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Synthesis, *in silico* Studies, and Anticonvulsant Activity of 1,3,4-Oxadiazole Derivatives

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Abstract

The title compounds 1,3,4-oxadiazole derivatives **(C1-C5)** were synthesized by the cyclization of 4-hydroxy benzhydrazide **(1)** with various substituted aromatic aldehydes **(2)** in the presence of ceric ammonium nitrate. The structures of the newly synthesized compounds were established based on FT-IR, ^{1}H-NMR, and Mass spectral data. *In silico* analysis was carried out using the Schrodinger 2018-3 suite device Maestro and docked to the binding site of the Human GABAA receptor (PDB ID:4COF). The toxicity of the compounds was predicted using the LAZAR (Lazy structure-activity relationship) program. The *invivo* anticonvulsant study was performed by means of a maximal electroshock test and pentylenetetrazole (PTZ)-induced seizures. Compounds **C4**&**C5** showed the highest docking score of −5.676 and −5.277, respectively, and compounds **C4**&**C5** showed the most increased *in vivo* anticonvulsant activity when compared with the reference drugs in both the PTZ and MES test methods.

Keywords: 1,3,4-oxadiazole, Anticonvulsant activity, GABAA receptor, Molecular docking

Introduction

1,3,4-Oxadiazoles are heterocyclic compounds with an oxygen atom and 2 nitrogen atoms at the third and fourth positions, and they are found to be an important class of compounds in pharmaceutical chemistry because of their potential pharmacophore with diverse biological activities [1]. They have been reported for various activities such as anti-inflammatory [2], anticonvulsant [3], antimicrobial [4], ulcerogenic [5], anti-HIV [6], antifungal [7], anticancer [8], antihypertensive [9], antitubercular [10] and analgesic [11] etc.

Drugs available therapeutically containing this heterocyclic nucleus are Zibotentan used as an anticancer agent [12], Fenadiazole as a hypnotic agent [13], Raltegravir as an HIV-integrase inhibitor drug [14], Furamizole as an antibacterial agent [15] and Tiodazosin as an alpha1-adrenergic antagonist [16].

Many works of literature report the presence of toxophoric –N=C–O– linkage in the 1,3,4-oxadiazole ring which isconsidered responsible for their potent biological activities. [17,18]. 1,3,4-oxadiazole heterocyclics participate in hydrogen bonding interactions with different receptors because of their ester functionalities and very good bioisosteres of amide, thereby, shows a significant increase in biological activity [19].

Epilepsy is a diverse group of illnesses affecting over 60 million people worldwide. It is distinguished by episodes of hypersynchronous neuronal firing and hyper excitability accompanied by motor, sensory, or autonomic occurrences with or without loss of consciousness [20]. Many alternative treatments are available for treating epilepsy, such as neuromodulation, palliative surgery, and a ketogenic diet (KD). However, regardless of its efficacy, the unpalatable and antagonistic features of the ketogenic diet do not meet patient compliance, especially children [21].

Moreover, antiepileptic drug therapy terminates or minimizes seizure frequency in only up to 60 - 70 % of patients, and long-term antiepileptic therapy (AEDs) remains the backbone of epilepsy treatment. Furthermore, 50 % of epilepsy patients experience undesirable side effects of the available drug treatments, rendering therapy difficult, so there is a high demand for new anticonvulsants with minimal or no side effects [22,23].

GABA receptors are the principal mediators of fast synaptic inhibitory transmission in the human brain. A reduction in GABA receptor signalling activates hyperactive neurological disorders such as epilepsy, anxiety, and insomnia [24]. Inadequacy in GABA transmission has often been involved in epilepsy in animal models and human syndromes [25].

The 1,3,4-oxadiazole nucleus has garnered remarkable recognition for acting as a selective GABA potentiating potent anticonvulsant agent having both GABA and sodium channel mechanisms. It has also been reported that some of the derivatives containing 1,3,4-oxadiazole nucleus are better tolerated than benzodiazepines, making themmore desirable drugs for the treatment of epilepsy [26].

From the above observations, the therapeutic importance of the 1,3,4-oxadiazole ring appeared to be of great interest for its synthesis andfor developing possible modifications of the structural components in the general framework of oxadiazole with respect to their binding efficacy, potency, and selectivity which would guide in the design of new oxadiazole derivatives as anticonvulsants.

Materials and methods

The Equiptronics digital melting point apparatus was employed for determining the melting points, which were uncorrected. Thin-layer chromatography (TLC) was used to monitor the reaction progress using plates of Silica gel G. Spots of the TLC were visualized under a UV light chamber. ^{1}H-NMR spectra were recorded using the Agilent-NMR 400-MR DD_2 spectrometer operating at 400 MHz using DMSO as a solvent. Chemical shift values are expressed in ppm (δ) values and TMS is used as an internal standard. An Alpha Bruker FT-IR Spectrometer was used for recording IR spectra (cm^{-1}) employing KBr disc. The Mass spectrum was recorded on the Waters LC-MS/MS instrument. All the chemical reagents as well as the required solvents were obtained from Sigma Aldrich, Bangalore, India.

General procedure for the synthesis of 1,3,4-oxadiazole derivatives (C1-5)

A mixture of p-hydroxybenzhydrazide **(1)** (0.01 mol) and substituted aromatic aldehydes **(2)** (0.01 mol) was taken in a round bottom flask and dissolved in dichloroformamide (30 mL). A pinch of ceric ammonium nitrate (5 mg) was added and the contents were refluxed for 10- 14 h and cooled to room temperature. The reaction contents were poured into crushed ice with constant stirring. The solid compound was filtered, washed with water, dried and recrystallized using alcohol. The physical data of title compounds is given in **Table 1** [27].

Table 1 Physical data of 1,3,4oxadiazole derivatives (C1-5).

Comp	Ar-CHO	Molecular formula	Molecular weight
C1	4-Cl	$C_{14}H_9Cl\,N_2O_2$	272.69
C2	2-NO_2	$C_{14}H_9N_3O_4$	283.24
C3	4-Br	$C_{14}H_9BrN_2O_2$	317.14
C4	4-OCH_3	$C_{15}H_{12}N_2O_3$	268.27
C5	3-OH	$C_{14}H_{10}N_2O_3$	254.24

4-(5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl) phenol(C1)

White solid, % yield: 81.09 %, m.p.: 258-260 ºC. FT-IR (KBr, v, cm^{-1}): 3236 (OH), 2908 (C-H), 1624 (C=N), 1554 (C=C), 1082 (C-O-C), 747 (C-Cl). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ ppm= 6.92-8.65(m, Ar-H, 8H), 11.82 (s, OH, 1H). MS (m/z): 273.32 (M+1).

4-(5-(2-nitrophenyl)-1,3,4-oxadiazol-2-yl) phenol(C2)

Light yellow solid, % yield: 78.04 %, m.p.: 191-193 ºC. FT-IR (KBr, v, cm^{-1}): 3205 (OH), 1603(C=N), 1525(C=C), 2905(C-H), 1067 (C-O-C), 1482(NO_2). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ ppm=6.92-8.84(m, Ar-H, 8H), 11.72 (s, OH, 1H). MS (m/z): 284.28 (M+1).

4-(5-(4-bromophenyl)-1,3,4-oxadiazol-2-yl) phenol (C3)

Dark brown solid, % yield: 82.67 %, m.p.: 183-185 ºC. FT-IR (KBr, v, cm^{-1}): 3251 (OH), 1625 (C=N), 1554(C=C), 2931 (C-H), 1072 (C-O-C), 750(C-Br). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ ppm=6.91-7.861(m, Ar-H, 8H), 11.86 (s, OH, 1H). MS (m/z): 317.14 (M+).

4-(5-(4-methoxyphenyl)-1,3,4-oxadiazol-2-yl) phenol (C4)

Brown solid, % yield: 83.05 %, m.p.: 242-244 ºC. FT-IR (KBr, v, cm^{-1}): 3245 (OH), 1607 (C=N), 1555 (C=C), 2905 (C-H), 1098 (C-O-C). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ ppm=3.78(s, OCH_3, 3H), 6.93-7.87 (m, Ar-H, 8H), 11.74 (s, OH, 1H). MS (m/z): 269.35(M+1).

3-(5-(2-hydroxyphenyl)-1,3,4-oxadiazol-2-yl) phenol (C5)

Light orange solid, % yield: 76.56 %, m.p.: 214-216 ºC. FT-IR (KBr, v, cm^{-1}): 3324 (OH), 1611 (C=N), 1551(C=C), 2926(C-H), 1097 (C-O-C). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ ppm= 6.92-8.02(m, Ar-H, 8H), 11.77 (s, OH, 1H), 12.06 (s, OH, 1H). MS (m/z): 255.30 (M+1).

***In silico* analysis**

In silico analysis (Lipinski's RO5, molecular docking, ADME properties was carried out using Schrodinger 2018-3 suite device Maestro 11.7.012, (Ligprep, Glide XP docking, QuikProp). The synthesized compounds were docked in the groove of the binding site in 4COF, which is the crystal structure of a human gamma-amino butyric acid receptor, the GABA(A)R-beta3 homopentamerwith a resolution of 2.97 °A which is the major inhibitory neurotransmitter in the human brain [28,29].

Anticonvulsant activity

Animals

Swiss albino mice (weighing 20 - 25 g) of either sex were used for the study. The animals were obtained a week before experimentation from the animal house facility of Nitte-Deemed to be University, Mangalore, Karnataka, and kept in the lab for acclimatization. They were housed and maintained under 12 h dark/light cycles at 27 ± 2 °C in polypropylene cages. They were fed with water and standard pellet feed except during the experiments. Pregnant females were excluded. Ethical clearance for experimentation on animals was taken from the IAEC (Certificate No: NGSMIPS/IAEC/120) prior to the start of the work.

Acute toxicity studies

Standard husbandry conditions were maintained for the acute toxicity studies of 1,3,4-oxadiazole derivatives (OECD, 2001) on female albino mice (20 - 25 g). Prior to the experimentation, the animals were fasted over-night and were treated with a single dose of 1,3,4-oxadiazole derivatives and monitored for a period of 48 h for mortality (short term toxicity). Based on the short-term toxicity profile, the next doses were determined according to the OECD guidelines No.425 [30].

Maximal electroshock method

Swiss albino mice of either sex with a bodyweight of 20 - 25 g were divided into 6 groups of 6 animals each. Group-I was given a1% aqueous solution of tween 80 as a control, while Group-II was administered phenytoin (25 mg/kg p.o) and served as a standard. Groups-III-VII were orally administered with respective 1,3,4-oxadiazole derivatives at a dose of 100 mg/kg, respectively. The experiment was started 45 min after the administration of vehicle or the test compounds and 30 min after the standard drug. To start the session, a 60 Hz alternate current for 0.2 s was applied to the animal through corneal electrodes. The different stages of convulsion flexion, hind limb extension, stupor, the total duration of the convulsion, and the recovery period taken were recorded (**Table 7** and **Figure 5**). The decrease in duration of the hind limb extension is considered as a protective action that implies anticonvulsant activity [31].

PTZ induced convulsions

Swiss albino mice of either sex with a bodyweight of 20 - 25 g were divided into 6 groups of 6 animals each. Group-I was given a1% aqueous solution of tween 80 served as a control, Group-II served as standard which was administered with diazepam (5 mg/kg i.p). Groups III-VII were given 1,3,4-oxadiazole derivatives orally at a dose of 100 mg/kg, respectively. After 45 min of the vehicle or test compounds and 30 min after the standard drug, the PTZ (80mg/kg weight s.c.) was given to the mice.

Immediately after PTZ administration, the mice were placed individually in a cage and observed for latency to clonic convulsions and mortality for the duration of 30 min (**Table 8** and **Figure 6**) [32].

Statistical analysis

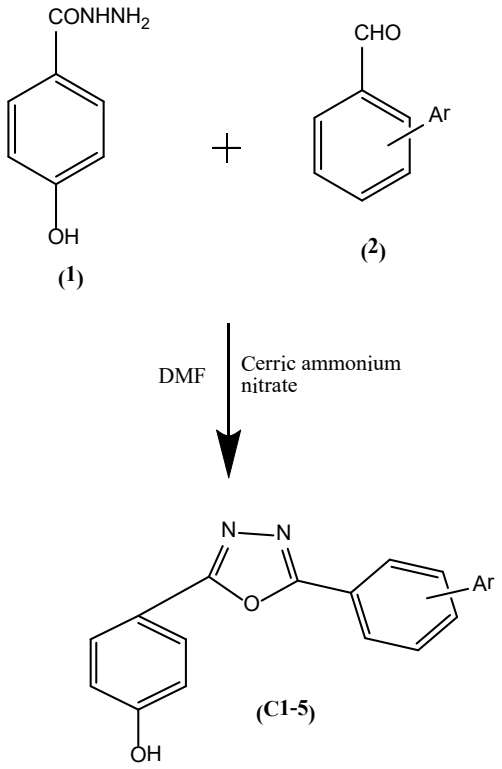
The results are presented as Mean ± SEM, n = 6. One-way ANOVA was used for a statistical demonstration followed by Dunnett's multiple comparison test, and $p < 0.05$ value was considered significant.

Results and discussion

Chemistry

1,3,4-oxadiazole derivatives were prepared in a satisfactory yield by refluxing 4-hydroxy benzhydrazide with various substituted aromatic aldehydes and adding a pinch of ceric ammonium nitrate which acts as a catalyst and dimethylformamide was used as solvent. All the title compounds were obtained in good yield. The reaction sequence followed is outlined in **Scheme-01**.The physical data of the final synthesized compounds were shown in **Table 1**. Based on FT-IR,^{1}H-NMR and Mass spectral data, all the new compounds were confirmed to have their assigned structure.

Scheme-01



Scheme 1

***In silico* analysis**

The compounds showed desired physicochemical properties with no violations ofthe standard ranges (**Table 2**). The number of rotatable bonds is less than 10 for all compounds. The tPSA values of all the compounds are within the limit indicating the cell permeability. The synthesized compounds obey Lipinski's rule of 5 (**Table 3**).

Table 2 Physicochemical properties of compounds (C1-C5).

Comp	MR[a] (cm^3/mol)	tPSA[b]	Polarizability (Å3)	Nrobs[c]	Volume (cm^3)
C1	70.7±69	54.18	28.0±0.5 10^{-24}	2	221.67
C2	72.34±0.3	104.98	28.68±0.5 10^{-24}	3	231.47
C3	73.49±0.3	59.15	29.13±0.5 10^{-24}	2	226.02
C4	72.47±0.3	68.39	28.73±0.5 10^{-24}	3	233.68
C5	67.68±0.3	79.38	26.83±0.5 10^{-24}	2	216.15
Standard (Diazepam)	80.91±0.5	32.47	32.07±0.5 10^{-24}	1	216.54

[a]molar refractivity
[b]total polar surface area
[c]number of rotatable bonds

Table 3 Lipinski's RO5 for compounds (C1-5).

Comp	Molecular weight	Log P	DonorHB	AcceptorHB
C1	272.69	3.44	1	3.250
C2	283.24	3.16	1	4.250
C3	317.14	3.71	1	3.250
C4	268.27	2.75	1	4.000
C5	254.24	2.49	2	4.000
Standard (Diazepam)	284.75	2.84	0	4.000

The new compounds were docked in the groove of the binding site in 4COF. **Table 4** shows the affinity of the compounds for the receptor as well as their docking score. The synthesized compounds have binding free energy in the range of −4.977 to 5.676 kcal/mol. The active residues in 4COF are ARG C:117, GLY D:158, TYR D:157, SER D:156, GLU D:155, ARG D:207, TYR D:97, LEU D:99, TYR D: 205, LEU D:99, TYR C:62, ASN C:41, GLN C:64, ASP C:43, THR D:202, ALA D:201, PHE D:200, THR C:176, GLY C:177, ARG C:180, MET C:115.The highest affinity as well as the binding energy of −5.676 kcal/mol is displayed by Compound C5 in comparison to the other synthesized compounds. Compounds C5, C4 and C1 fit into the binding cleft of 4COF receptor with dock score of −5.676, −5.277 and −5.191 kcal/mol respectively. The hydrogen bond interactions are formed with GLU D:155 in the case of both C5 (**Figure 1**) and C4 (**Figure 2**). The hydrophobic interaction between the ligand and the receptor also represents good interaction. The docking conformations of these 2 compounds are represented in **Figures 3** and **4**.

Table 4 Dock scores of compounds (C1-C5) at the active site of 4COF.

Comp	Dock score
Std (Diazepam)	**−4.554**
C1	−5.191*
C2	−5.039
C3	−5.149
C4	−5.277**
C5	**−5.676****

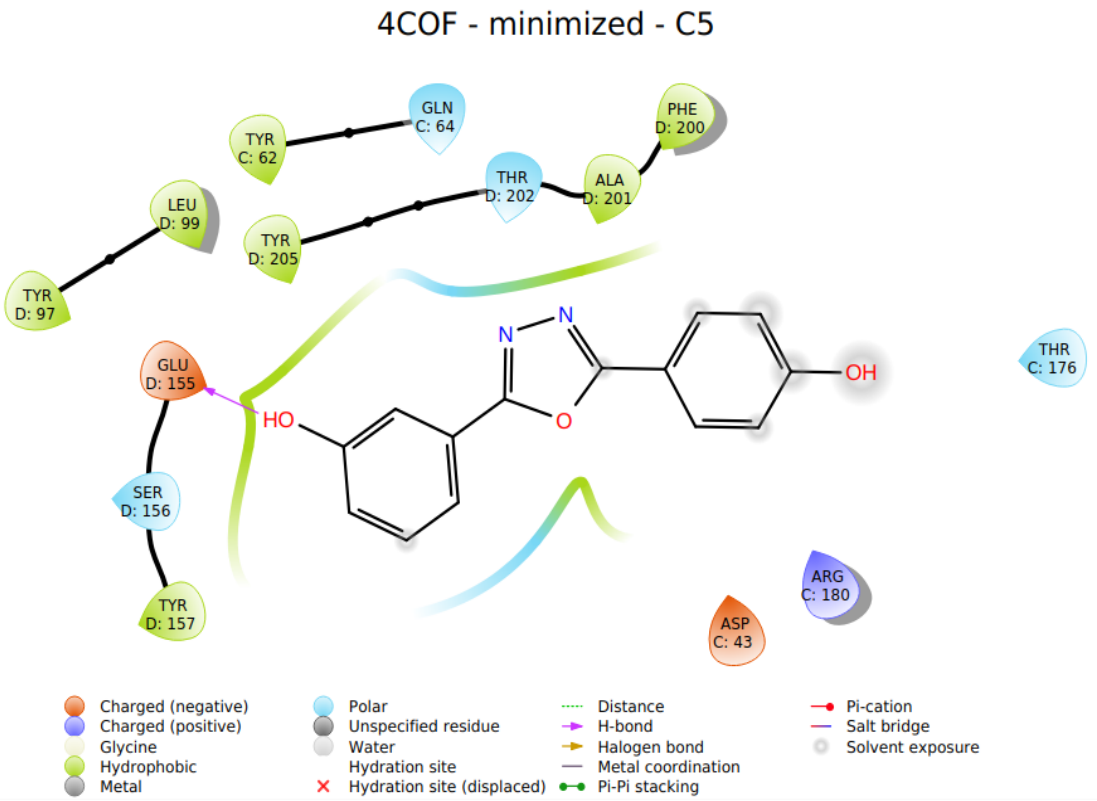


Figure 1 Interaction of compound C5 with 4COF.

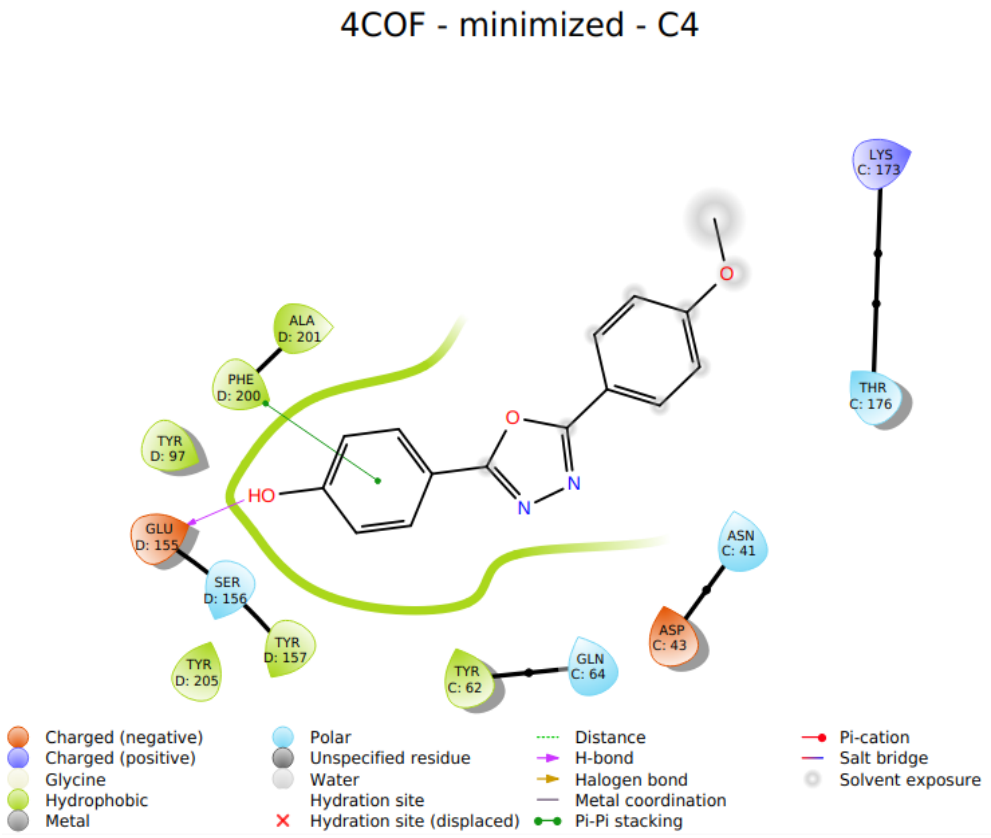


Figure 2 Interactions of compound C4 with 4COF.

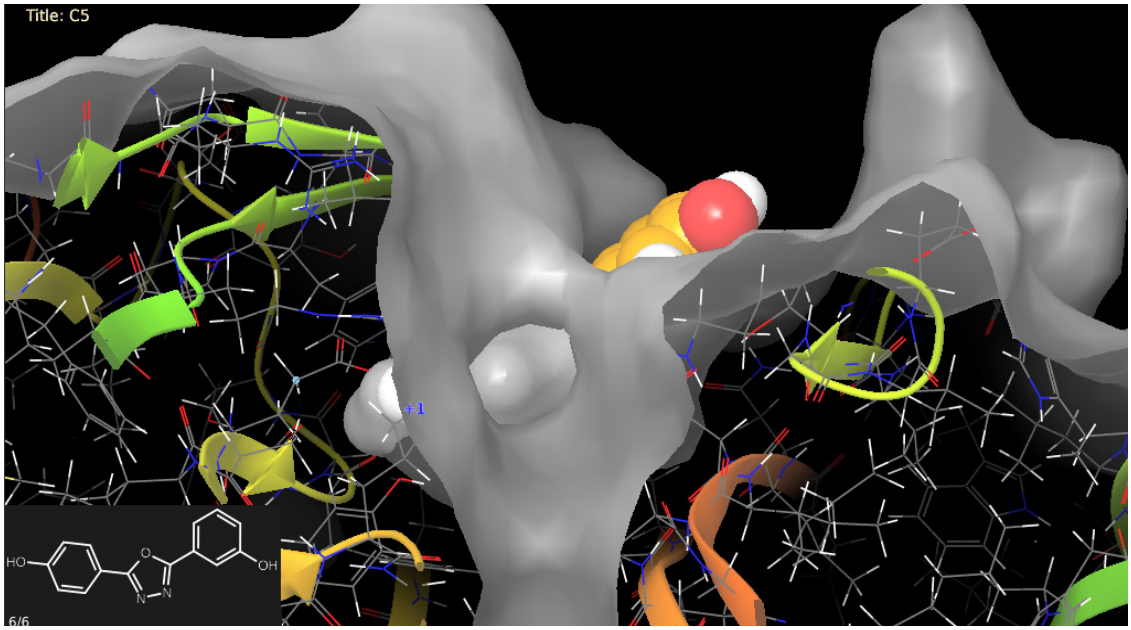


Figure 3 Docking interaction of C5 with 4COF.

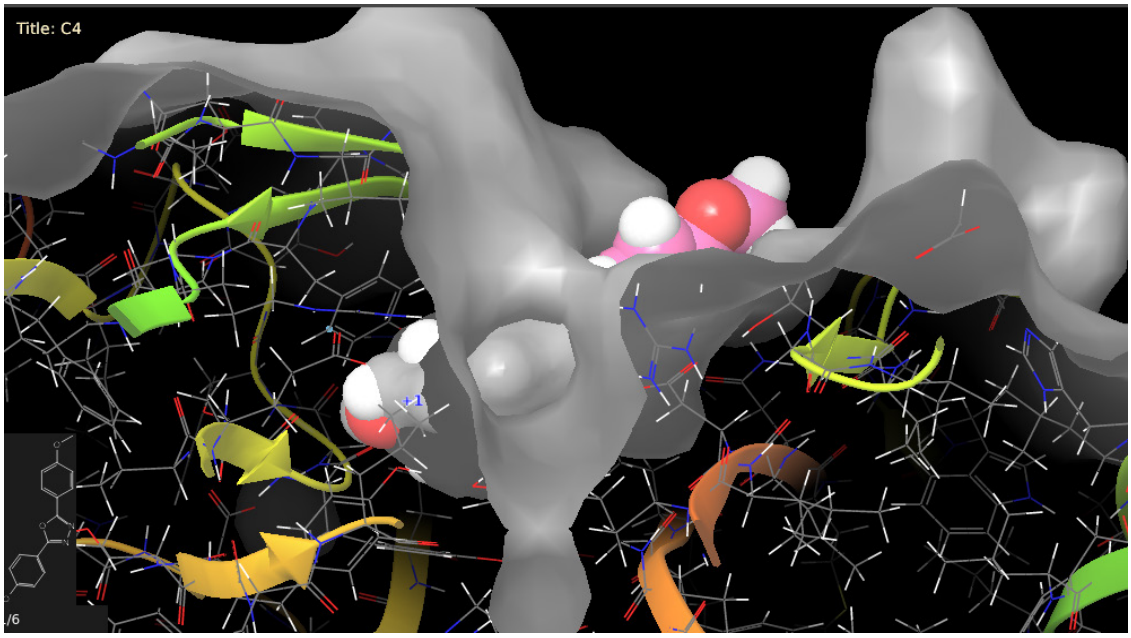


Figure 4 Docking interaction of C4 with 4COF.

The co-crystallized ligand was removed from the prepared protein (PDB: 4COF) and redocked at the binding site to validate the docking program. To compare the docked and reference conformations, the RMSD value is employed. When relocking or cross-docking is done, the RMSD number will be lower (ideally less than 1Å). The RMSD is primarily utilised to validate the docking study method. RMSD was calculated in molecular docking to compare the docked conformation of the reference ligand to its original conformation and to validate the protein-bound ligand docked in the same pocket to check the deviation. For the prepared PDB: 4COF, the RMSD values were found to be 0.098Å. The values found had the least RMSD value and were chosen for continued study. At the ligand-binding site, all of the produced ligands were docked with the protein. The XP docking dock score data were analyzed and summarised.

The ADME studies (**Table 5**) of the synthesized compounds helped in concluding that all the compounds have good BBB penetration as that of the standard shown in **Table 5**. The QPlogKp score indicates that all the test compounds and the standard have skin permeability. The QPlogKhsa score depicted that all the compounds were bound to the human serum albumin.

Table 5 ADME properties of compounds (C1-5).

Comp	QPlogBB[a]	QPlogKp[b]	QPPCaco[c]	QPlogKhsa[d]	Percent human oral absorption
C1	−0.291	−2.196	998.094	0.244	100.00
C2	−1.443	−3.931	119.539	0.093	75.50
C3	−0.280	−2.199	998.179	0.270	100.00
C4	−0.528	−2.130	997.311	0.149	96.88
C5	−1.009	−3.087	303.303	−0.026	82.91
Standard (Diazepam)	0.200	−1.669	2687.20	0.152	100.00

[a]Predicted brain/blood partition coefficient.
[b]Predicted skin permeability
[c]Predicted apparent Caco-2 cell permeability in nm/s.
[d]Prediction of binding to human serum albumin.

The Lazar program was used to predict the toxicity of the synthesized compounds (**Table 6**). The compounds of C2 were liable to cause mutagenicity. Whereas compounds C3 were liable to cause carcinogenicity, the remaining synthesized compounds were not found to be liable to cause any type of mutagenicity or carcinogenicity. All the synthesized compounds were not susceptible to causingacute toxicity and hence can be termed as safe.

Table 6 *In silico* toxicity prediction of compounds (C1-5).

Comp	Toxicity prediction using LAZAR			
	Maximum recommended daily dose (mmol/kg-bw/day)	**Mutagenicity**	**Acute toxicity LC50 (mmol/L) (Fathead Minnow)**	**Carcinogenic potency**
Std (Diazepam)	0.00757	−	0.15	−
C1	0.0825	−	0.0371	−
C2	0.00347	+	0.322	−
C3	-	−	0.00646	+
C4	0.0902	−	0.886	−
C5	0.00347	−	0.347	−

Anticonvulsant activity

All the newly synthesized compounds were screened for anticonvulsant activity by 2 models; namely, MES and PTZ induced convulsions (**Tables 7** and **8**). In both models, latency and duration are essential parameters to assess the anticonvulsant property. Compounds C4 [4-(5-(4-methoxyphenyl)-1,3,4-oxadiazol-2-yl) phenol] and C5 [3-(5-(4-hydroxyphenyl)-1,3,4-oxadiazol-2-yl) phenol] have shown a decrease in the flexion phase as well as in the extension phase. Also, compound **C5** has demonstrated a decline in the clonus phase, and compound **C4** has shown a decrease in the stupor phase. Overall, compounds **C4** and **C5** show convulsion duration times closer to thoseof standard drugs.

In the PTZ induced convulsion model, compounds **C5** and **C3** have shown the highest increasesin the latency phase, as well as compounds**C5** and **C1** showed a significant decrease in the duration time of the tonic-clonic seizure when compared to that of the control.

Remarkably, the presence of both electron-donating and withdrawing groups substituted at the 2- and 5- position of the 1,3,4-oxadiazole ring resulted in excellent anticonvulsant activity. Compound C4 with paramethoxy substituent illustrates the distal hydrophobic centre could be made to be more lipophilic than the phenyl ring, thus demonstrating more significant anticonvulsant potential.

Table 7 Data of anticonvulsant activity study by MES method.

Sl. No	Treatment	Dose	Duration of flexion phase	Duration of extension phase	Duration of clonus phase	Duration of stupor phase
1	Control	-	1.880±0.058	24.40±1.66	38.0±3.85	84.20±4.375
2	Standard (Phenytoin)	25 mg/kg (p.o)	0.52±0.049	8.400±5.40	3.40±0.51	9.40±1.939
3	C1	100mg/kg (p.o)	2.20±0.200	14.00±2.366	34.40±4.905	44.60±19.35**
4	C2	100mg/kg (p.o)	3.60±0.678	15.200±2.22	31.40±4.966	66.80± 24.105
5	C3	100mg/kg (p.o)	2.20±0.200	12.800±1.66	51.80±9.499	42.00±4.764**
6	C4	100mg/kg (p.o)	1.80±0.490	7.400±2.015	17.80±7.567	14.20±6.453****
7	C5	100mg/kg (p.o)	1.00±0.316	11.00±2.97	15.200±4.03	20.60±9.453****

The above data is presented as mean ± SEM, n = 6.

Table 8 Data of anticonvulsant activity study by PTZ induced convulsion method.

Sl. No	Treatment	Dose	Latency of tonic clonic seizures (s)	Duration of tonic clonic seizure (s)
1	Control	-	22.59±0.33	288.8±3.77
2	Standard	5.0 mg/kg (p.o)	287.6±4.61	67.40±3.076
3	C1	100mg/kg (p.o)	57.60±10.70	184.6±56.55
4	C2	100mg/kg (p.o)	44.00±4.743	285.2±27.18
5	C3	100mg/kg (p.o)	74.40±15.70*	317.0±18.13
6	C4	100mg/kg (p.o)	61.60±20.77	217.0±59.07
7	C5	100mg/kg (p.o)	81.00±20.22*	123.8±6.62*

The above data is presented as mean ± SEM, n = 6.

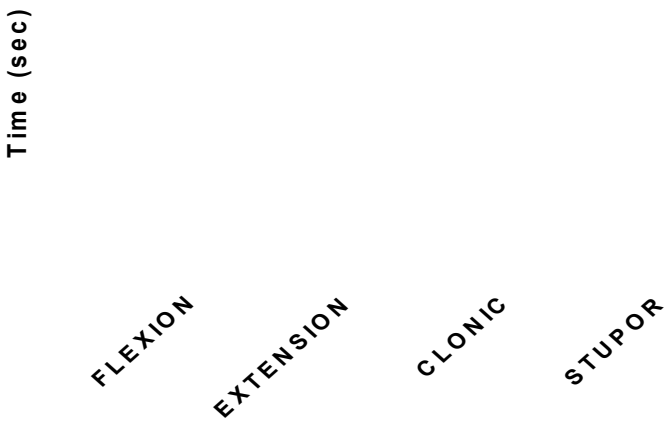


Figure 5 Duration of various phases of convulsion by MES induced convulsion.

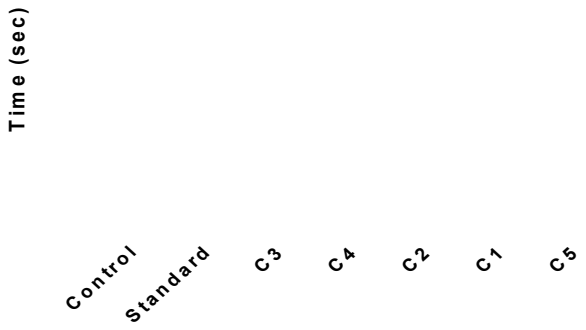


Figure 6 Latency of convulsion in PTZ induced convulsion.

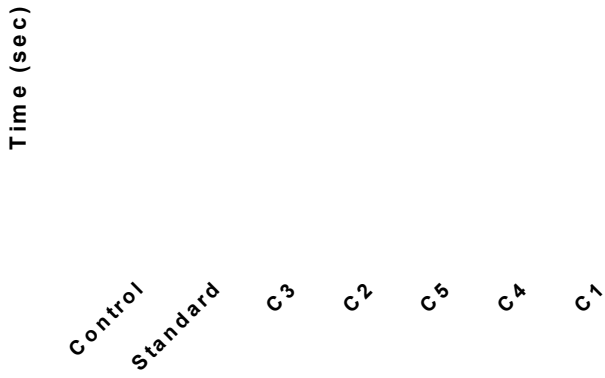


Figure 7 Duration of convulsion in PTZ induced convulsion.

Conclusions

In summary, a new series of 1,3,4-oxadiazole derivatives were conveniently synthesized from 4-hydroxy benzhydrazide and substituted aromatic aldehydes with good yields. Molecular docking studies represent a significant interaction between the ligand and the GABAA receptor. The *in silico* predictions resulted in supportive pharmacokinetic potency, and the Lazy structure-activity relationship (LAZAR) framework used for toxicity predictions revealed that the majority of the synthesized compounds were carcinogenic and mutagenic. Compounds C4 and C5 showed the highest affinity and binding energy, as well as the highest anticonvulsant activity in both electroshock and pentylenetetrazole-induced lethal convulsion tests. The results manifest that the compounds with the highest activity can be further used as a framework for design, alteration, and exploration to construct more effective analogs with lesser side effects.

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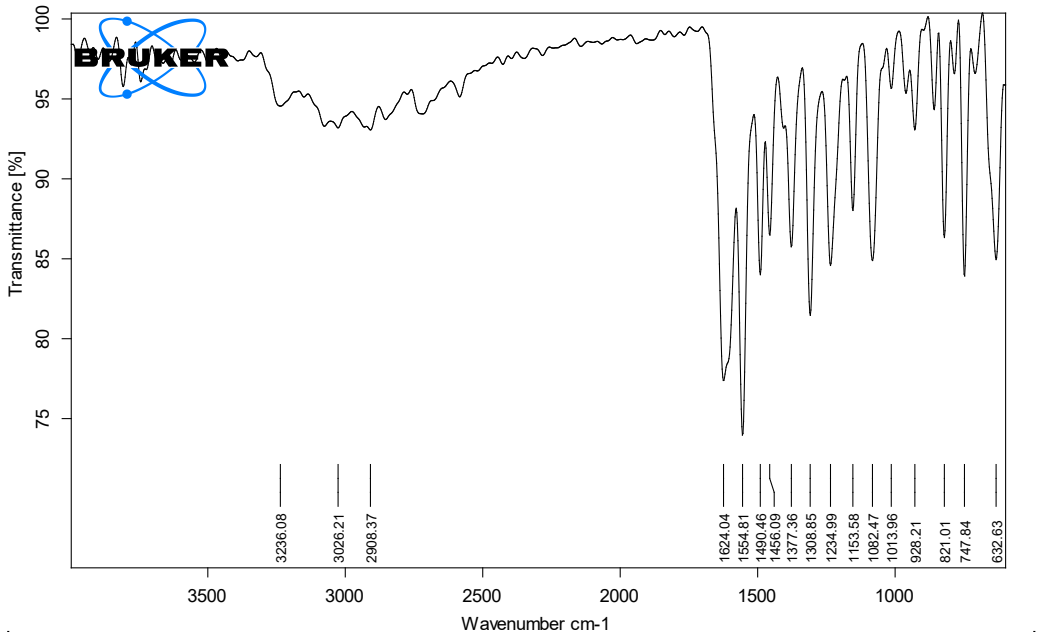
Appendixs

Mass spectral of compound C1:



D:\FTIR\C1.1	C1	Instrument type and / or accessory	8/29/2020

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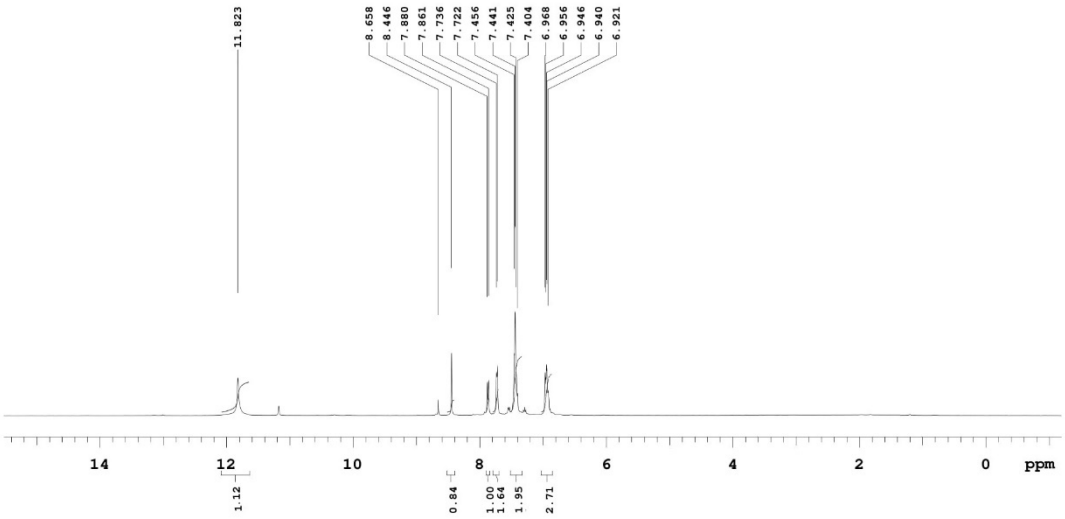
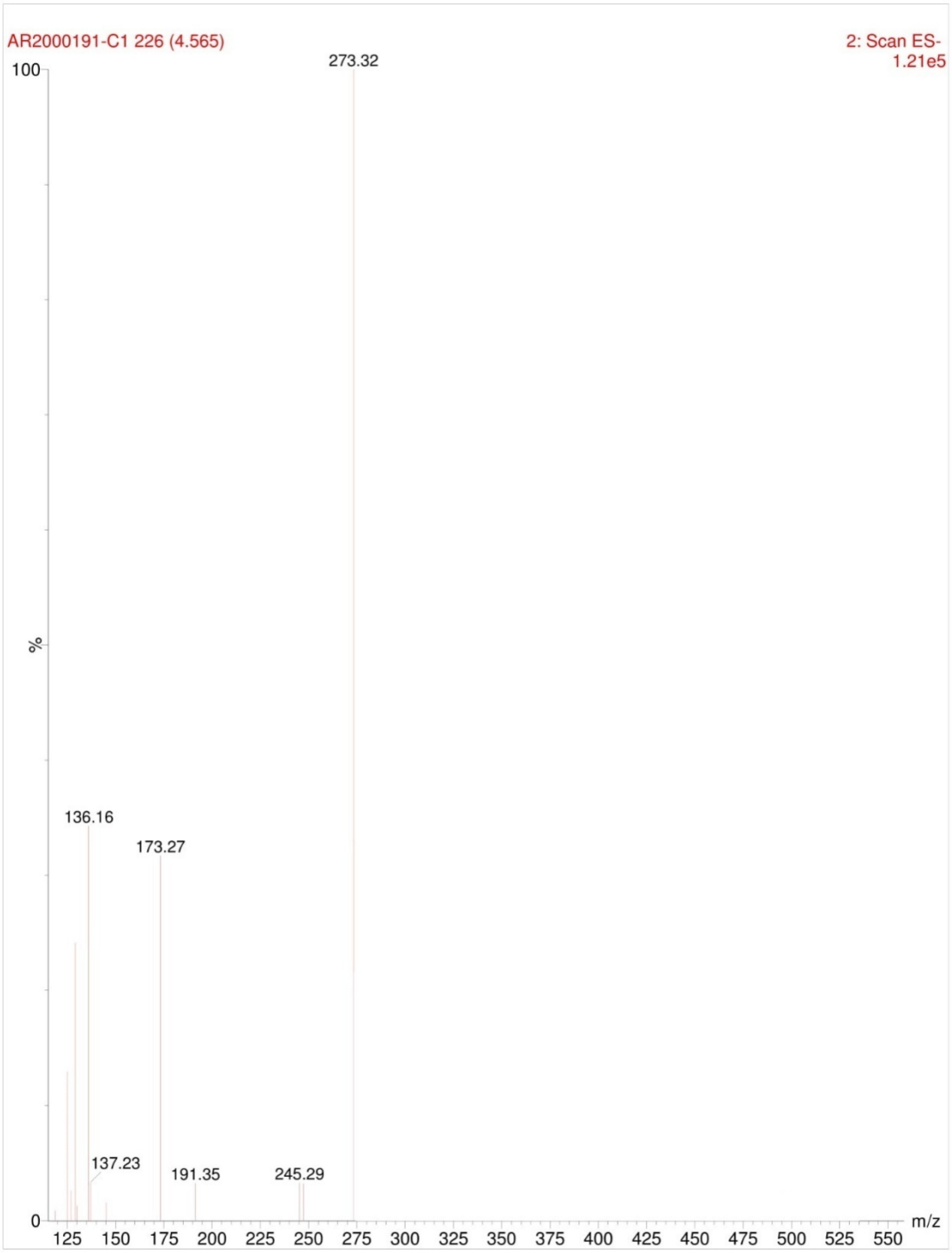
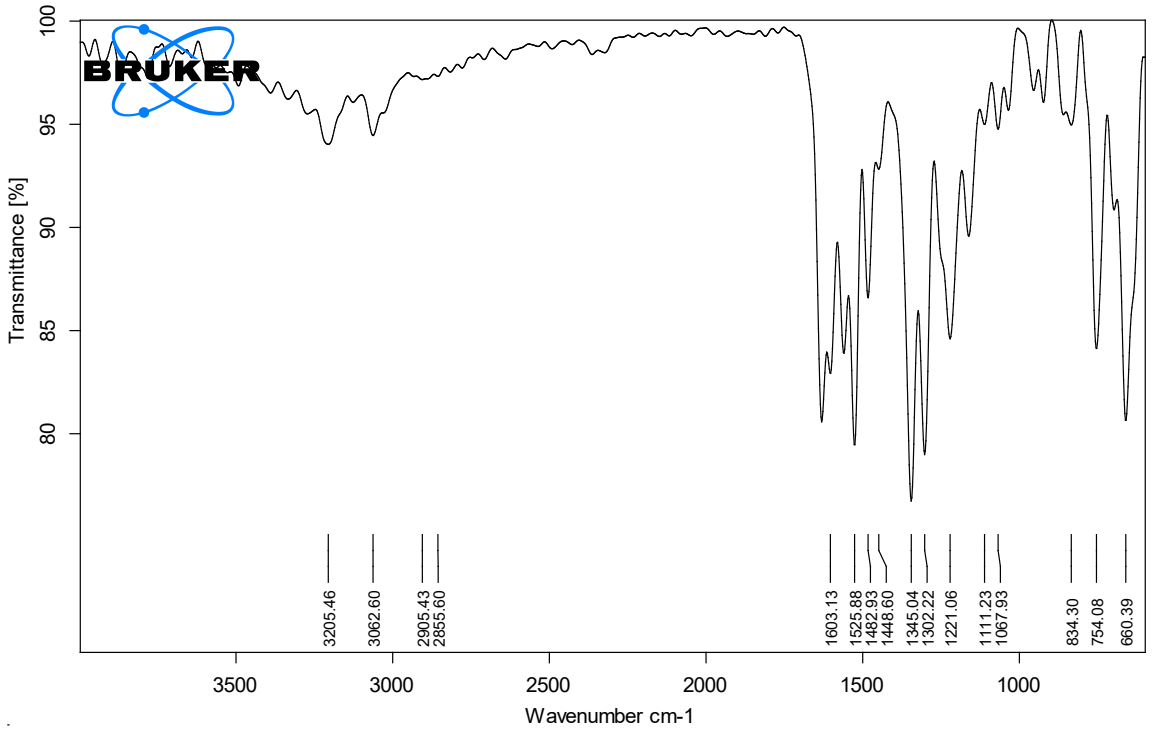


PULSE SEQUENCE Relax. delay 1.000 sec Pulse 45.0 degrees Acq. time 3.146 sec Width 10416.7 Hz 8 repetitions	OBSERVE H1, 399.8231903	DATA PROCESSING Line broadening 1.0 Hz FT size 65536 Total time 1 minute		C1-1H Solvent: dmso Ambient temperature Operator: vnmr1 VNMRS-400 "Agilent-NMR"


AR2000191-C1 226 (4.565)
2: Scan ES-
1.21e5
100
%
0
273.32
136.16
173.27
137.23
191.35
245.29
125 150 175 200 225 250 275 300 325 350 375 400 425 450 475 500 525 550
m/z

Mass spectral of compound C2:



D:\FTIR\C2.1	C2	Instrument type and / or accessory	8/29/2020

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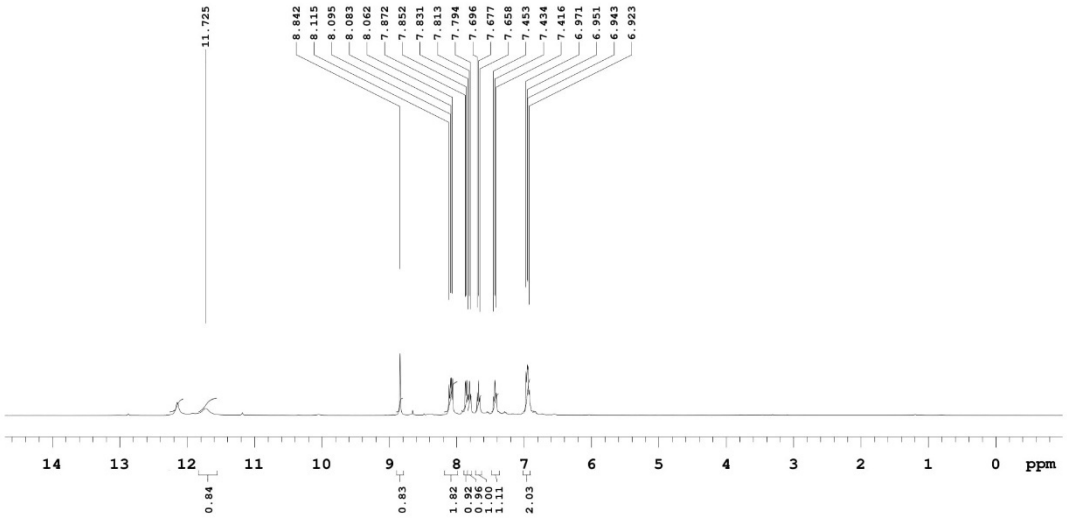
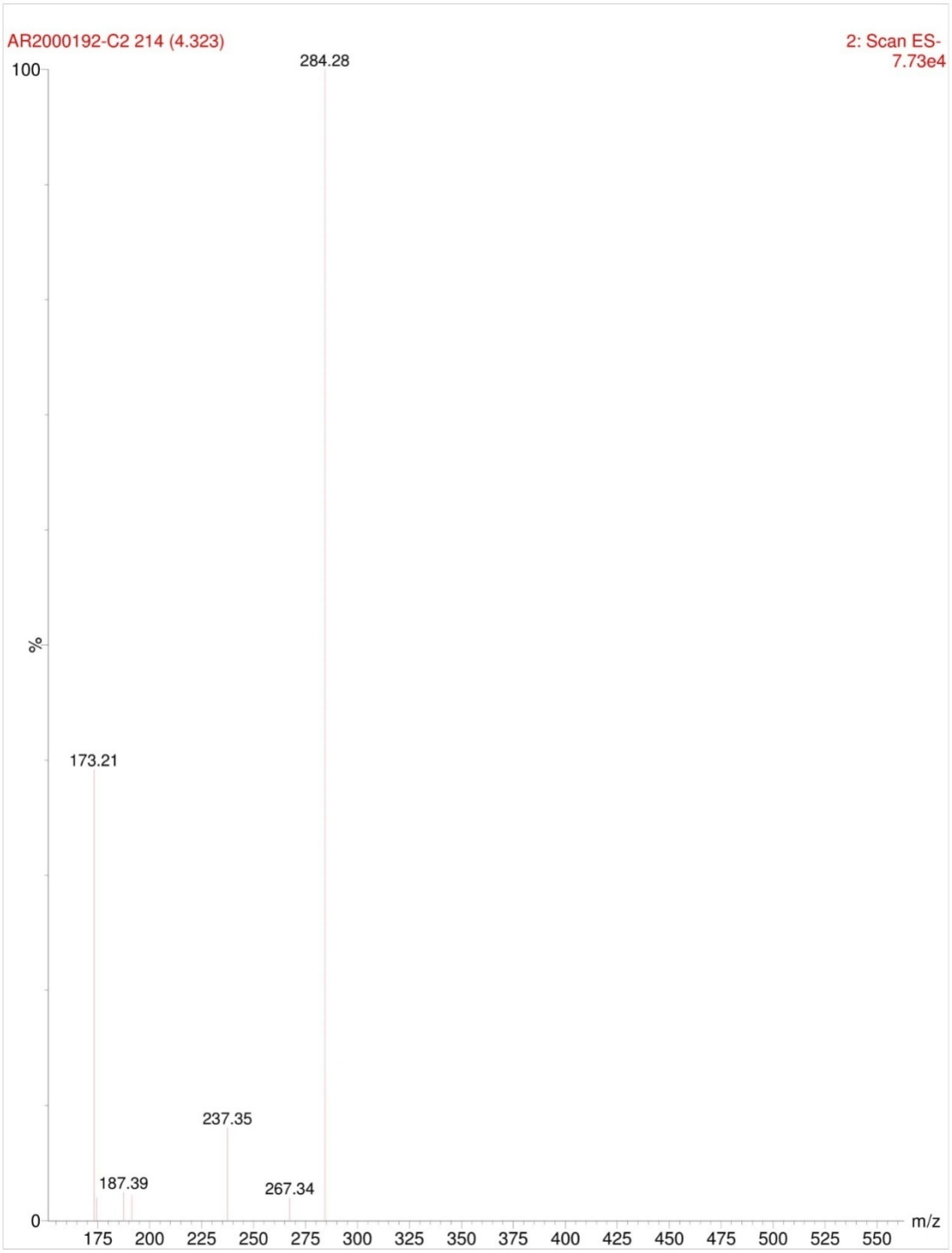
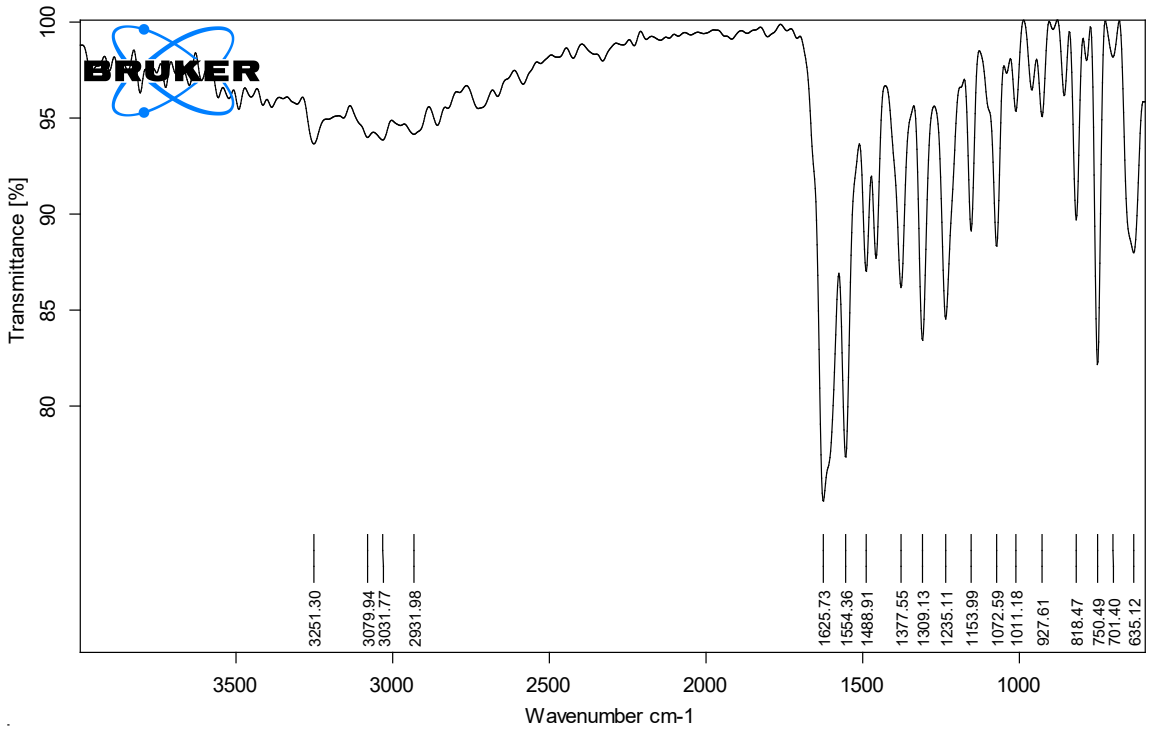


PULSE SEQUENCE Relax. delay 1.000 sec Pulse 45.0 degrees Acq. time 3.146 sec Width 10416.7 Hz 16 repetitions	OBSERVE H1, 399.8231903	DATA PROCESSING Line broadening 1.0 Hz FT size 65536 Total time 1 minute		C2-1H Solvent: dmso Ambient temperature Operator: vnmr1 File: C2-1H VNMRS-400 "Agilent-NMR"


AR2000192-C2 214 (4.323)
2: Scan ES-
7.73e4
100
%
0
284.28
173.21
237.35
187.39
267.34
175
200
225
250
275
300
325
350
375
400
425
450
475
500
525
550
m/z

Mass spectral of compound C3:



D:\FTIR\C3.1	C3	Instrument type and / or accessory	8/29/2020

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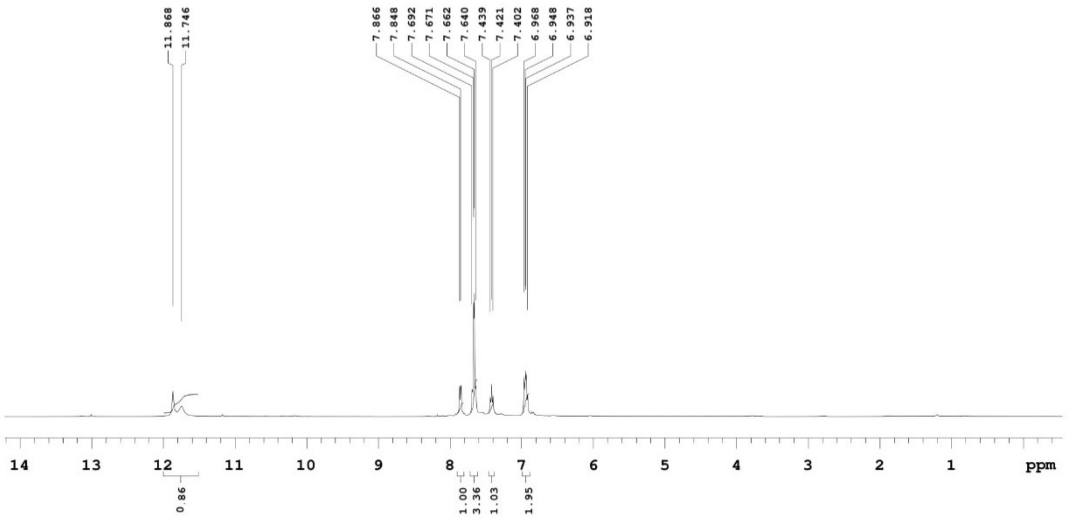
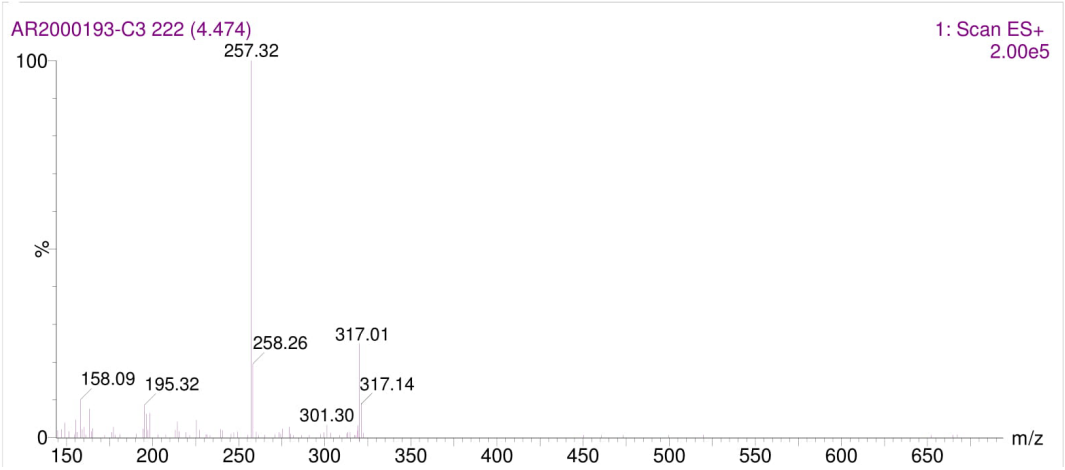
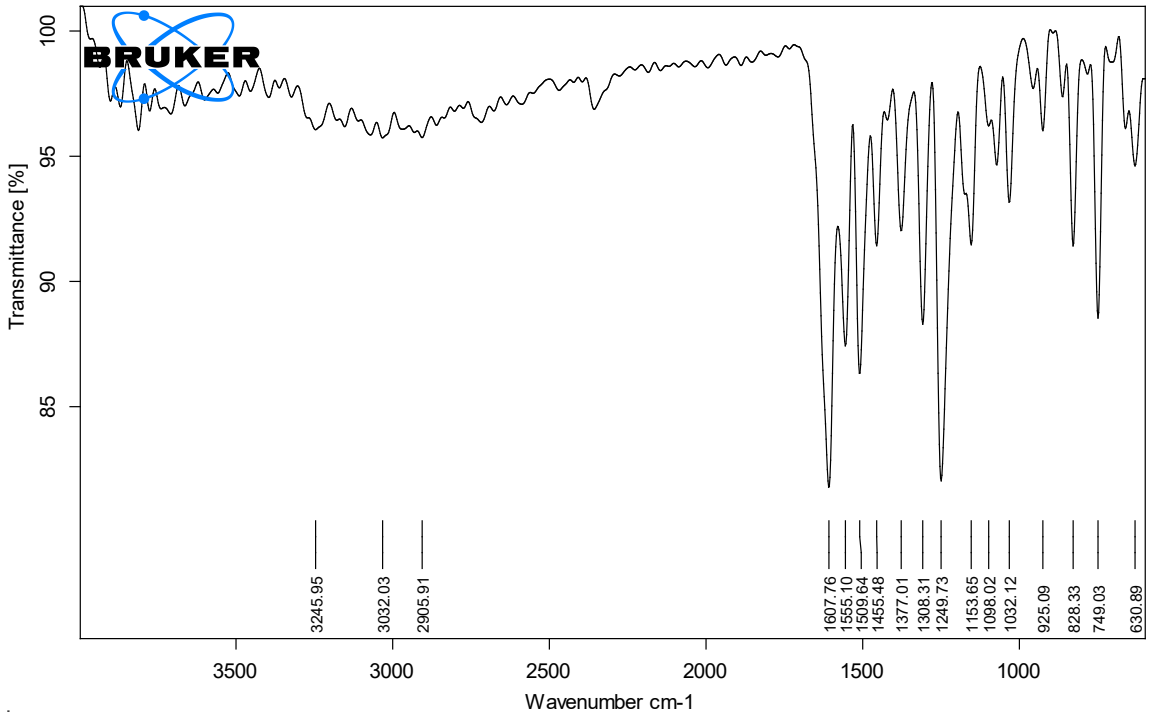


PULSE SEQUENCE Relax. delay 1.000 sec Pulse 45.0 degrees Acq. time 3.146 sec Width 10416.7 Hz 16 repetitions	OBSERVE H1, 399.8231903	DATA PROCESSING Line broadening 1.0 Hz FT size 65536 Total time 1 minute		C3-1H Solvent: dmso Ambient temperature Operator: vnmr1 File: C3-1H VNMRS-400 "Agilent-NMR"



Mass spectral of compound C4:



D:\FTIR\C4.2	C4	Instrument type and / or accessory	8/29/2020

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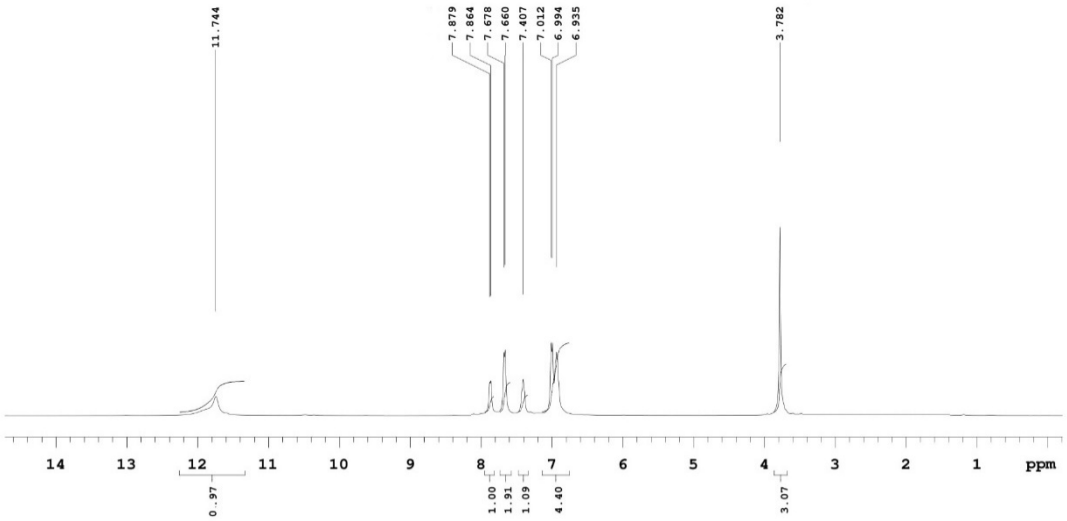
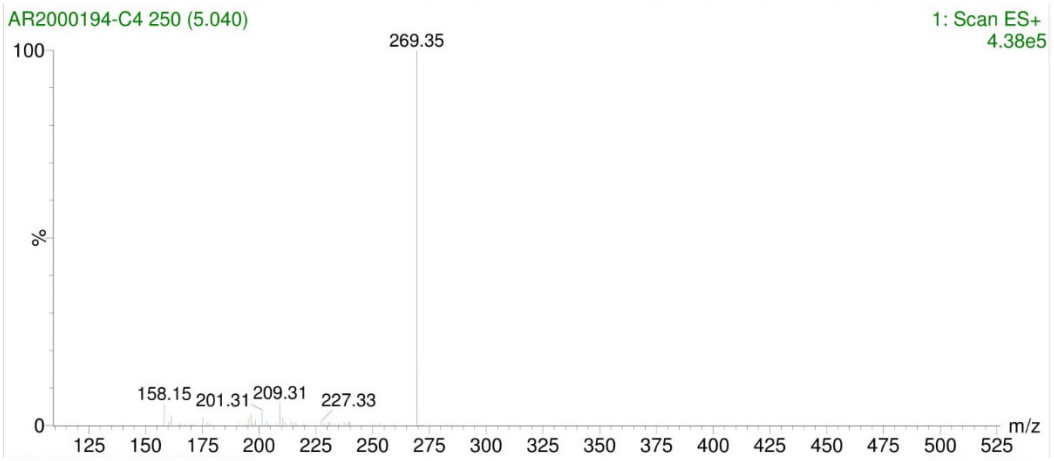
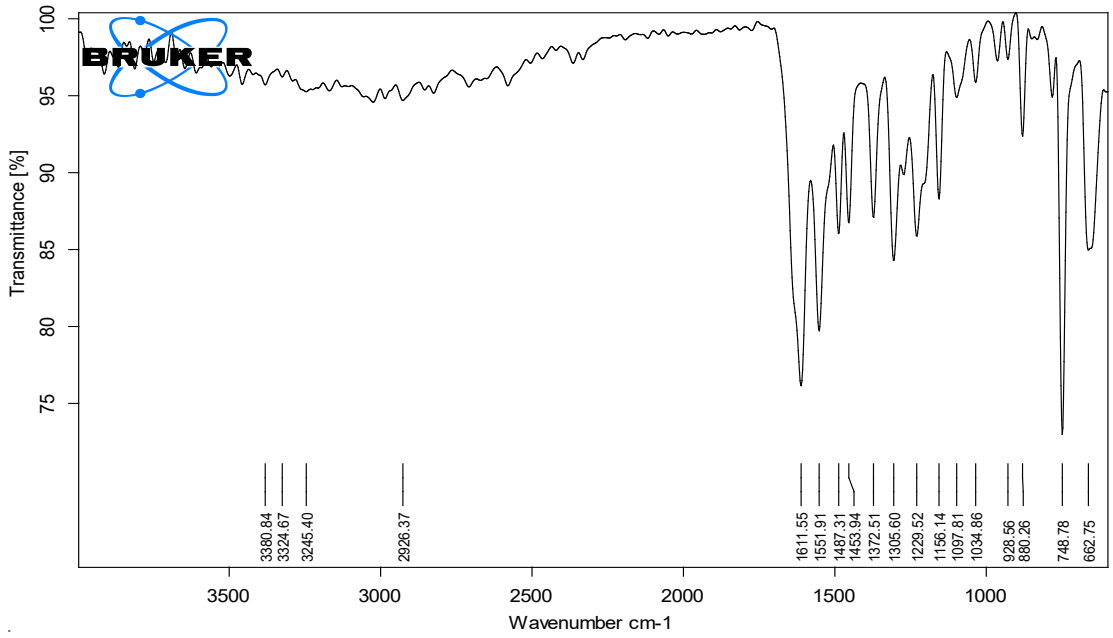

11.744
7.879
7.864
7.678
7.660
7.407
7.012
6.994
6.935
3.782
14 13 12 11 10 9 8 7 6 5 4 3 2 1 ppm
0.97
1.00
1.91
1.09
4.40
3.07
PULSE SEQUENCE
Relax. delay 1.000 sec
Pulse 45.0 degrees
Acq. time 3.146 sec
Width 10416.7 Hz
8 repetitions
OBSERVE H1, 399.8231903
DATA PROCESSING
Line broadening 1.0 Hz
FT size 65536
Total time 1 minute
C4-1H
Solvent: dmso
Ambient temperature
Operator: vnmr1
File: C4-1H
VNMRS-400 "Agilent-NMR"


AR2000194-C4 250 (5.040)
1: Scan ES+
4.38e5
100
%
0
269.35
158.15
201.31
209.31
227.33
125 150 175 200 225 250 275 300 325 350 375 400 425 450 475 500 525
m/z

Mass spectral of compound C5:



D:\FTIR\C5.1	C5	Instrument type and / or accessory	8/29/2020

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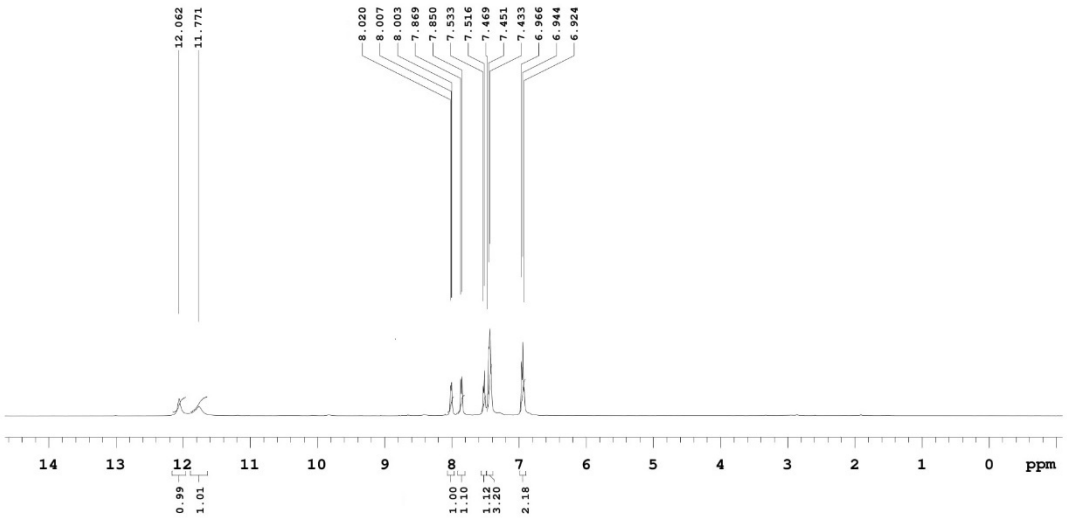
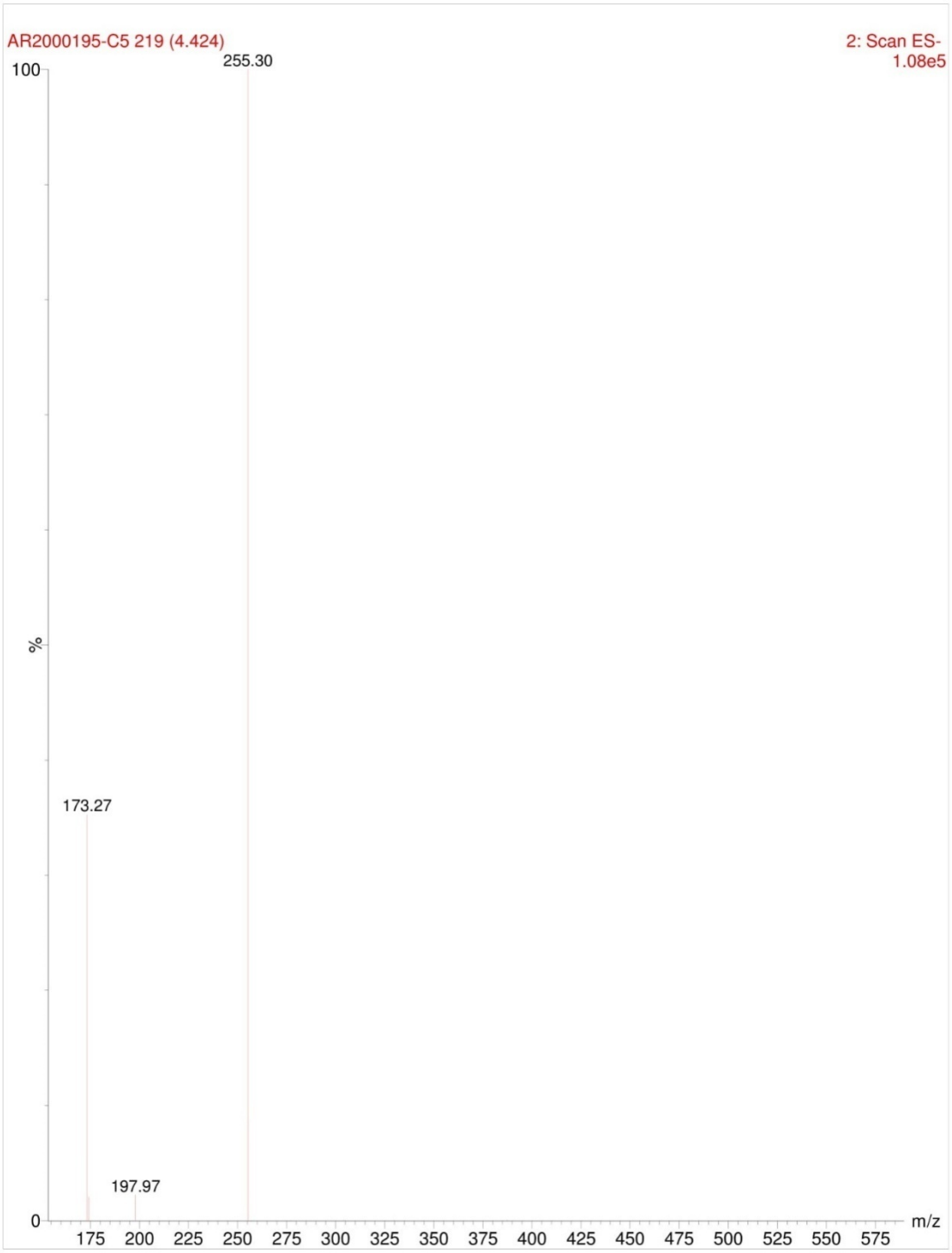


PULSE SEQUENCE Relax. delay 1.000 sec Pulse 45.0 degrees Acq. time 3.146 sec Width 10416.7 Hz 16 repetitions	OBSERVE H1, 399.8231903	DATA PROCESSING Line broadening 1.0 Hz FT size 65536 Total time 1 minute		C8-1H Solvent: dmso Ambient temperature Operator: vnmr1 File: C8-1H VNMRS-400 "Agilent-NMR"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100
%
0
255.30
173.27
197.97
175 200 225 250 275 300 325 350 375 400 425 450 475 500 525 550 575
m/z