79, 153.24, 150.24, 136.00, 135.79, 133.53, 131.84, 128.28, 127.03, 126.46, 124.19, 123.48, 121.76.

ESI-HRMS (m/z): [M+H]$^+$ calc’d. for C$_{15}$H$_9$F$_3$NO$_4$S$_2$ 387.9925, found 387.9921.

Synthesis of intermediate 3

Triflate 2 (3.0 g, 7.7 mmol) was dissolved in dry toluene (30 mL), to which was added sequentially the aniline (2.3 g, 9.2 mmol), Cs$_2$CO$_3$ (3.5 g, 10.8 mmol), BINAP (23 mg, 0.36 mmol), and Pd(OAc)$_2$ (52 mg, 0.23 mmol). The reaction was then heated to 80 °C under nitrogen atmosphere and was stirred for 16 hours. The reaction was then cooled to ambient temperature and diluted with EtOAc (30 mL). Solid precipitation was removed by filtration and the filtrate was concentrated by rotary evaporation under reduced pressure to yield the crude product which was purified by flash column chromatography on silica gel (SiO$_2$: PE/EtOAc, 25:1) to give the product as a yellow solid (3.5 g, 92% yield).

M.p.: 138.9-140.0 °C.

R$_f$ = 0.54 (1:25, EtOAc:PE).

IR (cm$^{-1}$): 3220, 2956, 2929, 2857, 1683, 1614, 1508, 1472, 1280, 913.

$^1$H NMR (500 MHz, CDCl$_3$): δ 9.82 (s, 1H), 8.29 (d, J = 1.7 Hz, 1H), 8.00 (d, J = 8.1 Hz, 1H), 7.93 (d, J = 7.8 Hz, 1H), 7.72 (dd, J = 8.8, 1.8 Hz, 1H), 7.49 (td, J = 7.8, 1.0 Hz, 1H), 7.43-7.40 (m, 1H), 7.11 (d, J = 8.8 Hz, 1H), 6.93 (d, J = 8.2 Hz, 1H), 6.86 (d, J = 2.3 Hz, 1H), 6.84 (dd, J = 8.2, 2.4 Hz, 1H), 3.83 (s, 3H), 1.04 (s, 9H), 0.21 (s, 6H).

$^{13}$C NMR (126 MHz, CDCl$_3$): δ 189.63, 168.46, 153.12, 151.88, 150.77, 143.50, 134.13, 133.23, 132.98, 132.70, 126.58, 126.23, 125.71, 122.69, 121.55, 121.53, 118.02, 115.11, 113.69, 110.14, 55.80, 25.87, 18.62, -4.42.

ESI-HRMS (m/z): [M+H]$^+$ calc’d. for C$_{27}$H$_{31}$N$_2$O$_3$Si 491.1825, found 491.1827.

Synthesis of intermediate 4

Compound 3 (1.4 g, 2.9 mmol) was dissolved in dry DMF (10 mL). The solution was cooled to 0°C in an ice bath and Cs$_2$CO$_3$ (1.4 g, 4.3 mmol) was added in one portion, followed by CH$_3$I (0.27 mL, 4.3 mmol). The mixture was warmed to ambient temperature slowly (by removing the ice bath). The reaction was monitored by TLC analysis till the disappearance of 3 (ca. 12 hrs). H$_2$O (10 mL) was added to quench the reaction and the mixture was diluted with EtOAc (20 mL). The biphasic mixture was
then transferred to a separatory funnel and the organic layer was washed sequentially with 
H₂O (4×10mL) and brine (1×10 mL), dried over anhydrous Na₂SO₄, filtered, and concentrated under 
reduced pressure. The crude product, without further purification, was directly subject to 
deprotection by being dissolved in THF (10 mL) and then treated with Bu₄NF・2H₂O (1.7 g, 5.4 mmol). 
After being stirred at ambient temperature for 20 minutes, the volatile organics were removed by 
rotary evaporation under reduced pressure and the resulting residue was purified by flash column 
chromatography (SiO₂: PE/EtOAc, 3:1) to give intermediate 4 as a yellow solid (0.8 g, 71% for the two 
sequential steps).

**M.p.:** 169.1-170.2 °C.  
**Rf =** 0.54 (1:3, EtOAc:PE).  
**IR (cm⁻¹):** 3163, 2962, 2834, 2818, 2363, 1702, 1596, 1512, 1371, 1263, 1103, 837, 795.  
**¹H NMR (500 MHz, CDCl₃):**  δ 10.09 (s, 1H), 8.85 (d, J = 1.9 Hz, 1H), 8.09 (d, J = 8.1 Hz, 1H), 7.96 (dd, J = 8.3, 2.0 Hz, 1H), 7.83 (d, J = 7.9 Hz, 1H), 7.52 - 7.47 (m, 1H), 7.34 (d, J = 8.3 Hz, 1H), 6.81 (d, J = 8.6 Hz, 1H), 6.44 (dd, J = 8.6, 2.6 Hz, 1H), 6.36 (d, J = 2.6 Hz, 1H), 3.71 (s, 3H), 3.20 (s, 3H).  
**¹³C NMR (126 MHz, CDCl₃):**  δ 190.98, 163.45, 154.41, 152.41, 147.08, 141.96, 140.86, 136.36, 134.11, 133.39, 131.00, 130.84, 126.77, 126.63, 123.25, 121.65, 114.76, 110.77, 102.66, 56.02, 41.84.  
**ESI-HRMS (m/z):** [M+H]^+ calc'd. for C₂₂H₁₉N₂O₃S 391.1116, found.391.1113.

### Synthesis of probe NP560

Intermediate 4 (50 mg, 0.13 mmol) and N-ethyl-2,3,3-trimethyl-3H indol bromide (24 mg, 0.13 
mmol) was dissolved in toluene (5.0 mL) and the mixture was cooled to 0 °C with an ice bath. 
Piperidine (12 µL, 0.13 mmol) was added to the mixture and the reaction was heated to reflux. The 
reaction was monitored by TLC analysis till the disappearance of 4. After cooling to ambient 
temperature, the mixture was diluted with EtOAc (20 mL) and was washed with with H₂O (1×10mL) 
and brine (1×10 mL), dried over anhydrous Na₂SO₄, and concentrated under reduced pressure. The 
resulting residue was purified by flash column chromatography (SiO₂: DCM/MeOH, 20:1) to give 
NP560 as a purple black solid (62 mg, 86%). 

**M.p.:** 240.1-241.5°C  
**Rf =** 0.34 (1:20, MeOH:DCM)  
**IR (cm⁻¹):** 3142, 2986, 2930, 2360, 1606, 1594, 1518, 1471, 1272, 791  
**¹H NMR (500 MHz, CDCl₃):**  δ 9.01 (d, J = 8.5 Hz, 1H), 8.37 (d, J = 1.6 Hz, 1H), 8.22 (d, J = 16.0 Hz, 
1H), 8.04 (d, J = 8.1 Hz, 1H), 7.89 (d, J = 16.0 Hz, 1H), 7.83 (d, J = 7.9 Hz, 1H), 7.68 (d, J = 7.7 Hz, 1H),  
7.61 – 7.55 (m, 3H), 7.51 – 7.44 (m, 2H), 7.38 (t, J = 7.5 Hz, 1H), 6.75 (d, J = 8.5 Hz, 1H), 6.47 (dd, J = 8.5,  
2.3 Hz, 1H), 6.42 (d, J = 2.2 Hz, 1H), 5.09 (q, J = 7.1 Hz, 2H), 3.72 (s, 3H), 3.27 (s, 3H), 1.85 (s, 6H), 1.63  
(t, J = 7.5 Hz, 3H)  
**¹³C NMR (126 MHz, CDCl₃):**  δ 180.79, 164.34, 154.56, 153.81, 152.39, 146.99, 143.42, 141.76,  
141.39, 140.59, 136.19, 133.67, 129.94, 129.83, 129.40, 129.03, 126.57, 126.38, 125.62, 122.99,  
122.78, 121.73, 114.95, 114.76, 112.92, 111.74, 104.26, 56.16, 52.36, 44.59 (d, J = 8.1 Hz), 42.54, 
27.28, 21.93, 14.62.  
**ESI-HRMS (m/z):** [M]^+ calc'd. for C₃₅H₂₆N₂O₂S⁺ 560.2372, found 560.2389.
General experimental for photophysical property characterization

All the photophysical characterization experiments were carried out in Tris-buffer solution (10 mM, pH 7.4) at ambient temperature. Probe NP560 was dissolved in DMSO to make a 5 mM stock solution. ONOO− and other bio-relevant species were prepared as described previously (J. Am. Chem. Soc. 2015, 137, 12296−12303).

To test the photophysical response of NP560 towards ONOO− or other reactive species, aliquots of probe stock solutions were diluted with Tris-buffer solution and treated with analytes to make sure both probes and analytes were kept at desired final concentrations. After quick and vigorous shaking, the mixture was allowed standing in the dark for 15 min and then the photophysical properties were recorded. All experiments were performed in triplicate, and data shown were the average.

Confocal fluorescence staining and analysis

EA.hy926 cells were used in the study. Briefly, the cells were grown in DMEM with 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere containing 5% CO₂. After culture on glass cover slips overnight, cells were treated with ONOO− or SIN-1 for indicated time, then stained with NP560 probe (5 μM) at 37 °C for 30 min before being fixed in 4 % PFA and imaged by confocal microscope (Nikon A1R).

For time-lapse confocal imaging in live cells, endothelial cells were cultured on glass-bottom plates overnight, then were incubated with NP560 probe (5 μM) at 37 °C for 30 min. The dynamic change of NP560 fluorescence in live cells with SIN-1 (2 mM) stimulation was captured by time-lapse confocal microscope for 60 min. The fluorescence density was analyzed using Image J software (NIH, Bethesda, MD, USA).

Cytotoxicity assay

The Cell Counting Kit-8 (CCK-8; Beyotime, Shanghai, China) was used to analyze the effects of NP560 on EA.hy926 endothelial cells viability according to the manufacturer’s protocols. Briefly, equal numbers of cultured EA.hy926 endothelial cells were incubated with or without NP560 (2.5, 5, 10, 15 μM) in a 96-well plate for 24 h. Then cells were incubated with CCK-8 solution (10 μl per well) at 37 °C with 5% CO₂ for 3 h. Absorbance at 450 nm was measured with a microplate reader (Elx800, Bio-Tek, Winooski, VT, USA). The well with medium and CCK-8 solution but without cells was used as a blank control. Cell viability of vehicle-treated control groups not exposed to NP560 was defined as 100%. Data are expressed as mean ± S.E.M. ns P>0.05 versus control.
Fig. S1 Liquid chromatography spectra of probe **NP560** (20 μM, peak a) after the treatment of various amounts of ONOO\(^-\). The intensity of peak a decreased in a ONOO\(^-\)-dose dependent way, accompanied by the emergence of peak b and peak c.
Fig. S2 Mass spectra of peak a in Fig S1. The m/z 560 signal was corresponding to the structure of NP560.

Fig. S3 Mass spectra of peak c in Fig S1. The m/z 438 signal was corresponding to the structure of NP560-I.

Fig. S4 Mass spectra of peak b in Fig S1. The m/z 212 signal was corresponding to the structure of 1-ethyl-3,3-trimethyloxindole ([M+Na]^+).
Fig. S5 Fluorescence response of NP560 towards various amounts of ONOO⁻. Data shown were the emission intensity at 583 nm or 475 nm of NP560 (5 µM) after the treatment of ONOO⁻ of indicated concentrations, λex 375 nm.

Fig. S6 Fluorescence response of NP560 towards the sequent treatment of small portions of ONOO⁻. Data shown were the time-lapsed emission at 583 nm or 475 nm of NP560 (5 µM) after the sequent treatment of ONOO⁻ (1 µM each time), λex 375 nm.
Step 1: Blank measurements (n=20). Mean: FB = 1.51433 ± 0.196429;

Step 2: Linear regression analysis on fluorescence intensity (583 nm) and the corresponding ONOO⁻ concentrations (0-1 μM):

![Linear Regression Graph](image)

\[ y = 1.31243 + 15.2676x \]

\[ R = 0.994 \]

Step 3: Detection limit calculation: C_{LOD} = 3σ/s

Where σ is the standard deviation of the blank measurements;
s is the slope of the calibration curve line obtained from the linear regression analysis.

Fig. S7 Procedures for limit of detection determination.

![Time-lapse Graph](image)

Fig. S8 Time-lapsed response of NP560 towards ONOO⁻. Data shown were the time-lapsed emission intensity change of NP560 at 583 nm after the treatment of ONOO⁻.
Fig. S9 Effects of NP560 on cell viability determined with the CCK-8 assay. The Cell Counting Kit-8 (CCK-8, Beyotime, Shanghai, China) was used to analyze the effects of NP560 on EA.hy926 endothelial cells viability according to the manufacturer’s protocols. Briefly, equal numbers of cultured EA.hy926 endothelial cells were incubated with or without NP560 (2.5, 5, 10, 15 μM) in a 96-well plate for 24 h. Then cells were incubated with CCK-8 solution (10 μl per well) at 37 °C with 5% CO₂ for 3 h. Absorbance at 450 nm was measured with a microplate reader (ELx800, Bio-Tek, Winooski, VT, USA). The well with medium and CCK-8 solution but without cells was used as a blank control. Cell viability of vehicle-treated control groups not exposed to NP560 was defined as 100%. Data are expressed as mean ± S.E.M. ns P>0.05 versus control.

Fig. S10 Intracellular fluorescence of NP560 in response to SIN-1 treatment. EA.hy926 endothelial cells were cultured on glass cover slips overnight and then incubated with SIN-1 at various concentrations for 1 h, followed by NP560 (5 μM) staining for 0.5 h. Cells were then fixed in 4 % PFA for 10 min and imaged by confocal microscope. The intracellular NP560 fluorescence was recorded with excitation at 405 nm and emission at 420-500 nm for the green channel colour, and excitation at 543 nm and emission at 560-630 nm for the red channel colour.
Fig. S11 Real-time tracking of endogenous ONOO⁻ formation by NP560. EA.hy926 endothelial cells were loaded with NP560 and then treated with SIN-1 (2 mM). Time-lapsed series of single confocal plane images were recorded with excitation at 405 nm and emission at 420-500 nm for the green channel colour, and excitation at 543 nm and emission at 560-630 nm for the red channel colour.

Fig. S12. Quantification of dynamic change of NP560 mean fluorescence intensity in Fig. S11. Data are presented as ratio to control.
Supplementary Movie 1. Time-lapse series of Fig. S11 with NP560 fluorescence being recorded with excitation at 405 nm and emission at 420-500 nm for the green channel colour.

Supplementary Movie 2. Time-lapse series of Fig. S11 with NP5605 fluorescence being recorded with excitation at 543 nm and emission at 560-630 nm for the red channel colour.