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Synthetic Methods for the Preparation of a Functional Analogue of Ru360, a Potent Inhibitor of Mitochondrial Calcium Uptake

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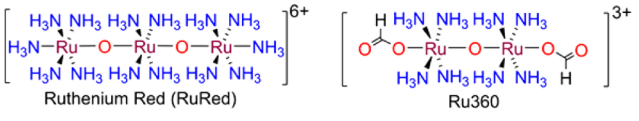
Supporting Information

ABSTRACT: The mixed-valent oxo-bridged ruthenium complex $[(HCO_2)(NH_3)_4Ru(\mu\text{-}O)Ru(NH_3)_4(O_2CH)]^{3+}$, known as Ru360, is a selective inhibitor of mitochondrial calcium uptake. Although this compound is useful for studying the role of mitochondrial calcium in biological processes, its widespread availability is limited because of challenges in purification and characterization. Here, we describe our investigations of three different synthetic methods for the preparation of a functional analogue of this valuable compound. We demonstrate that this analogue, isolated from our procedures, exhibits potent mitochondrial calcium uptake inhibitory properties in permeabilized HeLa cells and in isolated mitochondria.

The flux of mitochondrial calcium ions regulates cell death and survival.[1] Abnormalities in mitochondrial calcium levels have been implicated in a variety of diseases, such as ischemic heart disease, amyotrophic lateral sclerosis, and Alzheimer's disease.[2] The uptake of Ca^{2+} ions in the mitochondria is mediated by the mitochondrial calcium uniporter (MCU), a transmembrane protein that sits in the inner mitochondrial membrane.[3−5] The five subunits of this protein form a pentameric pore that mediates Ca^{2+} uptake.[6] Selective chemical inhibitors of the MCU are crucial tools for understanding the role of this transporter in mediating Ca^{2+}-dependent processes[7] and may also have significant therapeutic applications.[8,9]

The trinuclear oxo-bridged ruthenium complex ruthenium red (RuRed; Chart 1)[10] is a commercially available inhibitor of the MCU.[11] The use of RuRed, however, is limited by its poor selectivity for the MCU,[12] as well as by the large amount of impurities found in commercial sources of this compound.[13] One of the impurities found in the formulations of RuRed is a dinuclear oxo-bridged compound, $[(HCO_2)(NH_3)_4Ru(\mu\text{-}O)Ru(NH_3)_4(O_2CH)]^{3+}$ (Ru360; Chart 1). This "impurity" exhibits much greater potency and selectivity than RuRed for inhibiting the MCU, rendering it a valuable tool for studying the role of mitochondrial calcium in biological processes.[14−16] A key disadvantage of Ru360, however, is its limited commercial availability, as well as challenging synthesis, purification, and characterization.[15] As such, the use of impure RuRed as an inhibitor of the MCU in biological studies remains a common practice.

Chart 1. Structures of RuRed and Ru360



In this study, we investigate the synthesis and characterization of a functional analogue of Ru360. This analogue, $[(OH_2)(NH_3)_4Ru(\mu\text{-}O)Ru(NH_3)_4(OH_2)]^{5+}$ ($[\mathbf{1}]^{5+}$), is a simple variation of Ru360, where the axial formate ligands are replaced by water molecules. Although this compound has been characterized in solution,[15] we report three distinct synthetic methods for its isolation and detailed physical characterization. Additionally, we demonstrate that this cation is a potent inhibitor of mitochondrial calcium uptake in both permeabilized human cells and isolated yeast mitochondria that are genetically modified to express the human MCU and its essential regulator EMRE.[17,18]

We initially sought to synthesize Ru360 with the previously reported method.[15] Following this procedure, $RuCl_3{\cdot}nH_2O$ was dissolved in 6 M HCl containing 12% ethanol and heated at 90 °C to reduce ruthenium(IV) and nitrosyl impurities. The addition of concentrated NH_4OH precipitated a crude material, which was then resuspended in concentrated NH_4OH and heated at 40 °C overnight. Notably, this step of the reaction requires a sealed reaction vessel; heating in an open container does not produce the desired compound. The product is readily identified by a UV−vis absorbance band centered at 360 nm. The formation of RuRed as a byproduct is also evident from a broad absorbance band centered at 530 nm.

The previously reported purification of Ru360 was accomplished with cellulose-based, weakly acidic cation-exchange chromatography using Whatman CM-52 resin and an ammonium formate (NH_4HCO_2) buffer as the mobile phase.[15] This purification method gives Ru360 as the formate-capped form $[(HCO_2)(NH_3)_4Ru(\mu\text{-}O)Ru(NH_3)_4(O_2CH)]^{3+}$. Whatman CM-52 resin, however, is currently unavailable from commercial suppliers, prompting us to investigate alternative solid-phase supports for purification. A column packed with the strongly acidic cation-exchange resin, Dowex 50WX2, and a mobile phase of HCl were found to be effective for purification

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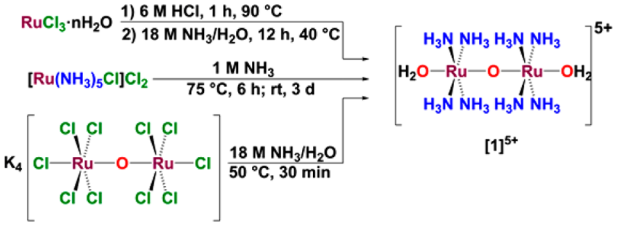
because these were previously used for isolating a chloride-capped analogue of Ru360.[15] At 2 M HCl, a byproduct of this reaction, confirmed to be $[Ru(NH_3)_5Cl]Cl_2$ by single-crystal X-ray diffraction (Figure S1 and Tables S1 and S2, Supporting Information, SI),[19] eluted as a yellow band. Upon an increase of the HCl concentration to 3 M, the Ru360 analogue, $[\mathbf{1}]^{5+}$, came off the column as a brown solution. At higher HCl concentrations (>4 M), RuRed and its oxidized form, ruthenium brown, could also be eluted. Rotary evaporation of the fractions containing $[\mathbf{1}]^{5+}$ afforded a brown solid, the elemental analysis of which was formulated to be $[\mathbf{1}]Cl_5{\cdot}1.5H_2O$, in typical yields of 10−20 mg (1.7−3.4%) starting from 250 mg of $RuCl_3{\cdot}nH_2O$.

The low yields and tedious purification prompted us to search for alternative synthetic methods for preparing this compound. We found that heating $[Ru(NH_3)_5Cl]Cl_2$[20] in 1 M NH_3 in a capped reaction vessel at 75 °C for 6 h, followed by stirring at room temperature for 3 days, gives rise to a green solution with an intense absorbance band at 360 nm, an indicator of a Ru360-like complex (Scheme 1). When the reaction vessel is capped, no

Scheme 1. Three Synthetic Methods for Obtaining $[\mathbf{1}]^{5+}$



detectable amount of RuRed is observed. Purification by ion-exchange chromatography with Dowex 50WX2 resin, as described above, afforded $[\mathbf{1}]^{5+}$ as a brown solid in yields of 10−20% from $[Ru(NH_3)_5Cl]Cl_2$.

We also explored the utility of $[Ru_2OCl_{10}]^{4-}$ as a synthon for complexes containing the Ru(μ-O)Ru core. The compound $K_4[Ru_2OCl_{10}]$ was prepared as described previously[21] and characterized by single-crystal and powder X-ray diffraction (Figures S2 and S3 and Tables S1 and S3, SI).[22] Accordingly, the reaction of $[Ru_2OCl_{10}]^{4-}$ with concentrated NH_4OH at 50 °C for 30 min afforded $[\mathbf{1}]^{5+}$ as the major product detected by UV−vis spectroscopy (Scheme 1). After purification by cation-exchange chromatography, $[\mathbf{1}]^{5+}$ can be isolated in typical yields of 4−8%.

Crystals grown from solutions of $[\mathbf{1}]^{5+}$ exhibited two different polymorphic forms (Figure S4, SI). The vapor diffusion of ethanol into aqueous solutions of $[\mathbf{1}]^{5+}$ yielded dark-red prisms, whereas the slow evaporation of 3 M HCl solutions of $[\mathbf{1}]^{5+}$ gave rise to green needles. Analysis of the red prisms revealed an oxo-bridged dinuclear ruthenium structure of the formula $[(OH)(NH_3)_4Ru(\mu\text{-}O)Ru(NH_3)_4(OH_2)]Cl_4$ (Figure 1 and Tables S1 and S4, SI). This formula differs from that of $[\mathbf{1}]^{5+}$ by the loss of 1 equiv of HCl to give a mixed aqua−hydroxo compound. The bridging μ-oxo-ligand sits on a crystallographic inversion center. As such, the ammine ligands assume an eclipsed conformation, and the Ru−O−Ru angle is exactly 180°. Such geometric features are also present in the formate-capped Ru360 ion, which lies on both a crystallographic inversion center and a mirror plane.[15,23] On the basis of hydrogen-bonding patterns in the lattice (vide infra), we assign the axial ligands as water and hydroxide. The inspection of intermolecular interactions between axial oxygen ligands on neighboring cations reveals the presence of a close contact of 2.446 Å (Figure S5, SI). These two oxygen atoms are

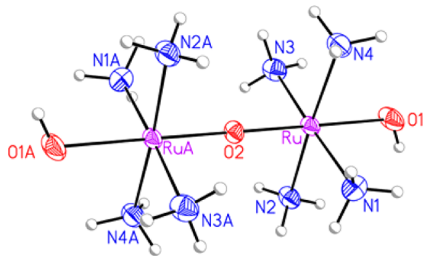


Figure 1. Crystal structure of $[(OH)(NH_3)_4Ru(\mu\text{-}O)Ru(NH_3)_4(OH_2)]^{4+}$. Thermal ellipsoids are shown at the 50% probability level.

related by a crystallographic inversion center; in addition, the distance between atoms is too short to constitute a normal hydrogen-bonding interaction. We hypothesize that this situation represents a symmetric hydrogen bond, where the hydrogen atom is shared equally between both oxygen-atom donors.[24] This hydrogen atom, which could not be located in the difference Fourier map, is inferred to lie on the crystallographic inversion center at the midpoint of the symmetric hydrogen bond. Accounting for this shared hydrogen atom, the net formula for the complex cation is $[(OH)(NH_3)_4Ru(\mu\text{-}O)Ru(NH_3)_4(OH_2)]^{4+}$. Four outer-sphere chloride ions are also present. The net balance of the charge, accounting for the hydrogen atom on the inversion center, results in the expected mixed-valent Ru^{III}/Ru^{IV} oxidation state, which is well characterized for Ru360.

The green needle polymorph (Table S5, SI) of this compound gave data that appeared suitable for X-ray diffraction studies. Although the initial structure solution was successful, full refinement of the structure was hindered by unresolved twinning. Basic atomic connectivity obtained from this data, however, reveal a structure largely similar to that of the red polymorph. Namely, a linear Ru−O−Ru complex with axial ligands believed to be oxygen-atom donors based on electron density contributions to the difference map and interatomic distances. This structure suggests that even in 3 M HCl chloride ions do not bind directly to the axial sites.

Cation $[\mathbf{1}]^{5+}$ was further characterized by cyclic voltammetry (Figure S6, SI) and spectroscopically. The [IV,III]/[III,III] and [III,III]/[III,II] redox potentials measured for $[\mathbf{1}]^{5+}$ at pH 7.6 are +120 and −420 mV vs Ag/AgCl, respectively. These potentials fall between those measured previously for the aqua- and hydroxo-capped analogues, which were measured at pH values of 2 and 8.[15] The UV−vis absorption spectrum (Figure 2a and

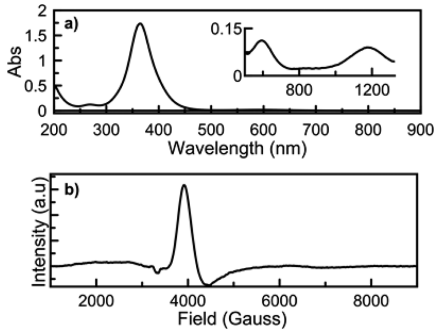


Figure 2. (a) UV−vis and near-IR (inset) spectra of $[\mathbf{1}]^{5+}$ in pH 7.4 PBS. (b) EPR spectra of $[\mathbf{1}]^{5+}$ obtained at 77 K, where g = 1.69 in 80:20 water/glycerol.

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Figure S7 and Table S6, SI) reveals a diagnostic absorbance band centered at 360 nm, a feature that gives Ru360 its name. A lower-energy transition centered at 600 nm gives rise to the observed green color of these compounds in solution. The intensity of this transition varied slightly depending on the synthetic method used to prepare the complex (Table S6, SI). We tentatively attribute this variance to the presence of small quantities of the RuRed ion, which has a large molar absorptivity (≈68000 M^{-1} cm^{-1}) at 530 nm. For the previously prepared aqua- and hydroxo-capped analogues, the absorbance maxima are at 335 and 593 nm and 360 and 612 nm, respectively.[15] Therefore, $[\mathbf{1}]^{5+}$ exhibits absorbance bands in pH 7.4 phosphate-buffered saline (PBS) that are intermediate between these two complexes. A near-IR transition, centered at 1180 nm (8480 cm^{-1}), arises from the intervalence charge-transfer state associated with this mixed-valent complex.[25] In the IR spectrum, the Ru−O−Ru stretching mode resonates at 850 cm^{-1} (Figures S8 and S9, SI). X-band electron paramagnetic resonance (EPR) spectroscopy of $[\mathbf{1}]^{5+}$ (Figure 2b and Figure S10, SI) at 77 K reveals a broad signal at $g = 1.69$, consistent with a paramagnetic Ru^{III}/Ru^{IV} mixed-valent ground state. In contrast, the EPR spectra of Ru360 is characterized by a signal at $g = 2.06$.[15] We note that mixed-valent oxo-bridged Ru^{III}/Ru^{IV} complexes bearing polypyridyl ligands display broad signals ranging from $g =$ 1.39 to 1.79,[26,27] which are consistent with our observation for $[\mathbf{1}]^{5+}$.

Having established $[\mathbf{1}]^{5+}$ as an analogue to Ru360, we sought to verify that it also inhibits mitochondrial calcium uptake. HeLa cells (7.5×10^6) were permeabilized with digitonin and incubated with 2 μM of the turn-on calcium-responsive dye Calcium Green-5N.[28] The fluorescence response of the dye was monitored over time after the addition of $CaCl_2$. The addition of Ca^{2+} ions leads to an immediate increase in the emission intensity (Figure 3a),

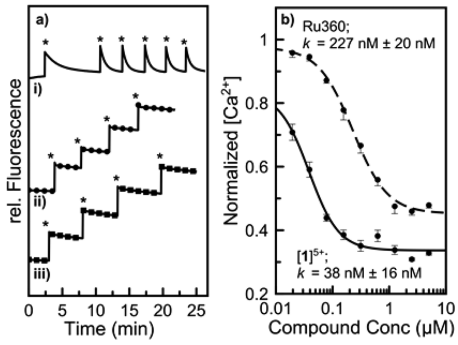

Figure 3. (a) Mitochondrial calcium uptake in digitonin-permeabilized HeLa cells at 37 °C, monitored with the fluorescent sensor Calcium Green-5N (i) in the absence of additional compound or in the presence of 5 μM (ii) Ru360, or (iii) $[\mathbf{1}]^{5+}$. The asterisk (*) signifies when a bolus of 10 μM Ca^{2+} was added. (b) Dose-dependent inhibition of mitochondrial calcium uptake in isolated yeast mitochondria.

which decays over the course of 3 min as the Ca^{2+} ions are buffered by the mitochondria. This process is reproducible for many (~10) pulses of Ca^{2+} ions. The addition of 5 μM $[\mathbf{1}]^{5+}$ to permeabilized HeLa cells inhibits mitochondrial calcium uptake (Figure 3a and Figure S11, SI). The addition of Ca^{2+} gives rise to an increase in the fluorescence intensity with no decay because the mitochondrial calcium uptake is impaired by $[\mathbf{1}]^{5+}$. Commercially available Ru360 exhibits similar properties in preventing mitochondrial calcium uptake (Figure 3a).

We confirmed these findings in yeast mitochondria expressing the human pore-forming subunit MCU, the essential regulator EMRE of the MCU, and a mitochondria-targeted aequorin as a luminescence-based calcium sensor.[18,29] We find that $[\mathbf{1}]^{5+}$ and Ru360 exert a concentration-dependent inhibitory effect on calcium uptake (Figure 3b and Figure S12, SI). The dose−response curves, fit with the Hill equation, reveal the Michaelis constant (k), a measure of the effectiveness of calcium uptake inhibition, for commercially available Ru360 to be 227 ± 20 nM. The analogue $[\mathbf{1}]^{5+}$ was found to be more potent, with k values for the three synthetic procedures ranging from 38 to 50 nM. Remarkably, although $[\mathbf{1}]^{5+}$ and Ru360 differ only by the nature of the axial ligand, $[\mathbf{1}]^{5+}$ demonstrates greater inhibitory properties. Different axial ligand substitution kinetics may play an important role in the relative activity of these species. It is known, for example, that the formate ligands of Ru360 aquate at a faster rate than the chlorido-capped analogue.[15] The complex chemical environment of biological media contains a range of potential ligands that may affect the formation of the true active species of Ru360. Further modifications of the axial ligands may lead to more effective MCU inhibitors by optimizing ligand substitution kinetics.

In summary, we described the synthesis and characterization of a structural and functional analogue of Ru360. We demonstrated that this compound can be prepared via three different synthetic routes. The two newly developed synthetic methods may be more broadly applicable for the preparation of new complexes with the Ru−O−Ru core, facilitating the discovery of new potent inhibitors of the MCU based on ruthenium.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.inorgchem.6b03108.

Experimental data and details (PDF)

Crystallographic data (CIF)

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Notes

The authors declare no competing financial interest.

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