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Alcohol Uncaging with Fluorescence Reporting: Evaluation of *o*-Acetoxyphenyl Methyloxazolone Precursors

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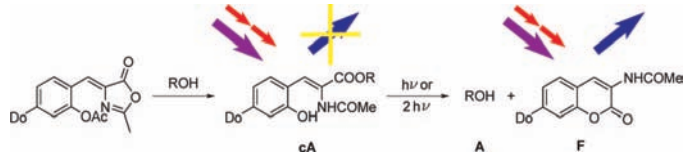
ABSTRACT



This paper evaluates a series of photolabile protecting groups with built-in fluorescence reporting. They rely on readily available *o*-acetoxyphenyl methyloxazolones as activated precursors. Alcohol substrates are easily caged. The resulting photoactivable esters exhibit large one- and two-photon uncaging cross sections. The alcohol substrates are quantitatively released in a 1:1 molar ratio with a strongly fluorescent coumarin coproduct that serves as a reporter to quantify substrate delivery.

Uncaging[1–3] with one- and two-photon excitation[4] is a method of choice for a precise delivery of chemicals both spatially and temporally in a noninvasive way. Its use in biological applications is now well-established.[5–10] However, because of the possible damaging of biological samples by light, illumination duration and power should ideally be kept low. There is thus a lower limit on the two-photon uncaging action cross section needed to deliver a given amount of molecules: values of 0.1 or 10 Goeppert-Mayer (GM; 1 GM = 10^{-50} cm^4 s/photon) are usually cited for a biological application.[6,11] In fact the 1 GM range has already been reached, leading to a successful use in biological systems.[6,12–14]

As biological samples often respond in a dose-dependent manner, a tool making it possible to vary, in a well-established way, the amount of delivered molecules would be of great interest. In theory, uncaging fulfills that aim, but it is often hampered by the need to characterize the uncaging rate in the biological system. Therefore an easy way to quantify the uncaging reaction would be extremely useful. One possibility is to use a fluorescent reporter.

Using the *o*-hydroxy cinnamate series that had been introduced by Porter et al.,[15] we showed that such photolabile

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protecting groups were good candidates to fulfill those goals. Indeed they exhibit a large two-photon uncaging action cross-section up to 4.7 GM at 750 nm in acetonitrile.[14] In addition, whereas they are intrinsically nonfluorescent, uncaging leads to the release of a fluorescent reporter and a chosen alcohol in a 1:1 molar ratio. The increase of fluorescence intensity at the uncaging site can be used as a probe for a quantitative analysis of the uncaging reaction (Figure 1).[14] This property is independent of the protected substrate.[16]

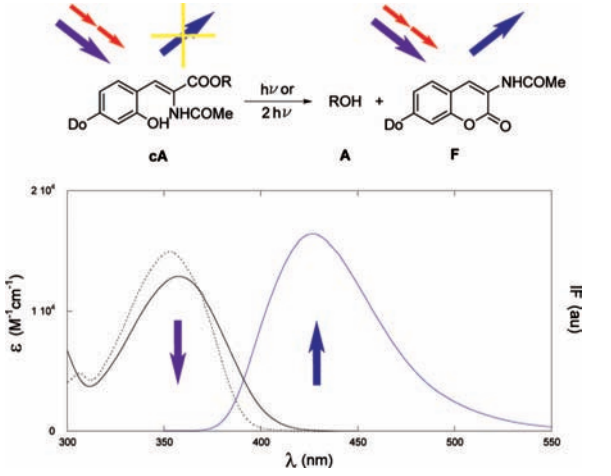


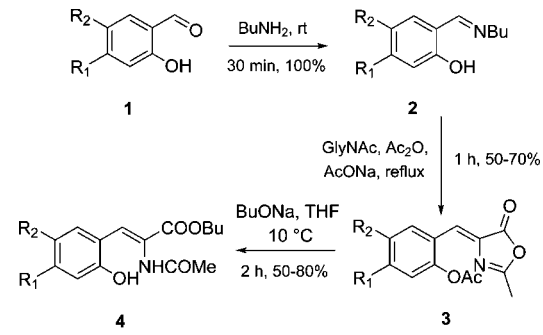
Figure 1. Uncaging with fluorescence reporting. Light absorption by the nonfluorescent *o*-hydroxyphenyl cinnamate **cA** resulting from oxazolone opening with the alcohol substrate **ROH** (violet and red arrows with one- and two-photon excitation, respectively) initiates a series of photochemical and thermal processes leading to release the **ROH** substrate together with a fluorescent coumarin **F** coproduct in a 1:1 stoichiometric ratio. **cA** and **F** absorb light in the same wavelength range (black solid and dotted lines, respectively), and the uncaging extent can be correspondingly extracted from the intensity of **F** fluorescence emission (blue solid line). The absorption and emission properties of the cinnamate **4c** at 293 K in Tris pH 7, 20 mM NaCl, 100 mM buffer/acetonitrile 1/1 (v/v) are used for a purpose of illustration.

In parallel to the development of the *o*-hydroxy cinnamate series, we considered another cinnamate series relying on the *o*-hydroxyphenyl oxazolone precursor. During the synthesis of those cinnamates, Cornforth et al. noticed that, upon heating, isomerization occurs around their trisubstituted double bond, leading to the formation of a coumarin.[17] Such a reaction could also be triggered by light, as later noted.[18] Their resemblance with Porter's series led us to hypothesize that this platform could be used to develop caging groups. We were especially interested in the easy and reliable coupling of the desired substrate. Indeed, the preparation of the carboxylic acid unit to cage a given alcohol in Porter's original series proved to be delicate from the synthetically accessible ethyl ester precursor. This feature could hamper further developments of the cinnamic platform to cage alcohols. In the series presented hereinafter, the direct reaction between the chosen alcohol and an appropriate oxazolone cycle (playing the role of an activated carboxylic acid) leads simply, in one step only, to the envisioned *o*-hydroxy cinnamate ester.

The relevance of the *o*-hydroxy oxazolone platform for uncaging applications was assayed using the original design of Cornforth et al. with a phenyl group as the oxazolone substituent. Results proved to be promising: (i) the syntheses of the *o*-hydroxyphenyl phenyloxazolone were easy, (ii) the oxazolone ring could be opened with stoichiometric amounts of a model alcohol, and (iii) the uncaging properties of the resulting cinnamates were attractive (see Supporting Information). However, the 3-benzoylamido coumarin coproduct formed upon uncaging was poorly fluorescent. We therefore prepared a series of 3-acyloylamido coumarins to identify a more appropriate fluorescent reporter for uncaging. Among these, we found that 3-acetamido coumarins were strongly fluorescent for all of the investigated electron-donating substituents (see Supporting Information). Hence we decided to focus on the *o*-hydroxyphenyl methyloxazolone platform.

The syntheses of the corresponding *o*-hydroxyphenyl methyloxazolone derivatives with the Plöchl−Erlenmeyer synthesis[19] were less straightforward than in the *o*-hydroxyphenyl phenyloxazolone series; this might be due to a poorer solubility, which would forbid product precipitation after oxazolone formation.[20−23] We therefore considered an alternative synthetic pathway (Scheme 1). We first activated

Scheme 1. Syntheses of Caged Model Alcohols **cA** (**4a**−**c**)



a: R_1 = OMe, R_2 = H; **b**: R_1 = $N(Et)_2$, R_2 = H; **c**: R_1, R_2 = OCH_2O

the aldehyde electrophilic center by alkyl imine formation which was shown to be especially efficient for reactions related to the nucleophilic condensation of *N*-acetyl glycine.[24] The butylimines **2a**−**c** were prepared quantitatively from butylamine and the parent easily accessible benzaldehydes **1a**−**c** (the benzaldehyde **1c** was obtained according to the literature).[25] We subsequently coupled *N*-acetyl glycine to the 2-hydroxyphenylbutylimines **2a**−**c** in acetic anhydride. The 2-acetoxyphenyl methyloxazolones **3a**−**c** were obtained

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Table 1. Photophysical and Photochemical Properties of Investigated (*Z*)-Cinnamates Relevant to the Uncaging Process with One- and Two-Photon Excitation[a]

	$\varepsilon_Z(\lambda_Z^{(1)})$, $mM^{-1}\,cm^{-1}$ (nm)	$\lambda_F^{(1)}[\Phi_F^{(1)}]$, nm[%]	$\lambda_{exc}^{(1)}$, nm	$10^3 Q_Z/Q_F$	$\Phi_{ZE}^{(1)}$, %	$\varepsilon_Z(\lambda_{exc}^{(1)})\Phi_{ZE}^{(1)}$, $mM^{-1}\,cm^{-1}$	$10^3\,k_2$, s^{-1}	$\delta_Z(750)\,\Phi_{ZE}^{(2)}$, GM	$\delta_F(750)\,\Phi_F^{(2)}$, GM
4a	17(330)	404[100]	330	1.8	4	0.7	35	*b*	*b*
4b	28(380)	483[70]	360	1.6	5	1.1	30	0.3	6.2
4c	17(356)	426[100]	350	4.4	7	1.1	130	2.0	4.6

[a] Maxima of single-photon absorption ($\lambda_Z^{(1)}$) and molar absorption coefficients for single-photon absorption at $\lambda_Z^{(1)}$, $\varepsilon_Z(\lambda_Z^{(1)})$ (±5%) of the (*Z*)-cinnamates; wavelength maxima ($\lambda_F^{(1)}$) and quantum yields ($\Phi_F^{(1)}$ ± 10%) of fluorescence emission of the coumarin coproducts upon uncaging after one-photon excitation; excitation wavelength used for the series of uncaging experiments with one-photon excitation, $\lambda_{exc}^{(1)}$; relative brightness of the starting (*Z*)-cinnamate with regard to brightness of the **F** coumarin coproduct at $\lambda_{exc}^{(1)}$, Q_Z/Q_F; quantum yield of (*Z*) to (*E*) photoisomerization after one-photon excitation at $\lambda_{exc}^{(1)}$, $\Phi_{ZE}^{(1)}$ (±10%); action cross section for (*Z*) to (*E*) photoisomerization with one-photon excitation at $\lambda_{exc}^{(1)}$, $\varepsilon_Z(\lambda_{exc}^{(1)})\Phi_{ZE}^{(1)}$ (±5%); rate constant for the thermal (*E*)-cinnamate to coumarin lactonization extracted from continuous illumination experiments, k_2 (±10%); action cross section for (*Z*) to (*E*) photoisomerization with two-photon excitation at $\lambda_{exc}^{(2)} = 750$ nm, $\delta_Z(750)\,\Phi_{ZE}^{(2)}$ (±20%; GM, 1 GM = 10^{-50} cm^4 s/photon) for the (*Z*)-cinnamate, and action cross section for fluorescence emission of the **F** coumarin with two-photon excitation at $\lambda_{exc}^{(2)} = 750$ nm, $\delta_F(750)\,\Phi_F^{(2)}$ (±20%; GM). Except for $\delta_Z(750)\,\Phi_{ZE}^{(2)}$ given in acetonitrile, acetonitrile/Tris pH 7, 20 mM NaCl, 100 mM buffer 1/1 (v/v) is the solvent. $T = 293$ K. See text and Supporting Information. [b] Not evaluated.

with reasonably good yields. These methyloxazolone precursors **3a**−**c** were eventually condensed with a model alcohol to characterize the photophysical and photochemical properties of the resulting cinnamates. *n*-Butanol was retained here because of the neutrality of the butyl chain with regards to the photophysical and photochemical properties of the photoactive backbone. The final model (*Z*)-cinnamates **4a**−**c** were easily obtained with moderate to good yields from condensing 2 equiv of sodium butylate in THF on **3a**−**c**.

The UV−vis absorption spectra of the investigated (*Z*)-cinnamates **4a**−**c** were used to determine their absorption properties upon one-photon excitation. In Tris pH 7, 20 mM NaCl, 100 mM buffer/acetonitrile 1/1 (v/v), the absorption spectrum of **4a**−**c** lies in the 300−400 nm range (Figure 1). Table 1 summarizes the absorption features of **4a**−**c**, maximum $\lambda_Z^{(1)}$ and molar absorption coefficient $\varepsilon_Z(\lambda_Z^{(1)})$ for single-photon absorption, associated with the band at the longest wavelength. The absorption maximum with one-photon excitation $\lambda_Z^{(1)}$ in **4a**−**c** is systematically red-shifted by 20 kJ mol^{-1} (12−18 nm) compared to that of the corresponding methyl-trisubstituted cinnamate in the Porter series.[14] This behavior is most probably related to the additional length of the conjugation pathway due to the *N*-acyl substituent on the double bond compared to the methyl group. As anticipated, $\lambda_Z^{(1)}$ shifts to the red when the donating power of the conjugated phenyl substituent increases. Hence, the longest maximal wavelength of absorption was observed for the diethylamino derivative **4b** ($\lambda_{4b}^{(1)} = 380$ nm) for which the absorption band significantly extends up to 420 nm. Eventually, the molar absorption coefficients at the wavelength of maximal absorption are satisfactorily large: $\varepsilon_Z(\lambda_Z^{(1)}) \approx 2 \times 10^4\ M^{-1}\,cm^{-1}$.

The photochemical properties of the present cinnamate series were first analyzed with one-photon excitation. We used H NMR and UV−vis absorption spectroscopy to analyze the species formed upon illumination of the caged (*Z*)-cinnamates. We showed using **4c** that the uncaging process obeys the reaction displayed in Figure 1: upon one-photon excitation, the caged alcohol **A** (here butanol) is released with the coumarin coproduct **F** in a 1:1 molar ratio (see Supporting Information). This behavior is similar to previous observations in the related Porter series.[14,15,26] Noticeably, the present (*Z*)-cinnamates and the corresponding coumarin coproduct **F** exhibit similar absorption properties; it is thus possible to perform uncaging and excitation of the reporting fluorescent molecule with the same excitation source.

We then analyzed the uncaging kinetics, which is critical in view of possible biological applications.[13,27] Figure 2 displays the typical temporal evolution of the fluorescence emission from a solution of a (*Z*)-cinnamate that is continuously illuminated at a wavelength $\lambda_{exc}^{(1)}$ close to $\lambda_Z^{(1)}$. A large increase of fluorescence emission as a function of time can be observed.

The curves displayed in Figure 2 were analyzed using the model introduced in our previous work.[14] We considered a three-step−six-reaction mechanism (Scheme 2a), which is reduced to a two-step−three-reaction mechanism involving only three rate constants (see Scheme 2b).

We derived the photochemical properties of the present caging groups with one-photon excitation from k_1 and k_{-1} (see Supporting Information). The fit also yields the relative brightness of the starting (*Z*)-cinnamate **Z** with regards to the one of the coumarin coproduct **F**. Table 1 displays the results of the different parameters extracted from these series of irradiation experiments. The coumarin coproduct emits a very strong fluorescence emission in the 400−500 nm range with quantum yields exceeding 60%. In contrast, the brightness of the starting *o*-hydroxycinnamates is uniformly very low at the excitation wavelength required for uncaging. Hence uncaging can be easily evidenced and quantified using the fluorescence increase with the present photolabile protecting groups.

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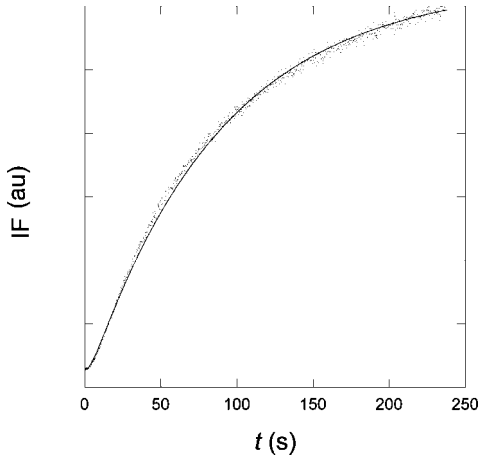


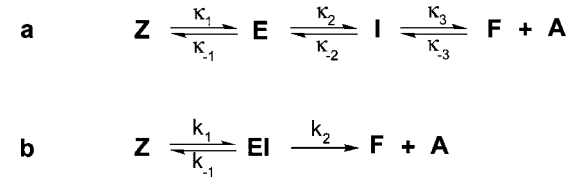
Figure 2. Temporal evolution of the fluorescence emission at λ_{em} = 450 nm from a 5 μM **4c** solution in acetonitrile/Tris pH 7, 20 mM NaCl, 100 mM buffer 1/1 (v/v) upon one-photon irradiation ($\lambda_{exc}^{(1)}$ = 350 nm) at light intensity $I_0(350) = 2.4 \times 10^{-8}$ einstein min $^{-1}$. Markers: experimental data. Line: fit (see Supporting Information). T = 293 K.

The quantum yields of (*Z*)- to (*E*)-cinnamate photoisomerization leading to alcohol uncaging are in the 5% range; this is significant compared to other uncaging photochemistries. Various values ranging from more than 10%[28–30] to less than 1%[31] have been reported for the most popular *o*-nitrobenzyl photoisomerization whereas similar values are reported in the case of the coumaryl photolabile protecting group.[6]

The values for the rate constant k_2 associated to the thermal cyclization of the (*E*)-cinnamates leading to alcohol release and the formation of the coumarin coproduct are in the 10 $^{-1}$ s $^{-1}$ range in the buffer−acetonitrile 1/1 (v/v) mixture.[32] This range is 10- to 100-fold larger than for Porter's cinnamates bearing a doubly substituted double bond.[14] From the latter point of view, the present observation supports the trend already observed in ref 14: the triple substitution pattern of the cinnamate double bond appears as the most favorable mean to accelerate substrate release.

We take advantage of the release upon uncaging of a strongly fluorescent coumarin coproduct in a 1:1 stoichiometry with the caged alcohol to measure the two-photon uncaging action cross section.[14] The kinetic analysis of the uncaging process of **4b** and **4c** leading to the formation of the coumarins **Fb** and **Fc** yields the action cross section for (*Z*)- to (*E*)-cinnamate photoisomerization with two-photon excitation for **4b** and **4c** at $\lambda_{exc}^{(2)}$ = 750 nm in acetonitrile (see Table 1). Both are larger than the 0.1 GM limit that was claimed to be the lowest value relevant for biological applications.[6] In fact, the **4c** value, which is equal to 2.0 GM at 750 nm, is among the largest reported to date.

Scheme 2. (a) Mechanism of uncaging yielding the caged alcohol **A** (here *n*-butanol) together with a coumarin **F** from illumination of the (*Z*) *o*-hydroxy cinnamate **cA** precursor. (b) Reduced mechanism for kinetic analysis. The rate constants associated to each elementary step (a) or to the reduced mechanism (b) are denoted, respectively, κ_i and k_i where the subscript i indicates the corresponding step.



Thus the present *o*-acetoxyphenyl methyloxazolones are favorable precursors yielding robust and attractive photolabile protecting groups with fluorescence reporting for alcohols upon one- and two-photon excitation. The corresponding *o*-hydroxy cinnamate incorporating the targeted alcohol can be easily synthesized. Their maximal wavelength for one-photon absorption can be tuned at the UV−vis limit where the cinnamates exhibit a strong absorption. The caged alcohol is essentially nonfluorescent and quantitatively releases upon one-photon excitation the alcohol substrate and a strongly fluorescent coumarin coproduct in a 1:1 molar ratio. In addition, uncaging and excitation of the reporting fluorescent molecule can be achieved using the same excitation source. We eventually measured two-photon uncaging action cross sections that compare favorably with the best reported values. In conclusion, the present *o*-acetoxy methyloxazolone precursors appear attractive to implement the "optical microsyringe" strategy in biological applications that require precise but not necessarily "fast" two-photon excitation-induced substrate release. In particular, our results suggest that quantitative control of substrate delivery could be achieved by recording the fluorescence emission from the coumarin **F** coproduct that acts as a reporter of the concentration of the photoreleased substrate.

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Supporting Information Available: Screening of the *o*-hydroxyphenyl phenyloxazolone platform and of the 3-acyloylamido coumarins; experimental section; identification of the products from the photochemical isomerization of the cinnamate **4c** with one-photon excitation. This material is available free of charge via the Internet at http://pubs.acs.org.

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