Electronic Supplementary Material (ESI) for Analytical Methods.

Development of bishydrazide-based fluorescent probes for the imaging of cellular peroxynitrite ($ONOO^-$) during ferroptosis

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Table of Contents

1. Materials and instruments

All solvents and reagents were commercially available and used without further purification. Doubly distilled water was used in all the experiments. Thin-layer chromatography (TLC) analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200-300), both of which were purchased from the Qingdao Ocean Chemicals. Fluorescence spectra and relative fluorescence intensity were measured with a Hitachi F-4600 spectrofluorimeter with a 10 mm quartz cuvette. UV/vis spectra were obtained with a Shimadzu UV-2700 spectrophotometer. High-resolution mass spectra (HRMS) for the characterization of structures were collected using a Bruker apex-Ultra mass spectrometer (Bruker Daltonics Corp., USA) in electrospray ionization (ESI) mode. ^{1}H and ^{13}C NMR spectra were recorded on an AVANCE III 400 MHz Digital NMR Spectrometer, using tetramethylsilane (TMS) as internal reference. LC-MS were collected using an Agilent 6510 Q-TOF LC/MS.

2. Preparation method of ONOO- solution:

Under the condition of ice bath, HCl solution (0.6 mol/L, 10mL) was added to the mixed solution containing $NaNO_2$ (0.6 mol/L, 10mL) and H_2O_2 (0.7 mol/L, 10mL), which was stirred vigorously, followed by NaOH solution (1.5 mol/L, 20mL). Finally, a very small amount of MnO_2 was added and filtered. Note that all the above solutions were prepared from deionized water, and the prepared $ONOO^-$ must be stored in the dark at -20 ° C.

$ONOO^-$ Concentration calibration: The UV spectrophotometer was used for testing. Firstly, 2mL of NaOH solution with a concentration of 0.1mol /L was used to sweep the baseline, and then X μL of prepared $ONOO^-$ solution was added to V mL of NaOH solution with a concentration of 0.1mol /L. The concentration of $ONOO^-$ solution can be obtained by the following equation. Where A_{302nm} is the absorbance of $ONOO^-$ at 302nm, V is the total volume of solution, and X is the volume of added $ONOO^-$ solution.

$$c = \frac{A_{302nm} * V}{1.67X}$$

3. Cytotoxicity experiments

HeLa cells were seeded in a 96-well plate at a cell density of 8000 cells/well, cultured for 24 hours, and then replaced with cultures containing different concentrations of **Rh-3** for 24 hours. After that, add 5 mg/mL of MTT solution 10 μL to each well to each well, continue to incubate for 3 h, then add 100 mL of dimethyl sulfoxide (DMSO) to dissolve the precipitate, and measure the absorbance of the resulting solution with a microplate reader.

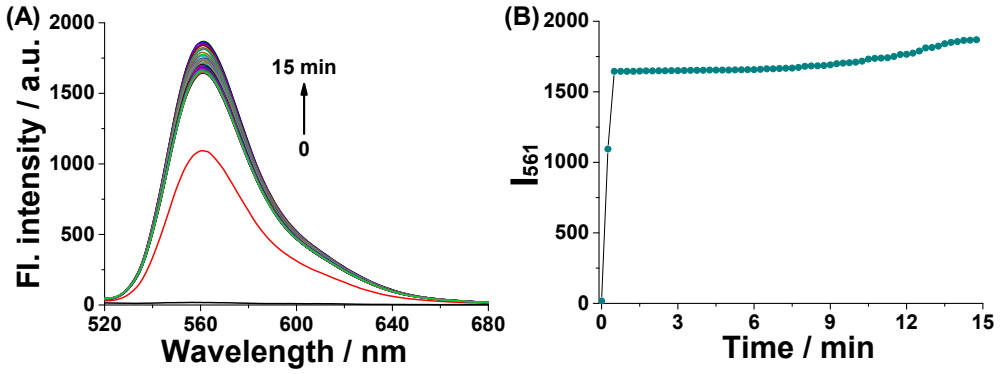

Fig. S1. Time-dependent fluorescence spectra (A) and fluorescent intensity (I_{561}) (B) of 5 μM **Rh-3** in the presence of 20 μM $ONOO^-$ in PBS (pH = 7.4, 20% DMF). λ_{ex} = 505 nm

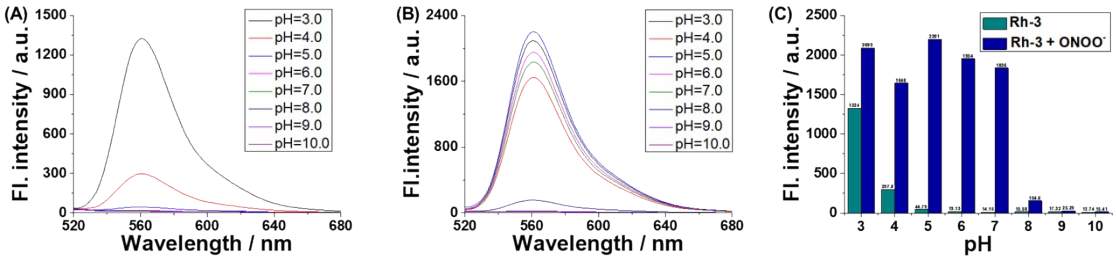

Fig. S2. (A) Fluorescence spectra of 5 μM **Rh-3** at various pH. (B) Fluorescence spectra of 5 μM **Rh-3** treated with 30 μM $ONOO^-$ at various pH. (C) Quantified fluorescence intensity (I_{561}) for (A) and (B). λ_{ex} = 505 nm.

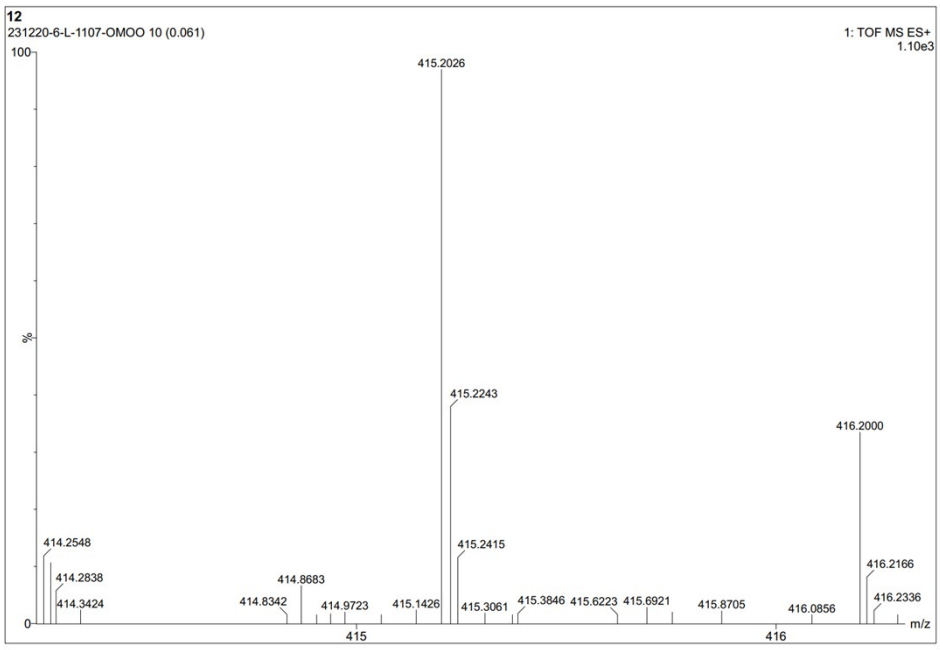

Fig. S3. HRMS data of the reaction products of **Rh-3** and $ONOO^-$.

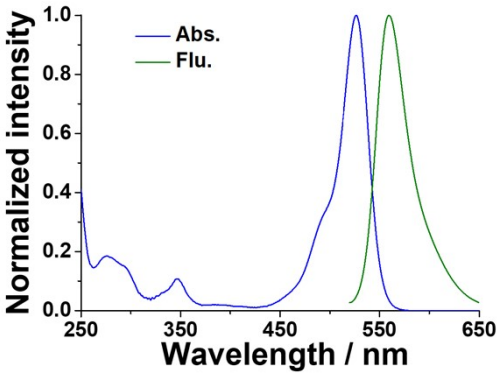

Fig. S4. Fluorescence spectra of 5 μM **Rh-3** in1;1 solution of acetic acid and water. λ_{ex} = 450 nm.

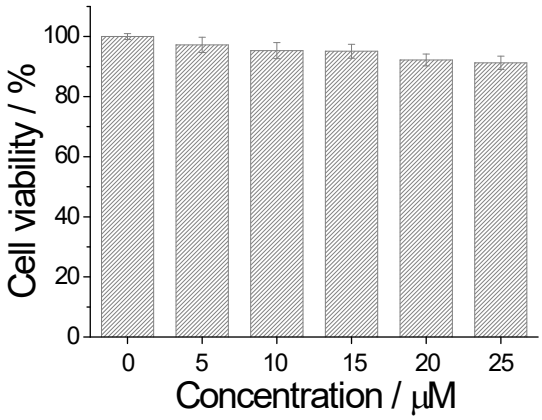

Fig. S5. Survial of HeLa cells in the presence of **Rh-3** at various concentrations measured using MTT assay.

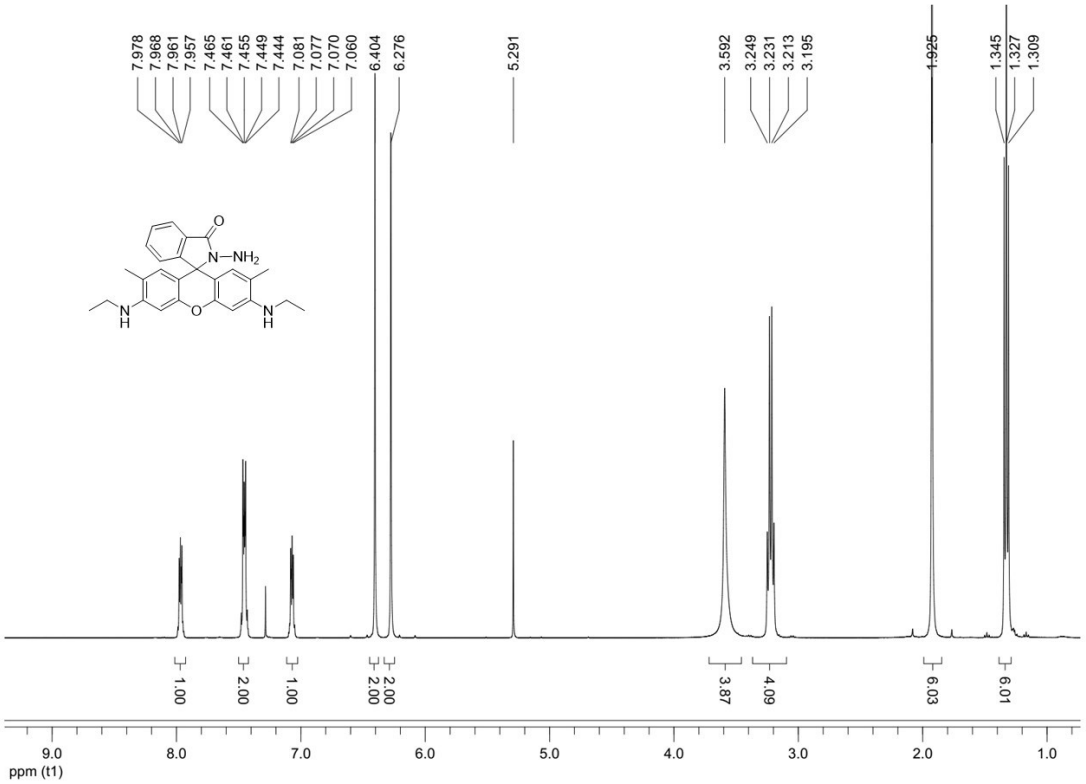

Fig. 6. ^{1}H NMR ($CDCl_3$) of **Rh-N**.

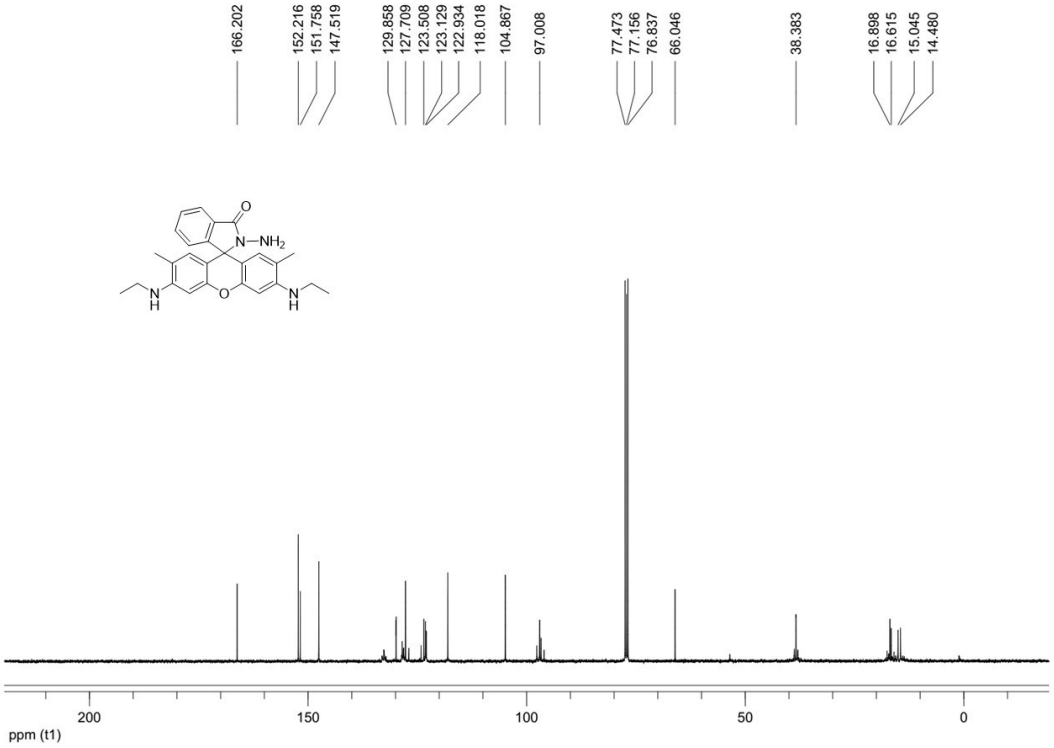

Fig. 7. ^{13}C NMR ($CDCl_3$) of **Rh-N**.

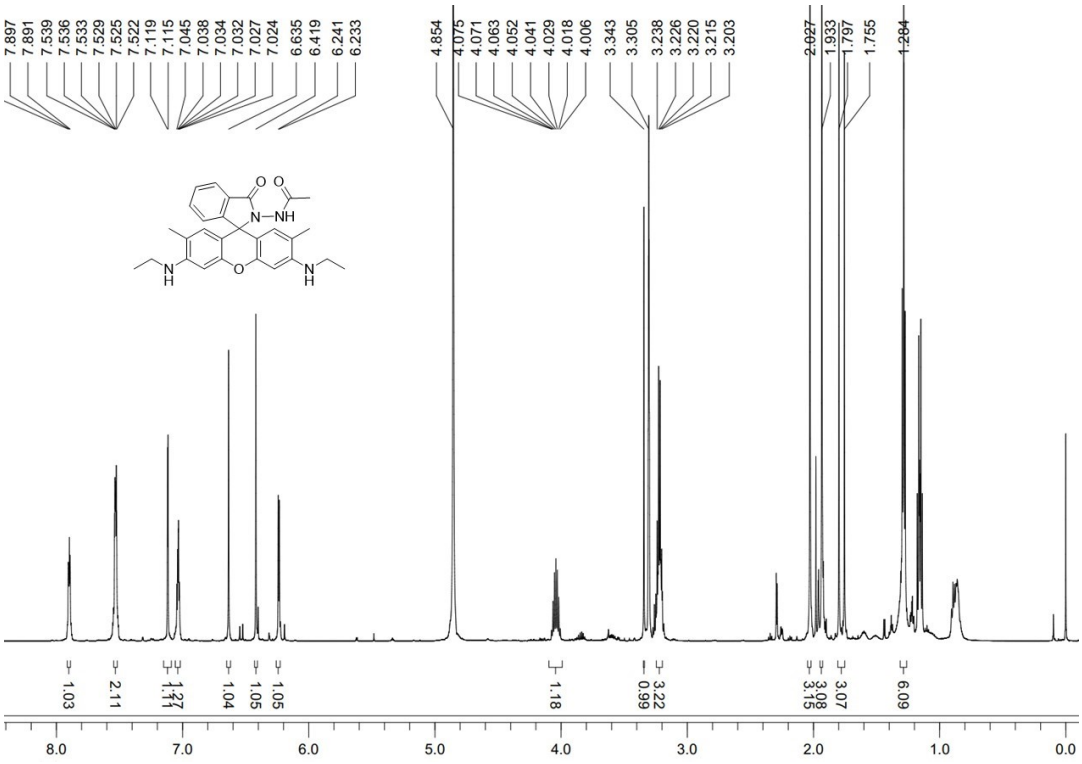

Fig. 8. ^{1}H NMR (MeOD-d_4) of **Rh-1**.

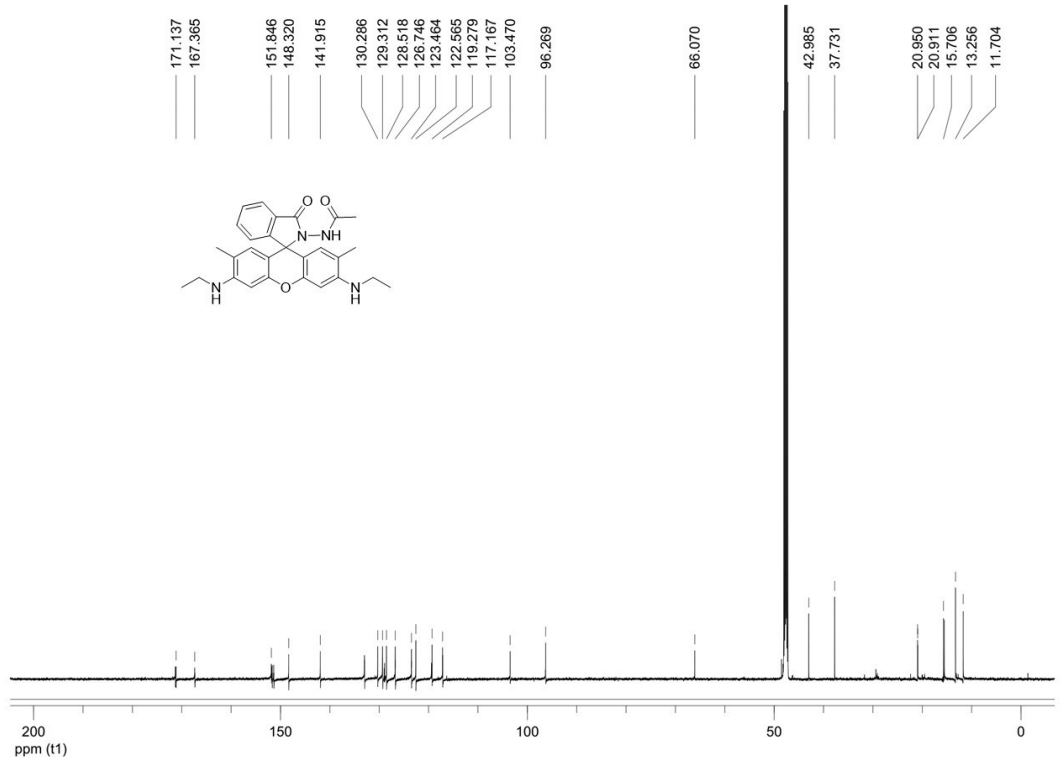

Fig. 9. ^{13}C NMR (MeOD-d_4) of **Rh-1**.

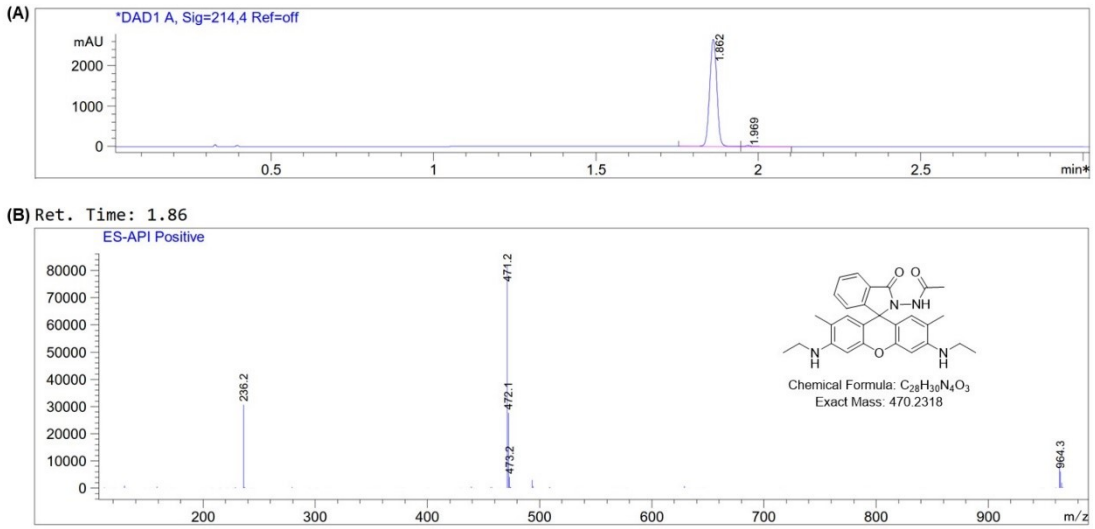

Fig. 10. LC-MS data of **Rh-1**.

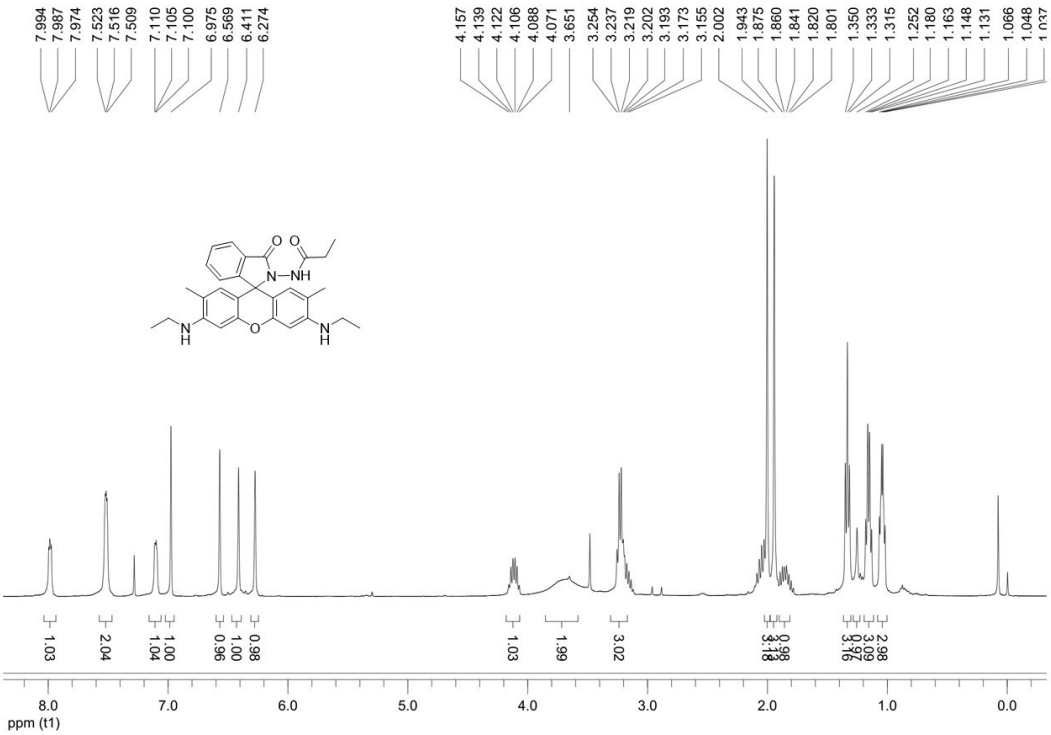

Fig. 11. ^{1}H NMR ($CDCl_3$) of **Rh-2**.

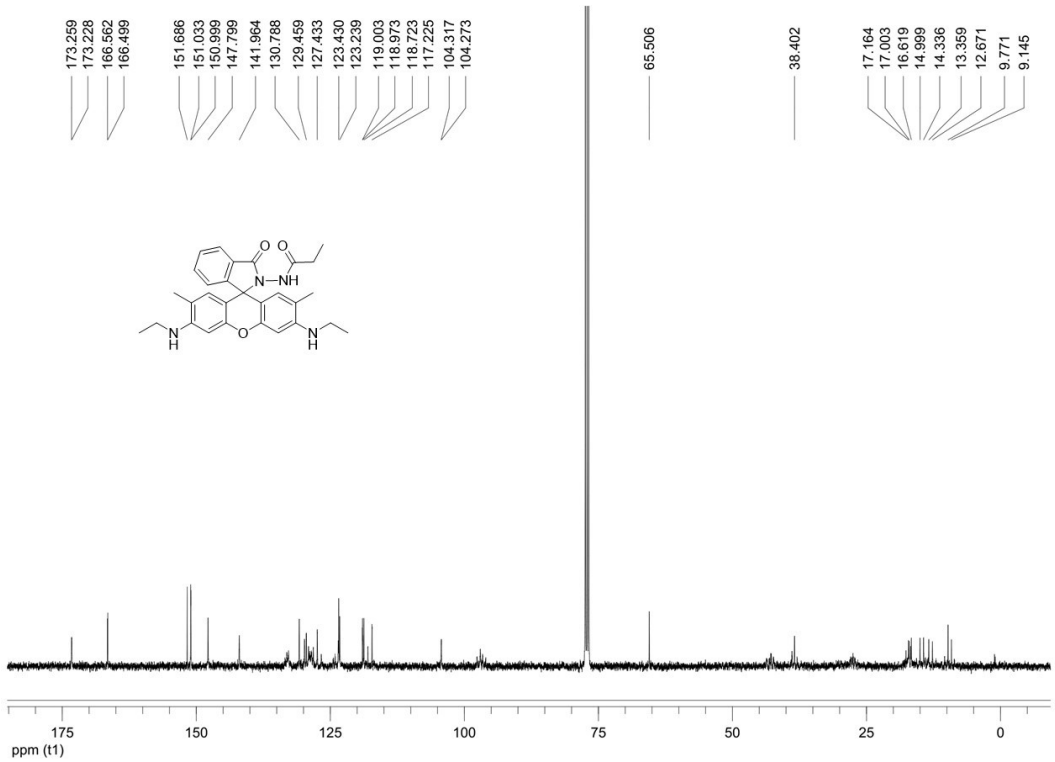

Fig. 12. ^{13}C NMR ($CDCl_3$) of **Rh-2**.

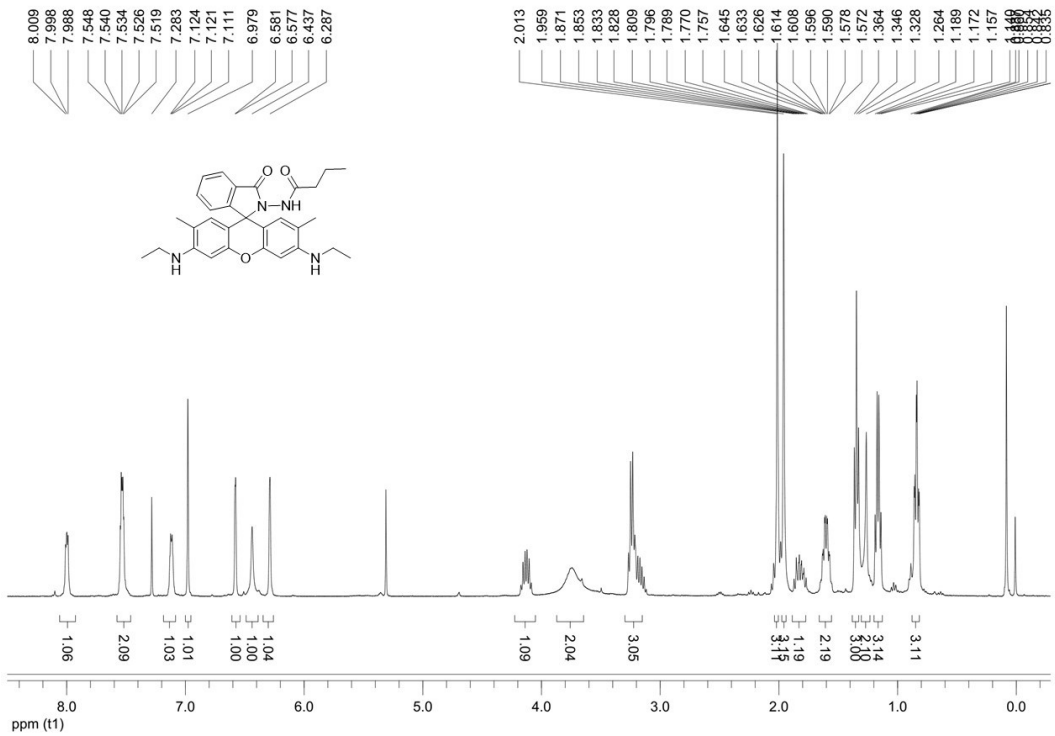

Fig. 13. ^{1}H NMR ($CDCl_3$) of **Rh-3**.

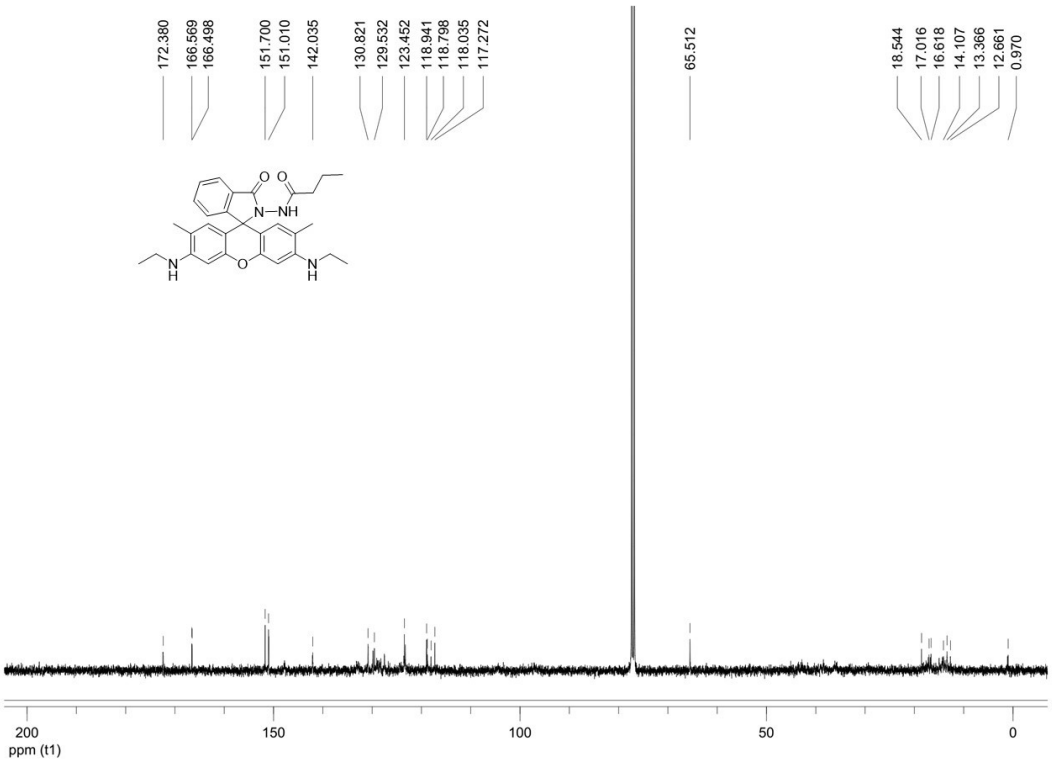

Fig. 14. ^{13}C NMR ($CDCl_3$) of **Rh-3**.

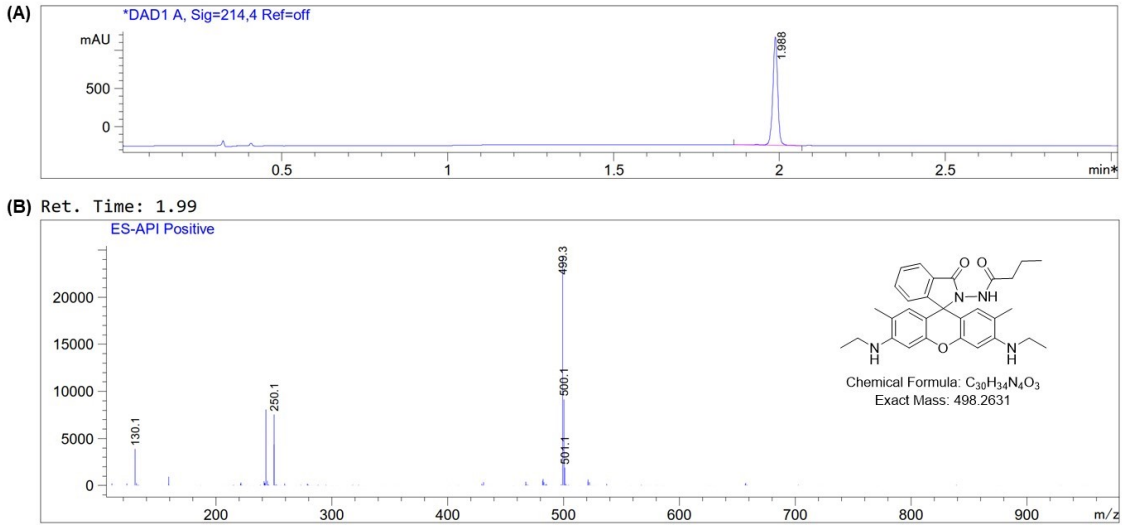

Fig. 15. LC-MS data of **Rh-3**.

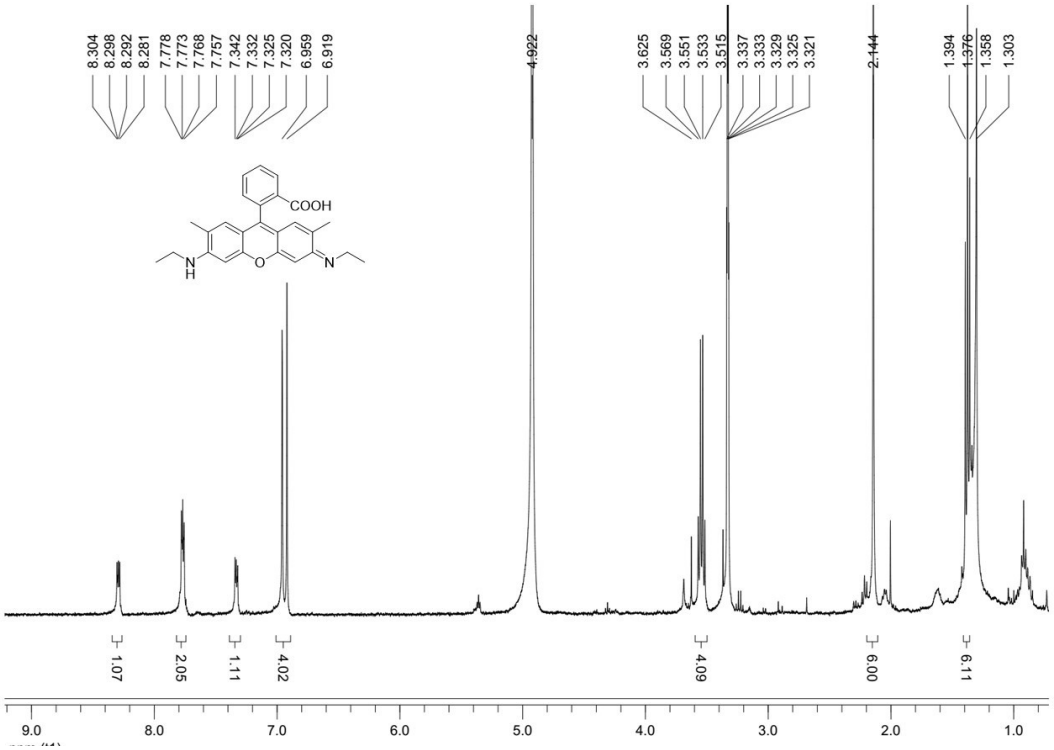

Fig. 16. ^{1}H NMR (MeOD-d_4) of **Rh-COOH**.

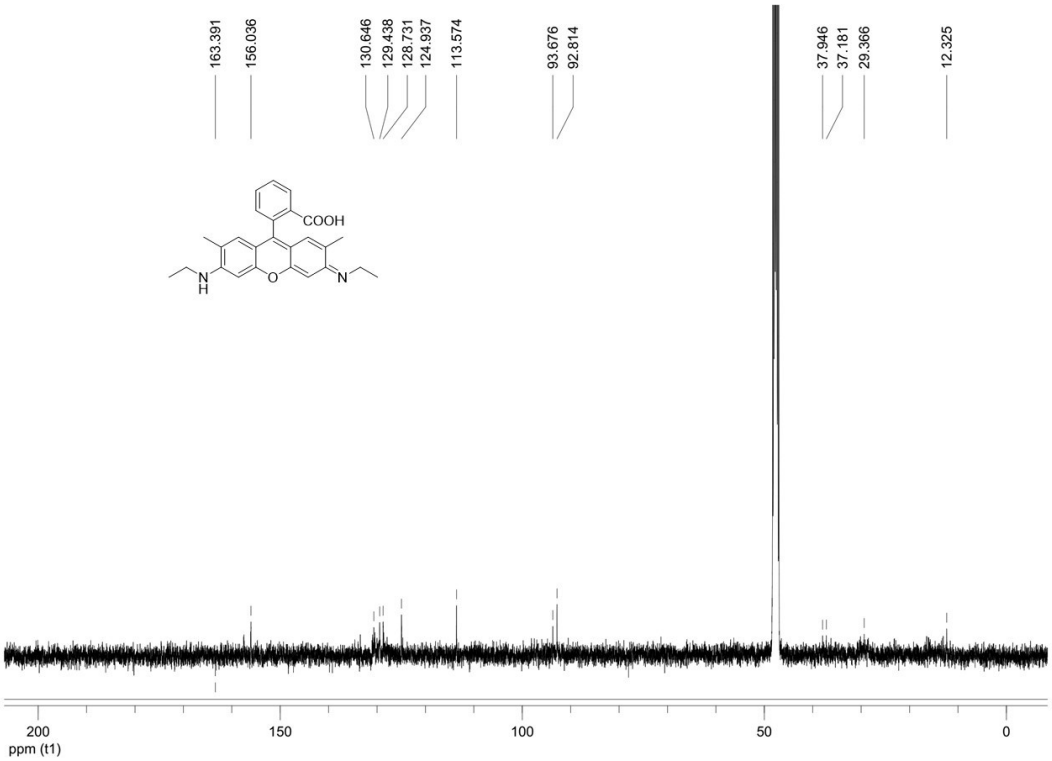

Fig. 17. ^{13}C NMR (MeOD-d_4) of **Rh-COOH**.

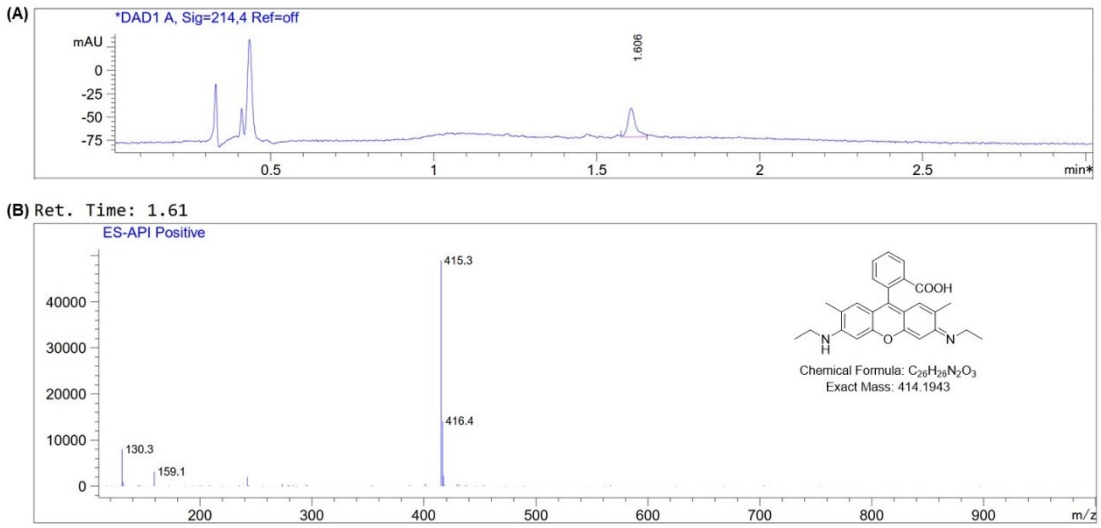

Fig. 18. LC-MS data of **Rh-COOH**.