Supporting Information

Efficient Two-Photon Fluorescent Probe for Human NAD(P)H:Quinone Oxidoreductase (hNQO1) Detection and Imaging in Tumor Cells

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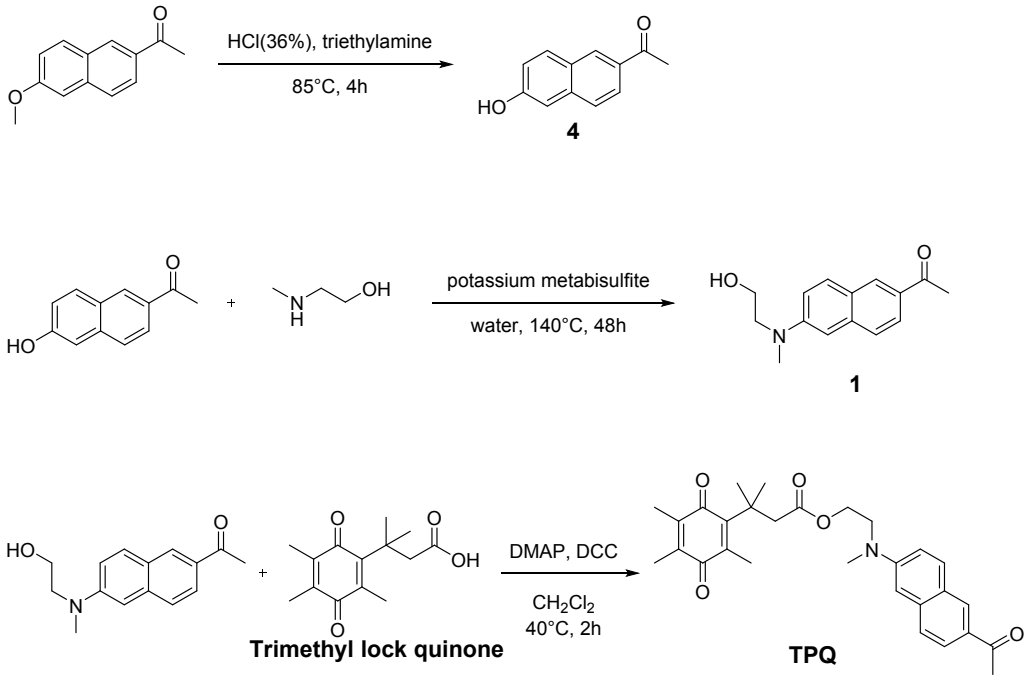
1. Materials and general methods.

All chemicals were obtained from Sigma-Aldrich and used as received. ^{1}H NMR and ^{13}C NMR spectra were recorded using Bruker 300MHz or Varian 500MHz. Chemical shifts were expressed in ppm using tetramethylsilane as an internal reference, and coupling constants (J) are reported in Hz. Mass spectra were measured in the ESI mode. Fluorescence emission spectra were recorded on FS-2 spectrophotometer (Scinco). UV absorption spectra were obtained on Evolution 201 (Thermo Scientific). All the spectroscopic experiments were performed in a 1cm X 1cm quartz cuvette. Thin layer chromatography was conducted with 60 F_{254} silica plates from Merck. Silica gel 60 (0.040-0.063 mm) was used for column chromatography. 9, 10-diphenylanthracene (Sigma-Aldrich; Φ = 0.9) in ethanol was used as a reference for quantum yield measurements.

2. Synthesis

The synthesis routes of probe TPQ were as follow, the synthesis methods were followed the published research[1].



Scheme S1. Synthesis route for probe **TPQ**.

1-(6-hydroxynaphthalen-2-yl)ethan-1-one (4)

To a 100ml screw cap round bottom flask, 6-Acetyl-2-methoxynaphthalene, **1** (1.00 g, 5 mmol)

dissolved in 4 mL of CH_2Cl_2 was added dropwise into 36% HCl (80 mL, 0.93 mol) under stirring. Then triethylamine (0.75 mL, 5.4 mmol) was added dropwise into the solution. The mixture was stirred at 85 ℃ for 4 h and chilled using an ice bath. Neutralize excess acid using solid NaOH. The solution was extracted three times with ethyl acetate and washed with brine. The organic layer was separated and dried with anhydrous $MgSO_4$. Solvent was removed under vacuum, and the resulting residue was purified by column chromatography (hexane : ethyl acetate = 1:1) to give compound **1** as a brown solid (0.46g, 50%). ^{1}H NMR (300 MHz, MeOD): δ (ppm): 2.699 (s, 3H), 7.174 (q, *J* = 2.57 Hz, 2H), 7.709 (d, *J* = 8.65 Hz, 1H), 7.933 (m, 2H), 8.506 (d, *J* = 1.39 Hz, 1H).

1-(6-((2-hydroxyethyl)(methyl)amino)naphthalen-2-yl)ethan-1-one (1)

In a 50ml round-bottom flask equipped with a condenser, compound **4** (372 mg, 2 mmol) and 2-(methylamino)ethanol (3.25mL, 40mmol) were mixed with potassium metabisulfite (2.2 g, 10 mmol) and 17mL of water. The mixture was stirred at 140 ℃ for 48 hours. The mixture was allowed to cool to room temperature and extracted three times with CH_2Cl_2 (25mL). The organic extracts were combined and dried over MgSO4. Solvent was removed under reduced pressure and the crude product was purified by column chromatography (hexane : ethyl acetate = 1:1) to give compound **2** as yellow solid (0.042g, 65%). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 2.690 (s, 3H), 3.156 (s, 3H), 3.673 (t, *J* = 6.02 Hz, 2H), 3.926 (q, *J* = 4.82 Hz, 2H) , 6.961 (d, *J* = 2.35 Hz, 1H) , 7.249 (dd, *J* = 9.02, 2.74 Hz, 1H) , 7.653 (d, *J* = 8.63 Hz, 1H) , 7.819 (d, *J* = 9.41 Hz, 1H) , 7.947 (d, *J* = 8.73, 1.87 Hz, 1H) , 8.333 (d, *J* = 1.33 Hz, 1H).

Probe TPQ

Quinone propionic acid (0.1mg, 0.4 mmol) was dissolved in anhydrous dichloromethane (10mL) and reacted with compound **1** (0.0254g, 0.1mmol) under nitrogen gas. To this was 4-(N,N-dimethylamino)pyridine (DMAP) (0.0096g, 0.08 mmol) and N,N -dicyclohexylcarbodiimide (DCC) (0.107g, 0.52 mmol). The resulting reaction mixture was stirred at 40 °C for 3 h. Solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel (hexane : ethyl acetate = 2:1) to afford a sticky brown solid of probe **TPQ** (0.058g, 100%). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 1.384 (s, 6H), 1.932 (dd, *J* = 10.07, 0.17 Hz, 6H), 2.130 (s, 3H), 2.695 (s, 3H), 2.970 (s, 2H), 3.116 (s, 3H), 3.697 (t, *J* = 6.37 Hz, 2H), 4.246 (t, *J* = 5.57 Hz, 2H), 6.889 (d, *J* = 2.52 Hz, 1H), 7.288 (dd, *J* = 9.01, 2.52 Hz, 1H), 7.645 (d, *J* = 8.65 Hz, 1H), 7.819 (d, *J* = 9.37 Hz, 1H), 7.949 (dd, *J* = 8.71, 1.78 Hz, 1H), 8.335 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm):197.97,

191.06, 187.63, 172.94, 152.48, 149.06, 143.15, 139.19, 138.73, 137.91, 131.29, 131.14, 130.50, 126.47, 125.48, 124.96, 116.18, 105.67, 61.50, 51.25, 47.70, 39.05, 38.24, 29.94, 29.02, 26.66, 14.54, 12.81, 12.36 . ESI MS m/z = 476.2964 $[M+H]^+$, calc. For $C_{29}H_{33}NO_2$ = 476.24.

3. Spectroscopic Measurements

Absorption spectra were recorded on S-3100 UV-Vis spectrophotometer and fluorescence spectra were obtained with FluoroMate FS-2 fluorescence spectrophotometer with a 1 cm standard quartz cell.

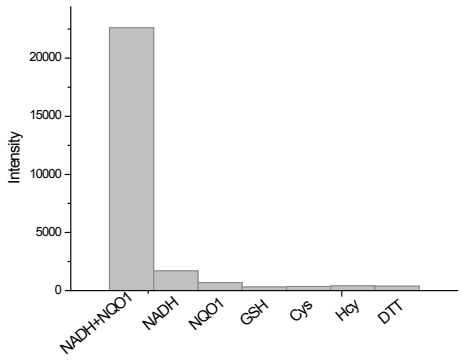


Figure. S1 Selectivity of probe **1** to various species. Experiments were performed in 0.1M PBS buffer containing 0.1M KCl and 0.007% BSA at room temperature. Bars represent the fluorescence intensity after the reaction for 2min. (λ_{ex} = 375nm, λ_{em} = 512nm).

4. Enzymatic Kinetics Assays

Enzymatic kinetics experiments were performed by using Varioskan Flash micro plate reader (6~1536 well) with 96 well plate. Various concentrations of **TPQ** (0−40 μM) was prepared in PBS buffer solution (10 mM, pH = 7.4) containing 0.007% BSA and 100 μM NADH. hNQO1 enzyme was added to a final concentration of 15 μg/mL, the fluorescence intensity was collected at 515 nm (λ_{ex} = 400 nm) with 1 min intervals from 0 to 30 min at 37 °C. The kinetic parameters of Michaelis-Menten equation were calculated with hyperbolic function by the nonlinear fitting algorithm (OriginPro 8.0).

5. Measurement of Two-Photon Cross Section

The two-photon cross section (δ) was determined by using femto second (fs) fluorescence measurement technique as described.[1] **TPQ** and **1** (1.0×10^{-6} M) was dissolved in PBS buffer (10 mM,

pH = 7.4) and the two-photon induced fluorescence intensity was measured at 720−880 nm by using rhodamine 6G as the reference, whose two-photon property has been well characterized in the literature.[1] The intensities of the two-photon induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The TPA cross section was calculated by using following equation

$$\delta = \frac{\delta_r\left(S_s\Phi_r\phi_r c_r\right)}{\left(S_r\Phi_s\phi_s c_s\right)}$$

Where the subscripts *s* and *r* stand for the sample and reference molecules. The intensity of the signal collected by a CCD detector was denoted as *S*. Φ is the fluorescence quantum yield. ϕ is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as c. δ_r is the TPA cross section of the reference molecule.

6. Cell Culture

All the cells were passed and plated on glass-bottomed dishes (NEST) for two days before imaging. They were maintained in a humidified atmosphere of 5/95 (v/v) of CO_2/air at 37 °C. The cells were treated and incubated with 1 μM Probe 1 at 37 °C under 5 % CO_2 for 30 min, washed three times with phosphate buffered saline (PBS; Gibco), and then imaged after further incubation in colorless serum-free media for 30 min. The culture mediums for each cell are as below.

HeLa human cervical carcinoma cells (KCLB, Seoul, Korea): MEM (WelGene Inc, Seoul, Korea) supplemented with 10 % FBS (WelGene), penicillin (100 units per mL), and streptomycin (100 μg/mL).

HT-29 cells (KCLB, Seoul, Korea): RPMI1640 (WelGene Inc, Seoul, Korea) supplemented with 10 % FBS (WelGene), penicillin (100 units per mL), and streptomycin (100 μg /mL).

MDA-MB 231 cells (KCLB, Seoul, Korea): RPMI1640 (WelGene Inc, Seoul, Korea) supplemented with 10 % FBS (WelGene), penicillin (100 units per mL), and streptomycin (100 μg/mL).

MDA-MB 468 cells (ATCC, Manassas, VA, USA): RPMI1640 (WelGene Inc, Seoul, Korea) supplemented with 10 % FBS (WelGene), penicillin (100 units per mL), and streptomycin (100 μg g/mL).

7. Two-Photon Fluorescence Microscopy

Two-photon fluorescence microscopy images of Probe 1-labeled cells were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP8 MP) with ×10 dry, ×40 oil and ×100 oil objectives, numerical aperture (NA) = 0.30, 1.30, and 1.30. The two-photon fluorescence microscopy images were obtained with a DMI6000B Microscope (Leica) by exciting the probes with a mode-locked titanium-sapphire laser source (Mai Tai HP; Spectra Physics, 80 MHz, 100 fs) set at wavelength 740 nm and output power 2215 mW, which corresponded to approximately 1.62×10^8 mW/cm^2 average power in the focal plane. The lateral resolution is approximately 200-300 nm, estimated by the equation, $r_{xy} = (0.46 \times \lambda_{ex})/NA$, where λ_{ex} = excitation wavelength (740 nm) and NA = numerical aperture (1.3). To obtain images at 400−600 nm range, internal PMTs were used to collect the signals in an 8 bit 1024 × 1024 pixels 200 Hz scan speed, respectively.

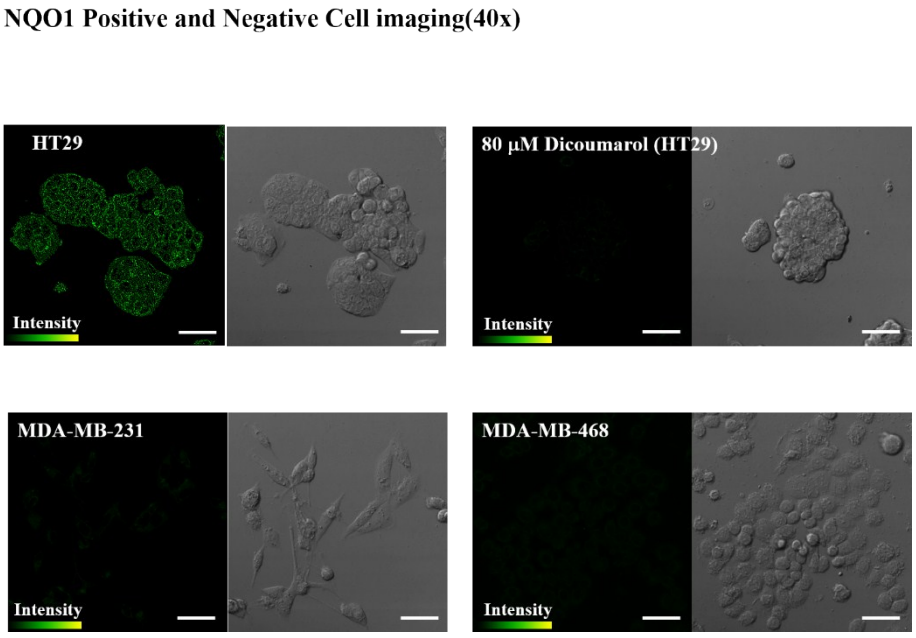


Figure S2 Microscopy images of hNQO1-positive HT29 colon cell (a), HT29 colon cell with reductase inhibitor dicoumarol (80μM, b), hNQO1-negative breast cancer cell MDA-MB-231(c) and MDA-MB-231(d) after incubation for 30 min at 37 °C with 1 μM probe TPQ. The images were taken in optical windows between at 400−600 nm range, internal PMTs were used to collect the signals 200 Hz scan speed. λ_{ex}:740 nm.

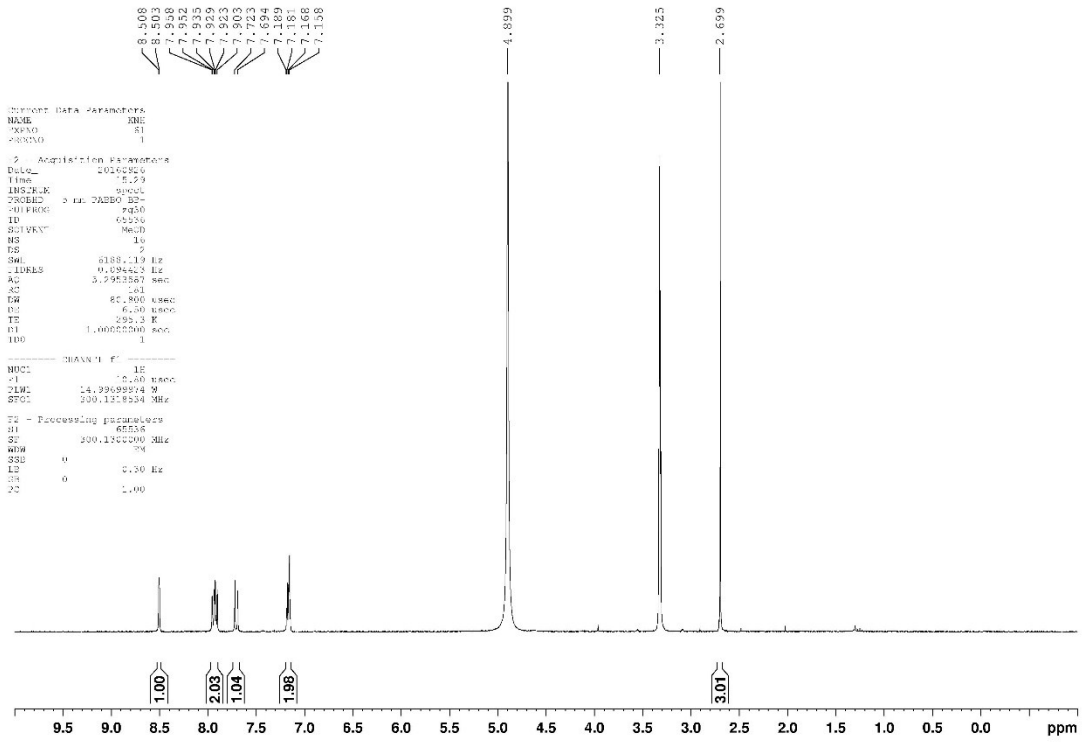
8. NQO1 assay of Probe 1

NQO1 assay were performed by Molecular Devices SpectraMax M5 and 96-well plates. All fluorescence measurements were performed in 0.1M PBS (pH 7.4, 0.1M KCl, 0.007% bovine serum albumin) at room temperature. Solutions of β-nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH, Sigma-Aldrich) were made using the PBS buffer. Total volume per well was

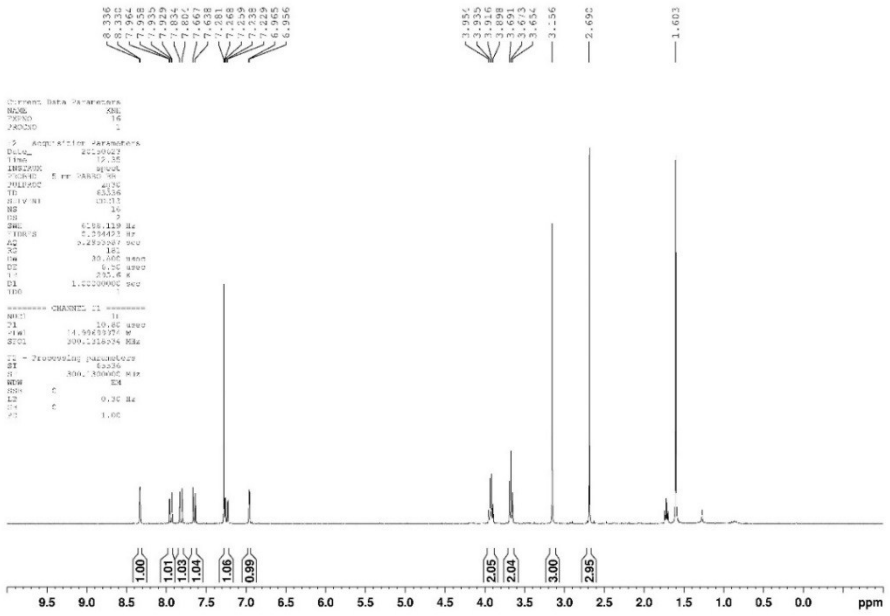
200 x 10^{-6}L with a final β-NADH concentration 1 x 10^{-4}M in each assay. Stock solutions of Probe TPQ were prepared in NADH solution to a final concentration between 0μM and 50μM. Assays were initiated by the injection of NQO1 so as final NQO1 content of 8μg. Releasement of the Reporter 2 from Probe 1 was monitored by time dependent fluorescence measurement (λ_{ex} = 355 nm and λ_{em} = 538 nm) and data were collected every 30 s for 10 min.

9. NMR Spectrum

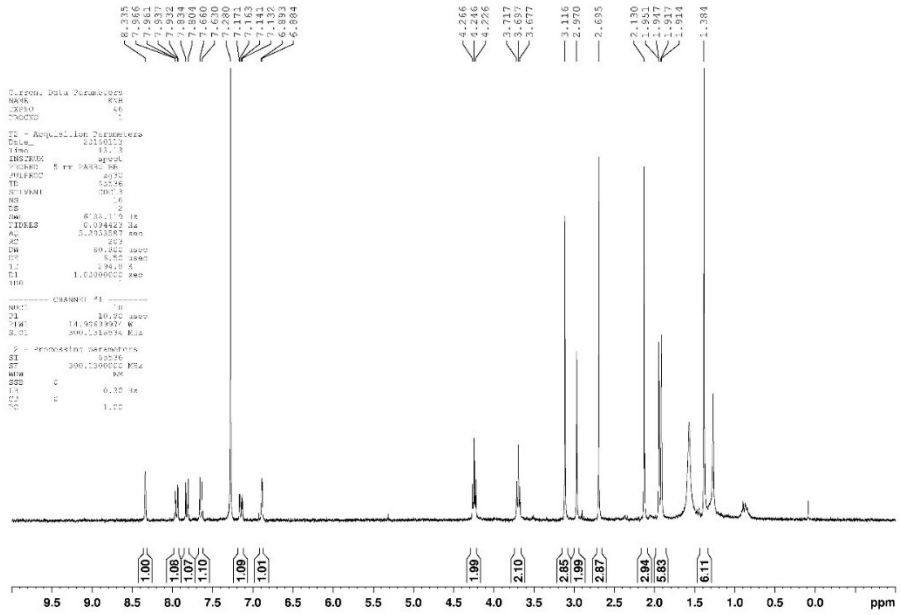
^{1}H NMR of Compound **4**



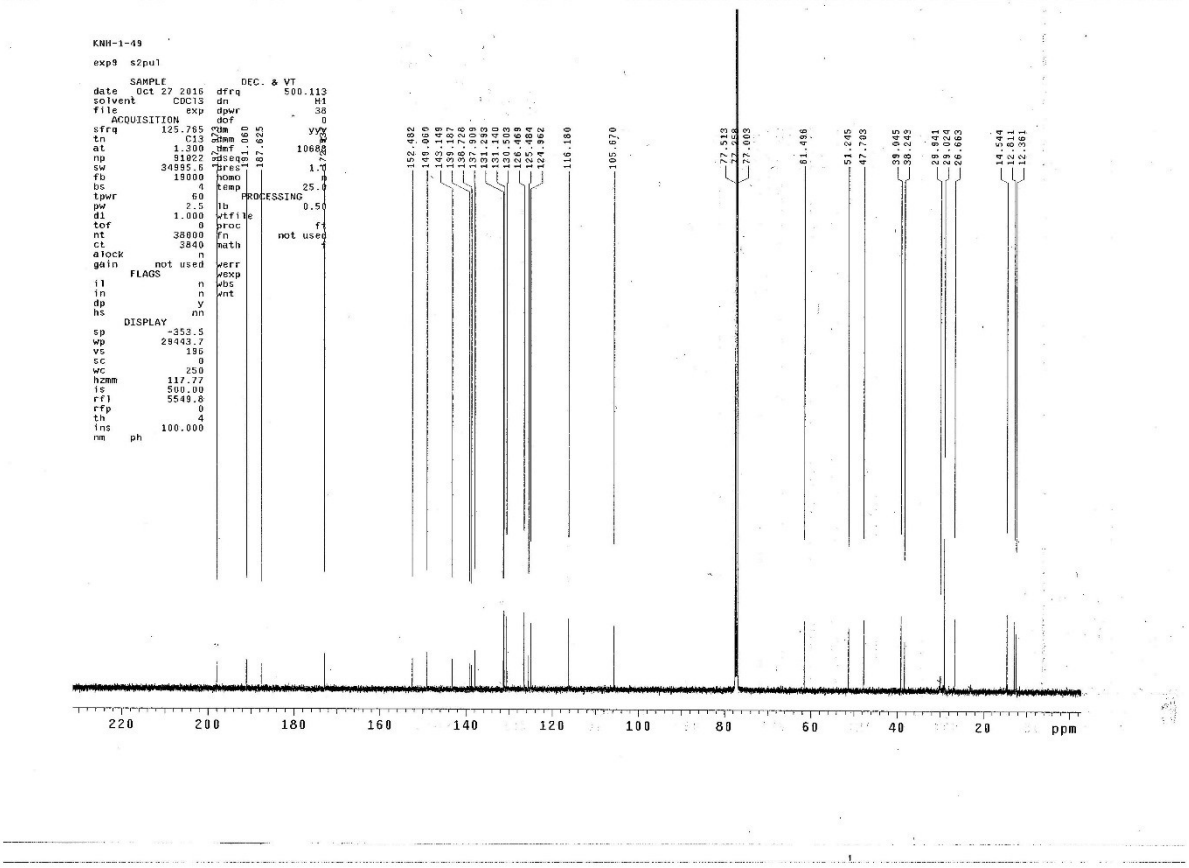
^{1}H NMR of Compound **1**



^{1}H NMR of Probe **TPQ**



^{13}C NMR of Probe **TPQ**



10. References

1. (a) A. S. Rao, S. Singha, W. Choi and K. H. Ahn, *Org. Biomol. Chem.*, 2012, **10**, 8410; (b) N. Weerapreeyakul, R. Anorach, T. Khuansawad, C. Yenjai, and M. Isaka, *Chem. Pharm. Bull.*, 2007, **55**, 930; (c)Yuan, L.; Wang, L.; Agrawalla, B. K.; Park, S. J.; Zhu, H.; Sivaraman, B.; Peng, J.; Xu, Q. H.; Chang, Y. T.* *J. Am. Chem. Soc.* 2015, **137**, 5930-5938.