Electronic Supplementary Information

Fluorescence Probe for the Fast and Sensitive Detection and Imaging of $ONOO^-$ *via* Regulation of Charge Distribution

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Contents:

1. Materials and general methods

1.1. Chemicals

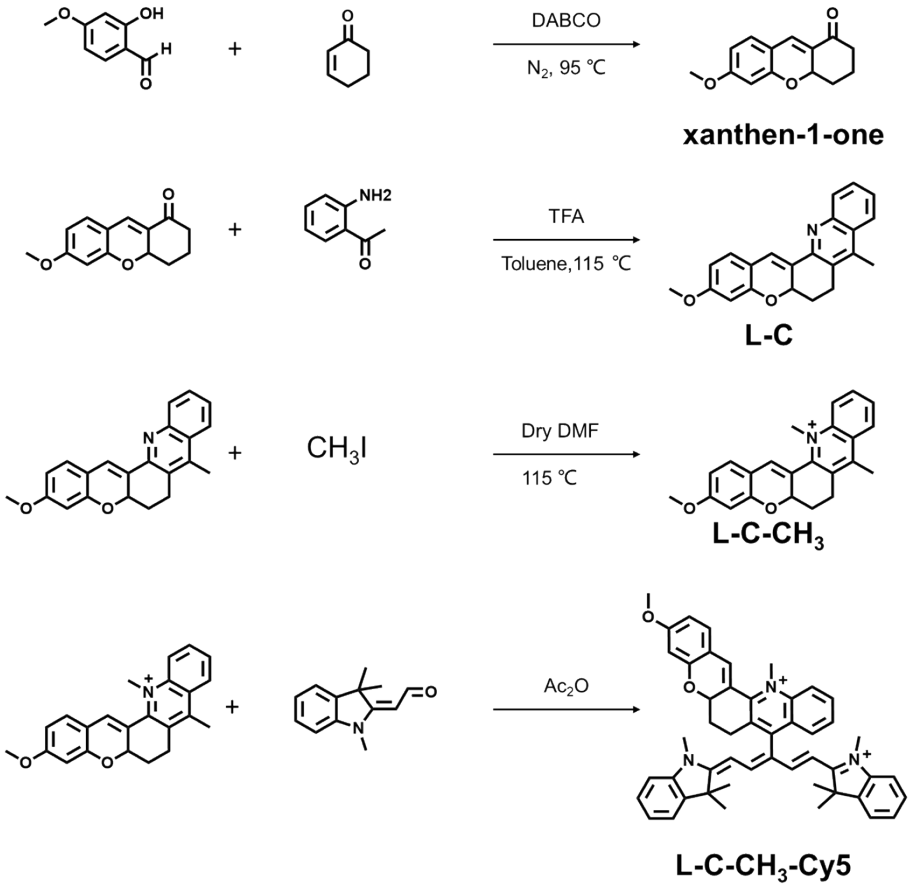
Unless otherwise stated, all reagents for synthesis were obtained commercially and used without further purification. 2-Hydroxy-4-methoxybenzaldehyde, 2-cyclohexen-1-one and trifluoroacetic acid (TFA) were purchased from Adamas, 2-aminoacetophenone, 1,3,3-trimethyl-2-(formylmethylene)indoline were purchased from Bidepharm, iodomethane was purchased from Energy Chemical, tert-butyl methyl ether was purchased from Innochem, triethylenediamine was purchased from Tianjin Jinke Fine Chemical Company,

Mito-Green Tracker was obtained from Beyotime. Dulbecco's modified Eagle's media (DMEM) was purchased from Gibco. Other chemical reagents were purchased from Beijing Chemical Plant, Tianjin Fuchen or Tianjin Damao.

1.2. Instrumentation

^{1}H NMR and ^{13}C NMR spectra were measured with a Bruker Avance 400 MHz spectrometer. The fluorescence spectra were measured by the Hitachi F-4600 fluorescence spectrophotometer. Mass spectra were obtained by the liquid chromatography-mass spectrometer (Thermo Finnigan LTQ XL). UV absorption spectra were obtained by UV-3600 ultraviolet visible near infrared spectrophotometer. The cell images were obtained by using a laser scanning confocal microscope (Leica SP8). Calculation of Pearson correlation coefficient and fluorescence intensity of cells were done by Image J.

1.3. Synthesis.



Scheme S1. Synthetic route for probe **L-C-CH_3-Cy5**

Xanthene-1-one

Xanthene-1-one was synthesized on the basis of previous work.[1] A mixture of compound 2-hydroxy-4-methoxybenzaldehyde (3040 mg, 20 mmol), 2-cyclohexen-1-one (1920 mg, 20 mmol), triethylenediamine (DABCO, 2243.4 mg, 20 mmol) in water/1,4-dioxane (30 mL, 1:1, V/V) was stirred at 95 °C for 2 days at N_2 atmosphere. The solvent was then removed under

reduced pressure to give the solids, which were dissolved with ethyl acetate, washed with saturated ammonium chloride aqueous solution and saturated salt water. After drying with anhydrous sodium sulfate, the solvent was evaporated to obtain the pure product, which was purified by column chromatography using petroleum ether/ethyl acetate from 10:1 to 3:1 to afford **xanthen-1-one**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 (d, *J* = 2.3 Hz, 1H), 7.14 (d, *J* = 8.4 Hz, 1H), 6.52 (dd, *J* = 8.5, 2.4 Hz, 1H), 6.43 (dd, *J* = 2.4, 0.7 Hz, 1H), 4.97 (ddd, *J* = 10.6, 6.0, 2.3 Hz, 1H), 3.80 (s, 3H), 2.62-2.43 (m, 2H), 2.43-2.29 (m, 1H), 2.13-1.92 (m, 1H), 1.69 (qdd, *J* = 13.0, 4.6, 2.4 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 197.19, 163.15, 157.64, 131.92, 131.08, 127.28, 115.51, 108.95, 101.26, 74.91, 55.50, 38.66, 29.63, 18.07.

Synthesis of L-C. A mixture of compound **xanthene-1-one** (460 mg, 2 mmol), 2-aminoacetophenone (405 mg, 3 mmol) and trifluoroacetic acid (0.25 mL) in methylbenzene (0.5 mL) was stirred at 115 °C for 3h. After being cold down to temperature, the solvent was dropped into methyl tert-butyl ether and solids were precipitated. The solids were washed three times with ethyl acetate, the precipitation was centrifuged. Then the precipitation was dissolved with dichloromethane, washed with saturated sodium carbonate solution and saturated salt water. After drying with anhydrous sodium sulfate, the solvent was evaporated to obtain the pure product **L-C**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (d, *J* = 8.4 Hz, 1H), 7.94 (dd, *J* = 8.8, 1.4 Hz, 1H), 7.91 (s, 1H), 7.62 (ddd, *J* = 8.3, 6.8, 1.4 Hz, 1H), 7.46 (ddd, *J* = 8.2, 6.8, 1.3 Hz, 1H), 7.18 (d, *J* = 8.3 Hz, 1H), 6.52 (dd, *J* = 8.3, 2.5 Hz, 1H), 6.48 (d, *J* = 2.4 Hz, 1H), 5.19 (ddd, *J* = 11.7, 5.0, 2.3 Hz, 1H), 3.80 (s, 2H), 3.32-3.16 (m, 1H), 2.83 (ddd, *J* = 17.2, 13.5, 4.5 Hz, 1H), 2.62 (q, *J* = 4.2 Hz, 1H), 2.58 (s, 3H), 2.21 (dtd, *J* = 13.4, 12.0, 4.6 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.24, 156.01, 150.30, 146.87, 140.92, 129.71, 129.02, 128.73, 128.62, 127.42, 127.08, 125.75, 123.44, 122.49, 117.21, 107.88, 101.30, 74.78, 55.42, 28.61, 23.83, 13.88. MS (ESI): calculated for $C_{22}H_{19}NO_2$([M]$^+$): 330.1; found 330.1.

Synthesis of L-C-CH_3. A mixture of compound **L-C** (329 mg, 1 mmol), MeI (710 mg, 5 mmol) in dry DMF (3 mL) was stirred at 115 °C for 12h. After the reaction was completed, monitored by thin-layer chromatography (TLC), the mixture was dropped into methyl tert-butyl ether and solids were precipitated. and **L-C-CH_3** was purified by column chromatography using dichloromethane: methanol= 20:1 (V/V). ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.54 (dd, *J* = 8.5, 1.4 Hz, 1H), 8.48 (d, *J* = 8.9 Hz, 1H), 8.18 (ddd, *J* = 8.7, 7.0, 1.4 Hz, 1H), 7.97 (ddd, *J* = 8.1, 6.9, 0.9 Hz, 1H), 7.62 (d, *J* = 1.8 Hz, 1H), 7.47 (d, *J* = 8.5 Hz, 1H), 6.72 (dd, *J* = 8.5, 2.5 Hz, 1H), 6.68 (d, *J* = 2.4 Hz, 1H), 5.21 (d, *J* = 5.1 Hz, 1H), 4.63 (s, 3H), 3.83 (s, 3H), 3.49-3.42 (m, 2H), 2.92 (s, 3H), 2.76 (td, *J* = 14.5, 3.4 Hz, 1H), 2.47-2.41 (m, 2H), 2.02 (dddd, *J* = 14.3, 12.7, 5.3, 3.3 Hz, 1H). ^{13}C NMR (101 MHz, d_6-DMSO) δ 163.95, 156.51, 154.72, 150.47, 138.88, 137.61, 134.21, 133.01, 131.66, 129.12, 127.12, 127.01, 120.29, 120.04, 115.89, 110.01, 101.58, 73.10, 56.23, 44.11, 27.90, 23.31, 16.26. MS (ESI) m/z calculated for $C_{23}H_{22}NO_2^+$, [M$^+$]: 344.2; found 344.2.

Synthesis of L-C-CH_3-Cy5. A mixture of **L-C-CH_3** (141 mg，0.3 mmol) and 1,3,3-trimethyl-2-(formylmethylene)indoline (150 mg，0.75 mmol) in acetic anhydride (3 mL) were stirred at 115 °C for 12h. After the reaction was completed, monitored by thin-layer chromatography (TLC), the mixture was dropped into methyl tert-butyl ether and solids were precipitated. Then **L-C-**

CH_3-Cy5 was purified by column chromatography using dichloromethane: methanol = 10:1 (V/V). ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.74 (d, *J* = 14.5 Hz, 2H), 8.65 (d, *J* = 8.9 Hz, 1H), 8.20 (ddd, *J* = 8.7, 6.3, 2.1 Hz, 1H), 7.98-7.86 (m, 2H), 7.85-7.78 (m, 1H), 7.69-7.64 (m, 2H), 7.62 (d, *J* = 8.6 Hz, 1H), 7.42-7.33 (m, 3H), 7.31-7.24 (m, 3H), 6.77 (dd, *J* = 8.6, 2.4 Hz, 1H), 6.66 (d, J = 2.4 Hz, 1H), 5.34-5.27 (m, 1H), 5.17 (dd, *J* = 14.5, 8.1 Hz, 2H), 4.80 (s, 3H), 3.82 (s, 3H), 3.26 (s, 3H), 3.16 (s, 3H), 2.89 (d, *J* = 15.3 Hz, 1H), 2.70 (t, *J* = 13.6 Hz, 1H), 2.40 (d, *J* = 14.3 Hz, 1H), 2.12 (ddd, *J* = 14.0, 10.4, 4.8 Hz,1H), 1.82-1.66 (m, 12H). ^{13}C NMR (101 MHz, d_6-DMSO) δ 174.69, 174.40, 163.70, 156.17, 155.54, 152.34, 151.57, 146.74, 142.34, 141.13, 139.59, 138.04, 133.99, 131.53, 128.35, 127.19, 125.84, 125.41, 123.77, 122.32, 120.25, 119.01, 115.54, 111.51, 109.64, 101.06, 100.25, 99.85, 72.84, 55.79, 54.91, 49.39, 44.25, 31.82, 31.53, 27.33, 23.63. MS (ESI) m/z calculated for $C_{49}H_{49}N_3O_2^{2+}$, [M^{2+}]: 355.7; found 355.7.

2. General Procedure for Spectral Measurements.

Unless otherwise noted, all the spectral measurements were performed in 10 mM phosphate buffer saline (PBS, pH = 7.4) according to the following procedure. To a test tube, a certain amount of 10 mM PBS was added, followed by addition of 10 μL **L-C-CH_3-Cy5** stock solution in DMSO (0.5 mM) and appropriate volume of $ONOO^-$ solution, and the final volume was 1 mL with 1% DMSO (v/v). After incubation on shaker at 37 °C in thermostat for 5 min, the absorption and fluorescence spectra were measured.

3. Computation Detail.

The lowest singlet excited state structures of **L-C-CH_3-Cy5** and **L-C-CH_3-CHO** in implicit CPCM water solvent were first optimized by TD-DFT method at the ωB97XD/6-31G* level of accuracy using Gaussian 16 package, respectively.[2] Then for **L-C-CH_3-Cy5**, we conducted the CASSCF calculation with Ahlrichs' def2-SVP basis set and CPCM water solvent to investigate the properties of low-lying excited states.[3] The chosen active space consists of 10 electrons in 9 orbitals, including both π and π* orbitals in different conjugated fragments. The resolution of identity approximation in the form of the chain of spheres exchange (RIJCOSX), in conjunction with def2/J and def2-SVP/C auxiliary basis set, were used to speed up the CASSCF calculation.[4] All CASSCF calculations were carried out in ORCA 5 program.[5]

4. Colocalization Fluorescence Imaging of HepG2 cells.

The DMEM was added 10% fetal bovine serum, 1% penicillin, 1% streptomycin as cell culture medium, and cells were cultured in an environment at 37 °C with 5% CO_2 and 95% air. Cells

were seeded onto commercially available sterilized confocal dishes and incubated with cell culture medium for 24 h until the cells adhered to the well plate, then washed with PBS for 3 times. Mito-Tracker Green (40 nM) and **L-C-CH_3-Cy5** (5 μM) were added in serum free media to incubate for 30 min at 37 °C, and then washed with PBS for 3 times. After refilling the culture medium, the cells were applied for confocal fluorescence imaging. In the experiments, Mito-Tracker Green was excited at 488 nm, and the emissions were collected at 500-540 nm; **L-C-CH_3-Cy5** was excited at 638 nm and emissions were collected at 645-685 nm.

5. Cytotoxicity Assay.

HepG2 cells were placed in 96-well plates and incubated in an incubator for 24 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used to determine cytotoxicity. Cells in 96-well plates were added with different concentrations of **L-C-CH_3-Cy5** and incubated for 24 h. Cells were treated with 20 μL MTT solution (5 mg/mL) for 4 h. Finally, the supernatant was removed, 100 μL DMSO was added to each well, and the UV absorption at 492 nm was measured.

6. Imaging of exogenous $^-$ and endogenous $ONOO^-$ in RAW 264.7 cells.

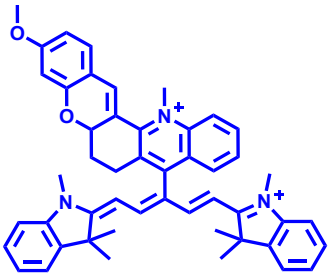
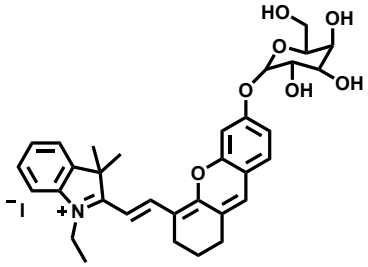
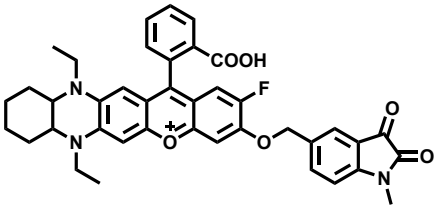
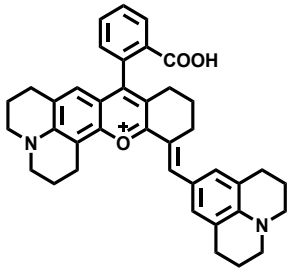
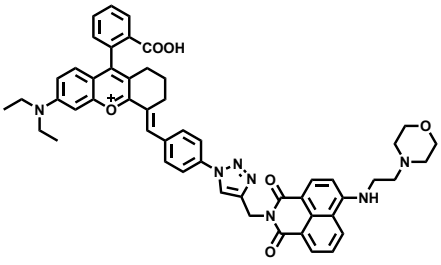
RAW264.7 cells were seeded onto commercially available sterilized confocal dishes and incubated with cell culture medium for 24 h until the cells adhered to the well plate. The culture medium was aspirated and washed 3 times with PBS. Then different reagents were added into the culture medium to trigger the upregulation of $ONOO^-$ levels in the cells. At last, the cells were incubated with **L-C-CH_3-Cy5** (10 μM) for another 30 min, and then washed with PBS for 3 times. After refilling the culture medium, the cells were applied for confocal fluorescence imaging to indicate the $ONOO^-$ levels. **L-C-CH_3-Cy5** was excited at 488 nm and emissions were collected at 550-700 nm.

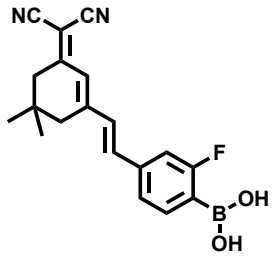
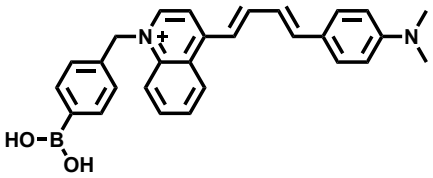
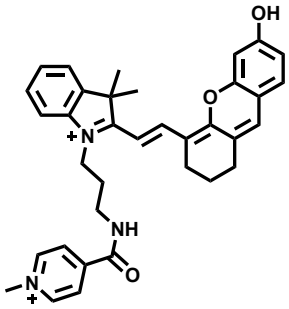
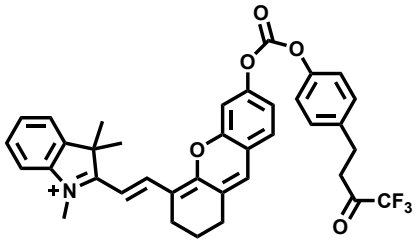
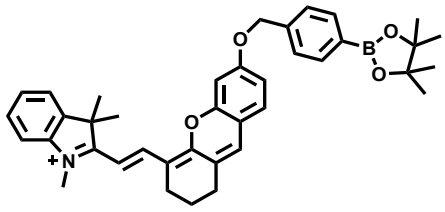
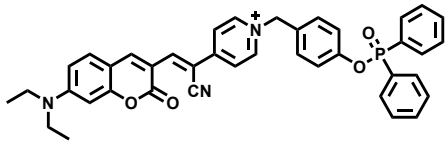
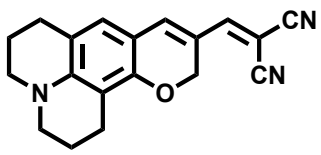
The Upregulation Procedures for Exogenous $ONOO^-$. Group I: no reagent was added (control group). Group II: cells were incubated with 100 μM of lincidomine hydrochloride (SIN-1) for 30 min. Group III: cells were incubated with 500 μM of SIN-1 for 30 min. Group IV: Cells were incubated with 100 μM SIN-1 and 1 mM N-acetylcysteine (NAC) for 30 min.

The Upregulation Procedures for Endogenous $ONOO^-$. Group I: no reagent was added (control group). Group II: cells were incubated with 6 μg/mL lipopolysaccharides (LPS) and 300 ng/mL phorbol myristate acetate (PMA) for 8 h in advance. Group III: 6 μg/mL LPS and 300 ng/mL PMA were incubated in advance for 8 h, then 1 mM NAC was incubated for 1 h.

7. Properties of $ONOO^-$ fluorescence probes.

Table S1: Properties of $ONOO^-$ fluorescence probes.

Probe	Limit of detection	Reaction time	Reference
	43 nM	< 10s	***This work***
	0.18 μM	150 s	*Microchem. J.*, 2025, **208**, 112566.
	72 nM	240 s	*Anal. Chem.*, 2020, **92**, 13305–13312.
	18 nM	about 30 min	*Sens. Actuators B Chem.,* 2025, **422**, 136686.
	153 nM	No mentioned	*Anal. Chem.*, 2021, **93**, 9064–9073.

	No mentioned	only 60 s	*Spectrochim. Acta A*, 2023, **295**, 122624.
	1.69 nM	< 5 min	*Anal. Chem.*, 2020, **92**, 14667–14675.
	216 nM	< 100 min	*Sens. Actuators B Chem.,* 2025, **426**, 137068.
	89.4 nM	< 5 min	*Chem. Sci.*, 2018, **9**, 6340–6347.
	71.7 nM	< 5 min	*Chem. Sci.*, 2018, **9**, 6340–6347.
	101.4 nM.	100 s,	*Biosens. Bioelectron.,* 2024, **254**, 116233.
	0.21 μM	< 5 min	*Sens. Actuators B Chem.*, 2021, **334**, 129603.

8. Fluorescence analysis, NMR, MS and cell experiments.

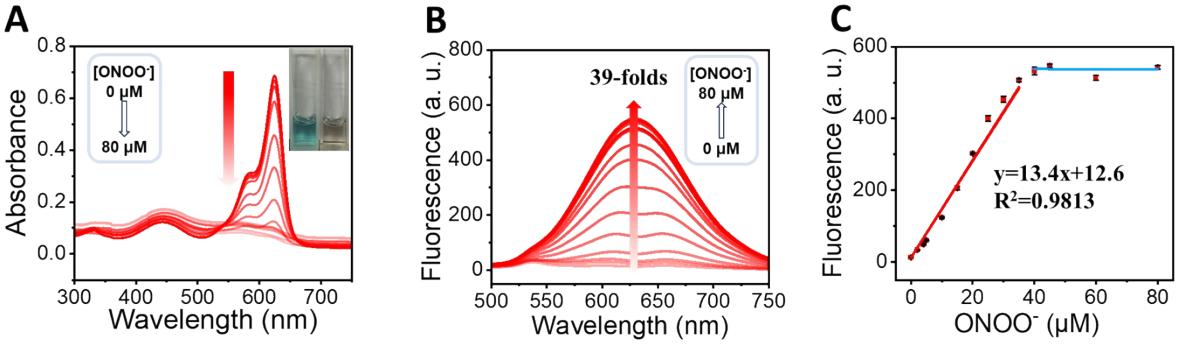


Fig. S1. The absorption **(A)** and fluorescent **(B)** spectra of **L-C-CH$_3$-Cy5** (5 μM) in the presence of different concentrations of ONOO$^-$ (0-80 μM). **(C)** Fluorescence intensity at 625 nm versus ONOO$^-$ concentration.

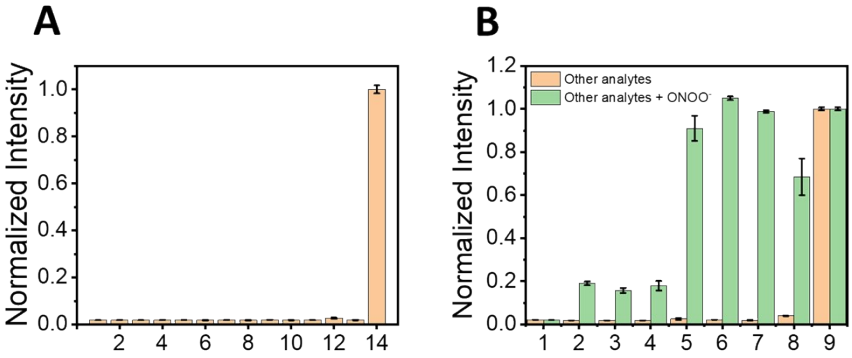


Fig. S2. (A) Fluorescence intensity of **L-C-CH$_3$-Cy5** responding to various biological species, including 1 (blank), 2-13 (500 μM: NaOAc, NaHS, $NaHCO_3$, NaCl, Na_2SO_4, Na_2SO_3, Na_2CO_3, $MgCl_2$, KNO_3, KCl, $FeCl_3$, $CaCl_2$), 14 (40 μM: ONOO$^-$). **L-C-CH$_3$-Cy5**: 5 μM, λ_{ex} = 450 nm. **(B)** Fluorescence intensity of **L-C-CH$_3$-Cy5** (5 μM) at 625 nm responding to ONOO$^-$ (40 μM) in the presence of various analytes (40 μM of each unless otherwise stated) including: (1) Blank, (2) Na_2S, (3) Cys, (4) GSH, (5) $^1O_2$, (6) ClO$^-$, (7) H_2O_2, (8) ·OH, (9) ONOO$^-$ in PBS.

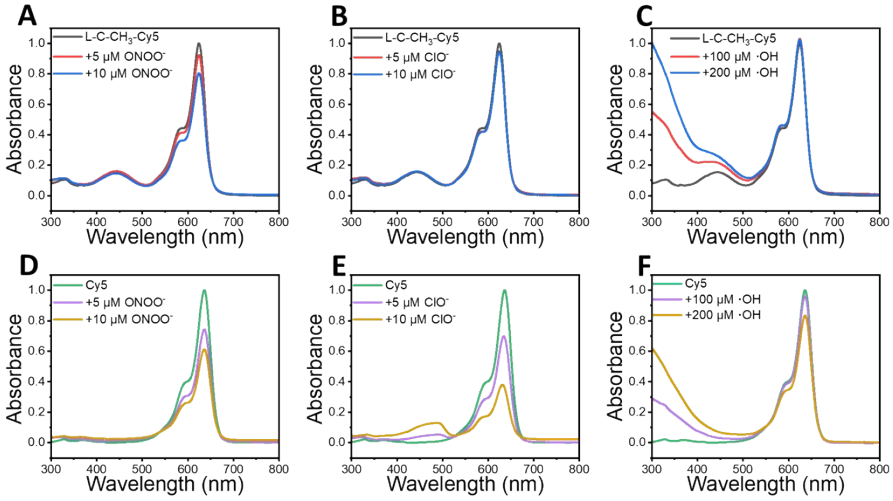


Fig. S3. The selectivity between **L-C-CH$_3$-Cy5** and **Cy5** towards different ROSs.

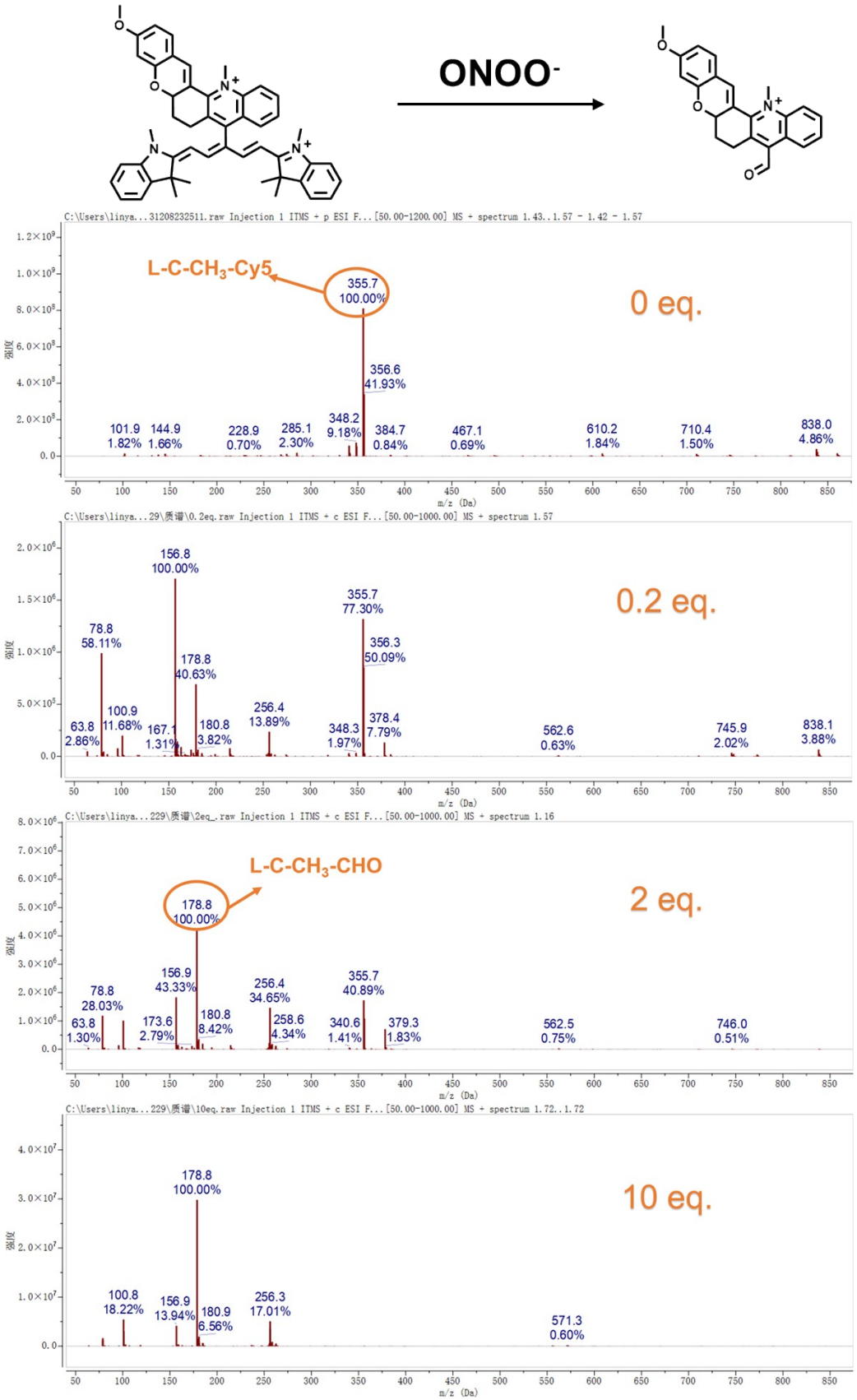


Fig. S4. The response mechanism by using the MS with the different equivalents (0, 0.2, 2, 10 eq.) of $ONOO^-$.

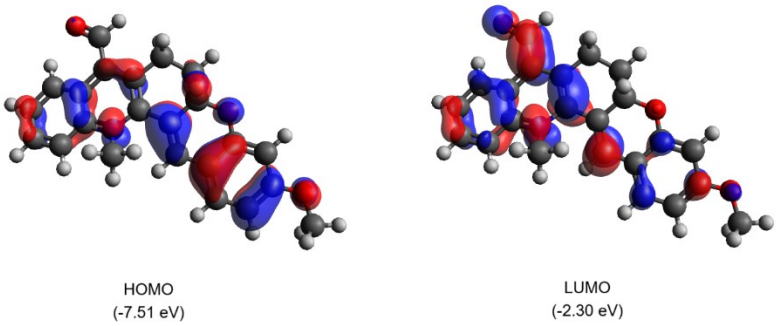


Fig. S5 HOMO-LUMO distributions of **L-C-CH_3-CHO**.

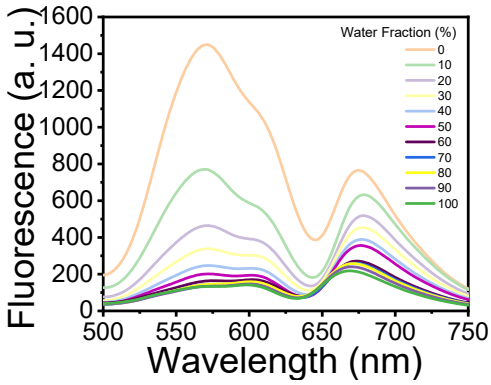


Fig. S6 Fluorescence spectra of **L-C-CH_3-Cy5** in a mixture of water-glycerol with different water fractions.

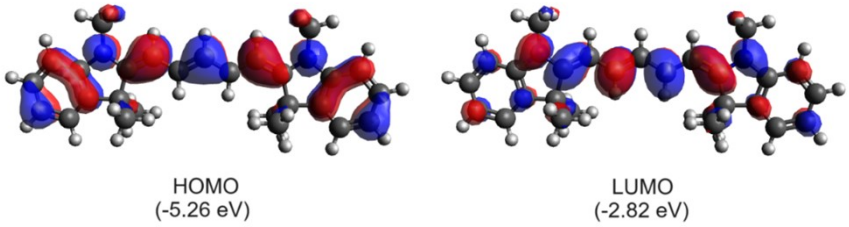


Fig. S7. HOMO-LUMO distributions of **Cy5**.

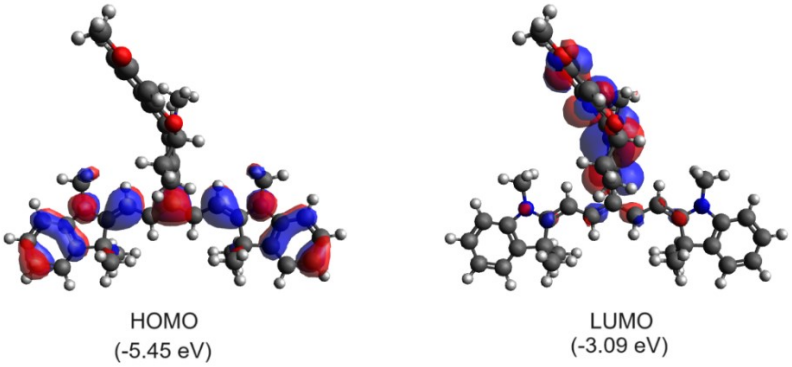


Fig. S8. HOMO-LUMO distributions of **L-C-CH_3-Cy5**.

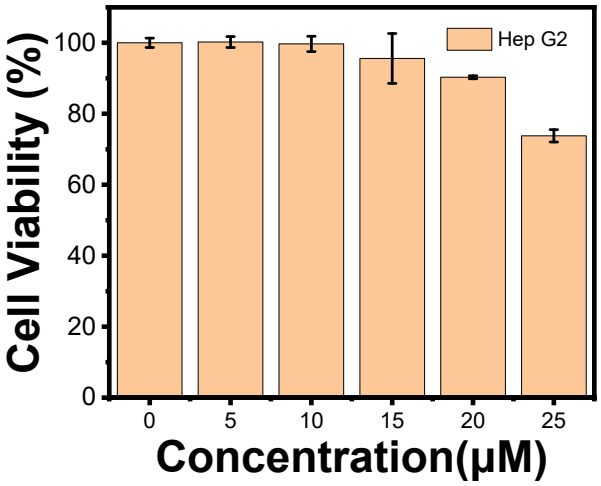


Fig. S9. The cell viability of **L-C-CH_3-Cy5** at different concentrations for HepG2 cells.

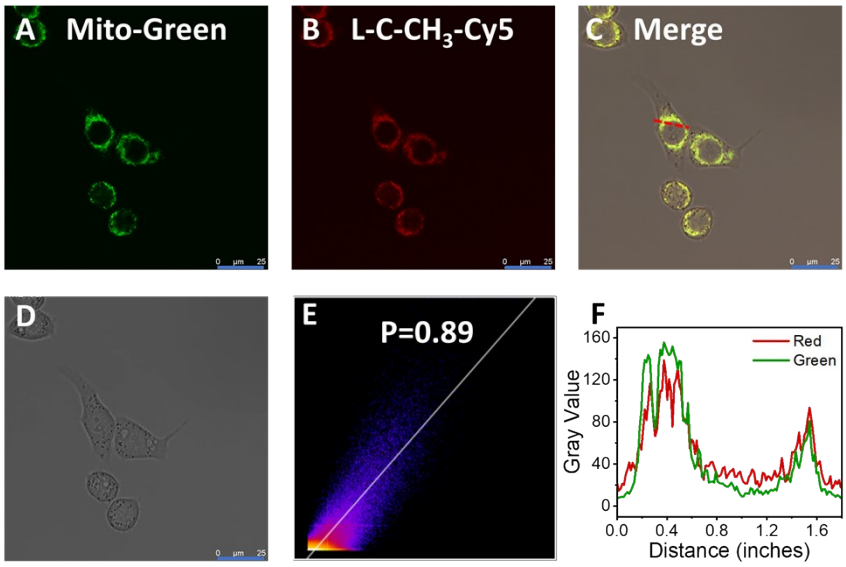


Fig. S10. Co-localization images of Mito-Tracker Green (40 nM) and **L-C-CH_3-Cy5** (5 μM) in HepG2 cells. (A) Mito-Tracker Green channel: λ_{em} = 500-540 nm, λ_{ex} = 488 nm; (B) Red channel of **L-C-CH_3-Cy5**: λ_{em} = 650-695 nm, λ_{ex} = 638 nm. (C) Merged image. (D) Corresponding DIC image. (E) Intensity correlation plot of Mito-Tracker Green and **L-C-CH_3-Cy5.**
(F) Intensity profiles of the linear region in (C). scale bar: 25 μm.

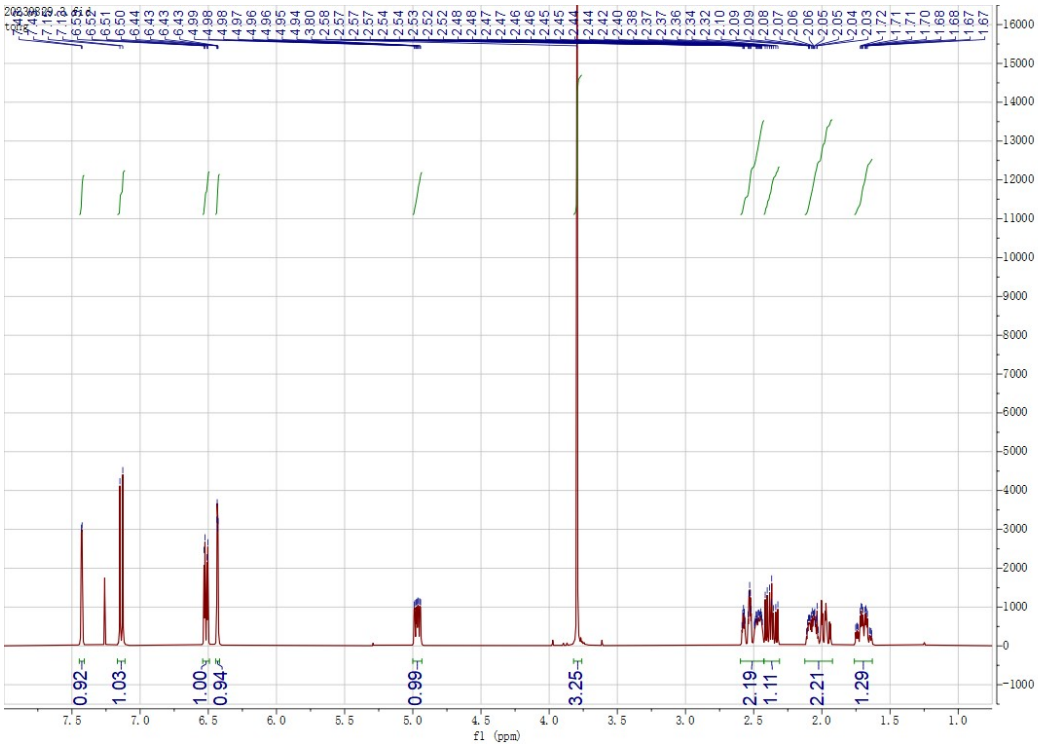


Fig. S11. ^{1}H NMR spectrum of **xanthen-1-one** in $CDCl_3$.

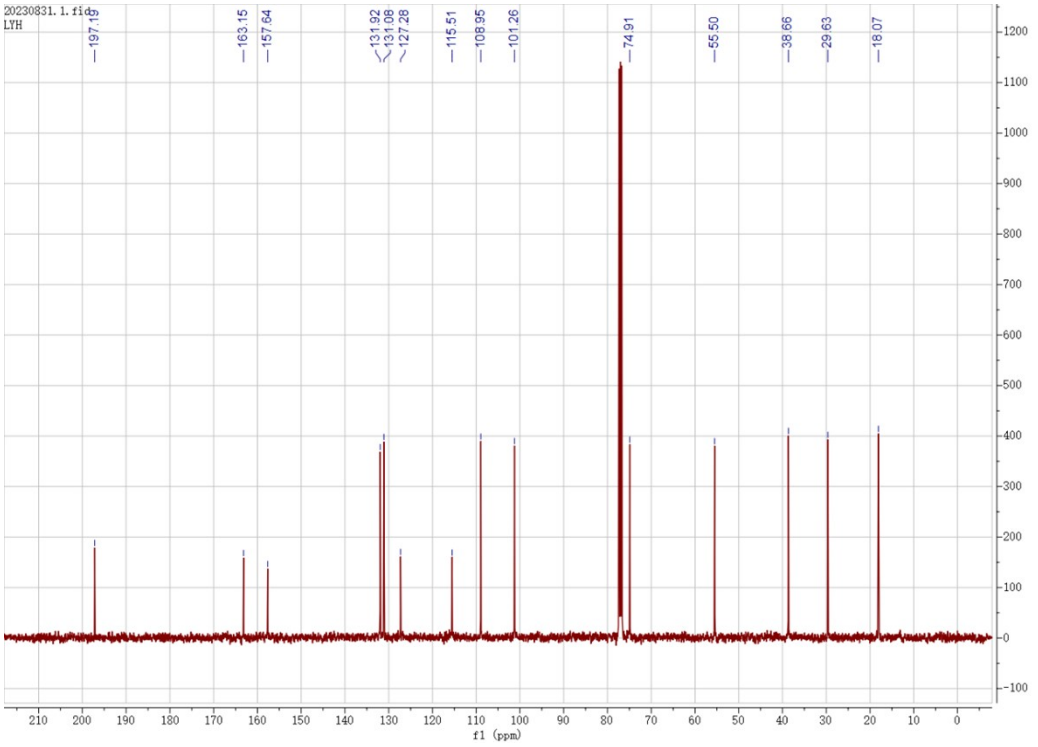


Fig. S12. ^{13}C NMR spectrum of **xanthen-1-one** in $CDCl_3$.

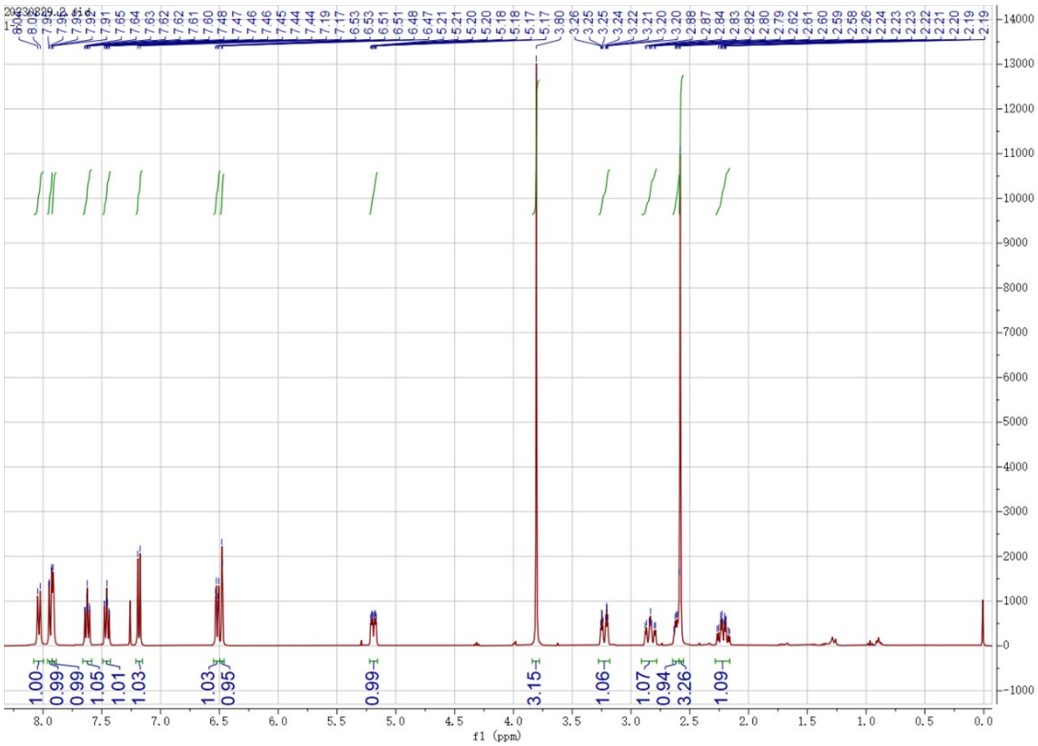


Fig. S13. ^{1}H NMR spectrum of **L-C** in $CDCl_3$.

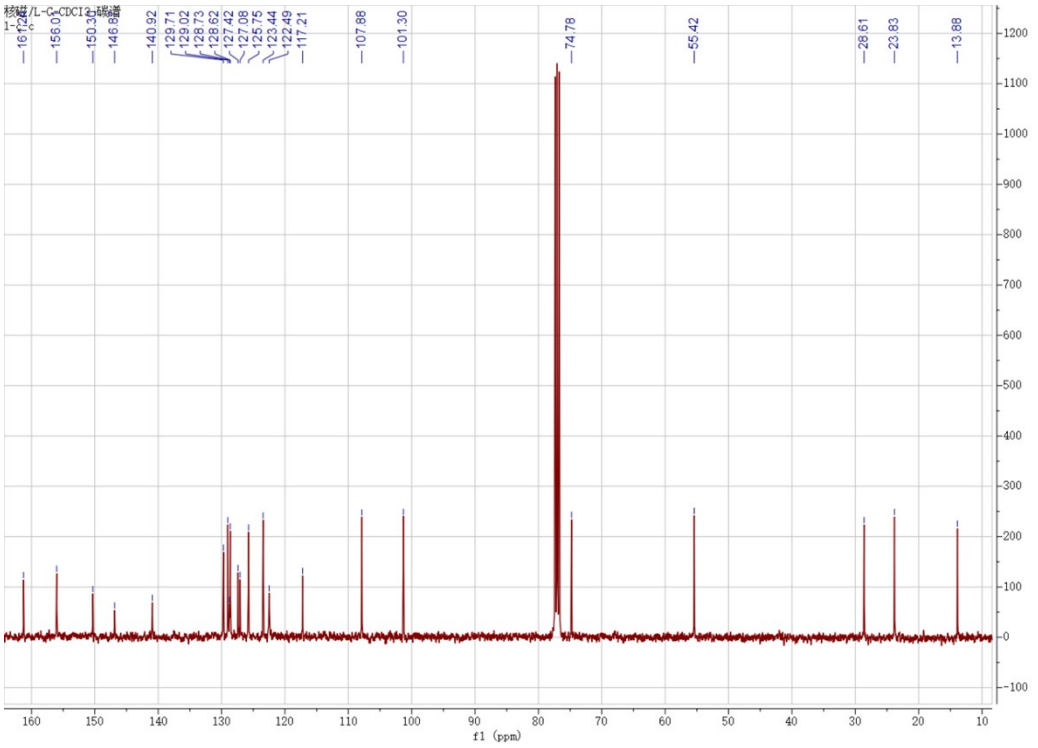


Fig. S14. ^{13}C NMR spectrum of **L-C** in $CDCl_3$.

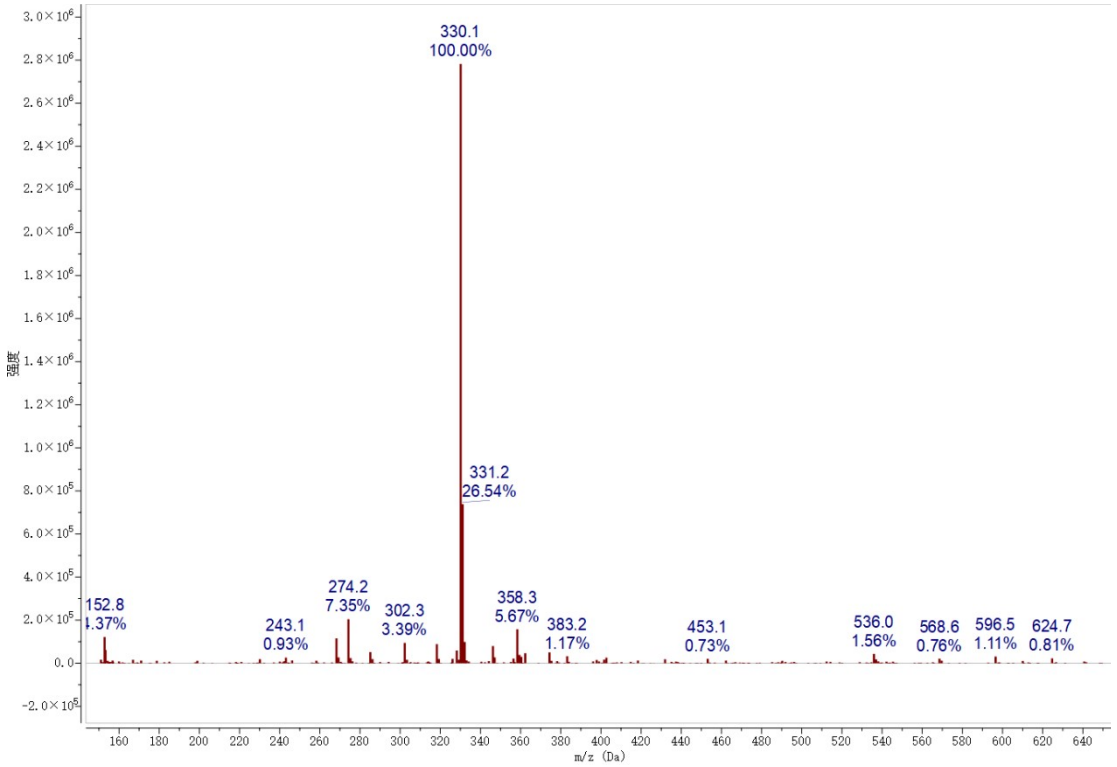


Fig. S15. The MS of **L-C.**

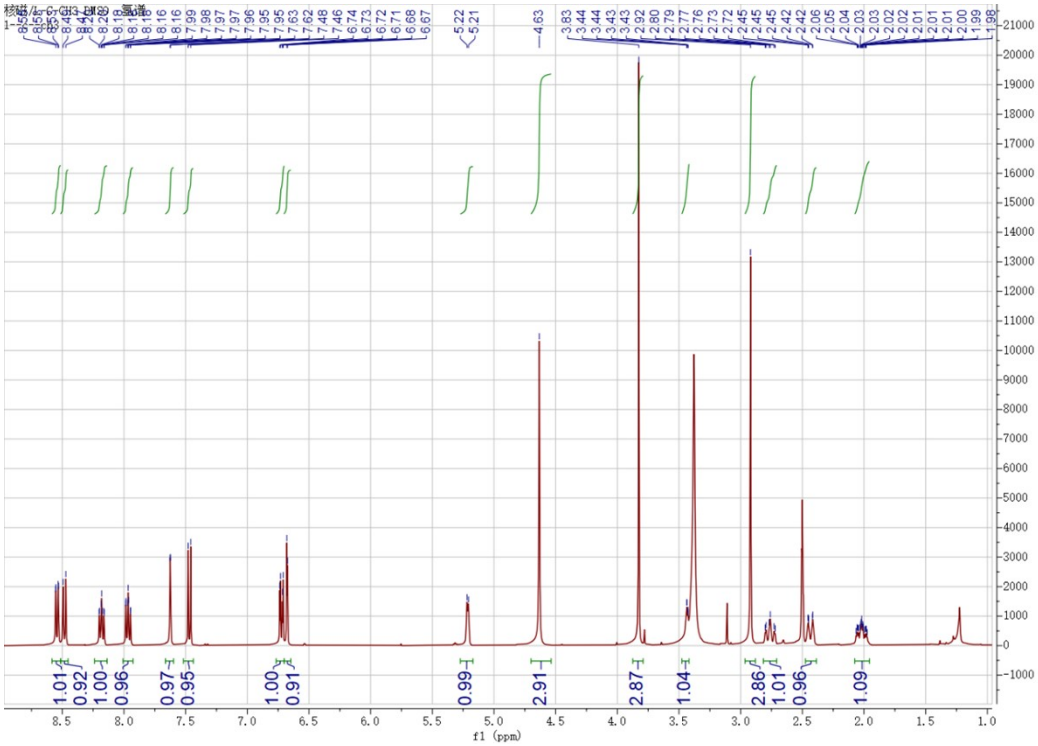


Fig. S16. ^{1}H NMR spectrum of **L-C-CH$_3$** in d_6-DMSO.

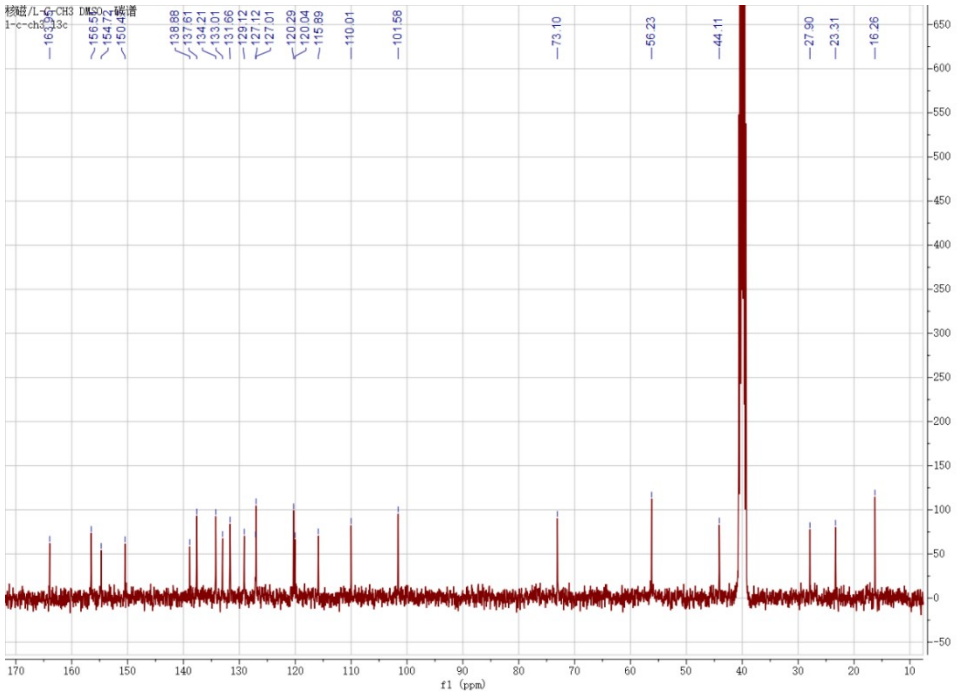


Fig. S17. ^{13}C NMR spectrum of **L-C-CH$_3$** in d_6-DMSO.

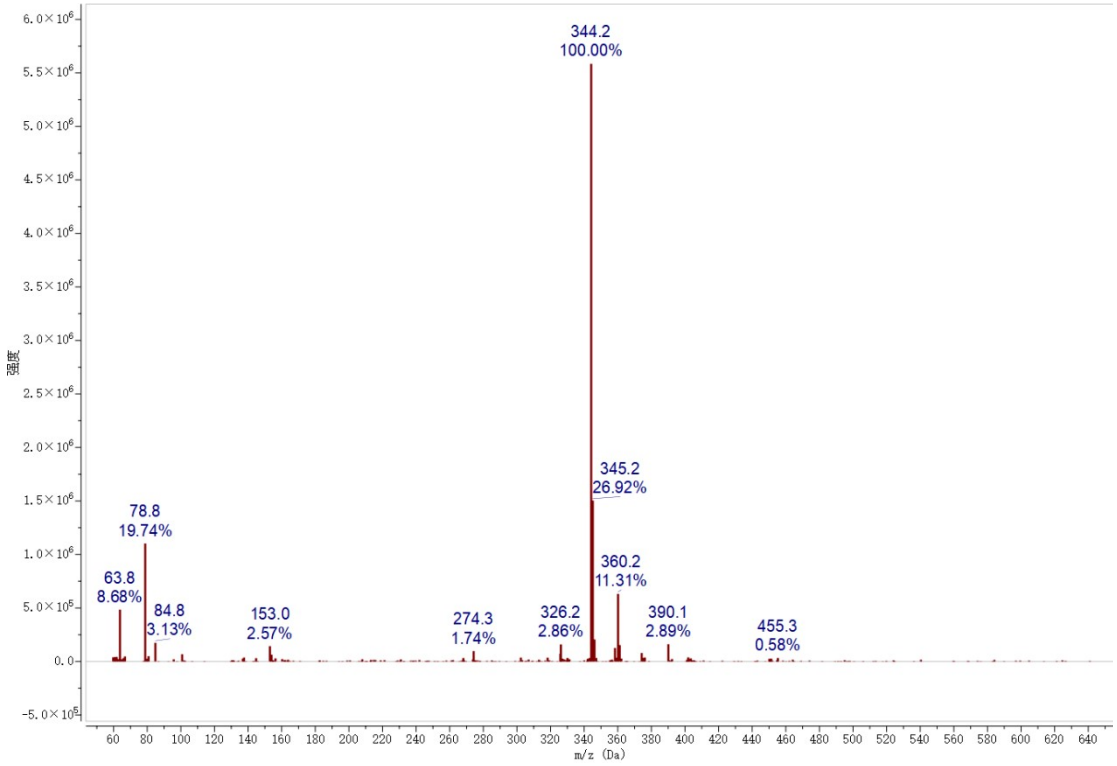


Fig. S18. The MS of **L-C-CH$_3$**.

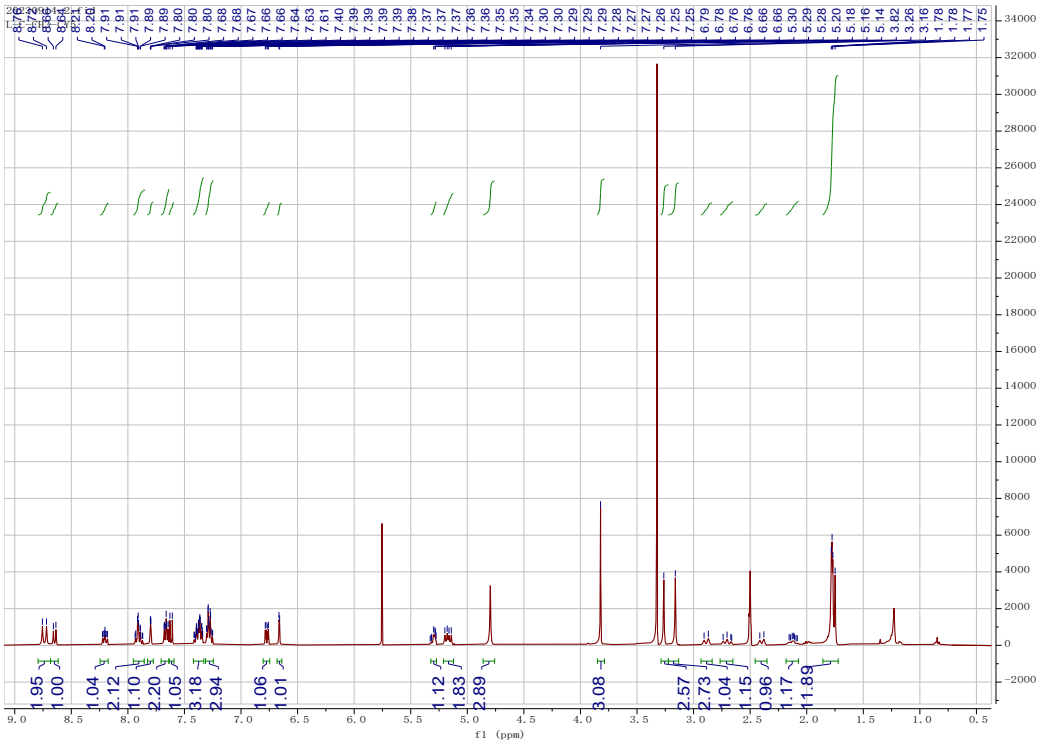


Fig. S19. ^{1}H NMR spectrum of **L-C-CH$_3$-Cy5** in d_6-DMSO.

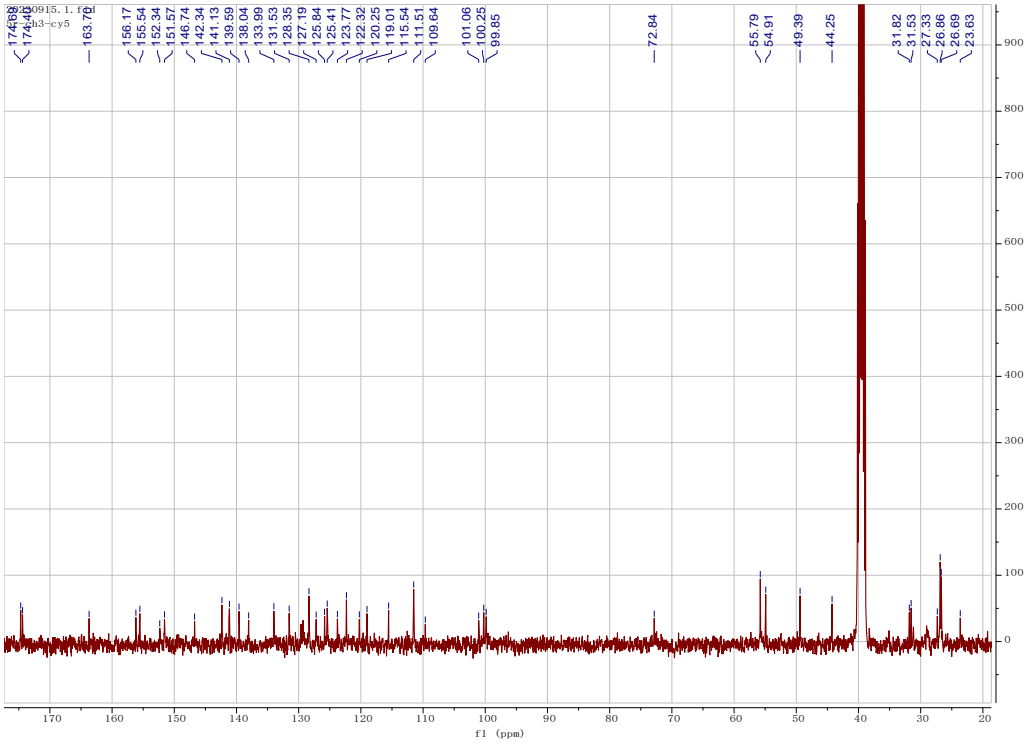


Fig. S20. ^{13}C NMR spectrum of **L-C-CH$_3$-Cy5** in d_6-DMSO.

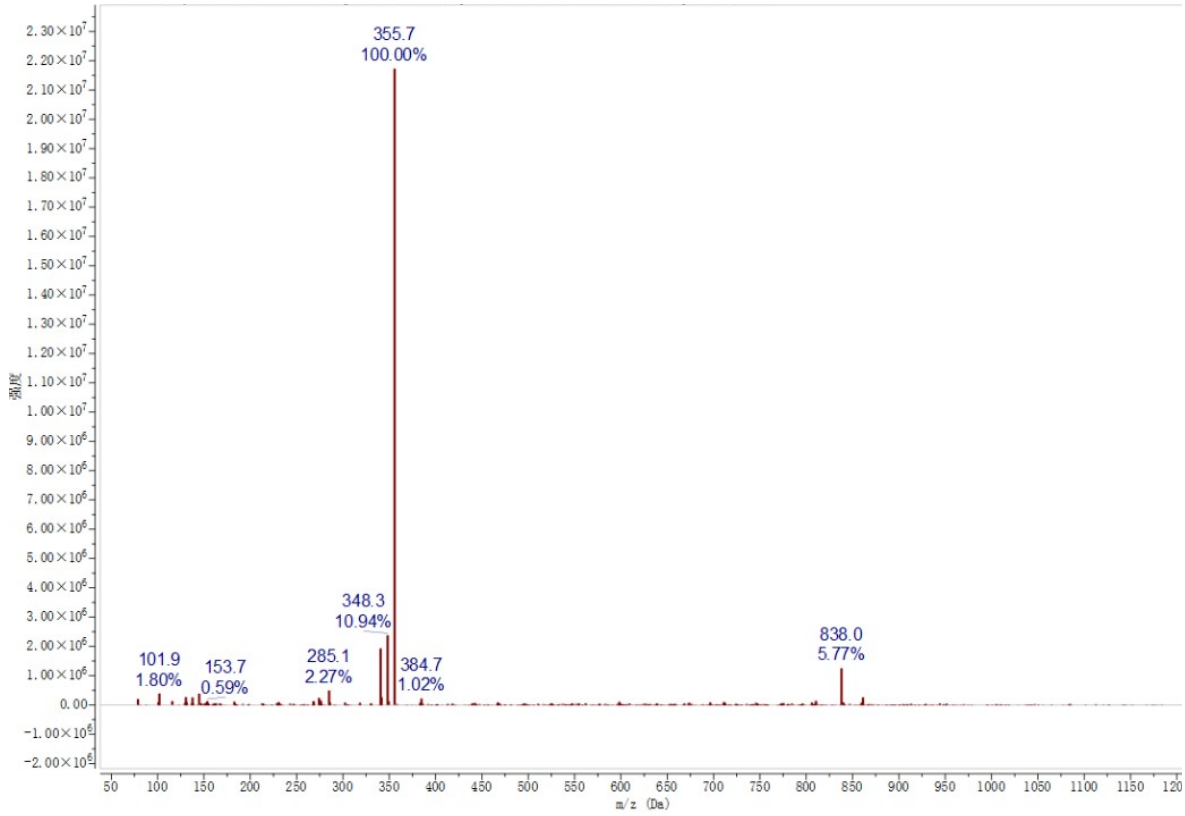


Fig. S21. The MS of **L-C-CH_3-Cy5.**

9. Reference

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