Synthesis, potential antitumor activity, cell cycle analysis, and multitarget mechanisms of novel hydrazones incorporating a 4-methylsulfonylbenzene scaffold: A molecular docking study

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1. NMR spectrum
2. Materials and methods
    2.1. Biological evaluation

2.1.1. *In vitro* antitumor screening

2.1.2. Apoptosis assay

2.1.3. Cell cycle analysis

2.1.4. *In vitro* cyclooxygenase (COX) inhibition assay

2.1.5. EGFR and Her2 assay

    2.2. Molecular docking method

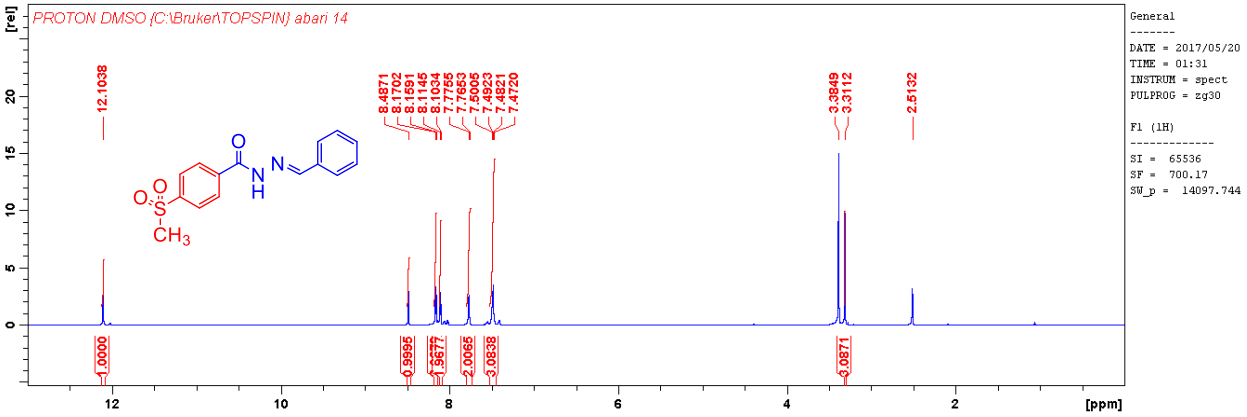
2.2.1. Selection of protein crystal structure

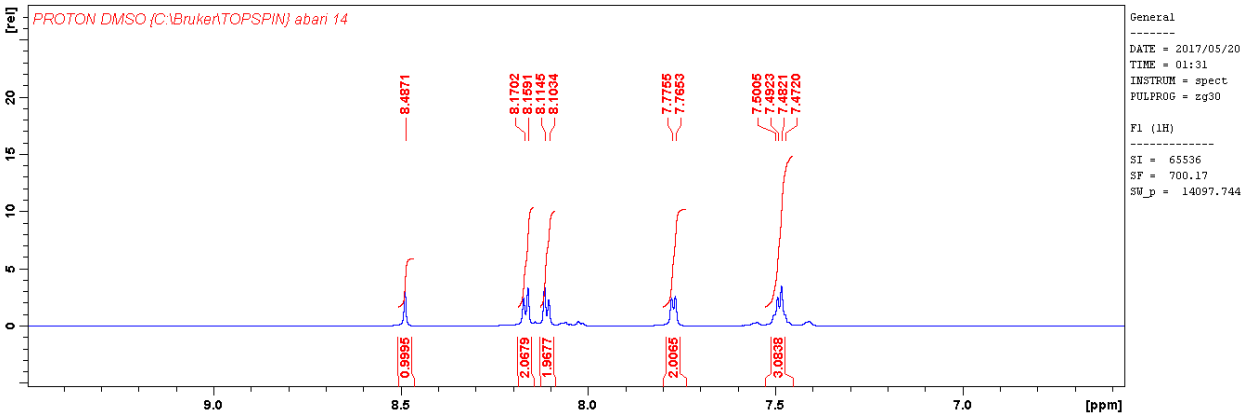
2.2.2. Preparation of the ligand

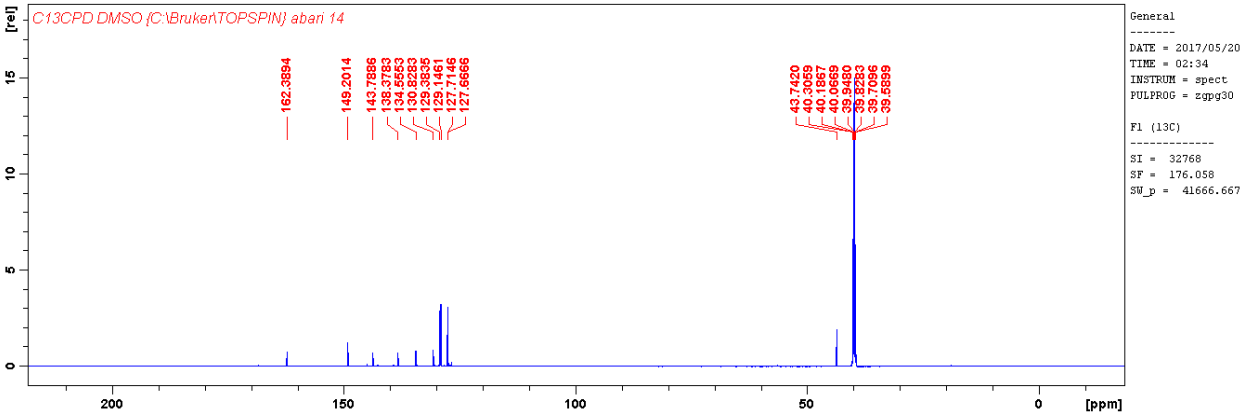
2.2.3. Docking experiment

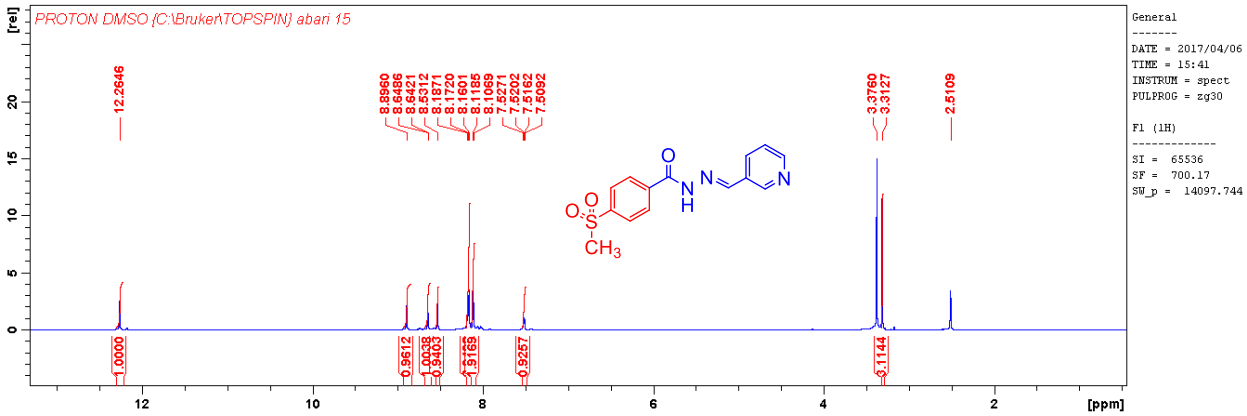
Figure S1. NCI Dose-Response Curves for compound **20**

1. NMR spectrum





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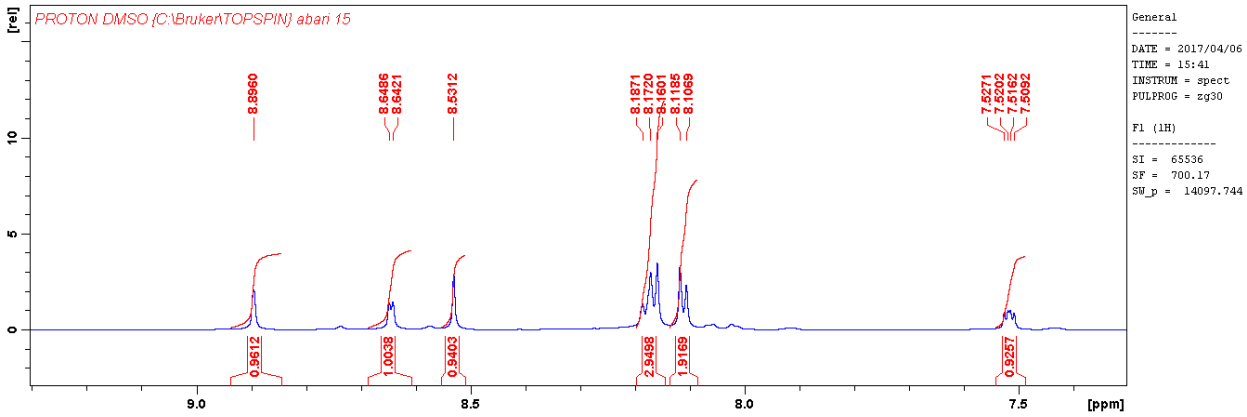
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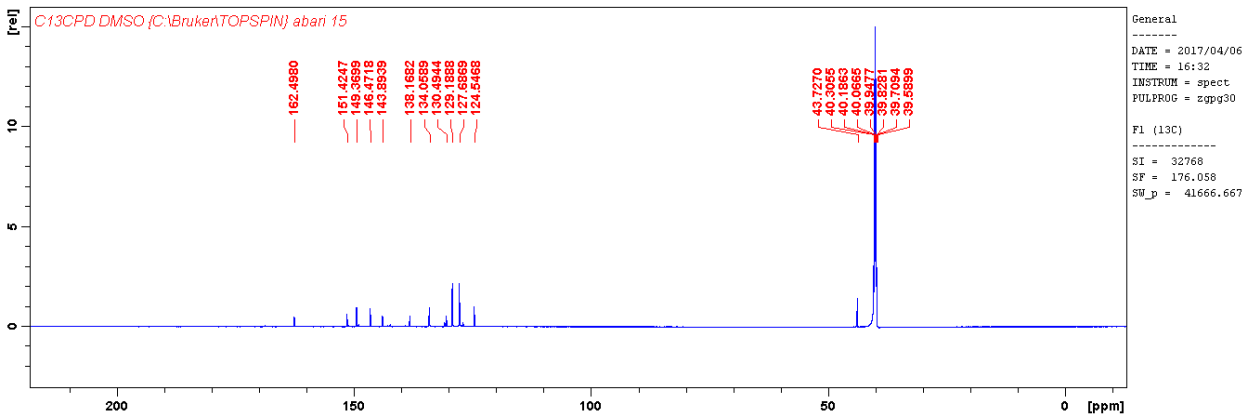
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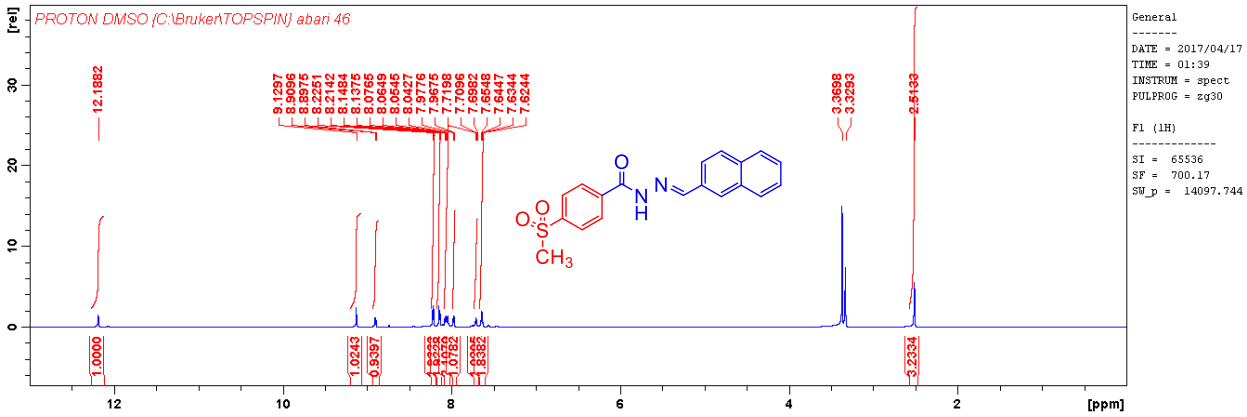
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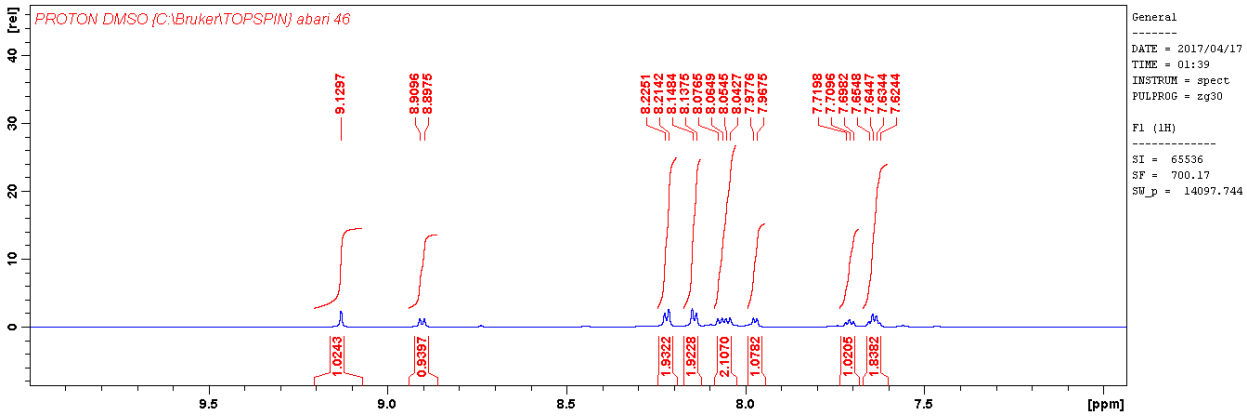
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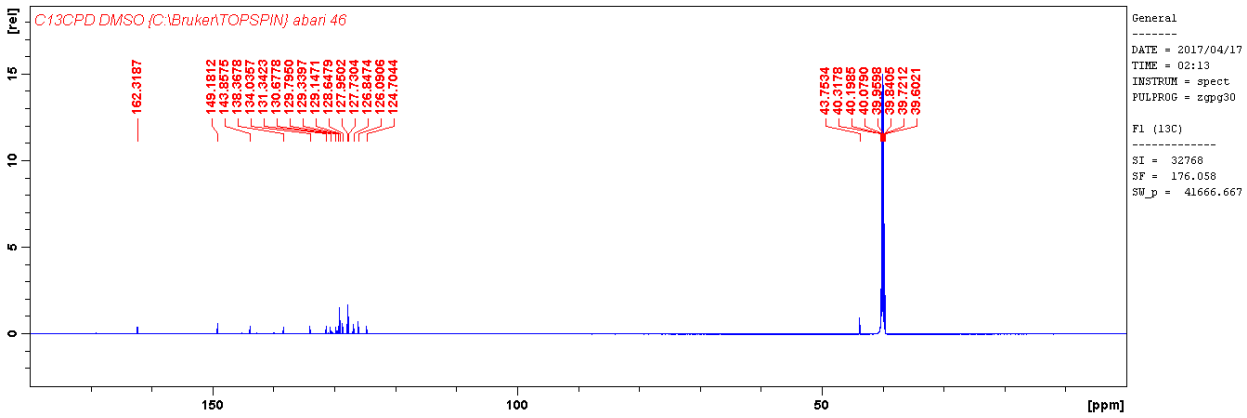
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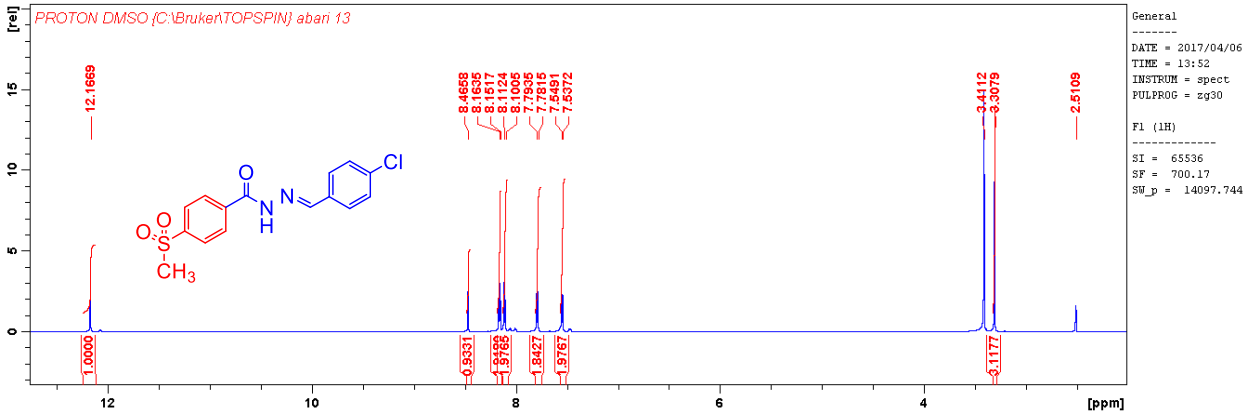
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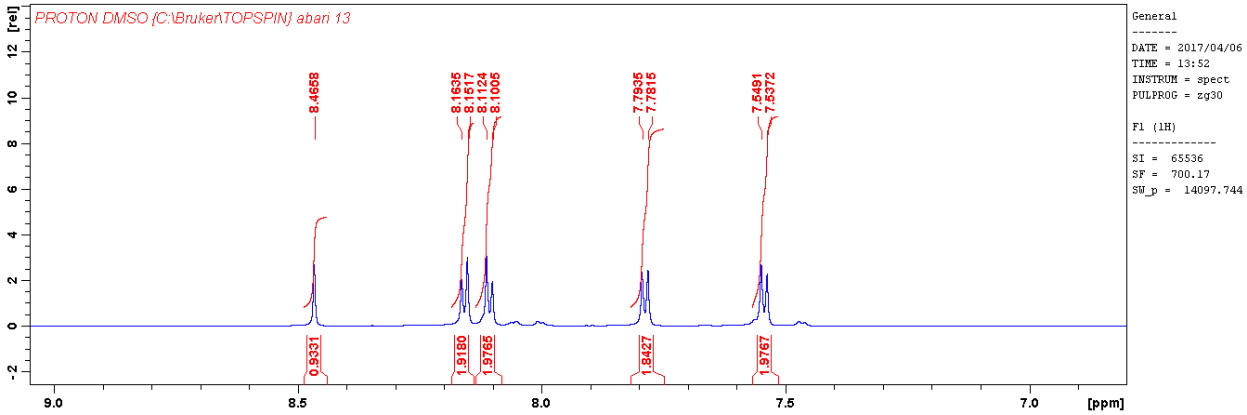
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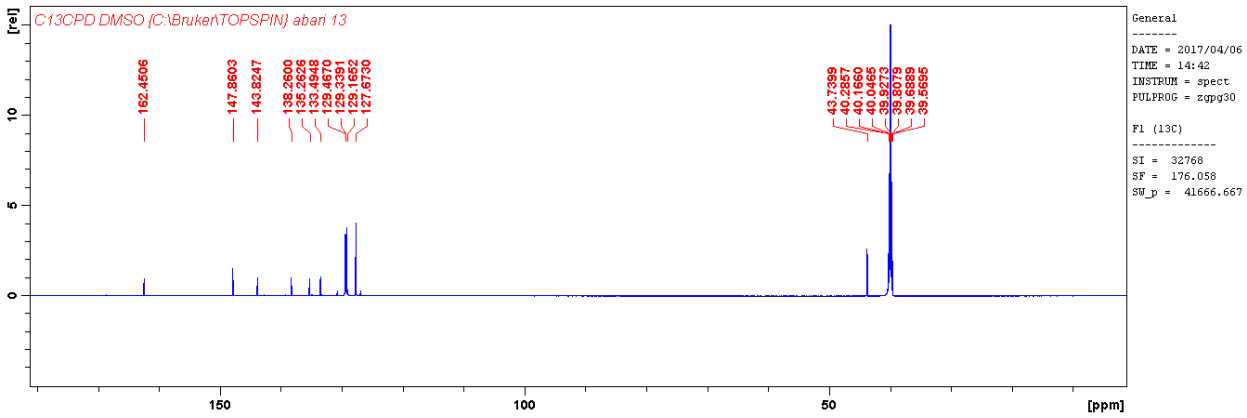
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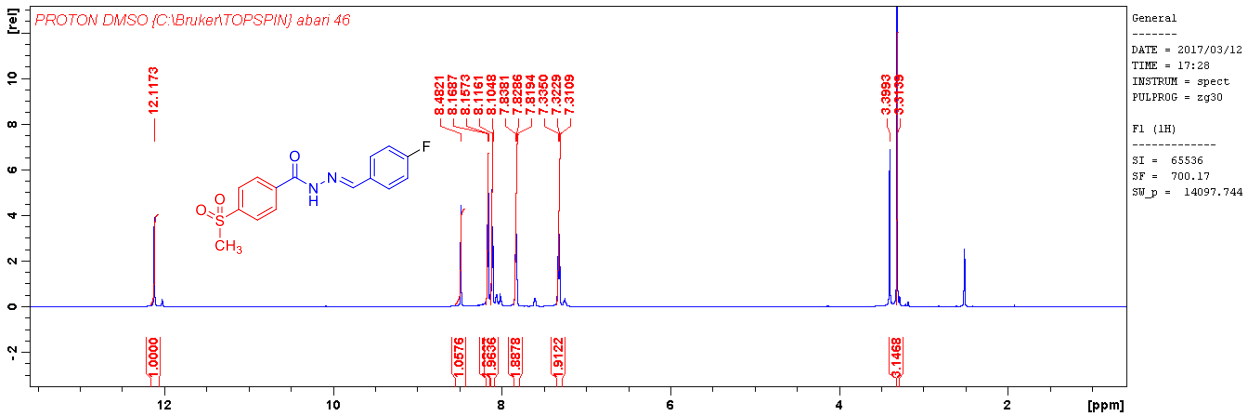
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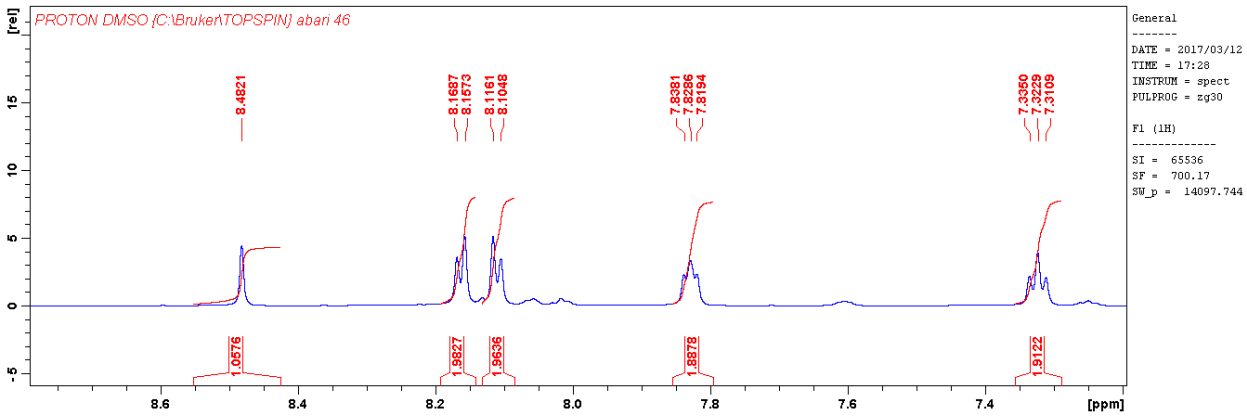
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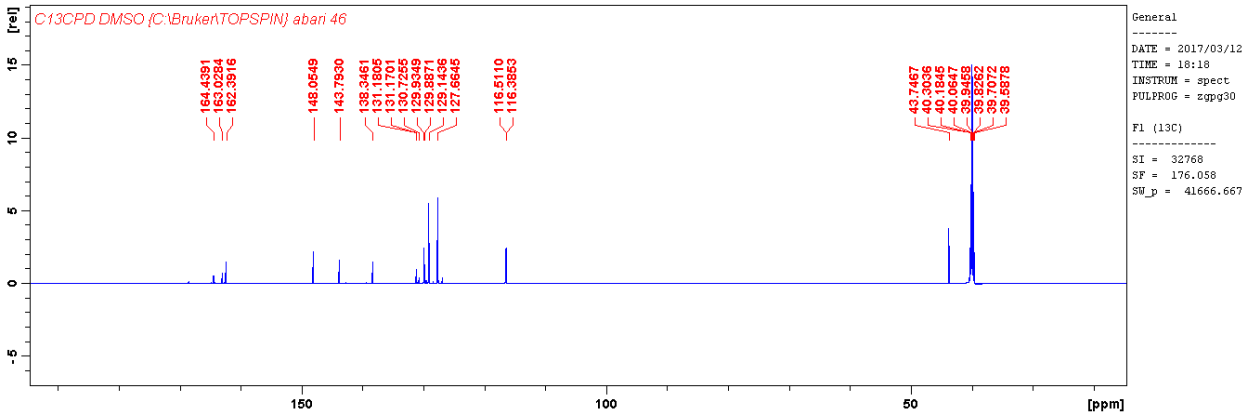
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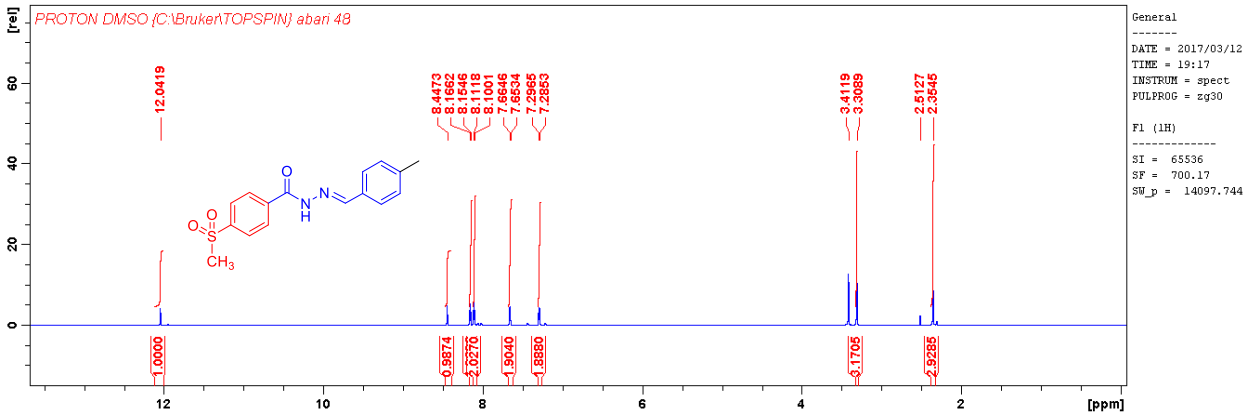
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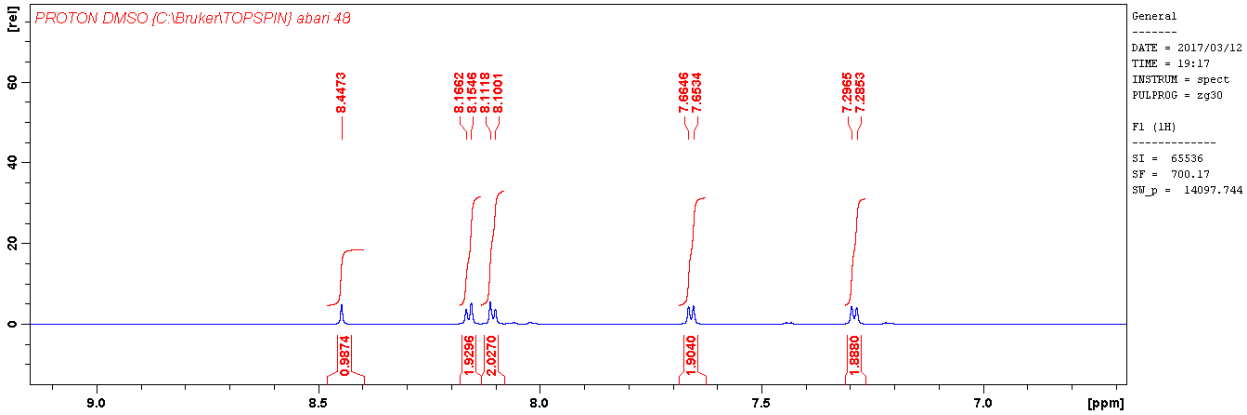
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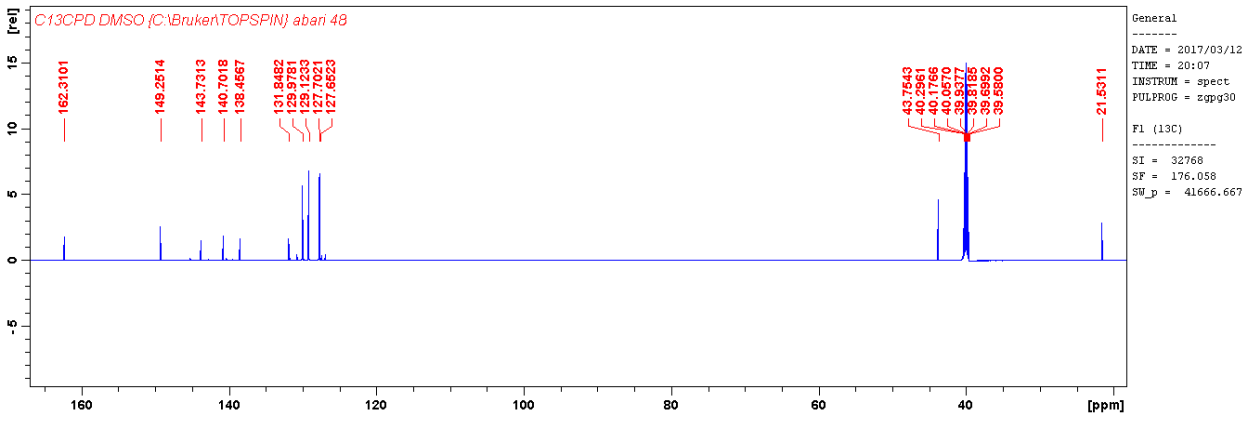
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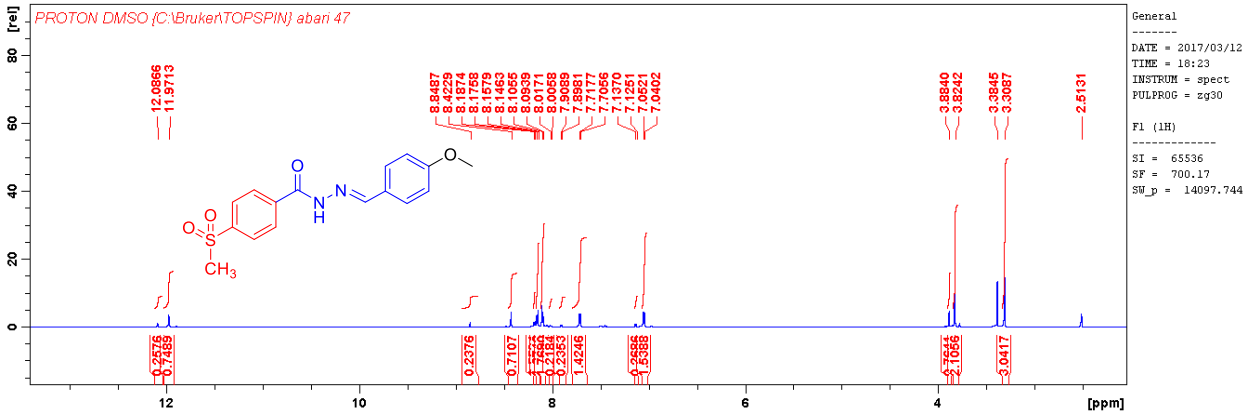
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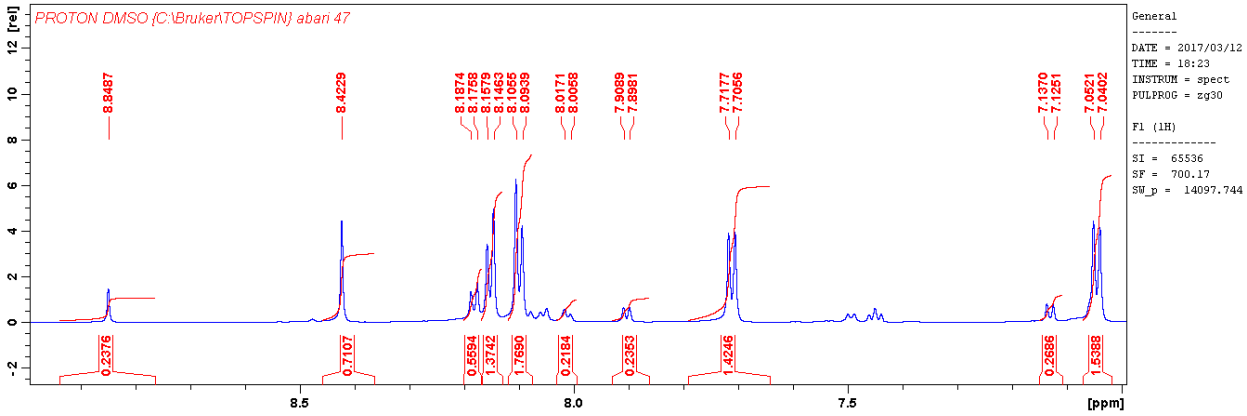
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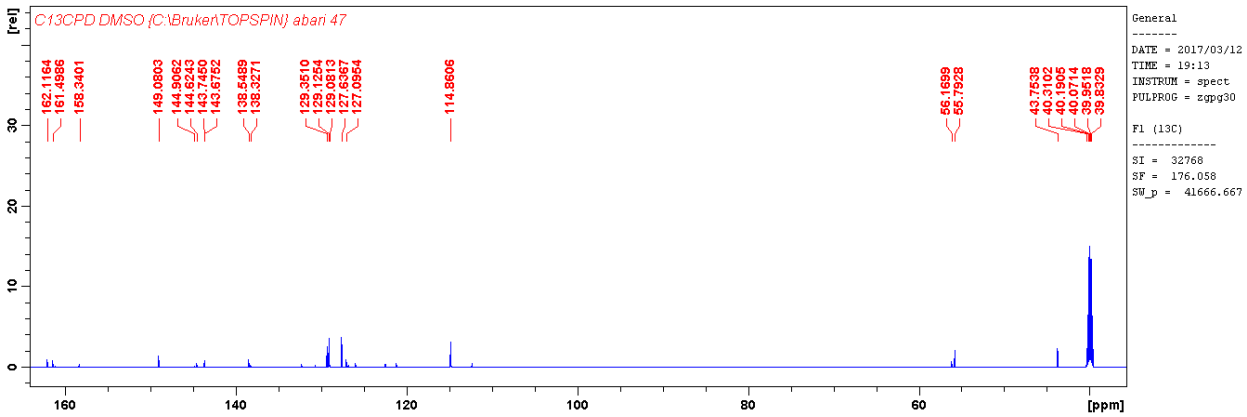
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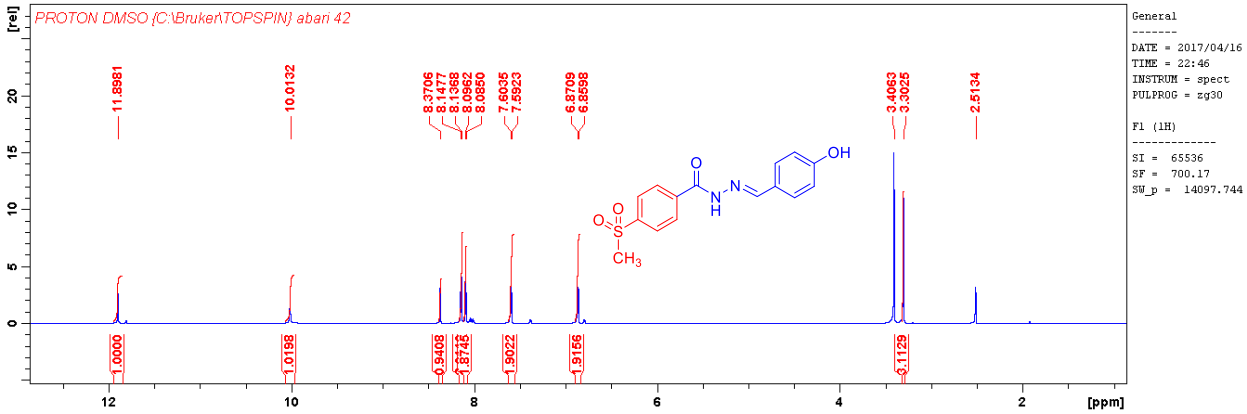
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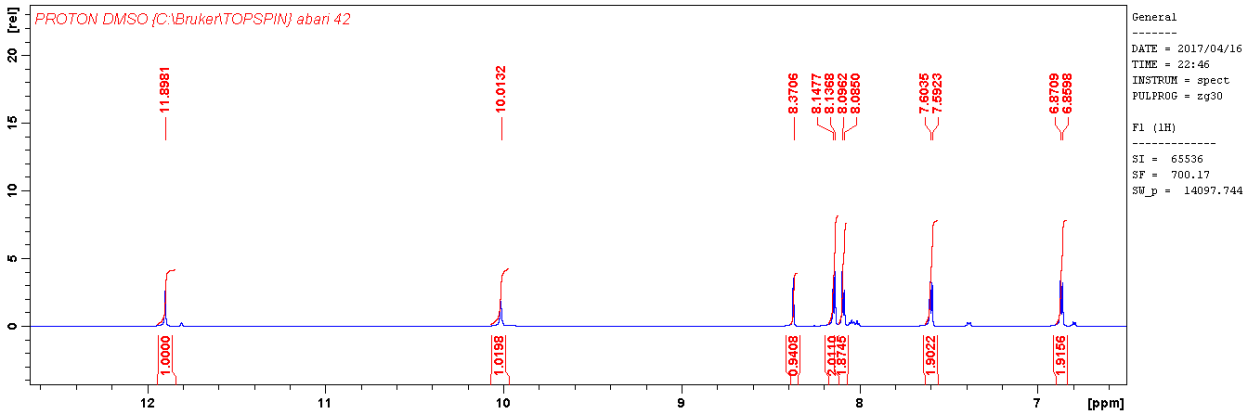
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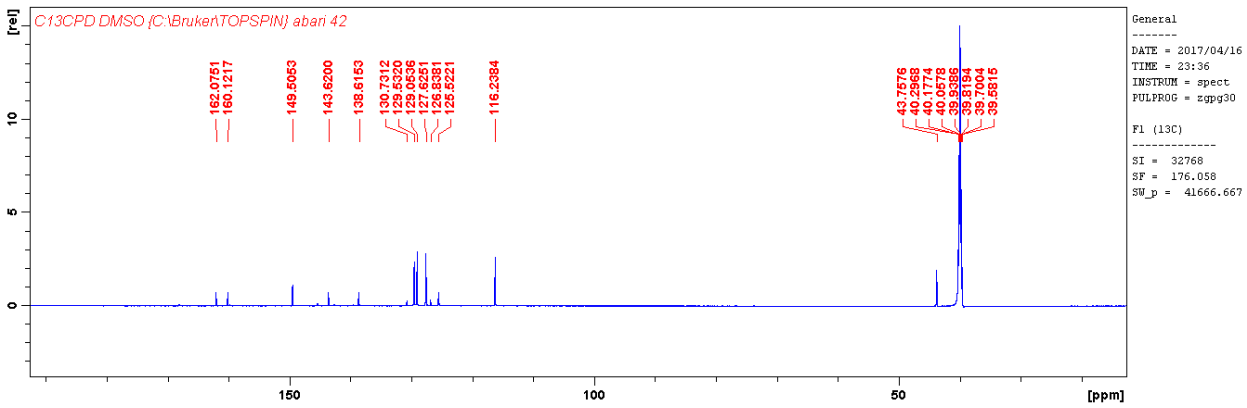
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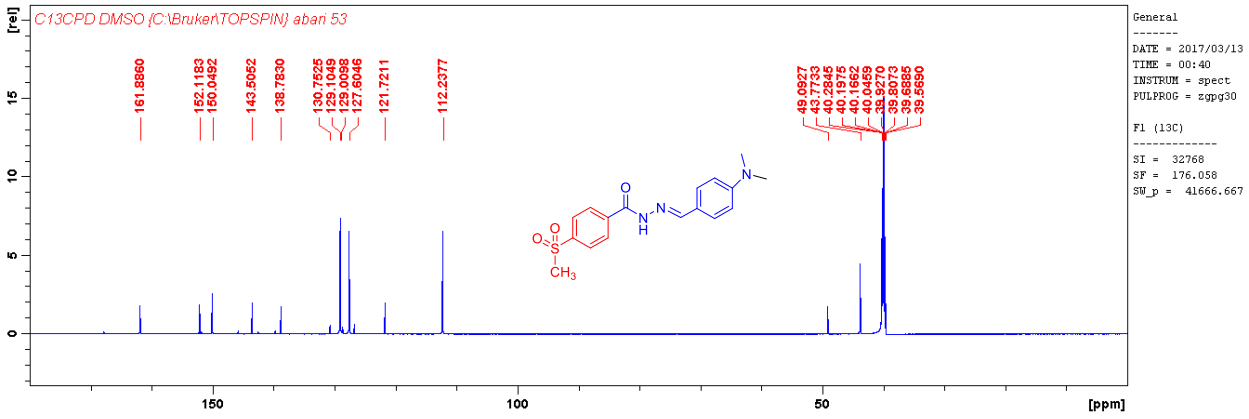
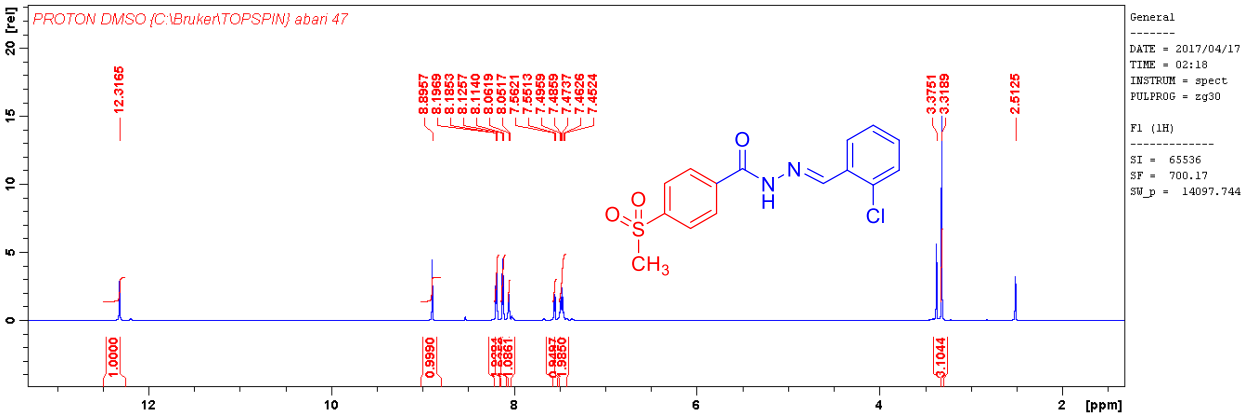
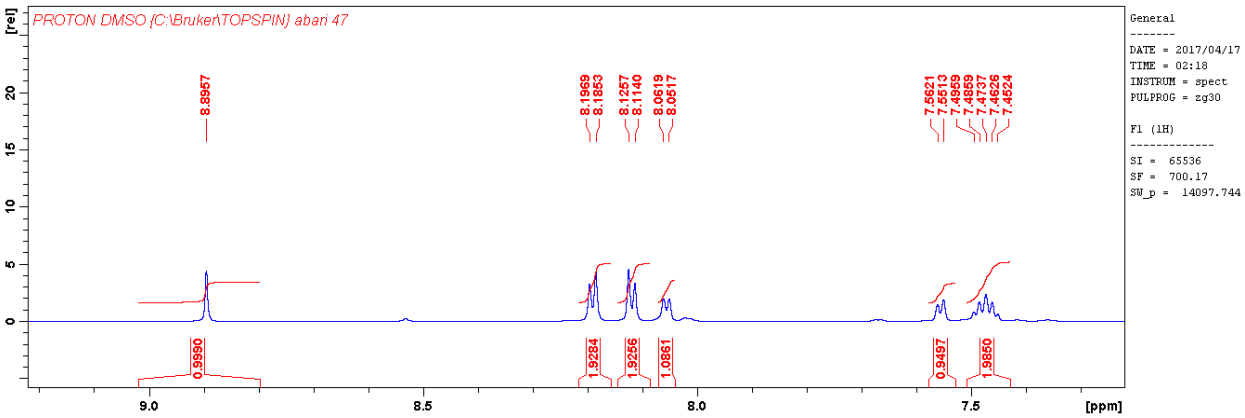
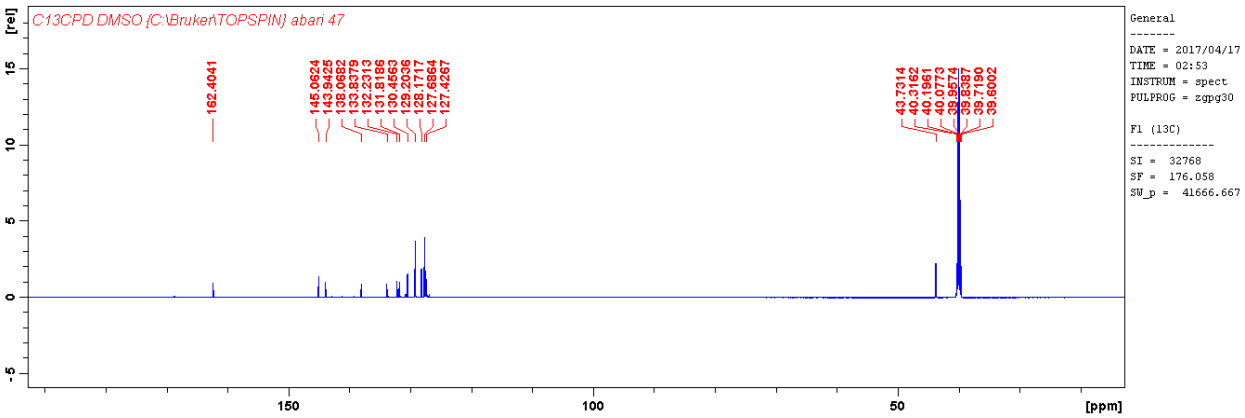
DATE = 2017/04/16
TIME = 23:36
INSTRUM = spect
PULPROG = zgpg30
F1 (13C)
SI = 32768
SF = 176.058
SW_p = 41666.667


C13CPD DMSO {C:\Bruker\TOPSPIN} aban 53
[rel]
15
10
5
0
161.8860
152.1183
150.0492
143.5052
138.7830
130.7525
129.1049
129.0098
127.6046
121.7211
112.2377
49.0827
43.7733
40.2846
40.1975
40.1662
40.0459
39.9270
39.8073
39.6885
39.5690
O
N
H
N
O
S
O
CH3
150
100
50
[ppm]
General
DATE = 2017/03/13
TIME = 00:40
INSTRUM = spect
PULPROG = zgpg30
F1 (13C)
SI = 32768
SF = 176.058
SW_p = 41666.667


PROTON DMSO {C:\Bruker\TOPSPIN} abari 47
12.3165
8.8957
8.1969
8.1853
8.1257
8.1140
8.0619
8.0517
7.5621
7.5513
7.4959
7.4859
7.4737
7.4626
7.4524
3.3751
3.3189
2.5125
1.0000
0.9990
1.9284
1.9256
1.0861
0.8857
1.9850
0.1044
[rel]
[ppm]
General
DATE = 2017/04/17
TIME = 02:18
INSTRUM = spect
PULPROG = zg30
F1 (1H)
SI = 65536
SF = 700.17
SW_p = 14097.744


PROTON DMSO {C:\Bruker\TOPSPIN} abari 47
8.8957
8.1969
8.1853
8.1257
8.1140
8.0619
8.0517
7.5621
7.5513
7.4859
7.4737
7.4626
7.4524
0.9990
1.9284
1.9256
1.0861
0.9497
1.9850
[rel]
[ppm]
General
DATE = 2017/04/17
TIME = 02:18
INSTRUM = spect
PULPROG = zg30
F1 (1H)
SI = 65536
SF = 700.17
SW_p = 14097.744


C13CPD DMSO {C:\Bruker\TOPSPIN} abari 47
162.4041
145.0624
143.9425
138.0682
130.8379
132.2313
131.8966
130.4566
129.2036
128.1717
127.6864
127.4267
43.7314
40.3162
40.1961
40.0773
39.9574
39.8387
39.7190
39.6002
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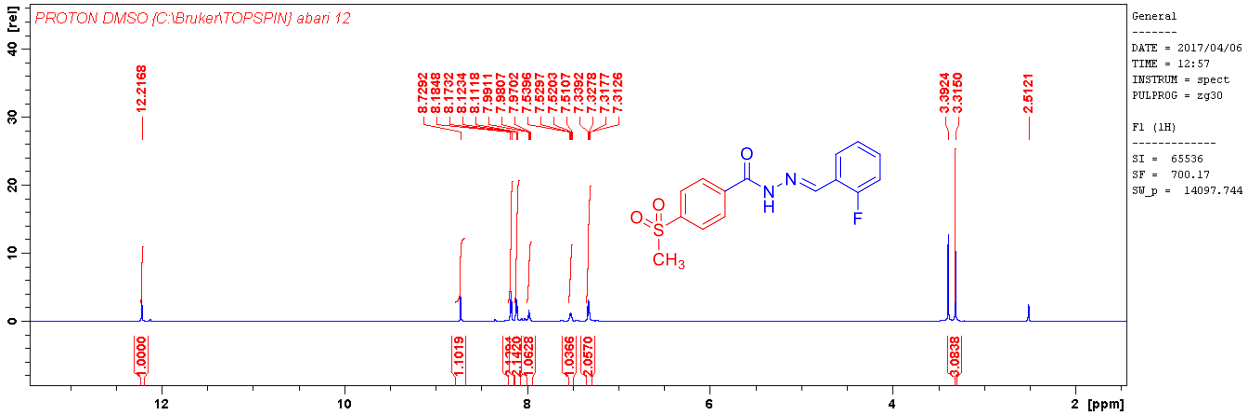
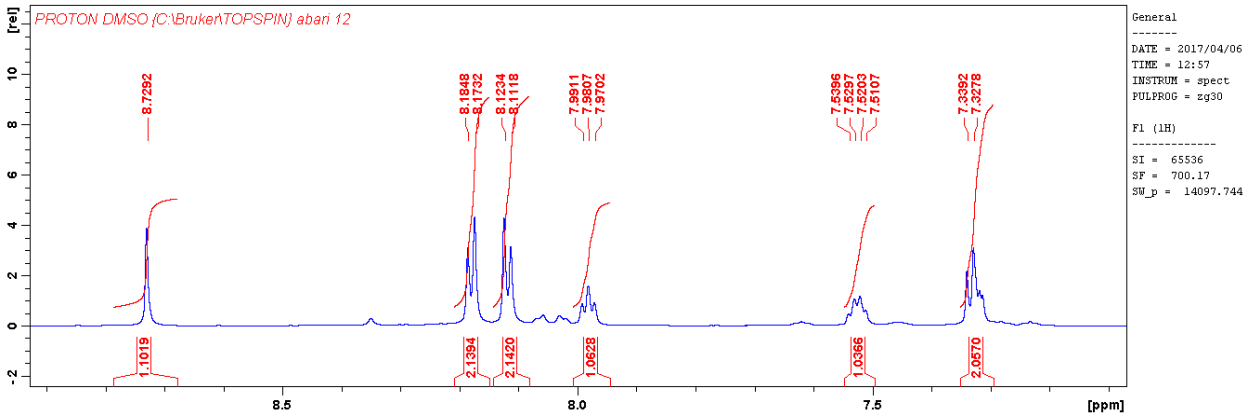
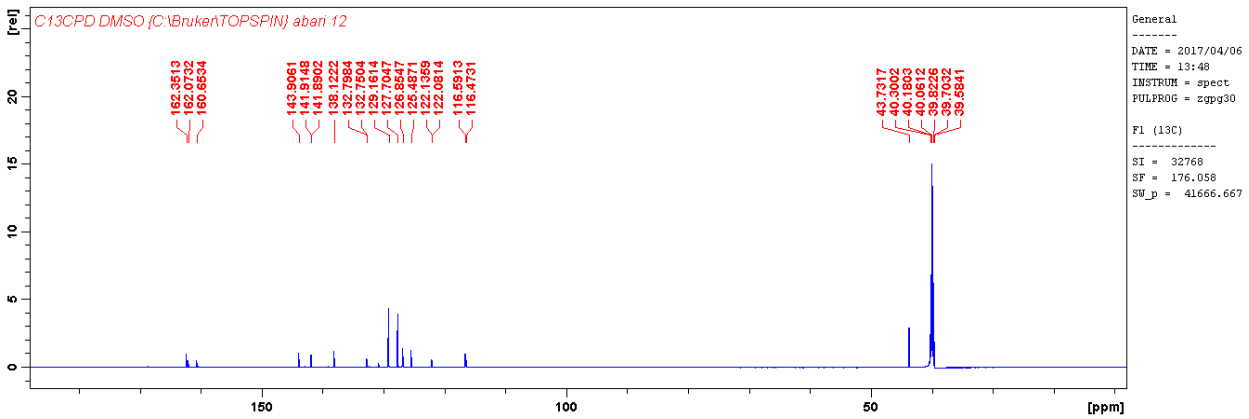
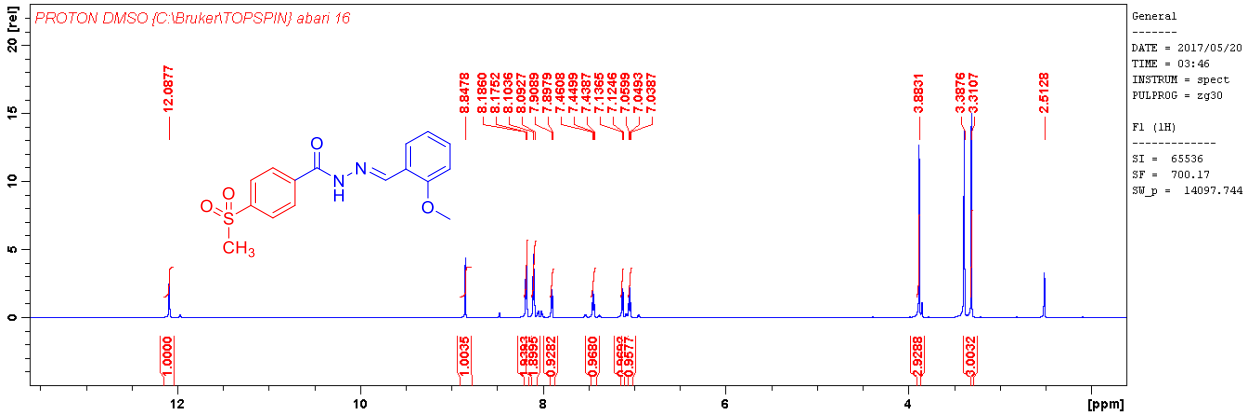
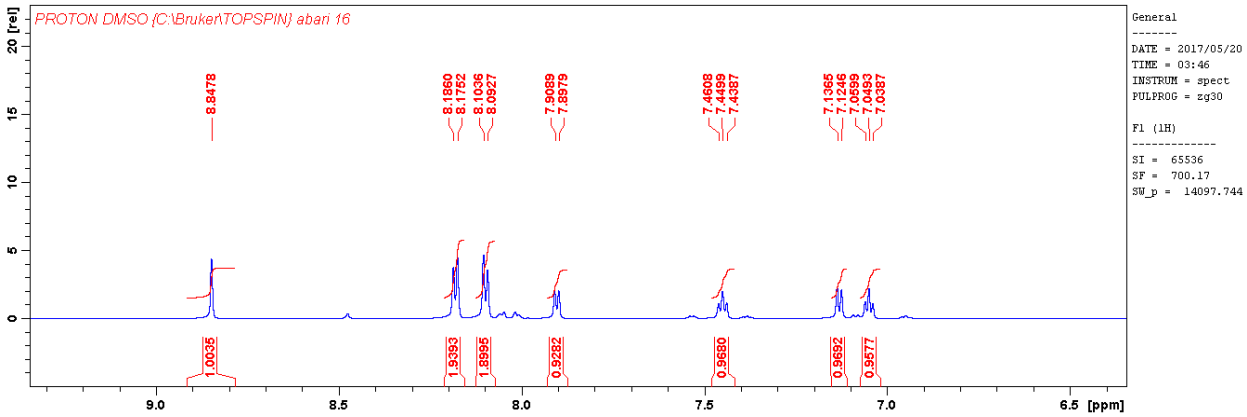
[ppm]
General
DATE = 2017/04/17
TIME = 02:53
INSTRUM = spect
PULPROG = zgpg30
F1 (13C)
SI = 32768
SF = 176.058
SW_p = 41666.667

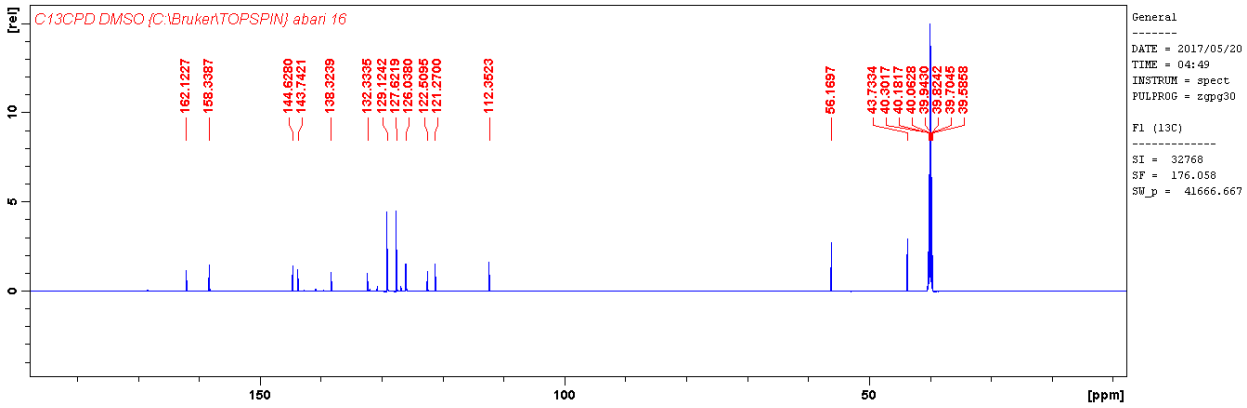

PROTON DMSO {C:\Bruker\TOPSPIN} abari 12
12.2168
8.7292
8.1848
8.1732
8.1234
8.1118
7.9911
7.9807
7.9702
7.5396
7.5297
7.5203
7.5107
7.3392
7.3278
7.3177
7.3126
3.3924
3.3150
2.5121
1.0000
1.1019
2.1394
2.1420
1.0628
1.0366
2.0570
1.0838
[rel]
[ppm]
General
DATE = 2017/04/06
TIME = 12:57
INSTRUM = spect
PULPROG = zg30
F1 (1H)
SI = 65536
SF = 700.17
SW_p = 14097.744

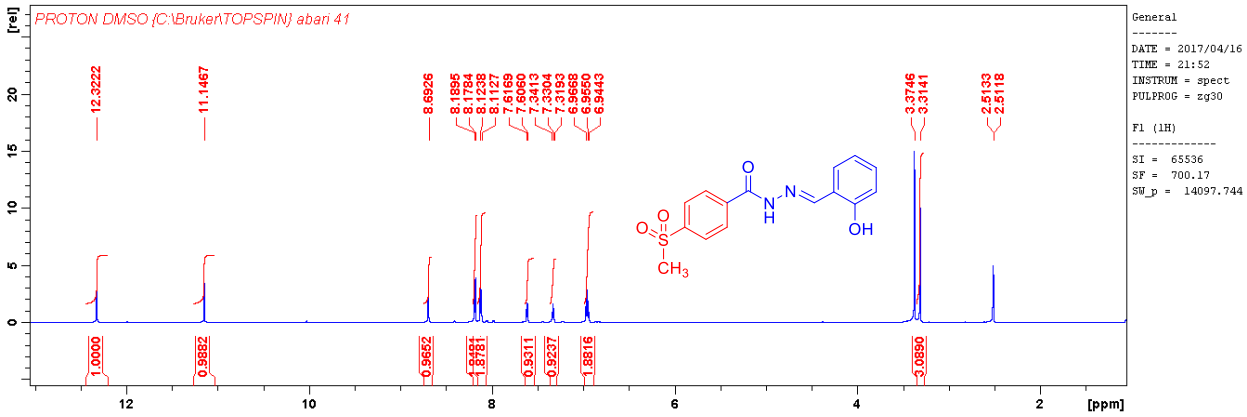

PROTON DMSO {C:\Bruker\TOPSPIN} abari 12
8.7292
8.1848
8.1732
8.1234
8.1118
7.9911
7.9807
7.9702
7.5396
7.5297
7.5203
7.5107
7.3392
7.3278
1.1019
2.1394
2.1420
1.0628
1.0366
2.0570
[rel]
[ppm]
General
DATE = 2017/04/06
TIME = 12:57
INSTRUM = spect
PULPROG = zg30
F1 (1H)
SI = 65536
SF = 700.17
SW_p = 14097.744

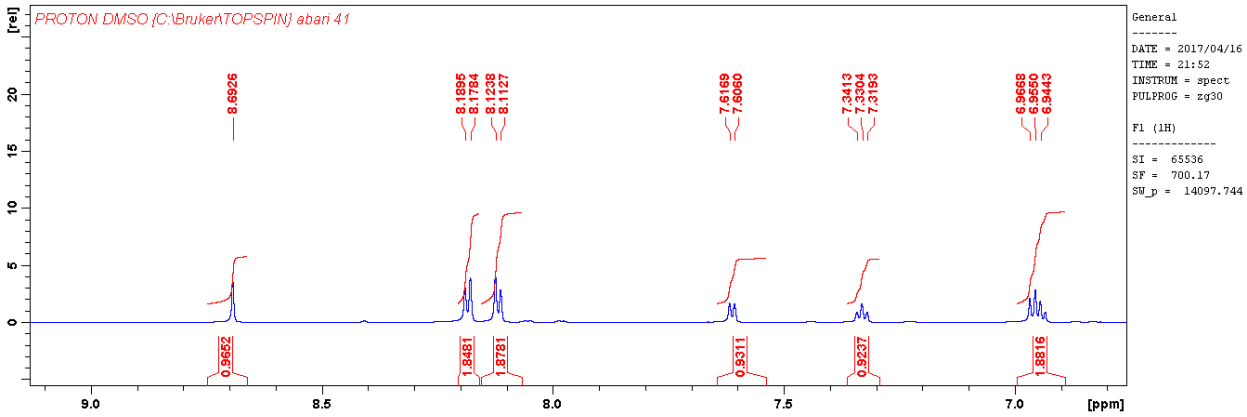

C13CPD DMSO {C:\Bruker\TOPSPIN} abari 12
162.3513
162.0732
160.6534
143.9061
141.9148
141.8902
138.1222
132.7984
132.7504
129.1614
127.7047
126.8547
125.4871
122.1359
122.0814
116.5913
116.4731
43.7317
40.3002
40.1803
40.0612
39.8226
39.7032
39.5841
[rel]
[ppm]
General
DATE = 2017/04/06
TIME = 13:48
INSTRUM = spect
PULPROG = zgpg30
F1 (13C)
SI = 32768
SF = 176.058
SW_p = 41666.667

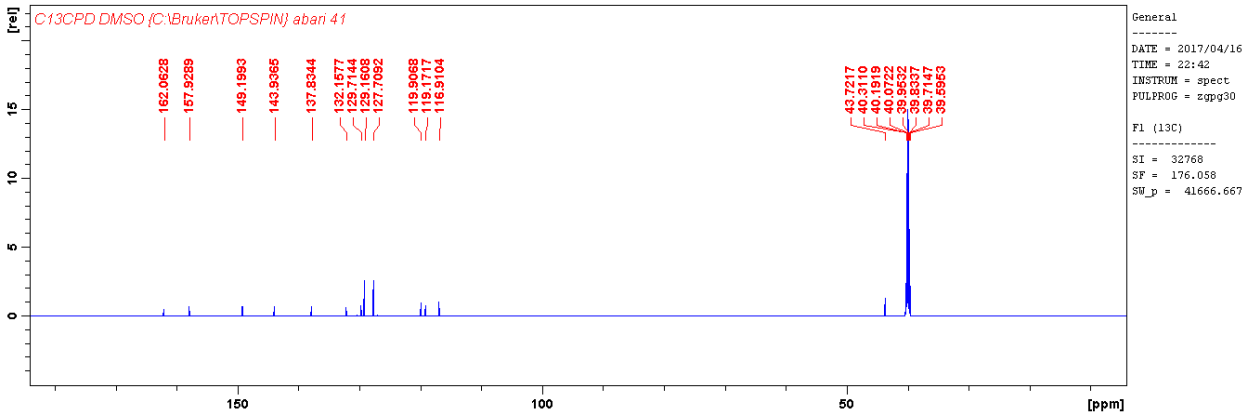

PROTON DMSO {C:\Bruker\TOPSPIN} abari 16
12.0877
8.8478
8.1860
8.1752
8.1036
8.0927
7.9089
7.8979
7.4608
7.4499
7.4387
7.1365
7.1246
7.0599
7.0493
7.0387
3.8831
3.3876
3.3107
2.5128
1.0000
1.0035
1.9393
1.8995
0.9282
0.9680
0.9692
0.9577
2.9288
6.0032
[ppm]
General
DATE = 2017/05/20
TIME = 03:46
INSTRUM = spect
PULPROG = zg30
F1 (1H)
SI = 65536
SF = 700.17
SW_p = 14097.744

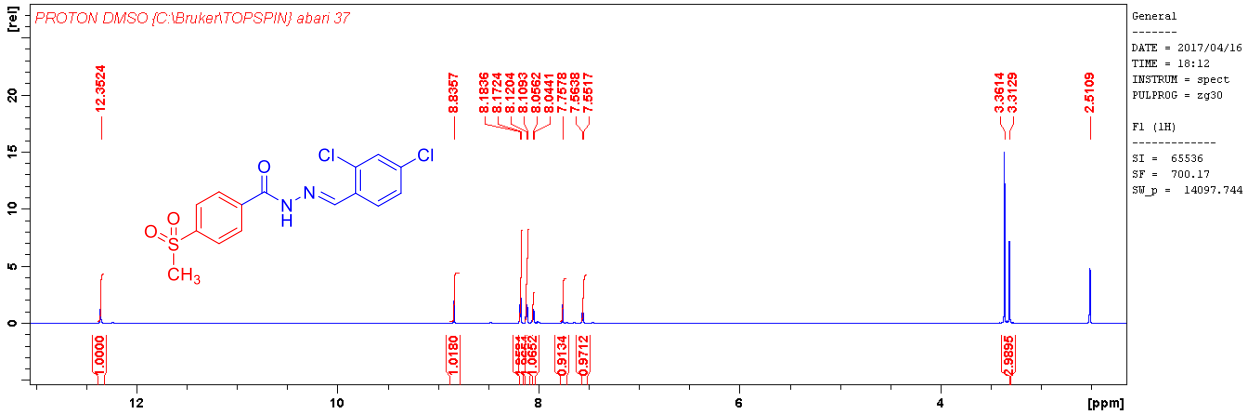

PROTON DMSO {C:\Bruker\TOPSPIN} abari 16
8.8478
8.1860
8.1752
8.1036
8.0927
7.9089
7.8979
7.4608
7.4499
7.4387
7.1365
7.1246
7.0599
7.0493
7.0387
1.0035
1.9393
1.8995
0.9282
0.9680
0.9692
0.9577
[ppm]
General
DATE = 2017/05/20
TIME = 03:46
INSTRUM = spect
PULPROG = zg30
F1 (1H)
SI = 65536
SF = 700.17
SW_p = 14097.744

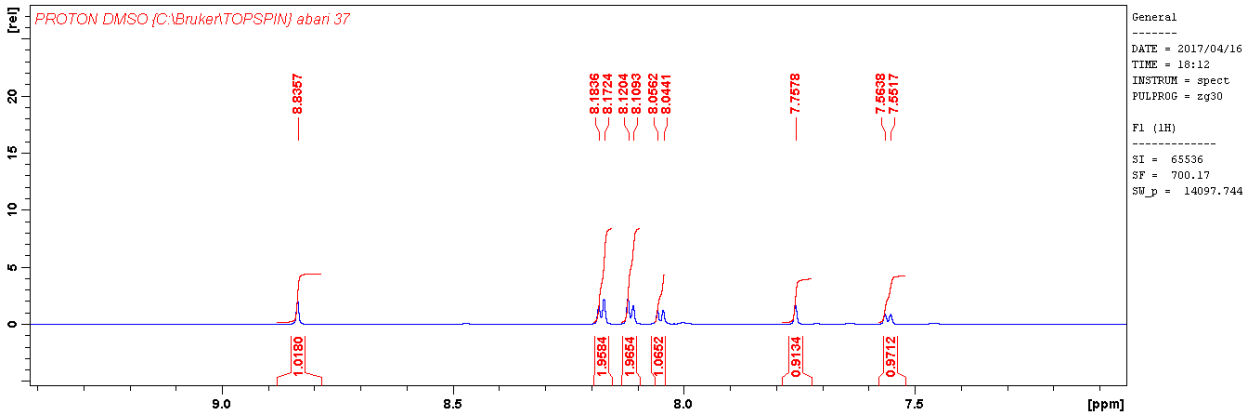

C13CPD DMSO {C:\Bruker\TOPSPIN} abari 16
162.1227
158.3387
144.6280
143.7421
138.3239
132.3335
129.1242
127.6219
126.0386
122.5096
121.2700
112.3523
56.1697
43.7334
40.3017
40.1817
40.0628
39.9430
39.8242
39.7045
39.5858
[ppm]
General
DATE = 2017/05/20
TIME = 04:49
INSTRUM = spect
PULPROG = zgpg30
F1 (13C)
SI = 32768
SF = 176.058
SW_p = 41666.667

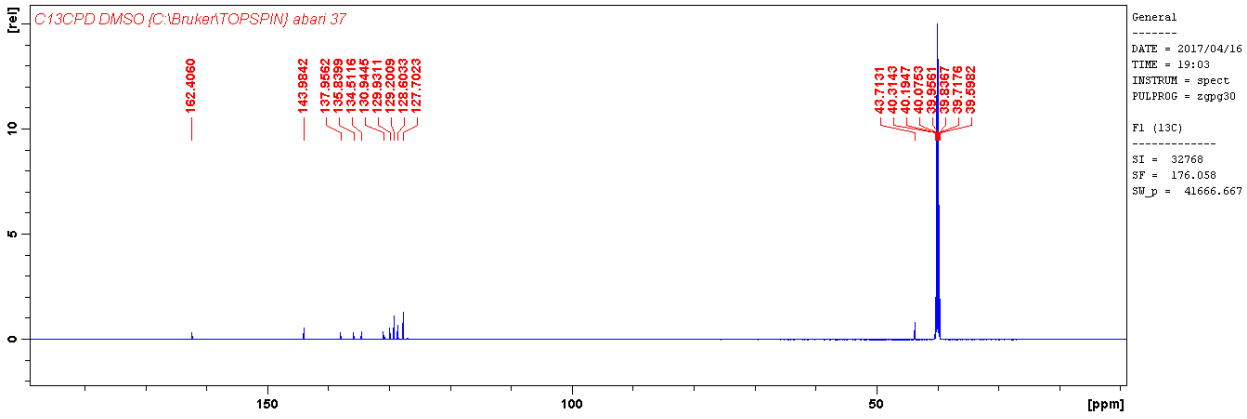

PROTON DMSO {C:\Bruker\TOPSPIN} abari 41
[rel]
12.3222
11.1467
8.6926
8.1895
8.1784
8.1238
8.1127
7.6189
7.6060
7.3413
7.3304
7.3193
6.9668
6.9550
6.9443
3.3746
3.3141
2.5133
2.5118
1.0000
0.9882
0.9652
1.8481
1.8781
0.9311
0.9237
1.8816
3.0890
[ppm]
General
DATE = 2017/04/16
TIME = 21:52
INSTRUM = spect
PULPROG = zg30
F1 (1H)
SI = 65536
SF = 700.17
SW_p = 14097.744

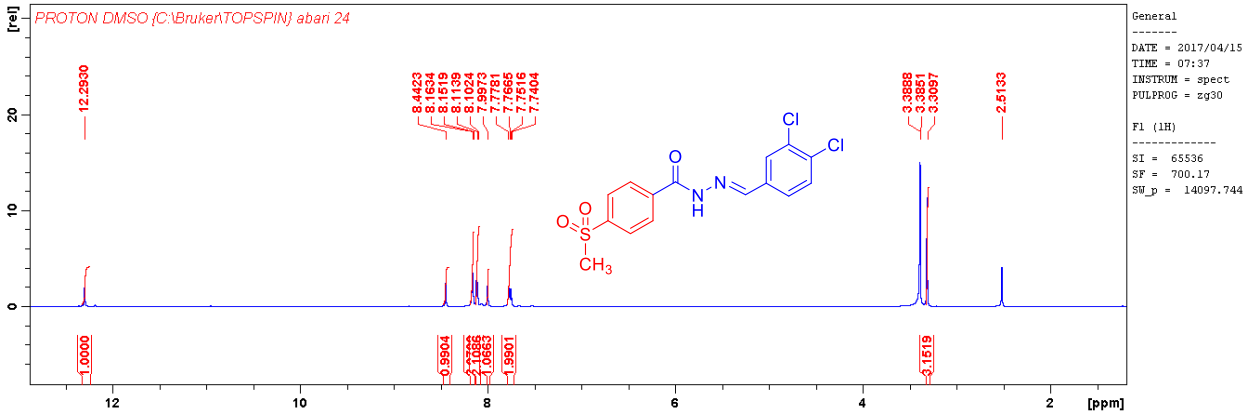

PROTON DMSO {C:\Bruker\TOPSPIN} abari 41
[rel]
8.6926
8.1895
8.1784
8.1238
8.1127
7.6189
7.6060
7.3413
7.3304
7.3193
6.9668
6.9550
6.9443
0.9652
1.8481
1.8781
0.9311
0.9237
1.8816
[ppm]
General
DATE = 2017/04/16
TIME = 21:52
INSTRUM = spect
PULPROG = zg30
F1 (1H)
SI = 65536
SF = 700.17
SW_p = 14097.744

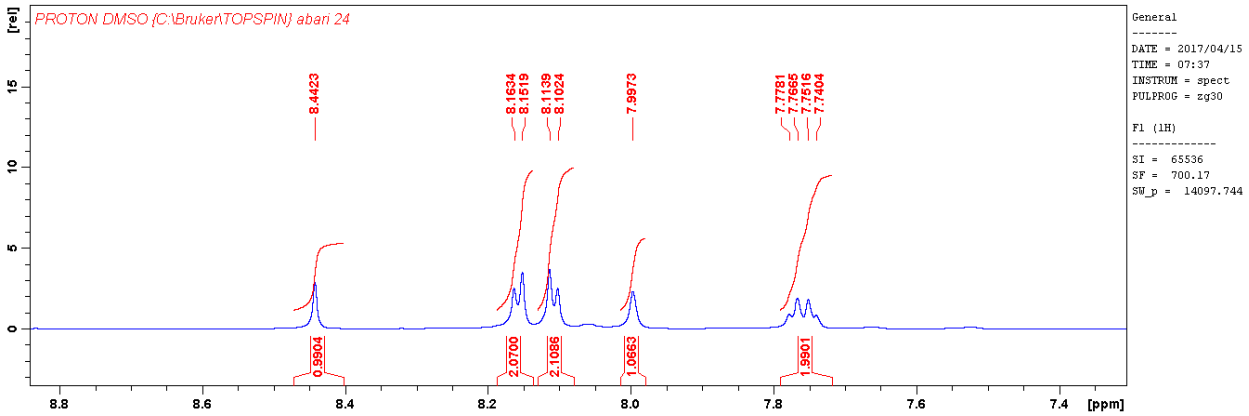

C13CPD DMSO {C:\Bruker\TOPSPIN} abari 41
[rel]
162.0628
157.9289
149.1993
143.9365
137.8344
132.1577
129.7144
129.1608
127.7092
119.9068
119.1717
116.9104
43.7217
40.3110
40.1919
40.0722
39.9532
39.8337
39.7147
39.5953
[ppm]
General
DATE = 2017/04/16
TIME = 22:42
INSTRUM = spect
PULPROG = zgpg30
F1 (13C)
SI = 32768
SF = 176.058
SW_p = 41666.667

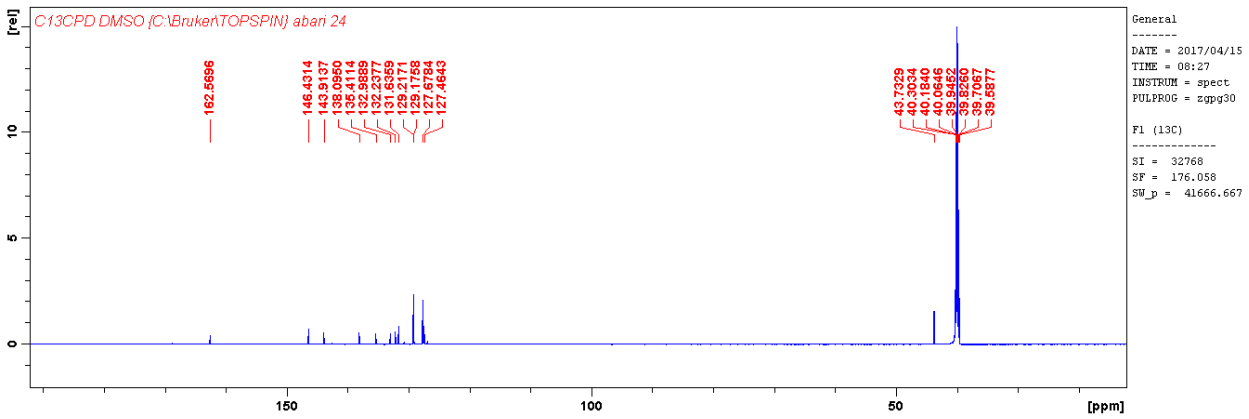

PROTON DMSO {C:\Bruker\TOPSPIN} abari 37
[rel]
12.3524
8.8357
8.1836
8.1724
8.1204
8.1093
8.0562
8.0441
7.7578
7.5638
7.5517
3.3614
3.3129
2.5109
1.0000
1.0180
1.9584
1.9654
1.0652
0.9134
0.9712
2.9895
[ppm]
General
DATE = 2017/04/16
TIME = 18:12
INSTRUM = spect
PULPROG = zg30
F1 (1H)
SI = 65536
SF = 700.17
SW_p = 14097.744

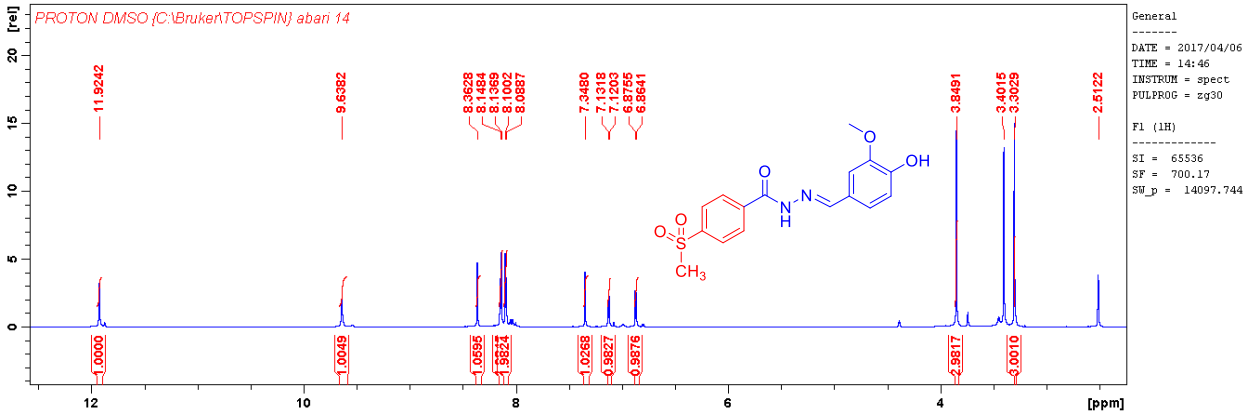

PROTON DMSO {C:\Bruker\TOPSPIN} abari 37
[rel]
8.8357
8.1836
8.1724
8.1204
8.1093
8.0562
8.0441
7.7578
7.5638
7.5517
1.0180
1.9584
1.9654
1.0652
0.9134
0.9712
[ppm]
General
DATE = 2017/04/16
TIME = 18:12
INSTRUM = spect
PULPROG = zg30
F1 (1H)
SI = 65536
SF = 700.17
SW_p = 14097.744

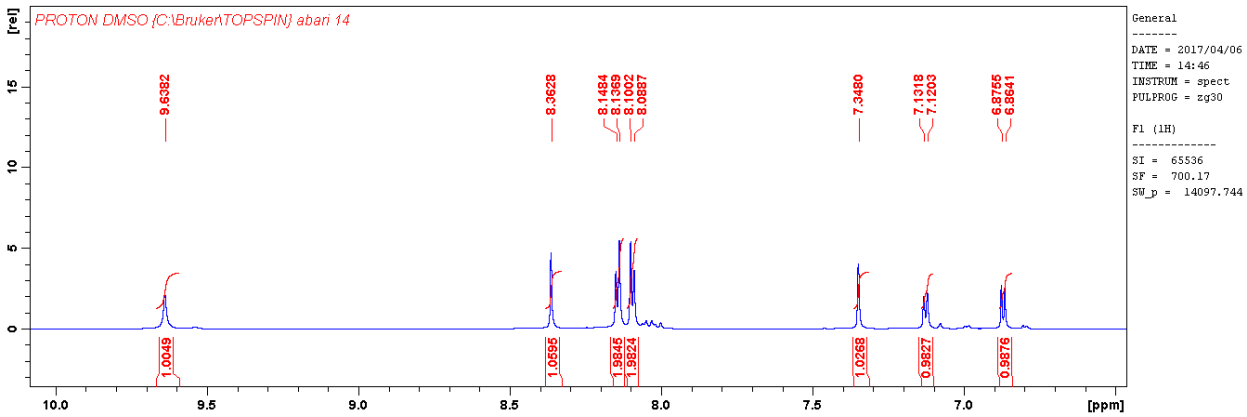

C13CPD DMSO {C:\Bruker\TOPSPIN} abari 37
[rel]
162.4060
143.9842
137.9562
136.8399
134.5116
130.9445
129.9311
129.2009
128.8033
127.7023
43.7131
40.3143
40.1947
40.0753
39.9561
39.8367
39.7176
39.5982
[ppm]
General
DATE = 2017/04/16
TIME = 19:03
INSTRUM = spect
PULPROG = zgpg30
F1 (13C)
SI = 32768
SF = 176.058
SW_p = 41666.667

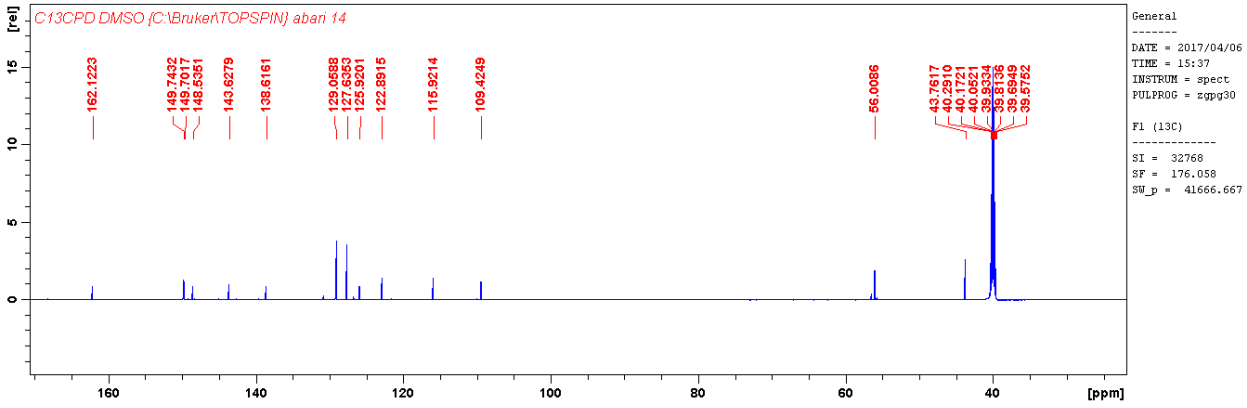

PROTON DMSO {C:\Bruker\TOPSPIN} abari 24
[rel]
12.2930
8.4423
8.1634
8.1519
8.1139
8.1024
7.9973
7.7781
7.7665
7.7516
7.7404
3.3888
3.3851
3.3097
2.5133
1.0000
0.9904
2.1086
1.0663
1.9901
3.1619
12
10
8
6
4
2
[ppm]
General
DATE = 2017/04/15
TIME = 07:37
INSTRUM = spect
PULPROG = zg30
F1 (1H)
SI = 65536
SF = 700.17
SW_p = 14097.744

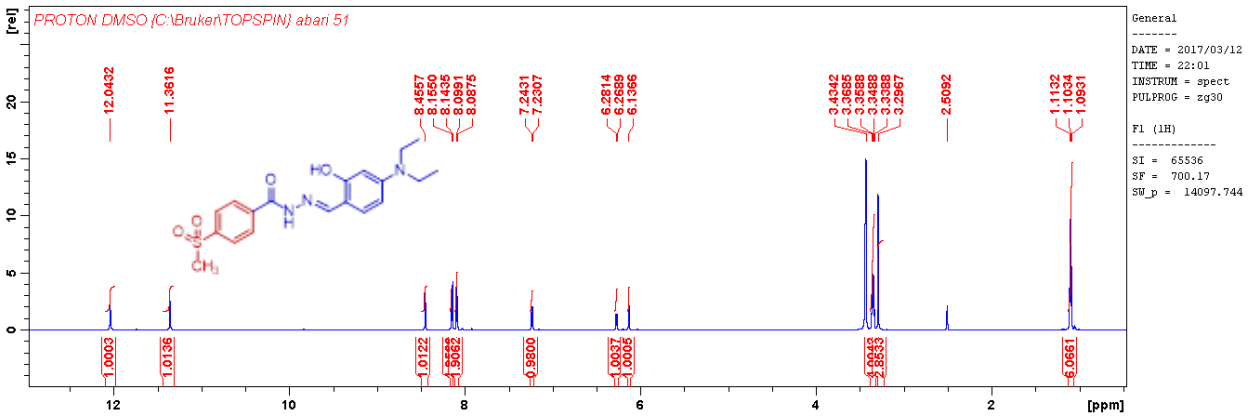

PROTON DMSO {C:\Bruker\TOPSPIN} abari 24
[rel]
8.4423
8.1634
8.1519
8.1139
8.1024
7.9973
7.7781
7.7665
7.7516
7.7404
0.9904
2.0700
2.1086
1.0663
1.9901
8.8
8.6
8.4
8.2
8.0
7.8
7.6
7.4
[ppm]
General
DATE = 2017/04/15
TIME = 07:37
INSTRUM = spect
PULPROG = zg30
F1 (1H)
SI = 65536
SF = 700.17
SW_p = 14097.744


C13CPD DMSO {C:\Bruker\TOPSPIN} abari 24
[rel]
162.5696
146.4314
143.9137
138.0950
135.4114
132.9889
132.2377
131.6359
129.2171
129.1758
127.6784
127.4643
43.7329
40.3034
40.1840
40.0646
39.9452
39.8260
39.7067
39.5877
150
100
50
[ppm]
General
DATE = 2017/04/15
TIME = 08:27
INSTRUM = spect
PULPROG = zgpg30
F1 (13C)
SI = 32768
SF = 176.058
SW_p = 41666.667


PROTON DMSO {C:\Bruker\TOPSPIN} abari 14
[rel]
11.9242
9.6382
8.3628
8.1484
8.1369
8.1002
8.0887
7.3480
7.1318
7.1203
6.8755
6.8641
3.8491
3.4015
3.3029
2.5122
1.0000
1.0049
1.0595
1.9824
1.0268
0.9827
0.9876
2.9817
3.0010
[ppm]
General
DATE = 2017/04/06
TIME = 14:46
INSTRUM = spect
PULPROG = zg30
F1 (1H)
SI = 65536
SF = 700.17
SW_p = 14097.744


PROTON DMSO {C:\Bruker\TOPSPIN} abari 14
[rel]
9.6382
8.3628
8.1484
8.1369
8.1002
8.0887
7.3480
7.1318
7.1203
6.8755
6.8641
1.0049
1.0595
1.9845
1.9824
1.0268
0.9827
0.9876
[ppm]
General
DATE = 2017/04/06
TIME = 14:46
INSTRUM = spect
PULPROG = zg30
F1 (1H)
SI = 65536
SF = 700.17
SW_p = 14097.744


C13CPD DMSO {C:\Bruker\TOPSPIN} abari 14
[rel]
162.1223
149.7432
149.7017
148.5351
143.6279
138.6161
129.0588
127.6353
125.9201
122.8915
115.9214
109.4249
56.0086
43.7617
40.2910
40.1721
40.0521
39.9334
39.8136
39.6949
39.5752
[ppm]
General
DATE = 2017/04/06
TIME = 15:37
INSTRUM = spect
PULPROG = zgpg30
F1 (13C)
SI = 32768
SF = 176.058
SW_p = 41666.667


PROTON DMSO {C:\Bruker\TOPSPIN} abari 51
[rel]
12.0432
11.3616
8.4557
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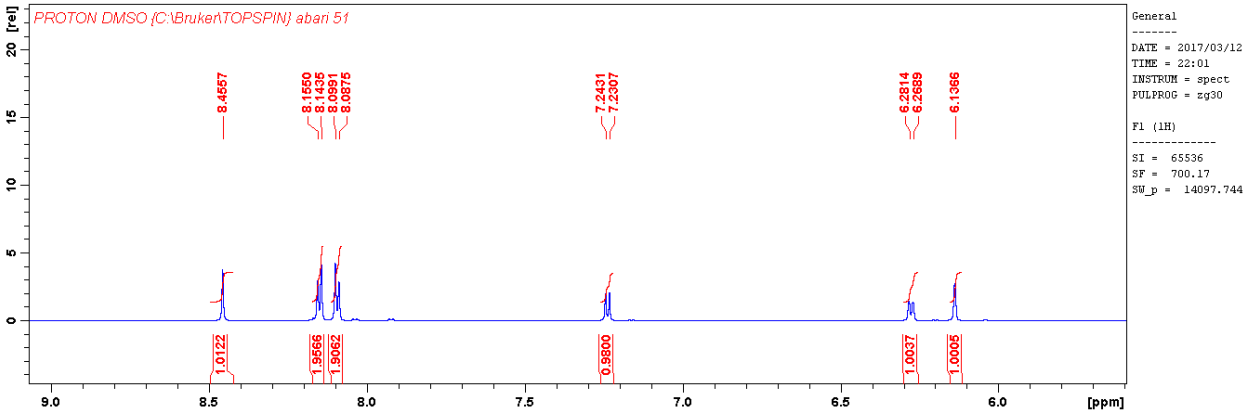
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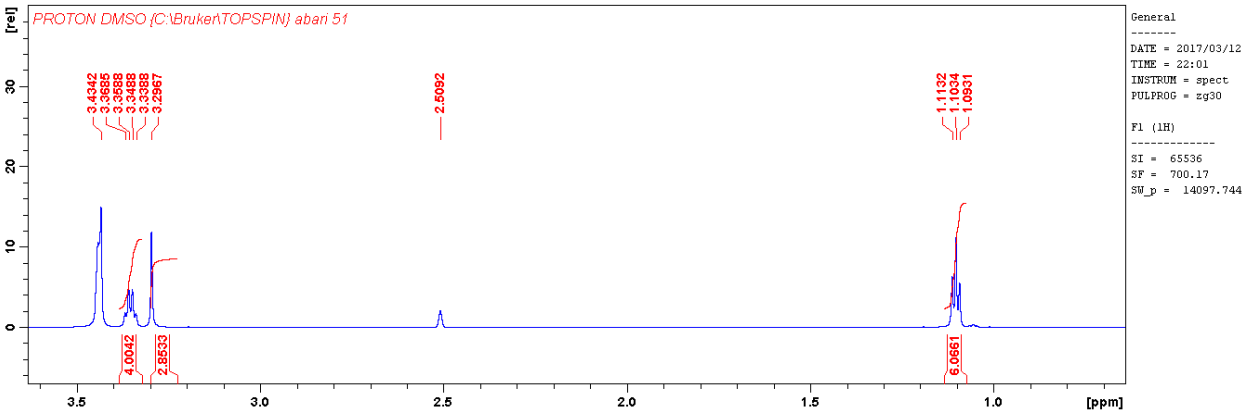
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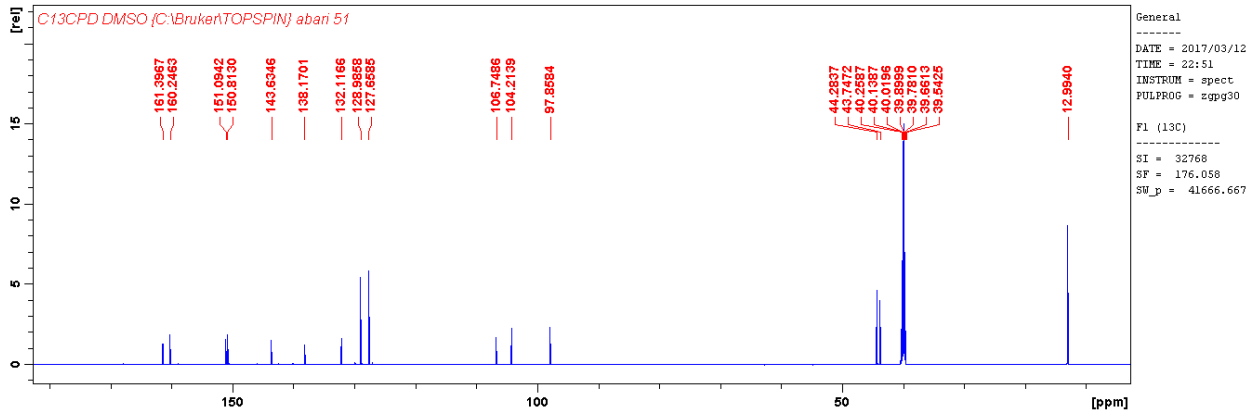
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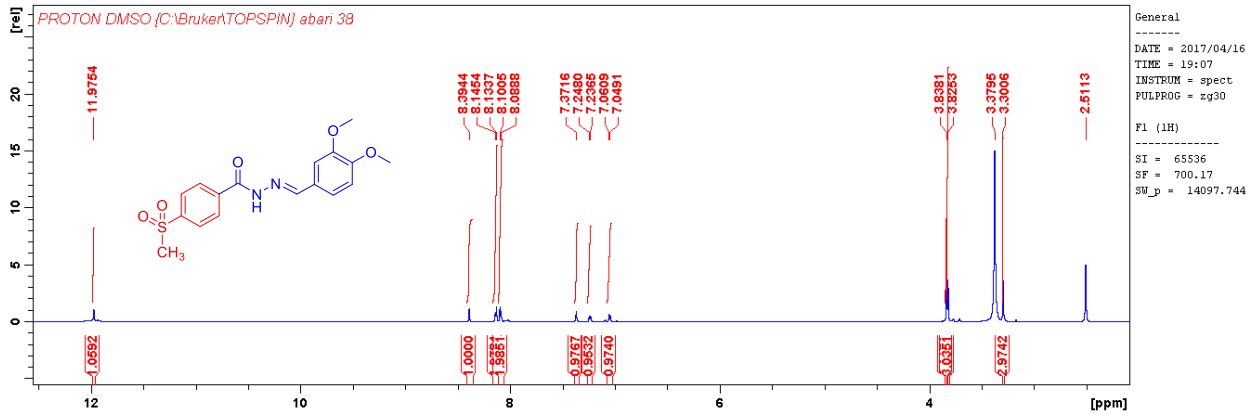
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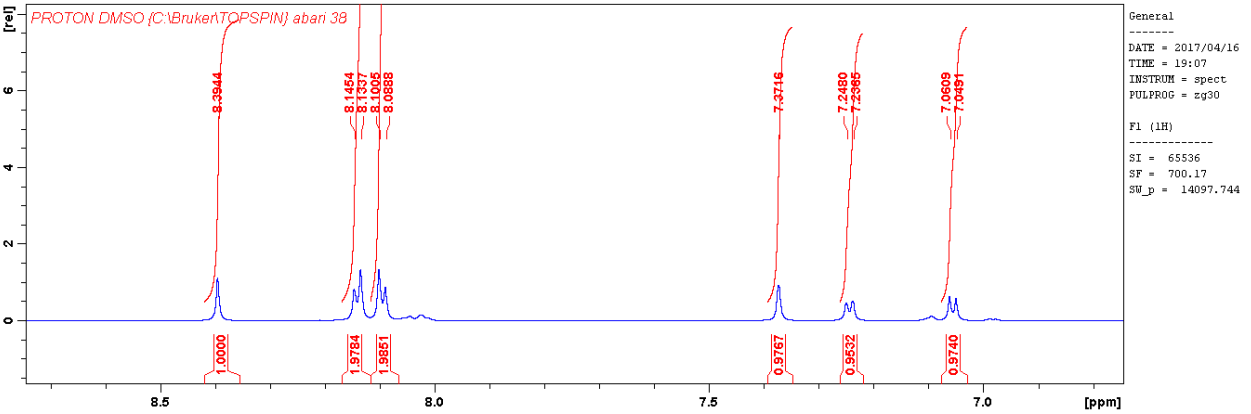
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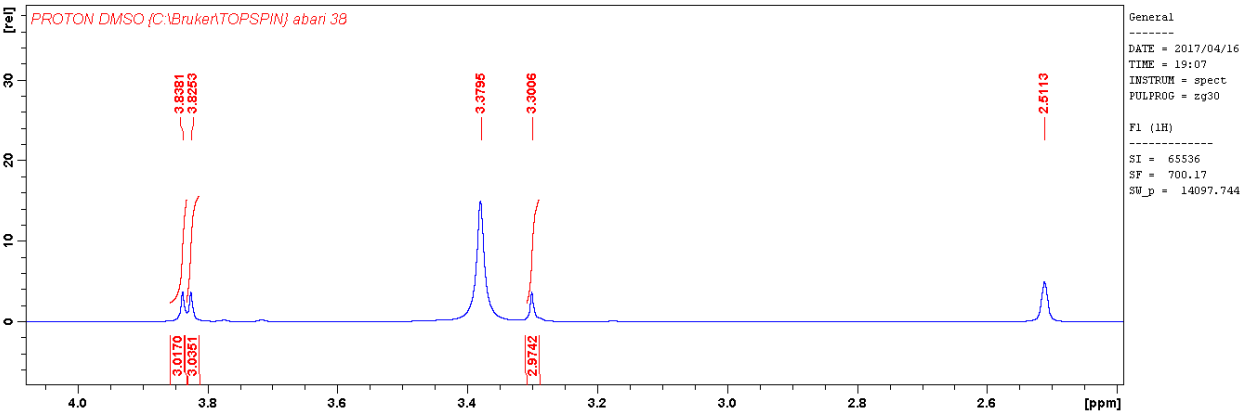
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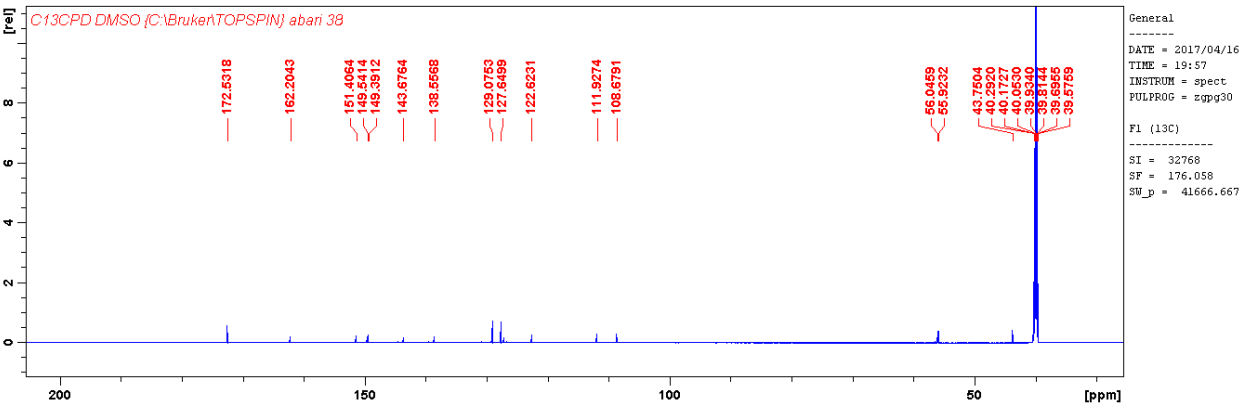
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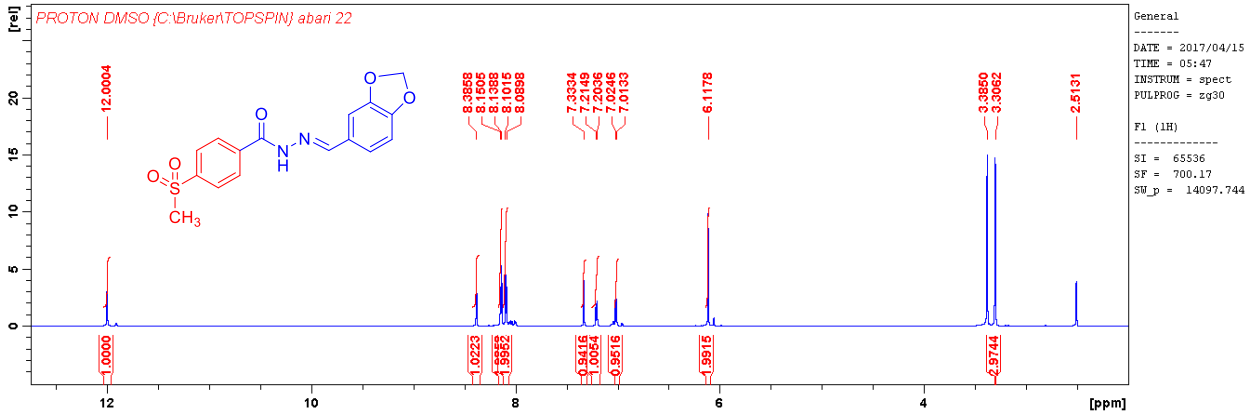
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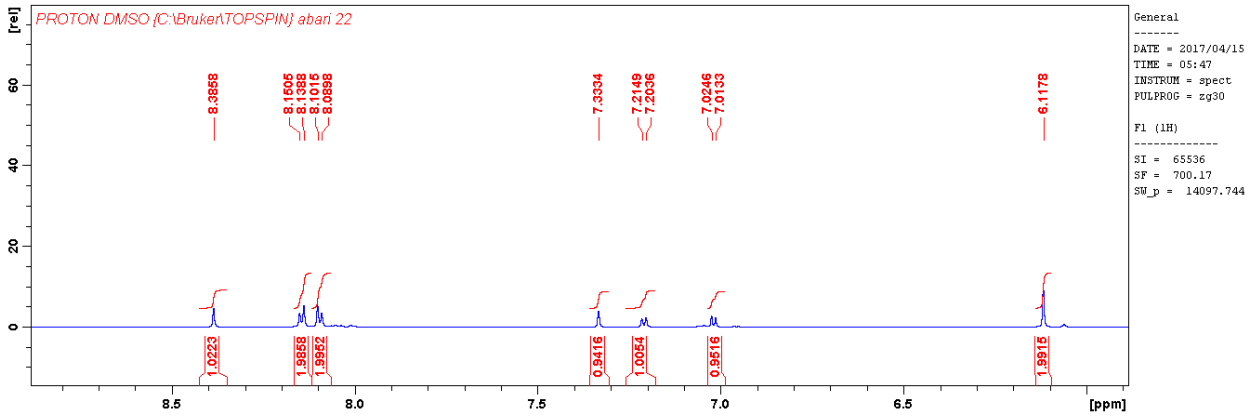
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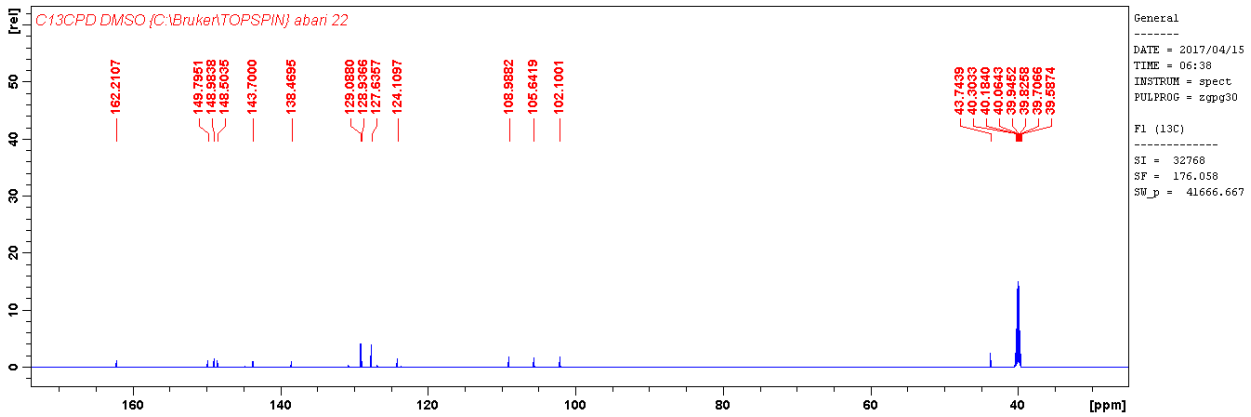
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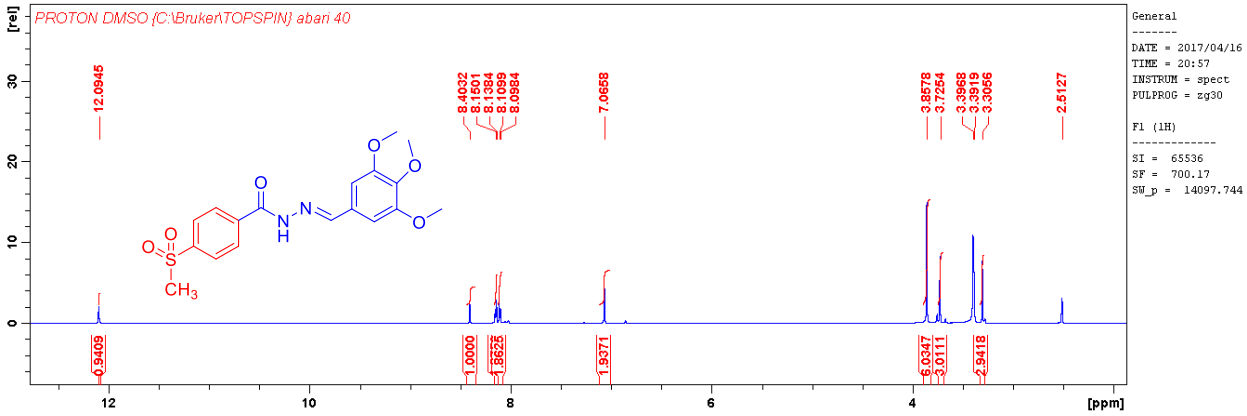
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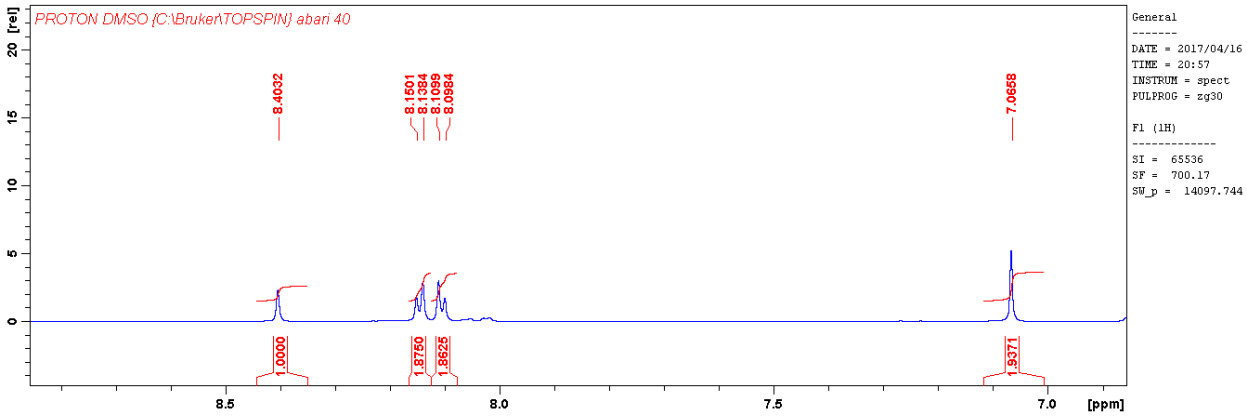
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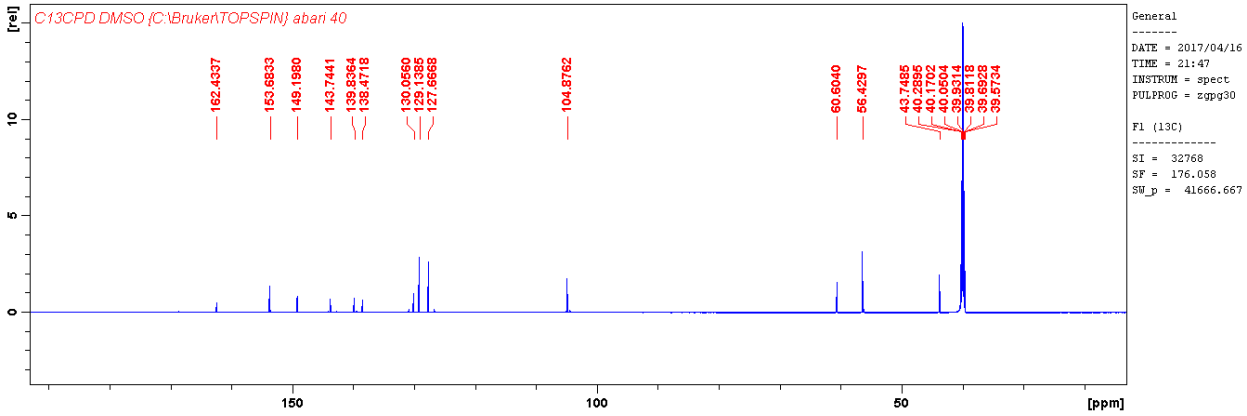
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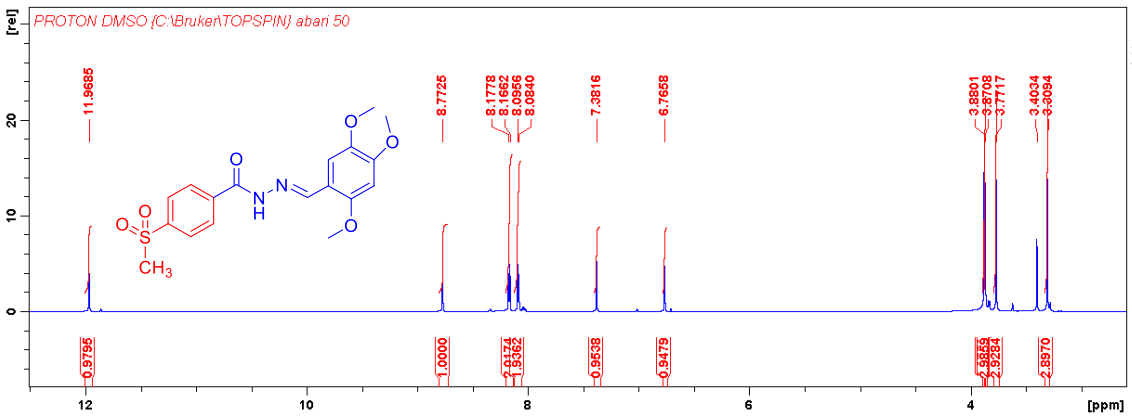
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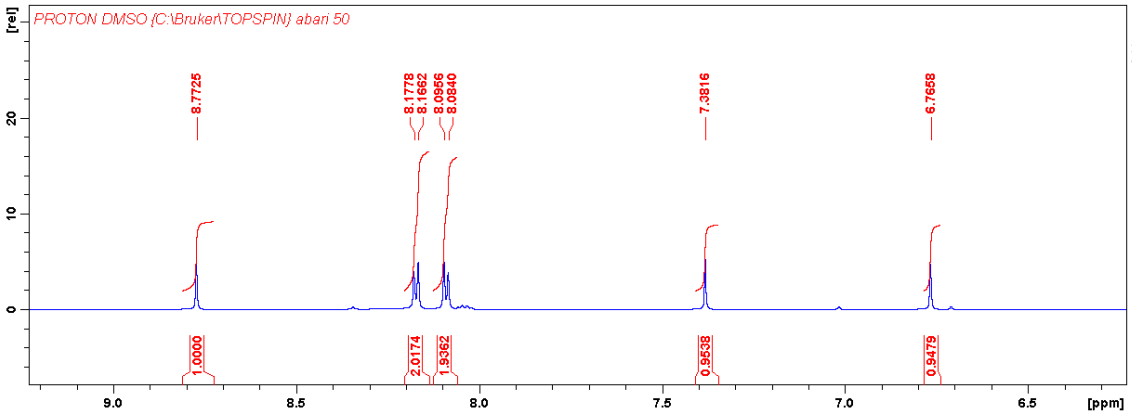
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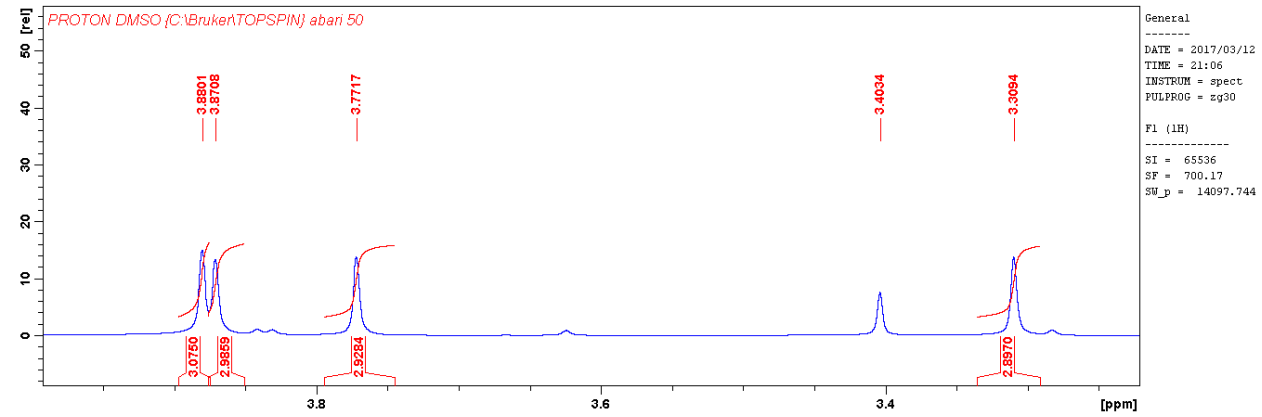
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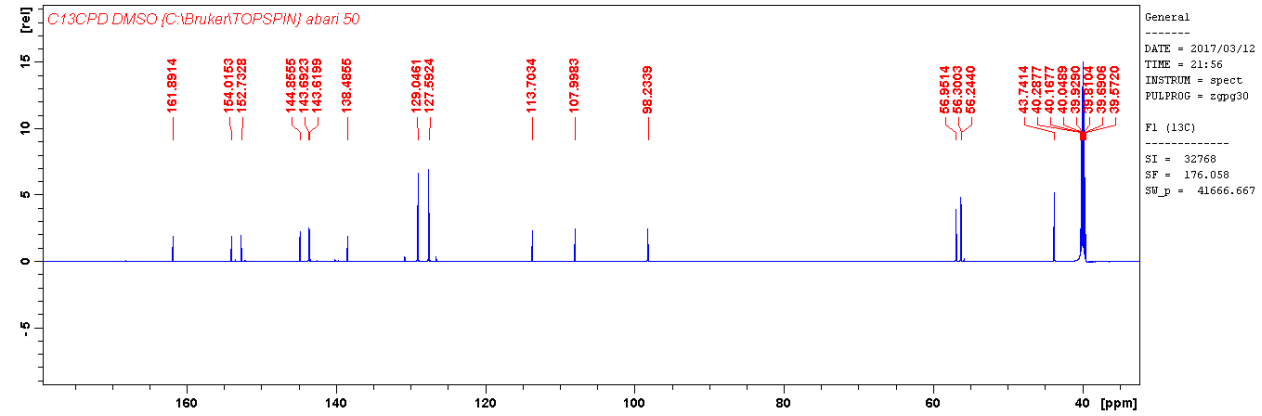
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2. Materials and methods

2.1. Biological evaluation

2.1.1. *In vitro* antitumor screening

The antitumor assay was performed on approximately 59 human tumor cell lines obtained from nine organs following the protocol of the Drug Evaluation Branch, National Cancer Institute, Bethesda, MD. Three dose-response parameters: GI50, TGI, and LC50, were calculated for each compound. Initially, the tested compounds were added to the culture at a single concentration (10 mM), and the cultures were incubated for 48 h. Endpoint determinations were made with a protein-binding dye, sulforhodamine B (SRB). The results for each tested compound were reported as the growth percentage of the treated cells compared to those of the untreated control cells. The percentage of growth was evaluated spectrophotometrically against controls not treated with test agents. The growth inhibitory effects of the most active compounds were tested in vitro against the full panel of approximately 60 human tumor cell lines at 10-fold dilutions of five concentrations, ranging from 10-4 to 10-8 M. A 48 h continuous drug exposure protocol was followed, and an SRB protein assay was used to estimate cell viability or growth. Using the seven absorbance measurements [time zero, (Tz), control growth in the absence of drug (C), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage growth was calculated at each the drug concentrations levels. Percentage growth inhibition was calculated as $[(T_i-T_z)/(C-T_z) \times 100]$ for concentration of which $T_i \geq T_z$,

$[(T_i-T_z)/T_z)] \times 100$ for concentration of which $T_i \leq T_z$.

2.1.2. Apoptosis assay

The induction of apoptosis was performed according to our previous report using Leukemia HL-60 cell line and well-established Annexin 5-FITC/PI detection kit. The cell line samples were analyzed using a FACSCalibur flow cytometer.

2.1.3. Cell cycle analysis

Using a method similar to that of our previous report, cell cycle analysis was carried out using Leukemia HL-60 cell line, stained with the DNA fluorochrome PI, and analyzed by FACSCalibur flow cytometer. Leukemia HL-60 cell was seeded at a density of 2 × 105 cells/well and incubated for 24 h in six-well plates. Fetal bovine serum (10%) was added, and the cells were incubated at 37°C in an atmosphere of 5% CO2. The medium was replaced with 1% (v/v) dimethyl sulfoxide (DMSO), containing 10.0 μM of compound **9**. Next, the cells were incubated for 48 h, washed with phosphate-buffered saline, fixed with 70% ethanol, rinsed with phosphate-buffered saline, and then stained with the DNA fluorochrome PI for 15 min at 37°C. Afterward, the samples were analyzed using a FACSCalibur flow cytometer.

2.1.4. *In vitro* cyclooxygenase (COX) inhibition assay

The colorimetric COX-2 inhibition assay (kit catalog number 560101, Cayman Chemical, Ann Arbour, MI) was used to measure the ability of the tested derivatives, and celecoxib was used to inhibit COX-2 isozyme following the manufacturer's instructions. The tested compounds' ability to inhibit ovine COX-2 was determined using an enzyme immunoassay (EIA) (kit catalog number 560101, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Cyclooxygenase catalyzes the first reaction in the biosynthesis of arachidonic acid (AA) to PGH2. PGF2a, produced from PGH2 by reduction with stannous chloride, was measured by an enzyme immunoassay (ACE™ competitive EIA). Stock solutions of the tested compounds were dissolved in a minimum volume of DMSO. Subsequently, 10 μl of various concentrations of test drug solutions (0.01, 0.1, 1, 10, 50, and 100 lM in a final volume of 1 mL) were added to a series of supplied reaction buffer solutions (960 μl, 0.1 MTris–HCl pH 8.0, containing 5 mM EDTA and 2 mM phenol) with either COX-1 or COX-2 (10 μl) enzyme in the presence of heme (10 μl) for 5 min at 37°C; after which 10 μl of AA (100 lM) solution was added, and the COX reaction was stopped by the addition of 50 μl of 1 M HCl after 2 min. PGF2a, produced from PGH2 by reduction with stannous chloride, was measured by enzyme immunoassay. This assay was based on the competition between PGs and a PG-acetylcholinesterase conjugate (PG tracer) for a limited amount of PG antiserum. The PG tracer amount that can bind to the PG antiserum is inversely proportional to the concentration of the PGs in the wells since the concentration of PG tracer is held constant while the concentration of PGs varies. This antibody, PG complex, binds to a mouse anti-rabbit monoclonal antibody that

had been previously attached to the well. The plate was washed to remove any unbound reagents, and then Ellman's reagent, containing the substrate of acetylcholine esterase, was added to the well. This enzymatic reaction produced a distinct yellow color that was absorbed in 410 nm. The intensity of the color, determined spectrophotometrically, was proportional to the amount of PG tracer bound to the well, which was inversely proportional to PGs present in the well during the incubation: Absorbance $\propto$ [Bound PG Tracer] a1/PGs. Percent inhibition was calculated by comparing the treated compound to various control incubations. The test compound concentration, causing 50% inhibition (IC50, lM), was calculated from the concentration-inhibition response curve (duplicate determinations).

2.1.5. EGFR and Her2 assays

In vitro luminescent EGFR tyrosine kinase assay, using Kinase-Glo® MAX as a detection reagent, and *In vitro* HER2 tyrosine kinase assay, using DP-Glo™ reagent that measures the adenosine diphosphate (ADP) formed from a kinase reaction, were performed; this luminescent signal positively correlates with ADP amount and kinase activity. In 17 µl of distilled water, 6 µl of Kinase assay buffer and 1 µl ATP and 1 µl PTK substrate were mixed (master mixture). In every well, mix 20 µl of the master mixture and 5 µl of Inhibitor solution (Test Inhibitor) for positive control, and use 5 µl of the same solution without inhibitor (Inhibitor buffer) as the blank solution; add 20 µl of diluted EGFR or Her2 enzyme and incubate at 30°C for 40 min. Add 50 µl of Kinase-Glo Max reagent to each well, cover the plate with aluminum foil and incubate the plate at room temperature for 15 min. Afterward, measure luminescence using the microplate reader. "Blank" value is subtracted from all readings. All samples and controls should be tested in duplicates.

2.2. Molecular docking method

Molecular docking protocols were carried out using the MOE 2008.10 software from Chemical Computing Group Inc. (Montreal, QC, Canada) following previously established methods. The crystal structures of COX-2 (PDB code: 1CX2), EGFR (PDB Code: 1M17), and HER2 (PDB Code: 3PP0) were retrieved from the protein data bank. The SwissTargetPrediction and the

SwissADME online tools were used to predict the test compounds' physicochemical, pharmacokinetics, and drug-likeness properties and used reference drugs.

2.2.1. Selection of protein crystal structure

The ligand-bound crystallographic structures of COX-2, EGFR, and Her2 are available in the Protein Data Bank (http://www.rcsb.org/). In this study, COX-2 (PDB Code: 1CX2), EGFR (PDB code: 1M17), and Her2 (PDB code: 3PP0) with bound inhibitors were evaluated and selected for docking. The structure preparation process in MOE corrected the errors of the protein. The first step in the generation of suitable protein structures is the assignment of hydrogen positions based on default rules. All bound waters and cofactors contained in the PDB file were removed. Finally, partial charges (the Gasteiger methodology) were calculated, and the active site of the ensemble was defined as the collection of residues within 10.0 Å of the bound inhibitors and comprised the union of all the ligands in the ensemble. All atoms located less than 10.0 Å from any ligand atom were considered.

2.2.2. Preparation of the ligand

The ligand coordinates were built using the builder tool of the MOE program. Next, the correct atom types (including hybridization states) and correct bond types were defined, hydrogen atoms were added, charges were assigned to each atom, and finally, the energies of the structures were minimized (MMFF94x, gradient: 0.01). The energies of the ligand structures were previously minimized using the semi-empirical AM1 method with MOE program; point charges were assigned to the ligands.

2.2.3. Docking experiment

The docking experiments on COX-2, EGFR, and Her2 were carried out by superimposing the energy-minimized ligand on bound inhibitors in the PDB files; after which, co-crystallized inhibitors were deleted. The default Triangle Matcher placement method was used for docking. GBVI/WSA dG scoring function, which estimates the ligand's free binding energy from a given pose, was used to rank the final poses. The geometry of the resulting complexes was studied

using the MOE's Pose Viewer utility. The ligand-enzyme complex with thelowest S score was selected.

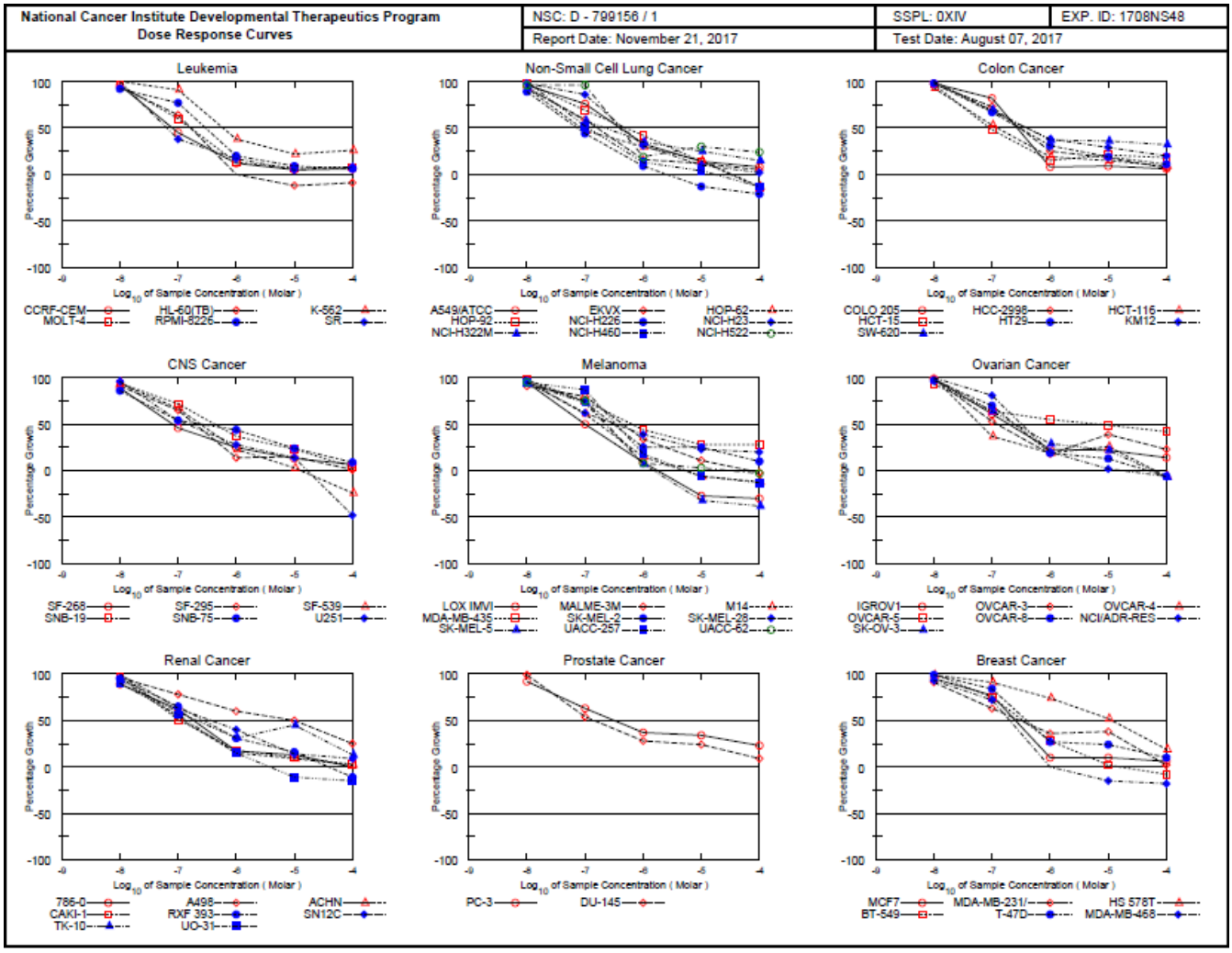

Figure S1. NCI Dose-Response Curves for compound **20**.