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Photolabile Glutamate Protecting Group with High One- and Two-Photon Uncaging Efficiencies

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A π-extended [2-(2-nitrophenyl)propoxy]carbonyl (NPPOC) derivative has been prepared as an efficient UV and near-IR photolabile protecting group for glutamate. This glutamate cage compound exhibits efficient photorelease upon one-photon excitation ($\varepsilon\Phi = 990\ M^{-1}cm^{-1}$ at 315 nm). In addition, it also shows efficient photorelease in activation of glutamate receptors in electrophysiological recordings. Combined with a high two-photon uncaging cross-section ($\delta\Phi = 0.45$ GM at 800 nm), its overall properties make this new cage—3-(2-propyl)-4′-methoxy-4-nitrobiphenyl (PMNB)—for glutamate a very promising tool for two-photon neuronal studies.

Introduction

Photolabile protecting groups are efficient tools for revealing intimate dynamic processes in living cells. This is particularly the case in neurosciences, where the controlled liberation of neurotransmitters leads to better understanding of the neuronal circuitry.[1–4] Nevertheless, the classically used one-photon excitation process permits only very limited spatial control over the release of neurotransmitters, as the photochemical reaction occurs along the entire light pathway. Better spatial resolution through a "photochemical two-photon uncaging" process was demonstrated a few years ago.[5,6] In that case, the biologically active substance was caged with two photoremovable protecting groups, so the liberation of the targeted active compound required two photolytic reactions to occur, which can take place simultaneously on the same molecule only in a reduced volume at the focus of a laser beam. However, this technique does not suppress the light absorption along the optical pathway by the liquid medium containing the caged molecule.

The chemical "two-photon process" can also be achieved by a physical nonlinear optical property: two-photon absorption (TPA).[7] In this case, the excited state is populated not by classical absorption of a single photon of energy $h\nu$, but by the simultaneous absorption of two photons of half the excitation energy ($h\nu/2$ each). The quadratic dependence of TPA versus light intensity induces sufficient excitation only at the focal point of a femtosecond pulsed laser. This intrinsically generates high spatial control (with one-micron precision in the three dimensions) for the release of active substance.[8] Furthermore, two-photon excitation involves the use of lower-energy, IR light rather than UV excitation. This provides two fundamental advantages for biological applications: reduced photodamage to the cells or organs and better penetration of the light beam in living tissues.[7] Many efficient caging groups for glutamate, using excitation wavelengths ranging from UV to visible light, have been described in the literature.[9–11] Among these photoremovable groups, only a few have successfully been used in a two-photon uncaging process; they include the 6-bromo-7-hydroxycoumarin series,[12] the nitroindoline series,[13] the dinitroindoline series,[14,15] and more recently the DMNPB (3-(4,5-dimethoxy-2-nitrophenyl)-2-butyl) caging group.[11] However, none of these photolabile protecting groups combines a high two-photon uncaging action cross-section together with high quantum efficiency for glutamate release. Here we report the synthesis and characterization of an efficient glutamate cage derived from the [2-(2-nitrophenyl)propoxy]carbonyl (NPPOC) series[16] and displaying efficient one- and two-photon photorelease in the near UV and in the 800 nm range, respectively.

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Results and Discussion

Molecular engineering and synthesis

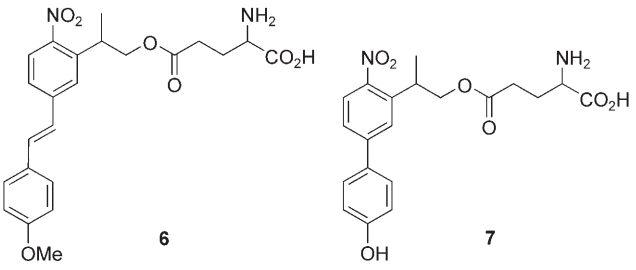
It is well known in molecular engineering of 1D donor–donor or donor–acceptor nonlinear optical chromophores that the optimization of the two-photon absorption cross-section (δ) requires an elongation of the conjugated π system and/or an increase in the power of the donor or acceptor side groups. Here, in addition, we have to keep the acceptor nitro group, which is crucial for the uncaging photochemical reaction. Classical ways to increase the length of a conjugated system starting from a single phenyl ring include the addition variously of a π bond, such as in styrene derivatives, of a phenyl ring, to provide a biphenyl system, or of a styrene moiety, to provide a stilbene derivative. Starting from the known 3-(4,5-dimethoxy-2-nitrophenyl)-2-butyl (DMNPB) structure,[11] lengthening of the π system appeared to be more promising than increasing the donor group efficiency (here, for example, by replacing the methoxy group by an amino group).[17] At this point, however, although this strategy would without doubt increase the two-photon absorption cross-section, care had to be taken about the efficiency of the photochemical reaction (in particular the uncaging quantum yield Φ). The introduction of an additional π bond such as in stilbene derivatives illustrates the importance of this parameter. Indeed, it has been reported—and we have observed, as shown later—that the uncaging quantum yields decrease with such structures.[17]

The efficiency of the photochemical reaction has also been improved by modifying the link between the caged molecule and the protecting group. We thus prepared an analogue of DMNPP containing two directly linked phenyl rings, initially described for one-photon photoliberation of deoxynucleosides.[16] This chromophore should have a high two-photon absorption cross-section, while photochemical side-reactions during the photolysis should be minimized. The methoxynitrobiphenyl key intermediate **1** was prepared by a modification of a described procedure (Scheme 1).[16] 2-(5-Iodo-2-nitrophenyl)propan-1-ol (**2**) was coupled to phenylboronic ester derivative **3** to give the corresponding methoxy derivative **1**. Protected glutamate (N-α-*tert*-butyloxycarbonyl-L-glutamic acid α-*tert*-butyl ester) was then grafted to this cage to give **4**, which was deprotected in acidic media to give the caged glutamate **5**. New compounds were characterized by ^{1}H and ^{13}C NMR spectroscopy, UV-visible spectroscopy, and mass spectrometry. All experimental details are given in the Supporting Information. The solubility of **5** at room temperature in phosphate buffer (pH 7.4) containing DMSO (1%) was measured as 1 mmol L^{-1}.

One- and two-photon photophysics and uncaging

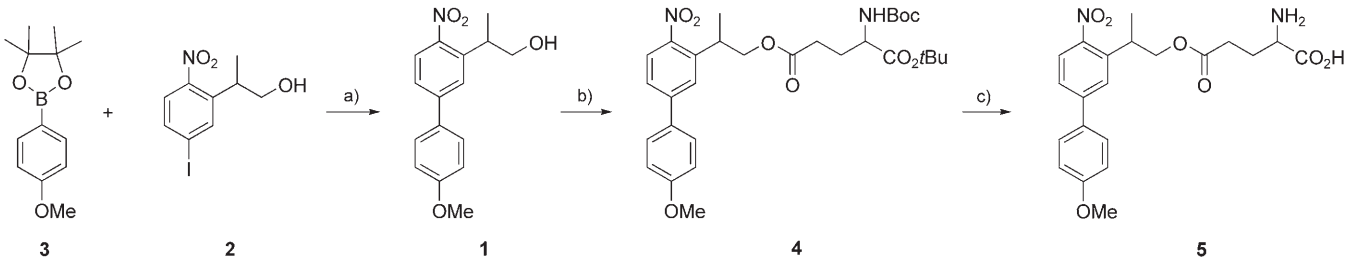
One-photon properties: The one-photon properties of caged glutamate **5** were investigated by UV-visible spectroscopy. The absorption maximum and molar absorption coefficient are 317 nm and 9900 M^{-1} cm^{-1}, respectively. The photolytic release of glutamate (as a chromophoric derivative that was formed quantitatively after condensation with *o*-phthaldialdehyde and mercaptoethanol) after irradiation in neutral buffered medium was analyzed quantitatively by HPLC.[18] Caged glutamate **5** afforded a 90% yield of glutamate release. This value is in the same range as that provided by the DMNPB-caged glutamate described previously.[11]

The percentages of released glutamate were also measured for the stilbenic derivative **6** and for compound **5**'s phenolic



analogue **7**. Compound **7** was prepared in order to check the efficiency of its phenolate salts, which present red shifts of the UV absorption transition, and were supposed to have better TPA cross-sections due to the better donor effect of O$^-$. The percentages of released glutamate were only 48% for **6** and <10% for **7**, indicating that these chemical functional groups induce some competitive photochemical pathways that do not contribute to the release of glutamate. We did not further characterize these two caging platforms.[19] The disappearance quantum yield of 0.1 for **5** was determined by comparison with the 1-(2-nitrophenyl)ethyl-ATP reference molecule at 315 nm and HPLC analysis.[20]

Overall, the high photolysis quantum yield and the significant molar absorption coefficient ($\varepsilon\Phi = 990$ M^{-1} cm^{-1} at 315 nm), together with the very efficient release of glutamate



Scheme 1. Synthesis of caged glutamate **5**. a) Toluene, $Pd(PPh_3)_4$, $NaHCO_3$ (aq.), 5 h, 110 °C, 65%; b) CH_2Cl_2, DMAP, Boc-L-Glu-O-*t*Bu, DCC, 59%; c) CH_2Cl_2, TFA, 91%.

(90%), make this molecule an efficient glutamate cage in near-UV photolysis. The fragmentation mechanism for the DMNPP cage has already been postulated,[11] and Figure 1 shows the changes in the UV-visible spectrum of **5** during photolysis in

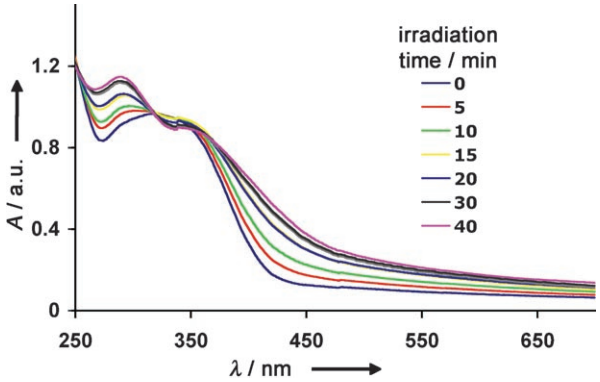


Figure 1. Changes in the UV-visible spectrum of **5** during photolysis ($\lambda_{ex}=364$ nm) in phosphate buffer (pH 7.4, 100 mM).

phosphate buffer. The nearly isobestic points (254, 325, and 380 nm) indicate a clean photochemical reaction, consistent with the 90% glutamate release. The light scattering occurring during the photolysis was due to insoluble photolysis residue. Flash photolysis experiments were performed on **5** (photolysis at 350 nm and analysis of the decay of transient at 410 nm) at room temperature. The rate-limiting step corresponding to the decomposition of the *o*-quinoid *aci*-nitro intermediate is 2000 s^{-1} ($t_{1/2}=0.34$ ms). This value indicates that the introduction of a supplementary phenyl ring directly linked to the molecule does not significantly perturb the one-photon photochemical parameters in relation to those of the related DMNPB-caged glutamate molecule.[11] The hydrolytic stability was also explored by HPLC in phosphate buffer (pH 7.4) at room temperature. There was 3% degradation after 24 h ($t_{1/2}=$ 16 days).

Two-photon properties: The two-photon photolysis curves, measured as described in the Experimental Section, are presented in Figure 2. By comparing the results obtained for compound **5** and for the reference 7-hydroxycoumarin-4-ylmethyl acetate (CouOAc),[12] the two-photon uncaging cross-section of compound **5** was determined to be 0.45 GM at 800 nm (1 GM = 10^{50} cm^4 s per photon). For comparison, we have also plotted the photolysis of the DMNPB-glutamate, which has a two-photon uncaging cross-section of 0.17 GM at 720 nm,[11] similarly to the MNI-glutamate molecule.[21] The two-photon uncaging cross-section ($\delta\Phi$) of compound **5** is, to the best of our knowledge, the highest value reported for a two-photon caged glutamate at 800 nm. The quadratic dependence of the uncaging rate, indicating a true two-photon process, was verified for peak intensities larger than 10 GW cm^{-2} (Figure 3).

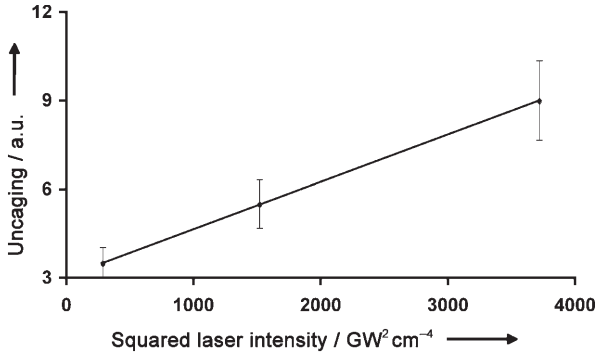


Figure 3. Quadratic dependence of the uncaging rate as a function of the laser intensity.

Electrophysiological experiments

In hippocampal neurons with whole cell patch clamp recording at −70 mV, addition of **5** (at 1 mM) from a freshly prepared solution in DMSO (100 mM) produced a small inward current similar to that produced by free glutamate (1–10 μM). At 0.5 mM no inward current was detected. At both 1 mM and 0.5 mM, addition of **5** produced turbidity in the bath solution. Photolysis with a flashlamp pulse (300–380 nm, 1 ms) at −70 mV (Figure 4) produced a rapidly rising inward current, with a rise time similar to the flash duration, with subsequent rapid decay to a constant level. This constant late current could be reversed by washing or by addition of 10 mM $MgCl_2$.

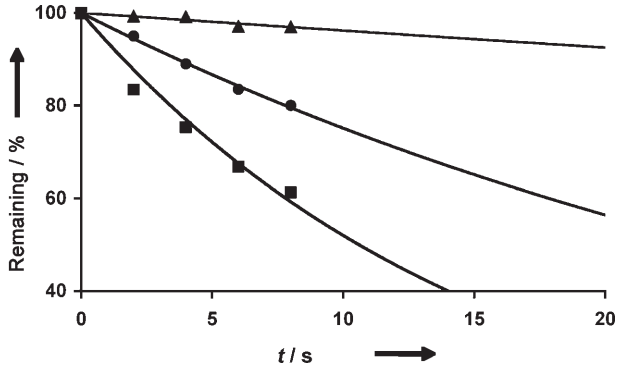


Figure 2. Determination of two-photon uncaging cross-section of **5** (■), CouOAc (●), and DMNPPE (▲) at $\lambda_{ex}=800$ nm in a water/acetonitrile solution (1.5% acetonitrile).

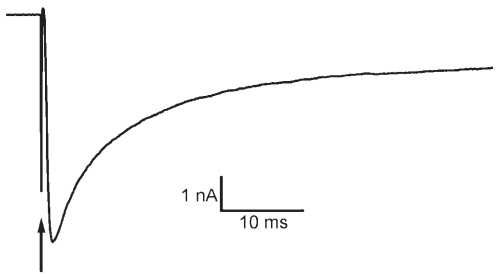


Figure 4. Response of a hippocampal pyramidal neuron to a 1 ms flash photoreleasing L-glutamate from caged L-glutamate (0.5 mM) at the time indicated by the arrow. Whole cell voltage clamp at −70 mV with a K-gluconate-based internal solution and a HEPES-buffered external Ringer with Ca (2 mM), zero Mg, glycine (100 μM), and TTX (tetrodotoxin, 1 μM). 10–90% rise time 0.5 ms.

This suggests that the steady-state level was due to residual activation of NMDA (*N*-methyl-D-aspartate) glutamate receptors by glutamate remaining in the vicinity of the cell. The fast rise was most likely due to activation of fast AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) subtype glutamate receptors, which show rapid desensitization. The fast activation of the receptors for glutamate in the presence of a high cage concentration, 0.5 or 1 mM, indicates that the cage has a low affinity and equilibrates quickly with glutamate receptors. However, no direct tests of the steady state occupancy of receptors by application of exogenous glutamate or other neurotransmitters were made in these experiments.

Conclusions

The photoremovable 3-(2-propyl)-4′-methoxy-4-nitrobiphenyl (PMNB) group, for which an improved synthesis has been developed, was demonstrated to be a very efficient caging group for glutamate with unprecedented two-photon sensitivity ($\delta\Phi =$ 0.45 GM at 800 nm). The preliminary electrophysiological experiments are encouraging, and work to increase the aqueous solubility of this new two-photon uncaging chromophore is in progress in order to allow the preparation of the high concentrations of the caged glutamate (over 5 mM) necessary for efficient neurophysiological experiments.

Experimental Section

Synthesis: All chemicals and reagents were purchased from Aldrich or Acros Organics and were used as received unless specified. The NPE-ATP was purchased from Jena Bioscience. THF was distilled over sodium and under argon, methylene chloride was distilled over calcium hydride under argon, triethylamine was distilled over potassium hydroxide under argon, and DMSO was distilled over calcium hydride under vacuum and conditioned under argon prior to use. ^{1}H and ^{13}C spectra were recorded with a 300 MHz Bruker Advance 300 instrument in $CDCl_3$ (internal standard 7.24 ppm for ^{1}H and 77 ppm, middle of the three peaks, for ^{13}C spectra) or [D_6]DMSO (internal standard 2.26 ppm and 39.5 ppm for ^{13}C spectra). FAB mass spectra were recorded with a ZA-HF instrument with 4-nitrobenzyl alcohol as a matrix, and ESI spectra were obtained on a Bruker HTC ultra (ESI-IT). TLC were run on Merck precoated aluminum plates (Si 60 F254). Column chromatography was run on Merck silica gel (60–120 mesh). 2-Ethyl-4-iodo-1-nitrobenzene and 2-(5-iodo-2-nitrophenyl)propan-1-ol (**2**) were prepared by the procedure reported in the literature.[16,23] The reference 7-hydroxycoumarin-4-ylmethyl acetate (CouOAc) was prepared by the protocol described by Furuta et al.[12]

One-photon photolysis: A solution of **5** (0.2 mM) in phosphate buffer (pH 7.4, 100 mM, 4 mL) was exposed to a 1000 W Hg Lamp (Hanovia) focused on the entrance slit of a monochromator at 364 nm (±0.2 nm). The reaction was monitored by UV, and aliquots of samples (100 μL) were analyzed by HPLC after derivatization with *o*-phthaldialdehyde and mercaptoethanol (detection by absorbance at 340 nm) to determine the percentage of released glutamate. The quantum yield for the photoconversion was determined (at 315 ± 0.2 nm) by comparison with the photolysis of 1-(2-nitrophenyl)ethyl-ATP (NPE-ATP, $\Phi = 0.63$),[20] which was taken as reference in a phosphate buffer (0.1 mM, pH 7.4) at 25 °C. These compounds were tested at identical optical densities at the irradiation wavelengths used. This mixture was photolyzed by continuous irradiation at 315 nm, and aliquots were subjected to reversed-phase HPLC to determine the extent of the photolytic conversions. Quantum yields were calculated by considering conversions up to 30%, to limit errors due to undesired light absorption during photolysis as much as possible.

Two-photon photolysis: The two-photon uncaging cross-section ($\delta\Phi$) was determined by comparison with that of CouOAc (0.13 GM at 800 nm)[12] as a known reference, under the same illumination conditions. An amplified Ti:Sapphire laser system delivering 50 fs, 800 nm pulses at a 5 kHz repetition rate was used to illuminate a spectrophotometric cuvette, specially designed for low volumes (Hellma 105.202.QS) and containing a water/acetonitrile solution either of the caged compound or of CouOAc (50 μL). With a collimated laser beam of cross-section 4 mm^2, the laser pulse delivered by our amplified laser system had a peak intensity in the range of several 10^{10} W cm^{-2} (corresponding to a few 10^{29} photons per cm^2 per s) that is similar to that obtained at the focus of a conventional Ti:sapphire femtosecond laser oscillator, but over a much larger area (and volume). This allowed illumination of the entire entrance window of the cuvette so as to photolyze the solution without the need for stirring. Illumination was carried out for 0 to 10 s. The global disappearance of caged glutamate in the solution was then measured by HPLC, as a function of laser exposure time. The laser peak intensity used for these in vitro experiments was much higher than those classically used for in vivo experiments, to allow chromatographic detection of the disappearance of the caged molecule by HPLC and an accurate two-photon uncaging cross-section determination. For in vivo experiments, the peak intensity used to induce a neuron response is generally much lower, due to toxicity problems.[24]

Electrophysiological experiments: Electrophysiological recordings were made in pyramidal neurons of primary hippocampal cultures in external solution containing NaCl (135 mM), KCl (4 or 10 mM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10 mM), $NaHCO_3$ (2.5 mM), glucose (10 mM), $CaCl_2$ (2 mM), glycine (0.1 mM), pH 7.3, with zero $MgCl_2$ to permit activation of NMDA receptors at negative potential. In most experiments tetrodotoxin (1 μM) was present. The internal solution contained potassium gluconate (135 mM), HEPES (10 mM), pH 7.3 with KOH, ethyleneglycol-bis-(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (0.5 mM), MgATP (4 mM), and GTP (0.1 mM). Caged glutamate was dissolved in dry DMSO at 100 mM and diluted in two steps into the bath solution at 0.5 or 1 mM. Photolysis was performed with a xenon arc flashlamp (Rapp Optoelectronic) with UG11 filter and neutral density filters in the light path. Full photolysis intensity was sufficient to uncage approximately 50% of NPE-caged ATP.

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Keywords: glutamate cage · neurotransmitters · photolabile protecting groups · photolysis · two-photon uncaging

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