Electronic Supplementary Material (ESI) for RSC Advances.

Identification of 3-amidoquinoline derivatives as PI3K/mTOR dual inhibitors with potential for cancer therapy

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1. Biological assays and molecular docking study

Class I PI3Ks enzyme assay

The inhibition of PI3Ks (P110α/85α, Promege; P110β, Millipore; P110γ, Invitrogen; P110δ, Millipore) activity was determined using the Kinase-Glo Plus Luminescent Kinase assay (PI3Kα, Promege) and ADP-Glo Kinase assay (PI3Kβ, γ and δ, Promege), respectively. Test compounds were serially diluted to the desired concentrations and then 2.5 μL of each of them was added to a 384-well plate (Corning) as assay plate. 1x kinase buffer was prepared contained 50 mM HEPES (pH 7.5), 3 mM $MgCl_2$, 1 mM EGTA, 100 mM NaCl, 0.03% CHAPS, 2 mM DTT. PI3K enzyme was diluted in the 1x kinase buffer to give 4x kinase solutions. PI3Kα, β, γ and δ were diluted to the final concentrations of 1.65 nM, 4.8 nM, 7.6 nM and 5.7 nM, respectively. 2.5 μL of kinase solution was then added to each well of the assay plate, except for control well without enzyme (add 2.5 μL of 1x kinase buffer instead). Meanwhile, PIP2 substrate and ATP were diluted in the 1x kinase buffer to give 2x substrate solution with final concentrations of 50 μM of PIP2 and 25 μM of ATP. After that, 5 μL of substrate solution was added to each well of the assay plate to start reaction. The assay plate was covered and incubated at room temperature for 1 h. As for PI3Kα, 10 μL of Kinase-Glo reagent was then added to each well of the assay plate to stop the reaction. Subsequently, the mixture was treated briefly with centrifuge, shaked slowly on the shaker for 15 min before reading on a plate reader for luminescence. As for PI3Kβ, γ and δ, 5 μL reaction mixture was transferred from 384-well to a new 384 plate and 5 μL of ADP-Glo reagent was added to each well to stop the reaction. The mixture was treated briefly with centrifuge, shaked slowly on the shaker and equilibrated for 40 min. 10 μL Kinase Detection reagent was added to each wells, shaked for 1 min and equilibrated for 1 h before reading on a plate reader for luminescence. Finally, conversion data was collected on Flex station and RLU values were converted to inhibition values using the formula of (sample RLU -min)/(max-min) ×100. Herein, "min" means the RLU of no enzyme control and "max" means the RLU of DMSO control.

mTOR enzyme assay

The inhibition of mTOR (1362-end, Millipore) activity was determined by using the Lance Ultra assay. Test compounds were serially diluted to the desired concentrations and then 2.5 μL of each of them was added to a 384-well plate (Corning) as assay plate. 1x kinase buffer was prepared contained 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 3 mM $MnCl_2$, 0.01% Tween-20, 2 mM DTT. mTOR was diluted in the 1x kinase buffer to give 4x kinase solution with a final concentration of 2.5 nM. 2.5 μL of kinase solution was then added to each well of the assay plate, except for control well without enzyme. Meanwhile, ULight-4E-BP1 peptide substrate (Thr37/46, PE) and ATP were diluted in the 1x kinase buffer to give 2x substrate solution with final concentrations of 50 μM of ULight-4E-BP1 peptide and 10.8 μM of ATP. 5 μL of substrate solution was then added to each well of the assay plate to start reaction. The assay plate was coved and incubated at room temperature for 1 hour. Subsequently, 10 μL of prepared detection solution buffer containing EDTA and Eu-anti-phospho-4E-BP1 antibody (Thr37/46, PE) was added to each

well of the assay plate. After treating briefly with centrifuge, allow the plate to equilibrate for 60 minutes before reading on a plate reader. Finally, conversion data was collected on Envision and Lance signal (665nm) values were converted to inhibition values using the formula of (Lance signal-min)/(max-min) × 100. Herein, "min" means the Lance signal of no enzyme control and "max" means the Lance signal of DMSO control.

Anti-proliferative assay

All new synthesized derivatives were screened for their anti-proliferative activity by sulforhodamin B (SRB) assay. PC-3 cells were seeded into 96-well plates and cultured for 10 h. Subsequently, it was exposed to serial concentrations of compound for 72 h. Cells were then washed with PBS and fixed with 10% (w/v) trichloroacetic acid at 4 °C for 1 hour. After washing, the cells were stained for 30 min with 0.4% SRB dissolved in 1% acetic acid and then washed by 1% acetic acid for 5 times. Finally, the protein-bound dye was extracted using 10 mM unbuffered Tris base. The absorbance was obtained at 515 nm on a multiscan spectrum (Thermo Fisher). The inhibition rate on cell proliferation of each well was calculated using the formula of (A515 control cells - A515 treated cells)/A515 control cells × 100%.

Western blot assay

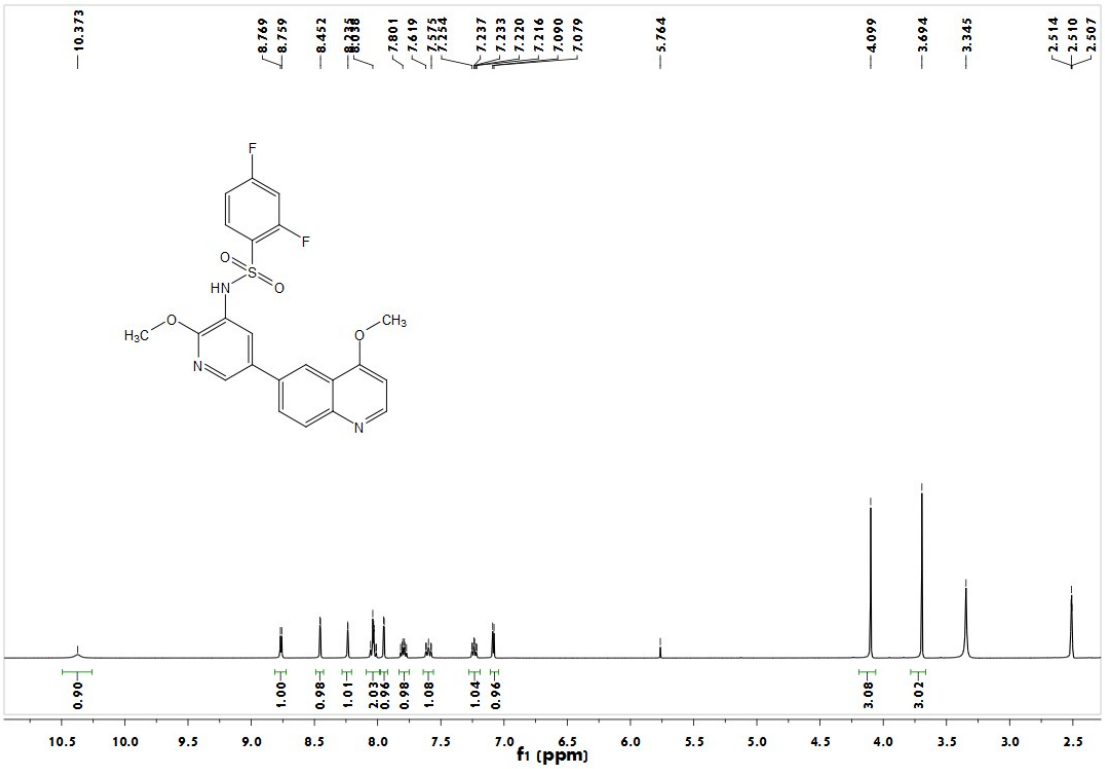
The suppressive activities of Akt and pAkt(ser473) in PC-3 cells were determined by western blot analysis. GAPDH was employed as the internal control. PC-3 cells were seeded into six-well plate at 8×10^5 cells per well and then incubated at 37 °C overnight prior to drug exposure. Cells were treated with **15a** at various concentrations of 10 nM, 20 nM, 40 nM and 80 nM, and incubated at 37 °C for 3 h, respectively. After compound treatment, culture medium was discarded and cells were rinsed with pre-chilled PBS 3 times immediately. Subsequently, cell lysates were collected by adding RIPA lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease and phosphatase inhibitor (Roche) onto cells. The cell lysates were clarified by centrifugation at 12,000 rpm for 15 min at 4 °C and the supernatant was collected as protein sample. The total protein concentration in protein sample was quantified using BCA protein Assay Kit (Pierce). Following this, the normalized protein samples were mixed with 4x NuPAGE LDS sample buffer (Life Tech) and boiled at 95˚C for 5 minute, then loaded and electrophoresed in NuPAGE 4-12 % Novex Bis-Tris gel (Life Tech). Subsequently, using Life Technologies IBLOT transfer system (Life Tech, IB301002), proteins were transferred from gel to a nitrocellulose membrane, which was blocked in SuperBlock blocking buffer (Thermo Fisher) for 1 h then in primary antibody dilution (rabbit anti-pAkt(ser473), rabbit anti-Akt, mouse anti-GAPDH, Abcam) overnight at 4 °C. After washing in TBST buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% Tween-20), membrane was incubated in diluted secondary antibodies for 2 h at room temperature, and washed in TBST again. Finally, membrane was imaged using LICOR Odyssey system.

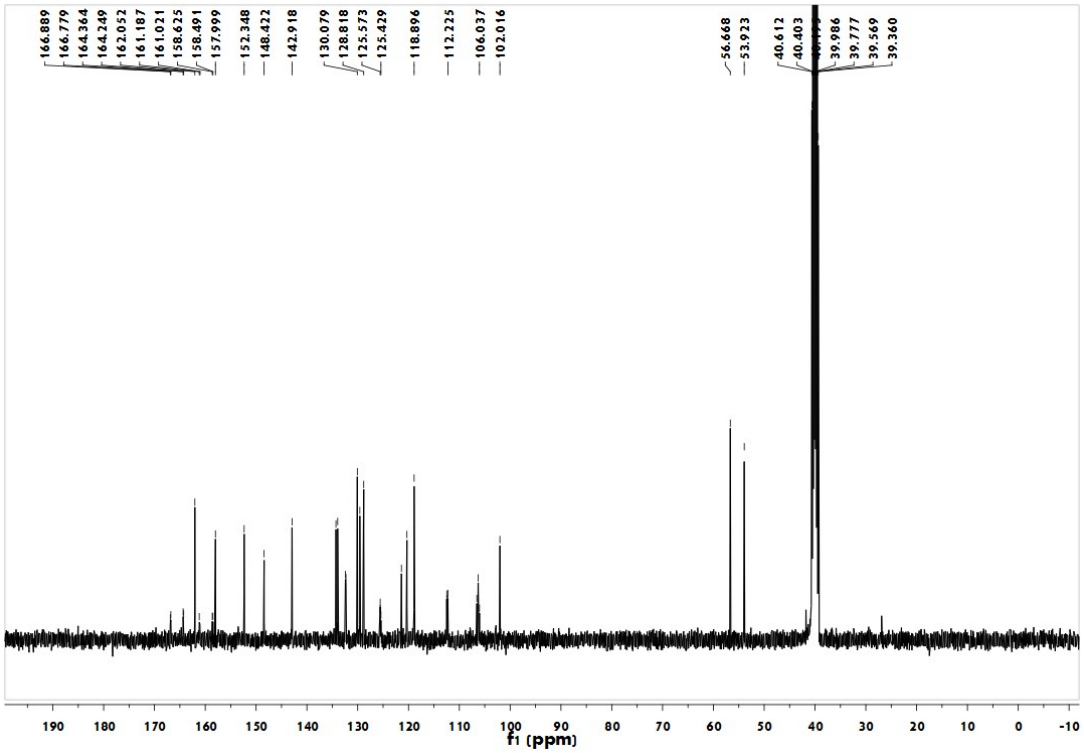
Molecular docking study

The co-crystal structure of PI3Kα in complex with BYL-719 (PDB code 4JPS) was chosen as the template to generate the docking modes. For the preparation of ligands, the 3D structures were generated and their energy minimization was performed by using Discovery Studio 2.1. For the preparation of protein, the hydrogen atoms were added, and the CHARMm-force field was employed. The whole PI3Kα enzyme was defined as a receptor and the site sphere was selected based on the BYL-719 binding location of PI3Kα, then the molecule BYL-719 was removed and **15a** was placed during the molecular docking procedure. Types of interactions of the docked PI3Kα with ligand were analyzed and then the docking conformations were selected and saved based on calculated energy.

2. Copies NMR Spectra of representative compounds

compound 5





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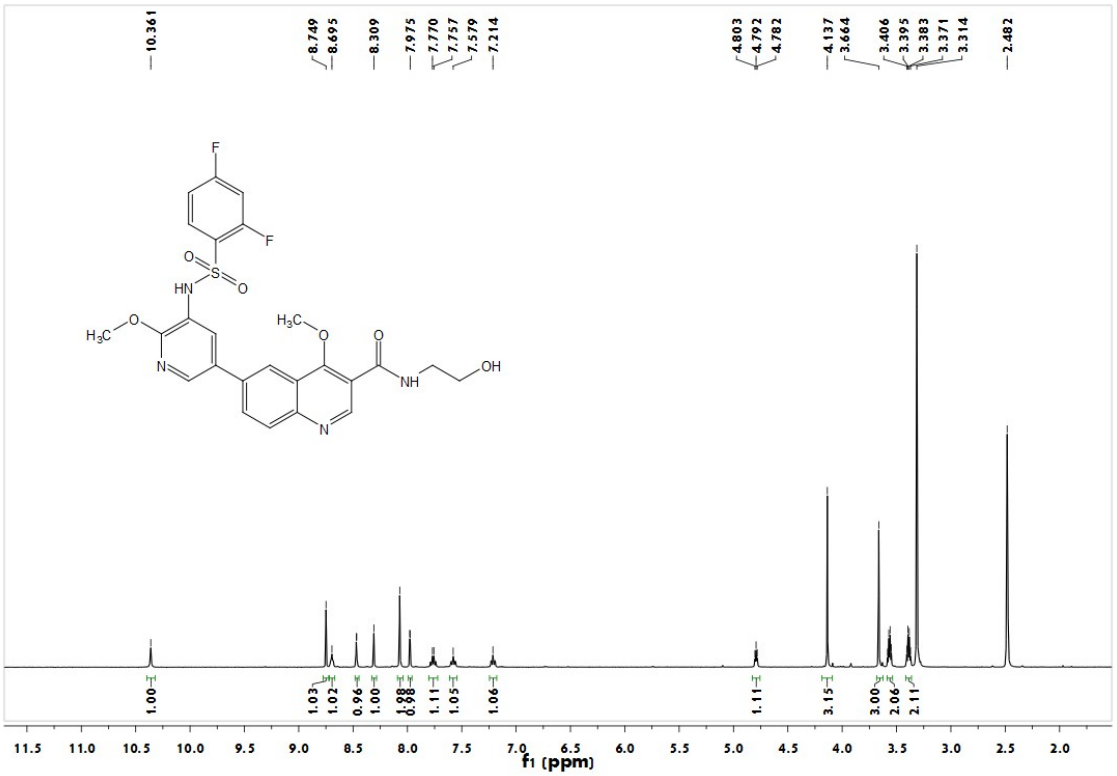

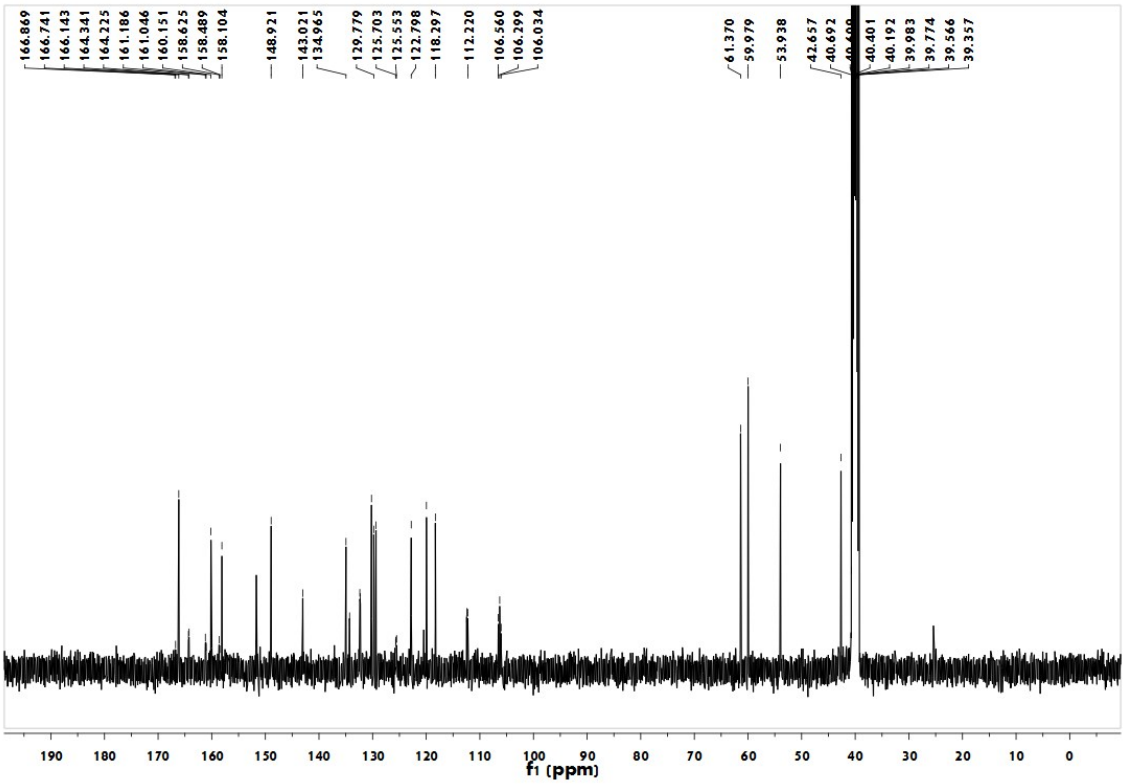

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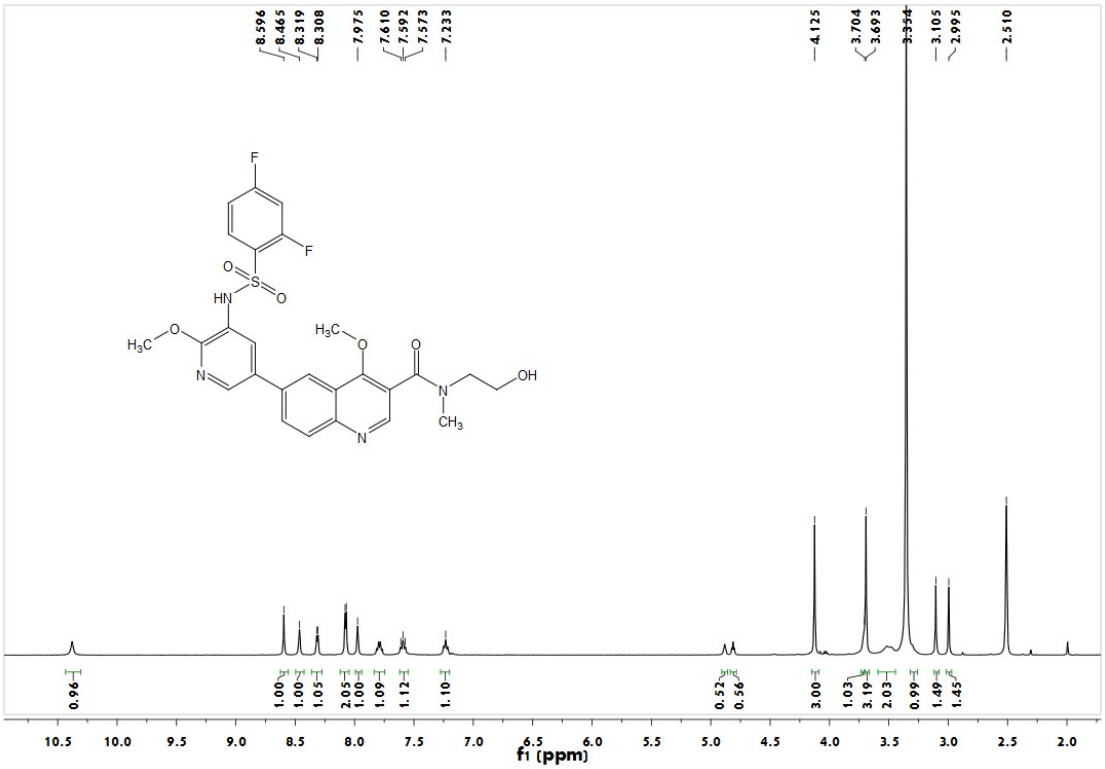

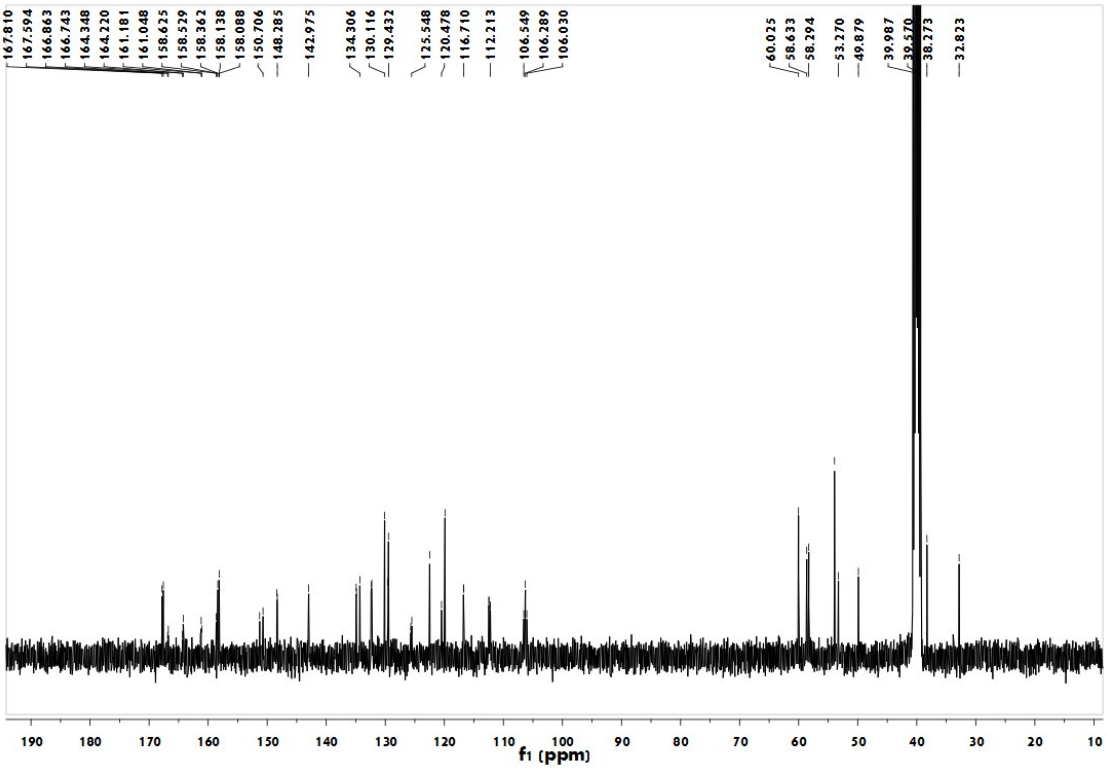

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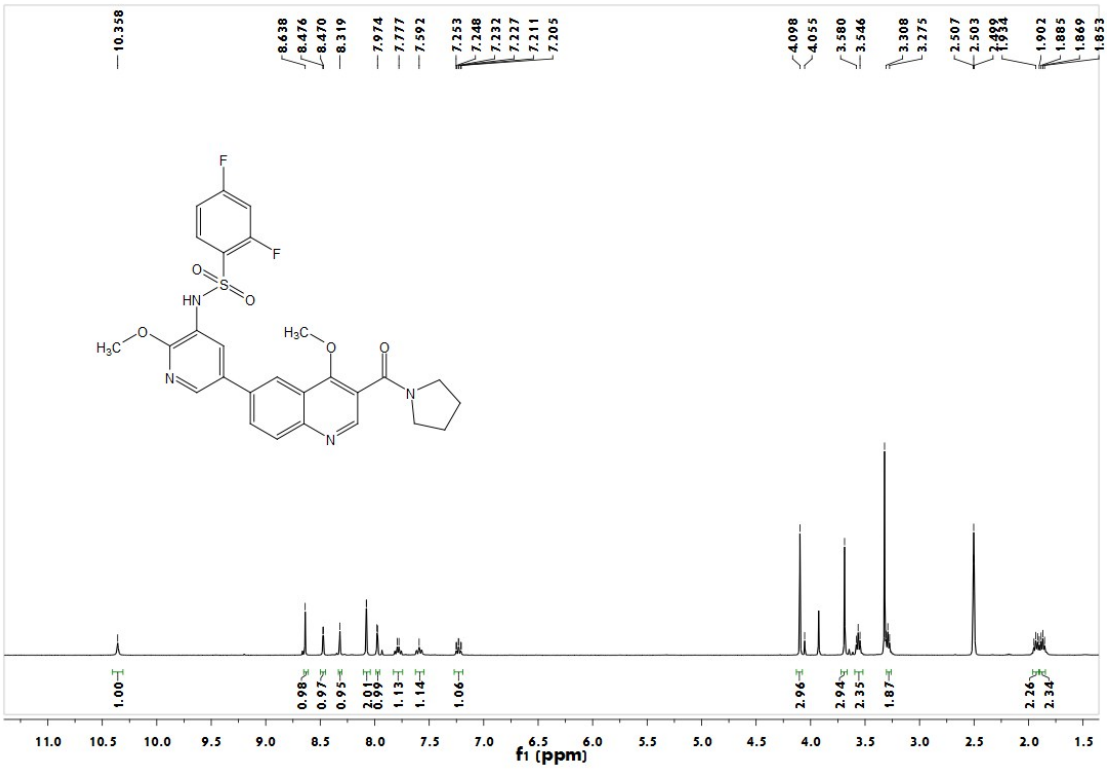

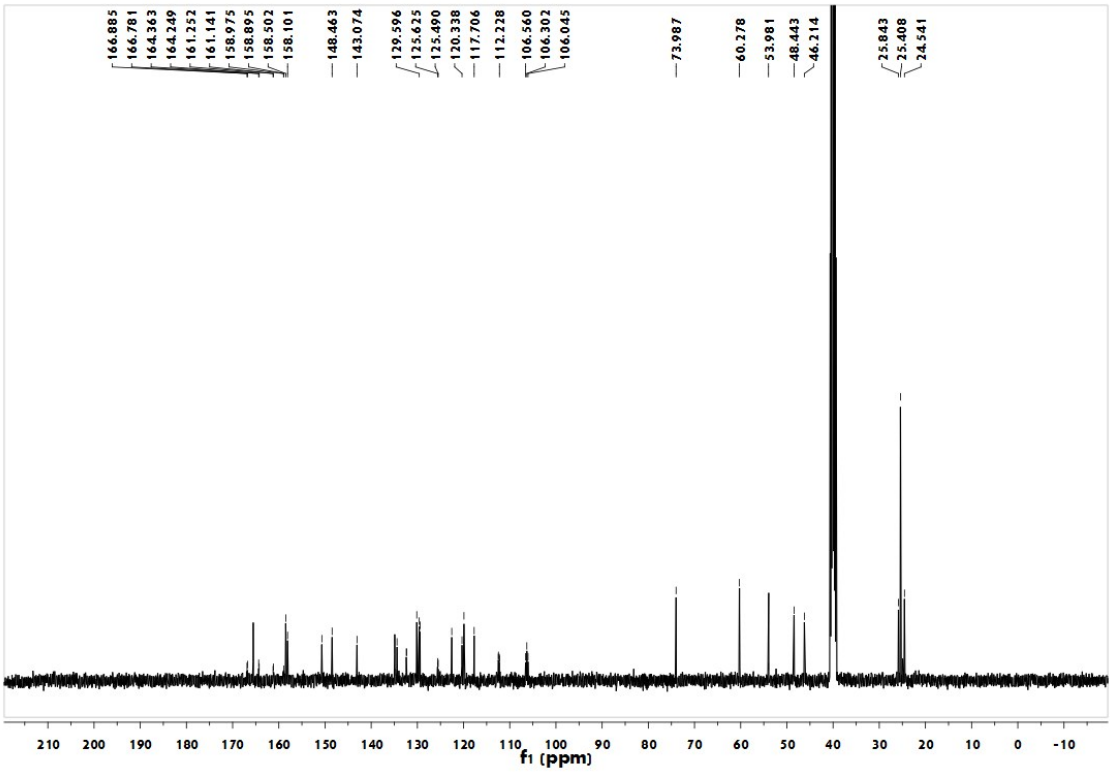

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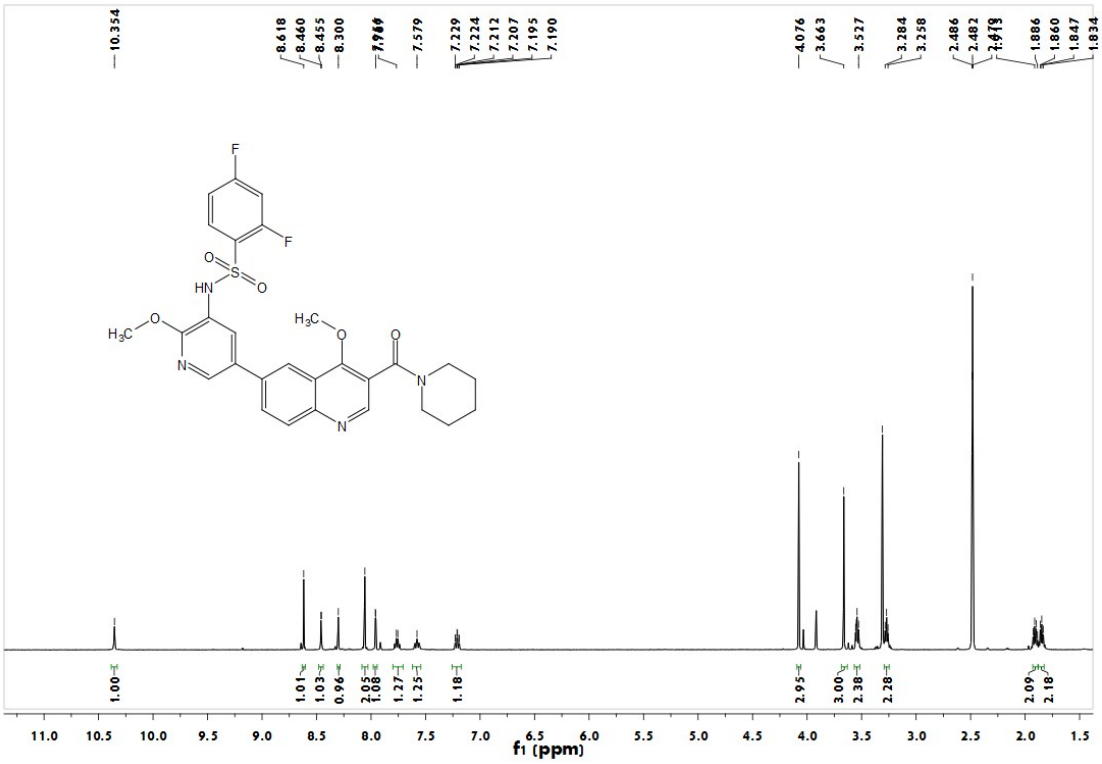

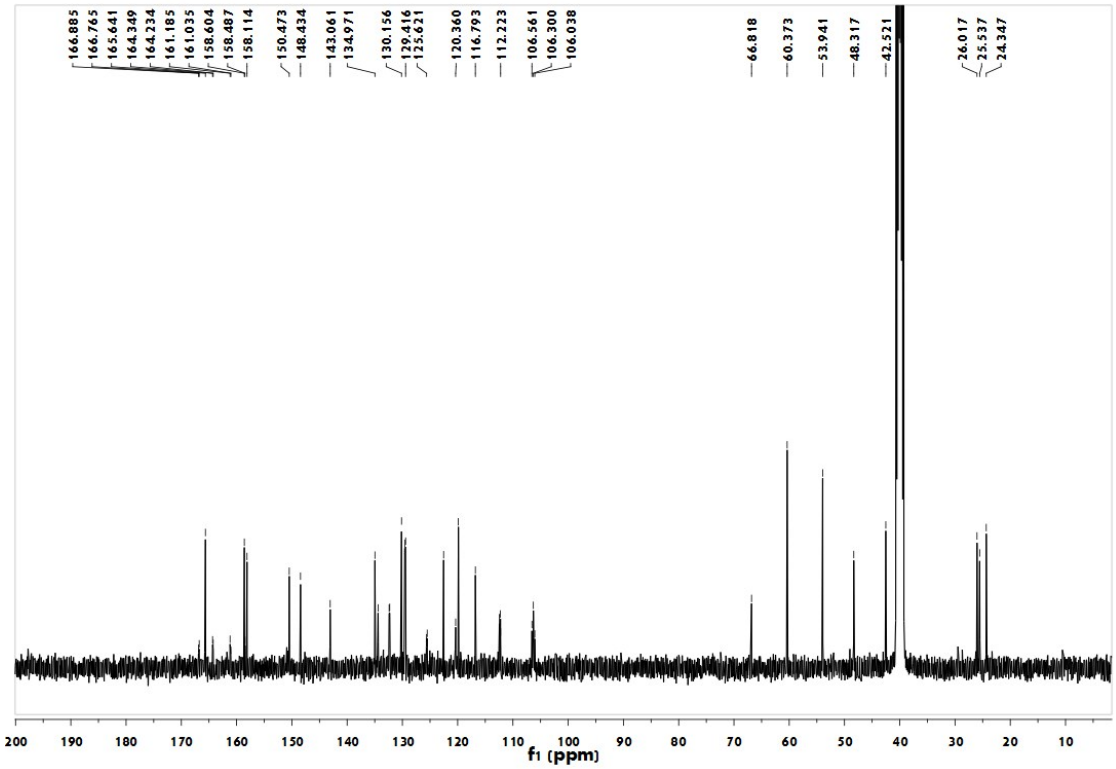

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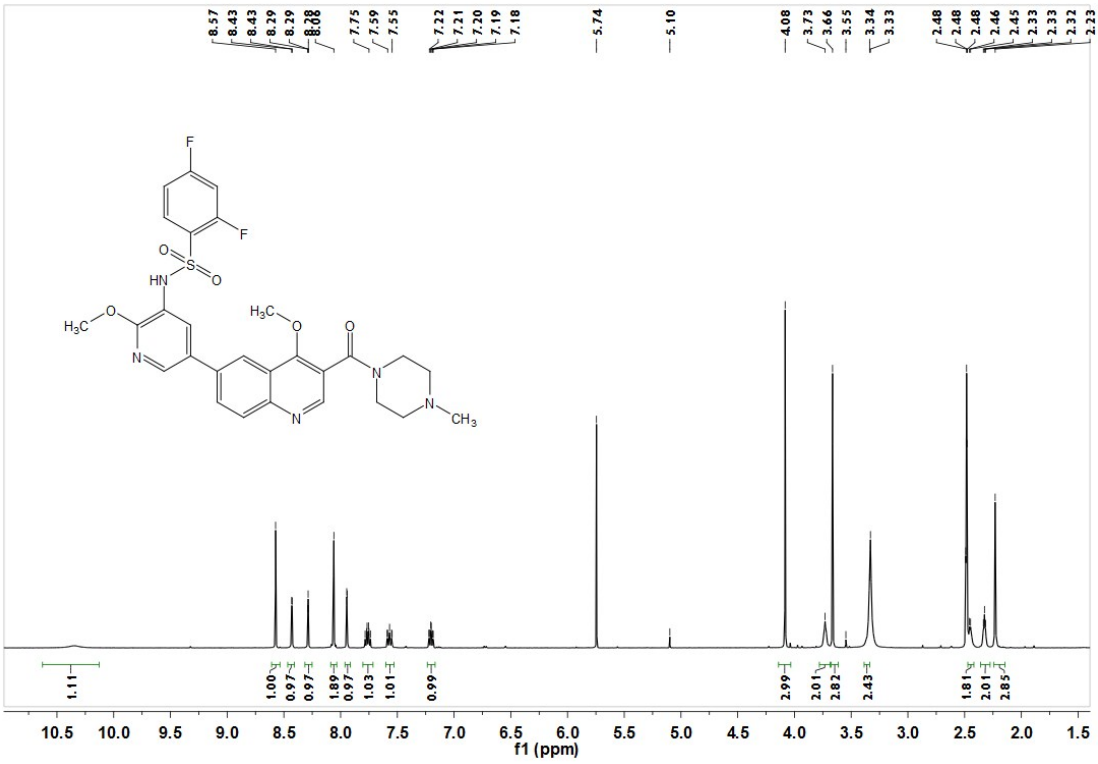

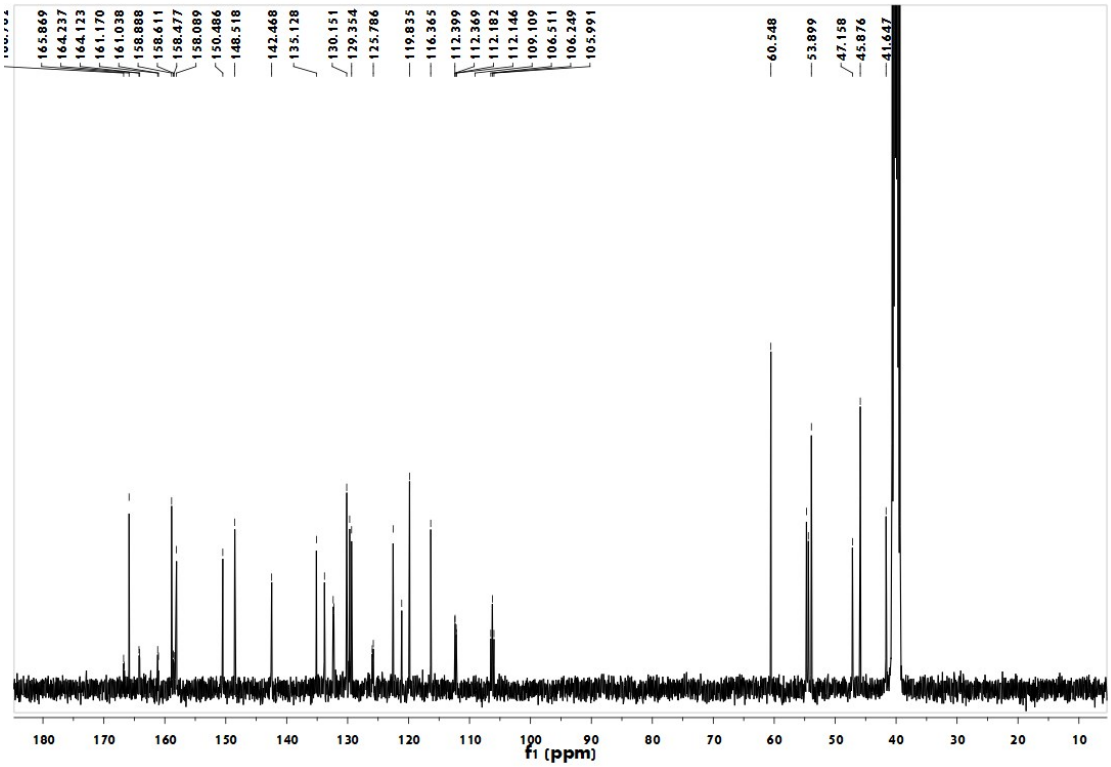

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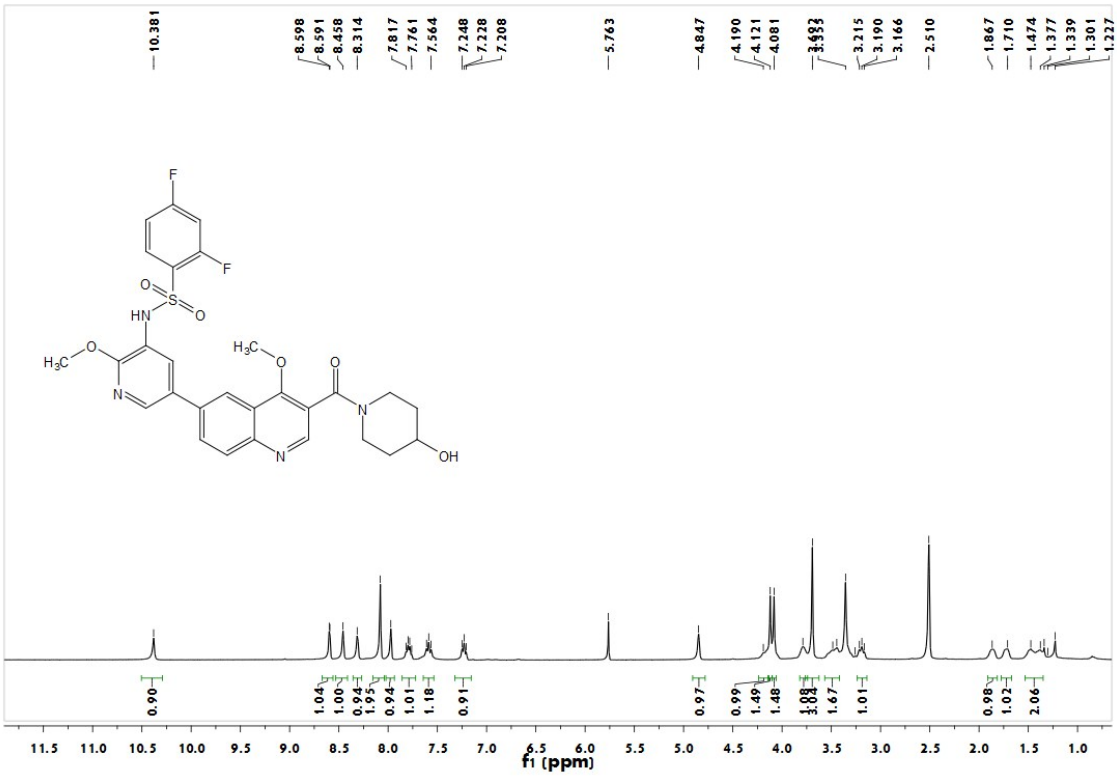

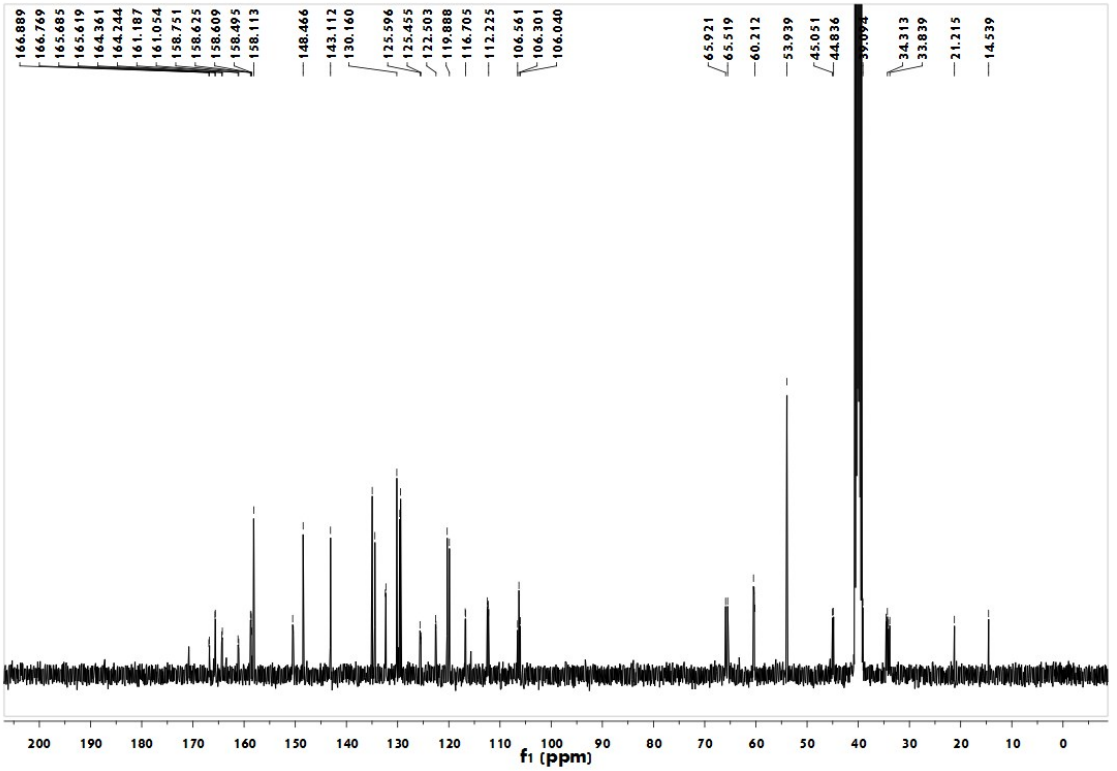

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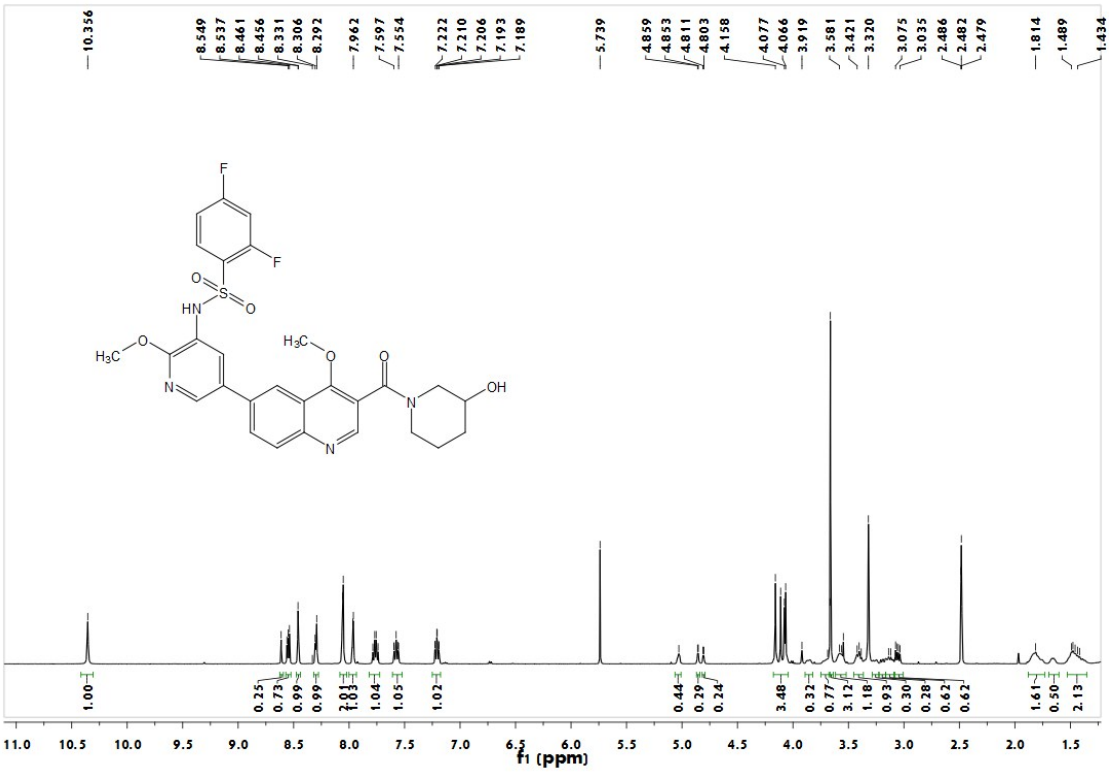

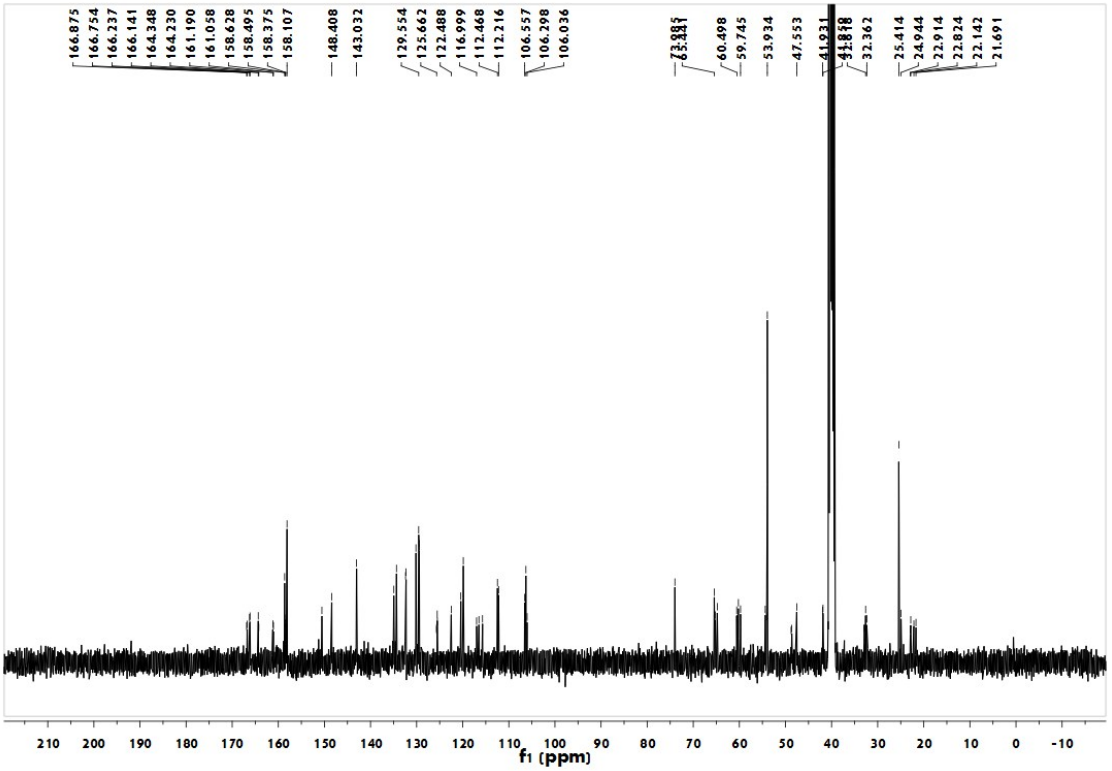

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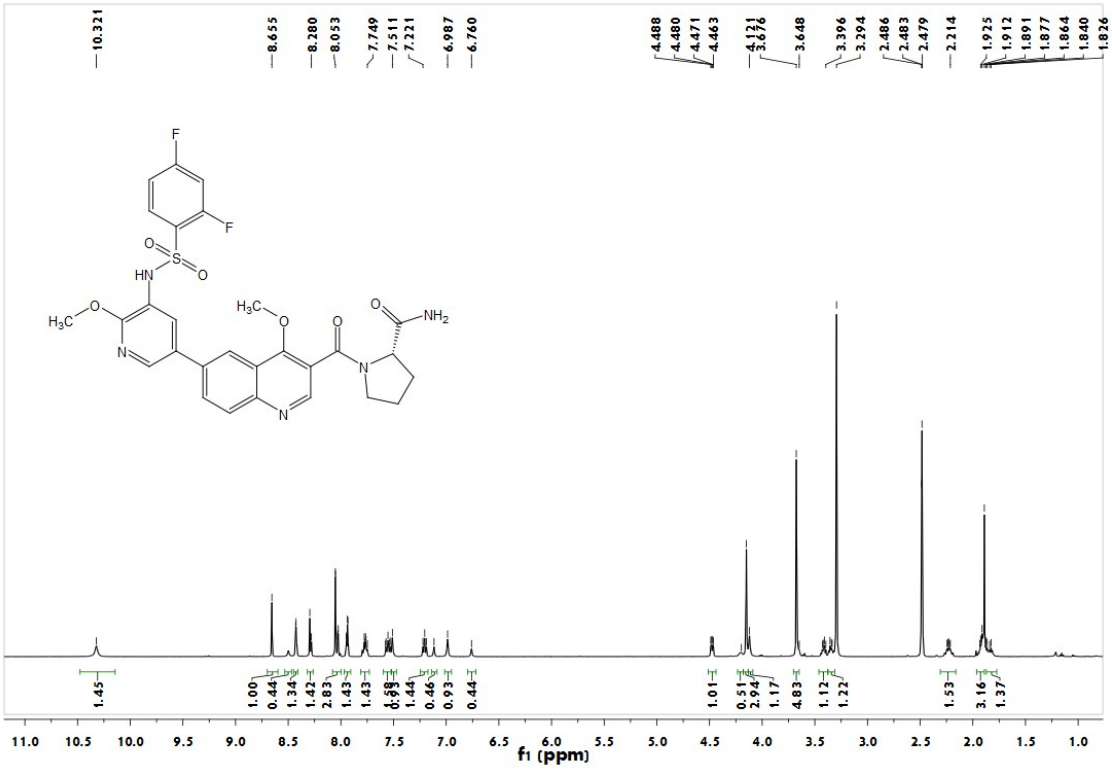

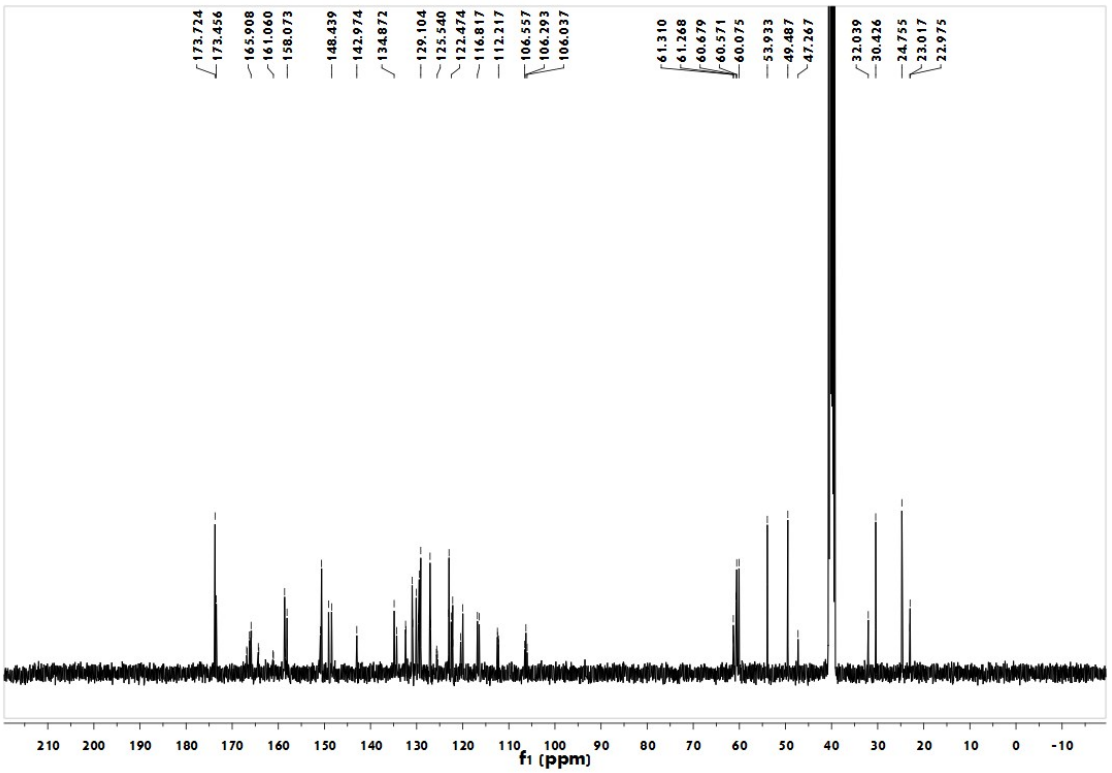

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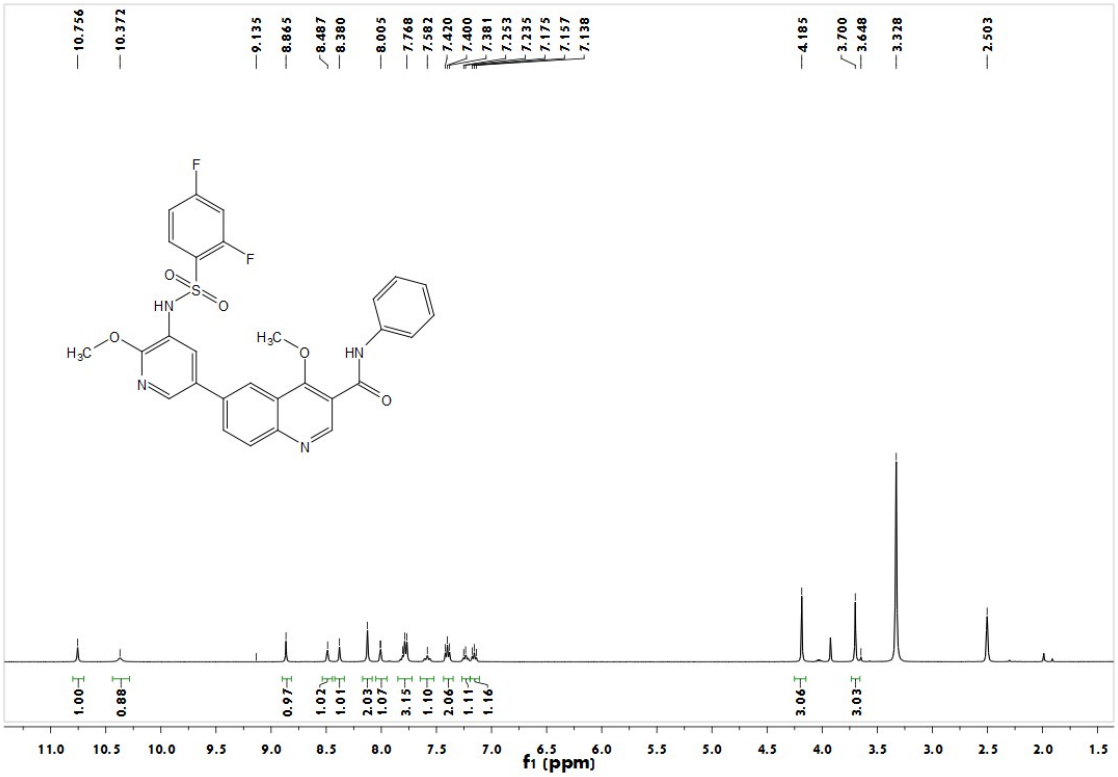

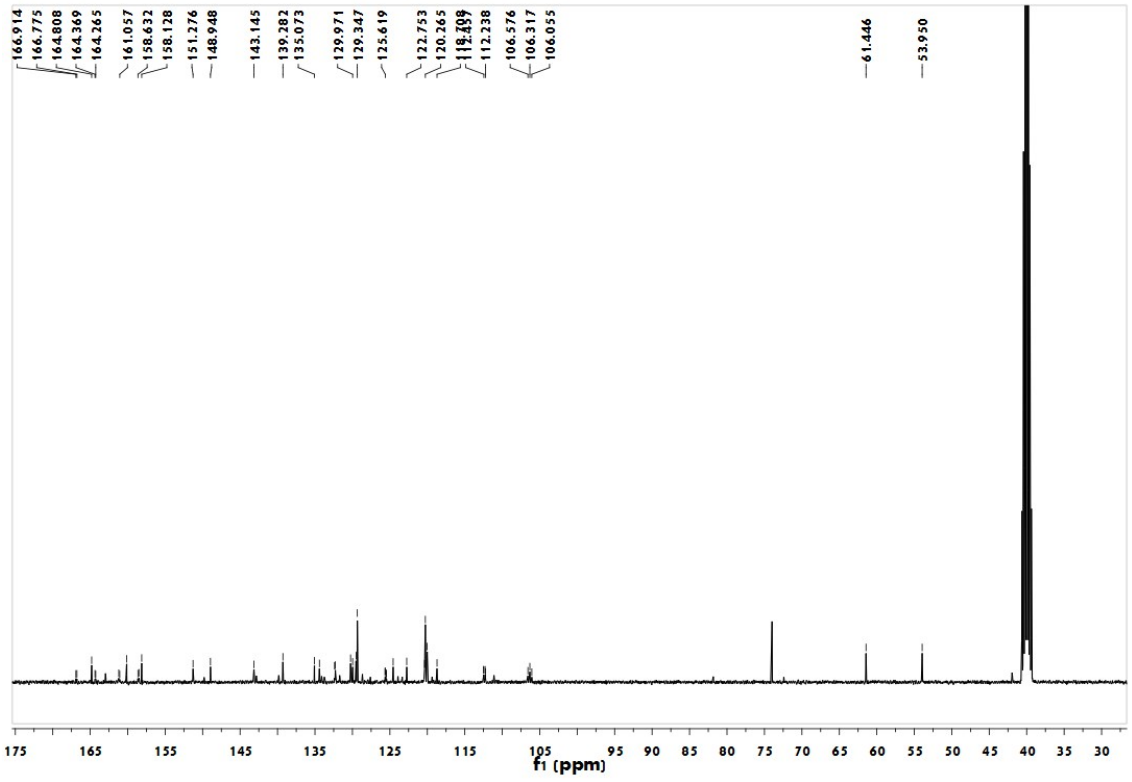

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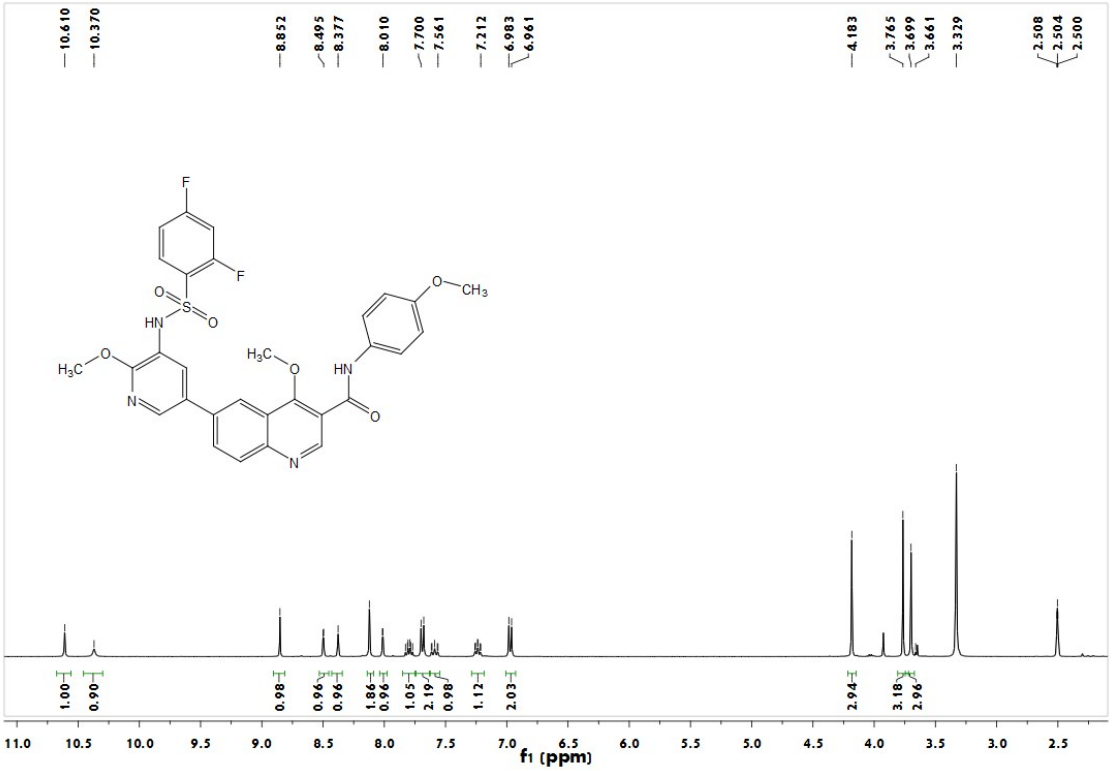

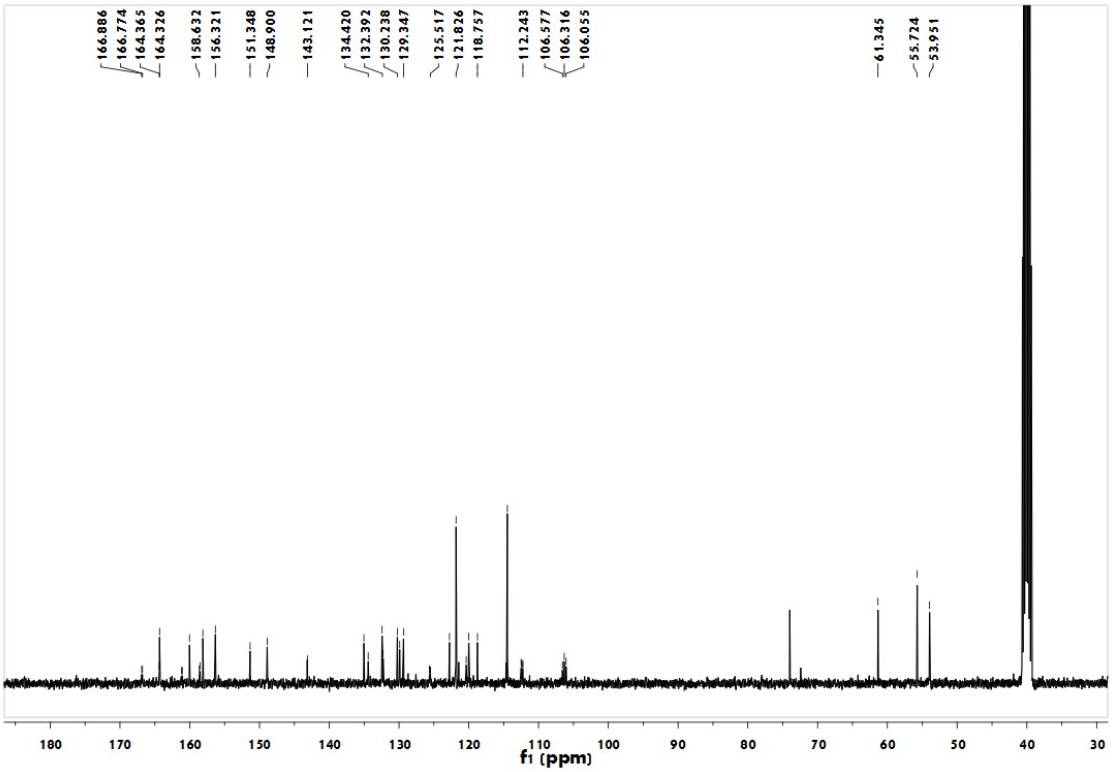

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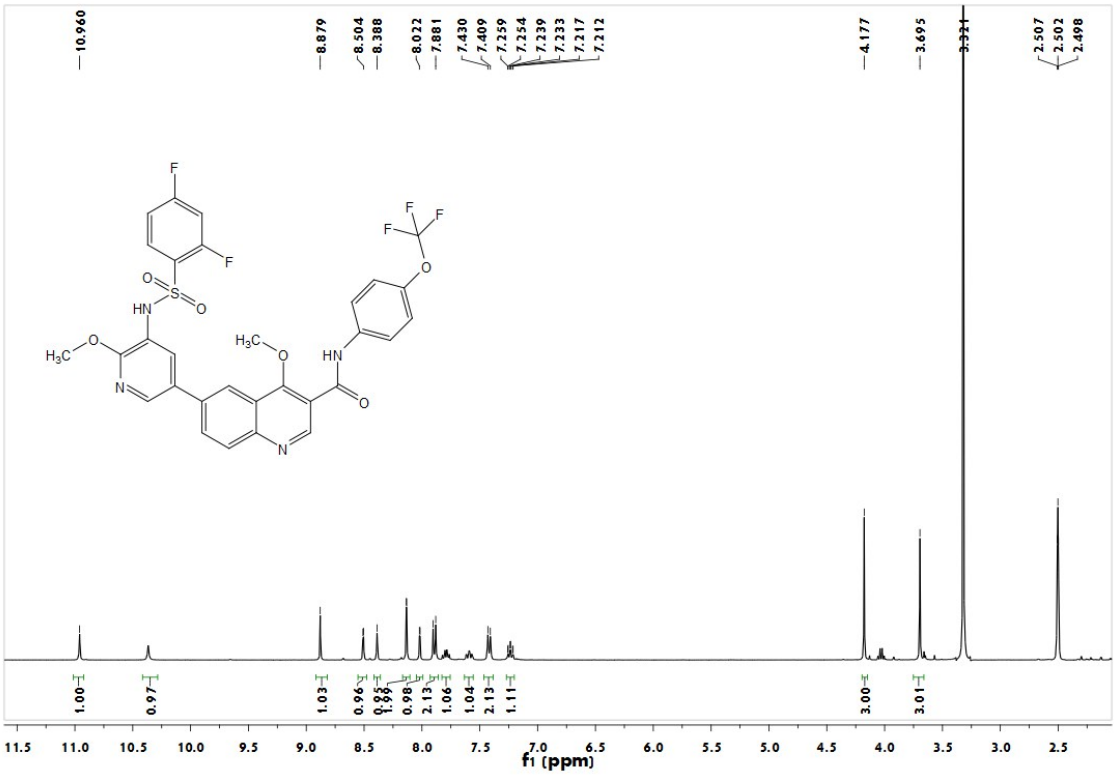
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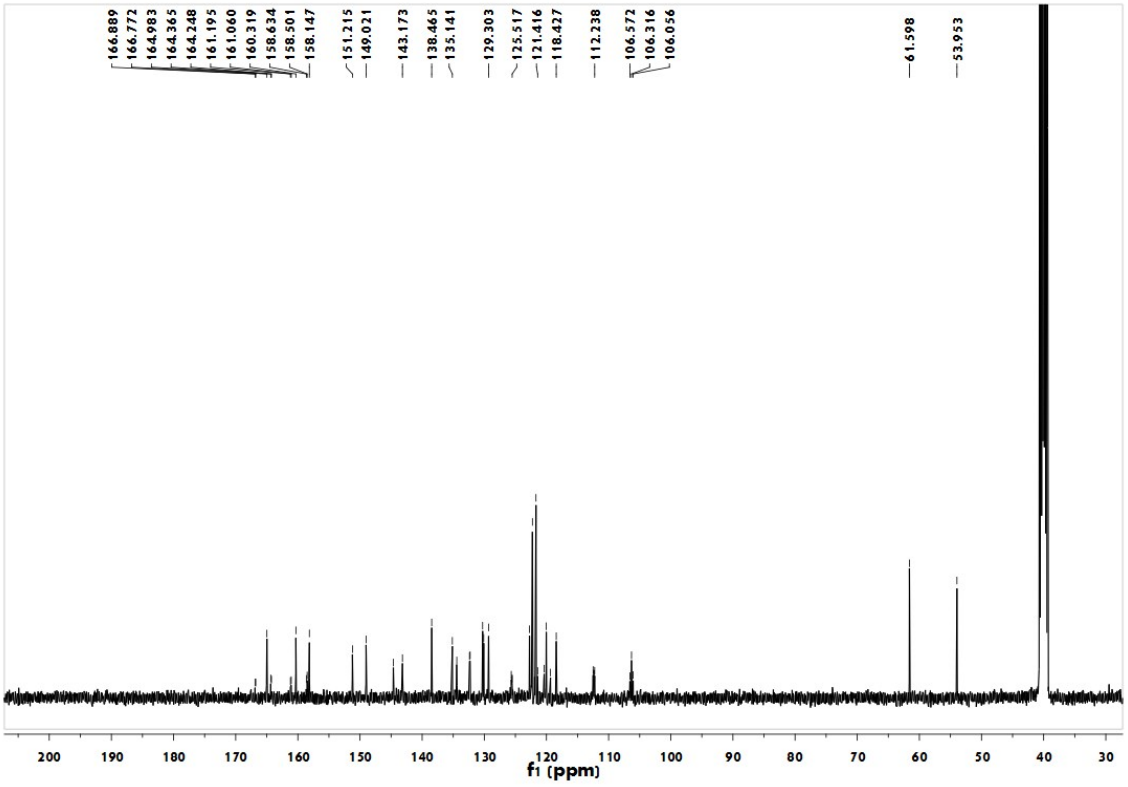
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