SUPPORTING INFORMATION

Identification of 5-amino-1,3,4-thiadiazole appended isatins as bioactive small molecules with polypharmacological activities

Uzma Azam,[a] Waqar Ahmed Humayun,[b] Amrutha K. Avathan Veettil,[c,d,e] Yang Liu,[c,d,e] Oguz Hastürk,[c,d,e] Mao Jiang,[c,d,e] Sonja Sievers,[d,f] Peng Wu[c,d,e]* and Muhammad Moazzam Naseer[a,c]*

[a]*Department of Chemistry, Quaid-i-Azam University, Islamabad 45320, Pakistan*

[b]*Department of Medical Oncology & Radiotherapy, King Edward Medical University, Lahore 54000, Pakistan*

[c]*Chemical Genomics Centre, Max Planck Institute of Molecular Physiology, Dortmund, 44227 Germany*

[d]*Department of Chemical Biology, Max Planck Institute of Molecular Physiology, Dortmund, 44227 Germany*

[e]*Faculty of Chemistry and Chemical Biology, TU Dortmund University, Dortmund, 44227 Germany*

[f]*Compound Management and Screening Center, Dortmund, 44227 Germany*

*Corresponding authors: peng.wu@mpi-dortmund.mpg.de (P. Wu); moazzam@qau.edu.pk (M. M. Naseer)

Table of Contents

1 Materials and methods

All the reactions were performed in washed, rinsed and oven-dried apparatus. Freshly distilled and dried solvents were used, and the reactions were carried out in an inert atmosphere of nitrogen. Thin layer chromatography (TLC) was performed to monitor the progress of reactions. Merck silica gel-60F_{254} 0.2 nm pre-coated aluminium plates were used for TLC. Physical and spectroscopic techniques were used as a tool to characterize the synthesized compounds. The capillary tube method was used to measure the melting point of the synthesized compounds using a Gallenkamp melting point apparatus MPD350.BM3.5 (UK). Bruker Avance 300 MHz NMR spectrophotometer was used to record ^{1}H and 75 MHz to record ^{13}C NMR spectra of the synthesized compounds. The solvent used for recording ^{1}H and ^{13}C NMR spectra was DMSO-*d*6. The chemical shift values were calibrated with reference to the residual solvent signal. To visualize the UV active compounds on TLC plates, a UV lamp at 254 nm wavelength was used. To visualize the UV inactive compounds different staining agents were used.

2 Synthesis of 5-amino-1,3,4-thiadiazole-2-thiol (H1)

The reported procedure was followed for the synthesis of 5-amino-1,3,4-thiadiazole **(H1)**. [1] In a 100 mL round bottom flask thiosemicarbazide (54.87 mmol, 5g) was dissolved in anhydrous ethanol (15 mL), anhydrous sodium carbonate (27.43 mmol, 2.91 g) was added together with carbon disulfide (54.87 mmol, 4.18 g). The reaction mixture was allowed to stir on reflux for 8 h. The completion of the reaction was indicated by TLC (*n*-hexane: ethyl acetate / 1:1). The solvent was largely removed under reduced pressure by using a rotary evaporator. The residue was dissolved in water (50 mL) and acidified with concentrated hydrochloric acid 37 % (4.4 mL). The crude product was then recrystallized from methanol to give the pure compound. Appearance: Yellowish white crystals; Yield: 90 %; Melting Point: 234-236 °C (Lit. 232-234 °C) [2]; R_f: 0.52 (*n*-hexane: ethyl acetate / 1:1)

3 Synthesis of 1-(2-bromoethyl)indoline-2,3-dione (H2)

The 1-(2-bromoethyl)indoline-2,3-dione **(H2)** was synthesized by following a reported procedure.[3] In a 100 mL of RBF, isatin (33.98 mmol, 5 g) and potassium carbonate (50.97 mmol, 7.04 g) in DMF (5 mL) were allowed to stir at room temperature for 10 to 15 min. to generate isatin anion. To the stirred solution of 1,2-dibromoethane, the isatin anion was added dropwise and the reaction mixture was stirred at 50-60 ˚C. The progress of the reaction was monitored by TLC. After the completion of the reaction, the reaction mixture was added to ice-cold water (150 mL) and extracted with EtOAc (3×60 mL). The organic layers were combined

and dried over magnesium sulphate ($MgSO_4$) then the solvent is removed through rotavapor. The extracted product was then purified by column chromatography using *n*-hexane: ethyl acetate / 85:15 solvent system to get pure orange-red crystals of the pure product. Appearance: Orange-red crystals; Yield: 75 %; Melting Point: 126-128 ℃ (Lit. 128-130 ℃) [4]; R_f: 0.56 (*n*-hexane: ethyl acetate / 7:3)

4 General method for the synthesis of benzohydrazides (HZ1-20)

Reported method for benzohydrazides synthesis was adopted. [5] In 250 mL round bottom flask having (2.4 mol) dry distilled ethanol, 0.246 mol of appropriate substituted benzoic acid and 0.5 mL of sulfuric acid was added and subjected to reflux for 20-24 h. Excess of ethanol was removed by rotavapor, and mixture was cooled. Water (50 mL) was added into the mixture. The water layer was extracted thrice with EtOAc and organic layer was washed with $NaHCO_3$ solution to remove acid from solution and after removing solvent esters was collected. Further these esters (0.0066 mol) were refluxed with hydrazine (0.0466 mol) in ethanol to get desired benzohydrazides **(HZ1-20)**. Product was confirmed with TLC and melting point (compared with lit.) [5-8]

4.1 Benzohydrazide (HZ1)

Appearance: White crystalline solid; Yield: 60 %; Melting Point: 112-113 ^oC (Lit. 112-114 ^oC) [5]; R_f: 0.56 (*n*-hexane: ethyl acetate / 3:7)

4.2 4-Hydroxybenzohydrazide (HZ2)

Appearance: White powdered solid; Yield: 53 %; Melting Point: 263-265 ^oC (Lit. 264-266 ^oC) [5]; R_f: 0.48 (*n*-hexane: ethyl acetate / 3:7)

4.3 4-Methylbenzohydrazide (HZ3)

Appearance: White crystalline solid; Yield: 60 %; Melting Point: 117-119 ^oC (Lit. 116-118 ^oC) [5]; R_f: 0.52 (*n*-hexane: ethyl acetate / 3:7)

4.4 4-Chlorobenzohydrazide (HZ4)

Appearance: White crystalline solid; Yield: 58 %; Melting Point: 164-166 ^oC (Lit. 162-164 ^oC) [5]; R_f: 0.50 (*n*-hexane: ethyl acetate / 3:7)

4.5 4-Bromobenzohydrazide (HZ5)

Appearance: White powdered solid; Yield: 60 %; Melting Point: 168-170 ^oC (Lit. 167-169 ^oC) [5]; R_f: 0.51 (*n*-hexane: ethyl acetate / 3:7)

4.6 4-Nitrobenzohydrazide (HZ6)

Appearance: Light yellow powdered solid; Yield: 62%: Melting Point: 213-215 ^oC (Lit. 216-218 ^oC) [6]; R_f: 0.49 (*n*-hexane: ethyl acetate / 3:7)

4.7 3-Methoxybenzohydrazide (HZ7)

Appearance: White crystalline solid; Yield: 57 %; Melting Point: 92-94 ^{o}C (Lit. 93-95 ^{o}C) [6]; R_f: 0.53 (*n*-hexane: ethyl acetate / 3:7)

4.8 3-Methylbenzohydrazide (HZ8)

Appearance: White solid; Yield: 60 %; Melting Point: 96-98 ^{o}C (Lit. 97-99 ^{o}C) [6]; R_f: 0.54 (*n*-hexane: ethyl acetate / 3:7)

4.9 3-Bromobenzohydrazide (HZ9)

Appearance: White powered solid; Yield: 58 %; Melting Point: 159-161 ^{o}C (Lit. 157-159 ^{o}C) [5]; R_f: 0.47 (*n*-hexane: ethyl acetate / 3:7)

4.10 3-Fluorobenzohydrazide (HZ10)

Appearance: White crystalline solid; Yield: 60 %; Melting Point: 138-140 ^{o}C (Lit. 137-139 ^{o}C) [6]; R_f: 0.45 (*n*-hexane: ethyl acetate / 3:7)

4.11 3-Nitrobenzohydrazide (HZ11)

Appearance: Light yellow solid; Yield: 61 %; Melting Point: 151-153 ^{o}C (Lit. 153 ^{o}C) [6]; R_f: 0.50 (*n*-hexane: ethyl acetate / 3:7)

4.12 2-Methoxybenzohydrazide (HZ12)

Appearance: White powdered solid; Yield: 56 %; Melting Point: 81-83 ^{o}C (Lit. 78-80 ^{o}C) [6]; R_f: 0.51 (*n*-hexane: ethyl acetate / 3:7)

4.13 2-Methylbenzohydrazide (HZ13)

Appearance: White crystalline solid; Yield: 65 %; Melting Point: 120-122 ^{o}C (Lit. 122-124 ^{o}C) [5]; R_f: 0.55 (*n*-hexane: ethyl acetate / 3:7)

4.14 2-Chlorobenzohydrazide (HZ14)

Appearance: White crystalline solid; Yield: 57 %; Melting Point: 116-118 ^{o}C (Lit. 118-120 ^{o}C) [5]; R_f: 0.52 (*n*-hexane: ethyl acetate / 3:7)

4.15 2-Bromobenzohydrazide (HZ15)

Appearance: White powered solid; Yield: 60 %; Melting Point: 153-155 ^{o}C (Lit. 152-154 ^{o}C) [5]; R_f: 0.46 (*n*-hexane: ethyl acetate / 3:7)

4.16 2-Nitrobenzohydrazide (HZ16)

Appearance: Light yellow solid; Yield: 66 %; Melting Point: 119-121 ^{o}C (Lit. 121-123 ^{o}C) [6]; R_f: 0.48 (*n*-hexane: ethyl acetate / 3:7)

4.17 3,5-dimethoxybenzohydrazide (HZ17)

Appearance: White crystalline solid; Yield: 70 %; Melting Point: 143-144 ^{o}C (Lit. 145-147 ^{o}C) [6]; R_f: 0.44 (*n*-hexane: ethyl acetate / 3:7)

4.18 Nicotinic hydrazide (HZ18)

Appearance: White crystalline solid; Yield: 58 %; Melting Point: 160-162 ^{o}C (Lit. 159-161 ^{o}C)[7]; R_f: 0.39 (*n*-hexane: ethyl acetate / 3:7)

4.19 Isoniazid (HZ19)

Appearance: White crystalline solid; Yield: 60 %; Melting Point: 171-173 ^{o}C (Lit. 170-172 ^{o}C) [8]; R_f: 0.40 (*n*-hexane: ethyl acetate / 3:7)

4.20 2-Chloroisonicotinic hydrazide (HZ20)

Appearance: White powdered solid; Yield: 65 %; Melting Point: 166-168 ^{o}C (Lit. 168-170 ^{o}C) [8]; R_f: 0.41 (*n*-hexane: ethyl acetate / 3:7)

5 Biological methods

5.1 Cell painting assay

The cell painting assay was performed following the previously described methods.[9, 10] Initially, 5 µL U2OS medium was added to each well of a 384-well plate (PerkinElmer CellCarrier-384 Ultra). Subsequently, U2OS cells were seeded with a density of 1600 cells per well in 20 µL medium. The plate was incubated for 10 min at the ambient temperature, followed by an additional 4 h incubation (37 °C, 5% CO_2). Compound treatment was performed with the Echo 520 acoustic dispenser (Labcyte) at final concentrations of 10 µM, 30 µM or 50 µM. Incubation with the compound was performed for 20 h (37 °C, 5% CO_2). Subsequently, mitochondria were stained with Mito Tracker Deep Red (Thermo Fisher Scientific, Cat. No. M22426). The Mito Tracker Deep Red stock solution (1 mM) was diluted to a final concentration of 100 nM in prewarmed medium. The medium was removed from the plate leaving 10 µL residual volume and 25 µL of the Mito Tracker solution were added to each well. The plate was incubated for 30 min in darkness (37 °C, 5% CO_2). To fix the cells, 7 µL of 18.5 % formaldehyde in PBS were added, resulting in a final formaldehyde concentration of 3.7 %. Subsequently, the plate was incubated for another 20 min in darkness (RT) and washed three times with 70 µL of PBS. (Biotek Washer Elx405). Cells were permeabilized by the addition of 25 µL 0.1% Triton X-100 to each well, followed by 15 min incubation (RT) in darkness. The cells were washed three times with PBS leaving a final volume of 10 µL. To each well 25 µL of a staining solution was added, which contains 1% BSA, 5 µL/mL Phalloidin (Alexa594 conjugate, Thermo Fisher Scientific, A12381), 25 µg/mL Concanavalin A (Alexa488 conjugate, Thermo Fisher Scientific, Cat. No. C11252), 5 µg/mL Hoechst 33342 (Sigma, Cat. No. B2261-25mg), 1.5 µg/mL WGAAlexa594 conjugate (Thermo Fisher Scientific, Cat. No. W11262) and 1.5 µM SYTO 14 solution (Thermo Fisher Scientific, Cat. No. S7576). The plate

is incubated for 30 min (RT) in darkness and washed three times with 70 µL PBS. After the final washing step, the PBS was not aspirated. The plates were sealed and centrifuged for 1 min at 500 rpm. The plates were prepared in triplicates with shifted layouts to reduce plate effects and imaged using a Micro XL High-Content Screening System (Molecular Devices) in 5 channels (DAPI: Ex350-400/ Em410-480; FITC: Ex470-500/ Em510-540; Spectrum Gold: Ex520-545/ Em560-585; TxRed: Ex535-585/ Em600-650; Cy5: Ex605-650/ Em670-715) with 9 sites per well and 20x magnification (binning 2). The generated images were processed with the *CellProfiler* package (https://cellprofiler.org/, version 3.0.0) on a computing cluster of the Max Planck Society to extract 1716 cell features per microscope site. The data was then further aggregated as medians per well (9 sites per 1 well), then over the three replicates. Further analysis was performed with custom *Python* (https://www.python.org/) scripts using the *Pandas* (https://pandas.pydata.org/) and *Dask* (https://dask.org/) data processing libraries as well as the *ScientificPython* (https://scipy.org/) package (separate publication to follow).

From the total set of 1716 features, a subset of highly reproducible and robust features was determined using the procedure described by Woehrmann *et al*.[11] Two biological repeats of one plate containing reference compounds were analyzed. For every feature, its full profile over each whole plate was calculated. If the profiles from the two repeats showed a similarity of no less than 0.8 (see below), the feature was added to the set. This procedure was only performed once and resulted in a set of 579 robust features out of the total of 1716 that were used for all further analyses. The phenotypic profiles were compiled from the Z-scores of all individual cellular features, where the Z-score is a measure of how far away a data point is from a median value. Specifically, Z-scores of test compounds were calculated relative to the Median of DMSO controls. The phenotypic compound profile is then determined as the list of Z-scores of all features for one compound. In addition to the phenotypic profile, an induction value was determined for each compound as the fraction of significantly changed features, in percent. Similarities of phenotypic profiles (termed *Biosimilarity*) were calculated from the correlation distances (CD) between two profiles (https://docs.scipy.org/doc/scipy/reference/generated/scipy.spatial.distance.correlation.html). Biosimilarity is then defined as: $Biosimilarity = 1 - CD$. Biosimilarity values smaller than 0 are set to 0 and the Biosimilarity is expressed in percent (0-100).

5.2 MTT assay

Cancer cells (obtained from DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were seeded in 96-well plates with 2000 cells/well and were incubated overnight. The medium was discarded and the media with compounds were added to the 96-well plates. Data were normalized to the medium with 0.5% DMSO. The cells were then incubated for 72 h. MTT solution (5 mg/mL in DPBS, 20 µL) was added per well in the dark and incubated at 37 °C for 4 h. Then the old medium with the MTT solution was removed, followed by the addition of 150 µL DMSO per well. The absorbance of the well was then measured at 492 nm via a TECAN plate reader.

6 Supplementary Figures

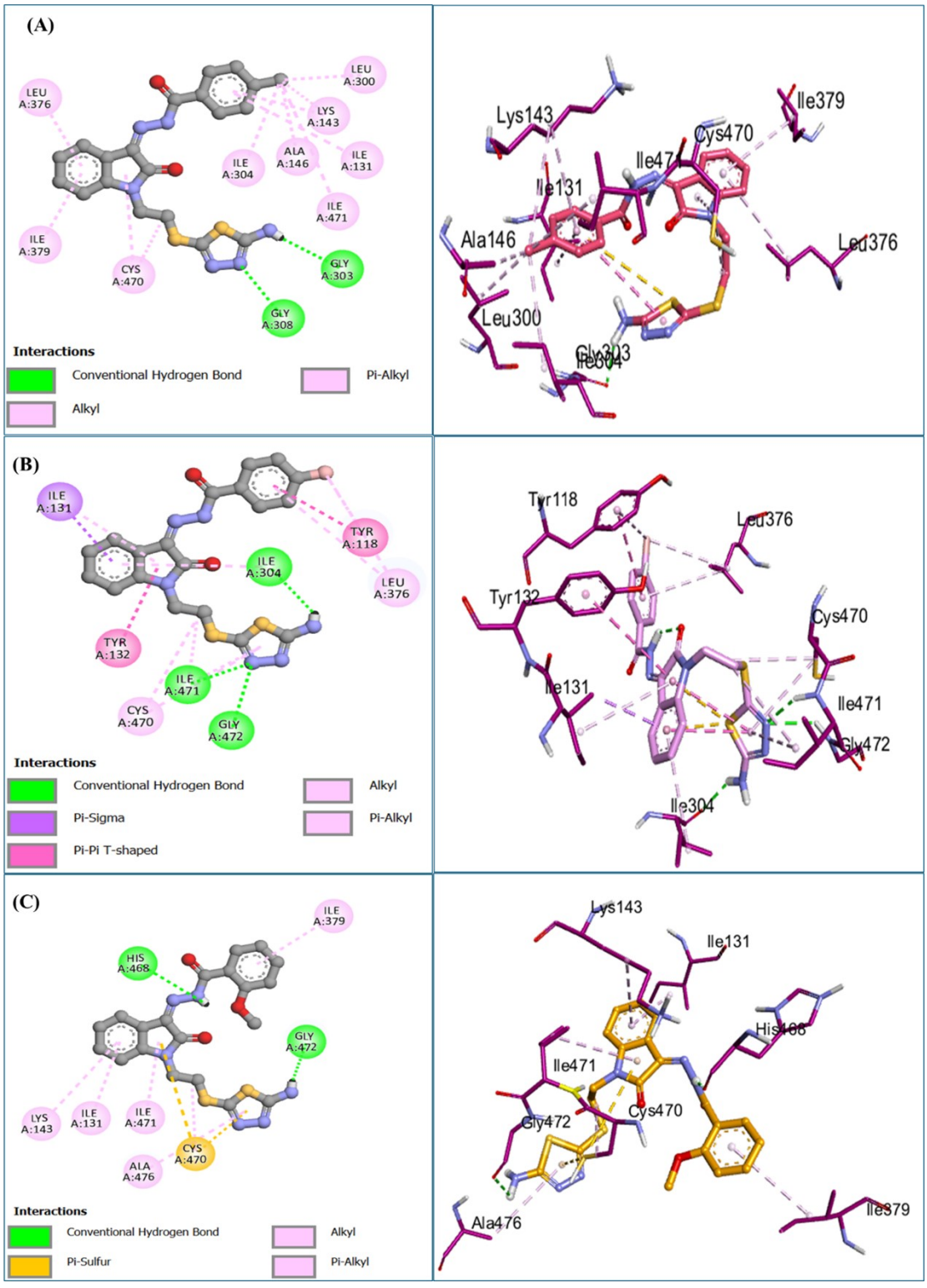


Figure S1: 2D and 3D protein ligand interactions of (A) **UZ-3**; (B) **UZ-5**; (C) **UZ-12** in the binding pocket of the protein (5V5Z).

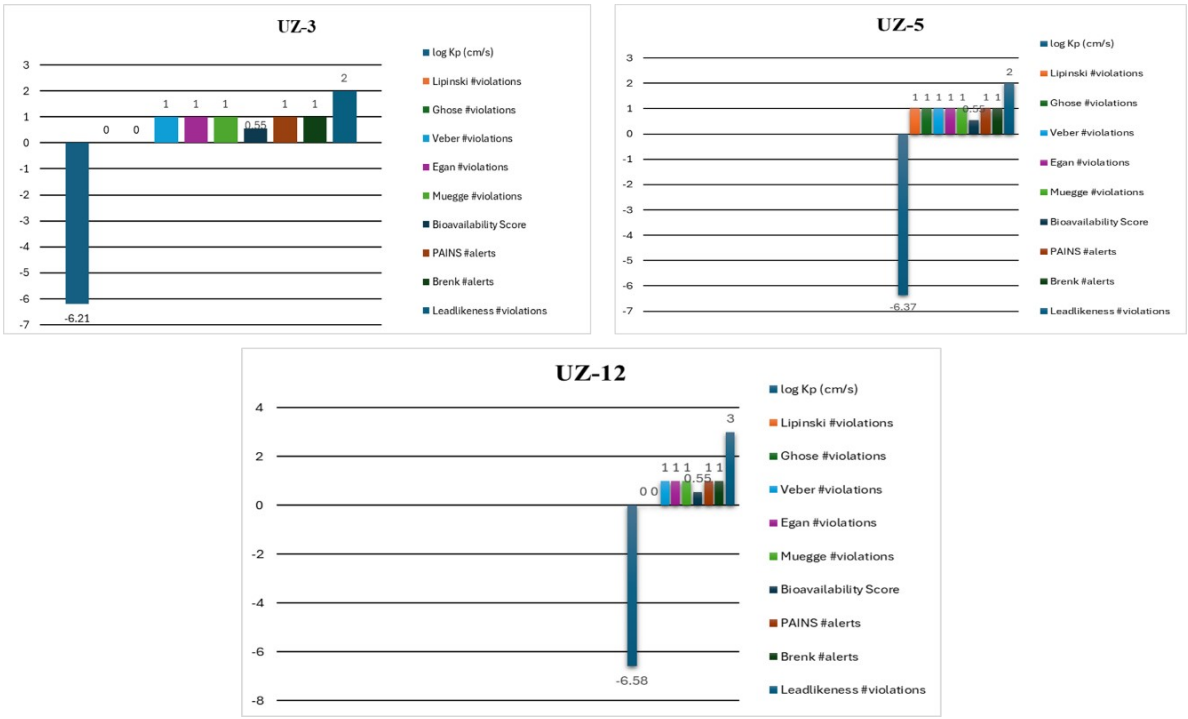


Figure S2: Representation of ADME predications for compounds **UZ-3, UZ-5** & **UZ-12**

References

1. M. Yusuf, R. A. Khan and B. Ahmed, *Bioorg. Med. Chem.*, 2008, **16**, 8029-8034.

2. S. H. Z. Al-Abdeen and A. J. Qasir, *Iraqi J. Pharm. Sci. (P-ISSN 1683-3597 E-ISSN 2521-3512)*, 2014, **23**, 42-50.

3. O. Radul, G. Zhungietu, M. Rekhter and S. Bukhanyuk, *Chem. Heterocycle. Cmpd.*, 1983, **19**, 286-288.

4. O. M. Radul, G. I. Zhungietu, M. A. Rekhter and S. M. Bukhanyuk, *Chem. Heterocycle. Compd.*, 1983, **19**, 286-288.

5. D. Kumari and H. Bansal, *Pharma Innov. J*, 2018, **7**, 543-550.

6. S. Veeramanikandan and H. Benita Sherine, *Der Pharma Chem*, 2015, **7**, 70-84.

7. W. M. Eldehna, M. Fares, M. M. Abdel-Aziz and H. A. Abdel-Aziz, *Molecules*, 2015, **20**, 8800-8815.

8. V. Judge, B. Narasimhan, M. Ahuja, D. Sriram, P. Yogeeswari, E. De Clercq, C. Pannecouque and J. Balzarini, *Med. Chem. Res.*, 2012, **21**, 1451-1470.

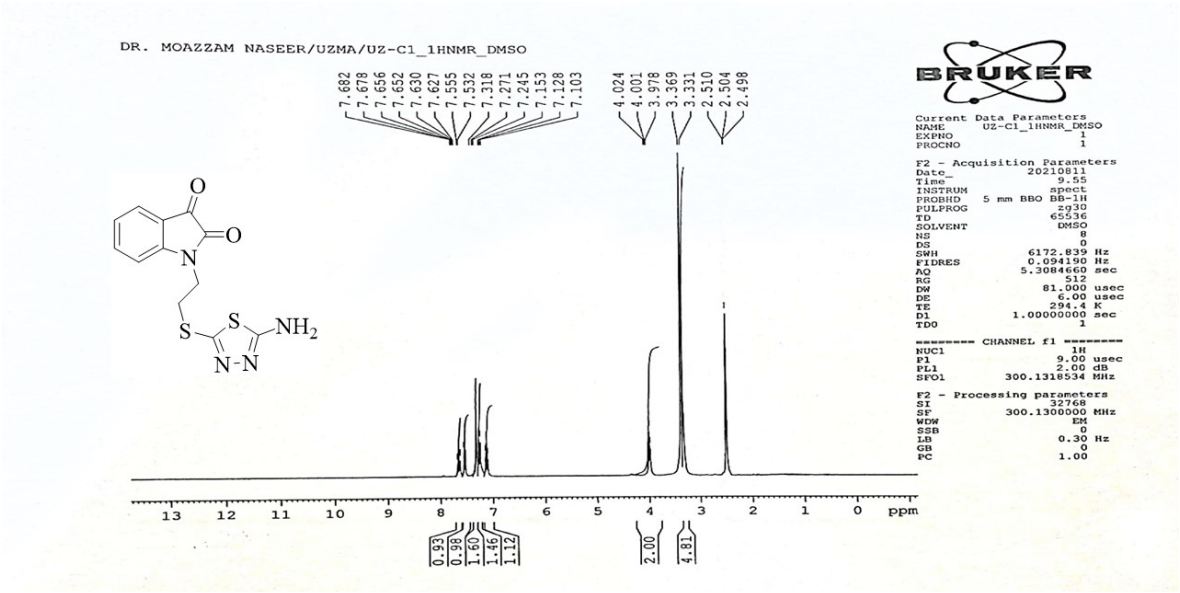
9. J. Xie, A. Pahl, A. Krzyzanowski, A. Krupp, J. Liu, S. Koska, B. Schölermann, R. Zhang, J. Bonowski, S. Sievers, C. Strohmann, S. Ziegler, M. Grigalunas and H. Waldmann, *Angew. Chem. Int. Ed.*, 2023, e202310222.

10. J. Liu, S. Mallick, Y. Xie, C. Grassin, B. Lucas, B. Schölermann, A. Pahl, R. Scheel, C. Strohmann, C. Protzel, T. Berg, C. Merten, S. Ziegler and H. Waldmann, *Angew. Chem. Int. Ed.*, 2023, **62**, e202301955.

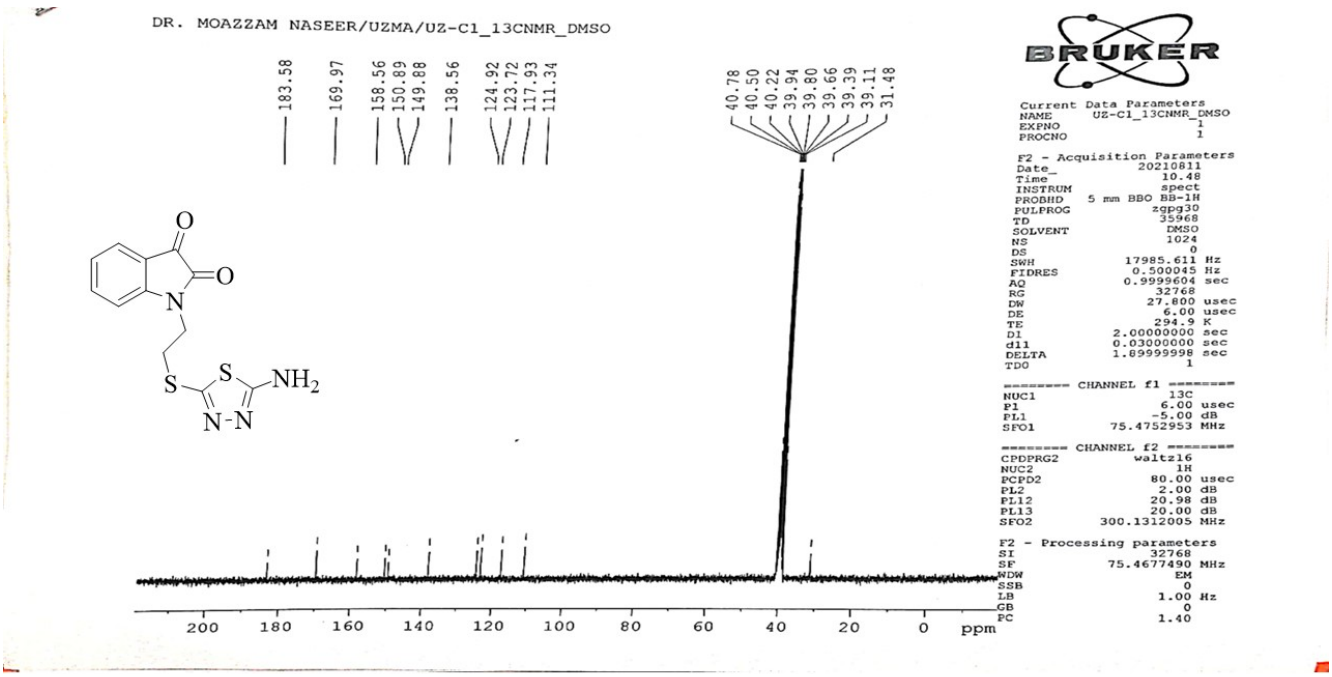
11. M. H. Woehrmann, W. M. Bray, J. K. Durbin, S. C. Nisam, A. K. Michael, E. Glassey, J. M. Stuart and R. S. Lokey, *Mol. BioSyst.*, 2013, **9**, 2604-2617.

Copies of NMR spectra of compounds (UZ1-20)

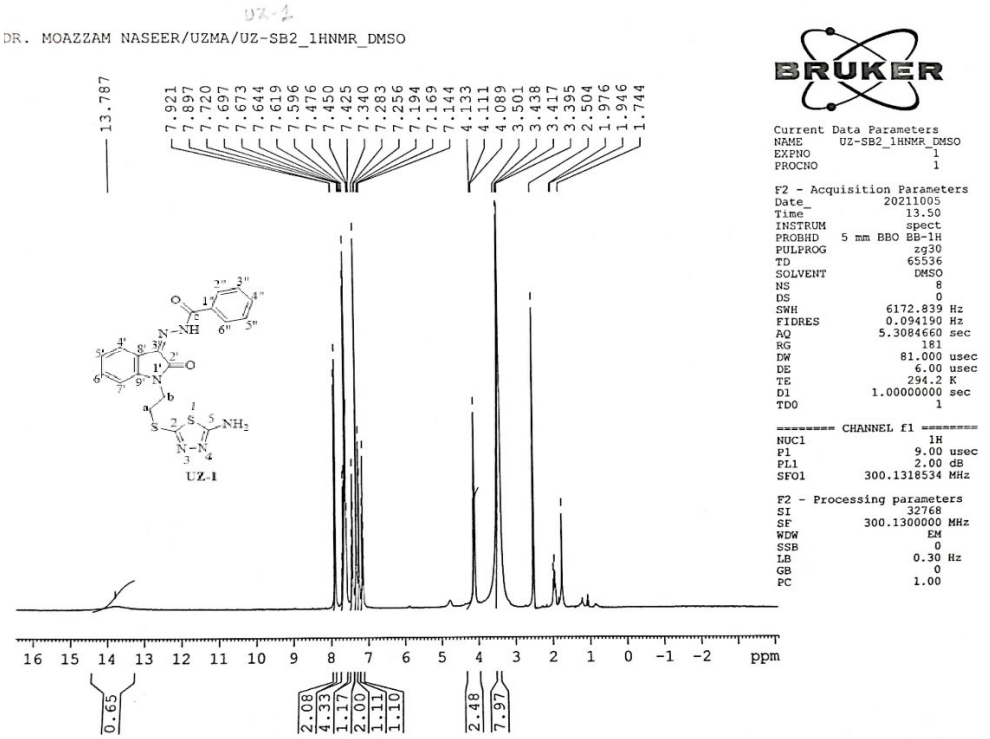
^{1}H NMR spectrum of compound UZ-C1



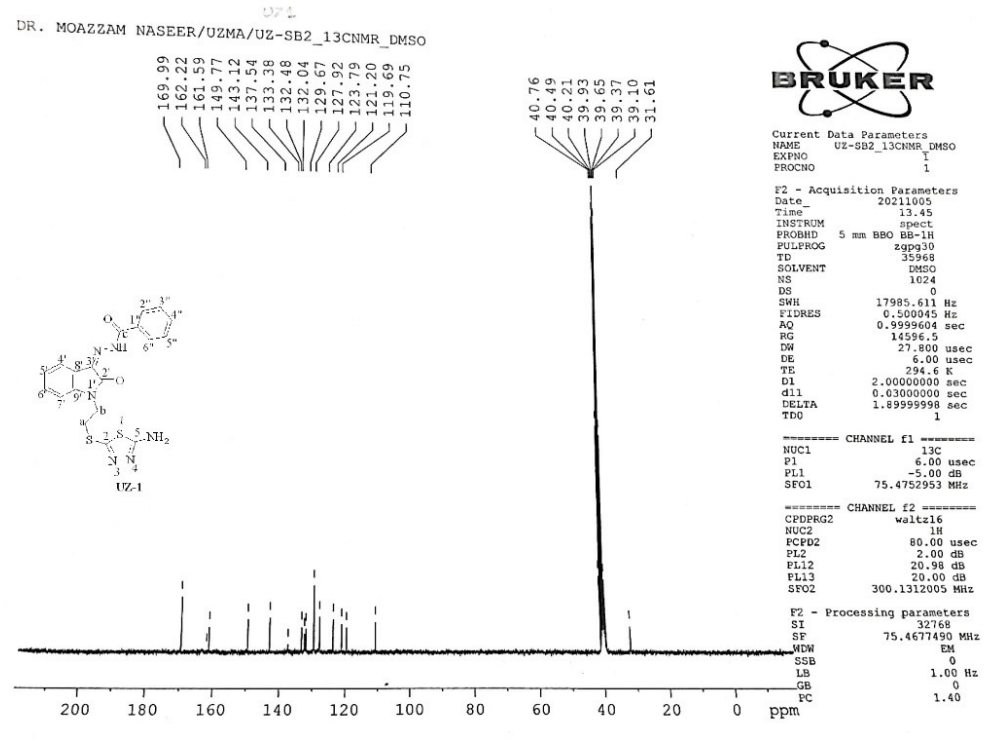
^{13}C NMR spectrum of compound UZ-C1



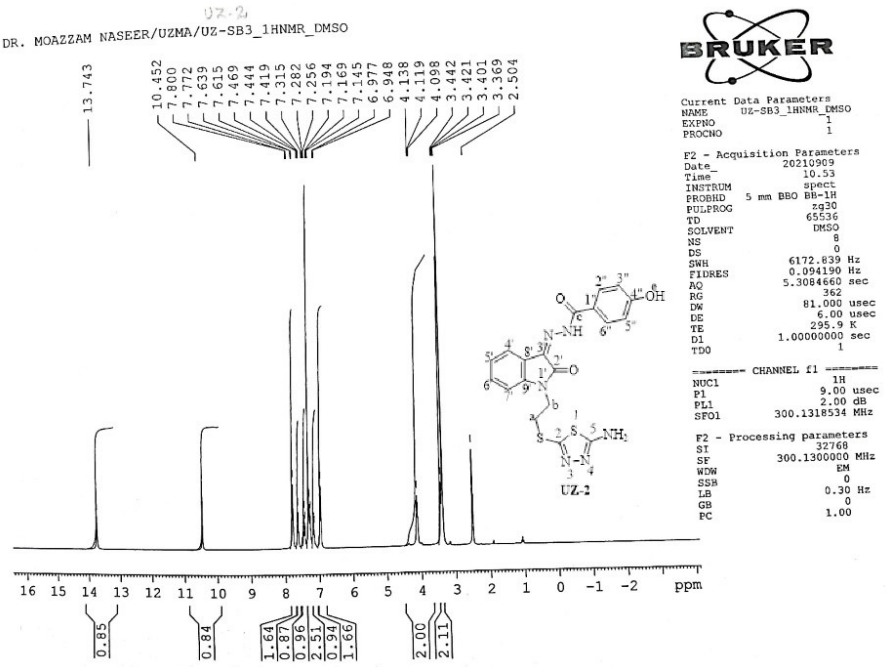
^{1}H NMR spectrum of compound UZ-1



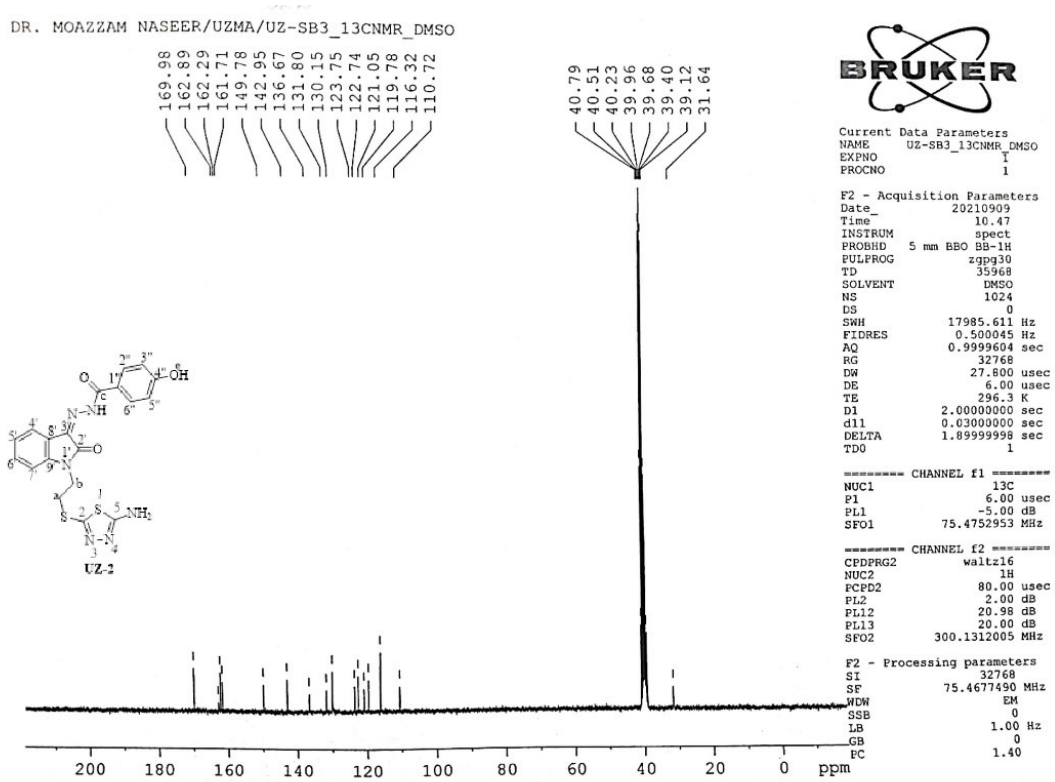
^{13}C NMR spectrum of compound UZ-1



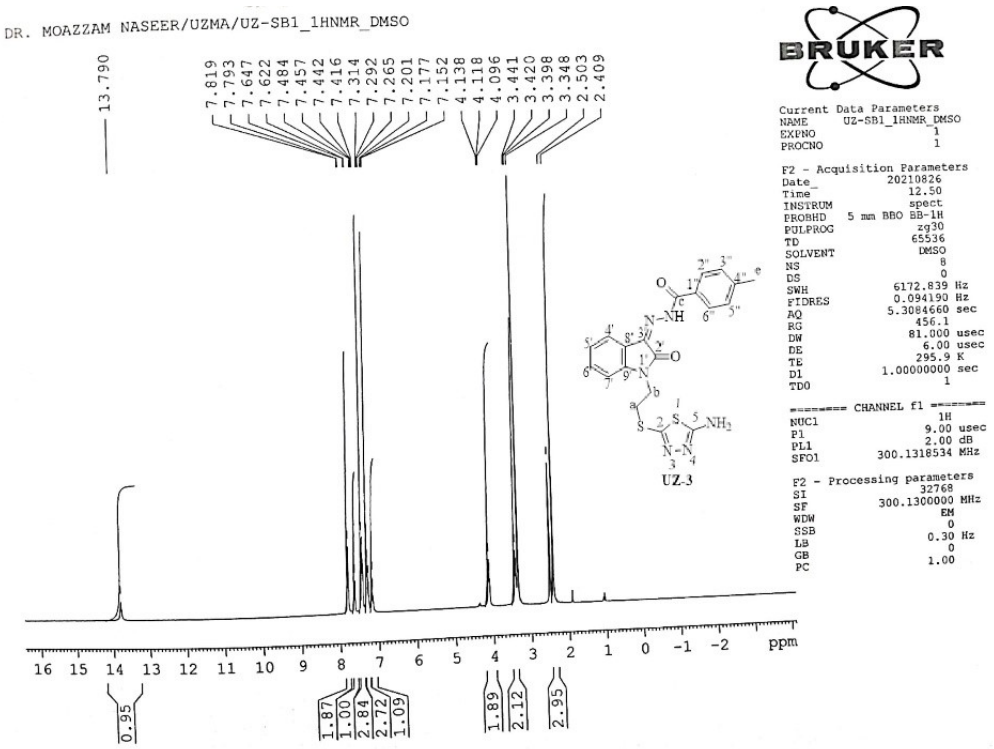
^{1}H NMR spectrum of compound UZ-2



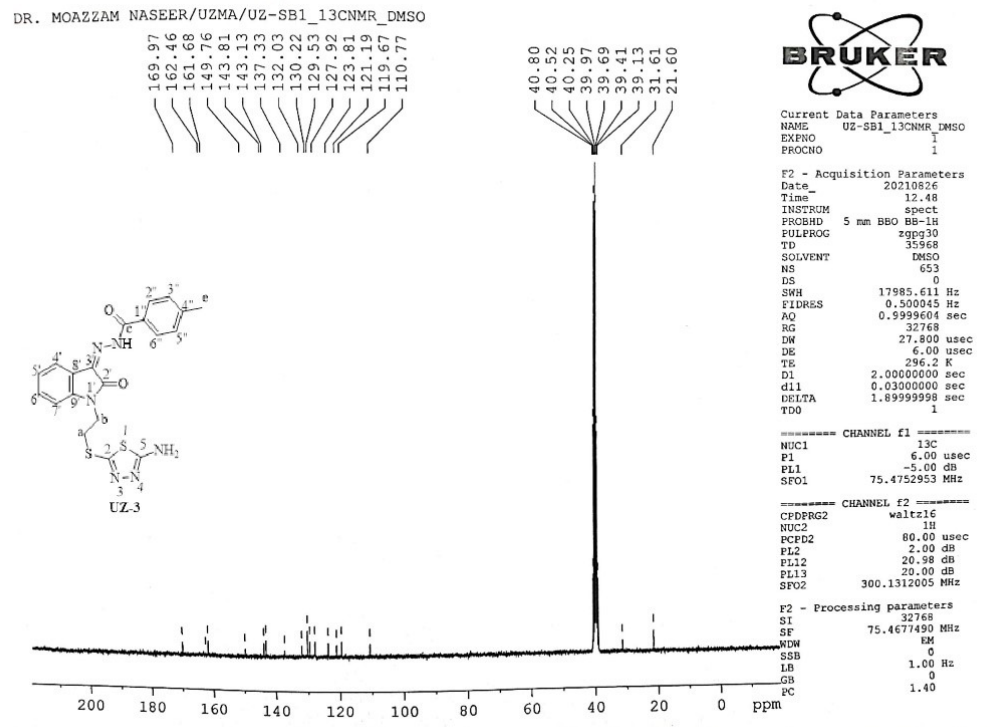
^{13}C NMR spectrum of compound UZ-2



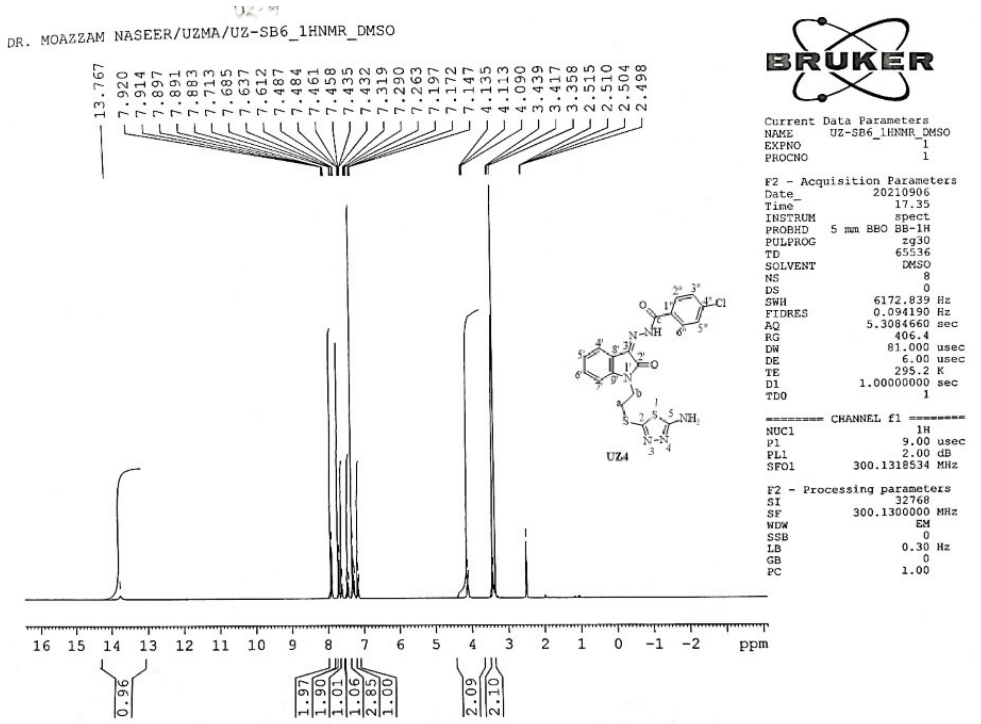
^{1}H NMR spectrum of compound UZ-3



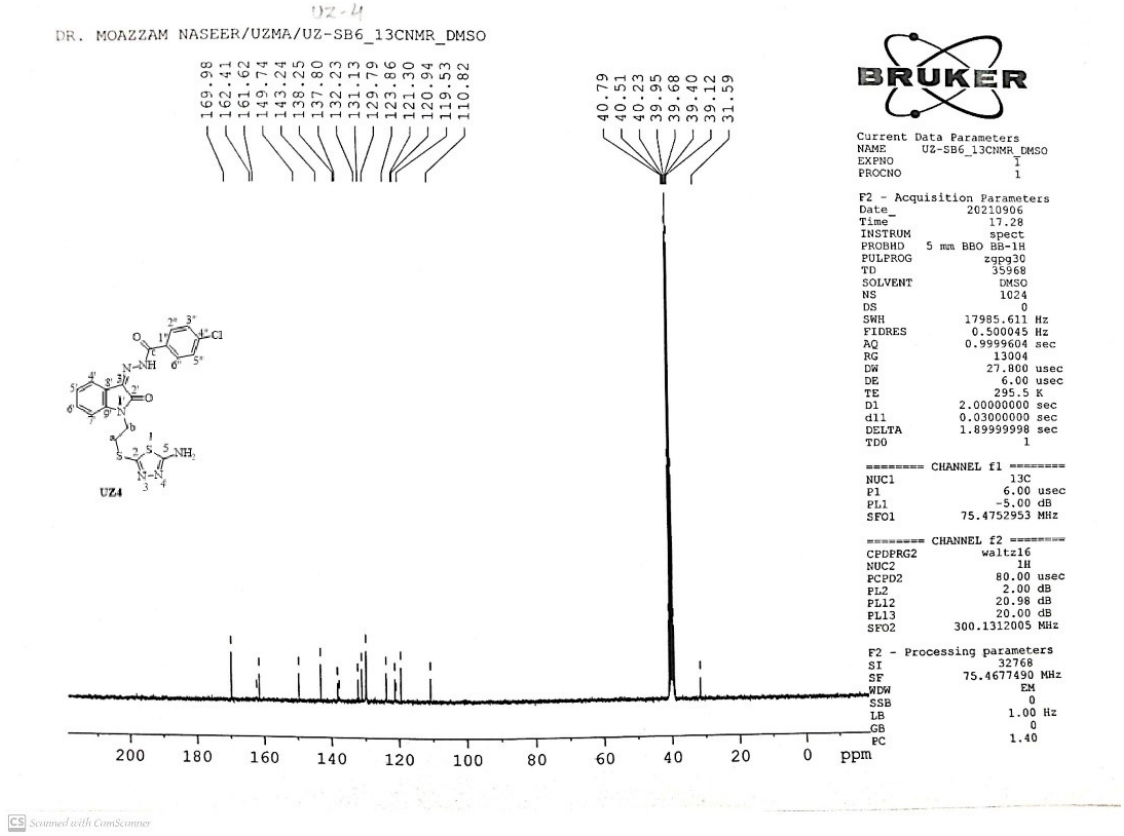
^{13}C NMR spectrum of compound UZ-3



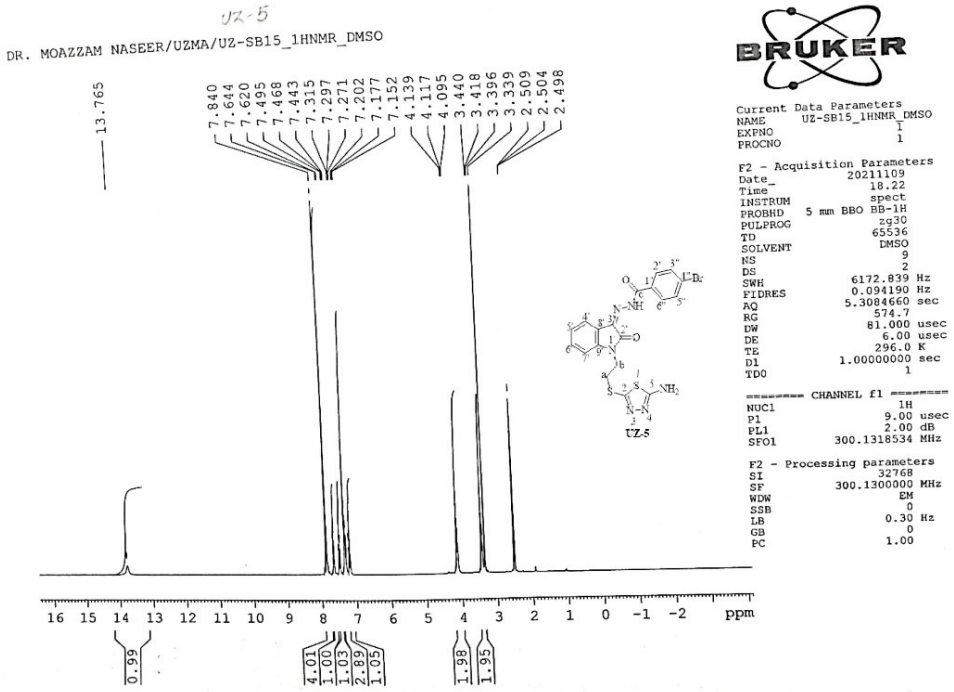
^{1}H NMR spectrum of compound UZ-4



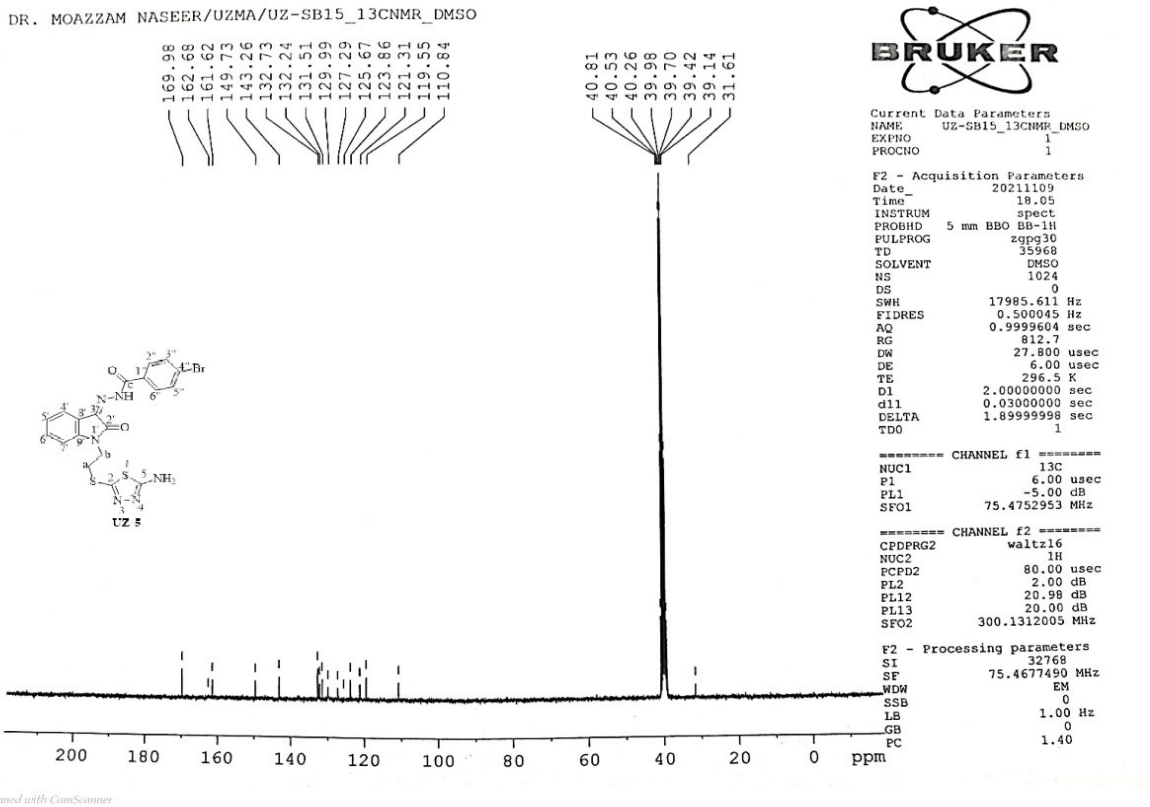
^{13}C NMR spectrum of compound UZ-4



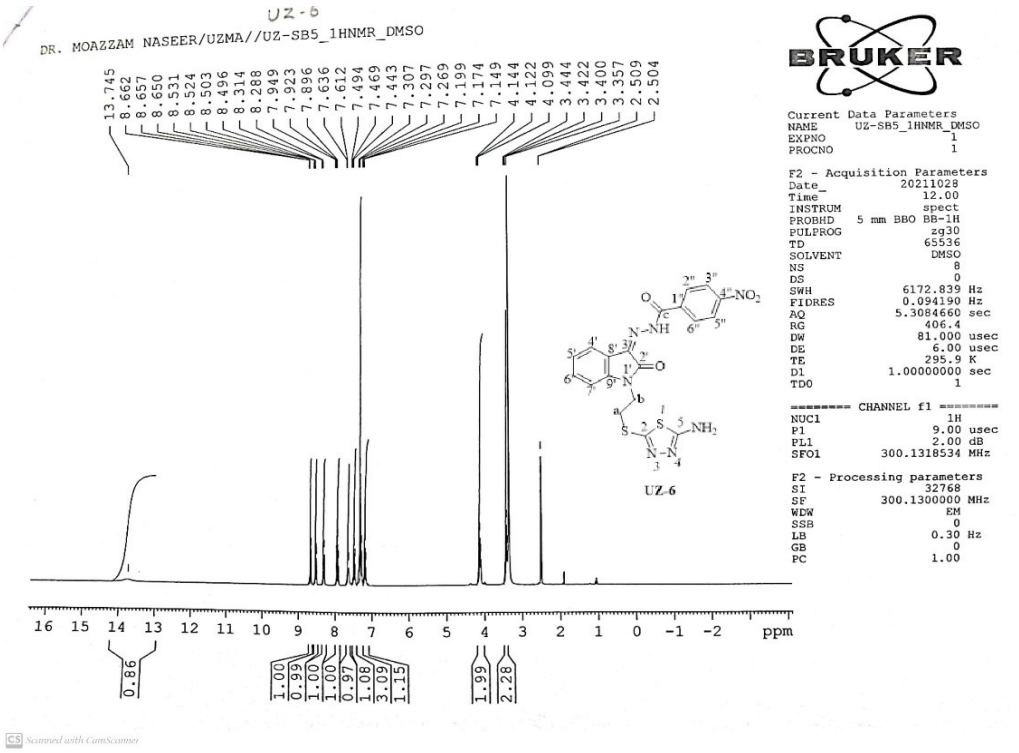
^{1}H NMR spectrum of compound UZ-5



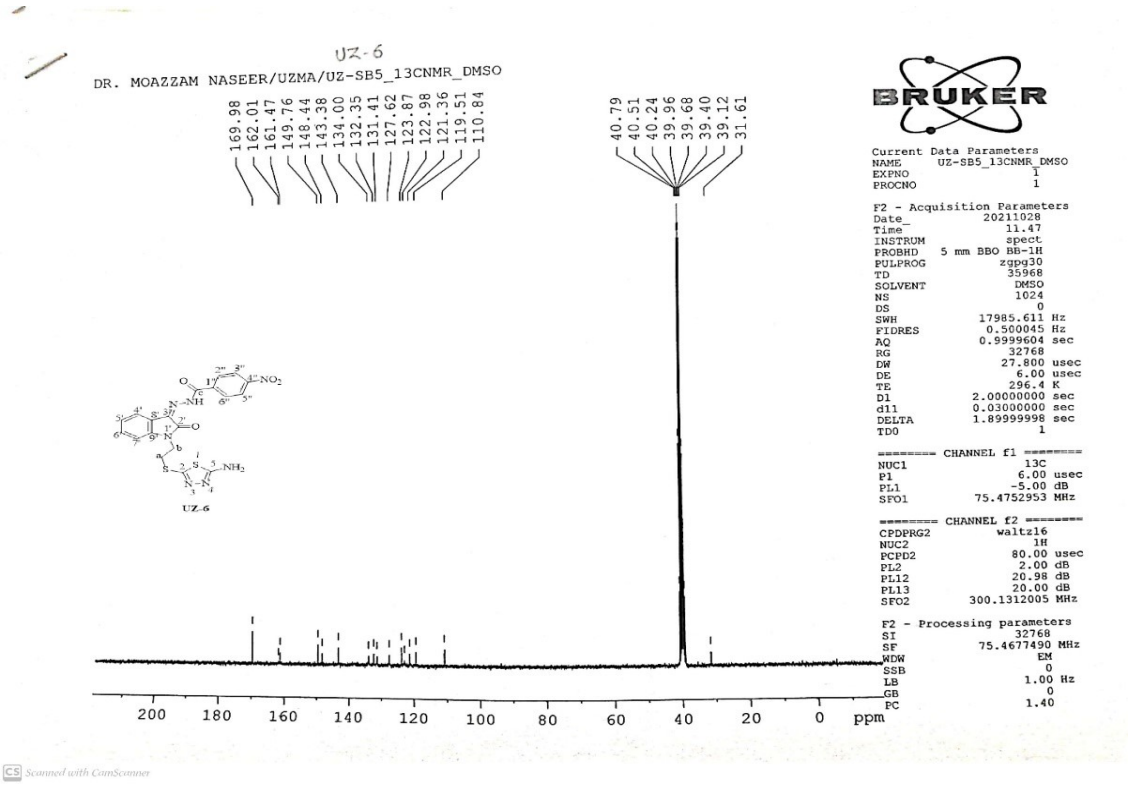
^{13}C NMR spectrum of compound UZ-5



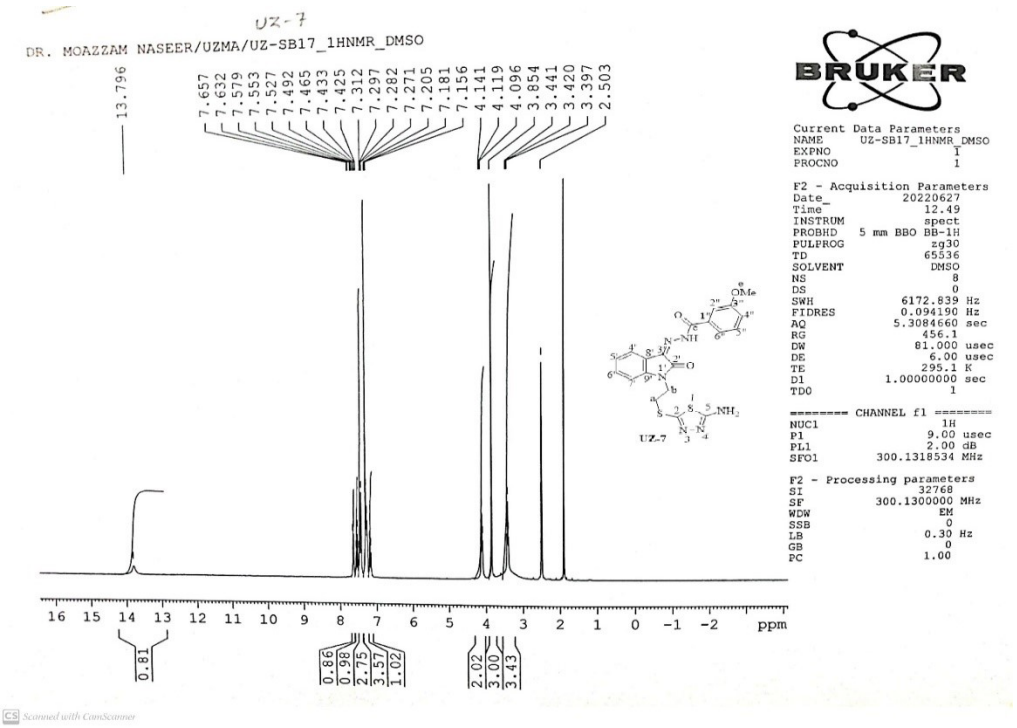
^{1}H NMR spectrum of compound UZ-6



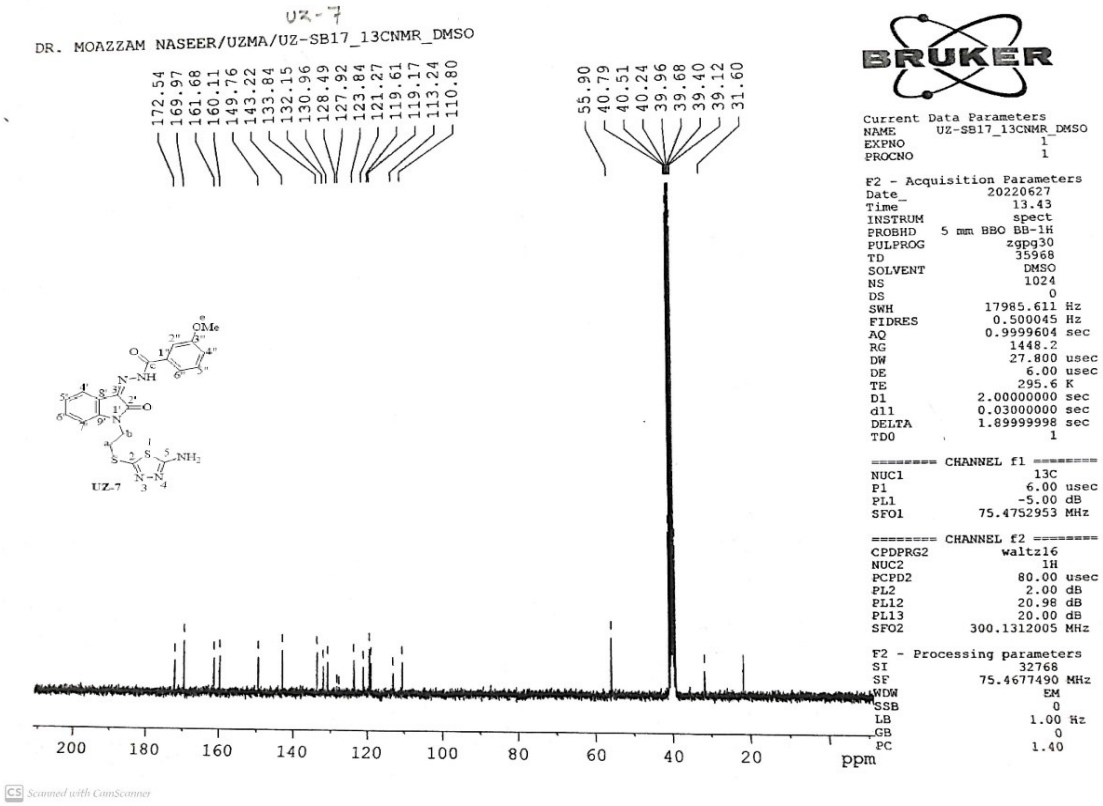
^{13}C NMR spectrum of compound UZ-6



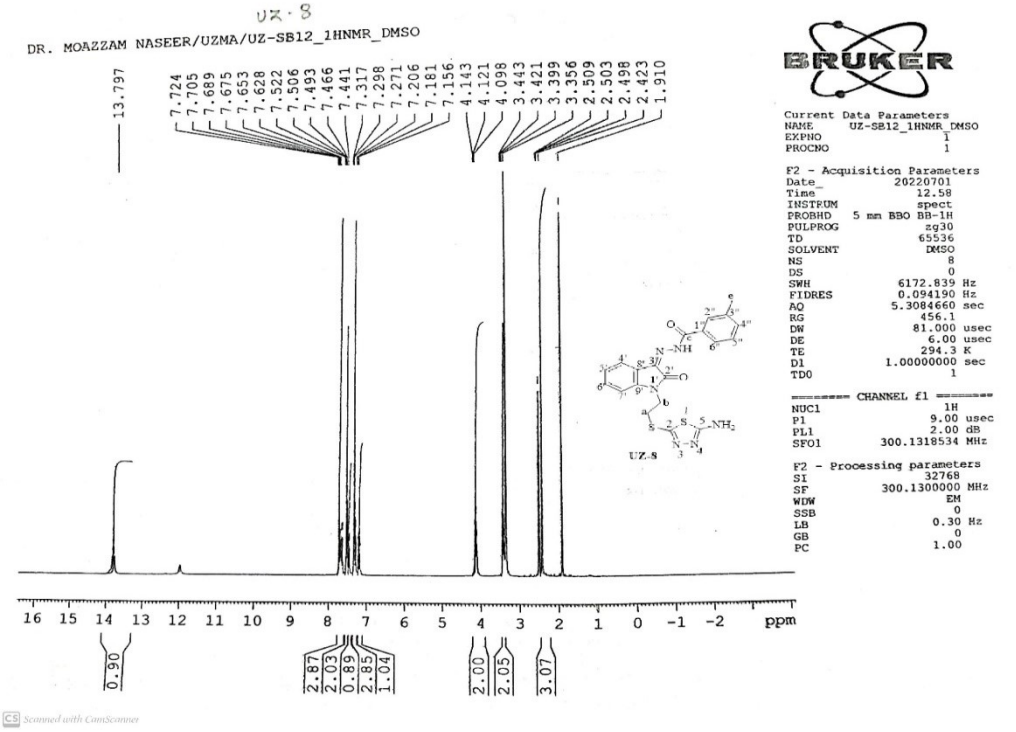
^{1}H NMR spectrum of compound UZ-7



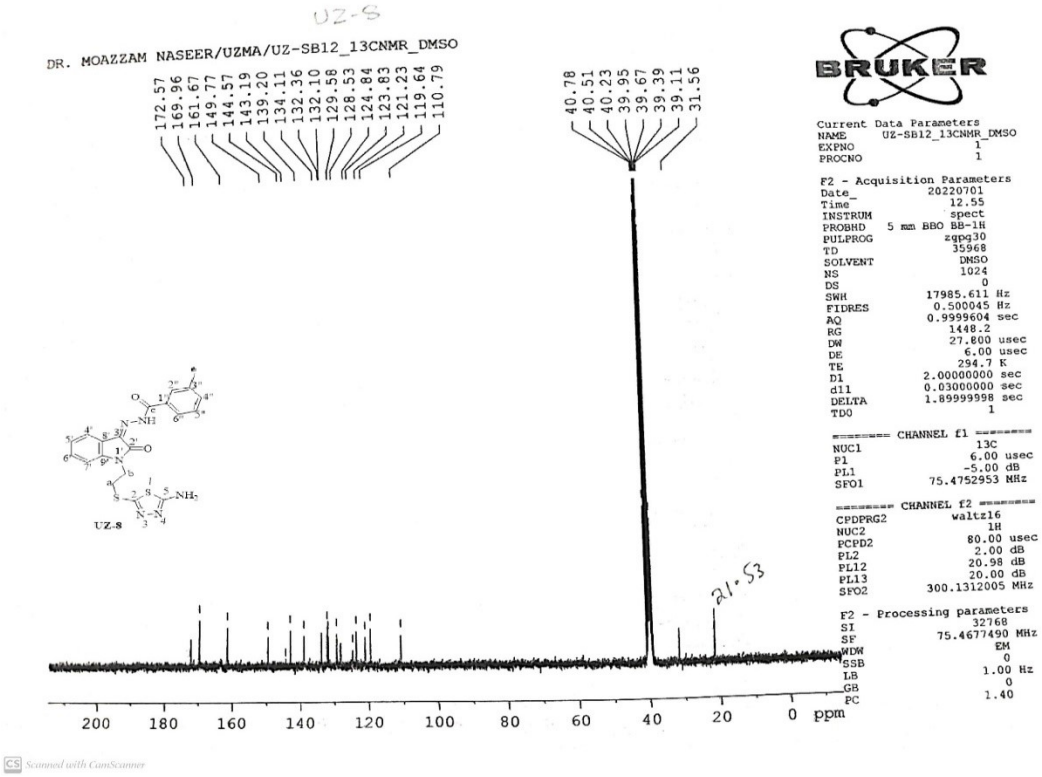
^{13}C NMR spectrum of compound UZ-7



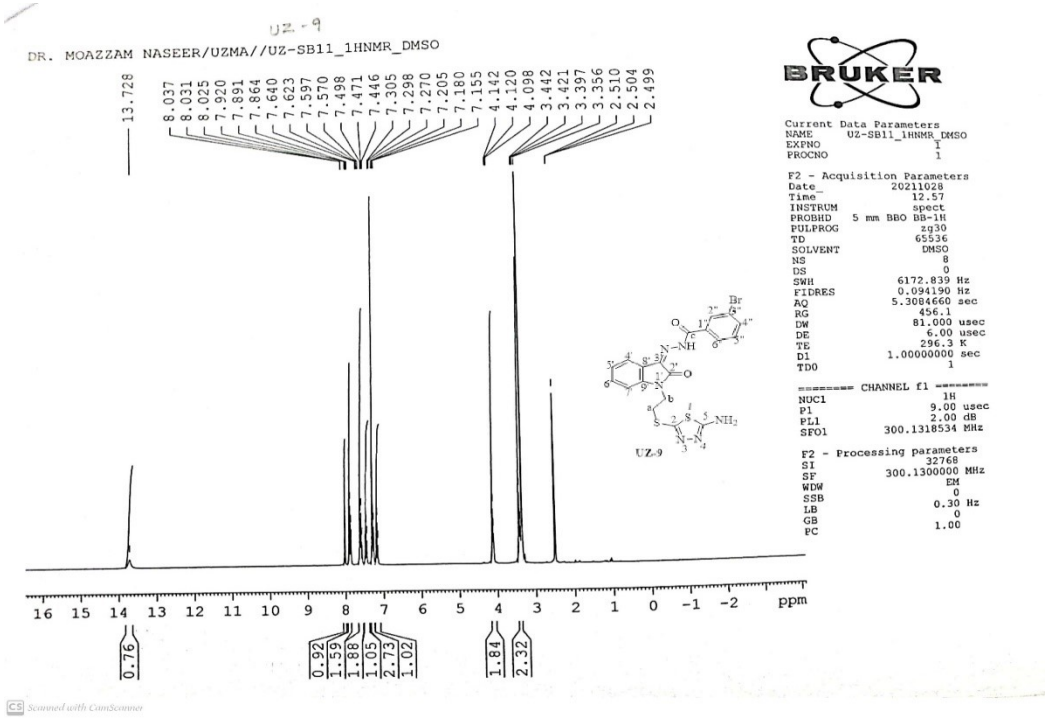
^{1}H NMR spectrum of compound UZ-8



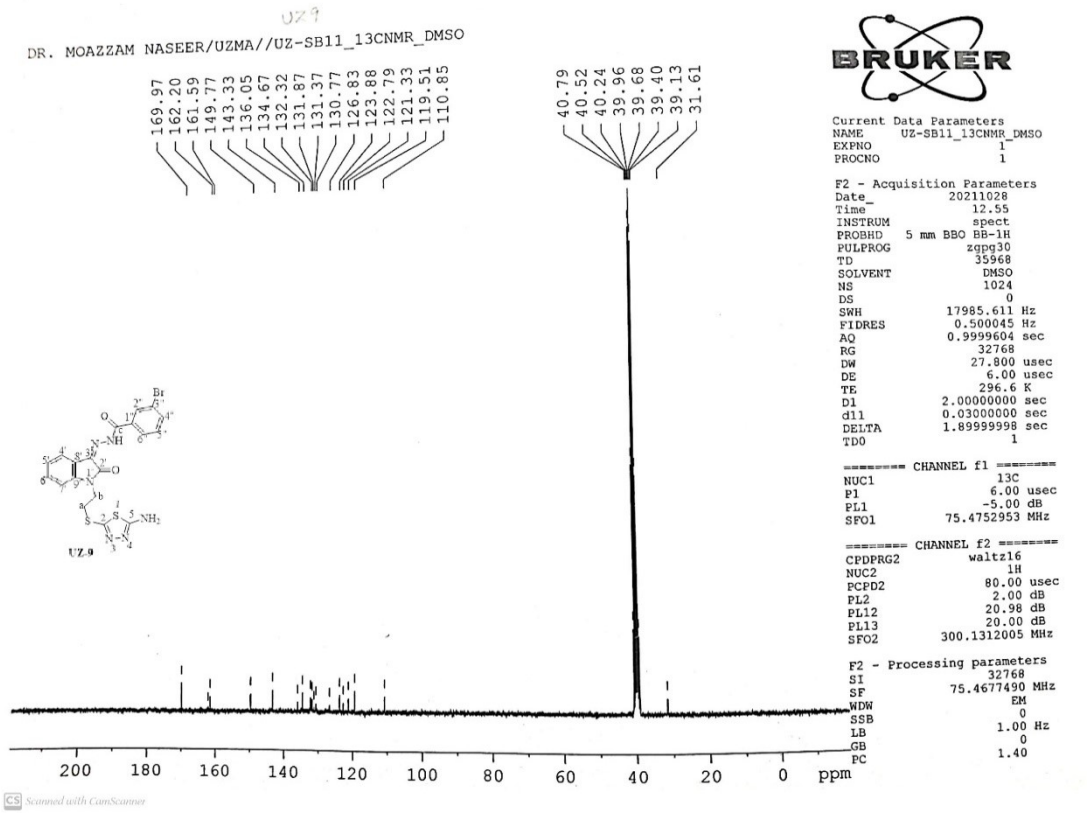
^{13}C NMR spectrum of compound UZ-8



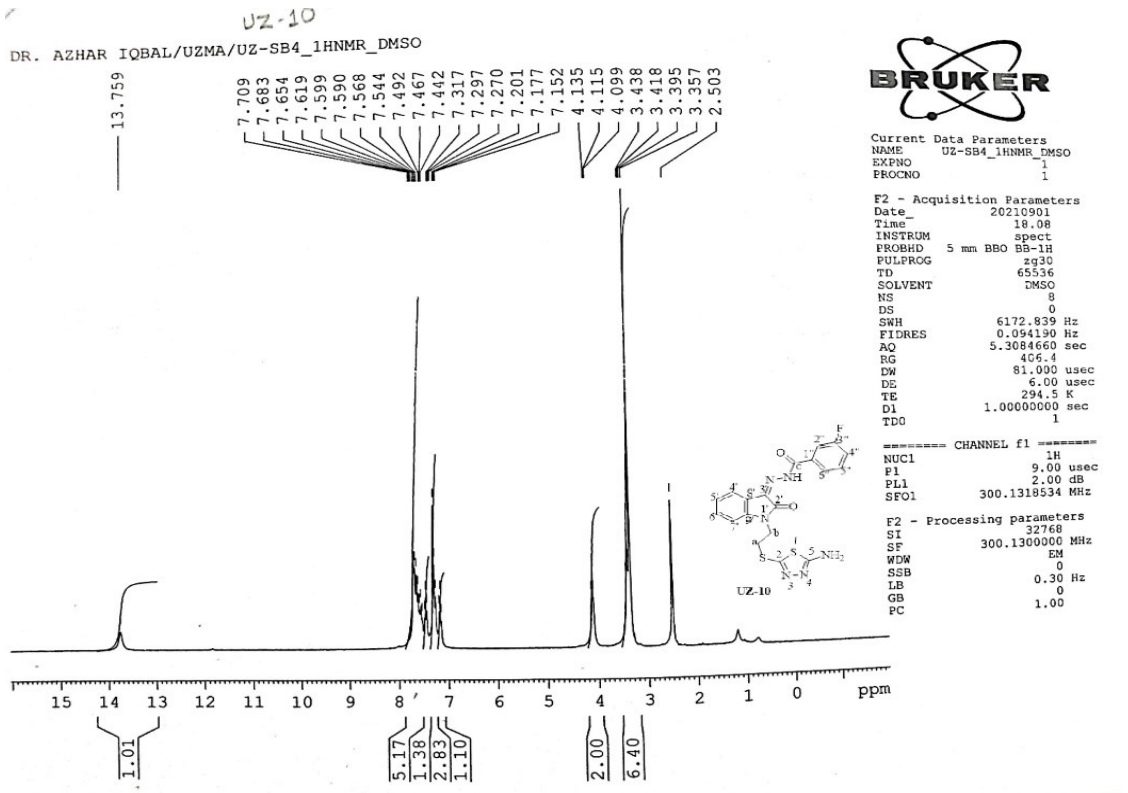
^{1}H NMR spectrum of compound UZ-9



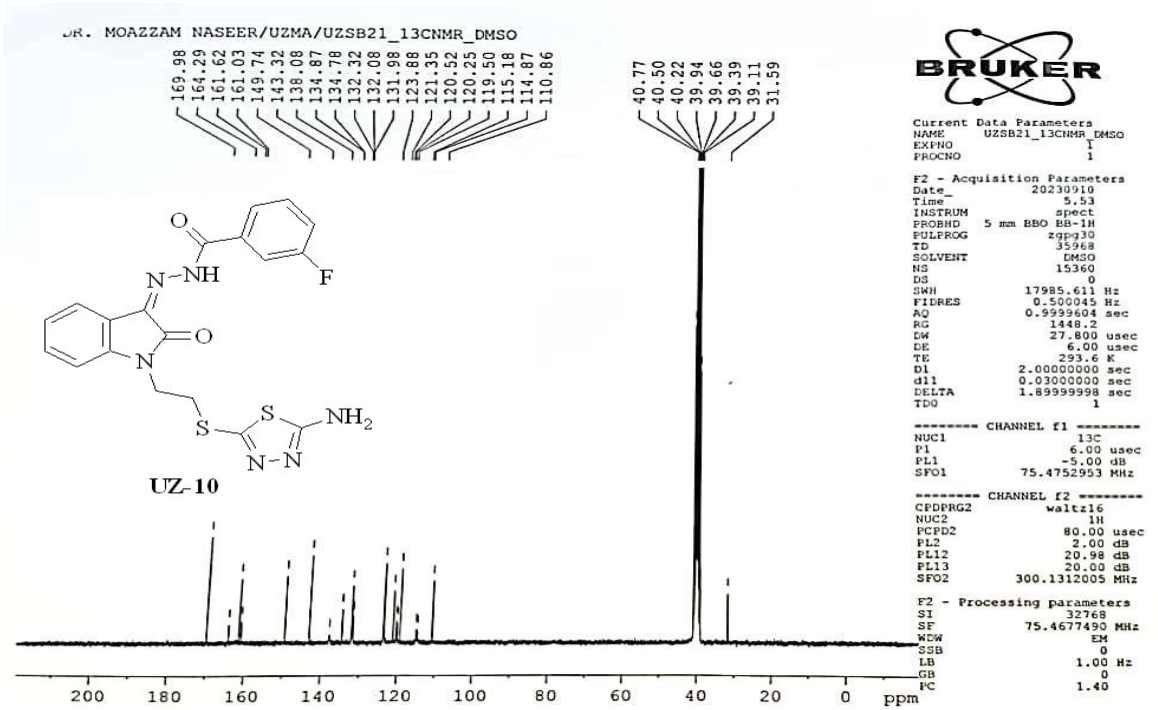
^{13}C NMR spectrum of compound UZ-9



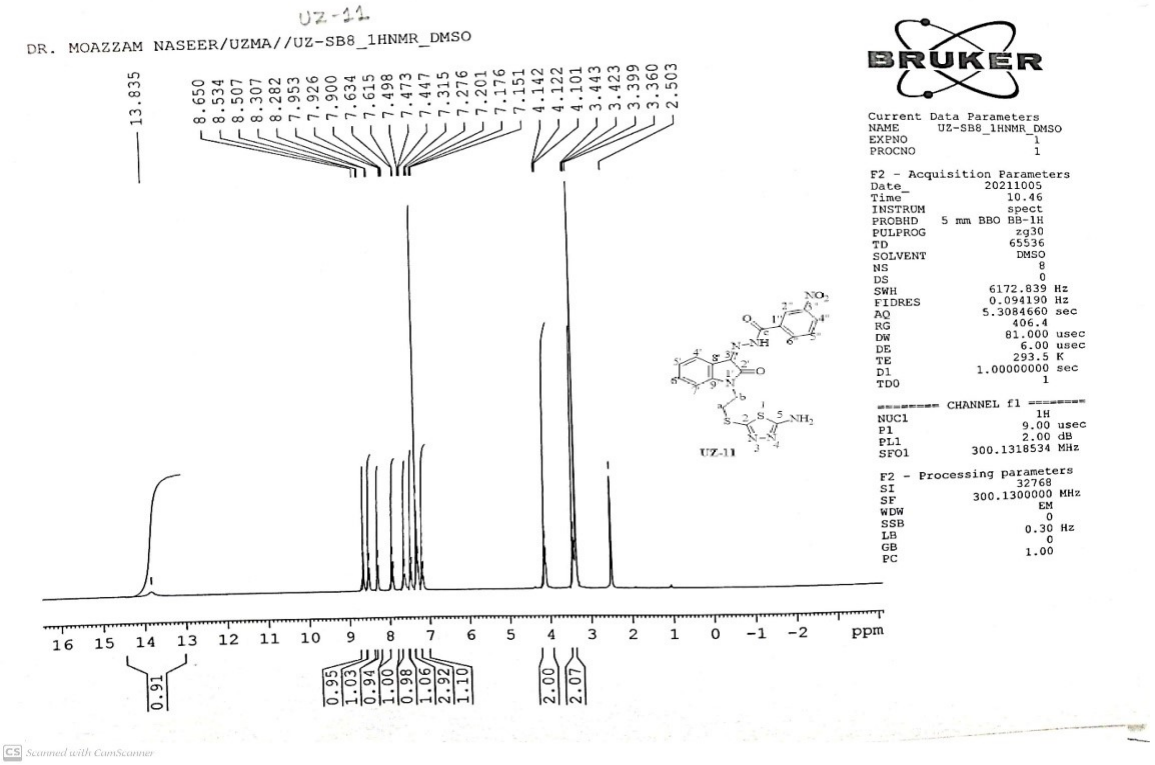
[1]H NMR spectrum of compound UZ-10



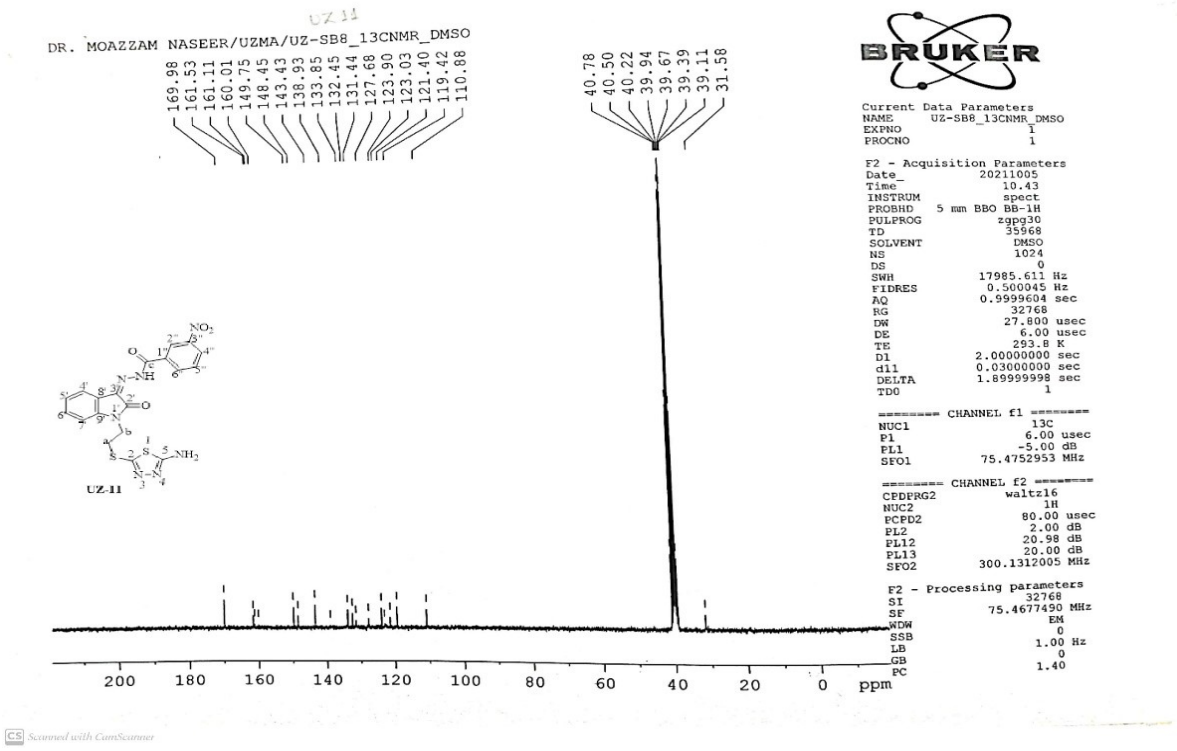
[13]C NMR spectrum of compound UZ-10



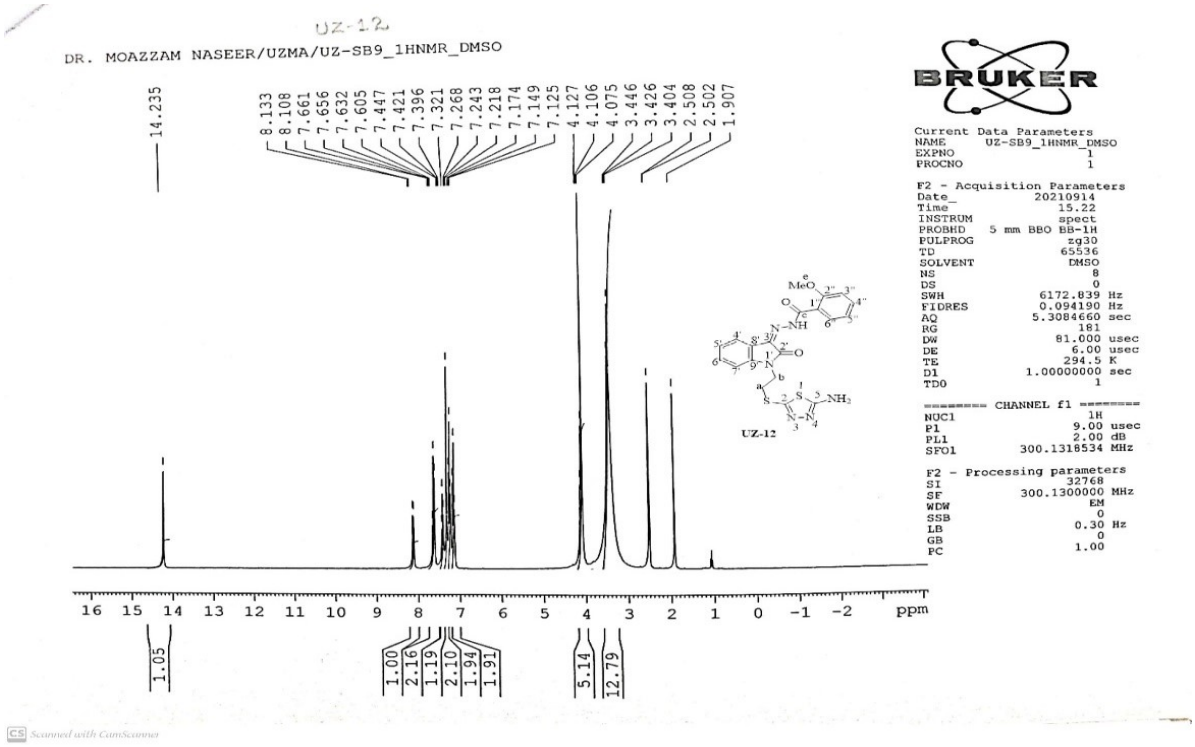
[1]H NMR spectrum of compound UZ-11



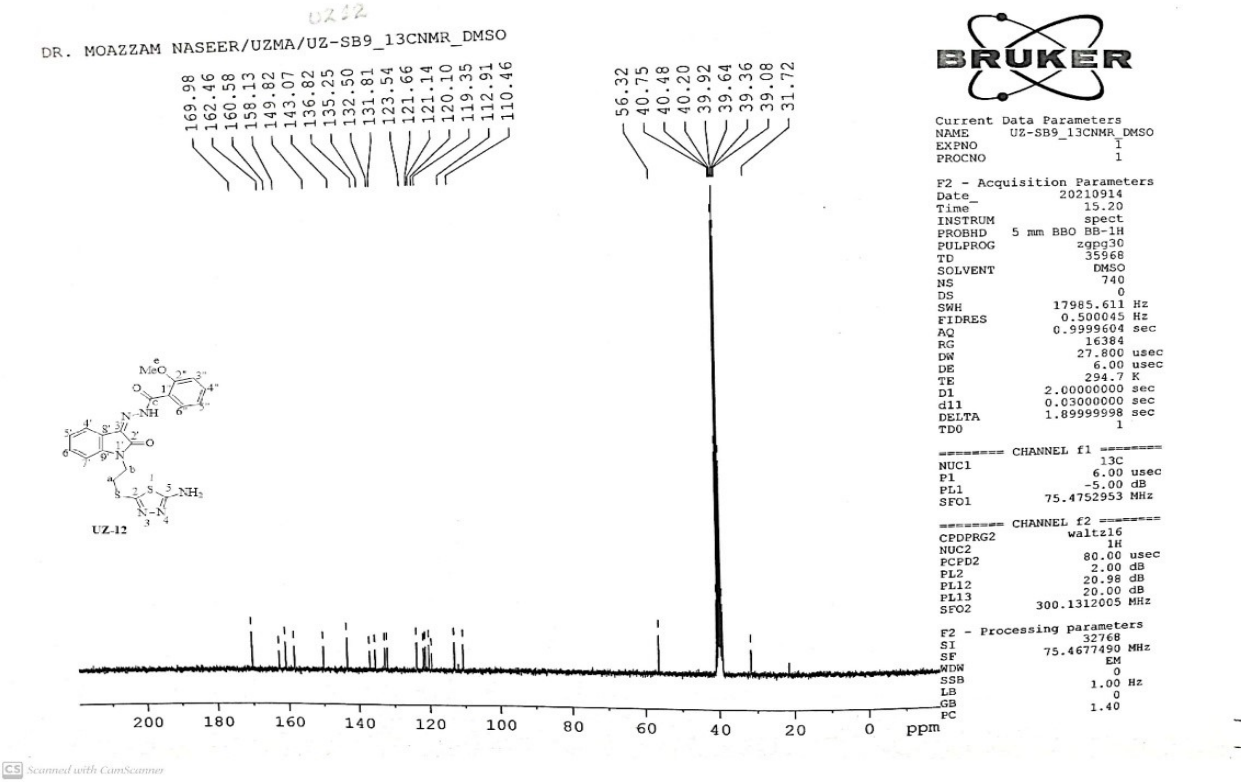
[13]C NMR spectrum of compound UZ-11



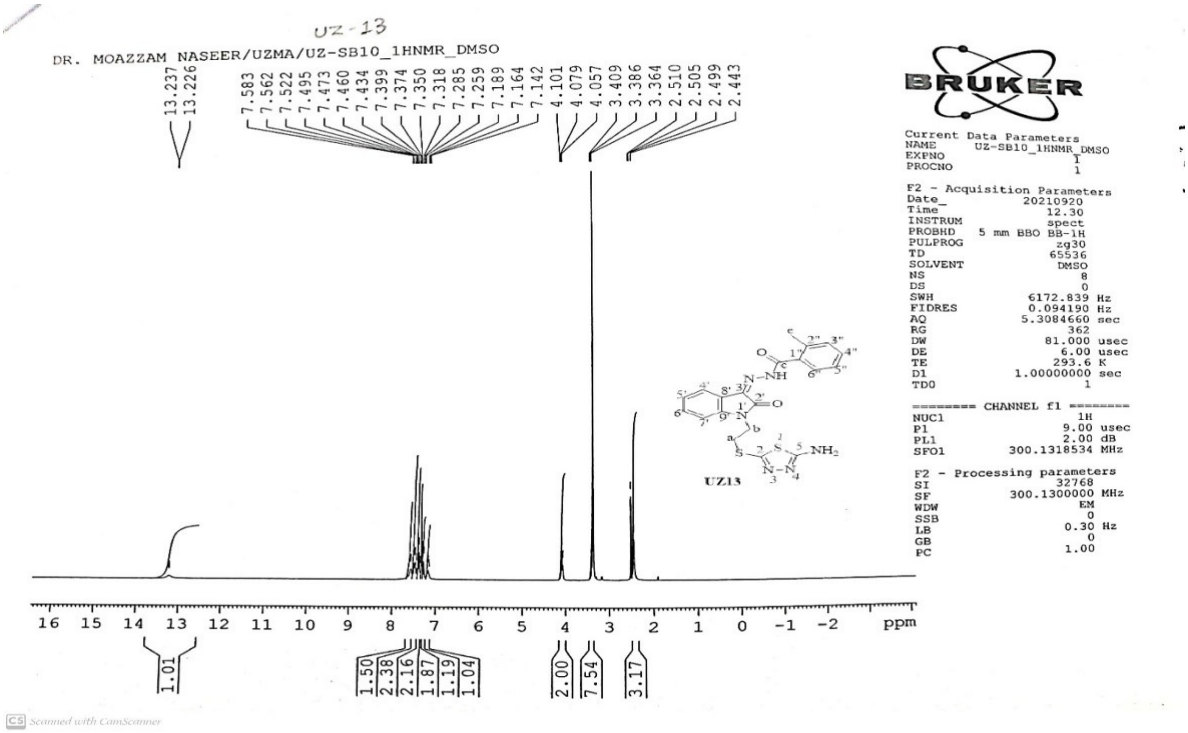
[1]H NMR spectrum of compound UZ-12



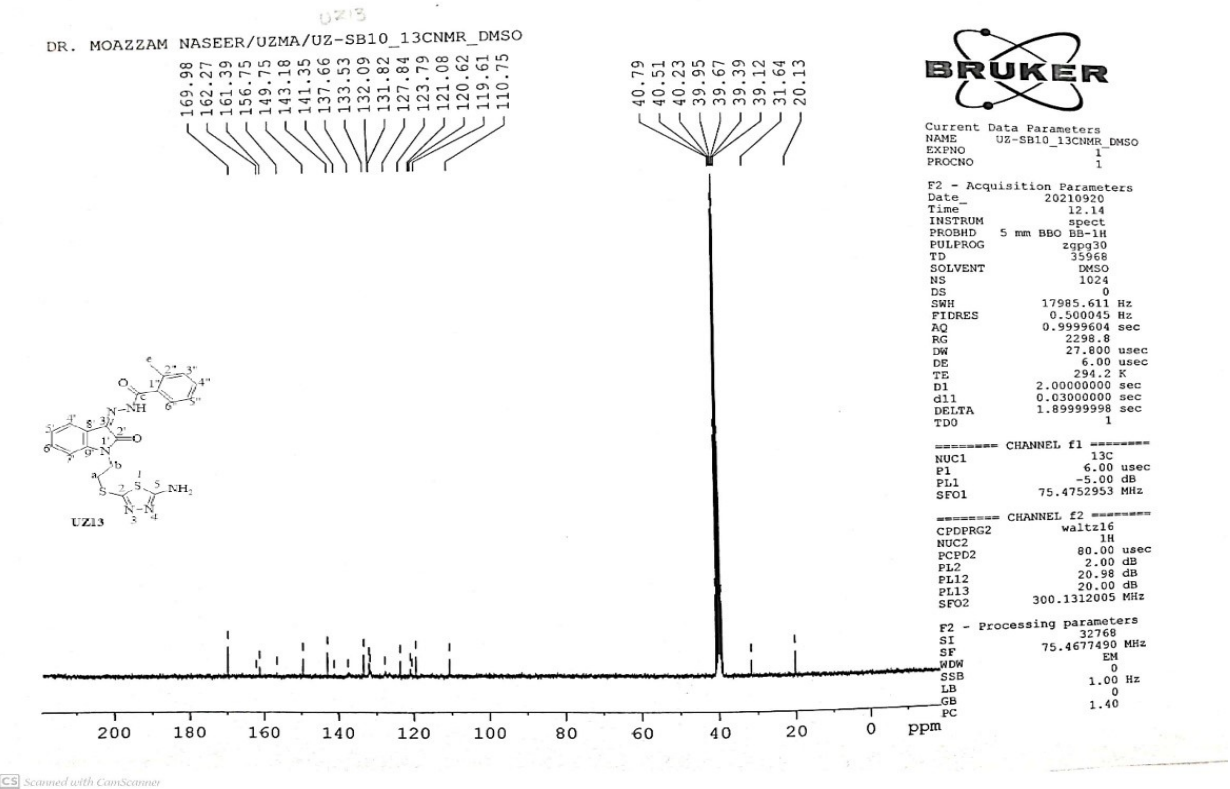
[13]C NMR spectrum of compound UZ-12



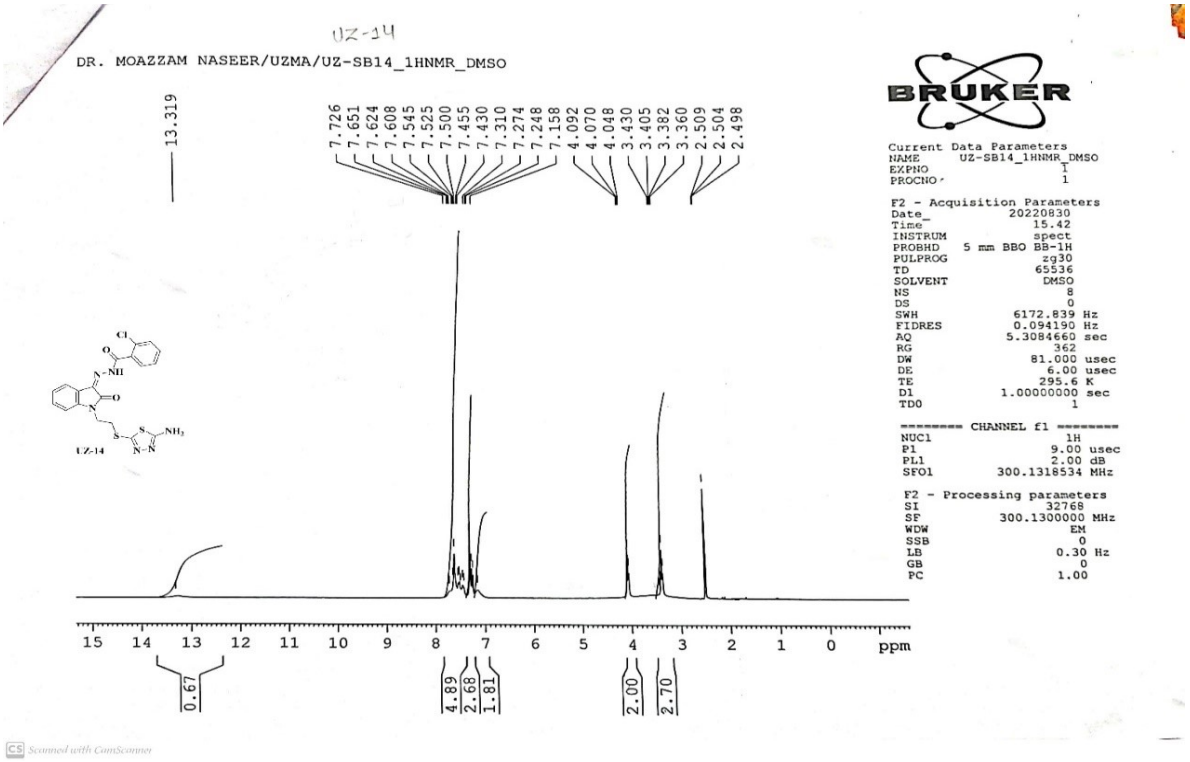
^{1}H NMR spectrum of compound UZ-13



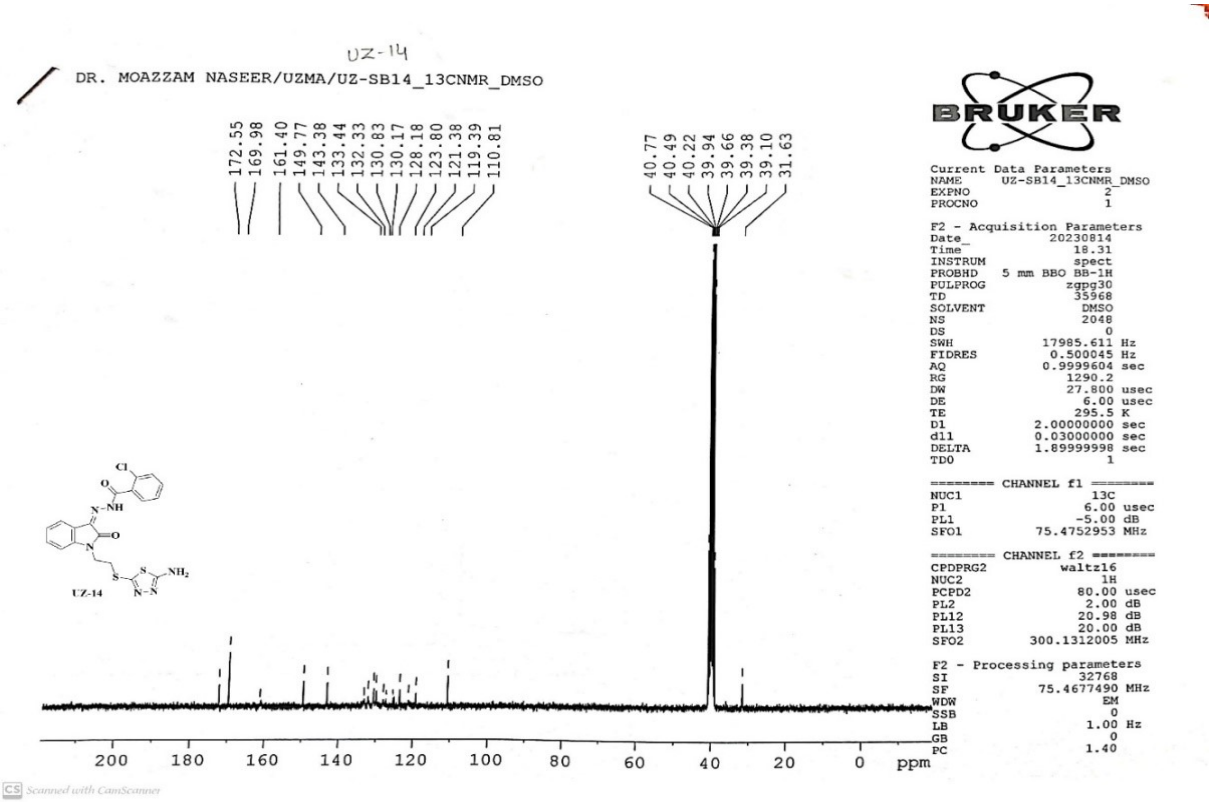
^{13}C NMR spectrum of compound UZ-13



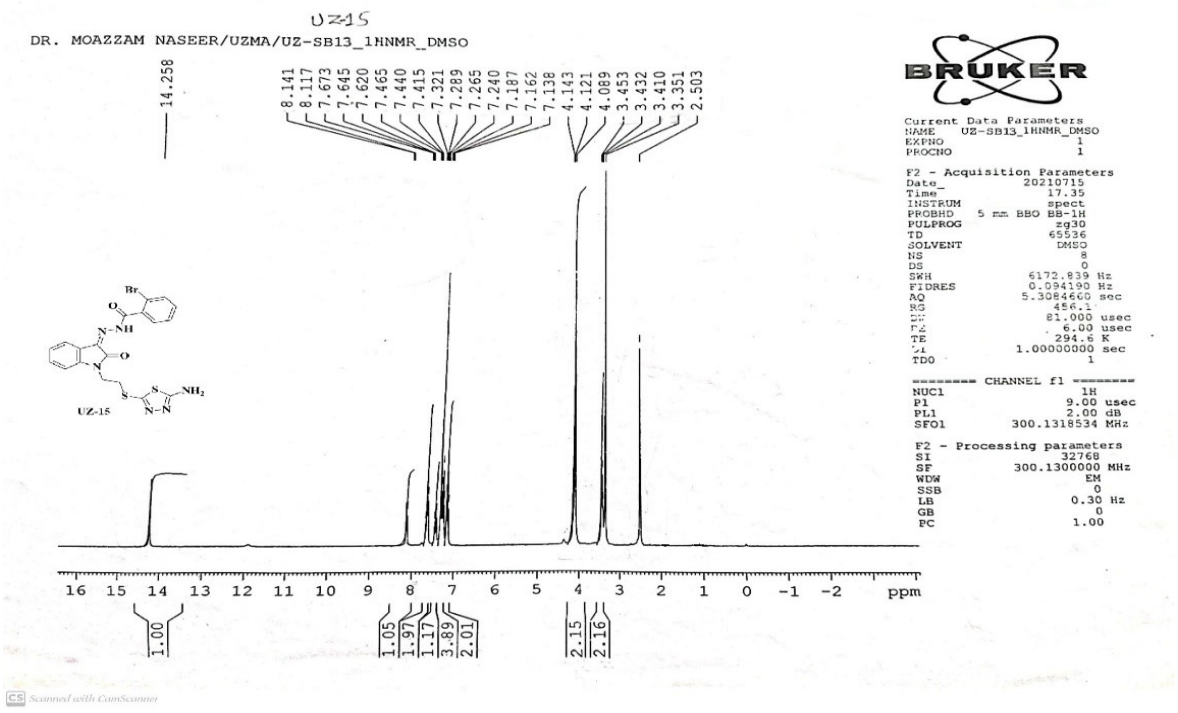
[1]H NMR spectrum of compound UZ-14



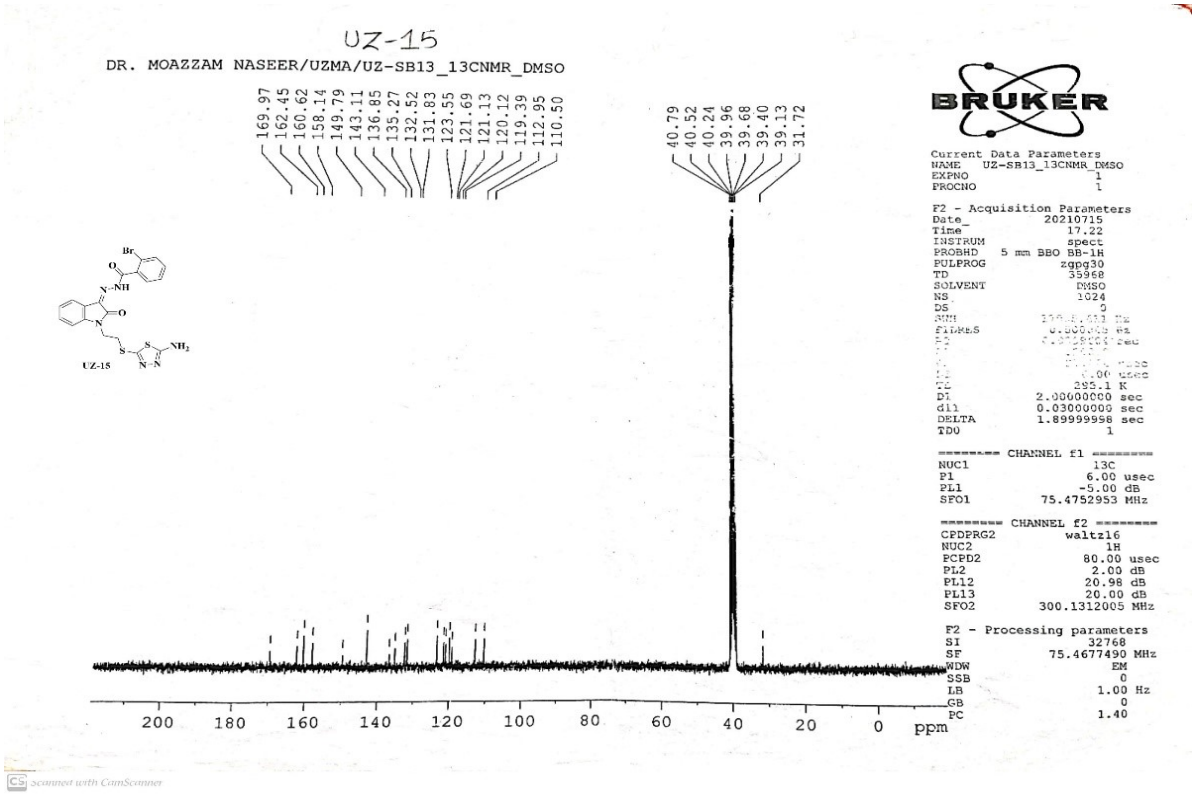
[13]C NMR spectrum of compound UZ-14



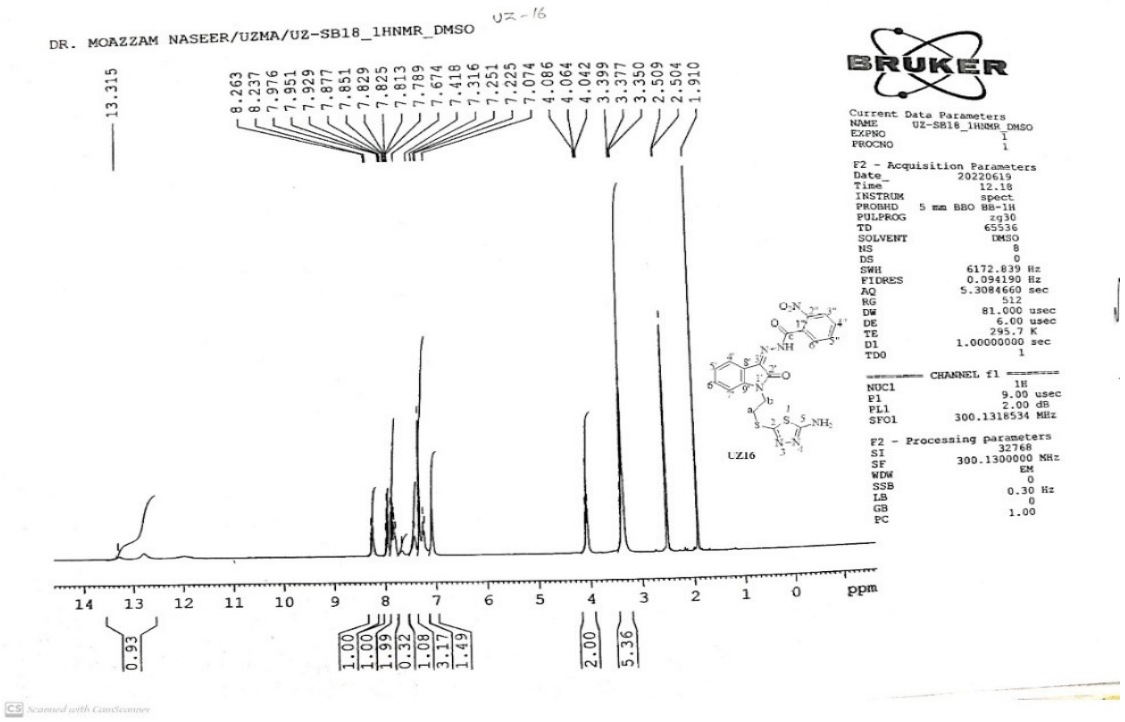
^{1}H NMR spectrum of compound UZ-15



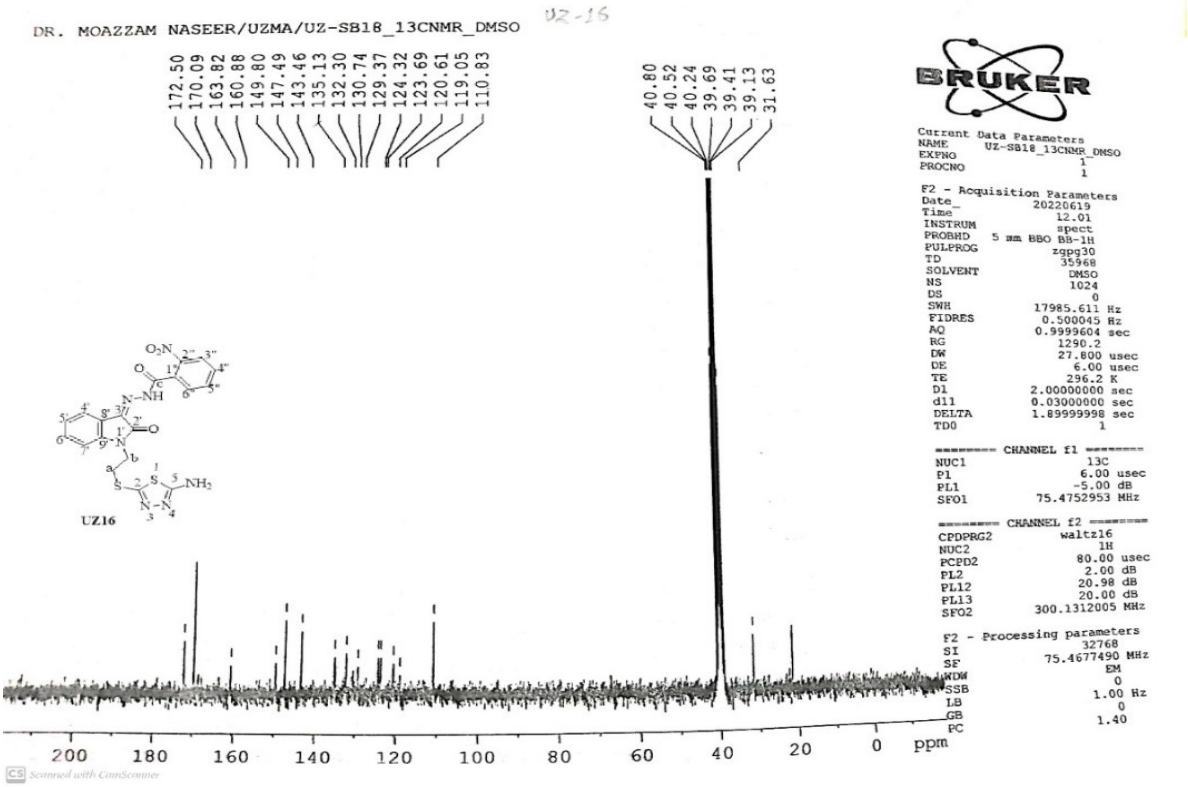
^{13}C NMR spectrum of compound UZ-15



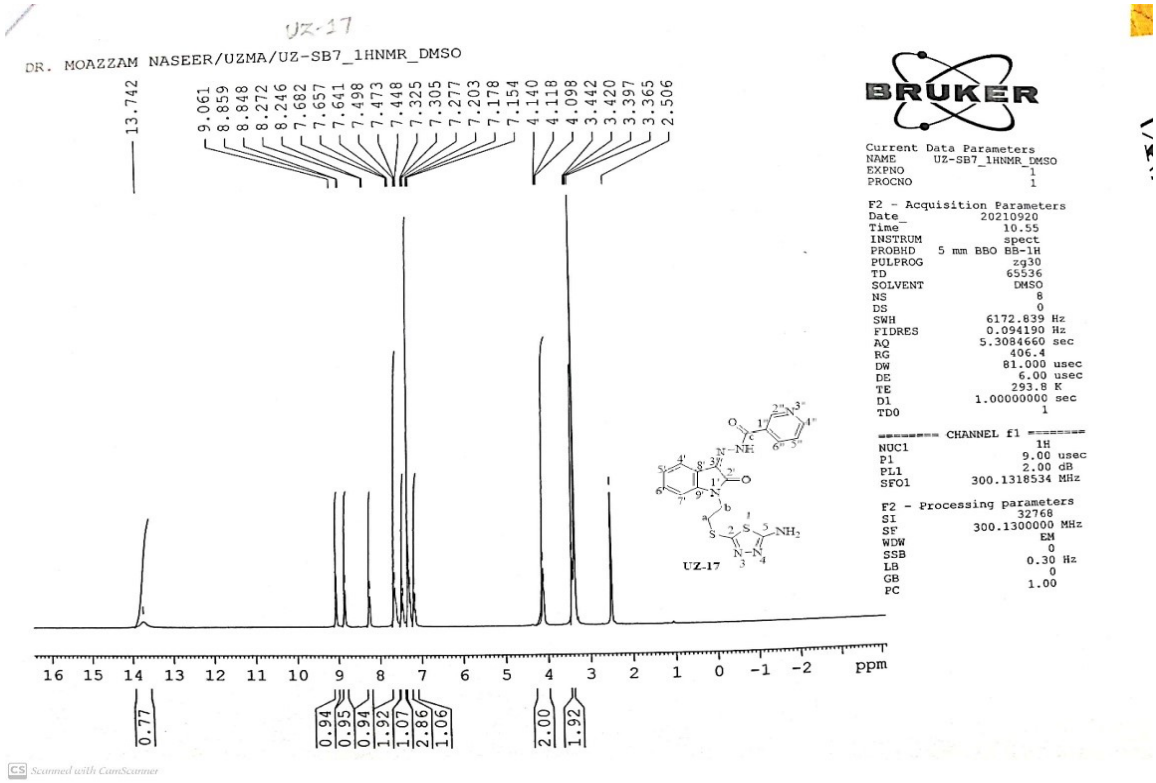
^{1}H NMR spectrum of compound UZ-16



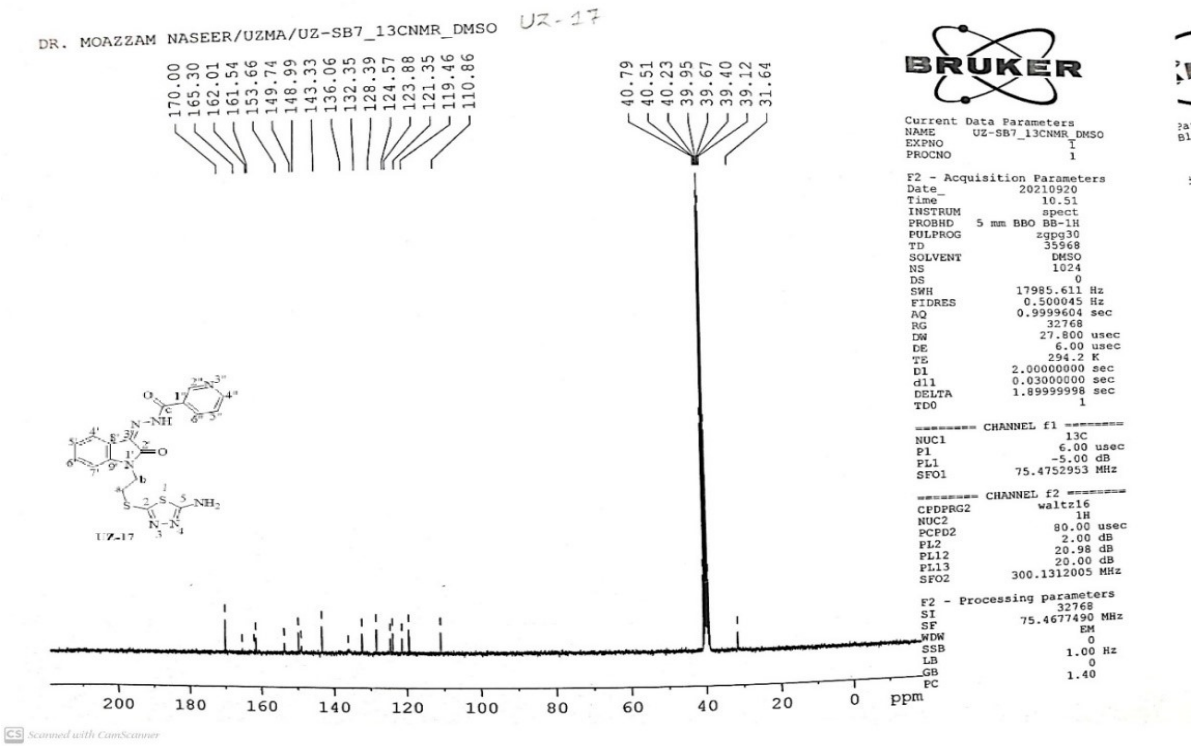
^{13}C NMR spectrum of compound UZ-16



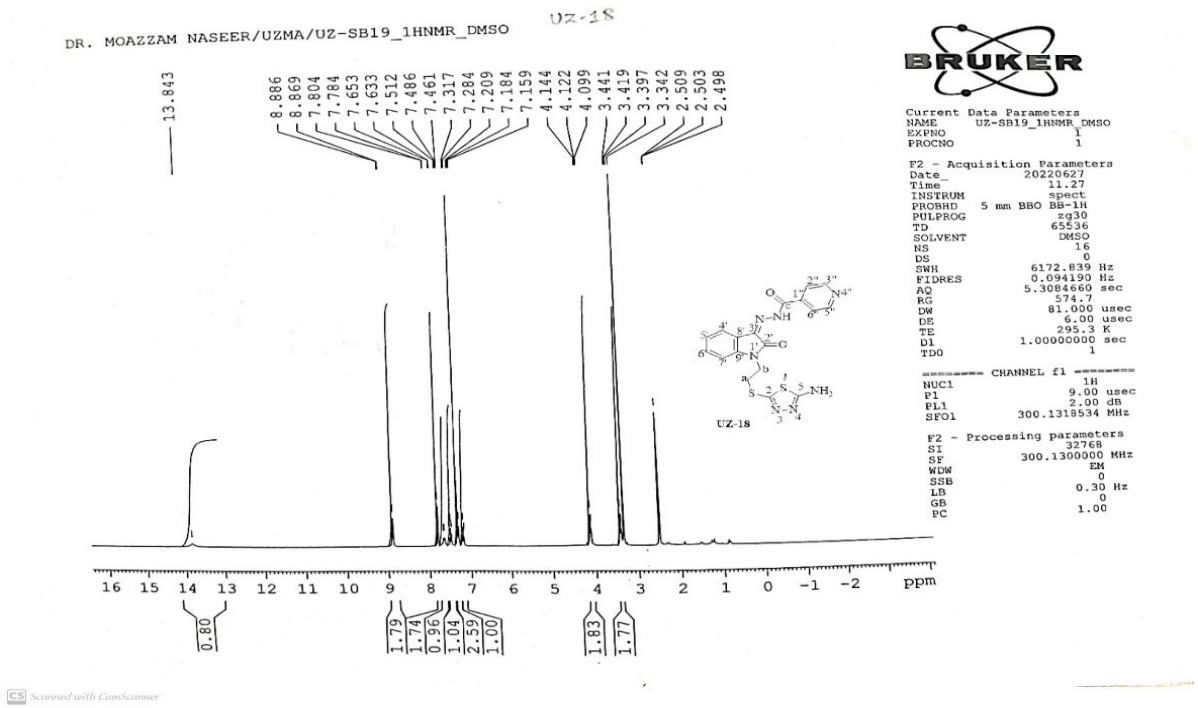
[1]H NMR spectrum of compound UZ-17



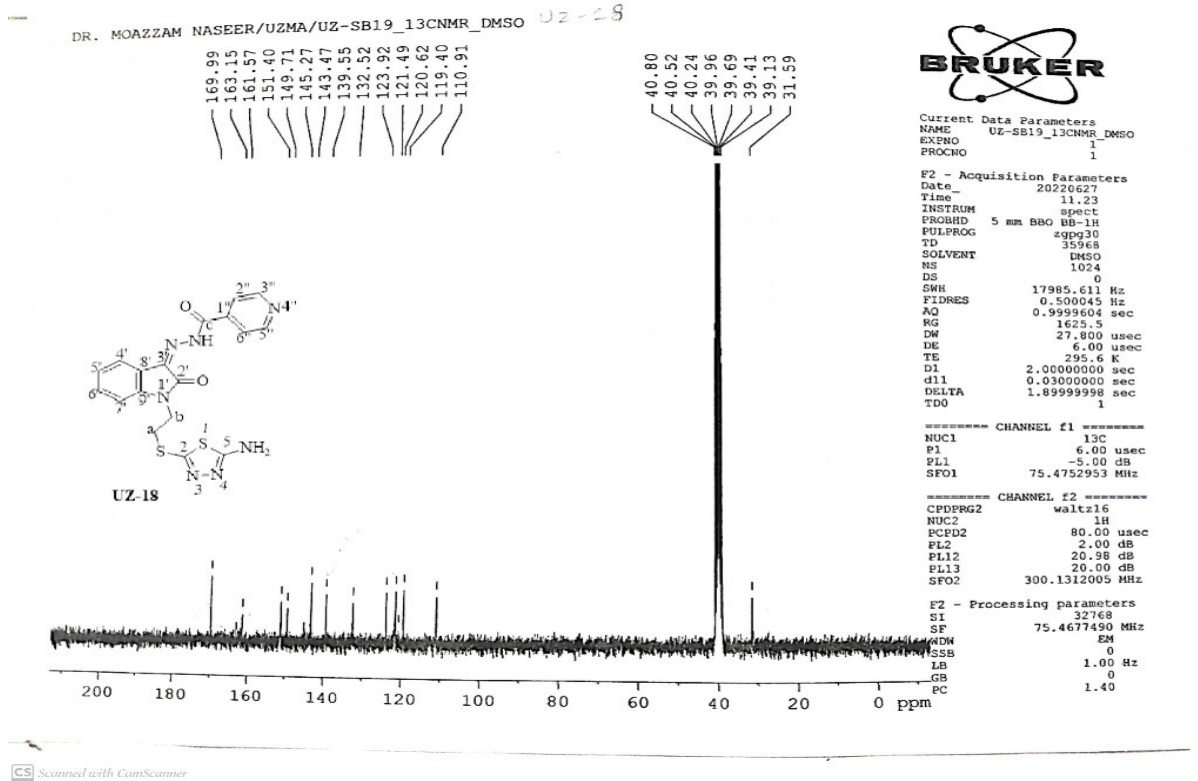
[13]C NMR spectrum of compound UZ-17



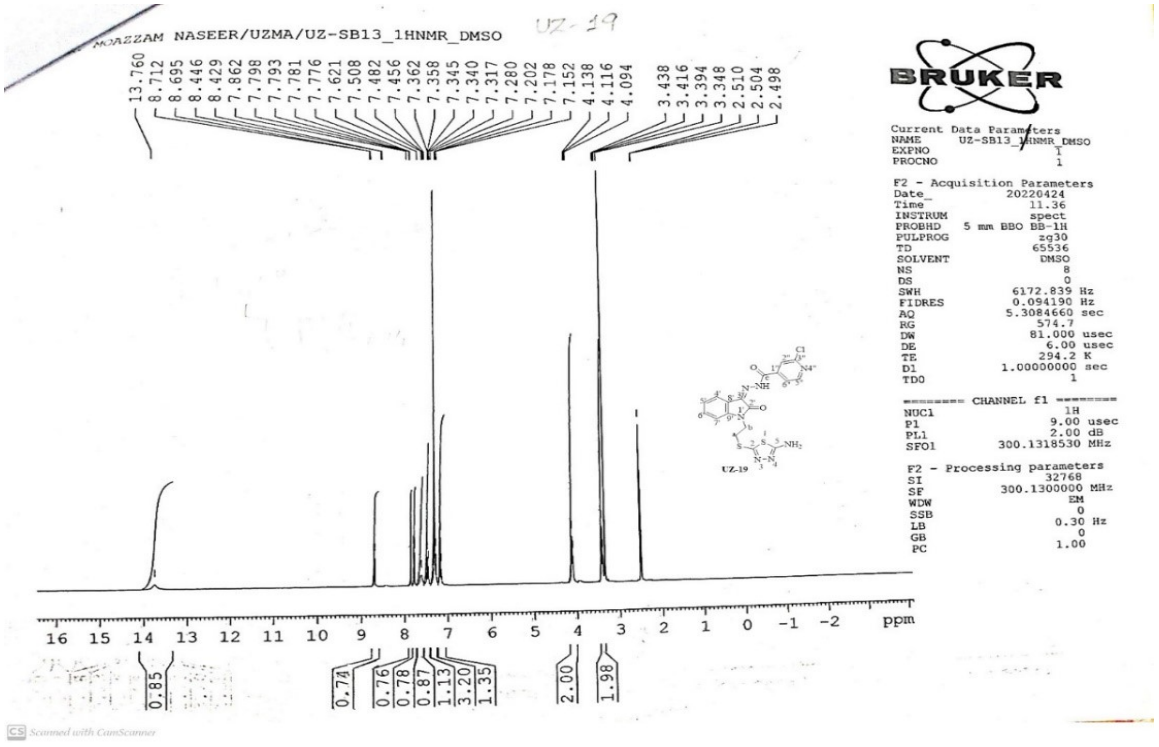
[1]H NMR spectrum of compound UZ-18



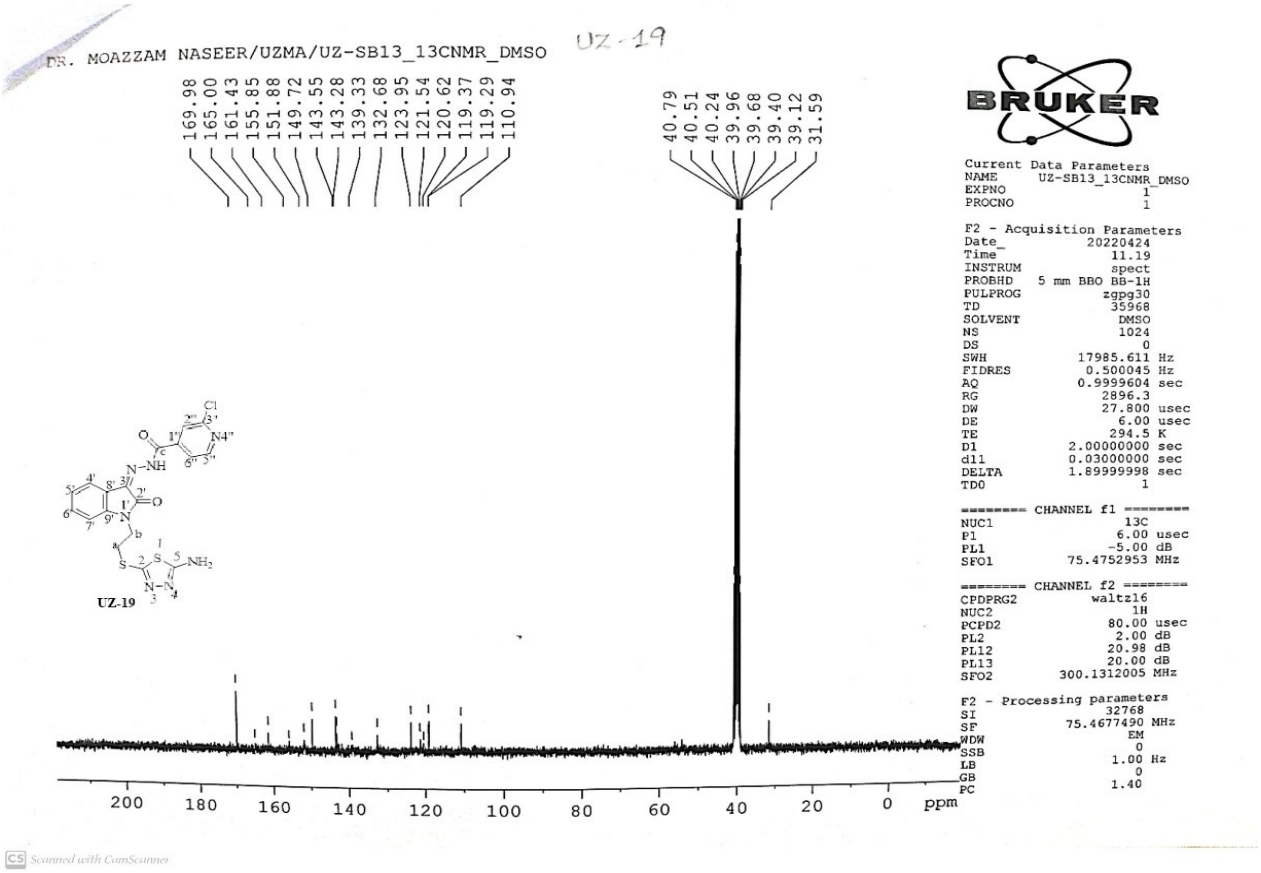
[13]C NMR spectrum of compound UZ-18



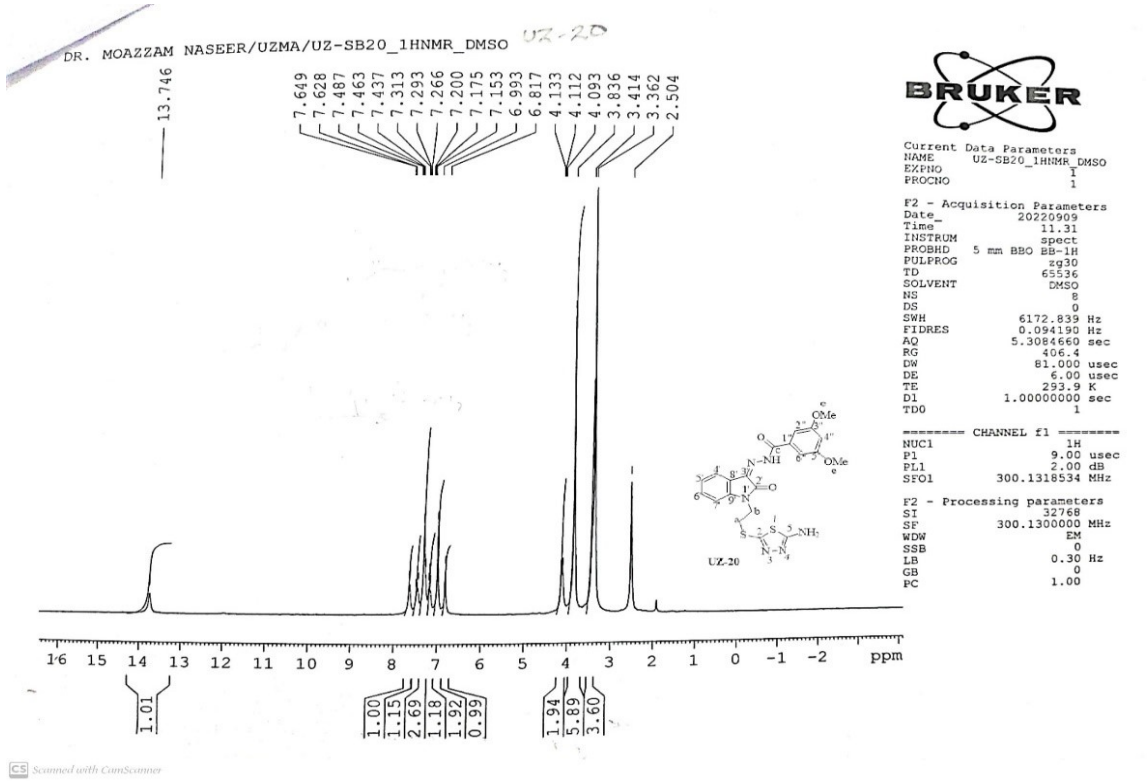
[1]H NMR spectrum of compound UZ-19



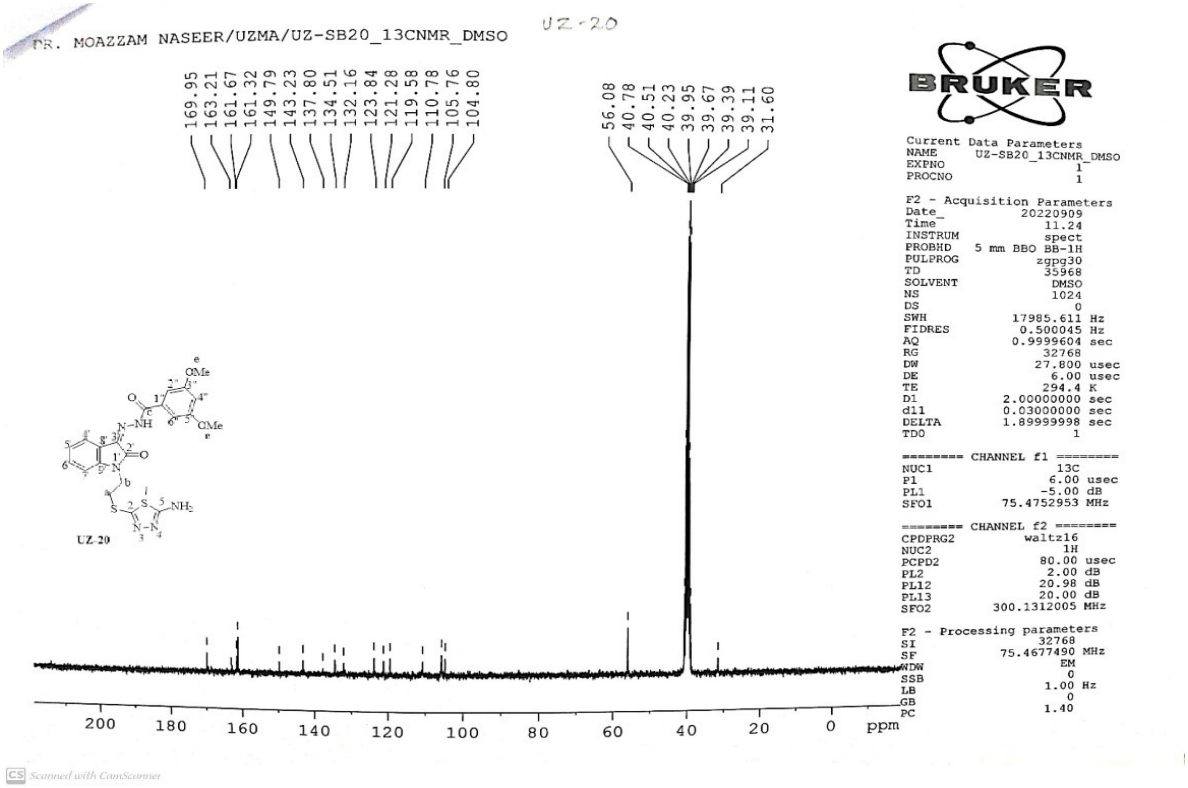
[13]C NMR spectrum of compound UZ-19



^{1}H NMR spectrum of compound UZ-20

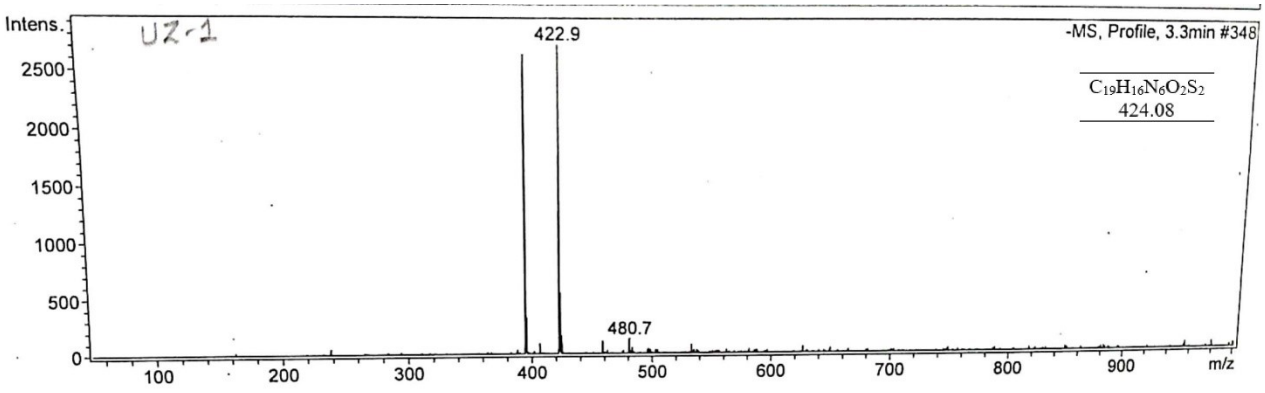


^{13}C NMR spectrum of compound UZ-20

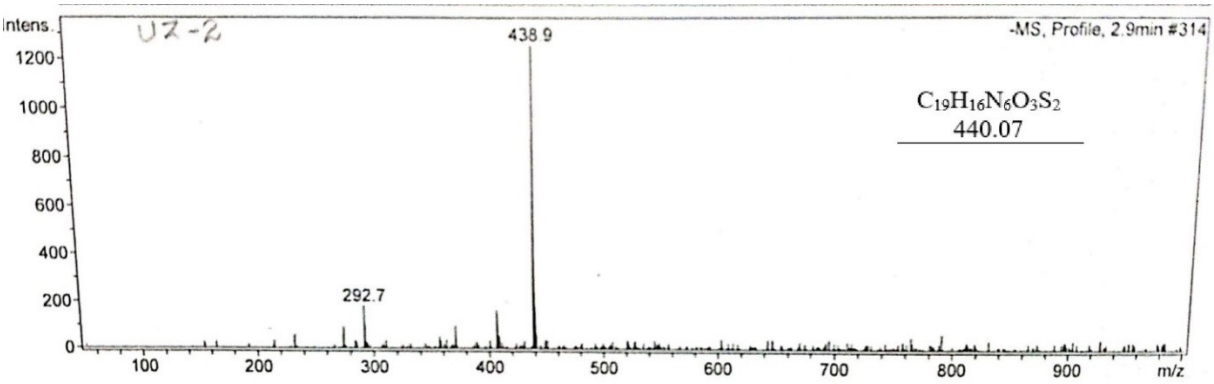


7 Copies of Mass spectra of compounds (UZ1-20)

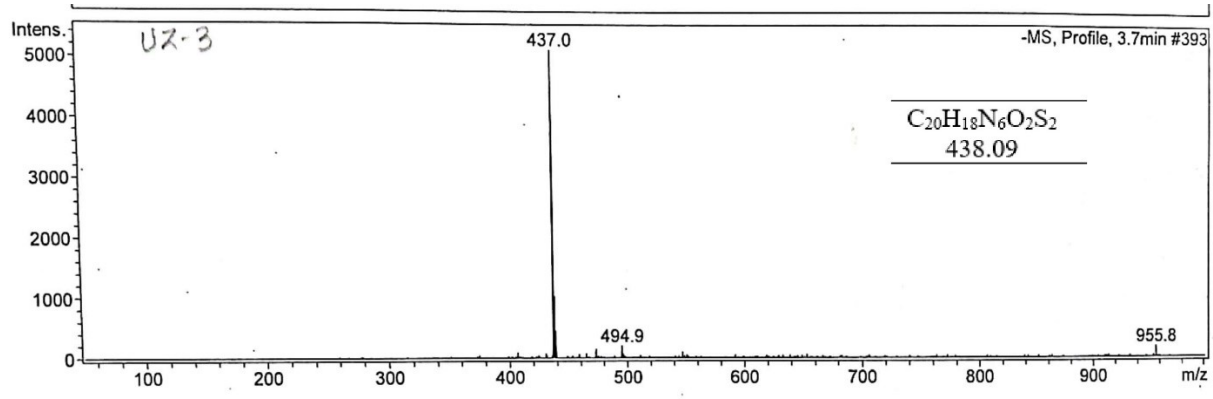
LC-MS of the compound UZ-1



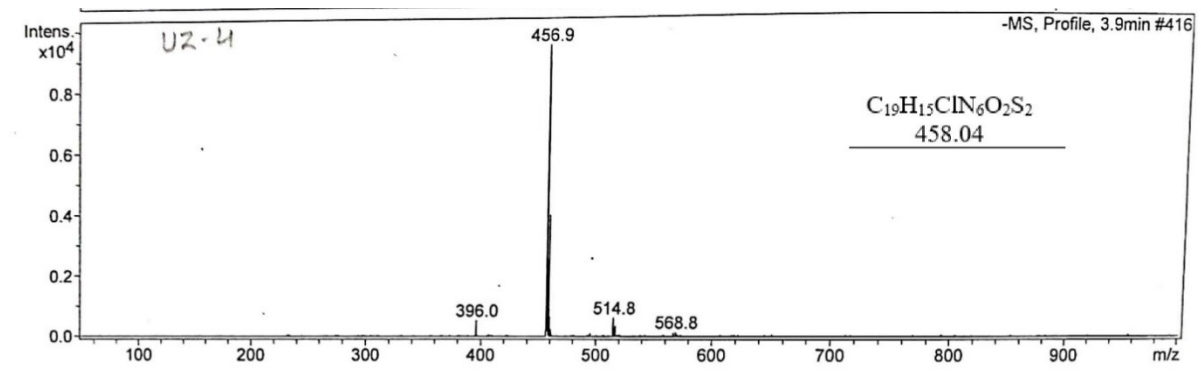
LC-MS of the compound UZ-2



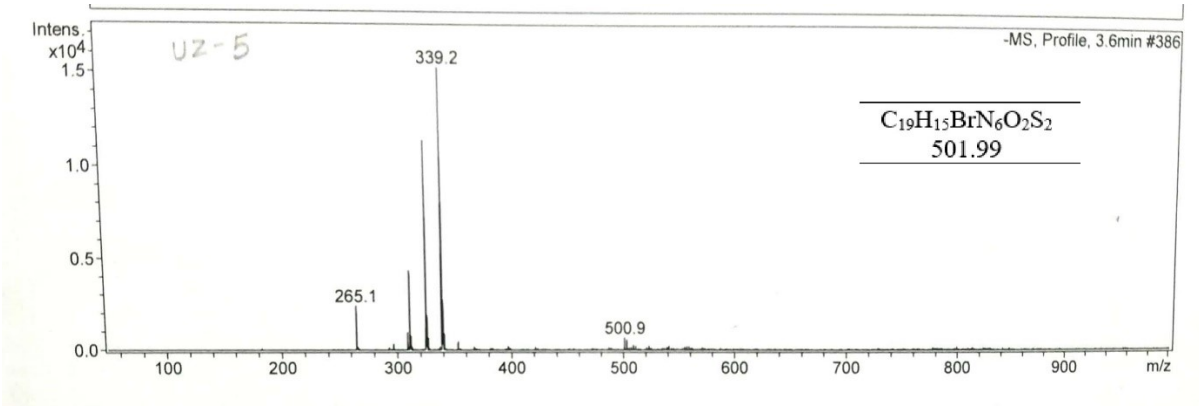
LC-MS of the compound UZ-3



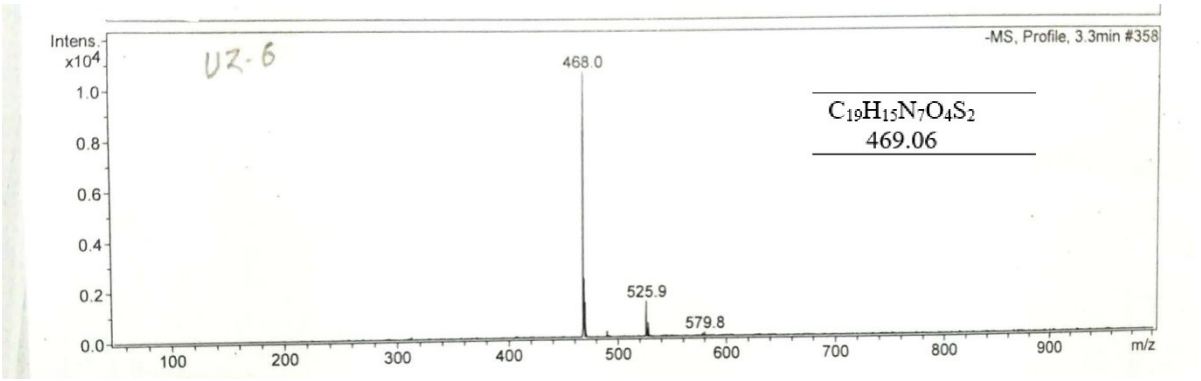
LC-MS of the compound UZ-4



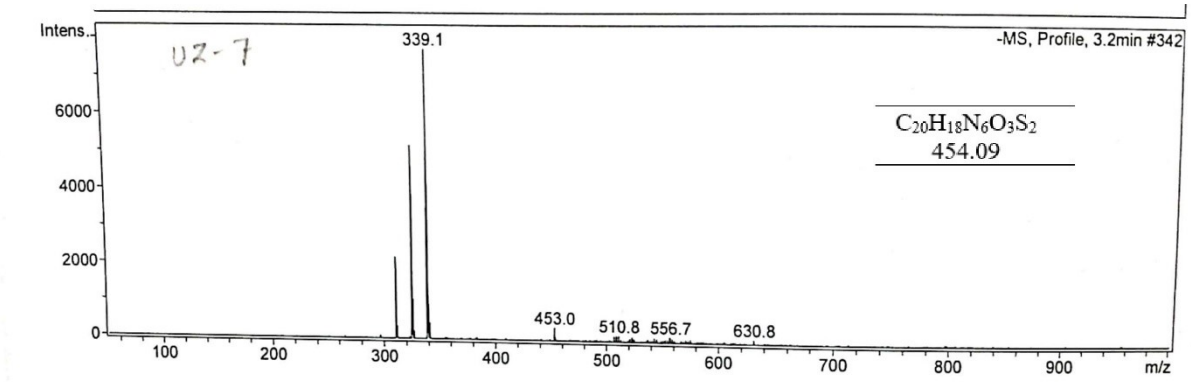
LC-MS of the compound UZ-5



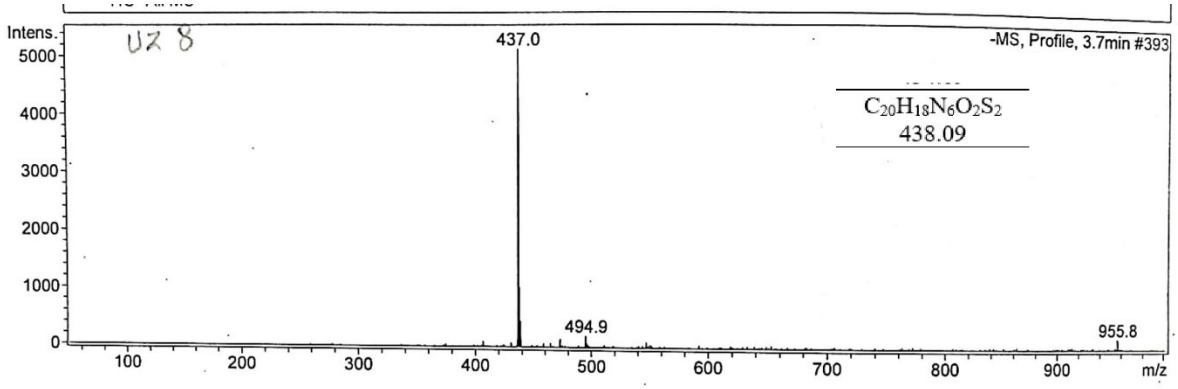
LC-MS of the compound UZ-6



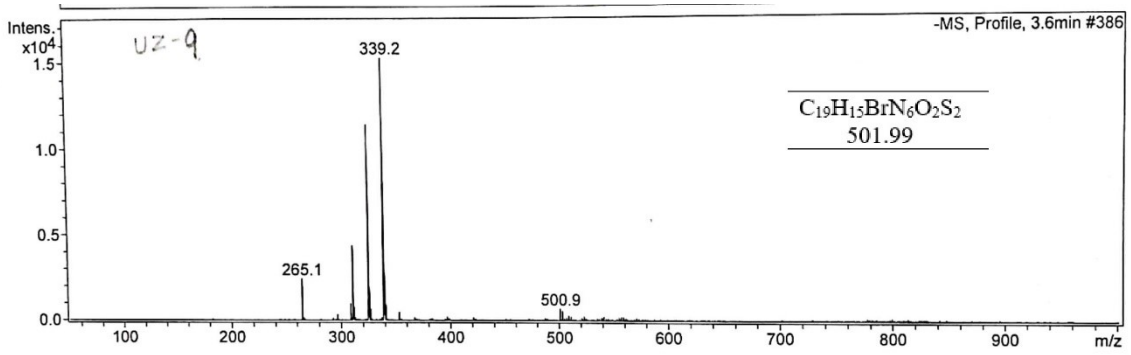
LC-MS of the compound UZ-7



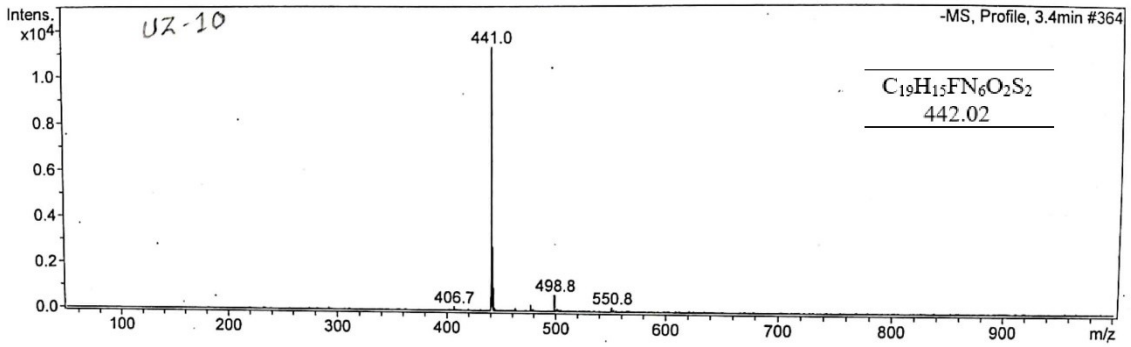
LC-MS of the compound UZ-8



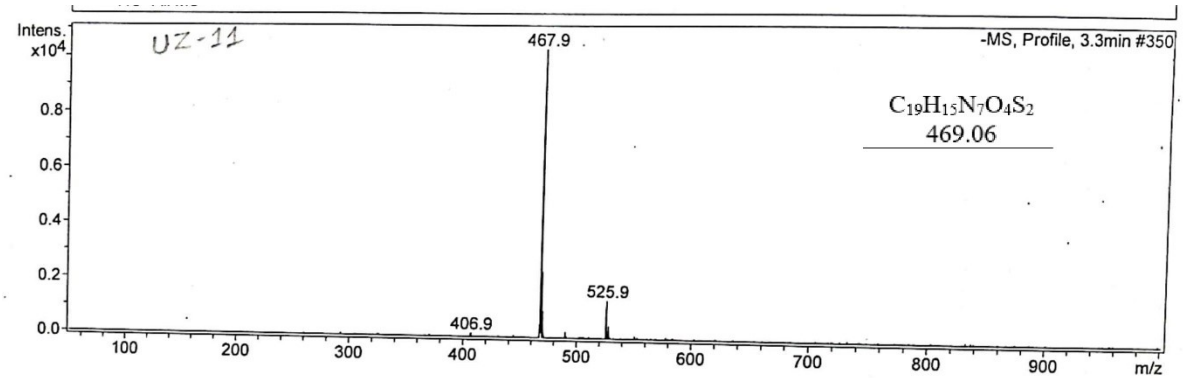
LC-MS of the compound UZ-9



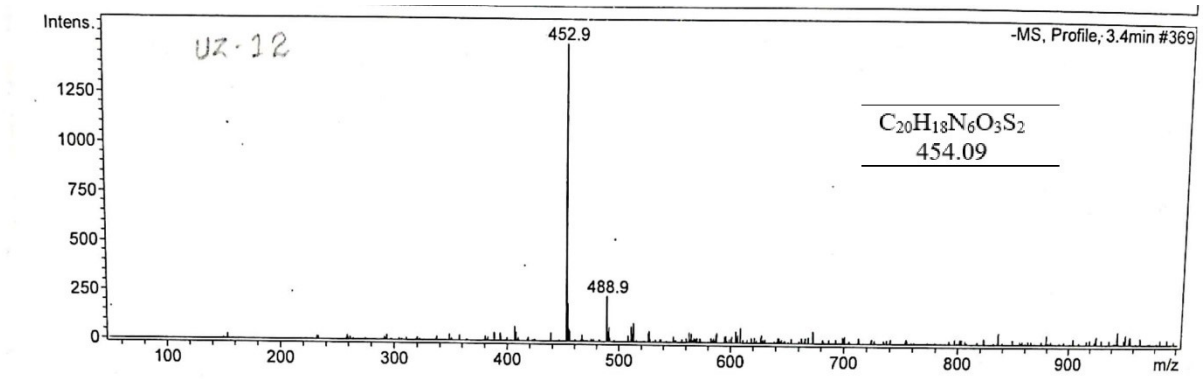
LC-MS of the compound UZ-10



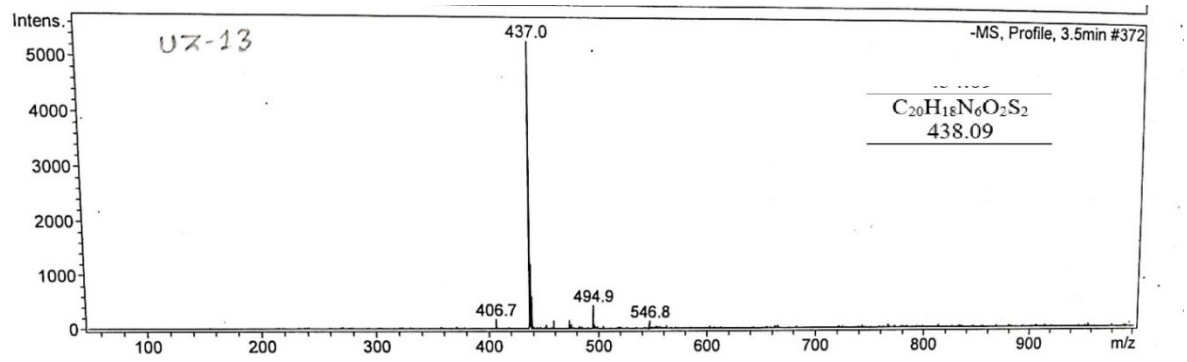
LC-MS of the compound UZ-11



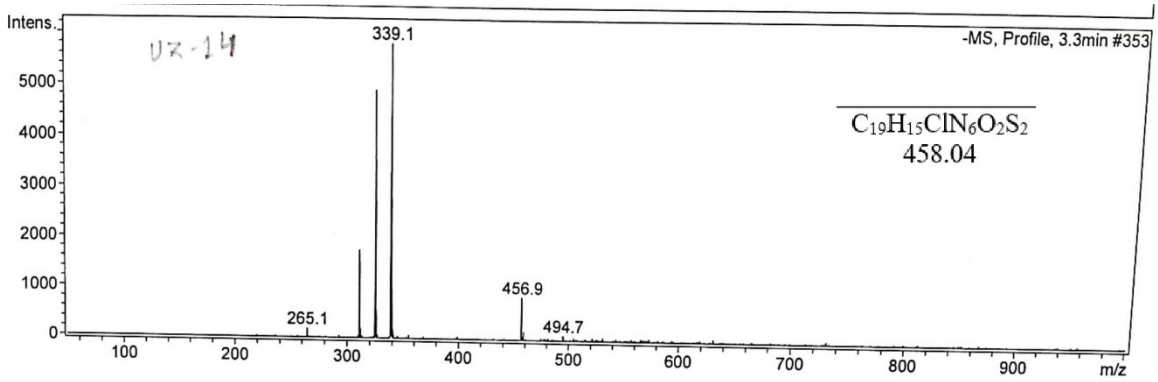
LC-MS of the compound UZ-12



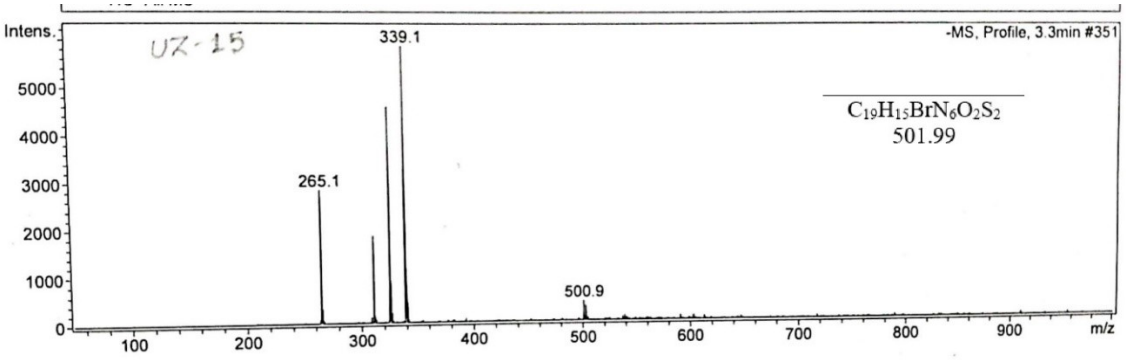
LC-MS of the compound UZ-13



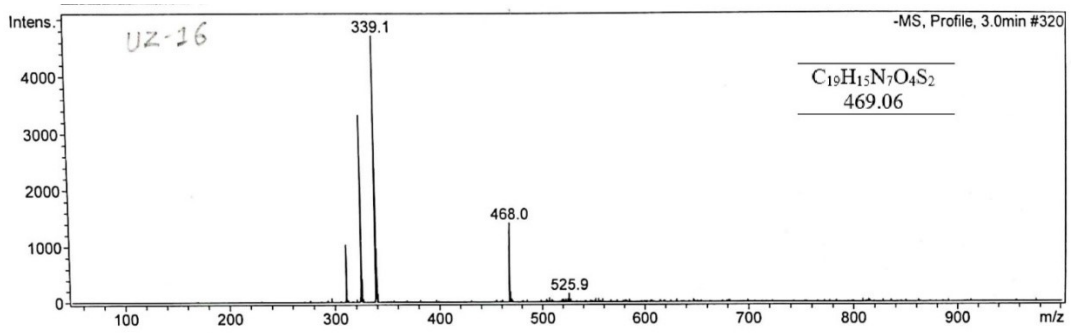
LC-MS of the compound UZ-14



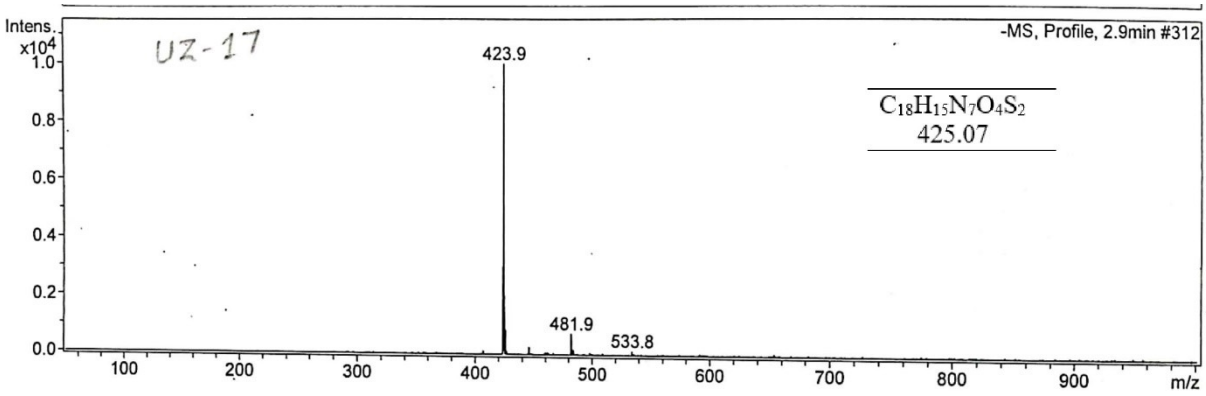
LC-MS of the compound UZ-15



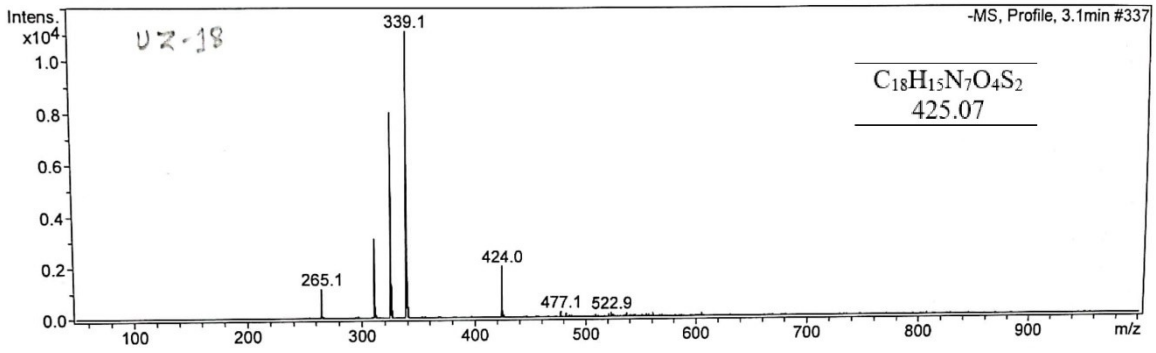
LC-MS of the compound UZ-16



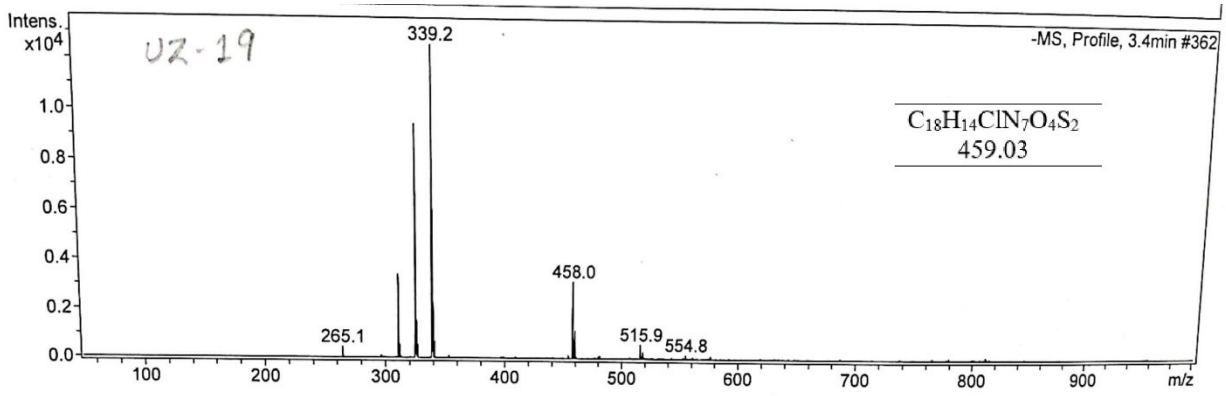
LC-MS of the compound UZ-17



LC-MS of the compound UZ-18



LC-MS of the compound UZ-19



LC-MS of the compound UZ-20