

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. ^1H NMR (300 MHz) and ^{13}C NMR (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 $\text{DMSO-}d_6$ 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 $\pm 0.4\%$ 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 $37\text{ }^\circ\text{C}$, 5% CO_2 and 95% humidity. Also, incubation of control cells occurred at $37\text{ }^\circ\text{C}$. However, after incubation for 24 h different concentrations of sample (50, 25, 12.5, 6.25, 3.125, 1.56 and $0\text{ }\mu\text{g L}^{-1}$) 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 ($\mu\text{g /mL}$), 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 $\mu\text{g/ml}$), 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 = $100 - [(\text{activity of enzyme with tested compounds} - \text{min}) / (\text{max} - \text{min})] \times 100$ (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). IC₅₀ values were processed by SPSS 19.0 statistical software from the inhibition curves. The experimental results were expressed by mean \pm 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 cytometric 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. KHO1091 invitrogen 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].

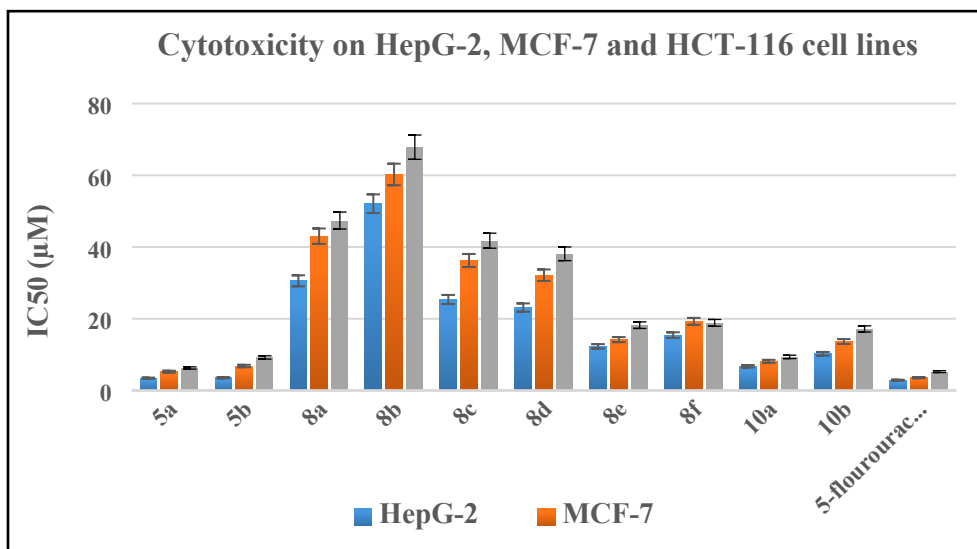
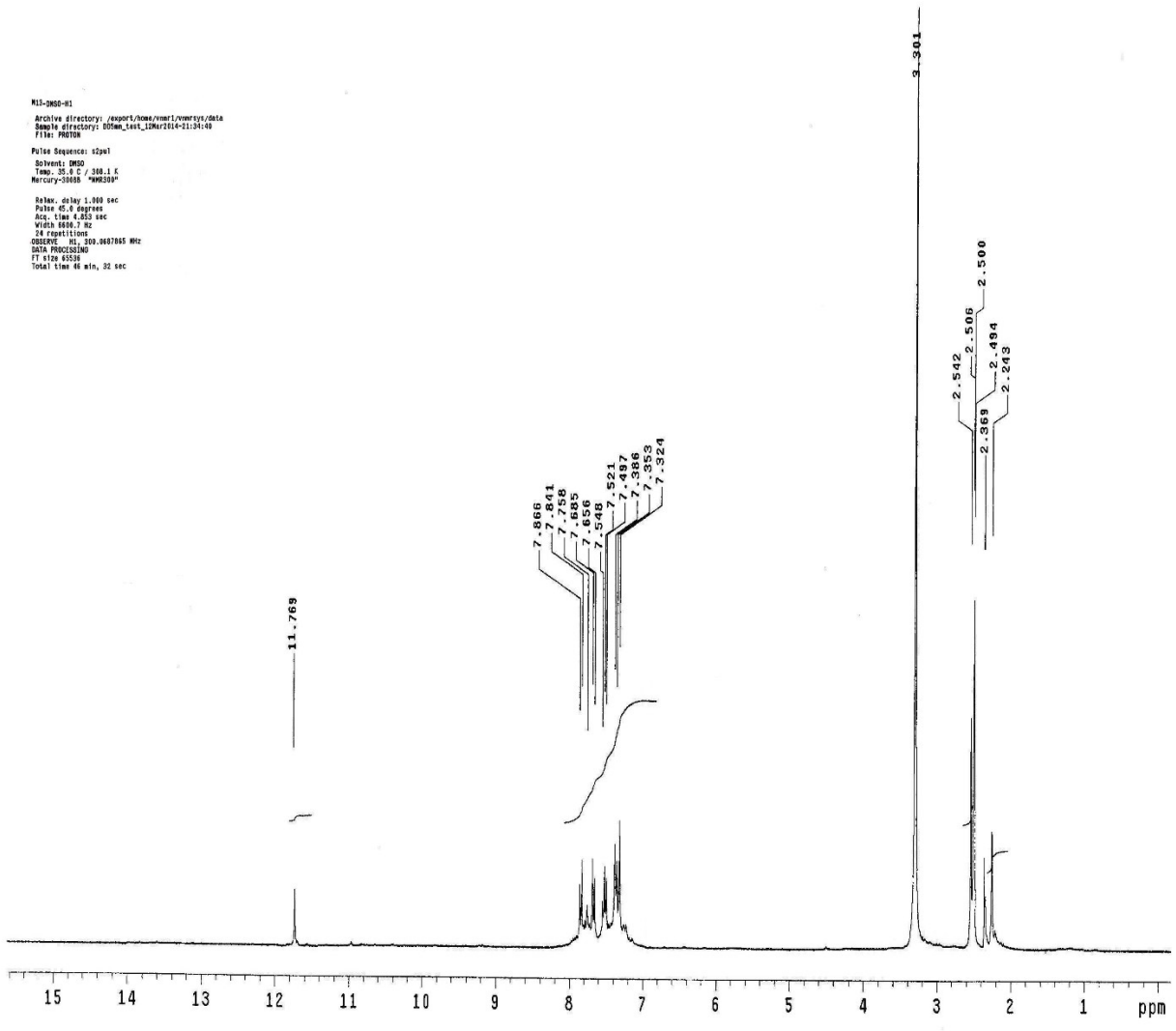


Fig. 1. Cytotoxic activity of the new target compounds against HepG-2, MCF-7, HCT-116 cancer cell lines

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

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Solvent: DMSO
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Mercury-30000 "MMS200"
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Pulse: 60.0 degrees
Acq. Time: 4.853 sec
Width: 8606.7 Hz
24 repetitions
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DATA PROCESSING
F1 size: 65536
Total time: 46 min, 32 sec



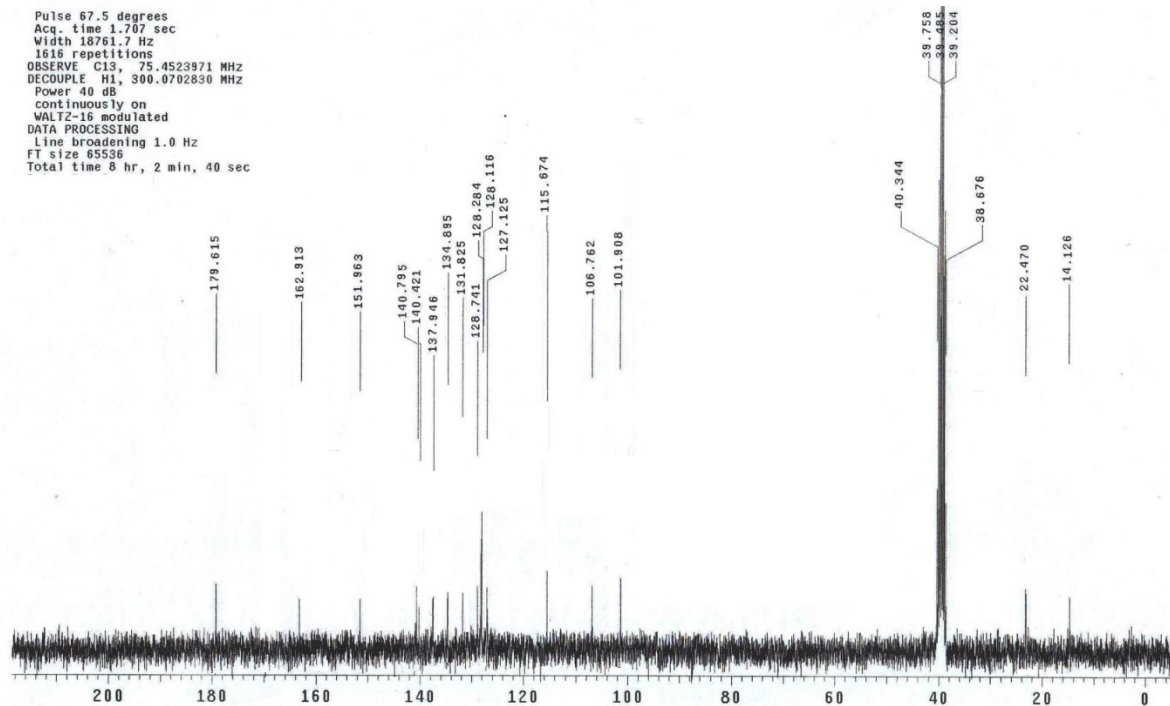
S1. ¹H NMR of compound 5a

M4-DMSO-C13

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Pulse Sequence: s2pu1
Solvent: DMSO
Temp. 30.0 C / 303.1 K
Mercury-300BB "NMR300"

Pulse 67.5 degrees
Acq. time 1.707 sec
Width 18761.7 Hz
1616 repetitions
OBSERVE C13, 75.4523971 MHz
DECOUPLE H1, 300.0702830 MHz
Power 40 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.0 Hz
FT size 65536
Total time 8 hr, 2 min, 40 sec



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

Mercury-300BB "NMR300"

Relax. delay 1.000 sec

Pulse 74.1 degrees

Acq. time 4.004 sec

Width 8000.0 Hz

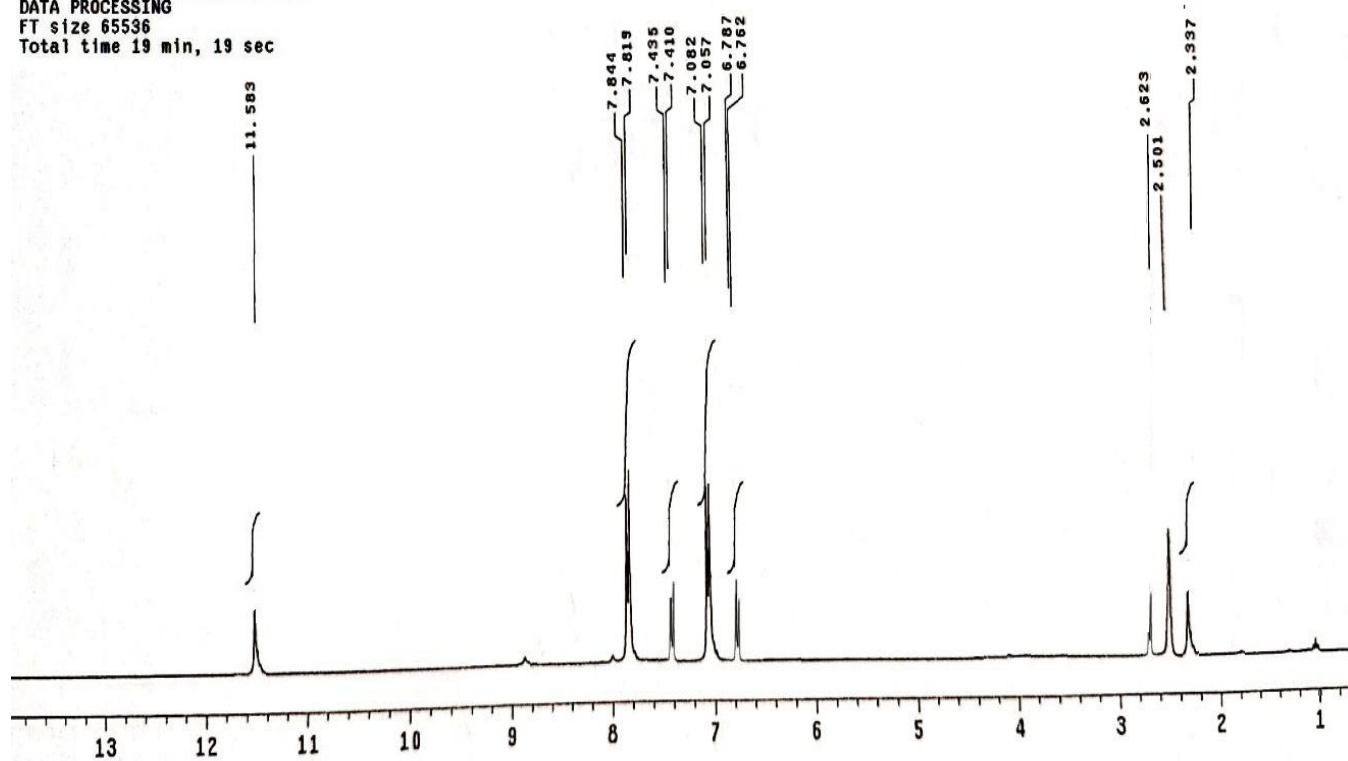
7 repetitions

OBSERVE H1, 300.0687875 MHz

DATA PROCESSING

FT size 65536

Total time 19 min, 19 sec



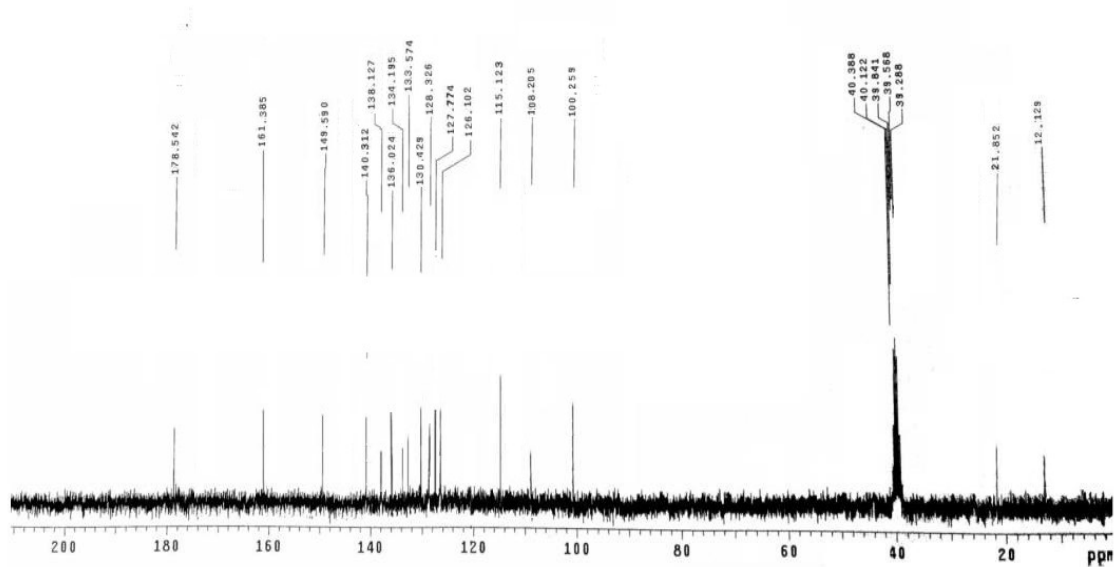
S3. ¹H NMR of compound 5b

A2-DMSO-C13

Archive directory: /export/home/vnmr1/vnmrsys/data
Sample directory: D05mm_test_12Mar2014-21:34:40

Pulse Sequence: s2pul
Solvent: DMSO
Temp. 40.0 C / 313.1 K
Mercury-300BB

Pulse 45.0 degrees
Acq. time 1.815 sec
Width 16761.7 Hz
440 repetitions
OBSERVE C13, 75.4523965 MHz
DECOUPLE H1, 300.6702830 MHz
Power 33 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.0 Hz
FT size 131072
Total time 32 hr, 58 min, 37 sec



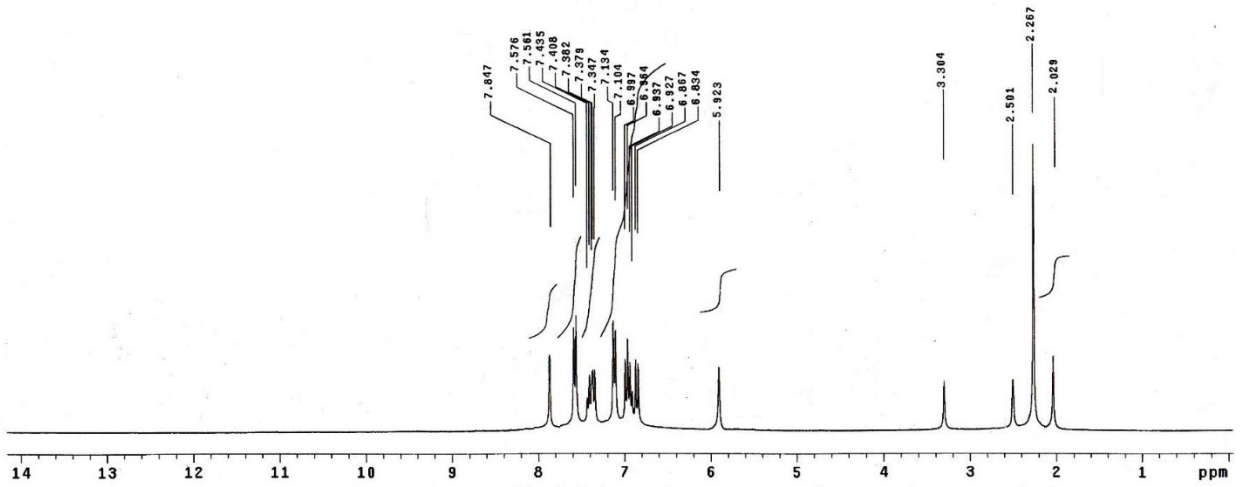
S4. ¹³C NMR of compound 5b

M3-DMSO-H1

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File: PROTON

Pulse Sequence: s2pu1
Solvent: DMSO
Temp: 35.0 C / 308.1 K
Mercury-300SB "NMR300"

Relax. delay 1.000 sec
Pulse 45.0 degrees
Acq. time 4.855 sec
Width 6600.7 Hz
24 repetitions
OBSERVE H1, 300.0687865 MHz
DATA PROCESSING
FT size 65536
Total time 46 min, 38 sec

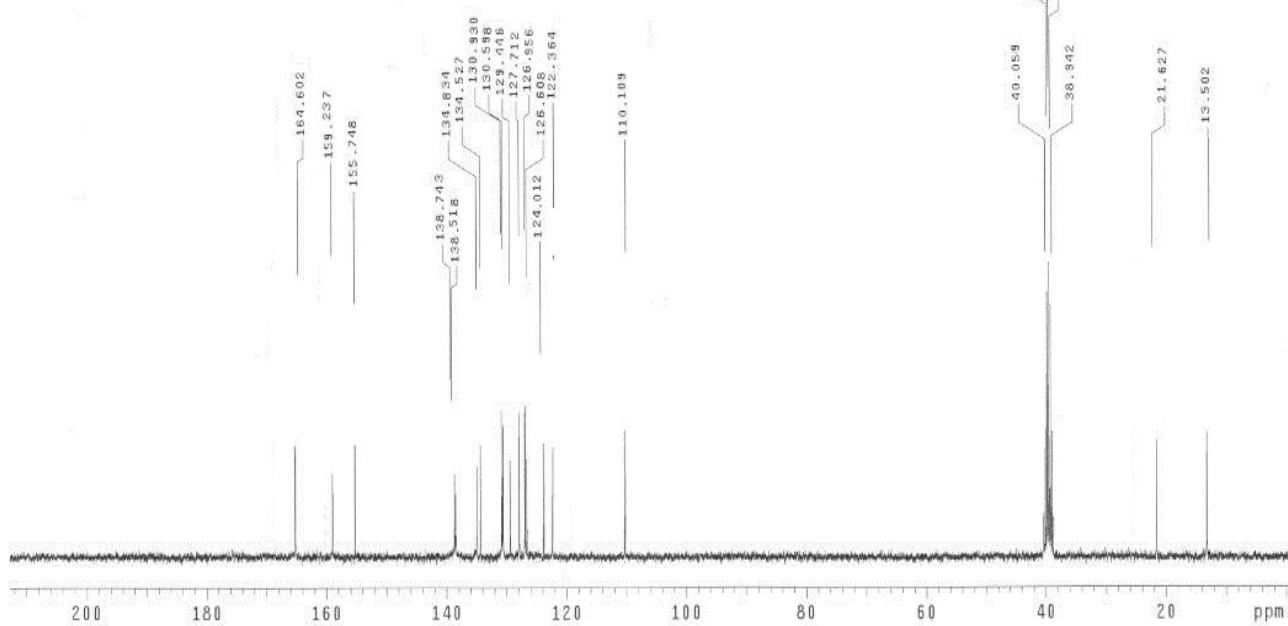


S5. ¹H NMR of compound 8a

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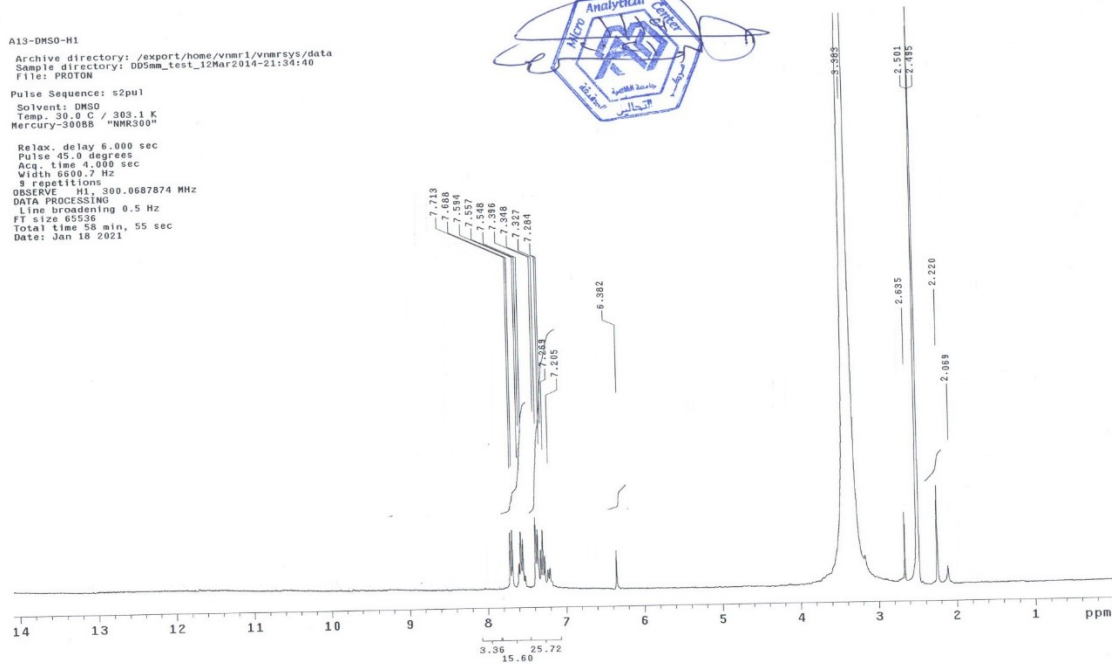
Pulse Sequence: s2pul
Solvent: DMSO
Temp. 40.0 C / 313.1 K
Mercury-300BB "NMR300"

Pulse 45.0 degrees
Acq. time 1.815 sec
Width 18761.7 Hz
1112 repetitions
OBSERVE C13, 75.4523997 MHz
DECOUPLE H1, 300.9702630 MHz
Power 33 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.0 Hz
FT size 131072
Total time 30 hr, 54 min, 37 sec



S6. ¹³C NMR of compound 8a

A13-DMSO-H1
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Pulse Sequence: s2pu1
Solvent: DMSO
Temp: 30.0 C / 303.1 K
Mercury-300SB "NMR300"
Relax. delay 6.000 sec
Pulse 45.0 degrees
Acq. time 4.000 sec
Width 6600.7 Hz
9 repetitions
OBSERVE H1, 300.0687074 MHz
DATA PROCESSING
Line broadening 0.5 Hz
FT size 65536
Total time 58 min, 55 sec
Date: Jan 18 2021

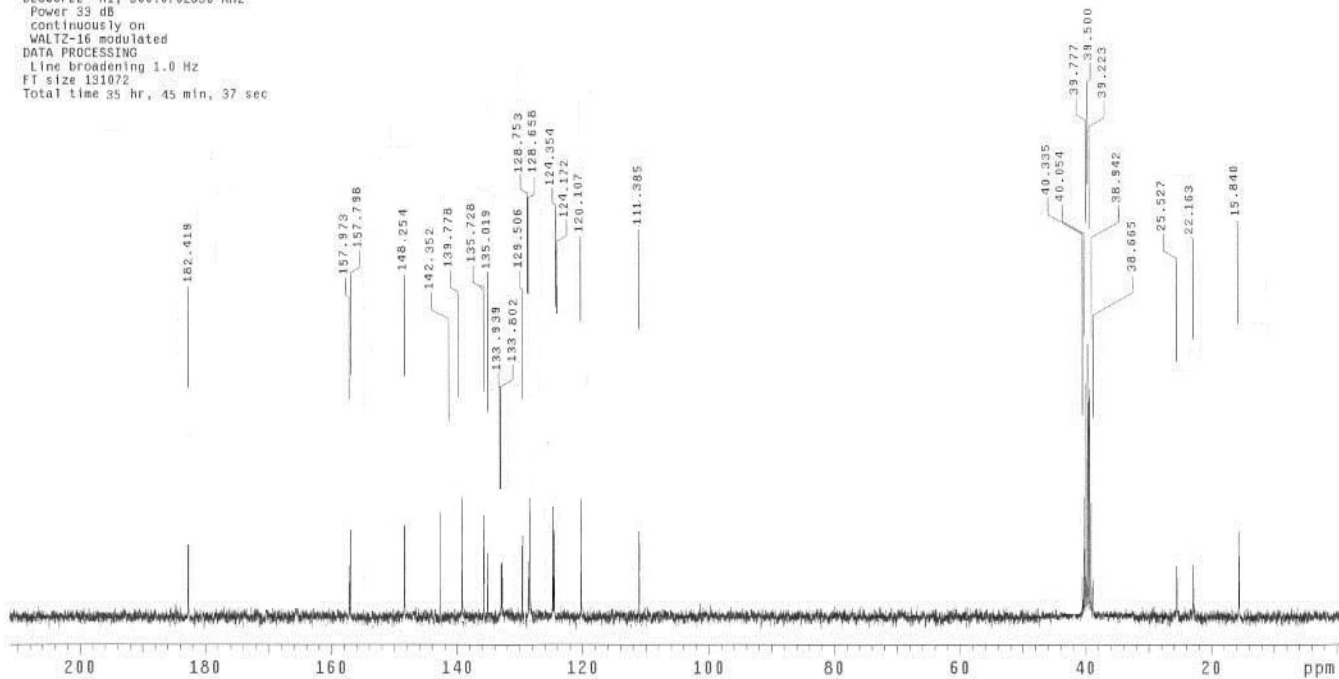


S7. ^1H NMR of compound **8b**

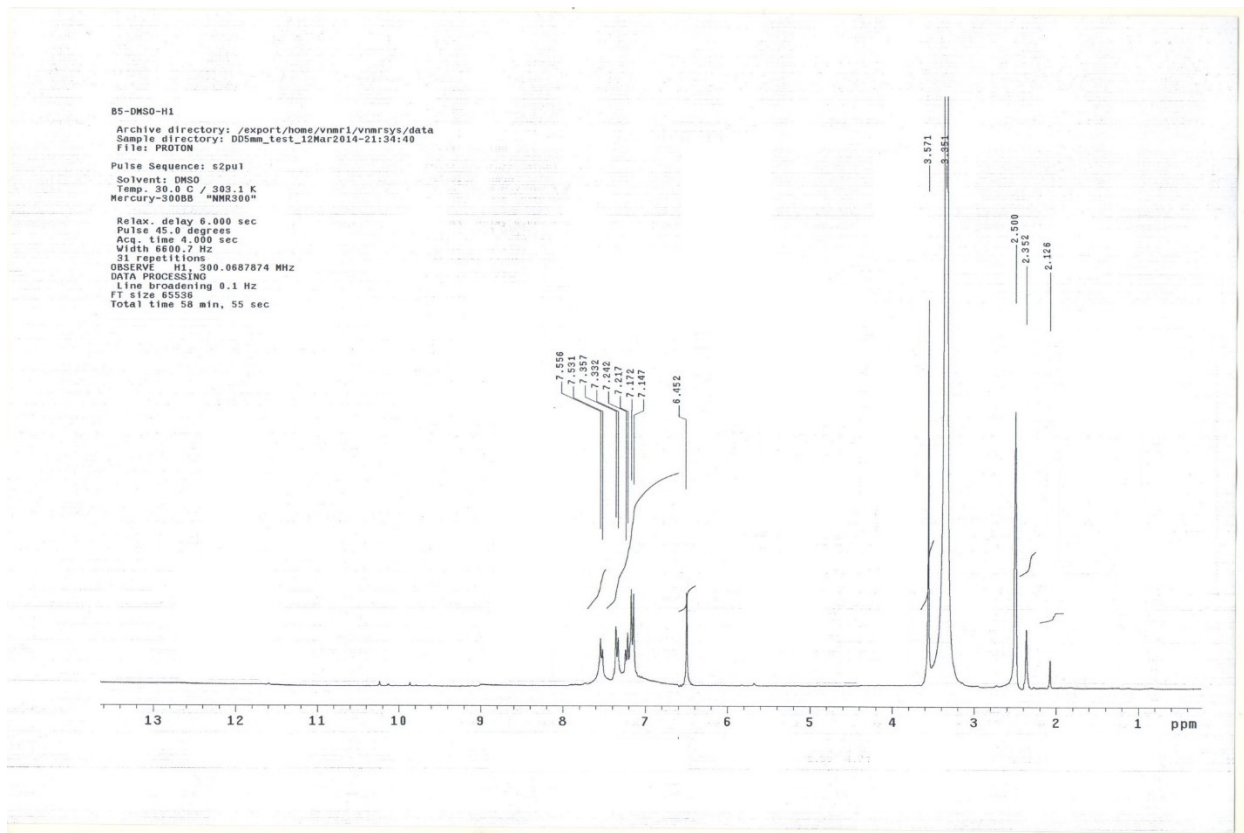
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File: PROTON

Pulse Sequence: s2pul
Solvent: DMSO
Temp. 30.0 C / 303.1 K
Mercury-300BB "NMR300"

Pulse 45.0 degrees
Acq. time 1.815 sec
Width 18761.7 Hz
560 repetitions
OBSERVE C13, 75.4523599 MHz
DECOUPLE H1, 300.0702830 MHz
Power 39 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.0 Hz
FT size 131072
Total time 35 hr, 45 min, 37 sec



S8. ¹³C NMR of compound 8b



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

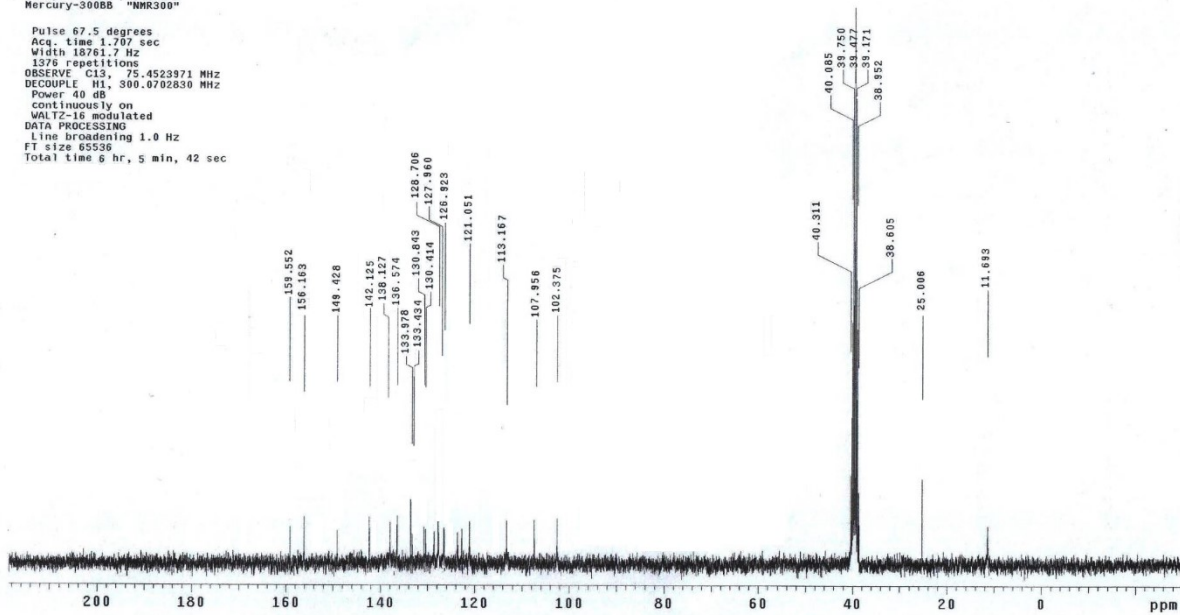
B4-DMSO-C13

Archive directory: /export/home/vmar1/vmrsys/data
Sample directory: DD5mm_test_12Mar2014-21:34:40
File: PROTON

Pulse Sequence: s2pul

Solvent: DMSO
Temp. 30.0 C / 303.1 K
Mercury-300BB "NMR300"

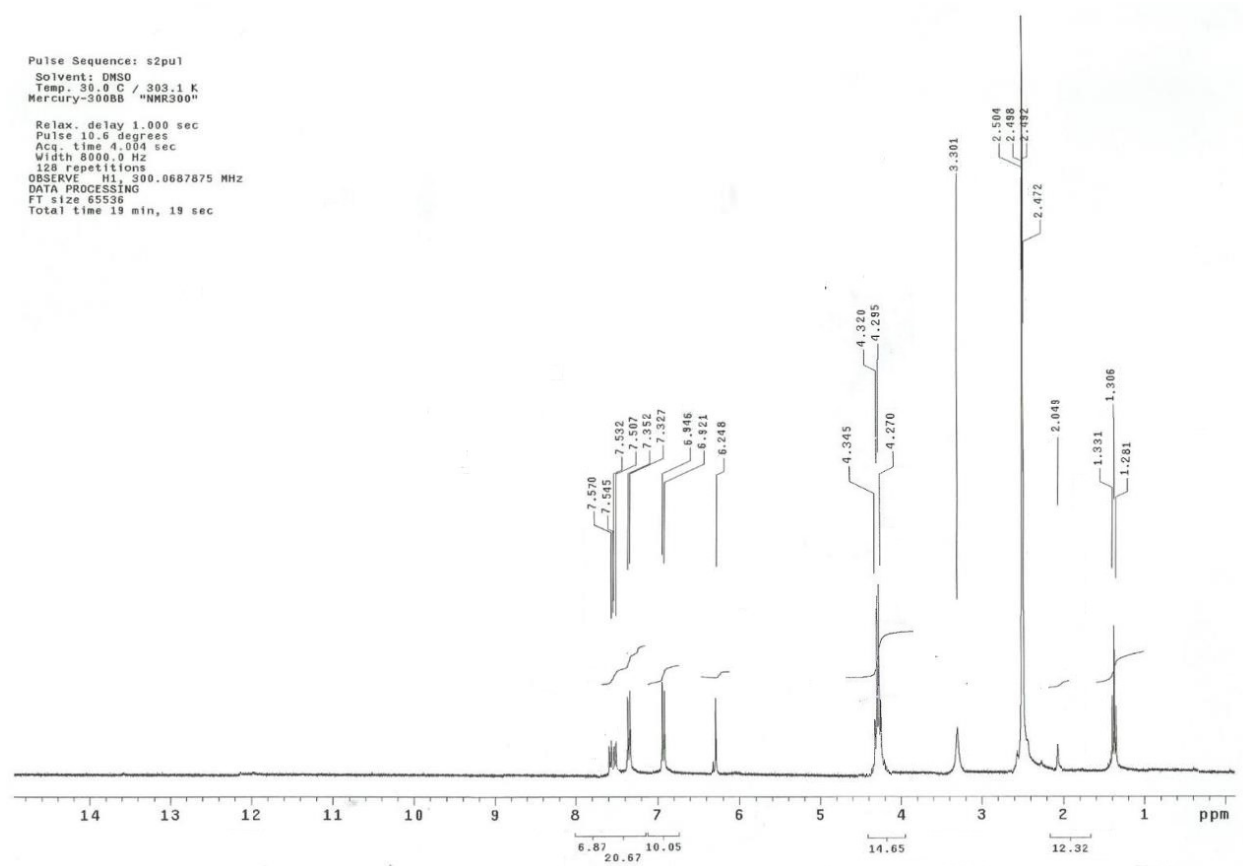
Pulse 67.5 degrees
Acq. time 1.707 sec
Width 16761.7 Hz
1376 repetitions
OBSERVE C13, 75.4523971 MHz
DECOUPLE H1, 300.0702830 MHz
Power 40 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.0 Hz
FT size 65536
Total time 6 hr, 5 min, 42 sec



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

Pulse Sequence: s2pu1
Solvent: DMSO
Temp. 30.0 C / 303.1 K
Mercury-300BB "NMR300"

Relax. delay 1.000 sec
Pulse 10.6 degrees
Acq. time 4.004 sec
Width 8000.0 Hz
128 repetitions
OBSERVE H1, 300.0687875 MHz
DATA PROCESSING
FT size 65536
Total time 19 min, 19 sec

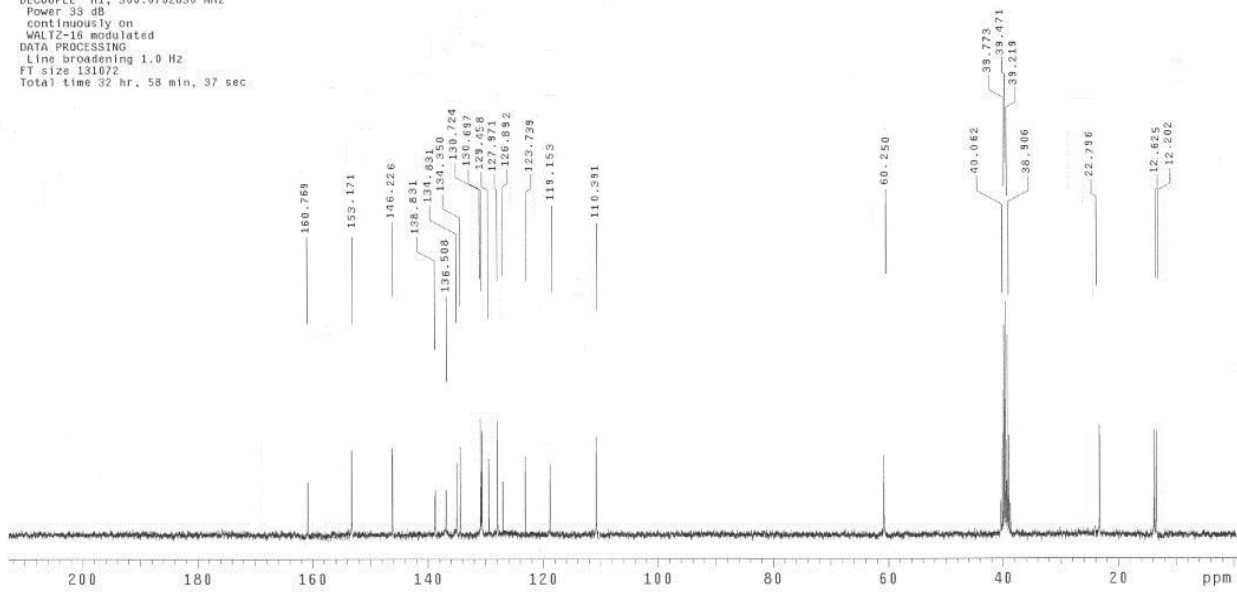


S11. ¹H NMR of compound 8d

Archive directory: /export/home/vnmr1/vnmrsys/data
Sample directory: B05mm_test_12Mar2014-21:34:40
File: PROTON

Pulse Sequence: s2pul
Solvent: DMSO
Temp. 40.0 C / 313.1 K
Mercury-300BB "NMR300"

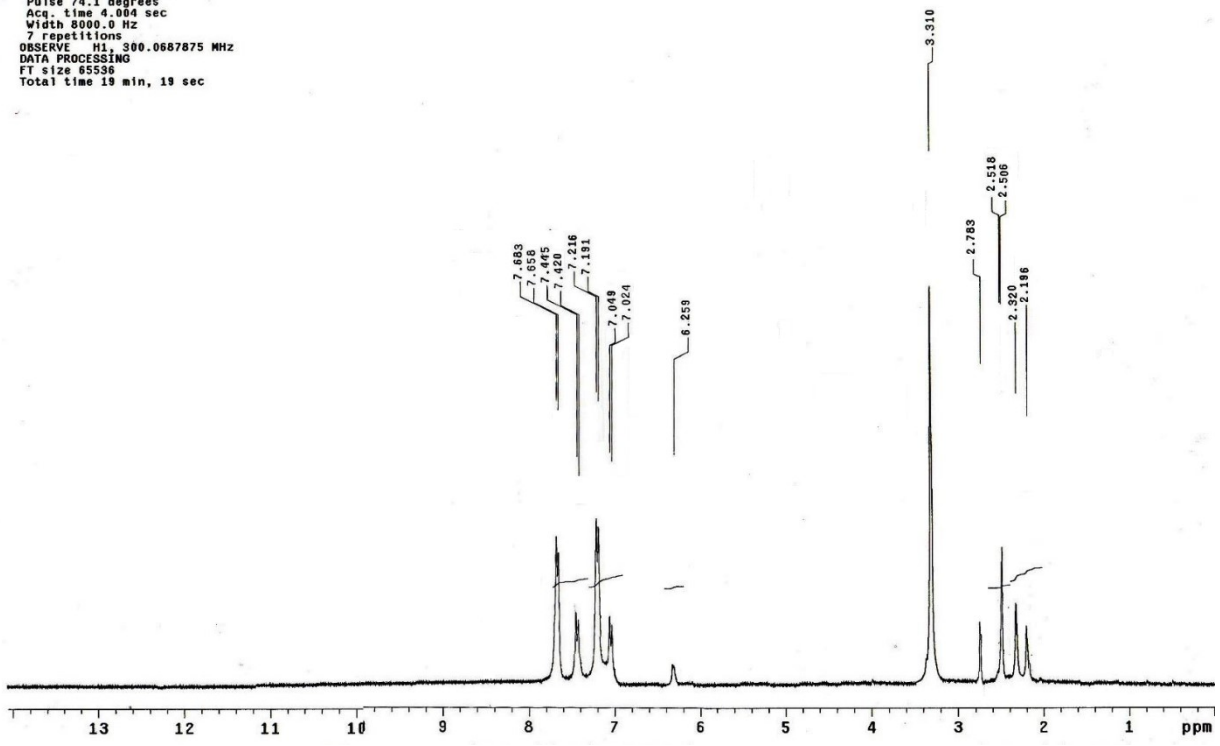
Pulse 45.0 degrees
Acq. time 1.815 sec
Width 18761.7 Hz
1112 repetitions
OBSERVE C13, 75.4523997 MHz
DICOUPLE H1, 300.0702830 MHz
Power 33 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.0 Hz
FT size 131072
Total time 32 hr, 58 min, 37 sec



S12. ¹³C NMR of compound **8d**

Pulse Sequence: s2pul
Solvent: DMSO
Temp. 30.0 C / 303.1 K
Mercury-300BB "NMR300"

Relax. delay 1.000 sec
Pulse 74.1 degrees
Acq. time 4.004 sec
Width 8000.0 Hz
7 repetitions
OBSERVE H1, 300.0687875 MHz
DATA PROCESSING
FT size 65536
Total time 19 min, 19 sec



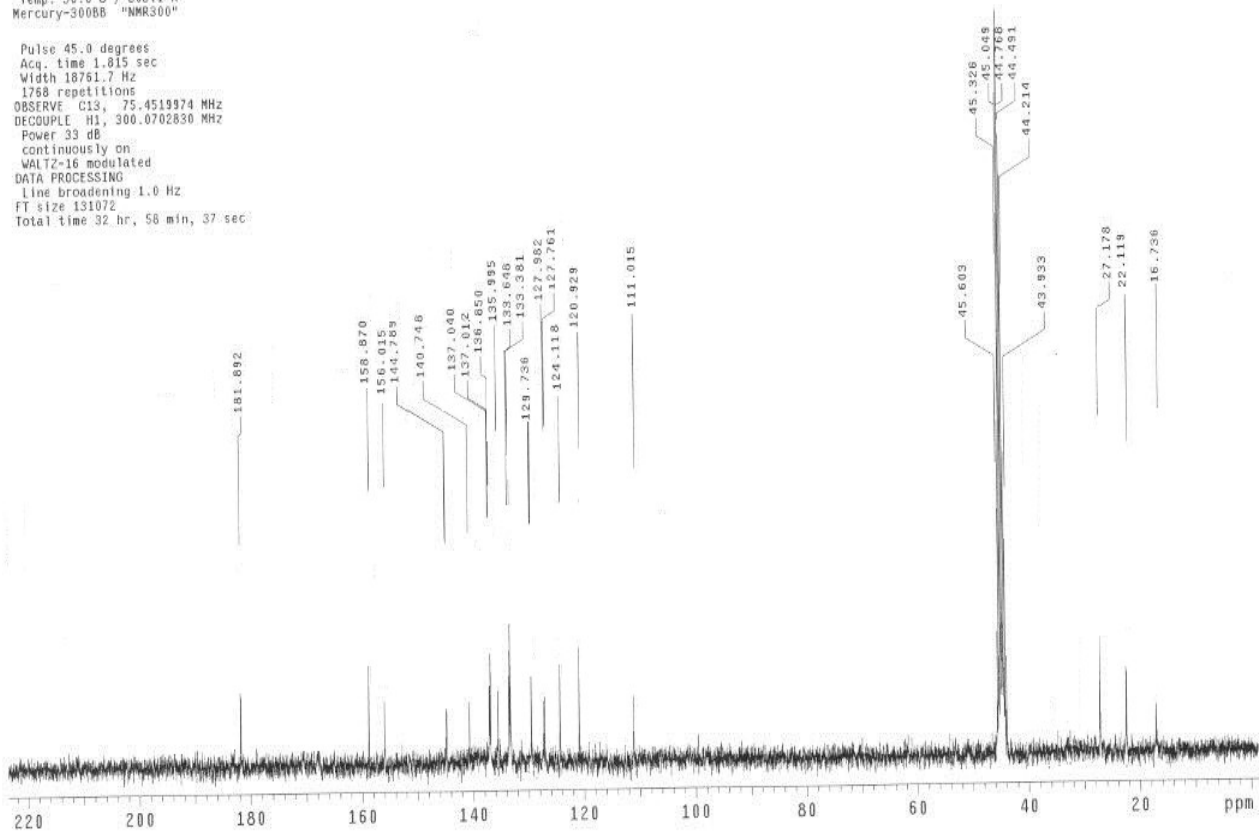
S13. ¹H NMR of compound 8e

Archive directory: /export/home/vnmr1/vnmrsys/data
Sample directory: DD5mm_test_12Mar2014-21:34:40
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Pulse Sequence: s2pu1

Solvent: DMSO
Temp. 30.0 C / 303.1 K
Mercury-300BB "NMR300"

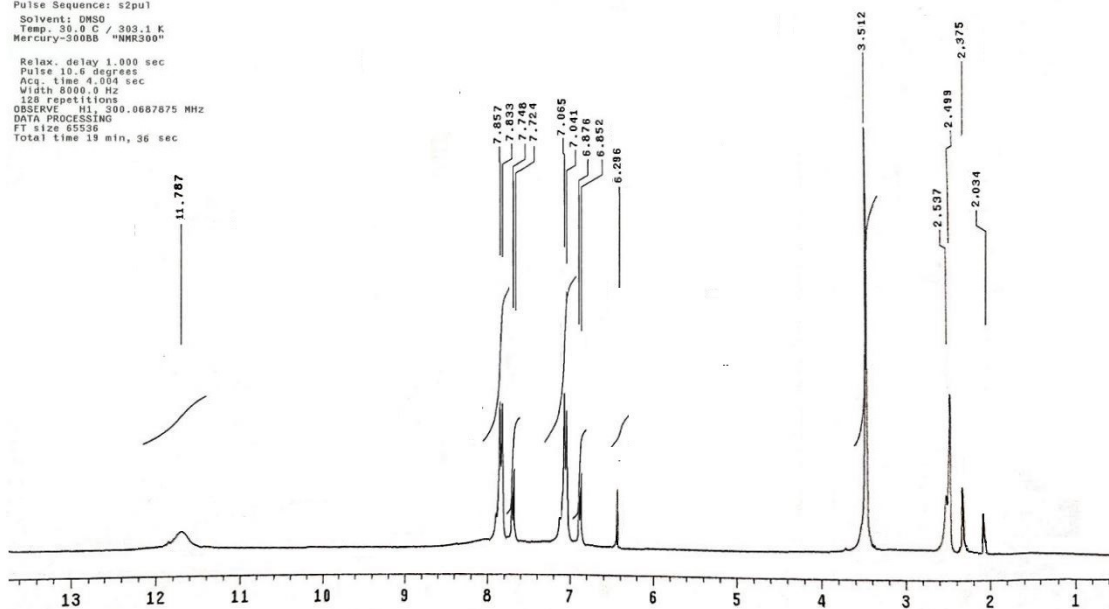
Pulse 45.0 degrees
Acq. time 1.815 sec
Width 18761.7 Hz
1768 repetitions
OBSERVE C13, 75.4519974 MHz
DECOUPLE H1, 300.0702830 MHz
Power 33 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.0 Hz
FT size 131072
Total time 32 hr, 58 min, 37 sec



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

Pulse Sequence: s2pu1
Solvent: DMSO
Temp: 30.0 C / 293.1 K
Mercury-3000B "NMR300"

Relax. delay 1.000 sec
Pulse 10.6 degrees
Acq. time 4.004 sec
Width 8000.0 Hz
128 repetitions
OBSERVE: H1, 300.0687875 MHz
DATA PROCESSING
F1 size 65536
Total time 19 min, 36 sec

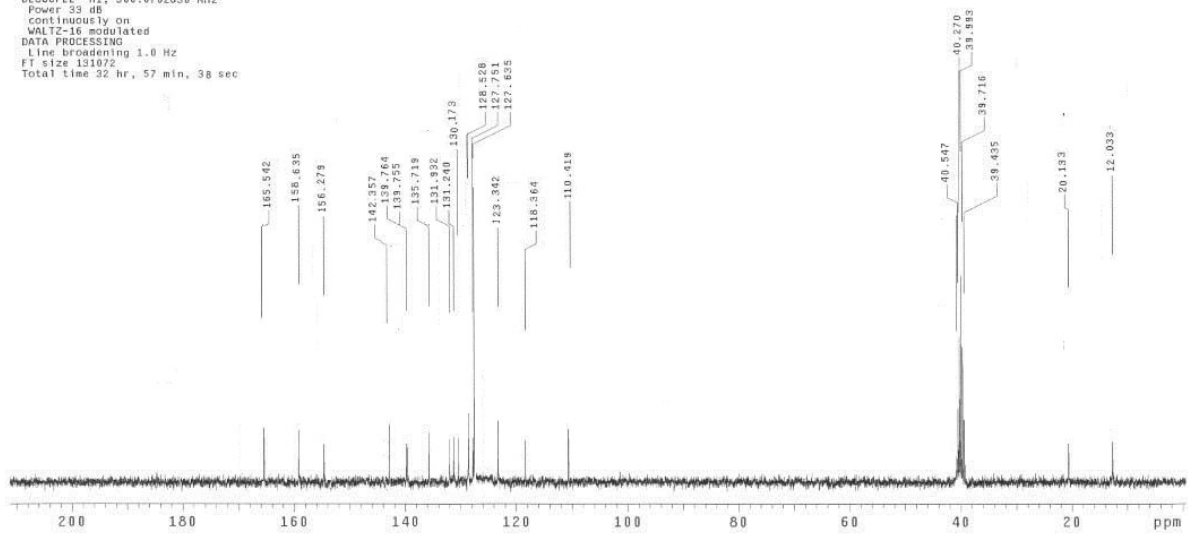


S15. ¹H NMR of compound 8f

Archive directory: /export/home/vnmr1/vnmrsys/data
Sample directory: 005mm_test_12Mar2014-21:34:49
File: PROTON

Pulse Sequence: s2pu1
Solvent: DMSO
Temp. 30.0 C / 303.1 K
Mercury-300BB "NMR300"

Pulse 45.0 degrees
Acq. time 1.815 sec
Width 18761.7 Hz
580 repetitions
OBSERVE C13, 75.4523599 MHz
DECOUPLE H1, 300.0702830 MHz
Power 33 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.0 Hz
FI size 131072
Total time 22 hr, 57 min, 38 sec



S16. ¹³C NMR of compound 8f

M23-DMSO-H

Archive directory: /export/home/vnmri/vnmrsys/data
Sample directory: D05mm_test_12Mar2014-21:34:40
File: PROTON

Pulse Sequence: s2pu1

Solvent: DMSO
Temp. 30.9 C / 303.1 K

Relax. delay 1.000 sec

Pulse 45.0 degrees

Acq. time 4.853 sec

Width 6600.7 Hz

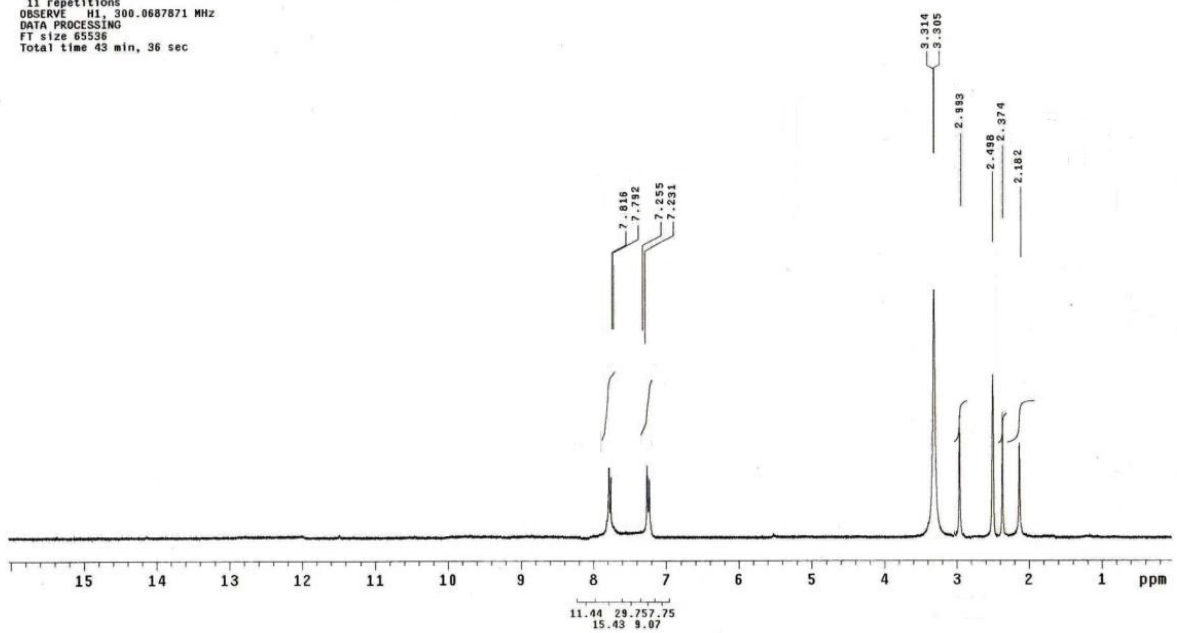
11 repetitions

OBSERVE H1, 300.0687871 MHz

DATA PROCESSING

FT size 65536

Total time 43 min, 36 sec



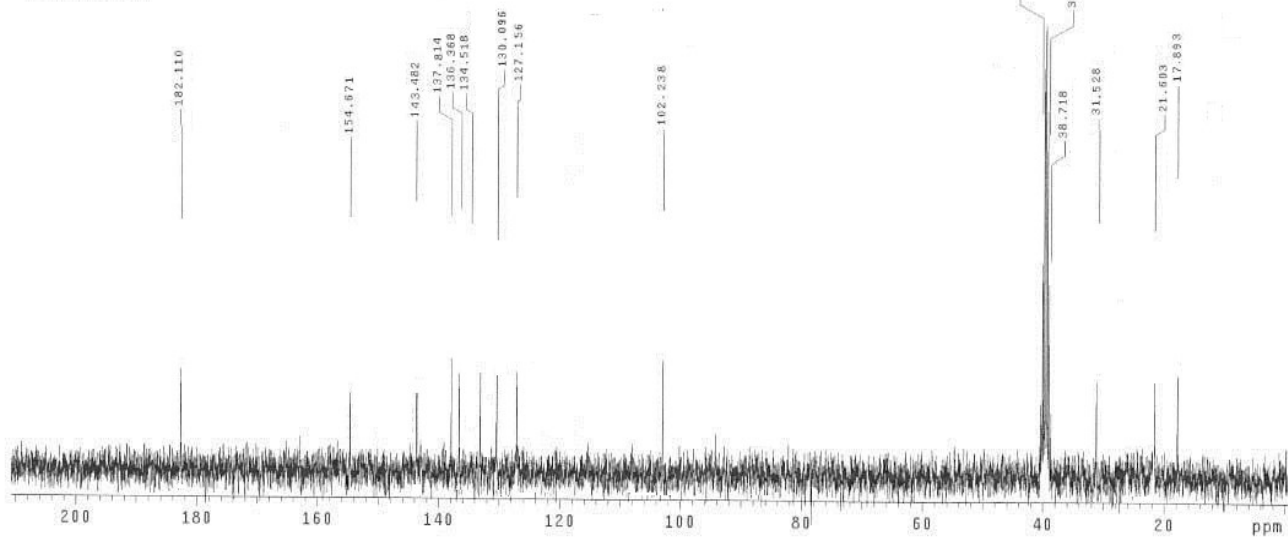
S17. ^1H NMR of compound **10a**

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Sample directory: D05mm_test_12Mar2014-21:34:40

Pulse Sequence: s2pu1
Solvent: DMSO
Temp. 40.0 C / 313.1 K

Mercury-300BB "NMR300"

Pulse 45.0 degrees
Acq. time 1.815 sec
Width 18761.7 Hz
344 repetitions
OBSERVE C13, 75.4523988 MHz
DECOUPLE H1, 300.6702830 MHz
Power 33 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.0 Hz
FT size 131072
Total time 32 hr, 53 min, 37 sec



S18. ¹³C NMR of compound 10a

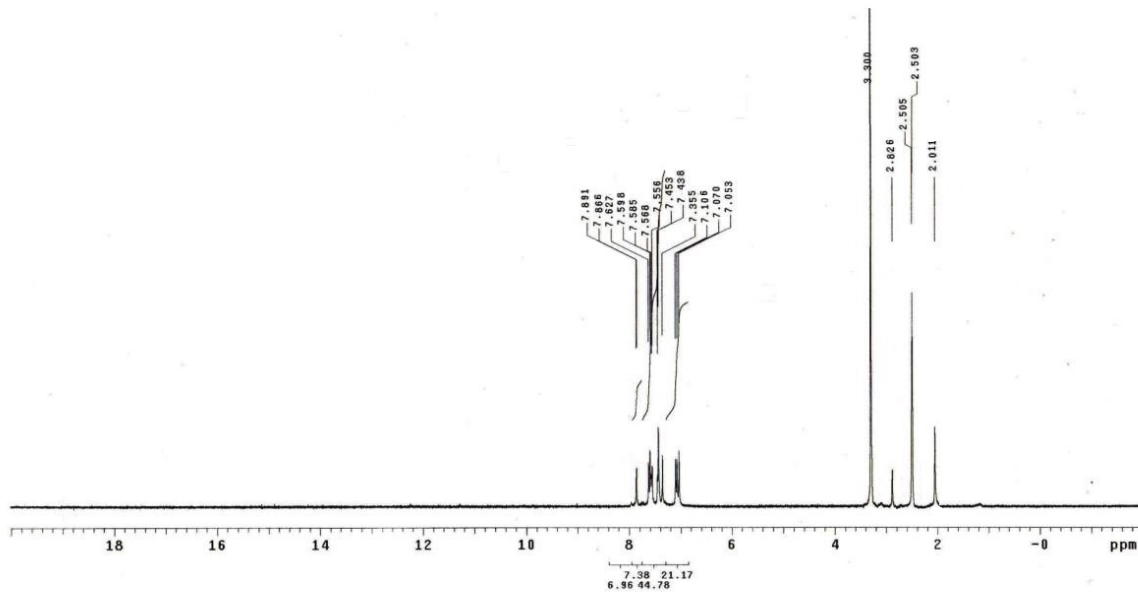
M17-DMSO-H

Archive directory: /export/home/vnmr1/vnmrSYS/data
Sample directory: DD5mm_test_12Mar2014-21:54:40
File: PROTON

Pulse Sequence: s2pu1
Solvent: DMSO
Temp. 30.0 C / 303.1 K

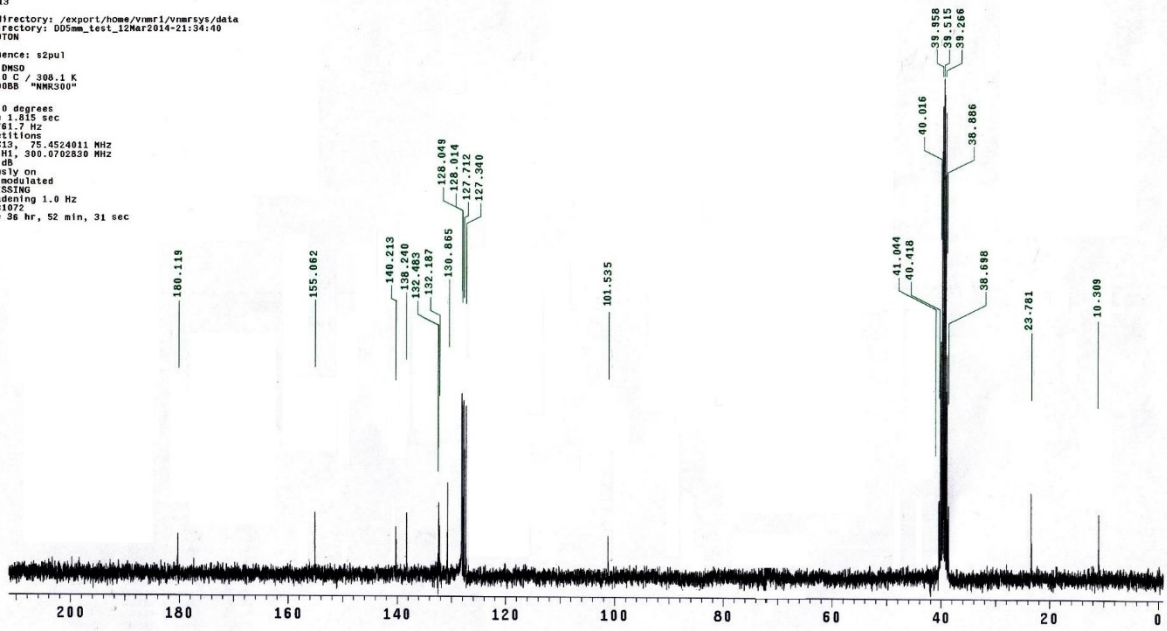
Relax. delay 1.000 sec
Pulse 45.0 degrees
Acq. time 4.853 sec
Width 6600.7 Hz

14 repetitions
OBSERVE N1, 300.0687671 MHz
DATA PROCESSING
F1 size 65536
Total time 45 min, 37 sec



S19. ¹H NMR of compound 10b

M8-DMSO-C13
Archive directory: /export/home/vnmr1/vnmrSYS/data
Sample directory: D05sm_test_12Mar2014-21:34:40
File: PROTON
Pulse Sequence: s2pul
Solvent: DMSO
Temp: 35.0 C / 308.1 K
Mercury-300SB "MMS300"
Pulse 45.0 degree
Acq. time 1.815 sec
Width 18761.7 Hz
2384 repetitions
OBSERVE C13, 75.4524011 MHz
DECOUPLE H1, 300.0702830 MHz
Power 33 dB
Continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.0 Hz
FT size 131072
Total time 36 hr, 52 min, 31 sec



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