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Caged Amino Acids for Visible-Light Photodelivery

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We present a photocleavable protecting group based on ruthenium bipyridyl complexes and suitable for amino acid photodelivery. We discuss the photochemical properties of the caged glutamate, which is a major neurotransmitter in the CNS. The molar absorptivity for the caged glutamate at 450 nm is $\varepsilon = 4200\ M^{-1}\ cm^{-1}$ and its quantum yield of photorelease at 450 nm is $\Phi_{PD} = 0.035$, which is about 17 times higher than that of the most active organic caged compound at this wavelength. Similar figures are obtained for other α-amino acids.

Introduction

Caged compounds or phototriggers composed of a molecule to be photoreleased and a protecting group are important tools for neurophysiology and other areas of biological research. The possibility to deliver precise amounts of biomolecules, that is, neurotransmitters, by means of a focused light spot[1–3] in a small area of living tissue allows the researcher to substitute the invasive current injection or picosyringe-based techniques. Nitrobenzyls and related molecules are the most widely employed photolabile protecting groups for caging biomolecules.[1] In this kind of phototrigger, UV light ($\lambda < 350$ nm) is used to start a chemical reaction that yields the free active compound and a residue containing the caging group.

An ideal caged compound should be soluble and stable in water at physiological temperatures and pH, present a large enough quantum yield of photorelease that is suitable for biological studies, and be harmless for the system under study. For mid- and long-term studies, the compound should be photolyzable by using visible rather than UV light in order to prevent cellular damage. Irradiation at 450 nm or higher wavelengths also enables the use of simple and inexpensive light sources, such as DPSS-YAG lasers or InGaN/AlGaN blue LEDs. Most organic caged compounds lack one or more of these characteristics. Banerjee et al. presented two photolabile protecting groups for carboxylic acids that can be used with visible light. The DANP-caged alanine[4] presents a quantum yield of photodissociation $\Phi_{PD} = 0.03$ at 360 nm that decreases to 0.002 at 450 nm. Moreover, it is thermally hydrolyzed in about 100 min, even at low temperatures such as 22 °C, which makes this compound unsuitable for many biological experiments. The α-amino acid glutamate is the major excitatory neurotransmitter in the central nervous system of mammals; therefore, it is one of the most important biomolecules to be caged. In all known types of caged glutamates, the photodetachable protecting group is bound to the carboxylic group of the amino acid. The new compound DECM glutamate[5] shows much better stability and a good yield of photodissociation and $\Phi_{PD} = 0.11$ at 400 nm. Coordination compounds have been used for targeted photodelivery,[6] and in some of these cases, visible-light uncaging of biologically relevant molecules such as nitric oxide is achieved.

We previously reported the first caged compound based on ruthenium bipyridyl complexes that are suitable for uncaging organic biomolecules such as 4-aminopyridine by using 450 nm light.[7] These phototriggers can be used with harmless visible light instead of injurious UV light, and the wavelength of the irradiation and the chemical and physical properties are easily tunable by means of derivatization of the bipyridine units surrounding the metal center. The same metal core can be used in order to make a caged amine compound.[8]

In these kinds of complexes, photorelease is achieved through an excitation pathway that involves irradiation of the MLCT band, which formally promotes a $[Ru^{III}(bpy^{-})]$ excited state; this further decays to a dissociative d–d state that expels the monodentate ligand, which in this case is the biomolecule.

Unfortunately, the photolabile protecting group $[Ru(bpy)_2XY]^{2+}$ contains two coordination positions and therefore any potential bidentate ligand such as an α-amino acid can attach very strongly to the metal center; this reduces the quantum yield of photodelivery. Specifically, the neurotransmitter glutamate can coordinate through both the

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amino and the α-carboxylate groups, and the quantum yield of photodissociation of the $[Ru(bpy)_2GlutH]^+$ complex is extremely low.

The obvious strategy to prevent the formation of the photoinert $[Ru(bpy)_2GlutH]^+$ complex, in which the glutamate ligand is coordinated in a bidentate manner, is the replacement of one of the bipyridine units by a tridentate ligand that occupies one more coordination position.

In this work, we present a method for α-amino acid caging that is based on ruthenium bipyridyl complexes, and we discuss the different photochemical properties of complexes having the tridentate ligands terpyridine and tris(pyrazolylmethane). The complex containing the latter ligand is the first caged amino acid based on inorganic complexes and photocleavable with visible light.

Results and Discussion

Figure 1 shows the UV/Vis spectra of the $[Ru(bpy)(tpy)(GlutH_2)]^{2+}$ complex in aqueous solution before and after irradiation with a 450-nm high-power LED. Changes in the spectra are minimal, which suggests that no photoreaction took place. This behavior is very different to that found in $[Ru(bpy)_2L_2]^{2+}$ complexes, where L is an aliphatic or aromatic amine that undergoes photocleavage to yield $[Ru(bpy)_2L(H_2O)]^{2+}$ and the free amine L.[8] It is known that [Ru(bpy)(tpy)L] complexes present much lower quantum yield of photosubstitution than their bis(bpy) analogues,[9] possibly due to the imperfect matching of the terpyridine coordination angles with those of the metal center, which increases the probability of vibrational decay of the d–d excited state.

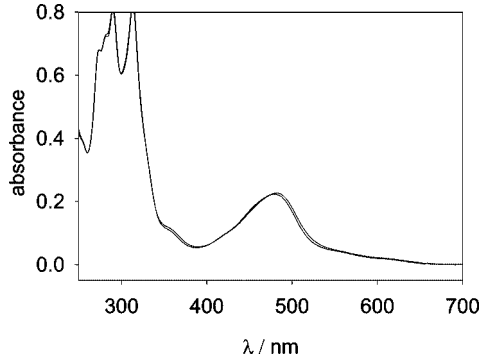

Figure 1. Electronic spectra of the $[Ru(bpy)(tpy)(GlutH)](PF_6)$ complex in H_2O at pH 7 before and after irradiation for 2 h with a 450-nm light of about 50 mW. No photodissociation is apparent ($\Phi_{PD} < 10^{-6}$).

The second compound having the tripodal tris(pyrazolylmethane) ligand (tpm) instead of the tpy ligand behaves differently. On one side, the redox properties of both compounds are very similar, which is in agreement with previous results from similar complexes. The $E_{1/2}$ for the Ru^{II}/Ru^{III} couple in $[Ru(bpy)(tpy)(GlutH_2)](PF_6)_2$ is 1.132 V vs. NHE in acetonitrile. A second quasireversible process can be seen at 1.317 V vs. NHE, and it appears to be the Ru^{II}/Ru^{III} couple for the oxidized amine species.[10] The same redox processes are visible in $[Ru(tpm)(bpy)(GlutH_2)](PF_6)_2$ and appear at 1.111 V vs. NHE and 1.300 V vs. NHE (see Supporting Information).

On the other side, whereas the photolability of the tpy complex is negligible, $[Ru(tpm)(bpy)]^{2+}$ is an efficient photolabile protecting group for the amino group. Figure 2a shows the aliphatic region of the ^1H NMR spectrum of $[Ru(tpm)(bpy)(GlutH_2)](PF_6)_2$ in D_2O. The signals of the amino protons of the coordinated glutamate can be seen at δ = 3.72 and 3.93 ppm, which indicates that proton exchange through D^+ addition cannot occur, as can be expected for amino-coordinated glutamate. Attempts to coordinate carboxylic acids to the $[Ru(tpm)(bpy)]^{2+}$ moiety under the same conditions as those for glutamate gave negative results, whereas complexes of several aliphatic and aromatic amines could be obtained. This evidence indicates that glutamate is coordinated through the amino group.

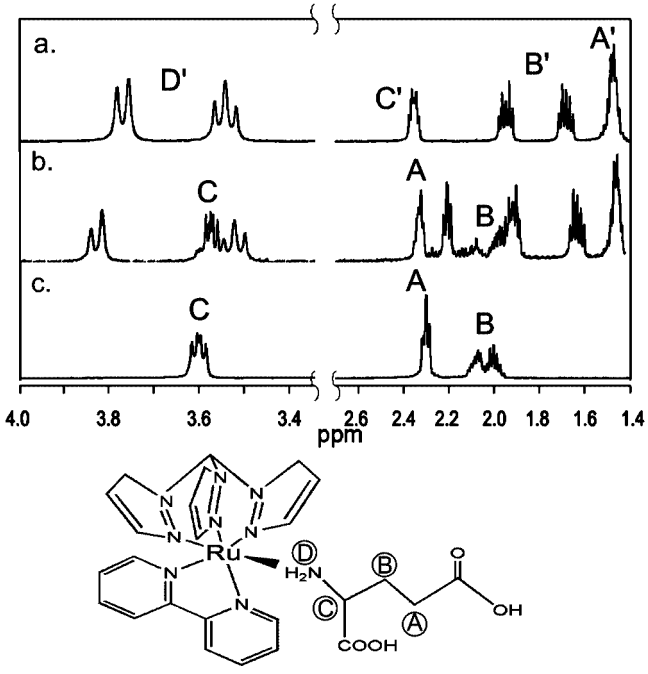

Figure 2. ^1H NMR spectrum of the $[Ru(tpm)(bpy)(GlutH_2)](PF_6)_2$ complex in D_2O (a) before irradiation, (b) after irradiation with a 450-nm source, and (c) after exhaustive photolysis (free glutamate). Signals are slightly shifted due to pH changes.

After irradiation with 450 nm light, the obtained ^1H NMR spectrum changes dramatically and the signals for the free glutamate can be seen (Figure 2b). Exhaustive photolysis gave the free glutamate (Figure 2c).

To determine the quantum yield of photorelease, irradiation of a dilute complex solution with the 450-nm LED source was performed. The used LED presents a narrow spectral width of 20 nm at half height. A glass cuvette containing 2 mL of a 74-μM aqueous solution of $[Ru(bpy)_2(py)_2](PF_6)_2$ as a standard[11] was used to calibrate the source. UV/Vis spectra were taken at different irradiation times. Spectra analysis was performed by considering that only the aqua complex $[Ru(bpy)_2(py)(H_2O)](PF_6)_2$ is obtained as a

photoproduct with a quantum yield of photosubstitution of 0.26. The light power in the cuvette was calculated as 9.4 mW at a center wavelength of 450 nm, which corresponds to 35.4×10^{-9} photon s^{-1}. Once calibrated, the same source was used immediately to irradiate a solution of [Ru(tpm)(bpy)(GlutH)](PF_6) at pH 7. The results can be seen in Figure 3.

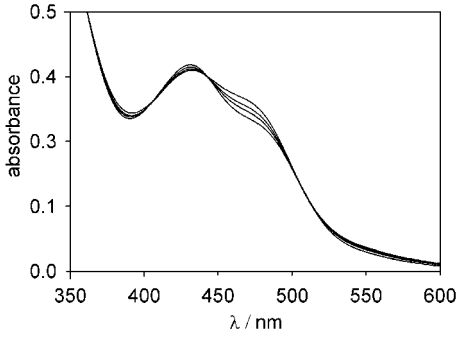

Figure 3. Electronic spectra of a 67-μM aqueous solution of the [Ru(tpm)(bpy)(GlutH)](PF_6) complex in H_2O irradiated with a 450-nm light. The initial shoulder at 475 nm grows as the photoaquation proceeds.

Complete spectral analysis during photodecomposition was performed. A linear combination of two species, in this case the original complex and the aqua complex $[Ru(tpm)(bpy)(H_2O)]^{2+}$, was fitted to the experimental spectra at each conversion degree. The latter aqua complex can be easily obtained by direct aquation of [Ru(tpm)(bpy)Cl]Cl in warm water. This analysis showed a perfect match between the experimental and the calculated spectra, which was in accord with the presence of only two absorbing species, as confirmed by NMR spectroscopic studies. The degree of photodecomposition was also obtained and the results are displayed in Figure 4. The quantum yield of photorelease for the glutamate complex can be calculated from the initial slope (Φ_{PD} = 0.035 at 450 nm), and it was found to be about 17 times higher than that of the DANP protecting group at the same wavelength.[4] The complex shows no thermal decomposition after a week at room temperature in the dark. These properties make this compound suitable for biological studies by using a mild 450-nm irradiation source. Similar results were obtained for all the other tested caged α-amino acids.

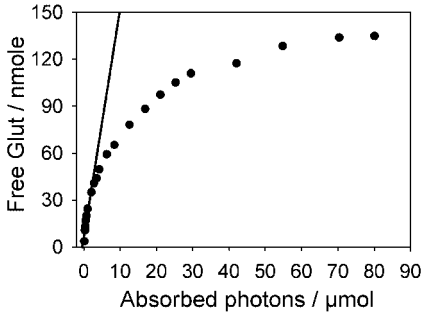

Figure 4. Yield of glutamate photouncaging by irradiation with a 450-nm light source (calculated from data in Figure 3).

To conclude, we devised the first inorganic-based caged compounds capable of delivering α-amino acids, including the major neurotransmitter glutamate. Their quantum efficiency of photouncaging with the use of 450-nm light is higher than that of better-known organic caged compounds. These compounds would be useful for photoelectrophysiological experiments in vitro and in vivo. Irradiation at 450 nm allows long-term experiments to be performed, given that blue light does not damage cells or tissues as UV light does. However, for in vivo experiments the penetration depth is not optimal. Cell viability tests (see Experimental Section) show that the complex is nontoxic for BHK cell cultures at physiologically useful concentrations (≈1 mM).

Experimental Section

All reagents were purchased from Sigma–Aldrich and used as received. $Ru(bpy)_2Cl_2$[12] and [Ru(bpy)(tpy)Cl]PF_6[13] were synthesized according to the literature. The UV/Vis spectra were recorded with a HP8452A diode-array spectrometer. NMR spectra were measured with a 500 MHz Bruker AM-500. Voltammograms were obtained in CH_3CN/0.1 M $TBAPF_6$ by using a three-electrode potentiostat based on an operational amplifier TL071 in current-to-voltage configuration,[14] and an acquisition software written in QuickBasic 4.5. A Pt wire with a diameter of 500 μM was used as a working electrode. Visible-light irradiation of samples for quantum yield determination was performed with a Luxeon Star III Royal Blue high-power light-emitting diode (LED). Irradiation power was chosen between 9 and 50 mW by changing the DC current through the LED. BHK-21, a hamster cell line, was grown in DMEM with 10% FBS, 2 mM glutamine, and 1 mM sodium pyruvate. Culture medium also contained 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 250 ng mL^{-1} amphotericin B. The cells were cultured in an autonomous Petri dish system for microscopy observations.[15] Temperature, moisture, and pCO_2 were kept constant during the viability tests.

Syntheses

Tris(pyrazolylmethane) (tpm): To an aqueous solution (30 mL) containing pyrazole (2.14 g) and tetrabutylammonium chloride (0.5 g) was added Na_2CO_3 (20 g) with vigorous stirring. Once dissolved, $CHCl_3$ (15 mL) was added. Nitrogen gas was bubbled through the solution for 10 min, and the mixture was heated at reflux for 72 h. The product was found in the solid precipitate and in the $CHCl_3$ layer. The solid and organic phases were separated and washed several times with more $CHCl_3$ to extract the tpm. The organic layer was evaporated to dryness and dissolved in boiling water. NaOH was added to reach pH ≈ 12, and the solution was ice cooled. The product precipitated in 60% yield (99% purity).

Ru(tpm)Cl_3: The ruthenium–tpm complexes were prepared following a slightly modified procedure given by Llobet et al.[16] A mixture containing $RuCl_3$ (633 mg), tpm (518 mg), and absolute EtOH (70 mL) was heated at reflux for 4 h. After filtration, the complex was obtained as a brown powder in 82% yield.

[Ru(tpm)(bpy)Cl]Cl: To a suspension of Ru(tpm)Cl_3 (300 mg) and 2,2′-bipyridine (111 mg) in EtOH (96%, 25 mL) was added ascorbic acid (130 mg, 1.2 equiv.). The reddish solution was heated at reflux for 40 min, cooled, and filtered to eliminate any undissolved Ru(tpm)Cl_3, which can be reused. Precipitation with saturated KPF_6 yielded a pure complex, which was washed with water and dried. [Ru(tpm)(bpy)Cl](PF_6): ^{1}H NMR (500 MHz, [D_6]acetone): δ

= 6.38 (t, J = 2.6 Hz, 1 H), 6.77 (t, J = 2.5 Hz, 2 H), 6.87 (d, J = 2.3 Hz, 1 H), 7.59 (t, J = 7.3 Hz, 2 H), 8.11 (t, J = 7.8 Hz, 2 H), 8.37 (d, J = 2.3 Hz, 2 H), 8.52 (d, J = 2.5 Hz, 1 H), 8.61 (d, J = 2.5 Hz, 2 H), 8.71 (d, J = 7.8 Hz, 2 H), 8.87 (d, J = 5.6 Hz, 2 H), 9.65 (s, 1 H) ppm. This PF_6 salt was dissolved in a minimum amount of acetone. Drops of tetrabutylammonium chloride saturated in acetone were added until total precipitation of the chloride salt. The precipitate was washed several times with acetone and then dried.

[Ru(bpy)(tpy)(GlutH$_2$)](PF$_6$)$_2$: A suspension of [Ru(bpy)(tpy)Cl](PF$_6$) (100 mg) in water (5 mL) was heated at 80 °C under an atmosphere of nitrogen for 30 min. Monosodium glutamate (10 equiv.) was then added. The pH was maintained around 9 by adding NaOH. The reaction was followed by UV/Vis spectroscopy for 2–3 h. The final product was precipitated through the addition of an excess amount of HPF_6 (1 M), washed several times with water, and dried with silica gel.

[Ru(tpm)(bpy)(AA)](PF$_6$)$_2$ (AA = α-amino acid): The syntheses of these compounds were performed in a similar manner to that used for the preparation of [Ru(bpy)(tpy)(GlutH$_2$)](PF$_6$)$_2$, but [Ru(tpm)(bpy)Cl]Cl was used as a starting compound in addition to the corresponding enantiomerically pure L-amino acid. Crystalline red powders, soluble in water at neutral or basic pH, were obtained. The counteranion can be changed by dissolving the PF_6 salt in acetone and reprecipitating with TBACl saturated in acetone.

[Ru(tpm)(bpy)(GlutH$_2$)](PF$_6$)$_2$·H$_2$O: Yield: 23%. ^{1}H NMR (500 MHz, D_2O): δ = 1.61 (m, 2 H), 1.82 (m, 1 H), 2.08 (m, 1 H), 2.49 (m, 1 H), 3.72 (t, J = 10.8 Hz, 1 H), 3.93 (d, J = 12.3 Hz, 1 H), 6.17 (t, J = 2.6 Hz, 1 H), 6.54 (d, J = 2.3 Hz, 1 H), 6.77 (t, J = 2.5 Hz, 2 H), 7.45 (t, J = 6.7 Hz, 1 H), 7.51 (t, J = 6.7 Hz, 1 H), 8.06 (t, J = 9.0 Hz, 1 H), 8.09 (t, J = 8.0 Hz, 1 H), 8.24 (d, J = 3.0 Hz, 1 H), 8.46 (d, J = 3.0 Hz, 2 H), 8.52 (m, 3 H), 8.59 (d, J = 2.2 Hz, 1 H), 8.67 (d, J = 5.6 Hz, 1 H), 8.76 (d, J = 5.6 Hz, 1 H), 9.46 (s, <1 H) ppm. $C_{25}H_{29}F_{12}N_9O_5P_2Ru$ (926.55): calcd. C 32.3, H 3.2, N 13.6, O 8.6; found C 33.0, H 3.0, N 14.2, O 8.9.

[Ru(tpm)(bpy)(ValH)](PF$_6$)$_2$·H$_2$O: Yield: 44%. ^{1}H NMR (500 MHz, D_2O): δ = 0.65 (d, J = 6.9 Hz, 3 H), 0.67 (d, J = 6.9 Hz, 3 H), 1.70 (m, 1 H), 2.40 (dd, J = 9.5, 3.5 Hz, 1 H), 2.89 (d, J = 12.0 Hz, 1 H), 3.76 (t, J = 10.7 Hz, 1 H), 6.21 (t, J = 2.5 Hz, 1 H), 6.50 (d, J = 1.8 Hz, 1 H), 6.79 (t, J = 2.4 Hz, 1 H), 6.80 (t, J = 2.9 Hz, 1 H), 7.50 (t, J = 6.3 Hz, 1 H), 7.54 (t, J = 6.6 Hz, 1 H), 8.10 (t, J = 7.9 Hz, 1 H), 8.12 (t, J = 7.2 Hz, 1 H), 8.28 (d, J = 3.0 Hz, 1 H), 8.42 (d, J = 1.8 Hz, 1 H), 8.49 (d, J = 2.5 Hz, 1 H), 8.50 (d, J = 3.0 Hz, 1 H), 8.56 (m, 3 H), 8.71 (d, J = 5.5 Hz, 1 H), 8.79 (d, J = 5.1 Hz, 1 H), 9.49 (s, <1 H) ppm. $C_{25}H_{31}F_{12}N_9O_3P_2Ru$ (896.57): calcd. C 33.4, H 3.5, N 14.1, O 5.4; found C 31.2, H 3.7, N 13.7, O 5.8.

[Ru(tpm)(bpy)(HisH)](PF$_6$)$_2$·2H$_2$O: Yield: 21%. ^{1}H NMR (500 MHz, D_2O): δ = 2.22 (s, 1 H), 2.68 (m, 1 H), 2.89 (m, 2 H), 3.26 (t, J = 6.4 Hz, 1 H), 3.52 (dd, J = 7.6, 5.5 Hz, 1 H), 5.94 (s, 1 H), 6.26 (t, J = 2.5 Hz, 1 H), 6.66 (d, J = 2.2 Hz, 1 H), 6.73 (t, J = 2.5 Hz, 2 H), 6.85 (d, J = 1.0 Hz, 1 H), 6.91 (s, 1 H), 7.45 (t, J = 6.6 Hz, 2 H), 7.66 (s, 1 H), 8.03 (d, J = 2.2 Hz, 1 H), 8.04 (d, J = 2.3 Hz, 1 H), 8.11 (t, J = 8.0 Hz, 2 H), 8.33 (d, J = 3.0 Hz, 1 H), 8.49 (d, J = 2.9 Hz, 2 H), 8.67 (t, J = 7.0 Hz, 3 H) ppm. $C_{26}H_{31}F_{12}N_{11}O_4P_2Ru$ (952.60): calcd. C 32.7, H 3.3, N 16.1, O 6.7; found C 31.2, H 3.3, N 17.4, O 7.7.

[Ru(tpm)(bpy)(GlnH)](PF$_6$)$_2$: Yield: 31%. ^{1}H NMR (500 MHz, [D$_6$]acetone): δ = 1.75 (m, 1 H), 1.90 (m, 1 H), 2.02 (m, 1 H), 2.50 (m, 1 H), 2.68 (m, 1 H), 4.10 (t, J = 11.5 Hz, 1 H), 5.78 (d, J = 11.9 Hz, 1 H), 6.37 (t, J = 2.6 Hz, 1 H), 6.64 (s, 1 H), 6.77 (d, J = 2.3 Hz, 1 H), 6.92 (m, 1 H), 7.15 (s, 1 H), 7.64 (t, J = 6.6 Hz, 1 H), 7.75 (t, J = 6.6 Hz, 1 H), 8.22 (t, J = 7.9 Hz, 1 H), 8.28 (t, J = 7.8 Hz, 1 H), 8.52 (d, J = 3.0 Hz, 1 H), 8.71 (d, J = 2.9 Hz, 1 H), 8.73 (d, J = 2.9 Hz, 1 H), 8.83 (m, 3 H), 8.97 (d, J = 2.0 Hz, 1 H), 9.02 (d, J = 5.6 Hz, 1 H), 9.15 (d, J = 1.9 Hz, 1 H), 9.67 (s, <1 H) ppm.

[Ru(tpm)(bpy)(TyrH)](PF$_6$)$_2$·H$_2$O: Yield: 19%. ^{1}H NMR (500 MHz, D_2O): δ = 2.43 (dd, J = 13.8, 10.8 Hz, 1 H), 2.69 (dt, J = 10.6, 3.6 Hz, 1 H), 2.75 (d, J = 12.3 Hz, 1 H), 2.88 (dd, J = 13.9, 3.6 Hz, 1 H), 3.96 (t, J = 11.4 Hz, 1 H), 6.17 (t, J = 2.5 Hz, 1 H), 6.44 (d, J = 2.0 Hz, 1 H), 6.68 (t, J = 2.5 Hz, 1 H), 6.77 (t, J = 2.8 Hz, 1 H), 6.79 (m, 4 H), 7.48 (m, 2 H), 8.10 (t, J = 7.8 Hz, 1 H), 8.14 (t, J = 7.8 Hz, 1 H), 8.23 (d, J = 2.9 Hz, 1 H), 8.41 (d, J = 5.5 Hz, 1 H), 8.43 (d, J = 2.9 Hz, 2 H), 8.45 (d, J = 3.0 Hz, 1 H), 8.49 (d, J = 2.0 Hz, 1 H), 8.53 (d, J = 8.0 Hz, 1 H), 8.58 (d, J = 8.2 Hz, 1 H), 8.66 (d, J = 5.4 Hz, 1 H), 9.46 (s, <1 H) ppm. $C_{29}H_{31}F_{12}N_9O_4P_2Ru$ (960.62): calcd. C 36.2, H 3.3, N 13.1, O 6.7; found C 34.2, H 3.5, N 13.4, O 6.1.

Supporting information (see footnote on the first page of this article): ^{1}H NMR spectra of the complexes and voltammogram of the [Ru(tpm)(bpy)GlutH$_2$](PF$_6$)$_2$ complex.

Acknowledgments

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