Electronic Supplementary Material (ESI) for RSC Medicinal Chemistry.

Supplementary materials

New pyrazole-pyridazine hybrids as selective COX-2 inhibitors: Design, synthesis, molecular docking, *in silico* studies and investigation of the anti-inflammatory potential by evaluation of TNF-α, IL-6, PGE-2 and NO in LPS-induced RAW264.7 macrophages

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Supplementary data

(Experimental of pharamcological activity studies and molecular docking , in addition to IR, ^{1}HNMR and ^{13}CNMR spectra of new compounds)

1. Experimental

1.1 Pharamcological activity studies

1.1.1 Cell viability assay

RAW264.7 cells ($6x10^{3}$ cells/per) were placed into a 96-well plate and kept for 24 h. Five different concentrations of the investigated compounds were used (100, 25, 6.3, 1.6, and 0.4 µM/L). The cells were pretreated with the different concentrations for 1 h and then incubated with LPS (0.5 µg/ml) for 24 h. To each well, MTT solution (5 mg/ml) was added and then incubated for 4 h at 37 °C. The media containing MTT was removed, then treated with 150 µL of DMSO. The absorbance was detected by a microplate reader at 500-600 nm .

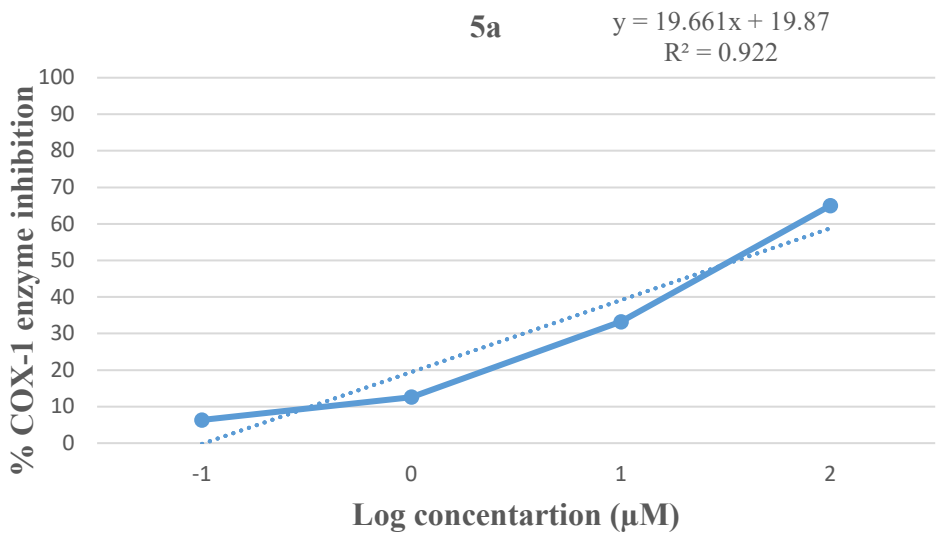
*Cytotoxicity results

Code	IC_{50} (µM)	SD (±)
5a	246	13
5b	61.6	3.2
5c	100	5.2
5d	158	8.2
5e	265	14
5f	328	17
6a	311	16
6b	138	7.2
6c	97.5	5.1
6d	212	11
6e	265	14
6f	169	8.8
Celecoxib	201	10

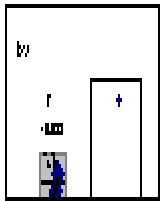
1.1.2 *In vitro* COX-1 and COX-2 inhibitory assay

In this assay, celecoxib and indomethacin were used as reference medications to investigate the ability of the new compounds to suppress human COX-1 and COX-2 using ten folds serial dilutions (1, 0.1, 0.01, 0.001 μg/mL) (**Table 1**). This was achieved using human COX-1 and COX-2 inhibitor screening kit supplied by Cayman chemicals (catalog numbers 701,070 and 701080, respectively, Ann Arbor, MI, USA). Dimethylsulfoxide (DMSO) was used to solubilize the new derivatives. Briefly, a mixture containing COX-1 or COX-2 enzyme (10 μL), heme (10 μL) and DMSO solutions of tested compounds samples (20 μL) was added to the supplied reaction buffer solution [160 μL, 0.1 M Tris–HCl, pH 8 containing 5 mM ethylenediamine tetra acetate (EDTA) and 2 mM phenol] then incubated for 10 min at 37 °C. Meanwhile, arachidonic acid was added (10 μL). To initiate the reaction, the final reaction mixture concentration was 100 μM. Later, stannous chloride (30 μL) was added to stop the COX reactions, then incubated at an ambient temperature for 5 min. Thereafter, quantification of $PGF_{2\alpha}$ produced in the samples by COX reactions was carried out by adopting an enzyme-linked immunosorbent assay (ELISA). The samples were transferred to a 96-well plate and then incubated at room temperature for 18 h. Any unbound reagent was removed by washing. Ellman's reagent (200 μL) was added and incubated for 60–90 min at room temperature. Finally, the plate was read by an ELISA plate reader, and the IC_{50} values for inhibition of both COX-1 and COX-2 enzymes were determined.

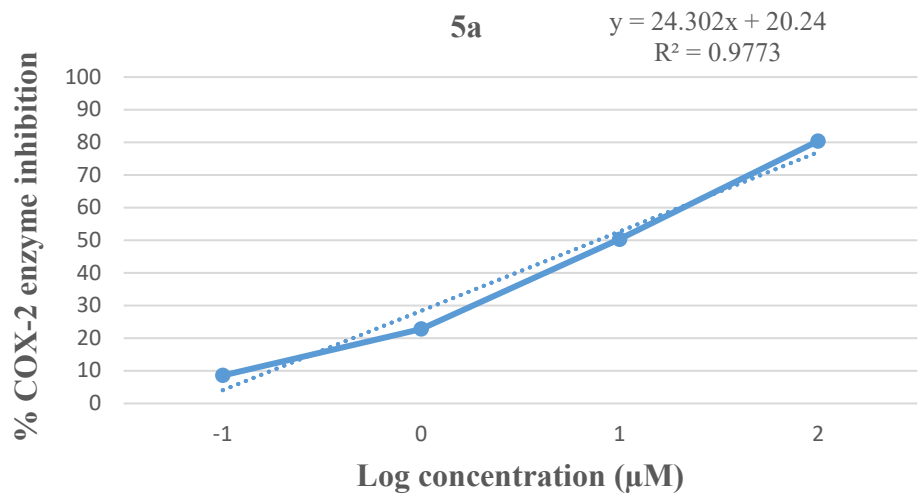
COX-1



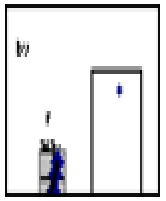
Code	IC_{50}	Conc.	Log	% Inhibition	K activity
5a	20.11	100	2	65	42.0402
		10	1	33.2	80.192
		1	0	12.6	10.938
		0.1	-1	6.33	112.343
EC				0	120



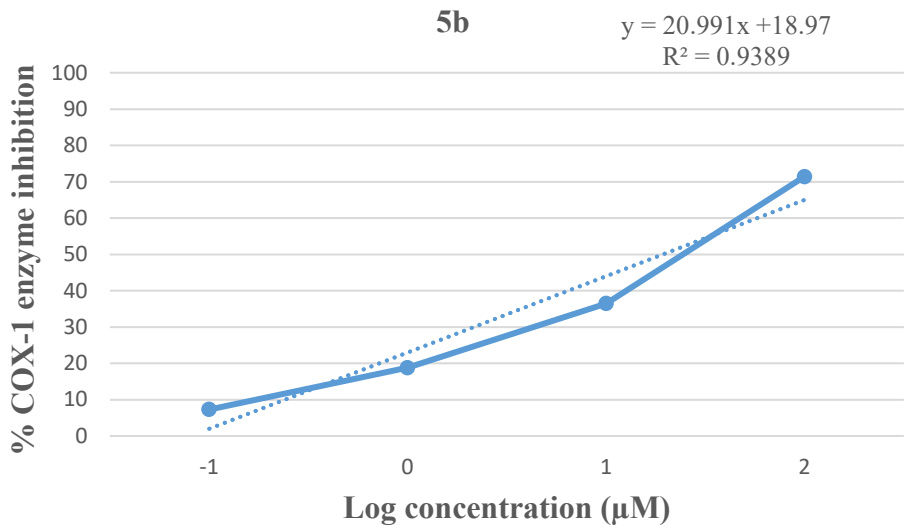
COX-2



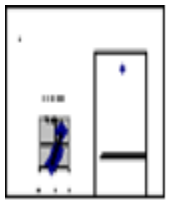
Code	IC_{50}	Conc.	Log	% Inhibition	K activity
5a	5.40	100	2	80.4	23.4623
		10	1	50.3	59.682
		1	0	92.6253	97.6658
		0.1	-1	8.56	109.727
EC				0	120



COX-1



Code	IC_{50}	Conc.	Log	% Inhibition	K activity
5b	4.58	100	2	73.9	34.2874
		10	1	40.5	76.2196
		1	0	21.4	97.4377
		0.1	-1	8.96	111.203
EC				0	120



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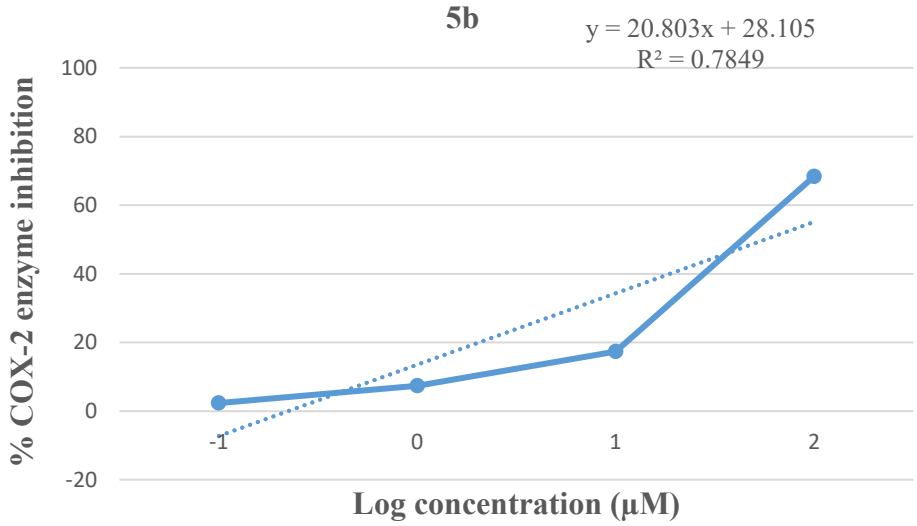
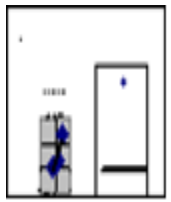
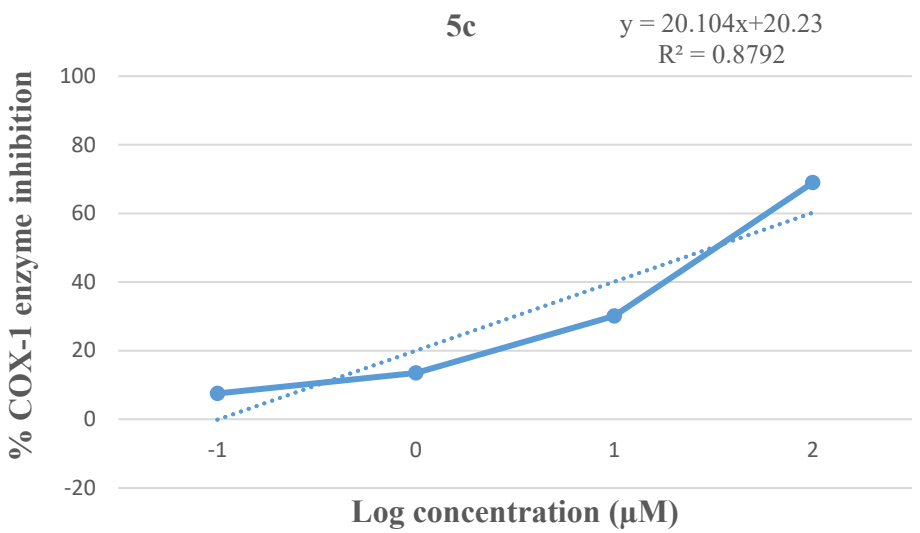


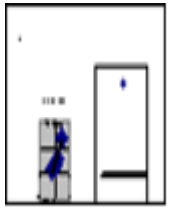
Code	IC_{50}	Conc.	Log	% Inhibition	K activity
5b	4.58	100	2	68.4	37.3638
		10	1	17.4	99.1659
		1	0	7.43	111.083
		0.1	-1	2.38	117.144
EC				0	120



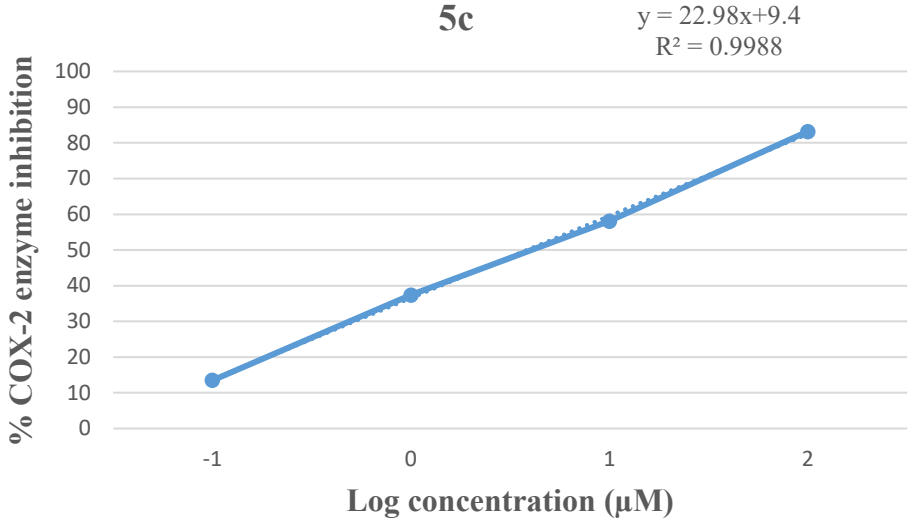
COX-1



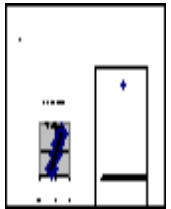
Code	IC_{50}	Conc.	Log	% Inhibition	K activity
5c	18.00	100	2	69	37.2277
		10	1	30.1	83.8644
		1	0	13.5	103.858
		0.1	-1	7.52	110.975
EC				0	120



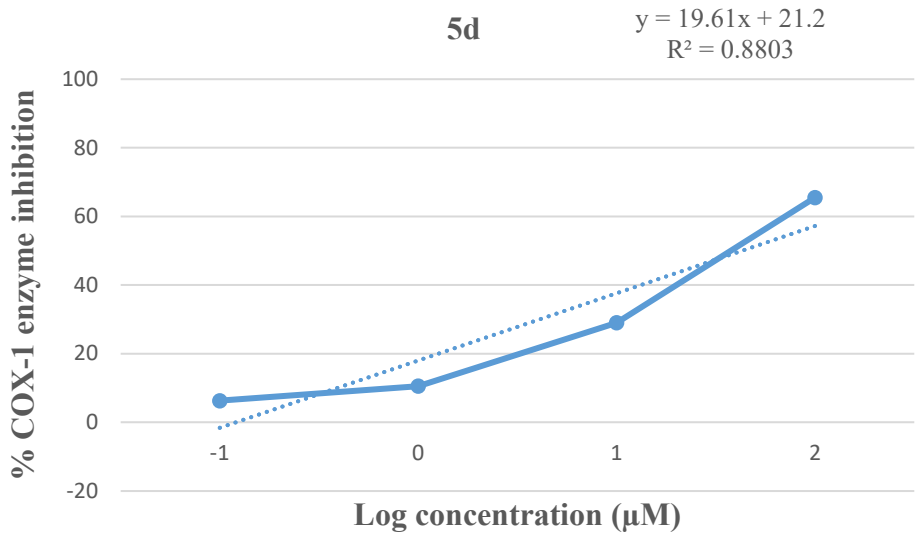
COX-2



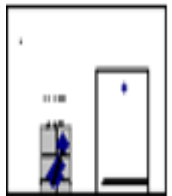
Code	IC_{50}	Conc.	Log	% Inhibition	K activity
5c	9.04	100	2	83.2	20.15
		10	1	58.1	50.309
		1	0	37.4	75.1395
		0.1	-1	13.5	103.846
EC				0	120



COX-1



Code	IC_{50}	Conc.	Log	% Inhibition	K activity
5d	4.15	100	2	65.5	41.4281
		10	1	29	85.2445
		1	0	10.5	107.459
		0.1	-1	6.3	112.439
EC				0	120



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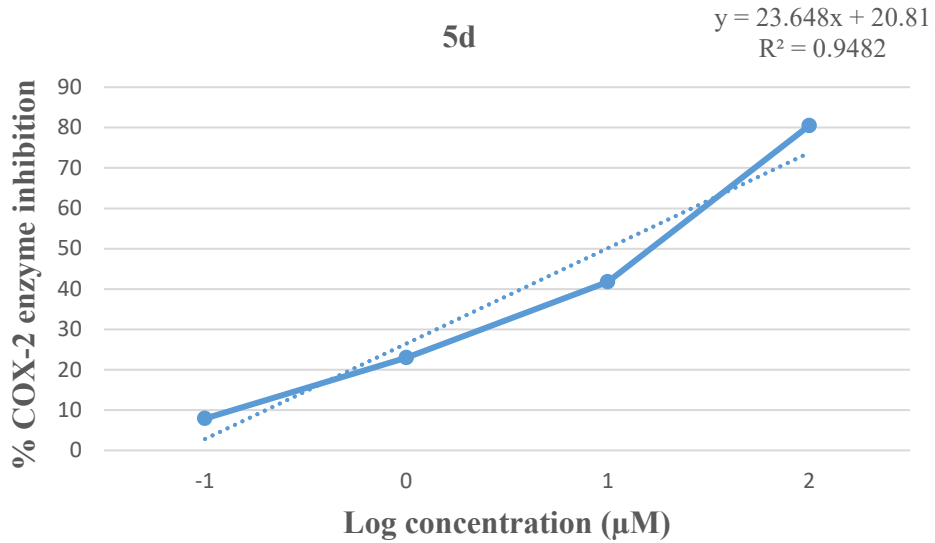
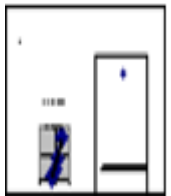


Code	IC_{50}	Conc.	Log	% Inhibition	K activity
5d	20.71	100	2	80.5	23.4143
		10	1	41.8	69.835
		1	0	23	92.3972
		0.1	7.94	35.2	110.471
EC				0	120



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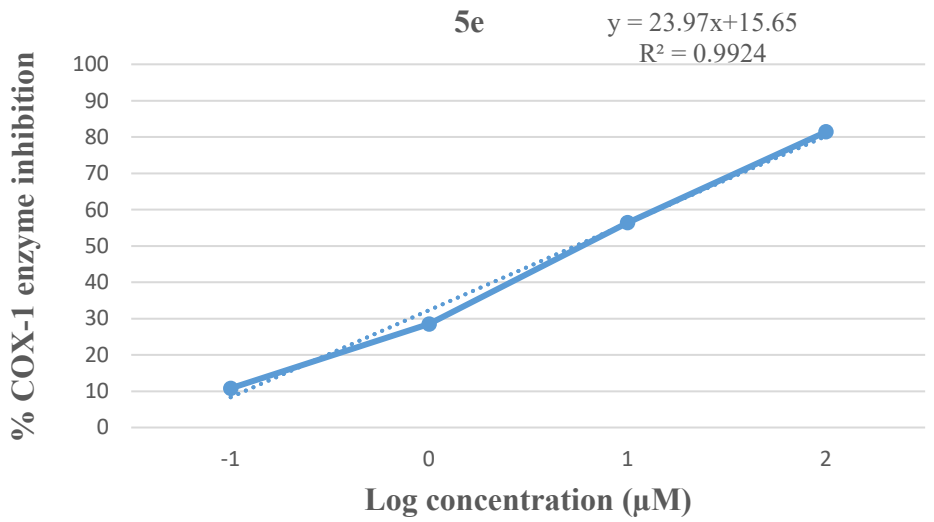
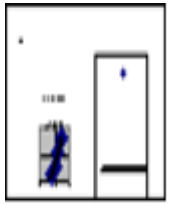


Code	IC_{50}	Conc.	Log	% Inhibition	K activity
5e	2.42	100	2	81.4	22.3342
		10	1	56.4	52.3372
		1	0	28.5	85.8326
		0.1	-1	10.8	107.003
EC				0	120



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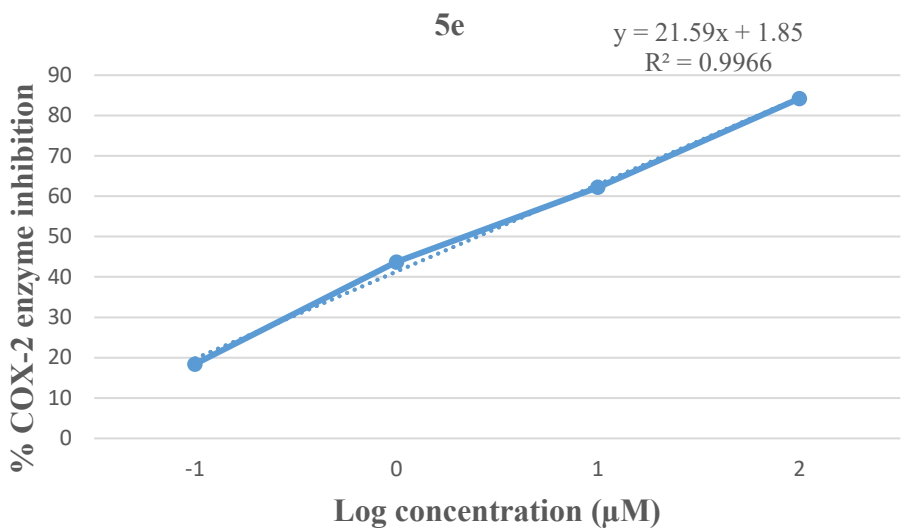
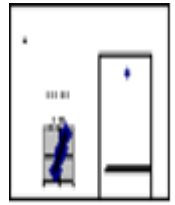


Code	IC_{50}	Conc.	Log	% Inhibition	K activity
5e	3.92	100	2	84.2	18.9019
		10	1	62.2	45.3885
		1	0	43.7	67.5428
		0.1	-1	18.4	97.9178
EC				0	120



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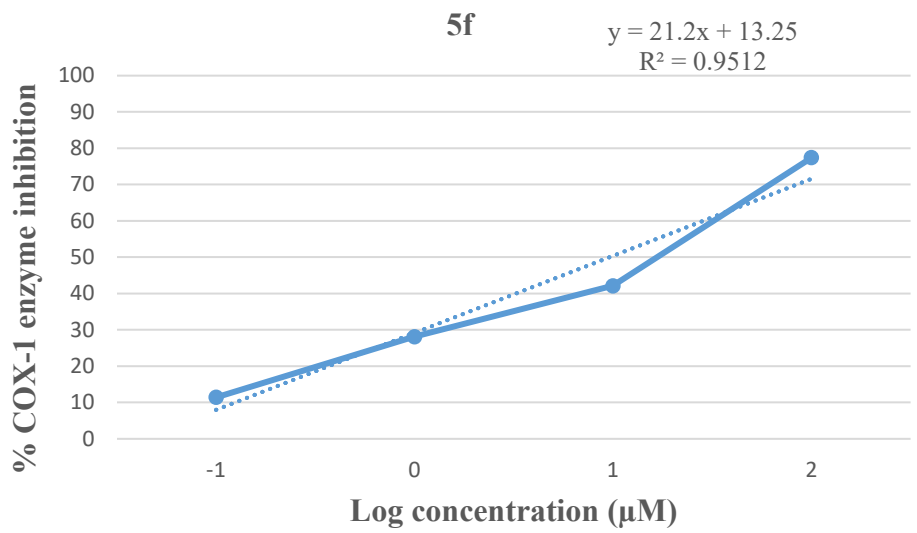
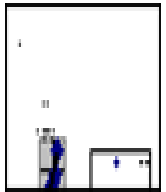


Code	IC_{50}	Conc.	Log	% Inhibition	K activity
5f	14.38	100	2	77.4	27.0987
		10	1	42.1	69.4989
		1	12.6	28.1	86.2286
		0.1	-1	11.4	106.343
EC				0	120



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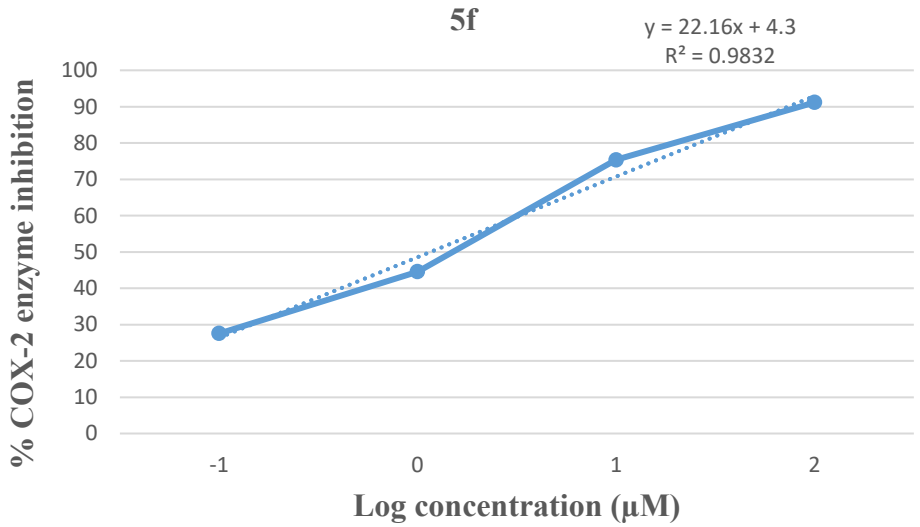
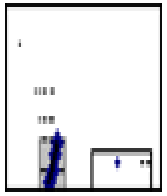


Code	IC_{50}	Conc.	Log	% Inhibition	K activity
5f	1.50	100	2	91.2	10.6091
		10	1	75.4	29.535
		1	0	44.6	66.4746
		0.1	-1	27.6	86.9367
EC				0	120



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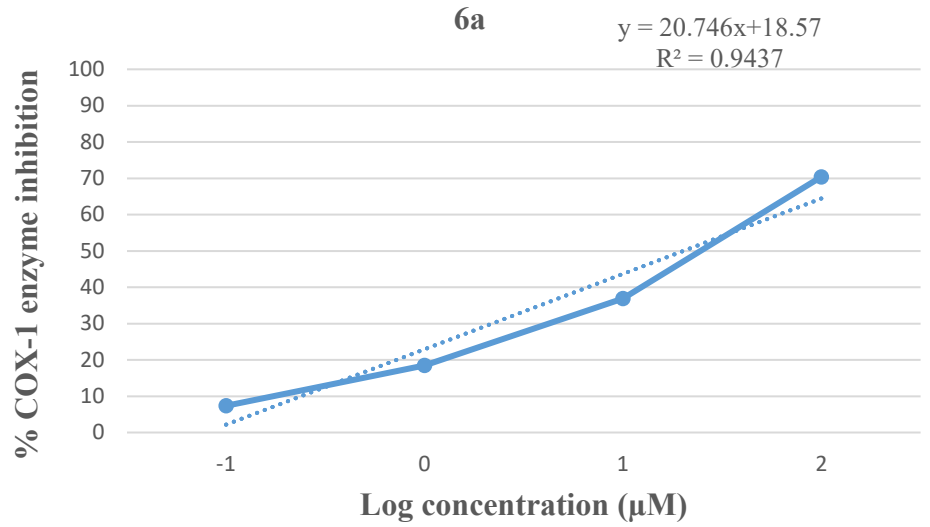
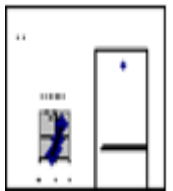


Code	IC_{50}	Conc.	Log	% Inhibition	K activity
6a	35.95	100	2	70.4	35.4635
		10	1	36.9	75.6796
		1	12.6	18.5	97.7618
		0.1	-1	7.38	111.143
EC				0	120



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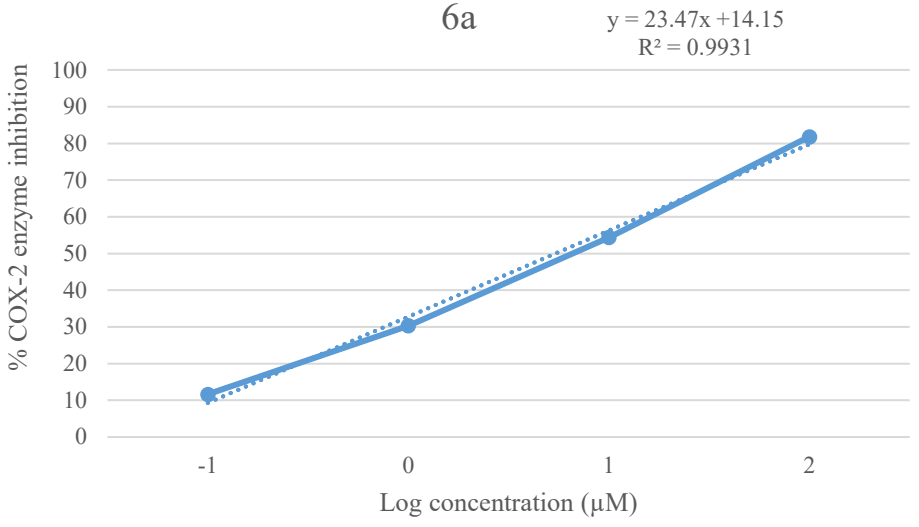
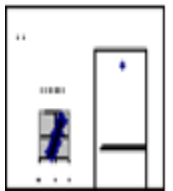


Code	IC_{50}	Conc.	Log	% Inhibition	K activity
6a	7.76	100	2	81.8	21.7942
		10	1	54.4	54.7375
		1	0	30.3	83.6244
		0.1	-1	11.6	106.127
EC				0	120



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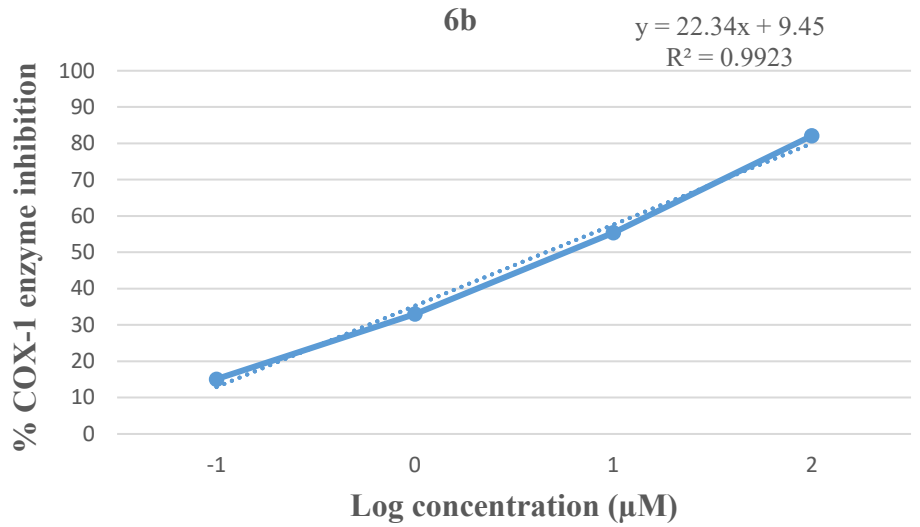

Code	IC_{50}	Conc.	Log	% Inhibition	K activity
6b	19.29	100	2	82.1	21.4341
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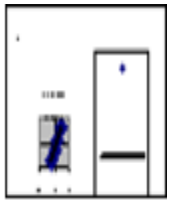
		1	12.6	33	80.444
		0.1	-1	15.1	101.902
EC				0	120



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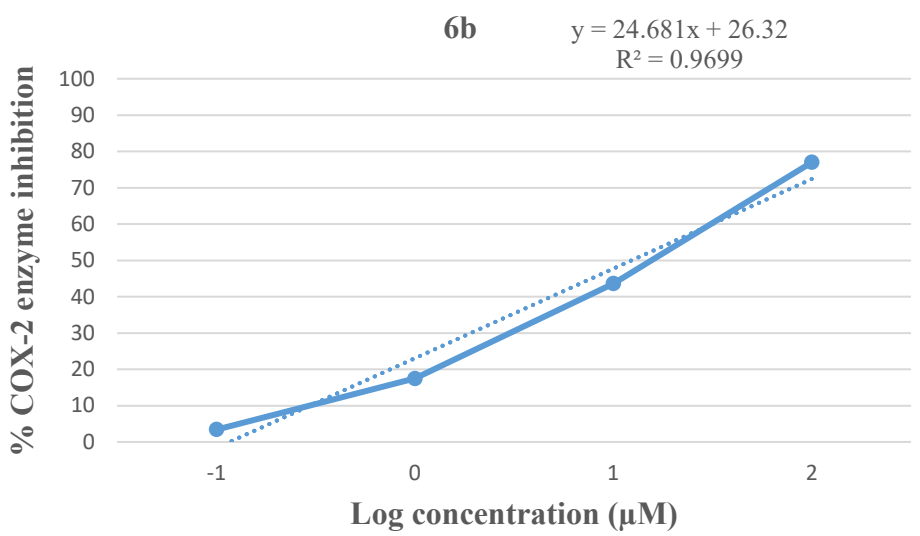
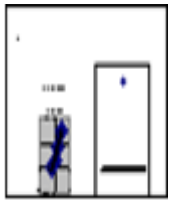


Code	IC_{50}	Conc.	Log	% Inhibition	K activity
6b	56.3	100	2	77	27.6268
		10	1	43.6	67.7228
		1	0	17.5	99.0219
		0.1	-1	3.43	115.884
EC				0	120



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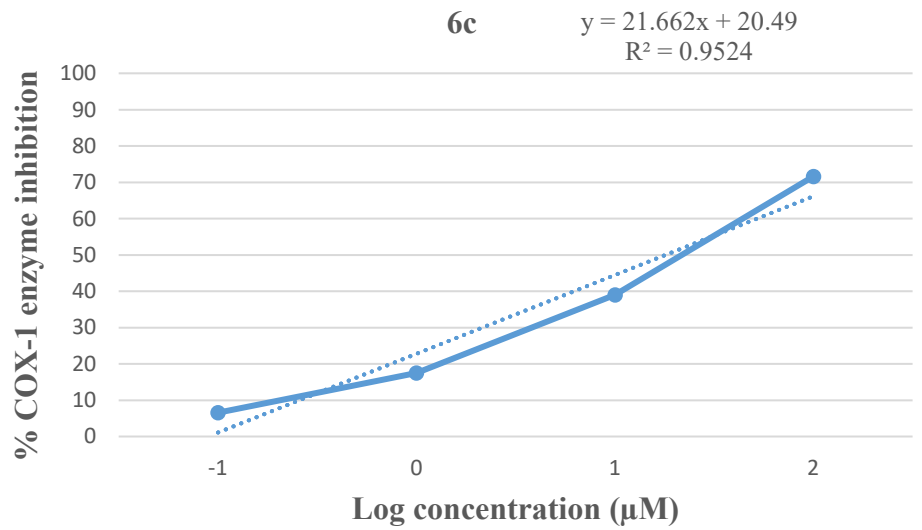
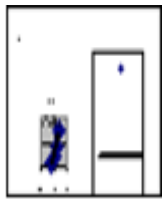


Code	IC_{50}	Conc.	Log	% Inhibition	K activity
6c	31.20	100	2	71.6	34.0954
		10	1	39	73.2553
		1	0	17.5	99.0219
		0.1	-1	6.56	112.127
EC				0	120



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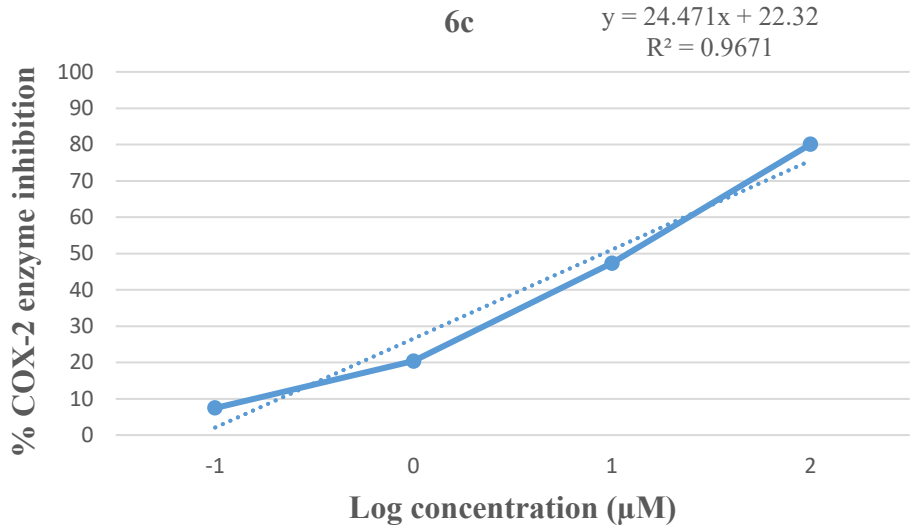
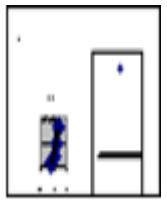
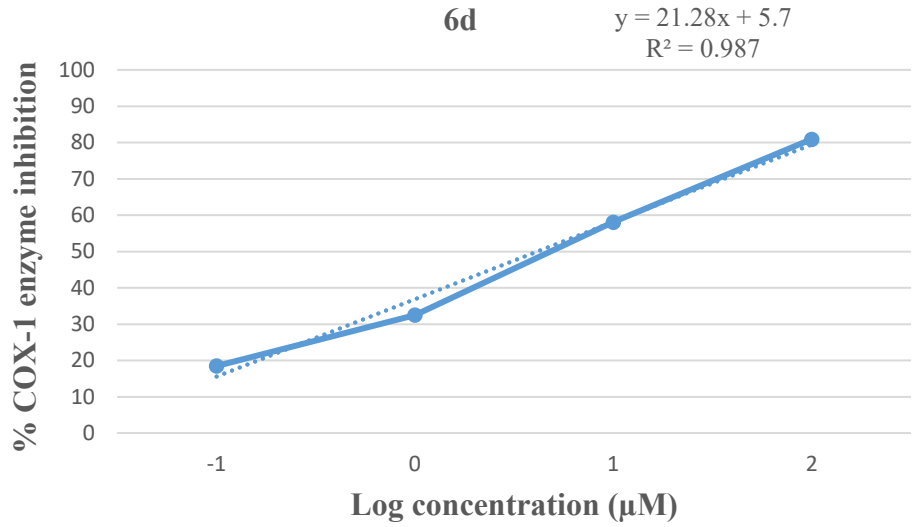


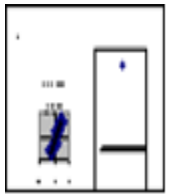
Code	IC_{50}	Conc.	Log	% Inhibition	K activity
6c	3.85	100	2	80.1	23.9064
		10	1	47.4	63.1383
		1	0	20.4	95.5776
		0.1	-1	7.53	110.963
EC				0	120



COX-1



Code	IC_{50}	Conc.	Log	% Inhibition	K activity
6d	42.91	100	2	80.9	22.9703
		10	1	58.1	50.261
		1	0	32.5	81.0321
		0.1	-1	18.5	97.8218
EC				0	120



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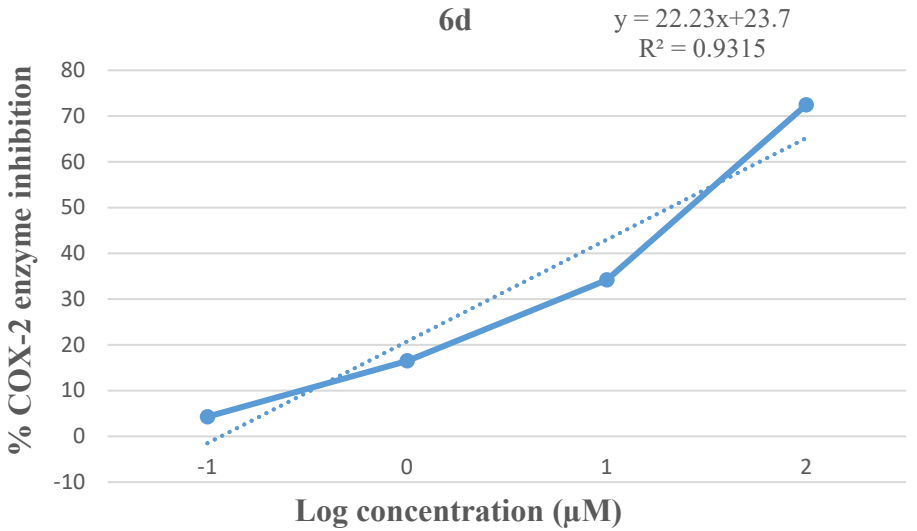
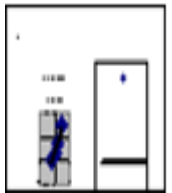
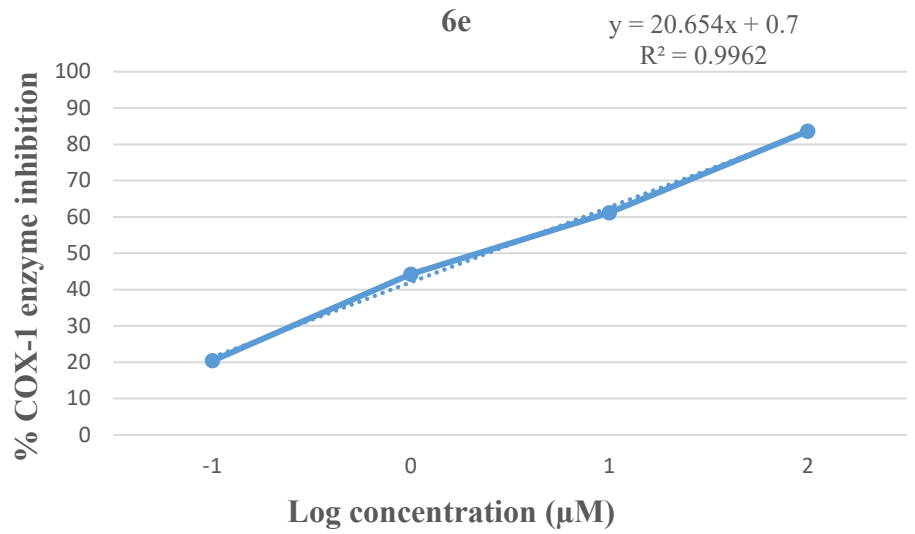


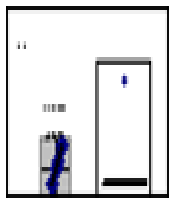
Code	IC_{50}	Conc.	Log	% Inhibition	K activity
6d	9.87	100	2	72.5	33.0153
		10	1	34.2	78.9919
		1	0	16.5	100.222
		0.1	-1	4.3	114.839
EC				0	120



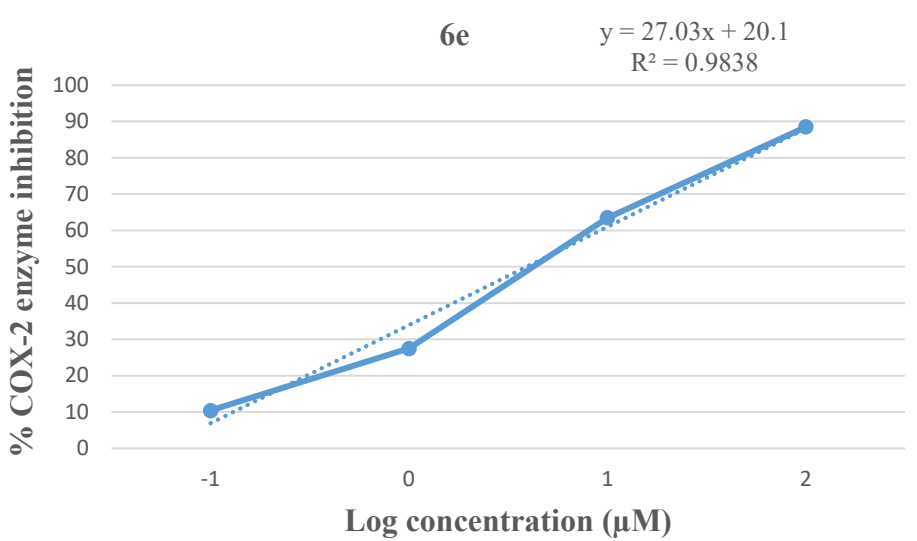
COX-1



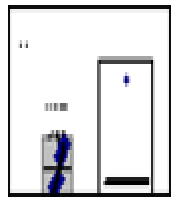
Code	IC_{50}	Conc.	Log	% Inhibition	K activity
6e	5.48	100	2	83.6	19.634
		10	1	61.4	46.3366
		1	12.6	44.2	66.9547
		0.1	-1	20.4	95.5416
EC				0	120



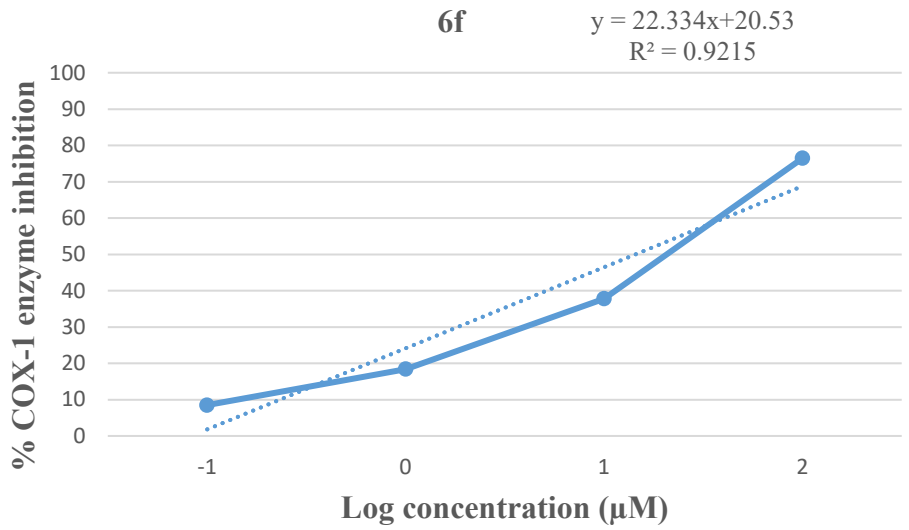
COX-2



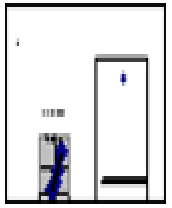
Code	IC_{50}	Conc.	Log	% Inhibition	K activity
6e	2.51	100	2	88.5	13.8254
		10	63.5	83	43.8284
		1	0	27.5	87.0207
		0.1	-1	10.4	107.543
EC				0	120



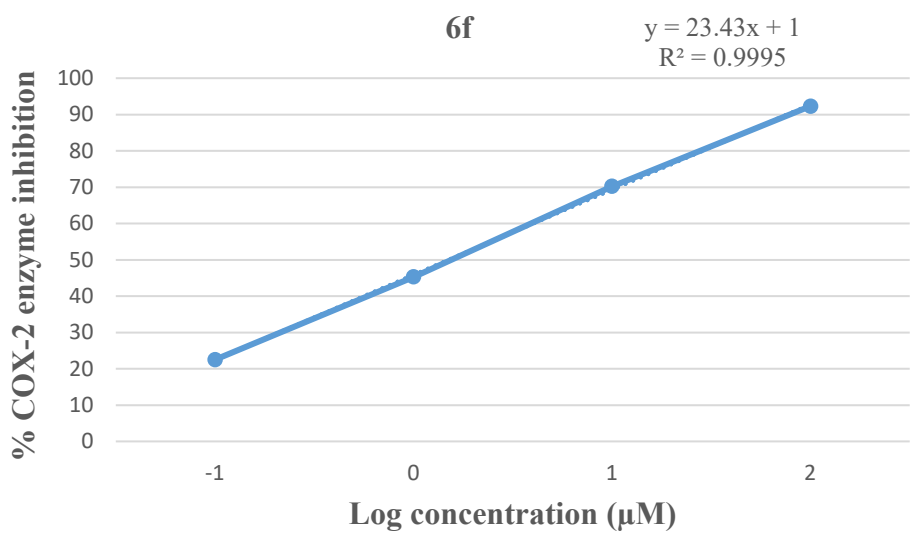
COX-1



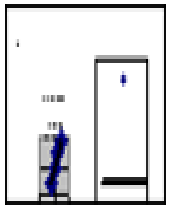
Code	IC_{50}	Conc.	Log	% Inhibition	K activity
6f	9.61	100	2	76.5	28.2148
		10	1	37.8	74.5995
		1	12.6	18.4	97.9178
		0.1	-1	8.52	109.775
EC				0	120



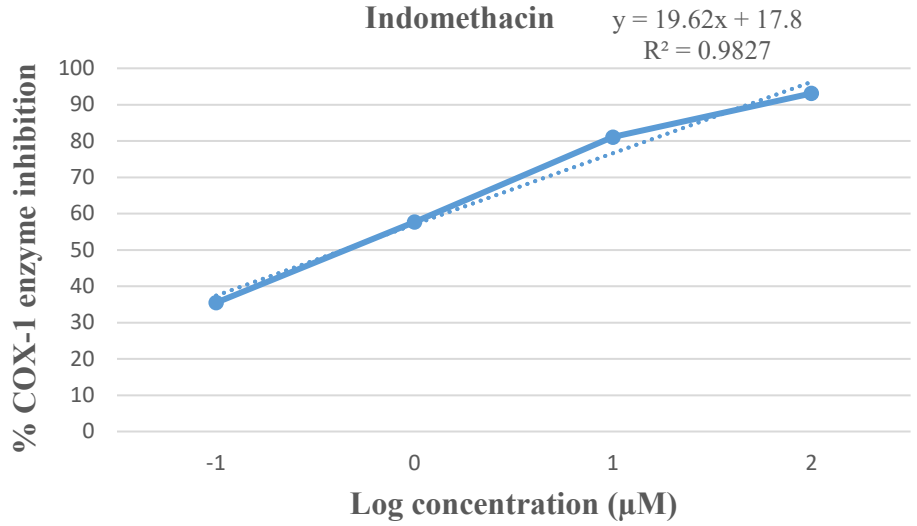
COX-2



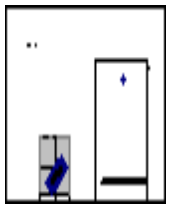
Code	IC_{50}	Conc.	Log	% Inhibition	K activity
6f	1.15	100	2	92.3	9.25293
		10	1	70.2	35..7756
		1	0	45.3	65.6706
		0.1	-1	22.5	92.9973
EC				0	120



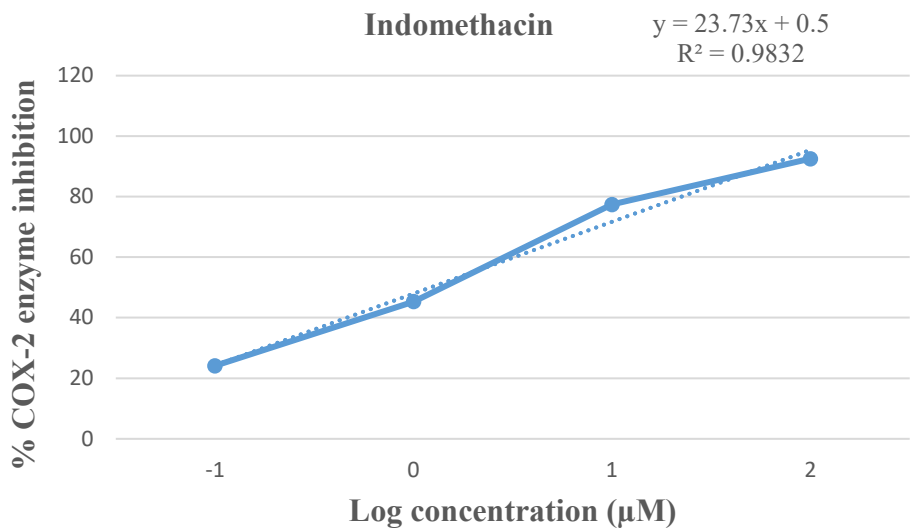
COX-1



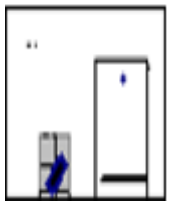
Code	IC_{50}	Conc.	Log	% Inhibition	K activity
Indomethacin	0.43	100	2	93.2	8.25683
		10	1	81.1	22.7303
		1	12.6	57.7	50.7531
		0.1	-1	35.5	77.4557
EC				0	120



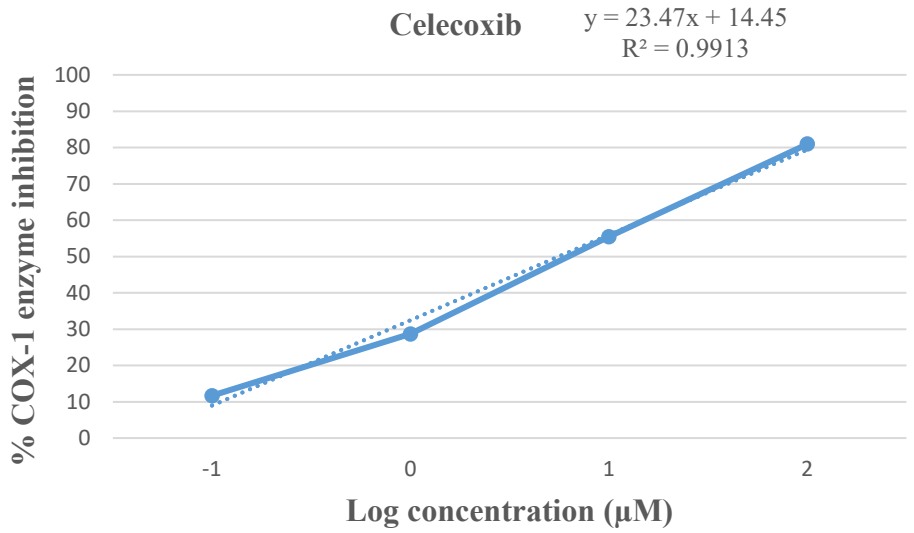
COX-2



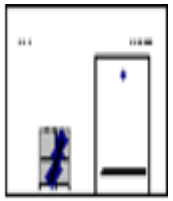
Code	IC_{50}	Conc.	Log	% Inhibition	K activity
Indomethacin	1.22	100	2	92.5	9.0129
		10	1	77.4	27.1587
		1	0	45.3	65.6346
		0.1	-1	24.1	91.1131
EC				0	120



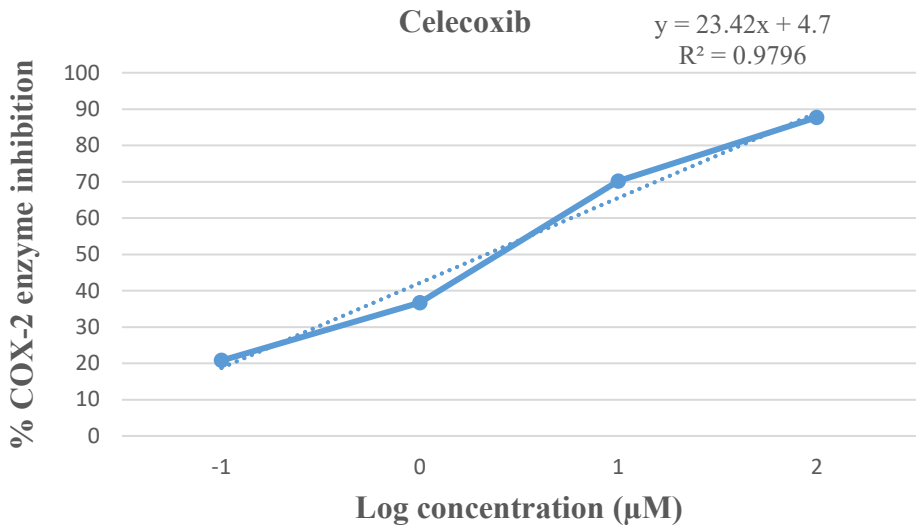
COX-1



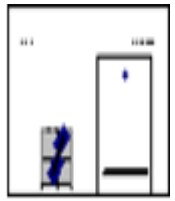
Code	IC_{50}	Conc.	Log	% Inhibition	K activity
Celecoxib	5.43	100	2	81.8	21.8542
		10	1	55.5	53.4413
		1	12.6	28.7	85.5206
		0.1	-1	11.7	105.983
EC				0	120



COX-2



Code	IC_{50}	Conc.	Log	% Inhibition	K activity
Celecoxib	2.16	100	2	87.7	14.7975
		10	1	70.2	35.7516
		1	0	36.7	75.9196
		0.1	-1	20.8	95.0255
EC				0	120



1.1.3 Inhibition of TNF-α, IL-6, and PGE-2 expression in LPS-induced RAW264.7 cell

RAW 264.7 cells were incubated in 96 well-plates (5×10^3 cells/well) in 5% CO_2 at 37 °C for 24 h. Thereafter, the cells were treated with the tested compounds (50 μM) in the presence of LPS (1 μg/mL). The untreated cells, acted as the blank control, whereas the negative control group (LPS group) was represented by the treated cells with LPS + DMSO (final concentration of 0.1% (v/v)). TNF-α, PGE-2, and IL-6 were quantified 24 h after treatment, utilizing specified ELISA kits.

1.1.4 Inhibition of NO expression LPS-induced RAW264.7 cells

Aiming to measure NO expression in the culture medium, RAW264.7 cells pretreated with the test compounds for 2 h were treated with LPS (1 μg/mL) for 24 h. To the culture medium (100 L), Griess reagent (100 L) (Sigma-Aldrich, St. Louis, MO, USA) was added, then incubated at room temperature for 10 min. ELISA reader at 540 nm was used to measure the optical density (OD) as an indicator of NO.

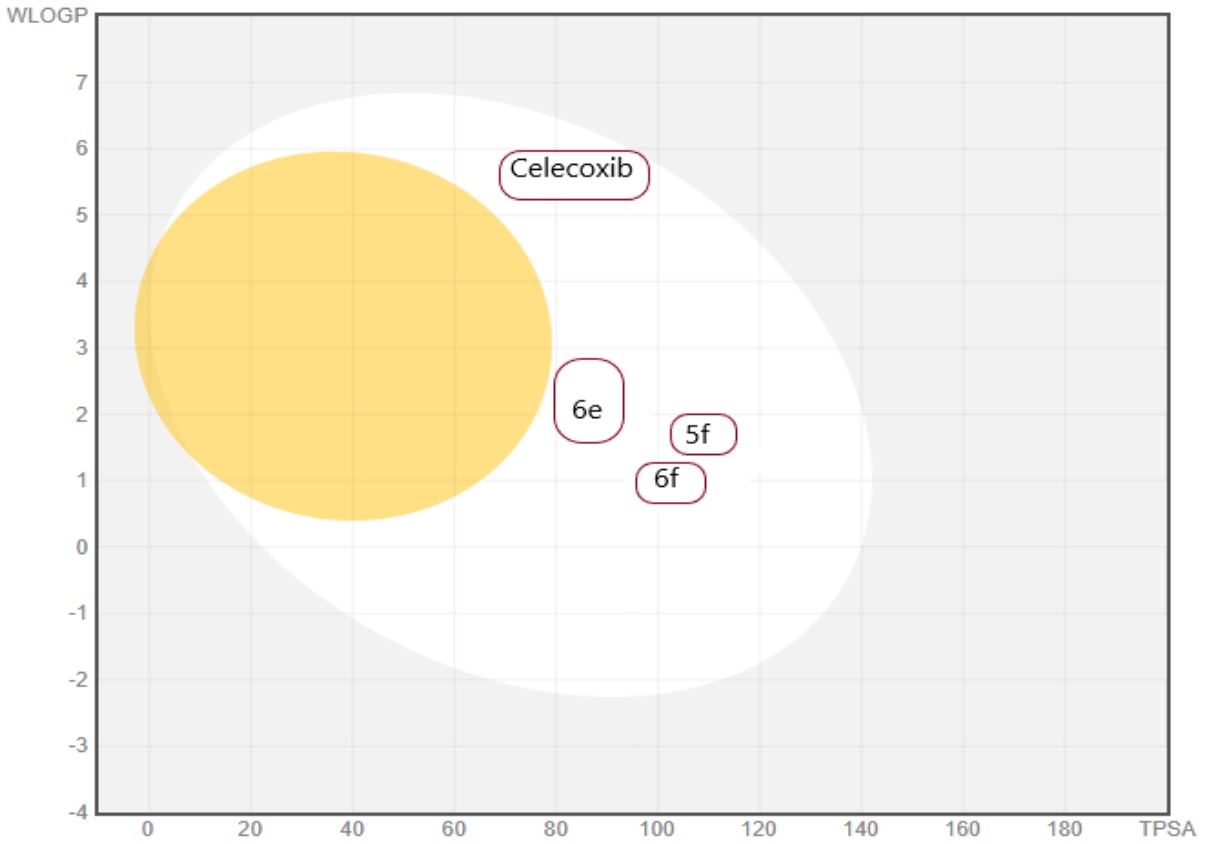
1.2 Molecular docking of compounds **5f**, **6e** and **6f** in the active site of COX-2

Molecular Operating Environment (MOE Version 2015.10) software was used to study the molecular modeling for the new active compounds **5f**, **6e** and **6f**. The COX-2 enzyme X-ray crystal structure (PDB entry 3LN1) was downloaded from the RCSB protein data bank website (http://www.rcsb.org). Aiming to prepare protein structure, the repeating chains and water molecules were removed from the enzyme active site. Protonate 3D process was applied to add hydrogen atoms to the atoms of the receptor. The determined pocket was isolated, and the partial charges were calculated. Hiding backbone was performed to give a final prepared protein. Before performing the docking, the tested compounds and celecoxib were prepared automatically by certain steps including 3D protonation, calculating partial charges and minimizing energy with Merck Molecular force field.

Ligands were outlined using ChemDraw office (ChemDraw office version 12.0.2) and saved as mol extension, then prepared by protonation and energy minimization.

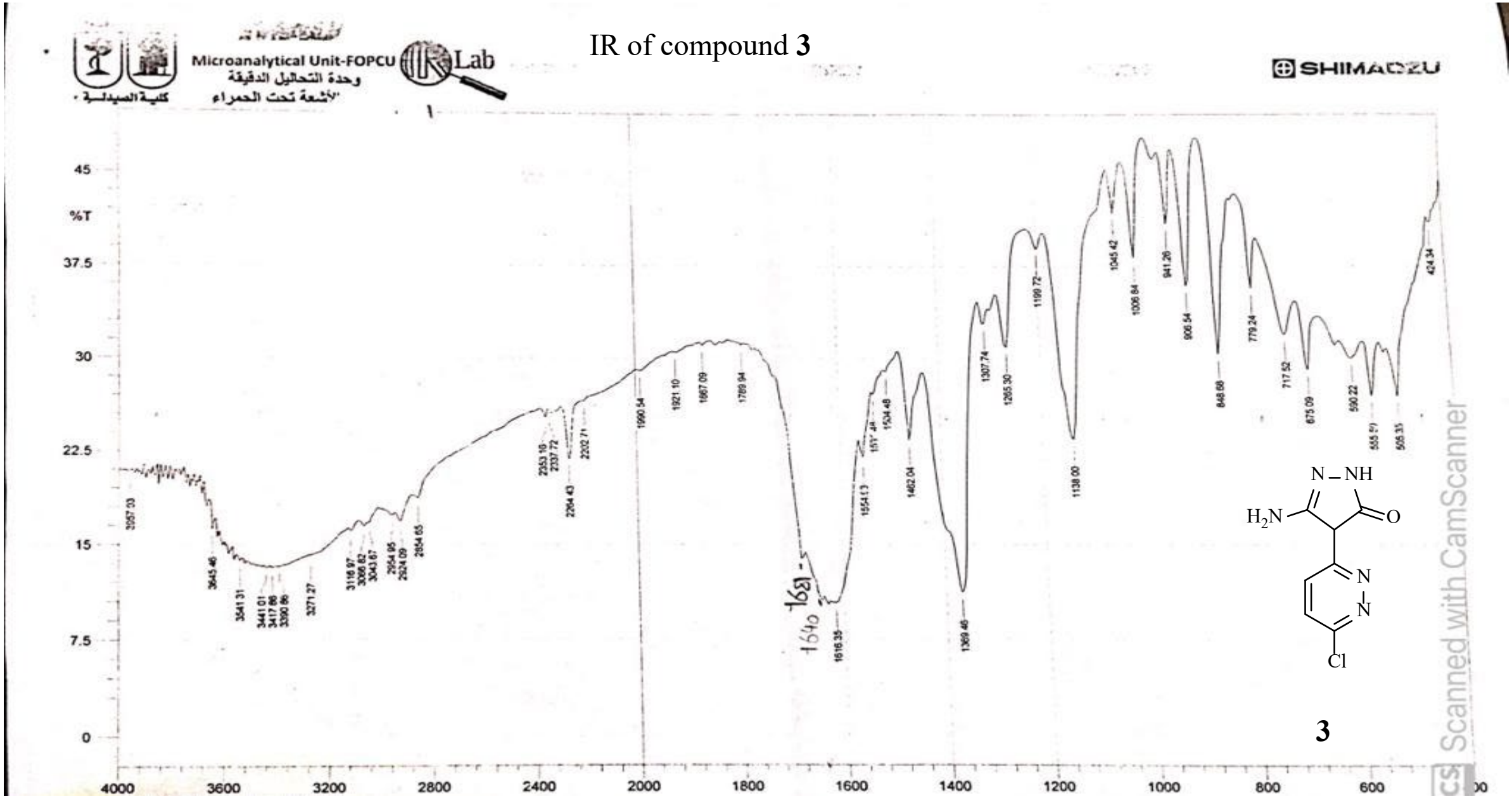
2.7. Physicochemical, ADME, and pharmacokinetic properties in *silico* prediction

The BOILED-Egg graphical representation of the WLOGP *vs*. topological polar surface area for the tested compounds



3. IR, ^{1}H NMR and ^{13}C NMR spectra of the new derivatives.

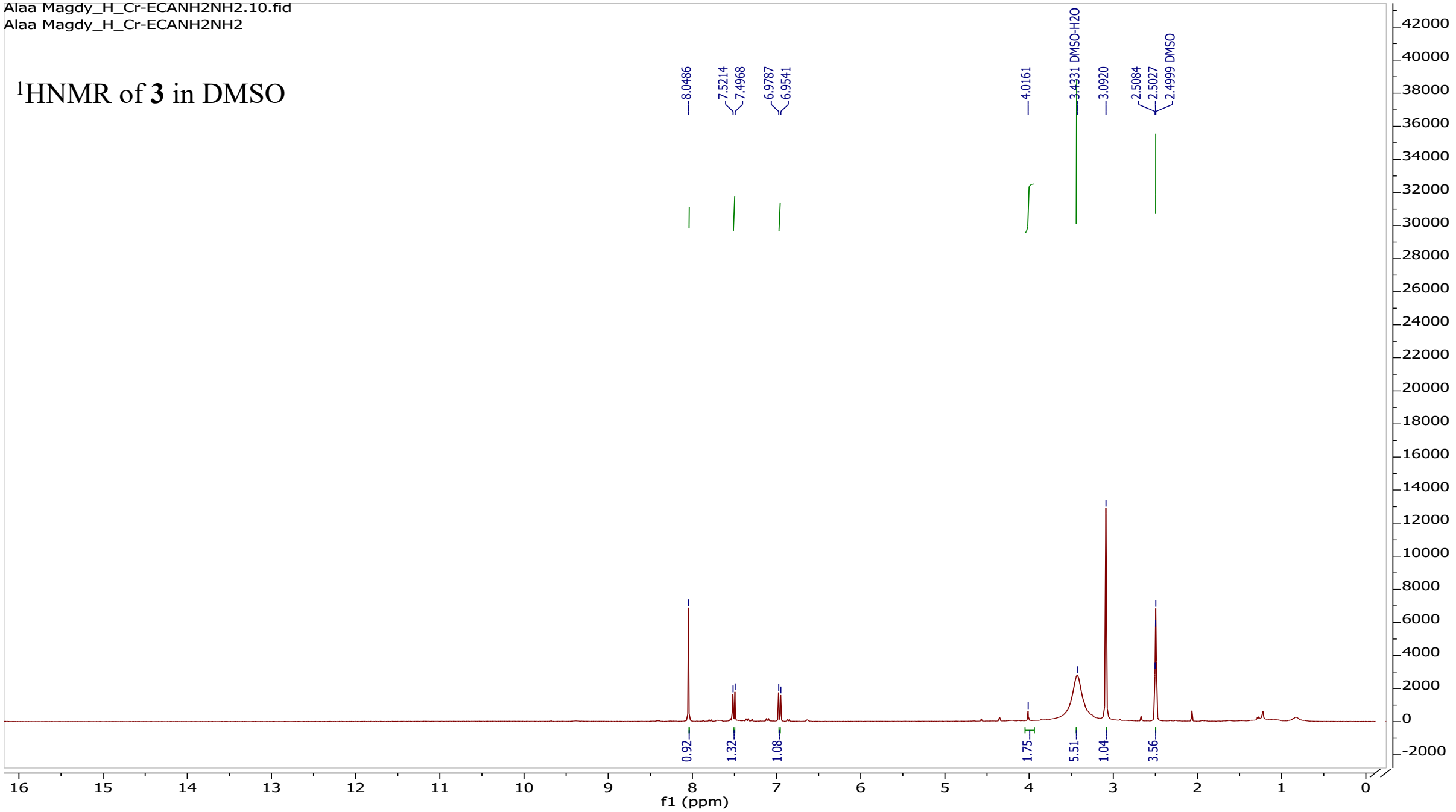
IR of compound **3**



3

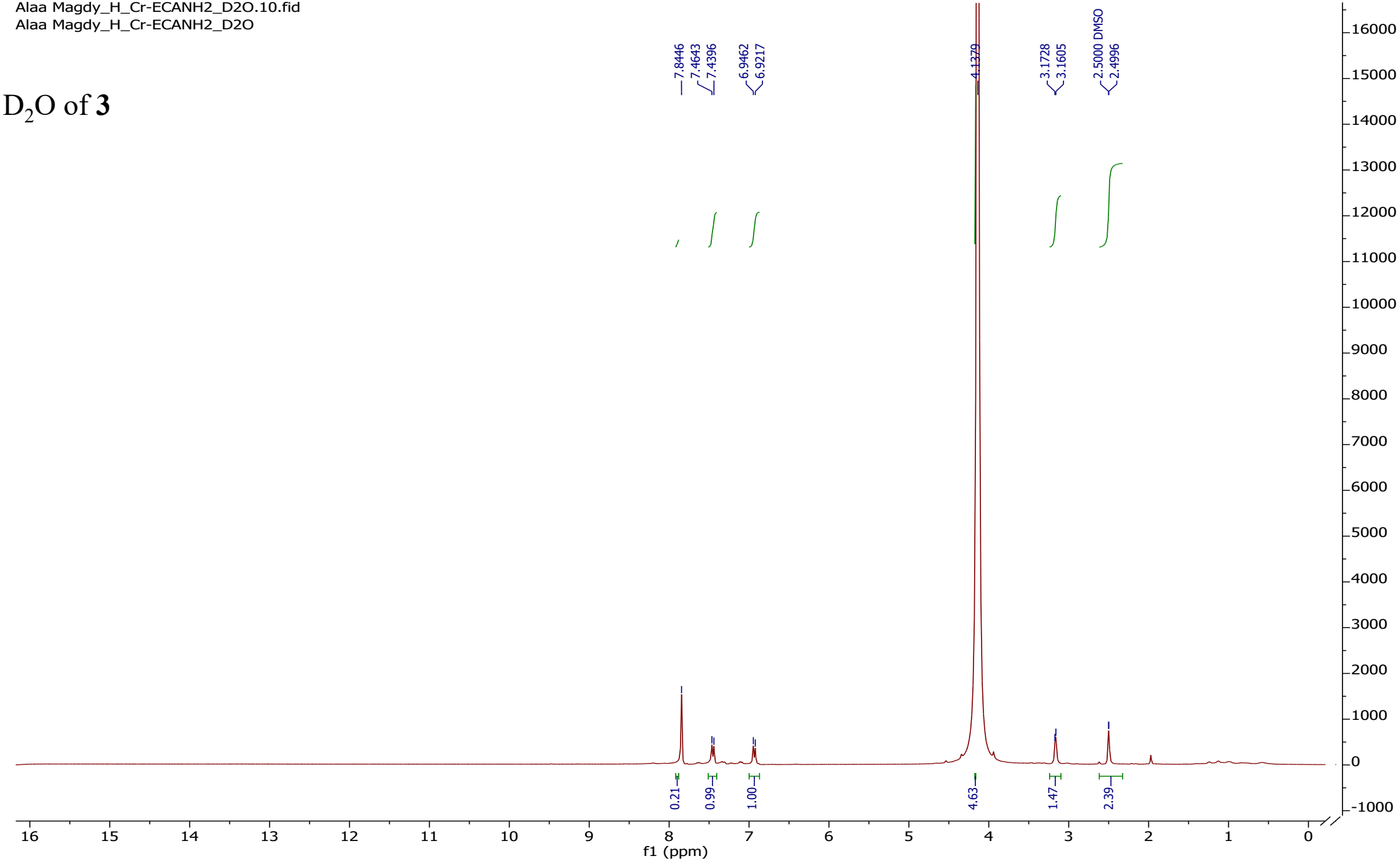
Alaa Magdy_H_Cr-ECANH2NH2.10.fid
Alaa Magdy_H_Cr-ECANH2NH2

^{1}HNMR of **3** in DMSO

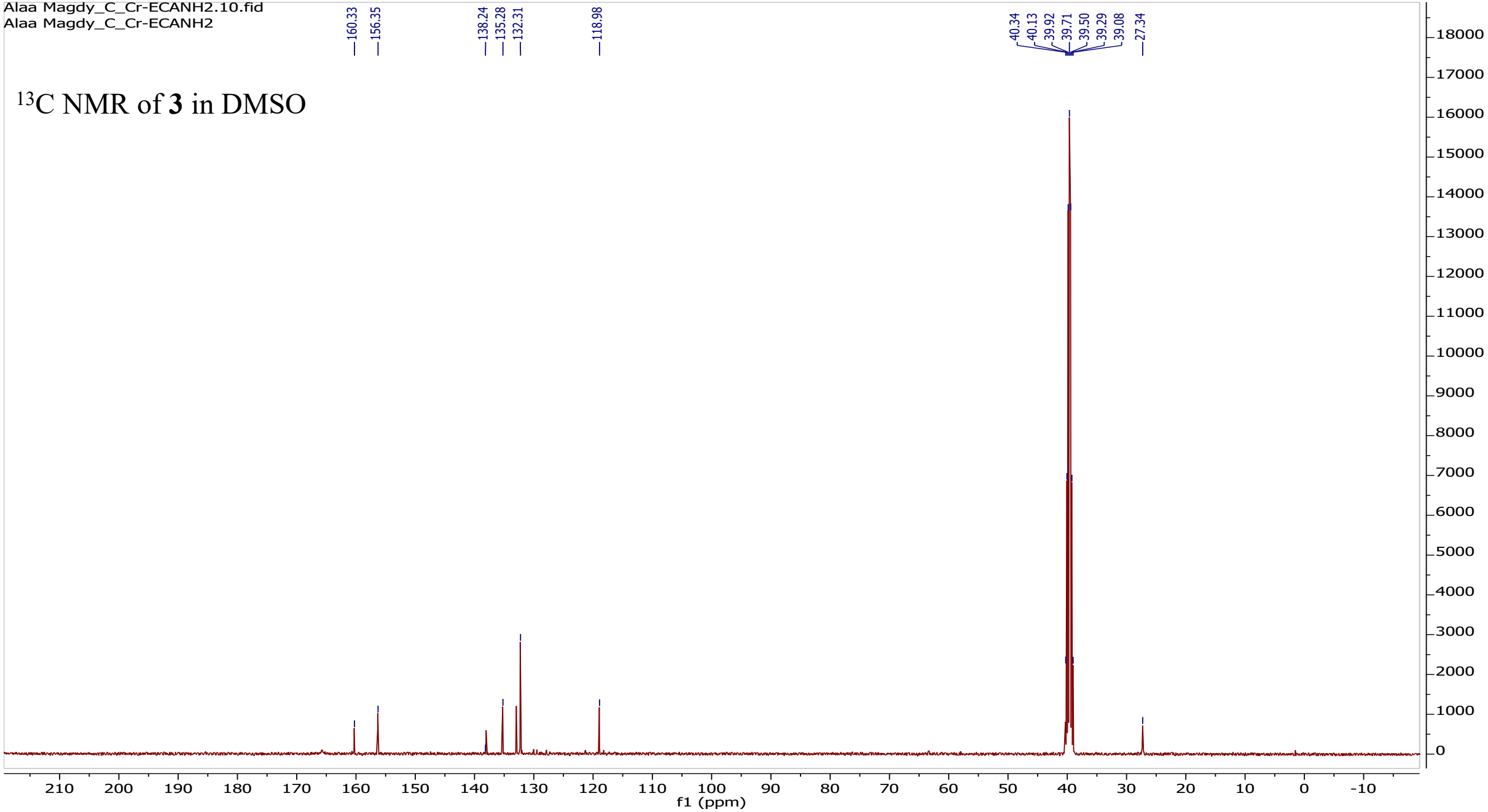


Alaa Magdy_H_Cr-ECANH2_D2O.10.fid
Alaa Magdy_H_Cr-ECANH2_D2O

D_2O of **3**

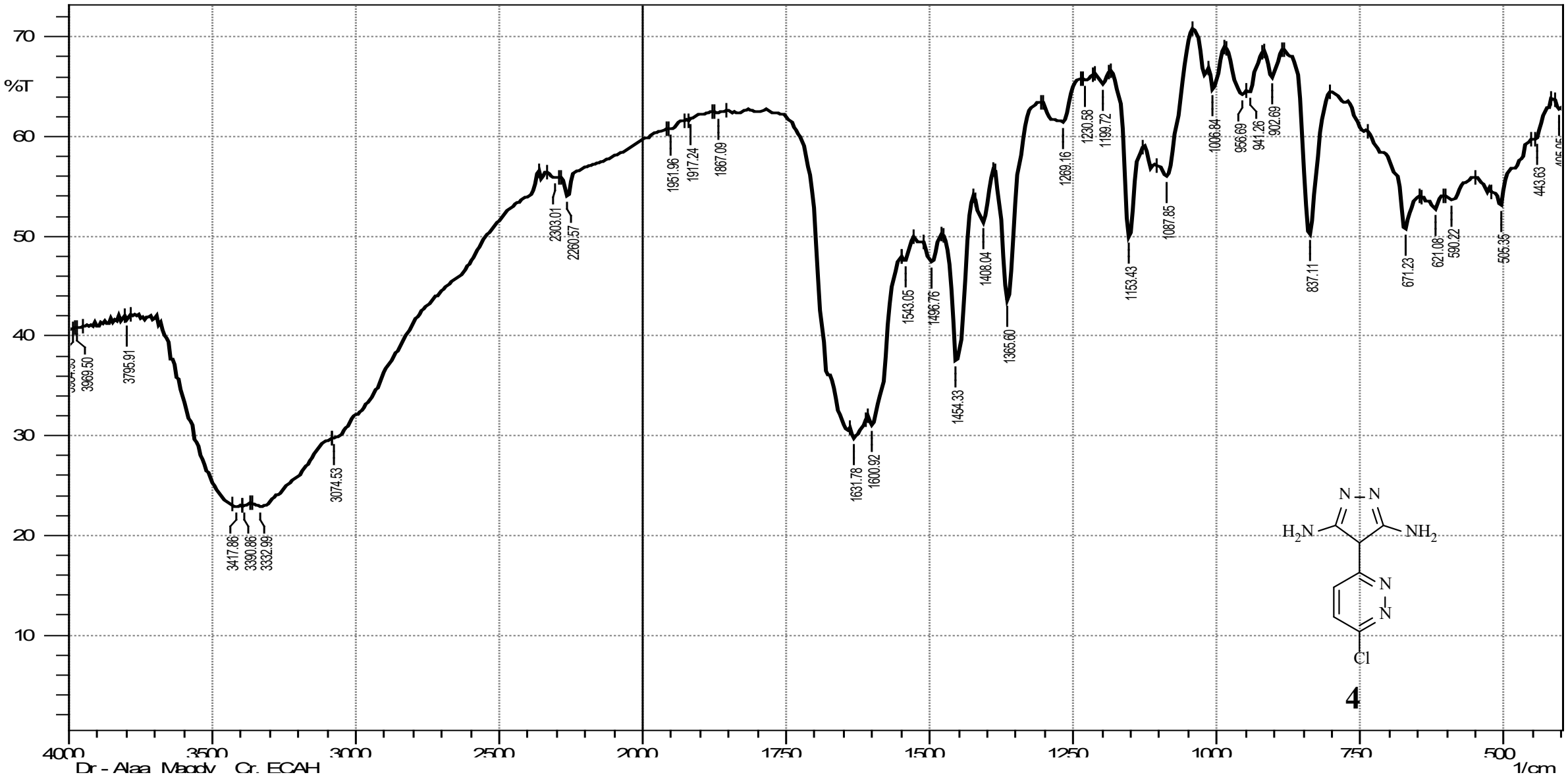


Alaa Magdy_C_Cr-ECANH2.10.fid
Alaa Magdy_C_Cr-ECANH2



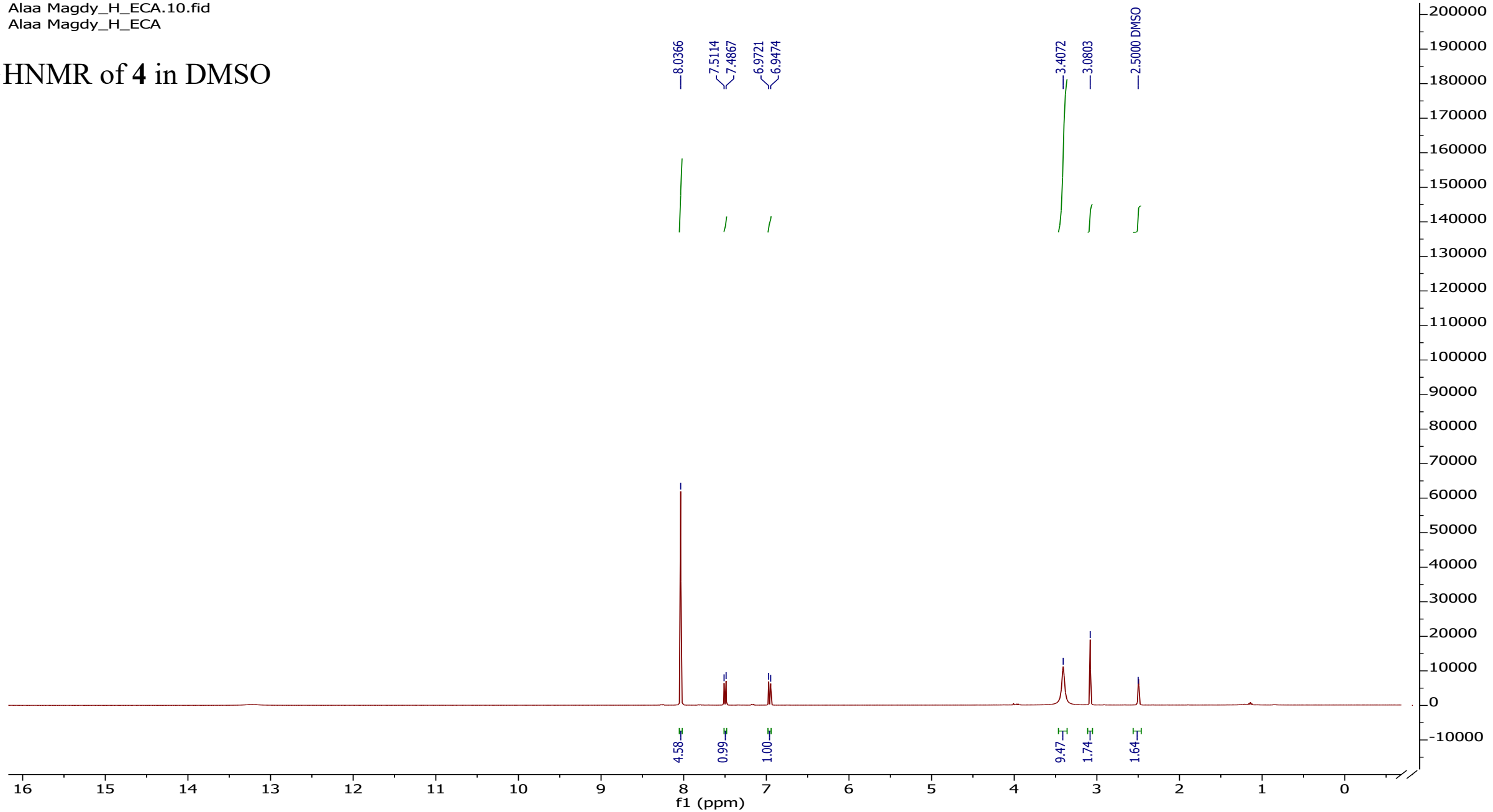
^{13}C NMR of **3** in DMSO

IR of compound **4**



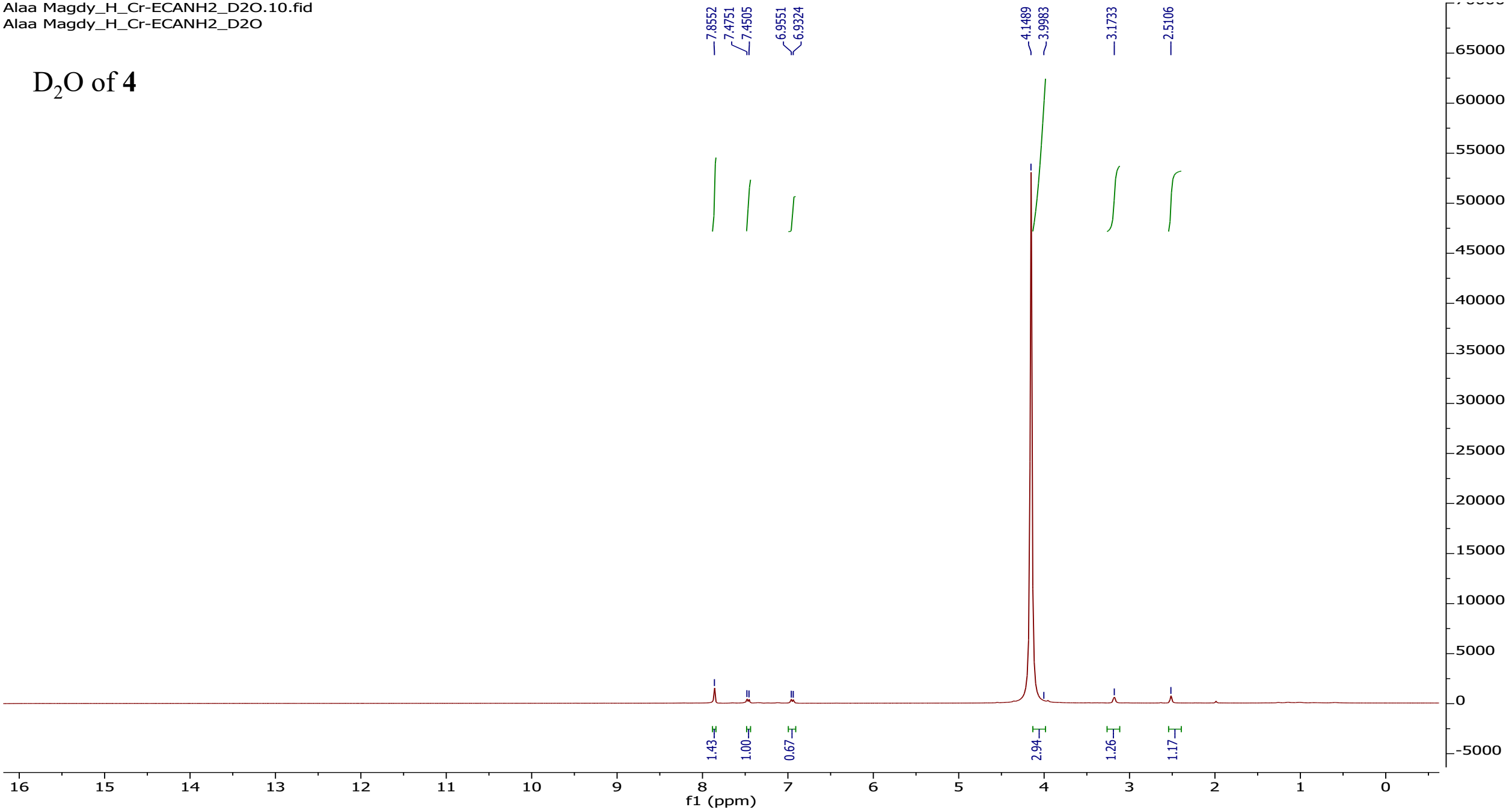
Alaa Magdy_H_ECA.10.fid
Alaa Magdy_H_ECA

^{1}HNMR of **4** in DMSO



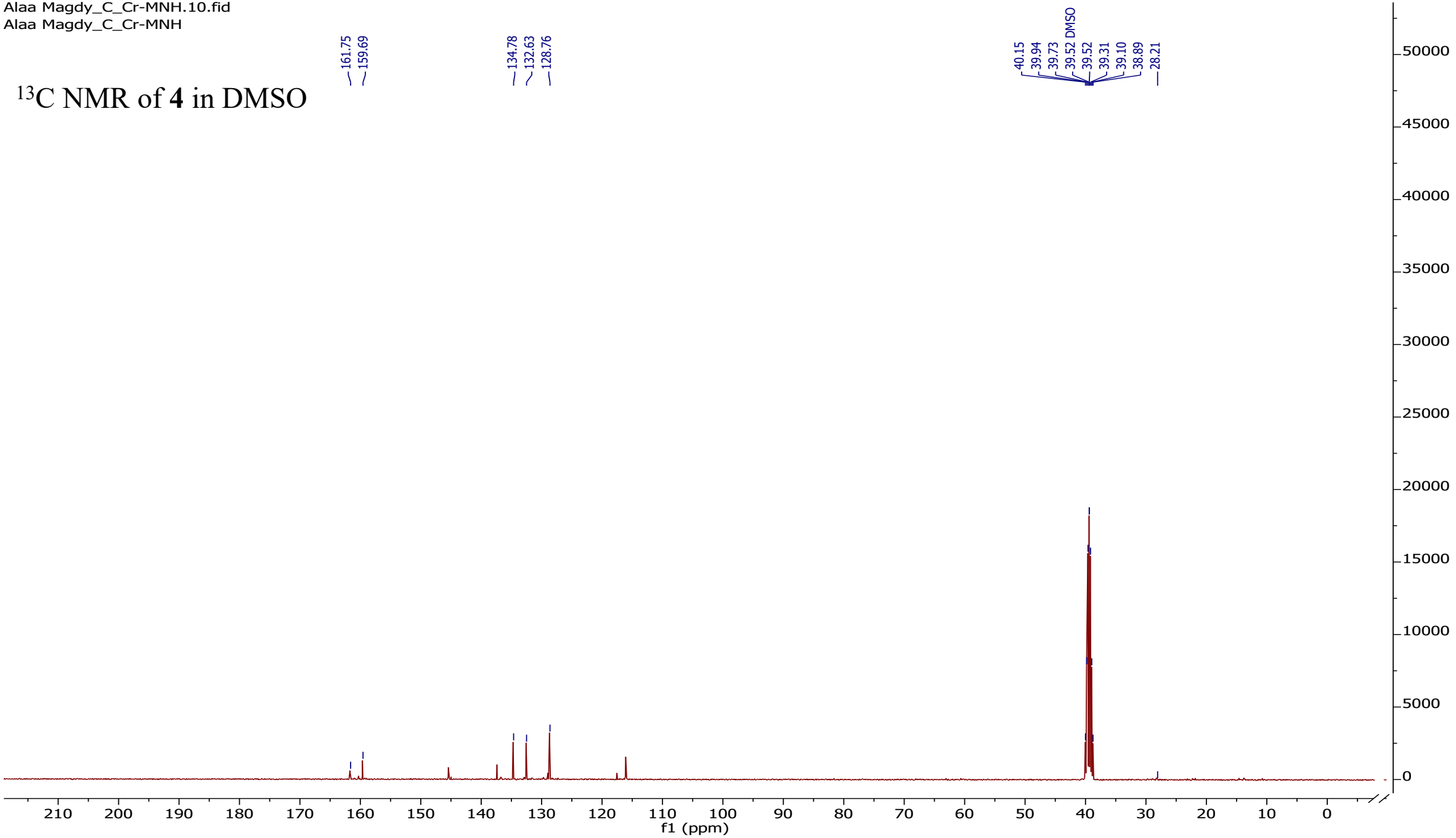
Alaa Magdy_H_Cr-ECANH2_D2O.10.fid
Alaa Magdy_H_Cr-ECANH2_D2O

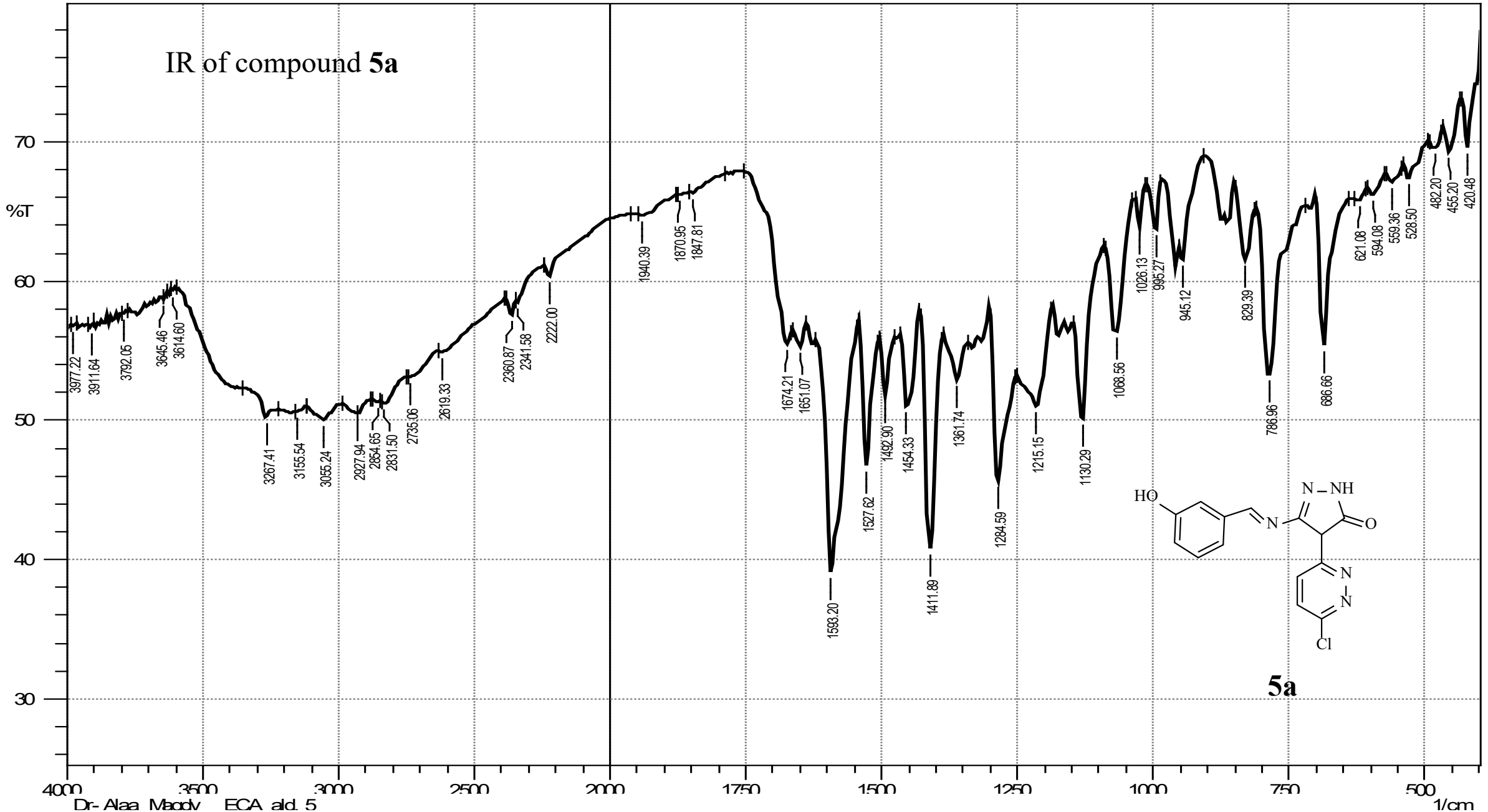
D_2O of **4**

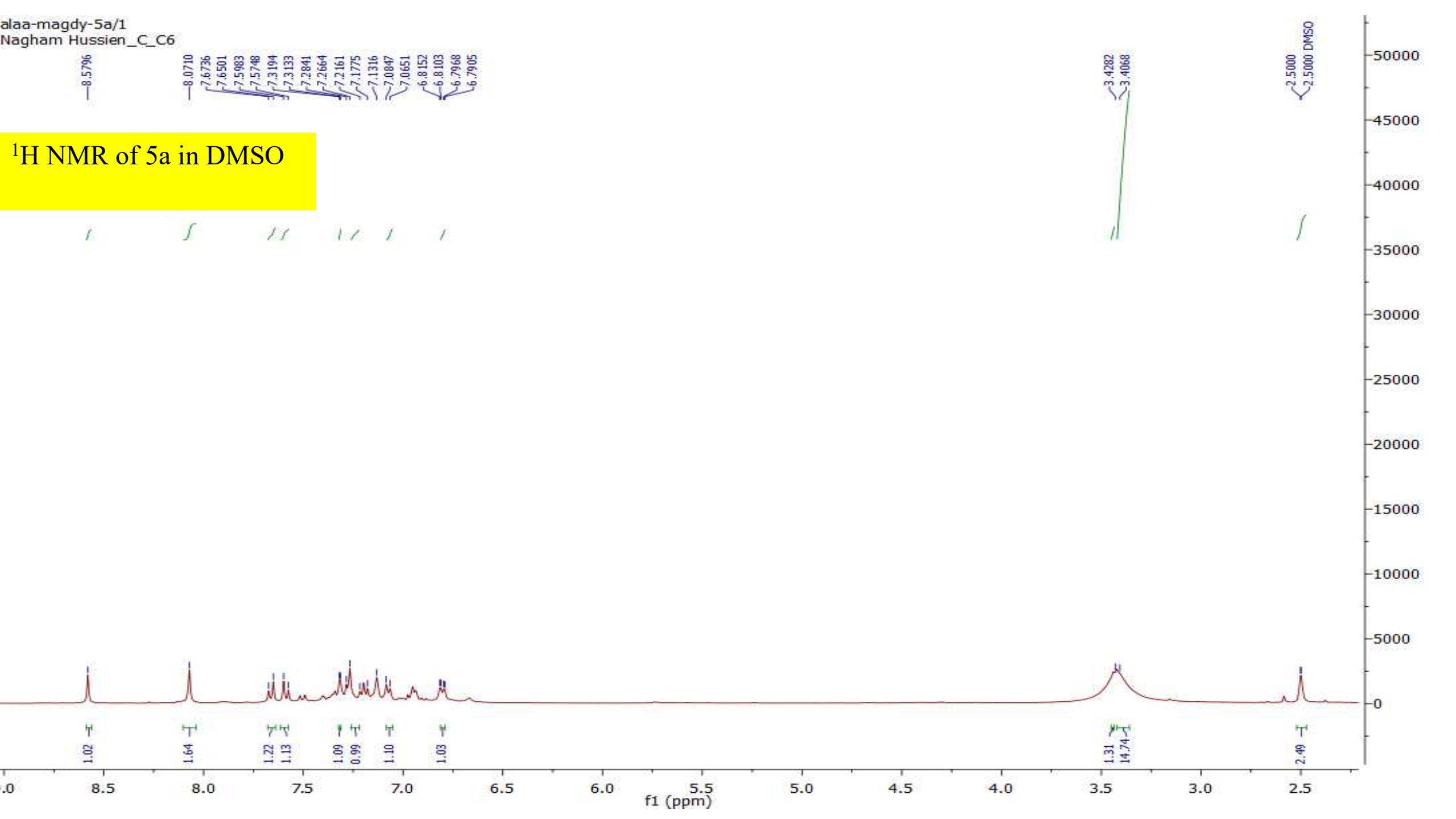


Alaa Magdy_C_Cr-MNH.10.fid
Alaa Magdy_C_Cr-MNH

^{13}C NMR of **4** in DMSO



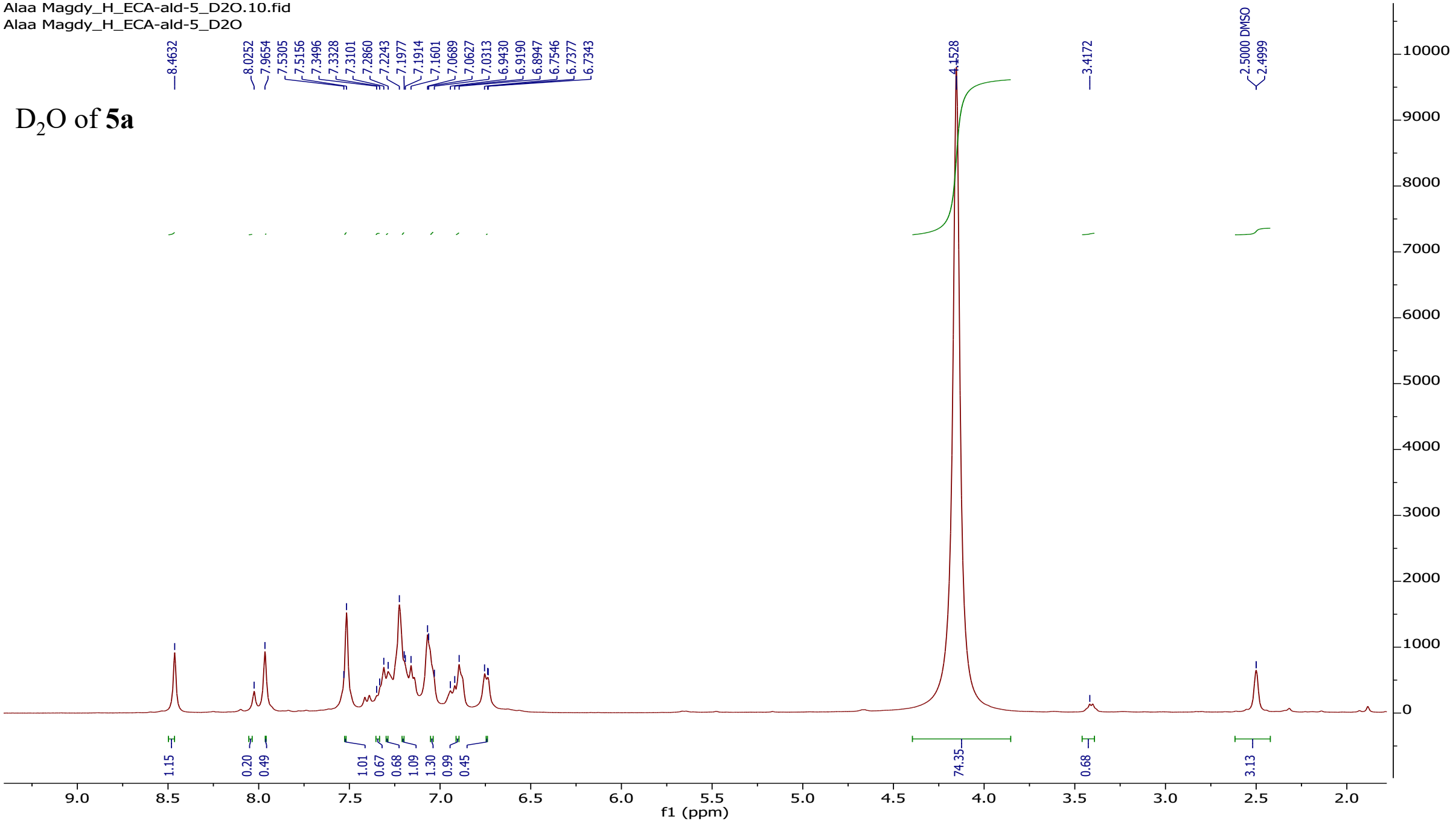

IR of compound 5a
%T
70
60
50
40
30
4000
3500
3000
2500
2000
1750
1500
1250
1000
750
500
1/cm
3977.22
3911.64
3792.05
3645.46
3614.60
3267.41
3155.54
3055.24
2927.94
2854.65
2831.50
2735.06
2619.33
2360.87
2341.58
2222.00
1940.39
1870.95
1847.81
1674.21
1651.07
1593.20
1527.62
1492.90
1454.33
1411.89
1361.74
1284.59
1215.15
1130.29
1068.56
1026.13
995.27
945.12
829.39
786.96
686.66
621.08
594.08
559.36
528.50
482.20
455.20
420.48
HO
N
N – NH
O
N
N
Cl
5a
Dr- Alaa Maody ECA ald 5



[1]H NMR of 5a in DMSO

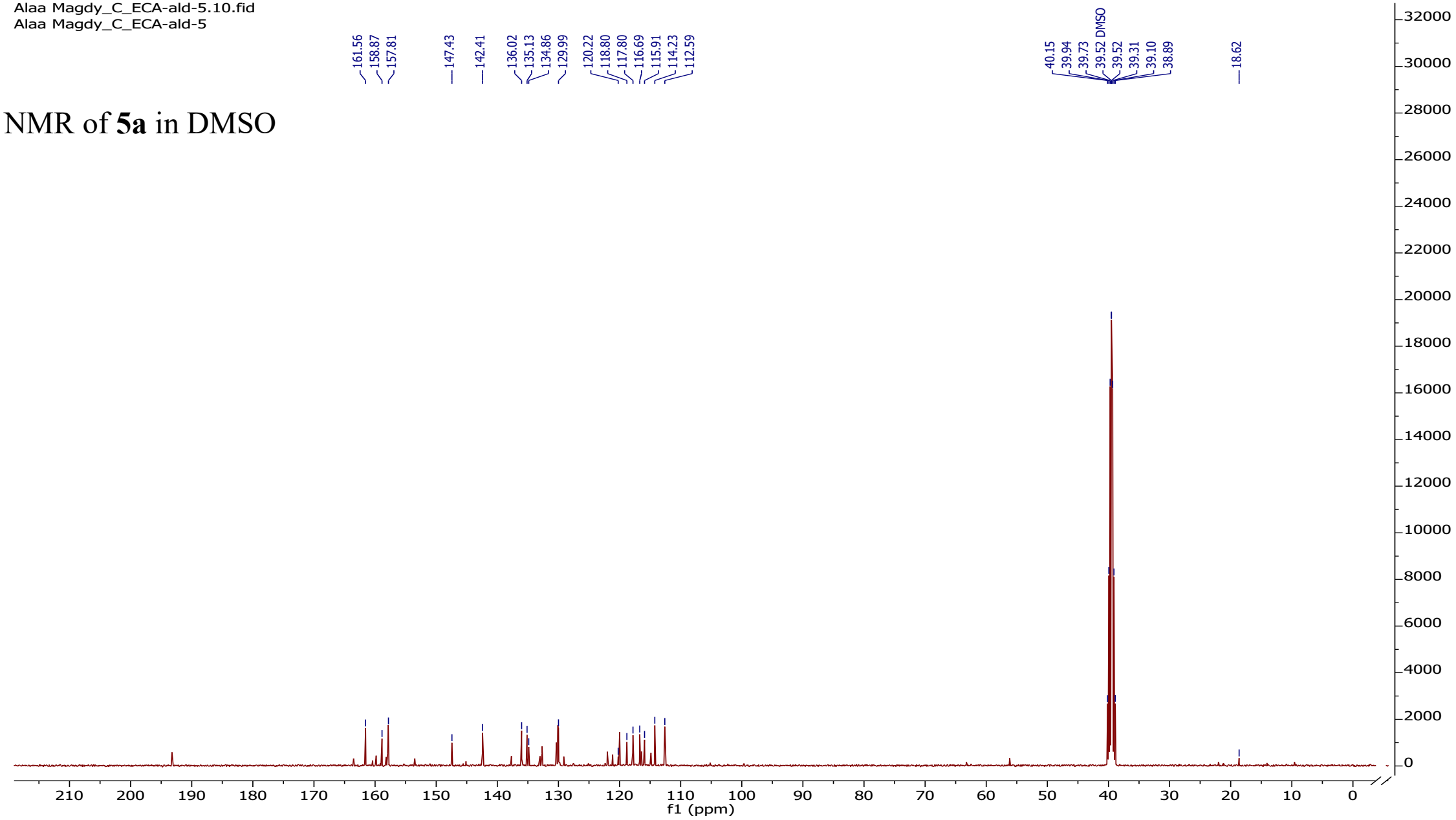
Alaa Magdy_H_ECA-ald-5_D2O.10.fid
Alaa Magdy_H_ECA-ald-5_D2O

D_2O of **5a**

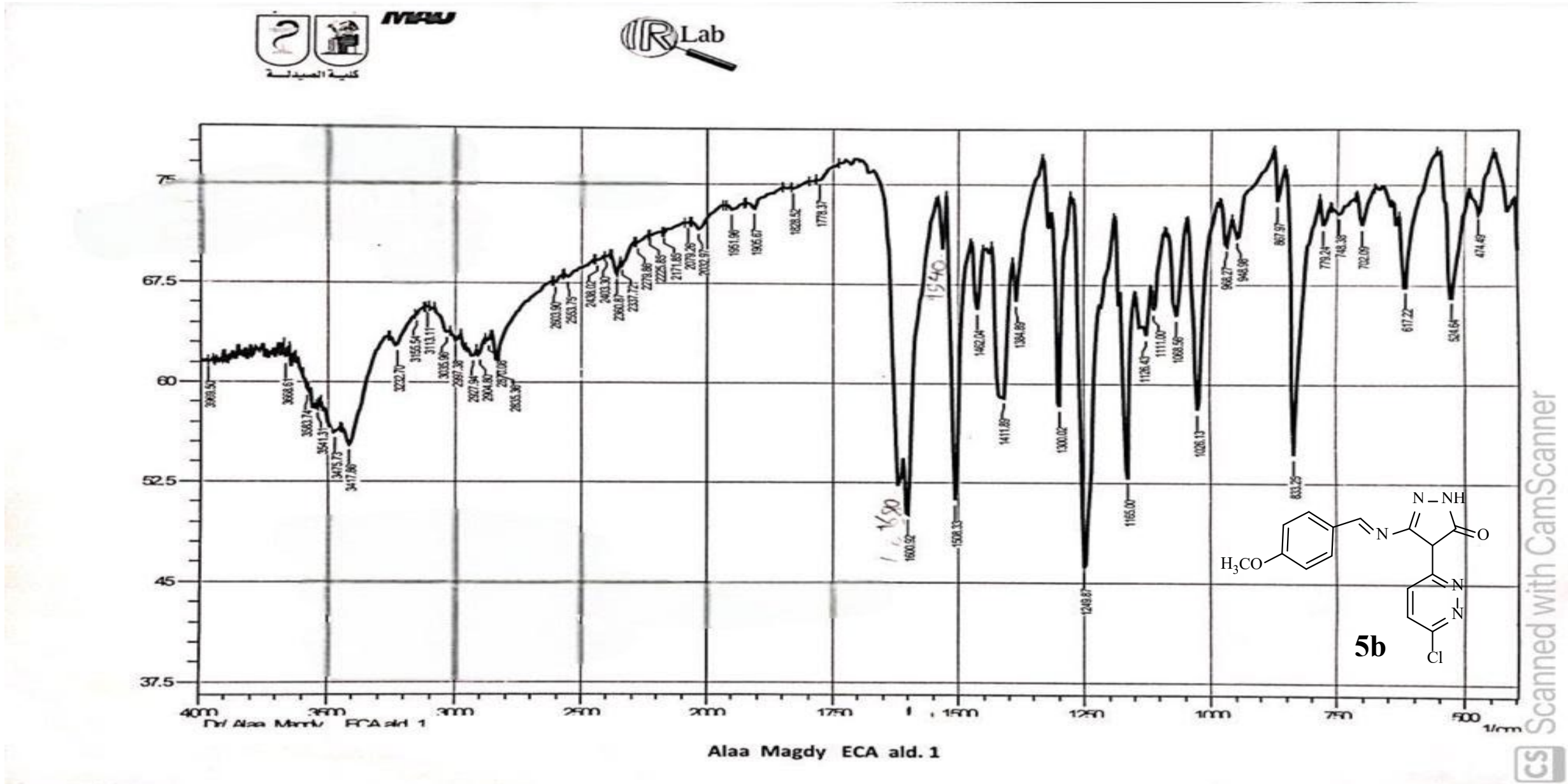


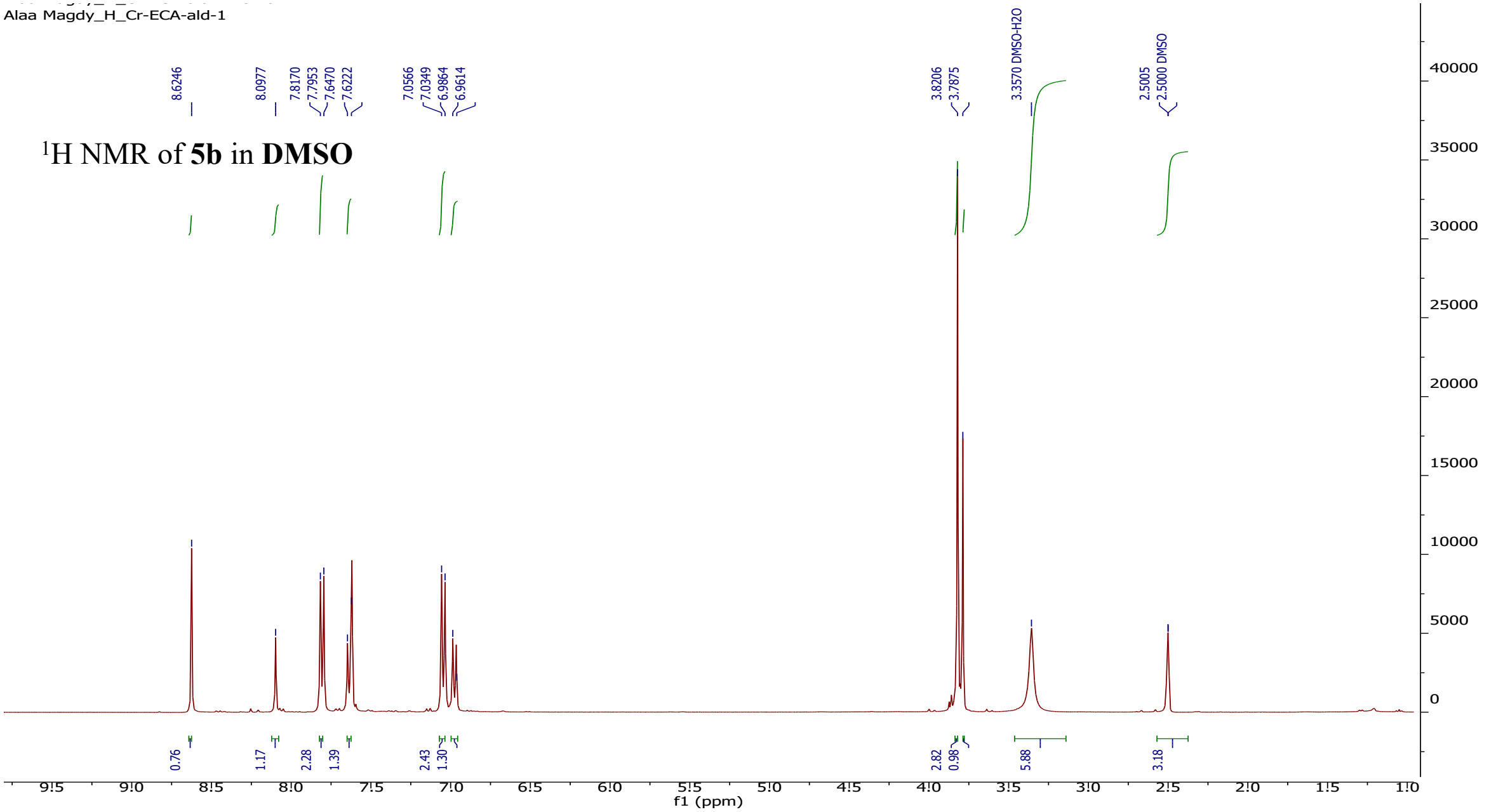
Alaa Magdy_C_ECA-ald-5.10.fid
Alaa Magdy_C_ECA-ald-5

^{13}C NMR of **5a** in DMSO



IR of compound **5b**

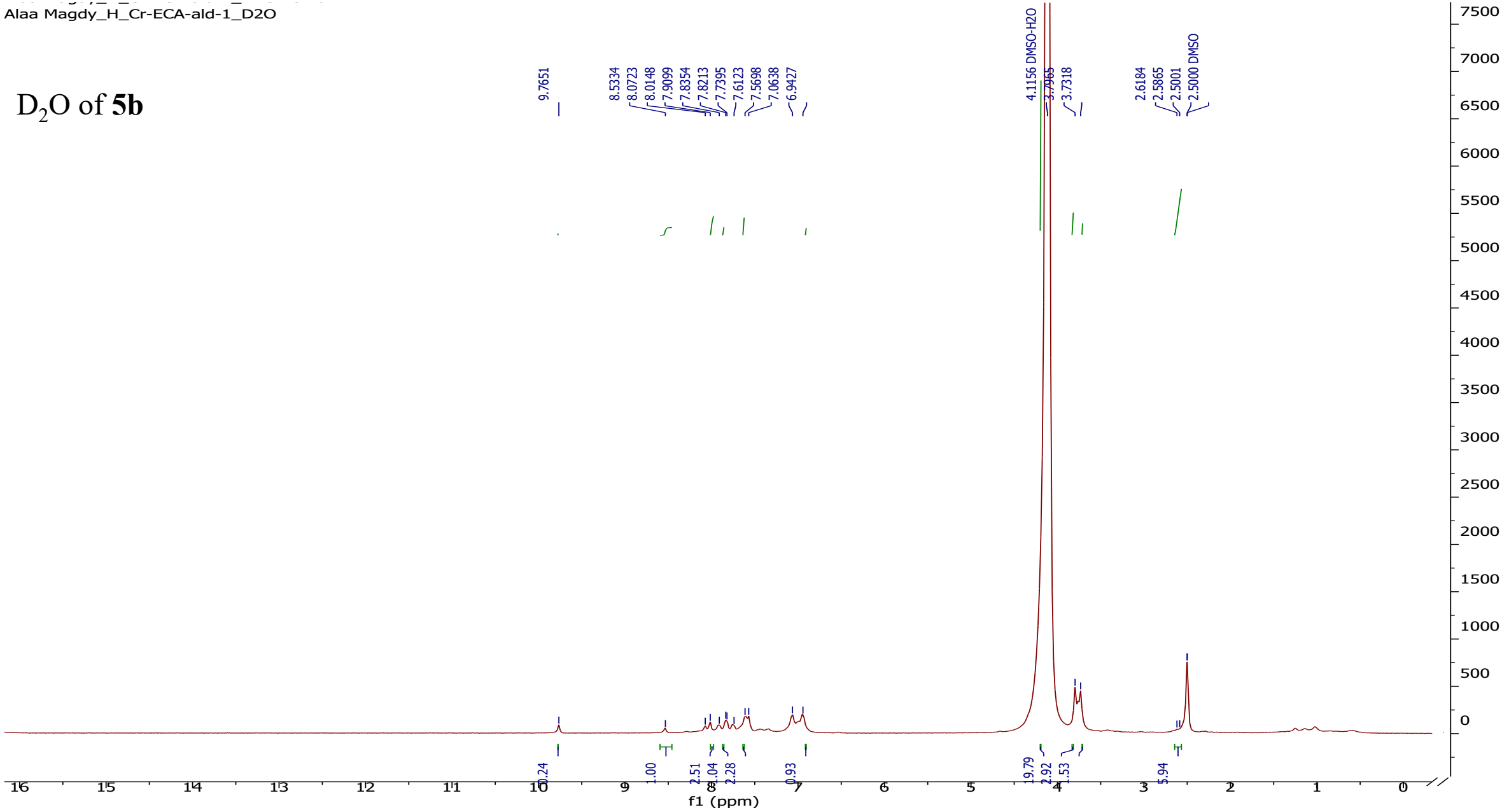




^{1}H NMR of **5b** in **DMSO**

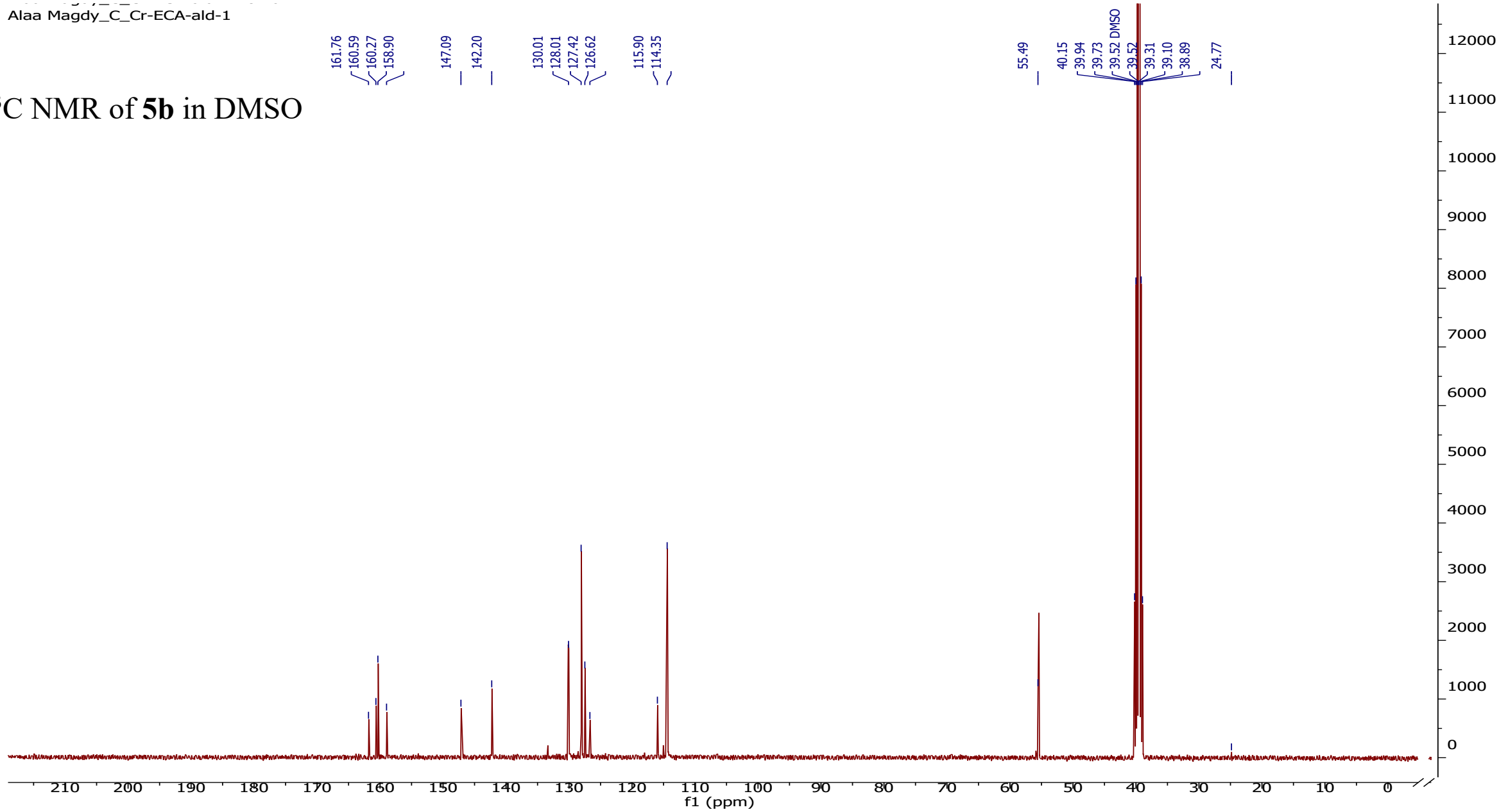
Alaa Magdy_H_Cr-ECA-ald-1_D2O

D_2O of **5b**

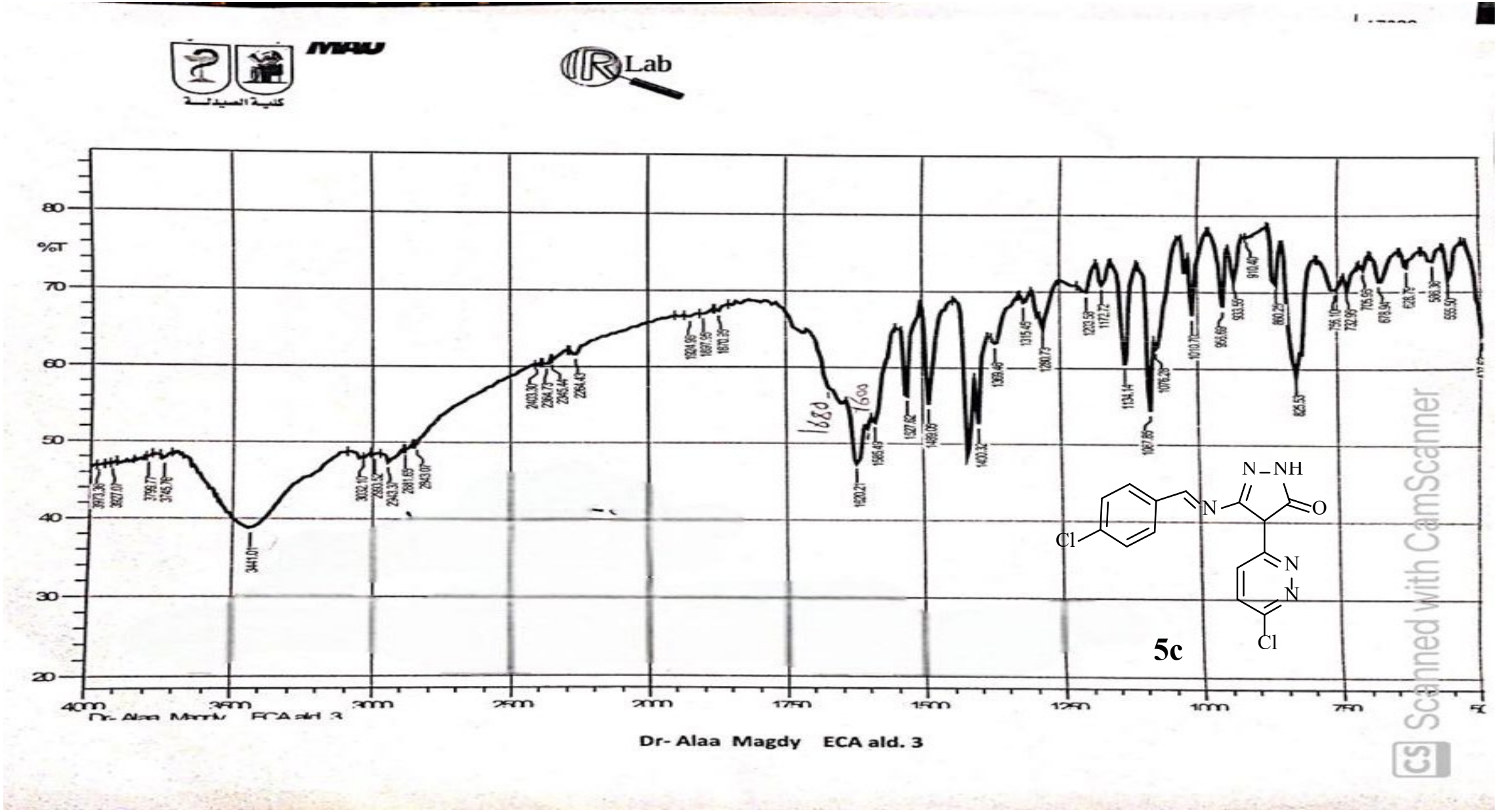


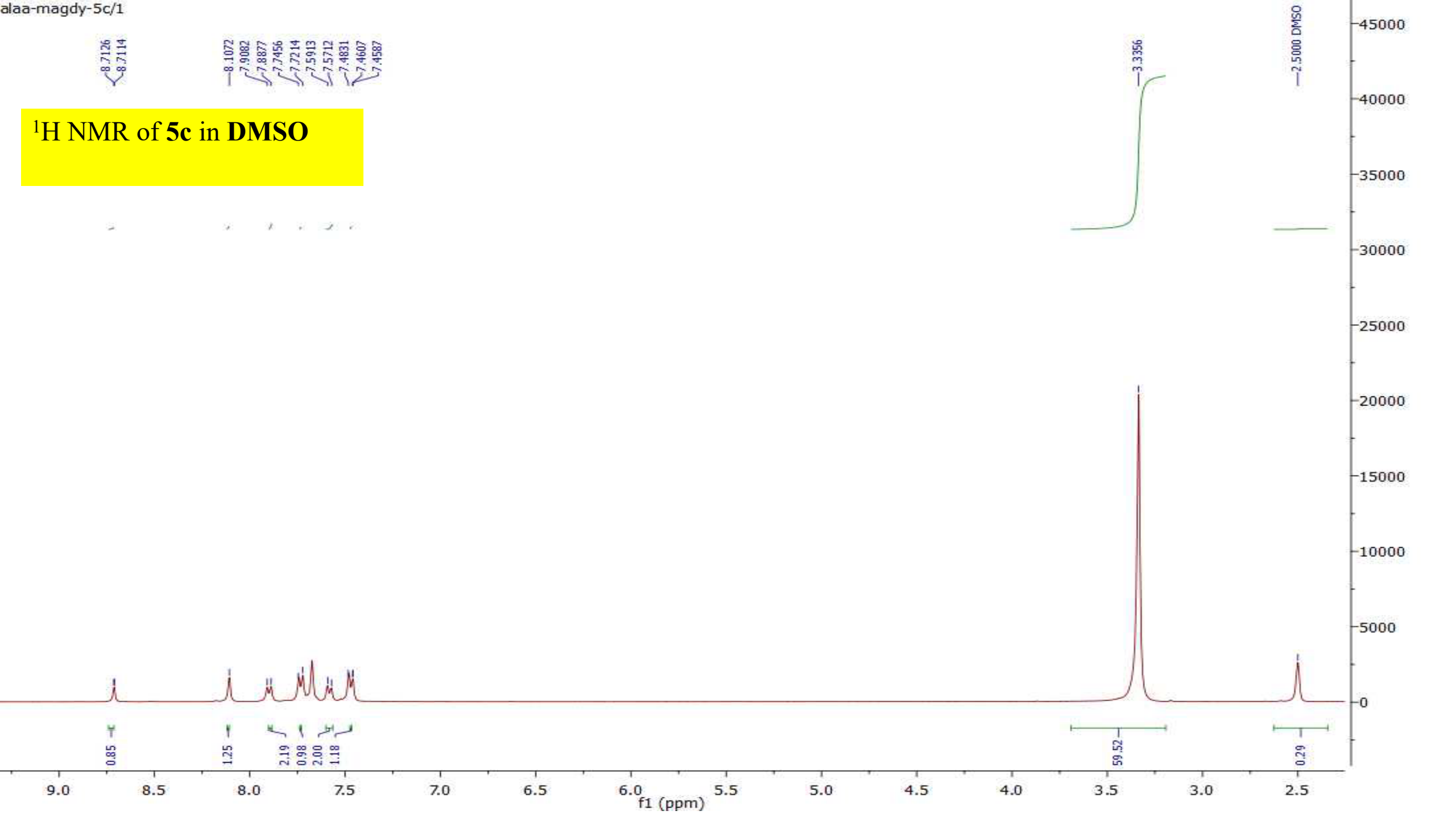
Alaa Magdy_C_Cr-ECA-ald-1

^{13}C NMR of **5b** in DMSO

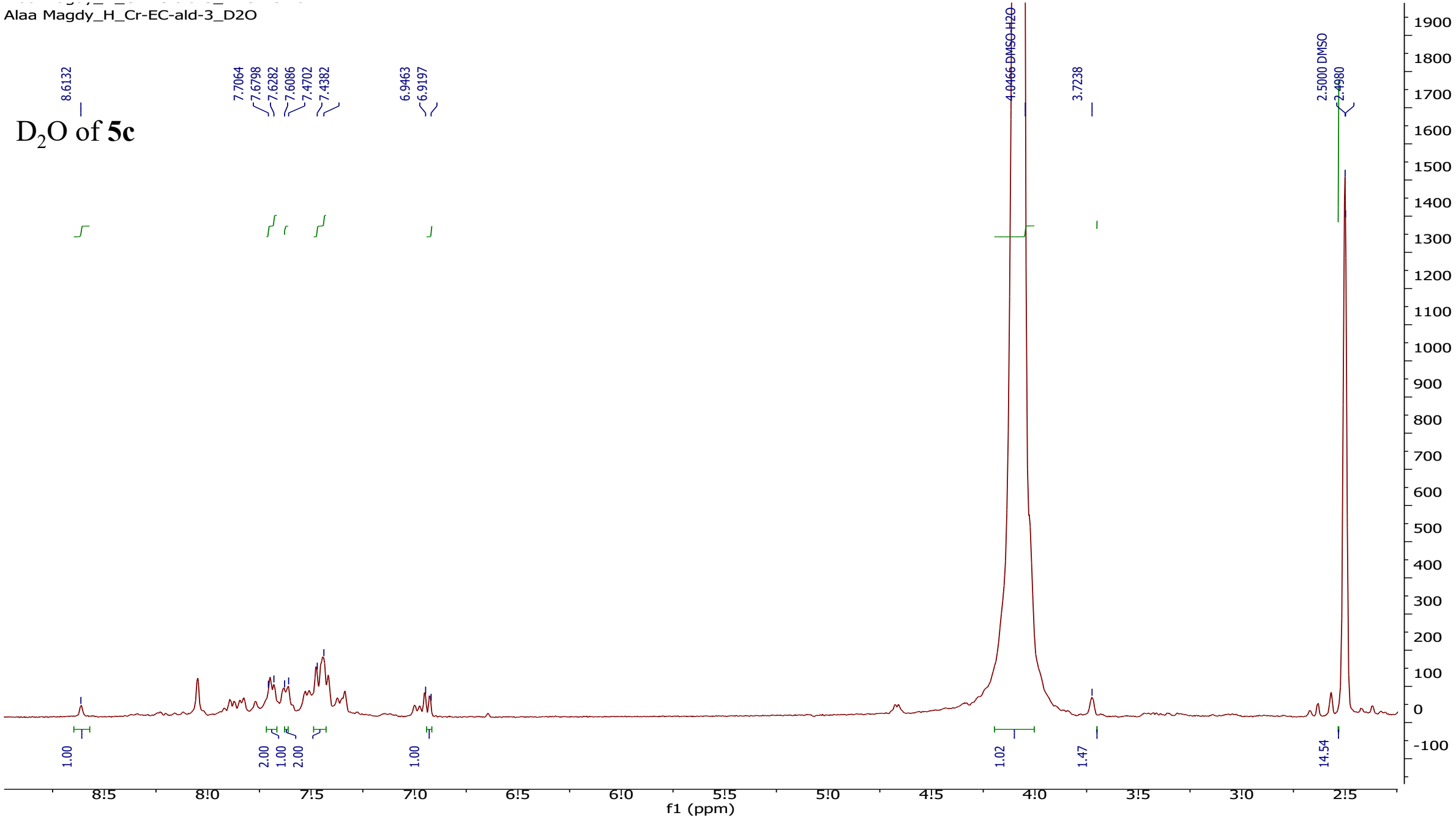


IR of compound **5c**



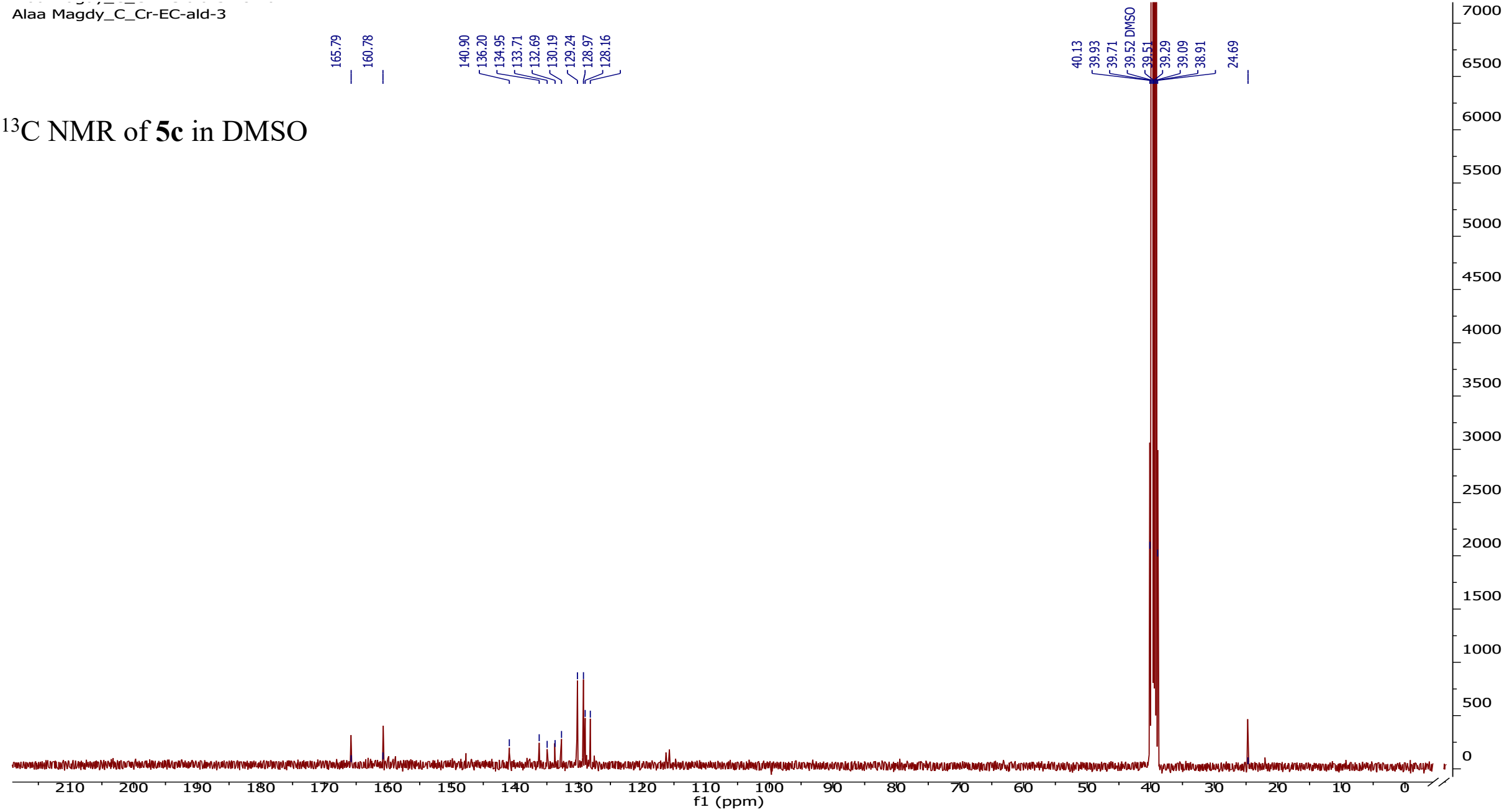


^{1}H NMR of **5c** in **DMSO**

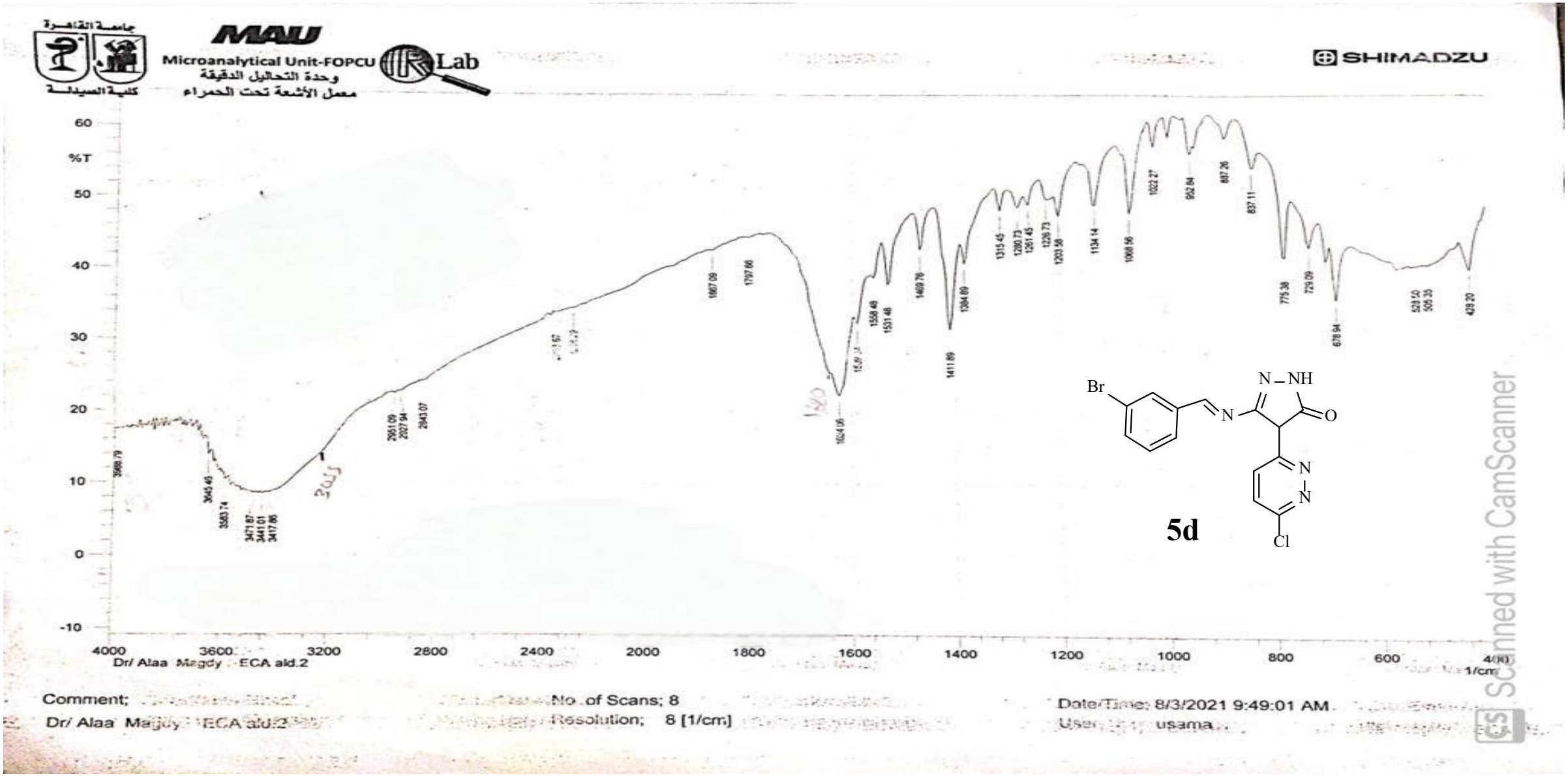

Alaa Magdy_H_Cr-EC-ald-3_D2O
D2O of 5c
8.6132
7.7064
7.6798
7.6282
7.6086
7.4702
7.4382
6.9463
6.9197
4.0466 DMSO H2O
3.7238
2.5000 DMSO
2.4980
1.00
2.00
1.00
2.00
1.00
1.02
1.47
14.54
f1 (ppm)

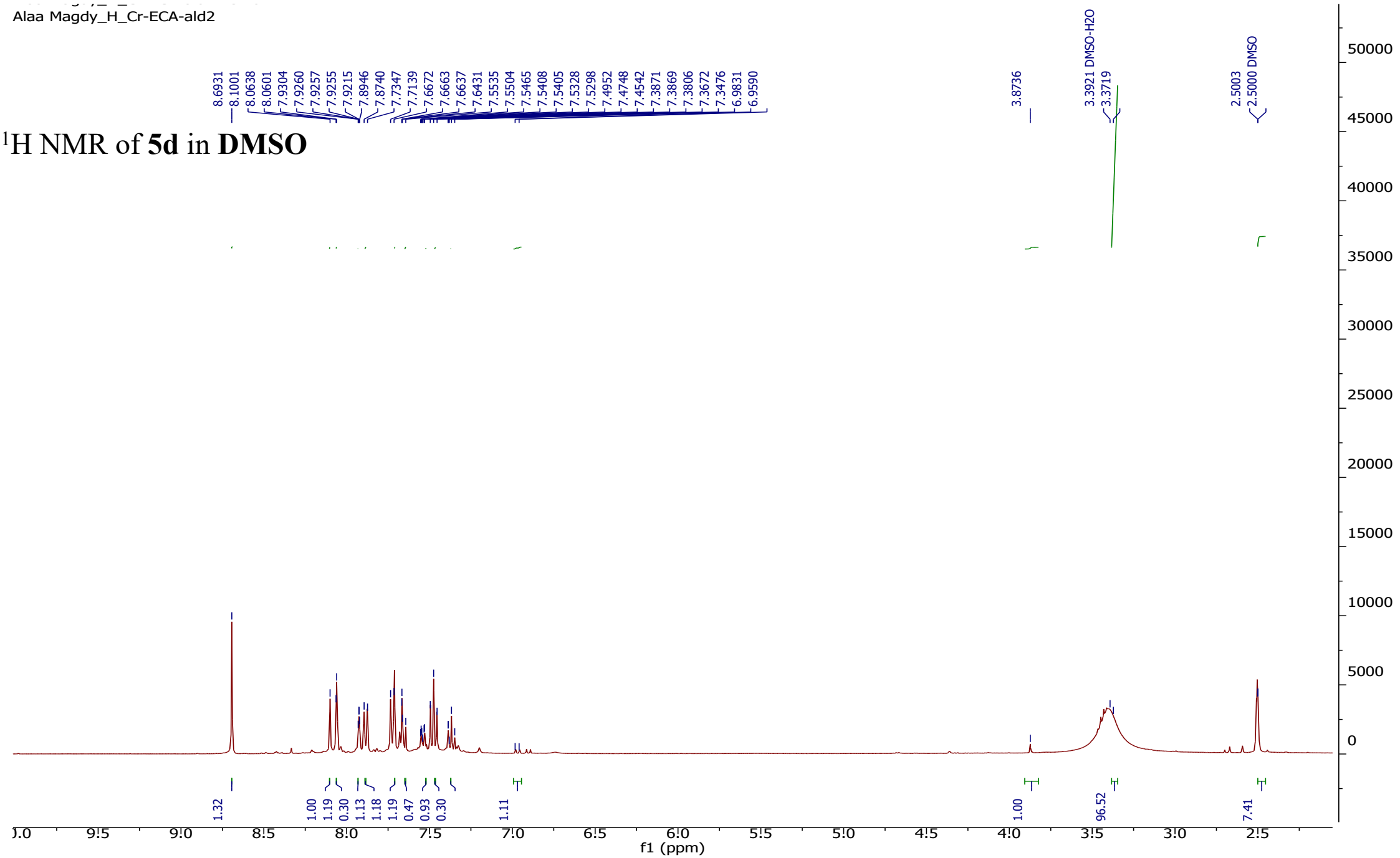
Alaa Magdy_C_Cr-EC-ald-3

^{13}C NMR of **5c** in DMSO



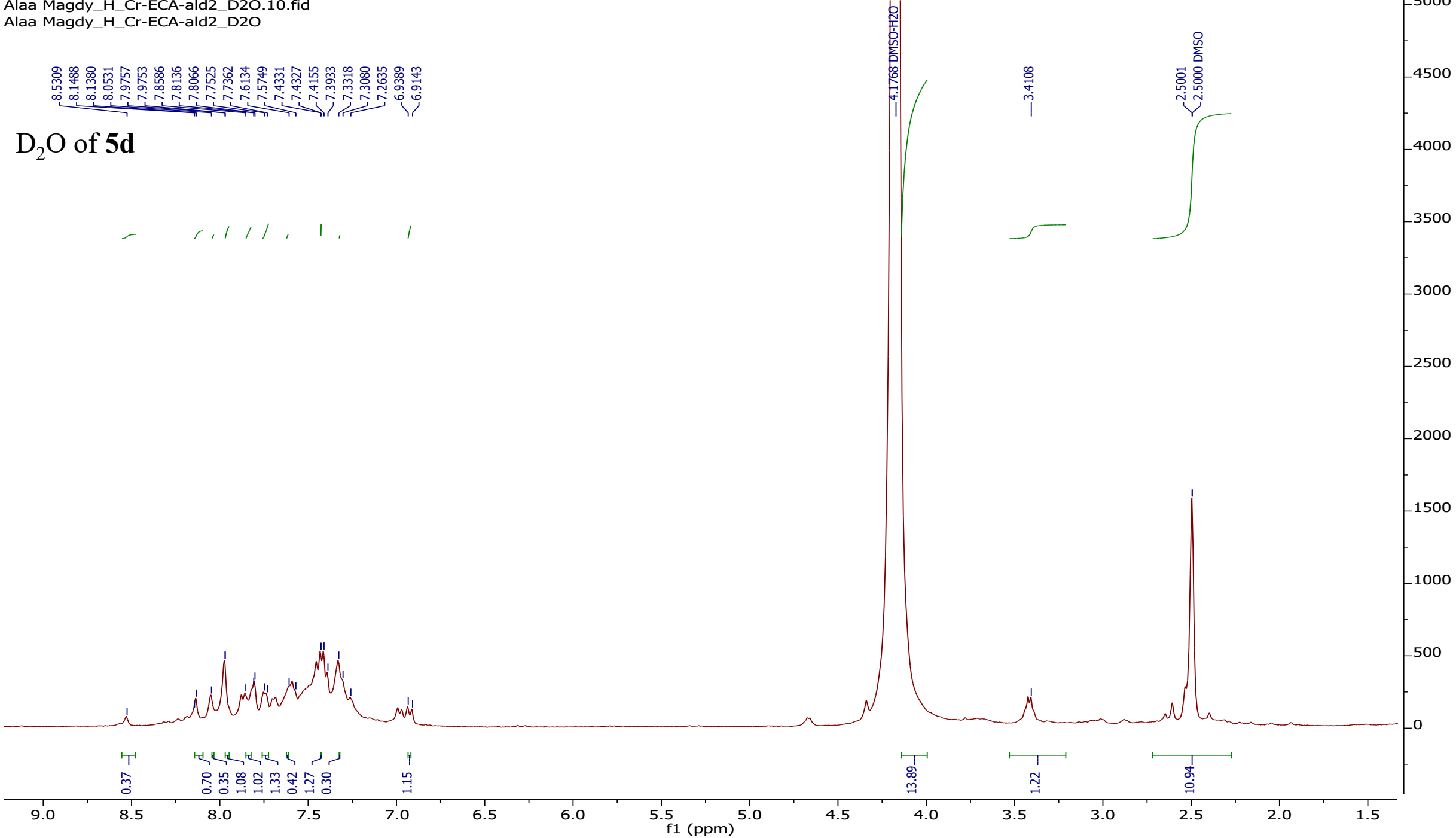
IR of compound **5d**





^{1}H NMR of **5d** in **DMSO**

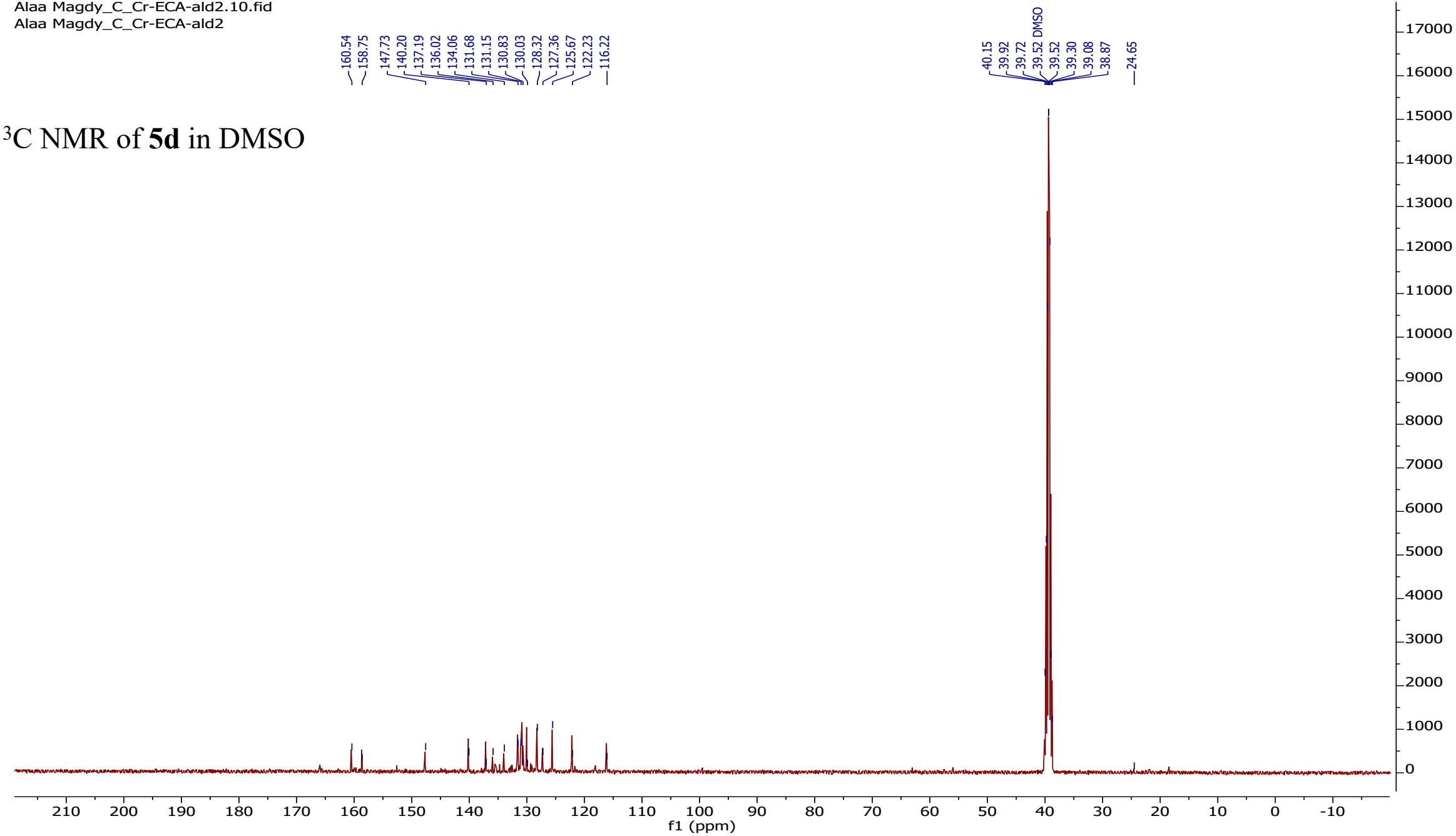
Alaa Magdy_H_Cr-ECA-ald2_D2O.10.fid
Alaa Magdy_H_Cr-ECA-ald2_D2O



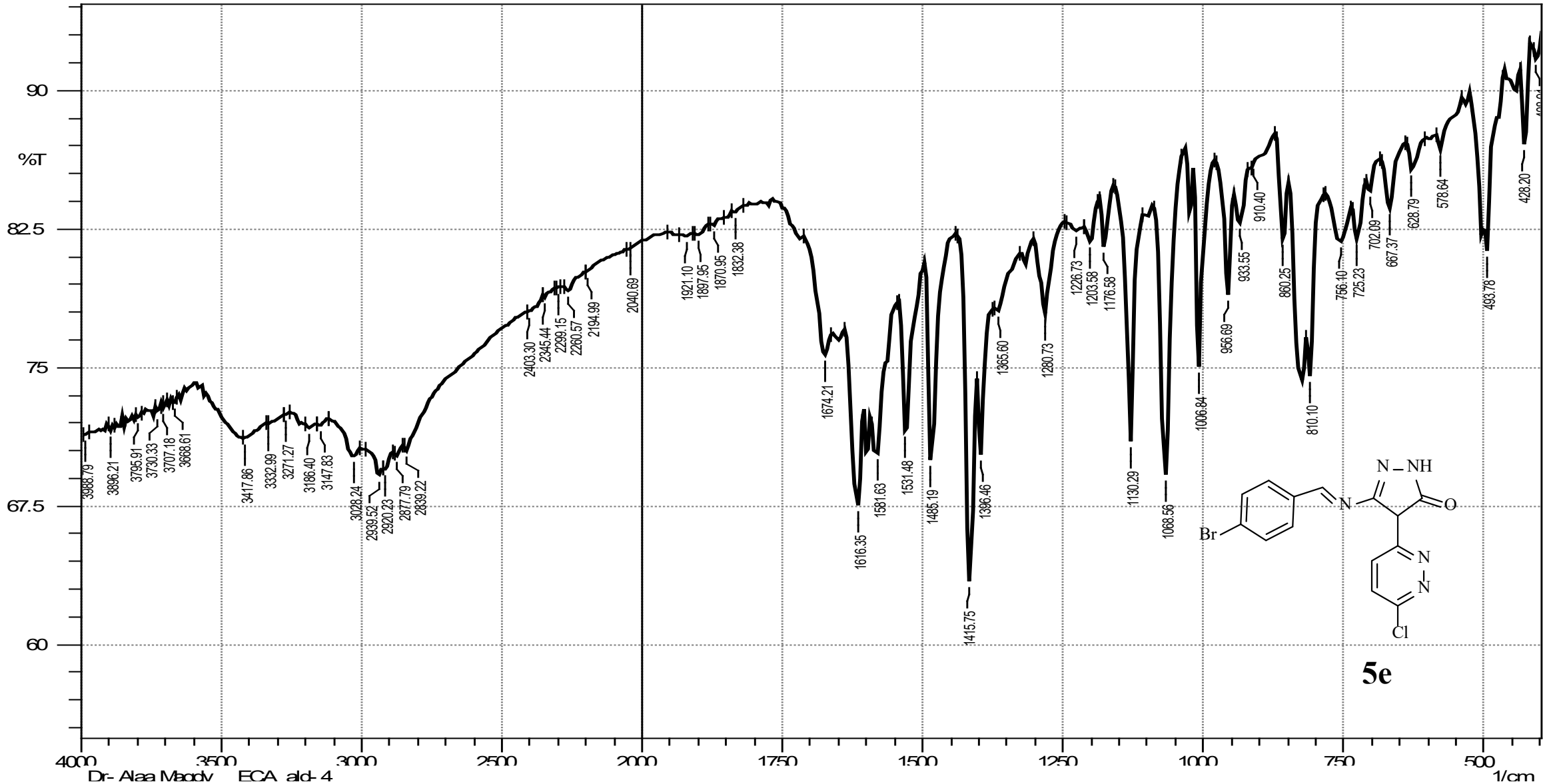
D_2O of **5d**

Alaa Magdy_C_Cr-ECA-ald2.10.fid
Alaa Magdy_C_Cr-ECA-ald2

^{13}C NMR of **5d** in DMSO

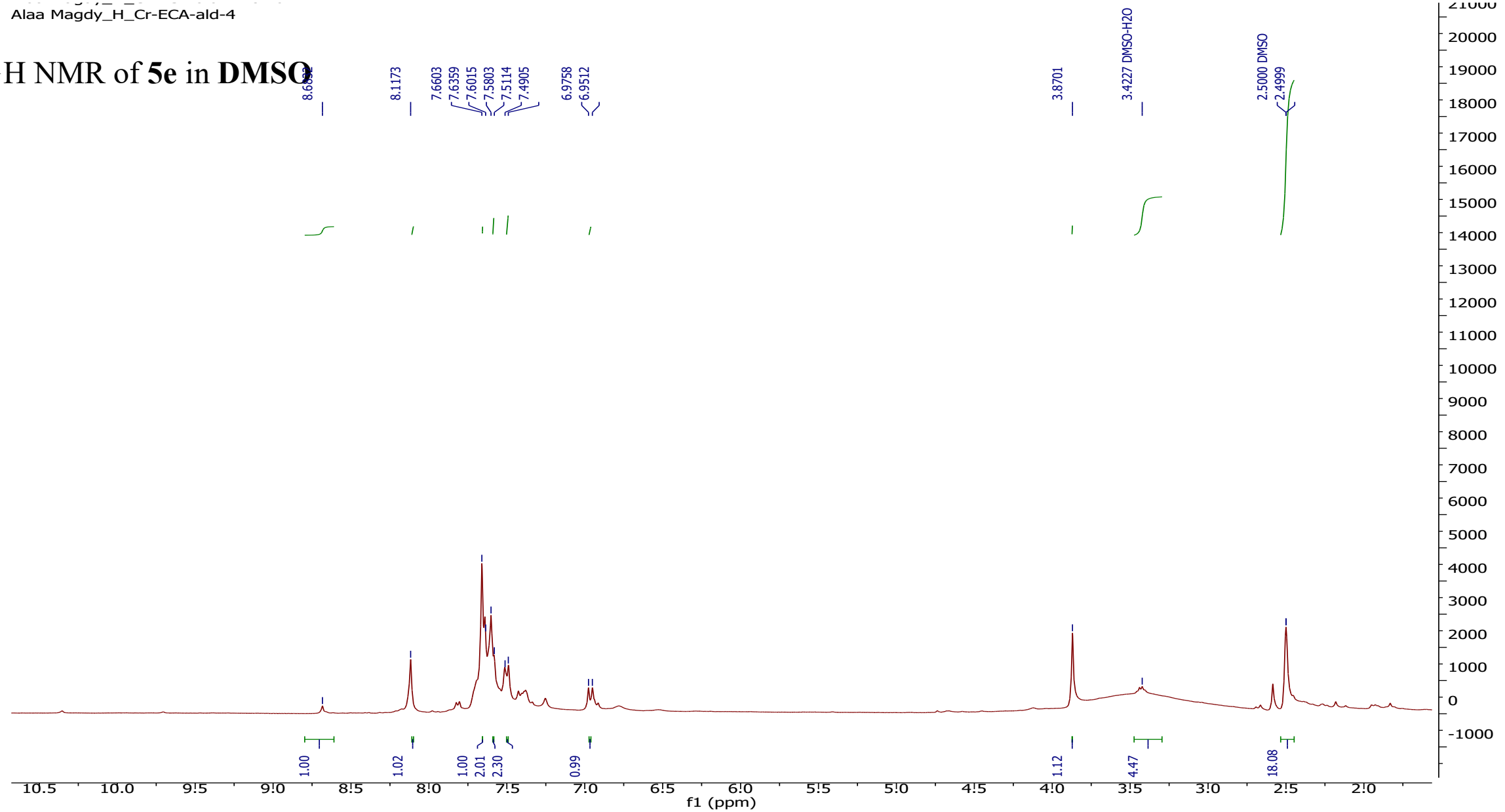


IR of compound **5e**



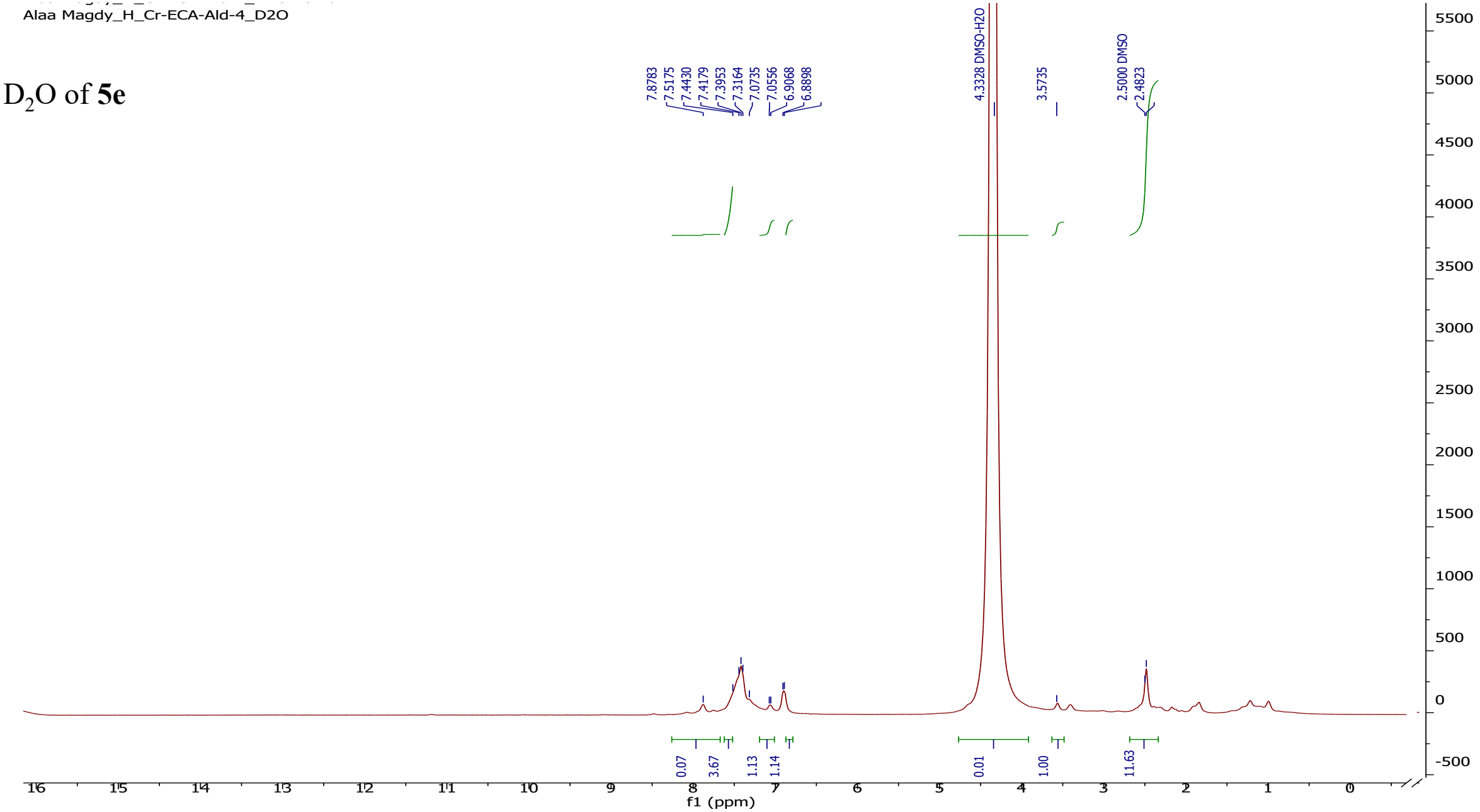
Alaa Magdy_H_Cr-ECA-ald-4

^{1}H NMR of **5e** in **DMSO**



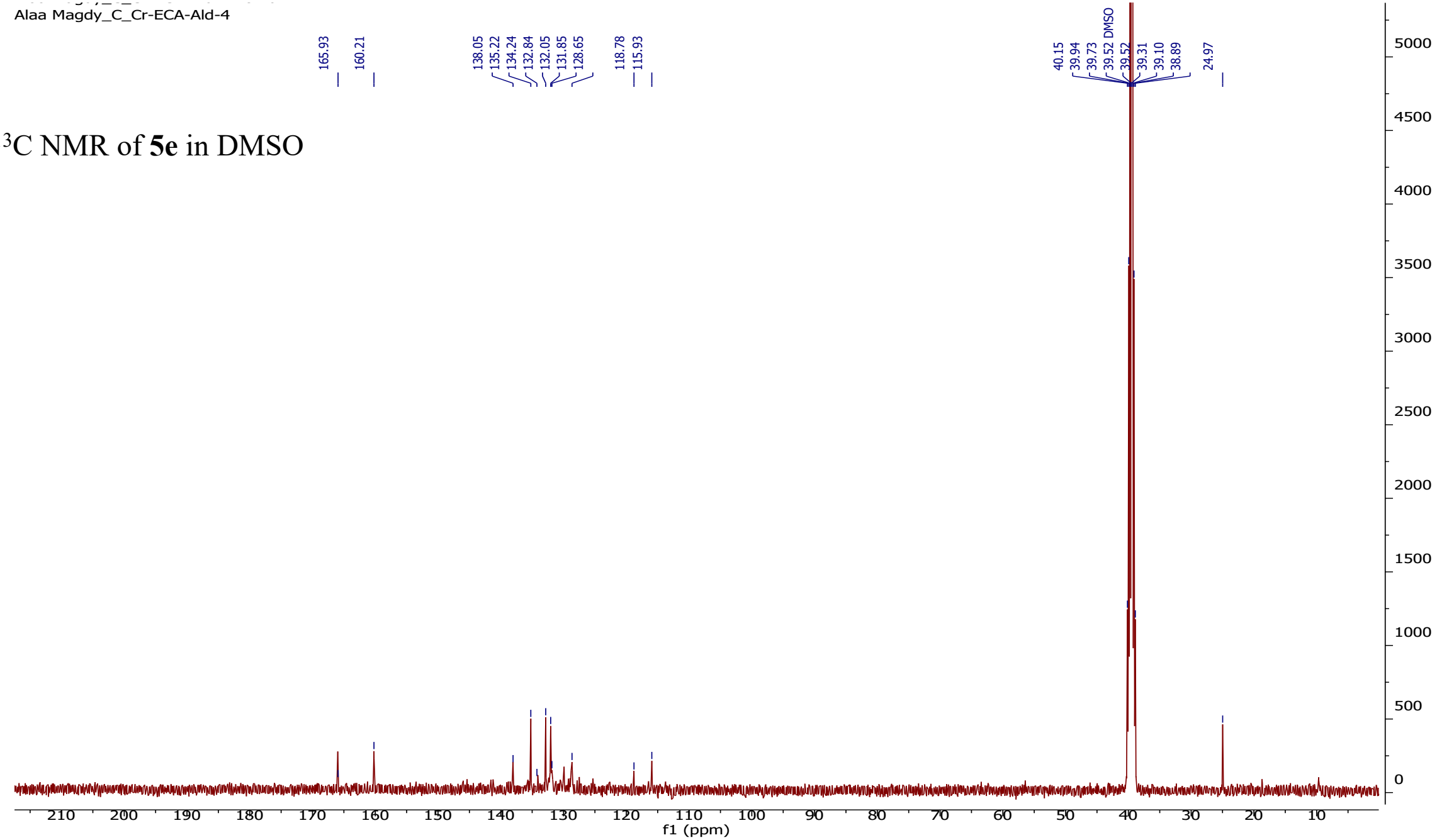
Alaa Magdy_H_Cr-ECA-Ald-4_D2O

D_2O of **5e**

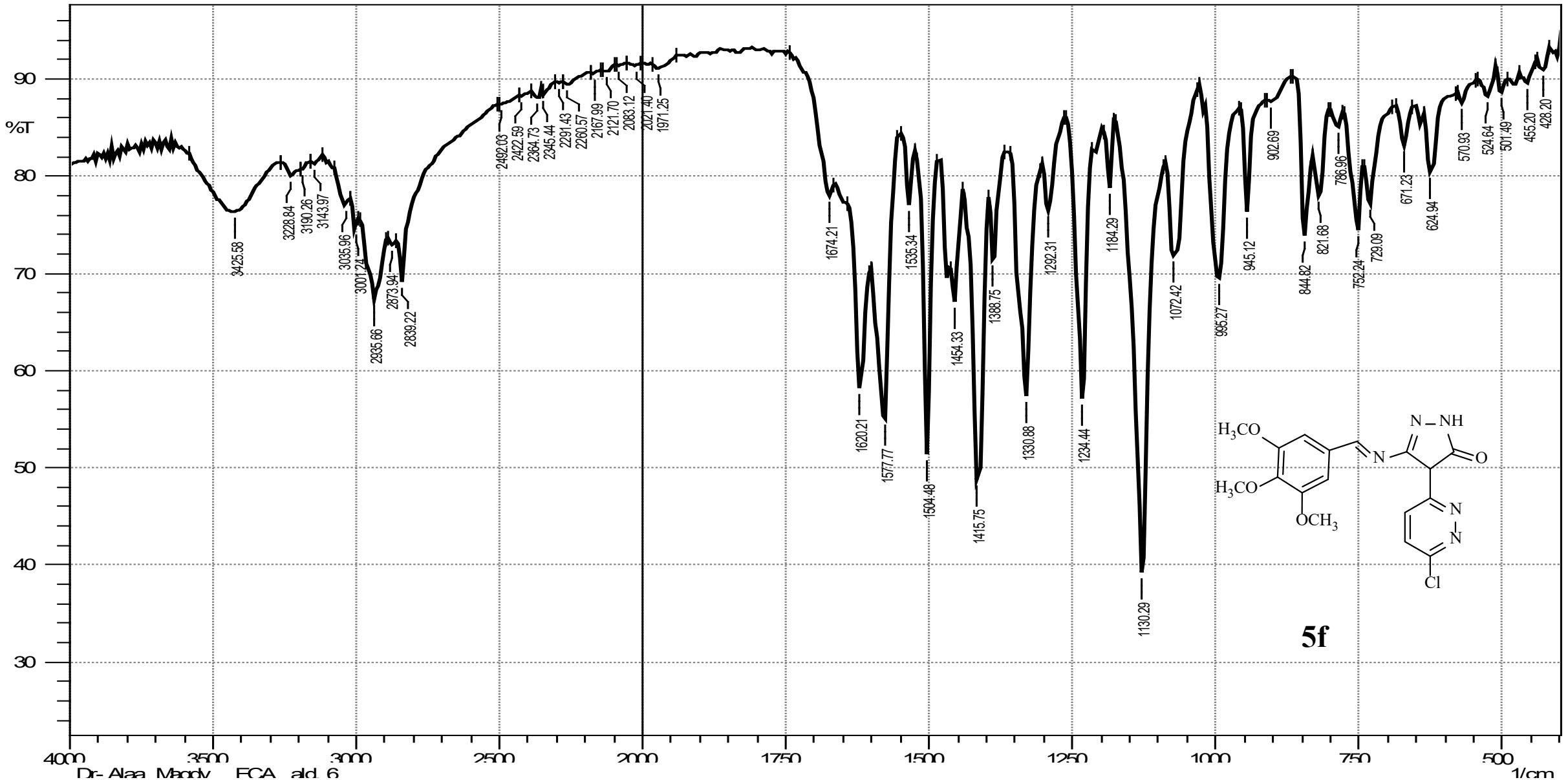


Alaa Magdy_C_Cr-ECA-Ald-4

^{13}C NMR of **5e** in DMSO

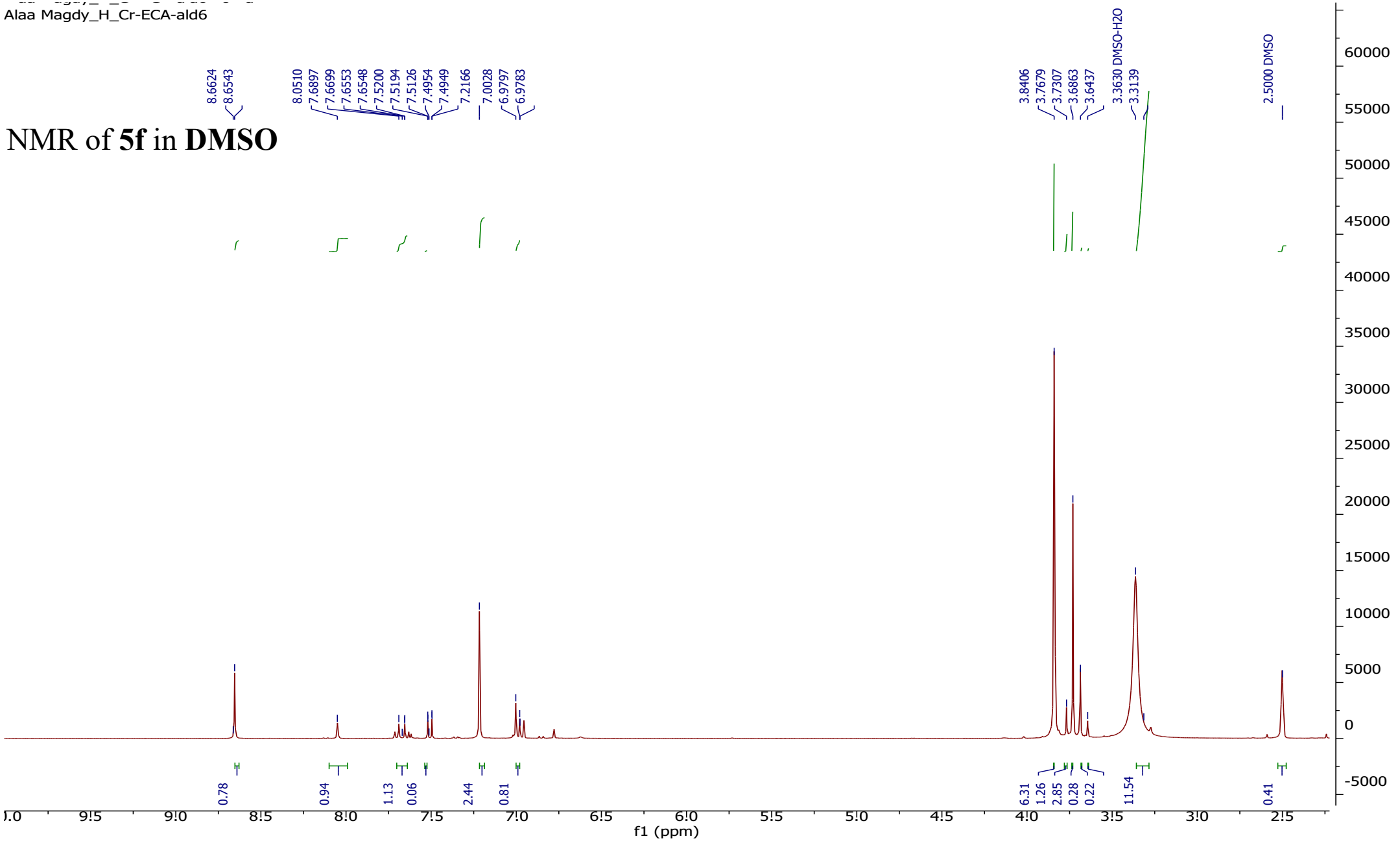


IR of compound **5f**



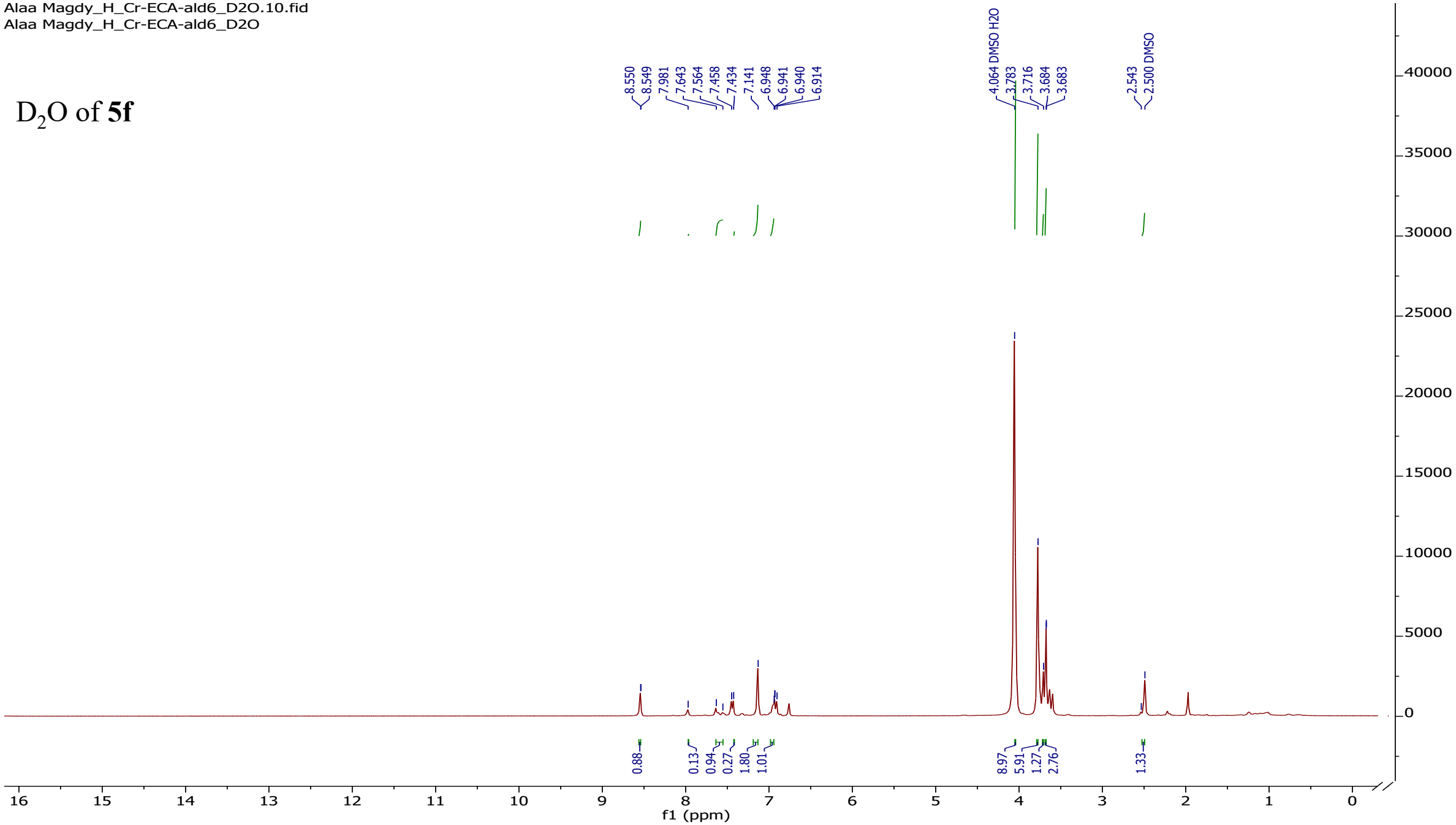
Alaa Magdy_H_Cr-ECA-ald6

^{1}H NMR of **5f** in **DMSO**



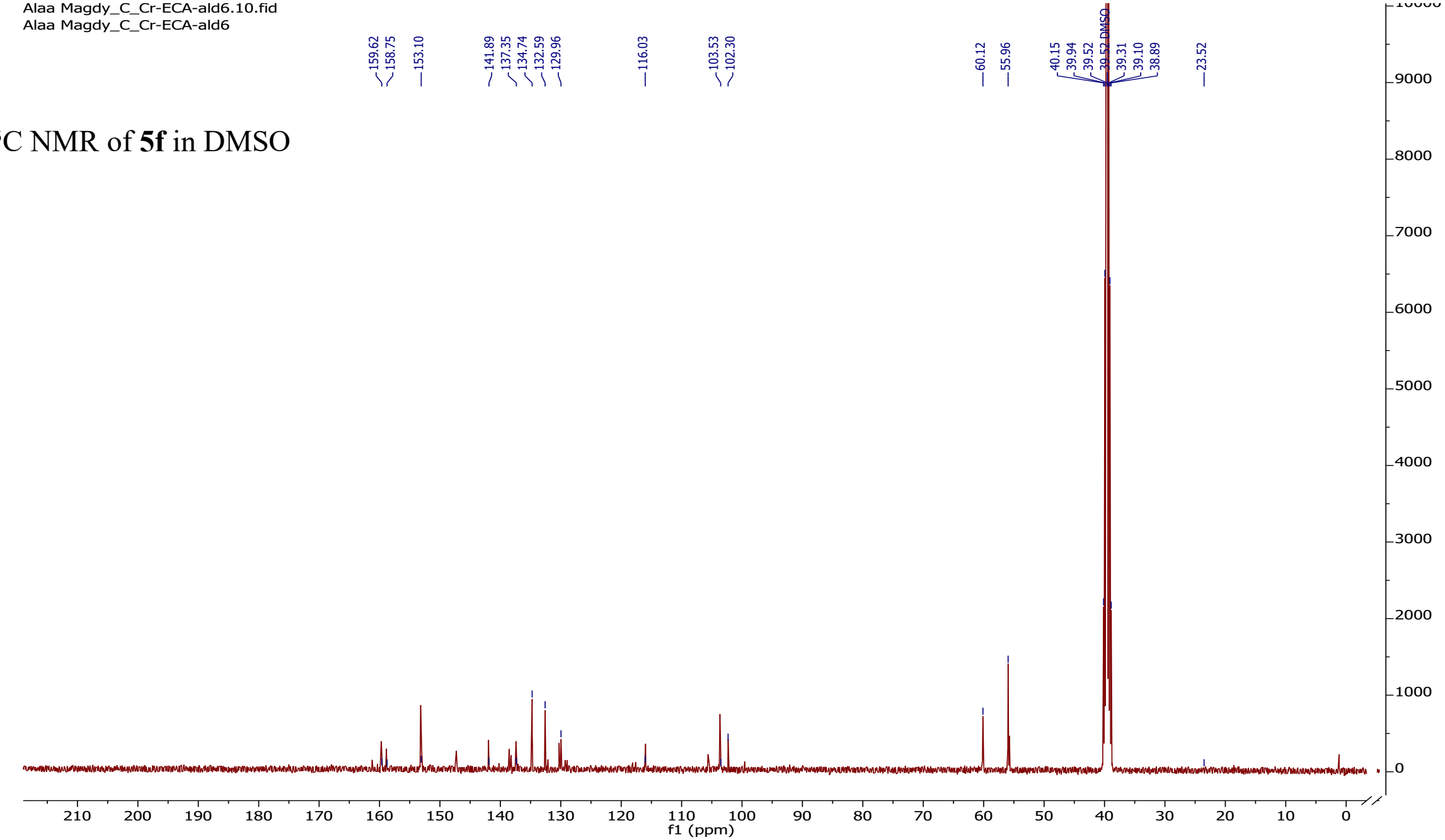
Alaa Magdy_H_Cr-ECA-ald6_D2O.10.fid
Alaa Magdy_H_Cr-ECA-ald6_D2O

D_2O of **5f**

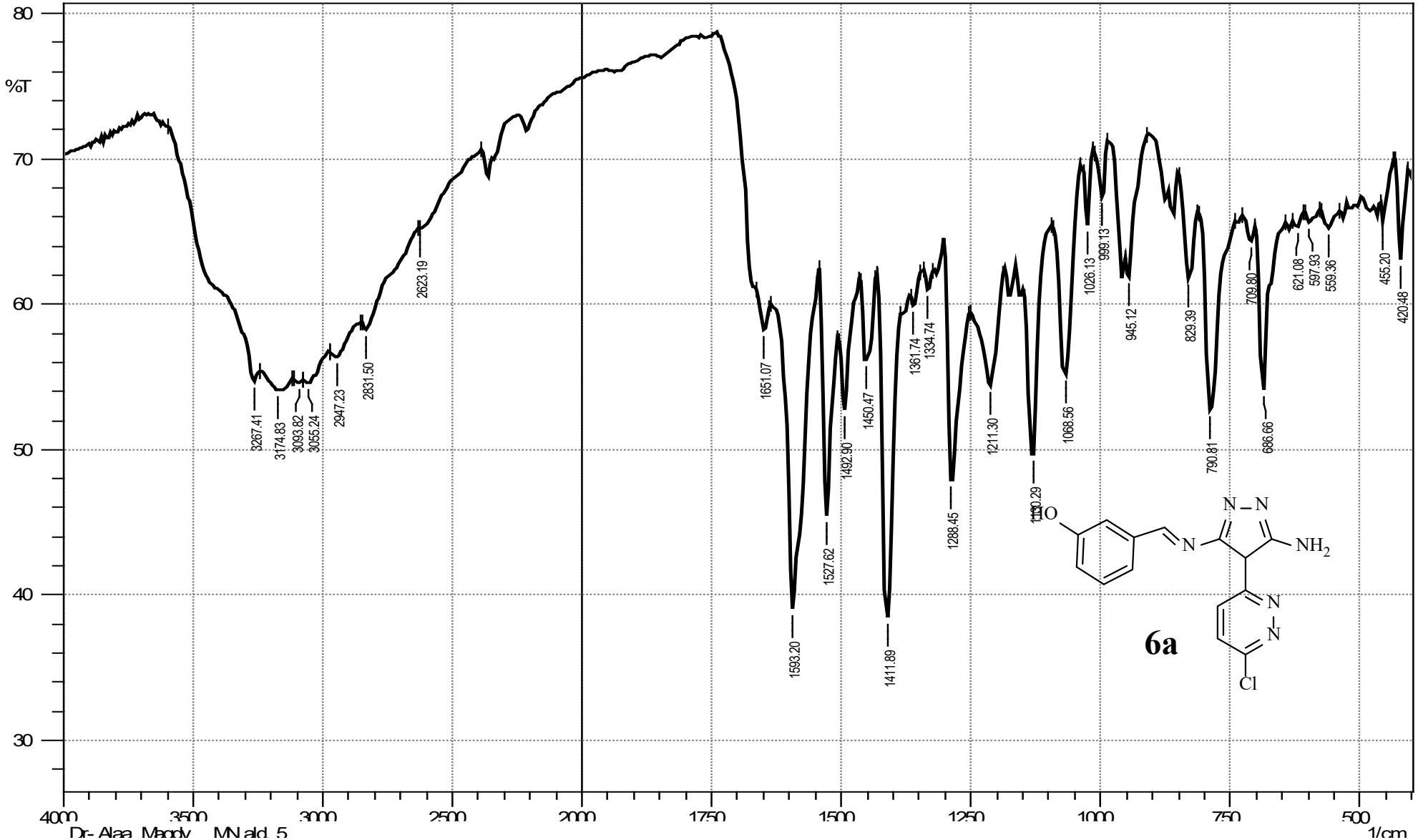


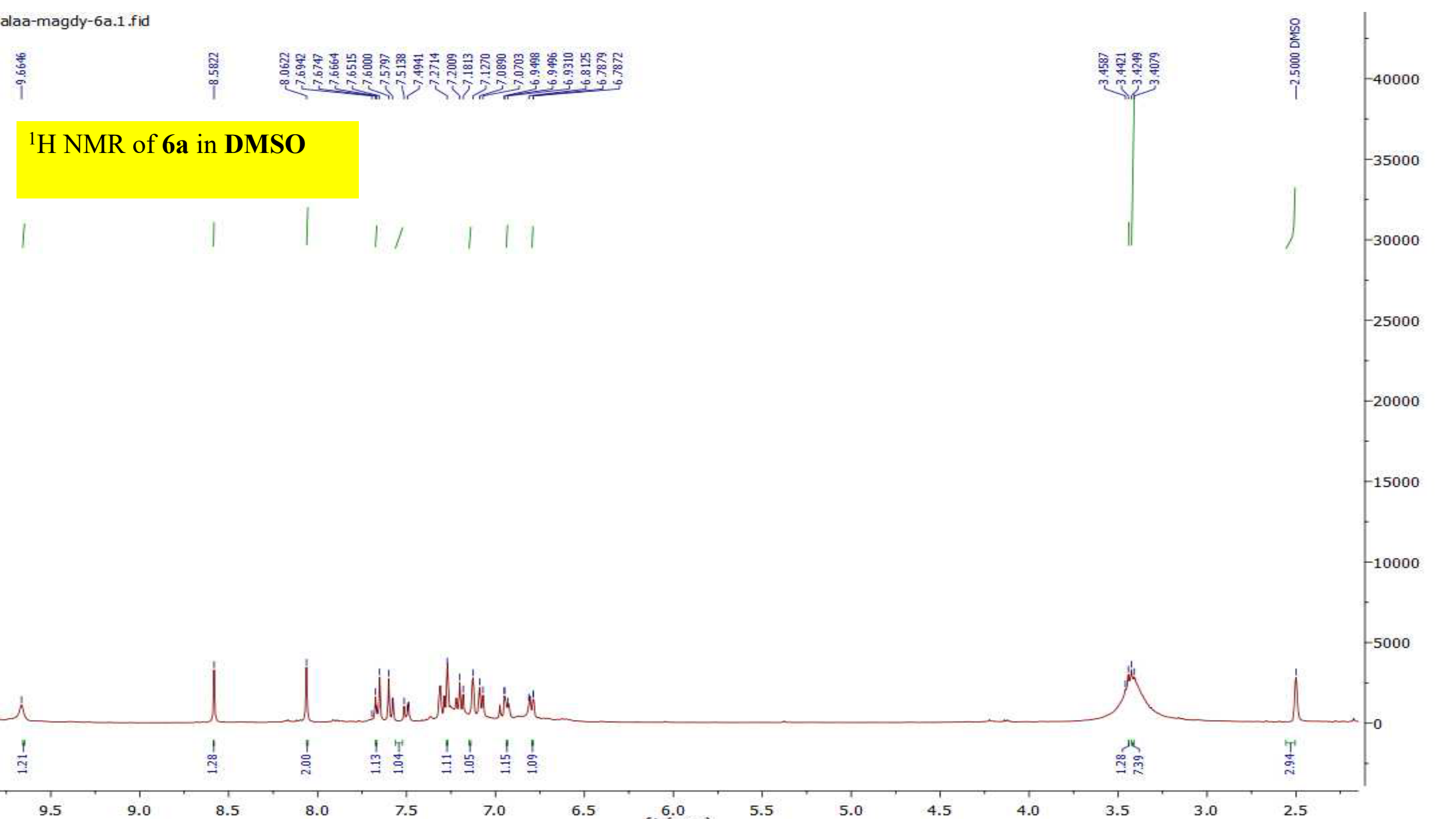
Alaa Magdy_C_Cr-ECA-ald6.10.fid
Alaa Magdy_C_Cr-ECA-ald6

^{13}C NMR of **5f** in DMSO



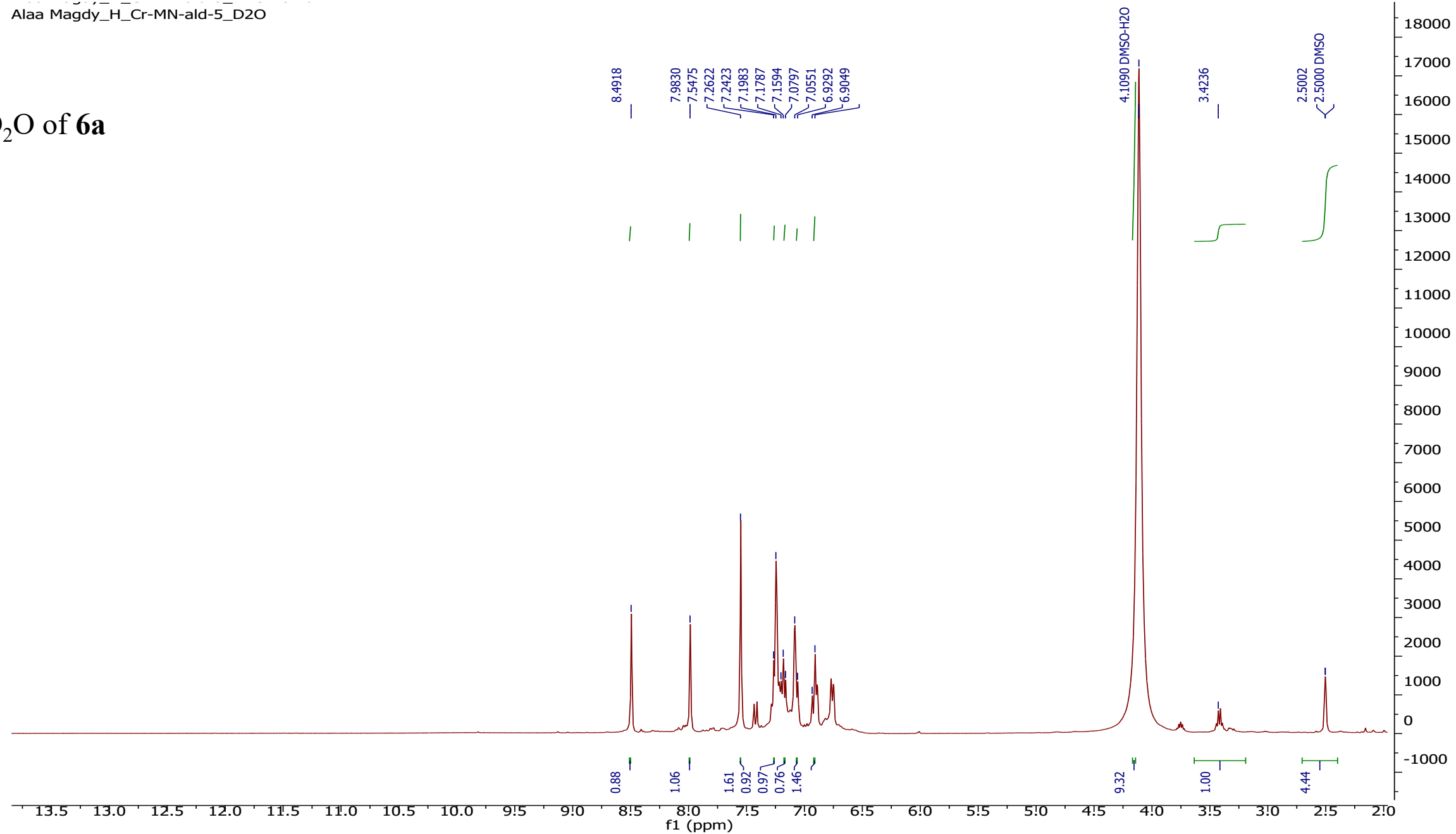
IR of compound **6a**




alaa-magdy-6a.1.fid
9.6646
8.5822
8.0622
7.6942
7.6747
7.6664
7.6515
7.6000
7.5797
7.5138
7.4941
7.2714
7.2009
7.1813
7.1270
7.0890
7.0703
6.9498
6.9496
6.9310
6.8125
6.7879
6.7872
3.4587
3.4421
3.4249
3.4079
2.5000 DMSO
1H NMR of 6a in DMSO
1.21
1.28
2.00
1.13
1.04
1.11
1.05
1.15
1.09
1.28
7.39
2.94

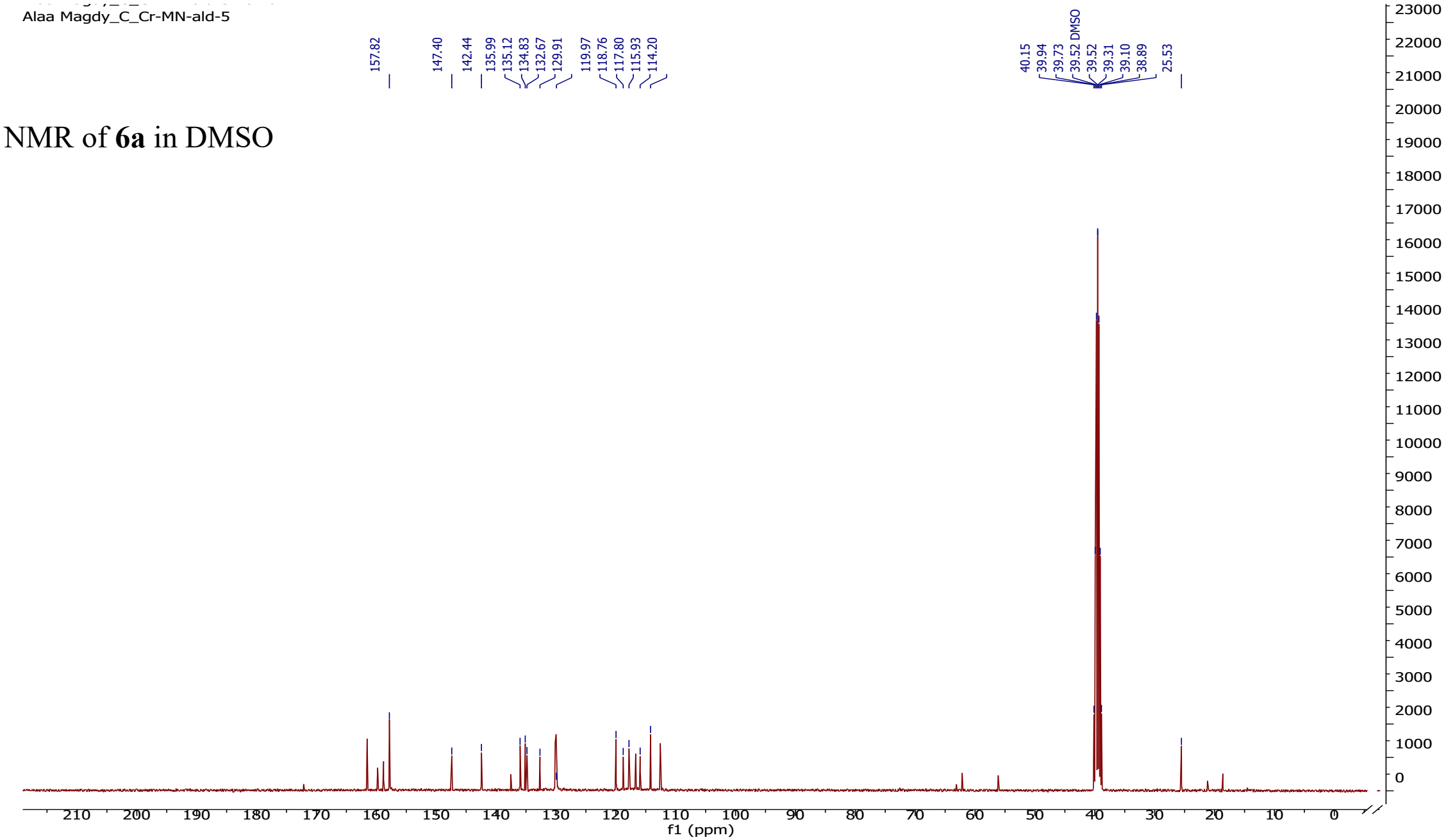
Alaa Magdy_H_Cr-MN-ald-5_D2O

D_2O of **6a**

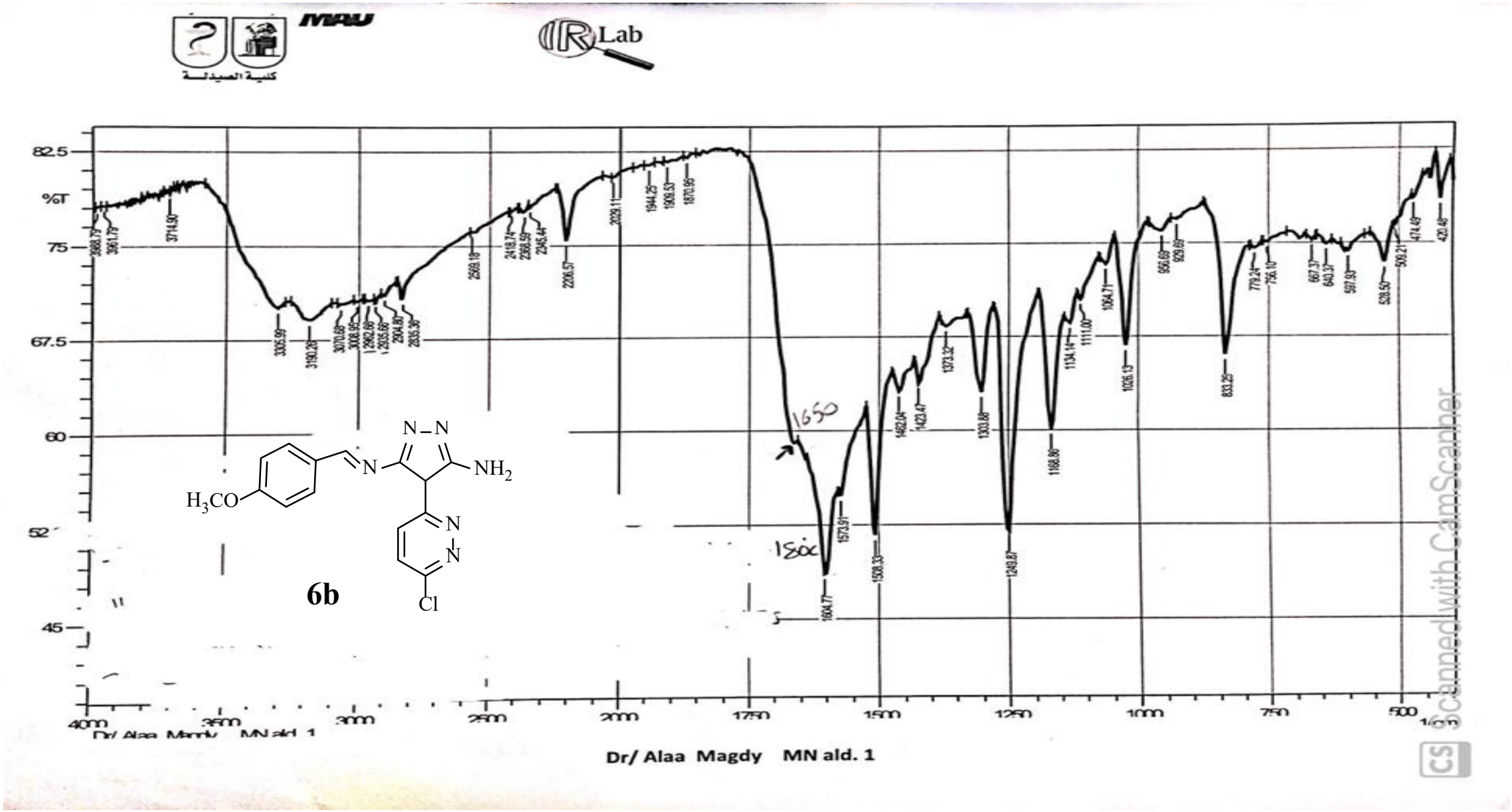


Alaa Magdy_C_Cr-MN-ald-5

^{13}C NMR of **6a** in DMSO

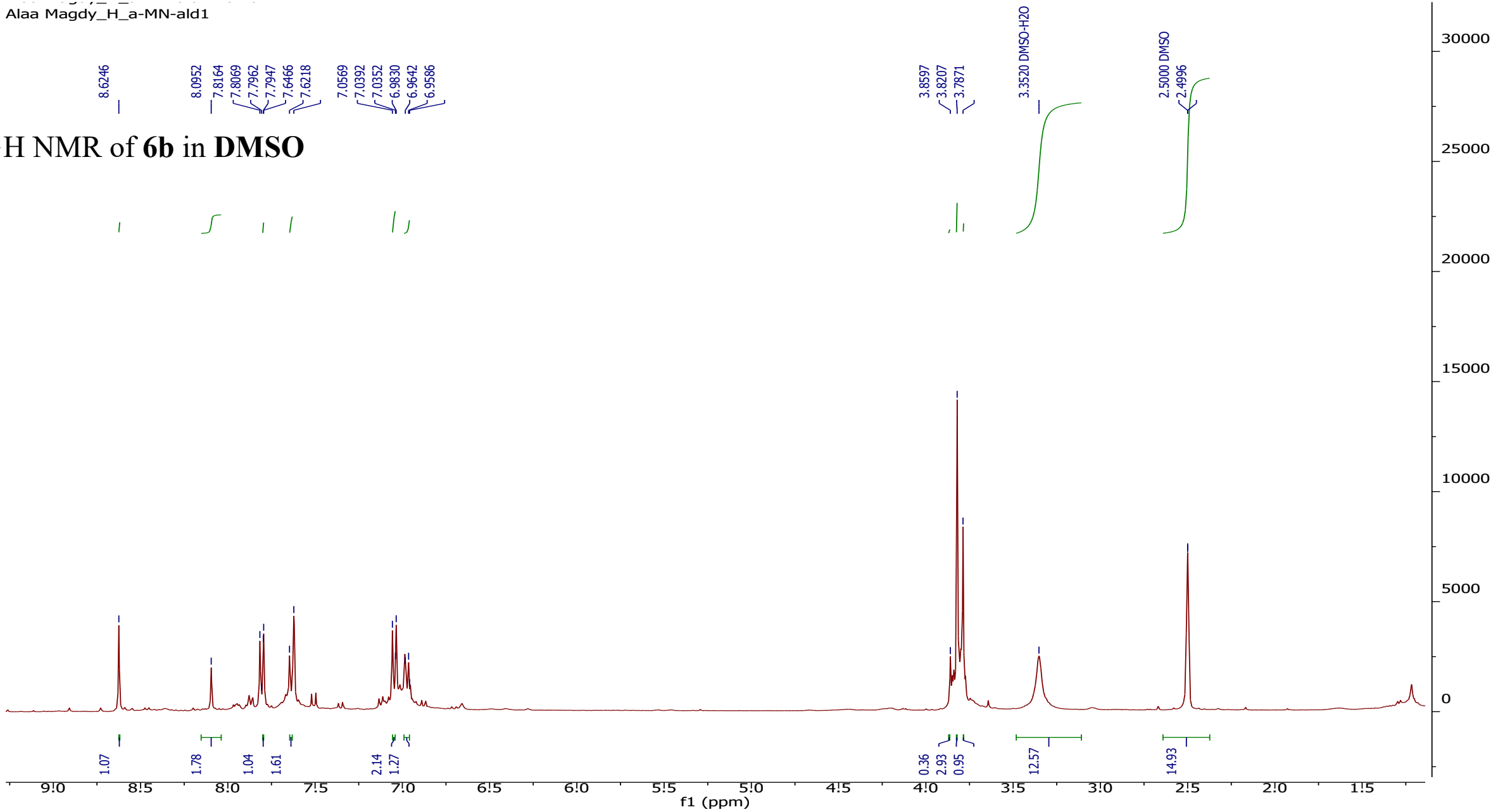


IR of compound **6b**

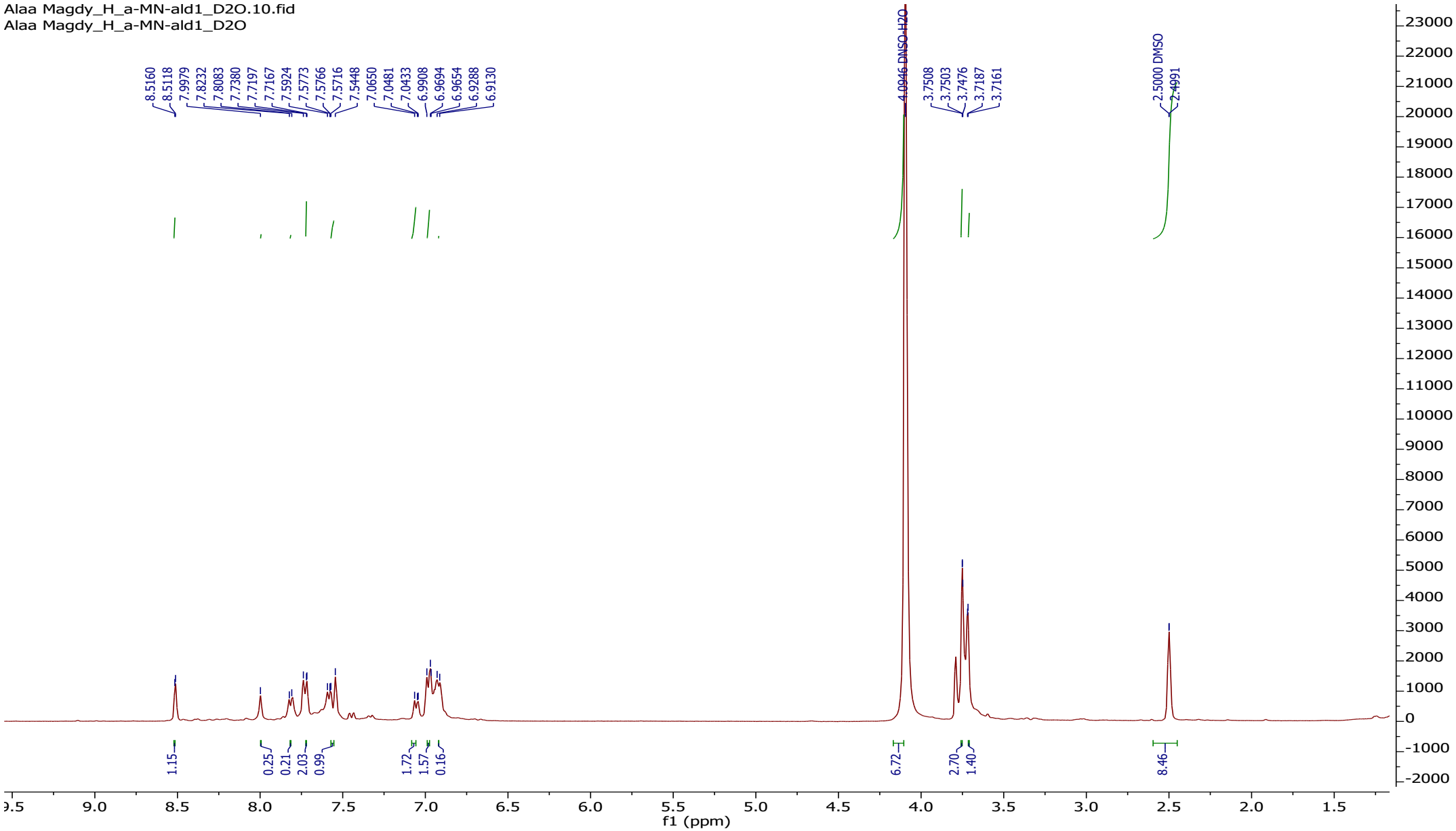


Alaa Magdy_H_a-MN-ald1

^{1}H NMR of **6b** in **DMSO**

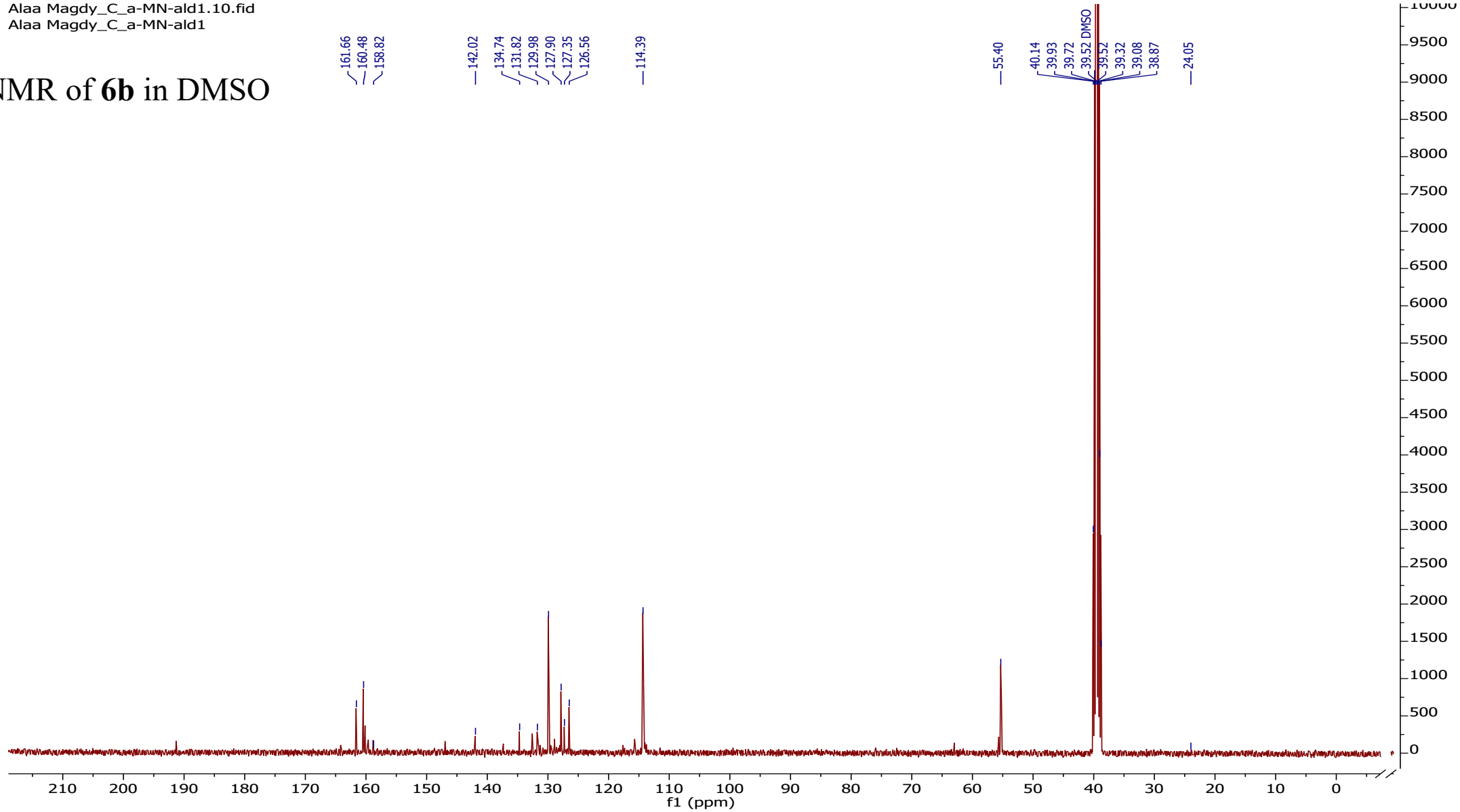


D_2O of **6b**

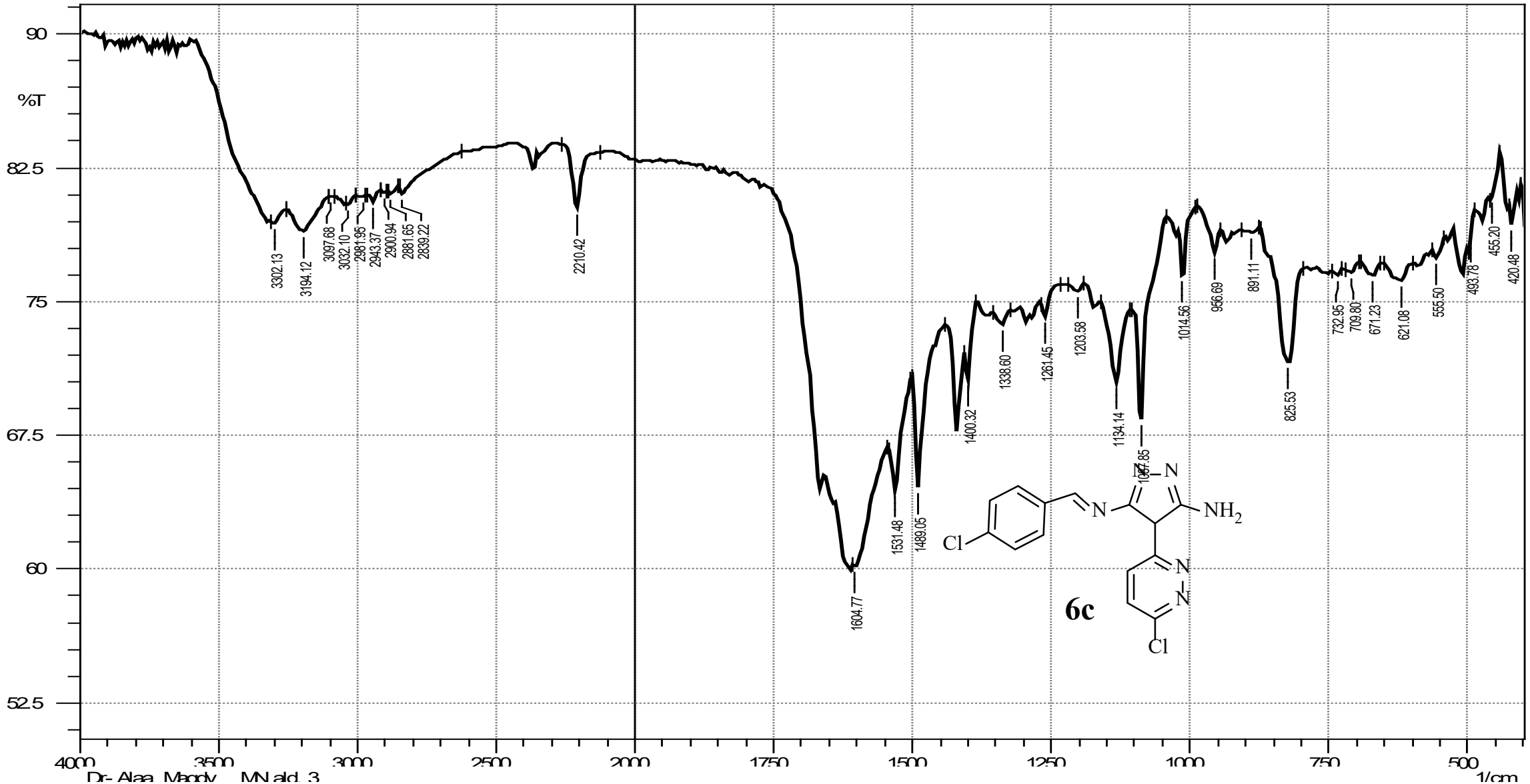

Alaa Magdy_H_a-MN-ald1_D2O.10.fid
Alaa Magdy_H_a-MN-ald1_D2O
8.5160
8.5118
7.9979
7.8232
7.8083
7.7380
7.7197
7.7167
7.5924
7.5773
7.5766
7.5716
7.5448
7.0650
7.0481
7.0433
6.9908
6.9694
6.9654
6.9288
6.9130
4.0946 DMSO-H2O
3.7508
3.7503
3.7476
3.7187
3.7161
2.5000 DMSO
2.4991
1.15
0.25
0.21
2.03
0.99
1.72
1.57
0.16
6.72
2.70
1.40
8.46
f1 (ppm)

Alaa Magdy_C_a-MN-ald1.10.fid
Alaa Magdy_C_a-MN-ald1

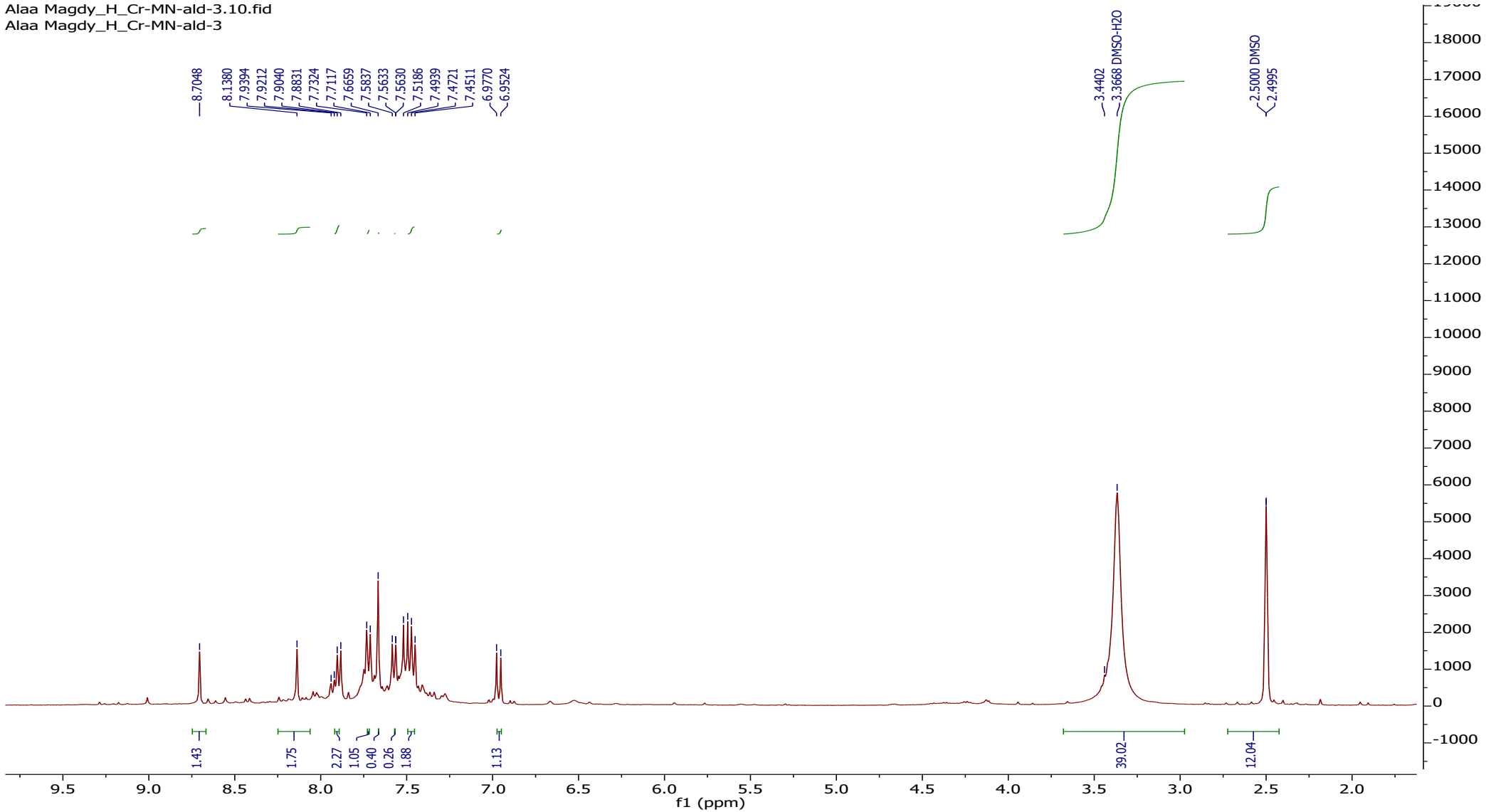
^{13}C NMR of **6b** in DMSO



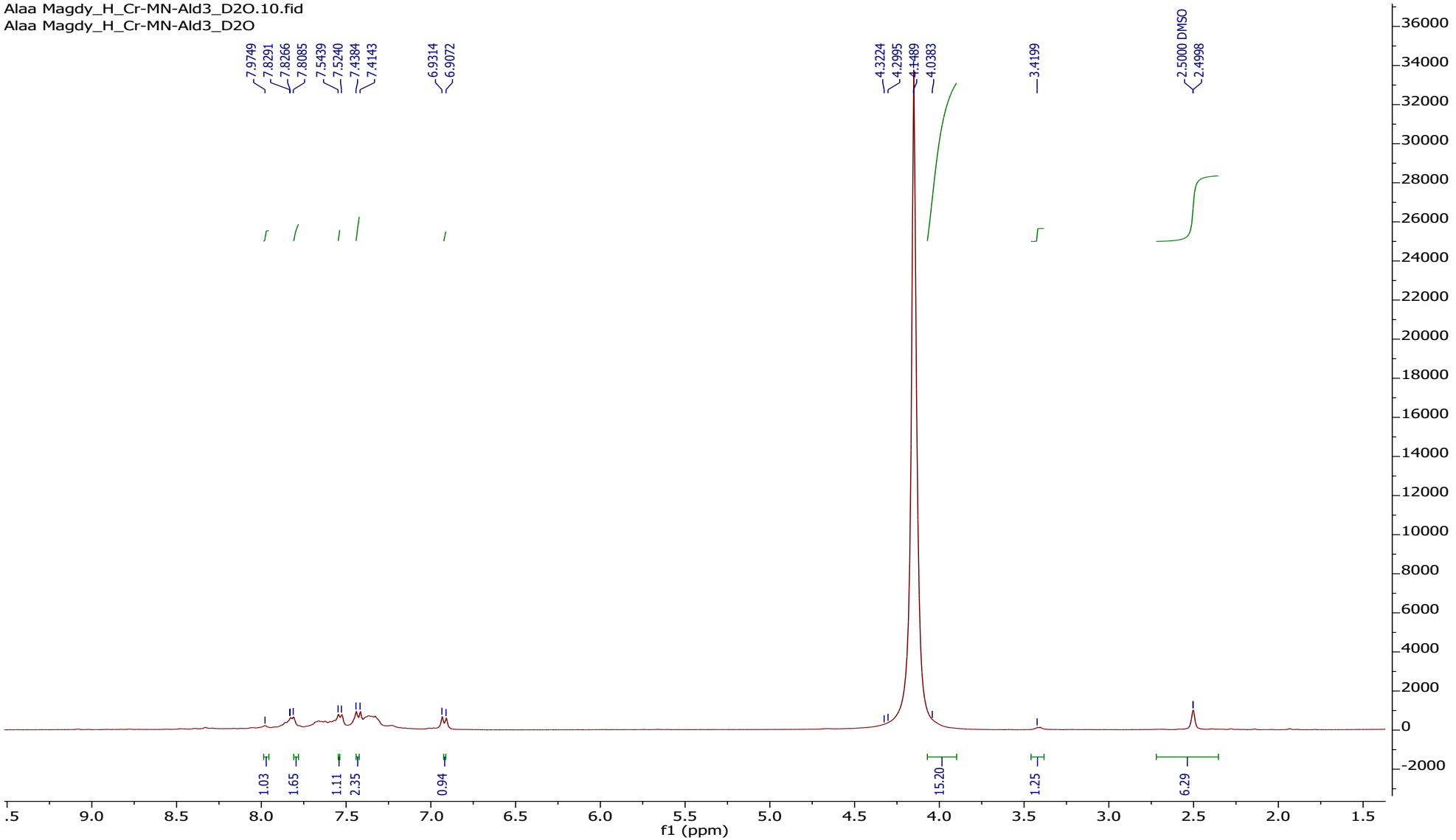
IR of compound **6c**



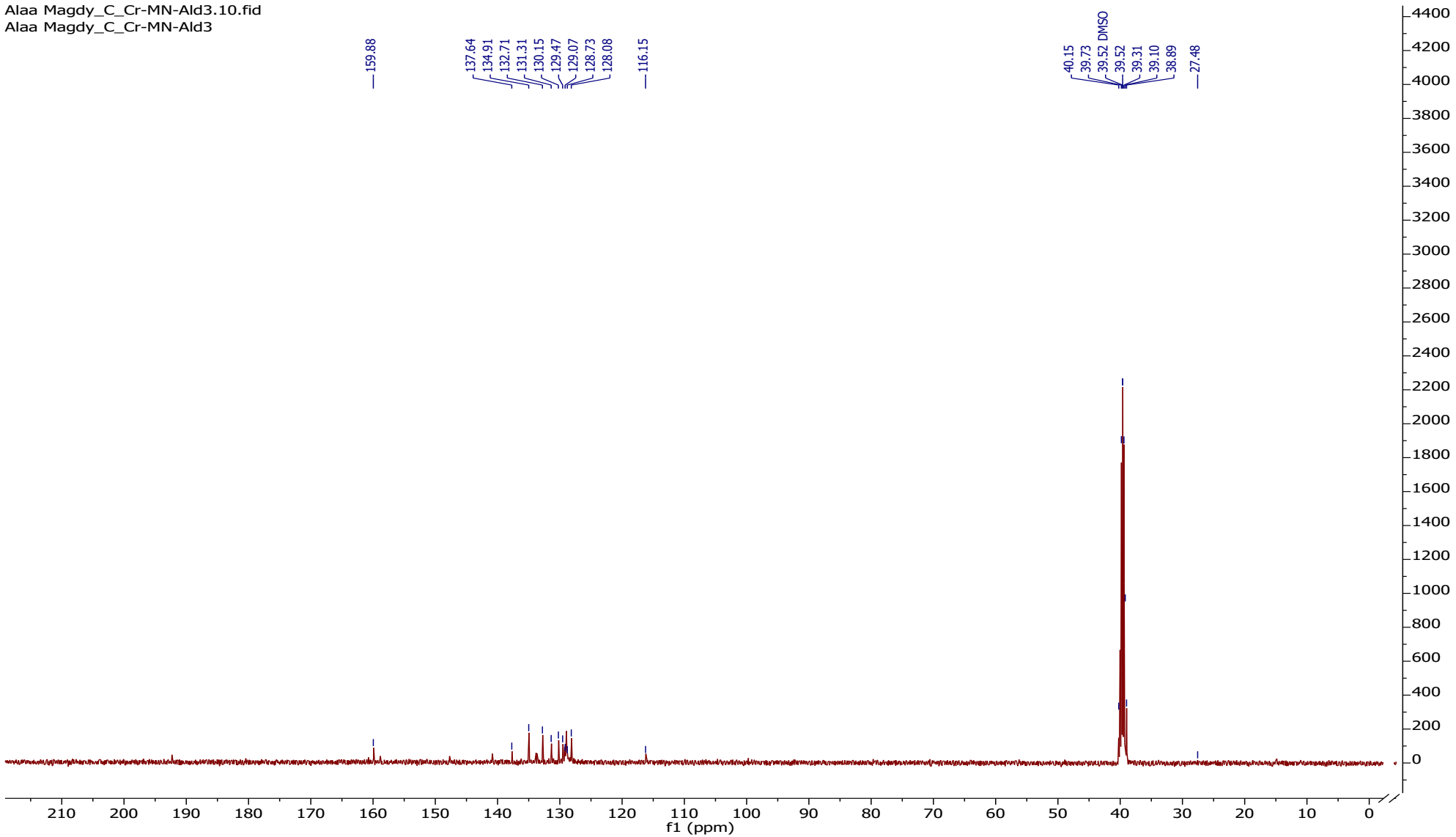
^{1}H NMR of **6c** in **DMSO**



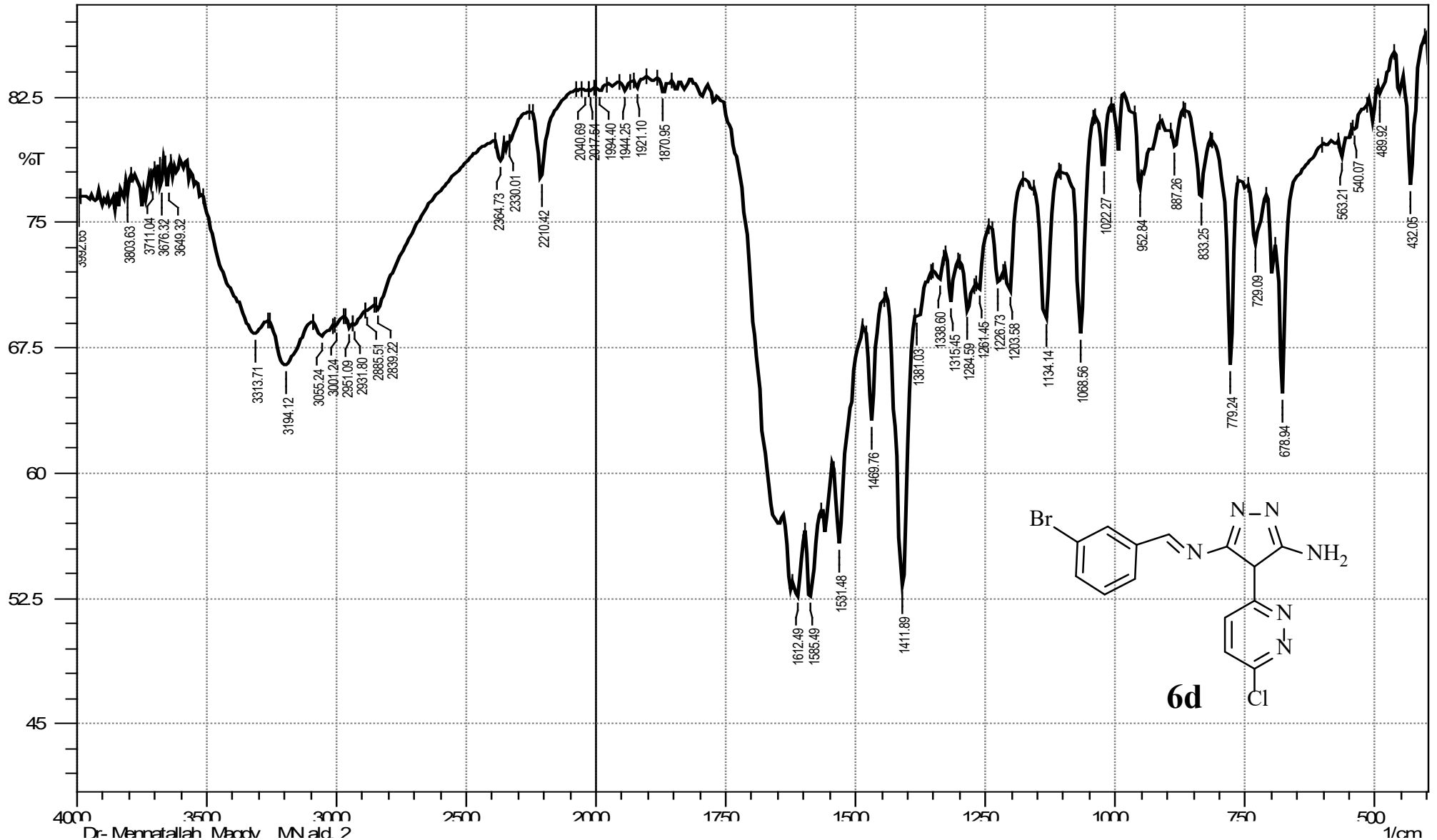
D_2O of **6c**

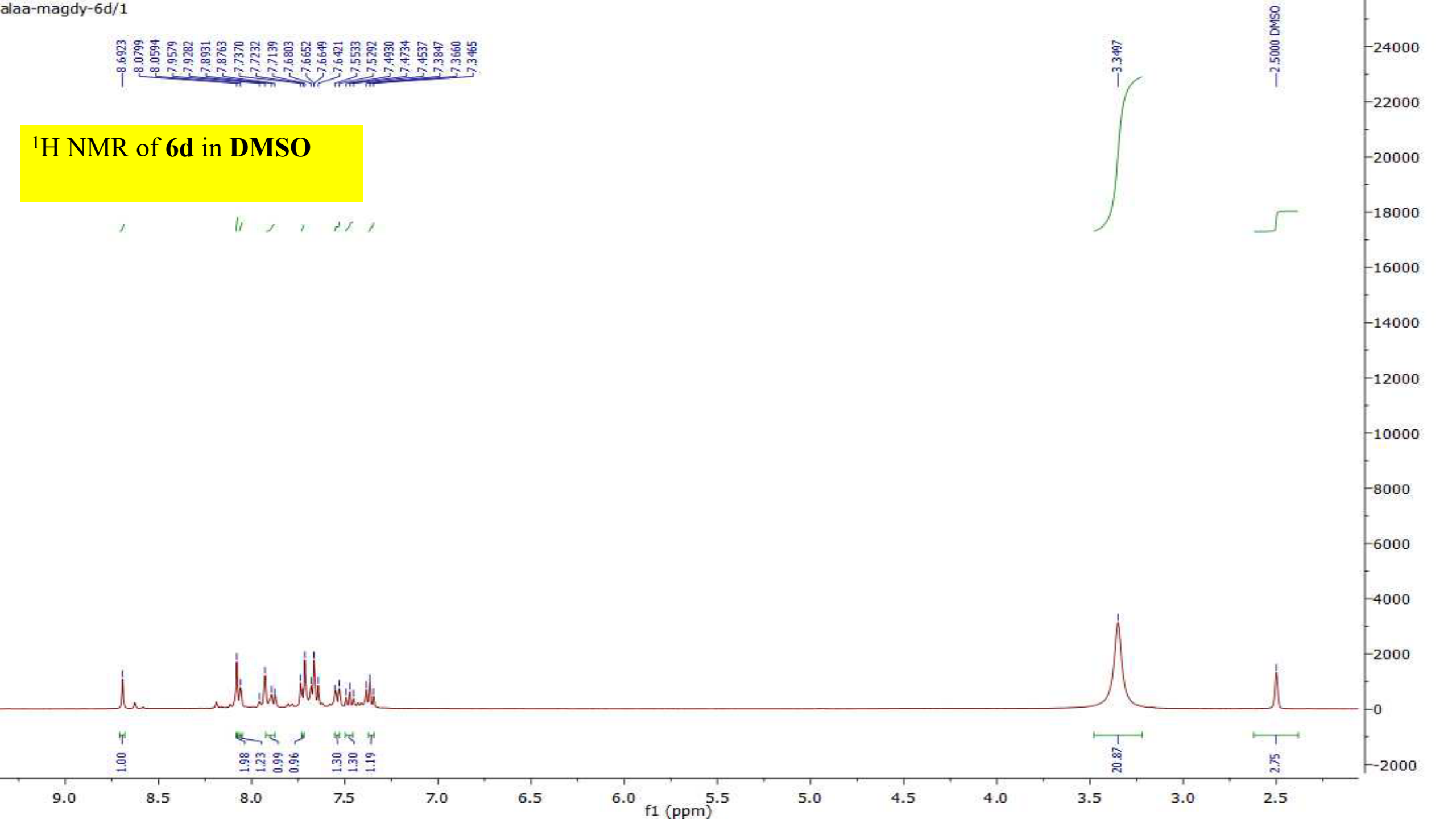

Alaa Magdy_H_Cr-MN-Ald3_D2O.10.fid
Alaa Magdy_H_Cr-MN-Ald3_D2O
7.9749
7.8291
7.8266
7.8085
7.5439
7.5240
7.4384
7.4143
6.9314
6.9072
4.3224
4.2995
4.1489
4.0383
3.4199
2.5000 DMSO
2.4998
1.03
1.65
1.11
2.35
0.94
15.20
1.25
6.29
f1 (ppm)

^{13}C NMR of **6c** in DMSO



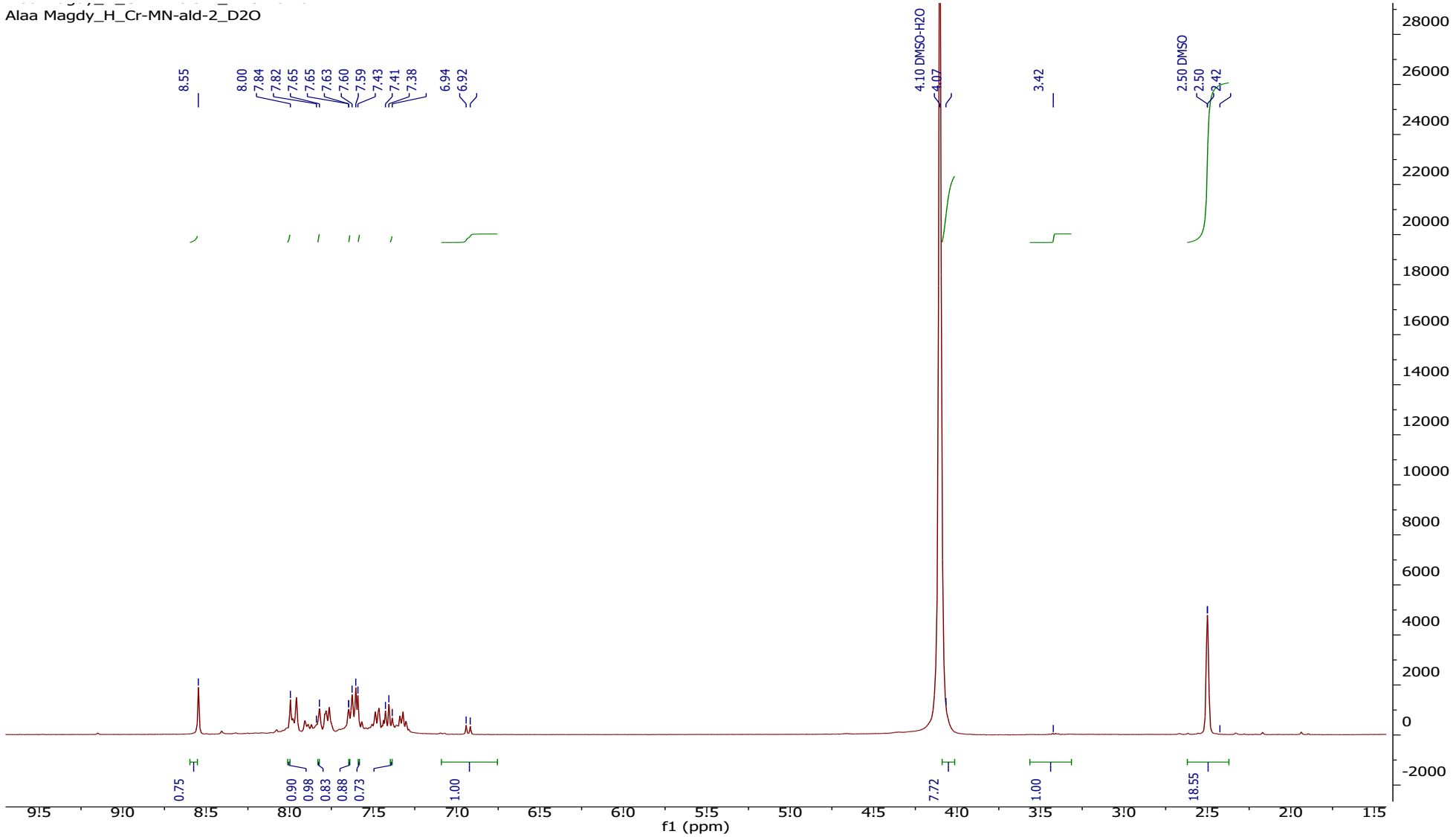
IR of compound **6d**



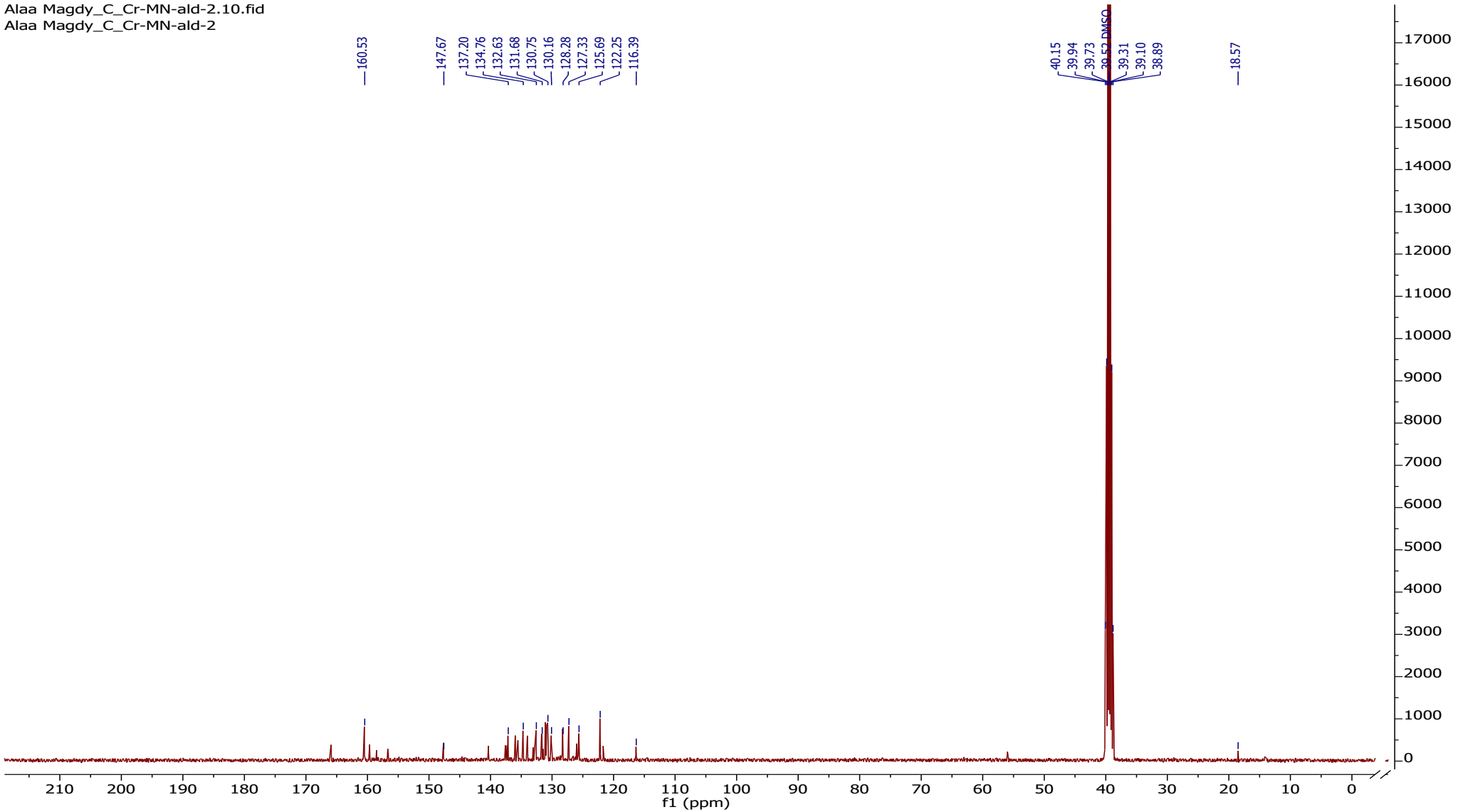


[1]H NMR of **6d** in **DMSO**

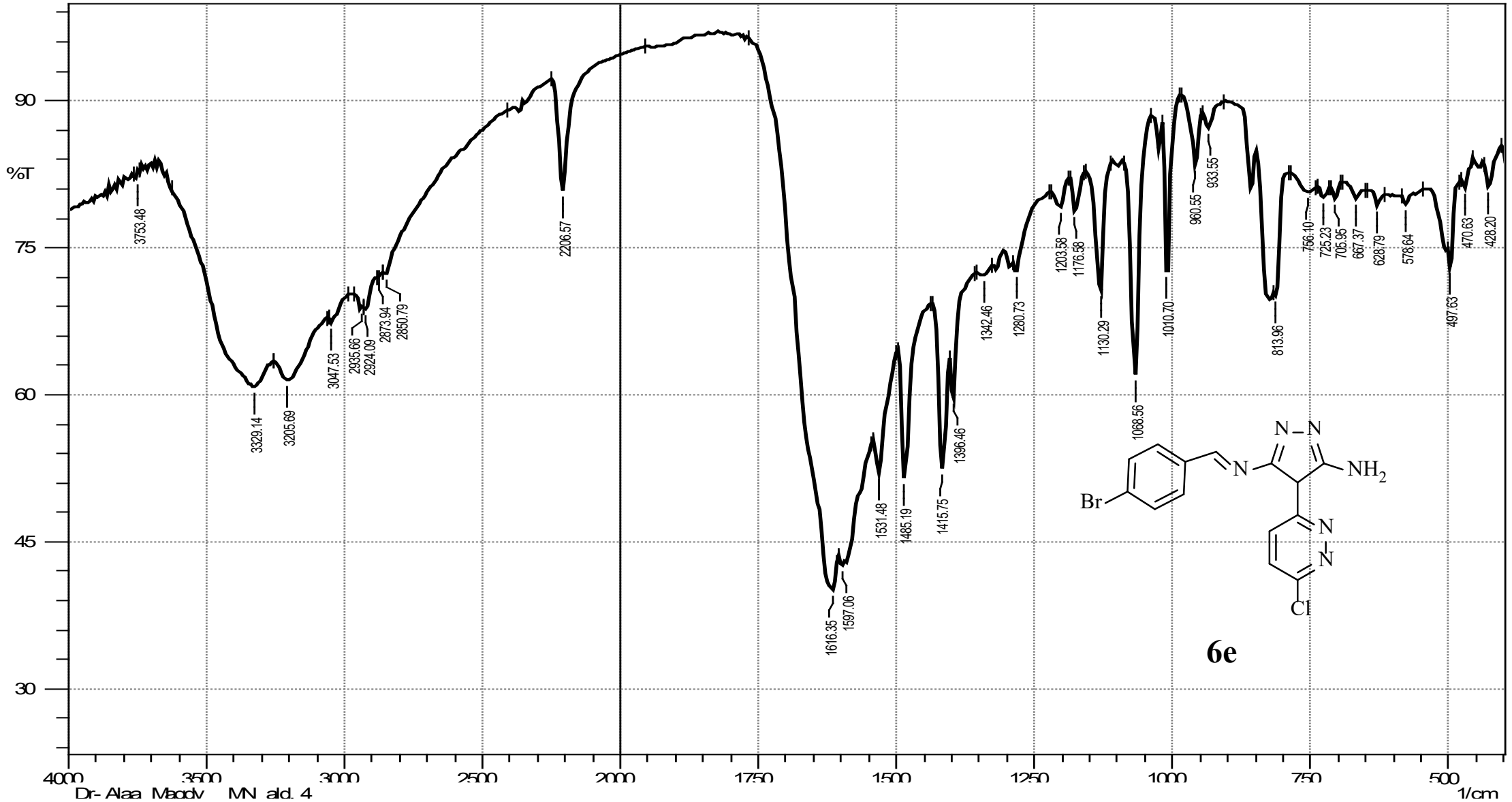
D_2O of **6d**


Alaa Magdy_H_Cr-MN-ald-2_D2O
8.55
8.00
7.84
7.82
7.65
7.65
7.63
7.60
7.59
7.43
7.41
7.38
6.94
6.92
4.10 DMSO-H2O
4.07
3.42
2.50 DMSO
2.50
2.42
0.75
0.90
0.98
0.83
0.88
0.73
1.00
7.72
1.00
18.55
f1 (ppm)

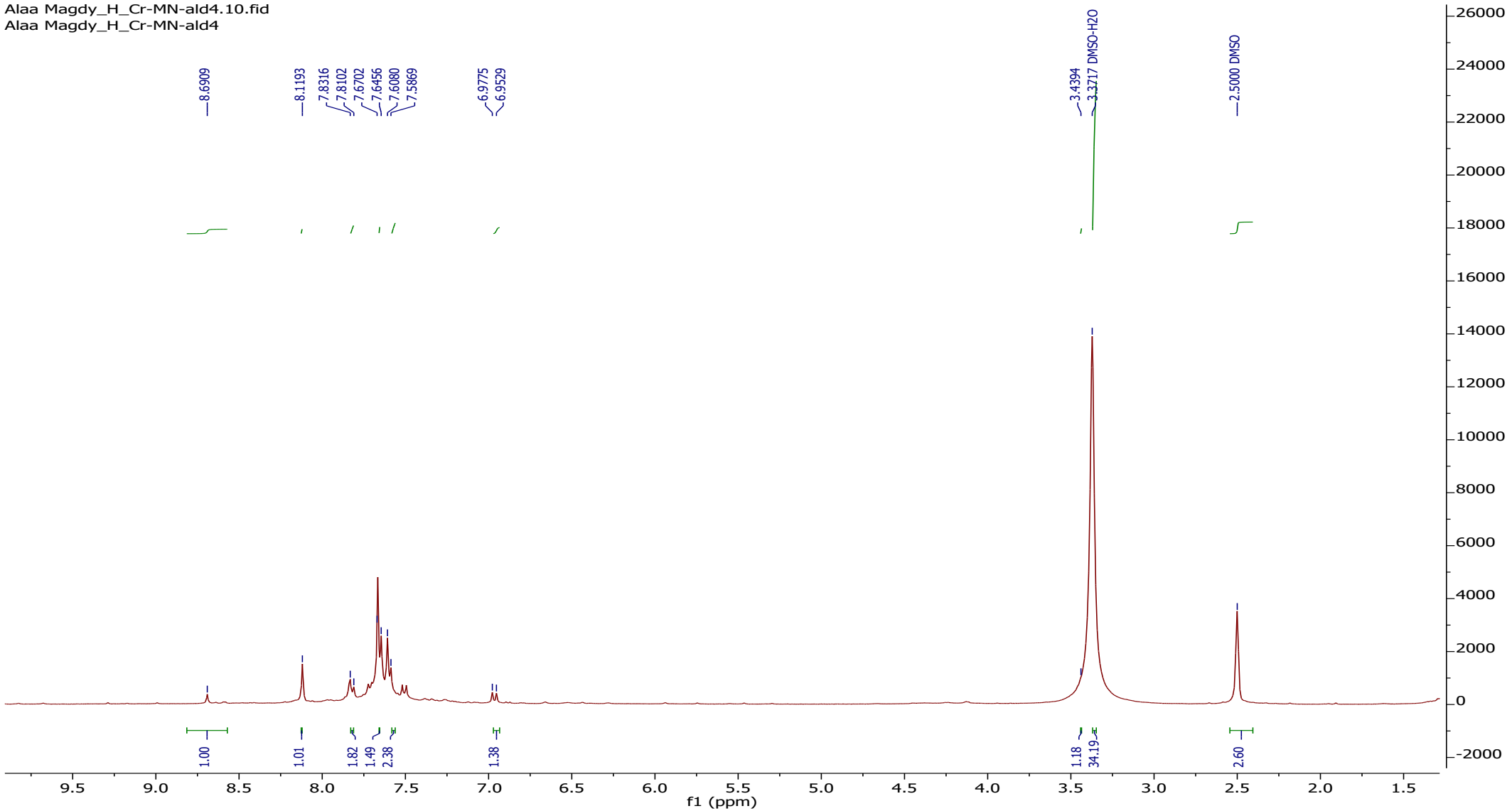
^{13}C NMR of **6d** in DMSO



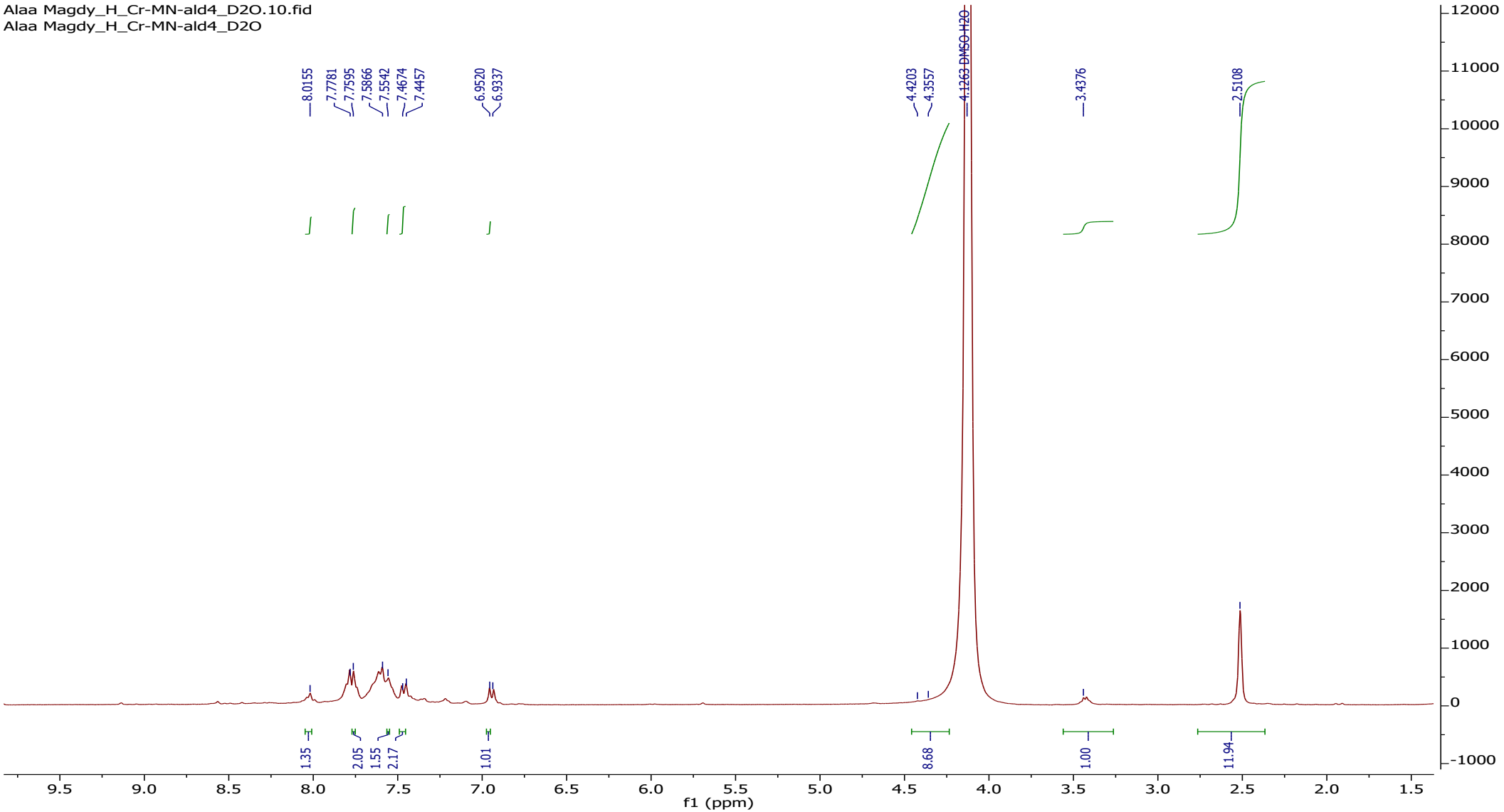
IR of compound **6e**



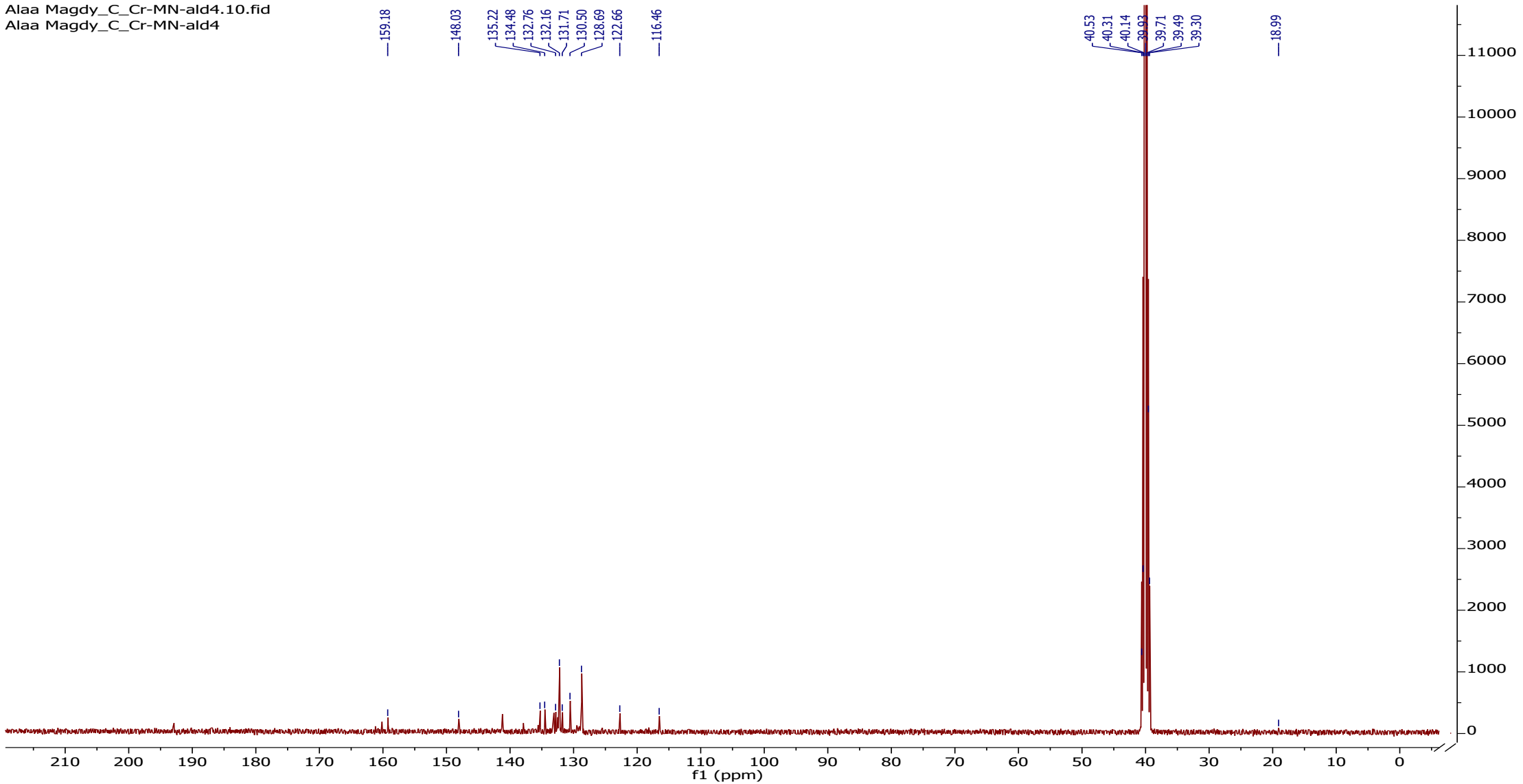
^{1}H NMR of **6e** in **DMSO**



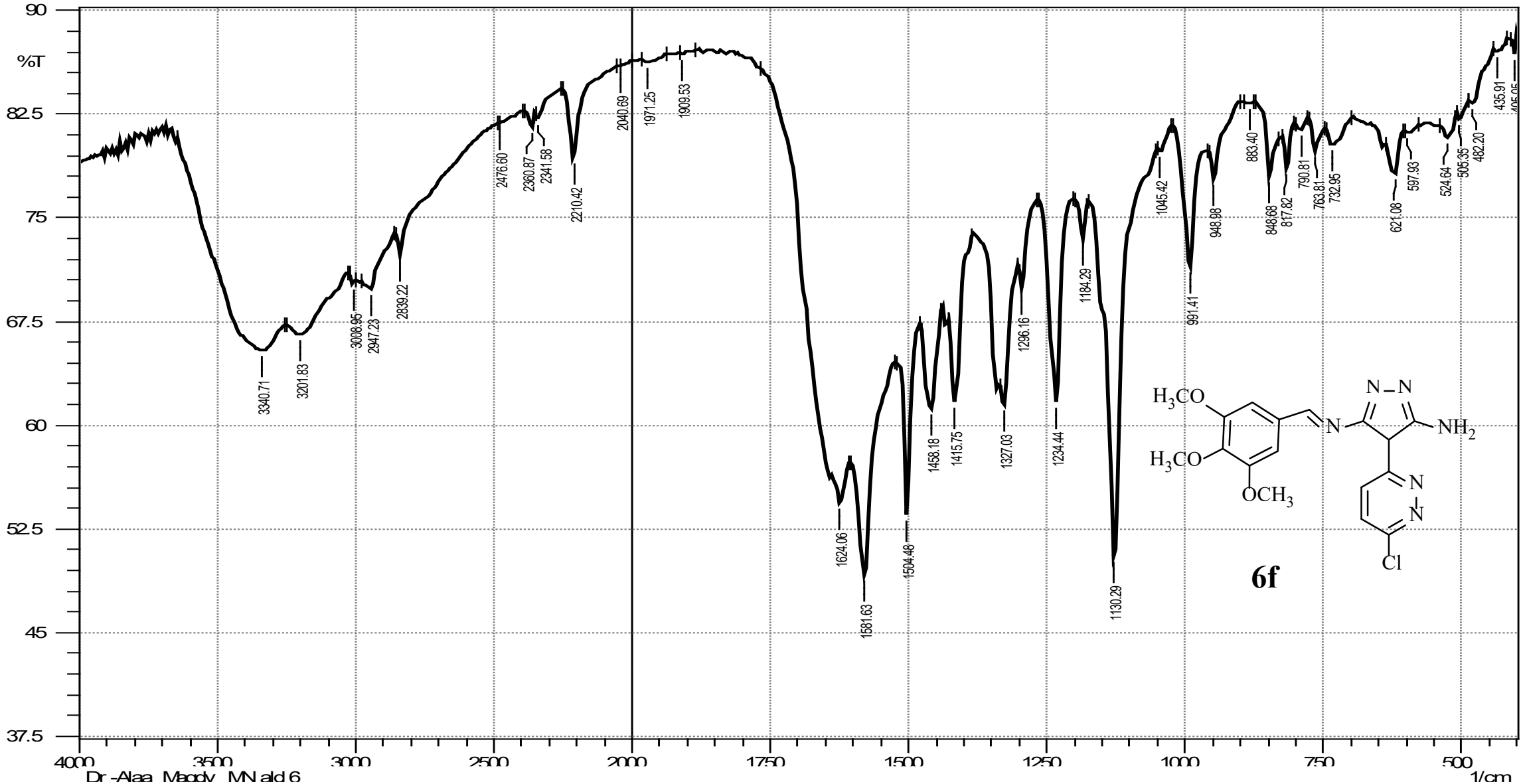
D_2O of **6e**


Alaa Magdy_H_Cr-MN-ald4_D2O.10.fid
Alaa Magdy_H_Cr-MN-ald4_D2O
8.0155
7.7781
7.7595
7.5866
7.5542
7.4674
7.4457
6.9520
6.9337
4.4203
4.3557
4.1263 DMSO H2O
3.4376
2.5108
1.35
2.05
1.55
2.17
1.01
8.68
1.00
11.94
f1 (ppm)

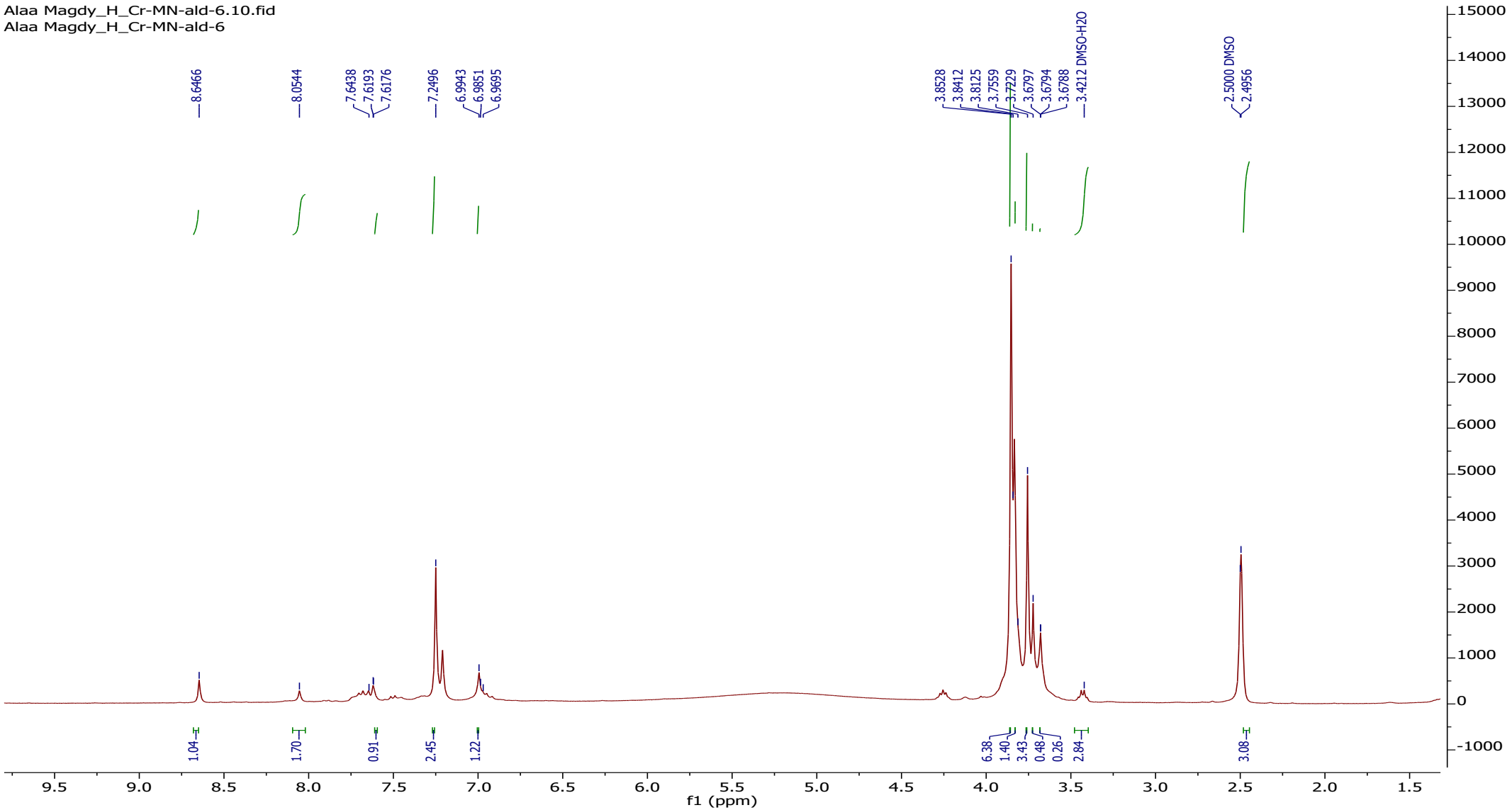
^{13}C NMR of **6e** in DMSO



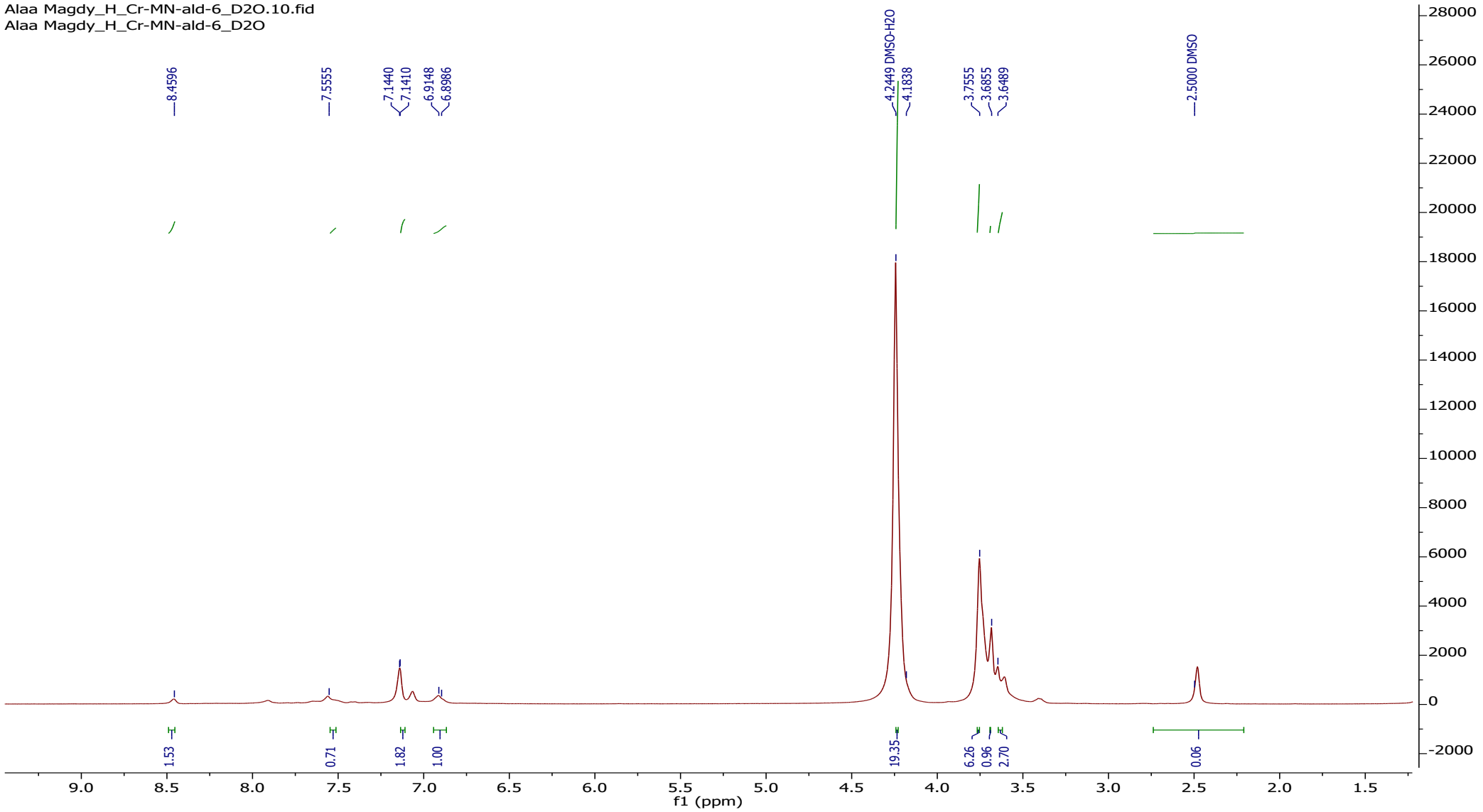
IR of compound **6f**



^{1}H NMR of **6f** in **DMSO**



D_2O of **6f**


Alaa Magdy_H_Cr-MN-ald-6_D2O.10.fid
Alaa Magdy_H_Cr-MN-ald-6_D2O
8.4596
7.5555
7.1440
7.1410
6.9148
6.8986
4.2449 DMSO-H2O
4.1838
3.7555
3.6855
3.6489
2.5000 DMSO
1.53
0.71
1.82
1.00
19.35
6.26
0.96
2.70
0.06
f1 (ppm)

^{13}C NMR of **6f** in DMSO