Design and Synthesis of a Highly Selective Fluorescent Turn-on Probe for Thiol Bioimaging in Living Cells

Xin Li,[a] Shijing Qian,[b] Qiaojun He,[b] Bo Yang,[b] Jia Li,[c] and Yongzhou Hu[*, a]

[a]ZJU-ENS Joint Laboratory of Medicinal Chemistry, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China

[b]Institute of Pharmacology and Toxicology, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China

[c]State key laboratory of drug research, Shanghai Institute of Material Medica, Chinese Academy of Science, Shanghai 201203, China

huyz@zju.edu.cn

Tel/Fax: 086-571-88208460

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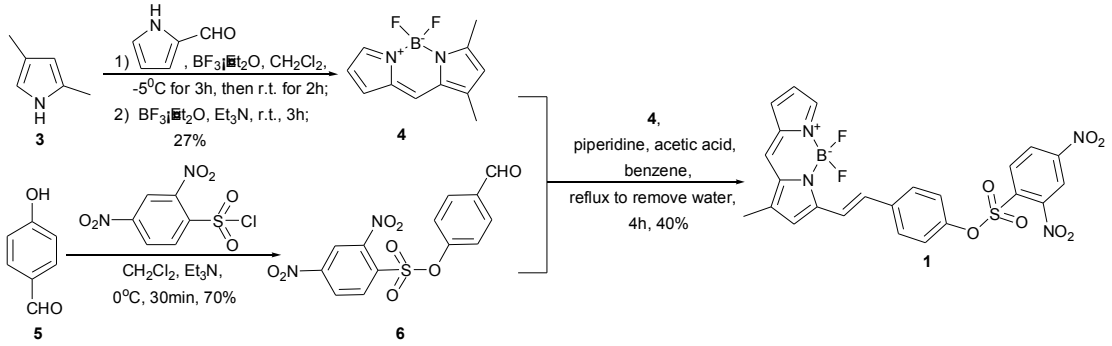
Synthesis and spectroscopic data of 1, 2, 4, 6

General: Unless otherwise noted, all reagents were purchased from commercial suppliers and used without further purification. Anhydrous CH_2Cl_2 was obtained by being distilled from GaH_2 and anhydrous benzene distilled from Na prior to use. Reactions were monitored by thin layer chromatography using TLC Silica gel 60 F254 supplied by Qingdao Puke Seperation Meterial Corporation, Qingdao, P. R. Chin. Silica gel for column chromatography was 200-300 mesh and was supplied by Qingdao Marine Chemical Factory, Qingdao, P. R. China. Characterization of intermediates and final compounds was done using IR, NMR spectroscopy and mass spectrometry, final purity of **1** was controlled using two different HPLC systems. Melting points were measured on BÜCHI B-540 and uncorrected. IR spectra were obtained on a Bruker Vector 22 spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker DPX-400 or Bruker DPX-500 Fourier transform spectrometer or with *d*-$CHCl_3$ or d_6-DMSO as solvents and tetramethylsilane (TMS) as the internal standard. All spectra were recorded at 25^{o}C and chemical shifts were given in ppm and coupling constants (*J*) in Hz. Low-resolution mass data were obtained on a Finnigan LCQ DECA XP plus LCMS spectrometer. High-resolution mass data were obtained on a Micromass Q-Tof Ultima^{TM} spectrometer. HPLC chromatograms were recorded on an Agilent 1100 series LC system (Agilent ChemStation A.08.03) equipped with a VWD (G1314A) and C18 column (4.6 ×200 mm × 5 μm, DiamonsilTM). Absorption spectra were acquired using a Hitachi U-3010 spectrophotometer. Fluorescence measurements were carried out on a PE LS45 fluorescence spectrometer.

Synthesis

Scheme S1. Synthesis of probe **1**



1,3-Dimethyl 4,4-Difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene (4). Pyrrole 2-carboxyaldehyde (500 mg, 5.26 mmol) was dissolved in dry dichloromethane (30 mL) and cooled down to -5^{o}C under nitrogen atmosphere. 2,4- Dimethylpyrrole (**3**, 542 μL, 5.26 mmol) was then added to the mixture slowly and stirred for 5 min, followed by dropwise addition of $BF_3{\cdot}Et_2O$ (660 μL, 5.26 mmol). After being stirred at -5 ^{o}C for 3 h, the reaction was allowed to stand at rt and stirred for 2 h. Then another portion of $BF_3{\cdot}Et_2O$ (1.98 mL, 9.45 mmol) and Et_3N (2.19 ml, 15.8 mmol) were added subsequently at rt. After 3 h, the reaction mixture was quenched with H_2O (10 mL) and diluted with CH_2Cl_2 (20 mL). The organic phase was then washed with H_2O (×3), brine (×1) and dried over Na_2SO_4. The solvent was evaporated, and the residue was purified by silica gel column chromatography eluted with petrol ether (PE)/EtOAc (10:1) to yield 314 mg (1.43 mmol, 27%) of **4** as a dark red solid.

Mp (from PE/EtOAc = 10:1) 136-138^{o}C.

IR (KBr): ν_{max} = 3069, 2921, 1600, 1531, 1466, 1399, 1270, 985 cm^{-1}.

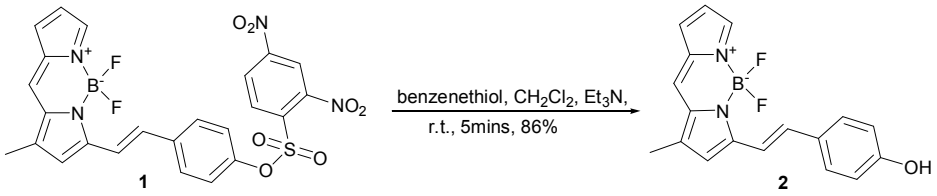
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.64 (s, 1H), 7.20 (s, 1H), 6.92 (s, 1H), 6.43 (s, 1H), 6.16 (s, 1H), 2.58 (s, 3H), 2.27 (s, 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 163.13, 145.79, 139.25, 136.51, 132.65, 126.51, 124.75, 121.26, 116.33, 15.18, 11.40.
ESI-MS *m*/*z* 219.3 $[M-H]^-$.

4-Formylphenyl 2,4-dinitrobenzenesulfonate (6). To a stirred solution of 4-hydroxybenzaldehyde (**5**, 200 mg, 1.64 mmol) in dry CH_2Cl_2 (15 mL) was added Et_3N (250 μL, 1.8 mmol). The mixture was then cooled to 0°C and a solution of 2,4-dinitrobenzenesulfonyl chloride (479 mg, 1.8 mmol) in dry CH_2Cl_2 (5 mL) was added dropwise. After being stirred at 0°C for 30 min, the starting materials were found to be totally consumed by TLC monitoring. H_2O was then added to quench the reaction. The mixture was transferred to separatory funnel and the organic phase was washed with H_2O (×3) and brine (×3) subsequently. After being dried over Na_2SO_4, the solvent was evaporated under reduced pressure to give the crude product, which was purified by recrystallization from CH_2Cl_2 to give **6** as a colorless crystal (400 mg, 1.14 mmol, 70%).
Mp (from CH_2Cl_2) 128-130°C.
IR (KBr): ν_{max} = 3099, 2873, 1694, 1595, 1540, 1390, 1347, 1194, 886, 843 cm^{-1}.
^{1}H NMR (400 MHz, $CDCl_3$): δ 10.02 (s, 1H), 8.70 (s, 1H), 8.55 (d, *J* = 8.6 Hz, 1H), 8.26 (d, *J* = 8.6 Hz, 1H), 7.95 (d, *J* = 8.3 Hz, 2H), 7.42 (d, *J* = 8.3 Hz, 2H).
^{13}C NMR (100 MHz, $CDCl_3$): δ 189.97, 152.35, 150.87, 148.75, 135.41, 133.54, 133.12, 131.45 (2C), 126.33, 122.54 (2C), 120.26.
ESI-MS *m*/*z* 375.0 $[M+Na]^+$

1-Methyl-3-(4-(2,4-dinitrophenylsulfonyloxy)styrenyl)-4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene (1). Aldehyde **6** (80 mg, 0.23 mmol) was added to a solution of **4** (50 mg, 0.23 mmol) in dry benzene (45 mL) under nitrogen atmosphere. The mixture was cooled to 0°C, piperidine (0.28 mL) and AcOH (0.28 mL) were then added dropwise with stirring. After the addition, the reaction was refluxed for 4 h with continuous removal of water using a Dean-Stark water separator. The mixture was then cooled to r.t., transferred to a separatory funnel, washed with H_2O (×3) and brine (×1), and dried over Na_2SO_4. Afer evaporation of the solvent, the crude product was purified on a silica gel column eluted with PE/EtOAc (3:1) to yield **1** as a dark amaranthine solid (50 mg, 0.09 mmol, 40%).
Mp (from PE/EtOAc = 3:1) 235-236°C.
IR (KBr): ν_{max} = 2917, 1592, 1523, 1449, 1402, 1278, 1152, 964, 894, 817 cm^{-1}.
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.68 (s, 1H), 8.51 (d, *J* = 8.3 Hz, 1H), 8.18 (d, *J* = 8.8 Hz, 1H), 7.71 (s, 1H), 7.62-7.51 (m, 3H), 7.31 (d, *J* = 15.2 Hz, 1H), 7.24 (m, 3H), 7.00 (s, 1H), 6.73 (s, 1H), 6.49 (s, 1H), 2.34 (s, 3H).
^{13}C NMR (125 MHz, DMSO): δ 170.59, 157.32, 151.82, 149.24, 148.34, 145.61, 139.99, 138.49, 137.41, 135.84, 133.94, 133.47, 130.73, 129.66 (2C), 127.73 (2C), 125.97, 123.14 (2C), 121.32, 119.05, 117.91, 117.30, 11.43.
HRMS *m*/*z* calc: 577.0777, found: 577.0768 $[M + Na]^+$.
HPLC purity, system A: 96.5%; system B:95.6%.

Scheme S2. Synthesis of free fluorophore **2**



1-Methyl-3-(4-hydroxyl-styrenyl)-4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene (2). To a stirred solution of **1** (12 mg, 0.022 mmol) in CH_2Cl_2 (30 mL) was added Et_3N (2.7 μL, 0.02 mmol) and benzenethiol (3 μL, 0.03 mmol) subsequently. After the addition of benzenethiol, the reaction solution instantly turned from mauve to pink and TLC monitoring revealed the disappearance of **1** and the appearance of a fluorescent product. The mixture was then washed with H_2O (×3) and brine (×1), dried over Na_2SO_4, concentrated under reduced pressure and purified by silica gel column chromatography (PE/EtOAc = 2:1) to give **2** (6 mg, 0.018 mmol, 86%) as a black solid.

Mp (from PE/EtOAc = 2:1) 181-183^oC.

IR (KBr): ν_{max} = 3447, 3108, 1596, 1543, 1460, 1394, 1281, 1140, 988, 829 cm^{-1}.

^1H NMR (500 MHz, $CDCl_3$): δ 7.70 (s, 1H), 7.56 (m, 3H), 7.39 (d, J = 16.3 Hz, 1H), 7.19 (s, 1H), 6.95 (d, J = 3.7 Hz, 1H), 6.91 (d, J = 8.6 Hz, 2H), 6.79 (s, 1H), 6.49 (dd, J = 3.7, 2.1 Hz, 1H), 2.37 (s, 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 159.44, 157.51, 144.60, 140.04, 138.29, 137.99, 132.97, 132.33, 129.91(2C), 128.84, 125.38, 122.49, 117.18, 116.46, 116.02 (2C), 11.50.

ESI-MS m/z 323.3 $[M-H]^-$.

Quantum Yield Measurements

Determination of the quantum yield is accomplished by comparison with standards of known quantumn yield. For probe **1**, quinine sulfate dissolved in 0.1 M H_2SO_4 ($Ø_F$ 0.53, λex 360 nm, 22^oC) was selected as the standard. While fluorescein dissolved in 0.1 M NaOH ($Ø_F$ 0.95, λex 460 nm, 22^oC) was the standard for **2**. Quantum yields were calculated by measuring the integrated emission area of the fluorescent spectra of **1** or **2** and comparing that value to the area measured for standards and were obtained with the following equation where $\sum[F]$ was the integrated fluorescence intensity and Abs is absorbance at excitation wavelength.

$\Phi_F^{sample}=\Phi_F^{standard} \cdot Abs^{standard} \cdot \Sigma[F^{sample}] / Abs^{sample} / \Sigma[F^{standard}]$

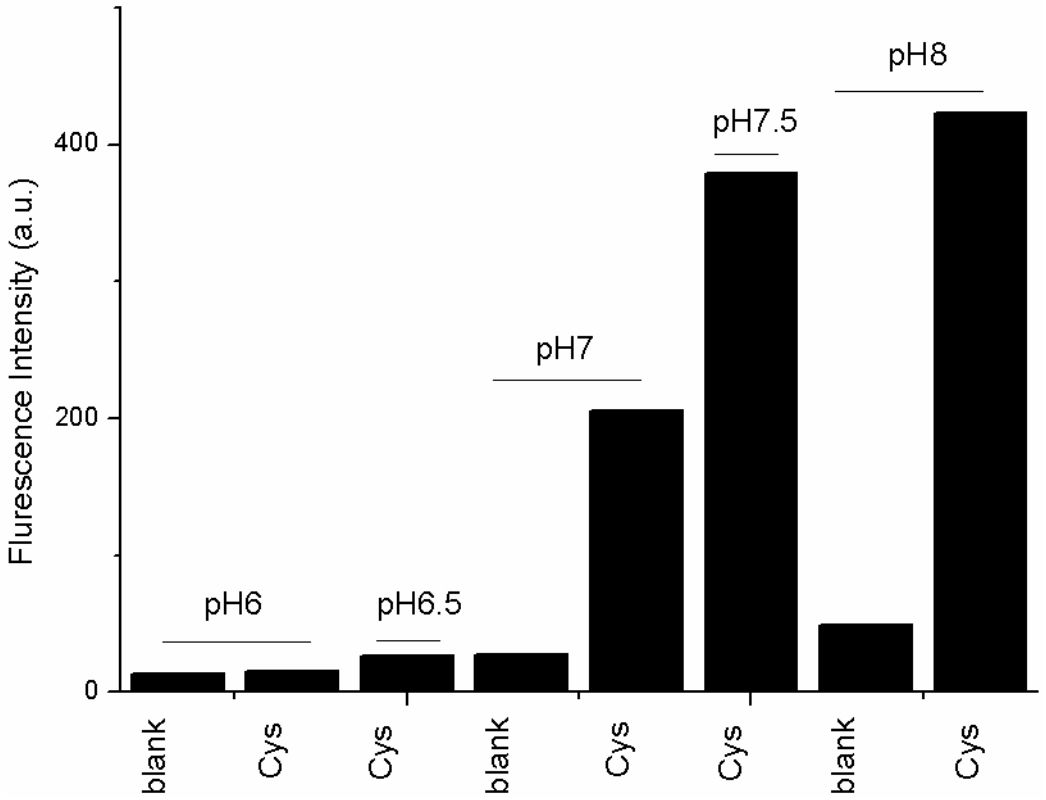


Figure S1. Stability of probe **1** at various pH and dependency of the present fluorometric assay on pH. Working solution of probe **1** in EtOH was diluted with aqueous PBS buffer (pH 6, 6.5, 7, 7.5, 8, respectively) to make a final concentration of 40 μM. For the study of its stability, the solutions at pH 6, 7, 8 were diluted with equal volume of PBS (pH 6, 7, 8; 0.01M containing 1% EtOH). For the examination of dependency of the present fluorometric assay on pH, the solutions (pH 6, 6.5, 7, 7.5, 8) were added equal volume of PBS (pH 6, 6.5, 7, 7.5, 8; 0.01M containing 1% EtOH) containing 400μM of cysteine. After 1h of incubation at room temperature, the solutions were then sampled for fluorescence measurement at λex = 527 nm and the fluorescence intensity at λem=570 nm is plotted. ("blank" for dilution with PBS and "Cys" for dilution with cysteine solution).

HPLC data of probe 1

Probe **1**	mobile phase	purity
system A	CH_3CN/H_2O (80:20)	96.5%
system B	CH_3OH/H_2O (80:20)	95.6%

HPLC conditions and traces for probe 1.

General conditions: C18 column (4.6 ×200 mm × 5 μm, Diamonsil^{TM}); ambient temperature; flow rate: 1.0 mL/min; injection volume: 15 μL; wavelength detection: 530 nm; mobile phase: CH_3CN/H_2O 80:20 for system A and CH_3OH/H_2O 80:20 for system B.

HPLC traces of probe 1 (Figure S2 and S3).

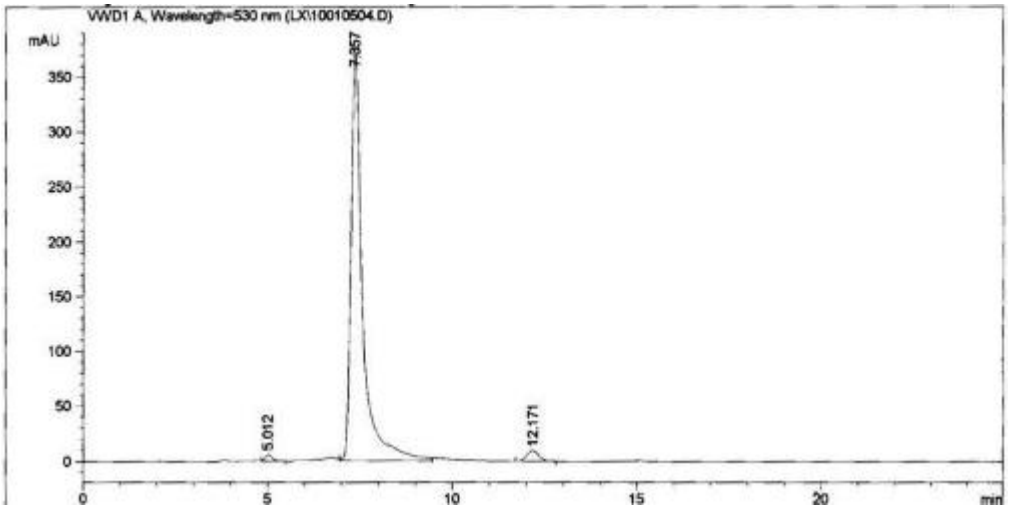


Figure S2. HPLC trace of **1** using the general conditions above and system A.

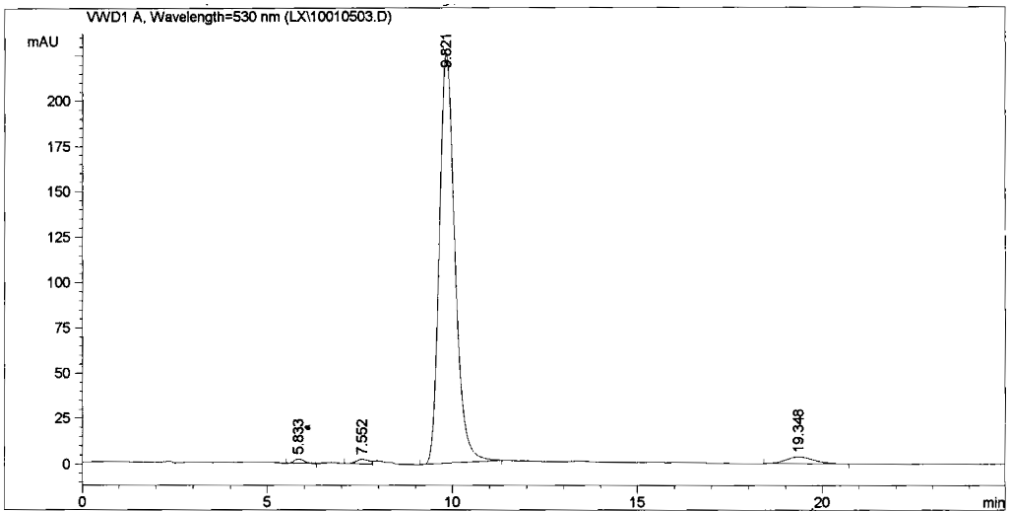


Figure S3. HPLC trace of **1** using the general conditions above and system B.

Spectra of key compounds