Two-photon uncaging of bioactive compounds: Starter guide to an efficient IR light switch

Two-Photon Uncaging of Bioactive Compounds: Starter Guide to an Efficient IR Light Switch

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Abstract:

Triggering physiological responses with a light switch has become a reality with the development of smart molecular probes such as photolabile protecting groups (PPGs), able to "uncage" biological ligands on demand. To make the light switch virtually harmless and confine the excitation to the single-cell level, the caged ligands can be released using two-photon (2P) absorption and 2P microscopy using red/infrared light. This exceptional level of precision however comes at the cost of a reduced photosensitivity and a poor compatibility of early PPGs with 2P excitation. This review aims to provide a tutorial guidebook to the design of 2P-sensitive PPGs suitable for optobiology by discussing challenges, strategies and progress in uncaging of bioactive compounds. To do so, we first recall the photo-physical principles governing 2P absorption, and the resulting ground rules in the design of efficient 2P absorbing organic dyes. We then detail how following these guidelines has led to tremendous progress in the development of a new generation of caged compounds, and the implications in the fields of biophotonics, from neurology to targeted therapy.

1. Introduction

At the frontier with biology, the design of molecular probes containing light-responsive moieties allows the use of light as an input, and collect valuable information on tissues (imaging), control physiological signals (optoneurology), or deliver bioactive ligands (therapy) as output. Among such tools, some excited chromophores undergo a photochemical cascade leading to the cleavage of a covalent bond. From the point of view of the released function, this cleavable chromophore can thus be described as a photolabile protecting group (PPG).[1–4] This "protection-deprotection" strategy is suitable for masking not only chemical, but also biological functions in bioactive ligands; in which case the molecule, trapped by the PPG in the form of an inactive precursor, is often said to be metaphorically “caged” by this covalent bond. Shining light on the caged compound releases the ligand under its active form by restoring its original functionality, as a conventional deprotection reaction would do.

Although PPGs have opened avenues towards the control of physiological responses in tissues with light, the criteria for their use in biological environments are dire. In particular, the need to increase spatial resolution and avoid the photo-toxicity of UV light gradually led the research towards a promising alternative technique: two-photon excitation (2PE). This non-linear optical phenomenon makes it possible to excite a chromophore by simultaneous absorption of two half-energy photons instead of one, which doubles the wavelength of irradiation **(Figure 1)**. The trigger then becomes red or near-infrared (NIR) light, whose wavelengths penetrate better into tissues and reduce the risk of photodamage. Shortly after the introduction of the first 2P microscopes,[5] and even before their use in cell biology became widely accessible, Adams and Tsien predicted in 1993 the substantial progress that would rise from the combination of 2P microscopes and the photorelease of biomolecules.[6] In almost 30 years, with the advent of Ti:Sapphire lasers and 2P microscopy techniques, the field of 2P uncaging has not only come into being, but has also largely grown. The capacity for a chromophore to absorb two photons

simultaneously is however dictated by very different physical rules from the traditional single-photon (1P) irradiation, and even to this day, the 2P photosensitivity of PPGs described in literature remains a barrier to widespread biological applications. The aim of this review is to provide guidelines and rationality to the design of new 2P-sensitive "cages" based on theoretical rules, and empirical evidence found in literature.[7–10] To this end, we will first introduce the fundamental notions of photophysics at play in 2P absorption, and provide explanation for the design of 2P absorbing chromophores. We will then review how such engineering strategies were applied to the main families of PPGs, and the implications in selected biological applications.

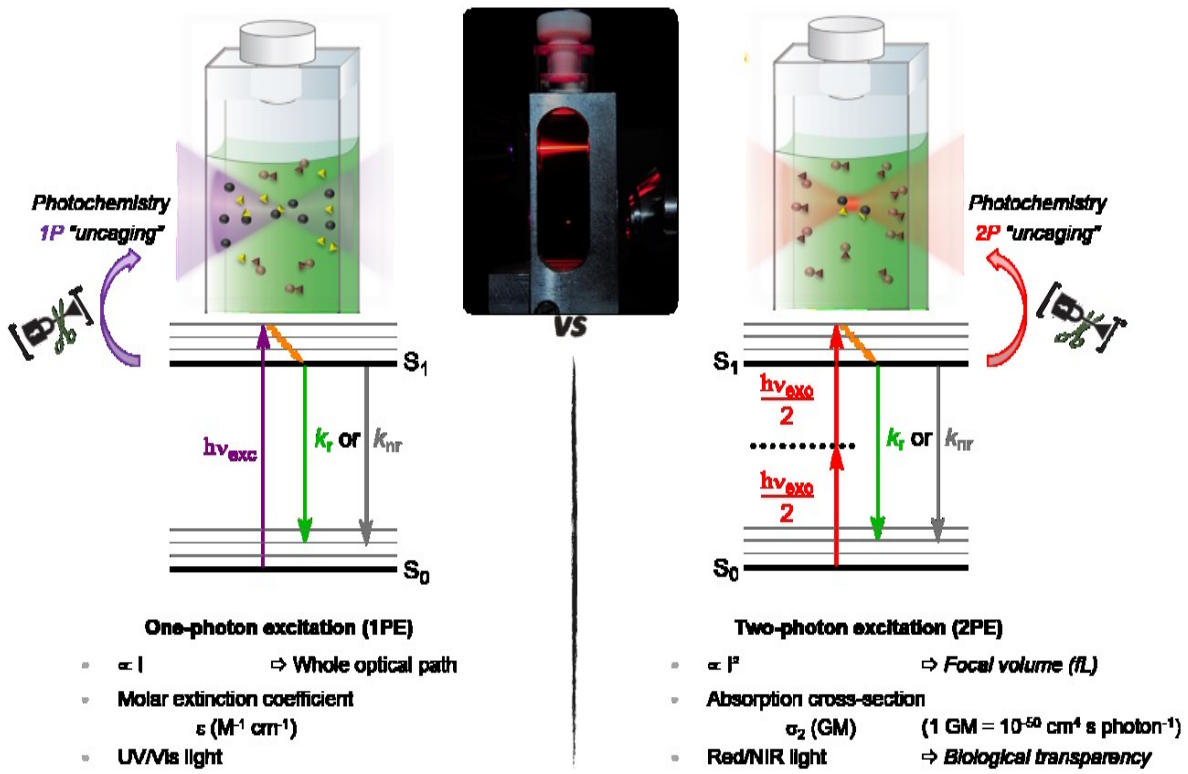


Figure 1. Simplified Jablonski diagrams and key features of the 1PA (left) and 2PA phenomena (right). The photo-deprotection phenomenon is illustrated in cuvettes with the case of an active substrate (triangle) protected with a PPG (circle). and the resulting volume of photorelease in each case. The center picture illustrate the difference in spatial resolution between both types of excitation with the example of red-emissive dye excited with a focalised light beam (405 nm) in 1PA (top) and a pulsed laser beam (1050 nm) in 2PA (bottom)

2. Molecular engineering of two-photon dyes: from theory to application

2.1. From linear to non-linear optics

Light-matter interactions must be considered from two complementary perspectives. When light, as an electromagnetic wave, interacts with matter, its electric field $\vec{E}$ also causes a displacement of the charges within the material, and therefore the creation of dipoles. The resulting macroscopic polarization field $\vec{P}$ is directly related to the amplitude of $\vec{E}$ in the light beam, and can generally be expressed as a Taylor polynome (Equation (1)).

$$\vec{P} = \overrightarrow{P_0} + \varepsilon_0 \chi^{(1)} \vec{E} + \varepsilon_0 \chi^{(2)} \vec{E}^2 + \varepsilon_0 \chi^{(3)} \vec{E}^3 + \cdots \quad (1)$$

In which ε_0 is the vacuum permittivity constant, and $\chi^{(n)}$ is a tensor of rank n + 1 describing the electrical susceptibility of the n^{th} order of the material. At the molecular level, the resulting dipole moment $\vec{\mu}$ can be expressed in a similar way according to the local field $\vec{F}$ (Equation (2)).

$$\vec{\mu} = \overrightarrow{\mu_0} + \alpha\vec{F} + \beta F^2 + \gamma F^3 + \cdots \quad (2)$$

In this expression, $\overrightarrow{\mu_0}$ is the permanent dipole moment of the molecule, and α is the polarisability. In *linear* optics, only this first-order term is taken into account. Other terms can be neglected, and the resulting polarisation expression is *linear*. When the intensity of $\vec{E}$ increases, this approximation of linearity is no longer valid. Higher order terms such as those involving the quadratic hyperpolarisability (or second order polarizability) β, cubic hyperpolarisability (or third order polarizability) γ etc., become significant in the Taylor polynome. This is the field of *non-linear* optics (NLO).[11–13]

Quadratic hyperpolarisability β governs non-resonant second-order NLO phenomena disrupting the ground state without promoting an electron to an electronic excited state, such as second harmonic generation (SGH).[14] Cubic hyperpolarisability γ reflects third-order phenomena, such as third harmonic generation (TGH) and the 2P absorption (2PA).

Indeed, in a quantum physics description, if the incident energy is high enough to fill the gap between the ground state and the excited state, and if the oscillation is efficient enough to "push" the electron towards a higher energy MO, resonant NLO can occur with the simultaneous absorption of two photons (or more[13]). The chromophore is then promoted from its ground state S_0 to an excited state, as illustrated on Figure 1. This was first theorised by Maria Göppert-Mayer in 1929,[15,16] but could only be observed experimentally in 1961 by Kaiser and Garrett[17] after the development of the first pulsed lasers. The real part of hyperpolarisability γ will influence the nonlinear refractive index of the medium, while its imaginary part will define the 2PA cross section σ_2.[18,19]

Absorption cross-sections σ_n define the absorption capacities of 1PA and 2PA dyes as the effective "absorption surface" (or section) on which photons can be captured as light passes. As exemplified in the case of 1PA **(Figure 2)**, each molecule absorbs on a fraction σ/S of the surface beam S. On the totality of the infinitesimal section considered, the total absorbed surface Σ, is equal to this fraction multiplied by the number of chromophores in the corresponding volume, and therefore the concentration of the sample. In 2PA, the cross-section σ_2 therefore quantifies the capacity of a dye to absorb two photons simultaneously, and is expressed in Göppert-Mayer (1 GM = 10^{-50}cm^4 s photon^{-1}) as a tribute to its original theoretician.

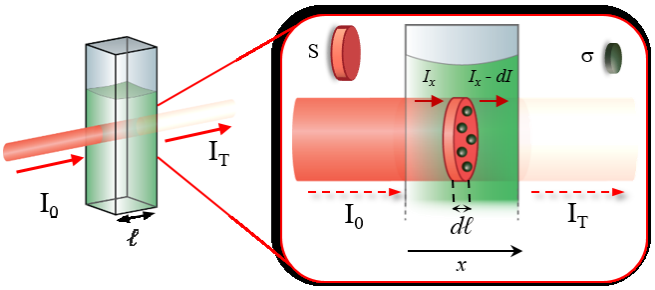


Figure 2. Illustration of the concept of absorption cross-section for a dye in solution. The incident beam I_0 can be decomposed, along the optical pathway, into an infinity of irradiated sections S of infinitesimal length dℓ. At each position x of the optical path, the light intensity is I_x before the section, and I_x - dI at the exit. The absorption cross-section of each molecule is represented as dark green discs.

In a common model describing the 2PA phenomenon, the absorption of a first photon causes the creation of a very short-lived (10^{-15} s) non-resonant excited state, called "virtual state". To further promote the electron on a real electronic level, the absorption of the second photon must occur on the hundreds of attoseconds to tens of femtoseconds timescale,[12] during the lifetime of the virtual state, which explains the use of pulsed lasers. The probability to encounter a second photon on this timeframe also depends on the spatial and temporal overlap with the incident photons, which implies a quadratic dependence on light intensity. Thus, by applying hypotheses equivalent to Beer-Lambert's law,[20] the light intensity attenuation following 2PA can be described according to Equation (3), where I is the intensity expressed as photon flux, ℓ the optical path, C_N the number of molecules per cm^3, and σ and σ_2 respectively the 1P and 2P absorption cross-sections at a given wavelength.

$$\frac{\partial I}{\partial \ell} = -C_N \sigma I - C_N \sigma_2 I^2 \quad (3)$$

Considering that the energy of photons used for 2PA is largely insufficient to resonate alone with an excited state and cause 1PA, only the second quadratic term of Equation (3) remains. This quadraticity confines the excitation exclusively to the focal point of the laser, and provides spatial resolution on the femtolitre level, i.e. the size of *E. coli* bacteria or individual human synapses. Although it is theoretically possible to use two photons of different energy,[21–23] 2PE experimental setups generally use two photons of identical "degenerate" energy $h\nu_{exc}/2$, which consequently doubles the excitation wavelength used in 1PE, and shifts it to red or NIR wavelengths. Phototoxicity of such wavelengths is far lesser than UV radiations and their tissue penetration is largely improved thanks to poorer absorption of endogenic chromophores and scattering. With a penetration of up to a few cm without particular damage,[24] this wavelength range is called the "biological transparency window".[25]

As selection rules differ in NLO, the excited state reached upon quasi-simultaneous double absorption may be different from the one achieved by single-photon absorption. Selection

rules dictate whether an electronic transition is allowed or forbidden based on the overlap between the two MOs involved and the so-called transition dipole moment μ_{gx} (Equation (4)).

$$\mu_{gx} = \int_{-\infty}^{\infty} \Psi_g \, \hat{\mu} \, \Psi^*_x \, d^3r \quad (4)$$

Ψ_g and Ψ^*_x are the wave functions respectively associated with the MO of the ground state S_0 and the excited state S_x, $\hat{\mu}$ is the dipole moment operator which corresponds to the charge of the electron multiplied by its position vector, and d^3r indicates that the integral is calculated over the entire space coordinates. Transitions are called forbidden if the norm of their transition dipole moment is zero. In 2PA, selection rules have important consequences on the absorption wavelength and the absorption capacity of a chromophore.

2.2. From quantum models to ground rules

Based on the physical foundations of NLO, several ground rules apply to the development of non-linear materials. Among these, two key factors influence particularly the 2PA response of a material: internal charge transfer (ICT) and centrosymmetry. The latter is known to be a restrictive parameter towards even order polarisabilities (such as β). As a third order phenomenon, 2PA can theoretically be observed with any chromophore irrespective of its symmetry. The symmetry of the dye will however alter the nature of the electronic transitions observed based on their "allowed" or "forbidden" nature. Quantum models[11,26–28] have been developed to rationalise such properties with regards to the symmetry of orbitals and wavefunctions. In particular, "essential states models"[29–32] allow the description of the 2PA cross-section σ_2 as a function of the complex tensor of the microscopic hyperpolarisability γ. Such models consider that, in a perturbative description[28] of γ, the main contributions are provided by the lower-energy 1P-allowed excited state of the linear optical response, and by a few additional (i.e. "essential") states that are strongly dipole-coupled to it. Therefore, only the

interaction between the ground state $|g\rangle$, the final two-photon-allowed excited state $|f\rangle$ and the major one-photon-allowed state $|i\rangle$ are considered. Each of those electronic states MOs belong to a certain symmetry group, and consequently, selection rules may apply restrictions to certain transitions **(Figure 3)**. In non-centrosymmetric systems (Figure 3 (a)), the transition to the first excited state is generally both 1P- and 2P-allowed. Only two states, $|g\rangle$ and $|f\rangle$, can be considered in this description of the 2P phenomenon ("two-state model").

In the case of centrosymmetric chromophores (Figure 3 (b) and (c)), the ground state $|g\rangle$ is of even symmetry, i.e. symmetrical with respect to the centre of inversion (*gerade*). Following Laporte's rule, an electron can undergo a transition to a 1P-allowed state $|i\rangle$ of odd symmetry (*ungerade*) strongly coupled by transition dipole moment. In 2PA however, the frequency ν (or angular frequency $\omega = 2\pi\nu$) does not resonate with this transition and creates a virtual state below $|i\rangle$ which preserves some features of the resonant 1P-allowed state $|i\rangle$, such as an *ungerade* symmetry. The second photon generates a new dipolar transition towards the lowest lying *gerade* final state $|f\rangle$, higher in energy and strongly coupled to $|i\rangle$ by transition dipole moment. Thanks to this double inversion, the transition $g \rightarrow f$ is 2P-allowed while being symmetry-forbidden in 1PA. This inversion of the selection rules between 1PA and 2PA can be generalised to all centrosymmetric chromophores, in which λ_{2PA}^{max} is located at higher energies than $2\lambda_{1PA}^{max}$. Therefore, three "essential states" are considered in this description.

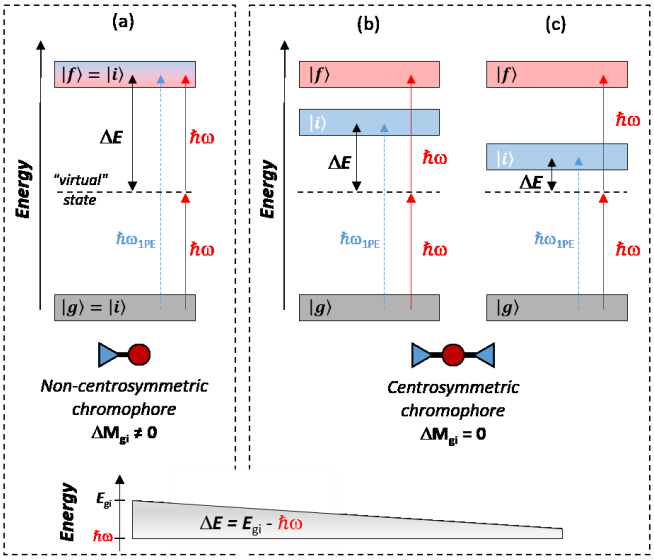


Figure 3. Energy diagram following the essential states model in the case of (a) non-centrosymmetric chromophores; (b) a centrosymmetric chromophore with high detuning energy; (c) a centrosymmetric chromophore with small detuning energy.

The key to essential state models is to rely on the lifetime of the virtual state and its potential interaction with the lowest-lying resonant excited state. Indeed, the uncertainty principle states that the lifetime Δt of a photon $\hbar\omega$ in this intermediate state will be superior to the ratio $\hbar/\Delta E$, where ΔE is the so-called "detuning resonance energy", i.e. the difference between the energy level of the virtual state and that of the 1P resonant state $|i\rangle$ (Figure 3). Consequently, if ΔE decreases, the lifetime of the virtual state increases, which increases the probability to absorb a second photon, and therefore the σ_2 of the chromophore.

As stated above, the 2PA cross-section is directly correlated to the cubic hyperpolarisability of the material, and more specifically its imaginary part $\mathrm{Im}\left(\boldsymbol{\gamma}(-\omega,\omega,-\omega,\omega)\right)$. Although the demonstration goes beyond the scope of this review,[18,28,33–35] the expression of the fourth rank tensor γ[28] introduced in the context of the essential states model provides a proportionality relation for σ_2[34,36] (Equation (5)).

$$\sigma_2 \propto \mathrm{Im}\left(\begin{array}{ll} & \dfrac{\mu_{gi}^2\,\Delta M_{gi}^2}{\left(E_{gi}-\hbar\omega-j\Gamma_{gi}\right)^2\left(E_{gi}-2\hbar\omega-j\Gamma_{gf}\right)} \quad "D" \\ + \sum_{i\neq f,g} & \dfrac{\mu_{gi}^2\,\mu_{if}^2}{\left(E_{gi}-\hbar\omega-j\Gamma_{gi}\right)^2\left(E_{gf}-2\hbar\omega-j\Gamma_{gf}\right)} \quad "T" \\ - & \dfrac{\mu_{gi}^4}{\left(E_{gi}-\hbar\omega-j\Gamma_{gi}\right)^2\left(E_{gi}+\hbar\omega-j\Gamma_{gi}\right)} \quad "N" \end{array} \right) \tag{5}$$

In Equation (5), the imaginary unit is called j for the sake of clarity, the transition dipole moments associated with the transition $x \rightarrow y$ are called μ_{xy}, and the difference between static dipole moments of states $|x\rangle$ and $|y\rangle$ is written ΔM_{xy} for a better distinction. E_{xy} is the energy difference between both states, and Γ_{xy} is a damping factor associated with the transition. The three components of this expression contribute differently to the value of σ_2. The *D*-term is named for its "dipolar" contribution, as it involves mainly static dipole moments ΔM_{xy}. The second positive contribution is called “Two-photon-term” (*T*-term) as it is always present in 2PA. Finally, the negative-term (*N*-term) can be subtracted to this expression when the photon $\hbar\omega$ is too close to the resonant energy,[35] which is usually not the case. Thus, by neglecting the *N*-term, considering that the damping factor Γ is identical for all transitions,[36] and isolating the imaginary part, Equation (5) can be further simplified to the following expression.[37]

$$\sigma_2 \propto \left(\frac{\mu_{gi}^2\,\Delta M_{gi}^2}{(\hbar\omega)^2} + \sum_{i\neq f,g} \frac{\mu_{gi}^2\,\mu_{if}^2}{\Delta E^2} \right) \tag{6}$$

Equation (5) and (6) therefore create a distinction between two cases, depending on the existence or not of a difference between dipole moments in the ground state and in the excited state ΔM_{gi}; and therefore on the symmetry of the chromophore.

In non-centrosymmetric dipolar chromophores (Figure 3 (a)), the presence of static dipole moments in the ground state $|g\rangle$ and in the excited state $|f\rangle$ ($\Delta M_{gi} \neq 0$) implies that the dipolar term of Equation (5) and (6) will be non-zero, and that both the *D*- and the *T*-term will contribute to the σ_2. However, as shown in Figure 3, the intermediate state $|i\rangle$ is merged with or higher

than $|f\rangle$, $(E_{gi} - \hbar\omega) \geq \hbar\omega$. Therefore, assuming that the static and transition dipoles are all of the same order of magnitude, the *D*-term contribution to the expression of σ_2 will generally be greater than that of the *T*-term.

In centrosymmetric chromophores however ($\Delta M_{gi} = 0$), the dipolar term is zero, and the 2PA cross-section is directly governed by the *T*-term. The lower energy level of $|i\rangle$ compared $|f\rangle$ brings out two direct consequences. First, when energy gap between the virtual state and the 1P-allowed state $|i\rangle$ decreases (Figure 3 (b) and (c)), the denominator of the *T*-term ΔE^2 decreases quadratically. Consequently, very high σ_2 values can be reached in centrosymmetric "quadrupolar" compounds, which is mathematically impossible in the case of dipoles. Secondly, and as opposed to non-centrosymmetric chromophores, $\Delta E < \hbar\omega$. Therefore, for equal dipole moments, i.e. equal π-conjugated structure and *push-pull* strength, the *T*-term in a centrosymmetric chromophore will necessarily be greater than the *D*-term in its non-centrosymmetric analogue. Therefore, provided that the detuning energy is small enough, the 2P response of a quadrupolar compound will be inherently stronger than the one of its corresponding dipole.

Based on Equation (5) and (6), general trends in the molecular engineering of 2P absorbing materials have been established. In practice, some terms of the equation such as μ and M are difficult to access experimentally,[38] but the following rules can be followed:[35,39–42]

(i) *Increasing the static and transition dipole moments.* According to Equation (6), the increase in ΔM_{gi} leads to a quadratic increase of σ_2, as does the increase of transition dipole moments μ. In practice, this can be done in two ways. The first is to tune the electron-donating (ED) or electron-withdrawing (EW) capacity of the end-groups (and/or the core, if relevant) of the molecule. The intramolecular charge transfer (ICT) is therefore a major driving force of 2PA, and any modification in this favour should affect the σ_2 positively. The second is to increase the length of the π-

conjugated system. Indeed, according to Equation (4), the transition dipole moment is calculated by integration over the three coordinates of the radius *r*, i.e. the distance over which the charge moves during a transition. Intuitively, μ will increase when its vector coordinates are located farther away from the origin, which is achieved with longer conjugation. There is however an upper limit to this chain length beyond which the wavefunction becomes incoherent, especially because of the loss of planarity in the molecule.[39]

(ii) *Prepare centrosymmetric chromophores.* For equal donor and acceptor groups, a quadrupole (D-π-A-π-D or A-π-D-π-A) will have a higher 2P response than the corresponding dipole (D-π-A or A-π- D).

(iii) *Approaching resonance for the virtual state.* In centrosymmetric systems, if the 1P-allowed state is located near the virtual state (i.e., the wavelength of the laser), the 2PA cross section can be significantly increased, as observed for instance on porphyrin derivatives.[43] This effect is called intermediate state resonance enhancement (ISRE).[22,43,44] However, at wavelengths close to 1P resonance, linear absorption may not be negligible any longer, and additional factors such as 1PA line shape and 2PA to 1PA ratio may have to be taken into account to determine the actual 2P response.

(iv) *Minimising the bandwidth.* The damping factor Γ influences the bandwidth of the transition; and a sharper band will result in a higher σ_2^{max} than a flattened band.

Importantly, these key factors are interdependent, and they often need to be considered holistically to anticipate effects on σ_2. In certain cases, quantitative predictions remain hard to achieve to this day, as the current theoretical models fail to provide a complete understanding of the transitions observed experimentally. As an example, in non-centrosymmetric

chromophores, intense transitions can sometimes be observed in the lower wavelengths of the 2PA spectrum without being prominent at $\lambda_{2PA}/2$ in linear absorption, which is sometimes attributed to important vibronic contributions. In centrosymmetric systems, the theoretical parity selection rule, albeit predicting the nature of the 2P-allowed transitions, sometimes falls short of quantitation and intensity prediction. Quantum chemical calculations can provide assistance in this case, but experimental measurements of the 2PA spectra remain the most reliable source of quantitative information.[45,46]

Nonetheless, since the first demonstration of the phenomenon on organic chromophores,[47] the rational design of 2P-responsive chromophores has progressed dramatically.[39,40,48] They are composed mainly of three components: an electron-donor group (D), and an electron-withdrawing group (A) linked via a polarizable π-bridge (or "core", for centrosymmetric systems).

2.3. Engineering of 2P absorbing molecules

The ICT is generated by ED and EW groups located either at the ends, or on the core of the chromophore. Hammett coefficients[49] can be useful indexes to evaluate the strength of such groups, and their effect on the 2PA cross-section. The most common terminal groups are dialkyl- or diphenylamino moieties, that provide high donating strength and retain a certain stability to oxidation.[50] Oxygen groups (-OH and -OR) have lower donor capacity, but phenolates are commonly used to design pH-responsive dyes.

Prasad *et al.* were among the first to report 2P-responsive dipolar organic dyes with different polarizable π-bridges based on a diphenylamino-vinylpyridine *push-pull* combination. Their 2PA cross-sections were modest, but the extension of the conjugation allowed them to reach 115 GM values in THF.[51] The importance of the ED group in ICT-induced 2PA was demonstrated by replacing the diphenylamino group with a poorly donating thiophene group,

which completely annihilated the 2P response. Ehrlich *et al.* reported as early as 1997 the increase in ICT caused by modification of the ED and terminal EW groups in centrosymmetric chromophores, and the consequent effect on the 2PA cross-section in stilbene derivatives **(Figure 4 (a))**. The addition of dibutylamino (**2**) and diphenylamino (**3**) donor groups on stilbene **1** increased the σ_2 value respectively by 100 GM and 330 GM.[52] Deeper structural changes were then carried out.[53] Replacing the vinyl bond of stilbene **1** with a *p*-divinylbenzene core, which resulted in a jump of 500 GM for compound **4**, thus highlighting the importance of an extended π-conjugated system. Importantly, a dramatic increase in σ_2 was reported upon addition of EW nitrile groups on the divinylbenzene core of this centrosymmetric chromophore. 2PA cross-sections as high as 4000 GM have been reported in this work by molecular engineering of dyes taking advantage of all the driving forces mentioned above.

In centrosymmetric systems such as **4**, terminal ED groups are often more efficient than terminal EW groups; and conversely an acceptor character is generally more favourable at the core of the system.[50] In this sense, bis-acceptor A-π-D-π-A compound **6a** is much less 2P-responsive than D-π-A-π-D compounds like **6c**, or even than its oxygenated counterpart **6b**. Significant 2PA cross-sections have nonetheless been achieved with bis-acceptor structures, such as bis-pyridinium quadrupoles.[54]

The π-conjugated bridge is the key element enabling the charge transfer between the aforementioned ED and EW groups. Four factors are taken into account in the choice of the link: its donor or acceptor character (vide supra), size, conformation (rigid or flexible, plane or not), and its nature (alkyne, alkene, aryl, etc ...).

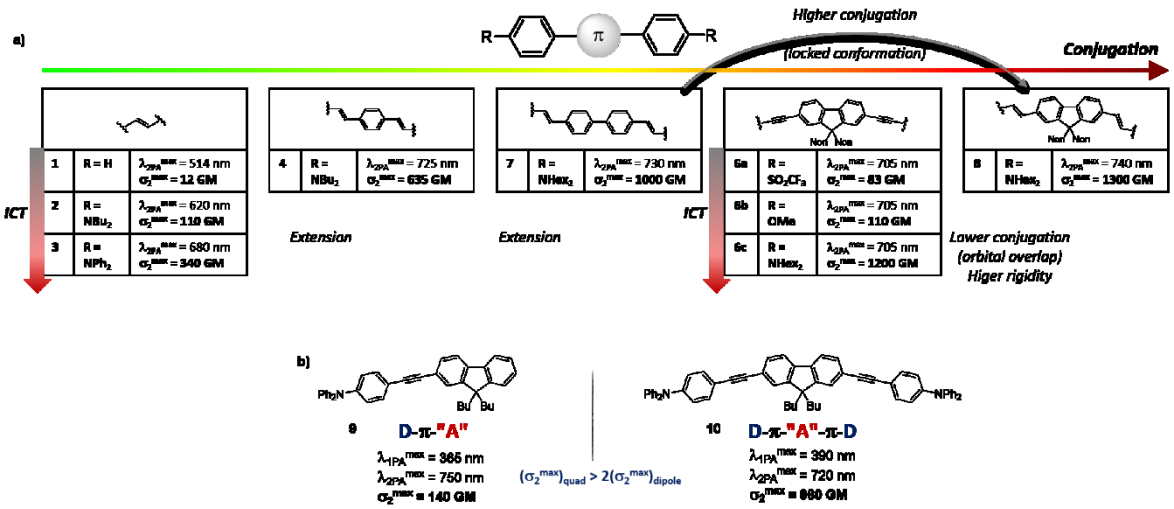


Figure 4. a) Roadmap of the structural modifications performed on centrosymmetric chromophores and stilbene derivatives, and evolution of the σ_2 as a function of ICT, and conjugation (length and planarity).[39,52,53] Non = *n*-C_9H_{18}. b) Comparison of σ_2^{max} and λ_{2PA}^{max} values in dipole **9** quadrupole **10** of equivalent strength.[55]

The length positively influences the σ_2 by transferring the electrons over longer distances, which increases dipole moments. In this sense, replacing the phenyl ring in quadrupole **4** (Figure 4 (a)) with a biphenyl pattern (compound **7**) allows a 60% increase in the 2PA cross-section.

To optimise the orbital overlap, and therefore the electronic coupling in a π-conjugated system, planarity is also essential. This is demonstrated by the replacement of the biphenyl bridge in **7** by a constrained 2,7-fluorenyl core in **8**, which prevents free rotation and causes an additional 30% increase of the σ_2 value. Thus, fluorene and dihydrophenanthrene cores are commonly used to block the conformation and maximize planarity. Their benzylic position(s) are commonly functionalised with bulky chains for enhanced solubility and π-stacking prevention. In a similar way, the nature of the π-conjugated linkers between constitutive modules of the chromophore is important, and one of the most striking examples is the comparison of *trans*-vinylic bridges with ethynyl bridges. In general, the conjugation character is less pronounced in triple bonds than double bonds because the orbital overlap of sp hybridized carbons with neighbouring sp^2 carbons is less favourable. Compounds **8** and **6c** show a perceptible, albeit

limited, effect on σ_2 in this sense. However, in some particular cases, especially in highly sterically-hindered chromophores, a more rigid acetylenic bridge can freeze the conformation of the chromophore and enhance conjugation, as compared to a more freely-rotating double bond which can move away from the plane. The geometry of the chromophore can therefore affect immensely its 2PA properties. This feature has been notably highlighted in the case of porphyrin dimers, [56,57] porphyrin arrays with controlled dihedral angle,[58] and bis-acceptor acetylenic quadrupoles with inhibited rotation.[59]

The abovementioned elements can be combined within non-symmetric dipolar structures, (dipole A-π-D) or symmetrical chromophores (quadrupole: A-π-A, D-π-D, A-π-D-π-A , D-π-A-π-D or octupole $D(-\pi-A)_3$, $A(-\pi-D)_3$.[39,40,48] The benefits of centrosymmetric chromophores over dipoles, theorised by Equation (6), was confirmed experimentally, sometimes with spectacular results.[56,60,61] The comparison of two chromophores bearing identical D and A groups embedded in the same conjugated structure, but arranged in different symmetries, allows a simple illustration of this fact. Even considering the increase in conjugation caused by the introduction of symmetry, the 2PA cross-section of dipolar compound **9** is multiplied by a factor of 7 when the push-pull system is embedded in an equivalent quadrupolar compound **10 (Figure 4 (b))**. As explained by symmetry selection rules, the λ_{2PA}^{max} of compound **10** is also shifted away from $2\lambda_{1PA}^{max}$, while this transition remains allowed in the case of dipole **9**. Even larger differences in σ_2^{max} between quadrupoles and their corresponding dipoles have been reported.[54]

The 7-fold enhancement of σ_2 in compound **10** is thus superior to the number of branches (i.e. 2). Studies then focused on the potential benefits of multi-branched systems with more complex symmetry, for which more complex theoretical models had to be developed to take into account inter-branch interactions such as exciton couplings.[62,63] Adding an axis of delocalisation for the charge transfer provides compounds with multidirectional conjugation called octupoles with

favourable cooperative effects between the axes. Such cooperation can be evidenced by the determination of σ_2/n where n is the number of branches. Comparing 4,4'

-bis(diphenylamino)stilbene (DPAS) **3**[52] with equivalent octupole **11**[64] thus proves that the cross-section value is greater than three times 340 GM **(Figure 5)**.

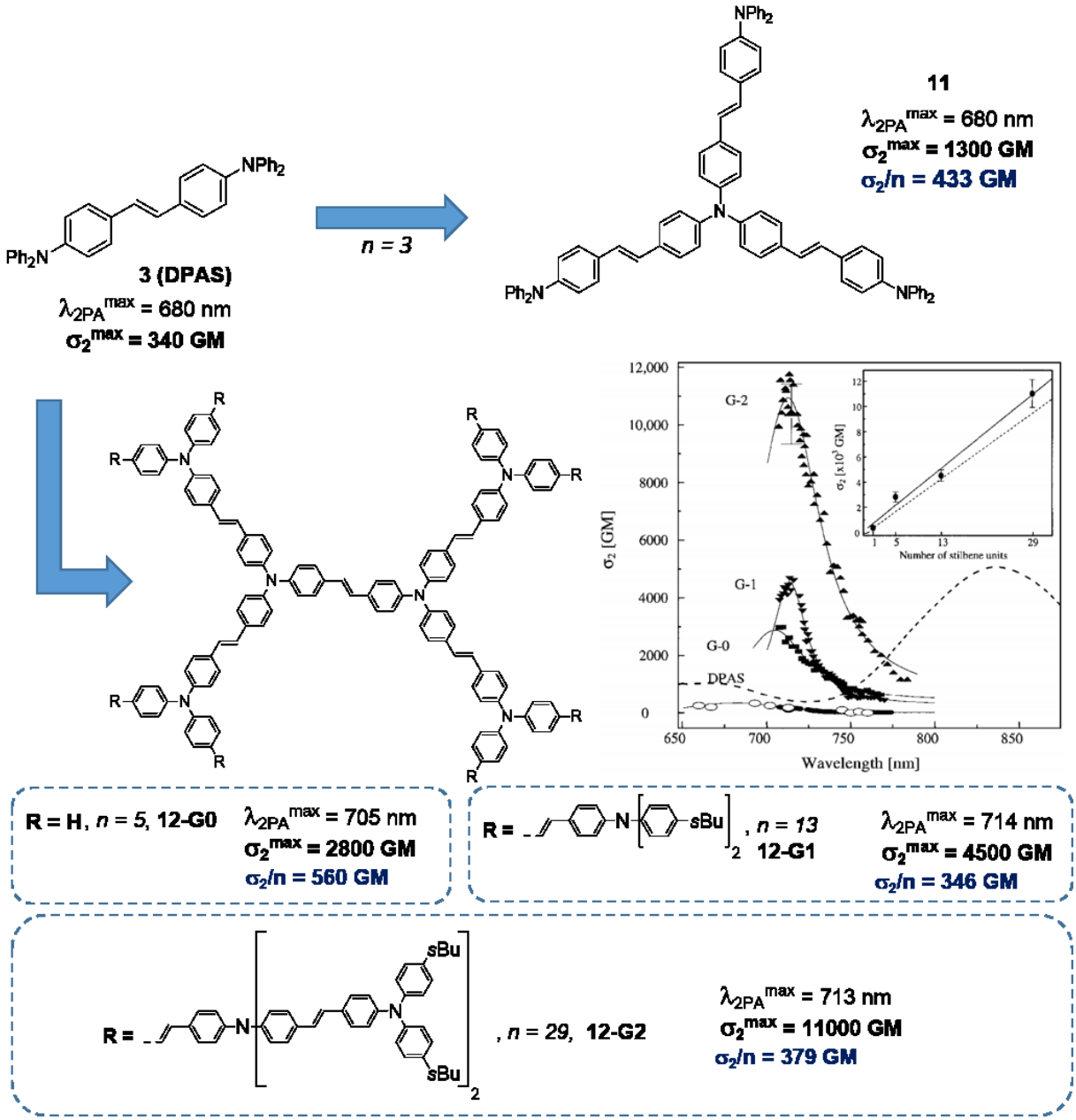


Figure 5. Effect of the degree of branching on σ_2 and σ_2/n values in compound **3**, corresponding octupole D(-π-D)$_3$ **11**[52] and different generations (G0, G1 and G2) of the multibranched compound **12**.[64] The 2PA spectra of dendrimers **12-G0** (squares), **-G1** (downward triangles), and **-G2** (upward triangles) and constitutive stilbene unit DPAS (circles), and the linear absorption of dendrimer **12-G2** (dashed curve, half abscissa values) are shown for comparison. The inset on the spectrum shows the dependence of the σ_2^{max} on the total number of stilbene branches with a linear fit (solid line), and with the thermodynamic

limit of a collection of non-interacting chromophores (dashed line). Adapted with permission from ref. [65] © The Optical Society.

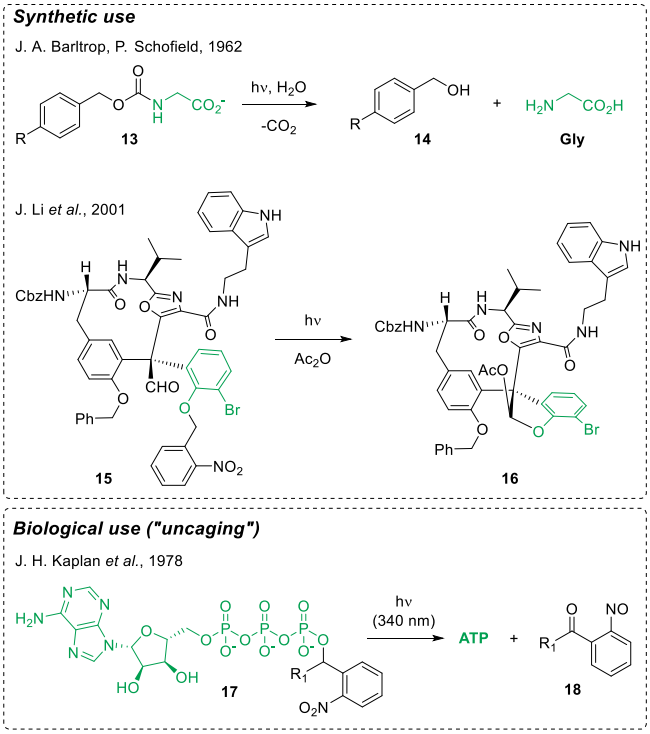
In the same design strategy, further increasing the number of conjugation paths in the molecule enables the accumulation of tens of 2P absorbing dipolar branches in dendritic structures (Figure 5). Multi-branched compound **12** reached a σ_2 of 11 000 GM with a σ_2/n value of 393 GM,[65] which is still higher than the constitutive stilbene unit alone (compound **3**). However, this strategy is limited by a saturation of the cooperative effects which generally occurs beyond the second generation, as demonstrated by comparing the different generations of dendrimer **12 (G0, G1 and G2)**. In these compounds, the cross-sections of the dendrimers are slightly larger than the theoretical case where stilbene sub-units contribution would simply add together (Figure 5, see inset on the spectrum), which denotes a beneficial cooperative effect between branches. However, although a marked increase in σ_2/n is observed for the G0 dendrimer, subsequent increase in branching is less beneficial with a σ_2/n saturating around 350 GM. It should also be noted that these interactions can be deleterious and degrade the response in 2PA. Such design strategies have allowed many research groups to synthesize chromophores capable of efficiently reaching the excited state by 2P irradiation. It is worth mentioning that several other advanced strategies allow the enhancement of the 2PA response, such as the preparation of dyes with a singlet diradical nature.[66,67]

Importantly, relaxation processes usually remain independent from the mode of excitation (i.e. 1PA or 2PA), and from the particular excited state reached upon absorption. It is therefore commonly accepted that 2P dyes follow Kasha's rule as they would in 1PA, and that the excited state energy can then be used for different applications, including photochemistry and uncaging with same rate constants and efficiencies. Anti-Kasha effects have been reported for a number of excited state phenomena, including bond-breaking reactions,[68] but remain uncommon in

2P uncaging (2PU) applications to this day. Thus, the above-mentioned guidelines have been widely applied to the design of PPGs sensitive to red/NIR light.

3. Photolabile protecting groups: structures, strategies and state of the art

Historically, the first application of light-induced bond cleavage was organic synthesis. In 1962, Barltrop[69] and Barton[70] both reported successively the first use of PPGs for the deprotection of carboxylic acids and amino-acids in peptide synthesis. In this early work, carbamate **13** is cleaved under UV light irradiation, triggering the release of deprotected glycine (Gly) along with the alcohol **14** as a PPG residue formed after a decarboxylation reaction **(Scheme 1)**. In synthesis, PPGs provide "greener" and milder alternatives than standard chemical deprotection, while being orthogonal to all standard protecting groups, and have been used for instance in total synthesis. This was exemplified by the investigation of J. Li *et al.* on the structure of (-)-Diazonamide A, which led them to prepare compound **16** by a chemical cascade triggered by the photodeprotection of **15**.[71]



Scheme 1. Early examples of photo-deprotection of glycine[69] and of phenol intermediate **15**[71] for synthetic purposes, and of ATP in biological applications.[72]

Thus grew the idea of preparing inactive photosensitive precursors of bioactive substances, and releasing them in vitro or in vivo upon irradiation. Schlaeger *et al.* with cyclic adenosine monophosphate (cAMP)[73] and Kaplan *et al.* with adenosine triphosphate (ATP)[72] were the first to study the release of bioactive compounds other than amino-acids for biological purposes with use of *ortho*-nitrobenzyl (*o*NB) PPGs (Scheme 1). This work introduced for the first time the figurative term of "caged" ATP for compound **28**, and of "uncaging".[72]

Photo-delivery of bioactive molecules is a very large field, and the term "cage" is sometimes deemed confusing and controversial, especially in comparison with non-covalent molecular cages and cavitands. Nonetheless, "uncaging" is now commonly accepted to describe a photo-induced bond cleavage, and even tends to spread beyond the scope of biological applications.

Controlling physiological phenomena is a challenging task with many constraints, and a PPG must meet a series of specifications to be applicable in biology **(Figure 6)**.

(i) The chromophore must absorb light efficiently. This efficiency is expressed by the absorption coefficient ε, or by σ_2 in the case of 2PE.

(ii) The excitation wavelength must be compatible with physiological media in order to avoid absorption of light by biological chromophores and potential photodamage.

(iii) The photolysis reaction must be clean. The masked ligand, noted Z in the rest of this section, must be released quantitatively (i.e. with a chemical yield close to 100%) and effectively (i.e. with high photochemical quantum yield Φ_u). The quantum yield of the photocleavage reaction Φ_u ("u" standing for "uncaging") is defined as the number of molecules released per 100 photons absorbed.

(iv) Compounds must be compatible with a photolysis in aqueous oxygenated solution. The chemically modified "caged" messenger, must be designed to be soluble in

physiological media, and depending on the application, it may be necessary to go through other cell barriers, or to bind to specific receptors (cancer cells, enzyme active sites etc...).

(v) In its caged form, the biomolecule must be inert in biological media, and stable "in the dark" (i.e. prior to irradiation) in this environment. Both the chemical intermediates and the photolysis residue must also be inert and non-toxic to the biological environment.

(vi) In biological media, the caged compound must regain its activity on a timescale compatible with the cellular process involved. This is particularly important in the case of neuronal applications in which the re-activation kinetics must be fast enough to generate a concentration peak in biomessenger, and activate the appropriate cell receptors. It must also be faster than the diffusion phenomenon (110-900 μs, depending on the nature of the medium, the cage and the ligand).[74]

Among these criteria, the hydrosolubility of such aromatic chromophores is one of the most critical points of development, along with the photosensitivity and uncaging efficiency. As such, the photocleavage efficiency of a PPG at a specific wavelength is dictated both by its capacity to absorb light (ε or σ_2), and by its intrinsic photochemical efficiency in a specific medium, i.e. its uncaging quantum yield Φ_u. Similarly to the definition of brightness for fluorophores ($\varepsilon\Phi_f$ or $\sigma_2\Phi_f$), the overall "photosensitivity" is hence determined by the product of both abovementioned parameters. The so-called "uncaging cross-section" is noted ε_u (expressed in $M^{-1}cm^{-1}$) for 1PE, and δ_u (expressed in GM) for 2PE, and expressed as:

$$\varepsilon_u = \varepsilon\ \Phi_u \quad (7)$$

$$\delta_u = \sigma_2\ \Phi_u \quad (8)$$

Since the first examples of photodeprotection in the 1970s, many families of cleavable compounds have been reported (Figure 6).[1–4] Applied successfully in optobiology until the

early 2000s, these early PPGs nonetheless proved to have insufficient 2P photosensitivity for practical use without tissue damage. As laser powers above the order of ten milliwatts are poorly tolerated by tissues,[75,76] high 2P photosensitivities δ_u are required in compensation, and a minimal threshold of 3 to 30 GM has been established to perform 2PU under physiologically compatible conditions.[2] Ogden *et al.* thus reported that photodamage to micrometric synapses could occur with average powers above 5 mW with 5 ms exposures. At this maximum power, using a pulsed laser of 200 fs and 76 MHz, and taking into account the diffusion coefficient a caged neurotransmitter molecule in water, they estimated that the minimum 2PU cross-section needed to achieve 50% steady-state photolysis was as high as 31 GM.[75] The recent years have been focused on the development of a second generation of PPGs based on such original structures with a 2PU cross-section falling in or beyond this range.[7–10]

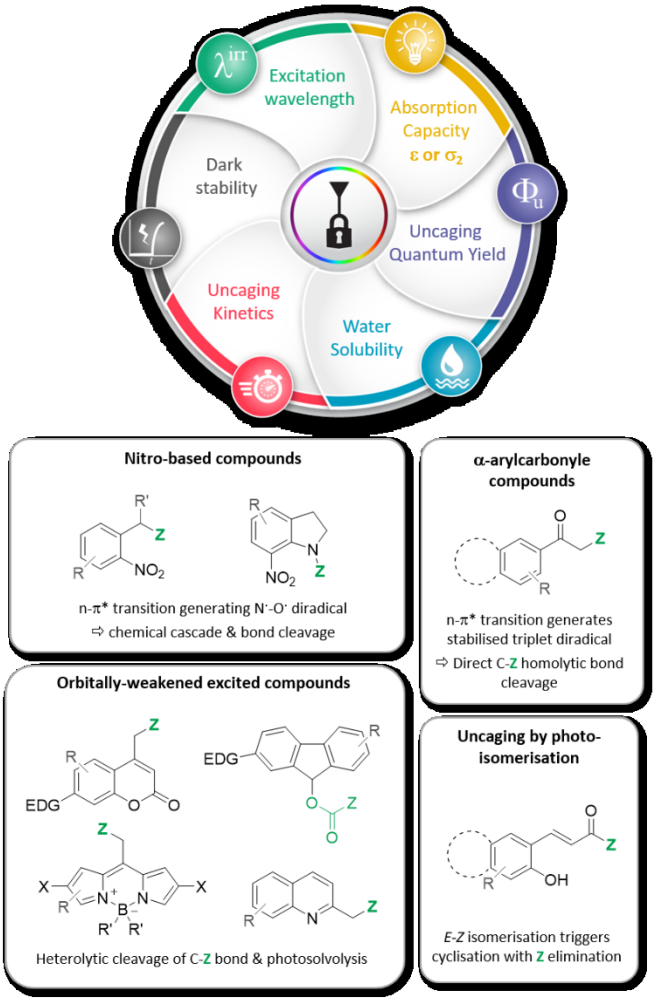
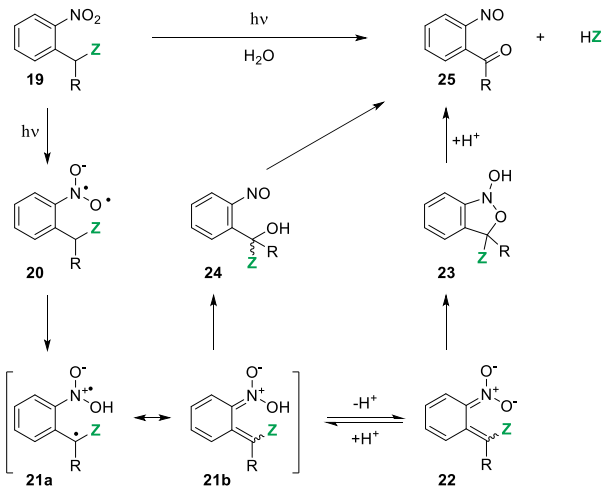


Figure 6. Summary of the specifications required for use of PPGs in biology, and of the main families of photosensitive moieties classified by type of mechanism. The Z group represents the biological substrate protected by the PPG (note that each PPG family can uncage specific chemical functions).

3.1. Nitro-based compounds: the nitrobenzyl family

Derivatives of the *o*NB family are by far the most widely studied. Initially developed for use in synthesis,[77] their application to biochemistry by Kaplan *et al.* for the release of ATP[72] in 1978 paved the way for several derivatives, many of which are now commercially available. The mechanism of release has been largely reported in literature **(Scheme 2)**.[1,78–80] The n→π* transition observed during the excitation of nitroaryls is often responsible for a decrease in the polarization of the bonds and the generation of radicals on the oxygen atom. In *o*NB derivative **19**, the formation of diradical **20** triggers a photochemical cascade leading to bond cleavage in the nearby benzylic substituent.[78] In the first step of this cascade, aci-nitro tautomers **21a** and **21b** are formed upon [1,5]-migration of an hydrogen radical from the *ortho*-benzyl substituent in **20**. The pKa of isomer **21b** as well as the nature of the R group then determine whether the cyclic intermediate **23** (for R = H) or the hemiacetal **24** (for R = alkyl) are formed. Both intermediates lead to the release of the caged substrate in a final rate-determining step.



Scheme 2. Photofragmentation mechanism reported for *o*NB derivatives in aqueous media.

The synthetic accessibility of *o*NB derivatives as well as their ability to protect a wide range of chemical functions (alcohols, phenols, thiols, phosphates, acids, or amines via a carbamate function) make them both user-convenient and versatile.[1,6] However, certain drawbacks have been highlighted, such as the toxicity of the nitroso byproduct **25**.[6] This derivative can also act as a "trap" for light radiation by internal filter effect, and react with the chemical species present in the medium, in particular endogenic amines. This reactivity was suspected in early reports by Kaplan *et al.* who described a quantitative chemical yield for the deprotection of inorganic phosphates, but a dramatic drop to 25% for the release of ATP.[72] Amino acids caged by *o*NB derivatives also have moderate stabilities to hydrolysis,[81] and exhibit slow activation kinetics. Katz and Dalva thus report that the release of glutamate by an *o*NB derivative only generates the action potential in synapses after 15 to 30 ms, which limits the applications in neurology.[82]

Early structural modifications aimed to overcome such drawbacks, but also to improve water solubility and absorption capacities **(Figure 7)**. As a general trend, substituents on the benzyl position tend to affect the uncaging quantum efficiency, while modifying the ring affects mostly the 1P and 2PA capacities. As such, the addition of a methyl at the benzylic position causes a strong increase of Φ_u for the release of phosphates[72] or acids.[83] The resulting 1P photosensitivity of **NPE-[Ac]** is more than 3 times higher than parent compound ***o*NB-[Ac]**. Many benzyl-substituted derivatives (phenyl,[84] nitrile[85]) were reported without necessarily improving the photolytic efficiency, but the α-carboxy-ortho-nitrobenzyl **CNB-[Glu]** notably improved solubility in biological media.[86]

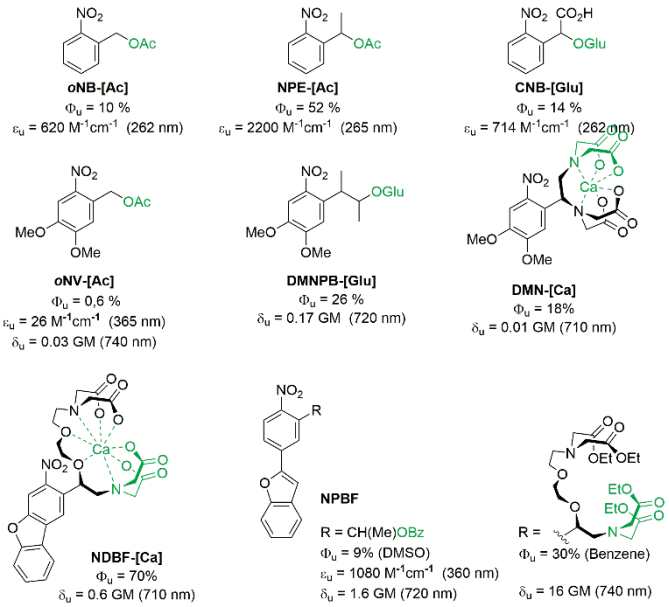
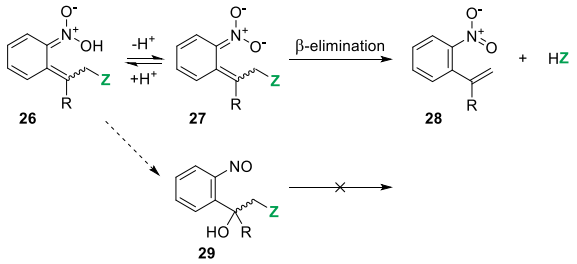


Figure 7. Structures and key photophysical properties of the first PPGs of the nitrobenzyl family cleaved by 1P or 2PE.

One of the most renowned compounds of the *o*NB family, *ortho*-nitroveratrile (*o*NV), contains two additional methoxy electron-donating groups on the ring. This increases ICT, causing a bathochromic shift of the absorption band by a hundred nanometres, and a higher oscillator strength. However, this change in the push-pull system is accompanied by a decrease of more than one order of magnitude of the Φ_u, as well as a lesser dark hydrolytic stability. Nonetheless, *o*NV remains one of the most widely used analogues of the nitrobenzyl family.

Subsequent studies postulated that replacing the benzyl group to a mono- or disubstituted homobenzyl would restore the uncaging efficiency.[87–89] In this sub-family of (nitrophenyl) propoxycarbonyls (NPPOC), **DMNPB-[Glu]**, directly derived from the veratryle derivative ***o*NV-[Ac]**, proved to release glutamate quantitatively in the same range of wavelength, with a Φ_u of 26%.[89] This improvement is partially rationalised by a modification of the fragmentation mechanism **(Scheme 3)**.[87] The deprotonation of the aci tautomer **26** leads this time to a β-elimination step yielding the *o*-nitrostyrene derivative **28**. The path leading to the

nitroso byproduct **29** ends up being a mechanistic impasse that does not lead to the release of the caged molecule.



Scheme 3. β-elimination mechanism occurring during the photofragmentation of *ortho*-nitrohomobenzyl derivatives.

Historically, *o*NB derivatives were among the first to be investigated under 2PE with lasers at the end of the 1990s for the photorelease of calcium ions,[90,91] followed by glutamate and acetic acid (Figure 7).[81] In this pioneer work, Tsien and co-workers report the first δ_u value of 0.03 GM for ***o*NV-[Ac]**.[81] Its homobenzyl analogue **DMNPB-[Glu]** later proved approximately 6 times more efficient when irradiated at the maximum of its 2PA band, however such photosensitivities remain very limited.[89] Limited 2P photosensitivities were also recorded for two photocleavable calcium chelating ligands, dimethoxy-nitrophene **DMN-[Ca]**[92] and nitrodibenzofurane derivative **NDBF-[Ca]**,[91] able to release Ca^{2+} ions in vitro upon ligand cleavage. An extended dibenzofuran backbone allows a 60-fold increase in 2PU cross-section in **NDBF-[Ca]** compared to **DMN-[Ca]** thanks to a large increase in Φ_u, but reported δ_u values remain below 1 GM. This class of compounds was later extended with 2-(4-nitrophenyl)benzofuran (NPBF) derivatives, which reportedly released a model carboxylic acid (benzoic acid) with a Φ_u of 9% in DMSO, and a model calcium ligand (ethylene glycol tetraacetic acid tetraethyl ester) with a Φ_u of 30% in benzene.[93] These results gave extrapolated δ_u values between 1.6 and 16 GM in their respective solvents, which highlights the importance of the leaving group and solvent in the photolysis reaction. NPBF has also

recently been used for 2P-photorelease of the 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) radical for targeted anticancer therapy.[94]

A new generation of π-extended *o*NB cages was developed over the last ten years by following the molecular engineering principles presented in section 2.3. Jullien and co-workers[85] were the first to report both π-extensions and tuning of the auxochromic moieties on *o*NB cages for the release of phenol and carboxylic acid derivatives **(Figure 8)**. Among relevant examples in their library of compounds, such modifications showed a poor influence on the 2PU properties. Substitution of the benzyl carbon with a bromine atom (**30**) induced a 2 to 3-fold increase in uncaging quantum yields and δ_u, while the nitrile derivative **31** proved inefficient. Interestingly, the stronger amino donor in compound **32** made the photolysis too slow to extract a Φ_u. In this work, the authors were also the first to extend the π-conjugated system of such cages, by preparing nitrobiphenyls (NBPs), but also stilbene and tolane derivatives **33** and **34**.

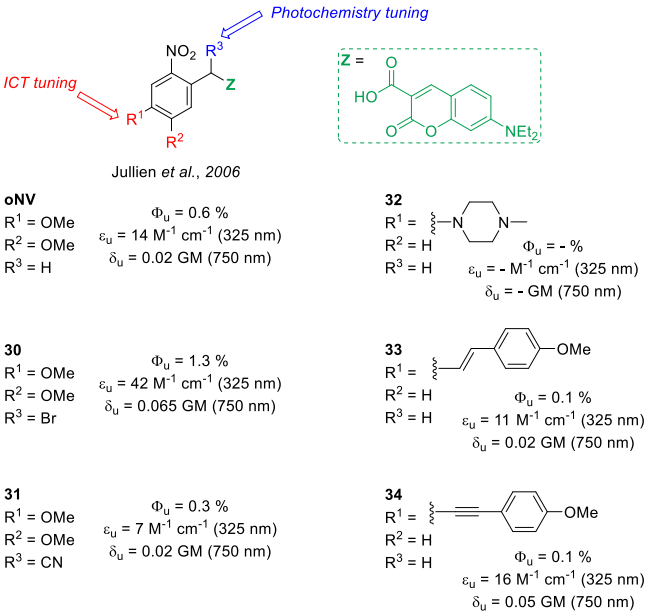


Figure 8. Examples of modified *o*NB derivatives reported by Jullien and co-workers for the photorelease of a caged fluorophore, and their photochemical properties in MeCN:TRIS buffer (1:1, v:v).[85]

Although the nitrodiphenylacetylene **34** proved the most efficient in the library, all δ_u values remained in the order of magnitude of the parent *o*NV. Later on, Boinapally et al. also reported extended oNB derivatives with a similar structure to styrene **33**, but locking the double bond inside a constrained dihydronaphthalene structure.[95] This resulted in a quantitative chemical yield for the photorelease reaction, and a Φ_u of 1% in MeOD. Following their work on the NPPOC family and its monomethylated analogue,[89] Goeldner and co-workers also explored this strategy and expanded DMNPB's π-conjugated system by synthesizing NBP derivatives.[96,97] The series of structural modifications can be summarised on a roadmap **(Figure 9)**. The first *para*-alkoxy biphenyl analogue **PENB-[Glu]** undergoes a significant drop in Φ_u, which is more than compensated by the expected increase in σ_2. A photosensitivity of 3.1 GM is obtained with this strategy. The methoxy donor can be replaced by a diethylene glycol chain to reach the solubility threshold of 5 mM required for use in neuroscience.[97] PENB retains the advantages of parent DMNPB while improving significantly the δ_u. In contrast, this strategy proved counterproductive in the case of the compounds **35** and **36**. Indeed, replacing the alkoxy donor with a stronger ED and hydrophilic phenol group creates competitive photochemical pathways in compound **35**, which released glutamate in only 10% chemical yield. Similar observations were made on the stilbene derivative **36** which released only 48% of glutamate.[96,97] Both compounds proved unsuitable for use as PPG for this reason. Similar modifications proved however beneficial in the case of the dialkylamino and nitrodiphenylacetylene derivatives **EANBP-[Glu]** and **NDPA-[Gib]**. Following the work carried out on PENB, Donato *et al.* further replaced the alkoxy donor with a stronger dialkylamino ED group on the NBP. This structural change made of **EANBP-[Glu]** the first PPG to break the 10 GM mark.[98] Reactivation of caged Glu occurs over a period of about 5 μs after the light pulse, and the two diethylene glycol chains on the nitrogen atom provide excellent 10 mM solubility in physiological media, which makes it compatible with

neuroscience experiments. The absorption band also undergoes an 80 nm bathochromic shift, both in the 1P and 2PA spectra. This shift was also reported in other aminobiphenyl PPGs used for calcium uncaging.[99]

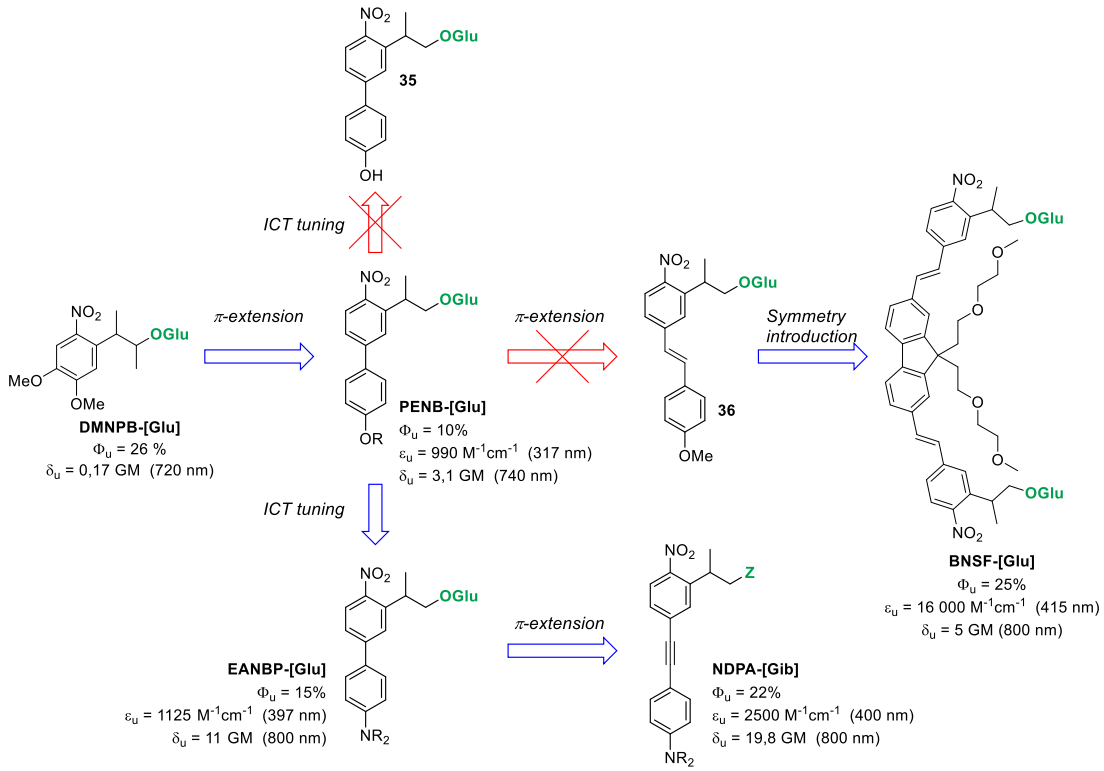


Figure 9. Roadmap of the structural modifications performed on NPPOC derivatives in order to optimise their 2PU cross-section. R = di(ethyleneglycol) ; Z = gibberellic acid.

Schelkle *et al.* continued the roadmap of structural modifications by increasing the conjugation length. Unlike compound **36**, the more planar and rigid triple bond in **NDPA-[Gib]** allows this time a significant improvement of both the Φ_u and the δ_u. With 19.8 GM for gibberellic acid (Gib) release, NDPA is still the most efficient 2P-sensitive PPG of the extended *o*NB family to this day.[100] Recently, Pham et al. pursued a similar strategy by investigating even more extend terphenyl cages.[101] Their methoxy and dimethylamino derivatives efficiently uncaged calcium ions under 2P excitation, with a σ_2 of 220 GM for the amino derivative, but 2PU cross-sections remained in the 5 GM range as none of their derivative combined both high σ_2 and Φ_u. Gug *et al.* reported an alternative approach by incorporating the photochemically sensitive

motif of NPPOC into a centrosymmetric A-π-A quadrupolar structure. The resulting compound, **BNSF-[Glu]**, is able to release two equivalents of glutamate with excellent 1P and 2PU properties, which validates the symmetry-based strategy. The high quantum yield Φ_u is however counterbalanced by a chemical yield of only 60%, in relation with the strong structural similarity between BNSF and compound **36**. Its solubility of 0.1 mM is also a limiting factor for use in neurons.[102]

3.2. Nitro-based compounds: the nitroindoline family

Since the 1970s, Amit *et al.* reported that *ortho*-nitroanilide compounds, and more particularly *N*-substituted 7-nitroindolines (NI) **(Figure 10)**, undergo a photosolvolysis in wet organic solvents resulting in the cleavage of the amide bond and the release of a carboxylic acid.[103] The 7-nitroindolines **37a**, **b** and 8-nitrotetrahydroquinoline **37c** were therefore described as potential PPGs for peptide synthesis. This class of compound was subsequently studied in depth by Papageorgiou and Corrie[104–108] who focused on the NI derivative, more effective than the tetrahydroquinolines analogues. Studying different substitution patterns of the aromatic cycle allowed them to significantly improve the photochemical properties for the release of amino acids in aqueous solution, with a focus on neurological applications. Ester **38**, which proved more water-soluble than **37a** and **37b**, was 2.5 times more effective than the compounds initially described by Amit *et al.*[104] Introducing a methoxy donor group at the 4-position of the heterocycle caused a further 3-fold increase in photochemical efficiency, yielding to the well-known **MNI-[Ac]**. Interestingly however, the dimethylamino donor in compound **39** suppresses all photochemical reactivity in indolines, supposedly because of a low-energy triplet excited state.[105]

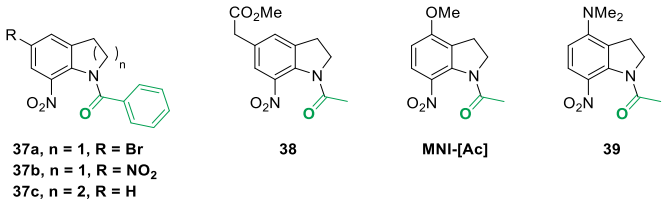
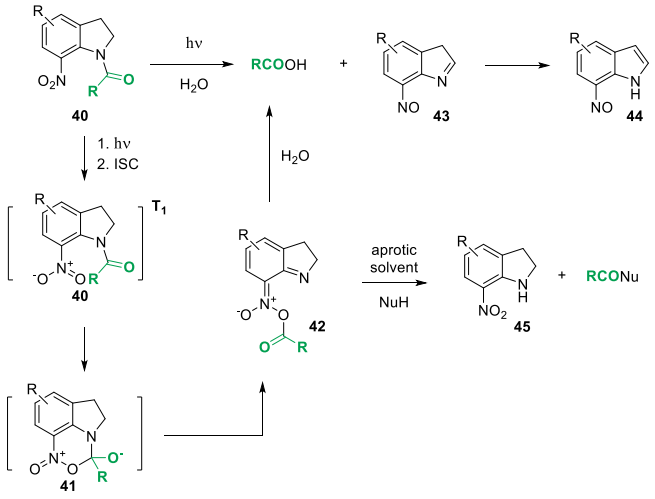


Figure 10. First examples of *ortho*-nitroanilides compounds studied for the photorelease of carboxylic acids by Amit *et al.* (**37a,c**)[103] then Papageorgiou and Corrie (**38**, **39** and **MNI-[Ac]**).[104,105]

On this occasion, they also reported that the photolytic mechanism in an aqueous media is no longer a solvolysis but a rearrangement[109] **(Scheme 4)**, which was supported by photolysis experiments performed in $H_2{}^{18}O$. In this sense, NI derivatives like compound **40** belong mechanistically to the category of nitro-based PPGs, like *o*NBs. The photochemistry of NIs occurs from a triplet state,[109,110] from which the acyl group of the amide migrates towards the oxygen of the nitro group at the *ortho* position via a cyclic intermediate **41**. Depending on the solvent, intermediate **42** can further react in two distinct ways. In water, deprotonation at the 2-position of the ring results in the release of the caged carboxylic acid and formation of nistrosoindolenine **43**, which slowly isomerises to its more stable indole form **44**. In organic solvents and in the presence of a nucleophile, the highly electrophilic acyl group of intermediate **42** undergoes a direct attack yielding nitroindoline **45**.



Scheme 4. Photofragmentation mechanism reported for NI derivatives in organic and aqueous media.

NIs are among the most widely-used cages in neuroscience. Unlike their nitrobenzyl analogues, aminoacids caged by NIs are stable in physiological media and released in quantitative chemical yield upon irradiation. Their kinetics are among the fastest, with a half-reaction time of only 200 ns. Above all, unlike the vast majority of available cages, NI-caged glutamates are perfectly inert towards glutamatergic receptors in synapses, and completely mask the neurotransmitatory activity of glutamate until release.[106] For all these reasons, the first 2PE experiments on indoline cages were successfully conducted on **MNI-[Glu]**,[111–119] showing a 2PU cross-section of only 0.06 GM at 720 nm **(Figure 11)**.[111] In this case however, the drawback of a low 2P-photosensitivity is compensated by the possibility of using very high concentrations of caged neurotransmitter without interference with the receptors. Trigo *et al.* further developed a 4-alkoxy bis-phosphate analogue of MNI called **DNPI-[GABA]**,[120] in an effort to improve water solubility, and reduce interference with GABAergic receptors by means of increased steric hindrance and ionic character . Several combinations of substituents have been attempted to improve the 2P photosensitivity of NI cages. Fedoryak *et al.* reported that, with a Φ_u reaching 47%, 4-methoxy-5,7-dinitroindolinyl glutamate (**MDNI-[Glu]**) was ten times as photosensitive as **MNI-[Glu]** under 1PE, but the 2PU cross-section was measured as identical to the parent compound (Figure 11).[121] The photolysis reaction of MDNI is also less clean, leading to a mixture of nitro and nitroso-type by-products, and a chemical yield in released species of only 70%.[108,121] Nonetheless, Ellis-Davies *et al.* developed **CDNI-[Glu]**, a carboxylate analogue with improved water solubility.[122]

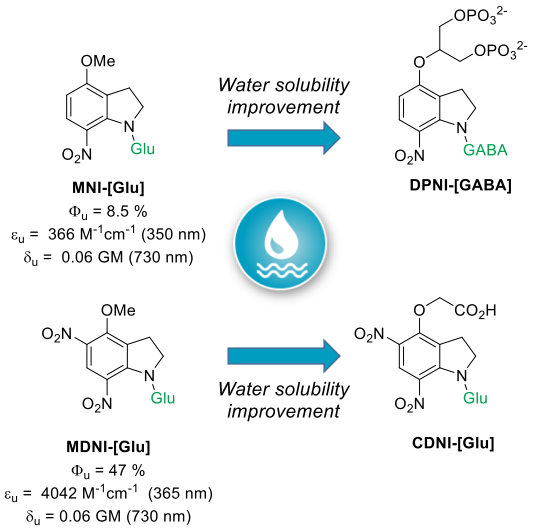


Figure 11: Structures of the most common 2P-sensitive NI-caged neurotransmitters.

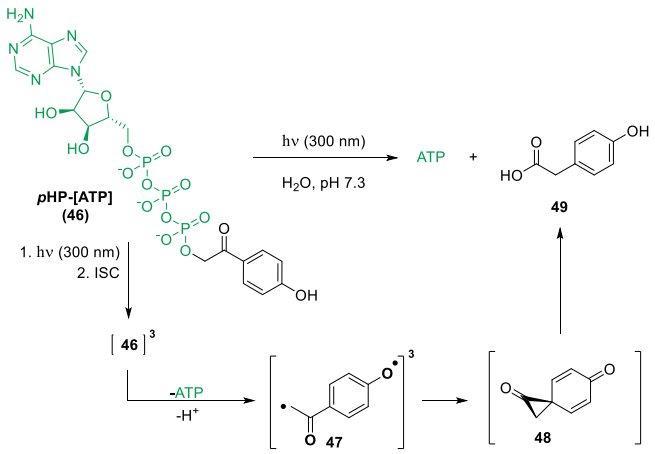
As the photochemistry of *ortho*-nitroanilides is extremely sensitive to substituents, the extension of the π-conjugate system has not been a popular strategy so far, and **MNI-[Glu]** remains by far, and despite its low δ_u, the most widely used reference in neuroscience for all the reasons stated above. These PPGs suffer however from a lack of versatility in terms of masked functions, and of synthetic accessibility because of poorly selective nitration reactions.

3.3. α-arylcarbonyl compounds: the phenacyl family

Although the first mentions of the phenacyl photochemistry date back to 1973,[123] this class of compound was introduced to uncaging by Givens *et al.* in the early 1990s to address some of the drawbacks of *o*NBs.[124] Actetophenone derivatives are synthetically accessible and present a photoreactivity based on their n→π* transition, which promotes either abstraction of a hydrogen atom, or of an electron from a donor molecule or neighbouring substituent. The photolysis mechanism and by-products thus depend strongly on the substituents present on the aromatic ring. The photochemical cascade leads to the release of the group located at the α-position of the carbonyl.

The first phenacyls used an unsubstituted acetophenone pattern and suffered from poor solubility in physiological media.[124] The introduction of a hydroxyl group at the *para*-position solved this problem while maintaining good photocleavage quantum yields. Givens and Park were the first to implement this structural modification on PPGs, which induces an equilibrium between the neutral *p*-hydroxyphenacyl (*p*HP) compound, absorbing at 282 nm, and its anionic form pHP^- at physiological pH. The superior donating capacity of the phenolate causes the appearance of a red-shifted absorption band at 330 nm. This PPG was able to release various phosphates, including cyclic adenosine monophosphate *c*AMP **(Scheme 5)**, in aqueous media with an uncaging quantum yield of 37%.[125] Further experiments were extended to other biological messengers, such as GABA and glutamate,[126] or peptides,[127] with quantum yields ranging between 12 and 40%. This pH-sensitive phenol-based strategy was later extended to other families of PPGs (vide infra).

The postulated photolysis mechanism in aqueous media (Scheme 5) involves the formation of a diradical in the triplet state **47** upon absorption of light followed by intersystem crossing (ISC). Thus, the active species is immediately released from the excited triplet state, which places phenacyl's kinetics among the fastest (10^9 s^{-1}), and makes them ideal for neurological studies. The remaining spirodiketone byproduct **48**, called "photo-Favorskii" intermediate, further hydrolyses to *p*-hydroxyphenylacetic acid (**49**),[128] whose absorption spectrum is very blue-shifted compared to PPG **46**. The PPG residue **49** is then completely transparent at the wavelength of the photolysis, which limits energy loss. A wide range of chemical functions can be protected with phenacyls.[129]



Scheme 5. Photofragmentation mechanism of ***p*HP-[ATP]** in aqueous buffer.

Despite such advantages, phenacyls have barely spread to 2PE techniques because of their deep-UV 1PA properties (280 nm in their neutral form). Houk *et al.* recently performed 2PA measurements on *p*HP-protected phosphates, showing a σ_2 value of 11 GM upon excitation at 550 nm **(Figure 12)**.[130] Therefore, the use of green laser excitation considerably limits further 2P applications. A mono- and di-methoxy derivative such as **50** effectively showed a 1PA band shifted to 350 nm, but reduced the Φ_u down to 3 or 4% respectively for the release of GABA via an ester bond. No 2PA measurements were performed in these studies.[131]

Despite a certain lack of versatility in the early structures of such derivatives, P. Singh *et al.* successfully incorporated phenacyl moieties within π-extended naphthalene[132] and carbazole rings (Figure 12).[133] In the "smart" naphthalene derivative **51**, glutathione activation in vitro switches on the ICT by cleaving the S-O bond and releasing SO_2. The resulting extended *p*HP derivative can be further activated by 1P and 2PE to release the anticancer agent chlorambucil via the same cleavage mechanism. The naphthalene skeleton hereby shifts the 2PE to the red, with a maximum σ_2 of 77 GM at 650 nm. This reasonable 2PA cross-section is combined with an excellent Φ_u, in accordance with related pHP derivative, to give a δ_u of 21 GM. This value is among the highest to date for any 2P-active PPG, and obtained after very limited structural modification. Interestingly, the uncaging quantum yield was reported to increase in low pH

environments. In a similar strategy, mono- and bisacetylcarbazoles with higher ED groups were prepared. Acetylcarbazole PPGs such as **52** are able to release respectively one or two similar (Z = Z ') or different (Z ≠ Z') substrates upon excitation. As in the case of standard phenacyls, it was postulated that photolysis takes place from the triplet excited state, but that the bond cleavage can be either heterolytic, or homolytic followed by electron transfer. Chemically quantitative photolysis was reported on a dozen of compounds, with quantum yields in the 10-15% range in aqueous buffer with 30% of acetonitrile. Such compounds have been designed and applied to anticancer therapy, but no 2P measurements were carried out so far.[134,135]

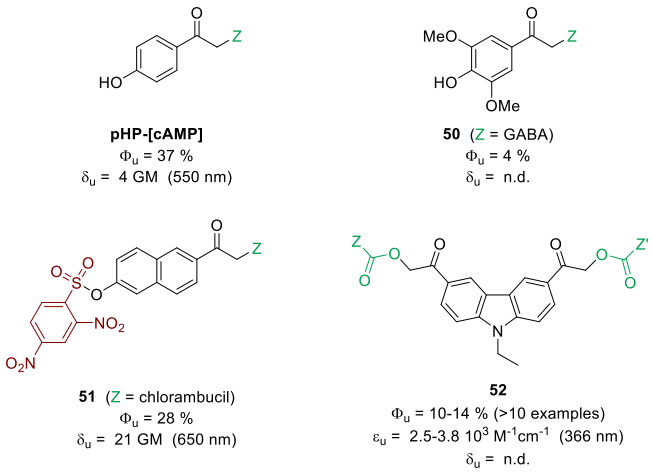


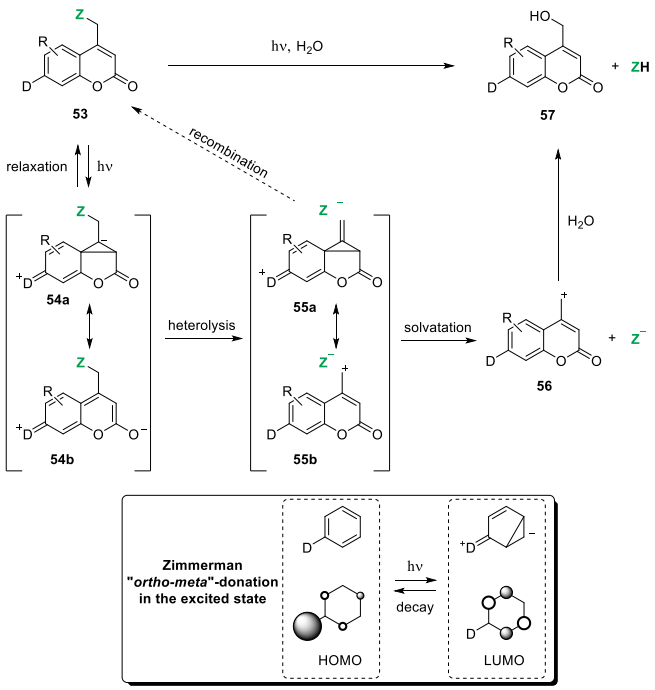
Figure 12. Structures of PPGs containing the phenacyl photosensitive unit and corresponding 1P and 2PU properties.

3.4. Excited-state weakened rings: the coumarin family

During the course of their investigations on light-induced formation of benzyl carbocations, Givens and Matuszewski showed in the 1980s that coumarin-4-ylmethylphosphates undergo a C-O bond heterolysis leading to the release of the phosphate and the formation of a by-product from the trapping of the carbocation.[136] Direct heterolytic bond cleavage in the excited state is related to two intertwined particularities of certain electron-rich cycles with *meta*- or "pseudo-*meta*" substitution **(Scheme 6)**.[137] First, upon promotion of an electron to the excited state, a substantial charge density is displaced from the ED group at the 7-position of

the ring to the 4-position, which ends up being strongly enriched.[81] Secondly, and as a result, while carbon C(4) possesses a very weak bonding coefficient in its ground state orbital, its anti-bonding contribution in the excited state is significant because of the charge transfer. Thus, when C(4) is substituted with an sp^2 benzylic carbon, the σ-antibonding orbital mixes with the chromophore's LUMO. Finally, if a leaving group Z is present in this position, the C-Z bond is weakened in the excited state, and cleaved with rate constants of the order of 10^9 to 10^{10} s^{-1}.[138] Such distinctive electronic attributes were first reported on *meta*-enriched benzyl acetates, and is commonly called "Zimmerman *meta*-effect".[137] This rationale has been used recently in the design of several new photosensitive moieties[139] such as BODIPY[140,141] of fluorenyl[142] derivatives, but coumarin-4-ylmethyls remain the most widely used in 2PE among them.

The resulting photocleavage mechanism[1,138,143] is then an excited state solvolysis (Scheme 6) occurring from the zwitterionic forms **54a**, representing formally the Zimmerman effect in the singlet excited state, and **54b**. The C-Z bond usually undergoes a heterolytic cleavage yielding an unseparated stabilised ion pair represented as **55a** and **55b**. Homolytic cleavage followed by electron transfer may also be observed in certain conditions, but should be disfavoured compared to an ionic mechanism.[144]



Scheme 6. Photo-solvolysis mechanism reported for coumarin-4-ylemethyl PPGs, and representation of the Zimmerman *meta*-effect occuring upon excitation of *meta*-enriched rings.

Competitive recombination of this ion pair may occur and inhibit the efficiency of the photolysis by reforming the starting material **53**. Solvatation will otherwise separate the ions, thus exposing carbocation **56** to nucleophilic attack. In aqueous media, **56** is trapped by water, to yield the hydroxymethylcoumarin byproduct **57**, along with the protonated molecule ZH. The overall mechanism can be assimilated to an S_N1 from the excited state. The photo-release efficiency is therefore highly dependent on the nature of the leaving group. The recombination step can be kinetically very competitive in the case of certain caged functional groups, such as phenols, that may also undergo photo-Claisen rearrangement.[145]

Years after the first report on coumarinylmethyl photosensitivity,[136] Furuta *et al.* developed the 6-bromo-7-hydroxycoumarin (BHC) analogue, and pioneered the field of 2PU by demonstrating for the first time utility of PPGs in 2P neurological experiments. The *ortho*-bromo phenol pattern lowers the pKa of the hydroxyl group to shift the equilibrium towards the phenolate form at physiological pH. In this ambivalent strategy, the anionic form promotes the

ICT and induces a bathochromic shift of the absorption band while improving strongly the solubility in water; while the more lipophilic neutral form is more likely to cross the lipid bilayers in biological media and provides increased stability for storage.[81] Moreover, as the triplet state is not unlikely to be involved in the photolysis mechanism, the bromine substituent increases the efficiency of the cleavage by promoting ISC via heavy atom effect. **BHC-[Ac**] was reported with a long-standing record 2PU photosensitivity of 2 GM **(Figure 13)**. Yet, amino-acids caged via their carboxylic acid functionality were shown to be slightly unstable to hydrolysis. This could be further improved by caging glutamate via a carbamate function on the α-nitrogen, which affected both the Φ_u and kinetics of **BHC-[Glu]**, and relegated the δ_u below 1 GM. **BHC-[Glu]** still remains a reference compound for the release of amino-acids to this day.

Many structural analogues of BHC, modified with different methoxy,[136,138] 6,7-dimethoxy,[146] amino[147] and dialkylamino[148–151] electron-donating patterns, were reported, which highlighted an important versatility in this family of PPGs (Figure 13). Changes at the 7-position were the most extensively studied in literature, and 7-diethylaminocoumarin derivatives (DEAC) have gained popularity for 1P and 2PU of neuro-messengers.[148–151] With such derivatives, Shembekar *et al.* reported uncaging of glycine with a Φ_u of 12%, more than three times higher than BHC, and with a 1PA band shifted to 390 nm. The neurotransmitter is reported to be biologically inert, and the half-reaction time is 2.5 μs.[151] The loss of solubility generated by the diethylamino group was solved with the use of two carboxymethyl chains in the work of Hagen *et al.* on 7-dicarboxymethylaminocoumarin (DCMAC).[148] The 2P photolysis of caged cyclic guanosine monophosphate (*c*GMP) is reported as about 1.3 times faster than **BHC-[Glu]** under the same conditions. No extrapolated δ_u value was determined considering the difference in leaving group. A sulfonated variant of DCMAC was also reported.[152] The extension of the π-conjugated system at the 7-position with an electron-

donating *p*-dimethylaminostyryl group was also examined.[153] A 2PA cross-section of 309 GM was measured at 800 nm for compound **58** (Figure 13), thus validating the expected effect on σ_2 as compared with DEAC. However, the uncaging quantum yield proved 60 times lower than the parent compound under the same irradiation conditions, which limits the δ_u value to only 0.24 GM.

Strong absorption shifts were recorded after chemical transformations on the endocyclic EW carbonyl (C(2)) of the lactone ring. Costa *et al.*[154] followed by Jullien *et al.*[155,156] first reported the thionation of DEAC into 7-diethylaminothiocoumarin (DEATC, Figure 13). Thiocarbonyls are known to provide strong bathochromic shifts due to lower energy transitions,[157] and the maximum absorption of DEATC was measured at 472 nm. The compound was used *in vivo* for orthogonal activation of Cyclofen at 488 nm.[156] The Φ_u of DEATC is, however, almost an order of magnitude lower than for unmodified DEAC, and suffers from bad photostability of its photolysis by-product.[155] The library of compounds synthesised by Fournier *et al.* in this work contains a number of green-absorbing analogues, including dicyanovinylcoumarins used since then by Marchán *et al.* in a photo-activatable RGD-targeted drug delivery system.[158] Although no 2P data was reported to date on C(2) modified compounds, it is worth mentioning that the 1PA curves can sometimes extend beyond 650 nm. This significant bathochromic shift was notably evidenced by Marchán *et al.* on C(2)-cyano-(4-nitrophenyl)-methylene derivatives[159] (Figure 13), but causes a dramatic drop in uncaging efficiency, with a Φ_u falling in the 10^{-6} range in methanol.

The 3-position of the coumarin backbone is another strategic position in terms of molecular engineering since it is at the enriched end of the π-conjugated system, and many commercially available dyes exploit this push-pull pattern. Ellis-Davies *et al.* were the first to extend the π-conjugated system of coumarinylmethyl PPGs in this position by adding an electron-withdrawing acrylamide moiety to DEAC.[160] The pronounced push-pull character shifts the

1PA band to 450 nm, which explained the acronym “DEAC450” given to the PPG. The 2PA band of this dipolar compound is also shifted to $2\lambda_{1PA}^{max}$, i.e. 900 nm. Notably, DEAC450 also shows a marked increase in uncaging quantum yield, reaching a value of 39% for carboxylic acids (Glu,[160] GABA[161]), and up to 78% for phosphates (*c*AMP,[162] *c*GMP[163]). A solubility of 0.25 mM in biological medium was reported after modulation of the solubilising units on the amide end (aspartate,[160,162] or oligoethyleneglycol[161]).

Further modifications at the 3-position were performed by Chitose et al. with the extension of electron-depleted[164] and electron-rich[165] benzene moieties. The best results were obtained with the dimethylaminobenzene derivative, reportedly thanks to a D-π-D character within the PPG conjugated structure, with a 2PA cross-section of 18 GM at 680 nm in toluene, and an uncaging quantum yield of 16% for benzoic acid release. Even higher σ_2 values may be reached at lower wavelengths for such derivatives.[165] Preserving the double bond extension present in DEAC450 and the dimethylaminobenzene ED group introduced by Chitose *et al.*, Lin *et al.* introduced styryl moieties at the 3-position.[166] Postulating an altered mechanism in which the carbocation is trapped by an enriched styryl double bond in an intramolecular cyclisation, the authors prepared a library of extended coumarin and thiocoumarin derivatives with absorptions shifted up to 515 nm and uncaging quantum yields reaching up to 70%. In spite of a lowered Φ_u compared to DEAC450, compound **59** proved the most efficient upon 2PE thanks to an enhanced ICT and an increased σ_2. Klausen *et al.* also investigated the effect of ICT on extended coumarins by introducing strongly EW thiazolyl-type moieties at the 3-position.[167] Interestingly, an inverse correlation between the EW strength and the Φ_u values was shown. However, introducing an intermediate fluorenyl π-bridge between the coumarin backbone and the EW extension increased dramatically both the σ_2 and the Φ_u thanks to a mitigated ICT strength. This possible correlation between the “space-density” of ICT and the uncaging quantum yield was supported by DFT calculations. Following this molecular engineering

strategy, compound **60** was the first PPG reported with a 2P-photosensitivity above 100 GM. Without any direct influence on the σ_2, other attempts have been reported to improve the Φ_u of coumarin PPG rather than its 2PA capacity. Grafting an extra methyl group on benzylic carbon is expected to provide stabilisation of the intermediate carbocation **56** during the course of the photolysis mechanism. First reported by Weis *et al.*,[168] this modification was further exploited by Specht *et al.* who replaced the methyl group with an oligoethylene glycol chain improving water solubility.[169] The Φ_u reported for the release of aminodoxycycline via a carbamate function is almost doubled compared to parent DEAC (21%), which is consistent with a stabilised carbocation, and increases the δ_u value to 4 GM.

To this day, coumarins are among the most widely used PPGs in neurobiology, but also in materials science,[170,171] or in therapy.[172–174] Coumarin cages po interesting release kinetics (of the order of 10^8 s^{-1}), and above all an exceptional degree of structural modularity. The quantum efficiency is therefore strongly dependent on the caged functionality; and very large disparities are reported between carbamates, acids, and phosphates, or even between two different amino acids.[150]

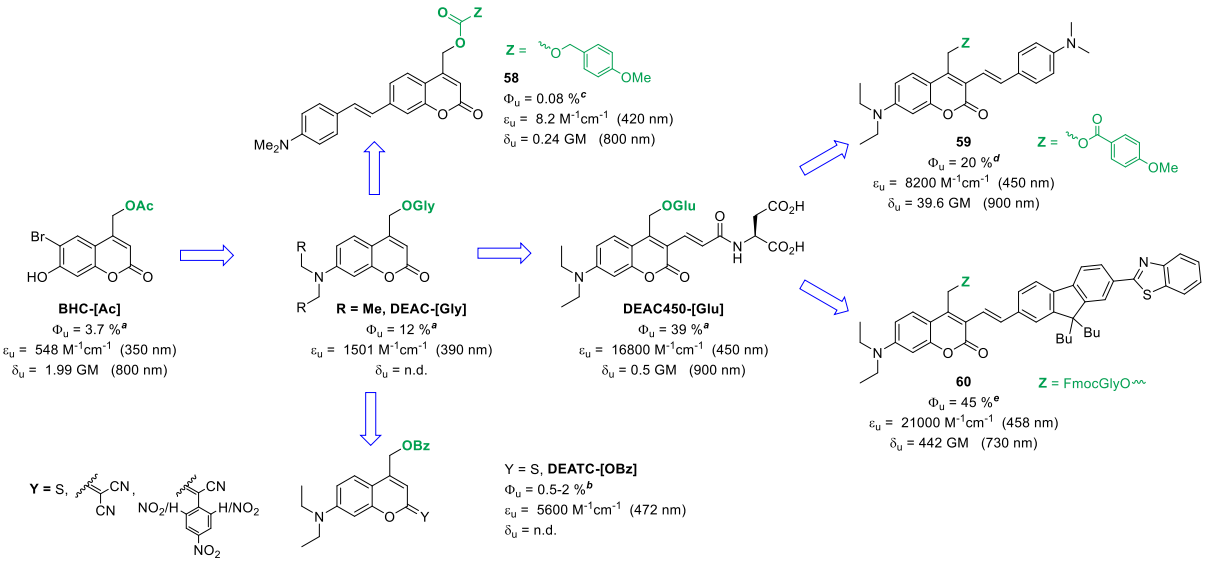


Figure 13. Roadmap of the structural modifications performed on the coumarin backbone, and their consequences on the 1P and 2PU properties. Values are reported for photolysis in [a]

phosphate buffer; [b] phosphate buffer with 50% MeCN; [c] phosphate buffer with 90% MeCN [d] MeOH:H_2O (9:1, v:v) [e] MeCN:H_2O (9:1, v:v).

3.5. The quinoline family

Following the first reports on 2P-sensitive coumarin PPGs,[81] a new family of heterocyclic cages based on a quinoline skeleton was reported. Fedoryak and Dore introduced the 8-Bromo-7-hydroxyquinoline (BHQ) PPG in 2002 for uncaging of carboxylic acid, with a δ_u of 0.59 GM at 740 nm **(Figure 14)**.[175] Carboxylates, phosphates and diols were successfully deprotected under the same conditions with δ_u ranging between 0.4 and 0.9 GM.[176] These early studies showed that the **BHQ-[Ac]** cage is cleaved more rapidly than its **BHC-[Ac]** analogue, and that both solubility and dark stability to hydrolysis are compatible with studies in a physiological media, although their dark stability is much lower.[175]

Although no evidence of *meta*-donation was reported in quinoline, the fragmentation mechanism was also first described as an heterolytic cleavage of the C-Z bond followed by excited-state solvolysis; which was supported by the incorporation of ^{18}O atoms into the byproduct upon photolysis in $H_2{}^{18}O$.[176] At first, this S_N1-type mechanism was postulated to occur from a singlet state, in which case the heavy atom effect implemented by the 8-bromo substitution would be detrimental to the photolysis efficiency. In order to increase the δ_u, other substituents able to lower the pKa of the 7-hydroxy group, as reported in previous PPGs, but unable to promote ISC to the triplet state were considered.[177] Structural modifications carried out in this study by Dore *et al.* in 2009 along with their effects on Φ_u and δ_u are summarised on Figure 14.

A chlorinated analogue of BHQ was prepared in accordance with this strategy, but surprisingly, the Φ_u was divided by 3. This decrease was first attributed to the concomitant increase in fluorescence, which is competitive towards photolysis, but eventually highlighted an important

role for the bromine atom of BHQ in the photocleavage process. Nitrile substitution caused a very strong decrease in pKa, thus shifting the equilibrium almost exclusively to the anionic form in physiological media. This modification preserved the Φ_u, and tripled the 1P photosensitivity, but did not affect 2PU significantly. Finally, an 8-nitro substitution completely quenched the photochemistry of the quinoline, presumably because of a low-energy triplet state. Recent studies by Dore *et al.* suggest that, within a library of 22 PPGs substituted at the 4-position, 4-aryl and electron-rich derivatives increase the δ_u to 2.6 GM while maintaining excellent uncaging quantum yields, with an interesting correlation between the photolysis efficiency and Hammett coefficients.[178]

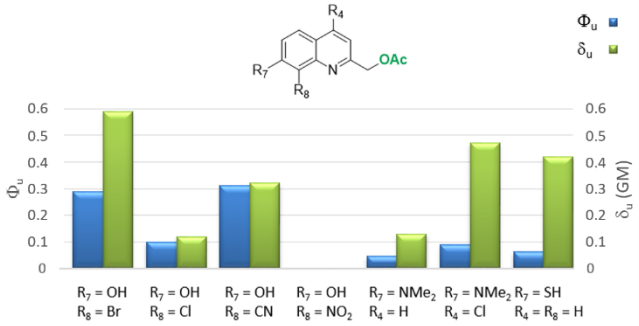
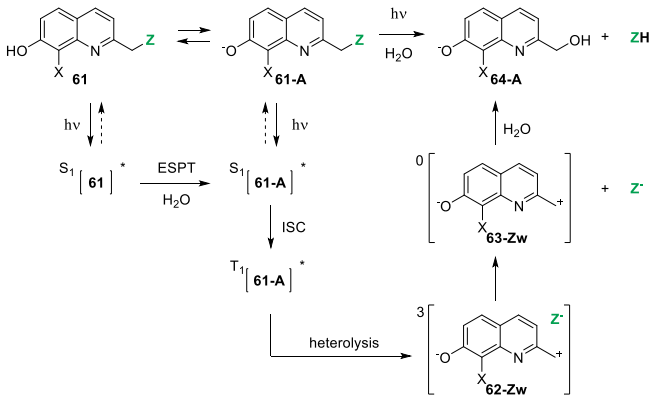


Figure 14. Evolution of the uncaging quantum yield (blue) and the 2PU cross-section (green) in substituted quinoline PPGs for the release of acetic acid.[177]

The ED capacity at the 7-position of the quinoline backbone was then modulated by replacing the hydroxyl group with dimethylamino or thiol groups. The effect on the 2PA response was positive, especially when a chlorine substituent was added at the 4-position, but strongly counterproductive on the uncaging quantum yield.[177] Out of all compounds studied in this library, BHQ retained the highest δ_u.

Thanks to this study and the comparison between bromo- and chloroquinoline, the photolysis mechanism of 8-halogeno-7-hydroxyquinolines could be elucidated **(Scheme 7)**.[179] It was thus reported to involve a transition to the triplet state of the phenolate **61-A**, either by direct excitation or by excitation of the neutral compound **61** followed by excited state proton transfer

(ESPT). Heterolytic bond cleavage then occurs from the triplet state of **61-A**, which induces the formation of zwitterion **63-Zw**. Trapping of the carbocation yields the photolysis byproduct **64-A** along with the released substrate. Photofragmentation takes place below the microsecond timescale.[176,179] It should be noted that the photolysis process of BHQ competes with an excited state dehalogenation reaction, which reduces the chemical yield of the deprotection reaction to 70%. This side reaction is not observed with the chlorinated derivative.[179] Since then, BHQ and 8-cyano-7-hydroxyquinoline have been used successfully for 2PU of biologically-active phenols, such as serotonin, octopamine or tyrosine.[180,181]



Scheme 7. Reported mechanism for the photo-solvolysis of BHQ derivatives.

Quinolines provide particularly relevant examples of 2P molecular engineering. Dalko *et al.* first focused on increasing the ICT in quinoline cages. In early work, positional isomers of Dore's 7-DMAQ[177] were investigated. The photolysis of 8-DMAQ isomer **(Figure 15 (a))** reached completion 6 times faster than 7-DMAQ in a TRIS/acetonitrile solvent mixture.[182] Under these conditions, **8-DMAQ-[Ac]** presents a δ_u of 1.7 GM at its maximum of 2PA, i.e. 700 nm **(Figure 15 (b))**. Based on this observation, further modifications have been performed in order to increase the ICT in the dipolar push-pull system,[183] but also to incorporate the photosensitive pattern within quadrupolar[184,185] or octupolar[186,187] symmetric systems (Figure 15 (a)).

In the dipolar strategy, an EW carboxylic acid was introduced on 8-DMAQ. Surprisingly, and in a similar way to coumarin-benzothiazolyl PPG **60**, although 5-carboxy-8DMAQ is 6 times less effective in 2PU than the parent compound 8-DMAQ under the same conditions, the introduction of an intermediate phenyl bridge eventually validated the strategy by tripling the value of the δ_u in the case of compound **65**.[183]

The design of quinoline-based centrosymmetric systems led to various outcomes. Quinoline dimer **66** showed slightly lower performance than 8-DMAQ alone, with only 0.40 GM at 730 nm.[184] The extension of the conjugation with a 2,7-fluorenyl core in compound **67** allowed a marked improvement of the δ_u,[185] however, it remains in the range of dipolar compound **65** which limits the benefit of this modification. Nonetheless, it should be noted that these values were evaluated by comparative photolysis at 730 nm. Yet, as observed on the 2PA spectrum of compound **67 (Figure 15 (c))**, and in accordance with the parity selection rule, the maximum of the 2P-allowed band in these centrosymmetric compounds is located at shorter wavelengths, with a σ_2^{max} value of 25 GM at 690 nm for **67**.

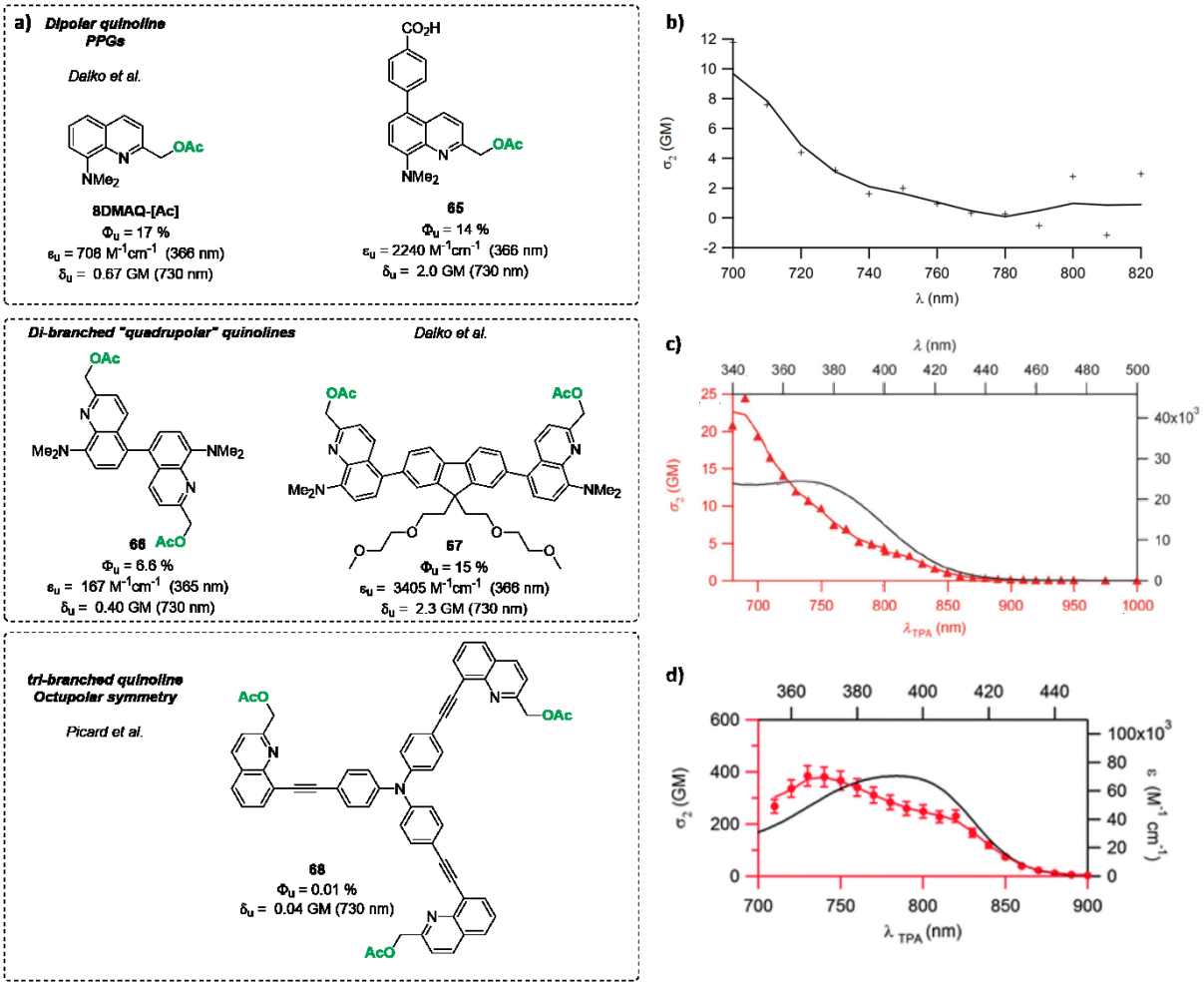


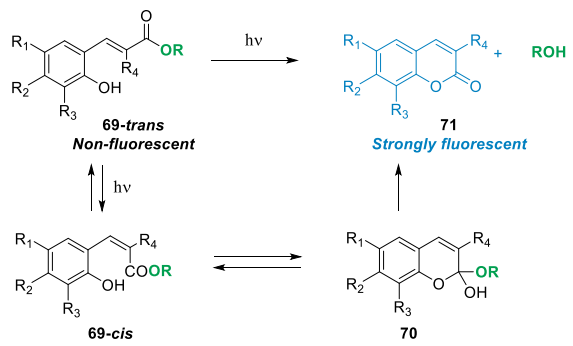
Figure 15. a) Structures of dipolar, quadrupolar and octupolar quinoline derivatives and the resulting effect of the modifications on the 2PU properties. b) 2PA spectrum of dipole 8DMAQ-[Ac] in MeCN/Tris buffer (1/1). Reprinted with permission from ref. [182]. Copyright 2012 American Chemical Society. c) 2PA spectrum (red) of quadrupole **67** in MeCN. Linear absorption spectrum (black) is given for comparison. Reprinted from ref. [185] with permission from John Wiley and Sons. d) 2PA spectrum (red) of octupole **68** in THF. Linear absorption spectrum (black) is given for comparison. Reprinted from ref. [187] with permission from The Royal Society of Chemistry.

Octupolar compound **68** reported by Picard *et al.* was designed as a combination of three quinoline moieties coupled at the C(8) positions to a triphenylamine group via ethynyl bridges. Although the σ_2 of **68** reached 390 GM at 730 nm **(Figure 15 (d))**, the uncaging quantum yield was reduced by several orders of magnitude, which considerably limited the 2P photosensitivity, and proved to be an example of counterproductive molecular engineering.[187] As such, reports show that the ethynyl bridges used in multipolar quinoline-based compounds tend to impede

the photochemical efficiency.[185,187] Similar quinoline trimers synthesised by Dalko *et al.* were indeed reported with higher δ_u values between 0.5 and 2 GM without triple-bond linkage.[186]

3.6. Uncaging via photo-isomerisation: the 2-hydroxycinnamyl family

Ortho-hydroxycinnamic (*o*HC) esters were introduced by Porter and co-workers in 1988 for reversible light-induced protease inhibition.[188] In this early work, this is achieved by photo-cleavage of ester bonds in synthetic inhibitor compounds, which restores the enzyme activity. Uncagers of the *o*HC family hold several interesting features, such as good cell permeability due to a certain hydrophilic/lipophilicity ambivalence.[189] The peculiarity of their fragmentation mechanism also lies in the formation of a highly fluorescent coumarin dye **71** during the course of the photorelease **(Scheme 8)**, starting from a non-fluorescent cinnamic acid derivative. This is explained by the stepwise formation of the **69-*cis*** isomer upon excitation, which enables spontaneous intramolecular cyclisation followed by elimination of the alcohol from hemiester **70**. This generation of luminescence opens the door to convenient photolysis monitoring, and to theranostic applications. Yet, *o*HC compounds suffer from slower activation kinetics than other derivatives (10^{-2} s^{-1} range),[189] which is their main limiting factor. It should be noted that the thermal cyclisation step can be accelerated when using 2PE because of localised heating at the focal point, which makes the isomerisation step rate-determining.



Scheme 8. Reported mechanism for the photorelease of alcohols with *o*HC derivatives involving sequential *E-Z* photo-isomerisation and lactonisation.

The influence of substituents on the π-conjugated system of the *o*HC platform was studied by Jullien *et al.* for photodeprotection of model alcohols (EtOH, BuOH).[189–191] Compound **72** **(Figure 16)** is another example of the abovementioned bromophenol pattern. Indeed, the 3,5-dibromo-4-hydroxy electron-donating motif shifts the excitation band of the compound to 350 nm at physiological pH, while increasing its water solubility via the phenol/phenolate equilibrium. Without significantly altering the Φ_u, the increase in electron density on the aromatic ring (**73** and **74**) boosts the ICT, and consequently the δ_u, which reaches 4.7 GM for compound **74**. A bis-carboxylated analogue of compound **74** has been reported since then with a Φ_u of 18% and a solubility in the millimolar range in aqueous buffer, but no 2PU experiment was carried out.[192]

Substituents on the cinnamate double bond have a significant effect on the kinetics, which is doubled in the case of the compound **75**.[191] However, such derivatives sometimes proved unstable at room temperature because of a spontaneous lactonisation triggering the alcohol release.[189] Steric hindrance can also be the cause of hypsochromic shifts of the absorption band due to a loss of planarity.

The extension of the π-conjugated system of *o*HCs has been reported on several occasions. Replacing the phenol ring with a naphthol (**76**) caused a very strong increase in photoisomerisation quantum yield, but the δ_u did not increase accordingly. Recently, Paul *et al.* also extended the π-conjugated system of *o*HC derivatives with an EW heterocycle (R_1 = Me, R_3 = 2-benzothiazolyl).[193] The resulting cage was used for 1PU of methylsalicylate in HeLa cells, with an estimated quantum yield of 10%, without performing 2P measurements. Interestingly, P. Singh *et al.* fused the *o*HC pattern with a carbazole heterocycle,[194] as they previously reported for phenacyl derivatives.[133] All reported derivatives showed an uncaging

quantum yield in the 4.5% range in a water/acetonitrile mixture (7:3, v:v). The sequential alcohol release upon irradiation of compound **77** can be monitored in a first instance by an increase in its green emission intensity, then by a blue shift of the emission band due to reduced ICT in the final fused-biscoumarin by-product. Subsequent z-scan 2PA measurements showed that the 2P photosensitivity of all derivatives was in the 0.4 GM range. The values are about an order of magnitude lower than for amino derivative **74**, but different photolysis media and measurement techniques prevent direct comparison.

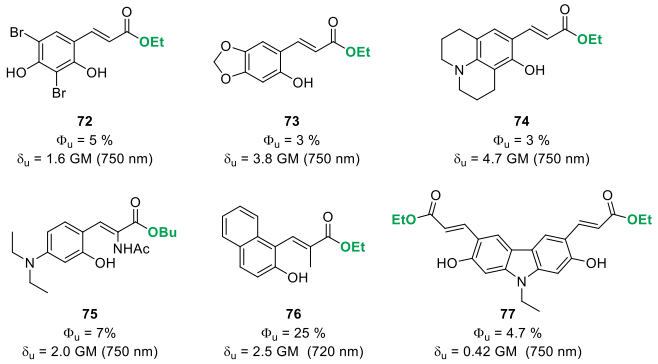


Figure 16. Structures and photochemical properties of *o*HC compounds caging model alcohols.

Eventually, compound **72** was used in zebrafish embryos under 2PE at 750 nm,[190] which validated the use of *o*HC cages *in vivo.* Several key advantages were highlighted in this family of PPGs for biological applications, however, they are exclusively suited for the release of alcohols, and for the study of physiological phenomena compatible with slow substrate release.

3.7. Other photosensitive structures

Although the literature surrounding 2PU is focused almost entirely on the above-mentioned structures, a multitude of other photo-cleavable moieties have been reported.[1] In the midst of organic PPGs, it is worth mentioning that the ruthenium (II) bipyridine complex RuBi, developed in the mid-2000s,[195] is able to release one of its amine ligands by excitation in its

metal-ligand charge transfer (MLCT) band, located at 450 nm and under 2PE at 800 nm in neurons.[196] Comparative photolysis experiments showed that **RuBi-[Glu]** triggered the action potential twice faster than **MNI-[Glu]**, with a δ_u of 0.14 GM. Bond cleavage is completed in less than 50 ns, which places the complex among the fastest cages available.[196] Pyridinium moieties were also reported to undergo photochemical cleavage,[197] and were used in an antenna-sensitized 2PU system.[198] It is worth highlighting that several more photocleavable structures[1] including BODIPY,[140,141] fluorenes,[142] bimanes,[199] hydroxyinden-3-ylmethyls,[200] xanthenes[201] and methylene blue derivatives[202] have been reported in literature, but have neither been developed for nor tested under 2PE, which opens more avenues in terms of molecular engineering of 2P cages.

3.8. Antenna-sensitized uncaging

Rather than optimising the photochemical properties and uncaging performance by direct structural modification of the PPG backbone, the light absorption capacity can be indirectly improved by introducing a second chromophore acting as an energy-harvesting antenna. The design of highly efficient 2P-absorbing antennas following the rules described in section 2.3 thus bypasses the issue encountered during the direct modification of PPGs. The energy of the antenna in its excited state must be transferred to the photochemistry module via an appropriate photophysical sensitization process such as Förster Resonance Energy Transfer (FRET), photo-induced electron transfer (PET) or triplet-triplet energy transfer (TT-ET).[1,203] Although beyond the scope of this review, several multi-chromophoric systems for 1P[110,204,205] and 2PU[198,206–208] have been reported and used successfully by following this alternative strategy.

4. Biological applications and underlying challenges

The intrinsic advantages of 2PU can be grasped by considering the spatio-temporal dimensions involved in physiological processes. Indeed, upon light excitation, ligand-receptor interactions must be reproduced artificially with utmost precision and specificity, and in this sense, 2PU applications all have different requirements. A wide panel of biological functions can be considered, and as a full overview of this domain goes beyond the scope of this manuscript, this section will focus on two leading applications: the controlled release of neurotransmitters and of drugs.

4.1. Uncaging of neurotransmitters: optoneurobiology

The nervous system consists of a network of polarized neuronal cells, carrying information through chemoelectric activity. Nerve impulses are converted to chemical message and back via small organic molecules acting as neurotransmitters. The challenge of 2PU in optoneurobiology is to mimic artificially the mechanics of a chemical synapse.[209] This implies a photolysis performed on the millisecond scale, and the release of thousands of neurotransmitters in the sub-micrometric synaptic cleft to cause the required millimolar concentration jump and trigger the action potential.[210,211] In this sense, the excitation volume of focalised lasers is perfectly adapted since the beam can be modelled by two Gaussian widths at half axial and lateral half-length respectively of the order of 1 μm and 0.30 μm.[111] Synapses can be excited individually by the laser,[112] which greatly facilitates the study of molecular and biological dynamics.

To preserve the three-dimensional resolution at the cellular level, both diffusion and photolysis kinetics are critical points to consider since the spatial confinement can be lost when the diffusional exchange is faster than the photolysis reaction itself. To that end, the excited

intermediates must remain concentrated within the irradiated volume and complete the photolytic reaction before they are replaced by a caged neuromessenger in the ground state; which implies rate constants greater than 10^5 s^{-1}.[212] The kinetics of the photolysis is also a preponderant factor to prevent potential photo-desensitisation of the neurons by prolonged laser pulses. The size of the PPG also bears important implications, as small objects will be more compatible with the sub-micrometric synaptic cleft. Finally, mimicking the concentration jump of a chemical synapse implies that the caged neurotransmitter must be soluble at high concentrations of the order of 1 mM in physiological media.

Examples of caged neurotransmitters, hormones and neuromediators, or calcium ligands developed for neuroscience are legion, and some of them are now commercially available. RuBi-caged glutamate,[213] or **MNI-[Glu]**[111–119] are now commonly used in neuroscience for the mapping of nerve pathways that can be chemically identified by the presence of neuromodulators,[111,115] for structural study of neurons and their activation sequences,[112,113] or for the study of synaptic plasticity.[119,214] Optoneurobiology has been a major driving application in the research of new high-performance 2P-sensitive structures. Important work has also been carried out to develop wavelength-selective "orthogonal" PPGs for multimodal photo-activation,[215] or to reduce the interaction of caged neurotransmitters with synaptic receptors.[216]

4.2 Uncaging of drugs: targeted therapy

In drug-delivery applications, the time, space and performance scales involved in cellular responses are somewhat less demanding as the main objective is to reduce off-target effects. However, other key factors such as dark stability, clearance and biological barriers must be

taken into account. Photoactivation can be performed either at the molecular level with so-called “pro-drugs”, or with the use of light-triggered nanomedicine.[217,218]

Cytotoxic anticancer agents have been caged with different 2P-sensitive PPGs,[133,172] and sometimes incorporated in molecular theranostic system.[158,219] No direct 2P experiments have been reported in either case, but such structures are known to be 2P-sensitive (*vide supra*). Recently, DEAC has been used at the nanoscale level for the photorelease of chlorambucil and doxorubicin, respectively with silica[220] and gold nanoparticles upon 2PE.[221] Aminocoumarin derivatives have also been incorporated in the structure of IR-disrupted micelles[222] and nanocomposites[223] for cargo release.

5. Summary and Outlook

The design of organic dyes compatible with the range of wavelengths of the “biological transparency windows” is arguably one of the most prevalent challenges in optobiology nowadays. The intricacy of such work further intensifies in the case of PPGs. Shifting the 1PE band of such light-cleavable moieties to biologically compatible wavelength is possible, but highly challenging. So far, only a couple of red-absorbing BODIPY[141] coumarin[159] and phenothiazinium[202] PPGs have been reported, and unfortunately, such massive bathochromic shifts often come at the cost of reduced bond-cleavage efficiencies and quantum yields. In addition, the tridimensional resolution of the excitation, and therefore the precision of the photo-activation, also remain very limited. The non-linear nature of 2PA bypasses this drawback, while providing easy access to red and IR wavelengths. Highly responsive 2P dyes are now commonly accessible via the molecular engineering strategies described above. Reports on PPGs with 2PA cross-sections of several hundreds of GM are now common, and δ_u records have increased by orders of magnitude over the last few years, from BHC (2 GM)[81]

to NDPA (20 GM)[100] and extended coumarin derivatives (440 GM).[167] Following the seminal work carried out on the *o*NB family, coumarin PPGs have lately appeared as the most promising and popular dyes thanks to their versatility, and may open up even more avenues to both specialists and newcomers to the field. Other original designs have been reported recently with the incorporation of the less-common phenacyl and *o*-hydroxycinnamate families into more conjugated structures. This strategy instigated new prospects for these families of dyes, and holds great potential for future research. In addition, a dormant potential resides in the number of new photosensitive structures that were reported without focus on 2P applications, and further collaboration between research groups should be encouraged in order to investigate their response under non-linear excitation.

However, many challenges remain, as the optimisation of PPGs to 2PE is a viable but tedious strategy. Extensions of the π-conjugated system – and therefore the molecular weight – of the dyes often lead to poor water solubility, and excessive modification may thus be counterproductive. In this regard, although size and symmetry empirically show an increase in 2PA, the ratio of σ_2^{max} to molecular weight of dyes rarely exceeds 1 or 2. Large PPGs are also more expensive and difficult to synthesize, less atom-economical, and less suited to certain applications. Furthermore, many examples relate an unpredictable Φ_u parameter upon structural modification. Such considerations must be kept in mind, and the key is eventually to find the optimal balance between performance, size, and practicality.

Two-photon uncaging remains a young but quickly expanding field, and exciting opportunities may arise if certain barriers are broken down by future research. Any work towards a rationalisation of the uncaging quantum yield in different PPG families, and towards a consensus over the measurement techniques used for δ_u – which still prevents direct performance comparison in some cases, would be greatly beneficial to the field. In addition, the translational potential of such probes is considerable but not entirely fulfilled, and the eye of all

communities involved in their development is of utmost importance for further progress towards high clinical relevance. New modular and point-of-care laser sources are being developed for more clinically friendly use of 2PA; and future progress in this domain, combined with the development of 2P-sensitive caged bio-modulators, may open new exciting avenues in biology and light therapies.

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