Supporting Information

Real-time Monitoring of Abnormal Mitochondrial Viscosity in Glioblastoma with A Novel Mitochondria-targeting Fluorescent Probe

Abbreviations:

Abbreviation	Full Name
GBM	Glioblastoma Multiforme
BBB	Blood Brain Barrier
FLI	Fluorescence Imaging
NMR	Nuclear Magnetic Resonance
TEM	Transmission Electron Microscope
IVIS	In Vivo Imaging System
CLSM	Confocal Laser Scanning Microscope
TICT	Twisted Internal Charge Transfer
LOD	Limits of Detection
U87MG	Human Brain Astrocyte Cell
bEnd.3	Mouse Brain Microvascular Endothelial Cell
H_2O_2	Hydrogen Peroxide
ROS	Reactive Oxygen Species

$^1O_2$	Singlet Oxygen
·OH	Hydroxyl Radical
GSH	Glutathione
Cys	L-Cysteine
Hcy	Homocysteine
Tyr	Tyrosine
Ser	Tryptophan
ASP	Aspartate
PBS	Phosphate Buffer Saline
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-
DMSO	Dimethyl Sulfoxide
DMF	N, N-Dimethylformamide
THF	Tetrahydrofolate
EtOH	Ethanol
DCM	Dichlorometha

Experimental Instruments:

Related Experiments	Instrument Name	Producer
^{1}H NMR, ^{13}C NMR	600MHz/Bruker	Germany
LC-MS	4600 UPLC/Triple TOF	America

HPLC	Agilent 1260 infinity II	Germany
UV-vis spectrums	SHIMADZU UV2550	Japan
FL spectrums	Hitachi F-7000	Japan
Flow cytometry assay	Attune NxT	America
Laser confocal assay Co-localization assay Cell Ball Experiment	Zeiss LSM 880 with Airyscan	Germany
In vivo imaging	IVIS Lumina XR Perkinelmer	America

Materials and instruments:

All chemicals used were purchased from professional suppliers and are analytical grade without further purification unless otherwise stated. The commercial mitochondrial localization probe was purchased from Beyotime (Shanghai, China). ^{1}H and ^{13}C NMR spectra were obtained by a Bruker 600MHz NMR spectrometer (Germany), while mass spectra were obtained using a 4600 HPLC-MS spectrometer (AB SCIEX, USA). The Shimadzu UV2550 (Japan) and Hitachi F-7000 (Japan) were used to determine the spectral properties of the probe. Inverted laser confocal microscopy (Zeiss LSM 880 with Airyscan, Germany) and IVIS Lumina XR PerkinElmer (USA) were used for fluorescence imaging of live cells and mice, respectively.

Synthesis method of 2-(3,5,5-trimethylcyclohex-2-en-1-ylidene) malononitrile (Compound 1):

Isophorone (4.15 g, 30 mmol) was dissolved in ethanol (10 mL), then malononitrile (1.98 g, 30 mmol) and piperidine (0.2 mL) were added to the mixture and stirred at 85℃ for 4h. After the reaction, the product was poured into ice water, filtered by vacuum, and the crude product was recrystallized with petroleum ether to obtain a purple-red solid. Yield: 5.085 g (91%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 6.56 (s, 1H), 2.54 (s, 2H), 2.23 (s, 2H), 2.05 (s, 3H), 0.94 (s, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 171.94, 163.02, 119.87, 113.95, 113.14, 76.47, 45.28, 42.43, 32.43, 27.75, 25.44. ESI-MS calcd for $C_{12}H_{14}N_2$: $[M+H]^+$ 187.12, found 187.1230.

Synthesis method of (E)-2-(3-(4-aminostyryl)-5,5-dimethylcyclohex-2-en-1-ylidene) malononitrile (Compound 2):

Dicyanoisophorone (1.862 g, 10 mmol) and 4-aminobenzaldehyde (1.211 g, 10 mmol) were dissolved in ethanol (10 mL). And then 1 mL of piperidine was added to the mixture and stirred at 80℃ for 5h. After the reaction, the mixture was dried under vacuum and purified by column chromatography (Petroleum ether: ethyl acetate = 10:1, *v*/*v*) to obtain purplish black solid. Yield: 1.823 g (63%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.41 (s, 2H), 7.17 (s, 1H), 7.07 (s, 1H), 6.71 (s, 1H), 6.57 (s, 2H), 5.87 (s, 2H), 2.58 (s, 2H), 2.51 (s, 2H), 1.01 (s, 6H). ^{13}C NMR (151 MHz, DMSO-

d$_6$) ^{13}C NMR (151 MHz, DMSO-*d*$_6$) δ 170.40, 158.09, 151.86, 140.34, 130.66, 123.94, 123.85, 120.42, 114.24, 73.11, 42.79, 38.69, 32.13, 27.94. ESI-MS calcd for $C_{19}H_{19}N_3$: $[M+H]^+$ 290.16, found 290.1647.

Synthesis method of (5-iodopentyl)triphenylphosphonium (Compound 3):

Triphenyl phosphine (262 mg, 1 mmol) and 1,5-diiodopentane (646 mg, 2 mmol) were dissolved in toluene (5 mL). The reaction was stirred under the protection of nitrogen at 110 ℃ for 12 h. After the reaction, the powder was filtered and washed with ethyl acetate, and dried to get a yellow oily substance. Yield: 330 mg (72%). ^{1}H NMR (600 MHz, DMSO-*d*$_6$) δ 7.91 (s, 3H), 7.86 – 7.76 (m, 12H), 3.65 (s, 2H), 3.23 (s, 2H), 1.79 (s, 2H), 1.57 (s, 4H). ^{13}C NMR (151 MHz, DMSO-*d*$_6$) δ 135.40, 135.38, 134.14, 134.08, 119.26, 118.69, 32.36, 31.30, 31.19, 21.27, 21.24, 20.83, 20.50, 8.99. ESI-MS calcd for $C_{23}H_{25}IP^+$: $[M+H]^+$ 459.07, found 459.0724.

Determination of fluorescence quantum yield

The fluorescence quantum yield (Φ*u*) is obtained by measuring the absorbance and fluorescence intensity of **ZVGQ** and Rhodamine B in ethanol solution (Φs=0.69, λ_{ex}=365 nm, 5 μM) respectively, and then calculating by the formula as follows：

$$\Phi u = [(AsFun_u^2)/(AuFsn_s^2)]\ \Phi s.$$

As and Au represent the absorbance of the reference substance and the substance to be measured at excitation wavelength, the absorbance should be less than 0.05. Fs and Fu are the fluorescence intensity of the reference substance and the substance to be measured, n_s and n_u are the refractive index of the solvent, respectively. And Φs is the quantum yield of the reference substance.

2.5. Cell culture and cytotoxicity assay

Cell culture: U87MG and bEnd.3 cells were cultured in Dulbecco′s modified Eagle medium (DMEM, P AN Biotech, Germany) supplemented with 10% (v/v) fetal bovine serum (FBS, Life Technologies, USA) and 1% antibiotics (penicillin-streptomycin). The cells were incubated at 37°C with 5% CO2.

Cytotoxicity assay: The cytotoxicity of the probe **ZVGQ** on U87MG and bEnd.3 cells was evaluated by using MTT assay. After incubation of cells in 96-well plates for 24 h, the 10% DMEM culture medium was removed, and 100 μL of 2% DMEM culture medium containing different concentrations of **ZVGQ** (0, 1, 2.5, 5, 10, 20 μM) was introduced for another 12 and 24 h. Then, weighed an appropriate amount of thiazolyl blue (MTT: 5 mg/mL) solid in a dark environment, and added PBS to dissolve completely, shaked and mixed, and the solution was transferred to a microporous filter through a sterile syringe for filtration. Finally, the 96-

well plate was taken out of the incubator, and 10 μL of filtered MTT solution was directly added to each well in a dark environment, wrapped with tin foil, and placed in the incubator for 4 h. After 4 h, the drug solution in the well plate was removed with a syringe, and then 150 μL of DMSO solution was added, and the absorbance value was detected using a microplate reader.

2.6. Flow cytometry assay

U87MG cells were evenly seeded into 6-well plates (1×10^5 cells per well) and incubated over night at 37 °C. Concurrently, U87MG cells were incubated with **ZVGQ** (5 μM) for 0, 30, 60, 90, 120 and 180 min and pretreated with different concentrations of **ZVGQ** (1, 5, 10, 15, 20, 30 μM), where the fluorescence intensity was continuously monitored during this period respectively. After washing the cells three times with PBS, the fluorescence intensity of the cells was collected on Attune NxT 4 flow cytometer (Thermo Fisher Scientific). We then analyzed the data on Flowjo V10 to determine the mean value for the corresponding channels, with λ_{ex}=425 nm and λ_{em}=548 nm.

2.7. Cell imaging

Imaging of viscosity in U87MG cells with stimulated by nystatin and rotenone. U87MG cells were pretreated with nystatin (20 μM, 30 min) and

rotetone (20 μM, 30 min) at 37°C. After washing the cells with PBS, they were co-incubated with **ZVGQ** (5 μM) for 60 min, with imaging performed every 10 min. Fluorescence signals were collected at 600-700 nm, λ_{ex}=522 nm. The fluorescence intensity of the cells was measured with Zeiss LSM 880 and airscan and evaluated on Zen (Zeiss, Blue version) through the computation of the mean total field of vision of the relevant channels.

Colocalization experiments. For the co-location experiment, LD tracker, Mito tracker and Lyso tracker were utilized as commercial probes with specific $\lambda_{ex}/\lambda_{em}$ wavelengths (LD tracker: 504/511 nm, Mito tracker: 579/599 nm, Lyso-tracker: 504/511 nm). U87MG cells were incubated at 37 ℃ for 30 min. After PBS washing, U87MG cells were treated with **ZVGQ** (5 μM) for 2 h, fluorescence signal was collected at 600-700 nm, λ_{ex}=522 nm. The fluorescence intensity of cells was obtained by Zeiss LSM 880 with Airyscan and analyzed on Zen (ZEISS, Blue version) by calculating the mean of the total fields of view in the corresponding channels.

2.8. 3D Cell sphere penetration assay

Establishment of 3D tumor sphere model in vitro. An appropriate amount of low melting point agarose was weighed, then DMEM culture solution was added, dissolved in a 90°C water bath for 1 h, and DMEM culture solution was supplemented to ensure that the agarose concentration

was 2% (w/v). After sterilization, 30 μL per well was added to a 96-well plate and refrigerated for later use. U87MG cells in the logarithmic growth phase were seeded into agarose-coated 96-well plates (5000 cells/well) and then cultured at 37°C in a 5% CO2 incubator after gentle shaking. The culture medium was supplemented every 2 days and used after 7 days of routine culture.

Tumor sphere penetration assay. The U87MG tumor spheres were observed under a microscope 7 days after inoculation, and the following experiments were performed on tumor spheres with uniform shape and size, round and dense. **ZVGQ** (5 μM) was added to each well, and then cultured at 37 ℃ in a 5% CO2 incubator for 2 h. After incubation, the culture solution was aspirated and discarded. The tumor spheres were rinsed 2-3 times with pre-cooled PBS and placed in a petri dish, and then tomography was scanned and photographed by a confocal laser microscope.

2.9. Establishing and fluorescence imaging of tumor-bearing mouse model

The 6-week-old BALB/c nude mice were purchased from the Hangzhou Ziyuan Laboratory Animal Technology Co. Ltd. (Hangzhou, China). All animal operations complied with the protocols approved by the Institute of Animal Care and Use Committee. This study has received approval from

the Institutional Animal Care and Use Committee of Nanjing University.

Before establishing the model, U87MG-Luc cells were trypsinized, resuspended in 10% cell culture medium and the concentration of viable cells was controlled to 1×10^8 cells/mL. BALB/c nu mice (6 weeks) were anesthetized by intraperitoneal injection of chloral hydrate (4%, 0.01 mL/g), and U87MG-Luc cells (5 μL) were inoculated into the right striatal region of the brain (1 mm anterior skull, 2 mm lateral, 3 mm depth and 5 min needle dwell time). After surgery, the scalp was sutured, and the penicillin sodium (80 U/mL, 0.1 mL) was injected intramuscularly to prevent infection. 7 days after tumor implantation, the tumor formation was observed by *in vivo* imaging system (IVIS lumina XR, USA).

The 6-week-old BALB/c nude mice were randomly assigned to one of three groups: a normal saline group, a 1.5 mg/kg **ZVGQ** group and a 3 mg/kg **ZVGQ** group. Fluorescence signals were captured from the mice at 30, 60, 90, 120, 150 and 180 mins using the IVIS Spectrum imaging system and were collected between 600-700 nm with excitation at 522 nm. Fluorescence intensity of glioma tissue in vivo was obtained using the IVIS Lumina XR in vivo imaging system from PerkinElmer and analyzed using the Living Image software by calculating the average irradiance (p/sec/cm^2/sr/(μW/cm^2)).

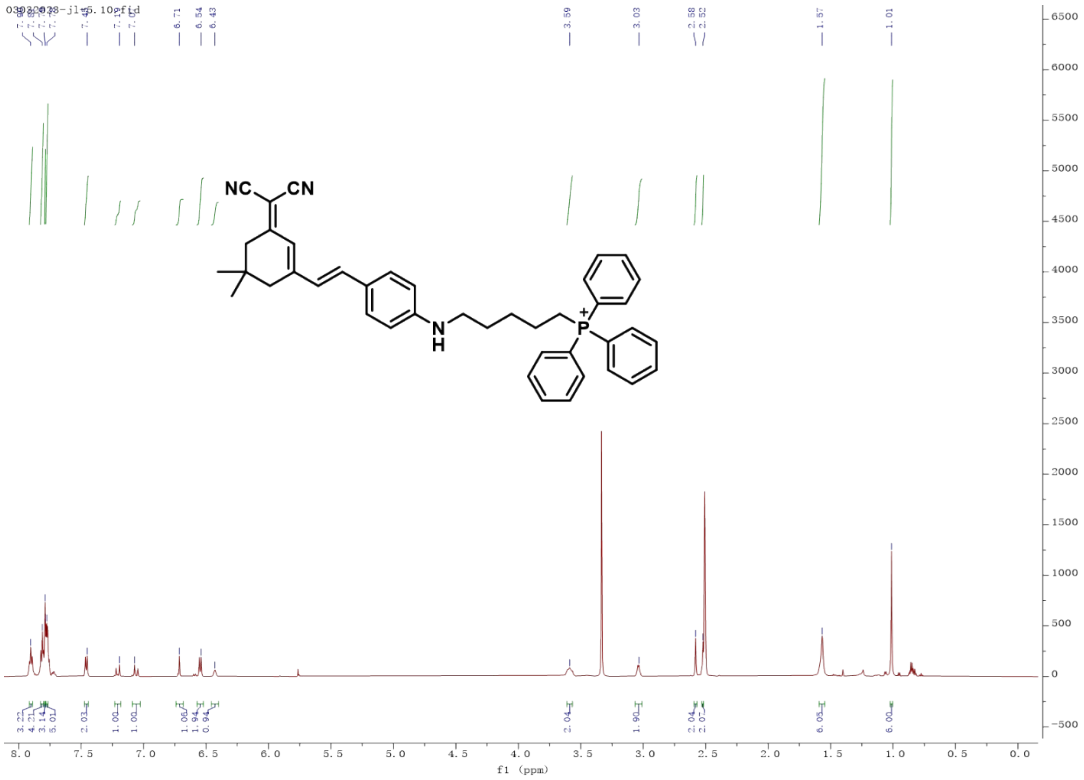


Fig. S1. ^{1}H NMR of **ZVGQ** (600 MHz, DMSO-d_6)

^{1}H NMR (600 MHz, DMSO-d_6) δ 7.90 (s, 3H), 7.81 (s, 4H), 7.79 (s, 3H), 7.78 (s, 5H), 7.45 (s, 2H), 7.19 (s, 1H), 7.07 (s, 1H), 6.71 (s, 1H), 6.54 (s, 2H), 6.43 (s, 1H), 3.59 (s, 2H), 3.03 (s, 2H), 2.58 (s, 2H), 2.52 (s, 2H), 1.57 (s, 6H), 1.01 (s, 6H).

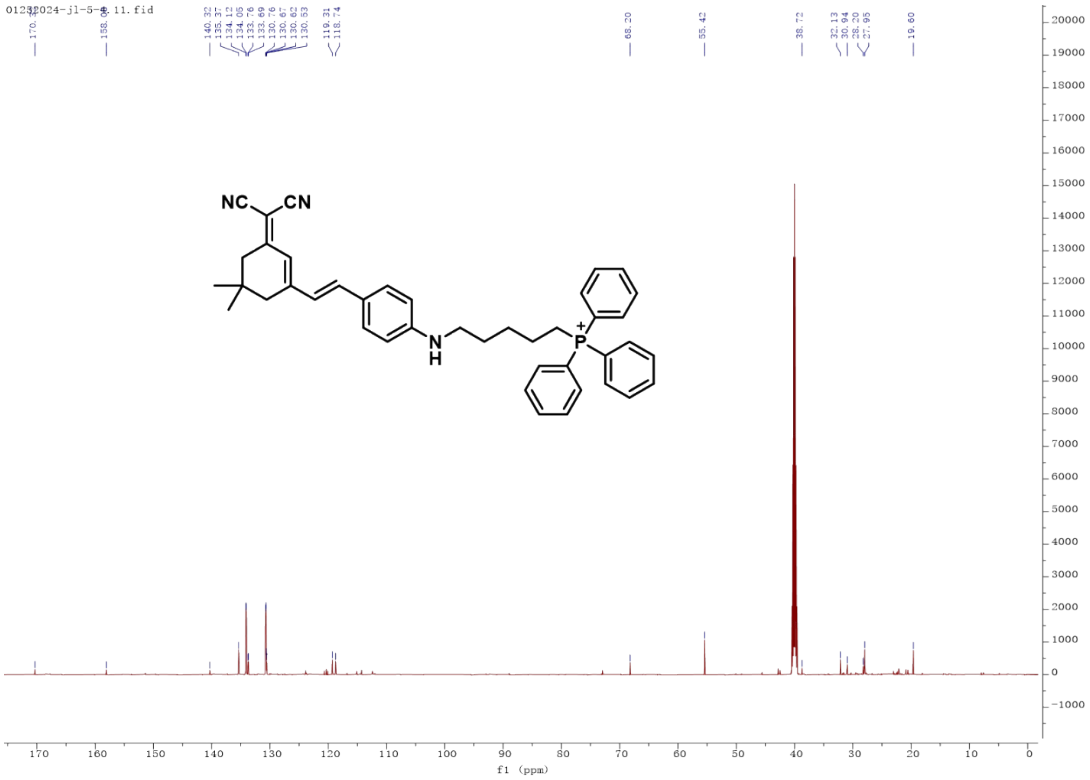


Fig. S2. ^{13}C NMR of **ZVGQ** (151 MHz, DMSO-*d*6)

^{13}C NMR (151 MHz, DMSO-*d*6) δ 170.32, 158.08, 140.32, 135.37, 134.12, 134.05, 133.76, 133.69, 130.76, 130.67, 130.62, 130.53, 119.31, 118.74, 68.20, 55.42, 38.72, 32.13, 30.94, 28.20, 27.95, 19.60.

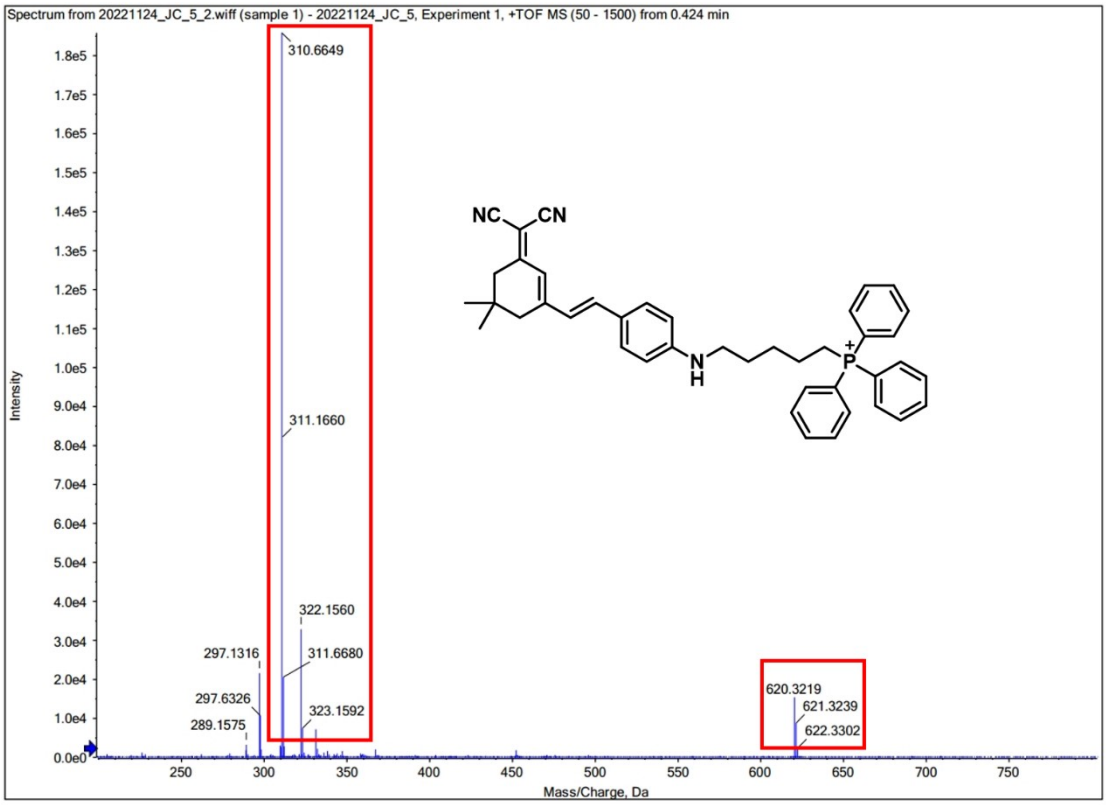


Fig. S3. The ESI-MS spectrum of **ZVGQ** (calculated for $C_{42}H_{43}N_3P^+$ $[M+H]^+$ 620.32, found 620.3219).

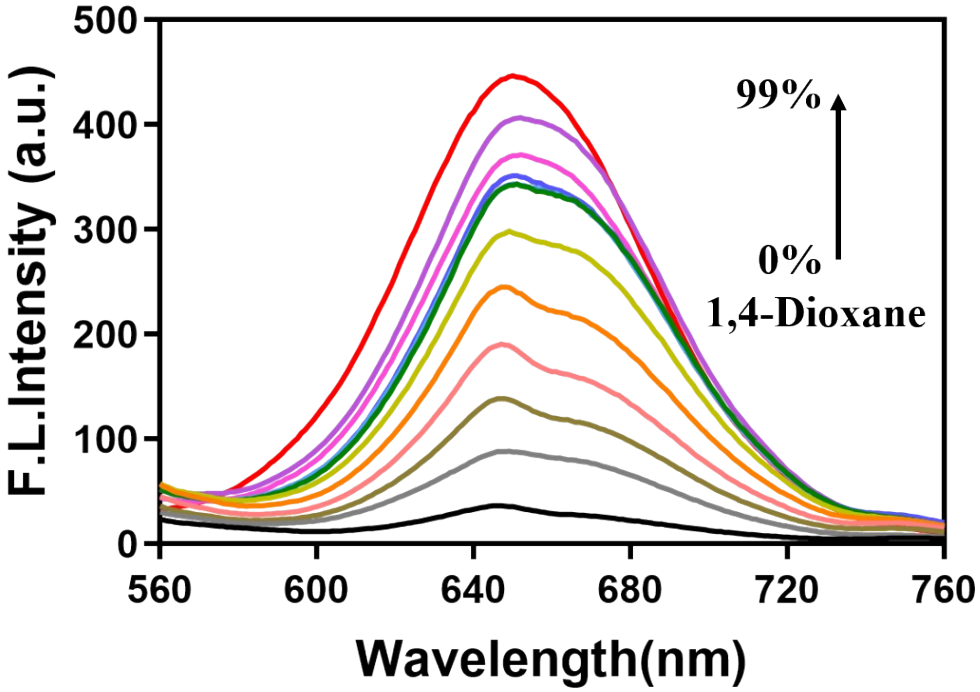


Fig. S4. Fluorescence intensity of **ZVGQ** in different 1,4-dioxane/water mixtures.

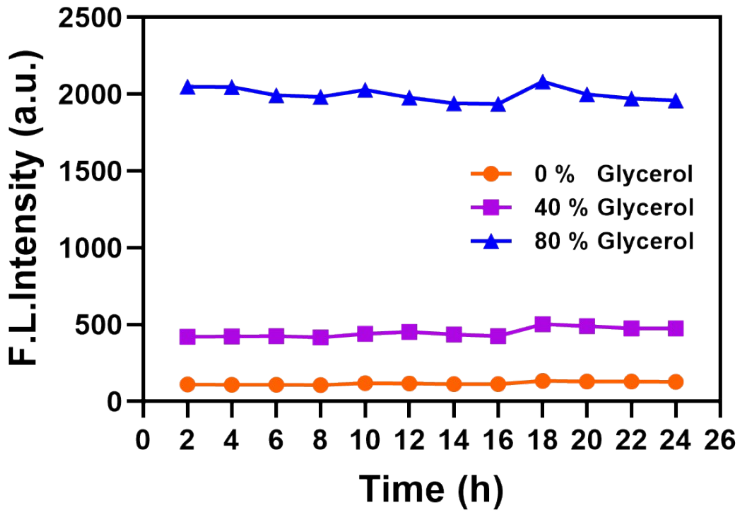


Fig. S5. Photostability experiment of **ZVGQ** *in vitro*.

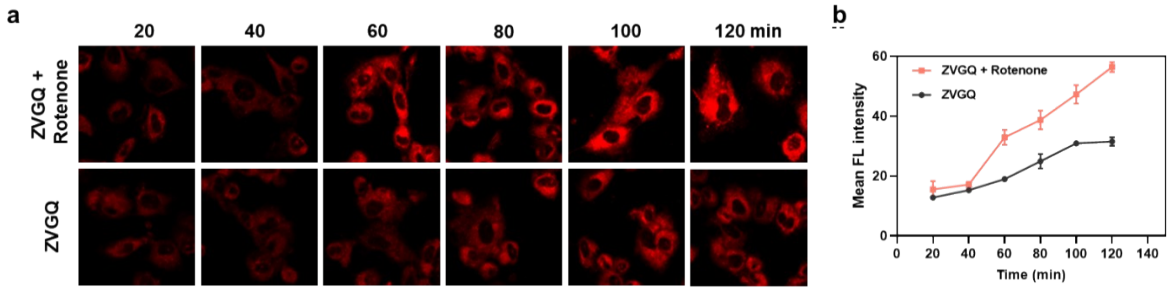


Fig. S6. Confocal Imaging of ZVGQ in Living U87MG Cells. (a) CLSM images of U87MG cells after treatment with/without rotenone stained with **ZVGQ** (10 μM). (b) Fluorescence intensity at different time periods in (a).

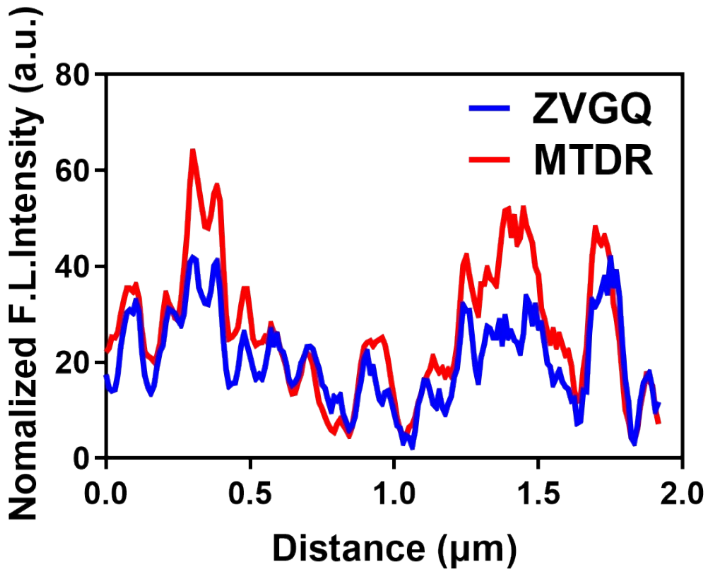
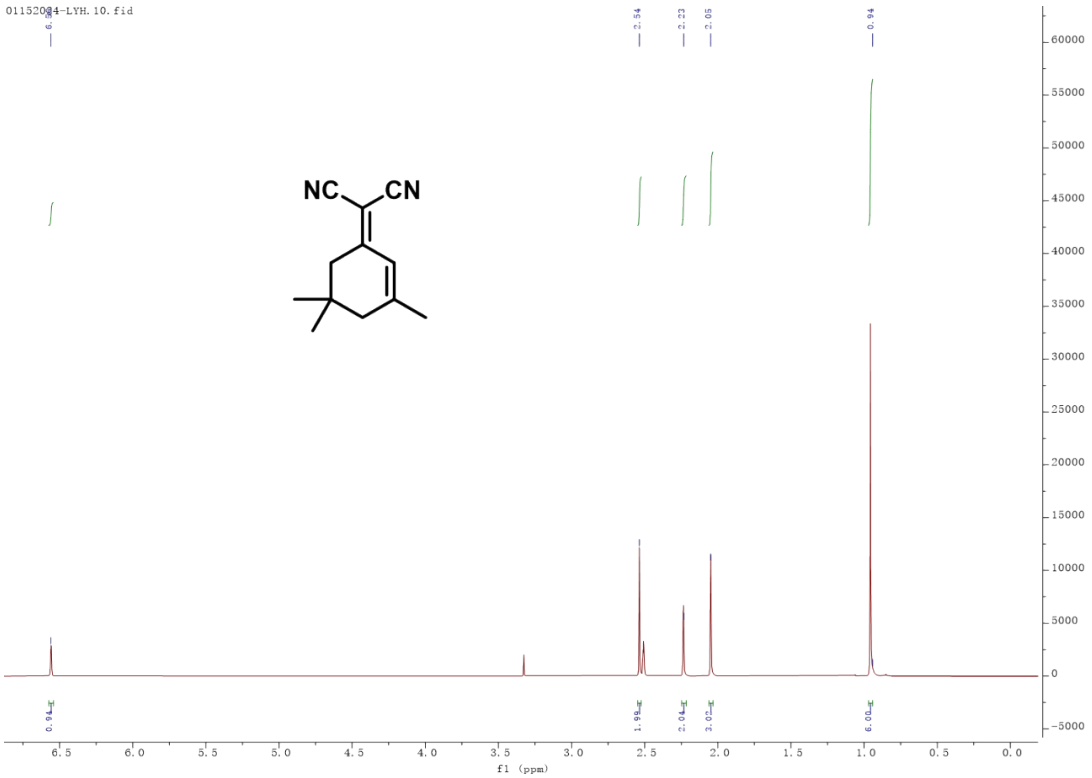
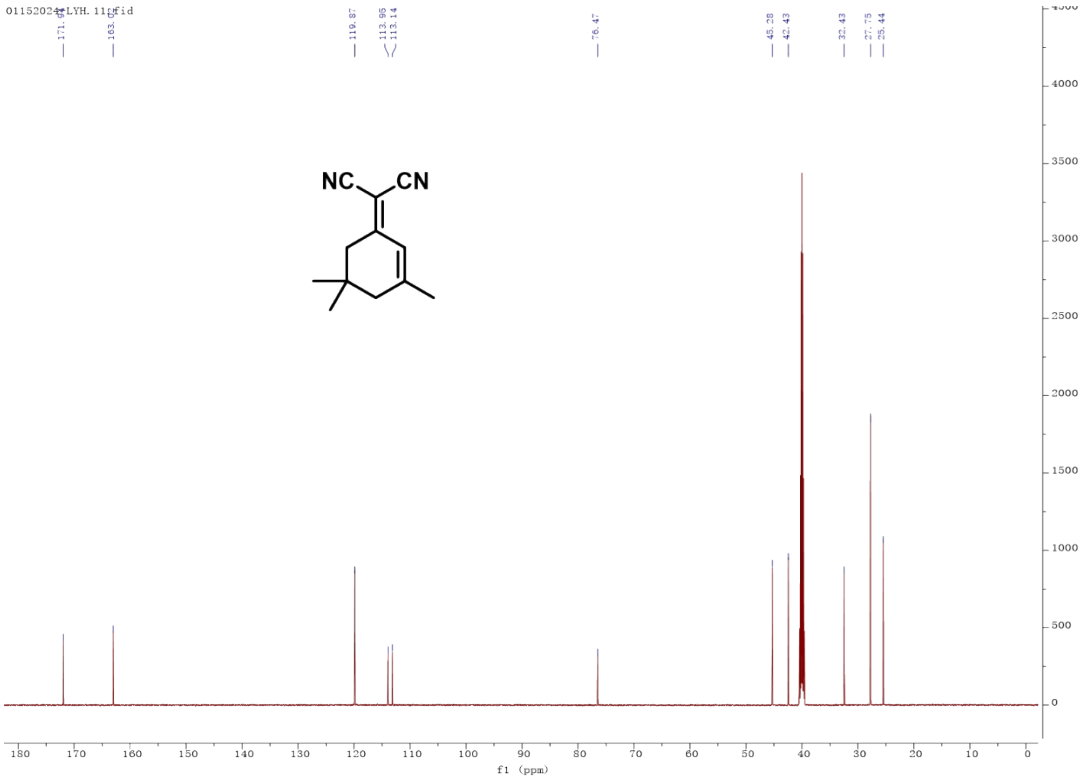


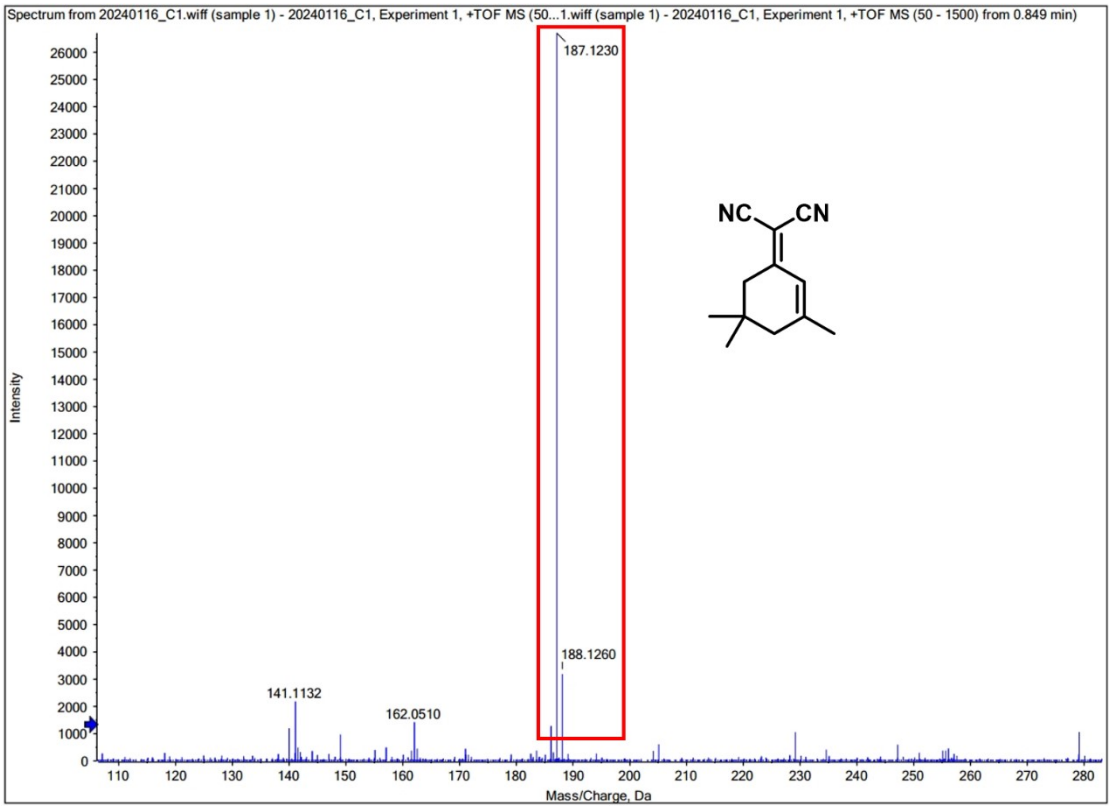
Fig. S7. The fitting curve of FL intensity in the underlined part in **Fig. 5a**



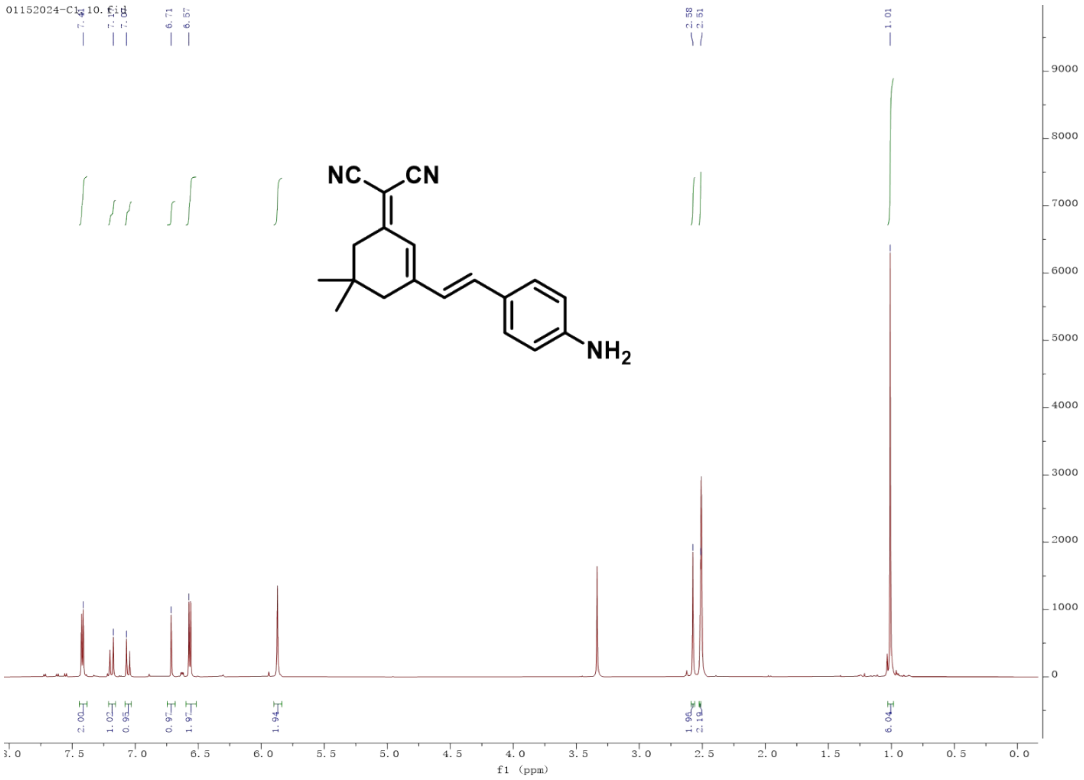
^{1}H NMR of Compound 1 (600 MHz, DMSO-d_6)



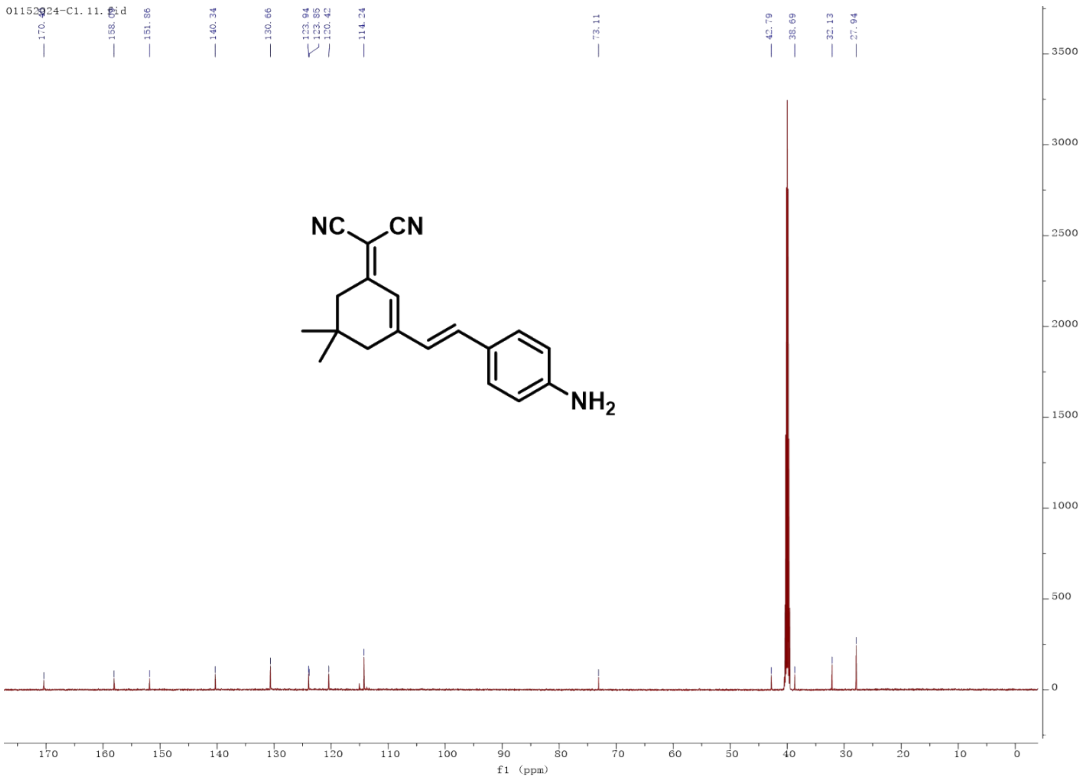
^{13}C NMR of Compound 1 (151 MHz, DMSO-d_6)



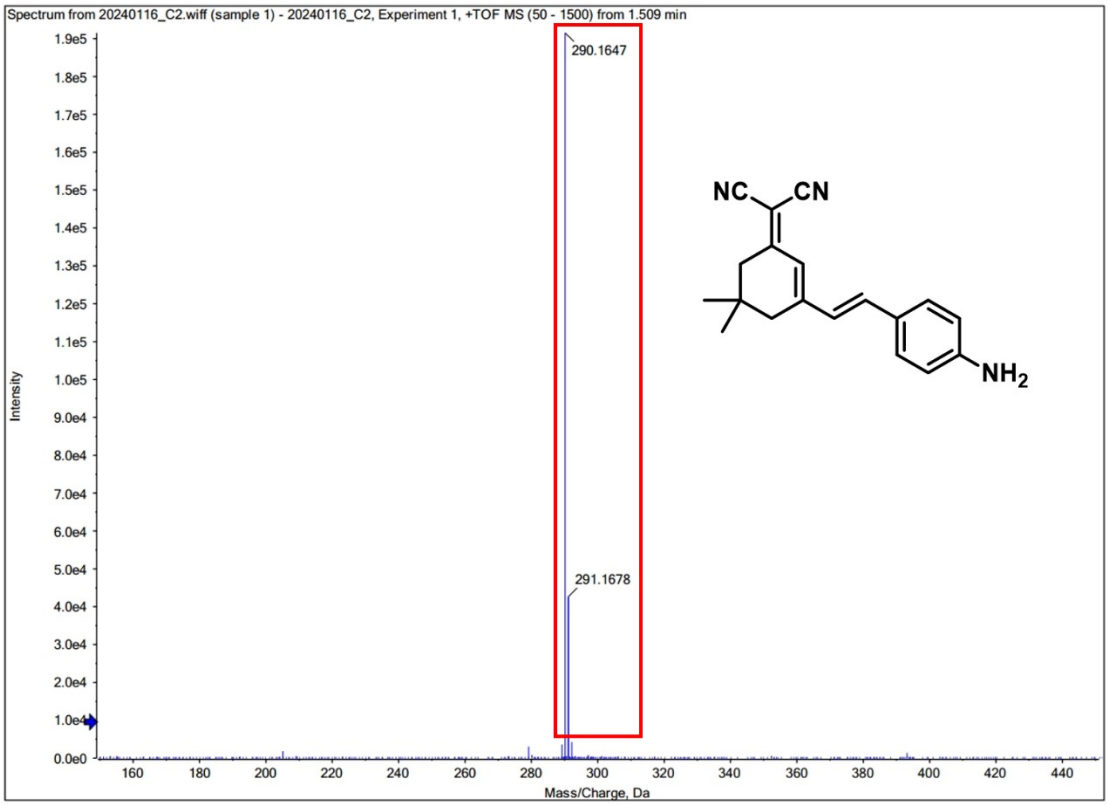
The ESI-MS spectrum of Compound 1 (calculated for $C_{12}H_{14}N_2$ [M+H]+ 187.12, found 187.1230).



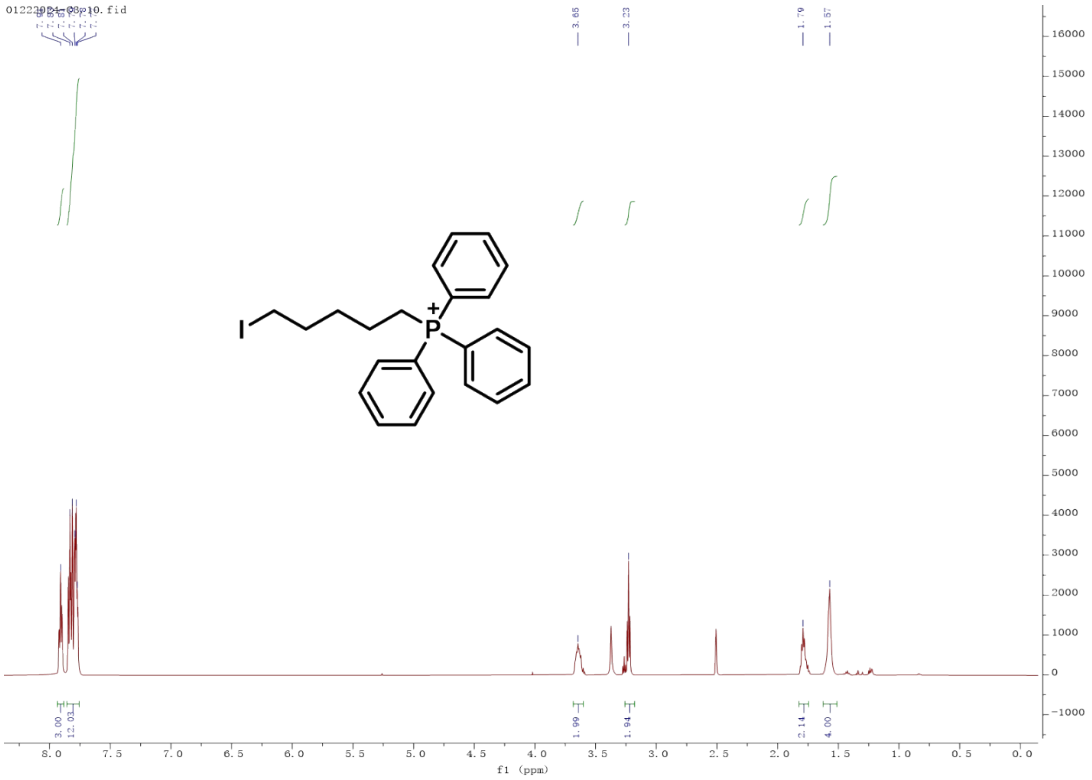
^{1}H NMR of Compound 2 (600 MHz, DMSO-d_6)



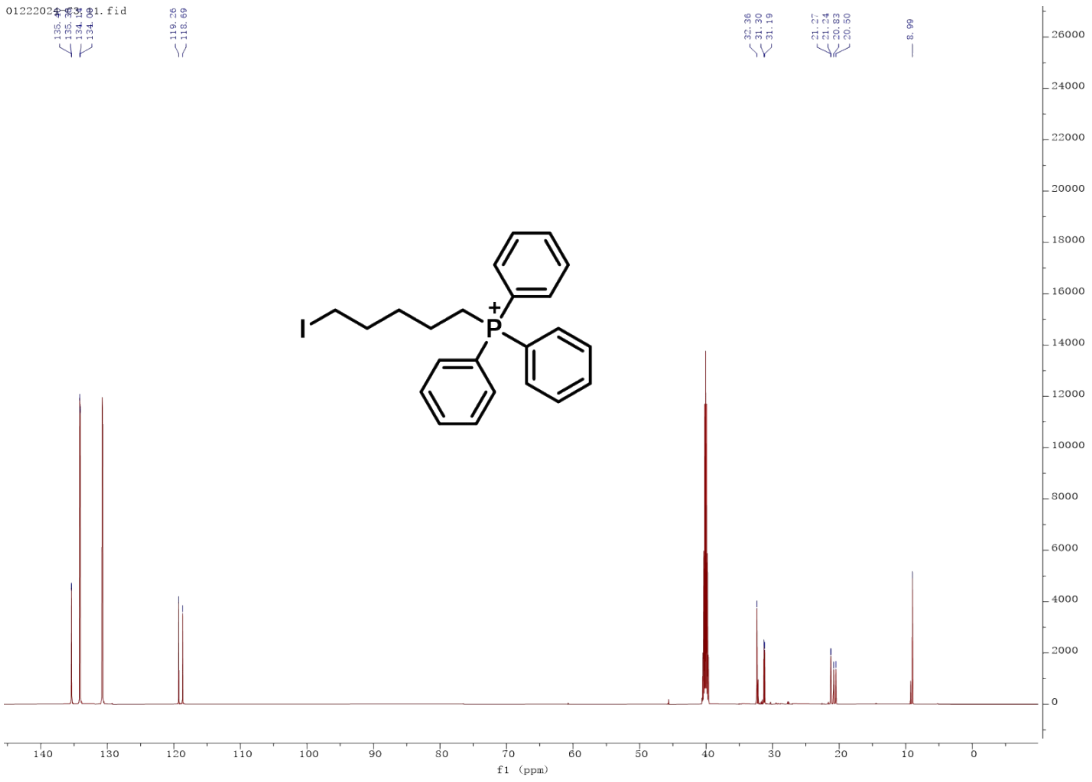
^{13}C NMR of Compound 2 (151 MHz, DMSO-d_6)



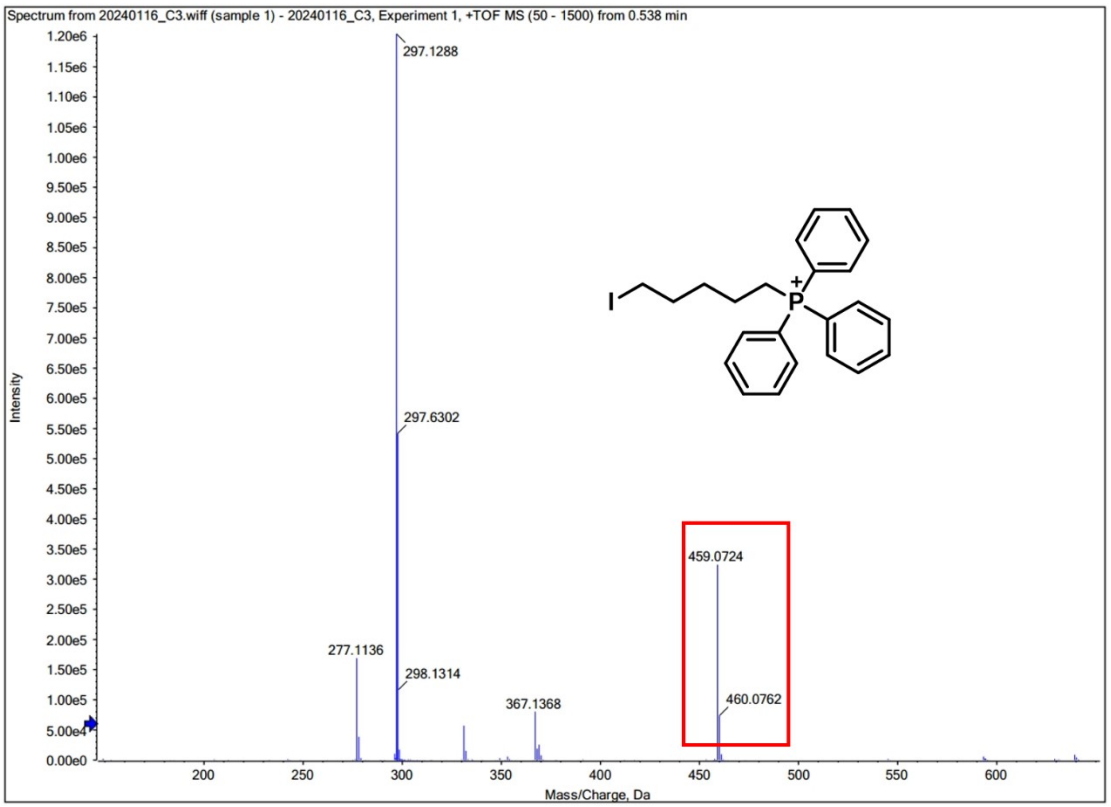
The ESI-MS spectrum of Compound 2 (calculated for $C_{19}H_{19}N_3$ [M+H]+ 290.16, found 290.1647).



^{1}H NMR of Compound 3 (600 MHz, DMSO-d_6)



^{13}C NMR of Compound 3 (151 MHz, DMSO-d_6)



The ESI-MS spectrum of Compound 3 (calculated for $C_{23}H_{25}IP^{+}$ [M+H]+ 459.07, found 459.0724).