Supplementary data

Novel pyrazolo[3,4-*d*]pyrimidine derivatives: Design, synthesis, anticancer evaluation, VEGFR-2 inhibition, and antiangiogenic activity

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Supplementary data

(Biological evaluation- Molecular docking- Chemistry)

1 Biological evaluation

1.1. Antiproliferative activity evaluation

Anticancer activity screening of the newly synthesized compounds was measured *in vitro* utilizing 60 different human cancer cell lines provided by US NCI according to previously reported standard procedure as follows:

- Cells were seeded into 96-well microtiter plates in a density of 5,000-1,000 cells per 100 µL/well. Cells were then incubated at 37°C, 5% CO_2, 95% air, and 100% relative humidity for 24 h before the addition of experimental compounds. After 24 h, two plates of each cell line were fixed in situ with trichloroacetic acid (TCA), to present a measurement of the cell population for each cell line at the time of compound exposure (Tz).
- Experimental compounds were solubilized in DMSO at 400-fold, the desired final maximum test concentration, and stored frozen before use. At the time of compound addition, an aliquot of frozen concentrate was thawed and diluted to twice the desired final maximum test concentration with a complete medium containing 50 µg/mL gentamicin.
- Additional four, 10-fold or ½ log serial dilutions were made to provide a total of five compound concentrations plus control. Aliquots of 100 µL of these different compound dilutions were added to the appropriate microtiter wells containing 100 µL of the medium, resulting in the required final compound concentrations.
- Following compound addition, the plates were incubated for an additional 48 h at 37°C, 5% CO_2, 95% air, and 100% relative humidity. For adherent cells, the assay is terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50 µL of cold 50% (w/v) TCA (final concentration, 10% TCA and incubated for 60 min at 4°C. The supernatant was discarded, and the plates were washed five times with tap water and air-dried.
- Sulforhodamine B (SRB) solution (100 µL) at 0.4% (w/v) in 1% acetic acid was added to each well and plates were incubated for 10 min at room temperature. After staining, the unbound dye was removed by washing five times with 1% acetic acid and the plates were air-dried. The bound stain was subsequently solubilized with 10 mM Trizma base, and the absorbance was obtained using an automated plate reader at a wavelength of 515 nm. For suspension cells, we used the same methodology except that the assay was terminated by fixing settled cells at the bottom of the wells by gently adding 50 µL of 80% TCA (final concentration, 16% TCA). Using the seven absorbance measurements [time zero (Tz), control growth (C), and test growth in the

presence of a compound at the five concentration levels (Ti)], the percentage growth was calculated at each of the compound concentration levels. Percentage growth inhibition is calculated as follows:

$$[(Ti - Tz)/(C - Tz)] \times 100 \text{ for concentrations for which } Ti \geq Tz,$$

$$[(Ti - Tz)/Tz] \times 100 \text{ for concentrations for which } Ti > Tz$$

1.2. Measurement of IC_{50} of compounds 12a–d against breast cancer cell lines (MDA-MB-468 and T-47D) and normal breast cell line MCF-10a using MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method of monitoring *in vitro* cytotoxicity is well suited for use with multiwell plates. The assessment of cell population growth is based on the capability of living cells to reduce the yellow product MTT to a blue product, formazan, by a reduction reaction occurring in the mitochondria. The cell lines were incubated for 24 h in 96-microwell plates. The number of living cells in the presence or absence (control) of the test compound is directly proportional to the intensity of the blue color, measured by spectrophotometry at a wavelength of 570 nm. Measure the background absorbance of multiwell plates at 690 nm and subtract from the 570 nm measurement. Five concentrations ranging from 0.01 μM to 100 μM (with a semi-log decrease in concentration) were tested for the compound under study. Each experiment was carried out in triplicate. The IC_{50} values [the concentration required for 50% inhibition of cell viability] were calculated using sigmoidal dose-response curve-fitting models. The selectivity index (SI) was calculated as the ratio of cytotoxicity (IC_{50}) on normal cells (MCF-10a) to breast cancer cells (MDA-MB-468 and T47D).

1.3. Assessment of VEGFR-2 inhibitory activity

Briefly, 25 μL of the prepared master mixture was poured into each well. 5 μL of the "Test Inhibitor" inhibitor solution was given to each well. The "Blank" group received 5 μL of the same solution as the "Positive Control" group but without the inhibitor. 600 μL of kinase buffer and 2400 μL of water were mixed to make 3 mL of kinase buffer. The blank wells received 20 μL of kinase buffer. The VEGFR-2 needed for the test was measured, and kinase buffer was used to dilute the enzyme to 1 ng/ μL. The reaction was started by adding 20 μL of diluted VEGFR-2 enzyme to the wells labeled as "Test Inhibitor Control" and "Positive Control," and the mixtures were then incubated at 30 °C for 45 min. Each well received 50 μL of Kinase-Glo Max reagent after 45 min., and the plate was incubated for 15 min. at room temperature. A microplate reader was used to measure the luminescence.

1.4. Wound healing test

After warming up at room temperature for 10 min., the 24-well plate wound healing inserts were filled with 500 mL suspension of HUVECs (1.0 × 106) in 10% fetal bovine serum (FBS) media. The cells were incubated in a cell culture incubator until they formed a monolayer. The inserts were carefully removed from the well. The media was gradually aspirated from the wells and discarded. The wells were washed with media to get rid of dead cells and remain. Finally, the media was added to the wells to keep the cells hydrated, and

then the wells were examined under a light microscope. The wells were subsequently filled with media containing the IC_{50} value of the tested drug **12b** or sunitinib for 72 h. The cells were incubated until they formed a monolayer in a cell culture incubator. The inserts were carefully removed from the well. The media was gradually inhaled and thrown from the wells. The wells were cleaned with a medium to remove dead cells and debris. Eventually, the wells received medium to maintain the cells hydrated, and the wells were examined under a light microscope. The wells were subsequently filled with media containing the stated concentrations of the tested drug **12d** or sunitinib. The wound closure was examined, and the percentage of cells that had closed into the wound site was measured using a light microscope

1.5. Analysis of cell cycle

Flow cytometry was used to analyze the cell cycle using ab139418 propidium iodide flow cytometry kit/BD (Abcam, Cambridge, UK), as directed by the manufacturer guidelines. MDA-MB-468 cells were treated with compound **12b** at its IC_{50} value (3.343 μM) for 24 h. The cells were washed twice with ice-cold phosphate buffer saline (PBS) and collected by centrifugation. The cells were then fixed using ice-cold 66% (v/v) ethanol, washed with PBS, and re-suspended with 0.1 mg/mL RNase to digest cellular RNA and thus minimize stained RNA in the background. The cells were next stained with PI, a fluorescent molecule that may bind to nucleic acid, at a concentration of 40 mg/mL. In cells, PI attaches to DNA in proportion to its amount. Because the DNA content of cells at different stages of the cell cycle differs, the fluorescence intensity can be used to assess the stage of cell growth. FacsCalibur (BD Biosciences, USA) was used to estimate cell fluorescence, which was then examined using Cell-Quest software (Becton Dickinson). Cell cycle analysis of MDA-MB-468 cells without any treatment was used as a control.

1.6. Evaluation of apoptosis by Annexin V

According to the manufacturer's instructions, flow cytometry was used to analyze apoptosis using Annexin V and propidium iodide double-staining apoptosis detection kit (Biovision, USA). Annexin V is a phosphatidylserine (PS) binding protein with a high affinity. After beginning apoptosis, the latter is a cell membrane component that translocates from the inner face of the plasma membrane to the cell surface. PS can be detected on the cell surface using a fluorescent Annexin V conjugate. MDA-MB-468 cells ($5x10^5$) were exposed to compound **12b** at its IC_{50} value, and then subsequently incubated for 24 h. After that, the cells were centrifuged and resuspended in 500 mL of binding buffer. Annexin V and PI double staining was accomplished by mixing 5 μL of Annexin V with 5 μL of PI. The cells were then incubated for 15 min. in the dark at room temperature. FacsCalibur was used to assess cell fluorescence after incubation (BD Biosciences, USA). The results were represented using dot-plot graphs.

1.7. Caspase-3 enzyme assay

The level of the apoptotic marker caspase-3 was measured using Invitrogen ELISA Kit Catalog # KHO1091. The procedure of the used kit was performed according to the manufacturer's instructions. Let all components reach room temperature before use. Gently combine all liquid reagents before use. Add 100 µL of the standard diluent buffer to the zero standard wells. Add 100 µL of standards and controls or diluted samples to the appropriate microtiter wells. Incubate for 2 h at room temperature. Pipette 100 µL of caspase-3 (Active) detection antibody solution into each well. Incubate for 1 h at room temperature. Add 100 µL anti-rabbit IgG HRP working solution to each well. Prepare the working dilution and incubate for 30 min. at room temperature. Add 100 µL of stabilized chromogen to each well. The liquid in the wells will begin to turn blue. Incubate for 30 min. at room temperature and in the dark. Add 100 µL of stop solution to each well. The solution in the wells should change from blue to yellow. Read the absorbance of each well at 450 nm. Read the plate within 2 h after adding the stop solution. Use curve fitting software to generate the standard curve. Read the concentrations for unknown samples and controls from the standard curve.

2- Molecular docking

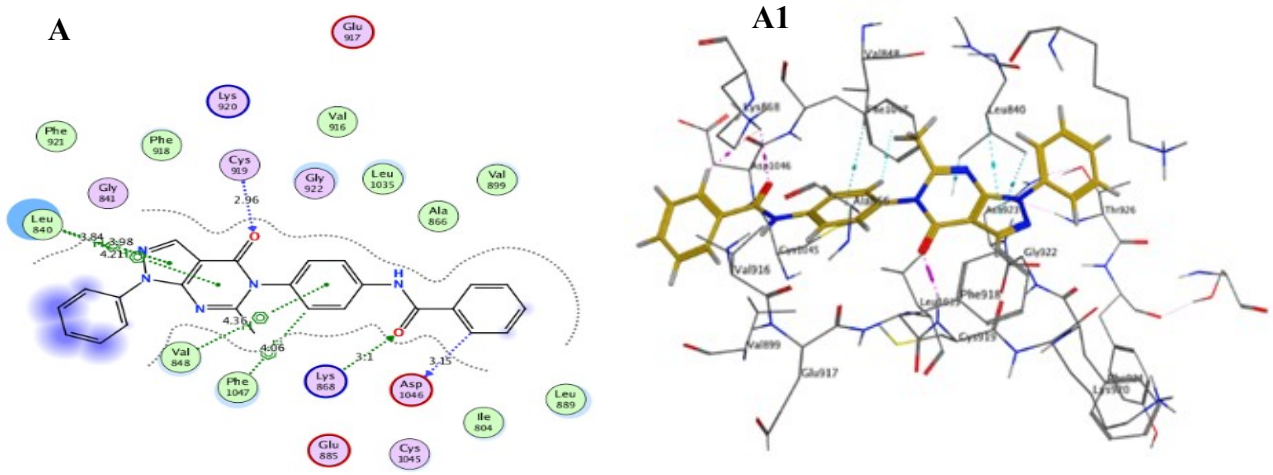
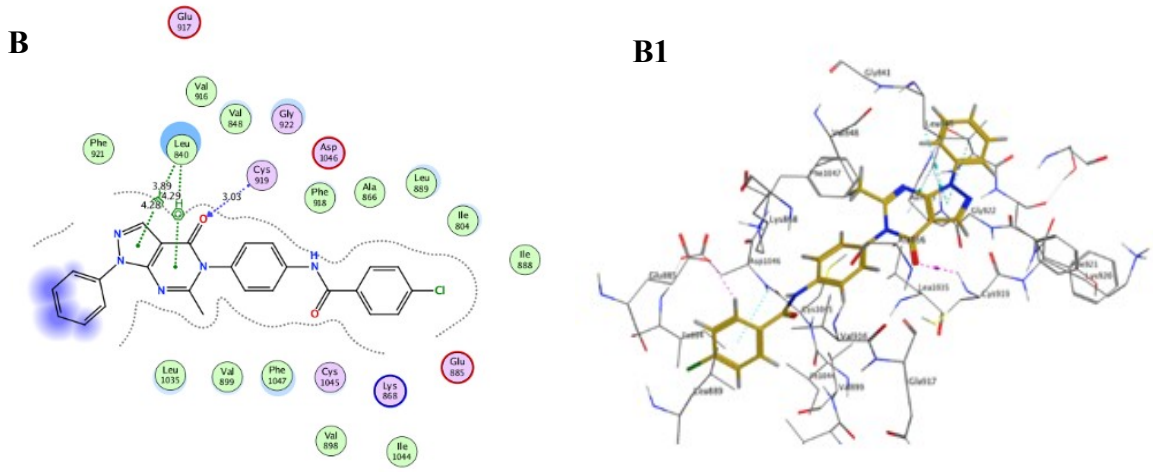


Fig. S1. **A**, **A1**) 2D and 3D representations of molecular docking of compound **12a** within VEGFR-2 binding site



Fig, S2. **B**, **B1)** 2D and 3D representations of molecular docking of compound **12c** within VEGFR-2 binding site.

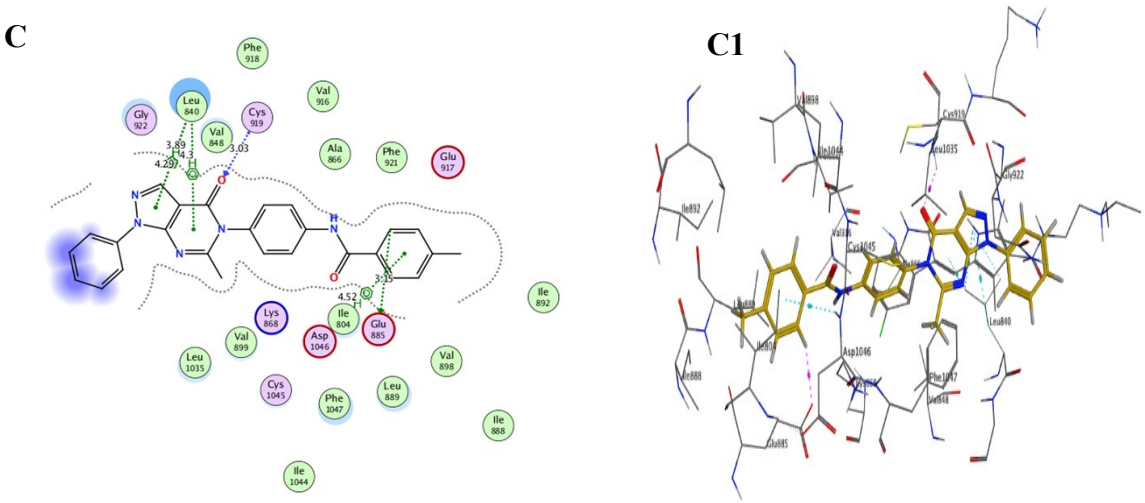
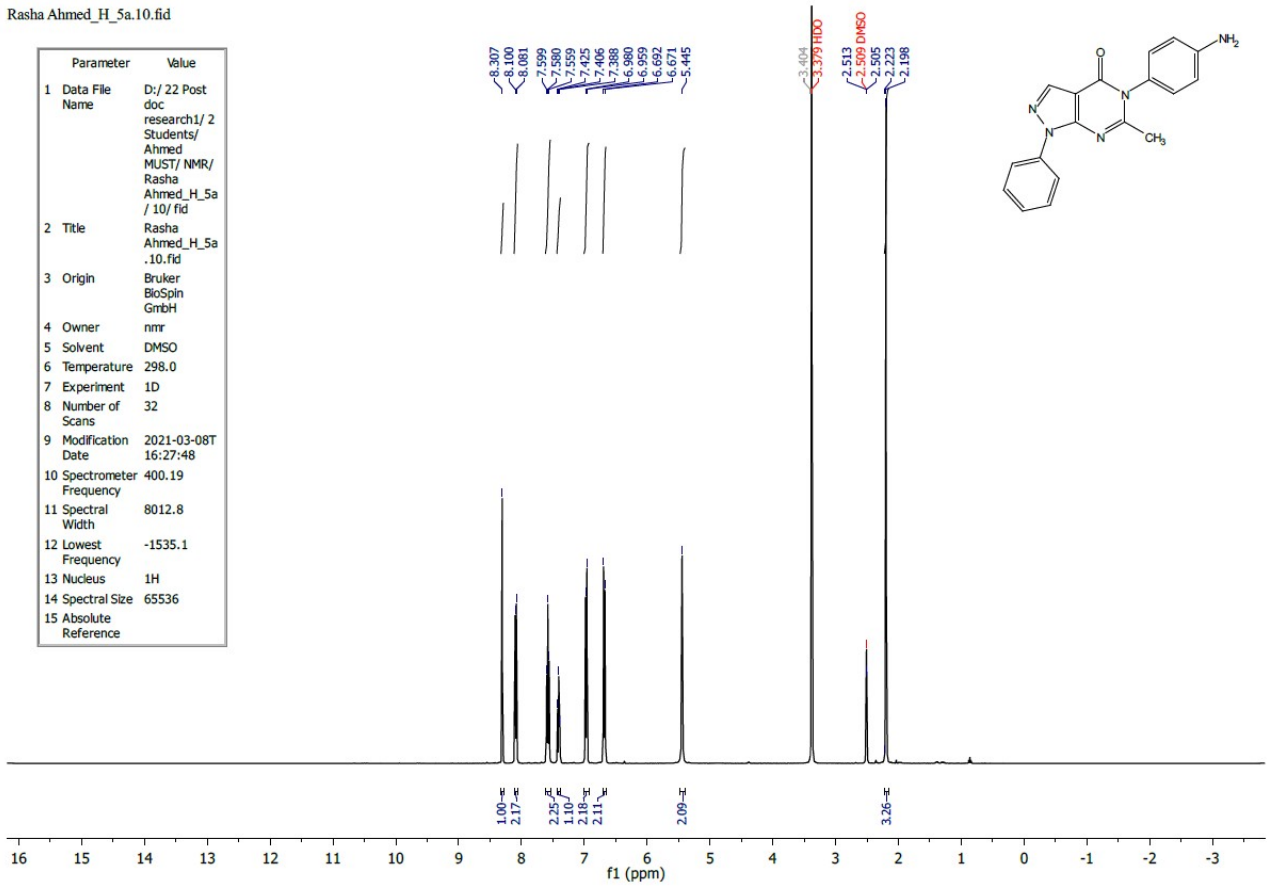


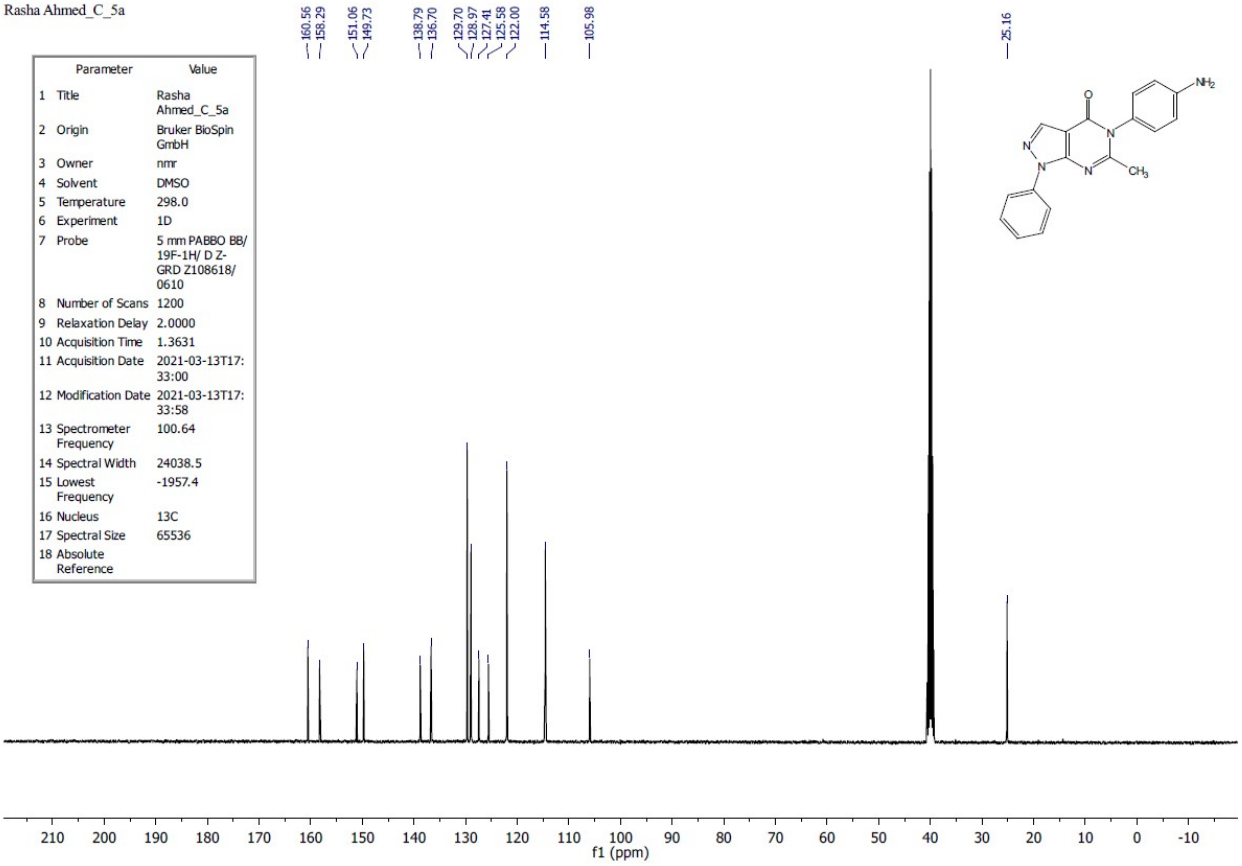
Fig. S3. **C**, **C1**) 2D and 3D representation of molecular docking of compound **12d** within VEGFR-2 binding site.

3- Chemistry: ^{1}H NMR, ^{13}C NMR, and Mass spectra of the new derivatives.

Compound 4



Rasha Ahmed_C_5a



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16	Nucleus	13C
17	Spectral Size	65536
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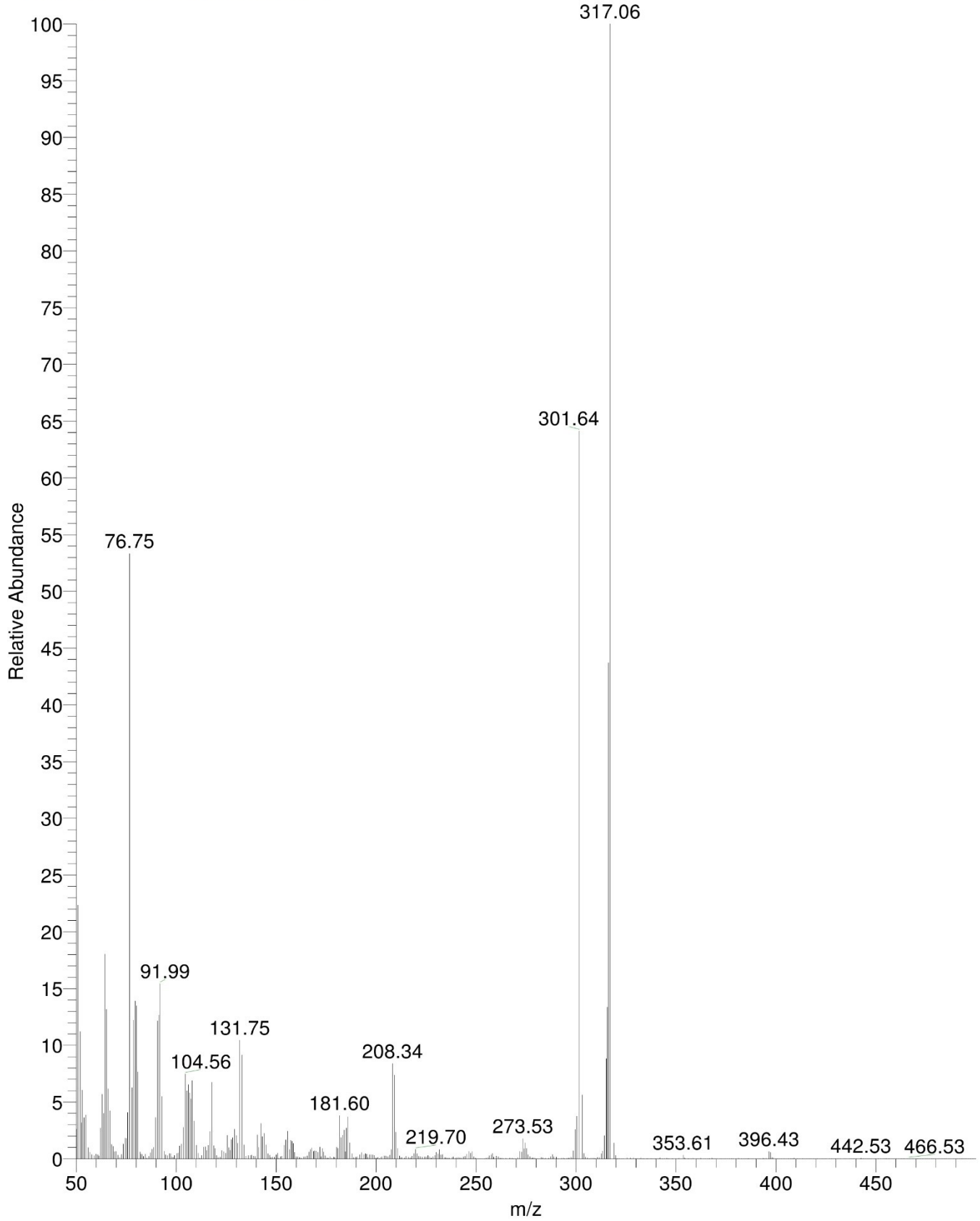

Compound 6

Ahmed M-AbdElHamied_H_Ch

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3	Owner	nmr
4	Solvent	DMSO
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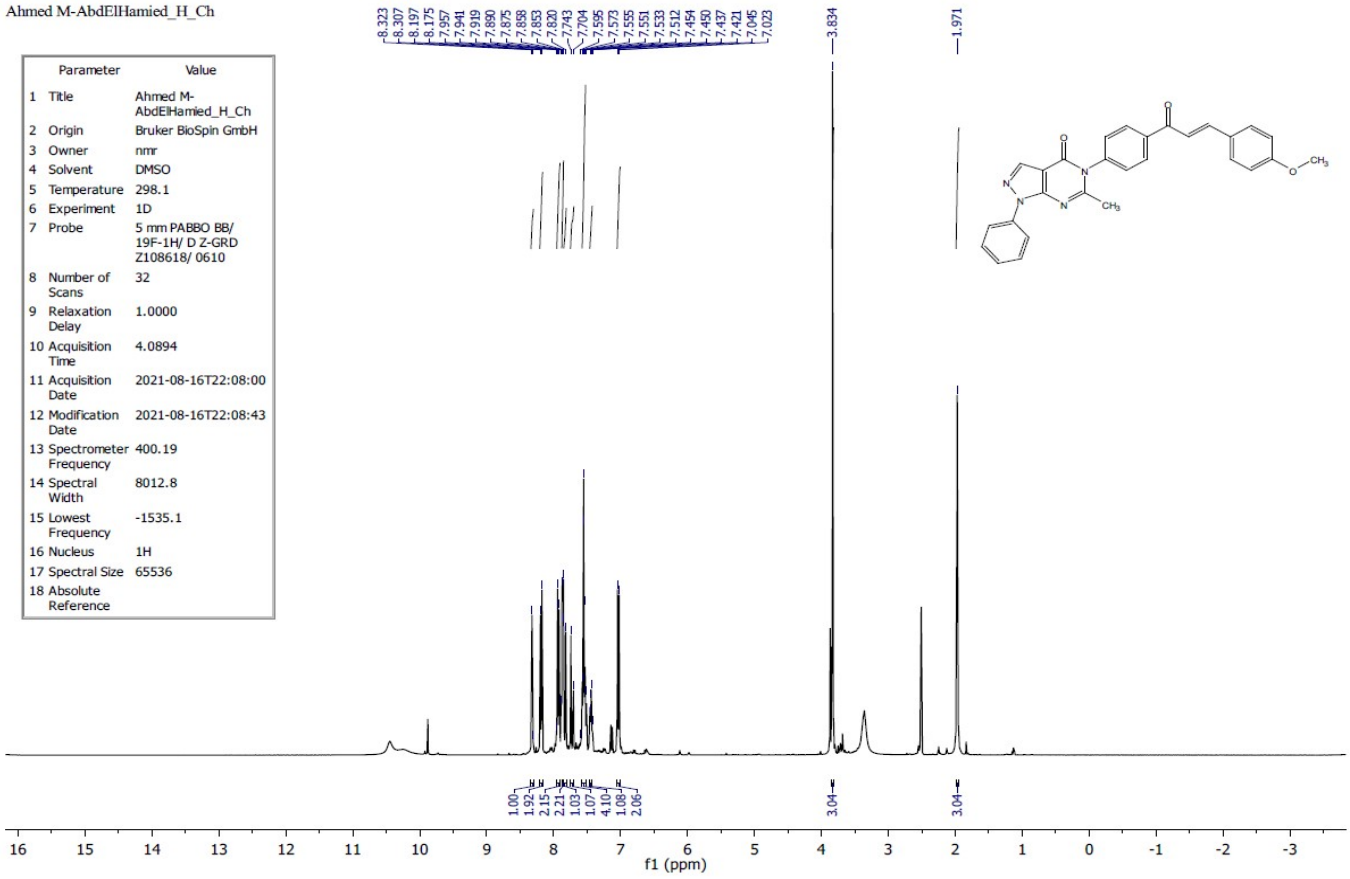
17	Spectral Size	65536
18	Absolute Reference	



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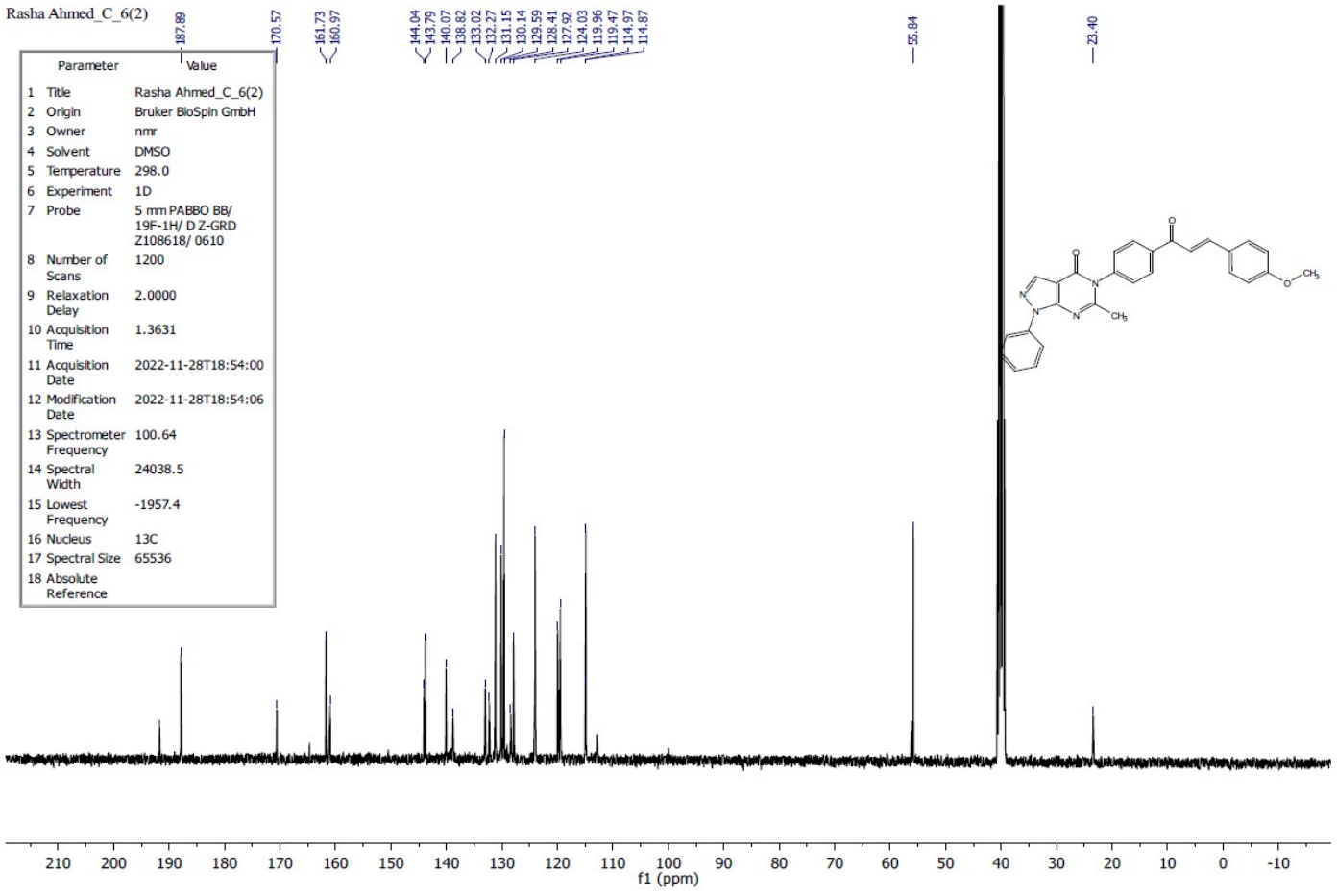
16	Nucleus	13C
17	Spectral Size	65536
18	Absolute Reference	



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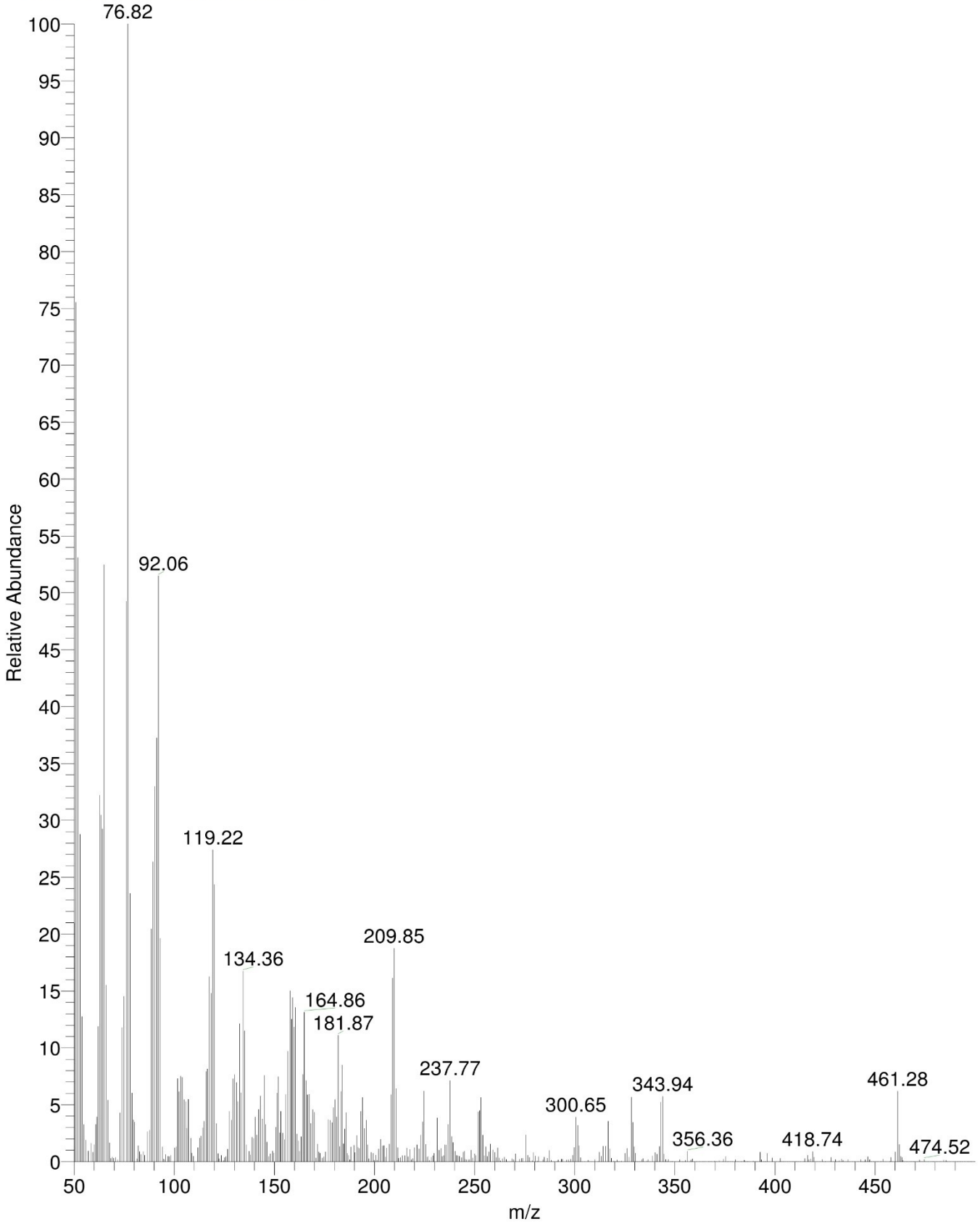

Compound 7

Rasha Ahmed_H_P-OCH3

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4	Solvent	DMSO
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6	Experiment	1D
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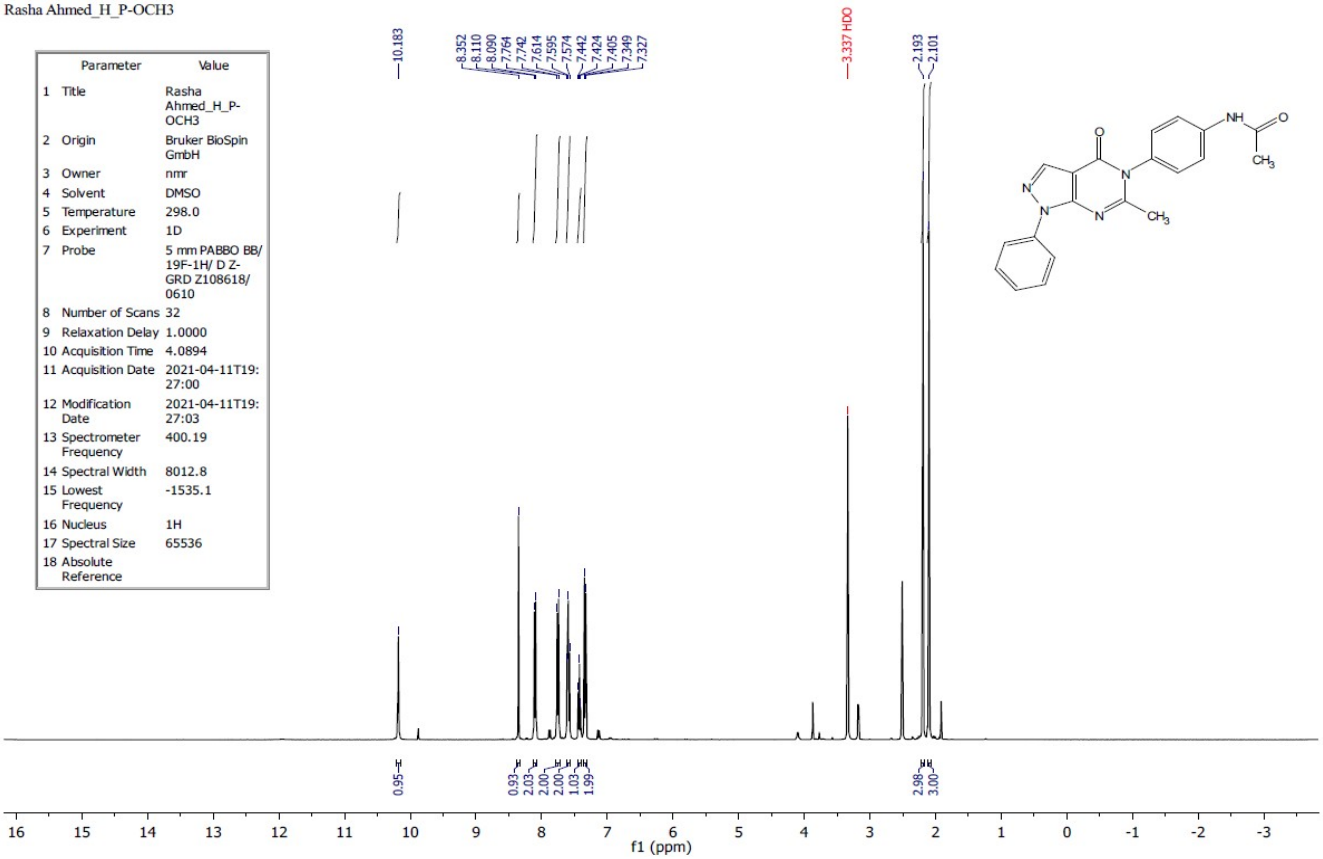
17	Spectral Size	65536
18	Absolute Reference	



Rasha Ahmed_C_P-OCH3

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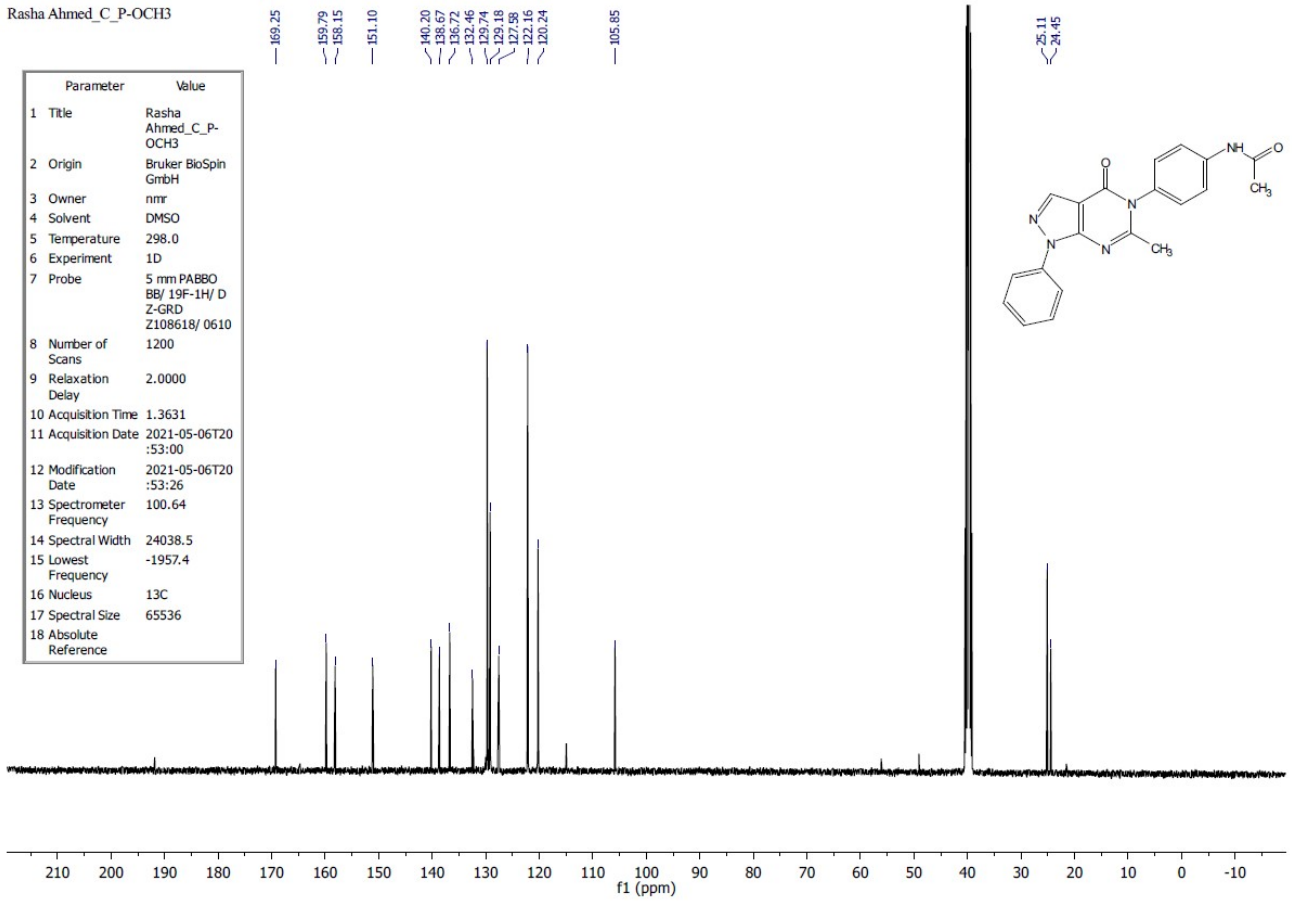

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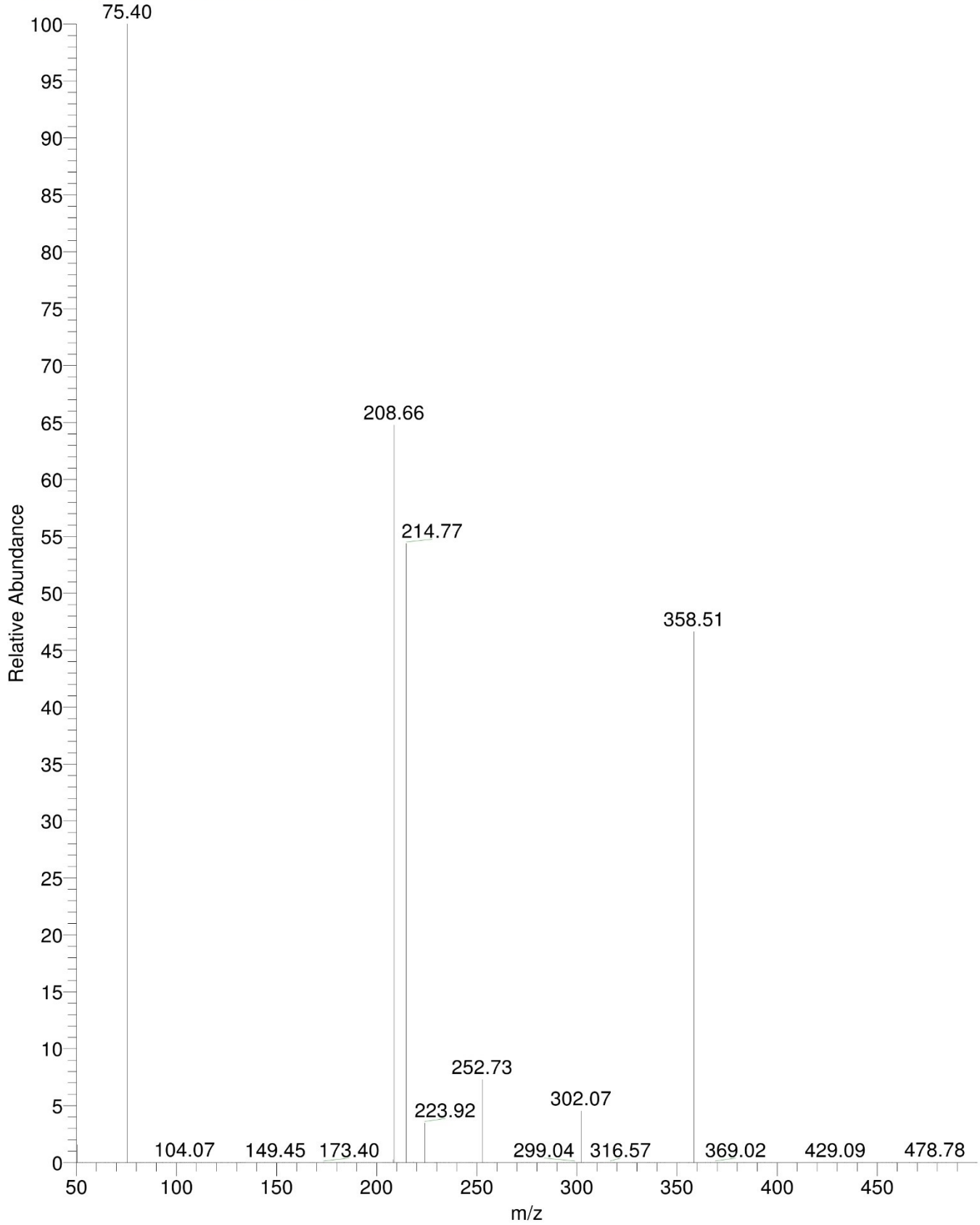

Compound 8

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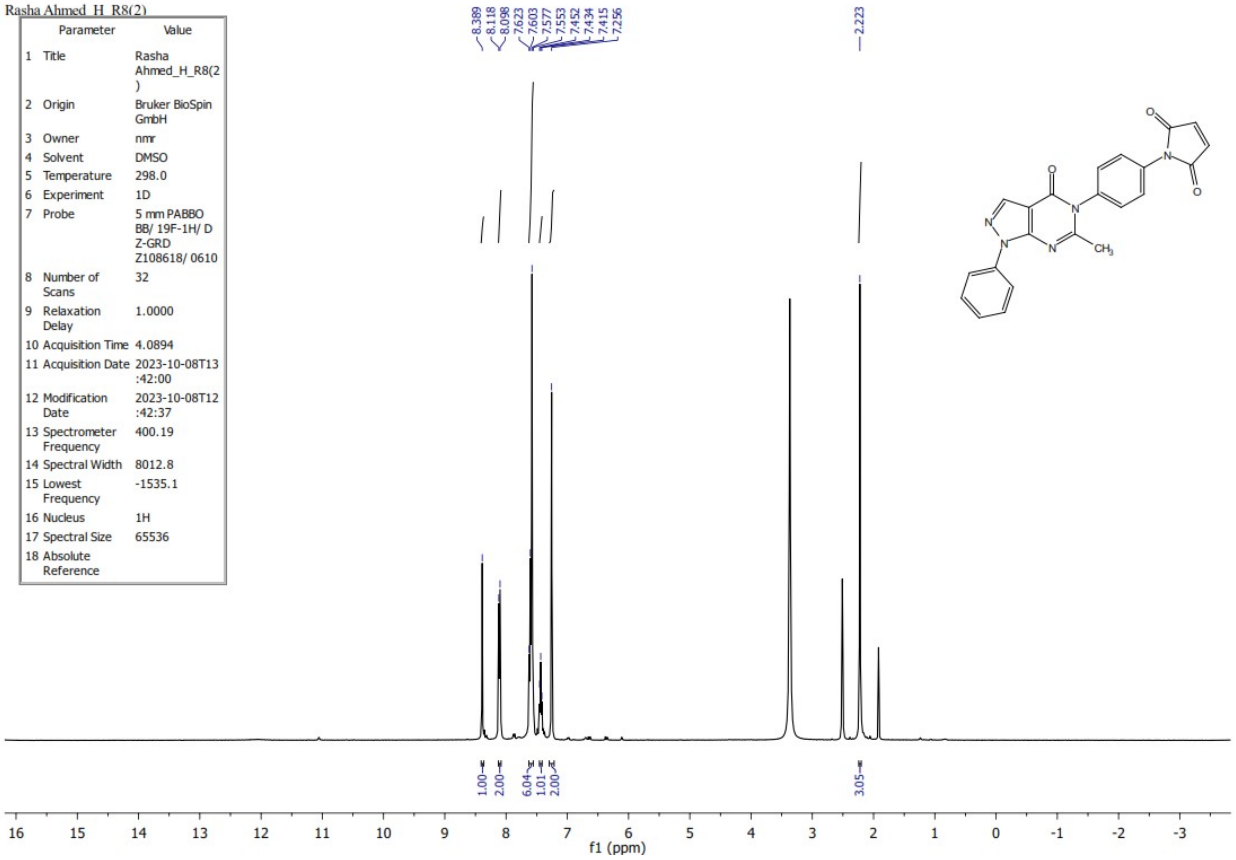

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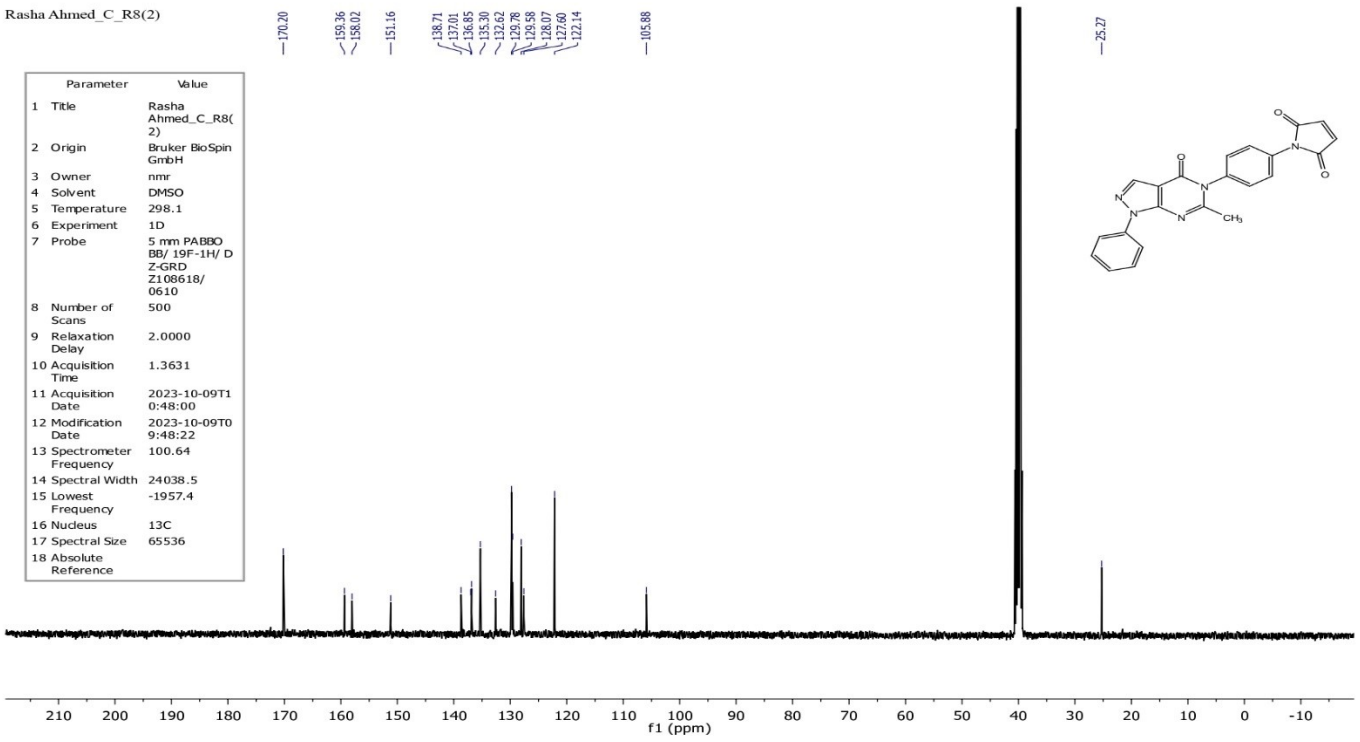

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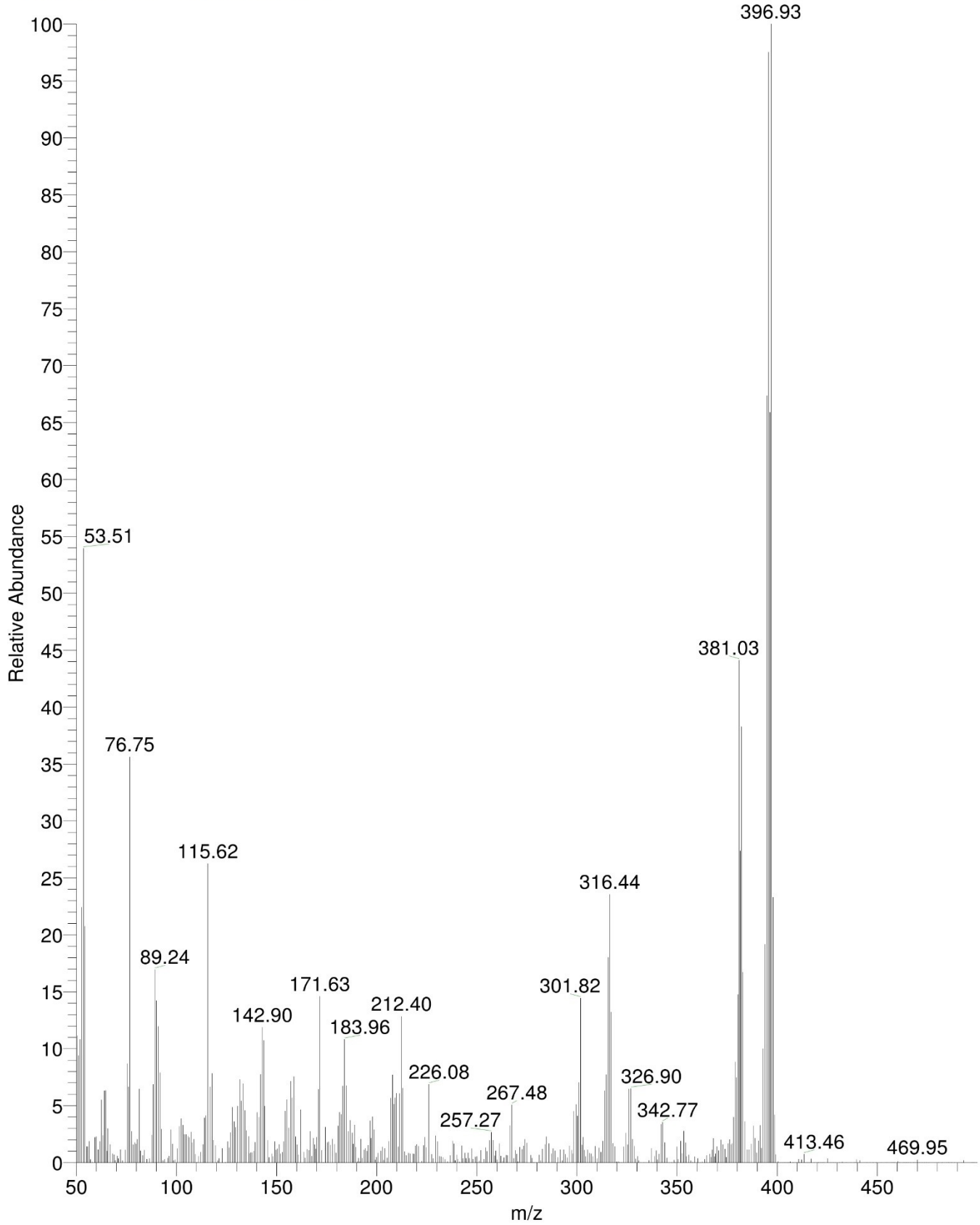
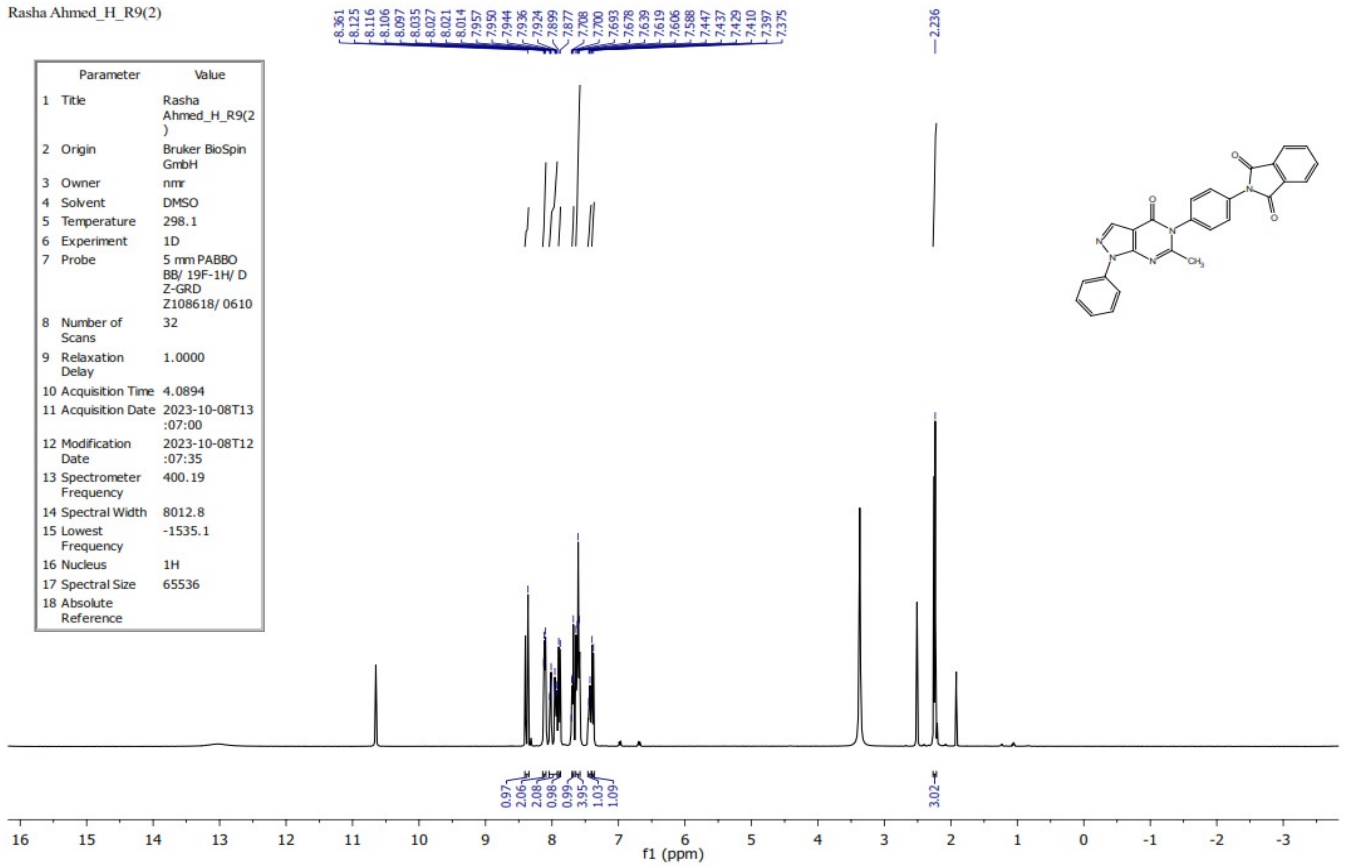


Compound 9



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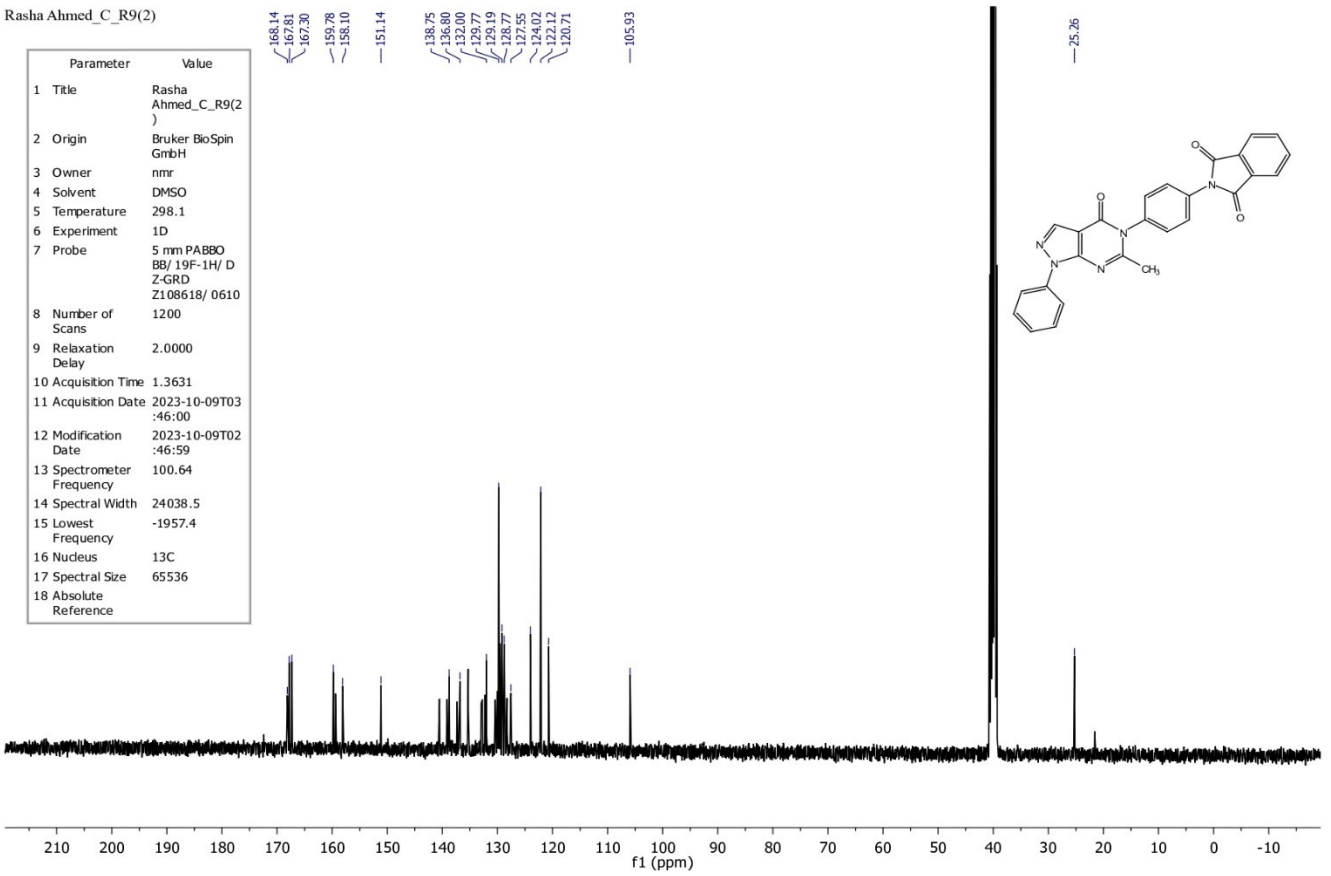

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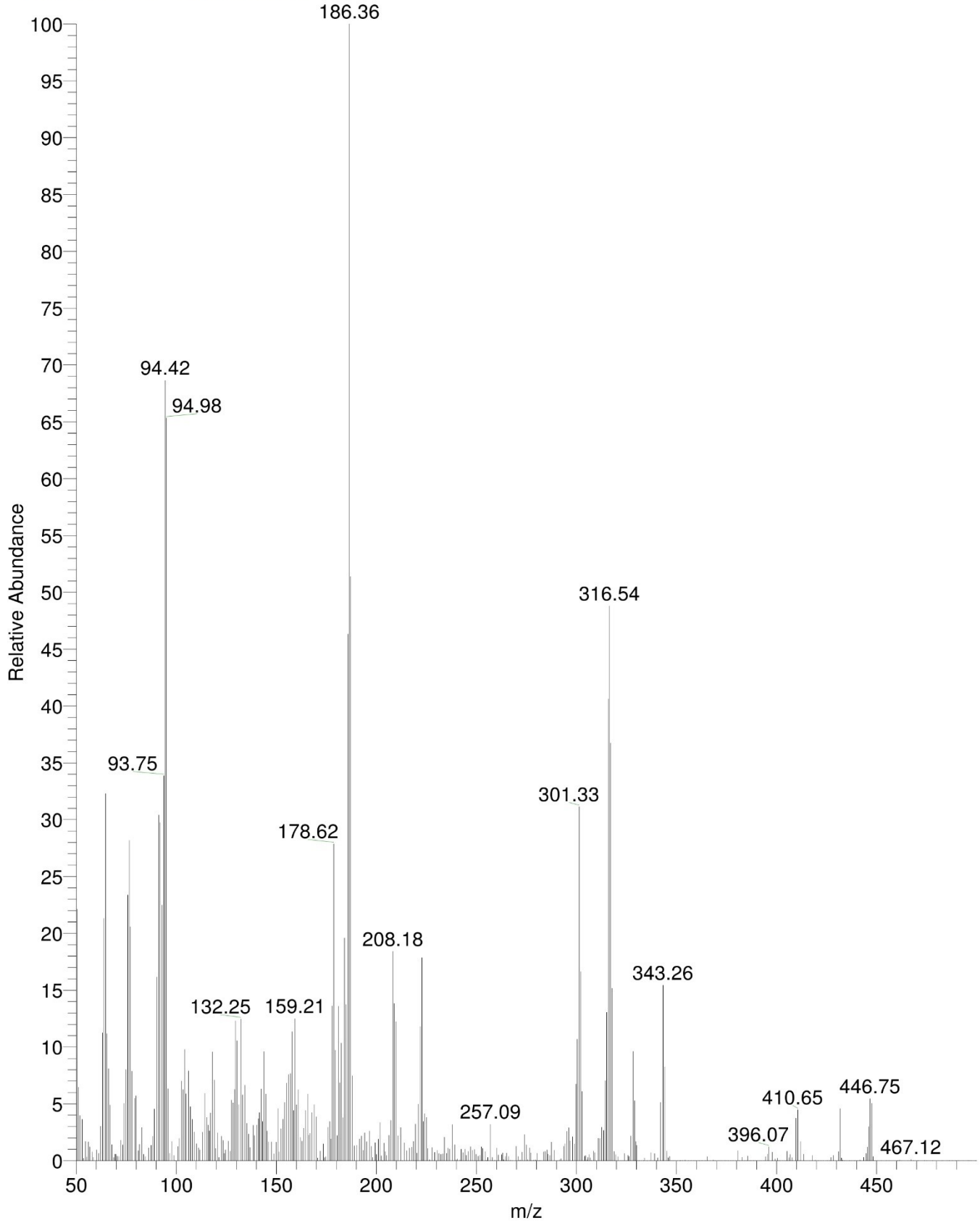
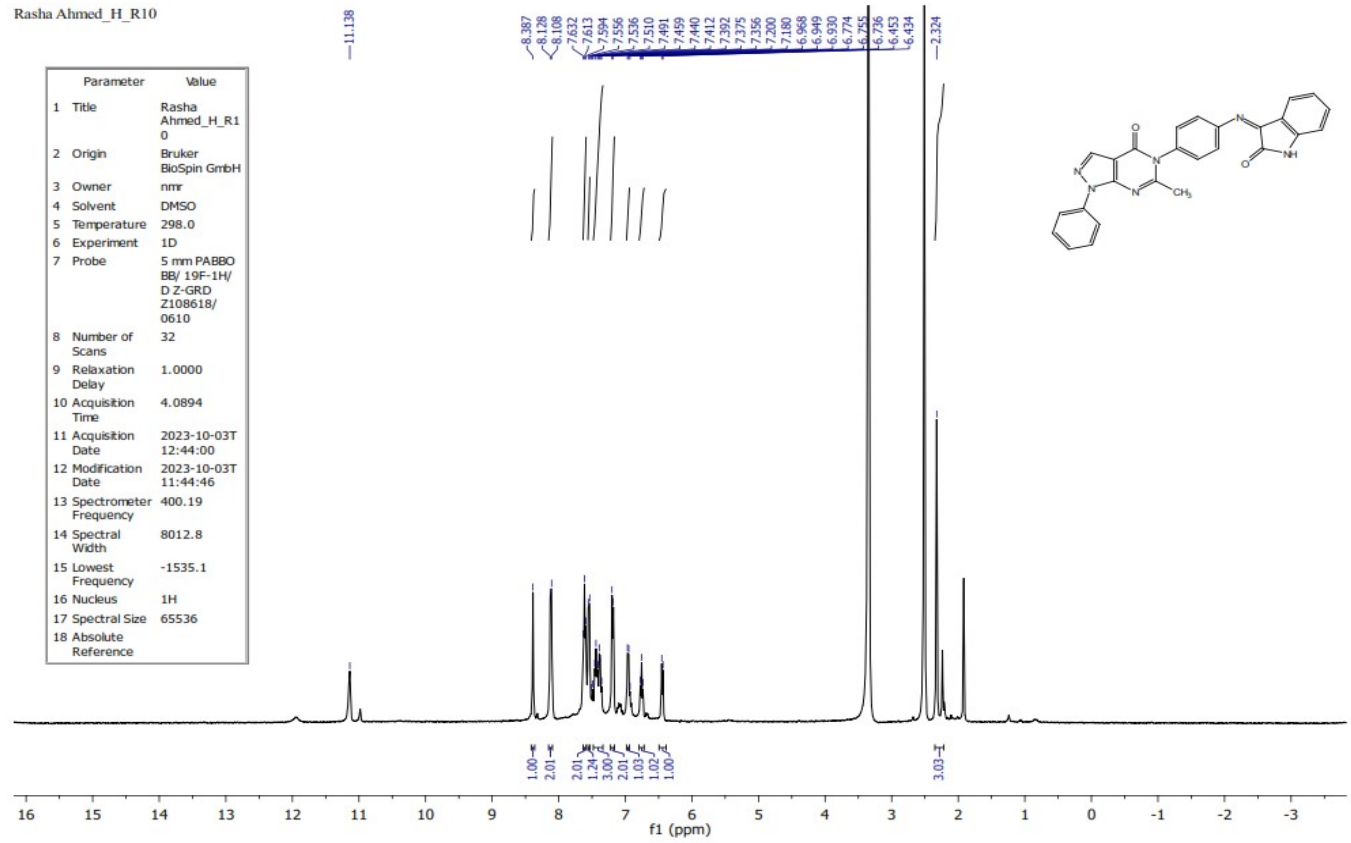


Compound 10



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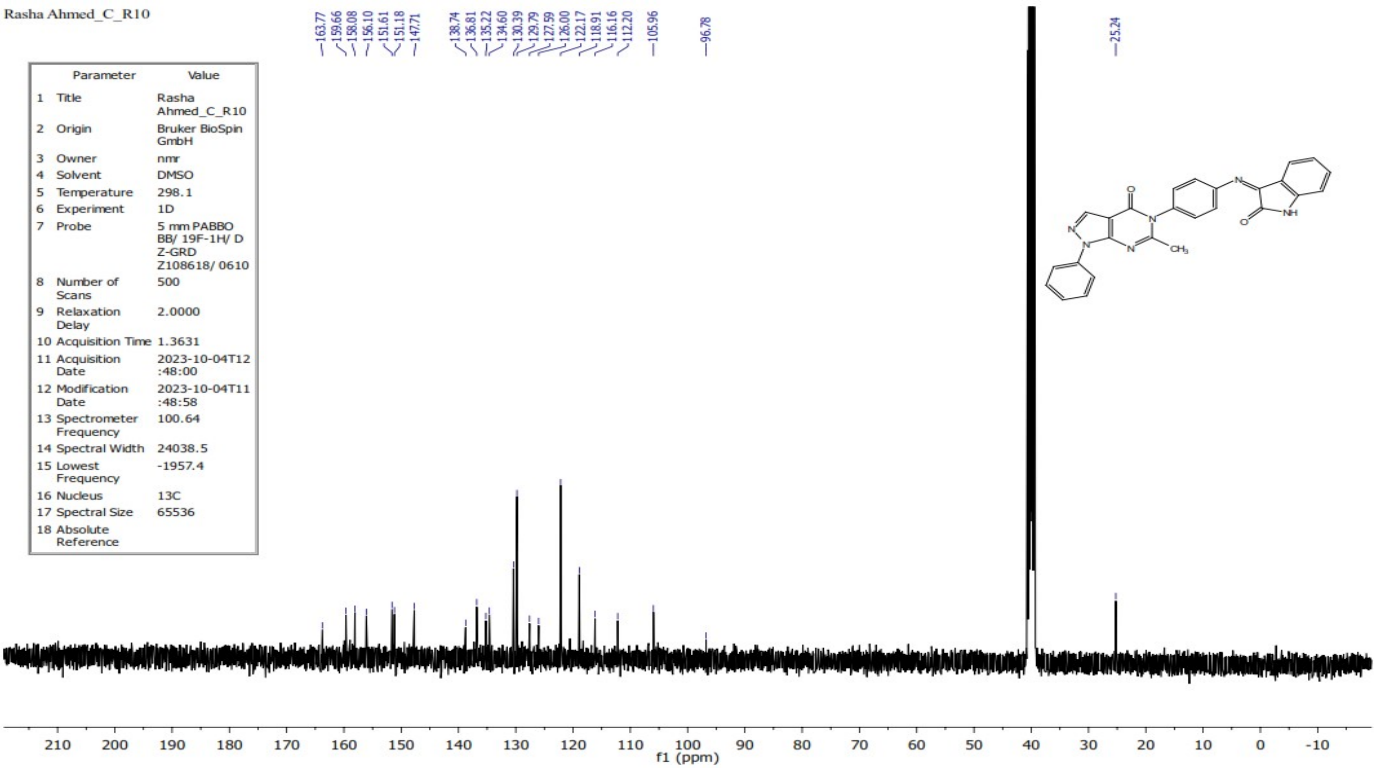
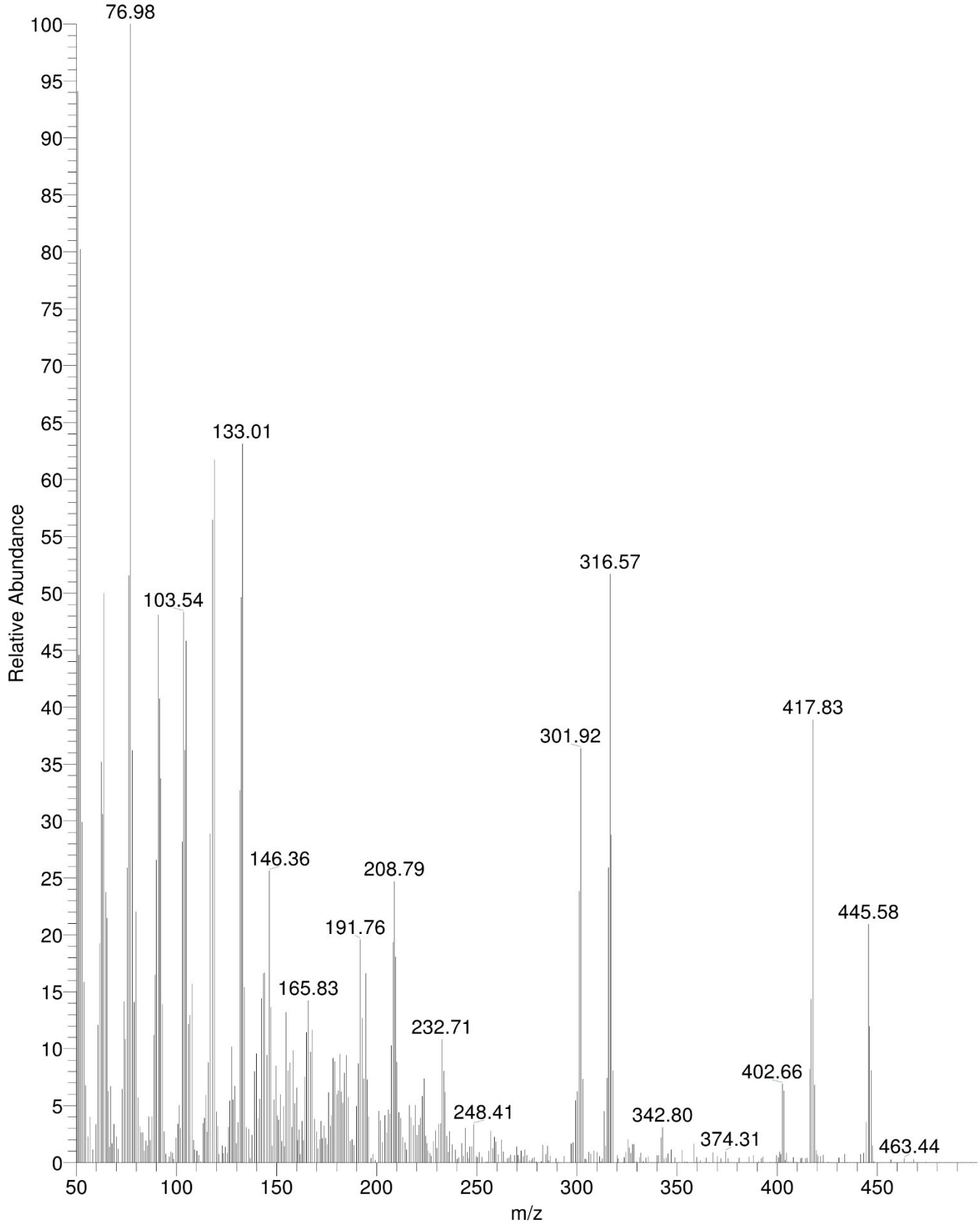
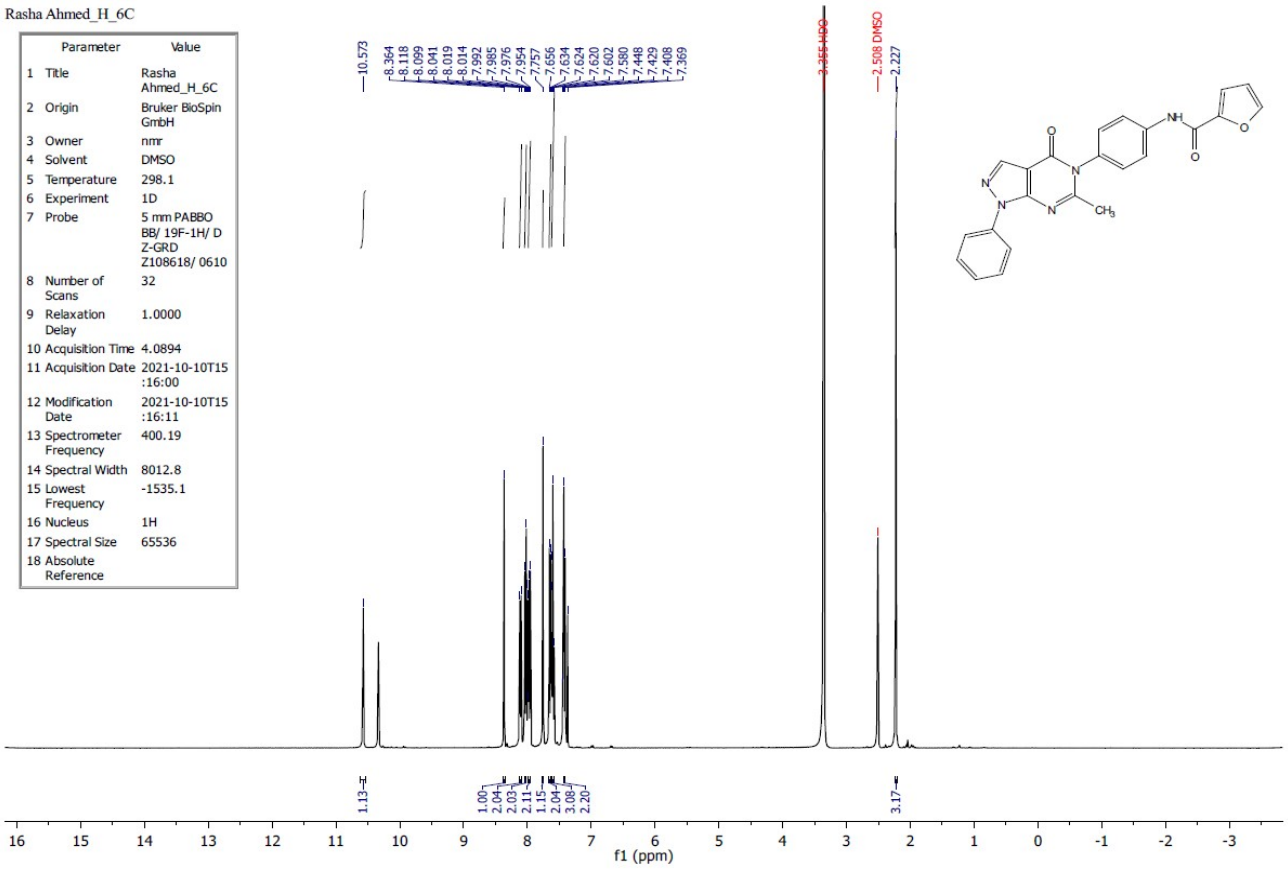


	Parameter	Value
1	Title	Rasha Ahmed_C_R10
2	Origin	Bruker BioSpin GmbH
3	Owner	nmr
4	Solvent	DMSO
5	Temperature	298.1
6	Experiment	1D
7	Probe	5 mm PABBO BB/ 19F-1H/ D Z-GRD Z108618/ 0610
8	Number of Scans	500
9	Relaxation Delay	2.0000
10	Acquisition Time	1.3631
11	Acquisition Date	2023-10-04T12 :48:00
12	Modification Date	2023-10-04T11 :48:58
13	Spectrometer Frequency	100.64
14	Spectral Width	24038.5
15	Lowest Frequency	-1957.4
16	Nucleus	13C
17	Spectral Size	65536
18	Absolute Reference	

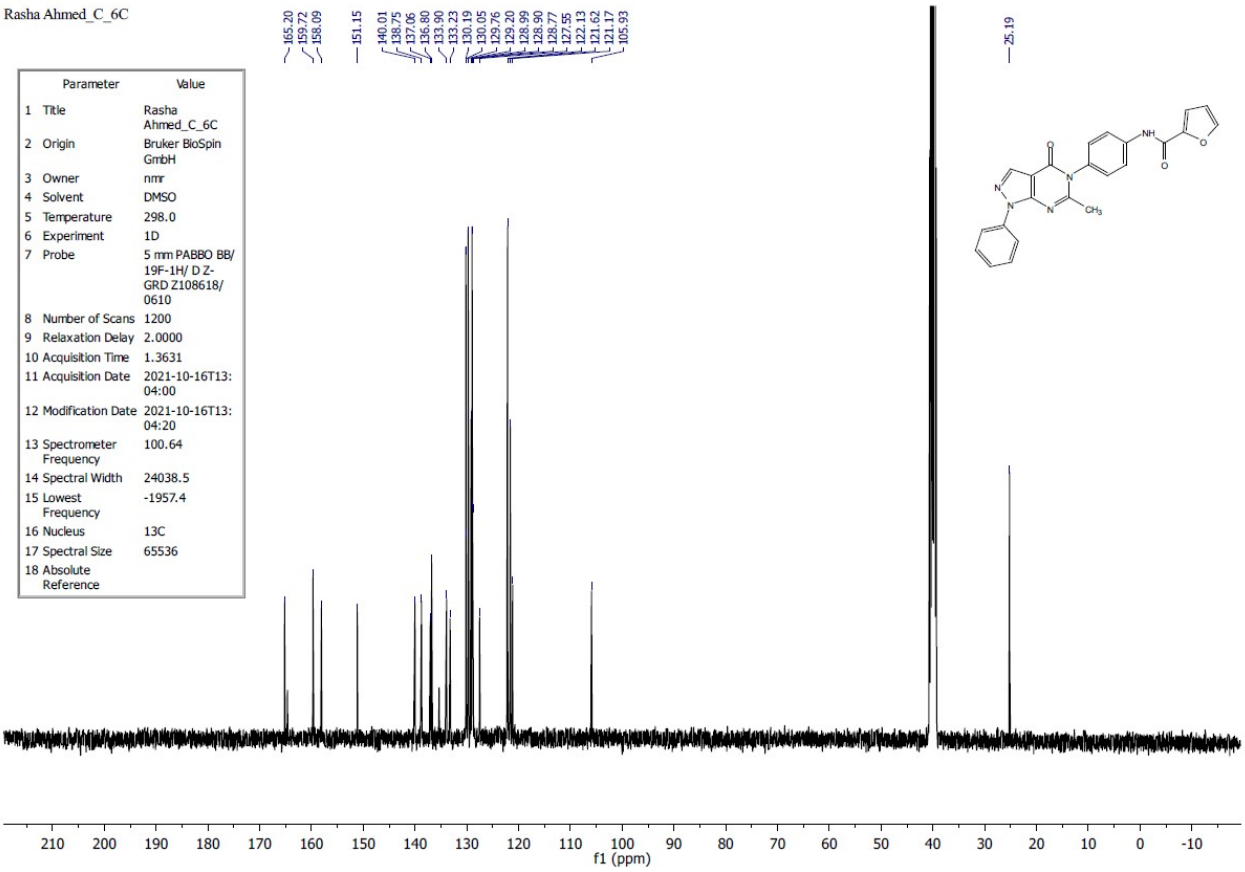
Ahamed-10 #1142 RT: 3.91 AV: 1 NL: 4.19E4
T: {0,0} + c EI Full ms [50.00-500.00]



Compound 11

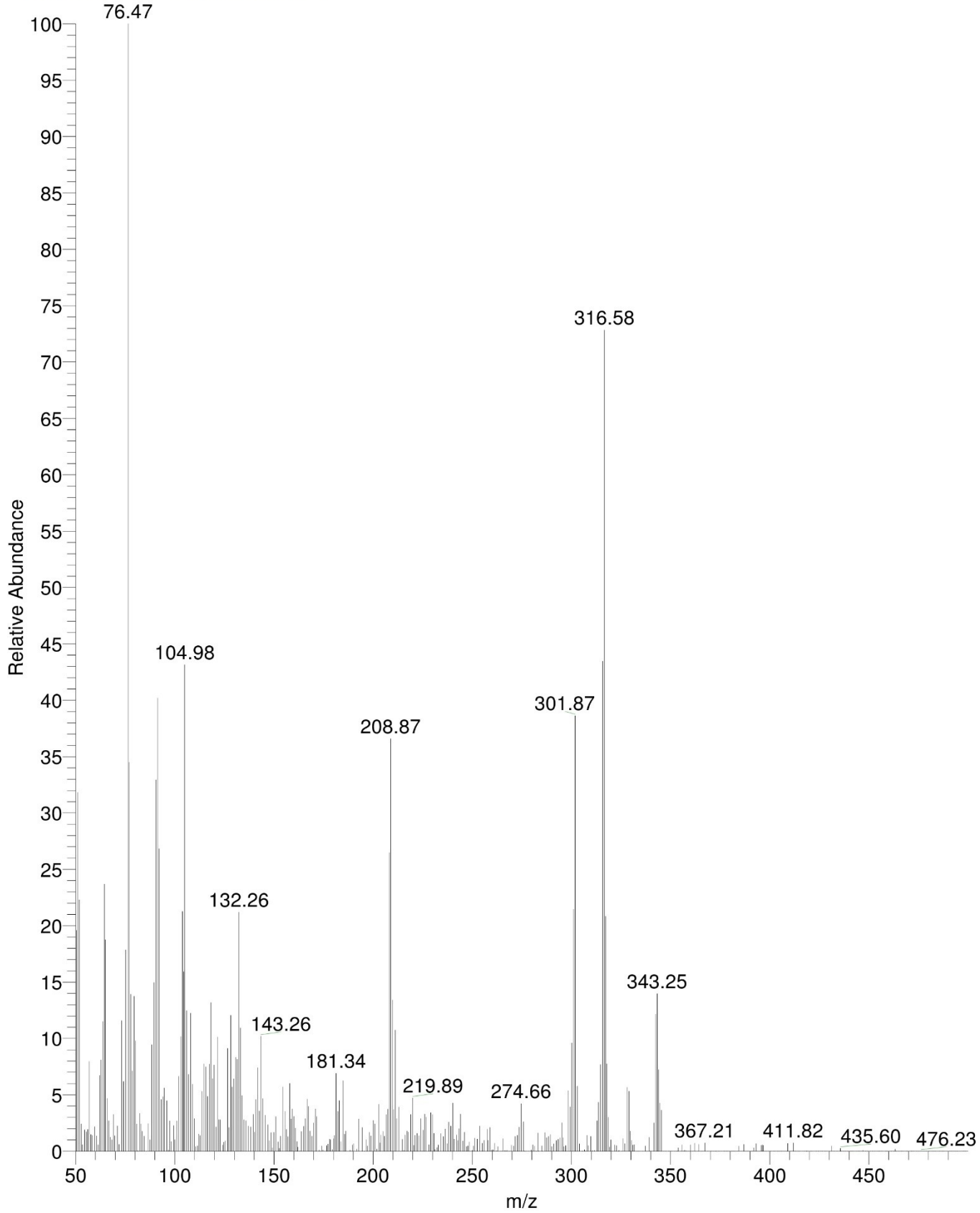

Rasha Ahmed_H_6C
Parameter Value
1 Title Rasha Ahmed_H_6C
2 Origin Bruker BioSpin GmbH
3 Owner nmr
4 Solvent DMSO
5 Temperature 298.1
6 Experiment 1D
7 Probe 5 mm PABBO BB/ 19F-1H/ D Z-GRD Z108618/ 0610
8 Number of Scans 32
9 Relaxation Delay 1.0000
10 Acquisition Time 4.0894
11 Acquisition Date 2021-10-10T15:16:00
12 Modification Date 2021-10-10T15:16:11
13 Spectrometer Frequency 400.19
14 Spectral Width 8012.8
15 Lowest Frequency -1535.1
16 Nucleus 1H
17 Spectral Size 65536
18 Absolute Reference
10.573
8.364
8.118
8.099
8.041
8.019
8.014
7.992
7.985
7.976
7.954
7.757
7.656
7.634
7.624
7.620
7.602
7.580
7.448
7.429
7.408
7.369
3.355 H2O
2.508 DMSO
2.227
1.13
1.00
2.04
2.03
2.11
1.15
2.04
3.08
2.20
3.17
f1 (ppm)

Rasha Ahmed_C_6C



	Parameter	Value
1	Title	Rasha Ahmed_C_6C
2	Origin	Bruker BioSpin GmbH
3	Owner	nmr
4	Solvent	DMSO
5	Temperature	298.0
6	Experiment	1D
7	Probe	5 mm PABBO BB/ 19F-1H/ D Z-GRD Z108618/ 0610
8	Number of Scans	1200
9	Relaxation Delay	2.0000
10	Acquisition Time	1.3631
11	Acquisition Date	2021-10-16T13:04:00
12	Modification Date	2021-10-16T13:04:20
13	Spectrometer Frequency	100.64
14	Spectral Width	24038.5
15	Lowest Frequency	-1957.4
16	Nucleus	13C
17	Spectral Size	65536
18	Absolute Reference	

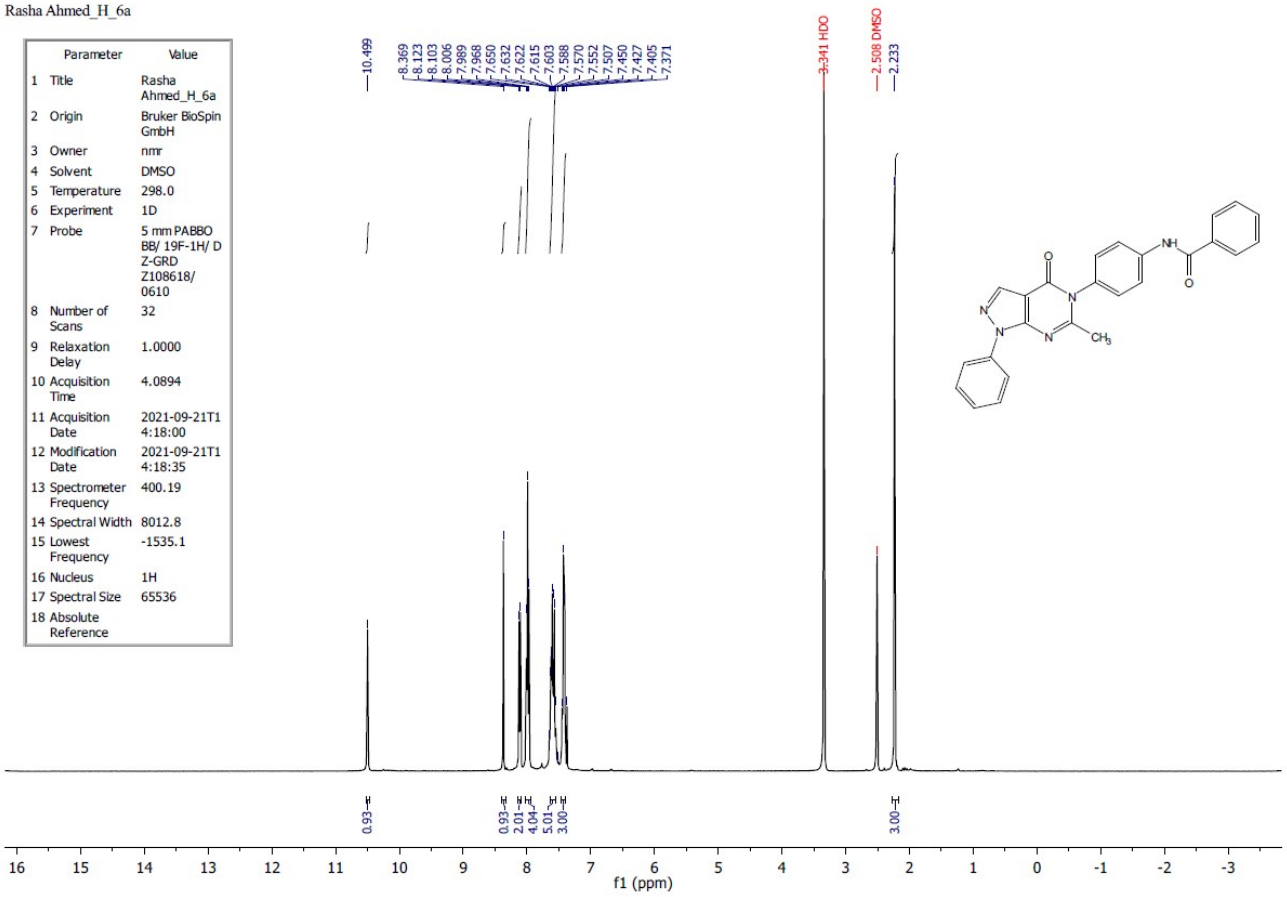
Ahamed-11 #672 RT: 2.32 AV: 1 NL: 3.42E4
T: {0,0} + c EI Full ms [50.00-500.00]

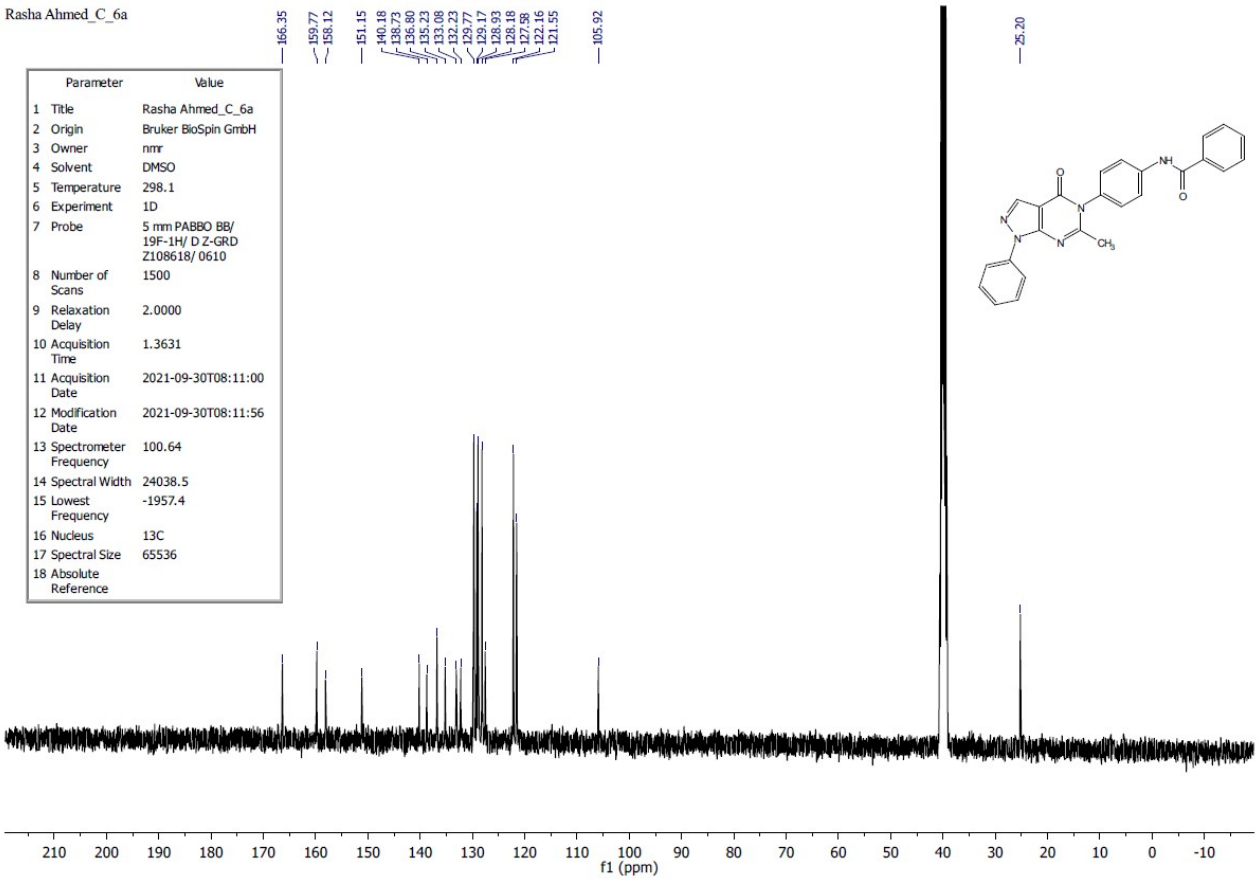


Compound 12a

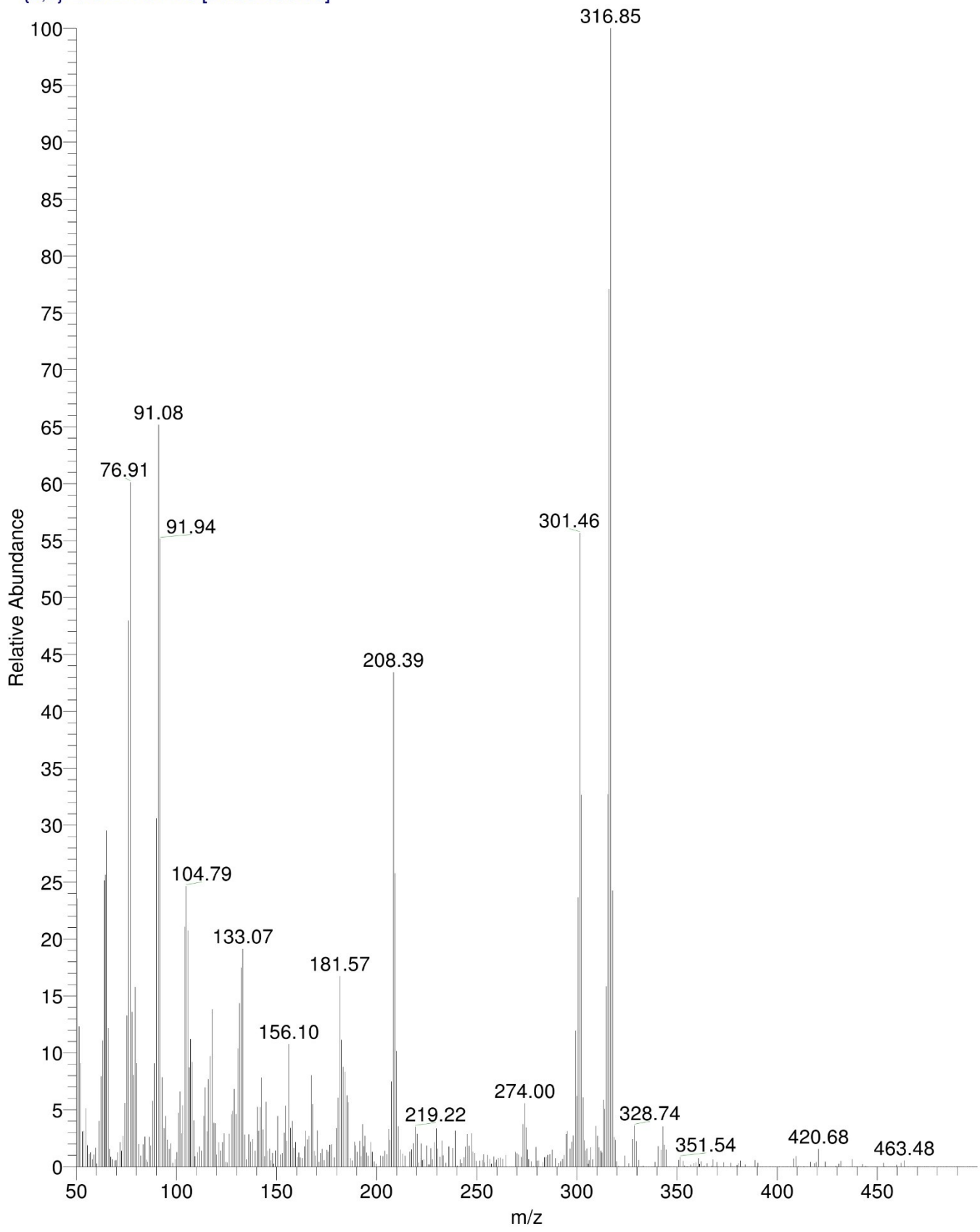
Rasha Ahmed_H_6a

	Parameter	Value
1	Title	Rasha Ahmed_H_6a
2	Origin	Bruker BioSpin GmbH
3	Owner	nmr
4	Solvent	DMSO
5	Temperature	298.0
6	Experiment	1D
7	Probe	5 mm PABBO BB/ 19F-1H/ D Z-GRD Z108618/ 0610
8	Number of Scans	32
9	Relaxation Delay	1.0000
10	Acquisition Time	4.0894
11	Acquisition Date	2021-09-21T14:18:00
12	Modification Date	2021-09-21T14:18:35
13	Spectrometer Frequency	400.19
14	Spectral Width	8012.8
15	Lowest Frequency	-1535.1
16	Nucleus	1H
17	Spectral Size	65536
18	Absolute Reference	

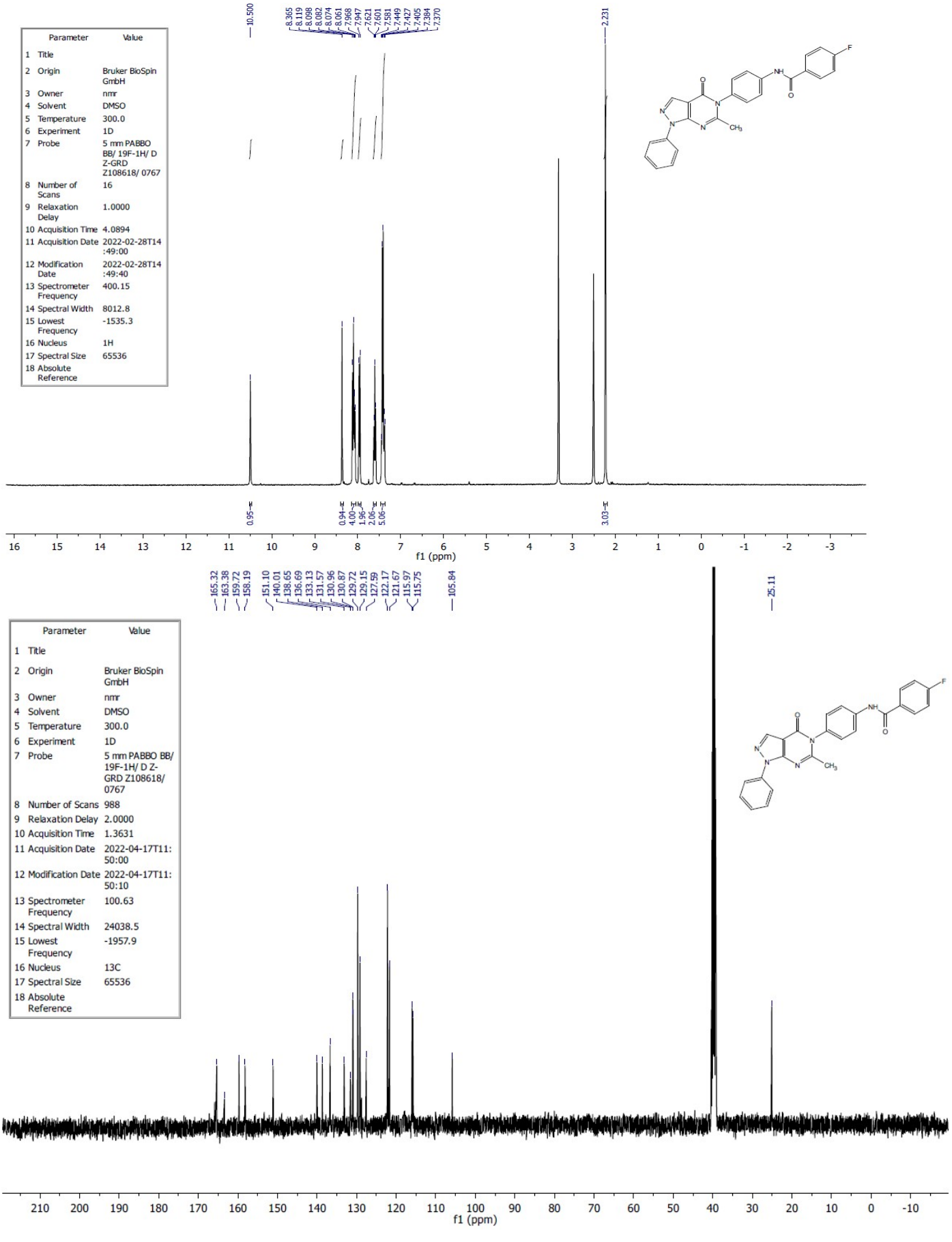



Rasha Ahmed_C_6a
166.35
159.77
158.12
151.15
140.18
138.73
136.80
135.23
133.08
132.23
129.77
129.17
128.93
128.18
127.58
122.16
121.55
105.92
25.20
Parameter Value
1 Title Rasha Ahmed_C_6a
2 Origin Bruker BioSpin GmbH
3 Owner nmr
4 Solvent DMSO
5 Temperature 298.1
6 Experiment 1D
7 Probe 5 mm PABBO BB/ 19F-1H/ D Z-GRD Z108618/ 0610
8 Number of Scans 1500
9 Relaxation Delay 2.0000
10 Acquisition Time 1.3631
11 Acquisition Date 2021-09-30T08:11:00
12 Modification Date 2021-09-30T08:11:56
13 Spectrometer Frequency 100.64
14 Spectral Width 24038.5
15 Lowest Frequency -1957.4
16 Nucleus 13C
17 Spectral Size 65536
18 Absolute Reference
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

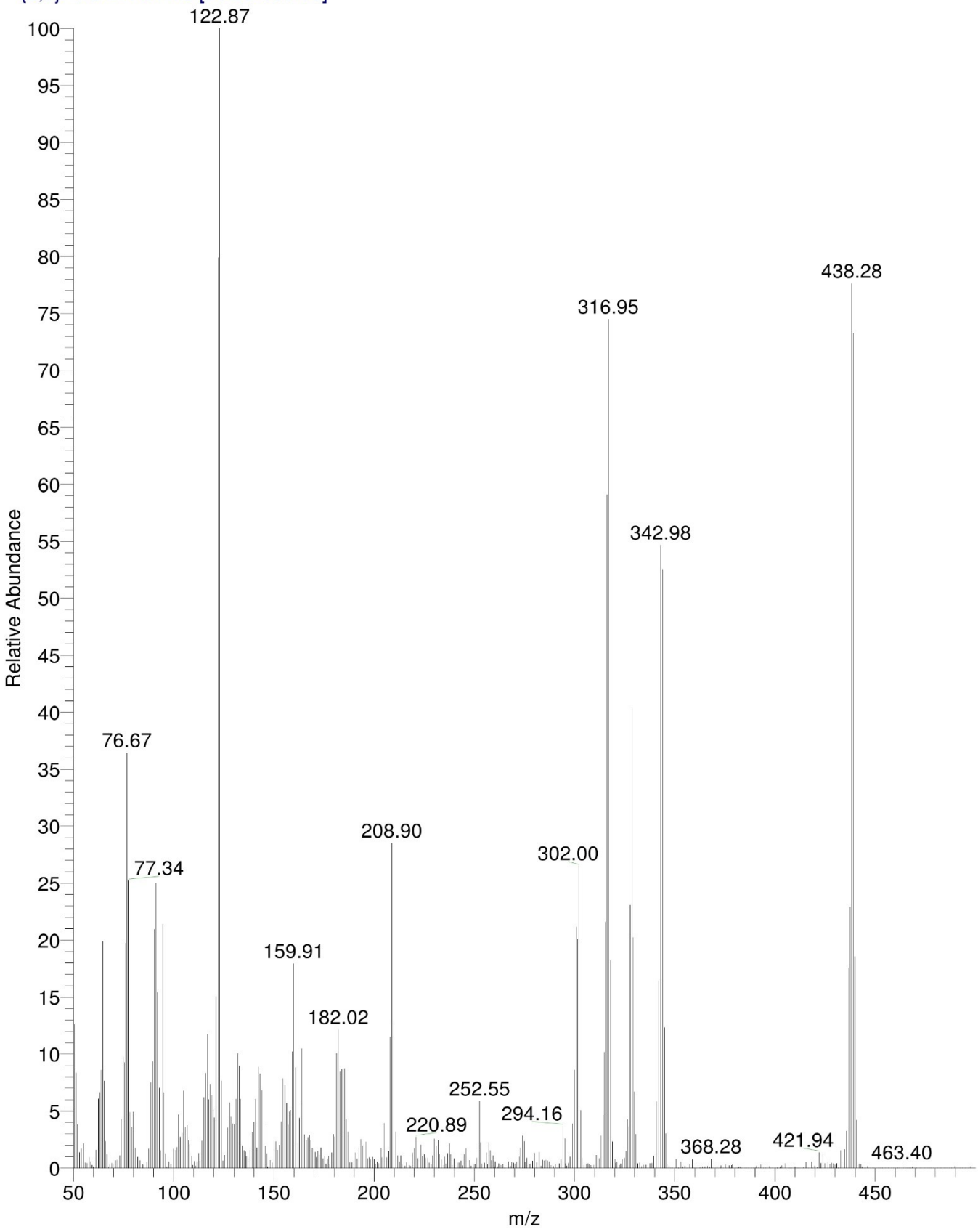
Ahamed-12a #776 RT: 2.67 AV: 1 NL: 4.36E4
T: {0,0} + c EI Full ms [50.00-500.00]



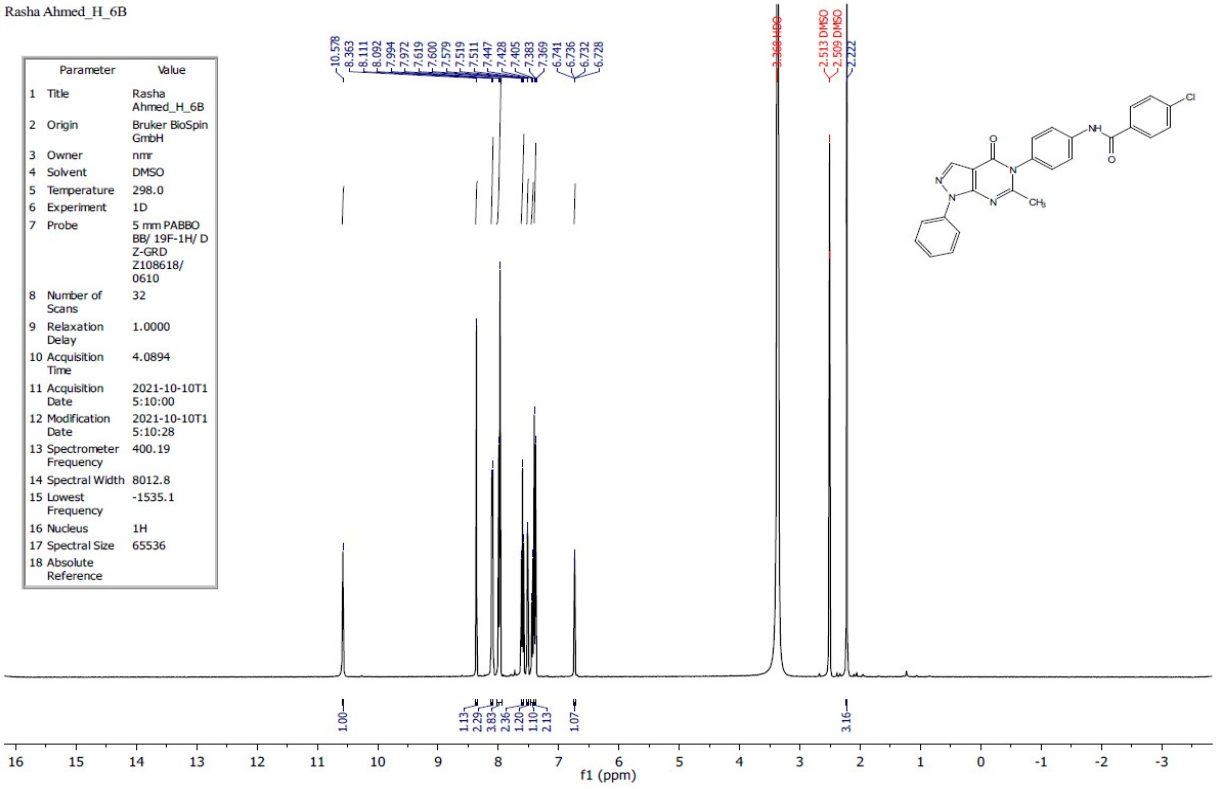
Compound 12b

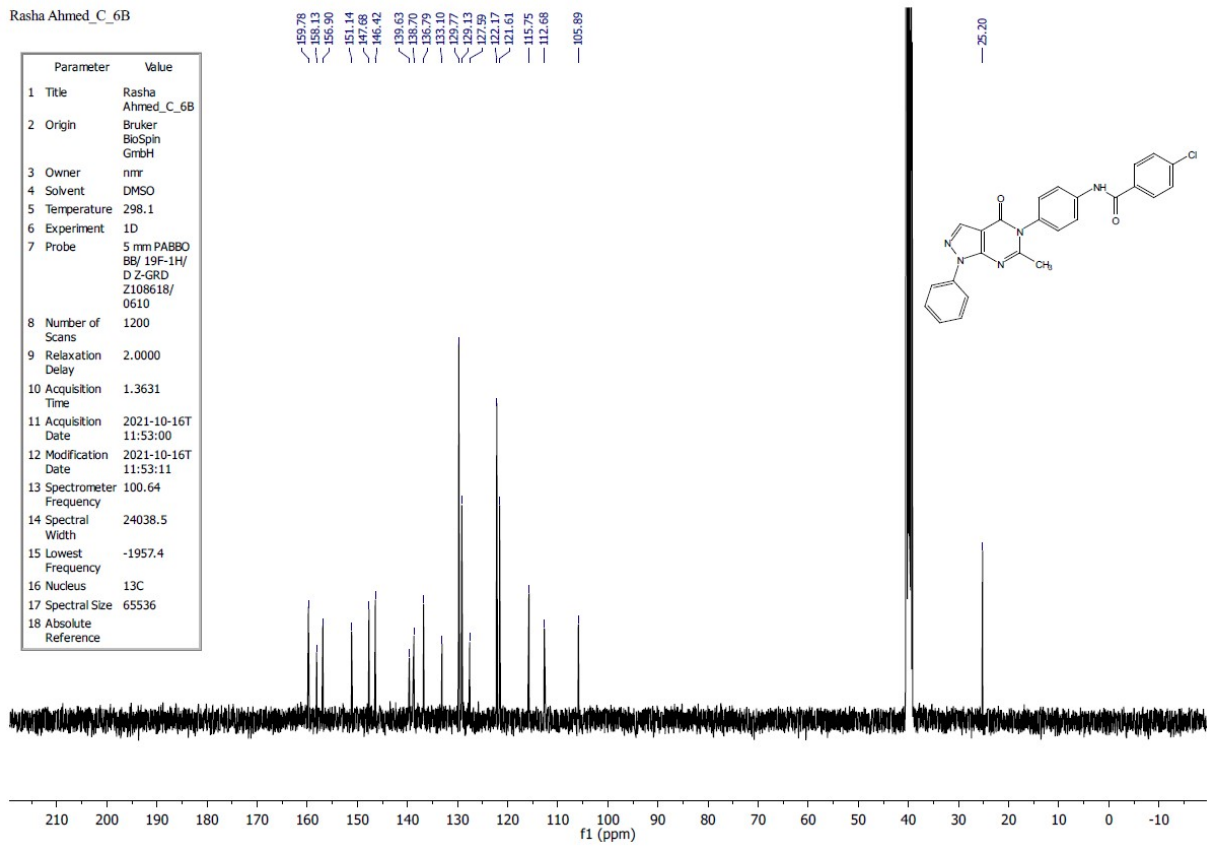


Ahamed-12b #1195 RT: 4.10 AV: 1 NL: 1.23E5
T: {0,0} + c EI Full ms [50.00-500.00]

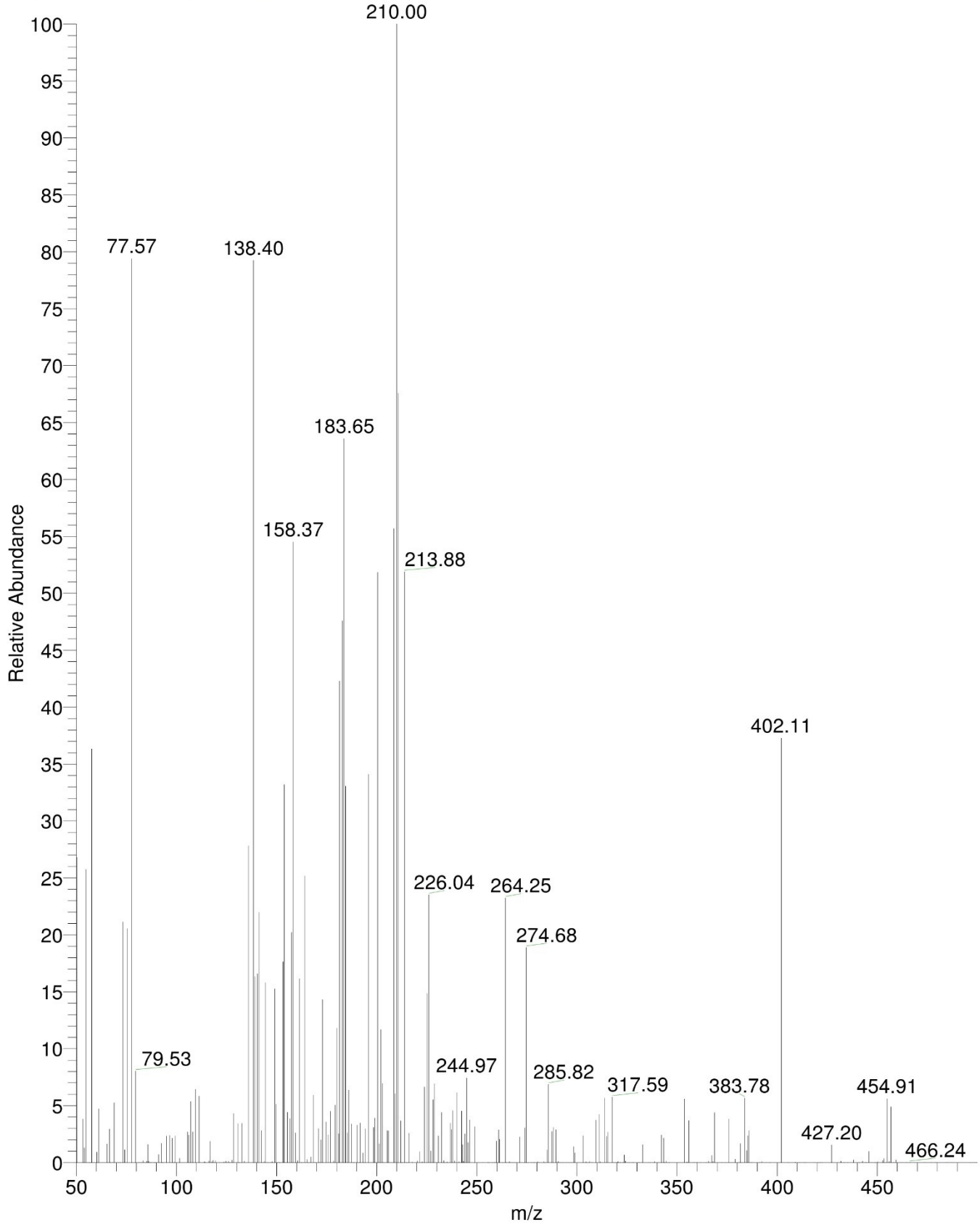


Compound 12c

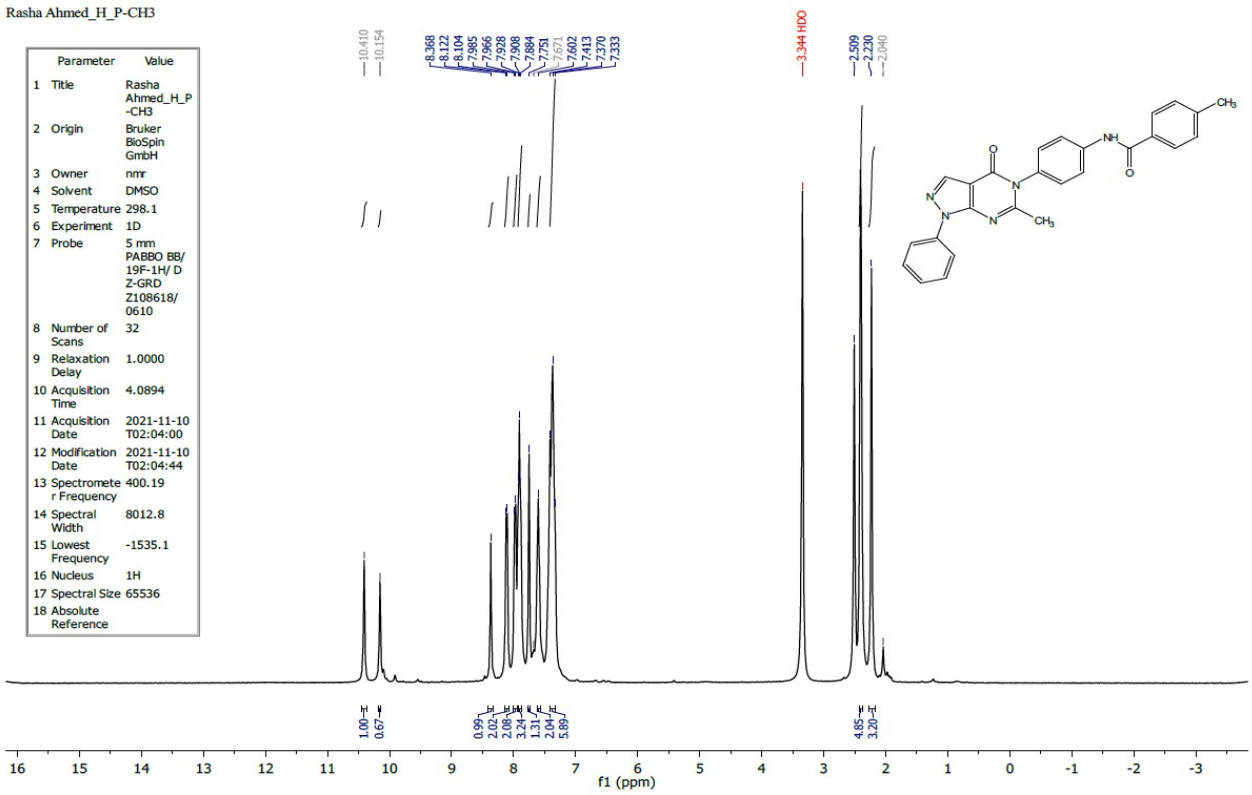




Ahamed-12c #1010 RT: 3.47 AV: 1 NL: 7.42E2
T: {0,0} + c EI Full ms [50.00-500.00]

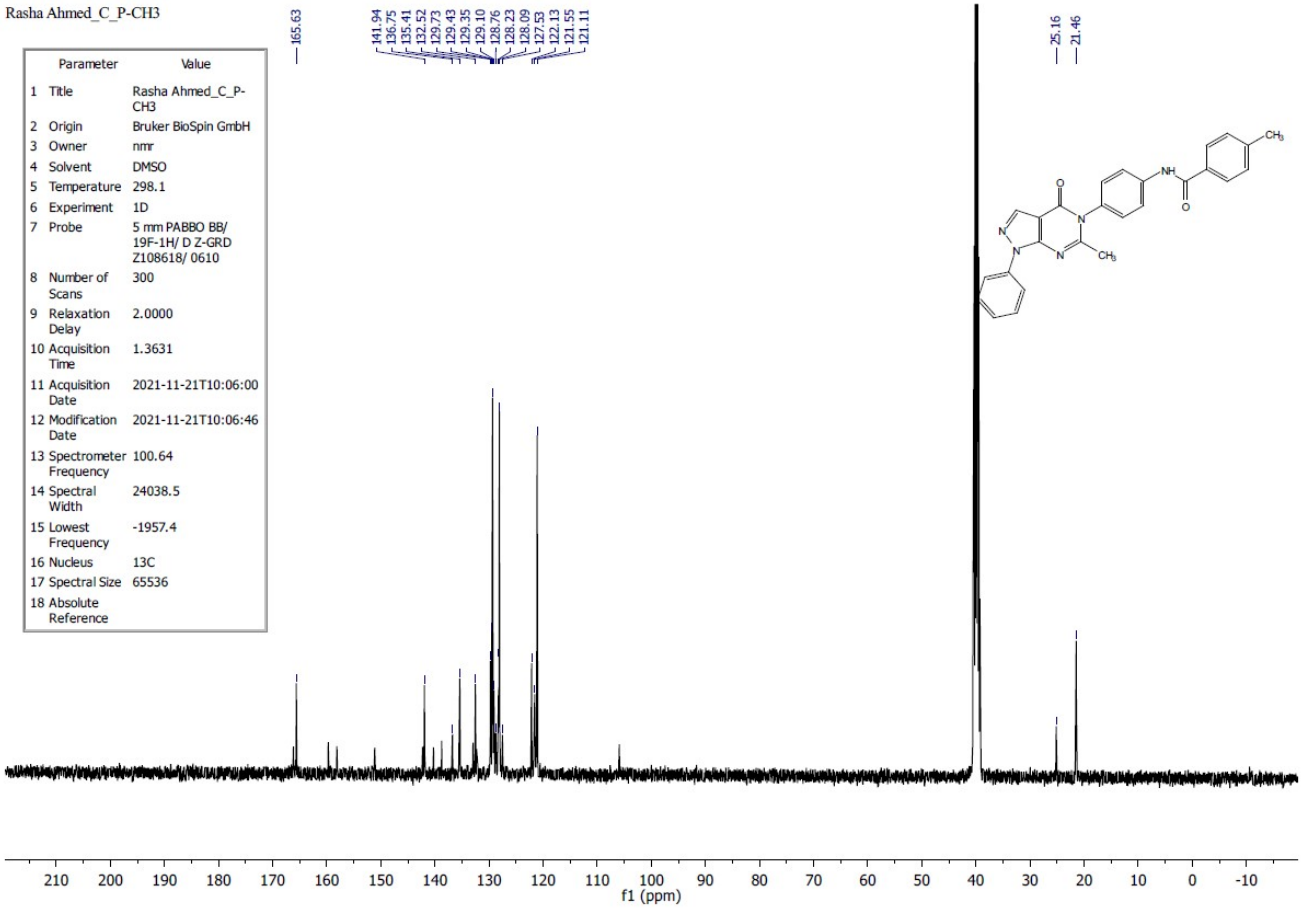


Compound 12d

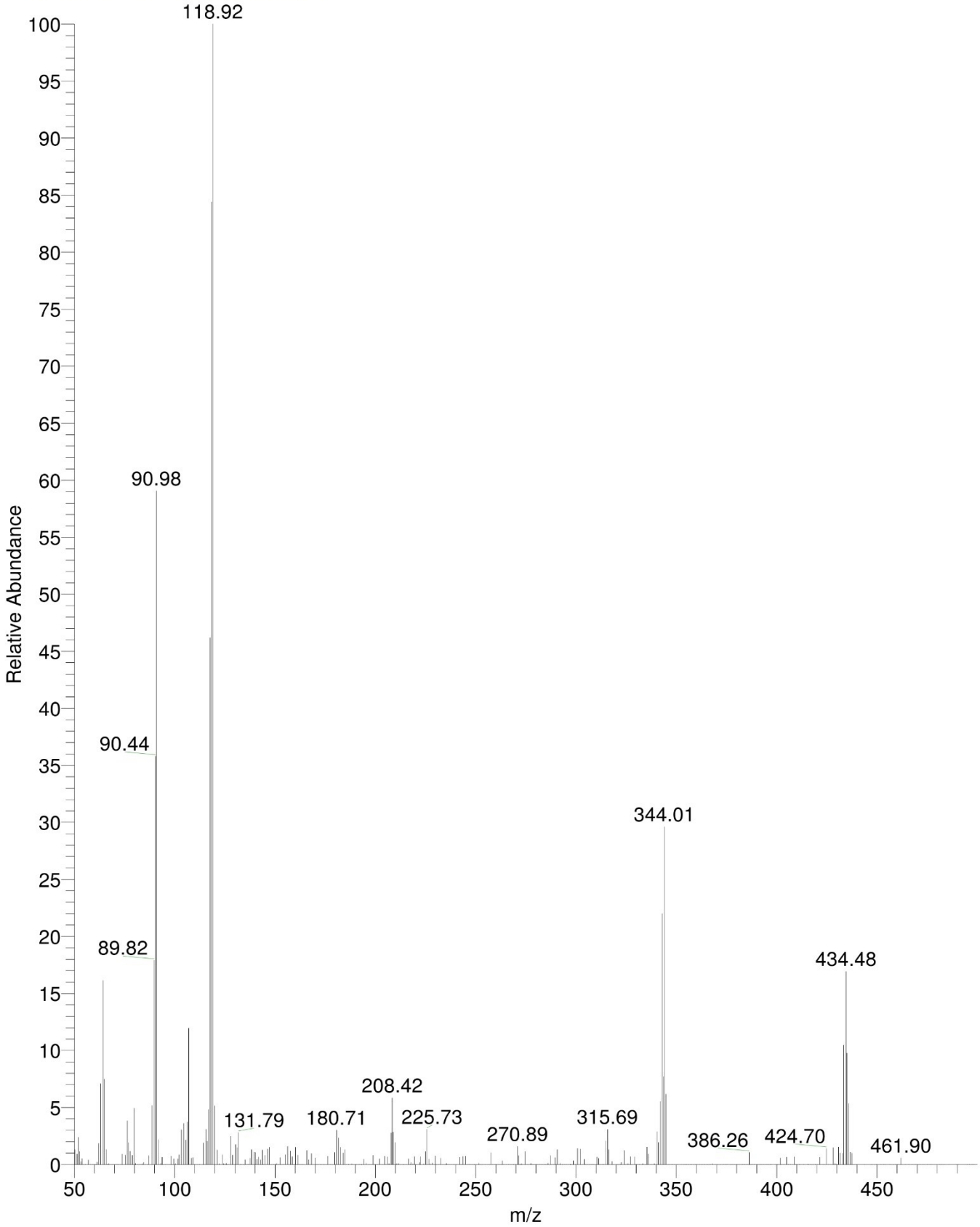


Rasha Ahmed_C_P-CH3

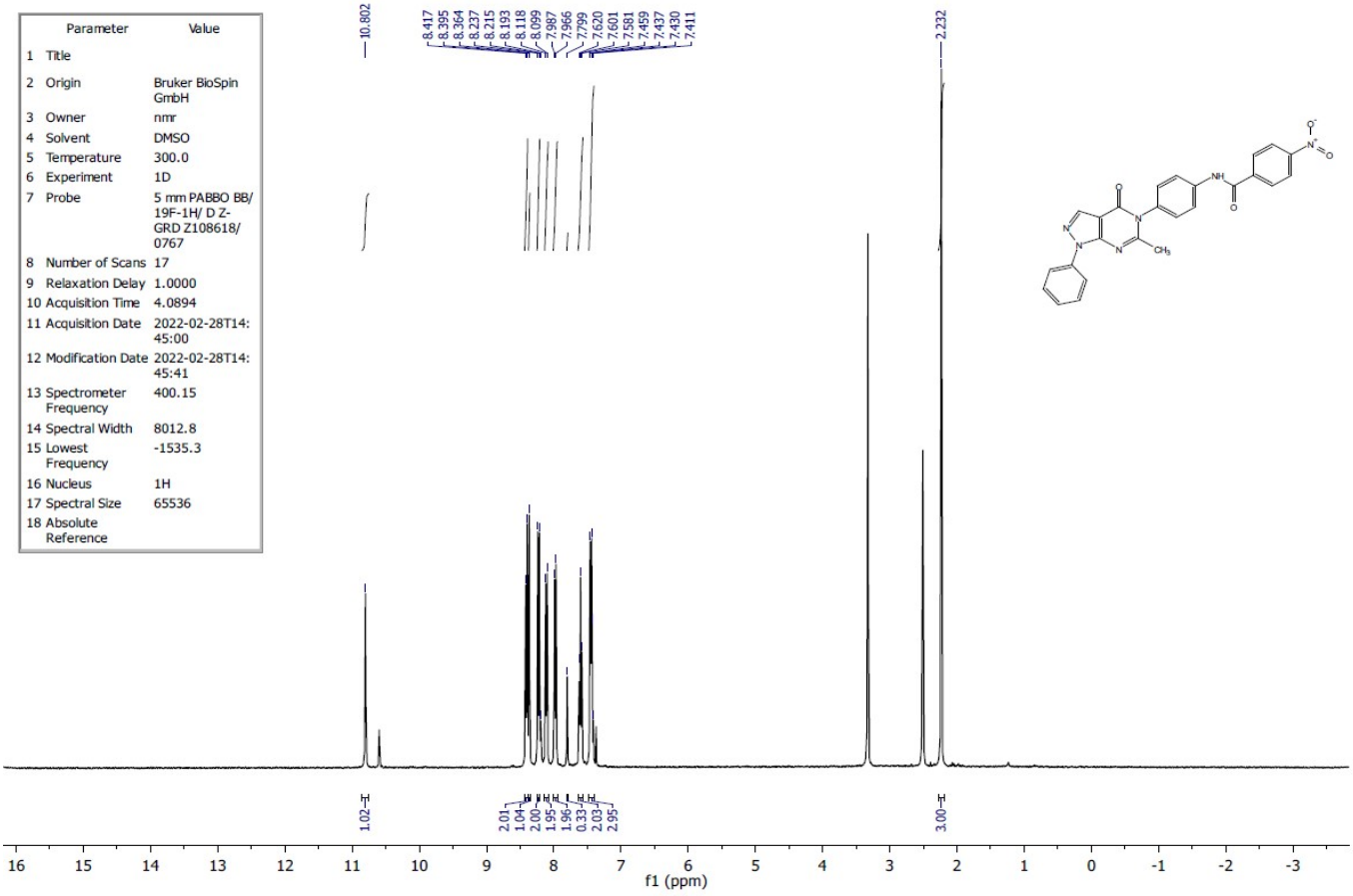
	Parameter	Value
1	Title	Rasha Ahmed_C_P-CH3
2	Origin	Bruker BioSpin GmbH
3	Owner	nmr
4	Solvent	DMSO
5	Temperature	298.1
6	Experiment	1D
7	Probe	5 mm PABBO BB/ 19F-1H/ D Z-GRD Z108618/ 0610
8	Number of Scans	300
9	Relaxation Delay	2.0000
10	Acquisition Time	1.3631
11	Acquisition Date	2021-11-21T10:06:00
12	Modification Date	2021-11-21T10:06:46
13	Spectrometer Frequency	100.64
14	Spectral Width	24038.5
15	Lowest Frequency	-1957.4
16	Nucleus	13C
17	Spectral Size	65536
18	Absolute Reference	

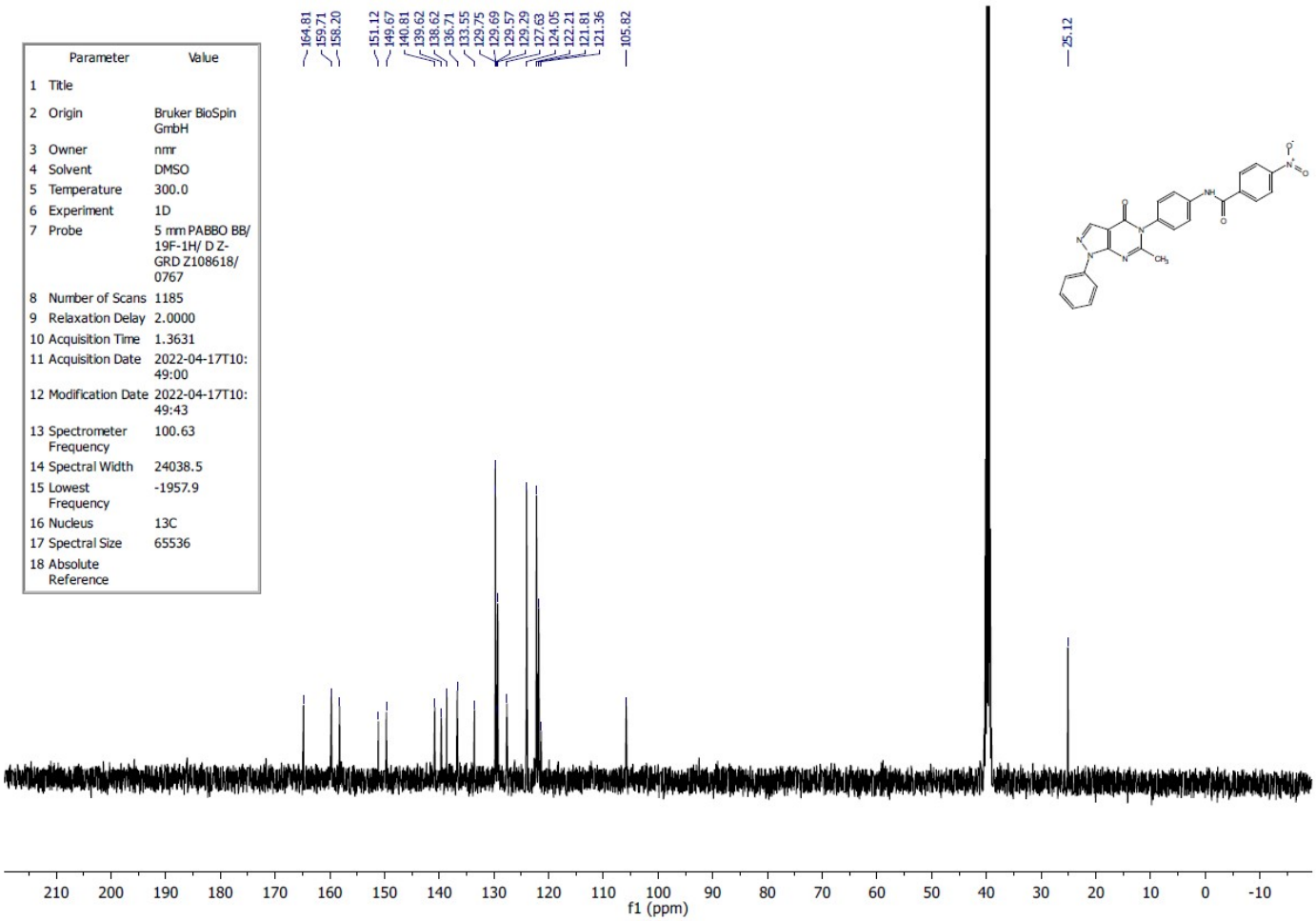


Ahamed-12d #978 RT: 3.36 AV: 1 NL: 3.43E4
T: {0,0} + c EI Full ms [50.00-500.00]



Compound 12e




Parameter Value
1 Title
2 Origin Bruker BioSpin GmbH
3 Owner nmr
4 Solvent DMSO
5 Temperature 300.0
6 Experiment 1D
7 Probe 5 mm PABBO BB/ 19F-1H/ D Z-GRD Z108618/ 0767
8 Number of Scans 1185
9 Relaxation Delay 2.0000
10 Acquisition Time 1.3631
11 Acquisition Date 2022-04-17T10:49:00
12 Modification Date 2022-04-17T10:49:43
13 Spectrometer Frequency 100.63
14 Spectral Width 24038.5
15 Lowest Frequency -1957.9
16 Nucleus 13C
17 Spectral Size 65536
18 Absolute Reference
164.81
159.71
158.20
151.12
149.67
140.81
139.62
138.62
136.71
133.55
129.75
129.69
129.57
129.29
127.63
124.05
122.21
121.81
121.36
105.82
25.12
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

Ahamed-12e #18 RT: 0.09 AV: 1 NL: 6.33E3
T: {0,0} + c EI Full ms [50.00-500.00]