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## ARTICLE

## Near-infrared emission of dibenzoxanthenium and its application in the design of nitric oxide probes

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Two dibenzoxanthenium derivatives bearing a 3,4-diaminophenyl group, compounds **1a** and **1b**, have been prepared and evaluated as near-infrared probes for the detection of nitric oxide. Compound **1a** gave a 3-fold emission enhancement towards nitric oxide at 681 nm, whereas compound **1b** exhibited OFF–ON emission behavior towards nitric oxide, resulting in a 35-fold increase in the fluorescent intensity with an emission maximum of 750 nm. Probe **1b** showed independent emission in the presence of common cations, amino acids, reactive oxygen and nitrogen species; furthermore, it can work at acidic and neutral conditions.

## Introduction

Despite a growing number of potential applications for near-infrared (NIR) chromophores, progress towards the development of new fluorophores has been limited. Although several new design theories have been developed in this field,<sup>1</sup> the main method currently used for the preparation of long wavelength excitation and emission candidates is the expansion of the conjugated systems of classic chromophores. For example, polymethylene cyanine dyes were developed by extending the conjugated double bonds,<sup>2</sup> and new BODIPY dyes were prepared by extending the fused heterocycle systems or by connecting double bond-containing structures.<sup>3–5</sup>

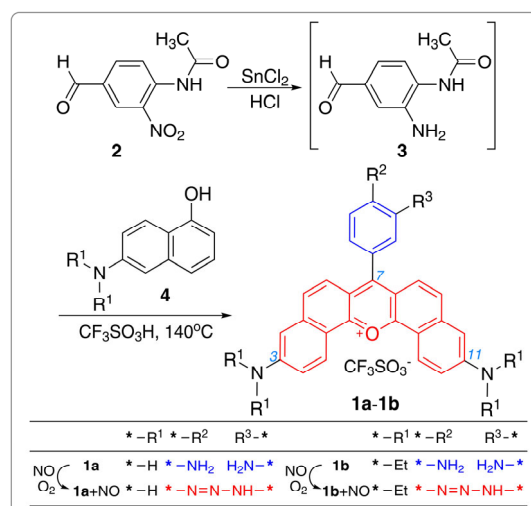
Rhodamine is one of the best fluorophores and has been used extensively in numerous applications.<sup>6,7</sup> Notwithstanding there have been very few reports pertaining the use of naphthorhodamine and the extension of its fused skeleton, this design strategy has been used to achieve an optically responsive lactone ring opening-closing equilibrium. It is noteworthy, however, that the compounds reported in this case were limited to dibenzo[*c,h*]xanthenium scaffolds bearing carboxyphenyl groups at their 7-position.<sup>8</sup> The development of a new strategy for the preparation of benzoxanthenium compounds bearing diaminophenyl groups would therefore provide access to novel series of functional dyes with diaminophenyl-benzoxanthenium photoinduced electron transfer (PET) abilities. It was envisaged that a 3,4-diaminophenyl group could be used as a suitable moiety for the development of benzoxanthenium compounds bearing diaminophenyl groups, because this group has been used previously as a reactive unit and applied to the detection of nitric oxide in probes based on some fluorophores,<sup>9–11</sup> and only one NIR nitric oxide probe based on polymethylene cyanine was

worked at pH 6–11 in neutral alkaline conditions.<sup>12</sup> Given that anilines can be protonated under acidic conditions, there is strong need for the development of pH-independent<sup>13</sup> NIR probes<sup>14</sup> for the detection of nitric oxide that could be used to complement existing probes.

## Results and discussion

## Design and synthesis of probes 1a–b

The vast majority of the reported nitric oxide probes were prepared from the corresponding nitrobenzyl compounds, with the final products being obtained by reduction,<sup>11,15</sup> however, the method was

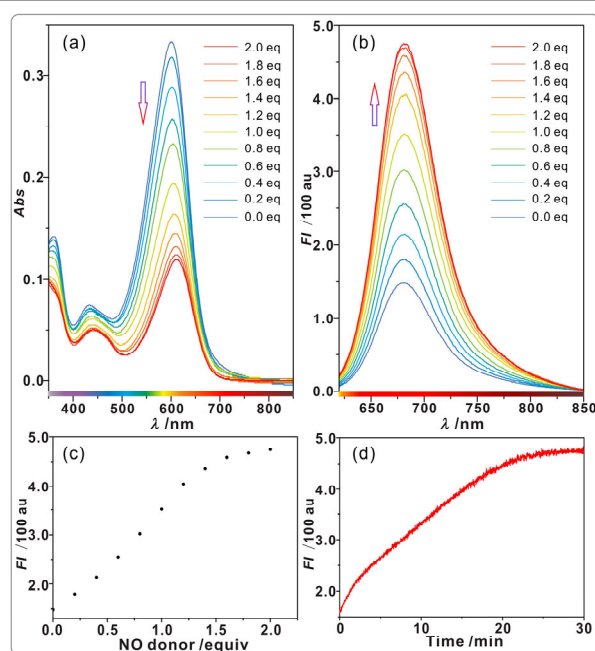
Scheme 1. Preparation and the proposed detection mechanism of probes **1a–b**.

not effective in this case since the dibenzoxanthenium will be reduced to colorless solutions. The synthetic route used for the construction of the dibenzoxanthenium compounds evaluated in this study is shown in Scheme 1. Briefly, *N*-(4-formyl-2-nitrophenyl)acetamide (**2**) was treated with stannous chloride under acidic conditions to give *N*-(2-amino-4-formylphenyl)acetamide (**3**), which was found to be unstable. Compound **3** was therefore immediately reacted with 6-aminonaphthalen-1-ol derivatives (**4**) in trifluoromethanesulfonic acid to give the target molecules **1a** and **1b** via a semi-*in situ* synthetic process.

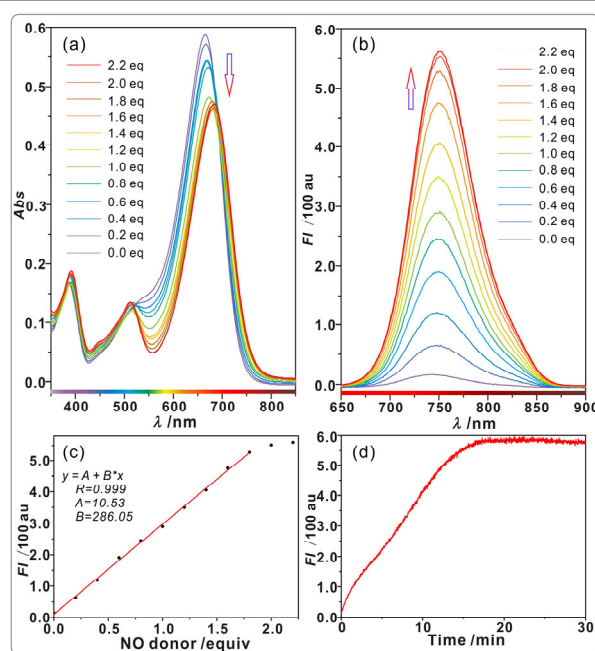
### Optical responses of probes **1a**–**b** towards nitric oxide

The optical response of probe **1a** to nitric oxide was tested using 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-methyl-1-triazene (NOC13) as a nitric oxide donor.<sup>14</sup> The optical properties of probe **1a** (10  $\mu$ M) evaluated in the presence of different concentrations of NOC13 (0.0–2.0 equiv) in a 20 mM HEPES buffer–DMSO (1/1, v/v) solution. The absorption maxima of probe **1a** (Fig. 1(a)) was slightly red-shift from 600 to 612 nm when it was measured in the presence of 2.0 equivalents of NOC13. The emission signal of probe **1a** (Fig. 1(b)) was determined to be in the range of 620–850 nm following its excitation at 600 nm (Fig. S1(a)), and there was a 3-fold increase in the emission maxima at ca. 681 nm. The maximum response time was reached within 25 min when probe **1a** was treated with 2.0 equivalents of NOC13 (Fig. 1(d)) and its fluorescence quantum yield increased from 0.027 to 0.16 (Table 1). Although the reaction of 3,4-diaminophenyl towards nitric oxide (Fig. S11) resulted in an emission response, the fluorescent intensity enhancement (3-fold) at 681 nm observed for probe **1a** with and without the nitric oxide donor mean that this probe would be unsuitable for use in the detection of nitric oxide; therefore, based on the poor properties of probe **1a**, we proceeded to synthesize and evaluate probe **1b** bearing diethylamino groups at the 3- and 11-positions of its dibenzoxanthenium core in an attempt to provide improved optical properties.

The absorption maximum of probe **1b** (10  $\mu$ M, Fig. 2(a)) was 683 nm when it was treated with 2.2 equivalents of NOC13. Furthermore, the weak emission maximum of probe **1b** at 750 nm was dramatically increased by 35-fold across its entire range of 650–870 nm following the addition of 2.2 equivalent of NOC13 (Fig. 2(b)), and its fluorescence quantum yield increased from 0.015 to 0.13 (Table 1). The test reaction between probe **1b** and the nitric oxide donor reached completion within 20 min (Fig. 2(d)), and linear relationship was observed between the fluorescence intensity of probe **1b** and the concentration of NOC13 at concentrations in the range of 0.0–1.8 equivalents (Fig. 2(c)). The absorption and emission maxima of probe **1b** were red shift by about 70 nm relative to those of probe **1a**, and probe **1b** also exhibited an OFF–ON type of detection behavior. Based on the excitation properties of **1b** (Fig. S1(b)), an emission response titration experiment was conducted, which gave an emission in the range of 670–870 nm following excitation at 663 nm (Fig. S2). Furthermore, the emission maximum at 750 nm showed a good linear relationship with the amount of NOC13. Taken together, these results indicated that probe **1b** was suitable for use as a nitric oxide probe that could be excited in red or NIR regions. Compared with other nitric oxide probes reported in the literature, probe **1b** is



**Fig. 1** Optical responses of probe **1a** (10  $\mu$ M) towards different amount of NOC13 (0–2.0 equiv.) in HEPES buffer (20 mM, pH = 7.4)–DMSO (1/1, v/v) solutions; (a) absorption spectra; (b) emission spectra ( $\lambda_{\text{ex}} = 600$  nm); (c) the linear response of fluorescence intensities ( $\lambda_{\text{em}} = 681$  nm) towards NOC13 concentrations (0–1.6 equiv.); (d) time-dependent response of probe **1a** to NOC13 (2.0 equiv.) within 30 min.



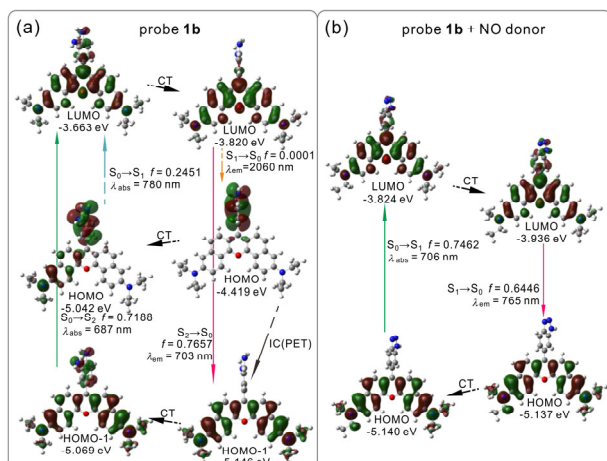
**Fig. 2** Optical responses of probe **1b** (10  $\mu$ M) towards different amount of NOC13 (0–2.2 equiv.) in HEPES buffer (20 mM, pH = 7.4)–DMSO (1/1, v/v) solutions; (a) absorption spectra; (b) emission spectra ( $\lambda_{\text{ex}} = 633$  nm); (c) the linear response of fluorescence intensities ( $\lambda_{\text{em}} = 750$  nm) towards NOC13 concentrations (0–1.8 equiv.); (d) time-dependent response of probe **1b** to NOC13 (2.0 equiv.) within 30 min.

one of the nitric oxide probes that the excitation and emission wavelengths are in long wavelength region.<sup>14</sup>

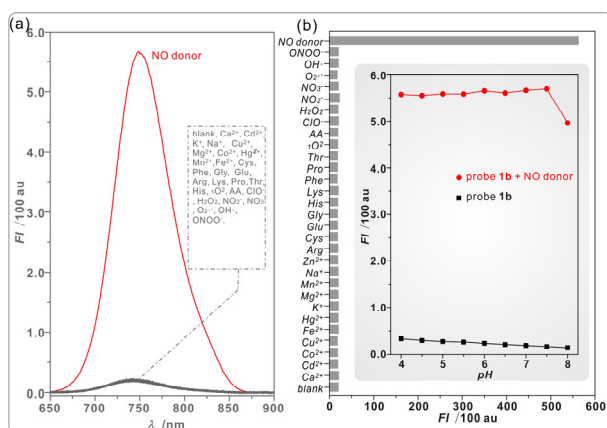
Table 1 Photophysical properties of probes **1a** and **1b** in 20 mM pH 7.4 HEPES buffer-DMSO (1/1, v/v) solution.

	$\lambda_{\text{Abs}}$ , max (nm)	$\lambda_{\text{Em}}$ , max (nm)	Stokes Shift (nm)	$\epsilon$ ( $\text{M}^{-1}\cdot\text{cm}^{-1}$ )	$\Phi^a$
<b>1a</b>	600	681	81	$3.3 \times 10^4$	0.027
<b>1a</b> +NO <sup>b</sup>	612	681	69	$1.2 \times 10^4$	0.16
<b>1b</b>	666	745	79	$5.9 \times 10^4$	0.015
<b>1b</b> +NO <sup>b</sup>	683	750	67	$4.6 \times 10^4$	0.13

<sup>a</sup> Cyanine dye HPDITCP ( $\Phi = 0.16$  in ethanol, ref. <sup>16</sup>) was used as the reference for fluorescence quantum yields. <sup>b</sup> With NOC13 (2.0 equiv.).



**Fig. 3** The frontier molecular orbitals (FMOs) involved in the vertical excitation and emission of probe **1b** (left) and **1b** + NO (right). CT stands for conformation transformation, and IC (PET) stands for internal conversion (photoinduced electron transfer). Excitation and radiative processes are marked as solid lines and the non-radiative processes are marked by dotted lines.



**Fig. 4** Fluorescent responses of probe **1b** (10  $\mu\text{M}$ ) to different analytes; (a) emission spectra; (b) fluorescent intensities, insert is the pH dependent emissions of probe **1b** before and after addition of the nitric oxide donor. NO donor (25  $\mu\text{M}$ ); common cations:  $\text{K}^+$  (100 mM),  $\text{Na}^+$  (100 mM),  $\text{Ca}^{2+}$  (0.5 mM),  $\text{Cd}^{2+}$  (0.3 mM),  $\text{Cu}^{2+}$  (0.3 mM),  $\text{Mg}^{2+}$  (0.5 mM),  $\text{Co}^{2+}$  (0.3 mM),  $\text{Hg}^{2+}$  (0.3 mM),  $\text{Mn}^{2+}$  (0.3 mM),  $\text{Fe}^{2+}$  (0.3 mM); bioactive amino acids (0.1 mM): Cys, Phe, Gly, Glu, Arg, Lys, Pro, Try, His; reactive oxygen and nitrogen species (50  $\mu\text{M}$ ):  $\text{HO}\cdot$ ,  $\text{H}_2\text{O}_2$ ,  $\text{ClO}^-$ ,  $\text{O}_2^{\cdot-}$ , and  $\text{ONOO}^-$ ; oxidized forms of NO (50  $\mu\text{M}$ ):  $\text{NO}_2^-$  and  $\text{NO}_3^-$ ; and ascorbic acid (AA, 50  $\mu\text{M}$ ).

### Theoretical calculations

(TD)DFT calculations were performed to develop a better understanding of the differences in the optical properties of probes **1a** and **1b**. As shown in Fig. 3, the phenyl ring of probe **1b** was

almost perpendicular to the plane of its dibenzoxanthenium moiety when it was in its lowest excited state, with the dihedral angle changing from its ground state value of  $58^\circ$  to  $89^\circ$ . This change made the electronic redistribution, and the HOMO orbital energy of diaminophenyl was higher than that of benzoxanthenium, so the PET process occurred, which resulted in fluorescent quenching, and **1b** consequently exhibited a very weak fluorescence signal (Fig. 2(b)). The addition of the NOC13 to the system results in the formation of a five-membered ring, which would restrict the rotation of the benzene ring and the HOMO and LUMOs of **1b** + NO located almost entirely on the fluorophores, which would lead to an enhancement in fluorescence attributed to no PET but an intramolecular charge transfer (ICT) process on the contrary. Meanwhile, the fluorescent band had a bathochromic shift due to the increase of molecular conjugated degree.

Although there was a slight difference in dihedral angles observed in the ground and excited states of probe **1a** (i.e.,  $55^\circ$  in the ground state to  $51^\circ$  in the excited state), no PET was observed (Fig. S3(a)); and probe **1a** gave emission (Fig. 1(b)). Detailed results of the excitation and emission spectra are shown in Tables S1–2.

### The selectivity of probes

The selectivity of probes **1a** and **1b** was also evaluated in terms of the ability to react preferentially with nitric oxide over numerous other potential interferents under biological conditions. No discernible differences were observed in the emission spectra of probes **1a** and **1b** (Fig. 4, S4) when they were recorded at pH 7.4 in the presence of several different species.

The pH-dependent emission properties of probe **1b** were also evaluated both in the presence and absence of the (Fig. 4(b, insert)), it is clear that probe **1b** can be applied successfully for the detection of nitric oxide at pH values in the range of 4.0–7.5, which covers most intracellular pH conditions.

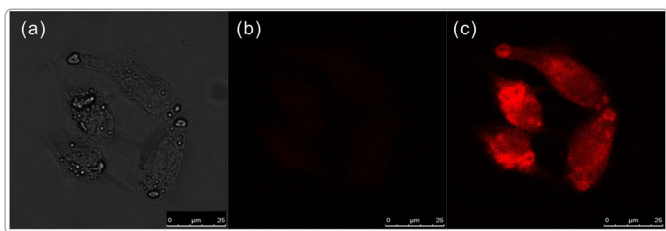
### Fluorescence imaging of living cells with probe 1b

Confocal microscopy images were also used to evaluate the ability of probe **1b** to detect nitric oxide in HeLa cells (Fig. 5). HeLa cells were initially cultured with probe **1b** at a final concentration of 10  $\mu\text{M}$  for 20 min, and the level of fluorescence intensity within the cells was found to be rather weak (Fig. 5(b)); in contrast, it was then cultured with the at a final concentration of 100  $\mu\text{M}$  by a further 20 min,<sup>14</sup> a significant increase in their fluorescence intensity (Fig. 5(c)) was showed. This result therefore suggests that probe **1b** could be used for the *in cellulo* imaging of nitric oxide.

### Conclusions

In summary, new dibenzoxanthenium derivatives bearing a diaminophenyl group at their 7-position have been designed and prepared for *in cellulo* imaging applications. Two dibenzo[c,h]xanthenium derivatives (probes **1a** and **1b**) with a 3,4-diaminophenyl group at their 7-position were prepared and evaluated as probes for the detection of nitric oxide. Probe **1a** with primary amino groups attached to the 3- and 11-positions of the fluorophore showed a red–NIR emission ( $\lambda_{\text{em}} = 681$  nm), whereas probe **1b** bearing tertiary amino groups at the same positions of the





**Fig. 5** Fluorescence confocal imaging of probe **1b** in HeLa cells; (a) bright field; (b) fluorescence imaging after cultured with probe **1b** (10  $\mu$ M) for 20 min; (c) fluorescence imaging after cultured with NOC13 (100  $\mu$ M) for another 20 min.

fluorophore exhibited an OFF-ON type NIR emission ( $\lambda_{em} = 750$  nm) in the absence and presence of the NO donor, which was attributed to a PET effect, and could be excited by red ( $\lambda_{ex} = 633$  nm) or NIR ( $\lambda_{ex} = 663$  nm) light. Probe **1b** also exhibited good sensitivity and selectivity, and was successfully applied to the *in cellulo* imaging of nitric oxide in HeLa cells. The results of this research therefore represent a good example of the preparation of dibenzoxanthenium fluorophores bearing aminophenyl groups for the chemo sensors application in more acidic biological systems.

## Experimental section

### Materials and apparatus

All reagents and solvents (synthetic or analytical grade) were purchased from TCI Development Co., Ltd. (Tokyo, Japan), Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and Energy Chemical Co., Ltd. (Shanghai, China). Flash chromatography was performed with silica gel (300-400 mesh). NO donor (NOC 13) was synthesized as described.<sup>17</sup> The cyanine dye HPDITCP as standard to determine quantum yield was synthesized as reported.<sup>18</sup> The HEPES buffer was prepared with 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (20 mM), and adjusted with concentrated NaOH (1.0 M) or HCl (1.0 M) to various pH values measured by digital pH-meter.

<sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectra were recorded on Varian or Agilent spectrometers, solvent peaks were used as internal standards, which were underlined in supporting spectra. High-resolution mass spectra were recorded on a Finnigan MAT95 mass spectrometer (ESI<sup>+</sup>). Infrared (IR) spectra were recorded on a Nicolet 5200 IR spectrometer using solid samples dispersed in KBr pellets. Melting points were determined on an X-4 microscope electron thermal apparatus (Taike, China).

UV-vis spectra were obtained with a Shimadzu UV-1800 spectrophotometer. Fluorescence emission spectra were performed with fused quartz cuvette (10 mm  $\times$  10 mm) on Shimadzu RF-5301PC spectroscopy with R-928 photomultiplier at room temperature; the excitation and emission slits width are all set at 3 nm. The pH values were measured with a Lei-Ci (pH-3C) digital pH meter (Shanghai, China) using a combined glass-calomel electrode.

Cells were cultured in Roswell Park Memorial Instrument (RPMI-1640). Fluorescence confocal images were recorded with a Leica TCS SP5 II confocal laser scanning microscope, the images were handled with its attached software.

### Synthesis of 3,11-diamino-7-(3,4-diaminophenyl)dibenzo[c,h]xanthen-14-ium trifluoromethanesulfonate (**1a**)

4-Acetamido-3-nitrobenzaldehyde (1.0 mmol, 208.2 mg) was dissolved in 30 mL dichloromethane (DCM) saturated with 10 mL concentrated HCl. The solution was stirred at 0  $^{\circ}$ C for 20 min, then SnCl<sub>2</sub>·2H<sub>2</sub>O (20.0 mmol, 4.52 g) was added slowly, and the reaction was continued at room temperature until TLC monitoring showed complete consumption of starting material, the solution was neutralized by saturated sodium bicarbonate, filtered, washed by acetone, and the filtrate was extracted by a mixture of DCM/methanol (10:1, v/v), organic layer was combined and dried over Na<sub>2</sub>SO<sub>4</sub>, evaporated to afford compound **3** as a crude product (70.0 mg), which was used directly without further purifying since it is unstable. Compound **3** (70.0 mg) and 6-amino-1-naphthol (0.79 mmol, 125.0 mg) were combined in trifluoromethanesulfonic acid (2.0 mL). The reaction mixture was stirred at 100  $^{\circ}$ C for 2 h and at 140  $^{\circ}$ C for another 2 h, then the reaction was cooled to room temperature and poured into 40 mL brine. The product was extracted into CH<sub>2</sub>Cl<sub>2</sub>/MeOH (5:1). The organic layer was combined and washed with brine, dried by Na<sub>2</sub>SO<sub>4</sub>, concentrated, and subjected to flash column chromatography (silica gel, 9:1 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to give **1a** as a black powder (41.0 mg), yield 19 % (based on compound **4**), mp 177–180  $^{\circ}$ C. IR  $\nu$  (KBr, cm<sup>-1</sup>): 3444, 2924, 1628, 1612, 1462, 1385, 1257, 1130, 1115, 1038. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  8.85 (d, *J* = 9.0 Hz, 2H, 2 $\times$ Ar-*H*), 7.67 (d, *J* = 9.2 Hz, 2H, 2 $\times$ Ar-*H*), 7.62 (d, *J* = 9.4 Hz, 2H, 2 $\times$ Ar-*H*), 7.27 (d, *J* = 9.4 Hz, 2H, 2 $\times$ Ar-*H*), 7.09 (br, 4H, 2 $\times$ NH<sub>2</sub>), 6.99 (s, 2H, 2 $\times$ Ar-*H*), 6.84 (br, 2H, 2 $\times$ Ar-*H*), 6.72 (d, *J* = 7.9 Hz, 1H, Ar-*H*), 5.58 (br, 2H, NH<sub>2</sub>), 5.02 (br, 2H, NH<sub>2</sub>). <sup>13</sup>C NMR (151 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  161.5, 155.0, 155.0, 140.3, 139.6, 135.2, 127.4, 126.9, 124.8, 122.6, 120.5, 119.3, 117.6, 116.6, 113.9, 113.0, 107.8. HRMS-ESI<sup>+</sup>: *m/z* = 417.1721 (calcd for [M-CF<sub>3</sub>SO<sub>3</sub>]<sup>+</sup>, 417.1715).

### Synthesis of 7-(3,4-diaminophenyl)-3,11-bis(diethylamino)dibenzo[c,h]xanthen-14-ium trifluoromethane-sulfonate (**1b**)

Compound **1b** was synthesized by condensing **3** (70.0 mg) and 6-*N*, *N*-diethylamino-1-naphthol (0.78 mmol, 169.0 mg) using a similar procedure to that described for **1a**. Compound **1b** (60.0 mg) was obtained as a black powder, yield 23 % (based on compound **4**), mp 219–222  $^{\circ}$ C. IR  $\nu$  (KBr, cm<sup>-1</sup>): 3448, 3375, 2974, 1608, 1512, 1454, 1358, 1257, 1153, 1030. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  8.73 (dd, *J* = 9.6, 3.7 Hz, 2H, 2 $\times$ Ar-*H*), 7.69 (d, *J* = 8.9 Hz, 2H, 2 $\times$ Ar-*H*), 7.55 (dd, *J* = 9.4, 1.3 Hz, 2H, 2 $\times$ Ar-*H*), 7.34 (d, *J* = 9.4 Hz, 2H, 2 $\times$ Ar-*H*), 7.12 (s, 2H, 2 $\times$ Ar-*H*), 6.84 (d, *J* = 7.9 Hz, 1H, Ar-*H*), 6.83 (s, 1H, Ar-*H*), 6.71 (d, *J* = 7.8 Hz, 1H, 2 $\times$ Ar-*H*), 5.58 (br, 1H, NH), 5.02 (br, 1H, NH), 3.59 (q, *J* = 6.7 Hz, 8H), 1.23 (t, *J* = 6.8 Hz, 2H, 4 $\times$ CH<sub>3</sub>). <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  159.7, 154.0, 151.0, 139.4, 139.2, 134.7, 126.9, 126.3, 124.2, 122.4, 119.9, 117.1, 116.3, 115.8, 113.5, 111.7, 106.1, 44.5, 12.6. HRMS-ESI<sup>+</sup>: *m/z* = 529.2959 (calcd for [M-CF<sub>3</sub>SO<sub>3</sub>]<sup>+</sup>, 529.2967).

### Absorption and fluorescence titration

Stock solutions (100  $\mu$ M) of probes **1a–1b** were prepared in volumetric flask (100 mL) with DMSO. Stock solutions (1 mM) of NO donor were prepared in volumetric flask (100 mL) with 0.01M NaOH solution. Each test solution (10  $\mu$ M) was prepared in a volumetric flask (10 mL) with 1 mL stock solution of probes, 4 mL DMSO, and corresponding volume of stock solution of NO donor, diluted with HEPES buffer solution (pH = 7.4) to give a total volume of 10.0 mL

with various concentration of NO donor. Fluorescence and absorption spectra were measured after 30 mins.

### Selectivity experiment

Stock solutions of various ions were prepared in volumetric flasks (10 mL) with concentrations of NaCl (1 M), KCl (1 M), MgSO<sub>4</sub> (5 mM), CaCl<sub>2</sub> (5 mM), CoCl<sub>2</sub>·6H<sub>2</sub>O (3 mM), CuCl<sub>2</sub>·2H<sub>2</sub>O (3 mM), NiCl<sub>2</sub> (3 mM), CdCl<sub>2</sub>·2.5H<sub>2</sub>O (3 mM), HgCl<sub>2</sub> (3 mM) in doubly distilled water. Stock solutions of all kinds of amino acids were all prepared in volumetric flasks (100 mL) with concentrations of 1 mM in doubly distilled water. Solid KO<sub>2</sub> was used to provide superoxide (O<sub>2</sub><sup>•-</sup>). Hydroxylradical (OH<sup>•</sup>) was prepared by the Fenton reaction of H<sub>2</sub>O<sub>2</sub> and Fe<sup>2+</sup>. Singlet oxygen (<sup>1</sup>O<sub>2</sub>) was obtained from ClO<sup>-</sup> and H<sub>2</sub>O<sub>2</sub>. Peroxynitrite (ONOO<sup>-</sup>) was generated from sodium nitrite and hydrogen peroxide in a quenched-flow reactor. Solid NaClO, NaNO<sub>2</sub>, and NaNO<sub>3</sub> were dissolved in water to provide ClO<sup>-</sup>, NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup>, respectively. To test the selectivity of probes for NO, K<sup>+</sup> (100 mM), Na<sup>+</sup> (100 mM), Ca<sup>2+</sup> (0.5 mM), Cd<sup>2+</sup> (0.3 mM), Cu<sup>2+</sup> (0.3 mM), Mg<sup>2+</sup> (0.5 mM), Co<sup>2+</sup> (0.3 mM), Hg<sup>2+</sup> (0.3 mM), Mn<sup>2+</sup> (0.3 mM), Fe<sup>2+</sup> (0.3 mM), Cys (0.1 mM), Phe (0.1 mM), Gly (0.1 mM), Glu (0.1 mM), Arg (0.1 mM), Lys (0.1 mM), Pro (0.1 mM), Thr (0.1 mM), His (0.1 mM), 5 equiv. of NO<sub>2</sub><sup>-</sup> (50 μM), NO<sub>3</sub><sup>-</sup> (50 μM), ONOO<sup>-</sup> (50 μM), OH<sup>•</sup> (50 μM), H<sub>2</sub>O<sub>2</sub> (50 μM), <sup>1</sup>O<sub>2</sub> (50 μM), ClO<sup>-</sup> (50 μM), O<sub>2</sub><sup>•-</sup> (50 μM), ascorbic acid (AA) (50 μM) and 2.5 equiv. NO donor (25 μM) were added to the solution of probes (10 μM) in pH 7.4 HEPES buffer with 50% DMSO, respectively. Each test solution was prepared in a volumetric flask (10 mL) with 1 mL stock solution of probes, 4 mL DMSO, and 1 mL stock solution of corresponding ions, amino acids solutions, reactive oxygen and nitrogen species, diluted with 20 mM 7.4 HEPES buffer solution to give a total volume of 10 mL. Probe **1a** was excited at 600 nm, probe **1b** were excited at 633 nm and 663 nm.

### pH-dependent responses evaluation for 1b

The test of pH-dependent response of probe **1b** without NO donor: the test solution (10 μM) was prepared in a volumetric flask (10 mL) with 1 mL stock solution of probe **1b**, 4 mL DMSO, diluted with 20 mM HEPES buffer of various pH values (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) to give a total volume of 10.0 mL. Fluorescence spectra were then obtained.

The test of pH-dependent response with NO donor: solution (10 μM) of **1b** in a volumetric flask (250 mL) was prepared with 25 mL Stock solution, 100 mL DMSO, and 7.5 mL Stock solution of NO donor, diluted with 20 mM HEPES buffer (pH = 7.4) to give a total volume of 250.0 mL. Each test solution (10 μM) was prepared by adding 10 mL solution above, adjusted with concentrated NaOH (1.0 M) or HCl (1.0 M) to various pH values (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) measured by digital pH-meter. Fluorescence spectra were then obtained.

### Determination of quantum yield

All the relative fluorescence quantum yields were determined and calculated with the following equation:

$$\Phi_x/\Phi_{st} = [A_{st}/A_x][n_x^2/n_{st}^2][D_x/D_{st}]$$

Where st: standard; x: sample; Φ: quantum yield; A: absorbance at the excitation wavelength, D: area under the fluorescence spectra on an energy scale; n: the refractive index of the solution, and the refractive

index of mixed solution of DMSO and water can be calculated from the reported data.<sup>3</sup> The cyanine dye HPDITCP (Φ = 0.16 in ethanol) was used as standard.<sup>19</sup>

### Calculation details

The geometries of probes **1a-1b** and **1a/b** + NO at the ground state (S<sub>0</sub> state) were optimized by density functional theory (DFT) at the B3LYP/6-31G(d) level,<sup>20</sup> and the geometries at the lowest singlet excited state (S<sub>1</sub> state) were optimized by TDDFT methods with the same basis set using the Gaussian program package. In some cases, higher excited states were optimized. There are no imaginary frequencies in frequency analysis of all the calculated structures; therefore, each calculated structures are in the local energy minimum. The energy gap between the S<sub>0</sub> state and the lowest excited state was calculated with the (TD)DFT/BP86/TZVP method based on the optimized S<sub>0</sub> state geometry (for absorption) and the lowest singlet excited state geometry (for fluorescence), respectively. Water was used as the solvent for the (TD)DFT calculations (CPCM model).

### Cell culture

HeLa Cells were cultured with 10% calf serum, penicillin (100 U·mL<sup>-1</sup>), streptomycin (100 μg·mL<sup>-1</sup>) and L-glutamine (250 μM) at 37°C in a 5:95 CO<sub>2</sub>-air incubator. Cells with 2 × 10<sup>5</sup> density were loaded onto a glass-bottomed coverslip with a diameter of 35 mm and cultured for 48 h before use.

### Fluorescent imaging of HeLa cells

Probes **1b** (10 μM, cells medium (1 mL) containing 10 % DMSO) were used in the fluorescent imaging of NO in HeLa cells. HeLa cells was firstly cultured with probe **1b** for 20 min, after that, the cells were washed with PBS (pH = 7.4) for three times, then NO donor was added with a final concentration of 100 μM. The cells were incubated for another 20 min. The red channel emission with probe **1b** was measured at 650–795 nm with excitation at 633 nm.

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### Notes and references

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1. N. Karton-Lifshin, L. Albertazzi, M. Bendikov, P. S. Baran and D. Shabat, *J. Am. Chem. Soc.*, 2012, **134**, 20412-20420.
2. J. Han and K. Burgess, *Chem. Rev.*, 2010, **110**, 2709-2728.
3. S. Atilgan, T. Ozdemir and E. U. Akkaya, *Org. Lett.*, 2008, **10**, 4065.
4. E. Deniz, G. C. Isbasar, O. A. Bozdemir, L. T. Yildirim, A. Siemiarczuk and E. U. Akkaya, *Org. Lett.*, 2008, **10**, 3401-3403.
5. Z. Xu, X. Chen, H. N. Kim and J. Yoon, *Chem. Soc. Rev.*, 2010, **39**, 127-137.
6. M. Beija, C. A. M. Afonso and J. M. G. Martinho, *Chem. Soc. Rev.*, 2009, **38**, 2410-2433.
7. H. Kobayashi, M. Ogawa, R. Alford, P. L. Choyke and Y. Urano, *Chem. Rev.*, 2010, **110**, 2620-2640.
8. K. Furukawa, H. Abe, J. Wang, M. Uda, H. Koshino, S. Tsuneda and Y. Ito, *Org. Biomol. Chem.*, 2009, **7**, 671-677.
9. X. H. Dong, C. H. Heo, S. Y. Chen, H. M. Kim and Z. H. Liu, *Anal. Chem.*, 2014, **86**, 308-311.
10. Y. Q. Sun, J. Liu, H. X. Zhang, Y. Y. Huo, X. Lv, Y. W. Shi and W. Guo, *J. Am. Chem. Soc.*, 2014, **136**, 12520-12523.
11. H. X. Zhang, J. B. Chen, X. F. Guo, H. Wang and H. S. Zhang, *Anal. Chem.*, 2014, **86**, 3115-3123.
12. Y. Gabe, Y. Urano, K. Kikuchi, H. Kojima and T. Nagano, *J. Am. Chem. Soc.*, 2004, **126**, 3357-3367.
13. H. Yu, Y. Xiao and L. Jin, *J. Am. Chem. Soc.*, 2012, **134**, 17486-17489.
14. E. Sasaki, H. Kojima, H. Nishimatsu, Y. Urano, K. Kikuchi, Y. Hirata and T. Nagano, *J. Am. Chem. Soc.*, 2005, **127**, 3684-3685.
15. G. K. Vegesna, S. R. Sripathi, J. Zhang, S. Zhu, W. He, F. T. Luo, W. J. Jahng, M. Frost and H. Liu, *ACS Appl. Mater. Interfaces*, 2013, **5**, 4107-4112.
16. K. Rurack and M. Spieles, *Anal. Chem.*, 2011, **83**, 1232-1242.
17. J. A. Hrabie, J. R. Klose, D. A. Wink and L. K. Keefer, *J. Org. Chem.*, 1993, **58**, 1472-1476.
18. A. B. Descalzo and K. Rurack, *Chem-Eur. J.*, 2009, **15**, 3173-3185.
19. K. Rurack and M. Spieles, *Anal. Chem.*, 2011, **83**, 1232-1242.
20. M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. M. Jr, J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, T. Keith, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, O. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski and D. J. Fox, Gaussian, Inc., Wallingford CT, 2010.