Supplementary information

Novel halogenated pyrido[2,3-a]carbazole with enhanced aromaticity as potent anticancer and antioxidant: Rationale design and microwave assisted synthesis

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Experimental section:

Materials and methods: All the chemicals used were chemically pure and AR grade. Solvents were purified and dried according to the standard procedure[1]. Elemental analyses of synthesized compounds were performed on a vario EL 111 CHN analyzer. IR spectra were recorded by KBr pellet technique in the range 400 - 4000 cm^{-1} region using a Perkin Elmer FT-IR 8000 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV 111 500 MHz instrument using TMS as internal reference. Electron ionization mass spectra of the compounds were recorded on a JEOL GCMATEII mass spectrometer. Antioxidant and anticancer studies were carried out at the Kovai Medical Centre and Hospital Pharmacy College, Coimbatore, Tamilnadu. Molecular docking studies were carried out at Department of Animal Science, Bharathidasan University, Tiruchirappalli. Melting points were recorded using Veego VMP-DS heating table apparatus and are uncorrected.

Biological evaluation:

DNA binding - Titration experiments

The binding affinities of all the compounds with CT-DNA were carried out in doubly distilled water with Tris(hydroxymethyl)-aminomethane (Tris, 5 mM) and sodium chloride (50 mM) and the pH of the solution is adjusted to 7.2 with hydrochloric acid. A solution of CT-DNA in the buffer gave a ratio of UV absorbance of about 1.8-1.9 at 260 and 280 nm, which indicated that the DNA was sufficiently free of protein. The DNA concentration per nucleotide was determined by absorption spectroscopy using the molar extinction coefficient value of 6600 dm^3 mol^{-1} cm^{-1} at 260 nm. The compounds were dissolved in a solvent mixture, comprising of 5 % DMSO and 95 % Tris-HCl buffer for all the experiments. Stock solutions were stored at 4 ^oC and used within 4 days. Absorption titration experiments were performed with fixed concentration of the compounds (25 μM) with varying concentration of DNA (0-50 μM). While measuring the absorption spectrum, an equal amount of DNA was added to the all test solutions and the reference solution to eliminate the absorbance of DNA itself[2].

In order to illustrate quantitatively the consequence, the absorption data were analyzed to evaluate the intrinsic binding constant $(\mathbf{K_b})$[3] , where [DNA] is the concentration of DNA in base pairs, the apparent absorption coefficient ε_a, ε_f and ε_b corresponds to A_{obs}/ [compound], the extinction coefficient of the free compound and the extinction coefficient of the compound when fully bound to DNA, respectively.

$$DNA/(\varepsilon_a-\varepsilon_f) = [DNA]/(\varepsilon_b-\varepsilon_f) + 1/K_b(\varepsilon_b-\varepsilon_f) \qquad -(3)$$

Nuclease activity using gel electrophoresis:

The DNA cleavage activity of the pyrido[2,3-a]carbazole derivatives was monitored by agarose gel electrophoresis on pBR322 DNA. The tests were performed in the absence and presence of activating agent, H_2O_2 under aerobic conditions. Hydrolytic cleavage was monitored by 30 μM of pBR322 DNA and 50 μM of each compounds in 5 % DMSO and 95 % Tris-HCl buffer (5 mM, pH 7.2) with 50 mMNaCl.

For oxidative cleavage, each reaction mixture contained 30 μM of pBR322 DNA, 50 V of each compounds in 5 % DMSO and 95 % Tris-HCl buffer (5 mM, pH 7.2) with 50 mMNaCl and 60 μM of H_2O_2.

The samples with sufficient buffer were incubated for about 2 h at 37 ^oC. After incubation, 1 μL of loading buffer (0.25 % bromophenol blue, 0.25 % xylene cynol and 60 %

glycerol) were added to the reaction mixture and loaded onto a 1 % agarose gel containing 1.0 µg/mL of ethidium bromide. The electrophoresis was carried out for about 2 h at 50 V in Tris-acetic acid EDTA buffer. The bands were visualized under UV light and photographed.

Antioxidant activity

The 2,2-diphenyl-2-picryl-hydrazyl (DPPH) radical and hydroxyl (OH) radical scavenging activity of the compounds was measured according to the method of Elizabeth[4]. The DPPH radical is a stable free radical having a λ_{max} at 517 nm. A fixed concentration of the experimental compound (100 µL) was added to a solution of DPPH in methanol (0.3 mmol, 1 mL) and the final volume was made up to 4 mL with double distilled water. DPPH solution with methanol was used as a positive control and methanol alone acted as a blank. The solution was incubated at 37 °C for 30 min in the dark. The decrease in absorbance of DPPH was measured

The scavenging activity for hydroxyl radicals recommended by Yu *et al.,* with major changes[5]. Reaction mixture contained (0.6 mL of 1.0 mmol) deoxyreboses, phenylhydrazine (0.4 mL of 0.2 mM) and phosphate buffer 0.6 mL of 10 mmol (pH 7.4). It was incubated for one hour at room temperature. Then add 1 mL of 2-8 % TCA, 1 mL of 1% TBA and 0.4 mL of compounds at various concentrations and kept in water bath for 20 min. The absorbance of the mixture at 532 nm was measured with a spectrophotometer (HACH 4000 DU UV-Visible spectrophotometer). The hydroxyl radical scavenging activity was calculated.

For each of the above assays, the tests were run in triplicate, and various concentrations (20-100 µg/ mL). The percentage inhibition of absorbance was calculated and plotted as a function of the concentration of standard and sample to determine the antioxidant concentration. The percentage of activity was calculated using the formula,

$$\% \text{ of suppression ratio} = [(A_0 - A_c)/A_0] \text{ X } 100. \quad - (1)$$

Where, A_0 and A_c are the absorbance in the absence and presence of the tested compounds, respectively. The 50 % activity (IC_{50}) can be calculated using the percentage of activity[6,7].Ascorbic acid was used as a standard for all the above assays.

ABTS radical scavenging activity: ABTS radical cation was produced by reacting 7 mM ABTS solution with 2.45mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 16h. In the moment of use, the ABTS solution was diluted with ethanol to an absorbance at 734 nm. Each sample with various carbazole derivatives were added to 2.0 mL of

ABTS solution and mixed vigorously. The reaction mixture was allowed to stand at room temperature for 6 min and the absorbance at 734nm was immediately recorded.

In vitro anticancer activity assay

Cytotoxicity activity of the carbazolederivatives were carried out on human cervical cancer cells (HeLa), human breast cancer cell line (MCF-7) and human laryngeal epithelial carcinoma cell line (Hep-2) cancer cell lines was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). All cells were maintained at 37 ^{0}C, 5 % CO_2, 95 % air and 100 % relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week. Compounds capability to arrest the proliferation of tumor cells were evaluated after 48 h of incubation

Cell treatment procedure:

The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted by tryphan blue exclusion assay using a hemocytometer. The cell suspension was diluted with medium containing 5 % FBS to give final density of $1x10^5$ cells/ml. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/wells and incubated to allow for cell attachment at 37 ^{0}C, 5 % CO_2, 95 % air and 100 % relative humidity. After 24h the cells were treated with serial concentrations (0.1-100 μM) of the test samples. They were initially dissolved in neat DMSO and diluted to twice the desired final maximum test concentration with serum free medium. Additional four, serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 μl of these different sample dilutions was added to the appropriate wells already containing 100 μl of medium, resulted the required final sample concentrations. Following drug addition the plates were incubated for an additional 48h at 37 ^{0}C, 5 % CO_2, 95 % air and 100 % relative humidity. The medium containing without samples was served as control and triplicate were maintained for all concentrations.

MTT assays: The compounds were dissolved in DMSO and blank samples containing same volume of DMSO are taken as controls to identify the activity of solvent in this cytotoxicity experiment. After 48h of incubation, 15 μl of MTT (5 mg/ml) in phosphate buffered saline (PBS)

was added to each well and incubated at 37 ^{0}C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then measured the absorbance at 570 nm using micro plate readers. The % cell inhibition was determined using the following formula.

$$\% \text{ Cell Inhibition} = 100 - \text{Abs (sample)}/\text{Abs (control)} \times 100 \quad - (2)$$

Nonlinear regression graph was plotted between % Cell inhibition and Log concentration and IC_{50} was determined using GraphPad Prism software[8,9].

Viscosity measurements: The viscosity measurement was carried outusing an Ubbelodhe viscometer immersed in a thermostatic waterbath maintained at 25 (±0.l) ºC. DNA samples with approximately200 base pairs in length were prepared by sonication in order tominimize complexities arising from DNA flexibility. Titrationswere performed by addition of small volume of concentratedstock solutions of metal complexes to a solution of CT–DNA in the viscometer. Flow times were measured with a digitalstopwatch; each sample was measured three times and an averageflow time was calculated. Relative viscosities for CT-DNA inthe presence and absence of the complexes were calculated fromthe relation $\eta = (t - t0)/t0$, where t is the observed flow time ofDNA-containing solution and t0 is the flow time of Tris–HClbufferalone. Data are presented as $(\eta/\eta0)1/3$versus binding ratio(R = [complex]/[DNA] = 0.0–0.25), where η is the viscosity of CT-DNAin the presence of complexes and η0 is the viscosity of CT-DNAalone.

Molecular docking studies

In order to understand the interaction at molecular level, compounds **3a-3g** were docked with X-ray crystal structure of human protein kinase CK2 (PDB ID: 3OWJ) using AutoDock 4.2. Docking protocol reported earlier communications[10–12].Ligand structures were drawn using build panel & prepared using Ligprep module implemented Maestro-8.4 (Schrodinger LLC). Energy minimization is carried out using OPLS-2005 force field. Structures were saved in .pdb format and rewritten using open bablefor AutoDock compatible atom type. For docking, grid parameter file (.gpf) and docking parameter files (.dpf) were written using MGL Tools-1.5.6. Receptor grids were generated using 60×60×60 Grid points in xyz with grid spacing of 0.375Å. Grid box was generated by considering active residues ASN118, ILE174, VAL53, VAL116. Map types were generated using autogrid 4.2. Docking was carried out with following parameters with number of

runs: 50, population size: 150, number of evaluations: 2,500,000 and number of generations: 27,000, using autodock 4.2. Analysis of docking results was done using MGL Tools-1.5.6. Top scoring molecule in the largest cluster was analyzed for its interaction with the protein.

ADME properties prediction: The compounds were further carried out into ADME (absorption, distribution, metabolism and excretion) properties prediction using Qikprop[13].

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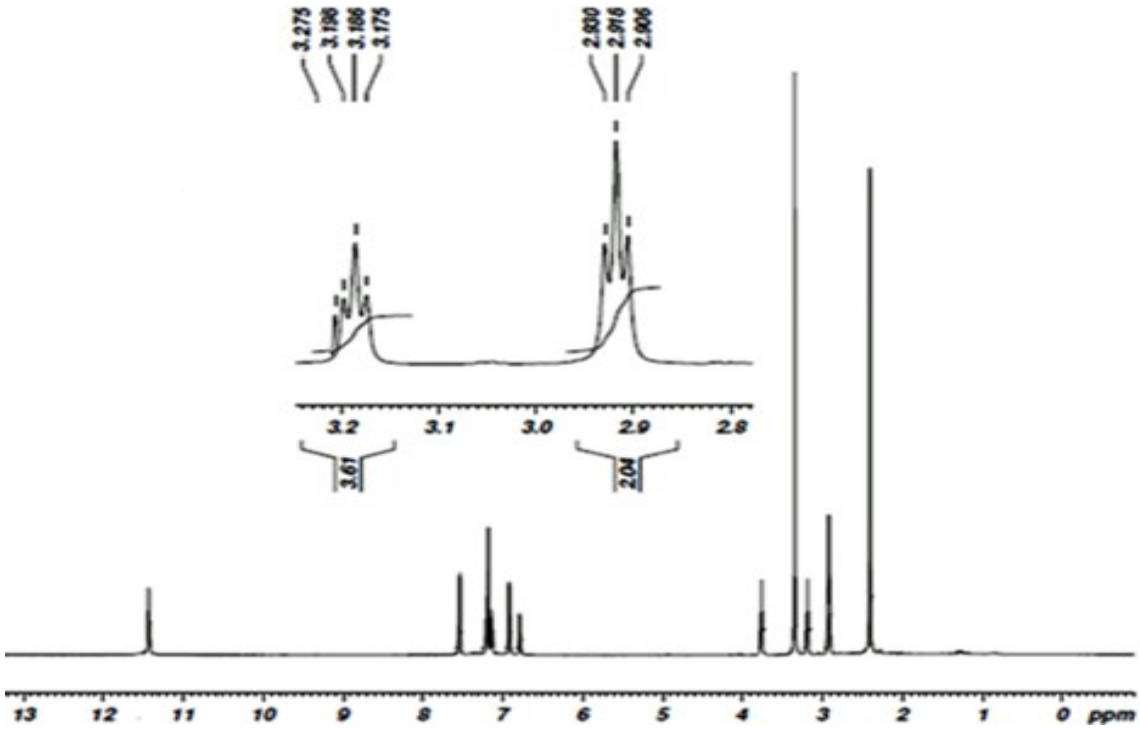
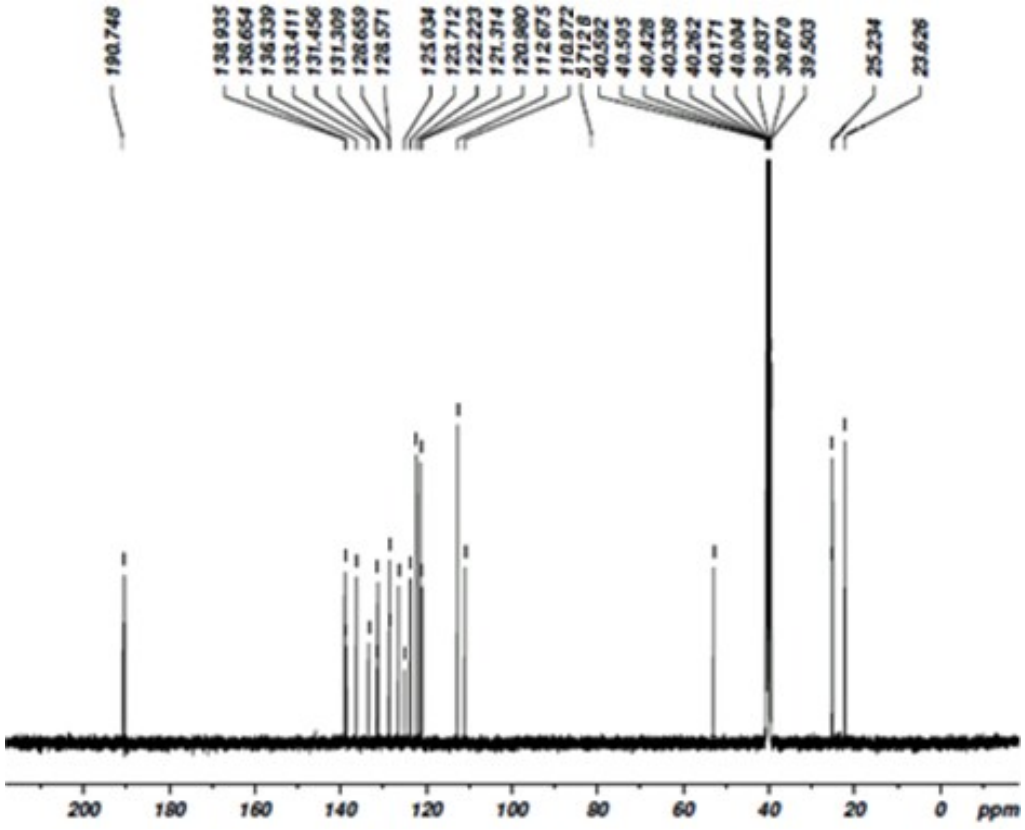
NMR characterization of compounds (3a-3g)



^{1}H NMR Spectrum of compound **3a**



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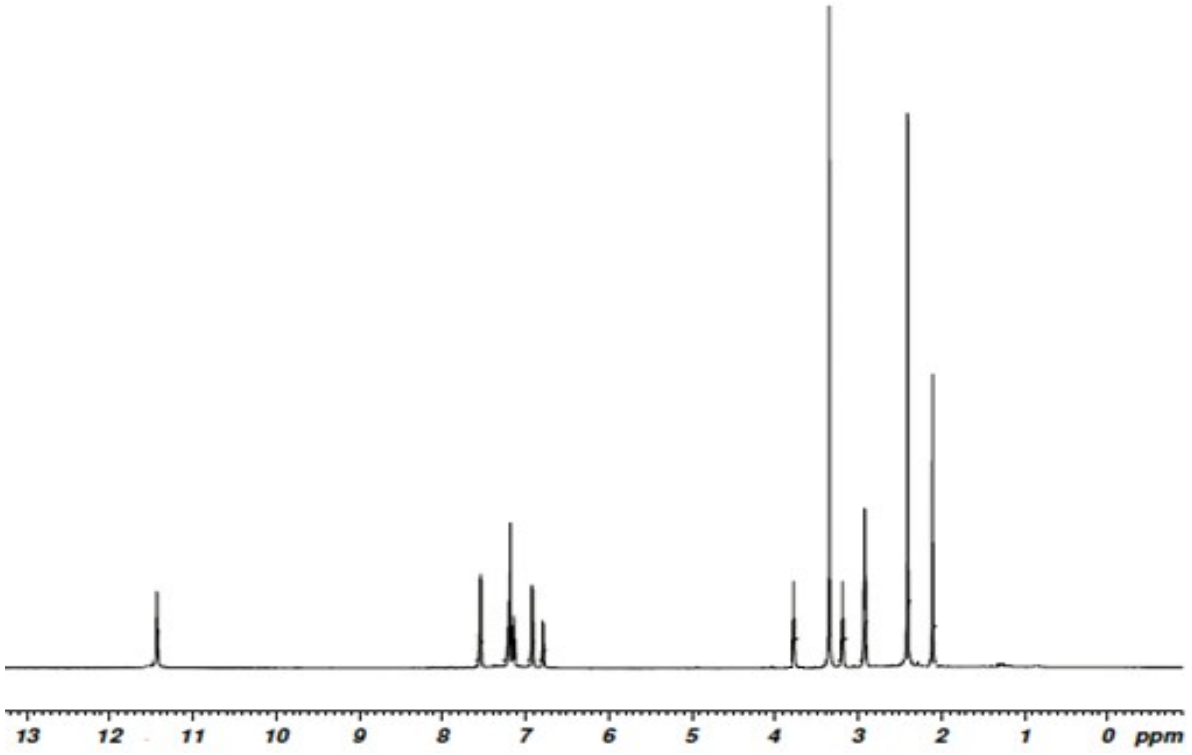

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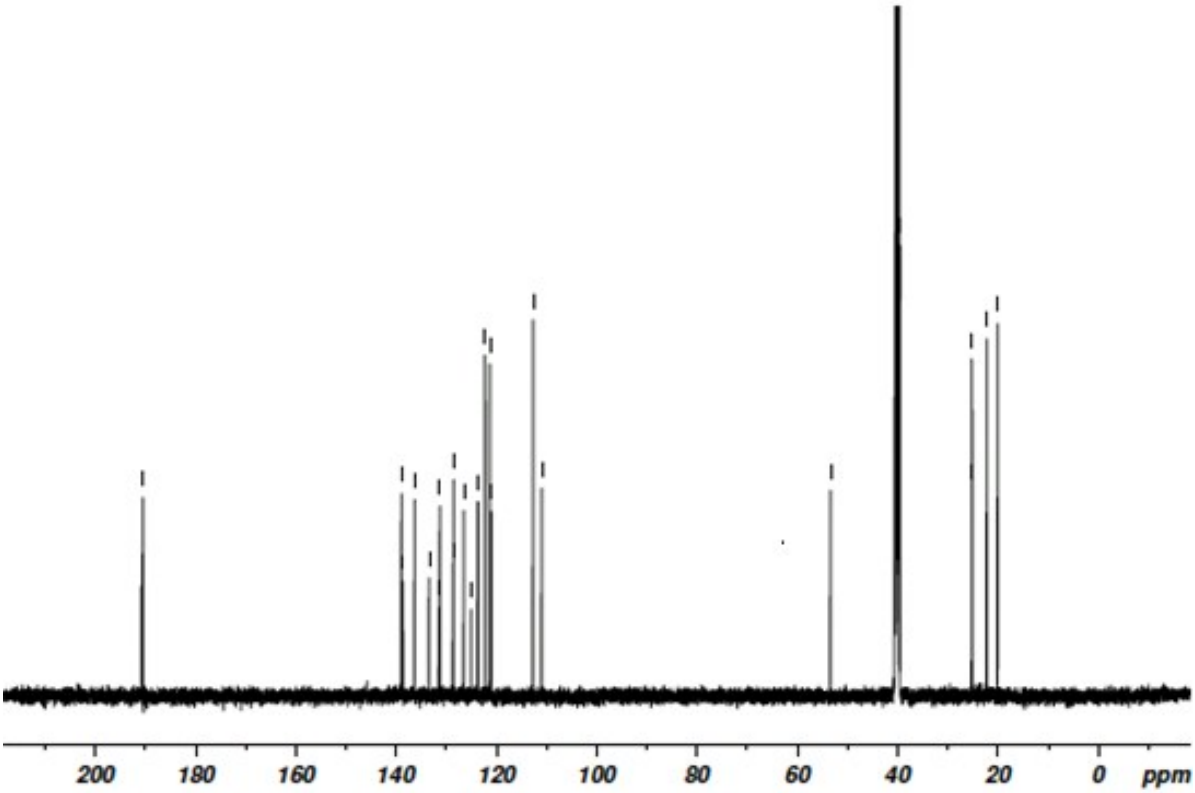

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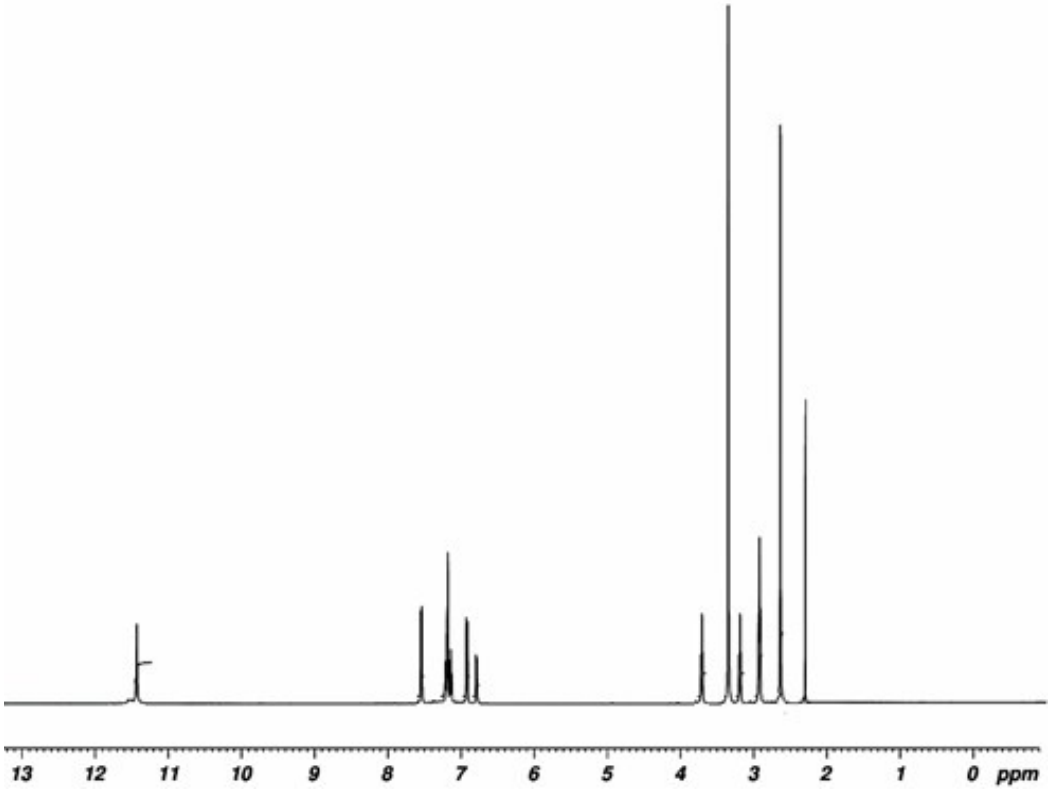

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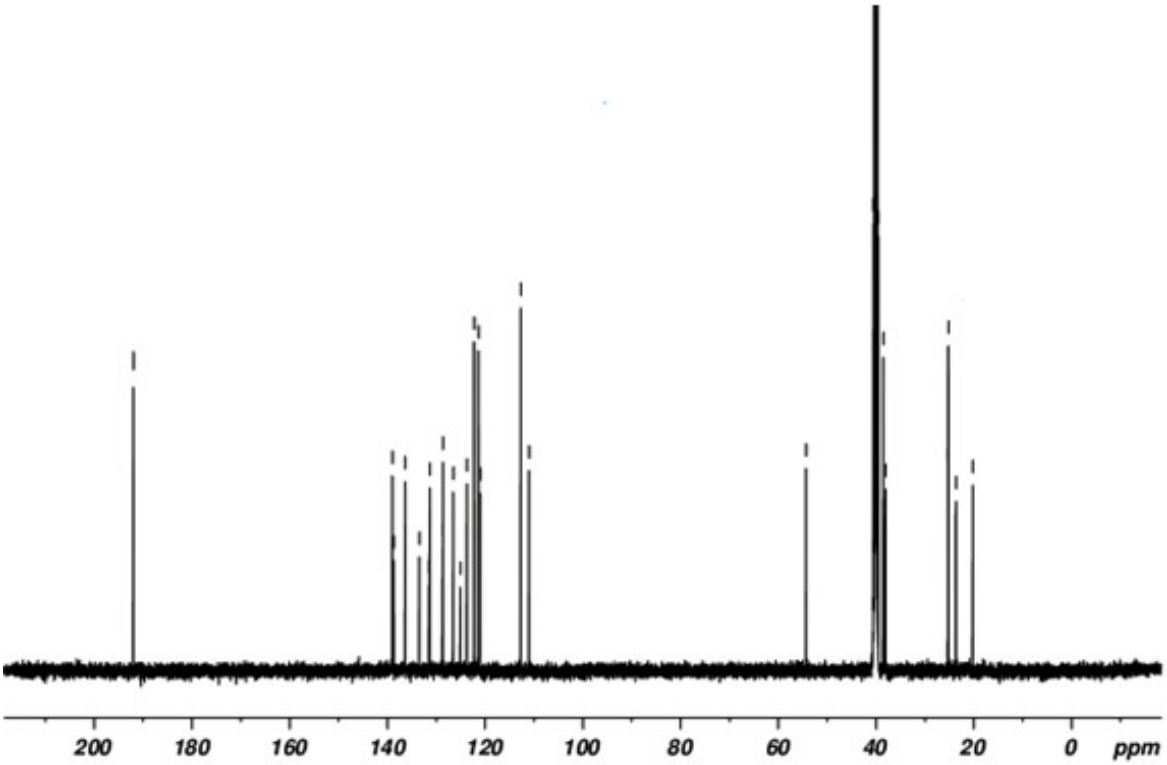
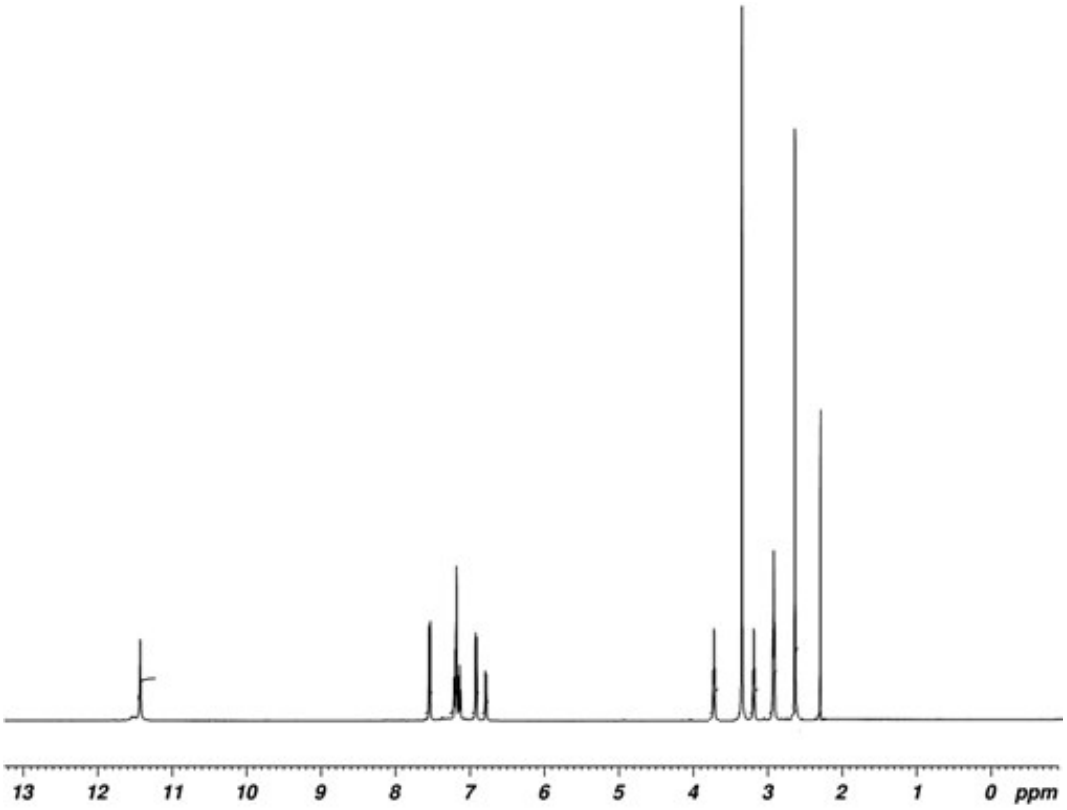
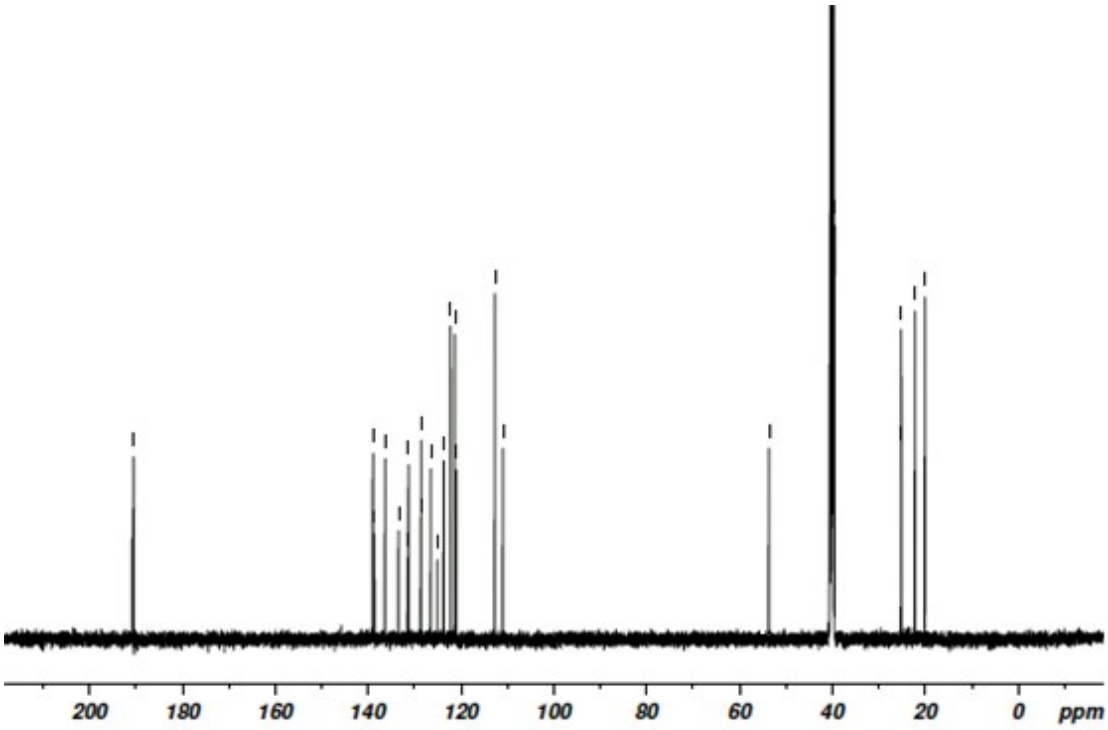


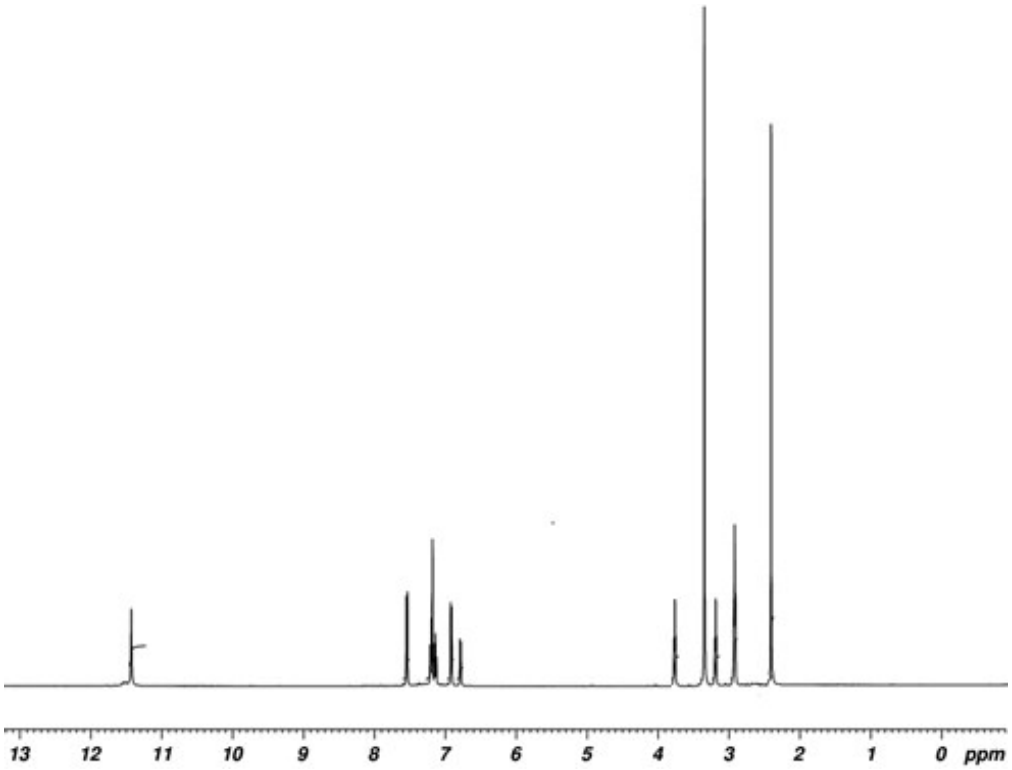
^{13}C NMR Spectrum of compound **3c**



^{1}H NMR Spectrum of compound **3d**



^{13}C NMR Spectrum of compound **3d**



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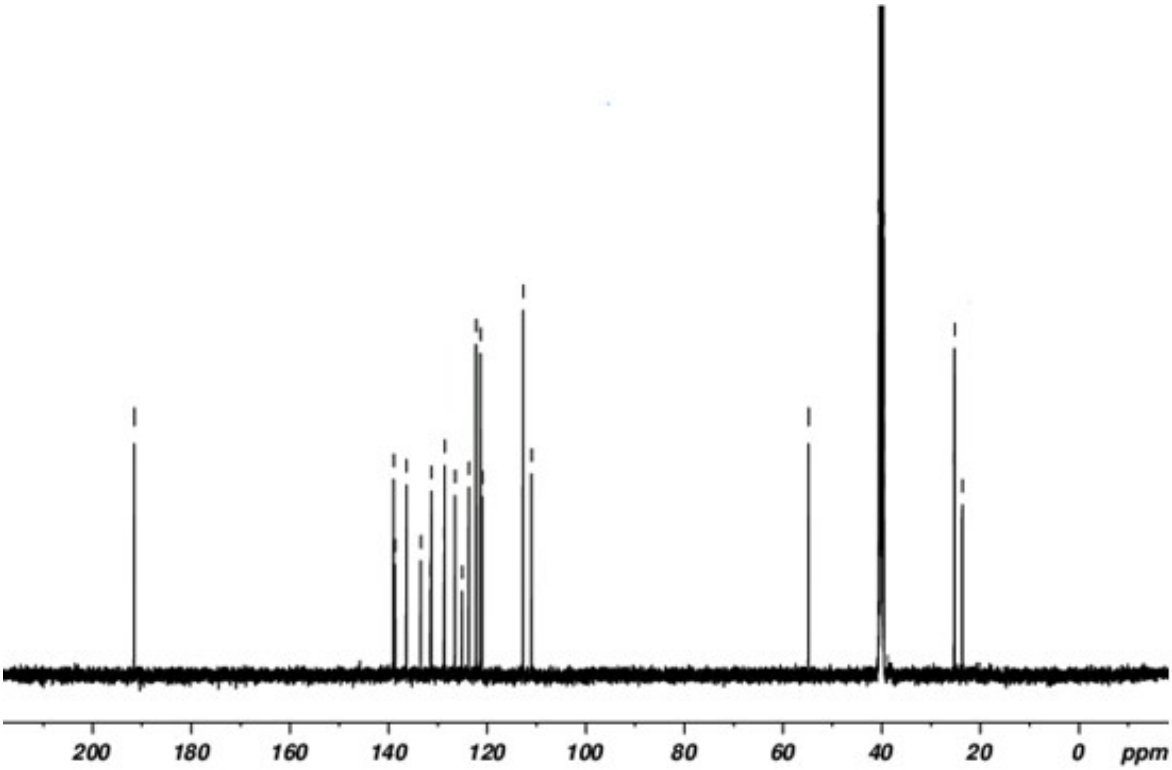
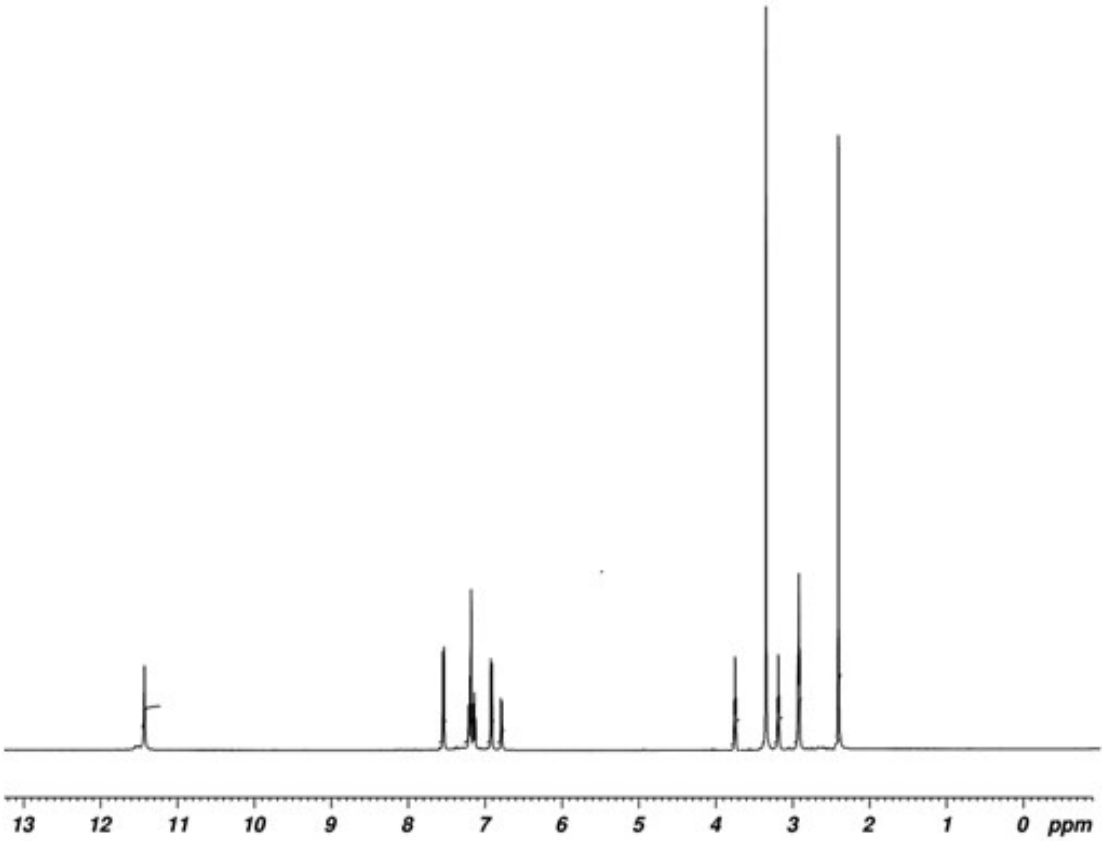
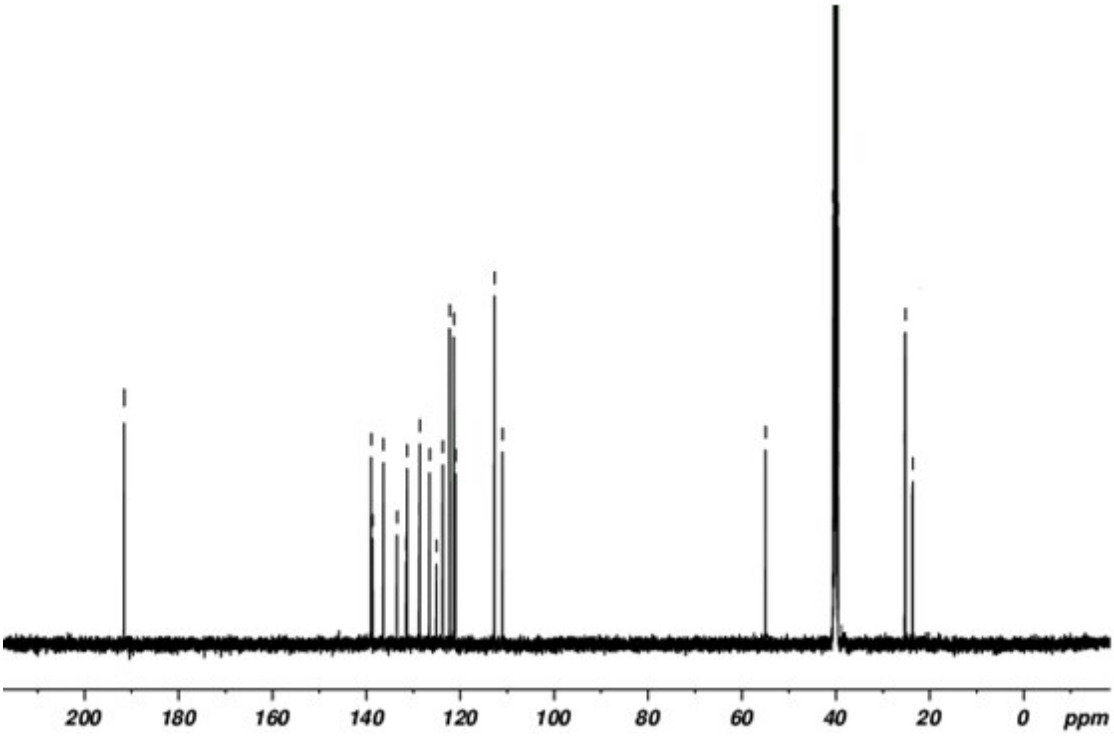


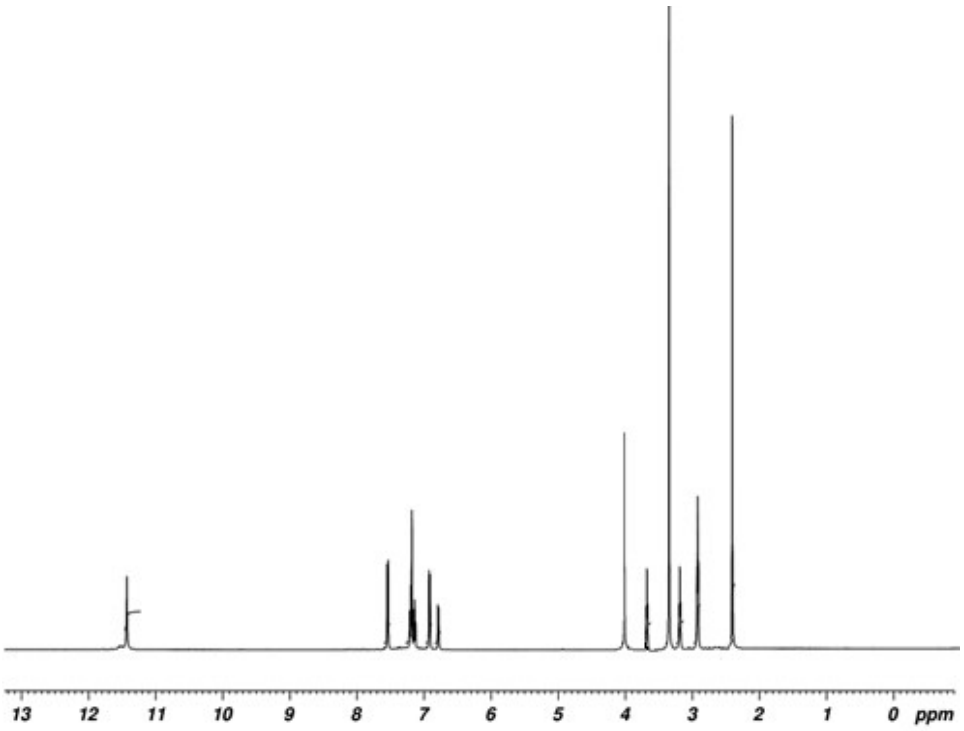
^{13}C NMR Spectrum of compound **3e**



^{1}H NMR Spectrum of compound **3f**



^{13}C NMR Spectrum of compound **3f**



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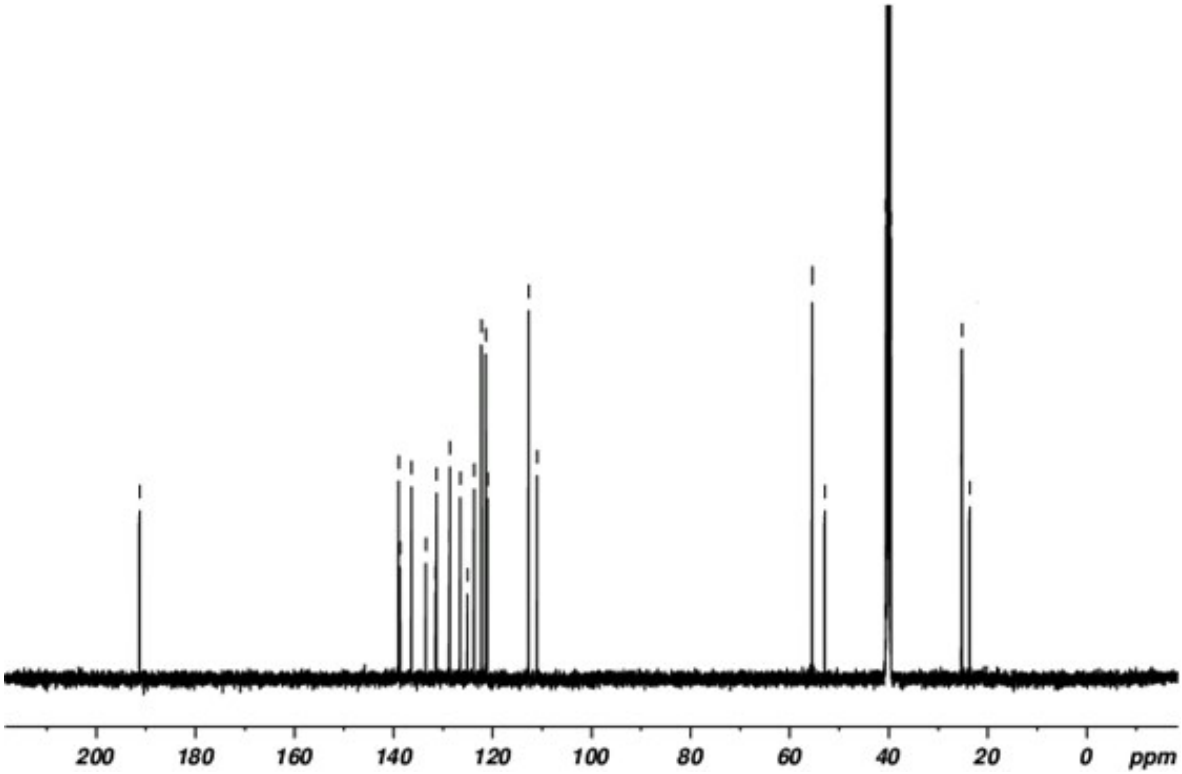

^{13}C NMR Spectrum of compound **3g**