Two-Photon Photolysis

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Molecular Engineering of Photoremovable Protecting Groups for Two-Photon Uncaging**

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Photoremovable protecting groups have become a mainstay for dynamic studies in various biological systems, from neuroscience to genetics,[1] mainly because photoinduced activation is orthogonal to other techniques used to detect biological responses.[2] The photochemical release of the active molecule is usually induced by an initial one-photon absorption process, leading to a limited spatial localization of the released substance. To overcome this obstacle, two-photon (TP) excitation has recently emerged as a very promising technique to obtain spatial control.[3,4] Indeed this nonlinear optical (NLO) process takes place only where the light intensity is at a maximum, typically by focusing an infrared pulsed laser beam. In this case, the excited state yields to the photolytic reaction by the simultaneous absorption of two low-energy photons (infrared instead of ultraviolet, in classical absorption), which also limits the phototoxicity of the excitation beam. Unfortunately, the various photoremovable groups ("cages") that have been developed for one-photon photoactivation exhibit very low efficiency in two-photon excitation.[5] Some chemical modifications have been performed on these chromophores to improve their TP sensitivity, and new platforms have also been described.[6–10] These approaches have led to moderately efficient TP cages with uncaging cross-sections ($\delta_u\phi_u$) of about 1 Goeppert-Mayer ($1\ \mathrm{GM} = 10^{-50}\ \mathrm{cm^4\,s\,photon^{-1}}$) at best. However, this value remains insufficient for use in biological studies, for which a 3 GM minimum value has been suggested.[11]

We report herein the design, synthesis, and characterization of highly efficient TP cages, and their application to glutamate photorelease. During the last decade, the optimization of chromophores for TP absorption (TPA) became an important goal for organic chemists,[12,13] and give rise to many applications in material[14,15] and biological[16,17] sciences. These efforts have led to various possible approaches for increasing the TPA properties of chromophores or fluorophores. Different chromophore geometries have been investigated, with linear (1D),[18,19] planar (2D),[20,21] and tetrahedral (3D)[22] structures. The typical dipolar architecture of a 1D TPA chromophore, the smallest system to be useful in biology, is generally composed of two electron-donor or electron-acceptor groups (D or A) linked to a central core by conjugated systems. Donor or acceptor groups can be added on the central core to give quadrupolar architectures (Figure 1).

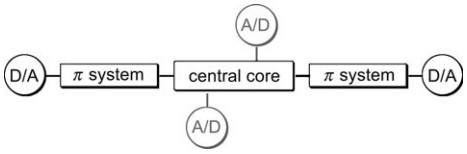

Figure 1. Typical structure of a 1D two-photon absorption chromophore (black = dipolar, gray = quadrupolar).

The TPA properties of such systems can be improved by lengthening the conjugated system and/or increasing the electron-donating or -withdrawing effect of the side groups. We recently described the 3-(2-propyl)-4′-methoxy-4-nitrobiphenyl (PMNB) cage as an efficient TP photolabile protecting group for glutamate ($\delta_u\Phi_u = 0.45$ GM at 800 nm).[23] Its uncaging cross-section has been increased in comparison with the well-known methoxynitrobenzyl platform by extending the π system. Another approach was proposed by Andraud, Baldeck, and co-workers, who pointed out that TPA cross-sections of oligomers can be enhanced by biexcitonic coupling between two weakly conjugated monomers.[24–26] We applied this concept to the molecular engineering of new linear caging platforms. The first designed molecule (4,4′-bis-{8-[4-nitro-3-(2-propyl)-styryl]}-3,3′-dimethoxybiphenyl or BNSMB, Figure 2) was composed of two vinylogues of PMNB linked together to take advantage of a possible interaction between the two monomers. A double bond was introduced in the system to improve its solubility in organic solvents. Clearly, this increase in the length of conjugation is beneficial to the TPA properties, but can be detrimental to the uncaging quantum yield, as it might induce photochemical side reactions. To obtain a linear bis(donor–acceptor) system (according to Figure 1), the methoxy group was moved from the *para* to the *ortho* position, allowing the two monomers to be linked by a single C–C bond. This

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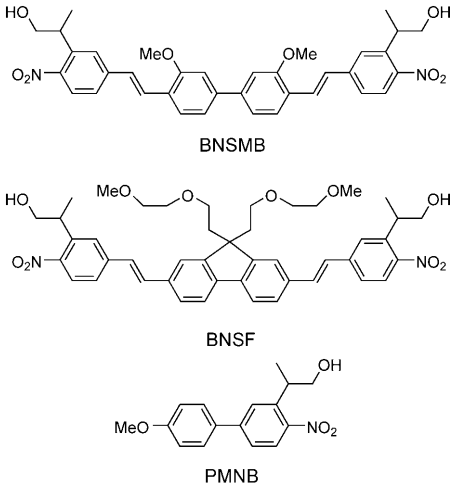


Figure 2. Structure of new TP photolabile cages: quadrupolar bis-(donor–acceptor) BNMSB (top), dipolar acceptor–acceptor BNSF (middle), and the parent molecule PMNB (bottom).

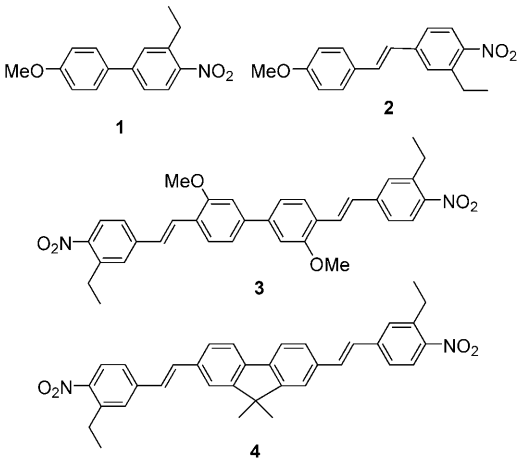


Figure 3. Molecular structures of model compounds used for semi-empirical AM1 calculations

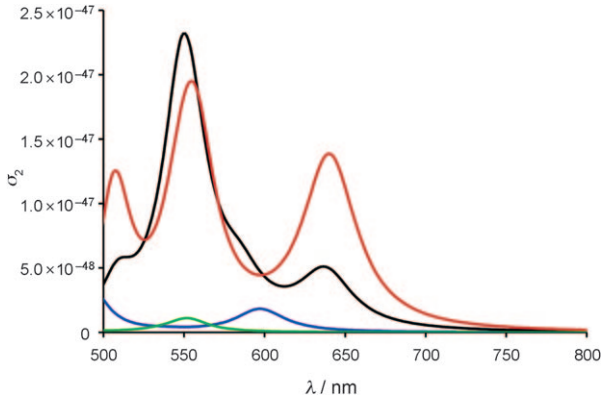


Figure 4. Calculated two-photon absorption spectra of model chromophores **1** (green), **2** (blue), **3** (black), and **4** (red).

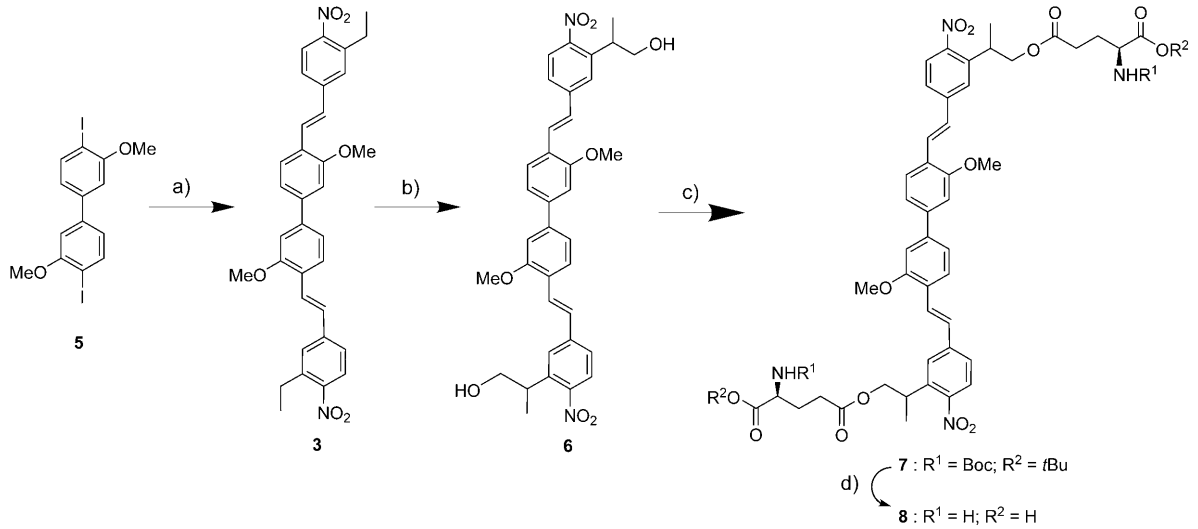
structure can be considered as a biphenyl core with two electron-donating methoxy groups, surrounded by two styrenic π systems bearing electron-withdrawing nitro groups. The nitro groups also play a fundamental role in the uncaging process, as they are involved in the mechanism of the photochemical reaction.[8] The presence of a noncentrosymmetric push–pull system, which is very important in quadratic NLO properties such as second harmonic generation, is not necessary for cubic NLO properties, such as TPA. Thus, we designed another original cage based on a dipolar architecture, in which the methoxy groups were removed and the interaction between the two acceptor groups was enforced by retaining the planarity of the central core. For this purpose, we designed a caging platform with a fluorenyl central core, substituted in the 9-position by 1-(3,6-dioxaheptyl) chains, to increase aqueous solubility (2,7-bis-{4-nitro-8-[3-(2-propyl)-styryl]}-9,9-bis-[1-(3,6-dioxaheptyl)]-fluorene or BNSF, Figure 2). A parent chromophore has been reported with a TPA cross-section in excess of 5000 GM at 520–570 nm.[27] In addition, such dipolar or quadrupolar structures seem promising for the uncaging process, as they carry two photoremovable moieties that can photorelease two biomolecules per cage.

To validate these strategies, TPA properties were tested on model chromophores **1**–**4** (Figure 3) by using a semi-empirical calculation method AM1 (Austin Method 1) for geometry optimization and a CNDO/S method (CNDO = complete neglect of differential overlap) modified by Andraud et al, for the calculation of TPA properties.[28] Chromophore **1** represents a model for the recently developed cage PMNB, which we used for TP uncaging of glutamate.[23] Chromophore **2** acts as a model compound of a vinylogue of **1**. The two other systems, **3** and **4**, represent models for the new cages reported in this paper. Compound **3** is a quasi-dimeric form of **2**, and **4** represents the symmetrical linear system designed in this work.

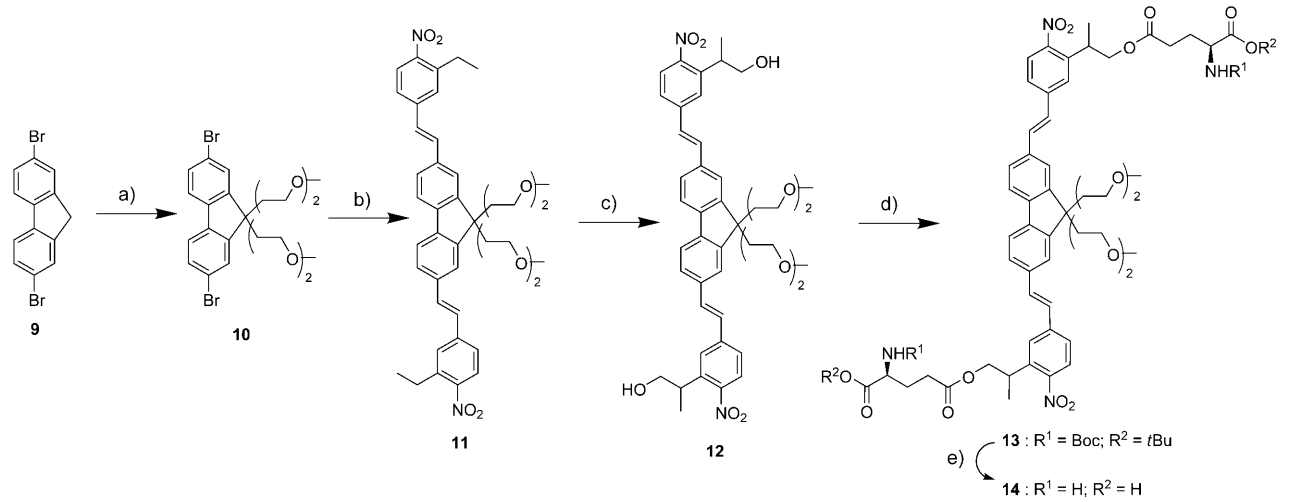
As expected, the calculated two-photon absorption spectrum for **2** (Figure 4) showed a red-shifted absorption and an increased TPA, in comparison with **1**. For the dimeric system **3**, there is a red shift, mainly resulting from the increased length of conjugation, and a threefold increase of the TPA cross-section compared to **2**. These effects are even more pronounced in the case of **4**, even in the absence of electron-donating methoxy groups. A threefold increase of the TPA cross-section is evident for **4**, in comparison with the quadrupolar chromophore **3**.

In this theoretical study, we compared only the TPA efficiencies of the model chromophores. However, in dealing with TP uncaging of biomolecules, the quantum yield of the photolytic reaction must also be taken into account.

Caged glutamates **8** (4,4′-bis-{8-[4-nitro-3-(2-propyl)-styryl]}-3,3′-di-methoxybiphenyl, BNSMB) and **14** (2,7-bis-{4-nitro-8-[3-(2-propyl)-styryl]}-9,9-bis-[1-(3,6-dioxaheptyl)]-fluorene, BNSF) were synthesized according to the methods outlined in Scheme 1 and Scheme 2, respectively. The bis-(stilbene) chromophore **3** was synthesized by the Heck coupling of 3-ethyl-4-nitrostyrene[6] and 4,4′-diiodo-3,3′-dimethoxybiphenyl **5**, itself readily obtained from the commercially available diazonium salt fast blue B. To synthesize the



Scheme 1. Synthesis of caged glutamate **8** (BNSMB–Glu). a) Xylenes, $Pd(OAc)_2$, EtN_3, tri-*o*-tolylphosphine, 3-ethyl-4-nitrostyrene; 5 days, 140 °C, 68 %; b) *t*BuOK/*t*BuOH, paraformaldehyde, dimethylsulfoxide (DMSO), 80 °C, 3 h; c) Boc-L-Glu-O-*t*Bu (boc = *tert*-butoxycarbonyl), 4-dimethylaminopyridine (DMAP), dicyclohexylcarbodiimide (DCC), CH_2Cl_2, room temperature, 2 h; d) 20 % trifluoroacetic acid (TFA), CH_2Cl_2, room temperature, 6 h.



Scheme 2. Synthesis of caged glutamate **14** (BNSF–Glu). a) NaH, *N*,*N*-dimethylformamide, 1-bromo-2-(2-methoxyethoxy)ethane; b) xylenes, $Pd(OAc)_2$, Et_3N, tri-*o*-tolylphosphine, 3-ethyl-4-nitrostyrene, 5 days, 140 °C, 68 %; c) *t*BuOK/*t*BuOH, paraformaldehyde, DMSO, 80 °C 3 h, 20 %; d) Boc-L-Glu-*O*-*t*Bu, DMAP, DCC, CH_2Cl_2, room temperature, 2 h; e) 20 % TFA, CH_2Cl_2, room temperature, 6 h.

fluorenyl chromophore **11**, the first step was the functionalization of 2,7-dibromofluorene **9** at the 9-position with two 1-(3,6-dioxaheptyl) chains, to improve the water solubility of the molecule. The Heck coupling was then carried out in the same conditions as for the biphenyl compound, to give **11**. Chromophores **3** and **11** were then treated with a *t*BuOK/*t*BuOH mixture in the presence of paraformaldehyde, to give cages **6** and **12**, respectively. Protected glutamate (*N*-α-*tert*-butyloxycarbonyl-L-glutamic acid-α-*tert*-butyl ester) was coupled to these cages, prior to deprotection in acidic media (20 % trifluoroacetic acid), to give caged glutamates **8** and **14**. All compounds were fully characterized by UV/Vis spectroscopy, HPLC, ^1H and ^{13}C NMR spectroscopy, and high-resolution mass spectrometry (see the Supporting information).

The one-photon photophysical properties of caged glutamates **8** and **14** were measured (Table 1) and compared to those of MNI–Glu (MNI = 4-methoxy-7-nitroindolinyl),[29] DMNPB–Glu (DMNPB = 3-(4,5)-dimethoxy-2-nitrophenyl)-2-butyl),[8] PMNB–Glu,[23] and Bhc–Glu (*N*-(6-*B*romo-7-hydroxycoumarin-4-yl)methoxycarbonyl-L-glutamic acid). A significant red-shift is evident for the two new cages, as well as a strong increase in molar extinction coefficient. The photolytic release of glutamate was analyzed quantitatively by HPLC.[30] Caged glutamates **8** and **14** afforded 60 % and 65 % yields of glutamate release per glutamate unit, respectively. As each caging system incorporates two caged glutamates, the overall yield of glutamate release reaches 120 % per molecule. The disappearance quantum yields (Φ) of 0.3 for **8** and 0.25 for **14**, respectively,

Table 1: One- and two-photon photophysical properties of caged glutamates **8** (BNSMB–Glu), **14** (BNSF–Glu), MNI–Glu, DMNPB–Glu, PMNB–Glu, and Bhc–Glu.

Caged glutamate	λ_{max} [nm]	ε (λ_{max}) [$M^{-1}cm^{-1}$]	Yield [% Glu][a,b]	Φ	$\varepsilon\Phi(\lambda_{max})$ [$M^{-1}cm^{-1}$]	$\delta_u\Phi_u$ [GM][c]
BNSMB–Glu (**8**)	400	39340	60	0.30	11800 (6600)[b]	0.9
BNSF–Glu (**14**)	415	63960	65	0.25	16000 (7500)[b]	5.0
MNI–Glu[30]	350	4300	–	0.08	366	0.06[d]
DMNPB–Glu[8]	350	4500	95	0.26	1170	0.17[d]
PMNB–Glu[23]	317	9900	90	0.1	990	0.45
Bhc–Glu[7]	368	17470	–	0.02	350	0.37

[a] Yield of glutamate release per glutamate unit. [b] At 354 nm. [c] At 800 nm. [d] At 740 nm.

were determined by comparison with the reference molecule, 2-(nitrophenyl)ethyl–ATP [31] at 315 nm, monitored by HPLC analysis. Clearly, the one-photon photolytic efficiencies of glutamate release at 354 nm is at least one order of magnitude higher for the new probes compared to the previously described cages (Table 1). This increase is even more accentuated at higher wavelengths ($\lambda \geq 400$ nm).

The two-photon uncaging cross-sections $\delta_u\Phi_u$ of **8** and **14** have been determined in the range 740–900 nm (Figure 5). At 800 nm, the two-photon uncaging cross-sections of **8** and **14**

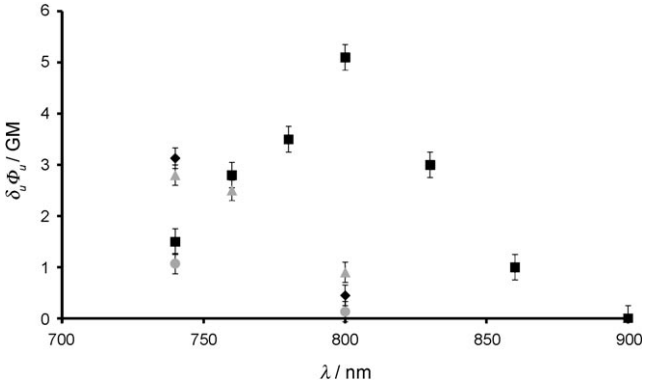


Figure 5. Two-photon photolysis cross-sections ($\delta_u\Phi_u$) of Bhc–Glu (●), PMNB–Glu (◆), BNSMB–Glu **8** (▲), and BNSF–Glu **14** (■) at 800 nm.

are 0.9 GM and 5.0 GM, respectively. For comparison, we also plotted the data for the well-known Bhc–Glu and PMNB–Glu (Figure 5). The $\delta_u\Phi_u$ value of 5 GM at 800 nm for BNFP–Glu **14** is unprecedented for a glutamate cage in the 800 nm range. Notably, the photolysis residue for such systems (nitrophenethyl series) is not a nitroso derivative (as for nitrobenzyle cages), but a nitro compound,[8] which means that the efficiency of the second release should not be affected by the first release. These results validate the new strategy of linear-elongated symmetric cages for TP photorelease of glutamate (see the Supporting Information for further details).

The molecular engineering strategy described herein has led successfully to two new caging platforms, which allow the photorelease of glutamate with high efficiency, both by one- and two-photon excitation: $\varepsilon\Phi > 10000\,M^{-1}cm^{-1}$ and $\delta_u\Phi_u$ up to 5.0 GM. Furthermore, these two new cages present a two-photon uncaging cross-section greater than 2 GM in the range 740–850 nm, the optimal window both for tissue transparency and classically available laser sources. The successful use of these new cages is promising, owing to adequate solubility in water, especially for BNSF–Glu, as a result of the presence of 1-(3,6-dioxaheptyl) chains (≥ 0.1 mM in 0.1M phosphate buffer). Further investigations on the effect of **8** and **14** on neurostimulation are underway.

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