Supporting Information

Ruthenium(II) arene NSAID complexes : inhibition of cyclooxygenase and antiproliferative activity against cancer cell lines

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Contents

Experimental Procedure

All the chemicals required are purchased from Sigma and used without further purification. NMR spectra were recorded on an AVANCE III 400 Ascend Bruker BioSpin machine at ambient temperature. Mass spectrometric analyses were done on Bruker-Daltonics, microTOF-Q II mass spectrometer and elemental analyses were carried out with a ThermoFlash 2000 elemental analyzer. Spectrophotometric measurements were performed on a Varian UV-Vis spectrophotometer (Model: Cary 100) (for absorption) and a Fluoromax-4p spectrofluorometer from Horiba JobinYvon (Model: FM-100) (for emission) using a quartz cuvette with path length of 1 cm. The sulforhodamine B (SRB) growth inhibition (GI_{50}) assays were carried out by Advanced Center for Treatment, Research and Education in Cancer (ACTREC), Mumbai by following the literature procedure.[1] COX and LOX inhibition study has been carried out according to the given protocol.[2,3]

EXPERIMENTAL PROCEDURE for SRB Assay

The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For present screening experiment, cells were inoculated into 96 well microtiter plates in 100 µL at plating densities as shown in the study details above, depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were

incubated at 37° C, 5 % CO_2, 95 % air and 100 % relative humidity for 24 h prior to addition of experimental drugs.

Samples were initially solubilized in dimethyl sulfoxide at 100mg/ml and diluted to 1mg/ml using water and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate (1mg/ml) was thawed and diluted to 100 µg/ml, 200 µg/ml, 400 µg/ml and 800 µg/ml with complete medium containing test article. Aliquots of 10 µl of these different sample dilutions were added to the appropriate microtiter wells already containing 90 µl of medium, resulting in the required final drug concentrations i.e.10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml.

After compound addition, plates were incubated at standard conditions for 48 hours and assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 µl of cold 30 % (w/v) TCA (final concentration, 10 % TCA) and incubated for 60 minutes at 4°C. The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (50 µl) at 0.4 % (w/v) in 1 % acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1 % acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm reference wavelength.

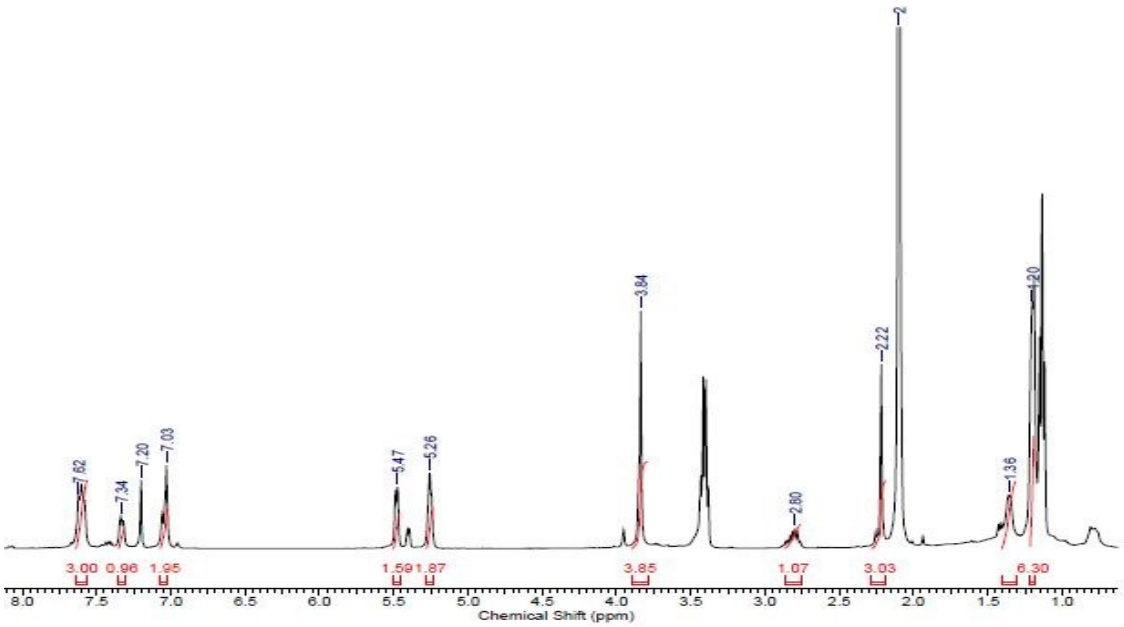


Figure S1: ^{1}H NMR of Compound 1

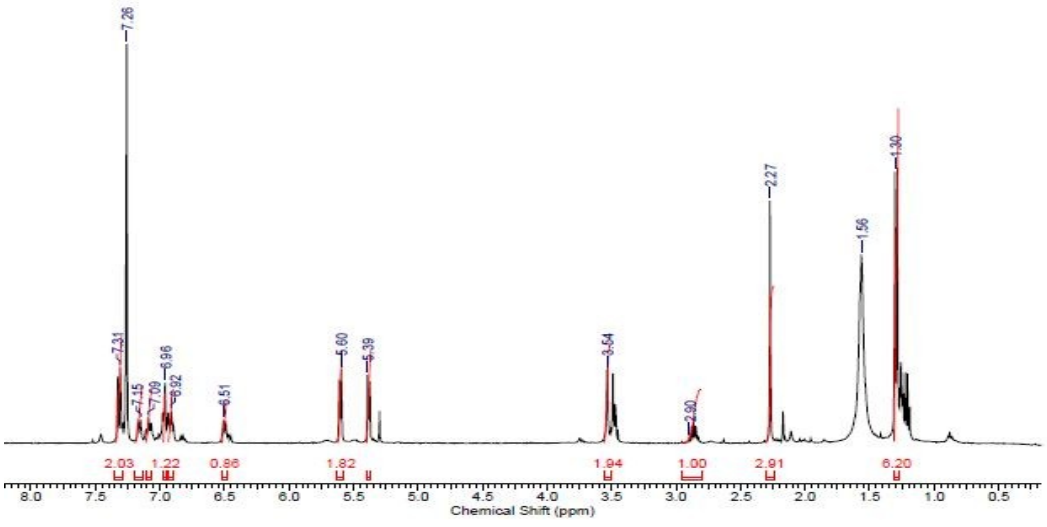


Figure S2: ^{1}H NMR of Compound 2

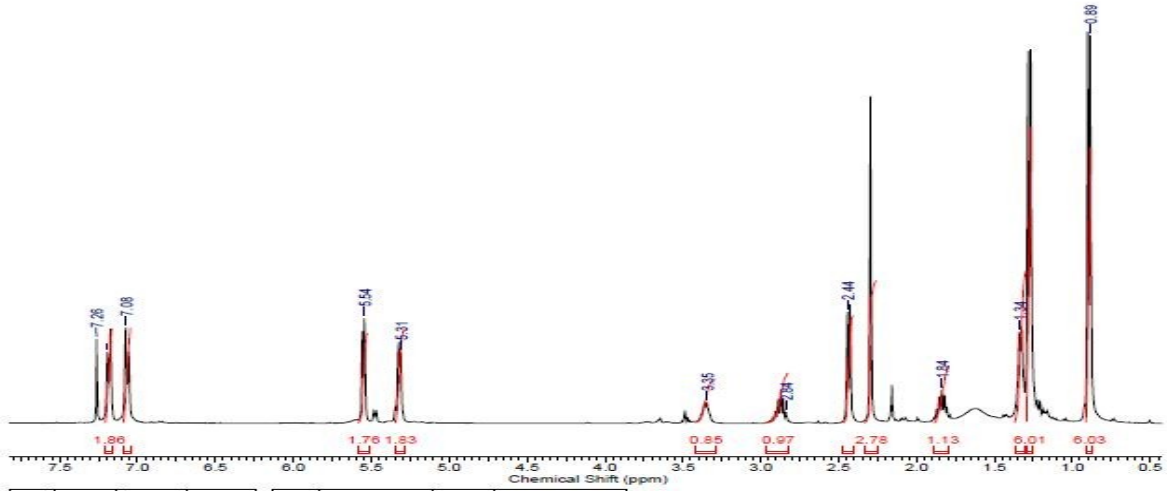


Figure S3: ^{1}H NMR of Compound 3

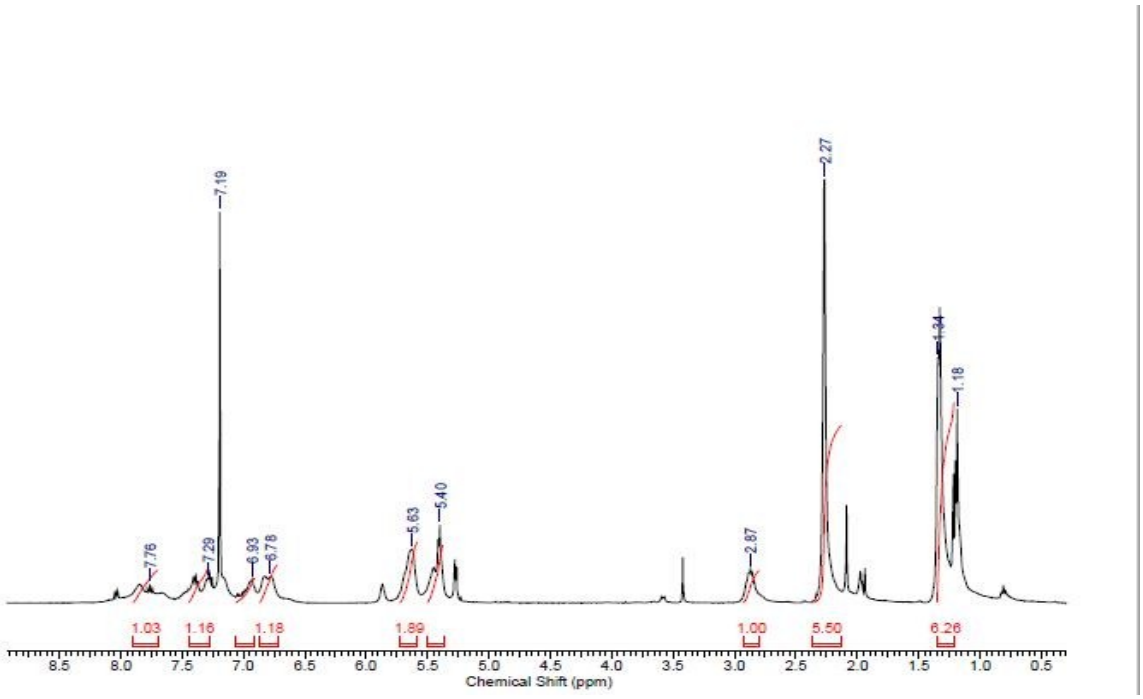


Figure S4: ^{1}H NMR of Compound 4

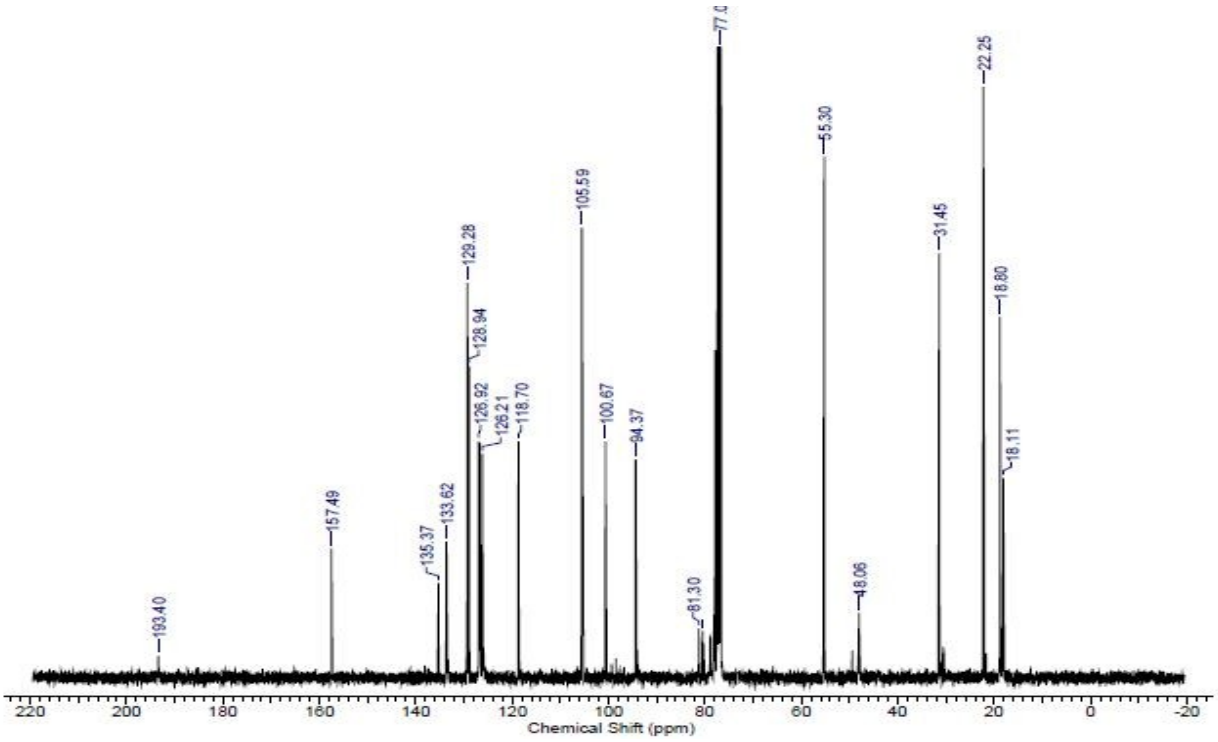


Figure S5: ^{13}C NMR of Compound 1

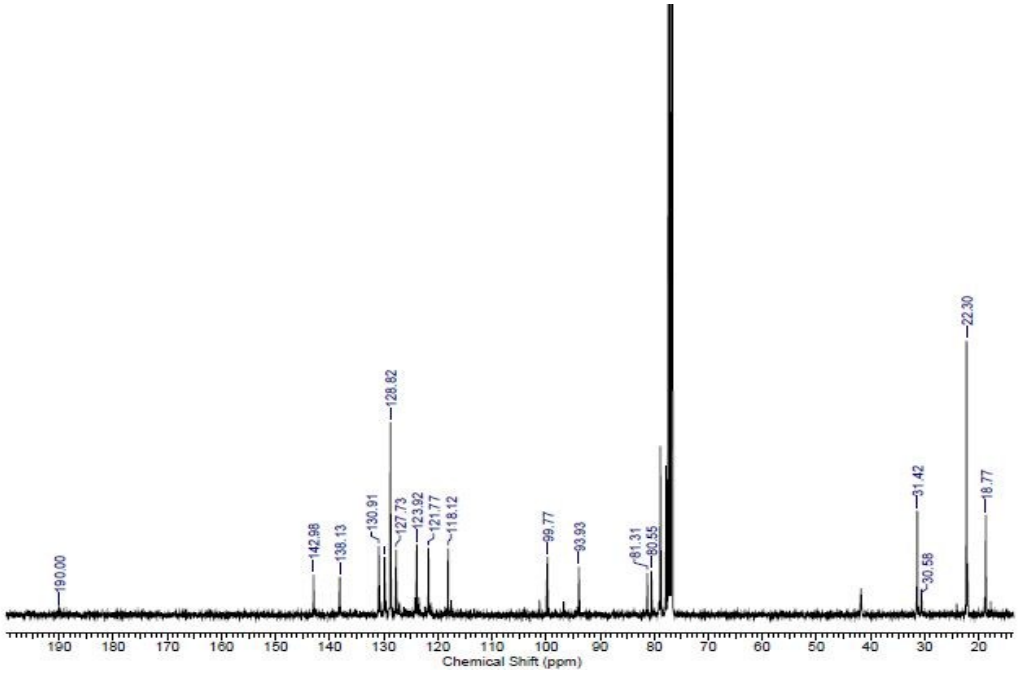


Figure S6: ^{13}C NMR of Compound 2

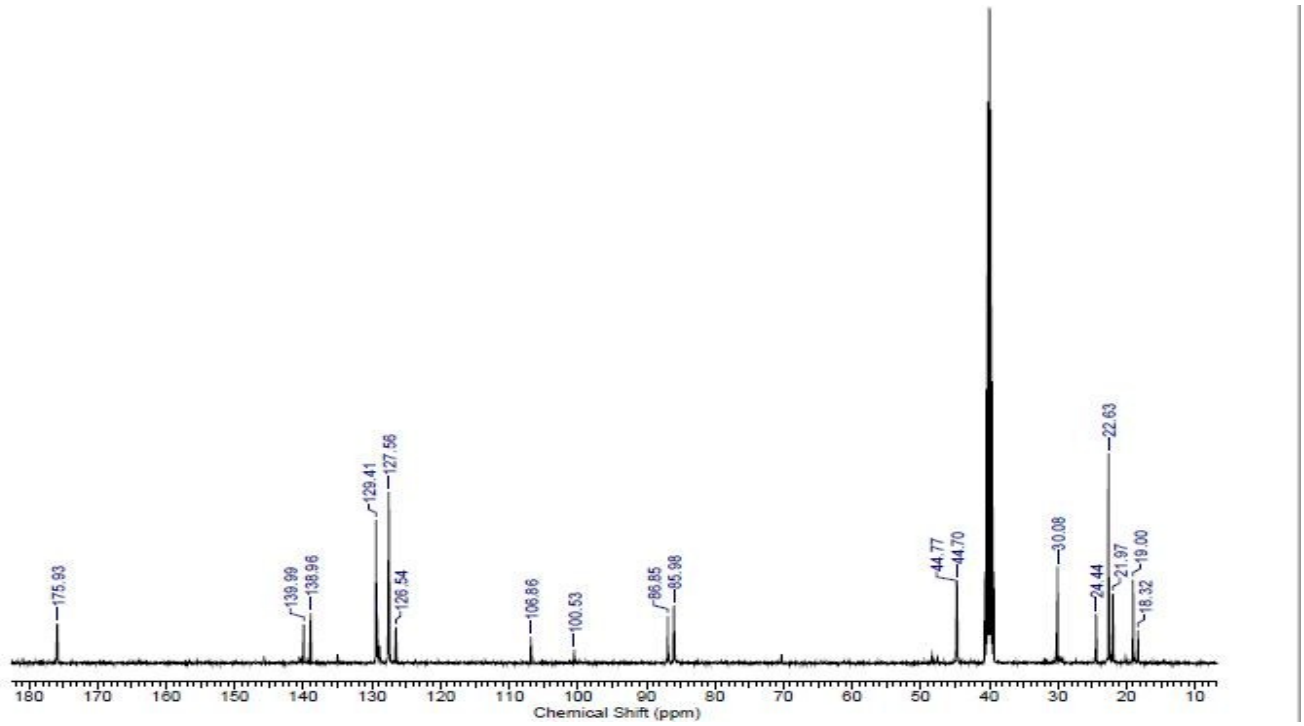


Figure S7: ^{13}C NMR of Compound 3

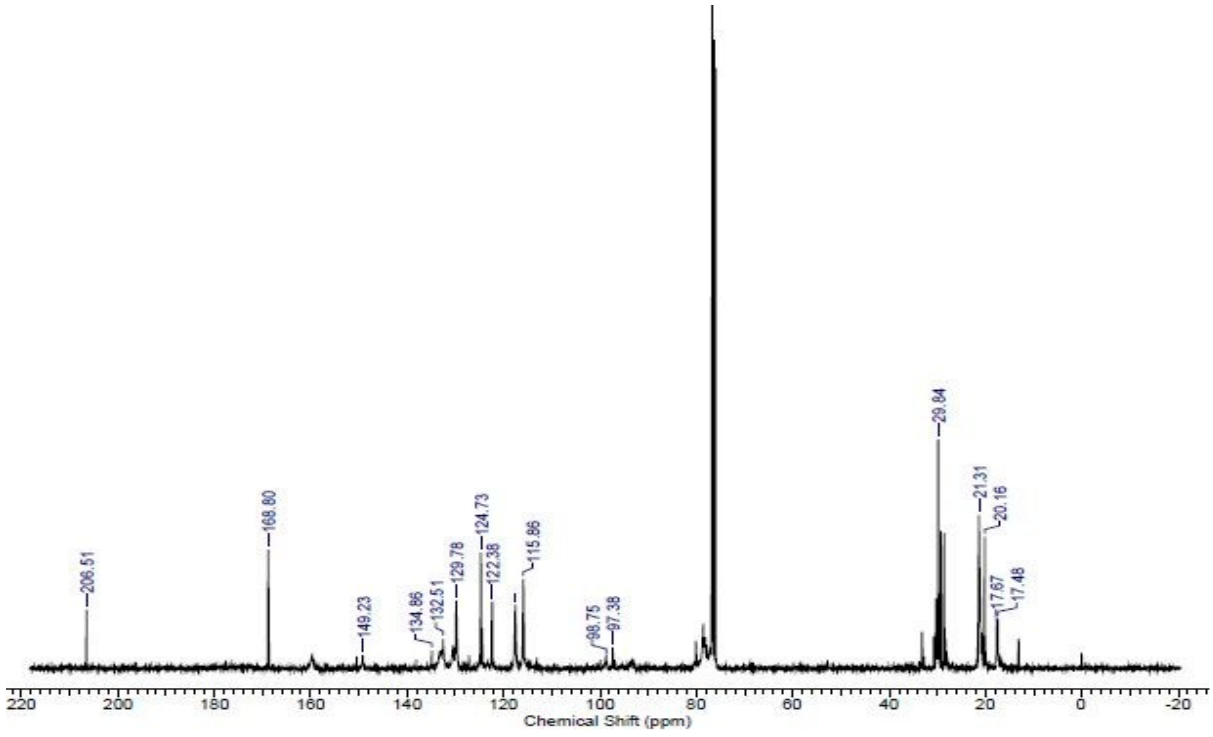


Figure S8: ^{13}C NMR of Compound 4

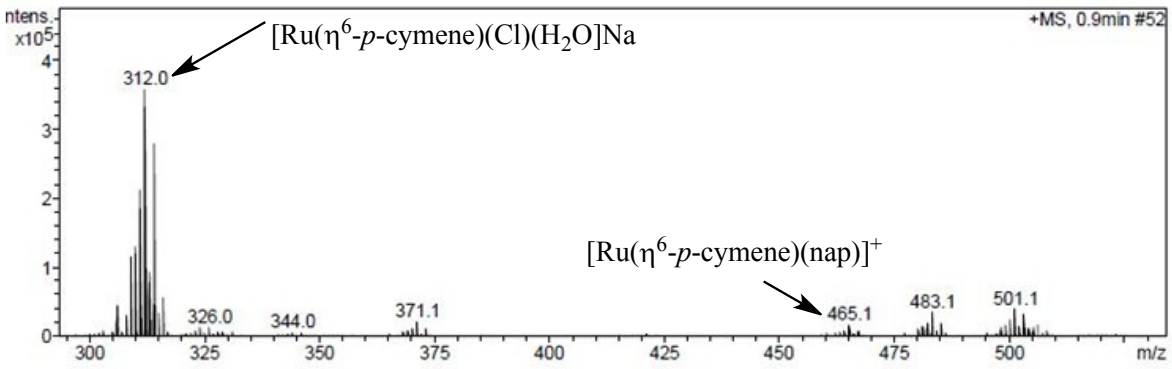


Figure S9: ESI-MS of Complex 1

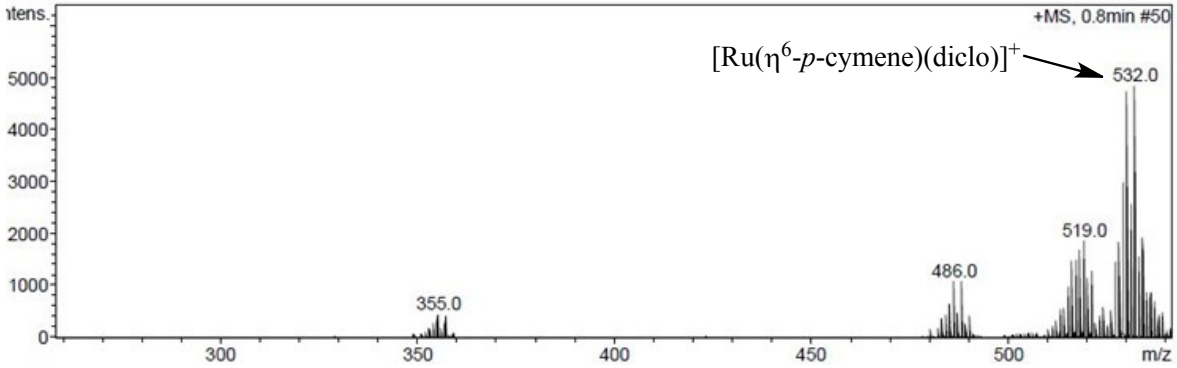


Figure S10: ESI-MS of Complex 2

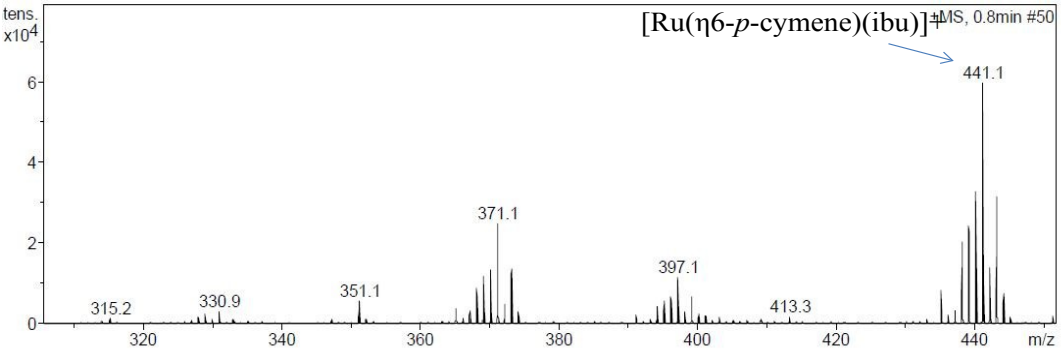


Figure S11: ESI-MS of Complex 3

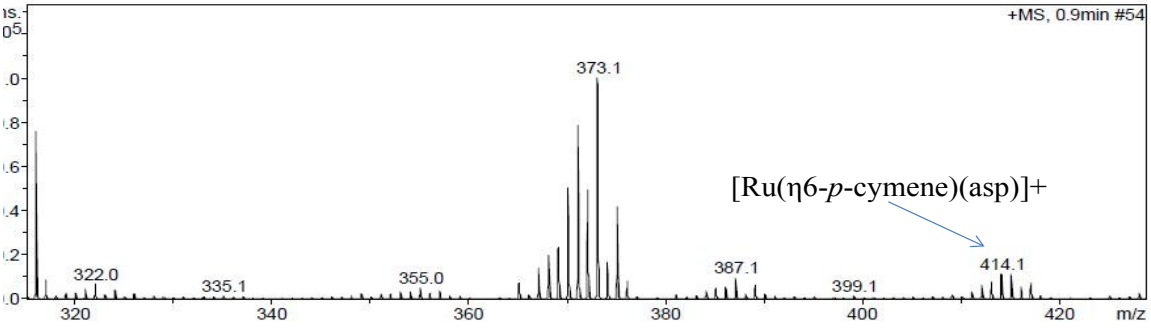


Figure S12: ESI-MS of Complex 4

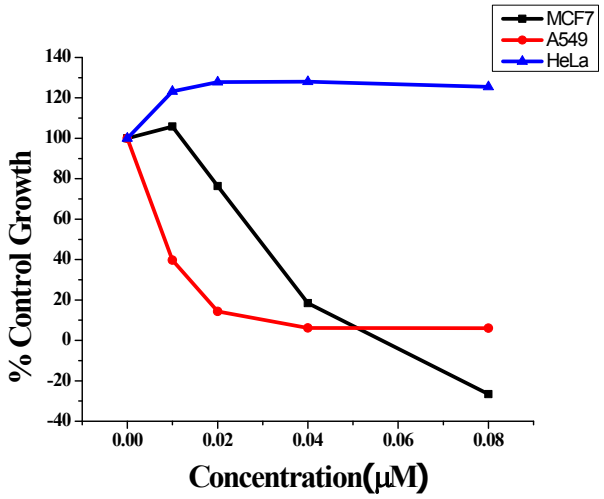


Figure S13: Analysis of the GI_{50} value of compound 1 against different cancer cell Lines

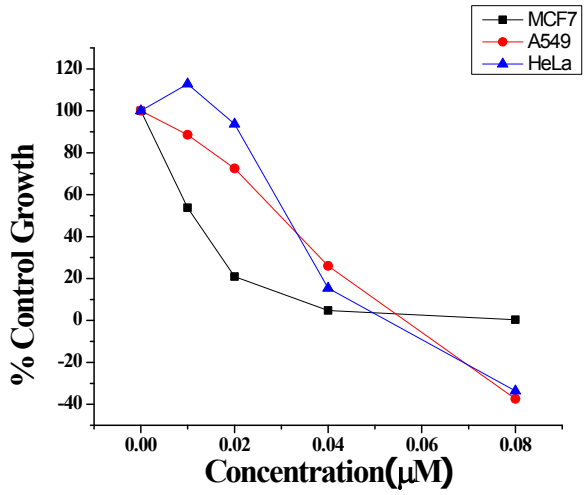


Figure S14: Analysis of the GI_{50} value of compound 3 against different cancer cell Lines

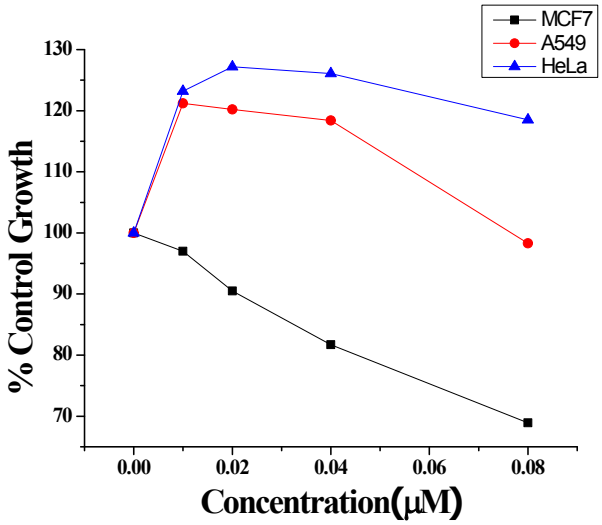


Figure S15: Analysis of the GI_{50} value of compound 4 against different cancer cell Lines

Stability studies in DMSO

In order to check the stability of the prepared compounds in solution state, 1-2 mg of the sample was dissolved in DMSO-d_6 and its NMR was recorded after 0.5, 3, 24 and 48 h. To check the stability of the prepared compounds by ESI-MS, the compounds were dissolved DMSO-MeOH mixture and ESI-MS was recorded after 15 mins.

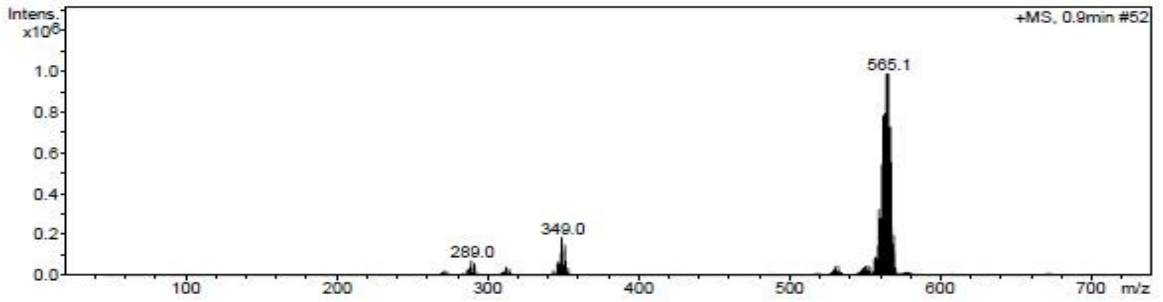


Figure S16: ESI-MS of Compound 2 in DMSO-MeOH.

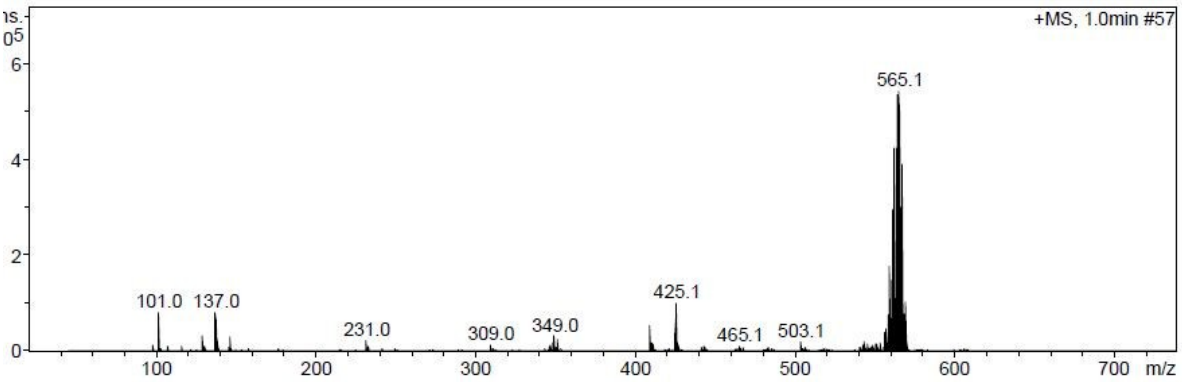


Figure S17: ESI-MS of Compound 4 in DMSO-MeOH.

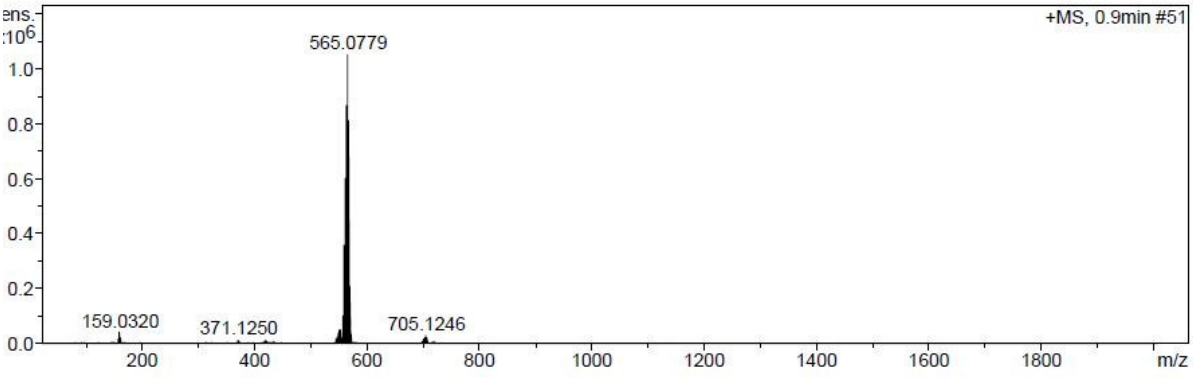


Figure S18: ESI-MS of Compound 4 in DMSO-MeOH.

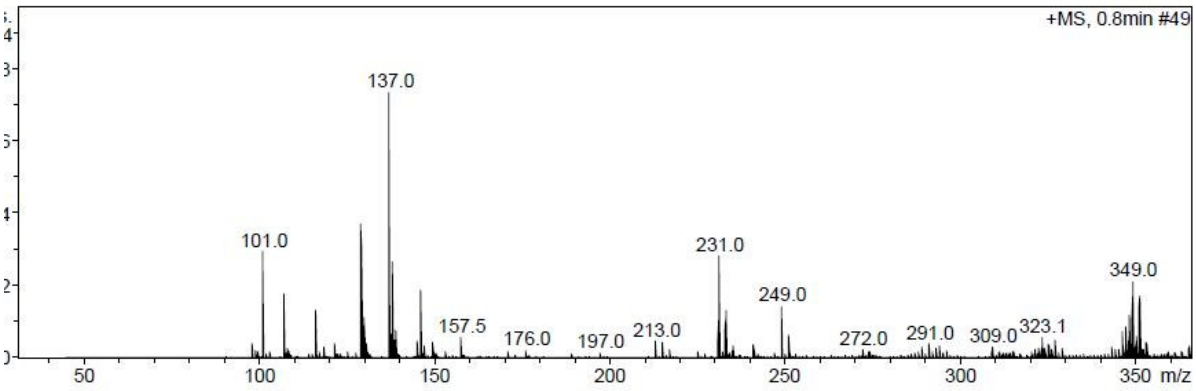


Figure S19.1: ESI-MS of Compound 1 in 1% DMSO-media

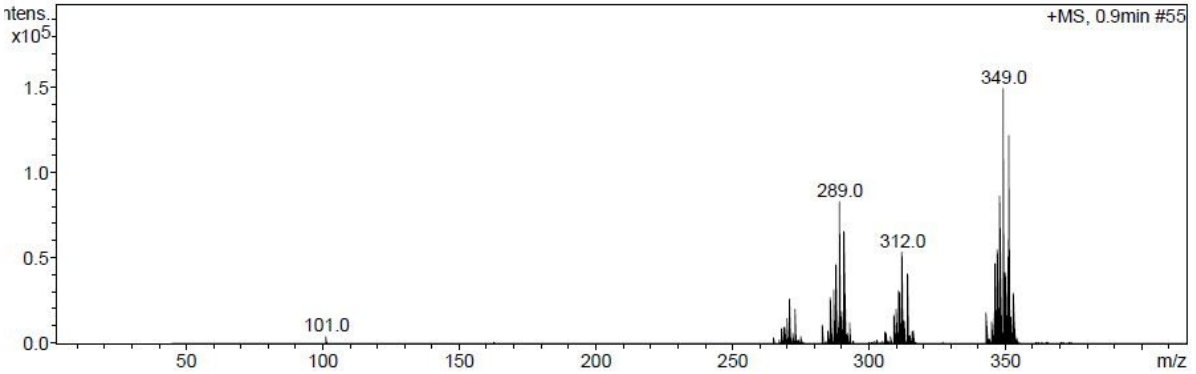


Figure S19.2: ESI-MS of Compound 2 in 1% DMSO-media

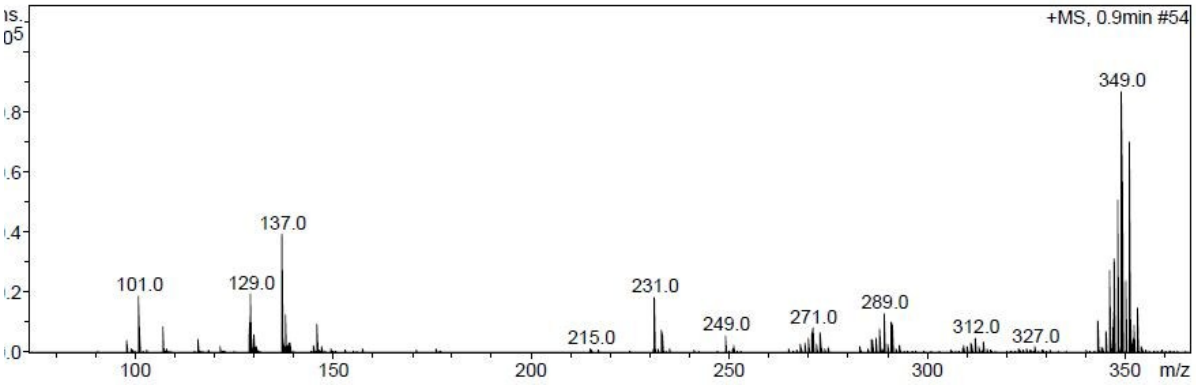


Figure S19.3: ESI-MS of Compound 3 in 1% DMSO-media

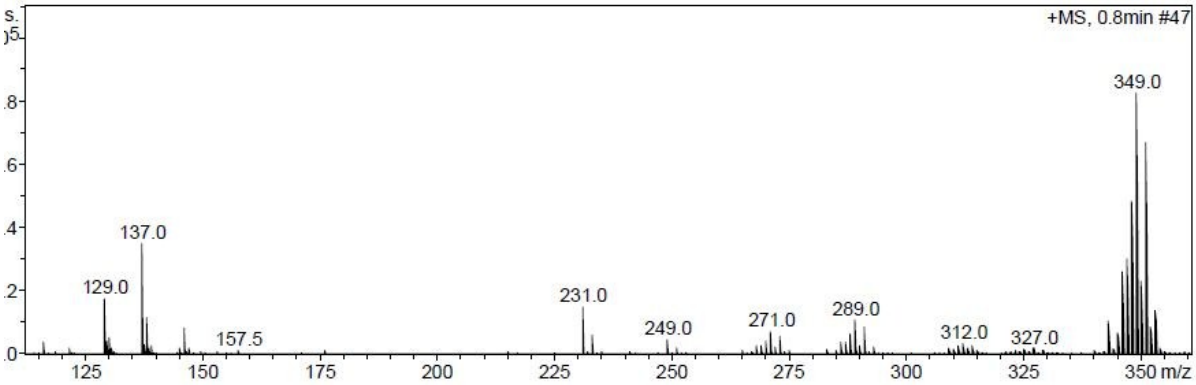


Figure S19.4: ESI-MS of Compound 4 in DMSO-MeOH.

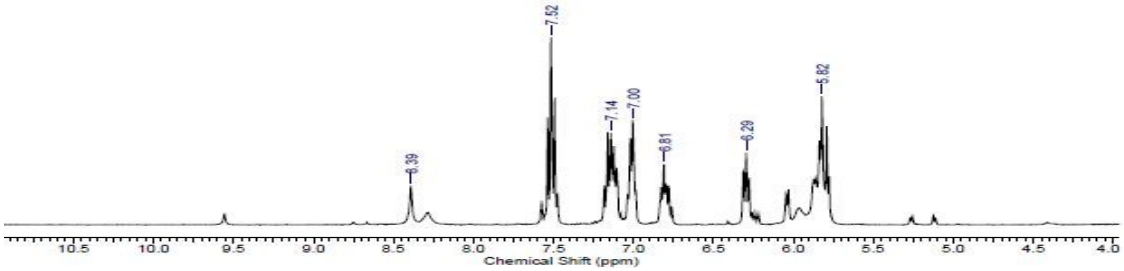


Figure S20: ^{1}H NMR of Compound 2 in DMSO-d_6.

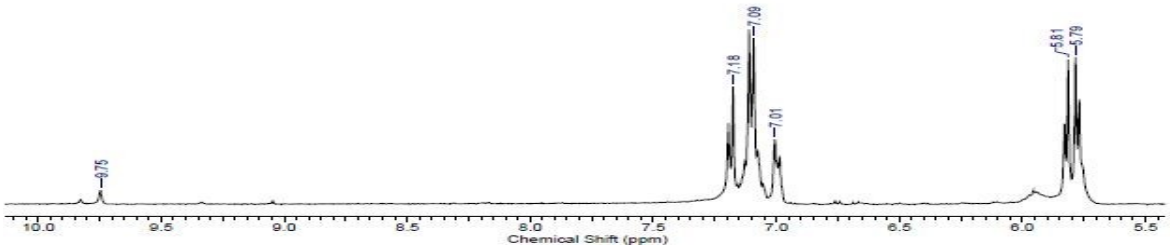


Figure S21.1 : ^{1}H NMR of Compound 3 in DMSO-d_6

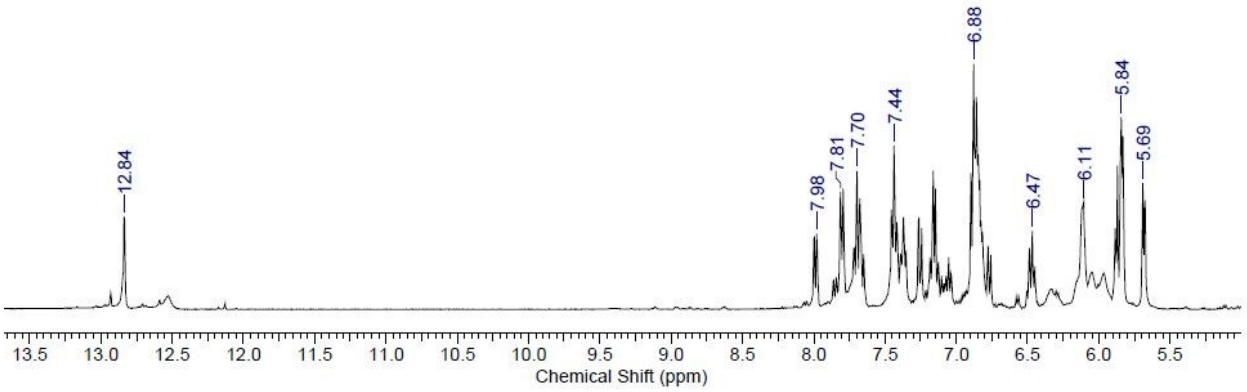


Figure S21.2 : ^{1}H NMR of Compound 4 in DMSO-d_6

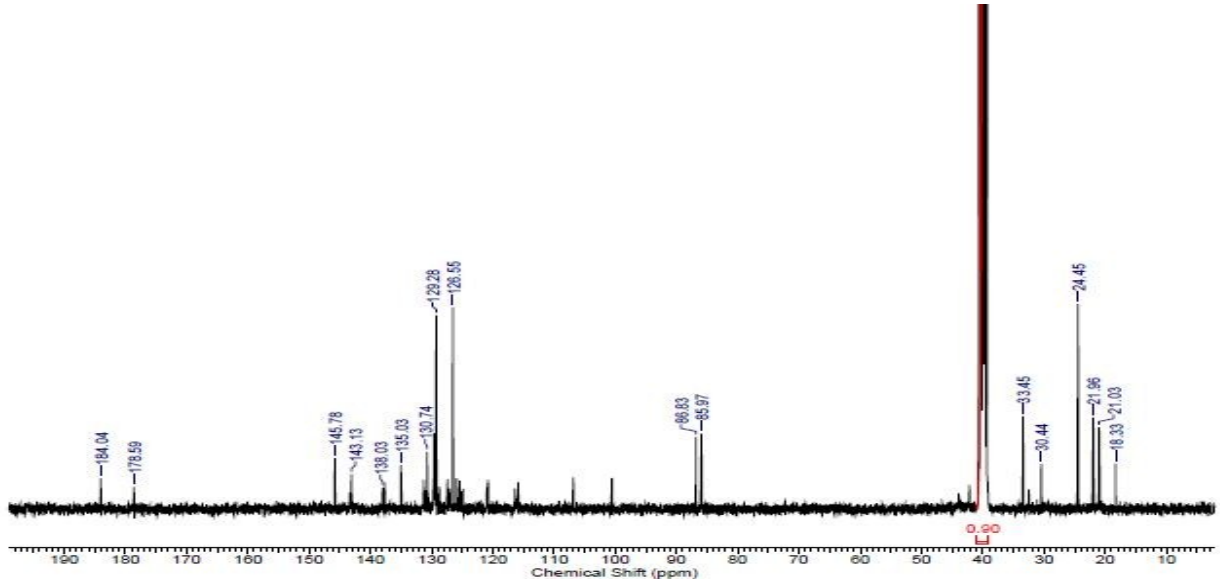


Figure S22: ^{13}C NMR of Compound 2 in DMSO-d_6

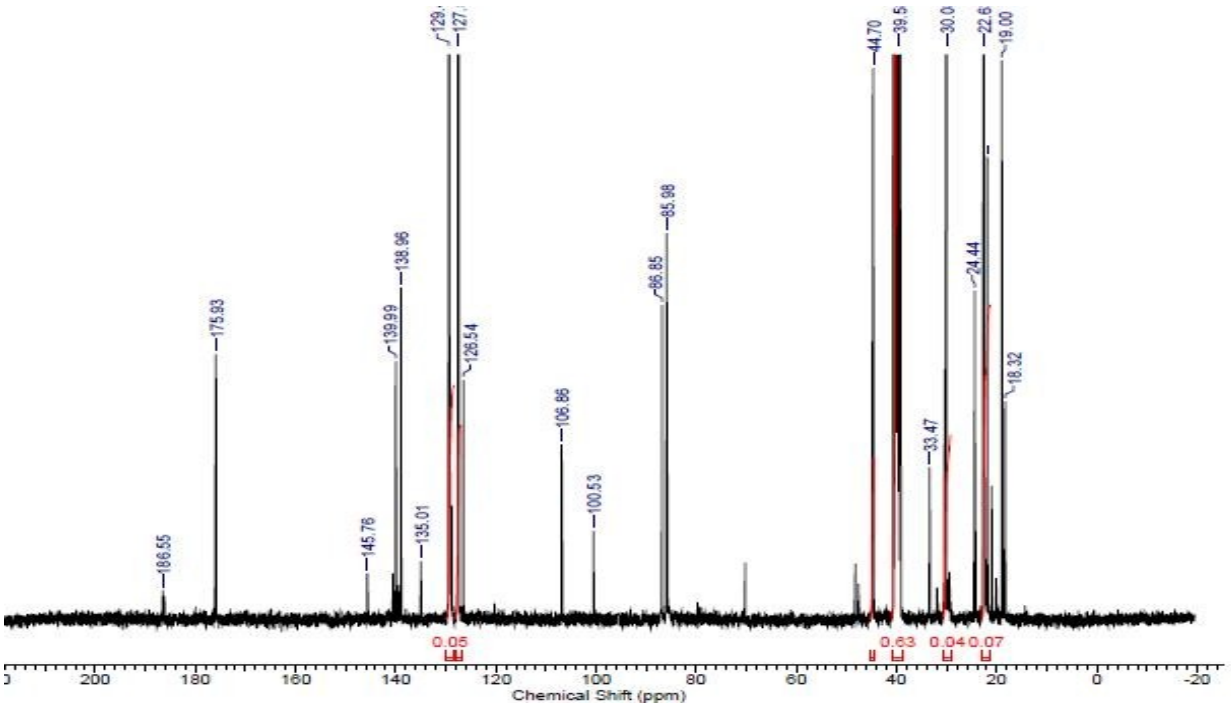


Figure S23: ^{13}C NMR of Compound 3 in DMSO-d_6

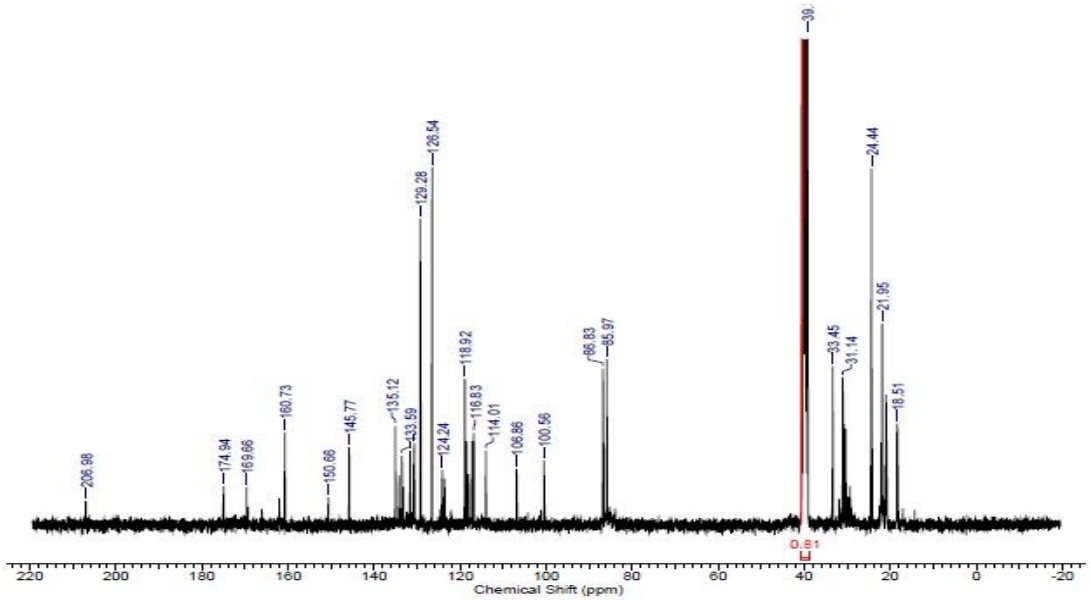


Figure S24: ^{13}C NMR of Compound 4 in DMSO-d_6

Assay of Cyclooxygenase (COX)

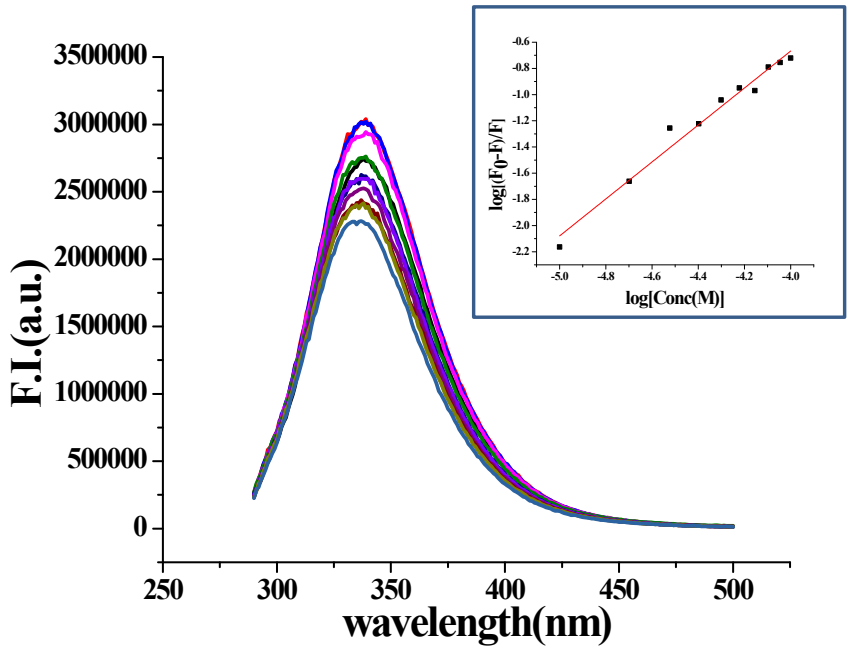
The assay mixture for COX contained Tris – HCl buffer, glutathione, hemoglobin and enzyme. The reaction was started by the addition of arachidonic acid. Then the reaction was stopped after 1 min incubation at 37˚C by addition of 0.2 mL of 10 % TCA in 1 N HCl, mixed and 0.2 mL of TBA was added. The contents were heated in a boiling water bath for 20 min, cooled and centrifuged at 1,000 rpm for 3 min. The supernatant was measured at 532 nm for COX activity. The experiments were carried out in triplicate manner for all the samples and graph was plotted taking the average of the three observations.

Assay of Lipoxygenase (LOX)

The assay mixture for 15-lipoxygenase contained 2.75 mL of phosphate buffer pH 6.5, 0.2 mL of sodium linoleate and 50 µl of enzyme. The increase in OD was measured at 280 nm. The experiments were carried out in triplicate manner for all the samples and graph was plotted taking the average of the three observations.

DNA binding experiments

DNA binding studies of the prepared compounds were carried out using ct-DNA by fluorescence spectroscopy. A stock solution of ct-DNA was prepared in tris-HCl buffer and Ethedium bromide was added to it . First, fluorescence spectra of DNA bound EB were obtained at the excitation and emission wavelengths of 515 nm and 602 nm respectively. The titration quenching experiment was carried out by keeping the concentration of DNA in buffer constant and adding 10 µL of the sample solution at a time. After addition of the sample solution, the solution was kept for 5 min and then fluorescence intensity was measured. The quenching efficiency was calculated from Stern-Volmer eq.



S25: Fluorescence quenching of ct-DNA by compound 3

Protein binding experiments

Protein binding studies of the synthesised compounds were performed by tryptophan fluorescence quenching experiments using Bovine serum albumin (BSA).The excitation wavelength for BSA was at 280 nm and the quenching of the emission intensity of the

tryptophan residues of BSA at 345 nm was monitored using the complexes as quencher with increased concentration. The excitation and emission slit widths and scan rates were kept constant throughout the experiment. A 10 μM stock solution of HSA was prepared using 50 mM tris buffer solution and stored at 4°C for further use. Stock solutions of 5 mM strength were made using synthesized compounds. Fluorometric titration was carried out taking 2 mL of the protein solution and fluorescence intensity was measured as blank. For titration, each time, 10 μL of the stock solution was added to the protein solution and fluorescence intensity was measured. For all the four complexes, up to 100μL of the solution was added to measure fluorescence quenching. The fluorescence quenching data was further analyzed by the Stern–Volmer equation, which again can be expressed in terms of bimolecular quenching rate constant and average life time of the fluorophore as shown in following equation.

$$\frac{F_0}{F} = 1 + Kq\tau_0[Q] = 1 + Ksv[Q]$$

where F_0 and F are the fluorescence intensities in the absence and the presence of a quencher, k_q is the bimolecular quenching rate constant, τ_0 is the average lifetime of fluorophore in the absence of a quencher and [Q] is the concentration of a quencher (metal complexes). K_{SV} is the Stern–Volmer quenching constant in M^{-1}.

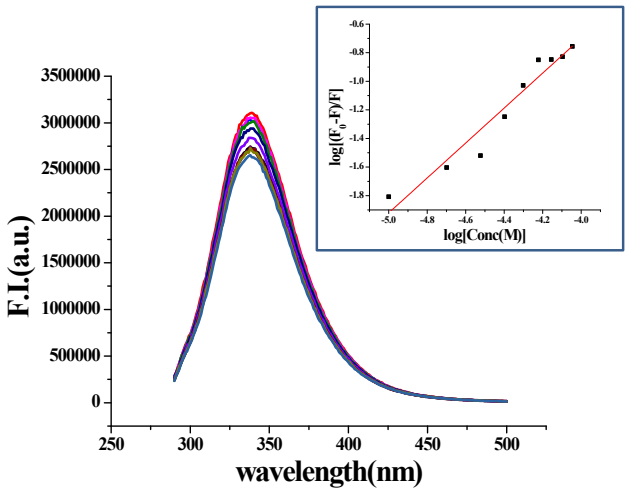


Figure S26: Fluorescence Quenching of BSA by Compound 1

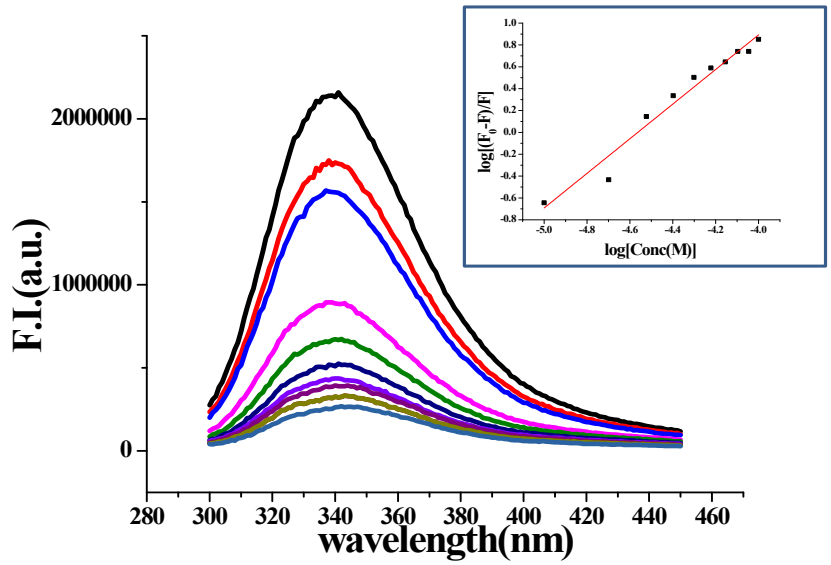


Figure S27: Fluorescence Quenching of BSA by Compound 2

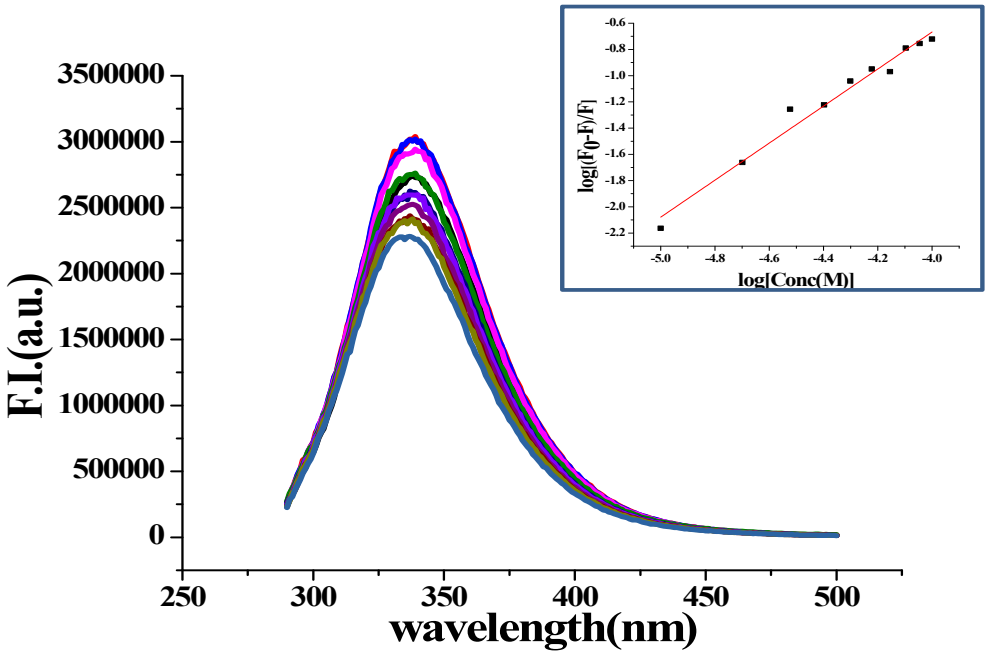


Figure S28: Fluorescence Quenching of BSA by Compound 3

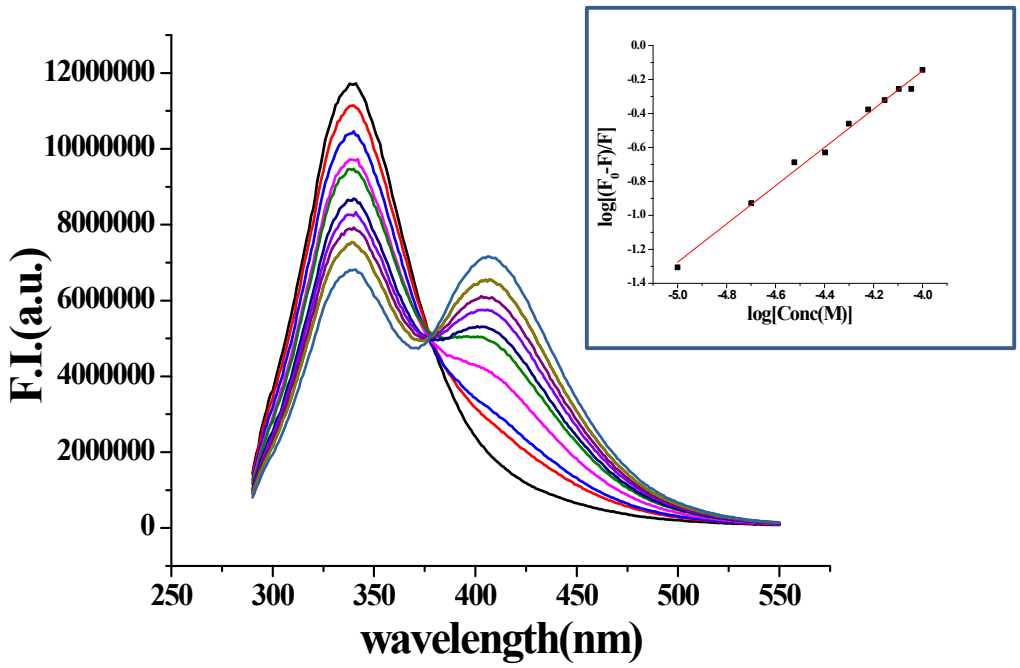


Figure S29: Fluorescence Quenching of BSA by Compound 4

Interaction with Amino acids

Reactivity of the prepared compounds with amino acids was examined using NMR spectroscopy. 2 mg of the compound was mixed with equimolar amount of different amino acids viz. His, Met, Cys .The mixture was dissolved in 1:1 DMSO-d_6/D_2O and NMR spectra was recorded after 0.5, 24, 48 h.

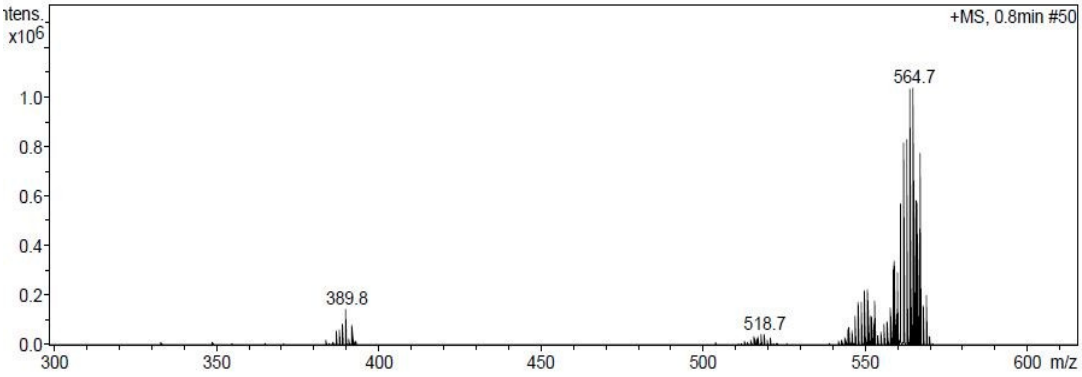


Figure S30.1: ESI-MS of Histidine Adduct by Compound 1

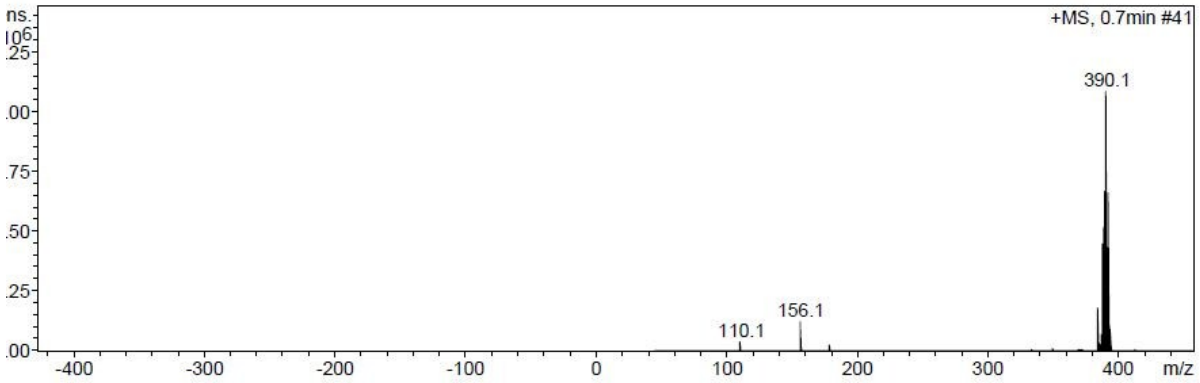


Figure S30.2: ESI-MS of Histidine Adduct by Compound 2

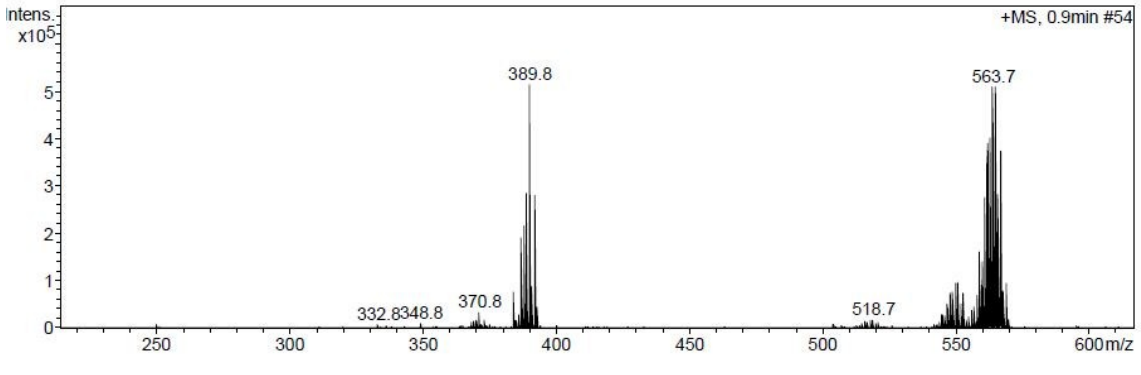


Figure S30.3: ESI-MS of Histidine Adduct by Compound 3

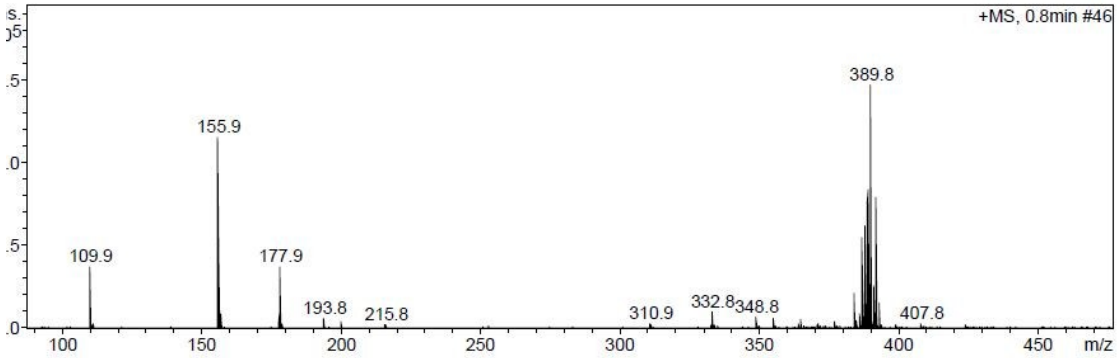


Figure S30.4: ESI-MS of Histidine Adduct by Compound 4

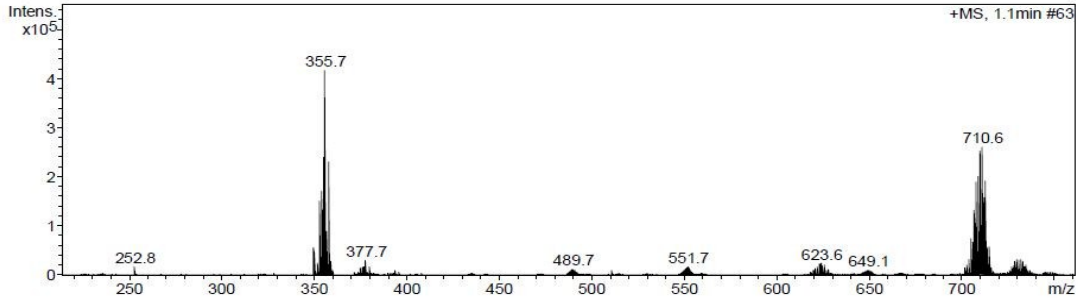


Figure S31.1: ESI-MS of Cysteine Adduct by Compound 1

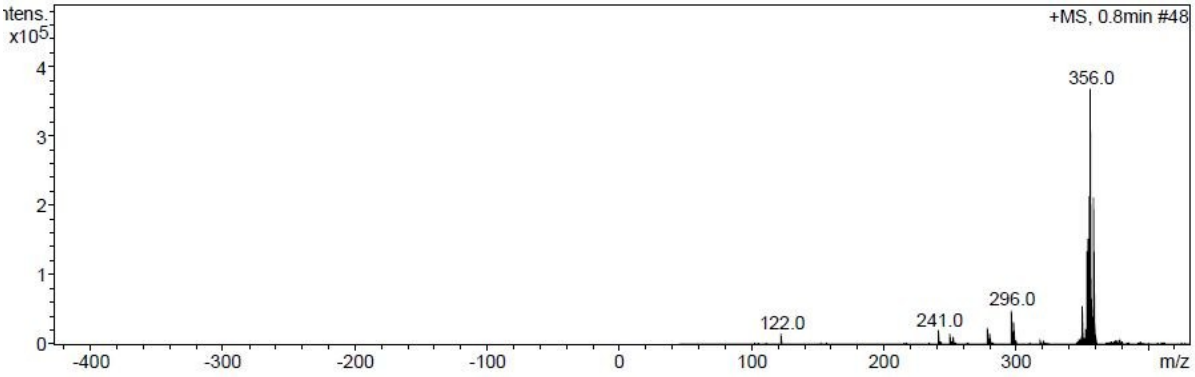


Figure S31.2: ESI-MS of Cysteine Adduct by Compound 2

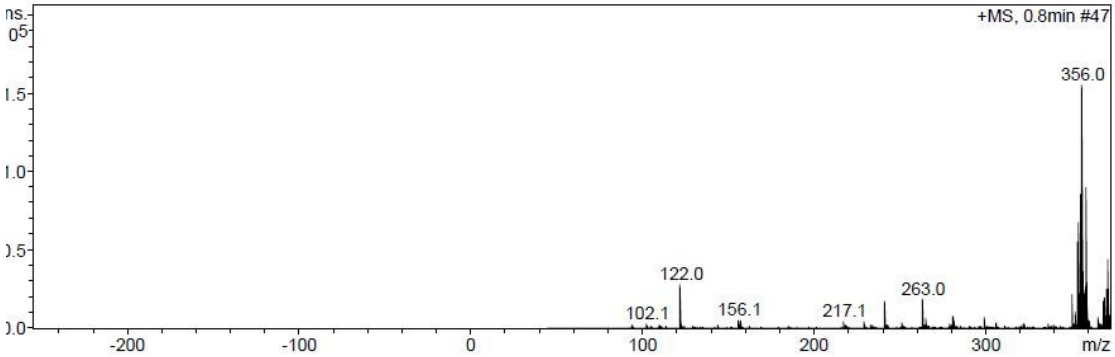


Figure S31.3: ESI-MS of Cysteine Adduct by Compound 3

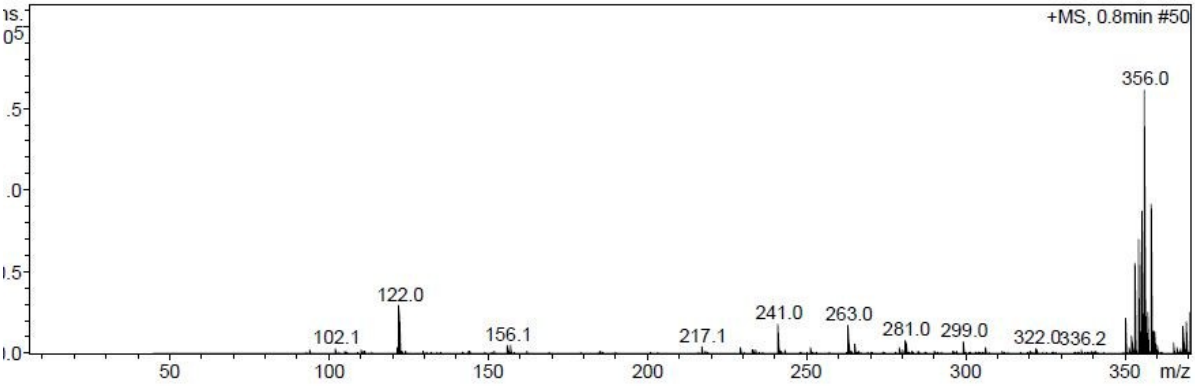


Figure S31.4: ESI-MS of Cysteine Adduct by Compound 4

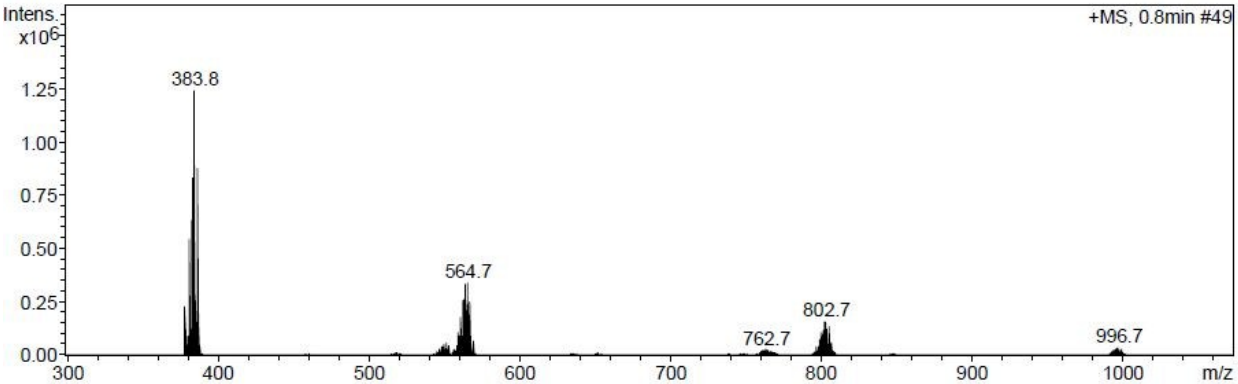


Figure S32.1: ESI-MS of Methionine Adduct by Compound 1

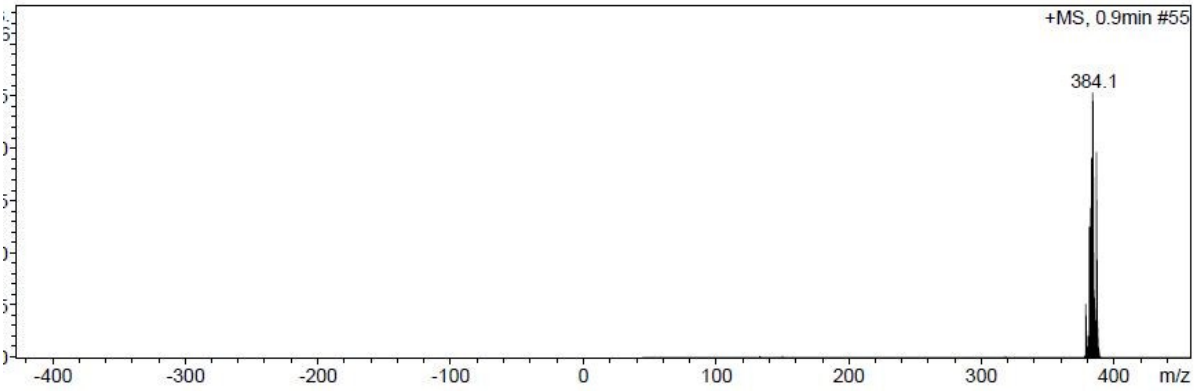


Figure S32.2: ESI-MS of Methionine Adduct by Compound 2

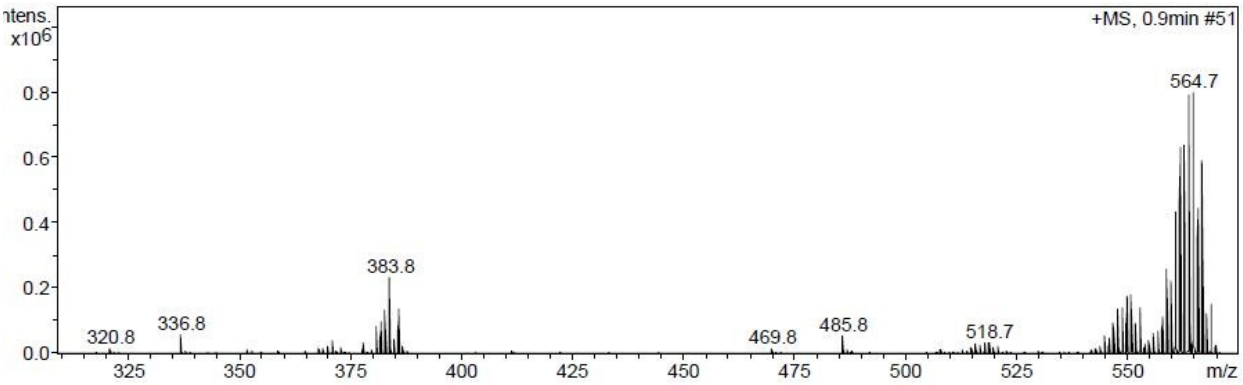


Figure S32.3: ESI-MS of Methionine Adduct by Compound 3

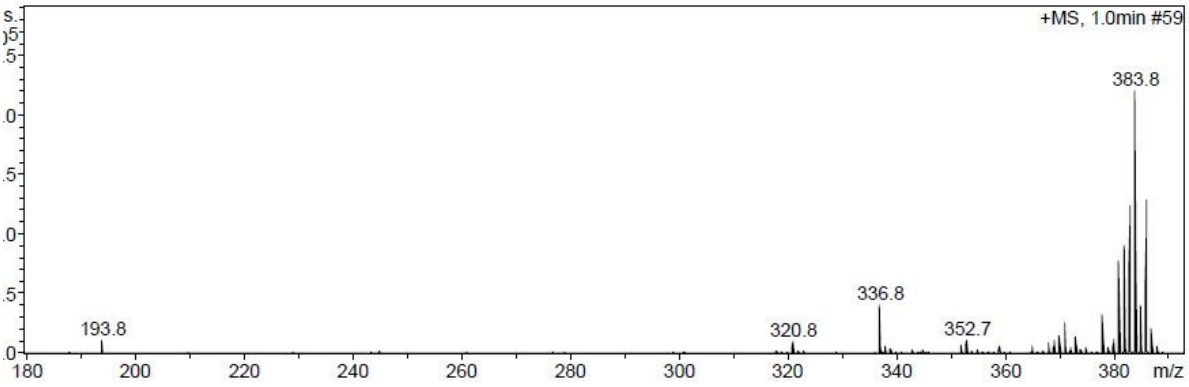


Figure S32.4: ESI-MS of Methionine Adduct by Compound 4

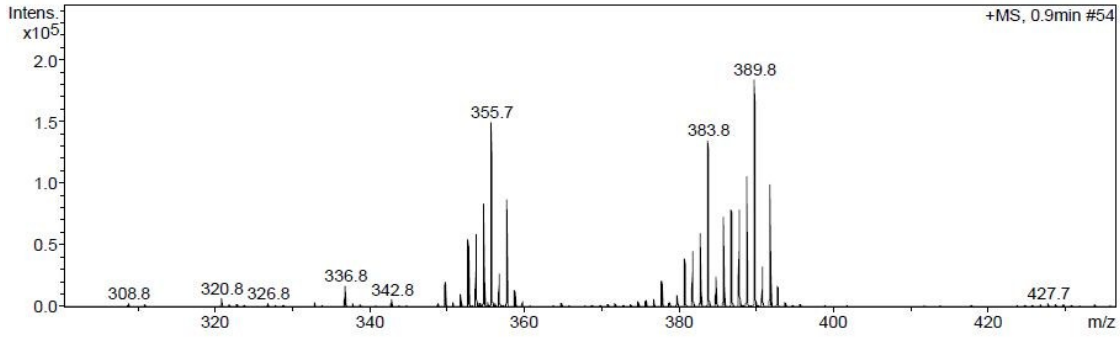


Figure S33.1:ESI-MS of Mixture of [Histidine+Cysteine+Methionine] Adduct by Compound 1

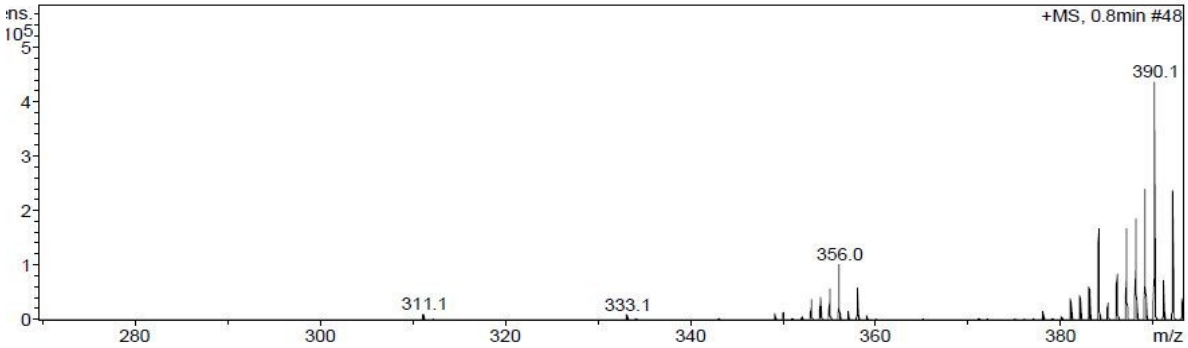


Figure S33.2: ESI-MS of Mixture of [Histidine+Cysteine+Methionine] Adduct by Compound 2

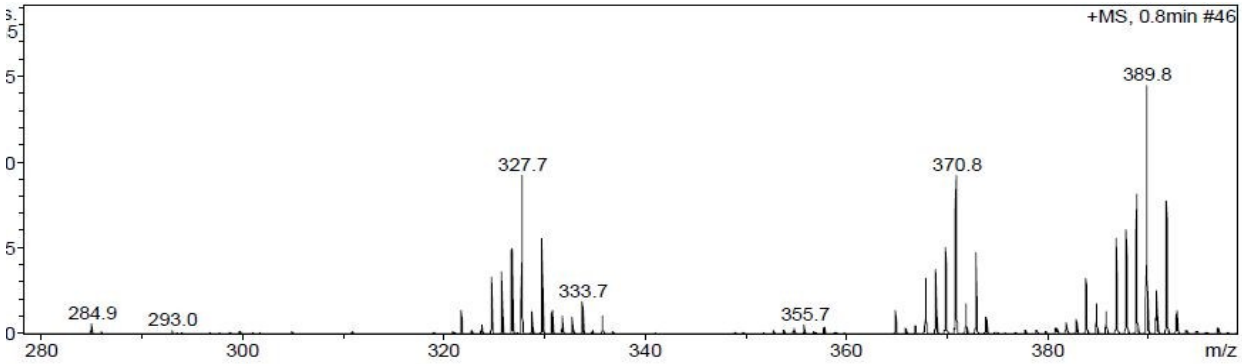


Figure S33.3: ESI-MS of Mixture of [Histidine+Cysteine+Methionine] Adduct by Compound 3

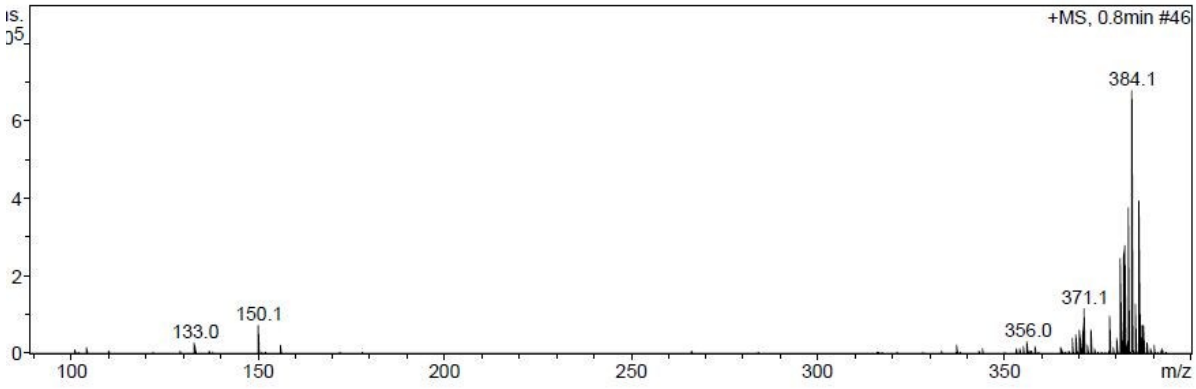


Figure S33.4:ESI-MS of Mixture of [Histidine+Cysteine+Methionine] Adduct by Compound 4

Docking Studies

AutoDock simulation method:

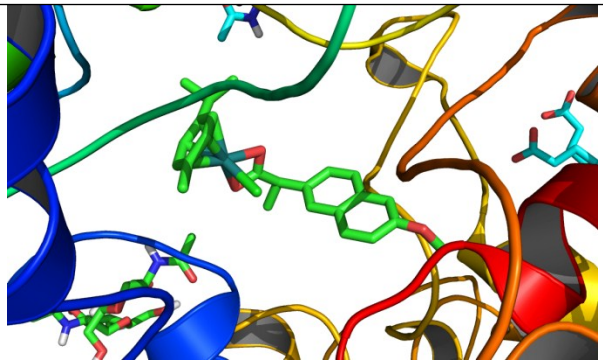
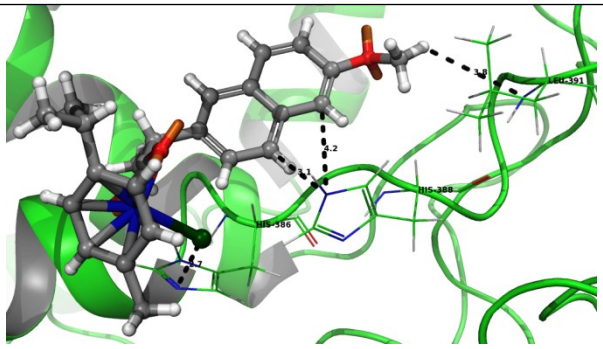
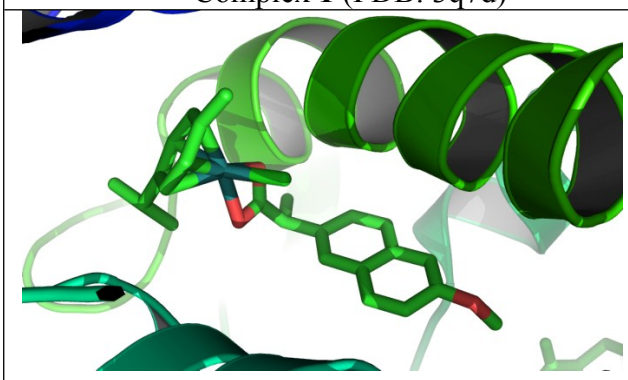
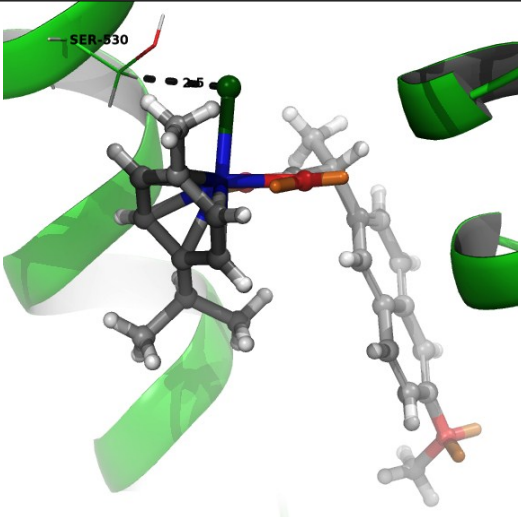
AutoDock 4.2 MGL tools and Lamarckian Generic algorithm (LGA) were used for protein-fixed ligand-flexible docking calculations.[4] The conformation of complexes were taken from their X-ray crystal structures. A two-step docking protocol was employed to perform the docking experiment. In the first step, both the ligand and protein were prepared. The torsion angle and the flexibility issues of the ligand were optimized. For the protein, all the water molecules and the metal ions were removed from the extracted crystal structure. All polar and non-polar hydrogen atoms and the Gasteiger charge were added in to the crystal structure of the protein. In the second step, all possible binding sites of the receptor were determined by the co-crystallized ligand present in the active site of the proteins (for naproxen PDB: 3q7d;[5] for diclofenac PDB: 5ikq[6]). Auto grid version 4.2 was used to calculate the grid parameters and the grid size was set to 40 × 40 × 40 points with grid spacing of 0.375 Å. Twenty search attempts (*i.e.*, ga_run parameter) were performed for each ligand. The maximum number of energy evaluations before the termination of LGA run was 250,000 and the maximum number of generations of LGA run before termination was 27,000. Other docking parameters were set to the default values of AutoDock 4.2 program.

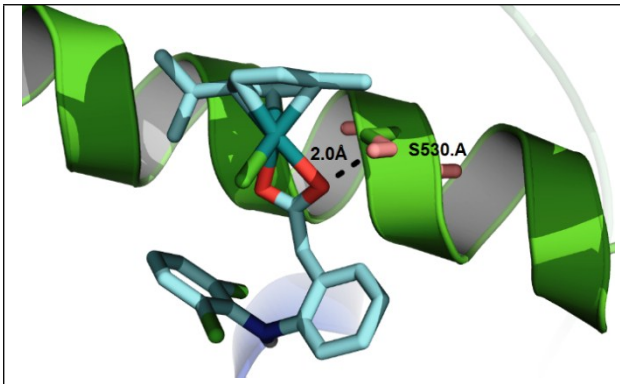
Gold simulation method:

The docking simulation was performed by using the Gold 5.5 (Hermes 1.8.2) simulation software. The X-Ray crystallographic structures of the both proteins (PDB id: 3q7d and 5ikq) were extracted from protein data bank. Both the proteins were prepared by Gold 5.5 software by removing the water molecules followed by structural refinement study, respectively. Additionally, hydrogen atoms were added up to the crystal structure. All possible binding

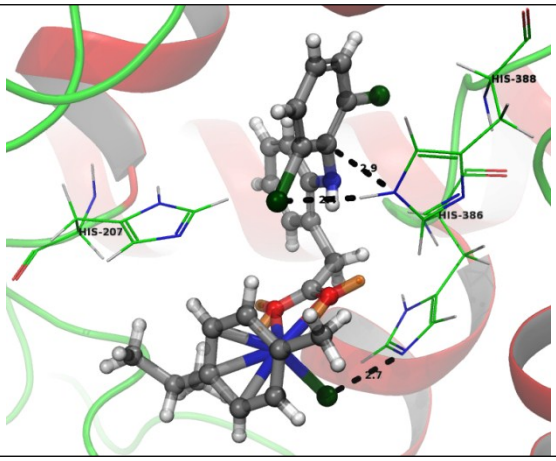
sites of the receptor were determined by the co-crystallized ligand present in the active site of the proteins. The grid was selected based on the ligands co-crystallized with the protein. The grid size for PDB 3q7d and 5ikq was considered as -33.555 × 41.327× 18.997, and 26.992 × 41.680 × 14.356, respectively. Thirty GA run was selected for all the docking experiments.[7] The docked complexes were visualized by Pymol 1.3 software.

Figureure S34.1: Docking interaction illustration of the selected ligands with COX-2

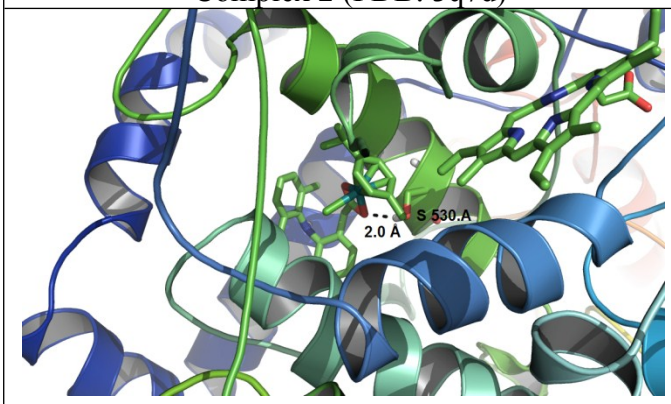
AutoDock 4.2	Gold 5.5
	
Complex **1** (PDB: 3q7d)	Complex **1** (PDB: 3q7d)
	
Complex **1** (PDB: 5ikq)	Complex **1** (PDB: 5ikq)



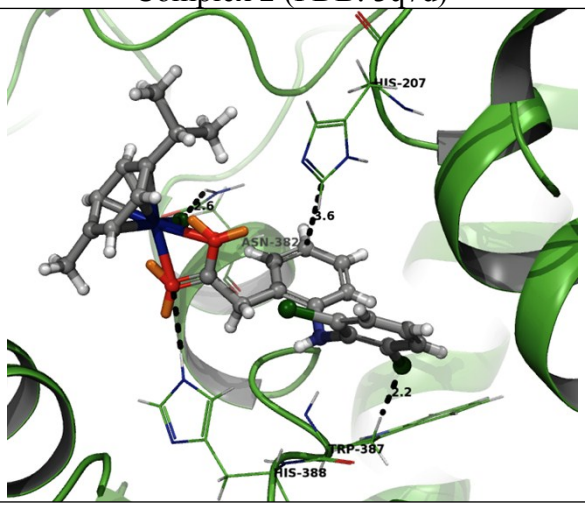
Complex **2** (PDB: 3q7d)



Complex **2** (PDB: 3q7d)

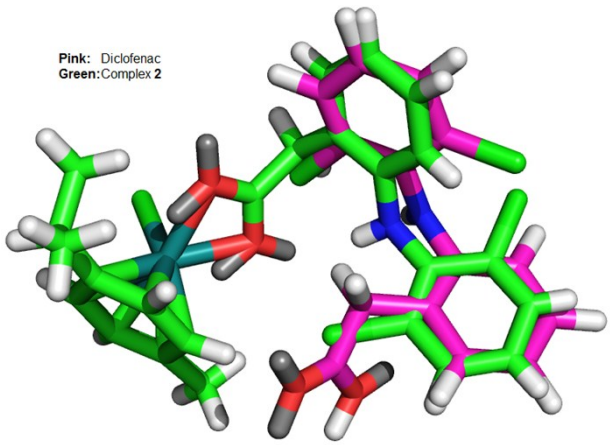


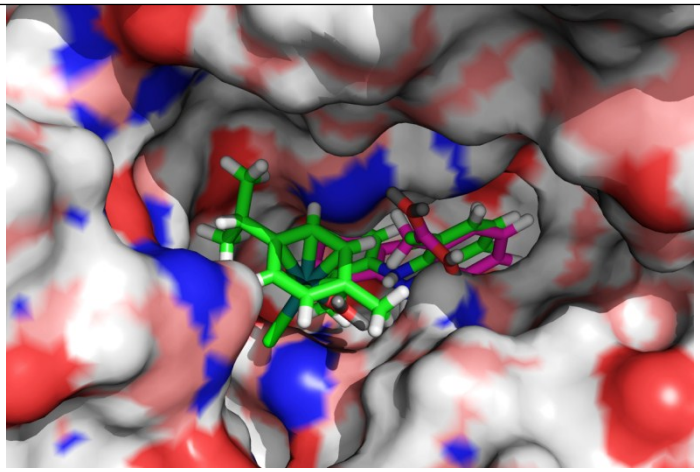
Complex **2** (PDB: 5ikq)



Complex **2** (PDB: 5ikq)

Figureure S34.2: Overlay diagrams for the binding of complex 2 and free diclofenac at the binding site of COX-2 (PDB: 5ikq) showing their binding precision as obtained by Gold simulation.





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X-ray crystallography

X-ray single crystal of complex **1** and **2** was immersed in cryo-oil, mounted in Nylon loops and measured at a temperature of 150 K in a Bruker AXS-KAPPA APEX II with graphite monochromated Mo-Kα (λ=0.71073) radiation. Data were collected using omega scans of 0.5º per frame and full sphere of data were obtained. Cell parameters were retrieved using Bruker SMART[8] software and refined using Bruker SAINT[9] on all the observed reflections. Absorption corrections were applied using SADABS.[10] Structures were solved by direct methods by using the SHELXS–97 package[11] and refined with SHELXL–2014/7.[12] Calculations were performed using the WinGX System–Version 2014.1.[13] Least square refinements with anisotropic thermal motion parameters for all the non-hydrogen atoms and isotropic for the remaining atoms were employed. All hydrogen atoms bonded to carbon were included in the model at geometrically calculated positions and refined using a riding model. Uiso(H) were defined as 1.2Ueq of the parent carbon atoms for phenyl and methylene residues and 1.5Ueq of the parent carbon atoms for the methyl groups. There was some void in the structure which, despite the negligible volume and number of electrons (63 Å^3 and 3 electrons per unit cell), was corrected with PLATON/SQUEEZE.[14]

	1	**2**
Formula	$[C_{24}H_{27}ClO_3Ru]$	$[C_{24}H_{24}Cl_3NO_2Ru]$
Formula weight	499.97	565.86
Crystal system	Orthorhombic	Orthorhombic

Space group	P212121	Pbca
a(Å)	9.328(5)	16.8227(12)
b (Å)	11.490(6)	11.3329(8)
c (Å)	21.33(10)	25.1614(17)
α (deg)	90	90
β (deg)	90	90
γ (deg)	90	90
V ($Å^3$)	2286.1(19)	4797.0(6)
Z, molecules/cell	4	8
Density(g/cm^3)	1.453	1.567
Absorption coefficient (mm^{-1})	0.824	1.009
λ [Mo-Kα] (Å)	0.71073	0.71073
T(K)	150	298
No. of reflections collected	18852	18726
No of independent reflections	6337	5987
No of observed reflections	5077	5064
R1	0.0542	0.0282
wR2	0.1454	0.0741
GOF	1.07	1.07

Table S1: Crystallographic data for complexes 1 and 2

References

(1) N. J. M. Sanghamitra, M. K. Adwankar, A. S. Juvekar, V. Khurajjam, C. Wycliff and A. G. Samuelson, *Indian J. Chem., Sect. A: Inorg., Bio-inorg., Phys., Theor. Anal. Chem.*, 2011, **50**, 465-473.

(2) T. Shimizu, K. Kondo and O. Hayaishi, *Arch Biochem Biophys.*, 1981, **206**, 271-276.

(3) B. Axelrod, T. M. Cheesbrough and S. Laakso, *Methods Enzymol.*, 1981, **71**, 441-451.

(4) G. M. Morris, D. S. Goodsell, R. S. Halliday, R. Huey, W. E. Hart, R. K. Belew and A. J. Olson, *J. Comput Sci.*, 1998, **19(14)**, 1639-1662.

(5) K. C. Duggan, D. J. Hermanson, J. Musee, J. J. Prusakiewicz, J. L. Scheib, B. D. Carter, S. Banerjee, J. A. Oates and L. Marnett, J. *Nat. Chem. Biol.*, 2011, **7**, 803-809.

(6) B. J. Orlando and M. G. Malkowski, *J. Biol. Chem.*, 2016, **291**, 15069-15081.

(7) G. Jones, P. Willet, R. C. Glen, A. R. Leach, R. Taylor, *J. Mol. Biol.*, 1997, **267**, 727−748.

(8) SAINT, Area Detector Integration Software, Siemens Analytical Instruments Inc., Madison, WI, USA, 1995.

(9) SMART & SAINT Software Reference manuals, version 5.0, Bruker AXS Inc., Madison, WI, 1998.

(10) G. M. Sheldrick, SADABS, Program for Semi-empirical Absorption Correction, University of Göttingen, Göttingen, Germany, 1997.

(11) G. M. Sheldrick and T. R. Schneider, [16] SHELXL: Highresolution refinement, in Methods in Enzymoogy, ed. C. W. Carter Jr., Academic Press, New York, 1997, **276**, 307–326.

(12) G. M. Sheldrick, Acta Crystallogr., Sect. A: Fundam. Crystallogr., 2008, **64**, 112-122.

(13) L. J. Farrugia, *J. Appl. Crystallogr.*, 1999, **32**, 837-838.

(14) A. J. Spek, *Appl. Crystallogr.*, 2003, **36**, 7-13.