Electronic Supplementary Material (ESI) for Metallomics.

Supporting Information

Conjugation of Gold (III) Complex with Vitamin B1 and Chlorambucil Derivatives: Anticancer Evaluation and Mechanistic Insights

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1. Experimental

1.1. Materials and instruments

All chemicals were obtained from commercial sources unless otherwise stated. ^{1}H and ^{13}C spectra were recorded on a 400 Mercury Plus Varian instrument. Elemental analyses were performed with an EA 3000 CHNS. ESI mass spectra were recorded on a Waters LCT Premier XE spectrometer. Electronic absorption spectra were recorded on a JASCO 7580 UV-vis-NIR. Emission intensity measurements were carried out using a Hitachi F 4500 spectrofluorometer.

1.2. Synthesis 4-(4-(bis(2-chloroethyl)amino)phenyl)-N-(2-phenylpyridin-4-yl)butanamide (Chlorambucil-phenylpyridine, CHL-N^C)

To chlorambucil (305 mg, 1 mmol) in dry dichloromethane (20 mL) was added oxalyl chloride (175 μL, 2 mmol) and DMF (3 drop). The solution was stirred at room temperature for 100 min, and evaporated in rotavap. The resulting compound was dissolved in dry THF (30 mL) and added 4-amino-2-phenylpyridine (205 mg, 1.2 mmol) and triethylamine (700 μL, 5 mmol) in dry THF (30 mL) at 0 ºC. After 15 min., the resulting solution was stirred at room temperature for 12 h and evaporated in the rotavap. The resulting compound was dissolved in ethyl acetate (50 mL), washed with brine (2 × 20 mL), and dried over $MgSO_4$ and finally concentrated in rotavap. The silica gel column chromatography was used to purify the crude compound by ethyl acetate: petroleum ether (2: 8) as mobile phase. Pale yellow solids (346 mg, 76% yield, and 1 mmol). Anal. Calc. (%) for $C_{25}H_{27}Cl_2N_3O$ (455.1531): C, 65.79; H, 5.96; N, 9.21. Found (%): C, 65.76; H, 5.94; N, 9.18. ESI-MS: 456.1609 $[M + H]^+$. ^{1}H NMR ($CDCl_3$): δ 8.29-8.35 (m, 3H, H-Ar), 7.88 (d, 1H, H-d, J = 7.2 Hz), 7.50-7.58 (m, 3H, H-Ar), 7.42 (d, 1H, H-a, J = 7.2 Hz), 7.34 (s, 1H, H-NH), 7.09 (d, 2H, H-6,10, J = 7.6 Hz), 6.80 (d, 2H, H-7,9, J = 7.6 Hz), 3.65-3.71 (m, 8H, H-11,12), 2.62 (t, 2H, H-4, J = 7.6 Hz), 2.32 (t, 2H, H-2, J = 7.6 Hz), 1.79-1.85 (m, 2H, H-3).

^{13}C NMR ($CDCl_3$): δ 178.8 (C=O), 155.1 (Ar), 144.9 (Ar), 140.0 (Ar), 137.7 (Ar), 134.9 (Ar), 130.0 (Ar), 126.8 (Ar), 113.1 (Ar), 99.9 (Ar), 98.1 (Ar), 52.2, 44.4, 35.9, 32.8, 27.6.

1.3. Synthesis sodium dithiocarbamate vitamin B1 (B1-DTC)

Vitamin B1 hydrochloride (337 mg, 1 mmol) was treated with sodium hydroxide (80 mg, 2 mmol) in methanol (3 mL), and the solutions were stirred at 0 °C for 20 min. Then, carbon disulfide (66.5 μL, 1.1 mmol) was added dropwise, and the mixture was stirred at room temperature for 2h. The solvents were removed under vacuum at room temperature, and the residues obtained were dissolved in methanol and filtered off trough Celite, and the filtrate was concentrated in vacuum (not more than 20 °C). Pale yellow solid (207 mg, 52% yield, and 1 mmol). Anal. Calc. (%) for $C_{13}H_{16}ClN_4NaOS3$ (398.0072): C, 39.14; H, 4.04; N, 14.04. Found (%): C, 39.11; H, 4.02; N, 14.01. ESI-MS: 375.0175 $[M - Na]^-$. ^{1}H NMR (D_2O): δ 8.18 (s, 1H, H-1'), 8.09 (s, 1H, H-5'), 7.97 (s, 1H, H-3'), 6.01 (s, 2H, H-4'), 3.84 (t, 2H, H-8', J = 7.6 Hz), 3.61 (s, 1H, H-9'), 2.94 (s, 3H, H-6'), 2.81 (d, 2H, H-7', J = 7.6 Hz), 2.68 (s, 3H, H-2'). ^{13}C NMR (D_2O): δ 210.0 (C-10'), 164.0 (Ar), 162.1 (Ar), 157.2 (C-5'), 145.1 (Ar), 142.2 (Ar), 138.0 (Ar), 106.2 (Ar), 59.9 (C-8'), 50.0 (C-4'), 30.0 (C-7'), 24.3 (C-2'), 13.0 (C-6').

1.4. Synthesis complex [(CHL-N^C)Au^{III}(B1-DTC)](Cl$_2$), 1

Sodium tetrachloroaurate(III) dihydrate ($Na[AuCl_4]. 2H_2O$) (398 mg, 1 mmol) and CHL-N^C (455 mg, 1 mmol) were added simultaneously in 20 mL of ethanol and mixture was stirred for 3 h at room temperature. Then, B1-CS2 (398 mg, 1 mmol) in 10 mL distilled water was added slowly in the pale yellow turbid solution obtained of first step. The reaction mixture was stirred for an additional 2 h at room temperature to obtain the clear solution. The solution was evaporated in rotavap, washed with fresh distilled water (2 × 5 mL) and dried at room temperature under vacuum. Pale yellow solid (893 mg, 84% yield, and 1 mmol). Anal. Calc. (%)

for $C_{38}H_{42}AuCl_4N_7O_2S_3$ (1063.7593): C, 42.91; H, 3.98; N, 9.22. Found (%): C, 42.89; H, 3.97; N, 9.19. ESI-MS: 1028.3063 $[M - Cl]^+$. ^1H NMR (D_2O): δ 8.15 (s, 1H, H-1'), 8.09 (s, 1H, H-5'), 7.99, (s, 1H, H-3'), 7.88 (d, 1H, H-Ar, *J* = 7.6 Hz), 7.73 (d, 1H, H-Ar, *J* = 7.6 Hz), 7.50-7.59 (m, 3H, H-Ar), 7.42 (d, 1H, H-Ar, *J* = 7.6 Hz), 7.48 (s, 1H, H-NH), 7.22 (d, 1H, H-b, *J* = 7.6 Hz), 7.10 (d, 2H, H-Ar, *J* = 7.6 Hz), 6.84 (d, 2H, H-Ar, *J* = 7.6 Hz), 5.80 (s, 2H, H-4'), 3.96 (t, 2H, H-8', *J* = 7.6 Hz), 3.76 (s, 1H, H-9'), 3.58-3.65 (m, 8H, H-11,12), 2.90 (s, 3H, H-6'), 2.80 (d, 2H, H-7', *J* = 7.6 Hz), 2.68 (s, 3H, H-2'), 2.52 (t, 2H, H-4, *J* = 7.6 Hz), 2.24 (t, 2H, H-2, *J* = 7.6 Hz), 1.63-1.80 (m, 2H, H-3). ^{13}C NMR (D_2O): δ 197.1 (C-10'), 177.3 (Ar), 165.0 (Ar), 161.9 (Ar), 157.1 (Ar), 145.0 (Ar), 141.9 (Ar), 138.9 (Ar), 136.3 (Ar), 129.1 (Ar), 125.1 (Ar), 112.3 (Ar), 108.3 (Ar), 98.8 (Ar), 97.1 (Ar), 59.9, 53.7, 51.2, 42.3, 36.4, 34.6, 31.1, 28.2, 24.9, 13.9.

1.5. Stability tests

The stability of the compounds was tested by dissolving the compounds in PBS buffer or DMEM (Dulbecco's Modified Eagle's Medium - high glucose) solution (for CHL-N^C dissolved in 1% DMSO, and for B1-DTC and complex **1** just in PBS buffer or DMEM that were both water soluble) and keeping them for 72 h at 37 °C. Briefly, a 10 μL of the solution was injected into an HPLC system (Thermo, USA) connected to a UV/Vis spectrophotometer. A Hypersil Gold Dim (100 × 2.1 mm, Thermo, USA) reversed-phase column was used at a flow rate of 0.5 mL min^{-1}. The mobile phase was 80:20 acetonitrile (0.1% trifluoroacetic acid): water (0.1% trifluoroacetic acid).

1.6. Cell culture

The human breast adenocarcinoma (MCF-7 and MDA-MB-231) and human colon cancer (HCT-116), as well as non-tumorigenic cells human lung fibroblasts (MRC-5), were obtained from the American Type Culture Collection (ATCC, USA). All reagents and cell culture media

were purchased from Gibco Company (Germany). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 IU mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin and 2 mM of Glutamax at 37 ˚C in a humidified incubator at 5% CO_2. The adherent cultures were grown as monolayers and were passaged once in 4-5 days by trypsinizing them with 0.25% Trypsin-EDTA.

1.7. Cytotoxicity

The MCF-7, MDA-MB-231, HCT-116, and MRC-5 were analyzed for viability post treatment using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay following previously reported procedures. [1] The stock solutions of free ligand CHL-N^C and auranofin were prepared by dissolving the compound in aqueous culture medium with 1% DMSO and for cisplatin by dissolving in 0.9% NaCl. The stock solutions of B1-DTC and complex **1** were prepared by dissolving the compounds in the aqueous culture medium. The stocks were further diluted with the respective medium containing 10% FBS (0.02–10 μM) before addition to the cells. Cells were trypsinized with 0.25% trypsin-EDTA and counted with 0.4% trypan blue. The cells were seeded at a concentration of (3-10) × 10^3 cells/well, dependent upon the growth characteristics of the cell line, and grown for 24 h at 37 °C in a humidified incubator. After 24 h, the medium was removed and replaced with fresh medium containing the compound to be studied at the appropriate concentration. Triplicate cultures were established for each treatment. After 72 h, each well was treated with 10 μl of a 5 mg/mL MTT saline solution, and, following 5 h of incubation, 100 μl of a sodium dodecylsulfate solution in HCl (0.01 M) was added. The optical absorbance of each well (96-well plates) was quantified using EnVision multilabel plate readers (PerkinElmer, Waltham, MA, USA) at 570 nm wavelength. The mean absorbance for each drug dose was expressed as a percentage of the control, untreated well absorbance and

plotted vs drug concentration. The IC_{50} values were determined by nonlinear regression analysis using GraphPad Prism software. The IC_{50} value was calculated as the concentration reducing the proliferation of the cells by 50% and is presented as a mean (±SE) of three independent experiments each with triplicates. $P < 0.05$ was considered to be statistically significant.

SI was obtained average IC_{50} for MRC-5 normal cells divided by average IC_{50} for MCF-7, MDA-MB-231, and HCT-116 cancer cells. The sign (>) indicates that IC_{50} value is not reached at the maximum concentration in the examined range.

1.8. Cellular uptake

MCF-7 cancer cells were seeded at a density of 10^6 cells/dish and maintained at 37 °C in a 5% CO_2 atmosphere. Cells were washed with phosphate-buffered saline (PBS), and fresh growth medium (5 mL) was added to each dish that freshly prepared complex **1** was added to cells at IC_{50} concentration. The dishes were incubated at 37 °C under for a period of 24 h. The growth medium was removed and each dish was washed with PBS. Trypsin (0.25% in PBS) was added, and cells were harvested from the plate by using ethylenediaminetetraacetic acid (EDTA)-trypsin and centrifuged. Dishes were washed with PBS, and this was added to the cell suspension to ensure that all the cells were collected. The resulting suspension was centrifuged, and the supernatant was discarded. The cell pellet was washed with PBS (2 mL) and saline (0.9% NaCl, 99.999% pure, 2 mL), and the supernatant was discarded each time. The number of cells per dish was determined by scoring one dish out of each series of four. The Au content was determined by graphite furnace atomic absorption spectroscopy, after digestion of the cells in concentrated HNO_3 (Tracepur, Merck) and dilution to 10.0 mL. All the measurements were completed in triplicate.

1.9. Preparation of thioredoxin reductases

The cytosolic thioredoxin reductase (TrxR1) and mitochondrial thioredoxin reductase (TrxR2) from rat liver were obtained from Sigma-Aldrich and used without further purification.

1.10. In vitro TrxR1 and TrxR2 inhibition

The assay was performed in 0.2 M Na, K-phosphate buffer (pH 7.2) containing 2 mM EDTA, 0.25 mM NADPH (Nicotinamide adenine dinucleotide phosphate) and about 0.5-2 μg of TrxR protein. The reaction was initiated by the addition of 3 mM DTNB (5,5'-dithiobis (2-nitrobenzoic acid)) to compounds (1-30 μM) and the increase of absorbance was monitored at 412 nm over 5 min at 25 ºC. Enzyme activity was calculated taking into account that 1 mole of NADPH yields 2 moles of CNTP anion (reduced DTNB).

1.11. Thioredoxin reductase inhibition studies in MCF-7 cells

Thioredoxin reductase inhibition studies in MCF-7 cells were performed as previously reported.[2,3] For thioredoxin reductase activity assays, the complete cell lysates was obtained from MCF-7 cells treated with the complex **1** and auranofin (1-30 μM) for 24 h. Control cells that were not treated with compounds were also prepared. Then, the cells were collected, washed three times in PBS, and centrifuged. The samples were lysed by douncing using scrapers and sheer force though syringe with a 34 gauge needle in assay buffer (Sigma-Aldrich, Thioredoxin Reductase Assay kit, CS0170) added to1mM protease inhibitor cocktail (Sigma-Aldrich, P1860). Then the lysates were centrifuged for 15 min to isolate insoluble material. Protein concentration in the cellular extracts was determined using the Bradford Reagent (Sigma-Aldrich) and bovine serum albumin as standard. The soluble lysates were incubated for 20 minutes in assay buffer before adding DTNB (5,5'-dithiobis(2-nitrobenzoic) acid) and absorbance was monitored at 412 nm over 30 min at 25 ºC. Lysates were tested in three independent experiments. TrxR activity was

calculated as micro-mole reduced DTNB per minute per mg of total protein at 25°C and pH7.0. The percentage of activity inhibition refers to control cells.

Moreover, cell lysate thioredoxin reductase was also assessed with a test based on insulin reduction. [4] Briefly, 12 μg of cell lysates were incubated in a final volume of 50 μl of 100 mM Hepes/Tris (pH 7.6), in presence of 12.5 mM EDTA, 1.5 mM NADPH, 0.25 mM insulin and 120 μM recombinant rat TrxR. The reaction was stopped at fixed time (30 min) by adding 1 mM DTNB dissolved in 7.2 M guanidine in Tris-HCl 0.1M (pH 8.1) and samples estimated at 412 nm. The reaction solutions without TrxR were used as the control, which was subtracted. The percentage of activity inhibition refers to control cells.

1.12. In vitro GR inhibition

Glutathione reductase activity was estimated at 25 ºC in 0.1 M Tris–HCl (pH 8.1) containing 0.2 mM NADPH. Reactions were started by the addition of 1 mM GSSG and followed spectrophotometrically at 340 nm.

1.13. In vitro GPx inhibition

Glutathione peroxidase activity was estimated at 25 ºC in 50 mMHepes/Tris (pH 7.0) and EDTA 3 mM, 0.3 mMNADPH, 5 mM GSH and 0.25 mM tert-butyl hydroperoxide according to Little et. al. [5]

1.14. Induction of apoptosis

Apoptosis assays were performed by treatment complex **1** and cisplatin (1 μM) in MCF-7 cells for 24h as previously reported. [6]

1.15. DNA binding studies

The DNA binding studies were carried out by dissolving complex **1** in Tris-HCl/NaCl buffer (10 mM Tris-HCl, 10 mM NaCl, pH 7.2). The stock solution of CT DNA gave a ratio of UV

absorbance at 260 and 280 nm (A_{260}/A_{280}) of 1.90, showing that the DNA sample was free from proteins. The UV spectra of CT DNA in the presence of complex **1** have been recorded for a constant complex concentration (10 μM) and by varying the concentration of CT DNA from 0 to 100 μM.

The binding constant (K_b) values were obtained using the following (eq. 1) from the ratio of the slope to intercept in plots [DNA] / (ε_a- ε_f) versus [DNA]. [7]

$$[DNA] / (\varepsilon_a - \varepsilon_f) = [DNA] / (\varepsilon_b - \varepsilon_f) + 1 / K_b (\varepsilon_b - \varepsilon_f) \quad \text{eq.1}$$

where, ε_a, ε_b, and ε_f are the apparent, bound and free compound extinction coefficients, respectively. [7]

In order to study the ability of the complex **1** to displace EB (Ethidium bromide) from the EB-DNA complex, the competitive studies of complex **1** with EB have been considered by fluorescence spectroscopy. The EB displacement experiments were performed by monitoring changes in the fluorescence intensity at 587 nm (450 nm excitation), after adding aliquots of the 10 μM test solution to an aqueous solution of EB-DNA.

The spectra were analyzed according to the Stern-Volmer equation (eq.2): [7]

$$I_0/I = 1 + K_q [Q] \quad \text{eq.2}$$

where, I_0 and I are the fluorescence intensities exhibited in the absence and presence of the compounds, respectively; [Q] corresponds to the concentration ratio of the compound to DNA. The slope of the plot of I_0/I versus [Q] gives K_q.

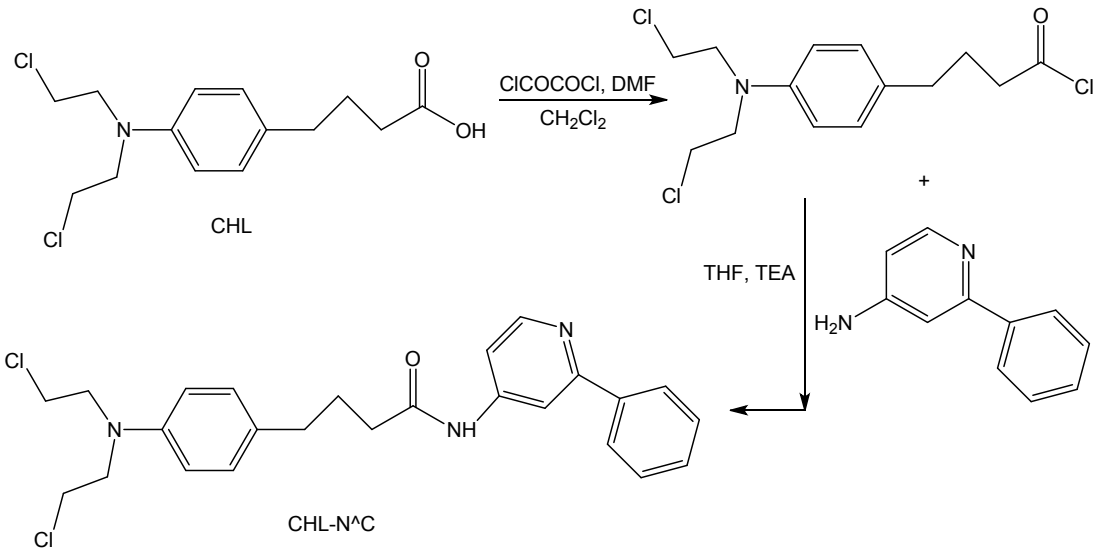
Viscosity measurements were carried out using an Ubbelohde viscometer, maintained at constant temperature (25 °C) in a thermostat by keeping the concentration of DNA constant (10 μM), and varying the concentration of the complex from 0 to 50 μM. Data are presented as $(\eta/\eta_0)^{1/3}$ versus the ratio of the concentration of complex to DNA, where η is the viscosity of

DNA in the presence of the compound and η_0 is the viscosity of DNA alone. Viscosity values were calculated according to the relation $\eta = (t - t_0)/ t_0$, where t was the flow time of the CT DNA solution in the presence or absence of the complex **1** and t_0 that of the buffer alone.

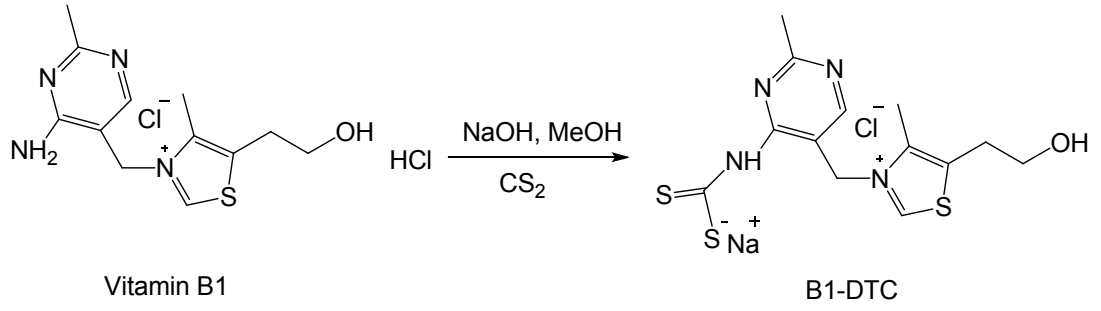
1.16. Computational methods

1.16.1. Optimization Structure

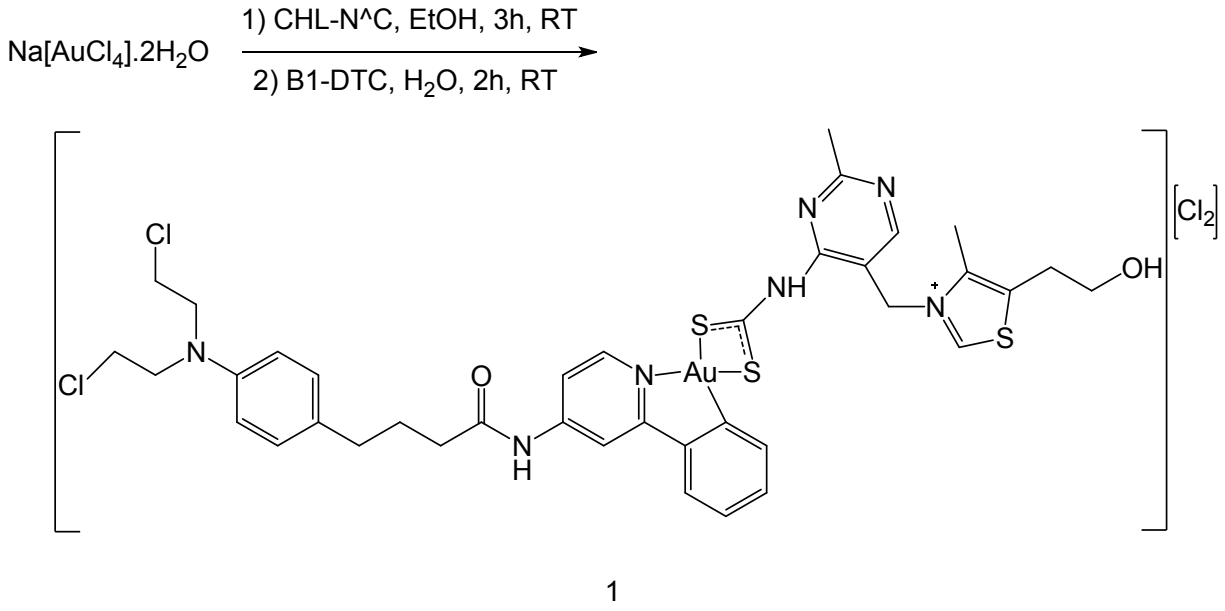
The M062X functional [8] with two basis sets such as LANL2DZ [9] and 6-31+G(d,p) were performed for optimizing transition metal atom and other atoms in the complex **1**. Gaussian 09 package [10] was used for this optimization calculation. Figure S14 shows that the optimized structure of complex **1** which this optimized geometry of complex **1** was performed as input for future computations.



Scheme S1. Synthetic pathways of the 4-(4-(bis(2-chloroethyl)amino)phenyl)-N-(2-phenylpyridin-4-yl)butanamide (Chlorambucil-phenylpyridine, CHL-N^C).



Scheme S2. Synthetic pathways of the sodium dithiocarbamate vitamin B1 (B1-DTC).



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Scheme S3. Synthetic pathways of the complex [(CHL-N^C)Au^{III}(B1-DTC)](Cl_2), **1**

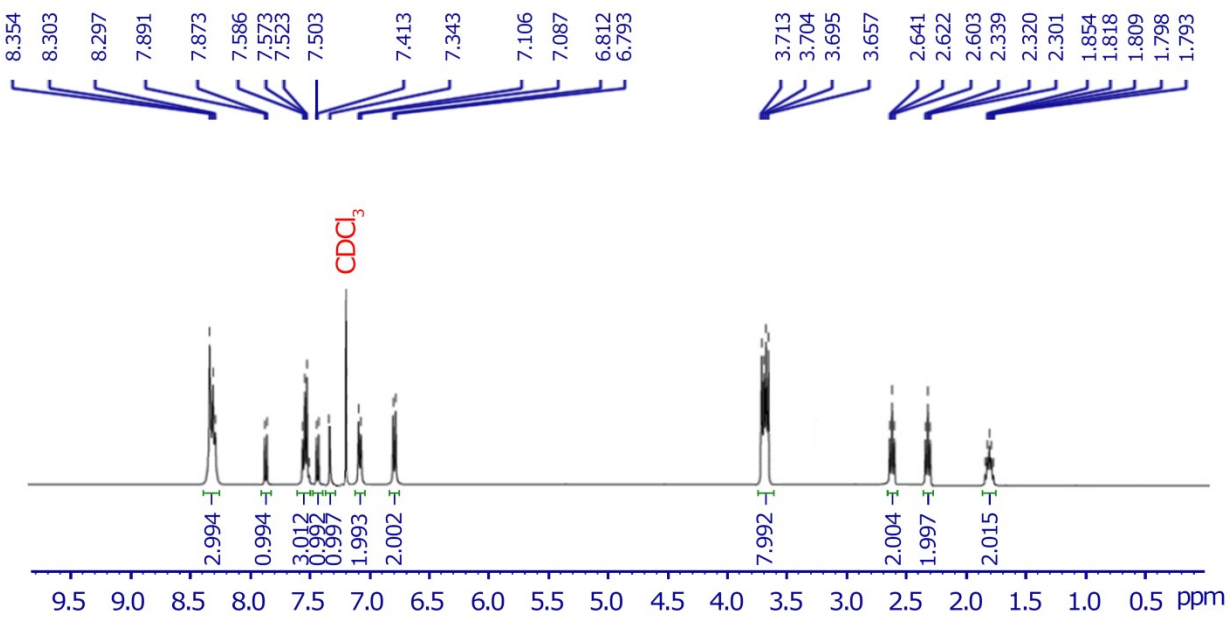


Figure S1. ^{1}H NMR spectrum of ligand **CHL-N^C** ($CDCl_3$).

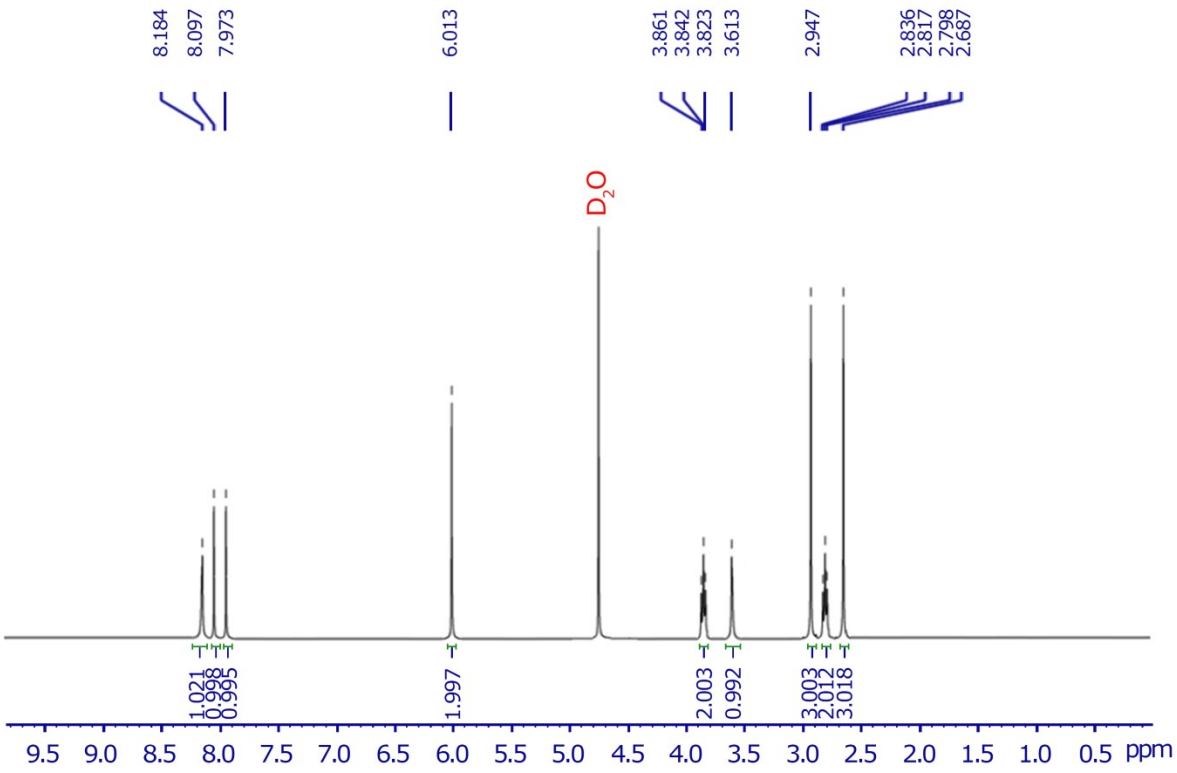


Figure S2. ^{1}H NMR spectrum of ligand **B1-DTC** (D_2O).

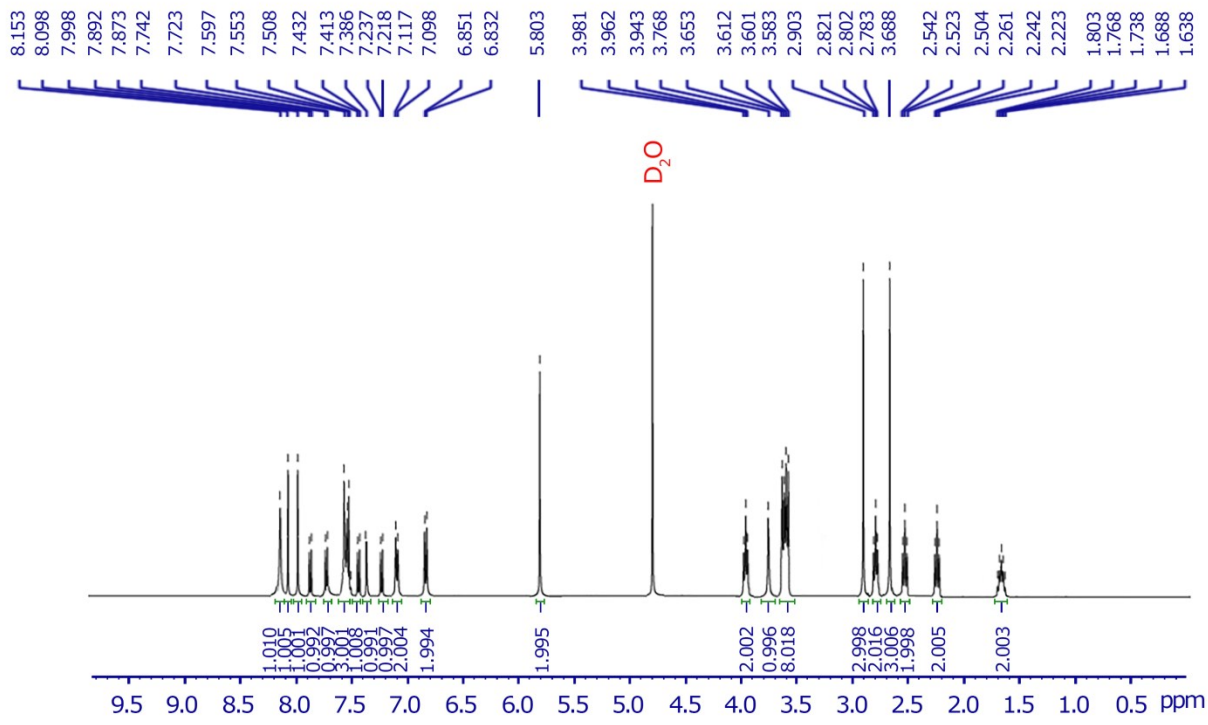


Figure S3. ^{1}H NMR spectrum of complex **1** (D_2O).

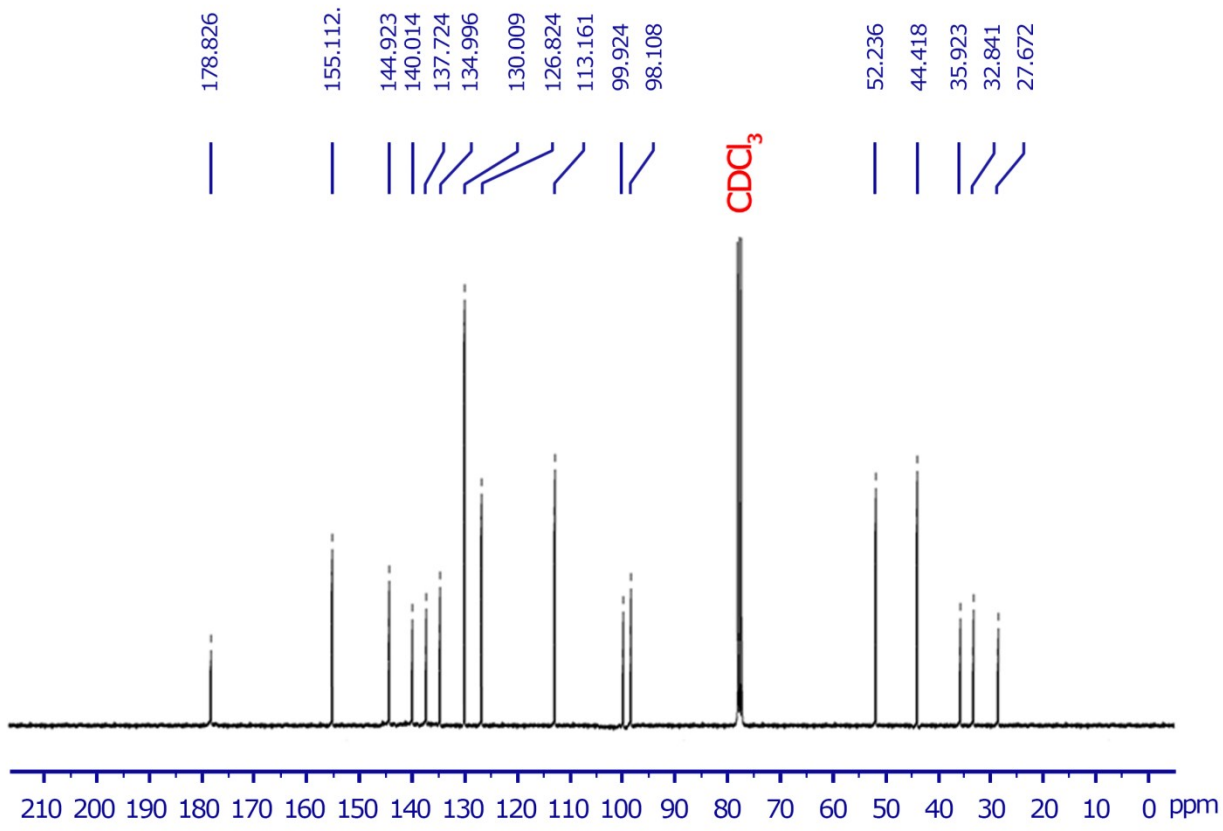


Figure S4. ^{13}C NMR spectrum of ligand **CHL-N^C** ($CDCl_3$).

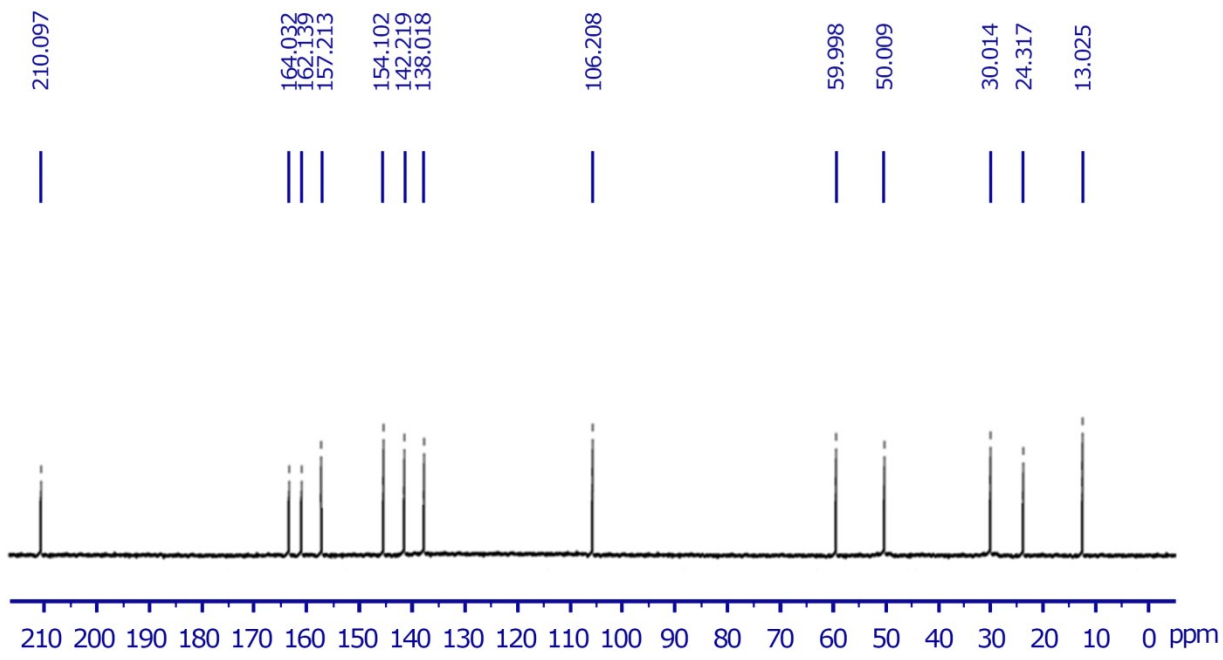


Figure S5. ^{13}C NMR spectrum of ligand **B1-DTC** (D_2O).

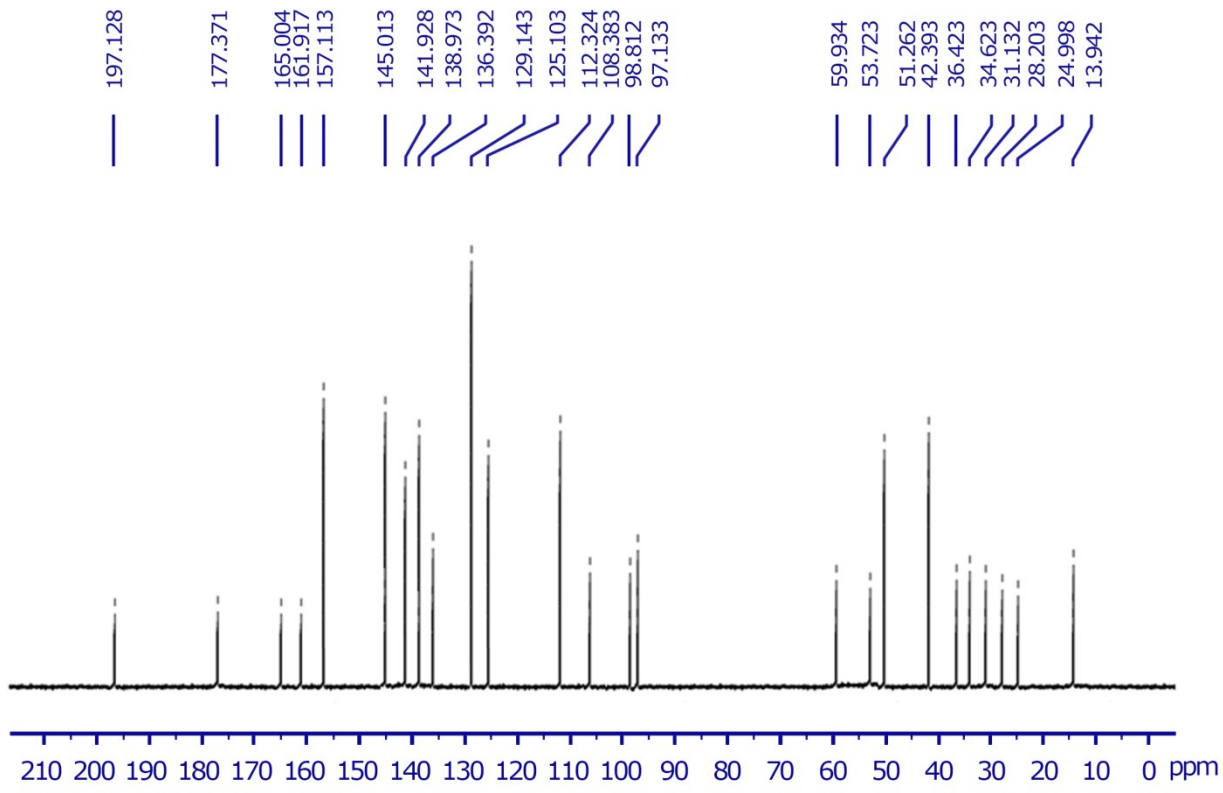


Figure S6. ^{13}C NMR spectrum of complex **1** (D_2O).

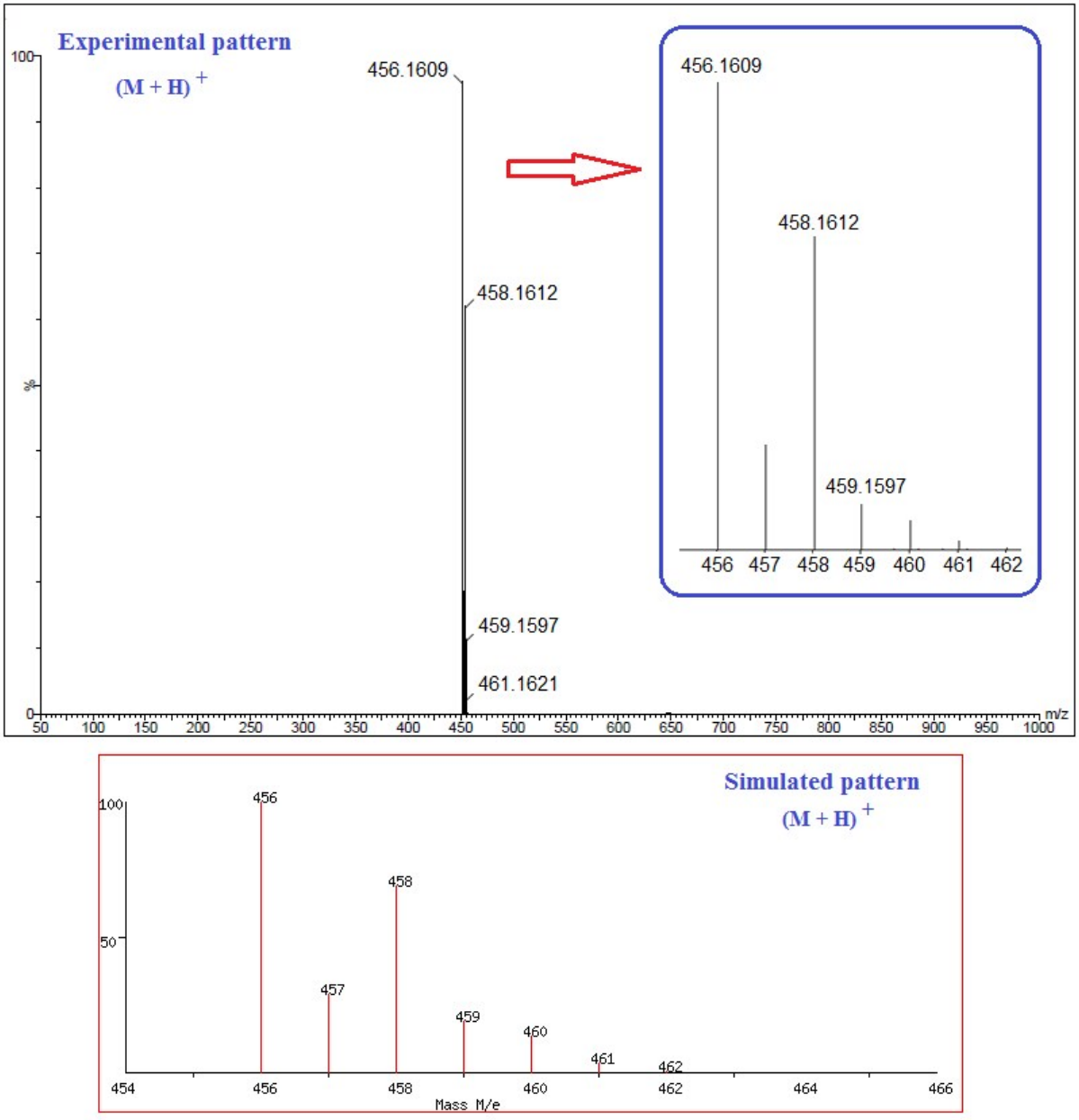


Figure S7. TOF MS spectrum of ligand **CHL-N^C**.

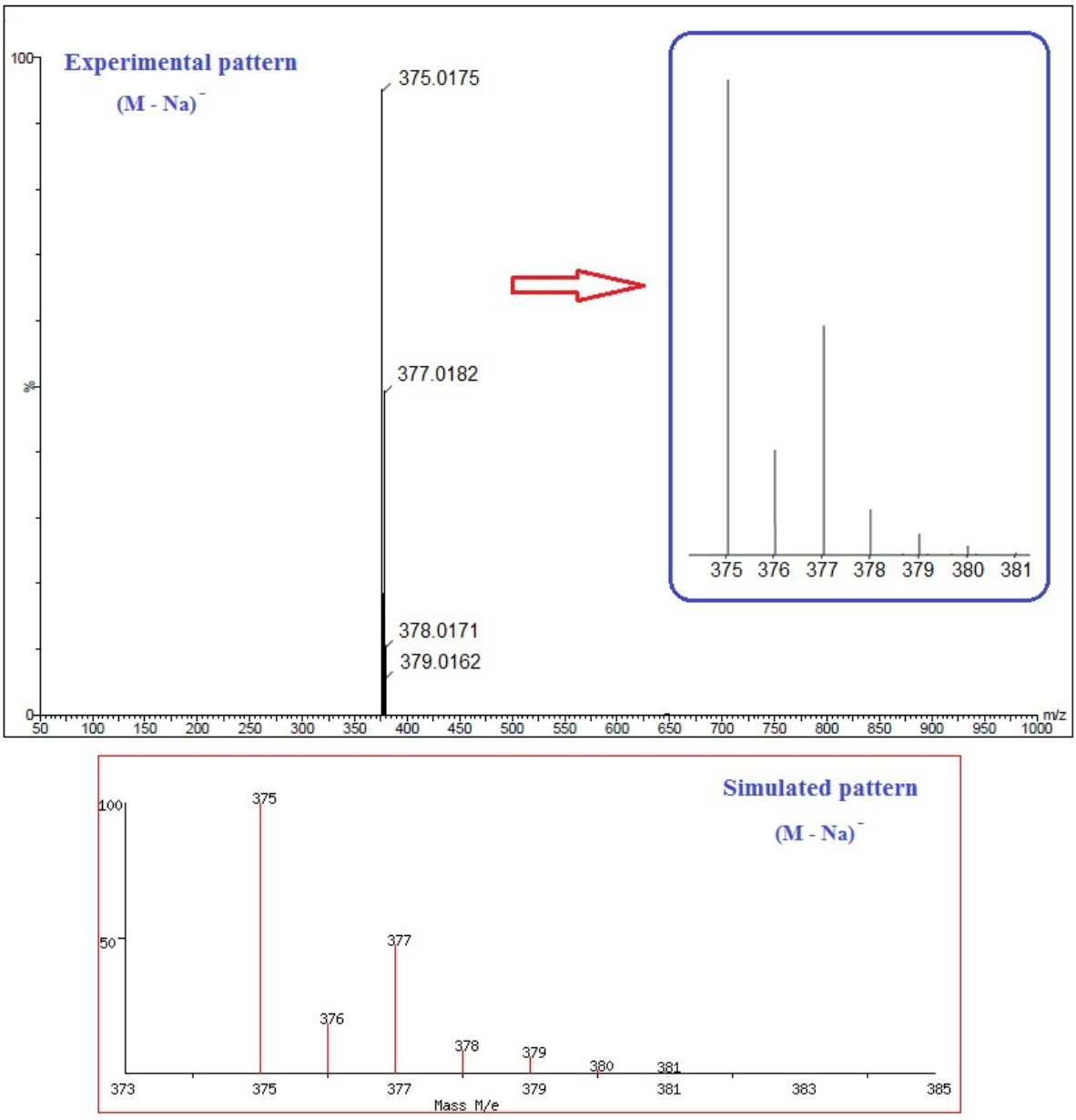


Figure S8. TOF MS spectrum of ligand **B1-DTC**.

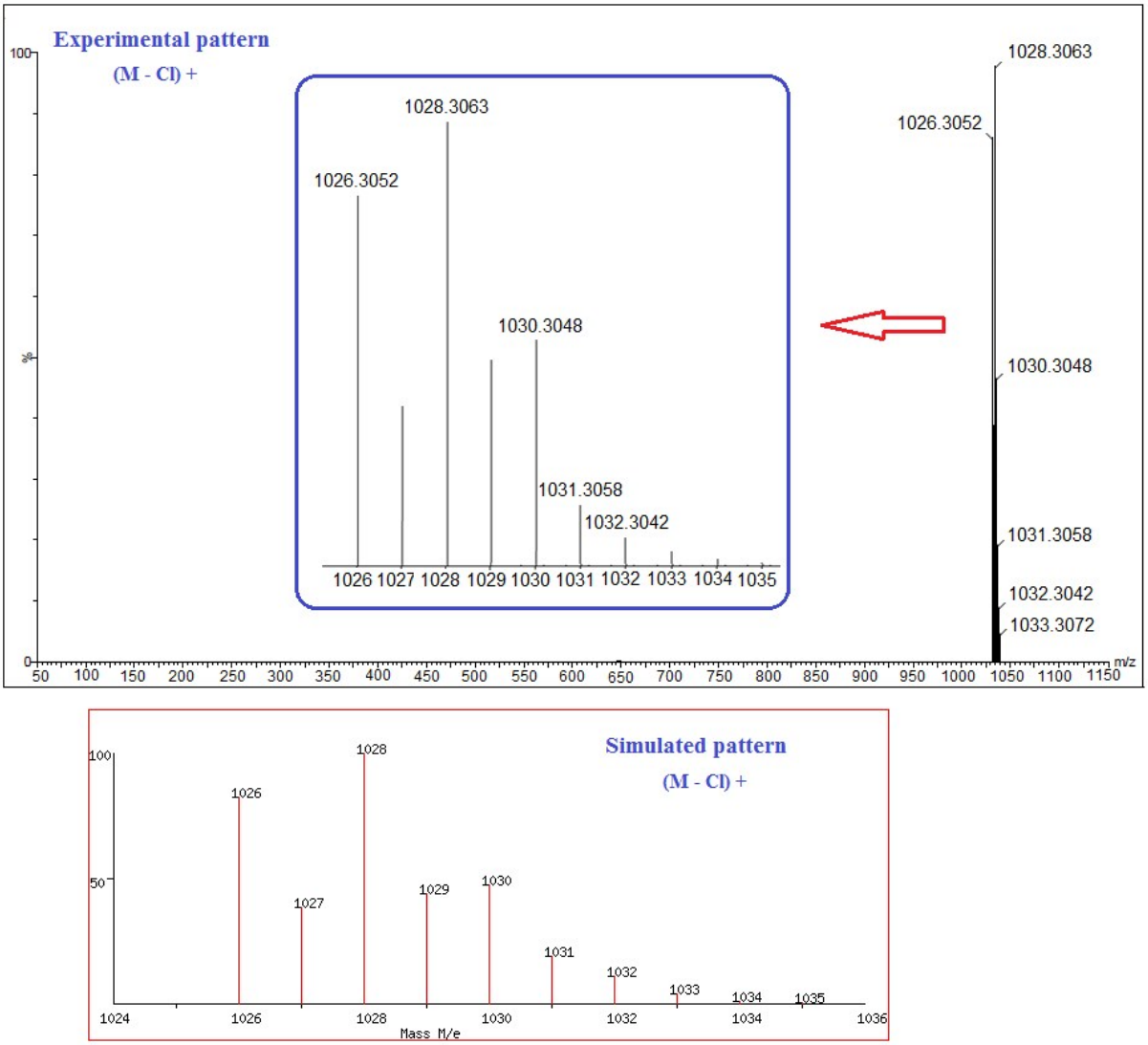


Figure S9. TOF MS spectrum of complex **1**.

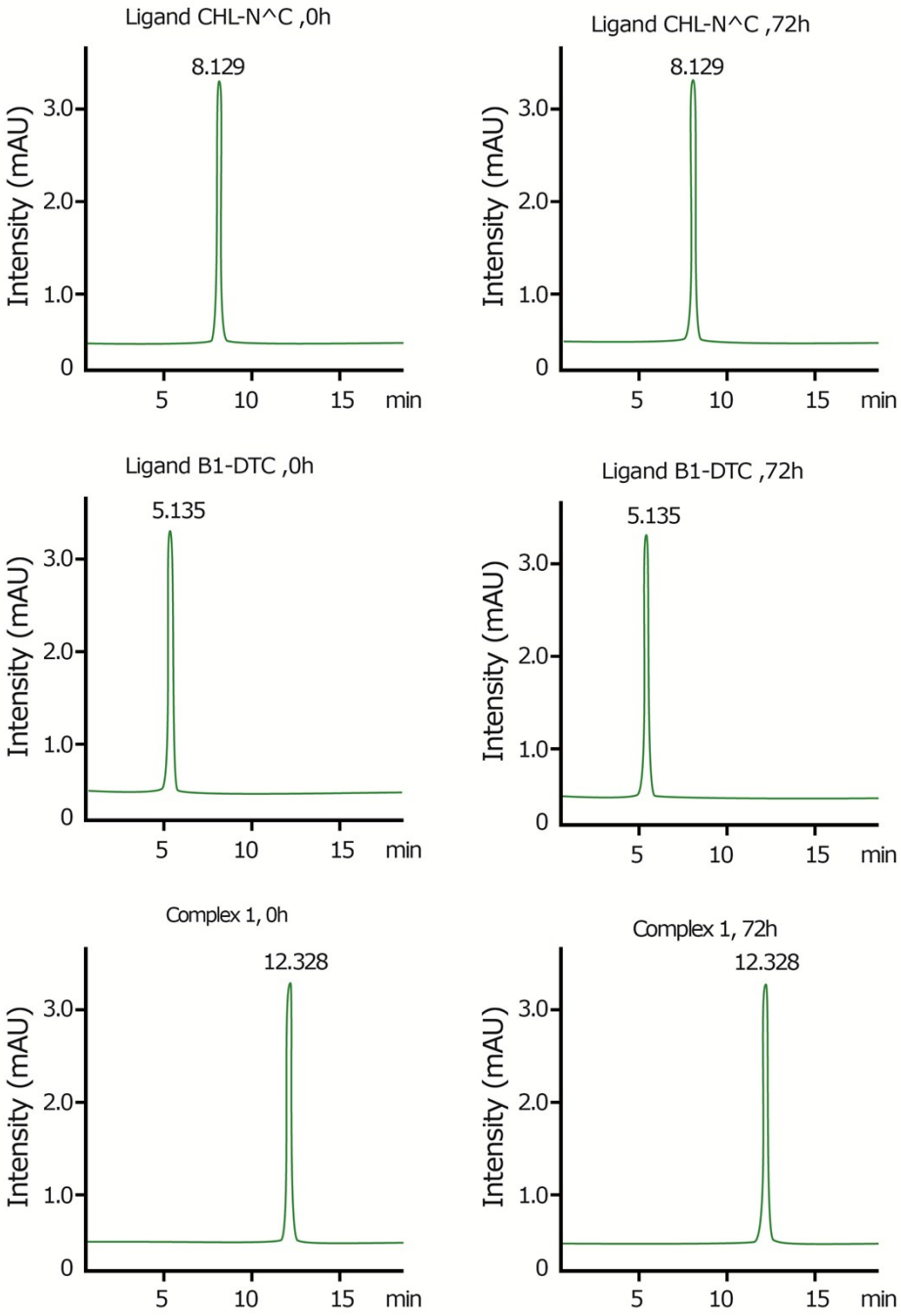


Figure S10. HPLC of the CHL-N^C in PBS buffer/1% DMSO and B1-DTC and complex **1** in PBS buffer solution recorded during 72 h of incubation time at 37 ˚C.

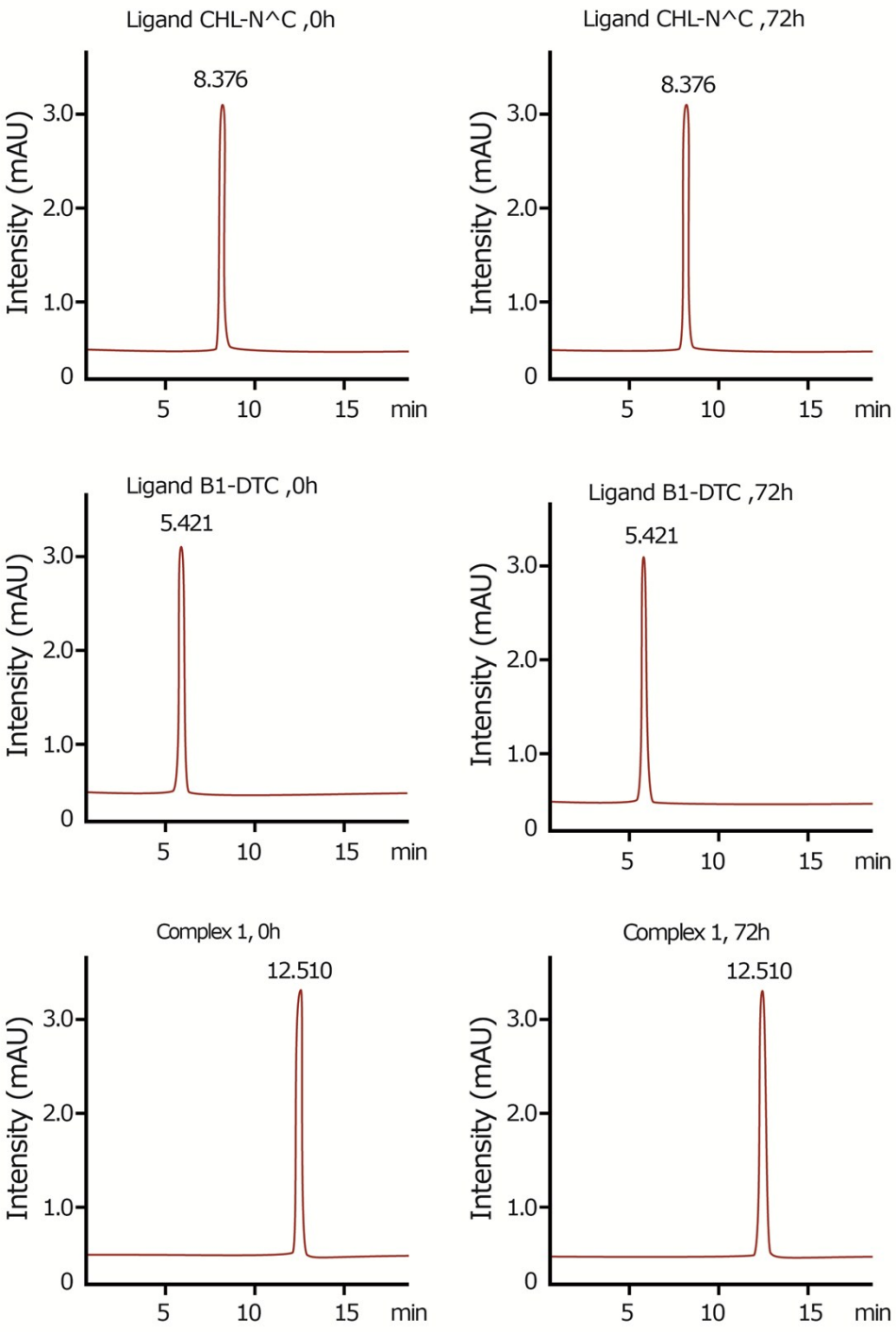


Figure S11. HPLC of the CHL-N^C in DMEM (Dulbecco's Modified Eagle's Medium - high glucose) /1% DMSO solution and B1-DTC and complex **1** in DMEM solution recorded during 72 h of incubation time at 37 ˚C.

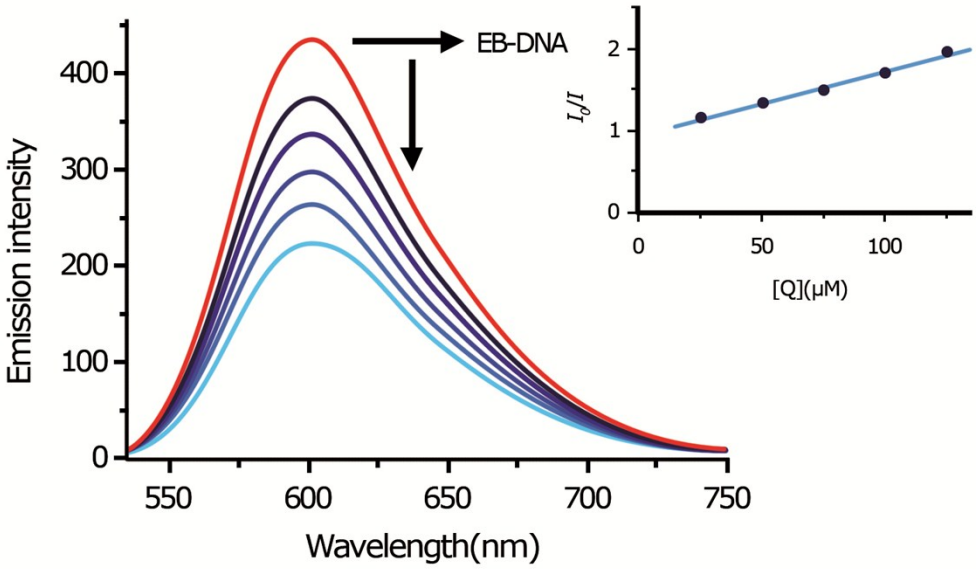


Figure S12. Fluorescence quenching of EB bound to DNA (10 μM) in the presence of the complex **1** (0-50 μM).

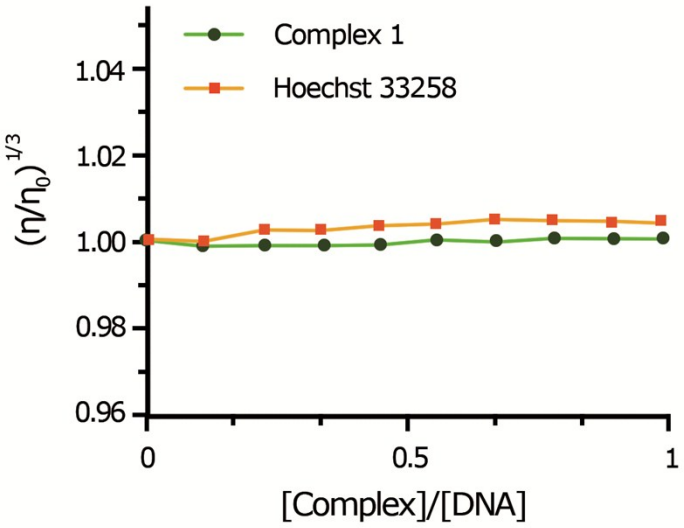


Figure S13. Effect of increasing amounts of Hoechst 33258, and the complex **1** on the relative viscosity of CT DNA at 25 ± 0.1 °C.

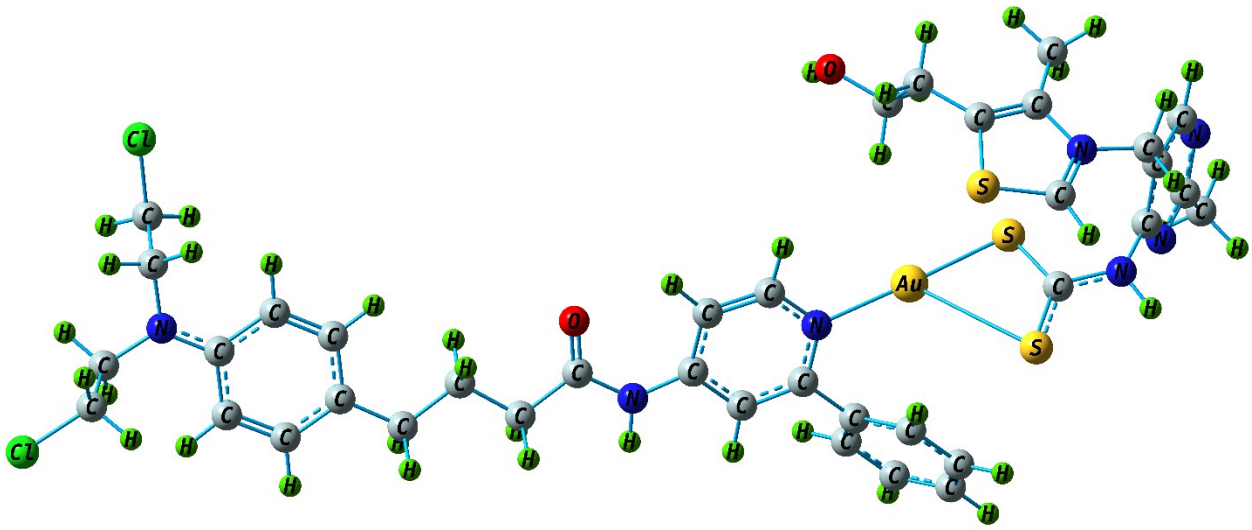
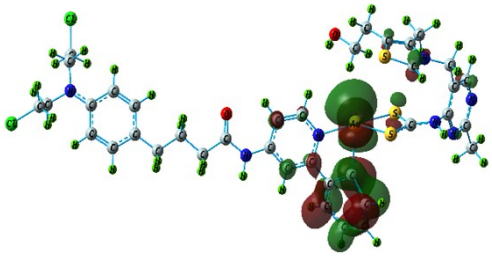
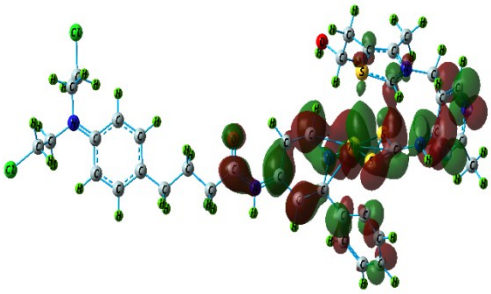


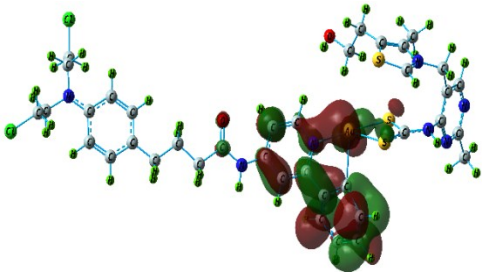
Figure S14. The structure of complex **1** optimized with M062X method also lanl2dz and 6-31G+(d,p) basis sets were used for Au atom and other atoms, respectively.



LUMO

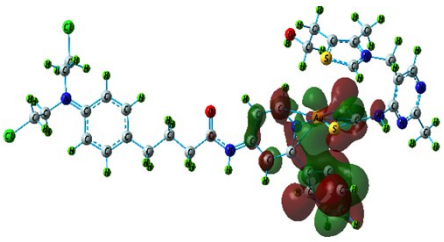


LUMO



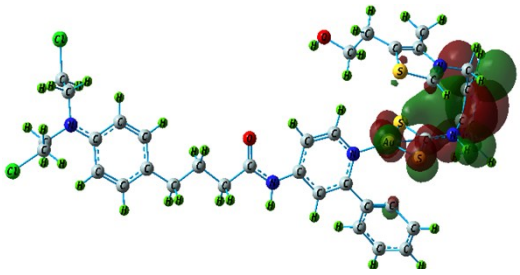
HOMO

(a)

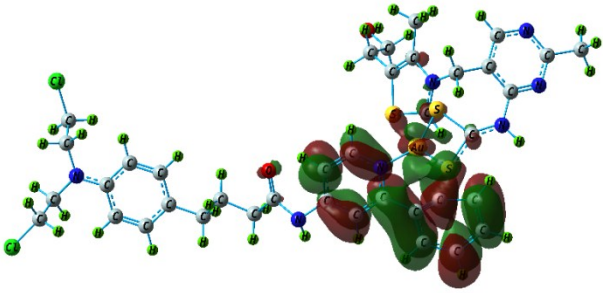


HOMO

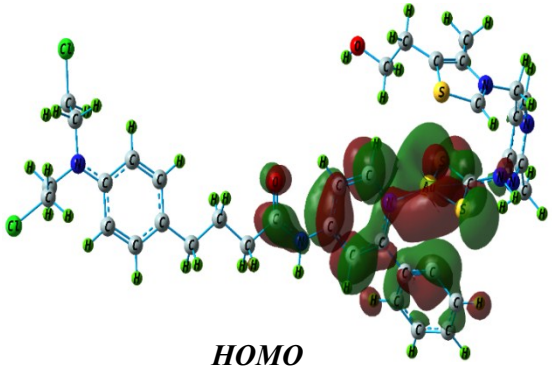
(b)



LUMO

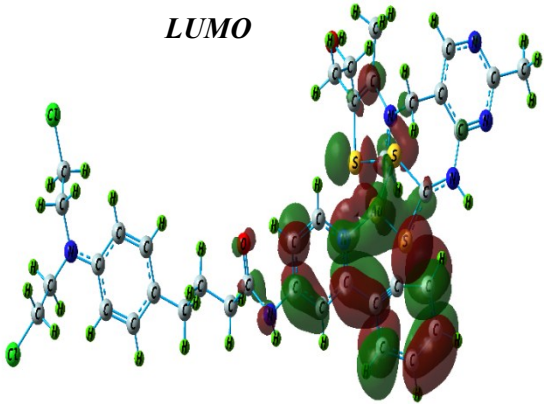


LUMO



HOMO

(c)



HOMO

(d)

Figure S15. The shapes of HOMO and LUMO molecular orbitals of (*a*) optimized structure of complex **1** (*b)* complex **1** after interaction with DNA (*c*) complex **1** extracted from interaction with TrxR1 and *(d)* complex after interaction with TrxR2 with indicated symbol atoms.

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