Supplementary material

2. Experimental

2.1. Chemistry

Melting points are uncorrected and were determined in open capillary tubes using electric melting point apparatus (G-K). Infrared spectra (KBr discs) were measured on a Shimadzu FTIR, 8300 PC IR spectrophotometer. ^{1}H NMR (300 MHz) and ^{13}CNMR (75 MHz) was recorded with a Bruker model Ultra Shield NMR spectrometer with TMS as the internal standard and chemical shifts were reported on a δ scale (ppm) using DMSO-d_6 as a solvent, while the coupling constants (J values) are given in Hz. Elemental analyses were determined on a PerkinElmer 240, and the values found were within ±0.4% of the theoretical. All reactions were monitored by TLC on Merck Silica Gel 60F254 and spots were detected using a UV lamp (254 nm). The biological activities were carried out in the Medical Mycology Laboratory of the Regional Center for Mycology and Biotechnology of Al-Azhar University, Cairo, Egypt.

2.2.*In vitro* anticancer screening

The cell lines were purchased from the American Type Culture collection as follows: liver carcinoma cell line (HepG2), breast carcinoma cell line (MCF-7), and colorectal cancer cell line (HCT). Cytotoxic activity screening was performed using MTT assay at Regional Center for Mycology and Biotechnology, Al- Azhar University. Exponentially, cells were placed in 10^4 cells/ well for 24 h, and then add fresh medium which containing different concentration of the tested sample. Serial two-fold dilution of the tested sample were added using a multichannel pipette. Moreover, all cells were cultivated at 37 °C, 5% CO_2 and 95% humidity. Also, incubation of control cells occurred at 37 °C. However, after incubation for 24 h different concentrations of sample (50, 25, 12.5, 6.25, 3.125, 1.56 and 0 μg L^{-1}) were added and continued the incubation for 48 h, then, add the crystal violet solution 1% to each well for 0.5 h to examine viable cells. Rinse the wells using water until no stain. After that, add 30% glacial acetic acid to all wells with shaking plates on Microplate reader (TECAN, Inc.) to measure the absorbance, using a test wavelength of 490 nm. Besides, compare the treated samples with the control cell. The cytotoxicity was estimated by IC_{50} in (μg /mL), the concentration that inhibits 50% of growth of cancer cell.

2.3. c-Met kinase assay

The c-Met kinase activity was determined in 384-well plates using homogenous timeresolved fluorescence (HTRF) assays following the manufacture's instruction. The compounds **5a** and **5b**

were dissolved in DMSO and diluted to different concentrations with kinase buffer. First, 4 µl of each compound solution, 2 µl of TK substrate solution (5 µM), 2 µl of c-met solution (0.3075 µg/ml), 2 µl of ATP solution (15 µM) were successively added to each well. Reactions were incubated for 40 min at 37 °C and stopped by the addition of 10 µl mixed solution containing 5 µl SA-XL665 (0.5 µM) and 5 µl TK Antibody, and Sealing plate incubation for 1 h at 37 °C. The fluorescence at 620 nm and 665 nm was measured with Mithras LB943 (Bethold, Germany) using the excitation light at 320 nm. The inhibition rate (%) was calculated using the following equation: % inhibition = 100-[(activity of enzyme with tested compounds-min)/(max–min)] × 100 (max: the observed enzyme activity measured in the presence of enzyme, substrates, and cofactors; min: the observed enzyme activity in the presence of substrates, cofactors and in the absence of enzyme). IC_{50} values were processed by SPSS 19.0 statistical software from the inhibition curves. The experimental results were expressed by mean ± SD of three independent experiments.

2.4. Cell cycle analysis

Further exploration of the cytotoxic activity of compound **5a** was performed using propidium iodide (PI) flow cytomertric analysis to measure the extent of PI that binds to DNA of dead cells with permeable plasma membranes to determine cell cycle status in tissue culture quantitate cell death at all cell phases. After incubation of HepG-2 cells with compound **5a**, cells were fixed with ethanol then dehydrated before staining with PI according to the reported methodology.

2.5. Annexin-V FTIC apoptotic study

Estimation of fractional DNA content (aka sub-G1 assay) is a widely used assay to determine apoptosis. Cleavage of genomic DNA into smaller fragments (180–200 bp lengths) is a hallmark for apoptosis in numerous cells. PI stained cells will stain less intensely and show a peak below the G1 peak (Sub-G1). In this work, flow cytometer with Annexin V-fluorescein isothiocyanate/propidium iodide FITC/PI double staining apoptosis detection kit (K101, Biovision) was used to study apoptosis of HepG-2 cells treated with compound 5a. Cells were incubated, collected by centrifugation, re-suspended in 500 µL of 1X binding buffer. Annexin V-FITC (5 µL) and PI (5 µL) was added and incubation was continued for additional 5 min in the dark at room temperature. Annexin V-FITC binding was analyzed by flow cytometry using FITC signal detector (FL1) and PI staining by FL2 phycoerythrin emission signal detector [75].

2.6. Estimation of Bax and Bcl-2 Levels

Quantitative determination of pro-apoptotic BAX and anti-apoptotic Bcl-2 proteins in human cell lysates was performed using DRG® Human Bax ELISA (EIA-4487) and Zymed® Bcl-2 ELISA Kit (99–0042). Procedures of the colorimetric kits were performed according to the manufacturer's instructions [76,77]. Protein of interest in the samples and standards binds to the antibody coated on the plate. A biotin-conjugated antibody is added and binds to protein captured by the first antibody. Streptavidin-HRP is added and binds to the biotin-conjugated antibody. The substrate solution is added to the wells to form the colored products. The reaction is then terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared to determine the protein concentration.

2.7. Estimation of Human p53 Level

Human p53 present in HepG-2 cells was determined; using Human p53 ELISA-Kit (CS0070 Sigma) read using spectrophotometer at 450 nm against untreated control cells (negative control) and Erlotinib (positive control) applying the standard protocols of the manufacturers [78]. The samples or standard having human p53 bind to antibodies adsorbed to the microwells. Addition of biotin-conjugated was followed by incubation and addition of dispense of unbound biotin-conjugated streptavidin HRP. Then, the reaction was terminated by adding acid, and the absorbance was measured at 450 nm.

2.8. Human CASP-3 (Caspase-3) Estimation

Sandwich enzyme linked immuno-sorbent ELISA assay kits rely upon containment of a certain protein in a sandwich of distinct antibodies conjugated to a colorimetric 3,3′ ,5,5′ -Tetramethylbenzidine (TMB) substrate. Antibody-substrate intensity is measured spectrophotometrically to weight up the caspase protein quantity. KHO1091invitrogen caspase-3 was used in this study to estimate caspase-3 activity. After dilution of cell lysates and detection of antibody protein linked to anti-rabbit-IgG-HRP, microwells were then incubated. A colored product was then produced upon addition of TMB Substrate Solution. A stop solution was added in the last step before measuring color intensity at 450 nm. [79].

2.9.Molecular docking study

The molecular docking simulation of the promising *in vitro* screened derivatives **5a** and **5b** against c-Met was done using the Molecular Operating Environment software (MOE-Dock) version 2014.0901 [70,71]. The co-crystallized structure of c-Met kinase complexed with its native ligand, *N*-{3-fluoro-4-[(7-methoxyquinolin-4-yl)oxy]phenyl}-1-[(2R)-2-hydroxypropyl]-5-

methyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazole-4-carboxamide, was downloaded from the protein data bank (PDB code: 3U6I) [72]. Initially, the original ligand was re-docked into the active binding site of c-Met kinase to assess the root-mean-square deviation value. Then, the docking studies of the newly targeted compounds were estimated following to the previously reported procedure [80,81].

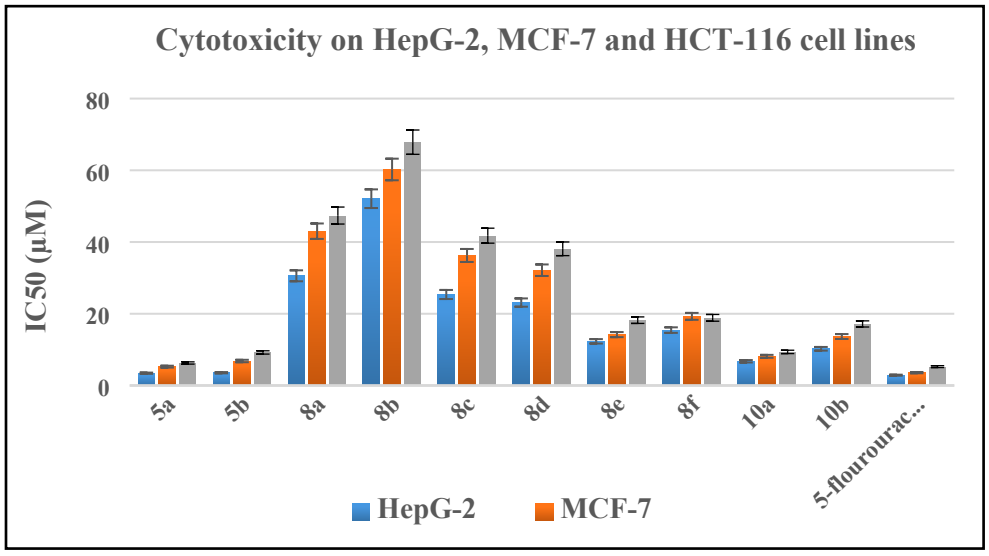
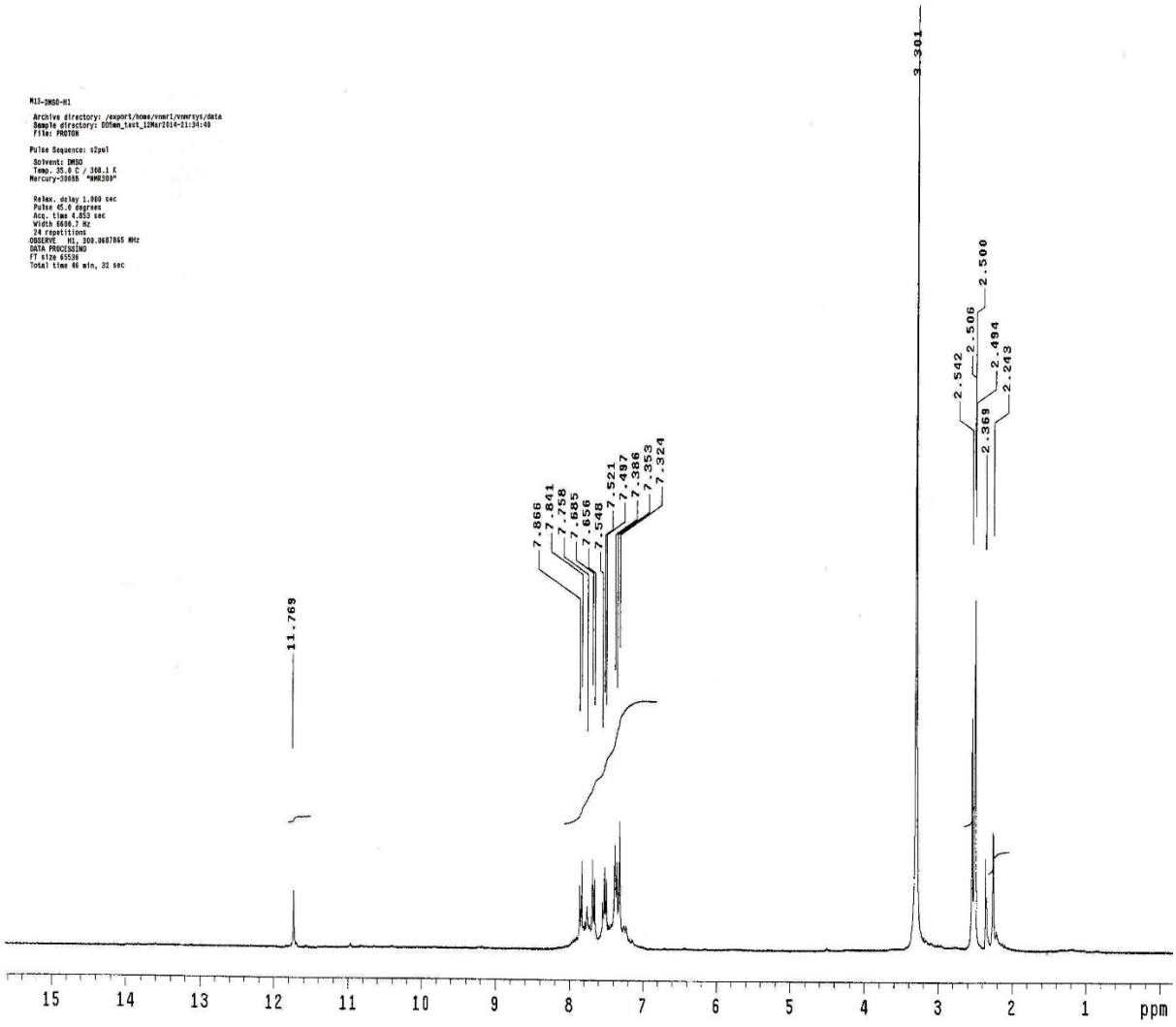


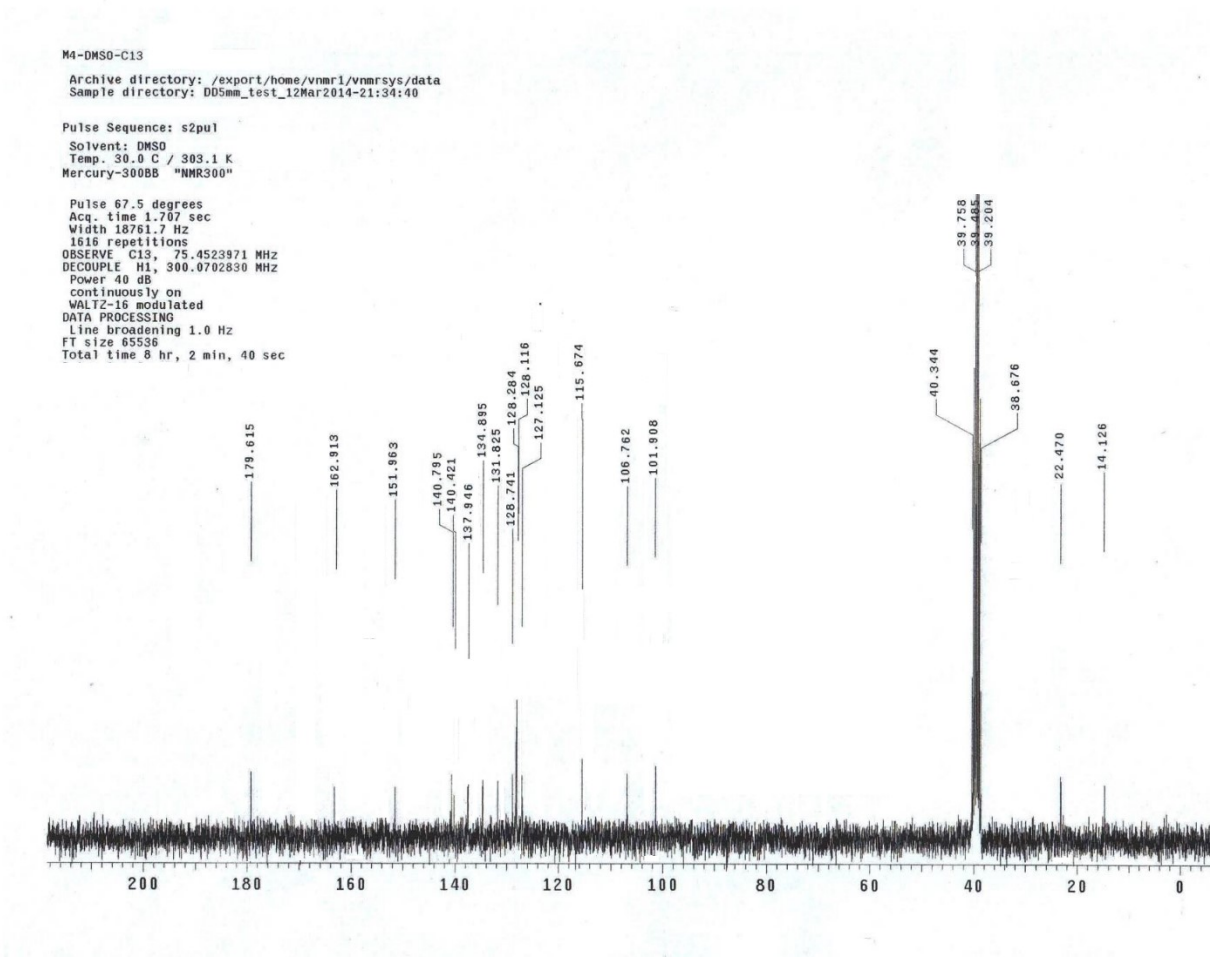
Fig. 1. Cytotoxic activity of the new target compounds against HepG-2, MCF-7, HCT-116 cancer cell lines

Table 1: Predicted Pharmacokinetic Properties of pyrazolo[3,4-*b*]pyridine-5-carbonitrile derivatives **5a** and **5b**.

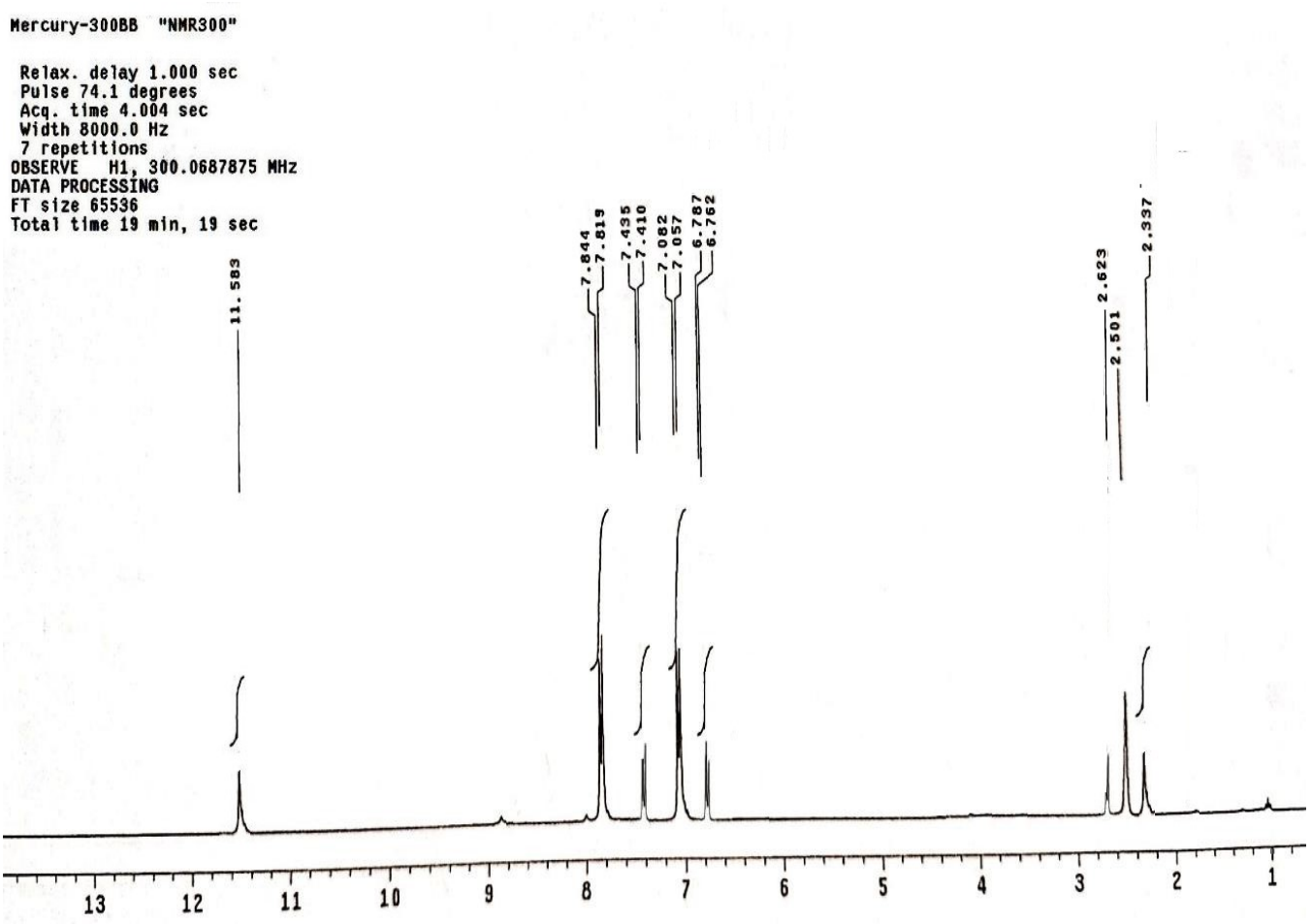
Comp. No.	GIT absorption	BBB permeability	P-gp substrate	Bioavailability score	PAINS alert
5a	low	NO	NO	0.55	0
5b	low	NO	NO	0.55	0



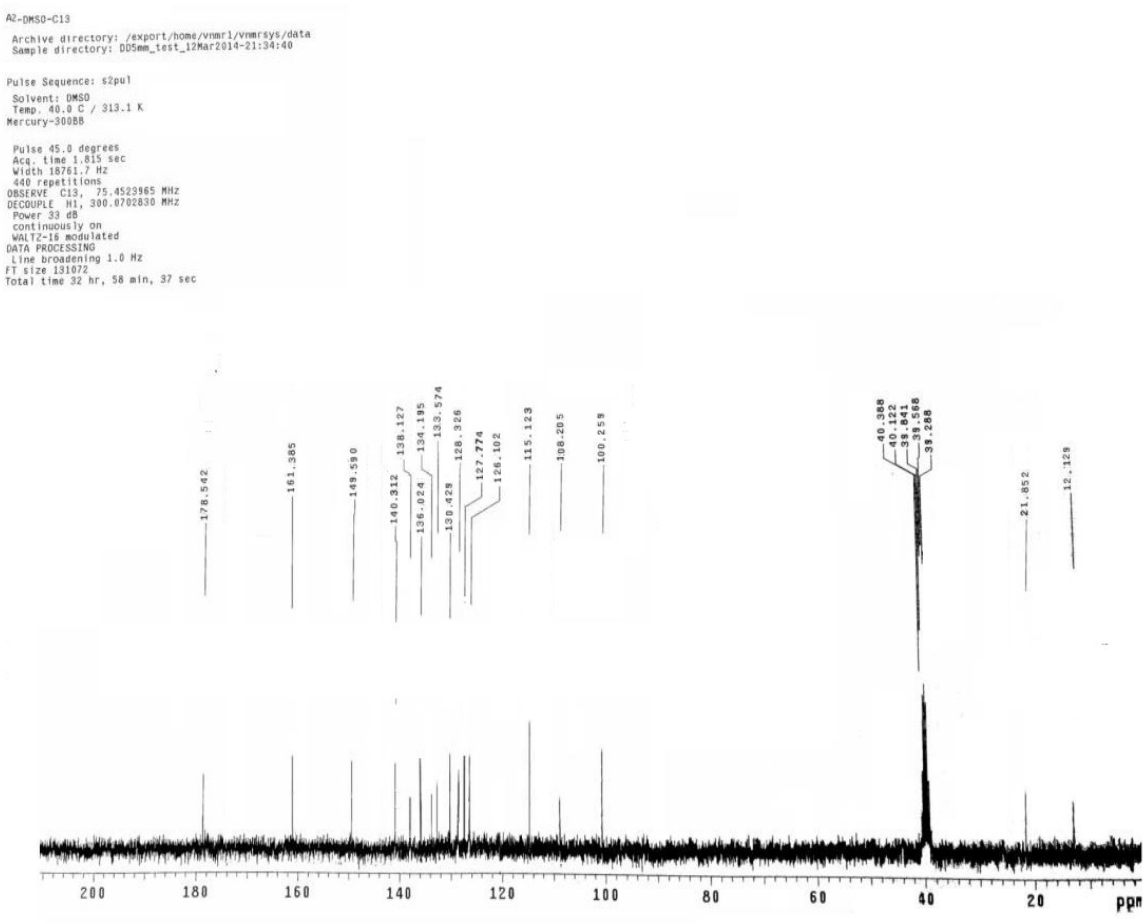
S1. ^{1}H NMR of compound **5a**



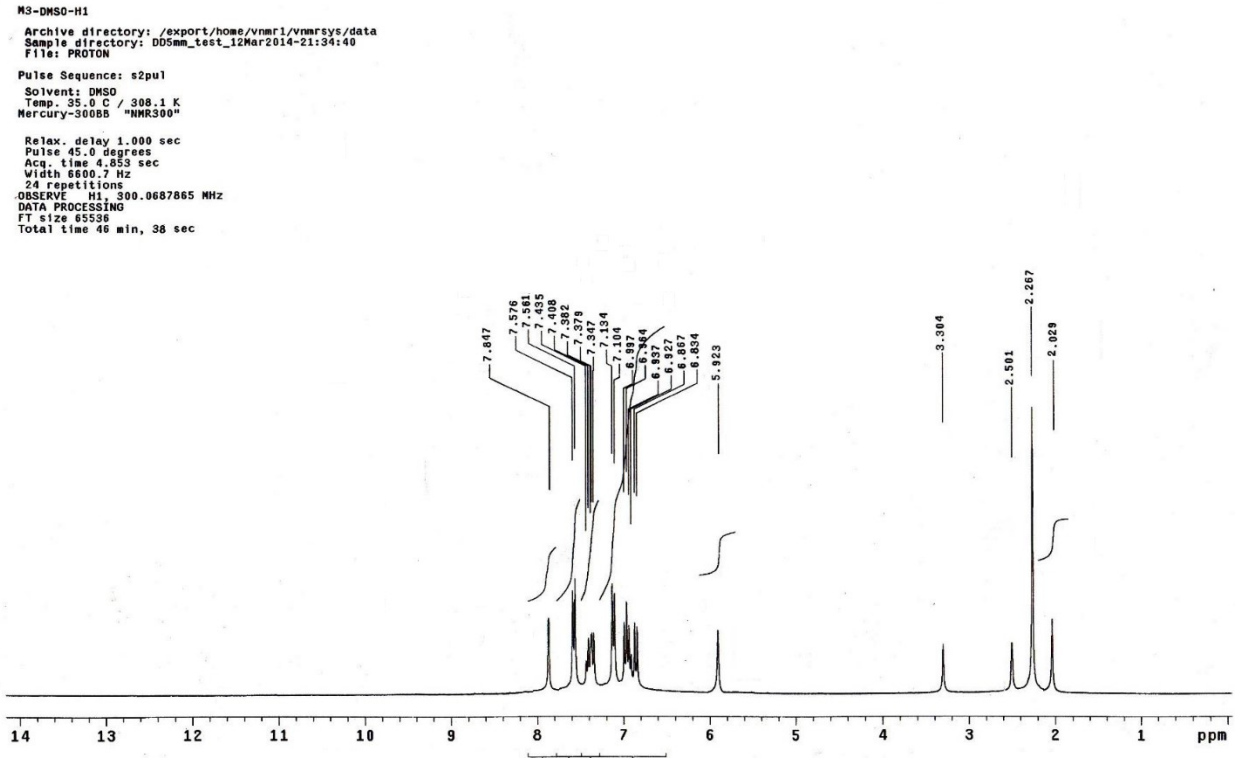
S2. ^{13}C NMR of compound **5a**



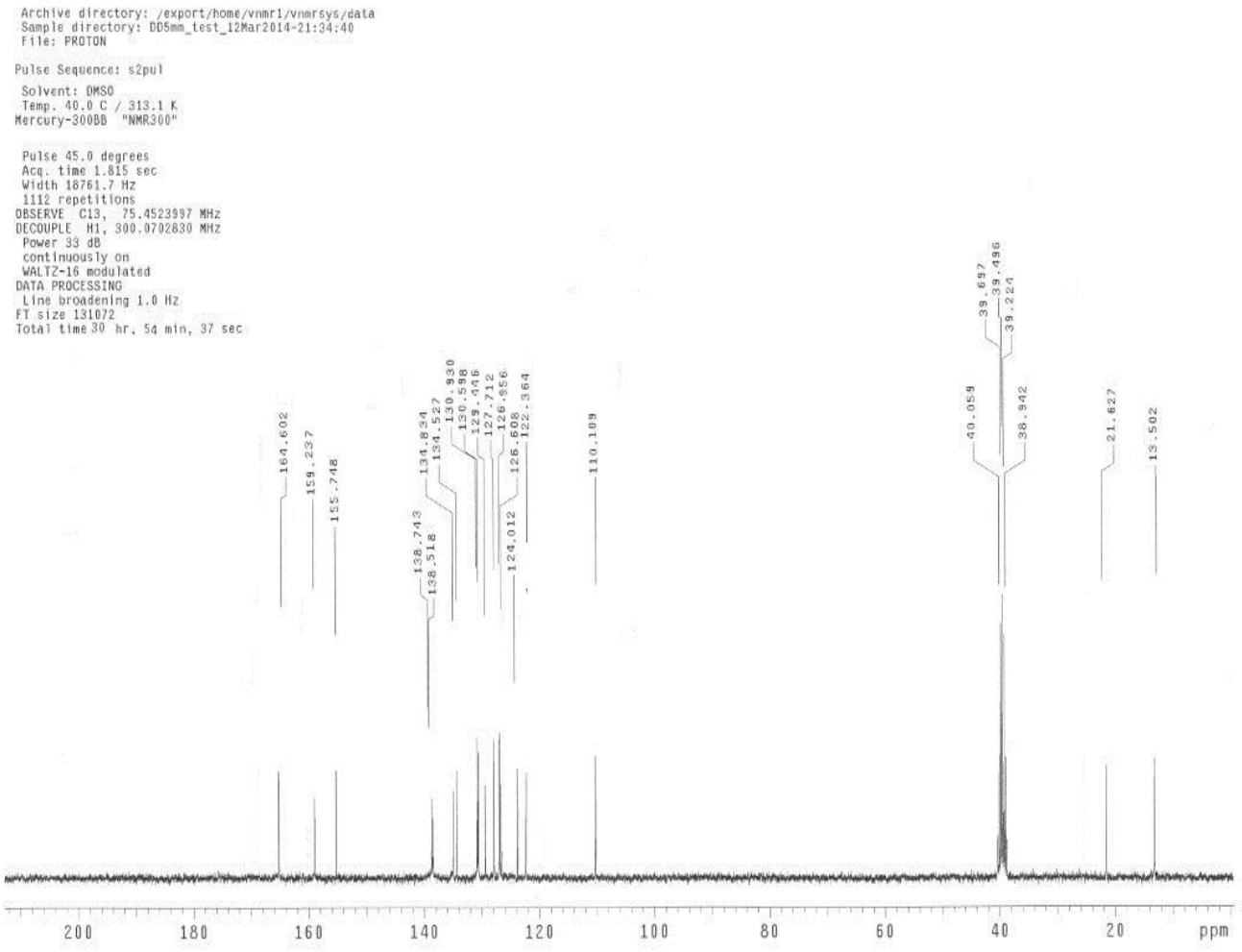
S3. ^{1}H NMR of compound **5b**



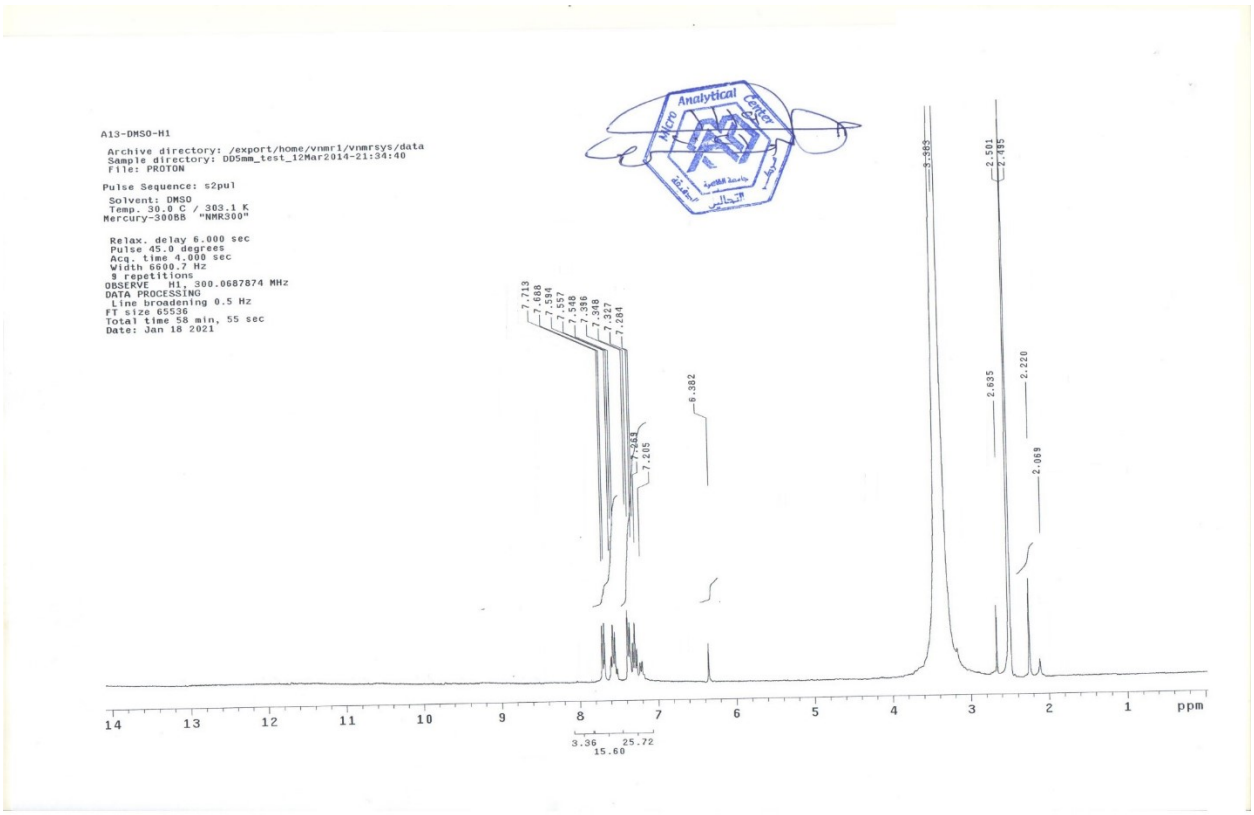
S4. ^{13}C NMR of compound **5b**



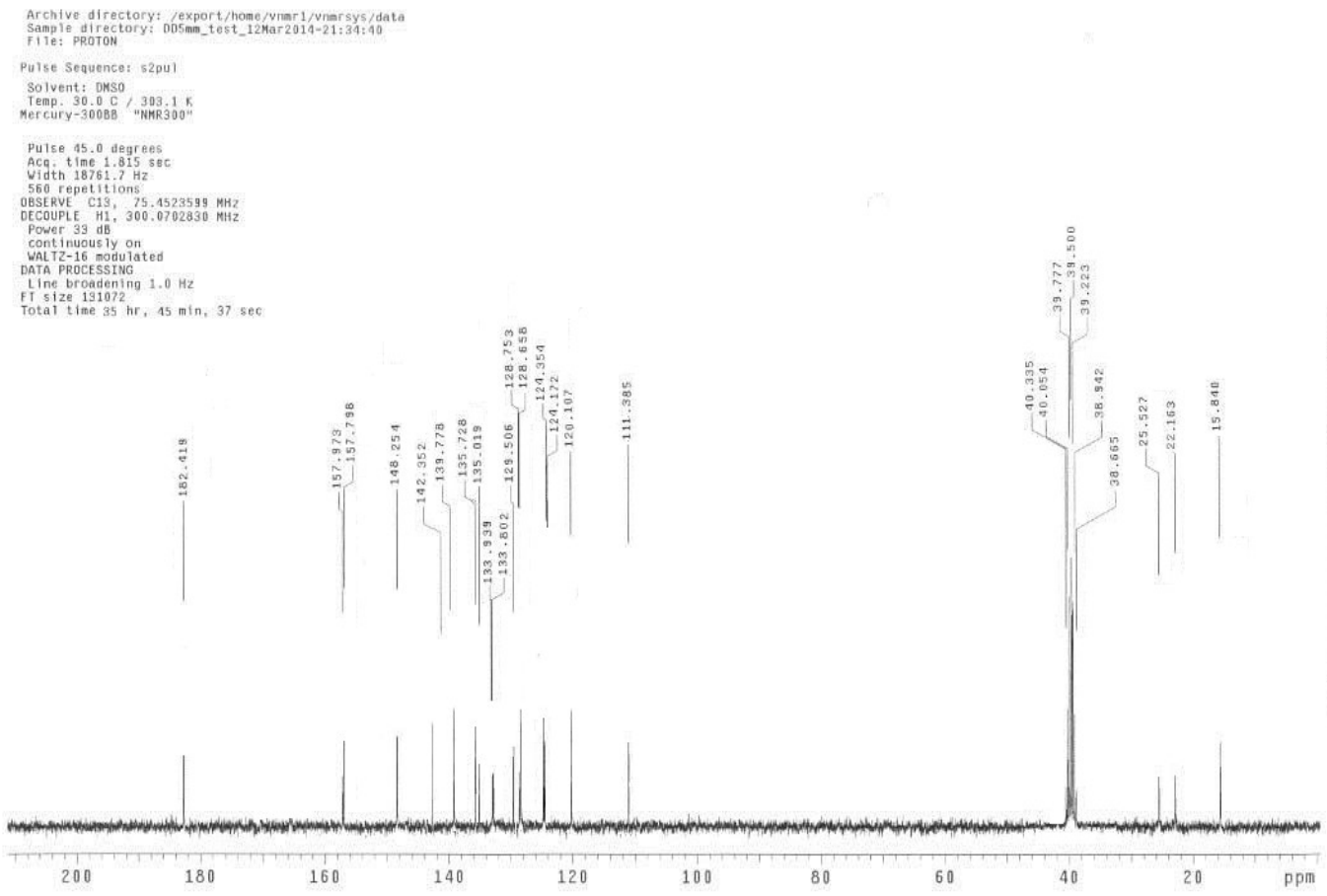
S5. ^{1}H NMR of compound **8a**



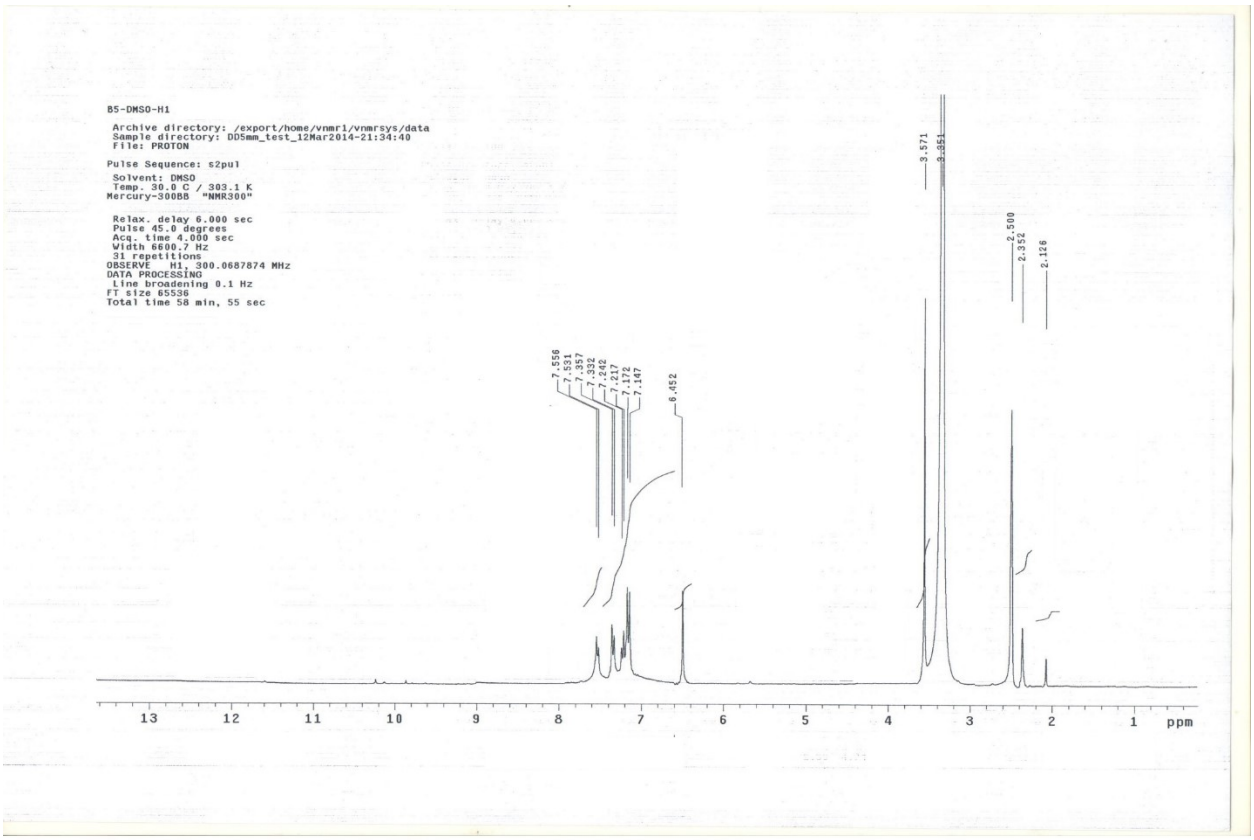
S6. ^{13}C NMR of compound **8a**



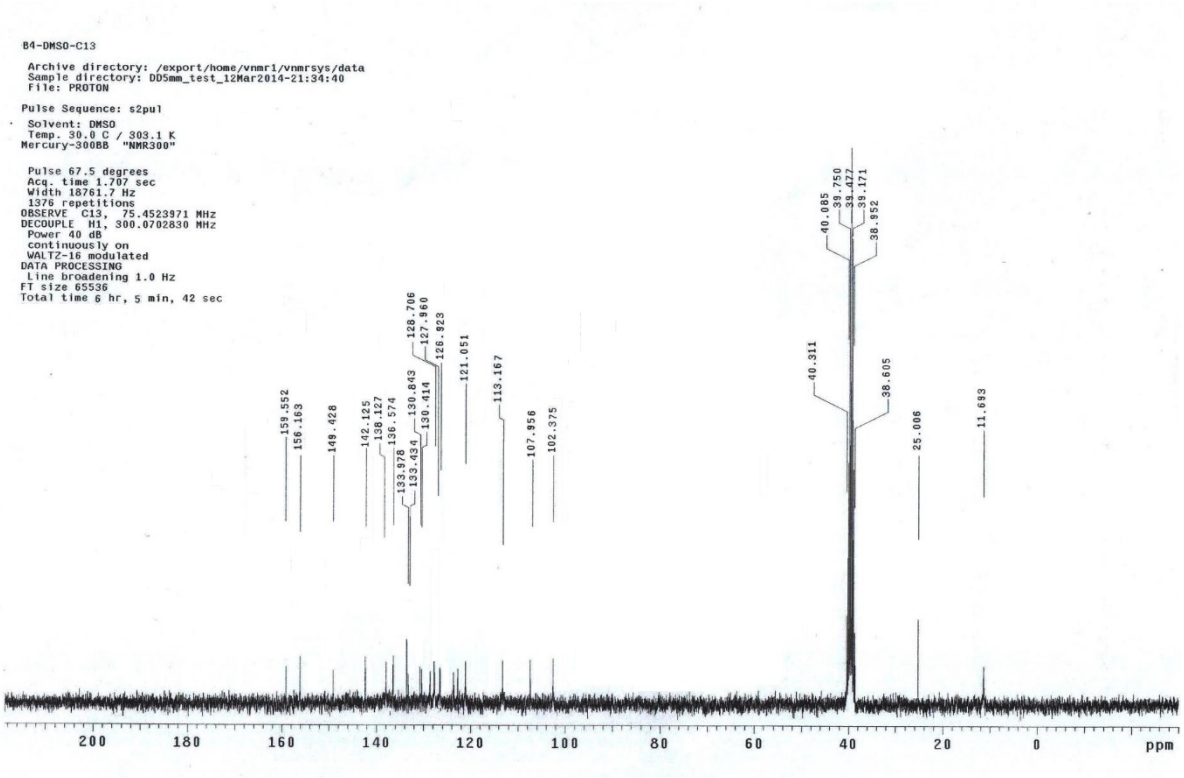
S7. ^{1}H NMR of compound **8b**



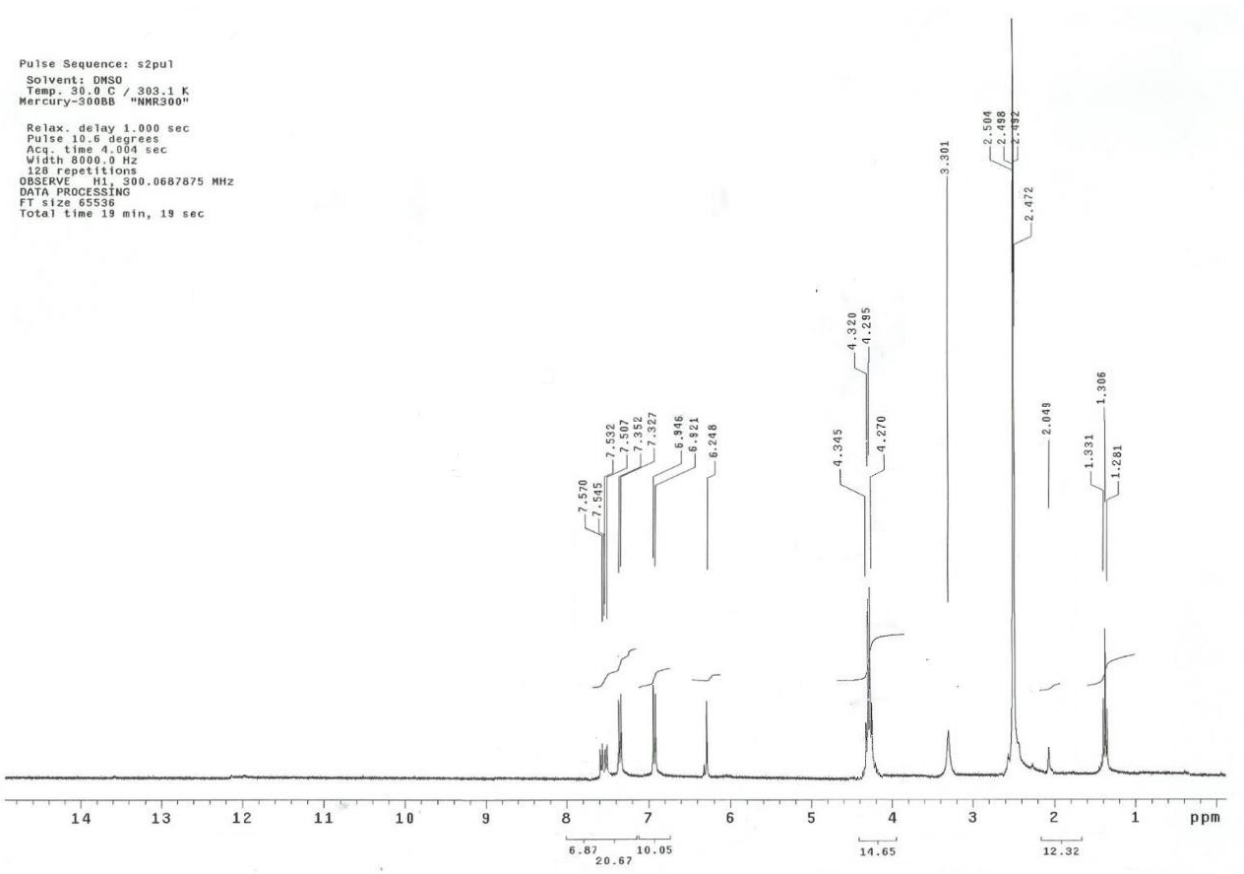
S8. ^{13}C NMR of compound **8b**



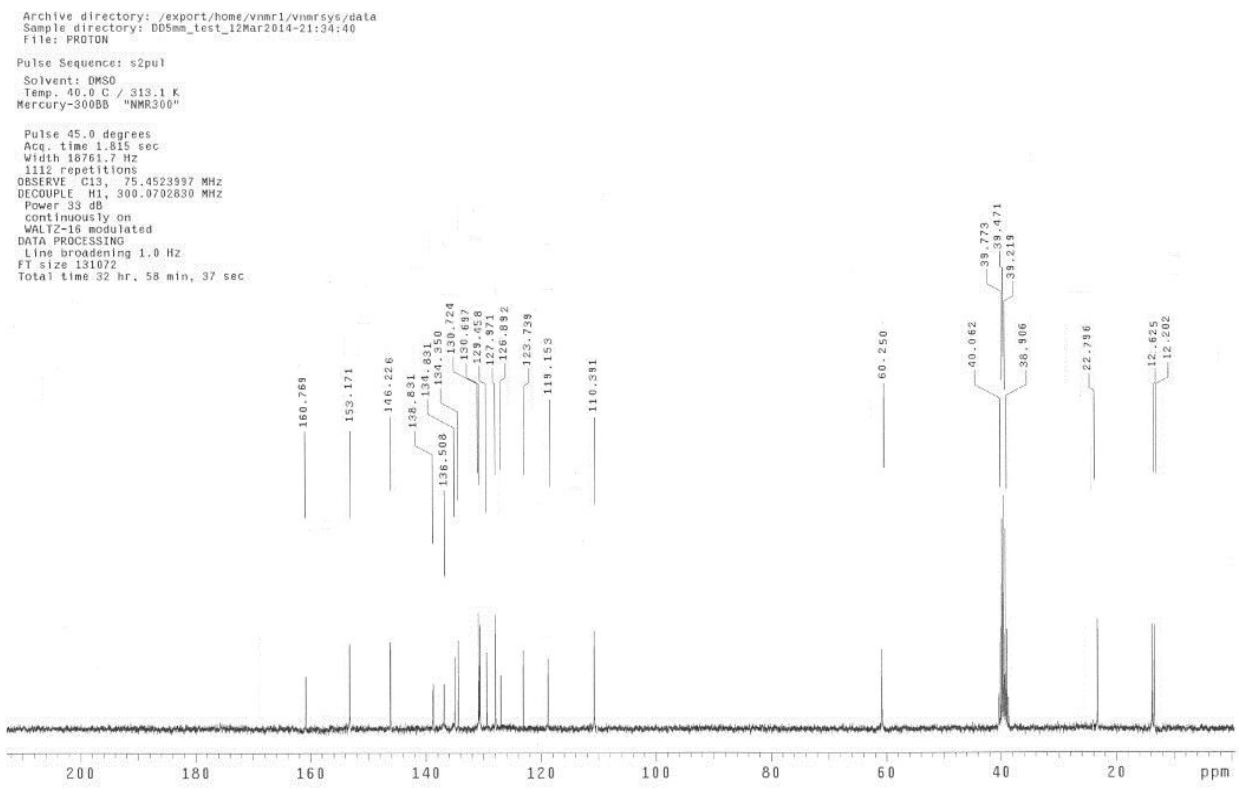
S9. ^{1}H NMR of compound **8c**



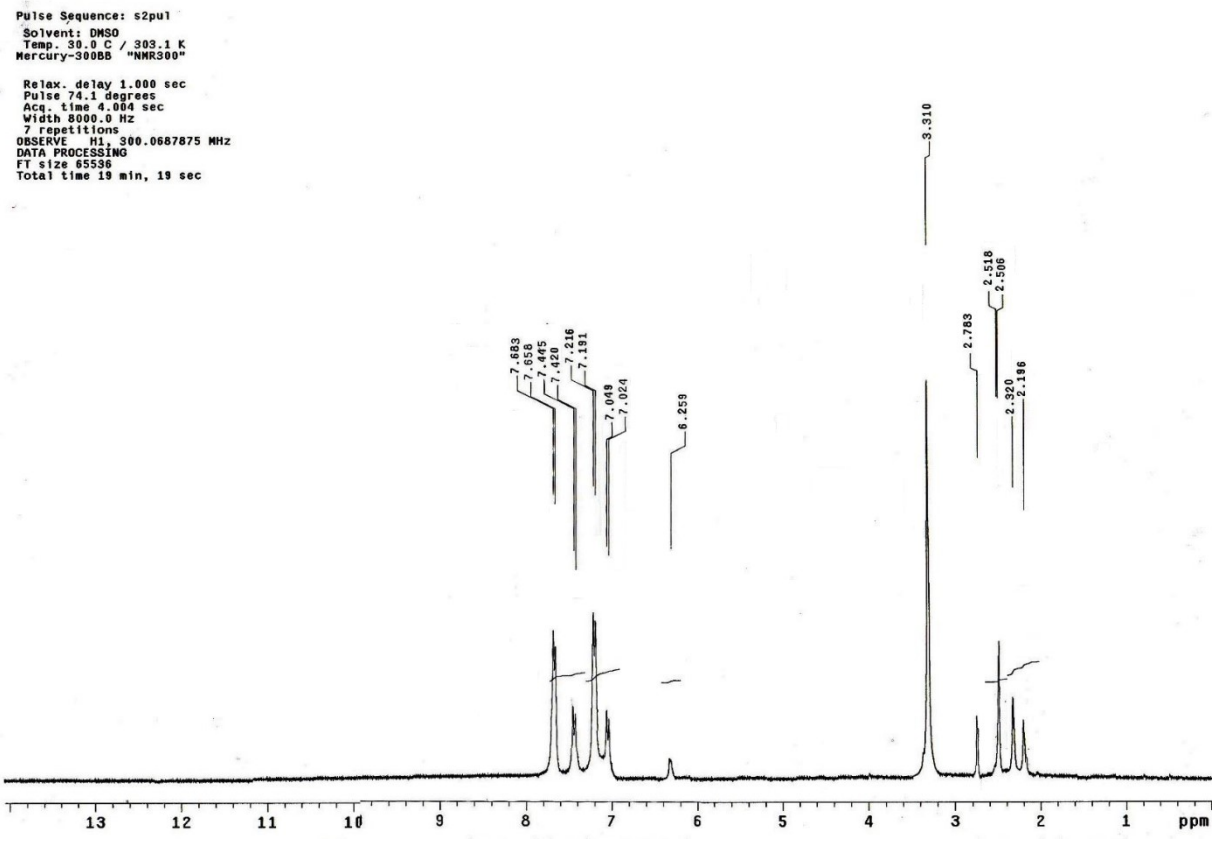
S10. ^{13}C NMR of compound **8c**



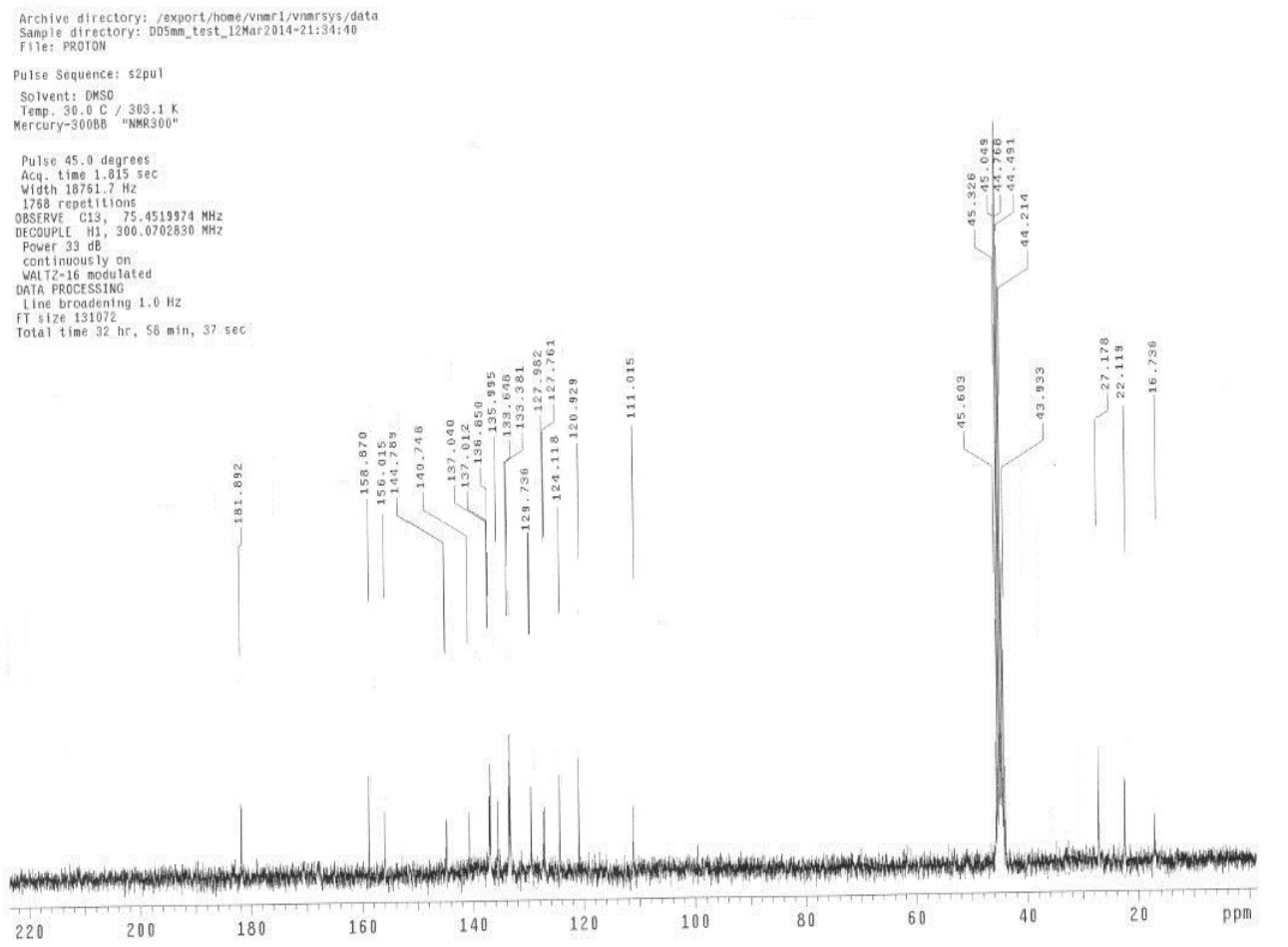
S11. ^{1}H NMR of compound **8d**



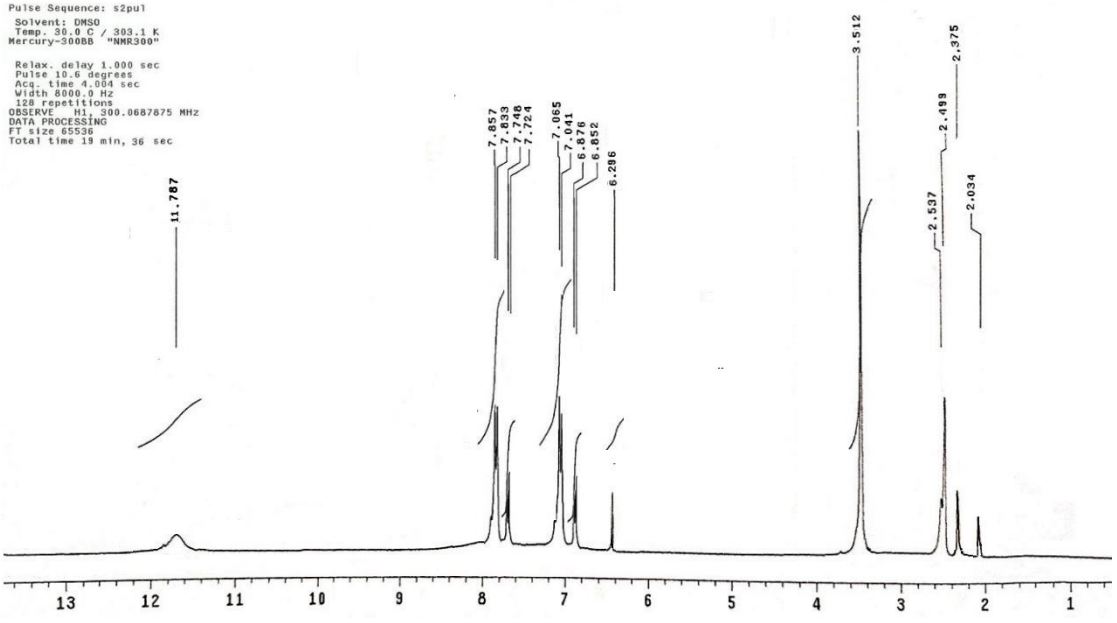
S12. ^{13}C NMR of compound **8d**



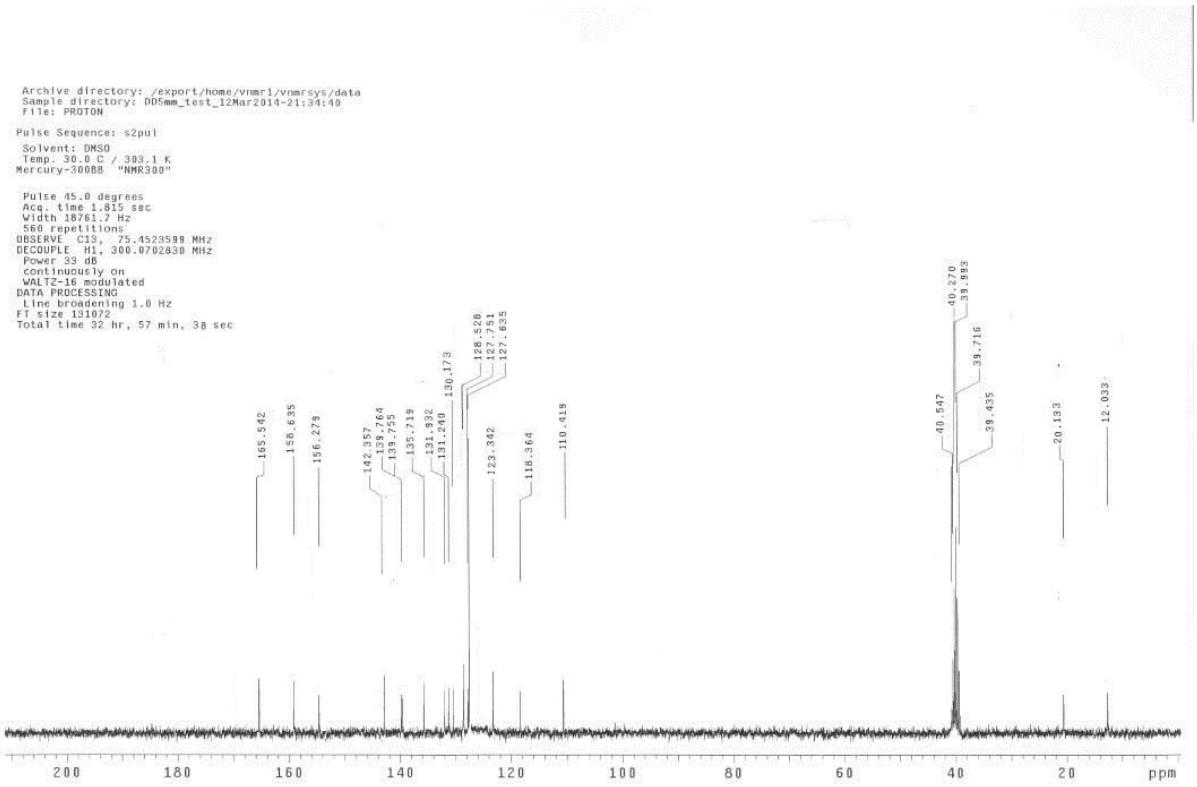
S13. ^{1}H NMR of compound **8e**



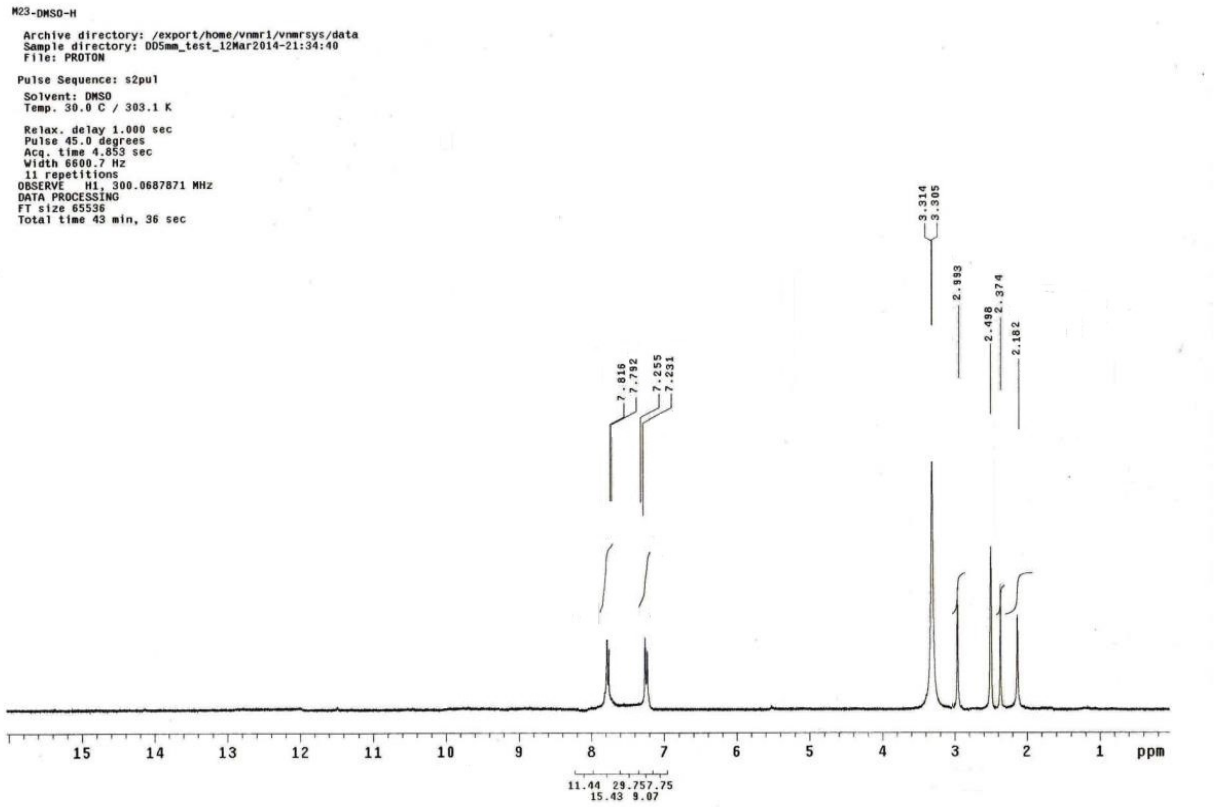
S14. ^{13}C NMR of compound **8e**



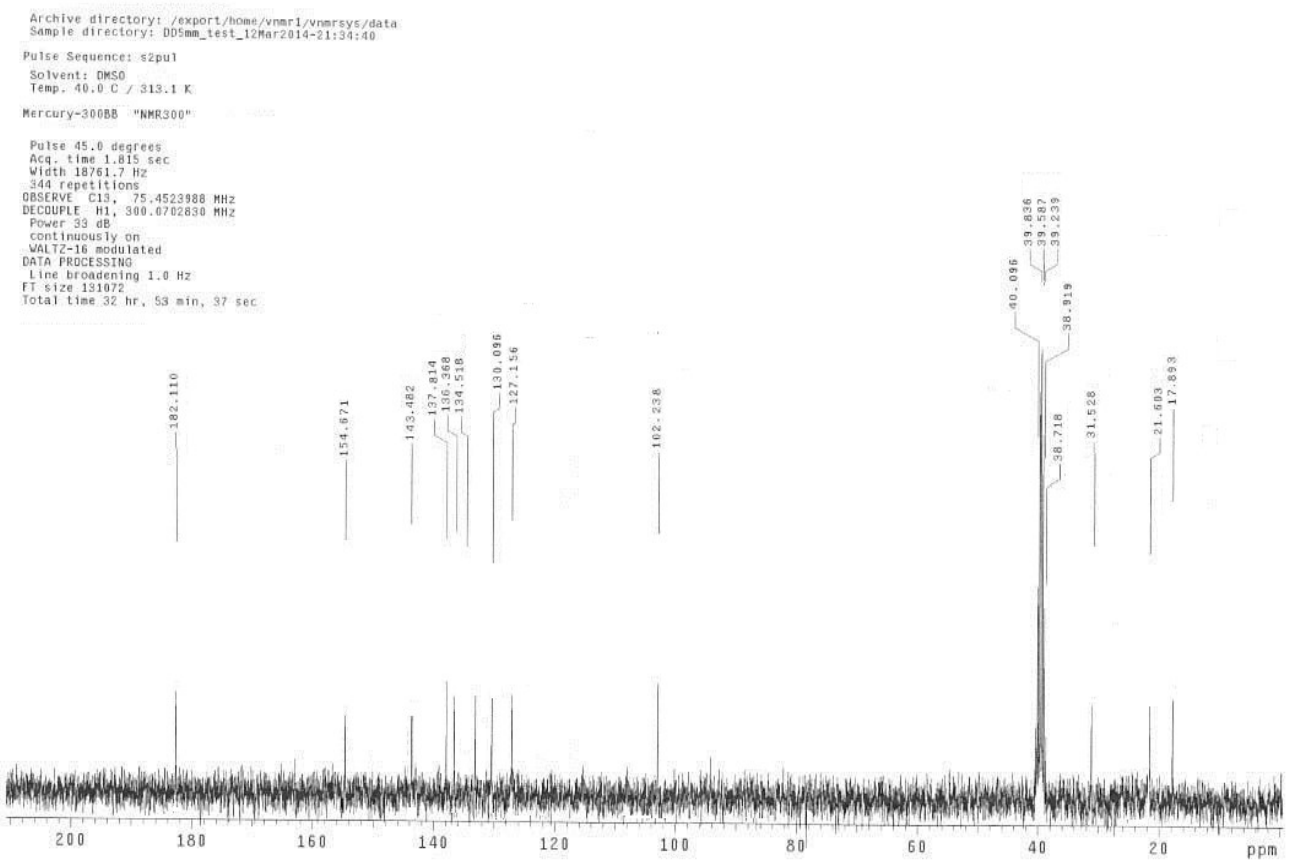
S15. ^{1}H NMR of compound **8f**



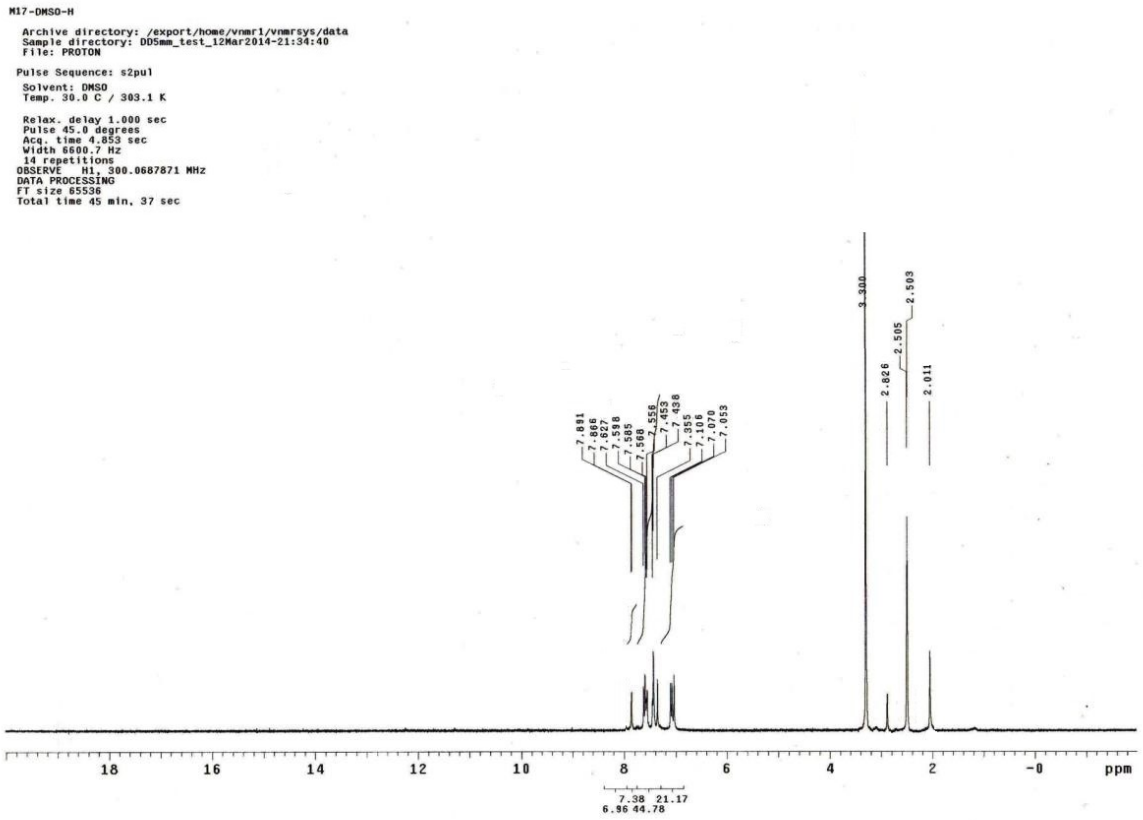
S16. ^{13}C NMR of compound **8f**



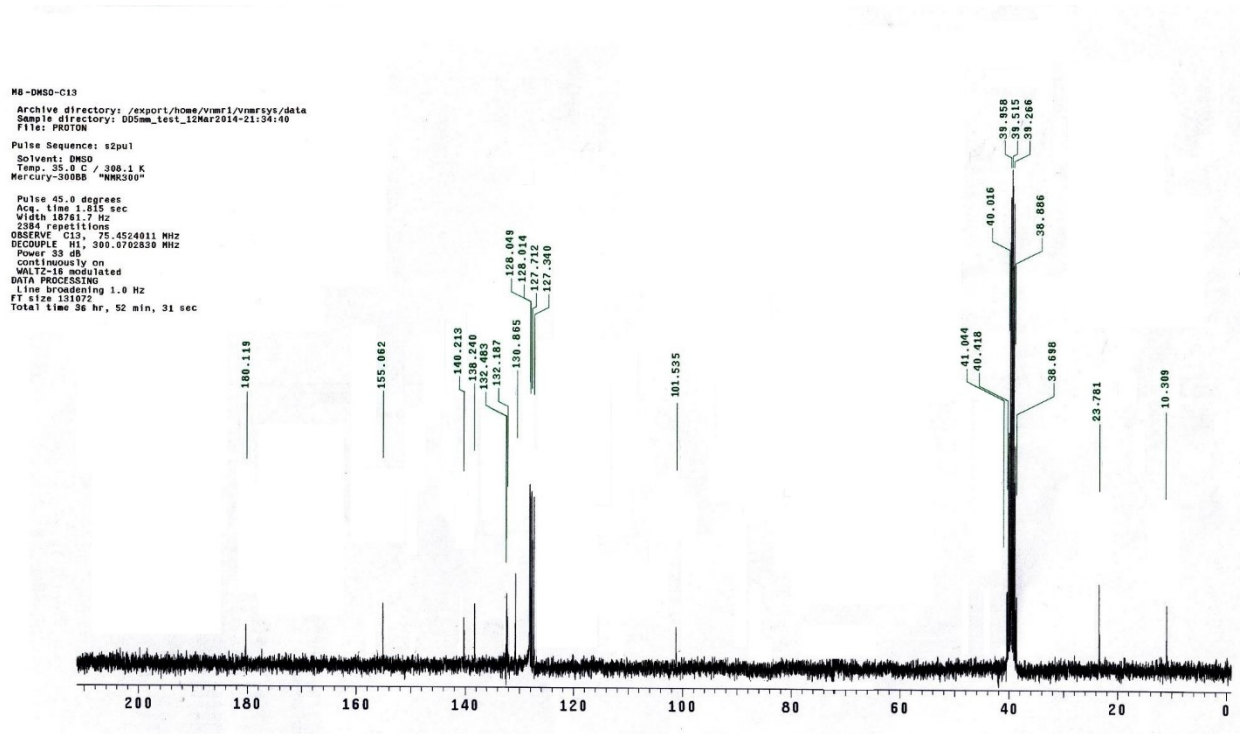
S17. ^{1}H NMR of compound **10a**



S18. ^{13}C NMR of compound **10a**



S19. ^{1}H NMR of compound **10b**



S20. ^{13}C NMR of compound **10b**