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Graphical Abstract

Synthesis of 5-substituted-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one analogs and their biological evaluation as anticancer agents: mTOR inhibitors

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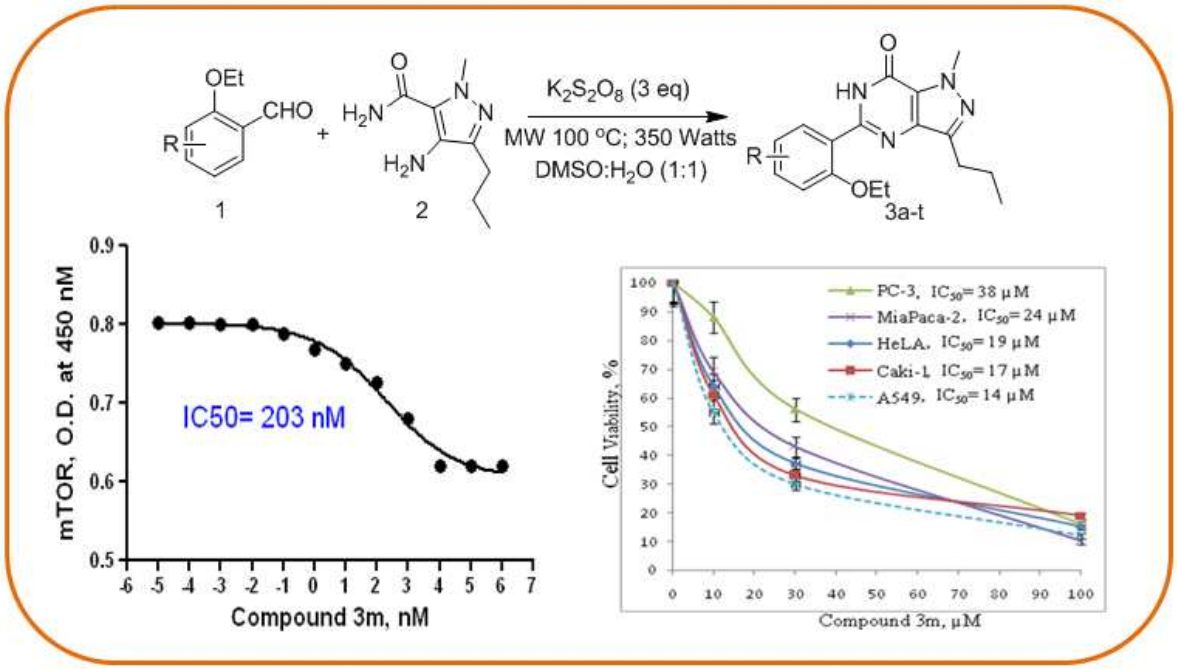

Series of 1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6H)-ones are synthesized using microwave assisted strategy. Screened against HeLa, CAKI-I, PC-3, MiaPaca-2, A549 for anticancer activity and 3m was found to be an mTOR inhibitor.

Synthesis of 5-substituted-1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one analogs and their biological evaluation as anticancer agents: mTOR inhibitors

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Abstract:

A microwave assisted strategy for synthesis of series of 1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-ones has been developed and their biological evaluation as anticancer agents is described. The synthetic protocol involves simple procedure by oxidative coupling of 4-amino-1-methyl-3-propyl-1*H*-pyrazole-5-carboxamide with different aldehydes in presence of $K_2S_2O_8$ offering 5-substituted-1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one compounds in excellent yields. The *in vitro* anticancer activity screening against human cancer cell lines HeLa, CAKI-I, PC-3, MiaPaca-2, A549 gave good results. The in detailed mechanistic correlation studies of compound **3m** revealed that the compound shows anticancer activity through apoptosis mechanism and also inhibits mTOR with nonomolar potency. The design was based on docking with mTOR protein. The concentration dependent cell cycle analysis, western blotting experiment and nuclear cell morphology studies have been described.

Keywords: Pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one, $K_2S_2O_8$ catalyst, Microwave irradiation, Cytotoxicity, mTOR inhibitor

1.0. Introduction

The pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-ones and their bioisosteres are heterocyclic compounds with important biological functions including antitumor activity and many other activities.[1] 6-cycloalkyl-pyrazolopyrimidinones are reported for CNS disorders,[2] GHS-R1a antagonists and inverse agonists for the treatment of obesity is also repoted.[3] Recently, imine-pyrazolopyrimidinones are presented as anticancer derivatives.[4] 1-aryl-4,5-dihydro-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ones were identified as inhibitors of cyclin-dependent kinase (CDK) with IC_{50} in the low micromolar range[5] and several other reports are available for various activities for this scaffold. On the basis of biological data reported in literature, pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one class of compounds are very important in the treatment of impotence, as can be evidenced by the top selling PDE5 inhibitory drug in market *i.e.*, sildenafil.[6] Few more to include in pyrazolopyrimidinone class other than sildenafil as PDE5 inhibitors are acetildenafil (hongdenafil), aildenafil (methisosildenafil), sulfoaildenafil (thioaildenafil), etc.

A pyrazolopyrimidine scaffold based molecule *i.e.* PP242, PP30 and some others[7] (Fig. 1), are reported as highly potent, selective and ATP-competitive mTORC1/mTORC2 inhibitor (IC_{50} = 8 nM for PP242 and 80 nM for PP30). PP242 has >10 folds selectivity over the other PI3K family kinases (IC_{50} 0.102 µM, 0.408 µM, 1.27 µM, 1.96 µM and 2.2µM for p110γ, DNA-PK, p110δ, p110α and p110β, respectively). PP242 is also reported to exhibits excellent selectivity over 215 other protein kinases. PP242 differentially inhibits insulin-stimulated phosphorylations of cellular proteins both *in vitro* and in *vivo* in a manner distinctly different from that seen in mTORC2-functional knockout $SIN1^{-/-}$ cells or in cultures treated with Rapamycin, which targets only mTORC1, but not mTORC2. Moreover, it is reported that PP242 can significantly enhance iPSC generation, which is experimentally confirmed by J.A Menendez *et. al.* that this mTOR inhibitor (PP242) is the most powerful longevity-promoting molecule that enhances iPSC generation,[8] and robustly decelerates the cellular senescence imposed by a DDR equivalent to senescence that is caused by pluripotency associated transcription factor expression. However, support for this hypothesis was evidenced by recent findings that well-characterized mTOR inhibitors and autophagy activators (e.g., PP242, rapamycin and resveratrol) notably improve the speed and efficiency of iPSC generation.[8]

Synthesis of pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one is well exploited and there are various methods already reported for the synthesis of pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one class of compounds using traditional as well as microwave mediated approaches.[9] The aim of this study was to synthesize and evaluate the biological potential of pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one analogs for their anticancer potential. In this direction, we initiated our efforts towards its synthesis and biological activity. The detailed chemistry and biological evaluation of these compounds as anticancer agents is explained in the present study.

2.0. Result and discussion

2.1. Chemistry:

2.1.1. Synthesis of 1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one analogs:

We intended to synthesize compounds based on 1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one scaffold by using microwave assisted protocol (Scheme 1). In this direction we started the studies for optimization of synthesis of 5-(2-ethoxyphenyl)-1-methyl-3-propyl-1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6H)-one **3a**.

The optimization studies were initiated by screening of different oxidizing agents as depicted in table 1 using DMSO:Water in 1:1 proportion to see the conversion in desired product. Amongst all oxidants, the best result was observed with $K_2S_2O_8$, in equivalence studies for catalyst, 3 eq. of catalyst has given maximum yields (Table 1, see Supplementary data). Therefore, all reactions were conducted using this condition after optimization of catalyst. However, oxone has also given the product **3a** with minor yields.

After screening of the catalyst we started study of selectivity for solvent that could affect the formation of 5-(2-ethoxyphenyl)-1-methyl-3-propyl-1*H*-pyrazolo[4,3-d]pyrimidin-7(6*H*)-one **3a**. The solvent screening was carried out to find out the best conversion, the mixture of DMSO:H_2O in 1:1 proportion has given the best results with excellent yields (Table 2, see Supplementary data).

The microwave protocols were optimized for this reaction as mentioned in the table 3; the reactions carried under different microwave Watt powers have given varied results. Wherein, entry 3(b) (Table 3, see Supplementary data) was found to be the best condition for maximum conversion.

A series of compounds based on 1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one scaffold was synthesized using these optimized conditions, wherein, all kind of substrates with diversity around aryl ring were chosen for conversion and in all cases products obtained in good to excellent yields (Table 4).

2.2. Biology:

2.2.1. *In vitro anticancer activity*

These compounds were taken up for *in vitro* cell based cytotoxicity screening against various human cancer cell lines and the results for this screening are mentioned in the Table 5.

2.2.2. *Compound **3m** inhibits significant cell growth inhibition in different panel of human cancer cell lines:*

Cytotoxicity assay was performed by using tetrazolium based calorimetric method (MTT assay) against human lung cancer cell lines A549, kidney cancer cell line Caki-1, pancreateic cancer cell line MiaPaCa-2, prostate cancer cell line PC-3 and cervical cancer cell line HeLa. Compound **3m** caused concentration dependent inhibition of cell proliferation in these cell lines in 48 h (Fig.2). Compound **3m** has IC_{50} value of approximately 14 µM in A549 cell line, 17 µM in Caki-1 cells, 24 µM in MiaPaCa-2 cells, 38 µM inPC-3 cells, in 19µM HeLa cells after 48 h.

2.2.3. *Compound **3m** altered whole cell and nuclear morphology*

Treatment of human leukaemia HL-60 cells with compound **3m** at 10, 30, 50, and 70µM concentrations caused cell wall deformation, shrinkage of cell size, nuclear condensation and formation of scattered apoptotic bodies as shown by arrows in the Fig. 3A and B, while the nuclei of untreated cells are healthy and round in shape. The number of apoptotic bodies increased with increased concentration of compound **3m** in A549 cells. This revealed that compound **3m** induce cell death through induction of apoptosis in A549 cells.

2.2.4. *Compound **3m** increases sub-G0 DNA fraction of cell cycle phase in concentration dependent manner*

A549 cells treated with compound **3m** exhibited concentration dependent increase in hypo diploid sub-G0 DNA fraction (<2nDNA) indicative of apoptotic population as analyzed by modfit software (Fig. 4). Control cells showed 1% sub-G0 DNA fraction while A549 cells treated with compound **3m** for 24 h exhibited continuous increase in sub-G0 fraction which may comprise both apoptotic and debris fraction implying together the extent of cell death.

The damage was more apparent with higher concentration of compound **3m** over the period of study. The sub-G0 fraction increased from ~1% of control to ~28% after 24 h of treatment of compound **3m**. There was hardly any significant effect after 24h of treatment on G1, S and G2/M, which indicated that decrease in DNA fluorescence is not cell cycle selective.

2.2.5. *Compound **3m** inhibits mTOR-p70S6Kinase signaling and induces apoptosis in A549 cells*

Compound **3m** at indicated concentrations inhibits phosphorylated (Serine 2448) and non phosphorylated form of mammalian target of rapamycin (mTOR), its two other subunits rictor and raptor and their two main substrate p70S6K and 4EBP1in A549 cells after 24h (Fig.5). Compound **3m** inhibits active phosphorylated form of eIF4E (Serine 209) and p-p70S6Kinase (T389) at all concentrations (Fig.5).

Compound **3m** also inhibits mTOR kinase in a cell free enzyme assay (K-LISA™ mTOR kit) and IC_{50} value was found to be 203 nM (Fig. 6). The mammalian target of rapamycin (mTOR) is a serine/threonine kinase present downstream of phosphatidylinositol 3-kinase/Akt signalling pathway and it involves in regulating basic cellular functions including cellular growth and proliferation (Wullschleger etal, 2006).[10] Aberrant activation of the PI3K/Akt/mTOR pathway is found in many types of cancer including small cell lung cancer (Marinov etal. 2009).[11] AKT can activate mTOR by phosphorylating at serine 2448. Activated mTOR have two well characterized downstream targets, ribosomal protein S6 kinase 1 (p70S6K) and eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), both of which are involve in the regulation of protein synthesis (Hay and Sonenberg, 2004).[12] Simultaneously, compound **3m** also alter the expression of key apoptotic proteins like caspas-3 activation and PARP-1 (Poly-ADP-ribose-polymerase) cleavage (Fig. 5).

2.3. Docking studies

Based on the docking studies, the molecule **3m** shows one H-bond interaction with Val2240 at a distance of 2.203 A^0 (Fig. 7). Further, this ligand also shows more stability within the binding pocket due to the hydrophobic cleft formed by Leu 2185, Trp 2239, Met 2345 and Ile 2356 around the ligand. The best dock score and the calculated binding energy of this complex were -6.8 and -80.95 respectively. Based on this evidence and the preliminary biological activity, the in detailed biological activity for **3m** was conducted.

3.0. Conclusion

In present study, 1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one scaffold have been constructed using microwave assisted protocol and the biological evaluation results as anticancer agents are promising. The synthetic protocol can be applied for preparation of analogs of active compound i.e. **3m** which involves a simple procedure to obtain 5-substituted-1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one compounds in excellent yields. Moreover, **3m** also acts as mTOR inhibitor and the concentration dependent cell cycle analysis, western blotting experiment and nuclear cell morphology studies suggests that the mechanism through which 3m acts as anticancer agent is apoptosis. 5-substituted-1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one is an excellent scaffold and can be exploited for further study in the field of cancer.

4.0. Experimental protocols

4.1.1. Chemistry

All reactions were performed in a sealed tube under mentioned microwave irradiation conditions. Analytical thin layer chromatography was performed using TLC pre-coated silica gel 60 F_{254} (20 x 20 cm). TLC plates were visualized by exposing UV light or by iodine vapors or immersion in anisaldehyde charring reagent or in 2,4-dinitrophenyl hydrazine or ninhydrin followed by heating on hot plate. Organic solvent were concentrated by rotary evaporation and dried using high vacuum suction pump. Compounds were purified by column chromatography using normal phase silica-gel (100-200 mesh size). ^{1}H NMR spectra were recorded with 400 and 500 MHz NMR instruments. Chemical data for protons are reported in parts per million (ppm, scale) downfield from tetramethylsilane and are referenced to the residual proton in the NMR solvent ($CDCl_3$: δ 7.26, DMSO-d_6 δ 2.50 or other solvents as mentioned).

4.1.1.1. Synthesis of 1-methyl-3-propyl-5-(2,4,5-trimethoxyphenyl)-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one

In a typical procedure, a solution of aromatic aldehyde i.e. 2,4,5-trimethoxybenzaldehyde (0.196 g, 1 eq.) and 4-amino-1-methyl-3-propyl-1*H*-pyrazole-5-carboxamide (0.191 g, 1.05 eq) in DMSO:H_2O (1:1) add $K_2S_2O_8$ (0.810 g, 3 eq.) was taken in a sealed reaction tube and the reaction mixture was irradiated under microwave conditions for 3 min with a power of 350 Watts at 100 ^oC. After completion, the reaction mass was diluted with EtOAc (20 mL) and added water (30 mL). Separated the organic layer and extracted with EtOAc (2x10ml). The combined

organic layer was then washed with brine solution, concentrated under vacuum and purified on silica-gel (100-200 mesh) column chromatography, affording white solid 1-methyl-3-propyl-5-(2,4,5-trimethoxyphenyl)-1*H*-pyrazolo[4,3-d]pyrimidin-7(6H)-one i.e., compound **3j** in (0.351 g) 98% yield.

*4.1.1.2. Representative analytical data for compound **3j** i.e.*, 1-methyl-3-propyl-5-(2,4,5-trimethoxyphenyl)-1*H*-pyrazolo[4,3-d]pyrimidin-7(6*H*)-one. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.99 (s, 1H), 8.03 (s, 1H), 6.59 (s, 1H), 4.27 (s, 3H), 4.05 (s, 3H), 3.97 (s, 6H), 2.93 (t, *J* = 7.5 Hz, 2H), 1.93 – 1.83 (m, 2H), 1.04 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 153.61, 152.09, 151.83, 149.06, 144.66, 142.60, 137.96, 123.88, 113.50, 112.81, 97.89, 56.57, 56.15, 55.89, 37.77, 27.07, 21.59, 13.81. HRMS (ESI) calcd for $C_{18}H_{23}N_4O_4$ [M-H$^+$] 359.17193, found 359.17139.

4.1.2. Molecular modeling studies

The computational studies on mTOR were carried out using the Schrodinger suite 2012 molecular modeling software. Crystal structure 4JT5 was taken for docking studies. The coordinates of mTOR protein in complex with the co-crystalized ligand (2-[4-amino-1-(propan-2-yl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl]-1H-indol-5-ol) that was obtained from protein data bank.[13] The protein was prepared for docking using the protein preparation wizard. Hydrogens were added to the protein and water residues were removed beyond 5A°from the heteroatom. Further, only those water residues, having interactions with the protein and heteroatom were kept, and the rest were deleted. Then the ligand was extracted and protein was refined by assigning H-bonds and minimization at OPLS 2005 force field. A grid was generated at active site, identified on the bases of already co-crystalised ligand to the receptor using receptor grid generation module. The docking protocol was standardized using the co-crytalized ligand conformation.

4.1.3. Biological Studies

a) Reagents/chemicals: RPMI-1640, streptomycin, kanamycin, penicillin, L-glutamine, phenylmethanesulfonyl fluoride (PMSF), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Propidium iodide (PI), Fetal bovine serum, pyruvic acid were purchased from Sigma-Aldrich (Bangalore, India). Anti-human antibodies to PARP-1, caspase-3 and β-actin were purchased from SantaCruz Biotechnology (SantaCruz, CA). Anti-human antibodies to

mTOR and its phosphorylated form (S2448), p-eIF4E (S209), Raptor, Rictor and p-p70S6Kinase (T389) were purchased from Cell signaling technology (Danvers, MA). K-LISA™ mTOR kit (#CBA055) was purchased from Calbiochem, USA. Electrophoresis reagents, Protein estimation kit and protein marker were from Bio-Rad Laboratories (Hercules, CA).

b) Cell culture and treatment: Human lung cancer cell lines A549, kidney cancer cell line Caki-1, pancreatic cancer cell line MIA-PaCa-2, prostate cancer cell line PC3 and cervical cancer cell line HeLa were purchased from ECACC. Cells were grown in RPMI/DMEM/MEM growth medium containing 10% FCS, 100U penicillin and 100 mg/ml streptomycin. Cells were grown in CO_2 incubator (Thermocon Electron Corporation, Houston, TX) at 37 ^oC temperature, 95% humidity and 5% CO_2 gas environment. Cells treated with compounds were dissolved in DMSO while the untreated control cultures received only the vehicle (DMSO<0.2%).

4.1.3.1. Cell proliferation assay

Cells were seeded in 96 well flat bottom plates. Next day when they attained 60-70% confluency, they were treated with compounds at different concentrations for 24h and 48h. MTT dye (2.5 mg/ml in PBS) was added 4 hours priors to experiment termination. The supernatant was discarded and the MTT formazan crystals were dissolved in 150 µL of DMSO. The OD measured at 570 nm with reference wavelength of 620 nm (Bhushan et al 2006).[14] The most active compound **3m** was taken up for in detailed biological evaluation.

4.1.3.2. Hoechst Staining

Human lung cancer A549 cells were treated with compound **3m** for 24 h at 10, 30, 50 and 70 µM concentrations. After treatment cells were collected, washed twice with PBS and fixed in 400 µl of fixing solution composed of cold acetic acid: methanol (1+3, v/v) overnight at 4 °C. Cells were washed with fixing solution and dispensed in 50 µl of fixing solution. Spread cells on a clean cold slide and dried overnight at room temperature. Cells were stained with Hoechst 33258 (5 µg/ml in 0.01 M citric acid and 0.45 M disodium phosphate containing 0.05% Tween 20) for 20-30 min at room temperature. After that slides were washed with distilled water followed by in PBS. While wet, pour 40 µl of mounting fluid (PBS: glycerol, 1/1) over the slide and covered with glass cover slip and sealed with nail polished. Cells were observed under microscope for any nuclear morphological changes occur in apoptosis (Saxena et al. 2010).[15]

4.1.3.3. Cell cycle analysis

Human lung cancer cell line A549 was seeded in 60mm^2 dishes and after attaining 60-70% confluency, treated with compound **3m** for 24 hours at 10, 30, 50 and 70 µM concentrations. Cells were washed with PBS twice after 24h and fixed overnight in 70% alcohol. Next day cells were washed twice with PBS and thereafter they are subjected to RNase digestion (200 µg/mL) at 37^{0}C for 1.30 h and then incubated with PI (10 µg/mL) for 30 min. Cells were analyzed immediately on flow cytometer (BD Biosciences, San Jose, CA). The data were collected in list mode on 10,000 events and illustrated in a histogram, where the number of cells (counts) is plotted against the relative fluorescence intensity of PI (FL-2; λem: 585 nm; red fluorescence). Resulting DNA distributions were analyzed by Modfit (Verity Software House Inc., Topsham, ME) for the proportions of cells in G_0-G_1, S- phase, and G_2-M phases of the cell cycle (Chanda et al 2012).[16]

4.1.3.4. Cell lysates preparation for western blots analysis

Human lung cancer cell line A549 was treated with compound **3m** for 24h at 10, 30, 50 and 70 µM concentrations. Cells were collected by centrifugation at 400×*g* at 4^{0}C, washed with PBS twice and processed for preparation of whole cell lysates. Cells were lysed with cold Cell lysis buffer (RIPA, Sigma with 50 mM NaF, 0.5 mM $NaVO_4$, 2 mM PMSF and 1% protease inhibitor cocktail) for 40 min. Cells were centrifuged at 12000× g for 15 min at 4^{0}C and the supernatant was collected as whole cell lysates for western blot analysis (Bhushan et al 2007).[17]

4.1.3.5. Western blot analysis

Protein was measured using Bio-Rad protein assay kit and protein lysates (30-70 µg) were subjected to SDS-PAGE analysis. They are transferred to PVDF membrane for 2 hours at 4^{0}C at 100V using Bio-Rad electrode assembly. The membrane was blocked by incubation with 3% BSA or 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature to prevent any non-specific binding. After an hour the blots were incubated with respective primary antibodies for 3-4 h at room temperature. They are washed three times with TBST and were incubated with horseradish peroxidase conjugated secondary antibodies for another 1 h. At the end, membranes were washed three times with TBST buffer with 15 min interval and signals detected using ECL plus chemiluminescence's kit on X-ray film (Bhushan et al 2007).[17]

4.1.3.6. mTOR kinase essay

mTOR inhibition of compound **3m** was found out by using K-LISA™ mTOR kit from Calbiochem (#CBA055). It is an ELISA-based assay that utilizes a p70S6K-GST fusion protein as a specific mTOR substrate. The assay was carried out according to the manufacturer's protocol. Briefly, 100 µl of recombinant p70S6K-GST fusion protein was pre-incubated at room temperature in the glutathione coated 96-well plate for 1h after that a mixture of 49µl of ice-chilled mTOR kinase and 1µl of test compounds or DMSO was added. The reaction was initiated by the addition of 50 µl of mTOR kinase assay buffer containing 100 µM ATP and 1µM DTT. The plate was treated first with 100 µl of anti-p70S6K-T389 for 1h and then with 100 µl of HRP-conjugated antibody for 1h to detect the T389-phosphorylated p70S6K. Absorbance was measured at 450 nm and 595 nm using microplate spectrophotometer. The IC_{50} values were calculated by analysis non linear regression with variable slope by using GraphPad Prism-5 software.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://

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Figure captions

Figure 1: Pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one scaffold based potential candidates and drugs

Figure 2: Compound 3m inhibits cell proliferation in different panel of human cancer cell lines. Cells were grown in 96-well culture plate and when 60-70% confluent was treated with 1, 10, 30, 50, 70 and 100 µM concentration for 48h. Cells were incubated with MTT and OD measured as described in Materials and Methods. Data are Mean ± SD (n= 8 wells), and representative of three similar experiments

Figure 3: Effect of compound 3m on the cell wall and nuclear morphology of A549 cells. A) A549 cells were treated with 10, 30, 50 and 70µM concentrations of compound 3m for 24h and visualized under phase contrast inverted microscope (Olympus 1X 70, 30X). B) Subsequently cells were stained with Hoechst 33258 and visualized for nuclear morphology and apoptotic bodies' formation. Compound 3m induced the formation of apoptotic bodies as indicated by arrows in concentration dependent manner.
Data are representative of one of three similar experiments

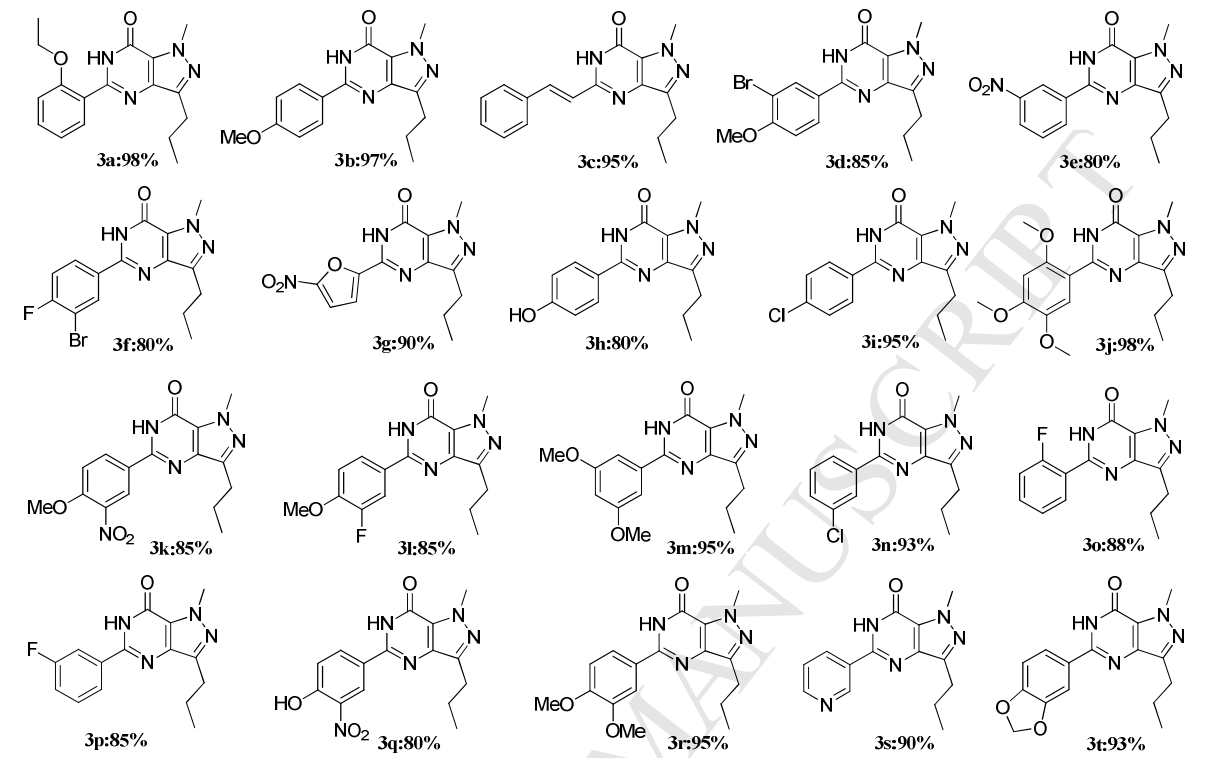
Figure 4: Cell cycle analyses of lung cancer A549 cells through PI staining. Cells were seeded in six well plate and after 60-70% confluence, treated with compounds 3m for 24h time period at indicated concentrations. After treatment cells were stained with PI (10µg/ml) to determine DNA Fluorescence and cell cycle phase distribution as described in Materials and methods. Data were analyzed by Modfit software (Verity Software House Inc., Topsham, ME) for the proportions of different cell cycle phases. Fraction of cells from apoptotic, G1, S and G2 phases analyzed from FL2- A vs. cell counts is shown in %. Data are representative of one of three similar experiments.

Figure 5: Inhibition of mTOR signalling cascade in lung cancer A549 cells by compounds 3m. A549 cells treated with compound 3m at 10, 30, 50 and 70 µM concentrations for 24 h. Total cell lysates were prepared and protein samples (40-80 µg) were loaded on SDS-PAGE gel for western blot analysis, β-actin was used as internal control to represent the same amount of proteins applied for SDS-PAGE. Specific antibodies were used for detection of mTOR, its activated phosphorylated form at serine 2448, Raptor, Rictor along with downstream substrates of mTOR, eIF4E and p70S6kinase. Compound 3m also induces apoptotic caspase-3 level and PARP cleavage. Immunoblots were representative of one of three similar experiments.

Figure 6: Compound 3m inhibits mTOR kinase in a cell free enzyme essay (K-LISA™ mTOR kit) showing IC_{50} value as 203 nM.

Figure 7: A cartoon showing the interactions of compound 3m with active site of mTOR using PDB4JT5 (The carbonyl group of molecule showing H-bonding with Val2240 (2.203 A^0))

Table 4: Synthesis of compounds based on 1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one scaffold

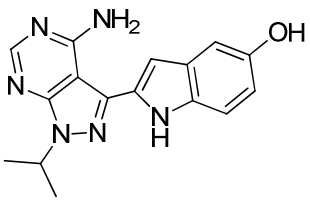


[a]All yields are isolated yields after column purification

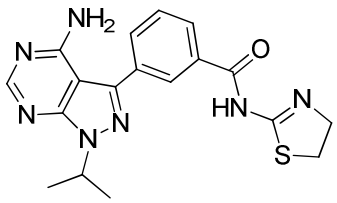
Table 5: *In vitro* cell based screening of 1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one compounds

Code	Cell lines														
	HeLa (Cervix cancer)			CAKI-I (Renal cancer)			PC-3 (Prostate Cancer)			MiaPaca-2 (Pancreatic Cancer)			A549 (Lung cancer)		
	Conc, (µM)	% GI	IC_{50} (µM)	Conc, (µM)	% GI	IC_{50} (µM)	Conc, (µM)	% GI	IC_{50} (µM)	Conc, (µM)	% GI	IC_{50} (µM)	Conc, (µM)	% GI	IC_{50} (µM)
3a	10	36	>100	10	12	>100	10	7	>100	10	7	>100	10	21	>100
	30	37		30	18		30	10		30	25		30	30	
	100	40		100	19		100	14		100	38		100	37	
3b	10	31	>100	10	8	>100	10	5	>100	10	18	>100	10	0	>100
	30	34		30	25		30	6		30	20		30	15	
	100	42		100	52		100	14		100	32		100	36	
3c	10	22	92	10	26	>100	10	33	32	10	11	>100	10	30	38
	30	43		30	33		30	48		30	20		30	45	
	100	59		100	46		100	98		100	44		100	80	
3d	10	0	>100	10	24	57	10	14	>100	10	11	>100	10	0	90
	30	21		30	38		30	16		30	15		30	17	
	100	36		100	74		100	19		100	22		100	58	
3e	10	21	>100	10	31	30	10	22	>100	10	14	>100	10	12	>100
	30	22		30	50		30	27		30	17		30	25	
	100	39		100	73		100	47		100	45		100	40	

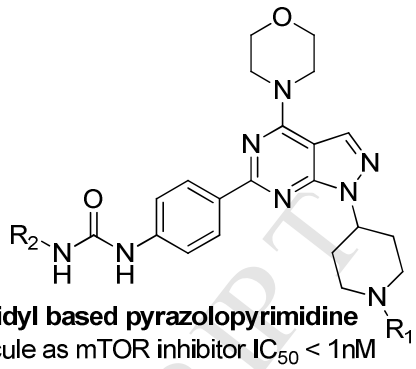
3f	10	34	24	10	30	18	10	32	27	10	34	38	10	20	28
	30	69		30	68		30	62		30	45		30	57	
	100	81		100	78		100	74		100	98		100	98	
3g	10	12	>100	10	27	>100	10	10	>100	10	6	>100	10	0	>100
	30	17		30	32		30	23		30	16		30	8	
	100	48		100	41		100	39		100	32		100	43	
3h	10	29	34	10	22	>100	10	19	>100	10	23	>100	10	11	90
	30	44		30	25		30	24		30	29		30	28	
	100	78		100	35		100	32		100	43		100	59	
3i	10	12	>100	10	34	28	10	22	>100	10	11	>100	10	19	87
	30	19		30	56		30	31		30	22		30	39	
	100	14		100	66		100	47		100	43		100	58	
3j	10	30	>100	10	16	>100	10	5	>100	10	12	>100	10	0	>100
	30	33		30	16		30	7		30	21		30	7	
	100	35		100	17		100	10		100	32		7	21	
3k	10	32	>100	10	25	>100	10	0	>100	10	3	>100	10	11	>100
	30	35		30	31		30	6		30	24		30	31	
	100	43		100	35		100	13		100	44		100	47	
3l	10	49	13	10	35	20	10	33	31	10	38	35	10	26	28
	30	66		30	65		30	78		30	57		30	57	
	100	87		100	87		100	97		100	87		100	90	
3m	10	36	19	10	39	17	10	12	37	10	31	24	10	45	14
	30	63		30	67		30	44		30	57		30	68	
	100	85		100	81		100	84		100	90		100	98	
3n	10	22	>100	10	35	29	10	16	>100	10	12	>100	10	0	87
	30	31		30	51		30	17		30	16		30	27	
	100	49		100	76		100	25		100	23		100	65	
3o	10	11	35	10	24	>100	10	17	>100	10	11	>100	10	0	>100
	30	43		30	26		30	23		30	20		30	8	
	100	65		100	49		100	43		100	43		100	23	
3p	10	1	>100	10	34	96	10	23	95	10	15	>100	10	22	86
	30	18		30	47		30	44		30	21		30	37	
	100	33		100	54		100	56		100	44		100	68	
3q	10	0	>100	10	23	>100	10	21	>100	10	17	>100	10	0	>100
	30	7		30	35		30	25		30	22		30	23	
	100	12		100	44		100	29		100	48		100	46	
3r	10	13	>100	10	21	>100	10	11	>100	10	21	96	10	9	>100
	30	21		30	28		30	14		30	32		30	14	
	100	44		100	36		100	27		100	55		100	48	
3s	10	0	>100	10	21	>100	10	1	>100	10	21	>100	10	0	>100
	30	23		30	22		30	3		30	32		30	10	
	100	38		100	31		100	14		100	45		100	29	
3t	10	32	88	10	46	27	10	23	98	10	11	>100	10	10	54
	30	44		30	55		30	31		30	13		30	37	
	100	61		100	61		100	51		100	34		100	78	



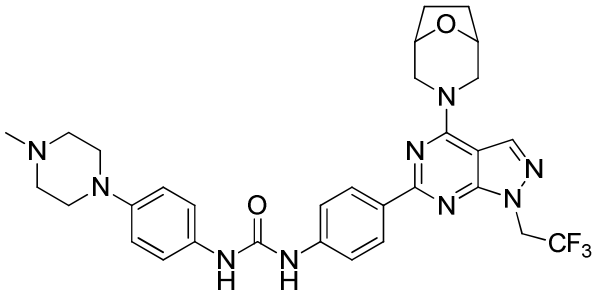
PP242 mTOR IC_{50} 8nM



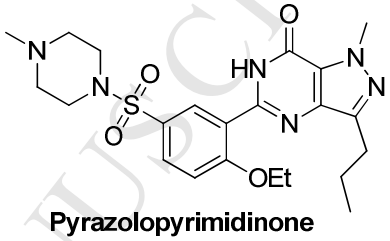
PP30 mTOR IC_{50} 80 nM



Ureidyl based pyrazolopyrimidine molecule as mTOR inhibitor IC_{50} < 1nM
Selectivity Vs. PI3K-α > 1000X



6-arylureidophenyl -pyrazolopyrimidine:
a mTOR inhibitor with enhanced cellular potency and improved stability towards human microsomes



Pyrazolopyrimidinone
scaffold based Sildenafi: PDE5 inhibitor

Figure 1

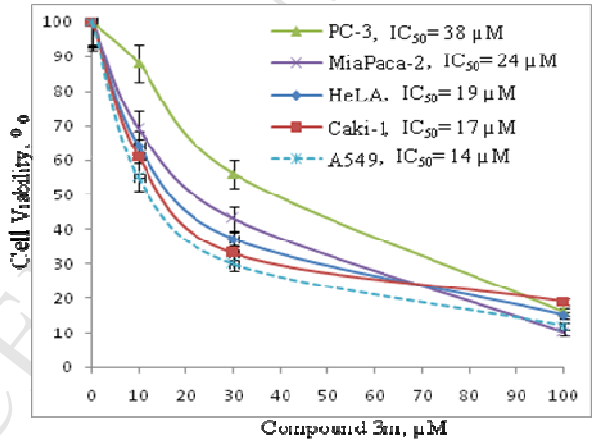


Figure 2

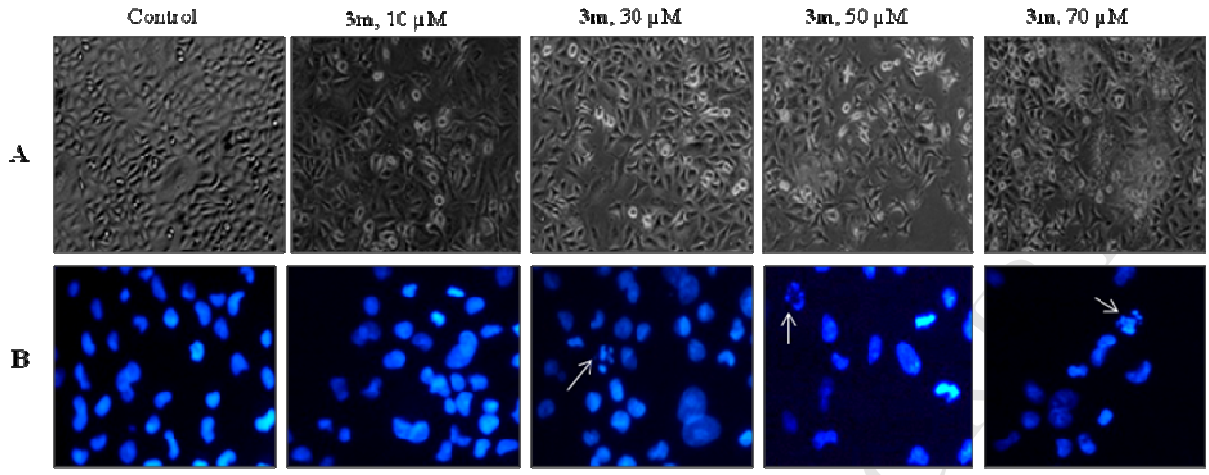
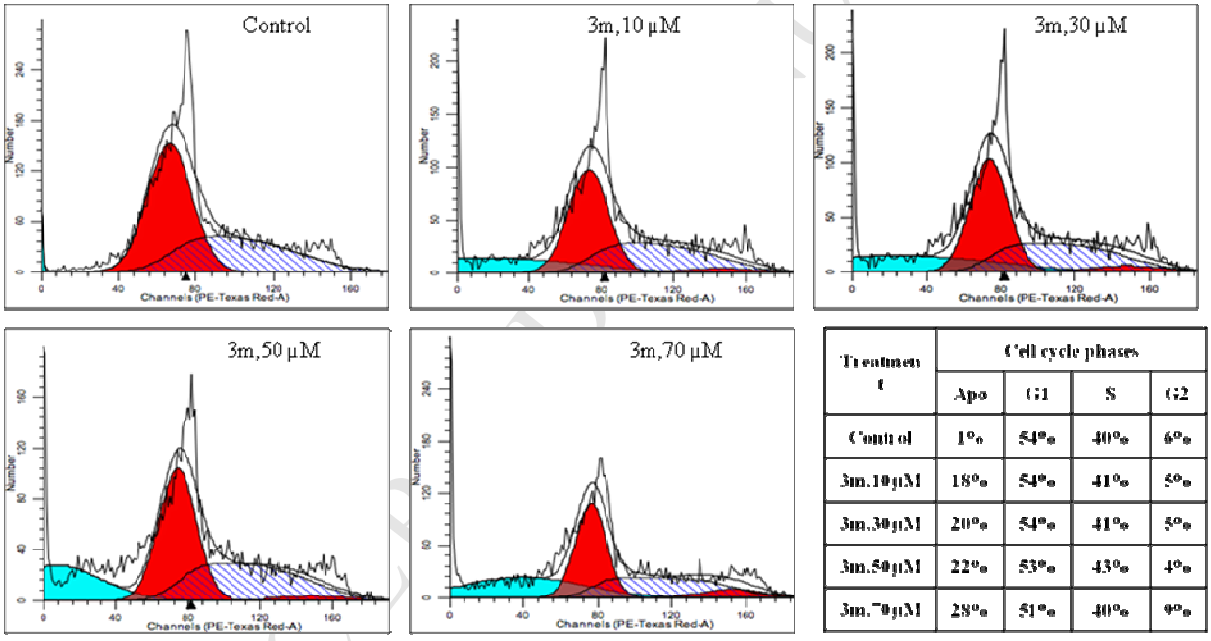


Figure 3



Treatment	Cell cycle phases			
	Apo	G1	S	G2
Control	1%	54%	40%	6%
3m,10µM	18%	54%	41%	5%
3m,30µM	20%	54%	41%	5%
3m,50µM	22%	53%	43%	4%
3m,70µM	28%	51%	40%	9%

Figure 4

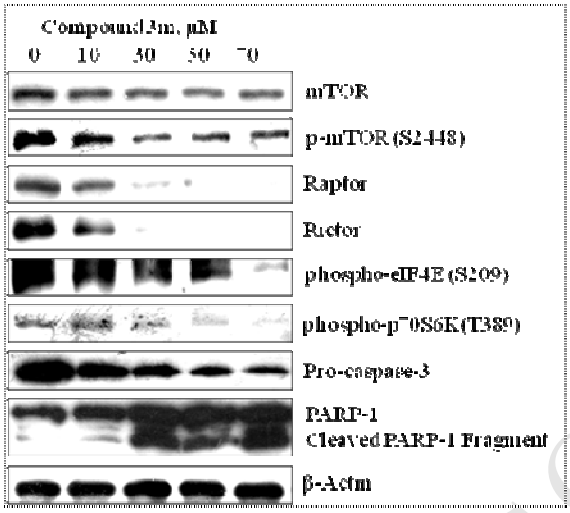


Figure 5

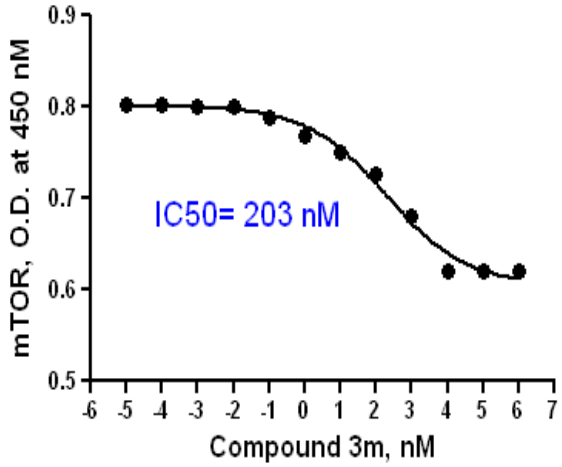


Figure 6

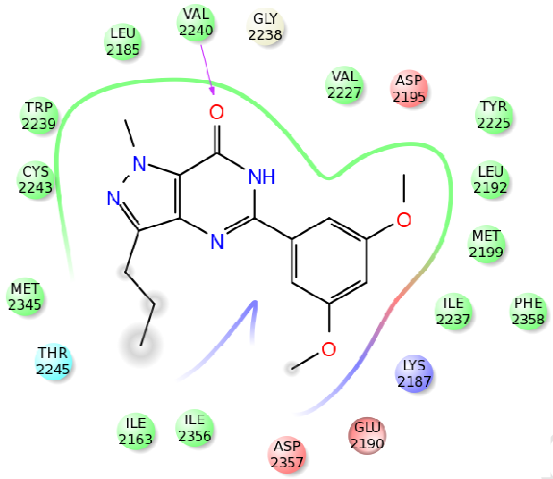
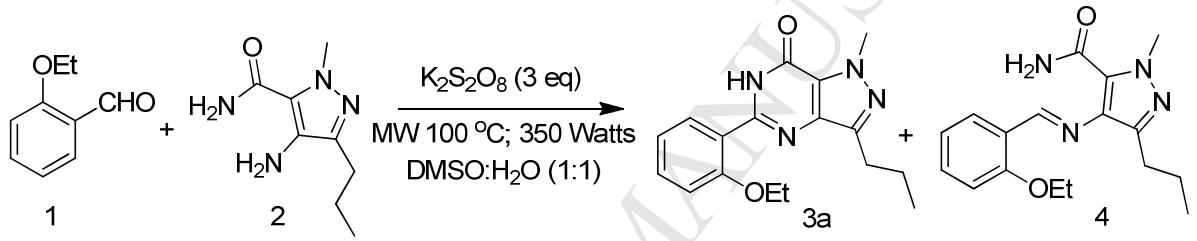


Figure 7



Scheme 1: Microwave assisted synthesis of 5-(2-ethoxyphenyl)-1-methyl-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one

Highlights

Synthesis of 5-substituted-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one analogs and their biological evaluation as anticancer agents: mTOR inhibitors

G. Lakshma Reddy, Santosh Kumar Guru, M. Srinivas, Anup Singh Pathania, Priya Mahajan, Amit Nargotra, Shashi Bhushan,* Ram A. Vishwakarma,* Sanghapal D. Sawant*

Highlights

- Microwave assisted synthesis of 1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one scaffold
- *In vitro* anticancer activity: HeLa, CAKI-I, PC-3, MiaPaca-2, A549 cancer cell lines
- Compound **3m:** cell cycle analysis, western blotting, nuclear cell morphology
- mTOR inhibitory potential of compound **3m** with nonomolar potency: IC_{50}= 203 nM

Appendix

Supplementary data

Synthesis of 5-substituted-1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one analogs and their biological evaluation as anticancer agents: mTOR inhibitors

G. Lakshma Reddy,[a,d] Santosh Kumar Guru,[b] M. Srinivas,[a,d] Anup Singh Pathania,[b,d] Priya Mahajan,[c,d] Amit Nargotra,[c,d] Shashi Bhushan,[b,d]* Ram A. Vishwakarma,[a,d,]* Sanghapal D. Sawant,[a,d,]*

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Table of Contents

1.1. Table 1: Screening of catalyst for oxidative cyclization and formation of 3a

S.No	Solvent	Oxidizing agent	Yield(%)	
			3a	4
1	DMSO/H_2O(1:1)	H_2O_2	0	75
2	DMSO/H_2O(1:1)	TBHP	0	70
3	DMSO/H_2O(1:1)	*m*-CPBA	0	75
4	DMSO/H_2O(1:1)	Oxone	15	15
5	**DMSO/H_2O(1:1)**	**$K_2S_2O_8$**	**98**	**0**

1.2. Table 2: Solvent screening for the formation of 3a

Sr. No	Solvent	Oxidant	Yield(%)[a]	
			3a	4
1	EtOH	$K_2S_2O_8$	0	95
2	ACN		0	90
3	THF		0	85
4	PhCl		0	50
5	H_2O		20	45
6	EtOH/H_2O(1:1)		20	75
7	ACN/H_2O(1:1)		85	10
8	THF/H_2O(1:1)		65	30
9	DMF		85	10
10	DMSO		90	5
11	DMF/H_2O(1:1)		90	5
12	**DMSO/H_2O(1:1)**		**98**	**0**

[a]All yields are isolated yields after column purification

1.3. Table 3: Optimization of microwave protocols for the synthesis of 3a

Entry	Microwave Power (Watts)	Temp (°C)	Time (Min)	Yield(%)[a] 3a	4
1	100	(a) 80	3	20	75
			5	25	70
		(b) 100	3	35	60
			5	40	55
2	250	(a) 80	3	40	50
			5	45	50
		(b) 100	3	55	40
			5	65	30
3	350	(a) 80	3	80	15
			5	85	10
		(b) 100	**3**	**98**	**0**

[a]All yields are isolated yields after column purification

1.4. Analytical data

3a) 5-(2-ethoxyphenyl)-1-methyl-3-propyl-1*H*-pyrazolo [4, 3-d] pyrimidin-7(6*H*)-one[1]: ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.14 (s, 1H), 8.48 (d, *J* = 8.0 Hz, 1H), 7.46 (m 1H), 7.15 (m 1H), 7.04 (d, *J* = 8.4Hz, 1H), 4.35 – 4.23 (m, 5H), 2.95 (t, *J* = 7.6 Hz, 2H), 1.95 – 1.83 (m, 2H), 1.61 (t, *J* = 7.2 Hz, 3H), 1.05 (t, *J* = 7.2Hz, 3H). HRMS (ESI) calcd for $C_{17}H_{21}N_4O_2$ ([M-H$^+$] 313.16645, found 313.16580.

3b) 5-(4-methoxyphenyl)-1-methyl-3-propyl-1*H*-pyrazolo [4, 3-d] pyrimidin-7(6*H*)-one[1,3]: ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.71 (s, 1H), 8.06 (d, *J* = 8.8Hz, 2H), 7.02 (d, *J* = 8.8 Hz, 2H), 4.28 (s, 3H), 3.89 (s, 3H), 2.93 (t, *J* = 7.6 Hz, 2H), 1.93 – 1.82 (m, 2H), 1.03 (t, *J* = 7.4 Hz, 3H). HRMS (ESI) calcd for $C_{16}H_{19}N_4O_2$ ([M-H$^+$] 299.15080, found 299.15035

3c) (E)-1-methyl-3-propyl-5-styryl-1*H*-pyrazolo [4, 3-d] pyrimidin-7(6*H*)-one: ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.50 (s, 1H), 7.75 (d, *J* = 16.4Hz, 1H), 7.59 (d, *J* = 8.0 Hz, 2H), 7.46 – 7.34 (m, 3H), 6.97 (d, *J* = 16.4 Hz, 1H), 4.29 (s, 3H), 2.92 (t, *J* = 7.6 Hz, 2H), 1.92 – 1.80 (m, 2H), 1.04 (t, *J* = 7.2 Hz, 3H).^{13}C NMR (126 MHz, DMSO) δ 154.15, 149.09, 144.71, 137.97, 136.22,

135.16, 129.31, 128.95, 127.31, 124.28, 120.99, 37.77, 27.09, 21.65, 13.85. HRMS (ESI) calcd for $C_{17}H_{19}N_4O$ [M-H$^+$] 295.15589, found 295.15622.

3d) 5-(3-bromo-4-methoxyphenyl)-1-methyl-3-propyl-1*H*-pyrazolo [4, 3-d] pyrimidin-7(6*H*)-one: ^{1}H NMR (400 MHz, DMSO) δ 11.80 (s, 1H), 8.26 (d, *J* = 1.8 Hz, 1H), 8.05 (dd, *J* = 8.8, 1.7 Hz, 1H), 7.18 (d, *J* = 8.8 Hz, 1H), 4.08 (s, 3H), 3.86 (s, 3H), 2.73 (t, *J* = 7.6 Hz, 2H), 1.77 – 1.62 (m, 2H), 0.89 (t, *J* = 7.2 Hz, 3H). HRMS (ESI) calcd for $C_{16}H_{18}BrN_4O_2$ [M-H$^+$] 377.06131, found 377.05963.

3e) 1-methyl-5-(3-nitrophenyl)-3-propyl-1*H*-pyrazolo [4, 3-d] pyrimidin-7(6*H*)-one[1]: ^{1}H NMR (400 MHz, DMSO) δ 8.90 (s, 1H), 8.50 (d, *J* = 8.0 Hz, 1H), 8.38 (dd, *J* = 8.0, 1.4 Hz, 1H), 7.81 (t, *J* = 8.0 Hz, 1H), 4.17 (s, 3H), 2.82 (t, *J* = 7.4 Hz, 2H), 1.85 – 1.71 (m, 2H), 0.96 (t, *J* = 7.4 Hz, 3H). HRMS (ESI) calcd for $C_{15}H_{16}N_5O_3$ [M-H$^+$] 314.12531, found 314.12430.

3f) 5-(3-bromo-4-fluorophenyl)-1-methyl-3-propyl-1*H*-pyrazolo [4, 3-d] pyrimidin-7(6*H*)-one: ^{1}H NMR (400 MHz, DMSO) δ 12.51 (s, 1H), 8.41 (d, *J* = 5.2 Hz, 1H), 8.13 (s, 1H), 7.54 (m 1H), 4.16 (s, 3H), 2.81 (t, *J* = 7.5 Hz, 2H), 1.84 – 1.69 (m, 2H), 0.96 (t, *J* = 7.4 Hz, 3H). HRMS (ESI) calcd for $C_{15}H_{15}BrFN_4O$ [M-H$^+$] 365.04133, found 365.04088.

3g) 1-methyl-5-(5-nitrofuran-2-yl)-3-propyl-1*H*-pyrazolo [4, 3-d] pyrimidin-7(6*H*)-one: ^{1}H NMR (400 MHz, DMSO) δ 8.32 (s, 1H), 7.88 (s, 1H), 4.16 (s, 3H), 2.82 (t, *J* = 7.4 Hz, 2H), 1.84 – 1.68 (m, 2H), 0.96 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 153.75, 152.07, 147.43, 145.67, 139.96, 136.88, 124.72, 115.02, 114.06, 37.92, 27.02, 21.52, 13.79. HRMS (ESI) calcd for $C_{13}H_{14}N_5O_4$ [M-H$^+$] 304.10458, found 304.10409.

3h) 5-(4-hydroxyphenyl)-1-methyl-3-propyl-1*H*-pyrazolo [4, 3-d] pyrimidin-7(6*H*)-one[1]: ^{1}H NMR (400 MHz, DMSO) δ 12.16 (s, 1H), 10.04 (s, 1H), 7.95 (d, *J* = 8.7 Hz, 2H), 6.87 (d, *J* = 8.7 Hz, 2H), 4.14 (s, 3H), 2.79 (t, *J* = 7.5 Hz, 2H), 1.88 – 1.62 (m, 2H), 0.95 (t, *J* = 7.4 Hz, 3H). HRMS (ESI) calcd for $C_{15}H_{17}N_4O_2$ [M-H$^+$] 285.13515, found 285.13382.

3i) 5-(4-chlorophenyl)-1-methyl-3-propyl-1*H*-pyrazolo [4, 3-d] pyrimidin-7(6*H*)-one[2, 3]: ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.94 (s, 1H), 8.08 (d, *J* = 8.4 Hz, 2H), 7.50 (d, *J* = 8.4 Hz, 2H), 4.30 (s, 3H), 2.93 ((t, *J* = 7.6 Hz, 3H), 1.92 – 1.81 (m, 2H), 1.03 (t, *J* = 7.4 Hz, 3H). HRMS (ESI) calcd for $C_{15}H_{16}ClN_4O$ [M-H$^+$] 303.10126, found 303.0991.

3j) 1-methyl-3-propyl-5-(2,4,5-trimethoxyphenyl)-1*H*-pyrazolo[4,3-d]pyrimidin-7(6*H*)-one: ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.99 (s, 1H), 8.03 (s, 1H), 6.59 (s, 1H), 4.27 (s, 3H), 4.05 (s,

3H), 3.97 (s, 6H), 2.93 (t, *J* = 7.5 Hz, 2H), 1.93 – 1.83 (m, 2H), 1.04 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 153.61, 152.09, 151.83, 149.06, 144.66, 142.60, 137.96, 123.88, 113.50, 112.81, 97.89, 56.57, 56.15, 55.89, 37.77, 27.07, 21.59, 13.81. HRMS (ESI) calcd for $C_{18}H_{23}N_4O_4$ [M-H$^+$] 359.17193, found 359.17139.

3k) 5-(4-methoxy-3-nitrophenyl)-1-methyl-3-propyl-1*H*-pyrazolo [4, 3-d] pyrimidin-7(6*H*)-one: ^{1}H NMR (400 MHz, DMSO) δ 12.54 (s, 1H), 8.62 (d, *J* = 2.3 Hz, 1H), 8.38 (dd, *J* = 8.9, 2.3 Hz, 1H), 7.52 (d, *J* = 9.0 Hz, 1H), 4.16 (s, 3H), 4.01 (s, 3H), 2.81 (t, *J* = 7.5 Hz, 2H), 1.83 – 1.71 (m, 2H), 0.96 (t, *J* = 7.4 Hz, 3H). HRMS (ESI) calcd for $C_{16}H_{18}N_5O_4$ [M-H$^+$] 344.13588, found 344.13441.

3l) 5-(3-fluoro-4-methoxyphenyl)-1-methyl-3-propyl-1*H*-pyrazolo [4, 3-d] pyrimidin-7(6*H*)-one: ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.36 (s, 1H), 8.07 (dd, *J* = 10.8 ,1.8Hz, 1H), 7.92 (d, *J* = 8.4 Hz, 1H), 7.07 (t, *J* = 8.4 Hz, 1H), 4.31 (s, 3H), 3.98 (s, 3H), 2.93 (t, *J* = 7.6 Hz, 2H), 1.96 – 1.76 (m, 2H), 1.04 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 154.55, 152.26, 149.25, 148.61, 144.85, 137.76, 125.44, 124.26, 114.90, 114.70, 113.57, 56.20, 37.80, 27.09, 21.61, 13.83; HRMS (ESI) calcd for $C_{16}H_{18}FN_4O_2$ [M-H$^+$] 317.14138, found 317.14025.

3m) 5-(3,5-dimethoxyphenyl)-1-methyl-3-propyl-1*H*-pyrazolo[4,3-d]pyrimidin-7(6*H*)-one: ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.92 (s, 1H), 7.25 (d, *J* = 2.0 Hz, 2H), 6.61 (t, *J* = 2.0 Hz, 1H), 4.30 (s, 3H), 3.89 (s, 6H), 2.98 – 2.89 (m, 2H), 1.93 – 1.80 (m, 2H), 1.03 (t, *J* = 7.4 Hz, 3H). ^{13}C (126 MHz, $CDCl_3$) δ 161.27, 155.58, 149.29, 146.87, 139.14, 134.88, 124.55, 105.53, 102.65, 55.60, 38.18, 27.73, 22.35, 14.05 HRMS (ESI) calcd for $C_{17}H_{21}N_4O_3$ [M-H$^+$] 329.16137, found 329.16031.

3n) 5-(3-chlorophenyl)-1-methyl-3-propyl-1*H*-pyrazolo [4, 3-d] pyrimidin-7(6*H*)-one[3]: ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.76 (s, 1H), 8.23 (s, 1H), 8.09 (d, *J* = 7.6 Hz, 1H), 7.54 – 7.43 (m, 2H), 4.33 (s, 3H), 2.94 (t, *J* = 7.6 Hz, 2H), 1.93 – 1.82 (m, 2H), 1.04 (t, *J* = 7.4 Hz, 3H). HRMS (ESI) calcd for $C_{15}H_{16}ClN_4O$ [M-H$^+$] 303.10126, found 303.09995.

3o) 5-(2-fluorophenyl)-1-methyl-3-propyl-1*H*-pyrazolo [4, 3-d] pyrimidin-7(6*H*)-one: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.73 (d, *J* = 10.4 Hz, 1H), 8.34-8.28 (m, 1H), 7.51 (dd, *J* = 13.7, 7.1 Hz, 1H), 7.37-7.31 (m,1H), 7.25-7.18 (m, 1H), 4.27 (s, 3H), 2.92 (d, *J* = 7.6 Hz, 2H), 1.92 – 1.81 (m, 2H), 1.03 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 160.70, 158.22, 154.01,

147.04, 144.99, 137.70, 132.39, 131.01, 124.52, 122.27, 116.13, 37.83, 27.09, 21.66, 13.76. HRMS (ESI) calcd for $C_{15}H_{16}FN_4O$ [M-H^+] 287.13081, found 287.12944.

3p) 5-(3-fluorophenyl)-1-methyl-3-propyl-1*H*-pyrazolo [4, 3-d] pyrimidin-7(6*H*)-one[4]: ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.71 (s, 1H), 8.05 -7.87(m, 2H), 7.54-7.46 (m, 1H), 7.26-7.20 (m, 1H), 4.31 (s, 3H), 2.94 (t, *J* = 7.6 Hz, 2H), 1.93 – 1.82 (m, 2H), 1.04 (t, *J* = 7.4 Hz, 3H). HRMS (ESI) calcd for $C_{15}H_{16}FN_4O$ [M-H^+] 287.13081, found 287.1304.

3q) 5-(4-hydroxy-3-nitrophenyl)-1-methyl-3-propyl-1*H*-pyrazolo[4,3-d]pyrimidin-7(6*H*)-one: ^{1}H NMR (400 MHz, DMSO) δ 12.49 (s, 1H), 8.64 (s, 1H), 8.25 (d, *J* = 8.0 Hz, 1H), 7.24 (d, *J* = 8.0 Hz, 1H), 4.14 (s, 3H), 2.79 (t, *J* = 7.5 Hz, 2H), 1.86 – 1.65 (m, 2H), 0.95 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 154.47, 153.82, 148.10, 144.86, 137.62, 136.67, 133.76, 124.47, 123.80, 119.21, 79.11, 37.78, 27.08, 21.57, 13.82. HRMS (ESI) calcd for $C_{15}H_{16}N_5O_4$ [M-H^+] 330.12023, found 330.11878.

3r) 5-(3, 4-dimethoxyphenyl)-1-methyl-3-propyl-1*H*-pyrazolo[4,3-d]pyrimidin-7(6*H*)-one[2]: 1H NMR (400 MHz, $CDCl_3$) δ 11.03 (s, 1H), 7.69 – 7.62 (m, 2H), 6.95 (d, *J* = 8.4 Hz, 1H), 4.26 (s, 3H), 4.01 (s, 3H), 3.95 (s, 3H), 2.93 (t, *J* = 7.6 Hz, 2H), 1.93 – 1.80 (m, 2H), 1.03 (t, *J* = 7.4 Hz, 3H). HRMS (ESI) calcd for $C_{17}H_{21}N_4O_3$ [M-H^+] 329.16137, found 329.16032.

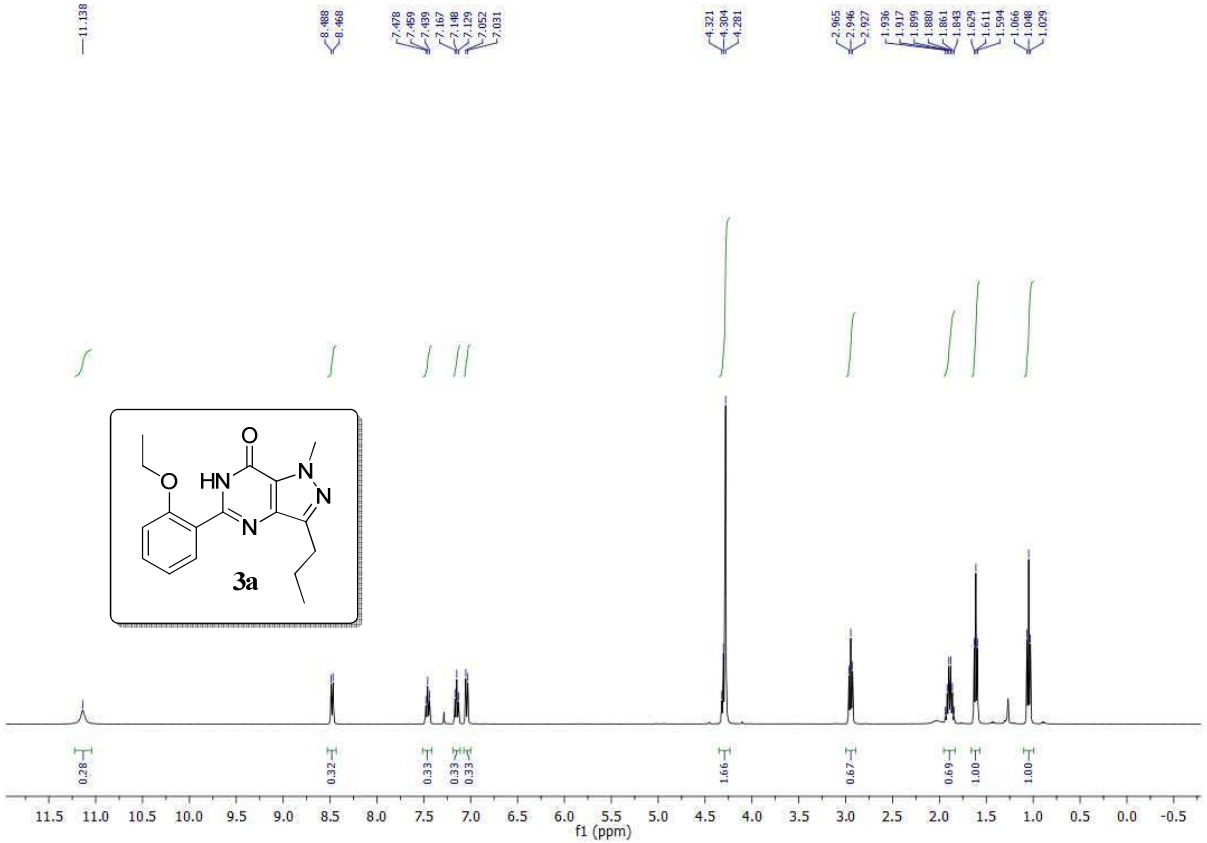
3s) 1-methyl-3-propyl-5-(pyridin-3-yl)-1*H*-pyrazolo [4, 3-d] pyrimidin-7(6*H*)-one[1]: ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.13 (s, 1H), 9.43 (d, *J* = 1.8 Hz, 1H), 8.78 (dd, *J* = 4.7, 1.1 Hz, 1H), 8.58–8.43 (m, 1H), 7.47 (dd, *J* = 7.9, 4.8 Hz, 1H), 4.32 (s, 3H), 2.95 (t, *J* = 7.6 Hz, 2H), 1.95 – 1.82 (m, 2H), 1.04 (t, *J* = 7.4 Hz, 3H). HRMS (ESI) calcd for $C_{14}H_{16}N_5O$ [M-H^+] 270.13549, found 270.13503.

3t) 5-(benzo[d][1,3]dioxol-5-yl)-1-methyl-3-propyl-1*H*-pyrazolo[4,3-d]pyrimidin-7(6*H*)-one: ^{1}H NMR (400 MHz, DMSO) δ 12.25 (s, 1H), 7.67 (dd, *J* = 8.2, 1.8 Hz, 1H), 7.62 (d, *J* = 1.7 Hz, 1H), 7.05 (d, *J* = 8.2 Hz, 1H), 6.12 (s, 2H), 4.14 (s, 3H), 2.79 (t, *J* = 7.5 Hz, 2H), 1.83 – 1.70 (m, 2H), 0.95 (t, *J* = 7.4 Hz, 3H). HRMS (ESI) calcd for $C_{16}H_{17}N_4O_3$ [M-H^+] 313.13007, found 313.12878.

1.2. Scanned Spectral Data:

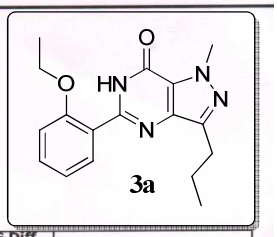
1. Compound 3a:

^{1}H NMR in $CDCl_3$



HRMS

Qualitative Compound Report



3a

Data File	GLR-OEt.d	**Sample Name**	GLR-OEt
Sample Type	Sample	**Position**	Vial 22
Instrument Name	Instrument 1	**User Name**	
Acq Method	vishal_MS_25072012.m	**Acquired Time**	11/19/2012 2:01:46 PM
IRM Calibration Status	Success	**DA Method**	as.m
Comment			

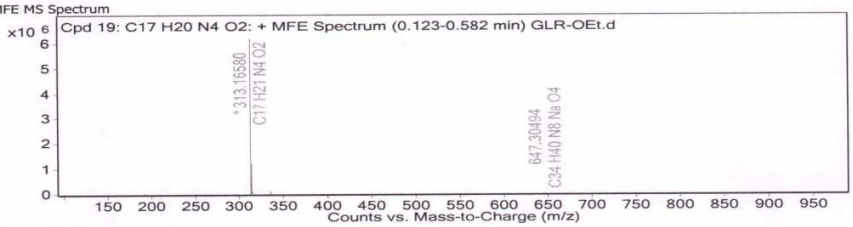
Sample Group **Info.**

Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 19: C17 H20 N4 O2	0.171	312.15852	C17 H20 N4 O2	C17 H20 N4 O2	0.33	C17 H20 N4 O2

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 19: C17 H20 N4 O2	313.1658	0.171	Find by Molecular Feature	312.15852

MFE MS Spectrum



MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
313.1658	1	6190320	C17 H21 N4 O2	(M+H)+
314.16901	1	1178716.9	C17 H21 N4 O2	(M+H)+
315.17101	1	132620.6	C17 H21 N4 O2	(M+H)+
316.17283	1	9972	C17 H21 N4 O2	(M+H)+
335.14715	1	119619.7	C17 H20 N4 Na O2	(M+Na)+
336.15019	1	22215.4	C17 H20 N4 Na O2	(M+Na)+
351.12206	1	3094.9	C17 H20 K N4 O2	(M+K)+
647.30494	1	29313.8	C34 H40 N8 Na O4	(2M+Na)+
648.30728	1	13061.5	C34 H40 N8 Na O4	(2M+Na)+
649.31084	1	3209	C34 H40 N8 Na O4	(2M+Na)+

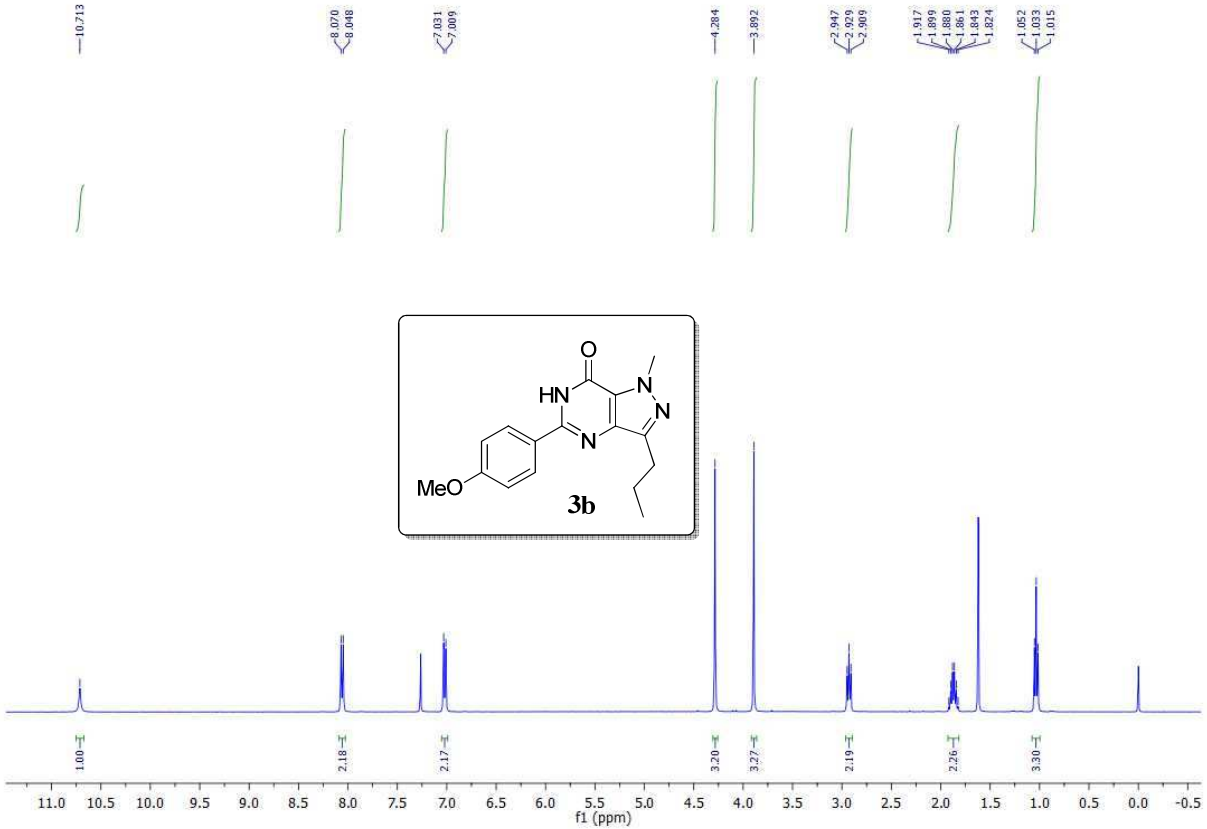
Predicted Isotope Match Table

Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	313.1658	313.1659	0.34	100	100	82.41	81.49
2	314.16901	314.16884	-0.55	19.04	20.17	15.69	16.43
3	315.17101	315.17147	1.45	2.14	2.34	1.77	1.91
4	316.17283	316.17398	3.66	0.16	0.2	0.13	0.16
5	317.17454	317.17649	6.15	0	0.01	0	0.01

--- End Of Report ---

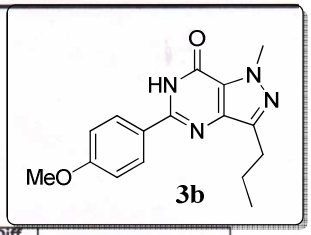
2. Compound 3b

^{1}H NMR in $CDCl_3$



HRMS

Qualitative Compound Report



3b

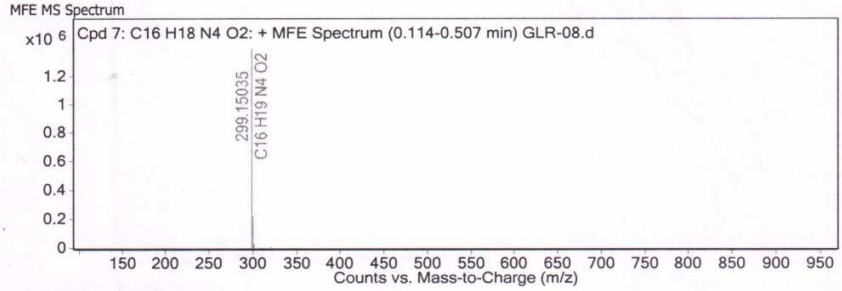
Data File	GLR-08.d	**Sample Name**	Unavailable
Sample Type	Unavailable	**Position**	Unavailable
Instrument Name	Unavailable	**User Name**	Unavailable
Acq Method		**Acquired Time**	Unavailable
IRM Calibration Status	Success	**DA Method**	as.m
Comment	Sample information is unavailable		

Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 7: C16 H18 N4 O2	0.171	298.14307	C16 H18 N4 O2	C16 H18 N4 O2	-0.33	C16 H18 N4 O2

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 7: C16 H18 N4 O2	299.15035	0.171	Find by Molecular Feature	298.14307

MFE MS Spectrum



MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
299.15035	1	1390353.4	C16 H19 N4 O2	(M+H)+
300.15282	1	224317	C16 H19 N4 O2	(M+H)+
301.15497	1	26806.6	C16 H19 N4 O2	(M+H)+
302.1558	1	3087.3	C16 H19 N4 O2	(M+H)+
321.13218	1	6009.2	C16 H18 N4 Na O2	(M+Na)+
322.13399	1	1351.4	C16 H18 N4 Na O2	(M+Na)+

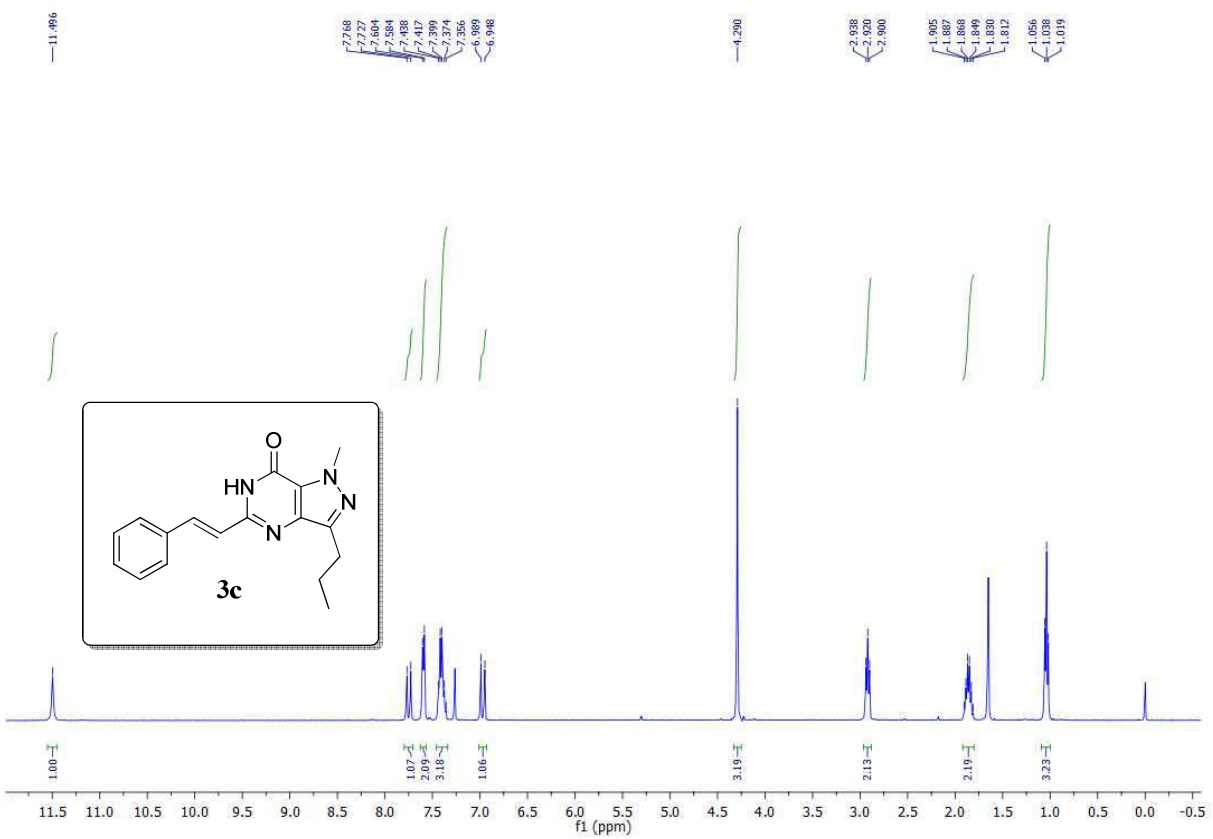
Predicted Isotope Match Table

Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	299.15035	299.15025	-0.34	100	100	84.54	82.39
2	300.15282	300.15316	1.12	16.13	19.06	13.64	15.71
3	301.15497	301.15574	2.56	1.93	2.13	1.63	1.76
4	302.1558	302.15822	8.02	0.22	0.18	0.19	0.14

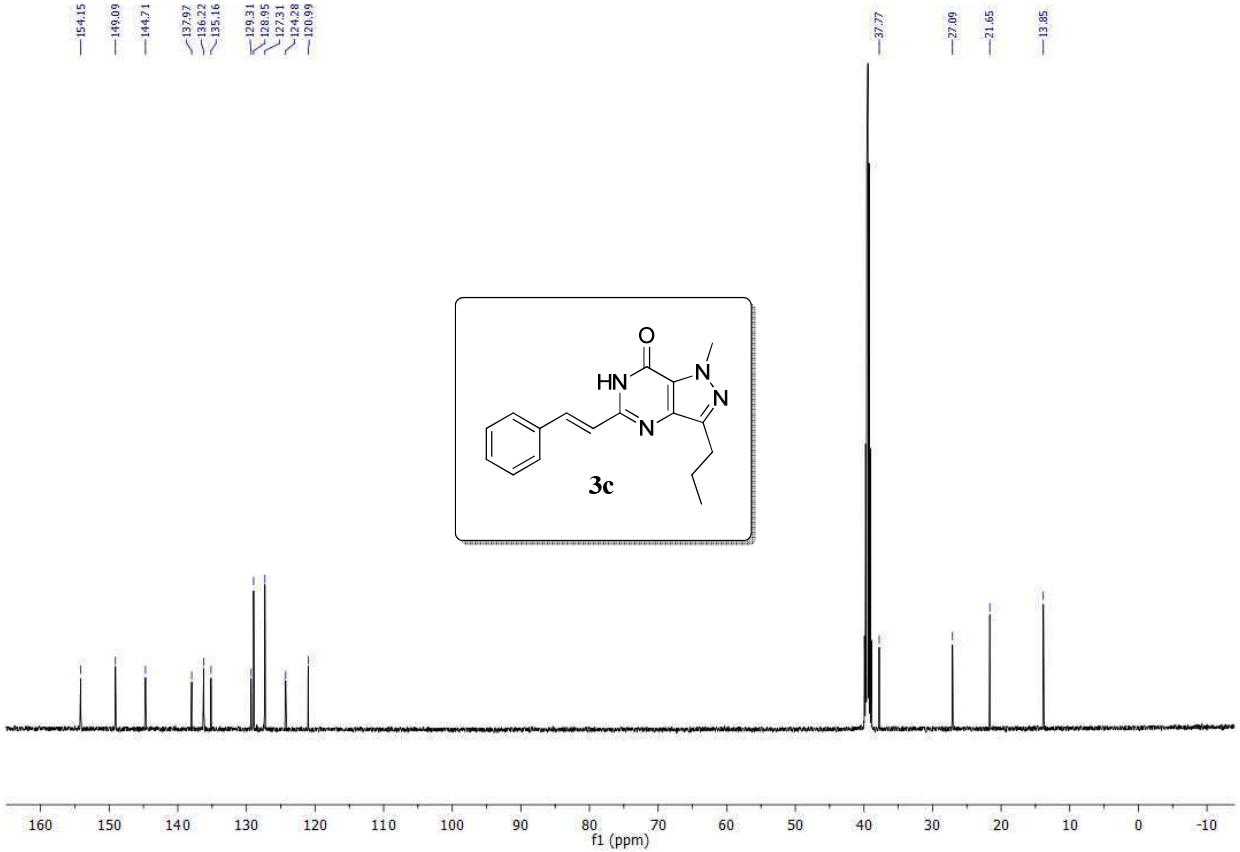
--- End Of Report ---

3. Compound 3c

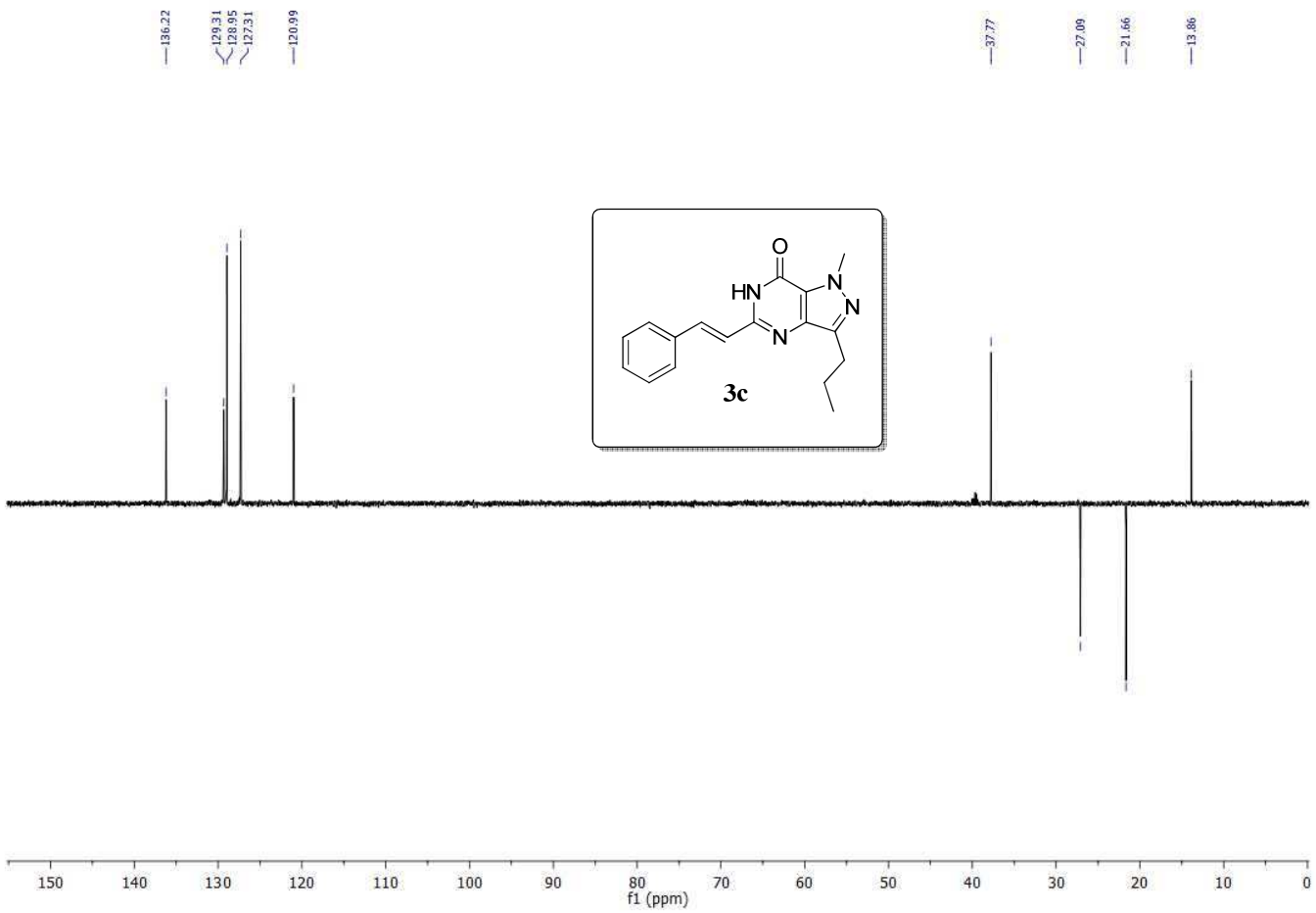
^{1}H NMR in $CDCl_3$



^{13}C NMR in DMSO-d_6



DEPT NMR in DMSO-d_6

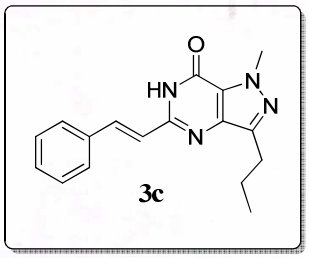


HRMS

Qualitative Compound Report

Data File	daily ms.d GLR-11.d	**Sample Name**	GLR-11
Sample Type	Sample	**Position**	57
Instrument Name	Instrument 1	**User Name**	
Acq Method	DAILY MS DESI.m	**Acquired Time**	2/17/2012 5:09:29 PM
IRM Calibration Status	All Ions Missed	**DA Method**	as.m
Comment			

Sample Group **Info.**



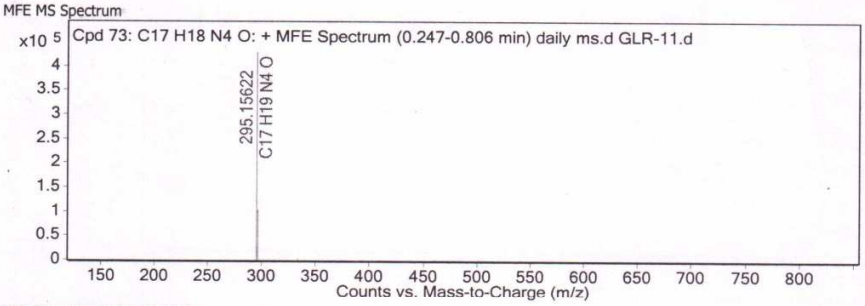
3c

Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 73: C17 H18 N4 O	0.338	294.14893	C17 H18 N4 O	C17 H18 N4 O	-2.96	C17 H18 N4 O

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 73: C17 H18 N4 O	295.15622	0.338	Find by Molecular Feature	294.14893

MFE MS Spectrum



MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
295.15622	1	428039.7	C17 H19 N4 O	(M+H)+
296.15817	1	103269.4	C17 H19 N4 O	(M+H)+
297.16111	1	9196.2	C17 H19 N4 O	(M+H)+
298.16345	1	858.2	C17 H19 N4 O	(M+H)+
333.11103	1	3055.8	C17 H18 K N4 O	(M+K)+
334.11312	1	521.5	C17 H18 K N4 O	(M+K)+
335.1149	1	341.6	C17 H18 K N4 O	(M+K)+

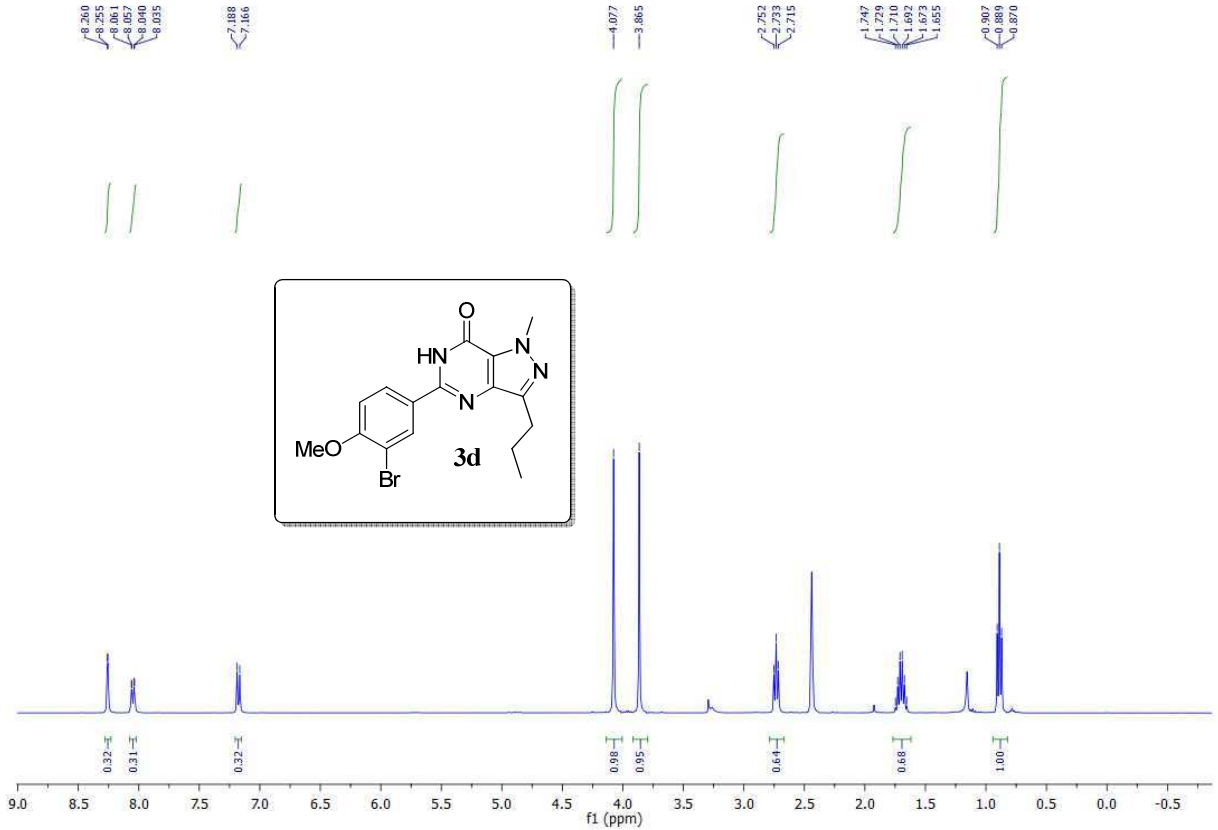
Predicted Isotope Match Table

Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	295.15622	295.15534	-2.99	100	100	79.07	81.71
2	296.15817	296.15827	0.33	24.13	20.1	19.08	16.43
3	297.16111	297.16101	-0.32	2.15	2.12	1.7	1.74
4	298.16345	298.16364	0.61	0.2	0.16	0.16	0.13

--- End Of Report ---

4. Compound 3d

^{1}H NMR in DMSO-d_6

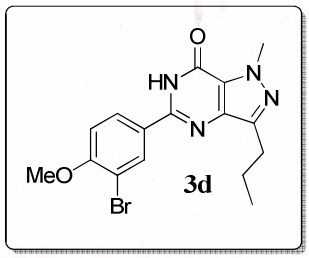


HRMS

Qualitative Compound Report

Data File	.d GLR-OO.d	**Sample Name**	GLR-OO
Sample Type	Sample	**Position**	51
Instrument Name	Instrument 1	**User Name**	
Acq Method	DAILY MS DESI.m	**Acquired Time**	2/23/2012 3:51:40 PM
IRM Calibration Status	All Ions Missed	**DA Method**	as.m
Comment			

Sample Group **Info.**



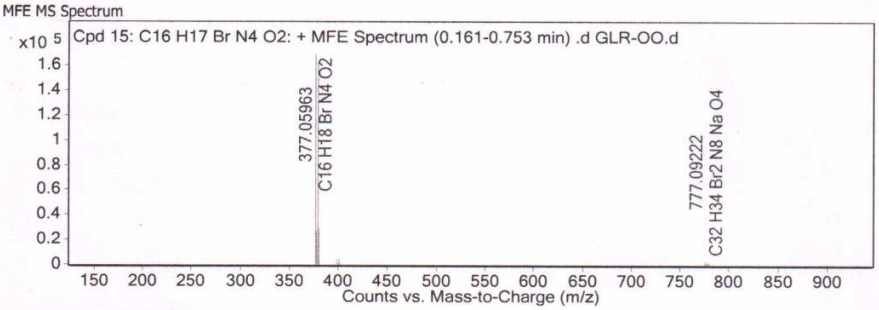
3d

Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 15: C16 H17 Br N4 O2	0.252	376.05237	C16 H17 Br N4 O2	C16 H17 Br N4 O2	2.98	C16 H17 Br N4 O2

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 15: C16 H17 Br N4 O2	377.05963	0.252	Find by Molecular Feature	376.05237

MFE MS Spectrum



MS Spectrum Peak List

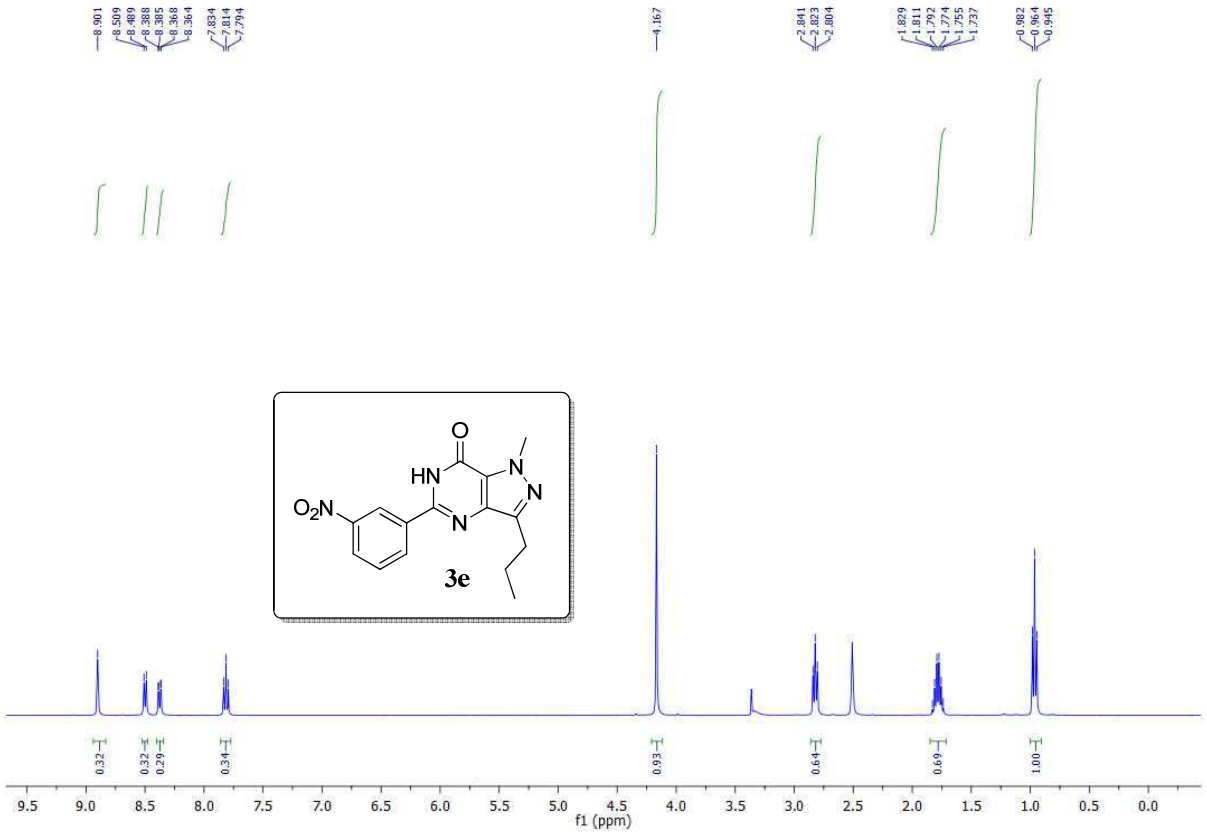
m/z	z	Abund	Formula	Ion
377.05963	1	169694.6	C16 H18 Br N4 O2	(M+H)+
378.06236	1	27309.6	C16 H18 Br N4 O2	(M+H)+
379.05778	1	163118.4	C16 H18 Br N4 O2	(M+H)+
380.06041	1	28652.1	C16 H18 Br N4 O2	(M+H)+
381.0631	1	3073.8	C16 H18 Br N4 O2	(M+H)+
399.04091	1	4508.3	C16 H17 Br N4 Na O2	(M+Na)+
401.03953	1	4469.6	C16 H17 Br N4 Na O2	(M+Na)+
775.09143	1	1253.9	C32 H34 Br2 N8 Na O4	(2M+Na)+
777.09222	1	2646	C32 H34 Br2 N8 Na O4	(2M+Na)+
779.08826	1	1461.1	C32 H34 Br2 N8 Na O4	(2M+Na)+

Predicted Isotope Match Table

Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	377.05963	377.06077	3.02	100	100	43.26	41.77
2	378.06236	378.06367	3.47	16.09	19.05	6.96	7.96
3	379.05778	379.05888	2.9	96.12	99.41	41.58	41.52
4	380.06041	380.06169	3.36	16.88	18.71	7.3	7.81
5	381.0631	381.06424	2.98	1.81	2.08	0.78	0.87
6	382.06532	382.06671	3.63	0.25	0.17	0.11	0.07

5. Compound 3e

^{1}H NMR in DMSO-d_6

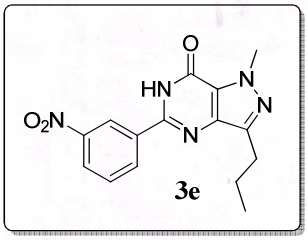


HRMS

Qualitative Compound Report

Data File	GLR-01.d	**Sample Name**	GLR-01
Sample Type	Sample	**Position**	Vial 29
Instrument Name	Instrument 1	**User Name**	
Acq Method	vishal_MS_25072012.m	**Acquired Time**	11/19/2012 3:47:53 PM
IRM Calibration Status	Success	**DA Method**	as.m
Comment			

Sample Group **Info.**



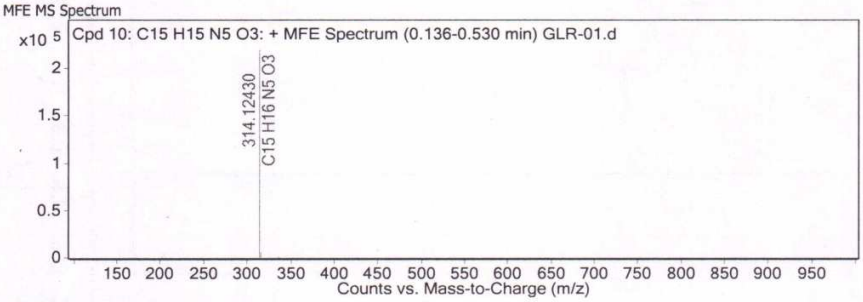
3e

Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 10: C15 H15 N5 O3	0.173	313.11702	C15 H15 N5 O3	C15 H15 N5 O3	1.49	C15 H15 N5 O3

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 10: C15 H15 N5 O3	314.1243	0.173	Find by Molecular Feature	313.11702

MFE MS Spectrum



MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
314.1243	1	219505.2	C15 H16 N5 O3	(M+H)+
315.12725	1	39115.9	C15 H16 N5 O3	(M+H)+
316.12967	1	5130.5	C15 H16 N5 O3	(M+H)+
336.10699	1	829.7	C15 H15 N5 Na O3	(M+Na)+

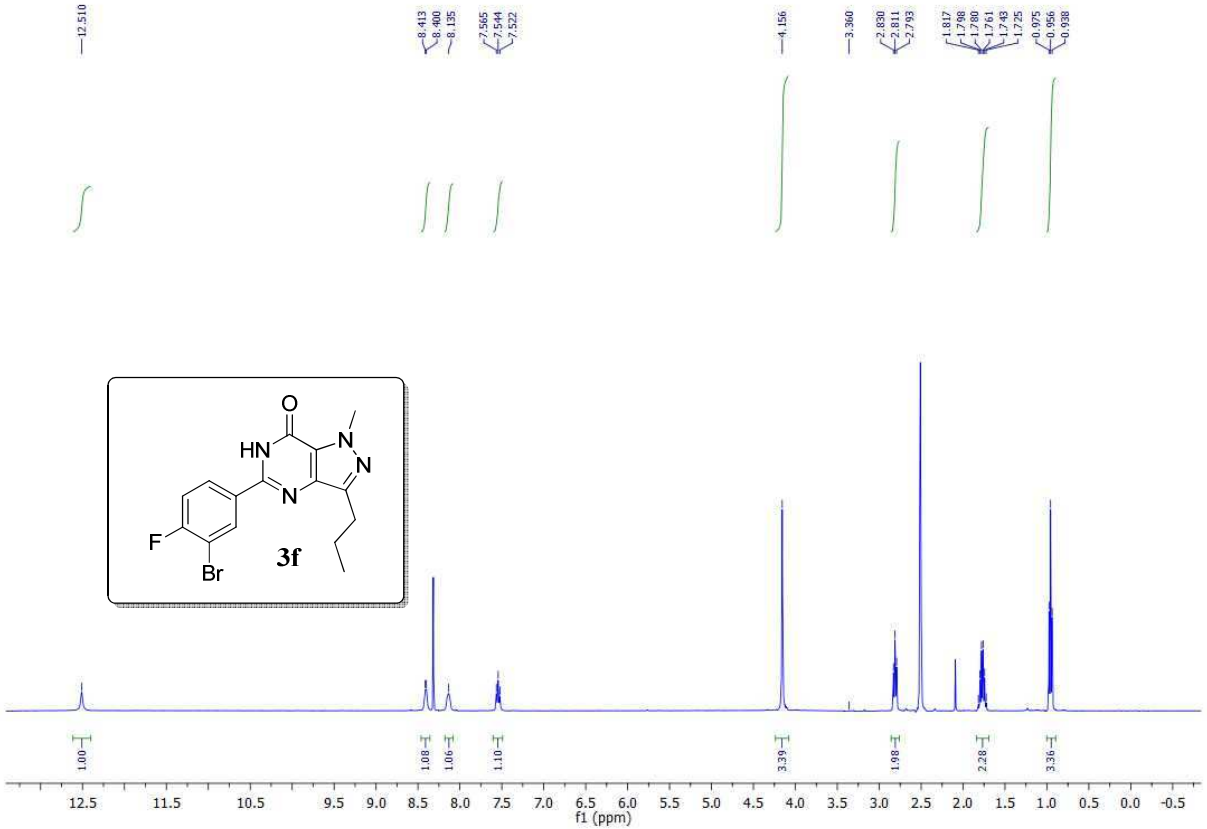
Predicted Isotope Match Table

Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	314.1243	314.12477	1.49	100	100	83.22	82.95
2	315.12725	315.12753	0.89	17.82	18.35	14.83	15.22
3	316.12967	316.1299	0.73	2.34	2.21	1.95	1.83

--- End Of Report ---

6. Compound 3f

^{1}H NMR in DMSO-d_6

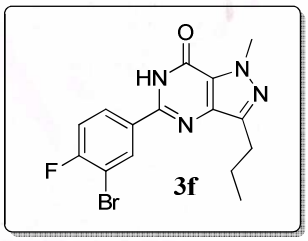


HRMS

Qualitative Compound Report

Data File	daily ms0044.d	**Sample Name**	3
Sample Type	Sample	**Position**	2
Instrument Name	Instrument 1	**User Name**	
Acq Method	DAILY MS DESI.m	**Acquired Time**	1/18/2012 12:20:28 PM
IRM Calibration Status	Success	**DA Method**	as.m
Comment			

Sample Group **Info.**

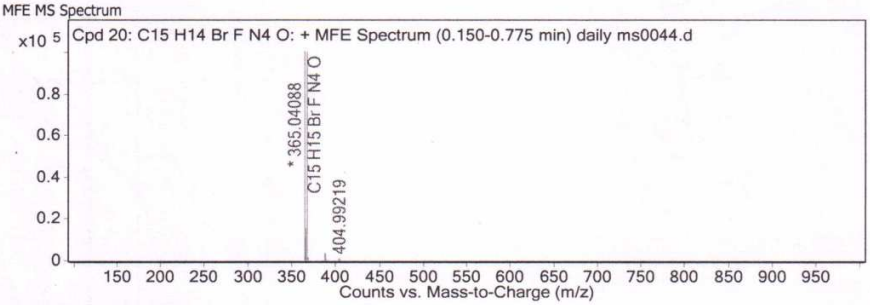


Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 20: C15 H14 Br F N4 O	0.272	364.0336	C15 H14 Br F N4 O	C15 H14 Br F N4 O	-0.26	C15 H14 Br F N4 O

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 20: C15 H14 Br F N4 O	365.04088	0.272	Find by Molecular Feature	364.0336

MFE MS Spectrum



MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
365.04088	1	100778	C15 H15 Br F N4 O	(M+H)+
366.04398	1	15676	C15 H15 Br F N4 O	(M+H)+
367.03895	1	100156	C15 H15 Br F N4 O	(M+H)+
368.04184	1	15596.4	C15 H15 Br F N4 O	(M+H)+
369.04489	1	1446.3	C15 H15 Br F N4 O	(M+H)+
387.02258	1	3596.3	C15 H14 Br F N4 Na O	(M+Na)+
388.02589	1	789.5	C15 H14 Br F N4 Na O	(M+Na)+
389.02051	1	3716.2	C15 H14 Br F N4 Na O	(M+Na)+
402.99514	1	868		(M+K)+
404.99219	1	918		(M+K)+

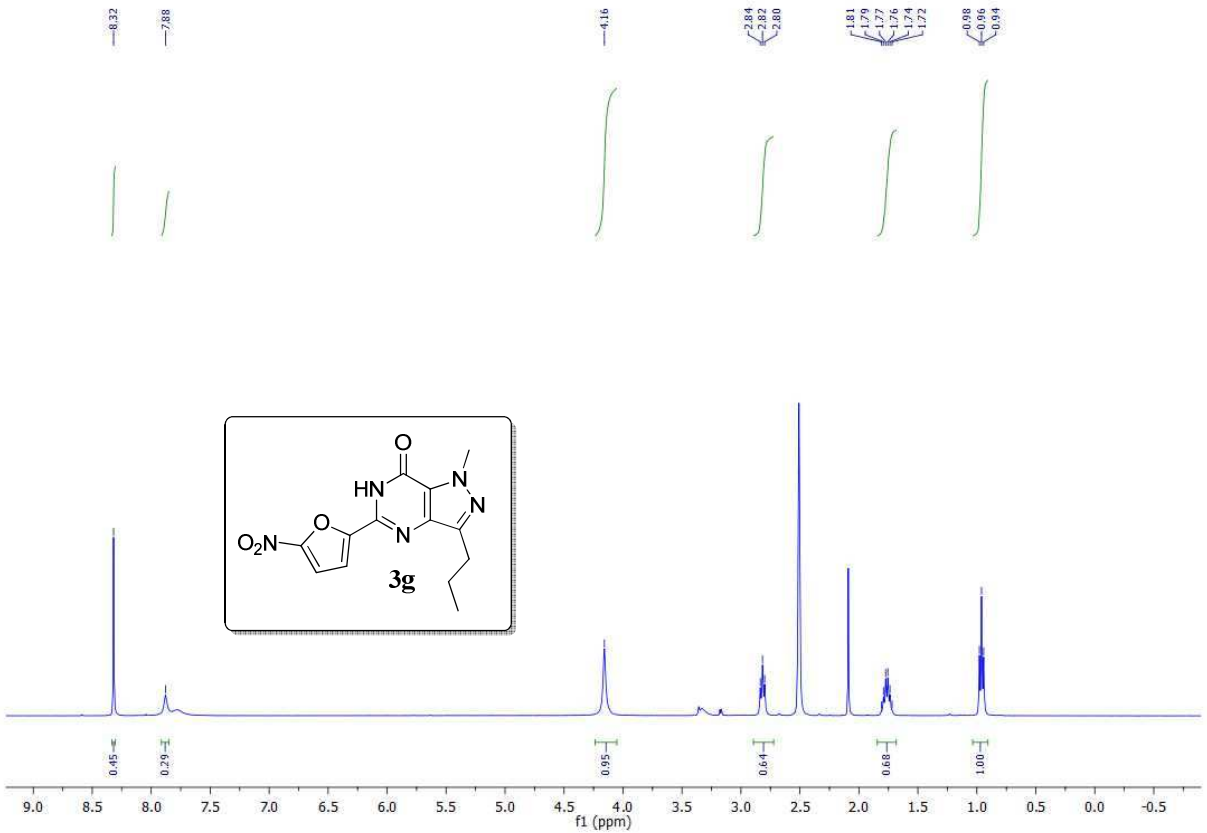
Predicted Isotope Match Table

Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	365.04088	365.04078	-0.28	100	100	43.11	42.34
2	366.04398	366.04365	-0.92	15.55	17.9	6.71	7.58
3	367.03895	367.03886	-0.24	99.38	98.99	42.85	41.91
4	368.04184	368.04165	-0.51	15.48	17.53	6.67	7.42
5	369.04489	369.04428	-1.63	1.44	1.68	0.62	0.71
6	370.04831	370.04681	-4.06	0.1	0.11	0.04	0.05

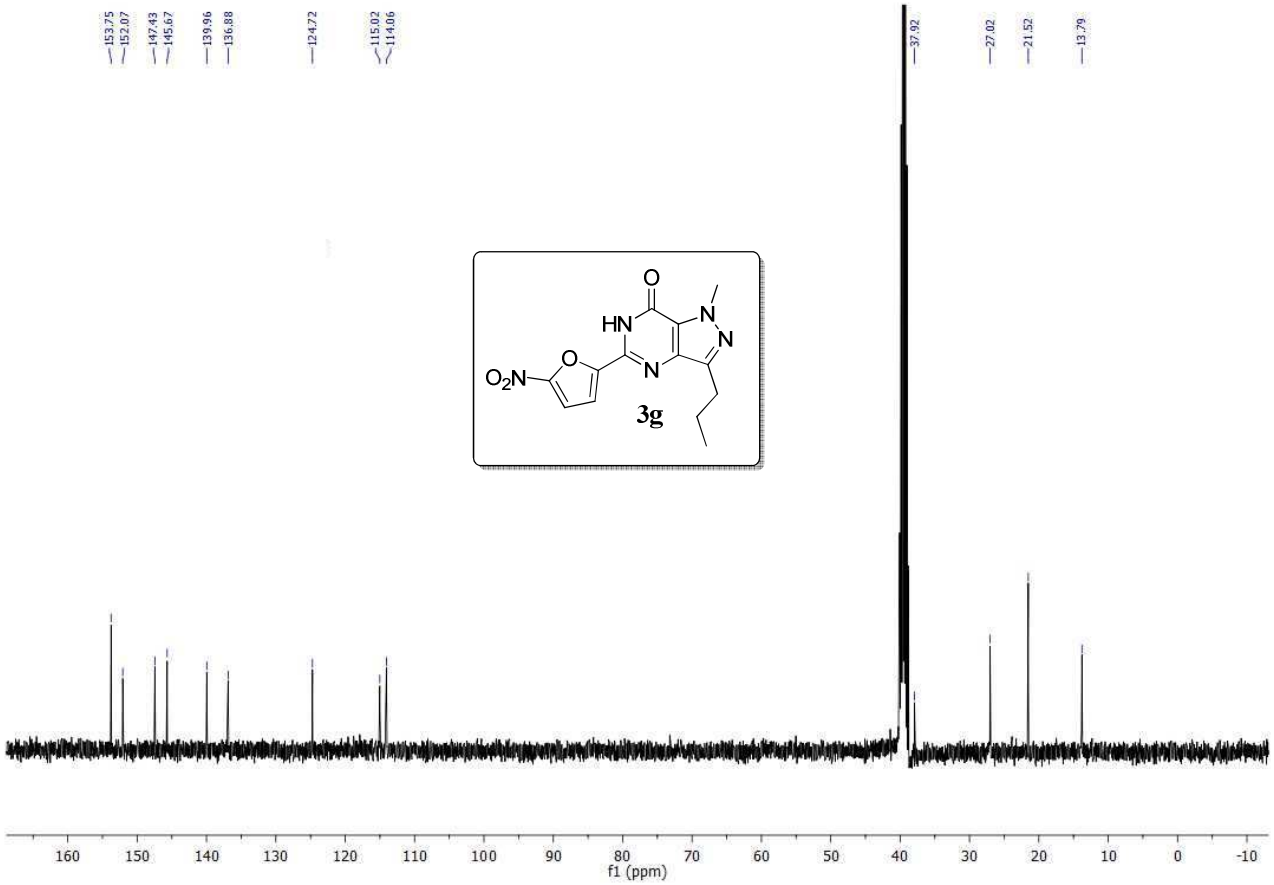
--- End Of Report ---

7. Compound 3g

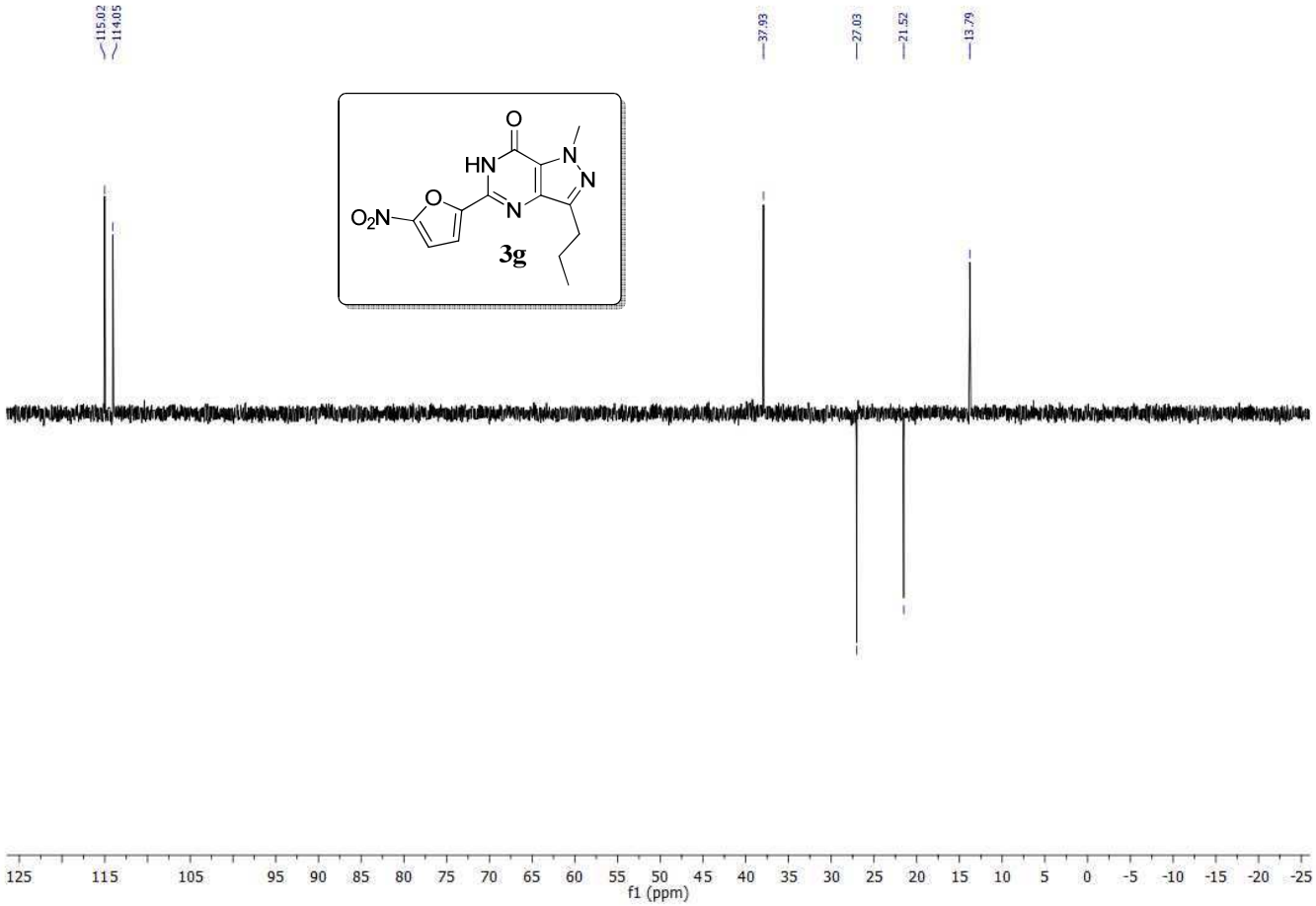
^{1}H NMR in DMSO-d_6



^{13}C NMR in DMSO-d_6



DEPT NMR in DMSO-d_6

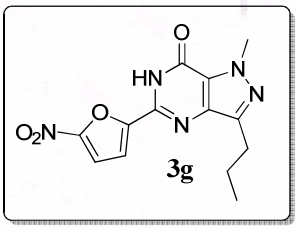


HRMS

Qualitative Compound Report

Data File	daily ms0032.d	**Sample Name**	4
Sample Type	Sample	**Position**	26
Instrument Name	Instrument 1	**User Name**	
Acq Method	DAILY MS DESI.m	**Acquired Time**	1/18/2012 11:41:39 AM
IRM Calibration Status	Success	**DA Method**	as.m
Comment			

Sample Group **Info.**

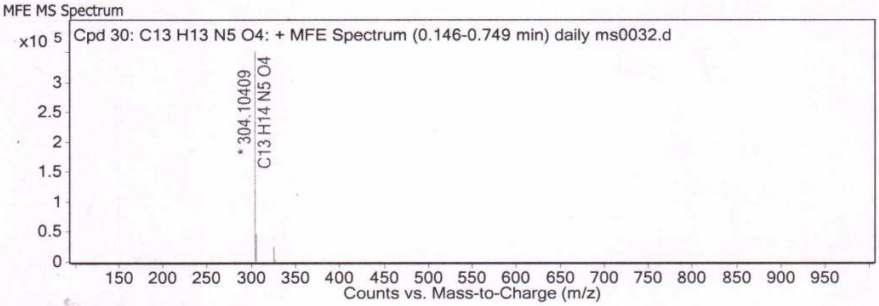


Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 30: C13 H13 N5 O4	0.236	303.09682	C13 H13 N5 O4	C13 H13 N5 O4	-0.21	C13 H13 N5 O4

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 30: C13 H13 N5 O4	304.10409	0.236	Find by Molecular Feature	303.09682

MFE MS Spectrum



MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
304.10409	1	352052.5	C13 H14 N5 O4	(M+H)+
305.10722	1	46982.8	C13 H14 N5 O4	(M+H)+
306.10937	1	5822.5	C13 H14 N5 O4	(M+H)+
307.11129	1	595.1	C13 H14 N5 O4	(M+H)+
326.08634	1	24963.2	C13 H13 N5 Na O4	(M+Na)+
327.08911	1	3812.6	C13 H13 N5 Na O4	(M+Na)+
328.0897	1	744	C13 H13 N5 Na O4	(M+Na)+

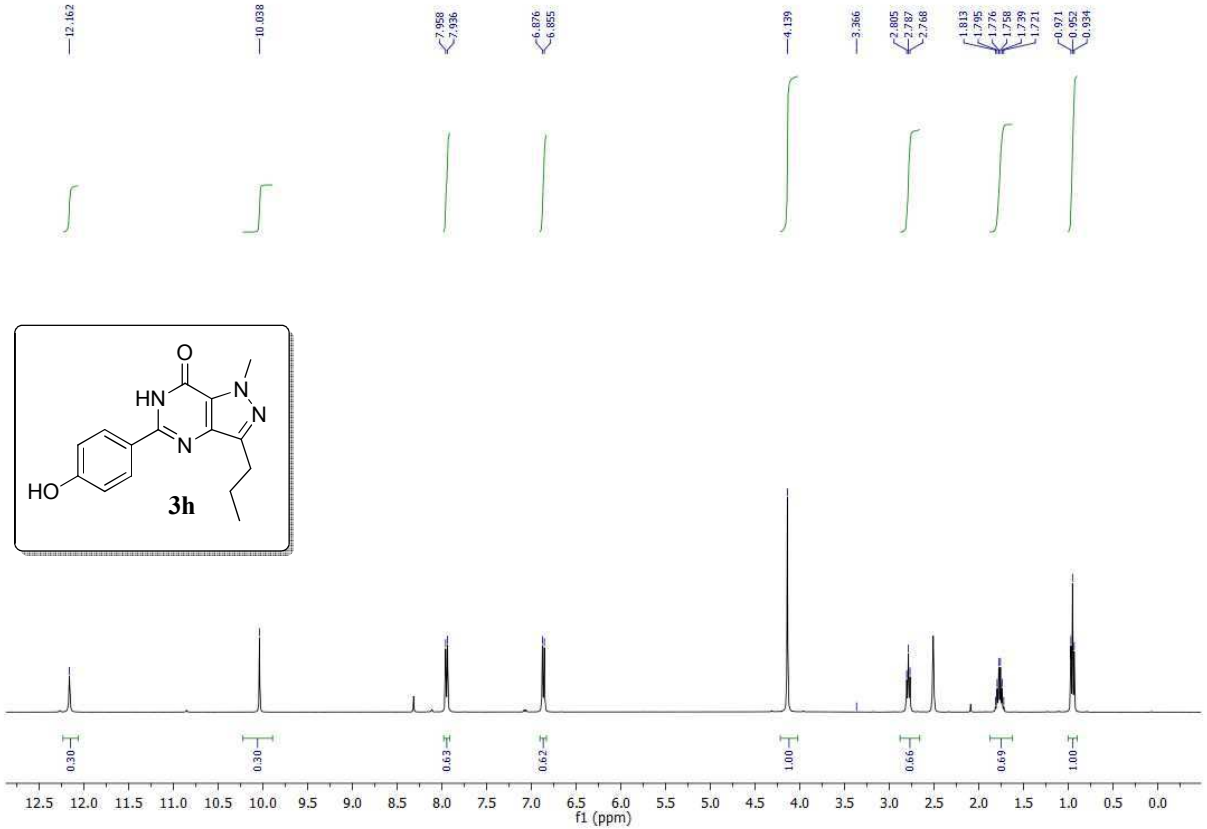
Predicted Isotope Match Table

Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	304.10409	304.10403	-0.21	100	100	86.83	84.43
2	305.10722	305.10671	-1.66	13.35	16.2	11.59	13.68
3	306.10937	306.10891	-1.48	1.65	2.05	1.44	1.73
4	307.11129	307.11124	-0.16	0.17	0.19	0.15	0.16

--- End Of Report ---

8. Compound 3h

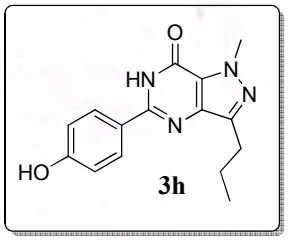
^{1}H NMR in DMSO-d_6



HRMS

Qualitative Compound Report

Data File	GLR-05.d	**Sample Name**	GLR-05
Sample Type	Sample	**Position**	Vial 19
Instrument Name	Instrument 1	**User Name**	
Acq Method	vishal_MS_25072012.m	**Acquired Time**	11/19/2012 1:47:31 PM
IRM Calibration Status	Success	**DA Method**	as.m
Comment			
Sample Group	**Info.**		



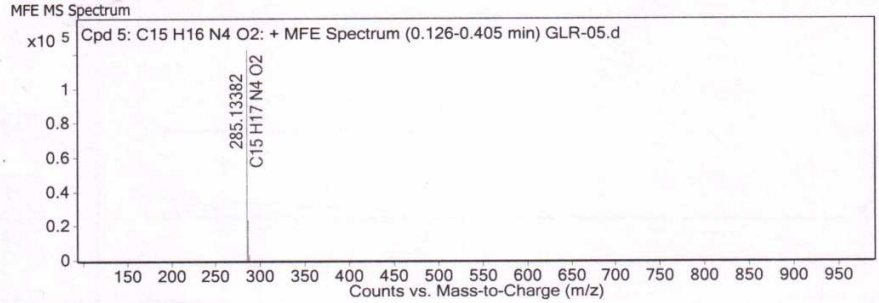
3h

Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 5: C15 H16 N4 O2	0.171	284.12654	C15 H16 N4 O2	C15 H16 N4 O2	2.75	C15 H16 N4 O2

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 5: C15 H16 N4 O2	285.13382	0.171	Find by Molecular Feature	284.12654

MFE MS Spectrum



MS Spectrum Peak List

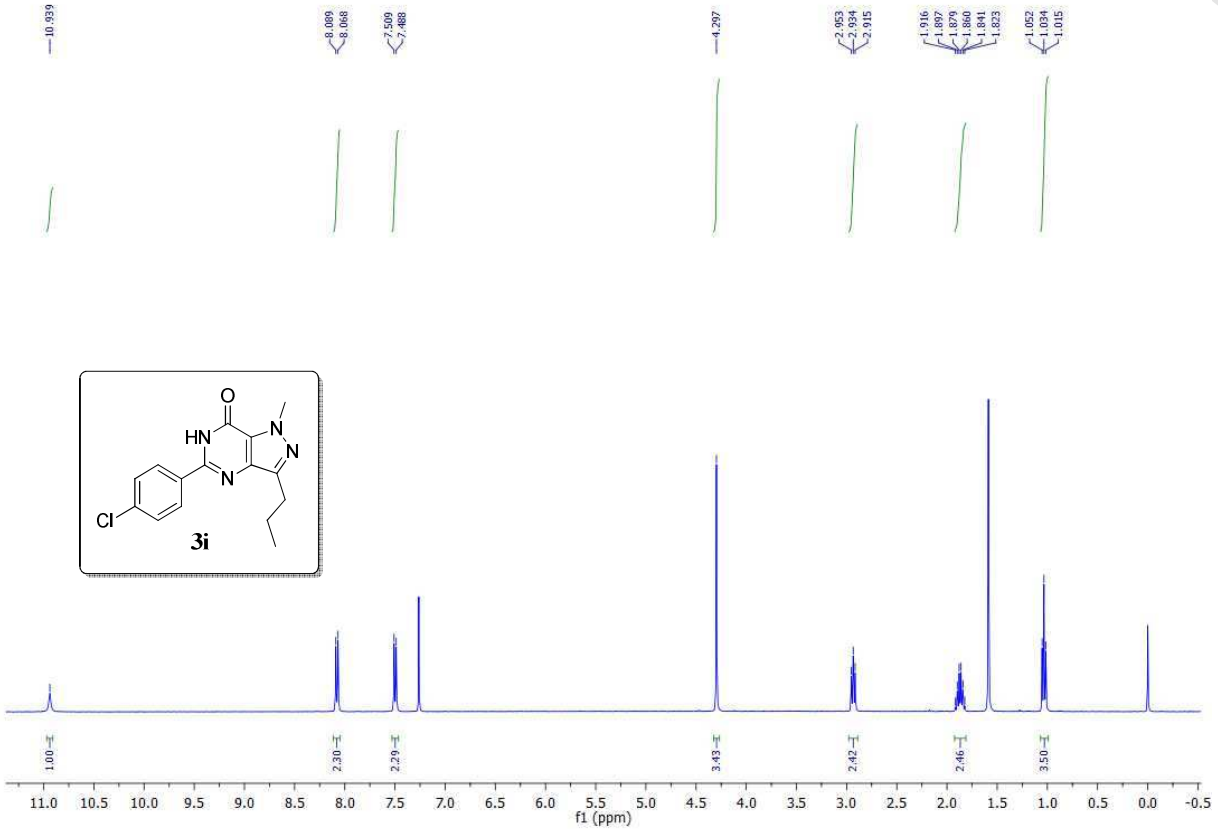
m/z	z	Abund	Formula	Ion
285.13382	1	122615.1	C15 H17 N4 O2	(M+H)+
286.13654	1	23372.1	C15 H17 N4 O2	(M+H)+
287.13921	1	3165.2	C15 H17 N4 O2	(M+H)+

Predicted Isotope Match Table

Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	285.13382	285.1346	2.74	100	100	82.21	83.41
2	286.13654	286.13748	3.29	19.06	17.96	15.67	14.98
3	287.13921	287.14001	2.77	2.58	1.93	2.12	1.61

9. Compound 3i

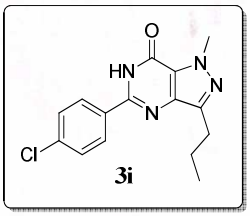
^{1}H NMR in $CDCl_3$



HRMS

Qualitative Compound Report

Data File	GLR-09.d	**Sample Name**	GLR-09
Sample Type	Sample	**Position**	Vial 28
Instrument Name	Instrument 1	**User Name**	
Acq Method	vishal_MS_25072012.m	**Acquired Time**	11/19/2012 2:29:59 PM
IRM Calibration Status	Success	**DA Method**	as.m
Comment			
Sample Group	**Info.**		



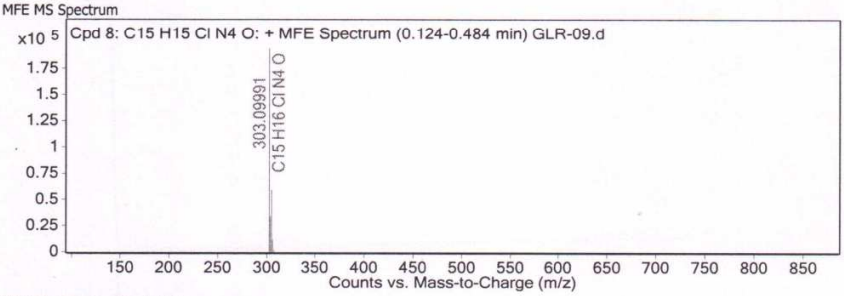
3i

Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 8: C15 H15 Cl N4 O	0.172	302.09264	C15 H15 Cl N4 O	C15 H15 Cl N4 O	2.64	C15 H15 Cl N4 O

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 8: C15 H15 Cl N4 O	303.09991	0.172	Find by Molecular Feature	302.09264

MFE MS Spectrum



MS Spectrum Peak List

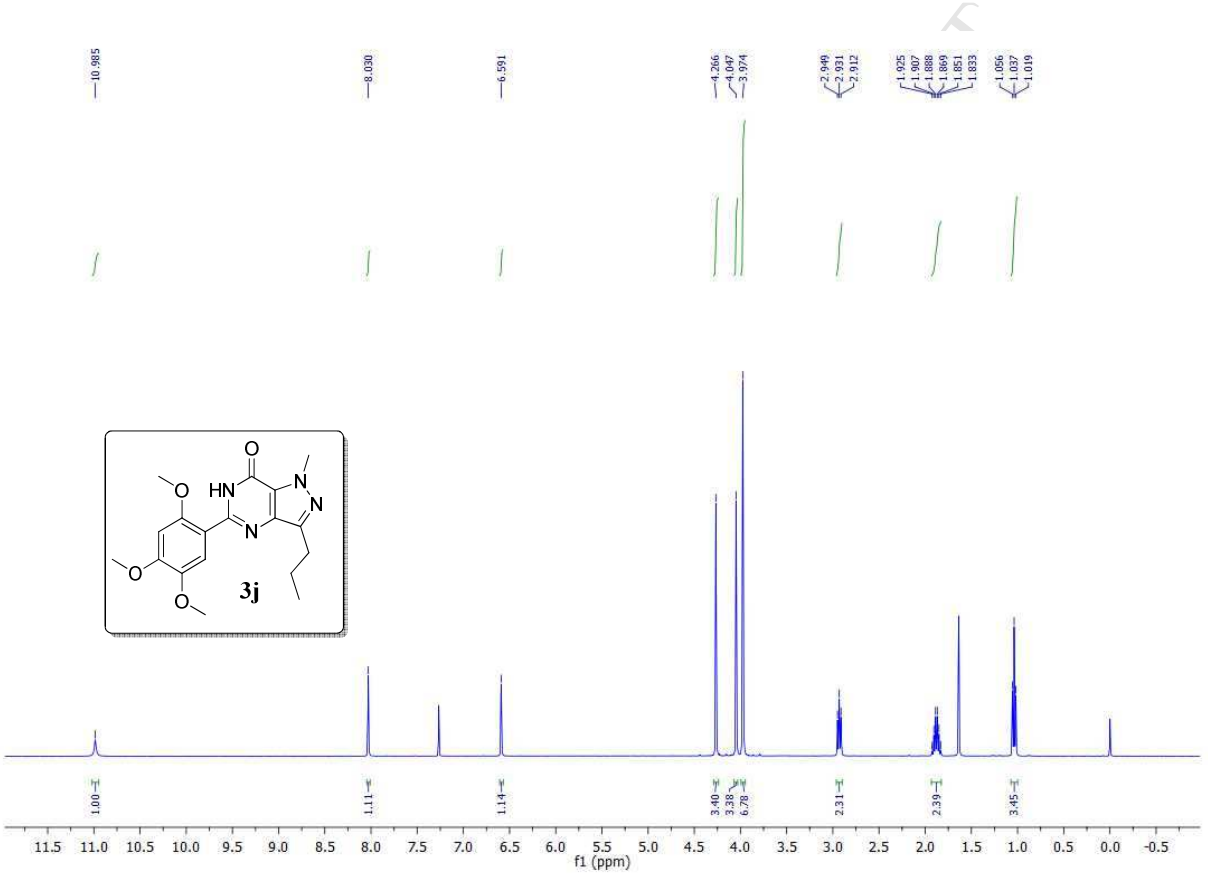
m/z	z	Abund	Formula	Ion
303.09991	1	194356	C15 H16 Cl N4 O	(M+H)+
304.10298	1	34317.9	C15 H16 Cl N4 O	(M+H)+
305.09761	1	59485.7	C15 H16 Cl N4 O	(M+H)+
306.10056	1	12004	C15 H16 Cl N4 O	(M+H)+
307.101	1	1578.7	C15 H16 Cl N4 O	(M+H)+

Predicted Isotope Match Table

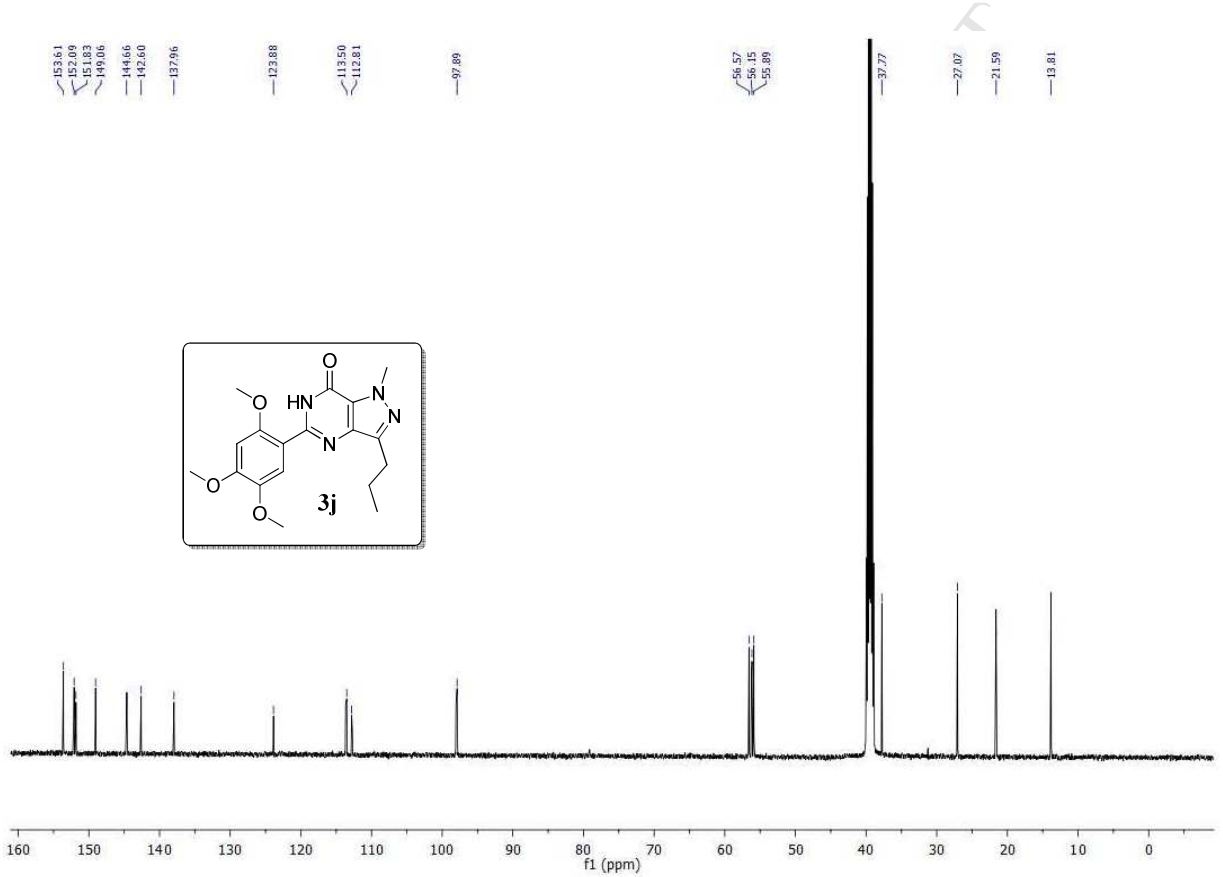
Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	303.09991	303.10072	2.65	100	100	64.41	63.28
2	304.10298	304.10359	1.99	17.66	17.91	11.37	11.33
3	305.09761	305.0982	1.92	30.61	33.71	19.71	21.33
4	306.10056	306.1008	0.8	6.18	5.85	3.98	3.7
5	307.101	307.10338	7.75	0.81	0.56	0.52	0.35

10. Compound 3j

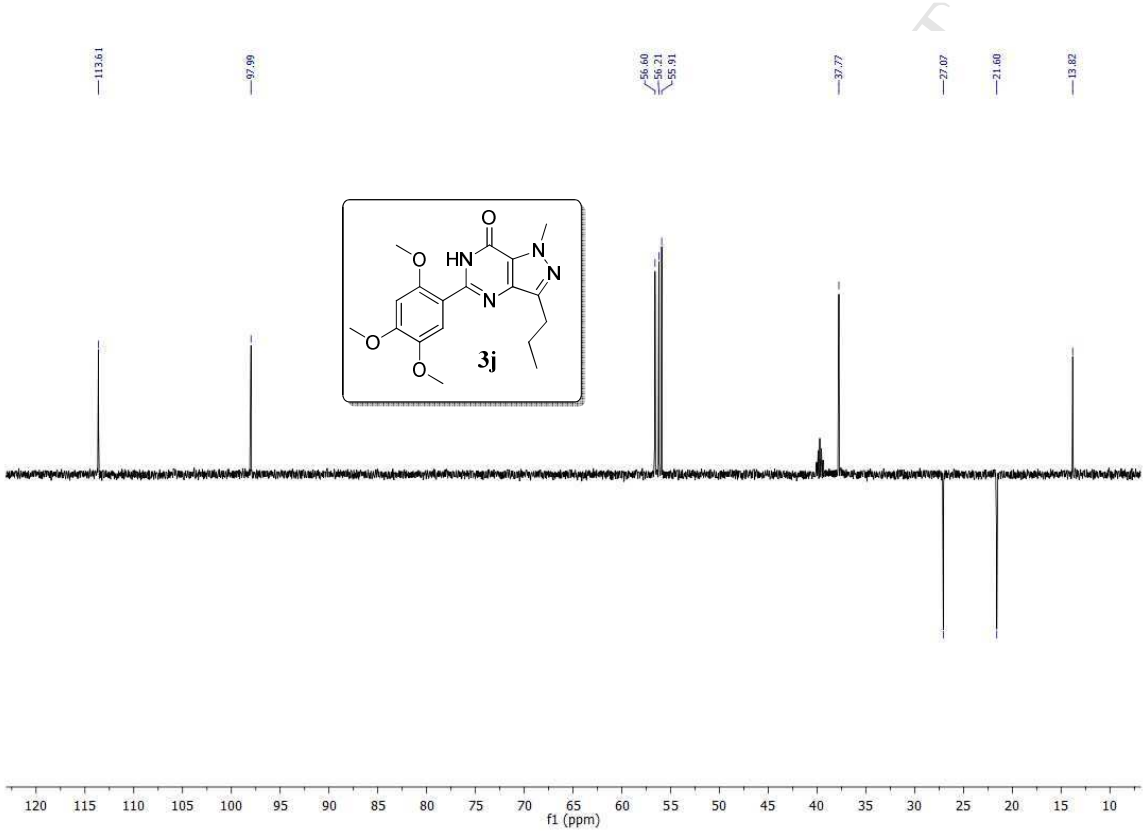
^{1}H NMR in $CDCl_3$



^{13}C NMR in DMSO-d_6



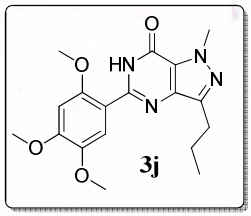
DEPT NMR in DMSO-d_6



HRMS

Qualitative Compound Report

Data File	GLR-10.d	**Sample Name**	GLR-10
Sample Type	Sample	**Position**	Vial 23
Instrument Name	Instrument 1	**User Name**	
Acq Method	vishal_MS_25072012.m	**Acquired Time**	11/19/2012 2:08:52 PM
IRM Calibration Status	Success	**DA Method**	as.m
Comment			
Sample Group	**Info.**		

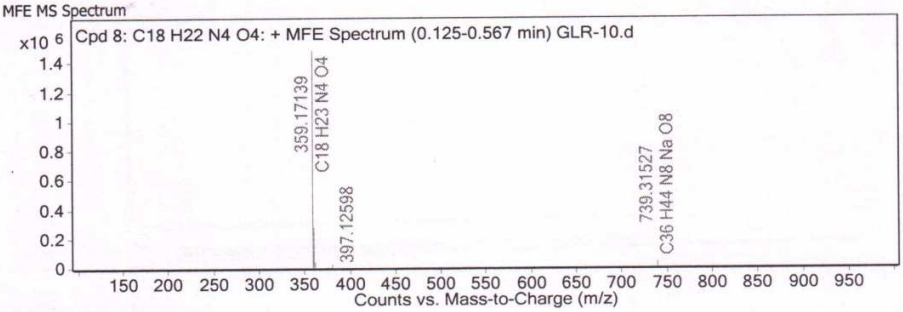


Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 8: C18 H22 N4 O4	0.172	358.16411	C18 H22 N4 O4	C18 H22 N4 O4	0	C18 H22 N4 O4

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 8: C18 H22 N4 O4	359.17139	0.172	Find by Molecular Feature	358.16411

MFE MS Spectrum



MS Spectrum Peak List

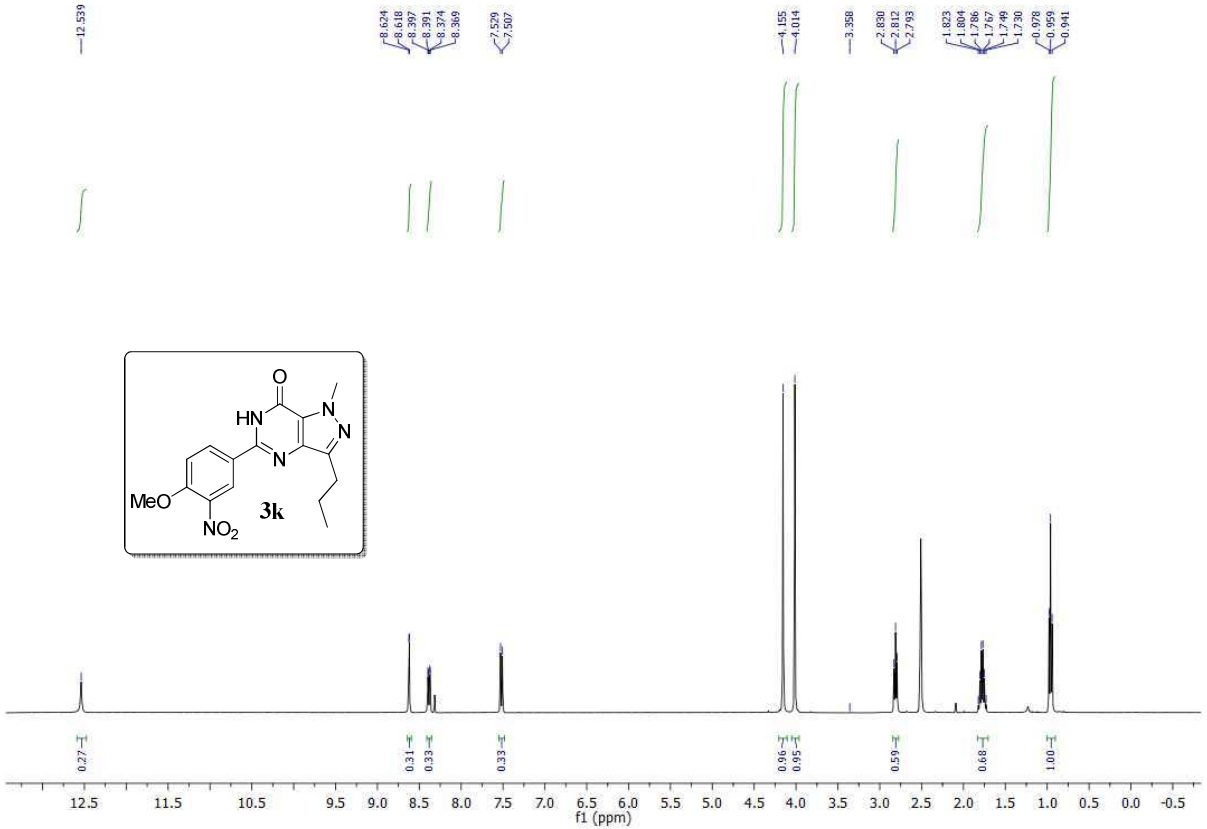
m/z	z	Abund	Formula	Ion
359.17139	1	1478702.6	C18 H23 N4 O4	(M+H)+
360.17386	1	276305.2	C18 H23 N4 O4	(M+H)+
361.17604	1	36368.8	C18 H23 N4 O4	(M+H)+
362.17862	1	3941.5	C18 H23 N4 O4	(M+H)+
381.15182	1	21637.3	C18 H22 N4 Na O4	(M+Na)+
382.15722	1	3238.3	C18 H22 N4 Na O4	(M+Na)+
397.12598	1	1378.5		(M+K)+
739.31527	1	35895.2	C36 H44 N8 Na O8	(2M+Na)+
740.31824	1	14522.2	C36 H44 N8 Na O8	(2M+Na)+
741.3231	1	3411.4	C36 H44 N8 Na O8	(2M+Na)+

Predicted Isotope Match Table

Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	359.17139	359.17138	-0.01	100	100	82.36	80.22
2	360.17386	360.17435	1.35	18.69	21.35	15.39	17.12
3	361.17604	361.17683	2.19	2.46	2.99	2.03	2.4
4	362.17862	362.17931	1.91	0.27	0.31	0.22	0.25

11. Compound 3k

^{1}H NMR in DMSO-d_6

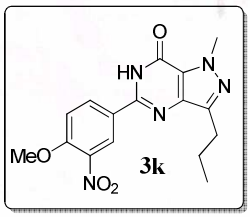


HRMS

Qualitative Compound Report

Data File	GLR-07.d	**Sample Name**	GLR-07
Sample Type	Sample	**Position**	Vial 27
Instrument Name	Instrument 1	**User Name**	
Acq Method	vishal_MS_25072012.m	**Acquired Time**	11/19/2012 2:26:30 PM
IRM Calibration Status	Success	**DA Method**	as.m
Comment			

Sample Group **Info.**

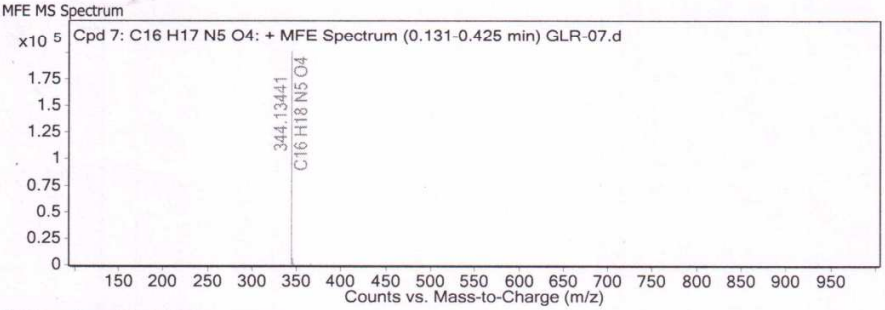


Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 7: C16 H17 N5 O4	0.171	343.12714	C16 H17 N5 O4	C16 H17 N5 O4	2.67	C16 H17 N5 O4

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 7: C16 H17 N5 O4	344.13441	0.171	Find by Molecular Feature	343.12714

MFE MS Spectrum



MS Spectrum Peak List

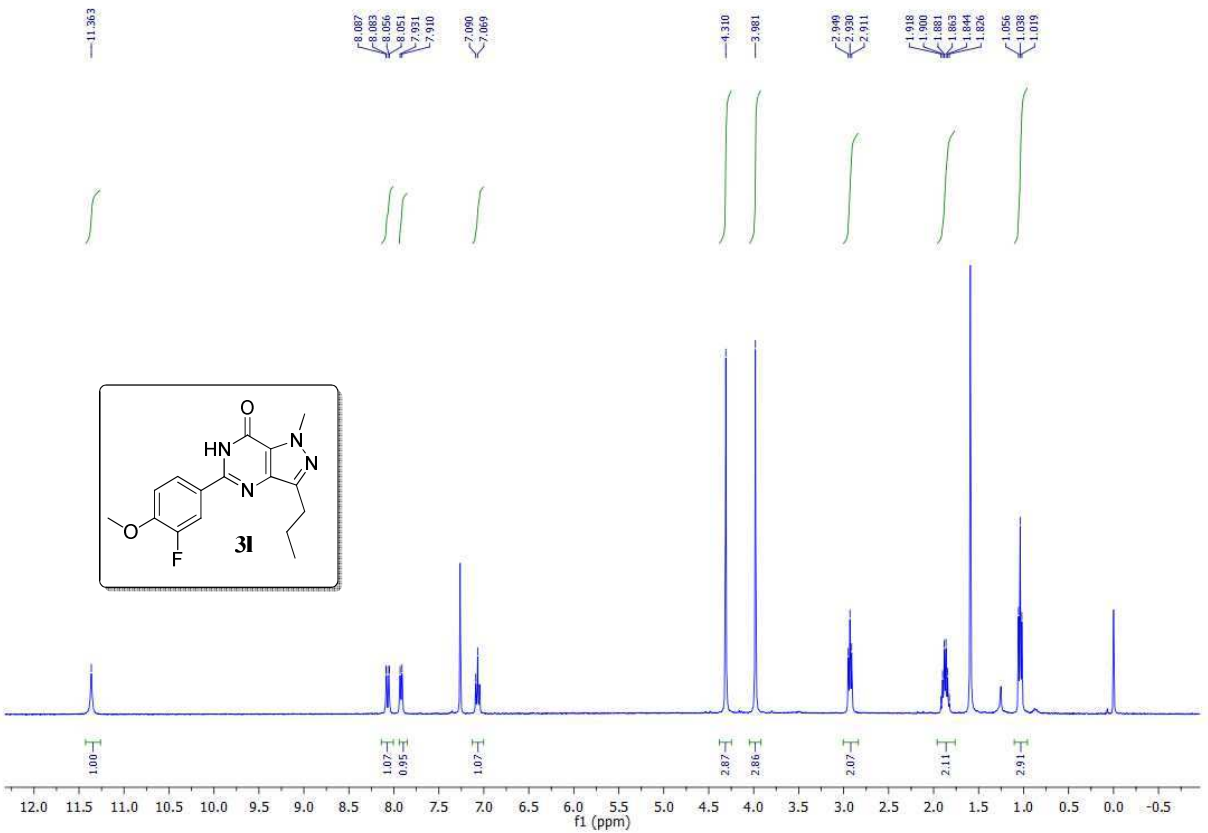
m/z	z	Abund	Formula	Ion
344.13441	1	200875.9	C16 H18 N5 O4	(M+H)+
345.13729	1	42815.5	C16 H18 N5 O4	(M+H)+
346.13909	1	6421.4	C16 H18 N5 O4	(M+H)+
347.14035	1	1001.6	C16 H18 N5 O4	(M+H)+
366.1184	1	1253.3	C16 H17 N5 Na O4	(M+Na)+

Predicted Isotope Match Table

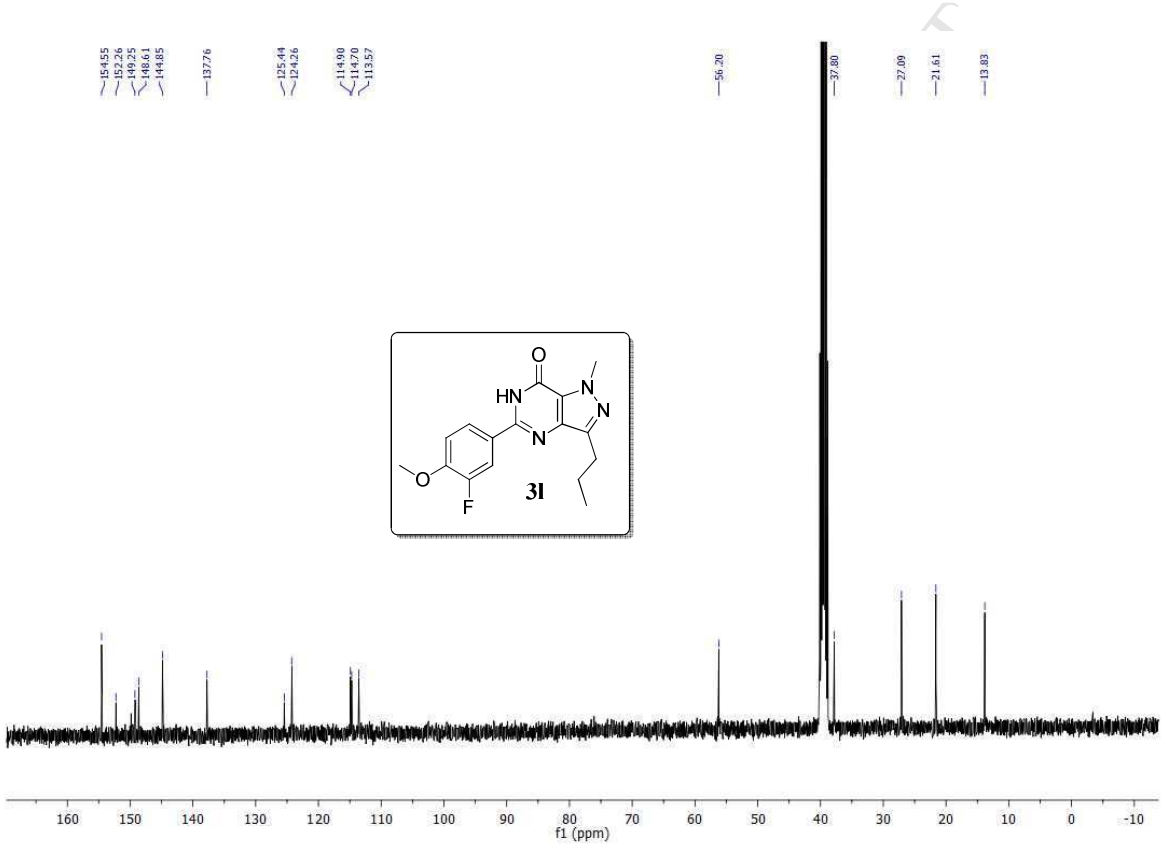
Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	344.13441	344.13533	2.66	100	100	79.99	81.71
2	345.13729	345.13813	2.43	21.31	19.49	17.05	15.93
3	346.13909	346.14048	4.01	3.2	2.62	2.56	2.14
4	347.14035	347.14287	7.24	0.5	0.27	0.4	0.22

12. Compound 3l

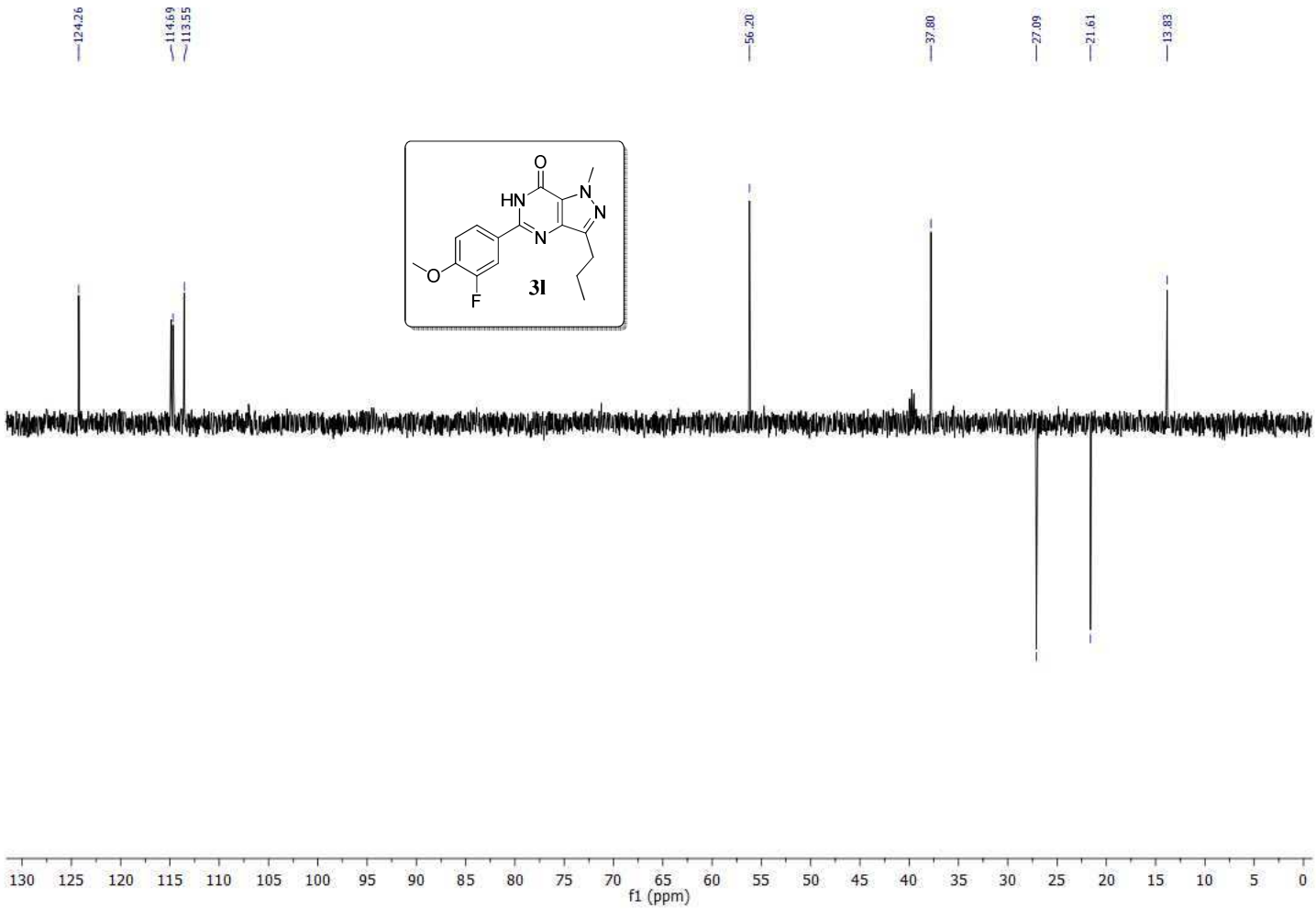
^{1}H NMR in $CDCl_3$



^{13}C NMR in DMSO-d_6



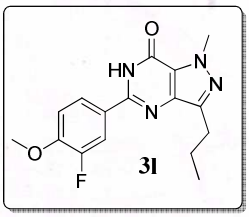
DEPT NMR in DMSO-d_6



HRMS

Qualitative Compound Report

Data File	GLR-12.d	**Sample Name**	GLR-12
Sample Type	Sample	**Position**	Vial 21
Instrument Name	Instrument 1	**User Name**	
Acq Method	vishal_MS_25072012.m	**Acquired Time**	11/19/2012 1:58:15 PM
IRM Calibration Status	Success	**DA Method**	as.m
Comment			
Sample Group	**Info.**		



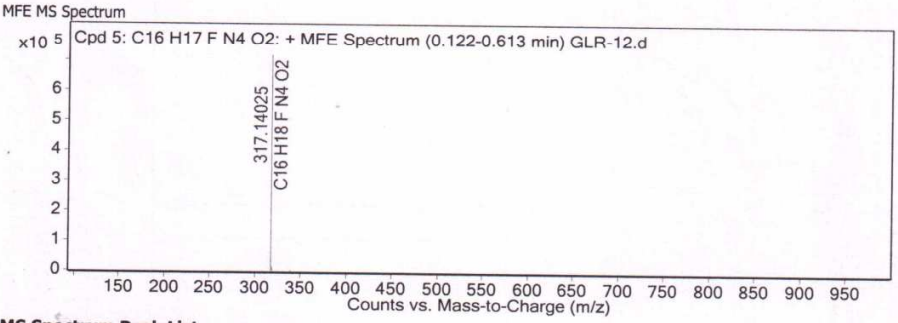
3l

Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 5: C16 H17 F N4 O2	0.171	316.13298	C16 H17 F N4 O2	C16 H17 F N4 O2	1.83	C16 H17 F N4 O2

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 5: C16 H17 F N4 O2	317.14025	0.171	Find by Molecular Feature	316.13298

MFE MS Spectrum



MS Spectrum Peak List

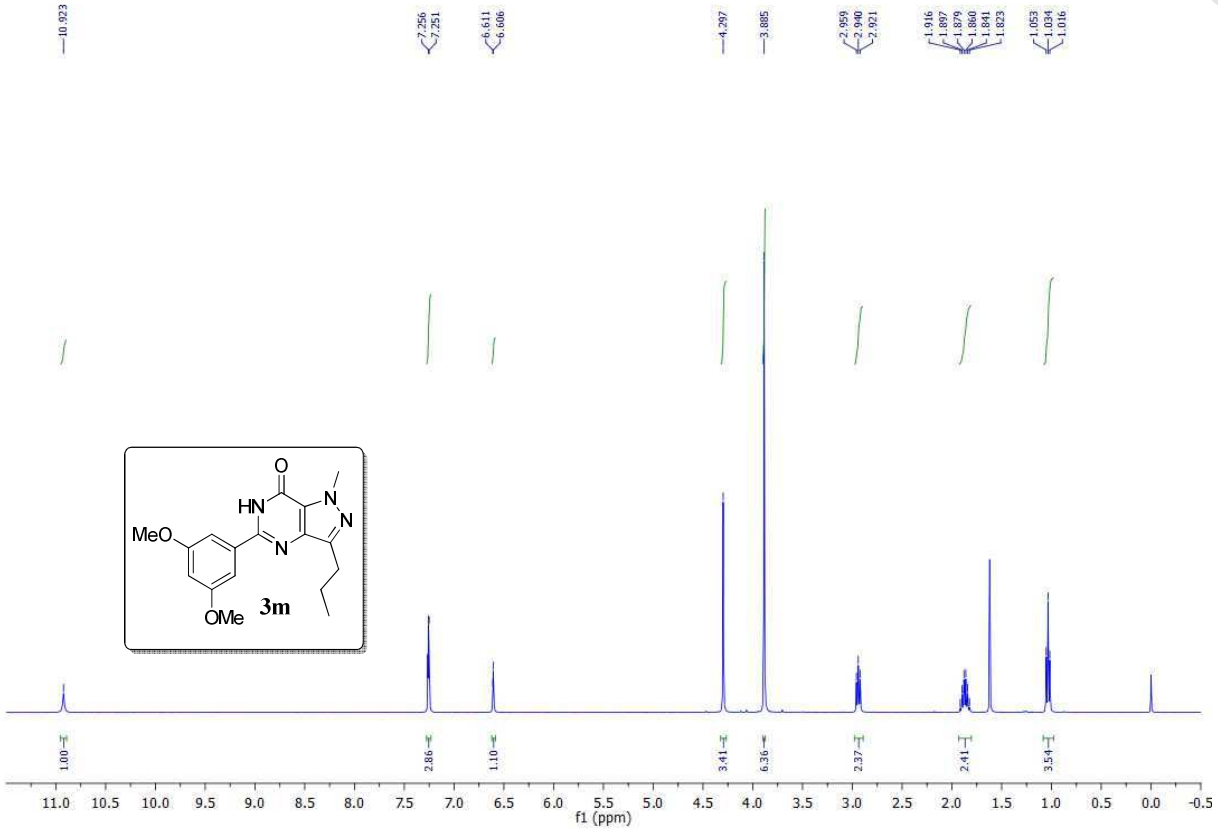
m/z	z	Abund	Formula	Ion
317.14025	1	719924.8	C16 H18 F N4 O2	(M+H)+
318.14312	1	127725.5	C16 H18 F N4 O2	(M+H)+
319.14513	1	16543	C16 H18 F N4 O2	(M+H)+
320.14798	1	1203.8	C16 H18 F N4 O2	(M+H)+
339.12253	1	3726.7	C16 H17 F N4 Na O2	(M+Na)+
340.12246	1	641.9	C16 H17 F N4 Na O2	(M+Na)+

Predicted Isotope Match Table

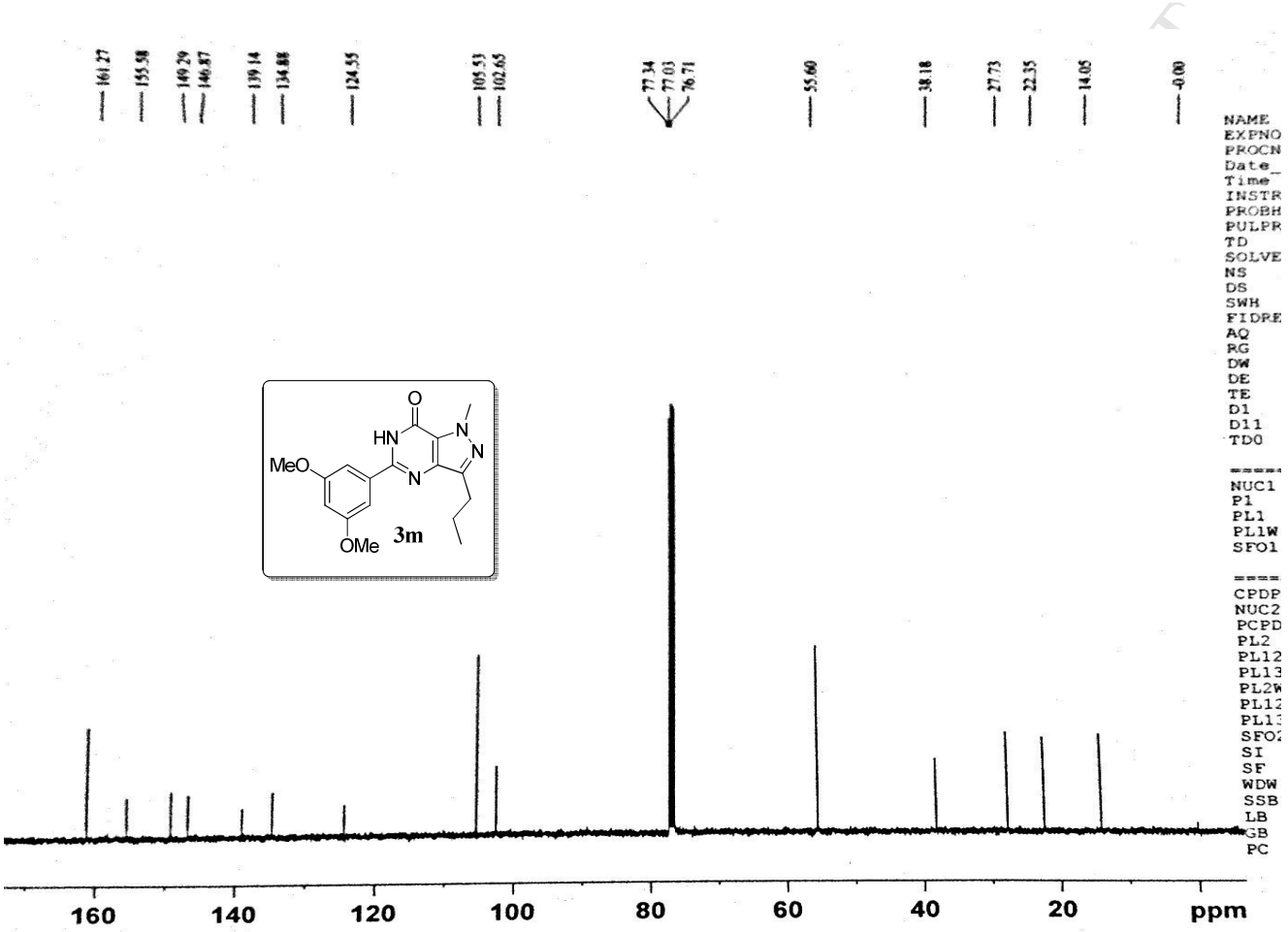
Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	317.14025	317.14083	1.82	100	100	83.19	82.4
2	318.14312	318.14374	1.93	17.74	19.05	14.76	15.7
3	319.14513	319.14631	3.7	2.3	2.13	1.91	1.75
4	320.14798	320.1488	2.54	0.17	0.18	0.14	0.14

13. Compound 3m

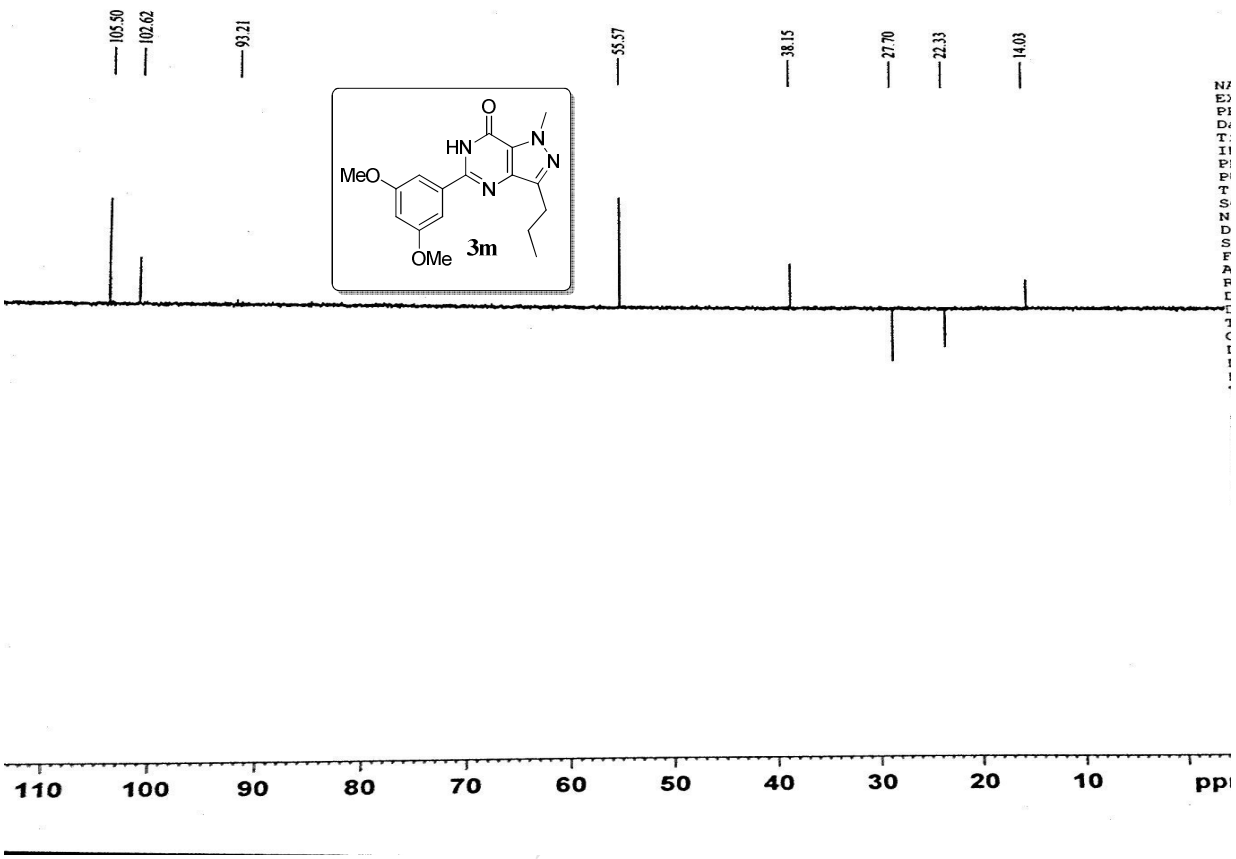
^{1}H NMR in $CDCl_3$



^{13}C NMR in $CDCl_3$



DEPT NMR in $CDCl_3$

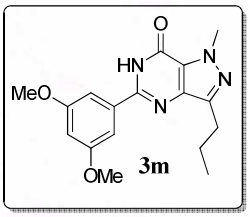


HRMS

Qualitative Compound Report

Data File	GLR-13.d	**Sample Name**	GLR-13
Sample Type	Sample	**Position**	Vial 17
Instrument Name	Instrument 1	**User Name**	
Acq Method	vishal_MS_25072012.m	**Acquired Time**	11/19/2012 1:40:29 PM
IRM Calibration Status	Success	**DA Method**	as.m
Comment			

Sample Group **Info.**

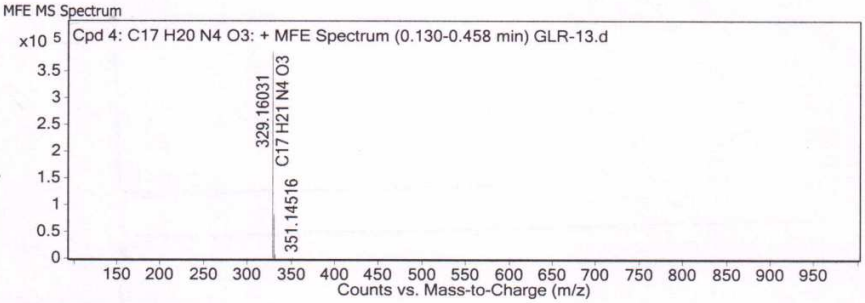


Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 4: C17 H20 N4 O3	0.171	328.15303	C17 H20 N4 O3	C17 H20 N4 O3	1.55	C17 H20 N4 O3

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 4: C17 H20 N4 O3	329.16031	0.171	Find by Molecular Feature	328.15303

MFE MS Spectrum



MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
329.16031	1	387320.4	C17 H21 N4 O3	(M+H)+
330.1629	1	81372.2	C17 H21 N4 O3	(M+H)+
331.16576	1	8449.1	C17 H21 N4 O3	(M+H)+
332.16928	1	1478.8	C17 H21 N4 O3	(M+H)+
351.14516	1	2754.7		(M+Na)+

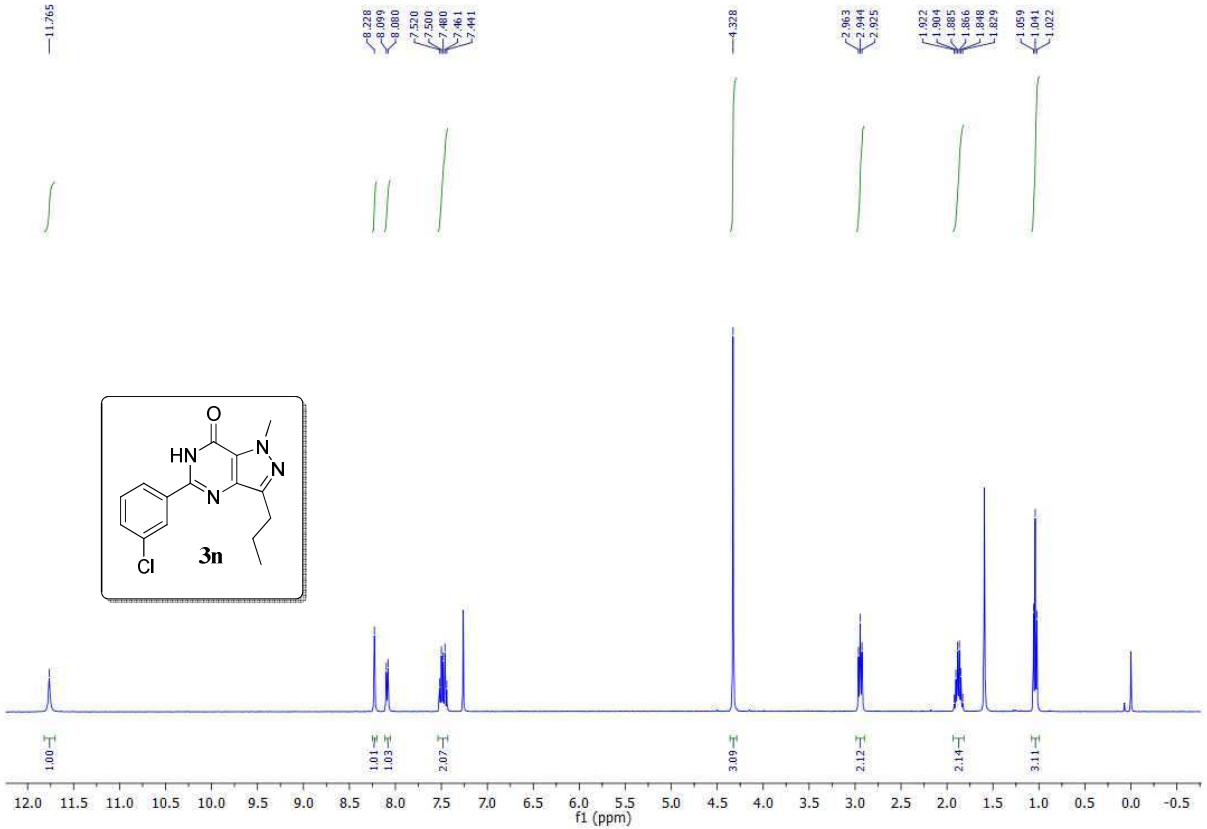
Predicted Isotope Match Table

Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	329.16031	329.16082	1.53	100	100	80.92	81.3
2	330.1629	330.16375	2.59	21.01	20.2	17	16.43
3	331.16576	331.16628	1.56	2.18	2.56	1.77	2.08
4	332.16928	332.16875	-1.59	0.38	0.24	0.31	0.2

--- End Of Report ---

14. Compound 3n

^{1}H NMR in $CDCl_3$

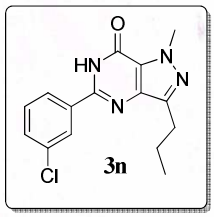


HRMS

Qualitative Compound Report

Data File	GLR-14.d	**Sample Name**	GLR-14
Sample Type	Sample	**Position**	Vial 20
Instrument Name	Instrument 1	**User Name**	
Acq Method	vishal_MS_25072012.m	**Acquired Time**	11/19/2012 1:54:41 PM
IRM Calibration Status	Success	**DA Method**	as.m
Comment			

Sample Group **Info.**

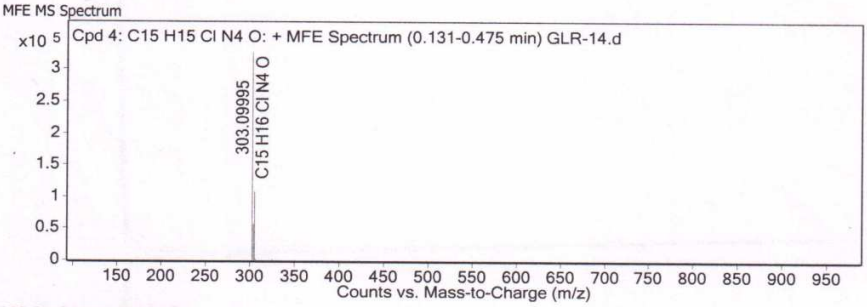


Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 4: C15 H15 Cl N4 O	0.172	302.09267	C15 H15 Cl N4 O	C15 H15 Cl N4 O	2.55	C15 H15 Cl N4 O

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 4: C15 H15 Cl N4 O	303.09995	0.172	Find by Molecular Feature	302.09267

MFE MS Spectrum



MS Spectrum Peak List

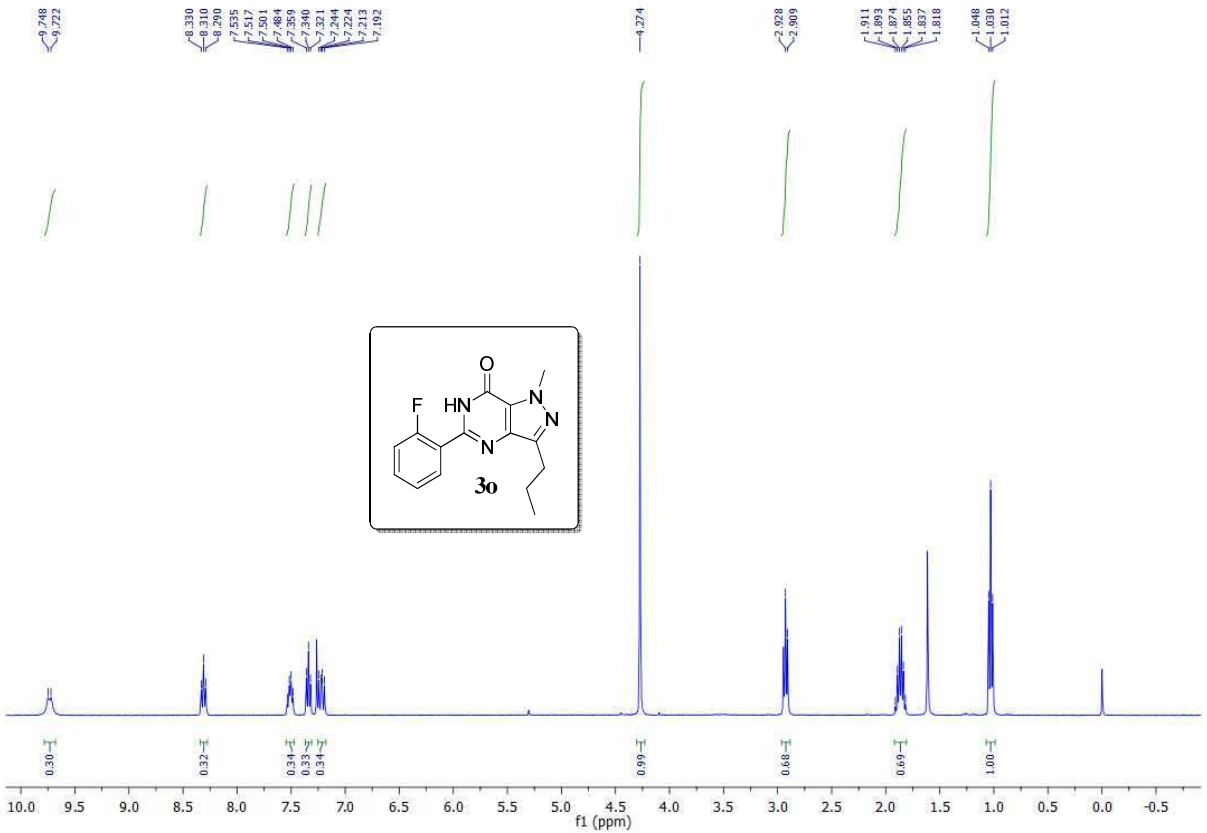
m/z	z	Abund	Formula	Ion
303.09995	1	327725.1	C15 H16 Cl N4 O	(M+H)+
304.10235	1	56778.1	C15 H16 Cl N4 O	(M+H)+
305.09724	1	108261.9	C15 H16 Cl N4 O	(M+H)+
306.09989	1	19209.3	C15 H16 Cl N4 O	(M+H)+
307.10223	1	1784.4	C15 H16 Cl N4 O	(M+H)+
325.08122	1	2002.6	C15 H15 Cl N4 Na O	(M+Na)+

Predicted Isotope Match Table

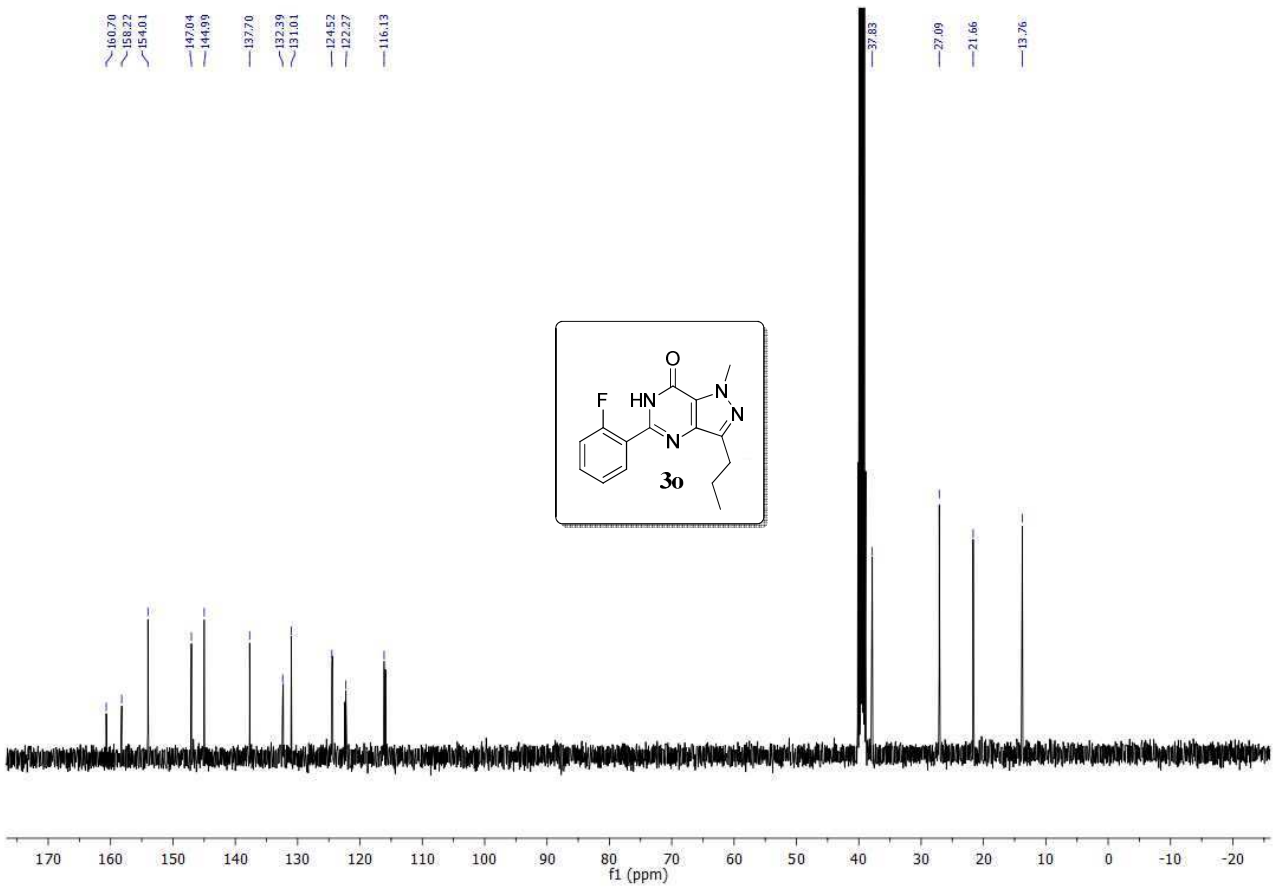
Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	303.09995	303.10072	2.51	100	100	63.79	63.28
2	304.10235	304.10359	4.08	17.32	17.91	11.05	11.33
3	305.09724	305.0982	3.15	33.03	33.71	21.07	21.33
4	306.09989	306.1008	2.96	5.86	5.85	3.74	3.7
5	307.10223	307.10338	3.75	0.54	0.56	0.35	0.35

15. Compound 3o

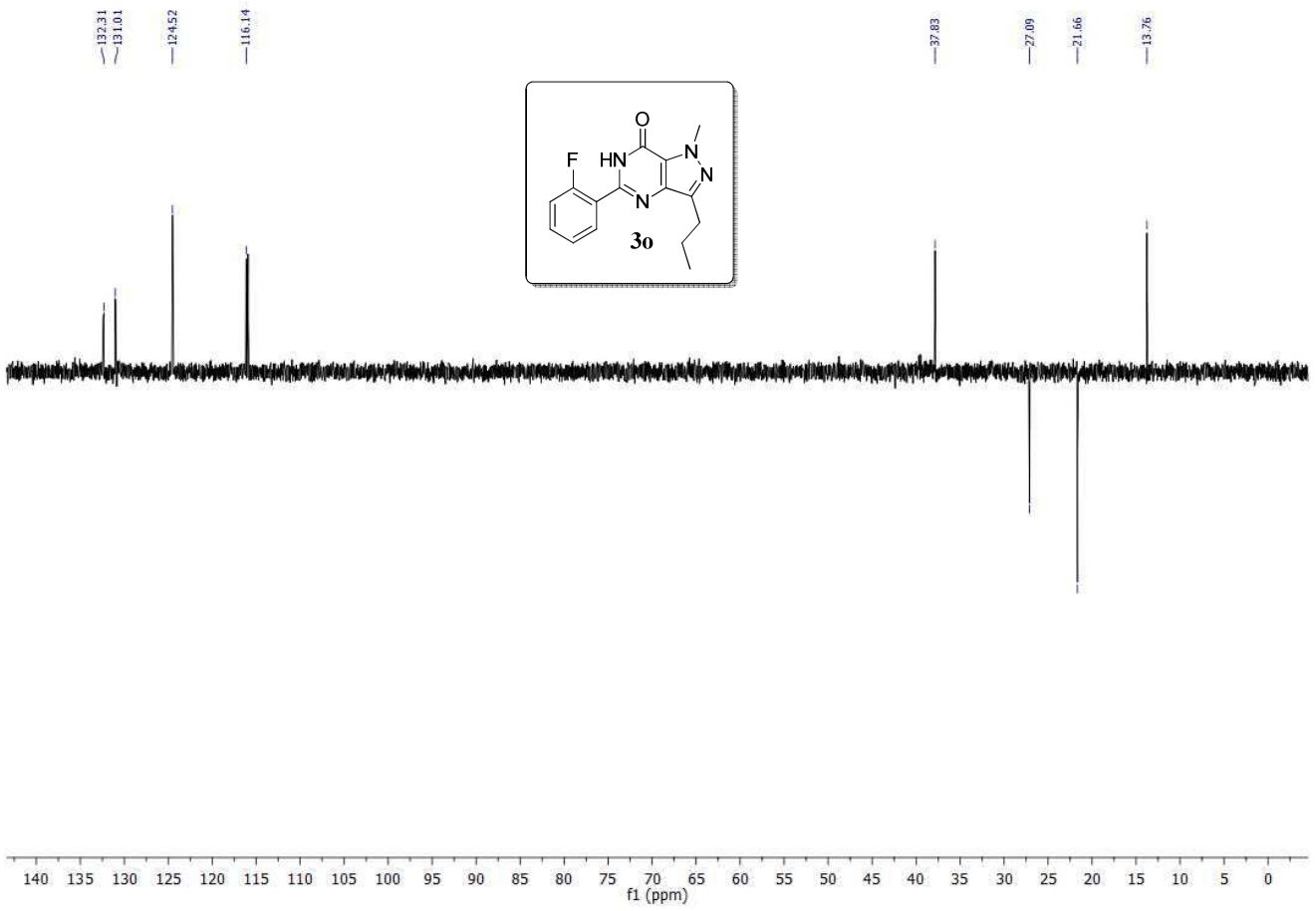
^{1}H NMR in $CDCl_3$



^{13}C NMR in DMSO-d_6



DEPT NMR in DMSO-d_6

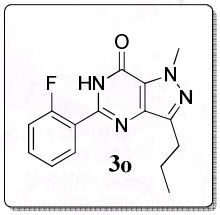


HRMS

Qualitative Compound Report

Data File	GLR-15.d	**Sample Name**	GLR-15
Sample Type	Sample	**Position**	Vial 25
Instrument Name	Instrument 1	**User Name**	
Acq Method	vishal_MS_25072012.m	**Acquired Time**	11/19/2012 2:15:56 PM
IRM Calibration Status	Success	**DA Method**	as.m
Comment			

Sample Group **Info.**

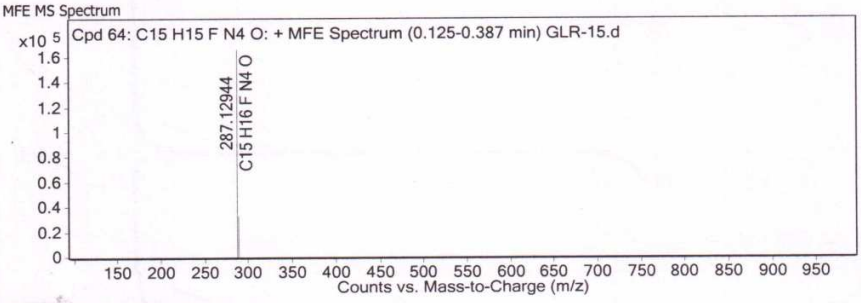


Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 64: C15 H15 F N4 O	0.176	286.12217	C15 H15 F N4 O	C15 H15 F N4 O	2.87	C15 H15 F N4 O

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 64: C15 H15 F N4 O	287.12944	0.176	Find by Molecular Feature	286.12217

MFE MS Spectrum



MS Spectrum Peak List

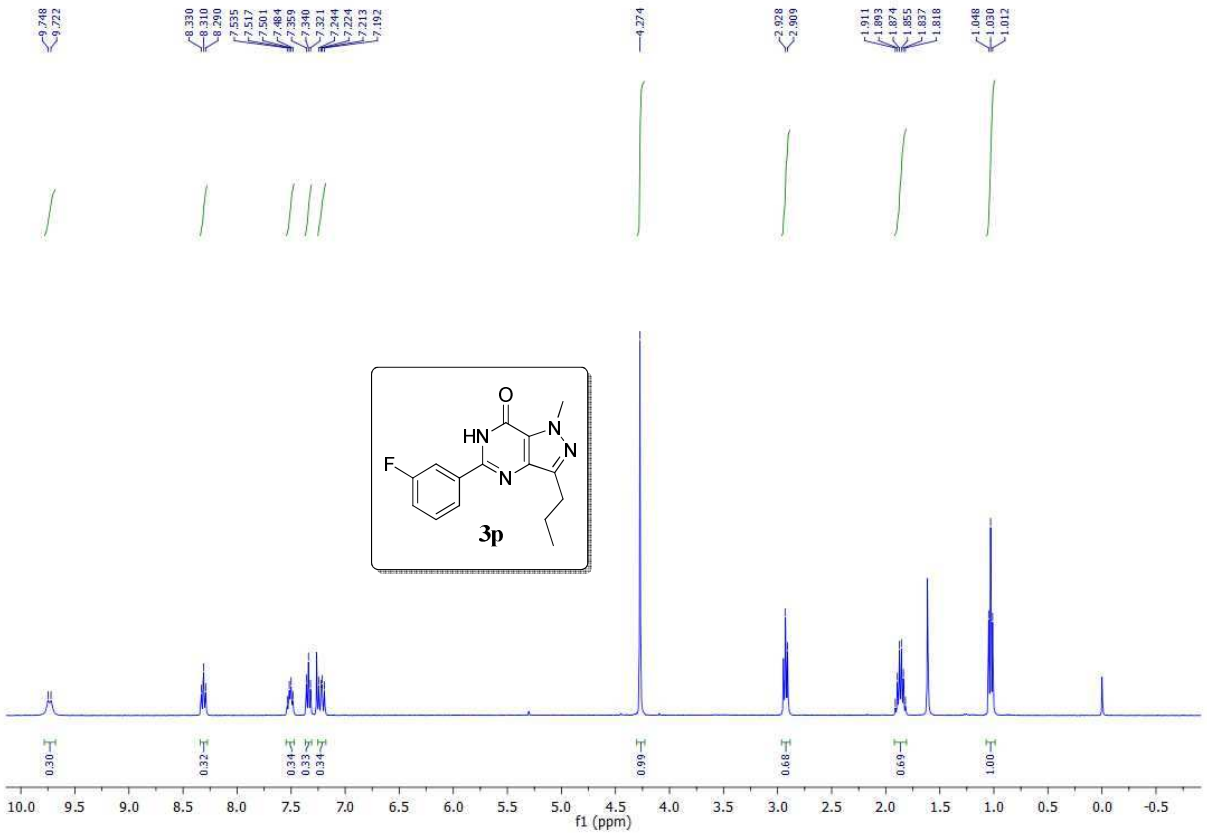
m/z	z	Abund	Formula	Ion
287.12944	1	165751.1	C15 H16 F N4 O	(M+H)+
288.13243	1	32258.4	C15 H16 F N4 O	(M+H)+
289.13631	1	3908.1	C15 H16 F N4 O	(M+H)+

Predicted Isotope Match Table

Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	287.12944	287.13027	2.86	100	100	82.09	83.59
2	288.13243	288.13314	2.47	19.46	17.91	15.98	14.97
3	289.13631	289.1358	-1.79	2.36	1.72	1.94	1.44

16. Compound 3p

^{1}H NMR in $CDCl_3$

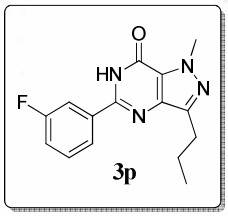


HRMS

Qualitative Compound Report

Data File	GLR-A-16.d	**Sample Name**	GLR-A-16
Sample Type	Sample	**Position**	Vial 12
Instrument Name	Instrument 1	**User Name**	
Acq Method	vishal_12-01-13.m	**Acquired Time**	09-07-2013 PM 1:18:59
IRM Calibration Status	Success	**DA Method**	daily_report.m
Comment			

Sample Group **Info.**
Acquisition SW Version 6200 series TOF/6500 series Q-TOF B.05.01 (B5125)



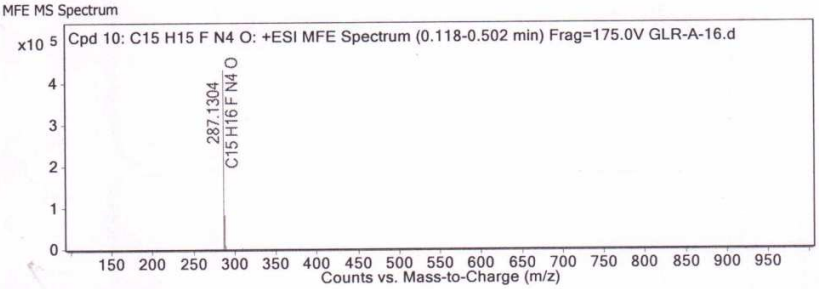
3p

Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 10: C15 H15 F N4 O	0.193	286.1231	C15 H15 F N4 O	C15 H15 F N4 O	-0.45	C15 H15 F N4 O

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 10: C15 H15 F N4 O	287.1304	0.193	Find by Molecular Feature	286.1231

MFE MS Spectrum



MS Spectrum Peak List

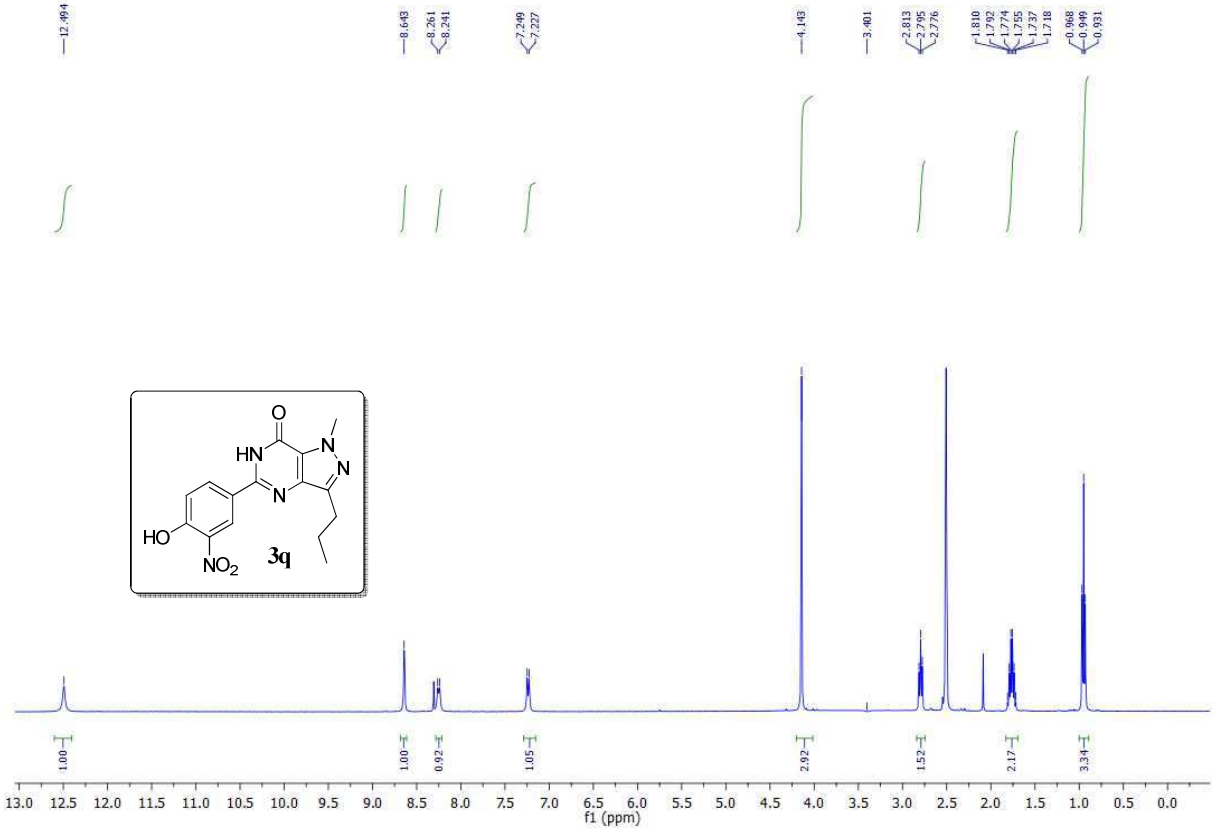
m/z	z	Abund	Formula	Ion
287.1304	1	431654.78	C15 H16 F N4 O	(M+H)+
288.1332	1	79701.38	C15 H16 F N4 O	(M+H)+
289.1356	1	7297.76	C15 H16 F N4 O	(M+H)+
290.1436	1	480.78	C15 H16 F N4 O	(M+H)+
309.1122	1	2202.81	C15 H15 F N4 Na O	(M+Na)+
310.1191	1	273.13	C15 H15 F N4 Na O	(M+Na)+

Predicted Isotope Match Table

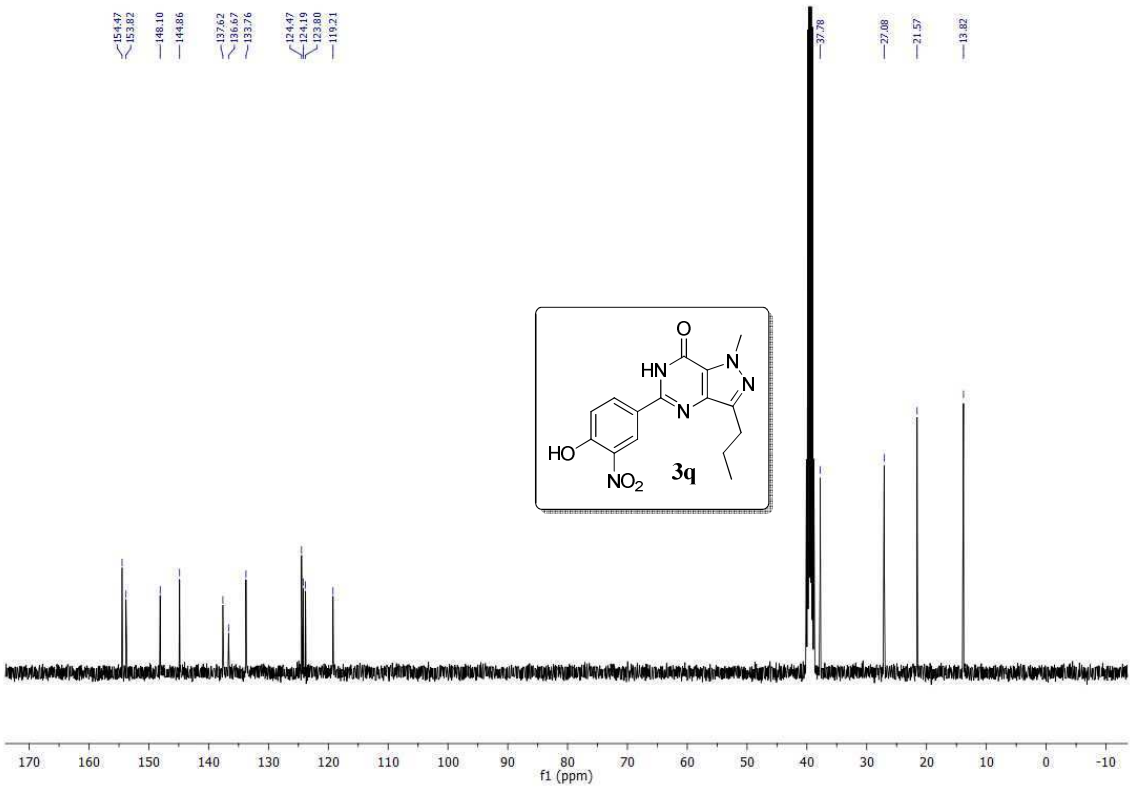
Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	287.1304	287.1303	-0.51	100	100	83.15	83.51
2	288.1332	288.1331	-0.11	18.46	17.91	15.35	14.95
3	289.1356	289.1358	0.63	1.69	1.72	1.41	1.44
4	290.1436	290.1383	-18.03	0.11	0.12	0.09	0.1

17. Compound 3q

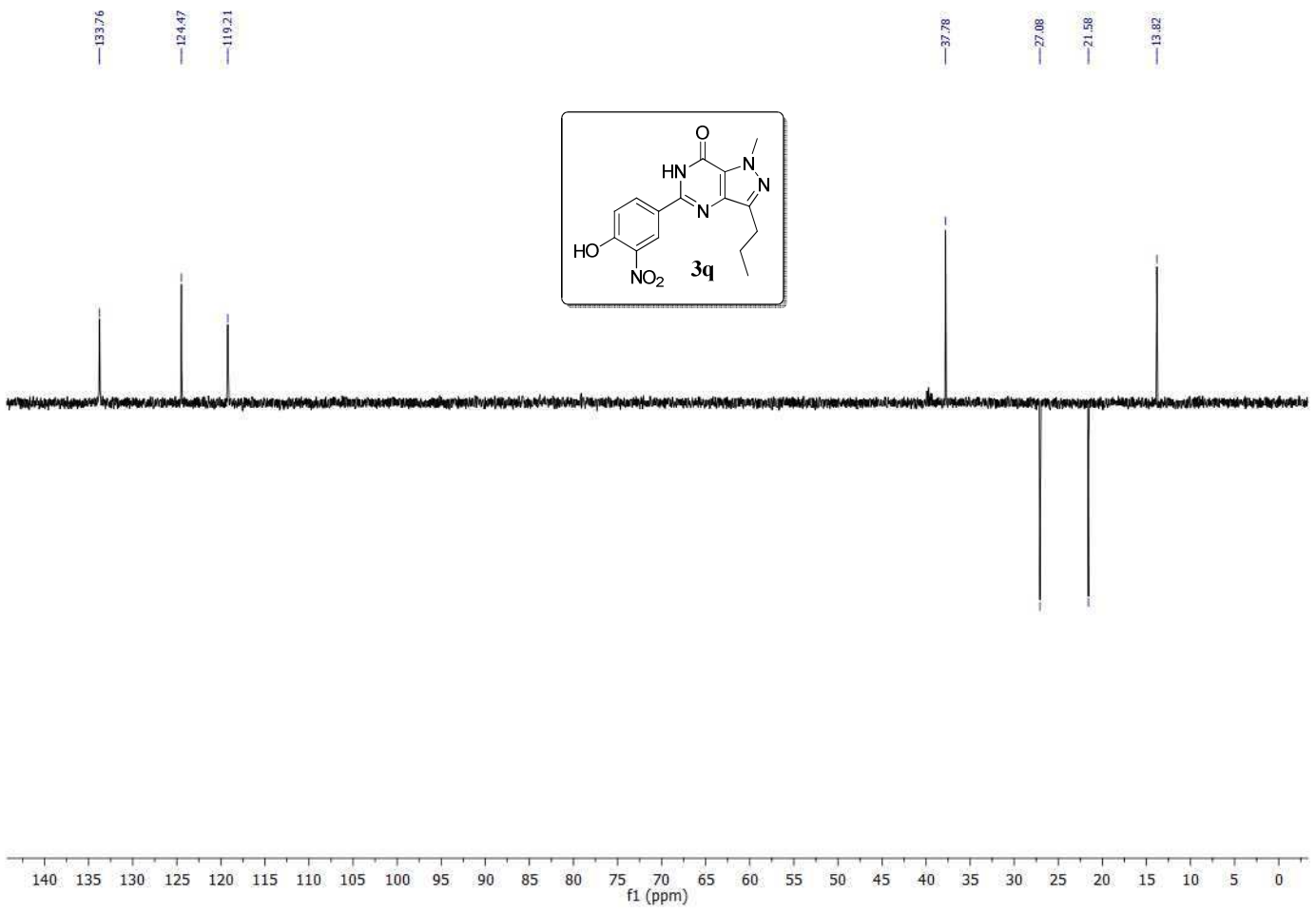
^{1}H NMR in DMSO-d_6



^{13}C NMR in DMSO-d_6



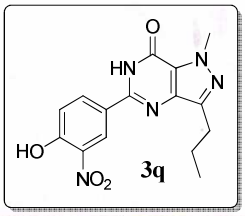
DEPT NMR in DMSO-d_6



HRMS

Qualitative Compound Report

Data File	GLR-17.d	**Sample Name**	Unavailable
Sample Type	Unavailable	**Position**	Unavailable
Instrument Name	Unavailable	**User Name**	Unavailable
Acq Method		**Acquired Time**	Unavailable
IRM Calibration Status	Success	**DA Method**	as.m
Comment	Sample information is unavailable		

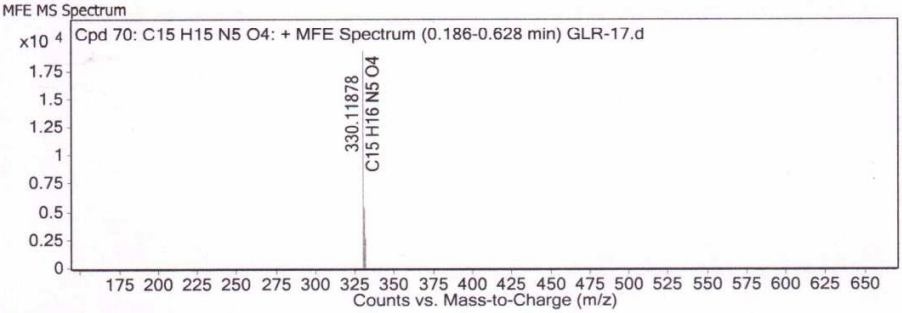


Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 70: C15 H15 N5 O4	0.568	329.11153	C15 H15 N5 O4	C15 H15 N5 O4	2.67	C15 H15 N5 O4

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 70: C15 H15 N5 O4	330.11878	0.568	Find by Molecular Feature	329.11153

MFE MS Spectrum



MS Spectrum Peak List

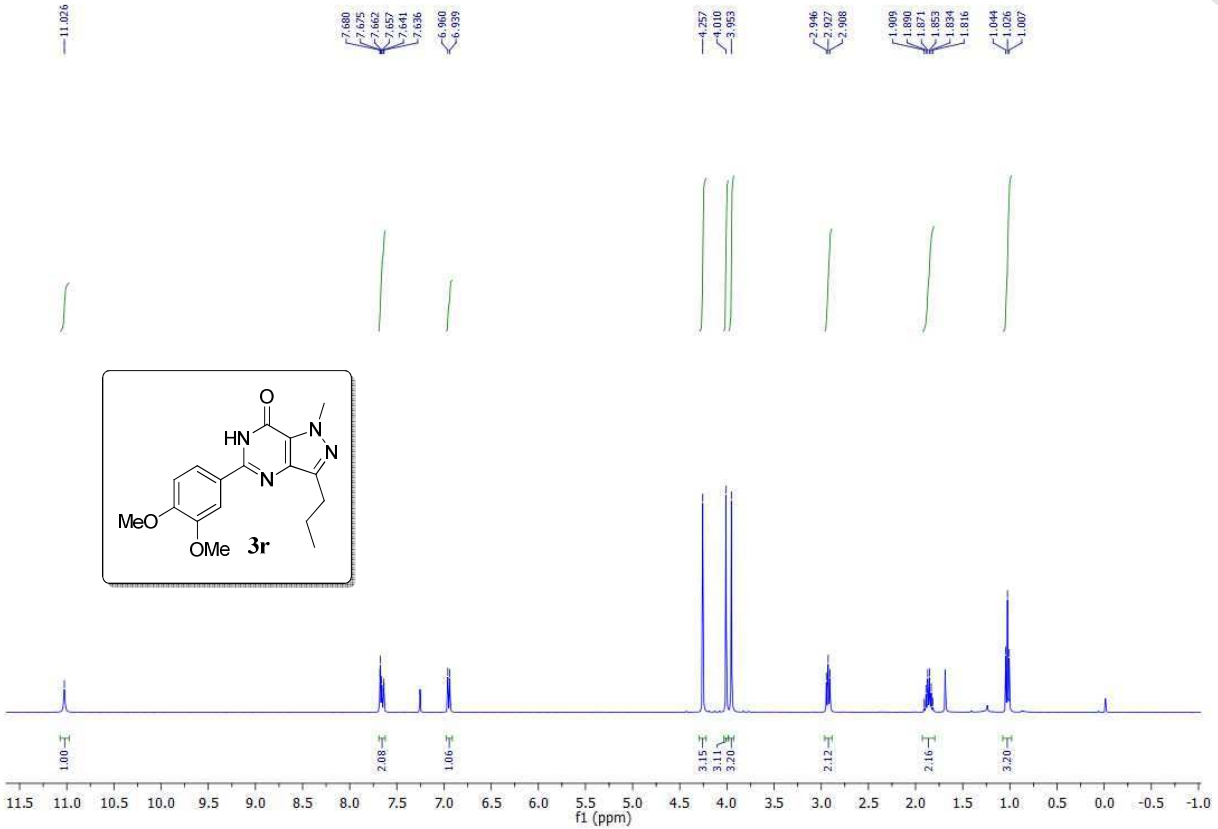
m/z	z	Abund	Formula	Ion
330.11878	1	19252.1	C15 H16 N5 O4	(M+H)+
331.12188	1	5363.5	C15 H16 N5 O4	(M+H)+
332.13058	1	2642.2	C15 H16 N5 O4	(M+H)+

Predicted Isotope Match Table

Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	330.11878	330.11968	2.73	100	100	70.63	82.78
2	331.12188	331.12244	1.71	27.86	18.39	19.68	15.22
3	332.13058	332.12475	-17.55	13.72	2.42	9.69	2

18. Compound 3r

^{1}H NMR in $CDCl_3$

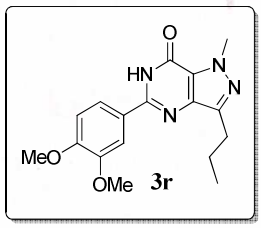


HRMS

Qualitative Compound Report

Data File	GLR-18.d	**Sample Name**	GLR-18
Sample Type	Sample	**Position**	Vial 16
Instrument Name	Instrument 1	**User Name**	
Acq Method	vishal_MS_25072012.m	**Acquired Time**	11/19/2012 1:33:25 PM
IRM Calibration Status	Success	**DA Method**	as.m
Comment			

Sample Group **Info.**

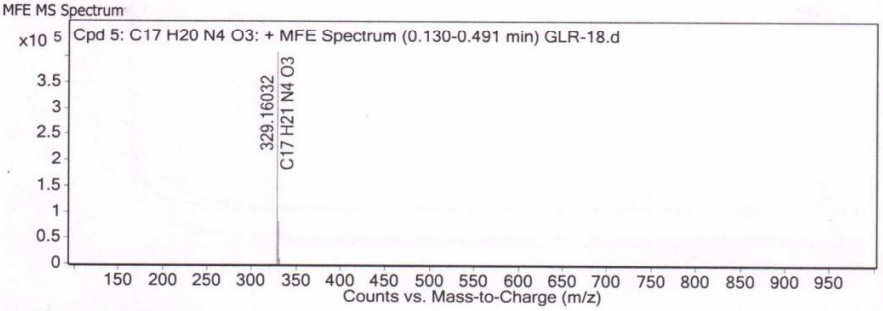


Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 5: C17 H20 N4 O3	0.171	328.15304	C17 H20 N4 O3	C17 H20 N4 O3	1.53	C17 H20 N4 O3

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 5: C17 H20 N4 O3	329.16032	0.171	Find by Molecular Feature	328.15304

MFE MS Spectrum



MS Spectrum Peak List

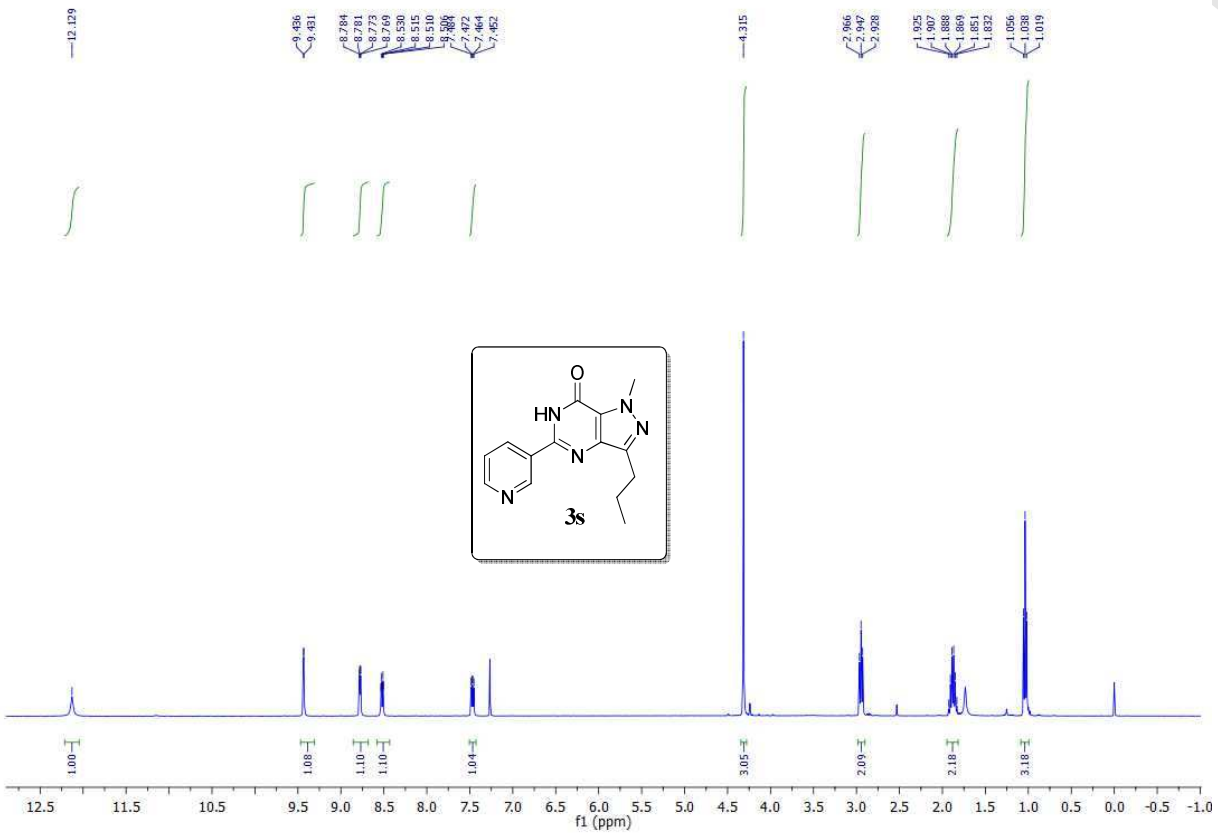
m/z	z	Abund	Formula	Ion
329.16032	1	409042.8	C17 H21 N4 O3	(M+H)+
330.16284	1	81408.9	C17 H21 N4 O3	(M+H)+
331.1662	1	11141.1	C17 H21 N4 O3	(M+H)+
332.16535	1	1211.2	C17 H21 N4 O3	(M+H)+

Predicted Isotope Match Table

Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	329.16032	329.16082	1.51	100	100	81.35	81.3
2	330.16284	330.16375	2.77	19.9	20.2	16.19	16.43
3	331.1662	331.16628	0.23	2.72	2.56	2.22	2.08
4	332.16535	332.16875	10.25	0.3	0.24	0.24	0.2

19. Compound 3s

^{1}H NMR in $CDCl_3$

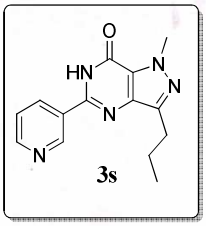


HRMS

Qualitative Compound Report

Data File	daily ms029.d	**Sample Name**	PY-2
Sample Type	Sample	**Position**	69
Instrument Name	Instrument 1	**User Name**	
Acq Method	DAILY MS DESI.m	**Acquired Time**	2/3/2012 5:05:14 PM
IRM Calibration Status	All Ions Missed	**DA Method**	as.m
Comment			

Sample Group **Info.**



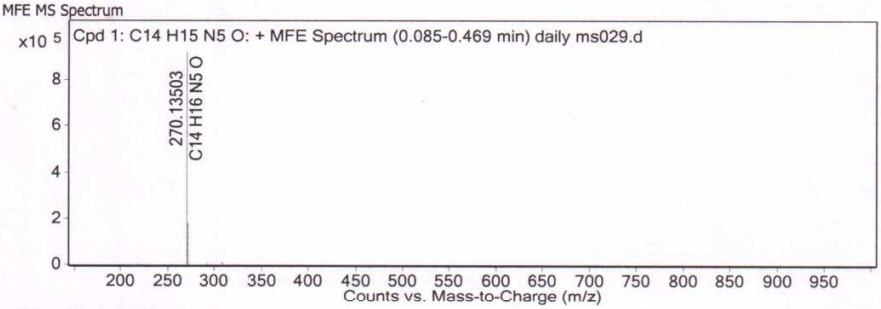
3s

Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 1: C14 H15 N5 O	0.121	269.12776	C14 H15 N5 O	C14 H15 N5 O	-0.36	C14 H15 N5 O

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 1: C14 H15 N5 O	270.13503	0.121	Find by Molecular Feature	269.12776

MFE MS Spectrum



MS Spectrum Peak List

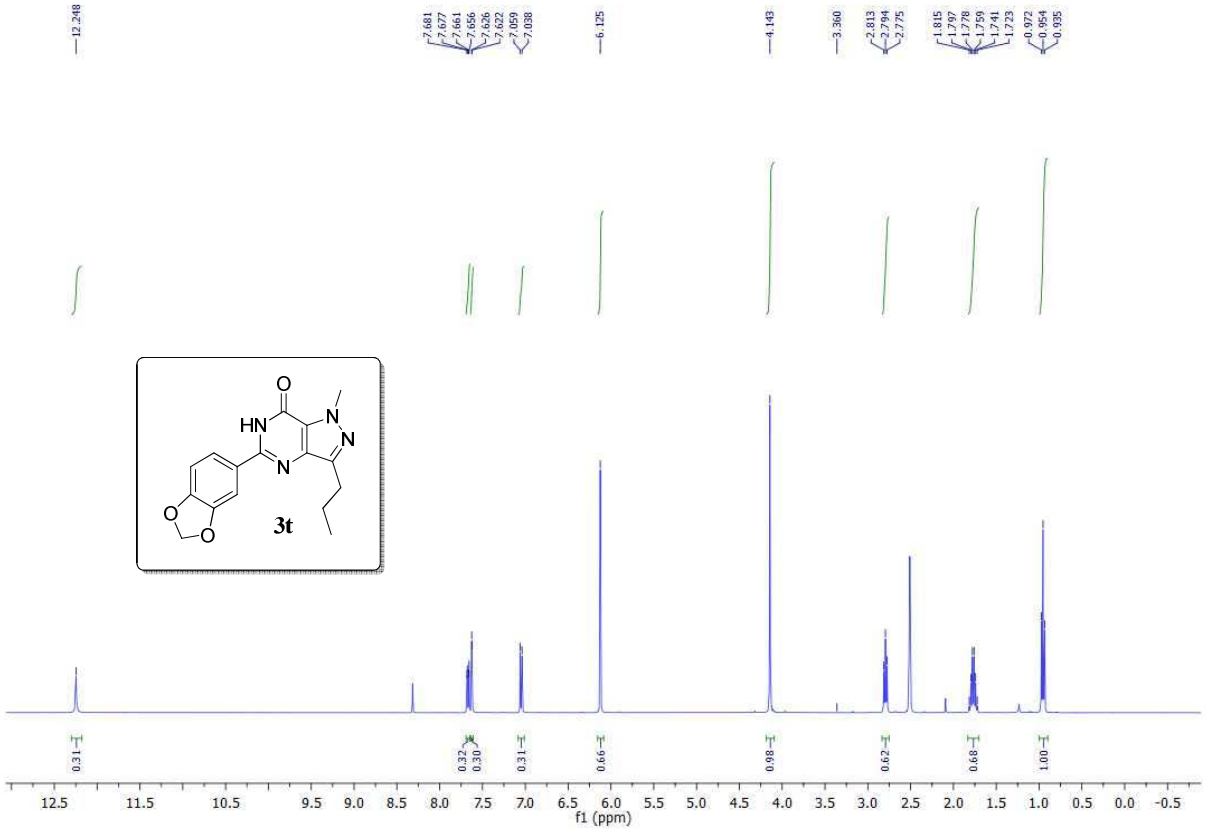
m/z	z	Abund	Formula	Ion
270.13503	1	919337.8	C14 H16 N5 O	(M+H)+
271.13807	1	178131.8	C14 H16 N5 O	(M+H)+
272.14014	1	13954.7	C14 H16 N5 O	(M+H)+
273.14255	1	822.6	C14 H16 N5 O	(M+H)+
292.11731	1	4681.6	C14 H15 N5 Na O	(M+Na)+
293.12003	1	988.6	C14 H15 N5 Na O	(M+Na)+
308.08798	1	7653.7	C14 H15 K N5 O	(M+K)+
309.09111	1	1644.3	C14 H15 K N5 O	(M+K)+
310.08961	1	642.8	C14 H15 K N5 O	(M+K)+

Predicted Isotope Match Table

Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	270.13503	270.13494	-0.35	100	100	82.66	84.11
2	271.13807	271.13765	-1.53	19.38	17.19	16.02	14.46
3	272.14014	272.14018	0.12	1.52	1.6	1.25	1.34
4	273.14255	273.14259	0.14	0.09	0.11	0.07	0.09

20. Compound 3t

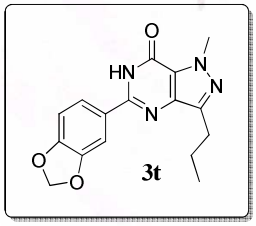
^{1}H NMR in DMSO-d_6



HRMS

Qualitative Compound Report

Data File	GLR-21.d	**Sample Name**	GLR-21
Sample Type	Sample	**Position**	Vial 24
Instrument Name	Instrument 1	**User Name**	
Acq Method	vishal_MS_25072012.m	**Acquired Time**	11/19/2012 2:12:25 PM
IRM Calibration Status	Success	**DA Method**	as.m
Comment			
Sample Group	**Info.**		

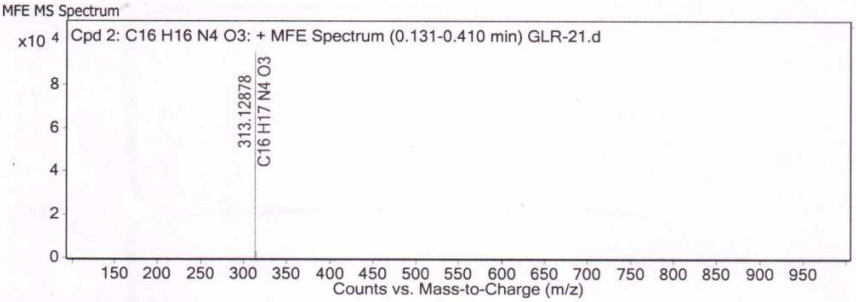


Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 2: C16 H16 N4 O3	0.171	312.12151	C16 H16 N4 O3	C16 H16 N4 O3	2.36	C16 H16 N4 O3

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 2: C16 H16 N4 O3	313.12878	0.171	Find by Molecular Feature	312.12151

MFE MS Spectrum



MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
313.12878	1	95061	C16 H17 N4 O3	(M+H)+
314.13235	1	17694.3	C16 H17 N4 O3	(M+H)+
315.13432	1	2834.8	C16 H17 N4 O3	(M+H)+

Predicted Isotope Match Table

Isotope	*m/z*	Calc m/z	Diff (ppm)	Abund %	Calc Abund %	Abund Sum %	Calc Abund Sum %
1	313.12878	313.12952	2.36	100	100	82.24	82.36
2	314.13235	314.13242	0.24	18.61	19.08	15.31	15.71
3	315.13432	315.13489	1.8	2.98	2.34	2.45	1.93

References:

1. K.M. Khan, G.M. Maharvia, M.I. Choudhary, A. Rahman, S. Perveen, J. Heterocyclic Chem. 42 (2005) 1085-1093.
2. N.R. Reddy, G.M. Reddy, B.S. Reddy, P.P. Reddy, J. Heterocyclic Chem. 42 (2005) 751-754.
3. N. Mulakayala, B. Kandagatla, Ismail, R.K. Rapolu, P. Rao, C. Mulakayala, C.S. Kumar, J. Iqbal, S. Oruganti, Bioorg. Med. Chem. Lett. 22 (2012) 5063–5066
4. R.J. Abdel-Jalil, M. Khanfar, S. Al-Gharabli, M.M. El-Abadelah, K. Eichele, M.U. Anwar, W. Voelter, Heterocycles 65(8) (2005) 1821-1827.