Supplementary Materials:

Novel Sunifiram-Carbamate Hybrids as Potential Dual Acetylcholinesterase Inhibitor and NMDAR Co-Agonist: Simulation-Guided Analogue Design and Pharmacological Screening

Khalid A. Agha,[a,b,*] Nader E. Abo-Dya,[b,c] Abdul Rashid Issahaku,[d] Clement Agoni,[d] Mahmoud E. S. Soliman,[d] Eatedal H. Abdel-Aal,[b] Zakaria K. Abdel-Samii,[b] and Tarek S. Ibrahim,[b,e]

[a] *Department of Organic and Medicinal Chemistry, Faculty of Pharmacy, Fayoum University, Fayoum, 63514, Egypt.*

[b] *Department of Pharmaceutical Organic Chemistry, Faculty of Pharmacy, Zagazig University, Zagazig, 44519, Egypt.*

[C] *Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Tabuk, Tabuk, 71491, Saudi Arabia.*

[d] *Molecular Bio-computation and Drug Design Laboratory, School of Health Sciences, University of KwaZulu-Natal, Westville Campus, Durban 4001, South Africa.*

[e] *Department of Pharmaceutical Chemistry, Faculty of Pharmacy, King Abdulaziz University, Jeddah, 21589, Saudi Arabia.*

*Corresponding author at: Department of Organic and Medicinal Chemistry, Faculty of Pharmacy, Fayoum University, Fayoum, 63514, Egypt.

e-mail:Aghanet2010@yahoo.com

Table of Contents

1. Computational methodology, Systems retrieval, preparation and molecular dynamics of the targets ability to bind to the glycine binding pocket of NMDA receptors .

The X-ray crystal structure of the ligand binding domain (LBD) of NMDA receptor (PDB ID: K4FQ) was retrieved from RSCB protein data bank and prepared for molecular docking [1–3] by removing all nonstandard residues including water using UCSF Chimera. [4] The missing residues were modelled using MODELLER. [5] Molegro Molecular Viewer [6] was then employed to resolve torsion discrepancies. The two dimensional structures of the synthesized analogues 3a-j were sketched using Marving Sketch software [7]. Universal Force Field incorporated into Avogadro 1.2.0 software [8] was then employed to optimise the energy on the 2D structures and to build their 3D structures. The molecular geometries of the compounds were optimised using the steepest descent algorithm and saved for molecular docking. The compounds were then docked into the glycine binding pocket of the binding domain of the NMDA receptor using Autodock vina [9]. The pharmacokinetics and physicochemical properties of the compounds were then evaluated using SwissADME.[10] The compound with the highest docking score, low toxicity, favourable ADMET properties and Sunifiram (3a) were then selected for molecular dynamics simulation. The simulation process was performed by employing the Graphical Processing Unit version of the Particle Mesh Ewald Molecular Dynamics (PMEMD) engine in AMBER18 package [11]. AMBER FF14SB protein forcefield and the integrated pdb4amber program were used to parameterise the protein and modify the protein respectively [12], whiles the selected compounds were parameterised using the ANTECHAMBER module [13] of the AMBER 18 package which as well created the atomic partial charges for the compounds. The tLeap module of the AMBER18 package was then used to combine the protein and ligands into their docked complexes, neutralise the complexes by adding Na^+ and Cl^- counter ions and solvate the neutralised complexes with Transferable Intermolecular Potential with 3 Points (TIP3P) water box size of 8Å. An initial partial minimisation for 2500 steps employing 500 kcal/mol Å restraint potential was performed for the selected systems. This was followed by a full minimisation without energy restraint for 5000 steps. Heating of the systems was then performed gradually for 50 ps from 0 k to 300 k in NTP ensemble using Langevin thermostat [14] and a harmonic restraint of 5 kcal/mol A2. Berendsen barostat was used to ensure constant atmospheric pressure at 1 bar whiles the systems were then equilibrated at 300k for 1000 ps without energy restraints. MD productions was then performed for 250ns with the SHAKE algorithm used to restrain all hydrogen bonds [15]. CPPTRAJ and

PTRAJ modules [16] integrated into the AMBER18 package were then used to analyse the trajectories and coordinates generated from the production. The data were then plotted and analysed using Origin data tool [17]. Structural visualisation was performed using Discovery Studio. [18]

Thermodynamics calculations.

The differential binding affinity and stability of the selected compounds as well as the free binding energy of the complexes were investigated. The Molecular Mechanics/Poisson-Boltzmann Area method [19] was applied for the investigation due to its efficiency and widely reported reliability [20]. The binding free energy of this approach is depicted as follows:

$$\Delta G_{bind} = G_{complex} - G_{receptor} - G_{ligand} \quad (1)$$

$$\Delta G_{bind} = E_{gas} + G_{sol} - T\Delta S \quad (2)$$

Where ΔG_{bind} is taken to be the sum of the gas phase and solvation energy terms less the entropy ($T\Delta S$) term

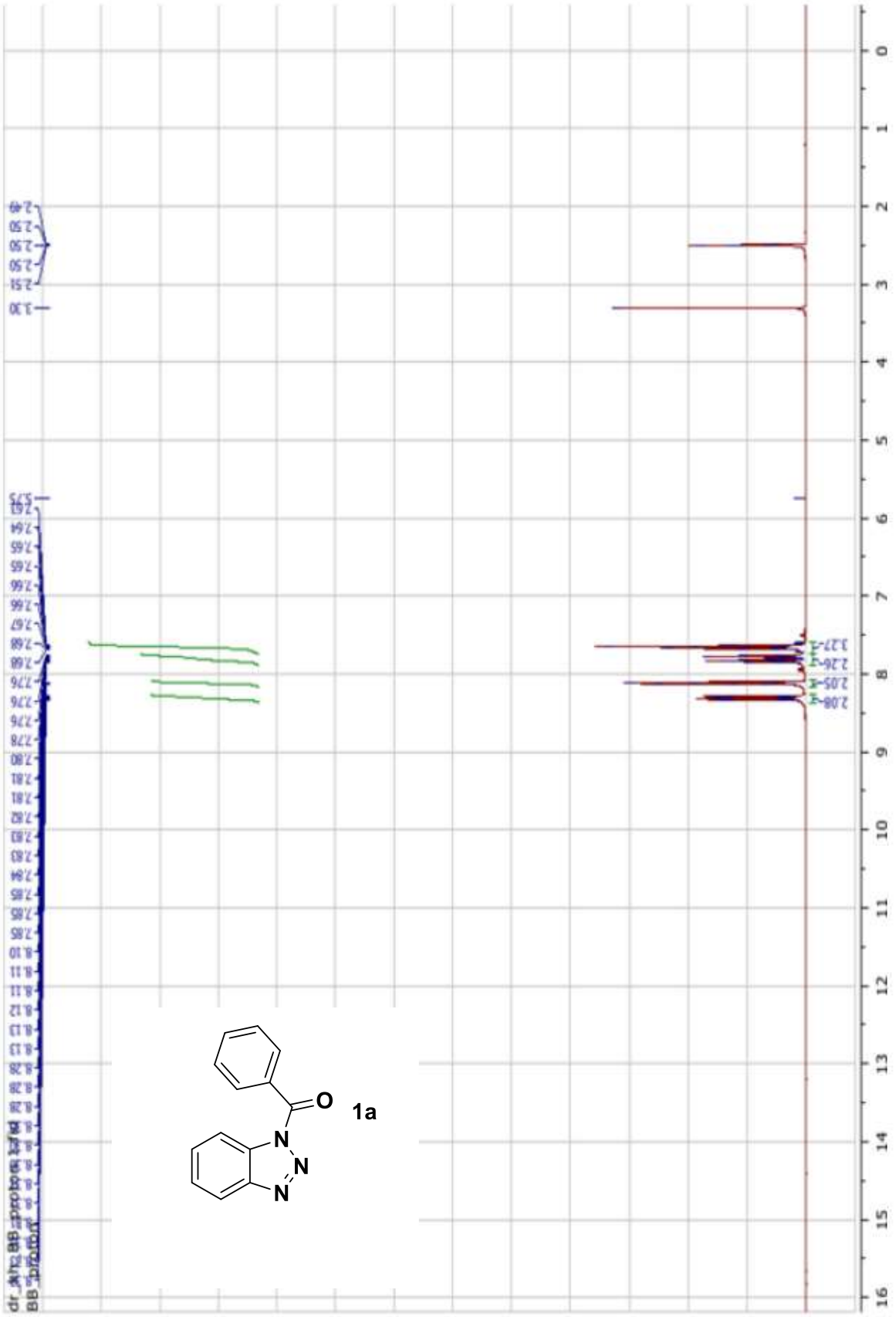
$$E_{gas} = E_{int} + E_{vdw} + E_{ele} \quad (3)$$

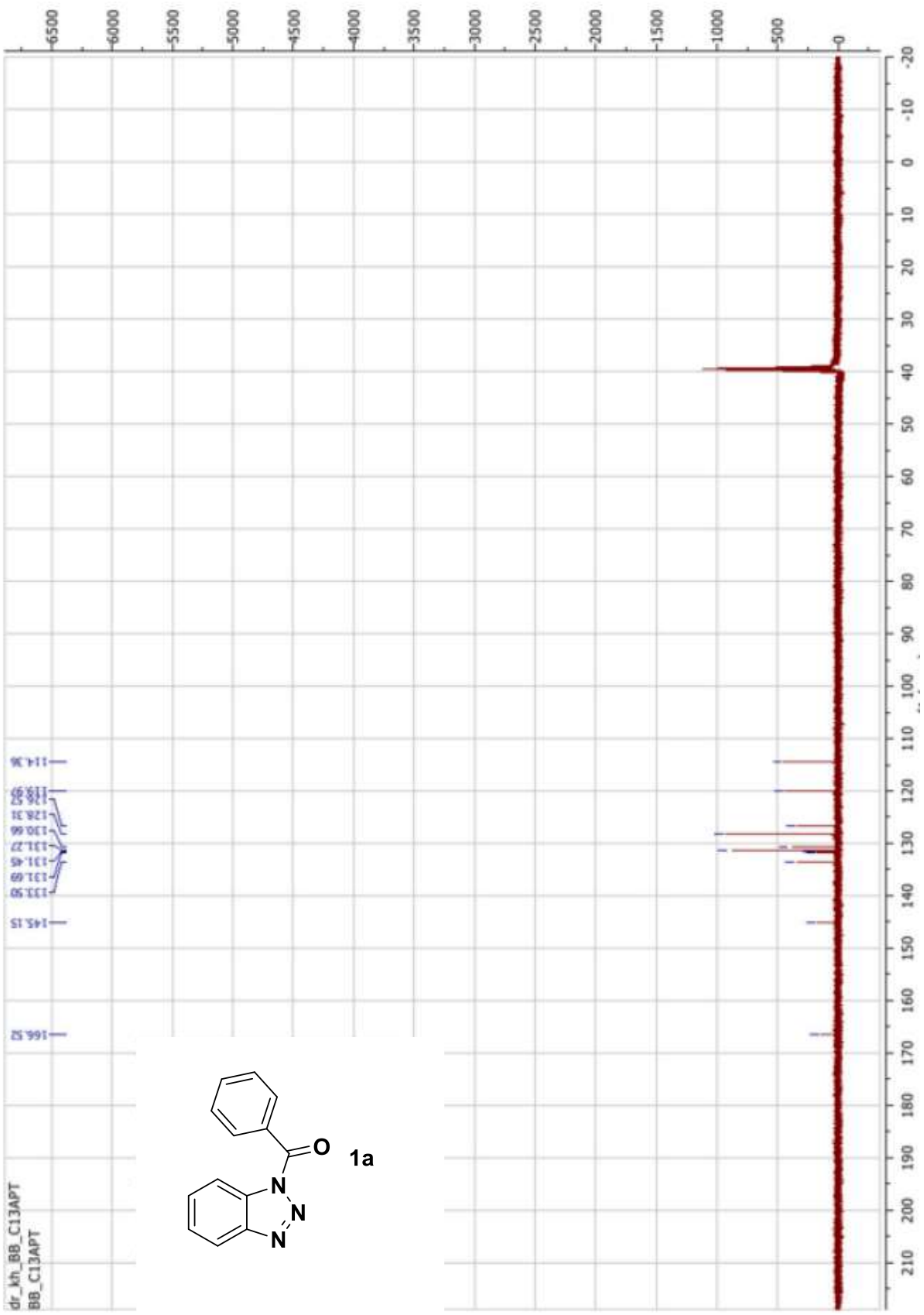
Where E_{gas} is the total of the AMBER force field internal energy terms. E_{int} (bond, angle and torsion), the covalent van der Waals (E_{vdw}) and the non-bonded electrostatic energy component (E_{ele}). The solvation energy is denoted by the equation:

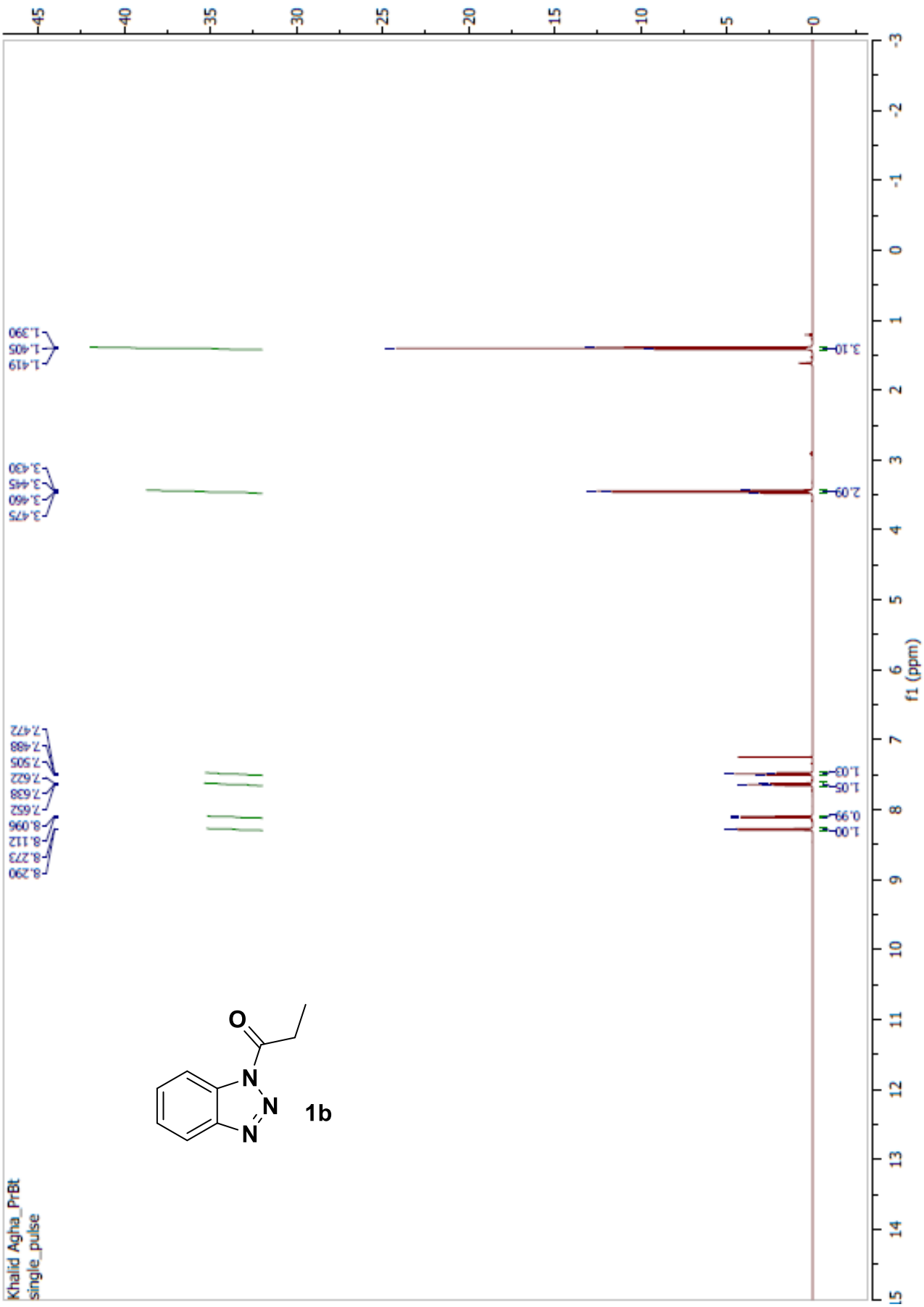
$$G_{sol} = G_{PB} + G_{non\text{-}polar} \quad (4)$$

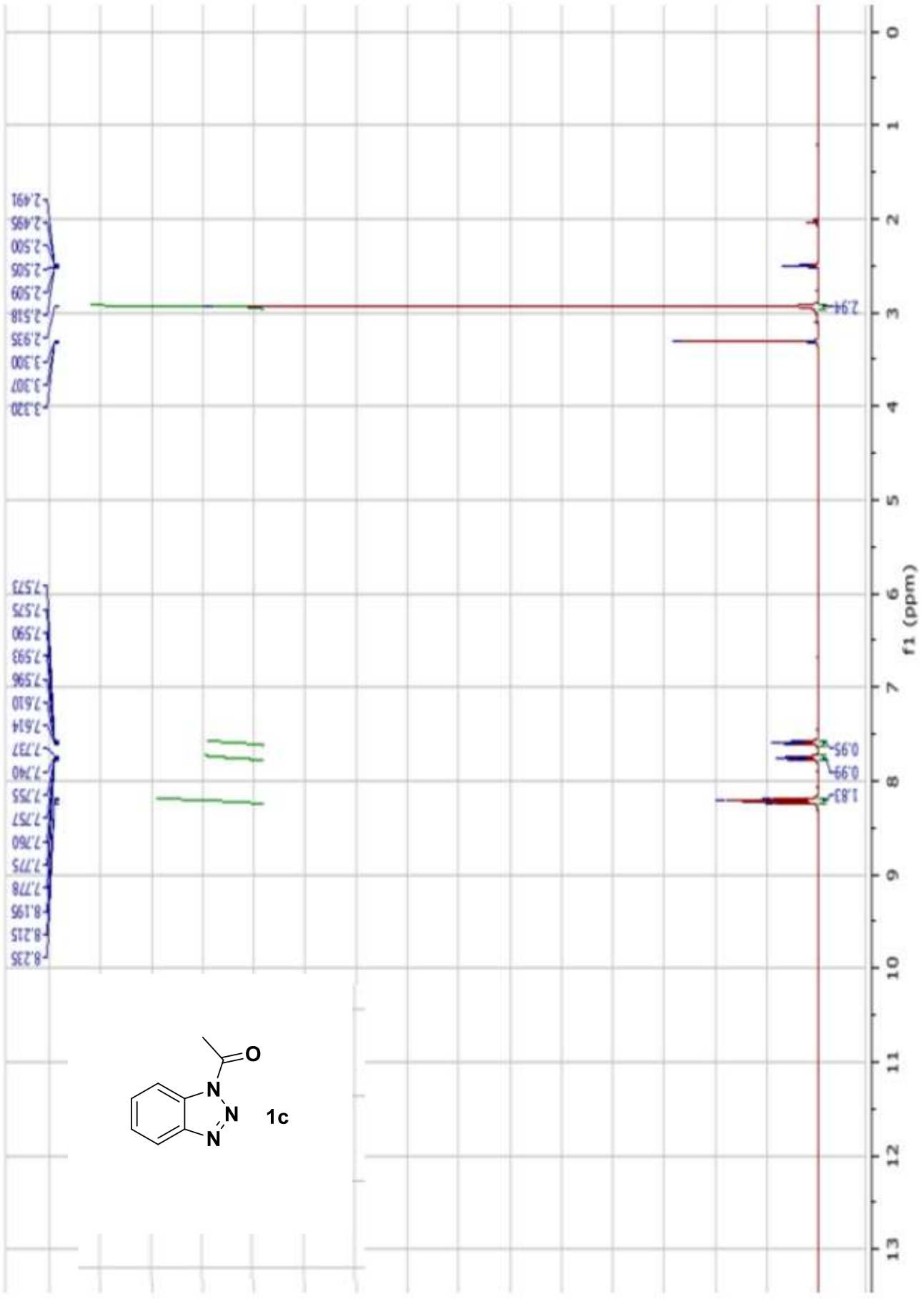
$$G_{non\text{-}polar} = \gamma SASA + b \quad (5)$$

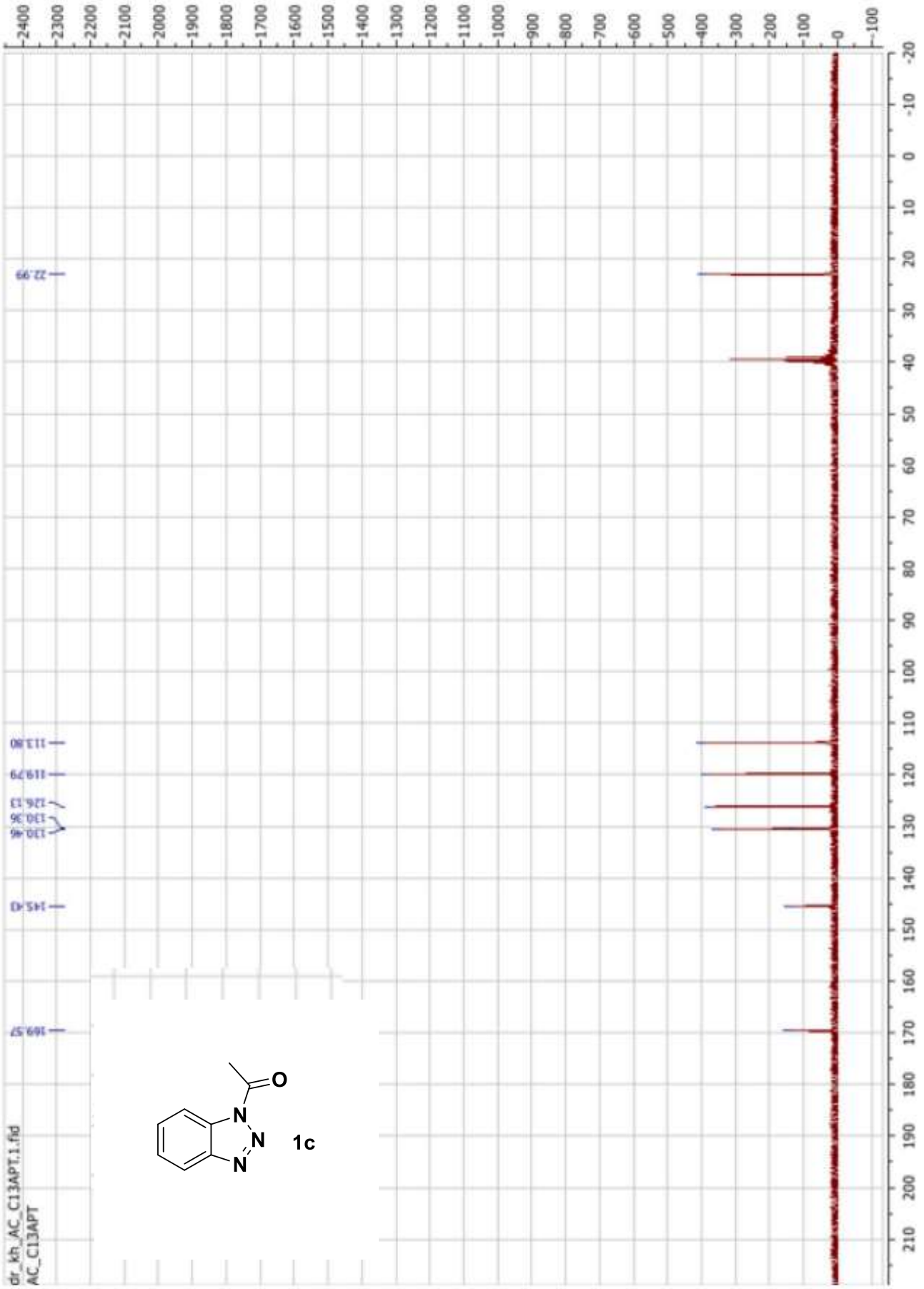
The polar solvation contribution is denoted as G_{PB} and $G_{non\text{-}polar}$ represents the non-polar contribution energy and is computed from the solvent assessable surface area (SASA). Which is obtained by the use of 1.4A water probe radius. Per-residue decomposition analyses were also performed to estimate individual energy contribution of the residues of the substrate pocket to the affinity and stabilisation of the compounds.

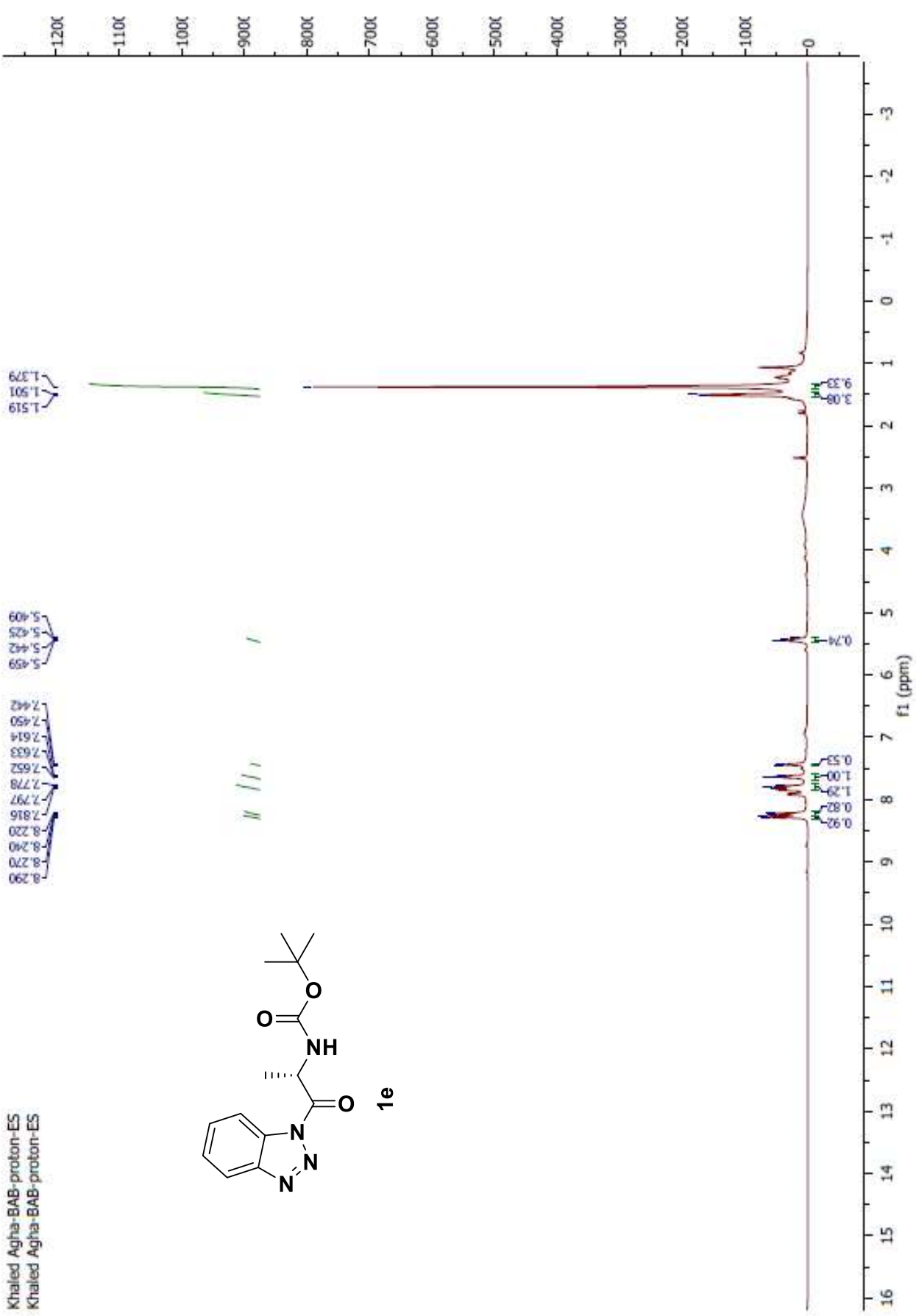

O
N
N
N
1a

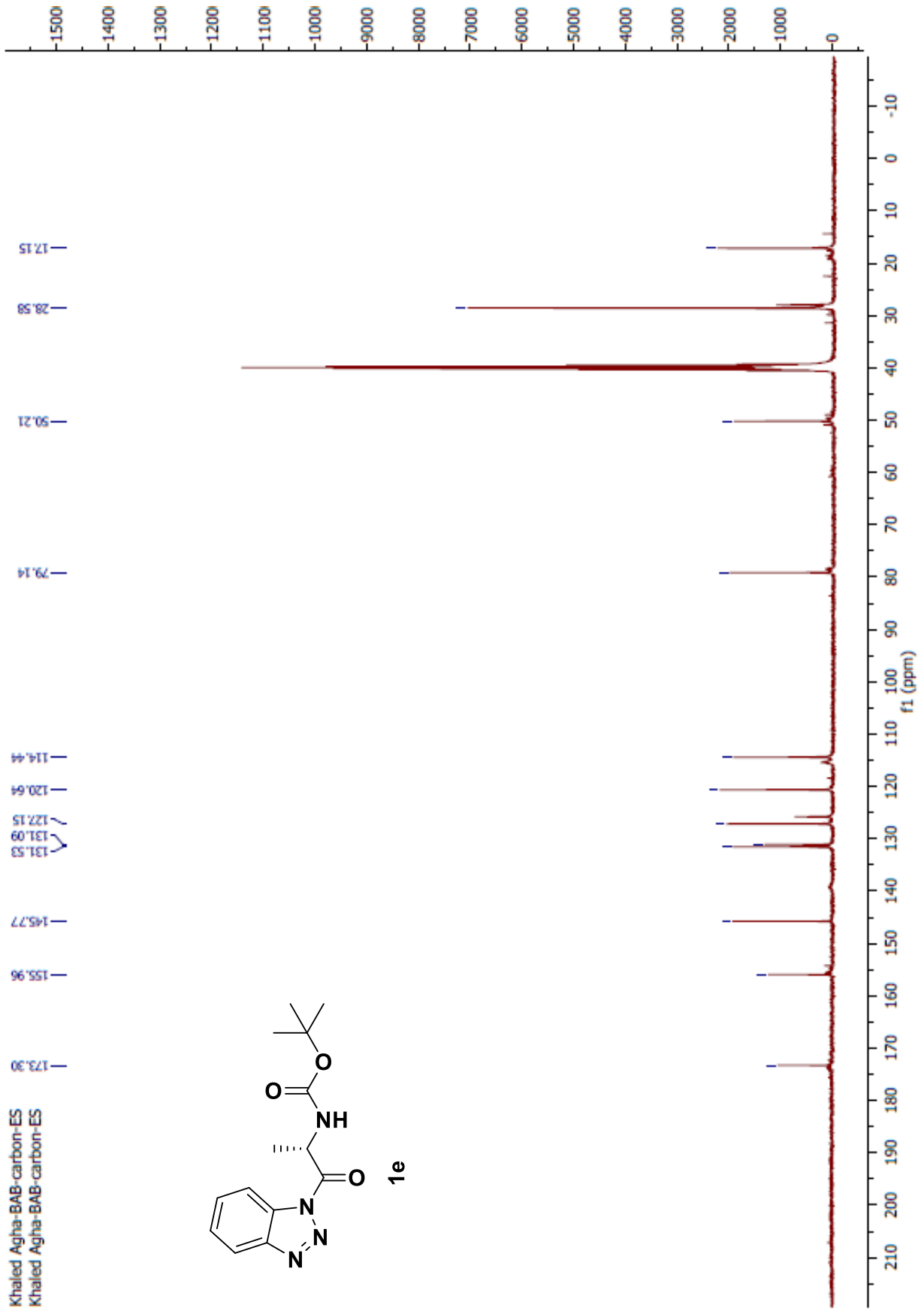

dr_kh_BB_C13APT
BB_C13APT
O
N
N
N
1a

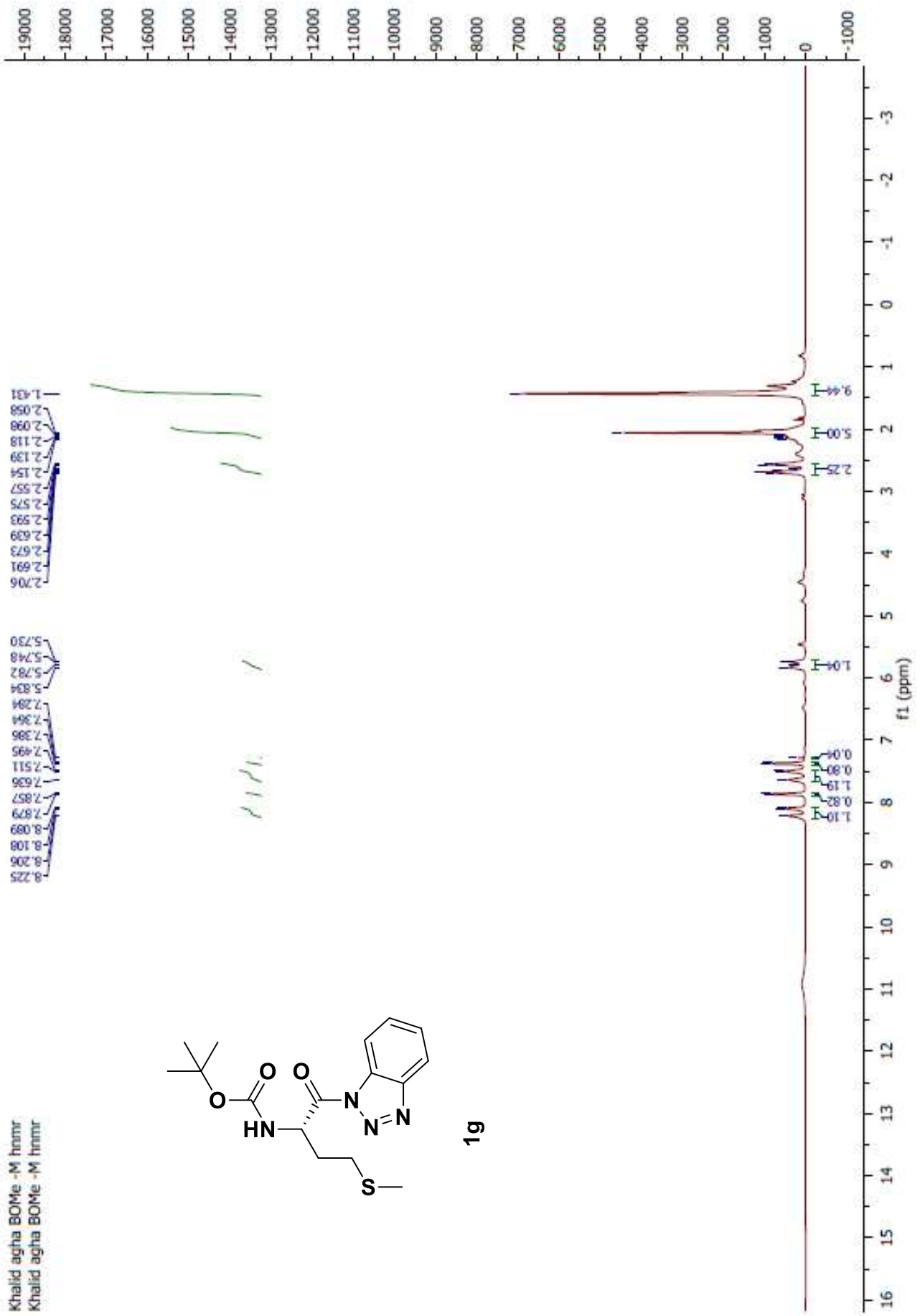

Khalid Agha_PrBt
single_pulse
f1 (ppm)
1b


f1 (ppm)
2.94
0.95
0.99
1.83
O
N
N
N
1c

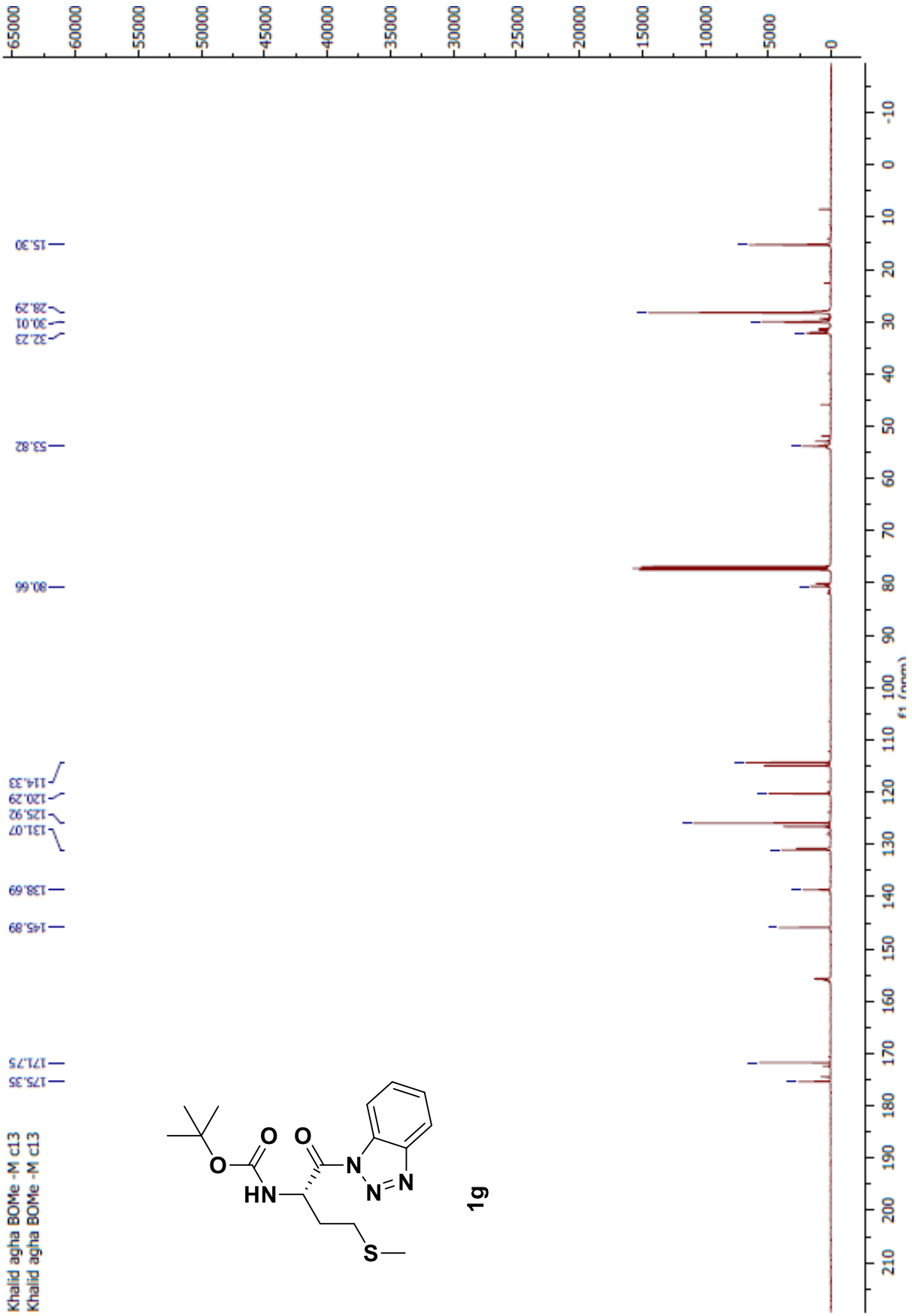

22.99
113.80
119.79
126.13
130.36
130.46
145.40
169.57
dr_kh_AC_C13APT.1.fid
AC_C13APT
O
N
N
N
1c

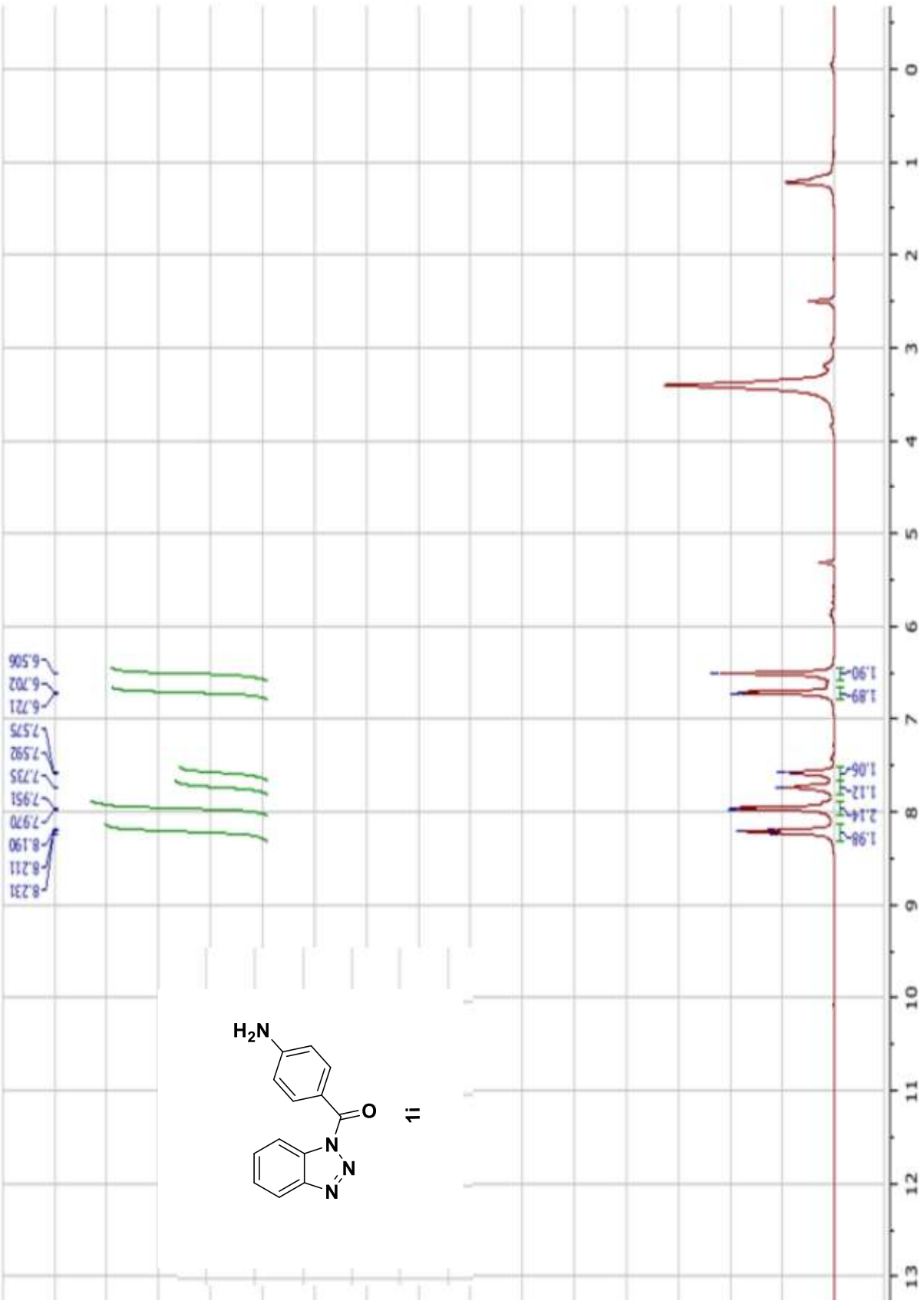

Khaled Agha-BAB-proton-ES
Khaled Agha-BAB-proton-ES
1.519
1.501
1.379
5.459
5.442
5.425
5.409
8.290
8.270
8.240
8.220
7.816
7.797
7.778
7.652
7.633
7.614
7.450
7.442
9.33
3.08
0.74
0.52
1.00
1.29
0.82
0.92
f1 (ppm)
1e

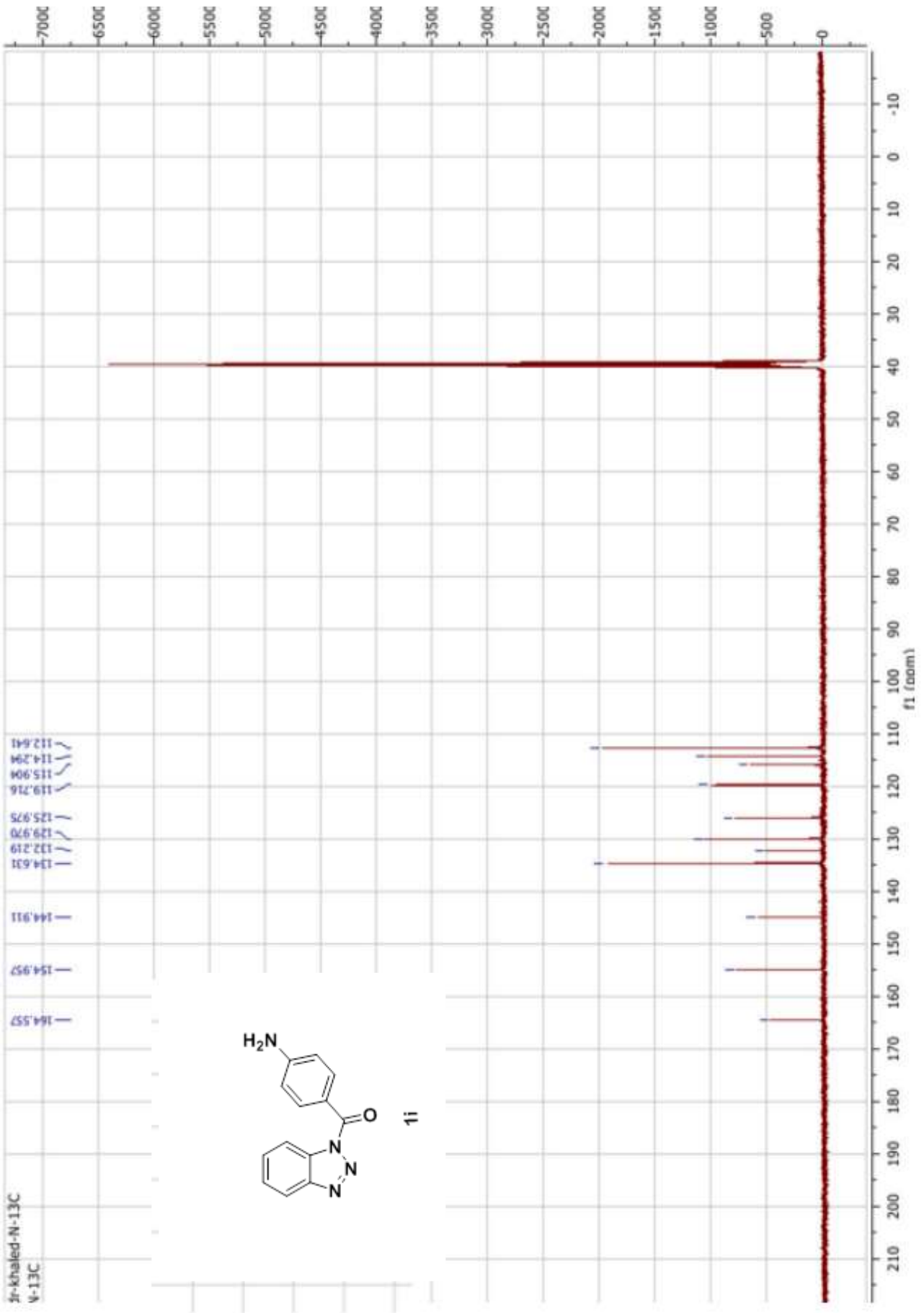

Khaled Agha-BAB-carbon-ES
Khaled Agha-BAB-carbon-ES
173.30
155.96
145.77
131.53
131.09
127.15
120.64
114.44
79.14
50.21
28.58
12.15
f1 (ppm)
1e

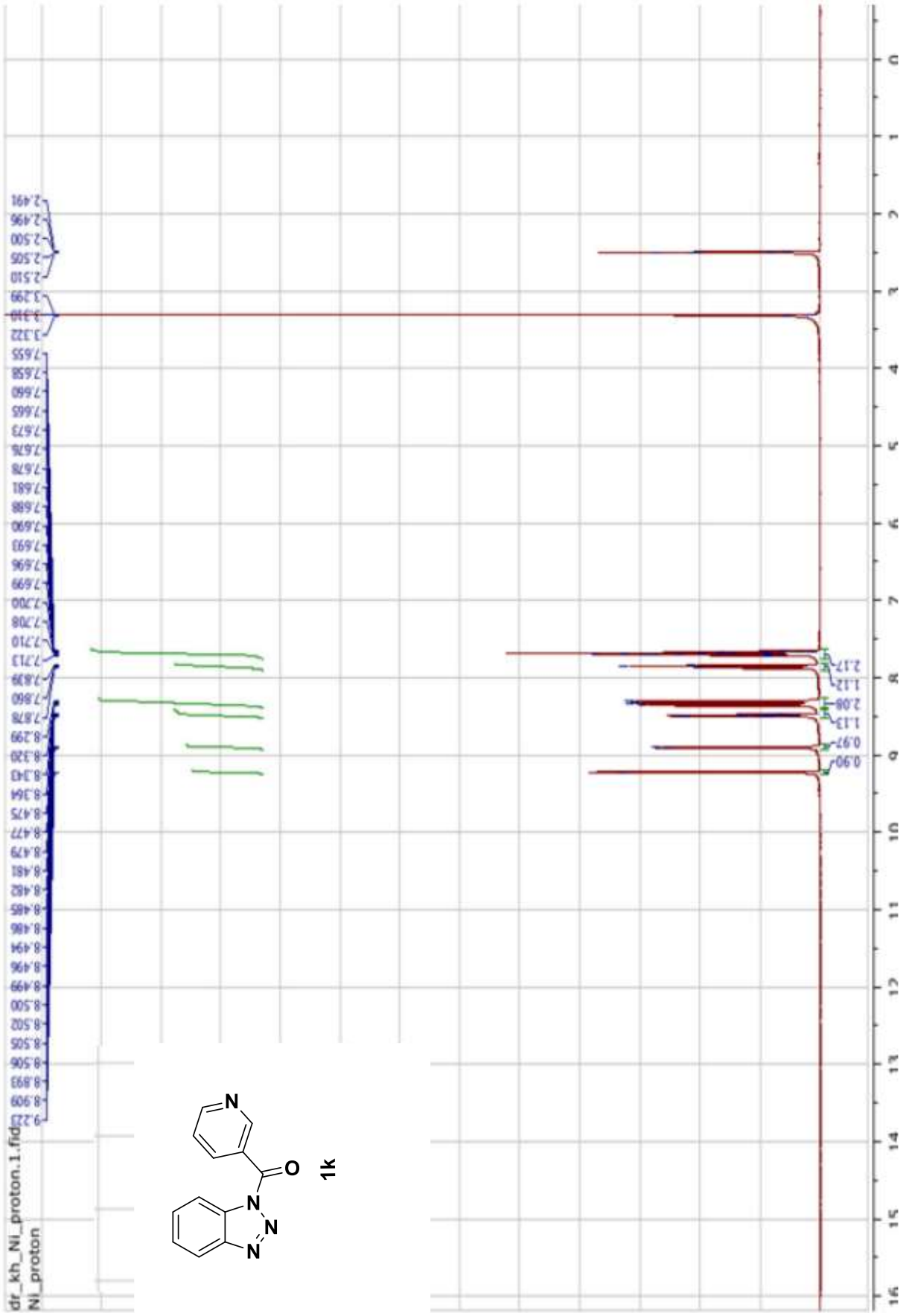

Khalid agha BOMe -M hmmr
Khalid agha BOMe -M hmmr
1.431
2.058
2.098
2.118
2.139
2.154
2.557
2.575
2.593
2.639
2.673
2.691
2.706
5.730
5.748
5.782
5.834
7.284
7.364
7.386
7.495
7.511
7.636
7.857
7.879
8.089
8.108
8.206
8.225
9.44
5.00
2.25
1.04
0.04
0.80
1.19
0.82
1.10
f1 (ppm)
O
O
O
HN
N
N
N
S

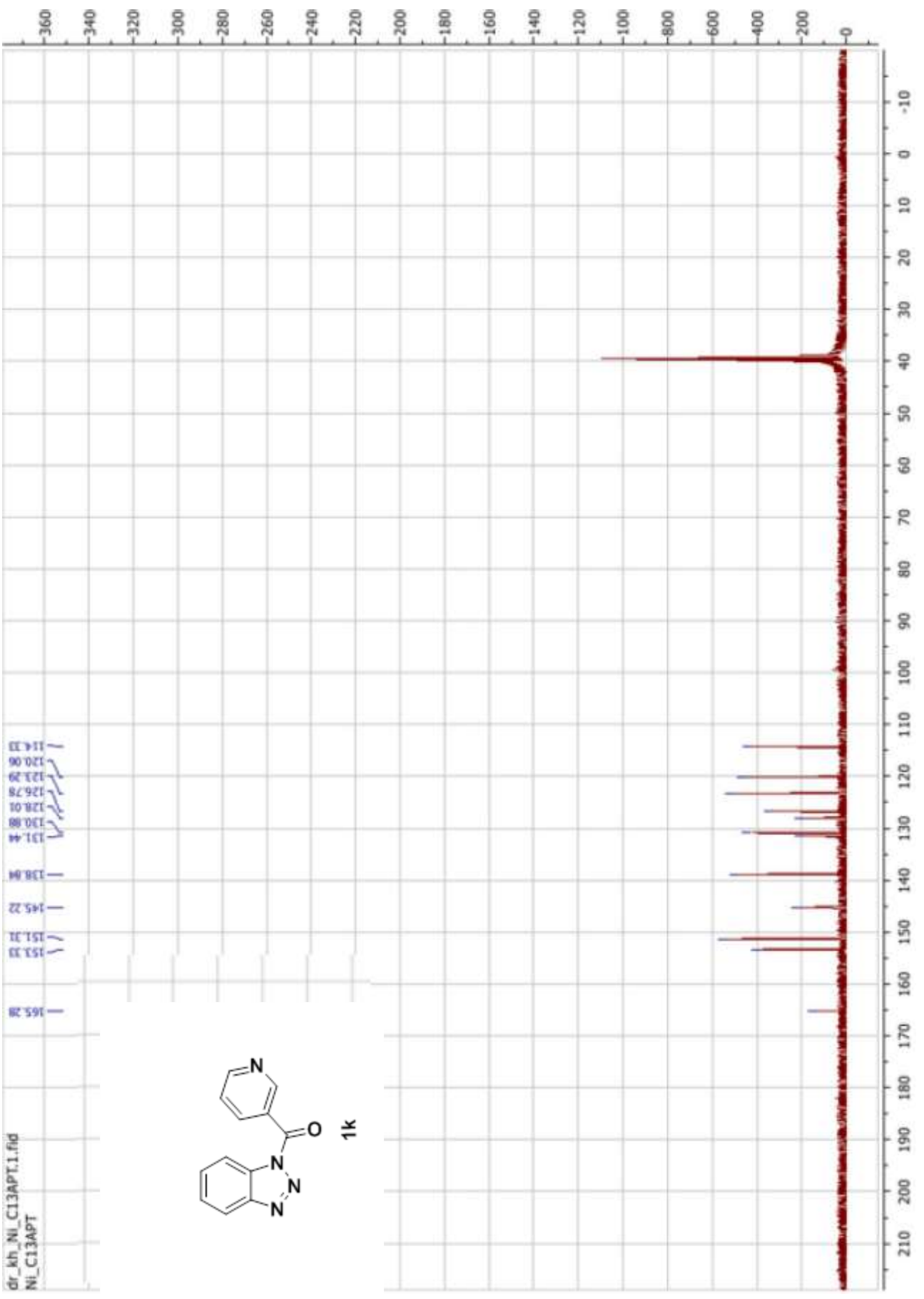
1g

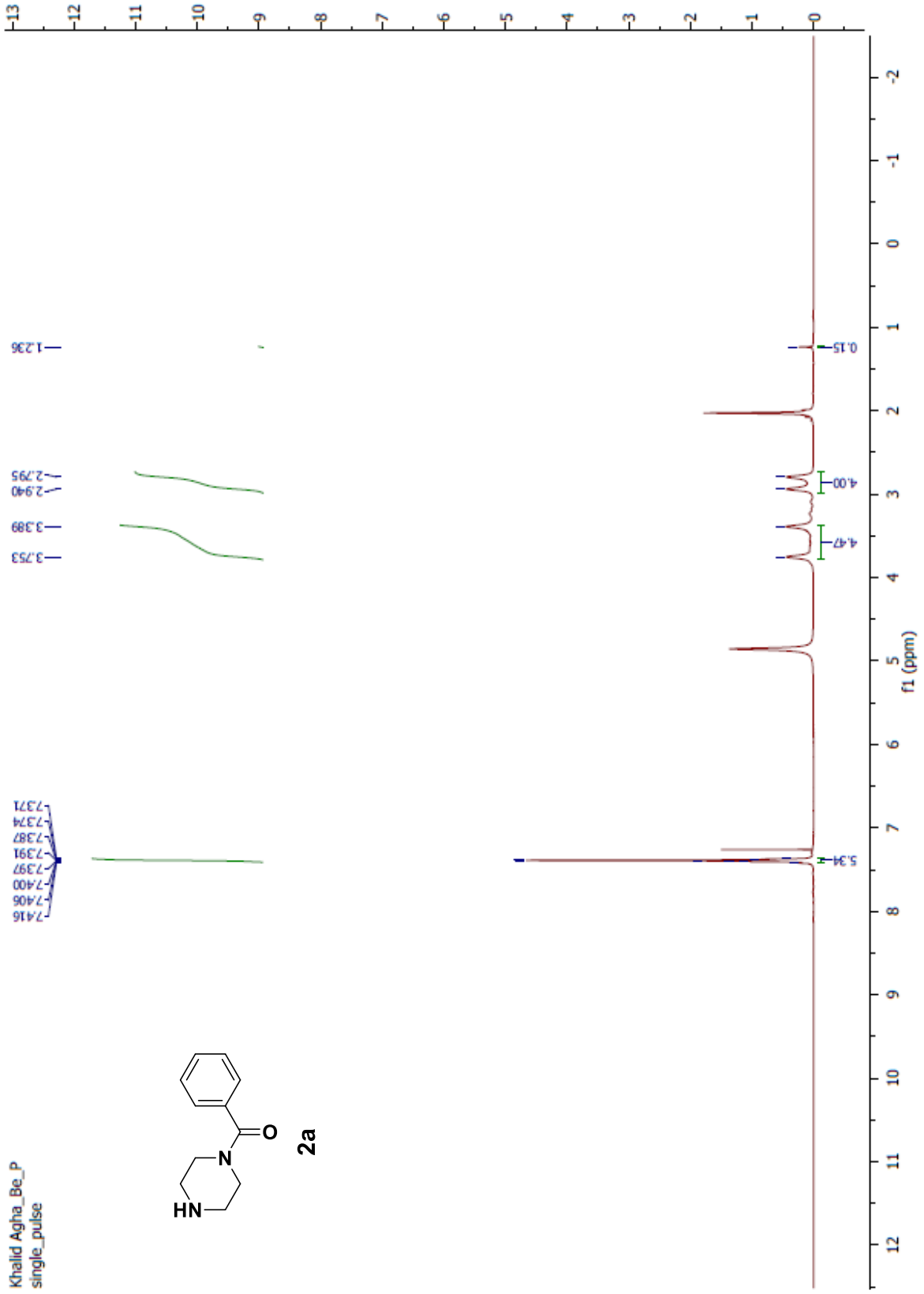

Khalid agha BOMe -M c13
Khalid agha BOMe -M c13
175.35
171.75
145.89
138.69
131.07
125.92
120.29
114.33
80.66
53.82
32.23
30.01
28.29
15.30
f1 (ppm)
1g

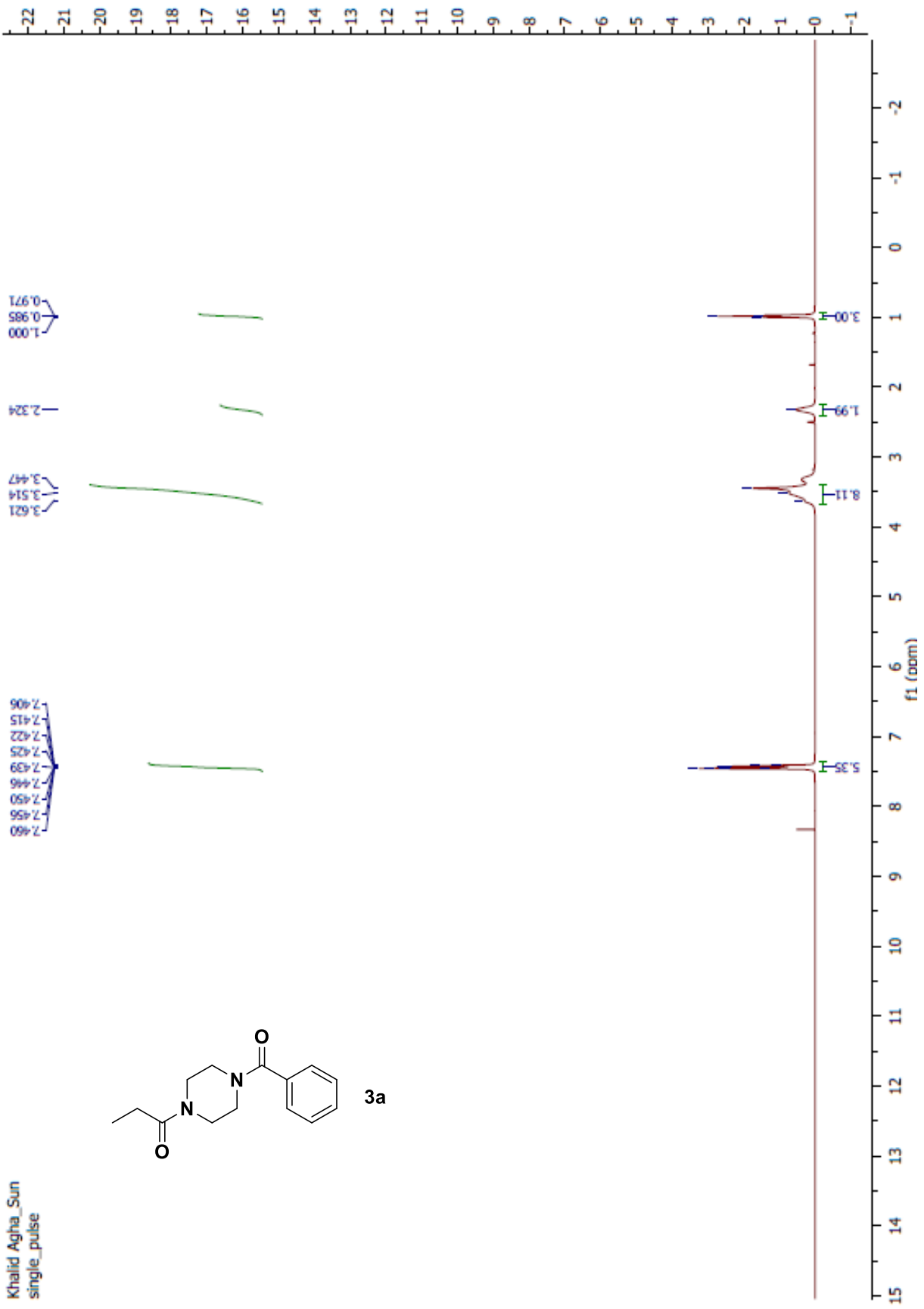

6.506
6.702
6.721
7.575
7.592
7.735
7.951
7.970
8.190
8.211
8.231
1.90
1.89
1.06
1.12
2.14
1.98
H_2N
O
N
N
N
1i


jr-khaled-N-13C
N-13C
f1 (ppm)
1i

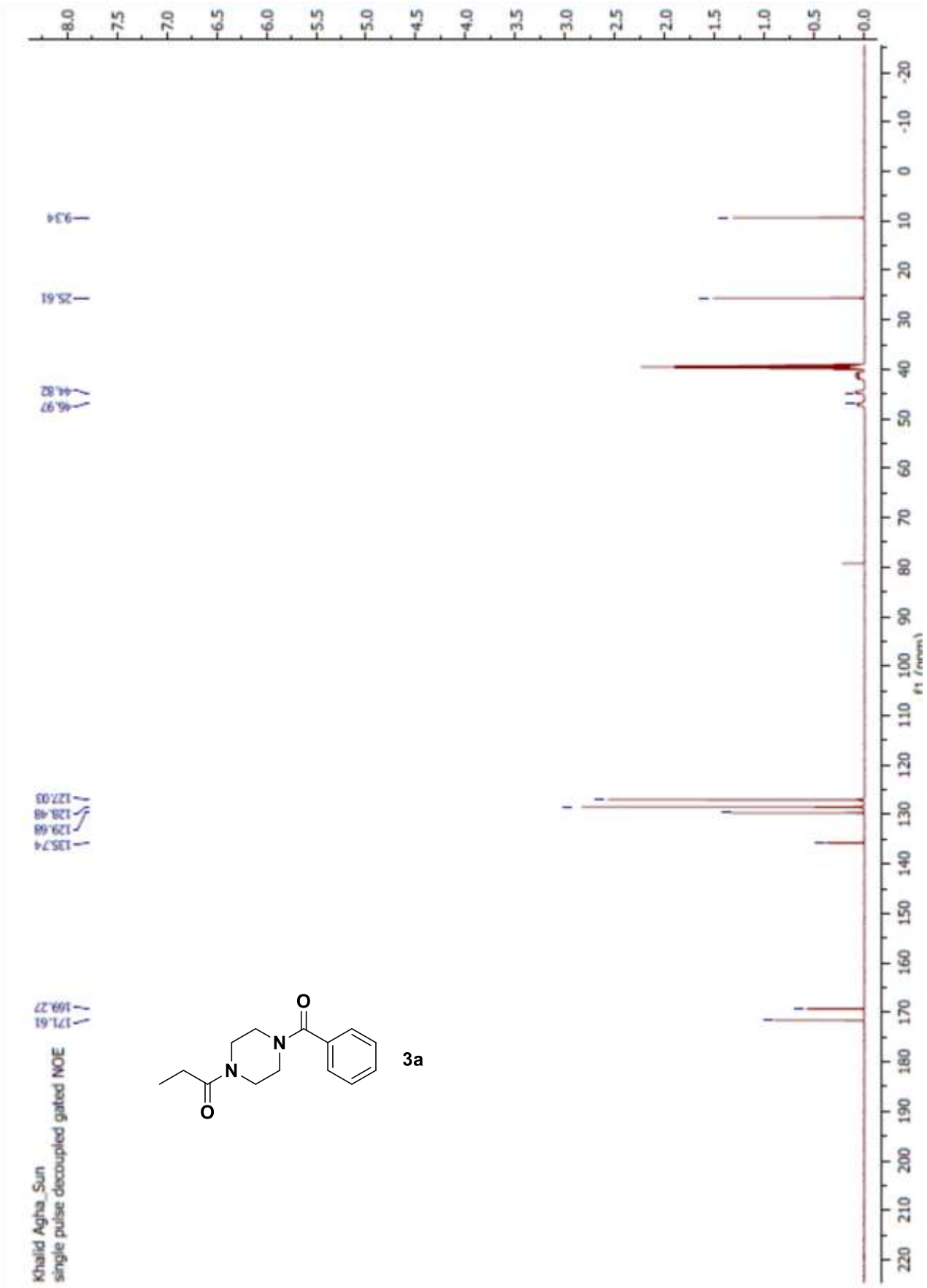

dr_kh_Ni_proton.1.fid
Ni_proton
N
O
N
N
N
1k


dr_kh_Ni_C13APT.1.fid
Ni_C13APT
165.28
153.33
151.31
145.22
138.94
131.44
130.88
128.01
126.78
123.29
120.06
114.33
1k


Khalid Agha_Be_P
single_pulse
2a
O
N
HN
f1 (ppm)
1.236
2.795
2.940
3.389
3.753
7.371
7.374
7.387
7.391
7.397
7.400
7.406
7.416
0.15
4.00
4.47
5.34

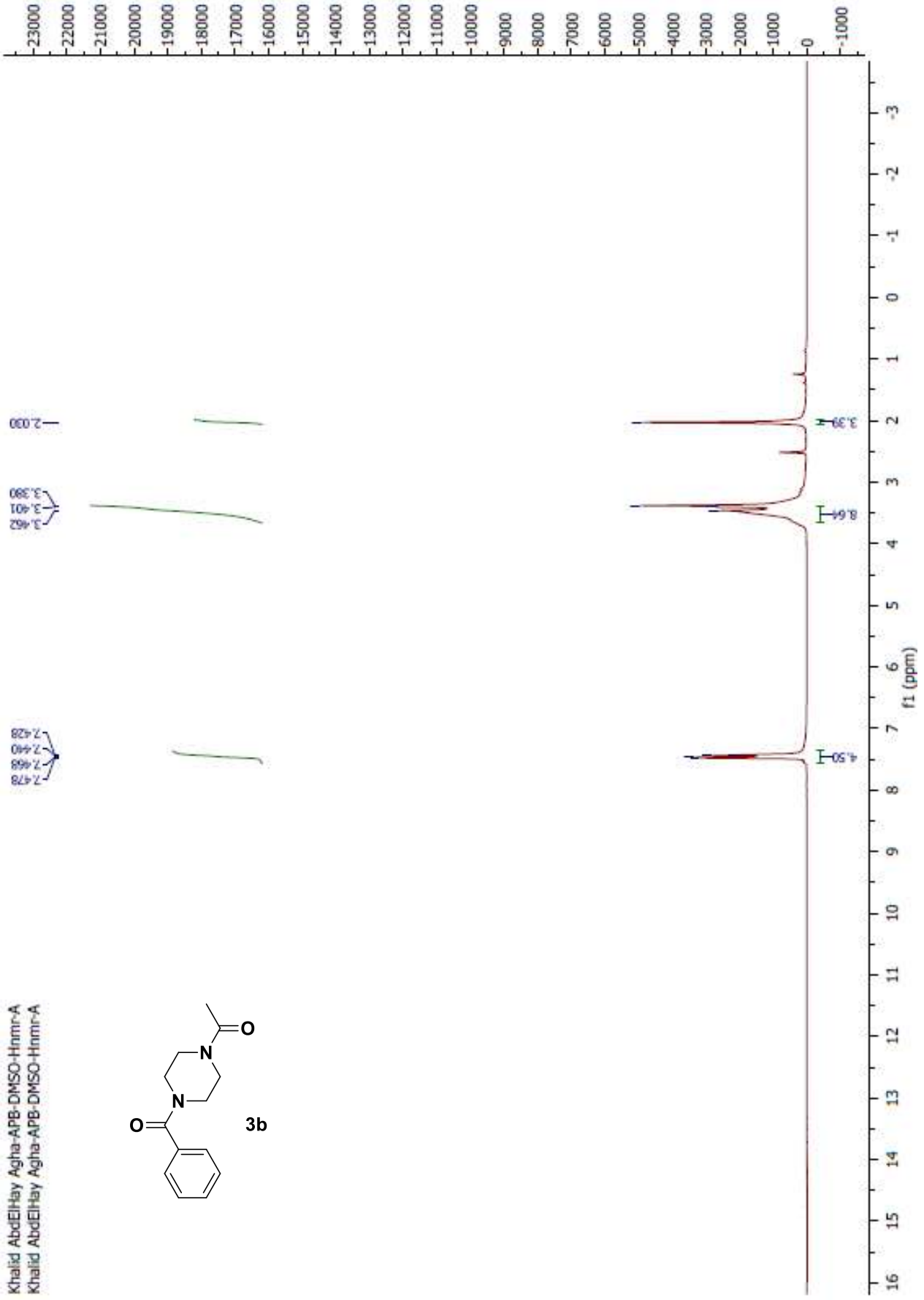

Khalid Agha_Sun
single_pulse
f1 (ppm)
3.00
1.99
8.11
5.35
1.000
0.985
0.971
2.324
3.621
3.514
3.447
7.460
7.456
7.450
7.446
7.439
7.425
7.422
7.415
7.406
O
N
N
O

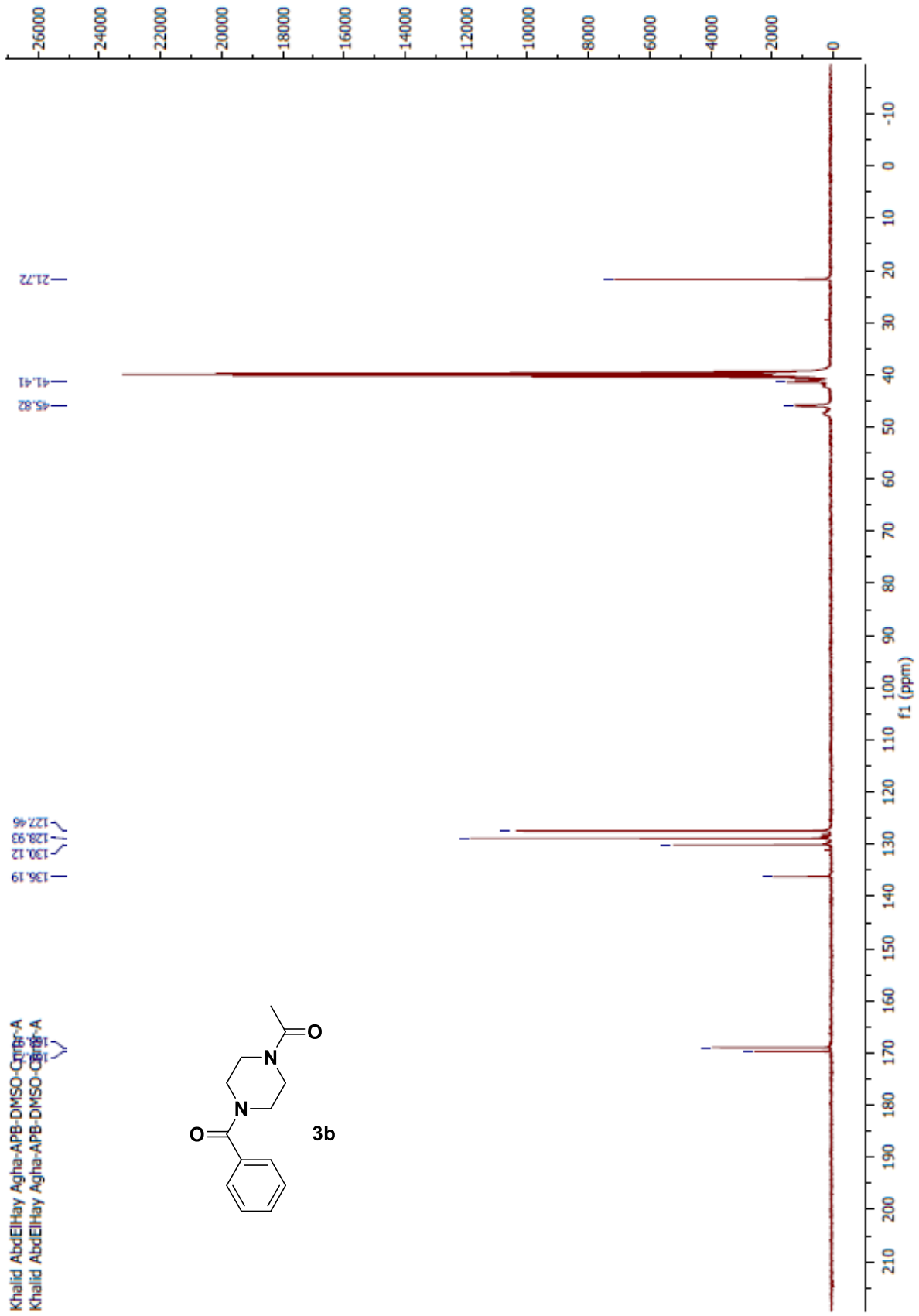
3a

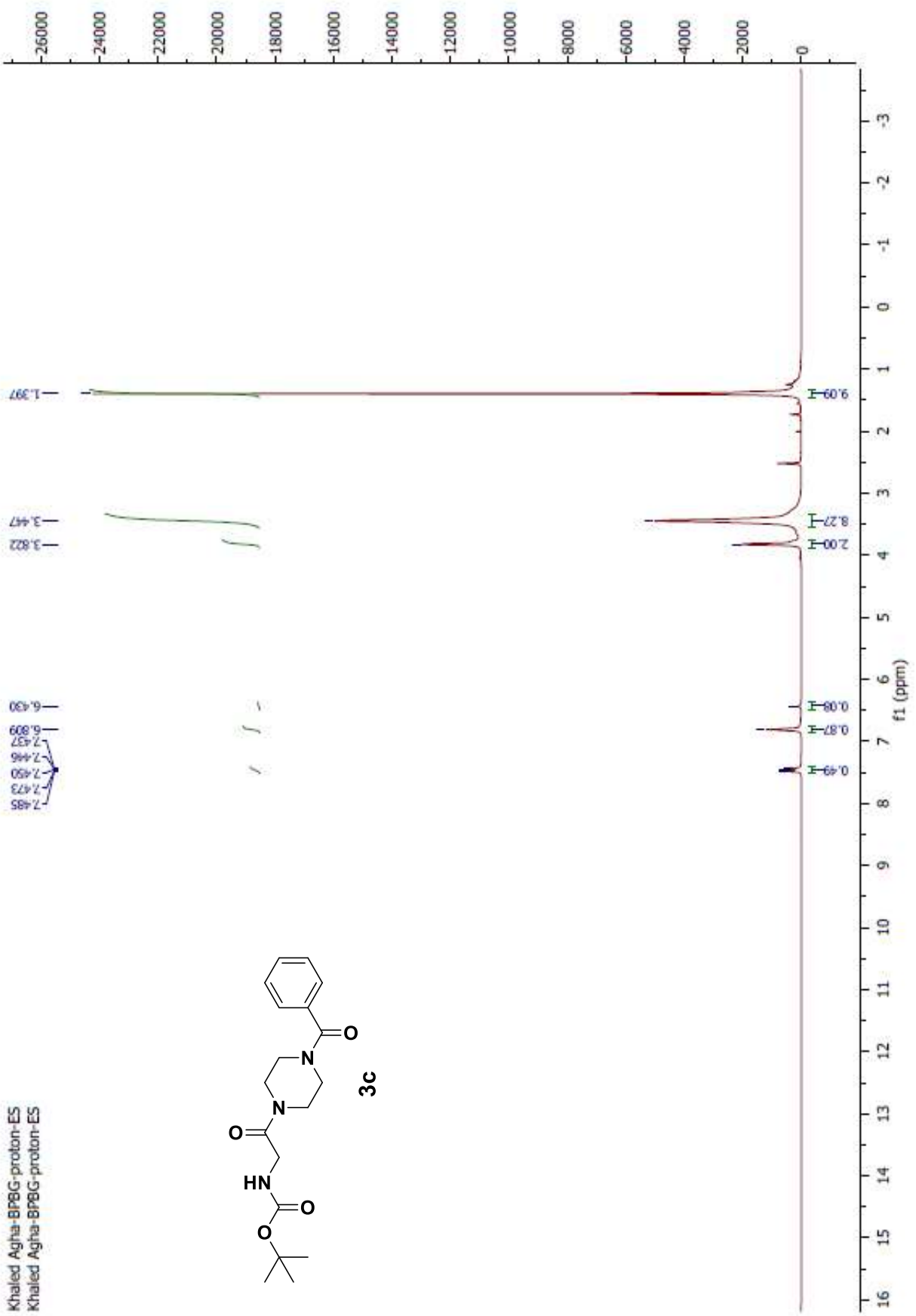

Khalid Agha_Sun
single pulse decoupled gated NOE
9.34
25.61
44.82
46.97
127.03
128.48
129.68
135.74
169.27
171.61
f1 (ppm)

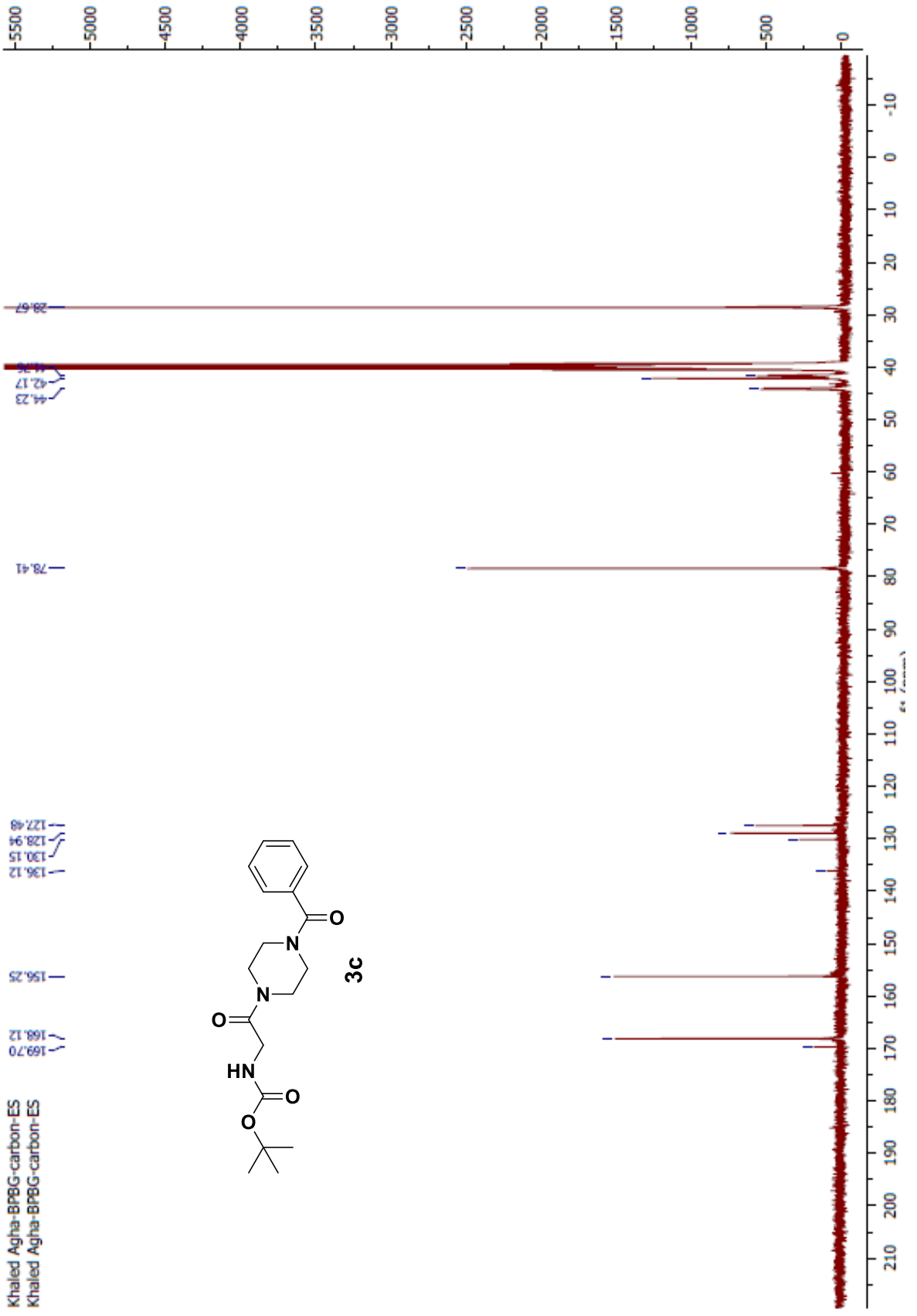
O
N
N
O

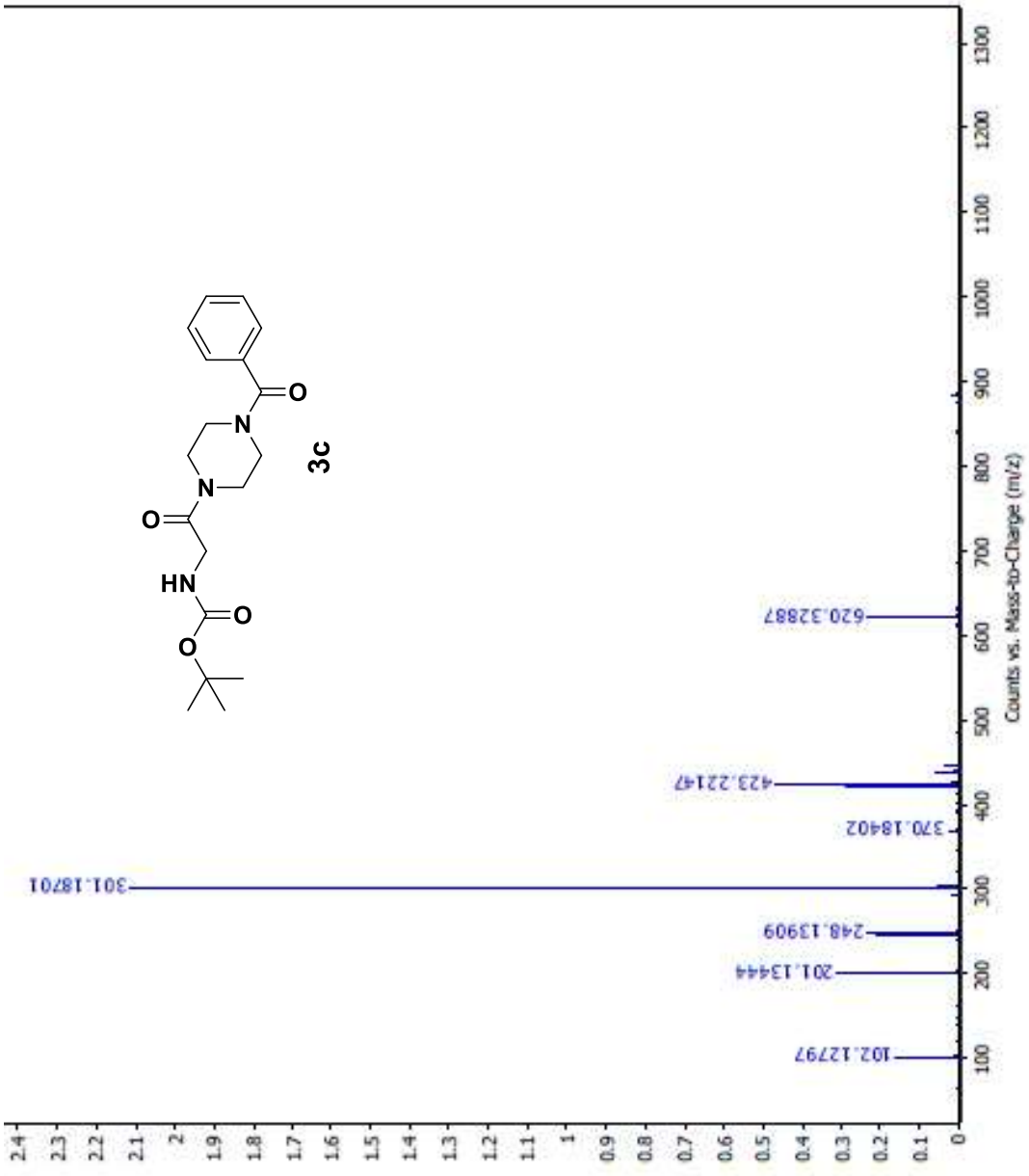
3a

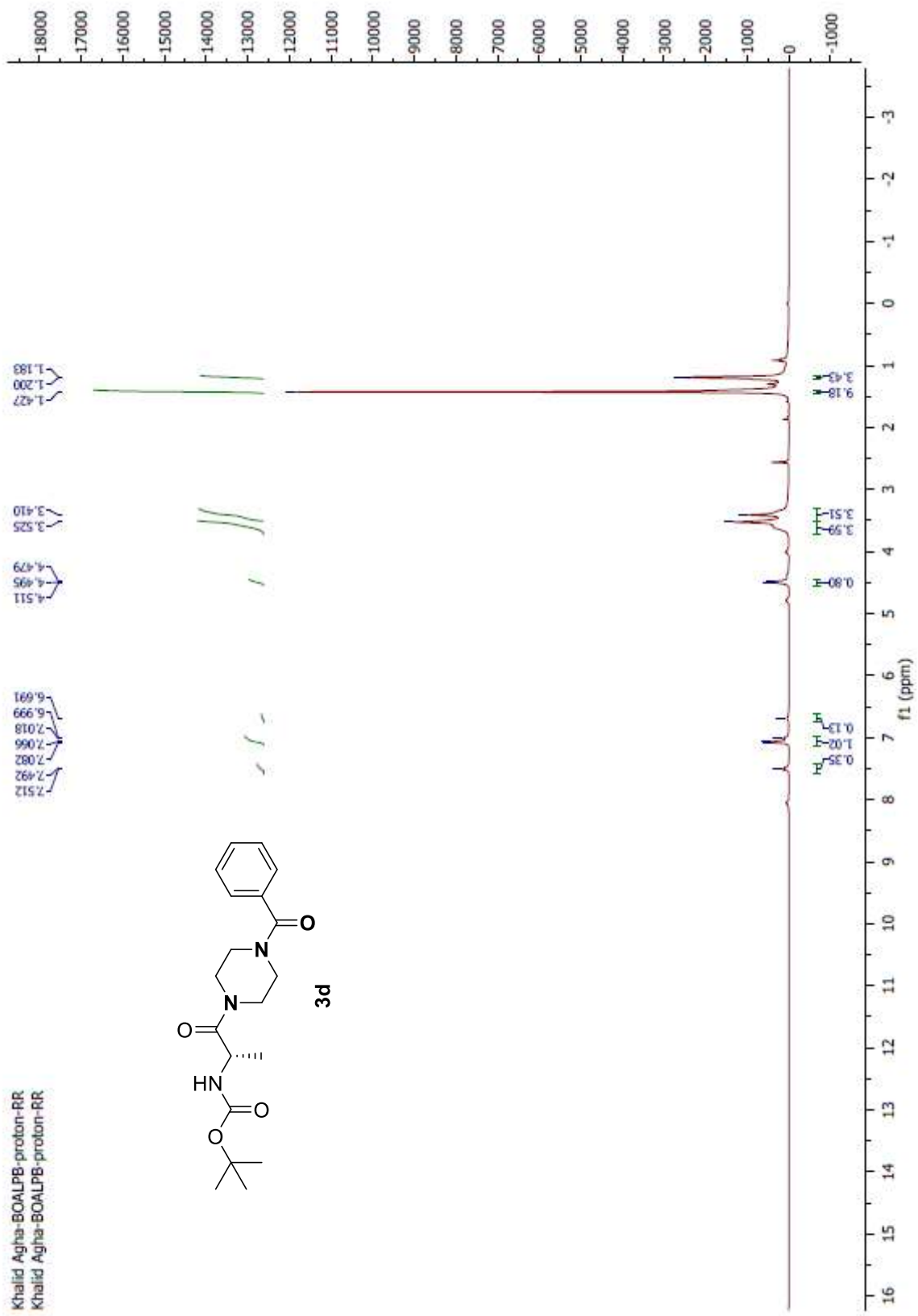

Khalid AbdElHay Agha-APB-DMSO-Hnmr-A
Khalid AbdElHay Agha-APB-DMSO-Hnmr-A
7.478
7.468
7.440
7.428
3.462
3.401
3.380
2.030
4.50
8.64
3.39
f1 (ppm)
O
N
N
O
3b

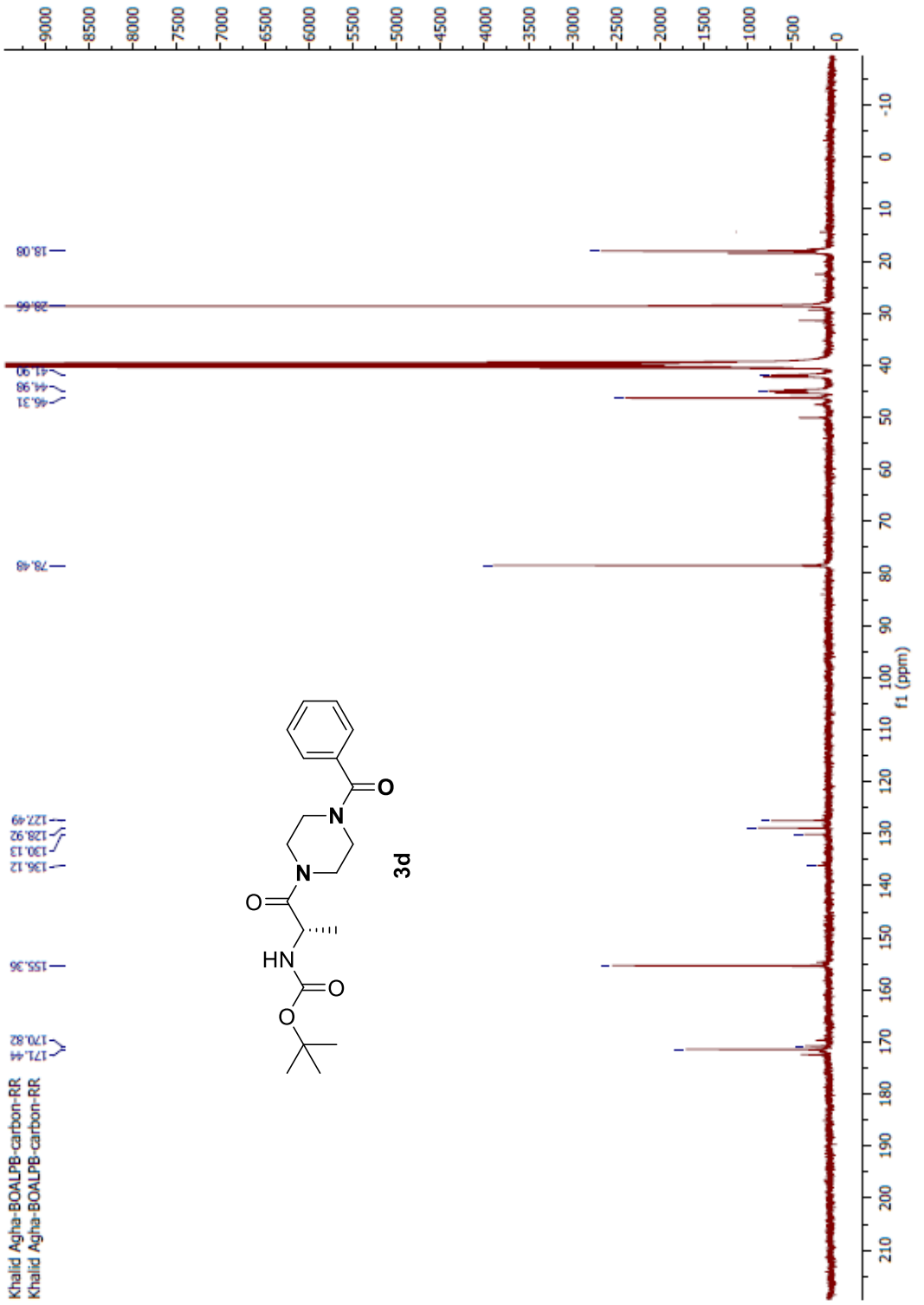

Khalid AbdElHay Agha-APB-DMSO-Cmpd-A
Khalid AbdElHay Agha-APB-DMSO-Cmpd-A
21.72
41.41
45.82
127.46
128.93
130.12
136.19
f1 (ppm)
3b

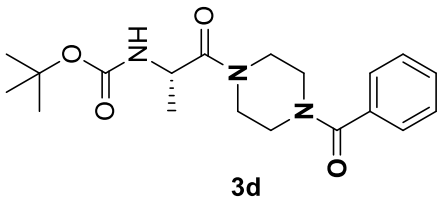

Khaled Agha-BPBG-proton-ES
Khaled Agha-BPBG-proton-ES
1.397
3.447
3.822
6.430
6.809
7.437
7.446
7.450
7.473
7.485
9.09
8.27
2.00
0.89
0.87
0.49
f1 (ppm)
3c


Khaled Agha-BPBG-carbon-ES
Khaled Agha-BPBG-carbon-ES
28.67
44.25
42.17
41.76
78.41
136.12
130.15
128.94
127.48
156.25
169.70
168.12
3c

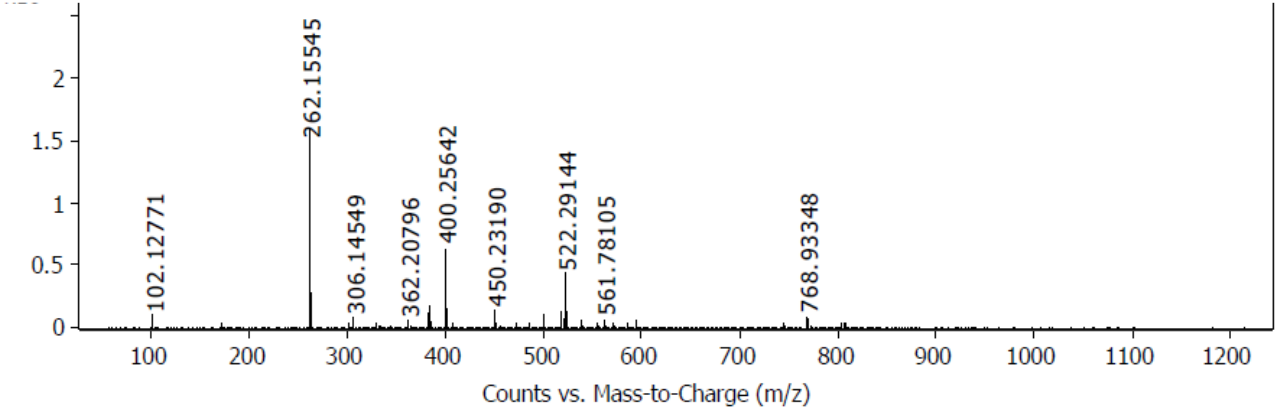

3c
620.32887
423.22147
370.18402
301.18701
248.13909
201.13444
102.12797
Counts vs. Mass-to-Charge (m/z)


Khalid Agha-BOALPB-proton-RR
Khalid Agha-BOALPB-proton-RR
3d
f1 (ppm)


Khalid Agha-BOALPB-carbon-RR
Khalid Agha-BOALPB-carbon-RR
18.08
28.66
41.90
44.98
46.31
78.48
122.49
128.92
130.13
136.12
155.36
170.82
171.44
f1 (ppm)
3d

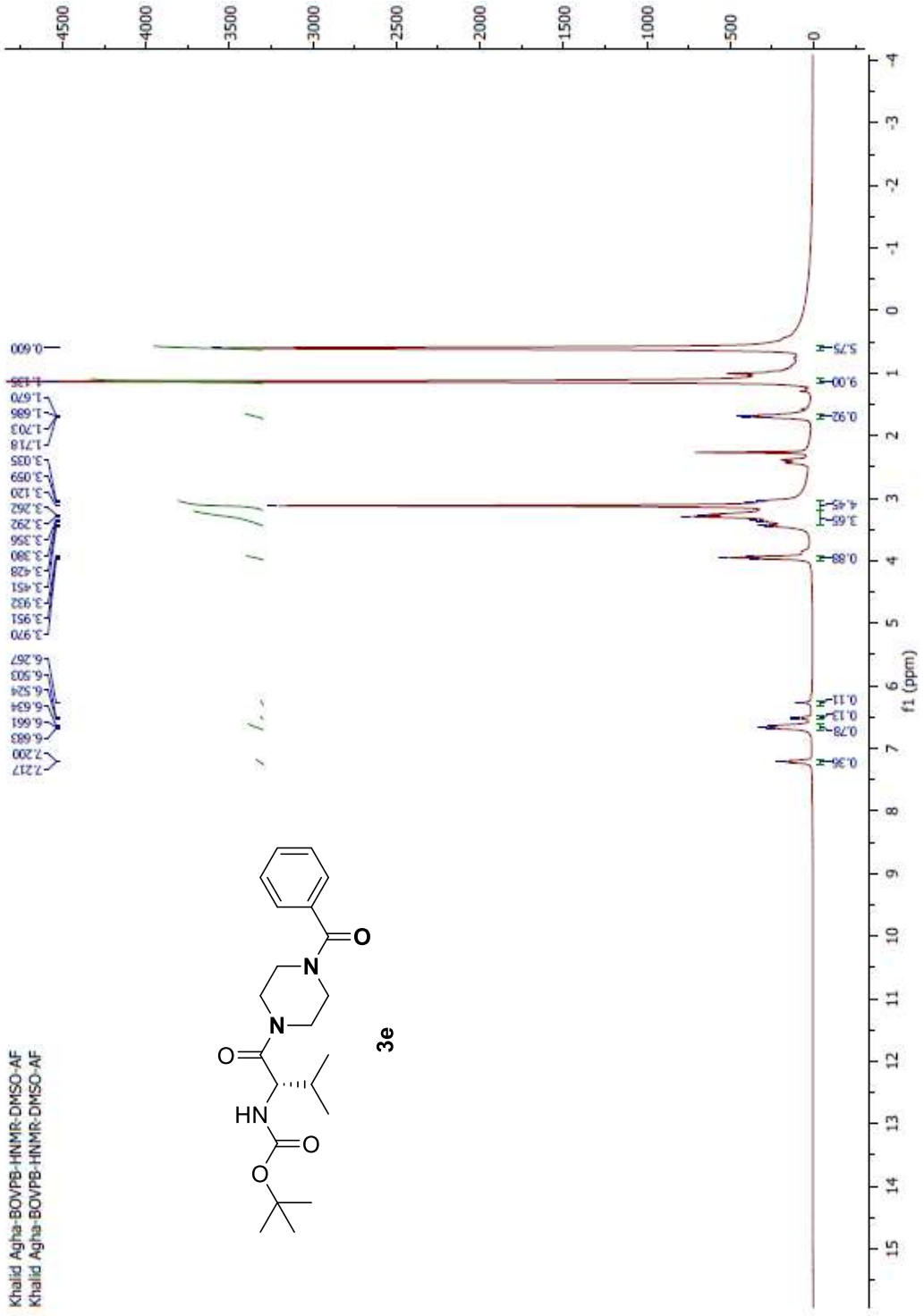


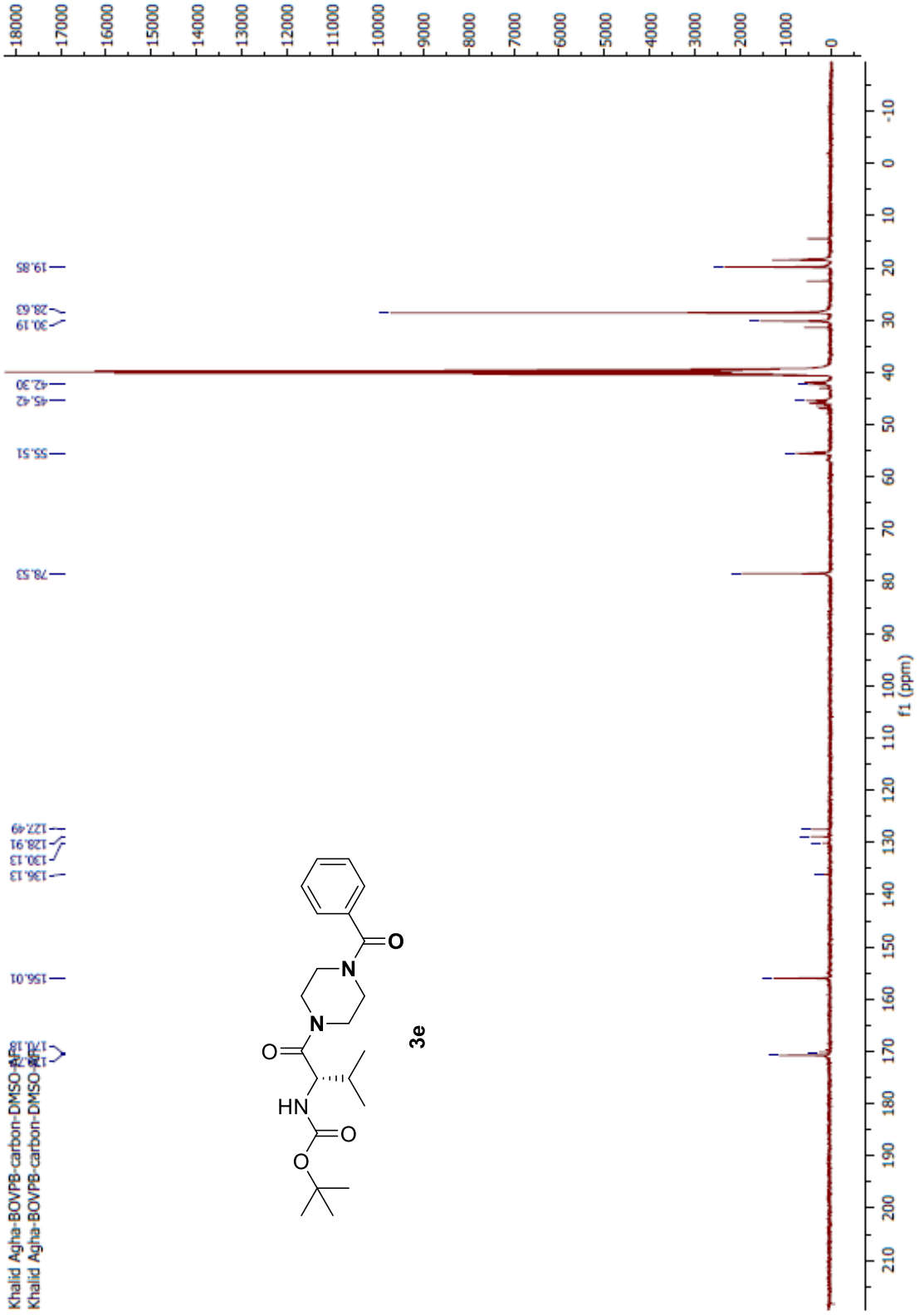
3d

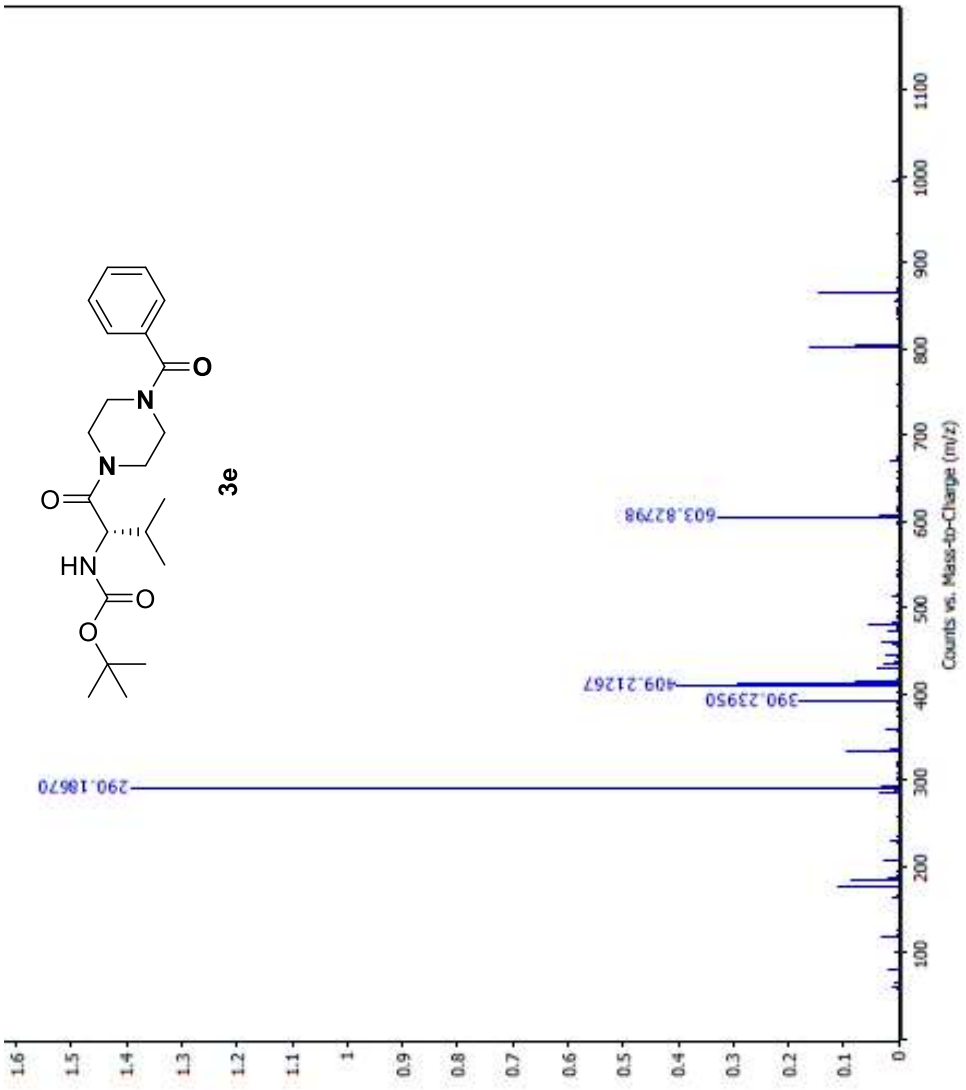


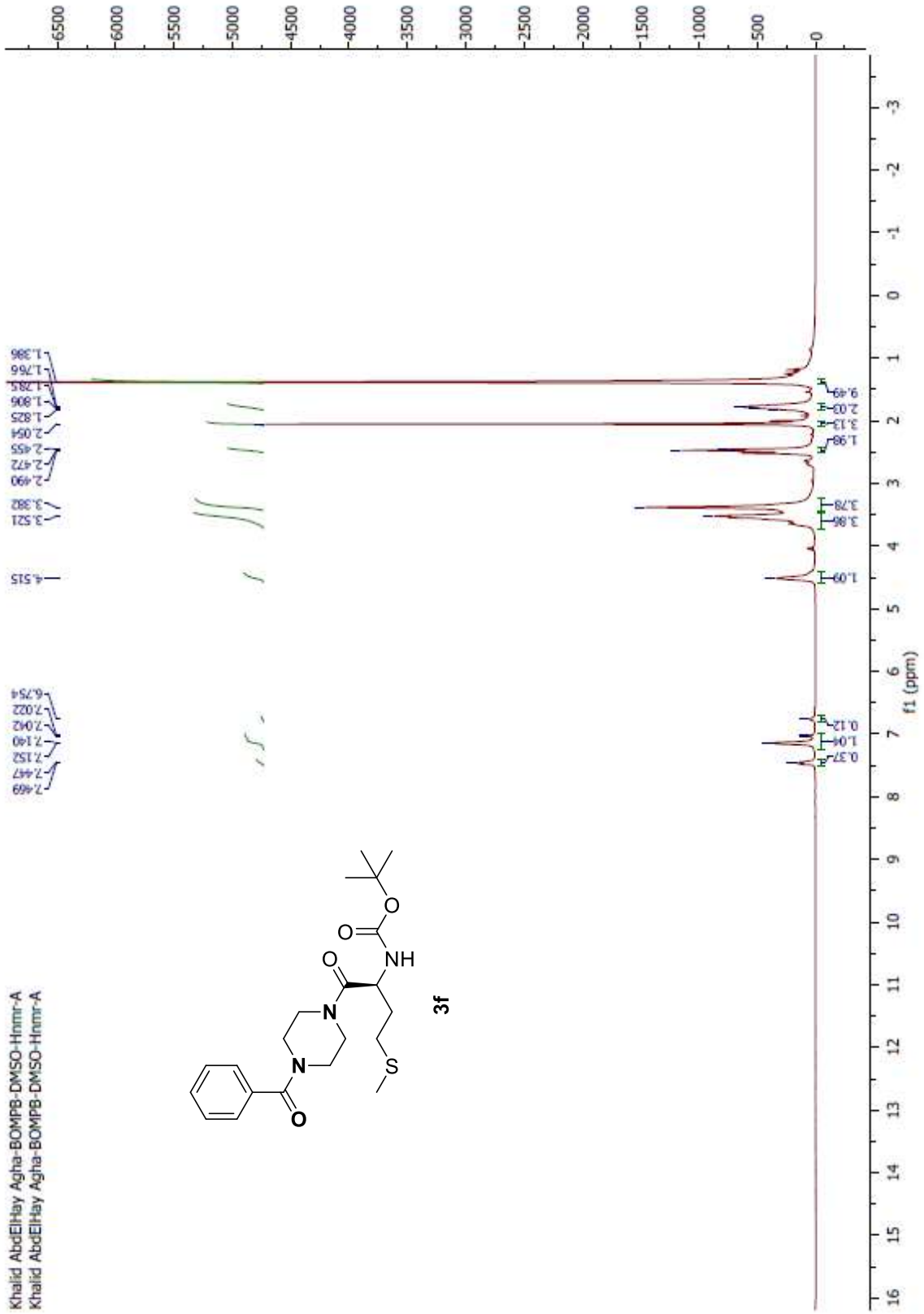
Peak List

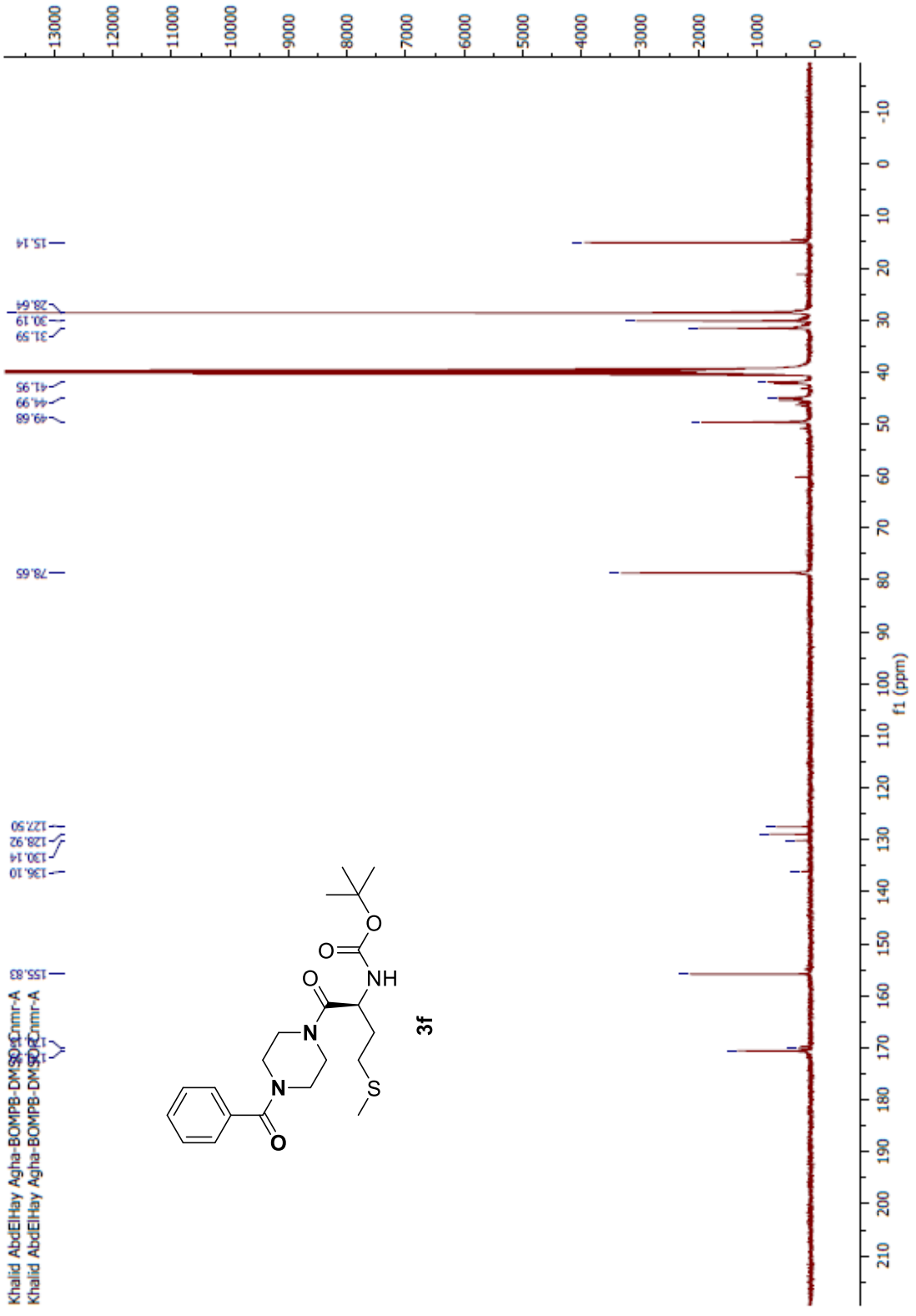
m/z	z	Abund
262.15545	1	1578466.38
263.15876	1	286410.34
381.18128	2	107872.13
384.1908	1	165550.41
400.25642	1	637177.56
401.2593	1	151639.13
450.2319	2	138375.19
519.28227	2	120170.31
522.29144	1	427703.19
523.29437	1	130557.73

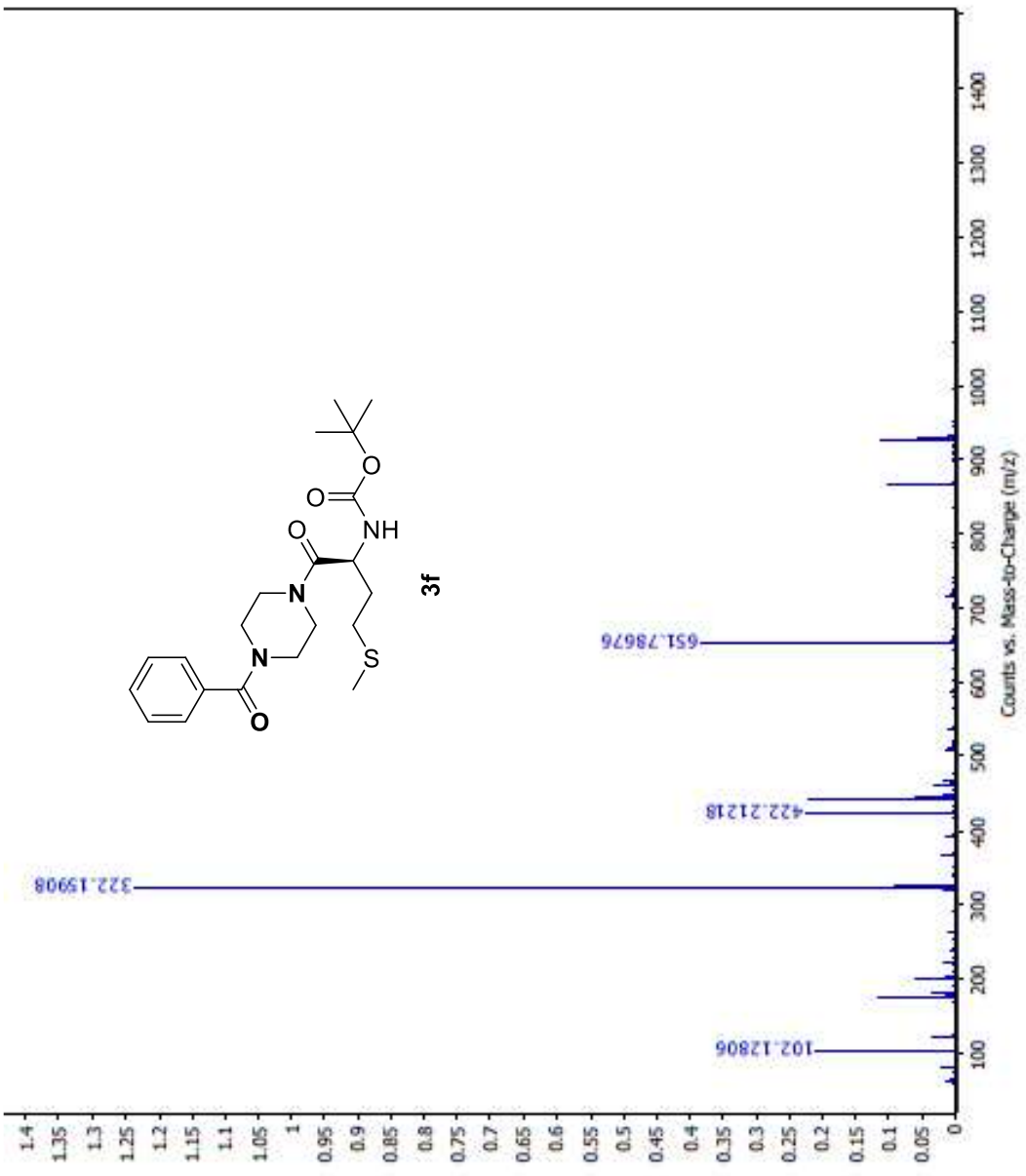

Khalid Agha-BOVPB-HNMR-DMSO-AF
Khalid Agha-BOVPB-HNMR-DMSO-AF
3e
f1 (ppm)

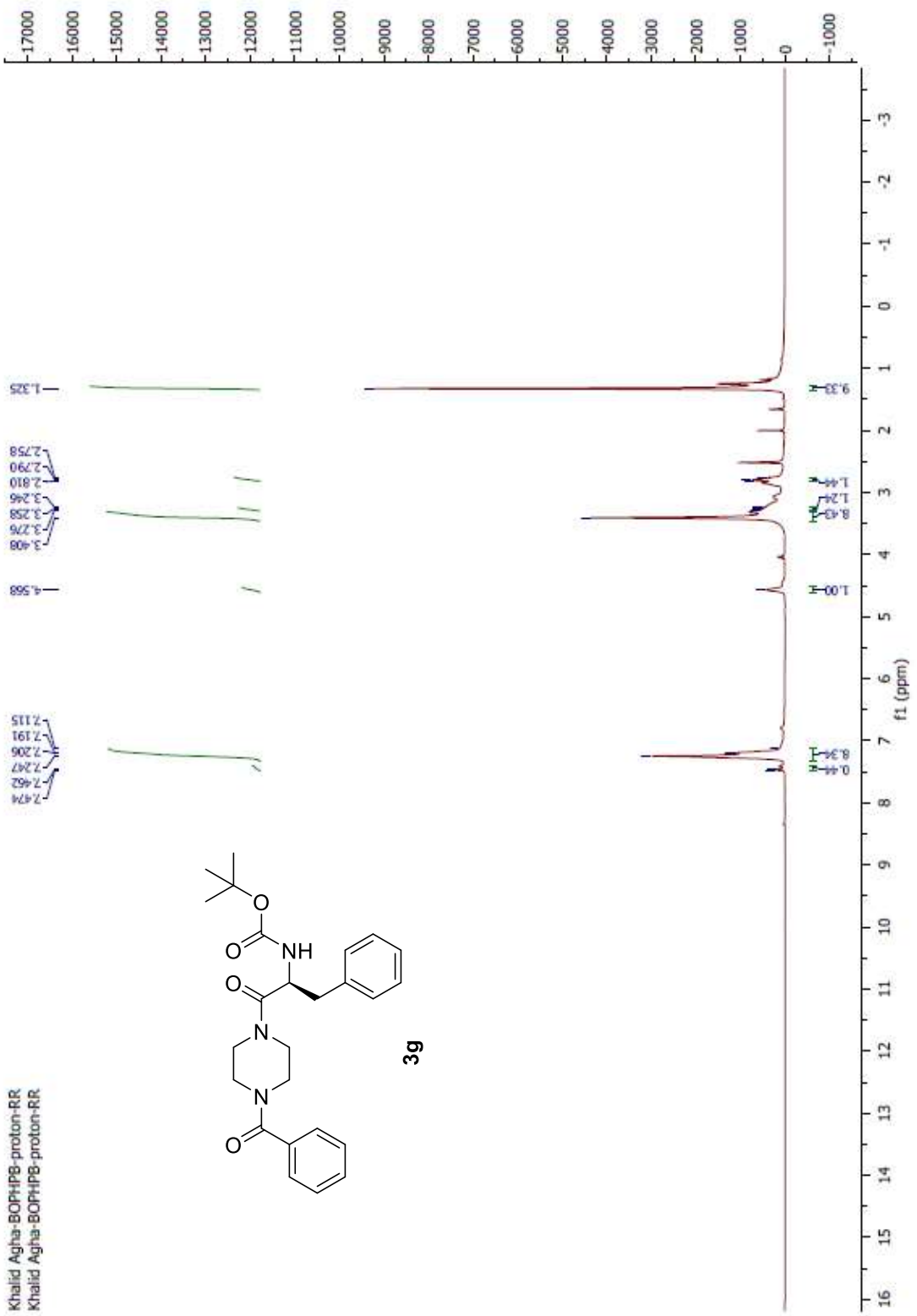

Khalid Agha-BOVPB-carbon-DMSO-AP
Khalid Agha-BOVPB-carbon-DMSO-AP
19.85
28.63
30.19
42.30
45.42
55.51
78.53
127.49
128.91
130.13
136.13
156.01
170.13
3e
f1 (ppm)

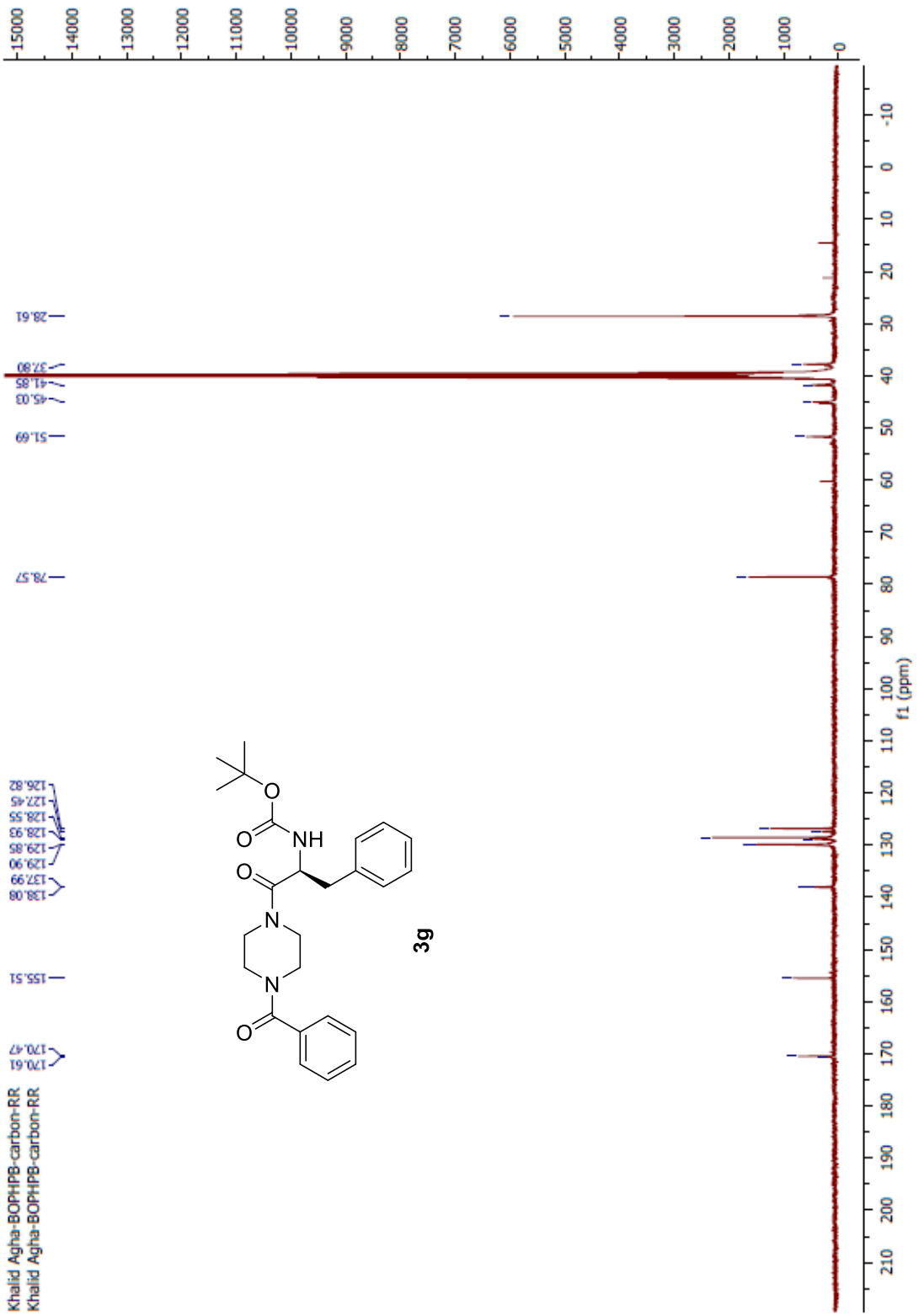

3e
290.18670
390.23950
409.21267
603.82798
Counts vs. Mass-to-Charge (m/z)

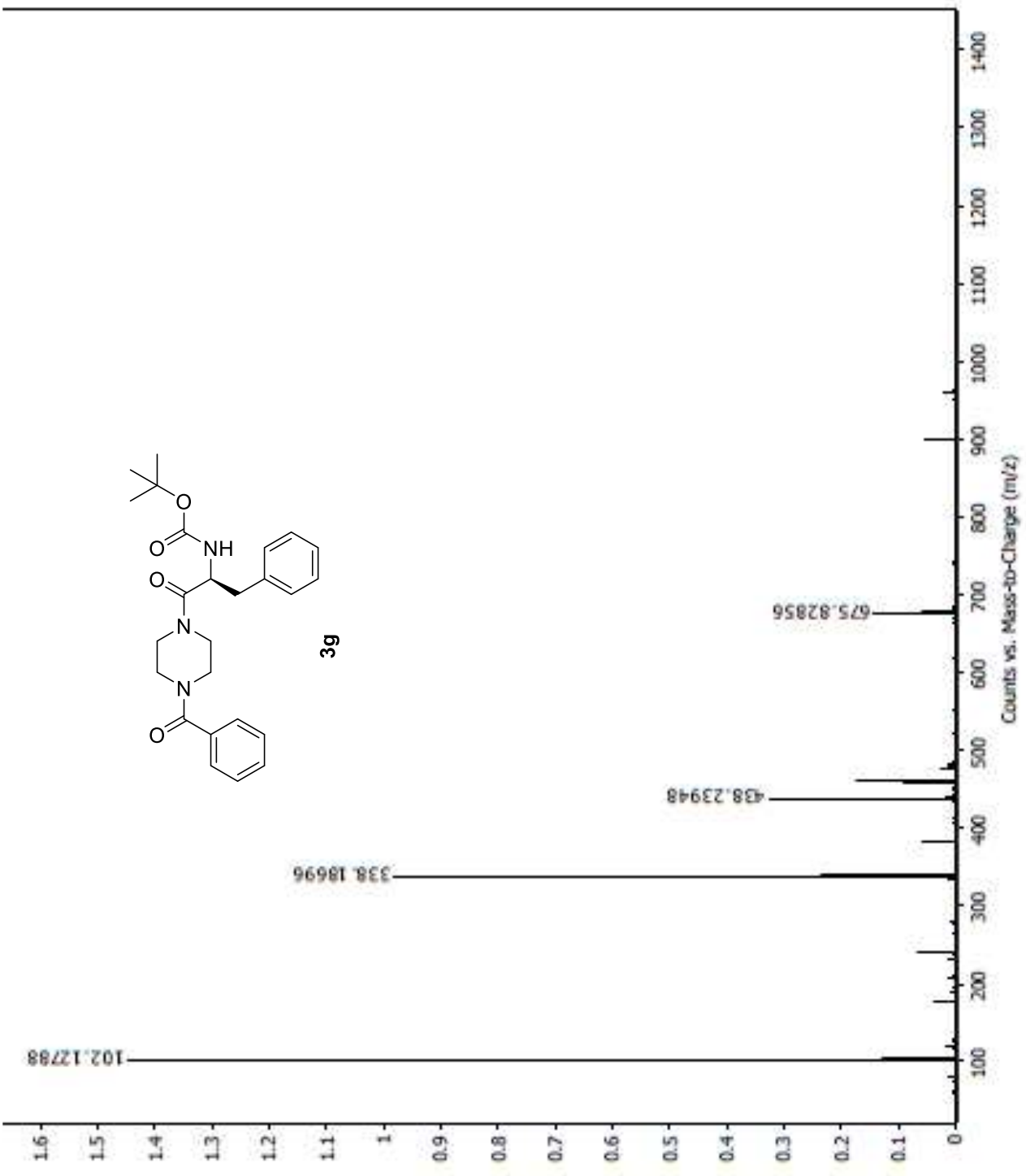

Khalid AbdElHay Agha-BOMPB-DMSO-Hnmr-A
Khalid AbdElHay Agha-BOMPB-DMSO-Hnmr-A
f1 (ppm)
3f

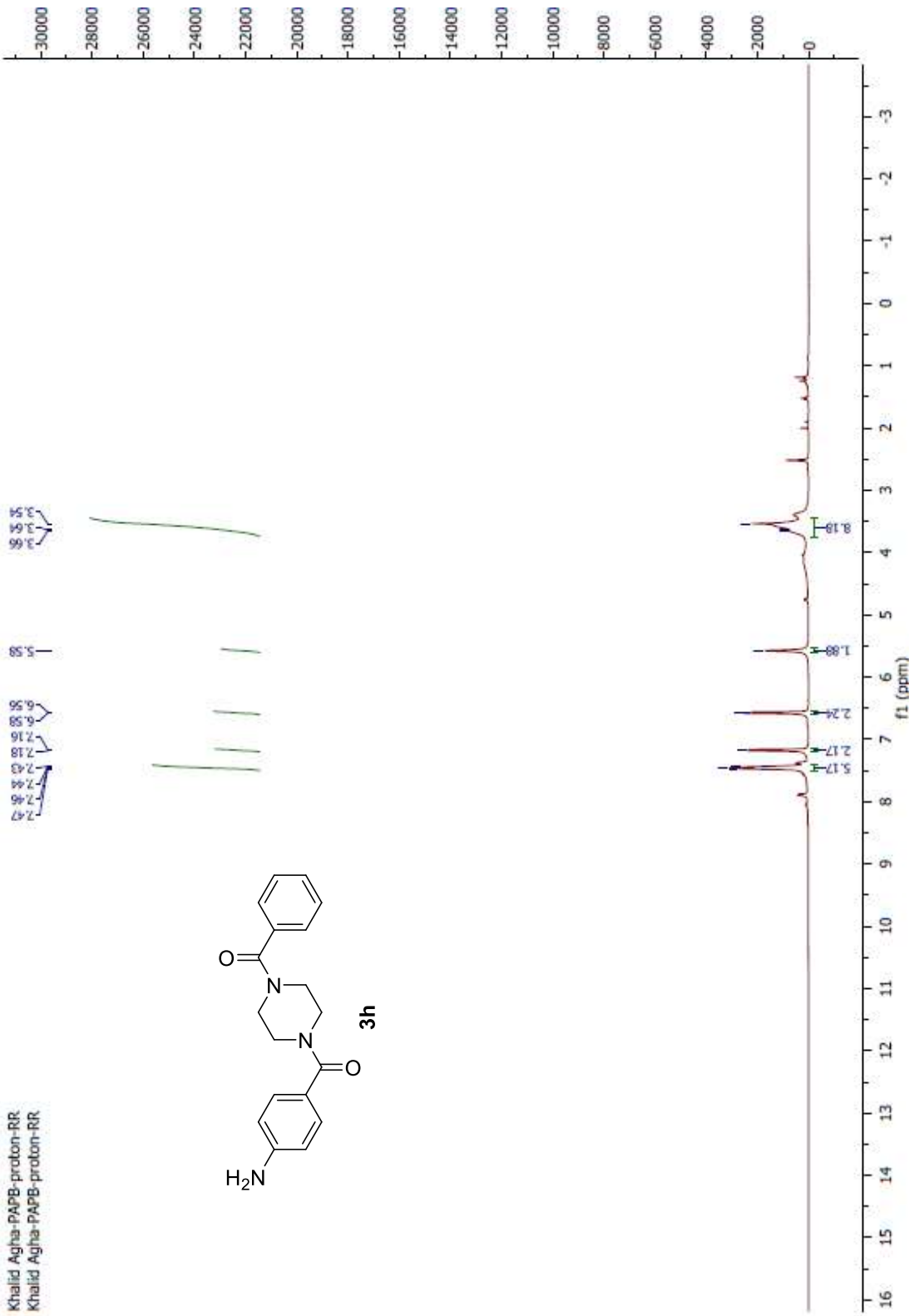

Khalid AbdElHay Agha-BOMPB-DMSO-1Cnmr-A
Khalid AbdElHay Agha-BOMPB-DMSO-2Cnmr-A
15.14
28.64
30.19
31.59
41.95
44.99
49.68
78.65
127.50
128.92
130.14
136.10
155.83
f1 (ppm)
3f

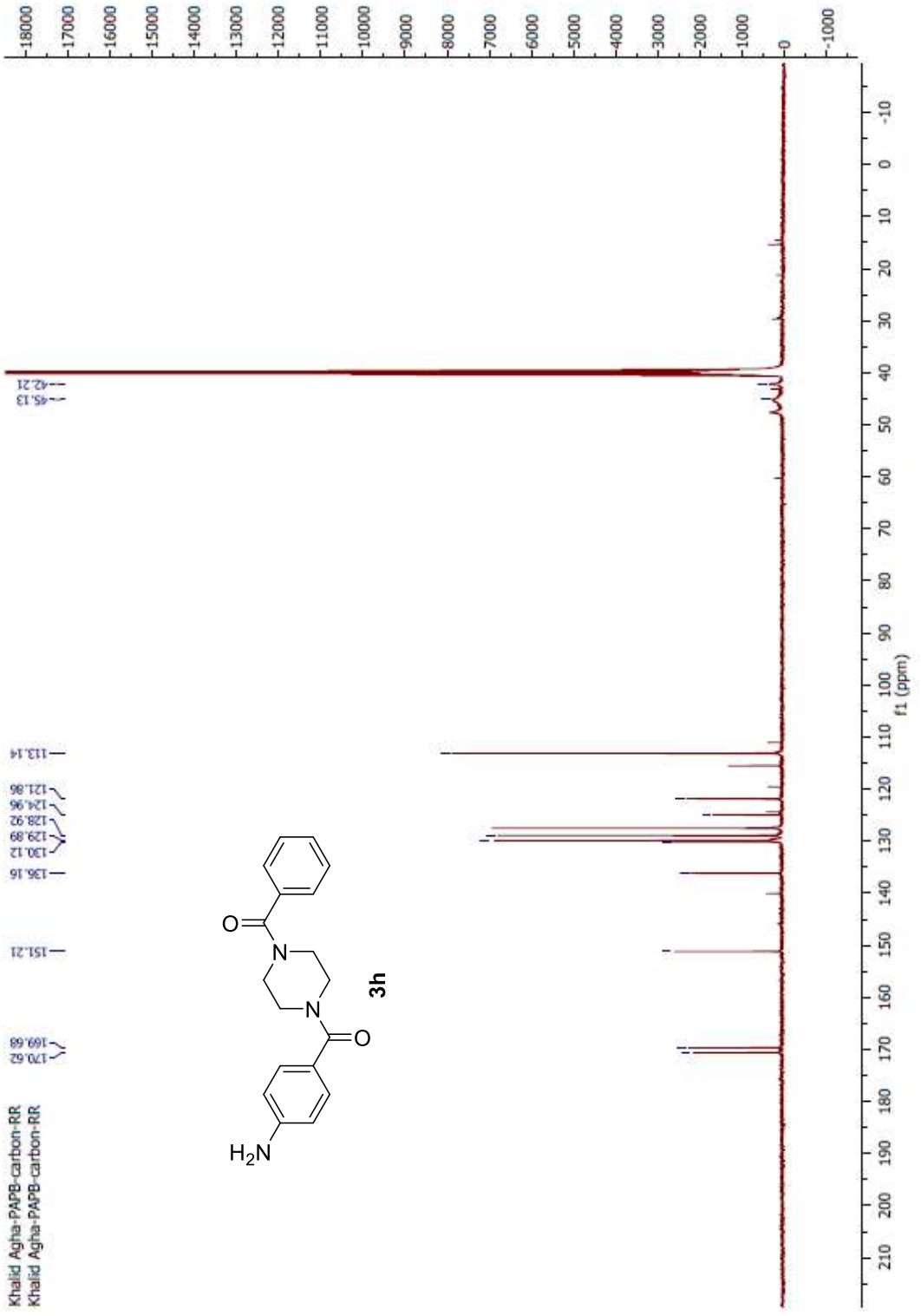

3f
322.15908
651.78676
422.21218
102.12806
Counts vs. Mass-to-Charge (m/z)

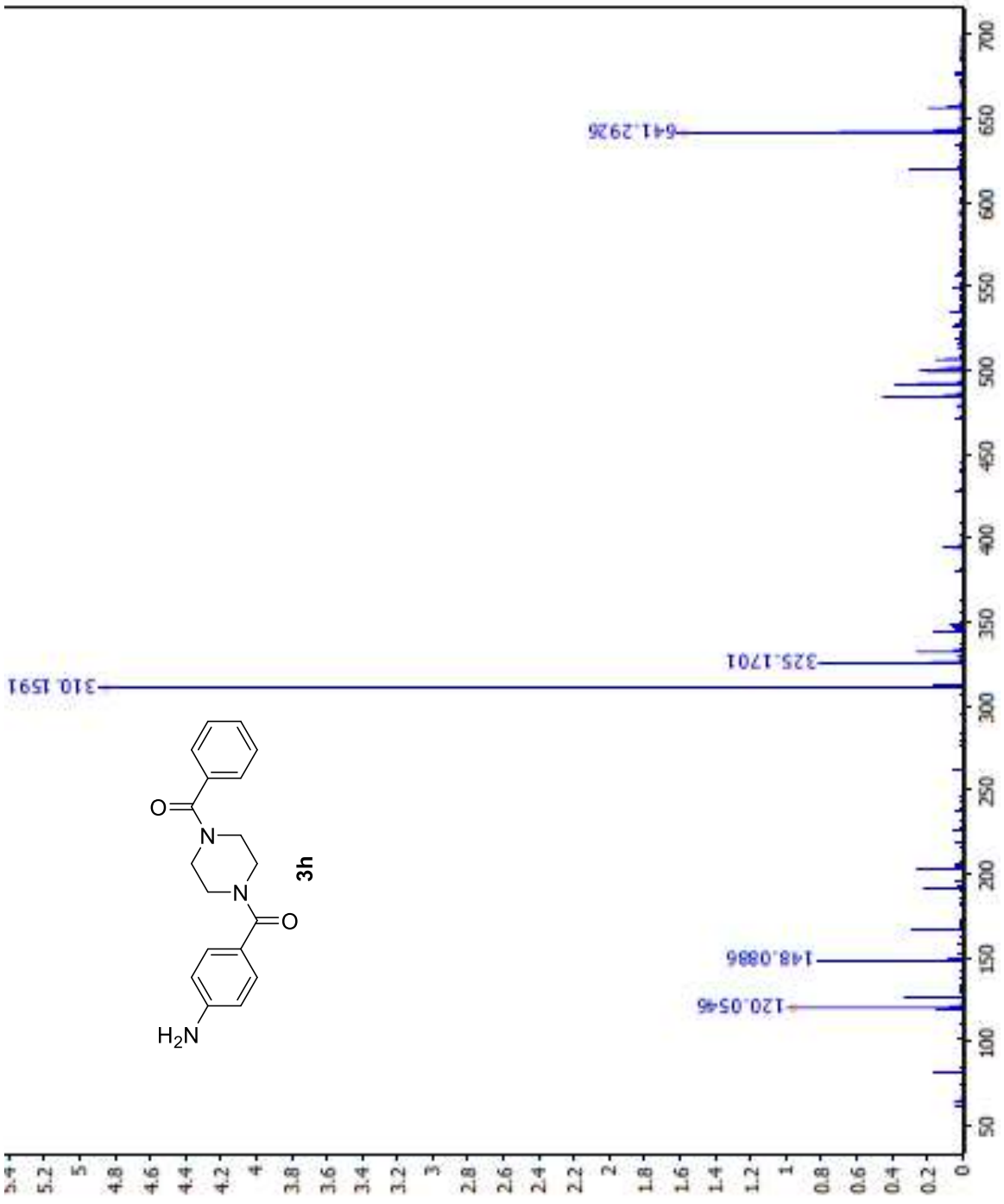

Khalid Agha-BOPHPB-proton-RR
Khalid Agha-BOPHPB-proton-RR
f1 (ppm)
3g

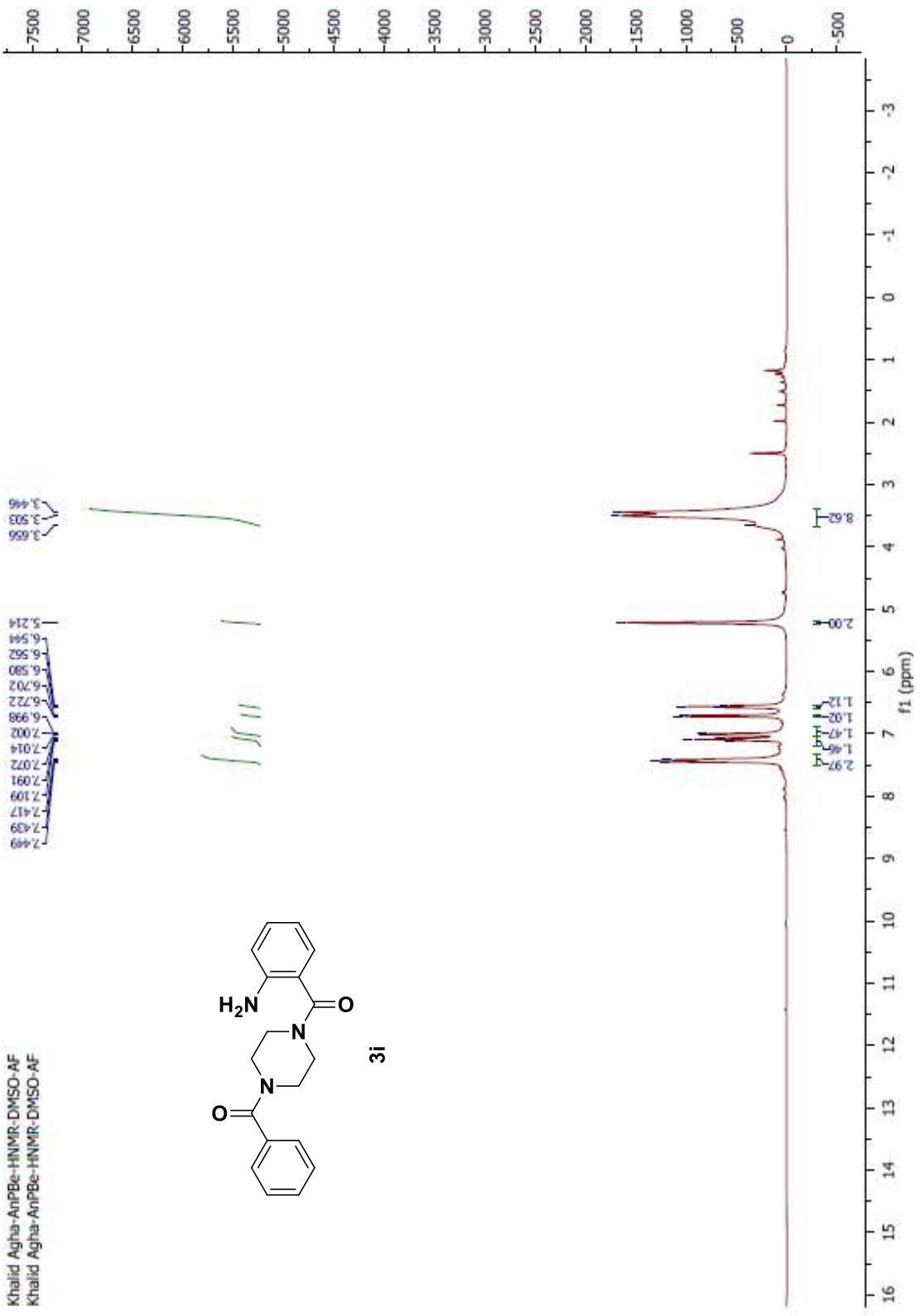

Khalid Agha-BOPHPB-carbon-RR
Khalid Agha-BOPHPB-carbon-RR
170.61
170.47
155.51
138.08
137.99
129.90
129.85
128.93
128.55
127.45
126.82
78.57
51.69
45.03
41.85
37.80
28.61
f1 (ppm)
3g

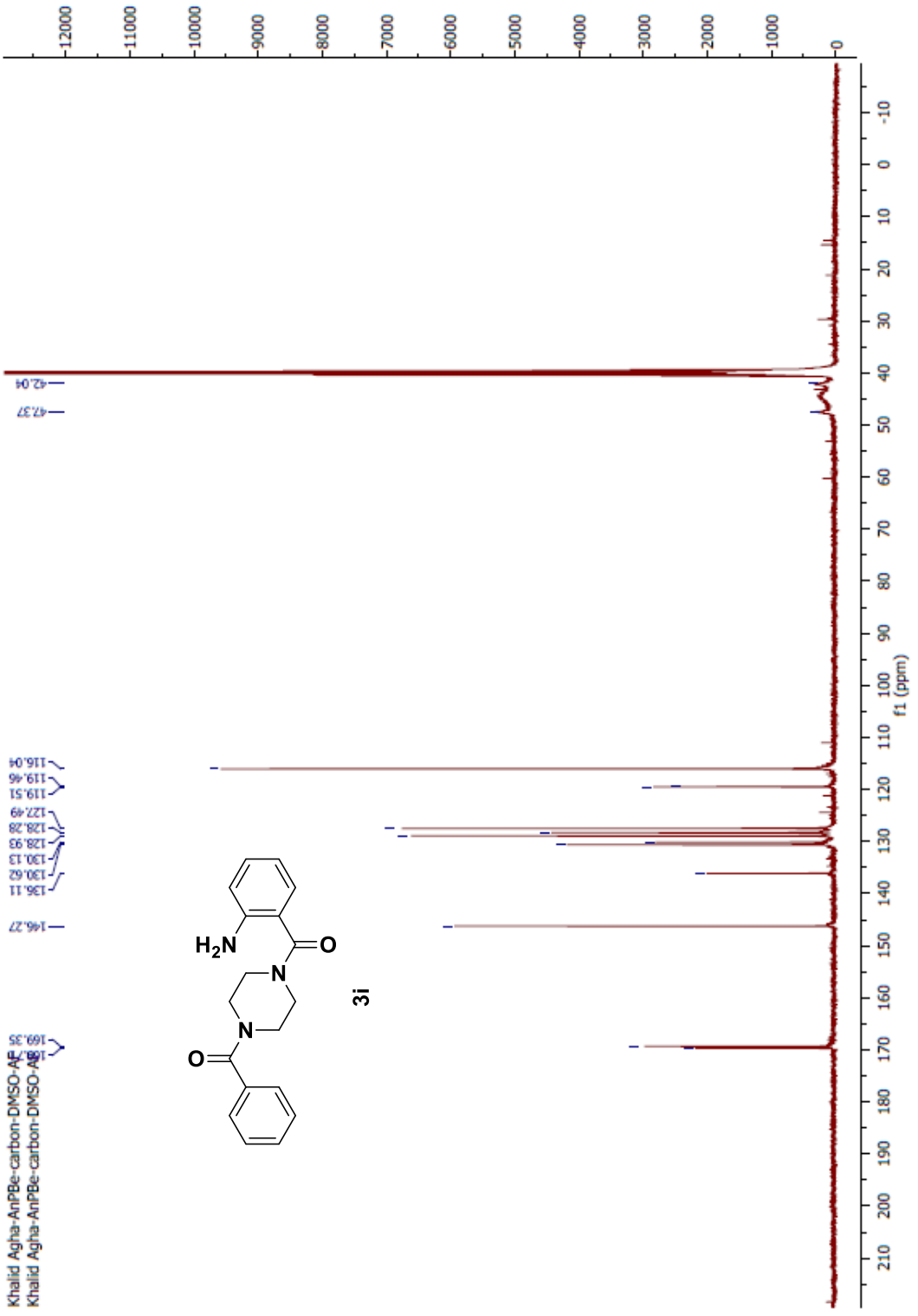

102.12788
338.18696
438.23948
675.82856
Counts vs. Mass-to-Charge (m/z)
3g

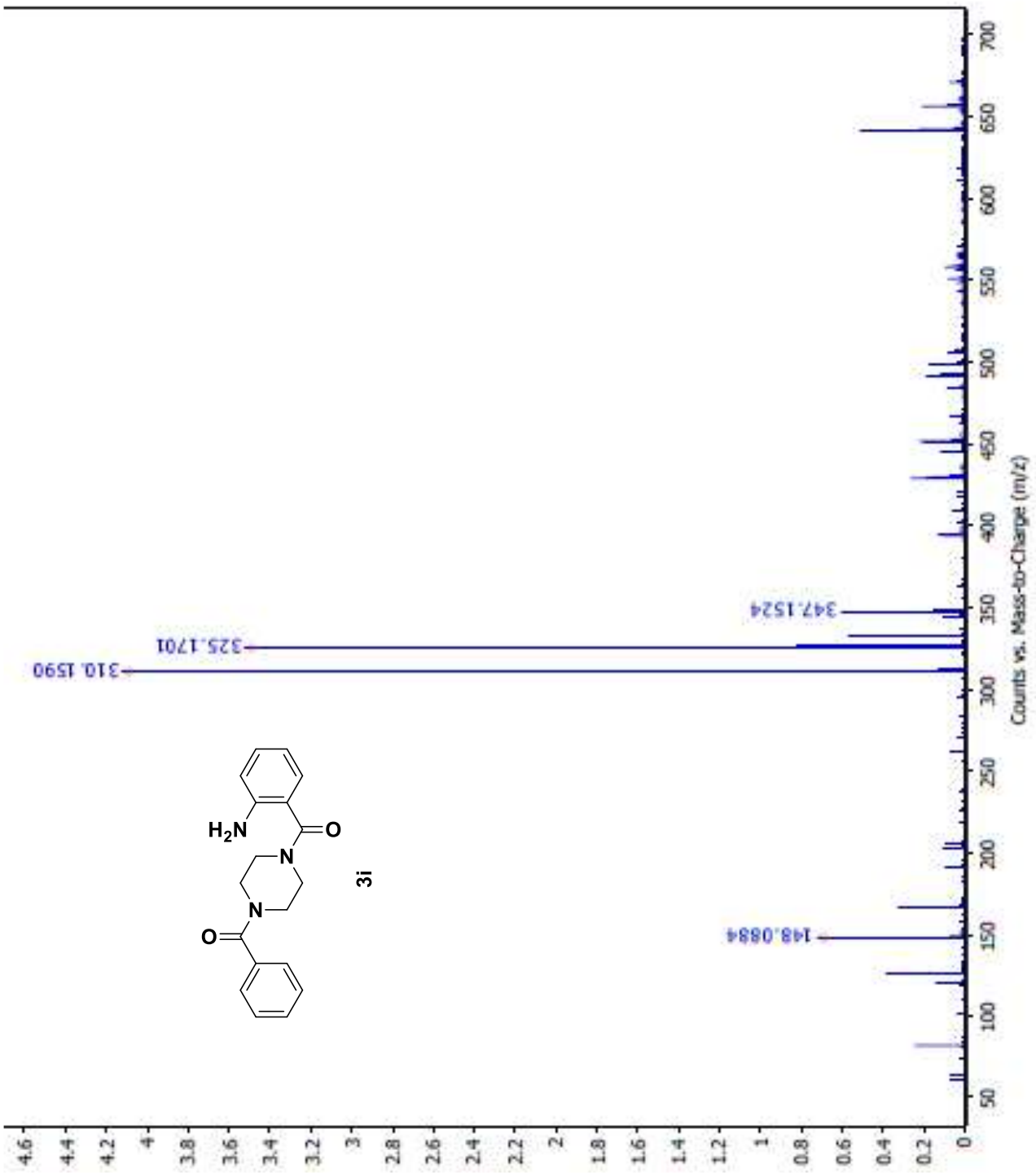

Khalid Agha-PAPB-proton-RR
Khalid Agha-PAPB-proton-RR
3h
f1 (ppm)

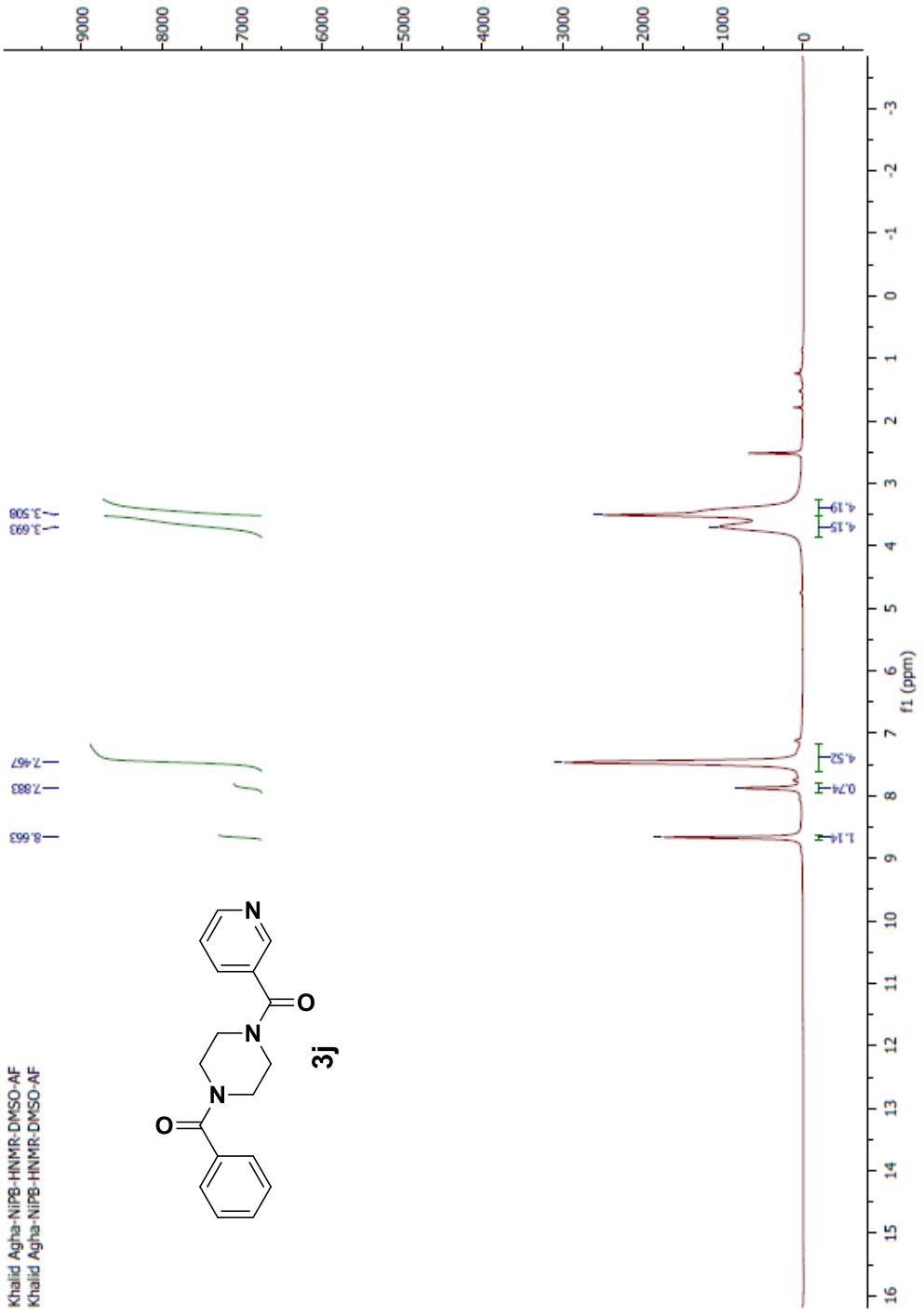

Khalid Agha-PAPB-carbon-RR
Khalid Agha-PAPB-carbon-RR
169.68
170.62
151.21
136.16
130.12
129.89
128.92
124.96
121.86
113.14
45.13
42.21
f1 (ppm)
3h

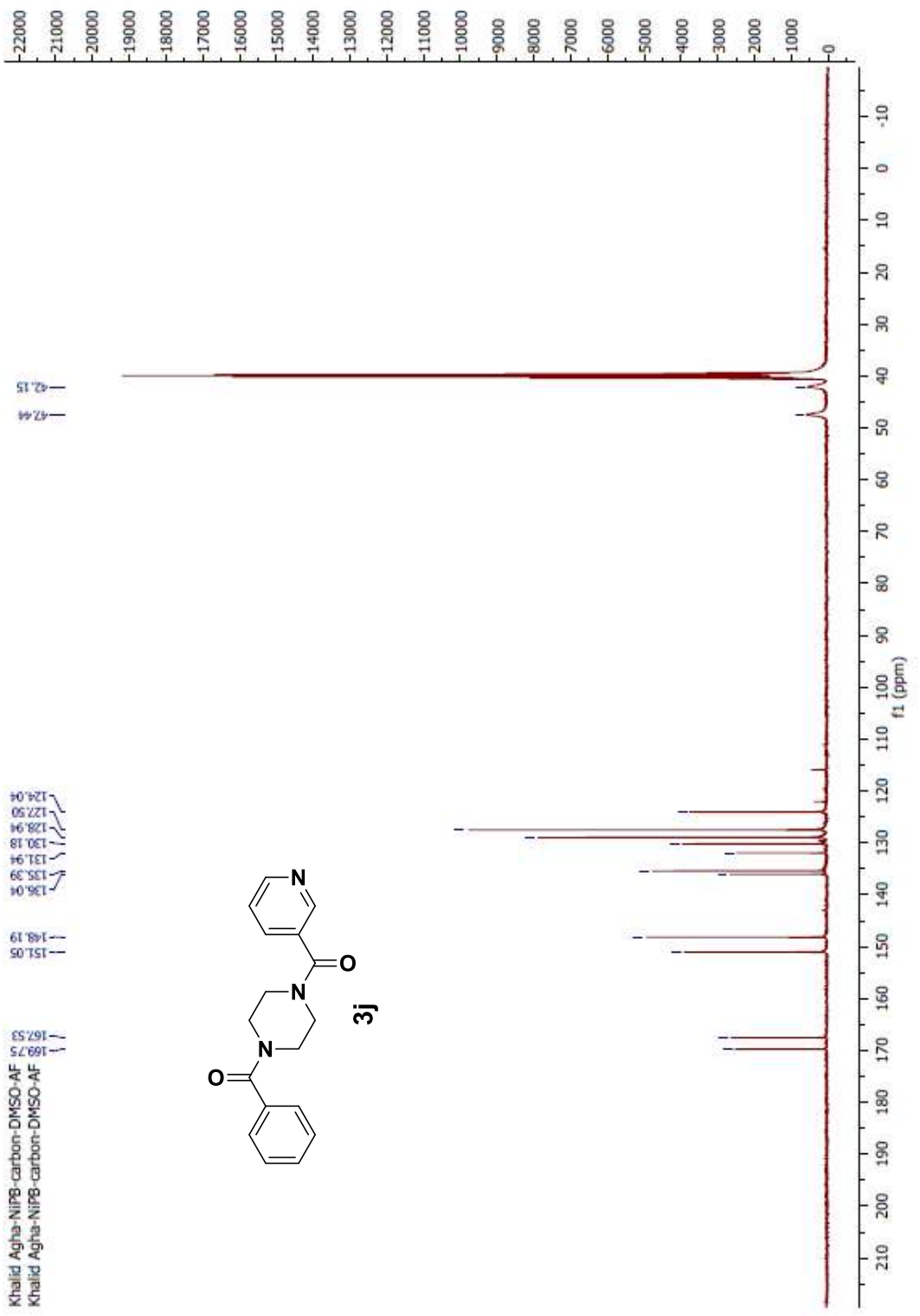

641.2926
325.1701
310.1591
148.0886
120.0546
3h
O
N
N
O
H2N

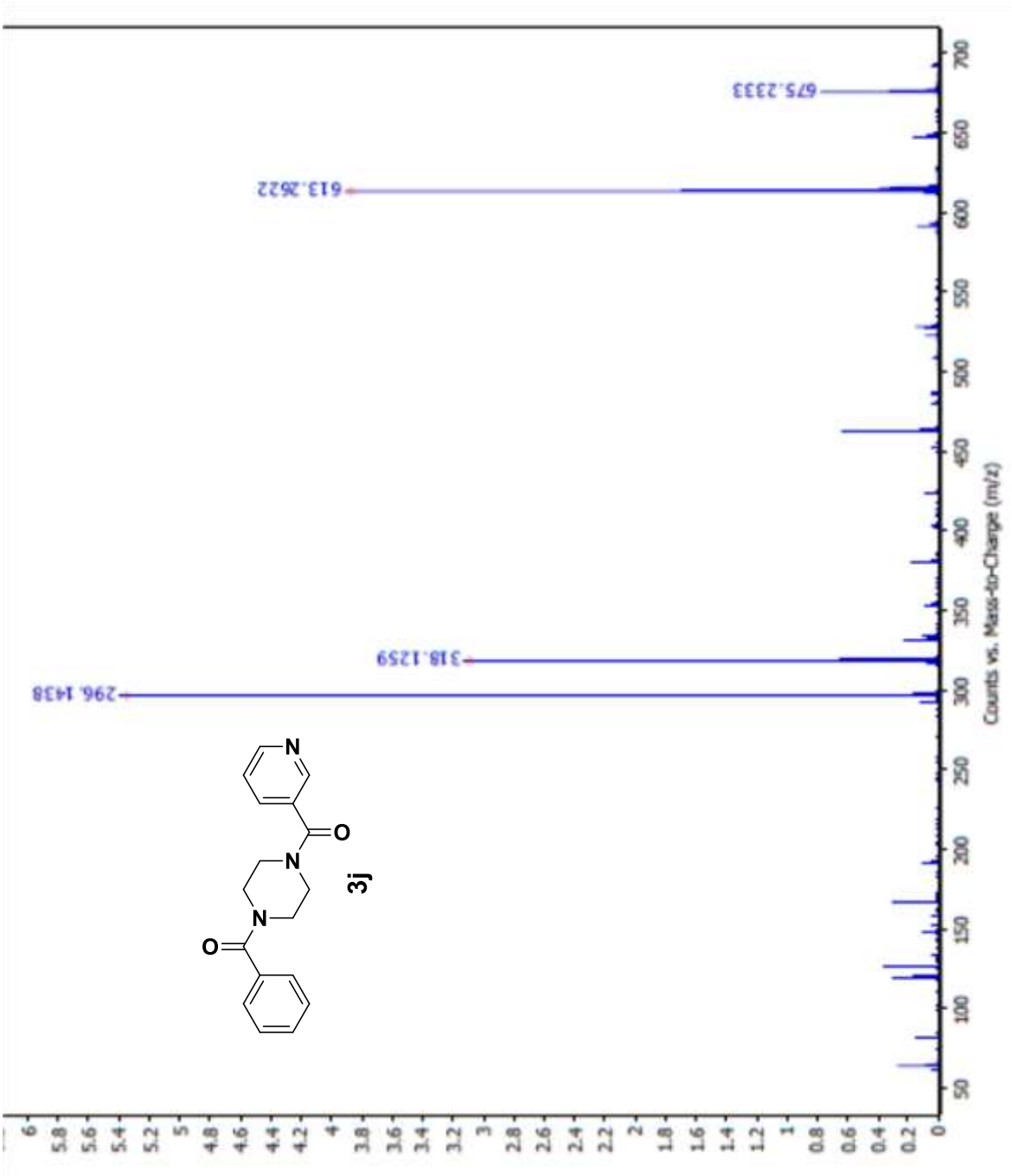

Khalid Agha-AnPBe-HNMR-DMSO-AF
Khalid Agha-AnPBe-HNMR-DMSO-AF
3i
f1 (ppm)


Khalid Agha-AnPBe-carbon-DMSO-AF
Khalid Agha-AnPBe-carbon-DMSO-AF
169.35
146.27
136.11
130.62
130.13
128.93
128.28
127.49
119.51
119.46
116.04
42.04
47.37
f1 (ppm)
3i


310.1590
325.1701
347.1524
148.0884
Counts vs. Mass-to-Charge (m/z)
H2N
O
N
N
O
3i


Khalid Agha-NiPB-HNMR-DMSO-AF
Khalid Agha-NiPB-HNMR-DMSO-AF
8.663
7.883
7.467
3.693
3.508
1.14
0.74
4.52
4.15
4.19
f1 (ppm)
3j


Khalid Agha-NiPB-carbon-DMSO-AF
Khalid Agha-NiPB-carbon-DMSO-AF
169.75
167.53
151.05
148.19
136.04
135.39
131.94
130.18
128.94
127.50
124.04
42.44
42.15
f1 (ppm)
3j


675.2333
613.2622
318.1259
296.1438
Counts vs. Mass-to-Charge (m/z)
3j

3. Pharmacological Screening

3.1 Cell Based Assay

The following codes were used for target compounds during cell based assay.

Code	Target
GPB	3c
APB	3d
VPB	3e
MPB	3f
PBB	3g
PAP	3h
ANP	3i
NPB	3j
DM235	Sunifram (3a)

General

Cell Line cells were obtained from American Type Culture Collection, cells were cultured using DMEM (Invitrogen/Life Technologies) supplemented with 10% FBS (Hyclone,), 10 ug/ml of insulin (Sigma), and 1% penicillin-streptomycin. All of the other chemicals and reagents were from Sigma, or Invitrogen. Plate cells (cells density 1.2 – 1.8 × 10,000 cells/well) in a volume of 100µl complete growth medium + 100 ul of the tested compound per well in a 96-well plate for 24 hours before the MTT assay .

Cell cuture protocol

1. Remove culture medium to a centrifuge tube.

2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin 0.53 mM EDTA solution to remove all traces of serum which contains Trypsin inhibitor.

3. Add 2.0 to 3.0 ml of Trypsin EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).

Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.

4. Add 6.0 to 8.0 mL of complete growth medium and aspirate cells by gently pipetting.

5. Transfer the cell suspension to the centrifuge tube with the medium and cells from step 1, and centrifuge at approximately 125 xg for 5 to 10 minutes. Discard the supernatant.

6. Resuspend the cell pellet in fresh growth medium. Add appropriate aliquots of the cell suspension to new culture vessels.

7. Incubate cultures at 37°C for 24 hrs.

8-After treatment of cells with the serial concentrations of the compound to be tested incubation is carried out for 48 h at 37ºC, then the plates are to be examined under the inverted microscope and proceed for the MTT assay.

3.1.1 MTT – Cytotoxicity assay protocol

The MTT method of monitoring in vitro cytotoxicity is well suited for use with multiwell plates. For best results, cells in the log phase of growth should be employed and final cell number should not exceed 106 cells/cm2. Each test should include a blank containing complete medium without cells.

1. Remove cultures from incubator into laminar flow hood or other sterile work area.

2. Reconstitute each vial of MTT [M-5655] to be used with 3 ml of medium or balanced salt solution without phenol red and serum. Add reconstituted MTT in an amount equal to 10% of the culture medium volume.

3. Return cultures to incubator for 2-4 hours depending on cell type and maximum cell density. (An incubation period of 2 hours is generally adequate but may be lengthened for low cell densities or cells with lower metabolic activity.) Incubation times should beconsistent when making comparisons.

4. After the incubation period, remove cultures from incubator and dissolve the resulting formazan crystals by adding an amount of MTT Solubilization Solution [M-8910] equal to the original culture medium volume.

5. Gentle mixing in a gyratory shaker will enhance dissolution. Occasionally, especially in dense cultures, pipetting up and down [trituration] may be required to completely dissolve the MTT formazan crystals.

6. Spectrophotometrically measure absorbance at a wavelength of 570 nm. Measure the background absorbance of multiwell plates at 690 nm and subtract from the 450 nm measurement. Tests performed in multiwell plates can be read using the appropriate type of plate reader or the contents of individual wells may be transferred to appropriate size cuvets for spectrophotometric measurement.

Detailed results

Dr.Khalid Agha MTT 25-Jul A549

	Blank	CC	Sample No. MPB/A549					Sample No. APB/A549				
	1	2	3	4	5	6	7	8	9	10	11	12
A	B	C	100uM	25uM	6.25uM	1.56uM	0.39uM	100uM	25uM	6.25uM	1.56uM	0.39uM
B	B	C	100uM	25uM	6.25uM	1.56uM	0.39uM	100uM	25uM	6.25uM	1.56uM	0.39uM
C	B	C	100uM	25uM	6.25uM	1.56uM	0.39uM	100uM	25uM	6.25uM	1.56uM	0.39uM

ROBONIK P2000 EIA READER

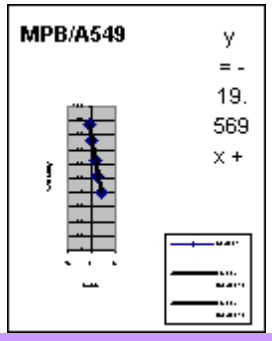
Wave length: 450 nm

Reference: 630 nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	0.454	0.178	0.229	0.282	0.342	0.394	0.203	0.242	0.295	0.343	0.405
B	0.001	0.433	0.183	0.234	0.278	0.336	0.399	0.215	0.254	0.284	0.361	0.416
C	0.001	0.457	0.189	0.236	0.283	0.347	0.386	0.194	0.248	0.296	0.339	0.411
mean	4E-04	0.448	0.1833	0.233	0.281	0.342	0.393	0.204	0.248	0.292	0.3477	0.4107
%		`	40.923	52.01	62.723	76.26	87.723	45.536	55.36	65.1	77.604	91.667

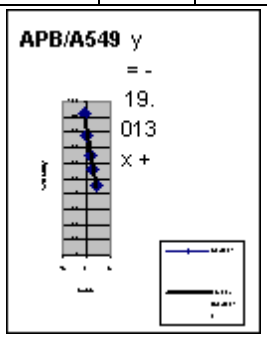
MPB/A549

log conc.	% viability
2	40.92
1.398	52.01
0.796	62.72
0.193	76.26
-0.409	87.72



APB/A549

log conc.	% viability
2	45.54
1.3979	55.36
0.7959	65.1
0.1931	77.6
-0.409	91.67



	Blank	CC	Sample No. ANP/A549					Sample No. PPB/A549				
	1	2	3	4	5	6	7	8	9	10	11	12
A	B	C	100uM	25uM	6.25uM	1.56uM	0.39uM	100uM	25uM	6.25uM	1.56uM	0.39uM
B	B	C	100ug	25uM	6.25uM	1.56uM	0.39uM	100uM	25uM	6.25uM	1.56uM	0.39uM
C	B	C	100ug	25uM	6.25uM	1.56uM	0.39uM	100uM	25uM	6.25uM	1.56uM	0.39uM

ROBONIK P2000 EIA READER

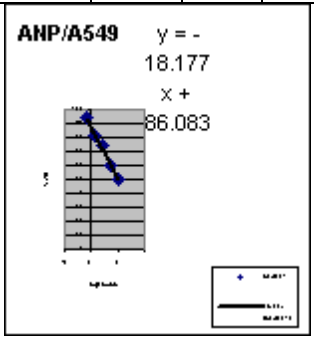
Wave length: 450 nm

Reference: 630 nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	0.462	0.229	0.271	0.338	0.364	0.418	0.171	0.242	0.311	0.357	0.392
B	0.001	0.454	0.218	0.267	0.325	0.363	0.427	0.179	0.238	0.306	0.352	0.388
C	0.001	0.435	0.226	0.269	0.331	0.369	0.423	0.185	0.245	0.314	0.349	0.384
mean	0.001	0.45	0.2243	0.269	0.3313	0.365	0.4227	0.1783	0.242	0.31	0.3527	0.388
% viability			49.815	59.73	73.575	81.13	93.856	39.6	53.66	68.91	78.312	86.158

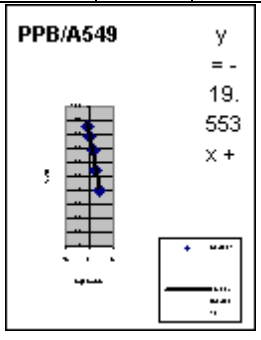
ANP/A549

log conc.	% viability



PPB/A549

log conc.	% viability



2	49.81
1.398	59.73
0.796	73.58
0.193	81.13
-0.409	93.86

2	39.6
1.3979	53.66
0.7959	68.91
0.1931	78.31
-0.409	86.16

	Blank	CC	Sample No. VPB/A549					Sample No. GPB/A549				
	1	2	3	4	5	6	7	8	9	10	11	12
A	B	C	100uM	25uM	6.25uM	1.56uM	0.39uM	100uM	25uM	6.25uM	1.56uM	0.39uM
B	B	C	100ug	25uM	6.25uM	1.56uM	0.39uM	100uM	25uM	6.25uM	1.56uM	0.39uM
C	B	C	100ug	25uM	6.25uM	1.56uM	0.39uM	100uM	25uM	6.25uM	1.56uM	0.39uM

ROBONIK P2000 EIA READER

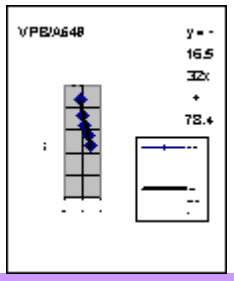
Wave length: 450 nm

Reference: 630 nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	0.475	0.213	0.261	0.298	0.342	0.398	0.229	0.282	0.342	0.394	0.447
B	0.003	0.447	0.222	0.252	0.289	0.338	0.408	0.234	0.278	0.336	0.399	0.452
C	0.001	0.461	0.208	0.254	0.297	0.336	0.401	0.236	0.283	0.347	0.386	0.443
mean	0.002	0.461	0.2143	0.256	0.2947	0.339	0.4023	0.233	0.281	0.342	0.393	0.4473
% viability			46.493	55.46	63.919	73.46	87.274	50.542	60.95	74.11	85.249	97.035

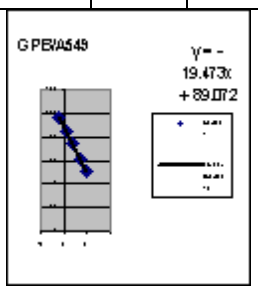
VPB/A549

2	46.49
1.398	55.46
0.796	63.92
0.193	73.46
-0.409	87.27



GPB/A549

2	50.54
1.3979	60.95
0.7959	74.11
0.1931	85.25
-0.409	97.04



	Blank	CC	Sample No. PAP/A549					Sample No. NPB/A549				
	1	2	3	4	5	6	7	8	9	10	11	12
A	B	C	100uM	25uM	6.25uM	1.56uM	0.39uM	100uM	25uM	6.25uM	1.56uM	0.39uM
B	B	C	100ug	25uM	6.25uM	1.56uM	0.39uM	100uM	25uM	6.25uM	1.56uM	0.39uM
C	B	C	100ug	25uM	6.25uM	1.56uM	0.39uM	100uM	25uM	6.25uM	1.56uM	0.39uM

ROBONIK P2000 EIA READER

Wave length: 450 nm

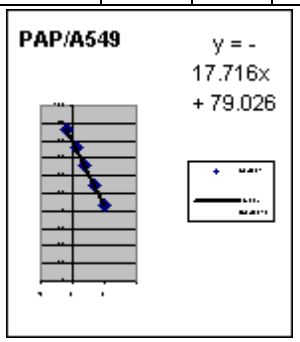
Reference: 630 nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	0.427	0.184	0.234	0.282	0.327	0.375	0.189	0.255	0.293	0.342	0.385
B	0.001	0.432	0.193	0.239	0.289	0.335	0.376	0.201	0.263	0.294	0.347	0.392
C	0.001	0.455	0.188	0.244	0.292	0.331	0.377	0.184	0.262	0.294	0.338	0.385
mean	0.001	0.438	0.1883	0.239	0.2877	0.331	0.376	0.1913	0.26	0.294	0.3423	0.3873

% viability			42.998	54.57	65.677	75.57	85.845	43.683	59.36	67.05	78.158	88.432

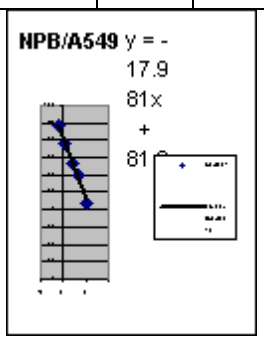
PAP/A549

log conc.	% viability
2	43
1.398	54.57
0.796	65.68
0.193	75.57
-0.409	85.84



NPB/A549

log conc.	% viability
2	43.68
1.3979	59.36
0.7959	67.05
0.1931	78.16
-0.409	88.43



	Blank	CC	Sample No.		DM235/A549			Sample No.				
	1	2	3	4	5	6	7	8	9	10	11	12
A	B	C	100uM	25uM	6.25uM	1.56uM	0.39uM					
B	B	C	100ug	25uM	6.25uM	1.56uM	0.39uM					
C	B	C	100ug	25uM	6.25uM	1.56uM	0.39uM					

ROBONIK P2000 EIA READER

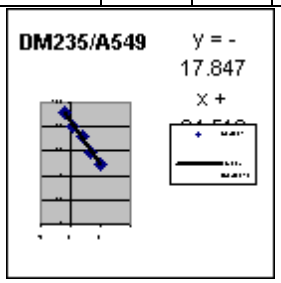
Wave length: 450 nm

Reference: 630 nm

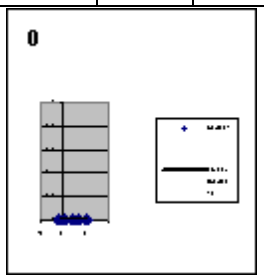
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	0.466	0.229	0.271	0.338	0.364	0.418					
B	0.003	0.439	0.218	0.267	0.325	0.363	0.427					
C	0.001	0.471	0.226	0.269	0.331	0.369	0.423					
mean	0.002	0.459	0.2243	0.269	0.3313	0.365	0.4227	0	0	0	0	0
% viability			48.91	58.65	72.238	79.65	92.151	0	0	0	0	0

DM235/A549

2	48.91
1.398	58.65
0.796	72.24
0.193	79.65
-0.409	92.15



2	0
1.3979	0
0.7959	0
0.1931	0
-0.409	0



3.1.2 *In vitro* acetyl Choline release assay

The relative amount of ACh released in response to the synthesized Targets **3a** and **3c-j** was determined in A549 lung adenocarcinoma cells by Song et al protocol as follow:

1. A549 cells were grown in 5 ml serum-free RPMI (SF-RPMI) to 90 % confluence in 60 mm cell culture dishes in a cell culture incubator set at 5 % CO_2 and 37 °C
2. On the day of the assay, 100 µM neostigmine (a chemical inhibitor of AChE in cells) was added to each plate for four hours at 37 °C. The plate contained 3 ml of media.
3. Four hours after the addition of neostigmine, the relevant concentration of test compunds **3a** and **3c-j** (1/2 IC_{50}) were added and the cells were incubated at 37 °C for 36 hrs.
4. The supernatant (medium) was collected and spun at 800 × g.
5. The supernatants were frozen at -80 °C and then lyophilized. The lyophilizer was set to a pressure of 10 micron Hg or below that value. The samples were lyophilized overnight.
6. Subsequently, the lyophilizate was reconstituted with 500 µl autoclaved distilled water, snap frozen in liquid nitrogen and stored at -80 °C for analysis.

The amount of ACh in the sample was measured using the Choline/acetylcholine Quantification Kit (BioVisio. Catalog #K615-100)

Detailed results

Ach STANDARDS	Conc. Pg/ml
St.1	500
St.2	400
St.3	300
St.4	200
St.5	100

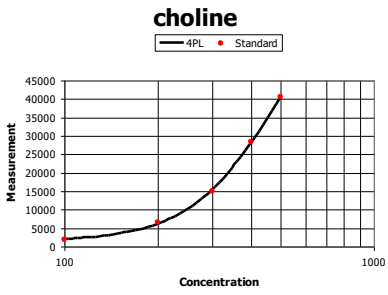


Plate map

	1	2	3	4	5	6	7	8	9	10	11	12
A	st1	MPB	ANP	GPB	DM235	MPB	ANP	GPB	DM235	-	-	-
B	st2	MPB	PPB	GPB	DM235	MPB	PPB	GPB	DM235	-	-	-
C	st3	MPB	PPB	PAP	DM235	MPB	PPB	PAP	DM235	-	-	-
D	st4	APB	PPB	PAP	cont	APB	PPB	PAP	cont	-	-	-
E	st5	APB	VPB	PAP	cont	APB	VPB	PAP	cont	-	-	-
F	st0	APB	VPB	NPB	cont	APB	VPB	NPB	cont	-	-	-
G	B	ANP	VPB	NPB	-	ANP	VPB	NPB	-	-	-	-

H	B	ANP	GPB	NPB	-	ANP	GPB	NPB	-	-	-	-

Samples OD results Total (+ACHE) Free(+neostagmine)

	1	2	3	4	5	6	7	8	9	10	11	12
A	40895	**26942**	**22154**	29262	**29182**	8136	9913	18155	11261	0	0	0
B	28736	**29161**	24362	28356	**28743**	8307	14572	18436	11554	0	0	0
C	15471	**28413**	26139	**26935**	**28519**	7992	15191	13056	11359	0	0	0
D	6944	25861	24941	**21773**	17642	9421	14757	12814	11459	0	0	0
E	2316	25918	**26829**	**22248**	18091	9197	17132	13461	11913	0	0	0
F	612	24796	**27914**	20157	17784	9318	16328	9463	10118	0	0	0
G	322	**22753**	**25775**	19554	0	9642	16819	9921	0	0	0	0
H	249	**21667**	28136	18943	0	9755	18316	9704	0	0	0	0

Calibrator	Wells	Conc.	Raw (Corrected)	Backfit	Recovery %
Standard1	A1	500	40500	499.4	99.88
Standard2	B1	400	28300	401.9	100.5
Standard3	C1	300	15100	296.6	98.86
Standard4	D1	200	6550	205.3	102.7
Standard5	E1	100	1920	91.1	91.1

Sample	Wells	Raw	Background Corrected	Conc.	Conc. (Average)	%CV	SD	SEM
Control1-T	D5	17600	17400	315.1	316.7	0.601	1.9	1.1
	E5	18100		318.8				
	F5	17800		316.3				
Control-F	D9	11500	10800	258.9	255.7	3.83	9.78	5.65
	E9	11900		263.4				
	F9	10100		244.7				
MPB (T)	A2	26900	27800	388.2	397.6	2.17	8.62	4.98
	B2	29200		405.1				
	C2	28400		399.4				
APB (T)	D2	25900	25100	379.9	377.4	1.28	4.84	2.79
	E2	25900		380.4				
	F2	24800		371.8				
ANP (T)	A3	22200	21800	351.4	351.6	1.21	4.24	2.45
	G2	22800		356				
	H2	21700		347.5				
PPB (T)	B3	24400	24800	368.4	374.5	1.85	6.94	4.01
	C3	26100		382.1				
	D3	24900		372.9				
VPB (T)	E3	26800	26400	387.3	387.4	2.11	8.17	4.72
	F3	27900		395.6				
	G3	25800		379.3				
GPB (T)	A4	29300	28200	405.9	400.7	1.14	4.56	2.63
	B4	28400		399				
	H3	28100		397.3				

PAP (T)	C4	26900	23300	388.1	362.9	6.05	22	12.7
	D4	21800		348.4				
	E4	22200		352.1				
NPB (T)	F4	20200	19200	335.6	330.7	1.48	4.89	2.82
	G4	19600		330.8				
	H4	18900		325.8				
DM235(Ref) (T)	A5	29200	28400	405.3	402.5	0.641	2.58	1.49
	B5	28700		401.9				
	C5	28500		400.2				
MPB (F)	A6	8140	7750	221.4	221.5	0.903	2	1.15
	B6	8310		223.5				
	C6	7990		219.5				
APB (F)	D6	9420	8920	236.9	235.6	0.551	1.3	0.75
	E6	9200		234.3				
	F6	9320		235.7				
ANP (F)	A7	9910	9380	242.4	240.8	0.634	1.53	0.882
	G6	9640		239.4				
	H6	9760		240.6				
PPB (F)	B7	14600	14400	288.6	291	0.974	2.83	1.64
	C7	15200		294.1				
	D7	14800		290.2				
VPB (F)	E7	17100	16400	310.8	307.7	1.12	3.44	1.99
	F7	16300		304				
	G7	16800		308.2				
GPB (F)	A8	18200	17900	319.4	320.6	0.362	1.16	0.67
	B8	18400		321.7				
	H7	18300		320.7				
PAP (F)	C8	13100	12700	274.6	275.1	1.13	3.1	1.79
	D8	12800		272.3				
	E8	13500		278.4				
NPB (F)	F8	9460	9300	237.3	240	1.08	2.59	1.49
	G8	9920		242.5				
	H8	9700		240.1				
DM235(Ref) (F)	A9	11300	11000	256.8	258.2	0.591	1.52	0.88
	B9	11600		259.8				
	C9	11400		257.8				
Blank	F1	612	0	< Curve	-	-	-	-
	G1	322		< Curve				
	H1	249		< Curve				

	choine					
sample	**Total**	**Free**	**ACH**		%	EC
MPB	397.6	221.5	176.1		2.886885	61
APB	377.4	235.6	141.8		2.32459	61
ANP	351.6	240.8	110.8		1.816393	61
PPB	374.5	291	83.5		1.368852	61

VPB	387.4	307.7	79.7		1.306557	61
GPB	400.7	320.6	80.1		1.313115	61
PAP	362.9	275.1	87.8		1.439344	61
NPB	330.7	240	90.7		1.486885	61
DM235(Ref)	402.5	258.2	144.3		2.365574	61
control	316.7	255.7	61		1	61

3.2 *In vitro* AChE inhibition assay.

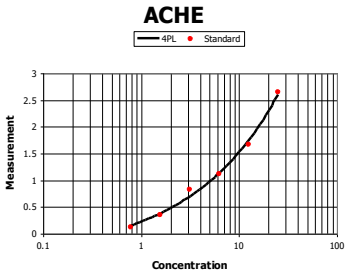
All chemicals required for the AChE inhibition assay were obtained from Sigma-Aldrich. The assay was performed according to Ellman's method.

3.3 *Ex-vivo* AChE inhibition assay (for target 3f)

Detailed results

s	Group	code	WT	dose ug/kg	Rat Brain ACHE ng/ml
1	control	1c	172	---	2.148
2		2c	155		2.778
3		3c	203		2.31
4		4c	169		2.847
5		5c	156		2.406
6	MPB (**3f**)	1s	218	10	1.39
7		2s	167		1.297
8		3s	188		1.196
9		4s	175		1.339
10		5s	184		1.107
11	Tacrine	1r	169	10	1.237
12		2r	176		1.108
13		3r	181		1.069
14		4r	206		1.115
15		5r	186		1.157

ACHE st	ng/ml
st1	25
st2	12.5
st3	6.25
st4	3.13
st5	1.56
st6	0.78



	1	2	3	4	5	6	7	8	9	10	11	12
A	st1	1c	5c	4s	3r	--	--	--	--	--	--	--
B	st2	1c	5c	4s	3r	--	--	--	--	--	--	--
C	st3	2c	1s	5s	4r	--	--	--	--	--	--	--
D	st4	2c	1s	5s	4r	--	--	--	--	--	--	--

E	st5	3c	2s	1r	5r	--	--	--	--	--	--	--
F	st6	3c	2s	1r	5r	--	--	--	--	--	--	--
G	B	4c	3s	2r	--	--	--	--	--	--	--	--
H	B	4c	3s	2r	--	--	--	--	--	--	--	--

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.664	0.543	0.594	0.338	0.257	0	0	0	0	0	0	0
B	1.685	0.522	0.575	0.351	0.281	0	0	0	0	0	0	0
C	1.139	0.661	0.364	0.268	0.273	0	0	0	0	0	0	0
D	0.842	0.648	0.352	0.292	0.292	0	0	0	0	0	0	0
E	0.371	0.572	0.339	0.323	0.304	0	0	0	0	0	0	0
F	0.145	0.559	0.328	0.311	0.285	0	0	0	0	0	0	0
G	0.018	0.671	0.295	0.288	0	0	0	0	0	0	0	0
H	0.024	0.663	0.316	0.273	0	0	0	0	0	0	0	0

Calibrator	Wells	Conc.	Raw (Corrected)	Backfit	Recovery %
Standard1	A1	25	2.64	25.63	102.5
Standard2	B1	12.5	1.66	11.5	92.03
Standard3	C1	6.25	1.12	6.114	97.82
Standard4	D1	3.125	0.821	3.91	125.1
Standard5	E1	1.563	0.35	1.441	92.21
Standard6	F1	0.7813	0.124	0.6929	88.69

Sample	Wells	Raw	Background Corrected	Conc.	Conc. (Average)	%CV	SD	SEM
1c	A2	0.543	0.512	2.198	2.148	3.34	0.0716	0.0507
	B2	0.522		2.097				
2c	C2	0.661	0.634	2.813	2.778	1.82	0.0506	0.0358
	D2	0.648		2.742				
3c	E2	0.572	0.545	2.342	2.31	1.99	0.046	0.0326
	F2	0.559		2.277				
4c	G2	0.671	0.646	2.869	2.847	1.11	0.0316	0.0223
	H2	0.663		2.825				
5c	A3	0.594	0.563	2.455	2.406	2.86	0.0687	0.0486
	B3	0.575		2.357				
1s	C3	0.364	0.337	1.413	1.39	2.35	0.0327	0.0231
	D3	0.352		1.367				
2s	E3	0.339	0.313	1.318	1.297	2.23	0.0289	0.0205
	F3	0.328		1.277				
3s	G3	0.295	0.285	1.158	1.196	4.43	0.053	0.0375
	H3	0.316		1.233				
4s	A4	0.338	0.324	1.314	1.339	2.6	0.0348	0.0246
	B4	0.351		1.363				
5s	C4	0.268	0.259	1.065	1.107	5.26	0.0582	0.0411
	D4	0.292		1.148				

1r	E4	0.323	0.296	1.259	1.237	2.49	0.0308	0.0218
	F4	0.311		1.215				
2r	G4	0.288	0.259	1.134	1.108	3.28	0.0364	0.0257
	H4	0.273		1.082				
3r	A5	0.257	0.248	1.029	1.069	5.35	0.0572	0.0404
	B5	0.281		1.11				
4r	C5	0.273	0.261	1.082	1.115	4.15	0.0462	0.0327
	D5	0.292		1.148				
5r	E5	0.304	0.273	1.19	1.157	4.07	0.0471	0.0333
	F5	0.285		1.123				
Blank	G1	0.018	0	0.3938	0.3999	2.16	0.00862	0.0061
	H1	0.024		0.406				

In vitro hepatotoxicity screening

researcher	assay	Date	cells	
Dr.Khaled Agha	MTT	03-Mar		THLE2

	Blank	CC	Sample No. 3F/THLE2					Sample No. 3i/THLE2				
	1	2	3	4	5	6	7	8	9	10	11	12
A	B	C	100ug	25ug	6.3ug	1.6ug	0.4ug	100ug	25ug	6.3ug	1.6ug	0.4ug
B	B	C	100ug	25ug	6.3ug	1.6ug	0.4ug	100ug	25ug	6.3ug	1.6ug	0.4ug
C	B	C	100ug	25ug	6.3ug	1.6ug	0.4ug	100ug	25ug	6.3ug	1.6ug	0.4ug

ROBONIK P2000 Eia reader

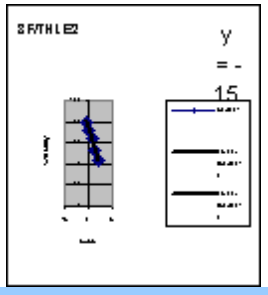
Wave length: 450 nm

Reference: 630 nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	0.539	0.244	0.292	0.352	0.389	0.442	0.243	0.275	0.319	0.355	0.418
B	0.001	0.554	0.235	0.303	0.349	0.393	0.439	0.257	0.269	0.321	0.357	0.405
C	0.001	0.567	0.239	0.292	0.355	0.397	0.441	0.229	0.278	0.325	0.346	0.424
mean	0.001	0.553	0.239	0.2957	0.352	0.393	0.4407	0.243	0.274	0.3217	0.3527	0.416
%		`	43.25	53.434	63.614	71.024	79.639	43.916	49.52	58.133	63.735	75.12

3F/THLE2

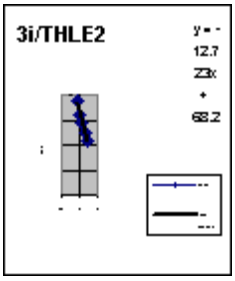
log conc.	% viability
2	43.25
1.398	53.43
0.796	63.61
0.193	71.02
-0.409	79.64



IC50=

3i/THLE2

log conc.	% viability
2	43.92
1.3979	49.52
0.7959	58.13
0.1931	63.73
-0.409	75.12



IC50=

	Blank	CC	Sample No. STA/THLE2					Sample No.				
	1	2	3	4	5	6	7	8	9	10	11	12
A	B	C	100ug	25ug	6.3ug	1.6ug	0.4ug					
B	B	C	100ug	25ug	6.3ug	1.6ug	0.4ug					
C	B	C	100ug	25ug	6.3ug	1.6ug	0.4ug					

ROBONIK P2000 Eia reader

Wave length: 450 nm

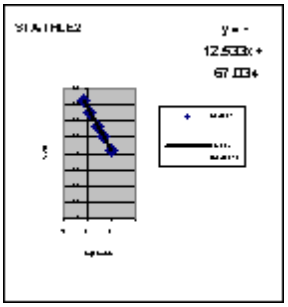
Reference: 630 nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	0.543	0.225	0.264	0.306	0.352	0.388					
B	0.001	0.525	0.231	0.271	0.302	0.344	0.389					
C	0.001	0.539	0.219	0.269	0.292	0.349	0.384					
mean	0.001	0.536	0.225	0.268	0.3	0.3483	0.387	0	0	0	0	0
% viability			42	50.031	56.005	65.028	72.246	0	0	0	0	0

STA/THLE2

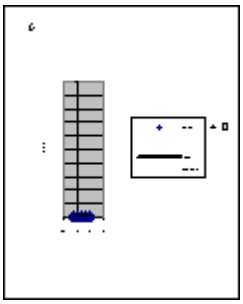
log conc.	% viability
2	42
1.398	50.03
0.796	56
0.193	65.03
-0.409	72.25

IC50=



log conc.	% viability
2	0
1.3979	0
0.7959	0
0.1931	0
-0.409	0

IC50=



References

[1] D. Ramírez. Computational Methods Applied to Rational Drug Design. *Open Med. Chem. J.* **2016,** 10, 7–20.

[2] A. M. Dar, S. Mir. Molecular Docking: Approaches, Types, Applications and Basic Challenges. *J. Anal. Bioanal. Tech.* **2017**, 8, 356.

[3] A. Das, S. Bhattacharya. Different Types of Molecular Docking Based on Variations of Interacting Molecules: Variations of Molecular Docking. in *Methods and Algorithms for Molecular Docking-Based Drug Design and Discovery*. **2016**, 148–172.

[4] E. F Pettersen , T. D. Goddard, C. C. Huang, G. S. Couch, D. M Greenblatt, E.C Meng, T. E. Ferrin. UCSF Chimera - A visualization system for exploratory research and analysis. *J. Comput. Chem.* **2004**, 25, 1605–1612.

[5] Z. Wang, H. Sun, X. Yao, D. Li,a L. Xu, Y. Li, S. Tiand, T. Hou. Comprehensive Evaluation of Ten Docking Programs on a Diverse Set of Protein. *Phys. Chem. Chem. Phys.* **2016,** 18, 12964.

[6] S. Kusumaningrum, E. Budianto, S. Kosela, W. Sumaryono, F.Juniarti, The molecular docking of 1,4-naphthoquinone derivatives as inhibitors of Polo-like kinase 1 using Molegro Virtual Docker. *J. Appl. Pharm. Sci.* **2014**, 4, 47–53.

[7] ChemAxon. Marvin Sketch. 2013.

[8] M. D Hanwell, D. E Curtis, D. C Lonie, T. Vandermeersch, E. Zurek , G. R Hutchison. Avogadro: an advanced semantic chemical editor, visualization, and analysis platform. *J. Cheminformatics*, **2012**. , 4:17.

[9] G. M. Morris, D. S. Goodsell, M. E. Pique, W Lindstrom, R. Huey, S. Forli, W. E. Hart, S. Halliday, R. Belew, A. J. Olson. AutoDock Version 4.2. *User Guid.* 2009, 1–49.

[10] A. Daina, , O. Michielin, V. Zoete, SwissADME: A free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Sci. Rep.* **2017**,**7**.

[11] Case, D. A. *et al.* Amber 18. **2018**.

[12] J. A. Maier, C. Martinez, K. Kasavajhala, L. Wickstrom, K. E. Hauser, C. Simmerling. ff14SB: Improving the Accuracy of Protein Side Chain and Backbone Parameters from ff99SB. *J. Chem. Theory Comput.* **2015,** 11, 3696–3713.

[13] H. J. C. Berendsen, J. P. M. Postma, W. F. van Gunsteren, a DiNola, J. R. Haak. Molecular dynamics with coupling to an external bath. *J. Chem. Phys.* **1984,** 81, 3684–3690.

[14] G. S. Grest, K. Kremer,. Molecular dynamics simulation for polymers in the presence of a heat bath. *Phys. Rev. A* **1986,** 33, 3628–3631.

[15] P. Gonnet. P-SHAKE: a quadratically convergent SHAKE in O (n2). *J. Comput. Phys.* **2007,** 220, 740–750.

[16] D. R. Roe T. E. Cheatham. PTRAJ and CPPTRAJ: Software for Processing and Analysis of Molecular Dynamics Trajectory Data. *J Chem Theory Com*, **2013**, **9**, 3084–3095.

[17] OriginLab Corporation. Origin 9.1 User Guide. *Http://Www.Originlab.Com/91* 1–210 (2013).

[18] D. S. BIOVIA. Discovery Studio. **2017**.

[19] B. Kuhn, P. Gerber, T. Schulz-Gasch, M. Stahl. Validation and use of the MM-PBSA approach for drug discovery. *J. Med. Chem.* **2005,** 48, 4040–4048.

[20] C. Wang, D. Greene, L. Xiao, R. Qi, R. Luo. Recent Developments and Applications of the MMPBSA Method. *Front. Mol. Biosci.* **2018,** 4.

[21] J. Cheung, M. J. Rudolph, F. Burshteyn, M. S. Cassidy, E. N. Gary, J. Love, M. C. Franklin, J. J. Height, 'Structures of Human Acetylcholinesterase in Complex with Pharmacologically Important Ligands', *J. Med. Chem.* **2012**, *55*, 10282–10286.

[22] O. Trott, A. J. Olson, 'AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading.', *J. Comput. Chem.* **2010**, *31*, 455–61.