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Palladium mediated C-O bond activation of benzopyrone in 4-oxo-4H-chromone-3-carbaldehyde-4(*N*)-substituted thiosemicarbazone: Synthesis, structure, nucleic acid/albumin interaction, DNA cleavage, antioxidant and cytotoxic studies

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Supporting information

Fig.S1-S8. ^{1}H-NMR spectra of the ligands (**HL$^{1-4}$**) and complexes (**1-4**).

Fig.S9-S16. ^{13}C-NMRspectra of the ligands (**HL$^{1-4}$**) and complexes (**1-4**).

Fig.S17-S19.ESI-MS spectra of the complexes **1, 2** and **4**.

Fig. S20. Synchronous spectra of BSA (1×10^{-6} M) in the absence and presence of complexes **1-4** (0-100 µM) in the wavelength difference of Δλ = 60 nm.

DNA binding studies

The experiments involving CT-DNA were performed in double distilled water with tris(hydroxymethyl)aminomethane (Tris, 5 mM) and sodium chloride (50 mM) and adjusted to pH 7.2 with hydrochloric acid. The concentration of CT-DNA was determined by UV absorbance at 260 nm. The molar absorption coefficient, $\varepsilon_{max}260$, was taken as 6600 M^{-1} cm^{-1}. CT-DNA solution of various concentrations (5-50 μM) dissolved in Tris-HCl (pH 7.2) were added to the nickel(II) complexes **1-4** (10 μM dissolved in a DMSO-H_2O mixture). Absorption spectra were recorded after equilibrium at 20 ºC for 10 min. The intrinsic binding constant K_b was determined by using the Stern-Volmer equation (1).[1]

$$([DNA] / [\varepsilon_a - \varepsilon_f]) = [DNA] / [\varepsilon_b - \varepsilon_f] + 1/ K_b[\varepsilon_b - \varepsilon_f] \qquad (1)$$

The absorption coefficients ε_a, ε_f, and ε_b correspond to A_{obsd}/[DNA], the extinction coefficient for the free complexes and the extinction coefficient for the complexes in the fully bound form, respectively. The intrinsic binding constant K_b can be obtained from the ratio of the slope to the intercept in plots of [DNA]/ [ε_a - ε_f] *versus* [DNA].

Competitive binding with ethidium bromide

To find out the exact mode of attachment of CT-DNA to the complexes, fluorescence quenching experiments of EB-DNA were carried out by adding 10 μL portion of 10 μM nickel(II) complexes every time to the sample containing 10 μM EB, 10 μM DNA and Tris buffer (pH 7.2). Before the measurements, the system was shaken and incubated at room temperature for ~5 min. The emission spectra were recorded at 530-750 nm. On the basis of the classical Stern-Volmer equation, the quenching constant has been analysed (2).

$$I_0/I = K_{sv}[Q] + 1 \qquad (2)$$

Where I_0 and I represent the emission intensities in the absence and presence of the complexes, respectively, K_{sv} is the quenching constant, and [Q] is the concentration ratio of the complex. The K_{sv} values have been obtained as a slope from the plot of I_o/I *versus* [Q].Further, the apparent DNA binding constant (K_{app}) were calculated using the following equation,

$$K_{EB}\,[EB] = K_{app}[\text{complex}]$$

(Where [complex] is the value at a 50% reduction in the fluorescence intensity of EB, K_{EB} ($1.0 \times 10^7\ M^{-1}$) is the DNA binding constant of EB, [EB] is the concentration of EB =10 μM).

DNA cleavage studies

The cleavage of DNA was monitored by using agarose gel electrophoresis. Supercoiled pBR322 DNA (100 mg) in 5% DMSO and 95% Tris buffer (5 mM, pH 7.2) with 50 MmNaCl was incubated at 37°C in the absence and presence of compounds. The DNA, compound and sufficient buffer were premixed in a vial, and the reaction was allowed to proceed for 2 h at 37°C. The samples were then analyzed by 1.5% agarose gel electrophoresis in tris-acetic acid-ethylenediaminetetraacetic acid buffer. The gel was stained with 0.5 μg cm^{-3}ethidium bromide before migration. After electrophoresis at 50 V for 3 h, the gel was illuminated and the digital images were analyzed by gel documentation system (SYNGEN USA).[2]

Bovine serum albumin binding study

BSA solution(10 μM) was prepared in phosphate buffer of pH 7.2 and stored in the dark at 4 °C for use. The excitation wavelength of BSA at 280 nm and the emission at 346 nm were monitored for the protein binding studies. The excitation and emission slit widths and scan rates were maintained constant for all of the experiments. Concentrated stock solution of complexes were prepared by dissolving the compounds in DMSO and diluted suitably with deionised water to required concentrations for all the experiments (1% DMSO in the final solution). Quenching of the emission intensity of tryptophan residues of BSA at 346 nm (excitation wavelength at 276 nm) was monitored using compound as quenchers with increasing compound concentration. The possible quenching mechanism has been interpreted using the Stern-Volmer equation (2).

When small molecule bind to the active site of BSA, the equilibrium binding constant and the number of binding sites can be analysed by using the Scatchard equation (4).

$$\log[(F_0 - F)/F] = \log K + n \log[Q] \qquad (4)$$

Where K is the binding constant of quencher with BSA, n is the number of binding sites, F_0 and F are the fluorescence intensity in the absence and the presence of the quencher. Which can be determined by the slope and the intercept of the double logarithm regression curve of log $[(F_0-F)/F]$ *versus* log[Q]. Synchronous fluorescence spectra of BSA with variousconcentrations of complexes (0-100 μM) were obtained from 300 to 500 nm when $\Delta\lambda$= 60 nm and from 290 to 500 nm when$\Delta\lambda$ = 15 nm. The excitation and emission slit widths were 5 and 6 nm, respectively. Fluorescence and synchronous measurements were performed by using a 1 cm quartz cell on a JASCO FP 6600 spectrofluorometer.

Three dimension fluorescence spectra measurement

The 3D fluorescence spectra of BSA were recorded with and without complexes. Protein solution at 10μM was transferred to a quartz cell, dilutedwith 2.0 mL phosphate buffer and mixed well. To this, 10μM of complexes was added and the 3D fluorescence spectra were recorded by scanningexcitation wavelength in the range of 200–350 nm and emissionwavelength from 220 to 500 nm at an interval of 10 nm. The scanningparameters were the same as in the fluorescence quenching experiments.

Evaluation of antioxidant assays

DPPH radical scavenging activity

The potential antioxidant activity of ligands and new nickel(II) complexes (**1-4**) was evaluated by diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was determined by Szabo method.[3]DPPH free radicals are used for rapid analysis of antioxidants. While scavenging the free radicals, the antioxidants donate hydrogen and form a stable DPPH molecule. Briefly, the various concentrations of the complexes (20-100 μg/ml) in 1 ml of 10 % aqueous DMSO were added to 5 ml of DPPH (0.1 mM in methanol) and were mixed rapidly. A negative control was prepared by adding 100 μl of 10% aqueous DMSO in 5 mL of 0.1 mM methanolic solution of DPPH. Radical scavenging capacity was measured in 10 min intervals using spectrophotometer by monitoring the decrease in absorbance at 517 nm. The IC_{50} values of DPPH decolonization of the complexes were calculated. Ascorbic acid was used as a positive control.

Nitric oxide scavenging assay[4]

The nitric oxide (NO) scavenging activity of the complexes has been investigated using the Green method[4],where sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10 mM) in phosphate buffered saline was mixed with a concentration of the compound (1 mL; 20–100 μM) and incubated at room temperature for 150 min. After the incubation period, 0.5 mL of Griess reagent containing 1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediaminedihydrochloride were added. The absorbance of the chromophore formed was measured at 546 nm.

Estimation of total antioxidant capacity

Total antioxidant was determined by phosphomolybdenum method[5] followed by Samples and standard (1 ml) was mixed with 2 ml of reagent solution [ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulphuric acid (0.6 M)]. All the reaction mixtures were incubated at 95 ºC for 90 min. The absorbance was measured at 695 nm.

For the above assay, all of the tests were run in triplicate and various concentrations of the compounds were used to fix a concentration at which the compounds showed in and around 50 % of activity. In addition, the percentage of activity was calculated using the formula: % of suppression ratio = $[(A_0 - A_c)/A_0] \times 100$. A_0 and Ac are the absorbance in the absence and presence of the tested compounds, respectively. The IC_{50} (half maximal inhibitory concentration) is a measure the effectiveness of the substance in inhibiting a specific biological or biochemical function. The 50 % activity (IC_{50}) can be calculated using the percentage of activity. All experimental results were expressed as the mean and (±) standard deviation (SD) of triplicate determinations.

Cytotoxicity studies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[6]

MTT, upon reduction by NAD(P)H-dependent cellular oxidoreductase enzymes present in the cytoplasm of metabolically active cells, forms formazan as a purple insoluble product. The amount of formazan estimated by a spectral method correlates with the number of live cells and provides a quantitative measurement of the cytotoxicity of the compound. The IC_{50} values were obtained from nonlinear regression using GraphPad Prism 5.[7] About 6000 cells of MCF-7 (human breast cancer cells) were taken in each well of a 96-well culture plate and incubated for 24 hours in a CO_2 incubator. After incubation, different concentrations (6, 12, 25, 50, 100μM) of the compounds dissolved in DMSO were added to the cells. After appropriate incubation time (24 h for complexes and ligands), the wells were treated with 20μl MTT (5 mg/ml phosphate-buffered saline, PBS) and incubated for 3 h. The purple formazan crystals obtained were dissolved in 200μl DMSO and the absorbance was measured at 570 nm in Molecular Devices Spectra Max M5 plate reader. Data were obtained as the average of three independent sets of experiments, all performed in triplicate for each concentration.[6]

Morphological Changes of MCF7 cells using phase contrast inverted microscope

Observation of morphological changes of apoptotic cells was performed according to the method with slight modifications.[8]Briefly, MCF-7 cells were seeded into 24-well plate and incubated overnight to attach. Then, the cells were treated with or without test compounds(control) at different concentrations of 12 and 25 μM for complexes**1-4** was incubated 24h at 37°C with 5% CO_2. The morphological changes of the cells were observed using an inverted light microscope at 100 X magnification.

Acridine orange/ethidium bromide staining

Acridine orange/ethidium bromide staining was carried out by the method of Gohel*et al*.[9]and MCF-7 cells were plated in a 24-well plates. They were allowed to grow at 37 °C in a humidified CO_2 incubator. Then the cells were treated with IC_{50} concentrations of complexes **1** and **4** for 24 h. The culture medium was aspirated from each well and the cells were gently rinsed twice with PBS at room temperature. Then equal volumes of cells from control and metal complexes treated were mixed with 100 ml of dye mixture (1:1) of acridine orange and ethidium bromide and viewed immediately by fluorescence microscopy.

References

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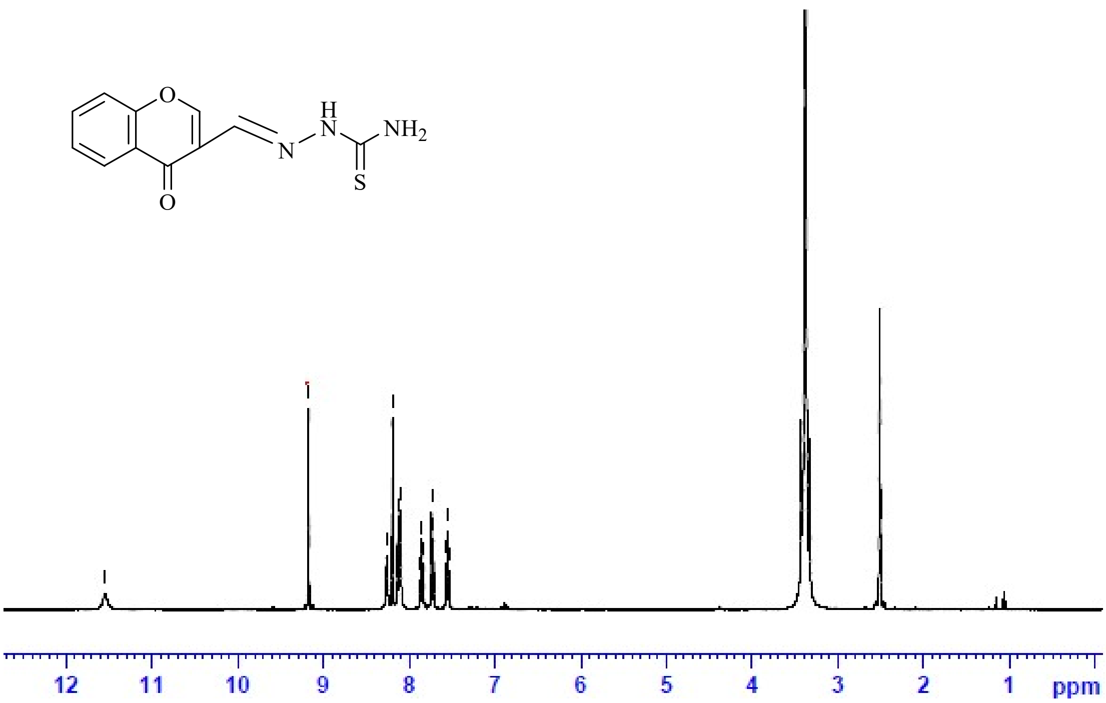


Fig. S1 ^{1}H-NMR spectrum of [H-Fcm-tsc] **(HL^{1})**

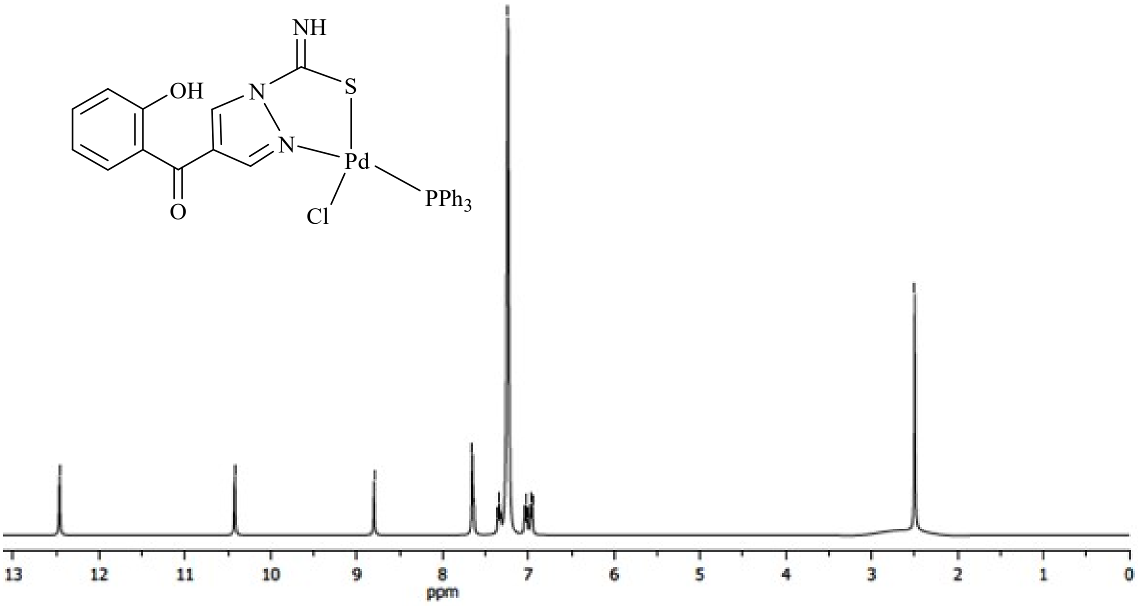


Fig. S2[1]H-NMR spectrum of [Pd(4(2Hbp-1-ca)(PPh$_3$)Cl] (**1**)

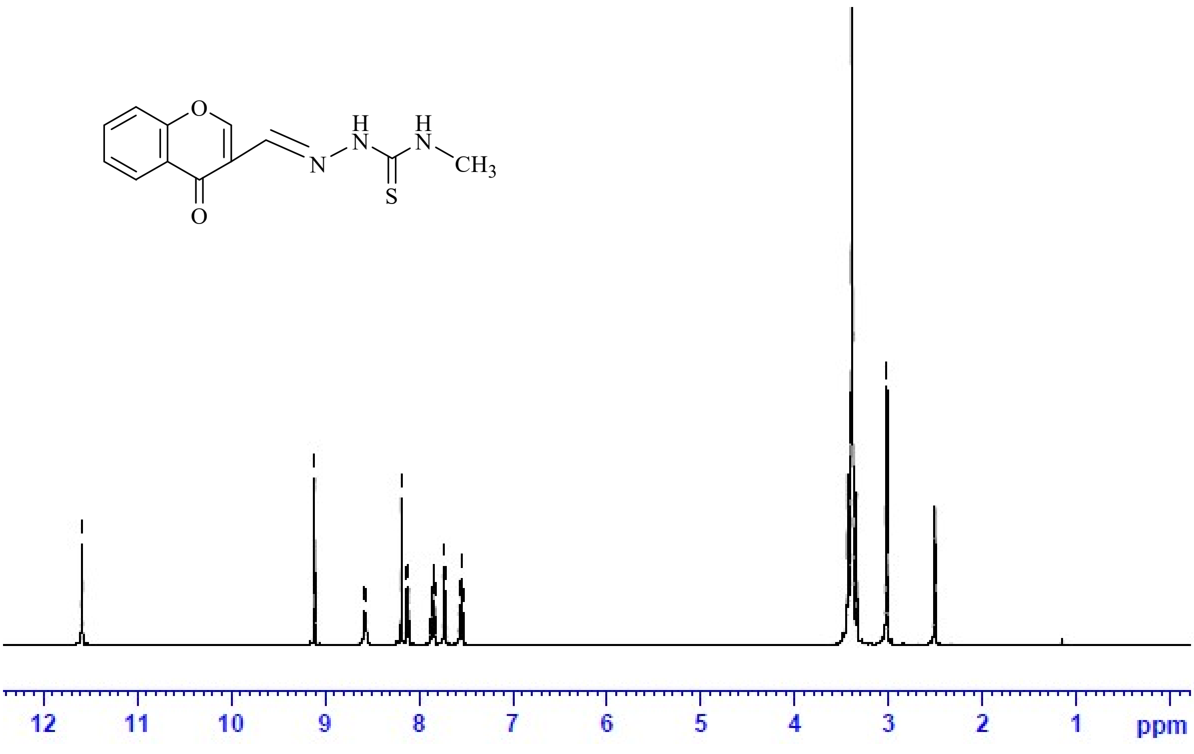


Fig. S3[1]H-NMR spectrum of [H-Fcm-mtsc] **(HL[2])**

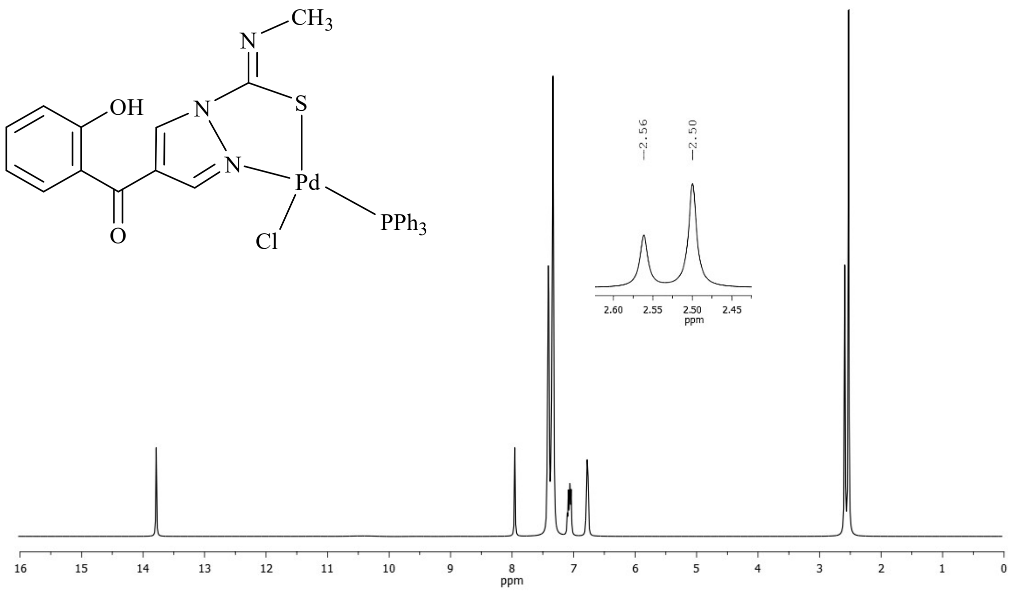


Fig. S4 [1]H-NMR spectrum of [Pd(4(2Hbmp-1-ca)(PPh_3)Cl] (**2**)

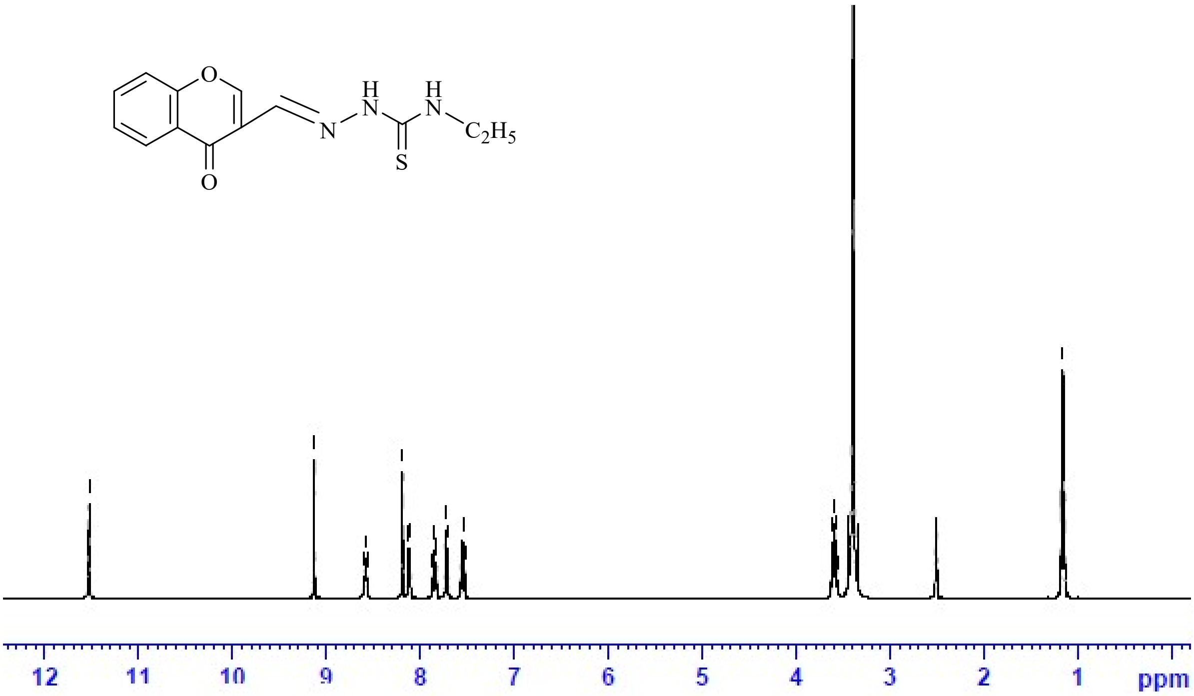


Fig. S5 [1]H-NMR spectrum of [H-Fcm-etsc] **(HL^3)**

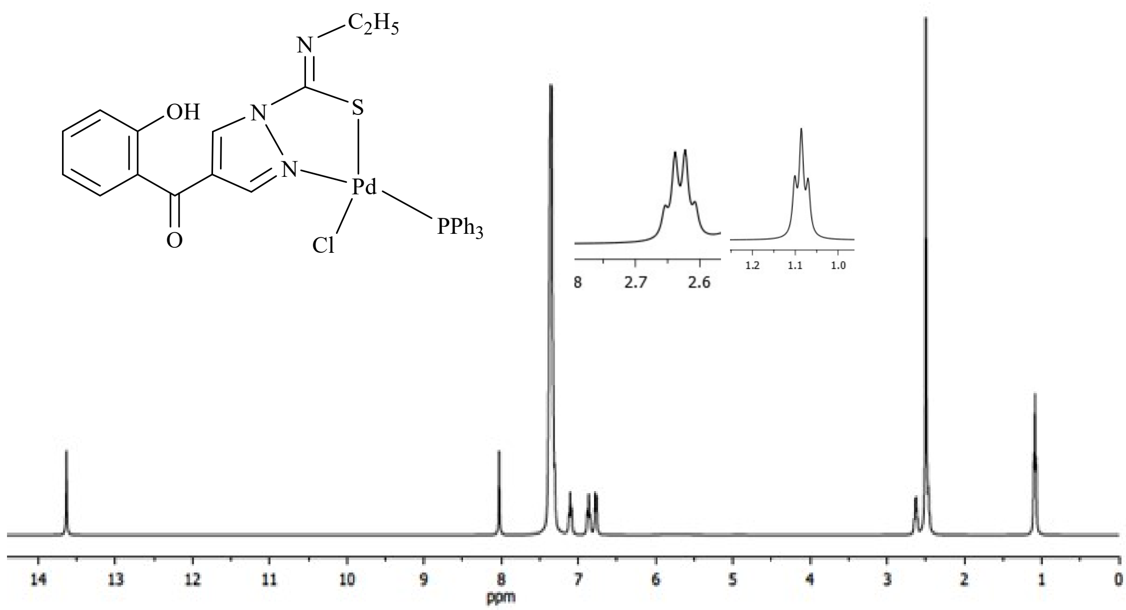


Fig. S6[1]H-NMR spectrum of Pd(4(2Hbep-1-ca)(PPh$_3$)Cl] (**3**)

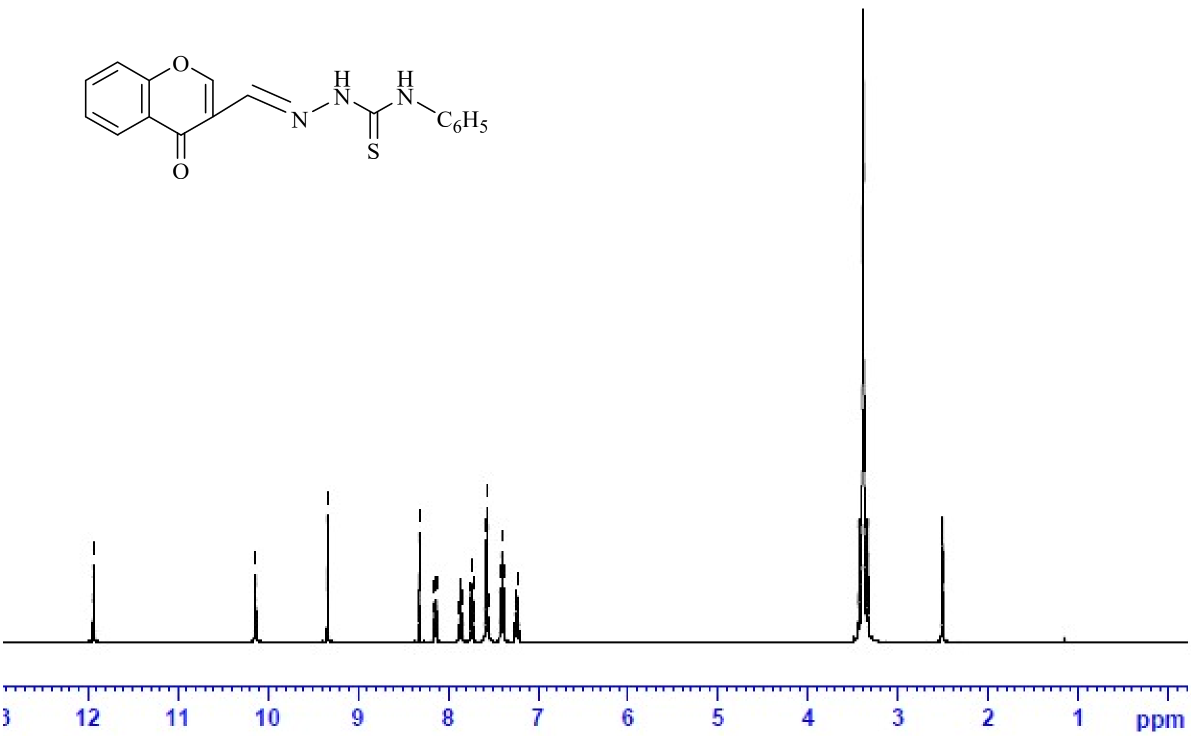


Fig. S7[1]H-NMR spectrum of [H-Fcm-ptsc] **(HL[4])**

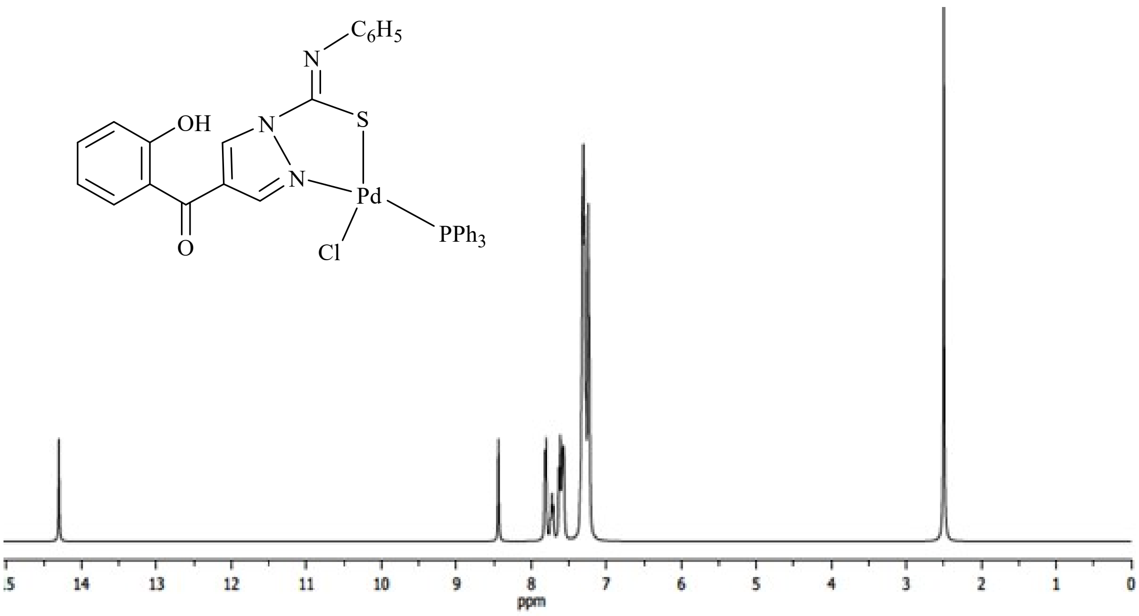


Fig. S8 ^{1}H-NMR spectrum of [Pd(4(2Hbpp-1-ca)(PPh_3)Cl] (**4**)

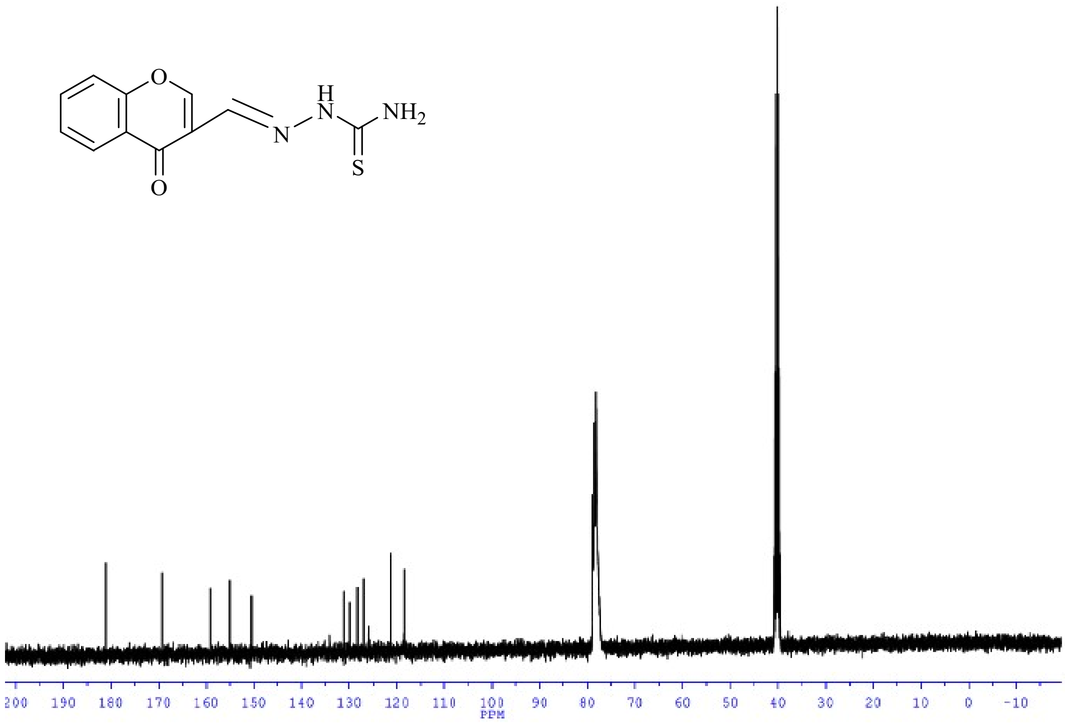


Fig.S9 ^{13}C-NMR spectrum of [H-Fcm-tsc] **(HL^{1})**

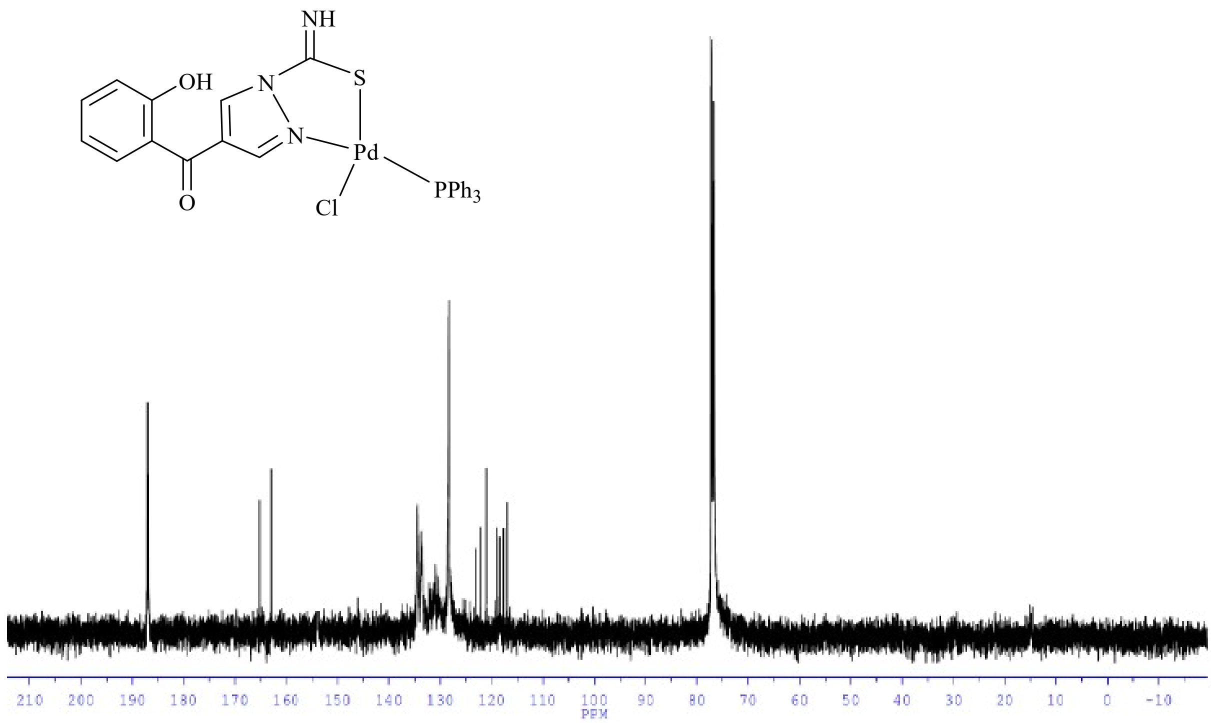


Fig. S10[13]C-NMR spectrum of [Pd(4(2Hbp-1-ca)(PPh_3)Cl] (**1**)

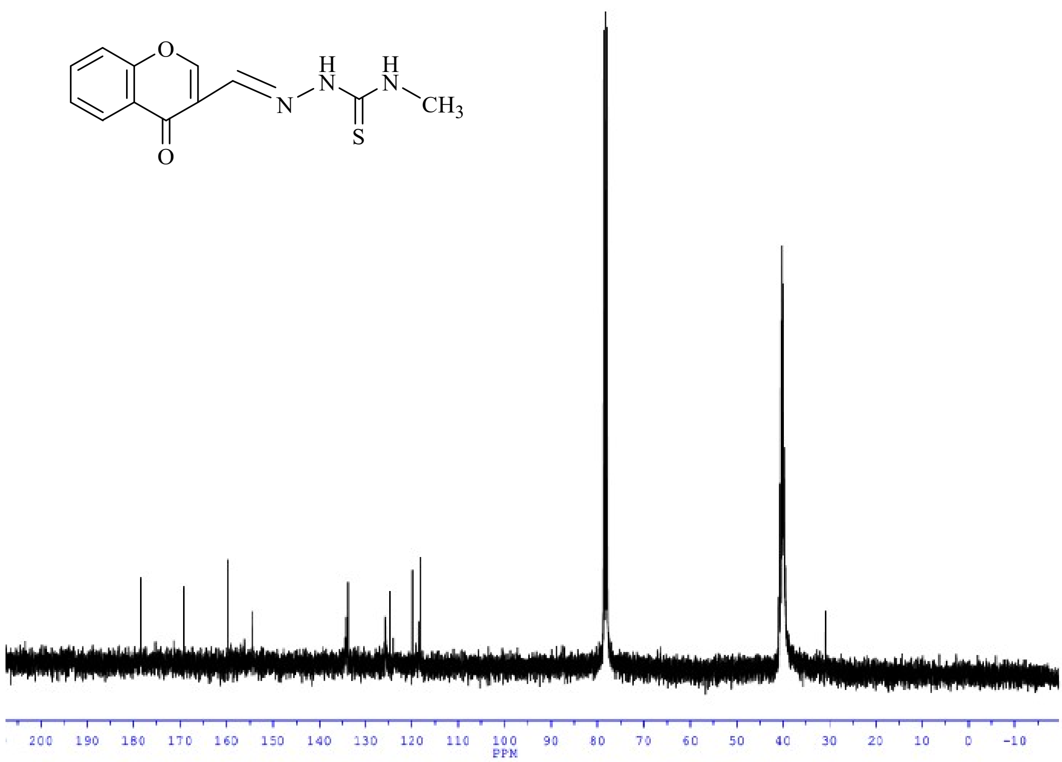


Fig.S11[13]C-NMR spectrum of [H-Fcm-mtsc] **(HL^2)**

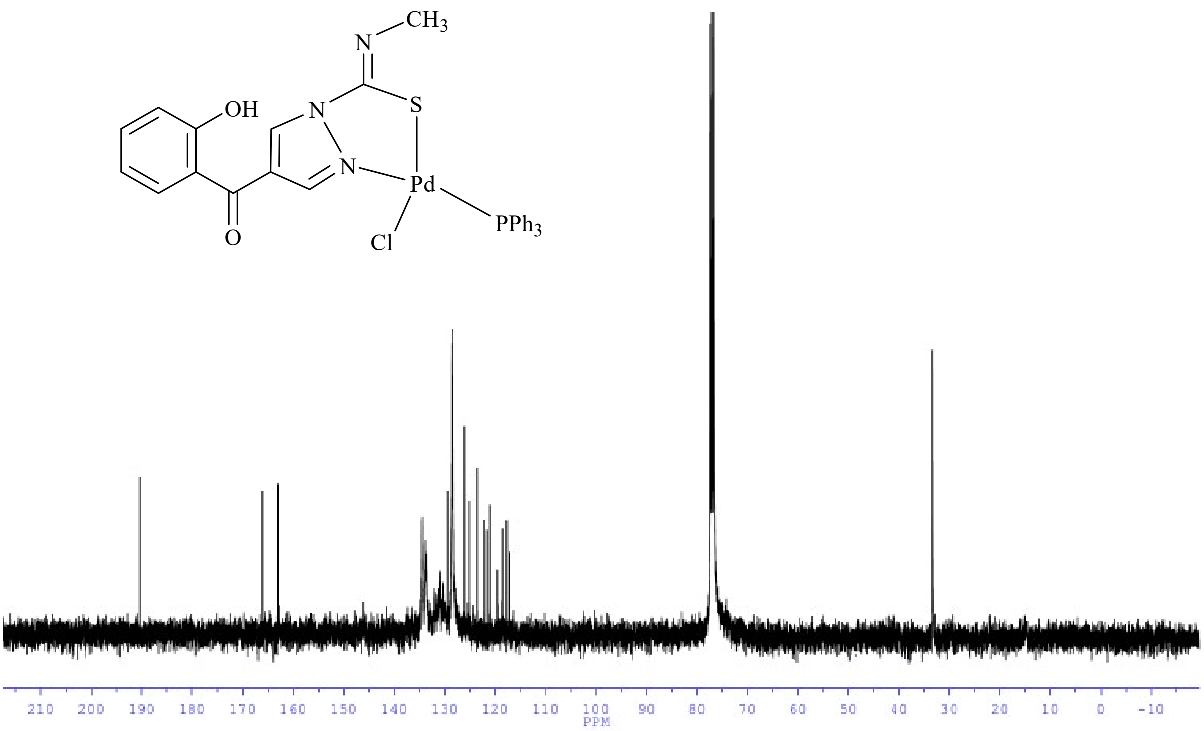


Fig. S12 [13]C-NMR spectrum of [Pd(4(2Hbmp-1-ca)(PPh_3)Cl] (**2**)

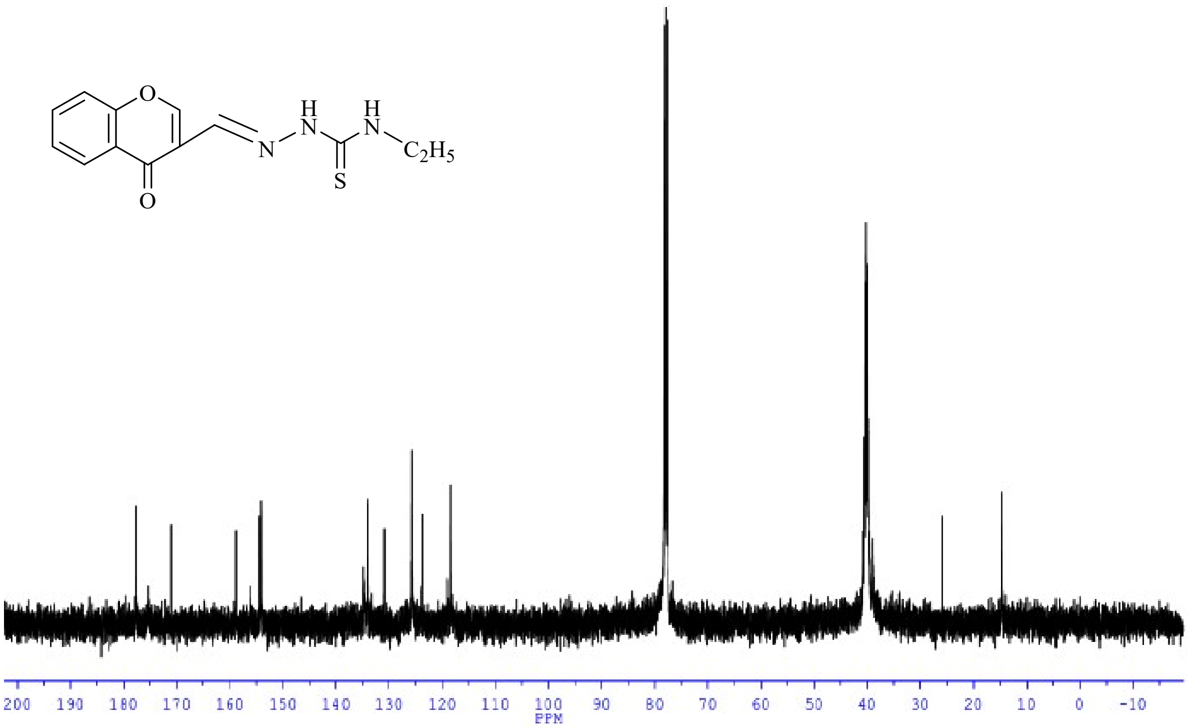
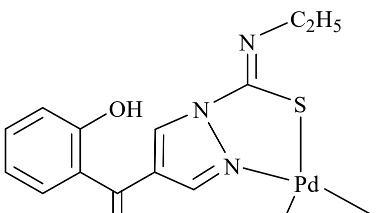


Fig.S13 [13]C-NMR spectrum of [H-Fcm-etsc] **(HL^3)**



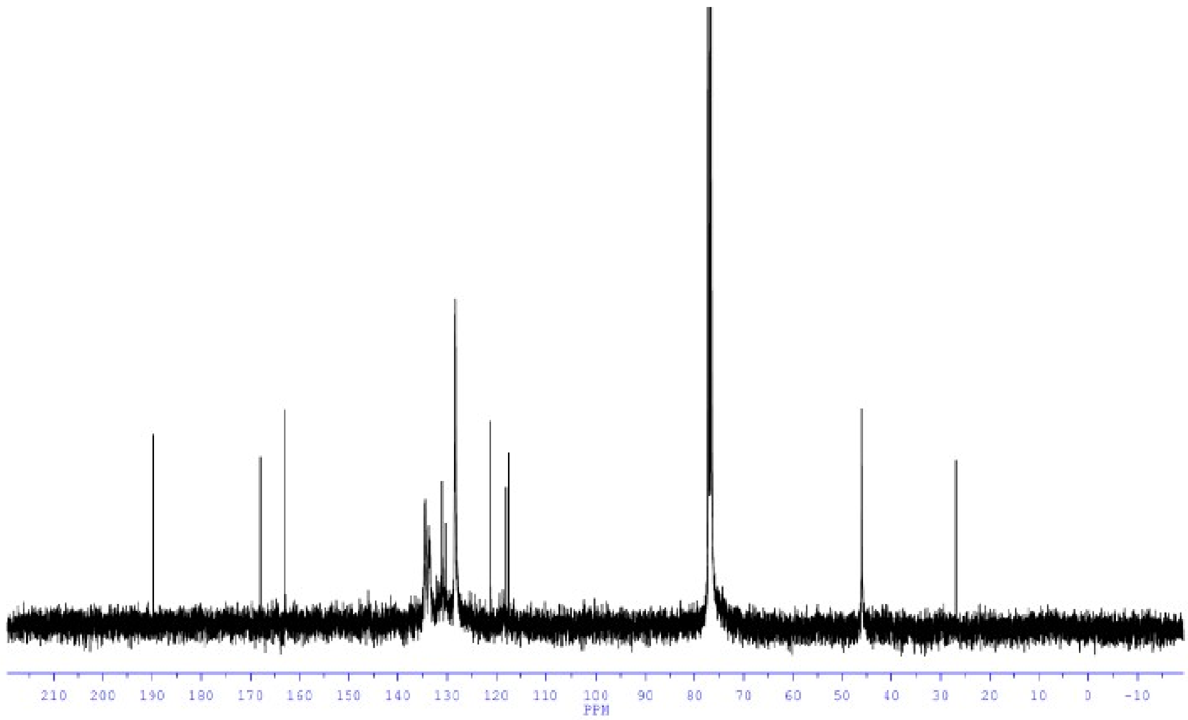


Fig. S14^{13}C-NMR spectrum of [Pd(4(2Hbep-1-ca)(PPh_3)Cl] (**3**)

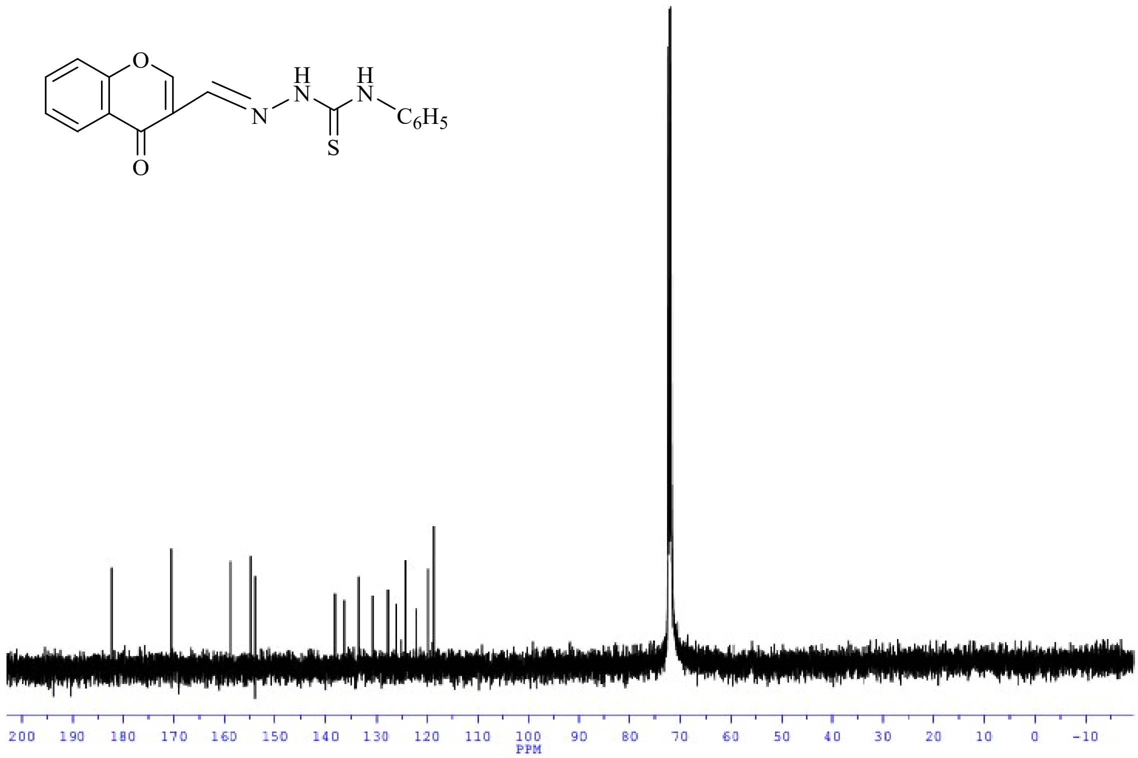


Fig.S15^{13}C-NMR spectrum of [H-Fcm-ptsc] **(HL^4)**

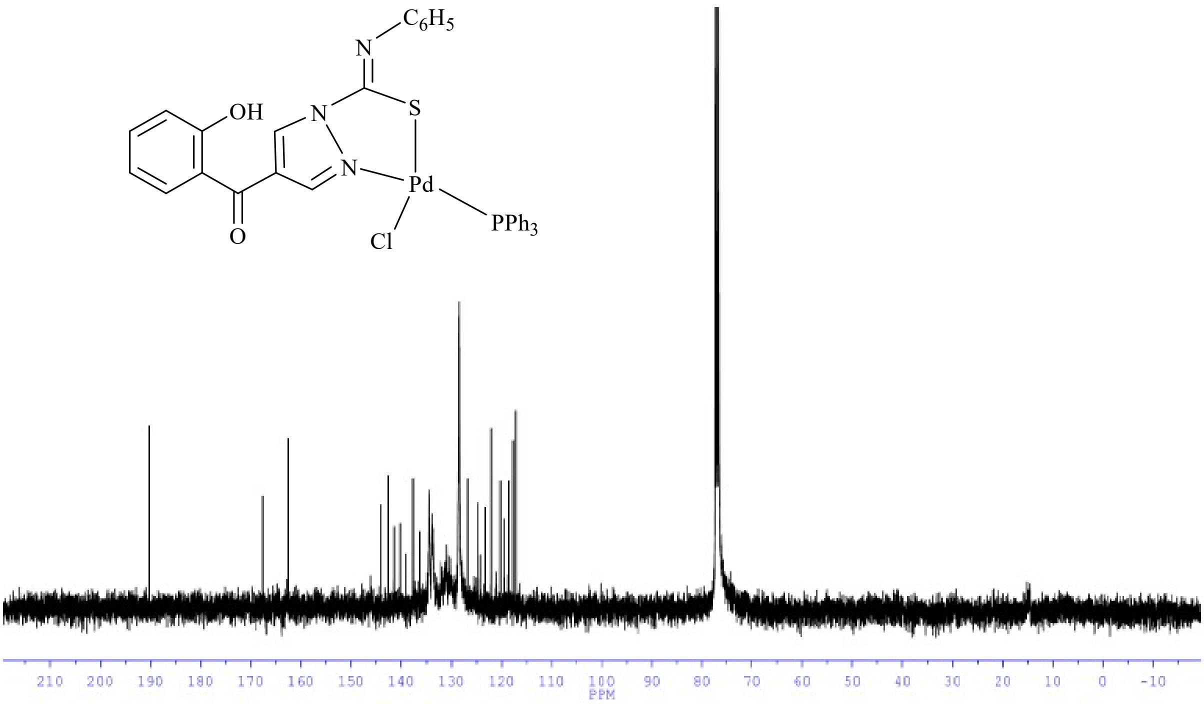


Fig. S16[13]C-NMR spectrum of [Pd(4(2Hbpp-1-ca)(PPh_3)Cl] (**4**)

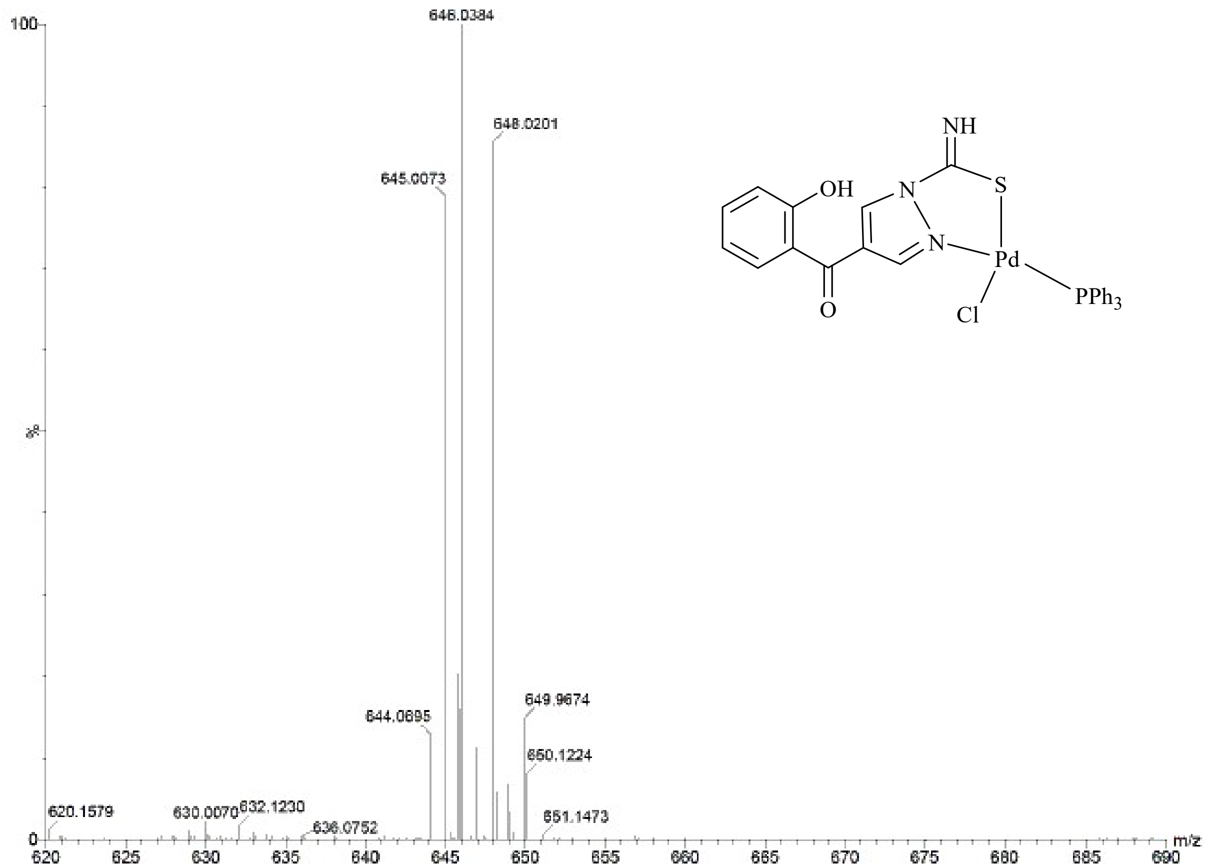


Fig. S17 ESI-MS spectrum of [Pd(4(2Hbp-1-ca)(PPh$_3$)Cl] (**1**)

Fig. S18 ESI-MS spectrum of [Pd(4(2Hbmp-1-ca)(PPh$_3$)Cl] (**2**)

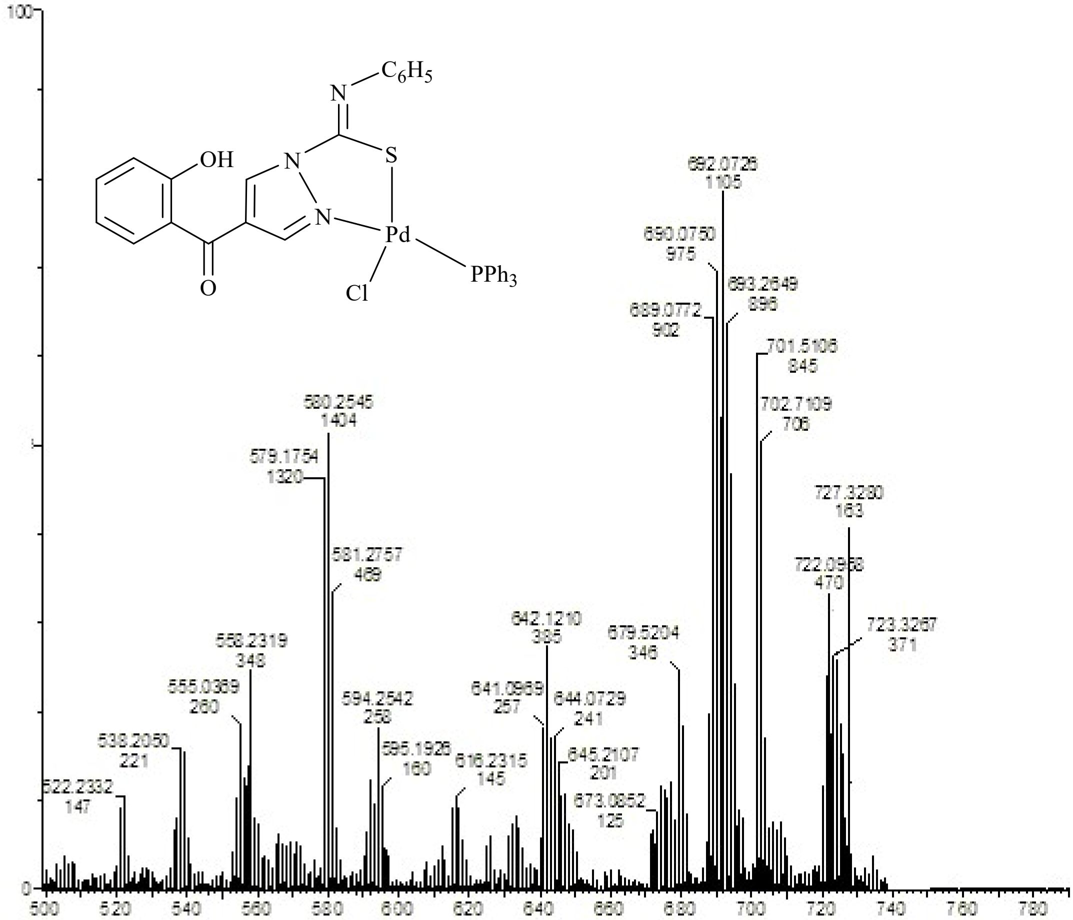
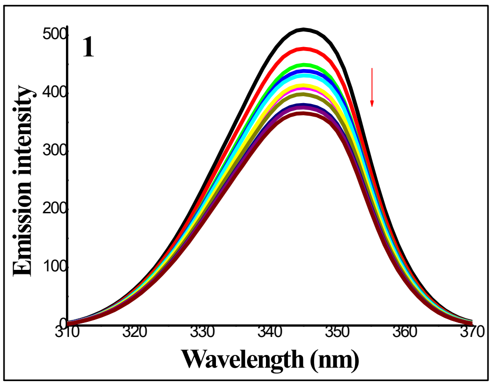
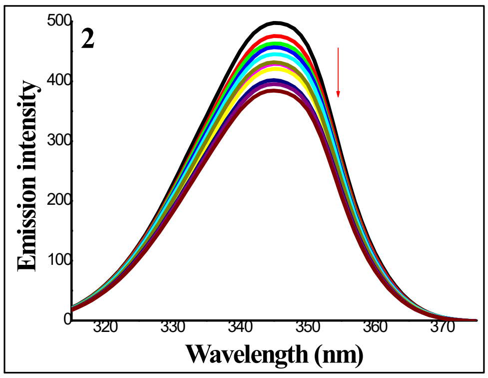


Fig. S19 ESI-MS spectrum of [Pd(4(2Hbpp-1-ca)(PPh$_3$)Cl] (**4**)





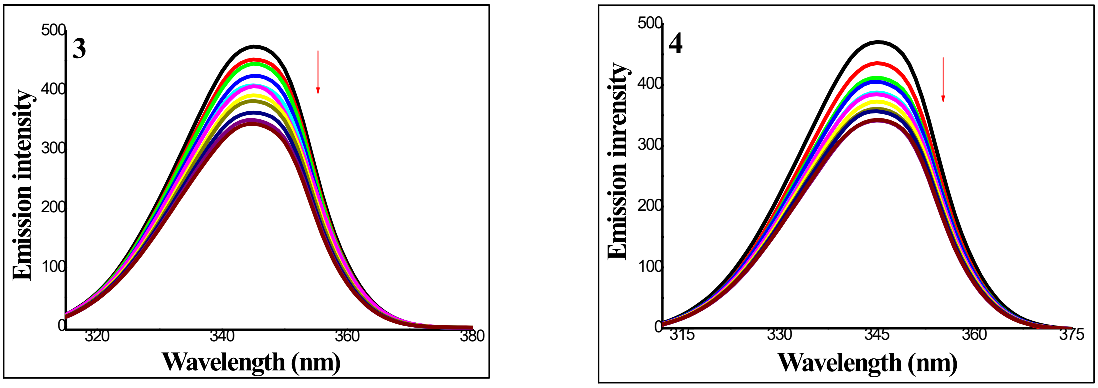


Fig. S20 Synchronous spectra of BSA (10 μM) in the absence and presence of complexes **1-4** (0-100 μM) in the wavelength difference of Δλ = 60 nm.