Supplementary Information

A fluorescent probe for simultaneously sensing NTR and hNQO1 and distinguishing cancer cells

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Contents:

1. General Experimental Section

Cell culture

HepG2 cells (human hepatocellular carcinoma cell line) and HeLa cells (human cervical carcinoma cell line) were grown in high glucose DMEM (Biological Industries) supplemented with fetal bovine serum (FBS, 10%), penicillin (100 U mL^{-1}) and streptomycin (100 U mL^{-1}). MDA-MB-231 (Human breast cancer cells) were grown in high glucose DMEM (Biological Industries) supplemented with fetal bovine serum (FBS, 20%), penicillin (100 U mL^{-1}) and streptomycin (100 U mL^{-1}). NCI-H446 (Human small cell lung cancer cell) were grown in RPIM-1640 (Biological Industries) supplemented with fetal bovine serum (FBS, 10%), penicillin (100 U mL^{-1}) and streptomycin (100 U mL^{-1}). HL-7702 normal liver cells were grown in RPMI 1640 (Biological Industries) supplemented with fetal bovine serum (FBS, 10%), penicillin (100 U mL^{-1}) and streptomycin (100 U mL^{-1}). Cells were incubated in an incubator at 37 °C with 5% CO_2/air environment.

Cytotoxicity assay

The in vitro cytotoxicity was measured using a standard methyl thiazolyl tetrazolium (MTT, MACKLIN) assay in HepG2 cell lines. Briefly, cells growing in log phase were seeded into 96-well cell-culture plate at 1×10^6 / well for 24h. The probe (200 μL/well) at concentrations of 5−30 μM was added to the wells of the treatment group, and 200 μL/well DMSO diluted in DMEM at final concentration of 0.5% to the negative control group, respectively. The cells were incubated for 12 h at 37 °C under 5% CO_2. Then the culture media were removed and MTT solution (0.5 mg/mL) was added to each well with 200 μL for 4 h at 37°C. After 4 h, the remaining MTT solution was removed, and 100 μL of DMSO was added to each well to dissolve the formazan crystals. After gently shaken for 10 min, absorbance was measured at 490 nm using a microliter plate reader. The following formula was used to calculate the viability of cell growth: viability (% T) = A1/A2 × 100 %, where A1 denotes absorbance value of treatment group, and A2 denotes absorbance value of control.

Confocal fluorescence imaging for living cells

The cells were incubated in confocal dishes for 12 h to allow them to adhere, and then

the solution of probe in DMSO was added to the adherent cells to give a final concentration of 10 μM probe. All fluorescence images were acquired with a Leica TCS SP8 confocal laser-scanning microscope with a 63 × oil objective lens.

For the endogenous NTR and hNQO1 imaging in cells, one group of HepG2 cells, HeLa cells, MDA-MB-231 and NCI-H446 cells were seeded in confocal culture dishes. After the cells were attached, they were incubated under anaerobic conditions for 4 hours, then incubated with a medium containing 10 μM probe for 30 minutes, and another group of HepG2 cells, HeLa cells, MDA-MB-231, NCI-H446 cells and HL-7702 cells were cultured under normoxia, and then contained with 10 μM. The culture medium of the probe was incubated for 30 minutes and washed three times with PBS buffer. For inhibitor-treated bioimaging, HepG2 cells were seeded in confocal culture dishes for cell attachment, incubated under anaerobic conditions for 4 hours, and then treated with 0.1 μM / L dicoumarin for 30 minutes. After incubating the culture solution containing the 10 μM probe for 30 minutes, the culture solution was removed and washed three times with PBS buffer. To present more apparent contrast, we normalized the data based on the lowest fluorescence intensity in each experiment.

Spectroscopic studies

Spectroscopic experiments were typically performed in phosphate buffered saline (PBS, 50 mM, pH 7.4, 50 μM NADH and 2.5% DMSO) at 37 °C. The probe was dissolved in DMSO to prepare a stock solution having a concentration of 0.2 mM. The probe was diluted in PBS buffer to give a final concentration of 1-10 μM. All measurements were performed in a 0.35 mL cuvette with 200μL of solution.

To assess the sensitivity of the probes, probes (5 μM) were first treated with different concentrations of NTR (0-2.4 μg / mL) for 20 minutes in the presence of hNQO1 (0.4 μg / mL). Subsequently, the probe (5 μM) was incubated with different levels of hNQO1 (0-0.5 μg / mL) for 20 minutes in the presence of NTR (2 μg / mL).

For the time-dependent fluorescence response of probes toward NTR and/or hNQO1, one group, into a 0.35 mL fluorescence quartz cell, 5 μM of probe was diluted in PBS, incubate for about 5 minutes, then 50 μM NADH was added and incubate for about 5

minutes, then 0.4 μg / mL hNQO1 was added and the mixture was incubate for about 10 minutes. Then 2 μ g / mL NTR were added and the mixture was incubate for about 20 minutes . Second, into a 0.35 mL fluorescence quartz cell, 5 μM of probe was diluted in PBS, incubate for about 5 minutes, then 50 μM NADH was added and incubate for about 5 minutes, then 2μg / mL NTR was added and the mixture was incubate for about 15 minutes. Then 0.4 μg / mL hNQO1 were added and the mixture was incubate for about 20 minutes .Third, into a 0.35 mL fluorescence quartz cell, 5 μM of probe was diluted in PBS, incubate for about 5 minutes, then 50 μM NADH was added and incubate for about 5 minutes. Then the mixture of 0.4 μg / mL hNQO1 and 2μg / mL NTR were added, incubate for about 30 minutes.

For the investigation on selectivity of probe, the probe (5 μM) was separately incubated with 2.5 mM inorganic salts (K^+, Na^+, Mg^{2+}, Ca^{2+}), 1.0 mM common amino acids (tyrosine, glycine, Tryptophan, Arginine, glutamic acid, lysine), 1.0 mM reducing agents (glutathione, L(+)-Cysteine, Homocysteine, dithiothreitol), related enzyme (5 μg / mL Human CES1, 5μg / mL Human CES2, 5 μg / mL glutathione reductase, 5 μg / mL NADPH-P450 Cytochrome Reductase), vitamin C (1 mM) and reactive oxygen and other analytes such as HClO (200 μM), H_2O_2 (1m M), NaHS (200 μM), Na_2SO_3 (10 mM), glucose (10 mM), and both of hNQO1 (0.4 μg / mL) and NTR (2 μg / mL) .

2. Determination of quantum yield

The quantum yields of the probe CNN after reacting with NTR and hNQO1 were ere measured to be 0.16, using quinine sulfate as a reference:

$$\Phi_x=\Phi_s(F_x/F_s)(A_s/A_x)(\lambda_{exs}/\lambda_{exx})(n_x/n_s)$$

Where Φ is quantum yield; F is the integrated area under the corrected emission spectrum; A is the absorbance at the excitation wavelength; λ_{ex} is the excitation wavelength; n is the refractive index of the solution; the subscripts x and s refer to the unknown and the standard, respectively. Quinine sulfate (Φ_F = 0.58) in 0.1mol / L H_2SO_4 was used as the standard.

3. Excitation and emission spectra of CNN with NTR and hNQO1

Excitation and emission spectra were recorded before and after the reaction of CNN with NTR and hNQO1 in phosphate buffer at pH 7.4.

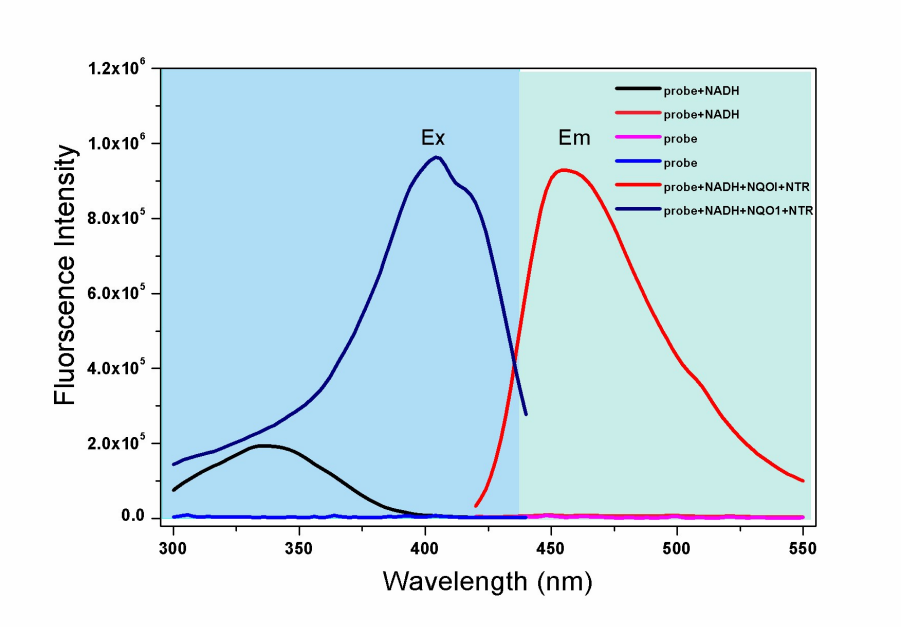


Figure S1. Fluorescence responses of 5 μM **CNN** to 50 μM NADH, 0.4 μg / mL of hNQO1, 2 μg / mL of NTR at pH 7.4 (PBS buffer, 10 mM) at 37°C, λ_{ex} / λ_{em} = 400 /454 nm.

4. Product Analysis of the reaction of CNN with NTR and hNQO1

The reactive products from the reaction of CNN and NTR and hNQO1 were examined by mass spectrometry, as shown in Figure S1. We first confirmed the products of the single analyte reactions of CNN with NTR or hNQO1 with HRMS. The hNQO1-triggered product **1** and NTR-triggered product **2** were observed as $[M-H]^-$ 320.0701 (calcd: 320.0665) and $[M-H]^-$ 417.1423 (calcd: 417.1444), respectively. Product **3** of the dual activation reaction was observed as $[M-H]^-$185.0351 (calcd:185.0345).

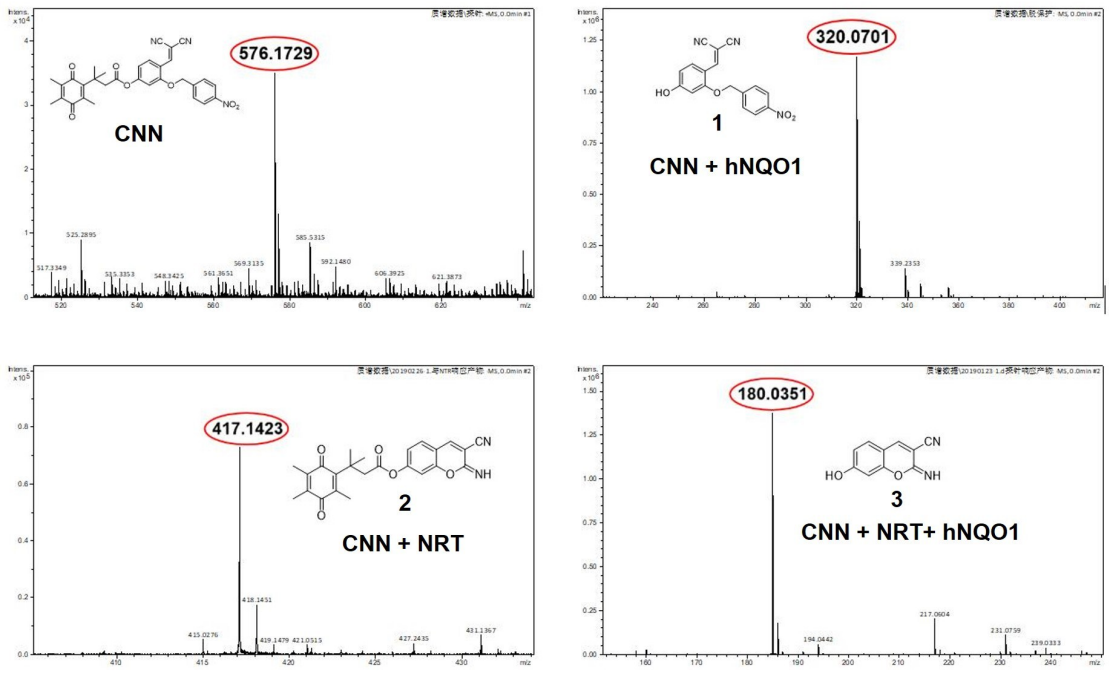


Figure S2. ESI-MS spectrum of the reactive products of CNN with NTR and hNQO1.

5. The interference test of CNN to two different kinds of biologically-relevant species

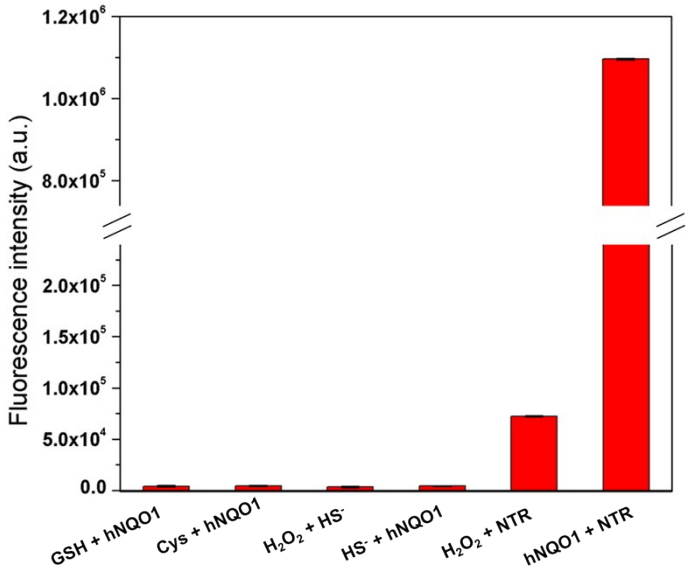


Figure S3. Fluorescence spectra of CNN (5 μM) upon treatment with two different kinds of biologically-relevant species. GSH (200 μM), Cys (200 μM), H_2O_2 (200 μM), NaHS (200 μM), hNQO1 (0.4 μg / mL) and NTR (2 μg / mL).

6. MTT assay

To investigate CNN cytotoxicity, MTT assay were carried out when the probe

existed HepG2 cells and HeLa cells in Figure S4. Cells (10^6 cell mL^{-1}) were dispersed within replicate 96-well microtiter plates to a total volume of 200 μL $well^{-1}$. Plates were maintained at 37 °C in a 5% CO_2 / 95% air incubator for 4 h. The probe CNN was diluted to different concentrations of solution with medium and added to each well after the original medium has been removed. HepG2 cells and HeLa cells were incubated with probe concentrations for 4 h. The concentrations of the probe were 0 μM to 30 μM, respectively. MTT solution (5.0 mg mL^{-1} in PBS) was then added to each well. After 4 h, the remaining MTT solution was removed and DMSO (150 μL) was added to each well to dissolve the formazan crystals. Absorbance was measured at 490 nm in a TRITURUS microplate reader. The result demonstrated that CNN should be a low cytotoxic probe under experimental conditions at the concentration of 10 μM.

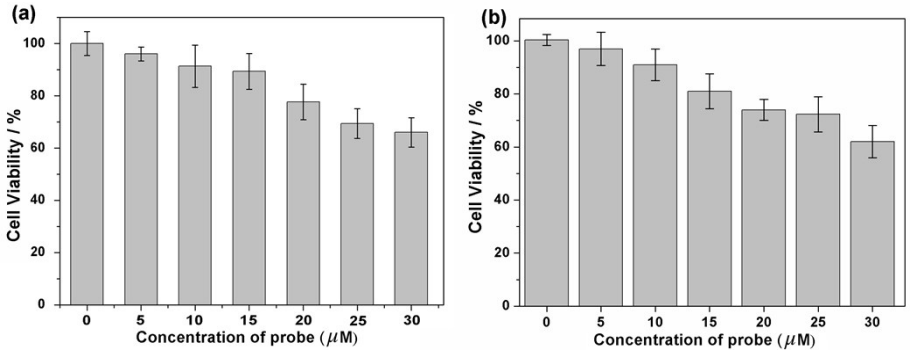


Figure S4. MTT assay of HepG2 cells (a) and HeLa cells (b) in the presence of different concentrations of **CNN**

7. Photo-bleaching test of the reaction product of CNN with endogenous NTR and hNQO1 in HepG2 cells

Photo-bleaching is an irreversible photochemical inversion of fluorescent molecule into a non-fluorescent state. The photo-stability of CNN was investigated by time-sequential scanning of the living cells. After 500 s of continuous irradiation with a 405-nm laser, no obvious changes were observed in fluorescence brightness of CNN. In order to quantitatively determine the photo-bleaching rate, we choose three regions, calculated the average intensity and obtained a curve with scanning time in Figure S4. The results showed that the intensities of CNN after 0 ~ 500 s of time-sequential scanning were about 90% of the initial value. These data indicated that probe CNN is

highly resistant to photo-bleaching.

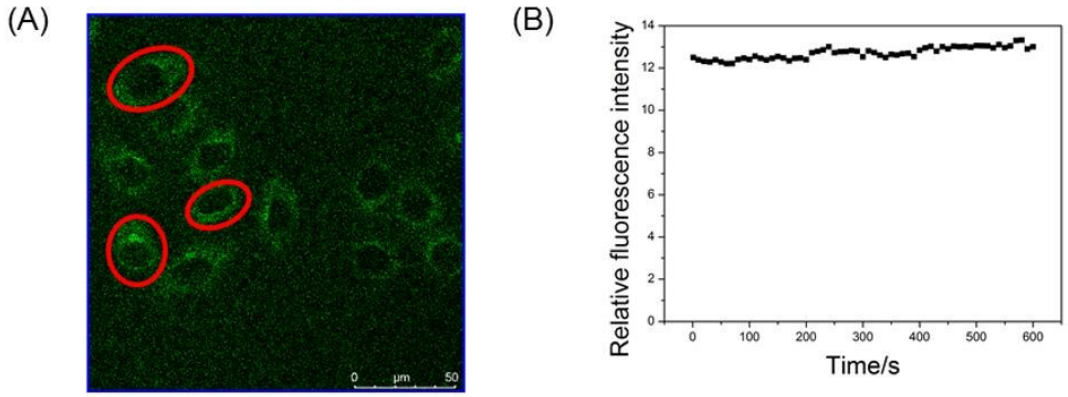


Figure S5. Test of photo-stability. Fluorescence images were achieved by means of time-sequential scanning of the probe-loaded HepG2 cells for 30 min. Normalized fluorescence intensity from three regions from 0 to 600 s of time-sequential scanning.

8. Endogenous NTR and hNQO1 detection in different cell lines in normoxic conditions

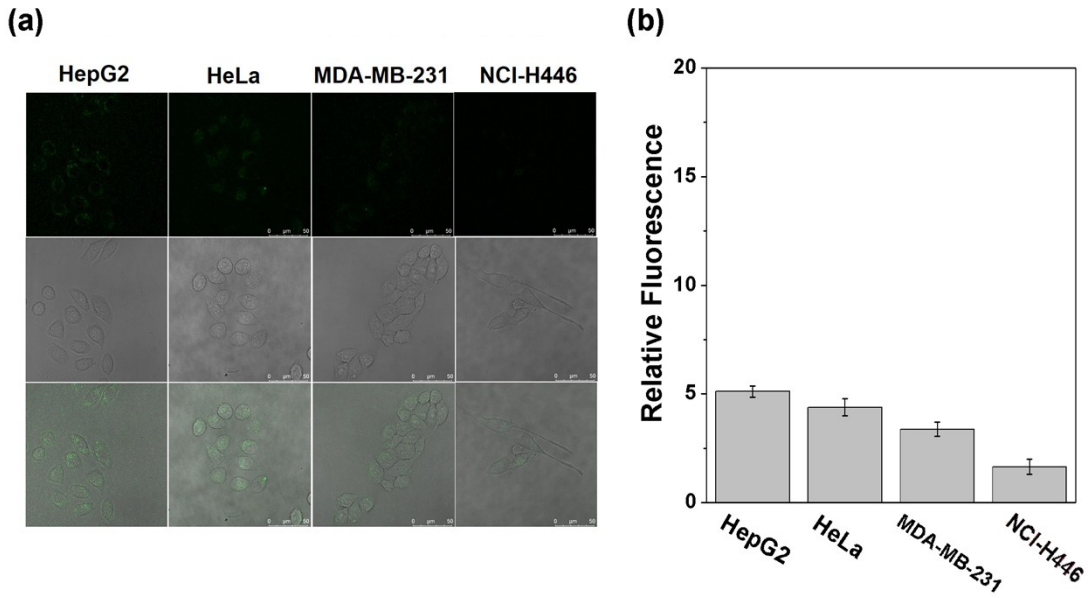
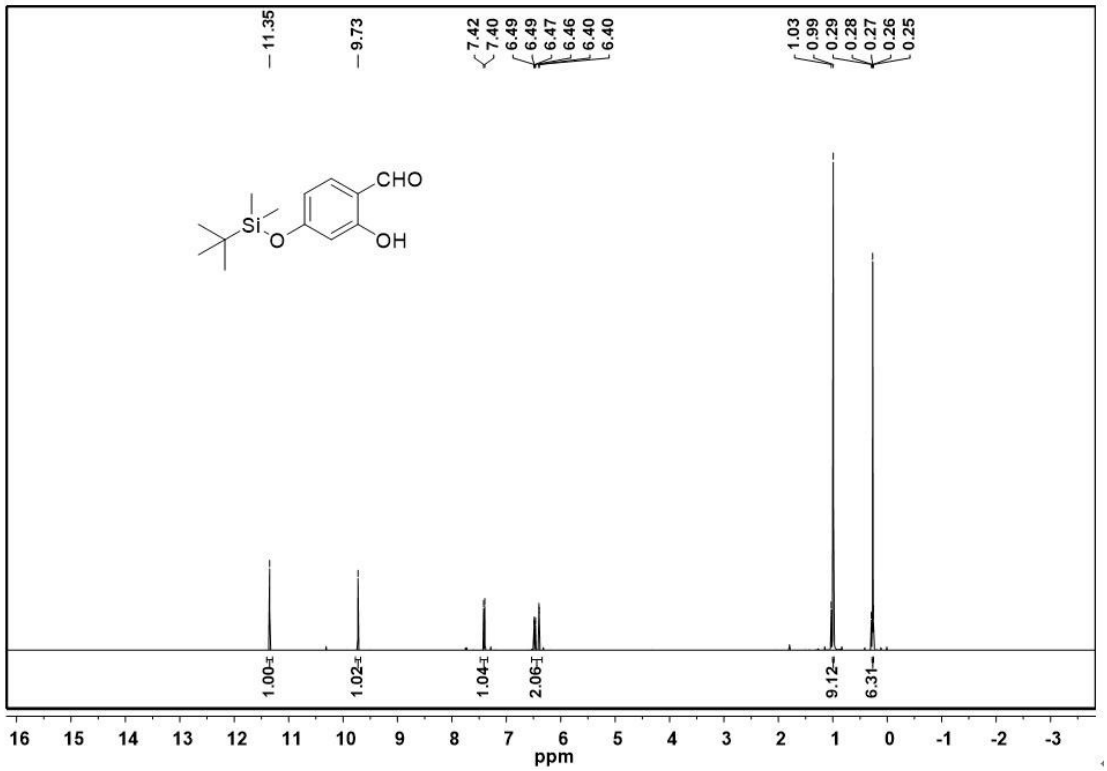


Figure S6 (a) Confocal fluorescence microscope images for endogenous NTR and hNQO1 detection in different cell lines using CNN (10μM) in normoxic conditions. (b) Relative fluorescence intensity of images from (a).

9. Spectrum of CNN and intermediates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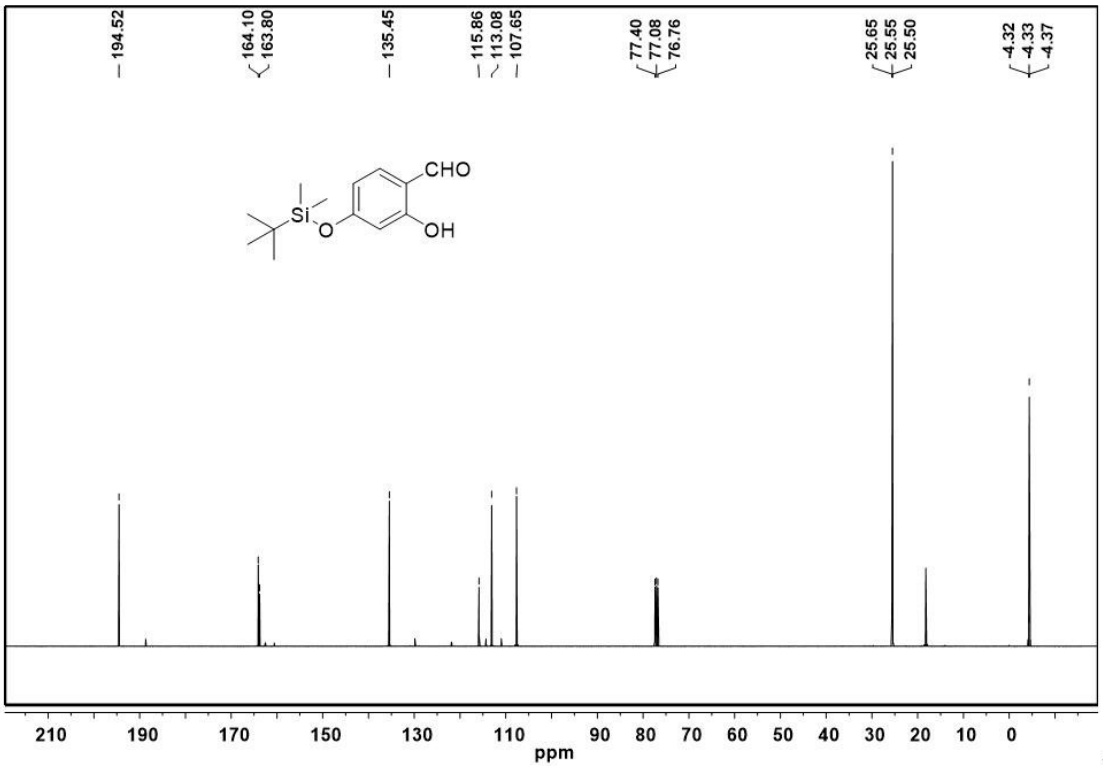
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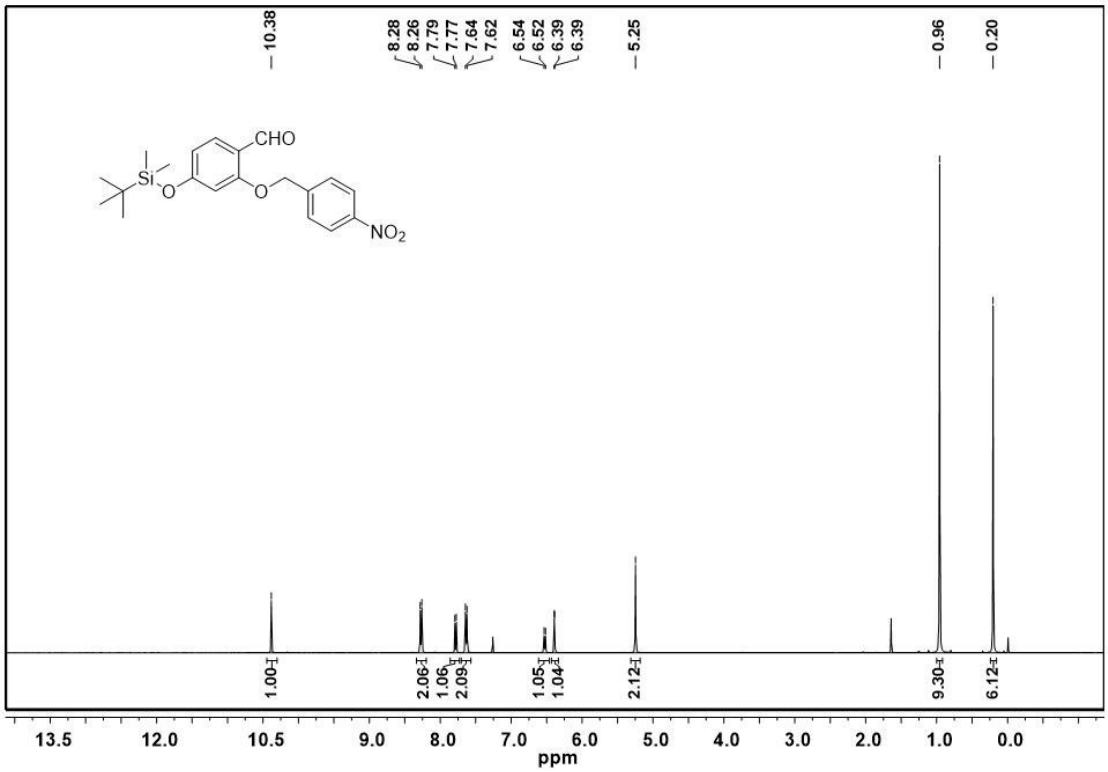
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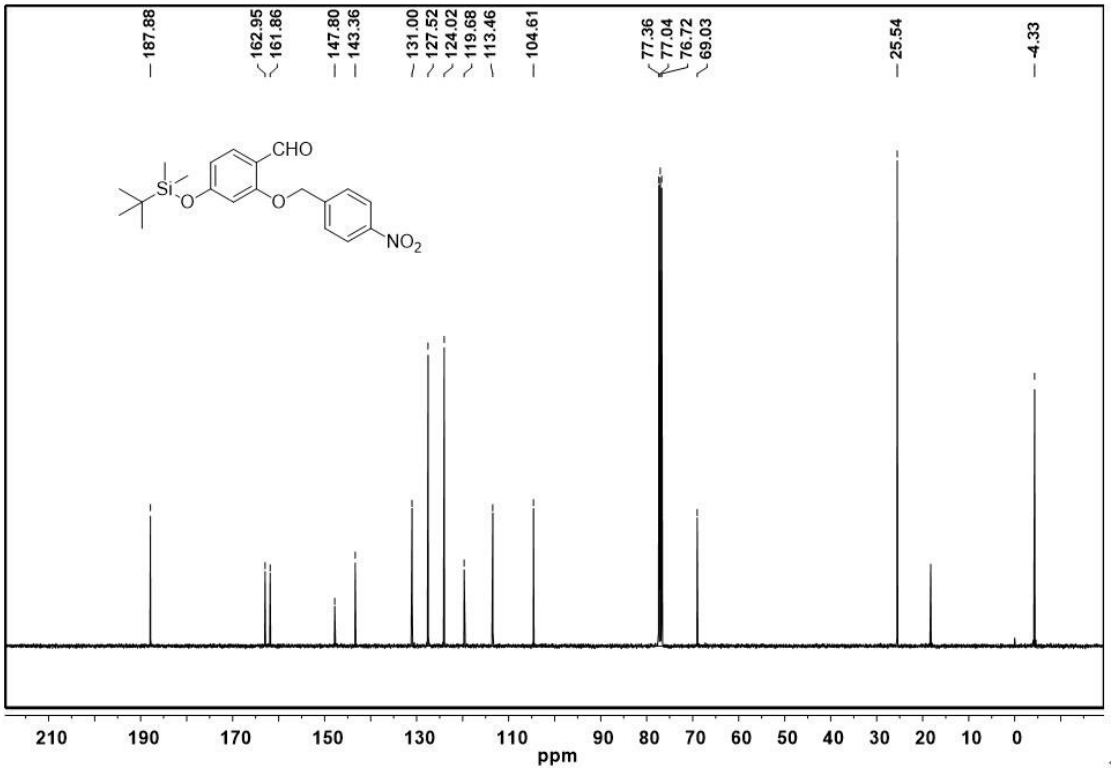
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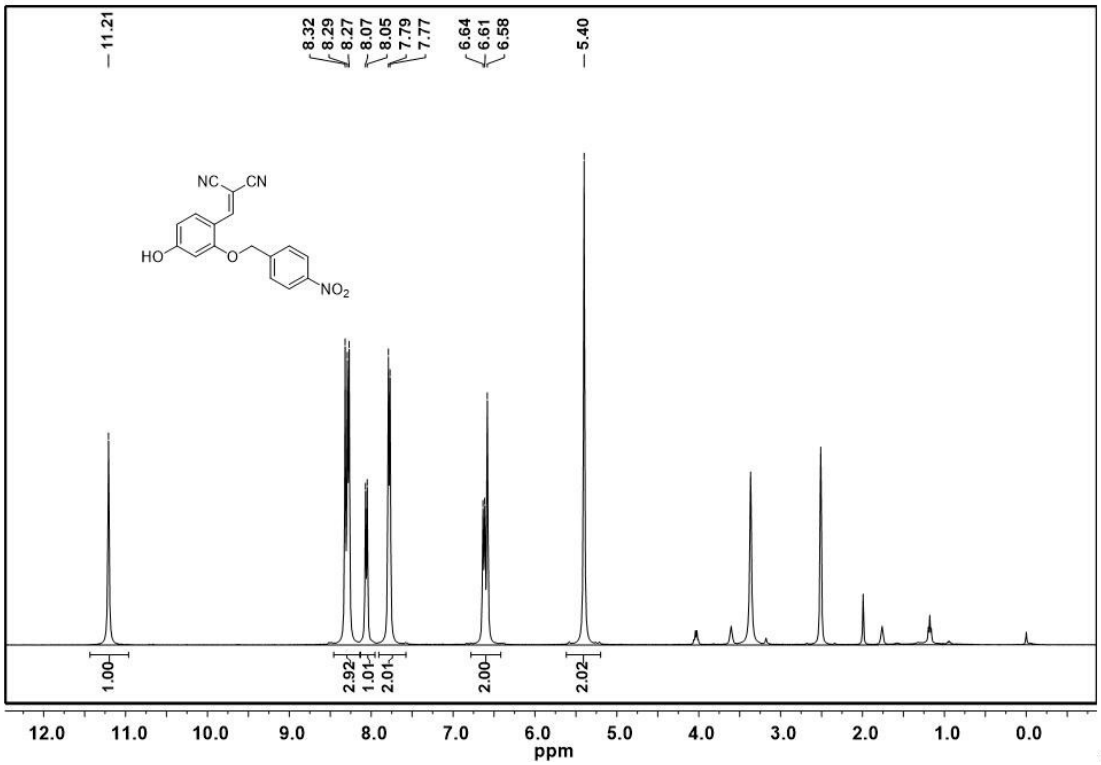
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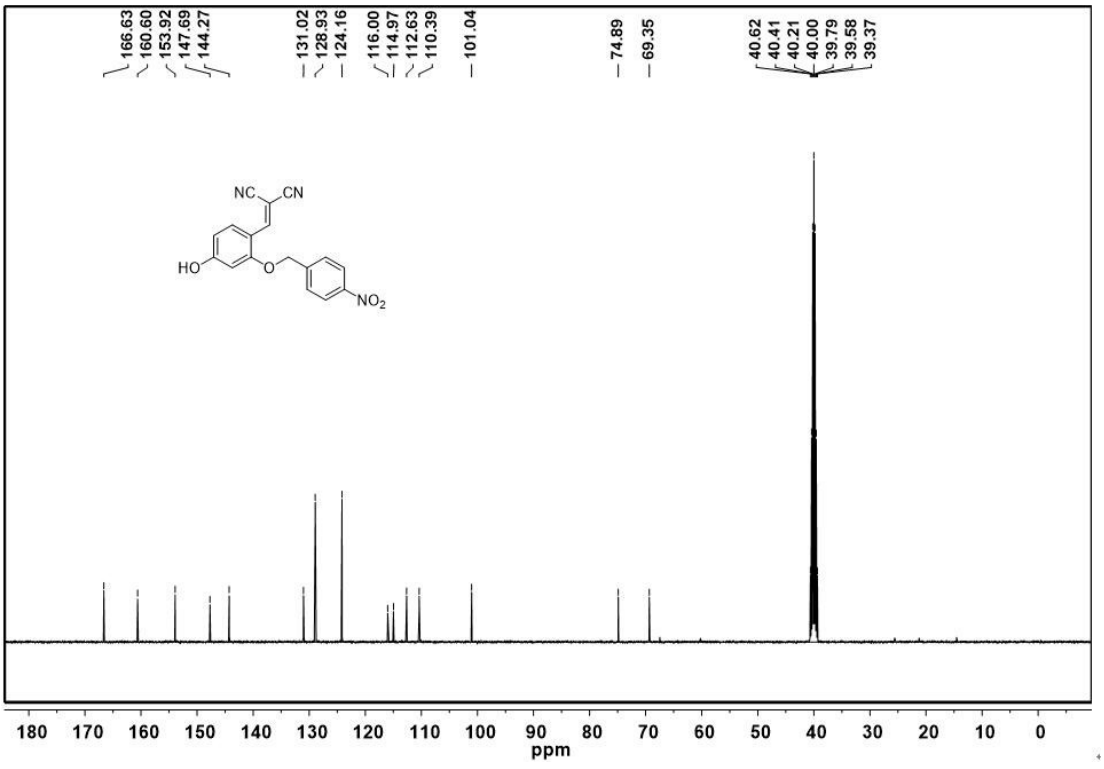
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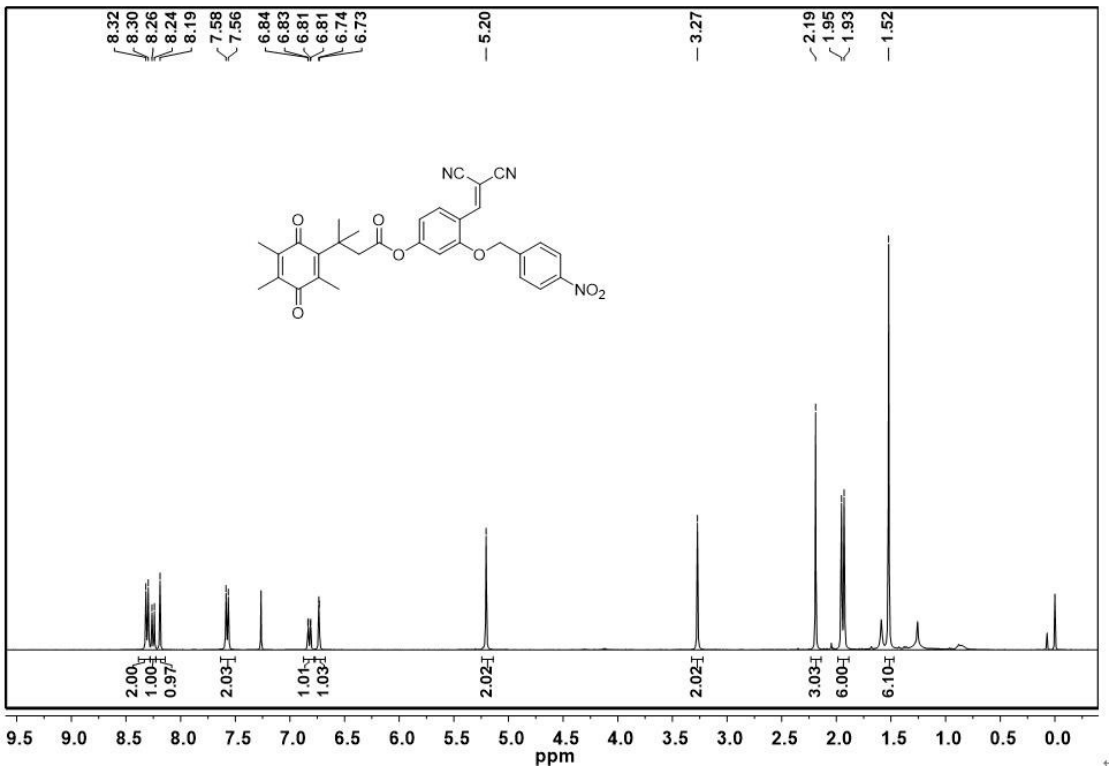
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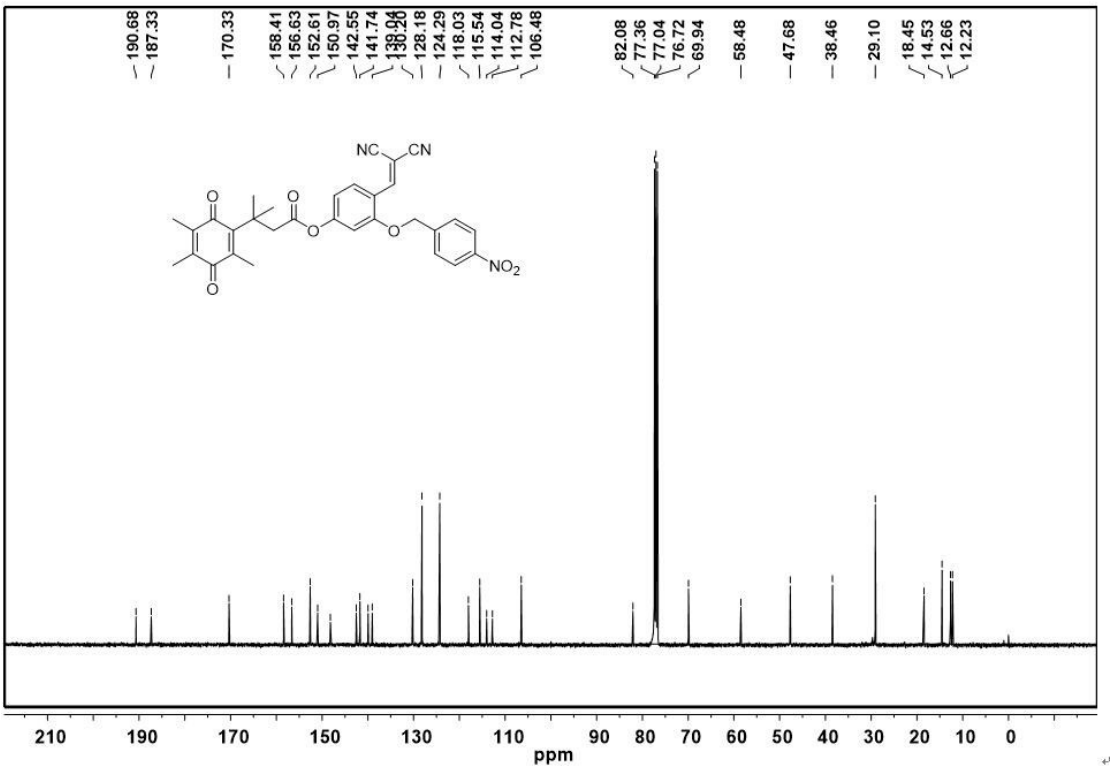
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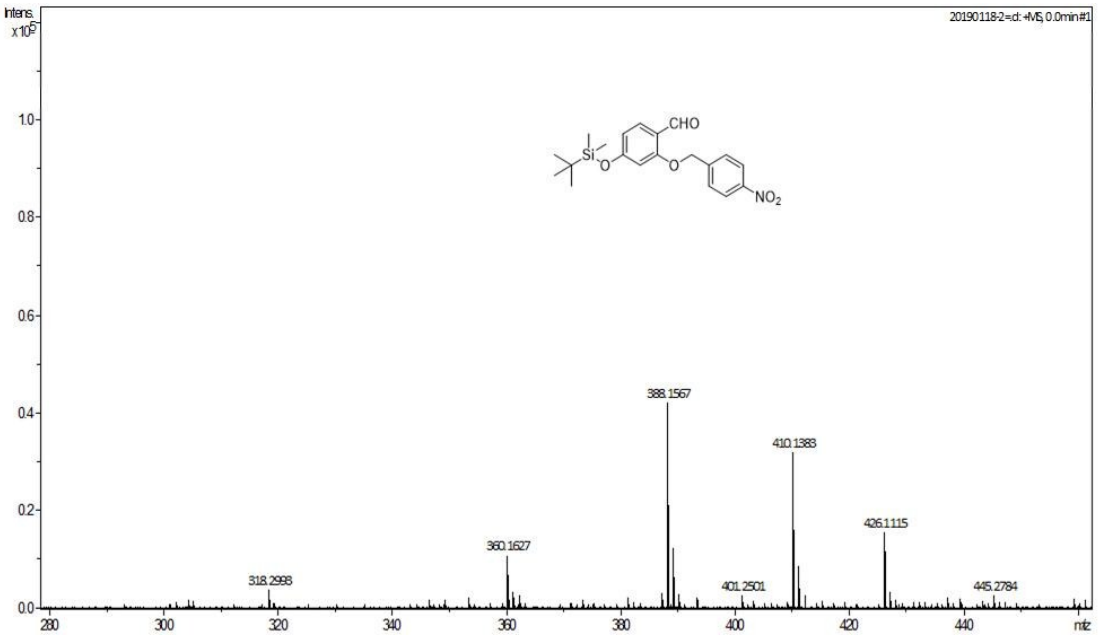
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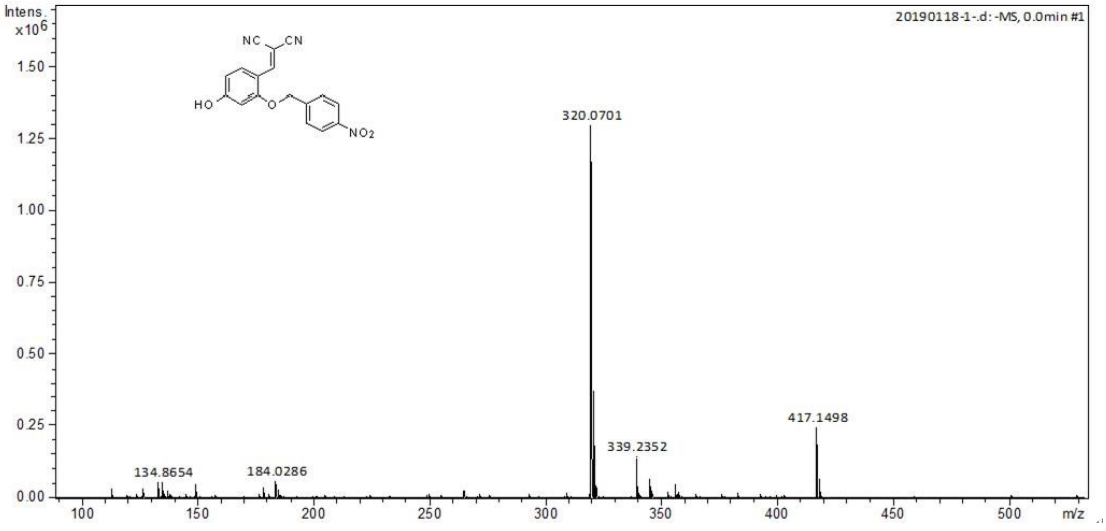
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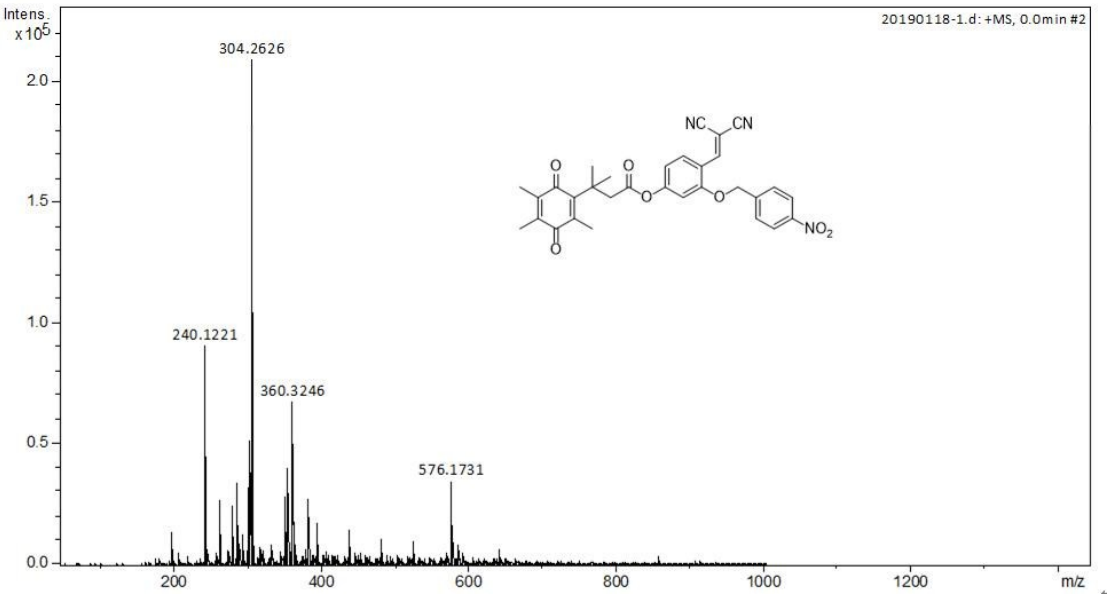
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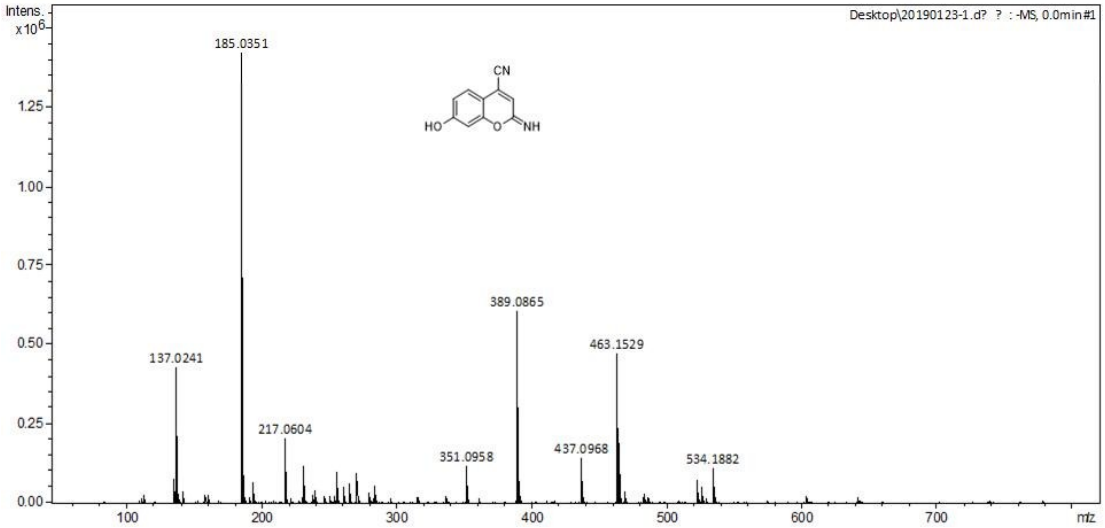
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