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# Trisubstituted Pyrazoles As Novel Inhibitor of Human Secretory Phospholipase $A_2$ with Antiinflammatory Activity SAR, and Molecular docking studies

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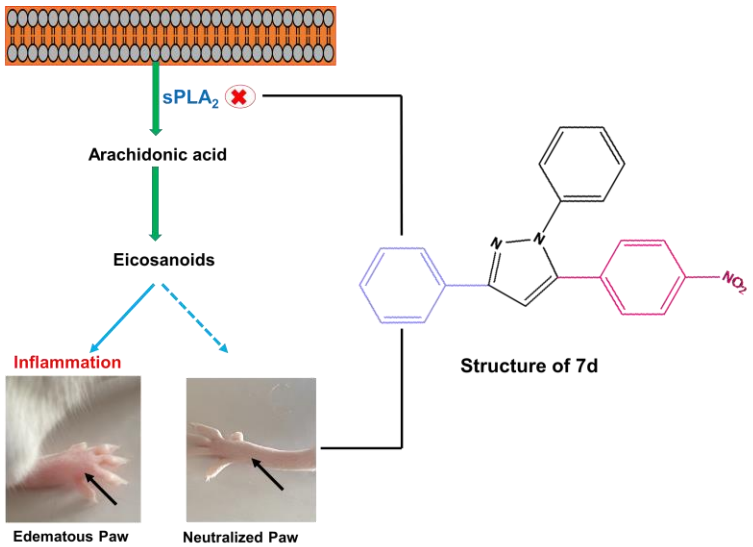
**Graphical abstract**



**ABSTRACT**

Phospholipases $A_2$ ($PLA_2$) are diverse group of enzymes, their main function involves hydrolysis of membrane phospholipids into arachidonic acid and lysophospholipids. These lipid mediators play critical roles in initiation, maintenance, and modulation of cell proliferation. The elevated intracellular $PLA_2$ activities are closely associated with several inflammation-associated diseases including Diabetic Macular Edema, inflammation and oxidative stress. Therefore $PLA_2$ enzymes are an important target for implicated in various inflammatory diseases. In the present study we report the identification of a potent, pyrazole-based small-molecule **7d** as $PLA_2$inhibitors shows significant inhibition and its $IC_{50}$ value was determined (HSF-$PLA_2$$IC_{50}$:23µM and NN-$PLA_2$$IC_{50}$:29µM). In addition to the in-vitro studies we have evaluated the efficacy of 7d, in *in-vivo* method using mouse paw edema model, here it significantly neutralizes the $sPLA_2$ induced edema in a dose dependent manner. Furthermore, it also reduces the MPO activity as well as suppression of inflammatory cells in edematous tissue. Apart from *in-vitro* and *in-vivo* method docking studies on $sPLA_2$ enzymes which include HSF-$PLA_2$ -8.4and NN-$PLA_2$-8with 7d confirms the binding interactions. So, in this study we are investigate a novels$PLA_2$ inhibitor and it might be exhibits as a good candidate to treat various inflammatory disease conditions after the several preclinical studies.

**Keywords:** Inflammation, Arachidonic acid, Secretory phospholipase $A_2$, Edema and Anti-inflammatory, pyrazole.

**Abbreviations:**$sPLA_2$; Secretory phospholipase $A_2$, HSF-$PLA_2$; Human synovial fluid phospholipase $A_2$, NN-$PLA_2$; *Naja naja* phospholipase $A_2$, AA; Arachidonic acid, SAR;Structure activity relationship.

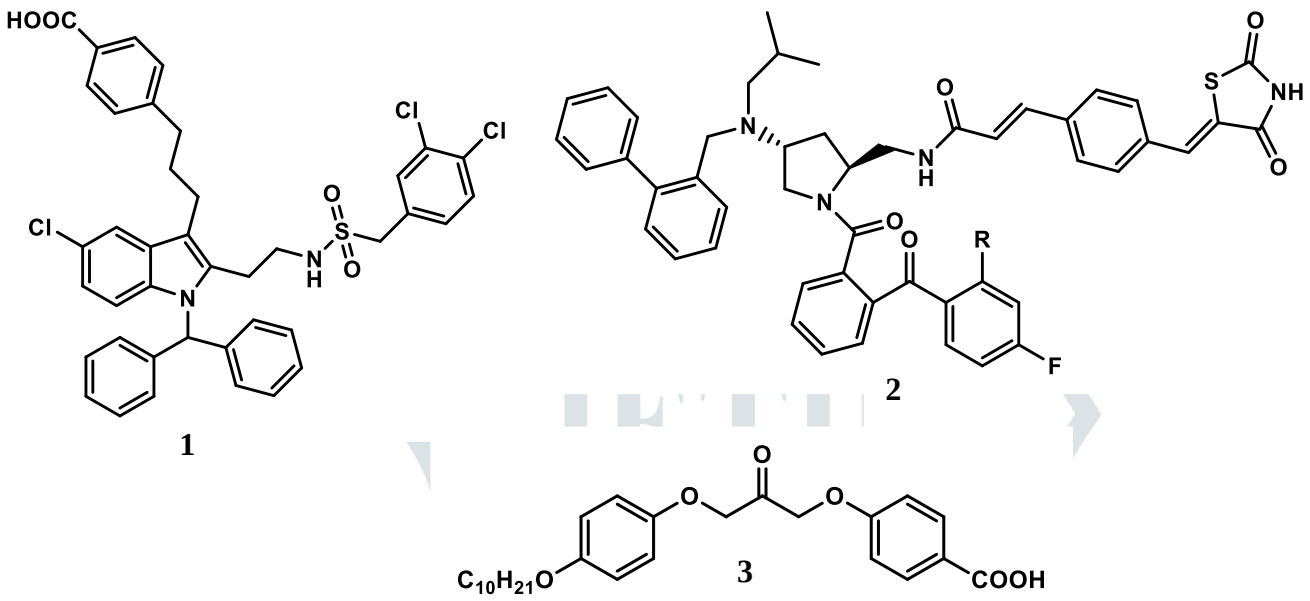
**Introduction**

Phospholipase $A_2$ ($PLA_2$) comprise super family enzymes consists of a broad range of phospholipid metabolizing enzymes, known for their ability to catalyze the ester bond at the sn-2 position of phospholipids[1] that releases arachidonic acid and the glycerol molecule. Arachidonic acid (AA) so released is further metabolized by cyclooxygenase (COX) and lipoxygenase (LO) into prostaglandins, leukotrienes, thromboxanes, and lipoxins.[2]Phospholipase $A_2$ enzymes family consist six subfamilies as cytosolic $PLA_2$s ($cPLA_2$s), calcium-independent $PLA_2$s ($iPLA_2$s), secreted $PLA_2$s ($sPLA_2$s), lysosomal $PLA_2$s, platelet-activating factor (PAF) acetylhydrolases, and adipose specific $PLA_2$s.[3-5] Each subfamily consists of several isozymes and they play crucial roles in diverse cellular responses, including phospholipid digestion and metabolism, host defense and signal transduction, inflammation,vasodilatation, vasoconstriction, apoptosis, and immune responses through interactions with eicosanoid receptors.[6]

They provides precursors for generation of eicosanoids, such as prostaglandins (PGa) and leukotrienes (LTs), when

the cleaved fatty acid is arachidonic acid, platelet-activating factor (PAF) when the sn-1 position of the phosphatidylcholine contains an alkyl ether linkage and some bioactive lysophospholipids, such as lysophosphatidic acid (lysoPA).[7]$PLA_2$also helps to maintain membrane structure and function, by removing oxidized and damaged phospholipids. However the increased activity of $PLA_2$ leads to increased generation of fatty acids and lysophospholipids, which in turn can be metabolized to second messengers and metabolites that contribute to neuroinflammation and propagation of neuronalinjury.[8-11]Thus $PLA_2$s attract significant attention in recent days for treatment of inflammation related disease and cell injuries.[12-17]Extensive studies are ongoing to find a single molecule that can inhibit eicosanoid-producing enzymes, which includes $PLA_2$and COX-2.Although some compounds have been reported as $PLA_2$ inhibitors, none have been found to be worthy in terms of potency, specificity, and material characteristics.[18-20]The difficulty in identification of $PLA_2$ inhibitors is associated by the fact that, the inhibitors have to partition into the phospholipid membrane to interfere with the enzyme in its active status. So, the inhibitors must have substantial lipophilicity for the enrichment in the phospholipid layer[21]. But the drawback is that such inhibitors suffer the lacking of suitable pharmaceutical properties for *in vivo* measurements of anti-inflammatory activity. The only $PLA_2$ inhibitor reported to undergo clinical development as an anti-inflammatory drugs are the indole derivative (**1**) from Wyeth.[21,22]pyrrolidinederivative (**2**)from Shionogi[23]and propan-2-one derivative(**3**)from AstraZeneca[24](Figure 1),though *In vivo* data for these compounds have not been available until now. In searching for nonpeptide and low-molecular-weight $PLA_2$ inhibitors, we have envision that Pyrazole, five-membered aromatic cyclic compound composed of three carbon atoms and two nitrogen atoms in adjacent positions will be the promising scaffold since it will satisfy all the essential pharmaceuticalproperties.[25,26]Moreover Pyrazole derivatives are reported to possess a wide range of biological activities[27-34]including anti-microbial, anti-fungal, anti-tubercular, anti-inflammatory, anti-convulsant, anticancer, anti-viral, angiotensin converting enzyme (ACE) inhibitor, neuroprotective, cholecystokinin-1 receptor antagonist, and estrogen receptor (ER) ligand activity, etc. The Pyrazole molecules and their derivatives performed well as anti-inflammatory agents but there are no such extensive studies were conducted on $PLA_2$ enzyme inhibition. In view of these in the present study we have reported the inhibition activity of 1, 3, 5 trisubstituted pyrazole derivatives against Phospholipases $A_2$.

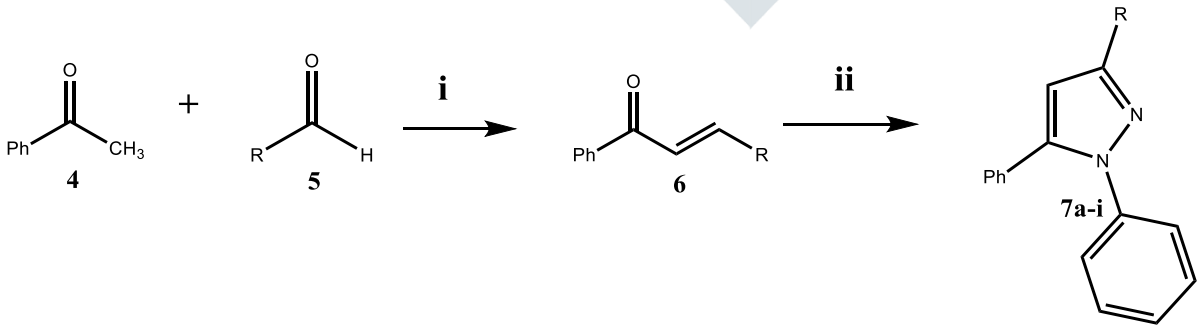
**Figure 1:**




## 1. Chemistry

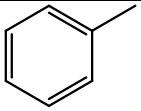
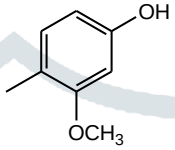
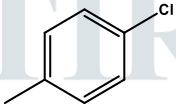
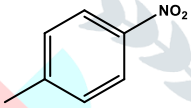
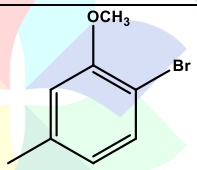
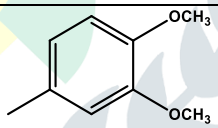
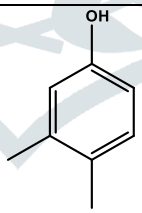
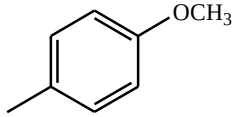
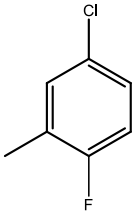
### 1.1 General Synthetic Procedure of 1, 3, 5-trisubstituted pyrazoles

In the present study we synthesized a 1, 3, 5 trisubstituted pyrazole compound by the route outlined in Scheme1. Refluxing the aromatic aldehydes (**2**) with acetophenone (**3**) inalkaine MeOHgive the intermediate chalcones (4) in excellent yield. The resulting chalcones obtained were refluxed with phenylhydrazine hydrochloride yields1, 3, 5-trisubstituted pyrazoles (**7a-i**). The solidpyrazoles derivative obatined was filtered and washed with water followed by recystalyzed with hot methanol. All the compounds are characterized by $^{1}$H-NMR and LC-MS.




**Scheme 1: Synthesis of 1, 3, 5 trisubstituted pyrazoles by condensation method**

**Table 1**

| Sl No | Compound No | R |
|---|---|---|
| 1 | **7a** |  |
| 2 | **7b** |   |
| 3 | **7c** |   |
| 4 | **7d** |   |
| 5 | **7e** |   |
| 6 | **7f** |   |
| 7 | **7g** |   |
| 8 | **7h** |   |
| 9 | **7i** |   |

## 2. Material and methods

### 2.1. Experimental section

All the reagents and chemicals used were reagent grade and were purchased from Sigma Aldrich and Avra Synthesisindia Pvt Ltd.Ketamine was purchased from the University Medical Facility with a prescription from the University authorized medical practitioner. 4-nitro-3-octanoyloxy-benzoicacid was purchased from Cambridge, MA (USA). Nordihydroguaiaretic Acid (NDGA) was procured from Sigma-Aldrich, St. Louis (USA). o-Dianisidine, bovine serum albumin (BSA), ethylene diamine tetra acetic acid (EDTA), dimethylsulphoxide (DMSO), and calcium chloride were bought from Sisco Research Laboratories Pvt. Ltd., Mumbai (India). TLC was performed on aluminium-backed silica plates (60F254, 0.2 mm) which were visualising underUV fluorescence (254 and 366 nm). $^{1}H$ and $^{13}C$ NMR spectra were recorded by Agilent WM Fourier transforms spectrophotometer and Bruker operating at 400 and 101 MHz respectively, using DMSO as a solvent and tetramethylsilane (TMS) as internal standard.The following abbreviations are used: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; m, multiplet and br, broad, for structural assignments of $^{1}H$ NMR. High Resolution Mass Spectra was recorded on waters, XEVO G2-XS QTof instrument.

### 2.2. General procedure for synthesis of chalcone derivatives

In a round bottom flask, 2.5 g of potassium hydroxide was taken and dissolved in water and ethanol mixture. The flask was immersed in an ice bath, and then substituted acetophenone (0.43 mol) and pure substituted benzaldehyde (0.43 mol) was added in to the flask. The mixture was stirred for 4-5 h at 15-20 $^{o}C$. The suspension formed was removed and kept in a refrigerator for 2-3 h. The product was filtered & washed with cold water until the washings were neutral to litmus. Further the product was washed with ice cold rectified spirit, dried and recrystallized from rectified spirit to afford the desired product.

### 2.3. General procedure for synthesis of1, 3, 5 trisubstituted pyrazoles

Chalcone (5.0 mmol 1.08 g) was dissolved in ethanol in 20 $cm^3$ at room temperature. An aqueous solution of sodium hydroxide (2.4 mol/$dm^3$, 8.3 $cm^3$) was added to the solution, and then phenylhydrazine (6.0 mmol) was dissolved in ethanol and added to the reaction mixture. The reaction was carried out under reflux conditions overnight. reaction was monitor by TLC, after complete of reaction ethanol was evaporated by vacuum pressure, compound was dissolve with ethyl acetate and washing with $MgSO_4$.The crude product was obtained by eluting the

mixture with ethyl acetate and then purified by column chromatography using petroleum ether/ethyl acetate (8:1) to obtain the compound. The yields of 1, 3, 5 trisubstituted pyrazoles 7a–7i were 65–84%.

### 2.4. Synthesis of 3-(4-methoxyphenyl)-1,5-diphenyl-1H-pyrazole (7a)

Yellow solid (75.7mg, 72%); mp138-140 °C; $^{1}$HNMR(400 MHz, DMSO-$d_6$) δ 8.74 (dd,*J*7.6Hz ,4H,ArH), 8.54 (d, *J*4.8Hz,4H,ArH ),8.2-8.16 (m,2H,ArH), 7.86-7.6(m,3H,ArH) ,7.60 (s,1H,ArH); $^{13}$CNMR ( 101 MHz, DMSO-$d_6$ ) δ 152.6,146.6,143.4,142.2,131.8, 131.3,131.0, 129.2,128.8,124.7,124.6,122.6,117.8; HRMS (ESI –TOF) m/z [ M+H]$^{+}$ Requires $C_{21}$ $H_{16}N_2$ 296.37 ; found 297.70.

### 2.5. Synthesis of 4-(1,5-diphenyl-1H-pyrazol-3-yl)-3-methylphenol (7b)

Brownish solid (72.6mg, 69%); mp160-162 °C; $^{1}$HNMR (400 MHz, DMSO-$d_6$) δ 8.84 (d, *J*12Hz, 2H,ArH), 8.46 ( d, *J*12Hz, 2H), 8.14-8.05 ( m,6H,ArH), 7.84-7.76 (m,4H,ArH),7.25 (t, *J*6Hz, ArH) ; $^{13}$CNMR ( 101 MHz, DMSO-$d_6$ ) δ 152.7, 151.0,146.6,145.7,144.1, 141.4,132.1, 131.3,129.3,128.9,124.3,124.0,117.7;HRMS (ESI –TOF) m/z [M+H]$^{+}$ Requires $C_{22}$ $H_{18}N_2O_2$ 342.39 ; found 343.10.

### 2.6. Synthesis of 3-(4-chlorophenyl)-1,5-diphenyl-1H-pyrazole (7c )

Cream white solid (84.1mg, 80%); mp 103-105 °C; $^{1}$HNMR (400 MHz, DMSO-$d_6$) δ 8.73 (d, J 3.2Hz,3H,ArH),8.59(d, J 4.8Hz,2H,ArH),8.24-8.2(m,4H,ArH),7.88(d,*J*8Hz,2H,ArH),7.74 (s,1H,ArH),7.6(d,J 4.8Hz,2H,ArH);$^{13}$CNMR(101MHz,DMSO-$d_6$)δ152.7,151.0,146.8,144.1, 141.3,134.1,131.6, 129.2,129.63,127.5,124.5,120.7,117.7;HRMS(ESI–TOF)m/z[M+H]$^{+}$ Requires $C_{21}$ $H_{15}$Cl $N_2$ 330.81 ; found 331.10.

### 2.7. Synthesis of 3-(4-nitrophenyl)-1,5-diphenyl-1H-pyrazole (7d)

Brownish solid (74.7mg, 71%); mp 140-142°C; $^{1}$HNMR (400 MHz, DMSO-$d_6$) δ 8.55( 2H,d,J 6Hz,ArH),8.34 (d ,J 8Hz,ArH),8.12-8.15(4H,m,ArH),8.06 (2H,d, *J*8Hz, ArH ), 7.84-7.80( 4H,m,ArH),7.24 (1H, *J*6Hz,s,ArH);$^{13}$CNMR ( 101 MHz, DMSO-$d_6$)δ152.7,151.0,146.7,145.7, 144.1,141.5,132.2,131.6,131.2,129.3,128.9,124.3,124.3,124.0,117.7;HRMS (ESI –TOF) m/z [M+H]$^{+}$ Requires $C_{21}$ $H_{15}N_3O_2$ 341.36 ; found 341.16.

### 2.8. Synthesis of 3-(4-bromo-3-methoxyphenyl)-1,5-diphenyl-1H-pyrazole (7e)

Brownish solid (70.5mg, 67%); mp 150-152 °C; $^{1}$HNMR (400 MHz, DMSO-$d_6$) δ 7.59 (s , 1H, ArH), 7.39-7.31 ( m, 2H,ArH),7.07-6.46 (m ,9H,ArH),6.5 (s,1H,ArH),4.18(s,3H,$OCH_3$,ArH); $^{13}$CNMR ( 101 MHz, DMSO-$d_6$ ) δ

145.7,143.5,138.4, 132.4,130.4,129.3,127.4,127.7, 120.8,118.7,116.5,116.3,54.1; HRMS (ESI –TOF) m/z $[M+H]^+$Requires $C_{22}$ $H_{17}$ $BrN_2O$405.29; found 405.20.

### 2.9. Synthesis of 3-(3,4-dimethoxyphenyl)-1,5-diphenyl-1H-pyrazole (7f)

Yellow solid (80mg, 76%); mp 162-164 °C; $^1$HNMR (400 MHz, DMSO-$d_6$) δ 7.44 (d,*J*4Hz ,4H),7.43-7.34 (m,6H,ArH), 7.32-7.25( m,3H,ArH),6.48 (s,1H,ArH), 4.22(s, 6H, $OCH_3$); $^{13}$CNMR ( 101 MHz, DMSO-$d_6$ ) δ150.1,142.0,138.7,133.7,133.9,130.5,128.3,125.5,125.7, 114.9, 53.4, 49.3 ; HRMS (ESI – TOF) m/z $[M+H]^+$ Requires $C_{23}$ $H_{20}N_2$ $O_2$ 356.42 ; found 357.13.

### 2.10. Synthesis of 3-(1,5-diphenyl-1H-pyrazol-3-yl)-4-methylphenol (7g)

Yellow solid (78.9mg, 75%); mp 160-162°C; $^1$HNMR (400 MHz, DMSO-$d_6$) δ 8.09-8.07 (1H,m,ArH), 7.92-7.86 (3H,m,ArH), 7.68 6 (d, 2H, *J* 8Hz,ArH), 7.5-7.46(4H,m,ArH) 7.43 (d, 2H, *J* 7.6Hz,ArH), 6.17( 1H,s,ArH); $^{13}$CNMR ( 101 MHz, DMSO-$d_6$ ) δ 151.1, 150.2,142.5 ,139.1 ,132.2 ,128.3 ,127.4 ,126.2,125.2 ,124.8,123.6 ,120.7 ,120.2,109.6,24.3; HRMS (ESI –TOF) m/z $[M+H]^+$ Requires $C_{22}H_{18}N_2$ $O_2$ 342.39 ; found 343.10

### 2.11. Synthesis of 5-(4-methoxyphenyl)-1,3-diphenyl-1H-pyrazole (7h)

Yellow solid (68.4mg, 65%); mp154-156 °C; $^1$HNMR (400 MHz, DMSO-$d_6$) δ 7.6-7.34(9H,m,ArH), 7.32-7.25 (5H,m,ArH), 6.17( 1H,s,ArH), 4.12 (3H,s,$OCH_3$) ; $^{13}$CNMR ( 101 MHz, DMSO-$d_6$ ) δ 142.5 ,139.1 ,132.2 ,128.3 ,127.4 ,126.2,125.2 ,124.8,123.6 ,120.7;δ HRMS (ESI –TOF) m/z $[M+H]^+$ Requires $C_{22}$ $H_{18}$ $N_2O$326.39; found 327.10.

### 2.12. Synthesis of 3-(5-chloro-2-fluorophenyl)-1,5-diphenyl-1H-pyrazole (7i)

Yellow solid (86.3.mg, 82%); mp °C; $^1$HNMR (400 MHz, DMSO-$d_6$)δ 8.73-8.56( m , 5H, ArH), 8.58-8.54 ( m, 3H,ArH), 8.22 (d ,2H,*J*8Hz, ArH), 7.85 (d ,2H, d, *J*7.6Hz, ArH), 7.62 (d ,2H,d, *J* 6.4Hz, ArH),7.59 (s,1H,ArH); $^{13}$CNMR (101 MHz, DMSO-$d_6$ )δ 151.4, 151.0, 149.0, 146.9, 145.7, 141.5, 135.2, 133.6, 132.9, 132.3, 131.6, 124.4, 124.2, 118.8, 114.5; HRMS (ESI –TOF) m/z $[M+H]^+$ Requires $C_{24}$ $H_{22}N_2O_3$ 348.08; found 349.18

## 3. Methodology

### 3.1 Animals

Swiss albino mice weighing 20 to 25 g were obtained from Central Animal House Facility, Department of Studies in Zoology, university of Mysore, Mysuru, India. The animal care and handling were conducted in compliance with National Regulations for Animal Research. The animal experiments were carried out after reviewing the protocols by the Animal Ethical Committee of the University of Mysore, Mysuru, India (Order

No: UOM/IAEC/17/ 2016-17).

### 3.2 Source of $sPLA_2$

$sPLA_2$ from *Naja naja*venom was partially purified according to our previous publication.[35]Human synovial fluid (HSF) was obtained by DRM multi-specialty hospital, Mysuru, India. $sPLA_2$ from HSF were purified according to published method.[36] All experiments involving human biological samples were conducted according to the protocols approved by the Institutional Human Ethical Committee, UOM, Mysuru (Sanction order no: IHEC-UOM No.17 /Ph. D/2016-17) and were in accordance with the regulations of the Indian Council of Medical Research, New Delhi, India.

### 3.3. Phospholipase $A_2$ assay and its inhibition

$PLA_2$ activity assay was carried out in 96-well plate method[37]with slight modifications. The standard assay mixture contained 220 µl of buffer (10 mM Tris–HCl, 10 mM $CaCl_2$, 100 mM NaCl, pH 7.8), 60 mM of substrate, and 30 µg of $sPLA_2$ were adding to a final volume of 260 µl. After the addition of $sPLA_2$, the reaction mixture was incubated for up to 40 min at 37 $^{\circ}$C and the absorbance was taken at 425 nm with 10 min interval. Enzyme activity was calculated based on the increase in absorbance after 40 m. For inhibition studies, pyrazole molecules were pre-incubated with the enzyme for 15 min at 37 $^{\circ}$C with different concentration. The $sPLA_2$ activity was measured and the results were expressed as % remaining activity. Since among screened pyrazole molecules, 7d shows better inhibition towards different sources of $sPLA_2$. In this regard we have selected 7d molecule for further studies and the $IC_{50}$ value was determined. NDGA a known standard inhibitor was taken as a positive control for the $sPLA_2$ assay.

**3.4. Effect of substrate concentration on $sPLA_2$ inhibition by7d:** The standard assay mixture contained 220 µl of buffer (10 mM Tris–HCl, 10 mM $CaCl_2$, 100 mMNaCl, pH 7.8), and 30 µg of $sPLA_2$ were adding to a final volume of 260 µl with $IC_{50}$ concentration of 7d. Adding various concentration of substrate started the reaction and further assay was carried out as described above.

**3.5. Effect of Calcium on inhibition of $sPLA_2$ by 7d:**The reaction mixture containing HSF-$PLA_2$ alone or with $IC_{50}$ concentration of 7d in 10 mM Tri-HCl buffer pH 7.8 was taken. Calcium concentration ranging from 0-15 mM was added to study the effect of calcium on the inhibition of $sPLA_2$ enzymes by 7d. The reaction was started by adding 60 mM of substrate and the assay was carried out as described above.

### 3.6. Neutralization of phospholipase $A_2$ induced mouse paw edema

The procedure ofVishwanath et, al[38] was followed for paw edema studies. Briefly, mice were given intraplantar injection with 40 µl of saline containing 5 µg of HSF-$PLA_2$to the right mice footpad. Respective left footpad was

injected with 40 µl of saline/vehicle and served as negative control. After 45 min, mice were sacrificed by using anesthesiaKetamine(30 mg/kg; i.p.) and both hind limbs were excised at the ankle joint and weighed individually. Animals treated with HSF-$PLA_2$was served as positive control. The edema ratio was calculated using the formula:

Edema ratio = (weight of edematous leg/weight of normal leg) × 100

For inhibition studies 5 µg of HSF-$PLA_2$was co-treatment with or without 7d, vehicle control (DMSO 1%) for 15 min at 37 $^{\circ}$C later injected to right paw and respective left paw received saline. The percentage of inhibition was calculated as described above.

### 3.7. Histopathological studies

HSF-$PLA_2$ induced edema in mouse tissues, with or without 7d was fixed in 10% formalin and routinely embedded in paraffin for histopathological examination. Tissues were cut into 4µm-thicks using a rotary microtome. Haematoxylin and Eosin (H & E) stains were used to observe the infiltration of inflammatory cells in epithelial and connective tissues.[39]

### 3.8. Myeloperoxidase assay

Myeloperoxidase (MPO) assay was performed according to the previously published method[40]with slight modification. MPO activity was estimated by mixing 50 µL of edema tissue homogenate with 2.95 mL of phosphate buffer (50 mM; pH 6.0) and 1 mL of 1.67 mM o-dianisidine hydrochloride containing 0.0005% $H_2O_2$ (v/v). The change in the absorbance at 450 nm was recorded and the activity was expressed as IU.

### 3.9. Proteinestimation

Protein content of all the samples was determined by the method of Lowry etal[41] using BSA as protein standard.

### 3.10. Computational studies

The aim of this study was to understand the binding mode of 7d with AA pathway enzyme protein such as *N. naja*$PLA_2$ and HSF-$PLA_2$ by the Auto docking vina method.[42]The crystal structure of human inflammatory s$PLA_2$ was recovered from PDB (PDB id: 1POE) and *N. naja* venom s$PLA_2$ (PDB id: 2WQ5). The PDB files were visualised using Discovery Studio, where the crystallographic water molecules and co-crystallised ligands were removed, and then loaded into the PyRx software, which automatically converted the PDB files to PDBQT files, with polar Hydrogens and Kollmann Charges addedTheAuto dock Vina module in PyRx was used for molecular docking of above-mentioned proteins against the ligand. Hydrogen bond network optimization and identification of proper ionization states (His tautomer's) were implemented. Ligand 7d pyrazole compound was drawn using chemdrawUltra 12.0. The structures were minimised using the MMFF94 forcefield using OpenBabel, for 10000

steps using steepest descent algorithm.[43]the ligands were saved as SDF files and converted to Auto dock PDBQT files using OpenBabel.Maximum of about 10 to 15 poses were attained for each ligand docking and the best of them was selected based on the glide score, energy and the interactions with the active site residues.

### 3.11. Statistical analysis

The results were expressed as mean ± SD. One-way ANOVA with Bonferroni's multiple comparison post hoc tests was performed to assess statistical significance using GraphPad Prism 5.0 software. The comparison between the groups was considered significant if data represents mean ± SD (n =3). *p < 0.1, *p < 0.01, ***p < 0.001as compared to control.

## 4. Results and Discussion

### 4.1Screening of pyrazole compounds against pro-inflammatory enzyme sPLA$_2$activity in vitro method

sPLA$_2$ plays a significant role in the pathogenesis of many diseases, therefore inhibition of this enzyme activity is one of the chemo therapeutic ways for treatment ofinflammatory related diseases. HSF- PLA$_2$ belongs to type II group of sPLA$_2$elevatedin the synovial fluid of arthritis patients. In an effort to establish structure activity relationship (SAR)data and identifying the likelihood of a potent PLA$_2$, inhibitor, we have screened the pyrazole derivatives **7a-I** against HSF-PLA$_2$by photometric method at two different concentrations of pyrazole compounds (30μM and 100μM).The result of this screeningis depicted in**Figure 2A**.It is evidenced from the **Figure 2** that, compound 7d displays significant inhibition(~90% inhibition at 100 μM) towards HSF-PLA$_2$ enzyme.

### 4.2. Determination of IC$_{50}$ value for 7d

Next we have performed the PLA$_2$inhibitionassayby compound 7din dose dependant mannerto determine the IC$_{50}$ value against two different sources of sPLA$_2$ which includes partially purified *Naja Naja* venom PLA$_2$ and HSF-PLA$_2$ belongs to toxins group and inflammatory marker respectively**(Figure 2B and 2C).** The IC$_{50}$ value was calculated by using XY scattered plot. The IC$_{50}$ of 7d against HSF-PLA$_2$ and NN-PLA$_2$is 23μM and 29μM respectively which were close to theIC$_{50}$ of control compound NDGAagainstHSF-PLA$_2$ 13μM and NN-PLA$_2$20μM respectively.

### 4.3. Effect of substrate and calcium concentration

Next we have examined the effect of substrate and calciumchloride concentrationon IC$_{50}$value of**7d**. For this we have examined the inhibition of HSF-PLA$_2$ activity at different concentration range of substrate in the absence and presence of **7d** at its IC$_{50}$ value. It was found that **7d**inhibitsHSF-PLA$_2$ activity approximately 50% at 25 nM substrate concentrationsand remained constant over the 100Nm range**(Figure 3A)**. This result revealed that, inhibition of HSF-PLA$_2$ by **7d** is independent of substrate concentration. To exclude the possibility of inhibitory effect of **7d** by metal chelation, we have tested the inhibitory effect of **7d** by increasing the concentration of calcium ion from 0–15 mM. It was found thatpresence of calcium ion did not alter the HSF-PLA$_2$ activity (~50%)

even at high concentration **(Figure 3B)**. This reveals that the inhibition of HSF-$PLA_2$ by **7d** is independent of calcium. Also,these results indicate that a pyrazole**7d**may directly interact with the active site of an enzyme and reduces its pro-inflammatory activity.

### 4.4. Neutralization of HSF-$PLA_2$ induced mouse paw edema for 7d

In addition to the *in vitro* inhibition studies, we have checked the *in-vivo*efficacy of **7d** by using awell-established inflammatory mouse paw edema model. We have performed the *invitro*studies using both NN-$PLA_2$ and HSF-$PLA_2$ since both are belongs to s$PLA_2$ group. However NN-$PLA_2$isa part of toxins group, therefore we have used HSF-$PLA_2$in the *in-vivo* studies by mouse paw edema model to assess the Anti-inflammatory activity of pyrazole derivative **7d**.High levels of extracellular $PLA_2$ activity are found in synovial fluid in patients with various kinds of arthritis but little is known about its direct contribution to the inflammatory process in affected joints. The edema induced by HSF-$PLA_2$ is rapid in onset and persistent. The peak inflammatory response was achieved by 45 min and was unchanged 6 h after injection. To examine the *in vivo* anti-inflammatory activity of **7d**, a well-established mouse paw edema model form our labwas used. We have showed that injection of HSF-$PLA_2$ into mouse paw induces edema which peaks the inflammatory response by 45 min and was unchanged 3 to 5 h after injection. The persistent edema and inflammation caused by HSF-$PLA_2$ may be due to their combined effect to hydrolyse membrane phospholipids resulting in loss of membrane integrity, production of eicosanoids which helps in amplifying the inflammation response. This type of rapid onset and persistent edema was also observed with injection of purified s$PLA_{2S}$ from *N. Naja* and *V. Russelli*venoms.[44, 45]In present experimentInjection of HSF-s$PLA_2$ (5 µg) into mouse hind paw resulted in swelling of the foot pad with an edema ratio of 175.73 ± 2.56%.However, when pyrazole derivative **7d** was co-injected with HSF-$PLA_2$, edema ratio decreased in a dose dependent manner(**Figure 4A)**.In this experiment is found that **7d**significantly subsides local acute inflammation of mouse paw edema.

Further the MPO assay was performed to assess the recruitment of inflammatory cells in edematous tissues. MPO is considered an important part of the innate immune systems and is secreted by neutrophils and macrophages. This enzyme has been plays an important role in the pathogenesis of various inflammatory diseases.[46]the activity of MPO was found to be increased in the inflamed paw after HSF-$PLA_2$ injection with significant decrease on treatment with **7d** (**Figure4B)**.In supporting to this we have performed Hand E staining to check the effect of 7d on assessing the recruitment of inflammatory cells at the site of tissue injury. Injection of HSF-$PLA_2$ alone causes the increase in recruitment of inflammatory cells. Whereas co-injected with 7d drastically reduces the number of cells in mouse paws edematous tissue **(Figure 4C)**. These studies further confirms that a pyrazole derivative compound **7d** prove to be a better anti-inflammatory molecule.

### 4.5. Docking studies

A molecular docking approach was used to elucidate the binding affinity and intermolecular interactions of compounds**7d**and**7h**with HSF-$PLA_2$ and NN-$PLA_2$.Compound **7d** exhibit the binding affinity of -8.4 (Kcal/mol) whereas **7h** exhibit the binding affinity of -7.1 (Kcal/mol) with HSF-$PLA_2$ (PDB ID: 1POE). In addition **7d** has a strong hydrogen bond with active site His: 47 (of the calcium binding domain region of amino acid nitro group) and various pi-electron cloud interactions with amino acids of other than calcium binding domino regions (**Figure 5A).**Suchhydrogen bond with active site His: 47 residue is not absorbed in case of compound 7h (**Figure 6A**) instead it only showsvarious pi-electron cloud interactions with amino acids ofother than calcium binding domino regions. Compounds **7d** and **7h** havecomparablebinding affinity of -8.0Kcal/mol and -8.1Kcal/mol respectively with NN-$PLA_2$ (PDBID: 2WQ5). Compound **7d**exhibit strong aromatic pi-electron cloud interaction (pi-pi interaction) with PHE :5, TRP :18 and PHE : 63 (**Figure 5B)**similarly compound 7h exhibit strong pi-sigma interaction with Asp :48, Val :30, Gly : 29 and Ala: 8**.**This docking study proved that compound **7d** is not blocking the calcium binding domain region, and this result is consistent with our observation thatinhibition of HSF-$PLA_2$ by **7d** is independent of calcium. Chloride studies proved that **7d**molecule as good interaction towards amino acid residues of as well as various catalytic sites of both s$PLA_2$s.

## 5. Conclusion

Here we have synthesized a novel pyrazole derivative **7d** which shows significant inhibitory property against pro-inflammatory s$PLA_2$enzyme. In addition, insilico method shows that **7d** has good binding interaction with s$PLA_2$ protein. The inhibition activity of this compound is attributed to be the formation of hydrogen bond with His: 47 residue of calcium binding domain. Further we validate its potency as an anti-inflammatory property using mouse paw edema model, here it neutralizes edema significantly. So,**7d**a s$PLA_2$inhibitor proven to be beneficial to treat various inflammatory disease conditions after the several preclinical studies

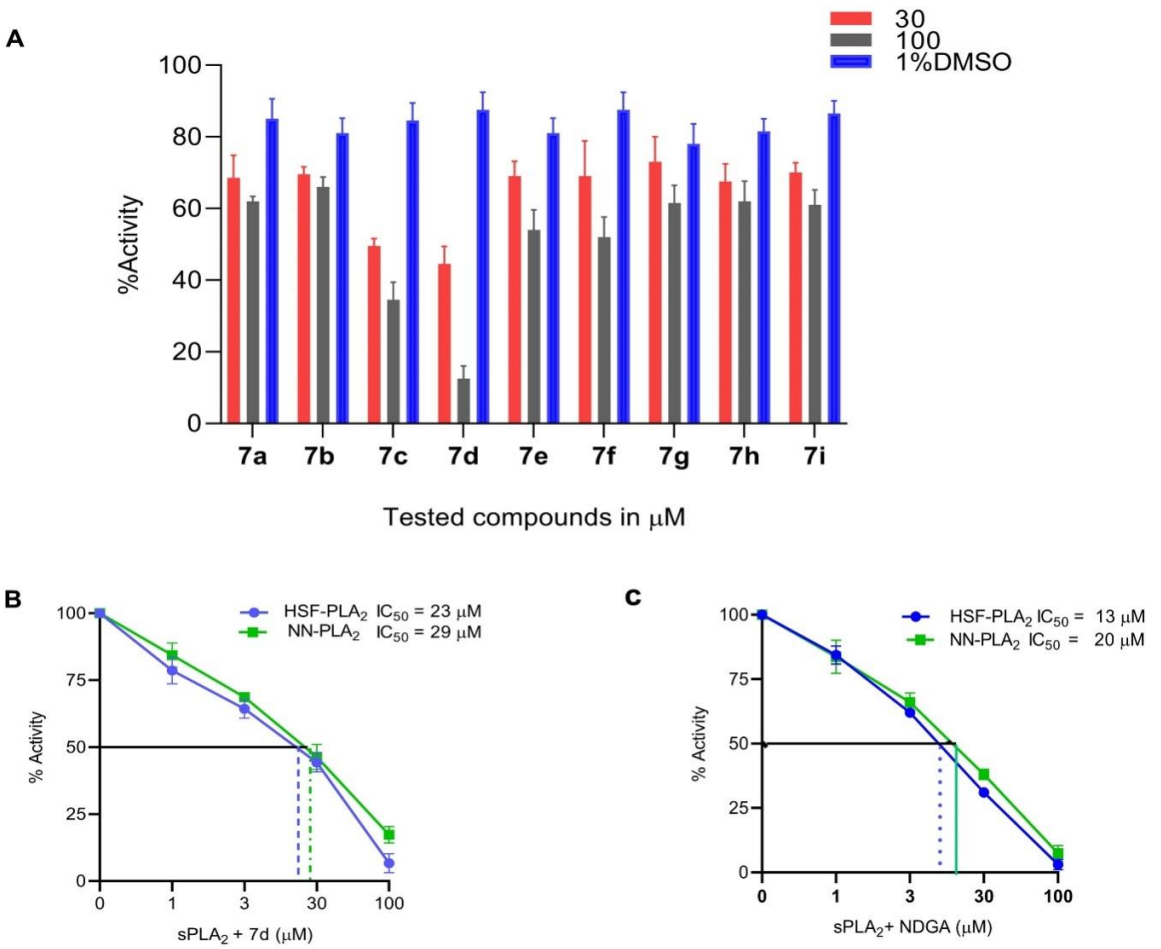



**Figure 2: Screening of pyrazole synthesized molecules ona proinflammatory HSF-PLA$_2$enzyme:(A)**Dose dependent Inhibition of HSF-PLA$_2$ by pyrazole compounds, DMSO was taken as a vehicle control. **(B)Determination of IC$_{50}$:**IC$_{50}$concentration of 7d for different sources of sPLAs$_2$was determined.**(C)**NDGA a known HSF-PLA$_2$ inhibitor taken as a positive control**.**

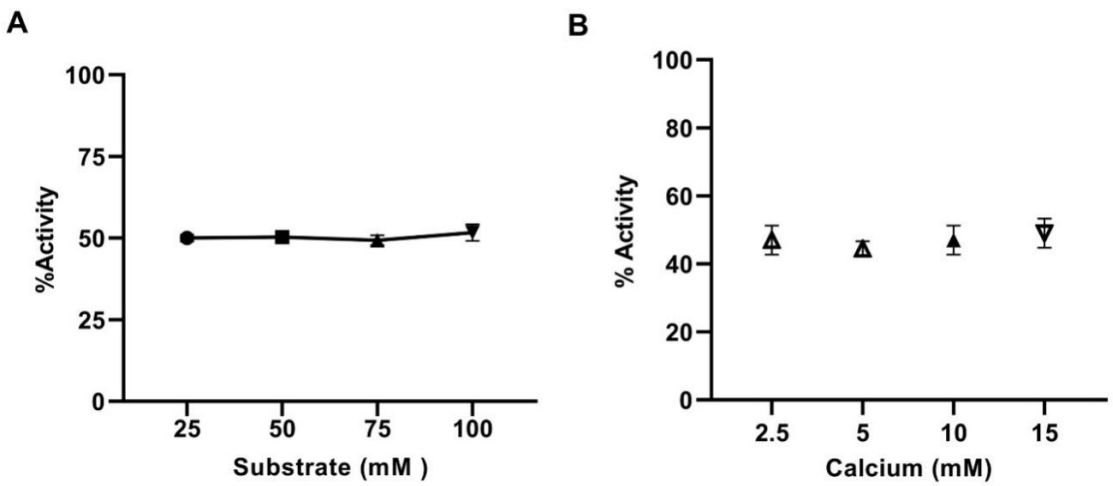



**Figure 3: Effect of substrate and calcium chloride concentration of 7dfor HSF-PLA$_2$ enzyme activity:HereFigure 3 (A) and (B)** represent the percent activity of HSF-PLA$_2$ at $IC_{50}$ concentration of 7d with varying concentrations of substrate or calcium (mM).

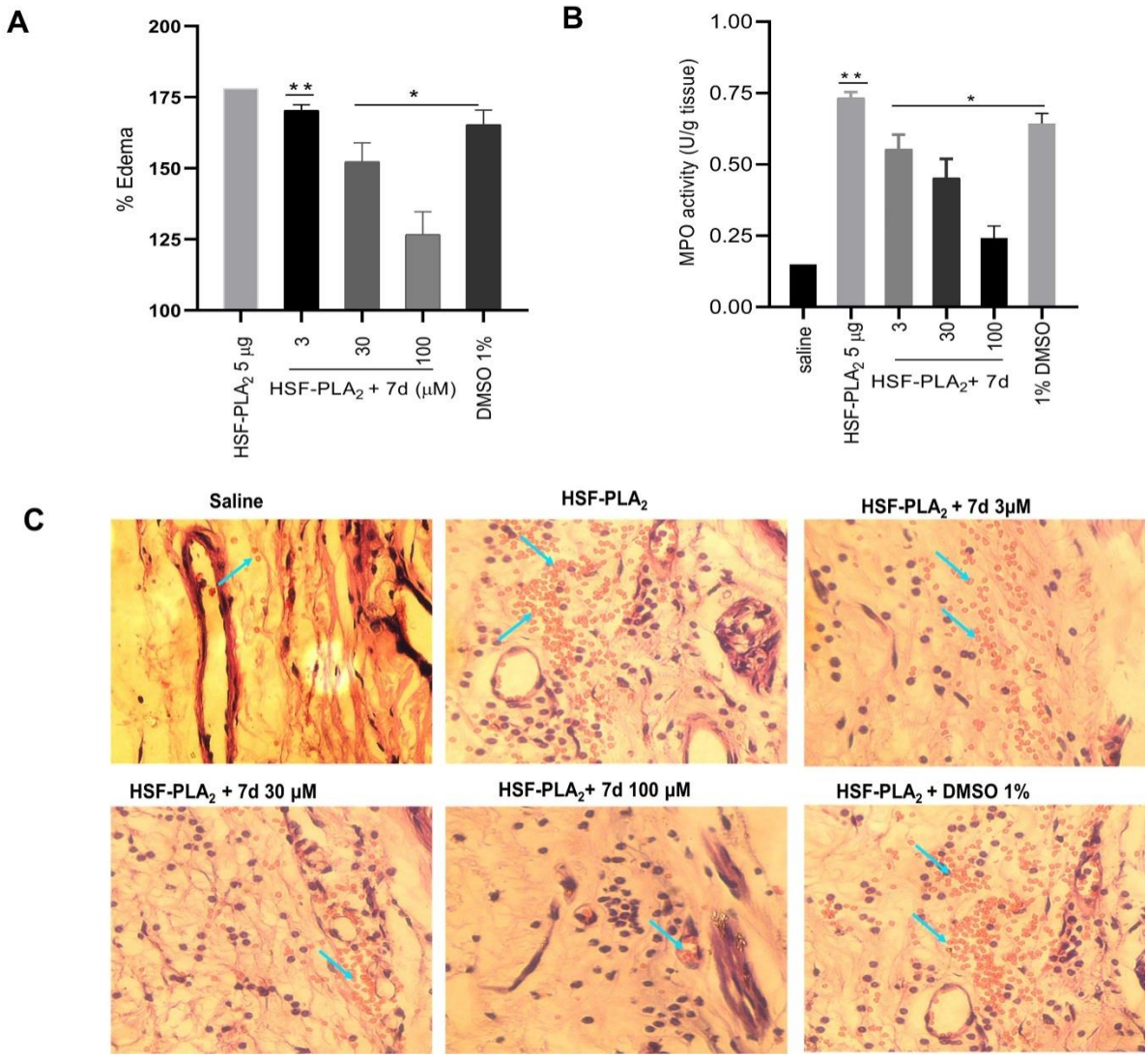
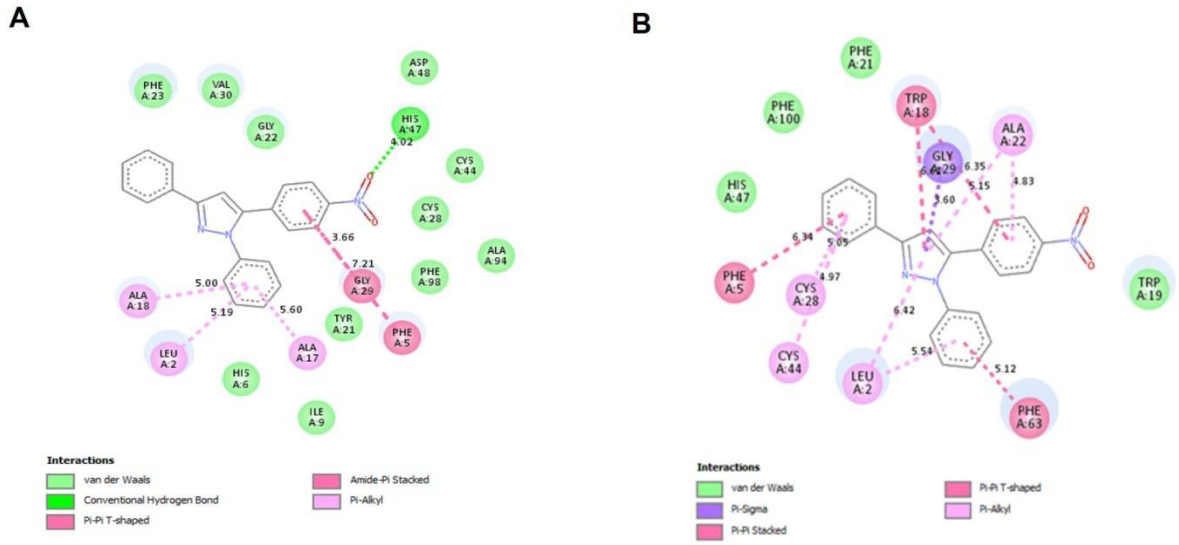
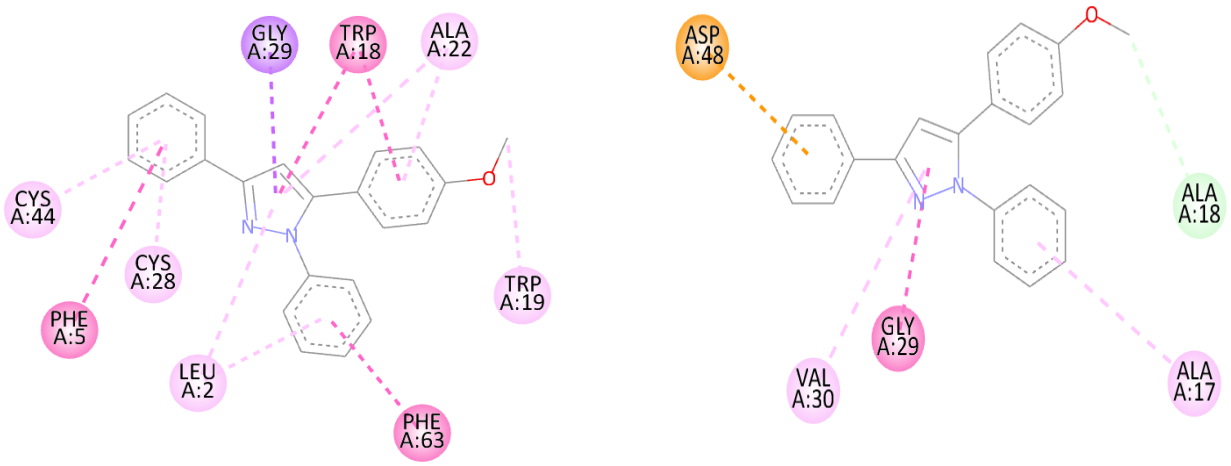



**Figure 4: Neutralization of HSF-PLA$_2$ induced mouse paw edema by 7d** (**A**) The reaction mixture contained 5 µg of HSF-PLA$_2$with or without different concentrations of 7d in a total volume of 40 µl and injected into mice right footpad. Injection of 40 µl of saline/vehicle to left footpad served as control. Data represents mean ± SD (n =3). *p 0.1, *p < 0.01, ***p < 0.001 as compared to HSF-PLA$_2$alone. **(B)MPO enzyme activity** was expressed as U/mg protein. Data represents mean ± SD (n =3). *p 0.1, *p < 0.01, ***p < 0.001 as compared to sPLA$_2$IIA alone. **(C)H and E staining**Haematoxylin and eosin (H&E) staining of paw edema tissue Magnification is 40 X.




| Target Protein | Docking score (kcal/mol) |
|---|---|
| A: 1POE (HSF-PLA) | -8.4 |
| B: 2WQ5 (NN-$PLA_2$) | -8 |

**Figure 5: Molecular Docking studies between 7d with HSF-$PLA_2$, NN-$PLA_2$:(A and B)**Energetically favorable binding mode of **7d** was calculated using induced fit molecular docking method. The hydrogen bonding and hydrophobic interactions between the 7d with HSF-$PLA_2$ and NN-$PLA_2$ are depicted using the PyRx software. Glide score and glide energy (calculated in kcal/mol) associated with best binding modes of 7d with the active site of HSF-$PLA_2$, NN-$PLA_2$is shown.




| Target Protein | Docking score (Kcal/mol) |
|---|---|
| **A: 1POE(HSF-PLA)** | **-7.1** |
| **B: 2WQ5(NN-PLA$_2$)** | **-8.1** |

**Figure6: Molecular Docking studies between 7h with HSF-PLA$_2$, NN-PLA$_2$:(A and B)**Energetically favorable binding mode of **7h** was calculated using induced fit molecular docking method. The hydrogen bonding and hydrophobic interactions between the 7h with HSF-PLA$_2$ and NN-PLA$_2$ are depicted using the PyRx software. Glide score and glide energy (calculated in kcal/mol) associated with best binding modes of 7h with the active site of HSF-PLA$_2$, NN-PLA$_2$ is shown

## 5. Declaration of Competing Interest

The authors confirm no contending monetary interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**SUPPORTING INFORMATION**

# Trisubstitutedpyrazoles as Novel Inhibitor of Human Secretory Phospholipase $A_2$ with Antiinflammatory Activity: SAR study and Molecular Modeling

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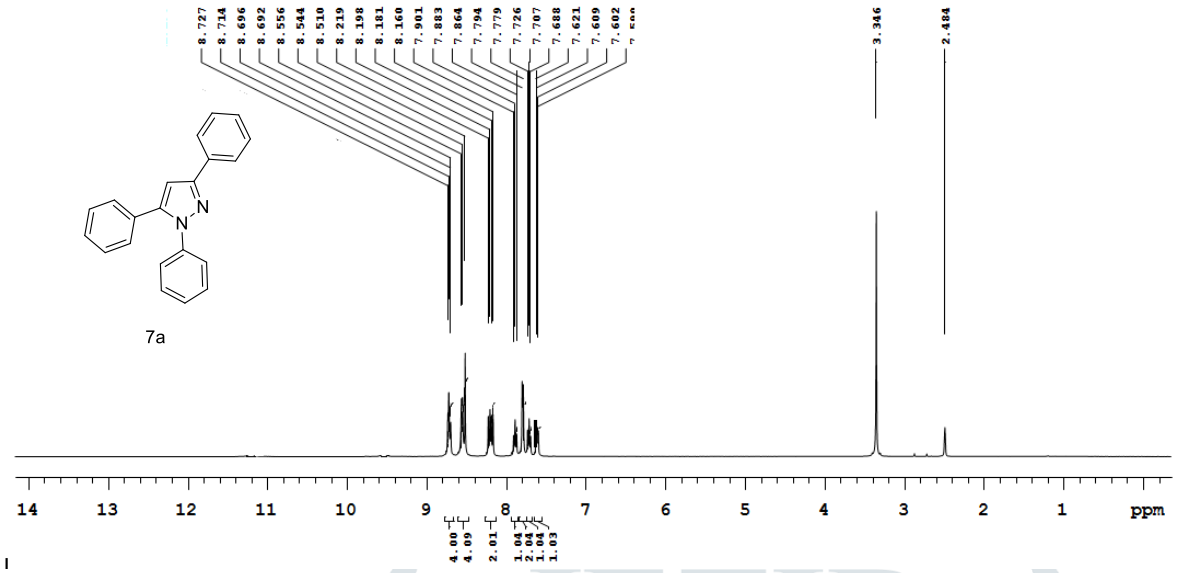
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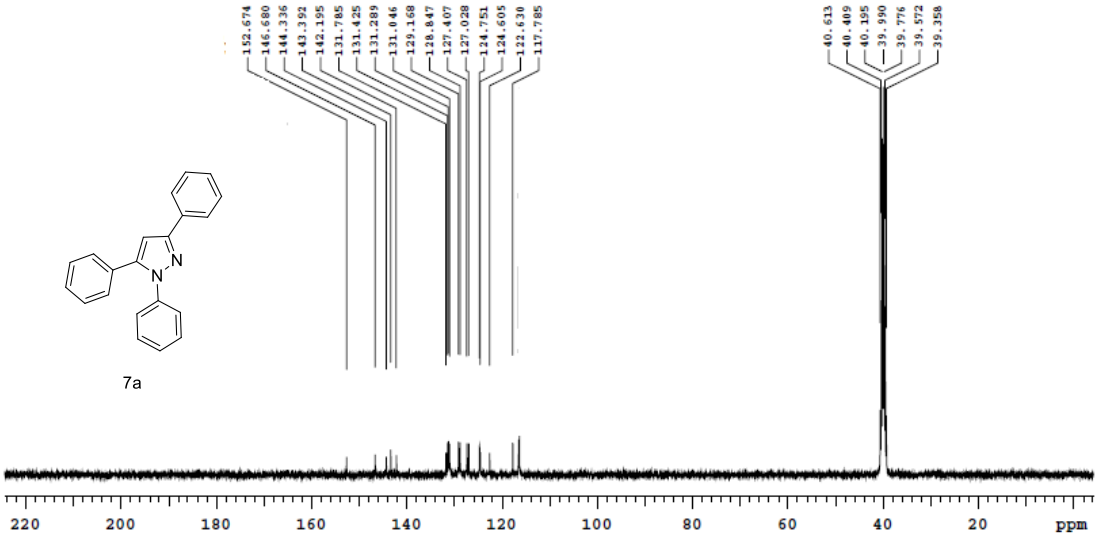
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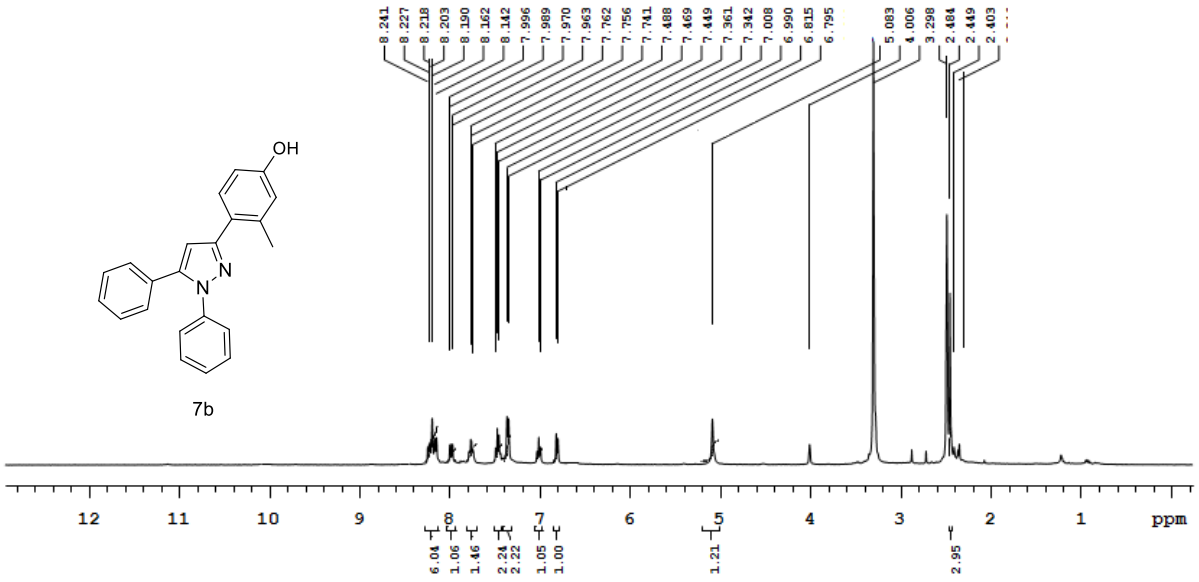
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7.756
7.741
7.488
7.469
7.449
7.361
7.342
7.008
6.990
6.815
6.795
5.083
4.006
3.298
2.484
2.449
2.403
OH
N
N
7b
12
11
10
9
8
7
6
5
4
3
2
1
ppm
6.04
1.06
1.46
2.24
2.22
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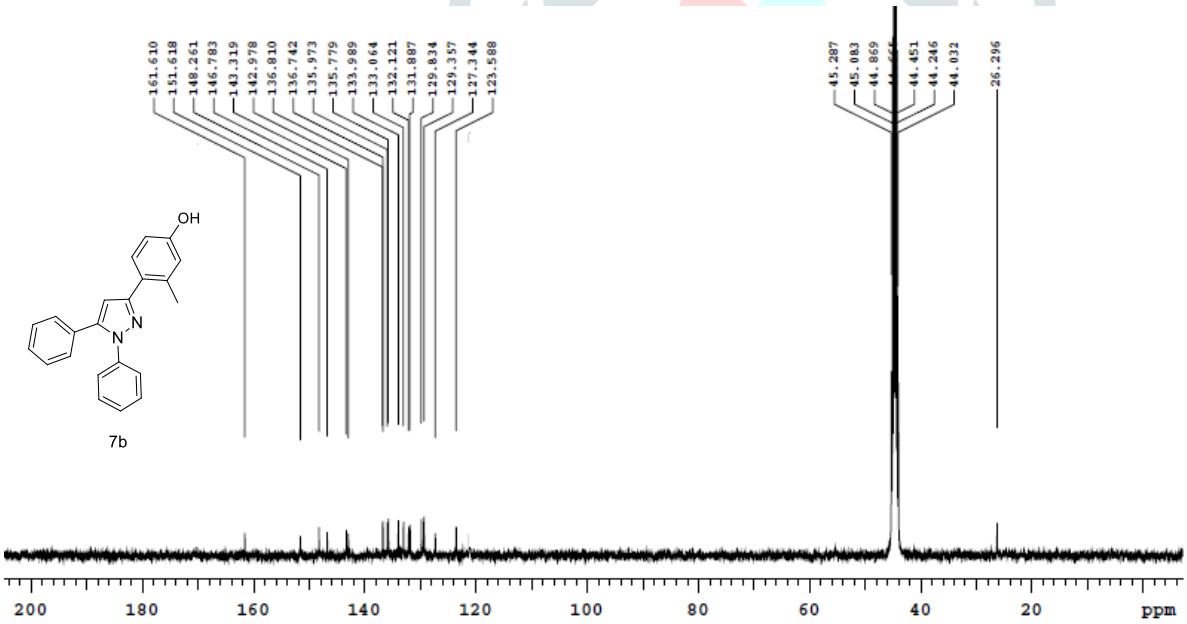
1.00
1.21
2.95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136.742
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135.779
133.989
133.064
132.121
131.887
129.834
129.357
127.344
123.588
45.287
45.083
44.869
44.451
44.246
44.032
26.296
OH
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N
7b
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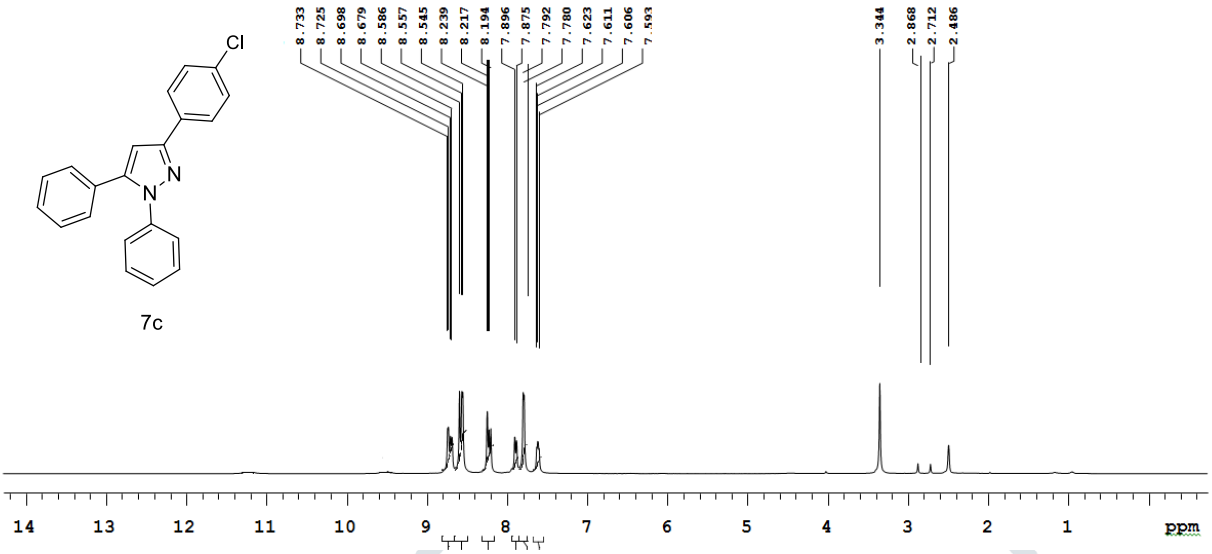
160
140
120
100
80
60
40
20
ppm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8.698
8.679
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8.545
8.239
8.217
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7.896
7.875
7.792
7.780
7.623
7.611
7.606
7.593
3.344
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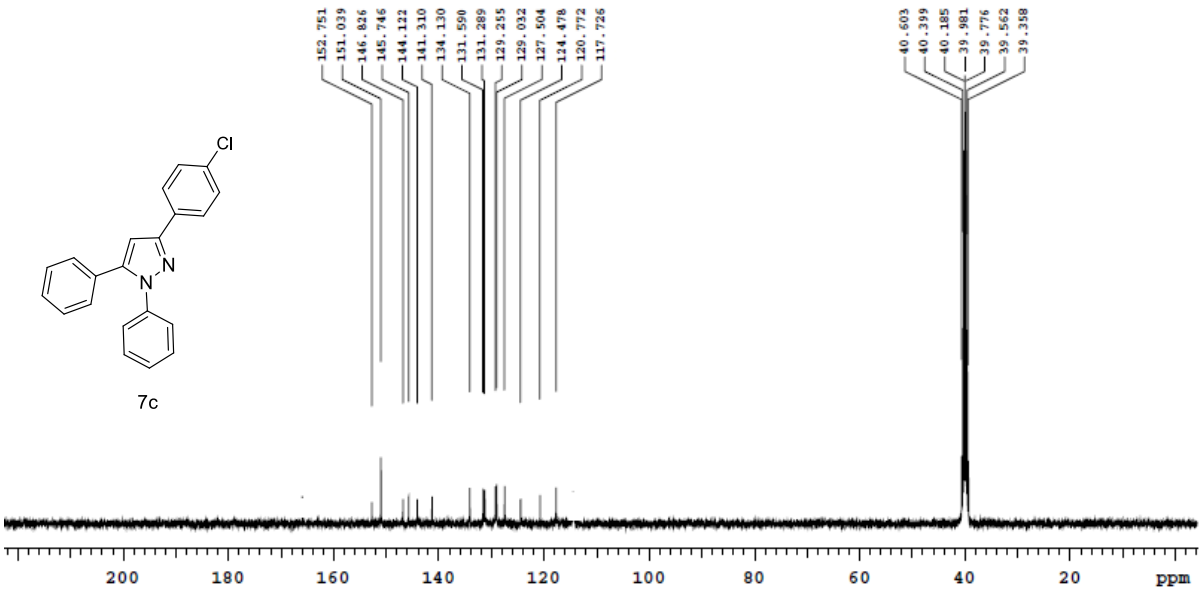
2.486
14
13
12
11
10
9
8
7
6
5
4
3
2
1
ppm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N
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7c
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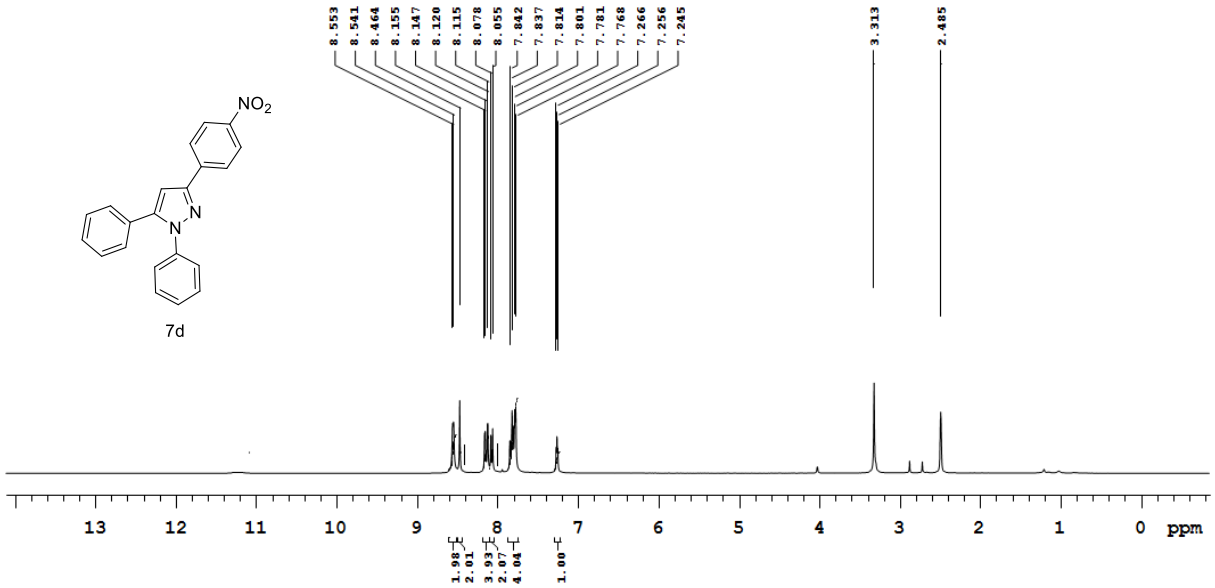
146.826
145.746
144.122
141.310
134.130
131.590
131.289
129.255
129.032
127.504
124.478
120.772
117.726
40.603
40.399
40.185
39.981
39.776
39.562
39.358
200
180
160
140
120
100
80
60
40
20
ppm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8.541
8.464
8.155
8.147
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8.115
8.078
8.055
7.842
7.837
7.814
7.801
7.781
7.768
7.266
7.256
7.245
3.313
2.485
NO2
N
N
7d
13
12
11
10
9
8
7
6
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4
3
2
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ppm
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2.01
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2.07
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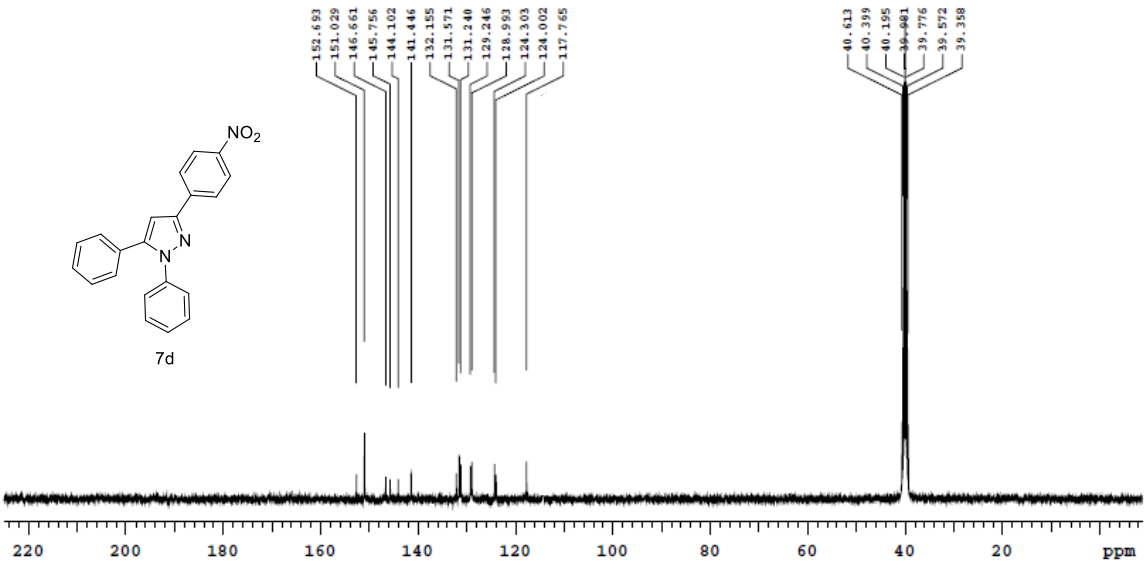
1.00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129.246
128.993
124.303
124.002
117.765
40.613
40.399
40.195
39.981
39.776
39.572
39.358
NO2
N
N
7d
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200
180
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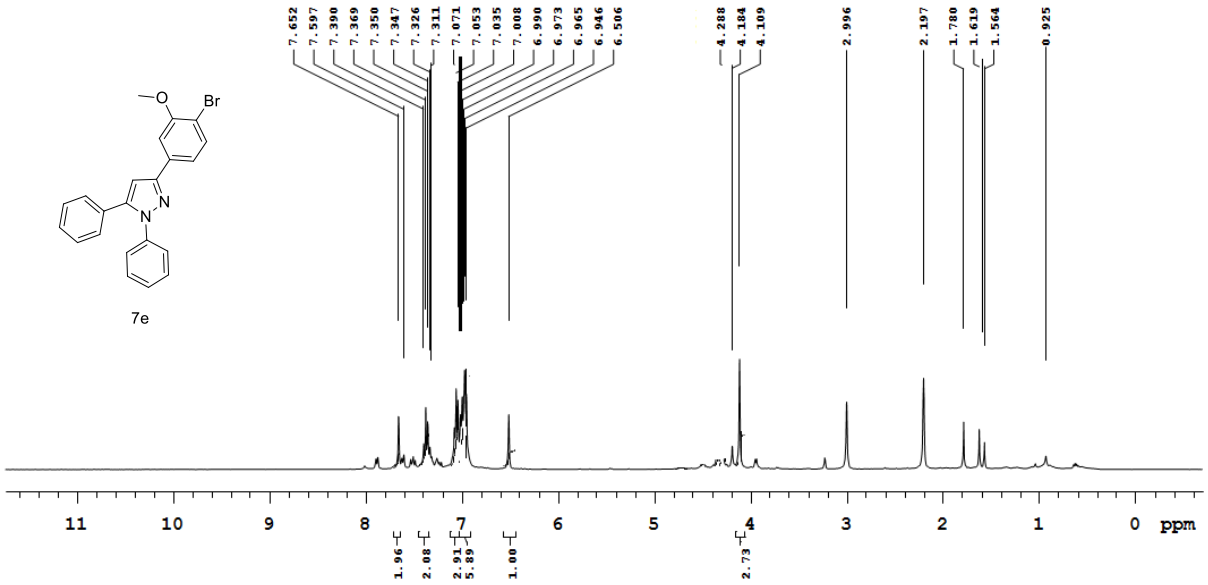
140
120
100
80
60
40
20
ppm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7.369
7.350
7.347
7.326
7.311
7.071
7.053
7.035
7.008
6.990
6.973
6.965
6.946
6.506
4.288
4.184
4.109
2.996
2.197
1.780
1.619
1.564
0.925
Br
O
N
N
7e
11
10
9
8
7
6
5
4
3
2
1
0
ppm
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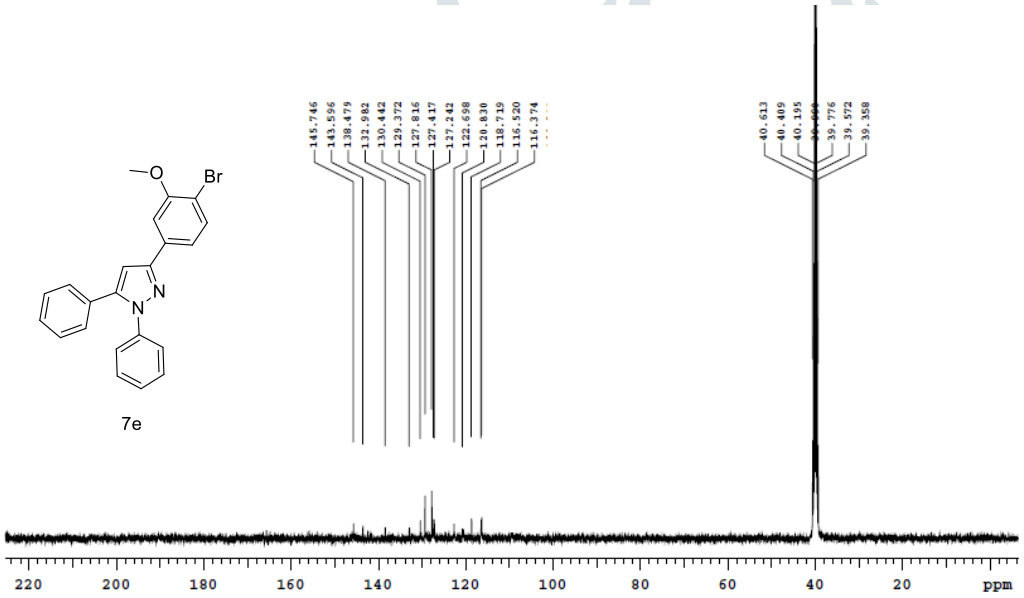
2.08
2.91
5.89
1.00
2.73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138.479
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130.442
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127.816
127.417
127.242
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116.520
116.374
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40.409
40.195
39.776
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Br
O
N
N
7e
220
200
180
160
140
120
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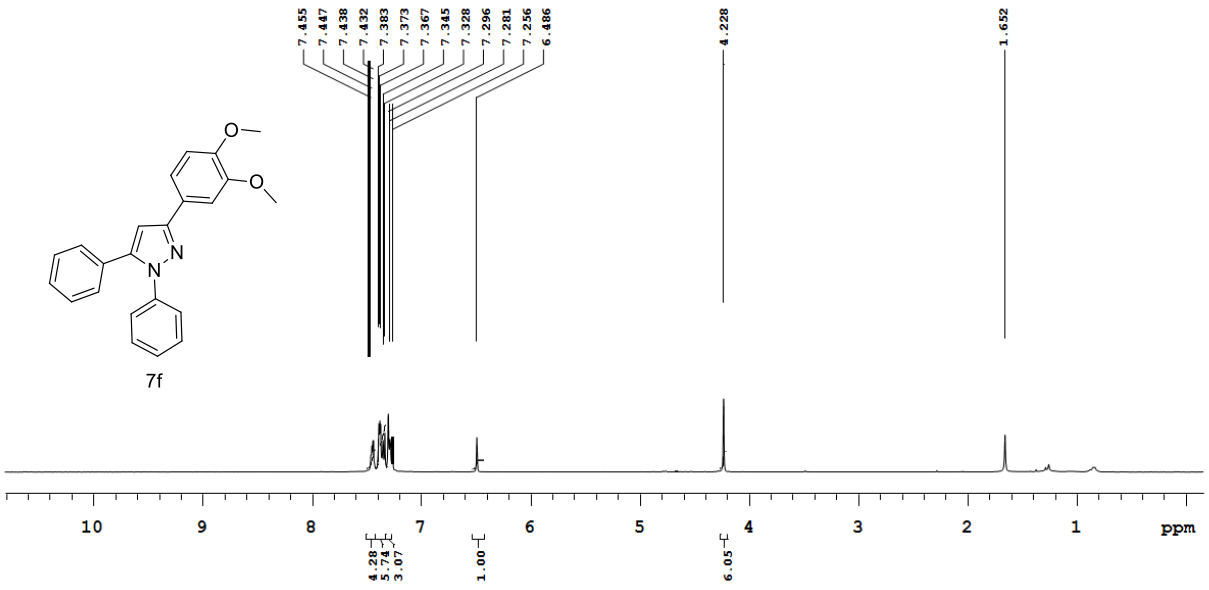
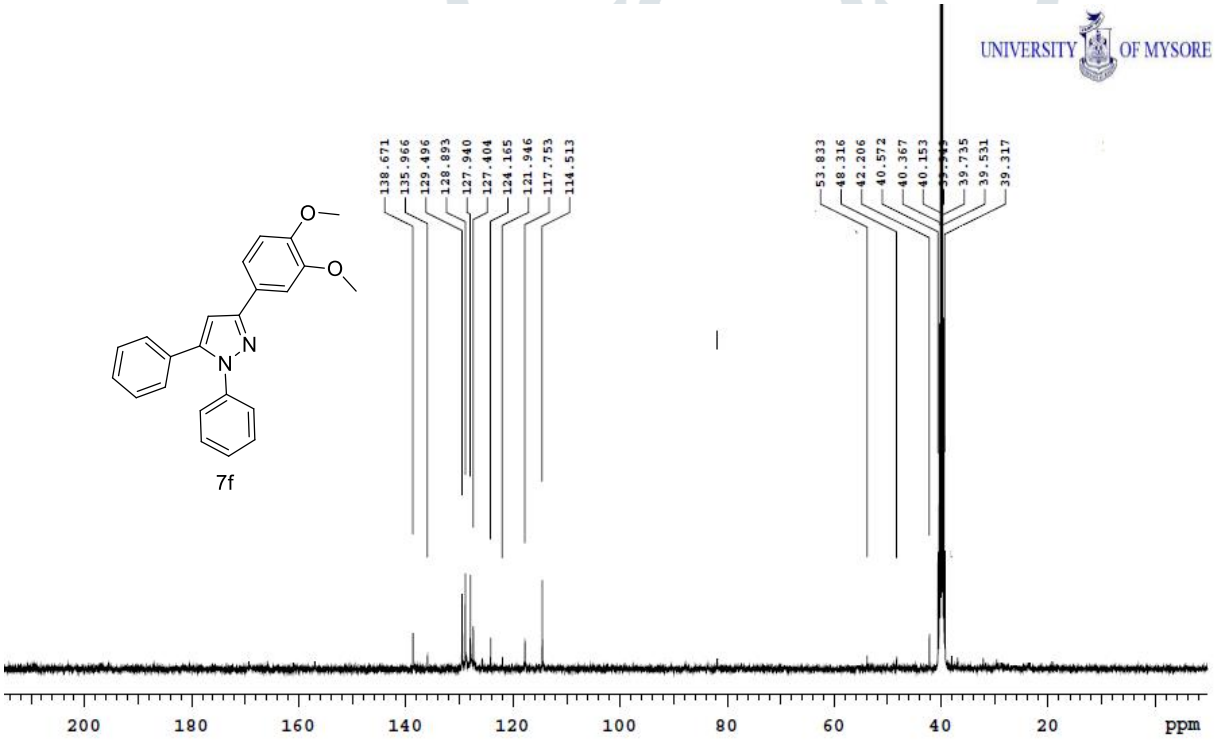
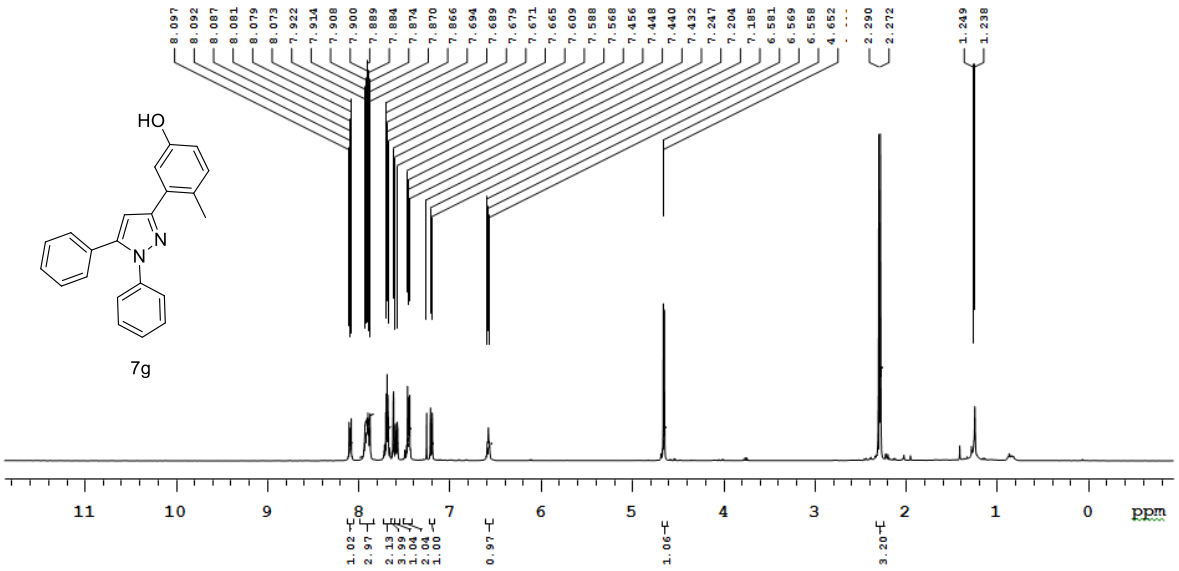
80
60
40
20
ppm

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8.087
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8.079
8.073
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7.914
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7.900
7.889
7.884
7.874
7.870
7.866
7.694
7.689
7.679
7.671
7.665
7.609
7.588
7.568
7.456
7.448
7.440
7.432
7.247
7.204
7.185
6.581
6.569
6.558
4.652
2.290
2.272
1.249
1.238
HO
N
N
7g
11
10
9
8
7
6
5
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3
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1
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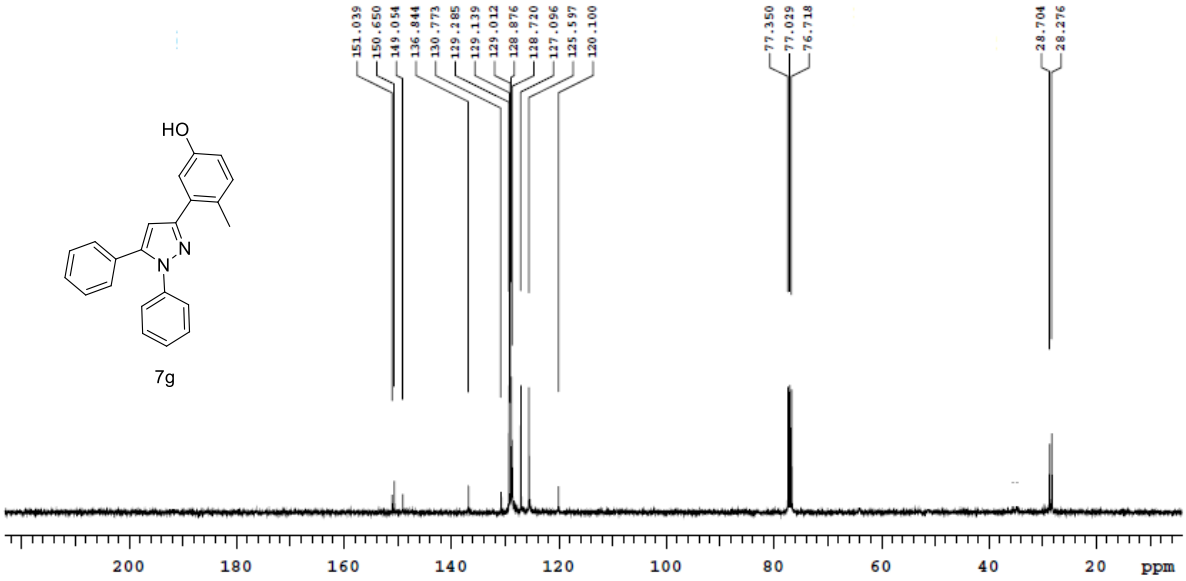
ppm
1.02
2.97
2.13
3.99
2.04
2.00
0.97
1.06
3.20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129.012
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128.720
127.056
125.597
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77.350
77.029
76.718
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28.276
HO
N
N
7g
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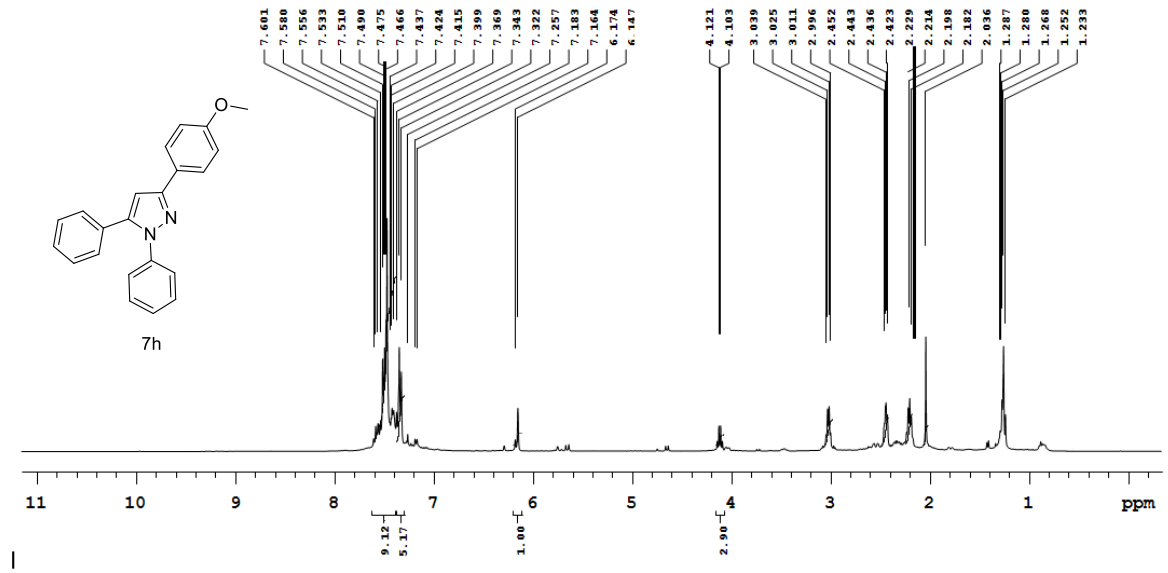
140
120
100
80
60
40
20
ppm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7.415
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7.369
7.343
7.322
7.257
7.183
7.164
6.174
4.121
4.103
3.039
3.025
3.011
2.996
2.452
2.443
2.436
2.423
2.229
2.214
2.198
2.182
2.036
1.287
1.280
1.268
1.252
1.233
O
N
N
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8
7
6
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4
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2
1
ppm
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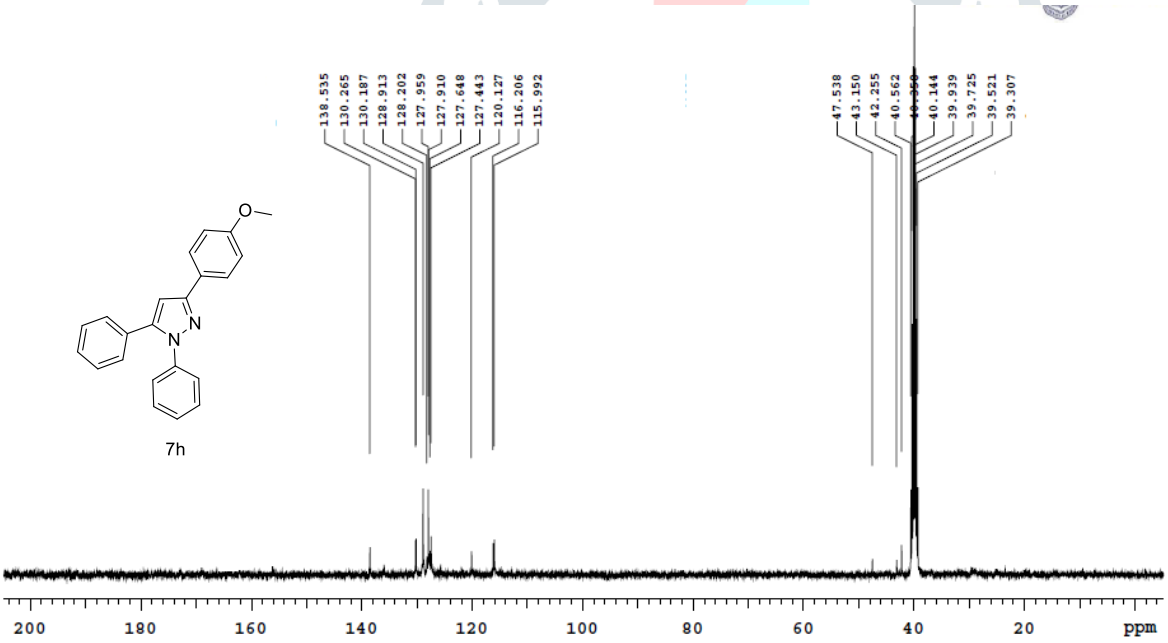
5.17
1.00
2.90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128.202
127.959
127.910
127.648
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120.127
116.206
115.992
47.538
43.150
42.255
40.562
40.350
40.144
39.939
39.725
39.521
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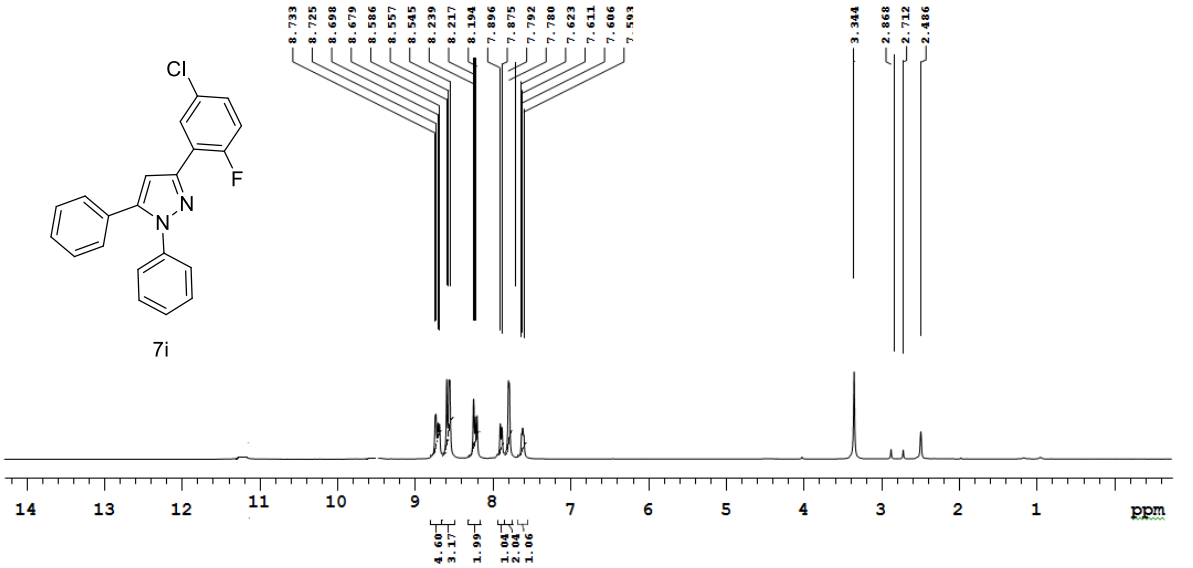
O
N
N
7h
200
180
160
140
120
100
80
60
40
20
ppm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8.725
8.698
8.679
8.586
8.557
8.545
8.239
8.217
8.194
7.896
7.875
7.792
7.780
7.623
7.611
7.606
7.593
3.344
2.868
2.712
2.486
Cl
F
N
N
7i
14
13
12
11
10
9
8
7
6
5
4
3
2
1
ppm
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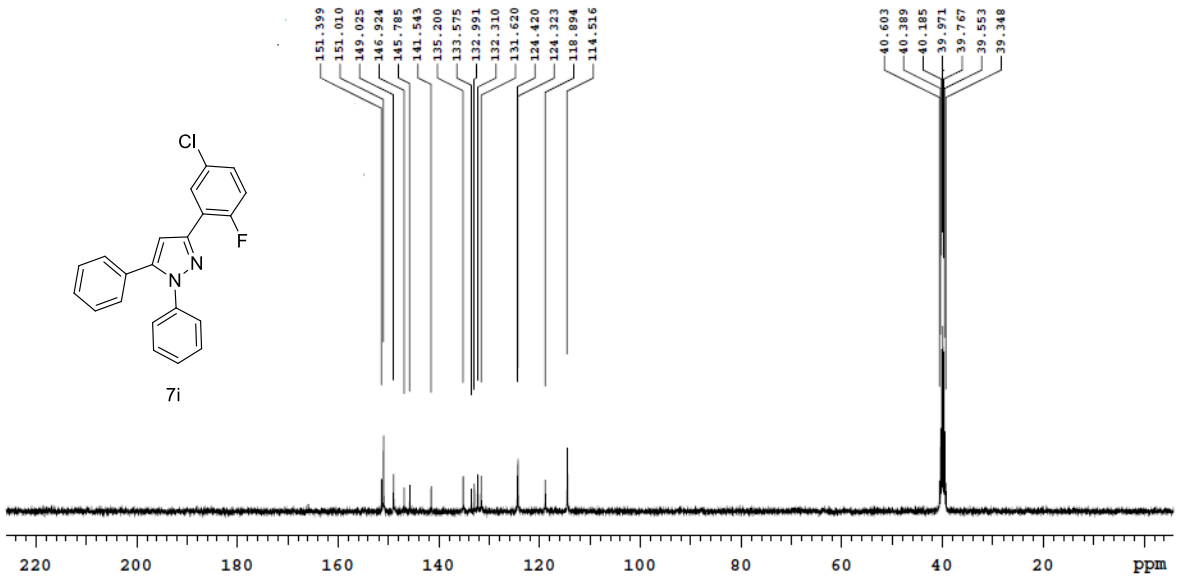
3.17
1.99
1.04
2.04
1.06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132.310
131.620
124.420
124.323
118.894
114.516
40.603
40.389
40.185
39.971
39.767
39.553
39.348
Cl
F
N
N
7i
220
200
180
160
140
120
100
80
60
40
20
ppm