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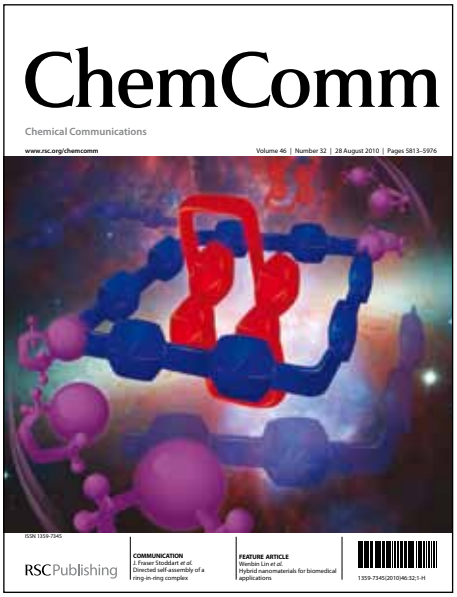

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COMMUNICATION

Tandem triad systems based on FRET for two-photon induced release of glutamate

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Tandem systems allowing enhanced two-photon (2P) absorption in a wavelength range permitting coupling of the primary excitation by energy transfer to an intramolecular cage known to have fragmentation properties suited to photolysis in neuroscience is demonstrated to lead to a 10-fold improvement in the 2P photolysis cross-section at experimentally compatible wavelengths.

Caged compounds, which can be irreversibly triggered by light, have attracted lot of interest over the years due to the many applications they offer in the fast developing field of neurosciences.[1] In particular, uncaging of the excitatory neurotransmitter L-glutamate (Glu) has been widely used in the activation and functional mapping of synaptic receptors and their signalling pathways, in studies of dendritic integration and of neuronal circuits. In parallel, two-photon (2P) excitation in the red-NIR region has emerged as a major tool for neurosciences, allowing much better spatial resolution deep within neural tissue than is possible with 1P excitation.[1a, 1c] Among the caging systems applied in neuroscience, the 7-nitroindolinyl (NI) and 4-methoxy-7-nitroindolinyl (MNI) photoremovable carbonyl protecting groups have become the most widely used because of their resistance to hydrolysis, their fast (sub-µs) release kinetics and lack of pharmacological interference in neural systems.[2] However, although efficient in 1P photolysis, the 2P uncaging cross-section ($\delta_u = \sigma_2\Phi_u$, the product of the 2P absorption cross-section (σ_2) and uncaging quantum yield (Φ_u)) remains relatively low (δ_u = 0.06 GM at 720 nm) preventing efficient 2P photolysis at non-toxic intensities and requiring high cage concentrations for *in vivo* studies.[3] Attempts to increase δ_u of NI derivatives via structural variations in the NI chromophore gave only small improvements of the 2P uncaging efficiency.[4] An approach in which the triplet states mediating NI photolysis were populated from an intramolecular sensitizer was found to be efficient but blue-shifted the wavelength range for 2P photolysis beyond that available from commonly used Ti-sapphire lasers.[5] Thus there is a strong motivation for the development of novel two-photon sensitive caged systems combining large δ_u in the red-NIR region with fast photolysis reactions for time-resolved release of neurotransmitters, and with excellent dark stability in biological conditions.

Most 2P uncaging systems developed so far relied on common uncaging systems (such as nitrobenzyl- or coumarinylmethyl-based cages) by including them in dipolar or quadrupolar conjugated compounds where they take part in the intramolecular charge redistribution responsible for large 2PA response.[1b, 1c, 6] Instead of trying to achieve both enhanced 2PA and efficient uncaging within a single chromophoric system, we implemented an alternative approach where the two processes are performed by two distinct units crafted to act in tandem.

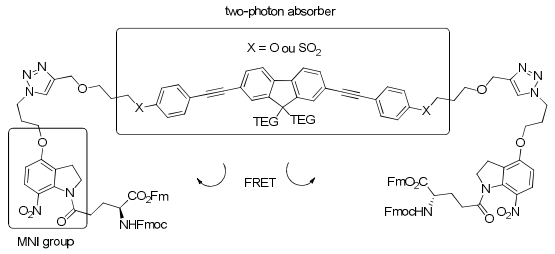
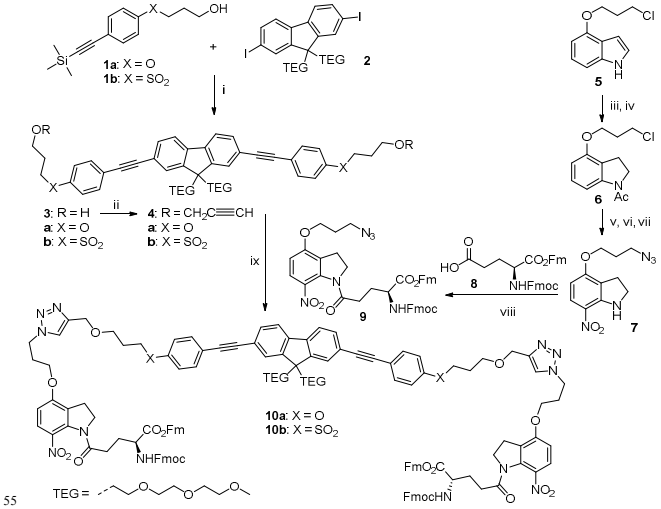


Fig. 1 Design of synergic triad structures 2P uncaging of glutamate



Scheme 1 *Reagents and conditions*: i) $PdCl_2(Ph_3P)_2$, CuI, CsF, THF/Et_3N, rt, **3a** 46%, **3b** 70%; ii) NaH, propargyl bromide, DMF, rt, **4a** 87%, **4b** 71%; iii) $NaBH_3CN$, AcOH, rt, then iv) DMAP, Et_3N, Ac_2O, CH_2Cl_2, rt, 95% (over 2 steps); v) $Cu(NO_3)_2$, CH_2Cl_2/Ac_2O, rt, 75% (5 and 7-nitro, ratio 1:1); vi) NaN_3, DMSO, 110 °C, then vii) KOH, THF/MeOH/Water, rt, 45% (over 2 steps); viii) oxalyl chloride, DMF, THF, rt, 57%; ix) sodium L-ascorbate, $CuSO_4$, DMSO, rt, **10a** 72%, **10b** 80%.

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Table 1 . Photophysical characteristics of the chromophores **3a-b** and the synergic triad systems **10a-b** in acetonitrile

	λ_{abs}^{max} [nm]	Log(ε^{max}) [$M^{-1}.cm^{-1}$]	λ_{em}^{max} [nm]	Stokes shift [10^3 cm^{-1}] [a)]	Φ_f [b)c)]	λ_{TPA}^{max} [nm]	σ_2 at λ_{2PA}^{max} [GM]
3a	354	4.81	383	2.1	0.67	714	48
3b	358	4.88	411	3.6	0.59	721	83
10a	355	4.93	382	2.0	0.10	710	42
10b	358	4.90	410	3.5	0.07	720	76

[a] Stokes shift = $1/\lambda_{abs} - 1/\lambda_{em}$; [b] Φ_f = fluorescence quantum yields ; [c] standard : quinine (Φ=0.546) in 0.5M aq H_2SO_4

The quadrupolar module is designed for improved 2PA in the wavelength range <800 nm, so as not to interfere in the range used for 2P imaging.[7] We chose to focus on the 4-alkyloxy-substituted NI uncaging unit similar to MNI-Glu in order to retain its hydrolytic stability and fast uncaging kinetics, of crucial importance for neuroscience investigations. Here we report the first synergic systems based on this strategy. They are composed of a hydrophilic quadrupolar platform built from a fluorenyl core[7] covalently grafted to two NI-caged glutamate moieties (Fig 1). We chose to use ethynyl linkers in order to preserve the photostability of the 2PA chromophoric module[7a] while adjusting its emission properties to allow FRET to the nitroindolines. Such synergic systems can in principle release *two* Glu neurotransmitters per molecule upon irradiation.

The synthesis of targeted synergic structures **10a** and **10b** is detailed in Scheme 1. The quadrupolar chromophoric modules bearing either two electron-releasing (D) or electron-withdrawing (A) groups, **4a** and **4b**, were prepared in two steps by means of an *in situ* deprotection / double Sonogashira coupling of the known 2,7-diiodofluorene derivative[8] **2** with the corresponding silylated alkynes **1a** or **1b** followed by the propargylation of the terminal hydroxyl groups in the presence of propargyl bromide with NaH in DMF. In parallel, the 4-substituted NI-Glu subunit **9** was synthesised following a six-step sequence from the known 4-(3-chloropropoxy)indole[9] **5** which was first reduced with $NaBH_3CN$ to afford, after acetylation, the protected indoline **6** with 95% of yield. A nitration reaction in the presence of copper nitrate was then applied to convert the intermediate **6** into an unseparable mixture of 5 and 7-nitro product (ratio 1:1) with 75% of yield.[10] Substitution of the chlorine atom by an azido moiety followed by based-promoted deprotection of the acetyl group afforded the desired derivative **7** as a pure regioisomer. The NI-Glu **9** was further obtained by coupling compound **7** and the acyl chloride generated *in situ* from the corresponding protected glutamate **8**. The MNI-Glu triads **10a** and **10b** were finally synthesised in very good yields using a two-fold Huisgen 1,3-dipolar cycloaddition of azide **9** with bis-alkyne **4**.

The photophysical characterisation and 2PA properties of the chromophoric quadrupolar modules **3a-b** and the related synergic systems **10a-b** are shown in Table 1 and Figures 2 and 3. All compounds display an intense absorption band in the near UV-region with maxima located around 355 nm and high molar extinction coefficients ranging from 6.4 to 8×10^4 $M^{-1}cm^{-1}$, at least one order of magnitude larger than the MNI cages (peak at 340 nm 4.3×10^3 $M^{-1}cm^{-1}$). Quadrupolar chromophores **3a** and **3b** exhibit also intense fluorescence emission in the near UV-blue visible region with fluorescence quantum yields of 0.67 and 0.59, respectively. Although small, an overlap is observed between the emission spectra of fluorophores **3a** or **3b** and the absorption spectrum of the NI chromophore, favouring FRET from the 2PA module to the nearby NI.

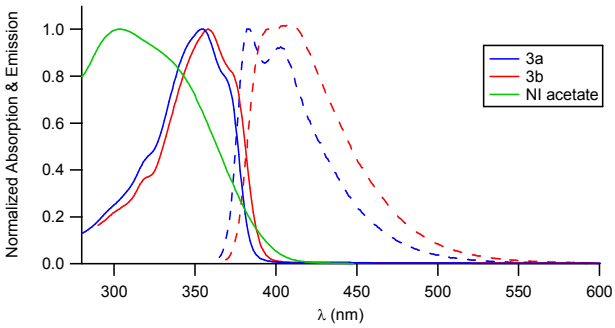

Fig. 2 Normalised absorption and fluorescence spectra of chromophoric moieties **3a, 3b** and NI in acetonitrile.

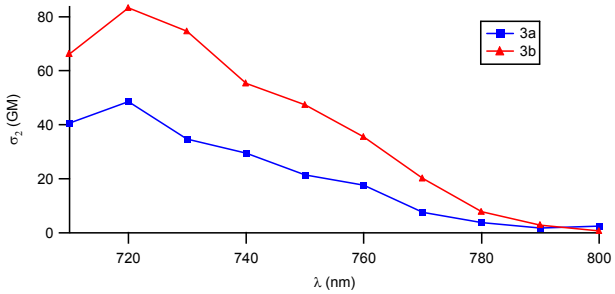

Fig. 3 2PA spectra of chromophoric moieties **3a** and **3b** in acetonitrile.

As shown in Table 1, compounds **10a** and **10b** show markedly reduced fluorescence compared to their quadrupolar modules **3a** and **3b**, respectively. This indicates that competing processes occur after excitation of the quadrupolar module. Since electrochemical studies indicate that intramolecular electron transfer is not favoured, we derived (majored) FRET quantum efficiencies values of 85% and 88%, for compounds **10a** and **10b**, using equation (1):

$$\Phi_{ET} = 1-(\Phi'_D/\ \Phi_D) \qquad (1)$$

where Φ'_D and Φ_D are the donor (quadrupoles) fluorescence quantum yields in the absence and presence of the acceptor (NI), respectively.[11]

In addition chromophores **3a** and **3b** were found to show markedly enhanced 2P absorption responses in the NIR region with respective σ_2^{max} values of 48 and 83 GM at 720 nm (Figure 3) which are 70-fold to 118-fold larger than that of MNI (0.7 GM). Hence 2PA modules **3** in tandem compounds **10** possess the required features to behave as suitable two-photon absorbers below 750 nm (as confirmed by their 2PA response, Table1) while permitting FRET to NI uncaging modules. Firstly, comparative 2P photolysis experiments were conducted on triad

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systems **10a, 10b** and NI reference sample **9,** using a Ti-sapphire laser at 740 nm (Figure 4). 2P uncaging was monitored by reverse phase HPLC-MS to quantify the extent of reaction.

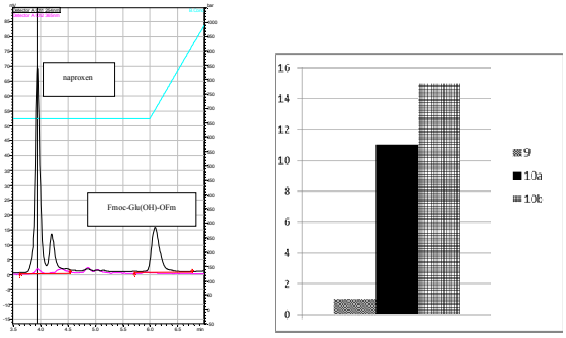

Fig. 4 Results obtained from the two-photon photolysis of compounds **9**, **10a** and **10b** after 24 hours of irradiation at 740 nm (left) HPLC chromatogramm obtained for the synergic system **10b**; (right) plot of the relative amount of released glutamate in the three cases (arbitrary units are used).

The production of the photo-released glutamic acid bearing the UV-responsive fluorenyl moieties was monitored by UV absorption (detection at 254 nm). We chose to use naproxen as an internal reference to allow the determination of the concentration of uncaged glutamate during the time course of the reaction. Whereas almost no fragmentation was recorded after 24 hours of 2P irradiation of the NI reference sample **9**, a noticeable HPLC peak corresponding to the photo-released glutamic acid was observed in both synergic samples **10a** and **10b**. Very interestingly, comparison of the percentage of released glutamate in the different experiments showed that glutamate uncaging efficiencies of the synergic systems **10a** and **10b** are respectively 11-fold and 15-fold greater compared to the NI cage **9**. Compound **10b** which has the larger 2PA response also lead to the best 2P uncaging efficiency. Importantly, the synergic caged MNI-Glu compounds **10a-b** displayed no dark hydrolysis under these conditions. In order to get an evaluation of the two-photon sensitivities, the absolute measurement of δ_u of compound **10b** was further performed using a Ti-sapphire laser with excitation at 730 nm (see SI). This led to (minored) δ_u value of about 0.5 GM at 730 nm. This is consistent with an enhancement of about one order of magnitude compared to MNI-Glu in the same conditions.

In conclusion, the triad systems investigated here, which allow a one-order of magnitude enhancement in 2P uncaging sensitivity compared to MNI provide proof of concept that the FRET route is highly promising for the design of effective 2P cages below 800 nm, allowing combined 2P bioimaging at λ_{2PE}>800 nm. This novel route will be further generalised to other caging groups and caged bioactive molecules.

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Notes and references

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