Electronic Supplementary Information

Small Quinolinium-Based Enzymatic Probes via Blue-to-Red Ratiometric Fluorescence

Pan Wang,[a] Jiajun Du,[a] Huijing Liu,[b] Guoqiang Bi*[b] and Guoqing Zhang*[a]

a. Heifei National Laboratory for Physical Sciences at the Microscale and Department of Polymer Science and Engineering, University of Science and Technology of China, 230026 Hefei, P. R. China.
b. CAS Key Laboratory of Brain Function and Disease and Department of Neurobiology and Biophysics, School of Life Sciences, University of Science and Technology of China, 230026 Hefei, P. R. China.

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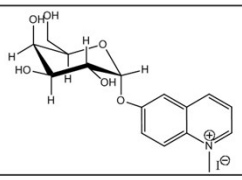
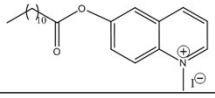
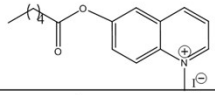
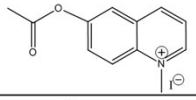
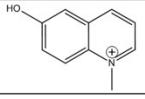
Email: gzhang@ustc.edu.cn

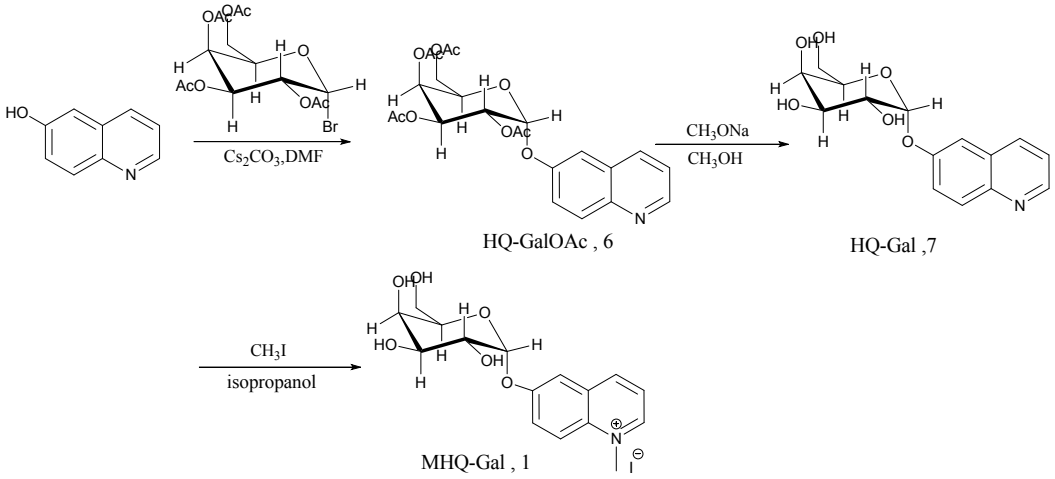
Part I. Materials and Instruments

Water was deionized with a Milli-Q SP reagent water system (Millipore) to a specific resistivity of 18.2 MΩ.cm. β-Galactosidase from *E.coli* (molecular weight 540,000 Da, EC3.2.1.23) and porcine pancreatic lipase (PPL, molecular weight 49,859 Da EC 3.1.1.3) were purchased from Sigma-Aldrich. DMF and CH_2Cl_2 were dried over KOH prior to use. All other chemicals and solvents were obtained from Aladdin Reagent Inc. (Shanghai) and were used as received without further purification. ^{1}H NMR (400 MHz) spectra were recorded on a Bruker AV300 NMR spectrometer operated in the Fourier transform mode. ^{1}H-NMR chemical shifts were reported in standard format as values in ppm relative to deuterated solvents. Mass spectral data (ESI/MS) were obtained on a Micromass auto spectrometer. UV/Vis absorption spectra were recorded on a Beijing Persee TU-1901 UV-Vis spectrometer. Excitation and steady-state fluorescence emission spectra and absolute quantum yield were recorded on a FluoroMax-4 spectrofluorometer (Horiba Scientific) and analyzed with an Origin integrated software FluoroEssence (v2.2). Fluorescence and lifetime data were acquired with a nanoLED laser with the excitation peak at 370 nm (NanoLED-370). Absolute fluorescence quantum yields were measured on a Hamamatsu Quantaurus-QY spectrometer.

Part II. Sample Synthesis

Number	Name	Structure
1	MHQ-Gal	
2	MHQ-12C	
3	MHQ-6C	
4	MHQ-2C	
5	MHQ	

Methylated 6-hydroxyquinolinium-β-galactose conjugate (MHQ-Gal, 1)

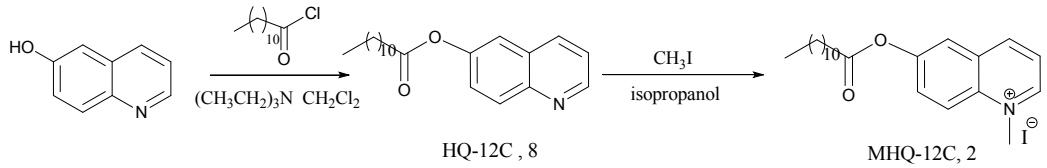


Synthesis of acylated 6-hydroxyquinoline-β-galactose (HQ-GalOAc, 6). 6-Hydroxyquinoline (0.600 g, 2.36 mmol) and cesium carbonate (1.00 g, 3.07 mmol) were dissolved in dry DMF (10 mL). Tetra-O-acetyl-α-D-galactopyranosyl-1-bromide (1.00 g, 2.43 mmol) in dry DMF (10 mL) was added dropwise and the solution was stirred at room temperature (20°C) for ~12 h. The reaction mixture was then poured into a hydrochloric acid solution (0.1 M, 200 mL) in an ice bath. The product was extracted with ethyl acetate (3×), and the combined organic layers were dried over sodium sulfate, filtered, and evaporated *in vacuo*. The crude product was purified by silica-gel column chromatography (ethyl acetate/dichloromethane, 1:1). The product was obtained as a white powder (0.39 g, 35%).^{1}H-NMR (400 MHz, Methanol-d_4) ,δ (TMS, ppm): 8.75 (dd, J = 4.4, 1.7 Hz, 1H), 8.35 – 8.26 (m, 1H), 7.99 (d, J = 9.0 Hz, 1H), 7.58 – 7.43 (m, 3H), 5.56 – 5.39 (m, 3H), 5.31 (dd, J = 10.3, 3.5 Hz, 1H), 4.41 (ddd, J = 7.3, 6.1, 1.2 Hz, 1H), 4.22 (dd, J = 6.5, 2.2 Hz, 2H), 2.19 (s, 3H), 2.07 (s, 3H), 2.03 (s, 3H), 1.99 (s, 3H).HRMS (ESI+): calcd for $[M+H]^+$, 476.1512; found, 476.1545.

Synthesis of 6-hydroxylquinoline-β-galactose (HQ-Gal, 7). HQ-GalOAc (6) (0.20 g, 0.42 mmol) was dissolved in methanol (10 mL) to which sodium methoxide (0.050 g, 0.93 mmol) was added at 0°C; the solution was stirred at room temperature (20°C) for ~10 h. The reaction mixture was neutralized with dilute hydrochloric acid and precipitated in 100-mL diethyl ether. The product was obtained as a white powder (0.12 g, 93%). ^{1}HNMR (400 MHz, Methanol-d_4), δ (TMS, ppm): 8.76 (dd, J = 4.6, 1.6 Hz, 1H), 8.40 (d, J = 8.3 Hz, 1H), 7.98 (d, J = 9.0 Hz, 1H), 7.63 (d, J = 9.1 Hz, 2H), 7.56 (dd, J = 8.4, 4.5 Hz, 1H), 5.07 (d, J = 7.7 Hz, 1H), 3.96 – 3.75 (m, 5H), 3.64 (dd, J = 9.7, 3.4 Hz, 1H). HRMS (ESI+): calcd for $[M+H]^+$, 308.1089; found, 308.1121.

Synthesis of methylated 6-hydroxyquinolinium-β-galactose (MHQ-Gal, 1). HQ-Gal (7) (120 mg, 0.39 mmol) and iodomethane (0.50 g, 3.52 mmol) were dissolved in isopropanol (5 mL) and the reaction mixture was heated at reflux overnight and then precipitated in 50 mL diethyl ether (3×) to afford a white powder. The product was purified by prep-scale HPLC (methanol:water = 1:1). The final product was a pale yellow solid (50 mg, 30%). ^{1}H-NMR (400 MHz, DMSO-d_6),δ (TMS, ppm): 9.35 (d, J = 5.7 Hz, 1H), 9.09 (d, J = 8.4 Hz, 1H), 8.53 – 8.43 (m, 1H), 8.11 (dd, J = 8.5, 5.7 Hz, 1H), 7.98 (d, J = 8.2 Hz, 2H), 5.17 (d, J = 7.6 Hz, 1H), 4.62 (s, 3H), 3.81 – 3.46 (m, 6H).HRMS (ESI+): calcd for $[M+H]^+$, 323.1324; found, 323.1315.

6-(Alkoyloxy)-1-methylquinolin-1-ium. (MHQ-12C, 2)

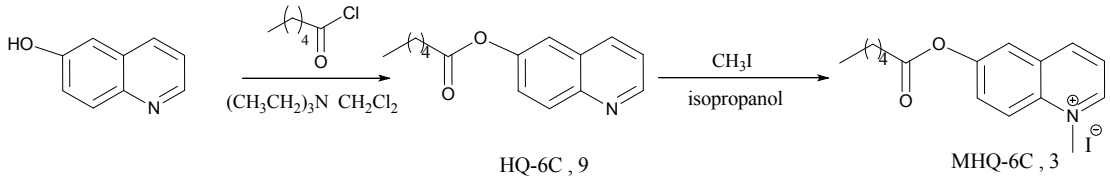


HQ-12C , 8 MHQ-12C, 2

Synthesis of quinolin-6-yl dodecanoate (HQ-12C, 8). Dodecanoyl chloride (2.30 g, 10.51 mmol) and 6-hydroxyquinoline (1.50 g, 10.34 mmol) were dissolved in 50-mL dry dichloromethane to which triethylamine (1.20 g, 11.88 mmol) was added dropwise. The mixture was allowed to react at 50°C overnight and was dried *in vacuo*. The crude product was purified by column chromatography over silica gel (ethyl acetate / hexane, 2:1) to give the final product as a brown solid (2.63 g, 78%).^{1}H-NMR (400 MHz, DMSO-d_6),δ (TMS, ppm):8.91 (dd, J = 4.1, 1.7 Hz, 1H), 8.38 (dd, J = 8.4, 1.7 Hz, 1H), 8.06 (d, J = 9.0 Hz, 1H), 7.75 (d, J = 2.6 Hz, 1H), 7.56 (dq, J = 9.5, 3.4, 2.6 Hz, 2H), 2.65 (t, J = 7.3 Hz, 2H), 1.68 (p, J = 7.4 Hz, 2H), 1.44 – 1.16 (m, 16H), 0.85 (t, J = 6.6 Hz, 3H). HRMS (ESI+): calcd for $[M+H]^+$, 328.2232; found, 328.2267.

Synthesis of 6-(dodecanoyloxy)-1-methylquinolin-1-ium (MHQ-12C, 2). HQ-12C (8) (0.900 g, 2.75 mmol) and iodomethane (1.00 g, 7.04 mmol) were dissolved in isopropanol (15 mL) and heated at reflux overnight. The reaction mixture was then precipitated in diethyl ether (200 mL, 3×) and dried *in vacuo* at 35 °C. The purified product was obtained as a yellow solid (1.13 g ,88%). ^{1}H-NMR (400 MHz, DMSO-d_6) ,δ (TMS, ppm): 9.50 (d, J = 5.7 Hz, 1H), 9.24 (d, J = 8.4 Hz, 1H), 8.60 (d, J = 9.5 Hz, 1H), 8.30 (d, J = 2.6 Hz, 1H), 8.20 (dd, J = 8.5, 5.7 Hz, 1H), 8.14 (dd, J = 9.6, 2.6 Hz, 1H), 4.65 (s, 3H), 2.72 (t, J = 7.4 Hz, 2H), 1.70 (p, J = 7.4 Hz, 2H), 1.45 – 1.20 (m, 16H), 0.93 – 0.81 (m, 3H). HRMS (ESI+): calcd for $[M+H]^+$, 343.2467; found, 343.2453.

6-(Alkoyloxy)-1-methylquinolin-1-ium (MHQ-6C, 3)



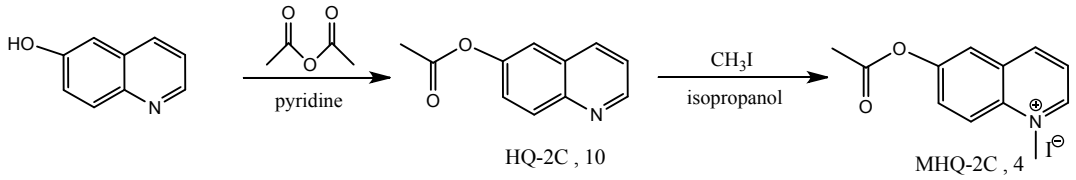
HQ-6C , 9 MHQ-6C , 3

Synthesis of quinolin-6-yl hexanoate (HQ-6C, 9). Hexanoyl chloride (1.50 g, 11.1 mmol) and 6-hydroxyquinoline (0.900 g, 6.21 mmol) were dissolved in 50-mL dry dichloromethane to which triethylamine (1.0 g, 9.90 mmol) was added dropwise. The mixture was allowed to react at 50°C overnight and was dried *in vacuo*. The crude product was purified by column chromatography over silica gel (ethyl acetate /

hexane, 2:1) to give the final product as a yellow solid (1.28 g, 85%). ^{1}H-NMR (400 MHz, DMSO-d_6) ,δ (TMS, ppm): 8.91 (dd, J = 4.2, 1.7 Hz, 1H), 8.37 (dd, J = 8.3, 1.7 Hz, 1H), 8.07 (d, J = 9.1 Hz, 1H), 7.76 (d, J = 2.6 Hz, 1H), 7.60 – 7.50 (m, 2H), 2.64 (t, J = 7.4 Hz, 2H), 1.67 (p, J = 7.3 Hz, 2H), 1.34 (tt, J = 7.0, 3.7 Hz, 4H), 0.89 (t, J = 6.8 Hz, 3H). HRMS (ESI+): calcd for $[M+H]^+$, 244.1293; found, 244.1329.

Synthesis of 6-(hexanoyloxy)-1-methylquinolin-1-ium (MHQ-6C, 3). HQ-6C (9) (0.600 g, 2.47 mmol) and iodomethane (1.00 g, 7.04mmol) were dissolved in isopropanol (10 mL). The mixture was heated at reflux overnight and then precipitated in diethyl ether (200 mL, 3×) and dried *in vacuo* at 35°C. The purified product was obtained as a yellow solid (0.57 g, 60%). ^{1}H-NMR (400 MHz, DMSO-d_6), δ (TMS, ppm):9.50 (d, J = 5.7 Hz, 1H), 9.24 (d, J = 8.5 Hz, 1H), 8.60 (d, J = 9.5 Hz, 1H), 8.30 (d, J = 2.6 Hz, 1H), 8.24 – 8.10 (m, 2H), 4.65 (s, 3H), 2.72 (t, J = 7.4 Hz, 2H), 1.71 (p, J = 7.3 Hz, 2H), 1.38 (hd, J = 9.8, 9.0, 4.4 Hz, 4H), 0.92 (t, J = 6.9 Hz, 3H). HRMS (ESI+): calcd for $[M+H]^+$, 259.1528; found, 259.1512.

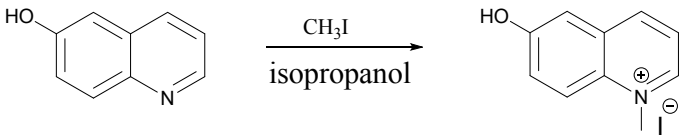
6-(Alkoyloxy)-1-methylquinolin-1-ium (MHQ-2C, 4)



Synthesis of quinolin-6-yl acetate (HQ-2C, 10). To a solution of 6-hydroxyquinoline (1.500 g, 10.34 mmol) in dry pyridine (30 mL), acetic anhydride (2.000 g, 19.61 mmol) was added. The reaction mixture was stirred at room temperature for ~3 h, then evaporated, and the residue was purified by column chromatography over silica gel (ethyl acetate / hexane, 1:2). The final product was brown solid (1.30 g, 67%). ^{1}H-NMR (400 MHz, DMSO-d_6), δ (TMS, ppm): 8.91 (dd, J = 4.2, 1.7 Hz, 1H), 8.44 – 8.31 (m, 1H), 8.07 (d, J = 9.1 Hz, 1H), 7.77 (d, J = 2.6 Hz, 1H), 7.58 (dt, J = 8.4, 3.2 Hz, 2H), 2.35 (s, 3H). HRMS (ESI+): calcd for $[M+H]^+$, 188.0667; found, 188.0704.

Synthesis of 6-acetoxy-1-methylquinolin-1-ium (MHQ-2C, 4). HQ-2C (10, 0.8 g, 4.28 mmol) and iodomethane (1 g, 7.04 mmol)were dissolved in isopropanol (10 mL) .The mixture was heated at reflux overnight and then precipitated in diethyl ether(200 mL, 3×) and dried *in vacuo* at 35°C .The purified product was luminous yellow solid (0.98 g, 70%). ^{1}H-NMR (400 MHz, DMSO-d_6), δ (TMS, ppm): 9.51 (d, J = 5.7 Hz, 1H), 9.24 (d, J = 8.4 Hz, 1H), 8.60 (d, J = 9.5 Hz, 1H), 8.30 (d, J = 2.6 Hz, 1H), 8.23 – 8.12 (m, 2H), 4.65 (s, 3H), 2.41 (s, 3H). HRMS (ESI+): calcd for $[M+H]^+$, 203.0902; found, 203.0896.

Methylated 6-hydroxylquinolinium (MHQ, 5)



MHQ , 5

6-Hydroxyquinoline (1.20 g, 8.27 mmol), iodomethane (1.76 g, 12.40 mmol), and isopropanol (10 mL) were added to a round bottom flask equipped with a magnetic stir bar. The mixture was heated at reflux overnight and precipitated (3×) in 200 mL diethyl ether. The precipitates were dried *in vacuo* overnight at 35°C to give pure product as a dark brown solid (2.13 g, 90%). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 11.12 (s, 1H), 9.23 (d, J = 5.6 Hz, 1H), 9.05 (d, J = 8.4 Hz, 1H), 8.39 (d, J = 9.5 Hz, 1H), 8.03 (dd, J = 8.5, 5.7 Hz, 1H), 7.78 (dd, J = 9.5, 2.8 Hz, 1H), 7.60 (d, J = 2.8 Hz, 1H), 4.58 (s, 3H). HRMS (ESI+): calcd for $[M+H]^+$, 161.0796; found, 161.0786.

Part III. Supplementary Figures

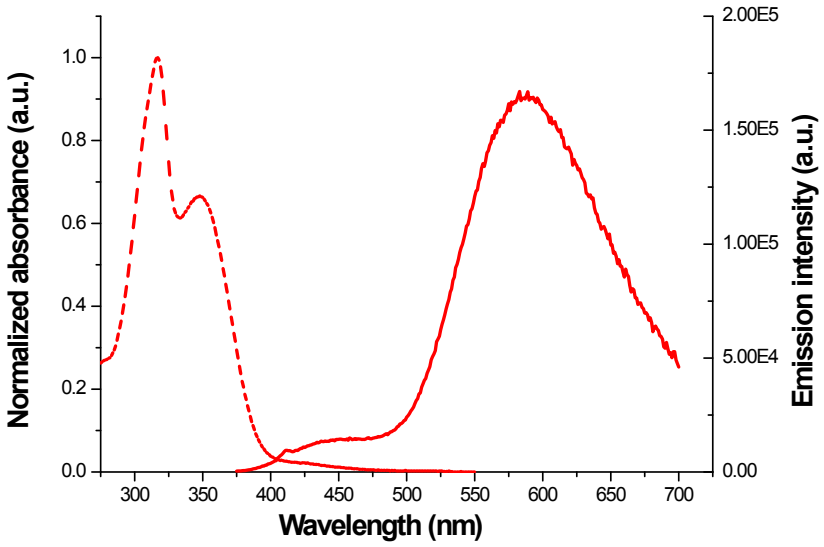


Figure S1. Normalized absorbance(dotted line) and steady-state emission(solid line) spectra of **MHQ** in phosphate buffer (pH 7.3).(λ_{ex} = 360 nm)

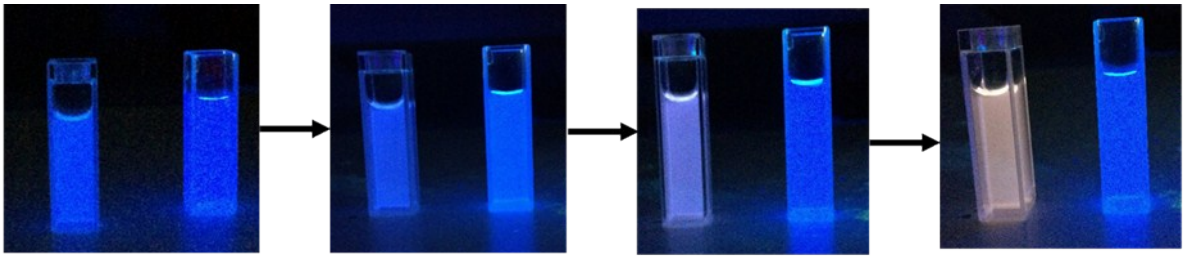


Figure S2. Images showing time-dependent fluorescence change of **MHQ-Gal** after the addition of β-galactosidase within 5 minutes at RT (25°C ± 2 °C). (Illumination source: a 365-nm hand)

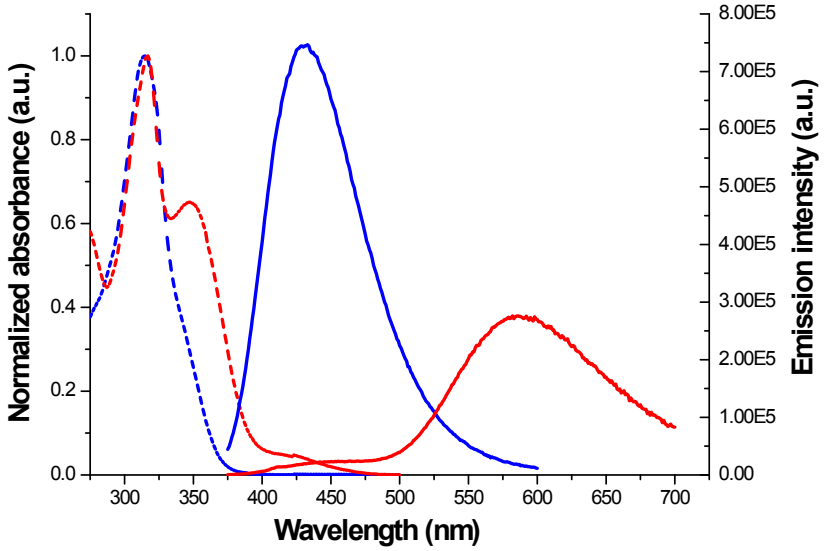


Figure S3. Normalized absorbance (dotted line) and steady-state emission (solid line) spectra of **MHQ-Gal** (100 μg/mL) before (blue line) and after (red line) the addition of β-galactosidase (0.05mg/mL) incubated for ~0.5 h in phosphate buffer (pH = 7.3) at 37 °C . (λ_{ex} = 360 nm)

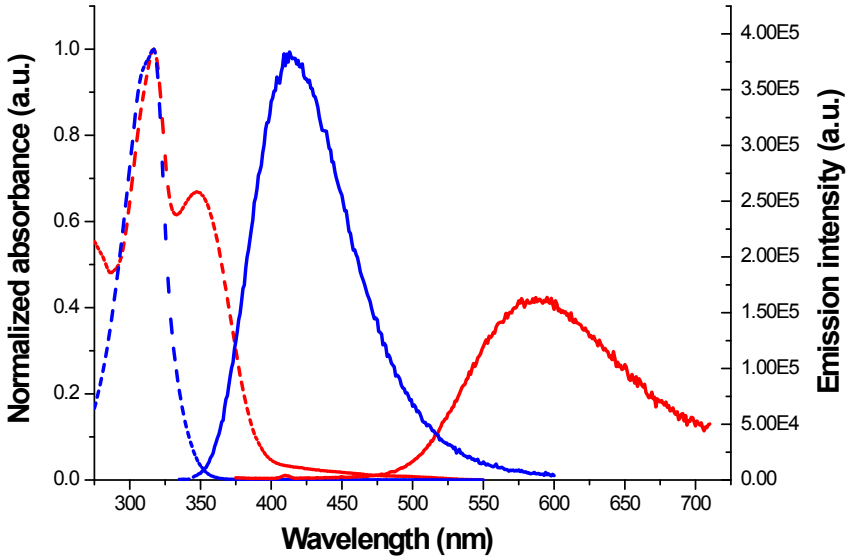


Figure S4. Normalized absorbance (dotted line) and steady-state emission (solid line) spectra of **MHQ-12C** (50 μg/mL) before (blue line) and after (red line) the addition of

PPL (0.1mg/mL) incubated for 1 h in phosphate buffer (pH = 7.3) at 37 °C ($\lambda_{ex,before}$ = 330 nm, $\lambda_{ex,after}$ = 360 nm).

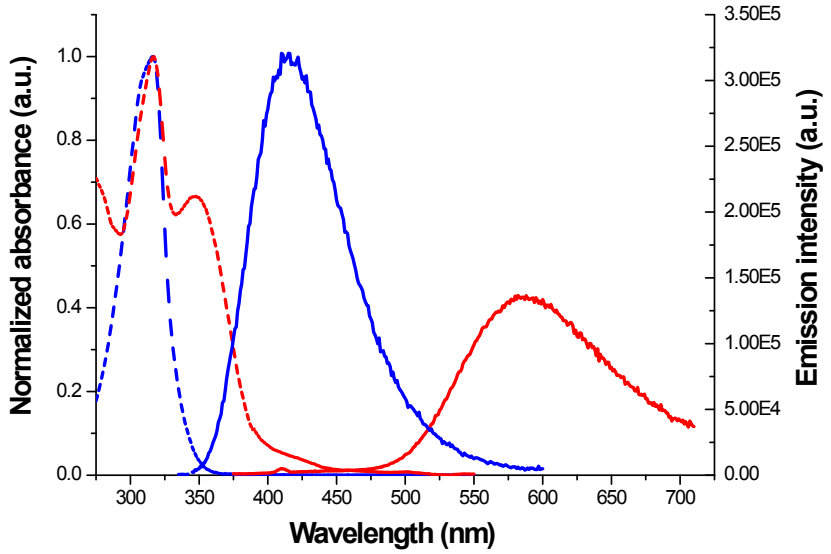


Figure S5. Normalized absorbance (dotted line) and steady-state emission (solid line) spectra of **MHQ-12C** (50 µg/mL) before (blue line) and after (red line) the addition of PPL (0.1mg/mL) incubated for 1 h in phosphate buffer (pH = 7.3) at 37 °C ($\lambda_{ex,before}$ = 330 nm, $\lambda_{ex,after}$ = 360 nm).

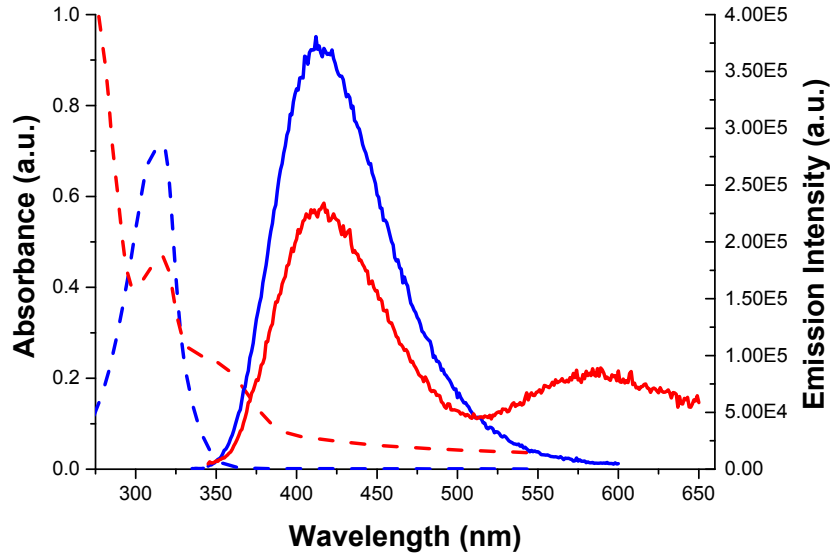


Figure S6. Absorbance (dotted line) and steady-state emission (solid line) spectra of **MHQ-2C** (50 µg/mL) before (blue line) and after (red line) the addition of PPL (0.1mg/mL) incubated for 1 h in phosphate buffer (pH = 7.3) at 37 °C ($\lambda_{ex,before}$ = 330 nm, $\lambda_{ex,after}$ = 340 nm).

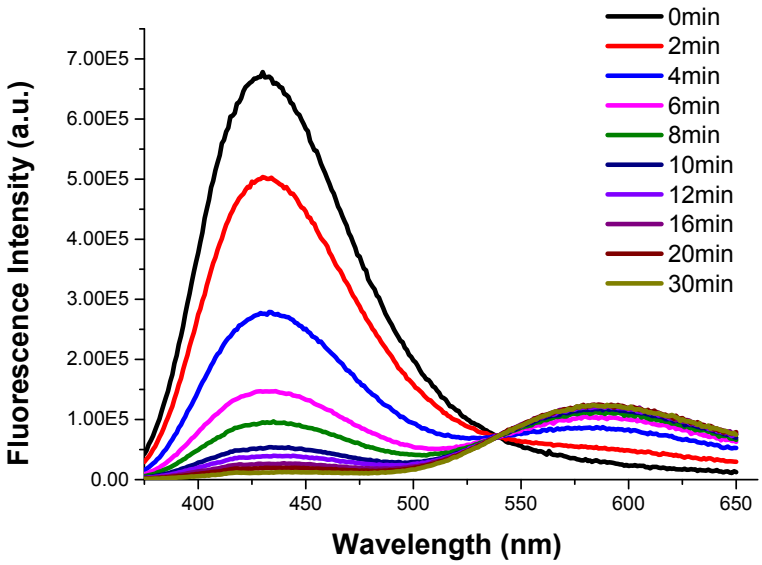


Figure S7. Time-dependent emission spectra of the solution of **MQ-Gal** (100 μg/mL) after the addition of β-galactosidase over 30 minutes in phosphate buffer (pH = 7.3) at RT (λ_{ex} = 360 nm).

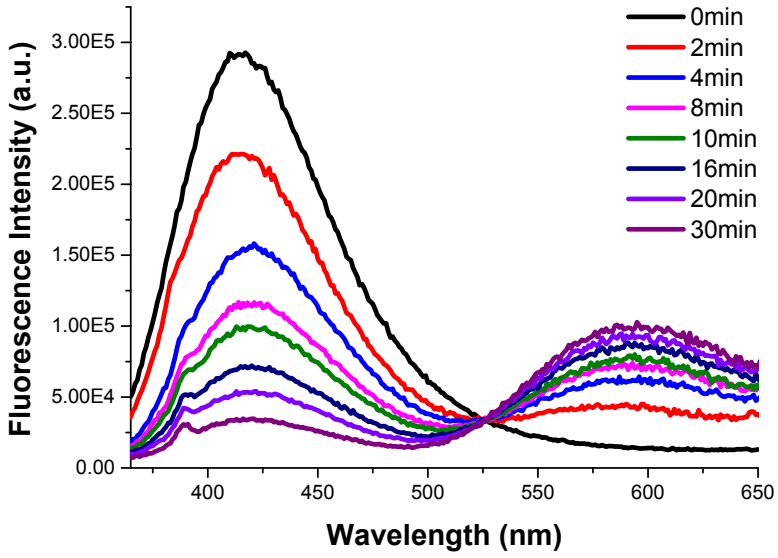


Figure S8. Time-dependent emission spectra of the solution of **MQ-12C** (50μg/mL) after the addition of PPL in 30 minutes in phosphate buffer (pH = 7.3) at RT (λ_{ex} = 340 nm).

Enzymatic Kinetics Assays. Fluorescence intensity vs. **MHQ** concentration standard curve was measured in PBS buffer solution (pH = 7.3). Various concentrations of **MHQ-Gal** (0-60 μM) and **MHQ-12C** (0-160 μM) were prepared in PBS buffer solution (pH = 7.3). β-Galactosidase enzyme was added to a final concentration of 0.1 mg/mL for **MHQ-Gal**. Porcine pancreatic lipase (PPL) was added to a final concentration of 0.1mg /mL for **MHQ-12C**. After incubation in 37°C for 180 s, the fluorescence intensity was collected at 588 nm (λ_{ex} = 360 nm). Kinetic constants such as k_{cat} and K_m were determined by linear fitting of the initial velocity vs. substrate concentration data to the Michaelis-Menten equation. Three independent experiments were performed.

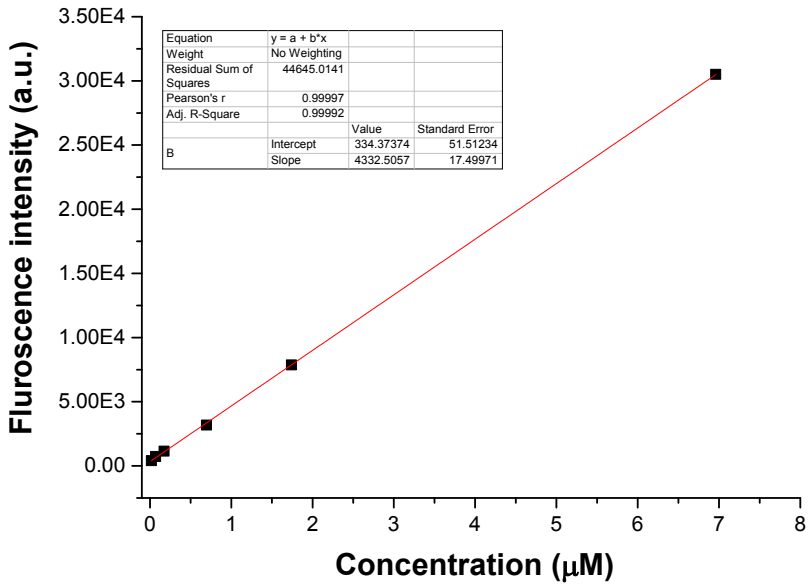


Figure S9. Fluorescence intensity to **MHQ** concentration standard curve (Abs<0.1).

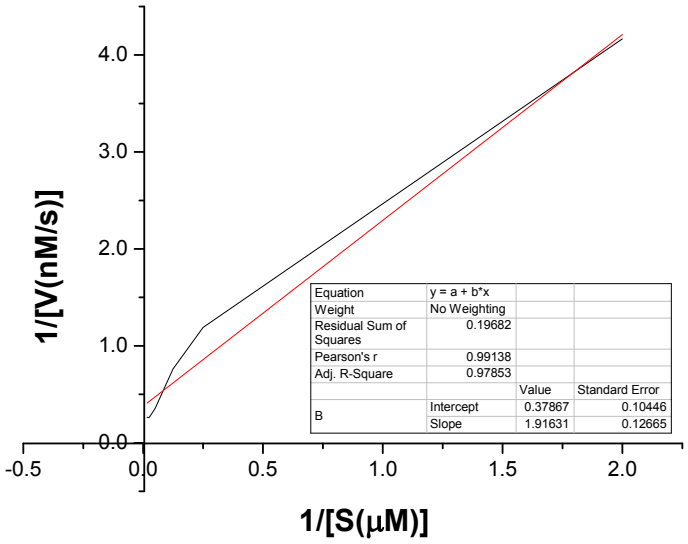


Figure S10. Lineweaver-Burk of Enzymatic Kinetics for **MHQ-Gal.**

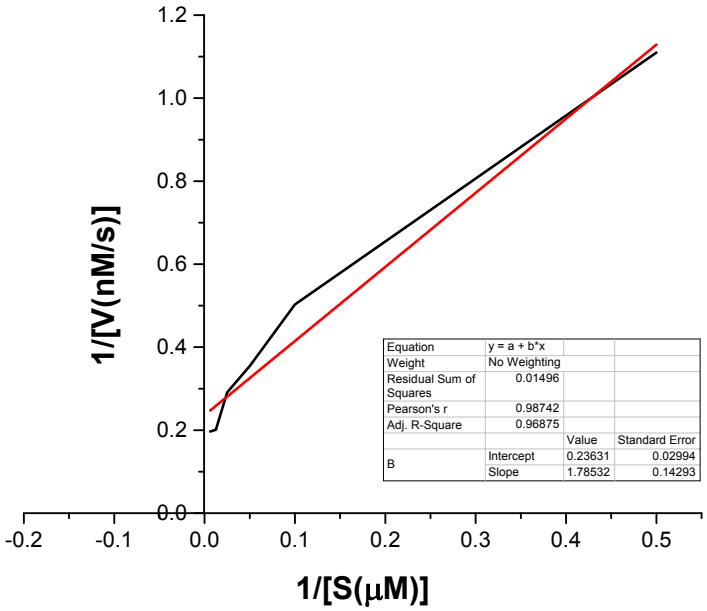


Figure S11. Lineweaver-Burk of Enzymatic Kinetics for **MHQ-12C.**

Response Time. The response time is related to enzyme concentration and substrate concentration. If the substrate concentration is fixed, the response time and the enzyme concentration are in an inversely proportional relationship. It means that if we have a higher enzyme concentration, then we have a higher speed of hydrolysis and a shorter response time. Consequently, the response time is not a fixed value, but depends on the specific *experiment and experience*. The general principle is to obtain the most dramatic fluorescence ratio in the shortest time possible. Two examples are given below.

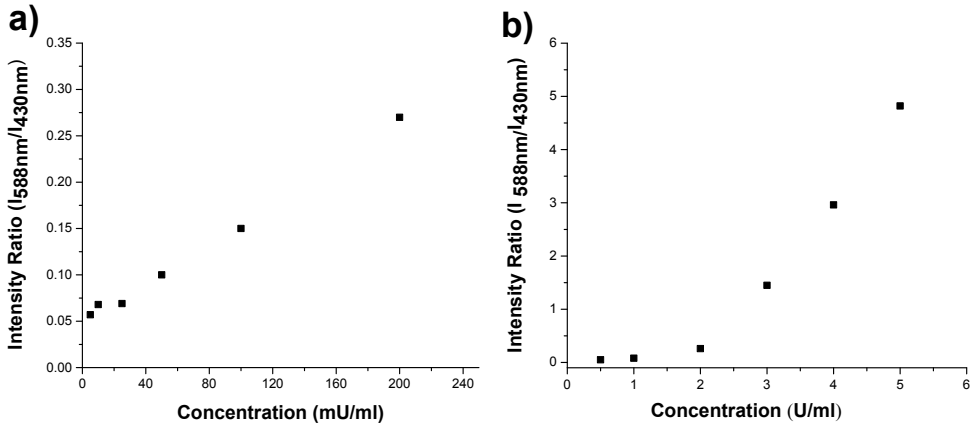


Figure S12. (a) Comparison of the fluorescence ratio 588nm/430nm after a 60-min incubation with the presence of 25 μg/mL **MHQ-Gal** at 37°C in a pH=7.3 buffer with increasing amounts of β-Galactosidase. **(b)** Comparison of the fluorescence ratio 588nm/430nm after a 30-min incubation with the presence of 50 μg/mL **MHQ-Gal** at 37°C in a pH=7.3 buffer with increasing amounts of β-Galactosidase.

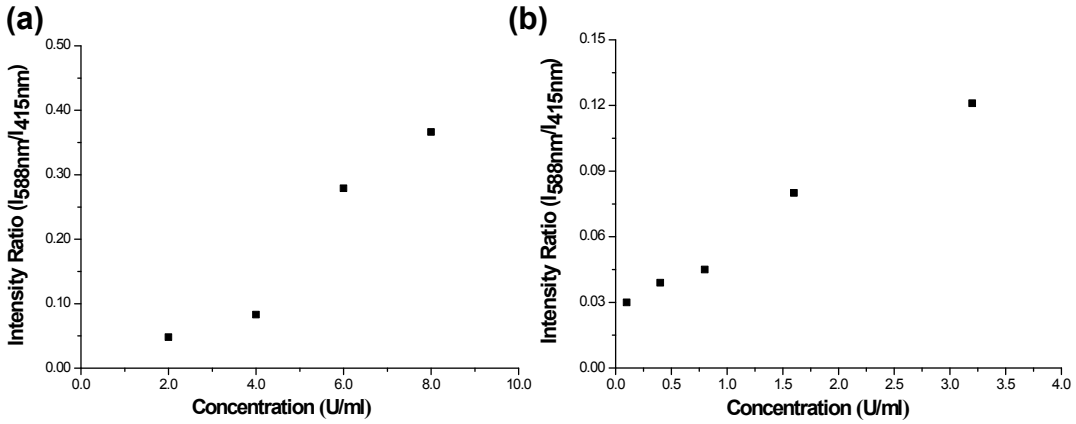


Figure S13. (a) Comparison of the fluorescence ratio 588nm/415nm after a 30-min incubation with the presence of 50 μg/mL **MHQ-12C** at 37°C in a pH=7.3 buffer with

increasing amounts of PPL. **(b)** Comparison of the fluorescence ratio 588nm/415nm after a 60-min incubation with the presence of 50 μg/mL **MHQ-12C** at 37°C in a pH=7.3 buffer with increasing amounts of PPL.

Table S1 . Spectroscopic Properties of dyes

	$\lambda_{Abs\text{-}max}$ /nm	λ_{em} /nm	ε ($M^{-1}cm^{-1}$) ($\lambda_{Abs\text{-}max}$)	Φ (Quantum yield)
MHQ	317	588	4850	0.0303
MHQ-Gal	314	431	5810	0.0987
MHQ-12C	317	416	5530	0.1668
MHQ-6C	317	415	9380	0.1644
MHQ-2C	317	415	4790	0.2018

Part IV. NMR and MS Figures

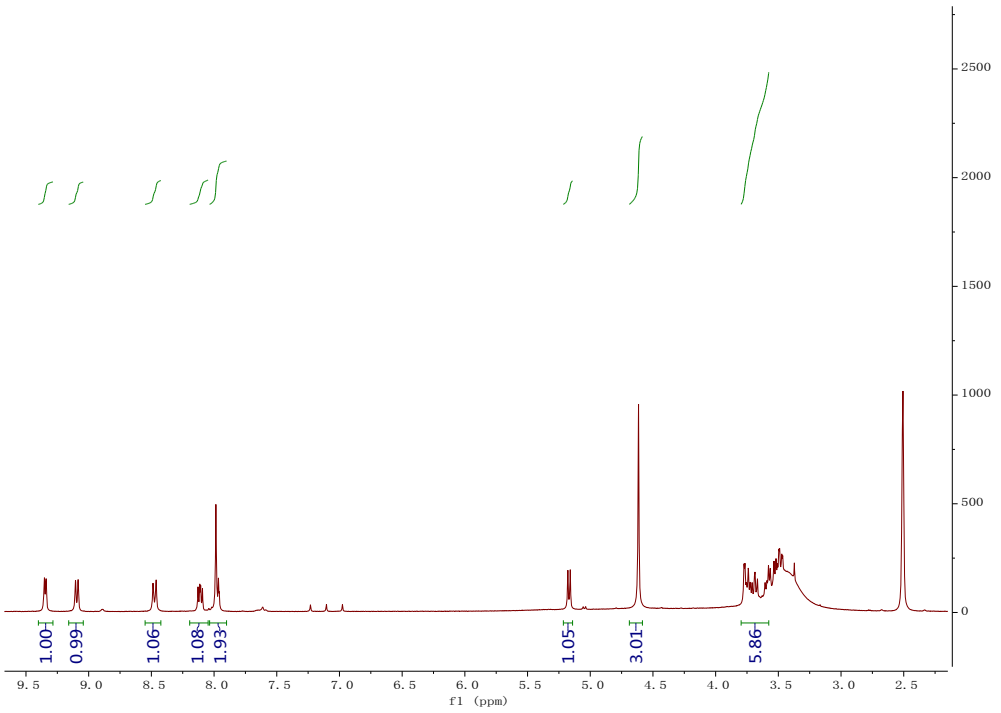


Figure S14. ^{1}H-NMR spectrum of **MHQ-Gal**(400 MHz, DMSO-d_6, 298 K)

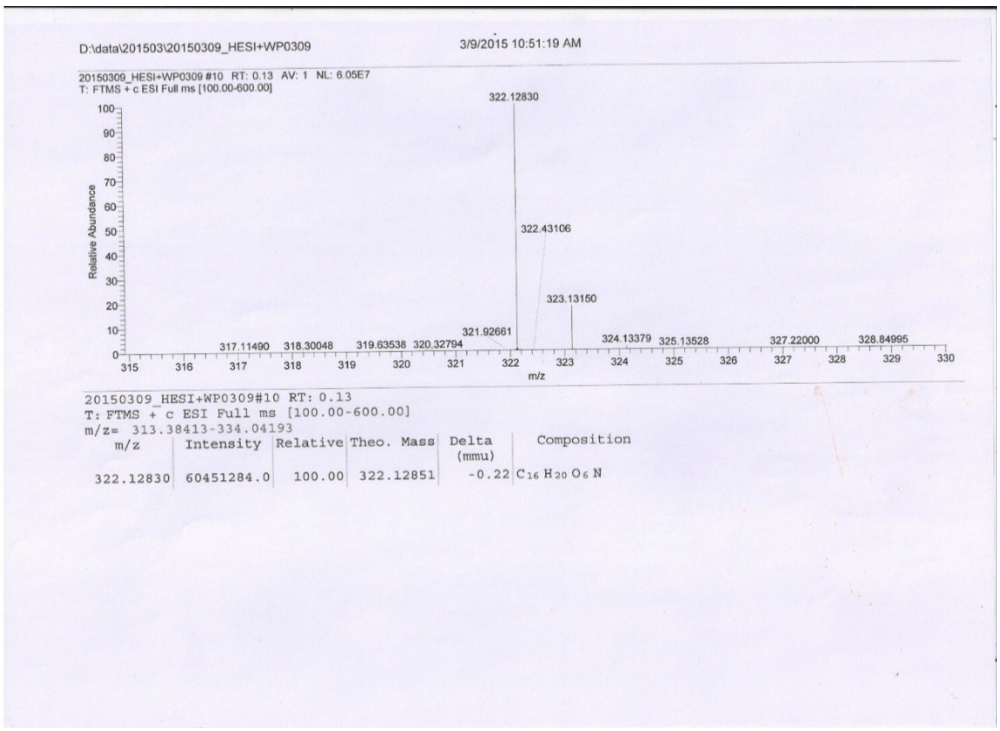


Figure S15.MS spectrum of **MHQ-Gal**

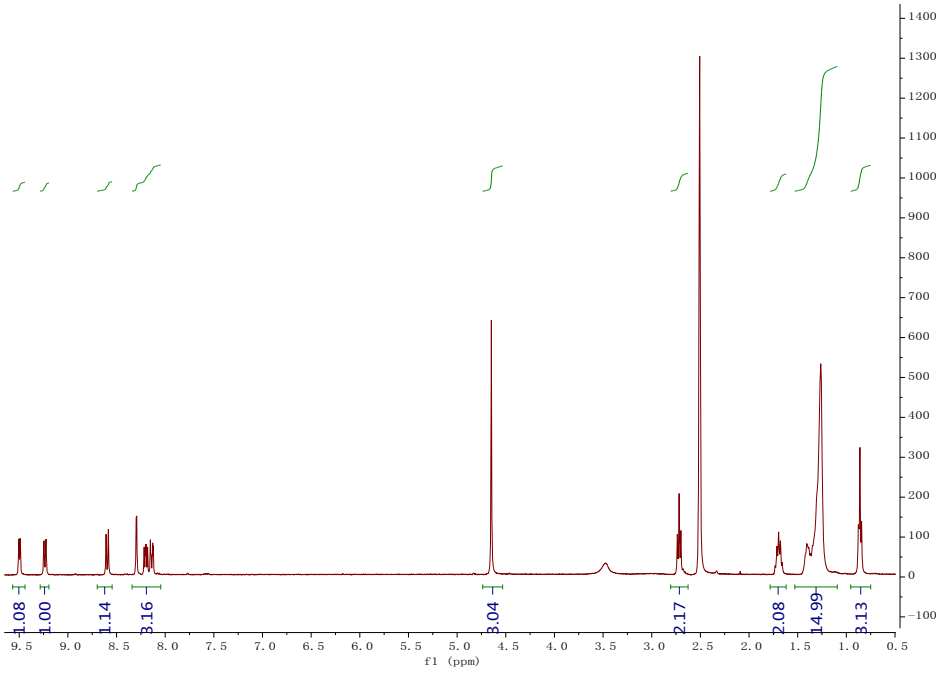


Figure S16. [1]H-NMR spectrum of **MHQ-12C**(400 MHz, DMSO-d_6, 298 K)

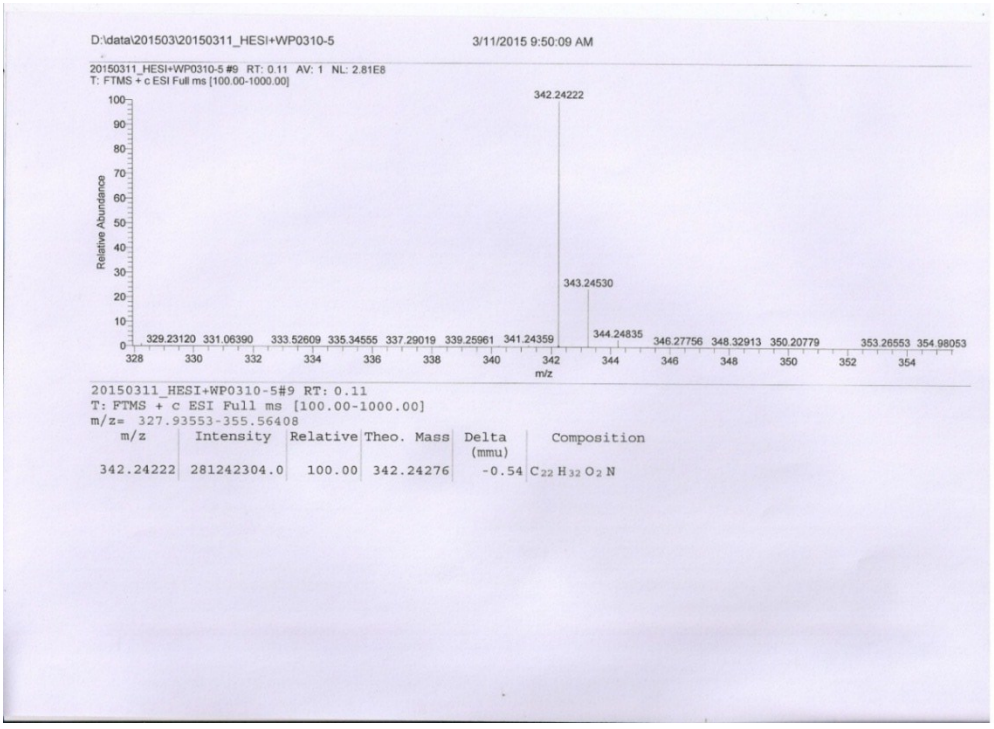


Figure S17. MS spectrum of **MHQ-12C**

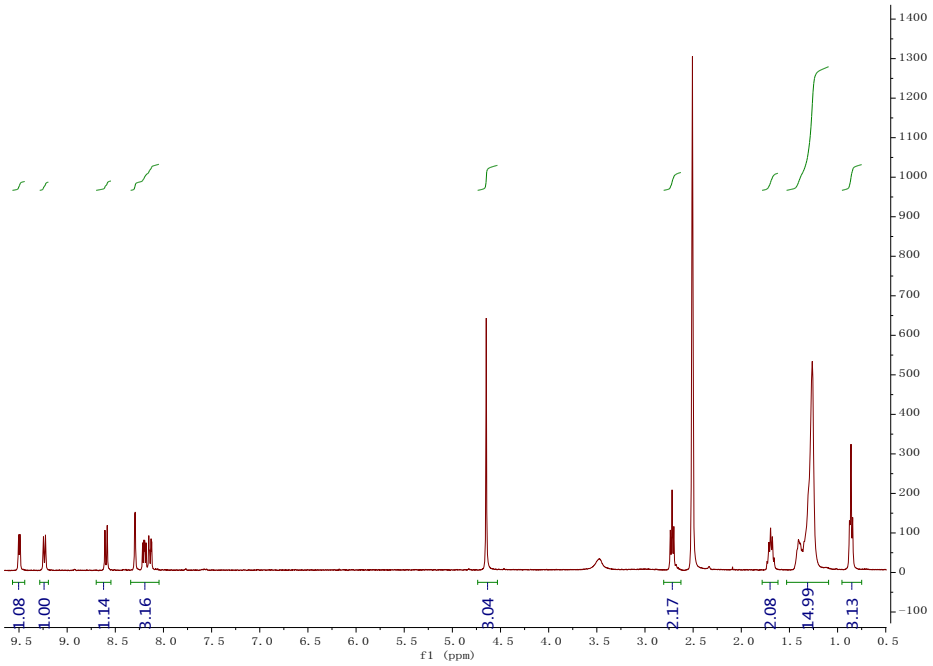


Figure S18. ^{1}H-NMR spectrum of **MHQ-6C**(400 MHz, DMSO-d_6, 298 K)

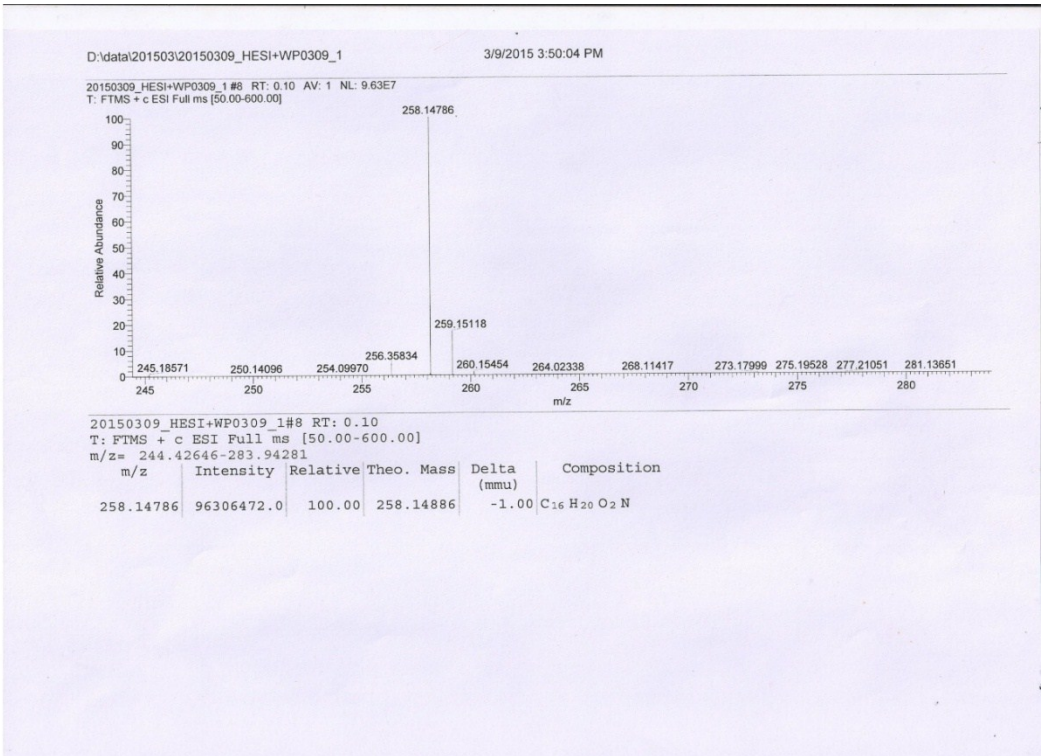


Figure S19. MS spectrum of **MHQ-6C**

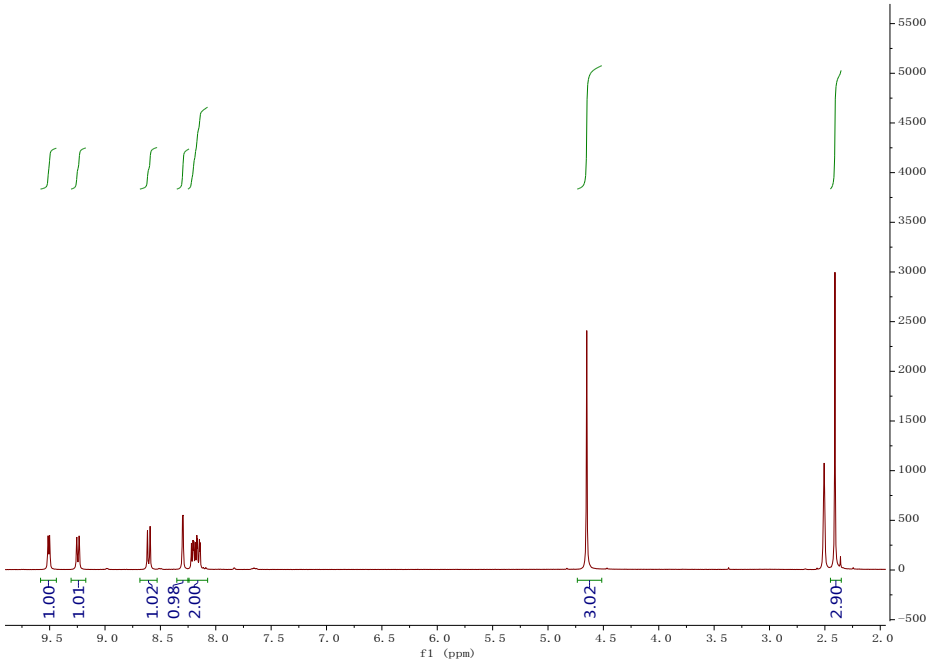


Figure S20. ^{1}H-NMR spectrum of **MHQ-2C**(400 MHz, DMSO-d_6, 298 K)

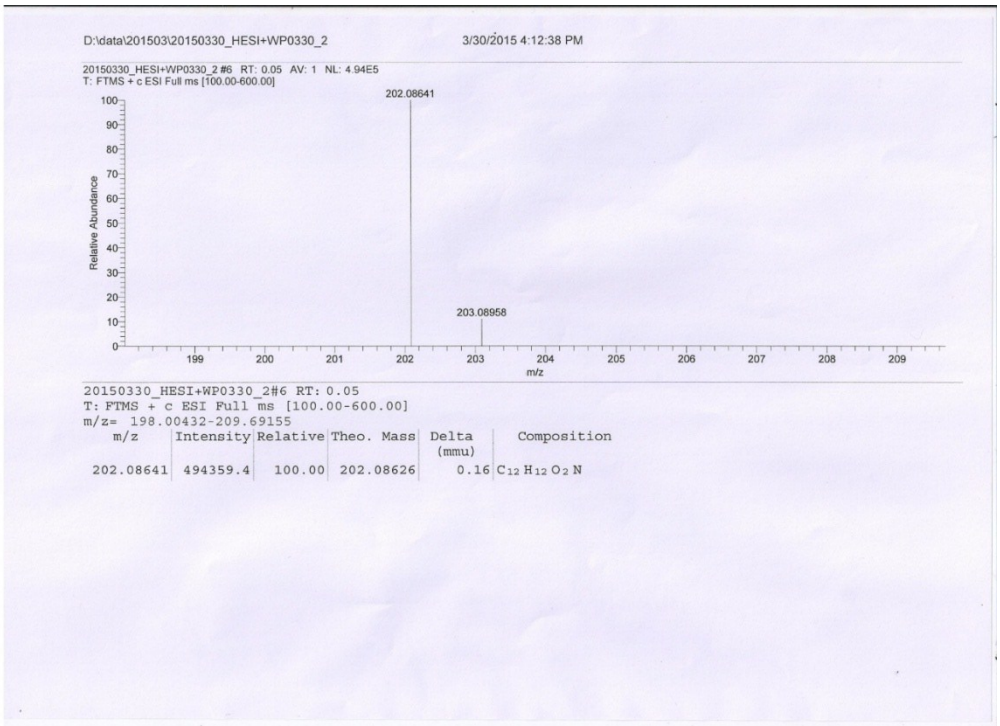


Figure S21. MS spectrum of **MHQ-2C**

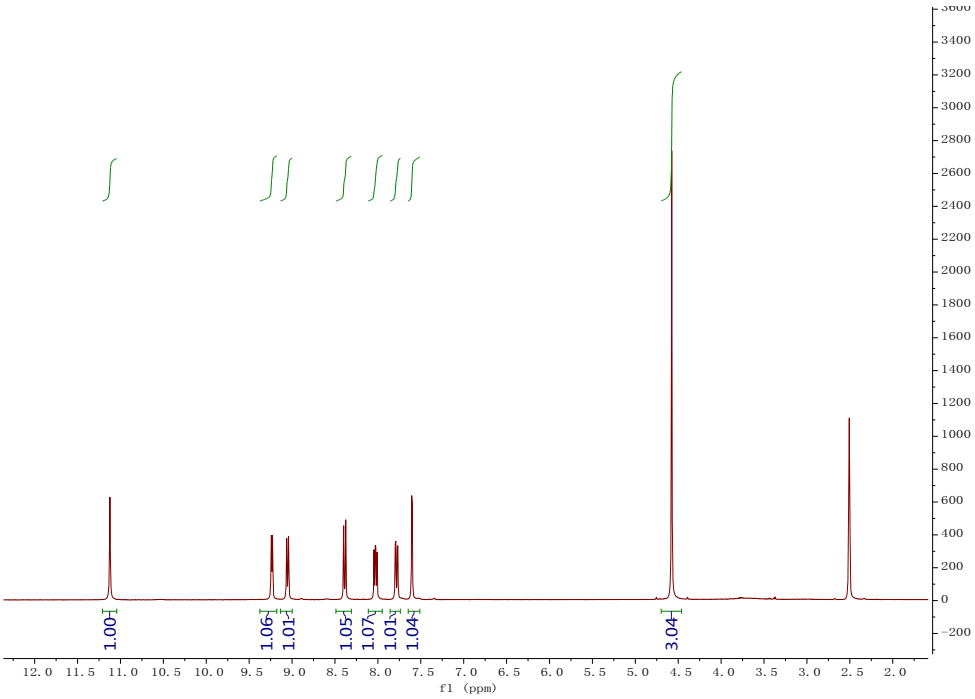


Figure S22. ^{1}H-NMR spectrum of **MHQ**(400 MHz, DMSO-d_6, 298 K)

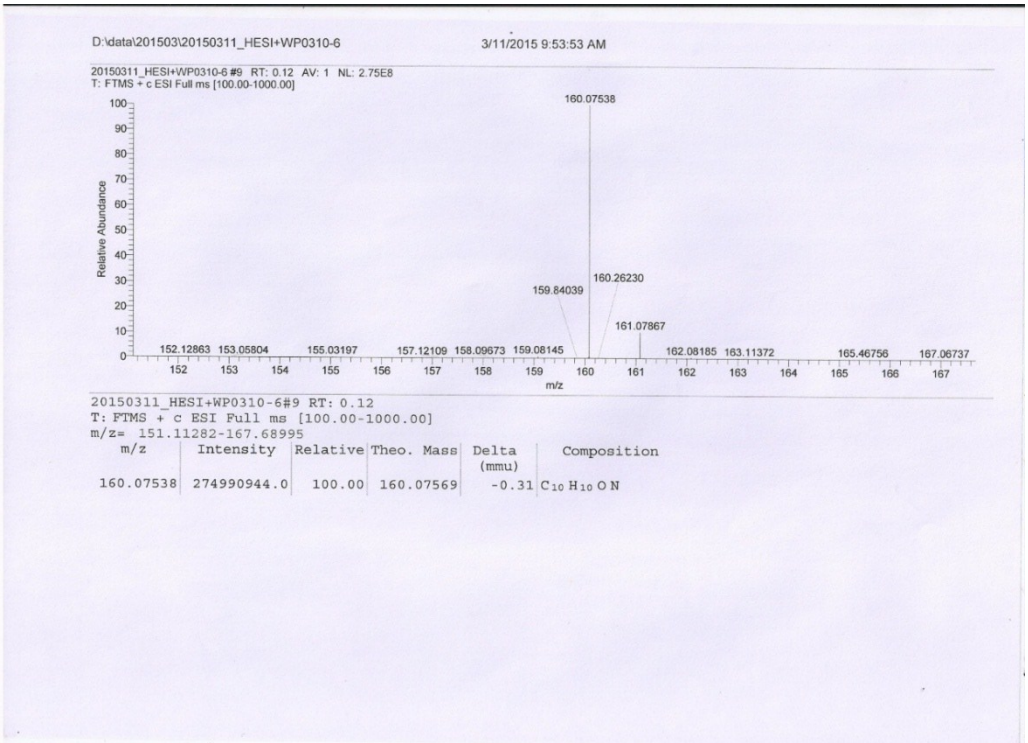


Figure S23. MS spectrum of **MHQ**