# Electronic Supplementary Information

# Ruthenium and Osmium-Arene-Based Paullones Bearing a TEMPO Free-Radical Unit as Potential Anticancer Drugs

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## Materials and Methods

All starting materials were purchased from Sigma-Aldrich and Acros and used as received unless specified otherwise. THF was distilled before use over molecular sieves 4 Å. 9-Carboxy-7,12-dihydroindolo[3,2-*d*][1]benzazepin-6(5H)-one (9-carboxy-paullone, **A**) was synthesised as described previously [1], $[Ru^{II}Cl(\mu\text{-}Cl)(\eta^6\text{-p-cymene})]_2$ and $[Os^{II}Cl(\mu\text{-}Cl)(\eta^6\text{-p-cymene})]_2$ were prepared by following the literature procedures [2, 3].

$^1$H NMR spectra were recorded on two Bruker Avance III spectrometers at 500.32 or 500.10 MHz, respectively. $^1$H shifts are quoted relative to the solvent residual signals. UV−vis absorption spectra were recorded on a Perkin-Elmer Lambda 650 spectrophotometer. ESI mass spectra were measured on a Bruker Esquire 3000 mass spectrometer by using methanol as solvent. The *m*/*z* values are quoted for the most abundant isotope. Infrared spectra were obtained on a Bruker Vertex 70 FT-IR spectrometer by means of the attenuated total reflection technique. EPR spectra were recorded on a Bruker EMX spectrometer. All elemental analyses were performed at the Microanalytical Laboratory of the University of Vienna with a Perkin Elmer 2400 CHN Elemental Analyser.

**Cell Lines and Cell Culture Conditions.** For cytotoxicity determination, six different human cancer cell lines were used: A549 (non-small cell lung), SW480 (colon carcinoma), both kindly provided by Brigitte Marian, Institute of Cancer Research, Department of Medicine I, Medical University Vienna, Austria, CH1 (ovarian carcinoma), kindly provided by Lloyd R. Kelland, CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, U.K., T47D (mammary ductal carcinoma), kindly provided by Evelyn Dittrich, Medical University of Vienna, as well as SK-Mel-28 (melanoma), kindly provided by Rodring Marculescu, Medical University of Vienna, and N87 (gastric carcinoma), purchased from ATCC. Cells were grown as adherent monolayer cultures in 75 cm² culture flasks (Iwaki/Asahi Technoglass) in Minimal Essential Medium (A549, CH1 and SW480) supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 1% v/v non-essential amino acids (from 100× stock) and 4 mM *L*-glutamine or RPMI 1640 medium (T47D, N87 and SK-Mel-28) supplemented with 10% heat-inactivated fetal bovine serum and 4 mM *L*-glutamine both without antibiotics at 37 °C under a moist atmosphere containing 5% $CO_2$ and 95% air. All cell culture media and reagents were purchased from Sigma-Aldrich Austria.

**Cytotoxicity Assay.** Cytotoxicity was determined by the colorimetric MTT assay (MTT = 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) as described previously [4]. Briefly, cells were harvested by trypsinisation and seeded into 96-well plates in volumes of 100 µL/well. Depending on the cell line, different cell densities were used to ensure exponential growth of the untreated controls during the experiment: $1.0\times10^3$ (CH1), $2.0\times10^3$ (SW480), $3.0\times10^3$ (A549), $6.0\times10^3$ (T47D and N87), $4.0\times10^3$ (SK-Mel-28). In the first 24 h the cells were allowed to settle and resume exponential growth. Then the test compounds were dissolved in DMSO, serially diluted in medium and added to the plates in volumes of 100 µL/well so that the DMSO content did not exceed 1%. After continuous exposure for 96 h (in the incubator at 37 °C and under 5% $CO_2$), the medium was replaced with 100 µL/well RPMI 1640 medium (supplemented with 10% heat-inactivated fetal bovine serum and 4 mM *L*-glutamine) and MTT solution (MTT reagent in phosphate-buffered saline, 5 mg/mL) in a ratio of 7:1, and plates were incubated for further 4 h. Then the medium/MTT mixture was removed and the formed formazan was dissolved in DMSO (150 µL/well). Optical densities at 550 nm were measured (reference wavelength 690 nm) with a microplate reader (Tecan). The quantity of viable cells was

expressed as a percentage of untreated controls, and 50% inhibitory concentrations ($IC_{50}$) were calculated from the concentration-effect curves by interpolation. Every test was repeated in at least three independent experiments, each consisting of three replicates per concentration level.

**Plasmid DNA Interaction Studies.** pUC19 DNA (2686 bp) plasmid was purchased from Fermentas Life Sciences. 500 ng of pUC19 plasmid was incubated with 10 µM and 50 µM of the test compounds in a 0.1× Tris-EDTA (TE) buffer for different time intervals (5 min up to 6 h) at 37 °C. The electrophoresis was performed in agarose (from Sigma-Aldrich) gel 1% w/v in 1× Tris-Borate-EDTA (TBE) buffer for 90 min at 80 V in 1× TBE buffer. Gels were stained with ethidium bromide (EtBr) in 1× TBE (0.75 µg/ml) for 20 min. Images were taken with the gel documentation system GelDoc-It Imaging System (UVP).

**Comet Assay.** The alkaline single cell gel electrophoresis (comet assay) was performed as described previously [5]. SW480 cells were seeded with a density of $4 \times 10^5$ cells in a total volume of 2 mL complete MEM medium in 6-well plates and allowed to settle for 24 h. Cells were treated with 50 µM methyl methanesulfonate (MMS) as a positive control as well as in co-incubation experiments. Test compounds were dissolved in DMSO and diluted with medium to a final concentration of 50 µM with less than 0.5% v/v of DMSO. The medium was removed and the cells were incubated with the test compounds for 2 or 4 h. Co-incubation of the respective compound and MMS (50 µM) was used to determine an impact of the complex on MMS-induced DNA damage. Afterwards cells were harvested by trypsinisation and centrifuged (3 min, 300× g). $2 \times 10^4$ cells were taken and suspended in low melting agarose (0.8%) and applied to frosted microscope slides pre-coated with a layer of normal melting agarose (0.5%). After removal of the cover slips, the slides were incubated in lysis buffer (89% lysis buffer stock solution: 2.5 mM sodium chloride, 100 mM EDTA, 10 mM Tris; 1% Triton-X-100, 10% DMSO) for 1 h at 37 °C. Thereafter the slides were placed in the electrophoresis chamber and DNA strands were allowed to segregate for 20 min in electrophoresis buffer (300 mM NaOH, 1 mM EDTA). After electrophoresis (25 V, 300 mA, 20 min) slides were washed with 0.4 M Tris-HCl, pH 7.5 and each sample was stained with 40 µL ethidium bromide (50 µg/mL). Analysis was done under a fluorescence microscope (Olympus BX40, $\lambda_{ex} \sim 550$ nm $\lambda_{em} \geq 590$ nm) by use of the Comet Assay IV system (Perceptive Instruments). 100 individual cells

randomly chosen were analysed from each sample. Experiments were carried out independently at least 3 times. The results were evaluated with respect to the tail intensity (intensity of DNA fluorescence in the comet tail relative to the overall DNA fluorescence intensity in the cell).

**Synthesis**

**9-Carboxy-7,12-dihydroindolo[3,2-*d*][1]benzazepin-6(5*H*)-thione, (B)** A mixture of 9-carboxy-7,12-dihydroindolo[3,2-*d*][1]benzazepin-6(5H)-one (10 g, 34 mmol), phosphorus pentasulfide (23 g, 52 mmol) and sodium hydrogen carbonate (34 g, 4.4 mmol) was refluxed in THF (700 ml) under argon atmosphere for 3 h. Warm solution was poured onto crushed ice (2 kg), the mixture was then stirred until the ice was molten, the precipitate formed was filtered off and washed with water and acetone. The yellow product was dissolved in methanol containing 1.5% of triethylamine, re-precipitated by addition of diluted acetic acid (30%), collected under suction, washed with methanol and dried in vacuo. Yield: 8.1 g, 67%. Anal. Calcd for $C_{17}H_{12}N_2O_2S(H_2O)_2 \cdot 0.15(C_4H_8O)$, ($M_r$ = 355.20 g/mol): C, 59.51; H, 4.88; N, 7.89; S, 9.03%. Found: C, 59.84; H, 4.83; N, 7.63; S, 9.43%. IR ($cm^{-1}$): 1671, 1284, 759. $^1$H NMR (DMSO-$d_6$, δ): 1.76 (m, 0.55 H, THF), 3.60 (m, 0.60 H, THF), 3.95 (s, 2H, $CH_2$), 7.41−7,46 (m, 3H), 7.52 (dd, 1H, 0.4 /6.8 Hz), 7.80−7.83 (m, 2H), 8.30 (s, 1H), 12.09 (s, 1H, NH), 12.11 (s, 1H, NH), 12.57 (br.s., 1H, COOH). ESI-MS in methanol (positive): 309 $[M+H]^+$, 331 $[M+Na]^+$.

**9-Carboxy-6-*N*-(2-methylaminopyridine)-7,12-dihydroindolo-[3,2-*d*][1]benzazepine (C).** To a suspension of 9-carboxy-7,12-dihydroindolo[3,2-*d*][1]benzazepin-6(5*H*)-thione (8.0 g, 22.5 mmol) in ethanol (200 ml) 2-aminomethylpyridine (8 ml, 78 mmol) was added and the mixture was refluxed under argon atmosphere for 2 h. The unreacted thione was filtered off and the solution was refluxed for further 15 h. After cooling the white precipitate formed was collected by filtration, washed with ethanol and dried in vacuo. Yield: 5.8 g, 55%. Anal. Calcd for $C_{23}H_{18}N_4O_2 \cdot 1.25H_2O$ ($M_r$ = 404.93 g/mol): C, 68.22; H, 4.10; N, 13.84%. Found: C, 68.10; H, 4.72; N, 13.67%. IR ($cm^{-1}$): 1649, 1344, 752. $^1$H NMR (DMSO-$d_6$, δ): 3.48 (s, 2H, $CH_2$), 4.51 (d, 2H, $CH_2$, 3.2 Hz), 7.04−7.10 (m, 2H), 7.21−7.29 (m, 3H), 7.45 (d, 1H, 6.8 Hz), 7.65−7.71 (m, 2H), 7.76 (d, 1H, 7.2 Hz), 7.95 (br.s., 1H, NH), 11.79 (s, 1H, indole-NH), 12.43 (br.s., 1H, COOH). ESI-MS in methanol (positive): 383 $[M+H]^+$.

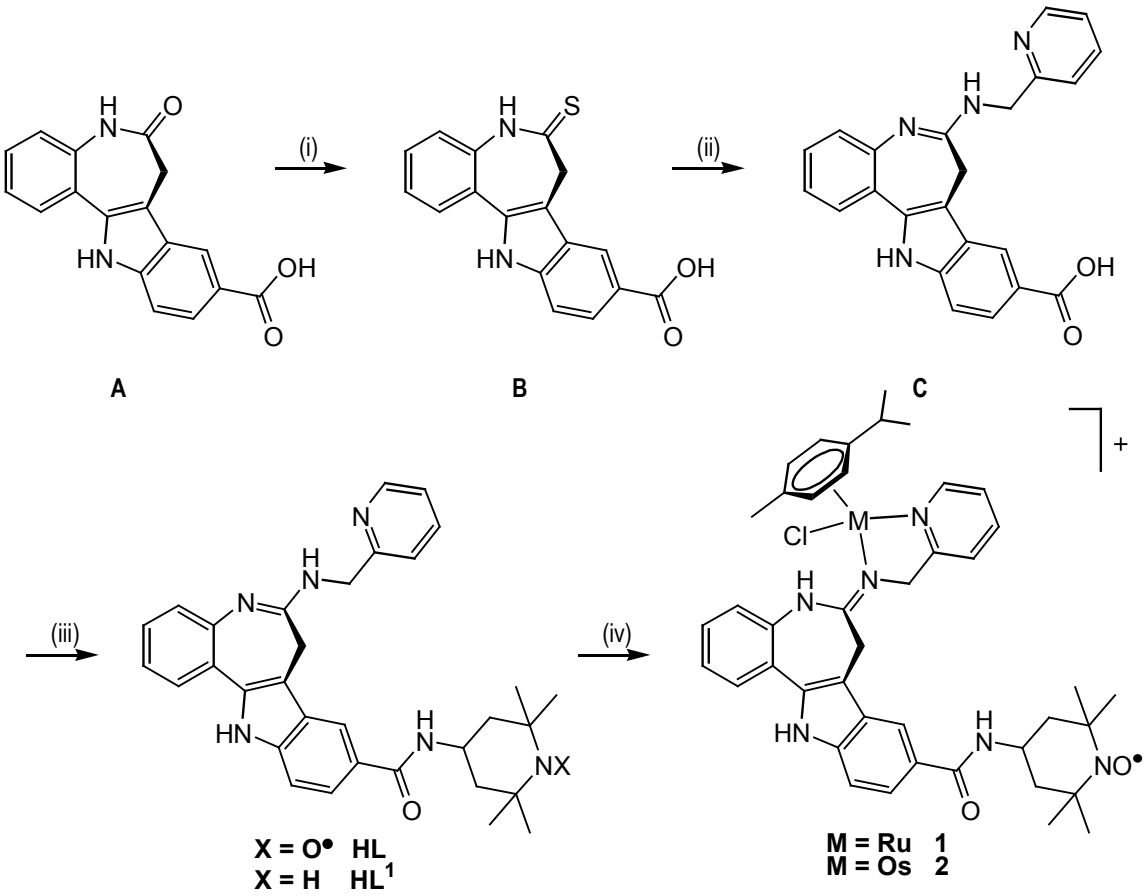
***N*-(4-amino-2,2,6,6-tetramethylpiperidine-*N*-oxyl)-6-*N*-(2-methylaminopyridine)-7,12-dihydroindolo-[3,2-d][1]benzazepine-9-carboxamide (HL).** To a solution of 9-carboxy-6-*N*-(2-methylaminopyridine)-7,12-dihydroindolo-[3,2-*d*][1]benzazepine (**C**) (1.0 g, 2.5 mmol) in DMF (90 ml) and triethylamine (10 ml) were added 1-hydroxybenzotriazole (0.75 g, 5.5 mmol), and then after 5 min 4-amino-TEMPO (0.7 g, 4.3 mmol) in DMF (5 ml) and EDCI (1.05 g, 5.5 mmol). The mixture was stirred at room temperature for 24 h and the solvent was evaporated under reduced pressure. The residue was dried in vacuo, dissolved in THF (30 ml) and purified by column chromatography on silica using THF as eluent. Fractions having in ESI-MS the only peak at *m*/*z* 536 were combined, evaporated under reduced pressure and the residue dissolved in methanol (5 ml). After 1 h crystals formed were collected under suction, washed with small amount of methanol and dried in vacuo. Yield: 0.386 g, 27%. Anal. Calcd for $C_{32}H_{35}N_6O_2 \cdot 0.5CH_3OH \cdot 1.5H_2O$, ($M_r$ = 578.70 g/mol): C, 67.45; H, 6.97; N, 14.52%. Found: C, 67.29; H, 6.58; N, 14.46%. IR ($cm^{-1}$): 1539, 1328, 762. ESI-MS in methanol (positive): 536 $[M+H]^+$. UV−vis ($1 \times 10^{-4}$ M in $CH_3OH$): $\lambda_{max}$, nm ($\varepsilon$, $M^{-1}cm^{-1}$): 260 (1300), 323 (300).

***N*-(4-amino-2,2,6,6-tetramethylpiperidine-*N*-oxyl)-6-*N*-(2-methylaminopyridine)-7,12-dihydroindolo-[3,2-*d*][1]benzazepine-9-carboxamide dihydrochloride hydrate ($HL^1 \cdot 1.3H_2O$).** The substance was prepared analogously to **HL** by using 4-amino-2,2,6,6-tetramethylpiperidine instead of 4-amino-TEMPO. Yield: 35%. Anal. Calcd for $C_{32}H_{36}N_6O \cdot 2HCl \cdot 1.33H_2O$, ($M_r$ = 617.55 g/mol): C, 62.24; H, 6.64; N, 13.61, Cl, 11.48%. Found: C, 62.18; H, 6.29; N, 13.51, Cl, 11.22%. IR ($cm^{-1}$): 1651, 671. $^1$H NMR (DMSO-$d_6$, δ; to reduce the influence of HCl on the spectrum an equimolar amount of $CH_3COONa$ was added): 1.35 (s, 6H), 1.40 (s, 6H), 1.56 (t, 2H, 10.0 Hz), 1.86 (dd, 2H, 2.4 /10.4 Hz), 3.48 (s, 2H), 4.37 (m, 1H), 4.50 (s, 2H), 7.02−7.08 (m, 2H), 7.20−7.24 (m, 2H), 7.28 (d, 1H, 6.4 Hz), 7.42 (d, 1H, 6.8 Hz), 7.63−7.67 (m, 2H), 7.71 (dd, 1H, 1.0/6.2 Hz), 7.97 (br.s., 1H), 8.28 (s, 2H), 8.49 (d, 1H, 3.6 Hz), 11.72 (s, 1H). ESI-MS in methanol (positive): 521 $[M+H]^+$.

**[($\eta^6$-*p*-Cymene)-*N*-(4-amino-2,2,6,6-tetramethylpiperidine-*N*-oxyl)-6-*N*-(2-methylamino-pyridine)-7,12-dihydroindolo-[3,2-*d*][1]benzazepine-9-carboxamide)chloridoruthenium(II)] chloride dihydrate (1).** To a solution of *N*-(4-amino-2,2,6,6-tetramethylpiperidine-*N*-oxyl)-6-*N*-

(2-methylaminopyridine)-7,12-dihydroindolo-[3,2-d][1]benzazepine-9-carboxamide (**HL**) (0.216 g, 0.4 mmol) in methylene chloride (20 ml) was added a solution of bis(($\eta^6$-*p*-cymene)(chlorido)(μ-chlorido)ruthenium(II)) (0.124 g, 2 mmol) in methylene chloride (5 ml) and the mixture was allowed to stand at room temperature for 2 h. The light-brown precipitate formed overnight at−20 °C was filtered off , washed with small amount of cold $CH_2Cl_2$ and dried in vacuo. Yield: 0.14 g, 41%. Anal. Calcd for $C_{42}H_{49}Cl_2N_6O_2Ru{\cdot}2H_2O$ ($M_r$ = 877.88 g/mol): C, 57.46; H, 6.09; N, 9.57; Cl, 8.08%. Found: C, 57.18; H, 5.86; N, 9.88; Cl, 8.04%. IR ($cm^{-1}$): 1621, 1321,763. ESI-MS in methanol (positive): 806 $[M{-}Cl]^+$, 770 $[M{-}Cl{-}HCl]^+$. UV−vis, $5 \times 10^{-5}$ M in $CH_3OH$: $\lambda_{max}$, nm ($\varepsilon$, $M^{-1}cm^{-1}$): 256 (55600), 322 (20300).

**[($\eta^6$-*p*-Cymene)-*N*-(4-amino-2,2,6,6-tetramethylpiperidine-*N*-oxyl)-6-*N*-(2-methylamino-pyridine)-7,12-dihydroindolo-[3,2-d][1]benzazepine-9-carboxamide)chloridoosmium(II) chloride trihydrate (2).** The procedure was the same as for **1**, but starting from bis[($\eta^6$-*p*-cymene)(chlorido)($\mu$-chlorido)osmium(II)]. Yield: 32%. Anal. Calcd for $C_{42}H_{49}Cl_2N_6O_2Os{\cdot}3H_2O$ ($M_r$ = 985.06 g/mol): C, 51.21; H, 5.63; N, 8.53%. Found: C, 51.25; H, 5.34; N, 8.71%. IR ($cm^{-1}$): 1616, 1321, 659. ESI-MS in methanol (positive): 896 $[M{-}Cl]^+$, 860 $[M{-}Cl{-}HCl]^+$. UV−vis ($CH_3OH$ : $H_2O$ 1:1), $\lambda_{max}$, nm ($\varepsilon$, $M^{-1}cm^{-1}$): 249 (59800), 321 (25200).




**Scheme S1.** Synthesis of **HL**, **1** and **2**; reagents and conditions: (i) $P_4S_{10}$, THF, reflux 3 h; (ii) 2-aminomethylpyridine, EtOH, reflux 15 h; (iii) 4-amino-2,2,6,6-tetramethylpiperidine-*N*-oxyl (or 4-amino-2,2,6,6-tetramethylpiperidine in case of **HL**[1]), 1-hydroxybenzotriazole, EDCI, $Et_3N$, DMF, RT, 24 h; (iv) $[MCl_2(p\text{-cymene})]_2$, $CH_2Cl_2$, RT, 2 h.

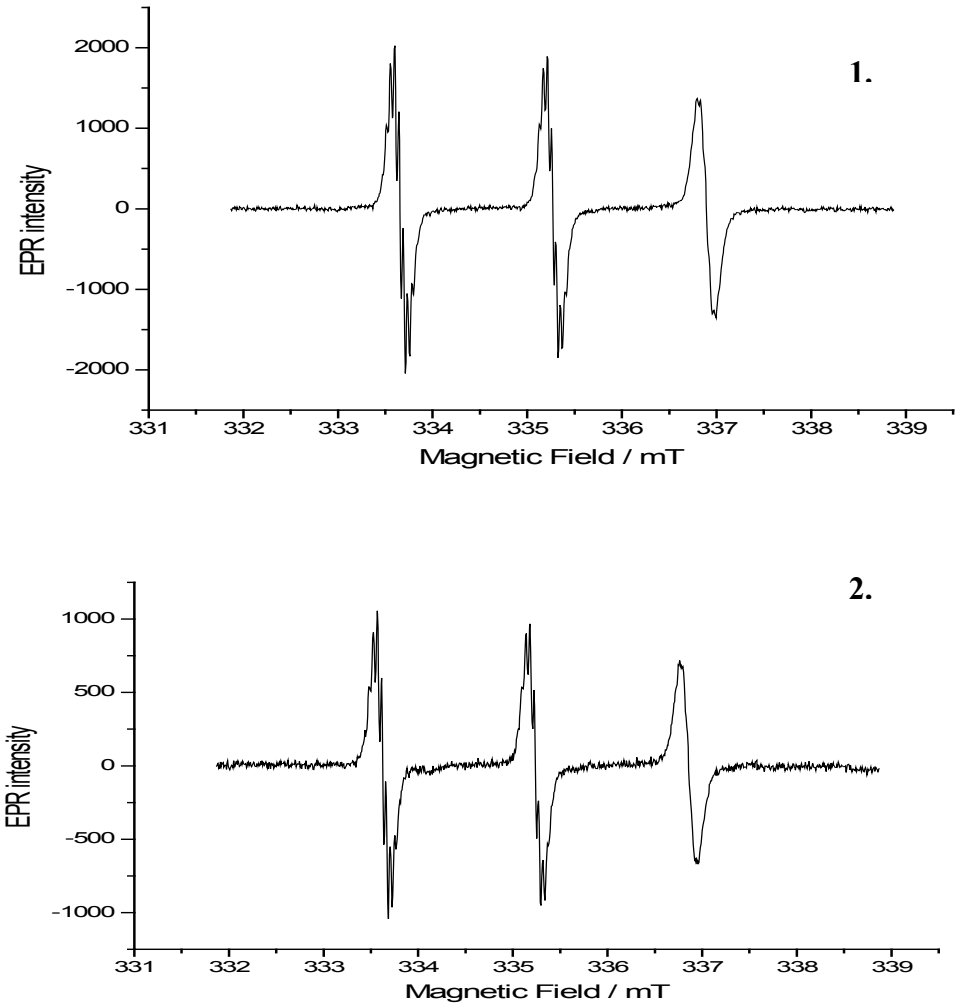
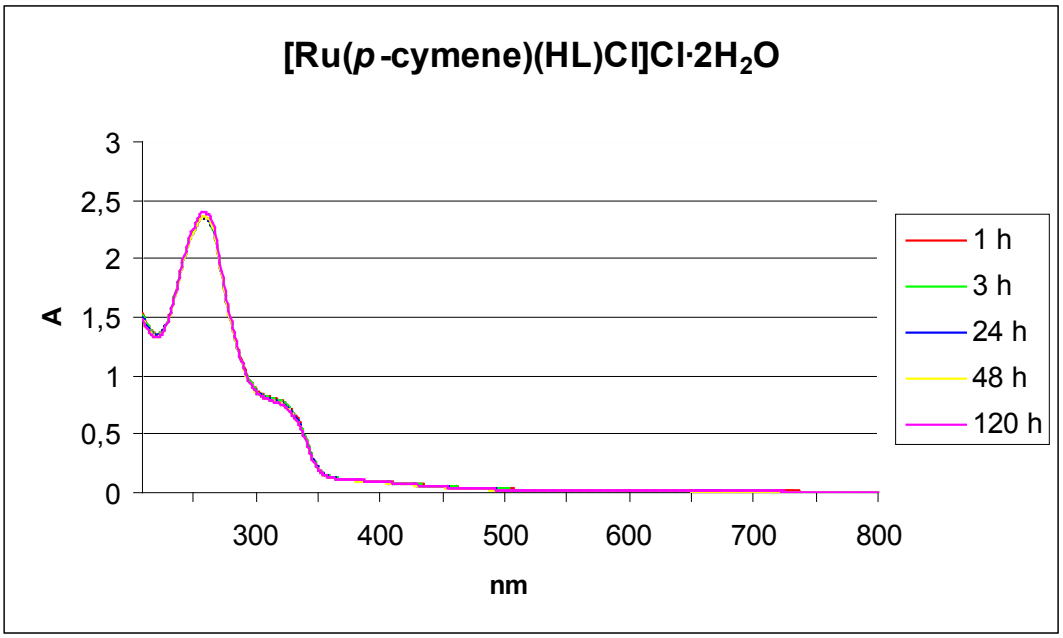



**Figure S1.** EPR spectra of $10^{-4}$ M solutions of (a) **1** and (b) **2** in $CH_3OH$ (EPR parameters: sweep width 70 G, modulation amplitude 0.1 G, microwave bridge frequency 9.482 GHz, power 10 mW).



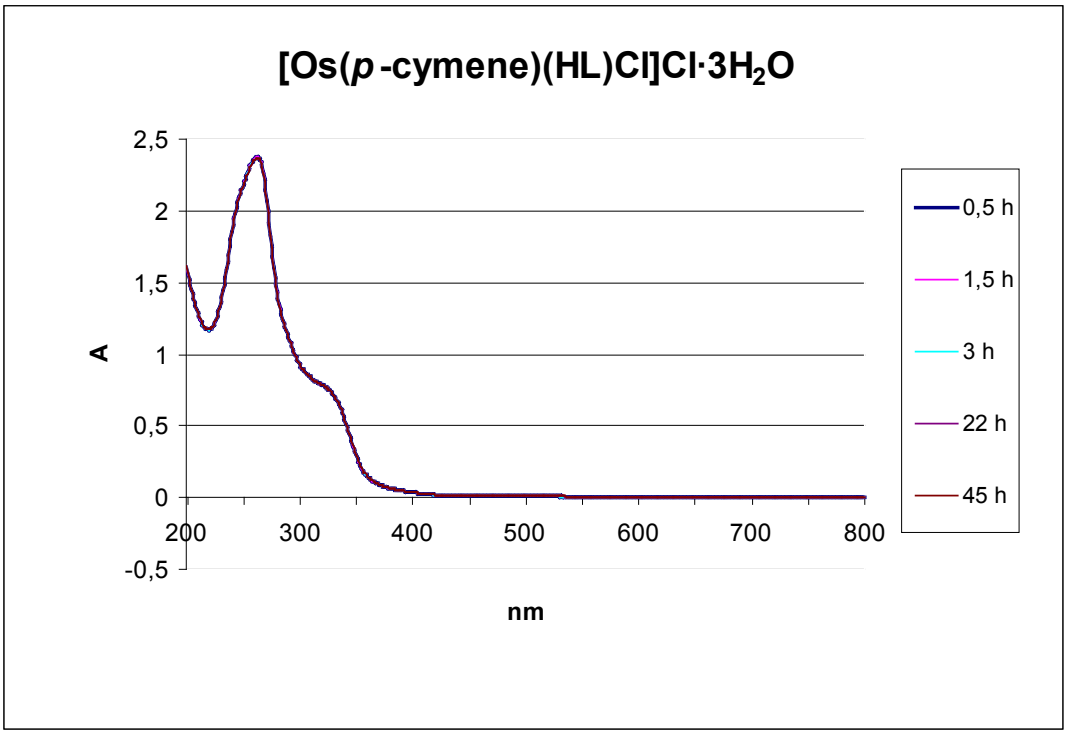




**Figure S2**. The UV−vis spectra of $4.5 \times 10^{-5}$ M solution of **1** and **2** measured over time in $CH_3OH/H_2O$ 1:1.

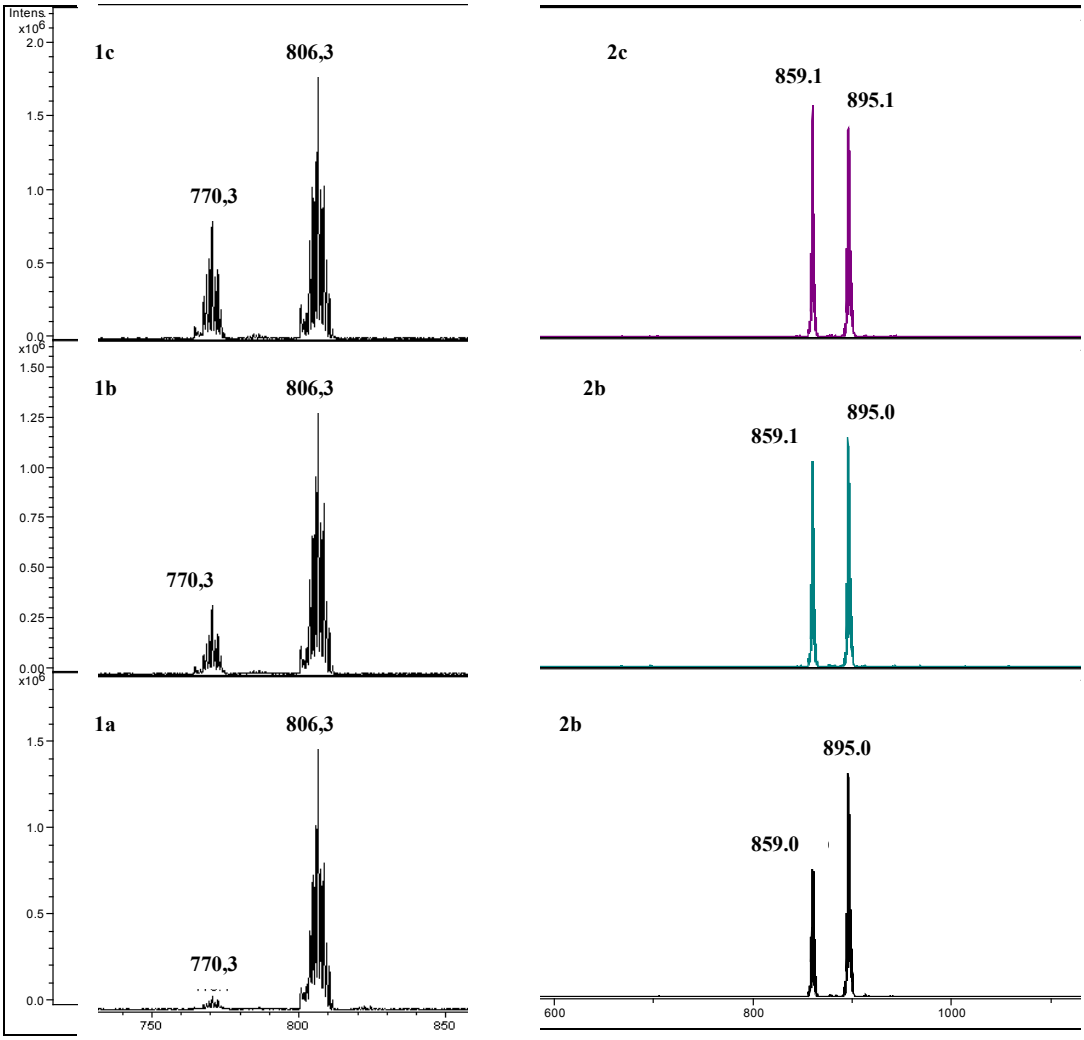



**Figure S3**. The time-resolved ESI MS spectra of $5.5 \times 10^{-5}$ M solutions of **1** in methanol/water 1:1 measured 5 min (1a), 2 h (1b) and 7 h (1c) and **2** measured 5 min (2a), 30 min (2b) and 90 min (2c) after preparation.

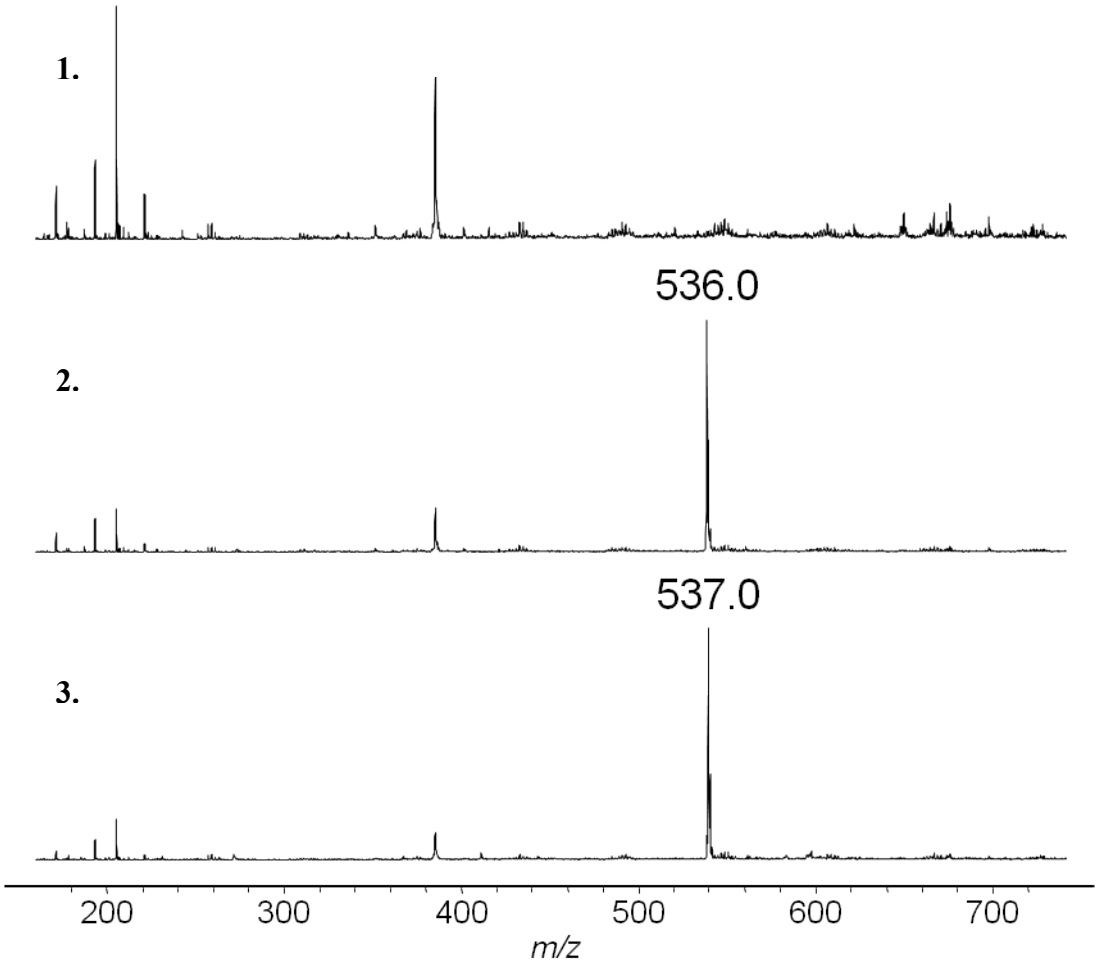



**Figure S4.** ESI mass spectra of Minimum Essential Medium (**MEM) – 1, MEM + HL – 2, MEM + HL + Ascorbic acid – 3.**

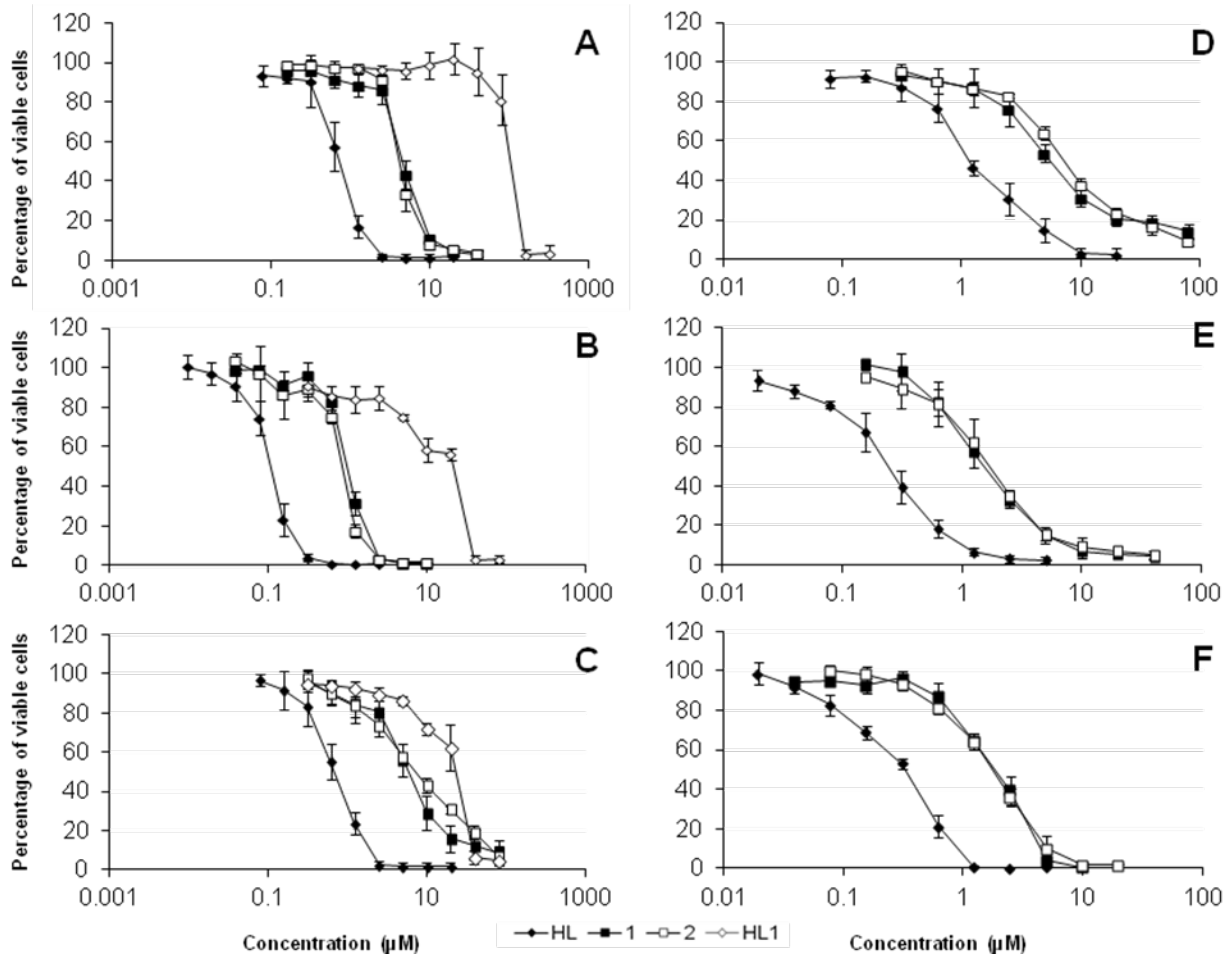



**Figure S5.** Concentration-effect curves of **HL**, **HL**[1], **1**, and **2** in the human cancer cell lines A549 (A), CH1 (B), SW480 (C), T47D (D), N87 (E) and SK-Mel-28 (F), all determined by the MTT assay using continuous exposure for 96 h.

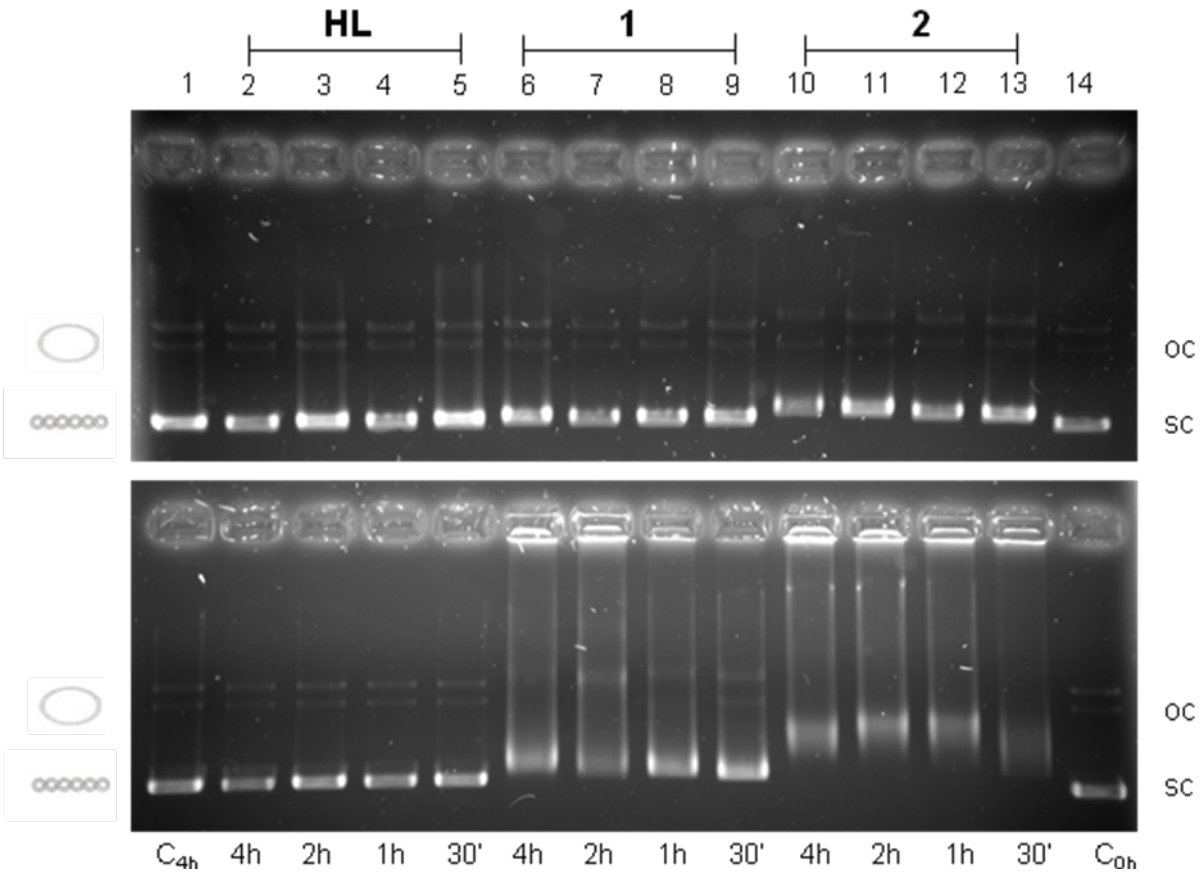



**Figure S6.** Electropherograms of dsDNA plasmid pUC19 after exposure to 10 μM (top) and 50 μM (bottom) of paullone ligand **HL** (lanes 2−5), ruthenium complex **1** (lanes 6−9) and osmium complex **2** (lanes 10−13) for different exposure times compared to untreated controls (C). Retardation of the supercoiled (sc) form by complexes **1** and **2** (but not **HL**) indicates untwisting by DNA binding. Disappearance of the band corresponding to the open-circular (oc) form of the plasmid and concurrent immobilisation of DNA in gel pockets suggest the formation of larger DNA aggregates, e.g. by interhelical cross-links.

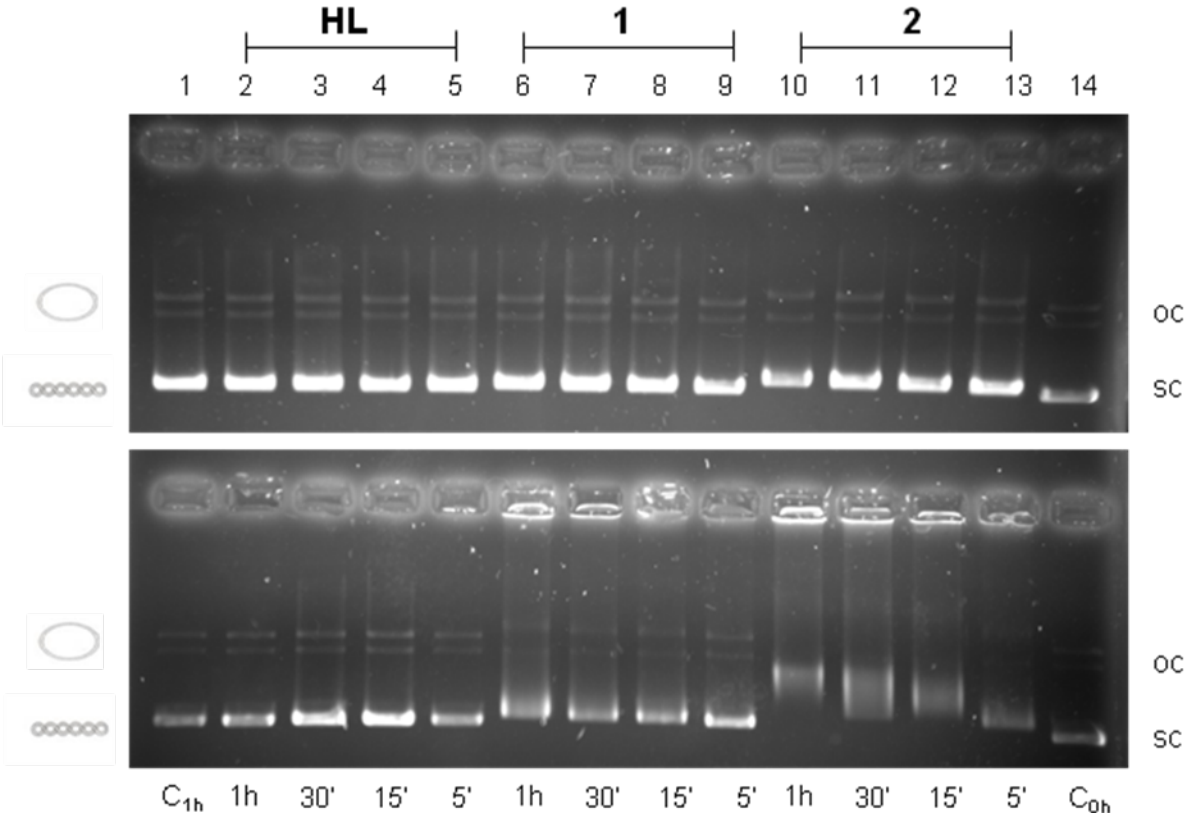



**Figure S7.** Electropherograms of dsDNA plasmid pUC19 after exposure to 10 μM (top) and 50 μM (bottom) of paullone ligand **HL** (lanes 2–5), ruthenium complex **1** (lanes 6–9) and osmium complex **2** (lanes 10−13) for different exposure times compared to untreated controls (C). Retardation of the supercoiled (sc) form by complexes **1** and **2** (but not **HL**) indicates untwisting by DNA binding. Disappearance of the band corresponding to the open-circular (oc) form of the plasmid and concurrent immobilisation of DNA in gel pockets suggest the formation of larger DNA aggregates, e.g. by interhelical cross-links.

**Table S1.** Tail intensities in [%] in SW480 cells treated with 50 µM of MMS as well as **HL**, **1** and **2** alone and in combination with 50 µM MMS for 2 and 4 h at 37 °C, determined by the comet assay.

| | **2 h** | **4 h** |
|---|---|---|
| **control** | 4.7 ± 0.6 | 3.5 ± 0.2 |
| **MMS** | 17.7 ± 1.5 | 28.3 ± 3.3 |
| **HL** | 5.6 ± 0.5 | 4.7 ± 0.9 |
| **1** | 5.6 ± 0.6 | 5.0 ± 1.3 |
| **2** | 6.0 ± 1.1 | 5.5 ± 0.9 |
| **HL + MMS** | 17.6 ± 3.0 | 29.6 ± 1.6 |
| **1 + MMS** | 21.7 ± 1.6 | 29.6 ± 4.0 |
| **2 + MMS** | 24.4 ± 3.4 | 32.5 ± 0.9 |

## References

1. T. Pies, Dissertation, Universität Hamburg, 2003.

2. M. A. Bennett, A. K. Smith, *J. Chem. Soc. Dalton Trans*., 1974, 233.

3. W. Kiel, G. Bal, A.G. Graham, *J. Organomet. Chem.* 1990, **383**, 481.

4. M.F. Primik, S. Göschl, M.A. Jakupec, A. Roller, B.K. Keppler, V.B. Arion, *Inorg. Chem*. 49 (2010) 11084–11095.

5. C. Bartel, A. K. Bytzek, *J. Biol. Inorg. Chem*., 2012, **17**, 465.