Synthesis, Kinetics and Cellular studies of new phenothiazine analogs as potent human-TLK inhibitors

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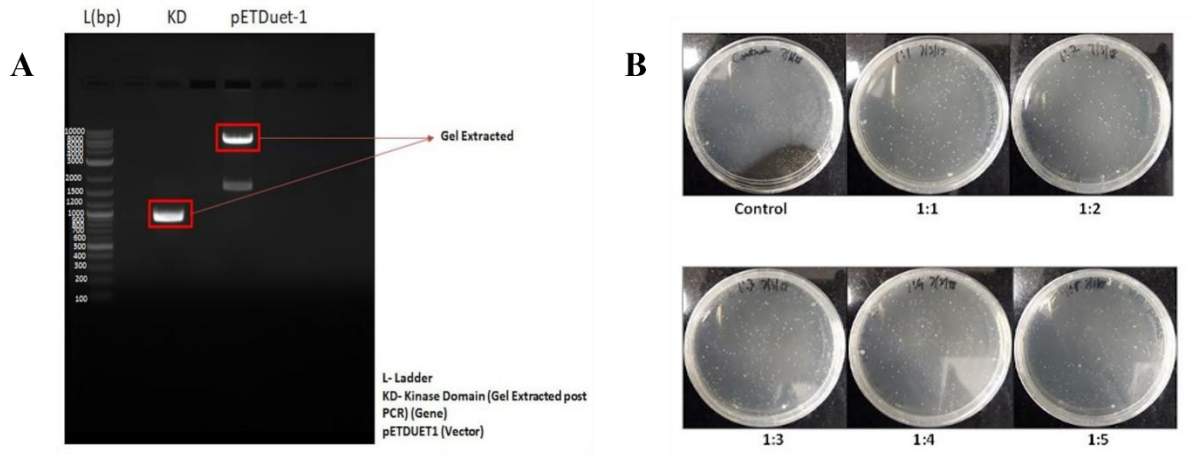


Figure S1: PCR amplification and ligation of TLK-kinase domain (A) PCR amplification and restriction digestion of hTLK1B_KD and pETDUET-1 vector. Lane 1: Ladder mark, Lane 2 (KD) describes the PCR amplification of 919 bp of hTLK1B_KD using pETDUET-KD_FOR, 5'-ACAGAGGATCCAAAGGACCACCCGACCCTG-3' and pETDUET-KD_REV, 5'-TCGTTGClonTCGTCGCCATTGGACAAGATCTTAAGACGACA-3' and followed by its restriction digestion using BamHI and EcoRI restriction enzymes, Lane 3 (pETDuet-1) describes the restriction digestion of the pTDUET-1 vector. The digested samples were extracted for ligation. The samples were run on 1% Agarose gel. **(B)** Bacterial transformation of the ligated product. The ligation was carried out in different vector: insert ratios (1:1-1:5). The ligation product was transformed into *Escherichia coli* DH5α™ cells. Individual colonies were picked to screen for positive clones from each ratio. Reaction mixture without insert was used as a re-ligation control to analyse the frequency of re-ligation of vector backbone to itself. 2-3 colonies of self-ligated vectors were observed.

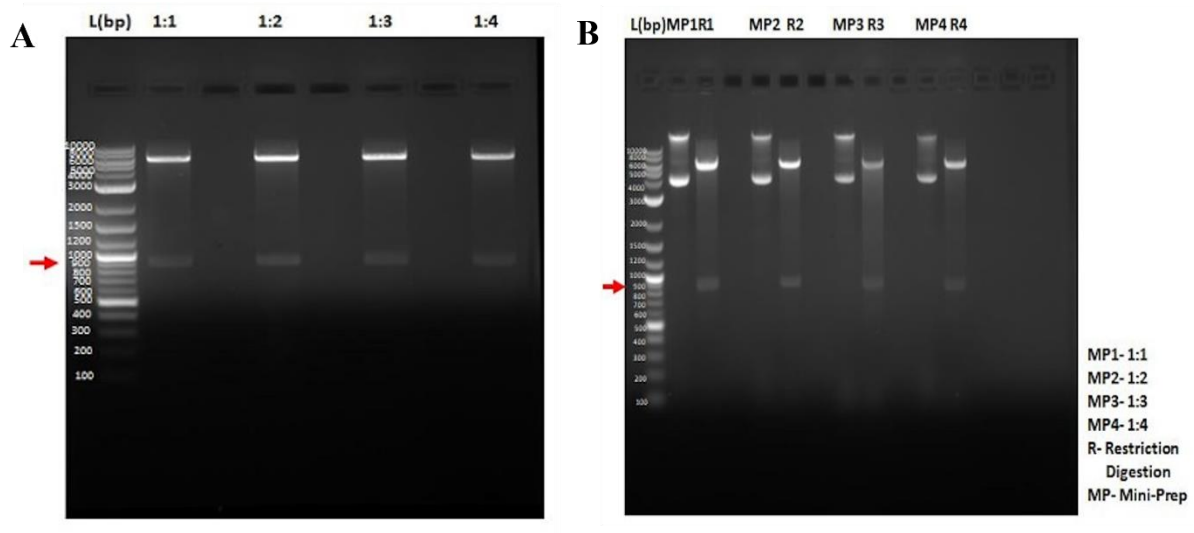


Figure S2: Restriction digestion of positive ligated colonies **(a)** First screening of the positive clones (Restriction digestion). The restriction digestion pattern was obtained as expected at 919 bp ladder mark (red arrow). All were positive clones. (b) Re-confirms the positive clones after plasmid scale-up. Undigested vector was used as a control. Restriction digestion yielded an

expected banding pattern of hTLK1B_KD at 919 bp ladder mark (red arrow). All were positive clones.

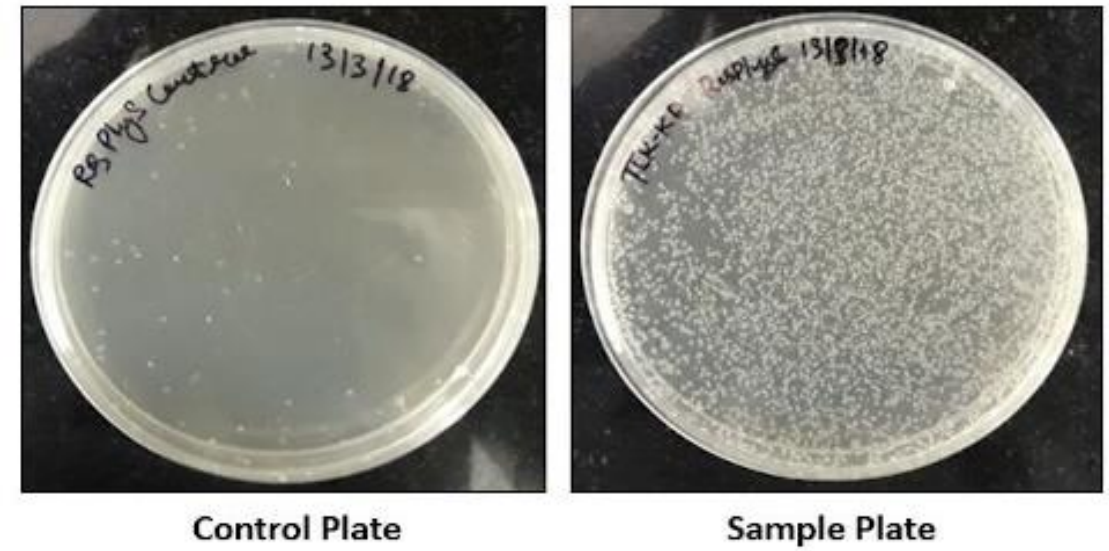


Figure S3: Bacterial transformation of constructed plasmid. The confirmed cloned plasmid was transformed into*Escherichia coli* Rosetta Gami 2™ (DE3) pLysS cells for the expression studies and other downstream applications. Control showed no colonies (the visible air bubbles are artifacts occurred as a result of preparative procedures)

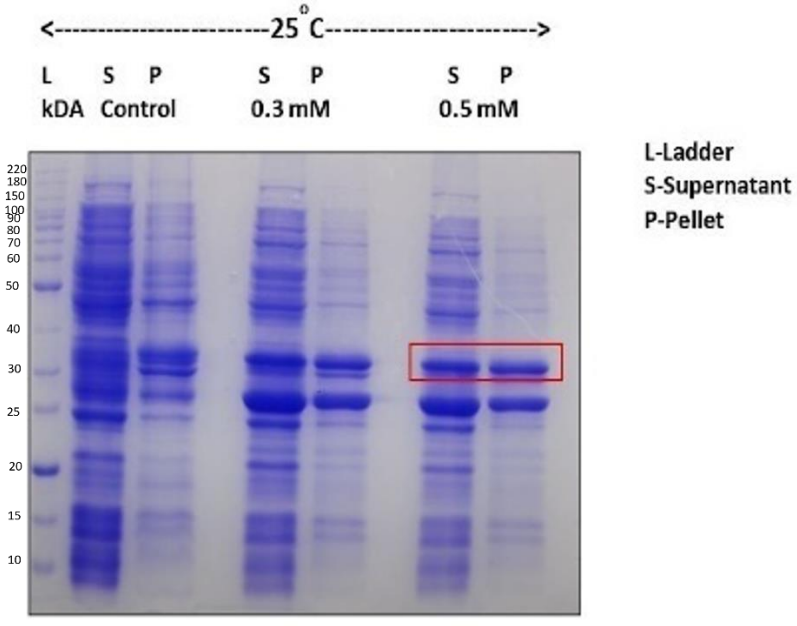


Figure S4: Optimisation of hTLK1B-KD expression. 12% SDS-PAGE gel image of the expression tests optimised at 25°C and at different IPTG concentrations (0.3mM and 0.5mM). Control was kept uninduced. The abbreviations are as follows, S, Supernatant; P, Pellet.

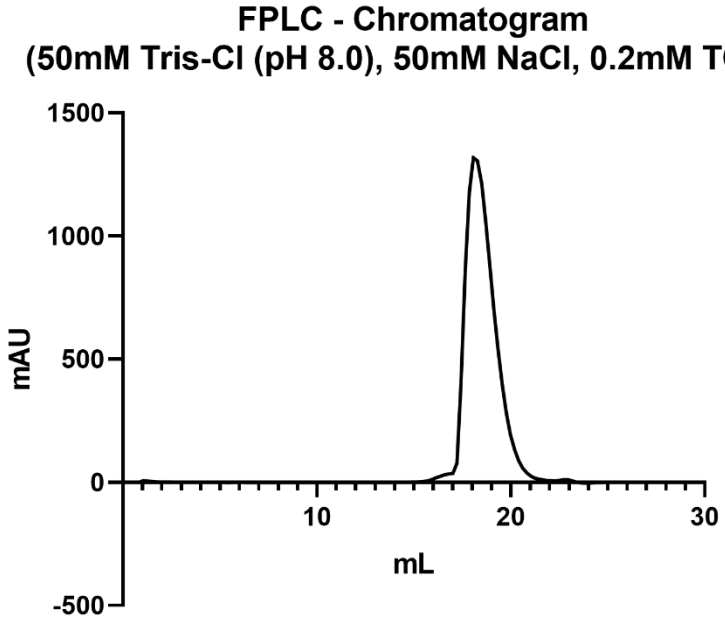
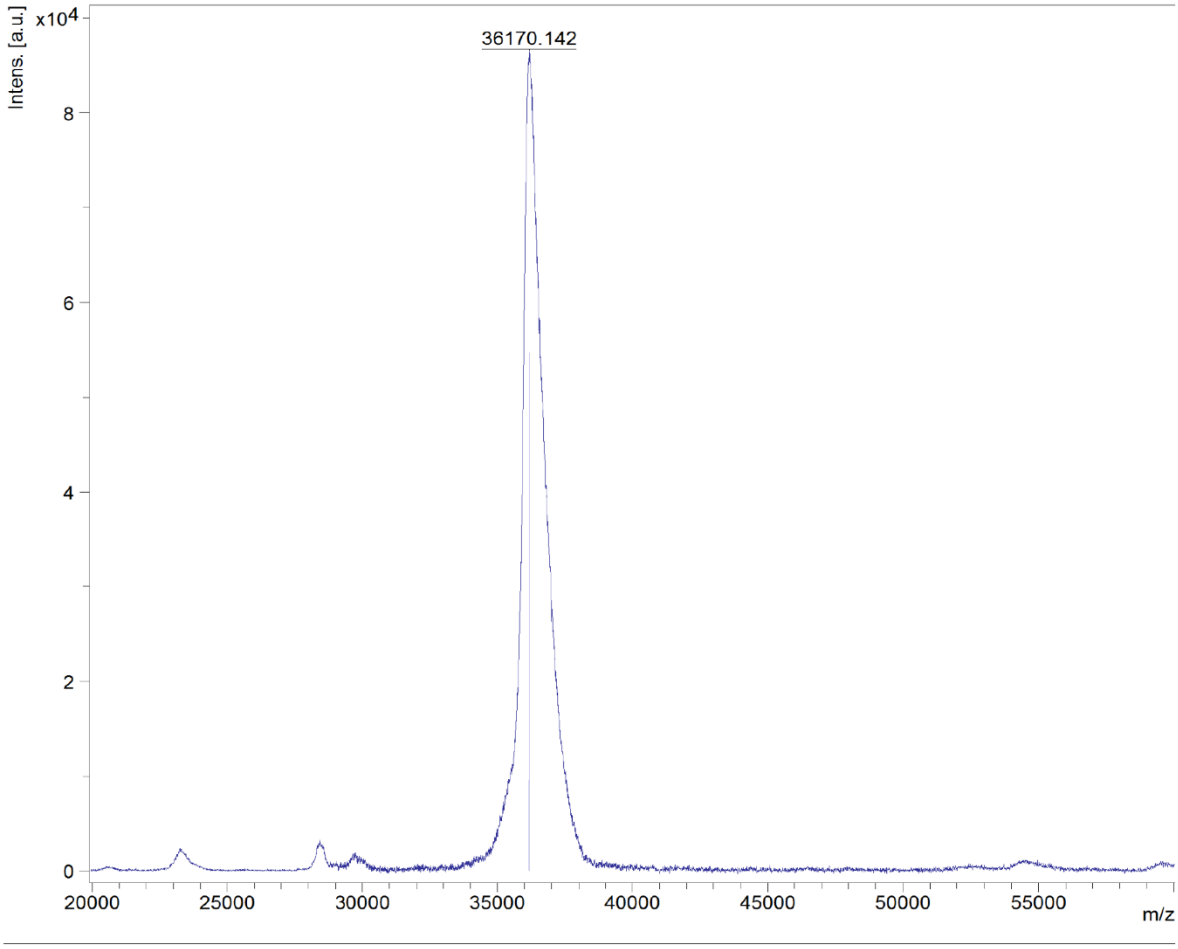


Figure S5: Size exclusion chromatography using FPLC of hTLK1B_KD. The concentrated protein was buffer-exchanged in 50 mM Tris-Cl (pH-8.0), 50 mM NaCl and 0.2 mM TCEP.



Acquisition Parameter

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Aquisition operation mode	Linear
Voltage polarity	POS
Number of shots	1500
Name of spectrum used for calibration	
Calibration reference list used	Protein_BSA

Figure S6: Mass spectrometry analysis of recombinant fusion protein. MALDI-TOF mass spectrometry analysis of hTLK1B-KD: The molecular weight of the protein was found to be 36.1 KDa, which was in agreement with the theoretically calculated molecular weight of monomeric TLK1B-KD.

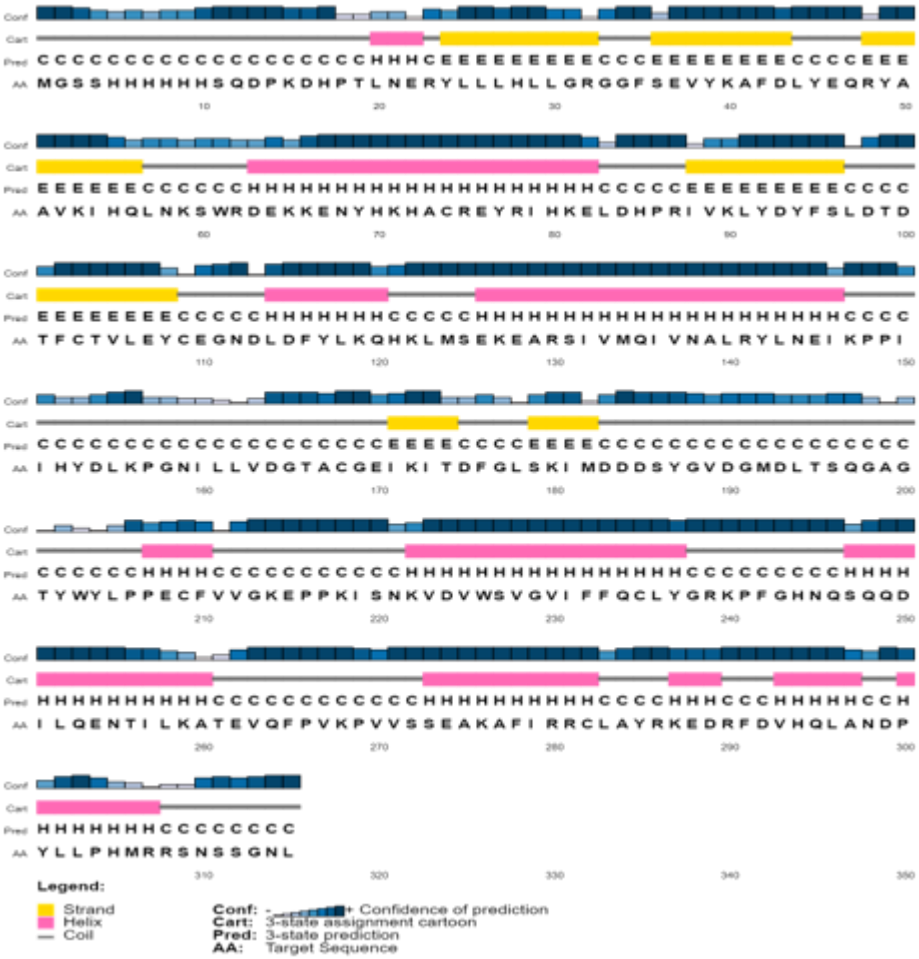


Figure S7: PSIPRED analysis of hTLK-KD. The data shows the presence of helix in the structure.

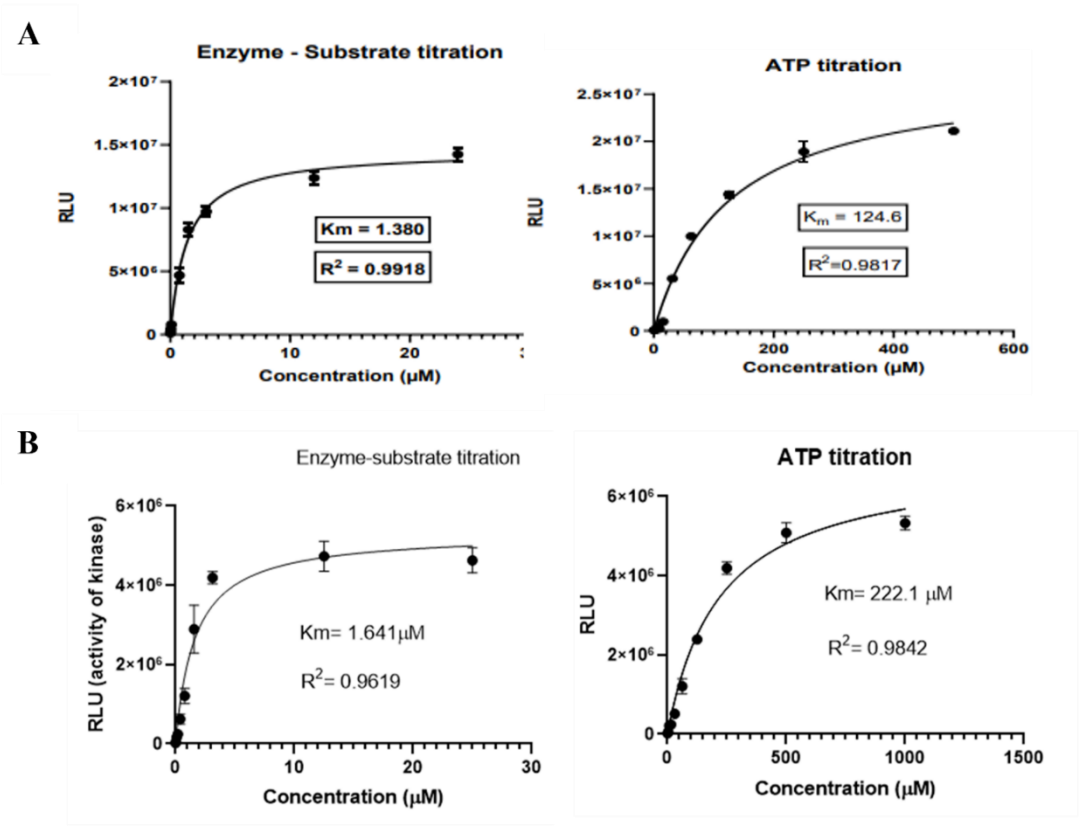


Figure S8: The titration curves with Histone and Nek1 as a substrate. (A) Determination of the Km value for Histone and ATP. The serial, twofold dilutions of ATP and kinase substrate were made respectively across the plate using 1X Kinase Reaction Buffer. The Km value of Histone H3 was found to be 1.38 μM. (B) Determination of the Km value for Nek1 and ATP. The serial, twofold dilutions of ATP and kinase substrate were made respectively across the plate using 1X Kinase Reaction Buffer. The Km value of Nek1 was found to be 1.64 μM.

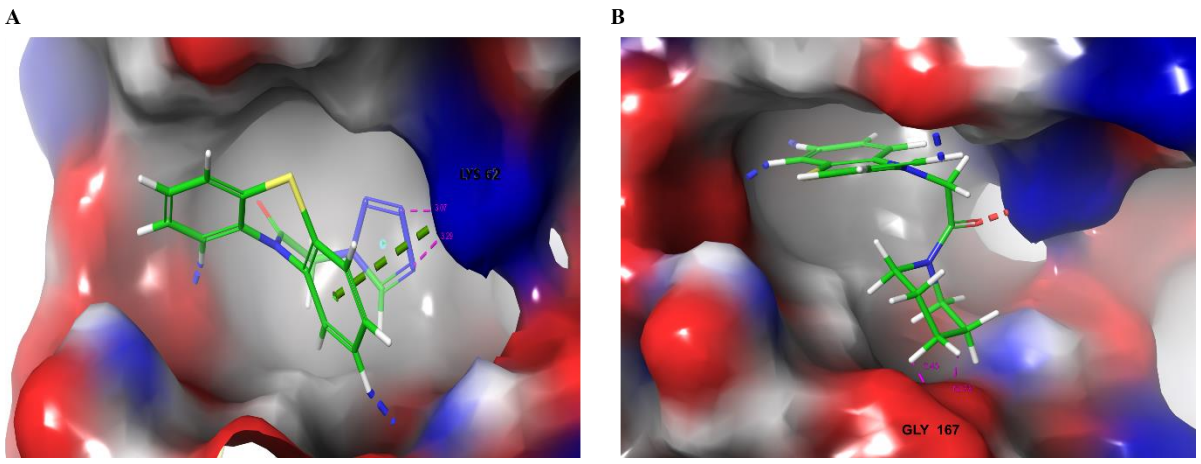


Figure S9: Docking studies of ligands with TLK-kinase domain. The docking pose of compound **9** (A) and compound **12** (B) in the ATP binding site. The tetrazole moiety forms an electrostatic interaction with Lys 62 (~3Å) and piperidine forms with Gly 167 (~2.7 Å).

Table S1: **ADME and drug-likeness of synthesized compounds**

Molecule	Consensus Log P	ESOL Class	Ali Class	GI absorption	BBB permeant	Pgp substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	Bio-availabilty
1	3.89	Moderately soluble	Moderately soluble	High	Yes	Yes	Yes	Yes	No	Yes	Yes	0.55
2	4.15	Moderately soluble	Moderately soluble	High	Yes	Yes	Yes	Yes	No	Yes	No	0.55
3	2.83	Soluble	Moderately soluble	High	Yes	Yes	Yes	Yes	No	Yes	Yes	0.55
4	2.41	Soluble	Soluble	High	Yes	Yes	Yes	No	No	Yes	Yes	0.55
5	2.74	Soluble	Soluble	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0.55
6	3.59	Moderately soluble	Moderately soluble	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0.55
7	2.69	Soluble	Soluble	High	Yes	Yes	No	Yes	Yes	Yes	Yes	0.55
8	3.3	Moderately soluble	Moderately soluble	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0.55
9	2.2	Soluble	Soluble	High	No	No	Yes	Yes	No	No	No	0.55
10	3.28	Moderately soluble	Moderately soluble	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0.55
11	2.67	Soluble	Soluble	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0.55
12	3.54	Moderately soluble	Moderately soluble	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0.55
13	2.19	Soluble	Soluble	High	Yes	Yes	No	Yes	No	Yes	Yes	0.55
14	1.86	Soluble	Soluble	High	Yes	Yes	No	Yes	Yes	Yes	Yes	0.55
15	2.72	Moderately soluble	Soluble	High	Yes	Yes	No	Yes	Yes	Yes	Yes	0.55
16	2.59	Soluble	Soluble	High	Yes	No	Yes	Yes	No	Yes	Yes	0.55
17	3.42	Moderately soluble	Moderately soluble	High	Yes	No	Yes	Yes	No	Yes	Yes	0.55
18	2.09	Soluble	Soluble	High	Yes	No	Yes	Yes	Yes	Yes	Yes	0.55
19	2.96	Moderately soluble	Moderately soluble	High	Yes	No	Yes	Yes	Yes	Yes	Yes	0.55
J54	3.27	Moderately soluble	Moderately soluble	High	Yes	Yes	Yes	No	No	Yes	Yes	0.55

Analytical Data: ^{1}HNMR, ^{13}CNMR, and mass spectrum of the compounds

Figure S10: ^{1}H NMR of Compound 1

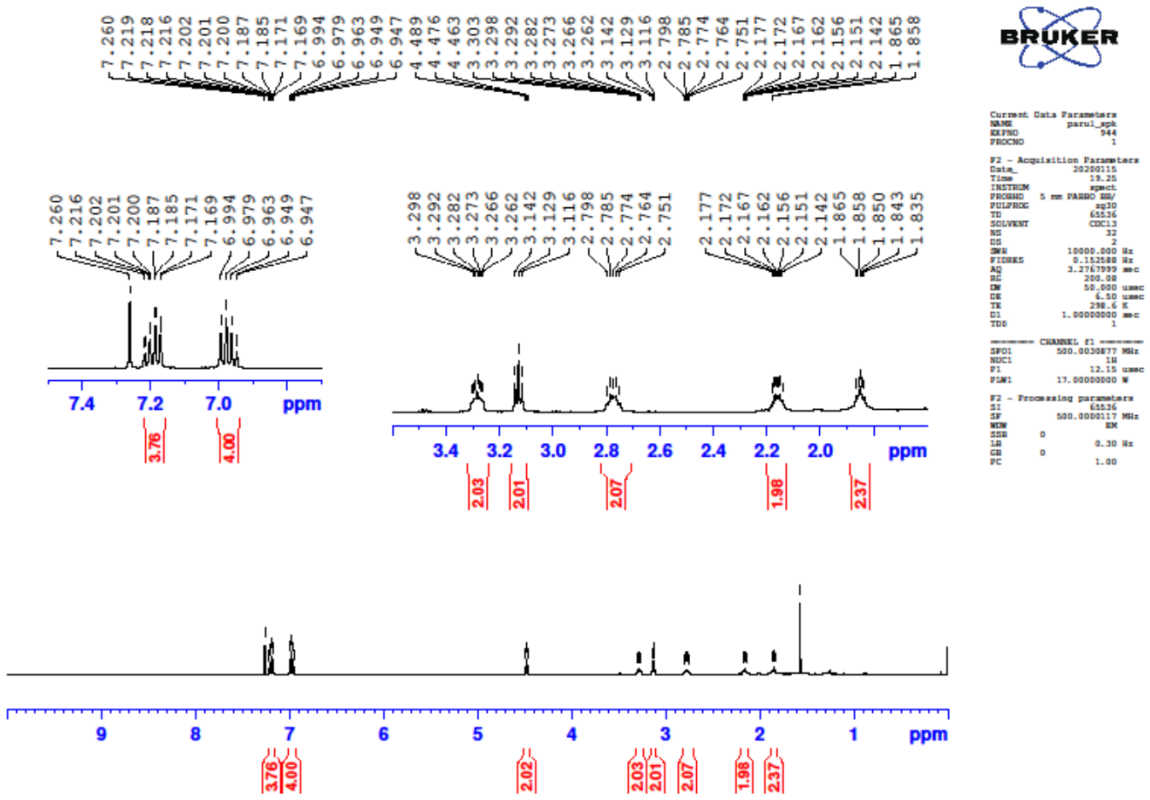


Figure S11: ^{13}C NMR of compound 1

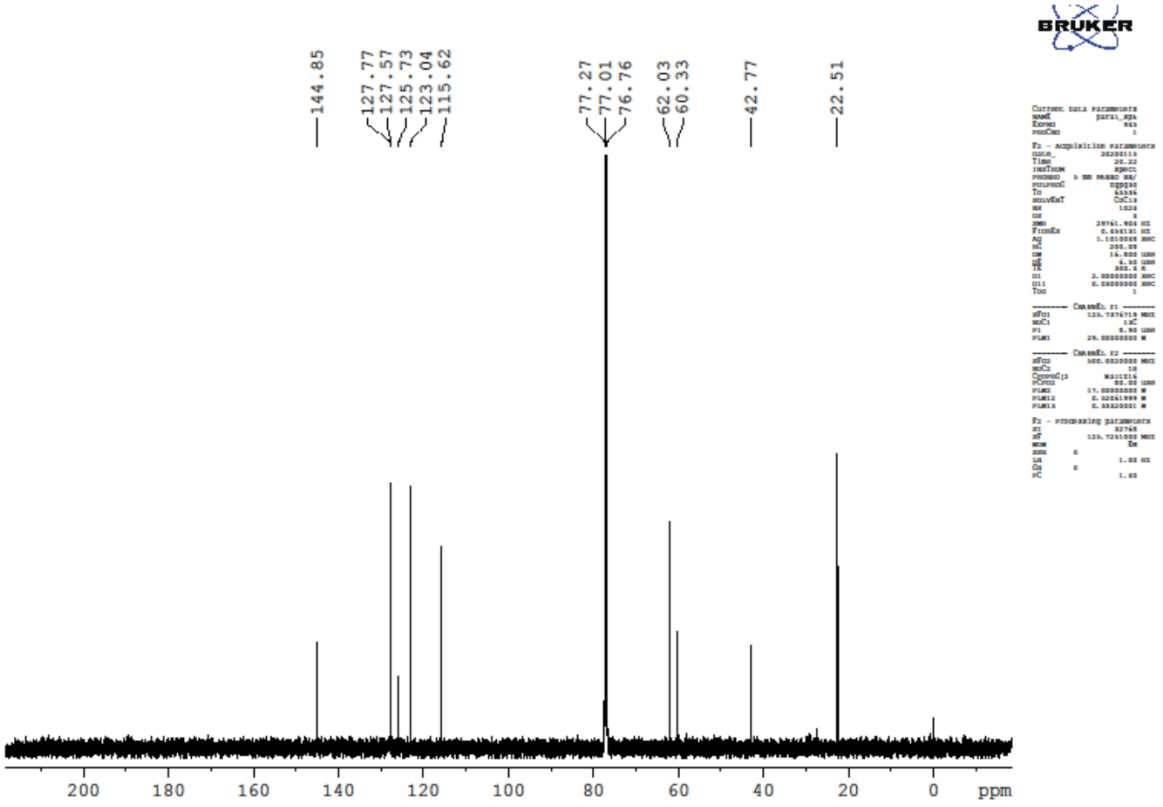


Figure S12: Mass of compound 1

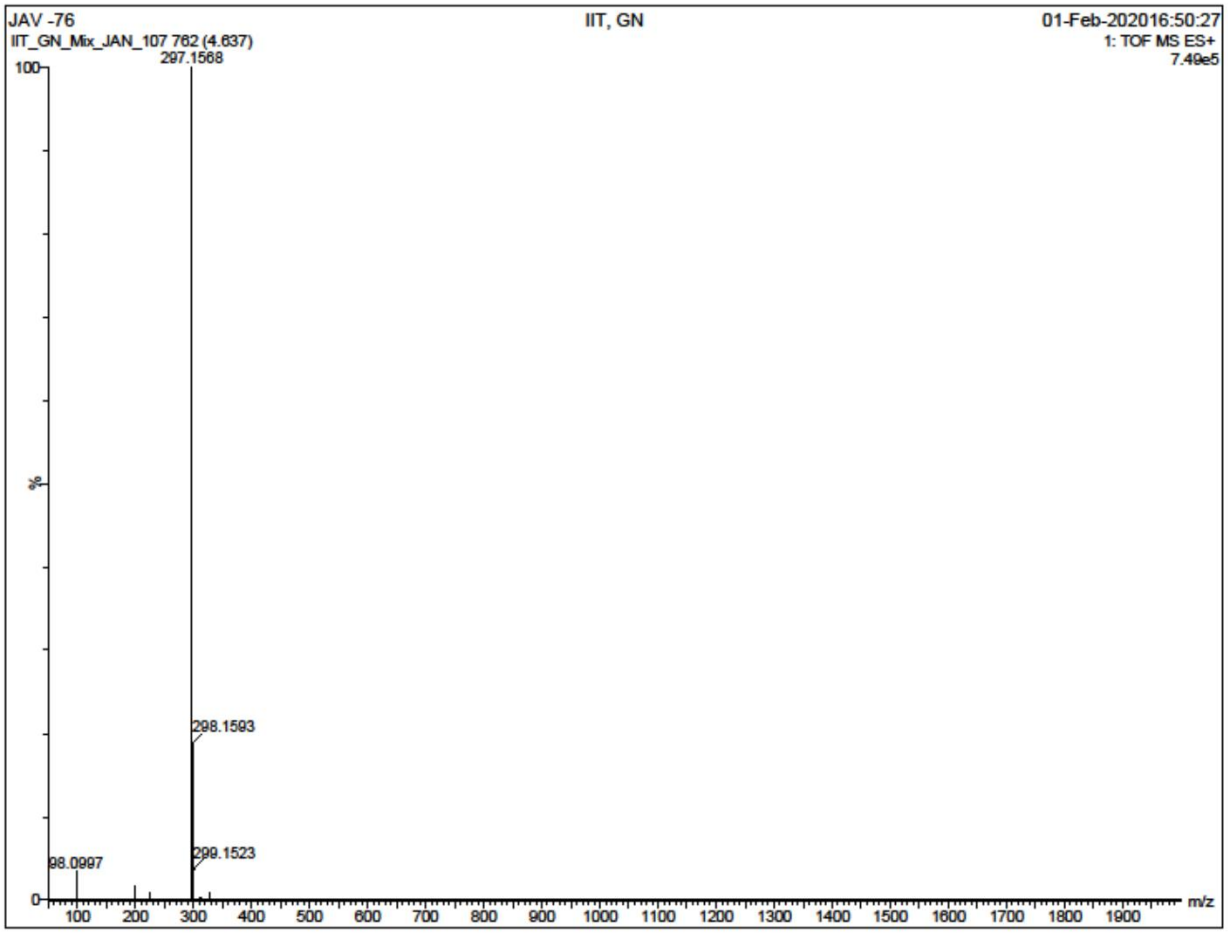


Figure S13: ^{1}H NMR of compound 2

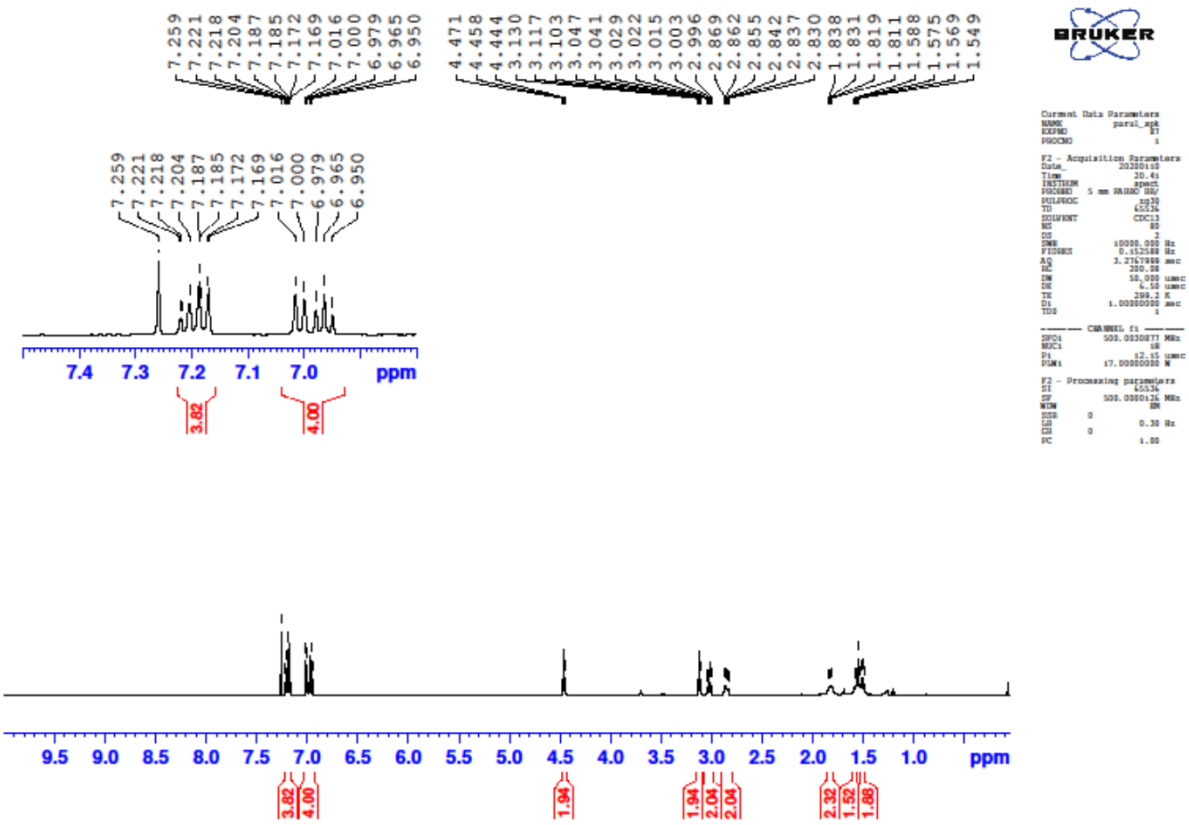


Figure S14: ^{13}C NMR of compound 2

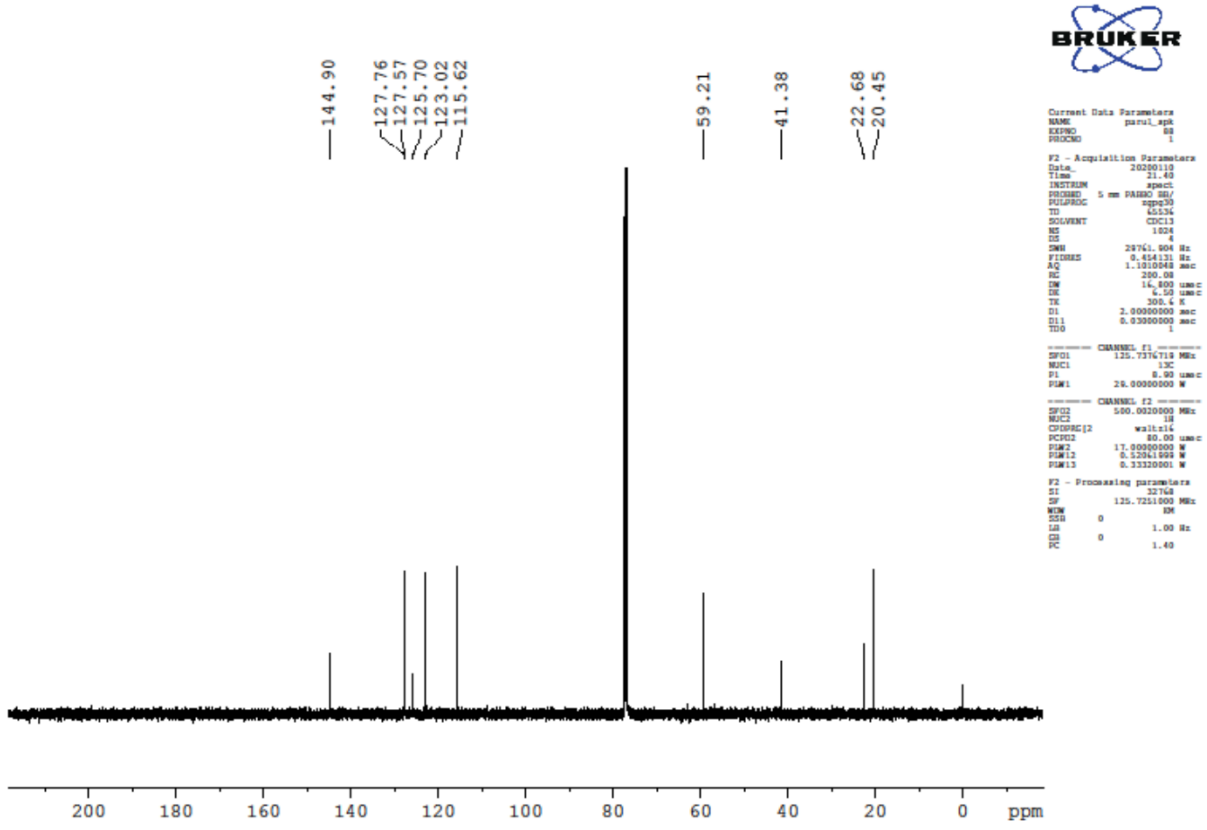


Figure S15: Mass of compound 2

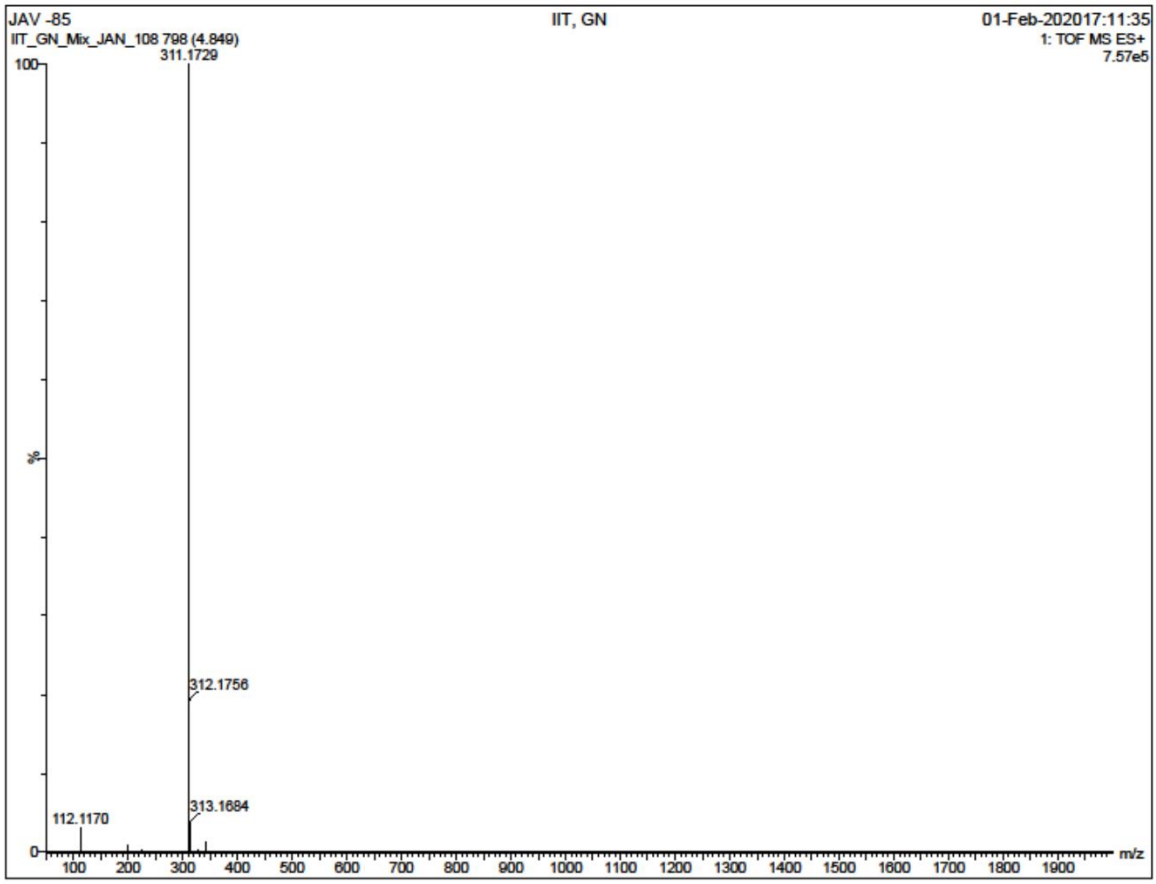


Figure S16: ^{1}H NMR of compound 3

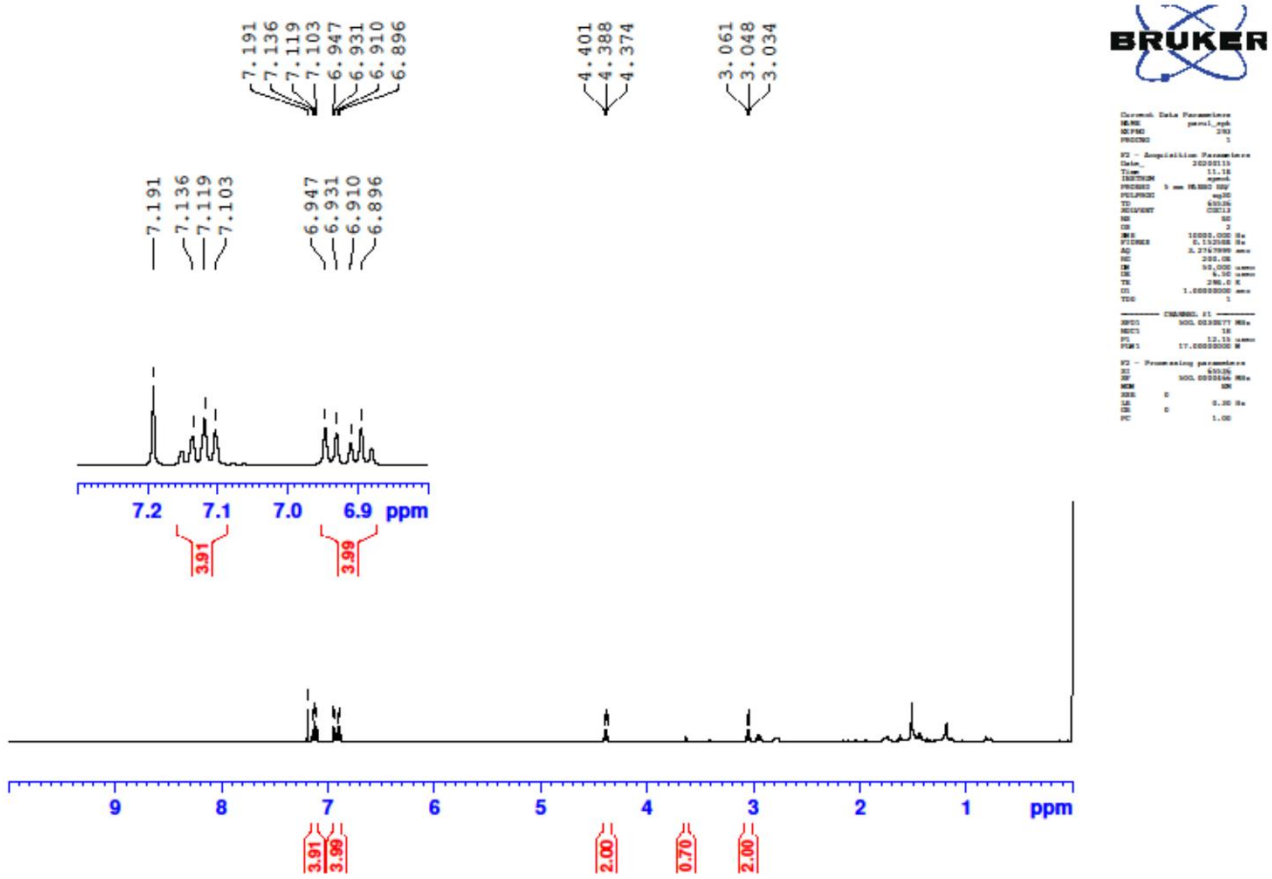


Figure S17: ^{13}C NMR of compound 3

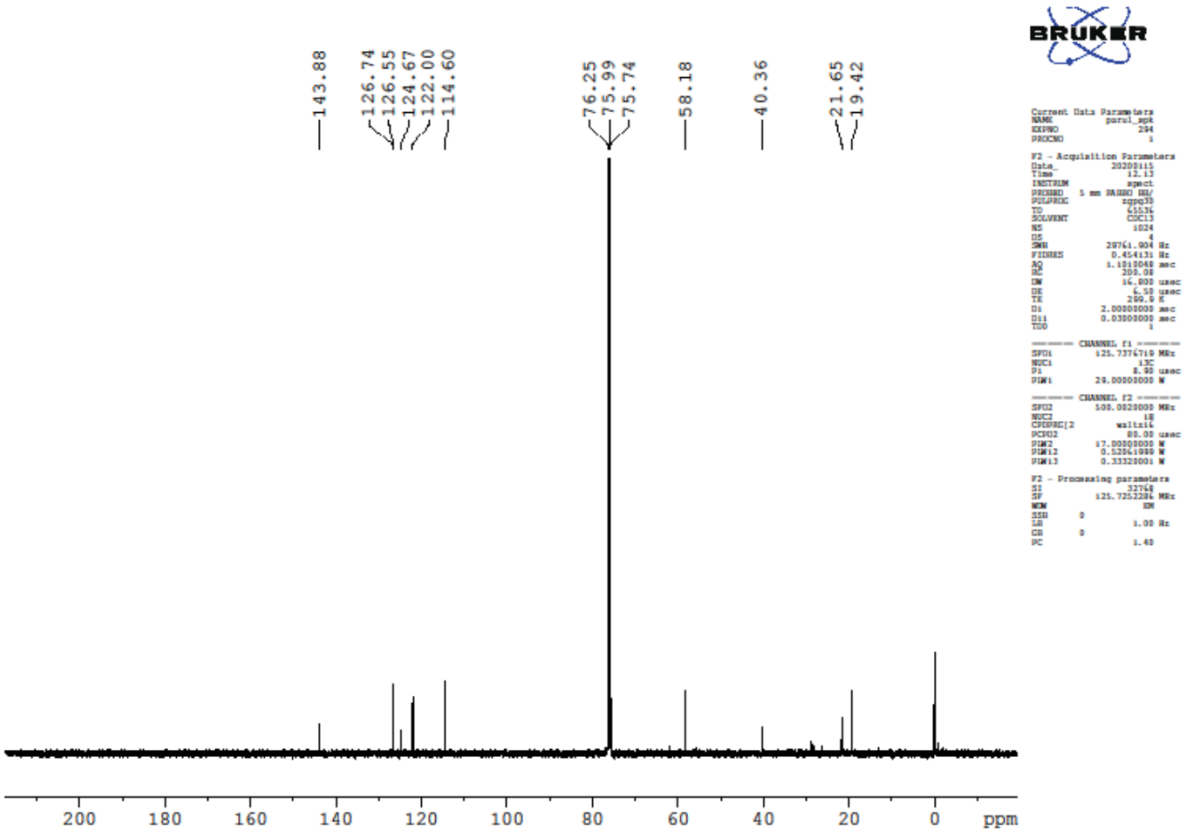


Figure S18: Mass of compound 3

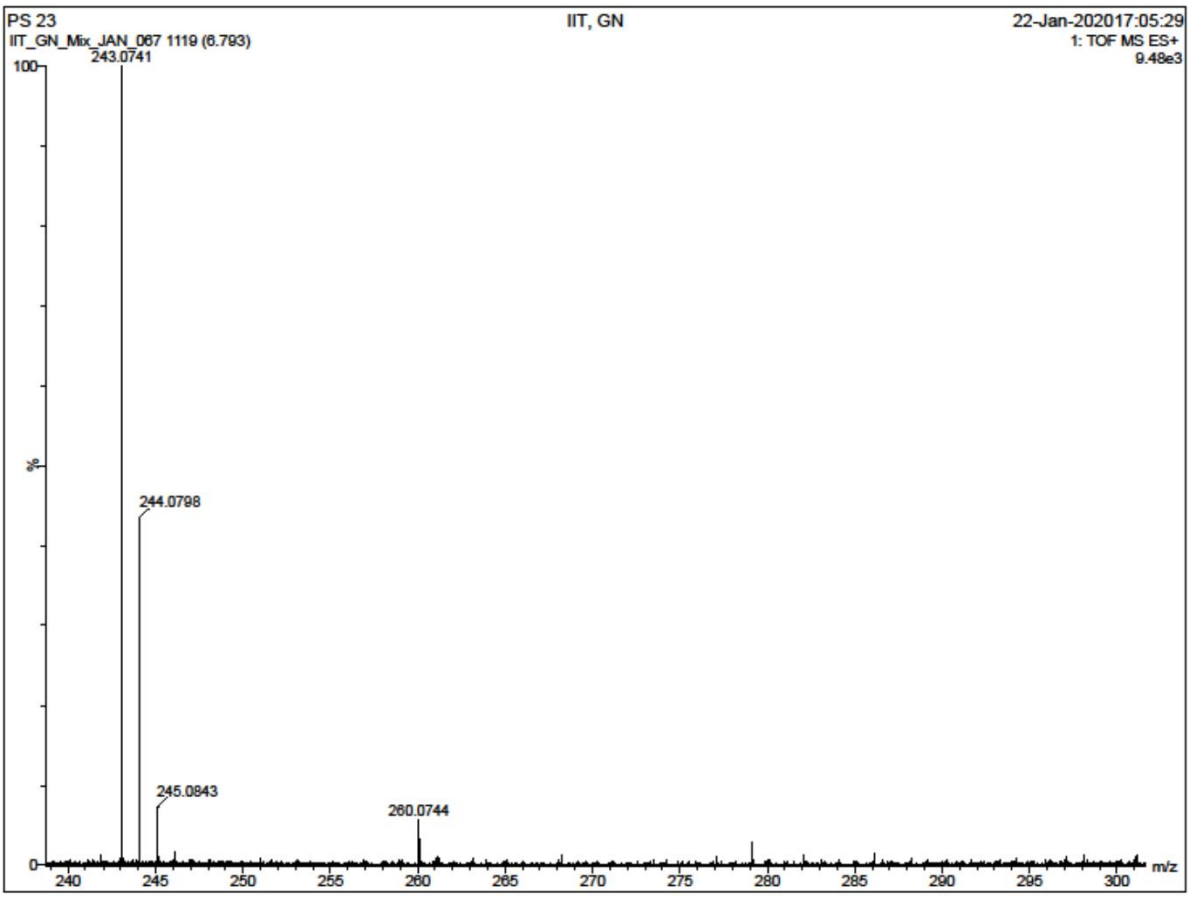


Figure S19: ^{1}H NMR of compound 4

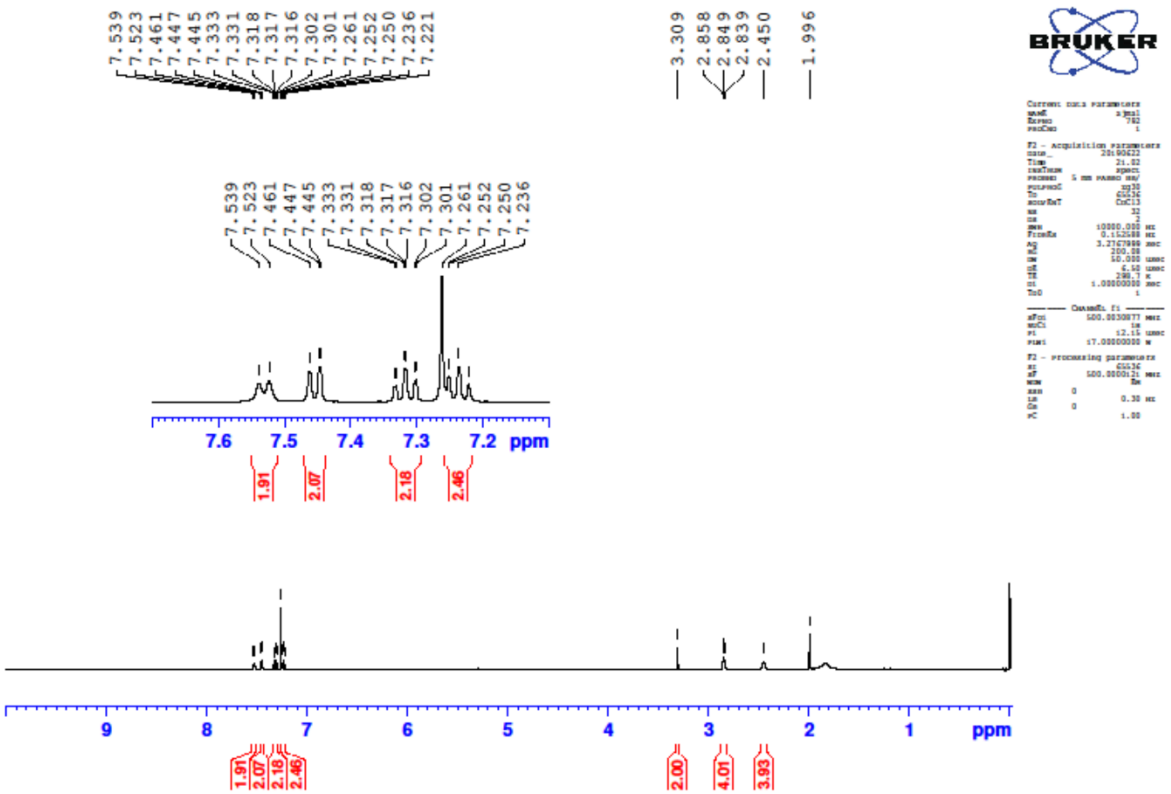


Figure S20: ^{13}C NMR of compound 4

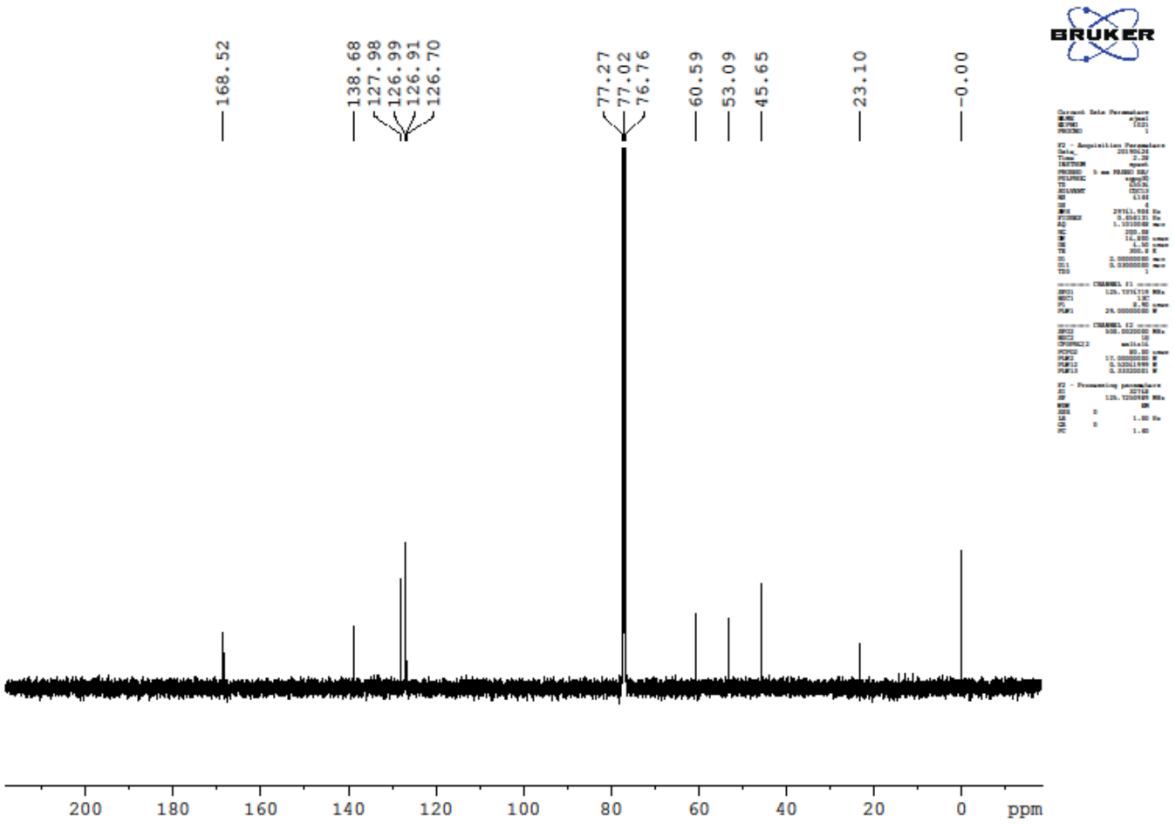


Figure S21: Mass of compound 4

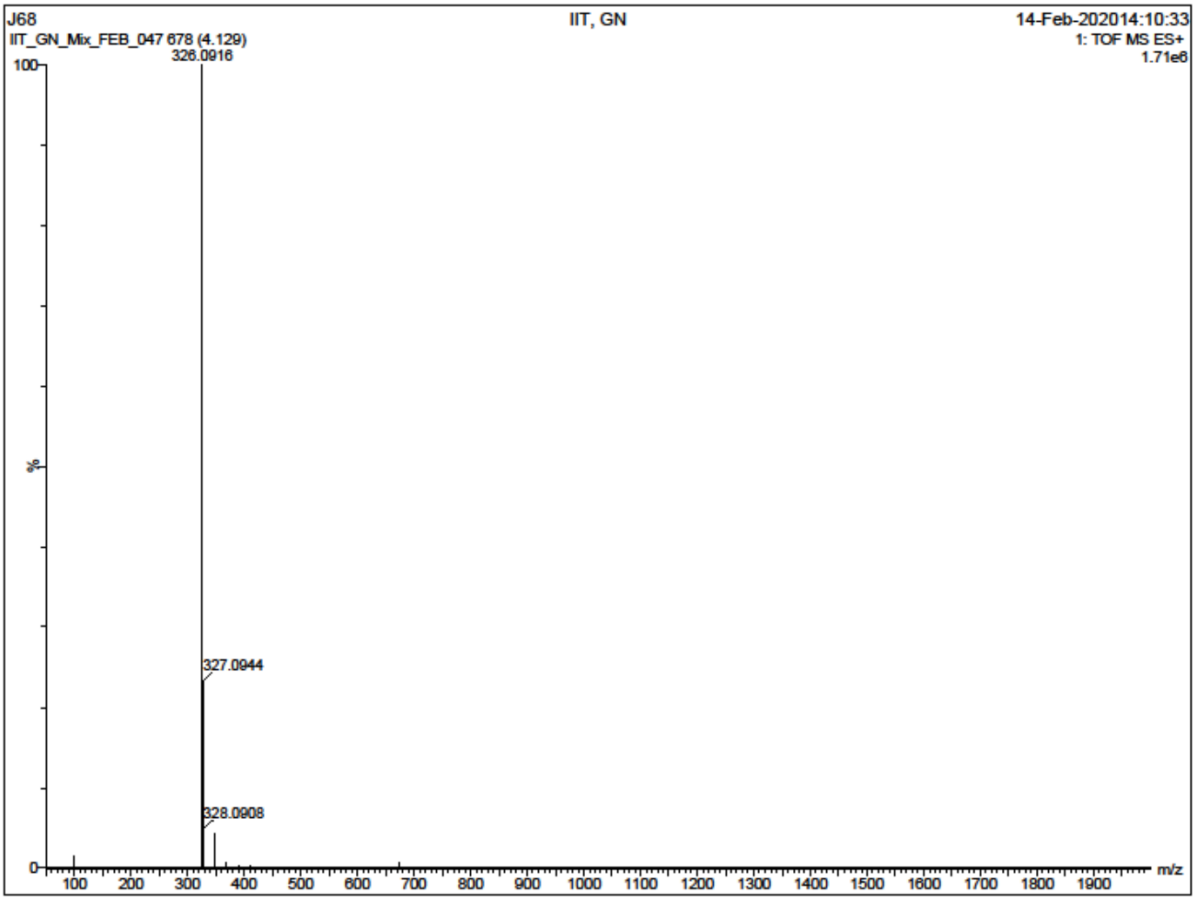


Figure S22: ^{1}H NMR of compound 7

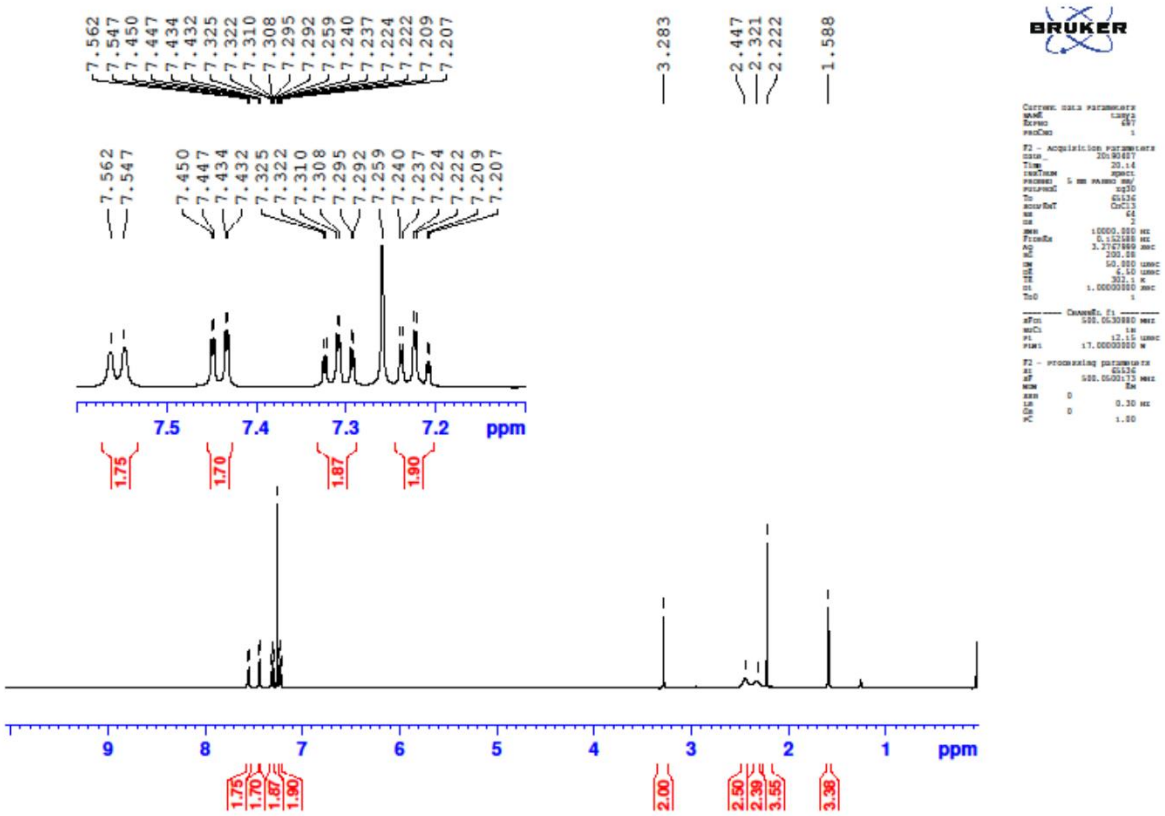


Figure S23: ^{13}C NMR of compound 7

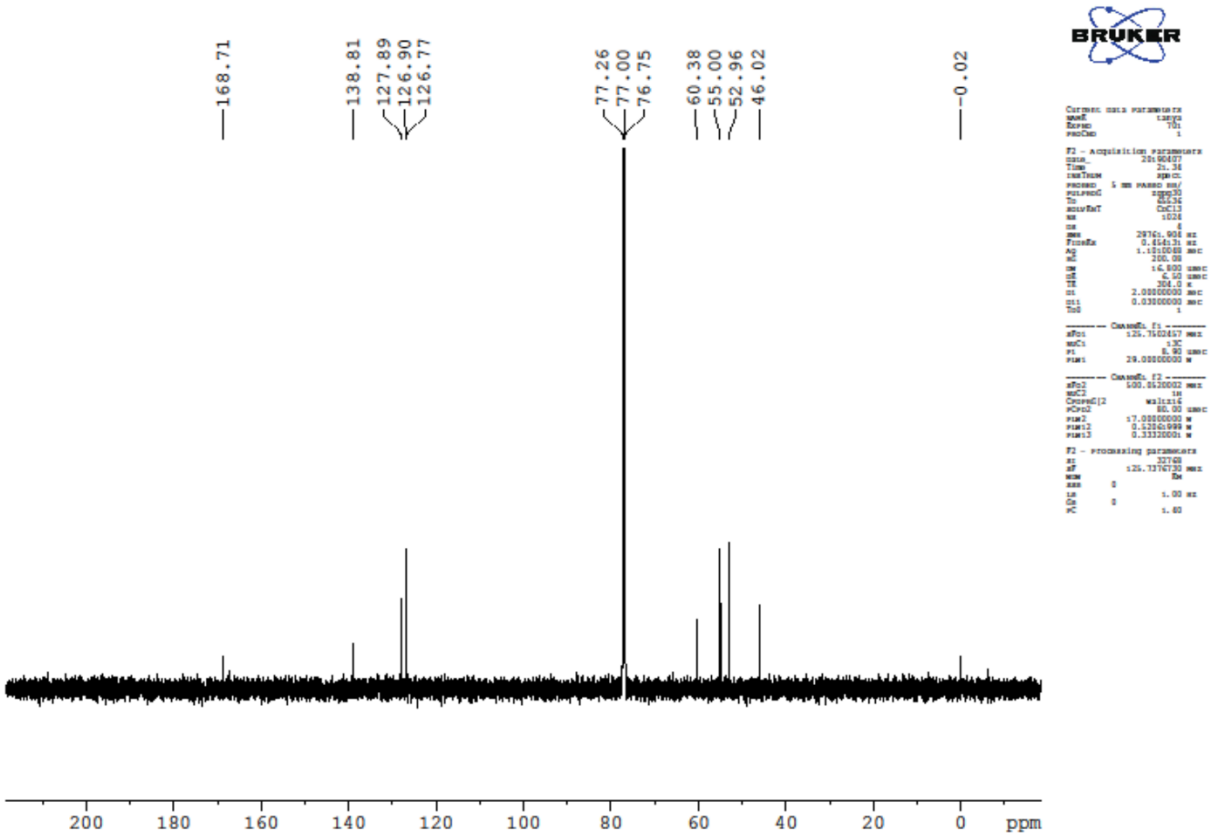


Figure S24: Mass of compound 7

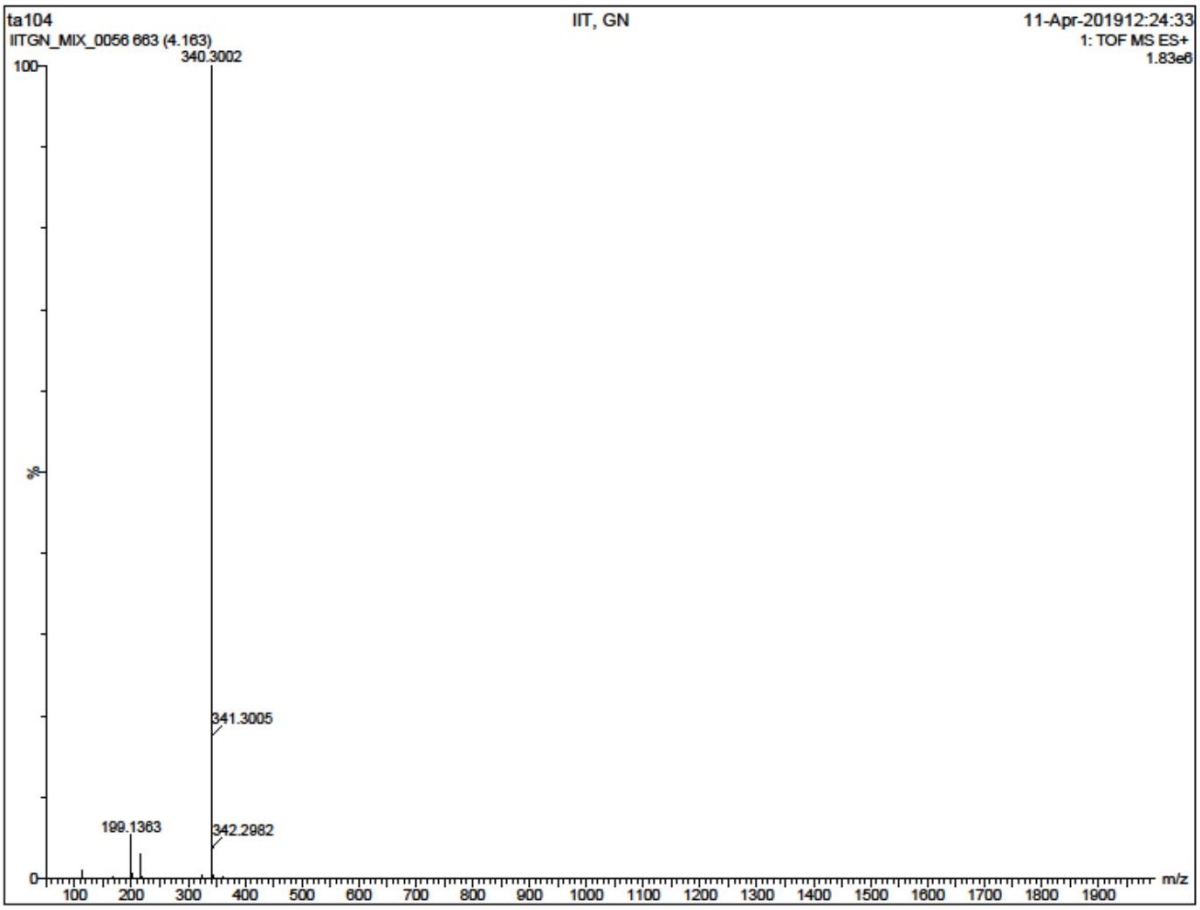


Figure S25: ^{1}H NMR of compound 8

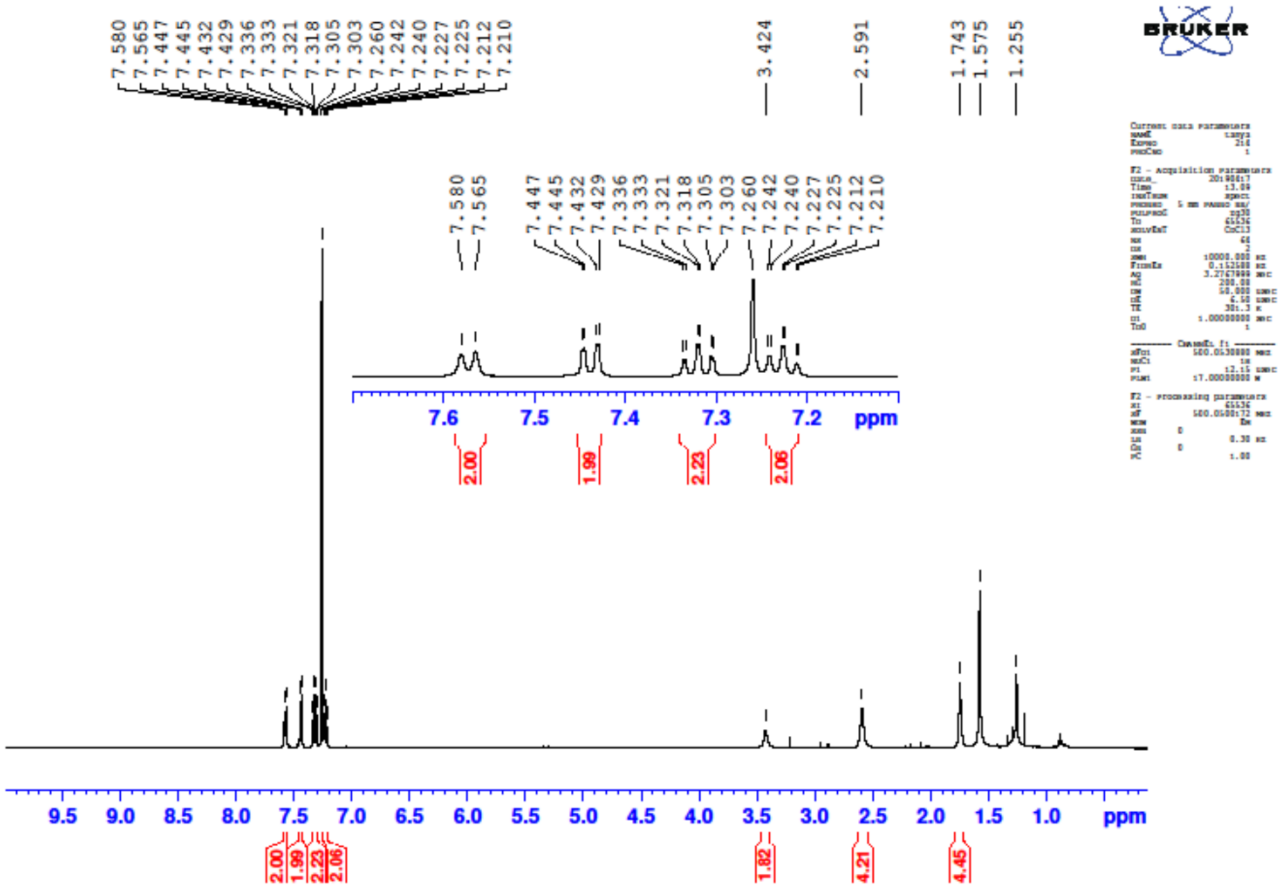


Figure S26: ^{13}C NMR of compound 8

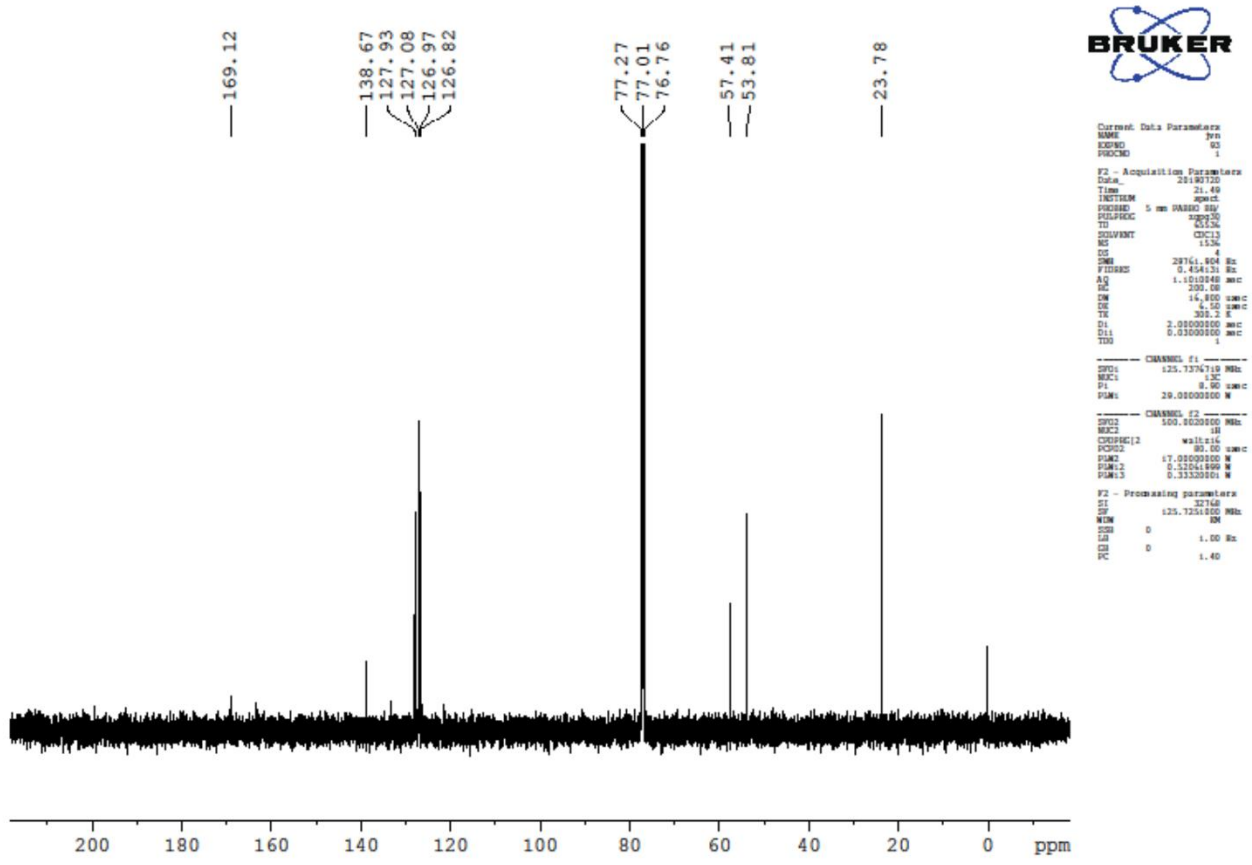


Figure S27: Mass of compound 8

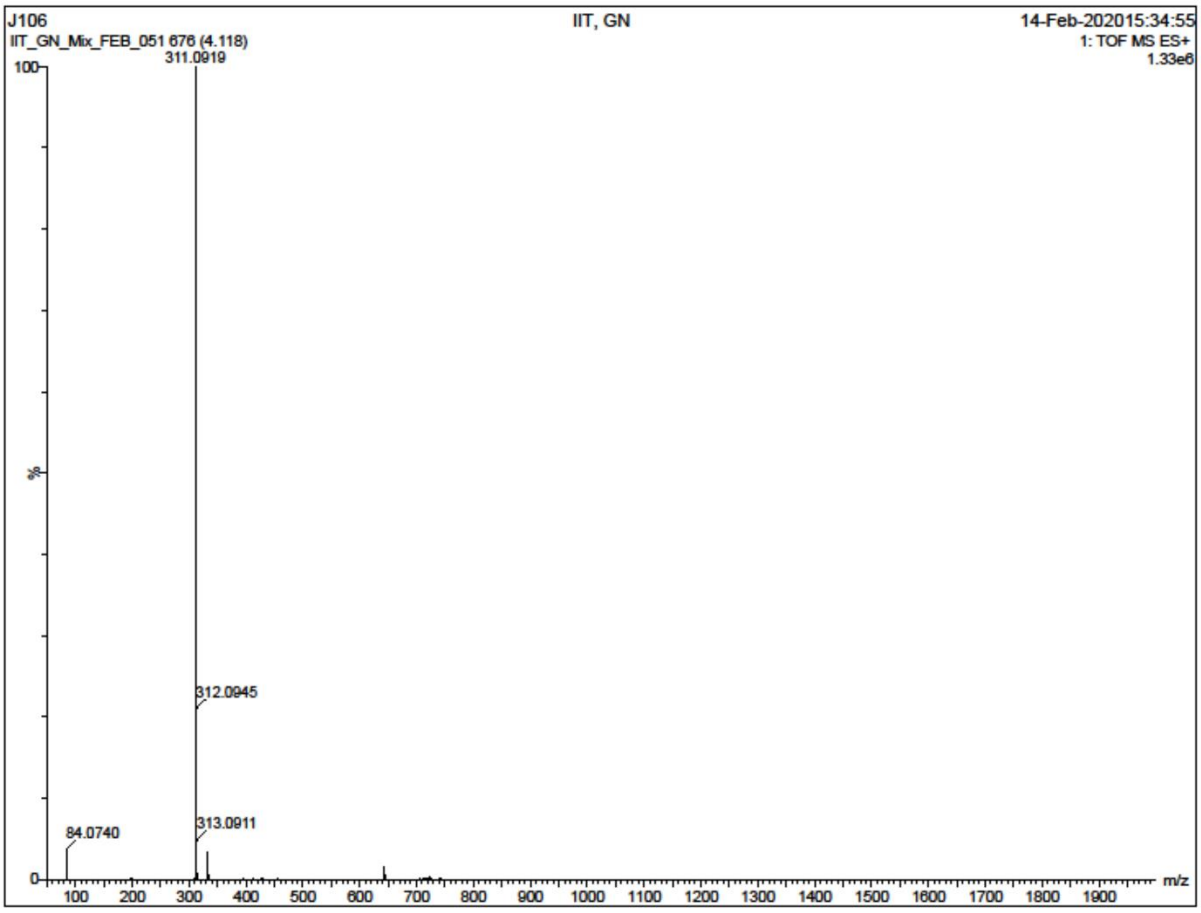


Figure S28: ^{1}H NMR of compound 9

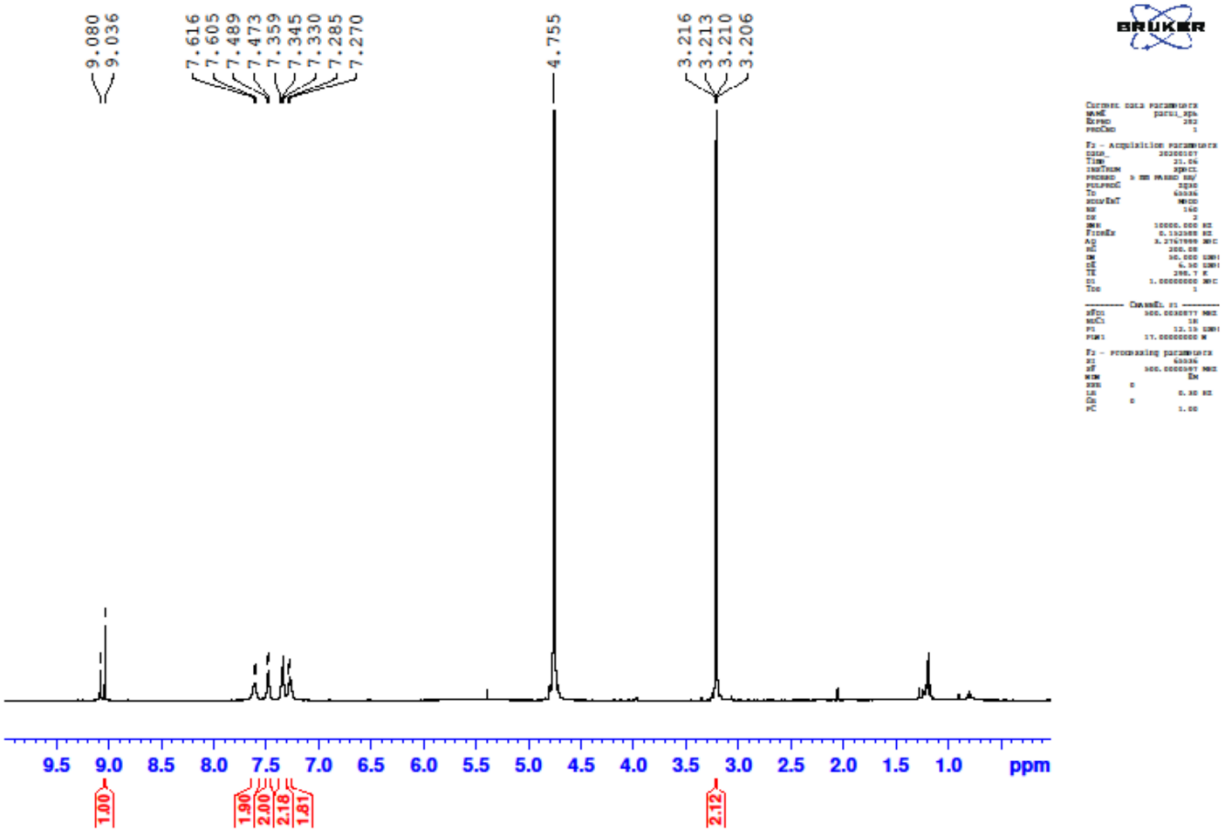


Figure S29: ^{13}C NMR of compound 9

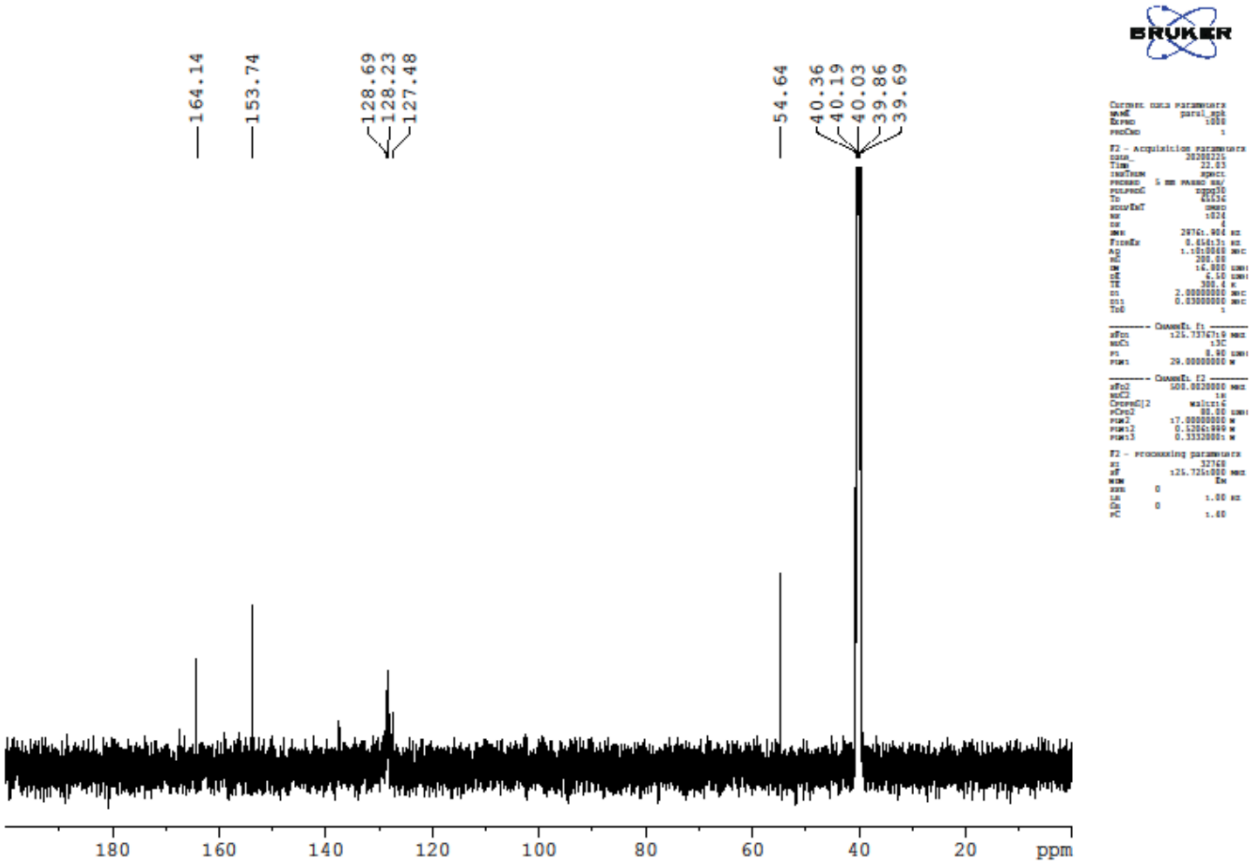


Figure S30: Mass of compound 9

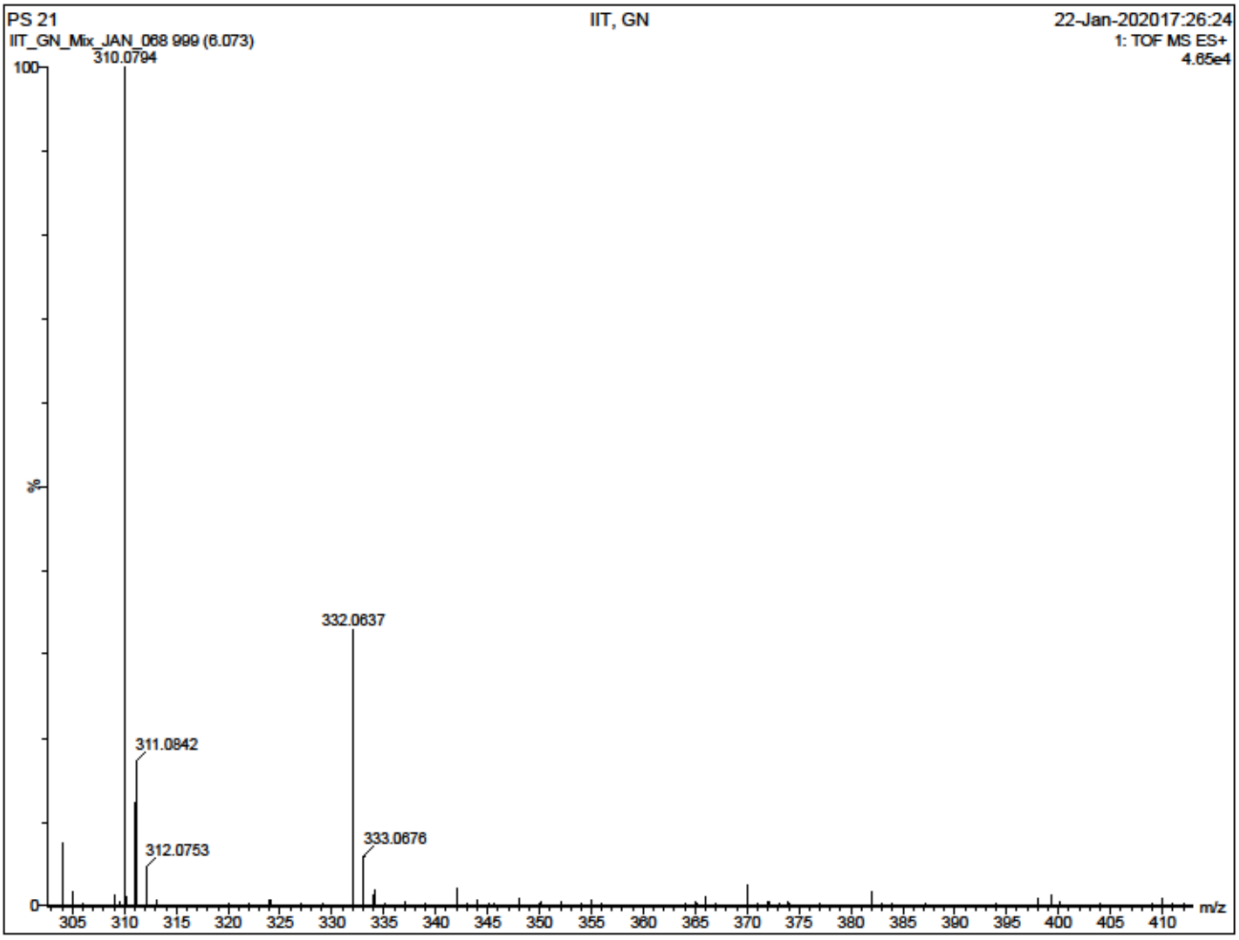


Figure S31: ^{1}H NMR of compound 10

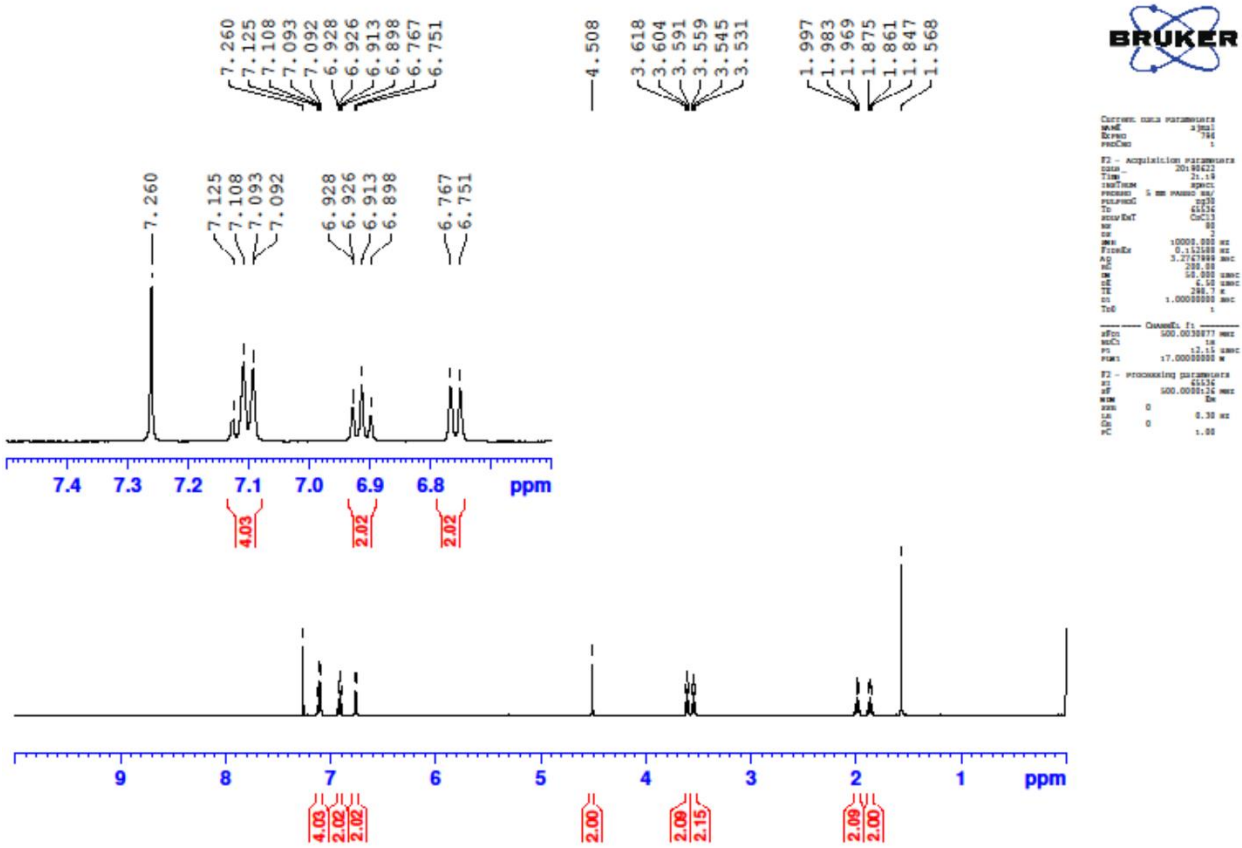


Figure S32: ^{13}C NMR of compound 10

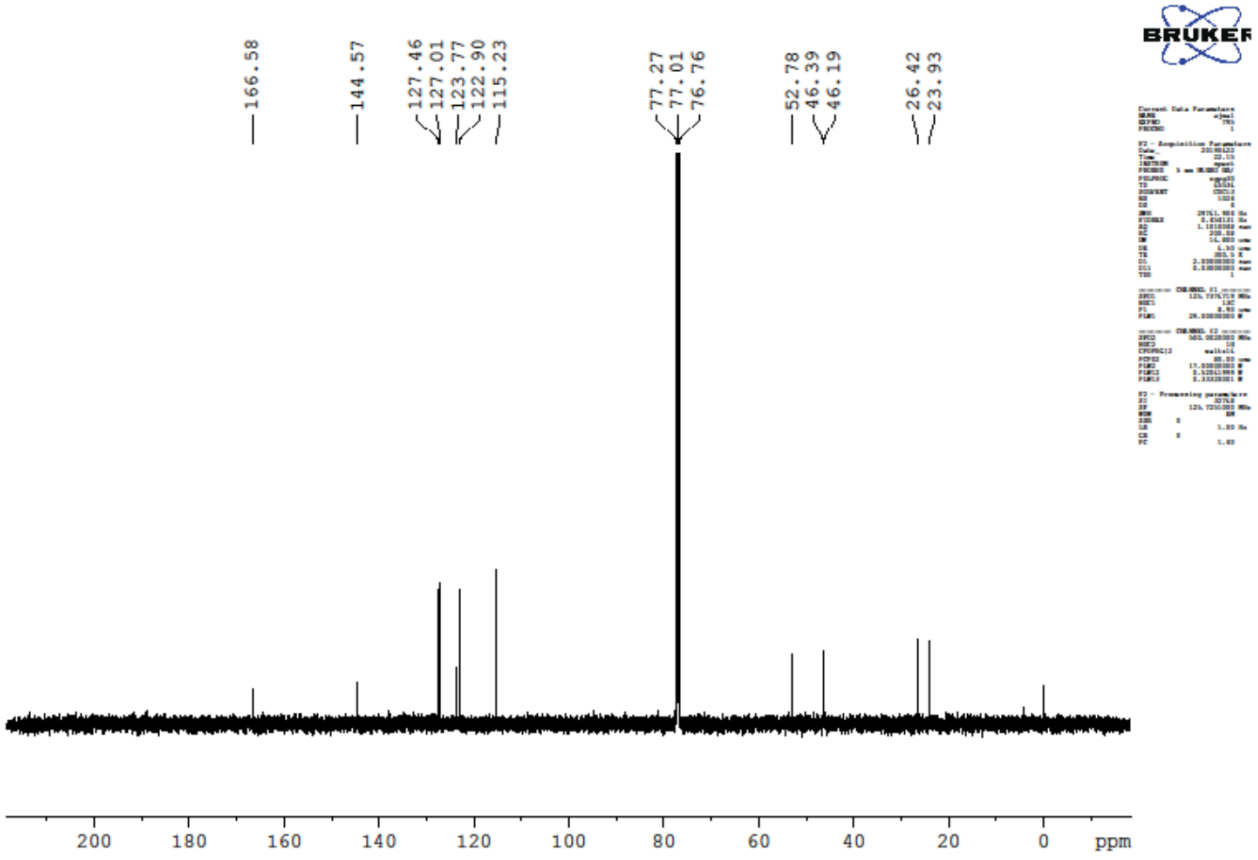


Figure S33: Mass of compound 10

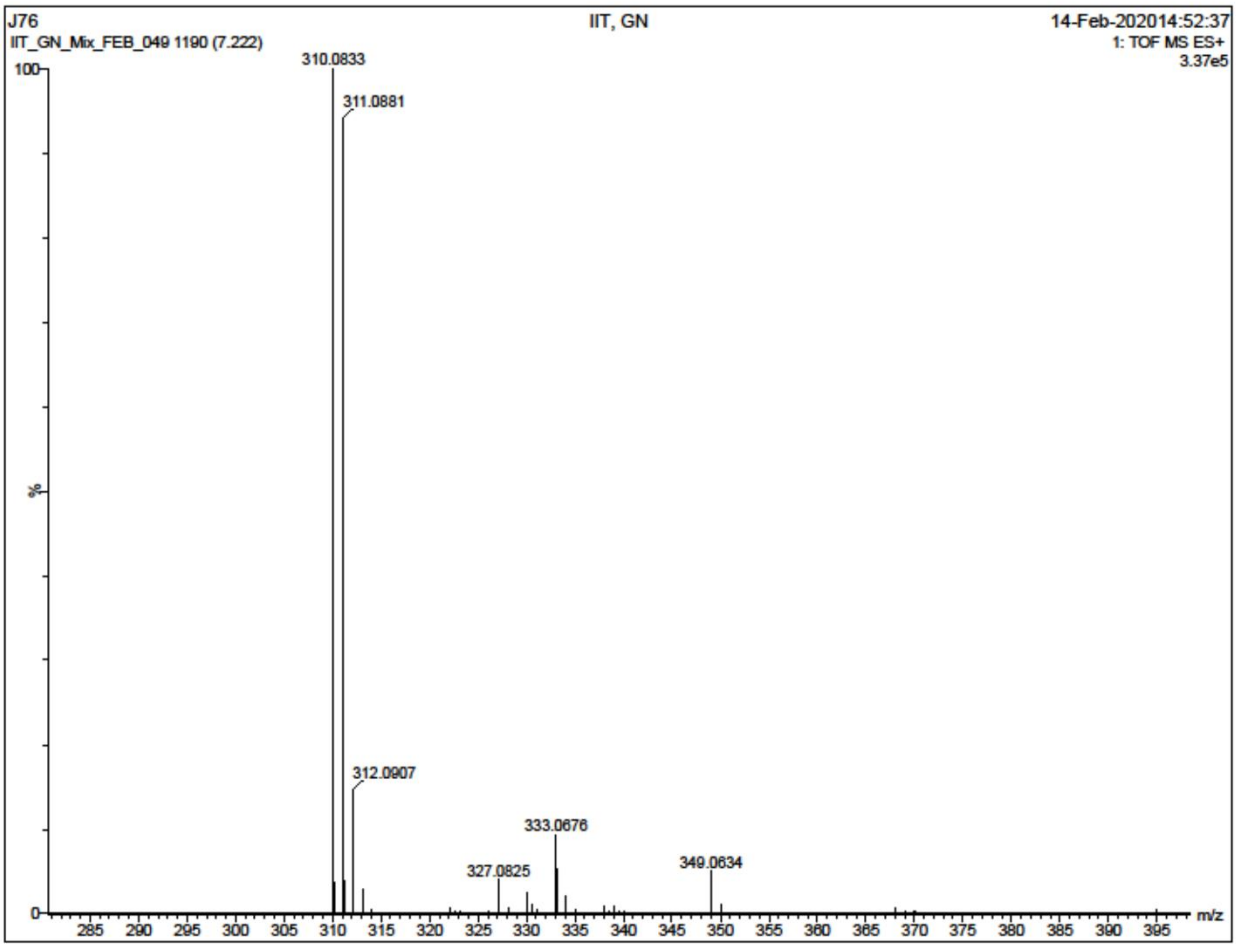


Figure S34: ^{1}H NMR of compound 13

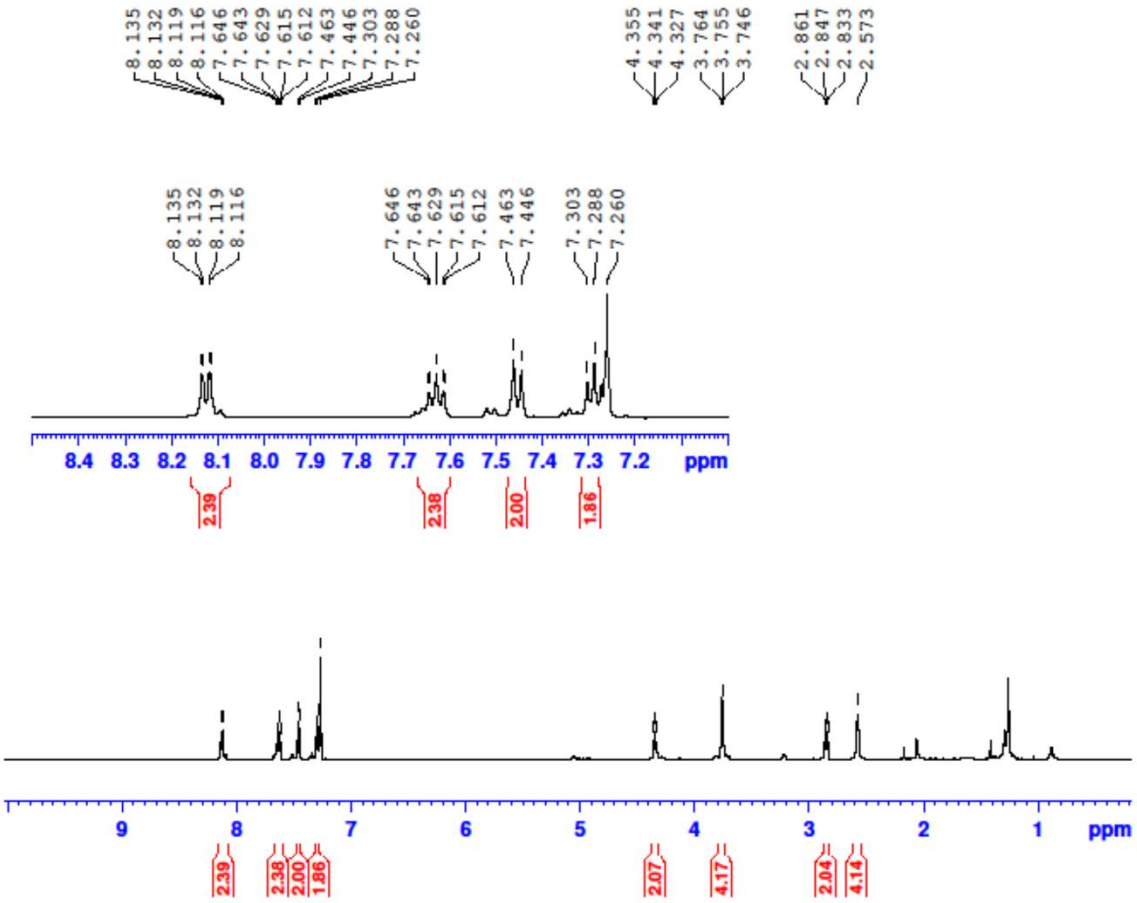


Figure S35: ^{13}C NMR of compound 13

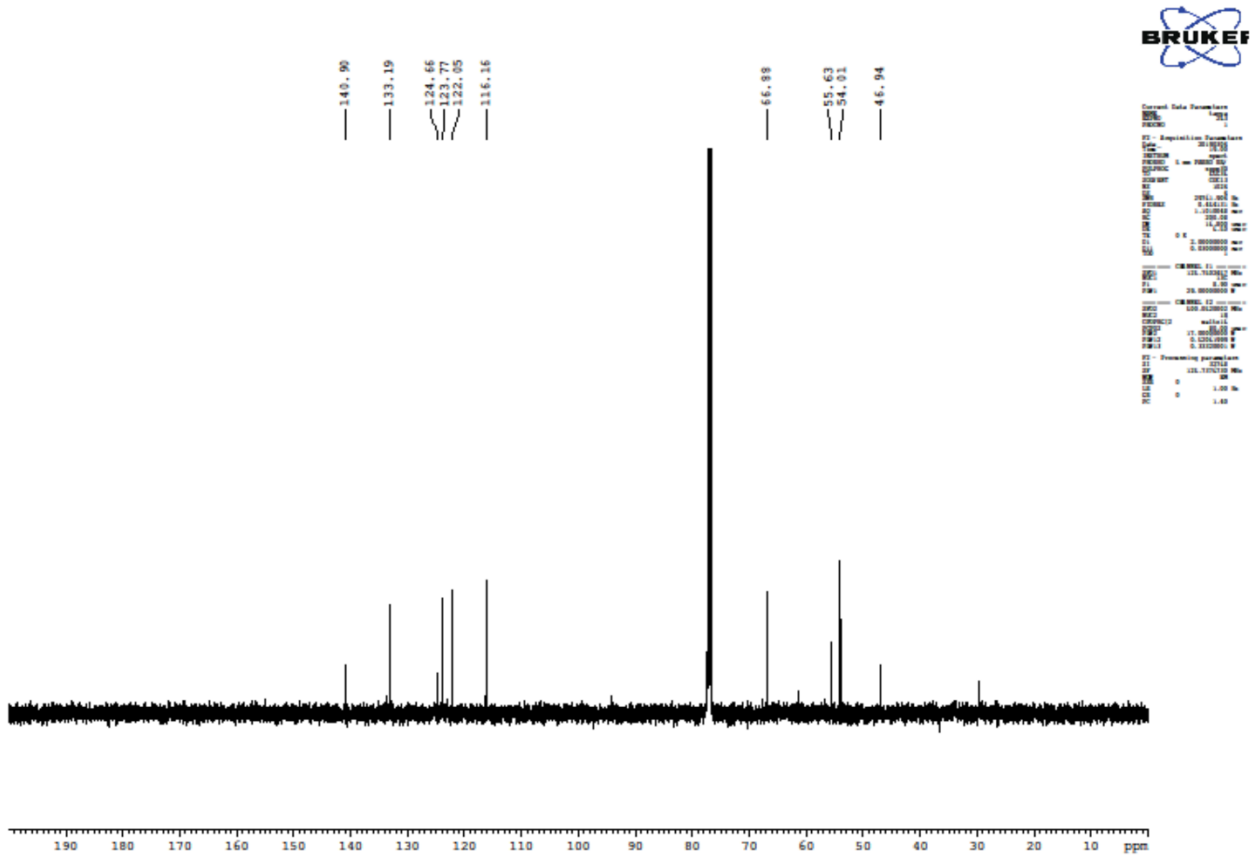


Figure S36: Mass of compound 13

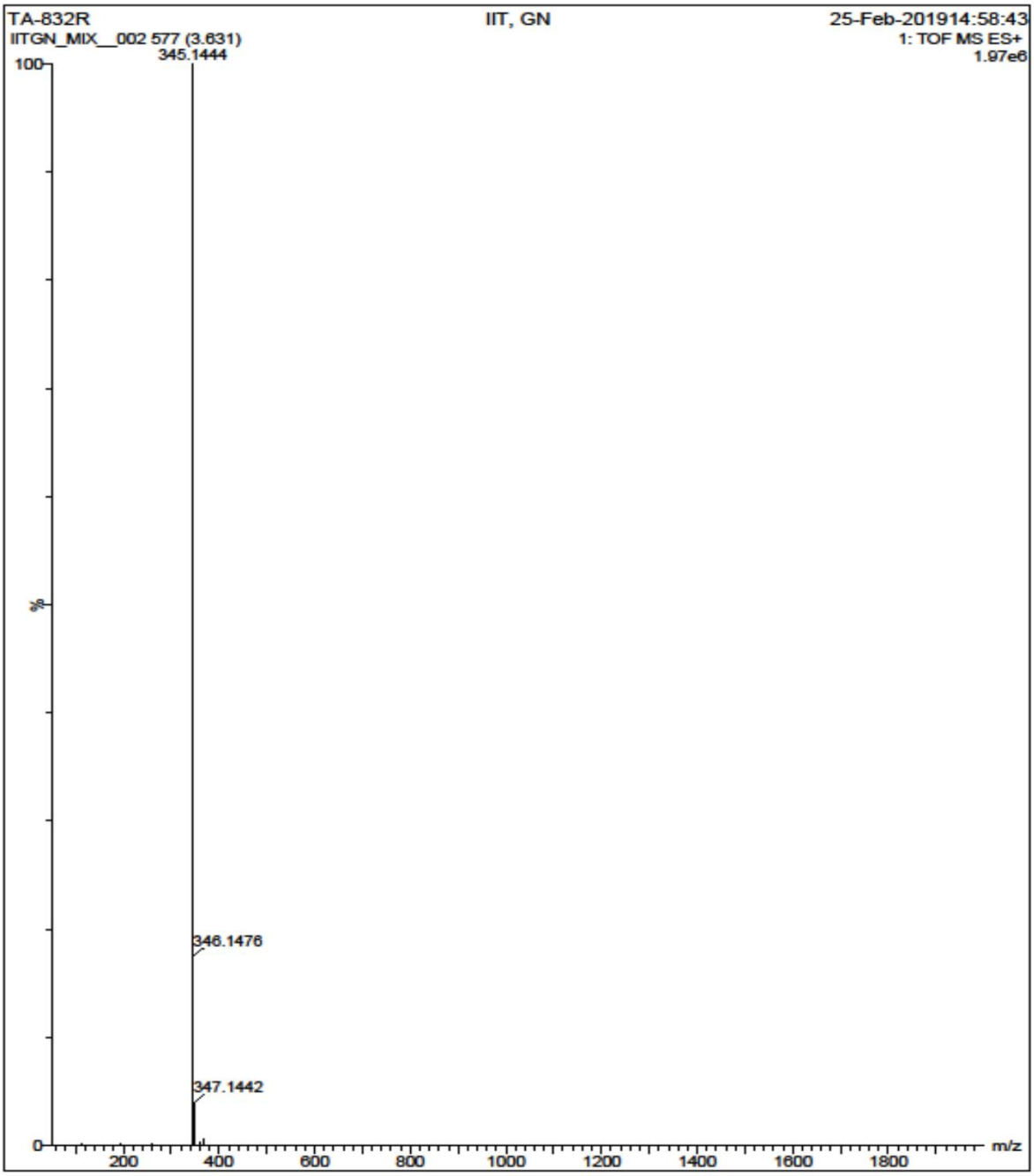


Figure S37: ^{1}H NMR of compound 14

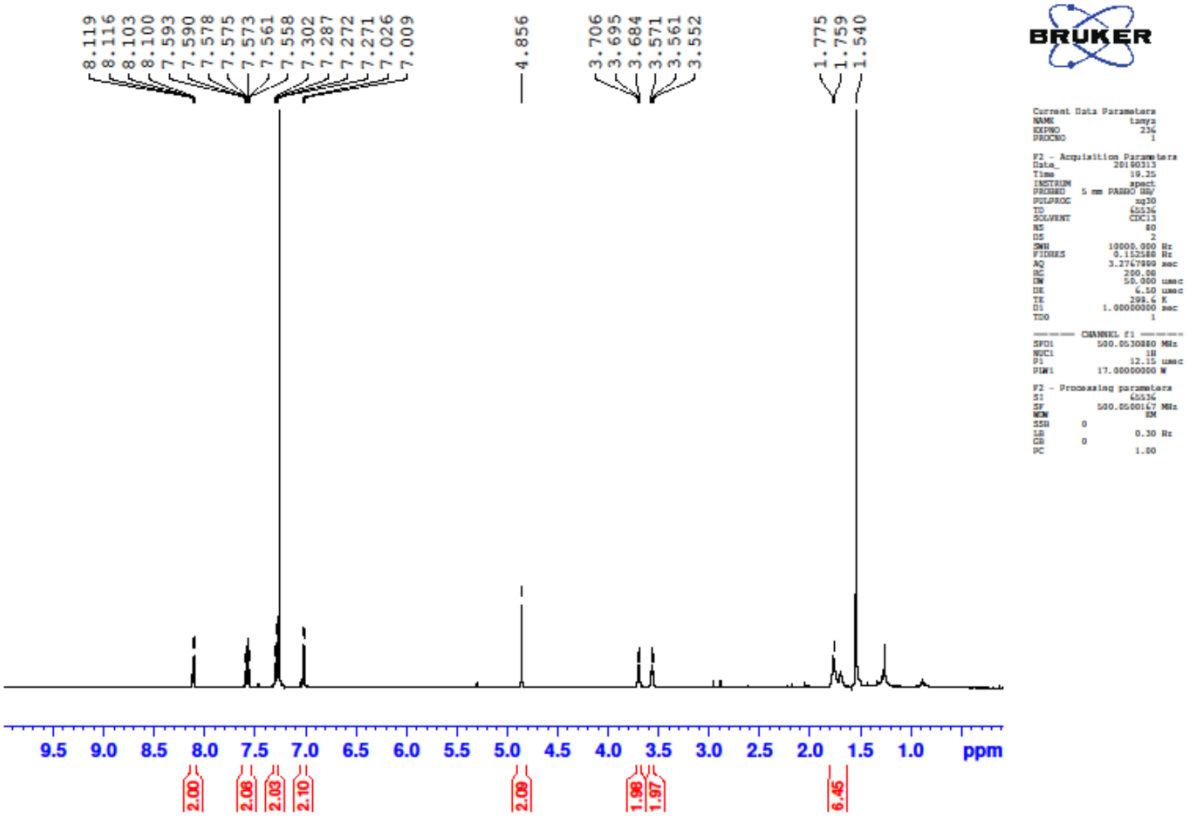


Figure S38: ^{13}C NMR of compound 14

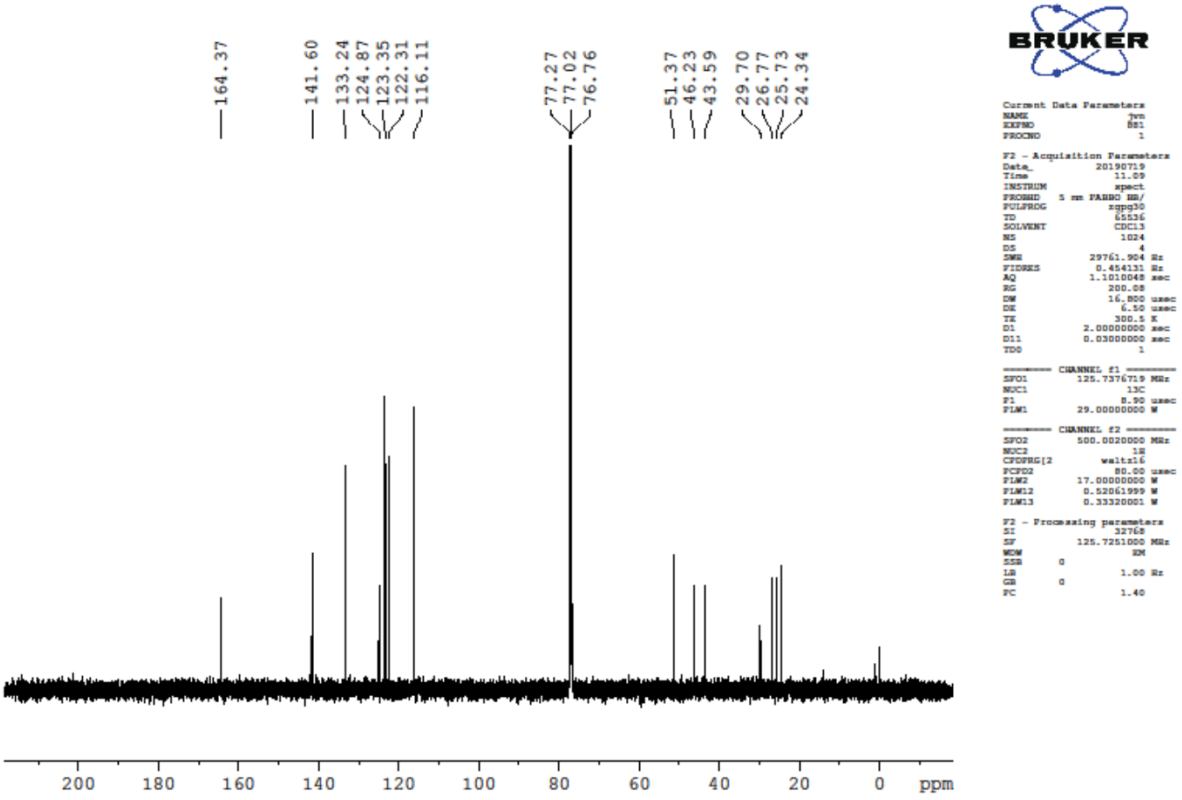


Figure S39: Mass of compound 14

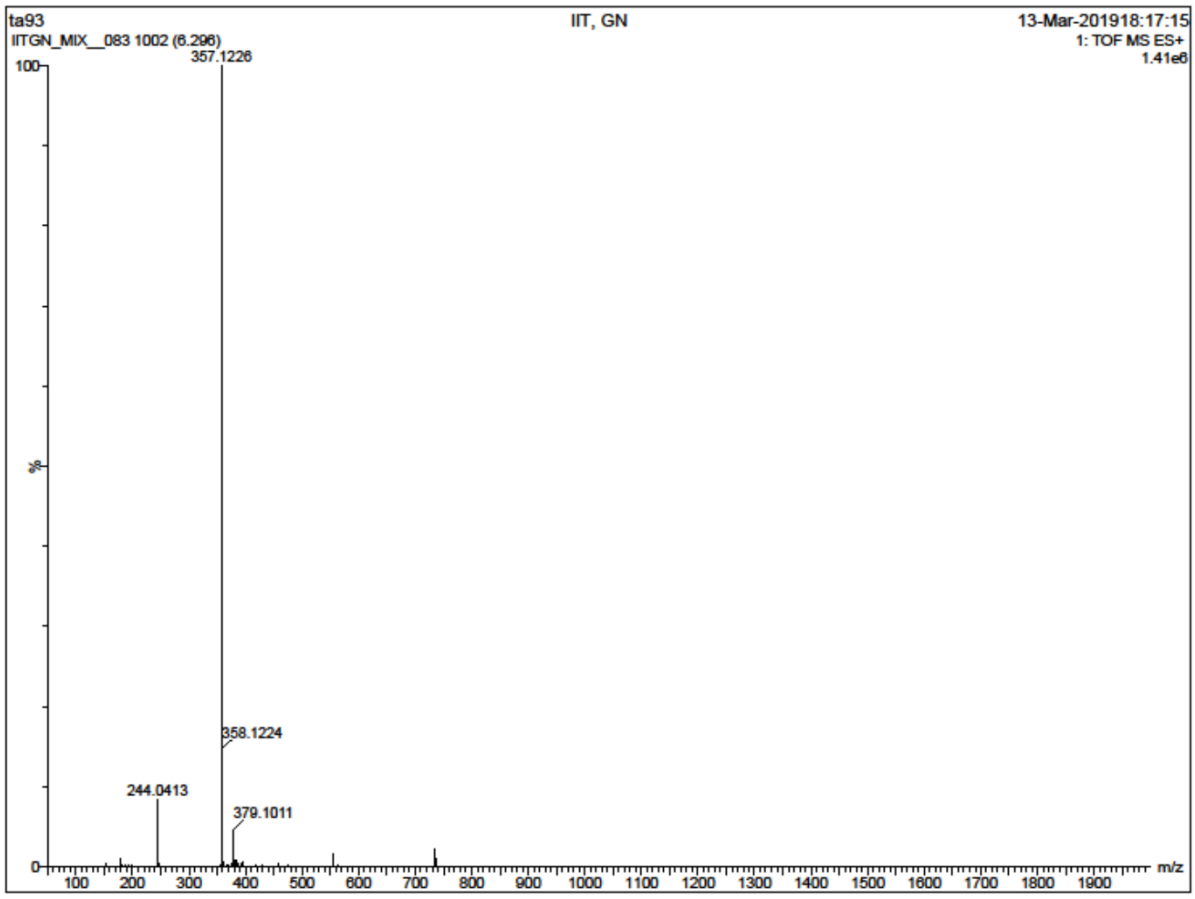


Figure S40: ^{1}H NMR of compound 15

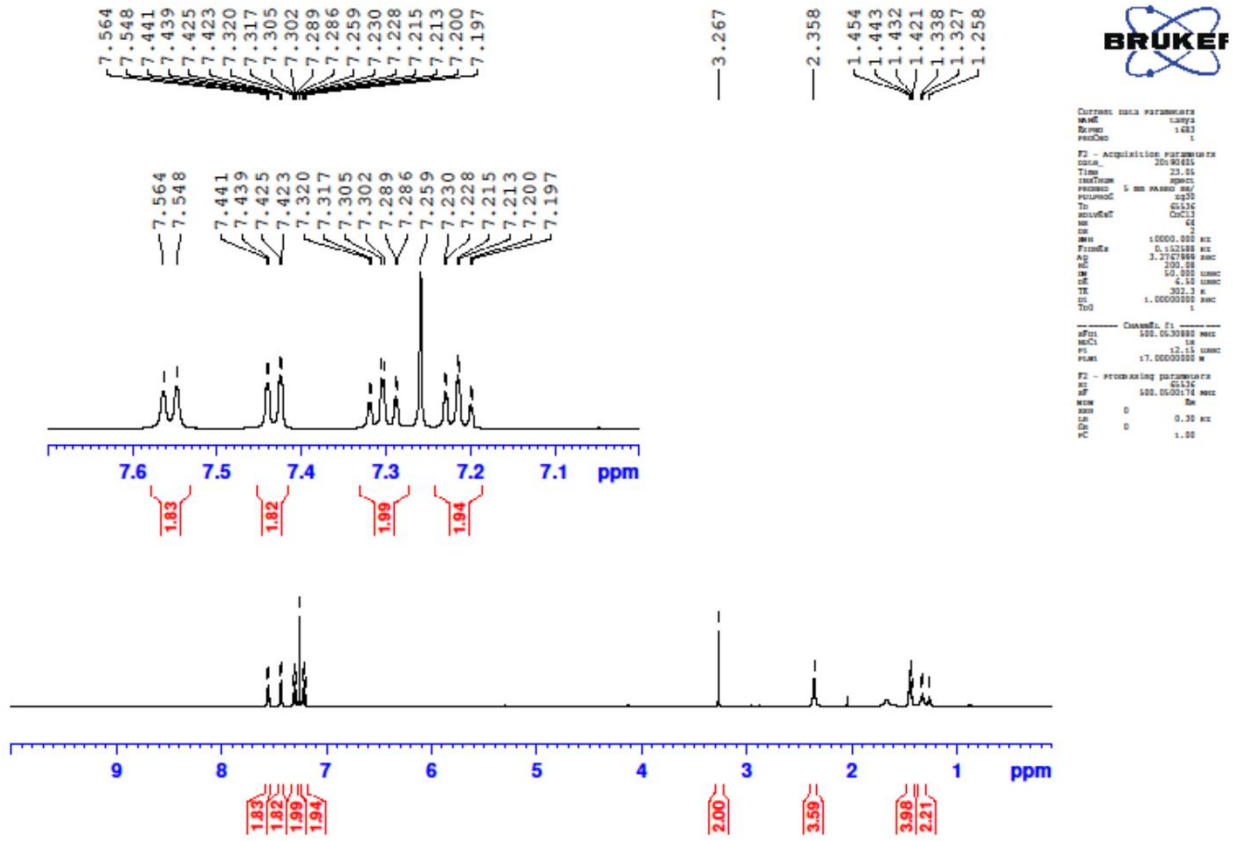


Figure S41: ^{13}C NMR of compound 15

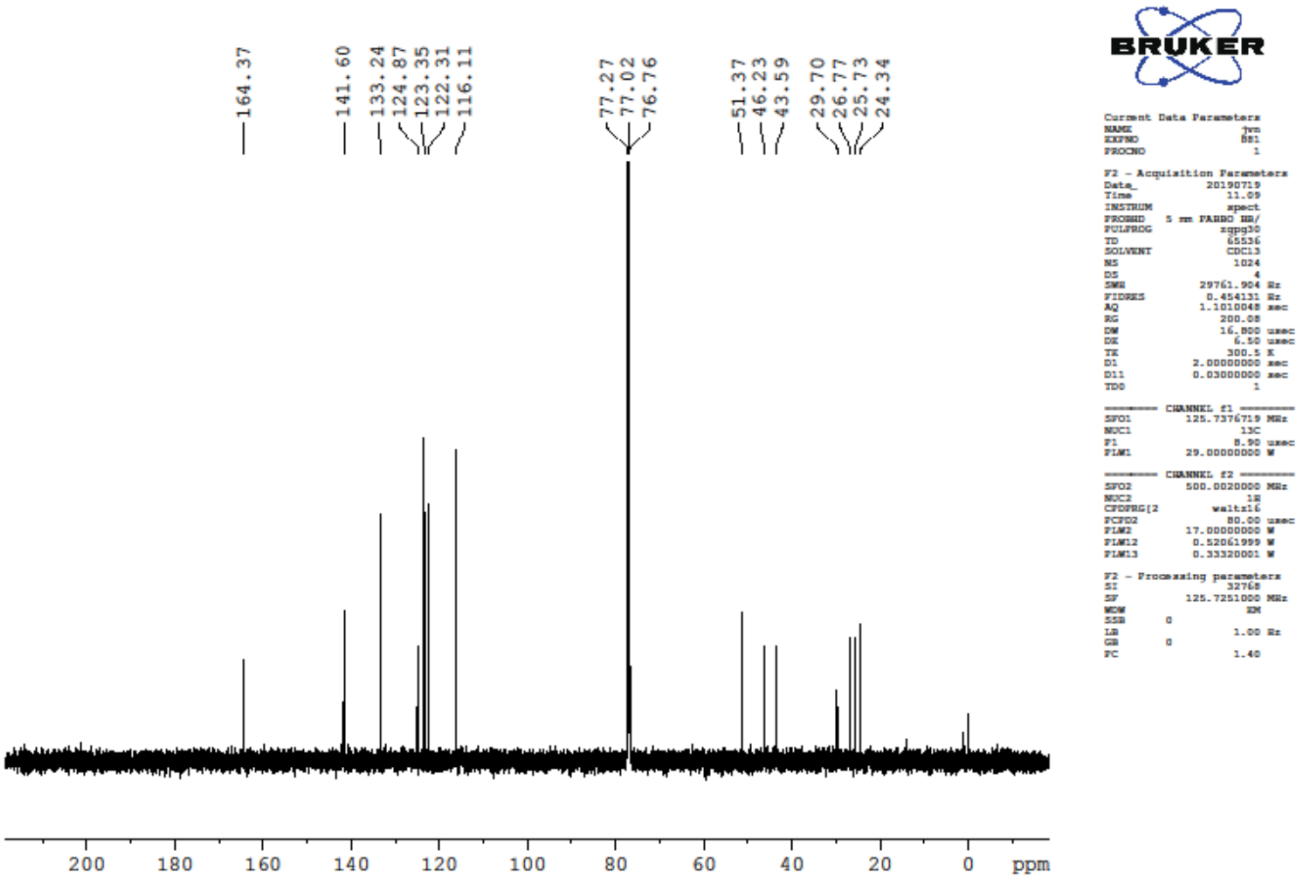
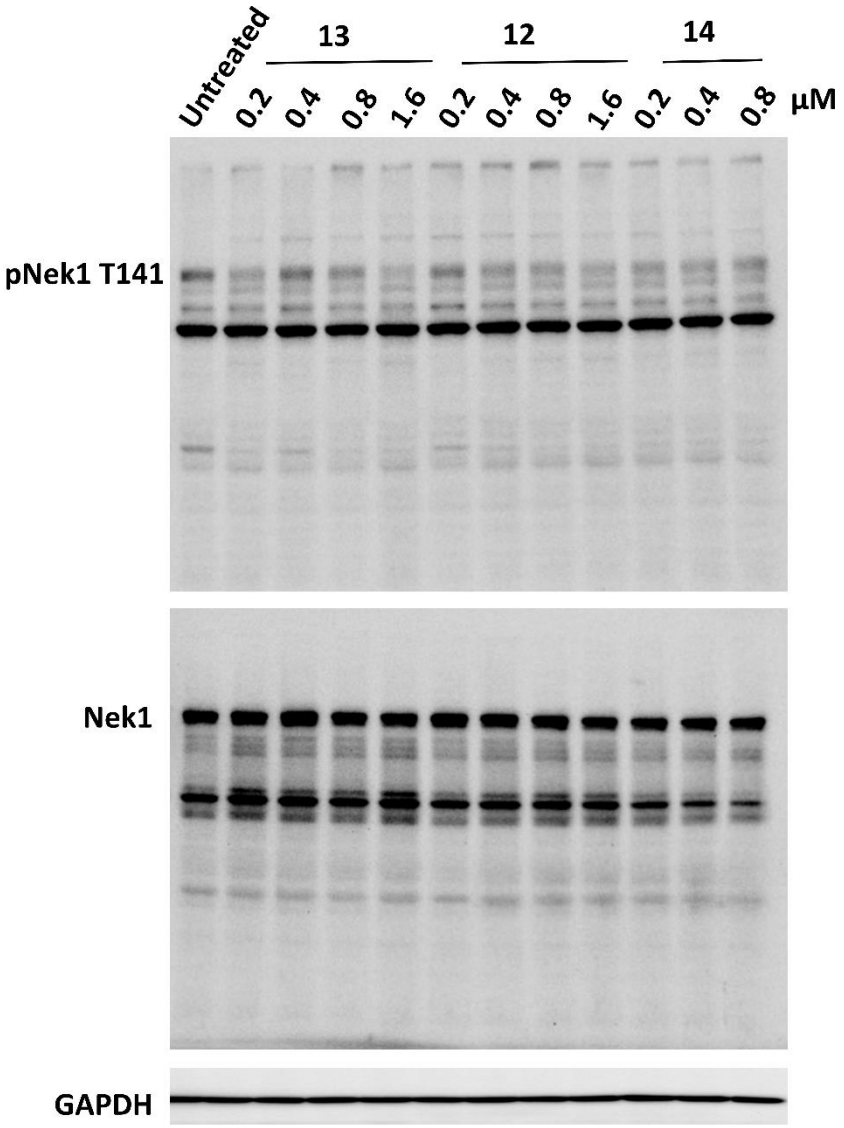


Figure S42. Raw Images of western blots:



The pNek1-T141 and Nek1 are replica-probed. GADPH blot itself was cut to avoid repeated probing.