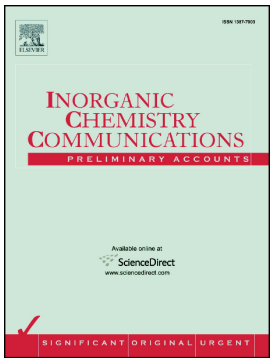

Design, synthesis and photobiological activity of novel ruthenium phthalocyanine complexes

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Design, synthesis and photobiological activity of novel ruthenium phthalocyanine complexes

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Ruthenium phthalocyanine complexes could function as novel photosensitizers for photodynamic therapy (PDT)[1-3]. Essentially PDT involves the combination of non-toxic photosensitizers undergoing irradiation with harmless visible light, that in the presence of oxygen, lead to the production of singlet oxygen as well other reactive oxygen species (ROS) that can induce cell death[4-6]. The cytotoxic efficiency of a photosensitizer is generally governed by the quantum yield and absorption coefficient, as well as the localization within the cell, because singlet oxygen can only diffuse a short distance[7-9]. The ideal photosensitizer should preferentially accumulate in cancer cells and tumors, which generally depends on the molecular structure of the photosensitizer. Understanding the relationship between the photosensitizer structure and cytotoxicity pathways is fundamental for the development of PDT for different types of cancer as well as non-cancer diseases[10]. In order to explore the use of ruthenium phthalocyanines as photosensitizers it is essential to develop synthetic methods to prepare different complexes with modifications on the phthalocyanine macrocycle[11]. However, the synthetic procedures and

purification procedures of these complexes tend to be difficult[12-13]. We have discovered a rather simple and elegant way to synthesize ruthenium-phthalocyanine compounds based on a template method. The template-assisted approach involves the use of *cis*-[$RuCl_2(DMSO)_4$] as a precursor that reacts with the appropriate dicyanobenzene to generate an intermediate with the nitrile ligand chelating the ruthenium ion.

The *cis*-[$RuCl_2(DMSO)_4$] starting material was synthesized according to a previously published procedure[14]. Reaction of this complex with 1.1 equivalents of 4-(3-carboxylic-benzyloxy)-phthalonitrile (DCBz) (named here dicyanobenzene-R) in ethanol under reflux for 3 h afforded *cis*-[$RuCl_2(DMSO)_2$(DCBz)], which was isolated in 76.0 % yield. The resulting structure of dicyanobenzene compound will drive the reaction to kinetically favorable monosubstituted phthalocyanine. The DCBz was obtained following a published procedure[15]. Briefly, the reaction of 4-nitrophthalonitrile with 4-hydroxybenzoic acid in dimethylsulfoxide for five days at 120 ºC afforded the desired product as 85.0 % in yield. The DCBz compound was characterized by mass spectrum. The ESI-MS spectra were obtained using ultrOTOFQ-ESI-TOF Mass Spectrometer (Bruker Daltonics, Billerica, MA, EUA). The electrospray solvent was 70:30 acetonitrile : milli Q water. For each analysis, 10 μL was introduced into the source at a flow rate of 5 μL min^{-1}. For $\{DCBz\}^-$ we found *m/z* = 263.0449, consistent with the calculated isotopic patterns (Supplementary material: Figure 1). Characterization by ^1H NMR was also carried out. Measurements were performed on a Bruker Avance III HD 400.15 MHz spectrometer in $DCCl_3$ and DMSO-d_6 as solvents at room temperature. Essentially the NMR samples were prepared by dissolving 5−15 mg of the compound in 0.5 mL of the appropriate solvent. The ^1H NMR spectra of DCBz were in good agreement with the proposed structure (Figure 1). The ^1H NMR spectrum showed signals for the aromatic hydrogens between 8.2 – 7.0 ppm. The proximity of ether as well

the acceptor character of carboxylic substituents causes downfield shifts in the plane of aromatic ring for H-9 and H-13. A similar effect was observed for H-4 and H-5, which experience the effect of the cyano group differently (depending on its position on the aromatic ring). A singlet signal at 13.04 ppm was observed and was assigned to the carboxylic acid substituent.

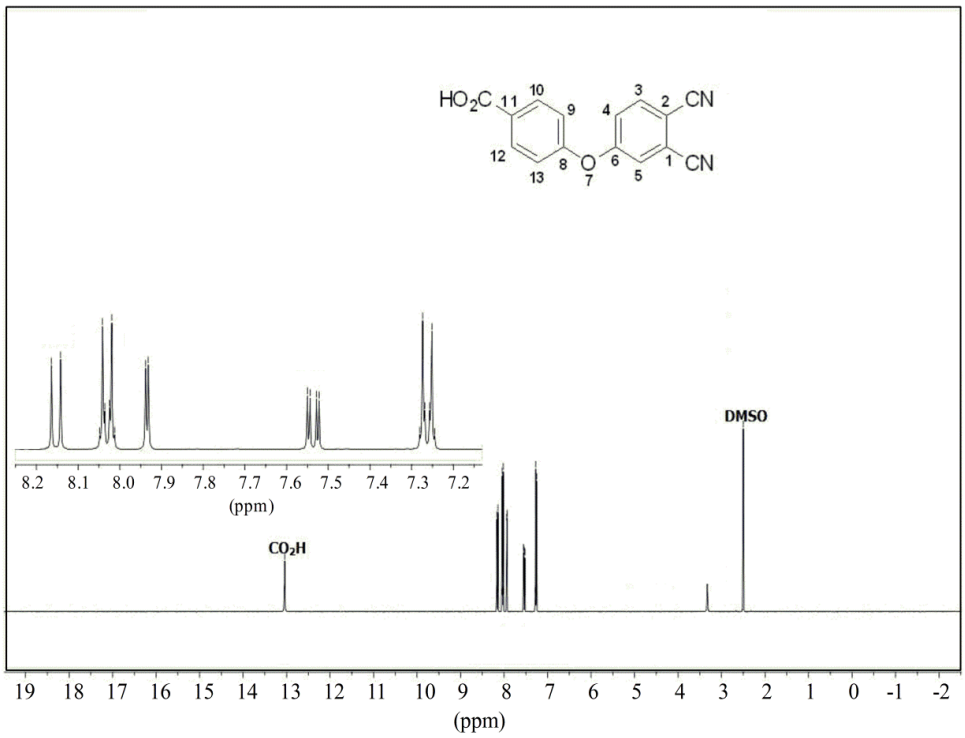
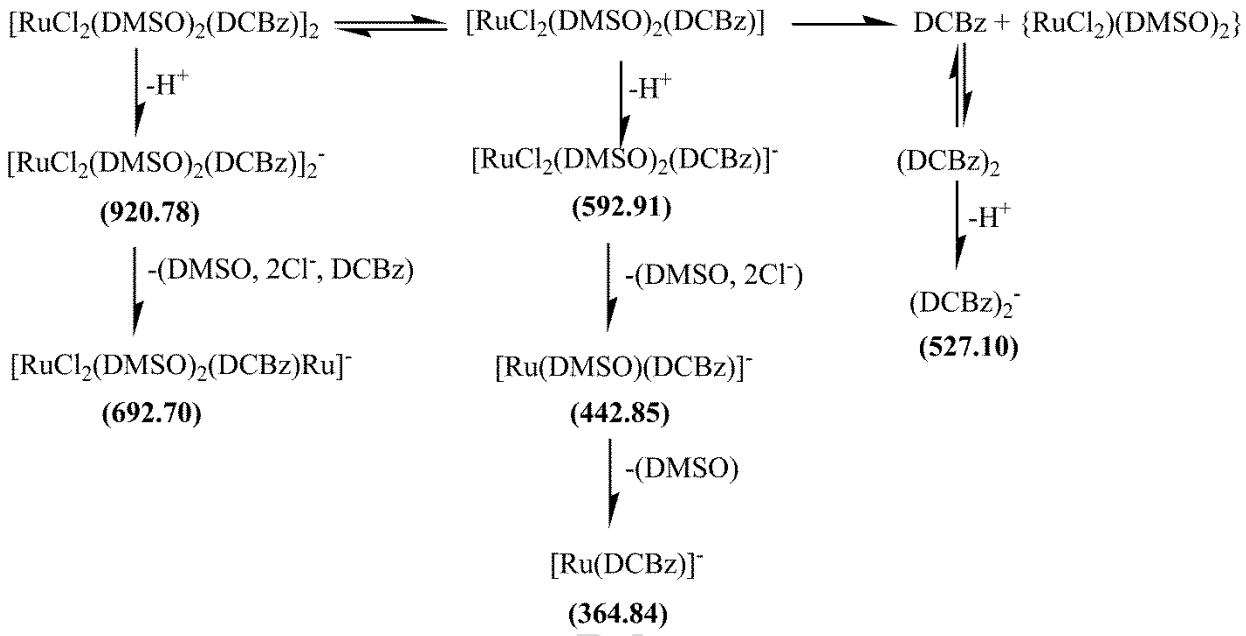


Figure 1: ^{1}H NMR spectrum of 4-(3-carboxylic-benzyloxy)-phthalonitrile (DCBz) recorded in DMSO-d6. δ(ppm): 7.25 (t, 1H, J_1=2.7 Hz); 7.27 (t, 1H, J_1=2.7 Hz); 7.54 (dd, 2H, J_1=8.7 Hz, J_2 = 2.6 Hz); 7.94 (d, 1H, J_1=2.5 Hz); 8.02 (t, 1H, J_1=2.7 Hz); 8.04 (t, 1H, J_1=2.7 Hz); 8.15 (d, 1H, J_1 = 8.8 Hz); 13.04 (s, 1H).

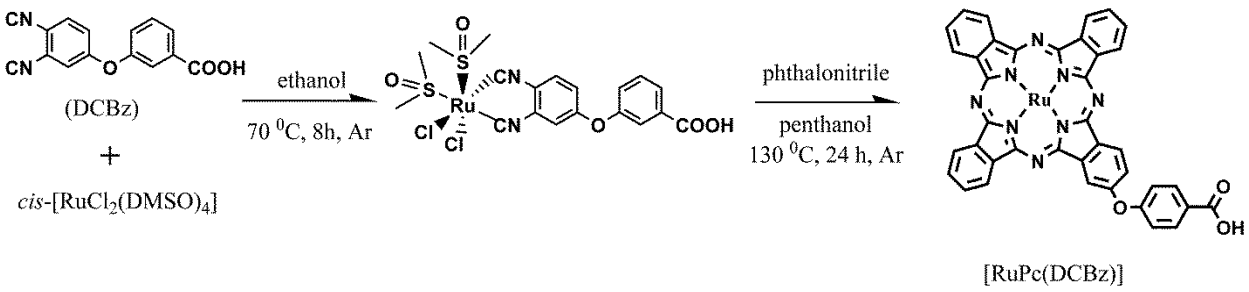
The isolated $[RuCl_2(DMSO)_2(DCBz)]$ was characterized by negative ion mode ESI-MS and showed *m/z* = 592.9156 consistent with the proposed formula (Supplementary material: Figure 2). The mass spectrum also generated by ionization sputtering the $[RuCl_2(DMSO)_2(DCBz)]$

complex to reveal the presence of a dimer forming a complex pattern suggesting fragmentation followed by an electron transfer process (Scheme 1).



Scheme 1: The proposed fragmentation route of $[RuCl_2(DMSO)_2(DCBz)]$ showing the formation of some key fragment ions.

The monosubstituted phthalocyanine ruthenium complex [Ru(Pc-DCBz)] was obtained by heating $[RuCl_2(DMSO)_2(DCBz)]$ in a 5-fold excess of phthalonitrile at 130 ºC in dry pentanol (Scheme 2). The crude product was obtained after 12 h at 0 °C. It was purified by column chromatography on basic silica eluting with tethrahydrofuran/toluene/dimethylformamide (70:20:10). After evaporation on a rotary evaporator, the blue solid was dried in vacuum for 4 h to give 54 % in yield based on the initial amount of $[RuCl_2(DMSO)_2(DCBz)]$.



Scheme 2: Template synthesis of [Ru(Pc-DCBz)] complex.

The [Ru(Pc-DCBz)] complex was characterized by mass, electronic absorption and FTIR spectra. The electronic absorption spectra of [Ru(Pc-DCBz)] displayed a typical spectrum for metallophthalocyanine compounds presenting intense π - π^* absorption in the visible spectra. It showed a band at 312 nm (ε = 2.8 10^5 $mol^{-1}L^{-1}$), generally called the Soret or B-band, and a band at 644 nm (ε = 1.4 10^5 $mol^{-1}L^{-1}$), generally termed the Q-band, with a shoulder at 586 nm in dichloromethane (DCM) solution. The [Ru(Pc-DCBz)] shows emission spectrum in DCM with λ_{max} = 690 nm after excitation at 640 nm. The FTIR spectrum was also similar to other ruthenium phthalocyanine species such [Ru(Pc)] (Pc = phthalocyanine). The formation of [Ru(Pc-DCBz)] was confirmed by the appearance of a carboxylic acid peak at 1670 cm^{-1}, which was compared to the FTIR spectrum of [Ru(Pc)] synthesized as described in a published procedure [15] (Supplementary material: Table 1).

MALDI-TOF analysis of [Ru(Pc-DCBz)] with dihydroxybenzene (DHB) as a matrix, gave mainly the two singly charged parent ions [Ru(Pc-DCBz)] (*m/z* = 749.89) and {[Ru(Pc-DCBz)]$_2$} (*m/z* = 1498.91). A third peak appeared with *m/z* = 1056.02 and was attributed to the association of [Ru(Pc-DCBz)] with 2 DHB. Signals of weaker intensity corresponding to different fragment ions were also observed (Supplementary material: Figure 3).

Singlet oxygen generation (Δ^1O_2) of [Ru(Pc-DCBz)] and [Ru(Pc)] were determined in DMSO using an earlier described procedure[16]. Briefly, the Φ^1O_2 values were calculated using 1,3-diphenylisobenzofuran (DPBF) as a singlet oxygen scavenger. A solution of 2.0 x 10^{-6} M sensitizer along with 2.5 x 10^{-5} M DPBF was irradiated using a 660 nm laser at 1 s time intervals from 0 to 10 s (Figure 2).

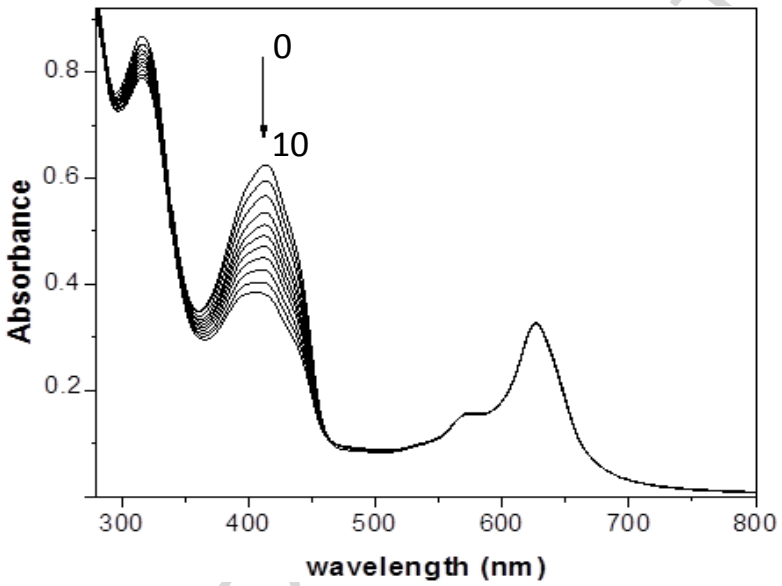


Figure 2: Changes in the absorption spectrum of DPBF in DMSO upon irradiation at 660 nm in the presence of [Ru(Pc-DCBz)]. Recorded at every 1 s interval.

Singlet oxygen quantum yields were determined in air observed by the decrease in the absorbance of DPBF monitored at 410 nm and compared with the decrease observed with the standard photosensitizer, zinc phthalocyanine under identical conditions (Φ_Δ = 0.67 in DMSO[17]). Values for the Φ^1O_2 of [Ru(Pc-DCBz)] was found 0.24 ± 0.03 and for [Ru(Pc)] was 0.35 ± 0.02 in DMSO. Cytotoxicity values of ruthenium-phthalocyanine compounds were measured by determination of cellular proliferation using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. To B16F10 and MCF7 cell lines were added both

ruthenium phthalocyanine sensitizers (1.0 x 10^{-5} M), incubated for 24 h, and irradiated at 660 nm using a dose of 8.93 Jcm^{-2}.

Figure 3 shows the cellular viability assays performed with [Ru(Pc-DCBz)] and [Ru(Pc)] in murine B16F10 melanoma and human breast cancer MCF7 cells. Essentially there was no cytotoxicity of either complexes in the studied cancer cell lines observed in the dark, although both compounds were active under light irradiation at 660 nm. At 1.0 x 10^{-5} M concentrations both [Ru(Pc-DCBz)] and [Ru(Pc)] yielded statistically the same value of the radiant exposure needed to cause 50 % death of the treated B16F10 cells. However in MCF7 cells, the PDT activity of [Ru(Pc-DCBz)] was higher than that of [Ru(Pc)]. The cell survival was found to be only 10 % when [Ru(Pc-DCBz)] was used, while it was 85 % for the [Ru(Pc)] complex.

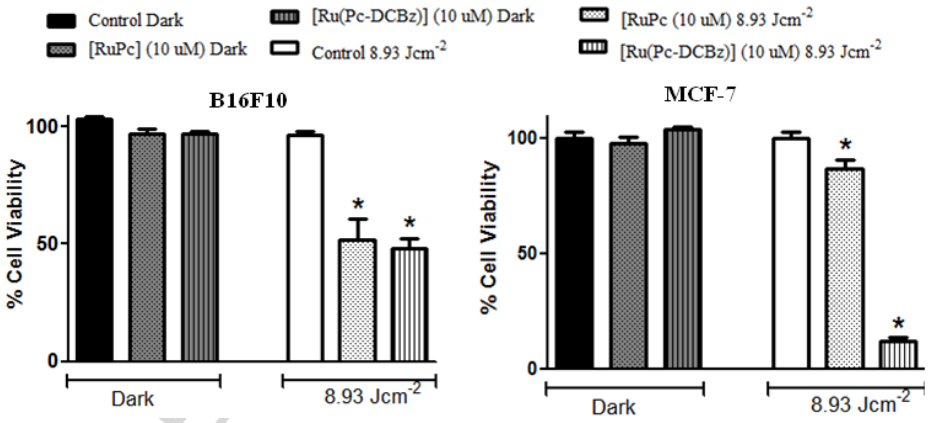


Figure 3: Cell viability plot of B16F10 and MCF-7 cancer cells treated with ruthenium phthalocyanine compounds (10 μM) for 24 h.

Moreover, [Ru(Pc-DCBz)] seemed to confer higher specificity and selectivity for breast cancer cells, compared to [Ru(Pc)]. MCF-7 breast cancer cells are more differentiated (known to be hormone responsive)[18] compared to B16F10 melanoma cells. Confocal microscopy images showed internalization of [Ru(Pc-DCBz)] in MCF-7 cells. A TRITC filter responding to red

fluorescence signals was employed to recognize the location of [Ru(Pc-DCBz)] with red fluorescence (Figure 4). The internalization is consistent with cell death process. That could be a critical determinant for the cytotoxicity efficacy.

Therefore, subcellular localization is indeed more important than singlet oxygen quantum yield in terms of overall cell killing[19]. New studies are underway to explore the cytotoxicity pathways of these ruthenium-phthalocyanine compounds and will be presented shortly.

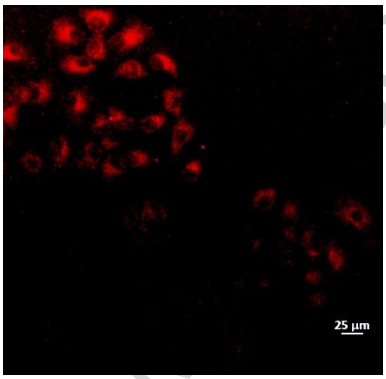


Figure 4: confocal laser scanning microscopy of MCF-7 breast cancer cells after incubation with [Ru(Pc-DCBz)] for 1 h at 37 °C (10 μM).

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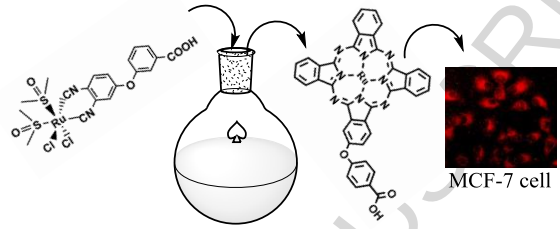
GRAPHYCAL ABSTRACT



The present work describes a novel and efficient synthetic method of ruthenium monosubstituted-phthalocyanine complexes and its application against cancer cell lines. It allows find relationship of subcellular localizations and cytotoxicity.

Highlights

- Synthesis of a ruthenium monosubstituted-phthalocyanine complex.
- *In vitro* cytotoxicity assays on cancer cells show complexes possible tumoricidal role.
- Confocal microscopy analysis on cancer cells was used to investigate cellular uptake.
- Subcellular localization is dependent on phthalocyanine substituent.