Supplementary information

In vivo imaging of hepatocellular nitric oxide using a hepatocyte-targeting fluorescent sensor

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Experimental section

Materials

4-Amino-3-nitrophenol, propargyl bromide, stannous chloride dihydrate ($SnCl_2$), Rhodamine B, 2,3,4,6-Tetra-O-acetyl-α-D-glactopyranosyl bromide and sodium ascorbate were purchased from Aladdin and used as received. Acetone, dioxane, DMSO, 1,2-dichloroehane and acetonitrile were analytically pure solvents and distilled before use. The water used throughout the experiments was the double-distilled water which was further treated by ion exchange columns and then by a Milli-Q water purification system. DEA/NONOate (diethylamine NONOate), tert-butylhydroperoxide (TBHP), Angeli's salt (AS) and KO_2 were obtained from commercial sources and used without additional purification. Unless specially mentioned, all other chemicals were used as received.

Characterization

^1H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 600 MHz NMR spectrometer. High resolution mass spectra (HR-MS) were obtained on Sciex X500R mass spectrometer. High-performance liquid chromatography (HPLC) analyses were performed on Agilent 1260. FTIR spectra were obtained by using a Nicolet Avatar 360 FTIR spectrophotometer. UV-vis spectra were recorded on a Hitachi U-3010 UV-vis spectrophotometer. Fluorescence spectra were recorded on a Hitachi F-4600 fluorescence spectrophotometer. Fluorescence images were obtained using an Olympus IX 71 with a DP72 color CCD.

Preparation of the sensor (Gal-RhB)

Synthesis of 1: The mixture of 4-amino-3-nitrophnol (462 mg, 3.0 mmol), K_2CO_3 (829 mg, 6.0 mmol) and propargyl bromide solution (0.5 mL, 4.5 mmol) in 20 mL of dry acetone was

heated to reflux for 12 h. After cooling to room temperature, the solution was filtered. The filtrate was concentrated under pressure and washed with an excess amount of petroleum ether to remove unreacted propargyl bromide. This crude product was further purified by column chromatography using petroleum ether/ethyl acetate (3:1, V/V) as the eluent to give an orange solid (409 mg, 75%). ^{1}H NMR (DMSO-d_6, 600 MHz, ppm): 7.51 (d, J = 2.9 Hz, 1H), 7.30 (s, 2H), 7.24-7.14 (m, 1H), 7.02 (d, J = 9.2 Hz, 1H), 4.77 (d, J = 2.2 Hz, 2H), 3.59 (d, J = 19 Hz, 1H). ^{13}C NMR (DMSO-d_6, 600 MHz, ppm): 146.80, 142.28, 128.90, 127.54, 120.65, 107.25, 78.95, 78.53, 56.10. HR-MS(ESI): m/z 193.0611 $[M+H]^+$.

Synthesis of 2: A cooled solution of stannous chloride dihydrate (1.35 g, 6.0 mmol) in concentrated HCl (3.0 mL) was added dropwise to a solution of **1** (200 mg, 1.2 mmol) in dioxane (15 mL) at 10 °C. After complete addition, the reaction mixture was allowed to stir for 48 h at room temperature, slowly becoming more yellow in color. After neutralizing with aqueous sodium hydroxide, the solution was extracted with dichloromethane (4 ×10 mL). The organic layer was then dried with anhydrous sodium sulfate and the solvent was evaporated in vacuo, yielding a yellowish brown viscous oil. This crude product was further purified by column chromatography using dichloromethane/ethyl acetate (5:1, V/V) as the eluent to afford a colorless and highly viscous oil (140 mg, 72%). ^{1}HNMR (DMSO-d_6, 600 MHz, ppm): 6.41 (d, J = 8.3 Hz, 1H), 6.20 (d, J = 2.8 Hz, 1H), 6.03 (dd, J = 8.4, 2.8 Hz, 1H), 4.52 (t, J = 5.6 Hz, 4H), 4.05 (m, 2H), 3.44 (t, J = 2.4 Hz, 1H). ^{13}C NMR (DMSO-d_6, 600 MHz, ppm): 170.33, 150.11, 136.44, 129.15, 114.91, 102.51, 80.18, 77.31, 55.68. HR-MS(ESI): m/z 163.0864 $[M+H]^+$.

Synthesis of 3: To a solution of 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl bromide (822 mg, 2.0 mmol) in dry DMSO (3 mL) was added sodium azide (650 mg, 10 mmol) and the reaction was allowed to stir at room temperature for 30 min. The reaction mixture was then diluted with water (20 mL) and extracted with EtOAc (50 mL). The organic layer was then dried with anhydrous sodium sulfate and the solvent was evaporated in vacuo. The crude product was further purified by flash chromatography using ethyl acetate/petroleum ether (1:2, V/V) as the eluent to give a white solid (750 mg, 90%). ^{1}HNMR ($CDCl_3$, 600 MHz, ppm): 5.38 (d, 1H), 5.13 (dd, J = 10.4, 8.6 Hz, 1H), 5.00 (dd, J = 10.4, 3.4 Hz, 1H), 4.57 (t, J = 8.6 Hz, 1H), 4.14 (m, 2H),

3.98 (dd, J = 7.0, 1.0 Hz, 1H), 2.14-1.98 (4xs, 12 H). ^{13}C NMR ($CDCl_3$, 600 MHz, ppm): 168.52 , 86.85, 71.44, 69.30, 66.66, 65.46, 59.82, 20.30. HR-MS(ESI): m/z 396.1020 $[M+Na]^+$.

Synthesis of 4: A solution of rhodamine B (60 mg, 0.07 mmol) in dry 1,2-dichloroehane (10 mL) was stirred until the solid dissolved completely, and phosphorus oxychloride (1 mL) was added with vigorous stirring at room temperature for 5 min. Then the solution was refluxed for 4 h. The reaction mixture was cooled and evaporated in vacuo to give acid chloride which was used without further purification.

Synthesis of 5: The compound **4** (100 mg, 0.21 mmol) was dissolved in acetonitrile (5 mL) and added dropwise to a solution of **2** (68 mg, 0.42 mmol) in CH_3CN (5 mL) and NEt_3 (1 mL). After stirring for 12 h at room temperature, the mixture was concentrated under vacuum and the crude product was purified by silica column chromatography using dichloromethane/ethyl acetate (15:1~10:1, V/V) as the eluent to give an off-white solid (62 mg, 51%). ^{1}HNMR (DMSO-d_6, 600 MHz, ppm): 7.87-7.84 (m, 1H), 7.58 (pd, J = 7.3, 1.2 Hz, 2H), 7.11 (d, J = 6.7 Hz, 1H), 6.58 (s, 2H), 6.37 (s, 2H), 6.23 (s, 2H), 6.14 (d, J = 2.1 Hz, 1H), 5.81 (d, J = 2.4 Hz, 2H), 4.57 (d, J = 2.3 Hz, 2H), 4.43 (s, 2H), 3.49 (t, J = 2.3 Hz, 1H), 3.31 (d, J = 5.6 Hz, 8H), 1.07 (t, J = 7.0 Hz, 12H). ^{13}C NMR (DMSO-d_6, 600 MHz, ppm): 164.73, 156.27, 152.10, 151.90, 147.44, 146.06, 131.91, 130.17, 128.04, 127.81, 127.47, 123.08, 121.73, 113.97, 106.99, 101.13, 100.23, 96.30, 78.38, 76.80, 65.84, 54.69, 42.86, 12.06. MS(ESI): m/z 587.3003 $[M+H]^+$.

Synthesis of 6: The compound **5** (58.7 mg, 0.10 mmol), **3** (83 mg, 0.20 mmol), sodium ascorbate (6.0 mg, 0.03 mmol) and $CuSO_4 \cdot 5H_2O$ (3.75 mg, 0.015 mmol) were suspended in a mixture of THF and water (V/V, 5 mL/2 mL) in a 50 mL round bottom flask equipped with a small magnetic stirring bar. Under a nitrogen atmosphere, the mixture was stirred at room temperature for 24 h. After the materials were consumed completely (monitored via thin-layer chromatography), the reaction mixture was washed three times with water and extracted with dichloromethane. The organic phase was dried over $MgSO_4$. The solvent was evaporated, and the residue was purified by silica column chromatography using dichloromethane/ethyl acetate (2:1, V/V) as the eluent to give an off-white solid (62 mg, 65%). ^{1}HNMR (DMSO-d_6, 600 MHz, ppm): 8.29 (s, 1H), 7.86 (d, *J* = 6.7 Hz, 1H), 7.62-7.55 (m, 2H), 7.12 (d, *J* = 7.0 Hz, 1H), 6.59 (s, 2H), 6.37 (s, 2H), 6.27-6.18 (m, 3H), 5.90 (dd, *J* = 8.8, 2.7 Hz, 1H), 5.83 (d, *J* = 8.8 Hz, 1H), 5.48 (d, *J*

= 9.2 Hz, 1H), 5.25 (d, *J* = 5.7 Hz, 1H), 5.01 (d, *J* = 5.2 Hz, 1H), 4.97-4.87 (m, 2H), 4.63 (d, *J* = 5.4 Hz, 1H), 4.40 (s, 2H), 4.02 (td, *J* = 9.2, 5.4 Hz, 1H), 3.78-3.73 (m, 1H), 3.70 (t, *J* = 6.0 Hz, 1H), 3.56-3.45 (m, 4H), 3.32 (m, 4H), 2.96 (m, 12H), 1.08 (t, *J* = 7.0 Hz, 12H). ^{13}C NMR (DMSO-d_6, 600 MHz, ppm): 169.92, 169.42, 168.50, 165.61, 157.87, 153.04, 152.77, 148.34, 146.97, 143.36, 132.80, 131.11, 128.92, 128.78, 123.94, 122.63, 114.71, 107.87, 106.11, 102.07, 101.11, 97.19, 84.20, 72.90, 70.41, 67.64, 67.28, 66.78, 61.52, 60.41, 48.57, 43.61, 20.44, 20.36, 20.27, 19.92, 12.35. MS(ESI): m/z 960.4139 $[M+H]^+$.

Synthesis of Gal-RhB: A solution of the compound **6** (100 mg, 0.105 mmol) in dry MeOH (8 mL) was added sodium methoxide (68 mg, 1.26 mmol). After stirring for 24 h at room temperature, The solution was then neutralized by addition of HCl (1 M) until pH 7, the mixture was concentrated under vacuum and the crude product was purified by silica column chromatography using dichloromethane/methanol (10:1~4:1, V/V) as the eluent to give an off-white solid (50 mg, 60%). ^{1}HNMR (DMSO-d_6, 600 MHz, ppm): 8.40 (s, 1H), 7.86 (d, *J* = 6.7 Hz, 1H), 7.62-7.55 (m, 2H), 7.12 (d, *J* = 7.5 Hz, 1H), 6.58 (s, 2H), 6.37 (s, 2H), 6.28-6.22 (m, 3H), 6.18 (d, *J* = 2.7 Hz, 1H), 5.91-5.80 (d, 1H), 5.82 (d, *J* = 8.8 Hz, 1H), 5.61 (t, *J* = 9.7 Hz, 1H), 5.48-5.40 (m, 2H), 4.93 (s, 2H), 4.58 (dt, *J* = 12.3, 6.3 Hz, 1H), 4.40 (s, 2H), 4.12 (dd, *J* = 11.6, 5.0 Hz, 1H), 4.02 (dd, *J* = 11.6, 7.3 Hz, 1H), 3.32 (m, 8H), 1.07 (t, *J* = 7.0 Hz, 12H). ^{13}C NMR (DMSO-d_6, 600 MHz, ppm): 165.68, 158.02, 153.04, 152.74, 148.36, 146.98, 142.71, 142.60, 132.84, 131.07, 128.86, 128.41, 123.96, 123.37, 122.65, 114.59, 107.89, 106.09, 102.12, 100.96, 97.20, 88.09, 78.43, 73.66, 69.27, 68.45, 66.81, 60.56, 60.43, 43.62, 12.34. IR (KBr), ν/cm^{-1}: 3370, 2973, 2923, 1673, 1612, 1513, 1465, 1378, 1263, 1220, 1114, 1089, 1020, 817, 786, 701, 628. MS(ESI): m/z 792.3716 $[M+H]^+$ and 815.3572 $[M+Na]^+$.

General Procedure for NO Detection

A stock solution of DEA/NONOate was prepared in 0.01M NaOH solution. Unless otherwise stated, all the fluorescence measurements were performed in 10 mM PBS buffered (pH 7.0) water solution, according to the following procedure. In a 5 mL cuvette, 2 mL of PBS and 15 μL of 1 mM Gal-RhB (final concentration, 5 μM) were mixed, followed by addition of an appropriate volume of stock solution. The final volume of liquid in the cuvette was adjusted to 3.0 mL with PBS. After incubation at 37 °C for several minutes in a thermostat, the solution was transferred to

a quartz cell of 1 cm optical length to measure the absorbance or fluorescence. Also, a solution containing Gal-RhB only was prepared as the control and its spectra were measured under the same conditions.

Selectivity evaluation of the probe

Superoxide ($O_2^{\bullet-}$) was added as solid KO_2. A stock solution of Angeli's salt (AS, a nitroxyl source) was prepared in 0.01 M NaOH solution. Hydroxyl radicals (OH•) and tert-butoxy radicals (•OtBu) were generated by reaction of Fe^{2+} with H_2O_2 or TBHP, respectively. Singlet oxygen ($^1O_2$) was generated from ClO^- and H_2O_2. Various analytes (5 μM DEA-NONOate, 100 μM for ROS and RNS, 5.0 mM for GSH, 1.0 mM for others) represented by K^+, Ca^{2+}, Na^+, Fe^{2+}, Mg^{2+}, Zn^{2+}, GSH, Cys, Hcy, H_2O_2, HNO, $O_2^{\bullet-}$, •OtBu, OH•, ascorbic acid (AA), NO_2^-, NO_3^-, SCN^-, HClO, $^1O_2$. Unless otherwise stated, all the fluorescence measurements were performed in 10 mM PBS buffered (pH 7.0) water solution, according to the following procedure. In a 5 mL tube, 2 mL of PBS and 15 μL of 1 mM Gal-RhB (final concentration, 5 μM) were mixed, followed by addition an appropriate volume of analyte solution. The final volume of the solution was adjusted to 3.0 mL with PBS, and the solution was mixed rapidly. After incubation at 37 °C for 10 min in a thermostat, the solution was transferred into a quartz cell of 1 cm optical length to measure fluorescence.

Cell viability assay

To examine the toxicity of the sensor Gal-RhB in living cells, HepG2 cells (human hepatoma cells), HeLa cells (human cervical cancer cells), A2780 cells (human ovarian cancer cells) and L929 cells (murine aneuploid fibrosarcoma cells) were incubated in DMEM medium supplemented with 10% fetal bovine serum (FBS) and allowed to grow for 24 h at 37 °C with 5% CO_2. After removal of the medium, cells were treated with the sensor Gal-RhB (0, 10, 20, 30, 40, 50 μM) and incubated for an additional 24 h. The cytotoxicity of the sensor against four cell lines was assessed by MTT assay according to ISO 10993-5.

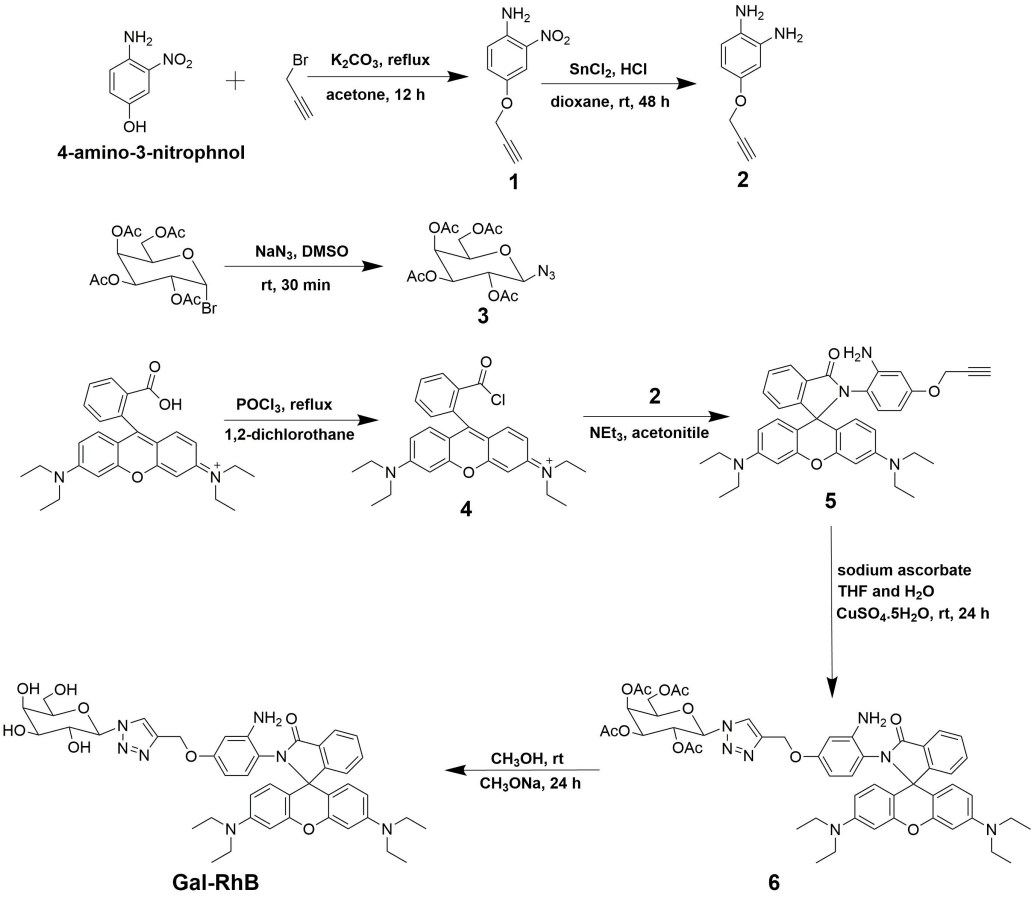
Cell imaging

For the experiments, four types of cells were incubated with **Gal-RhB** (final concentration 10 μM) or compound **5** (final concentration 10 μM) for 30 min at 37 ^oC, and then treated with or

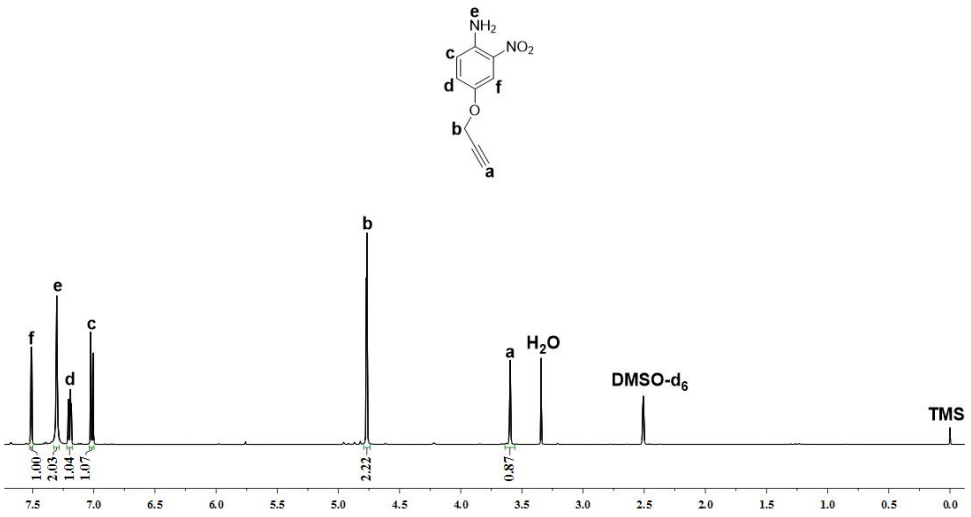
without DEA-NONOate (the donor of NO, 40 μM) for another 30 minutes. After that, the culture dishes were washed with PBS three times to remove the culture medium, then subject to an Olympus IX71 inverted fluorescence microscope equipped with a DP72 color CCD for cell imaging.

Fluorescence imaging in zebrafish

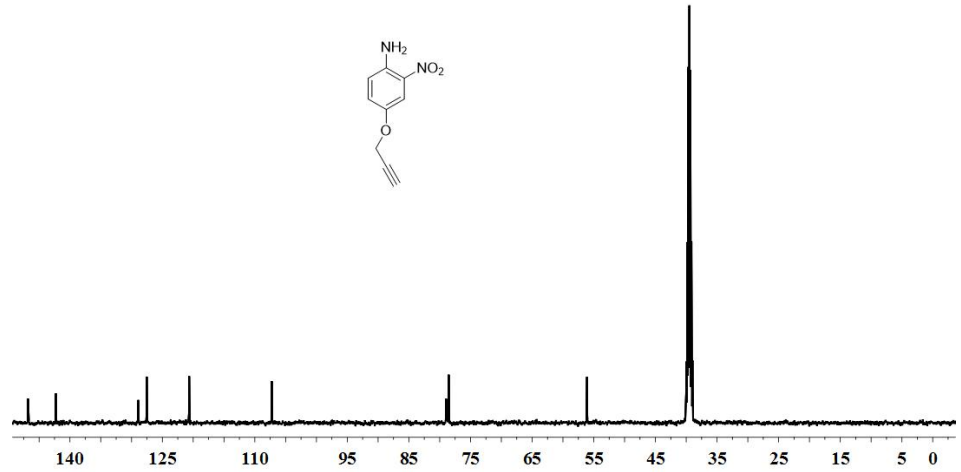
In this study, wild-type zebrafish were provided by Key Laboratory of Zebrafish Modeling and Drug Screening for Human Diseases of Guangdong Higher Education Institute, Southern Medical University. All zebrafish experiments were performed in full compliance with international ethics guidelines. Briefly, 5-day-old larvae were transferred into a 96-well microplate using a disposable transfer pipette, and then the larvae were incubated in 100 μL of E3 media containing **Gal-RhB** or compound **5** (final concentration: 10 μM) for 30 min. In contrast, the control group larvae were only incubated in 100 μL of E3 media. Then, the media solution was removed and the fish were washed three times with 100 μL E3 media. The fish were incubated with E3 media containing DEA-NONOate (the donor of NO, 0 μM, 20 μM and 40 μM respectively) for another 30 min at 28 ^{o}C before the removal of the media solution. After that, the fish were washed three times with 100 μL E3 media and observed on an Olympus IX71 inverted fluorescence microscope equipped with a DP72 color CCD. All the photographs were taken under identical exposure conditions.



Scheme S1. Synthetic route of the probe (**Gal-RhB**).



(A)



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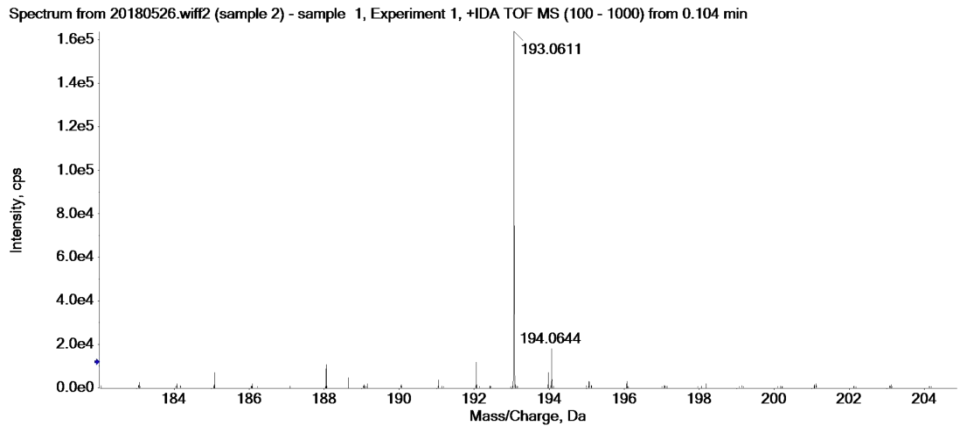
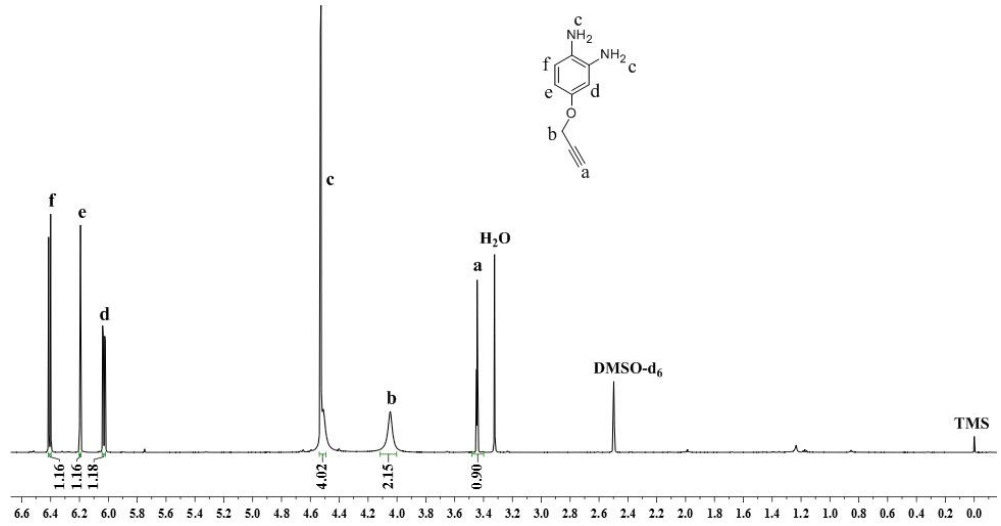
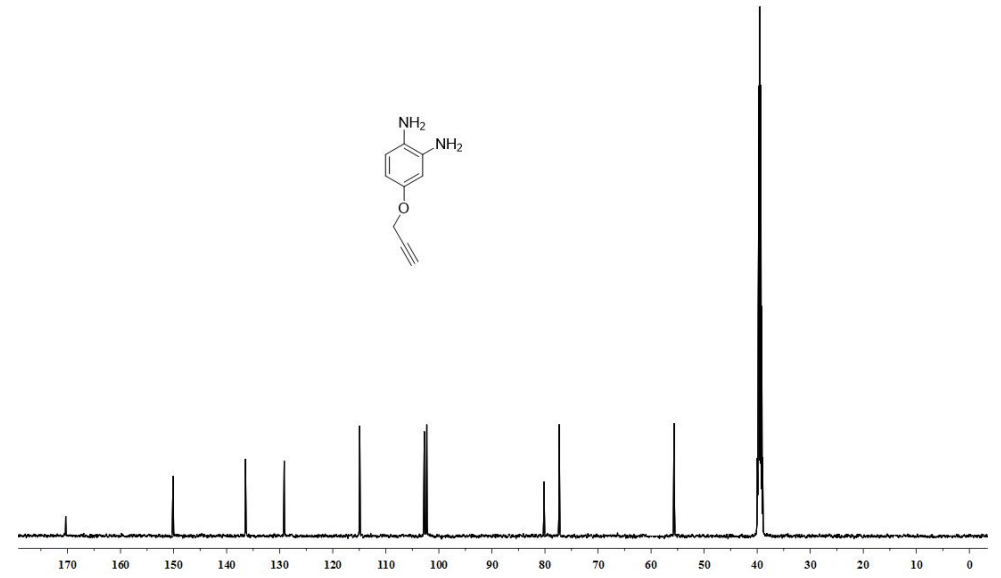


(C)

Figure S1. ^{1}H-NMR spectra (DMSO-d_6) (A), ^{13}C-NMR spectra (DMSO-d_6) (B) and HR-MS (C) for **1** (*m/z* 193.0611 $[M+H]^+$).



(A)



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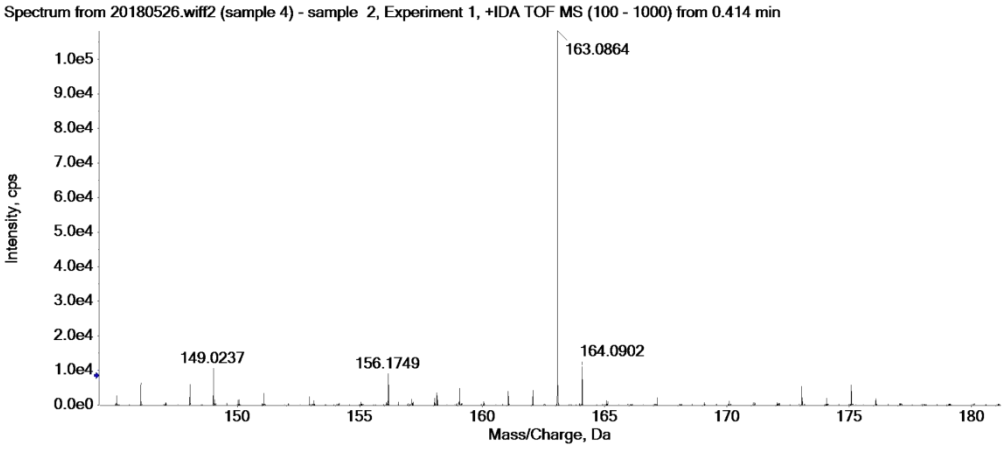
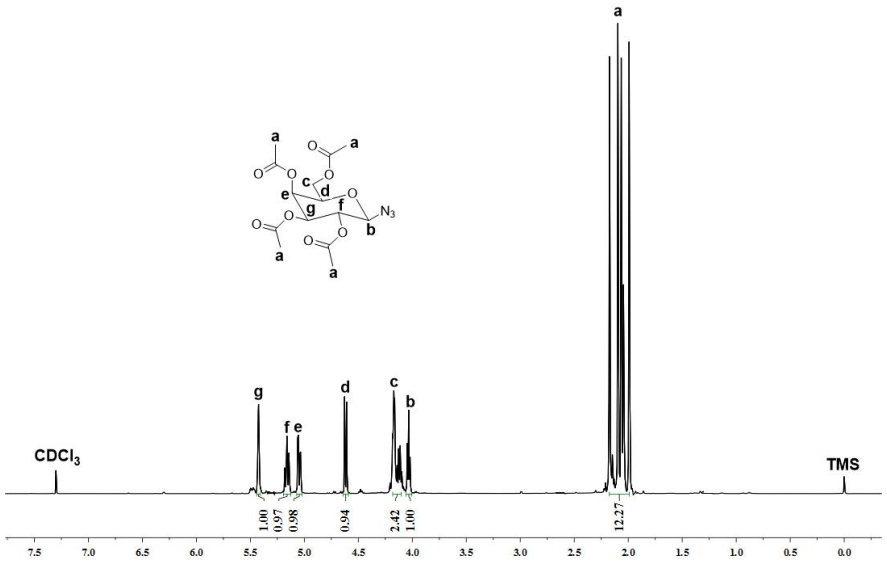


(C)

Figure S2. ^{1}H-NMR spectra (DMSO-d_6) (A), ^{13}C-NMR spectra (DMSO-d_6) (B) and HR-MS (C) for **2** (*m/z* 163.0864 $[M+H]^+$).



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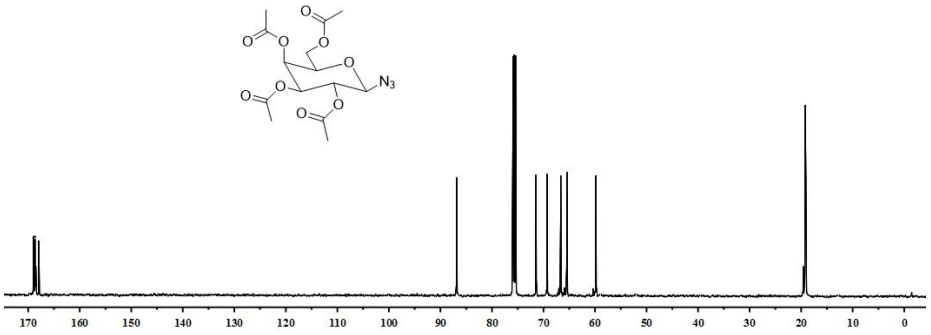

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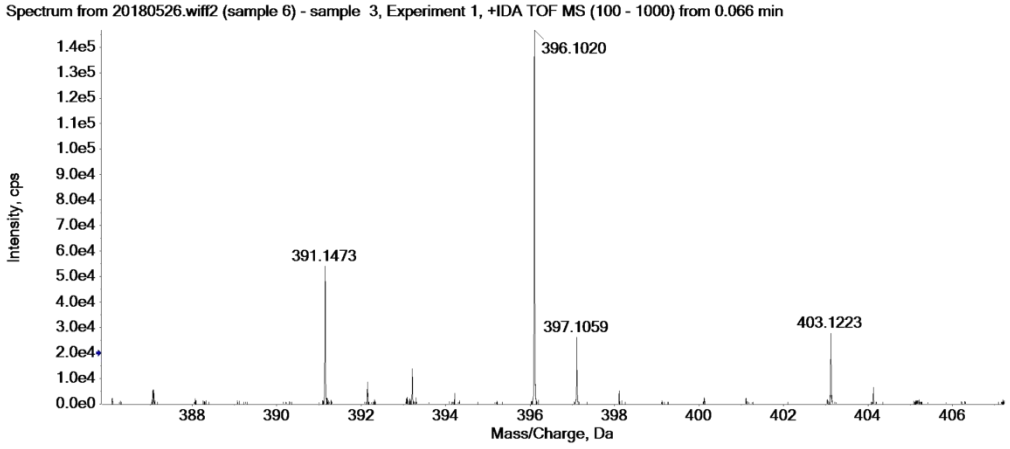
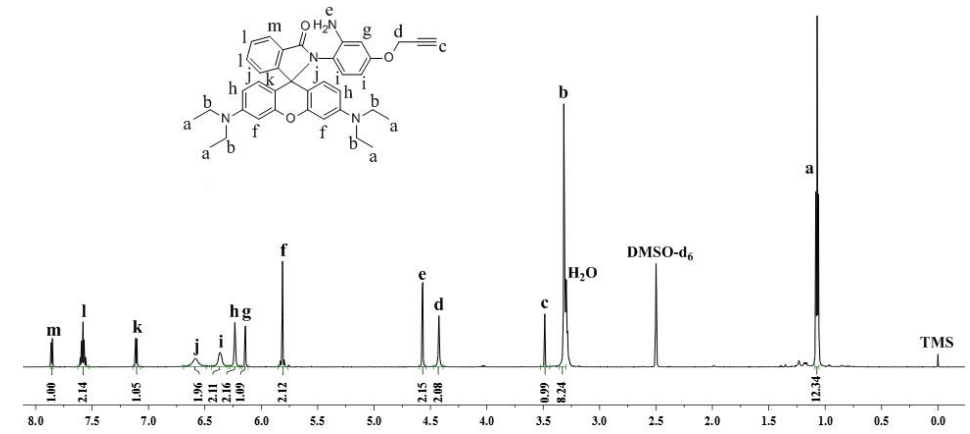


(C)

Figure S3. ^{1}H-NMR spectra ($CDCl_3$) (A), ^{13}C-NMR spectra ($CDCl_3$) (B) and HR-MS (C) for **3** (*m/z* 396.1020 $[M+Na]^+$).



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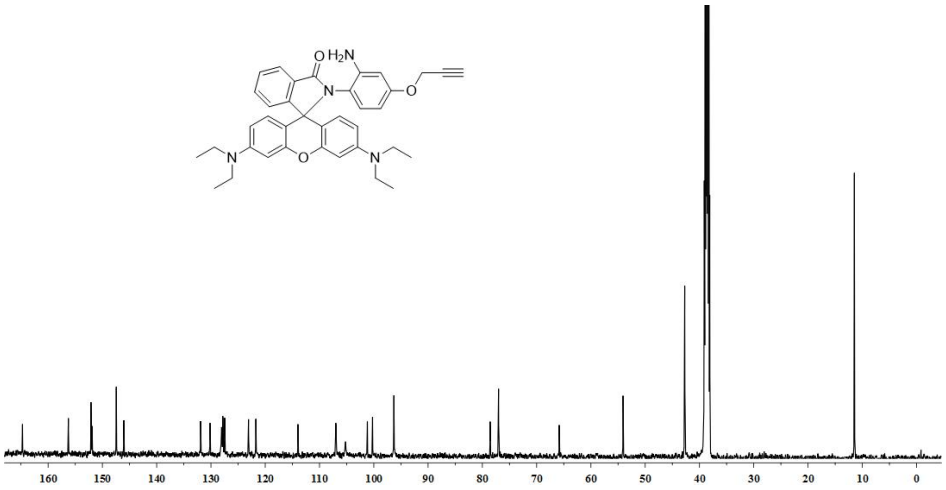

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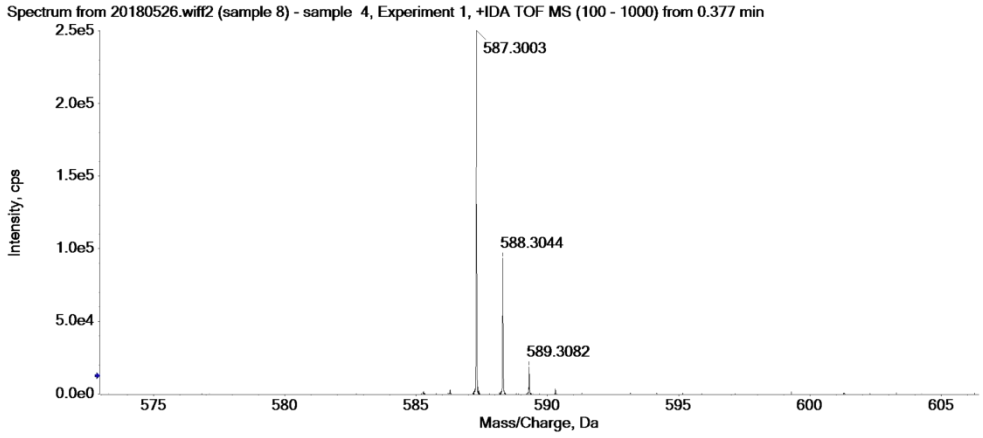
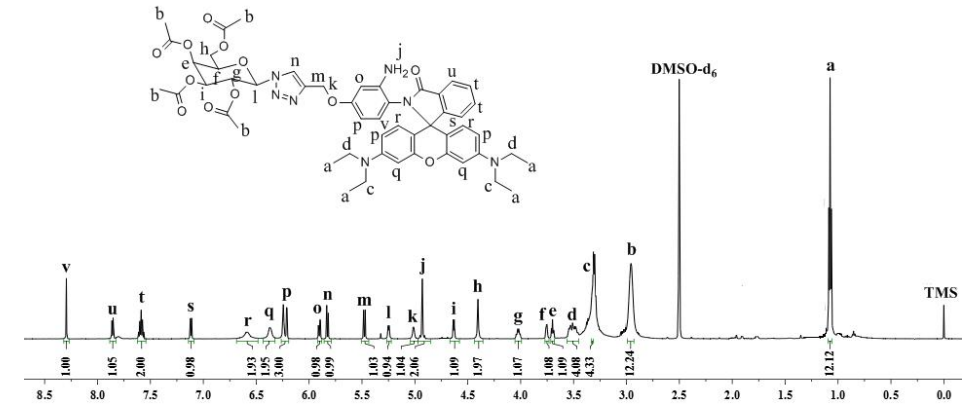


(C)

Figure S4. ^{1}H-NMR spectra (DMSO-d_6) (A), ^{13}C-NMR spectra (DMSO-d_6) (B) and HR-MS (C) for **5** (*m/z* 587.3003 $[M+H]^+$).



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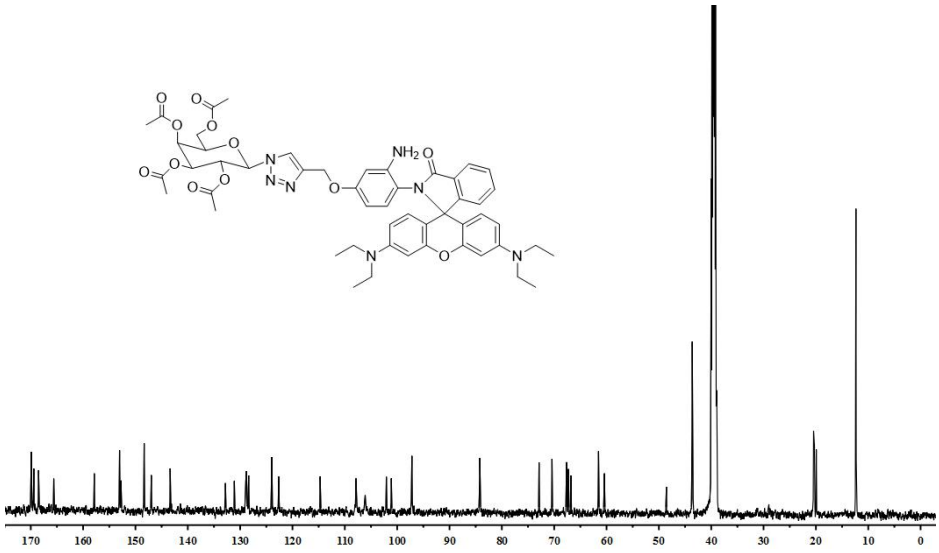

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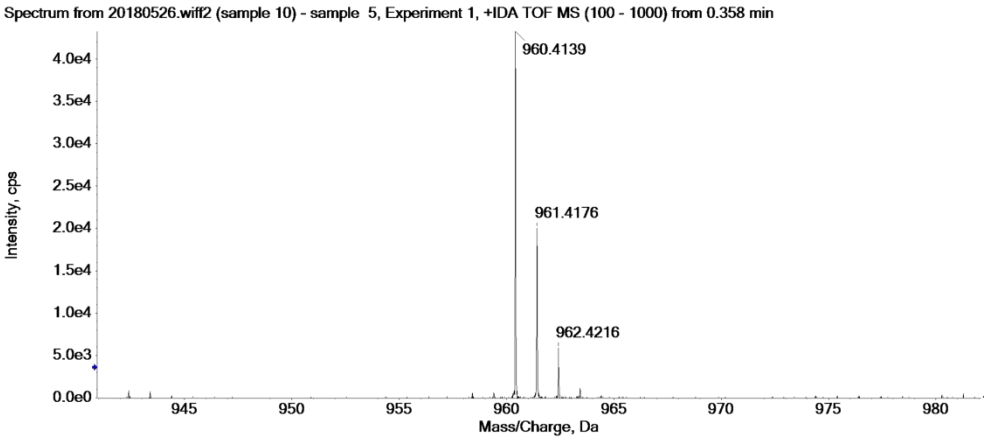
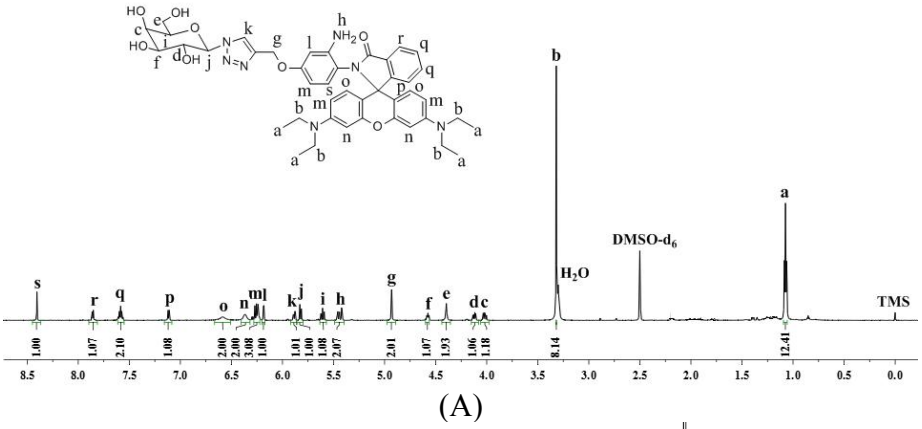


(C)

Figure S5. ^{1}H-NMR spectra (DMSO-d_6) (A), ^{13}C-NMR spectra (DMSO-d_6) (B) and HR-MS (C) for **6** (*m/z* 960.4139 $[M+H]^+$).



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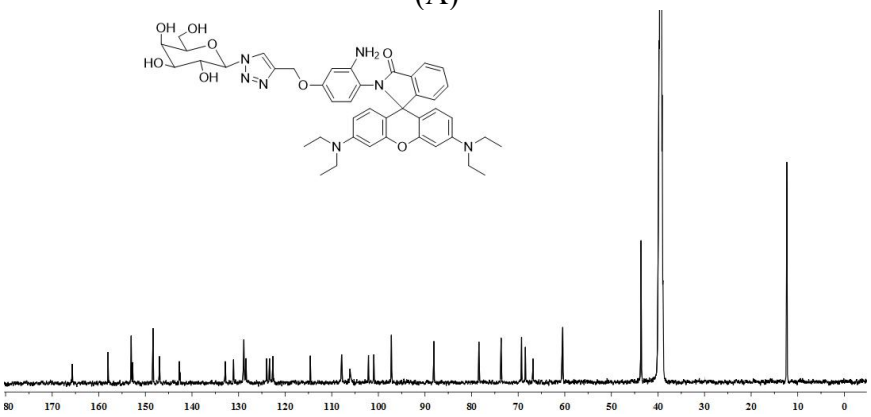

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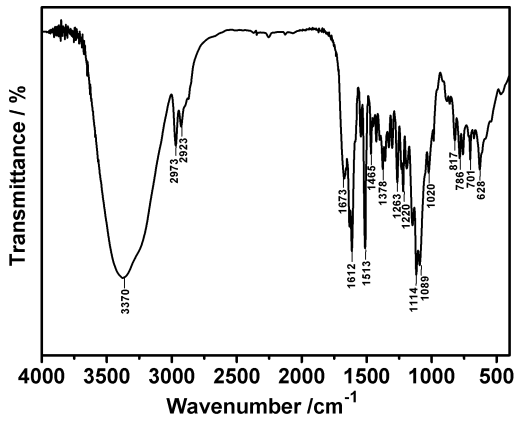

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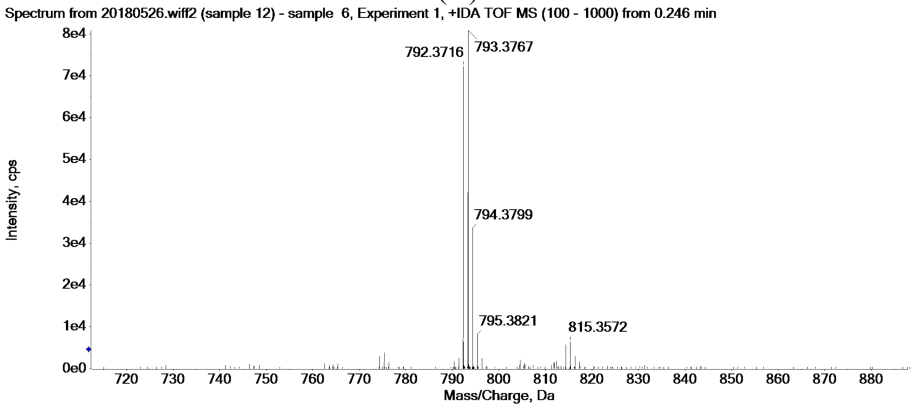

(D)

Figure S6. ^{1}H-NMR spectra (DMSO-d_6) (A), ^{13}C-NMR spectra (DMSO-d_6) (B), FTIR spectra and HR-MS (D) for **Gal-RhB** (*m/z* 792.3716 $[M+H]^+$ and 815.3572 $[M+Na]^+$).

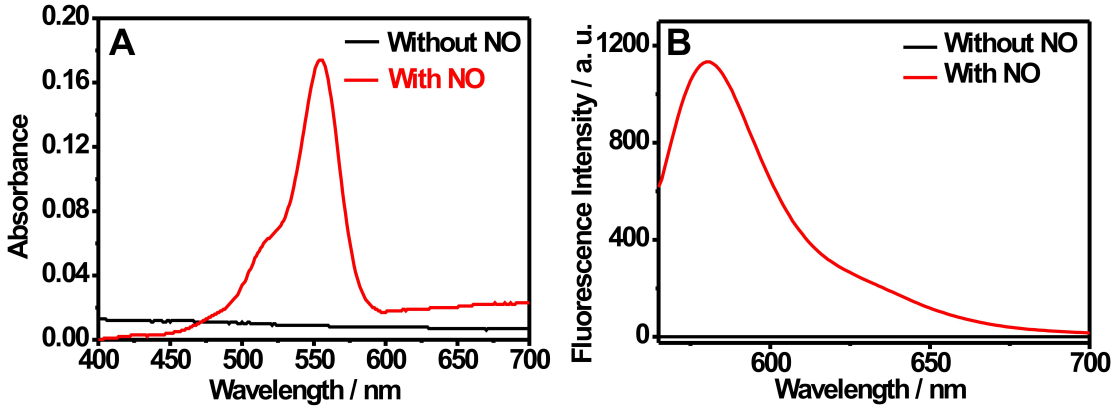

Figure S7. (A) Absorption and (B) fluorescence emission (λ_{ex} = 550 nm) spectra of the sensor (5 μM) before (black) and after (red) reaction with NO (100 μM) at 37 °C for 10 min.

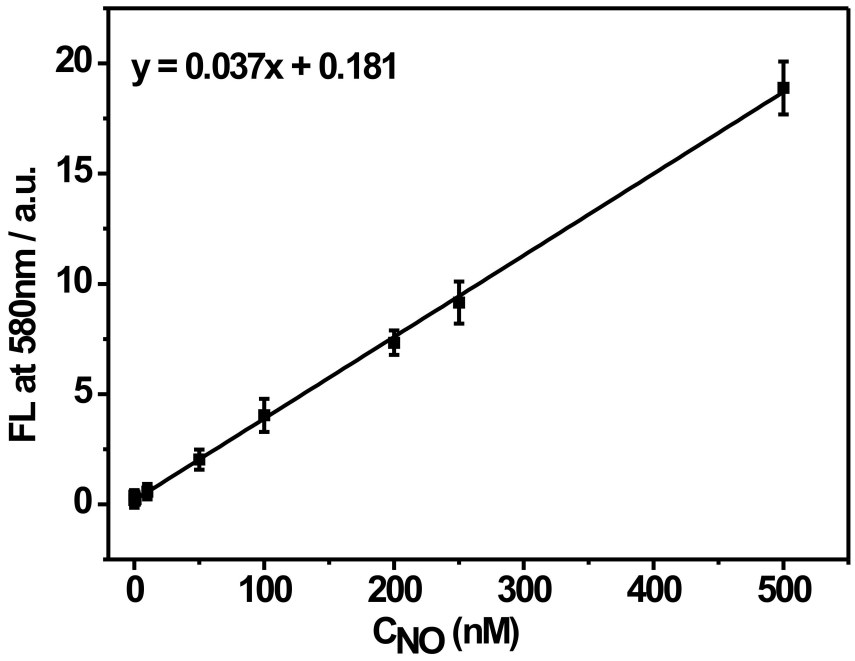

Figure S8. Fluorescence intensity at 580 nm for the sensor **Gal-RhB** (5 μM) in PBS buffered water (pH 7.0, 10 mM) as a function of NO concentration (upon 10 min of NO treatment). Excitation wavelength: 550 nm.

Determination of the detection limit:

First the calibration curve was obtained from the plot of fluorescence intensity at 580 nm as a function of the analyte concentration (NO). The regression curve equation was then obtained for the lower concentration part.

The detection limit = 3 × S.D. / k

where k is the slope of the curve equation, and S.D. represents the standard deviation for the fluorescence intensity at 580 nm of the sensor in the absence of NO.

I_{580nm} = 0.181 + 0.037 × [NO] (R = 0.999)

LOD = 3 × 0.020 / 0.037 = 1.62 nM

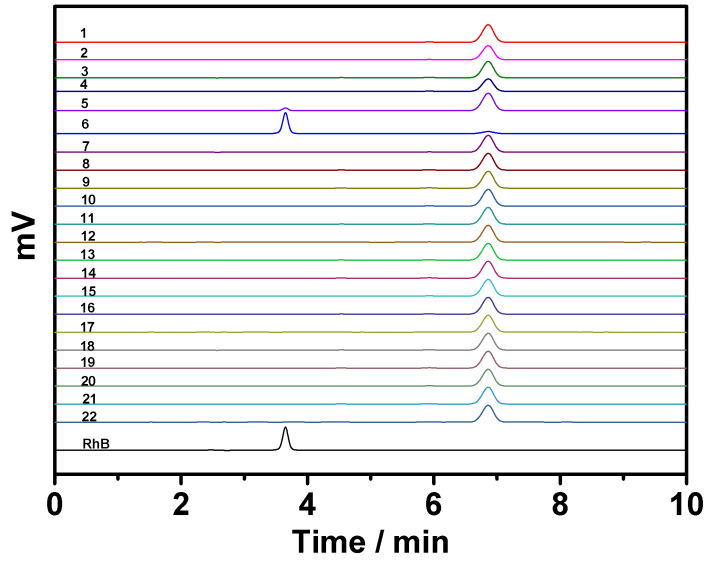

Figure S9. Typical HPLC chromatogram for the rhodamine B (RhB) and the sensor (**Gal-RhB**, 5 μM) incubated with various relevant species for 10 min: 1, blank; 2, H_2O_2; 3, HClO; 4, $^1O_2$; 5, HNO; 6, NO; 7, OH•; 8, O_2^-; 9, •OtBu; 10, GSH; 11, Cys; 12, Hcy; 13, NO_3^-; 14, NO_2^-; 15, SCN^-; 16, Ca^{2+}; 17, K^+; 18, Na^+; 19, Fe^{2+}; 20, Zn^{2+}; 21, Mg^{2+}; 22, AA (5 μM for DEA-NONOate, 100 μM for ROS and RNS, 5.0 mM for GSH, 1.0 mM for others). Peaks in the chromatograms were detected by monitoring the absorption at 220 nm or/and 554 nm. The mobile phase was 90/10 methanol/water at a flow rate of 1.0 mL/min.

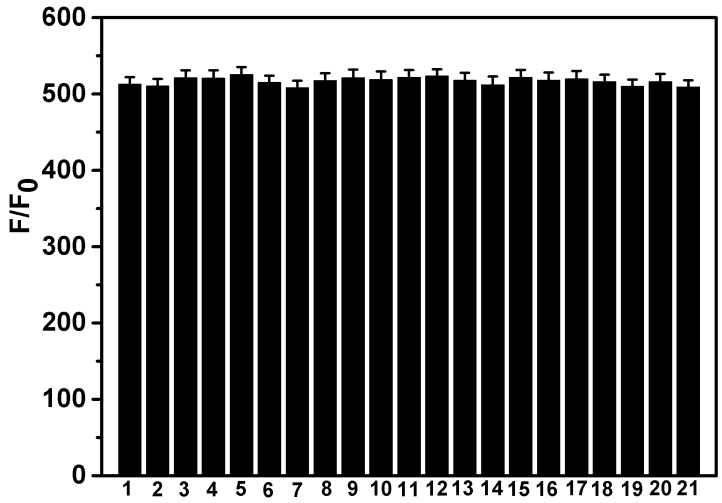

Figure S10. Fluorescence emission intensity ratio (F/F_0) of the sensor (**Gal-RhB**, 5 μM) at 580 nm in the presence of 5 μM of NO donor, as well as in the presence of different analytes respectively, 1, NO; 2, H_2O_2; 3, HClO; 4, $^1O_2$; 5, HNO; 6, OH•; 7, O_2^-; 8, •OtBu; 9, GSH; 10, Cys; 11, Hcy; 12, NO_3^-; 13, NO_2^-; 14, SCN^-; 15, Ca^{2+}; 16, K^+; 17, Na^+; 18, Fe^{2+}; 19, Zn^{2+}; 20, Mg^{2+}; 21, AA (5 μM for DEA-NONOate, 100 μM for ROS and RNS, 5.0 mM for GSH, 1.0 mM for others). All fluorescence intensities were measured for 10 min after the addition of the analytes.

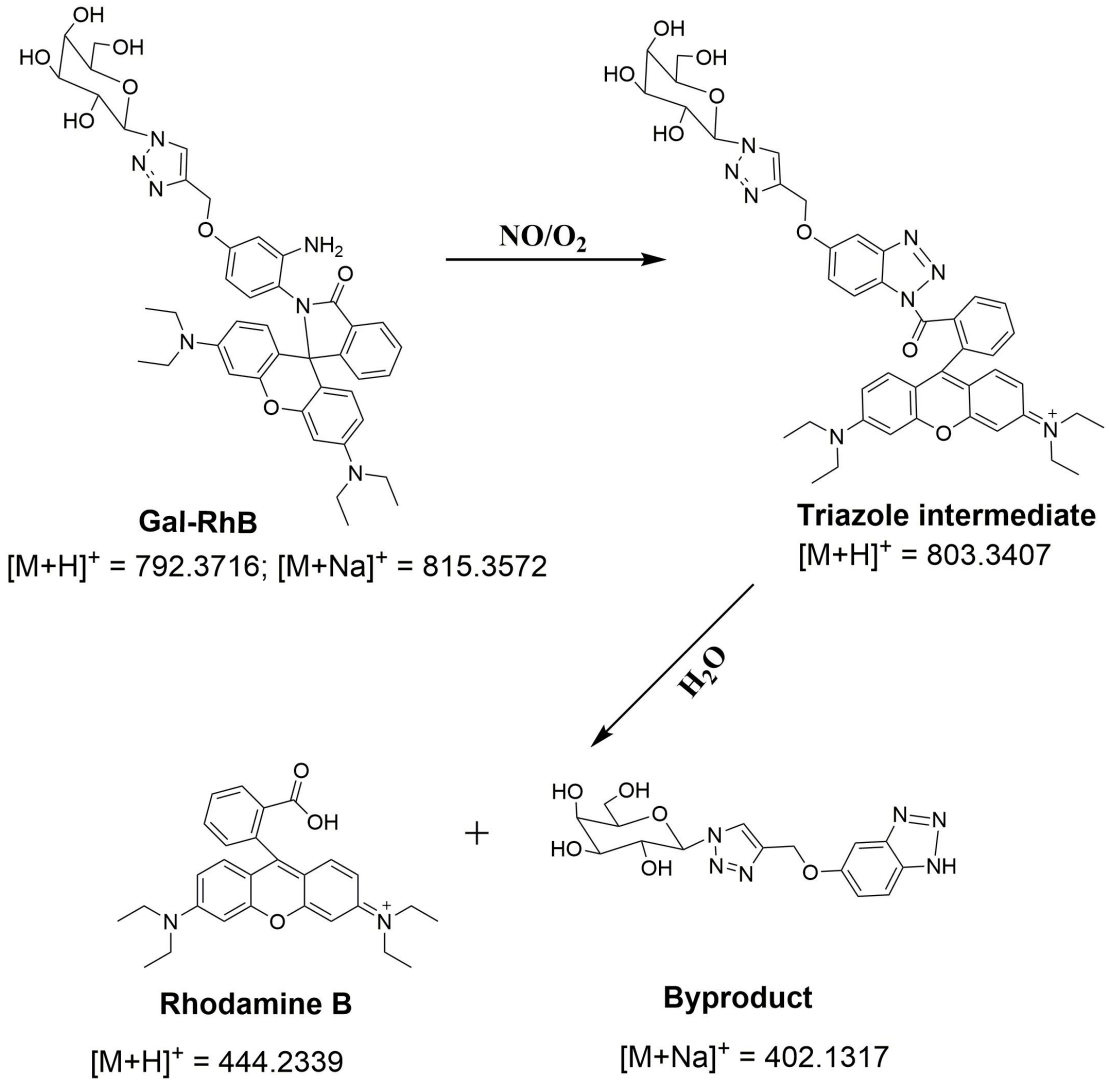

Figure S11. Proposed sensing mechanism of the sensor **Gal-RhB** with NO.

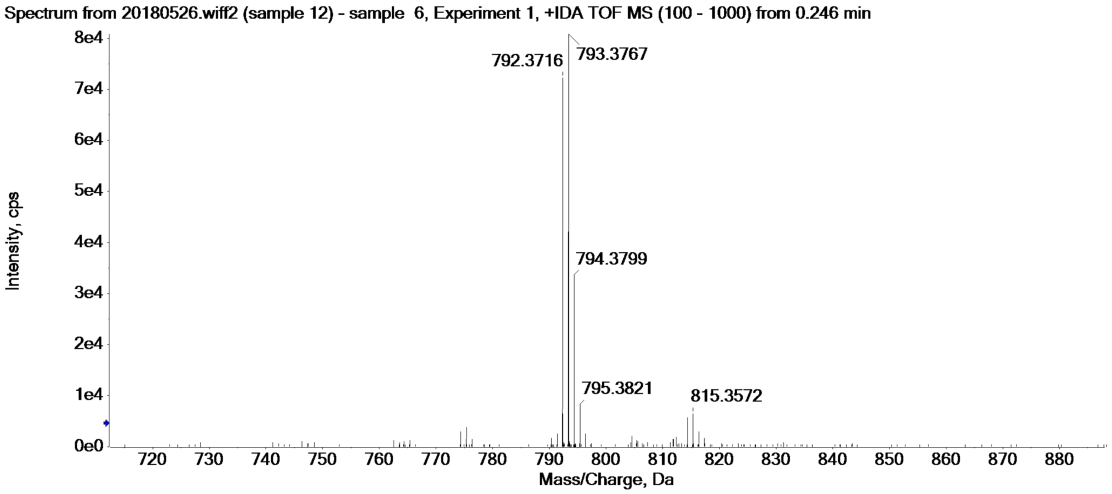

Figure S12. HR-MS of **Gal-RhB** in PBS buffered water (pH 7.0, 10 mM) under aerobic conditions. The peaks at m/z 792.3716 and 815.3572 correspond to (**Gal-RhB** + H)$^+$ and (**Gal-RhB** + Na)$^+$, respectively.

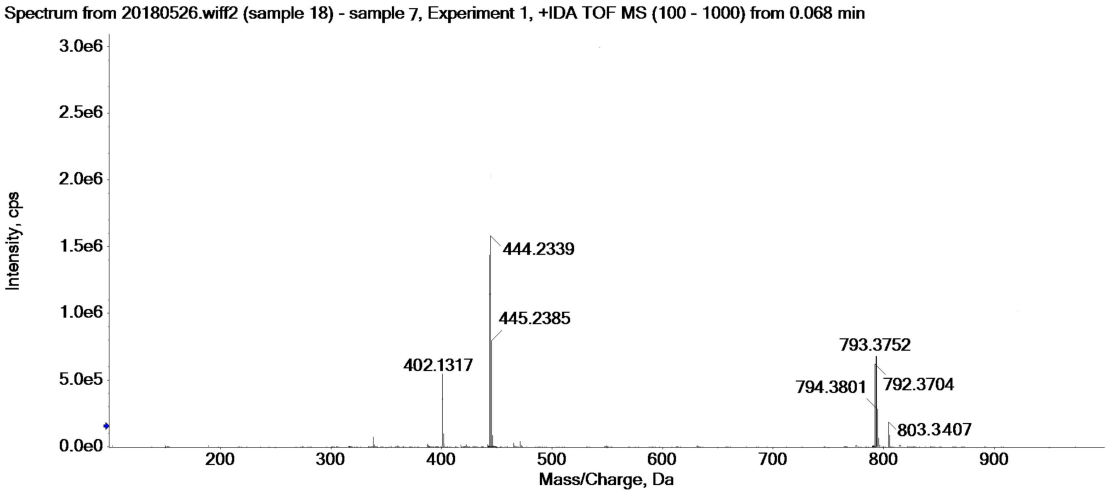

Figure S13. HR-MS of **Gal-RhB** after treatment with NO donor (DEA/NONOate) in PBS buffered water (pH 7.0, 10 mM) for 2 min under aerobic conditions. The peaks at m/z 792.3704 correspond to (**Gal-RhB**+H)$^+$; the peaks at m/z 803.3407 correspond to (triazole intermediate +H)$^+$; the peaks at m/z 444.2339 and 402.1317 are ascribed to (Rhodamine B + H)$^+$ and (Byproduct +Na)$^+$, respectively.

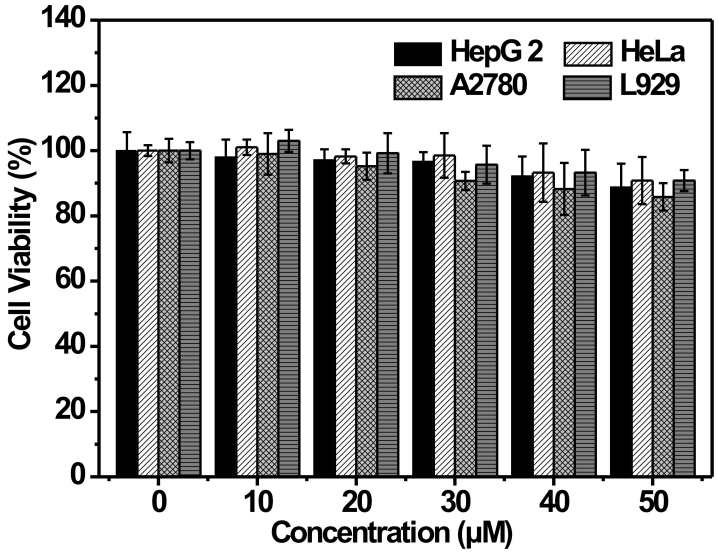

Figure S14. The effect of the sensor **Gal-RhB** on viabilities for four cell lines (HepG2, HeLa, A2780, L929). Cell viability was assessed by MTT assay upon 24 h of incubation after being treated with various concentrations of Gal-RhB. The results are the mean standard deviation of eight separate measurements.

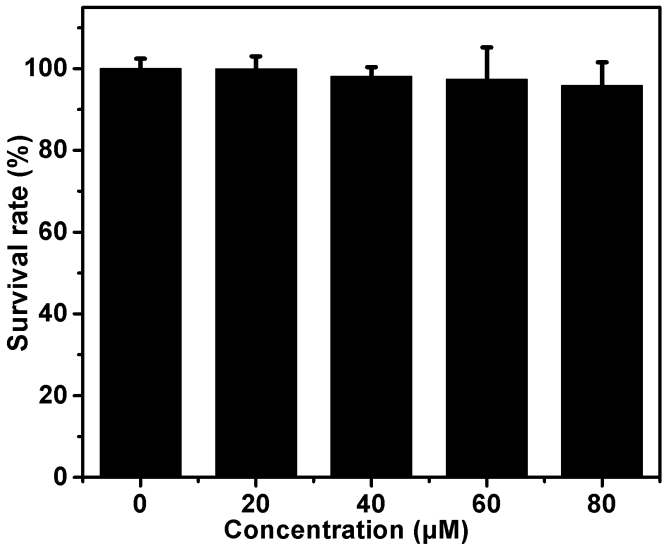

Figure S15. The survival rate of zebrafish larvae at different sensor (**Gal-RhB**) concentrations after 96 h exposure. Each concentration was performed independently for two times, and for each independent experiment, the assays were performed in triplicate. Data represent mean ± SD from two independent experiments.

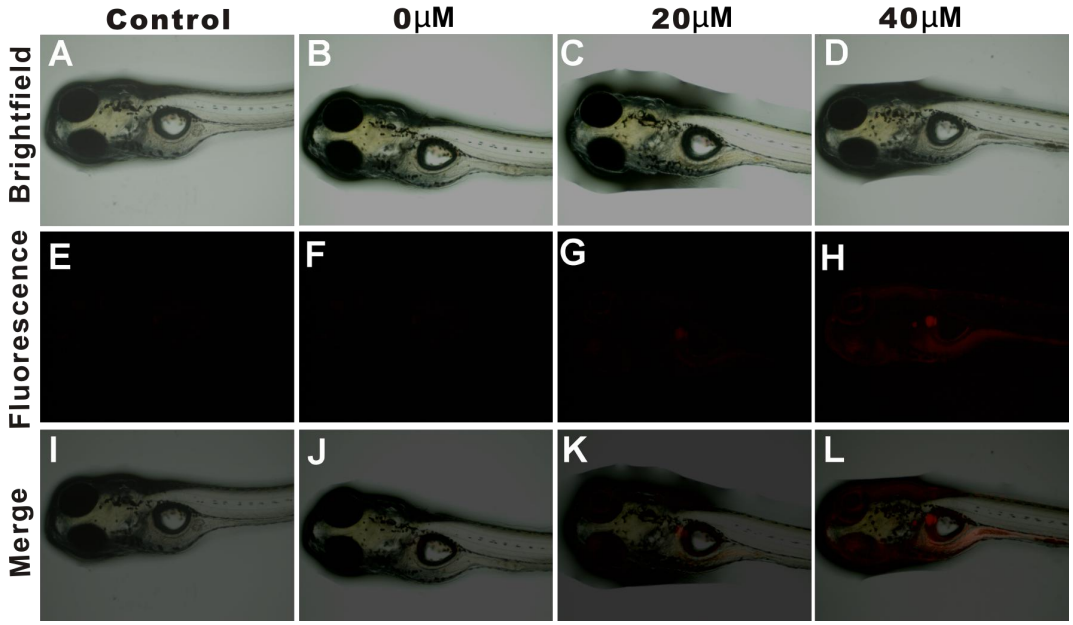

Figure S16. Fluorescent images of untreated zebrafish larvae (the control) (A, E and I), and zebrafish larvae pretreated with compound **5** (10 μM) and then incubated with 0 μM NO (B, F, and J), 20 μM NO (C, G and K) and 40 μM NO (D, H and L).