

## Supporting Information for

### **A FRET-based ratiometric two-photon fluorescent probe for dual-channel imaging of nitroxyl in living cells and tissues**

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## Table of contents

<b>Materials and apparatus.....</b>	<b>S3</b>
<b>General procedure for the spectra measurement.....</b>	<b>S3</b>
<b>Synthesis of donor 2 and probe P-Np-Rhod .....</b>	<b>S4</b>
<b>Cell cytotoxic assays and two-photon fluorescence imaging.....</b>	<b>S6</b>
<b>Two-photon fluorescence imaging of fresh mouse liver slices.....</b>	<b>S6</b>
<b>Figures S1-2.....</b>	<b>S7</b>
<b>Figures S3-4.....</b>	<b>S8</b>
<b>Figures S5-7.....</b>	<b>S9</b>
<b>Figure S8.....</b>	<b>S10</b>
<b>Scheme S1.....</b>	<b>S11</b>
<b>Figures S9-10.....</b>	<b>S12</b>
<b>References.....</b>	<b>S13</b>
<b>NMR spectrum and MS of all the new compounds.....</b>	<b>S14</b>

## Materials and apparatus

All chemical reagents were obtained from commercial suppliers and used without further purification. Thin-layer chromatography (TLC) was performed on precoated silica gel glass plates and column chromatography was conducted over silica gel (100-200 mesh), both of which were obtained from the Qingdao Ocean Chemicals (Qingdao, China). Water used in all experiments was doubly distilled and purified by a Milli-Q system (Millipore, USA). LC-MS analyses were performed using an Agilent 1100 HPLC/MSD spectrometer. Mass spectra were recorded with an LCQ Advantage ion trap mass spectrometer (Thermo Finnigan).  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were recorded on a Bruker DRX-400 spectrometer operating at 400 MHz. The chemical shifts ( $\delta$ ) are shown in ppm, multiplicities are indicated by s (singlet), d (doublet), t (triplet), dd (doublet of doublets), and m (multiplet). UV-vis absorption spectra were measured on a Shimadzu 2450 UV-visible Spectrometer with a 1 cm standard quartz cell. Fluorescence images of HeLa cells were obtained using Olympus FV1000-MPE multiphoton laser scanning confocal microscope (Japan). Fluorescence measurements were carried out on a Fluo-max-4 fluorescence spectrometer with both excitation and emission bandwidths set at 3.0 nm. All pH measurements were performed on a Mettler-Toledo Delta 320 pH meter.

## General procedure for the spectra measurement

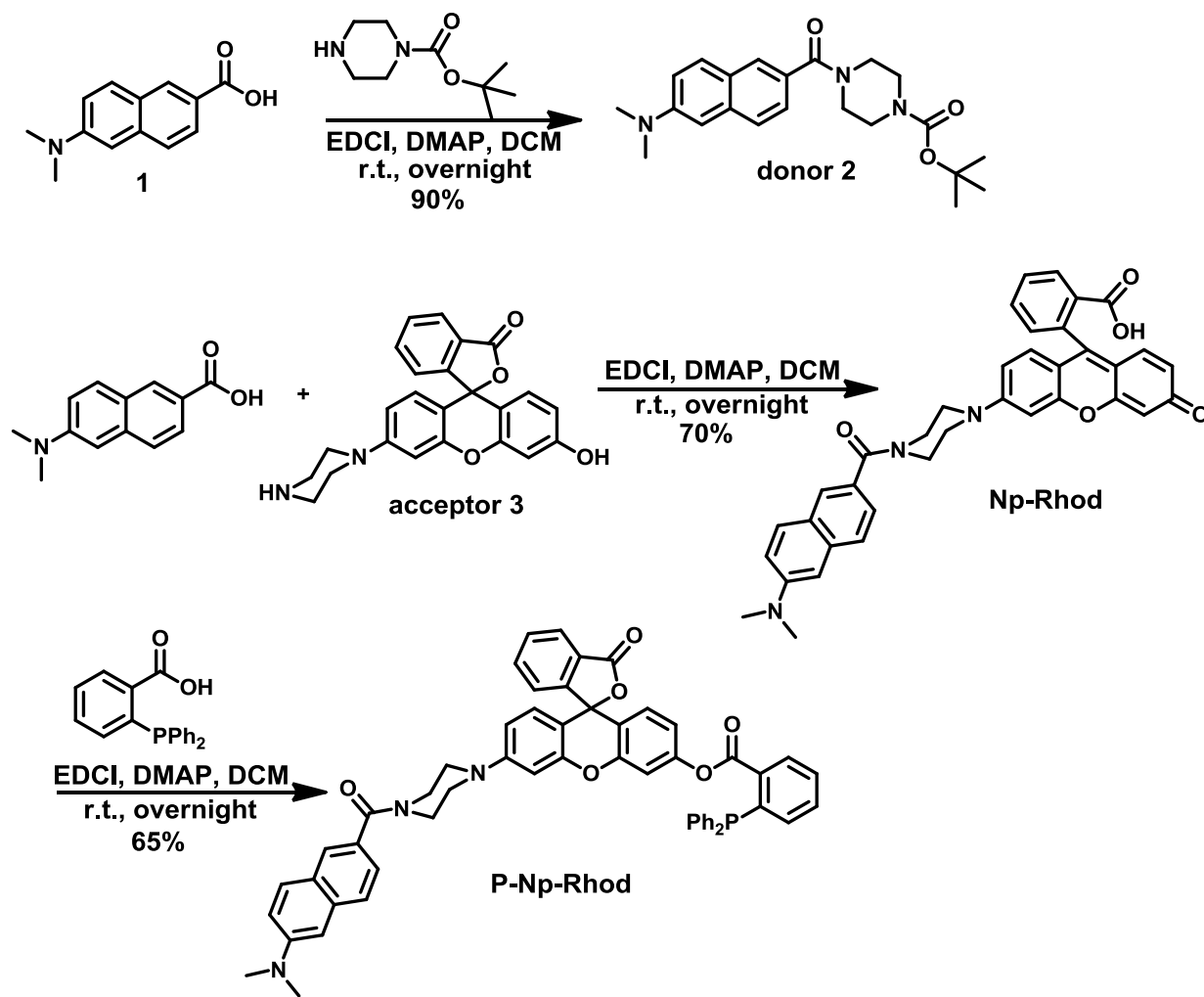
Both the measurement were performed in phosphate buffer solution (10 mM) with EtOH as the co-solvent ( $\text{H}_2\text{O}/\text{EtOH} = 1:1, \text{v/v}$ ). The fluorescence emission spectra were recorded at an excitation wavelength of 370 nm with emission wavelength range from 400 to 650 nm. Both the excitation and emission bandwidths were set at 3.0 nm. A stock solution of the probe **P-Np-Rhod** ( $1.0 \times 10^{-3} \text{ M}$ ) was prepared by dissolving the probe in EtOH. The solutions of various testing species were prepared from KCl, NaCl,  $\text{CaCl}_2$ ,  $\text{MgCl}_2$ ,  $\text{ZnCl}_2$ ,  $\text{FeCl}_3$ ,  $\text{NaNO}_2$ ,  $\text{NaNO}_3$ , NaClO,  $\text{H}_2\text{O}_2$ , glutathione (GSH), ascorbic acid (AA), and  $\text{Na}_2\text{S}$  (a hydrogen sulfide source) in twice-distilled water and Superoxide ( $\text{O}_2^-$ ) in DMSO. Both Angeli's salt (the HNO source) and DEA/NONOate (the NO source) were dissolved in 0.01M NaOH solution. Hydroxyl radical ( $\cdot\text{OH}$ ) and *tert*-butoxy radical ( $\cdot\text{OtBu}$ ) were generated by reacting 0.2 mM  $\text{Fe}^{2+}$  with 0.02 mM  $\text{H}_2\text{O}_2$  and 0.02 mM TBHP, respectively. The test solution of the probe **P-Np-Rhod** (15  $\mu\text{M}$ ) in 2 mL of 10 mM PBS buffer solution (pH 7.4) was prepared by placing 30  $\mu\text{L}$  of

the **P-Np-Rhod** stock solution ( $1 \times 10^{-3}$  M) in 1.97 mL of the various analytes buffer/EtOH solution ( $\text{H}_2\text{O}/\text{CH}_3\text{CH}_2\text{OH} = 1:1$ , v/v). The resulting solutions were shaken well and kept at ambient temperature for 30 min, and then the fluorescence intensities were measured. The two-photon excited fluorescence intensity was measured at 700-900 nm by using Rhodamine B as the reference, whose two-photon property has been well characterized in the literature.<sup>1</sup> **P-Np-Rhod** and **Np-Rhod** were dissolved in PBS buffered EtOH solution (10 mM, pH 7.4, containing 50% EtOH), then the intensities of the two-photon-induced fluorescence spectra of the samples and reference excited at the same wavelength were determined. The two-photon absorption cross-section ( $\delta$ ) was calculated by using the following formula:

$$\delta = \delta_r (S_s \Phi_r \phi_r C_r) / (S_r \Phi_s \phi_s C_s)$$

The subscripts *s* and *r* denote the sample and reference molecule, respectively.<sup>2</sup>

### Synthesis of donor 2 and probe **P-Np-Rhod**



**Scheme S1** Synthetic route of probe **P-Np-Rhod** and **donor 2**

Compound **1** and acceptor **3** were synthesized according to previous literatures.<sup>3</sup>

### Synthesis of donor **2**

EDCI (95.9 mg, 0.5mmol) and DMAP (31 mg, 0.25 mmol) were added to a solution of compound **1** (107.6 mg, 0.5 mmol) in 10 mL of dry CH<sub>2</sub>Cl<sub>2</sub>. The reaction mixture was stirred at room temperature for 30 min, and then *tert*-butyl piperazine-1-carboxylate (111.7 mg, 0.6 mmol) was added. The resulting solution was stirred at room temperature overnight. The solution was then washed with water, brine, and dried with anhydrous MgSO<sub>4</sub>. The organic solvent was concentrated under reduced pressure. The residue was purified by silica gel chromatography (petroleum ether/ ethyl acetate = 5:1, v/v) to afford donor **2** as a white solid (172.3 mg, 90%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.78 (s, 1H), 7.72 (d, *J* = 9.1 Hz, 1H), 7.66 (d, *J* = 8.5 Hz, 1H), 7.38 (dd, *J* = 8.5, 1.4 Hz, 1H), 7.20 (d, *J* = 7.6 Hz, 1H), 7.02-6.80 (m, 1H), 3.64 (s, 4H), 3.48 (s, 4H), 3.08 (s, 6H), 1.48 (s, 9H). MS (ESI): *m/z* 384.1 [M+H]<sup>+</sup>, 406.1 [M+Na]<sup>+</sup>, calcd 383.2.

**Synthesis of compound Np-Rhod.** EDCI (95.9 mg, 0.5mmol) and DMAP (31 mg, 0.25 mmol) were added to a solution of compound **1** (107.6 mg, 0.5 mmol) in 10 mL of dry CH<sub>2</sub>Cl<sub>2</sub>. The reaction mixture was stirred at room temperature for 30 min, and then compound **3** (240.5 mg, 0.6 mmol) was added. The resulting solution was stirred at room temperature overnight. The solution was then washed with water, brine, and dried with anhydrous MgSO<sub>4</sub>. The organic solvent was concentrated under reduced pressure. The residue was purified by silica gel chromatography (CH<sub>2</sub>Cl<sub>2</sub>/ EtOH=100:1, v/v) to afford compound **Np-Rhod** as a red solid (208.9 mg, 70%). <sup>1</sup>H NMR (400 MHz, *d*-DMSO) δ 10.15 (s, 1H), 8.01 (d, *J* = 7.5 Hz, 1H), 7.84 (s, 1H), 7.79 (m, 2H), 7.71 (t, *J* = 8.2 Hz, 2H), 7.39 (d, *J* = 9.7 Hz, 1H), 7.27 (m, 2H), 6.96 (s, 1H), 6.83 (s, 1H), 6.75 (d, *J* = 8.9 Hz, 1H), 6.70 (s, 1H), 6.56 (d, *J* = 7.0 Hz, 3H), 3.68 (s, 4H), 3.31 (s, 4H), 3.02 (s, 6H). <sup>13</sup>C NMR (100 MHz, *d*-DMSO) δ 169.94, 169.05, 159.79, 152.78, 152.58, 152.32, 152.15, 149.55, 135.84, 135.51, 130.35, 129.53, 129.36, 128.74, 128.49, 127.19, 126.54, 126.14, 125.27, 125.23, 124.90, 124.29, 117.04, 112.85, 112.24, 109.98, 108.89, 105.47, 102.53, 101.74, 83.48, 55.21, 47.78, 40.43. MS (ESI): *m/z* 596.0 [M-H]<sup>-</sup>, calcd 597.2.

**Synthesis of probe P-Np-Rhod.** A mixture of 2-(diphenylphosphino) benzoic acid (30.6 mg, 0.1 mmol), EDCI (19.2 mg, 0.1 mmol) and DMAP (6.2 mg, 0.05 mmol) were dissolved in dry DCM (3 mL), and

then the reaction solution was stirred at room temperature for 30 min. compound **Np-Rhod** (71.6 mg, 0.12 mmol) was then added to the mixture, and the resulting mixture was stirred at room temperature overnight. The solution was then washed with water, brine, and dried with anhydrous MgSO<sub>4</sub>. DCM was removed under reduced pressure and the crude product was purified by silica gel column chromatography (CH<sub>2</sub>Cl<sub>2</sub>/ EtOH=150:1, v/v) to afford compound **P-Np-Rhod** as a white solid (57.5 mg, 65%). <sup>1</sup>H NMR (400 MHz, *d*-DMSO) δ 8.27-8.23 (m, 1H), 8.04 (d, *J* = 7.4 Hz, 1H), 7.84-7.78 (m, 3H), 7.75 (d, *J* = 7.3 Hz, 1H), 7.71 (d, *J* = 8.8 Hz, 1H), 7.64-7.59 (m, 2H), 7.40 (s, 8H), 7.32 (d, *J* = 7.4 Hz, 1H), 7.25-7.20 (m, 4H), 7.07 (s, 1H), 6.97 (s, 1H), 6.95- 6.92 (m, 1H), 6.85 (s, 1H), 6.83-6.77 (m, 3H), 6.61 (d, *J* = 8.9 Hz, 1H), 3.68 (s, 4H), 3.30-3.40 (m, 4 H), 3.03 (s, 6H). <sup>13</sup>C NMR (100 MHz, *d*-DMSO) δ 170.13, 169.05, 164.84, 152.92, 152.77, 151.99, 151.75, 149.72, 140.96, 140.68, 137.57, 137.45, 136.23, 135.68, 134.28, 134.11, 133.90, 133.57, 133.21, 133.02, 131.72, 130.78, 129.70, 129.52, 129.38, 129.29, 129.22, 128.65, 127.35, 126.30, 125.43, 125.39, 124.51, 118.32, 117.39, 117.20, 112.76, 110.71, 108.43, 105.63, 101.77, 82.50, 55.36, 47.85, 40.63. <sup>31</sup>P NMR (162 MHz, *d*-DMSO) δ -2.92. MS (ESI): *m/z* 886.2 [M+H]<sup>+</sup>, calcd 885.3.

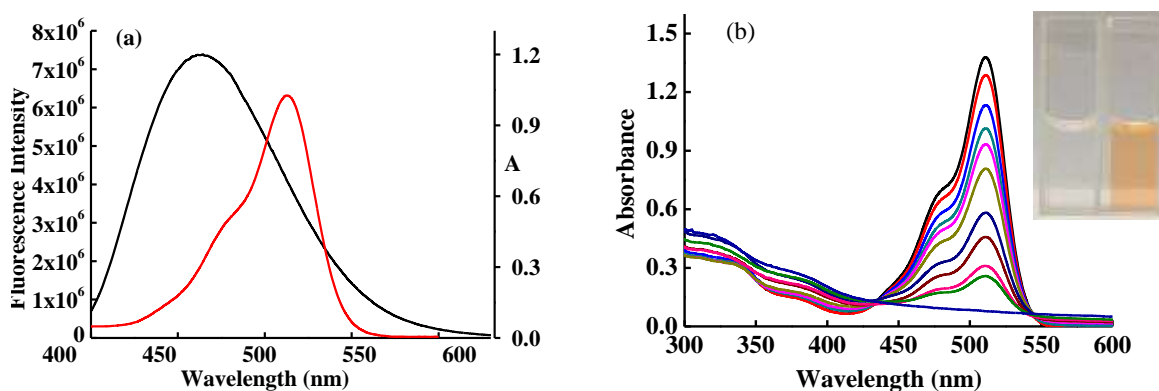
### Cell cytotoxic assays and two-photon fluorescence imaging

To evaluate the potential cytotoxicity of probe **P-Np-Rhod** and the product **Np-Rhod**, HeLa cells were seeded at 1×10<sup>5</sup> cells per well in 96-well plates and incubated for 24 h. After that different concentration (5-50 μM) of probe **P-Np-Rhod** and compound **Np-Rhod** were added to the cells and these cells were cultured for an additional 24 h. And then the cytotoxic effects of **P-Np-Rhod** and **Np-Rhod** were determined using MTT assays. For fluorescent imaging, HeLa cells were washed with Dulbecco's phosphate buffered saline (DPBS) three times and then incubated with 15 μM of **P-Np-Rhod** for 30 min. After the cells were washed with DPBS three times, 100 μM (or 200 μM) AS was added and the cells were incubated for another 30 min. The HeLa cells were washed with DPBS three times and imaged. The two-photon excitation wavelength of the femtosecond laser was fixed at 740 nm; the emission wavelengths were recorded at 470-530 and 550-650 nm respectively.

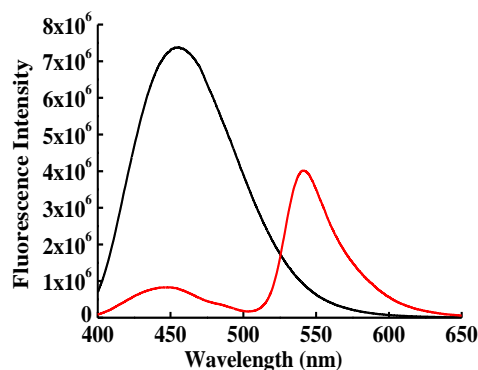
### Two-photon fluorescence imaging of fresh mouse liver slices

Frozen tissue slices were prepared from the livers of nude mice. The slices were incubated with

**P-Np-Rhod** (50  $\mu\text{M}$ ) in an incubator at 37  $^{\circ}\text{C}$  for 1 h and then washed with DPBS three times, and cultured with 1 mM AS for another 1h at 37  $^{\circ}\text{C}$ . Finally, the slices were washed with DPBS three times, and then two-photon fluorescence microscopy images were collected. The excitation wavelength of the femtosecond laser was set at 740 nm, the emission wavelengths were recorded at 470-530 and 550-650 nm, respectively



**Fig. S1** (a) Normalized emission spectra of donor derivative **2** (black line) and normalized absorption spectra of acceptor **3** (red line) in PBS/EtOH (1/1, v/v, pH= 7.4, 10 mM). (b) The absorption spectra of probe **P-Np-Rhod** (15  $\mu\text{M}$ ) in the presence of various concentrations of AS (0-100  $\mu\text{M}$ ) in PBS buffered (10 mM, pH = 7.4) aqueous EtOH solution (1:1, v/v).

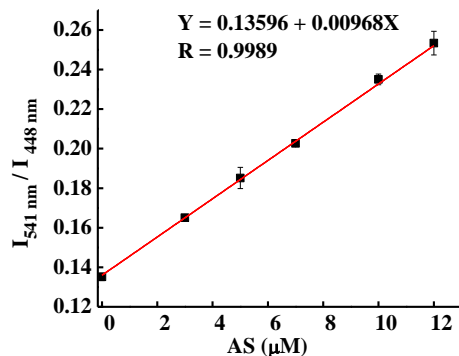


**Fig. S2** Normalized emission spectra. The black and red lines represent donor derivative **2** (15  $\mu\text{M}$ ) and **Np-Rhod** (15  $\mu\text{M}$ ) respectively, in PBS/EtOH (1/1, v/v, pH= 7.4, 10 mM), as the respective fluorescence responses;  $\lambda_{\text{ex}} = 370 \text{ nm}$ .

**Energy Transfer Efficiency (ETE)** = [(fluorescence of donor - fluorescence of donor in cassette)/fluorescence of donor]  $\times 100\%$ .<sup>4</sup>

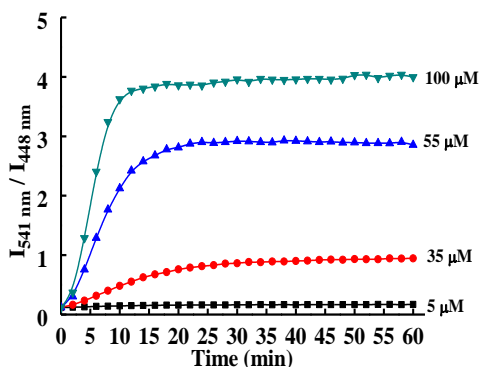
For **P-Np-Rhod**,  $ETE = (7241340 - 826170) / 7241340 \times 100\% = 88.6\%$

**Fluorescence titration and detection limit:** The detection limit was determined from the fluorescence titration data. The fluorescence intensity ratio ( $I_{541\text{ nm}}/I_{448\text{ nm}}$ ) increased linearly with the concentration of AS ranging from 3  $\mu\text{M}$  to 12  $\mu\text{M}$ . The detection limit was calculated to be  $5.9 \times 10^{-7}$  M based on  $3\sigma/\text{slope}$  method.



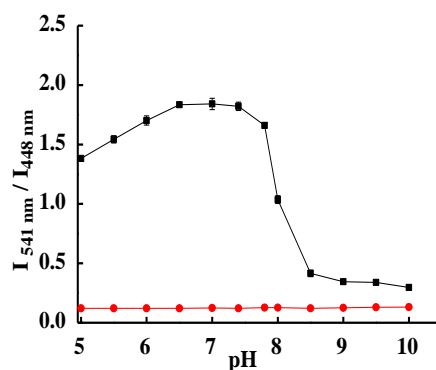
**Fig. S3** The linear responses at low AS concentrations (3-12  $\mu\text{M}$ ).  $\lambda_{\text{ex}} = 370$  nm.

**Kinetic studies:** Time-dependent fluorescence responses were obtained by monitoring the change of Fluorescent intensity ratio between the two wavelengths ( $I_{541\text{ nm}}/I_{448\text{ nm}}$ ) of the probe **P-Np-Rhod** after the addition of AS (5, 35, 55 or 100  $\mu\text{M}$ ) in buffered (PBS, pH = 7.4) water/ $\text{C}_2\text{H}_5\text{OH}$  (1/1, v/v) solution.

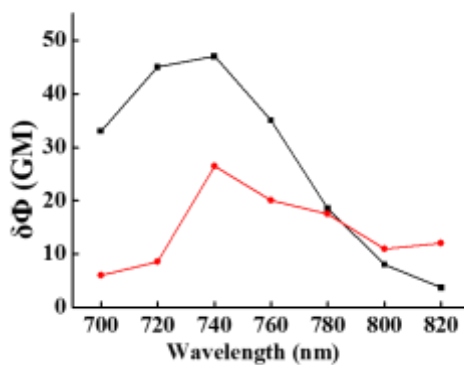


**Fig. S4** Time-dependent fluorescence spectral changes of **P-Np-Rhod** (15  $\mu\text{M}$ ) with AS (AS = 5, 35, 55 or 100  $\mu\text{M}$ ) in PBS buffered (10 mM, pH = 7.4) aqueous EtOH solution (1:1, v/v). The fluorescence intensity ratio  $I_{541\text{ nm}}/I_{448\text{ nm}}$  was plotted versus time. Time points represent 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, and 60 min,  $\lambda_{\text{ex}}=370$  nm.

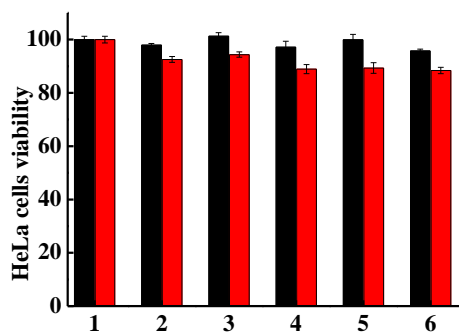




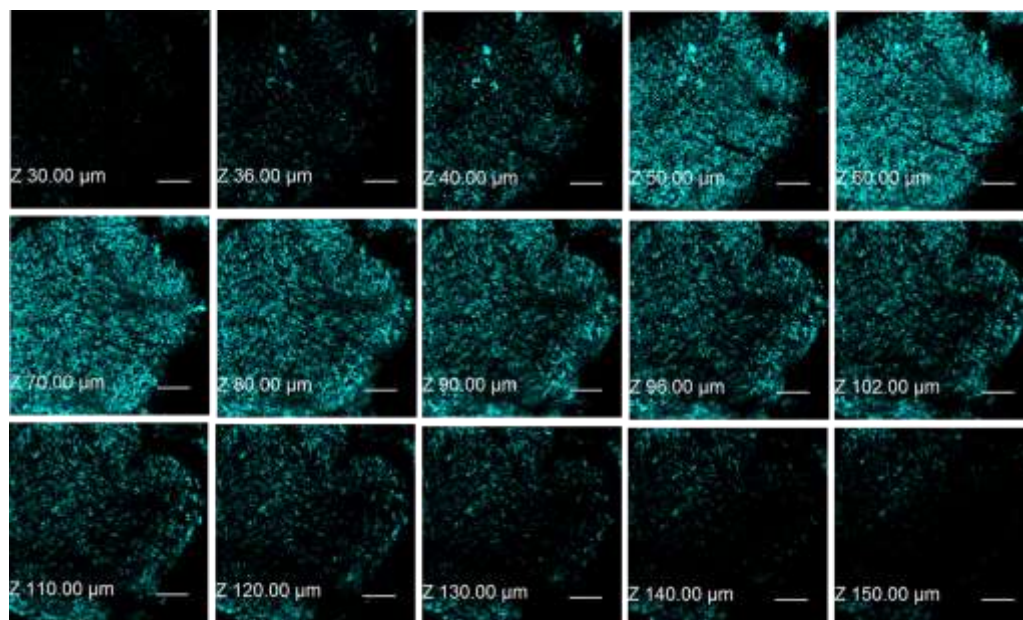
**Fig. S5** The influence of pH on ratiometric fluorescence responses ( $I_{541 \text{ nm}}/I_{448 \text{ nm}}$ ) of **P-Np-Rhod** (15  $\mu\text{M}$ ) in buffered/EtOH (1/1, v/v, pH = 5.0-10.0, 10 mM), the pH were adjusted by NaOH (aq, 1M) or HCl (aq, 1M),  $\lambda_{\text{ex}} = 370 \text{ nm}$ . Ratiometric fluorescence responses are shown before (red line) and after (black line) the addition of AS (50  $\mu\text{M}$ ), respectively



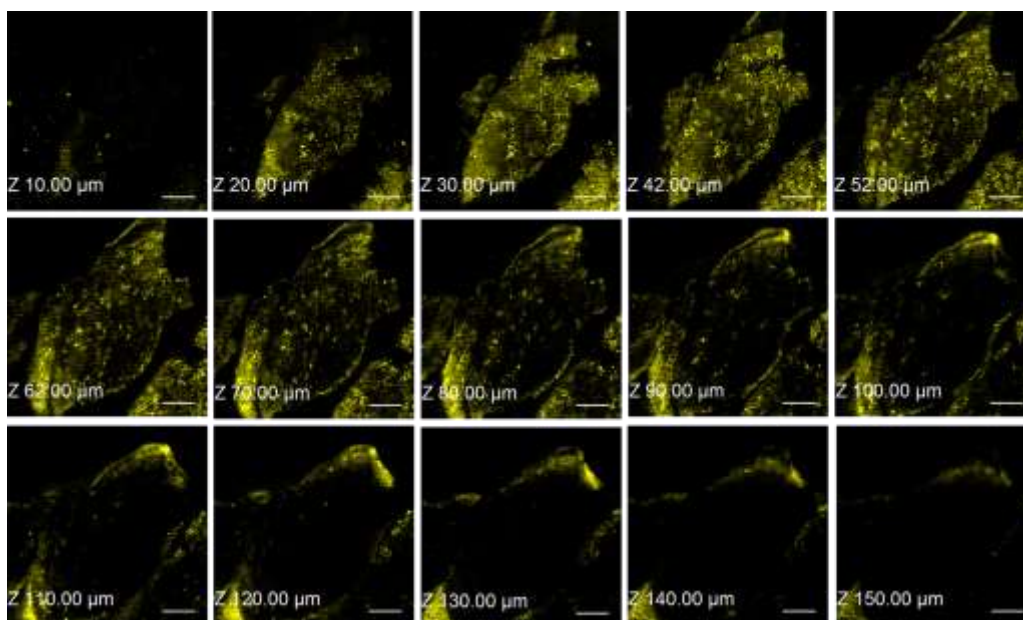
**Fig. S6** TP absorption cross-section of **P-Np-Rhod** and **Np-Rhod** in PBS buffered (10 mM, pH = 7.4) aqueous EtOH solution (1:1, v/v). Black line represents the absorption cross-section of **P-Np-Rhod** and red line represents the absorption cross-section of **Np-Rhod**.



**Fig. S7** Cytotoxicity assay of both **P-Np-Rhod** and **Np-Rhod** at different concentrations (1: 0  $\mu\text{M}$ ; 2: 5  $\mu\text{M}$ ; 3: 10  $\mu\text{M}$ ; 4: 20  $\mu\text{M}$ ; 5: 30  $\mu\text{M}$ ; 6: 50  $\mu\text{M}$ ) for HeLa cells. Black bar represents cytotoxicity of **P-Np-Rhod** and red bar represents cytotoxicity of **Np-Rhod**.



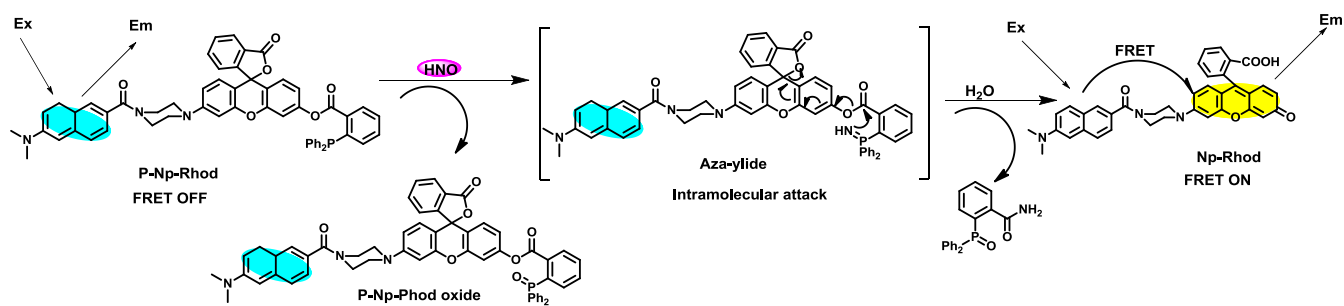
(a)



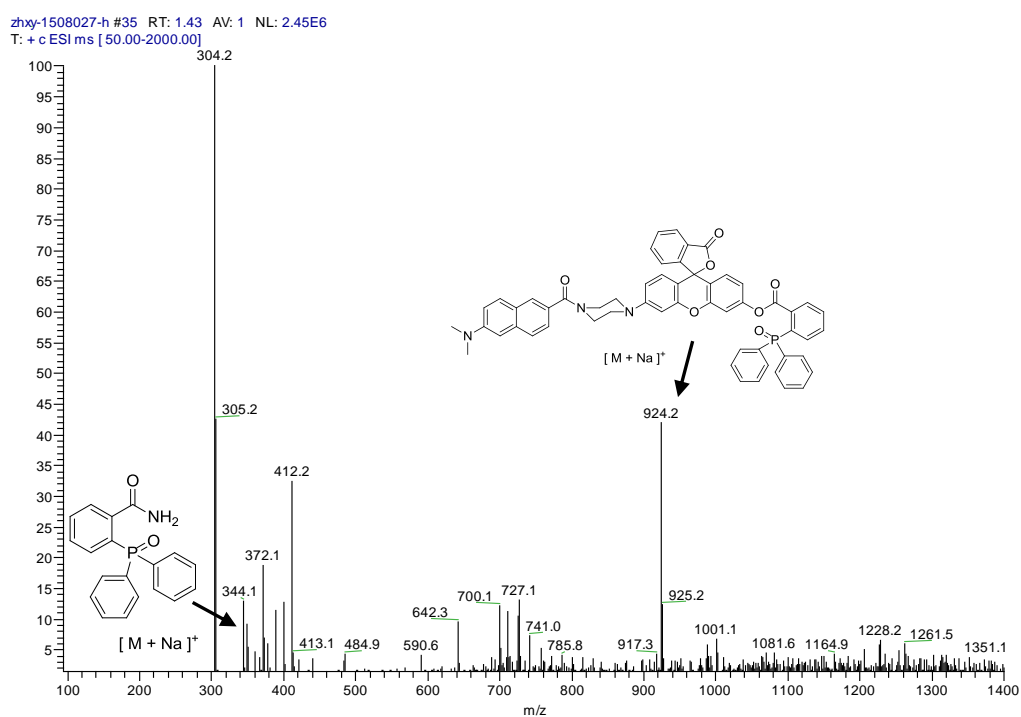
(b)

**Fig. S8** (a) Two-photon fluorescence images of a fresh rat liver slice incubated with **P-Np-Rhod** (50  $\mu\text{M}$ ) in the absence of AS at the depths of approximately 0-150  $\mu\text{m}$ ; (b) Two-photon fluorescence images of a fresh rat liver slice pretreated with **P-Np-Rhod** (50  $\mu\text{M}$ ) and then with AS (1 mM) at the

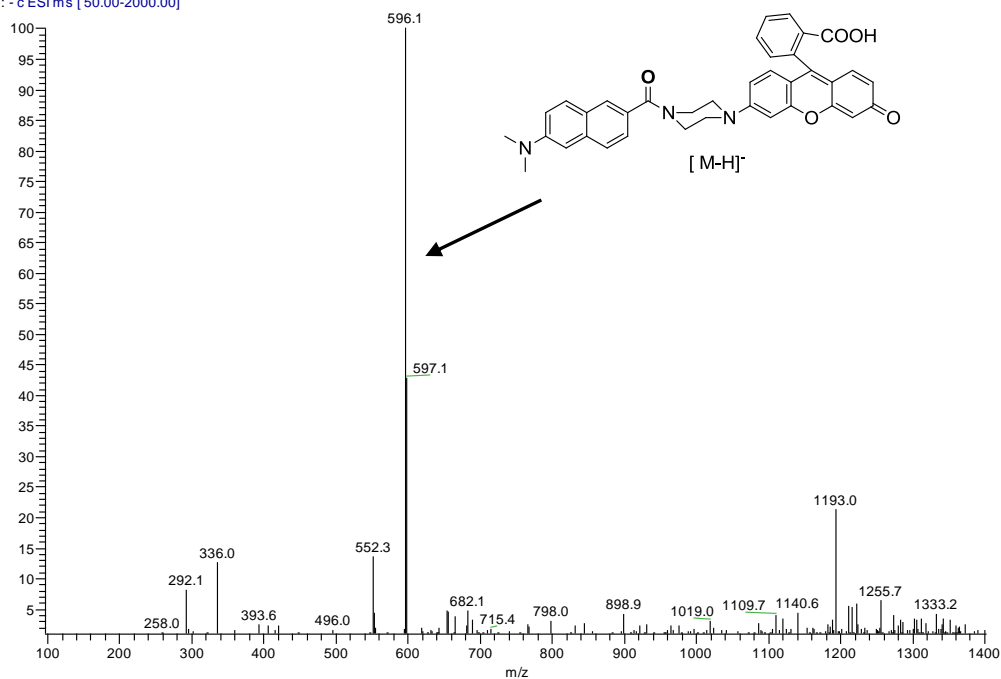
depths of approximately 0-152  $\mu\text{m}$ . Excitation at 740 nm, Scale bar = 200  $\mu\text{m}$ , Cyan channel:  $\lambda_{\text{em}}=470\text{-}530$  nm; yellow channel  $\lambda_{\text{em}}=550\text{-}650$  nm.



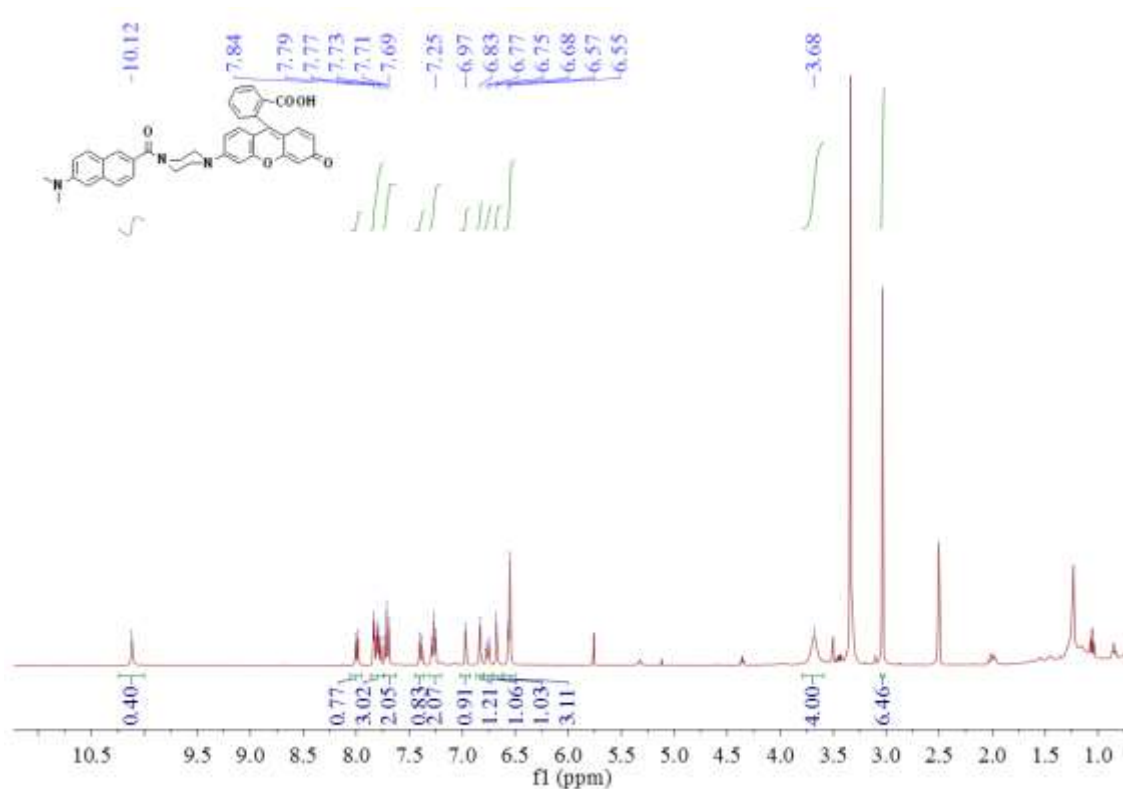
**Scheme S2** Reaction mechanism of **P-Np-Rhod** and HNO.



zhxy-1508027-h #28 RT: 1.17 AV: 1 NL: 1.04E6  
T: -c ESI ms [50.00-2000.00]



**Fig. S9** ESI-MS spectrum of **P-Np-Rhod** (200  $\mu$ M) with AS (1.5 mM) in PBS buffered (10 mM, pH = 7.4) aqueous EtOH solution (1:1, v/v). The ESI-MS spectrum was obtained 30 min after the addition of AS.

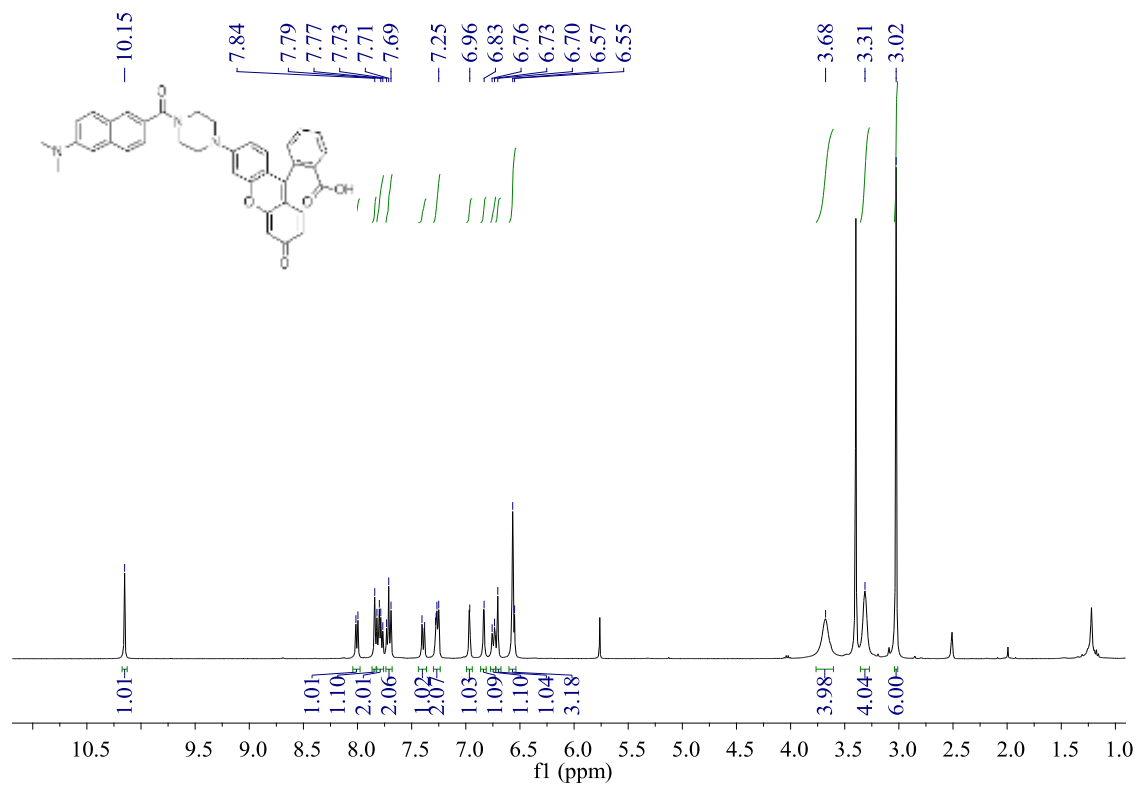


**Fig. S10** <sup>1</sup>H NMR spectrum of isolated product of probe **P-Np-Rhod** reacted with AS.

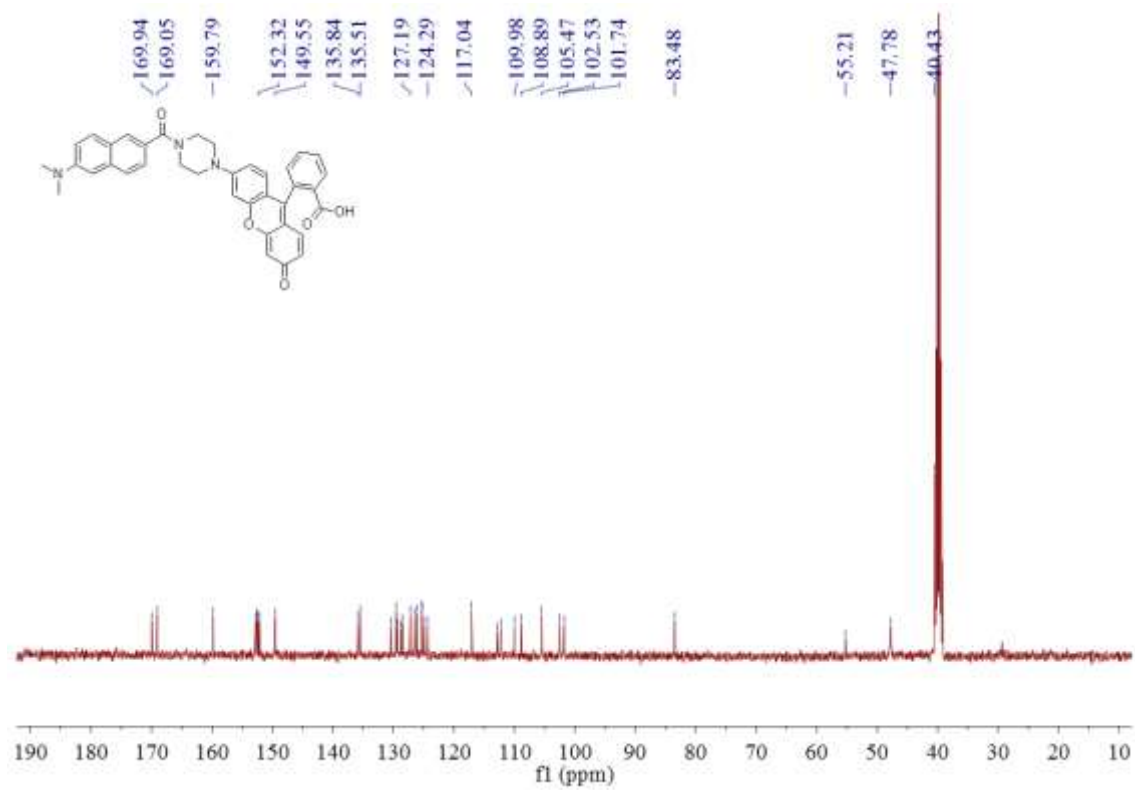
## References

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2. G. J. Mao, T. T. Wei, X. X. Wang, S. Y. Huan, D. Q. Lu, J. Zhang, X. B. Zhang, W. Tan, G. L. Shen and R. Q. Yu, *Anal. Chem.*, 2013, **85**, 7875.
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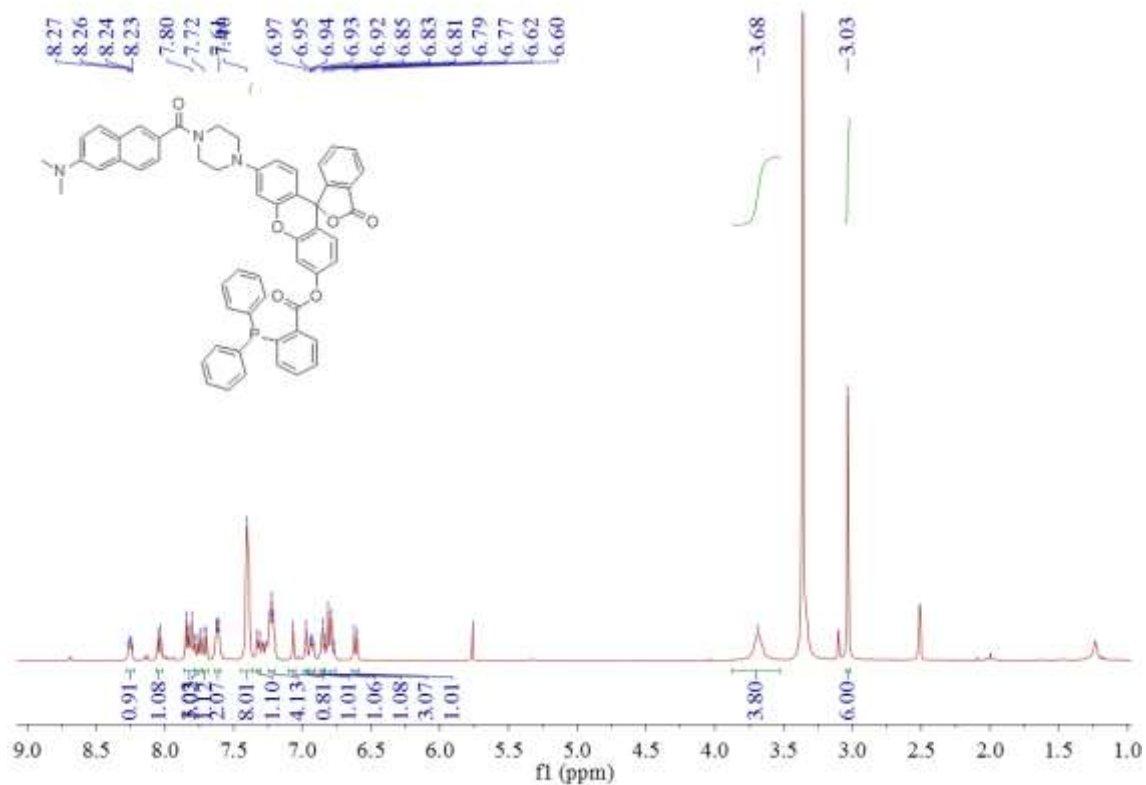
**NMR spectrum and MS of all the new compounds**



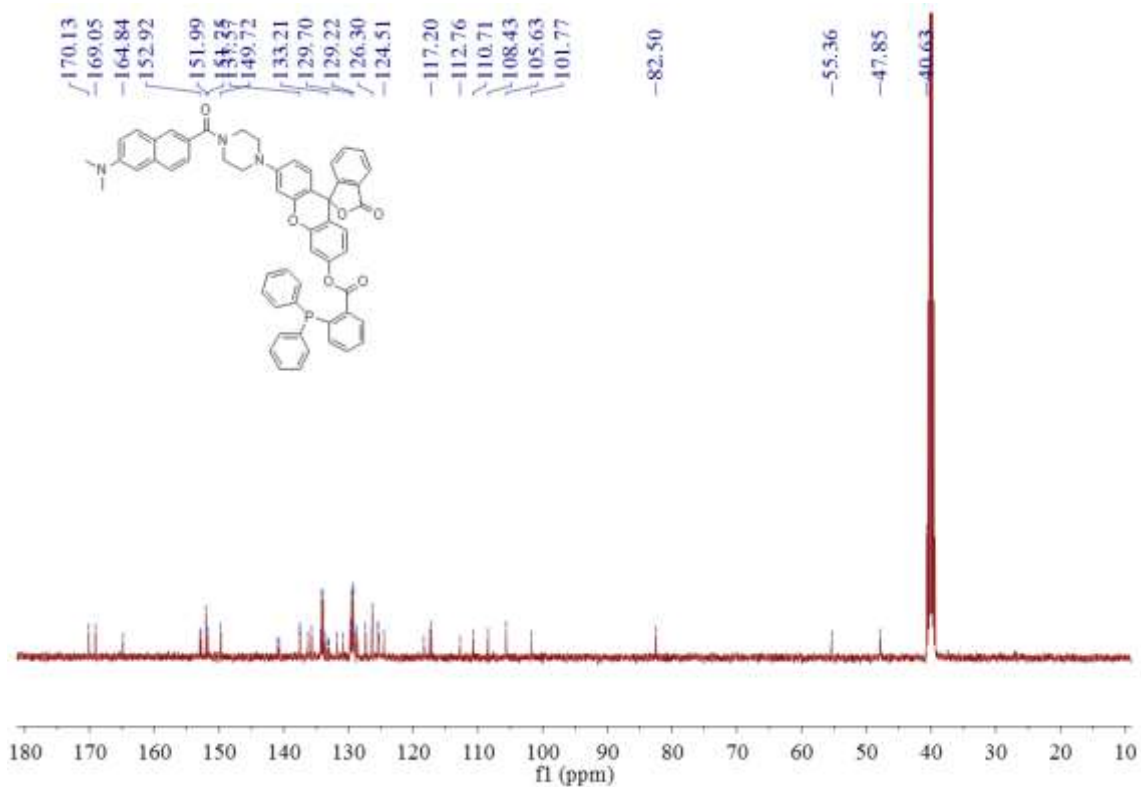
**Fig. S11**  $^1\text{H}$ NMR spectrum of the compound **Np-Rhod**



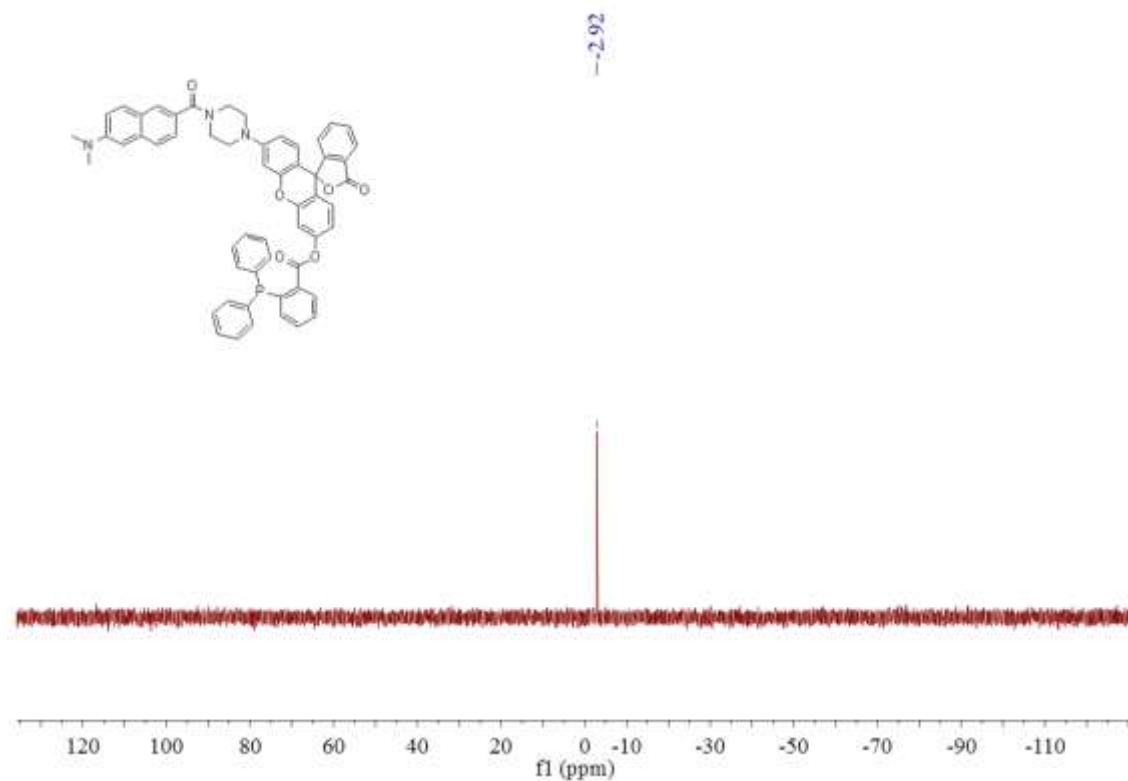
**Fig. S12**  $^{13}\text{C}$ NMR spectrum of the compound **Np-Rhod**



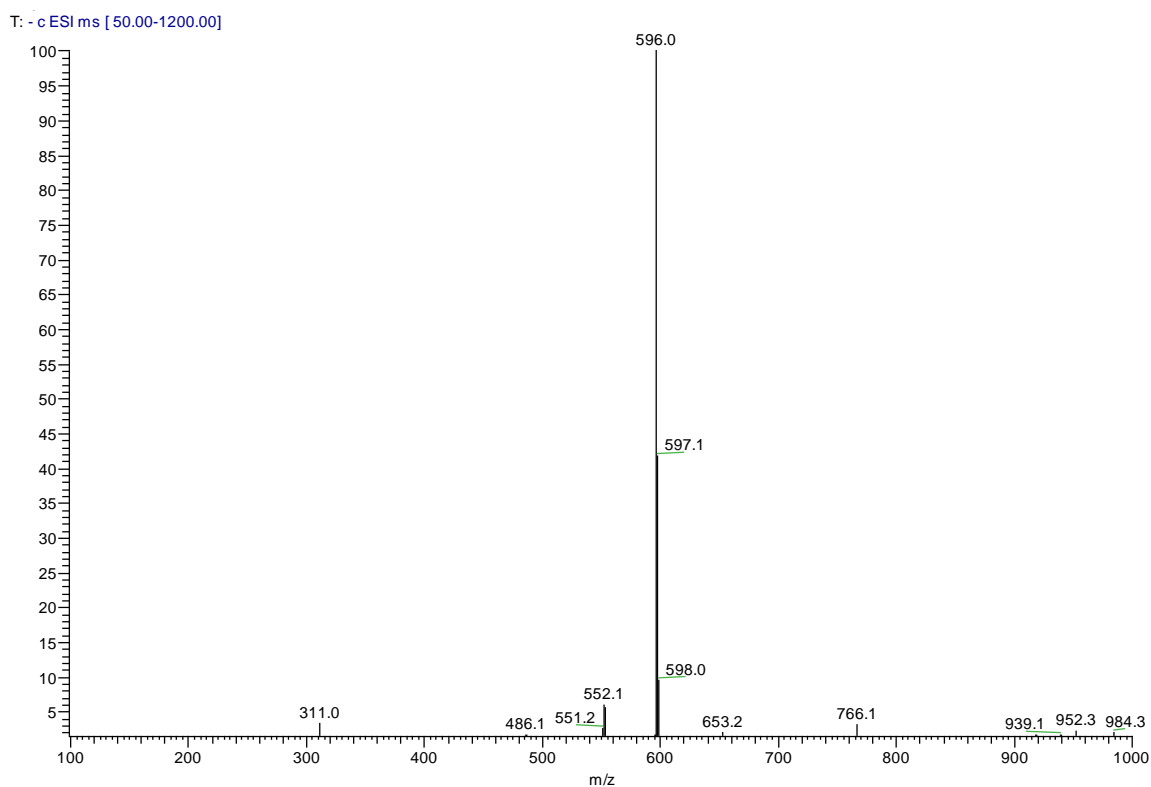
**Fig. S13**  $^1\text{H}$ NMR spectrum of the probe **P-Np-Rhod**



**Fig. S14**  $^{13}\text{C}$ NMR spectrum of the probe **P-Np-Rhod**



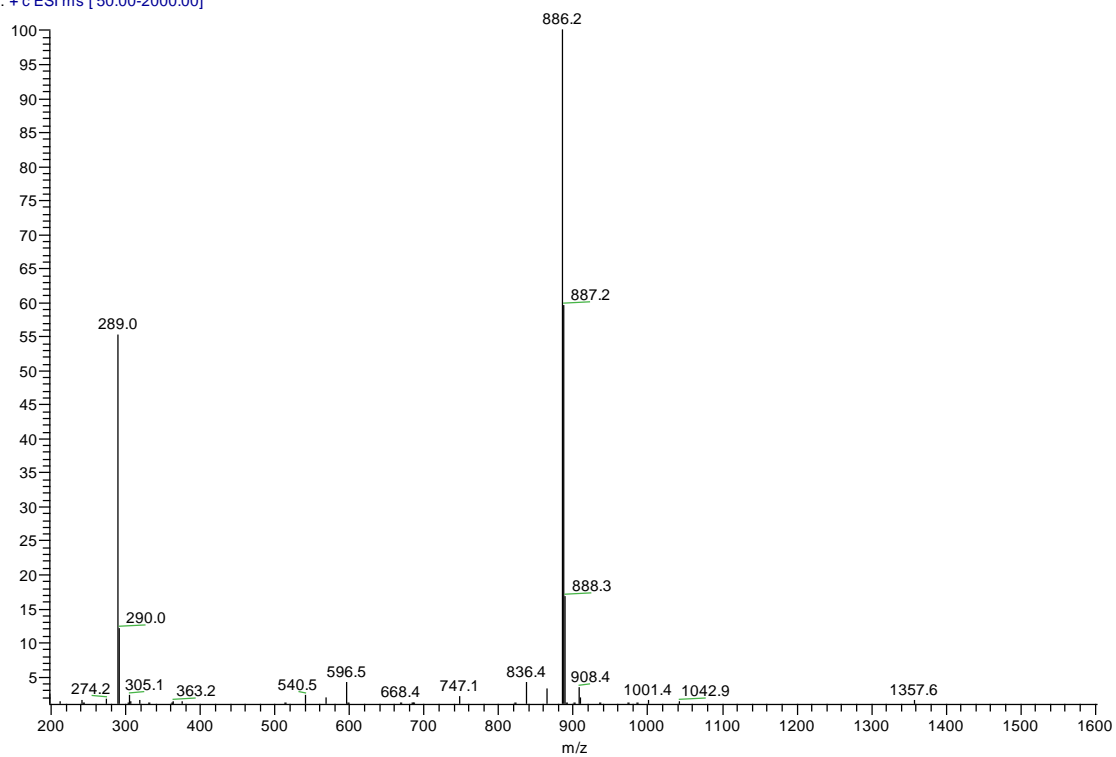
**Fig. S15**  $^{31}\text{P}$ NMR spectrum of the probe **P-Np-Rhod**



**Fig. S16** ESI mass spectrum of the compound **Np-Rhod**



zhxy-150707-885-h #13 RT: 0.47 AV: 1 SB: 2 0.17-0.20 NL: 1 67F7  
T: + c ESI ms [50.00-2000.00]



**Fig. S17** ESI mass spectrum of the probe **P-Np-Rhod**