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Caged Compounds

Two-Photon Uncaging with the Efficient 3,5-Dibromo-2,4-dihydroxycinnamic Caging Group**

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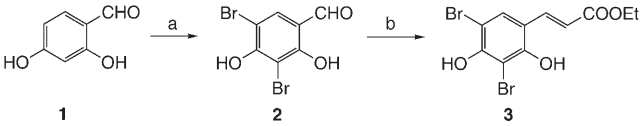
Cells constantly adjust the concentrations of their components in response to interactions with the environment. Therefore, influencing cell biology requires versatile tools to deliver a variety of chemicals. Apart from passive permeation, microinjection has been the most widely used delivery method. However, its spatial resolution is poor unless injection is performed invasively through the cell membrane. Two-photon photoactivation has been envisioned as a finely tunable noninvasive alternative[1–3] mimicking an "optical syringe" with submicrometer resolution. Here two major issues are the action cross section of two-photon uncaging and quantification of delivery. To avoid detrimental effects, the cross section should be as large as possible to reduce the illumination power needed to release a given amount of effector. For application in complex systems (e.g., a living organism) with unknown characteristics (e.g., volume, diffusion coefficients, optical homogeneity), quantification should ideally be performed during each uncaging event. Here we show how the above tasks can be performed with an appropriately designed photolabile moiety: Systematically nonfluorescent in the caged substrate, this photolabile moiety transforms on photoactivation into a water-soluble, strongly fluorescent coumarin that reports on the release of the desired chemical.[4] Quantitative analysis is thus simply achieved by recording fluorescence emission after uncaging at the targeted site.

There has been constant development of new caging groups aimed at improving their photophysical and photochemical characteristics.[5] Surprisingly, real-time quantification of uncaging has not attracted much attention: In contrast to in vitro conditions, studies are generally not quantitative in vivo. The *ortho*-hydroxycinnamic caging platform introduced by Porter et al.[6] provides an attractive means to fulfill this goal. In contrast to the photoreleased coumarin coproduct, the caged substrate does not fluoresce. Moreover, the *ortho*-hydroxycinnamic backbone is favorable for designing caging groups for specific demands. The photophysics/photochemistry with one- and two-photon excitation of such donor–acceptor ethylenic systems[7,8] and the formation of the fluorescent coumarin reporter[9] are well documented. In contrast to other systems,[10] syntheses are short and easy. We here report on the 3,5-dibromo-2,4-dihydroxycinnamic caging platform, which was optimized in terms of solubility and photophysical/photochemical properties for quantitative implementation of the optical-syringe strategy at the single-cell level. The relevance of these properties was tested by using zebrafish embryos as a model system.

Alcohols and amines can be photoreleased from their respective esters[6] and amides[11,12] by using the *ortho*-hydroxycinnamic strategy. For characterization of the caging moiety, we chose ethyl 3,5-dibromo-2,4-dihydroxycinnamate (**3**) as a model releasing an alcohol (e.g., ethanol, **5**) on illumination (Figure 1a). Ester **3** is efficiently synthesized in two steps (Scheme 1): Commercially available 2,4-dihydroxybenzaldehyde (**1**) is first brominated twice with bromine in acetic acid to give 3,5-dibromo-2,4-dihydroxybenzaldehyde (**2**) in 60% yield, which is subsequently condensed with ethyl triphenylphosphoranylideneacetate in toluene to provide **3** with the desired *E* configuration (60% yield).



1 2 3

Scheme 1. Two-step synthesis of ethyl 3,5-dibromo-2,4-dihydroxycinnamate (**3**). a) Br_2, AcOH, 60%; b) $Ph_3P{=}CHCOOEt$, toluene, 60%.

The 3,5-dibromo-2,4-dihydroxycinnamic caging group is weakly acidic: In water at 293 K it exhibits a first protonation constant of $pK_{a,1}(\mathbf{3}) = 5.0 \pm 0.1$, which is within the range of 4.5–7.4 relevant to biological media. Around neutral pH, as in a cell, caged substrate **3** exchanges between its acidic uncharged phenol state, which makes it soluble in a lipophilic environment such as a bilayer, and its basic, negatively charged phenolate form, which confers solubility in water. This dual solubility was introduced to facilitate the permeation of uncharged substrates caged with the 3,5-dibromo-2,4-dihydroxycinnamic moiety inside targeted cells embedded in a whole organism.

The caged substrate **3** forms thermally stable aqueous solutions at neutral pH at room temperature. These solutions strongly absorb light beyond 350 nm when **3** is in its anionic

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basic state: $\varepsilon_3(\lambda_{max}=369\ nm) = 2.5 \times 10^4\ M^{-1}cm^{-1}$ at pH 7.0 (Figure 2a). At the same pH, **3** is almost nonfluorescent on excitation at $\lambda_{exc} = 350$ nm: We obtained $\Phi_F^1(\mathbf{3}) = 0.01$ for its quantum yield of fluorescence. Illumination of **3** initiates a cascade of reactions:[6] *trans–cis* photoisomerization is followed by thermally driven lactonization leading to quantitative release of the desired substrate **5** and 6,8-dibromo-7-hydroxycoumarin (**4**; Figure 1a), as evidenced by ^{1}H NMR spectroscopy and UV/Vis absorption. The measured quantum yield of uncaging after one-photon excitation of $\Phi_u^{(1)} = 0.05$ gives rise to an uncaging action cross section for one-photon excitation at $\lambda_{max} = 369$ nm of $\varepsilon_3\Phi_u^{(1)} = 1250\ M^{-1}cm^{-1}$. Such a value exceeds by a factor of 40 the uncaging action cross section for the most popular 4,5-dimethoxy-2-nitrobenzyl photolabile protecting group[10] and satisfactorily compares with the most efficient caging groups reported to date.[2,3,13–15]

To facilitate implementation of the optical-syringe strategy, uncaging and excitation of the reporting fluorescent molecule should be done with the same excitation source. Coumarin **4** and caged substrate **3** do absorb light in the same wavelength range ($\varepsilon_4(\lambda_{max}=377\ nm) = 2.1 \times 10^4\ M^{-1}cm^{-1}$). However, **4** exhibits intense fluorescence emission (quantum yield of fluorescence $\Phi_F^{(1)}(\mathbf{4}) = 0.65 \pm 0.05$; emission peaks at $\lambda_{em} = 456$ nm, Figure 2b), in contrast to **3**. Note also that caged substrate **3** and coumarin coproduct **4** essentially exhibit similar acid–base behavior ($pK_a(\mathbf{4}) = 4.7 \pm 0.1$ in water at 293 K). In particular, the water solubility of **4** at neutral pH is significant for reliable quantification of uncaging based on fluorescence emission.

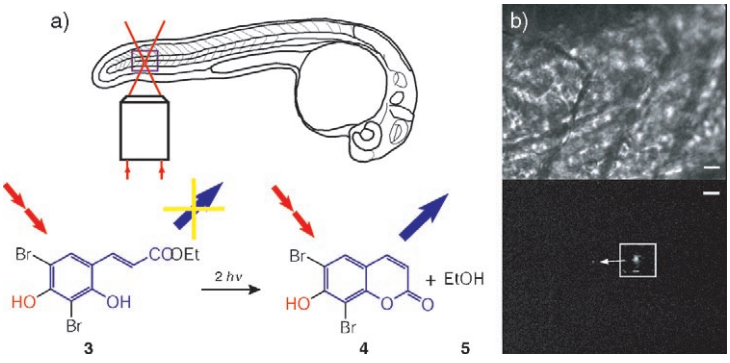

Figure 1. Optical-syringe strategy (a zebrafish embryo is used for illustration). The embryo is incubated in a solution of a nonfluorescent and biologically inactive caged precursor **3** (here 10 μM ethyl 3,5-dibromo-2,4-dihydroxycinnamate) that penetrates the whole organism. Then the embryo is submitted to two-photon excitation by focusing a laser beam (red arrows) in the caudal-fin area (a; the bonds involved in the *ortho*-hydroxycinnamic caging platform and the deprotonated hydroxy group at neutral pH are shown in blue and red, respectively). Local light absorption leads to uncaging of the biologically active substrate (here ethanol, **5**) in the targeted cell. Its final concentration is obtained by measuring the intensity of fluorescence emission from the 6,8-dibromo-7-hydroxycoumarin (**4**) that is formed as coproduct on uncaging in the single illuminated cell (b; top: bright-field image, scale bar: 10 μm; bottom: focal spot of two-photon excitation, scale bar 10 μm and 1 μm (inset); both images were taken with a regular CCD camera).

Similar behavior is observed on two-photon excitation in the wavelength range of 720–800 nm. Smooth formation of fluorescent coumarin **4** is evidenced by the analysis of the rise of fluorescence emission on illumination of a **3** solution with a Ti–sapphire pulsed laser. Its brightness $\delta\Phi_F^{(2)}$ after two-photon excitation lies in the 1-GM range ($1\ GM = 10^{-50}\ cm^4\ s\ photon^{-1}$) between 720 and 790 nm (see Figure 2a) and thus makes coumarin **4** a satisfactory uncaging reporter with two-photon excitation. Figure 2a also displays the uncaging-action cross-section spectrum of **3** with two-photon excitation in the same wavelength range: $\delta_u\Phi_u^{(2)}(\lambda_{exc}^{(2)},\mathbf{3})$ peaks at 1.6 GM at 750 nm, which is an order of magnitude larger than the limit of 0.1 GM that was claimed to be the lowest value relevant for biological applications.[2] This value makes the *ortho*-hydroxycinnamic caging platform one of the most efficient available to date.[2,3,14–16]

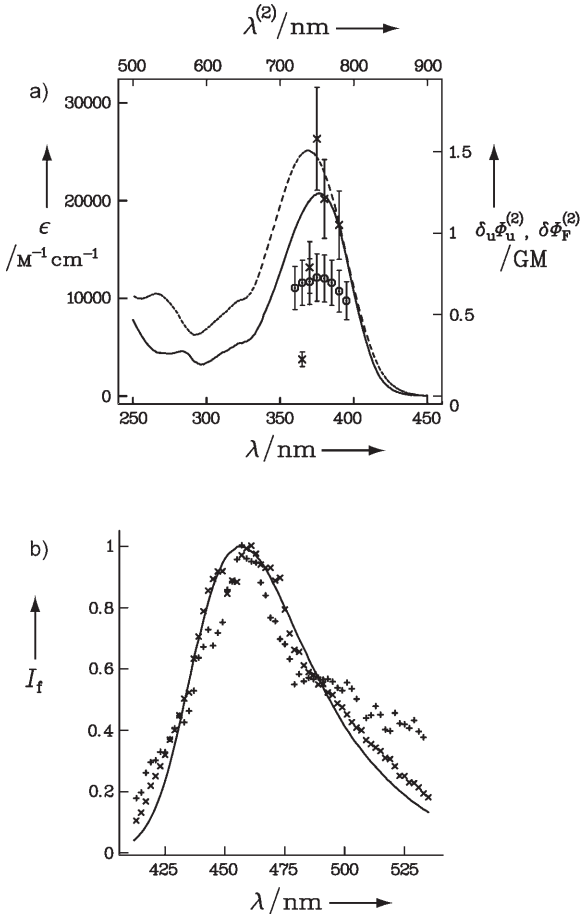

Figure 2. Photophysical and photochemical properties of **3** and **4** in vitro and in vivo. a) Single-photon absorption spectra: molar absorption coefficients $\varepsilon(\lambda)$ for **3** (dashed line) and **4** (solid line); cross sections of **3** uncaging and fluorescence after two-photon excitation: $\delta_u\Phi_u^{(2)}(\lambda^{(2)})$ (×) and $\delta\Phi_F^{(2)}(\lambda^{(2)})$ (○). b) Normalized steady-state fluorescence emission spectra of **4** with one- (——) and two-photon excitation (×) ($\lambda_{exc} = 350$ nm and $\lambda_{exc}^{(2)} = 750$ nm). The normalized final steady-state fluorescence emission spectrum with two-photon excitation arising from the targeted cell in vivo is displayed as plus signs (+).

Having quantitatively characterized the behavior of the caged substrate **3** in vitro, we checked that the protecting group was suitable for biological applications in a living organism. We chose the zebrafish, which seems particularly promising for the use of light to control biological activity: its embryo is transparent, unpigmented strains are available, and it has recently emerged as an attractive model animal for numerous studies.[17] We incubated a zebrafish embryo at the 10–15-somite stage in a 10 μM solution of **3** for 60 min and illuminated the caudal-fin area at 750 nm (Figure 1a). Figure 2b displays the fluorescence emission spectra with two-photon excitation recorded within the targeted embryo cell and the corresponding spectrum of **4** in water. The good agreement between the spectra suggests that the emitting fluorophore is indeed coumarin **4** in an aqueous environment. Then the emission intensity from the fluorescent cell can be converted to **4** concentration by using the **4** emission rate per molecule measured by fluorescence correlation spectroscopy after two-photon excitation. We found values in the 10 μM range which suggest that the initial intracellular concentration of caged precursor **3** was essentially similar to the concentration of the incubating solution. As shown in Figure 1b, the targeted single cell can be easily identified by its final strong fluorescence emission.

Thus, the 3,5-dibromo-2,4-dihydroxycinnamic caging platform seems promising for implementing the optical-syringe approach in vivo. The solubility of the 3,5-dibromo-2,4-dihydroxycinnamic moiety favors cell permeation. Its robust photophysical and photochemical properties after two-photon excitation put this caging group among the best available for substrate uncaging in biological samples. Quantitative control of delivery can be achieved by recording fluorescence emission from the water-soluble coumarin coproduct **4** that reports on the concentration of photoreleased substrate.

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