Caging ruthenium complexes with non-toxic ligands for photoactivated chemotherapy

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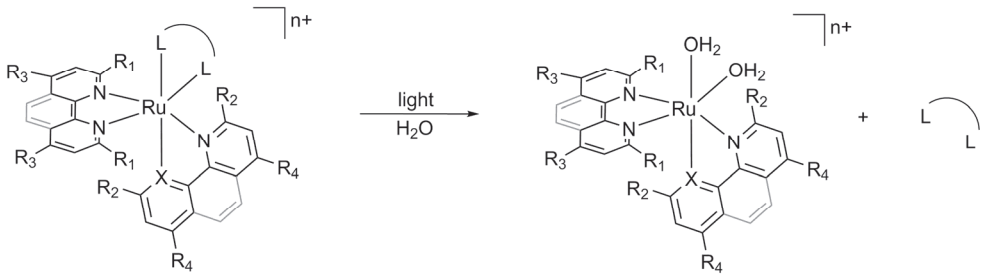
Summary, conclusions, and outlook

The main goal of the research described in this thesis was the development of new photoactivated chemotherapy (PACT) ruthenium(II) complexes bearing a non-toxic photolabile ligand. We first investigated whether non-toxic ligands such as L-proline, 2-(methylthio)methylpyridine (mtmp), or 3-(methylthio)propylamine (mtpa), once coordinated to ruthenium(II) complexes, could be photosubstituted upon visible light irradiation. The lipophilicity, and in some cases the strain of the ruthenium(II) complexes, were systematically varied and the effects of such variations on the cytotoxicity of the complexes in the dark and under light irradiation were studied. In the second part, the best ligand candidates (i.e. mtmp and mtpa) were coordinated to cyclometalated ruthenium complexes of the type [Ru(bpy)(phpy)(S,N)]PF_6 (bpy = 2,2'-bipyridine and phpy = 2-phenylpyridine), to shift the absorption of the complex to the red region of the spectrum. The photosubstitution properties of these cyclometallated complexes were investigated in detail. The most promising ruthenium complexes were tested in cancer cell monolayers under hypoxic conditions (1% O_2) to investigate their mode of action and distinguish between PACT and PDT.

7.1 Summary

The main goal of the research described in this thesis was the development of new photoactivated chemotherapy (PACT) ruthenium(II) complexes bearing a non-toxic photolabile ligand. The suitability of the natural amino acid L-proline as protecting ligand in a series of complexes of the type $[Ru(N,N)_2(L\text{-prolinate})]PF_6$ is reported in Chapter 2. The number of sterically hindering methyl groups increased from zero in $[Ru(bpy)_2(L\text{-prolinate})]PF_6$ (bpy = 2,2'-bipyridine, [**1**]PF_6) to two in $[Ru(bpy)(dmbpy)(L\text{-prolinate})]PF_6$ (dmbpy = 6,6'-dimethyl-2,2'-bipyridine, [**2**]PF_6), and up to four in $[Ru(dmbpy)_2(L\text{-prolinate})]PF_6$ ([**3**]PF_6). The photoreactivity of this type of complexes was found to be solvent dependent: while in water no substitution was observed upon light irradiation for any complex of the series, in CH_3CN the strained complexes [**2**]PF_6 and [**3**]PF_6 photosubstituted either L-proline or dmbpy in parallel. Interestingly, in water [**1**]PF_6 loses two hydrogens upon light irradiation in presence of aerial O_2, photooxidizing to the imino complex $[Ru(bpy)_2(L\text{-prolinate} - 2H)]PF_6$. The addition of electron-donating methyl groups in [**2**]PF_6 and [**3**]PF_6 decreases the acidity of the amine, preventing its oxidation to imine. Thus, due to the stability of the ruthenium-prolinate complexes in water and non-selective photosubstitution in CH_3CN, the negatively charged L-prolinate was discarded as protecting ligand, and by extension any natural amino acid with N,O coordination.



Scheme 7.1. Photosubstitution of a bidentate ligand upon light irradiation in water in a given ruthenium(II) polypyridyl complex. In the research described in this thesis we have tuned the strain and lipophilicity of the complexes by changing the functional groups in R_1, R_2, R_3, R_4. X = N or C.

Glazer *et al.* reported the photocytotoxicity of $[Ru(bpy)_2(dmbpy)]Cl_2$ against lung cancer cells (A549 cells), which was attributed, by analogy with cisplatin, to the photogenerated compound *cis*-$[Ru(bpy)_2(OH_2)_2]^{2+}$.[1] However, in Chapter 3 we show that dmbpy, which is also released, is cytotoxic by itself. Therefore, is $[Ru(bpy)_2(OH_2)_2]^{2+}$ cytotoxic? Can any PDT effect be discarded? In order to investigate the role of $[Ru(bpy)_2(OH_2)_2]^{2+}$ we compared the photocytotoxicity of

$[Ru(bpy)_2(dmbpy)]Cl_2$ with that of $[Ru(bpy)_2(mtmp)]Cl_2$ (mtmp = 2-(methylthio)methylpyridine), which has a neutral sulfur-based ligand. Both complexes are comparable: they generate $[Ru(bpy)_2(OH_2)_2]^{2+}$ upon light irradiation, they have low singlet oxygen generation quantum yields, and they have similar lipophilicity and low cellular uptake. The difference is that the released mtmp is not cytotoxic by itself. When treating lung cancer cells (A549 cell line) with $[Ru(bpy)_2(mtmp)]Cl_2$, no cytotoxic effect was observed either in the dark or upon light irradiation, thus we concluded that the photogenerated $[Ru(bpy)_2(OH_2)_2]^{2+}$ is not cytotoxic, and that the cytotoxicity observed after irradiation of $[Ru(bpy)_2(dmbpy)]Cl_2$ is caused by the released dmbpy. However, the more lipophilic $[Ru(Ph_2phen)_2(mtmp)]Cl_2$ (Ph_2phen = 4,7-Diphenyl-1,10-phenanthroline) shows enhanced cytotoxicity upon light irradiation. Thus, a ruthenium center can be cytotoxic, but the complex needs to be lipophilic enough to be taken up.

Knowing the suitability of N,S molecules as protecting ligands and the importance of a certain grade of lipophilicity to achieve cytotoxicity, a new series of complexes bearing the N,S ligand 3-(methylthio)propylamine (mtpa) was synthesized as described in Chapter 4. In this series the strain and the lipophilicity was increased by addition of methyl groups in positions 6 and 6' of bpy, as reported in Chapter 2 for L-prolinate complexes. The number of methyl groups has a crucial effect on the photochemistry and cytotoxicity of these complexes. While the non-strained complex $[Ru(bpy)_2(mtpa)](PF_6)_2$ does not fully photosubstitute mtpa and thus is not photocytotoxic against A549 cells, the more strained complex $[Ru(bpy)(dmbpy)(mtpa)](PF_6)_2$ shows efficient mtpa photosubstitution upon blue light irradiation, leading to photocytotoxicity. However, if the complex is too strained, as in $[Ru(dmbpy)_2(mtpa)](PF_6)_2$, it also activates thermally in the dark, losing the photoactivation feature. The characterization of these complexes was not an easy task. Besides the chirality of the octahedron (Δ or Λ), two other sources of isomerism are present: the configuration (*S* or *R*) of the sulfur atom, and the chair inversion of the six-membered ring resulting from the coordination of the N,S chelating ligand to the ruthenium center. The latter transforms an axial thioether methyl group (*ax*) into an equatorial one (*eq*) and *vice versa*, making a total of four possibles isomers for $[Ru(bpy)_2(mtpa)](PF_6)_2$ and $[Ru(dmbpy)_2(mtpa)](PF_6)_2$, and eight for $[Ru(bpy)(dmbpy)(mtpa)](PF_6)_2$, due to the thioether sulfur being *trans* either to bpy or to dmbpy. Despite the complexity of the stereochemical identification of these complexes, they were all fully characterized by a combination of 2D NMR spectroscopy and DFT calculations. The interligand interactions between the hydrogen

atoms in axial position of the mtpa chair conformation and the substitutents in the position 6 of the bpy appeared to be the main driving force in the stereoselectivity of the synthesis.

As N,S ligands seemed superior to N,O ligands due to their selective photosubstitution, they were chosen for the synthesis of photoactivatable ruthenium-based cyclometalated complexes of the type $[Ru(bpy)(phpy)(N,S)]PF_6$ ($phpy^-$ = 2-phenylpyridine, Chapter 5). The effect of the size of the chelate ring involving the N,S ligand and the ruthenium center (five- *vs.* six-membered ring) and of the nature of the nitrogen donor atom (primary amine *vs.* pyridine) was systematically investigated. Coordination of 2-(methylthio)ethyl-2-pyridine (mtep) or mtpa to the ruthenium(II) center, which results in a six-membered ring with chair conformation, leads to one isomer out of the 16 possible. However, when the N,S ligand leads to a five-membered chelate ring (2-(methylthio)ethylamine (mtea) or mtmp), two or three isomers were obtained, which were difficult to isolate. Thus, the size of the N,S chelating ligands can be chosen to tune the stereoselectivity of the reaction. Furthermore, complexes with N,S ligands leading to a six-membered chelate ring showed faster photosubstitution in CH_3CN than their five-membered chelate ring analogues. Probably rechelation is faster in the latter, lowering the overall photosubstitution rates. Finally, if the nitrogen of the N,S ligand is a pyridine, the complex was found to be less sensitive to oxidation than if it was a primary amine. We adscribe this effect to the location of the π-accepting pyridyl ligand *trans* to the carbon donor atom, which stabilizes the high electron density on the ruthenium center brought by cyclometalation. The complex $[Ru(bpy)(phpy)(mtep)]PF_6$, which bears a pyridyl-based N,S ligand forming a six-membered chelate ring, fulfills all criteria to become a promising PACT agent: it can be synthesized in a stereoselectively manner, it is stable under O_2 in the dark, and it photosubstitutes efficiently the non-toxic N,S ligand upon irradiation with green light.

In the last chapter, we questioned whether or not the ruthenium compounds described in this thesis are true PACT agents. In other words: can their low singlet oxygen production quantum yields (Φ_Δ) explain the observed cytotoxicity? First, as a control of the hypoxic cell irradiation setup we showed that the photocytotoxicity of the photosensistizers Rose Bengal and $[Ru(Ph_2phen)_2(bpy)]Cl_2$ is seriously impaired at 1% O_2. The low dioxygen concentration lowered the photo index (PI), *i.e.* the ratio of the EC_{50} value obtained obtained in a dark control and that after light irradiation, to 3.0 and 1.9, respectively, compared to the much higher values observed under normoxia (>400 and 29, respectively). Using the same set up, the cytotoxicity of the supposed PACT

complexes $[Ru(bpy)(Ph_2phen)(mtmp)](PF_6)_2$ and $[Ru(dmbpy)_2(mtmp)]Cl_2$ was tested, which showed a clear cytotoxicity enhancement after green light irradiation under hypoxia. The cytotoxicity in the dark was too low to establish a PI, which for a true PACT agent should be in the same range under normoxia and hypoxia.[2] Overall, the hypoxic conditions appear to be interesting for testing whether photosubstitutionally active compounds are indeed true, oxygen-independent PACT compounds, or weak but targeted PDT agents.

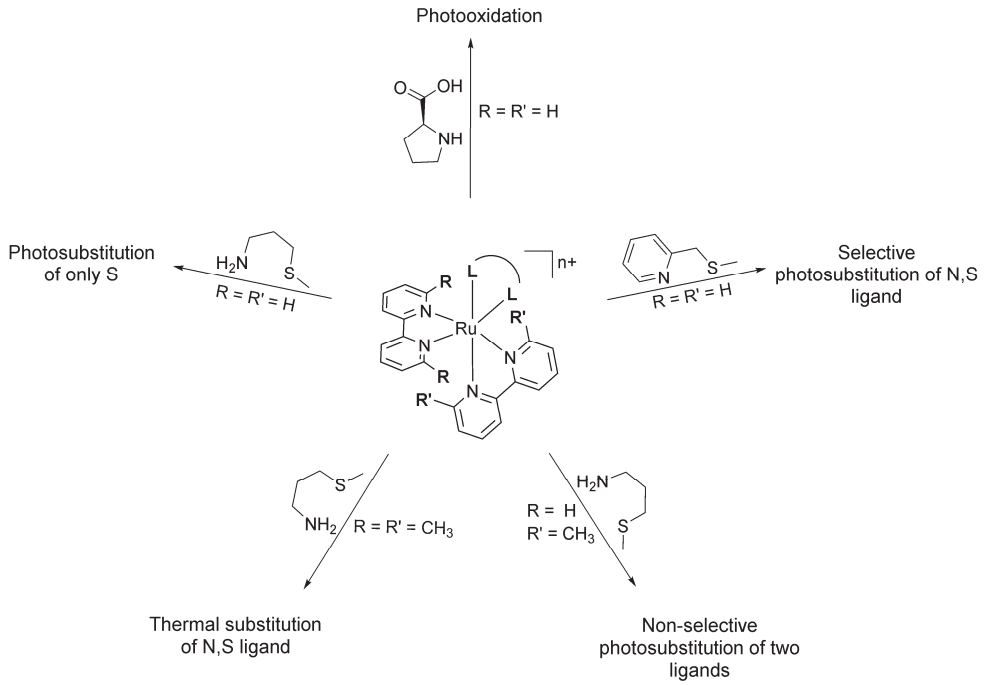
7.2 Discussion and conclusions

7.2.1 How to design a ruthenium complex capable of photosubstituting a bidentate ligand?

In the last decade, the photoreactivity of ruthenium polypyridyl complexes has been extensively studied. It is commonly accepted that thermal population of a triplet metal-centered state (^{3}MC) following photochemical generation of a triplet metal-to-ligand charge transfer state (^{3}MLCT) is the critical step to photosubstitute a ligand. Indeed, in the ^{3}MC state the coordination bond between one of the photolabile ligand L and the ruthenium center elongates, thus becoming weaker and more prone to cleavage by substitution of the leaving ligand by an entering solvent molecule.[3] In phosphorescent complexes such as $[Ru(bpy)_3]^{2+}$, the ^{3}MC state is very high in energy, which prevents photosubstitution to occur. As mentioned in previous chapters, there are two methods to make the ^{3}MC states more accessible. One is to distort the octahedral coordination sphere of the metal, upon which the crystal field splitting energy is decreased, making the thermal population of a metal-based e_g orbital from a half-filled ligand-based π^* orbital possible.[4] The other method is to tune the electronic structure of the complex by changing the nature of the ligand to be photosubstituted.[5]

In the research described in this thesis we have followed both approaches (Scheme 7.2). In absence of octahedral distortion, *i.e.* in a complex of the type $[Ru(bpy)_2(L)]^{n+}$, the nature of the coordinating atoms of ligand L has a great impact on photosubstitution. When L is L-proline, the complex $[Ru(bpy)_2(L\text{-prolinate})]^+$ does not photosubstitute any ligand due the strong σ-donor properties of the carboxylate moiety. However, when the negatively charged carboxylate group is substituted by a thioether donor group, photosubstitution of the sulfur donor atom by a solvent molecule does occur in water, as shown for $[Ru(bpy)_2(mtpa)](PF_6)_2$. Interestingly, upon light irradiation the photoproduct $[Ru(bpy)_2(mtpa\text{-}\kappa N)(OH_2)]^{2+}$ was obtained in the steady state, *i.e.* full photosubstitution of mtpa by two water molecules did not occur. When

the primary amine of mtpa was replaced by pyridine as in mtmp, the bis-aqua species $[Ru(bpy)_2(OH_2)_2]^{2+}$ was obtained upon light irradiation of $[Ru(bpy)_2(mtmp)]^{2+}$ in water. Although sulfur is clearly a more photolabile donor atom than a negatively charged oxygen ligand, and pyridine leads to full photosubstitution compared to the monosubstitution of the primary amine-based mtpa, a second factor has to be considered: the chelate ring size. As shown in Chapter 6, the chelate ring size has a major impact on the photoreactivity of N,S-based cyclometalated ruthenium complexes. Thus, we would expect that the different photoreactivity of mtpa and mtmp is not only due to the nature of the nitrogen donor atom, but also due to the different chelate ring sizes. In order to definitively solve this question, complexes bearing pyridine-based ligands resulting in a six-membered ring (*i.e.* $[Ru(bpy)_2(mtep)]^{2+}$) and primary amine-based ligands resulting in a five-membered ring ($[Ru(bpy)_2(mtea)]^{2+}$ should be synthesized and their photochemistry investigated.



Scheme 7.2. The photoreaction taking place upon irradiation of a ruthenium(II) complex depends on the nature of the photolabile ligand and on the strain of the complex.

The effect of the distortion of the octahedral geometry was also thoroughly investigated. In Chapter 2 and Chapter 4 we used varying numbers of hindering ligands such as dmbpy. Although octahedral distortion has a positive impact on the photosubstitution rate, it also has a negative impact on the selectivity of the

photosubstitution reaction, or even on the thermal stability of the complex in the dark. For example, $[Ru(bpy)(dmbpy)(L\text{-prolinate})]^{+}$ and $[Ru(bpy)(dmbpy)(mtpa)]^{2+}$ photosubstitued both dmbpy and L-proline or mtpa upon irradiation in CH_3CN and water, respectively, while $[Ru(dmbpy)_2(mtpa)]^{2+}$ is unstable in the dark. Thus, tris-heteroleptic complexes with only one hindering dmbpy ligand were considered, as they bring more thermal stability while keeping efficient photosubstitution. These heteroleptic complexes are significantly more challenging to synthesize, all the more when dissymmetric N,S or N,O ligands are introduced. The selectivity of photosubstitution reactions can also become problematic as several reactions may occur in parallel. To our knowledge, the non-selectivity in the photosubstitution of a ruthenium(II) complex is unprecedented, as well as the solvent-dependent selectivity, as shown for $[Ru(dmbpy)_2(mtmp)]^{2+}$ (Chapter 6). Indeed, understanding the fate of an excited state is not an easy task. Few groups have reported computational chemistry methods to predict and understand conical intersections, from where an excited complex in the ^{3}MLCT can follow different reaction pathways.[6-9] For example, the case of the bis-sulfoxide complex $[Ru(bpy)_2(OSSO)]^{2+}$ (OSSO = dimethylbis-(methylsulfinylmethyl)silane), which generates a mixture of mono- and bis-isomerized S→O complexes upon light irradiation, ahs been thoroughly studied.[10] In the case of a photosubstitution reaction, studies performed to the date usually focus on the ^{3}MC-^{3}MLCT gap, assuming that the entering ligand reacts quickly with any pentacoordinated intermediate state. In this thesis we show that a more complete method involving the nature of the entering ligand and including the effect of the solvent would be necessary.

7.2.2 Photocytotoxicity of ruthenium complexes photosubstituting a ligand

Light irradiation of ruthenium polypyridyl complexes might lead to several photoproducts, each of which may have its own biological effect. As shown in Figure 7.1, the excited state may lead by photosubstitution to an aquated ruthenium(II) complex and the free ligand, by electron transfer to superoxide radicals ($O_2^{\cdot -}$), and/or by energy transfer to singlet oxygen ($^1O_2$). Depending on which photoproduct is responsible for cell death, the mode of action can be considered as a PACT (metal-based or ligand-based), PDT type I, or PDT type II, respectively. However, it is also possible that several of these processes occur at the same time, making the identification of the predominant mode of action quite difficult.

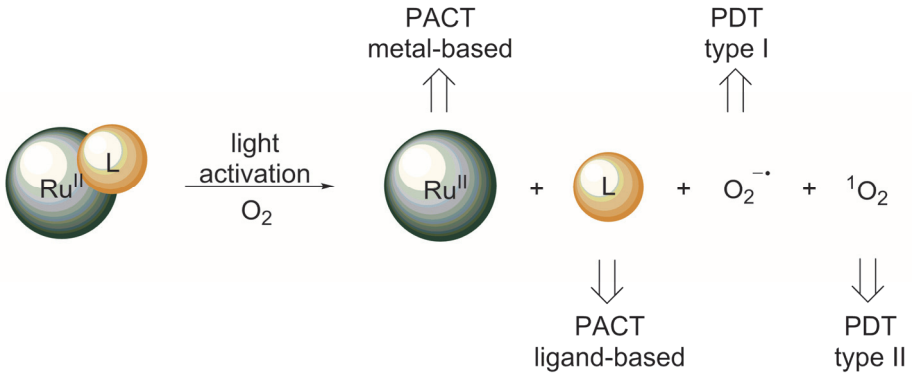


Figure 7.1. Possible photoproducts generated after light irradiation of a ruthenium(II) polypyridyl complex. Depending on which compound leads to cell death the mode of action will be associated to metal-based PACT, ligand-based PACT, PDT type I, or PDT type II.

In order to study specifically the photocytotoxicity of the metal center we first avoided the used of cytotoxic photolabile ligands and focused on L-proline, mtmp, and mtpa. Since dmbpy was found to be cytotoxic against A549 cells with an EC_{50} value of ~8 µM (Chapter 3), at least part of the cytotoxicity of all complexes partially releasing dmbpy may be attributed to the dissociated dmbpy. For Glazer's reference compound $[Ru(bpy)_2(dmbpy)]Cl_2$ for example,[1] we demonstrated that the photocytotoxicity is caused by the photoreleased dmbpy, while $[Ru(bpy)_2(OH_2)_2]^{2+}$ appeared to be incapable of penetrating the cell and do any harm. Some ambiguity between metal-based and ligand-based photocytotoxicity was also found for $[Ru(bpy)(dmbpy)(mtpa)](PF_6)_2$ (Chapter 4) and $[Ru(dmbpy)_2(mtmp)]Cl_2$ (Chapter 6), which can photosubstitute non-selectively both the N,S ligand and dmbpy.

For compounds in which the photocytotoxicity unambiguously comes from the metal center, distinguishing a PDT type II mechanism *vs.* a PACT mechanism led us to test our complexes under low dioxygen concentrations (1%), instead of the 21% typically used in the field. Even under 1% O_2, a complex like $[Ru(bpy)(Ph_2phen)(mtmp)]^{2+}$, which photosubstitutes mtmp by solvent molecules, showed enhanced cytotoxicity upon light irradiation. Clearly, the obtained photocytotoxicity remained mild under such demanding testing conditions, which entail the performance of the whole cytotoxicity assay under hypoxic conditions using 2D cancer cell monolayers that have been passaged twice under hypoxia before the assay. However, the fact that some photocytotoxicity was observed at all is encouraging, and justifies future research with different compounds and possibly different cancer models, ultimately aiming at demonstrating photoactivated anticancer activity *in vivo*.

7.2.3 On lipophilicity and dark cytotoxicity

Next to photocytotoxicity and its relation to the photochemical properties of the ruthenium complex, studying the impact of the lipophilicity of the metal-containing prodrug on its dark cytotoxicity has been highlighted many times across this thesis. Generally, bis(bipyridine)-based complexes, with *log P* values around −1.4 are not lipophilic enough to cross cell membranes and be taken up passively (Chapter 3), which also explains why $[Ru(bpy)_3]Cl_2$ is a poor PDT agent *in vitro* in spite of its excellent singlet oxygen generation properties (*data not shown*). Increasing the lipophilicity of the ruthenium complex by adding several methyl or phenyl groups, usually results in higher cell uptake, as has been described previously.[11] It also leads to a higher dark cytoxocity. $[Ru(dmbpy)_2(mtpa)](PF_6)_2$ or $[Ru(bpy)(Ph_2phen)(mtmp)](PF_6)_2$ are two examples of more lipophilic complexes showing significant dark cytotoxicity. Although the reason for such general cytotoxicity is unknown, many reports show that positively charged lipophilic compounds localize in the mitochondria, destabilizing the mitochondrial membrane potential, thus suggesting a general mechanism for dark cytotoxicity.[12] Finally, when the ruthenium complexes are too lipophilic, like in $[Ru(Ph_2phen)_2(mtmp)]Cl_2$ or the monocationic cyclometalated complex $[Ru(bpy)(phpy)(mtep)]PF_6$, the cytotoxicity observed in the dark is too high to be significantly improved by light irradiation, thus leading to overall disappointing photo indexes. In conclusion, the best metal-based PACT compounds require intermediate lipophilicity using tris-heteroleptic complexes and dissymmetric non-toxic protecting ligands, leading to stereochemically challenging chemical structures and interesting, solvent-dependent selectivity issues under light irradiation.

7.3 References

1. B. S. Howerton, D. K. Heidary and E. C. Glazer, *J. Am. Chem. Soc.*, **2012**, 134, 8324-8327.
2. L. N. Lameijer, D. Ernst, S. L. Hopkins, M. S. Meijer, S. H. C. Askes, S. E. Le Dévédec and S. Bonnet, *Angew. Chem., Int. Ed.*, **2017**, 56, 11549-11553.
3. A. J. Göttle, F. Alary, M. Boggio-Pasqua, I. M. Dixon, J.-L. Heully, A. Bahreman, S. H. C. Askes and S. Bonnet, *Inorg. Chem.*, **2016**, 55, 4448-4456.
4. A.-C. Laemmel, J.-P. Collin and J.-P. Sauvage, *Eur. J. Inorg. Chem.*, **1999**, 383-386.
5. R. N. Garner, L. E. Joyce and C. Turro, *Inorg. Chem.*, **2011**, 50, 4384-4391.
6. F. Alary, M. Boggio-Pasqua, J.-L. Heully, C. J. Marsden and P. Vicendo, *Inorg. Chem.*, **2008**, 47, 5259-5266.
7. V. I. Baranovskii, D. A. Mal'tsev and O. V. Sizova, *Russ. Chem. Bull.*, **2012**, 61, 973-979.
8. B. A. McClure, N. V. Mockus, D. P. Butcher Jr, D. A. Lutterman, C. Turro, J. L. Petersen and J. J. Rack, *Inorg. Chem.*, **2009**, 48, 8084-8091.
9. S. E. Greenough, G. M. Roberts, N. A. Smith, M. D. Horbury, R. G. McKinlay, J. M. Żurek, M. J. Paterson, P. J. Sadler and V. G. Stavros, *Phys. Chem. Chem. Phys.*, **2014**, 16, 19141-19155.
10. K. Garg, A. W. King and J. J. Rack, *J. Am. Chem. Soc.*, **2014**, 136, 1856-1863.
11. C. A. Puckett and J. K. Barton, *J. Am. Chem. Soc.*, **2007**, 129, 46-47.

12. M. Dickerson, Y. Sun, B. Howerton and E. C. Glazer, *Inorg. Chem.*, **2014**, 53, 10370-10377.