Supporting Information

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Synthesis, characterization, and biological activity of novel ıonic liquids with bis-imidazole moieties: Antitumor, antimicrobial effects, and molecular docking studies

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S1 :Instrumentation

^{1}H and ^{13}C-NMR spectra of ionic compounds were recorded on a Bruker 400 MHz spectrometer. These measurements used Tetra Methyl Silane (TMS) as an internal standard and Dimethyl sulfoxide (DMSO-d_6) as solvent. The FTIR spectra of compounds 2, 3(a-g) were recorded on a Perkin- Elmer Spectrum one Fourier-transform FTIR spectrometer (resolution 4) in KBr pellets. Melting point was measured by an electrothermal apparatus. All the chemicals were obtained from Fluka Chemie AG (Buchs, Switzerland) and Sigma-Aldrich (St. Louis, MO, USA).

S.2 : Material and Methods

2.1 Cancer Cell Lines and Cell Culture

In this paper, A549 (ATCC, CCL-185), Calu1 (ATCC, HTB-54), and H1650 (ATCC, CRL-5883) lung cancer cell lines, SW1353 (ATCC, HTB-94), MG63 (ATCC, CRL-1427), and Saos2 (ATCC, HTB-85) bone cancer cell lines, Beas2B (RRID, CVCL-0168) normal lung cell line, and HC (Sigma Aldrich, 402-05A) normal chondrocytes cell line were used. All cell preparation procedures were carried out in a sterile environment in a laminar cabinet. The cell lines were used after they were confluent in 37ºC, 5% CO_2 conditions in the supplemented DMEM or RPMI 1640 (Capricorn) medium containing 10% FBS (Capricorn) and 2% PenStrep (Gibco) solution. Measuring plates were seeded with 10,000 cells per well. After approximately 16 hours of pre-incubation, test molecules were added and measurements were performed after 24 hours of incubation.

2.2 Cell Proliferation Measurement and Determination of NCI-60 Survival Parameters

MTT (TCI) test was used to measure the effects of the synthesized test compounds on cell proliferation and NCI-60 survival parameter values. [1] This test protocol was applied after incubation of test substances and cancer cell lines for 24 hours. The results were reported as % cell inhibition and the optical density of the solvent (DMSO, at a max %0.5 final volume) treated cells was assumed to be 100%. The MTT method was used on cells of increasing concentrations of each test substance (1.96, 3.91, 7.81, 15.63, 31.25, 62.5, and 125.0 µg/mL) over a specified range to determine the NCI-60 survival parameters of test substances. It was analyzed by using a logarithmic function on the logarithmic curve prepared from the absorbance values obtained after the following formulas were used for the measurement of NCI-60 survival parameters (GI50, TGI, and LC50); Cell proliferation: [(Ti-Tz)/(C-Tz)]x100 if Ti>/=Tz (cytocytic effect) or [(Ti-Tz)/Tz]x100 if Ti<Tz (cytocidal or cytotoxic effect) (Tz; zero point, C; control growth, Ti; inhibition by test substance). GI50: Concentration value that reduces growth by 50% ([(Ti-Tz)/(C-Tz)]x100=50), TGI: Concentration value that reduces growth by 100% (Ti=Tz), LC50: concentration value that by 50% kill cells in the medium ([(Ti-Tz)/Tz]x100=-50).[2]

2.3 Cytotoxicity Test

Whether the test substances to be tested are cell cytotoxic or cytostatic was determined by the LDH method.[3] An increase in the amount of cells that die during the incubation period, depending on the substance tested, will result in an increase in LDH in the culture supernatant. Lactate dehydrogenase (LDH) is a cytoplasmic enzyme that is found in most cells and is stable. For this purpose, the LDH cell cytotoxicity kit (Elabscience) was used according to the manufacturer's procedure. Briefly, 100 µl of supernatant was collected from each sample and transferred to a new plate.100 µl of the working reagent was then added to each of the assay wells. To form formazan, the assay plates were then incubated at room temperature in the dark for 15 minutes. The change in the amount of formazan formed as a result of LDH enzyme activity was measured and evaluated according to the formula below; % Cytotoxicity = [(Substance Absorbance - Low Control / High Control - Low Control) x 100].

2.4. DNA Fragmentation by Agarose Gel Electrophoresis

DNA laddering activity of the compounds was evaluated by using a DNA laddering assay in accordance with the standard method.[4] Briefly, 7.5 x 10^5 cells were placed into 25 cm^2 culture flasks and treated with TGI concentrations of the compounds at 37°C with 5% CO_2 overnight. First, DNA-containing precipitate was extracted from the digest with a 50 µL phosphate-citrate buffer and centrifuged at 1500 x g for 5 minutes, and then 40 µL of supernatant was transferred to a micro centrifuge tube. Tween20 (5 µL) (ThermoFisher) solution (0.25% in ddH_2O) and RNase A (5 µL) (ThermoFisher) solution was added to the supernatant and then incubated at 37°C for 30 min. Next, proteinase K (5 µL) (ThermoFisher) was added to each tube and re-incubated at 37°C for 5 min. Finally, DNA-containing precipitate of the micro centrifuge tube was added to 5 µL of loading buffer and loaded to 1.5 % agarose gel containing 0.5 µg/mL ethidium bromide and electrophoresed at 200 mA for 40 min. After electrophoresis, DNA fragmentation on gels was imaged using a UVP gel imaging system.

2.5 Migration Assay

A wound-healing assay was used to determine the effect of the compounds on the migration of cells. It is a method based on measuring the proliferation ability of cells in the medium containing the test substance. Cells are loaded at 70,000 cells / 200 µL in each well of the 24-well plate with coated poly-L-lysine and incubated for 24 hours. After incubation, the cell monolayer in a straight line scraped to create a scratch with a sterile pipet tip. The cell debris was removed and the edge of the scratch was smooth by washing the cells with medium for the in vitro scratch assay. Then, the old medium is discarded and approximately 1 mL of fresh medium is added and test molecules are placed at the GI50 dose. From this moment, the specimens begin to be photographed. Photographs were taken on scratch, in which the test substances were put every 24 hours, until the gap in the scratch, which was used as a control, was filled.

S3: Molecular Docking Study

Multiple molecular docking software programs are available, each characterized by distinct capabilities and limitations. The accuracy of their results hinges on the quality of the underlying protein and small molecule structures, alongside the specific parameters and algorithms employed in the docking calculations.[5] In this study, molecular docking investigations were conducted to assess ligand-protein interactions and elucidate anti-cancer and inhibition mechanisms. These investigations centered on compound 3a. The crystal structures of the Epidermal Growth Factor Receptor (EGFR) (PDB ID: 1M17, Resolution: 2.60 Å) and Vascular Endothelial Growth Factor Receptor 1 (VEGFR1) (PDB ID: 3HNG, Resolution: 2.70 Å) were downloaded from the Protein Data Bank (PDB). Initial processing involved the removal of water molecules, ions, and other ligands from the complex using BIOVIA Discovery Studio Visualizer 2021.[6] Additionally, polar hydrogen atoms and Kollman charges were introduced to the receptor via AutoDock MGL Tools. AutoDock 4.2 software [7] was employed for the determination of ligand-protein interactions. Compound 3a was created using ChemDraw software, and reference drugs were acquired from the PubChem database (pubmed.ncbi.nlm.nih.gov). These compounds were subsequently optimized using the UFF force field within the Avogadro software.[8] Following this, all ligands were transferred to MGL Tools and converted into pdbqt files. During the docking process, the receptor molecules remained rigid while the ligands retained their flexibility. To facilitate the docking process, a grid was established to encompass the active site of the receptor molecule (protein). The lowest energy conformation was determined by allowing the ligands to explore this grid. The grid had dimensions of 60x60x60 points in the x, y, and z directions, with a grid spacing of 0.375 Å. The Lamarckian genetic algorithm method, implemented in AutoDock 4.2, was employed to process the docking studies. These studies involved a population of 150 individuals, a maximum of 2,500,000 energy evaluations, and a maximum of 54,000 generations. Result files were generated using AutoDock 4.2 software. Subsequently, the binding poses and energy calculations of

the docked structures were thoroughly analyzed and visualized using BIOVIA Discovery Studio Visualizer 2018. [6] The best poses were determined based on the binding scores with respect to the target proteins.

S4 : Antimicrobial Activity

The antimicrobial efficacy of the newly synthesized compounds was evaluated against nine distinct pathogenic microorganisms associated with various diseases. This assortment included eight microorganisms, encompassing Gram-negative bacteria (Escherichia coli ATCC 25922, Yersinia pseudotuberculosis ATCC 911, Pseudomonas aeruginosa ATCC 17853, Klebsiella pneumoniae ATCC 700603) and Gram-positive bacteria (Staphylococcus aureus ATCC 25923, Bacillus subtilis ATCC 6633, Bacillus cereus ATCC 10876, Enterococcus faecalis ATCC 29212), along with yeast-like fungi (Candida albicans ATCC 10231). These microorganisms were sourced from the American Type Culture Collection.

The minimal inhibitory concentration (MIC) values, expressed in micrograms per milliliter (μg/mL), of the newly synthesized compounds were determined using the microtitre broth dilution method, accompanied by the rapid INT colorimetric assay following the guidelines established by the Clinical and Laboratory Standards Institute (CLSI) as outlined in.[9] For the preparation of stock solutions of the synthesized compounds, all compounds were dissolved in DMSO (% 10). These stock concentrations underwent a two-fold serial dilution in 96-well microplates using Mueller–Hinton broth (MHB) as the diluent. Bacterial suspensions were prepared at a concentration of approximately 5 X 10^{-5} colony-forming units (CFU) per milliliter and inoculated into each well. The microplates were incubated at 37°C for 24 hours. After the 37°C incubation, each well received an addition of 40 μL of a 0.2 mg/mL solution of p-iodonitrotetrazolium chloride (INT) as an indicator of microbial growth. The microplates were further incubated for 30 minutes at 37°C, during which the MIC values were visually determined. The MIC value was recognized as the lowest concentration at which the growth of the tested microorganism was entirely inhibited, corresponding to the appearance of the first clear well. MIC values were determined at least in triplicate, with the value being considered the final MIC when repeated at least two times. Ampicillin (10 mg/mL) and fluconazole (2 mg/mL) served as standard commercial drugs for bacterial and yeast testing, respectively. A negative control experiment was conducted using only DMSO to account for any inhibitory effects stemming from the solvent.

References

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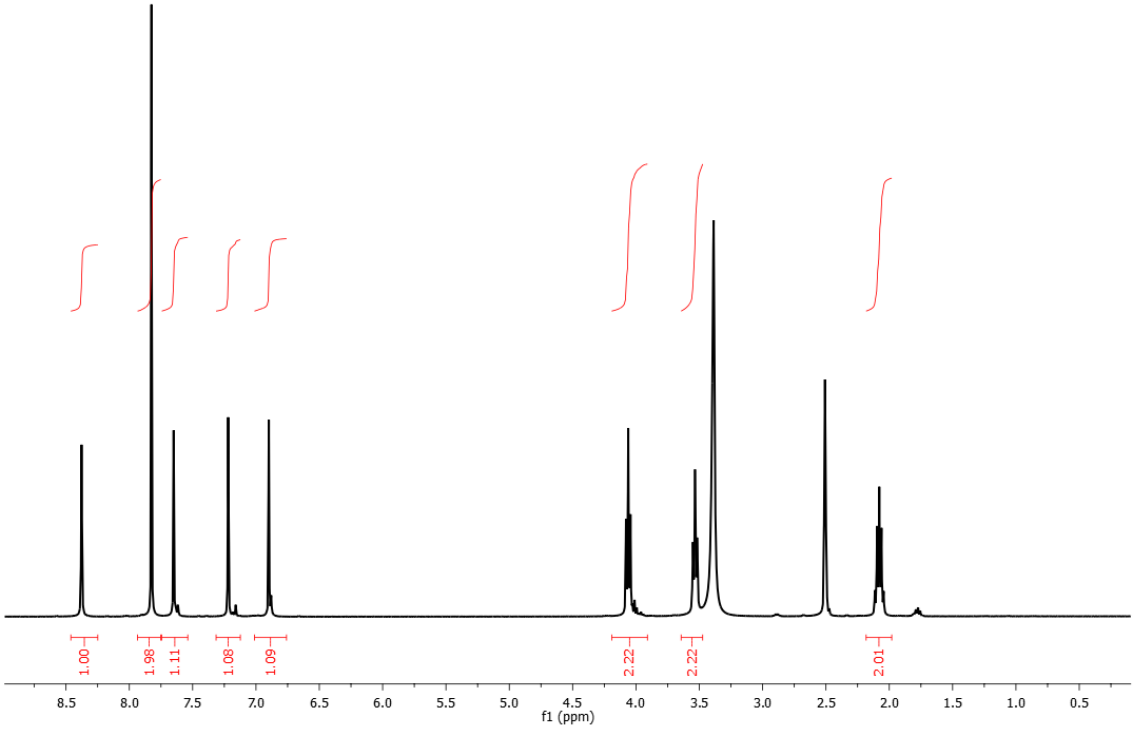
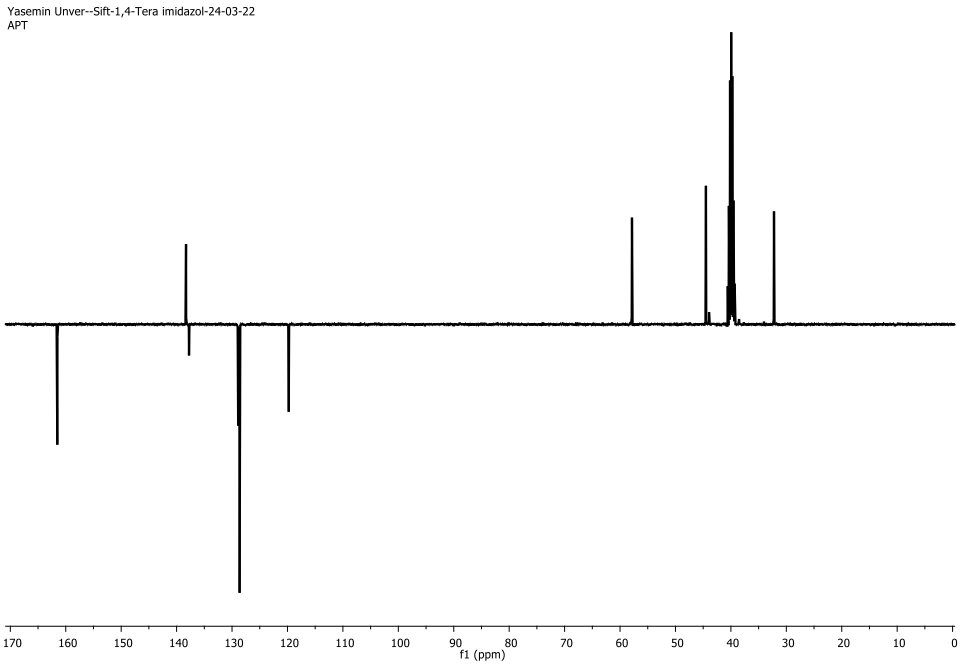


Figure S1: ^{1}H-NMR spectum of compound **2**



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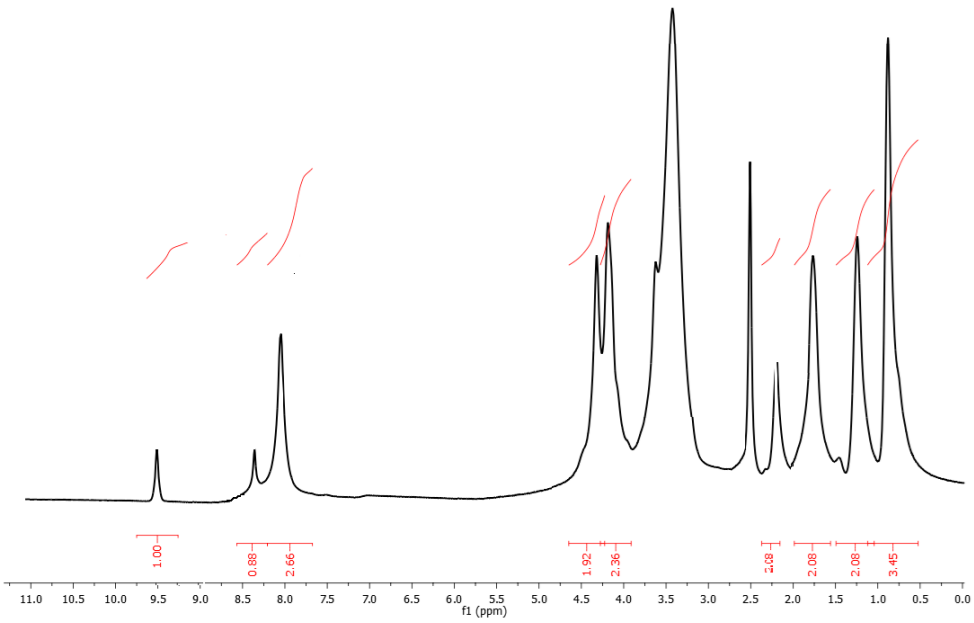
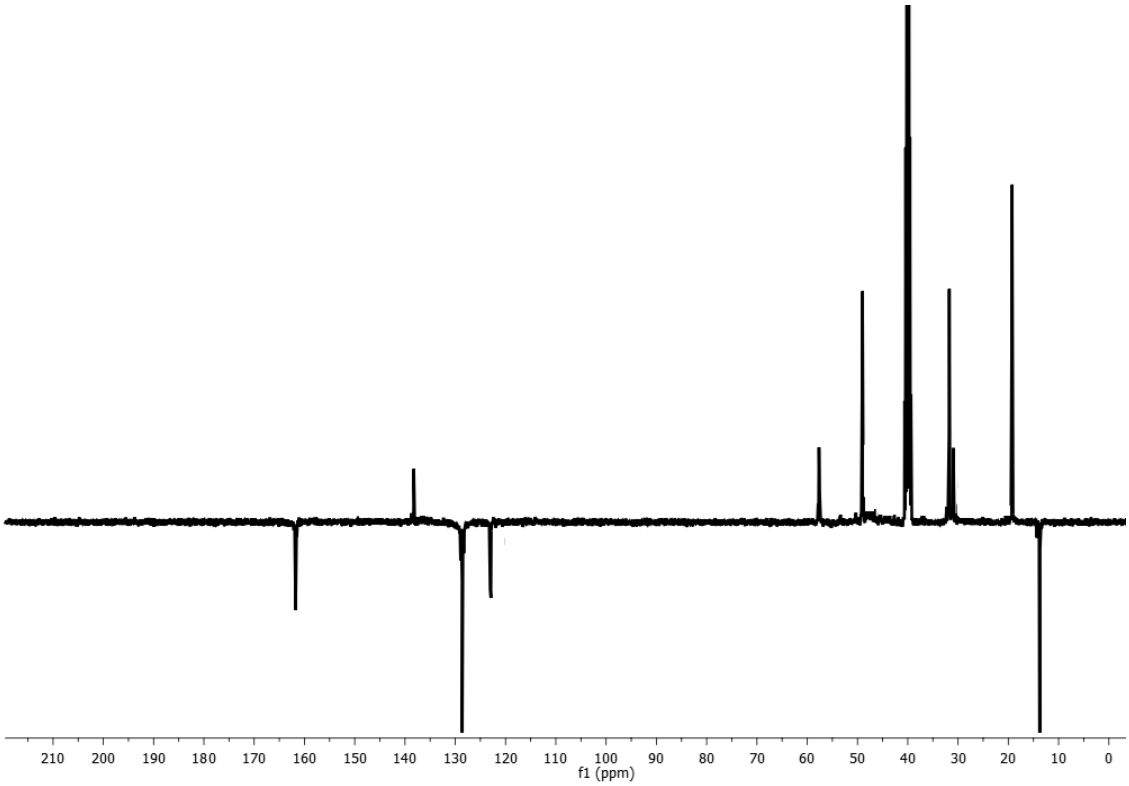


Figure S3: ^{1}H-NMR spectum of compound **3a**



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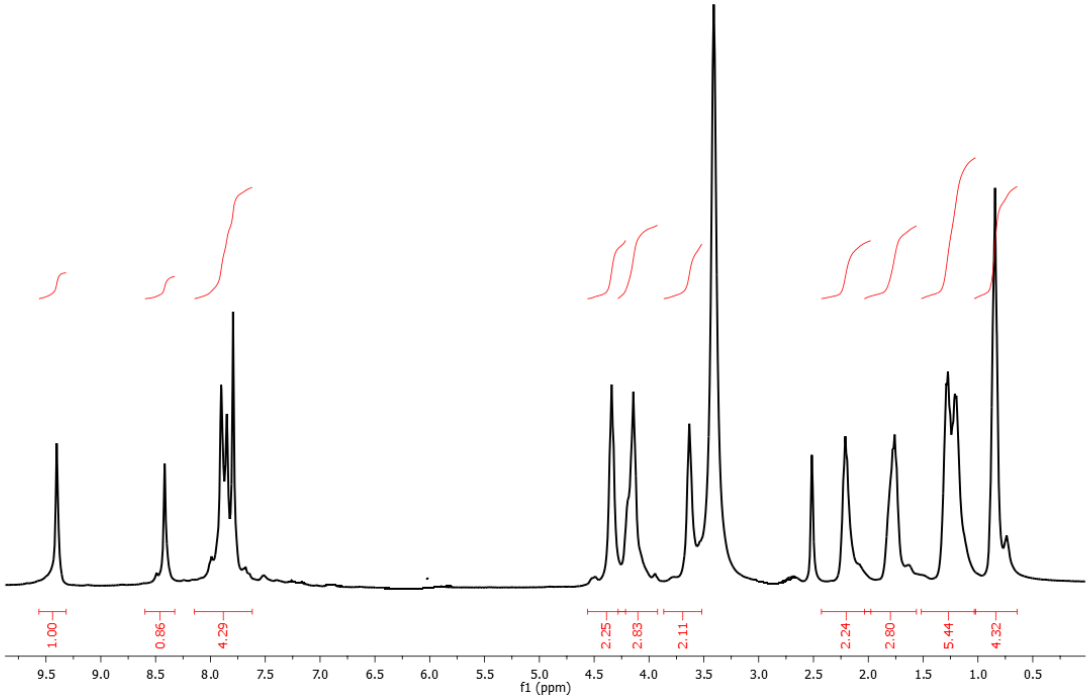

Figure S5: ^{1}H-NMR spectum of compound **3b**

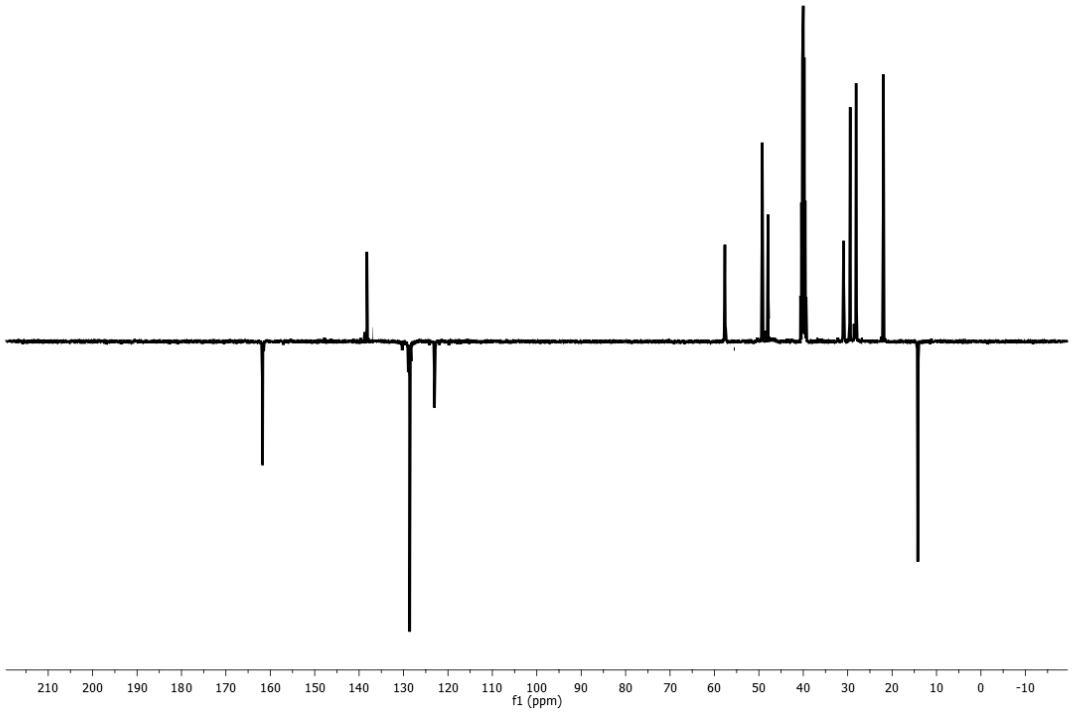

Figure S6: ^{13}C-NMR spectum of compound **3b**

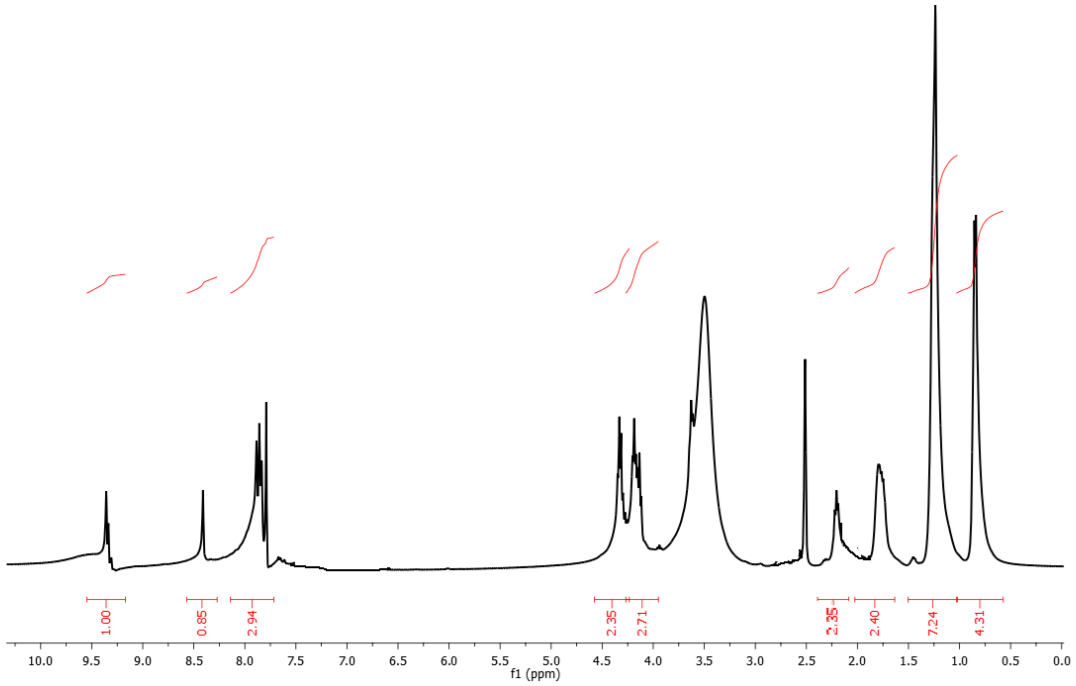

Figure S7: ^{1}H-NMR spectum of compound **3c**

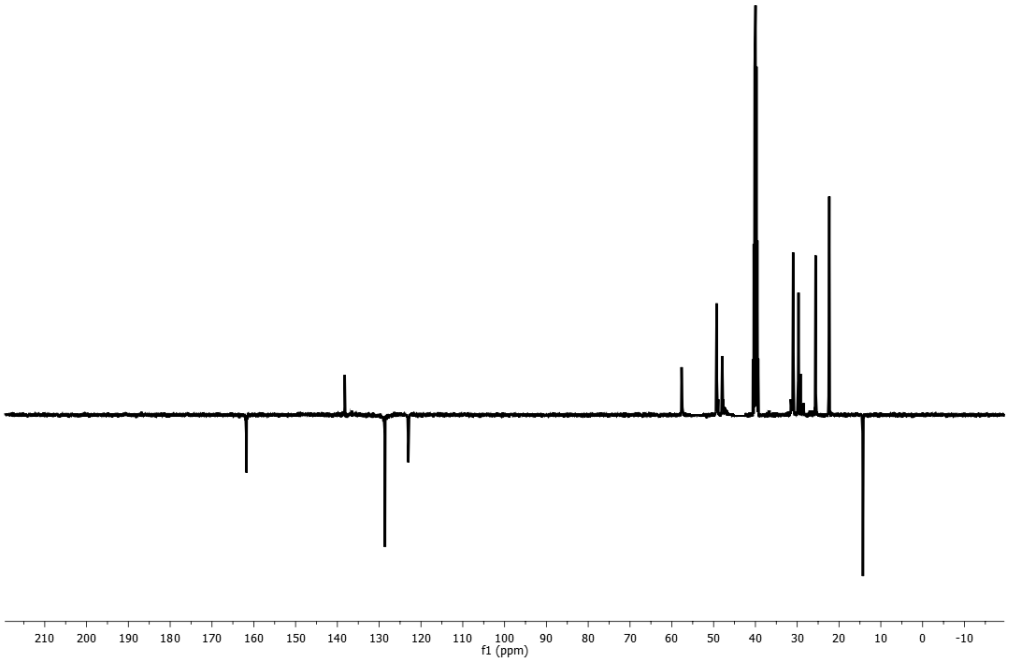

Figure S8: ^{13}C-NMR spectum of compound **3c**

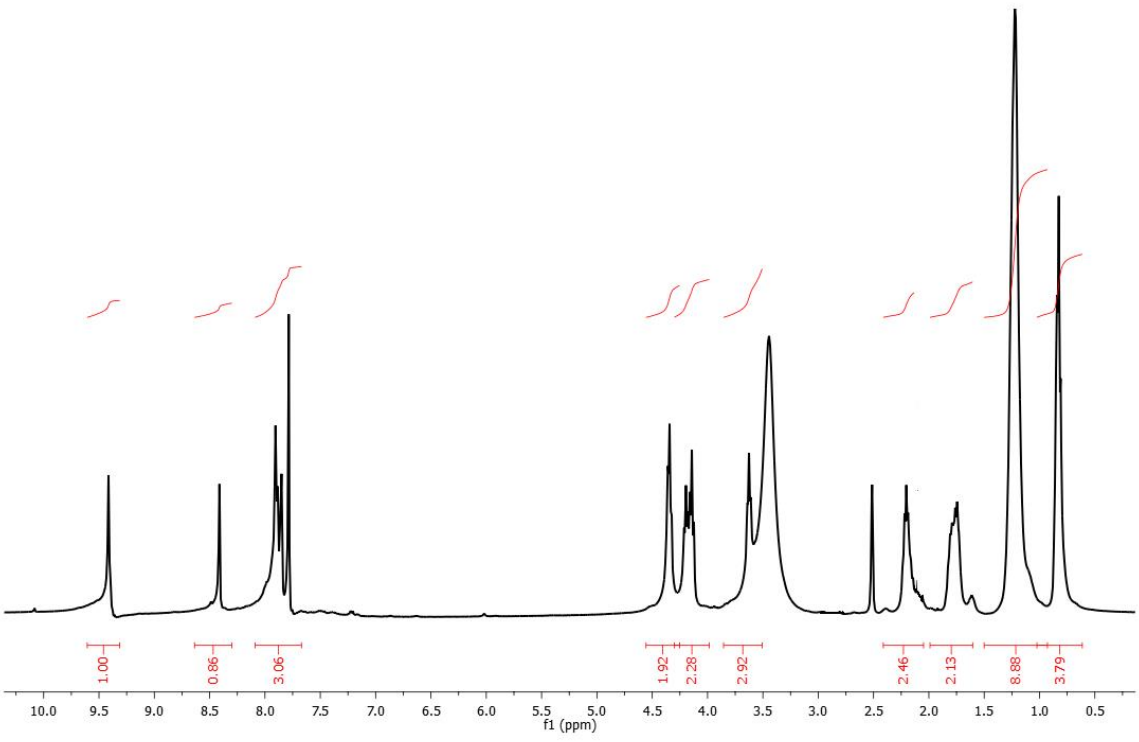

Figure S9: ^{1}H-NMR spectum of compound **3d**

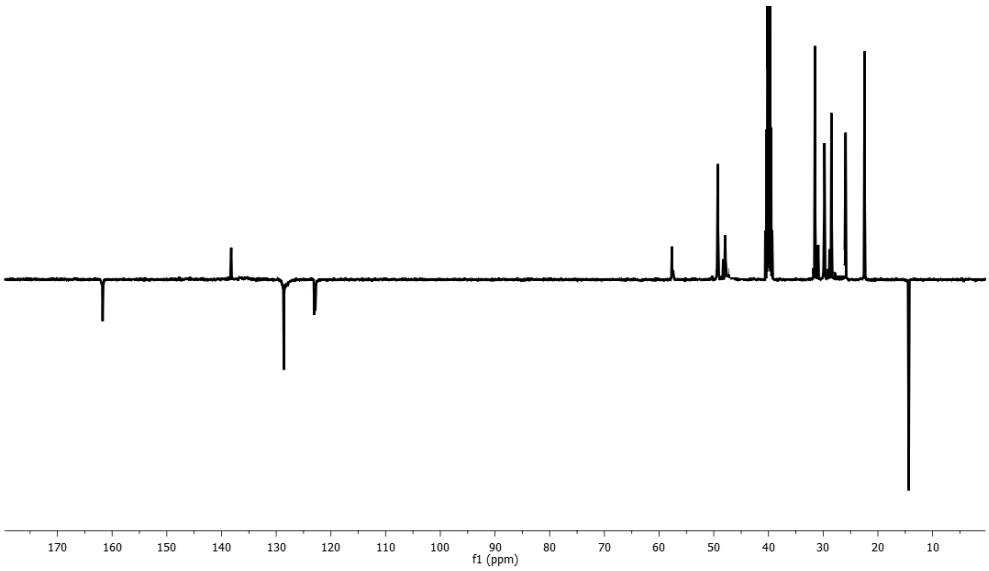

Figure S10: ^{13}C-NMR spectum of compound **3d**

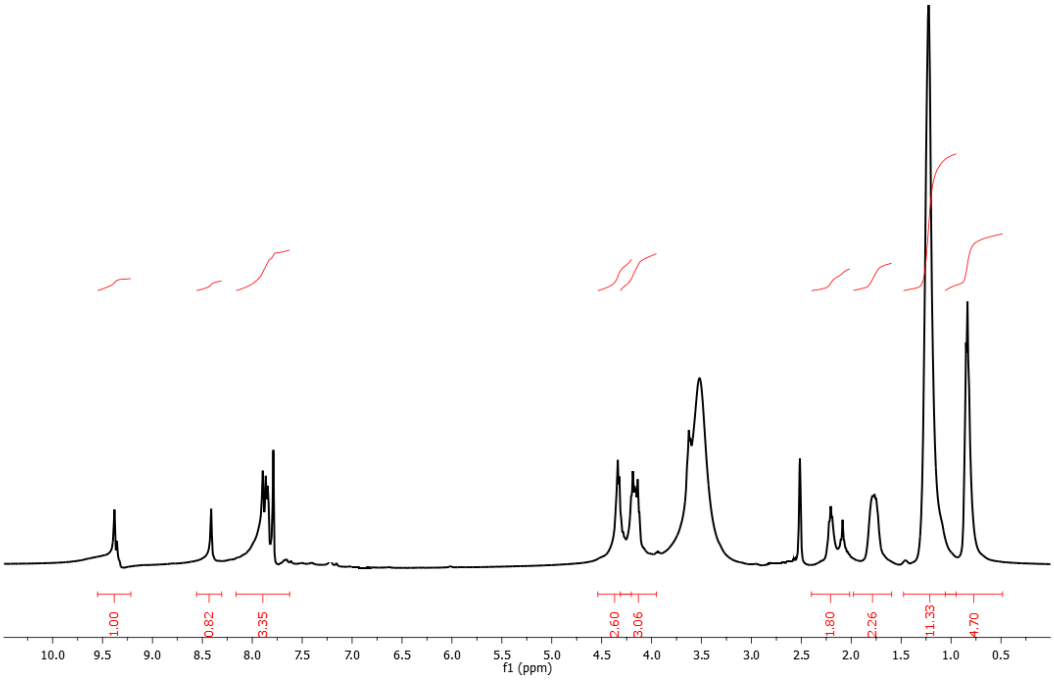

Figure S11: ^{1}H-NMR spectum of compound **3e**

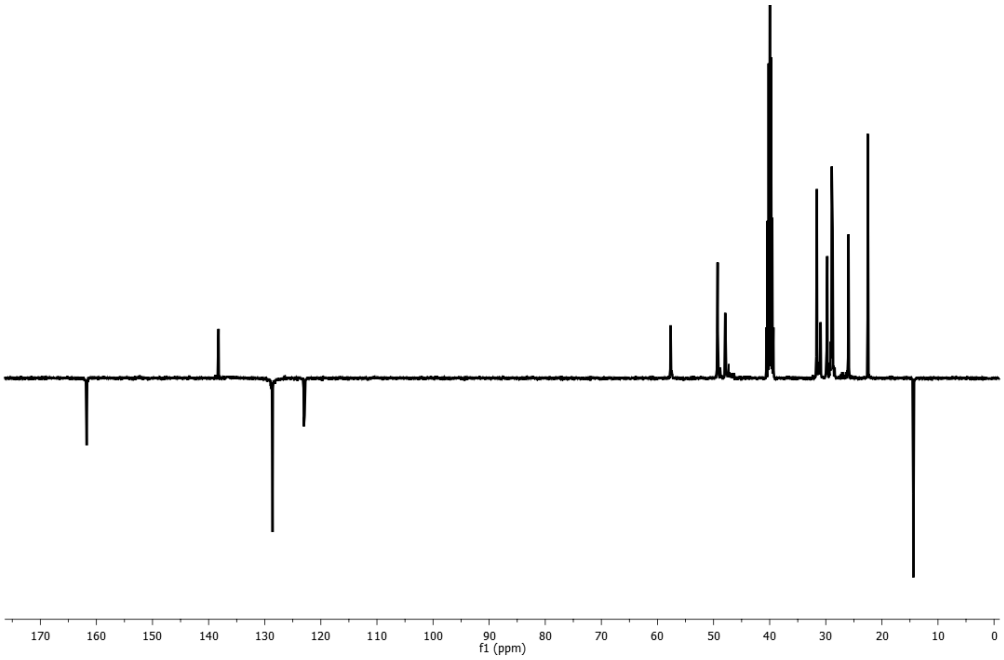

Figure S12: ^{13}C-NMR spectum of compound **3e**

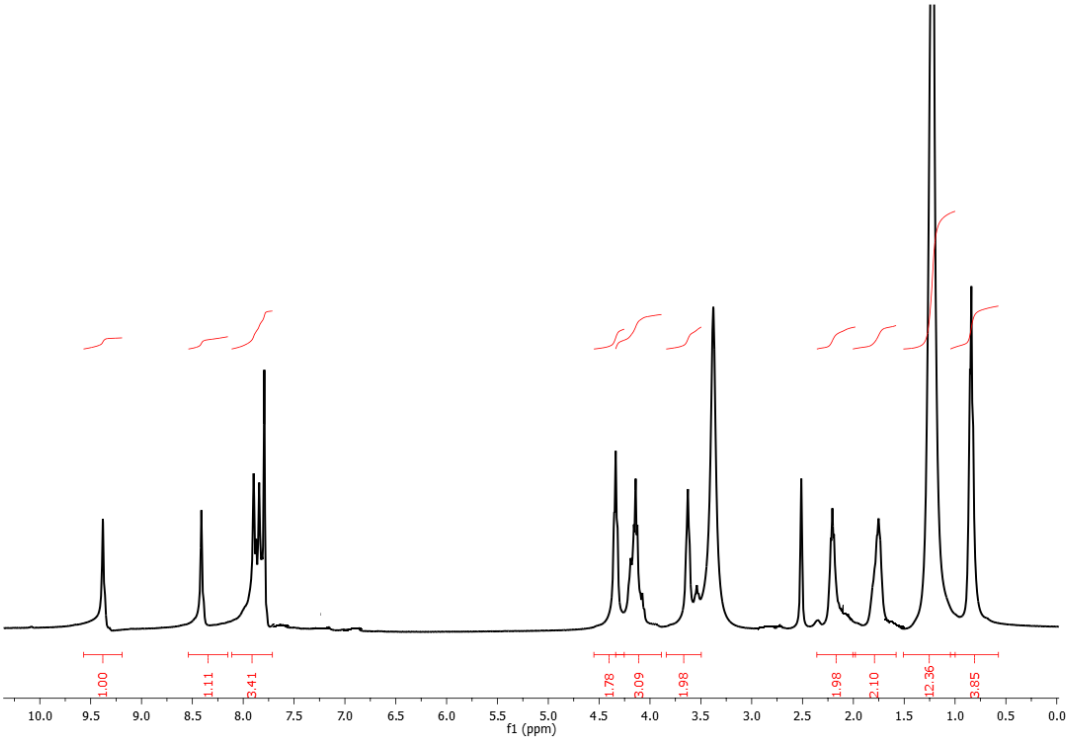

Figure S13: ^{1}H-NMR spectum of compound **3f**

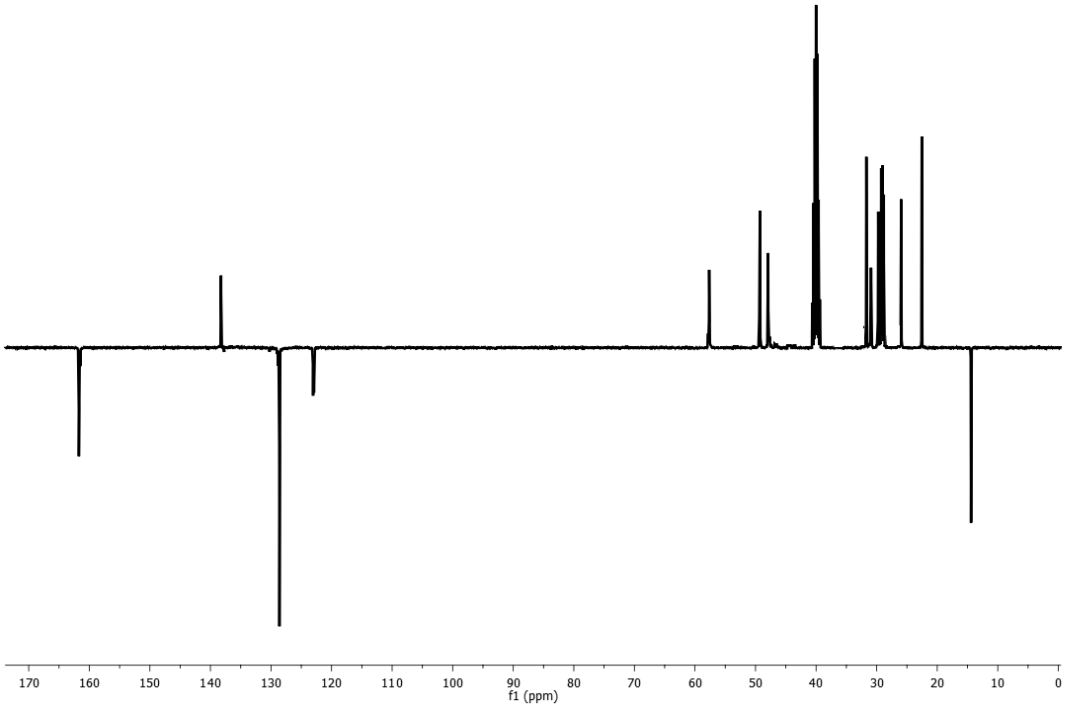

Figure S14: ^{13}C-NMR spectum of compound **3f**

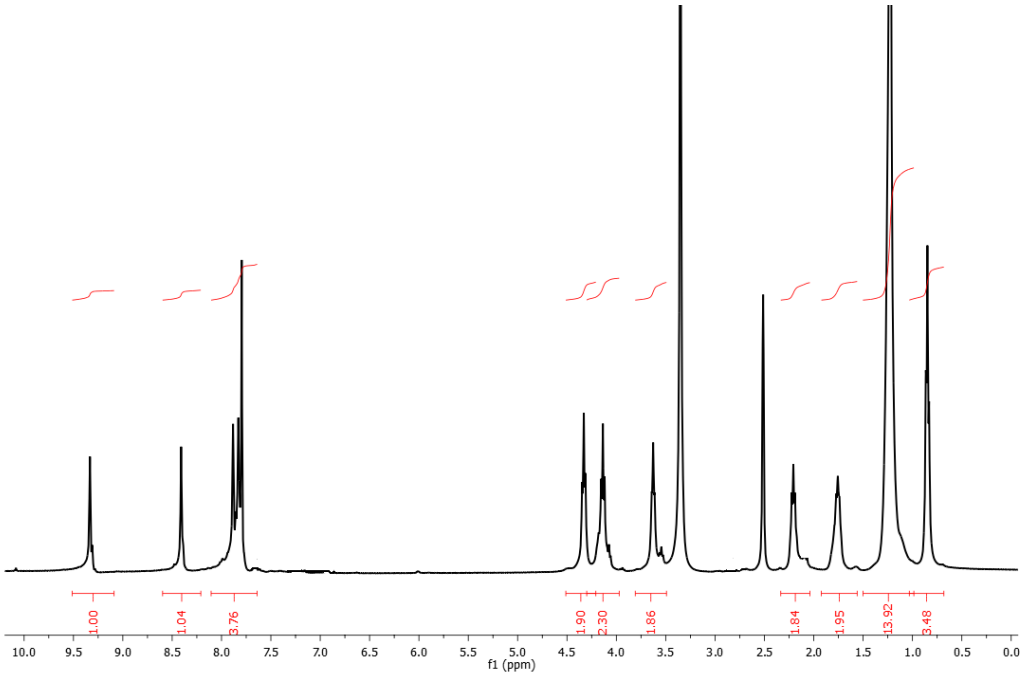

Figure S15: ^{1}H-NMR spectum of compound **3g**

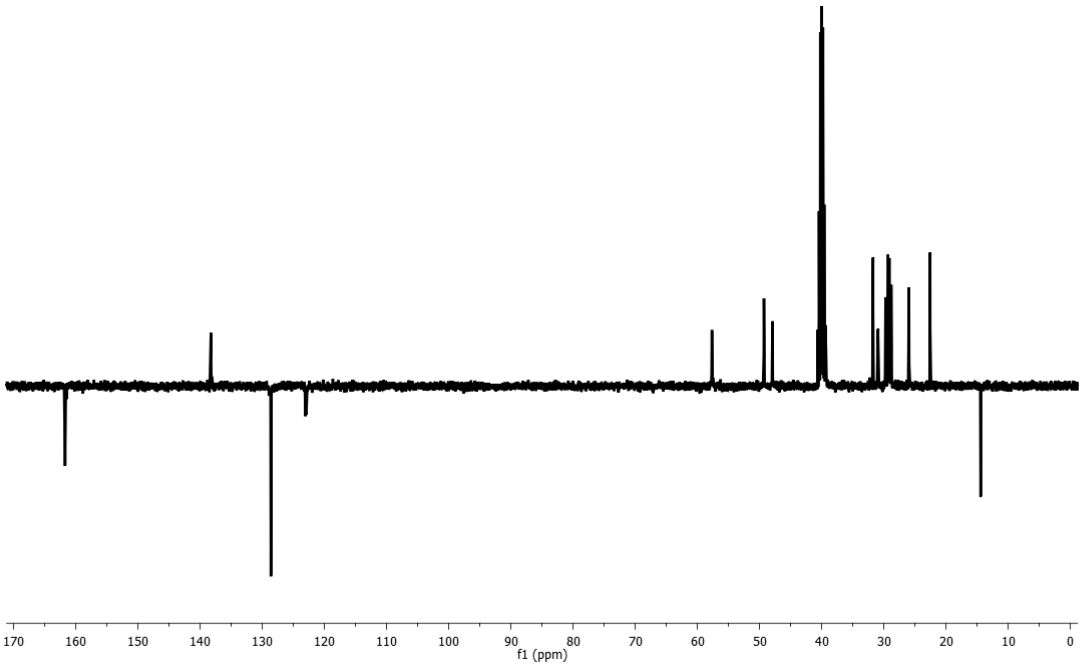

Figure S16: ^{13}C-NMR spectum of compound **3g**