Supplementary Information for

Rational Design of a Highly Sensitive and Selective Fluorogenic Probe for Detecting Nitric Oxide

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Table of Contents

Materials and methods..................................................................................S2
Experiments and characterizations...............................................................S4
Fig. S1 the crystal structures of 1 and 2.....................................................S7
Fig. S2 the pH dependence assay of 2.......................................................S8
Calculation of detection limit......................................................................S9
Calculation methods...................................................................................S10
Table S1 energy level of elected MOs.........................................................S11
Fig. S4 the selectivity of probe 1 to NO and other reactive nitrogen or oxygen species.................................................................S12
Cytotoxicity of probe 1 and 2......................................................................S13
NO imaging in He La cells..........................................................................S14
Appendix......................................................................................................S16
Materials and Methods

Solvents and reagents were purchased from commercial suppliers and used without further purification unless otherwise indicated. Spectroscopic grade DMSO were used for spectroscopic studies.

The $^1$HNMR and $^{13}$CNMR spectra were recorded on Varian BrÜker 400 MHz spectrometer. Mass spectra were obtained from a Quattro microtriple quadrupole mass spectrometer (Waters, Milford, MA, USA). UV-Vis spectra were recorded on a Cary-50 UV-Vis spectrophotometer. Fluorescence spectra were recorded on a Cary Eclipse Fluorescence spectrophotometer ($V = 600$ volts). The adjustable slit was set at 5 nm. Fluorescence quantum yield of samples were recorded on a Fluormax-4 spectrophotometer at room temperature with an integrating sphere system, and the machine was reevaluated using standard sample before measurement. Melting points were taken on a X-5B precise micro melting point apparatus. Single-crystal X-ray diffraction measurements were carried out on Rigaku MicroMax 002 CCD diffractometer at 298k using CuKα radiation. All structures were solved by direct methods and refined by full-matrix least squares on F$^2$ using the Shelxs-97 computer program package.

The nitric oxide (NO) stock solution in de-ionized water was prepared according to the literature. Peroxynitrite was generated from amyl nitrite and H$_2$O$_2$.

Quartz cuvettes with 10 mm path lengths and four faces polished were used. Stock solutions of probe 1 (5 mM) were prepared in phosphate buffer (50 mM, pH 7.4) with 20% DMSO.

Cell Culture and Imaging Materials and Methods. HeLa cells and Raw 264.7 cells were obtained from Cell Resource Center (IBMS, CAMS/PUMC) and cultured in Dulbecco’s Modified Eagle Medium (DMEM; Thermo Scientific) supplemented with 10% fetal bovine serum (FBS; GIBCO; Invitrogen), and 1% penicillin/streptomycin (Beijing Solarbio Scientific & Technology Co, Ltd). For imaging studies, cells were plated in Class Bottom Cell Culture Dish (Nest) containing 1 mL of complete DMEM and incubated at 37 ℃ under 5% CO$_2$ for one day. To induce NO production, Raw 264.7 cells were stimulated with 0.5 µg mL$^{-1}$ Lipopolysaccharide (LPS, Sigma-Aldrich) for 4 h. To induce NO production, Raw 264.7 cells were stimulated with 2
mM sodium nitroprusside (SNP) for 30-150 minutes. Bright filed and fluorescence images were taken with a Zeiss Abserver A1 inverted fluorescence microscope equipped with an EM-CCD camera (Hamamatsu) and an X-Cite 120 metal halide lamp (EXFP). Bright field image and fluorescence images were obtained using an 40× objective lens.


**Scheme S1.** Synthetic scheme of 1 and 2. (a) Ethyl acetoacetate, H$_3$PO$_4$, rt 12h; (b) Acetone, K$_2$CO$_3$, CH$_3$I, reflux, 6 h; (c) SeO$_2$, xylene, reflux, 10 h; (d) Methy acetoacetate, NH$_3$·H$_2$O, EtOH, reflux, 4 h.

**4-Methyl-7-hydroxylcoumarin (3).** 3-hydroxylphenol (11 g, 100 mmol) and ethyl acetoacetate (13 mL, 100 mmol) were added into concentrated phosphoric acid (52 mL, 85%). After stirring at room temperature for 12 hours, the reaction mixture was poured into 150 mL water. The crude product was collected by filtration and purified by recrystallization in ethanol to afford 3 as white crystal (23.7 g, yield 98%). Mp. 160~162°C, $^1$H NMR (400 MHz DMSO-$d_6$) δ 10.50 (s, 1H), 7.58 (t, 1H, $J$ = 5.3 Hz), 6.82 (t, 1H, $J$ = 7.1 Hz), 6.72 (d, 1H, $J$ = 1.5 Hz), 6.13 (s, 1H), 2.37 (d, 3H, $J$ = 0.8 Hz). $^{13}$C NMR (400 MHz DMSO-$d_6$) δ 161.04, 160.17, 154.71, 153.20, 126.26, 112.68, 111.86, 110.14, 102.06, 17.93; ESI MS calculated 176.1, found 177.1 (M+H$^+$).

**4-Methyl-7-methoxylcoumarin (4).** To a solution 30 mL acetone solution of 3 (3.5 g, 22 mmol), anhydrous potassium carbonate (6.0 g, 44 mmol) was added. The reaction mixture was refluxed for 10 min, and then methyl iodide (3.1 g, 22 mmol) was added dropwise. The resulting mixture was refluxed for 6 more hours and filtered without cooling. The filtrate was evaporated in vacuo to give the crude product which was purified by recrystallization in ethanol to afford 4 as white crystal. Mp. 163~165 °C, $^1$H NMR (400 MHz CDCl$_3$) δ 7.50 (d, 1H, $J$ = 8.7 Hz), 6.86 (m, 1H, $J$ = 2.5 Hz), 6.81 (d, 1H, $J$ = 2.4 Hz), 6.13 (d, 1H, $J$ = 0.9 Hz), 3.87 (s, 3H), 2.40 (d, 3H, $J$ = 1.0 Hz). $^{13}$C NMR (400 MHz CDCl$_3$) δ 161.05, 153.53, 152.44, 142.88, 125.34, 124.26, 117.52, 117.10, 113.92, 21.55, 18.56; ESI MS calculated 189.1, found 190.1 (M+H$^+$).
4-Formyl-7-methoxycoumarin (5). 4 (0.95 g, 5 mmol) was dissolved in 30 mL xylene and heated to reflux. After addition of selenium dioxide (1.1 g, 10 mmol), the reaction mixture was refluxed for 10 hours. The resulting suspension was filtrated without cooling. While filtrate was cooled to room temperature, crude product precipitated out of solution and was collected by filtration. Recrystallization in acetonitrile afforded 5 (0.85 g, 84%) as yellow needle-like crystal. Mp. 126~127℃, 

$^1$H NMR (400 MHz CDCl$_3$) δ 10.07 (s, 1H), 8.49 (d, 1H, $J$ = 5.0 Hz), 6.93 (m, 1H, $J$ = 2.5 Hz), 6.86 (d, 1H, $J$ = 2.4 Hz), 6.71 (s, 1H ), 3.89 (s, 3H ). $^{13}$C NMR (400 MHz DMSO-d$_6$) δ 193.70, 162.55, 160.37, 155.87, 143.50, 126.87, 121.28, 112.84, 108.16, 101.06, 55.93; ESI MS calculated 203.1, found 204.2 (M+H$^+$).

**Dimethyl 4-(7-methoxy-2-oxo-2H-chromen-4-yl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (1).** 5 (0.3 g, 1.47 mmol), methyl acetoacetate (1.1 g, 9.2 mmol) and ammonia solution (0.2 g, 9.2 mmol) were dissolved in 20 mL ethanol and refluxed for 4 hours. After cooling to room temperature, the reaction mixture was concentrated in vacuo to give crude product which was purified by recrystallization in ethanol to afford 1 (0.9 g, 42%) as yellowish crystal. Mp. 249~251℃, $^1$H NMR (400 MHz CDCl$_3$) δ 8.12 (d, 1H, $J$ = 9.1 Hz), 6.93 (d, 1H, $J$ = 2.5 Hz), 6.91(d, 1H, $J$ = 2.5 Hz), 6.79(d, 1H, $J$ = 2.4 Hz), 6.24 (s, 1H), 5.35 (s, 1H), 3.88 (s, 3H), 3.51 (s, 6H), 2.35 (s, 6H). $^{13}$C NMR (400 MHz DMF-d$_6$) δ 167.20,165.45, 155.38, 146.83, 127.83, 112.04, 11.98, 111.52, 101.67, 100.23, 55.68, 50.31, 17.90; ESI MS calculated 399.1, found 400.1 (M+H$^+$).

**Dimethyl 4-(7-methoxy-2-oxo-2H-chromen-4-yl)-2,6-dimethylpyridine-3,5-dicarboxylate (2).** NO gas was Bubbled through a CH$_2$Cl$_2$ solution containing 1 (0.2 g, 0.5 mmol) at room temperature and the reaction was monitored by TLC. The reaction mixture was sequentially washed with saturated NaHCO$_3$ and NaCl solutions, and dried over anhydrous MgSO$_4$ and evaporated in vacuo. The resulting residue as crude product was purified by flash chromatography with a mixture ethyl acetate and
petroleum ether (1:1) as eluent to afford 2 as yellowish crystalline (0.19 g, yield 95%).

Mp. 193 ～195 ℃, ¹H NMR (400 MHz CDCl₃) δ 6.96 (d, 1H, J = 8.8 Hz), 6.85 (d, 1H, J = 2.3 Hz), 6.78 (m, 1H, J = 2.4 Hz), 6.08 (s, 1H), 3.87 (s, 3H), 3.57 (s, 6H), 2.68 (s, 6H). ¹³C NMR (400 MHz DMSO-d₆) δ 166.25, 162.72, 159.12, 156.93, 154.60, 151.35, 140.23, 127.47, 124.70, 112.59, 111.15, 111.10, 100.87, 56.00, 52.47, 23.18; ESI MS calculated 397.1, found 398.3 (M+H⁺).
Figure S1. Crystal structures of (a) 1 and (b) 2.
Figure S2. The pH dependence of the fluorescence intensity of compound 2 (10 μM) in phosphate buffer solution with 20% DMSO. \( \lambda_{\text{ex}} = 334 \text{ nm} \), \( \lambda_{\text{em}} = 450 \text{ nm} \).
Calculation of the detection limit

The detection limit was calculated on the basis of literature previously reported. The fluorescence emission spectrum of probe 1 was determined by three times and the standard of blank measurement was achieved.

The detection limit was calculated by following formula:

\[
\text{Detection limit} = \frac{3s}{k}
\]

Where \(s\) is the standard deviation of blank measurement, \(k\) is the slope between the fluorescence intensity ratio versus NO concentration.


**Calculation methods**

In this work, the quantum chemical calculations were carried out using the Gaussian 09 program package. The possible ground state structures of probe 1 and compound 2 have been optimized with density functional theory (DFT) at B3LYP/6-31+G (d) level, in which the effects of solvent were considered using polarized continuum model (PCM) with 50 mM phosphate buffer (pH 7.4, 20% DMSO) as solvent. On the basis of optimized configuration for the ground state, TD-DFT calculations were performed using the B3LYP functional (TD-B3LYP-SCRF) within the adiabatic approximation to predict the excitation energies. In order to account for fluorescence properties, TD-B3LYP-SCRF calculations have also been performed to locate the excited state structures of probe 1 and compound 2.

![Figure S3](image)

**Figure S3.** (a). Molecular orbital plots for the HOMO-1, HOMO, LUMO and LUMO+1 of 1 and 2; (b). Jablonski diagram for PET pathways of 1; (c) Jablonski diagram for a local excited state (1LE) emission of 2. 1ET, electron transfer state.

**Table S1.** The energy level of selected molecular orbital of 4, 1 and 2
<table>
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<tr>
<th>Compound</th>
<th>HOMO-1 (eV)</th>
<th>HOMO (eV)</th>
<th>LUMO (eV)</th>
<th>LUMO+1 (eV)</th>
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</tr>
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<td>-2.27</td>
<td>-1.56</td>
</tr>
<tr>
<td>2</td>
<td>-7.20</td>
<td>-6.48</td>
<td>-2.36</td>
<td>-1.65</td>
</tr>
</tbody>
</table>


*Figure S4.* Fluorescence intensity of probe 1 (10 μM, in phosphate buffer 50 mM, pH 7.4) at 450 nm upon the addition of 100 equivalents of various oxidative species: NO, ascorbic acid (AA), H₂O₂, ¹O₂, KNO₃, NaNO₂, OONO⁻, ClO⁻: λₓₓ = 334 nm.
Cytotoxicity of probe 1 and compound 2

HeLa cells were grown in 96-well plates at an initial density $5 \times 10^3$ cells per well for 24 h. Subsequently, the 10 μM, 40 μM and 80 μM of probe 1 and compound 2 were incubated for 12 h, respectively. Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazoilum bromide (MTT) reduction assay. After incubation, MTT (20 μL, 5 mg mL$^{-1}$) assay was added to each well for 4 h. DMSO (100 μL) was added to each well after removing media. Absorption at 490 nm was measured on a plate reader.

Raw 264.7 cells were grown in 96-well plates at an initial density $5 \times 10^3$ cells per well for 24 h. Subsequently, the 10 μM, 40 μM and 80 μM of probe 1 and compound 2 were incubated for 12 h, respectively. Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazoilum bromide (MTT) reduction assay. After incubation, MTT (20 μL, 5 mg mL$^{-1}$) assay was added to each well for 4 h. DMSO (100 μL) was added to each well after removing media. Absorption at 490 nm was measured on a plate reader.

**Figure S5.** MTT assay of Raw 264.7 cells treated with probe 1

**Figure S6.** MTT assay of Raw 264.7 cells treated with compound 2
NO imaging in HeLa cells

Figure S7. Fluorescence response of probe 1 in HeLa cells. The fluorescence response were monitored for cells after 150 min of incubation with probe 1 (40 μM) (a) and for cells co-treated with SNP (2 mM) and probe 1 (40 μM) for 30 min (b), 60 min (c), 90 min (d), 120 min (e), 150 min (f). Images were taken with a Zeiss Abserver A1 inverted fluorescence microscope after removing DMEM and washing the cells three times with PBS.
**Figure S8.** MTT assay of HeLa cells treated with probe 1

**Figure S9.** MTT assay of HeLa cells treated with compound 2