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Ru^{II} and Ru^{III} complexes with 2,6-di-*tert*-butylphenol ligands: synthesis, electrochemical behaviour, antioxidant properties and antiproliferative activity

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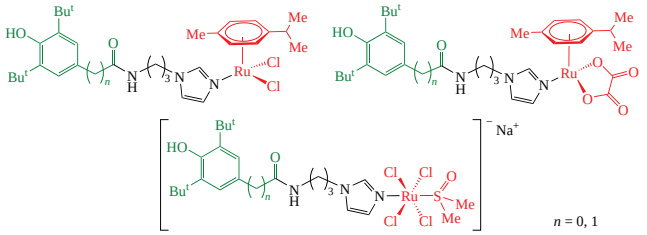
New ruthenium(II) and ruthenium(III) complexes with 2,6-di-*tert*-butylphenol as the antioxidant moiety were synthesized, and their antioxidant and antiproliferative activities were evaluated. Electrochemical behaviour and the potential of these compounds to act as inhibitors of lipid peroxidation in biological systems were explored.

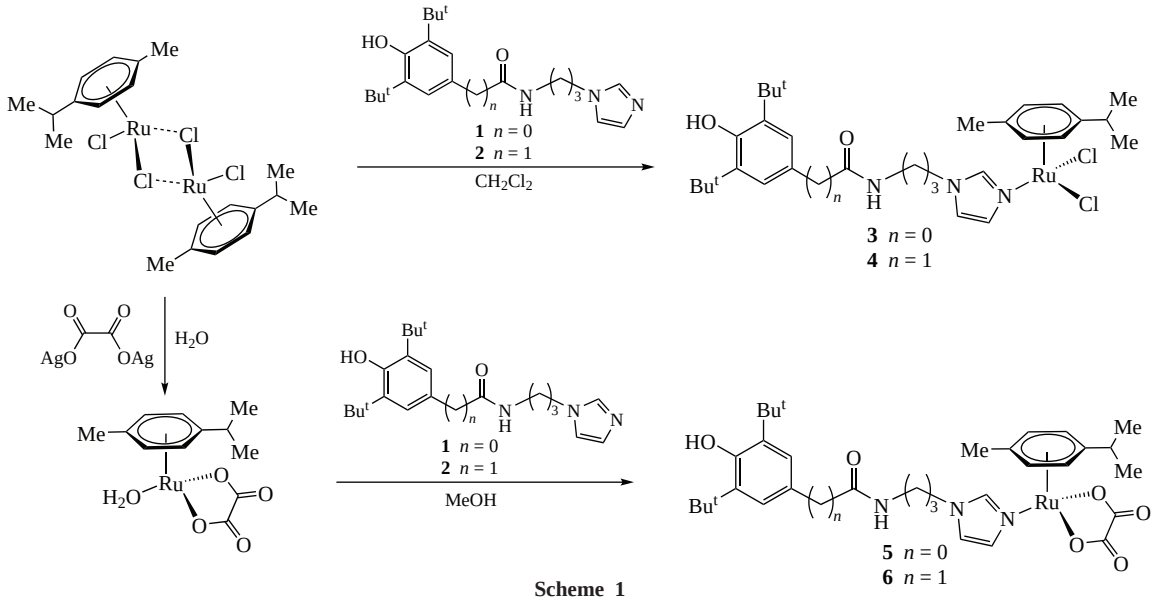


Keywords: ruthenium compounds, antioxidant activity, DPPH assay, CUPRAC assay, electrochemical behaviour, antiproliferative activity, lipid peroxidation.

Platinum-based chemotherapies, such as cisplatin, have been widely used as anticancer drugs for decades. They exert their anticancer effects by binding to DNA and causing damage, ultimately leading to cell death.[1–6] However, their usage is limited by their toxicity and the development of resistance in cancer cells. The development of alternative anticancer drugs to platinum-based chemotherapies has been a major focus in cancer research.[7–9] Ruthenium compounds have emerged as promising candidates due to their structural diversity and multi-targeting potential modes of action.[7,9–11] More diversity in structure allows for the design of ruthenium-based drugs with specific properties, such as increased selectivity for certain types of cancer cells or reduction in overall toxicity.[12] Additionally, the possibility of introducing substituents can also enhance the stability and bioavailability of these compounds, improving their efficacy as anticancer agents.[13–17] The development of ruthenium-based compounds as anticancer agents has garnered significant attention in recent years. These compounds have shown promising results in preclinical studies and have the potential to overcome some of the limitations of traditional chemotherapy drugs. One of the most well-known ruthenium-based anticancer agents is Ru^{III} complex NAMI-A.[18–20] Clinical trials of NAMI-A have shown promise, but it was rejected from stage II due to its toxicity profile and lack of convincing preliminary efficacy results.[21] Another promising ruthenium-based compound is BOLD-100 (formerly known as KP1339) which has been shown to induce cancer cell death through multiple mechanisms, including inhibition of DNA repair and induction of oxidative stress. Compound BOLD-100 recently received the FDA Orphan Drug Designation (ODD) for the treatment of gastric cancer which is a significant achievement in the ruthenium-based anticancer agents development.[22]

Ruthenium(II) compounds also represent a promising class of anticancer agents.[9,23] One of the most studied Ru^{II} compounds is RAPTA-C, which has been found to induce apoptosis in cancer cells by disrupting redox processes. This compound has shown efficacy against both solid tumours and hematological malignancies in preclinical studies. Another promising Ru^{II} compound is RM175 which has shown efficacy against various types of cancer, including breast, lung, and ovarian cancer.[24] As for Ru^{II} prodrugs, the mechanism was believed similar to that of cisplatin (activation of the complex *via* ligand exchange, followed by binding to the 7th nitrogen atom of guanine),[25,26] but now other significant proteins are thought to be key molecular targets. Compound BOLD-100 like almost all Ru complexes induces the formation of reactive oxygen species (ROS), which causes DNA damage and cell cycle arrest.[27] However, the non-specific nature of ROS generation can lead to increased toxicity of ruthenium complexes towards healthy cells. To mitigate this issue, antioxidant fragments could be used to provide cytoprotective effects to healthy cells and decrease general toxicity. Hindered phenols, the vitamin E mimetics, are well-studied antioxidants with broad cytoprotective effects[28] and they have been used to decrease the high toxicity of metal complexes by inhibiting radical oxidative processes.[29–32]

In the present study, 2,6-di-*tert*-butylphenol was used as an antioxidant protective moiety. The development of compounds is based on the variation of antioxidant activity of phenolic ligands between healthy and tumour cells. The protonation of the phenolic group in tumour cells, which occurs due to the increased acidity of the cellular environment, leads to a reduction in its antioxidant activity. This, in turn, means that these ligands will not be able to protect tumour cells from the toxic effects caused by an increase in the content of ROS. Conversely, in healthy



Scheme 1

cells, the same ligands will fully perform their protective functions. Therefore, the design of compounds with selective antioxidant activity in healthy cells is a promising approach to the development of new ruthenium-based anticancer agents.

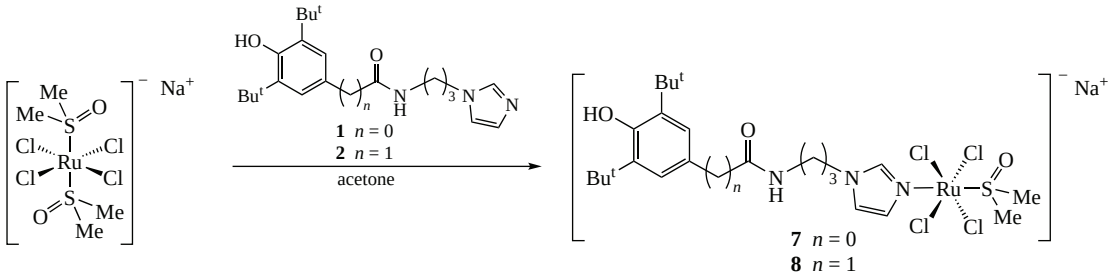
Ligands **1** and **2** were obtained using the previously described method with slight modifications (see Online Supplementary Materials, Scheme S1).[33] Ruthenium(II) chloride complexes **3** and **4** were prepared by the reaction of ligands with (η^6-*p*-cymene)ruthenium dichloride dimer (Scheme 1). Complex **3** was precipitated from the reaction mixture by the addition of ethyl acetate, and complex **4** was obtained by removing the solvent and washing the product. Ruthenium(II) complexes **5**, **6** with oxalate ligands were synthesized by treatment of the ligands with (η^6-*p*-cymene)Ru(C_2O_4)(H_2O) obtained *in situ via* the reaction between (η^6-*p*-cymene)ruthenium dichloride dimer and silver oxalate (see Scheme 1).[16] Complexes **5** and **6** were isolated by column chromatography on silica gel. All compounds were characterized by NMR spectroscopy (^{1}H), electrospray ionization mass spectrometry (ESI-MS) and elemental analyses (see Online Supplementary Materials, Figures S6–S15).

Ruthenium(III) complexes **7** and **8** were obtained by replacing one DMSO ligand in Na[Ru(Me_2SO)$_2$$Cl_4$] by an imidazole moiety of ligands **1** and **2** (Scheme 2). Complexes **7** and **8** were isolated by column chromatography on silica gel. The formation of desired Ru^{III} complexes was proved by ESI-MS (Figures S16, S17), and their purity was confirmed by elemental analysis.

The assessment of stability is an important step in investigating the pharmacokinetic properties of newly synthesized compounds. The analysis of compound stability under physiological conditions provides insight into possible ways of administration, distribution in the body and subsequent excretion. The stability of Ru^{III} complexes **7**, **8** was examined in a solution resembling physiological conditions (phosphate buffer, pH 7.4, 0.137 mM of NaCl and 0.0027 mM of KCl at 37 °C) using UV-VIS spectrophotometry. Complex **7** ($t_{1/2}$ = 240 ± 12 s) was found to be slightly more stable than complex **8** ($t_{1/2}$ = 87 ± 5 s).

The synthesized compounds contain organic ligands with a 2,6-di-*tert*-butylphenol moiety, which is known to possess antioxidant activity. This property is expected to provide protection to healthy cells against oxidative stress. The ability of compounds to undergo single electron transfer was estimated by CUPRAC assay. The activity was studied as the ability of compounds to reduce Cu^{2+} to Cu^{+} in complex with neocuproine (2,9-dimethyl-1,10-phenanthroline). This reaction is accompanied by colour changes from light blue to orange (λ_{max} = 450 nm) monitored by spectrophotometry. The results (Table 1) were presented in Trolox equivalents (known antioxidant 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). It was found that the activity of ligand **1** was lower than that of Trolox, whereas the activity of ligand **2** was similar to Trolox. Interestingly, all ruthenium complexes exhibited significantly higher antioxidant activity compared to Trolox, moreover, Ru^{II} complexes **3**, **4** with chloride ligands and Ru^{III} complexes **7**, **8** showed higher activity than Ru^{II} complexes **5**, **6** with oxalate ligands. The ability of compounds to participate in hydrogen atom transfer reactions was estimated by the DPPH assay. The reaction of stable DPPH radical with complexes under study was monitored spectrophotometrically *via* changing the colour of the solution from deep violet to pale yellow (λ_{max} = 517 nm). The antioxidant activity was estimated as EC_{50} (effective antioxidant concentration required to reduce the initial DPPH concentration by 50%) (see Table 1).

The obtained EC_{50} values indicated that ligands **1**, **2** and Ru^{II} complexes **5**, **6** with oxalate ligands did not show a significant activity toward DPPH radical, while Ru^{II} complexes **3**, **4** with chloride ligands and Ru^{III} complexes **7**, **8** were more active. For all compounds, the addition of CH_2 group between the phenolic ring and the amide group led to an increase in the antioxidant activity.



Scheme 2

Table 1 The activity of compounds in CUPRAC and DPPH assay.

Compound	TEAC (CUPRAC)	EC_{50}/μM (DPPH)
1	0.35 ± 0.03	>100
2	1.17 ± 0.04	94 ± 1
3	13.1 ± 0.9	13 ± 5
4	14.2 ± 0.3	9 ± 1
5	10.8 ± 0.4	>100
6	11.7 ± 0.5	72 ± 21
7	16.1 ± 0.8	6 ± 1
8	15.5 ± 0.6	1.9 ± 0.6

The redox properties of 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionic acid **1′**, *N*-[3-(1*H*-imidazol-1-yl)propyl]-3,5-di-*tert*-butyl-4-hydroxybenzamide **1″** (see Online Supplementary Materials, Figure S2), organic ligands **1**, **2** and the corresponding complexes **3**–**8** were studied by cyclic voltammetry (CV) on glassy carbon (GC) and Pt electrodes in MeCN with Bu_4NBF_4 as a supporting electrolyte. The electrochemical behaviour of compounds was generally similar on the GC and Pt electrodes. Values of the **1**–**8** redox potentials *vs.* Ag/AgCl are summarized in Table S1. Compounds **1′**, **1″** and **1**–**8** demonstrate the electrochemical activity both on Pt and GC electrodes (Figure S3) and are oxidized according to proposed Schemes S2 and S3. Organic ligands **1** and **2** demonstrated electrochemical behaviour that generally agrees with proposed (see Schemes S2 and S3).

Voltammograms of Ru^{II} complexes exhibit two or three irreversible peaks in the anodic range of potentials [Table S1, Figures S4(*b*),(*c*)], while the rest ones correspond to ligand oxidation according to Scheme S3. The electrochemical behaviour of Ru^{III} compounds **7** and **8** on the GC and Pt electrodes was more complicated [Figure S4(*d*)].

The one-electron wave observed in the cathodic range of potentials of –0.4 to –0.3 V (Figure S5) corresponds to the reduction of Ru^{III} to Ru^{II}. Upon potential scan-reversal following the Ru^{III} to Ru^{II} reduction process, quasi reversible peak of low intensity is detected being due to the oxidation of complex $[Ru^{II}Cl_3L(Me_2SO)(MeCN)]$ in which the Cl^- anion was displaced by the solvent. It is known that one of the possible ways of Ru^{III} complexes antitumour action is activation through the reduction mechanism,[34,35] according to which the Ru^{III} reduction would produce more labile, toward substitution, Ru^{II}–Cl species that would rapidly react with specific sites of proteins altering their activity. Replacement of Cl^- ligand by MeCN molecule results in a positive shift of the reduction potential (see Table S1, Figure S5), and this fact should be taken into account to design potential antitumour drugs able to follow the activation by the reduction pathway.

The antioxidant potential of new compounds in the biological system as inhibitors of lipid peroxidation (LP) in rat brain homogenate was investigated in light of the observation of radical-binding activity for several new ruthenium complexes. As can be seen from Figure 1, ligands **1** and **2**, as well as Ru^{II} complexes **3** with chloride ligand [part (*a*)] and oxalate-containing Ru^{II} complexes **5** and **6** [part (*b*)], practically do not suppress Fe^{III}-induced LP. However, Ru^{II} complex **4** [see part (*a*)] with chloride ligand and CH_2 group between the phenolic ring and the amide group as well as Ru^{III} complexes **7** and **8** [see part (*b*)] possess antioxidant activity against Fe^{III}-induced LP, and these compounds are more effective in comparison with Trolox or Fenozan. The same compounds reveal antioxidant potential against H_2O_2-induced LP (Table 2). These data correlated with the ability of compounds to participate in hydrogen atom transfer reactions (DPPH assay, see Table 1).

Using the MTT assay antiproliferative capability of the compounds against human cell lines that include breast carcinoma MCF7, lung adenocarcinoma A549, and colorectal carcinoma HCT116, as well as against non-malignant lung fibroblast WI38, was investigated (Table S2). All compounds demonstrated very mild antiproliferative activity. On the most sensitive HCT 116 cell lines, compounds **3**, **5** and **6** showed medium micromolar activity.

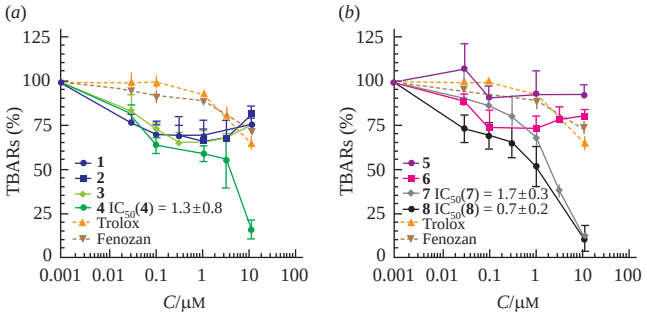


Figure 1 Influence of Trolox, Fenozan and (*a*) compounds **1**–**4** and (*b*) compounds **5**–**8** on Fe^{3+}-induced LP of rat brain homogenate. IC_{50} was calculated with nonlinear regression fit (GraphPad Prism v8.0).

Table 2 Influence of standard antioxidant Trolox, Fenozan and compounds **4**, **7** and **8** on H_2O_2-induced LP of rat brain homogenate.

Compound	LP (5 mM H_2O_2), IC_{50}/μM[a]
4	7.5 ± 6.7
7	1.35 ± 0.32
8	2.9 ± 2
Fenozan	33 ± 9
Trolox	7.8 ± 0.8

[a] Data presented as mean ± sd, IC_{50} were calculated with nonlinear regression fit (GraphPad Prism v8.0).

In conclusion, the series of new ruthenium complexes with 2,6-di-*tert*-butylphenol ligands were synthesized. The antioxidant behaviour of Ru^{II} and Ru^{III} complexes was examined in the model reactions using spectrophotometric and electrochemical methods, and high activity was found for Ru^{III} complexes. Also, the antioxidant properties of the new compounds were evaluated based on their ability to inhibit lipid peroxidation, and the complexes showed activity against Fe^{III}-induced LP. We found no direct correlation between antioxidant ability and antiproliferative activity of these Ru compounds, however search for new Ru complexes is still promising for further estimating their potential as anticancer drugs with dual actions.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2024.01.022.

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