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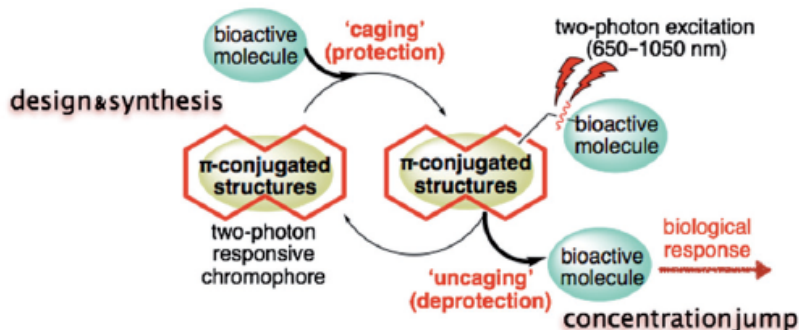
Design and Synthesis of Two-Photon Responsive Chromophores for Near-Infrared Light-Induced Uncaging Reactions

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Dedicated to Professor Herbert Mayr on the occasion of his 70th birthday

Abstract Near-infrared two-photon (TP)-induced photorelease (un-caging) of bioactive molecules such as drugs has attracted considerable attention because of its ability to elucidate mechanistic aspects of biological processes. This short review summarizes recent developments in the design and synthesis of TP-responsive chromophores.

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Key words caging reactions, uncaging reactions, photolabile protecting groups, two-photon absorption, time-dependent density functional theory

1. Introduction

In general, two principle methods are used to investigate the role of biologically active compounds and their associated mechanisms in biological processes: (1) imaging, and (2) caging & uncaging. Fluorescence microscopic techniques that use biological fluorescent stains, immunofluorescence, and fluorescent proteins can be utilized to generate real-time images of life phenomena, from which the active sites and functionalization of bioactive compounds can be clarified.¹ This

method is widely employed in biological and physiological studies.²

Another technique used to investigate *in vivo* activity involves concentration jumps of bioactive molecules.³ Since biological responses induced by such concentration jumps ceases within the micro- to millisecond timescale, spatio-temporal control of the release of bioactive molecules is necessary in physiological studies. Approximately 40 years ago, Schlaeger,⁴ Hoffmann⁵ and their co-workers, who were biochemists, developed a 'caging & uncaging' method for bioactive substances (Figure 1), in which the bioactive molecules are temporally inactivated (caged) by photolabile protecting groups (PPGs)^{6–11} and re-activated (uncaged) by flash photolysis of the caged compounds. For this reason, the meaning of 'caging & uncaging' is not derived from the shape of molecules, rather it signifies the 'masking (inactivation) & unmasking (activation)' of biologically active substances such as therapeutic drugs.

To date, several types of PPGs have been used in physiological studies investigating the phenomena of life (Figure 2), which can be categorized into two groups based on the photorelease uncaging mechanism: (1) Those in which the release is the consequence of an intramolecular rearrangement (nitrophenyl, or cinnamyl-type PPGs), and (2) PPGs that are cleaved directly by SN1-type solvent-assisted photolysis; the latter offering inherently faster release. The orthonitrobenzyl-type PPG (o-NB)^{12–14} was the first PPG in the 'caging & uncaging' technique in physiological studies.

However, this PPG has the potential to thermally hydrolyze at the benzylic position to produce bioactive substances during such studies. To avoid such a possibility, Specht, Goeldner and co-workers used an *ortho*-nitrophenethyl (*o*-NPE)^{15–18} PPG for their physiological studies. The PPG was developed by Hasan

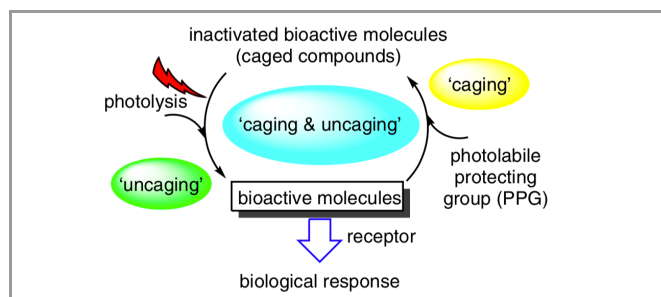


Figure 1 Concept of “caging & uncaging” of bioactive molecules

and co-workers.¹⁵ Nitro-substituted indoline derivatives^{19–24} have also been used for the uncaging reactions.

Givens and co-workers developed a photo-SN1-type of uncaging step using coumarin chromophores (Figure 2).^{25,26} Quinoline derivatives^{27–30} have also been used for the SN1-type photorelease of drugs. Kakiuchi and co-workers have discovered a novel chromophore employing a thiochromone for the uncaging step.³¹ Very recently, new visible-light-responsive PPGs with fluorescent character, i.e., BODIPY and bimeane, were developed by Smith, Winter, Klán, and Singh.³² In this case, acids [X = –OC(O)R] can be released by the visible light. Furthermore, Marchán et al. developed new coumarin-derived chromophores (DEDCC) having visible-light responsiveness.^{32d,e} Jana, Singh, and co-workers used a carbazole (CBZ) unit for the release of single and dual carboxylic acids.^{32f}

After the pioneering studies by Schlaeger and Hoffmann in the late 1970s, the ‘caging & uncaging’ technique has become a general tool for physiological studies, including neurological science, cell biology, gene expression, and structural biology. In the mid-1990s, the second generation of ‘caging & uncaging’ technology emerged, employing a two-photon (TP) excitation process with light at ~700–750 nm. Note that living tissues are almost transparent (low absorption) to light from the near-infrared (NIR) region, i.e. between 650–1050 nm. Denk,^{33,34} Lipp and Niggli,³⁵ Furuta and Tsien,³⁶ and Ellis-Davies and Kasai³⁷ were the contributors to this revolution, which provided this next generation of ‘caging & uncaging’ technology based on NIR-TP irradiation. For *in vivo* studies, caged compounds in general should be water-soluble and non-toxic. In addition to the basic conditions, it is becoming essential to have large TP responses from the chromophores.

The physicist Maria Göppert-Mayer laid down the theoretical foundation of the TP excitation process in 1931,³⁸ in which two photons are absorbed simultaneously by the same molecule. This is illustrated for Ar–X in Figure 3. Thanks to its strong one-photon (OP) absorption band at 400 nm (3.10 eV), the molecule can be electronically excited by irradiation at 400 nm to generate the corresponding excited state [Ar–X]* (S1) (Figure 3). Of course, the excited state molecule [Ar–X]* cannot be generated using an 800 nm OP excitation process, because the molecule has a vanishing OP absorption at 800 nm (1.55 eV), that is, the absorbance is zero at 800 nm (Figure 3). However, using ultra-short pulses with high light intensity (typically via a femtosecond pulsed laser), molecules having TP absorption character can absorb simultaneously two photons, in this case at 800 nm, to generate the same molecular excited state [Ar–X]*.

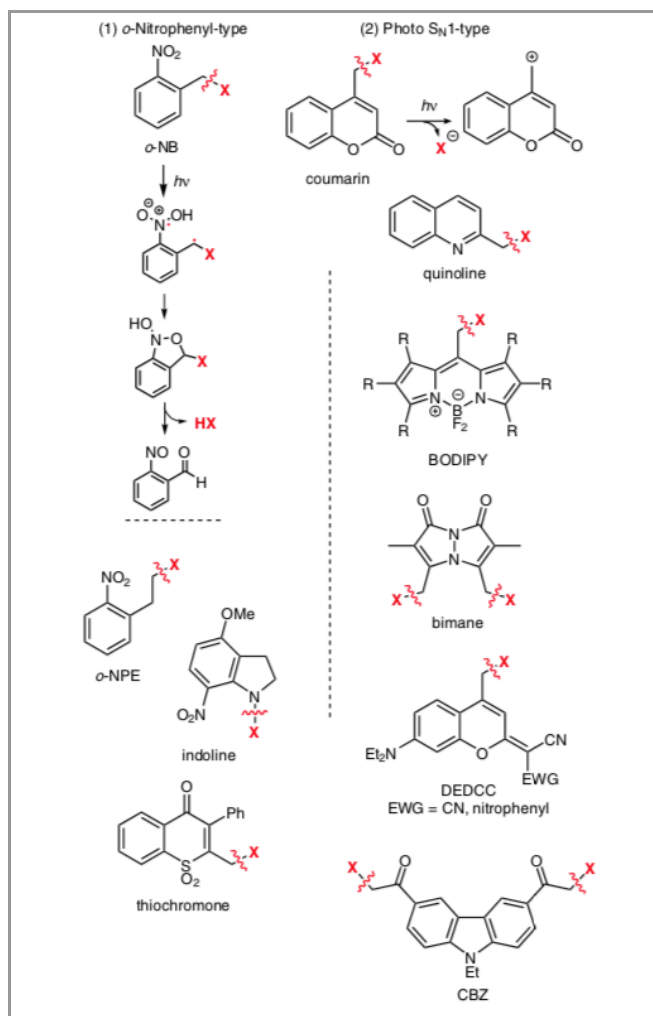


Figure 2 Selected types of photolabile protecting groups (PPGs)

Thus, in the TP excitation process, much lower energy photons can be used to bring molecules to their electronically excited state [Ar–X]* (S1). Half the energy ($h\nu' = 1/2 \times h\nu$) of the corresponding OP excitation ($E = h\nu$) can be used when the first electronic excitation band (S1) of the OP excitation process is allowed for the TP excitation process.

The efficiency of the TP absorption process is quantified by the TP cross-section (σ_2), its magnitude being expressed using the Göppert-Mayer (GM) unit, 1 GM = 10–50 cm⁴ s photon^{–1} molecule^{–1} (Figure 4, a). The uncaging efficiency ($\delta\eta$) of the TP excitation process is determined by the equation $\delta\eta = \sigma_2 \times \phi\eta$, where $\phi\eta$ is the quantum yield of the uncaging reaction. The advantages of using the NIR-TP excitation process in place of one-photon (OP) excitation with UV-vis light include: (1) less photo-damage to living tissue, (2) higher three-dimensional (3D) spatial resolution, (3) deeper penetration in turbid tissues, and (4) lower light scattering.

With a TP excitation process, only molecules located at the focal point will be excited (Figure 4, b), since the probability (P) of excitation is proportional to the light intensity (I) squared.³⁹ Conversely, all illuminated molecules will be excited with an OP excitation process. Thus, 3D spatial selective excitation is possible using the TP excitation technique, although a high-intensity femtosecond laser is required.^{40,41}

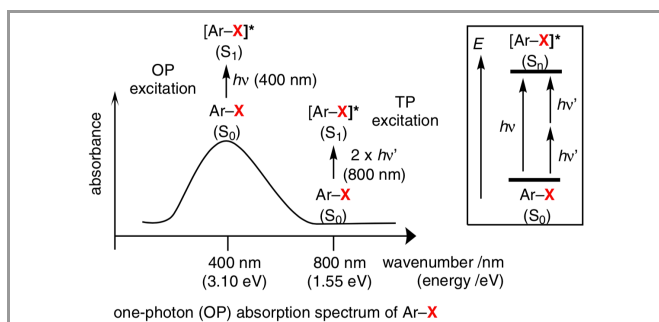


Figure 3 One-photon (OP) and two-photon (TP) excitation processes of Ar-X

In this short review, the basic strategies for designing TP-responsive chromophores for *in vivo* studies are first provided, followed by a summary of very recent developments in the TP uncaging technology using NIR light, including our contributions from the last three years, since excellent earlier reviews are also available.^{42–49}

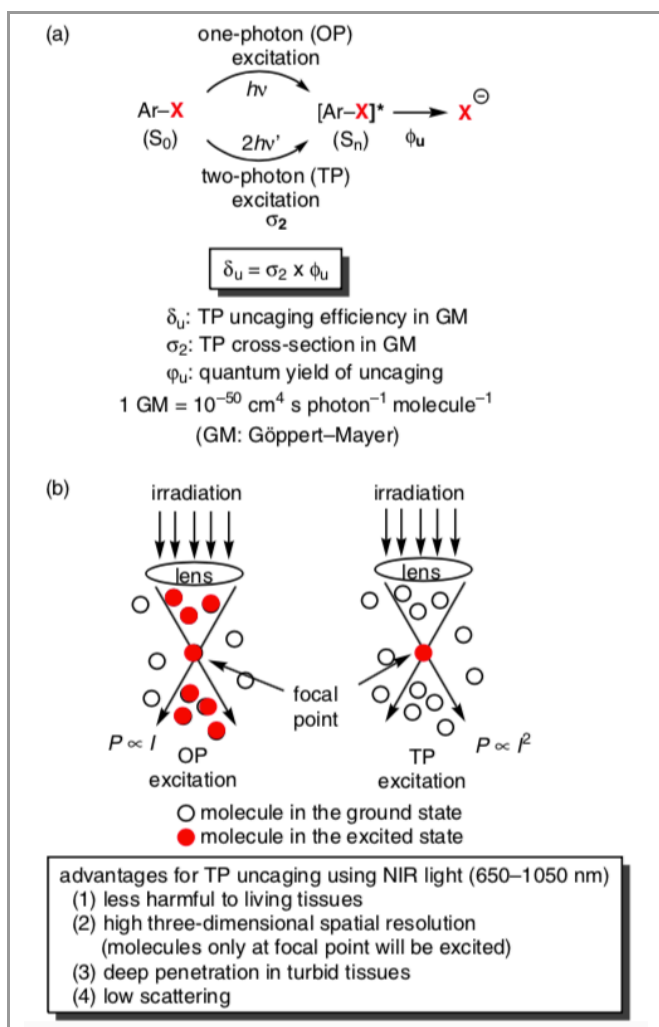


Figure 4 (a) TP uncaging reaction and (b) advantages of TP excitation versus OP excitation

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contributions, in the last three years, since excellent earlier reviews are also available.^{42–49}

2. Molecular Design of TP-responsive Organic Chromophores for “Caging & Uncaging”

Essentially, four strategies have been developed to design chromophores with smart TP absorption character: (1) π -conjugation, and (2) dipolar, (3) quadrupolar and (4) octupolar systems (Figure 5).⁵⁰ Besides, in terms of biological applications, it is useful to find out how the excitation wavelength in the NIR region of light (650–1050 nm) can be selected. In this regard, time-dependent density functional theory (TDDFT) has proved to be a powerful theoretical method for designing appropriate chromophores based on predicted TP absorption spectra.^{50–52}

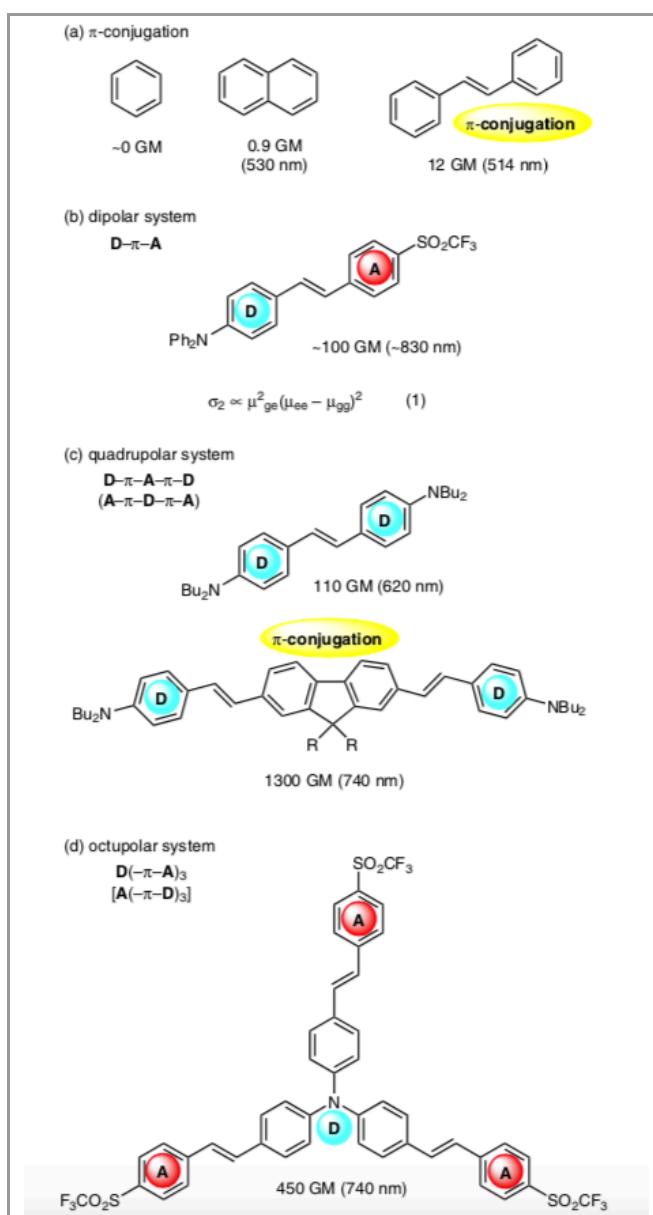


Figure 5 Strategies to induce TP absorption character into molecules

2.1. π -Conjugation

First, benzene possesses no TP-responsive character. Naphthalene shows slight TP absorption character, with 0.9 GM

at 530 nm.⁵³ Stilbene, in which two benzene rings are connected by a double bond, demonstrates a sizable TP response, 12 GM at 514 nm (Figure 5, a).⁵⁴ This basic trend clearly shows that the π -conjugation is directly correlated with TP absorption character.

2.2. A Dipolar System

The introduction of an electron-donating substituent (D) and an electron-withdrawing substituent (A), at either ends of stilbene, leads to a molecule with dipolar character (D- π -A). Figure 5 (b) highlights the dramatic increase in TP absorption cross-section, with 100 GM at \sim 830 nm, i.e., a wavelength of twice that of the OP absorption maximum at \sim 410 nm.⁵⁵ The one order of magnitude increase of the TP cross-section obtained with the push-pull structure can be rationalized within a two-state model, in which the cross section (σ_2) is proportional to the product of the squared transition dipole moment and the squared difference between the excited-state and ground-state dipole moments (Figure 5, b, eq 1).⁵⁰

2.3. A Quadrupolar System

Quadrupolar character (i.e. D- π -A- π -D or A- π -D- π -A) arises in a stilbene structure by introducing push-push or pull-pull substituent designs at either end of the core skeletal structure (Figure 5, c).⁵⁶ For example, a large σ_2 value of 110 GM was reported at 620 nm for a push-push structure. When the π -conjugation increases by introducing a fluorenyl core between two stilbenes moieties, σ_2 undergoes a more than tenfold enhancement accompanied by significant red-shift, leading for instance to 1300 GM at 740 nm.⁵⁷ A three-state model can be used to rationalize the TP response of such quadrupolar systems.

The TP absorption maxima of quadrupolar systems do not show up at twice the wavelength of the OP absorption peaks, since the selection rules for optical transitions are different for OP and TP processes in centrosymmetric structures.^{44,50} Often, the first TP absorption maximum shows up at a smaller wavelength than twice the first OP absorption maximum (i.e., $\lambda_{TP} < 2 \times \lambda_{OP}$).

2.4. An Octupolar System

Triangular structures such as 1,3,5-trisubstituted-benzene [i.e., D(- π -A)₃ or A(- π -D)₃] demonstrate an octupolar character via the introduction of push (D) or pull (A) substituents (Figure 5, d). Such octupolar structures also lead to sizable σ_2 values, for example, of more than 1000 GM above \sim 740 nm for the chromophores shown Figure 5 (d).^{55,58}

As briefly described above, both state and transition dipole moments of the molecule play crucial roles in producing molecules with high multi-photon absorption character. The absorption spectra of the TP excitation process can be predicted by TD-DFT calculations, at least qualitatively depending on available levels of theory.⁵⁰ For dipolar systems, the first TP absorption maxima is expected to show up at twice the wavelength of the first OP absorption maximum, both processes being symmetry allowed. Often this involves excitation to the first excited state (S1). For quadrupolar and octupolar structures, the first TP allowed electronic transition usually corresponds to a higher electronic excited state such as

S2 or S3, in which the transition dipole moments may be vanishing.^{50,55,58}

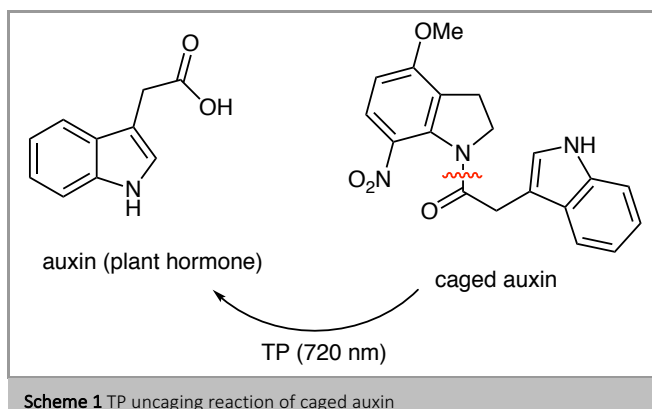
Increasing π -conjugation not only increases the TP absorption cross-section values of molecules, but also their hydrophobicity. However, caged compounds must possess suitable water solubility in physiological studies. Furthermore, the TP absorption maxima should be in the NIR region of 650–1050 nm. Thus, a balance of TP absorption character and water solubility must be considered when designing TP-responsive chromophores for physiological studies.

In addition to the above-mentioned requirements for state-of-the-art caged compounds, i.e., TP responsiveness, water solubility and non-toxicity, the rate of photorelease of bioactive molecules is also a very important issue for physiological experiments. The sharp concentration jump by photolysis, e.g., less than a microsecond timescale, is essential for observing fast biological responses.

3. Recent Developments of TP Uncaging Reactions

3.1. 4-Methoxy-7-nitroindolyl (MNI)-Caged Auxins

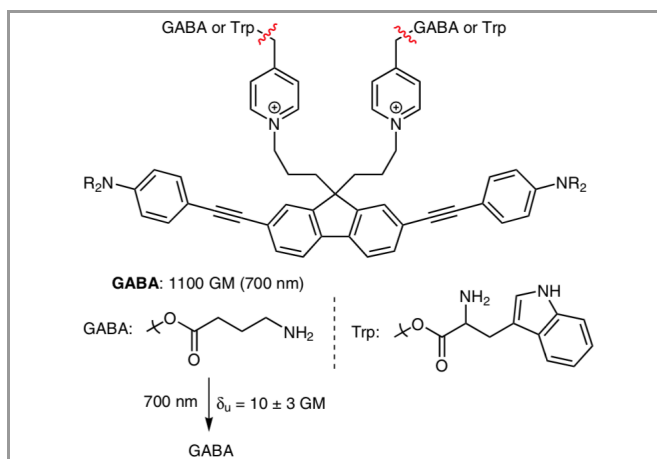
The plant hormone auxin (i.e., indole-3-acetic acid) plays a crucial role in almost every aspect of plant development. Hayashi and co-workers⁵⁹ have designed and synthesized a 4-methoxy-7-nitroindolyl (MNI)-caged auxin (Scheme 1). The caged auxin was found to be thermally stable in plants, and indole-3-acetic acid was released rapidly upon photolysis. However, the *o*-NPE-caged auxin was found to be unstable in plants. The authors determined optical control of auxin-responsive gene expression and auxin-related physiological responses using the TP-responsive caged auxin.



3.2. Uncaging of GABA and Tryptophan Using TP-induced Electron-Transfer Reactions

Anderson and co-workers⁶⁰ developed a new approach for the design of TP-responsive chromophores based on a photoinduced electron-transfer reaction (Scheme 2). They succeeded in synthesizing TP-responsive caged γ -amino butyric acid (GABA) and L-tryptophan (Trp) with an electron-donating fluorene-based chromophore and an electron-accepting pyridinium moiety. A high TP cross-section value of $\sigma_2 = 1100$ GM at 700 nm was found for GABA. Further, a clean photochemical release of GABA was observed at 700 nm irradiation with a chemical yield of >95% and a photochemical yield of 1% ($\phi_u = 0.01$). Thus, the efficiency of the TP-induced

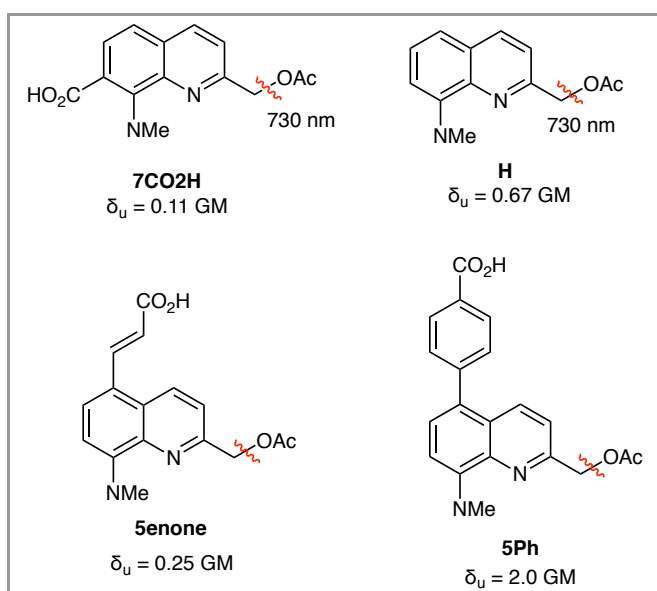
uncaging reaction of GABA was determined to be $\delta_u = 10 \pm 3$ GM.



Scheme 2 TP uncaging reaction of caged GABA and caged Trp

3.3. Effect of Position Isomerism in Aminoquinoline-Derived PPGs

Substituent effects on the TP-induced uncaging reaction were reported by Dalko and co-workers⁶¹ for TP-responsive aminoquinoline derivatives (Scheme 3). The TP uncaging efficiency at 730 nm for the C5-carboxy isomer **7CO2H**, $\delta_u = 0.11$ GM, was much smaller than the parent **H** ($\delta_u = 0.67$ GM), **5Bz** ($\delta_u = 2.0$ GM), and **5AcR** ($\delta_u = 0.25$ GM), even though the substituent is supposed to induce a higher intramolecular charge-transfer character than the parent **H**.

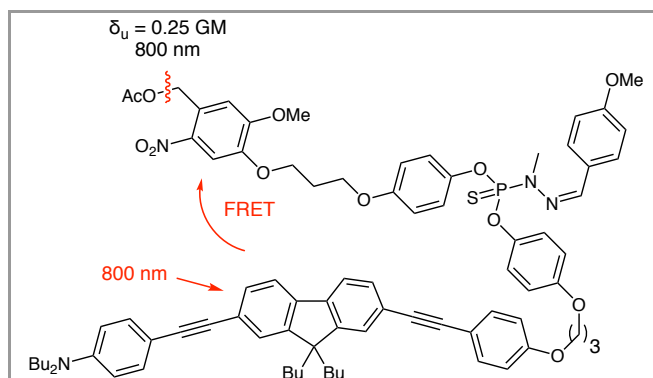


Scheme 3 TP uncaging reaction of quinolone derivatives

3.4. Cooperative Dyads for TP Uncaging

Blanchard-Desce and co-workers⁶² designed an intramolecular energy transfer system from the TP-responsive chromophore to the PPG unit. Donor-donor substituted ($R = NR_2$, OR) and acceptor-acceptor substituted ($R = SO_2CF_3$) fluorene structures with quadrupolar characters were chosen for the TP-responsive chromophore (Scheme 4), and the 4-methoxy-2-nitrophenyl unit was used for uncaging. The NIR-TP-

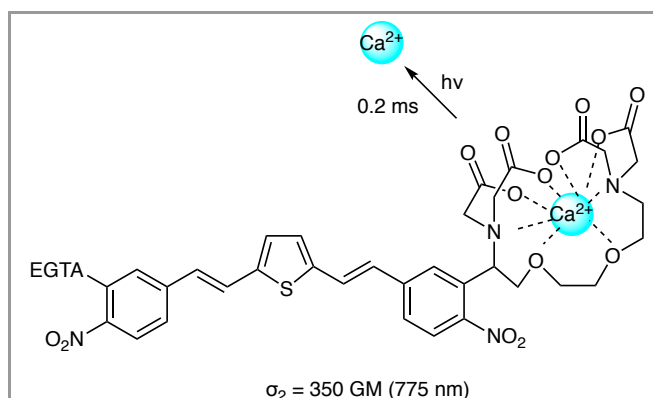
induced release of acetic acid (AcOH) was examined at 720–800 nm. The uncaging efficiency of the donor-donor substituted compound was found to provide the best TP uncaging efficiency of $\delta_u = 0.25$ GM ($\sigma_2 = 310$ GM) at 800 nm.



Scheme 4 TP-induced energy transfer and uncaging reaction of acetic acid. FRET: fluorescence resonance energy transfer

3.5. Caged Calcium with a Bisstyrylthiophene Backbone

Calcium ions (Ca^{2+}) have many crucial roles in the physiology and biochemistry of organisms and cells. For example, in signal transduction pathways, Ca^{2+} acts as a second messenger in neurotransmitter release from neurons. Therefore, caged-calcium compounds have attracted considerable attention in the field of neuroscience. Since Ca^{2+} cannot be covalently bound to organic chromophores, an ethylene glycol tetraacetic acid (EGTA) unit has been used as the *in vivo* Ca^{2+} -selective chelator. Ellis-Davies and co-workers⁶³ were the first to develop TP uncaging of Ca^{2+} using a nitrodibenzofuran structure. In 2016, this group developed a new EGTA-substituted chromophore with TP absorption character in the NIR region; specifically a dinitro derivative of bis-styrylthiophene (Scheme 5). The bis-nitro-styryl structure provided a sizable TP cross-section of 350 GM at 775 nm. Moreover, the Ca^{2+} chelator was found to release Ca^{2+} in <0.2 ms upon flash photolysis.

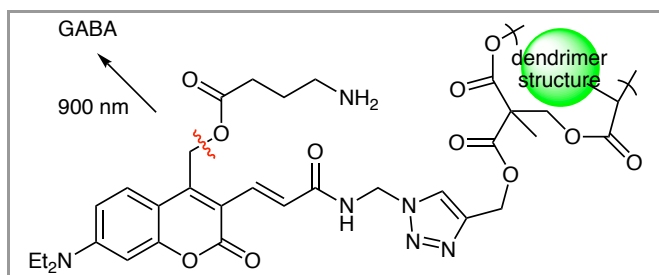


Scheme 5 Ca^{2+} uncaging reaction using a high TP-responsive chromophore

3.6. Cloaked Caged Compounds

Ellis-Davies and co-workers⁶⁴ invented cloaked caged compounds (e.g., **G5-DEAC450-GABA**), in which antagonists of ionotropic γ -aminobutyric acid (GABA) were cloaked in a fifth generation of a 2,2-bis(methylol)propionic acid dendrimer

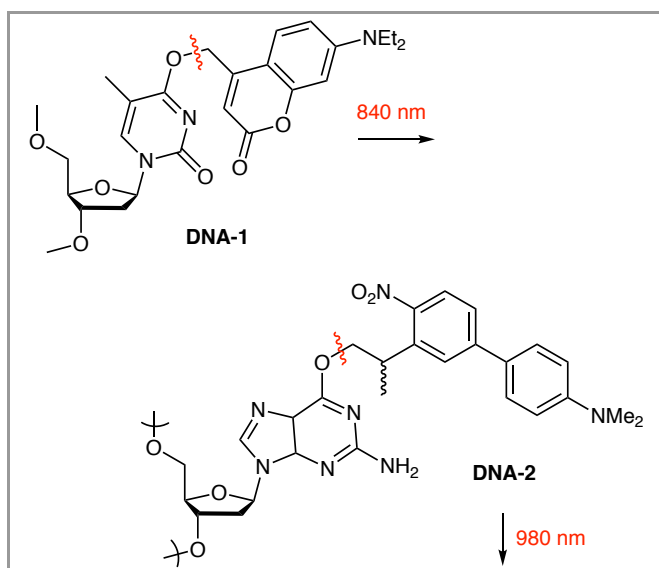
conjugated molecule (Scheme 6). Using the cloaked caged GABA compound, a physiological study of the signaling of the inhibitory neurotransmitter GABA became possible in its natural state for the first time.



Scheme 6 TP-induced (900 nm) uncaging reaction of GABA

3.7. Three-Dimensional Control of DNA Hybridization by Orthogonal Two-Color TP Uncaging

Heckel and co-workers⁶⁵ reported the three-dimensional control of DNA hybridization using TP uncaging reactions of **DNA-1** and **DNA-2** (Scheme 7). In this study, DNA strands were introduced into TP-responsive chromophores such as [7-(diethylamino)coumarin-4-yl]methyl and *p*-dialkylaminonitrophenyl chromophores. These 3D uncaging reactions demonstrated that the technique is feasible for more complex scenarios. **DNA-1** can be uncaged at 840 nm, and **DNA-2** can be uncaged at 980 nm, thus, selective uncaging was found to be possible by simply changing the excitation wavelength.

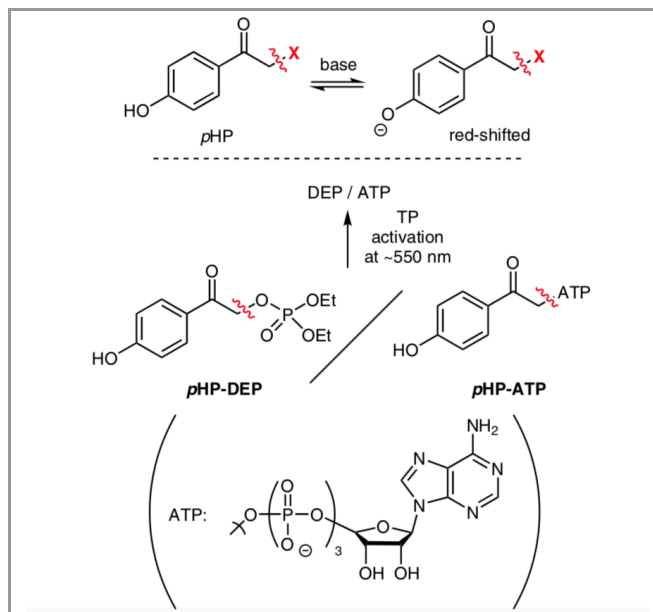


Scheme 7 Wavelength-dependent release of DNA using TP photolysis

3.8. TP-Induced Release of Diethyl Phosphate (DEP) and ATP

The first TP activation of *p*-hydroxyphenacyl (pHP) PPG^{66a,b} was reported by Houk, Givens, and Elles⁶⁷ (Scheme 8). A strong TP absorption (>10 GM) was observed at ~550 nm. Monitoring the progress of the uncaging reaction of diethyl phosphate (DEP) and adenosine triphosphate (ATP) revealed that the TP uncaging reactions were possible using visible light in the range 500–620 nm. The authors also suggested that the TP uncaging is also possible in the range 550–720 nm under

mild basic conditions, since the absorption band is shifted to lower energy (3.8 eV) in both the OP and TP absorption spectra.^{66c}



Scheme 8 TP uncaging of DEP and ATP using *p*-hydroxyphenacyl (pHP) PPG

4. Our Contribution to TP Uncaging Reactions

In collaboration with Professors Takayoshi Kobayashi (physicist) and Haruo Kasai (physiologist), the present authors Abe (organic chemist) and Katan (theoretical physicist) began investigating TP uncaging reactions in 2012 to better understand bioactive substrates in biological processes and clarify their roles. The target bioactive molecules were neurotransmitters such as glutamate (Glu), i.e., 2-aminopentanedioic acid.

Initially, the molecular design of TP-responsive PPGs was investigated. As noted in Section 2, the molecular size of TP-responsive chromophores should be small to enable water solubility and must also possess an appropriate TP absorption cross-section in the NIR region for utility in physiological experiments. Our target σ_2 value is at least over 100 GM in the NIR region. Although the quantum yields (ϕ_u) are difficult to predict and refine, exceeding a quantum yield over 5% is achievable. The reported largest value of TP uncaging efficiency, $\delta u = \sigma_2 \times \phi_u$, was approximately 1 GM when we began the study.

The stilbene core was selected as the platform to design a TP-responsive chromophore for physiological studies, because stilbene derivatives have relatively large σ_2 values despite their compact structures. However, stilbene derivatives are linear compounds, thus, it is possible for *cis/trans* isomerization to occur via their electronically excited states, which would diminish the quantum yield of the uncaging process of bioactive molecules. Therefore, we decided to use cyclic 7,8-dihydronaphthalene cores as the TP-responsive chromophores (Figure 6).⁶⁸ As newcomers to this field, we initially chose the well-known *o*-nitrobenzyl (*o*-NB) PPG group on account of the strong electron-withdrawing ability of the nitro group. To

impart water solubility to the chromophore, hydrophilic substituents such as carboxylic groups and polyethylene glycol units were introduced to the core structure.

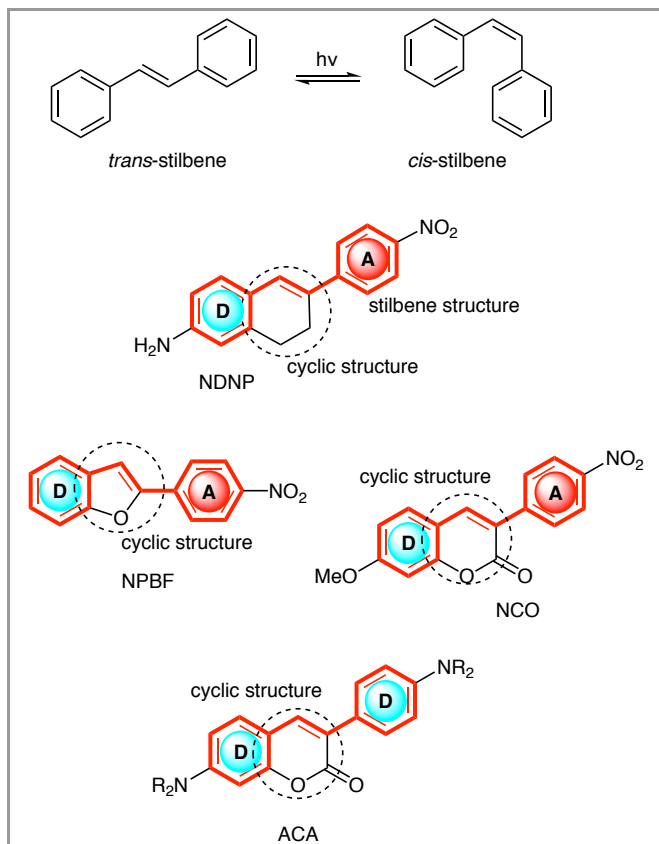
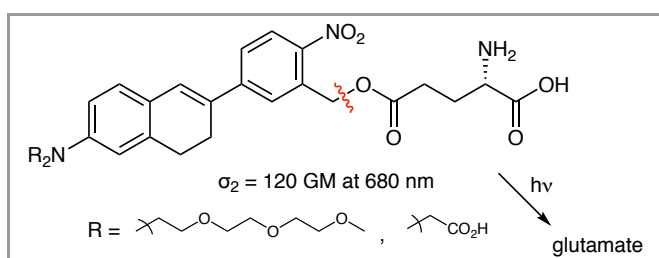


Figure 6 Our first molecular design for TP-responsive chromophores for physiological studies



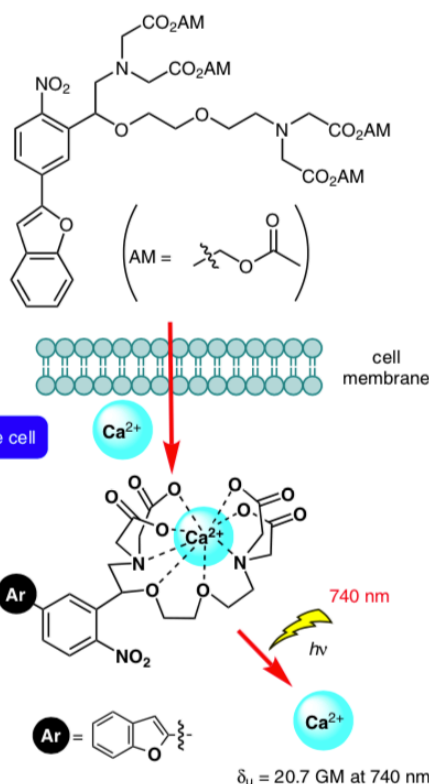
Scheme 9 TP-uncaging reaction of glutamate

The TP absorption spectrum of the newly designed TP-responsive chromophore, 6-(4-nitrophenyl)-7,8-dihydronaphthalen-2-amine, was computed at the TD-B3LYP/6-31G(d) level of theory. The TP absorption maximum was predicted to be 150 GM at ~ 810 nm. Therefore, the synthesis of the target molecules with a glutamate unit (Glu) began from 4-bromo β -tetralone and 3-bromobenzaldehyde (Scheme 9). Clean formation of Glu was observed using OP and TP photolysis. However, the quantum yield of the uncaging reaction was low, $\phi_u = 0.01$ (1%).

Next, we designed a new TP-responsive chromophore, 2-(4-nitrophenyl)benzofuran (NPBF) (Scheme 10).⁶⁹ Previously, Ellis-Davies reported that the 3-nitrodibenzofuran (NDBF) core possessed a TP uncaging efficiency (δ_u) of 0.6 GM with a high quantum yield of $\phi_u = 0.770$. The computed TP absorption

maximum of the NPBF chromophore was found to be 150 GM at 700 nm, while that for the NDBF structure amounted to 75 GM at 600 nm (i.e., twice smaller and blue-shifted). The TP uncaging efficiencies for the caged benzoic acid ($\phi_u = 0.09$) and caged Ca²⁺ with EGTA structure ($\phi_u = 0.3$) were determined to be 5 GM and 20.7 GM at 740 nm TP excitation, respectively. The NPBF chromophore was successfully applied *in vivo* for the caged Ca²⁺ ion.⁷¹

Subsequently, we investigated the donor-acceptor-substituted and donor-donor-substituted coumarin derivatives **NCO72** and **ACA73** as dipolar and quadrupolar systems, respectively (Scheme 11). The dipolar system **NCO** (Figure 6) was found to possess a sizable TP absorption maximum at 700 nm with 150 GM. A more than four-fold increase with a small blue-shift TP (700 GM at 650 nm) was predicted for the quadrupolar analogue **ACA**. The corresponding caged benzoic acids **NCO-BA** and **ACA-BA** were synthesized and their TP uncaging reactions were conducted with NIR irradiation. The TP uncaging efficiency for **NCO-BA** was found to be 3.4 GM at 710 nm, and ~ 16 GM at 650 nm for the quadrupolar system **ACA-BA**. This shows that quadrupolar structures are promising candidates for TP uncaging.

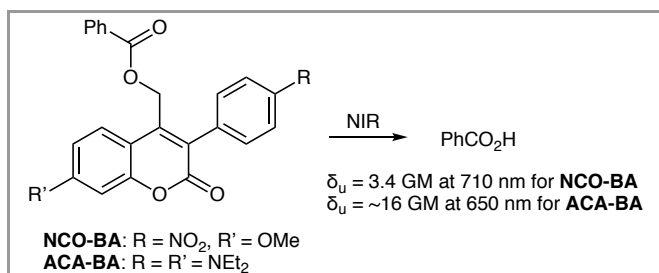


Scheme 9 TP uncaging of Ca²⁺ using NPBF chromophore

5. Summary

In this short review, recent developments of near-infrared (NIR) two-photon (TP) induced photorelease (uncaging) of bioactive molecules such as drugs have been summarized. The TP excitation process at 650–1050 nm is useful for physiological studies *in vivo*, since the spatiotemporal release of bioactive molecules can be achieved with high 3D control conditions in

deep areas of living tissues. The molecular engineering of the next generation of chromophores suitable for the ‘caging & uncaging’ technique should contribute to unravel more precisely the mechanisms of biological activity *in vivo*.



Scheme 11 TP uncaging reactions of benzoic acid using coumarin derivatives with dipolar and quadrupolar characters

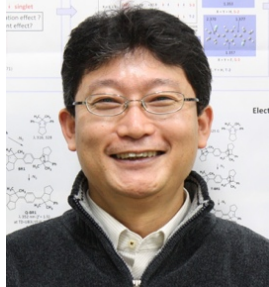
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