Supporting Information

A Non-peptide-based Fluorescence Probe, Capable of Sensitively Visualizing Asparagine Endopeptidase

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1Experimental Section

1.1 Reagents and Materials. Reagents used in this study were all of analytical grade, purchased from commercial suppliers and used as received unless otherwise stated. The reagents chemicals used in the work were bought from Energy-Chemical or Aladdin. All general chemicals for fluorescence detection including MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) were purchased from Energy Chemical Co. Mito-Tracker Green, ER-Tracker Green, Lysotracker Red and DAPI were purchased from Beyotime Biotech Inc. Mouse Legumain/ LGMN Protein (His Tag) was purchased from Sino Biological Inc. Chymotrypsin (CHT), Nitroreductase (NTR), Sulfatase (SULF), Cytochrome P450 family: CYP1A1 and CYP2J2, Gamma-glutamyl transpeptidase (GGT) were purchased from Sigma-Aldrich. Recombinant Proteinase 3 (PR3) was purchased from Wuhan Feiyue Biotechnology Co. Recombinant human Cathepsin G (CTSG) was purchased from Solarbio life sciences Co. Polyclonal Antibody to Legumain (LGMN) was purchased from Thermo Fisher Scientific for immunofluorescence staining. Monoclonal antibody Legumain (B-8) was purchased from Santa Cruz Biotechnology for western blot. Rat glioma C6 cells, and human glioma U251 cells were purchased from Procell Life Science& Technology Co.

1.2 Methods. ^{1}H-NMR and ^{13}C-NMR spectra of all compounds were performed with Bruker AvanceIII400/500/700 spectrometer. Mass spectrometric (MS) data were carried out using LTQ Orbit rap XL instruments. Absorption and emission spectra were performed with a Lambda 35 UV-visible spectrophotometer (PerkinElmer) and a VAEIAN CARY Eclipse fluorescence spectrophotometer (Serial No. FL0812-M018), respectively.

1.3 Synthesis of QMC11.

Compound11 was synthesized according to the literature.[1]

Compound②: **C11** (440 mg, 2 mmol) and K_2CO_3 (207 mg,1.5 mmol) were dissolved in DMF (1 mL) for 5 min. Then 3-bromopropyl (118 mg,1 mol) was added to the mixture and stirred 2.5 h under an argon atmosphere. The solution was washed water and extracted with ethyl acetate. The organic phase was removed under reduced pressure, and then the crude product was purified by silica gel chromatography using ethyl acetate and petroleum ether (PE: EA=10:1) as the eluent to afford Compound② as a dark red solid (380 mg): Yield 70%. 1H NMR (400 MHz, DMSO-d6) δ 6.70 (t, J = 6.1 Hz, 1H), 6.58 (d, J = 7.8 Hz, 1H), 6.25 (d, J = 7.8 Hz, 1H), 4.03 (dd, J = 6.1, 2.5 Hz, 2H), 3.86 – 3.72 (m, 4H), 3.27 – 3.18 (m, 4H), 3.12 – 3.09 (m, 1H); ^{13}C NMR (DMSO) δ(ppm)146.21, 146.11, 129.93, 129.56, 115.12, 105.08, 81.62, 74.06, 66.44, 50.65, 32.72. ESIMS (M+H) m/z = 259.118.

Compound③: was synthesized according to the literature.[2]

QMN3: Compound① (100 mg, 0.52 mmol) and Compound③ (176 mg, 0.5 mmol) were dissolved in DMF (1.5 mL) with piperidine (0.5 mL). Then the mixture was refluxed for 10 h under an argon atmosphere. The solvent was removed under reduced pressure, and then the crude product was purified by silica gel chromatography using dichloromethane and MeOH (DCM: MeOH=10:1) as the eluent to afford QMN3 as a dark orange solid (150 mg): Yield 60%.^{1}H NMR (400 MHz, DMSO-d_6) δ 8.94 (d, J = 1.4 Hz, 1H), 8.92 (d, J = 1.4 Hz, 1H), 8.24 (d, J = 8.9 Hz, 2H), 7.60 (s, 2H), 7.42 (d, J = 15.6 Hz, 2H), 7.09 (s, 2H), 7.02 (d, J = 2.0 Hz, 2H), 4.79 – 4.72 (m, 4H), 4.27 (t, J = 4.8 Hz, 4H), 3.72 – 3.66 (m, 4H), 3.02 – 2.96 (m, 13H), 2.70 (t, J = 6.2 Hz, 4H); ^{13}C NMR (DMSO) δ(ppm)159.88,

152.59, 150.14, 140.24, 138.65, 134.09, 130.87, 128.94, 125.52, 125.39, 121.13, 118.93, 118.87, 115.28,106.72, 67.44, 50.02, 48.12, 47.94, 46.75, 44.14, 25.03, 22.63, 22.12. ESIMS (M+H) m/z = 502.134.

QMC11: QMN3 (250 mg, 0.5 mmol) and Compound② (125 mg, 0.5 mmol) were dissolved in DMF (2 mL). Then added $CuSO_4 \cdot 5H_2O$ (0.062 g, 0.25 mmol; Dissolved in 0.5 mL water) and L-sodium ascorbate(0.099 g, 0.5 mmol) to the system. The mixture was stirred at 70 °C for 5 h under an argon atmosphere. The solvent was removed under reduced pressure, and then the crude product was purified by silica gel chromatography using dichloromethane and MeOH (DCM: MeOH=8:1) as the eluent to afford QMN3 as a dark orange-red solid (76 mg): Yield 20%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.94 (dd, J = 8.5, 1.4 Hz, 1H), 8.24 (d, J = 9.0 Hz, 1H), 7.62 (d, J = 6.7 Hz, 1H), 7.43 (d, J = 15.6 Hz, 1H), 7.09 (s, 1H), 7.03 (d, J = 8.8 Hz, 2H), 6.73 (d, J = 6.1 Hz, 1H), 6.58 (s, 1H), 6.25 (d, J = 7.8 Hz, 2H), 4.76 (t, J = 8.4 Hz, 2H), 4.26 (d, J = 4.9 Hz, 2H), 4.04 – 4.01 (m, 2H), 3.80 (t, J = 4.7 Hz, 7H), 3.69 (t, J = 4.8 Hz, 2H), 3.23 – 3.21 (m, 4H), 3.12 (d, J = 2.4 Hz, 1H), 2.69 (t, J = 6.2 Hz, 2H), 2.14 (s, 2H); ^{13}C NMR (DMSO) δ(ppm)159.87, 152.57, 150.12, 146.19, 146.09, 140.22, 138.63, 134.07, 130.86, 129.91, 129.54, 128.93, 125.51,125.37, 121.12, 118.92, 118.86, 115.27, 115.11, 106.71, 105.08, 81.61, 74.06, 67.43, 66.43, 50.64, 50.05, 48.11, 47.94, 46.76, 44.15, 32.72, 25.04, 22.65, 22.12. ESIMS (M-H) m/z = 759.106.

1.4 Fluorescence imaging in living cells. C6 cells, which overexpressed legumain, were selected for cell imaging. The cells were cultured in high-glucose DMEM medium containing 10% fetal bovine serum and kept in a humidified atmosphere of 5% CO_2 at 37 °C. The medium was replaced with fresh medium every day. Then, the cells were removed to confocal dishes at the density of 5×10^5 per dish in 2 mL of culture medium and incubated at the same condition for 8 h, and starved for one night before cell imaging experiments. To demonstrate the legumain-targeting ability of **QMC11**, C6 cells were treated with 20-40 μM of **QMC11** and QMN3 at 37 °C for 1 h, respectively. Moreover, another experiment was also designed to verify whether the probe was sensitive to the legumain. In brief, C6 cells were preincubated with the inhibitor **C11** (40 μM) at 37°C for 1 h before incubating with the **QMC11**. 30 min before fluorescence imaging, the lysosomal marker Lysotracker Red (20 nM) was added to the system. After washing three times by PBS, cell imaging was performed by the confocal microscopy.

1.5 Oil-water partition coefficient. We measured the oil-water partition coefficient using a shaking method. In the n-octanol and PBS buffered solution system, the solution of n-octanol and PBS buffered solution in a volume ratio of 1:1 was shaken on a shaker for 24 h and then placed in a centrifuge (2500 rpm) for 0.5 h. Next, the two phases were separated and the initial absorbance of the compound was measured on a UV-1750 spectrometer in PBS buffered solution (Abs_1). Then, an equal volume of probes solution was mixed with pre-saturated n-octanol and placed in a conventional shaker at room temperature for 6 h before being placed in a centrifuge (2500 rpm) for 0.5 h and the two phases were layered. The absorbance (Abs_2) of the probes in the PBS buffer layer was determined by a UV-1750 spectrometer. Finally, the measured oil-water partition coefficient was calculated according to the following formula:

$$\log P = \log [(Abs_1 - Abs_2)/Abs_2]$$

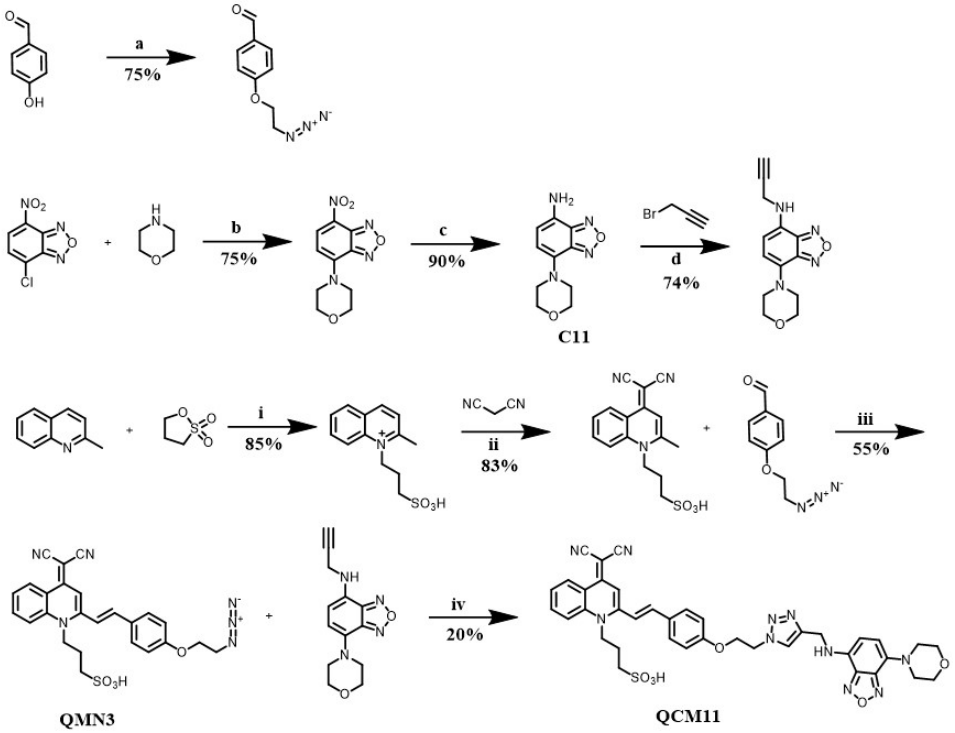
1.6 Cytotoxicity Assay. The cytotoxicity of **QMC11**(or QMN3) was evaluated with the 3-(4,5

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. C6 cells were seeded in 96-well plates at the density of 2×10^3 cells per well and cultured at 37 °C for 24 h to allow the cell attachment. After removing the medium, the solution of **QMC11**(or QMN3) at the concentration of 10, 20, 40, and 80 μM was added to each well. Then, cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 24 and 48 h. respectively. MTT dissolved in phosphate-buffered saline (PBS, pH 7.4) was added to each well. After incubating for another 4 h, the solution was removed. Finally, 150 μl of dimethyl sulfoxide was added to dissolve the formazan. The absorbance was obtained using microplate reader. The cell viability was calculated by the following formula: viability% = mean absorbance value of treatment group/mean absorbance value of control group×100%. All experiments were repeated three times independently.

1.7 Immunofluorescent staining. The cells were cultured in a confocal dish and incubated with probe molecules. Then the cells were fixed and closed, incubated with specific primary antibodies (Polyclonal Antibody to Legumain). After overnight, the primary antibodies were recovered and added to the secondary antibodies [Alexa Fluor 647-labeled Goat Anti-Rabbit IgG(H+L)]. Finally, we used laser confocal microscopy for imaging.

1.8 Western Blot Analysis. The cells were cultured in six-well plates until they reached 90%. The adherent cells were lysed with appropriate lysate. To prevent protein degradation, or to ensure the stability of phosphorylated or acetylated proteins, an additional mixture of protease inhibitors, phosphatase inhibitors, or deacetylase inhibitors is added. An appropriate amount of concentrated SDS-PAGE protein loading buffer was added to the collected protein samples and boiled at 95 °C for 15 min. Each sample was separated by SDS polyacrylamide gel electrophoresis (PAGE), followed by transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were taken out and blocked for 1 h at room temperature with QuickBlock™ Blocking Buffer for Western Blot. Then, the membranes were incubated overnight with monoclonal antibody Legumain (B-8) at 4 °C. Then, the membranes were taken out and rainsed with Western Wash buffer for 3 × 5 min and incubated with the HRP-conjugated goat anti-mouse anibody (1: 1000) for 1 h at room temperature. Bands were visualized with SuperSignal (Thermo Scientific Inc.).

2 Supplementary Scheme, Figures, and Tables.



Scheme S1. Synthetic route to **QMC11**: (a) ①1.2-Dibromoethane,②NaN_3, K_2CO_3, rt, 3 h; (b) Cs_2CO_3, acetonitrile, rt, 3 h; (c) Fe, conc. HCl, rt, 2 h; (d) K_2CO_3, DMF, 70 °C, 4 h; (i) benzene, 85 °C, 2 h; (ii) EtOH, Sodium ethoxide, ethanol, 0 °C, 6-8 h; (iii) Piperidine, DMF, 95 °C, 2 h; (iv) $CuSO_4$ (aq.), Sodium ascorbate, TBA, DMF, 70 °C, 2 h;

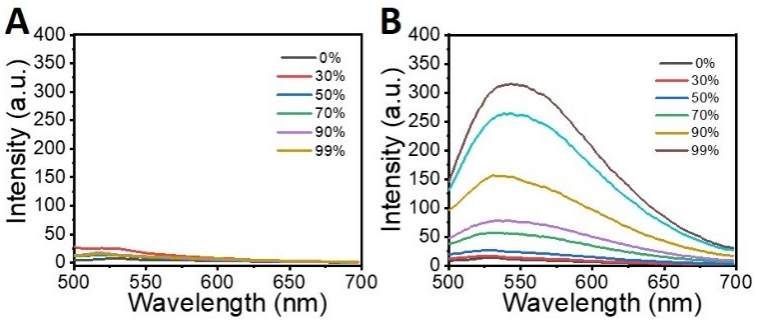


Figure S1. (A) Fluorescence spectra of **QMC11** in a mixture of water−tetrahydrofuran with different tetrahydrofuran fractions. (B) Fluorescence spectra of **QMC11** in a mixture of water−glycerin with different glycerin fractions.

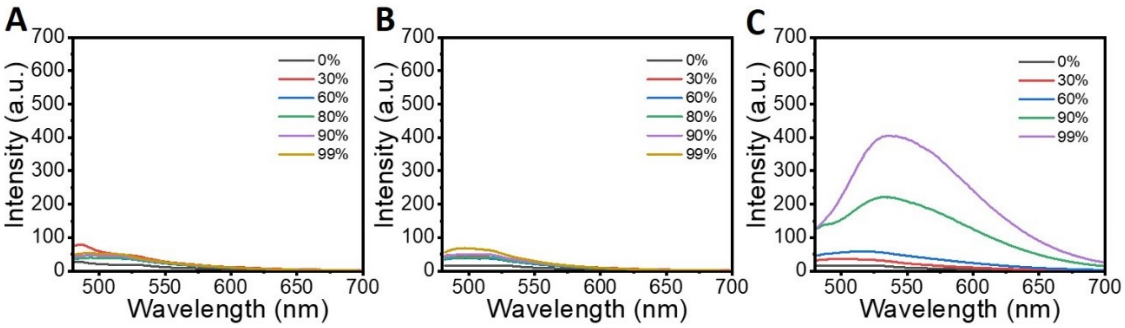


Figure S2. (A) Fluorescence spectra of QMN3 in a mixture of water−ethanol with different ethanol fractions. (B) Fluorescence spectra of QMN3 in a mixture of water−tetrahydrofuran with different tetrahydrofuran

fractions. (C) Fluorescence spectra of QMN3 in a mixture of water−glycerin with different glycerin fractions.

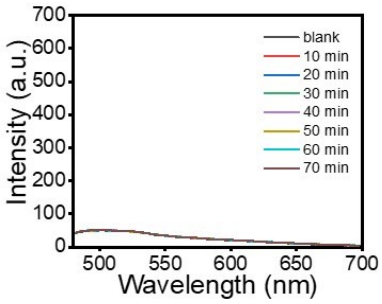


Figure S3. (A) Fluorescence spectra of QMN3 (20 μM) with incubation with AEP (200 ng/mL).

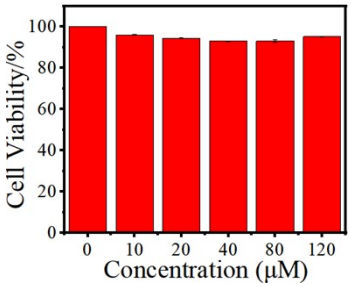


Figure S4. Cytotoxicity test of QMN3 (MTT).

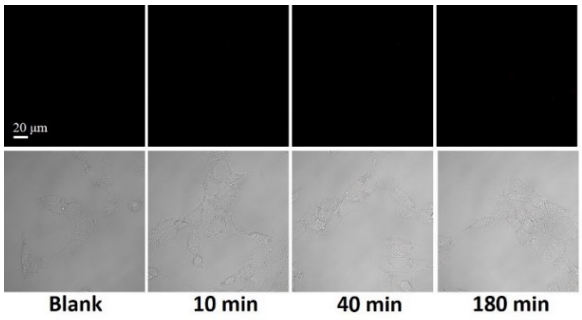


Figure S5. Image of C6 cells incubated with QMN3 (40 μM) for 3h (λ_{ex}: 478 nm, λ_{em}: 500-580 nm, scale bars: 20 μm).

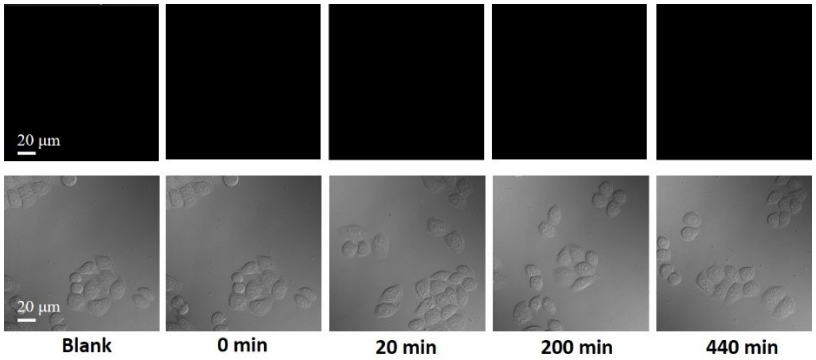


Figure S6. Image of LO2 cells incubated with **QMC11** (40 μM) for 7 h (λ_{ex}: 478 nm, λ_{em}: 500-580 nm, scale bars: 20 μm).

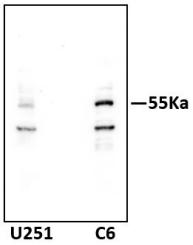


Figure S7. AEP expression by western blot in C6 cells and U251 cells.

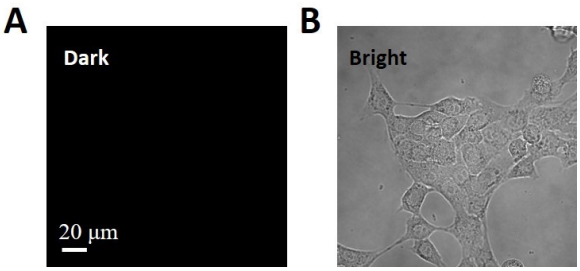


Figure S8. Image of 4T1 cells incubated with **QMC11** (40 μM) (λ_{ex}: 478 nm, λ_{em}: 500-580 nm, scale bars: 20 μm).

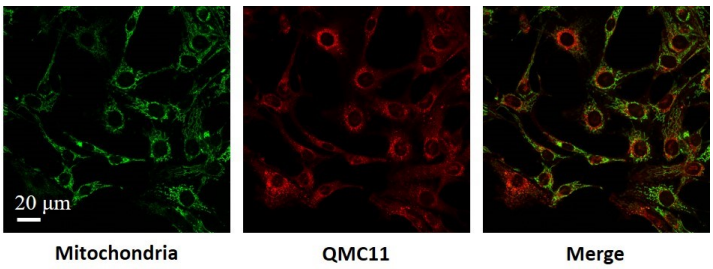


Figure S9. QMC11 (40 μM) was co-localized with Mito-Tracker Green in C6 cells (Pearson Correlation Coefficient: 0.513) (Mito-Tracker Green: λ_{ex}: 504 nm, λ_{em}: 510-540 nm, scale bars: 20 μm).

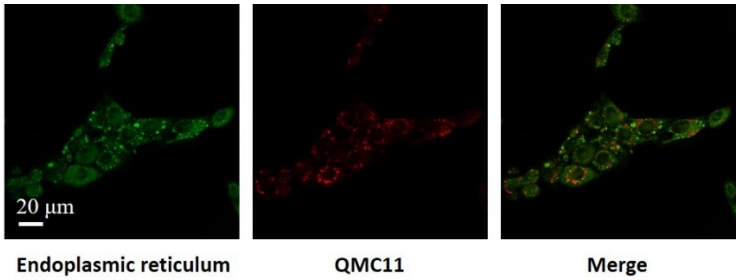


Figure S10. QMC11 (40 μM) was co-localized with ER-Tracker Green in C6 cells (Pearson Correlation Coefficient: 0.504) (ER-Tracker Green: λ_{ex}: 504 nm, λ_{em}: 510-540 nm, scale bars: 20 μm).

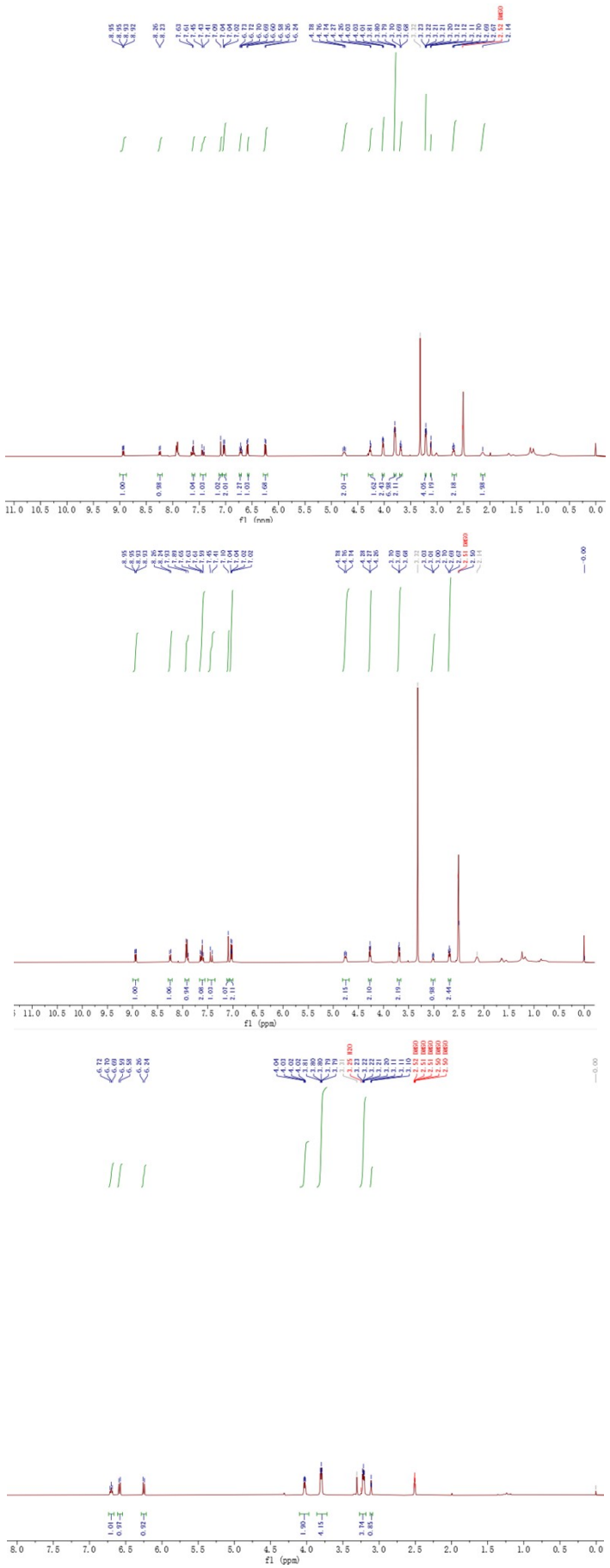


Figure S11. ^{1}H-NMR of **QMC11**, **QMN3** and **Compound②**.

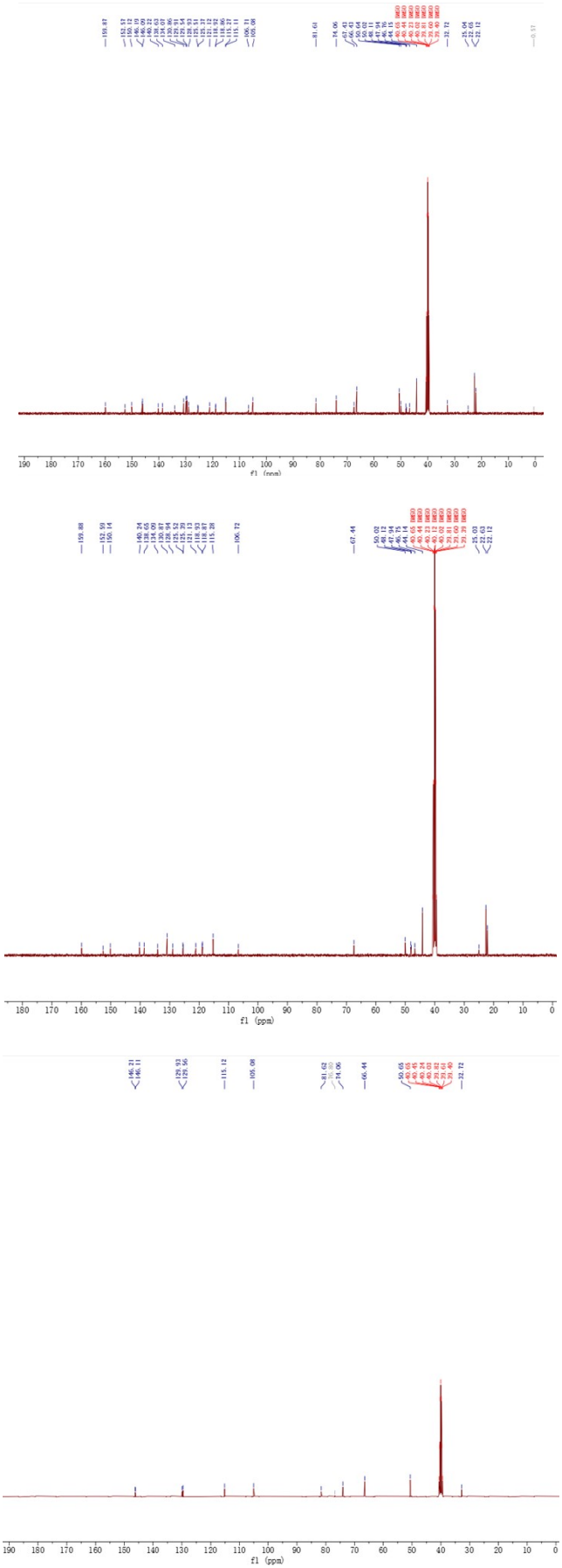


Figure S12. ^{13}C-NMR of **QMC11**, **QMN3** and **Compound②**.

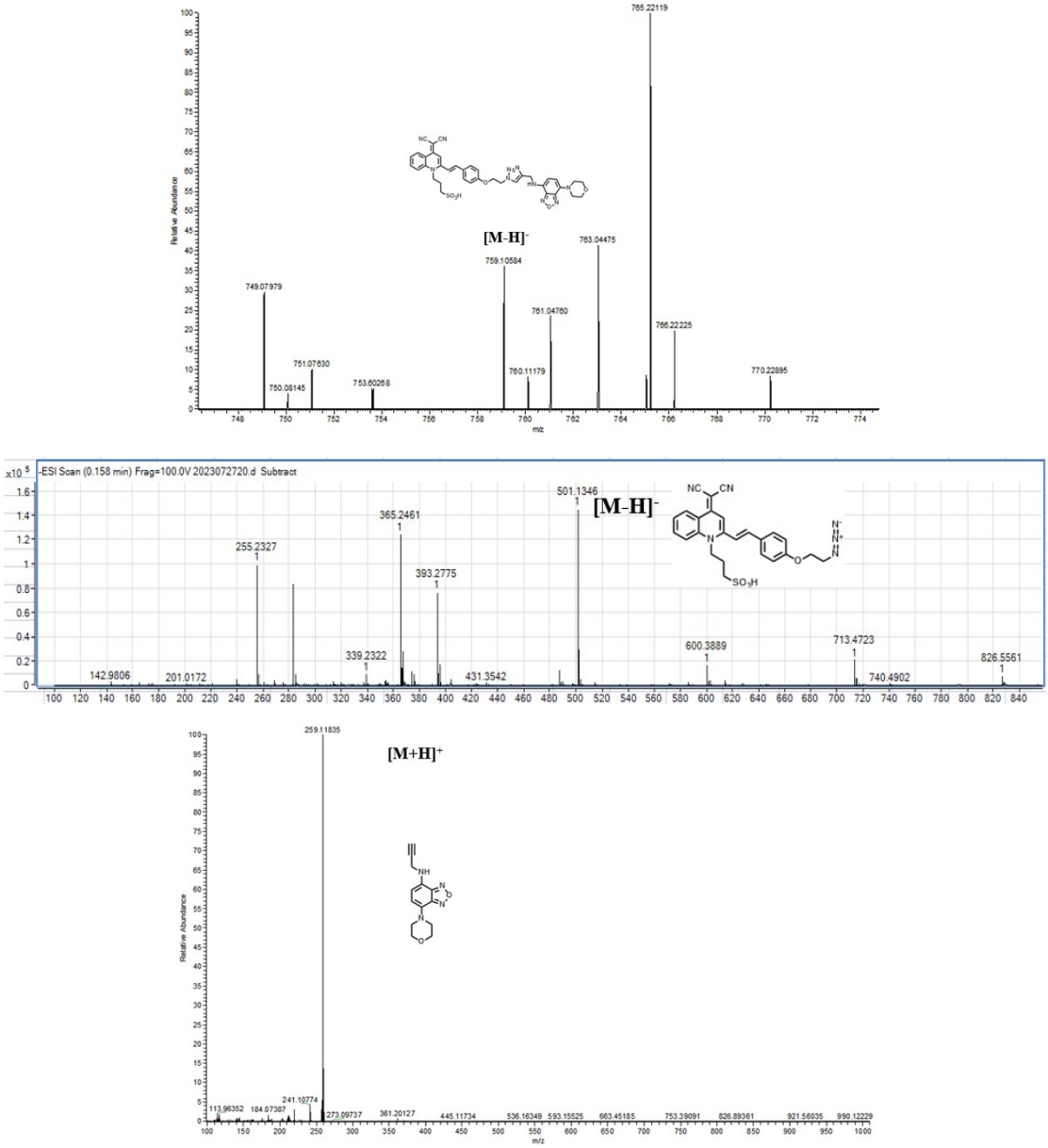


Figure S13. ESI-MS spectra for **QMC11, QMN3** and **Compound②**.

Supporting reference

1. K. Ye, *US Pat.,* US2022213087A1. 2022.
2. M. Xu, R. Li, X. Li, G. Lv, S. Li, A. Sun, Y. Zhou and T. Yi, *J Mater Chem B*, 2019, **7**, 5535-5540.