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Photorelease of a Metal-Binding Pharmacophore from a Ru(II) Polypyridine Complex

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Abstract

The adoption of compounds that target metalloenzymes comprises a relatively low (<5%) percentage of all FDA approved therapeutics. Metalloenzyme inhibitors typically coordinate to the active site metal ions and therefore contain ligands with charged or highly polar functional groups. While these groups may generate highly water-soluble compounds, this functionalization can also limit their pharmacological properties. To overcome this drawback, drug candidates can be formulated as prodrugs. While a variety of protecting groups have been developed, increasing efforts has been devoted towards the use of caging groups, which can be removed upon exposure to light, as this provides spatial and temporal control over the treatment. Among these, the application of Ru(II) polypyridine complexes is receiving increased attention based on their attractive biological and photophysical properties. Herein, a conjugate consisting of a metalloenzyme inhibitor and a Ru(II) polypyridine complex as a photo-cage is presented. The conjugate was designed using density functional theory calculations and docking studies. The conjugate is stable in an aqueous solution, but irradiation of the complex with 450 nm light releases the inhibitor within several minutes. As a model system, the biological properties were investigated against the endonucleolytic active site of the influenza virus. While showing no inhibition in the dark in an in vitro assay, the conjugate generated inhibition upon light exposure at 450 nm, demonstrating the ability to liberate the metalloenzyme inhibitor. The presented inhibitor-Ru(II) polypyridine conjugate is an example of computationally-guided drug design for light activated drug release and may help reveal new avenues for the prodrugging of metalloenzyme inhibitors.

Graphical Abstract

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Inhibition of Influenza Endonuclease
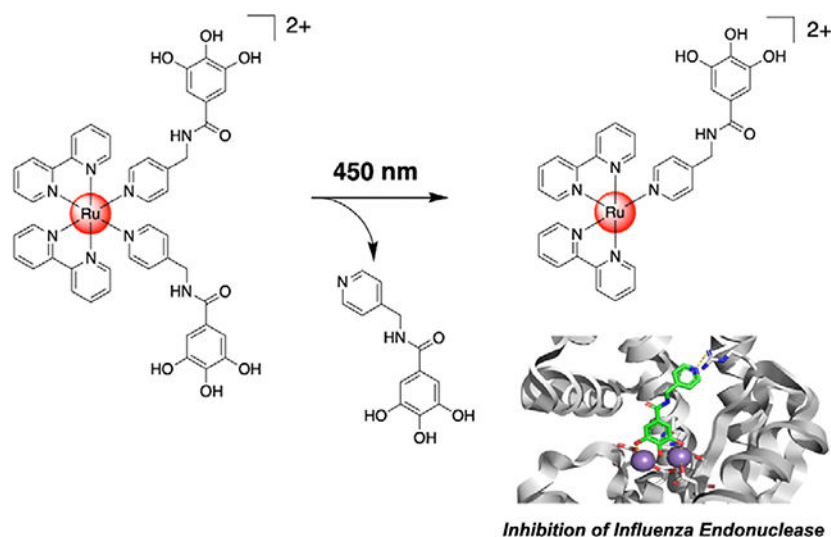
Keywords

Antiviral agents; Bioinorganic Chemistry; Medicinal Inorganic Chemistry; Photoactivation; Photocage

INTRODUCTION

About 40–50% of all identified and characterised enzymes require a metal ion to perform their biological function.[1–2] Despite comprising such a large space for drug development, less than 5% of recently approved therapies by the Food and Drug administration (FDA) target metalloenzymes,[3] making them attractive and underrepresented pharmacological targets.

While the metal ion can act as a structural domain, metal ions are also found in the active site to catalyse various transformations. The vast majority of compounds that act as metalloprotein inhibitors developed by academic, as well as pharmaceutical laboratories, are small molecules. These molecules typically bind to the catalytic active site metal ion, hindering the activity of the enzyme. To enable a tight coordination, compounds with charged or highly polar groups are necessary, which generates compounds that are generally hydrophilic. While improved water solubility can be desirable for a pharmacological modulator, these properties can also hamper their biological activity through various side effects or poor cellular permeability.[3–5]

Among different strategies to retain these polar functional groups of a promising inhibitor, the hydrophilic groups can be protected using a prodrug strategy. Notably, approximately 10% of all marketed drugs act through a prodrug mechanism.[6–7] To date, various protecting groups have been introduced and are currently utilized in clinically approved drugs. Despite their success, the regulation of the pharmacokinetic properties of the drug release represents a challenging research topic.[8–10] As a complementary strategy, the release by an external

trigger is considered favourable to regulate the dosing and location of a therapeutic, presenting the possibility to enable a selective treatment only at the target site.[11–12]

Over the last decades, increasing attention has been devoted towards the use of caging groups which can be removed upon exposure to light, providing complete spatial and temporal control over drug release. Within this field, Ru(II) polypyridine complexes are particularly promising photocaging groups, due to their attractive photophysical and biological properties (i.e., high stability, high water solubility, strong luminescence, large Stokes shift).[13–17] In addition, Ru(II) polypyridine complexes can be activated using visible light, while photoactive organic cages typically require UV light, which limits tissue penetration depth and can cause tissue damage.[18] To date, several Ru(II) polypyridine complex scaffolds based on $[Ru(2,2´;6´−2´´-terpyridine)(inhibitor)_3]^{2+}$,[19] [Ru(bidentate ligand)$_2$(inhibitor)$_2$]$^{2+}$,[20–24] and $[Ru(2,2´;6´−2´´-terpyridine)(bidentate ligand)(inhibitor)]^{2+}$,[25–33] with purine, thiol, pyridine and imidazole derivatives as enzyme inhibitors have been described. This strategy has been investigated for use as a combination treatment with other techniques, including chemotherapy or photodynamic therapy to treat cancer tumours.

Herein, the concept of combining a metalloenzyme inhibitor with a Ru(II) polypyridine complex as a caging agent has been explored. Using a combination of docking studies for prediction of the binding pose in the active site and density functional theory (DFT) calculations for investigation of the photorelease mechanism, a novel conjugate was designed. While the compound was found to be stable in an aqueous solution, the monodentate coordinated enzyme inhibitor was released within minutes upon visible light irradiation. Using a model metalloenzyme system, the conjugate demonstrated no inhibition in an in vitro assay in the dark, while significant inhibition upon irradiation at 450 nm was observed, presenting the possibility of a selective treatment. This study presents the first example of the computationally-guided design of compounds for light-activated drug release as well as the first example of a light-activated inhibitor against the influenza virus, and can serve as a model for the light-triggered release of metal-binding pharmacophores for various other diseases.

RESULTS AND DISCUSSION

Target Selection

The target of our effort is the RNA-dependent RNA polymerase of the influenza virus, which was used as a model metalloenzyme system.[34–38] The viral N-terminal domain (PA$_N$) of the PA polymerase subunit is a validated target with no known human homolog, and is highly conserved over all circulating influenza strains.[39] Inhibitors have been described that target the PA$_N$ by binding to its dinuclear Mn^{2+} or Mg^{2+} active site.[40–41] Indeed, the drug Baloxavir marboxil, which is administered as a carbonic acid ester prodrug, was recently FDA approved as a PA$_N$ inhibitor.[42–43]

Compound Design

The basis of the compound design is gallic acid that has a known IC$_{50}$ value (half maximal inhibitory concentration) of 4.2±1.0 μM against PA$_N$.[44] To enable elaboration of this

compound for conjugation with a photoactive metal center, the binding pose of this compound was predicted (Figure 1) in the PA_N active site. The three-dimensional geometry of the fragment was optimized using DFT calculations and the binding pose within the active site was anticipated using docking studies. As observed within the native protein, the coordination geometry of the Mn^{2+} ions was assumed to be octahedral. While the hydroxyl group in the 4-position coordinates to both metal centers, the binding is further supported by the axial coordination of the hydroxyl groups in positions 3 and 5. The coordination mode of gallic acid was found to be very similar to the crystal structures determined in metal-organic nanoclusters made of gallic acid derivatives with Mn(II) ions.[45] Furthermore, the binding pose of gallic acid was compared with structurally related compounds including hydroxypyridinones and 3-hydroxy-4-oxo-1,4-dihydropyridine-2-carboxylic acid derivatives which were crystallized in the active site of PA_N endonuclease.[39, 44, 46–50] The coordinating oxygen atoms of the predicted binding pose of gallic acid were found in the same positions as the those of the crystallographically described inhibitors.

To enable the coordination of a metal complex which can release the inhibitor upon a light trigger, a pyridine functionality was chosen as a conjugating group, which could be linked by a peptide bond. Docking studies of this molecule revealed (Figure 1) that the additional pyridyl moiety could potentially also interact within the active site by hydrogen bonding with Arg124.

As the basis for the photoreleasing moiety, Ru(II) complexes were investigated due to their high biocompatibility. Ru(II)/Ru(III) complexes such as RAPTA-C, KP-1019, KP-1339, NAMI-A and TLD-1433 have been or are currently being investigated in clinical trials as anticancer agents.[51–53] Of special interest as a scaffold for the photorelease of an enzyme inhibitor are Ru(II) polypyridine complexes, due to their attractive chemical and photophysical properties. As the compound $[Ru(2,2'\text{-bipyridine})_3]^{2+}$ is well known to be non-toxic,[54] a derivative of this compound was generated by exchanging one 2,2'-bipyridine ligand with two of the gallic acid-derived inhibitors. The mechanism of the photo-induced dissociation of the pyridine ligand from the scaffold was investigated by time-dependant DFT calculations (Figure 2). Upon irradiation, the Ru(II) polypyridine complex is excited to a singlet metal-to-ligand charge transfer (^{1}MLCT) state from which it can undergo an intersystem crossing process to a longer lived triplet ^{3}MLCT state. This excited state can decay back to the ground state upon emitting of a phosphorescence signal, making the complex also potentially suitable as an imaging probe. Alternatively, the excited triplet metal-centered- (^{3}MC) state can be populated, which can promote the photoinduced ligand loss of the monodentate pyridine derivative. The theoretical calculations indicate that the pyridine ligand has a distorted geometry and the Ru-N_{py} bond length is increased about 0.63 Å, which could explain the dissociation. The energy of the excited ^{3}MLCT and ^{3}MC states is highly dependent on the coordinated ligands and theoretically determined coordination bond length.[55] These findings are in agreement with the previous in-depth theoretical and experimental studies of the model compound $[Ru(2,2'\text{-bipyridine})_2(\text{pyridine})_2]^{2+}$.[55–57]

Compound Synthesis

The synthetic procedure for the conjugate complex is outlined in Scheme 1. In the first synthetic step, the metal salt Ru(III)Cl$_3$•×H$_2$O was reduced to Ru(II), forming Ru(dimethyl sulfoxide)$_4$(Cl)$_2$ (**1**). Upon addition of 2,2´-bipyridine and in the presence of an excess of lithium chloride, the asymmetric coordinated Ru(2,2´-bipyridine)$_2$(Cl)$_2$ (**2**) complex was obtained. The chloride ligands were replaced with labile acetonitrile capping ligands (**3**) upon treatment with silver(I) trifluoromethanesulfonate and removal of the precipitated silver(I) chloride. The addition of 4-(aminomethyl)pyridine resulted in an inseparable mixture of products with low solubility in many organic solvents. Based on these findings, the synthetic procedure was changed and the amine group of 4-(aminomethyl)pyridine protected with a *tert*-butyloxycarbonyl group (**4**). The coordination reaction of **4** with **3** successfully yielded the protected Ru(II) polypyridine complex **5**. Upon treatment with hydrochloric acid, the *tert*-butyloxycarbonyl protecting groups were removed and compound **6** isolated. Compound **7** was synthesized by preparing galloyl chloride from gallic acid and thionyl chloride. The addition of compound **6** to the acid chloride resulted in the formation of the conjugate **7**. Compound **8** was prepared similarly, using 4-(aminomethyl)pyridine. To determine the effects of the conjugation to the enzyme inhibitor, the unsubstituted complex [Ru(2,2´-bipyridine)$_2$(pyridine)$_2$][Cl]$_2$ (**9**) was synthesised in an analogous fashion to compound **5**. The identity of the resulting compounds was verified by NMR spectroscopy and high-resolution mass spectrometry (HR-MS) with purity of the final compounds confirmed by HPLC analysis (Figure S1-S22).

Photophysical Evaluation

The stability of the conjugate in an aqueous solution was investigated. The Ru(II) polypyridine complex **7** was incubated in water as well as phosphate buffered saline (PBS) at 37 °C for 48 h in the dark and then analyzed by HPLC. No changes in the chromatogram (Figure S23-S24) were observed, indicating a nominal stability of this metal complex under buffer conditions.

The absorption spectrum of **7** was measured in H$_2$O and compared with the theoretically predicted spectrum (Figure S25). While the ligand centred transitions at around 290 nm were reproduced, the predictions at longer wavelengths were red-shifted about 15 nm. The general overestimation of the absorption properties of Ru(II) polypyridine complexes using the same DFT functionals has been previously observed.[58–59]

The ability to release the enzyme inhibitor from the Ru(II) coordination sphere of **7**, predicted by DFT calculations, was investigated using various experimental techniques. Compound **7** was incubated in H$_2$O and the temporal change of the absorption spectrum was monitored in the dark as well as upon irradiation at 450 nm. While no changes in the dark were observed (Figure 3a), the absorption spectrum did show clear shifts after irradiation for 1 min with two isosbestic points (Figure 3b), indicating changes in the molecular structure of the compound. Within 3 min, new absorption characteristics were asymptotically reached. These results are comparable to the model system [Ru(2,2´-bipyridine)$_2$(pyridine)$_2$][Cl]$_2$ (**9**) which was found to release the pyridine ligands only slightly faster (Figure S26). The changes of **7** were also investigated by following the luminescence properties of the Ru(II)

polypyridine complex. Immediately after exposure to the light, a drastic decrease of the luminescence signal of the metal complex was observed (Figure 3c). After irradiation for 3 min, no emission could be detected, indicating that the generated photoproduct is photo-inactive. For verification that the measured emission signal originates from the prepared Ru(II) polypyridine complex, an excitation spectrum of **7** was measured (Figure S27). As expected, no significant differences to the absorption spectrum were observed. Following this assessment, the release of the enzyme inhibitor was studied by HPLC analysis (Figure 3d). After a 2 min irradiation, the HPLC trace showed some remaining Ru(II) polypyridine-inhibitor conjugate (R_t = 5.1 min), the released enzyme inhibitor (R_t = 11.0 min), as well as the appearance of a new Ru complex-based product peak (R_t = 9.9–10.1 min) as identified by the characteristic retention time and absorption spectrum. Upon doubling of the irradiation time to 4 min, full conversion was reached with the complete release of the enzyme inhibitor. Using an ESI-MS analysis of the light irradiated sample, the release of the enzyme inhibitor and the generation of [Ru(2,2´-bipyridine)$_2$(3,4,5-trihydroxy-*N*-(pyridin-4-ylmethyl)benzamide)]$^{2+}$ ([M – inhibitor + HCOO$^-$]$^+$ calcd. for $C_{34}H_{30}N_6O_6Ru$: 719.1, found: 718.9) was confirmed. Using an HPLC based method (see ESI for details), the quantum yield of the photoinduced ligand release was determined to be 16%.

Enzymatic Activity Evaluation

In order to determine the biological activity of the Ru(II) polypyridine-PA$_N$ inhibitor conjugate **7**, a Förster resonance energy transfer (FRET)-based enzymatic assay was utilized.[44] All Ru-based compounds **2**, **7**, and **9** (Figure 4) showed essential no significant inhibition (IC$_{50}$ >100 μM) in the dark, indicating that the 'metallocaged' compounds are effectively sequestered. Importantly, compound **8**, the presumed product of the ligand ejection, showed inhibitory activity (IC$_{50}$ = 9.3±3.7 μM) similar to gallic acid (IC$_{50}$ = 14.2±6.1 μM).

The effect on enzymatic activity of conjugate **7** upon irradiation was studied. The PA$_N$ enzyme-compound mixture was irradiated at 450 nm (0.84 J/cm^2) for 4 min, as previous investigations have shown that this light dose is necessary for a complete photorelease of the enzyme inhibitor from the Ru(II) center. It is important to note, that exposing the enzyme to a 4 min irradiation did not alter its activity. While complex **7** showed no inhibition effect in the dark (IC$_{50,dark}$ >100 μM), upon light exposure enzyme inhibition was observed (IC$_{50,450 nm}$ = 7.4±2.2 μM). These results suggest a selective treatment of the conjugate. The similar IC$_{50}$ values for the enzyme inhibitor **8** and the conjugate **7** (upon light irradiation) support the observation that the Ru(II) polypyridine inhibitor complex fully ejects one gallic acid derivative, and that this ligand acts as a potent inhibitor of the PA$_N$ endonuclease. Similar light irradiation of complex **9** showed negligible enzymatic inhibition, confirming that the Ru(II) polypyridine complex acts purely as a caging agents.

CONCLUSION

In summary, a conjugate consisting of a metalloenzyme inhibitor and a Ru(II) polypyridine photocage was designed and evaluated. Using a combination of docking studies and DFT calculations, the binding pose in the active site was predicted and the photo-ejection

mechanism of action investigated. While the conjugate remains intact in an aqueous solution, the enzyme inhibitor is ejected upon exposure to light. Using HPLC analysis, UV-visible, and emission spectroscopy the light triggered release at 450 nm was observed within several minutes at low light doses. After ejection of the inhibitor, the Ru(II) complex becomes photo-inactive. Utilizing an in vitro PA_N assay as a model system, the conjugate showed no inhibition in the dark, while releasing and therefore inhibiting the enzyme upon light exposure. Overall, we believe that the approach of a computationally guided compound design, as well as the combination of a photoactive metal complex with an organic inhibitor holds great potential for selective photo-triggered inhibitor release and can open avenues for the selective treatment of various other diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

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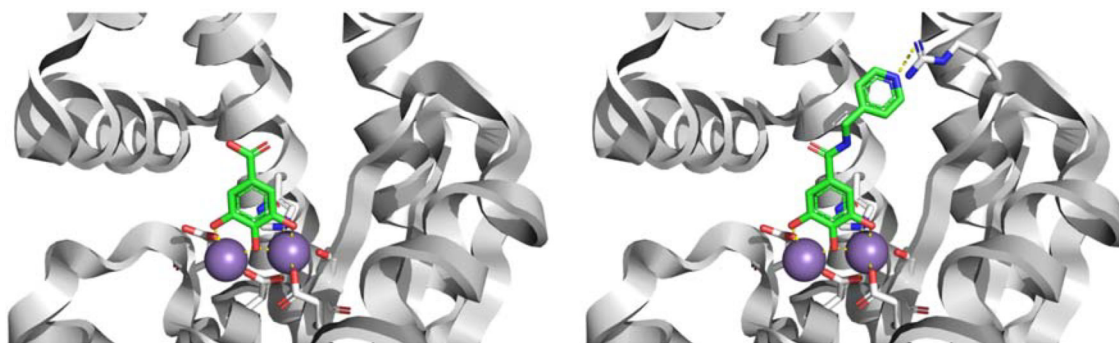
Figure 1.
Docking pose of the fragment gallic acid (left) and the extended inhibitor (right) within the dinuclear metal active site of PA_N endonuclease.
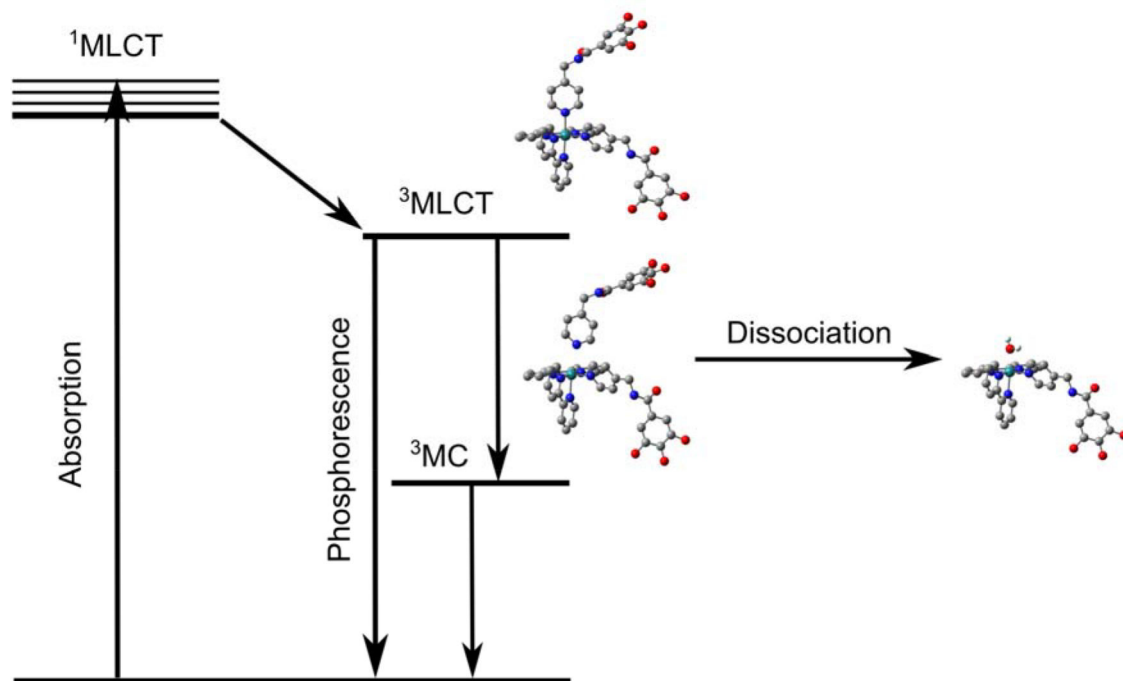
Figure 2.
Schematic diagram of the excited states leading to the dissociation of the pyridine ligand from the Ru(II) polypyridine complex.
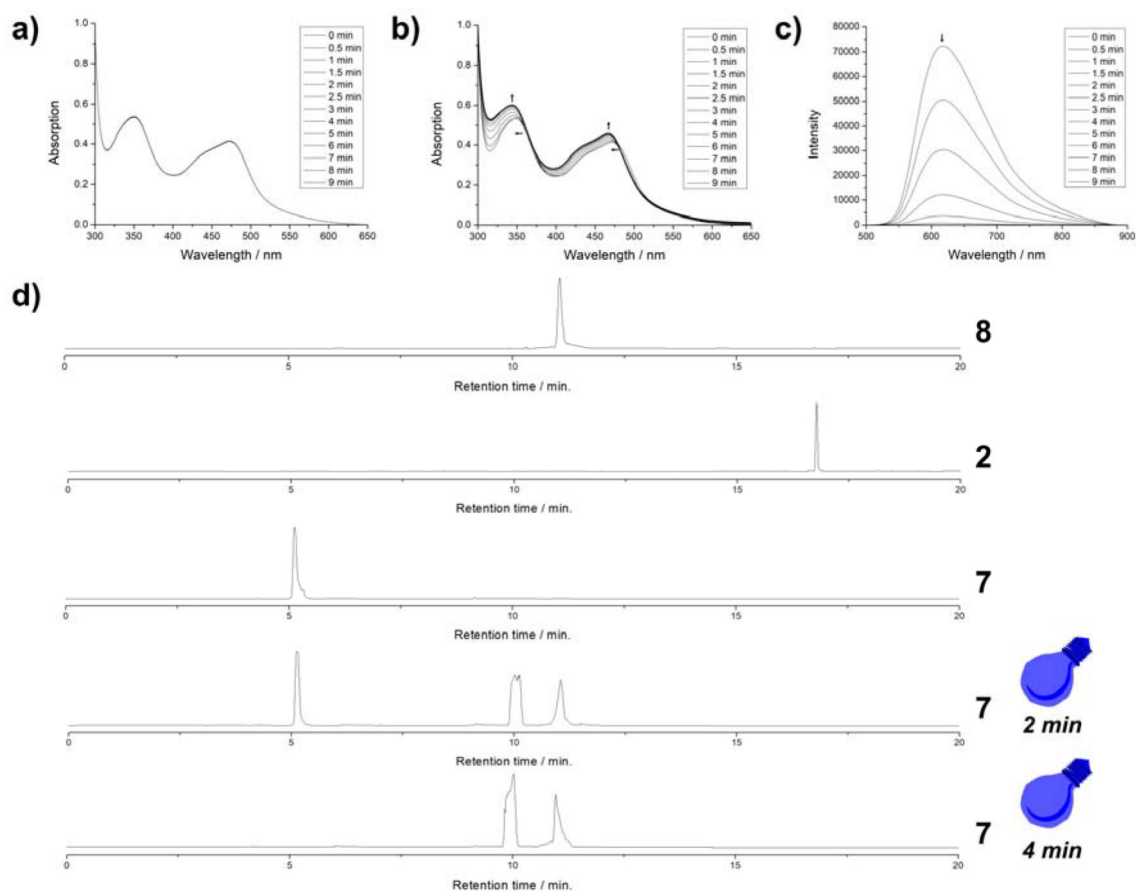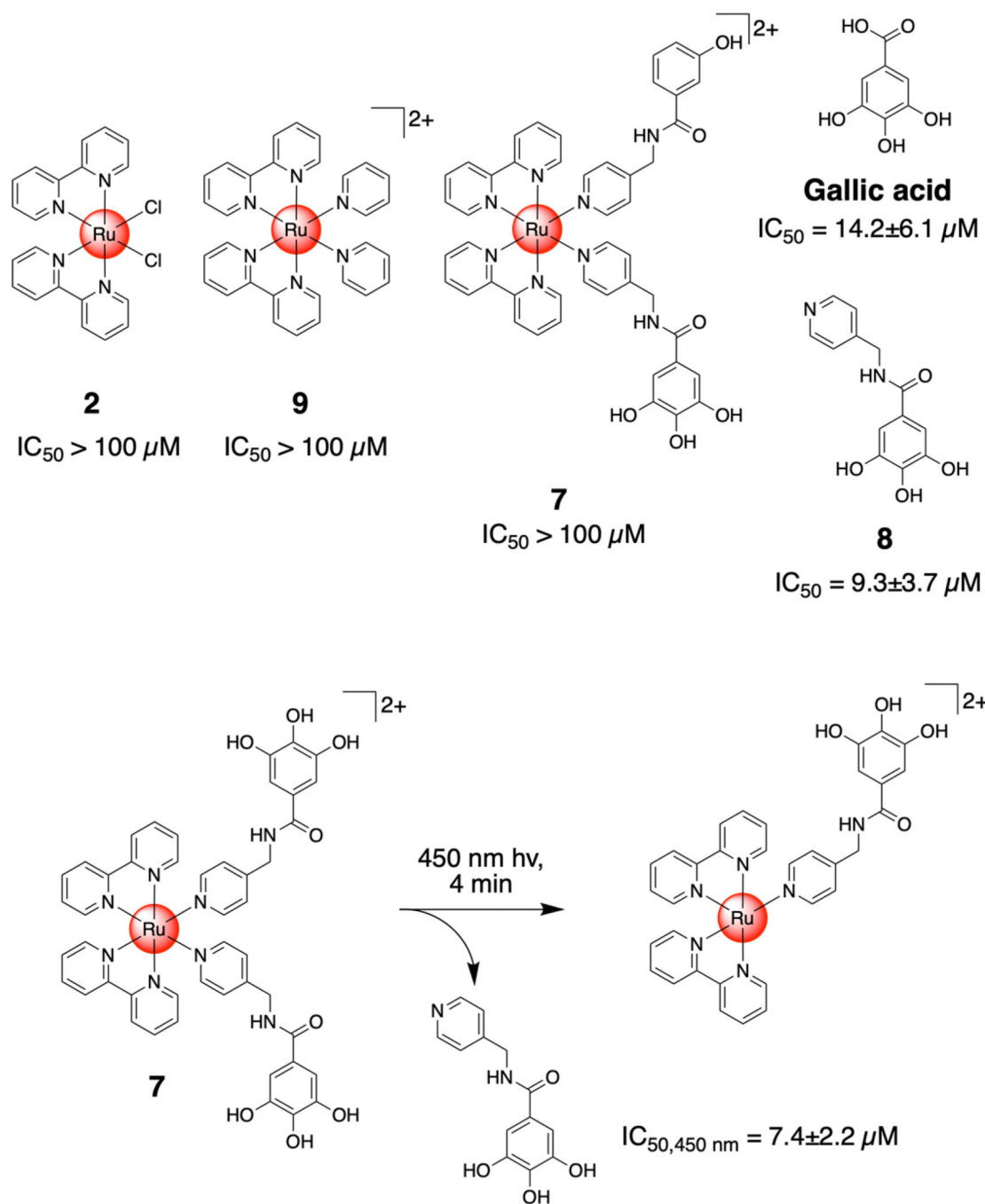
Figure 3.
Study of the release of the enzyme inhibitor from the Ru(II) coordination sphere of **7** upon irradiation at 450 nm. UV/VIS spectroscopy showing temporal change of the absorption spectrum of **7** upon incubation in H_2O a) in the dark; b) upon exposure to the light. c) temporal change of the emission spectrum of **7** upon incubation in H_2O and exposure to the light. d) HPLC traces of the enzyme inhibitor **8**, the $Ru(bipy)_2Cl_2$ intermediate **2** and the combined Ru(II) polypyridine-enzyme inhibitor complex **7** in the dark and upon exposure to the light for 2 and 4 min.

Figure 4.
Structure and inhibition values for compounds **2**, **7**, **8**, **9**, and gallic acid in the dark and for compound **7** upon irradiation at 450 nm.
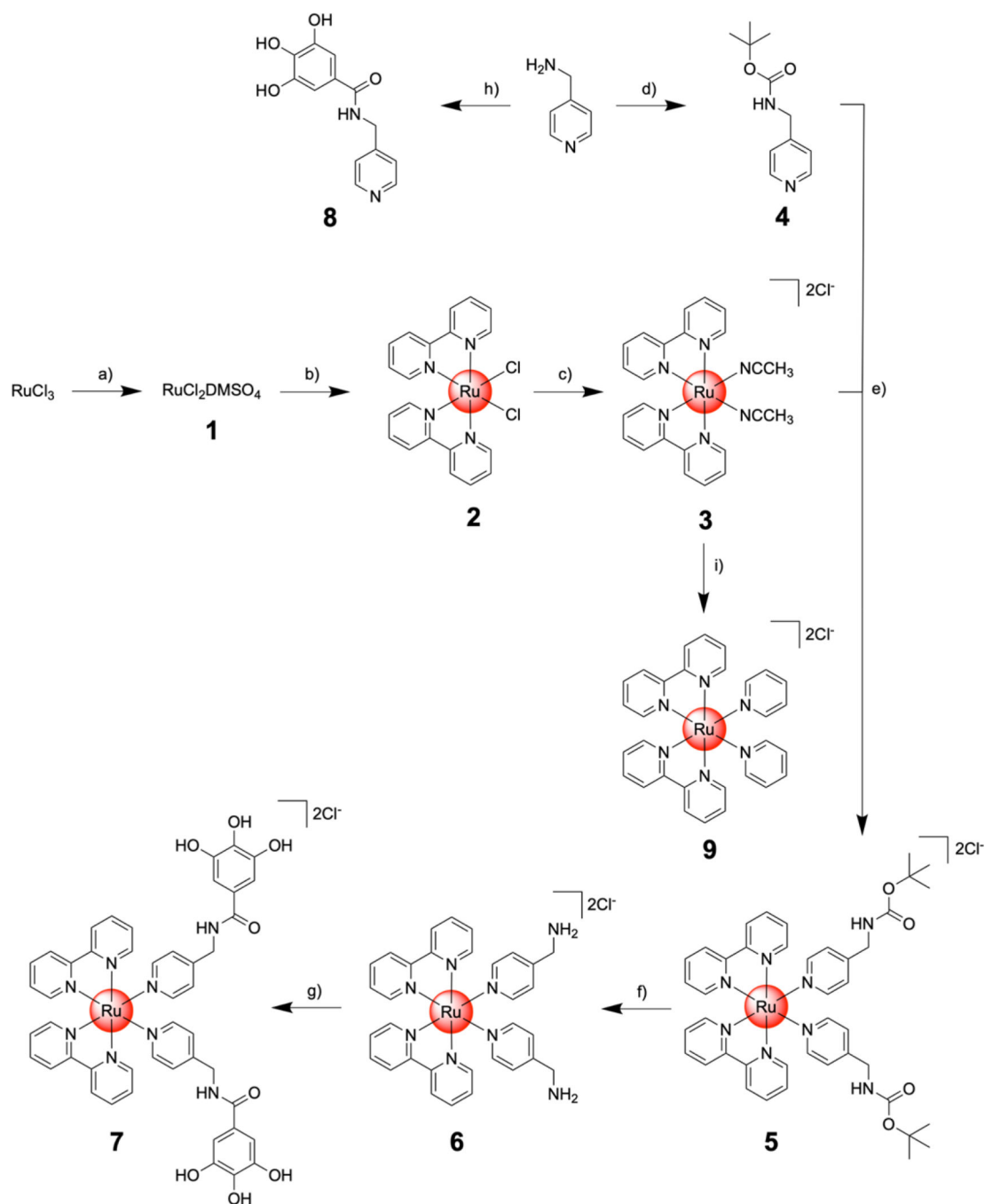
Scheme 1.
Synthetic strategy for the synthesis of the compounds. a) EtOH, 5h; DMSO, 150 °C, 3h; b) 2,2′bipyridine, LiCl, DMF, 4h, nitrogen atmosphere; c) AgOTf, CH$_3$CN, 3h; d) ZrCl$_4$, Boc$_2$O, CH$_3$CN, 1h; e) EtOH, 50 °C, 8h, nitrogen atmosphere; f) DCM/MeOH (4:1), HCl in Et$_2$O (2M), overnight; g) 1. gallic acid, SOCl$_2$, DCM, 2h, 2. DCM, 5h; h) gallic acid, SOCl$_2$, DCM, 2h; i) Pyridine, EtOH, 50 °C, overnight, nitrogen atmosphere.