Transforming the recognition site of 4-hydroxyaniline into 4-methoxyaniline grafted onto a BODIPY core switches the selective detection of peroxynitrite to hypochlorous acid

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1. Materials and instruments

Chemicals for synthesis were obtained from commercial suppliers and used without further purification unless otherwise stated. $^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker AV-400 spectrometer at ambient temperature. Mass spectra were measured on a HP 1100 LC-MS spectrometer. In the experiments of absorption or fluorescence measurements, compounds were dissolved in CH$_3$CN to obtain stock solutions (1.0 mM). These stock solutions were then diluted to the desired concentration. UV-vis absorption spectra were obtained on a Varian Cary 100 spectrophotometer. Fluorescence spectra were measured with a Varian Cary Eclipse Fluorescence spectrophotometer.

Cell Cultures. In a humidified atmosphere of 5/95 CO$_2$/air incubator, raw264.7 macrophages and hepatocytes were cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640) supplemented with 10% fetal bovine serum (FBS) at 37 °C.

Confocal image of cells. Cells were grown in a glass bottom dish for 24 h. For image of ONOO$^-$, cells were stained with BHAni (10 µM) in culture medium for 15 min at 37 °C, washed with D-Hanks. Then peroxynitrite donor 3-morpholinosydnonimine (SIN-1) (50 µM) was introduced for 30 min. For HOCl, cells were stimulated with lipopolysaccharides (LPS, 1 µg/mL) for 12 h, followed by phorbol myristate acetate (PMA, 1 µg/mL) for 1h, then loaded with BMAhi (10 µM) for 30 min. The excitation wavelength was 488 nm, and images were collected at 500-600 nm.

Synthesis of NC-B-Cl.
The mixture of 2-benzoyl-5-chloropyrrole (206 mg, 1 mmol) and POCl₃ (0.5 mL) in ClCH₂CH₂Cl (5 mL) was stirred for 12 h at room temperature. 2-(1H-2-pyrrolylmethylene) malononitrile (172 mg, 1.2 mmol) in ClCH₂CH₂Cl (15 mL) was then added to the reaction mixture, and further stirred at 85 °C for 12 h under N₂. After cooling to room temperature, the mixture was neutralized with saturated aqueous NaHCO₃, washed with water, and then dried over anhydrous Na₂SO₄. The solvent was evaporated in vacuo, and intermediate 1 was obtained, which was used for the next step without purification. Intermediate 1 was re-dissolved in CH₂Cl₂ (20 mL). Et₃N (0.8 mL) was then added to the mixture and further stirred for another 30 min, then BF₃·Et₂O (0.8 mL) was added, and the resulting mixture was stirred at room temperature overnight. The reaction mixture was washed with water, and dried over anhydrous Na₂SO₄. After evaporating the solvent in vacuo, the residue was purified by column chromatography to afford compound NC-B-Cl (176 mg, 47%). 

**1H NMR (CDCl₃, 400 MHz):** δ 8.29 (s, 1H), 7.69-7.65 (m, 2H), 7.61-7.57 (t, 2H), 7.53-7.52 (m, 2H), 7.16-7.15 (d, 1H), 6.90-6.89 (d, 1H), 6.67-6.66 (d, 1H); 

**13C NMR (CDCl₃, 100 MHz):** δ 153.1, 146.7, 145.5, 142.5, 137.6, 136.8, 135.9, 132.1, 131.7, 130.5, 128.9, 128.7, 123.0, 121.2, 113.7, 113.1, 82.8; 


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**S3**
Synthesis of BHAni.

Compound **NC-B-Cl** (37.8 mg, 0.1 mmol) and 4-hydroxyaniline (13.1 mg, 0.12 mmol) were mixed in CH$_2$Cl$_2$, and the mixture was stirred at room temperature for 30 min. The reaction mixture was washed with water, and dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated in vacuo, and the residue was purified by column chromatography to afford compound **BHAni** (36.2 mg, 80%). $^1$H NMR (d$_6$-DMSO, 400 MHz): δ 10.94 (s, 1H), 9.83 (s, 1H), 8.07 (s, 1H), 7.57-7.54 (m, 4H), 7.53-7.51 (m, 2H), 7.23-7.21 (d, 2H), 7.12-7.11 (d, 1H), 6.86-6.84 (d, 2H), 6.56-6.55 (d, 1H), 6.53-6.52 (d, 1H); $^{13}$C NMR (d$_6$-DMSO, 100 MHz): δ 161.8, 156.8, 145.8, 139.4, 138.0, 136.9, 132.9, 132.7, 130.1, 129.4, 128.6, 128.1, 127.0, 125.2, 120.8, 119.4, 118.6, 115.7, 115.5, 114.5, 71.3; HRMS (ESI): calcd for C$_{25}$H$_{16}$BF$_2$N$_5$ONa: 474.1314, Found: 474.1313 [M + Na]$^+$. 

Synthesis of BMAni.
The mixture of compound **NC-B-Cl** (37.8 mg, 0.1 mmol) and 4-methoxyaniline (14.8 mg, 0.12 mmol) was stirred at room temperature for 30 min, then washed with water, dried over anhydrous Na$_2$SO$_4$, evaporated. The residue was purified by column chromatography to afford **BMAri** (38.0 mg, 82%). $^1$H NMR (d$_6$-DMSO, 400 MHz): $\delta$ 10.83 (broad, 1H), 8.08 (s, 1H), 7.58-7.55 (m, 4H), 7.53-7.51 (m, 2H), 7.37-7.34 (d, 2H), 7.14-7.13 (d, 1H), 7.06-6.03 (d, 2H), 6.58-6.56 (d, 1H), 6.54-6.53 (d, 1H), 3.80 (s, 3H); $^{13}$C NMR (d$_6$-DMSO, 100MHz): $\delta$ 161.9, 158.4, 145.9, 139.5, 138.0, 137.0, 133.0, 132.8, 130.2, 129.8, 129.5, 128.7, 127.0, 125.6, 120.9, 119.4, 118.8, 115.5, 114.6, 114.5, 71.5, 55.4; HRMS (ESI): calcd for C$_{26}$H$_{17}$BF$_2$N$_5$O: 464.1494, Found: 464.1502 [M - H]$^-$.
2. Time-dependent fluorescence intensity changes of BHAni.

Figure S1. (a) and (b) Time-dependent fluorescence intensity changes of BHAni (1 µM) at 530 nm in the presence of ONOO⁻; Kinetic studies in b were explored using a stopped-flow spectrophotometer at 25 °C.
3. Fluorescence responses of BHAni toward ROS.

Figure S2. Fluorescence responses of BHAni (1 µM) toward ROS; ONOO⁻ (5 µM) and other ROS (10 µM).
4. Fluorescence responses of BMAni toward ROS.

Figure S3. Fluorescence responses of BMAni (1 µM) toward ROS. HOCl (5 µM), other ROS (10 µM).
Figure S4. (a) Time-dependent fluorescence changes of BMAni (1 µM) in the presence of 10 µM HOCl. (b) Time course of fluorescence intensity of BMAni at 528 nm in the presence of HOCl.
6. pH dependent fluorescence changes.

Figure S5. pH dependent fluorescence changes of (a) BHAni at 530 nm in the absence and presence of ONOO⁻ and (b) BMAni at 528 nm in the absence and presence of HOCl.

Figure S6. $^1$NMR (CD$_3$OD) and HRMS characterization of oxidation product from the reactions of BHAni + ONOO$^-$ or BMAni + HOCl.
8. Confocal fluorescence microscopy images of ONOO$^-$. 

Figure S7. Confocal fluorescence microscopy images of ONOO$^-$ in raw264.7 macrophages cells, the excitation wavelength was 488 nm and the emission was collected at 500-600 nm. a-c) cells stained with BHAni (10 µM) for 30 min; d-f) cells loaded with BHAni for 15 min, followed by treated with SIN-1 (50 µM) for 30 min; g-i) cells pretreated with urate (100 µM) for 30 min were incubated with BHAni for 15 min, subsequently loaded with SIN-1 for 30 min. The scale bars are 20 µm.
9. Confocal fluorescence microscopy images of HOCl.

Figure S8. Confocal fluorescence microscopy images of HOCl in raw264.7 macrophages cells, the excitation wavelength was 488 nm and the emission was collected at 500-600 nm. a-c) cells stained with BMAni (10 µM) for 30 min; d-f) cells were stimulated with LPS (1.0 µg/mL) for 12 h and followed by PMA (1.0 µg/mL) for 1 h, then treated with BMAni for 30 min; g-i) cells pretreated with ABH (200 µM) for 15 min were stimulated with LPS (1.0 µg/mL) for 12 h and followed by PMA (1.0 µg/mL) for 1 h, then stained with BMAni for 30 min. The scale bars are 20 µm.
10. A concentration-dependent fluorescence relationship
Figure S9 A) Image intensity of BMAni (10 µM) in the presence of various exogenous NaOCl; B) Image intensity of BHAni (10 µM) in the presence of various 3-morpholinosydnonimine hydrochloride (SIN-1); C) Image intensity changes of (a) BMAni (10 µM) (estimated from A) as a function of exogenous NaOCl increasing concentrations and (b) BHAni (10 µM) (estimated from B) as a function of SIN-1 increasing concentrations.
11. NMR and HRMS spectra.
Elemental Composition Report

Single Mass Analysis
Tolerance = 50.0 PPm / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
215 formula(s) evaluated with 2 results within limits (up to 1 best isotopic matches for each mass)
Elements Used:

ZhAO-CC
ZC-A/C/D 23 (0.750) Cm (19.55)

Minimum: 474.1513
Maximum: 474.1514

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Elemental Composition Report

Single Mass Analysis
Tolerance = 50.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
55 formulae(s) evaluated with 1 result(s) within limits (up to 1 best isotopic match for each mass)
Elements Used:
C: 0-20 H: 0-20 B: 0-1 N: 0-5 O: 0-1 F: 0-2

Minimum: 300.0 50.0 100.0

Mass  Calc. Mass  mDa  ppm  DBE  1-FIT  1-FIT (Horn)  Formula
464.1522  464.1494  0.8  1.7  20.5  39.6  0.0  C26 H17 B N5 O F2