Supporting Information

A Si-rhodamine-based Near-Infrared Fluorescent Probe for Visualizing Endogenous Peroxynitrite in Living Cells, Tissues, and Animals

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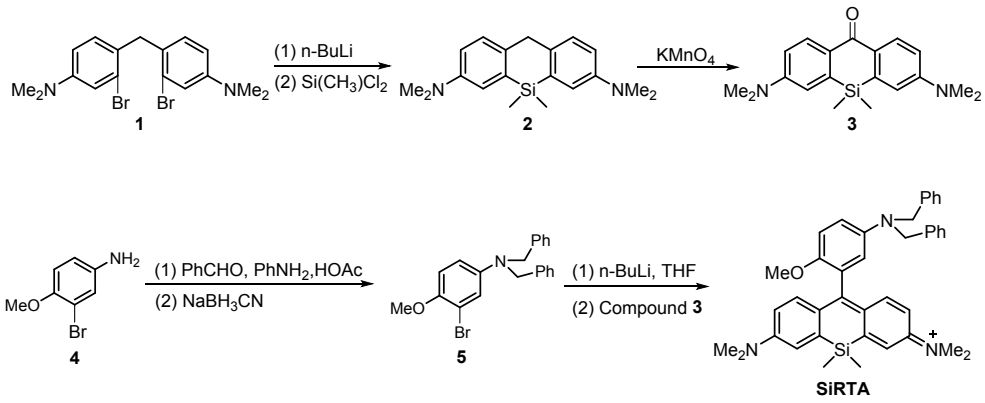
1. General information and methods

All reagents and solvents were purchased from commercial sources and were of the highest grade. Solvents were dried according to standard procedures. All reactions were magnetically stirred and monitored by thin-layer chromatography (TLC). Flash chromatography (FC) was performed using silica gel 60 (200–300 mesh). Absorption spectra were taken on Varian Carry 4000 spectrophotometer. Fluorescence spectra were taken on Hitachi F-7000 fluorescence spectrometer. The ^{1}H NMR and ^{13}C NMR spectra were taken on a Bruker spectrometer, and recorded at 600 and 150 MHz, respectively. The following abbreviations were used to explain the multiplicities: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad. High resolution mass spectra were obtained on a Varian QFT-ESI mass spectrometer. The imaging assays of cells were performed in Zeiss LSM 880+Airyscan Laser Scanning Confocal Microscope, unless otherwise mentioned. The imaging assays of living body were performed in Bruker In-Vivo FX Pro small animal optical imaging system with an excitation filter 630 nm and an emission filter 670 nm.

2. Synthesis



*Compound **3***:

4,4'-Methylenebis(3-bromo-N,N-dimethylaniline) **1** (2.00 g, 4.9 mmol) was dissolved in dry THF (100 ml) and stirred at -78 °C under N_2 atmosphere. n-Butyllithium (2.4 M solution in n-hexane, 5.1 ml, 12.2 mmol) was slowly added for 30 min to the solution and stirred for further 2 h at the same temperature. $SiMe_2Cl_2$ (0.67 ml, 6.1 mmol) was added to the reaction mixture and stirred at room temperature for 2 h. 2 N HCl aqueous solution was added carefully to neutralize the solution, and THF was evaporated. The resulting aqueous solution was extracted with EtOAc, and the organic phase was washed with saturated $NaHCO_3$ aqueous solution, water and brine, dried over Na_2SO_4, filtered and evaporated to obtain the crude **2**, which was directly applied to next step without purification.

The obtained crude **2** was dissolved in acetone (30 ml) and stirred at 0 °C. $KMnO_4$ (5.75 g) was added portionwise to the solution over the period of 2 h, and stirring was continued for further 2 h at the same temperature. The purple suspension was filtered through a Celite pad, and the yellow filtrate was evaporated. The residue was purified by silica gel column chromatography (petroleum ether/chloroform/ethyl acetate=7:1:1, v/v) to obtain **3** as a yellow solid (0.58 g, yield: 40.1%). ^{1}H NMR (600 Hz, $CDCl_3$) δ 8.43 (d, J = 9.0 Hz, 2H), 6.86 (d, J = 9.0 Hz, 2H), 6.83 (s, 2H), 3.11 (s, 12H), 0.50 (s, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 185.28, 151.44, 140.50, 131.62, 129.70, 114.29, 113.18, 40.06, -0.97; ESI-MS $[M+H]^+$: Calcd for 325.1736, Found 325.1734.

*Compound **5***:

A solution of 3-bromo-4-methoxyaniline **4** (100 mg, 0.139 mmol), benzaldehyde (73.67 mg, 0.695 mmol), and AcOH (0.2 mL) in CH_3OH (5 mL) was stirred at room temperature for 10 min, then $NaBH_3CN$ (27 mg, 0.417 mmol) was added to the mixture and stirring was continued at 60 ^oC until the reaction completion as confirmed by TLC (3 h). The reaction mixture was quenched with water and extracted with CH_2Cl_2. The organic layer was washed with brine and dried over Na_2SO_4. Filtration, evaporation and purification of the residue by silica gel flash chromatography (CH_2Cl_2: petroleum ether = 1:15) gave compound **5** as an white solid (52 mg, Yield: 41%).^{1}H NMR (600 MHz, $CDCl_3$) δ 7.35 (t, J = 7.0 Hz, 4H), 7.28 – 7.26 (m, 6H), 7.02 (b, 1H), 6.77 (d, J = 8.7 Hz, 1H), 6.66 (b, 1H), 4.57 (s, 4H), 3.82 (s, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 138.26, 128.68, 127.07, 126.87, 118.19, 113.63, 113.08, 112.73, 56.98, 54.83. ESI-MS $[M+H]^+$: Calcd for 382.0807, Found 382.0807.

*Compound ***SiRTA***:

Compound **5** (381 mg, 1.0 mmol) was dissolved in dry THF (10 ml) and stirred at -78 °C under N_2 atmosphere. n-Butyllithium (2.4 M solution in n-hexane, 0.42 ml, 1 mmol) was slowly added for 30 min to the solution and stirred for further 2 h at the same temperature. Compound **3** (100 mg, 0.31 mmol) in anhydrous THF (10 mL) was slowly added, and then the mixture was allowed to warm to room temperature and stirred overnight. The reaction was quenched by addition of 2 N HCl and then stirred at room temperature for 10 min. The resulting mixture was extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4 and evaporated. The crude product was purified by flash column chromatography (CH_2Cl_2:MeOH = 10:1) to afford pure **SiRTA** as a green solid (153 mg, yield: 76.5%). ^{1}H NMR (600 MHz, DMSO) δ7.37-7.29 (m, 6H), 7.28-7.21 (m, 6H), 7.05 – 6.96 (m, 3H), 6.91 (s, 1H), 6.75 (s, 2H), 6.49 (s, 1H), 4.60 (s, 4H), 3.51 (s, 3H), 3.29 (s, 12H), 0.53 (s, 6H); ^{13}C NMR (151 MHz, DMSO) δ 167.02, 154.08, 147.37, 141.08, 139.27, 128.91, 128.07, 127.46, 127.34, 127.22, 121.52, 116.58, 114.5, 113.33, 56.51, 55.19, 40.89. ESI-MS $[M]^+$: calcd for 610.3248, Found 610.3251.

3. Preparation of the test solution

Stock solution of **SiRTA** in CH_3CN (2 mM) was used to prepare the working solutions in PBS (50 mM, pH 7.4, containing 20% CH_3CN) with the final concentrations of 4.0 μM for assays in chemical system and 2.0 μM in biological systems. For assays in chemical system, $ONOO^-$ solution, synthesized according to a reported procedure,[1] was used, and its concentration was determined using an extinction coefficient of 1670 $M^{-1}cm^{-1}$ at 302 nm. For cell imaging assays, $ONOO^-$ was generated from a commercially available $ONOO^-$ donor SIN-1 (dissolved in deionized water). For assays in chemical system, the NO stock solution in deionized water, prepared by bubbling NO gas into a NaOH solution to eliminate NO_2 generated from the reaction of NO and O_2 and then into deoxygenated deionized water for 30 min, was used, whose concentration was determined to be 1.8 mM by Griess method. For assays in cells, a commercially available NO donor NOC-9 (dissolved in 0.1 M NaOH solution) was used. $O_2^{\bullet-}$ was prepared by adding KO_2 (7.1 mg) and 18-Crown-6 (1 equiv) to dry dimethyl sulfoxide (5 mL) and stirring vigorously for 10 min. $HO^{\bullet}$ was generated *in situ* by the Fenton reaction, and its concentration was equal to the Fe(II) concentration. $^1O_2$ was generated *in situ* by adding NaClO solution into H_2O_2 solution (10 eq), and its concentration was equal to the NaClO concentration. H_2O_2 solution was prepared by dilution of commercial H_2O_2 solution in deionized water, and its concentration was determined by using an extinction coefficient of 43.6 $M^{-1}cm^{-1}$ at 240 nm. NaClO solution was prepared by the dilution of commercial NaClO solution in deionized water, and its concentration was determined using an extinction coefficient of 350 $M^{-1}cm^{-1}$ at 292 nm. The aqueous solutions of $NaNO_2$ was freshly prepared and used as NO_2^- source. The aqueous solutions of K^+, Ca^{2+}, Na^+, Mg^{2+}, Al^{3+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Cu^+, and Cu^{2+} were freshly prepared from their chloride salts. The aqueous solutions of GSH and the DMSO solutions of DHA/AA/MGO were freshly prepared. For spectra studies, various analytes, except $^{\bullet}OH$ and $^1O_2$, were directly added to the solution of **SiRTA** (4 μM) in PBS (50 mM, pH 7.4, containing 20% CH_3CN), and then fluorescence spectra were recorded in the indicated time

points. For the cases of $^{\bullet}OH$ or $^1O_2$, **SiRTA** and H_2O_2 were premixed, and then Fe^{2+} or ClO^- was added to the mixture.

4. Cell culture and fluorescence imaging

4.1 Cell culture

The HeLa cell line, Raw 264.7 macrophage cell line, EA.hy 926 cell line, and pancreatic β-cell (INS-1) line were kindly provided by Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education (China). Cells were grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10 % FBS (Fetal Bovine Serum) at 37 ºC in humidified environment of 5% CO_2. Cells were plated on glass bottom cell culture dish (30 mm) and allowed to adhere for 12 hours. Before experiments, cells were washed with phosphate buffer saline (PBS) 3 times.

4.2 Imaging exogenous and endogenous NO in HeLa cells, RAW264.7 macrophages, and pancreatic β-cells (INS-1)

To test the selectivity of **SiRTA** for $ONOO^-$ in cell environment, HeLa cells were pretreated with **SiRTA** (2.0 μM) in PBS for 20 min, and then treated with SIN-1 and representative ROS, such as H_2O_2, ClO^-, NOC-9 (a commercially available NO donor), respectively, for 20 min. After washed with PBS three times, imaging assays were performed. For imaging of stimulator-induced $ONOO^-$, Raw 264.7 cells (or INS-1 cells) were pretreated with stimulator LPS (20 μg/mL)/INF-γ (150 units/mL) [or STZ (20 μg/mL)] for the indicated time, and then treated with **SiRTA** (2 μM, 20 min). For inhibition assays, Raw 264.7 cells (or INS-1 cells) were pretreated with LPS (20 μg/mL, 6 h)/INF-γ (150 units/mL, 6 h) [or STZ (20 μg/mL, 12 h)] in the presence of AG (0.5 mM), TEMPO (0.5 mM) , and FeTMPyP (50 μM), respectively, and then treated with **SiRTA** (2 μM, 20 min). After each treatment, the cells were washed with PBS 3 times. Emission was collected at 638−747 nm (λ_{ex}: 633 nm).

4.3 Imaging endogenous $ONOO^-$ in EA.hy926 endothelial cells after oxygen–glucose deprivation and reoxygenation (OGD/RO)

To mimic the ischemia–reperfusion (I/R) injury, EA.hy926 endothelial cells were subjected to OGD/RO condition. Briefly, the airtight hypoxia chamber was flushed with 95% N_2/5% CO_2 for 1 h before experiments. To obtain OGD, the standard culture medium was replaced with glucose-free Hank's balanced salt solution (HBSS), and then placed in hypoxia chamber flushed with 95% N_2/5% CO_2 at 37 °C. After 0.5 h, 1 h, and 2 h of OGD, respectively, the cells were returned to normal incubator for 1 h of RO, followed by incubation with **SiRTA** (2 μM, 20 min) and fluorescence imaging. The images were obtained in a EVOS FL Auto imaging system. For test the therapeutic effects of some phenolic acid antioxidants during the $ONOO^-$-induced I/R injury, EA.hy926 endothelial cells were subjected to OGD (2 h)/RO (1 h) condition in the presence of rosmarinic acid (50 μg/mL), Trolox (10 μg/mL), and caffeic acid (100 μg/mL), respectively, followed by incubation with **SiRTA** (2 μM, 20 min) and fluorescence imaging. The excitation and emission bandpasses of the standard Cy5 filter set were used.

4.4 Cell costaining studies

To evaluate the subcellular localization of **SiRTA**, HeLa cells were incubated with **SiRTA** (2.0 μM) and MitoTracker green FM (0.2 μM) (or LysoTracker green DN-26 (0.07 μM)) in PBS for 20 min. After washing with PBS 3 times, SIN-1 (10 μM) was added to light up the probe. For **SiRTA**, emission was collected at 638–747 nm (λ_{ex} = 633 nm). For MitoTracker green FM or LysoTracker green DN-26, emission was collected at 495-600 nm (λ_{ex} = 488 nm).

4.5 Photostability test

The photostability of **SiRTA** in the absence and presence of SIN-1 was tested in HeLa cells. The cells were plated on glass bottom cell culture dish (30 mm) and allowed to adhere for 12 hours. After washed with PBS 3 times, the cells were treated with 2 μM **SiRTA** for 20 min in DMEM. The **SiRTA**-loaded cells were then continuously irradiated by semiconductor laser under Zeiss LSM 880+Airyscan Laser Scanning Confocal Microscope for 60 min, and the representative images were obatined in different time points. Similarly, the cells treated with **SiRTA** (2 μM, 20 min) were

also subjected to the same irradiation treatment. Emission was collected at 638–747 nm (λ_{ex} = 633 nm).

5. MTT assays

HeLa Cells were seeded in 96-well microplates in DMEM medium supplemented with 10 % FBS (Fetal Bovine Serum) and 1% antibiotics at 37 ºC in humidified environment of 5% CO_2. After 24 h of cell attachment, the plates were washed with PBS, followed by addition of increasing concentrations of **SiRTA** (2–4 μM) in DMEM. The cells were then incubated at 37 ºC in an atmosphere of 5% CO_2 and 95% air for 24 h, followed by standard MTT assays (n= 6). Untreated assays (n = 6) were also conducted under the same conditions.

6. Imaging endogenous $ONOO^-$ in LPS-treated mouse models

All animal procedures were carried out under the guidelines approved by Animal Care and Use Committee of Shanxi University. In the assays, healthy ICR male rats were randomly divided into 3 groups: control group, diabetes group, and AG group. Rats in control group were fed with normal feeding. And rats in diabetes group and AG group were fed high-fat, refined-sugar diet for 6 weeks and then injected intraperitoneally (i.p.) with STZ (10 mg/kg) to induce diabetes. Diabetes in diabetes group and AG group was confirmed by a concentration of blood glucose higher than 13 mmol/L determined by glucometer. Then, rats in AG group were fed with AG (200 mg/kg), and rats in control group and diabetes group were were fed with equivalent water. After 24 h, rats in the three groups were injected i.p. with **SiRTA** solution (2 μM, 0.2 mL/100 g), followed by imaging at 30 min postinjection under a Bruker In-Vivo FX Pro small animal optical imaging system. Further, the rats in the three groups were sacrificed to harvest the kidney and liver, which were then cryo-sectioned as 5 μm thicknesses followed by observation under confocal microscope.

7. Supplementary Spectra

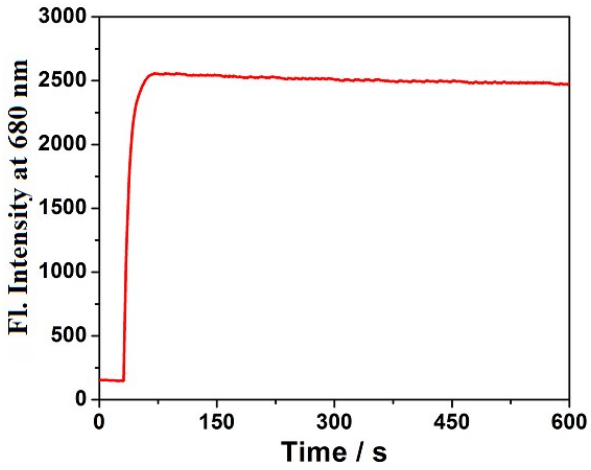


Figure S1. Time course of fluorescence intensities of **SiRTA** (4 μM) at 678 nm upon treated with $ONOO^-$ (20 μM). Conditions: (50 mM, pH 7.4); T = 25 °C; λ_{ex} = 650 nm; λ_{em} = 680 nm; Slits: 5/10 nm. Note that the excitation and emission slits of "5/10 nm" are the most appropriate for obtaining a sensitive fluorescence response of **SiRTA** for $ONOO^-$ in the detection condition. However, when the slits of "5/5 nm" was used, the obtained fluorescence signal was too low to fully reflect the sensitive fluorescence off-on response, and when the slits of "10/10 nm" was used, it was largely beyond the measurement range.

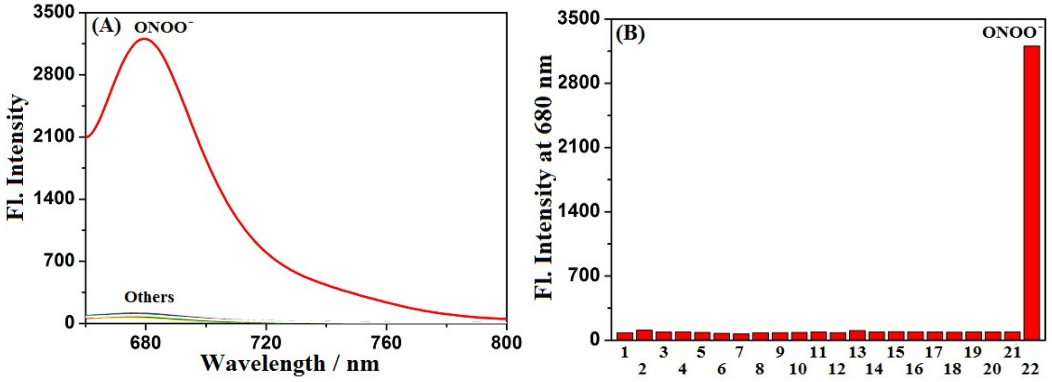


Figure S2. Fluorescence spectra (A) and intensities (B) of **SiRTA** (4 μM) treated with various competitive species at the time point of 5 min. (1) Probe only; (2) ClO^-; (3) H_2O_2; (4) $O_2^{\bullet-}$; (5) $^1O_2$; (6) $HO^{\bullet}$; (7) NO_2^-; (8) NOC-9 (a NO donor); (9) GSH; (10) AA; (11) DHA; (12) K^+; (13) Ca^{2+}; (14) Na^+; (15) Mg^{2+}; (16) Al^{3+}; (17) Zn^{2+}; (18) Fe^{2+}; (19) Fe^{3+}; (20) Cu^+; (21) Cu^{2+}; (22) $ONOO^-$. Concentrations for (2–8), 20 μM;

for (9–21), 1 mM; for (22), 20 μM. Conditions: (50 mM, pH 7.4, containing 20% CH_3CN); **T = 37 °C**; λ_{ex} = 650 nm; λ_{em} = 680 nm; Slits: 5/10 nm.

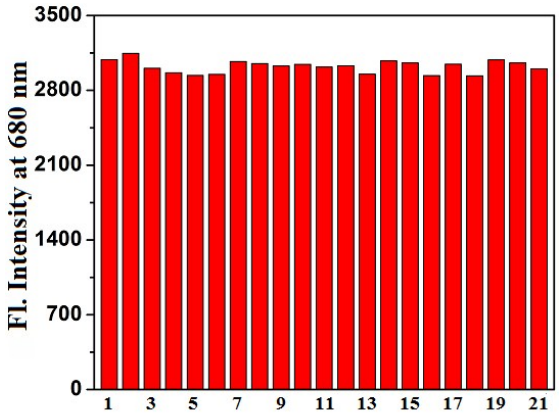


Figure S3. Fluorescence intensities of **SiRTA** (4 μM) pretreated with $ONOO^-$ (20 μM) for 1 min and then treated with various competitive species for 5 min, respectively. (1) Control; (2) ClO^-; (3) H_2O_2; (4) $O_2^{\bullet-}$; (5) $^1O_2$; (6) $HO^{\bullet}$; (7) NO_2^-; (8) NOC-9 (a NO donor); (9) GSH; (10) AA; (11) DHA; (12) K^+; (13) Ca^{2+}; (14) Na^+; (15) Mg^{2+}; (16) Al^{3+}; (17) Zn^{2+}; (18) Fe^{2+}; (19) Fe^{3+}; (20) Cu^+; (21) Cu^{2+}. Concentrations for (2–8), 20 μM; for (9–21), 1 mM. Conditions: (50 mM, pH 7.4, containing 20% CH_3CN); T = 25 °C; λ_{ex} = 650 nm; λ_{em} = 680 nm; Slits: 5/10 nm.

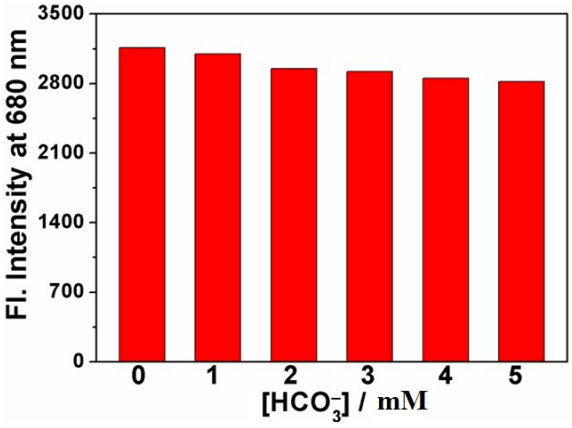


Figure S4. Fluorescence intensities of **SiRTA** (4 μM) treated with 10 equiv of $ONOO^-$ in the presence of varied amounts of HCO_3^- (1–5 mM). Conditions: PBS (50 mM, pH 7.4, containing 20% CH_3CN) at 25 °C. λ_{ex} = 650 nm; λ_{em} = 680 nm; Slits: 5/10 nm; voltage: 650 V.

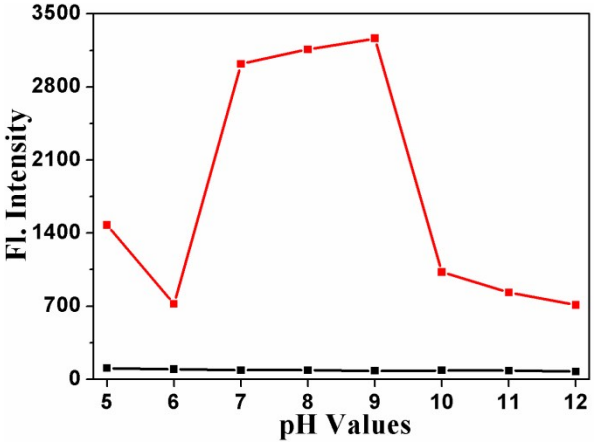


Figure S5. The fluorescence intensities at 680 nm for **SiRTA** (4 μM) in the absence and presence of $ONOO^-$ (20 μM) at varied pH values. Condition: B-R buffer (20 mM, pH = 5–12, containing 20% CH_3CN). λ_{ex} = 650 nm; λ_{em} = 680 nm; Slits: 5/10 nm; voltage: 650 V.

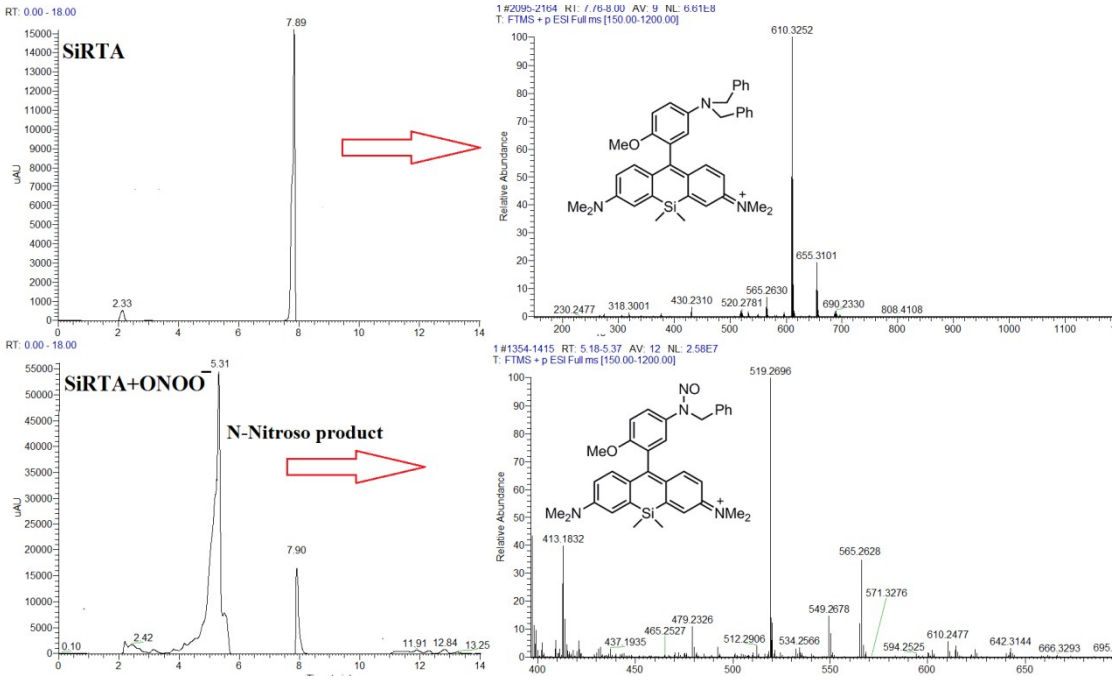


Figure S6. HPLC-MS results of **SiRTA** treated without (above) and with (below) $ONOO^-$ (40 μM).

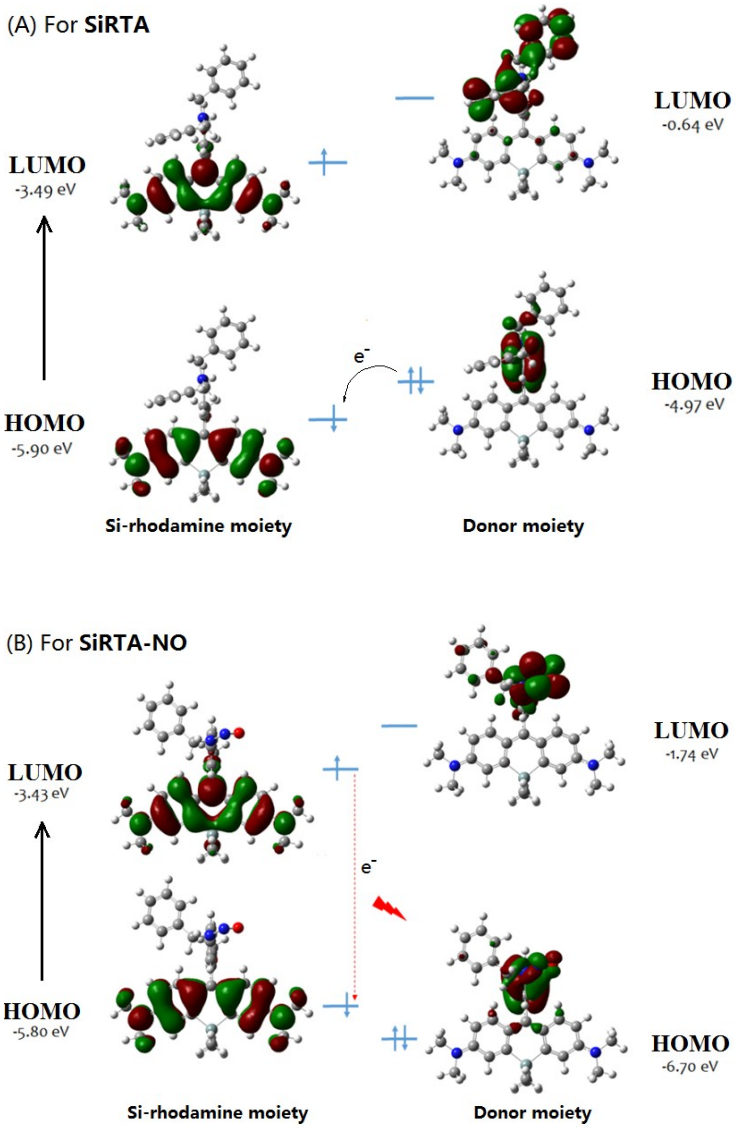


Figure S7. Frontier orbital energy representation of the PeT processes in **SiRTA** (A) and **SiRTA-NO** (B). All the theoretical studies were performed in PCM model in water by Gaussian 09 suite[2] with Becke's three-parameter hybrid exchange function with Lee-Yang-Parr gradient-corrected correlation functional (B3LYP functional) and 6-31+G* basis set.[3] All the local minima structures were confirmed by the absence of an imaginary mode in vibrational analysis calculations.

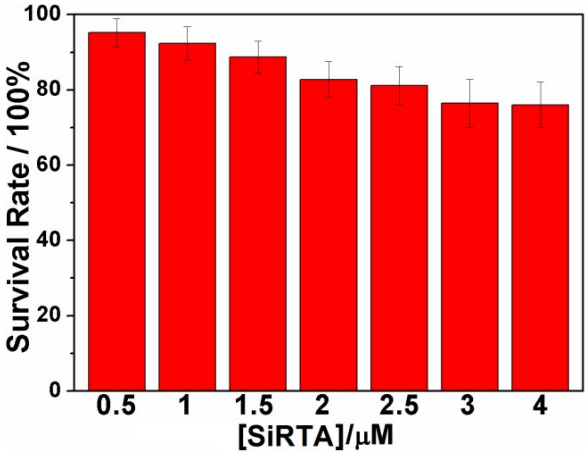


Figure S8. Percentage of viable HeLa cells after treated with increasing concentrations of **SiRTA** for 24 hours.

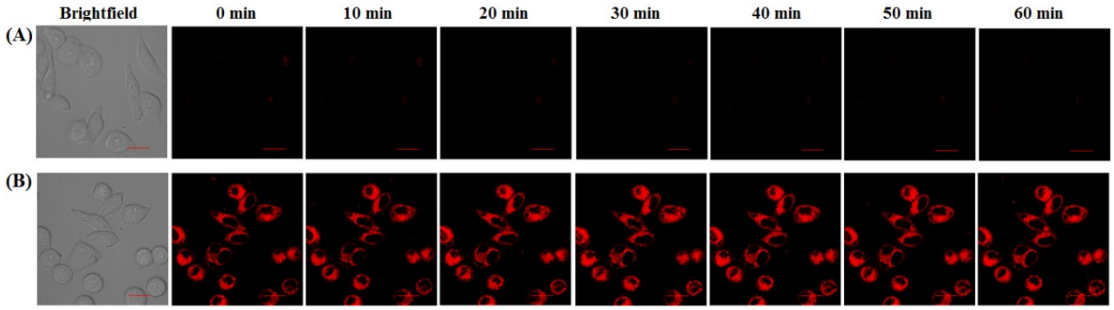


Figure S9. Confocal images of the **SiRTA** (2 μM)-loaded HeLa cells continuously irradiated by semiconductor laser (633 nm) for 60 min in the absence (A) and presence (B) of SIN-1 (500 μM). The representative images were obatined in the indicated time points. Emission was collected at 638−747 nm (λ_{ex}: 633 nm). Scale bar: 20 μm.

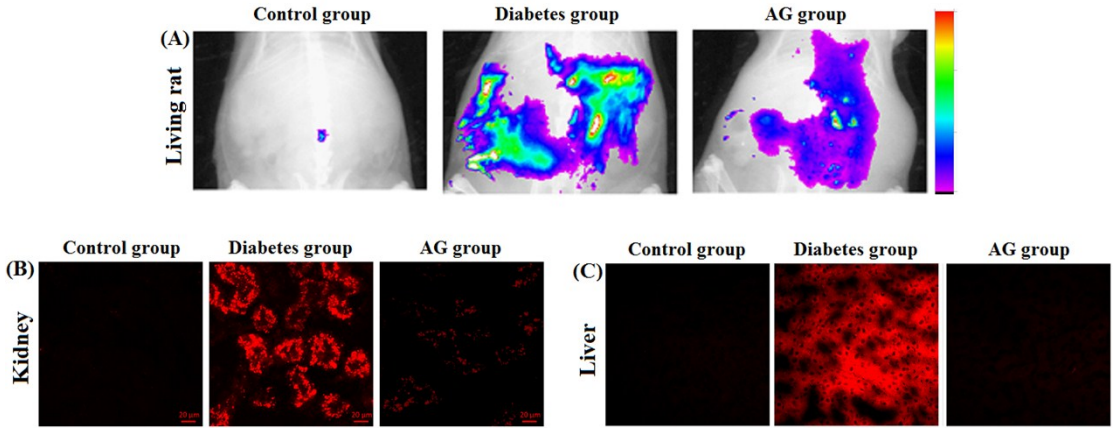


Figure 10. (A) Imaging of $ONOO^-$ in peritoneal cavity of rats in control group, diabetes group, and AG group by i.p. injection with **SiRTA** (100 μL, 2 μM). A Bruker In Vivo FX Pro small animal optical imaging system with an excitation filter of 630 nm and an emission filter of 720 nm was used. (B) The representative confocal images of kidney slices (5 μm thickness) of rats in control group, diabetes group, and AG group, respectively. (C) The representative confocal images of liver slices (5 μm thickness) of rats in control group, diabetes group, and AG group, respectively. Emission was collected at 638−747 nm (λ_{ex}: 633 nm).

8. ^{1}H NMR, ^{13}C NMR, and HRMS Charts

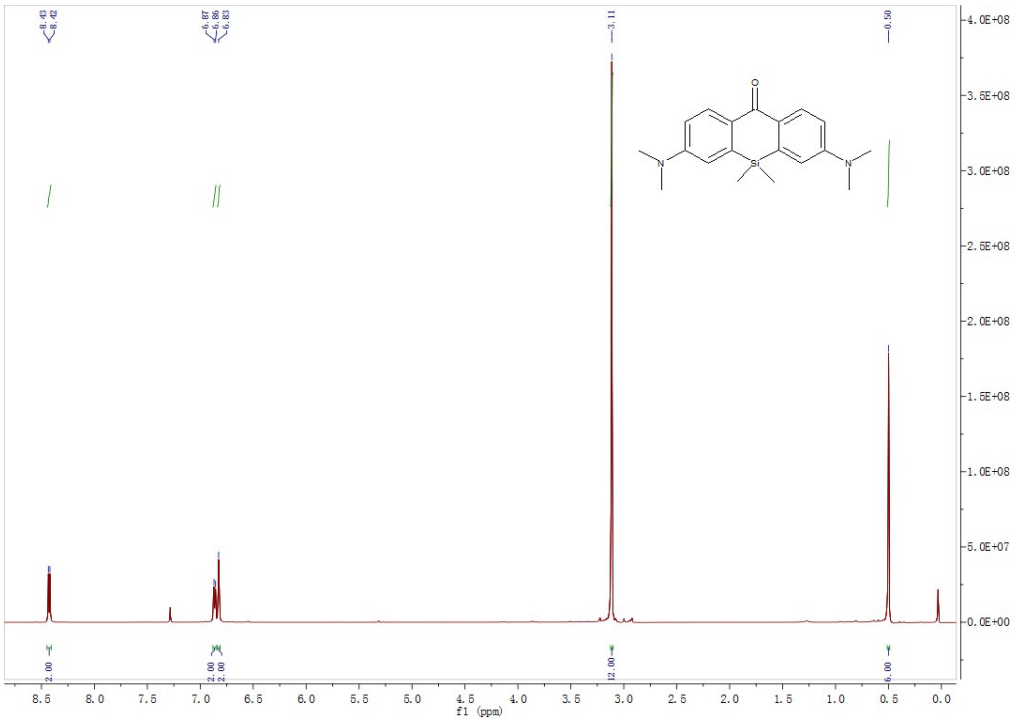


Figure S11. ^{1}H NMR chart of compound **3** ($CDCl_3$, 600 MHz).

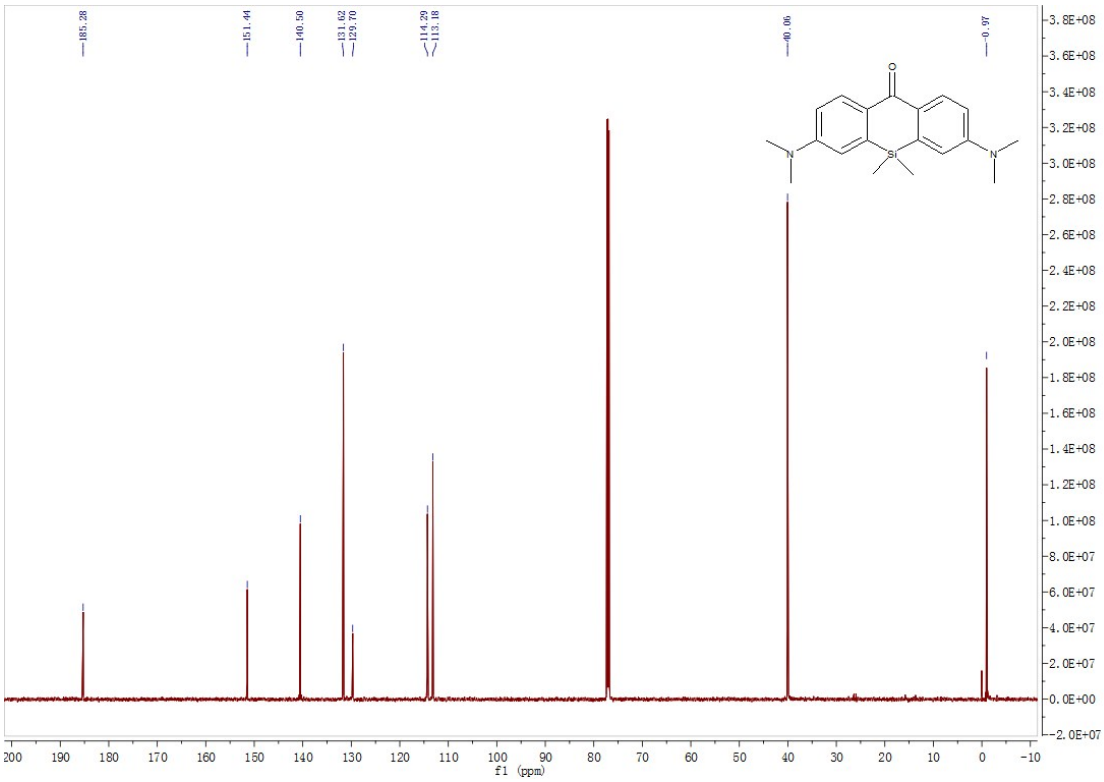


Figure S12. ^{13}C NMR chart of compound **3** ($CDCl_3$, 600 MHz).

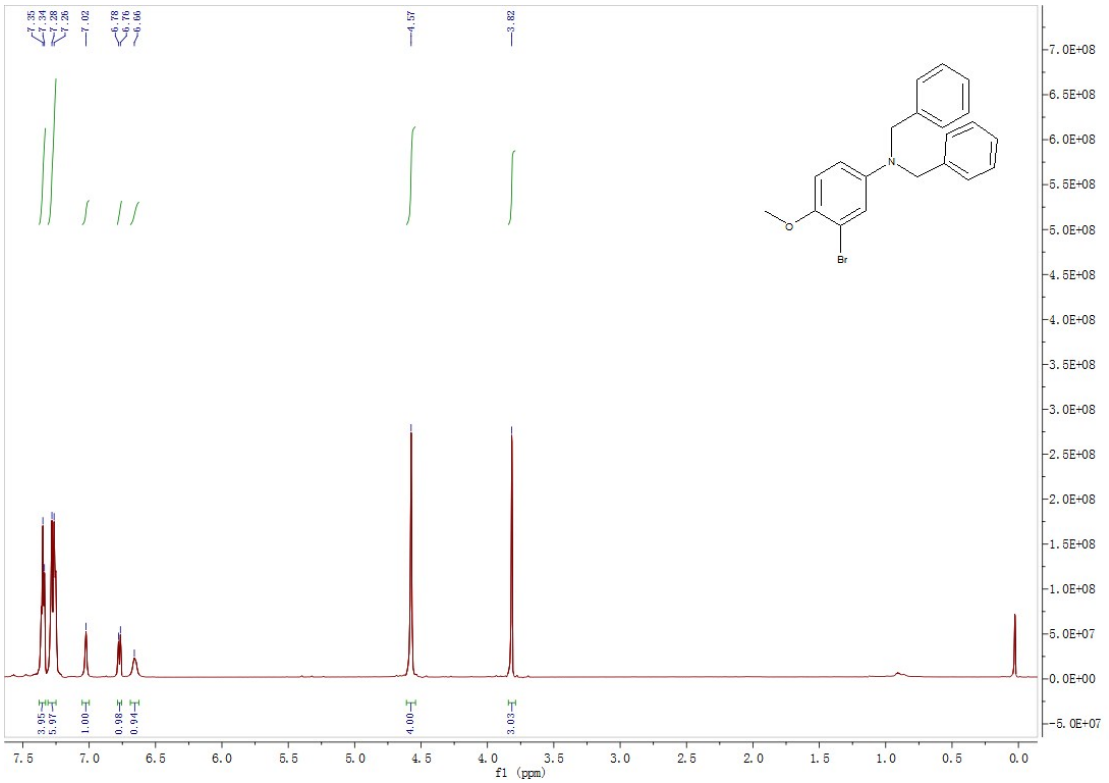


Figure S13. ^{1}H NMR chart of compound **5** ($CDCl_3$, 600 MHz).

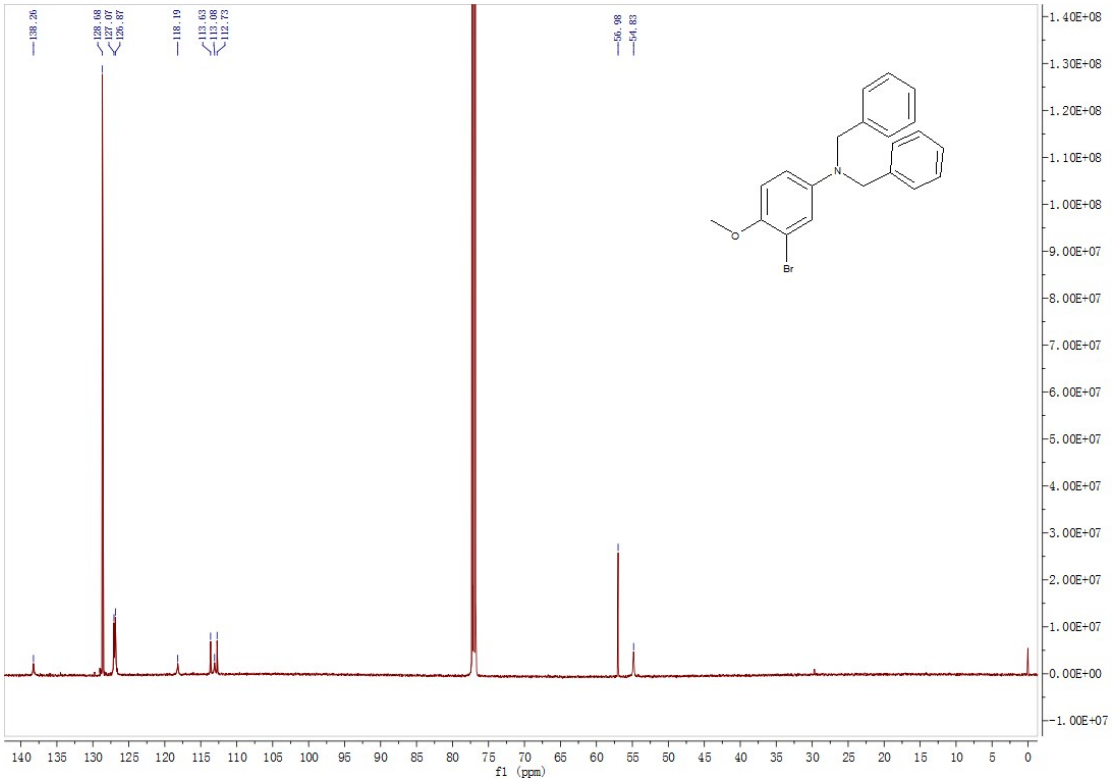


Figure S14. ^{13}C NMR chart of compound **5** ($CDCl_3$, 600 MHz).

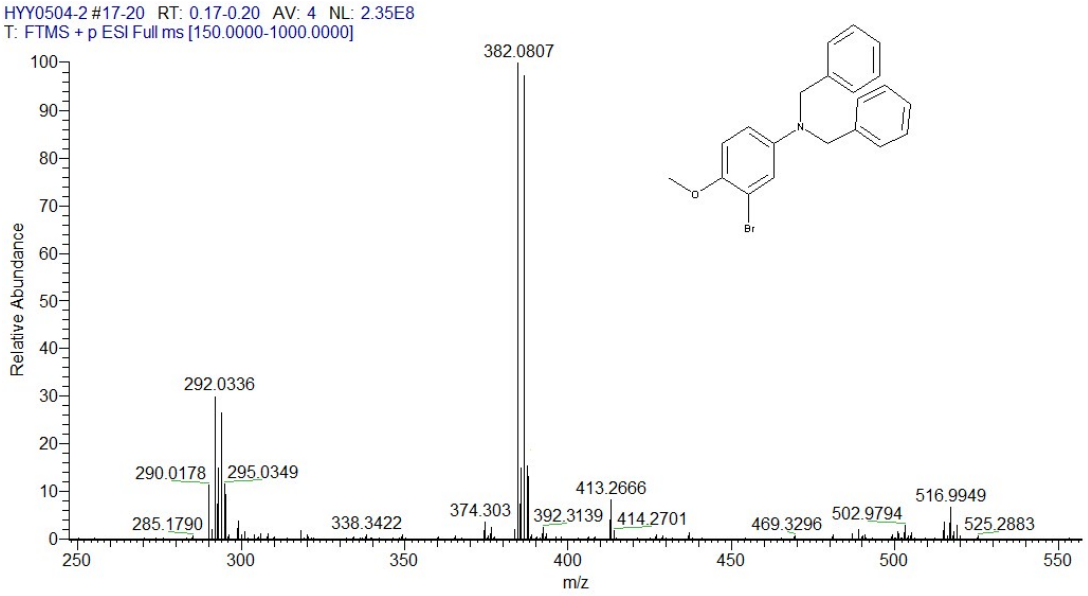


Figure S15. HRMS chart of compound **5**.

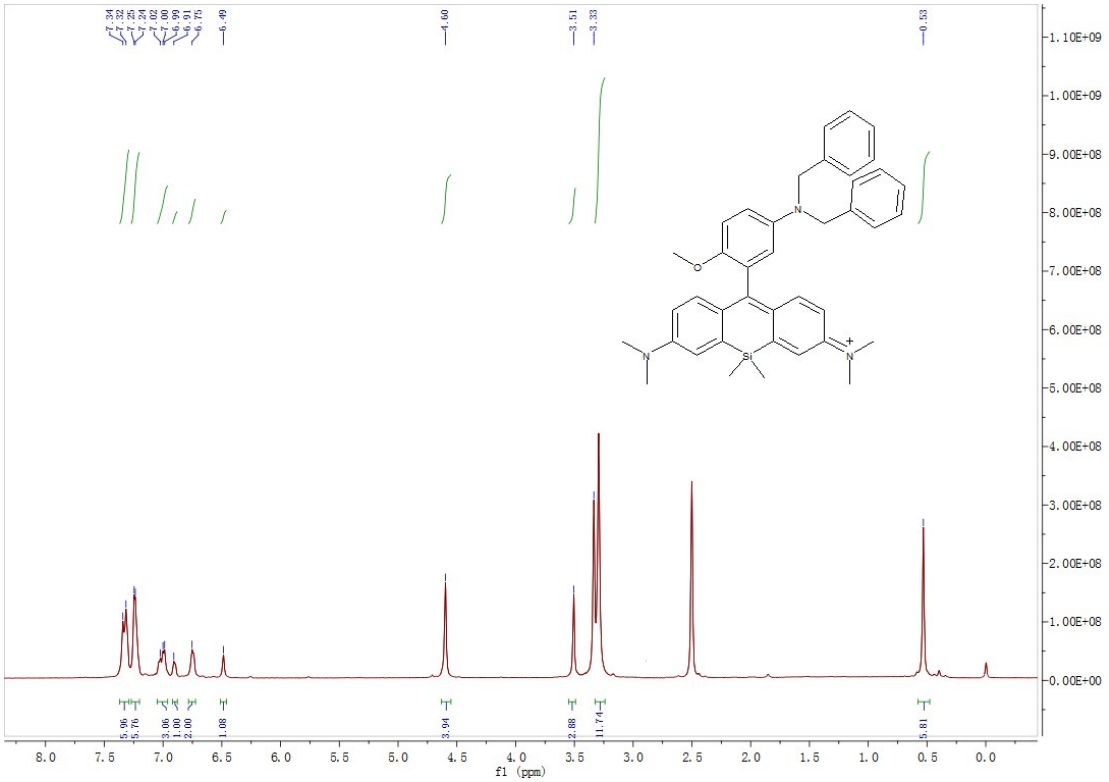


Figure S16. ^{1}H NMR chart of **SiRTA** (DMSO-d_6, 600 MHz).

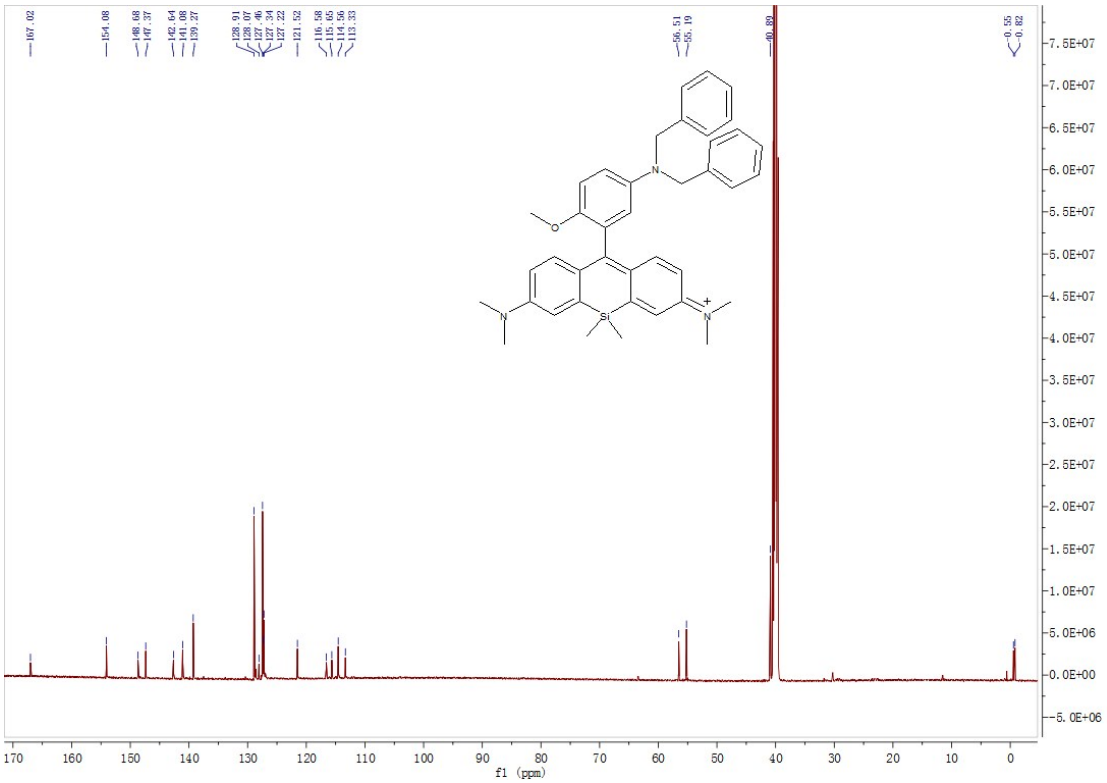


Figure S17. ^{13}C NMR chart of **SiRTA** (DMSO-d_6, 600 MHz).

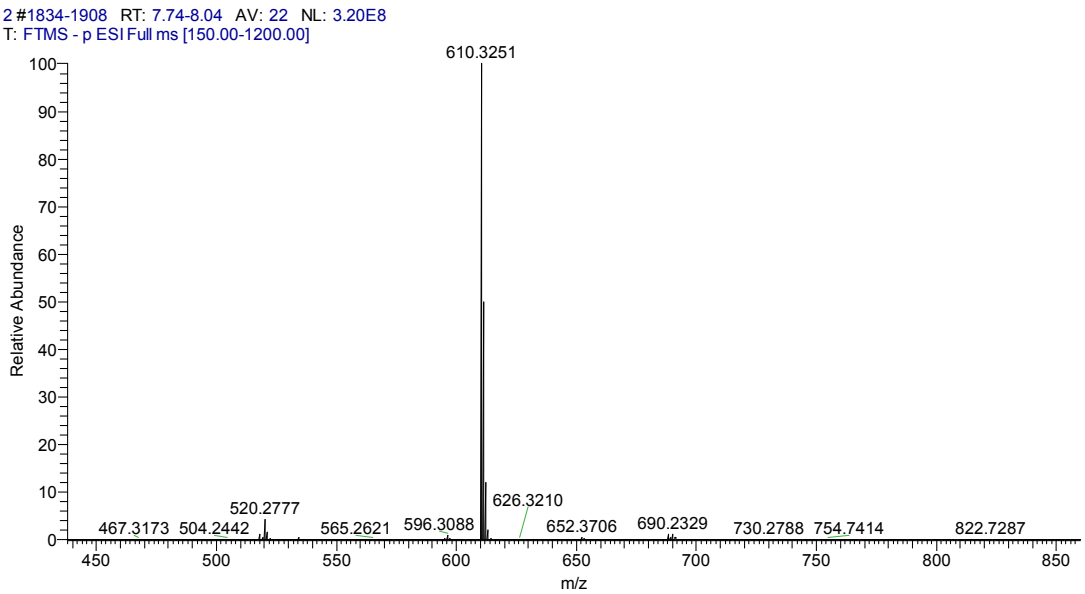


Figure S18 HRMS chart of **SiRTA**.

9. References

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