Electronic Supplementary Information (ESI)

Ratiometric Fluorescence Imaging for Distinguishing Chloride Concentration between Normal and Ischemic Ventricular Myocytes

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1. Experimental Section

Reagents and Apparatus.

Methyl 3-bromopropionate, 6-methoxyquinoline and 5-aminofluorescein were purchased from Sigma-Aldrich Co. Ltd. All other chemicals were from commercial sources and were of analytical reagent grade, unless indicated otherwise. Ultra-pure water purified with a Sartorius Arium 611 VF system (Sartorius AG, Germany) to a resistivity of 18.2 MΩ·cm was used throughout the experiment. Stock solution of MQAF (1.0 mM) was prepared in DMSO and stored at 4 °C in darkness . HEPES buffer was prepared by dissolving appropriate amount of HEPES in ultrapure water. The required pH was adjusted by adding appropriate amount of HCl or NaOH. The pH measurements were performed with a pH-3c digital pH-meter (Shanghai Lei Ci Device Works, China) with a combined glass-calomel electrode. ^{1}H NMR, ^{13}C NMR spectra were taken on a Bruker 300 MHz spectrometer.

Absorption Measurement.

Absorption spectra were recorded at room temperature with a pharmaspect UV-1700 UV-Visible spectrophotometer (Shimadzu, Japan) equipped with 1.0 cm quartz cells. The probe was diluted to 8.0 μM with 20 mM HEPES buffers at pH 7.4, then the mixture was equilibrated for 1 min before measurement.

Fluorescence Analysis.

All fluorescence measurements were carried out at Cary Eclipse Fluorescence Spectrophotometer with a xenon lamp and 1.0 cm quartz cells (Varian, Australia). The dual chromophores of the probe were excited at 318 nm and 494 nm, and the corresponding emission spectra were measured around 436 nm and 519 nm, respectively. The excitation and emission slits were both set to 5.0 nm.

Cell Culture.

Ventricular myocytes (H9c2 (2-1), purchased from the Committee on Type Culture Collection of the Chinese Academy of Sciences) were maintained following protocols provided by the American Type Tissue Culture Collection. Cells were grown in cell culture media and incubated at 37 °C in a 5% CO_2/ 95% air humidified incubator (MCO-15AC, SANYO). The cell culture medium was high glucose Dulbecco's Modified Eagle Medium (DMEM, 4.5 g of glucose/L) supplemented with 10% fetal bovine serum (Gibco, Invitrogen), $NaHCO_3$ (2.0 g/L) and 1% antibiotics (penicillin /streptomycin, 100 U/ml).

Confocal Imaging.

Fluorescent images were acquired on a Leica TCS SPE confocal laser-scanning microscope with an objective lens (×40). The emission was collected by the blue channel at 420-450 nm and the green channel at 510-540 nm respectively. Prior to imaging, cells were incubated with 20 μM probe for 10 min at 37 °C in a 5% CO_2/ 95% air humidified incubator, and then the medium was removed and washed with HEPES (pH 7.4, 20 mM) for three times.

Treatment of Normal and Ischemic Ventricular Myocytes.

Before treatment, the concentration of counted cells was adjusted to 10^5 cells mL^{-1}. Subsequently, cells were added to the sterilized glass coverslips in culture plates and cultured. After cell adherent, for the blank group: cells were incubated with chloride-free Tyrode solution for 3 hours at 37 °C under a humidified atmosphere containing 5% CO_2; for the control group: conditions were same as the blank group except for the normal Tyrode solution[1] was used instead; for the simulated

ischemia group: cells were incubated with simulated ischemia solution[2] in an airtight equipment for 3 hours at 37 °C under a humidified atmosphere filled with 5% CO_2 and 95% N_2.

Treatment of Different Extent of Myocardial Ischemia.

Cell adherent procedure was the same as that mentioned above. Then, for the SITS group: cells were dealt with the same method used in the simulated ischemia group, except that an additional chloride channel blocker SITS (0.1 mmol.L^{-1}) were added into the simulated ischemia solution; for the 50% Cl^- group, 25% Cl^- group and the Cl^- free group: cells were incubated with simulated ischemic solution containing 50% Cl^-, 25% Cl^-, no Cl^- contrast to the simulated ischemia group, respectively, the other conditions were the same.

MTT assay

Ventricular myocytes (1.0×10^6 cells mL^{-1}) were dispersed within replicate 96-well microtiter plates to a total volume of 200 μL/well respectively. Plates were maintained at 37 °C in a 5% CO_2/95% air incubator for 5 h. Ventricular myocytes were then incubated for 24 h upon different probe concentrations of 4.0×10^{-4}, 8.0×10^{-5}, 4.0×10^{-5}, 8.0×10^{-6} and 8.0×10^{-7} M, respectively. MTT solution (5.0 mg/mL, PBS, 150 μL) was then added to each well. After 4h, the remaining MTT solution was removed and 150 μL of DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at 490 nm in a TRITURUS microplate reader. Calculation of IC50 values were done according to Huber, W. et al.[3]

Imaging and photobleaching

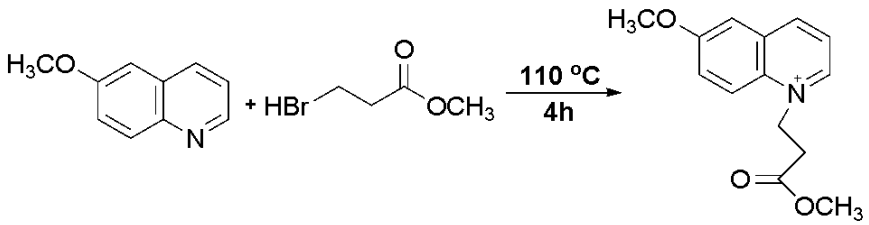
The treatment of cells was same as the blank group.

2. Synthesis and characterization of MQAF

Synthesis and characterization of 6-methoxyquiline-N-methyl-3 bromopropionate (MQMBP)

The starting step was preparation of the protonated form of 6-methoxyquinoline ramification. Using the methods described previously by Verkman,[4] we synthesized MQMBP.

Scheme 2.



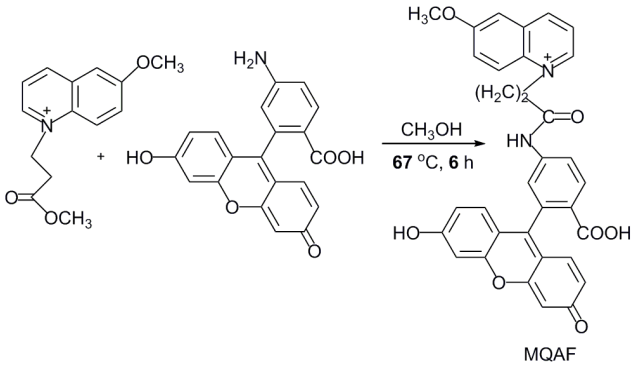
MQMBP

Briefly, methyl 3-bromopropionate (2.505 g, 15 mmol) was dissolved in 6-methoxyquinoline (1.592 g, 10 mmol). Keep the reaction temperature at 110 °C for 4 h to gain an orange viscous mass, then the mixture was cooled to room temperature and washed with large volumes of acetone overnight with stirring. The insoluble product was collected by centrifugation and an ivory-white product was collected. (2.03 g, 77.5%) ^{1}H NMR (300 MHz, DMSO): δ 3.13-3.18(t, 2H), 5.25-5.29(t,2H), 3.73(s, 3H), 3.99(s, 3H), 8.54-8.58(d,1H), 9.40-9.42(d,1H), 8.13-8.21(t,1H), 8.07(s,1H), 8.03-8.05(d,1H), 9.04-9.07(d,1H). ^{13}C NMR (75 MHz, DMSO): 171.8, 159.5, 146.3, 146.2, 142.6, 128.0, 127.6, 122.7, 121.0, 108.9, 56.7, 53.4, 52.3, 33.7.

Synthesis and characterization of MQAF

MQMBP (0.1311 g, 0.5 mmol) was dissolved in 10 mL methanol and stirred, then 5-amino-fluorescein (0.1736 g, 0.5 mmol) in 10 mL methanol was added to the above solution, subsequently, Na_2CO_3 (0.01251 g, 0.125 mmol) was added and the mixture was brought to a reflux with stirring at 67 ^oC. After 6 h, the solvent was evaporated and a red brown solid (0.2237 g, 79.8%) was obtained. ^1H NMR (300 MHz, DMSO): δ 1.03-1.08(t, 2H), 2.51(s,2H), 3.90(s, 3H), 4.00(d, 1H), 5.75(s,1H), 6.56-6.58(m,1H), 6.61-6.65(d,1H),6.84(m,1H), 6.87(s,1H), 6.94(s,1H), 6.97-6.99(d,1H), 7.37(s,1H). 7.41(s,1H), 7.45-7.47(d,1H), 7.90(d,1H), 7.93(t,1H), 8.24-8.27(m,1H), 8.72-8.74(d,1H), 9.37(d,1H), 10.02(s,1H). ^{13}C NMR (75 MHz, DMSO): 170.8, 169.9, 159.7, 157.6, 152.4, 151.0, 148,3, 144.2,140.1, 135.2, 130.8, 129.5, 128.2, 127.9, 124.6, 122.7, 122.1, 121.0, 112.9, 111.2, 108.8, 106.2, 102.6, 83.2, 56.8, 56.0, 53.5, 52.3, 40, 8, 39.3, 33.6.

Scheme 3.



3. Supplementary spectral figure

Absorption spectrum of MQAF.

Absorption and fluorescence spectral properties of MQAF were examined. As can be seen in Figure S1, the dual chromophores probe exhibits the characteristic absorption of MQMBP fragment at 308 nm， while exhibit intense absorption peaks of AF fragment at 450 nm and 478 nm.

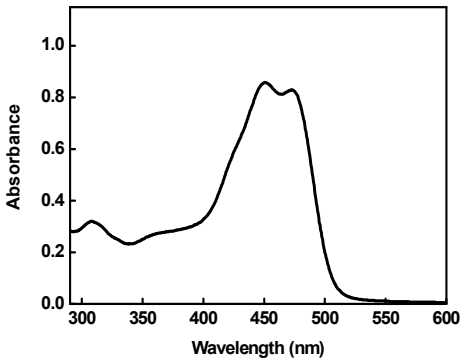


Figure S1. Absorption spectrum of MQAF (8.0 μM) in 20 mM HEPES (pH 7.4) at room temperature.

Full emission spectra of MQAF with excitation at 318 nm.

The dual-chromophore probe MQAF contains two fragments: the MQMBP fragment ($\lambda_{ex}/\lambda_{em}$= 318/436 nm) and AF fragment ($\lambda_{ex}/\lambda_{em}$= 494/519 nm). When the probe was excited by 318 nm, the AF wouldn't be excited well, since it was not the maximum excitation wavelength of AF. The corresponding full emission spectrum was showed in Figure S2. For this reason, MQAF was excited separately by 318 nm and 494 nm during the experiments.

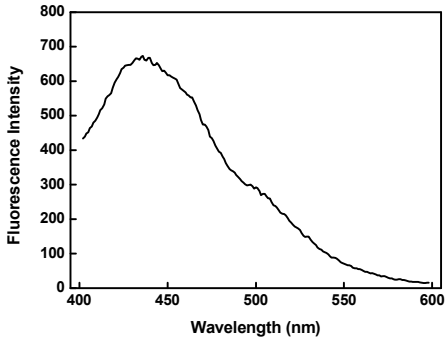


Figure S2. The full emission fluorescence spectra of 8.0 μM MQAF in HEPES buffer (20 mM, pH 7.4) under the excitation at 318 nm.

Fluorescence spectra of MQAF.

As is shown in Figure S3a and S3b, the maximum wavelengths of excitation and emission of the two chromophores of the MQAF lie at 318 nm/436 nm and 494 nm/519 nm, respectively. When chloride was added, an obvious fluorescence decrease of the MQMBP fragment was observed, while the fluorescence of the AF fragment keeps nearly unchanged.

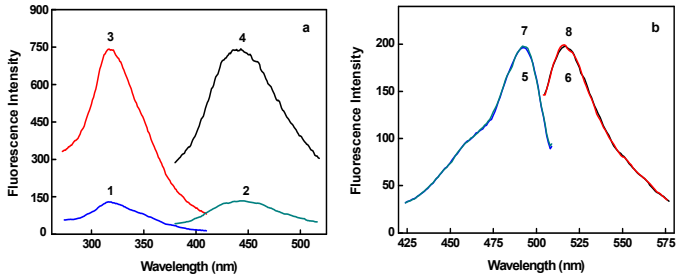


Figure S3. Fluorescence spectra of MQAF (8.0 μM). (a) Excitation spectra (lines 1 and 3) and the emission spectra (lines 2 and 4; excitation at 318 nm) either with (lower lines) or without (upper lines) Cl^-; (b) Excitation spectra (lines 5 and 7) and the emission spectra (lines 6 and 8; excitation at 494 nm) either with (line 7 and 8) or without (line 5 and 6) Cl^-; The spectra were acquired in 20 mM HEPES buffer (pH 7.4).

Influence of pH on fluorescence ratio.

The effect of pH on the reaction was studied (Figure S4). , The probe shows a slight pH-dependence before and after Cl^- addition within the physiological conditions (pH 6.8-8.2). We choose pH 7.4 throughout experiments.

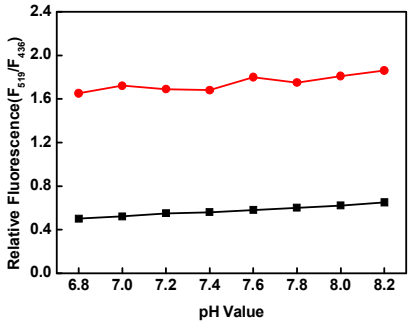


Figure S4. Effect of pH on fluorescence ratio before (square solid) and after (circle solid) addition of NaCl (75 mM) in 20 mM HEPES with a probe concentration of 8.0 μM.

Optimization of probe concentration.

We monitored the effect of probe concentration. The ratio of the fluorescent intensities at two wavelengths is less dependent on the probe concentration showed in Figure S5 .A concentration of MQAF at 8.0 μM was employed in experiments.

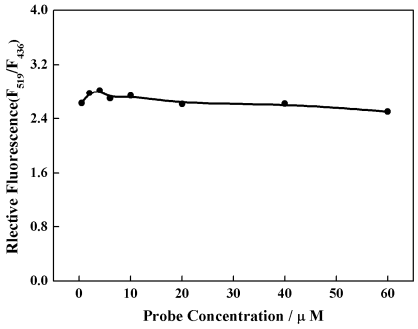


Figure S5. Effect of probe concentrations on the ratio of fluorescence intensity in 20 mM HEPES (pH 7.4) after the addition of 75 mM Cl^- at room temperature.

Kinetic assays

To test the photostability of the probe, the kinetic behavior of the reaction was investigated. The fluorescence signal was recorded as a function of reaction time for 60 min. Figure S6a and S6d were the fluorescence intensity of MQMBP fragment with time before and after Cl^- addition, the fluorescence intensity of AF fragment in the absence and presence of Cl^- can be seen from Figure S6b and S6c. The two fluorescence intensities of the probe remain almost constant with passage of time without and with Cl^-, which indicated that MQAF could instantly respond to the chloride, and the probe solution is stable to the medium such as light and air.

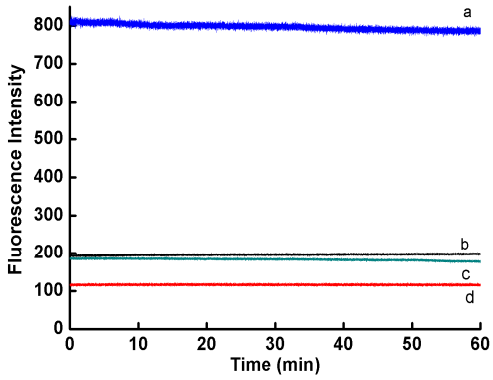


Figure S6. Time course of fluorescence intensity of 8.0 μM MQAF. The fluorescence intensity of MQMBP (line a and d) before and after Cl^- (100 mM) addition with the time change, the fluorescence intensity of AF (line b and c) without and with Cl^- (100 mM). The experiment was carried out in HEPES buffer (20 mM, pH 7.4).

4. MTT assay

MTT assays were performed in ventricular myocytes with five probe concentrations of 4.0×10^{-4}, 8.0×10^{-5}, 4.0×10^{-5}, 8.0×10^{-6} and 8.0×10^{-7} M. According to the experimental data, we calculated IC50 = 9.0×10^{-4} M. The result showed that our probe has low toxicity to ventricular myocytes under the experimental conditions at the concentration of 2.0×10^{-5} M.

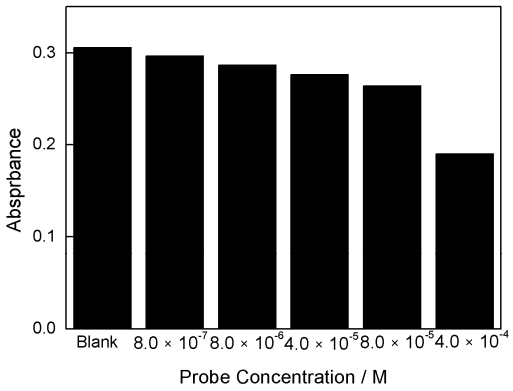


Figure S7. MTT assay of ventricular myocytes in the presence of the probe.

5. Imaging and photobleaching

To further confirm photostability of the probe, the photobleaching of the probe in cultured cells were investigated (Figure S8). Under different power sources, we observed the fluorescent brightness of the probe. The fluorescent intensity of 6-methoxyquinoline and 5-amino-fluorescein are both changeless, even if laser source power is increased, and these results show that the probe is photostable under experimental conditions.

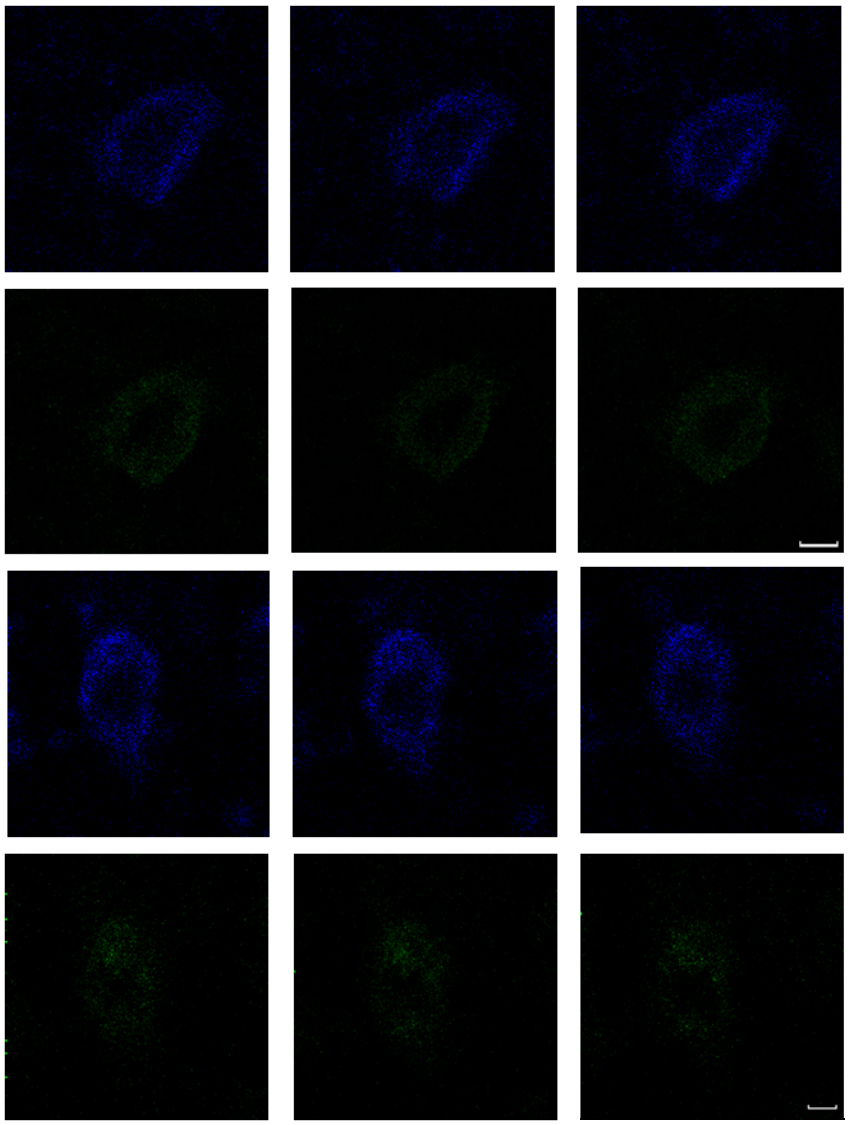


Figure S8. Confocal fluorescence imaging of ventricular myocytes with different time. Cells incubated with 20 μM probe for 10 min at 37 °C were washed with HEPES buffer (20 mM, pH 7.4) for three times before experiments. a and c were images of quinoline fragment under the power sources 40 mW and 50 mW, respectively; b and d were images of fluorescein fragment under the power sources 4.5 mW and 8.1 mW, respectively. The bar of a and b is 10 μM, c and d is 5 μM.

6. References

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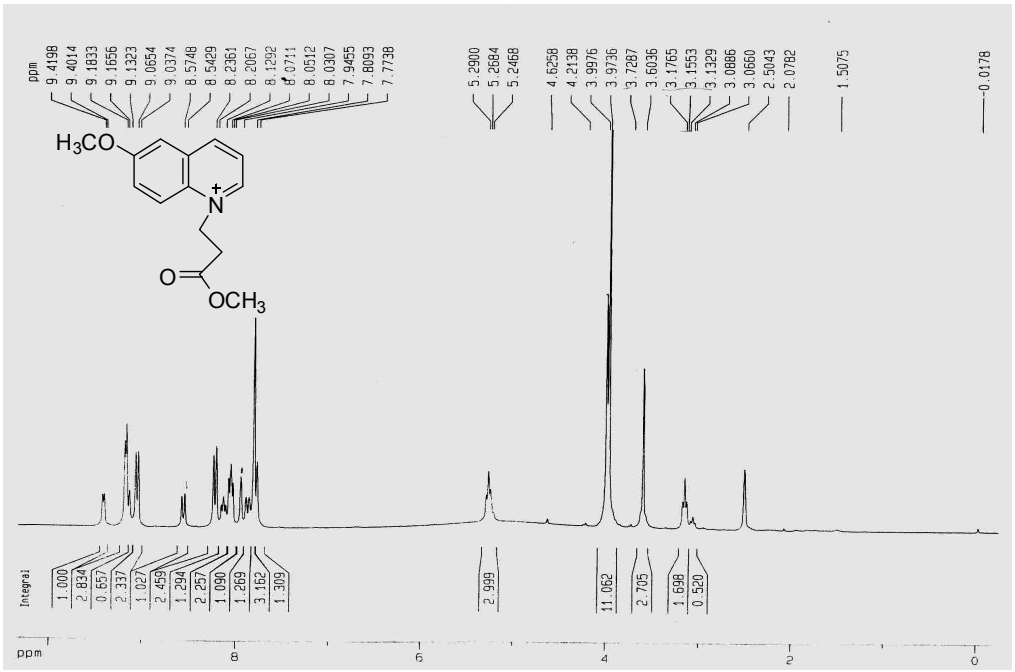
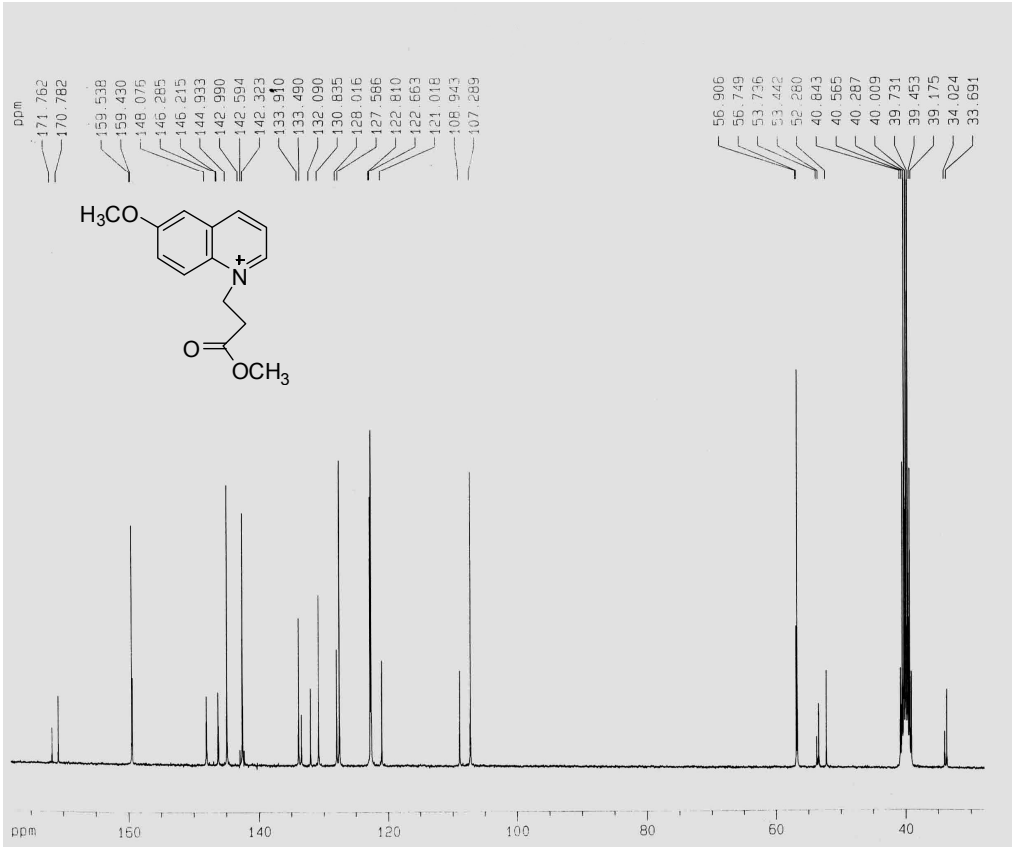
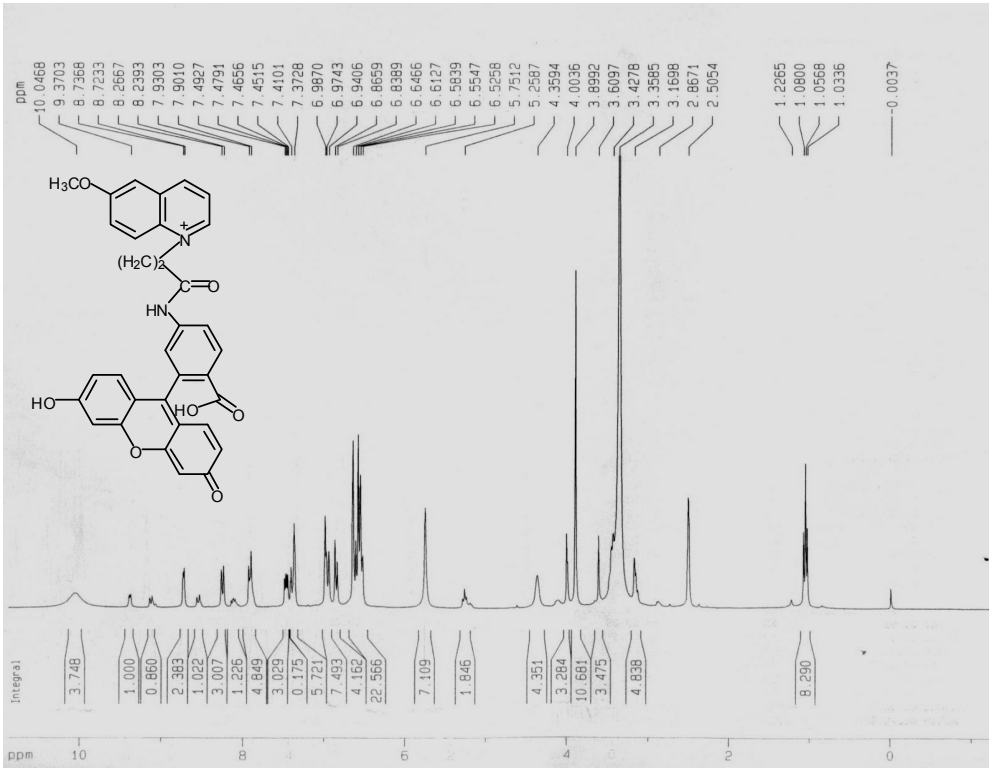
^{1}H NMR of MQMBP



^{13}C NMR of MQMBP



^{1}H NMR of MQAF



^{13}C NMR of MQAF