Supporting Information

Rhodol-Hemicyanine based ratiometric fluorescent probe for real-time monitoring of glutathione dynamics in living cells

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1. Experimental section

1.1 General information

All reagents and solvents in this study were purchased from commercial sources and were of the highest grade. Solvents were dried according to standard methods. Double-distilled water was used throughout the experiments. The **RdH** was dissolved into CH_3CN to prepare the stock solutions with a concentration of 2 mM. GSH was dissolved in degassed 10 mM sodium phosphate buffer (pH 7.4) to obtain 200 mM stock solution. All spectroscopic measurements were performed in PBS (10 mM, pH 7.4) buffer. Absorption spectra were performed on a Varian Carry 4000 spectrophotometer. Fluorescence spectra were measured on Hitachi F-7000 fluorescence spectrometer. The ^{1}H NMR and ^{13}C NMR spectra were recorded at 600

and 150 MHz, respectively. High resolution mass spectra were collected on a Varian QFT-ESI mass spectrometer. Live cell fluorescence images were acquired by Zeiss LSM 880+ Airyscan Laser Scanning Confocal Microscope with a 60×oil–immersion objective lens.

1.2 Synthesis of RdH

Compounds **1** and **2** were synthesized according to the reported method.[1]

Compound **RdH**: compound **2** (0.415 g, 1 mmol) and compound **3** (0.319 g, 1 mmol) were dissolved in ethanol (20 mL) and refluxed for 10 h. After being cooled to room temperature, the mixture was evaporated to dry under vacuum, and extracted with CH_2Cl_2 (30 mL×3). The combined organic extracts were dried over Na_2SO_4, filtered, and evaporated. The crude product was purified by column chromatography ($MeOH/CH_2Cl_2$ = 1:20) to afford compound **RdH** (0.412 g, yield: 61%). ^{1}H NMR (600 Hz, $CDCl_3$) δ 8.54 (d, J = 6.6 Hz, 1H), 8.23 (d, J = 7.8 Hz, 1H), 7.95 (d, J = 10.2 Hz, 1H), 7.74 (m, J_1 = 7.8 Hz, J_2 = 6.6 Hz, 2H) 7.69 (t, J = 7.8 Hz, 1H), 7.58 (t, J = 7.8 Hz, 1H)，7.52 (d, J = 15.0 Hz, 1H), 7.39 (t, J = 7.8 Hz, 1H) 7.28 (t, J = 6.6 Hz, 2H), 7.25 (d, J = 7.2 Hz, 1H)，7.16 (d, J = 7.8 Hz, 2H), 7.09 (t, J = 8.4 Hz, 2H) 6.76 (s, 1H), 6.54 (d, J = 8.4 Hz, H)，6.36 (d, J = 7.8 Hz, 1H), 6.16 (s, 1H),6.04 (s, J = 10.8 Hz，1H), 5.5 (d, J = 16.2 Hz，1H), 3.93 (m, J = 6.0 Hz, 4H), 1.20 (t, J = 7.2 Hz, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ171.7, 169.3, 155.0, 152.0, 149.7, 146.8, 140.6, 135.1, 134.4, 132.1, 130.2, 129.4, 129.2, 129.0, 128.9, 128.3, 128.2, 127.3, 126.1, 125.4, 124.0, 117.9, 116.0, 112.7, 112.4, 108.8, 105.0, 97.5, 51.4, 44.1, 12.4; ESI-MS $[M+H]^+$: calcd for 637.2161, Found 637.2158.

1.3 General information Determination of dissociation constant K_d of RdH with GSH

Absorption spectroscopy was used to determine the apparent dissociation constants (K_d') of **RdH** (5 μM) with GSH using the reported method.[2-4]

The following equation was used:

$$\frac{R - R_{min}}{R_{max} - R} = \frac{[GSH]}{K_d'}$$

The dissociation constants K_d was calculated based on the relationship between K_d and K_d':

$$K_d' = K_d \varepsilon_{RdH,580nm} / \varepsilon_{RdH-GSH,580nm}$$

The ratio between the absorbance at 530 nm and 580 nm (A_{530}/A_{580}), was calculated and recorded as R. R_{max} represents the ratio when GSH is 7 mM; R_{min} represents the ratio when GSH is 0 mM. Plotting $(R\text{-}R_{min})/(R_{max}\text{-}R)$ as a function of GSH concentration afforded a linear relationship. The reciprocal of the slope yields K_d'. ε is the molar absorption coefficient.

1.4 Kinetic analysis of the reaction of RdH with GSH

Kinetic parameters of **RdH** (5 μM) were determined by time-resolved fluorescence spectroscopy. The fluorescence increases upon addition of GSH (2 mM) was recorded at a scan rate of 0.2 s. The pseudo first-order constant (k_{obs}) was obtained by fitting the plots (Fig. 1F) with a pseudo-first-order reaction equation[5]:

$$F = F_\infty + (1 - F_\infty) \times e^{-k_{obs}t}$$

The half-life ($t_{1/2}$) was calculated by the following equation:

$$t_{1/2} = \frac{\ln 2}{k_{obs}} = \frac{0.693}{k_{obs}} [s]$$

1.5 Cyotoxicity assays

The CCK-8 cell proliferation assay was applied to determine the viabilities of the cells treated with **RdH**. The A549 cells were seeded into a 96-well plate with a density of 5.0×10^3 cells/well in 100 μL cell culture medium. After cell attachment, the cells were treated by **RdH** with different concentrations (0-10 μM). After cells were incubated 24 h and washed three times with PBS, CCK-8 (10 μL) in fresh medium was added. Then the plate was shaken for 30 min, and absorbance at 450 nm was measured by Synergy™ Mx Multi-Mode Microplate Reader. Cell viability (%) = ($A_{\text{with probe}} - A_{\text{blank}} / A_{\text{control}} - A_{\text{blank}}$) × 100%.

1.6 Cell culture and fluorescence imaging

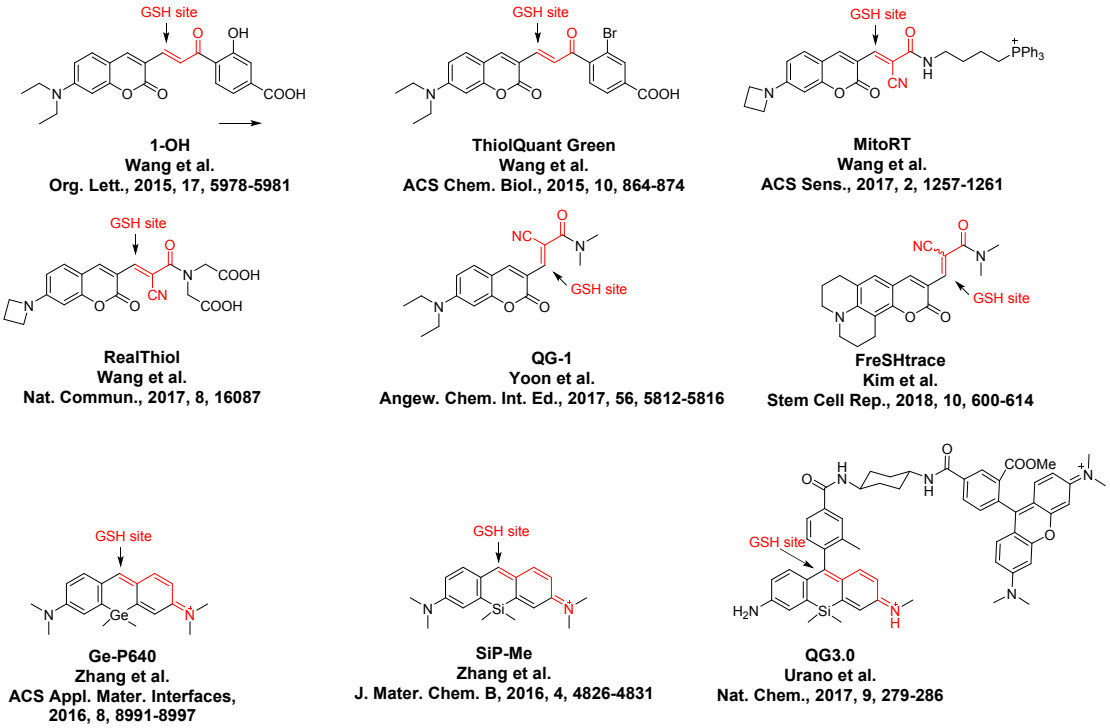
A549 cell lines were kindly provided by Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education (China). Cells were cultured in DMEM medium which were supplemented with 10 % FBS (Fetal Bovine Serum) and 1% antibiotics at 37 ºC in humidified environment of 5% CO_2. Cells were seeded in glass bottom cell culture dish (30 mm) and allowed to adhere for 12 hours. Before we started experiments, cells were washed with PBS 3 times.

To test the effect of inhibition of glutathione synthesis by BSO on GSH concentration, A549 cells were incubated with 50 μM buthionine sulfoximine (BSO, an inhibitor of γ-glutamylcysteine synthetize) for 24 hours. The cells were washed and treated with **RdH** (4 μM) in PBS for 20 min, and after washed with PBS 3 times, the fluorescence imaging assays were performed.

To illustrate the ability of **RdH** to monitor GSH dynamics, A549 cells were incubated with **RdH** for 20 min at 37 °C. After washed with PBS 3 times, A549 cells were imaged under a confocal microscope, NEM (1 mM) was added to the culture medium to decrease of GSH levels.

To test GSH dynamics under oxidative stress damage, A549 cells were incubated with LPS (1 μg/mL) in DMEM medium for 4h, 12h at 37 °C, respectively. The cells were washed and treated with **RdH** (4 μM) in PBS for 20 min, and after washed with PBS 3 times, the fluorescence imaging assays were performed.

2. Supplementary Spectra and Imaging date



Scheme S1. Molecular structures and nucleophilic reaction sites of the reversible GSH probe reported previously.

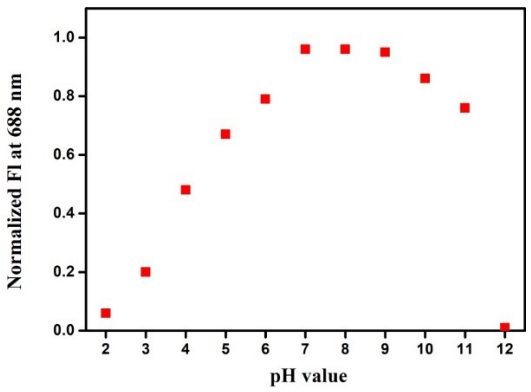


Figure S1. Normalized fluorescence intensity at 688 nm of **RdH** (10 μM) at different pH values in B-R buffer solution.

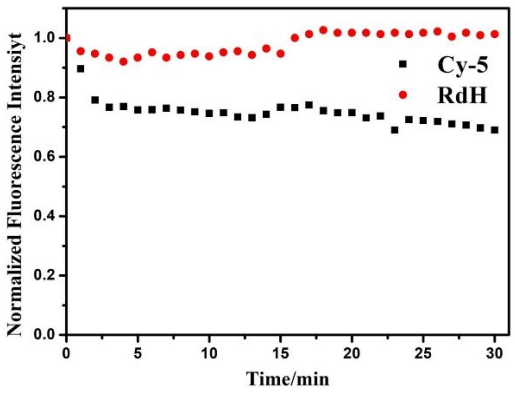


Figure S2. Normalized time-dependent fluorescence intensity changes of **RdH** and Cy-5 continuously excited by a 100 W Xe lamp for 30 min in PBS (10 mM, pH = 7.4).

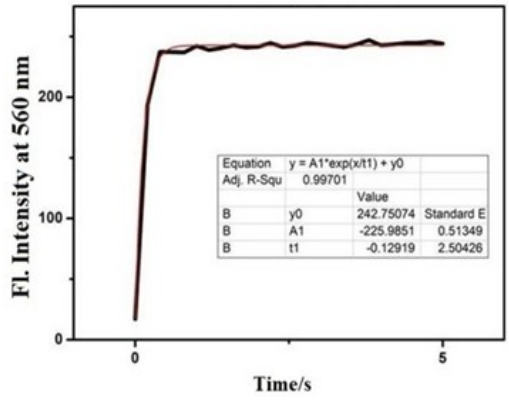
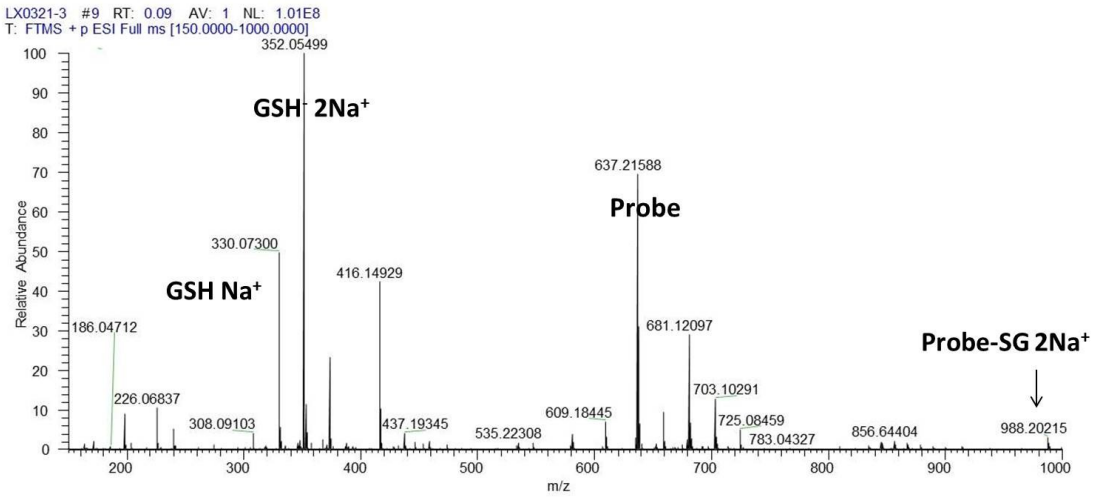
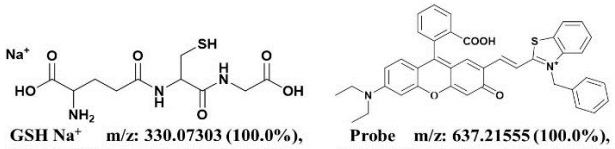


Figure S3. Time-dependent fluorescence intensity changes of **RdH** (5 μM) upon treated with GSH (2 mM) in PBS (pH = 7.4, 10 mM) (λ_{ex} = 510 nm, λ_{ex} = 560 nm, Slits: 5/5 nm). Inset describes the fitting equation, and the reciprocal of -t1 represents the observed rate constant (k_{obs}).

Table S1. Comparison of the kinetic parameters of **SiR_{610}**, **TQ Green**, **QG-1** and **RdH**.

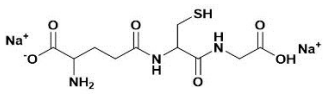
probe	k_{obs} [s^{-1}]	$t_{1/2}$ [s]	$K_{d,GSH}$ [mM]
SiR_{610}	1.99	0.35 (1 mM)	7.9
TQ Green	5.9×10^{-3}	116 (40 mM)	1.6
QG-1	0.12	5.82 (10 mM)	2.59
RdH	7.74	0.09 (2 mM)	1.42



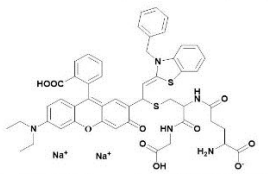


GSH Na^+ m/z: 330.07303 (100.0%), 331.07638 (10.8%), 332.06882 (4.5%), 332.07727 (1.2%), 331.07006 (1.1%)

Probe m/z: 637.21555 (100.0%), 638.21891 (43.3%), 639.22226 (9.1%), 639.21135 (4.5%), 640.21471 (2.0%)



GSH^- $2Na^+$ m/z: 352.05497 (100.0%), 353.05833 (10.8%), 354.05077 (4.5%), 354.05922 (1.2%)



Probe-GS $2Na^+$ m/z: 988.26325 (100.0%), 989.26660 (54.1%), 990.26996 (14.3%), 990.25905 (9.0%), 991.26240 (4.9%)

Figure S4. The ESI-MS spectra obtained by reaction of **RdH** and GSH.

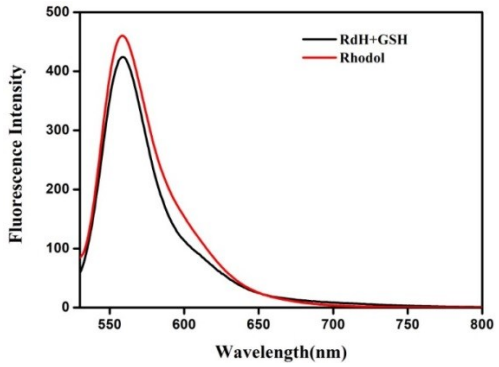


Figure S5. Fluorescence spectra of **RdH** (5 μM) in the presence of GSH (5 mM) compares with rhodol in PBS buffer (10 mM, pH = 7.4). λ_{ex}= 510 nm, slit: 5 nm/5 nm.

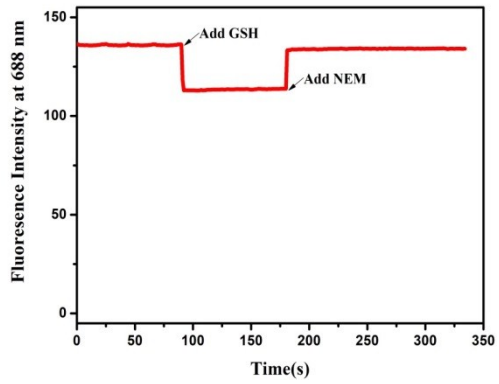


Figure S6. Time-course fluorescence changes of **RdH** (5 μM) upon addition of GSH (5 mM) then addition of NEM (5 mM) in PBS buffer (pH 7.4, 10 mM); λ_{ex}= 610 nm, λ_{em} = 688 nm, Slit: 5 nm/10 nm.

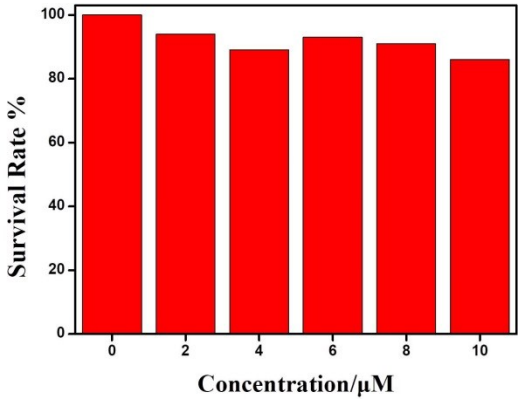


Figure S7. Percentage of viable A549 cells after treated with increasing concentrations of **RdH** for 24 h.

3.^{1}H NMR, ^{13}C NMR, and HRMS Charts of RdH.

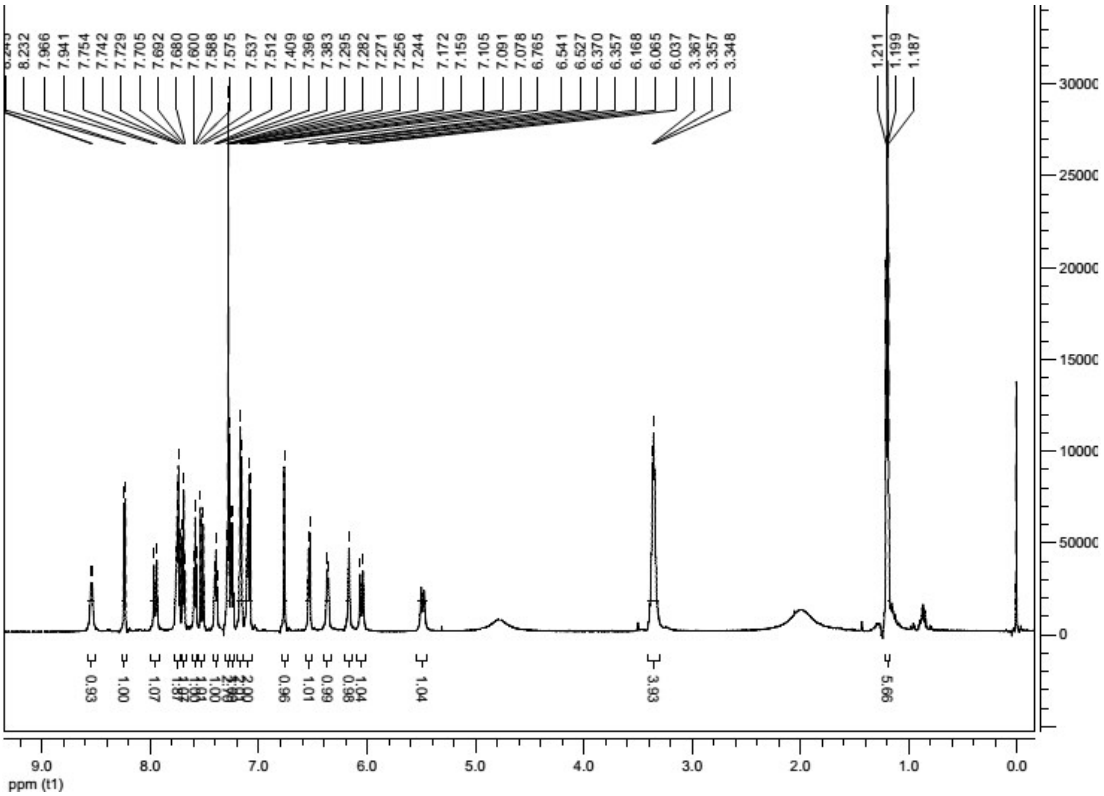


Figure S8. ^{1}H NMR spectra of **RdH**.

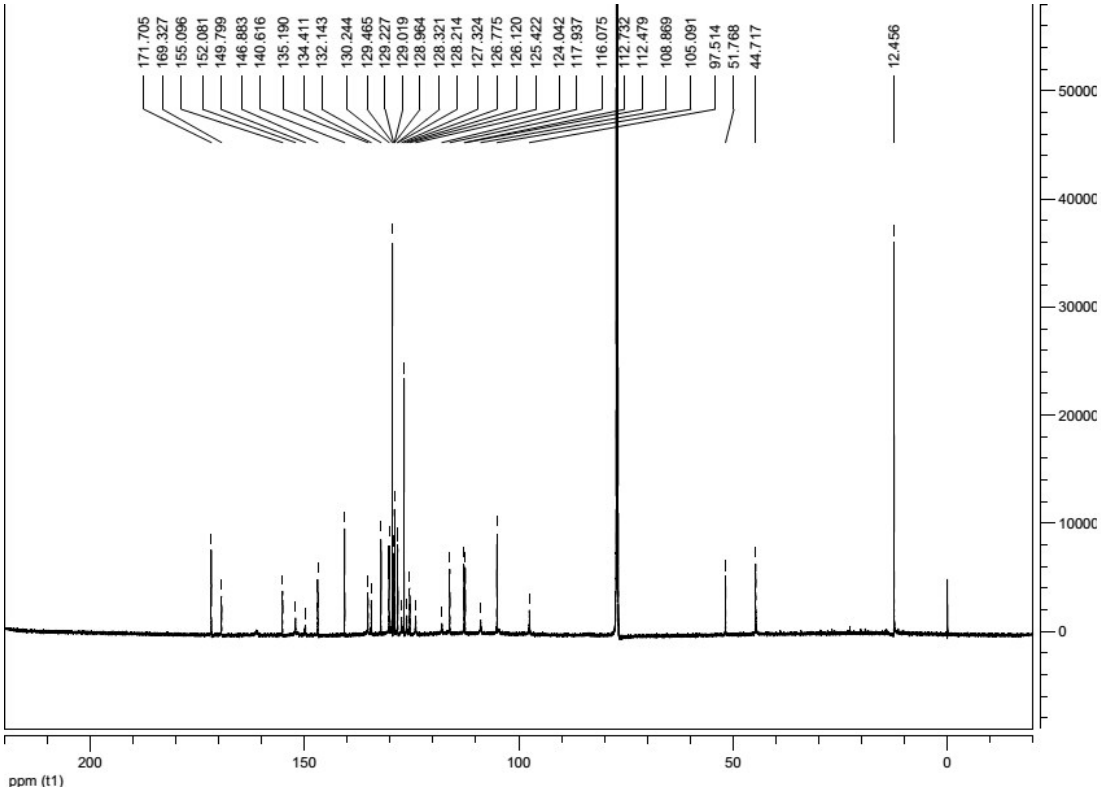


Figure S9. ^{13}C NMR spectra of **RdH**.

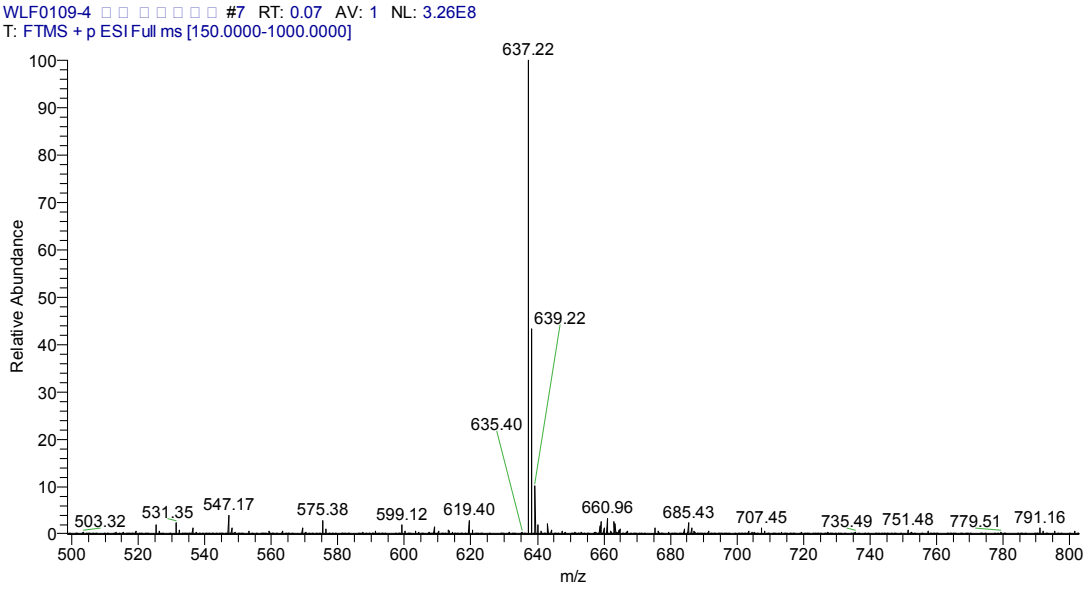


Figure S10. HRMS spectra of **RdH**.

4. References

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