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A New Inorganic Photolabile Protecting Group for Highly Efficient Visible Light GABA Uncaging

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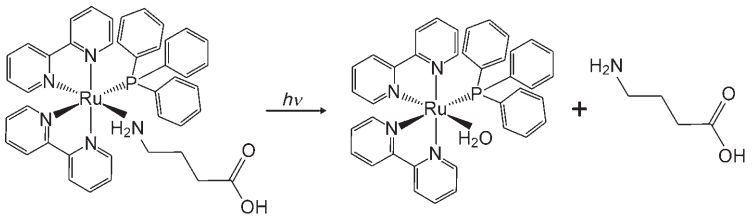
The field of cage compound design is continuously advancing, driven by the successful application of these tools in different biological experimental settings.[1] One area in which these compounds have proved particularly useful is neuroscience, where the rapid and highly localized release of neuroactive molecules that they permit has produced relevant results in studies of receptor distribution,[2] channel kinetics[3] and network circuitry.[4] Ideal caging groups should be noncytotoxic, establish enduring bonds with the biomolecules they protect, effectively block biomolecule activity when bonded, and undergo efficient removal by low-energy photons in order to minimize photodamage. Most photolabile protecting groups developed so far are organic compounds. Substituted nitrobenzyl and nitroindolinyl ester derivatives of many neuroactive substances including γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the brain, have been synthesized and characterized both chemically and biologically.[5] These protecting groups are removed upon irradiation with potentially harmful ultraviolet light, with uncaging quantum yields under 0.2. The longest photolization wavelength for an organic protecting group so far reported is 400 nm.[6]

In the last few years we have developed a new series of caged compounds that make use of ruthenium polypyridines as protecting groups. The link between the protecting group and the biomolecule is established through a coordination bond that can be broken with visible light. In the past, we have prepared caged compounds of the form $[Ru(bpy)^2L_2]^{2+}$ (bpy: 2,2′-bipyridine; L: 4-aminopyridine (a potassium channel blocker),[7] serotonin (a neuromodulator) or GABA[8]). The quantum efficiencies of photorelease measured for these compounds lie between 0.02 and 0.04 at 450 nm. In this work we report the synthesis, chemical properties and preliminary biological testing of an improved inorganic caged GABA compound in which one of the monodentate ligands has been replaced by a triphenylphosphine (PPh_3) moiety. The new caged compound, $[Ru(bpy)_2PPh_3GABA]^+$, releases the neurotransmitter with a quantum yield above 0.2 at 450 nm (Scheme 1).



Scheme 1. The scheme depicts the release of GABA after irradiation of $[Ru(bpy)_2PPh_3GABA]^+$.

Once synthesized, the compound was subjected to ^1H NMR spectroscopy in order to verify its chemical structure and purity, as well as to determine the absence of free GABA, either as a synthesis residue or arising from product decomposition in the solid state. The aromatic region of the spectrum showed the signals predicted for this complex alone (see the Supporting Information). The signals of coordinated GABA were found in the aliphatic portion of the spectrum (Figure 1).

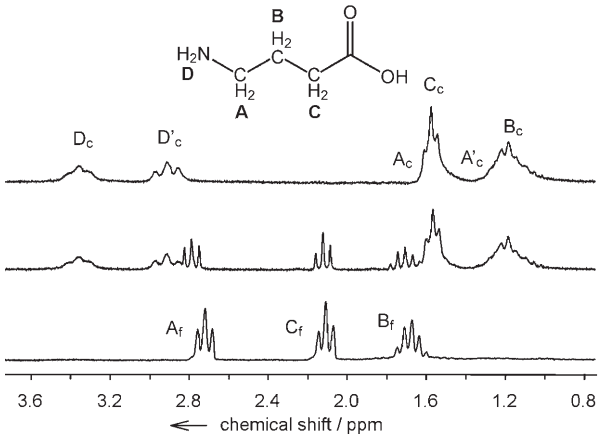


Figure 1. ^1H NMR spectra of $[Ru(bpy)_2PPh_3GABA]^+$ in D_2O. The upper trace shows the signals of coordinated (c) GABA in the dark complex. The middle trace corresponds to partial photolysis, and the lower trace shows the signal of free (f) GABA after total photolysis.

As found previously,[8] amine protons that belong to coordinated nitrogens do not undergo exchange for deuterium in D_2O; this shows that the bond is stable in water since nitrogen's free pair is restricted to the coordination bond. The upper trace in Figure 1 corresponds to the sample in the dark, and shows no signals other than those of coordinated GABA. The middle trace belongs to the same sample irradiated with a tungsten lamp to achieve partial photolysis, whilst exhaustive

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photolysis is shown in the lower trace. After irradiation, the signals of free GABA become apparent. These signals increased in intensity after direct addition of solid GABA to the sample. No photoproducts other than $[Ru(bpy)_2PPh_3H_2O]^{2+}$ and GABA were detected in the irradiated sample. The NMR analysis confirmed the 1:1 stoichiometry of the photoreaction.

The electrochemical behaviour of the complex was studied by cyclic voltammetry. We found three oxidation peaks at 1.67, 2.11, 2.34 V vs. NHE. This number of processes has also already been described for bis-primary amine complexes of ruthenium.[9] They are assigned to the Ru^{II}/Ru^{III} couples of the amine complex, its oxidation to the imine complex and finally to the nitrile complex, respectively. Similar results with lower potentials had been obtained for $[Ru(bpy)_2GABA_2]^+$.[8] The fact that $[Ru(bpy)_2PPh_3GABA]^+$ shows higher oxidation potentials is explained by the phosphine's lower basicity. These high values should prevent any cellular component from oxidizing the complex. To assess the photoreleasing performance of the compound, a series of UV/Vis absorption spectra were acquired while photolysis was conducted until all GABA was released. Superimposed spectra obtained from the photoreaction at neutral pH are depicted in Figure 2. The unvarying

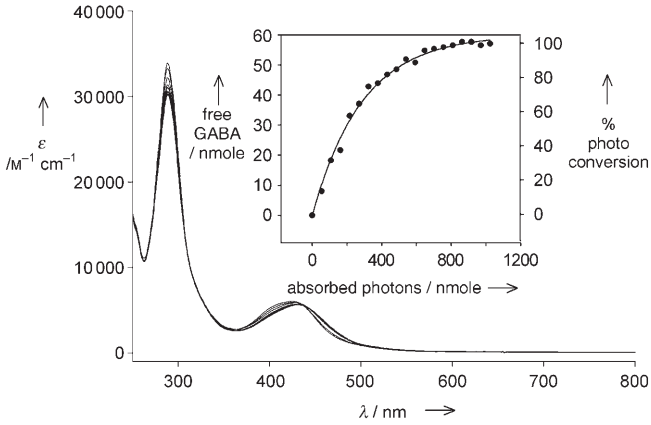

Figure 2. UV/Vis spectra of $[Ru(bpy)_2PPh_3GABA]^+$ in aqueous solution at pH 7, with irradiation at 450 nm. The inset shows the evolution of the photoreaction.

band at 290 nm is assigned to $\pi^* \leftarrow \pi$ transitions localized at the bipyridines.[10] The initial spectrum shows a maximum at 425 nm that corresponds to the metal-to-ligand charge transfer (MLCT) band. Absorption at this band populates a triplet state that is thermally activated to a dissociative d-d state, which leads to photoproducts.[11] Over the course of the reaction, the MLCT band is displaced to 430 nm in the $[Ru(bpy)_2PPh_3H_2O]^{2+}$ complex. The small differences between the initial and final spectra, associated with the similar basicities of H_2O and NH_2R, were enough to compute reaction progress, which is represented in the inset. As each molecule of the aqua complex corresponds to one of GABA, the production of the latter can be denoted.

Photolysis was also carried out at pH 12. Under these conditions, the reaction produces $[Ru(bpy)_2PPh_3OH]^+$ instead of $[Ru(bpy)_2PPh_3H_2O]^{2+}$ as one of the photoproducts. The MLCT band of the former is red-shifted to 450 nm, making differences with the starting species more evident. Stacked electronic spectra, together with the degree of photoconversion, are shown in Figure 3. Quantum yields of uncaging obtained from

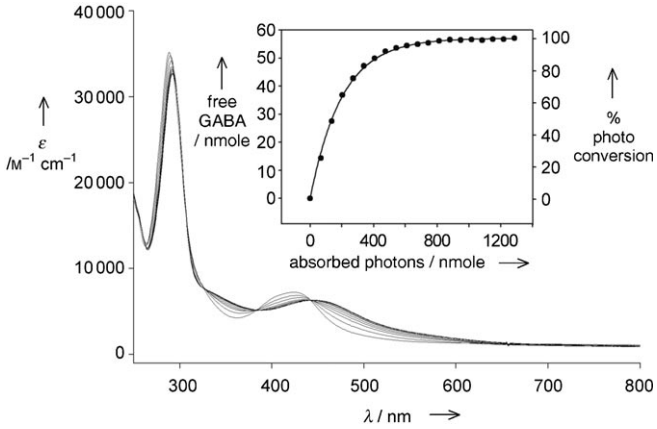

Figure 3. UV/Vis spectra of $[Ru(bpy)_2PPh_3GABA]^+$ in aqueous solution at pH 12, with irradiation at 450 nm. The inset shows the evolution of the photoreaction.

photolysis data can be found in Table 1, together with those of previously reported $[Ru(bpy)_2GABA_2]^+$ [8] and commercially available γ-aminobutyric acid α-carboxy-2-nitrobenzyl ester (O-(CNB-caged) GABA).[5b] Extinction coefficients at wavelength of maximal absorption are also provided.

Table 1. Wavelength of maximal absorption, extinction coefficient at maximal absorption, irradiation wavelength and quantum yield of photorelease of various caged forms of GABA.

Compound	pH	λ_{max} [nm]	ε_{max} [$M^{-1}cm^{-1}$]	λ_{irrad} [nm]	$\phi\lambda_{irrad}$
$[Ru(bpy)_2PPh_3GABA]^+$	7	424	6400	450	0.21
$[Ru(bpy)_2PPh_3GABA]^+$	12	424	6400	450	0.29
$[Ru(bpy)_2GABA_2]^{2+}$	7	488	8955	450	0.036
O-(CNB-caged) GABA	7	262	4500	308	0.16

It has already been established for $[Ru(bpy)_2XY]^{2+}$ complexes that higher energies for the MLCT band correlate positively to higher quantum yields of photosubstitution,[12] as is the case for $[Ru(bpy)_2PPh_3GABA]^+$ vs. $[Ru(bpy)_2GABA_2]^{2+}$. The ruthenium phosphine protecting group is removed with a quantum yield that surpasses that of the nitrobenzyl ester group, while the active absorption wavelength is kept in the visible range. The combination of extinction coefficients and quantum yields shows that $[Ru(bpy)_2PPh_3GABA]^+$ makes good use of incident photons, minimizing the amount of light needed to release a certain number of GABA molecules.

In order to study the physiological value of GABA photorelease from $[Ru(bpy)_2PPh_3GABA]^+$, experiments were performed in frog oocytes expressing $GABA_C$ receptors. These receptors

are ligand-gated ion channels that open a chloride (Cl^-) selective pore upon binding of GABA.[13] Thus, at membrane potentials near to the neuronal resting state, a measurable Cl^- current will flow through these channels in the presence of the neurotransmitter. Figure 4A illustrates ionic currents typically

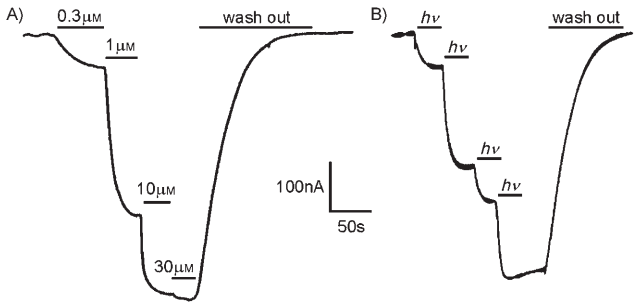

Figure 4. Membrane ionic currents recorded in frog oocytes expressing $GABA_C$ receptors during application of A) increasing concentrations of free GABA, or B) light pulses to a bath solution containing 30 μM $[Ru(bpy)_2PPh_3GABA]^+$.

observed in voltage-clamp experiments during a cumulative dose-response curve for free GABA. The ability of $[Ru(bpy)_2PPh_3GABA]^+$ to induce $GABA_C$ receptor-mediated Cl^- currents upon GABA photorelease was also evaluated by voltage-clamp recording. In these studies, the oocyte chamber was first equilibrated with frog Ringer's solution containing $[Ru(bpy)_2PPh_3GABA]^+$ (30 μM). Ambient light was kept to a minimum in order to avoid unwanted photorelease. Application of the caged compound without illumination was unable to evoke any membrane response or to change the recording baseline (not shown). Under these conditions, delivery of sequential light pulses to the bottom of the recording chamber evoked oocyte membrane current steps comparable to those observed during the direct application of increasing concentrations of free GABA (compare representative records in Figure 4A and B). Light-evoked membrane responses produced with $[Ru(bpy)_2PPh_3GABA]^+$ were washed out with frog Ringer's solution; this returned the oocyte to its initial baseline without any sign of cytotoxicity.

The inorganic-based GABA caged compound we have synthesized is a promising new tool in the field of neuroscience. The ruthenium phosphine protecting group is removed by visible light with a high quantum yield, contributing to reducing the risk of cell photodamage on irradiation of a biological preparation. Moreover, as light scattering is reduced with increasing irradiation wavelength[14] these low-energy photons should be able to penetrate further into biological tissue and allow for deeper neurotransmitter release. Additionally, the obligatory use of expensive optic materials or light sources can be avoided. Although UV-LEDs have become readily available, focusable short-wavelength lasers needed for localized uncaging are still of restricted accessibility. Biologically active neurotransmitter photodelivery from this compound has been shown in one-photon absorption mode.

Photochemical fragmentation kinetic studies on this compound require further investigation. However, photosubstitution kinetic constants determined for related $[Ru(bpy)_2XY]^{2+}$ compounds show values above $10^5\ s^{-1}$,[15] which if reproduced by $[Ru(bpy)_2PPh_3GABA]^+$, should allow the use of this photochemical tool in most neurophysiological studies. Photoelectrophysiological experiments with mouse cortical brain slices that show two-photon absorption promoted photorelease with this compound will be published elsewhere.

Experimental Section

Synthesis: Reagents and solvents were commercially available and were used as received. $Ru(bpy)_2Cl_2$ was synthesized according to the literature.[16] All solutions were degassed with N_2 prior to heating to prevent oxidation of the ruthenium aquo complexes.

$[Ru(bpy)_2PPh_3Cl]Cl$: $Ru(bpy)_2Cl_2$ (682 mg, 1.3 mmol) was dissolved in methanol (45 mL). Triphenylphosphine (410 mg, 1.5 mmol) was added, and the mixture was stirred until complete dissolution. After this, water (20 mL) was added, and the mixture was heated at reflux for 2 h. Once the reaction was complete, the solution was concentrated by rotary evaporation. The solid was resuspended in acetone (50 mL), which first produced the dissolution of $[Ru(bpy)_2PPh_3Cl]^+$ and then the precipitation of $[Ru(bpy)_2PPh_3Cl]Cl$. The solution was kept at 0 °C for one hour before filtration. The red solid was washed with portions of cold acetone and diethyl ether.

$[Ru(bpy)_2PPh_3GABA]PF_6$: $[Ru(bpy)_2PPh_3Cl]Cl$ (110 mg, 147 μmol) was dissolved in water (10 mL). The mixture was heated at 80 °C for half an hour to form $[Ru(bpy)_2PPh_3H_2O]^{2+}$. GABA (455 mg, 4.42 mmol) and aqueous NaOH (4.4 mL, 1 M) were then added. Heating was continued for 3 h, before complex precipitation with KPF_6 (0.8 mL, 1 M) in ice and centrifugation in a test tube. The pellet was twice resuspended in water (3 mL) and recentrifuged prior to desiccation. Further purification can be achieved through column filtration through OH^--loaded DEAE-Sephadex A-25 at pH 9.

NMR spectra were obtained with 200 MHz and 500 MHz Bruker NMR instruments. Complete NMR spectra are available in the Supporting Information. The voltagram was obtained in $CH_3CN/TBAPF_6$ (0.1 M) by use of a three-electrode potentiostat based on an operational amplifier TL071 in current-to-voltage configuration and acquisition software written in QB 4.5. A Pt wire with a diameter of 500 μm was used as the working electrode.

Photolysis: The UV/Vis spectra were measured with a HP8453 diode-array spectrometer. Visible light irradiation of samples for quantum yield determination was performed with a Luxeon Star III Royal Blue high-power light-emitting diode (LED). Light power was measured in a test photolysis of $[Ru(bpy)_2(py)_2]^{2+}$, by assuming a quantum yield photosubstitution of 0.26.[12] Photoconversion plots were fitted with a two-parameter single-exponential rise to maximum function $y=a(1-\exp(-bx))$; quantum yields were obtained as $a\times b$. Visible-light irradiation for biological studies was achieved with a fibre optic light source and a quartz halogen tungsten lamp.

RNA preparation, oocytes manipulation and electrophysiology: A human cDNA encoding the ρ1 $GABA_C$ receptor subunit cloned in the vector pBS (SK^-), suitable for in vitro transcription, was used as a template to synthesize cRNAs. cRNA solutions (0.1 to 0.3 $ng\,nL^{-1}$) were prepared in RNase-free H_2O and stored at −70 °C. *Xenopus*

laevis oocytes at stages V and VI were used for cRNA expression. Isolation and maintenance of oocytes were carried out as previously described.[17] Two-electrode voltage-clamp recordings were performed with an Axoclamp 2B amplifier 3 to 7 days after oocyte injection. Standard glass recording electrodes were made in a Narishige PB-7 puller and filled with KCl (3 M). Resistance values were approximately 1 MΩ. Holding potential was set to −70 mV, and current traces were acquired with an analogue to digital interface and stored on a PC with the aid of AXOTAPE software. Oocytes were placed in a chamber (volume 100 μL) containing frog Ringer's solution [NaCl (115 mM), KCl (2 mM), $CaCl_2$ (1.8 mM), HEPES (5 mM), pH 7.0], and drugs were dissolved in this solution and applied through the superfusion system. All the experiments were carried out at room temperature (23–24 °C).

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