Supporting Information for

Rhodamine Based Fluorescent Probe Validates Substrate and Cellular Hypoxia Specific NADH Expression

Arup Podder[‡,a], Seyoung Koo[‡,b], Jiyeong Lee[‡,c], Sora Mun[d], Sabina Khatun[e], Hee-Gyoo Kang*[,c,d], Sankarprasad Bhuniya*[,e] and Jong Seung Kim*[,b]

[a]Amrita Centre for Industrial Research & Innovation, Amrita School of engineering, Amrita Vishwa Vidyapeetham, Coimbatore, India 641112.

[b]Department of Chemistry, Korea University, Seoul 02841, Korea

[c]Department of Biomedical Laboratory Science, College of Health Sciences, Eulji University, Seongnam 13135, Korea.

[d]Department of Senior Healthcare, BK21 Plus Program, Graduate School, Eulji University, Seongnam 13135, Korea

[e]Department of Chemical Engineering & Materials Science, Amrita School of Engineering, Amrita Vishwa Vidyapeetham, Coimbatore, India 641112.

[‡]These authors contributed equally

*E-mail: jongskim@korea.ac.kr (JSK) or b_sankarprasad@cb.amrita.edu (SB) or kanghg@eulji.ac.kr (HGK).

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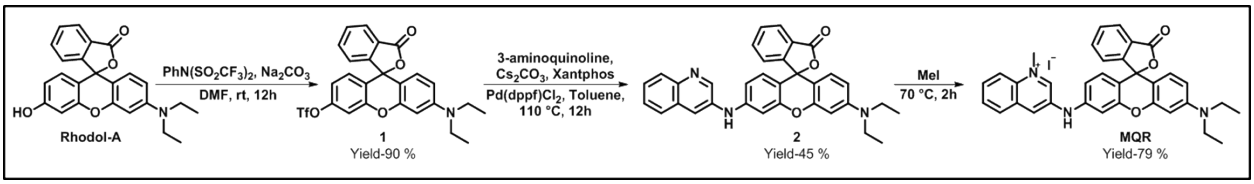
1. Synthesis, Spectroscopic Materials and Instruments.

3-aminoquinoline (Sigma Aldrich), [1, 1′-Bis (diphenylphosphino) ferrocene] dichloropalladium (II) complex with DCM (Aldrich), N-phenyl-bis (trifluoromethanesulfonimide) (Avra, India), aniline (Avra, India), Cs_2CO_3 (Avra, India), xantphos (Aldrich), Na_2CO_3 (Avra, India), DMF (Aldrich), ethanol (Merck), toluene (Loba chem., India) were purchased commercially and used without further purification. All the materials for UV/Vis and Fluorescence spectroscopy, Glutathione (Aldrich), Ascorbic Acid (Aldrich), Cystein (Aldrich), Pepsin (SRL), Trypsin (SRL), Tryrosinase (Aldrich), Lipase (Aldrich), Hydrogen peroxide (Avra Chem), Reduced flavin adenine dinucleotide (Aldrich), Fe^{2+} (Avra Chem), Ca^{2+} (Avra Chem), Na^{+} (Avra Chem), K^{+} (Avra Chem), Mg^{2+} (Avra Chem), β-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate (Aldrich), DMSO (J.T. Baker) were purchased from commercial suppliers and were used without further purification. Flash column chromatography was performed using Silica gel (100-200 mesh) and Analytical thin layer chromatography was performed using silica gel 60 (pre-coated sheets with 0.25 mm thickness). Mass spectra were recorded on anion SpecHiResESI mass spectrometer. NMR spectra were collected on a 400MHz spectrometer (Bruker, Germany).

2. Synthetic Procedures



Synthesis of 1

To a solution of diethyl amino rhodol[1] (500 mg, 1.29 mmol) in dry DMF (15 mL), N-phenyl-bis (trifluoromethane sulfonimide) (922 mg, 2.58 mmol) and Na_2CO_3 (684 mg, 6.45 mmol) were added and the reaction mixture was stirred for 12 h while keeping room temperature. After completion, the reaction mixture was poured into chilled water (50 mL) and extraction was done with ethyl acetate (2 × 25 mL). The organic layer was washed with an ice-cold water (3 × 50 mL) and a cold brine solution (1 × 50 mL), dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (100-200 mesh) using ethyl acetate in hexane (3:7) as an eluent to obtain **1** as a red solid (605 mg; 90 % yield). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.06 (s, 1H), 7.70 (q, 9.6 Hz, 1H), 7.65 (t, J = 4.8 Hz, 1H), 7.22 (t, J = 1.6 Hz, 2H), 6.92 (q, J = 5.6 Hz, 1H), 6.87 (d, J= 5.6 Hz, 1H), 6.59 (d, J = 6.0 Hz, 1H), 6.46 (d, J = 1.6 Hz, 1H), 6.40 (q, J = 6 Hz, 1H), 3.39 (q, J = 9.2 Hz, 4H), 1.19 (t, J = 4.0 Hz, 6H). ^{13}C-NMR (100 MHz, in $CDCl_3$): δ 169.16, 152.60, 149.89, 135.06, 130.97, 130.01, 128.80, 126.92, 125.12, 124.00, 120.28, 119.78, 117.65, 116.11, 110.31, 109.07, 104.42, 97.59, 82.79, 44.53, 12.45 ppm. ESI-HRMS m/z ($M+H^+$): calcd: 519.0963, found 520.1035.

Synthesis of 2

To a stirred solution of compound **1** (200 mg, 0.39 mmol) and 3-aminoquinoline (66 mg, 0.58 mmol) in toluene (10 mL), Cs_2CO_3 (378 mg, 1.16 mmol), xantphos (22.3 mg, 0.04 mmol) and Pd(dppf)Cl_2 (14.1 mg, 0.02 mmol) were mixed in sealed tube. The reaction was continued to stir for 12 h at 110 °C. After completion of the reaction, the reaction mixture was poured into water (10 mL) and extraction was done with ethyl acetate (3 × 10 mL). The organic layer was subsequently washed with water (2 × 20 mL) and brine (1 × 20 mL), dried with anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (100-200 mesh) using ethyl acetate in hexane (5:5) as the eluent to obtain ***2*** as a dark red solid (89 mg; 45 % yield). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.72 (s, 1H), 8.04 (d, J = 7.6 Hz, 2H), 7.86 (s, 1H), 7.70-7.55 (m, 4H), 7.52 (t, J = 8 Hz, 1H), 7.26 (t, J = 6.4 Hz, 2H), 7.00 (s, 1H), 6.76 (q, J = 18 Hz, 2H), 6.62 (d, J = 8.8 Hz, 1H), 6.42-6.35 (m, 3H), 3.37 (q, J = 14.4 Hz, 4 H), 1.17 (t, J = 7.2 Hz). ^{13}C-NMR (100MHz, DMSO-d6): δ 168.93, 152.42, 150.00, 136.25, 133.22, 131.55, 130.90, 129.12, 126.49, 125.30, 123.44, 120.74, 119.75, 117.63, 110.92, 109.76, 104.30, 97.35, 82.55, 44.29, 12.70 ppm. ESI- HRMS m/z (M^+) calcd: 513.2052, found 514.2123.

Synthesis of MQR

Compound **2** (100 mg, 0.19 mmol) and methyl iodide (2 mL) were poured in a sealed tube and heated at 70 °C for 2 h. Completion of the reaction was confirmed by checking TLC. Then reaction mixture was poured into water (10 mL) and extracted with ethyl acetate (3 × 10 mL). The organic layer was subsequently washed with water (2 × 20 mL) and brine (1 × 20 mL), dried with anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (100-200 mesh) using methanol in DCM (0.5:9.5) as the eluent to obtain *MQR* as a dark red solid (100 mg; 79 % yield). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 10.16 (s, 1H), 9.37 (s, 1H), 8.51 (d, J = 2 Hz, 1H), 8.05- 7.96 (m, 3H), 7.82-7.73 (m, 2H), 7.69-7.59 (m, 2H),7.21-7.16(m, 2H), 6.98 (q, J = 8.4 Hz, 1H), 6.59 (q, J = 11.2 Hz, 2H), 6.44 (d, J = 2.4 Hz, 1H), 6.37 (q, J = 8.8 Hz, 1H), 4.57 (s, 3H), 3.39 (q, J = 14 Hz, 4H), 1.19 (t, J = 6.8 Hz, 6H). ^{13}C-NMR (100MHz, DMSO-d6): δ 168.69, 152.20, 152.13, 149.26, 143.74, 143.26, 143.19, 137.10, 135.52, 133.87, 131.68, 130.11, 130.08, 129.84, 129.36, 129.23, 128.79, 128.66, 127.62, 127.58, 126.46, 124.57, 124.01, 118.70, 113.67, 112.71, 108.70, 104.50, 103.77, 96.86, 83.52, 43.62, 43.77, 12.27 ppm. ESI- HRMS m/z (M^+) calcd: 528.2282, found 528.2285.

3. Spectroscopy and Chromatography Methods

UV/Vis and Fluorescence Spectroscopy

UV/Vis and Fluorescence spectra were recorded using an S-3100 (Scinco) spectrophotometer and an RF-5301 PC spectrofluorometer (Shimadzu) fitted with a xenon lamp, respectively. For all experiment, 1 x 10^{-3} M stock solution of the MQR in DMSO was diluted in aqueous buffer (10 mM PBS, pH 7.4) to make 5 x 10^{-6} M solution and each MQR samples were excited at 525 nm. For studying time dependent UV/Vis spectra and Fluorescence spectra change of MQR, 80 equivalents of NADH was used. For studying correlation between NADH concentration and fluorescence intensity of MQR, aqueous buffer solution of MQR and NADH (0, 50, 75, 100, 125, 150, 200 μM) were prepared and incubated for 2 h at 37 ^{o}C before recording fluorescence intensity at 548 nm.

Reverse phase high performance liquid chromatography (RP-HPLC)
Reverse-phase HPLC experiments were performed on a VDSpher 100 C 18-E column (5μm, 250 × 4.6 mm) with a Young Lin HPLC system (YL9100) using a mobile phase consisting of a binary gradient of solvent A (water with 0.1% v/v TFA) and solvent B (Acetonitrile). Gradient: 30 % B to 100 % B for 20 min, then 100 % B for 5 min, then 20 % B for 5 min. Aqueous solution of MQR (100 μM) and NADH (400 μM) was prepared and aliquot of a solution were injected to column before and after incubation for 2 h at 37 ^{o}C.

4. *in vitro* studies Materials and Methods.

Cell culture and probe treatment
A549 cell line was purchased from the Korean Cell Line Bank (Seoul, Republic of Korea) and cultured in RPMI-1640 (Gibco, Life Technologies, CA, USA, #31800-022) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gemini Bio-Products, West Sacramento, CA, #100-500) at 37 ˚C with 5 % CO_2. For subculture, A549 was added by 0.25% Trypsin-EDTA (Gibco, Life Technologies, CA, USA, #25200-072).

Measurement of cell viability
WST-1 cell proliferation reagent (Roche, Indianapolis, IN, USA,#11644807001) was used for measurement of cell viability. We followed the methods of Verwilst et al.[2] Briefly, A549 cell line was seed in 96-well cell culture plates (Hyundai Micro Co., Ltd., Jung-ku, seoul, Korea) at a density of 10,000 cells per well and sustained at 37 ˚C with 5 % CO_2 for 24 h. After 24 h, probe was treated to A549 cell line (0, 2, 4, 8, 10, 16, 20, 30, 40, 50, 100 μM) for 24 h. Lastly, 10ul wst-1 reagent was added into each well for measurement of cell viability. After 30 min at 37 ˚C, cell viability was measured at 450 nm absorbance.

Confocal microscopy imaging
A549 cell line was seed on glass coverslips in 12-well tissue culture test-plate (SPL, Pocheon, Korea) at a density of 100,000 cells per well and sustained at 37 ˚C with 5 % CO_2 for 24 h. After 24 h, media were replaced with media containing 20 μM probe. And then, A549 cell line were incubated at 37 ˚C with 5 % CO_2 for 1 min, 3 min, 5 min, 10 min and 15 min. For subsequent experiments, MQR was treated to cells at last and images were taken after 15 min. To test NADH dose-dependent response of MQR, NADH was treated to A549 cell line for 30 min and then MQR was treated. Intensity of probe (20 μM) was measured after further incubation for 15 min. To test effect of LDH-A inhibitor, methyl 1-hydroxy6-phenyl-4-(trifluoromethyl)-1H-indole-2-carboxylate (NHI-2, TOCRIS), NHI-2 was treated to A549 cell line for 2 hours, and then the intensity of probe (20 μM) was measured. To test effect of metabolic perturbation, pyruvate (Aldrich) was treated to A549 cell line for 30 min and then the intensity of probe was measured. For further validation, D-(+) Glucose (Aldrich) was treated to A549 cell for 30 min and then images were collected. In addition, for hypoxia induction, A549 cell line was incubated at four different incubation condition for 16 h; normoxia (5 % CO_2, 21 % O_2) and hypoxia (5 %

CO_2 and 1 % O_2 / 3 % O_2 / 5 % O_2). For co-localization analysis, after probe (20 μM) were treated to A549 cell line, Mito-tracker Green FM (500 nM) (Thermo Fisher, New Hampshire, USA, Ex/Em 490/516nm), ER-tracker Green (1 μM) (Thermo Fisher, Ex/Em 374/430–640nm) and Lyso-tracker Green DND-26 (1 μM) (Thermo Fisher, Ex/Em 504/511nm) were used according to the manufacturer's instructions. LSM meta 510 Confocal microscopy (Carl Zeiss, Jena, Germany) was used to measure the fluorescence of probe. And then, glass coverslips were lifted from 12 well plate and were mounted in fluorescent mounting medium (DAKO, Glostrup, Denmark, #S3023).

Statistical analysis

Difference of the intensity of probe was measured by independent *t*-tests using the Statistical Package for Social Sciences software for windows, version 16.0 (SPSS 16; SPSS, Chicago, IL, USA). The bar graph data are expressed as mean ± standard deviation (SD). A p-value < 0.01 were considered significant.

5. Supplementary figures

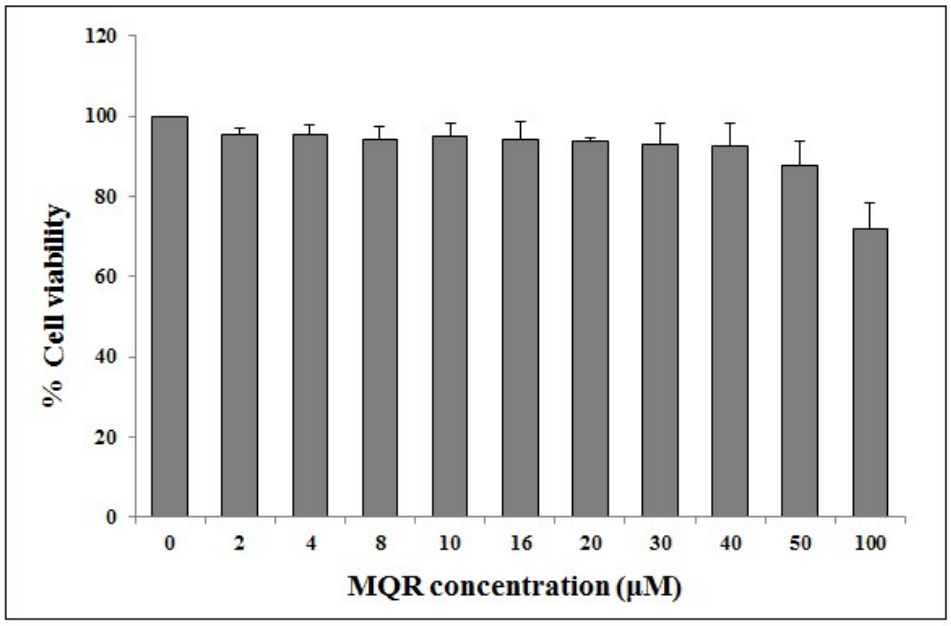


Figure S1. Cell viability of MQR. A549 cells were incubated with each concentration of MQR for 24h, then examined using WST-1 test. Graphs are representative of three separate experiments and show mean concentrations with standard error bars.

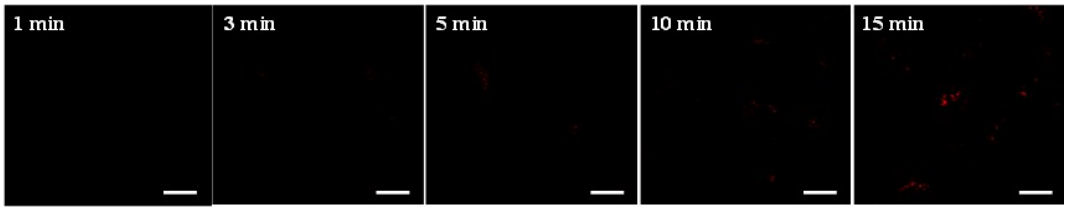


Figure S2. Time-dependent fluorescence changes of A549 cells that were incubated with 20 μM **MQR**. Scale bar, 5μm; magnification, x1000.

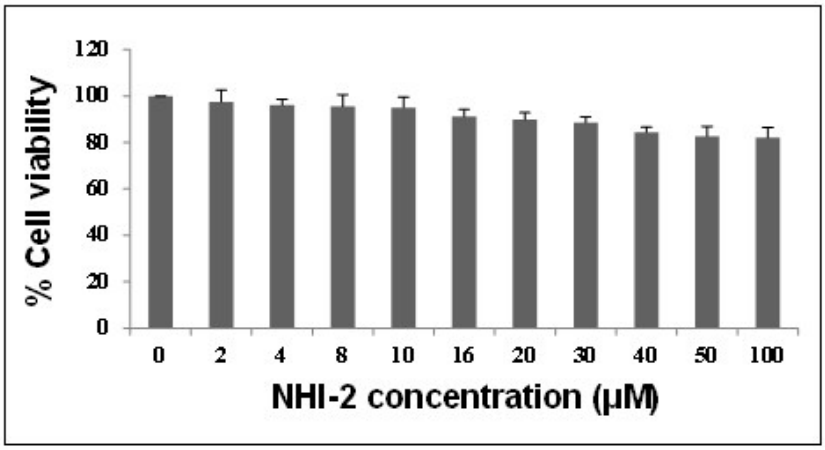


Fig. S3 Cell viability of NHI-2. A549 cells were incubated with each concentration of NHI-2 for 24h, then examined using WST-1 test. Graphs are representative of three separate experiments and show mean concentrations with standard error bars.

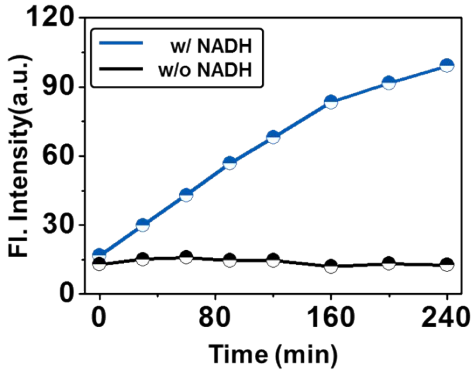


Fig. S4 Time-dependent fluorescence intensity change of MQR (5 μM) in the presence (400 μM) and absence of NADH.

6. ^{1}H, ^{13}C NMR and ESI-MS spectra

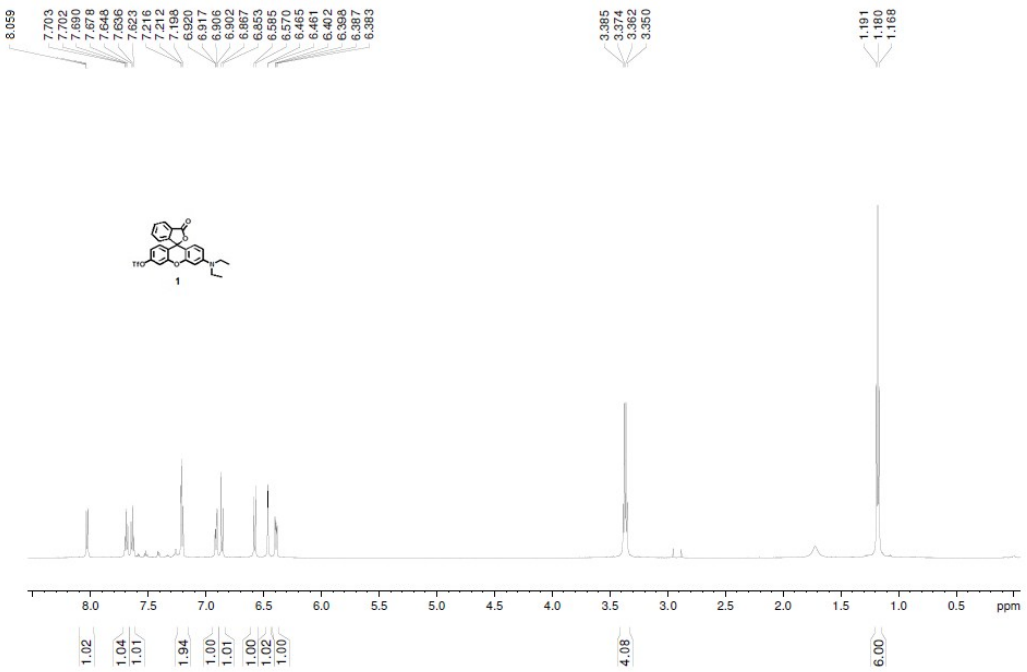


Fig. S5 ^{1}H-NMR of 1 in $CDCl_3$.

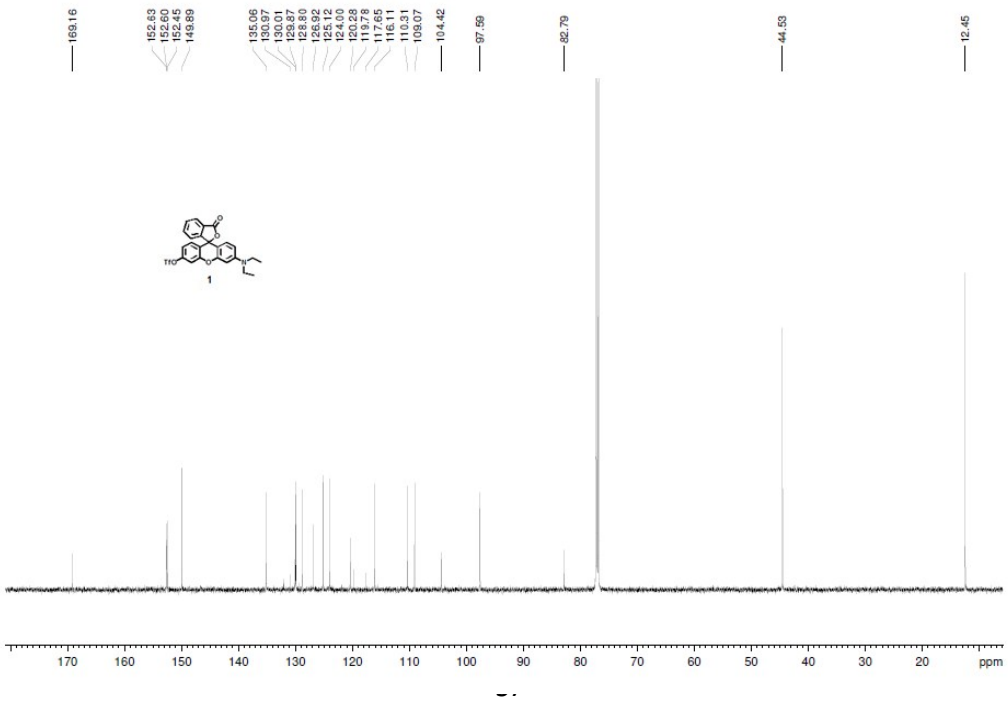


Fig. S6 ^{13}C-NMR of 1 in $CDCl_3$.

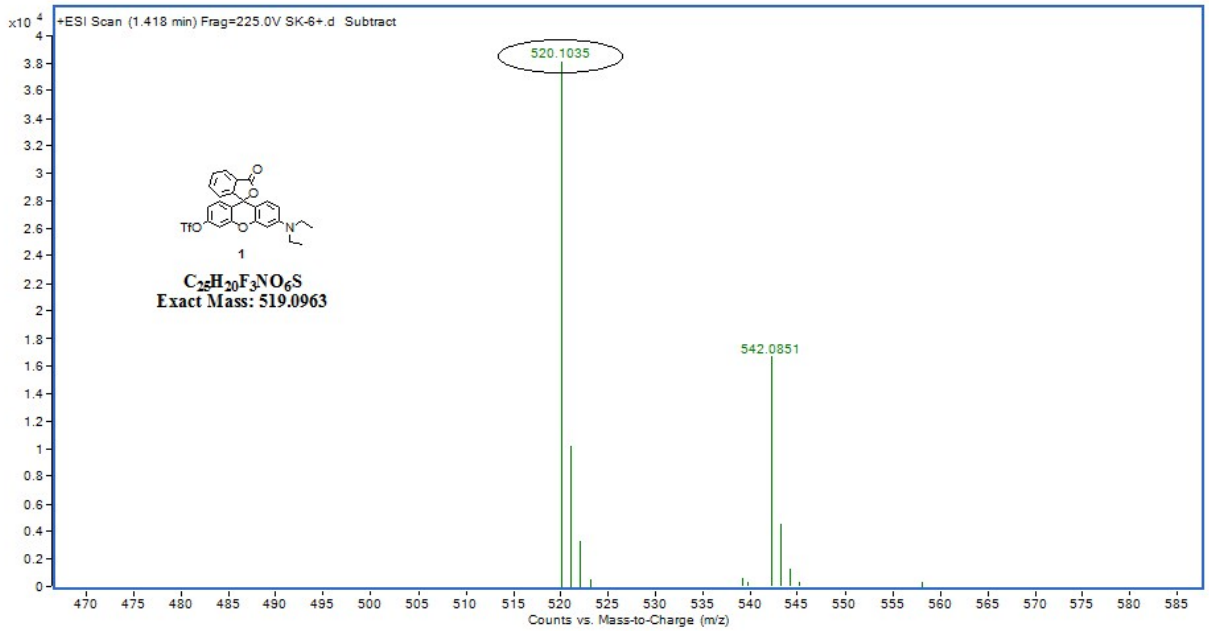


Fig. S7 ESI-HRMS of 1.

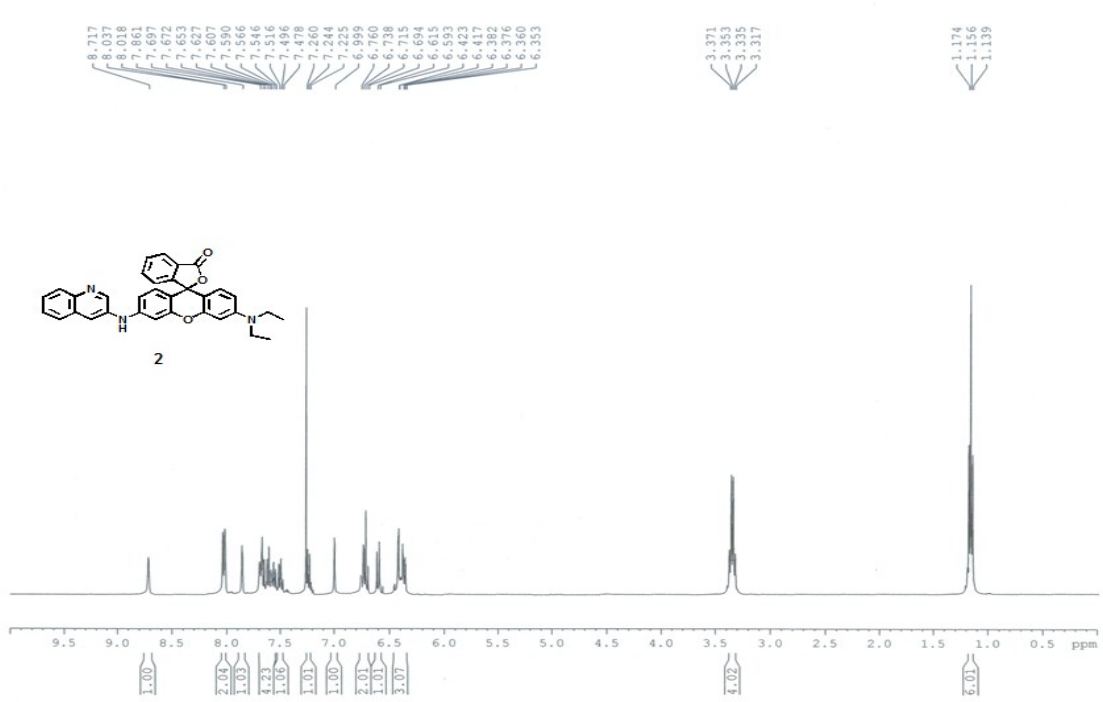


Fig. S8 ^{1}H-NMR of 2 in $CDCl_3$.

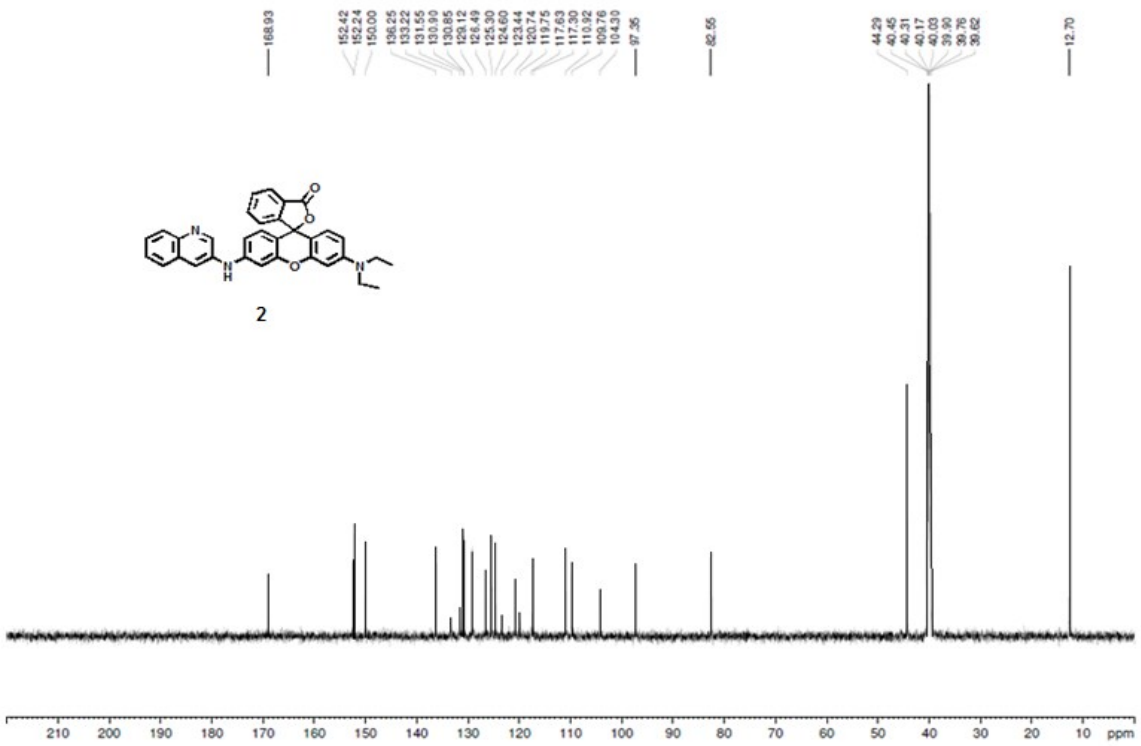


Fig. S9 ^{13}C-NMR of 2 in DMSO-d_6.

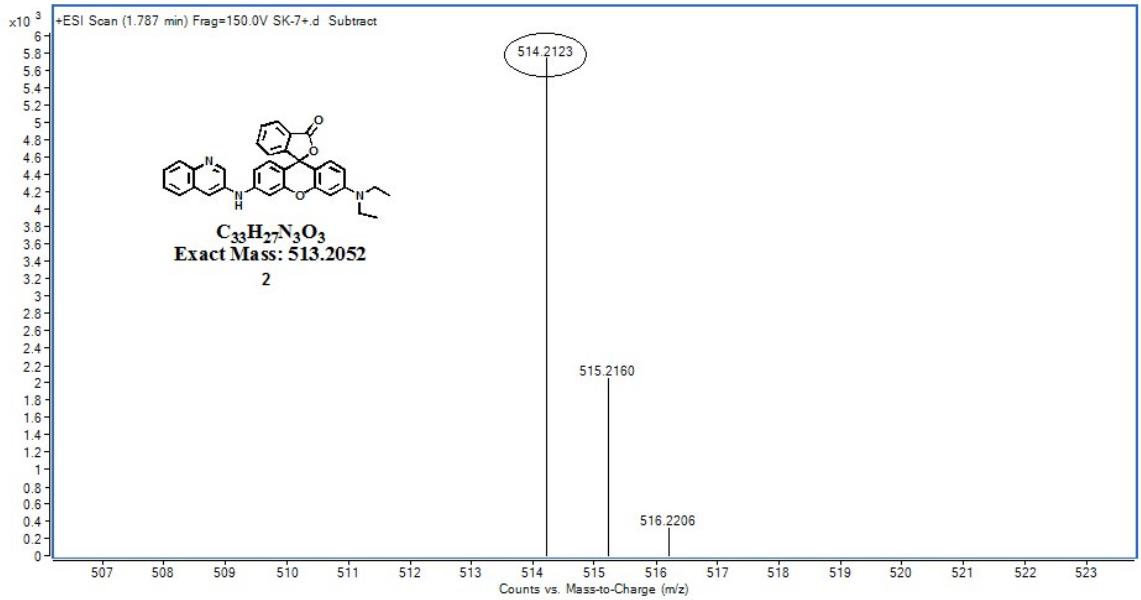


Fig. S10 ESI-HRMS of 2.

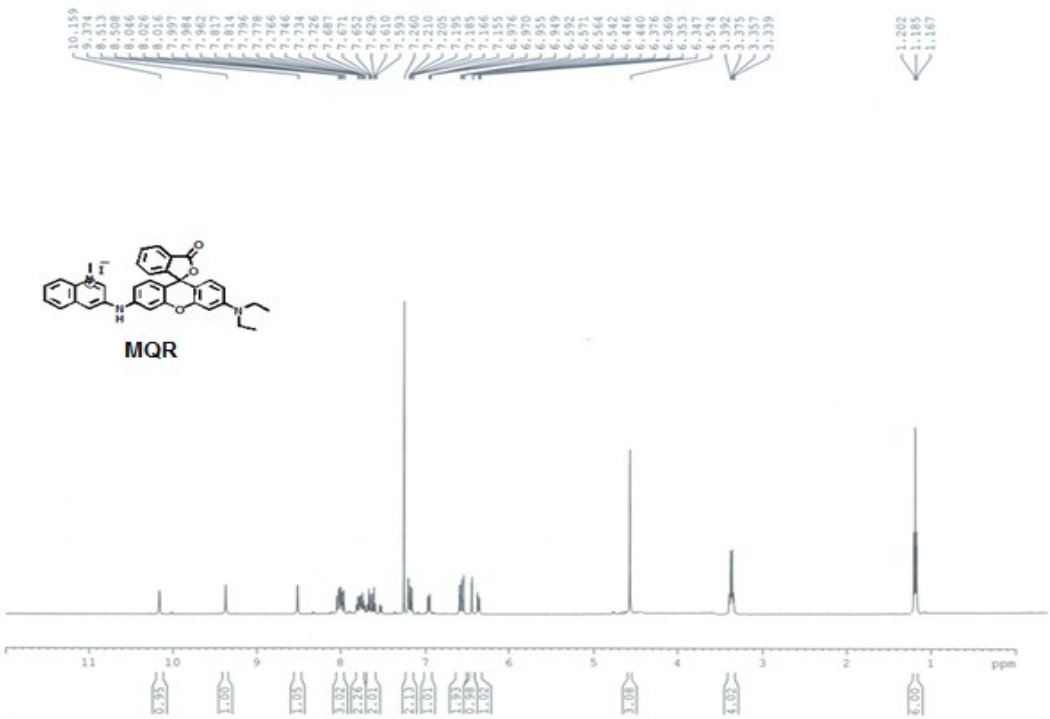


Fig. S11 ^{1}H-NMR of MQR in $CDCl_3$.

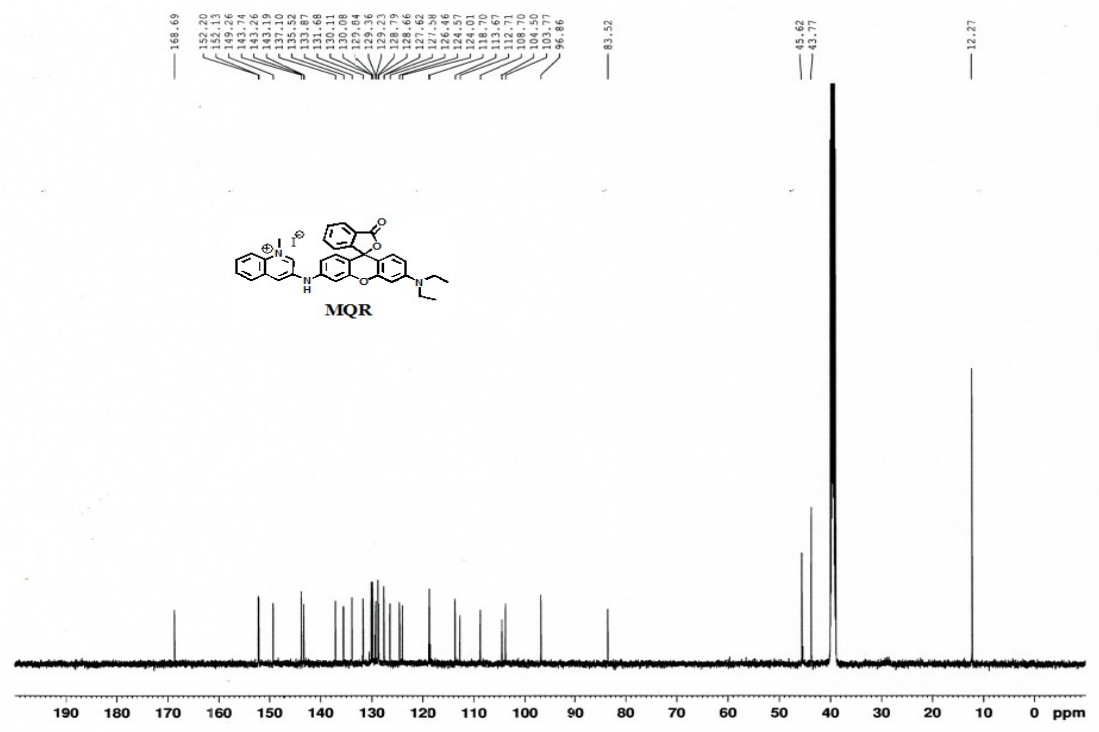
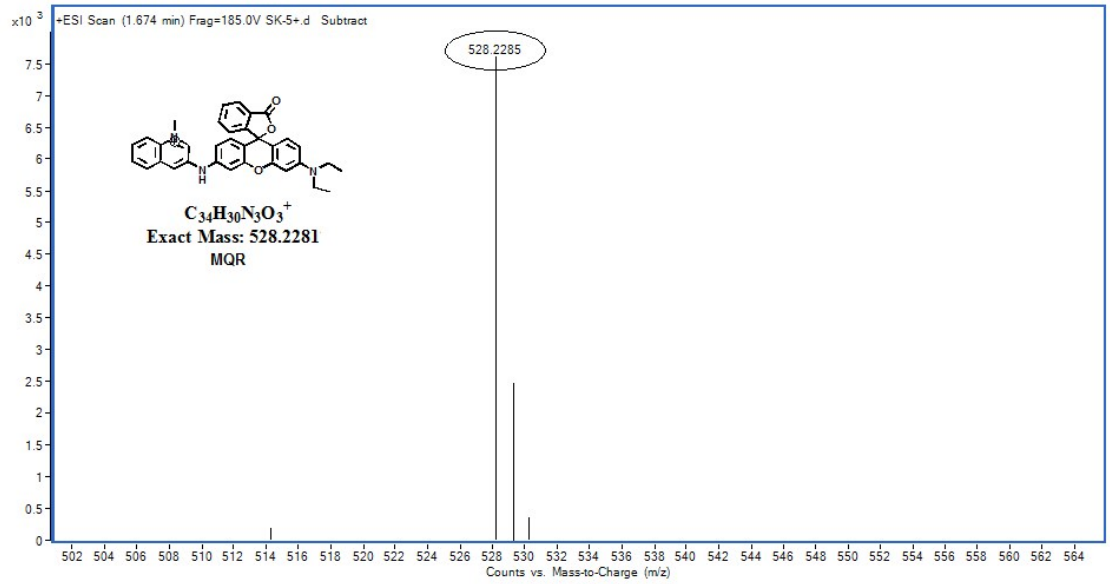


Fig. S12 ^{13}C-NMR of MQR in DMSO-d_6.



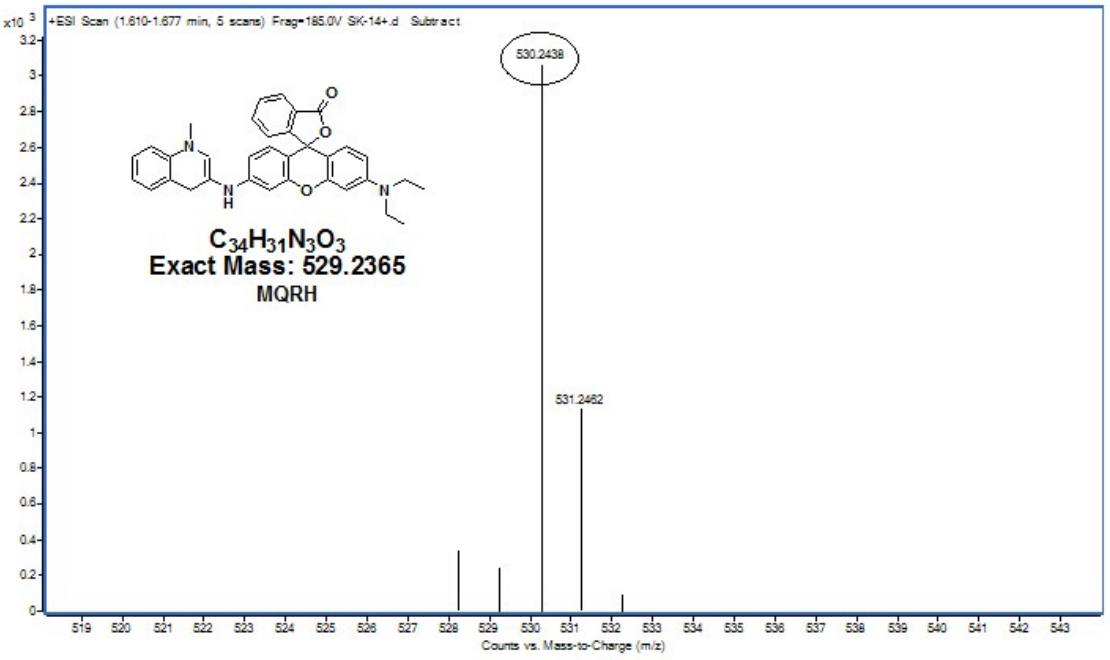


Fig. S13 ESI-HRMS of MQR.

Fig. S14 ESI-HRMS of MQRH after treatment with NADH.

7. Reference

1. H. G. Im, H. Y. Kim and S. K. Chang, *Sens. Actuators B*, 2014, **191**, 854-859.

2. P. Verwilst, J. Han, J. Lee, S. Mun, H.-G. Kang and J. S. Kim, *Biomaterials,* 2017, **115**, 104-114.