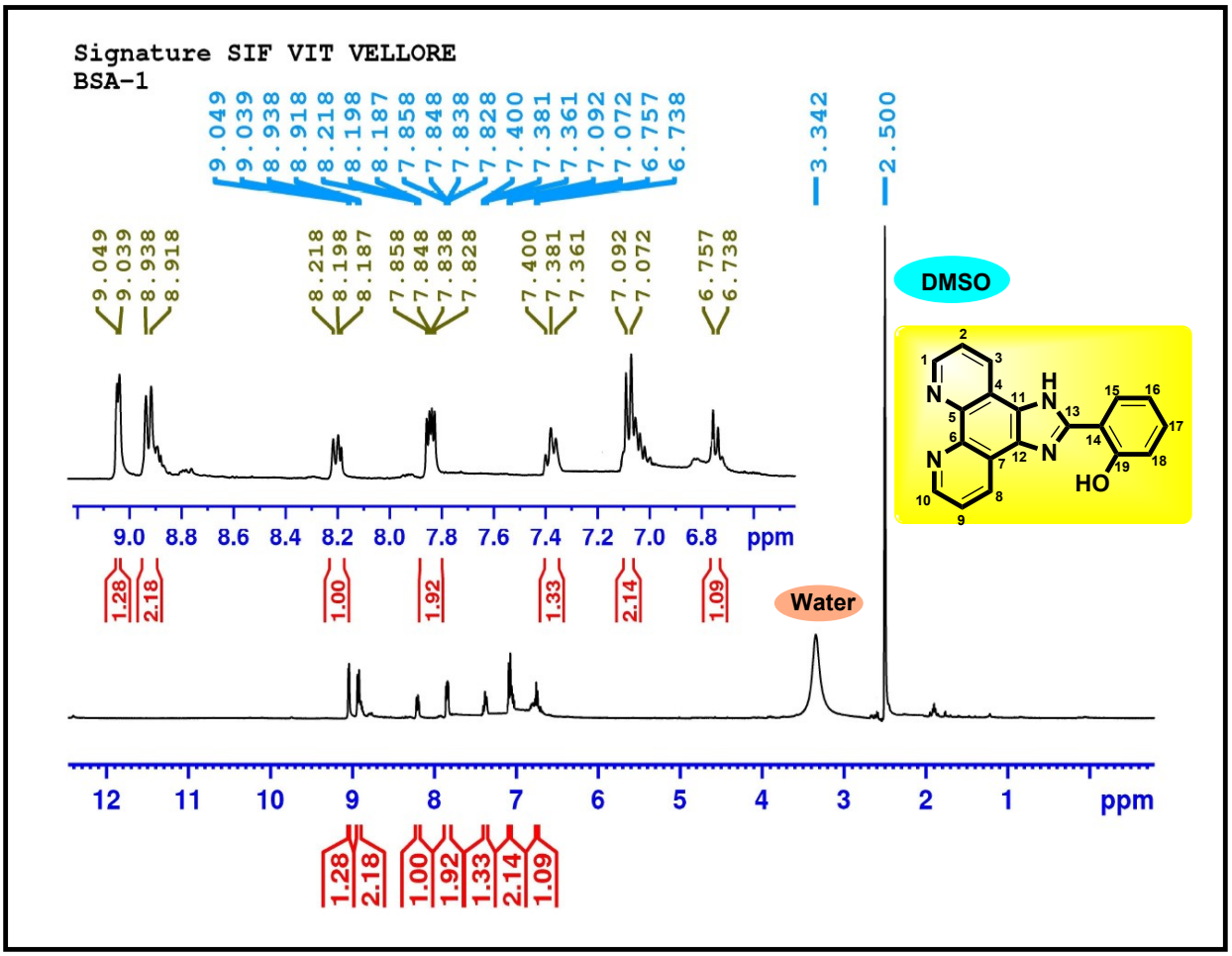
Supporting information

Iridium(III)-*Cp**-imidazo[4,5-f][1,10]phenanthrolin-2-yl)phenol analogues as hypoxia active, GSH-resistant cancer cytoselective and mitochondria targeting Cancer stem cell therapeutic agent‡

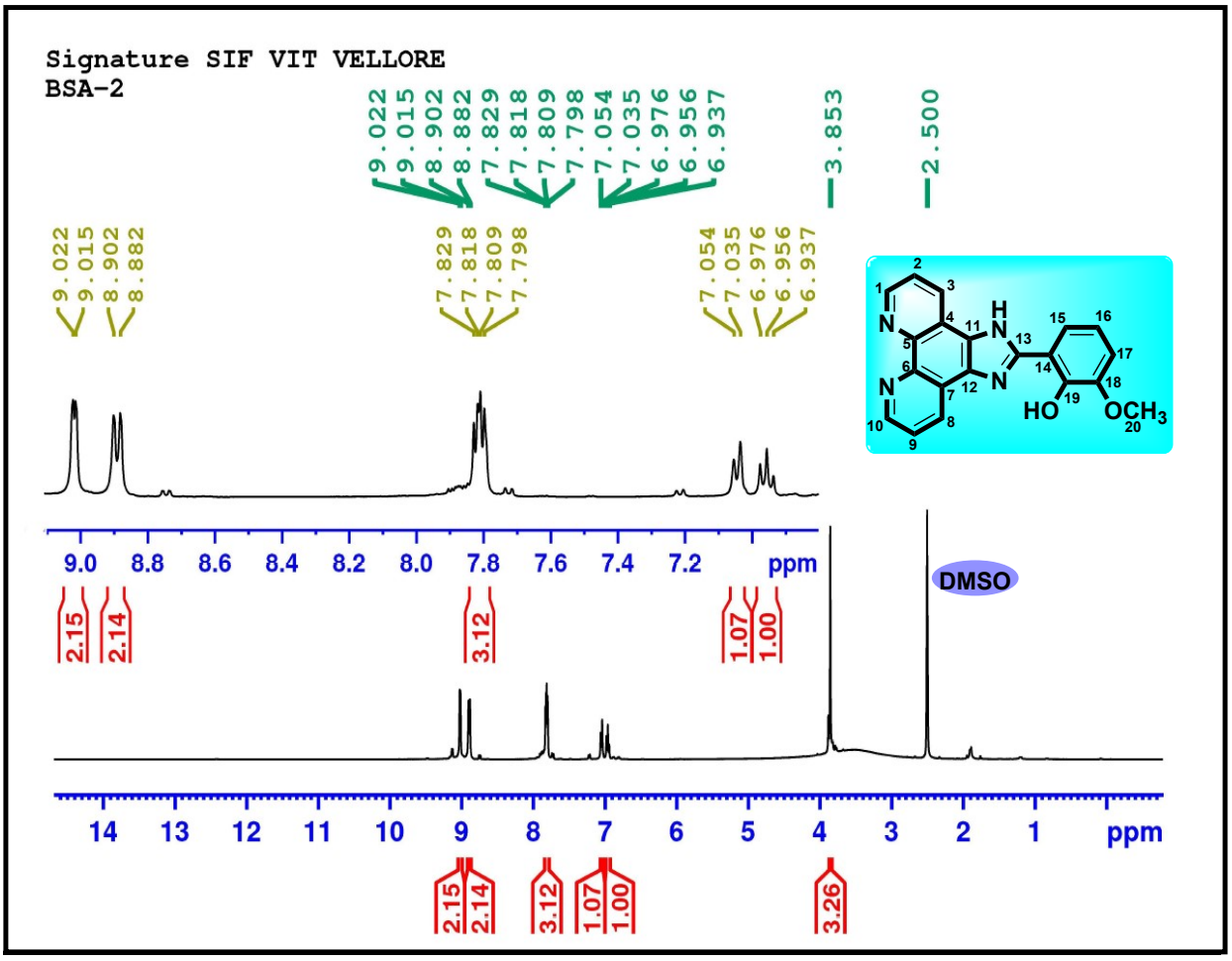
Binoy Kar,[†a] Shanooja Shanavas,[†b] Apoorva H. Nagendra,[b] Utpal Das,[a] Nilmadhab Roy,[a] Sudhindra Pete,[a] Ajay Sharma S,[a] Sourav De,[a] Ashok Kumar S K,[a] Suban K Sahoo,[c] Debashis Panda,[d] Sudheer Shenoy,[b] Bipasha Bose,[*b] Priyankar Paira[*a]

CHARACTERIZATION OF LIGANDS (L1-L7)

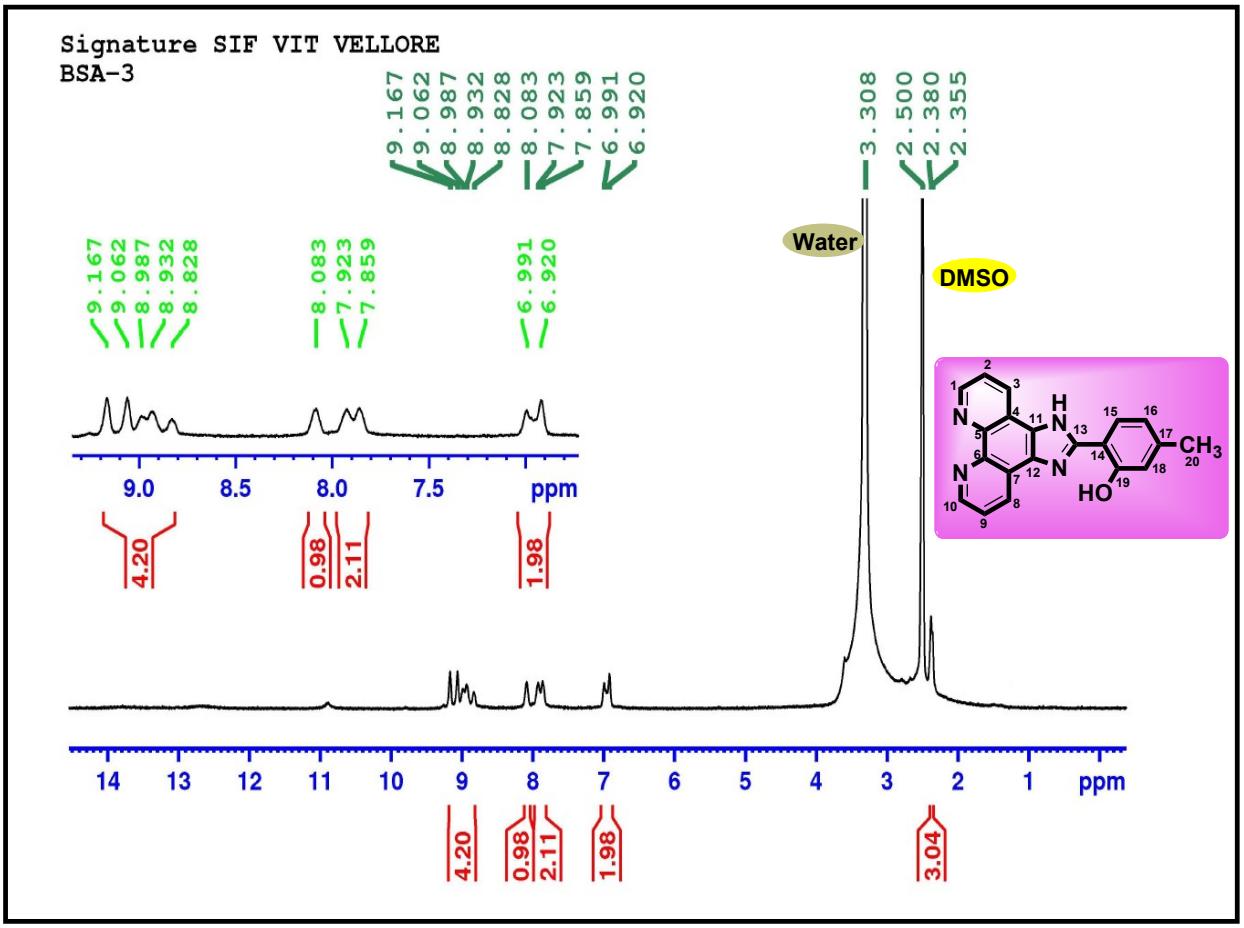
^{1}H NMR of ligand L1:



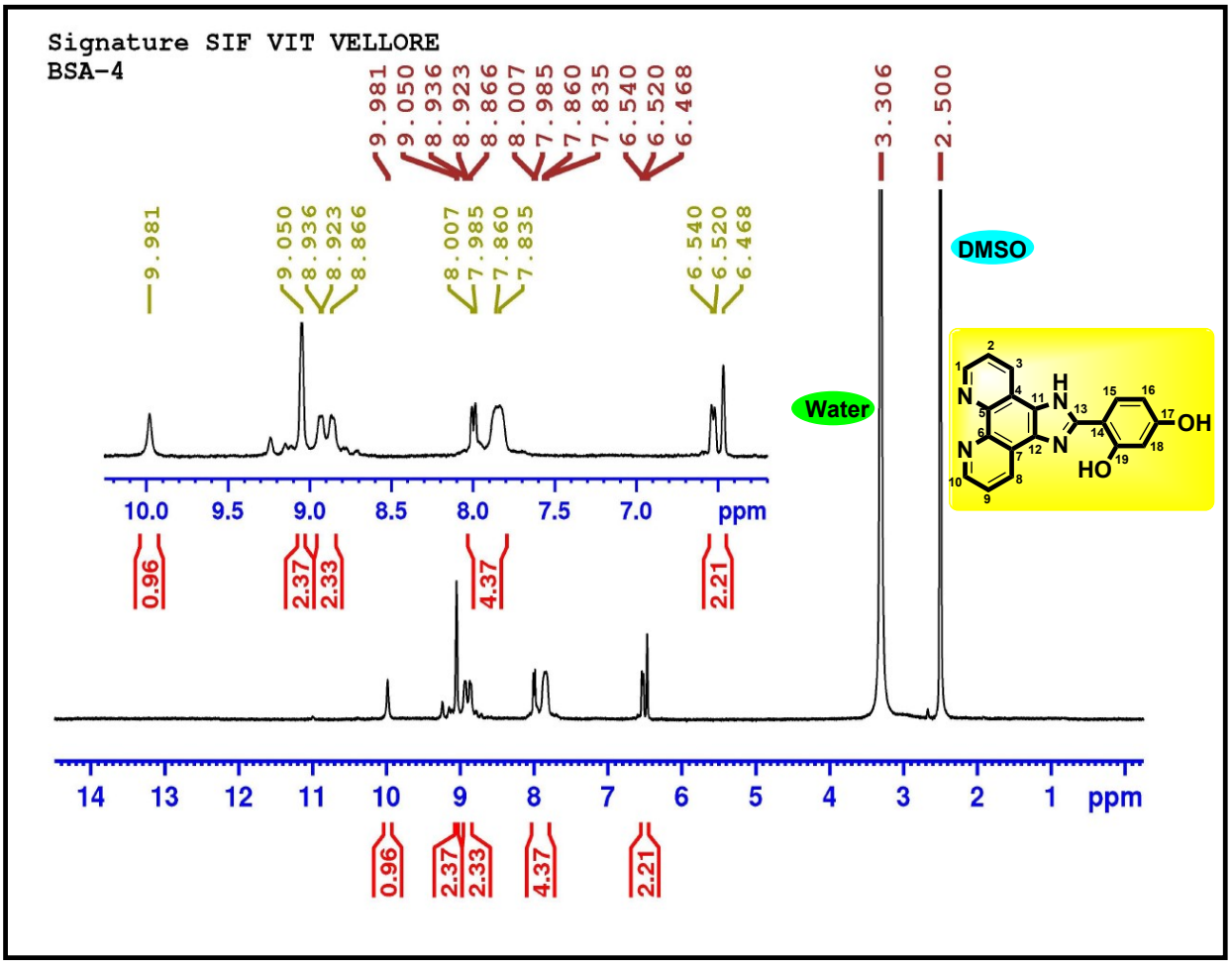
^{1}H NMR of ligand L2:



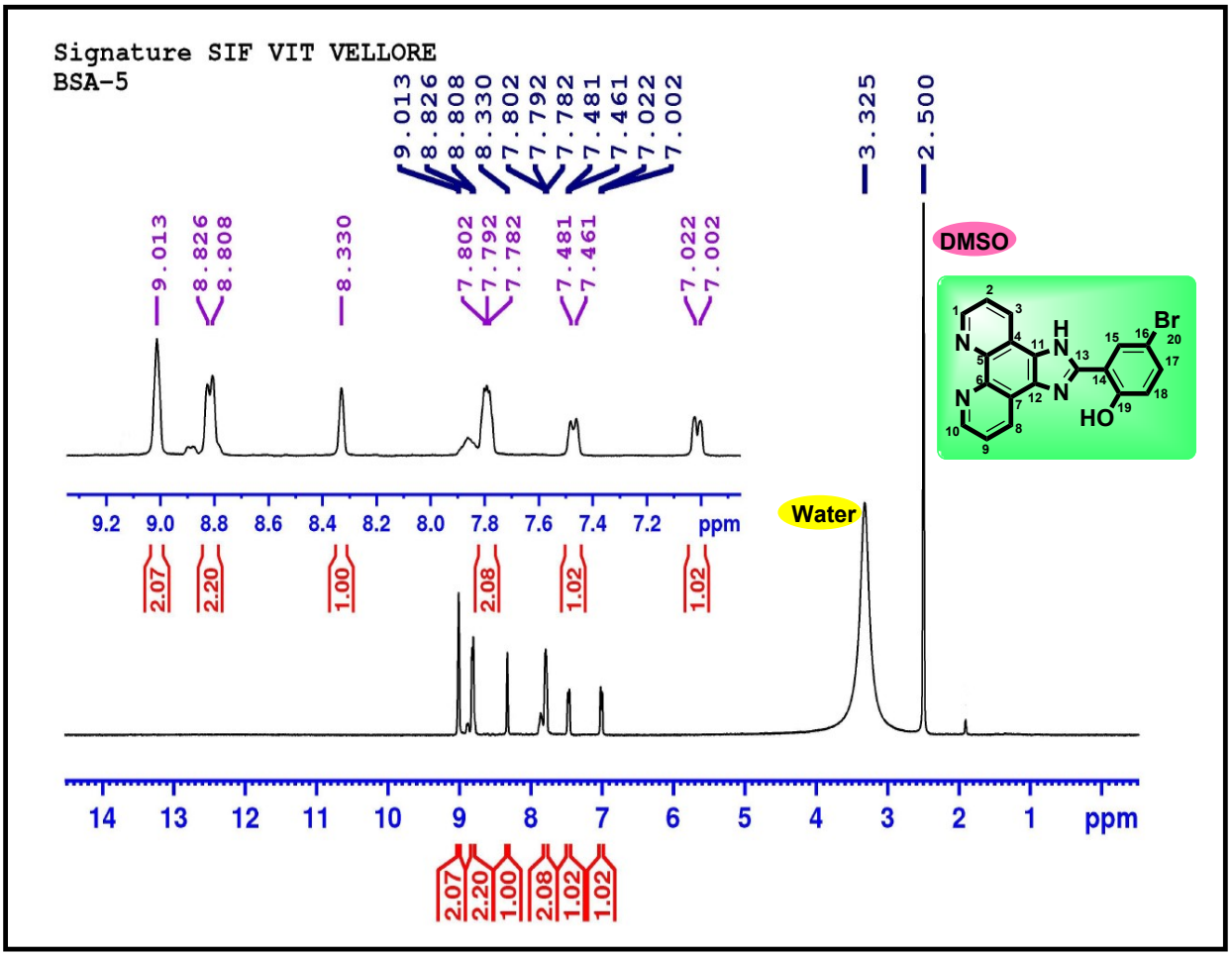
^{1}H NMR of ligand L3:



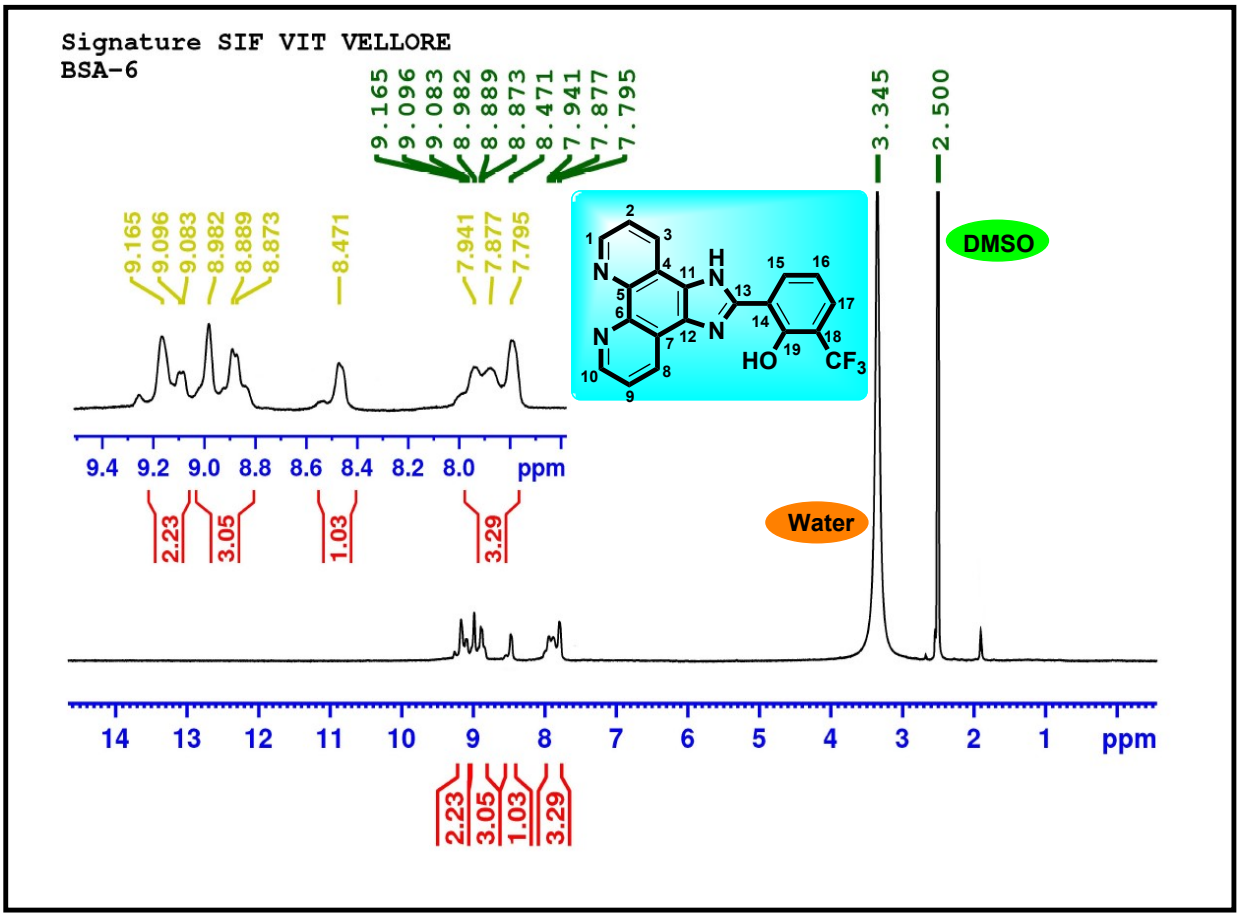
^{1}H NMR of ligand L4:



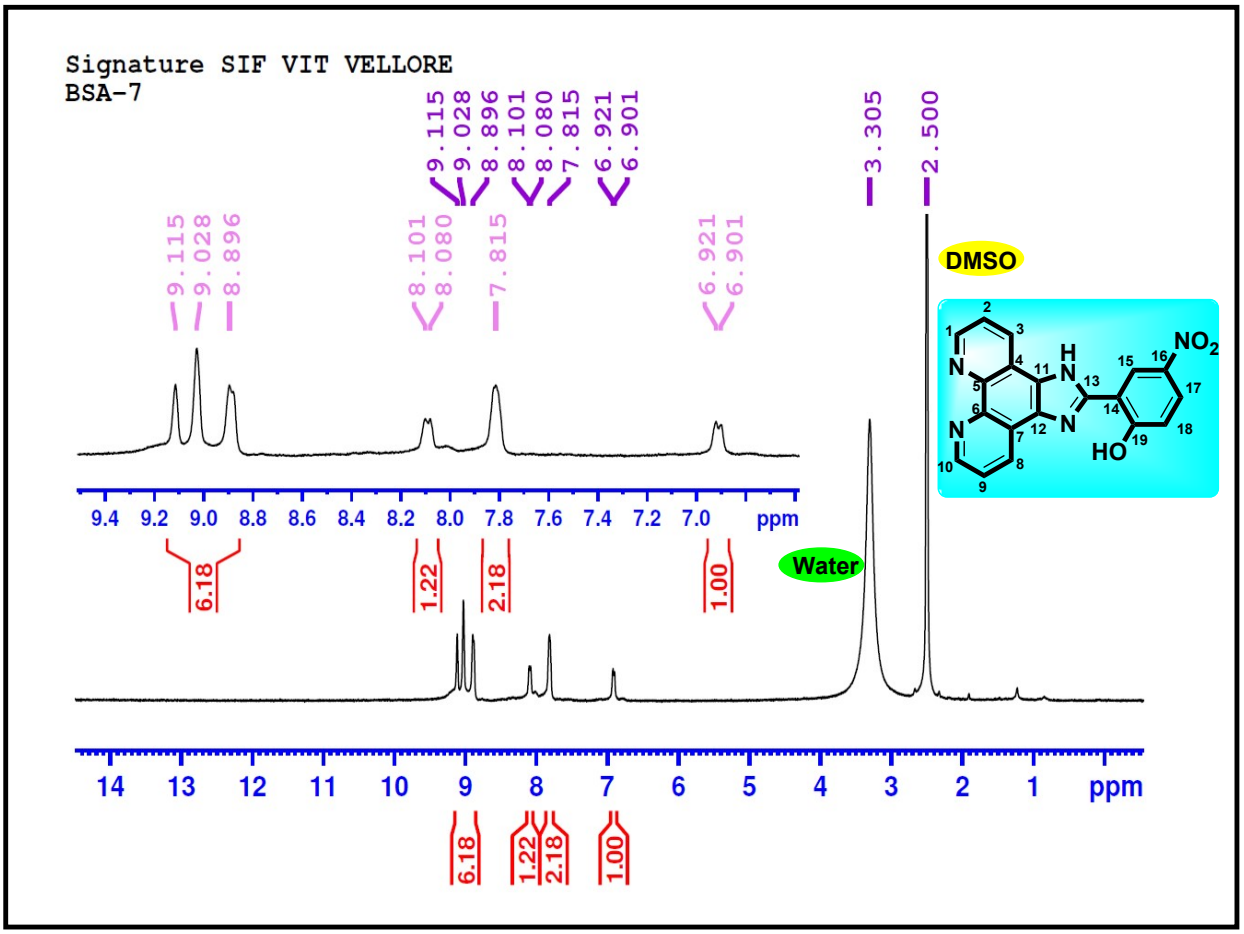
^{1}H NMR of ligand L5:



^{1}H NMR of ligand L6:

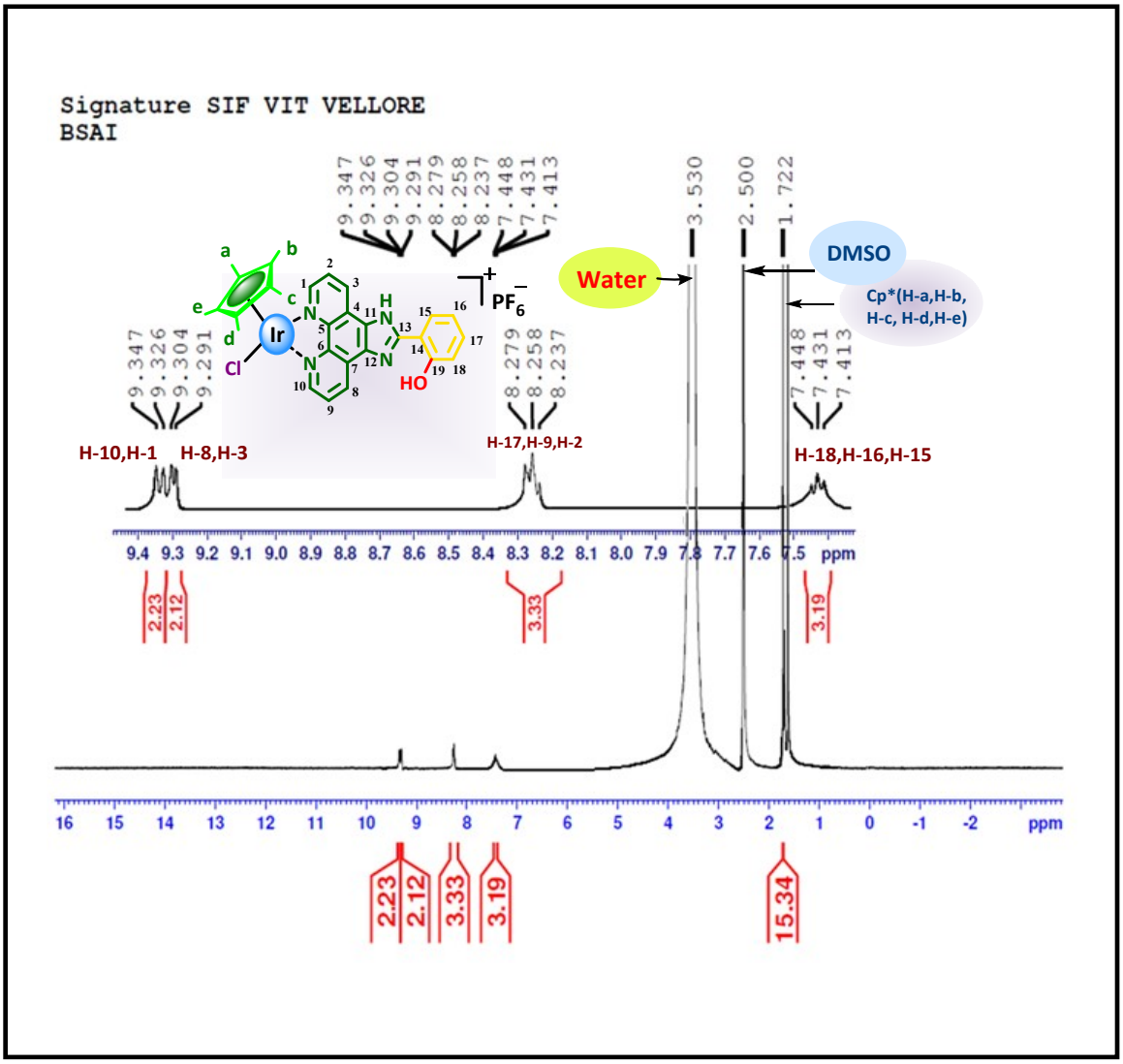


^{1}H NMR of ligand L7:

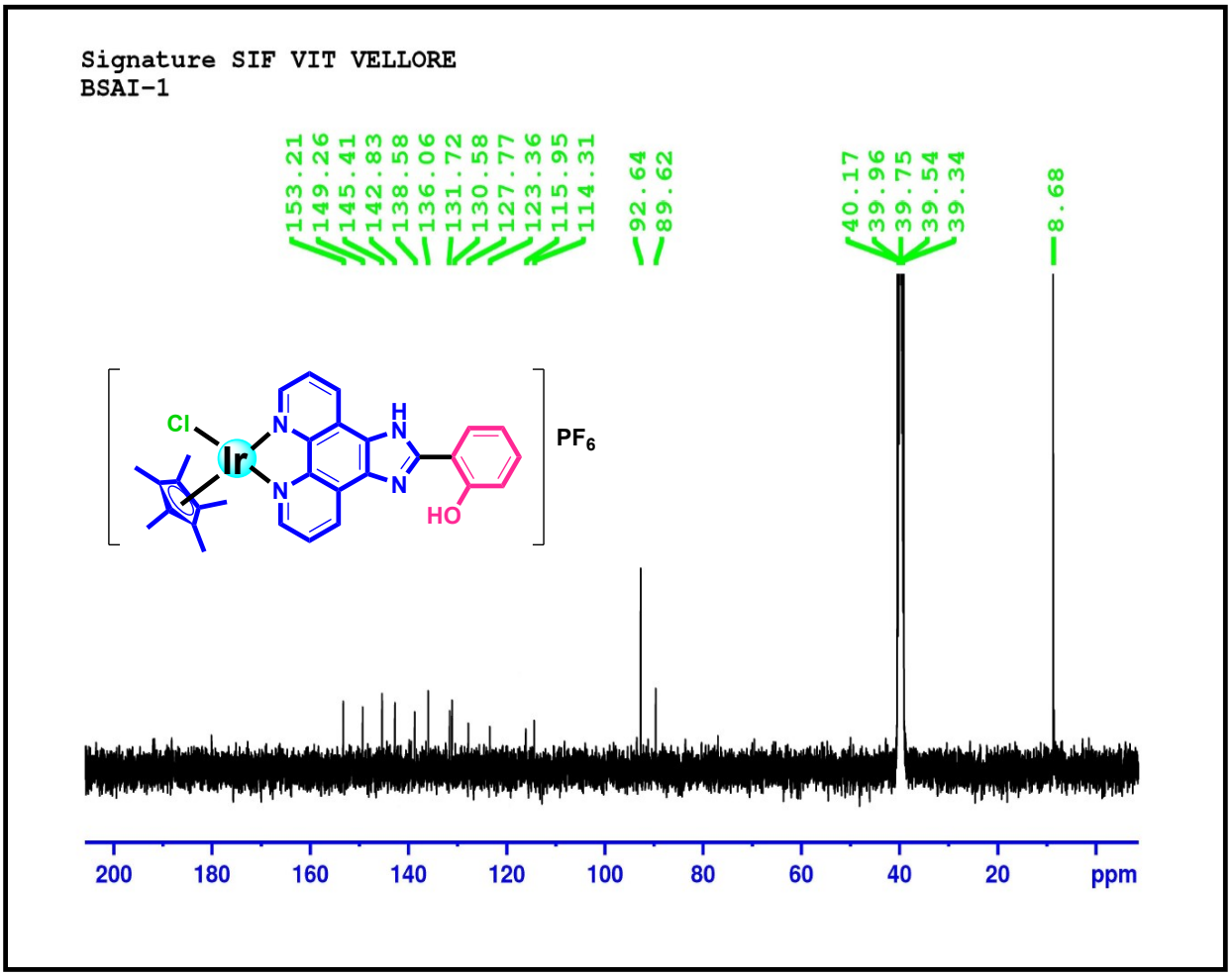


CHARACTERIZATION OF COMPLEXES (IrL1-IrL7):

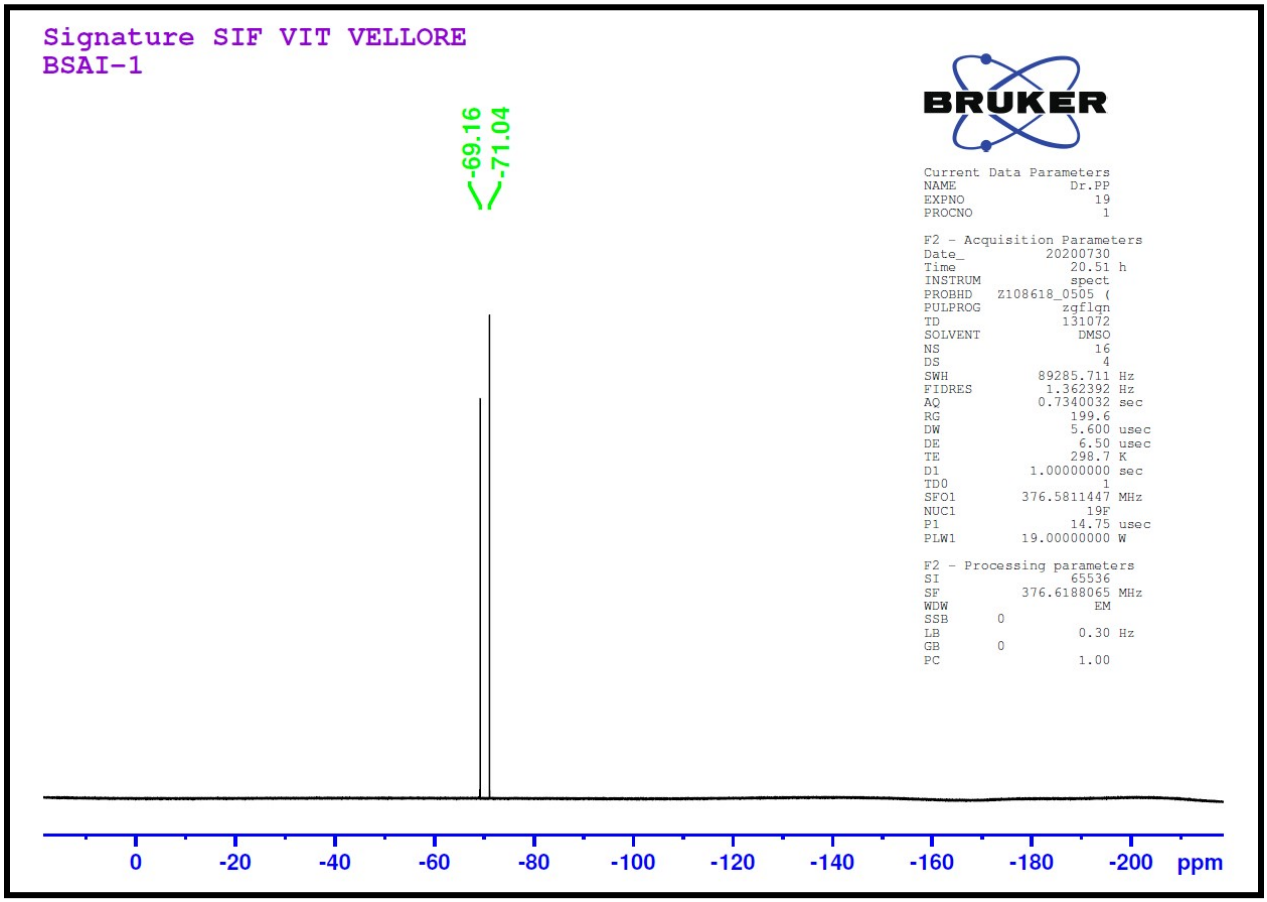
^{1}H NMR of complex IrL1:



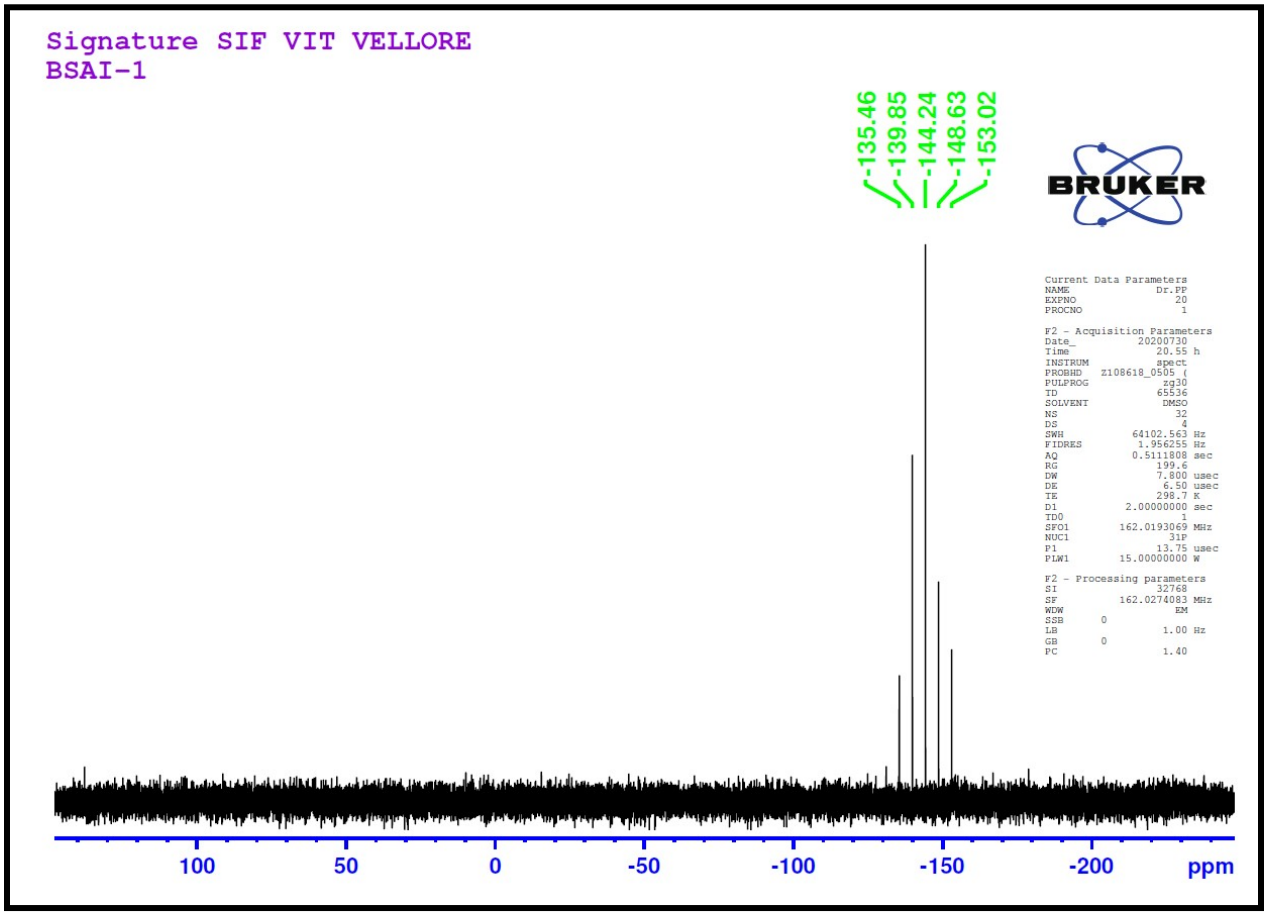
^{13}C NMR of complex IrL1:



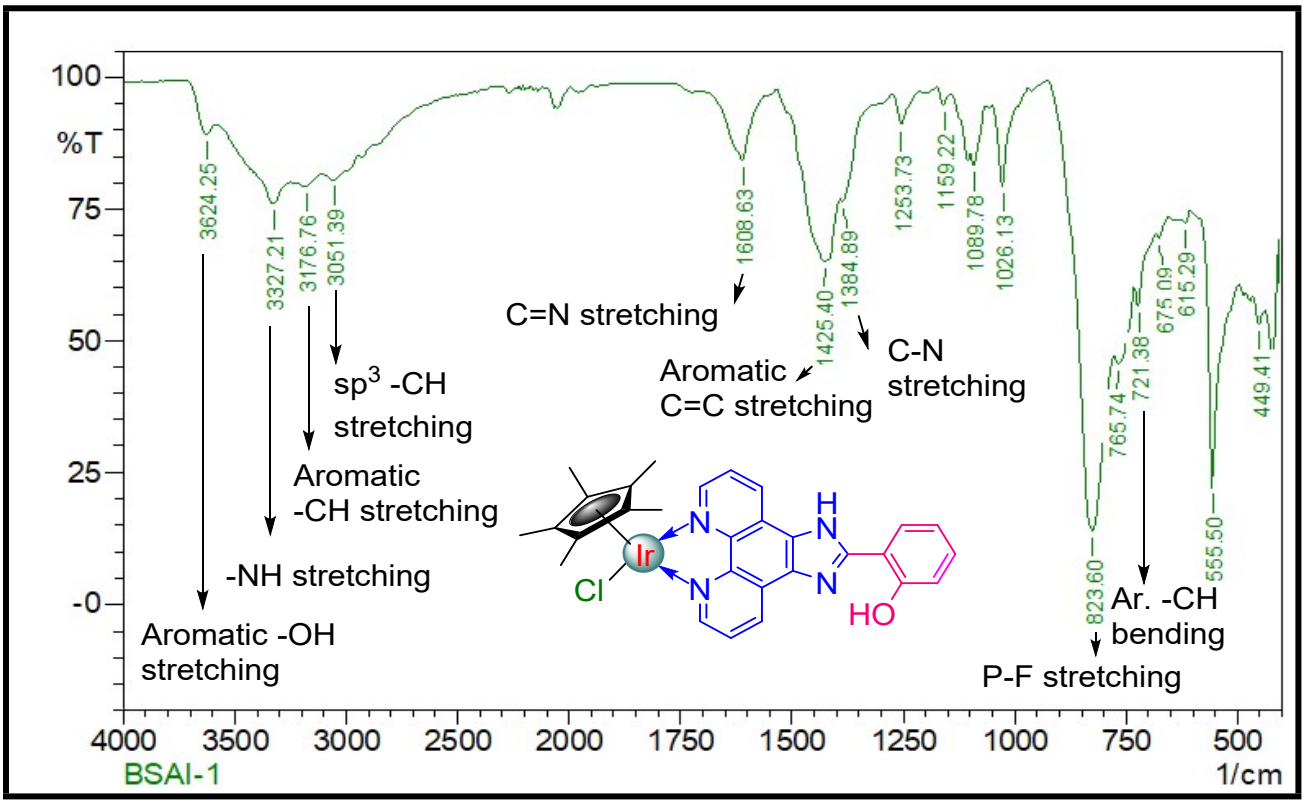
^{19}F NMR of complex IrL1:



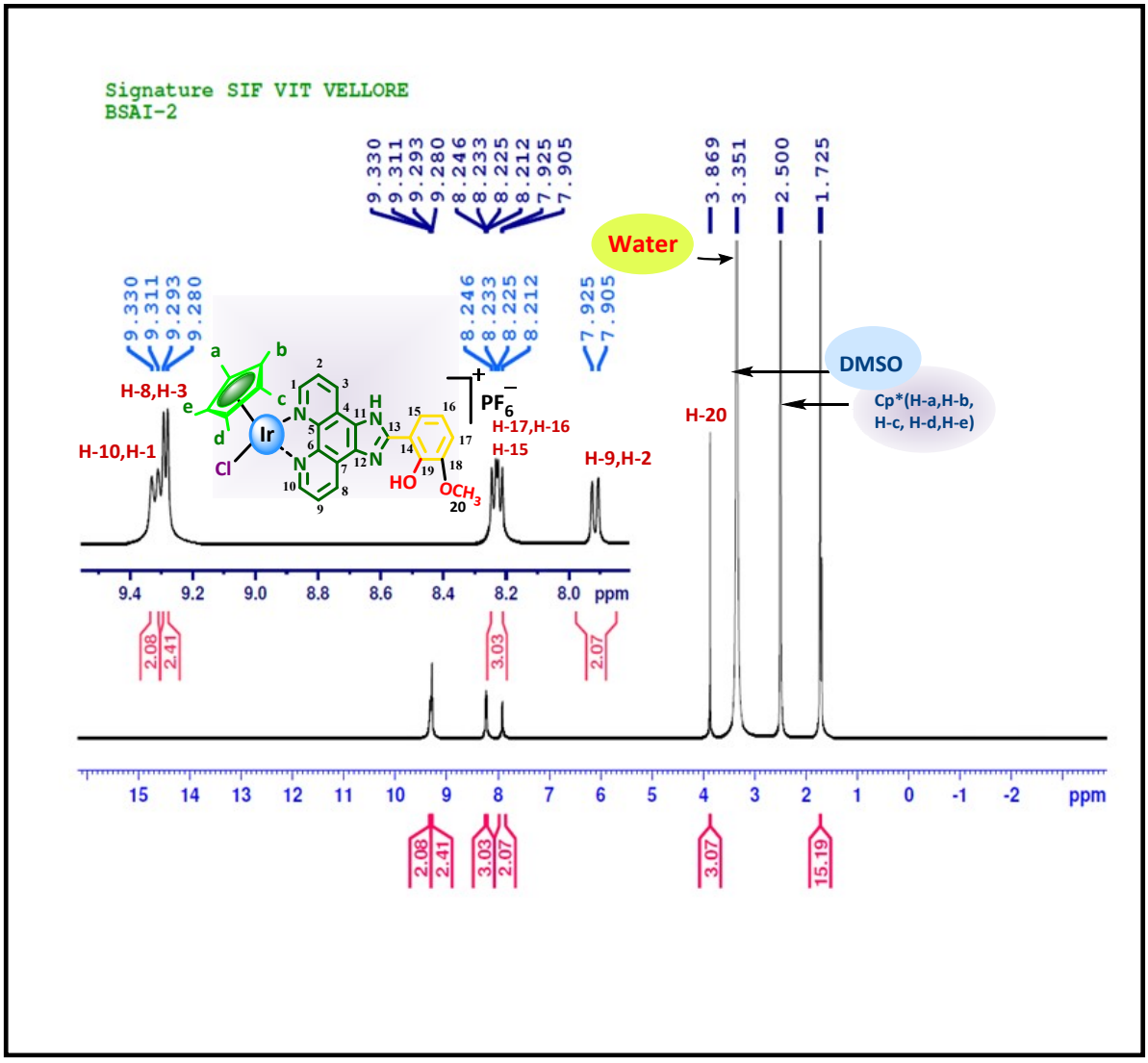
^{31}P NMR of complex IrL1:



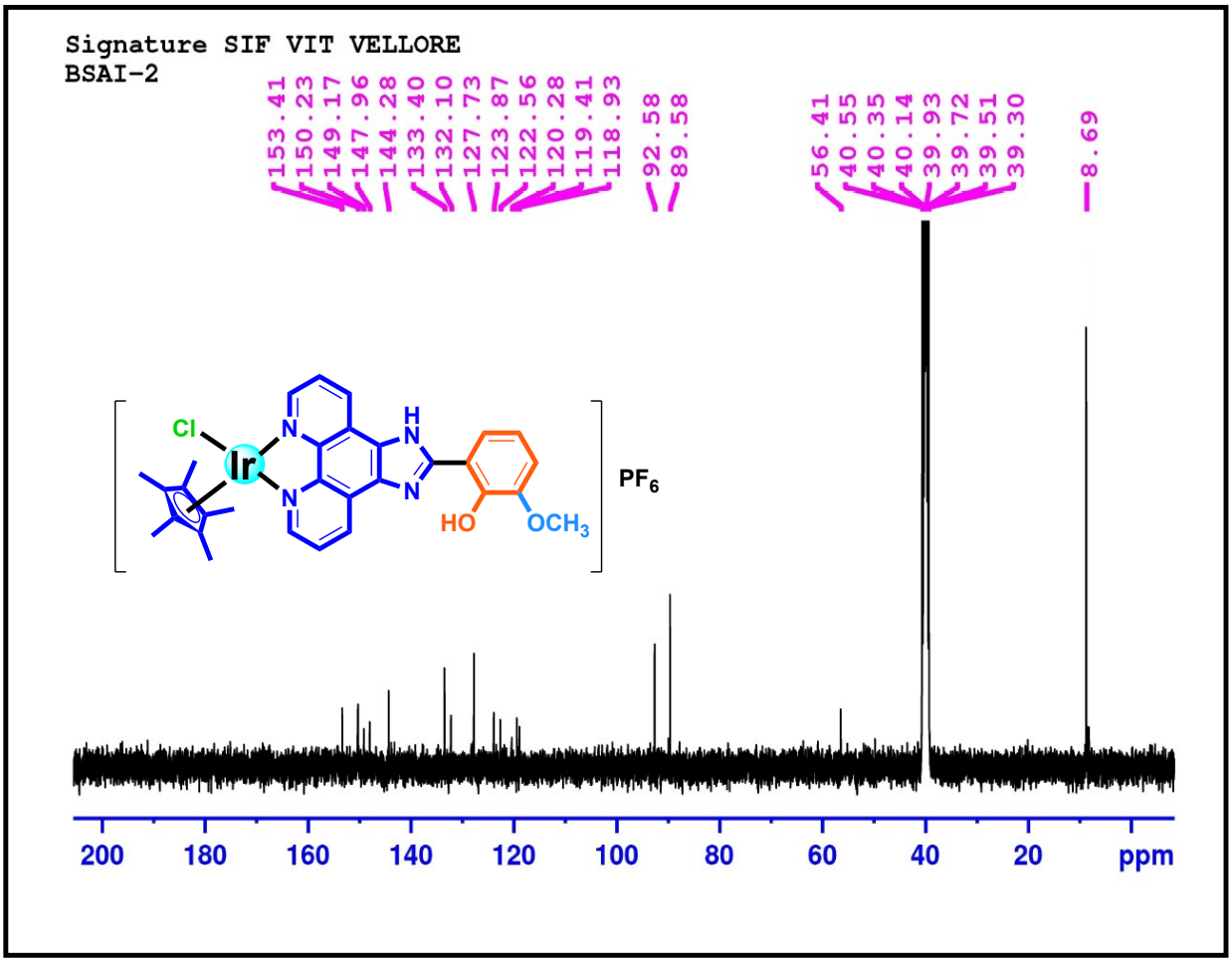
IR spectrum of IrL1:



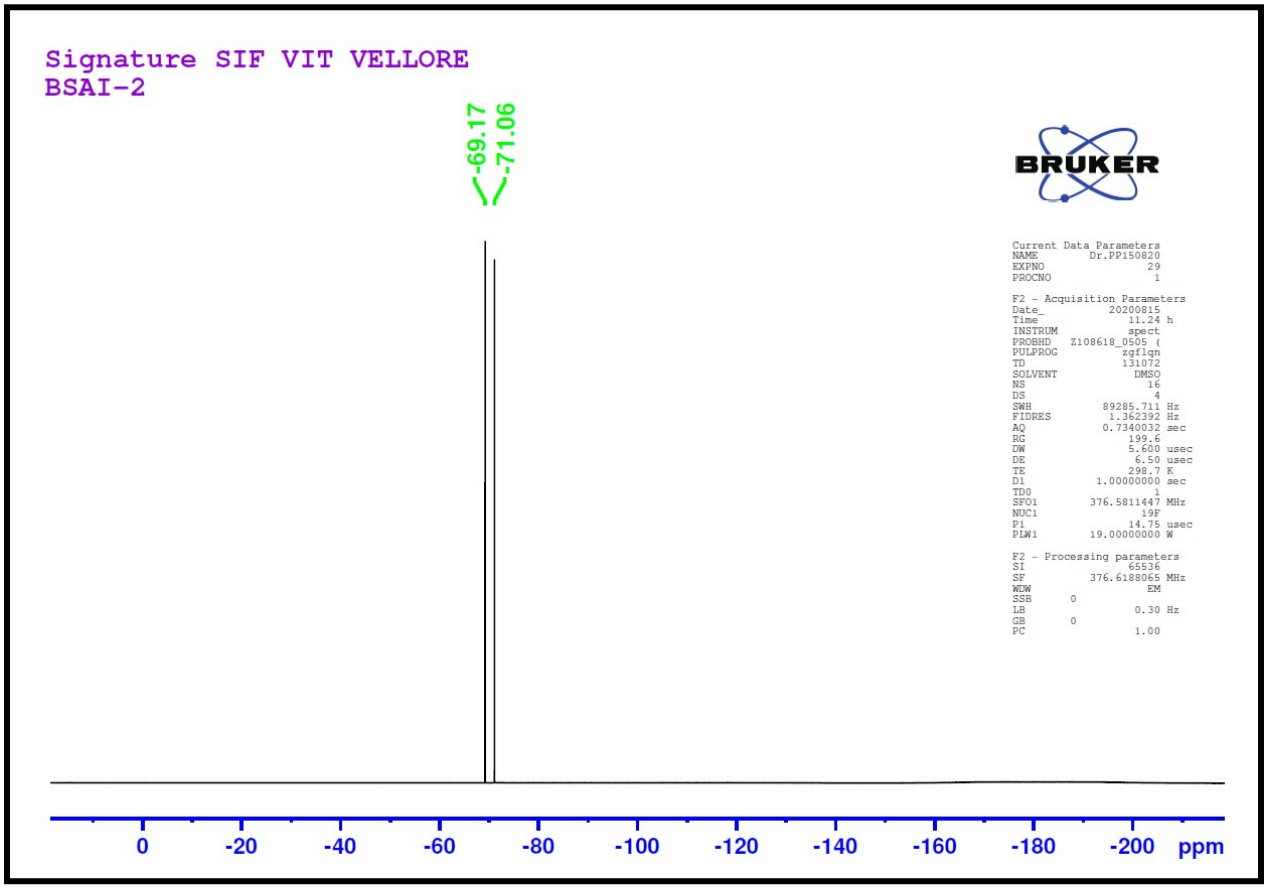
^{1}H NMR of complex IrL2:



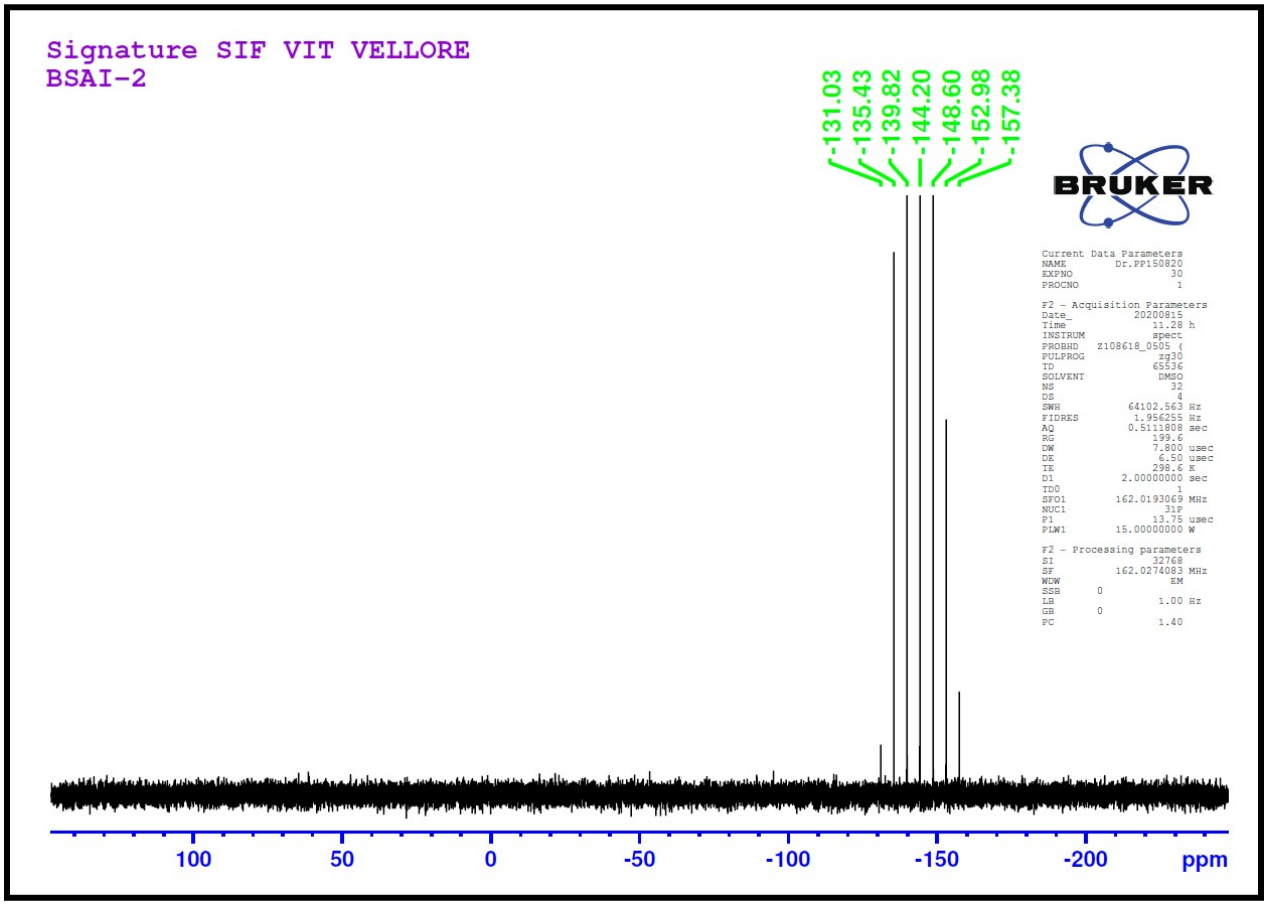
^{13}C NMR of complex IrL2:



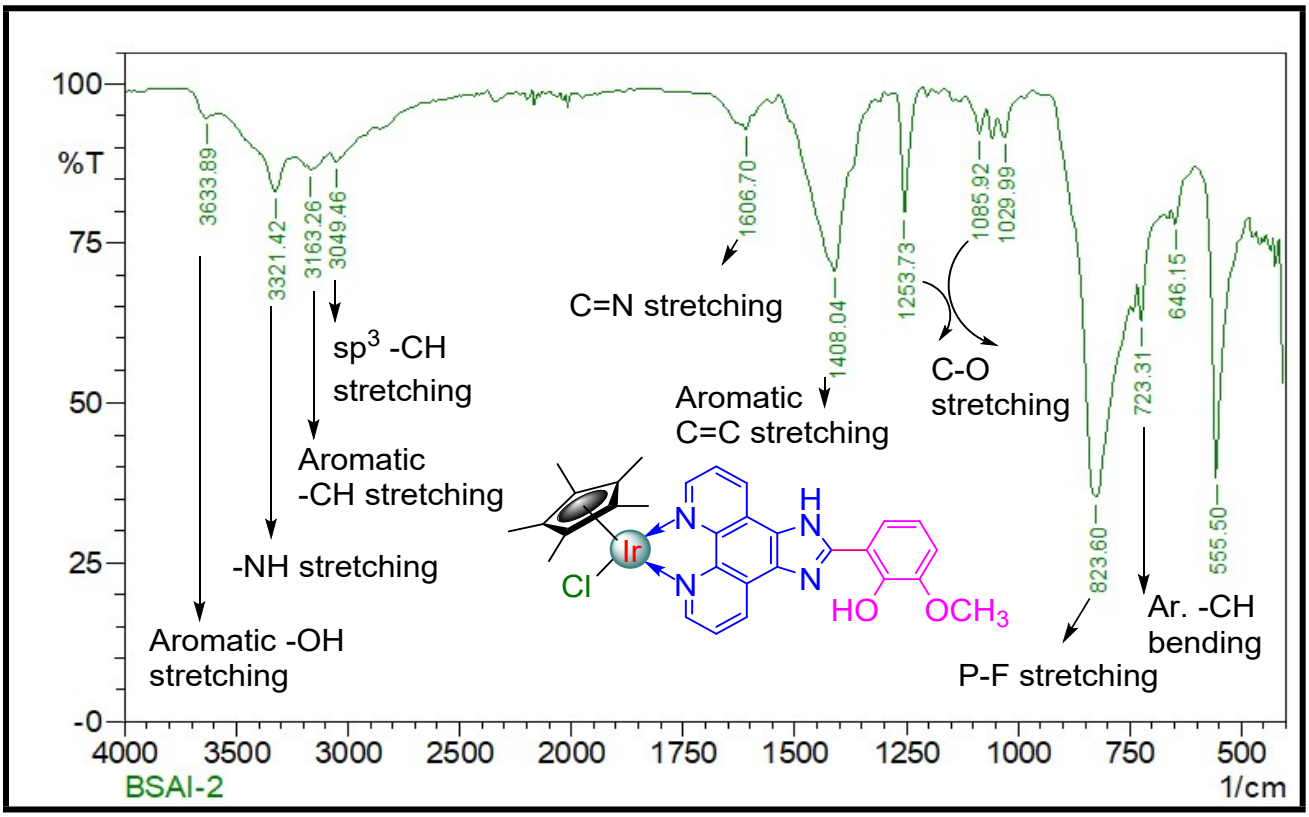
^{19}F NMR of complex IrL2:



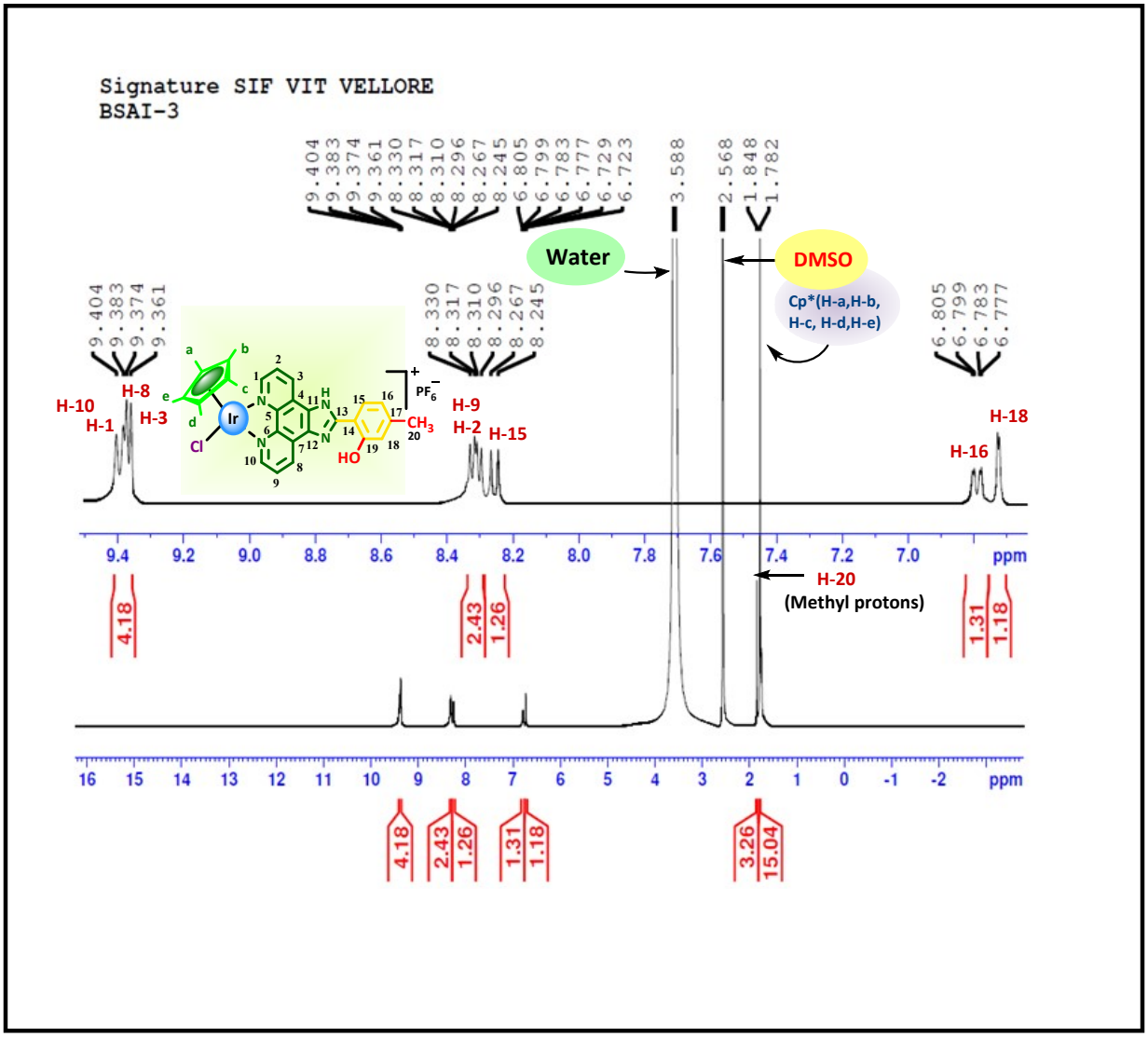
^{31}P NMR of complex IrL2:



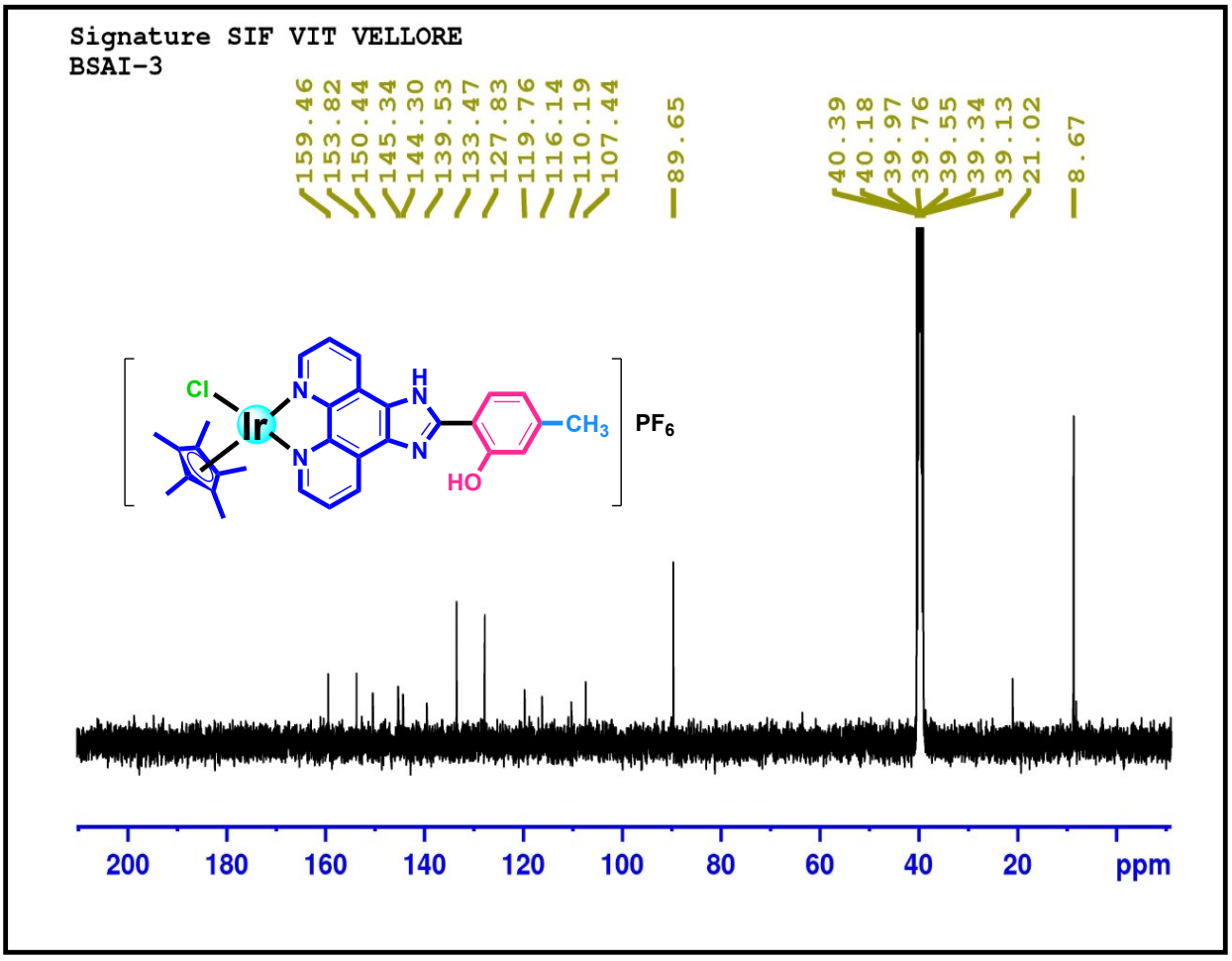
IR spectrum of IrL2:



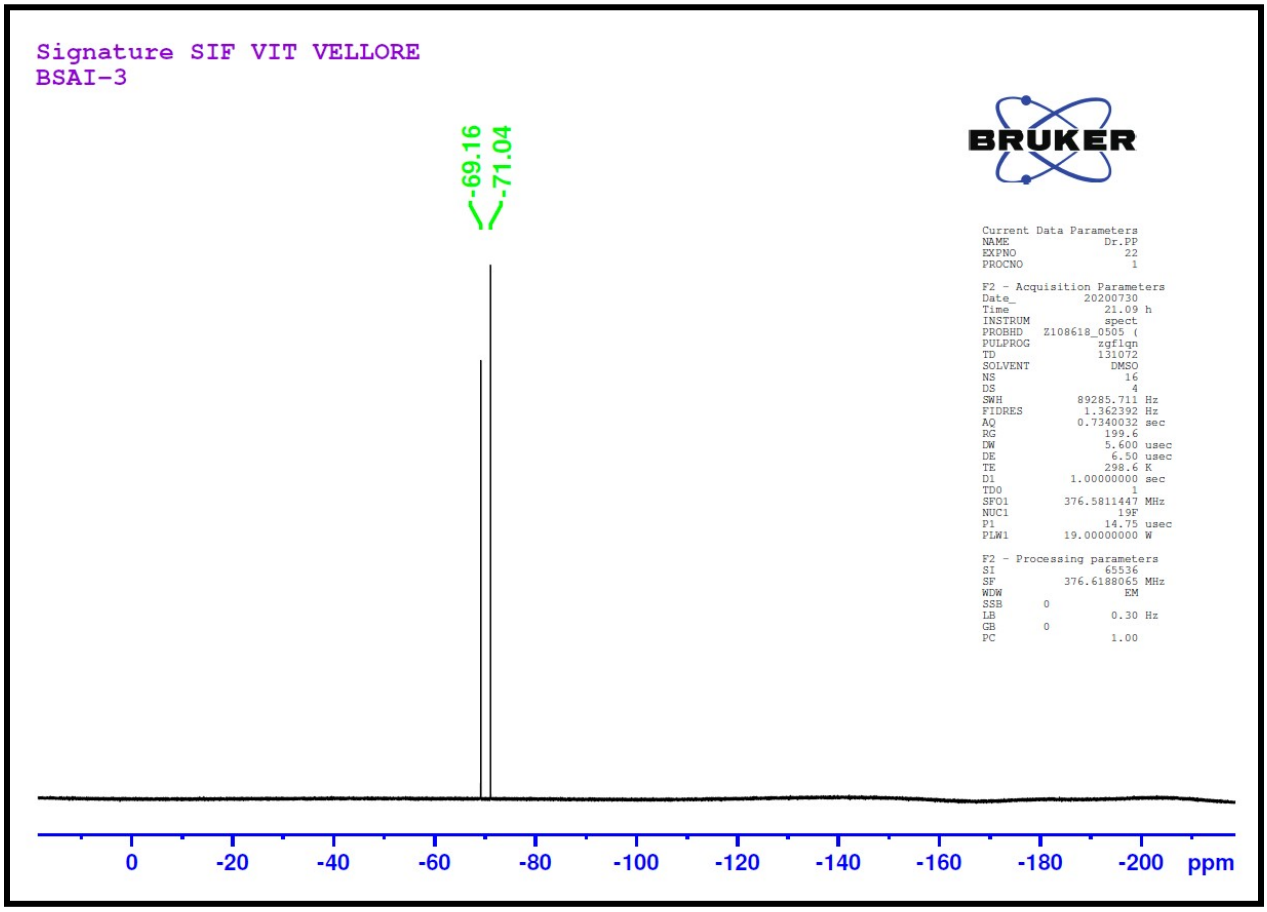
^{1}H NMR of complex IrL3:



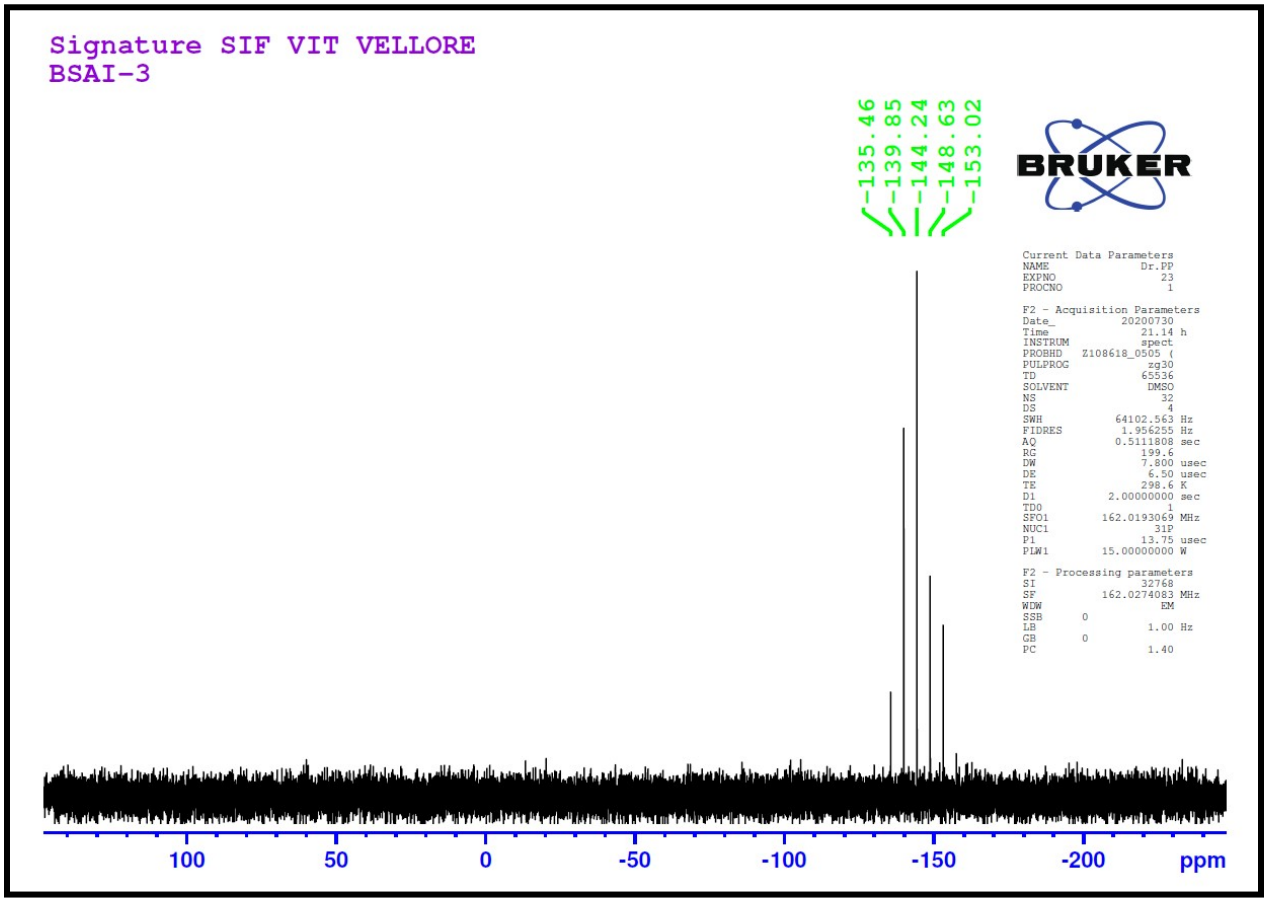
^{13}C NMR of complex IrL3:



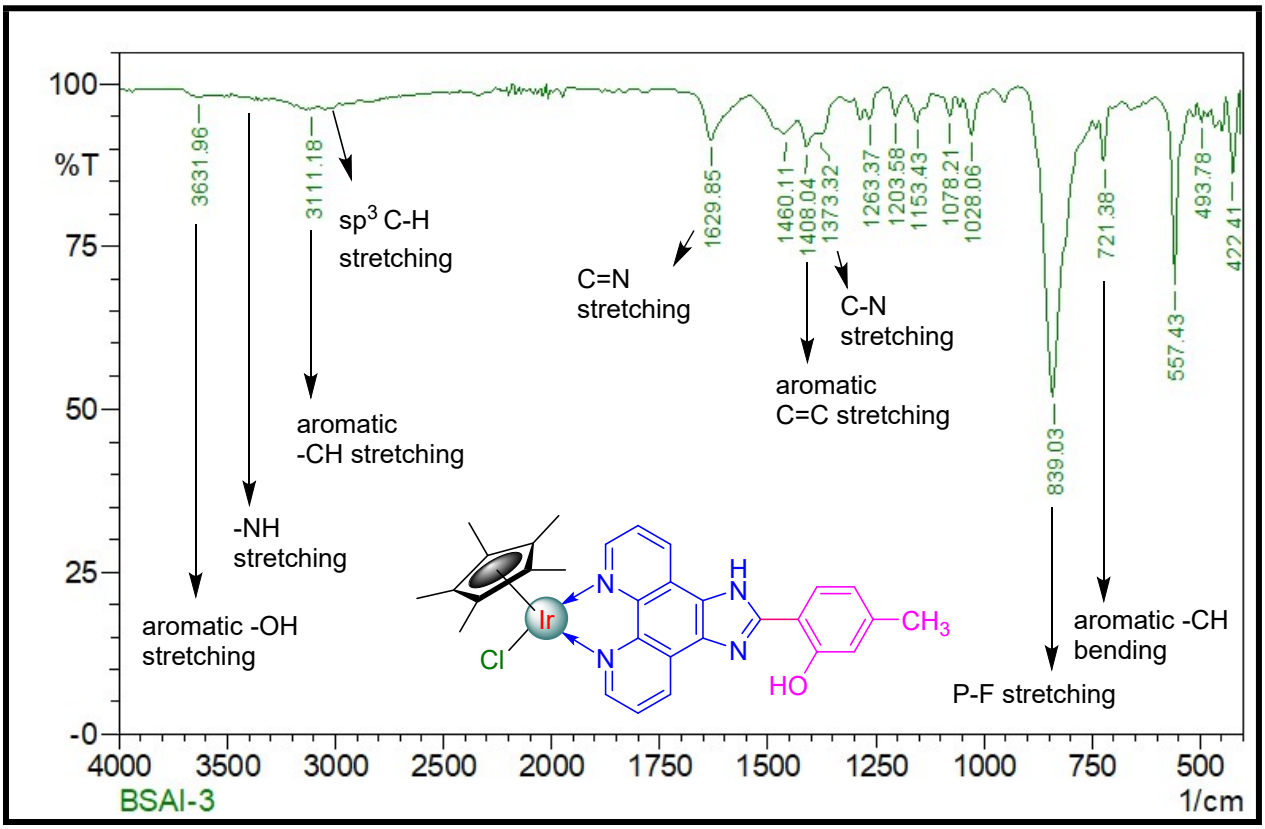
^{19}F NMR of complex IrL3:



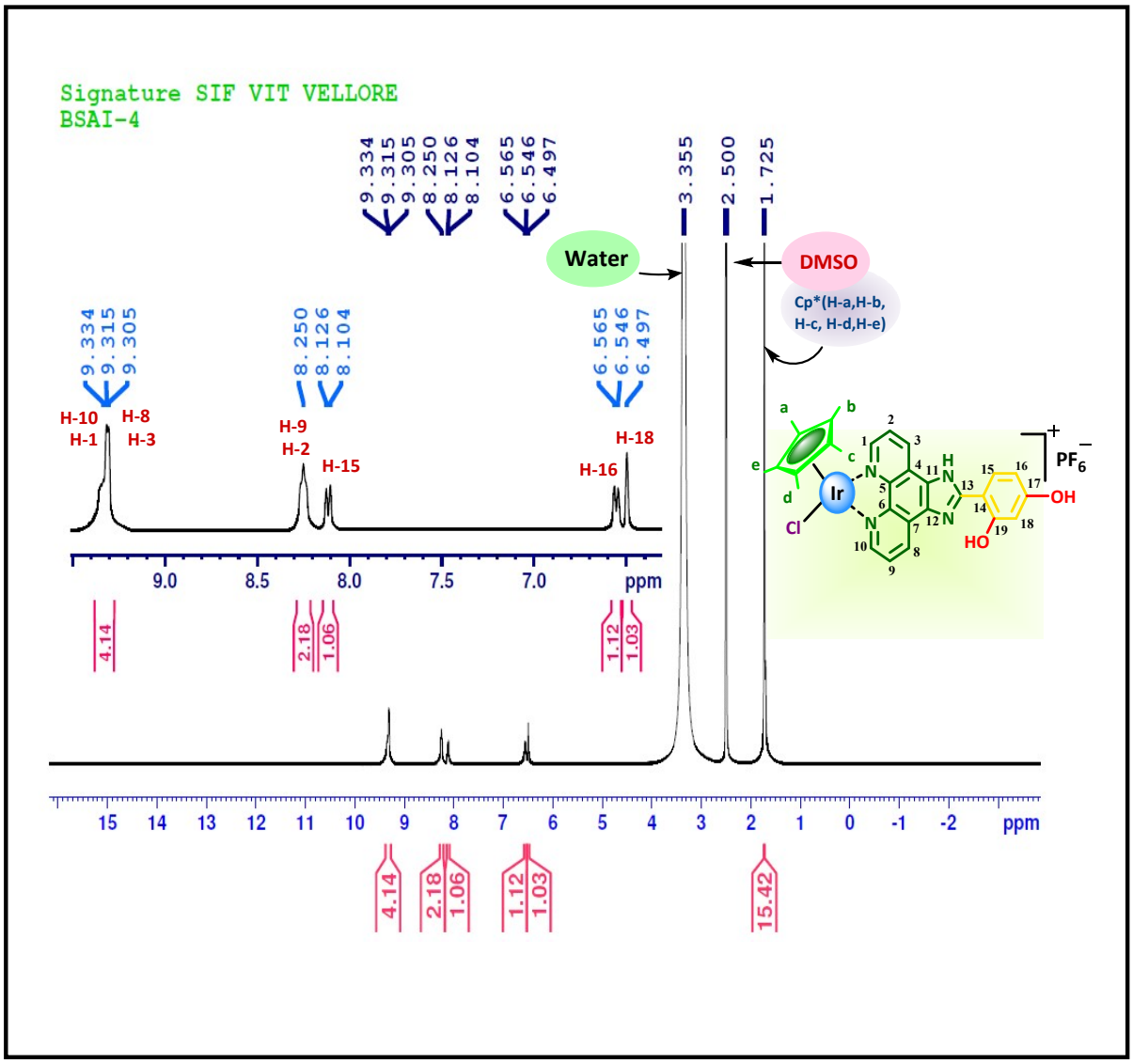
^{31}P NMR of complex IrL3:



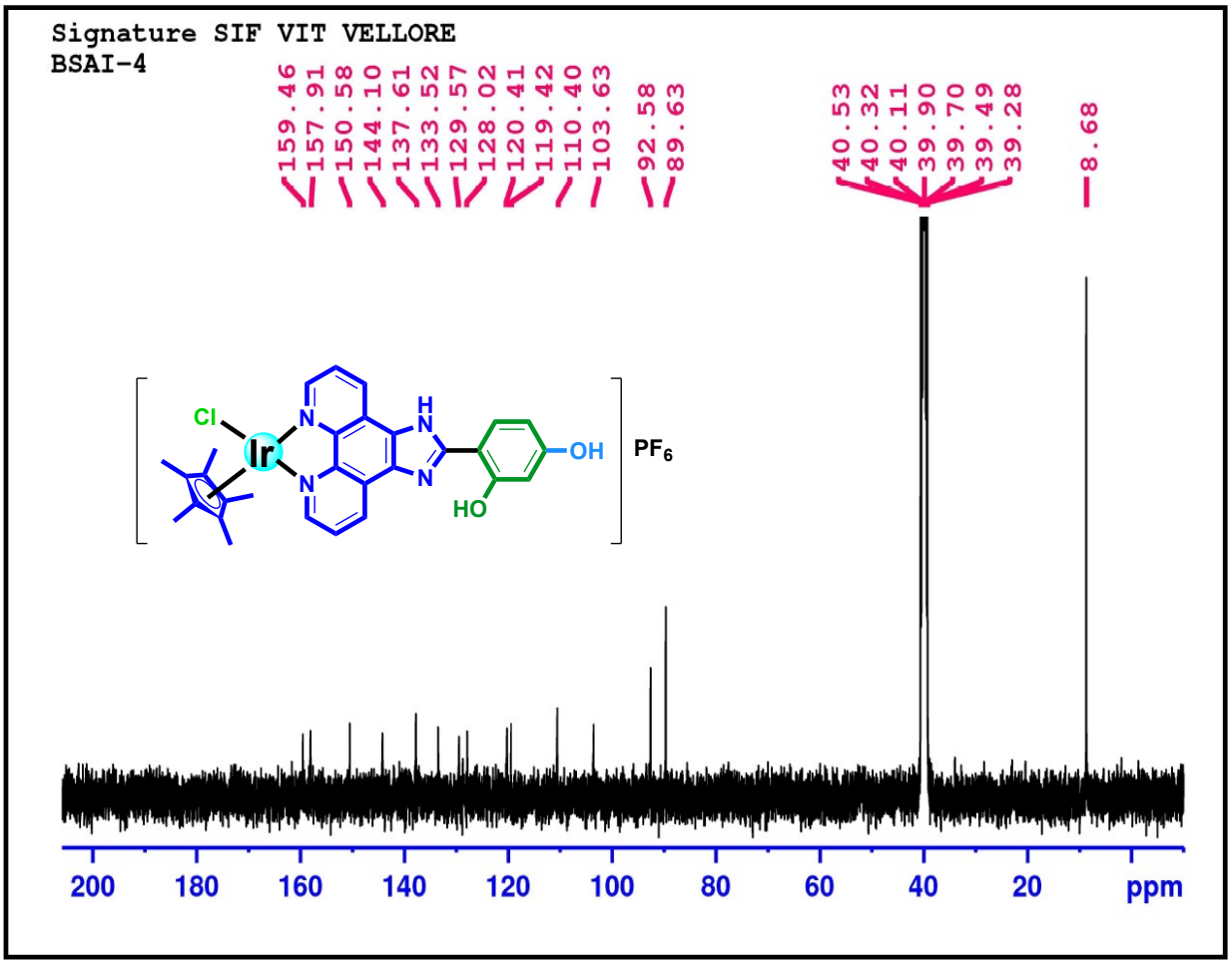
IR spectrum of IrL3:



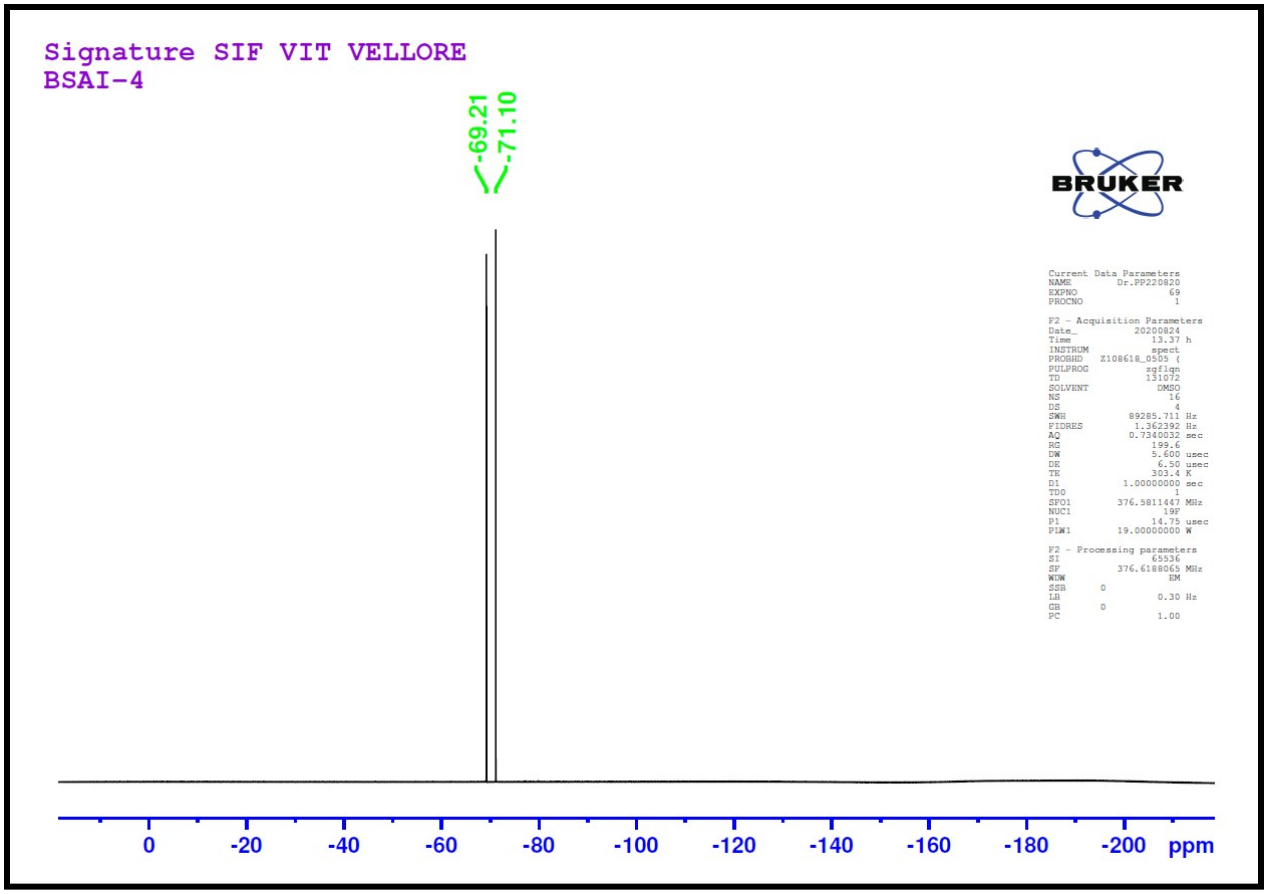
^{1}H NMR of complex IrL4:



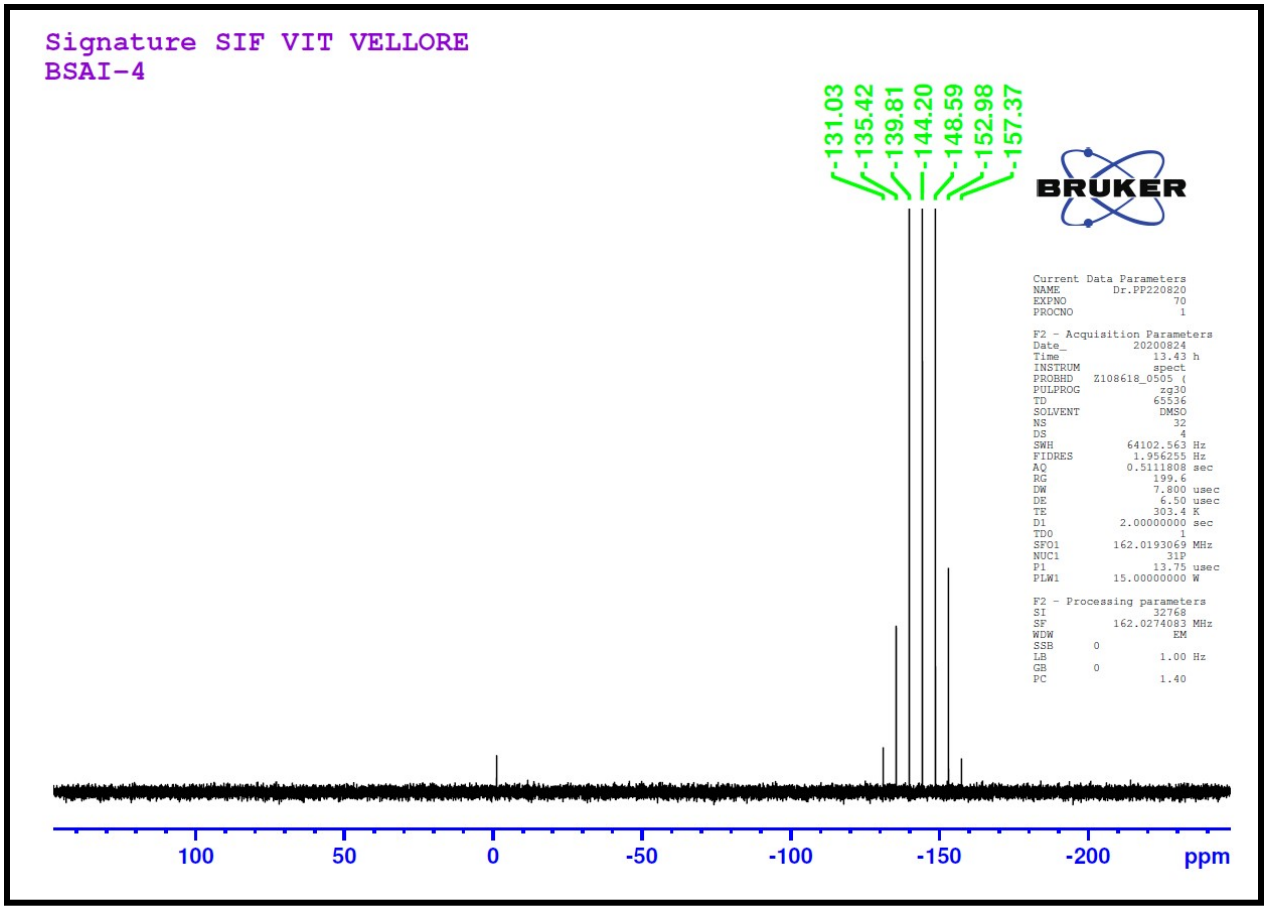
^{13}C NMR of complex IrL4:



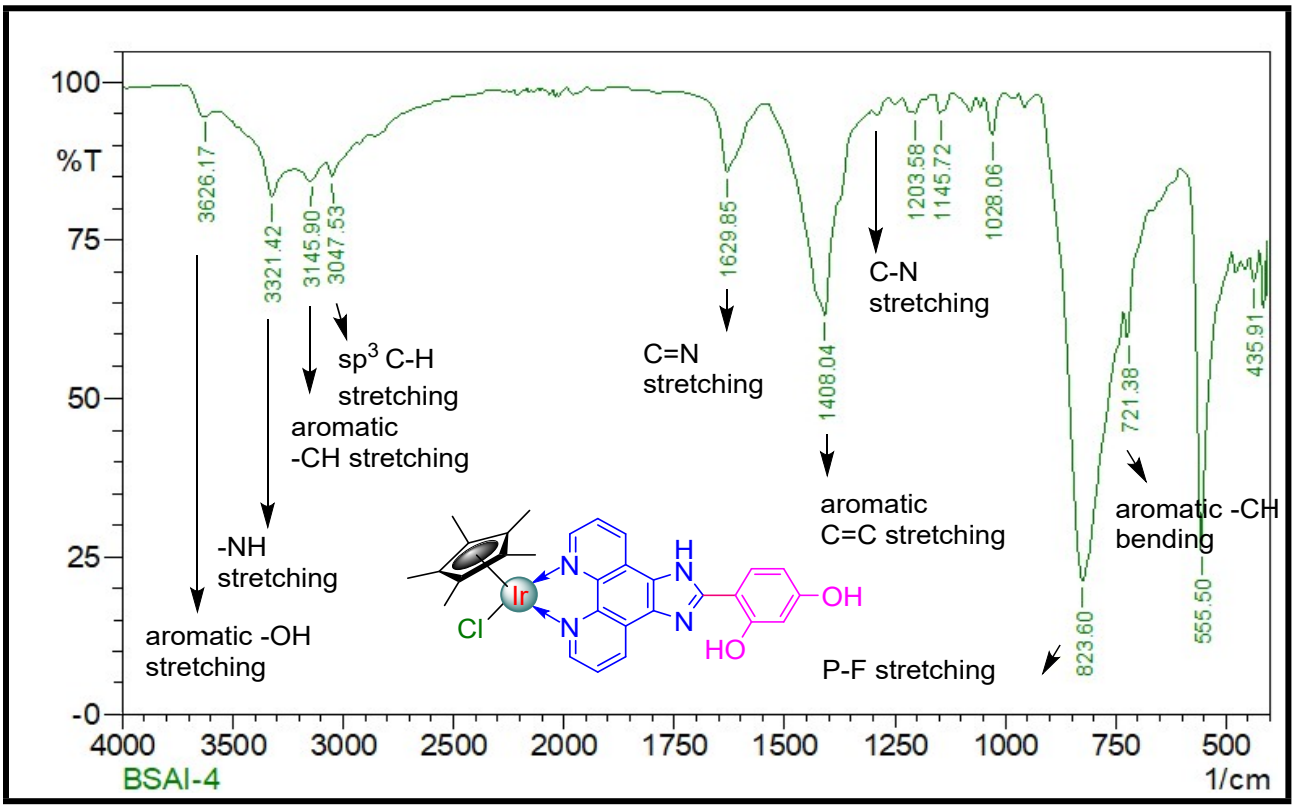
^{19}F NMR of complex IrL4:



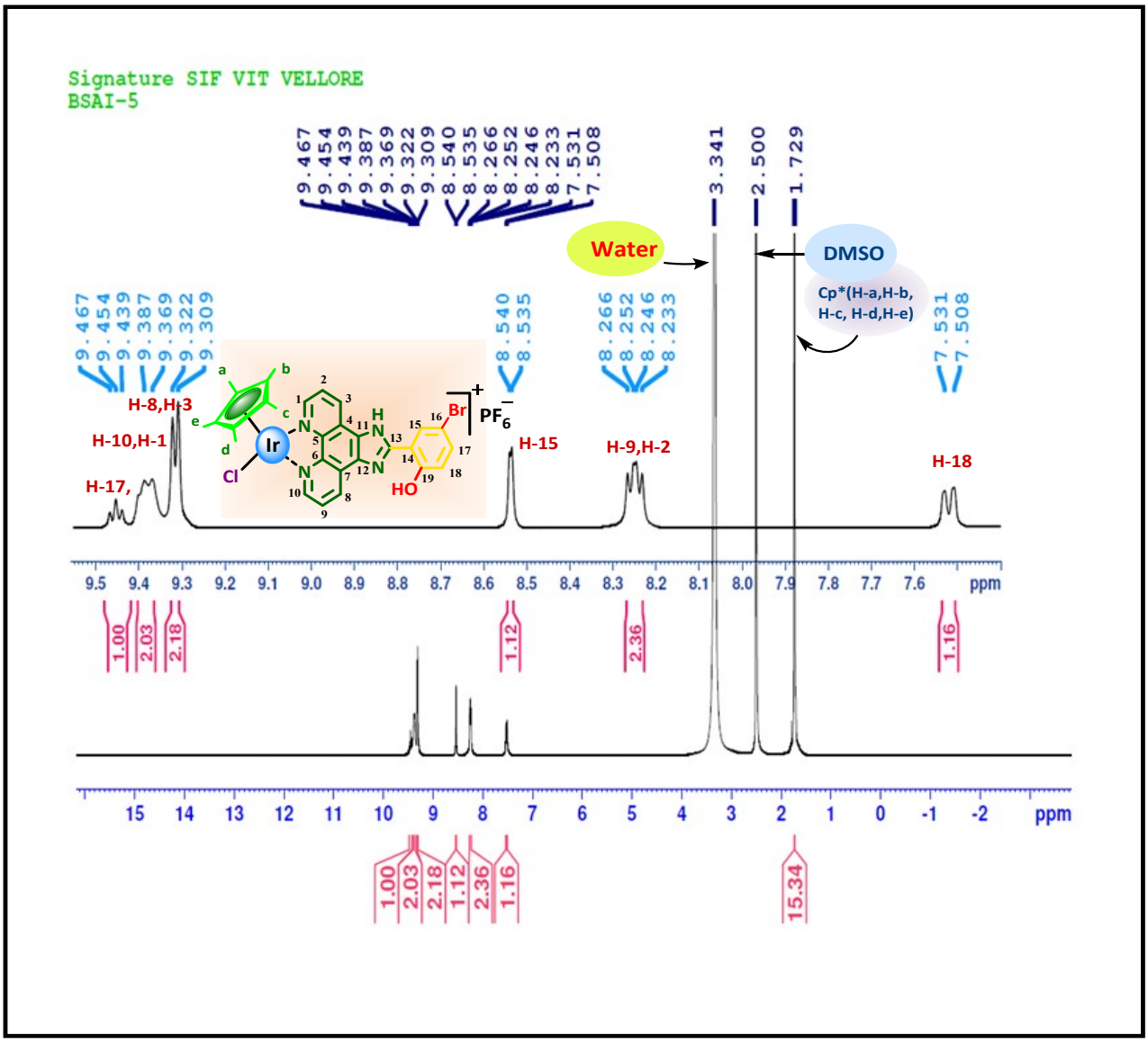
^{31}P NMR of complex IrL4:


Signature SIF VIT VELLORE
BSAI-4
-131.03
-135.42
-139.81
-144.20
-148.59
-152.98
-157.37
BRUKER
Current Data Parameters
NAME Dr.PP220820
EXPNO 70
PROCNO 1
F2 - Acquisition Parameters
Date_ 20200824
Time 13.43 h
INSTRUM spect
PROBHD Z108618_0505 (
PULPROG zg30
TD 65536
SOLVENT DMSO
NS 32
DS 4
SWH 64102.563 Hz
FIDRES 1.956255 Hz
AQ 0.5111808 sec
RG 199.6
DW 7.800 usec
DE 6.50 usec
TE 303.4 K
D1 2.00000000 sec
TD0 1
SFO1 162.0193069 MHz
NUC1 31P
P1 13.75 usec
PLW1 15.00000000 W
F2 - Processing parameters
SI 32768
SF 162.0274083 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
100
50
0
-50
-100
-150
-200
ppm

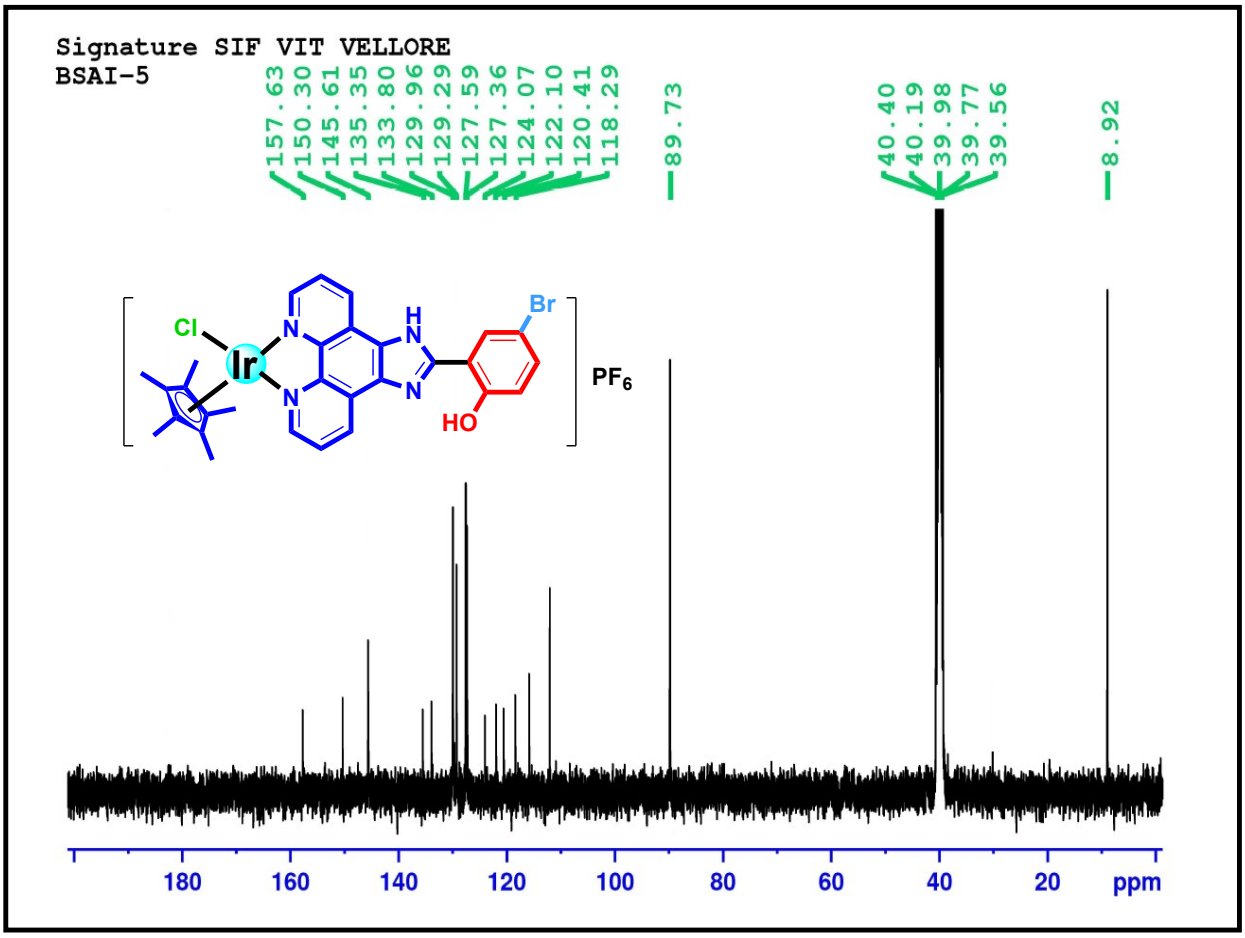
IR spectrum of IrL4:



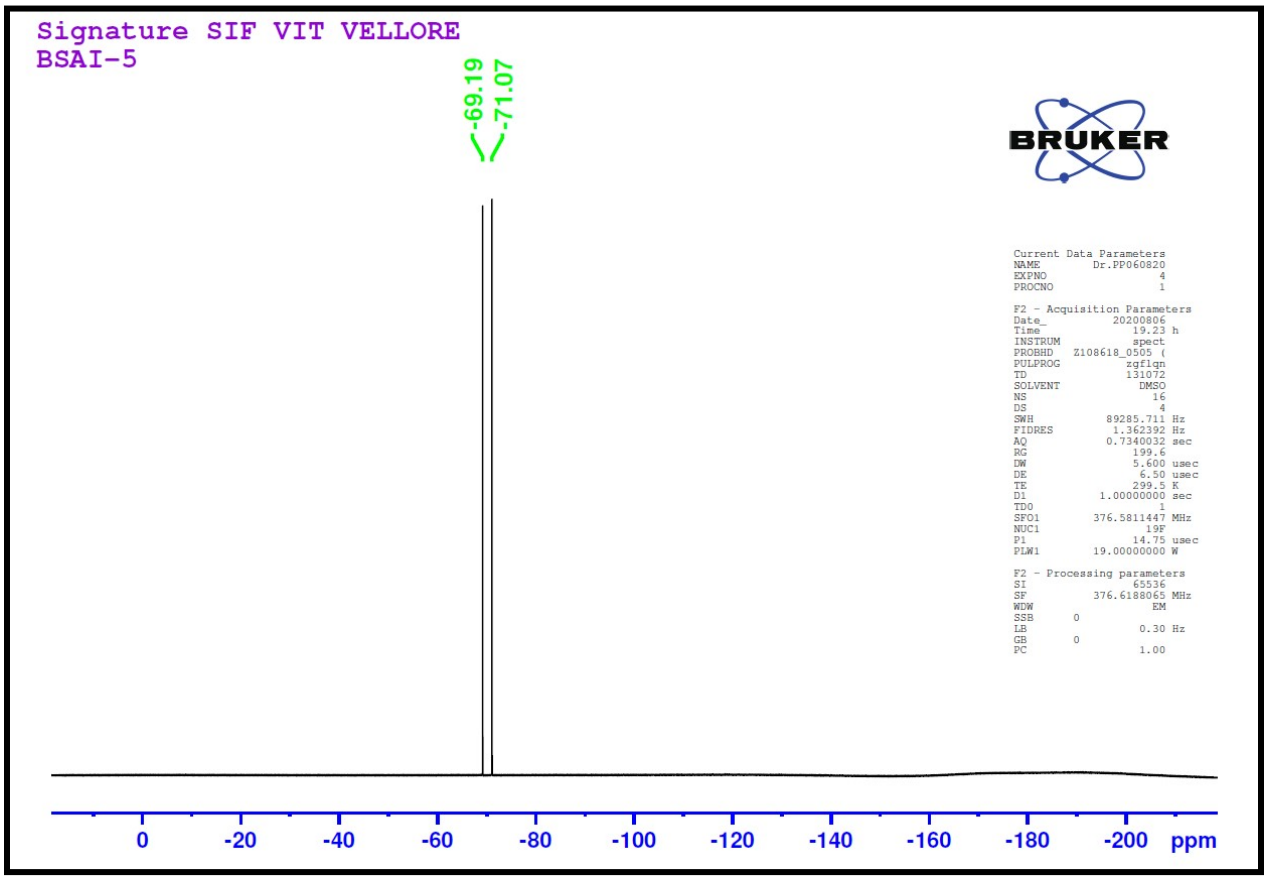
^{1}H NMR of complex IrL5:



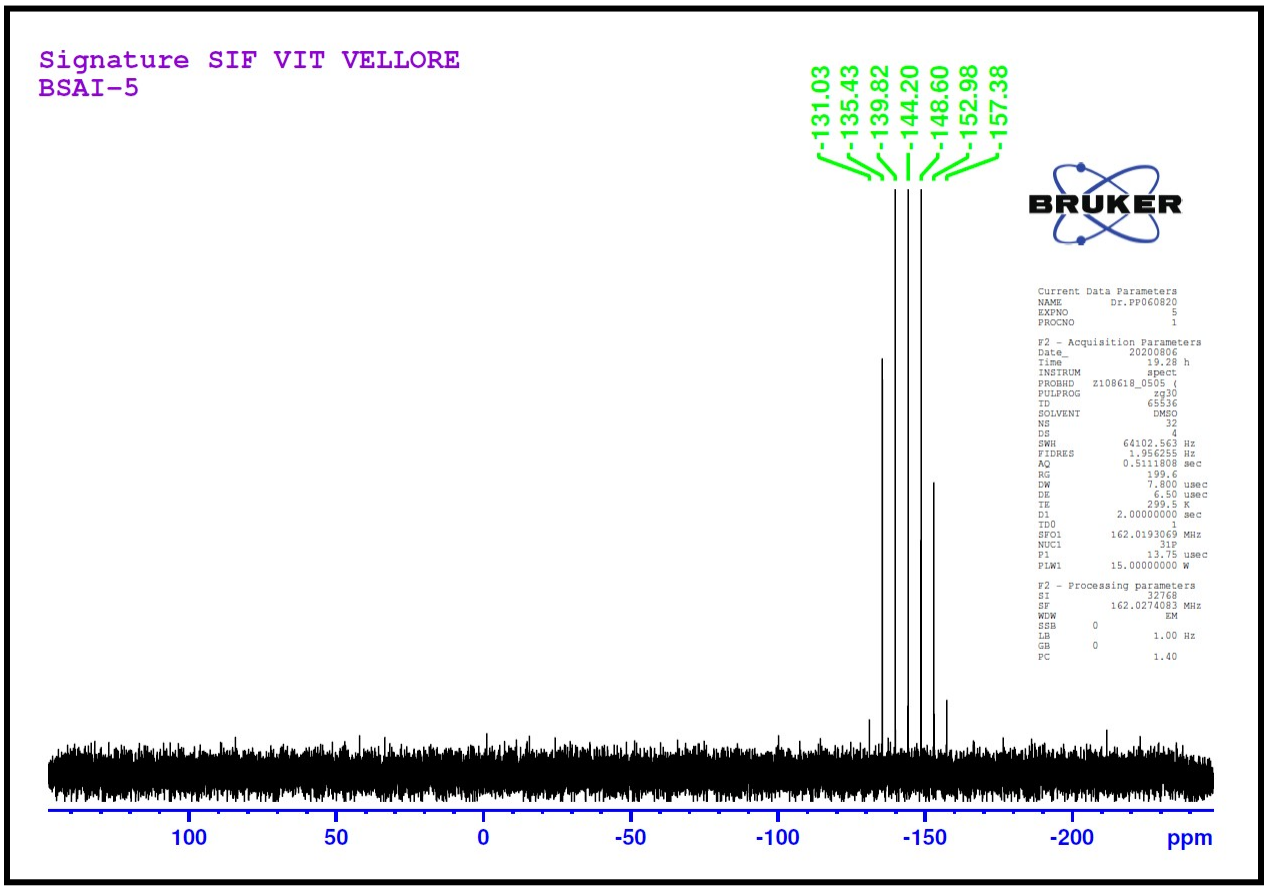
^{13}C NMR of complex IrL5:



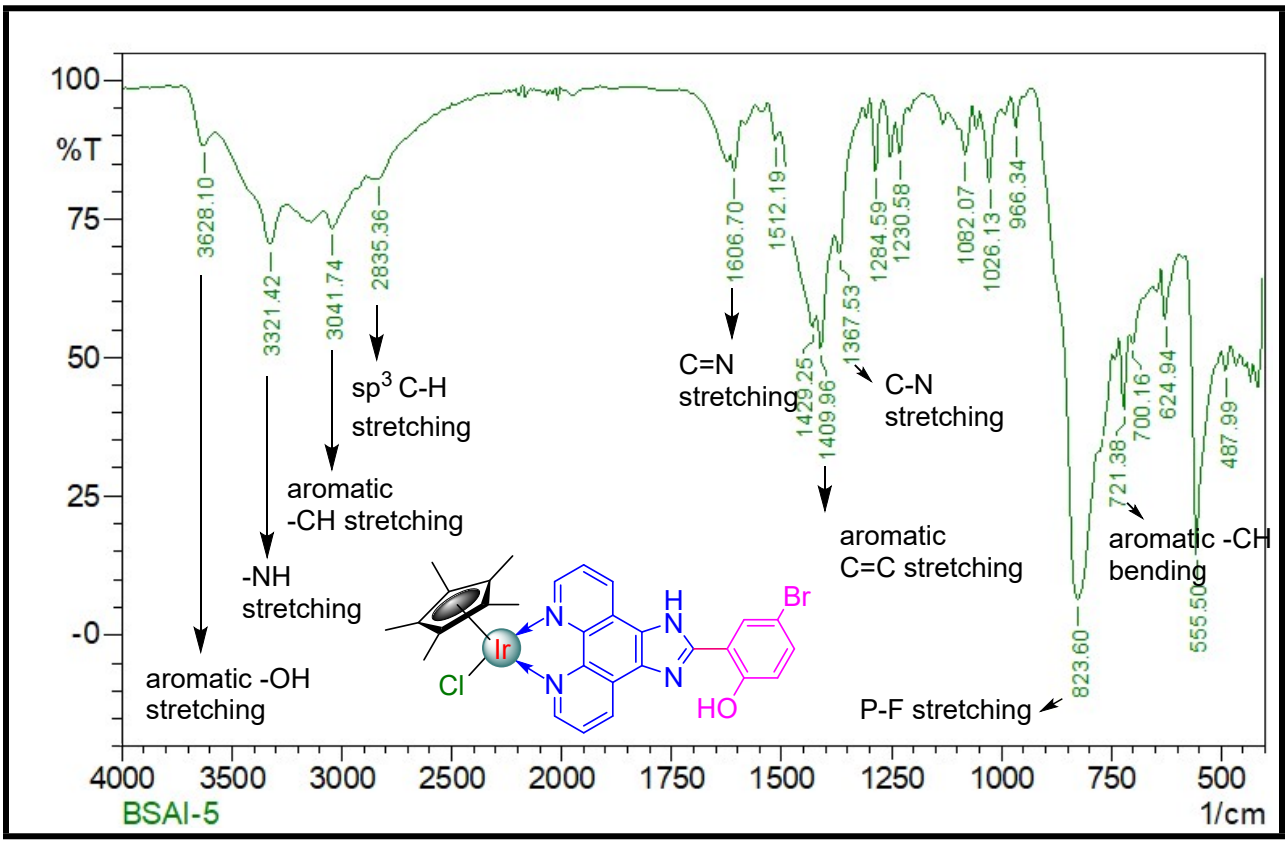
^{19}F NMR of complex IrL5:



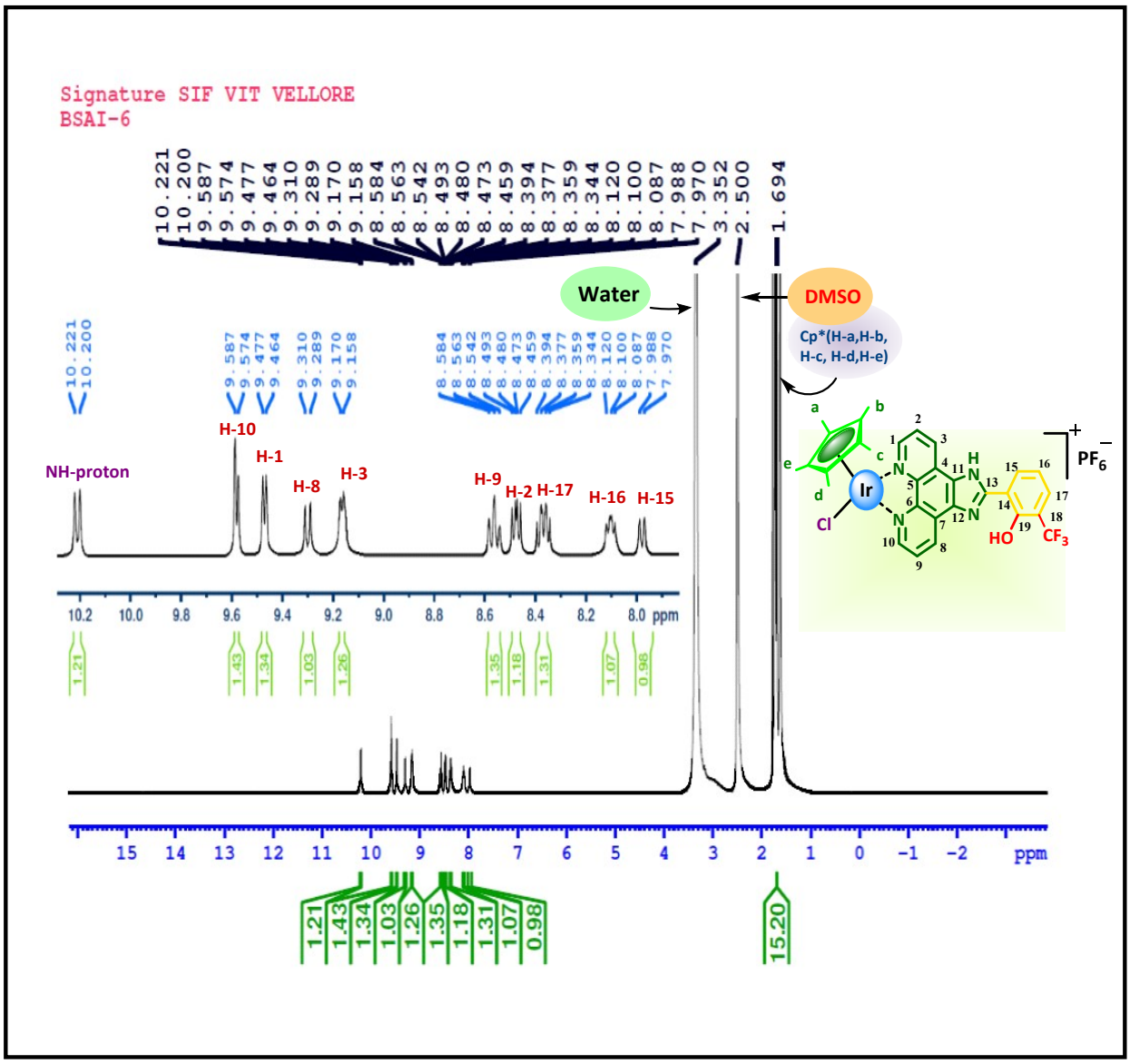
^{31}P NMR of complex IrL5:



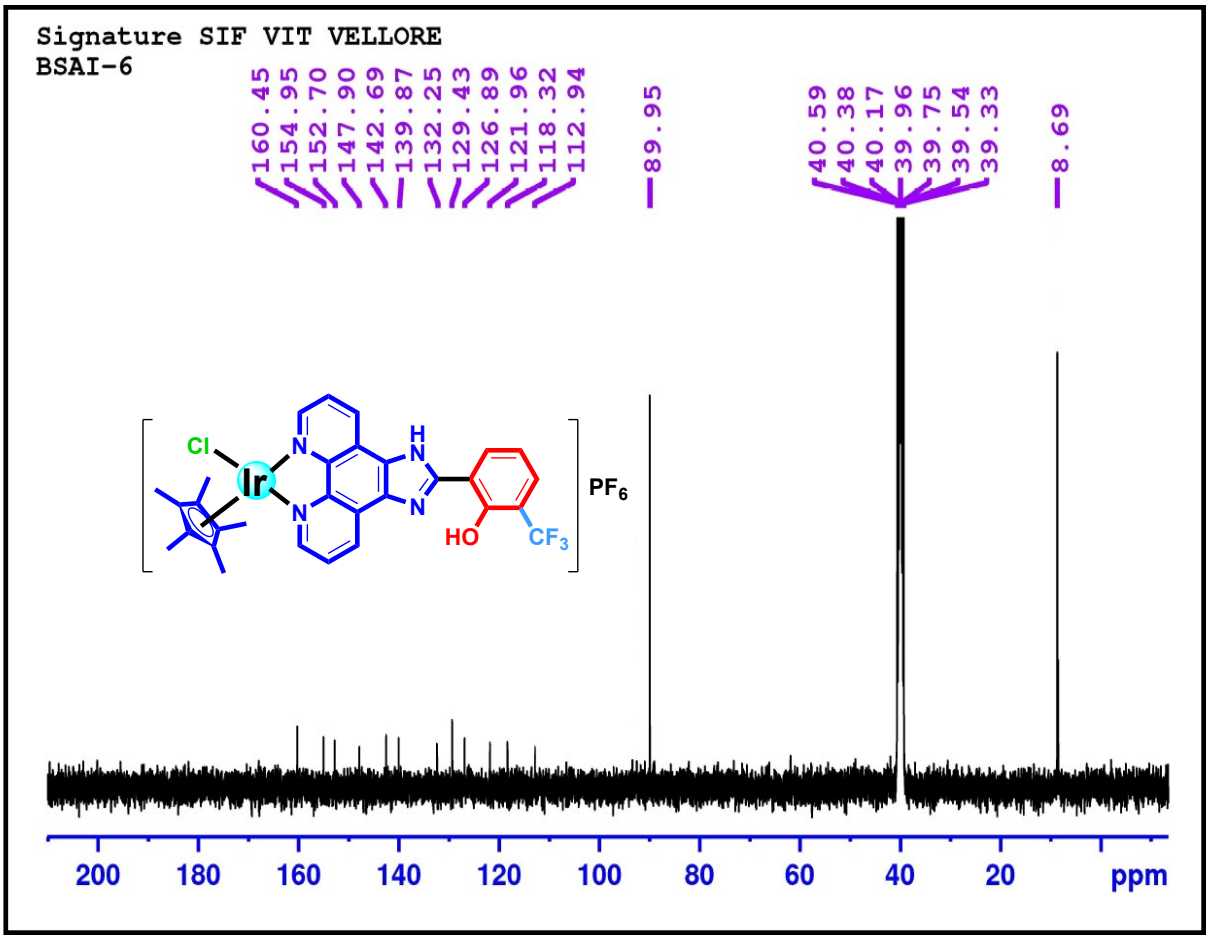
IR spectrum of IrL5:



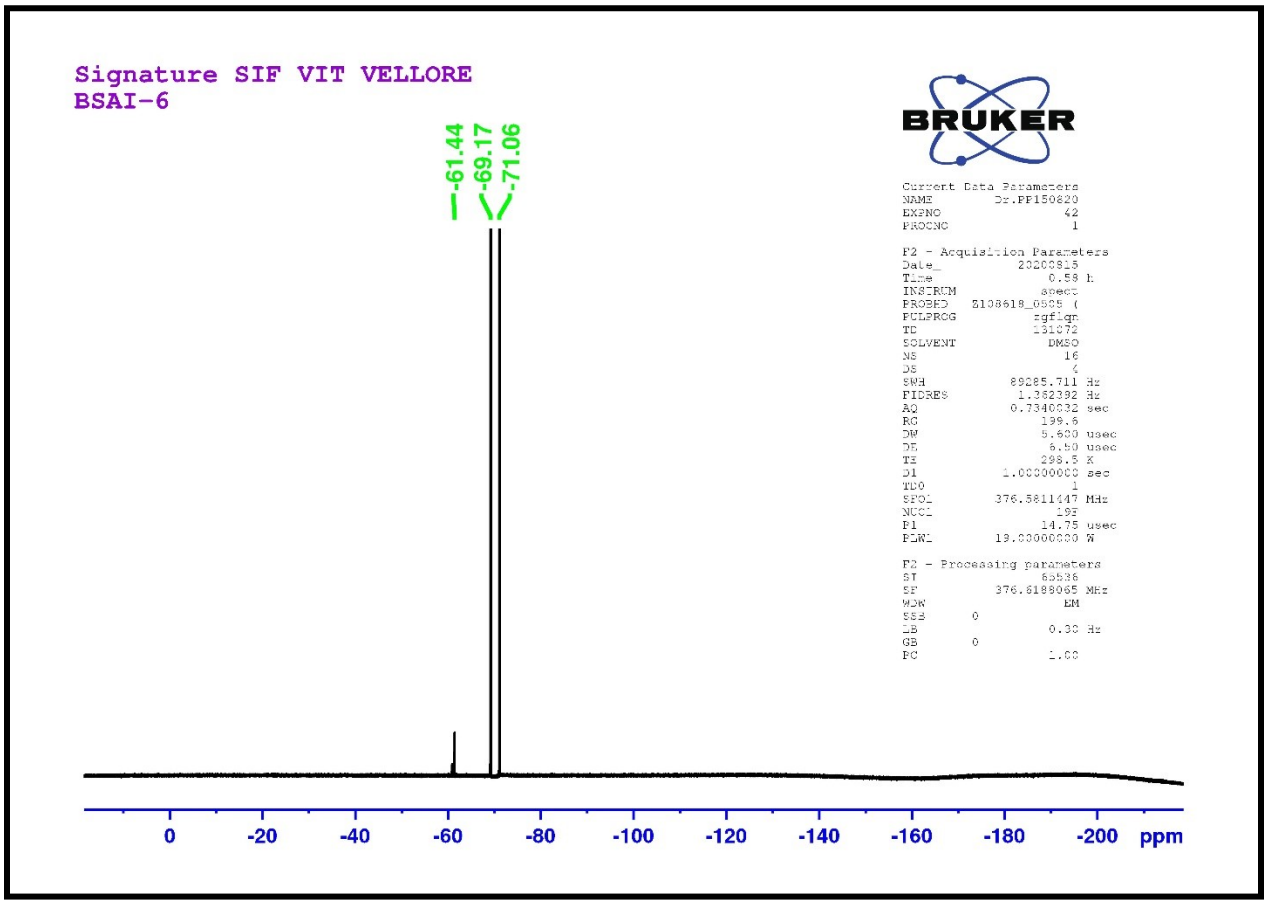
^{1}H NMR of complex IrL6:



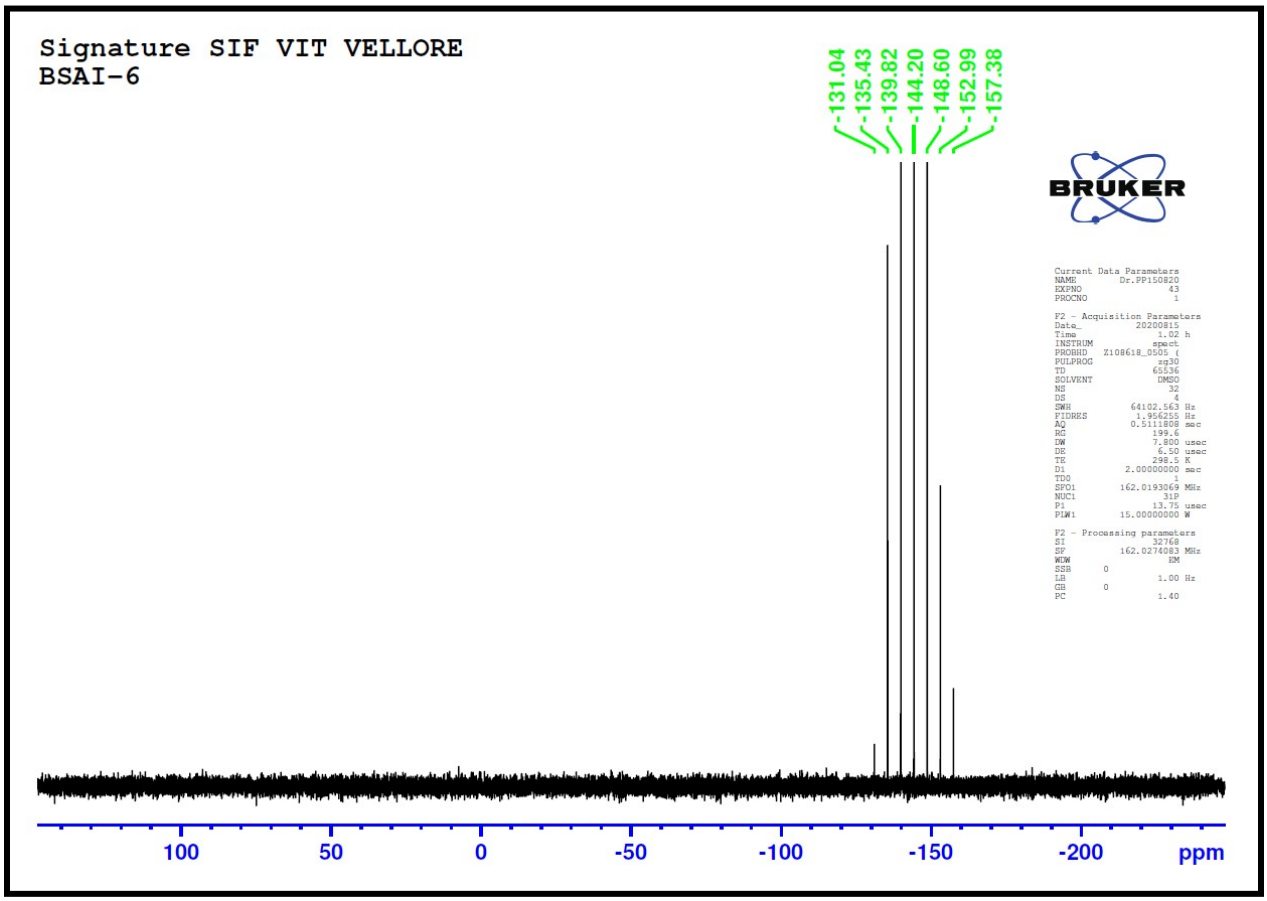
^{13}C NMR of complex IrL6:



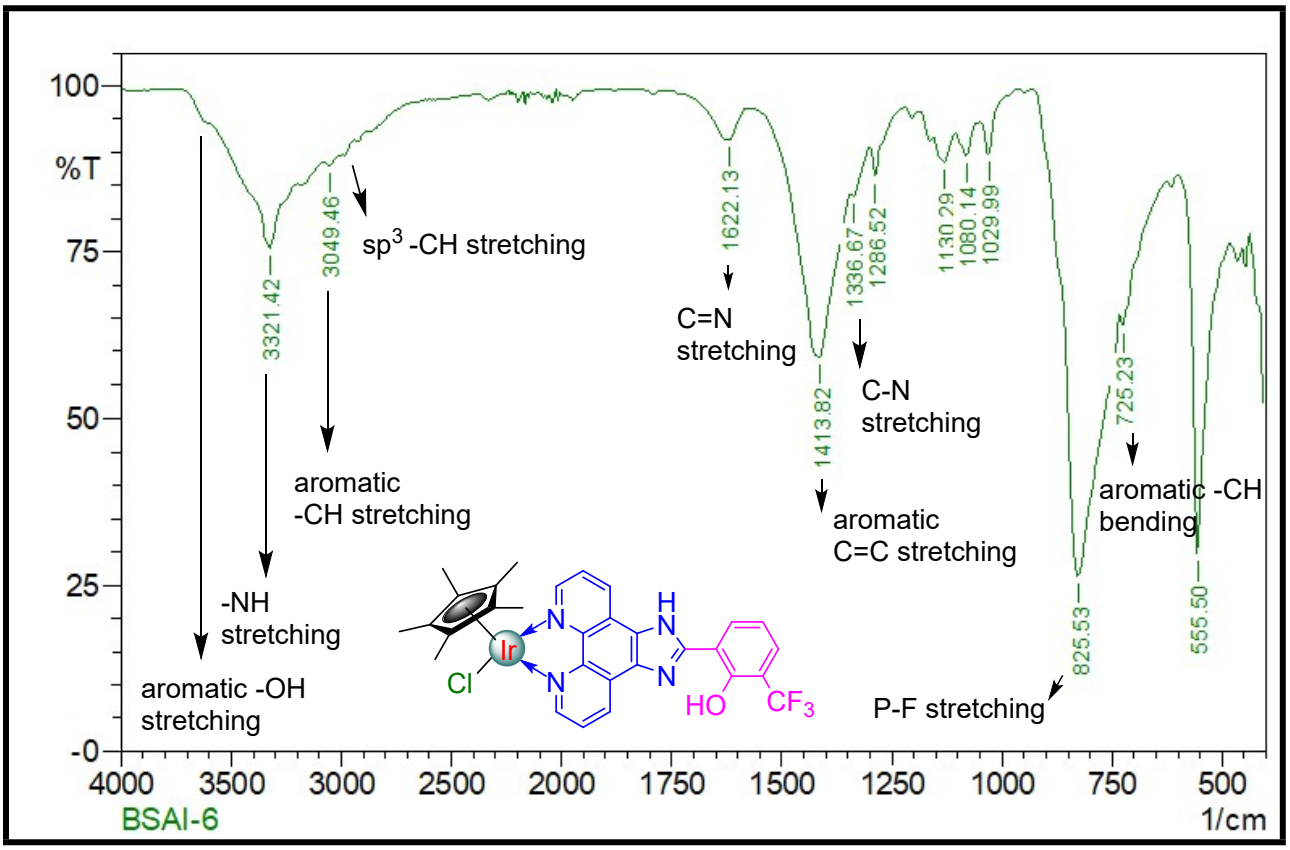
^{19}F NMR of complex IrL6:



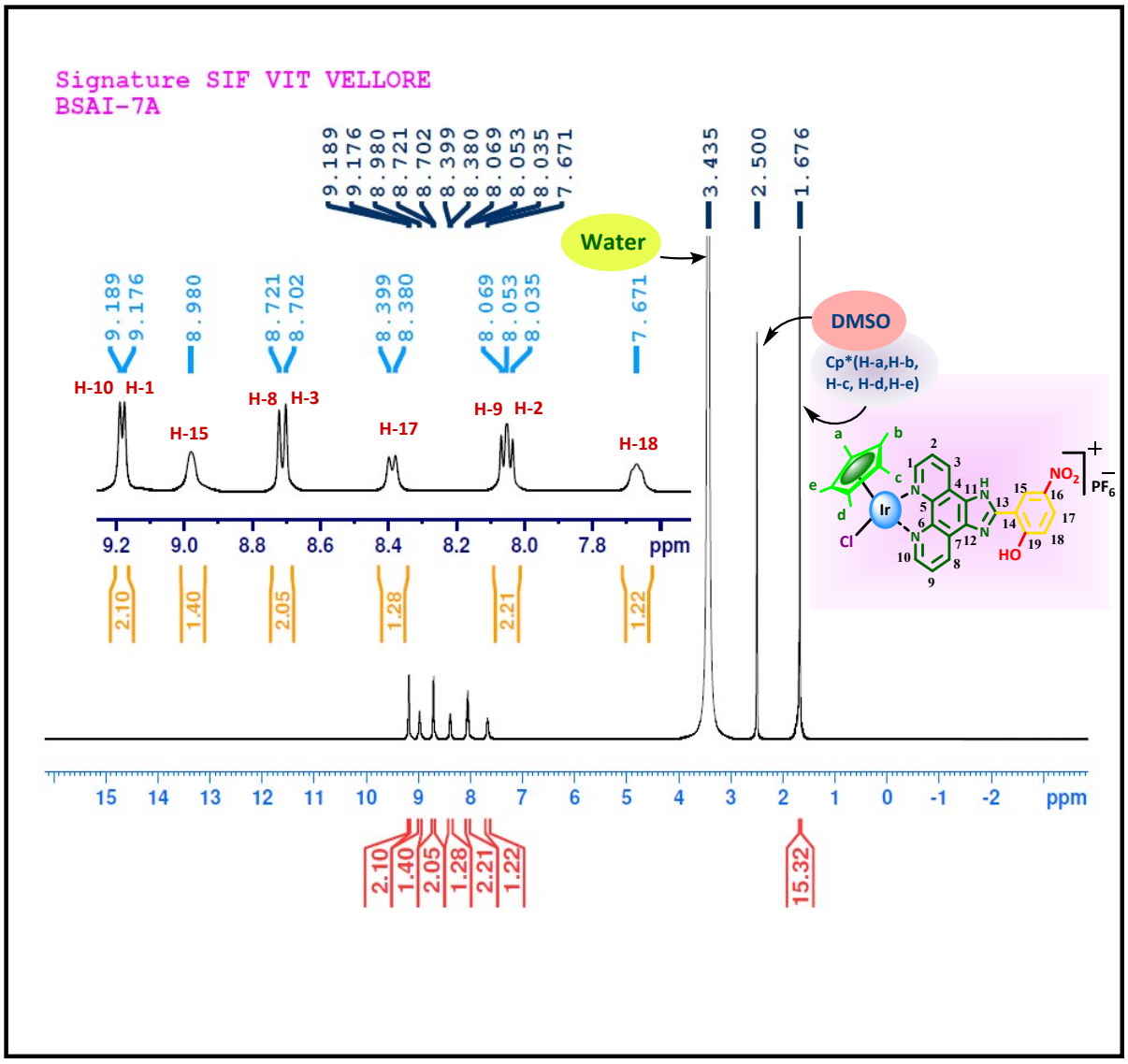
^{31}P NMR of complex IrL6:



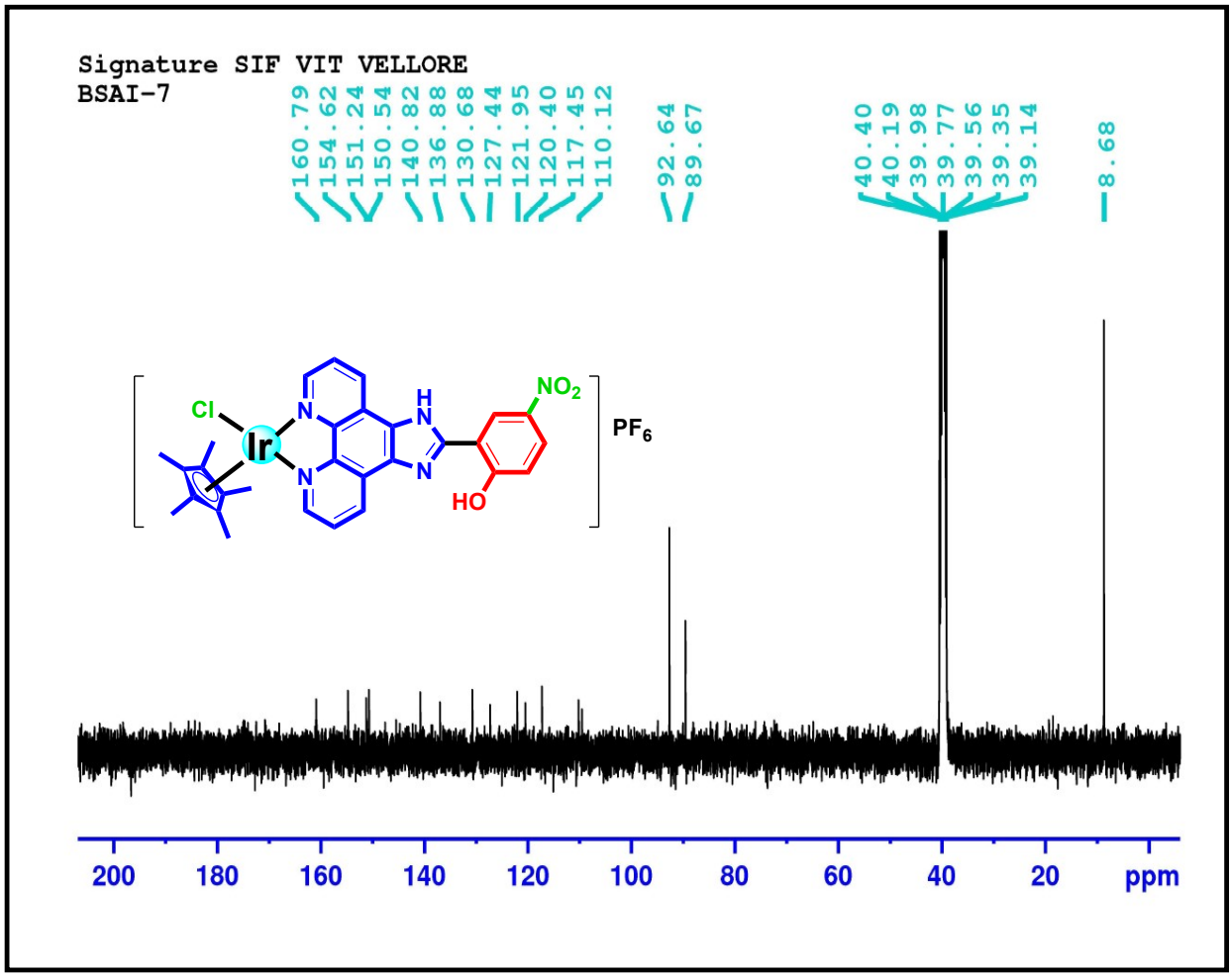
IR spectrum of IrL6:



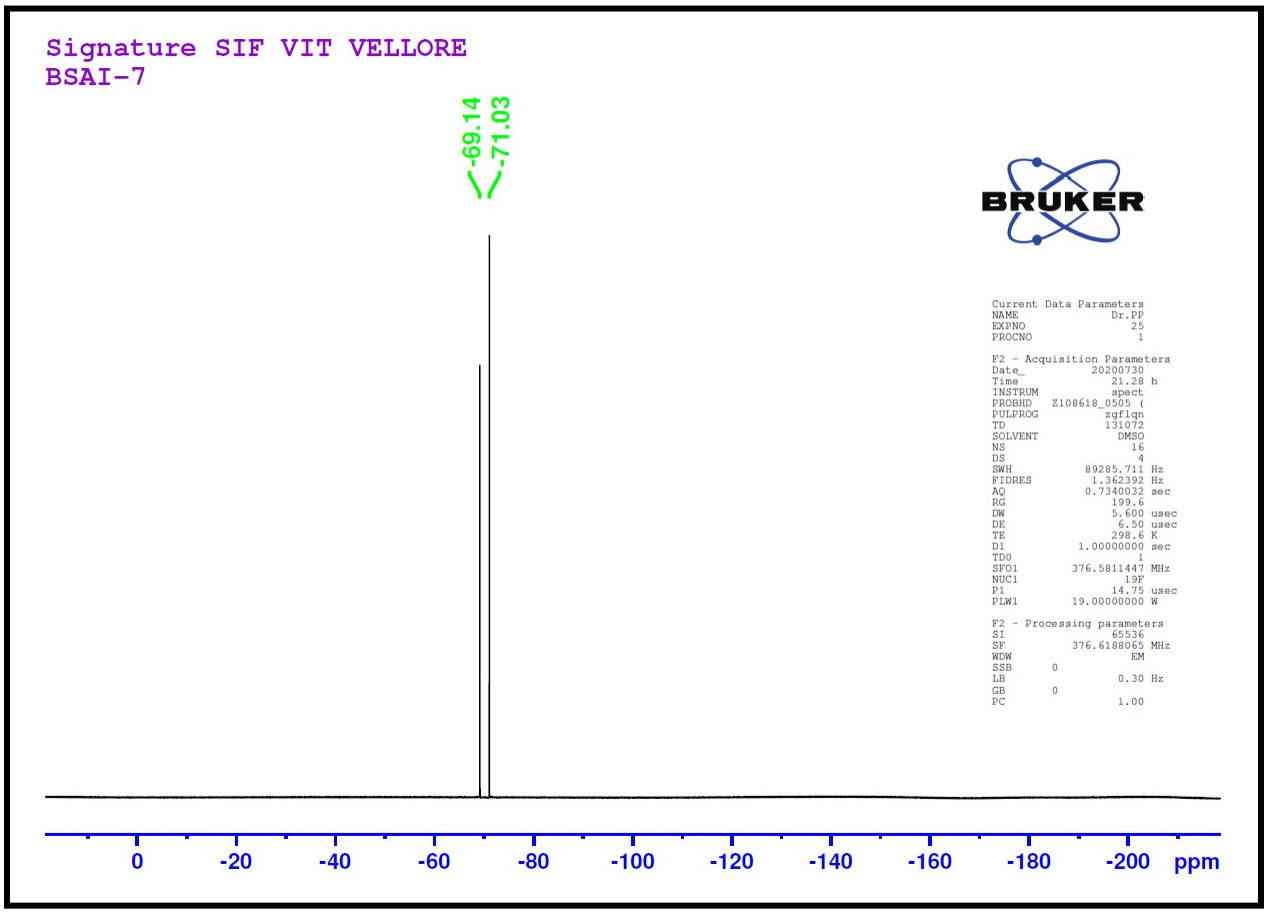
^{1}H NMR of complex IrL7:



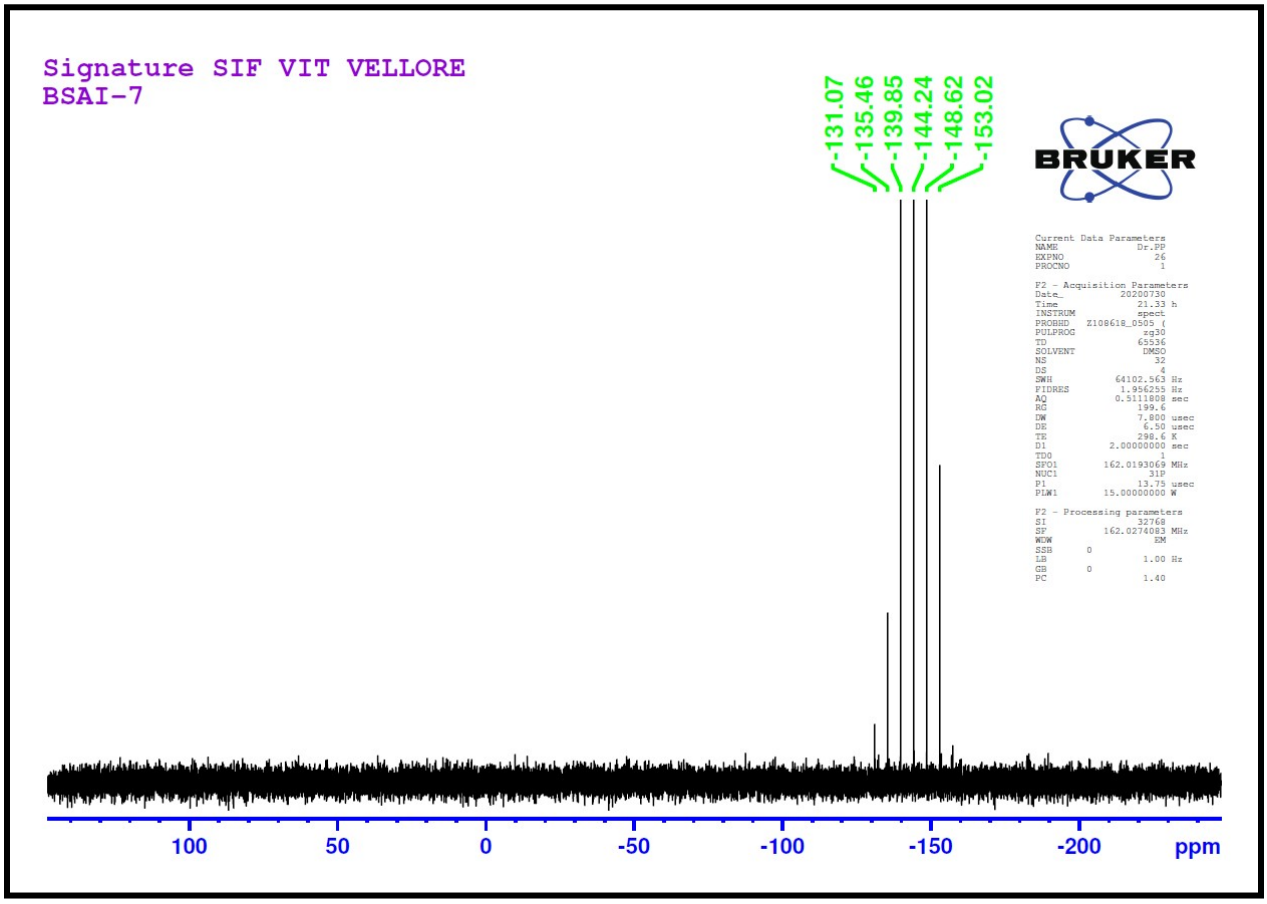
^{13}C NMR of complex IrL7:



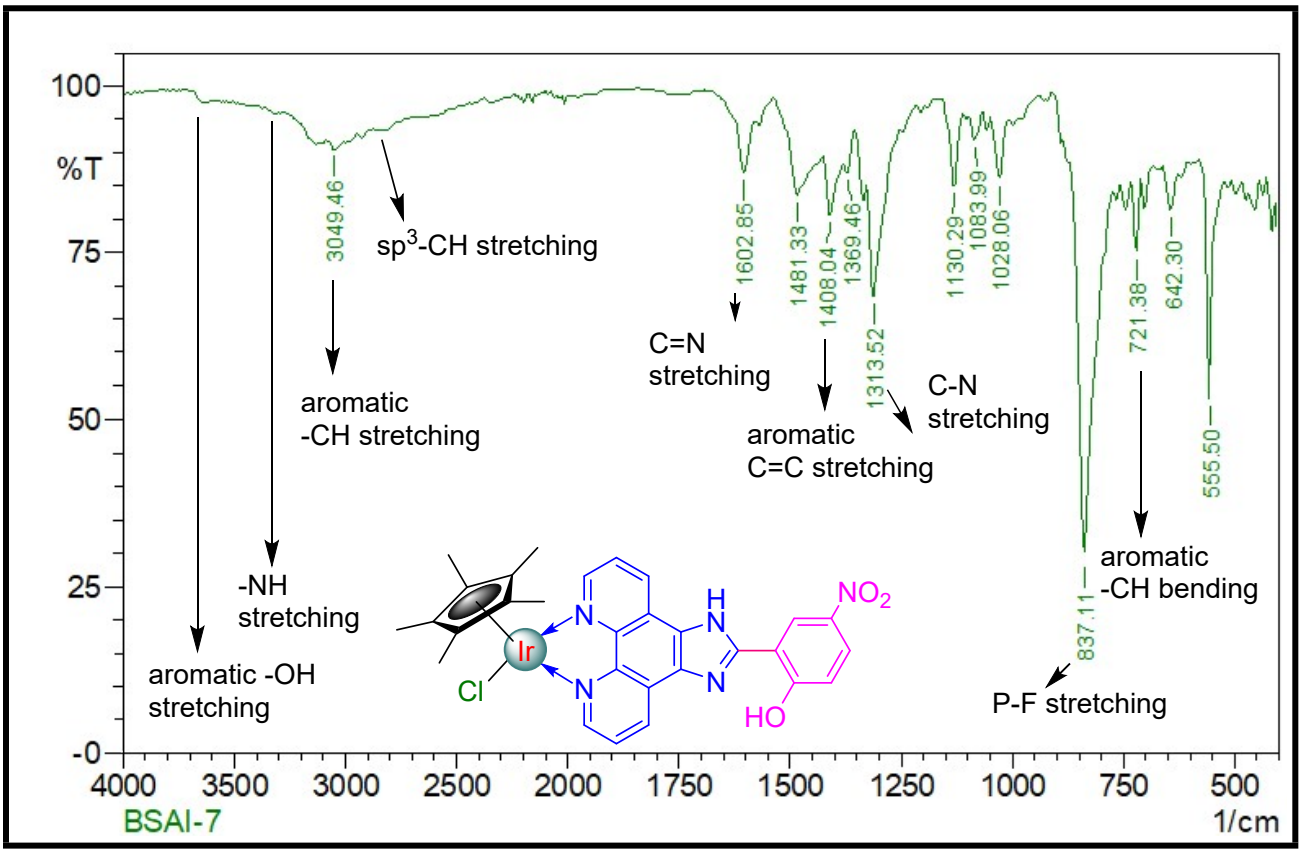
^{19}F NMR of complex IrL7:



^{31}P NMR of complex IrL7:

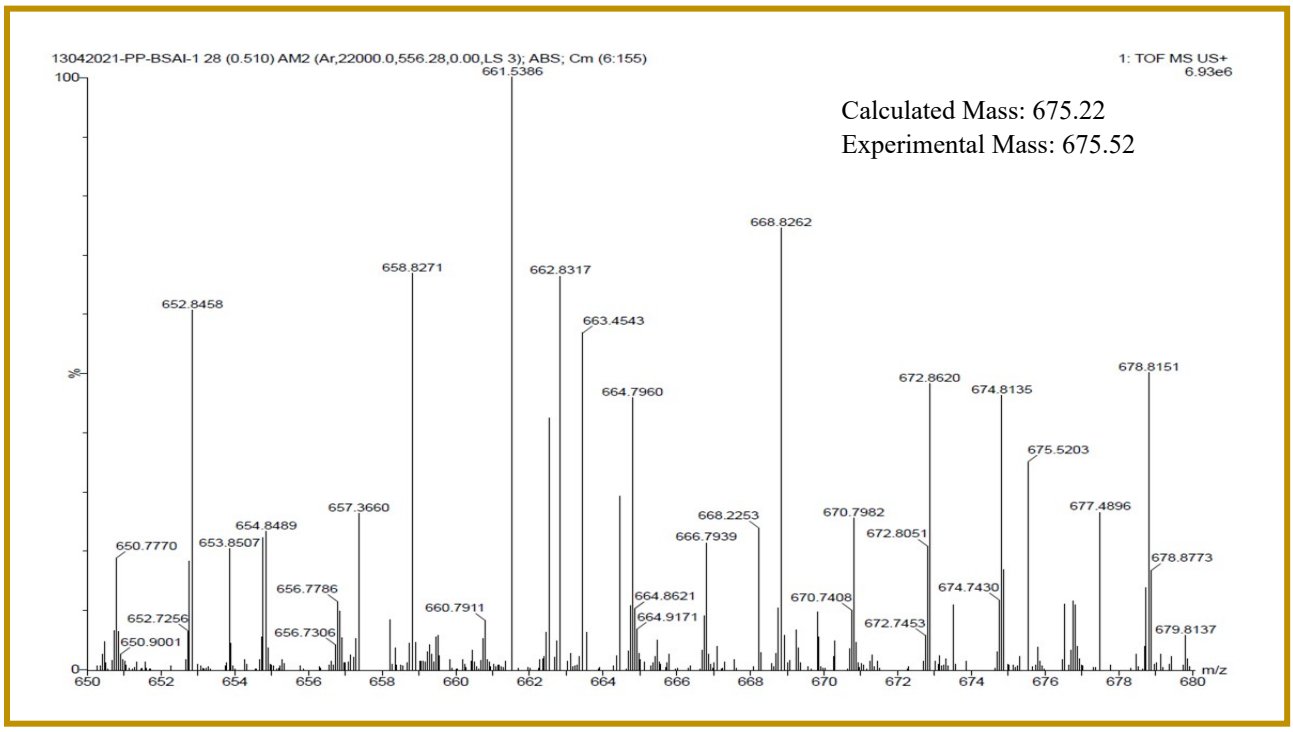


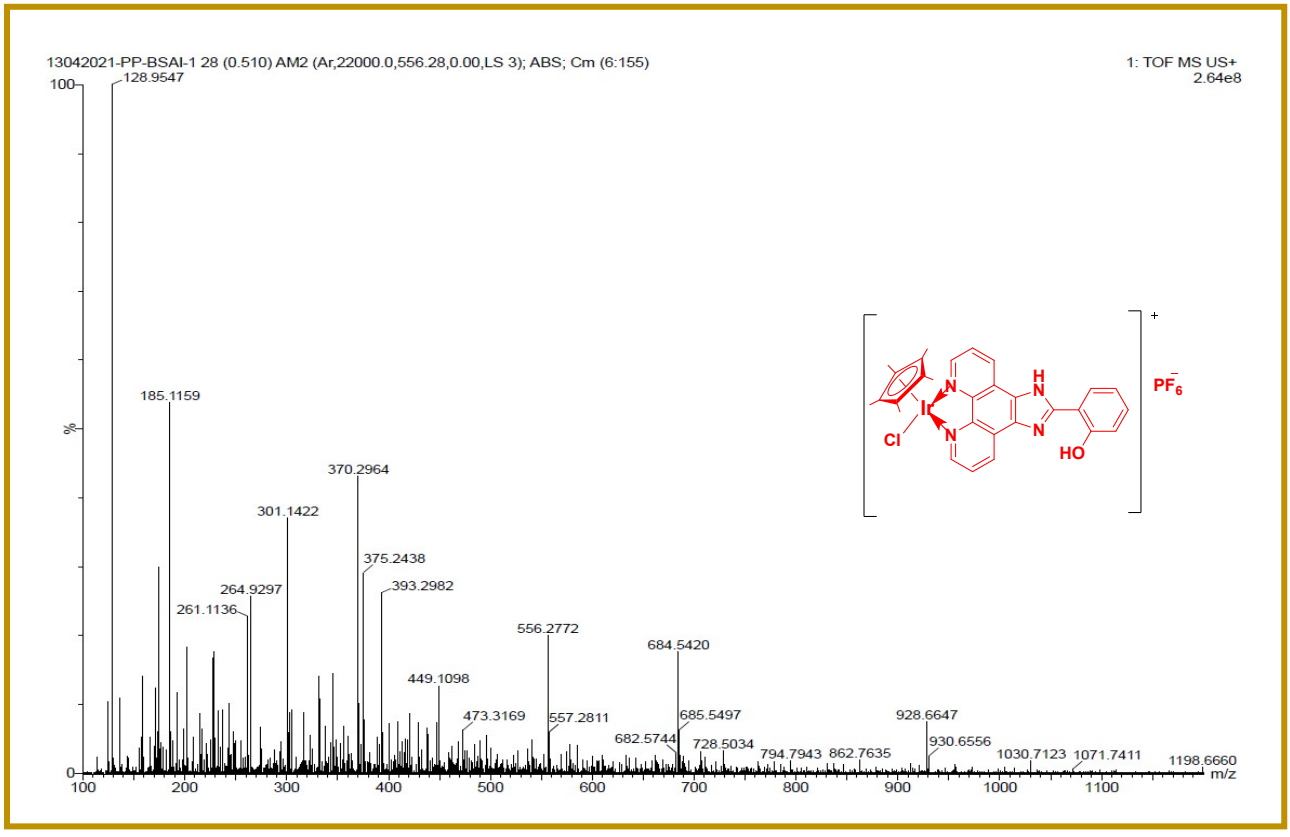
IR spectrum of IrL7:



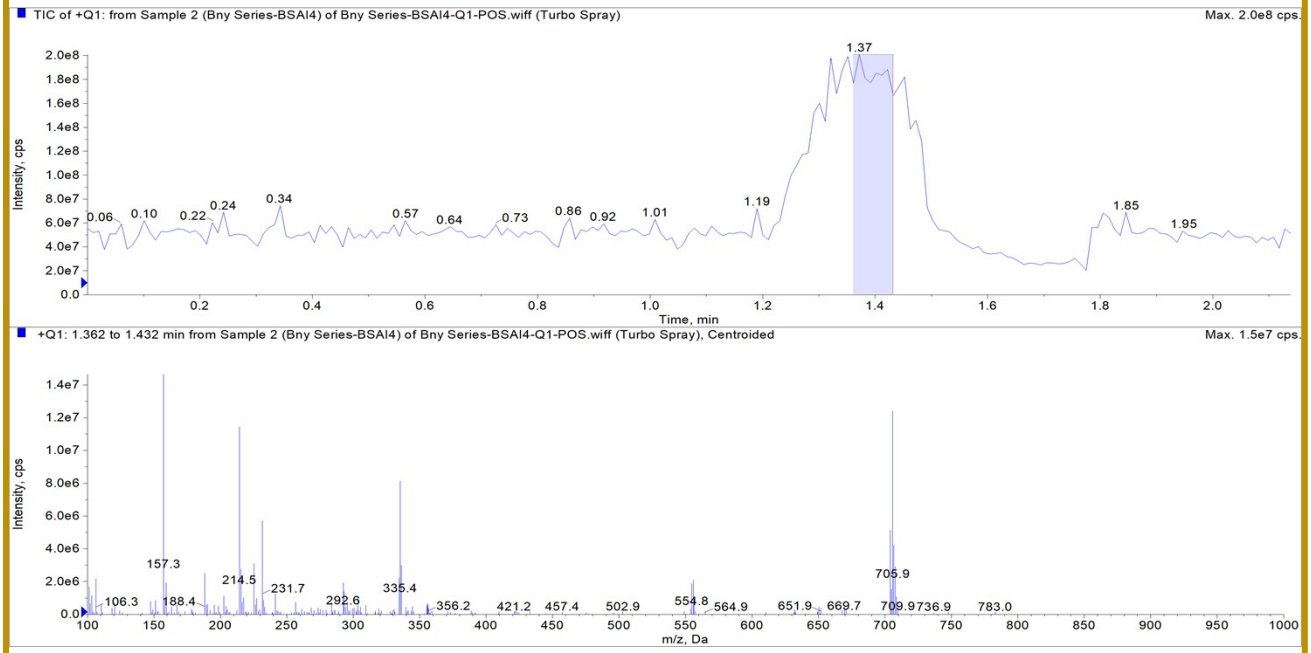
ESI-MS spectrum of complexes (IrL1 - IrL7):

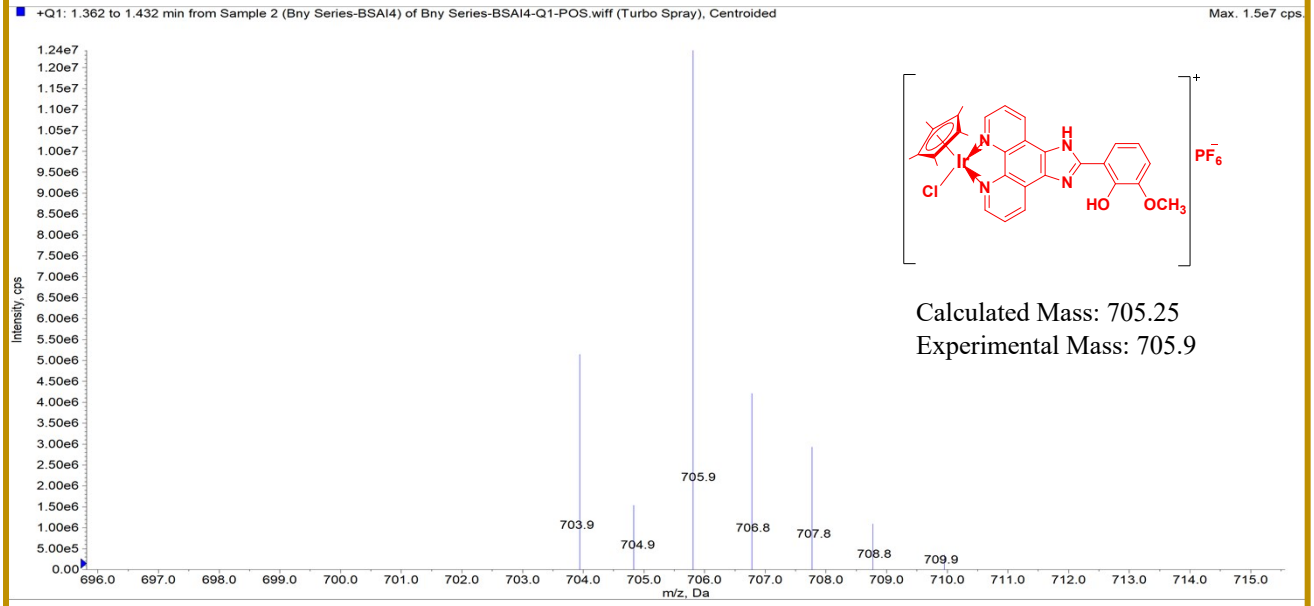
IrL1





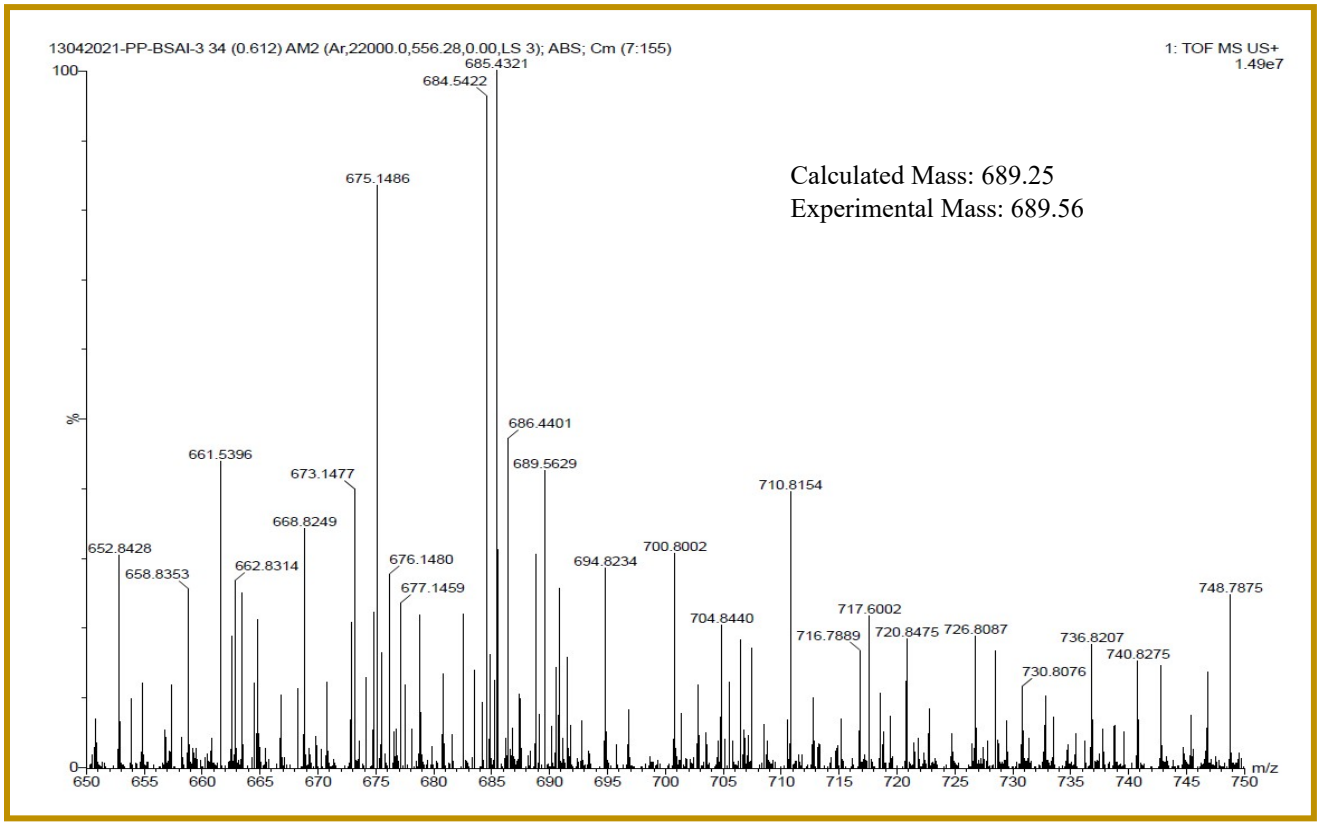
IrL2

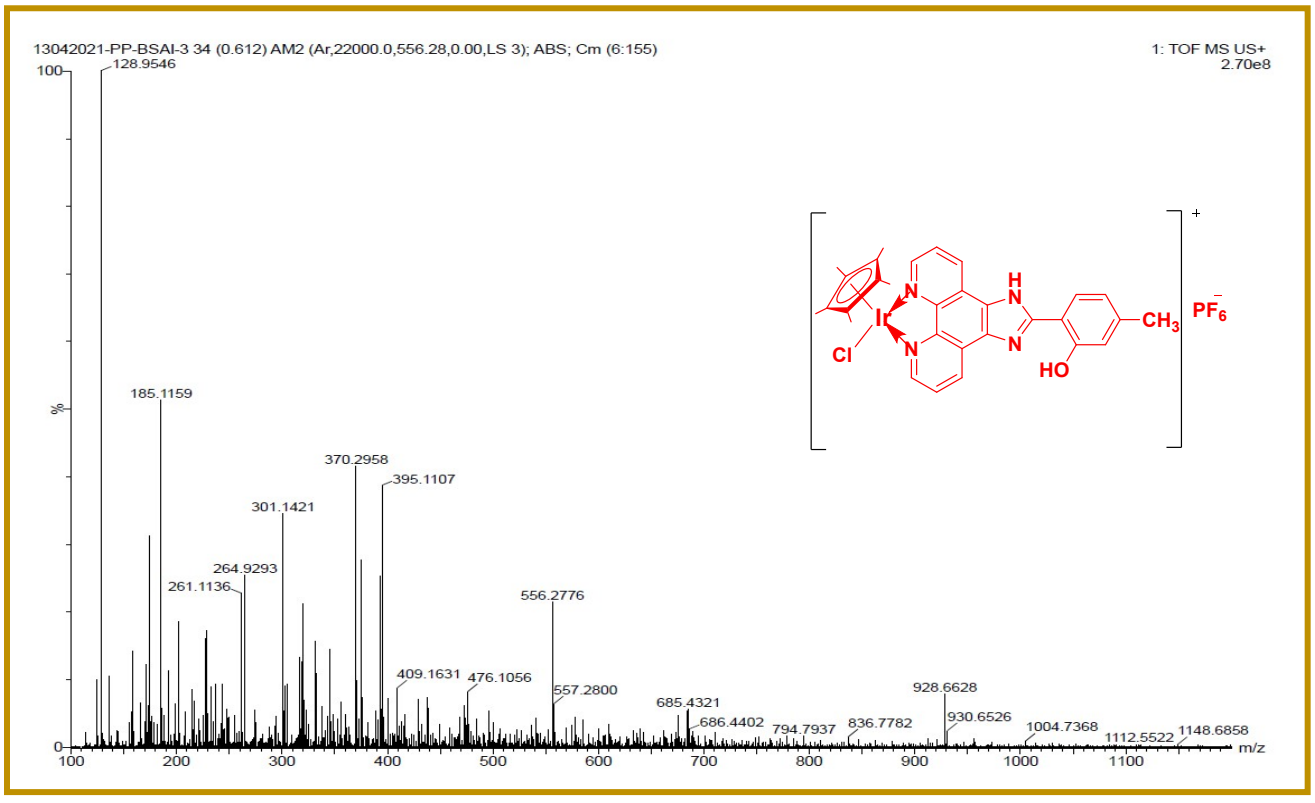




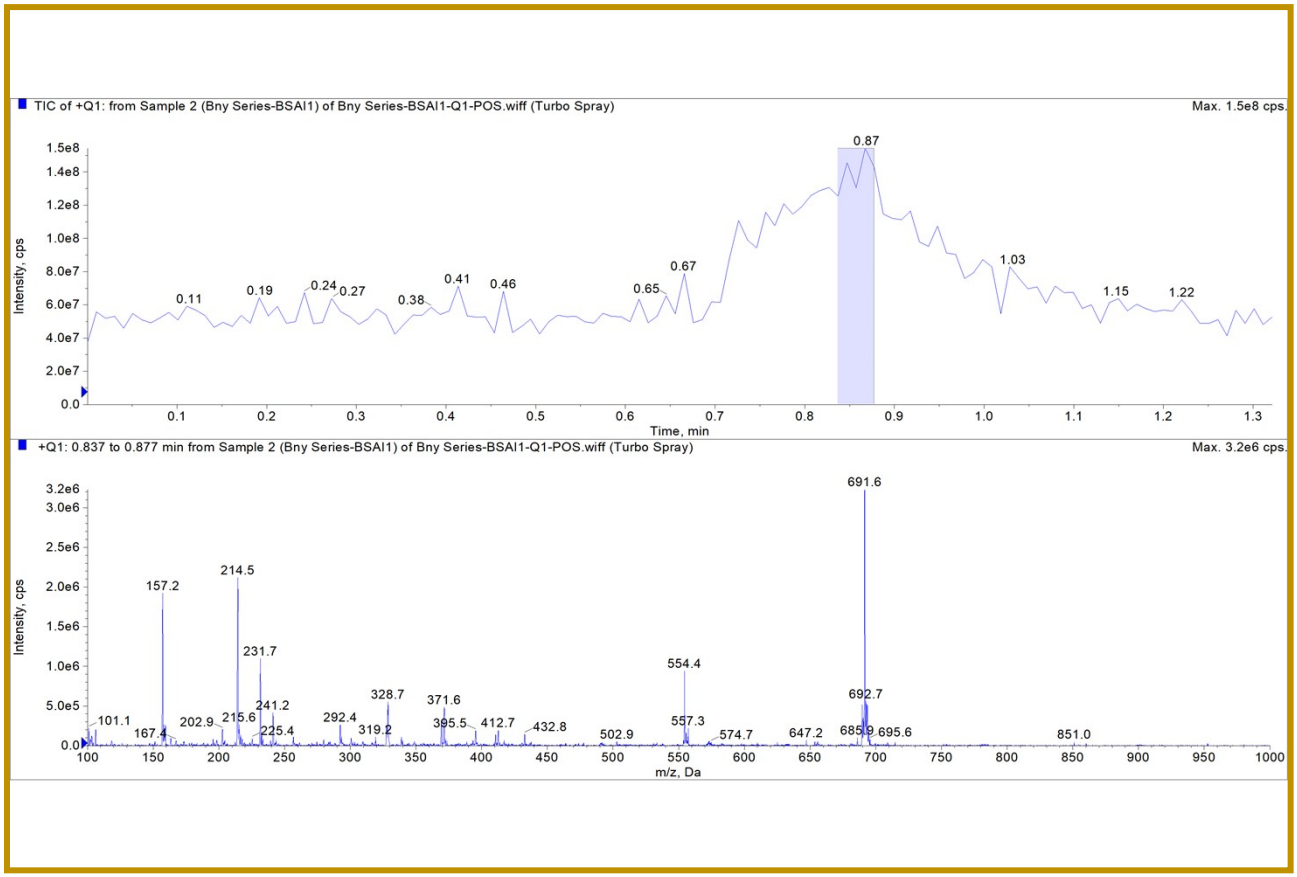
Calculated Mass: 705.25
Experimental Mass: 705.9

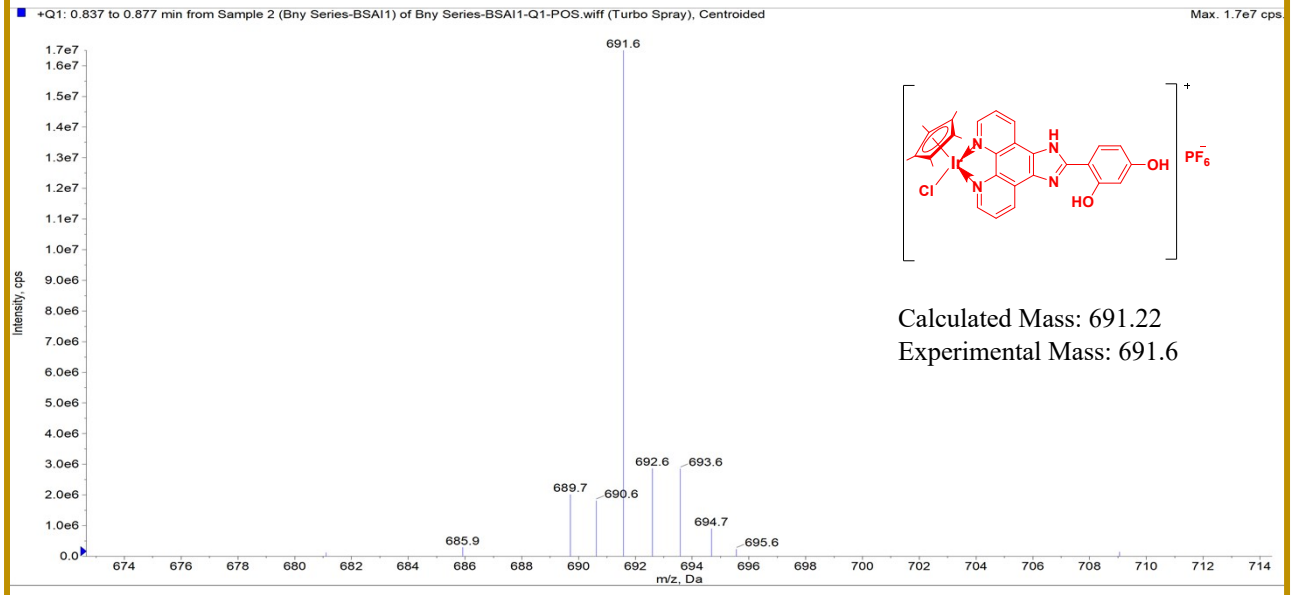
IrL3


13042021-PP-BSAI-3 34 (0.612) AM2 (Ar,22000.0,556.28,0.00,LS 3); ABS; Cm (7:155)
1: TOF MS US+
1.49e7
Calculated Mass: 689.25
Experimental Mass: 689.56
m/z


13042021-PP-BSAI-3 34 (0.612) AM2 (Ar,22000.0,556.28,0.00,LS 3); ABS; Cm (6:155)
1: TOF MS US+
2.70e8
m/z

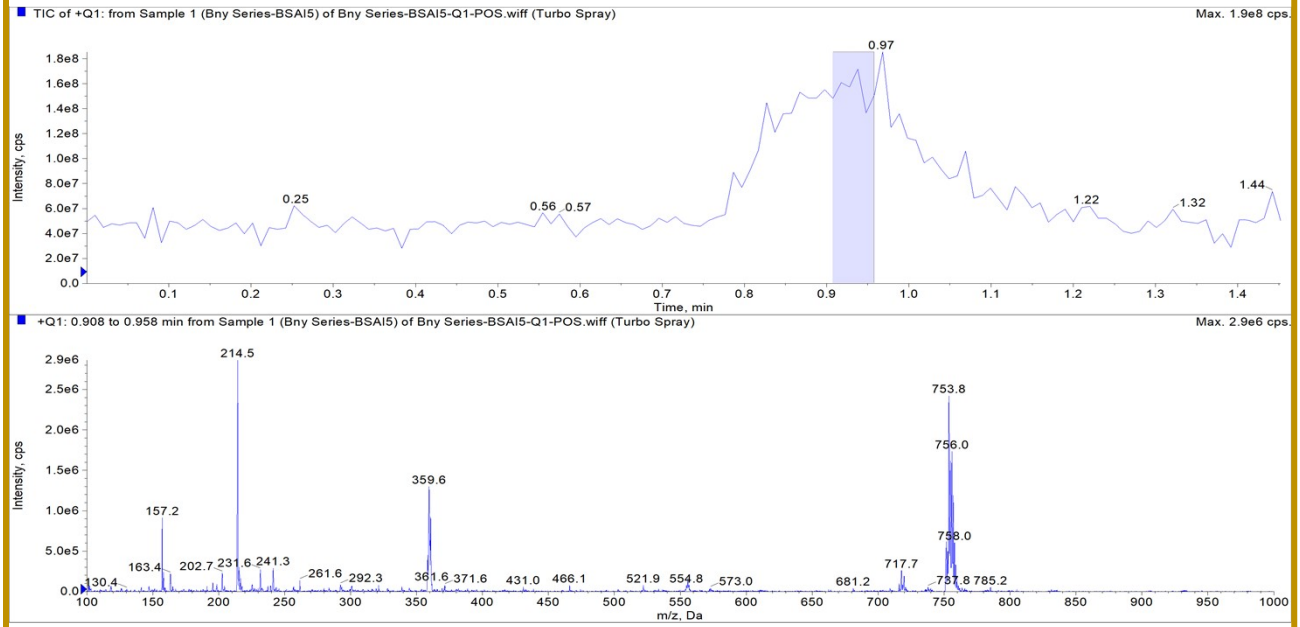
IrL4

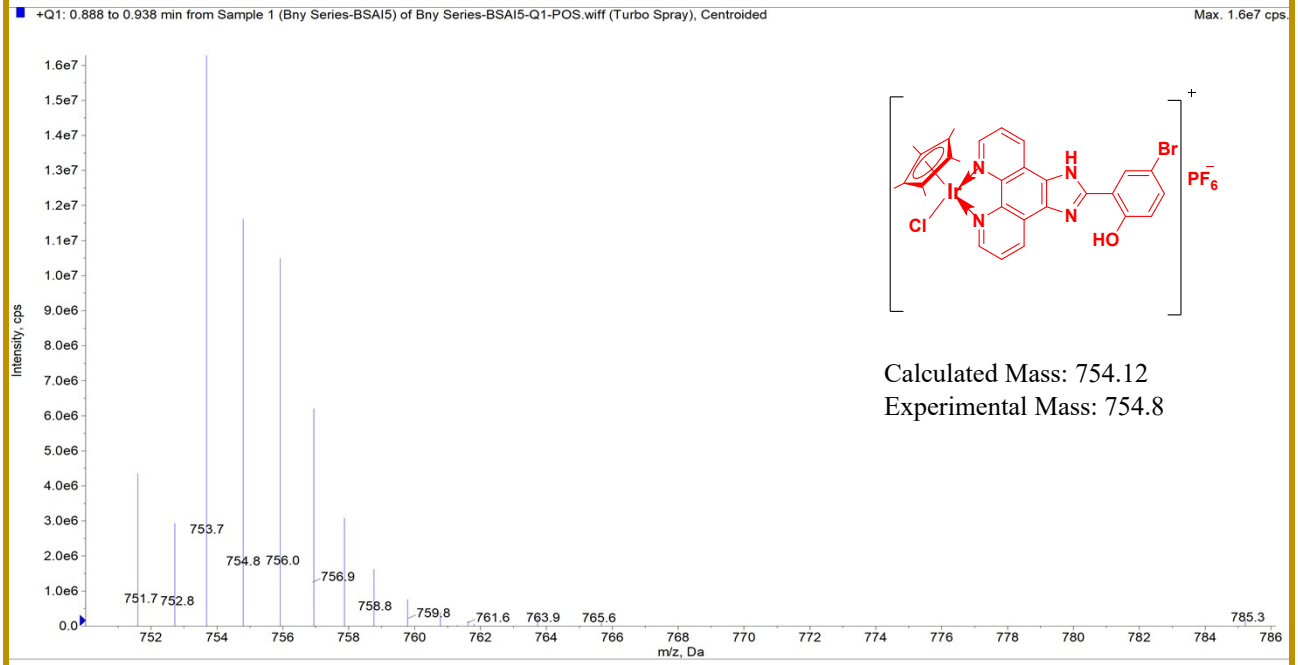




Calculated Mass: 691.22
Experimental Mass: 691.6

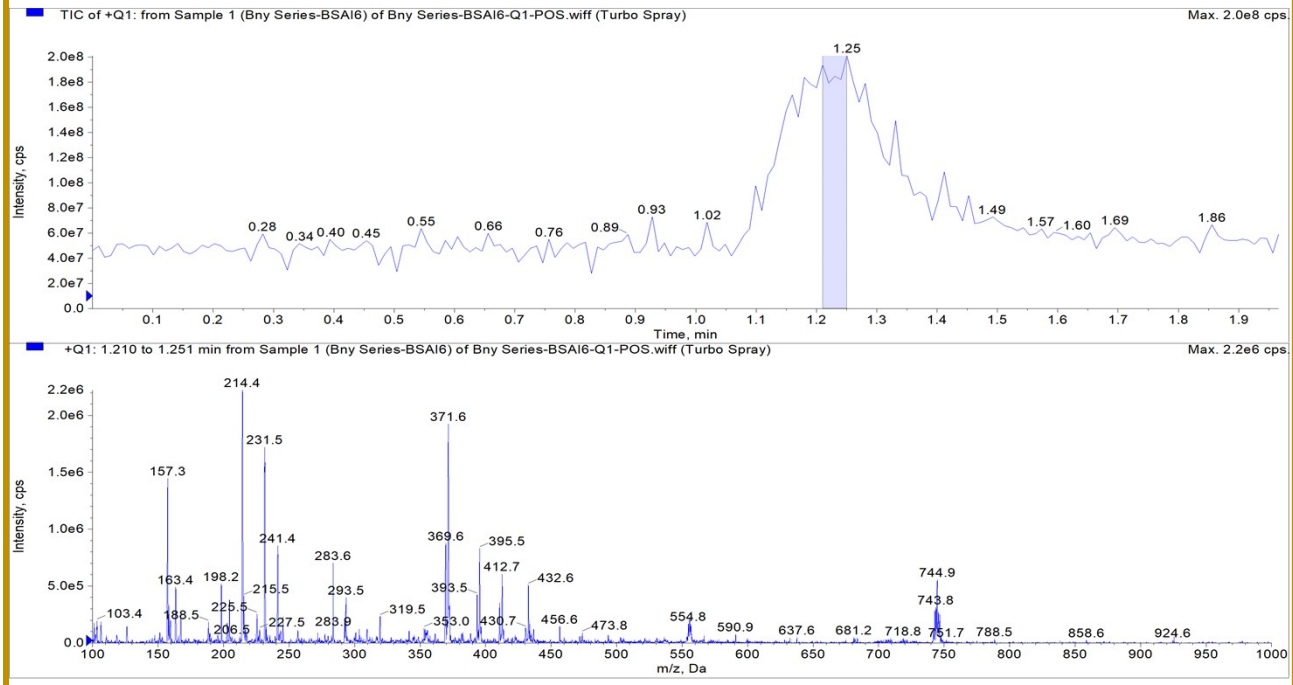
IrL5

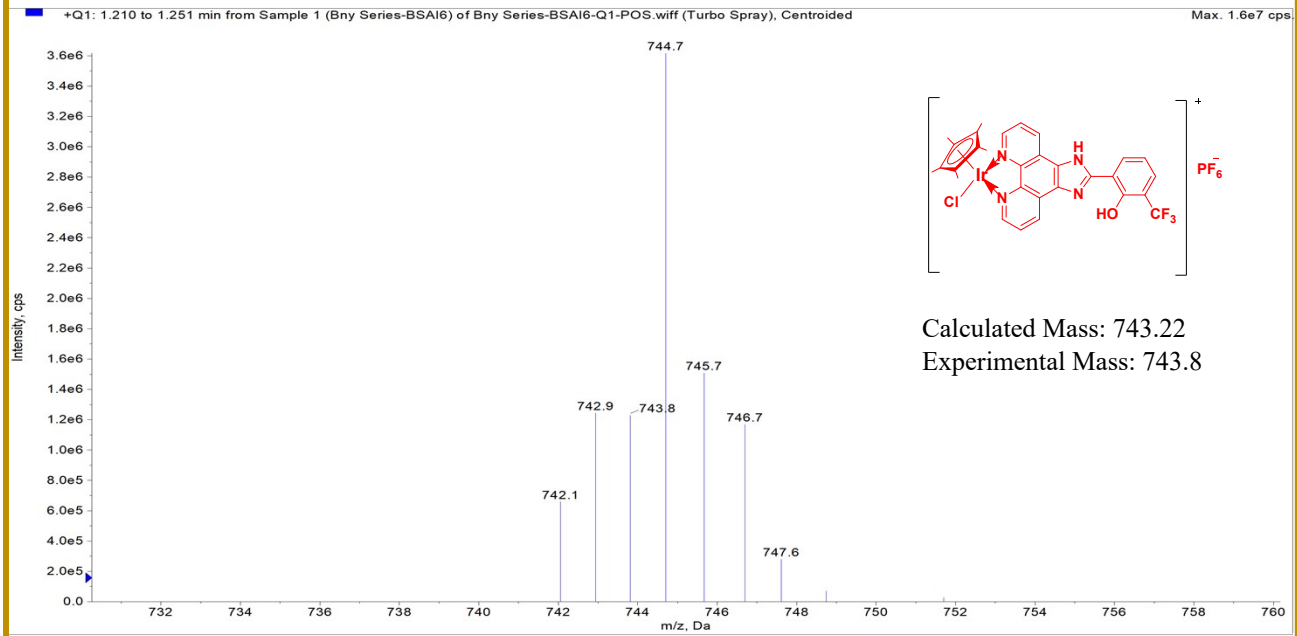




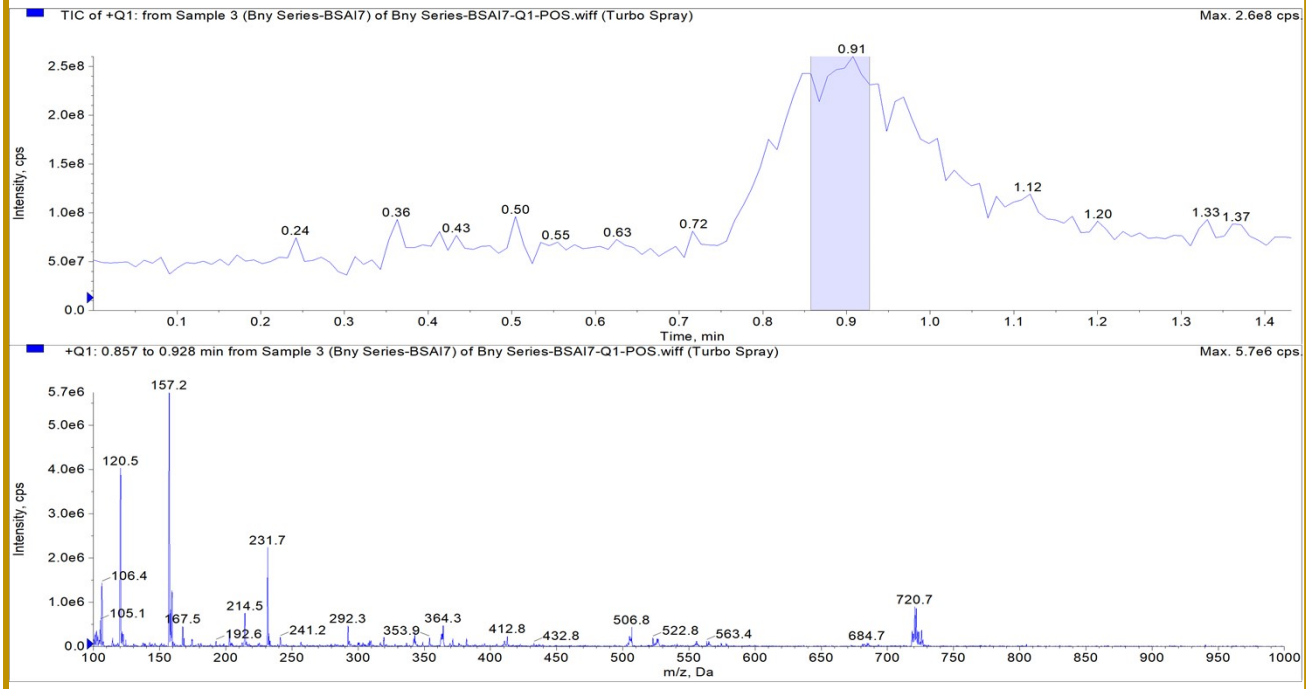
Calculated Mass: 754.12
Experimental Mass: 754.8

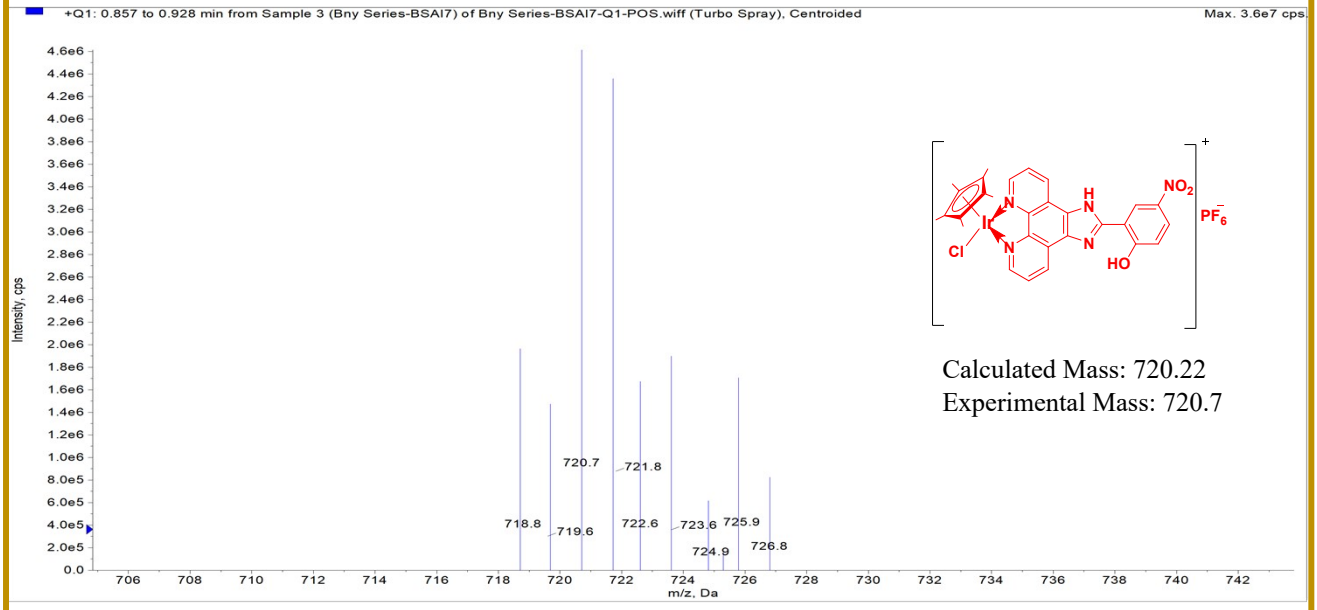
IrL6


TIC of +Q1: from Sample 1 (Bny Series-BSAI6) of Bny Series-BSAI6-Q1-POS.wiff (Turbo Spray)
Max. 2.0e8 cps.
Intensity, cps
Time, min
+Q1: 1.210 to 1.251 min from Sample 1 (Bny Series-BSAI6) of Bny Series-BSAI6-Q1-POS.wiff (Turbo Spray)
Max. 2.2e6 cps.
Intensity, cps
m/z, Da


+Q1: 1.210 to 1.251 min from Sample 1 (Bny Series-BSAI6) of Bny Series-BSAI6-Q1-POS.wiff (Turbo Spray), Centroided
Max. 1.6e7 cps.
Intensity, cps
m/z, Da
Calculated Mass: 743.22
Experimental Mass: 743.8

IrL7





Calculated Mass: 720.22
Experimental Mass: 720.7

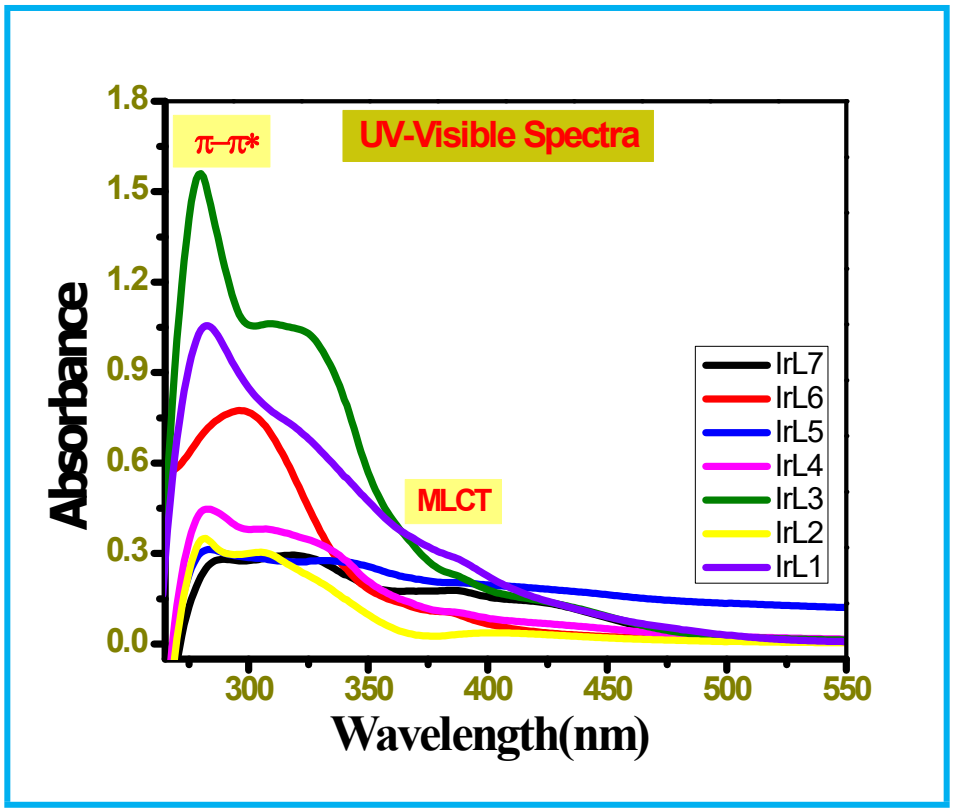
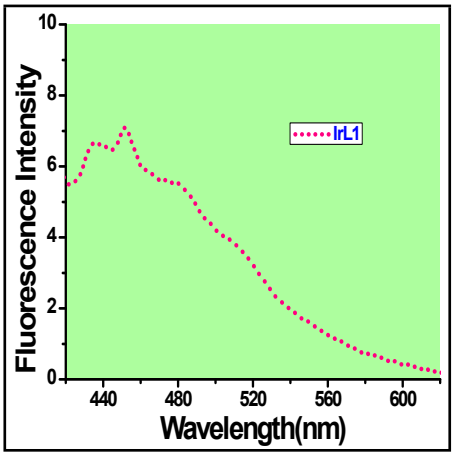
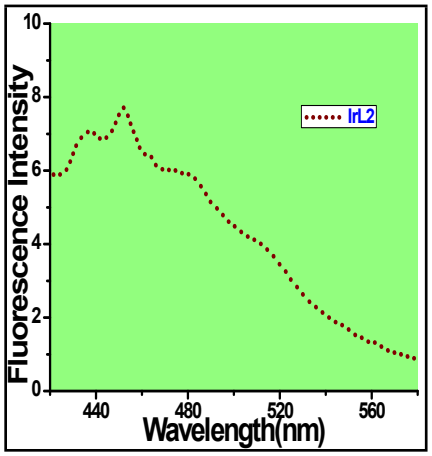


Fig. S1 UV-Vis spectra of complex IrL1-IrL7

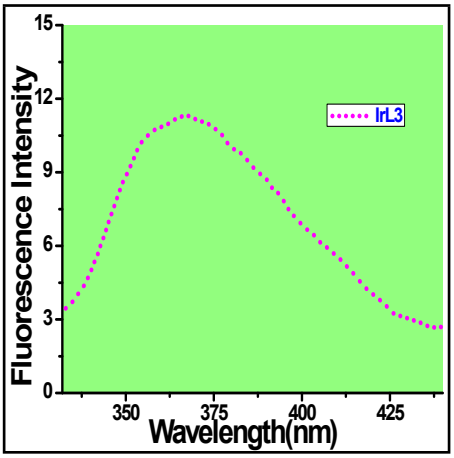
Fluorescence Emission Spectra



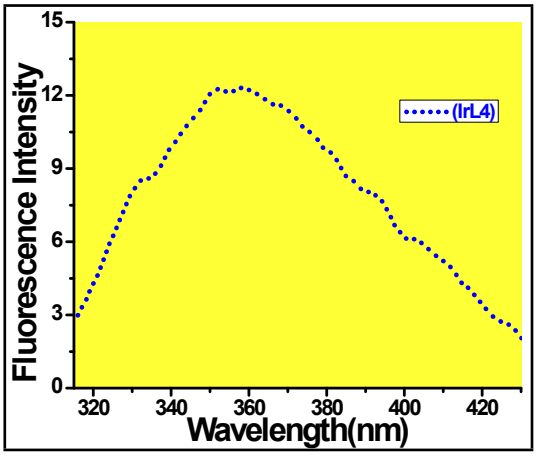
(a)



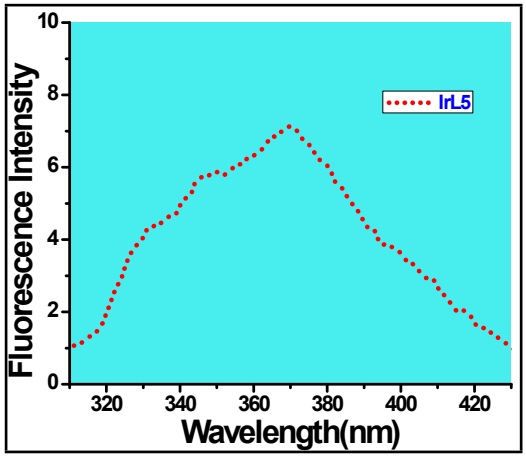
(b)



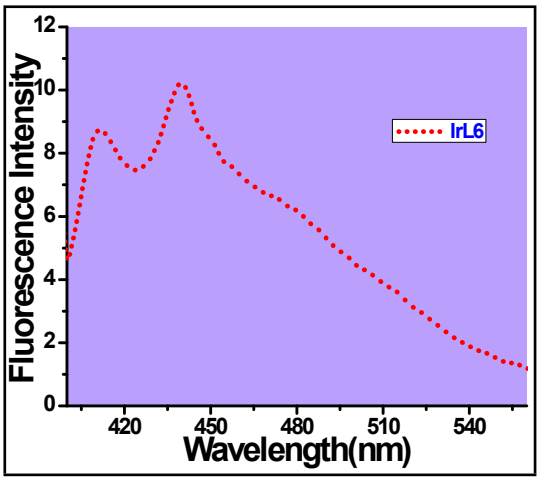
(c)



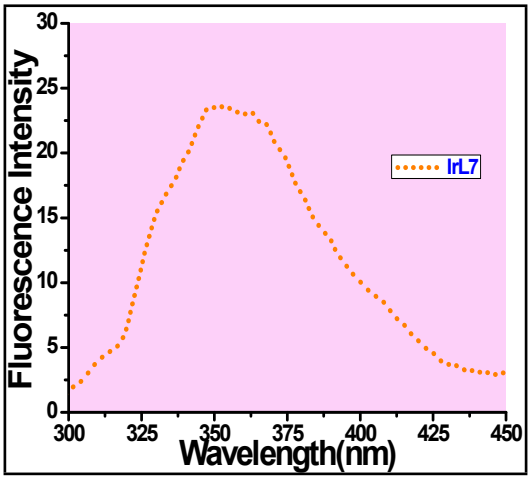
(d)



(e)



(f)



(g)

Fig. S2: Emission spectra of complexes IrL1–IrL7 in 10% DMSO-water

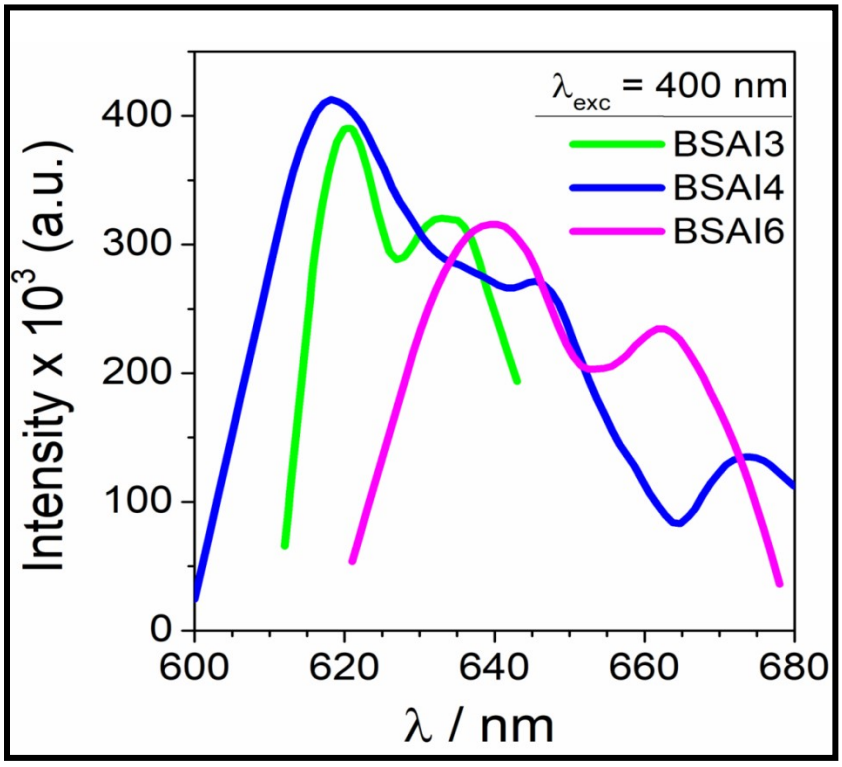


Fig. S3: Emission spectra of complexes IrL3 (green), IrL4 (blue), IrL6 (pink) in 100% DMSO

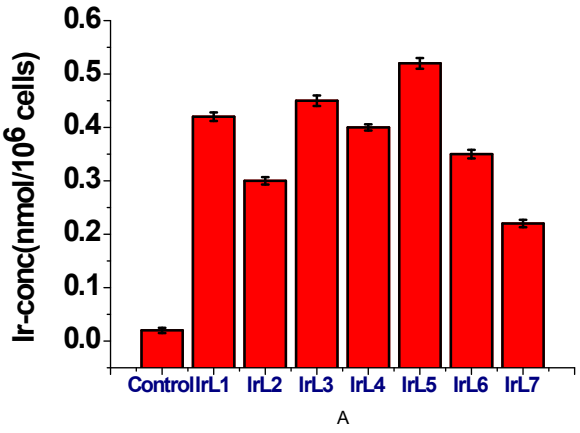


Fig. S4: Plot shows the amount of iridium present inside the cells with respect to control experiment of complexes. Each data is the mean of three independent experiments.

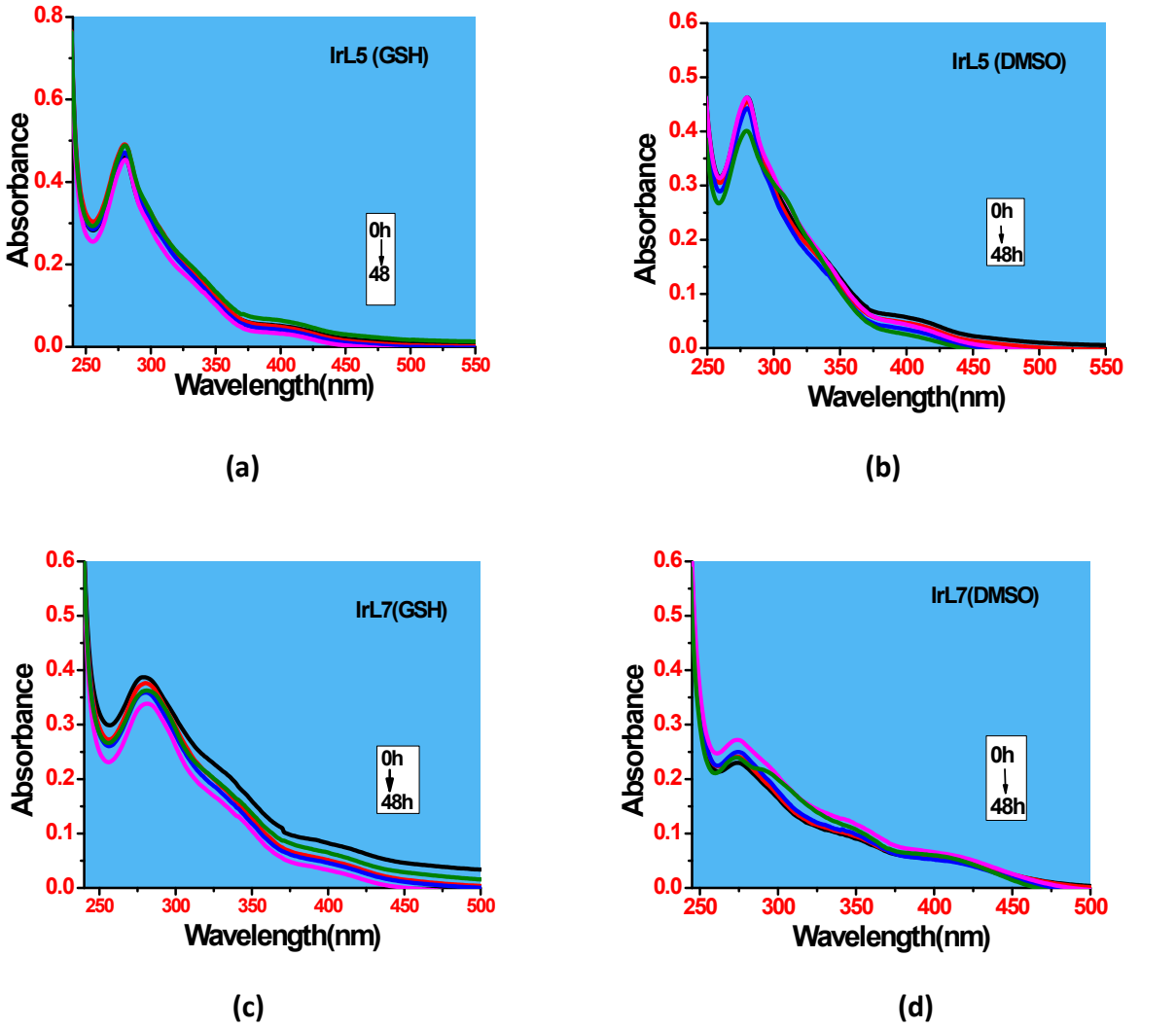


Fig. S5: Stability of selected Ir(III) complexes (IrL5 and IrL7) in aqueous 1mM GSH media [(a) and (c)] and also in 10% DMSO media [(b) and (d)]

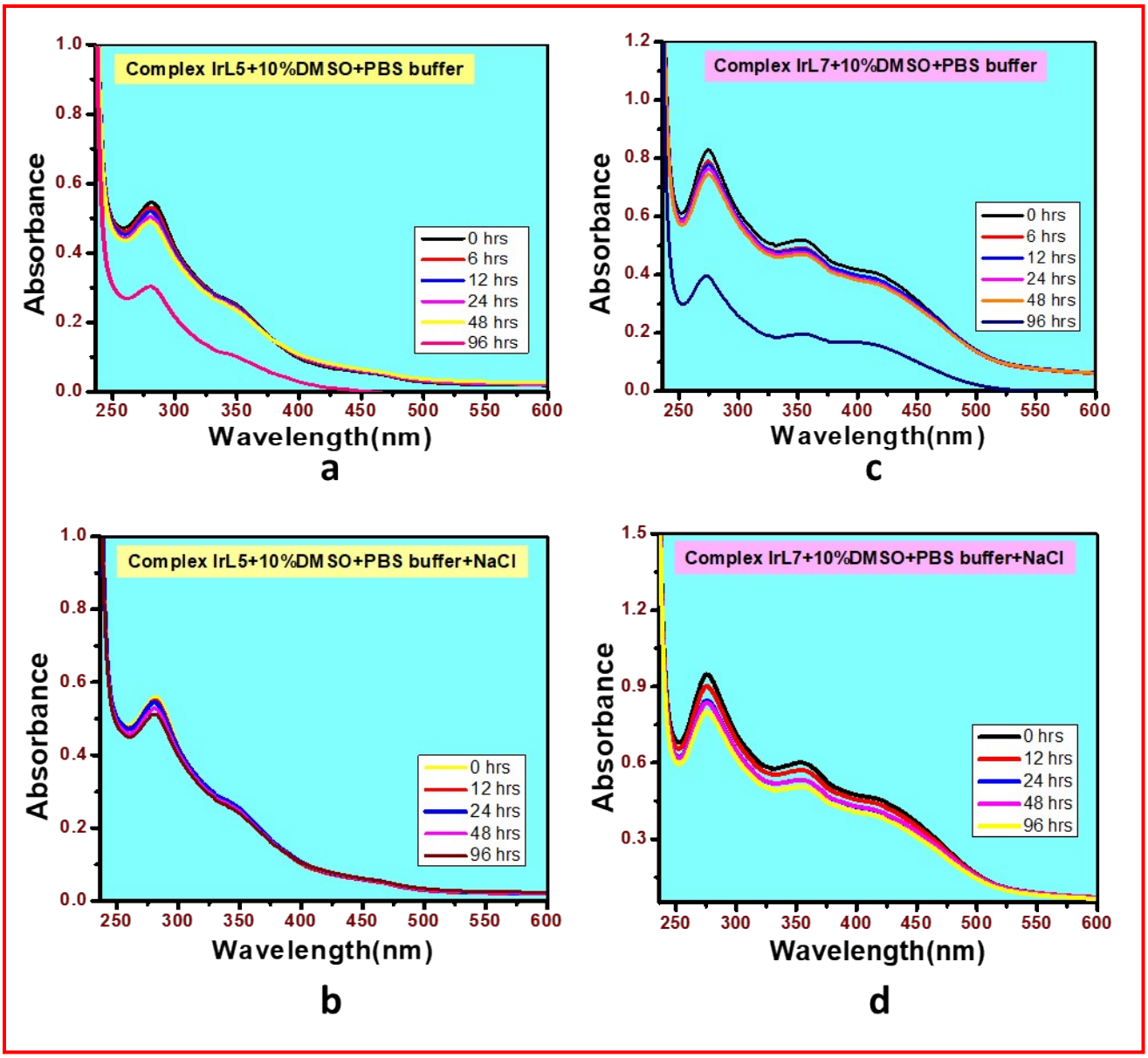
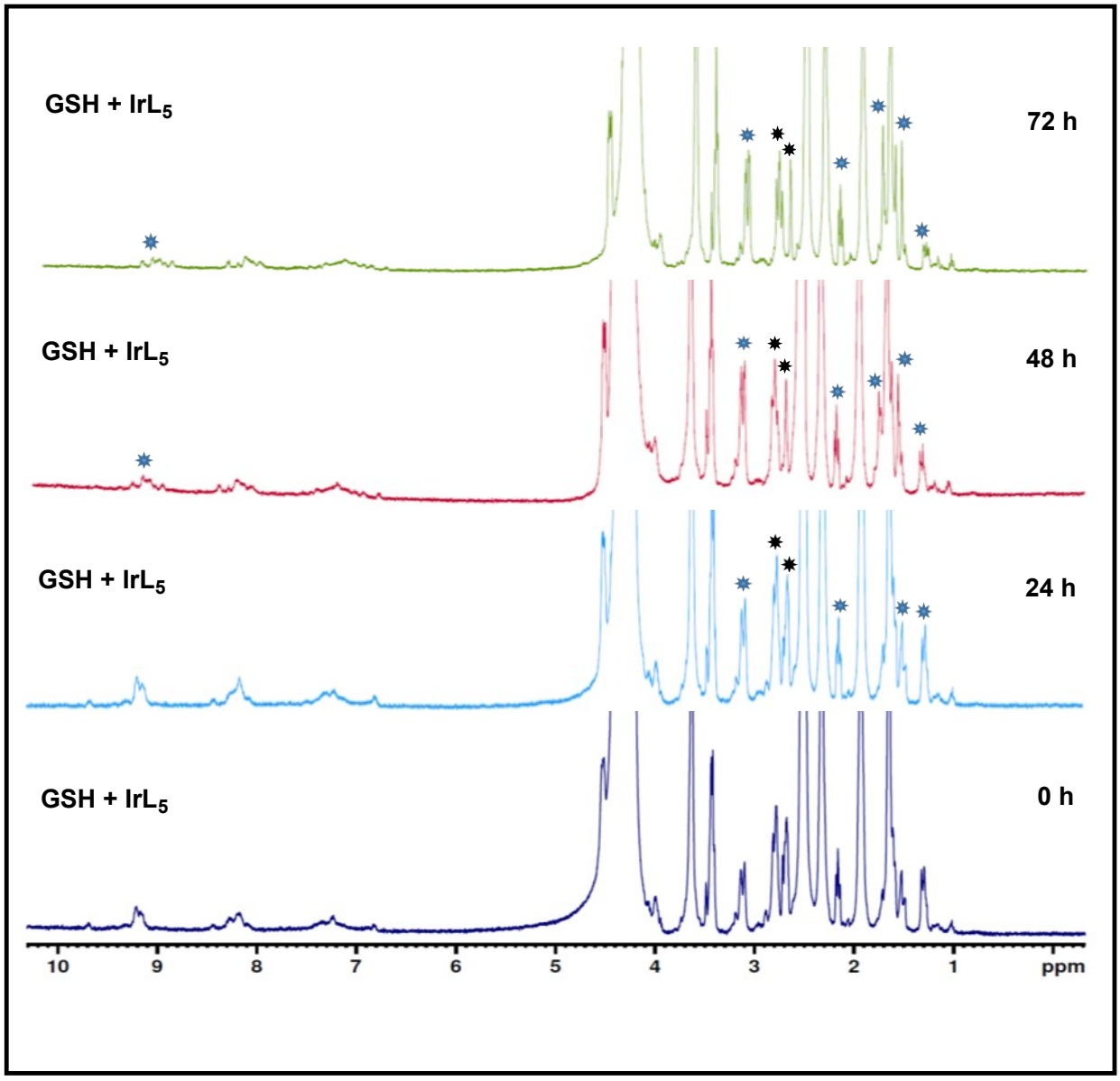
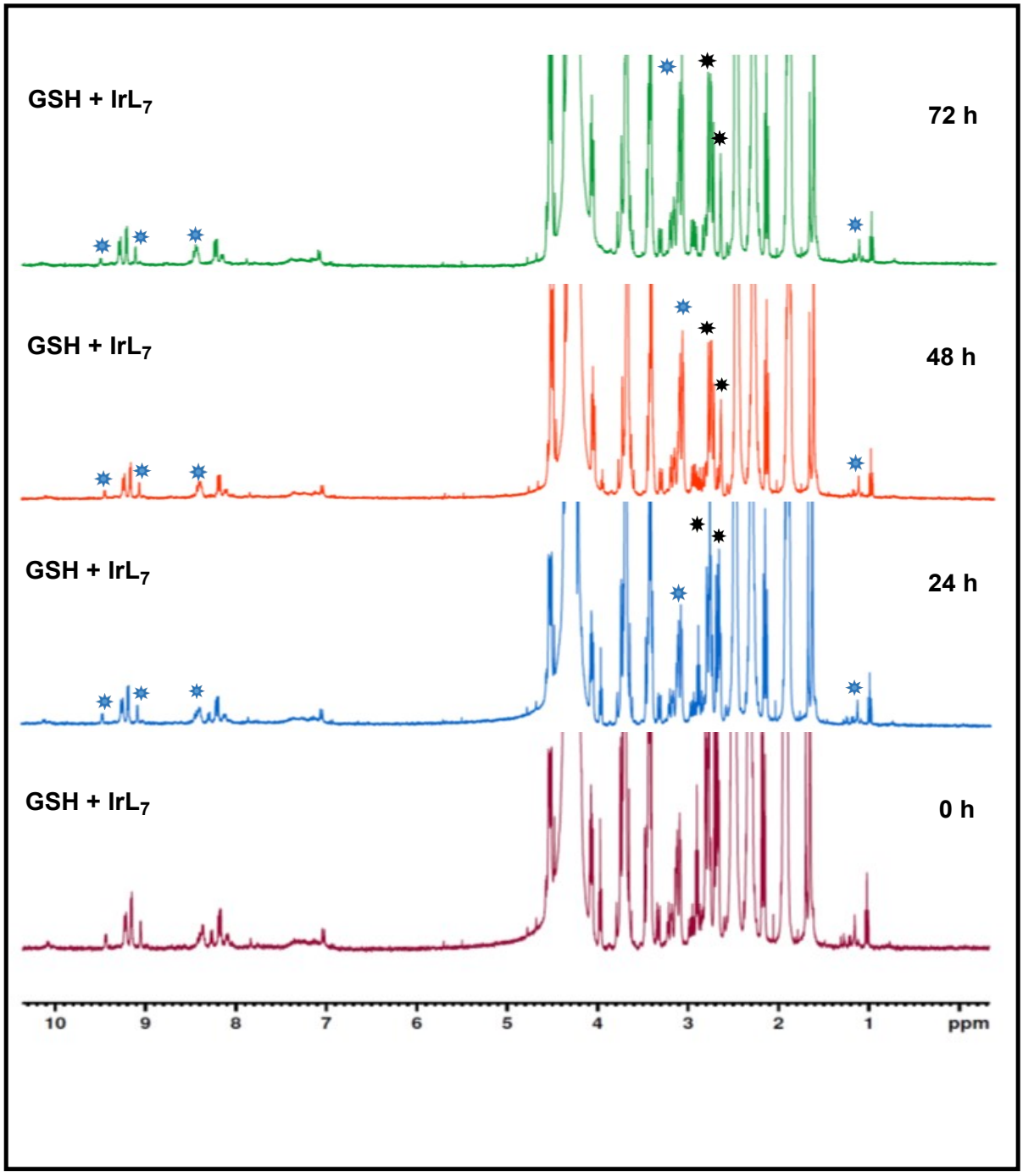


Fig. S6: Stability of selected Ir(III) complexes (IrL5 & IrL7) in 10% DMSO-PBS buffer media [(a) & (c)] and also in presence of NaCl [(b) & (d)].


GSH + IrL5
72 h
GSH + IrL5
48 h
GSH + IrL5
24 h
GSH + IrL5
0 h
10
9
8
7
6
5
4
3
2
1
ppm

(a)



(b)

Fig. S7 Stability study of complex (a) IrL5 and (b) IrL7 in reduced L-glutathione and water via ^{1}H NMR. Complexes are mixed with reduced L-glutathione (middle three) in 30% DMSO-d6/D2O mixture, recorded at different interval of time (0h, 12h, 24h and 48h) at 25°C. t = 0 h, stands for the spectra recorded immediately after dissolving reduced L-

glutathione and complex. *stands for hydrolysis product, *stands for GSH auto-oxidation product.

Table S1. Molecular docking estimated free energy of binding (kcal/mol) and the inhibition constant (K_i) of the complexes with the BSA and DNA.

Free Energy of Binding (kcal/mole)							
	IrL1	**IrL2**	**IrL3**	**IrL4**	**IrL5**	**IrL6**	**IrL7**
BSA	-6.54	-5.23	-5.33	-6.42	-5.71	-5.05	-6.49
DNA	-7.82	-7.82	-7.91	-7.28	-8.00	-7.76	-8.03
Inhibition Constant (K_i)							
BSA	16.08μM	147.69μM	124.76μM	19.64μM	64.93μM	198.85μM	17.38μM
DNA	1.84μM	1.85μM	1.60μM	4.64μM	1.36μM	2.04μM	1.29μM

Experimental Section:

Biology Experiment

Cell culture:

For doing the cell culture the cells were retained in DMEM media (Gibco), added with 10% fetal bovine serum (Himedia, India), 1% penicillin and streptomycin and 1% of Glutmax (Gibco, Thermo Scientific, USA) at 37°C in 5% CO_2. When the cells attained 70%-80% confluency they were trypsinized using 0.25% trypsin-EDTA (Thermo Fisher Scientific, USA).

***In vitro* cytotoxicity**

In vitro cytotoxicity study was measured by standard MTT assay protocol.[1] First the prepared complexes (**IrL1-IrL7**) were dissolved in 0.1% DMSO followed by dilution with DMEM medium. Two cancer cell lines *i.e.* human Epithelioid Cervix Carcinoma (HeLa), human epithelial colorectal adenocarcinoma cells (Caco-2), and one normal kidney cell (HEK 293) were used for this assay. Approximately 1×10^4 cells per well were cultured in 100 µl of a growth medium in 96-well plates and then incubated under 5% CO_2 atmosphere at 37°C temperature. Then the incubated cells were treated with different concentrations of the complexes (0-300 µM for HeLa cell and 0-150 µM for Caco-2 cell) in the volume of 100 µM/well. The cisplatin was taken as standard

positive control for this experiment. The Cells which were in the control wells, also engaged the same volume of medium containing 0.1% DMSO. After 48 h, the medium was superfluous and cell cultures were again incubated with 100 µL of MTT reagent (1 mg/ml) for 5 h at 37^{o} C. Then the resultant suspension was kept on micro vibrator for 10 min and the absorbance was recorded at λ = 570 nm in ELISA plate reader. Similar experiment was performed under hypoxic condition (1% O_2) in presence of excess GSH (1mM). For experiment in hypoxic condition the oxygen percentage of the CO2 incubator was maintained at 1 %. Compounds were loaded in 96 well micro plates in level-II bio safety cabinet under atmospheric conditions and then the drug loaded 96 well micro plate were placed in the incubator programmed to attain 1 % oxygen concentration. The incubator takes ca. 50-60 minutes to reach the 1 % oxygen level. The experiment was also performed in triplicate. The data were represented as the growth inhibition percentage i.e. % growth inhibition = 100 − [(AD × 100)/AB], where AD, measured absorbance in wells which contain samples and AB, measured absorbance for blank wells (cells with a medium and a vehicle).

Stability study

The stability of the Ir(III) complexes were tested in aqueous DMSO (H_2O: DMSO = 9:1), GSH (1mM) medium.

DNA binding study

Electronic absorption spectroscopy was used to study the binding capacity of the complexes with calf-thymus DNA (Ct-DNA) and competitive binding assay as studied using ethidium bromide (EtBr) as quencher by fluorescence spectroscopy.

UV–visible studies[2]

DNA binding assay was carried out by using complexes **IrL5** and **IrL7** in Tris-HCl buffer (5 mM Tris-HCl in water, pH 7.4) in aqueous medium. The concentration of Ct-DNA was calculated from its absorbance intensity at 260 nm and its known molar absorption coefficient value of 6600 M^{-1} cm^{-1}. Equal amount of DNA was added in both the sample and reference in cuvettes. Titration was carried out by increasing concentration of CT-DNA. On the eve of each measurement, sample was equilibrated with CT-DNA for about 5 min and then absorbance of the complex was measured. The intrinsic DNA binding constant (K_b) was calculated using the equation (i):

$$\frac{[DNA]}{(\varepsilon_a - \varepsilon_f)} = \frac{[DNA]}{(\varepsilon_b - \varepsilon_f)} + \frac{1}{K_b(\varepsilon_a - \varepsilon_f)} \quad (i)$$

Where [DNA] is the concentration of DNA in the base pairs, ε_a is the apparent extinction coefficient observed for the complex, ε_f corresponds to the extinction coefficient of the complex in its free form, and ε_b refers to the extinction coefficient of the complex when fully bound to DNA. The resultant data were plotted using Origin Lab, version 8.5 to obtain the [DNA]/(ε_a-ε_f) vs. [DNA] linear plot. The ratio of the slope to intercept from the linear fit gave the values of the intrinsic binding constants (K_b).

UV and Fluorescence study

UV and Fluorescence study of all these iridium (III) complexes, were executed in 10 % DMSO solution. Then the fluorescence quantum yields (Φ) were calculated by applying the comparative William's method which involves the use of well-characterized standard with known quantum yield value using 10% DMSO solution.[3] Quinine sulphate was used as a standard. Quantum yield was calculated according to the equation (ii):

$$\varphi = \varphi_R \times \frac{I_S}{I_R} \times \frac{OD_R}{OD_S} \times \frac{\eta_S}{\eta_R} \cdots\cdots\cdots (ii)$$

Where, φ = quantum yield, I = peak area, OD = absorbance at λmax, η = refractive index of solvent (s) and reference (R). Here, we have used quinine sulphate as a standard for calculating the quantum yield.

Ethidium bromide displacement assay

The ethidium bromide (EtBr) displacement assay was carried out to illustrate the mode of binding between the potent compounds with DNA.[4] The apparent binding constant (K_{app}) of the complexes **IrL5** and **IrL7** to Ct-DNA were calculated using ethidium bromide (EtBr) as a spectral probe in 5 mM Tris-HCl buffer (pH 7.4). EtBr was not able to exhibit any fluorescence in its free state as its fluorescence was quenched by the solvent molecules. But its fluorescence intensity was started to increase in presence of Ct-DNA, which suggested the intercalative mode of binding of EtBr with DNA grooves. The fluorescence intensity was found to decrease with further increase in concentration of the complexes. Thus it can be said that the complexes displaced EtBr from CT-

DNA grooves and the complexes themselves got bound to the DNA base pairs. The values of the apparent binding constant (K_{app}) were obtained by using the equation (iii):

$$K_{app} \times [Complex]_{50} = k_{EtBr} \times [EtBr] \cdots\cdots\cdots (iii)$$

Where K_{EtBr} is the EtBr binding constant (K_{EtBr} = 1.0 x 10^7 M^{-1}), and [EtBr] = 8 x 10^{-6} M. Stern-Volmer equation was followed for quantitative determination of the Stern-Volmer quenching constant (K_{SV}).[5] Origin (8.5) software was used to plot the fluorescence data to obtain linear plot of I_0/I vs. [complex]. The value of K_{SV} was calculated from the following equation.

$$I_0/I = 1 + K_{SV}[Q] \quad (iv)$$

Where I_0 = fluorescence intensity in absence of complex and I = fluorescence intensities in presence of complex of concentration [Q].

Protein binding studies

We are acquainted with the fact that serum albumin proteins are the main component. It is well known in blood plasma proteins and plays important roles in drug transport and metabolism, interaction of the drug with bovine serum albumin (BSA), a structural homologue of human serum albumin (HSA) was studied from tryptophan emission quenching experiment.[6] Tryptophan emission quenching experiment was performed to detect the interaction of the iridium complex **IrL5** and **IrL7** with protein BSA. Initially, BSA solution (2 x 10^{-6} M) was prepared in Tris-HCl/NaCl buffer. The aqueous solutions of the complexes were subsequently added to BSA solution with gradual increase of their concentrations. After each addition, the solutions were shaken slowly for 5 min before recording the fluorescence at a wavelength of 295 nm (λex = 295 nm). A gradual decrease in fluorescence intensity of BSA at λ = 340 nm was observed upon increasing the concentration of complex, which confirmed that the interaction between the complex and BSA was happened. Stern-Volmer equation was employed to quantitatively determine the quenching constant (K_{BSA}). Origin Lab, version 8.5 was used to plot the emission spectral data to obtain linear plot of I_0/I vs. [complex] using the equation (v) given below:

$$I_0/I = 1 + K_{BSA}[Q] = 1 + k_q \tau_0 [Q] \quad (v)$$

Where I_0 is the fluorescence intensity of BSA in absence of complex and I indicates the fluorescence intensity of BSA in presence of complex of concentration [Q], τ_0 = lifetime of the tryptophan in BSA found as 1 x 10^{-8} and kq is the quenching constant. Scatchard equation (vi) gives the binding properties of the complexes.[7] Where K = binding constant and n = number of binding sites.

$$\log\left(I_0 - I/I\right) = \log K + n\log\left[Q\right] \quad (vi)$$

Conductivity measurement[8]

For authenticating the interaction of the complexes with DMSO and aqueous DMSO, conductivity of the prepared complexes were performed using conductivity-TDS meter-307 (Systronics, India) and cell constant 1.0 cm^{-1}. Rate of conductivity was also estimated in different pH medium. Time dependent conductivity measurement was also carried out.

n-Octanol–water partition coefficient (log $P_{o/w}$)[9]

The log $P_{o/w}$ of the iridium complexes were adhering to shake flask method using the previously published procedure. A known amount of each complex (**IrL1-IrL7**) was suspended in water (pre-saturated with n-octanol) and shaken for 48 h on an orbital shaker. To allow the phase separation, the solution was centrifuged for 10 min at 3000 rpm. To obtain the partition coefficient, different ratios (0.5: 1, 1: 1, and 2: 1) of the saturated solutions were shaken with pre-saturated n-octanol for 20 min on an orbital shaker and followed the same procedure. Aliquots of the aqueous and octanol layers were pipetted out separately and the absorbances were measured with UV-Vis spectrophotometer using proper dilution. Each set was performed in triplicate, concentration of the substances in each layer was calculated using the respective molar extinction coefficients and the partition coefficient (log $P_{o/w}$) values were obtained from the ratio.

Cellular uptake study in HeLa cell[10]

At the outset, 1 × 10^6 Cells were seeded in each 90 mm dia petri-dish and incubated for 48 h. After incubation, equi-molar concentrations (5 μM) of each complex (**IrL1-IrL7**) were added and treated for 48 h. Then, the cells were washed with PBS (pH 7.2), followed by the treatment with trypsin-EDTA. After cell counting 1 × 10^6 numbers of cells were collected as cell pellets using centrifugation. Cell pellets were digested overnight in concentrated nitric acid (70% *v/v*) at 60˚C;

the resultant solutions were diluted by double-distilled water to make a final concentration of 5% HNO_3. The cellular accumulation of Ir(III) was determined by ICP–MS (Thermo Scientific XSERIES2). The standard solutions of iridium was freshly prepared and investigated while analyses for the samples were carried out in triplicates and the standard deviations were calculated.

Notes and References

1. P. Liu, B. Wu, J. Liu, Y. Dai, Y. Wang, K. Wang, DNA Binding and Photocleavage Properties, Cellular Uptake and Localization, and in-Vitro Cytotoxicity of Dinuclear Ruthenium(II) Complexes with Varying Lengths in Bridging Alkyl Linkers, *Inorg. Chem*., 2016, **55**, 1412−1422.
2. M. Sirajuddin, S. Ali, A. Badshah, Drug–DNA interactions and their study by UV–Visible, fluorescence spectroscopies and cyclic voltammetry, *J. Photochem. Photobio. B*., 2013, **124**, 1–19.
3. M. Shamsi-Sani, F. Hirini, SM. Abedini, M. Seddighi, Synthesis of benzimidazole and quinoxaline derivatives using reusable sulfonated rice husk ash (RHA-SO3H) as a green and efficient solid acid catalyst, *Res. Chem. Intermed*., 2016, **42**, 1091–1099.
4. S. Dasari, A. K. Patra, Luminescent europium and terbium complexes of dipyridoquinoxaline and dipyridophenazine ligands as photosensitizing antennae: structures and biological perspectives, *Dalton Trans*., 2015, **44**, 19844-19855.
5. J. Keizer, Nonlinear fluorescence quenching and the origin of positive curvature in Stern-Volmer plots, *J. Am. Chem. Soc*., 1983, **105**, 1494–1498.
6. V. D. Suryawanshi, L. S. Walekar, A. H. Gore, P. V. Anbhule, G. B. Kolekar, Spectroscopic analysis on the binding interaction of biologically active pyrimidine derivative with bovine serum albumin, *J. Pharm. Anal*., 2016, **6**, 56–63.
7. K. Jeyalakshmi, J. Haribabu, C. Balachandran, S. Swaminathan, N. S. P. Bhuvanesh, R. Karvembu, Coordination Behavior of N,N′,N″-Trisubstituted Guanidine Ligands in Their Ru–Arene Complexes: Synthetic, DNA/Protein Binding, and Cytotoxic Studies, *Organometallics,* 2019, **38**, 753−770.
8. S. Nikolić, L. Rangasamy, N. Gligorijević, S. Aranđelović, S. Radulović, G. Gasser, S. Grgurić-Šipka, Synthesis, characterization and biological evaluation of novel Ru(II)–arene complexes containing intercalating ligands, *J. Inorg. Biochem.* 2016, **160**, 156–165.
9. M. Kubanik, H. Holtkamp, T. Söhnel, S. M. F. Jamieson, C. G. Hartinger, Impact of the Halogen Substitution Pattern on the Biological Activity of Organoruthenium 8-Hydroxyquinoline Anticancer Agents, *Organometallics*, 2015, **34**, 5658–5668.
10. K. Purkait, S. Chatterjee, S. Karmakar, A. Mukherjee, Alteration of steric hindrance modulates glutathione resistance and cytotoxicity of three structurally related Ru(II)-*p*-cymene complexes, *Dalton Trans.*, 2016, **45**, 8541-8555.