

Biological testing

1. *In vitro* anti-proliferative activity

MTT assay protocol was applied as described previously to assess the anti-proliferative activity of the synthesized compounds. Three human cancer cell lines (HepG2, PC3 and MCF-7) were used in this test. Briefly, cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin and 100 µg/ml streptomycin, at 37°C in a 5% CO₂ incubator. Cells were seeded in a 96-well plate at a density of 5×10^3 cells / well and left overnight to attach. Cells were treated with different concentrations of the synthesized compounds and incubated for 72 h. 50 µl of MTT solution at 5mg/ml was then added and incubated for 4 h. 100 µl Dimethyl sulfoxide (DMSO) was added into each well to dissolve the purple formazan crystals. The developed color was measured at 570 nm using a plate reader (EXL 800, USA). The relative cell viability in percentage was calculated as (A₅₇₀ of treated samples/A₅₇₀ of untreated sample) X 100. The half maximal inhibitory concentration (IC₅₀) values were calculated using non-linear regression analysis of the data obtained from three independent experiments. Data were presented as mean ±SD.

2.1. Supernatant preparation

Cell culture supernatants were prepared from HepG2 cell line. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) , 100 U/ml of penicillin and 100 µg/ml of streptomycin at 37 °C in a humidified 5% CO₂ incubator. The candidate compounds and thalidomide were dissolved in DMSO as a stock solution at 100 mmol/L and diluted with an FBS-free medium to achieve the designated concentrations (IC₅₀). The same concentration of DMSO without any compounds was used as a control. 0.5×10^6 cells HepG2 cells were cultured into T25 flask, allowed to adhere overnight followed by treatment with the tested compounds. After 72 h, the media was collected and centrifuged for 15 min at 5000 rpm and equal volume of cell culture supernatants were collected and utilized for immunoassay using different kits [1].

2.2. Cell lysate preparation

The tested compounds and thalidomide were incubated for 72 h with HepG2 cells. Then, the cells were collected by trypsinization using 0.25 % trypsin/EDTA solution. Cell palettes then were washed three times with phosphate buffer saline (PBS) and lysed by three repetitive freezing/thawing cycles (thawing at 37 °C for 2 min and freezing at –80 °C for 15 min), followed by homogenization of the cells by passing through a 20G needle [2].

2.3 Estimation of NF- κ B P65 and Caspase-8 levels in HepG2 cell lysate.

50 μ L/well of cell lysate or standard were applied to the microtiter plates pre-coated with the specific monoclonal antibodies of caspase-8, and NF- κ B P65 and incubated at 37 °C for 90 min. All wells were washed three times using the wash buffer to remove any unbound proteins. Biotinylated detection antibody was then added, followed by incubation at 37 °C for 60 min. The solution was aspirated and washed three times with the wash buffer. Horseradish peroxidase (HRP) conjugated streptavidin was added to all wells and incubated for 30 min at 37 °C. At the end of the incubation period, the solution was removed and 90 μ L of substrate reagent was added to each well and incubated at 37 °C for 15 min. To stop the reaction, 50 μ L of stop solution was added and then the absorbance was measured immediately at 450 nm using the ELISA plate reader (FLUO star OPTIMA). Concentrations of caspase-8 and NF- κ B P65 were determined by interpolating the absorbance values with that of the standard curve obtained from the known concentrations of standard proteins. [2].

2.3 Estimation of VEGF and TNF- α in HepG2 levels cell culture supernatant.

In order to quantify the VEGF and TNF- α protein that was secreted extracellularly by HepG2 cells, following drug treatment, the media content was analyzed by ELISA using VEGF and TNF- α ELISA kits (E-EL-H1600, Elabscience and ab181421, abcam) respectively, as per manufacturer's instructions.

3. Cell cycle analysis

To determine the effect of the synthesized compounds on cell cycle distribution, cell cycle analysis was performed using propidium iodide (PI) staining and flow cytometry analysis for compound **XIVc** Flow Cytometry Kit for Cell Cycle Analysis (ab139418_Propidium Iodide Flow Cytometry Kit/BD) was used in this test. HepG2 cells were treated with the IC₅₀ of compound **XIVc** for 72 h. Then, the cell pellets were fixed in 70% ethanol at 4 °C for 12 h. After that, cells were washed with cold PBS, incubated with 100 μ L RNase A at 37 °C for 30 min, and stained with 400 μ L PI in the dark at room temperature for further 30 min. The

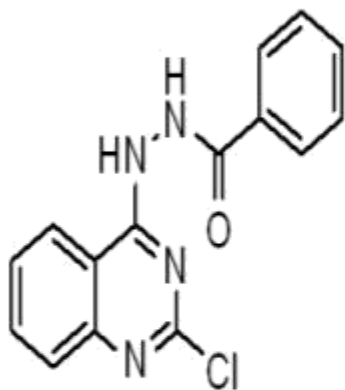
stained cells were measured using Epics XL-MCL™ Flow Cytometer (Beckman Coulter), and the data were analyzed using Flowing software (version 2.5.1, Turku Centre for Biotechnology, Turku, Finland).

4. Apoptosis assay

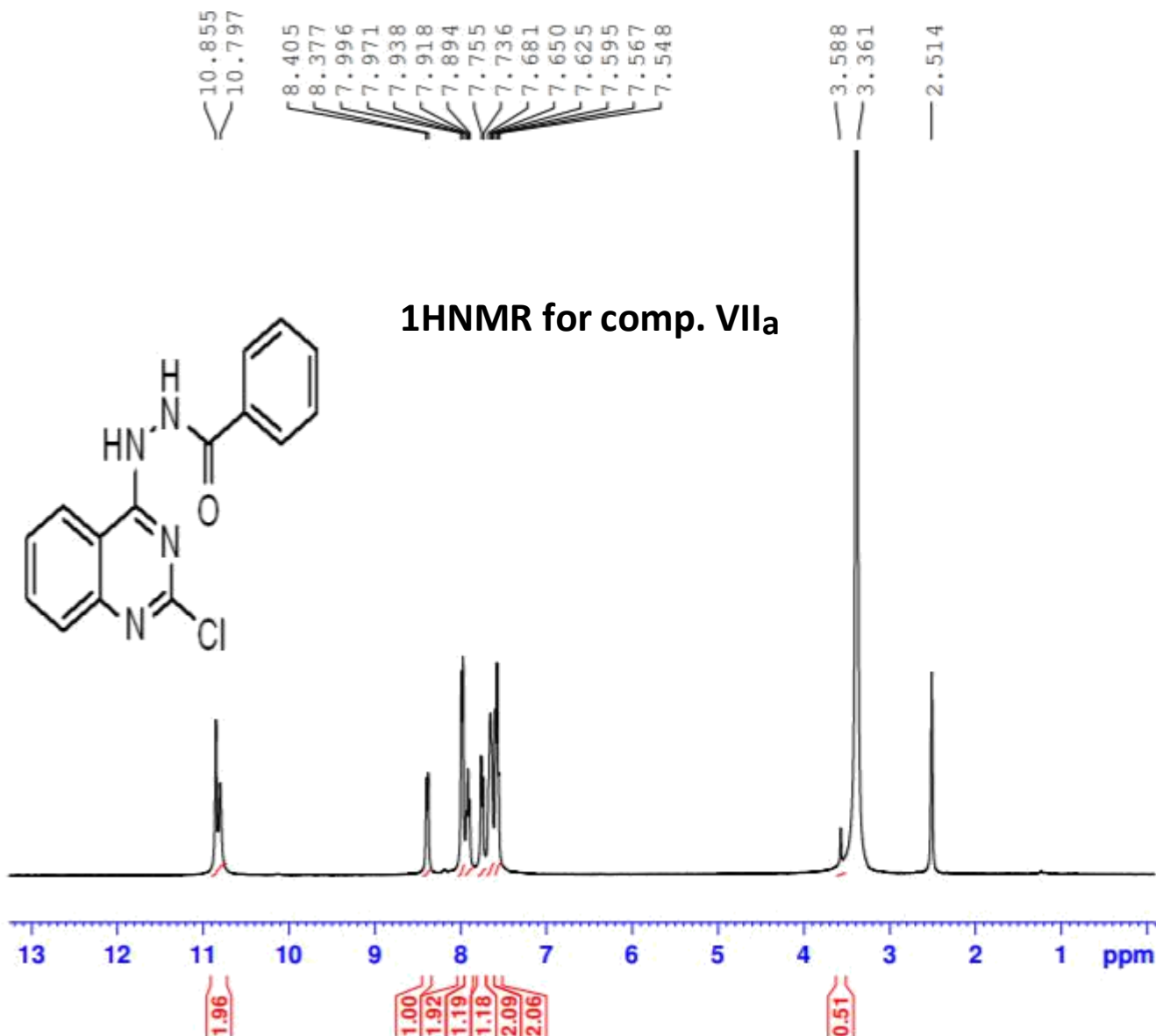
Flow cytometry was used to investigate the effect of synthesized compounds on cellular apoptosis. HepG2 cells were treated with compound **XIVc** at IC₅₀ for 72 h, cells then were collected by trypsinization, centrifuged, washed three successive times with PBS, suspended in 500 µl binding buffer, and double stained with 5 µl Annexin V-FITC and 5 µl PI in the dark at room temperature for 15 min. The stained cells were measured using Epics XL-MCL™ Flow Cytometer and analyzed using the machine provided software.

References

1. Dawoud, N.T.A.; El-Fakharany, E.M.; Abdallah, A.E.; El-Gendi, H.; Lotfy, D.R. Synthesis, and docking studies of novel heterocycles incorporating the indazolylthiazole moiety as antimicrobial and anticancer agents. *Sci. Rep.* 2022, 12, 3424.
2. Abdallah, A.E.; Mabrouk, R.R.; Elnagar, M.R.; Farrag, A.M.; Kalaba, M.H.; Sharaf, M.H.; El-Fakharany, E.M.; Bakhotmah, D.A.; Elkaeed, E.B.; Al Ward, M.M.S. New Series of VEGFR-2 Inhibitors and Apoptosis Enhancers: Design, Synthesis and Bi-ological Evaluation. *Drug Des. Devel. Ther.* 2022, 16, 587–606.



1HNMR for comp. VIIa



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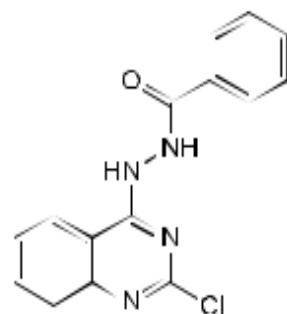
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 RG 205.37
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 DE 6.50 usec
 TE 298.0 K
 D1 1.00000000 sec
 TD0 1

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 PLW1 18.00000000 W

F2 - Processing parameters
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 WDW EM
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 LB 0.30 Hz
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BRUKER

Transmittance [%]



IR for comp. VIIa

3272.28

1669.10

1573.79

1523.11

1486.68

1421.30

1376.84

1281.43

1224.26

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799.02

758.00

704.25

685.22

601.37

3500

3000

2500

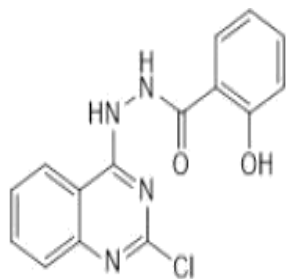
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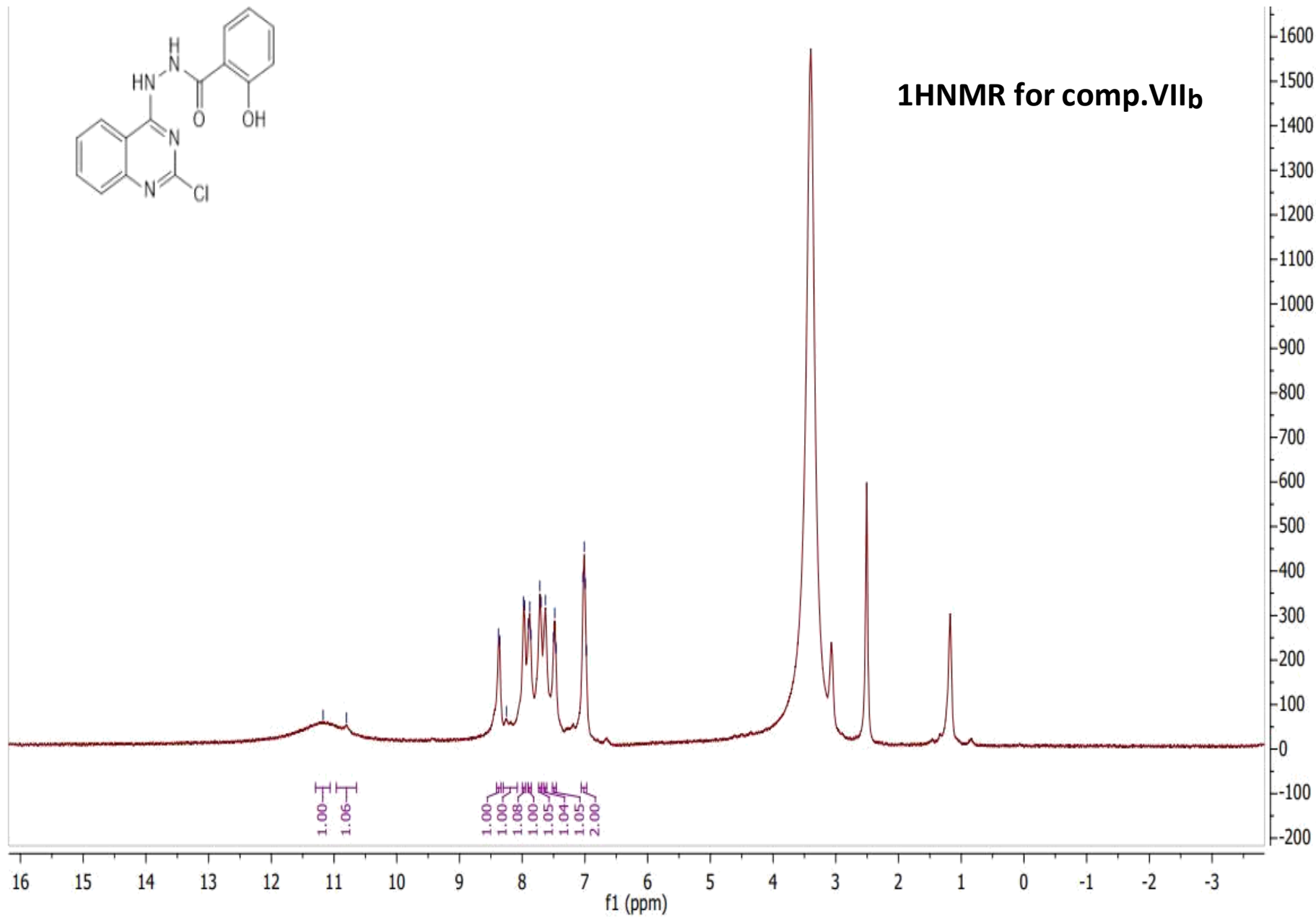
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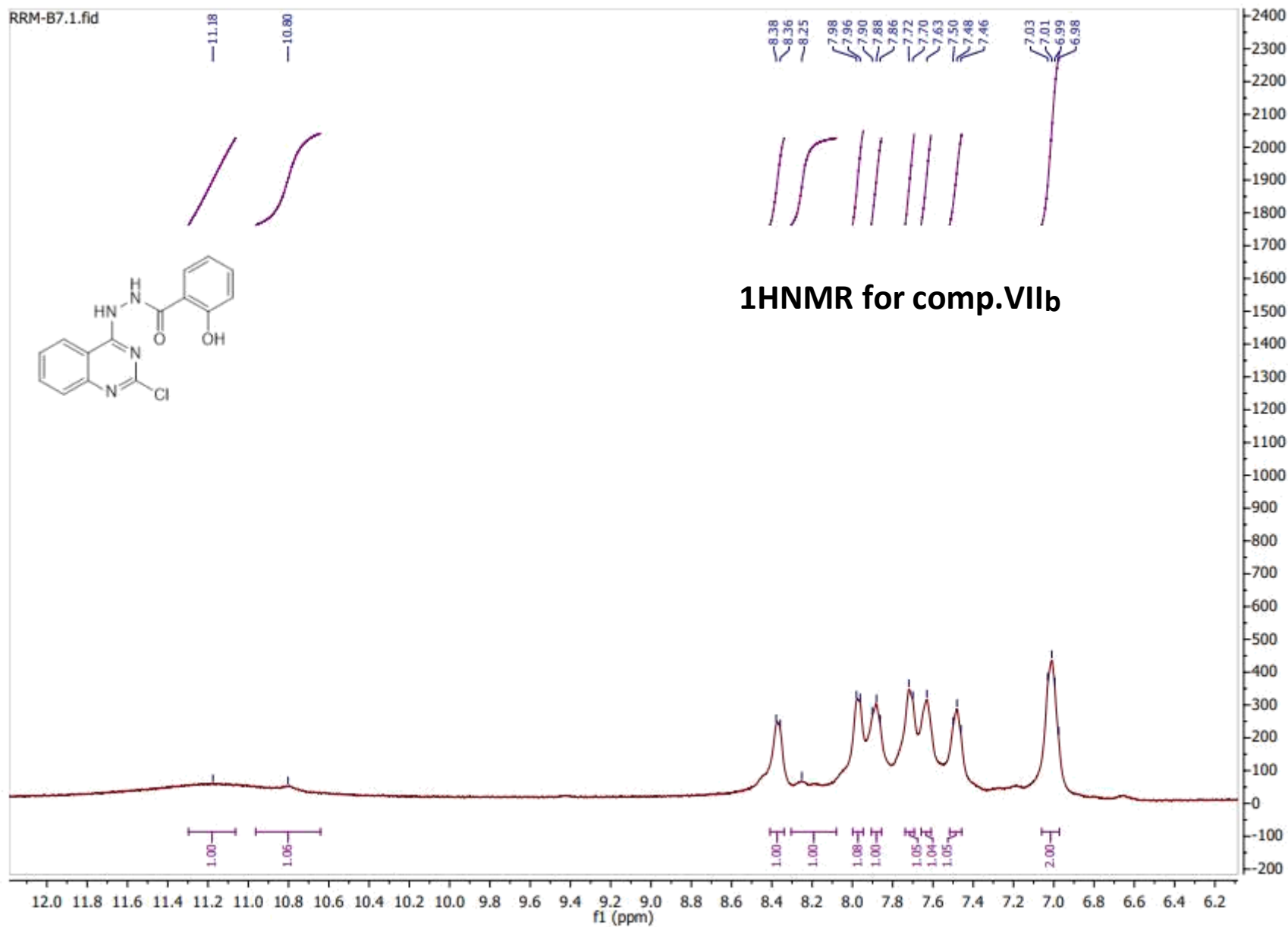
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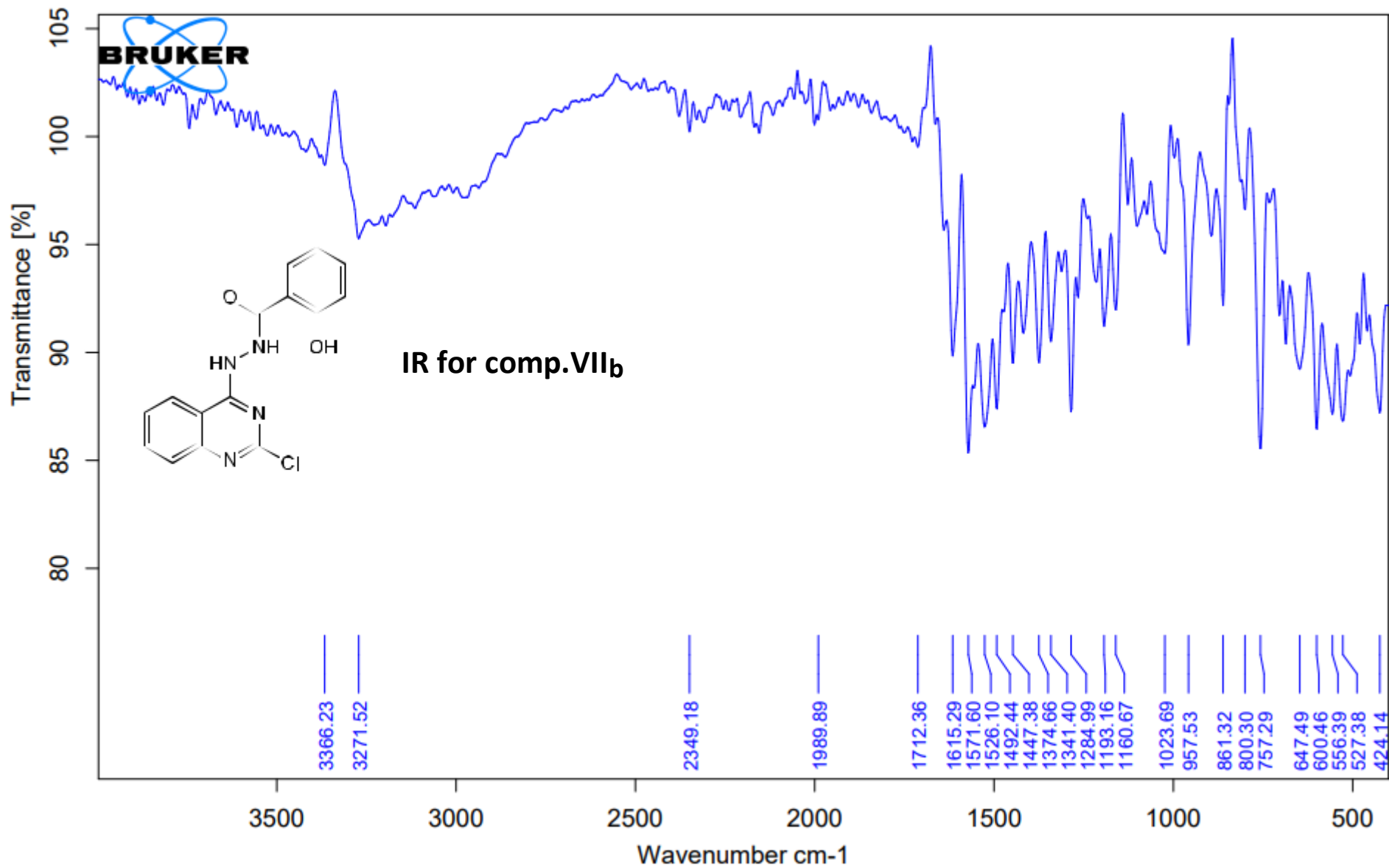


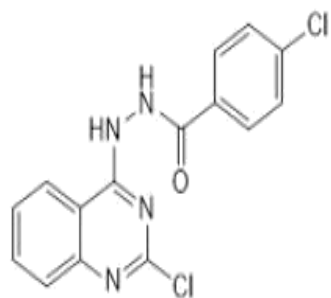
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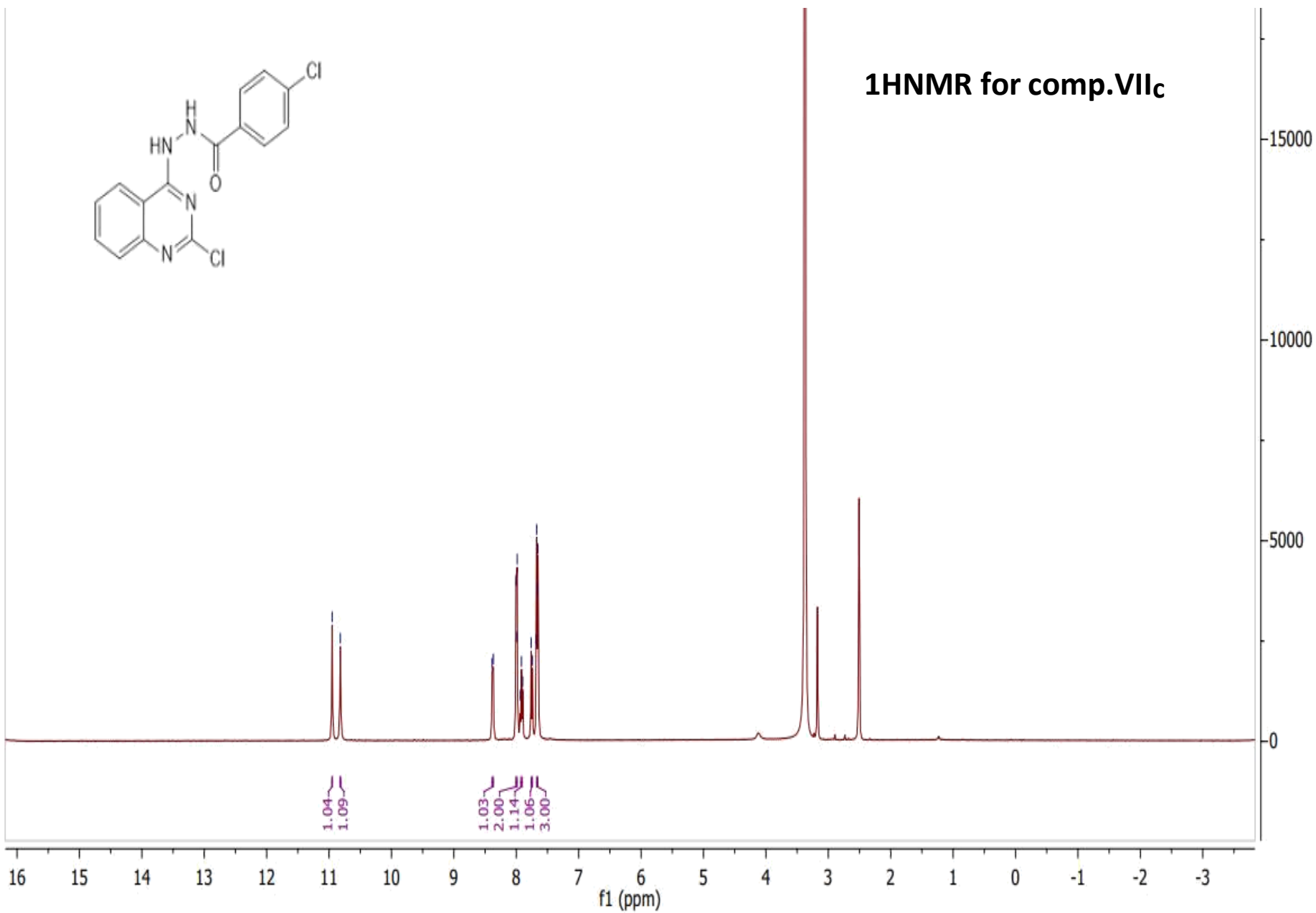
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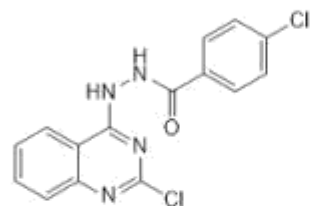




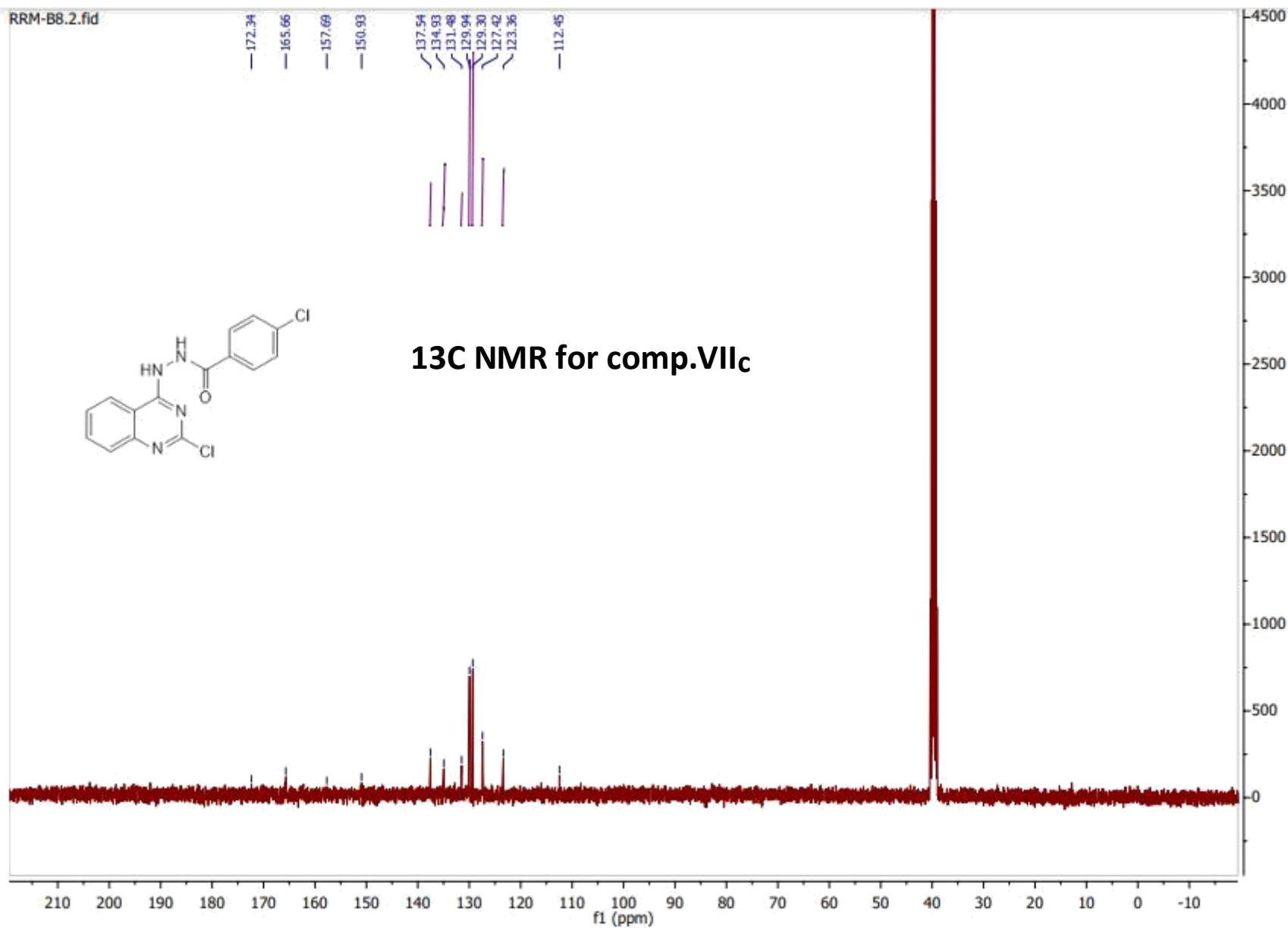
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RRM-B8.2.fid

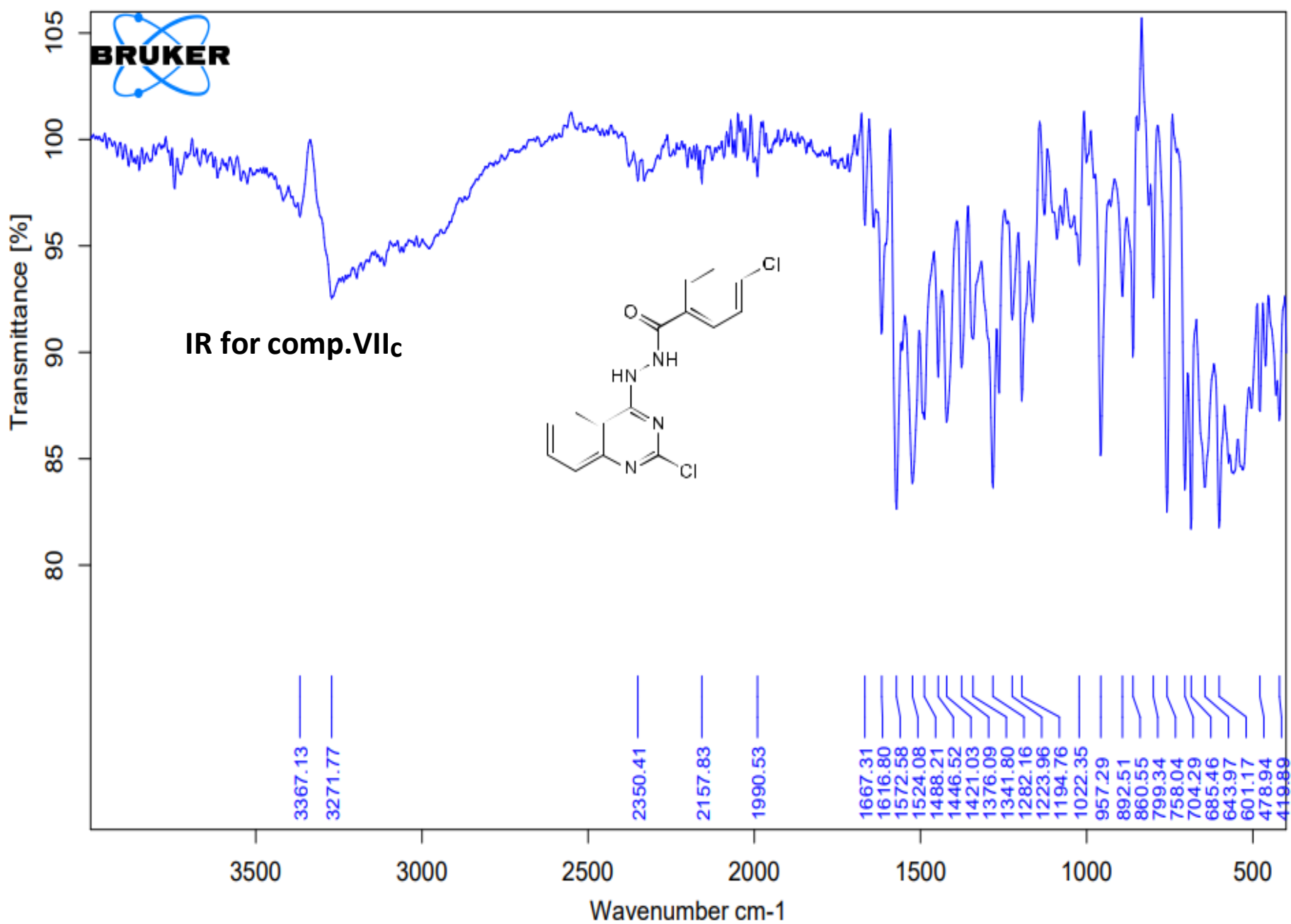
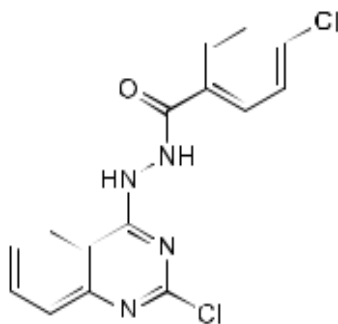


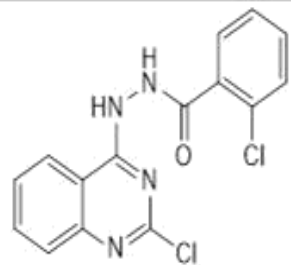
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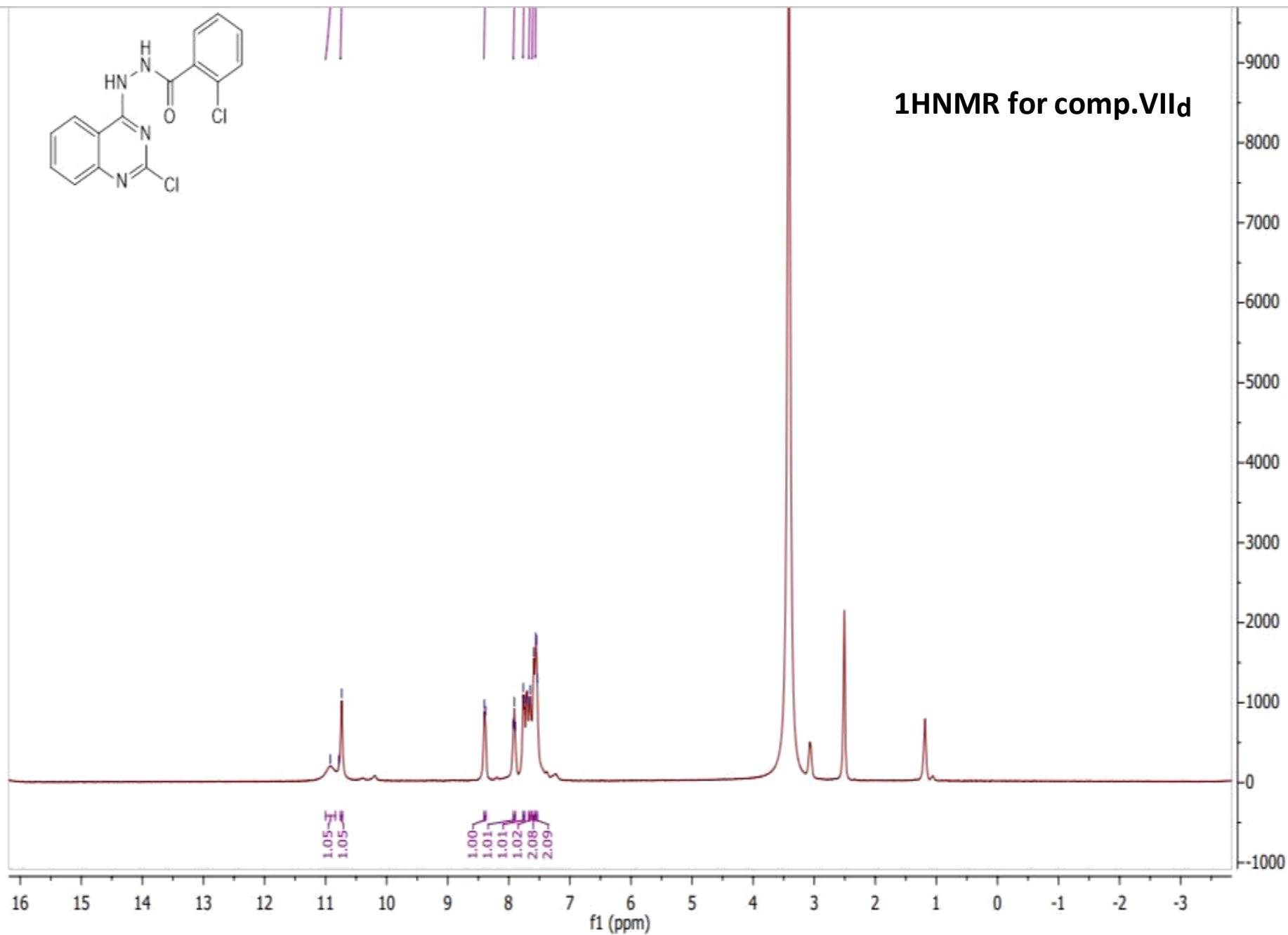


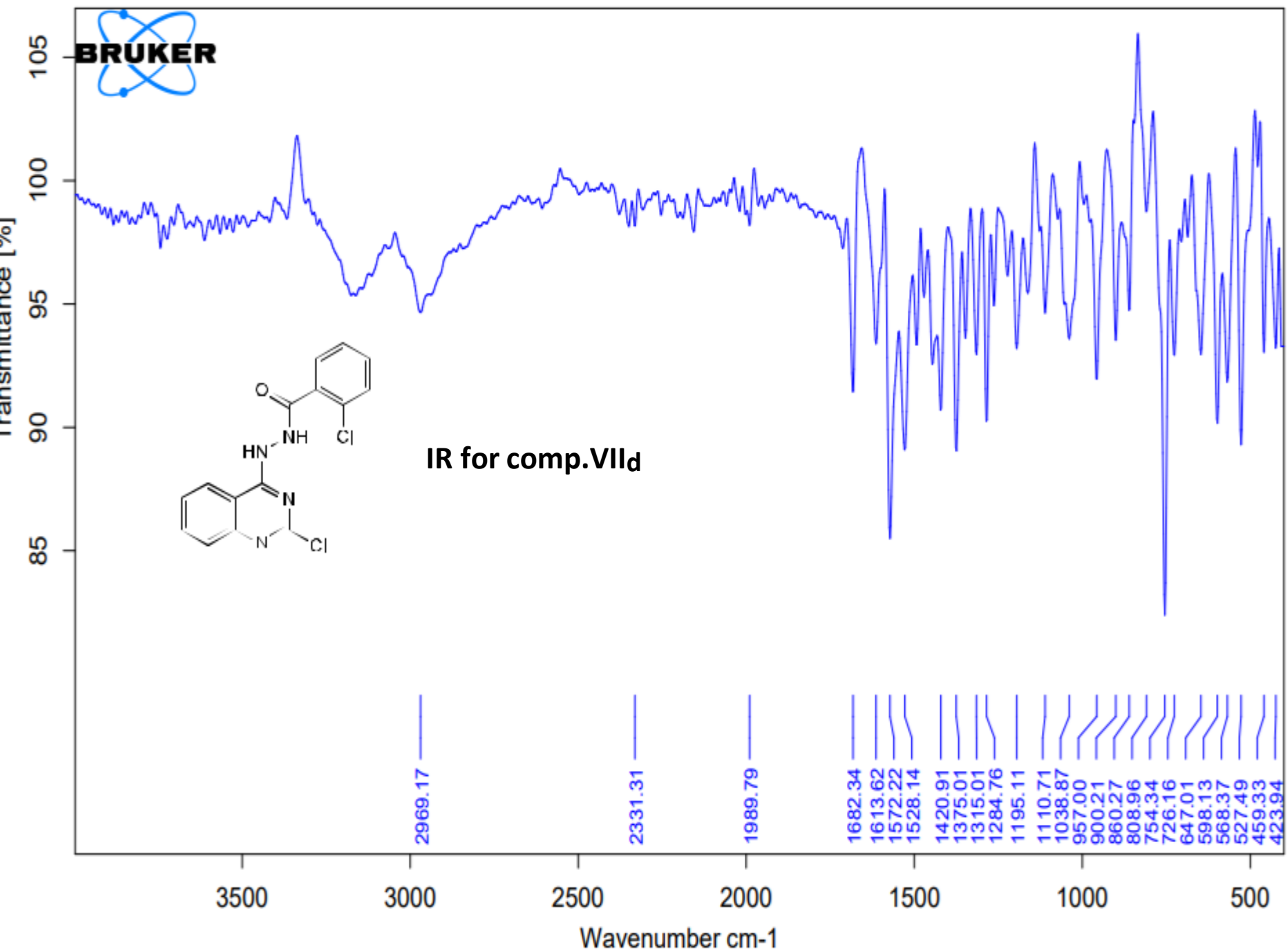
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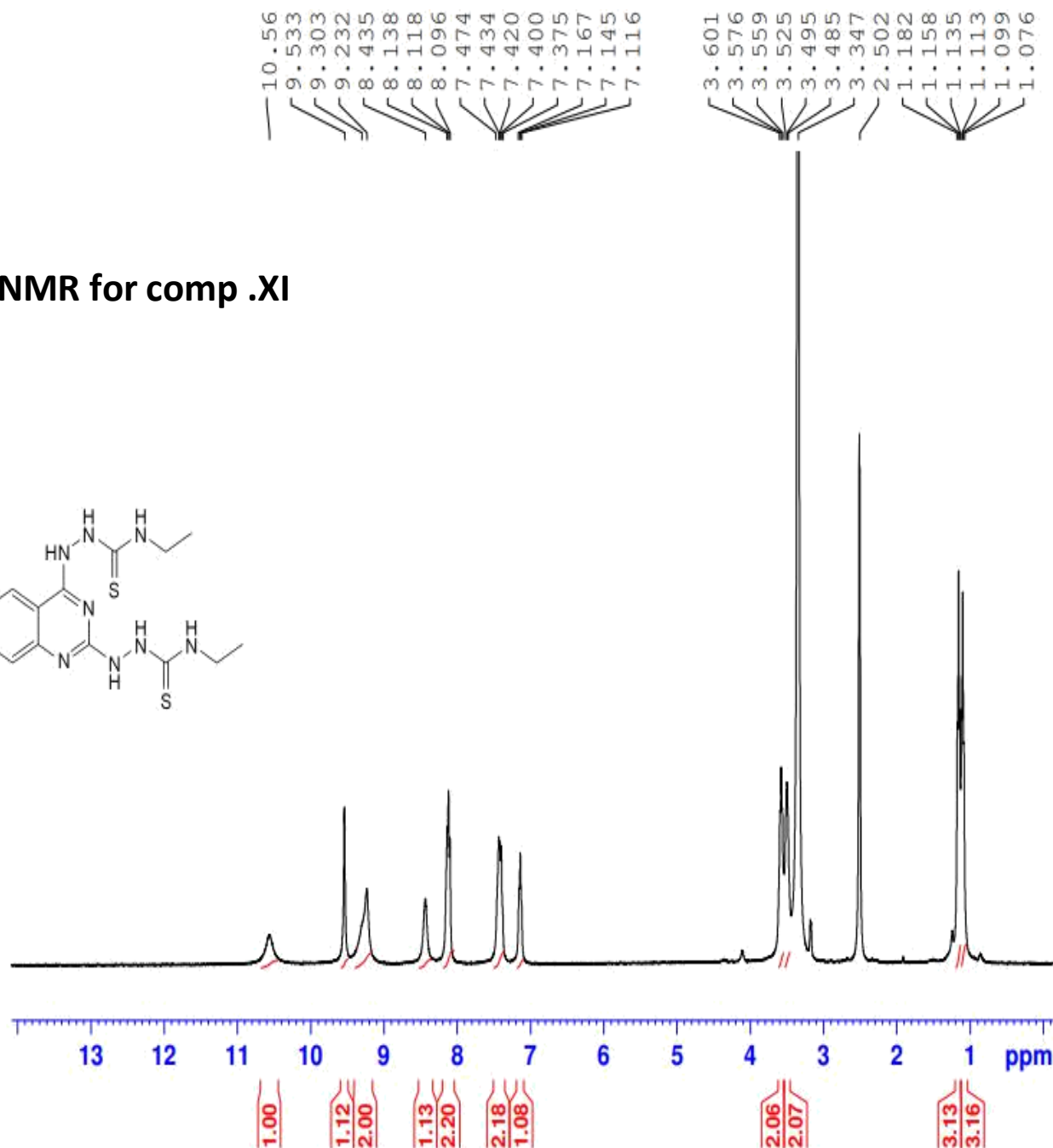
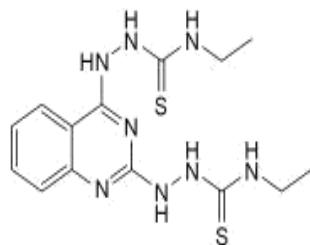


¹H NMR for comp.VII d





¹H NMR for comp .XI



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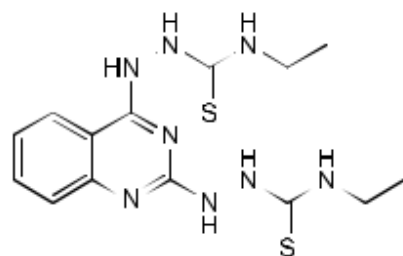
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 NUC1 1H
 P1 12.00 usec
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Transmittance [%]



IR for comp .XI

3345.64

3199.34

2971.97

1619.52

1536.70

1460.25

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465.96

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3000

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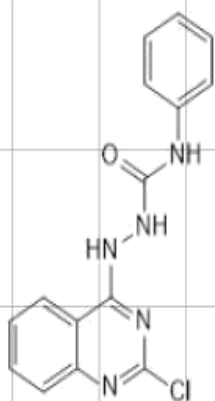
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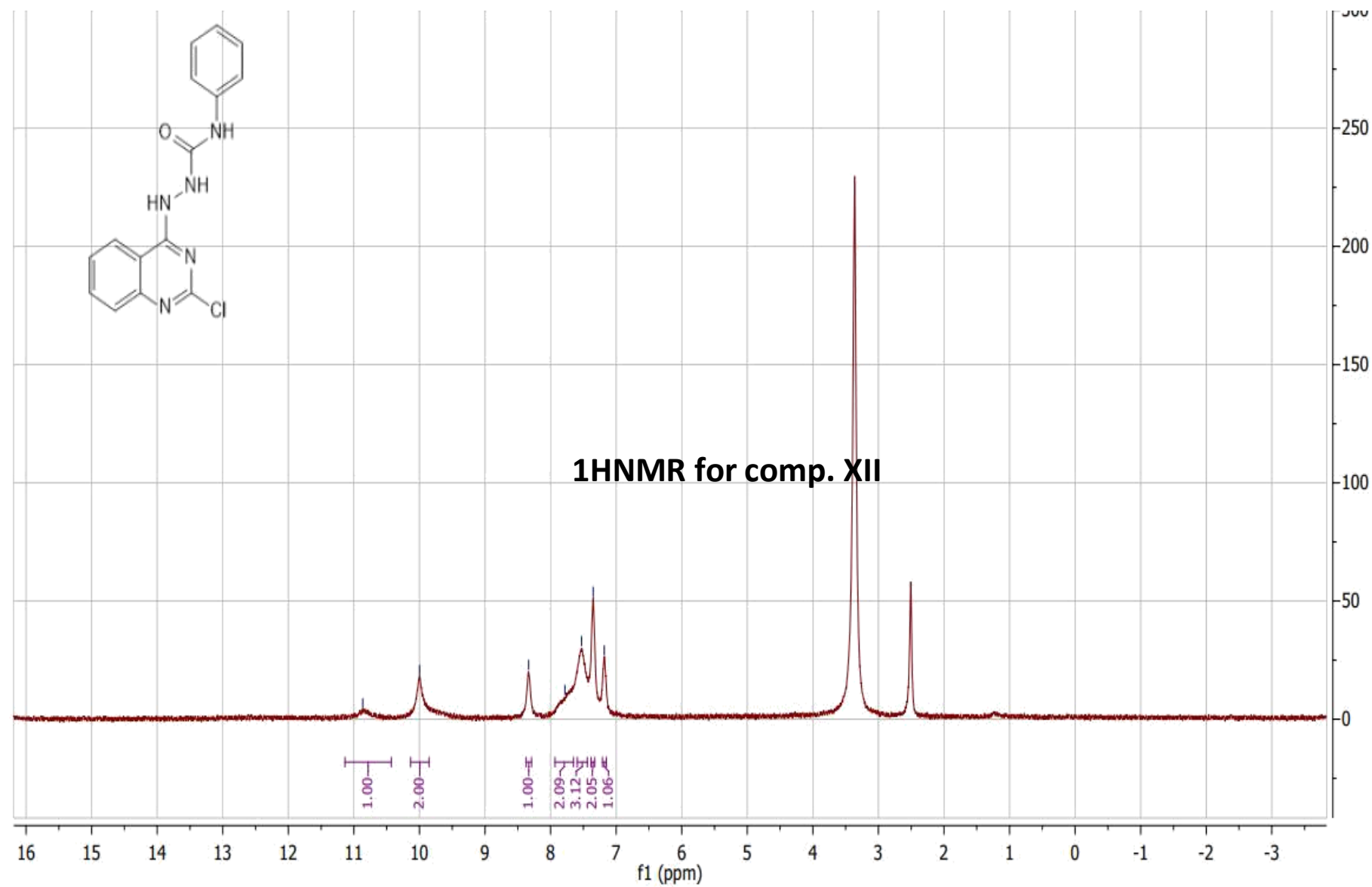
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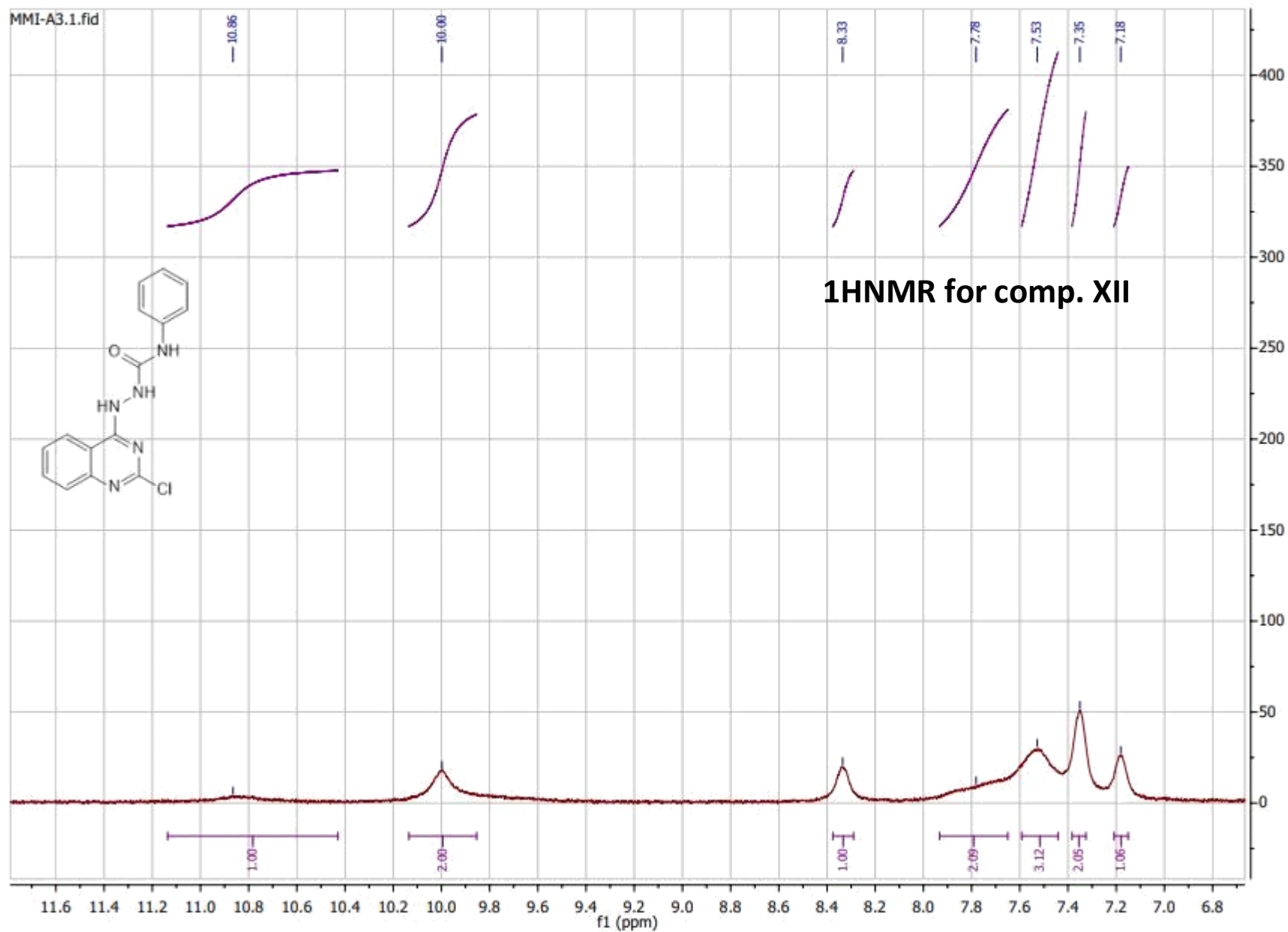
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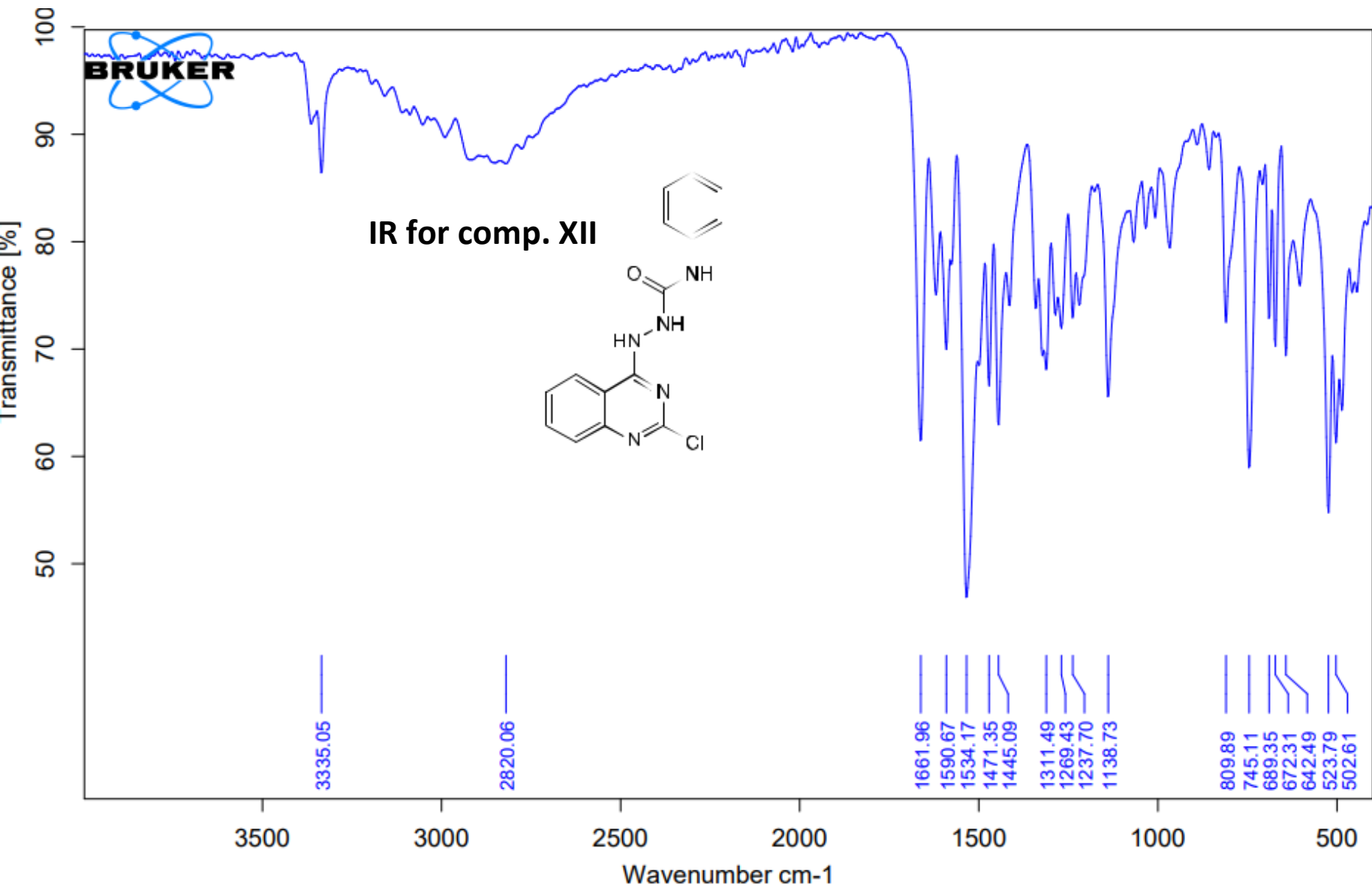


¹H NMR for comp. XII

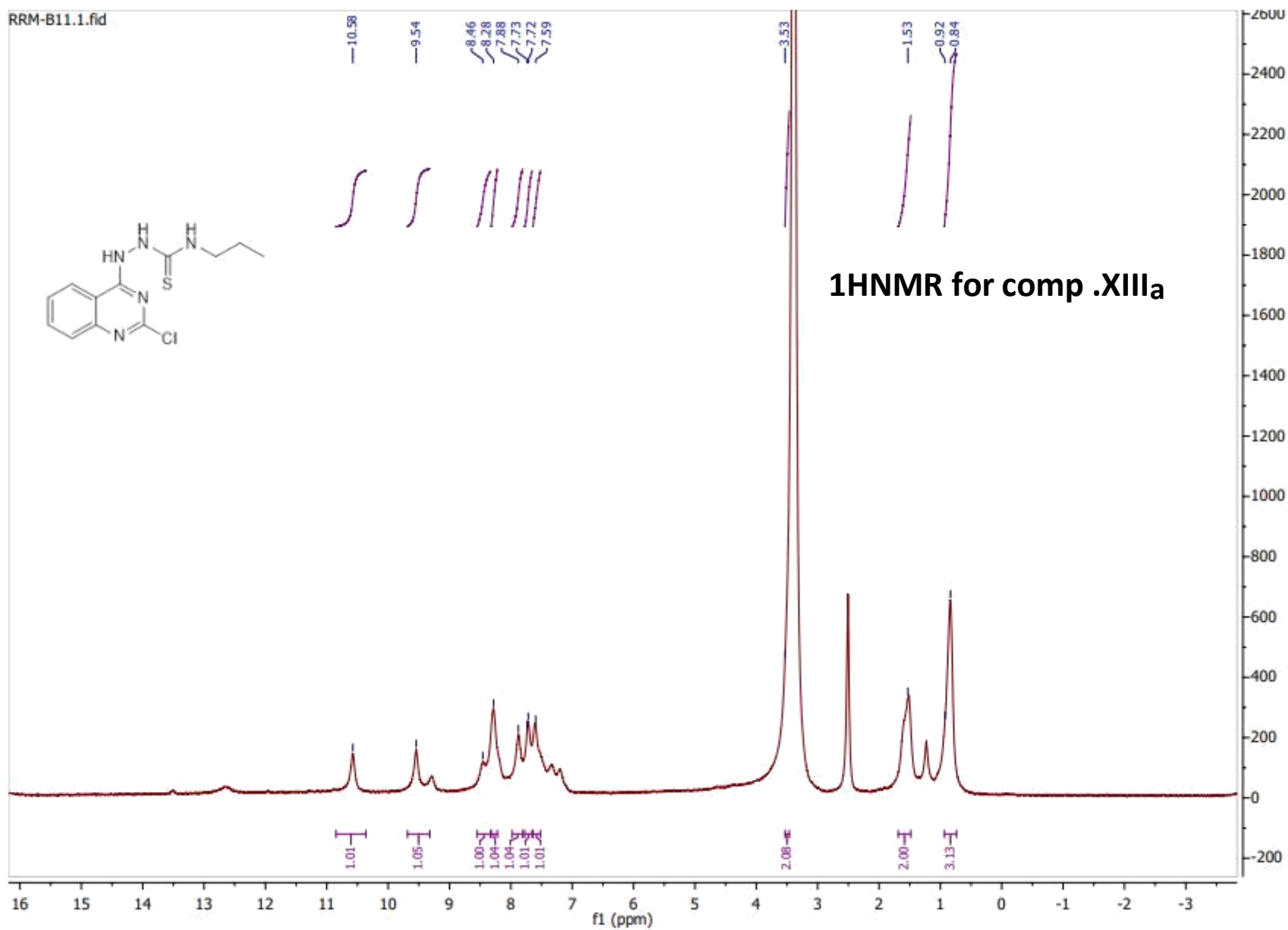
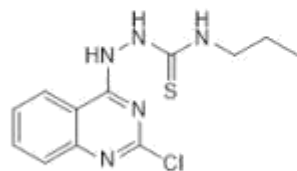


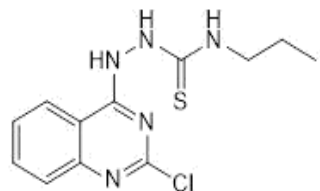
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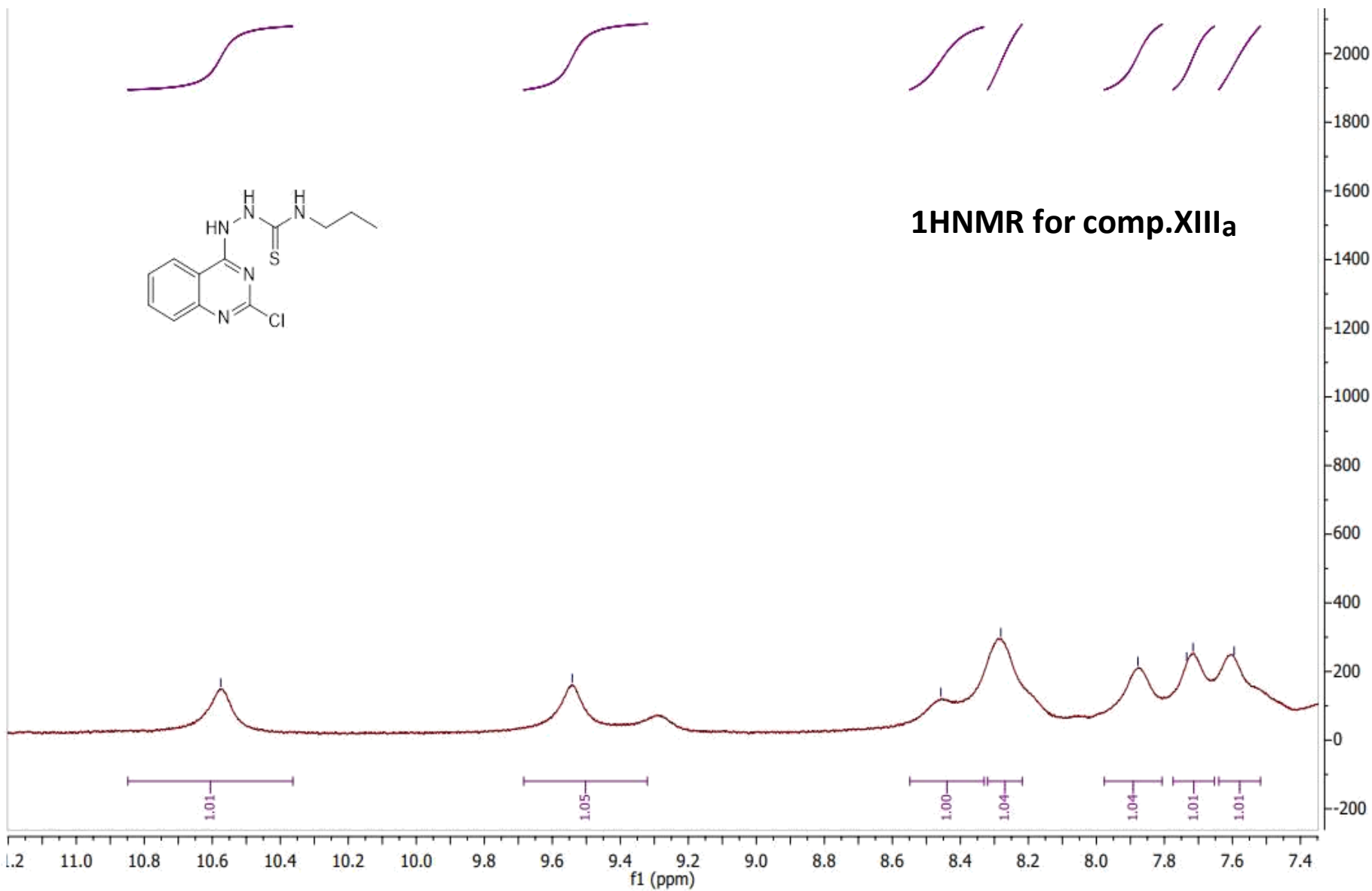


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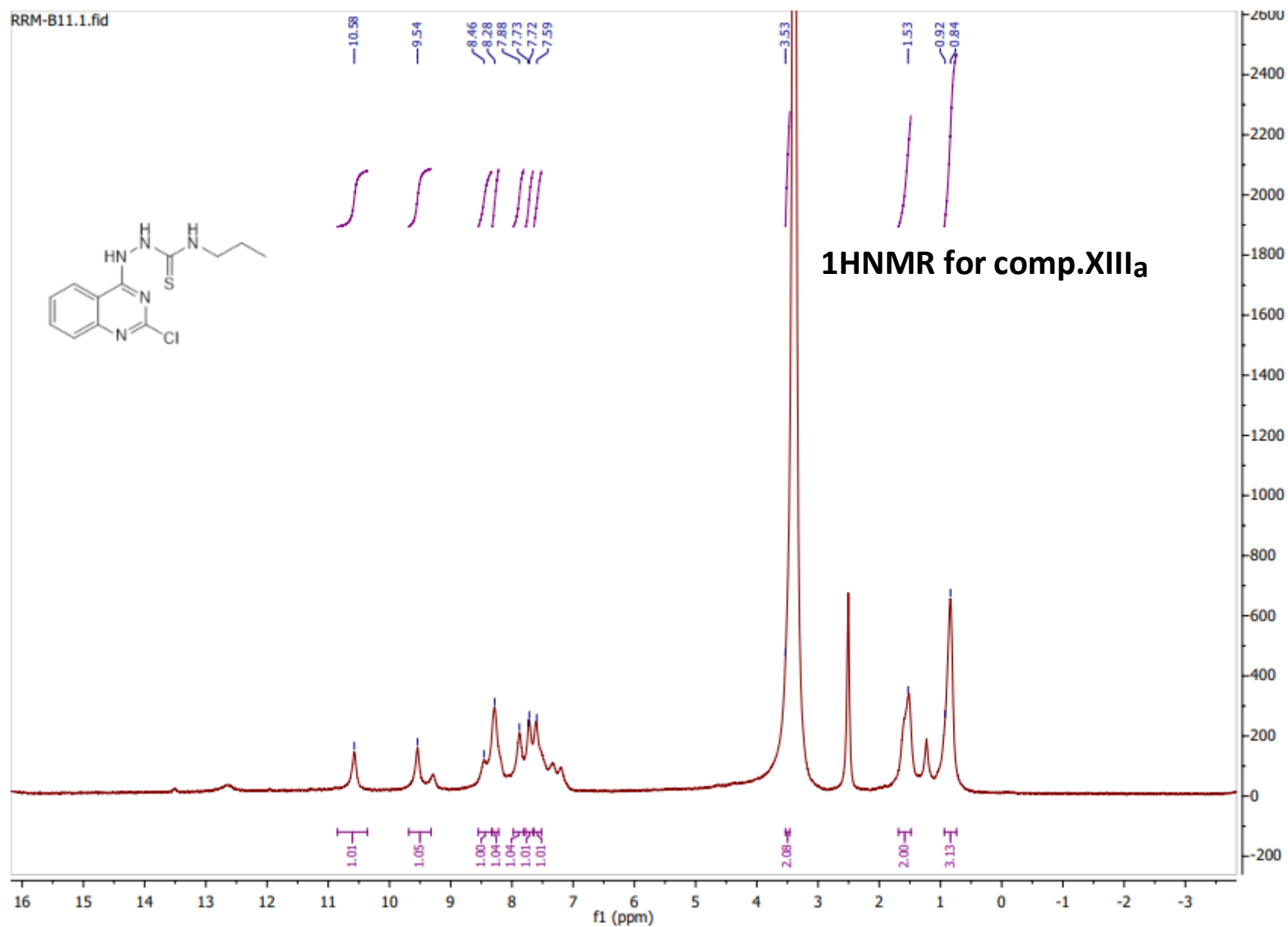


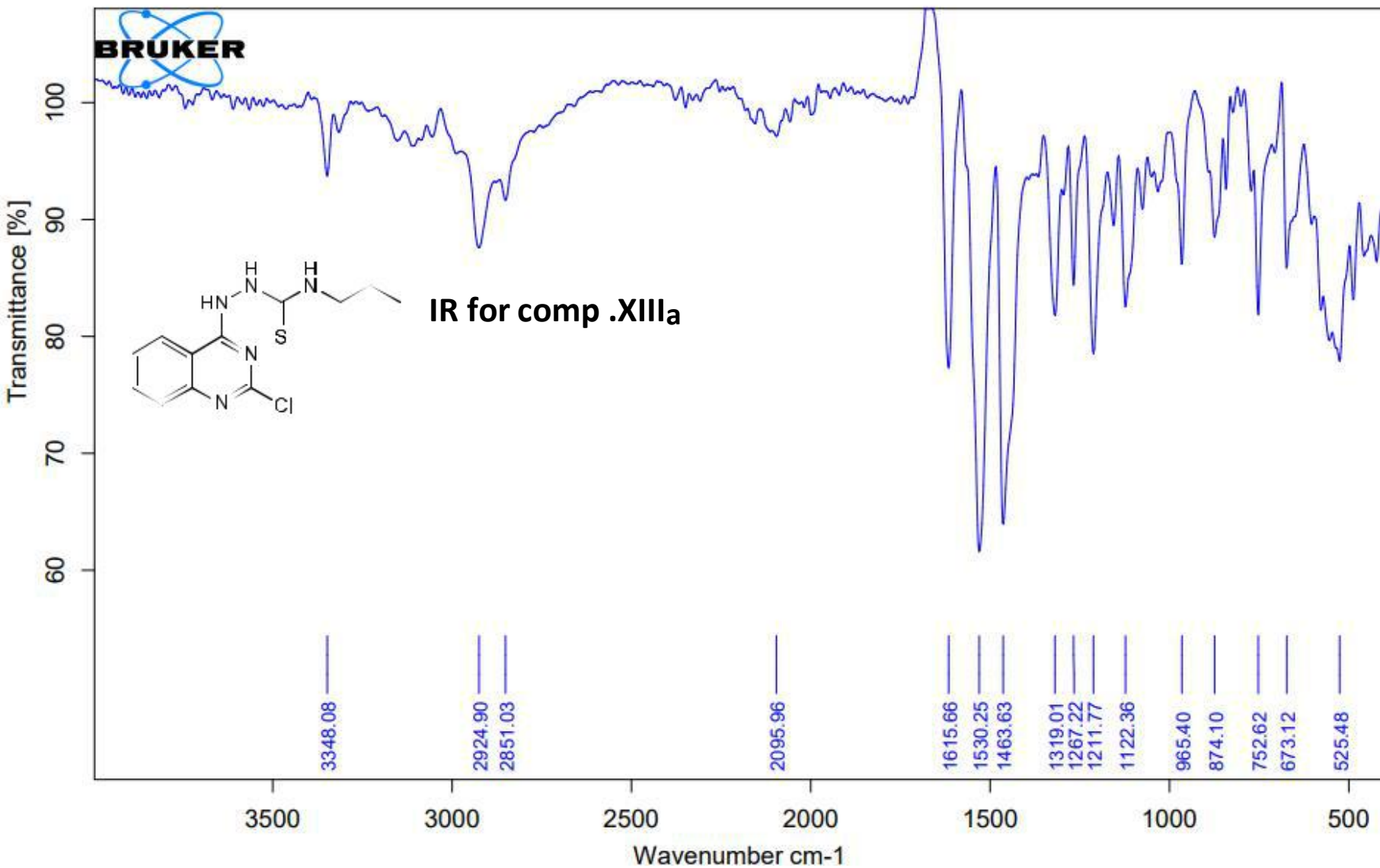


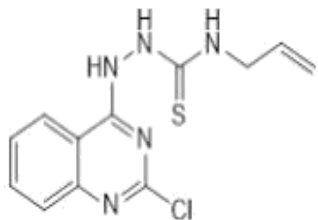
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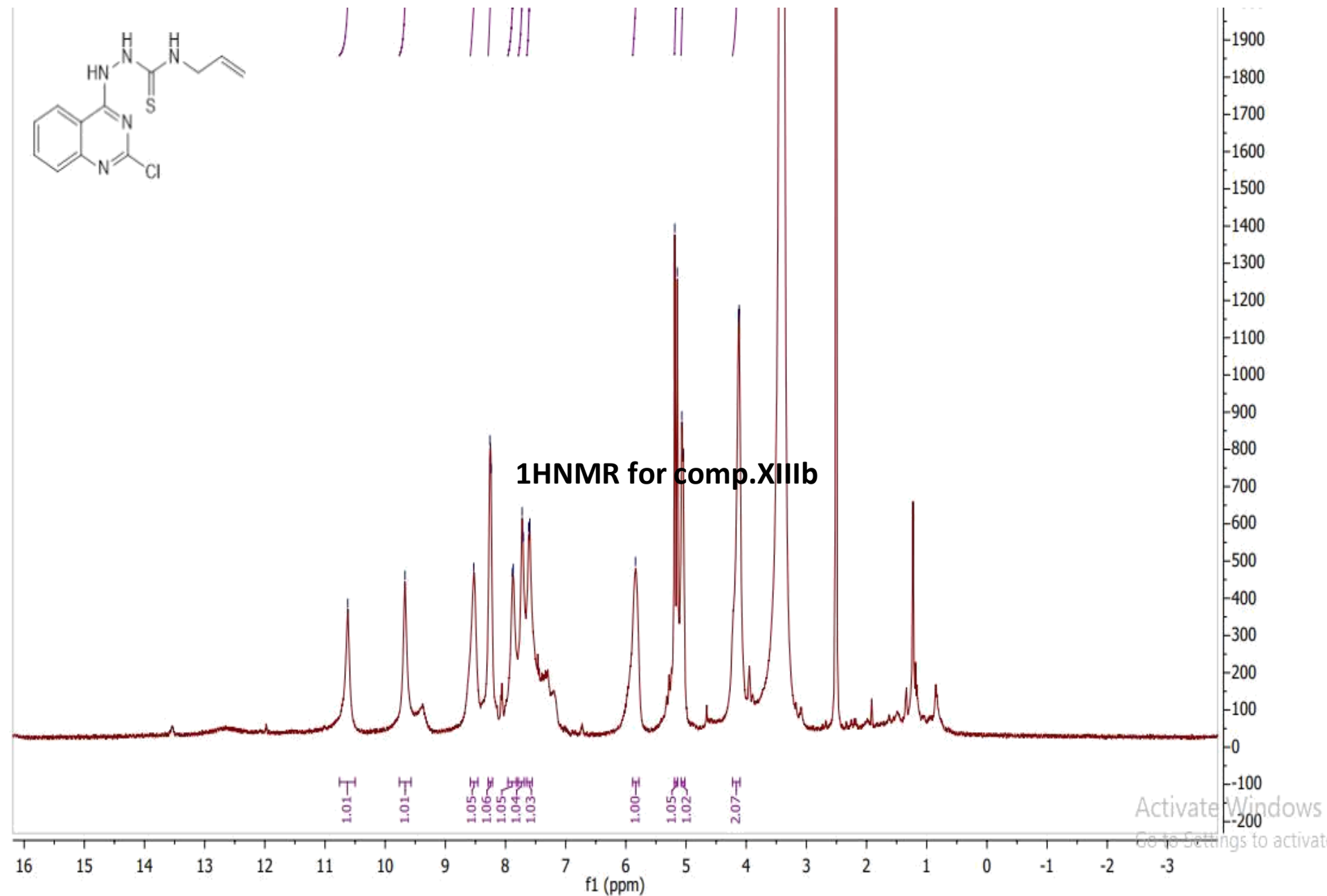
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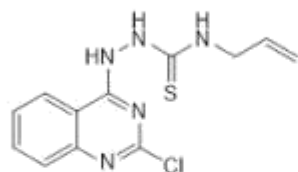




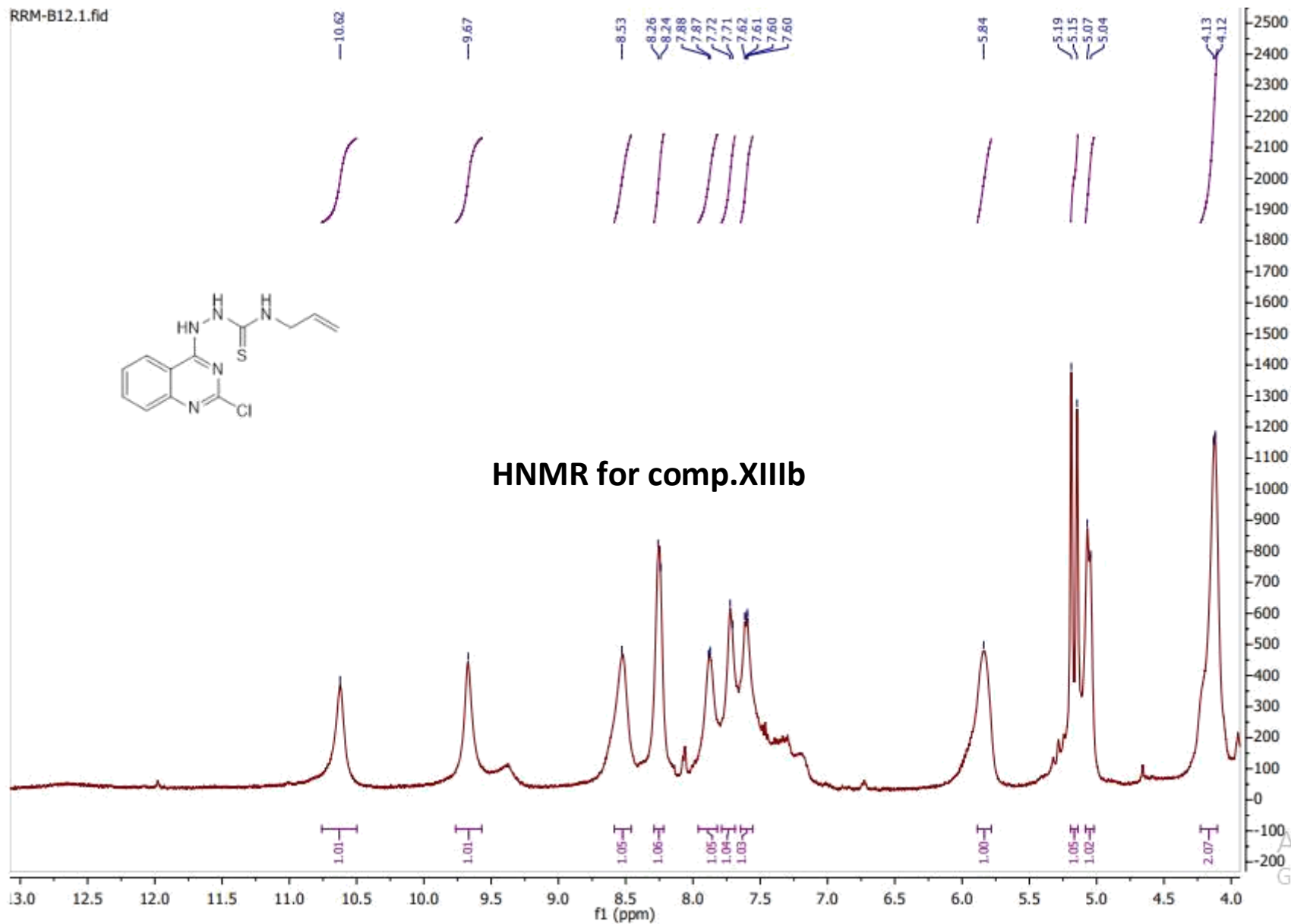
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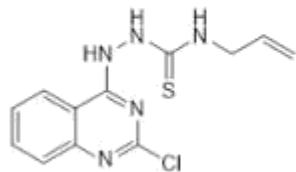
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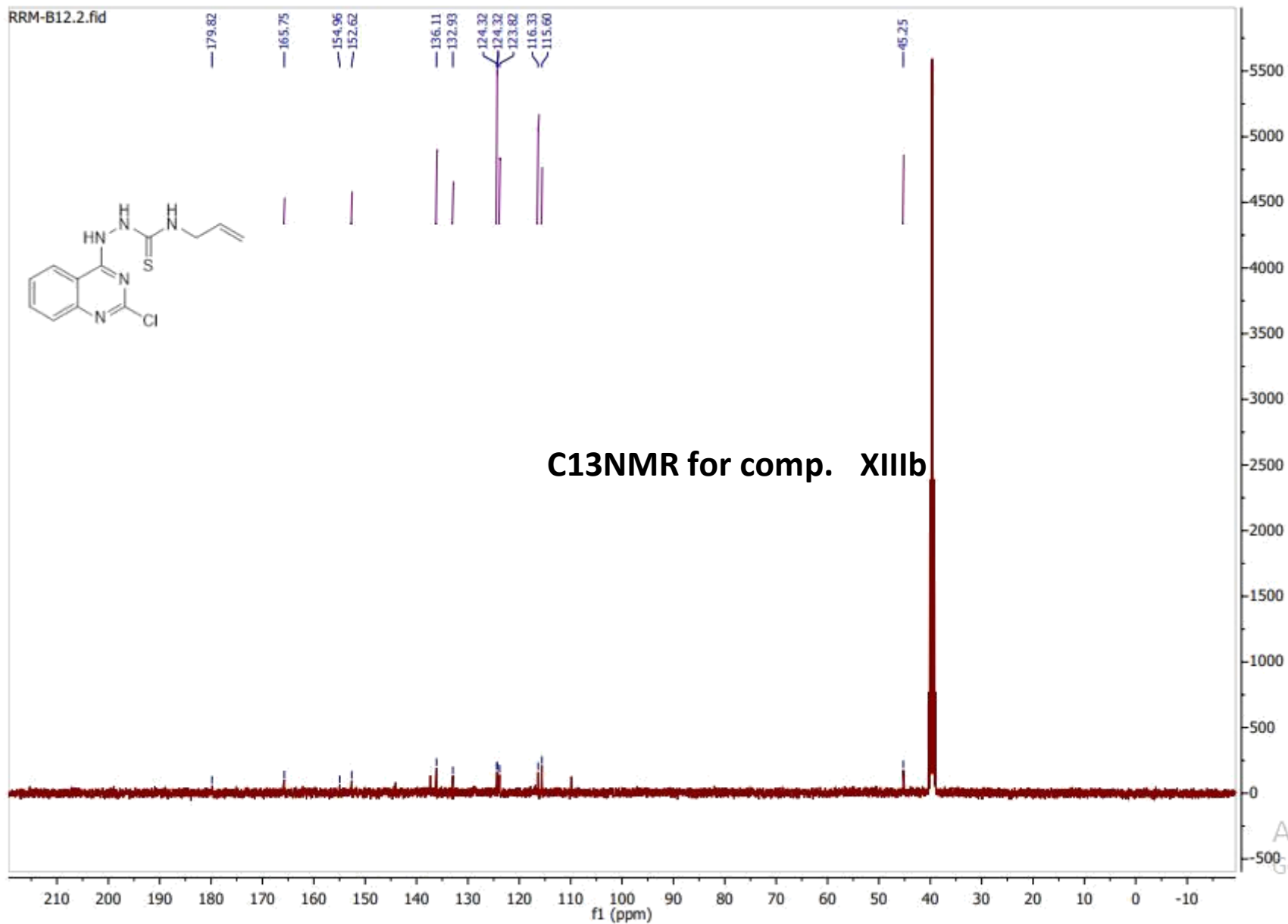
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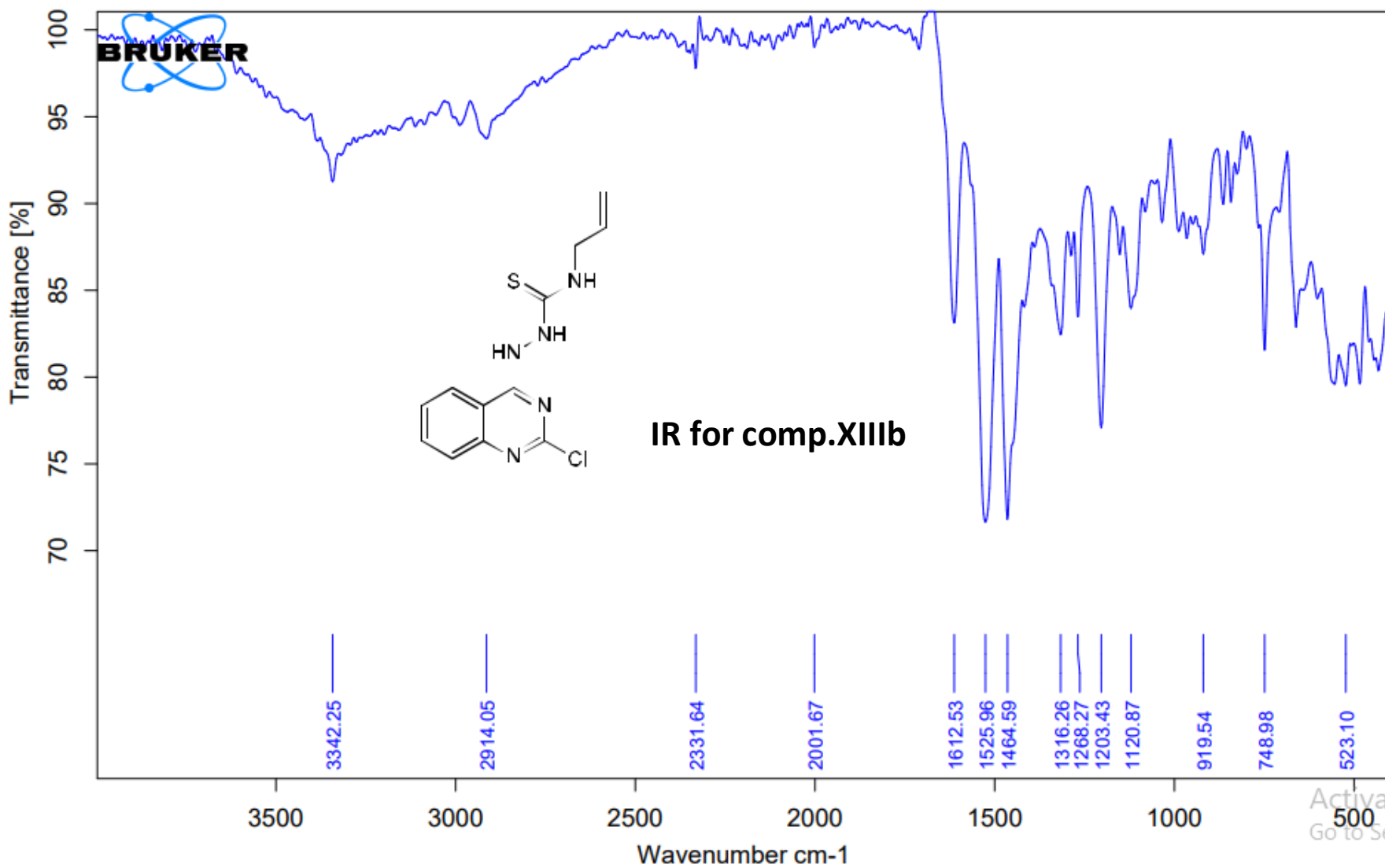
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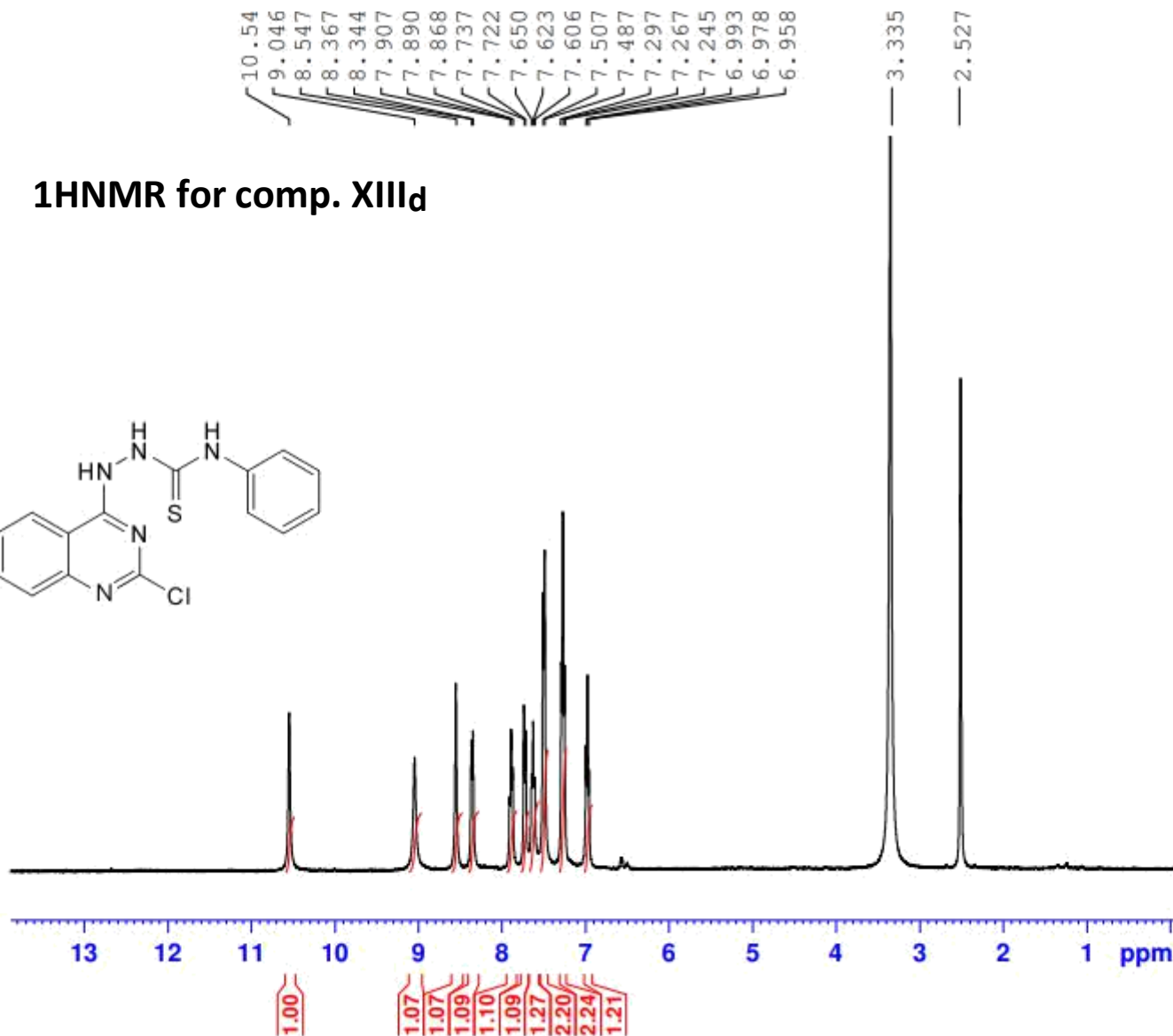
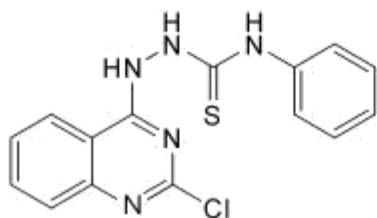
C13NMR for comp. XIIIb



Active
Go to S



¹H NMR for comp. XIIIId

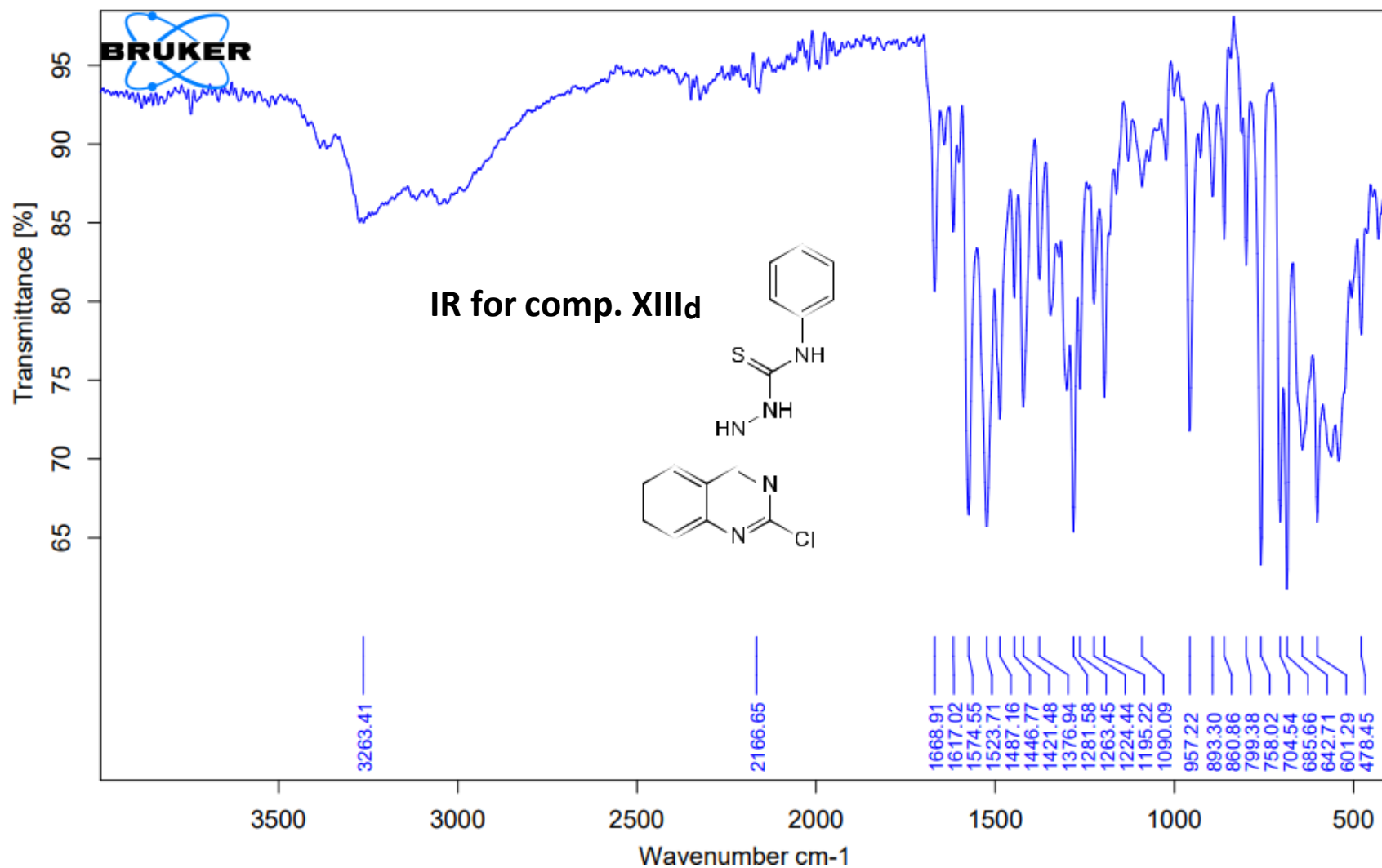


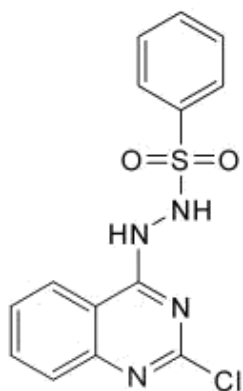
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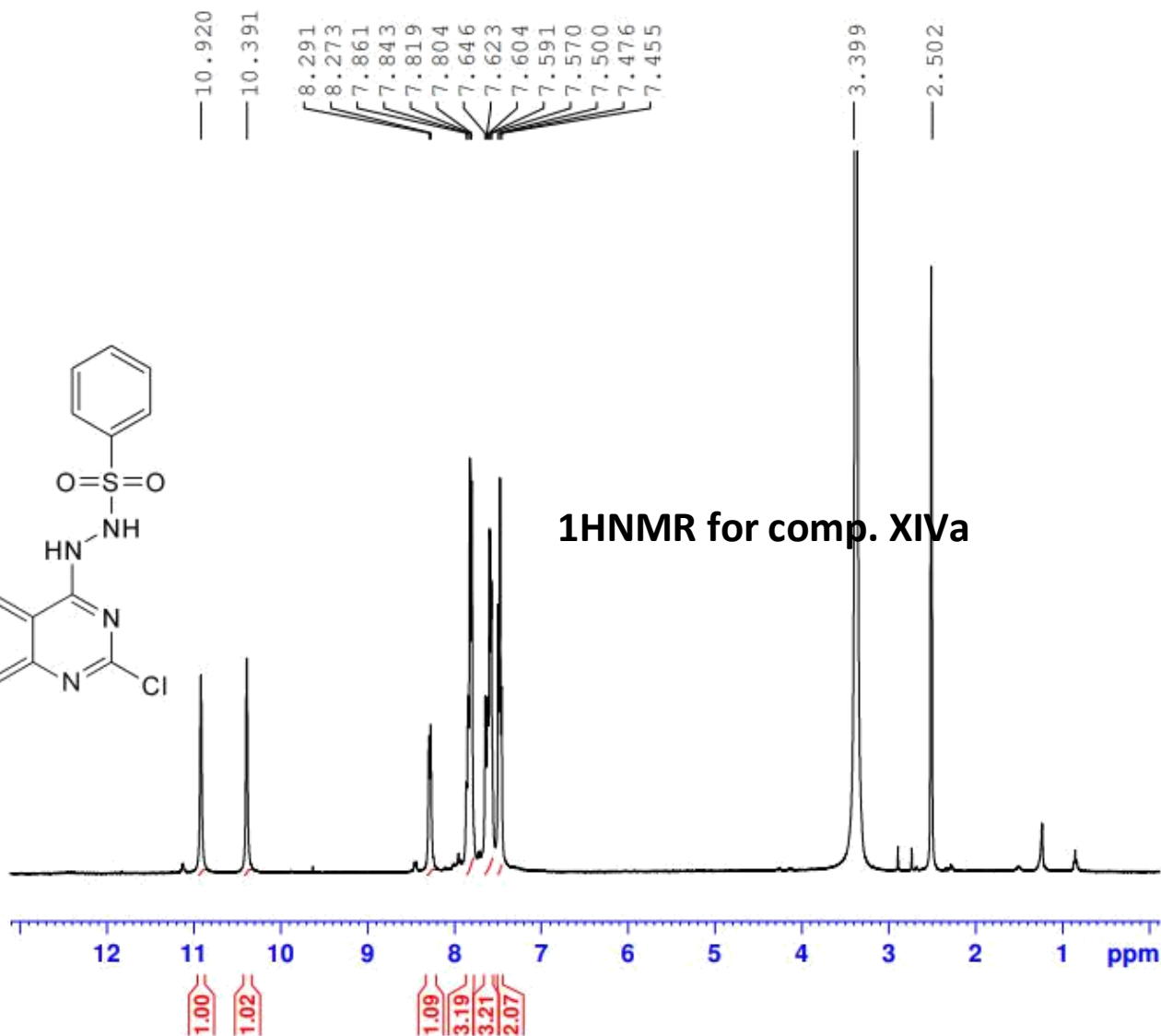
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¹HNMR for comp. XIVa

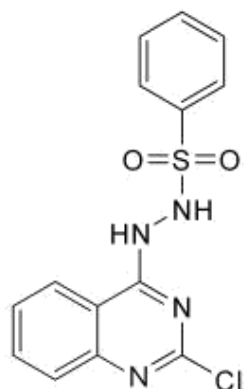


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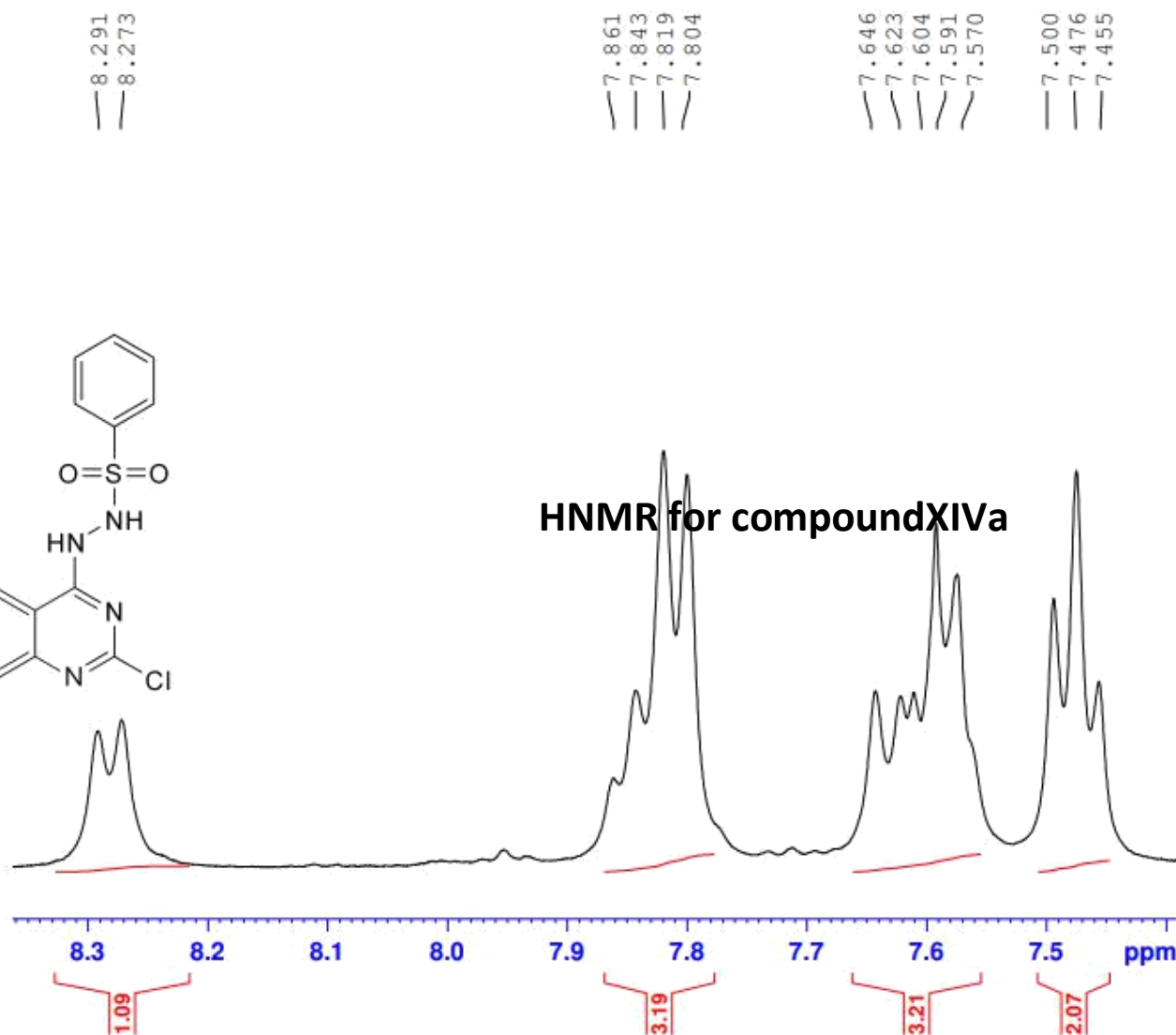
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----- CHANNEL f1 -----
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 P1 12.00 usec
 PLW1 18.00000000 W

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HNMR for compound XIVa

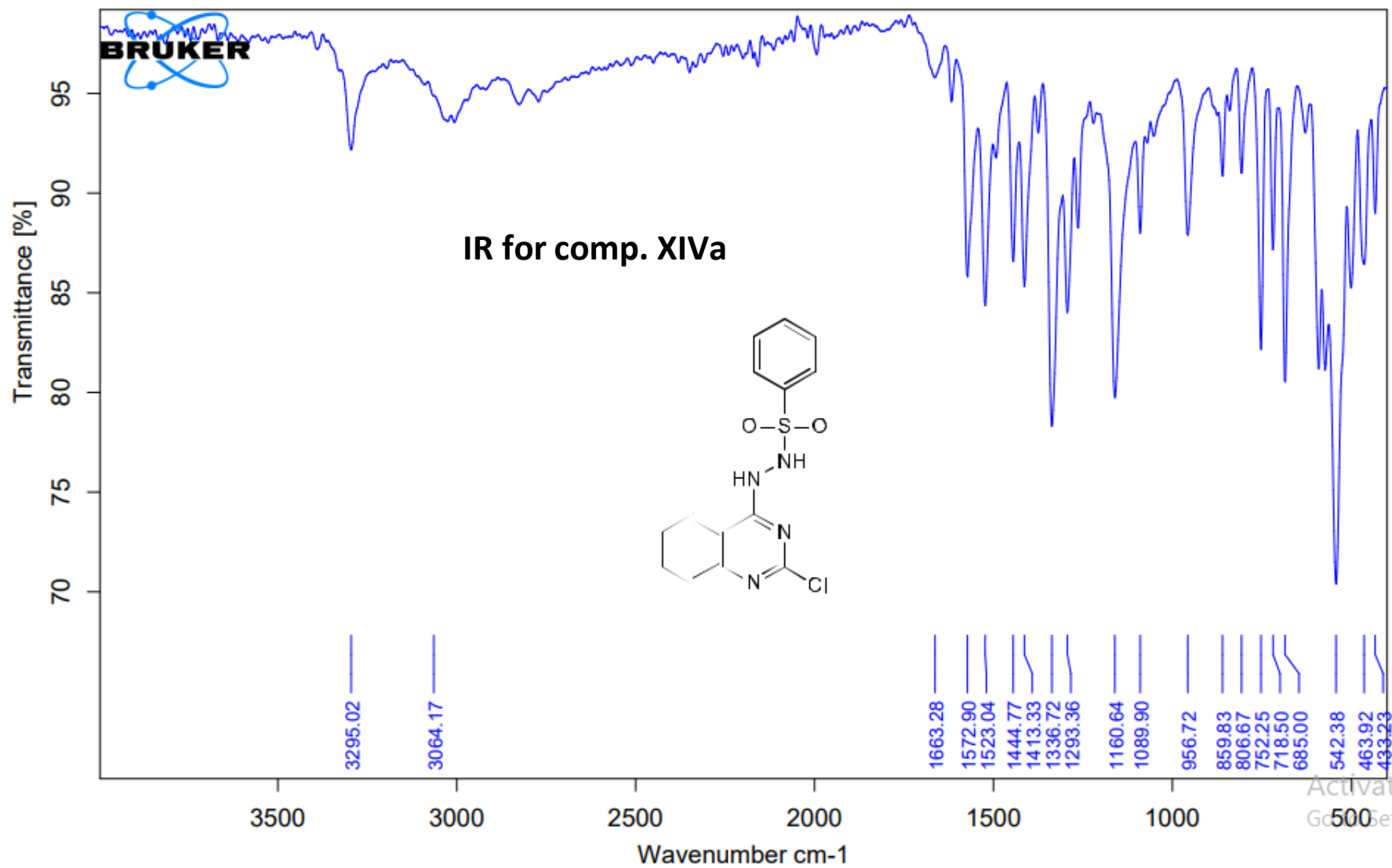


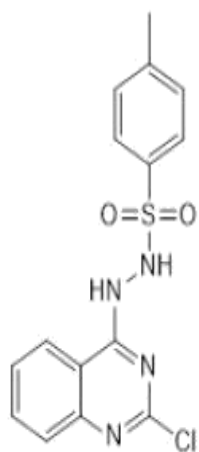
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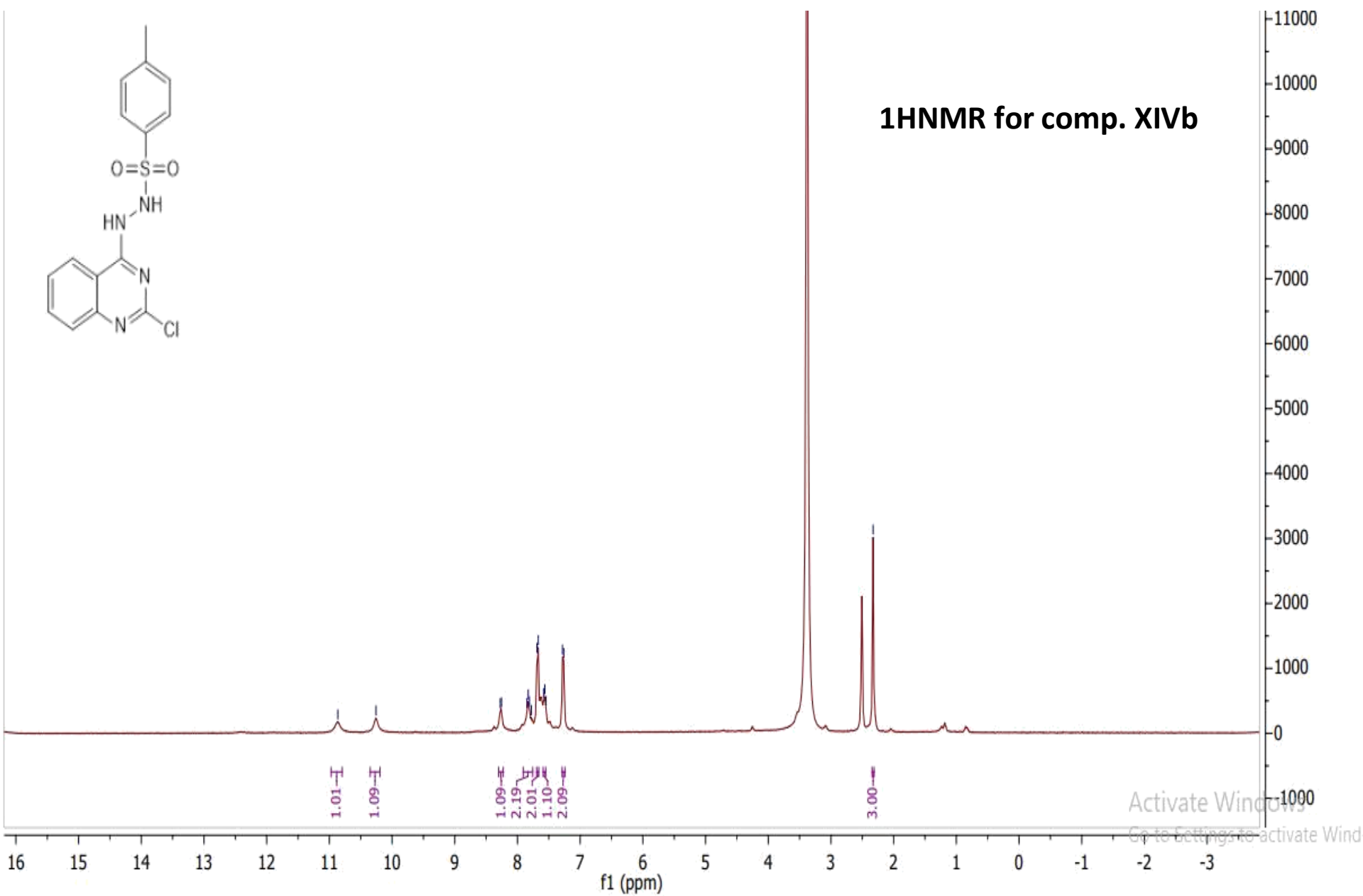
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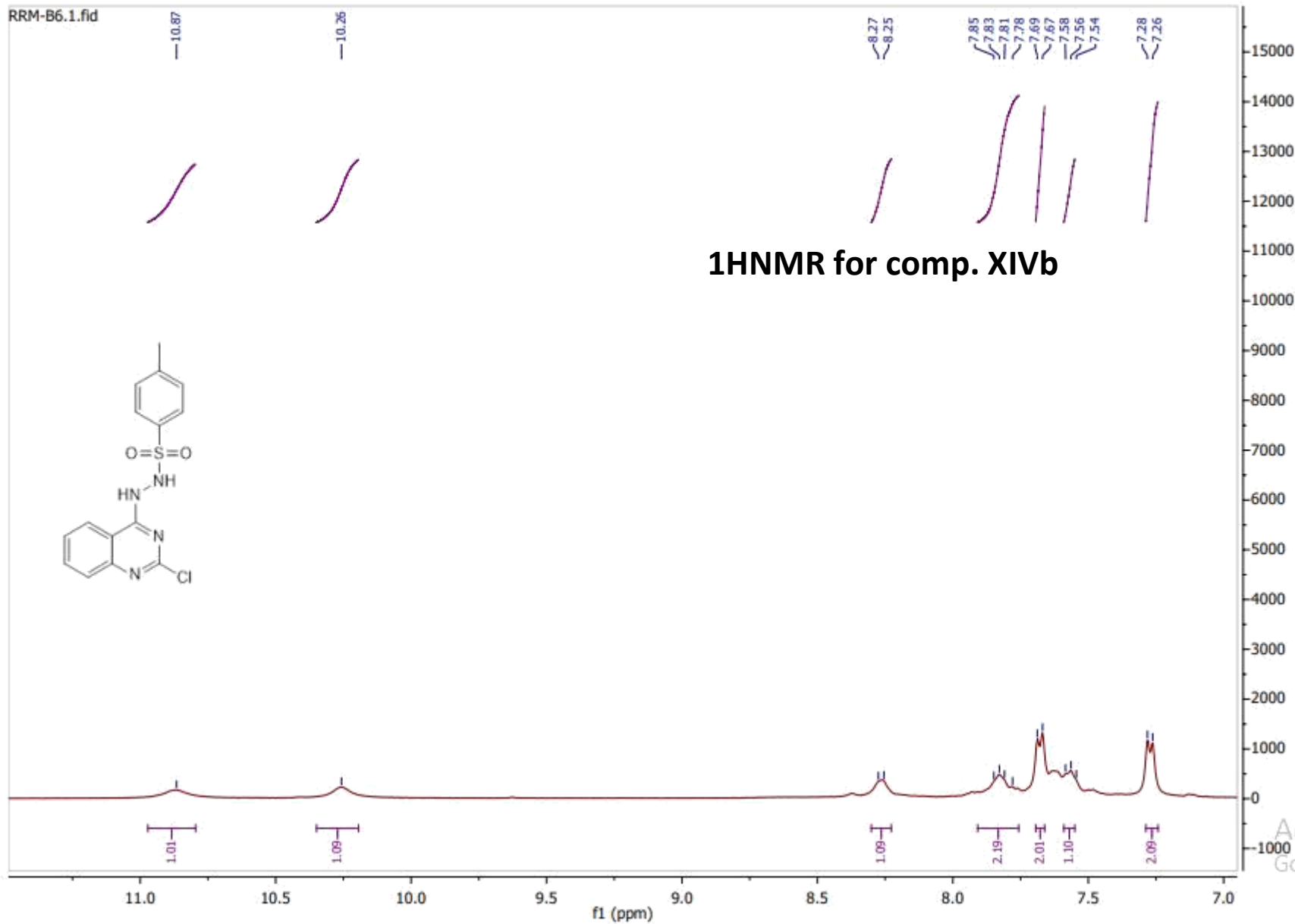


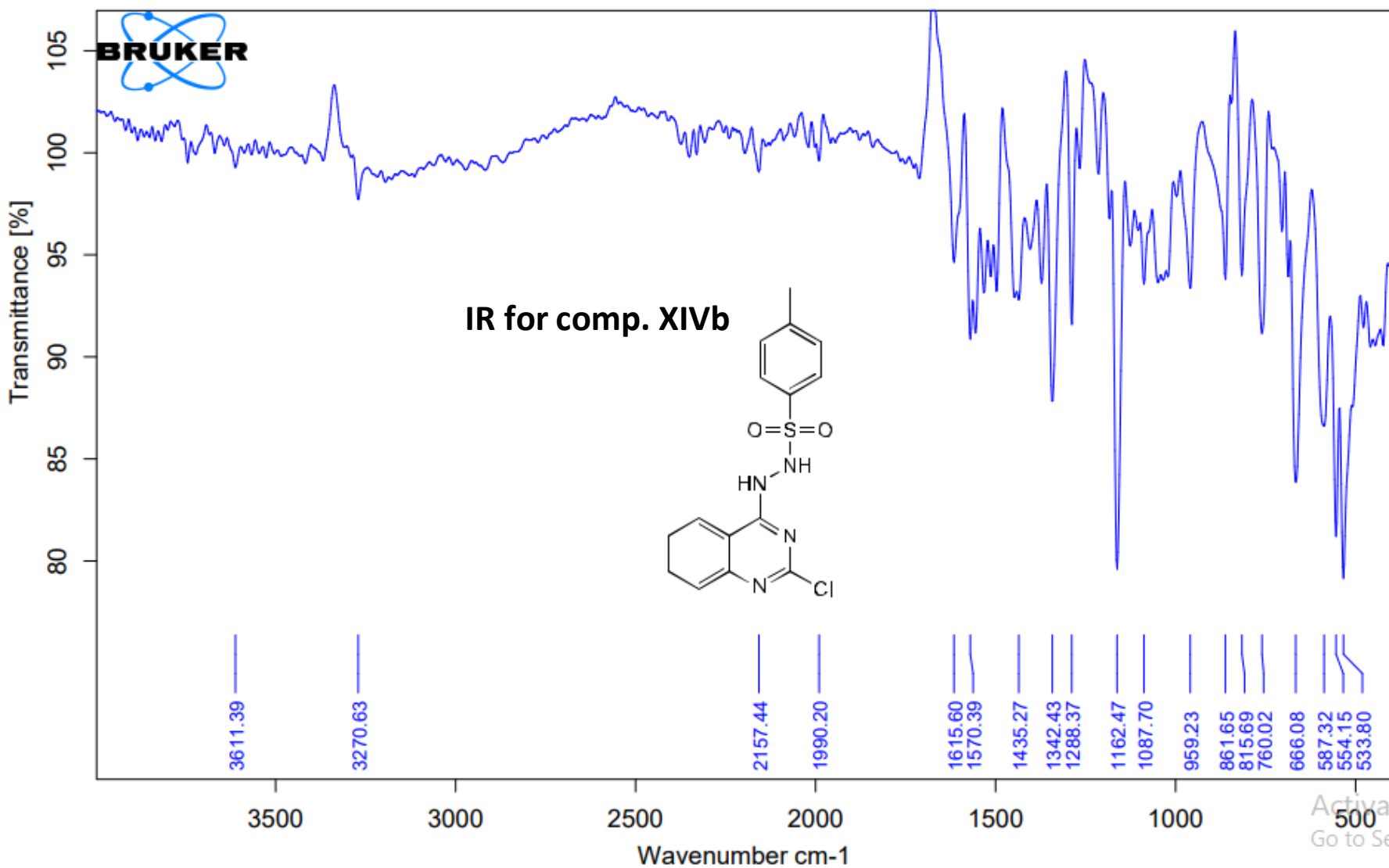


¹H NMR for comp. XIVb

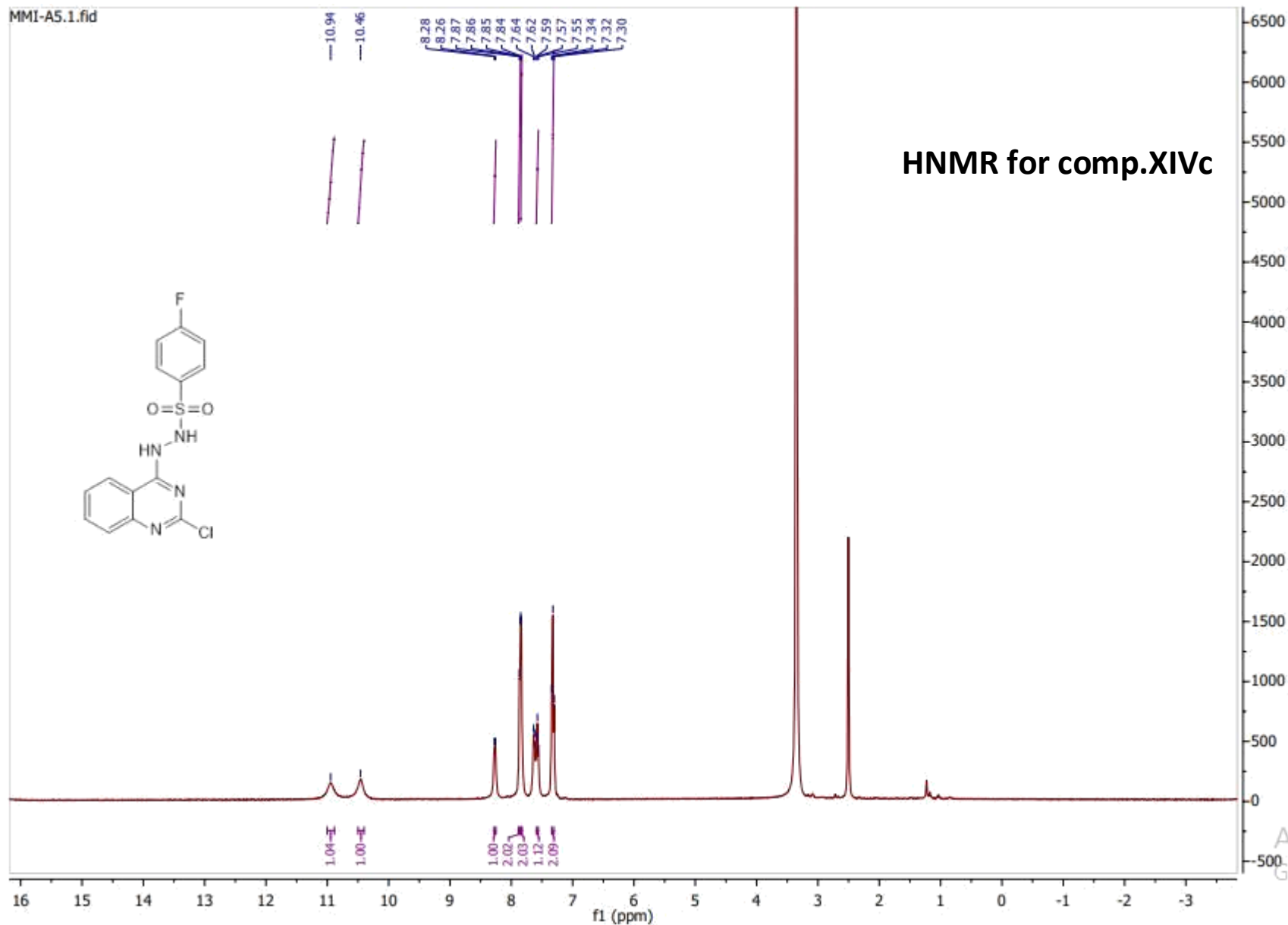


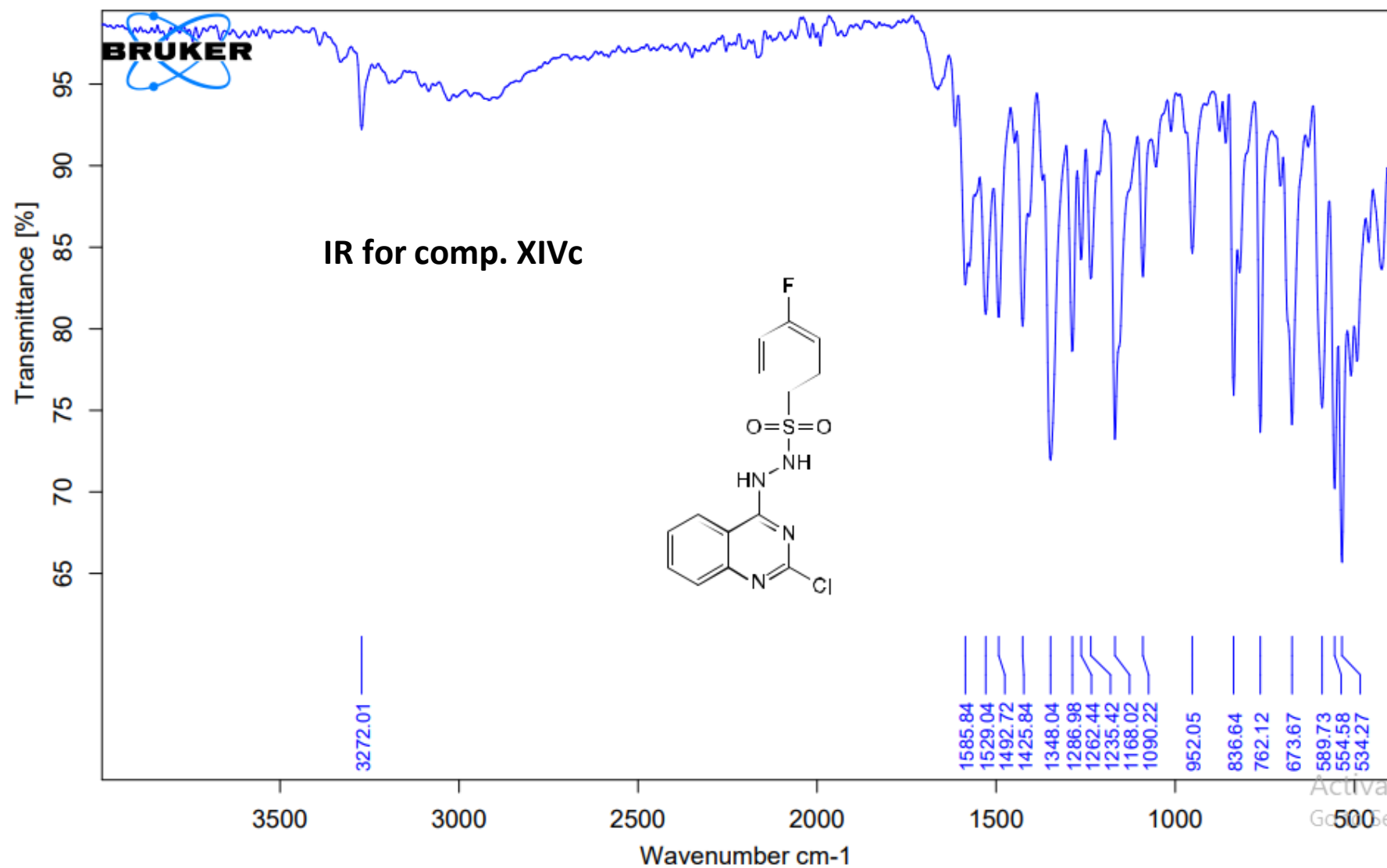
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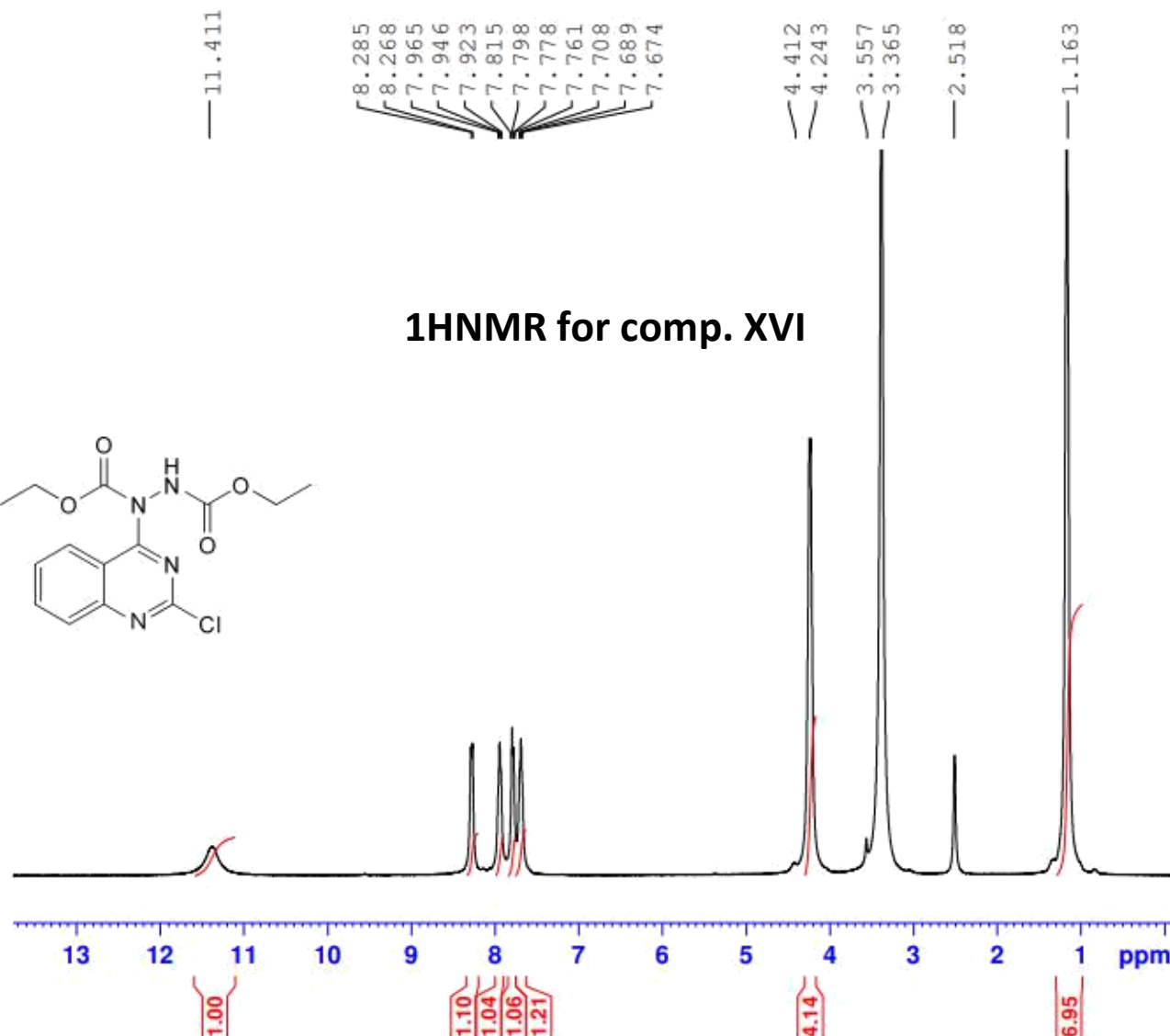
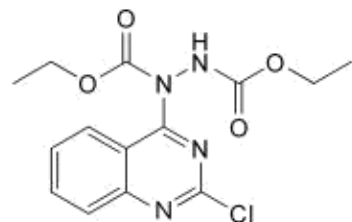


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1HNMR for comp. XVI

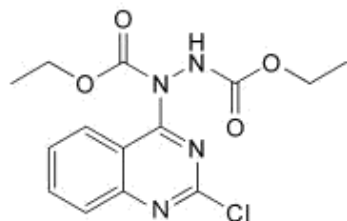


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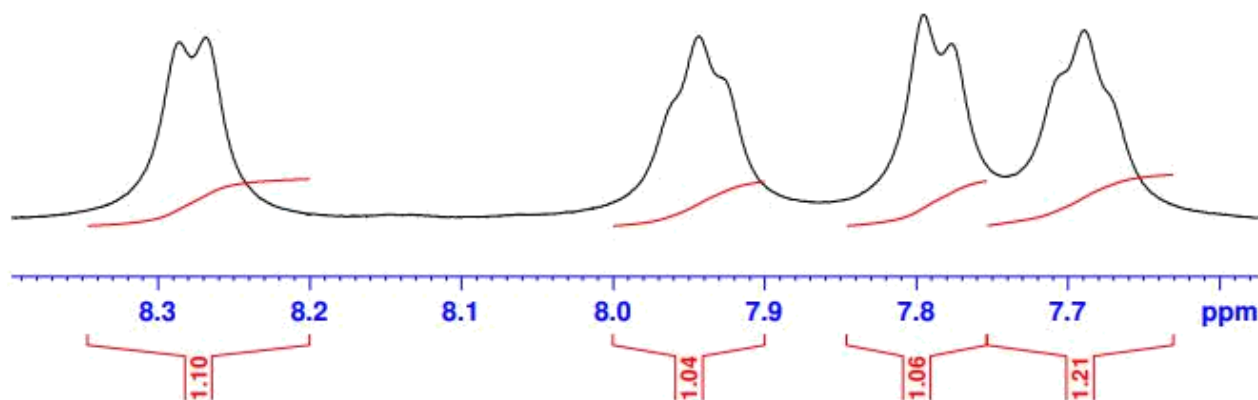
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 DE 6.50 usec
 TE 298.0 K
 D1 1.00000000 sec
 TD0 1

----- CHANNEL f1 -----
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 NUC1 1H
 P1 12.00 usec
 PLW1 18.00000000 W

F2 - Processing parameters
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 SF 400.1500000 MHz
 WDW EM
 SSB 0
 LB 0.30 Hz
 GB 0
 PC 1.00



1HNMR for comp.XVI



— 8.285
— 8.268

— 7.965
— 7.946
— 7.923

— 7.815
— 7.798
— 7.778
— 7.761

— 7.708
— 7.689
— 7.674

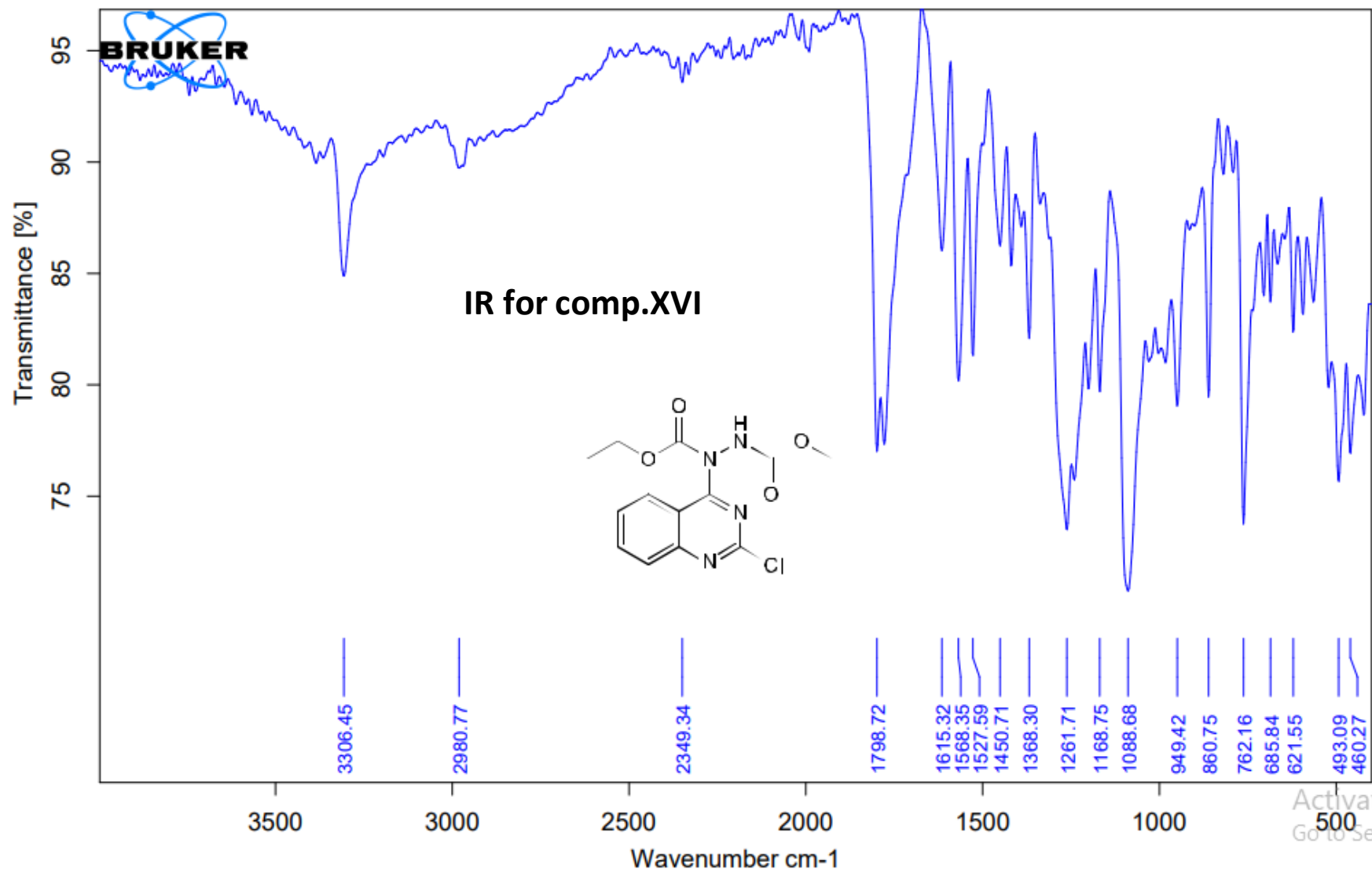


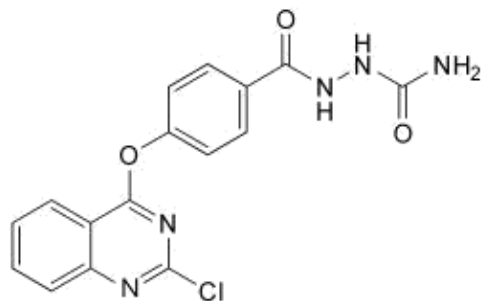
Current Data Parameters
NAME RRM-B5
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date_ 20200116
Time 10.42
INSTRUM spect
PROBHD 5 mm PABBO BB/
PULPROG zg30
TD 65536
SOLVENT DMSO
NS 59
DS 2
SWH 8012.820 Hz
FIDRES 0.122266 Hz
AQ 4.0894465 sec
RG 205.37
DW 62.400 usec
DE 6.50 usec
TE 298.0 K
D1 1.00000000 sec
TD0 1

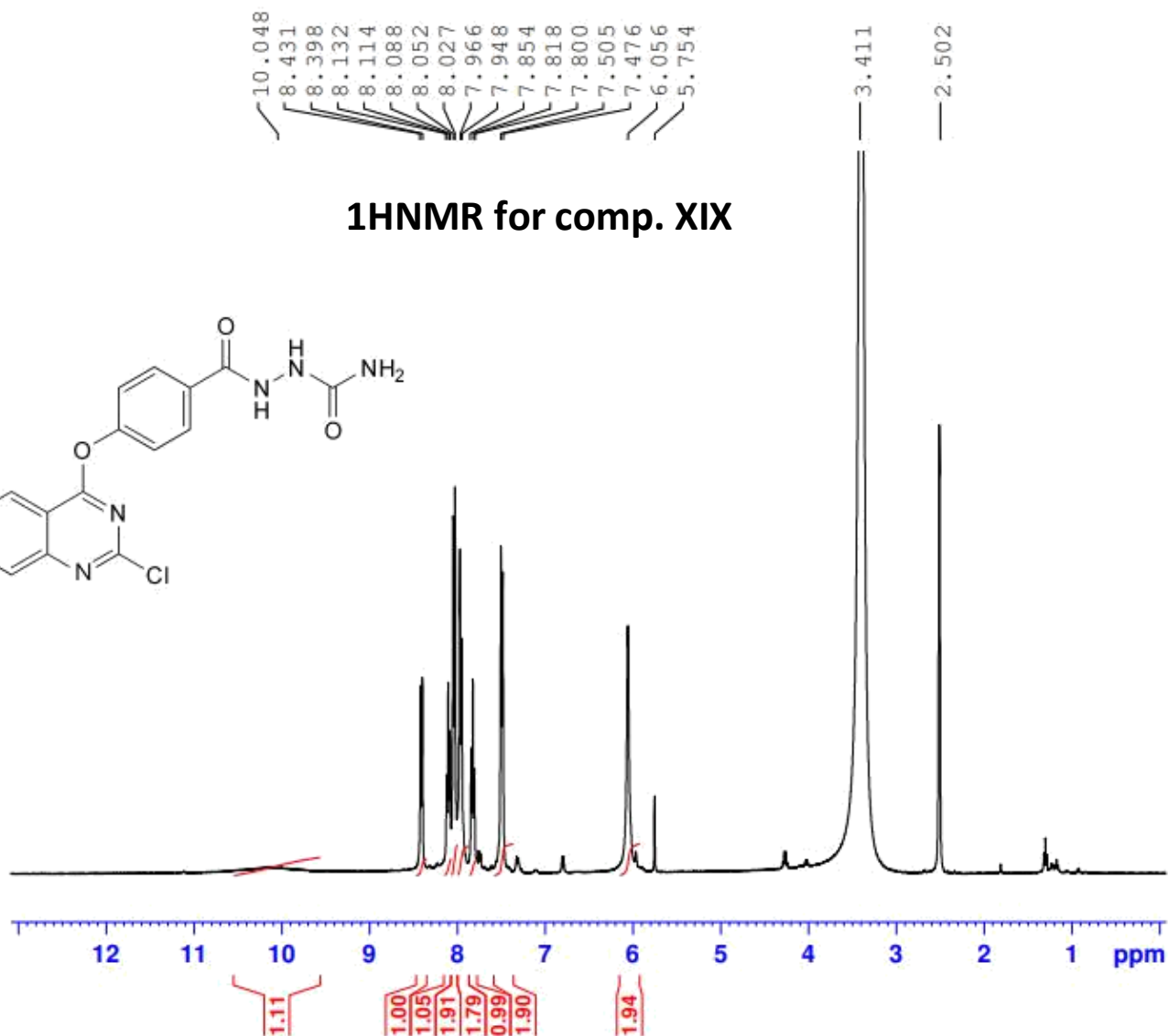
----- CHANNEL f1 -----
SFO1 400.1524711 MHz
NUC1 1H
P1 12.00 usec
PLW1 18.00000000 W

F2 - Processing parameters
SI 65536
SF 400.1500000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00





¹H NMR for comp. XIX



Current Data Parameters
 NAME RRM-B1
 EXPNO 1
 PROCNO 1

F2 - Acquisition Parameters
 Date_ 20200116
 Time 12.08
 INSTRUM spect
 PROBHD 5 mm PABBO BB/
 PULPROG zg30
 TD 65536
 SOLVENT DMSO
 NS 105
 DS 2
 SWH 8012.820 Hz
 FIDRES 0.122266 Hz
 AQ 4.0894465 sec
 RG 205.37
 DW 62.400 usec
 DE 6.50 usec
 TE 298.0 K
 D1 1.00000000 sec
 TD0 1

----- CHANNEL f1 -----
 SFO1 400.1524711 MHz
 NUC1 1H
 P1 12.00 usec
 PLW1 18.00000000 W

F2 - Processing parameters
 SI 65536
 SF 400.1500000 MHz
 WDW EM
 SSB 0
 LB 0.30 Hz
 GB 0
 PC 1.00

Activate Win
 Go to Settings t



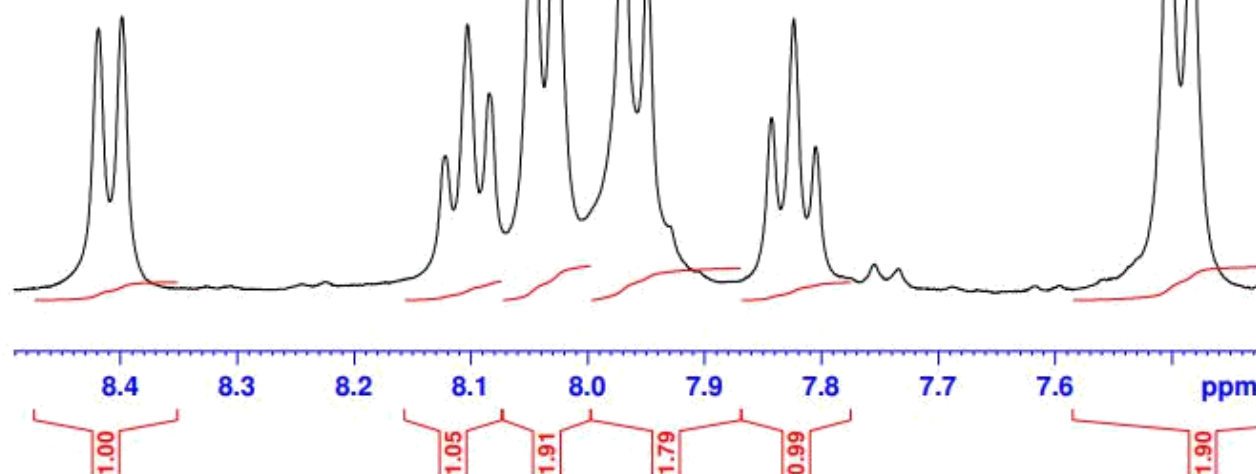
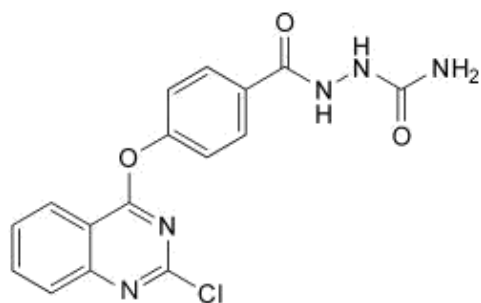
Current Data Parameters
NAME RRM-B1
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date_ 20200116
Time 12.08
INSTRUM spect
PROBHD 5 mm PABBO BB/
PULPROG zg30
TD 65536
SOLVENT DMSO
NS 105
DS 2
SWH 8012.820 Hz
FIDRES 0.122266 Hz
AQ 4.0894465 sec
RG 205.37
DW 62.400 usec
DE 6.50 usec
TE 298.0 K
D1 1.00000000 sec
TD0 1

----- CHANNEL f1 -----
SFO1 400.1524711 MHz
NUC1 1H
P1 12.00 usec
PLW1 18.00000000 W

F2 - Processing parameters
SI 65536
SF 400.1500000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

¹H NMR for comp. XIX



Activate Win
Go to Settings to

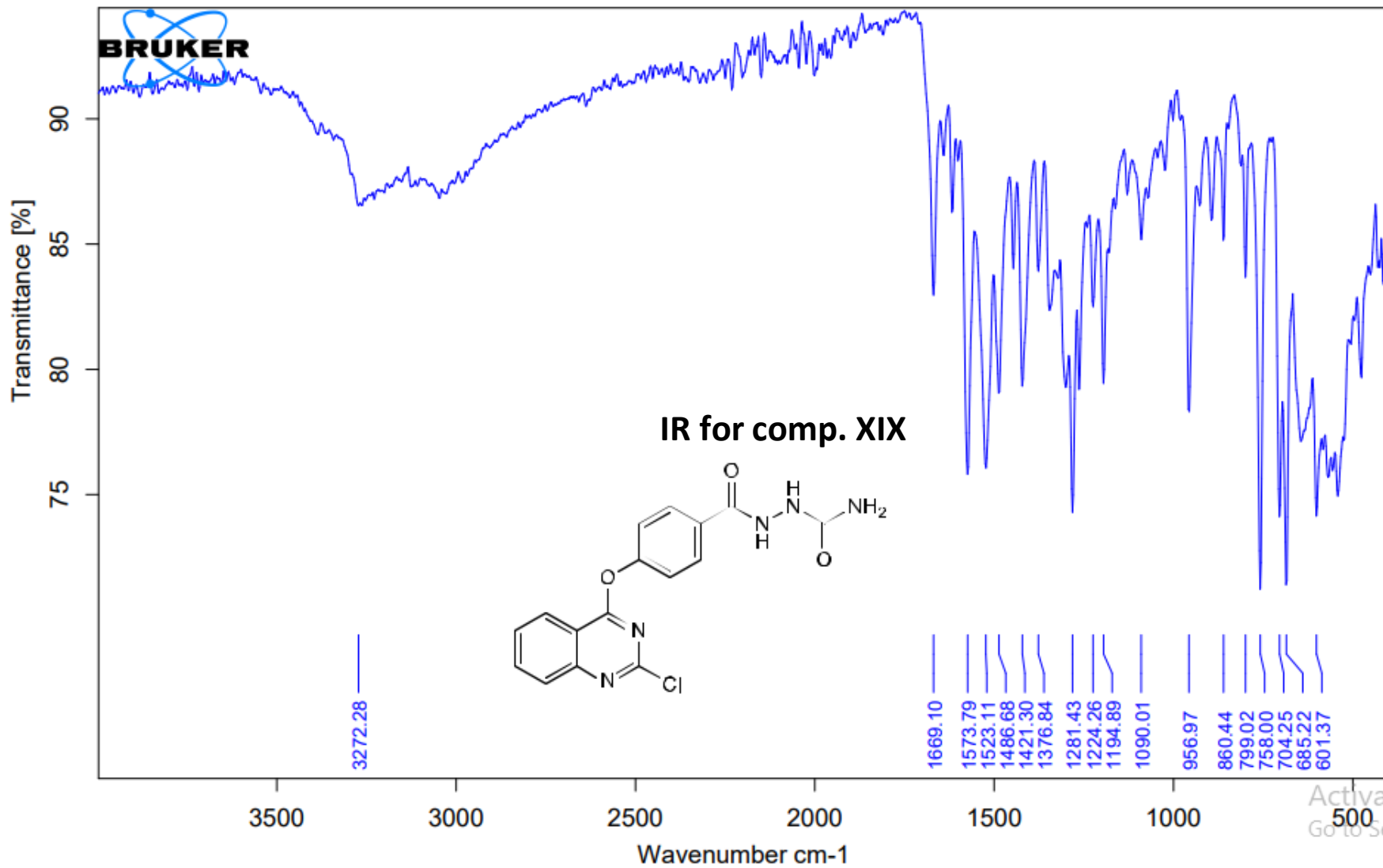
RRM-B1.2.fid

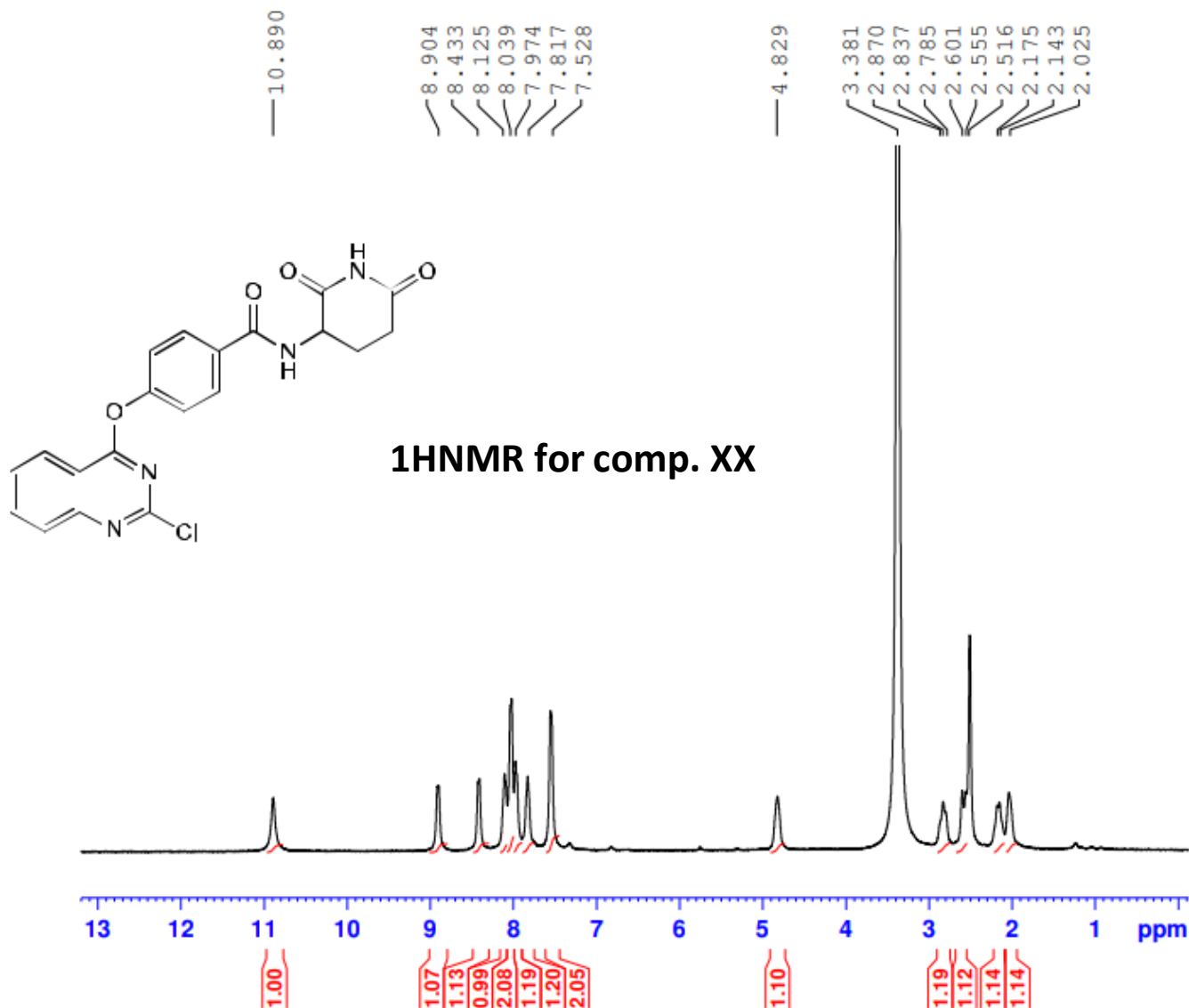
173.25
168.08
157.01
154.91
154.67
152.73
136.47
131.62
129.97
129.04
127.17
124.51
123.22
122.30
115.31

^{13}C NMR for comp. XIX

f1 (ppm)

Active
Go to S





Current Data Parameters
 NAME RRM-B2
 EXPNO 1
 PROCNO 1

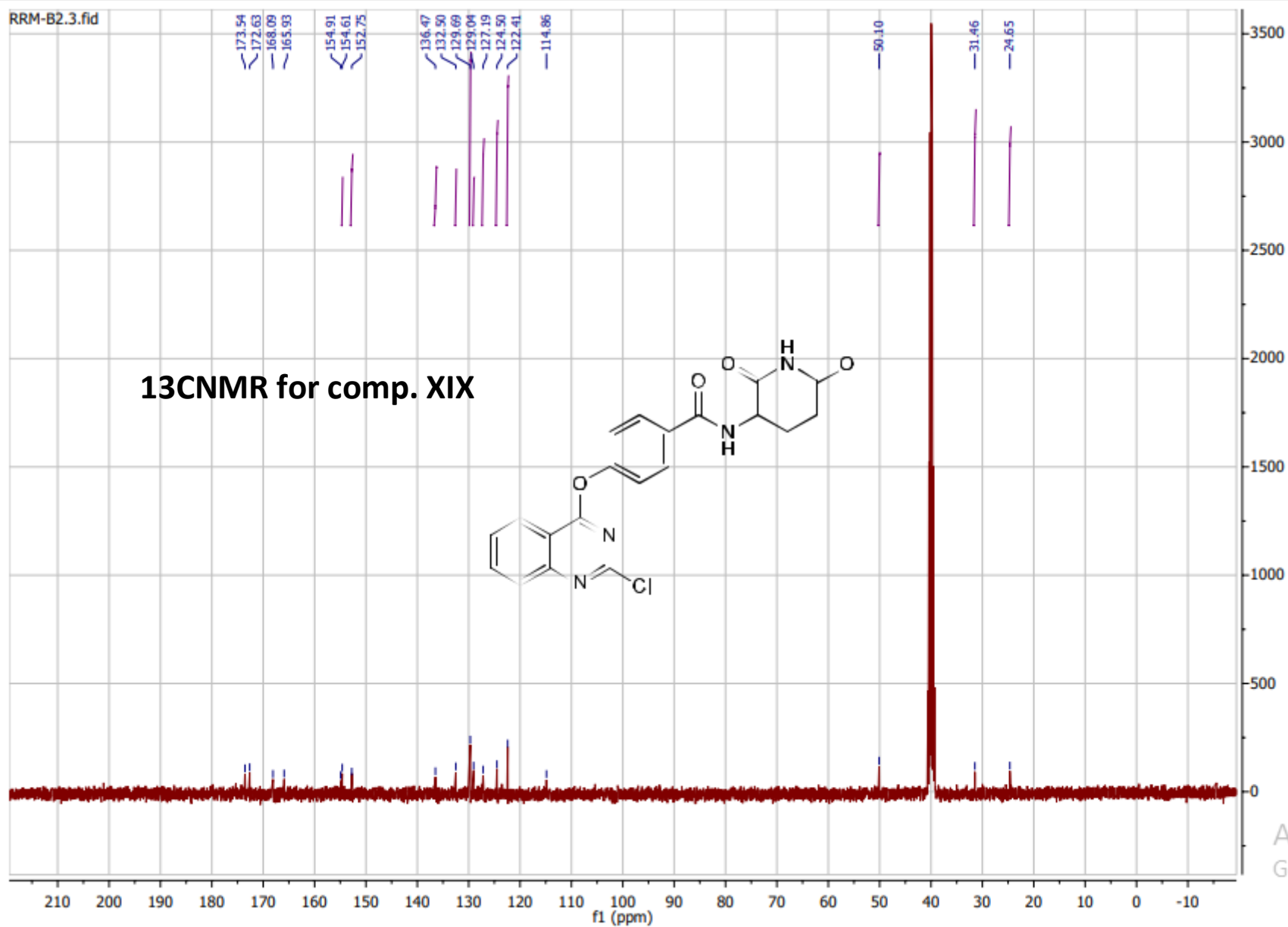
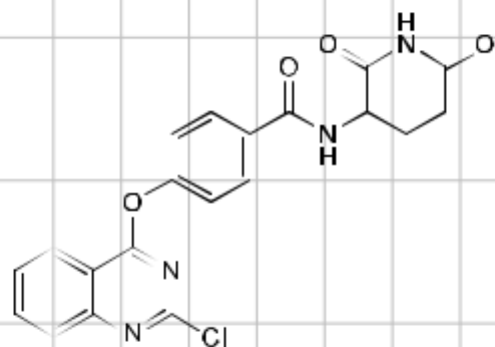
F2 - Acquisition Parameters
 Date_ 20200116
 Time 12.16
 INSTRUM spect
 PROBHD 5 mm PABBO BB/
 PULPROG zg30
 TD 65536
 SOLVENT DMSO
 NS 69
 DS 2
 SWH 8012.820 Hz
 FIDRES 0.122266 Hz
 AQ 4.0894465 sec
 RG 205.37
 DW 62.400 usec
 DE 6.50 usec
 TE 298.0 K
 D1 1.00000000 sec
 TD0 1

----- CHANNEL f1 -----
 SFO1 400.1524711 MHz
 NUC1 1H
 P1 12.00 usec
 PLW1 18.00000000 W

F2 - Processing parameters
 SI 65536
 SF 400.1500000 MHz
 WDW EM
 SSB 0
 LB 0.30 Hz
 GB 0
 PC 1.00

RRM-B2.3.fid

13CNMR for comp. XIX



Active
Go to S

