Supplementary Materials

3,5-DISUBSTITUTED TETRAHYDRO-2-*H*-1,3,5-THIADIAZINE-THIONE ESTER DERIVATIVES AND THEIR ANTIMICROBIAL EVALUATION

Irfanullah,[a,b] Jamshed Hashim,[a]* Nuzhat Arshad,[a,c]* Muhammad Yaseen,[b] Rasool Khan,[b] Tahseen Iqbal,[a] Syeda Zehra Hamid,[a] Afshan Kanwal,[a,c] and Iqbal Safi[a,b]

[a]*H.E.J.Research Institute of Chemistry, ICCBS, University of Karachi, Karachi-75270, Pakistan*
[b]*Institute of Chemical Sciences, University of Peshawar, Peshawar-25120, Pakistan*
[c]*Department of Chemistry, NED University of Engineering and Technology, Karachi-75270, Pakistan*

jamshedhashim@yahoo.co.uk; nuzhat@neduet.edu.pk

Table of Contents: **Page No:**

1. **Esterase Catalyzed Hydrolysis** **S2**
   1.1. Experimental detail of enzymatic hydrolysis S2

2. **Biological Screening Results** **S2-S4**
   2.1 Antifungal S2-S3
   2.2 Antibacterial S3-S4

3. **References** **S5**

4. **HPLC Chromatogram of Enzymatic Hydrolysis** **S6-S9**

5. **NMR Spectra** **S10-S25**

1. Esterase Catalyzed Hydrolysis

Hydrolysis of 3,5-disubstituted tetrahydro-2*H*-1,3,5-thiadiazine-6-thione (THTT) ester derivative (**9h**) was carried out by using Pig Liver Esterase (PLE) under phosphate buffer (pH 7.0-7.5) in a shaking incubator at 25 ˚C for 20 minutes duration. Acetone was used as a co-solvent and the reaction was monitored by analytical HPLC. The same enzymatic reaction conditions were employed to its acidic counterpart (**8e**), which after 20 minutes, delivered the same metabolite at 9.7 minute retention time.

1.1. Experimental detail of enzymatic hydrolysis

1.0 mL of substrate solution (1.0 mM solution in 1% phosphate buffer of pH 7.5 and 10% acetone) was placed in a 5 mL glass vessel. Then 0.5 ml (3 units total) of the PLE stock solution was added (stock solution was prepared by 4.0 mg of the PLE in 10 mL of 1% phosphate buffer of pH 7.5). The sample was placed in a shaking incubator at 25 ˚C for 20 minutes and monitored by analytical HPLC on a C18 reversed-phase analytical column (Athena CN, 150 × 4.6 mm, particle size 5 μm) at 40 ˚C using a mobile phase A (water/methanol 95:5 (v/v) + 0.1% Formic acid) and B (MeOH + 0.1% Formic acid) at a flow rate of 2.0 mL/min. The binary gradient starting with 5% B solution to 95% B in 15 minutes.

2. Biological Evaluation

2.1. Antifungal activity

Antifungal behaviors of synthesized THTT analogues were examined by employing the standard agar tube dilution protocol with the concentration of 200 μM in DMSO,[1-3] against five (05) human pathogens. Initial testing of THTT analogues was performed at 400 μg/mL concentration before final screening at 200 μg/mL. Three (03) replicate testings with same procedure were performed and the results are summarized in Table S1 for comparison purposes with IUPAC International Chemical Identifier (InChI) in extension of Table-4 of manuscript (See biological evaluation section of manuscript for detail method).

TABLE S1

Antifungal activities of THTT analogues (200 μg/mL) as % growth inhibition zone (± SEM).[a]

Entry	Compound	InChI Key	Fungi				
			Trichphyton rubrum	Candida albicans	Aspergillus niger	Microsporum canis	Fusarium lini
1.	**8a**	LWPSHVSZCPZNSZ-UHFFFAOYSA-N	100	100	–[b]	90.5 ± 0.5	100
2.	**8b**	LEGCJJRGTFMYDG-UHFFFAOYSA-N	–	80.2 ± 1.2	–	40.8 ± 2.4	50.2 ± 1.5
3.	**8c**	ZIALVUBNMFXWAZ-UHFFFAOYSA-N	–	10.8 ± 1.6	–	–	30.5 ± 2.0
4.	**8d**	KQSFMHKBKUMGPL-UHFFFAOYSA-N	50.6 ± 1.4	100	–	60.5 ± 1.0	30.8 ± 1.2
5.	**8e**	DIEWCCHSMJHFRH-UHFFFAOYSA-N	100	100	100	100	100
6.	**8f**	DEPIOVZXINNUPI-UHFFFAOYSA-N	30.2 ± 2.0	100	–	50.6 ± 1.6	80.2 ± 1.5
7.	**8g**	MGBAGUKJIQEWOE-UHFFFAOYSA-N	–	40.5 ± 2.5	–	–	20.5 ± 1.8
8.	**8h**	IISOAIXPVCMWNZ-UHFFFAOYSA-N	–	–	–	–	–
9.	**8i**	HJKOWWRXQOXAJD-UHFFFAOYSA-N	–	50.6 ± 1.2	–	–	90.4 ± 0.2
10.	**8j**	BEYBZIMOUIEKBO-UHFFFAOYSA-N	–	50.8 ± 1.0	–	–	–
11.	**9a**	AXCXOCWNLHTDRS-UHFFFAOYSA-N	ND[c]				
12.	**9b**	CJAHCGLBOXKKED-UHFFFAOYSA-N	–	–	–	–	–
13.	**9c**	PDVGVJSTPKUFPH-UHFFFAOYSA-N	–	–	–	–	80.6 ± 0.8
14.	**9d**	WZHYYQKEIRUVIG-UHFFFAOYSA-N	70.6 ± 0.8	20.2 ± 2.2	–	–	–
15.	**9e**	BZWQFMQXCFEUSO-UHFFFAOYSA-N	ND				
16.	**9f**	IJAHMCPXFQMCCT-UHFFFAOYSA-N	ND				
17.	**9g**	LQYLMVRXWBFASI-UHFFFAOYSA-N	–	40.4 ± 1.6	–	–	10.4 ± 2.2
18.	**9h**	RULGIDYOBNAEGP-UHFFFAOYSA-N	ND				
19.[d]	Miconazole		100*1	100*2	–	100*3	100*4
20.[e]	Amphotericin B				100		

[a] Result represents as mean of triplicate ± standard error of mean (SEM); testing Incubation period 7 days at 27 ±1 ºC.
[b] No inhibition observed.
[c] Not determined.
[d] MIC for miconazole; ∗1 = 97.8 μg mL^{-1}, ∗2 = 113.1 μg mL^{-1}, ∗3 = 98.1 μg mL^{-1}, ∗4 = 73.5 μg mL^{-1}.
[e] MIC for amphotericin B = 20.7 μg mL^{-1}.

2.2. Antibacterial activity

All the synthesized 3,5-disubstituted tetrahydro-2*H*-1,3,5-thiadiazine-6-thione (THTT) analogues (**8-9**) were also tested for their *in-vitro* antibacterial activity against four (04) bacterial cultures by using Microplate ALAMAR Blue Assay (MABA) literature protocols.[4] Results with three (03) replicate testings are summarized in Table S2 for comparison purposes with IUPAC International Chemical Identifier (InChI) in extension of Table-5 of manuscript (See biological evaluation section of manuscript for detail method).

TABLE S2

Antibacterial activities of THTT analogues (50 μg mL^{-1}).[a]

Entry	Compound	InChI Key	Growth inhibition zone (%)			
			Escherichia Coli	Shigella flexenari	Staphylococcus aureus	Pseudomonas aeruginosa
1.	**8a**	LWPSHVSZCPZNSZ-UHFFFAOYSA-N	–[b]	–	20.07 ± 0.5	–
2.	**8b**	LEGCJJRGTFMYDG-UHFFFAOYSA-N	–	–	22.37 ± 0.2	–
3.	**8c**	ZIALVUBNMFXWAZ-UHFFFAOYSA-N	–	–	27.75 ± 0	–
4.	**8d**	KQSFMHKBKUMGPL-UHFFFAOYSA-N	–	–	4.77 ± 0.8	–
5.	**8e**	DIEWCCHSMJHFRH-UHFFFAOYSA-N	–	–	48.34 ± 0.8	–
6.	**8f**	DEPIOVZXINNUPI-UHFFFAOYSA-N	–	3.51 ± 0.8	31.03 ± 0	–
7.	**8g**	MGBAGUKJIQEWOE-UHFFFAOYSA-N	–	–	–	–
8.	**8h**	IISOAIXPVCMWNZ-UHFFFAOYSA-N	–	–	4.27 ± 0.1	–
9.	**8i**	HJKOWWRXQOXAJD-UHFFFAOYSA-N	–	–	17.30 ± 0.4	–
10.	**8j**	BEYBZIMOUIEKBO-UHFFFAOYSA-N	–	6.88 ± 0.2	24.36 ± 0.5	–
11.	**9a**	AXCXOCWNLHTDRS-UHFFFAOYSA-N	ND[c]			
12.	**9b**	CJAHCGLBOXKKED-UHFFFAOYSA-N	–	1.84 ± 0.9	7.59 ± 0.1	–
13.	**9c**	PDVGVJSTPKUFPH-UHFFFAOYSA-N	–	–	16.25 ± 0.2	–
14.	**9d**	WZHYYQKEIRUVIG-UHFFFAOYSA-N	–	–	3.32 ± 0.3	–
15.	**9e**	BZWQFMQXCFEUSO-UHFFFAOYSA-N	ND			
16.	**9f**	IJAHMCPXFQMCCT-UHFFFAOYSA-N	ND			
17.	**9g**	LQYLMVRXWBFASI-UHFFFAOYSA-N	–	–	7.78 ± 0.1	–
18.	**9h**	RULGIDYOBNAEGP-UHFFFAOYSA-N	ND			
19.	Ofloxacin[d]		83.79 ± 0.8	85.24 ± 0.5	88.05 ± 0.6	82.45 ± 0.7

[a] Result represents as mean of triplicate ± standard error of mean (SEM).
[b] No inhibition observed.
[c] Not determined.
[d] Standard drug ofloxacin (50 μg/mL) is used.

3.0 REFERENCES

[1] Paxton JD (1991) In assay for antifungal activity; Hostettmann K (ed.), Dey PM, Harborne JB, Methods in plant biochemistry, London Academic Press 6: 33.

[2] Arfan M, Khan R, Khan MA, Anjum S, Choudhary MI, Ahmad M (2010) Synthesis and antileishmanial and antimicrobial activities of some 2,3-disubstituted 3*H*-quinazolin-4-ones. J. Enzyme Inhib. Med. Chem. 25: 451–458. doi: https://doi.org/10.3109/14756360903309412

[3] Choudhary MI, Shahwar D–E, Parveen Z, Jabbar A, Ali I, Rahman A–U (1995) Antifungal steroidal lactones from w*ithania coagulance*. Phytochemistry 40: 1243–1246. doi: https://doi.org/10.1016/0031-9422(95)00429-B

[4] Pettit RK, Weber CA, Kean MJ, Hoffmann H, Pettit GR, Tan R, Franks KS, Horton ML (2005) Microplate alamar blue assay for staphylococcus epidermidis biofilm susceptibility testing. Antimicrob. Agents Chemother., 49: 2612–2617. doi: 10.1128/AAC.49.7.2612-2617.2005

4.0 HPLC Chromatogram of Enzymatic Hydrolysis

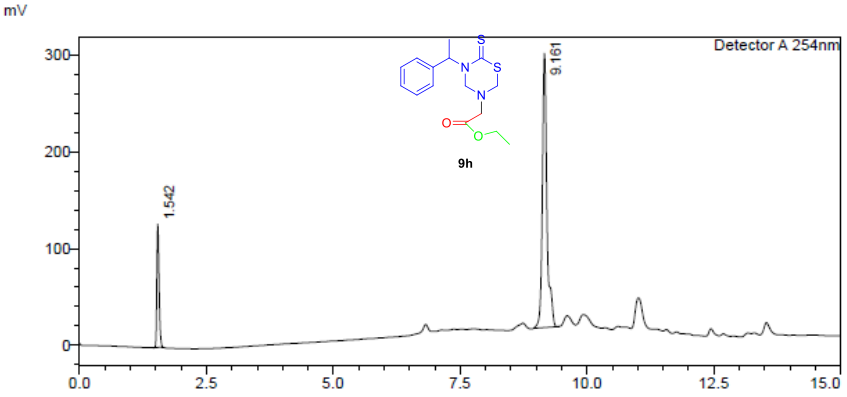
4.1. THTT ester derivative 9h (in buffer and acetone)

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	: Phenyl Glycine ester nmrpure		
Sample ID	: pure prodrug nmr pure		
Data Filename	: Phenyl Glycine ester nmrpure001_area.lcd		
Method Filename	: Prodrug_Method_file_JH_2.lcm		
Batch Filename	:		
Vial #	: -1	Sample Type	: Unknown
Injection Volume	: 10 uL		
Date Acquired	: 1/23/2021 5:20:48 PM	Acquired by	: System Administrator
Date Processed	: 1/23/2021 5:52:45 PM	Processed by	: System Administrator

<Chromatogram>



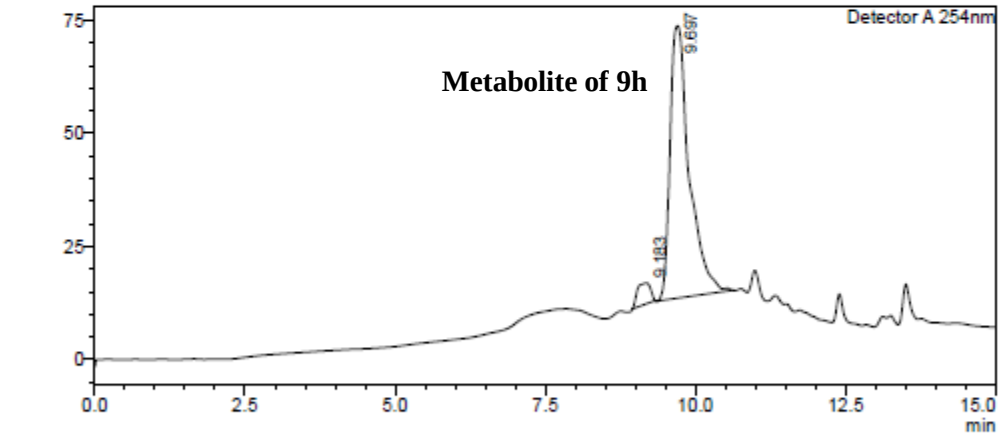
4.1.1. After 20 minutes; Esterase Hydrolysis of THTT ester derivative (9h)

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	: Phenyl ethyl ester20 min_revised		
Sample ID	: Phenyl ethyl ester20 min_revise		
Data Filename	: Phenyl ethyl ester20 min_revised001.lcd		
Method Filename	: Prodrug_Method_file_JH_2.lcm		
Batch Filename	:		
Vial #	: -1	Sample Type	: Unknown
Injection Volume	: 20 uL		
Date Acquired	: 1/23/2021 7:35:49 PM	Acquired by	: System Administrator
Date Processed	: 1/23/2021 7:50:51 PM	Processed by	: System Administrator

<Chromatogram>



<Peak Table>

Detector A 254nm

Peak#	Ret. Time	Area	Height	Area%
1	9.183	73234	4563	5.189
2	9.697	1338037	60179	94.811
Total		1411271	64742	100.000

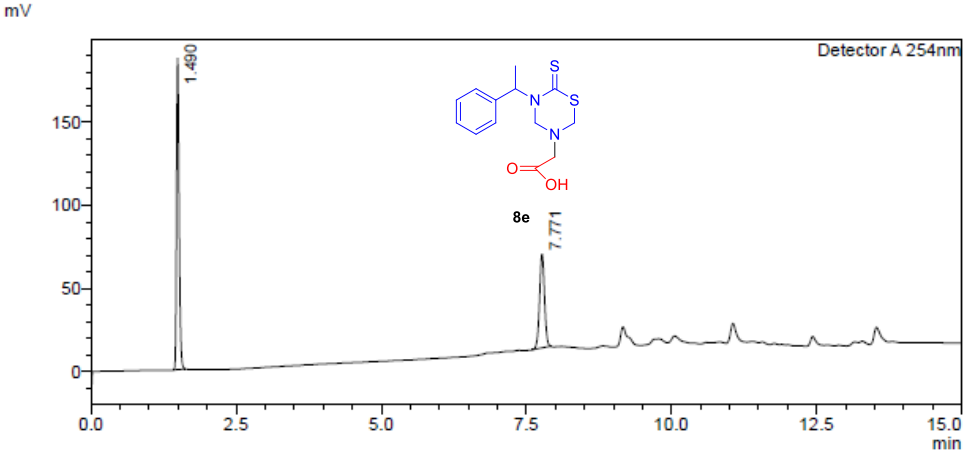
4.2. THTT (8e) acidic counterpart (in buffer and acetone)

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	: Phenyl Glycine acid nmrpure		
Sample ID	: pure acid nmr pure		
Data Filename	: Phenyl Glycine acid nmrpure001.lcd		
Method Filename	: Prodrug_Method_file_JH_2.lcm		
Batch Filename	:		
Vial #	: -1	Sample Type	: Unknown
Injection Volume	: 20 uL		
Date Acquired	: 1/23/2021 5:40:39 PM	Acquired by	: System Administrator
Date Processed	: 1/23/2021 5:55:41 PM	Processed by	: System Administrator

<Chromatogram>



<Peak Table>

Detector A 254nm

Peak#	Ret. Time	Area	Height	Area%
1	1.490	585554	187308	63.453
2	7.771	337255	56250	36.547
Total		922808	243559	100.000

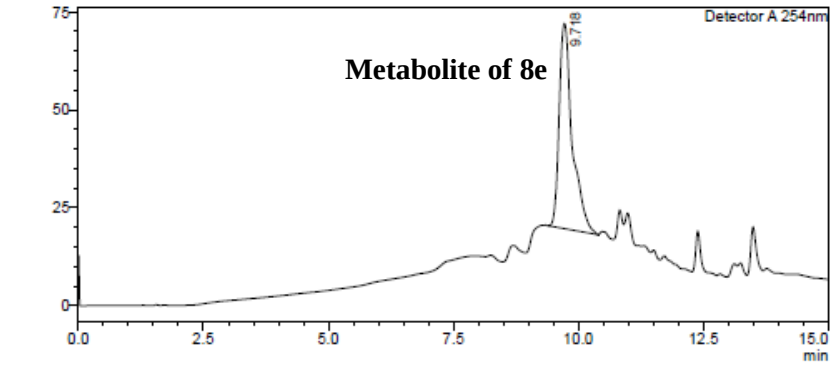
4.2.1. After 20 minutes; Esterase Hydrolysis of THTT (8e) Acidic Counterpart

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	: Phenyl Glycine acid10 min_f		
Sample ID	: pure acid RMIX 10 min_f		
Data Filename	: Phenyl Glycine acid10 min_f001.lcd		
Method Filename	: Prodrug_Method_file_JH_2.lcm		
Batch Filename	:		
Vial #	: -1	Sample Type	: Unknown
Injection Volume	: 20 uL		
Date Acquired	: 1/23/2021 6:56:08 PM	Acquired by	: System Administrator
Date Processed	: 1/23/2021 7:11:09 PM	Processed by	: System Administrator

<Chromatogram>



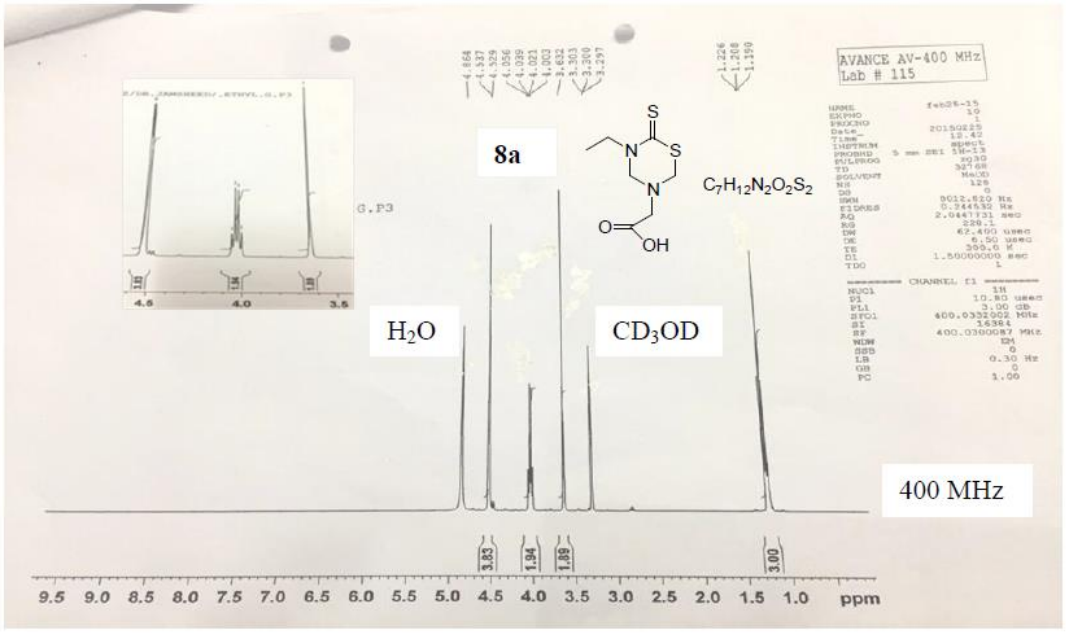
<Peak Table>

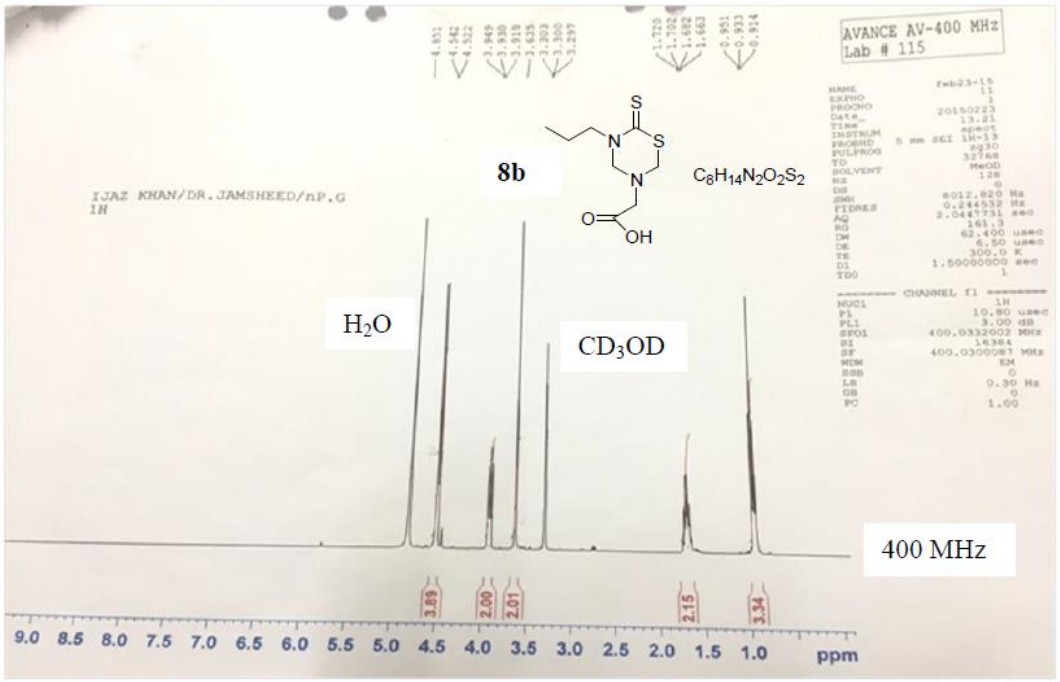
Detector A 254nm

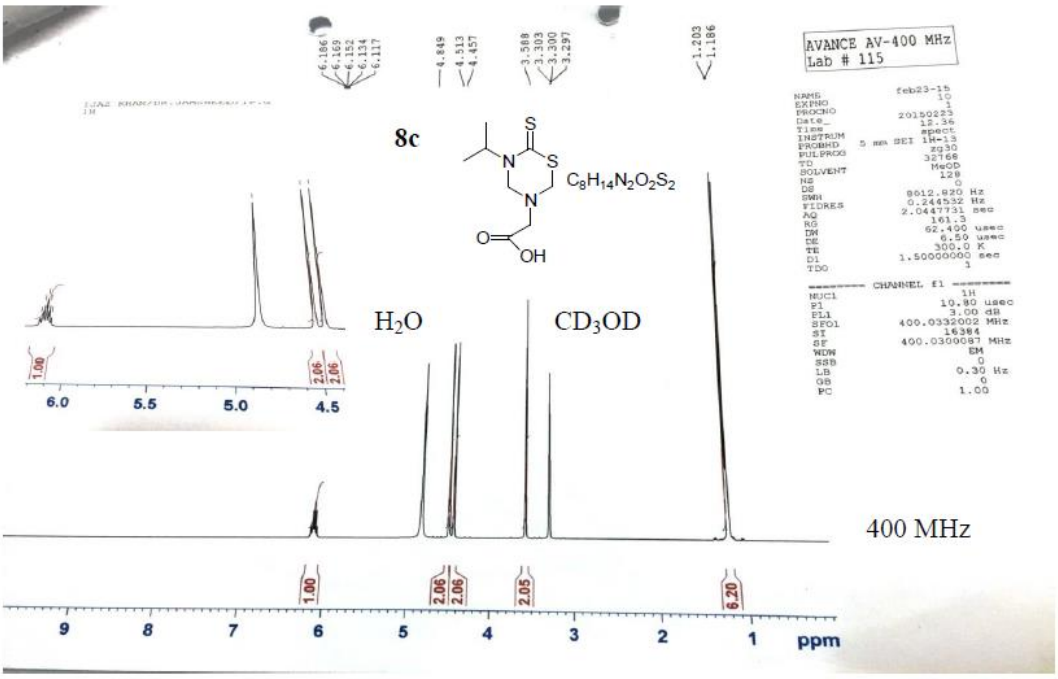
Peak#	Ret. Time	Area	Height	Area%
1	9.718	956548	52399	100.000
Total		956548	52399	100.000

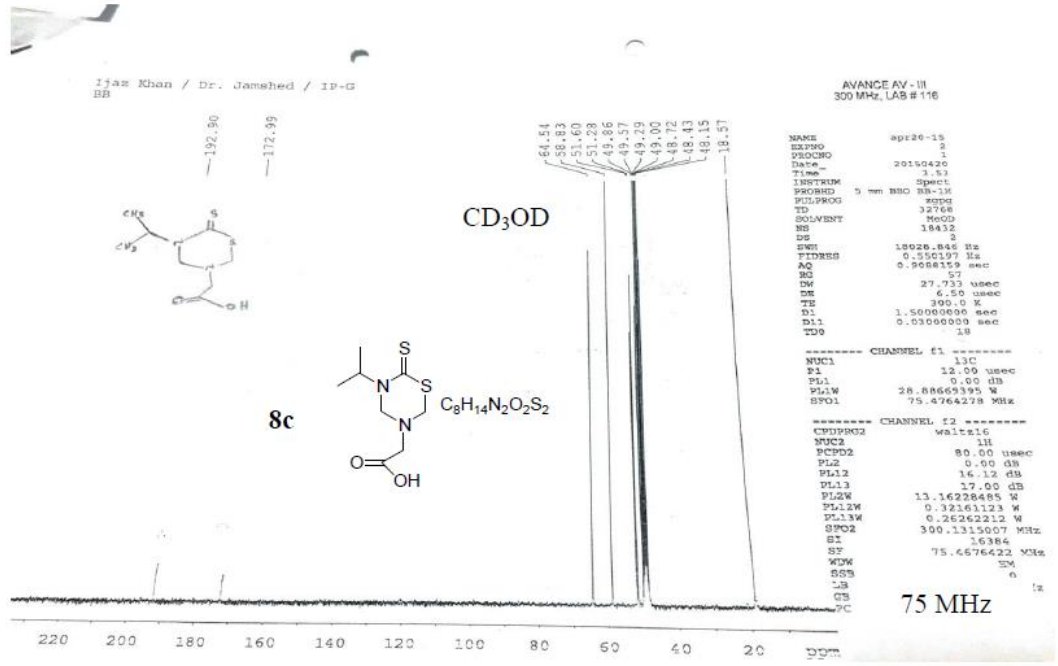
5. NMR Spectra

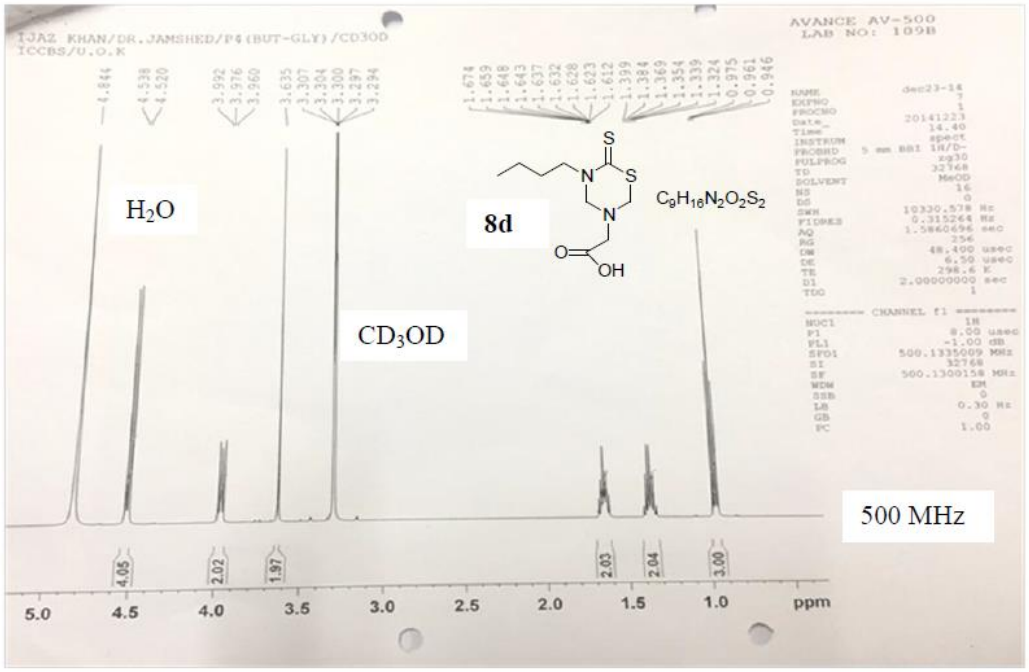
■ **NMR Spectra**

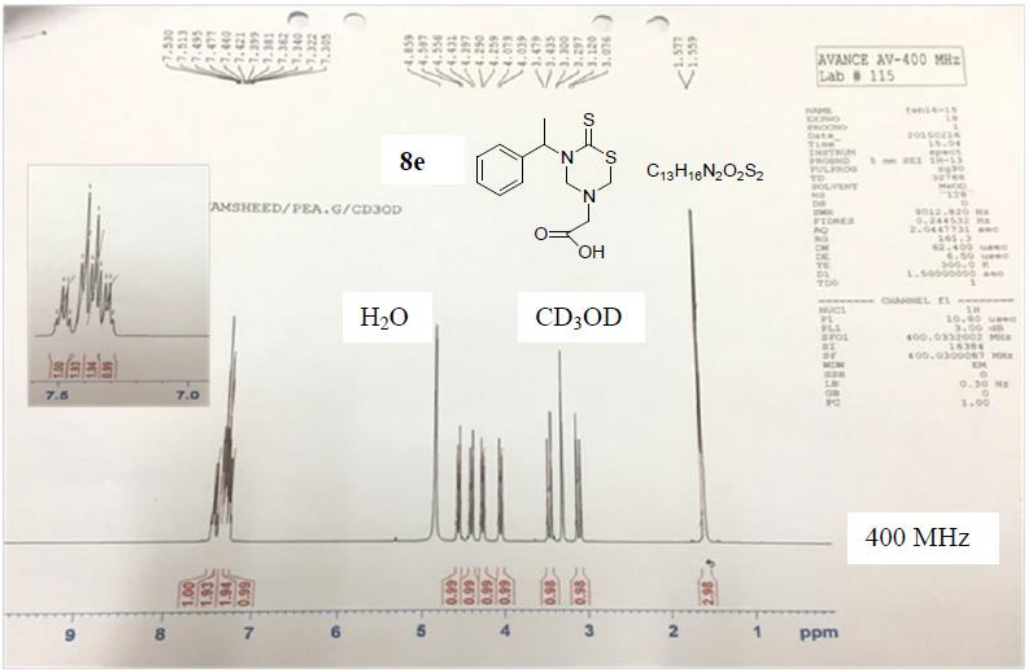


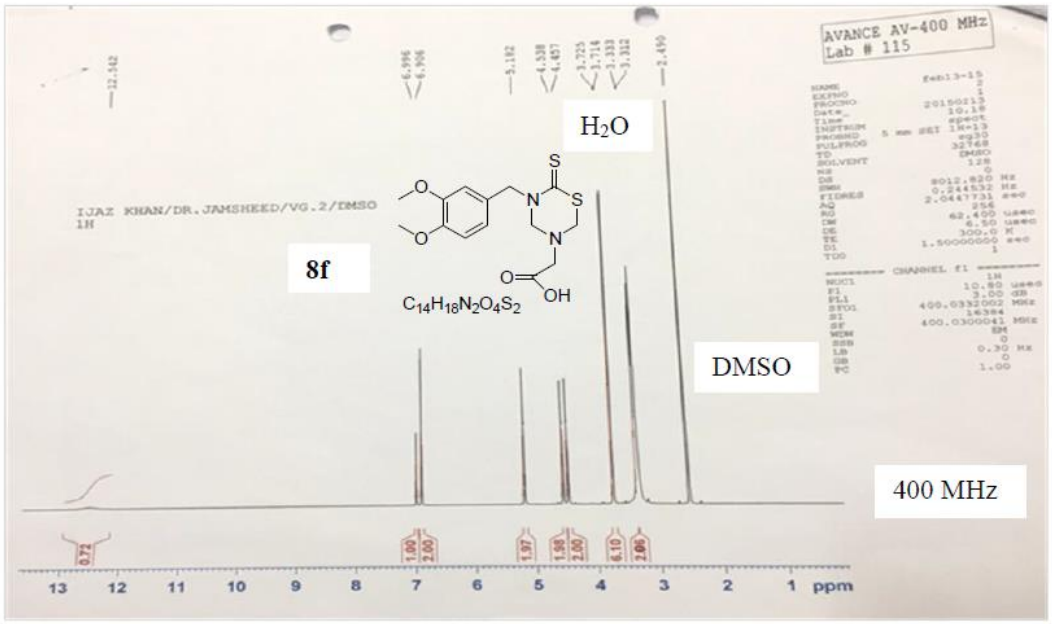


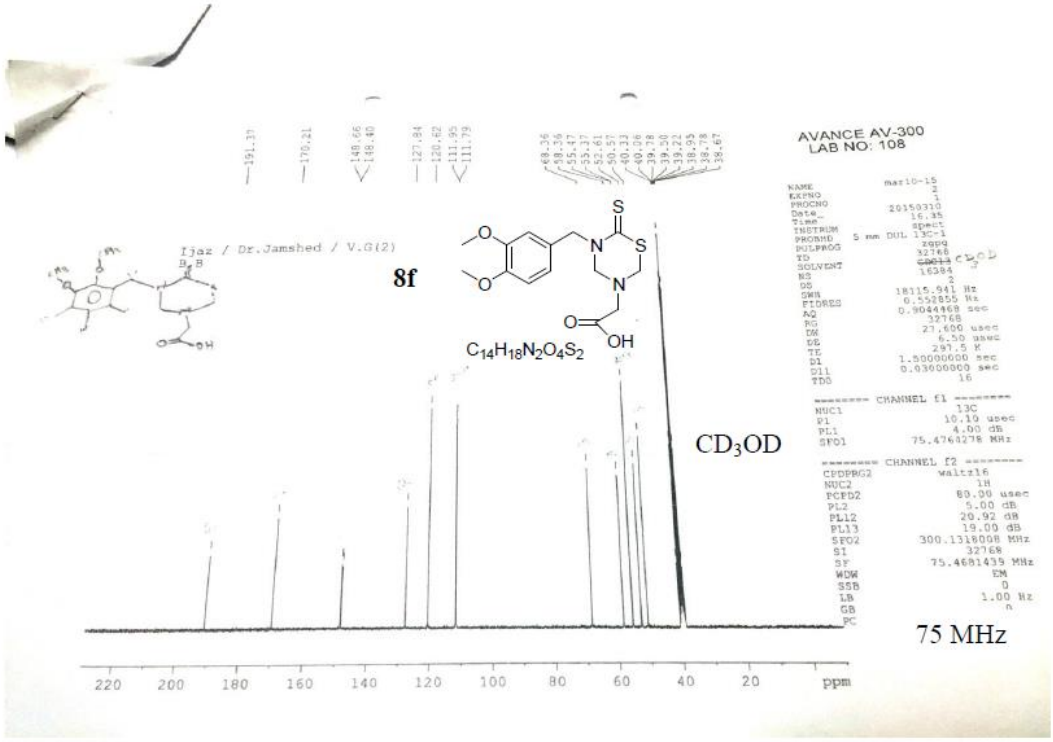

8c
$C_8H_{14}N_2O_2S_2$
H_2O
CD_3OD
AVANCE AV-400 MHz
Lab # 115
400 MHz
ppm

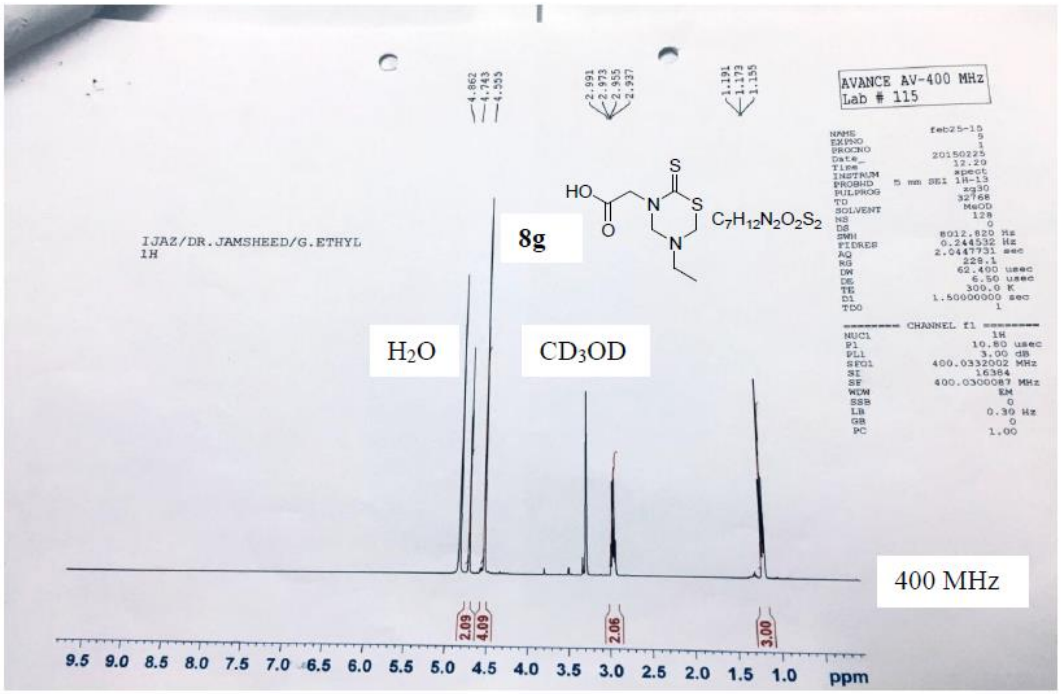

Ijaz Khan / Dr. Jamshed / IP-G
BB
AVANCE AV - III
300 MHz, LAB # 116
8c
$C_8H_{14}N_2O_2S_2$
CD_3OD
75 MHz
ppm

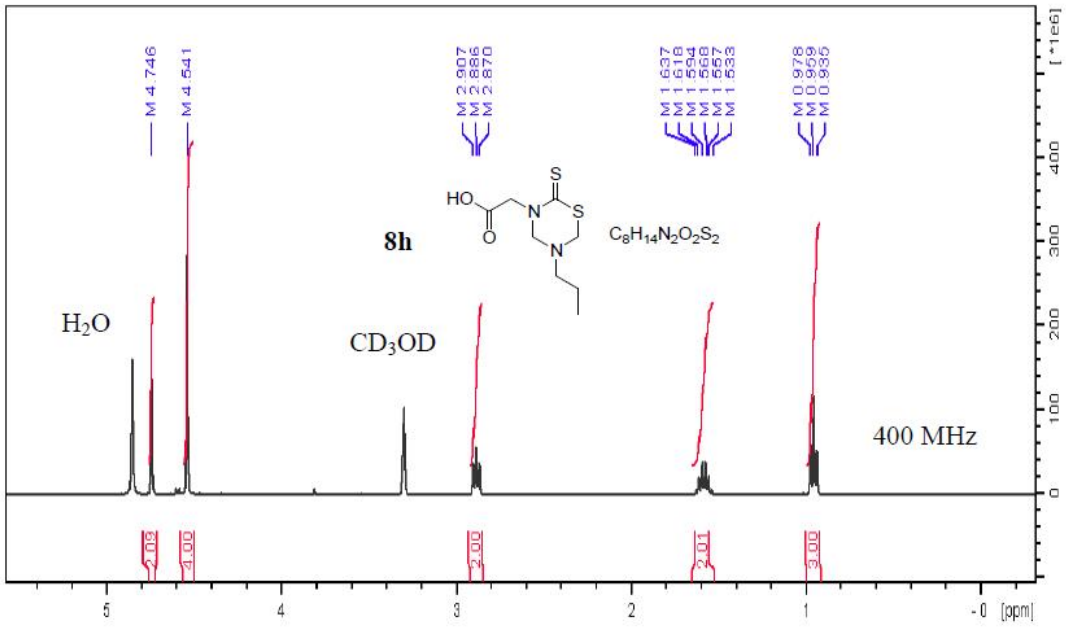

IJAZ KHAN/DR.JAMSHED/P6(BUT-GLY)/CD3OD
ICCBS/U.O.K
AVANCE AV-500
LAB NO: 109B
H2O
CD3OD
8d
C9H16N2O2S2
500 MHz
ppm

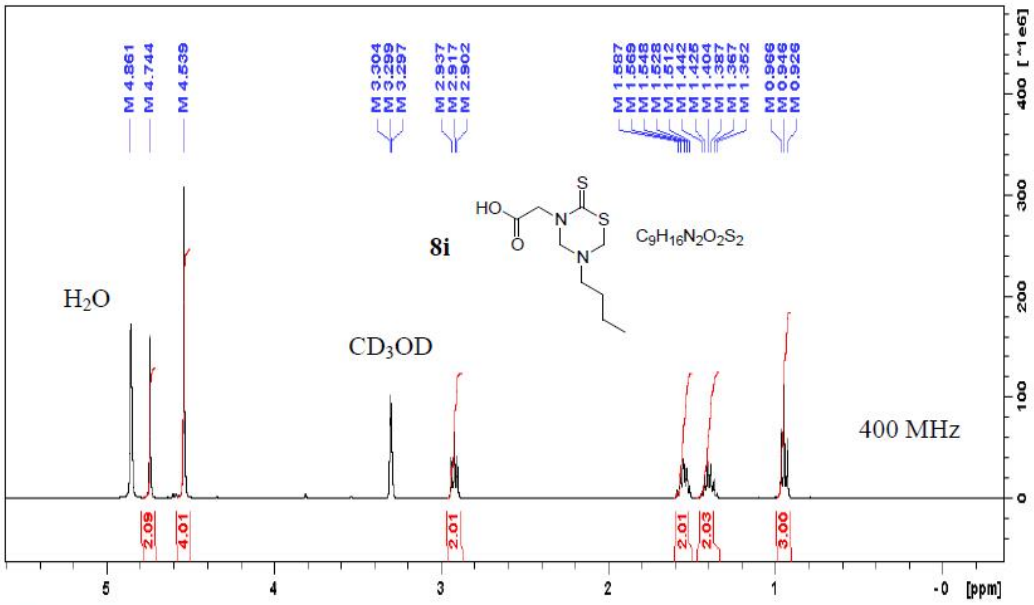

AVANCE AV-400 MHz
Lab # 115
8e
C13H16N2O2S2
H2O
CD3OD
400 MHz
ppm

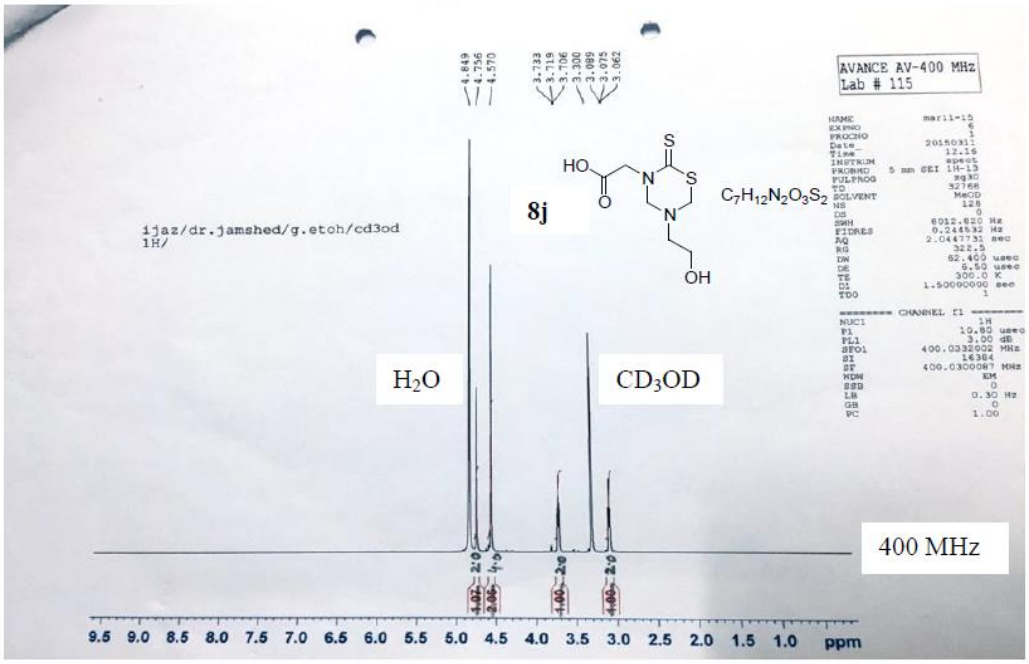

AVANCE AV-400 MHz
Lab # 115
IJAZ KHAN/DR.JAMSHEED/VG.2/DMSO
1H
H2O
8f
C14H18N2O4S2
DMSO
400 MHz
ppm

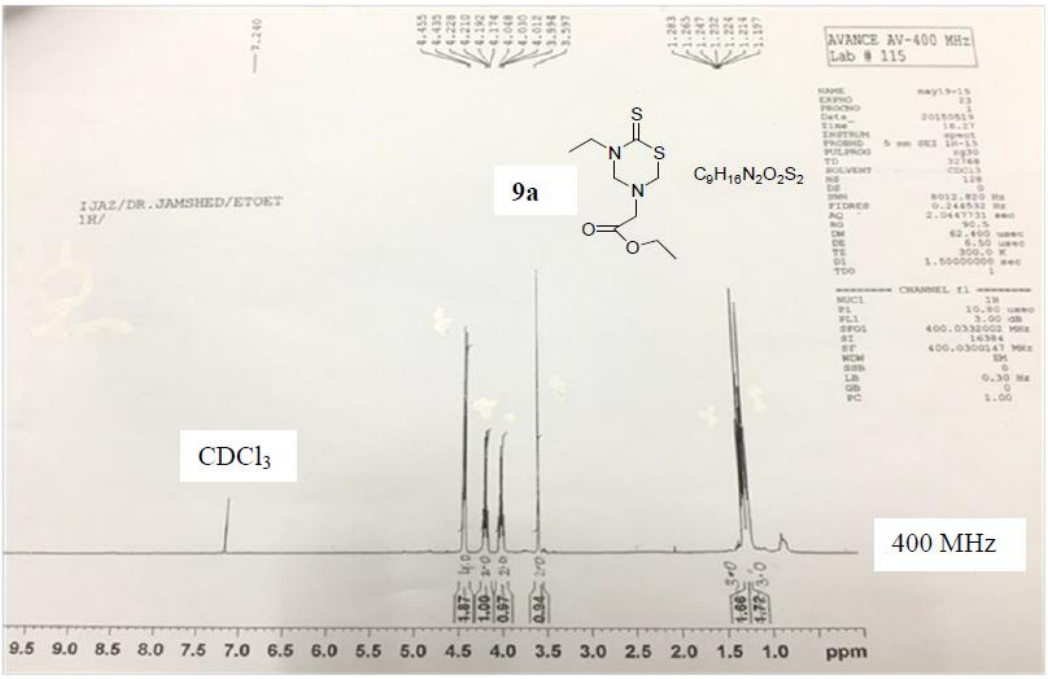

AVANCE AV-300
LAB NO: 108
Ijaz / Dr.Jamshed / V.G(2)
8f
C14H18N2O4S2
CD3OD
75 MHz
ppm

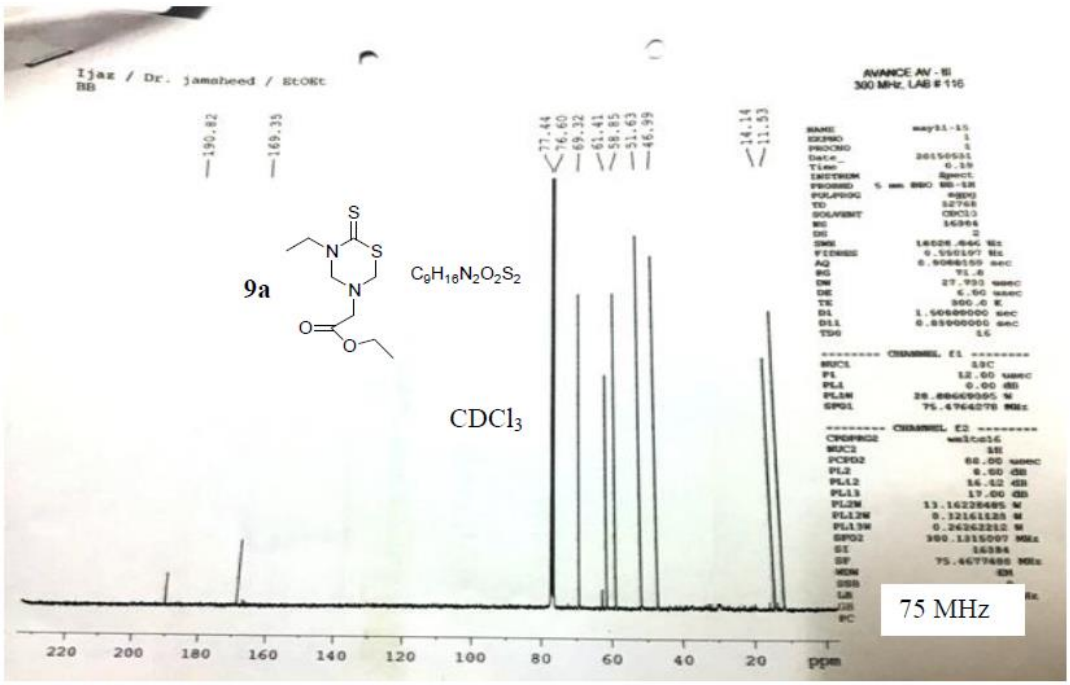

AVANCE AV-400 MHz
Lab # 115
IJAZ/DR.JAMSHEED/G.ETHYL
1H
8g
$C_7H_{12}N_2O_2S_2$
H_2O
CD_3OD
400 MHz
ppm

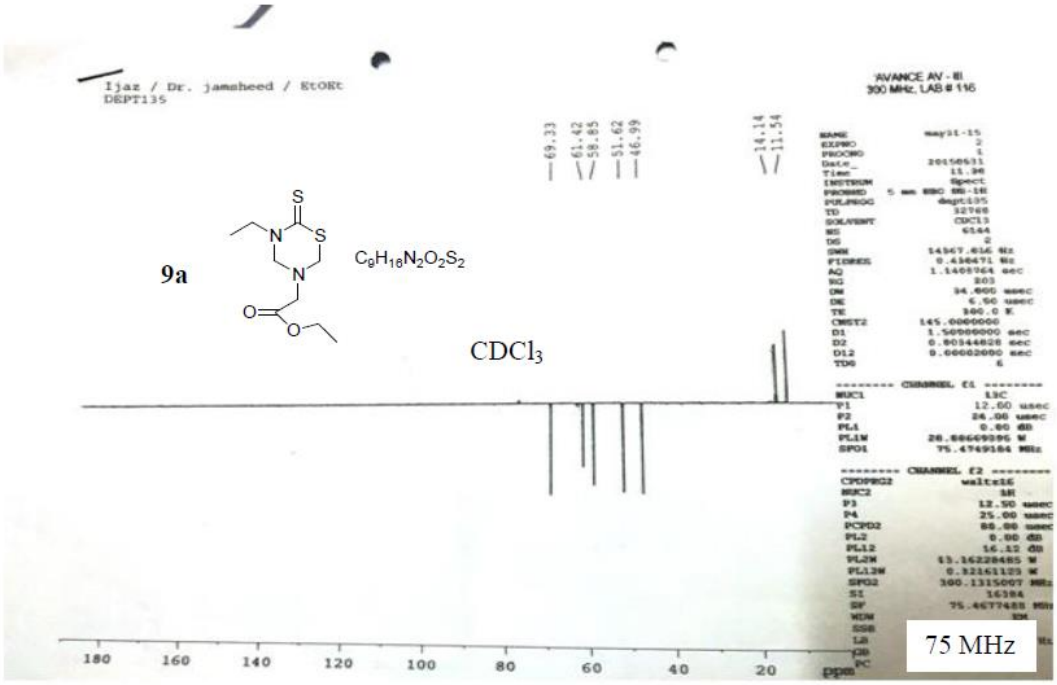

8h
$C_8H_{14}N_2O_2S_2$
H_2O
CD_3OD
400 MHz
[ppm]

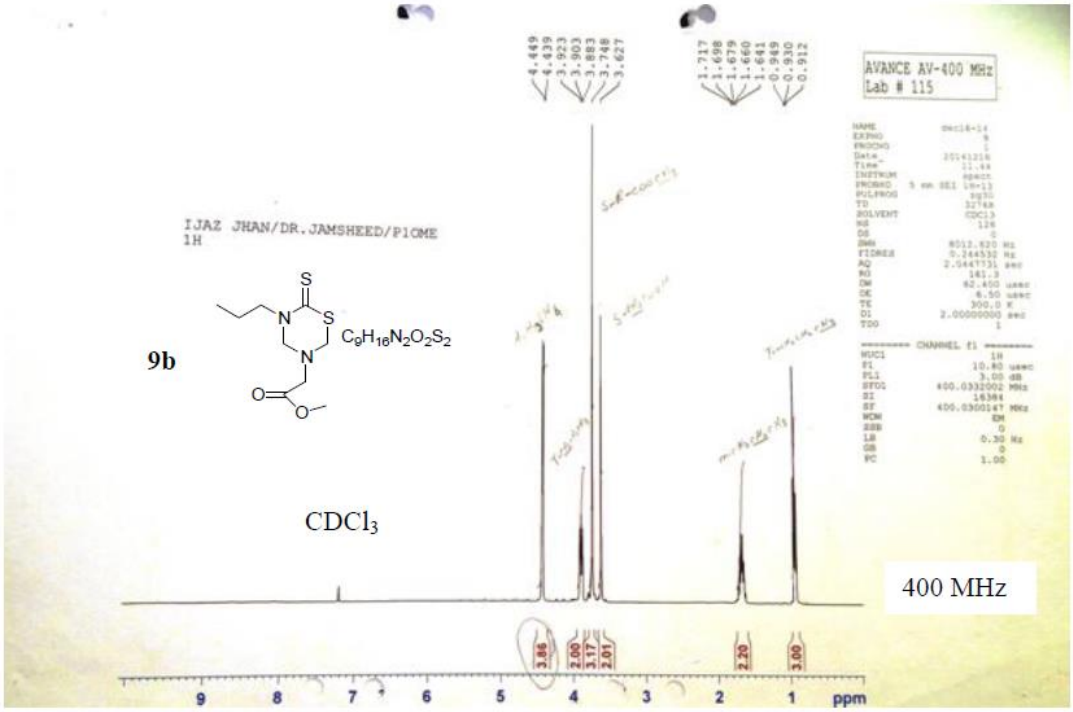

M 4.861
M 4.744
M 4.539
M 3.304
M 3.299
M 3.297
M 2.937
M 2.917
M 2.902
M 1.587
M 1.569
M 1.548
M 1.528
M 1.512
M 1.442
M 1.425
M 1.404
M 1.387
M 1.367
M 1.352
M 0.966
M 0.946
M 0.926
8i
$C_9H_{16}N_2O_2S_2$
H_2O
CD_3OD
400 MHz
2.09
4.01
2.01
2.01
2.03
3.00
[ppm]

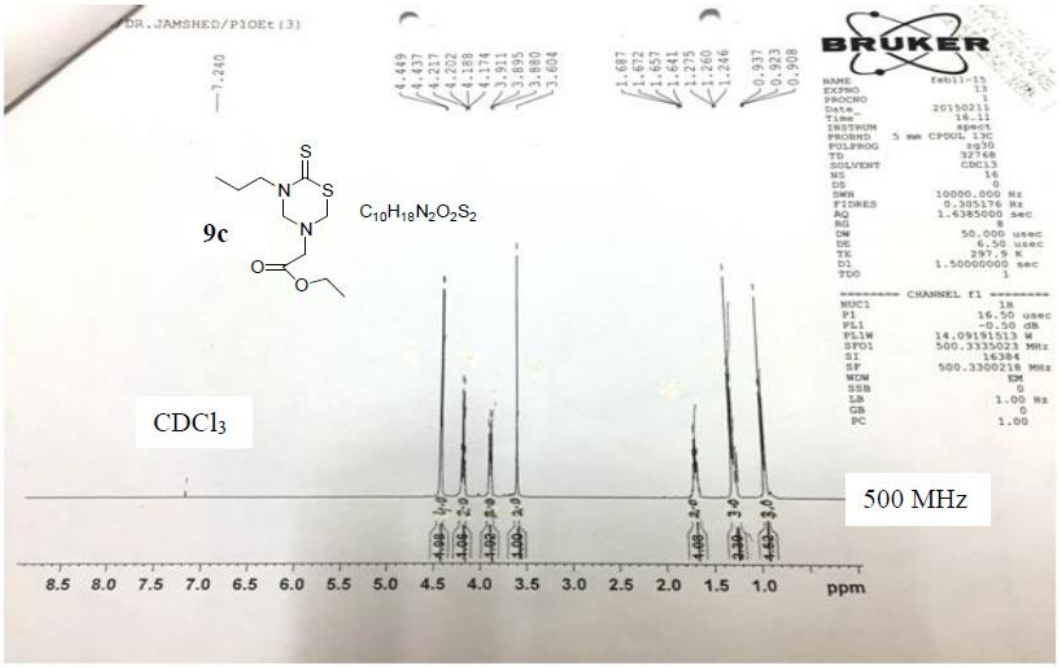

AVANCE AV-400 MHz
Lab # 115
ijaz/dr.jamshed/g.etoh/cd3od
1H/
8j
$C_7H_{12}N_2O_3S_2$
H_2O
CD_3OD
400 MHz
ppm

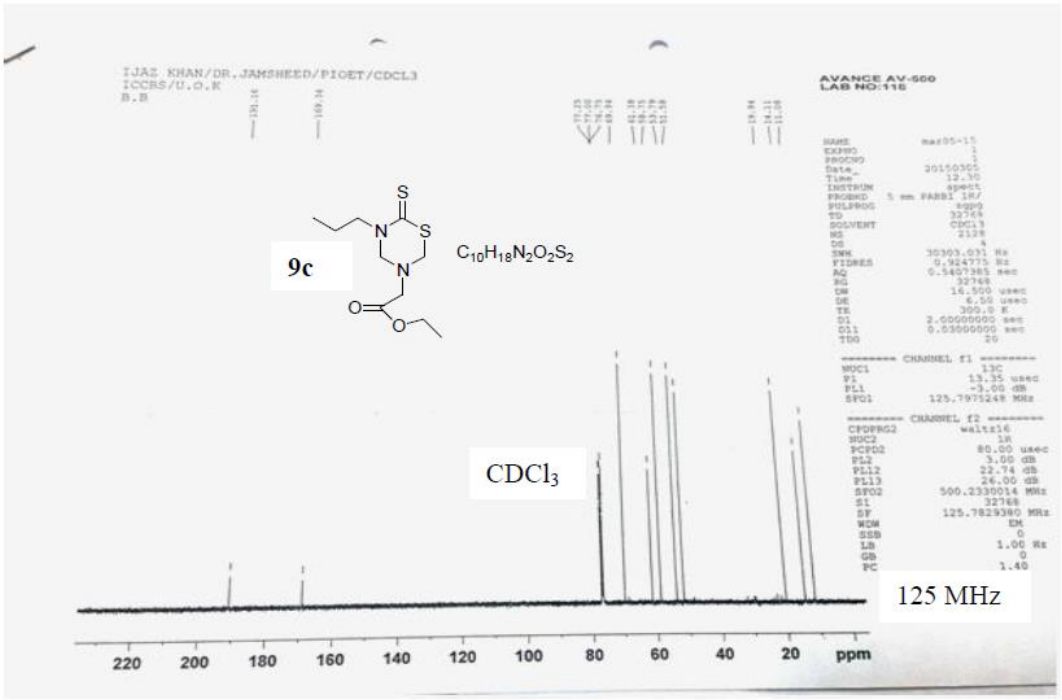

AVANCE AV-400 MHz
Lab # 115
IJAZ/DR.JAMSHED/ETOET
1H/
9a
$C_9H_{16}N_2O_2S_2$
$CDCl_3$
400 MHz
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm

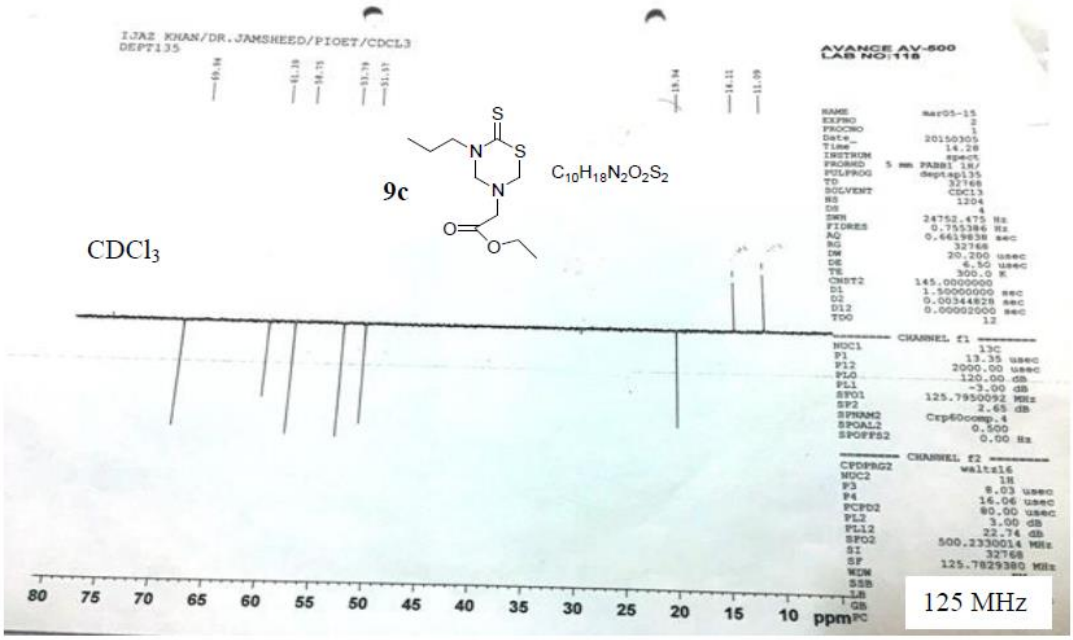

Ijaz / Dr. jamsheed / EtOEt
BB
AVANCE AV-
300 MHz, LAB # 116
9a
$C_9H_{16}N_2O_2S_2$
$CDCl_3$
75 MHz
220 200 180 160 140 120 100 80 60 40 20 ppm

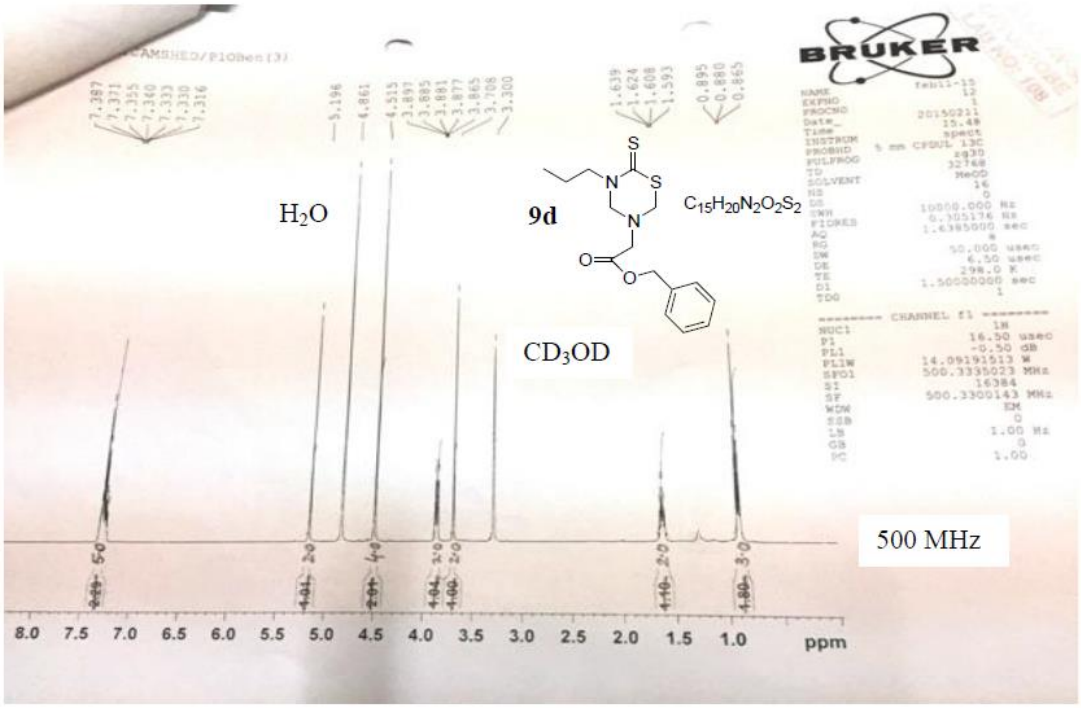

Ijaz / Dr. jamsheed / EtOAc
DEPT135
AVANCE AV - III
300 MHz, LAB # 116
69.33
61.42
58.85
51.62
46.99
14.14
11.54
9a
$C_9H_{16}N_2O_2S_2$
$CDCl_3$
75 MHz
180
160
140
120
100
80
60
40
20
ppm

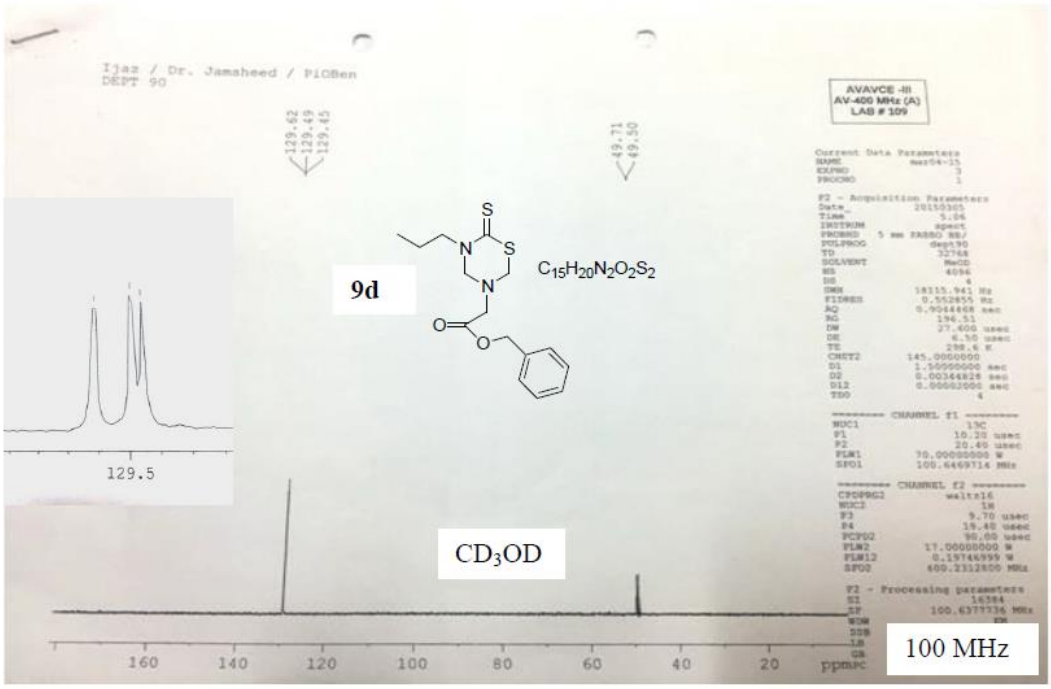

4.449
4.439
3.923
3.903
3.883
3.748
3.627
1.717
1.698
1.679
1.660
1.641
0.949
0.930
0.912
AVANCE AV-400 MHz
Lab # 115
IJAZ JHAN/DR.JAMSHEED/PICME
1H
9b
$C_9H_{16}N_2O_2S_2$
$CDCl_3$
400 MHz
3.86
2.00
3.17
2.01
2.20
3.00
9
8
7
6
5
4
3
2
1
ppm

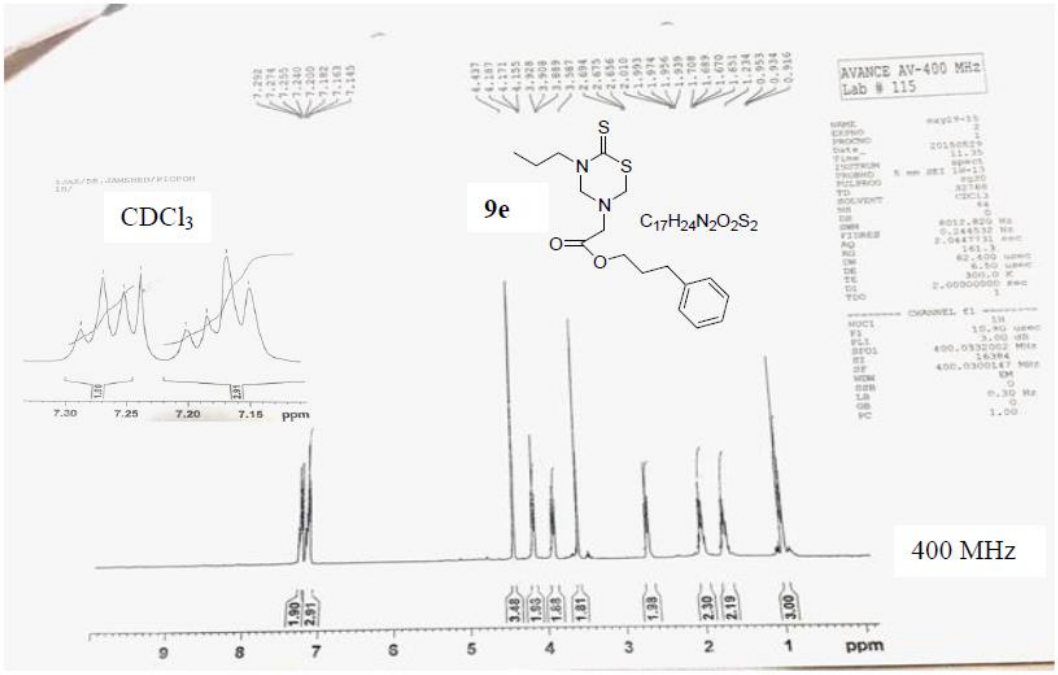

DR.JAMSHED/P1OEt (3)
BRUKER
9c
$C_{10}H_{18}N_2O_2S_2$
$CDCl_3$
500 MHz
ppm

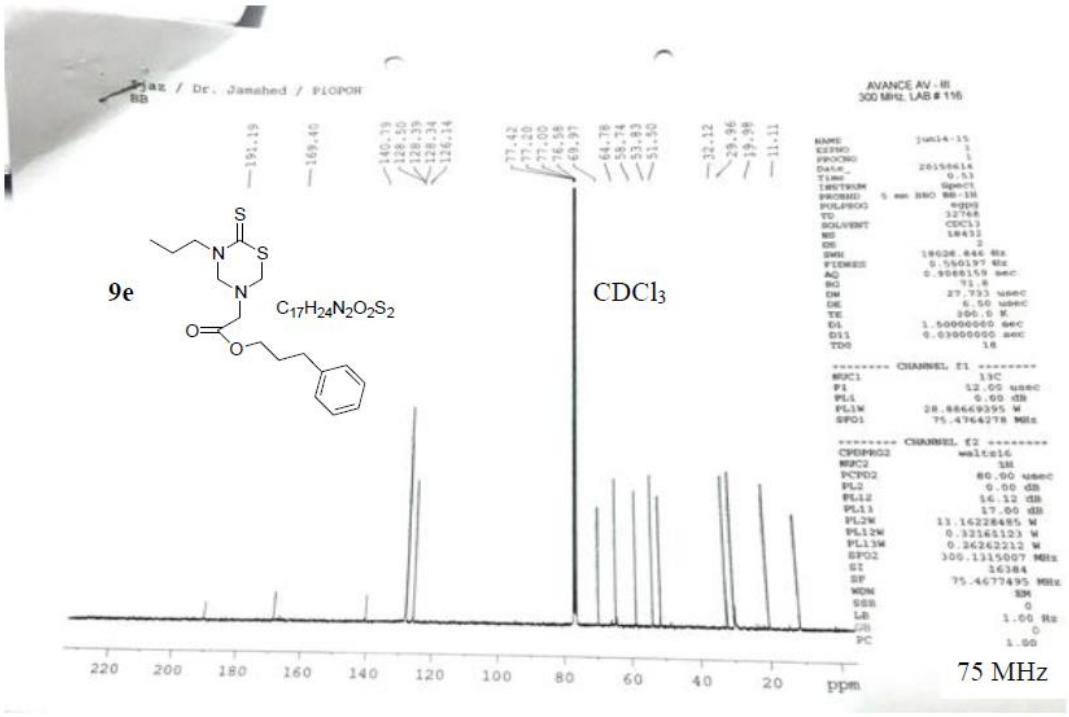

IJAZ KHAN/DR.JAMSHEED/P1OET/CDCL3
ICCBS/U.O.K
B.B
AVANCE AV-500
LAB NO:116
9c
$C_{10}H_{18}N_2O_2S_2$
$CDCl_3$
125 MHz
ppm

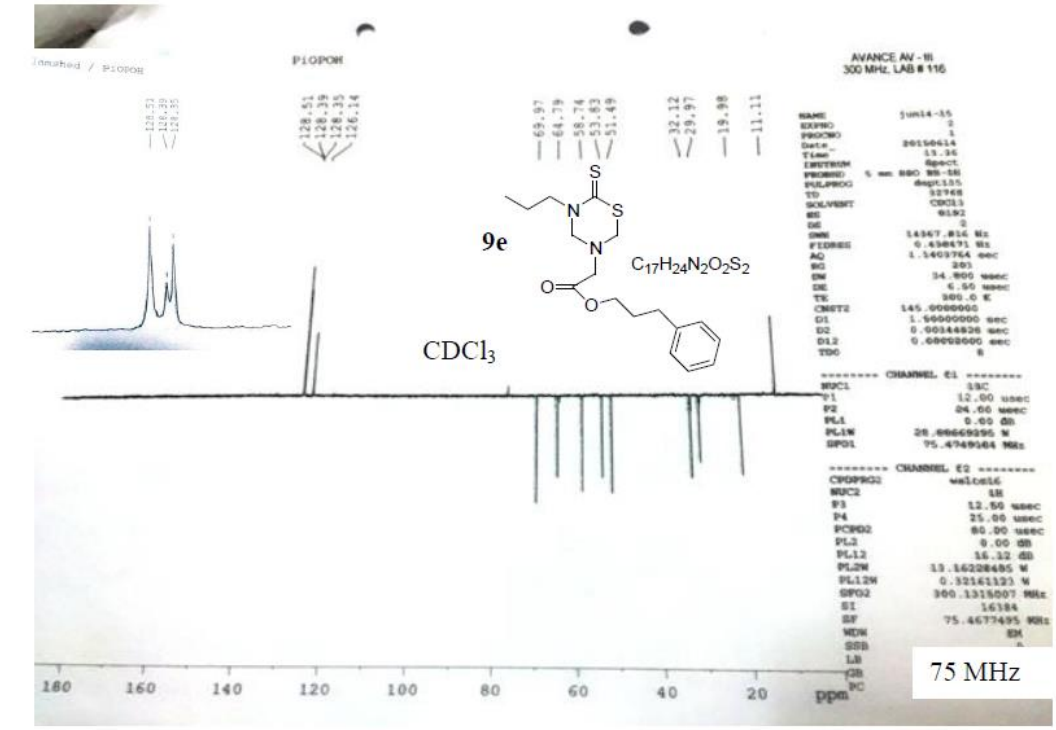

IJAZ KHAN/DR.JAMSHEED/PIOET/CDCL3
DEPT135
AVANCE AV-600
LAB NO:118
9c
$C_{10}H_{18}N_2O_2S_2$
$CDCl_3$
125 MHz

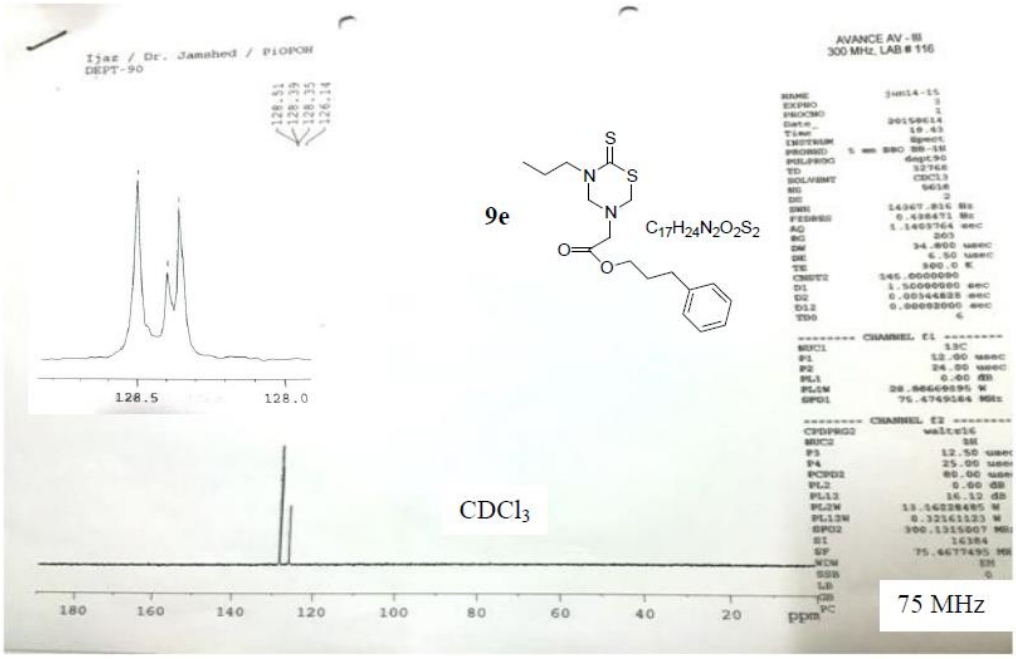

BRUKER
9d
$C_{15}H_{20}N_2O_2S_2$
H_2O
CD_3OD
500 MHz

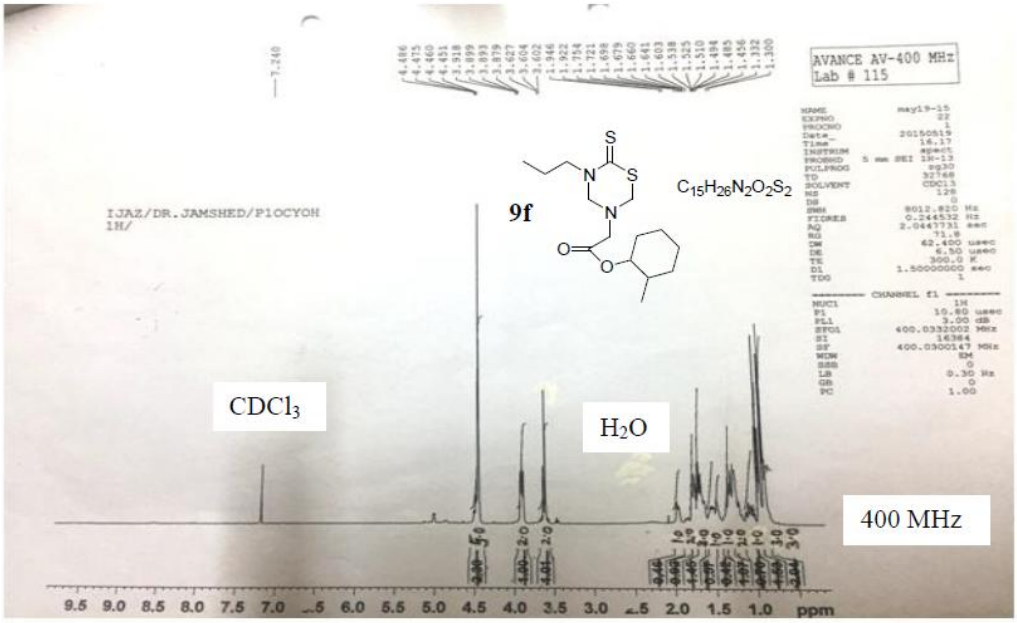

9d
$C_{15}H_{20}N_2O_2S_2$
CD_3OD
100 MHz

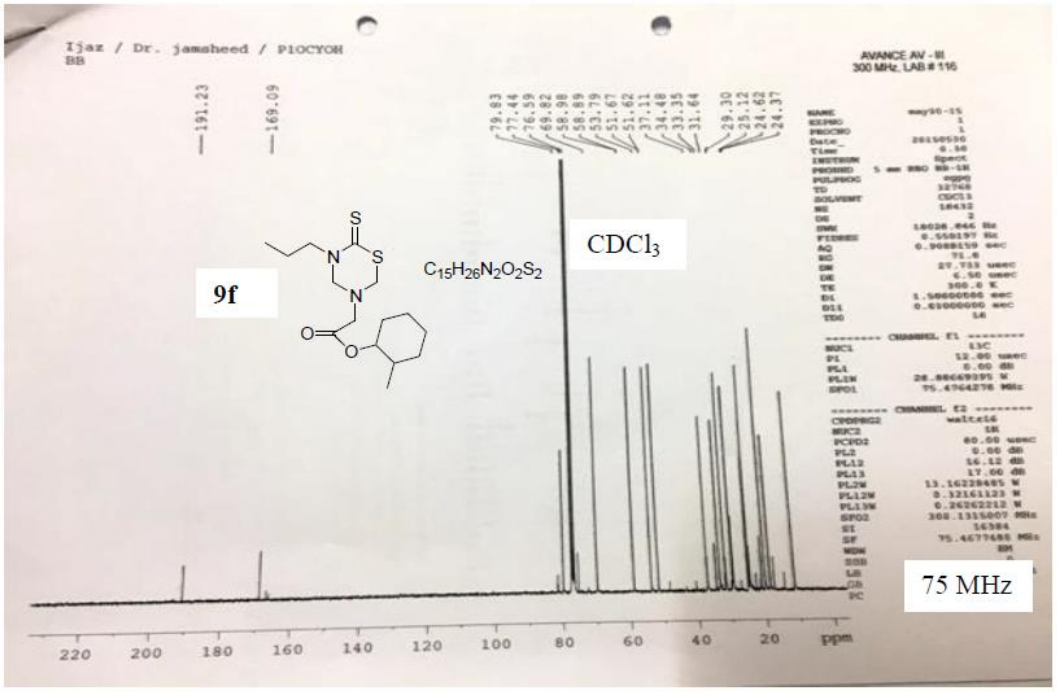

9e
$C_{17}H_{24}N_2O_2S_2$
$CDCl_3$
400 MHz

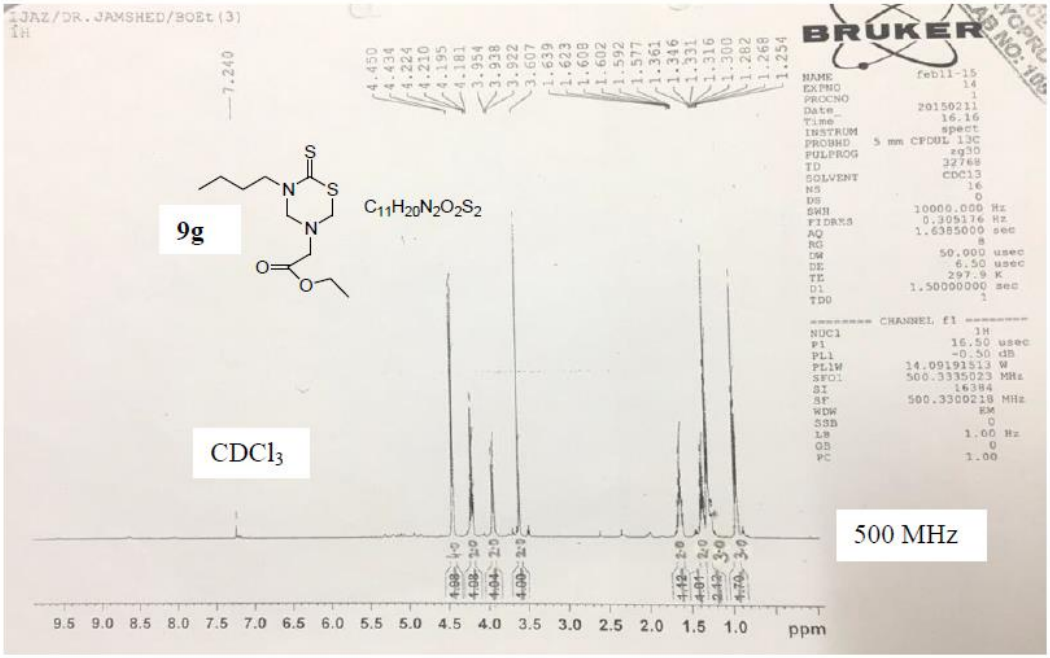

9e
$C_{17}H_{24}N_2O_2S_2$
$CDCl_3$
75 MHz

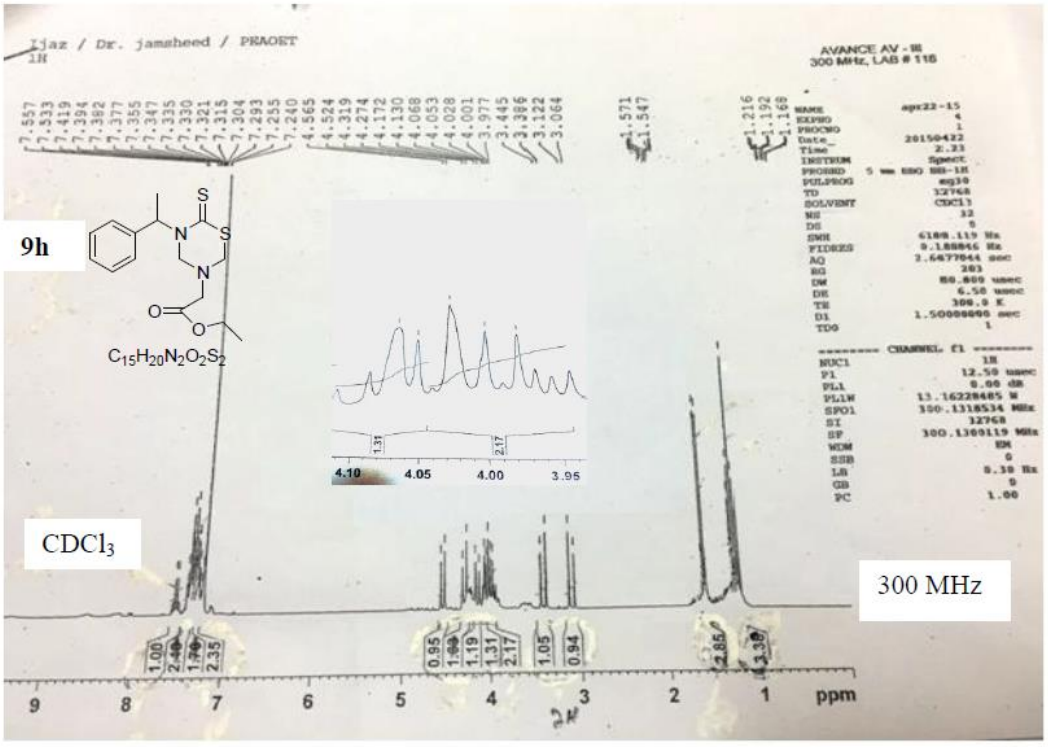

9e
$C_{17}H_{24}N_2O_2S_2$
$CDCl_3$
75 MHz

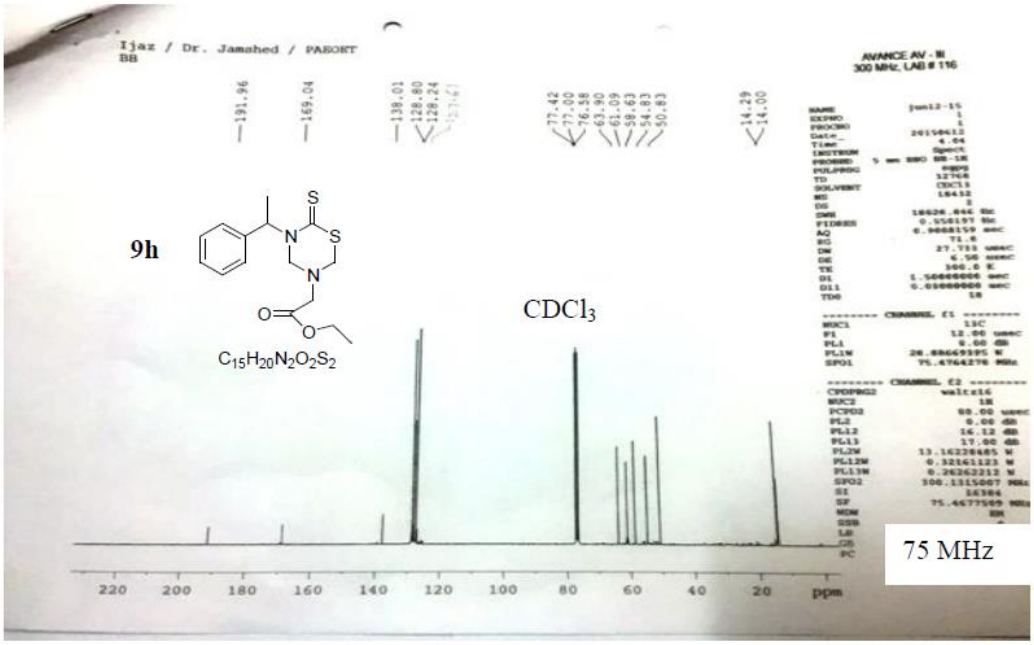

Ijaz / Dr. Jamshed / PiOPOH
DEPT-90
AVANCE AV - III
300 MHz, LAB # 116
9e
$C_{17}H_{24}N_2O_2S_2$
$CDCl_3$
75 MHz


AVANCE AV-400 MHz
Lab # 115
IJAZ/DR.JAMSHED/PiOCYOH
1H/
9f
$C_{15}H_{26}N_2O_2S_2$
$CDCl_3$
H_2O
400 MHz

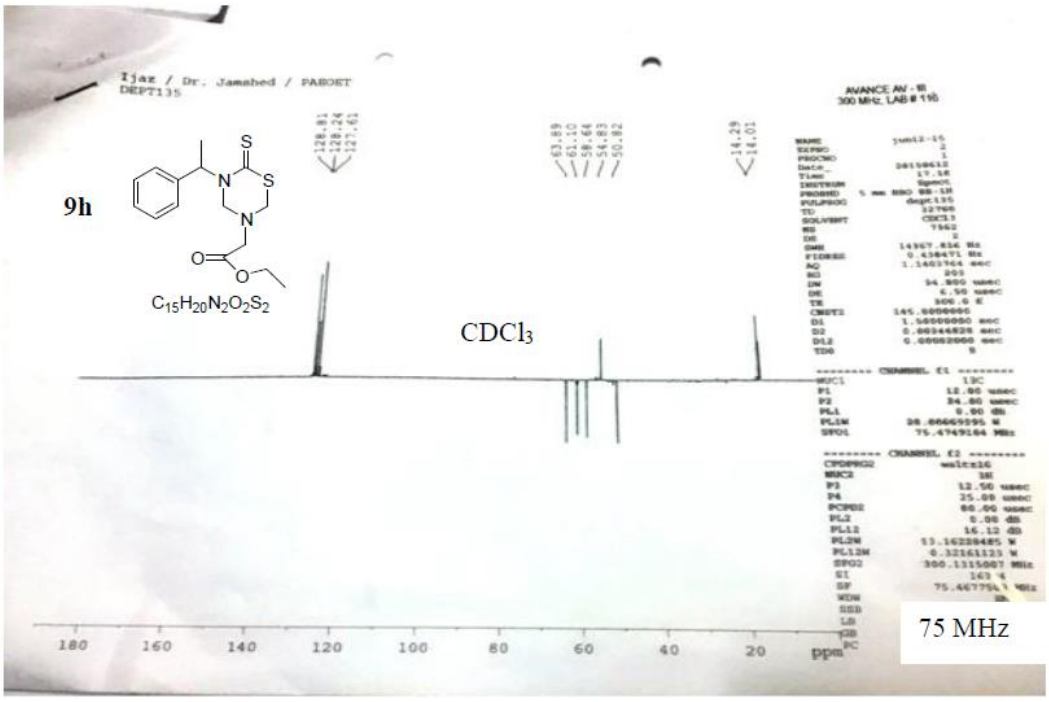

Ijaz / Dr. jamsheed / P1OCYOH
BB
AVANCE AV - III
300 MHz, LAB # 116
191.23
169.09
79.83
77.44
76.59
69.82
58.98
58.89
53.79
51.67
51.62
37.11
34.48
33.35
31.64
29.30
25.12
24.62
24.37
9f
$C_{15}H_{26}N_2O_2S_2$
$CDCl_3$
75 MHz
220 200 180 160 140 120 100 80 60 40 20 ppm

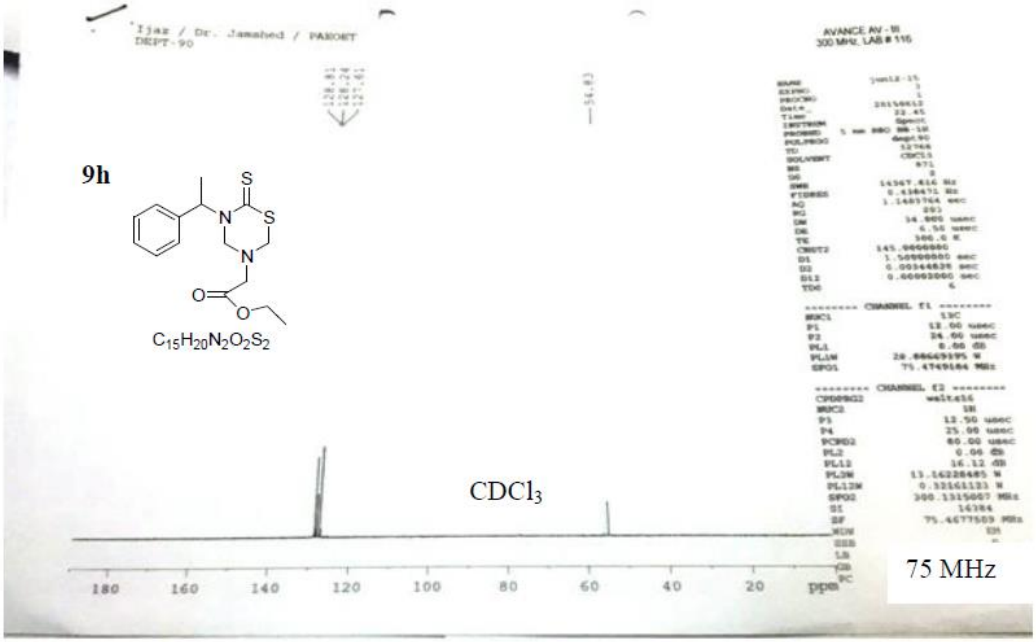

IJAZ/DR.JAMSHED/BOEt(3)
1H
BRUKER
7.240
4.450
4.434
4.224
4.210
4.195
4.181
3.954
3.938
3.922
3.607
1.639
1.623
1.608
1.602
1.592
1.577
1.361
1.346
1.331
1.316
1.300
1.282
1.268
1.254
9g
$C_{11}H_{20}N_2O_2S_2$
$CDCl_3$
500 MHz
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm


Ijaz / Dr. jamsheed / PRAOET
1H
AVANCE AV - III
300 MHz, LAB # 116
9h
$C_{15}H_{20}N_2O_2S_2$
$CDCl_3$
300 MHz
ppm


Ijaz / Dr. Jamshed / PASOET
BB
AVANCE AV - III
300 MHz, LAB # 116
9h
$C_{15}H_{20}N_2O_2S_2$
$CDCl_3$
75 MHz
ppm

S24


Ijaz / Dr. Jamshed / PAEC
DEPT135
9h
$C_{15}H_{20}N_2O_2S_2$
$CDCl_3$
75 MHz
ppm


Ijaz / Dr. Jamshed / PAEC
DEPT-90
9h
$C_{15}H_{20}N_2O_2S_2$
$CDCl_3$
75 MHz
ppm

S25