

Rhodamines NN: A Novel Class of Caged Fluorescent Dyes**

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Caged (that is, masked) fluorescent dyes are maintained in their nonfluorescent state by the incorporation of a photochemical labile group. The photosensitive masking group or “molecular cage” can be cleaved-off by irradiation with near-UV light, thereby rendering the dye fluorescent.^[1a-c] Caged fluorescent dyes are of enormous interest for biological imaging because they may be used, for example, for the analysis of protein dynamics,^[1d,e] multicolor fluorescence microscopy,^[1f] and far-field optical nanoscopy.^[1g] *o*-Nitrobenzyl groups are often used as masking groups;^[1a-c] however, the use of these dyes is limited because of their rather complex synthesis and the unwanted by-products liberated by photolysis.

Herein we report on the synthesis and characterization of a novel class of caged compounds—rhodamine NN dyes, which have a 2-diazoketone (COCNN) caging group incorporated into a spiro-9*H*-xanthene fragment (compounds **3** and **9-R** in Schemes 1 and 3, respectively). This very simple and small caging group is the core element of a new class of masked rhodamines that have remarkable properties. The rhodamine NN dyes can be easily prepared and conjugated with biomolecules, they undergo rapid uncaging under standard irradiation conditions (with wavelengths ≤ 420 nm) with formation of highly fluorescent rhodamine derivatives, and they can be used in aqueous buffers, as well as in various embedding media utilized in imaging applications.

In microscopy, these novel rhodamines may be used as labels alone or in combination with conventional fluorescent dyes and switchable rhodamine spiroamides.^[2] In the latter case, they enable new imaging protocols based on the stepwise activation and detection of several fluorescent markers. The combination of the new rhodamine NN derivative (**9-R**) with the photochromic spiroamide of rhodamine S^[2e] and a normal (uncaged) *N,N,N',N'*-tetramethylrhodamine resulted in a monochromatic multilabel imaging

scheme^[3] with low cross-talk, despite using three fluorophores with very similar absorption and emission spectra.^[2d,4]

Rhodamines are very photostable and bright fluorescent dyes which can readily be chemically modified^[5] and caged.^[6] Coumarines^[7] and fluorescein^[8] have also been used as caged fluorescent dyes.^[9] As a photocleavable unit, most of these caged compounds contain a 2-nitrobenzyl group or a derivative with an alkyl or a carboxy group in the α position to the phenyl ring (at the CH₂ group) and/or one or two methoxy groups in the aromatic ring.^[10] Compounds with a free carboxy group are required for bioconjugation. However, the synthesis of caged rhodamines with a free (“second”) carboxy group is difficult and their yield is low.^[6a]

The 2-nitrobenzyl group and its substitutes are bulky and generate toxic, colored, and highly reactive 2-nitrosobenzaldehyde or 2-nitrosobenzophenone derivatives upon photolysis. These compounds or their oligomers are expected to be poisonous to living cells, and they are also colored and interfere with optical measurements.

Other modern caging groups with the required absorption in the near-UV region are also bulky, rather lipophilic, and the procedures for their synthesis and introduction are often complex. For example, 2-(*N,N*-dimethylamino)-5-nitrophenol was reported to give photocleavable phenyl esters.^[11] 7-Diethylamino-4-(hydroxymethyl)-2*H*-chromen-2-one is known to form esters which can be cleaved easily by irradiation at 412 nm.^[12] Derivatives of 8-bromo-7-hydroxyquinoline^[13] and 6-bromo-7-hydroxycoumarines^[14] have also been proposed as light-sensitive protecting groups. The photolysis of these caged compounds generates light-absorbing by-products.

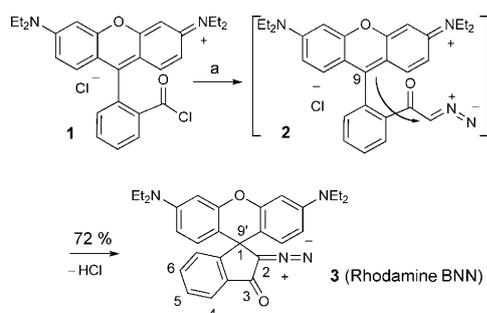
We set out to prepare masked fluorescent dyes without bulky caging groups. A very small 2-diazoketone fragment would be an ideal caging group, provided that it is still possible to integrate this group into the colorless form of a fluorescent dye and then restore the fluorescent state by photolysis. Rhodamines are ideal for this purpose, because they contain a carboxy group, which is known to form colorless and nonfluorescent lactones or lactams with the spiro-9*H*-xanthene fragment. Furthermore, this carboxy group may be transformed into a 2-diazoketone residue.

For the practical realization of this caging strategy, we used rhodamine B as a model compound and performed the reaction of diazomethane with its acid chloride **1**. The yellow crystalline diazoketone **3** was obtained in high yield (Scheme 1).^[15] In the course of the facile caging reaction, the positively charged C9 atom of the xanthene fragment attacks the negatively charged carbon atom of the diazomethane residue in the intermediate **2**. The simultaneous abstraction of a proton furnishes the stable five-membered ring.

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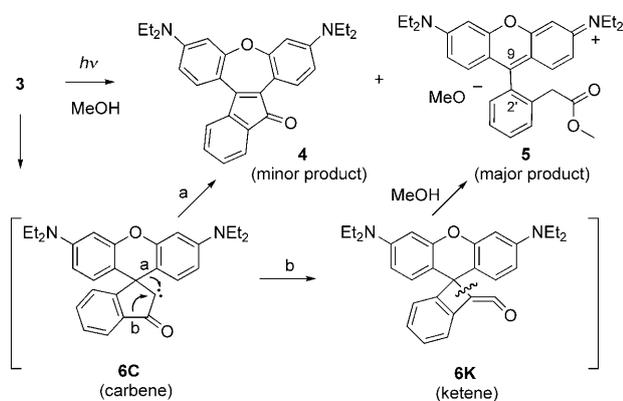
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Scheme 1. Synthesis of rhodamine BNN (**3**) by conversion of rhodamine B acid chloride (**1**) into the spiro-diazoketone form: a) CH_2N_2 in THF, Et_3N , 0°C –RT, 12 h.

Having the model caged compound **3** at hand, we studied its photolysis (Scheme 2). Irradiation of **3** with UV light resulted in two products **4** and **5** being detected and isolated

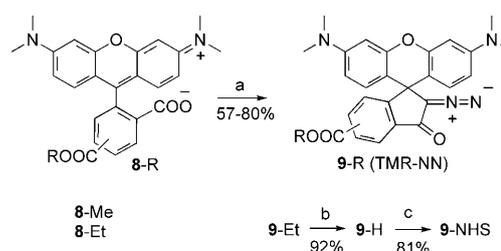


Scheme 2. Photolysis of caged rhodamine BNN (**3**) in methanol using UV light (320 nm) affords the highly fluorescent ester **5** as the main product. According to the HPLC and UV/Vis data, the molar ratio of **5/4** is ca. 3:1 (in methanol).

from the reaction mixture. The formation of the minor nonfluorescent ketone **4** may be explained by migration of the bond “a” in the intermediate carbene **6C**. The red and fluorescent major product **5** was formed by a Wolff rearrangement of carbene **6C** to ketene **6K** (with migration of the bond “b”) followed by addition of methanol to the highly reactive ketene group and breaking of the bond with the quaternary carbon atom.^[16a]

The bioconjugation procedures require reagents with an additional functional group that may be transformed further into an amino or a thiol reactive site. To provide caged rhodamines with an additional carboxy group in the benzoyl fragment of the diazoketone **9-R**, the esters of 5/6-carboxy *N,N,N',N'*-tetramethylrhodamines (TMR) **8** were prepared (Scheme 3).^[15] This approach allows esters with a caged rhodamine fragment to be synthesized and saponified while keeping the spiro diazoketone fragment intact. A mixture of regioisomeric *N*-hydroxysuccinimidyl esters **9-NHS** was used in the immunolabeling experiments.

The absorption spectrum of **3** (Figure 1) shows three maxima at 241 nm ($\epsilon = 60000$), 268 nm ($\epsilon = 33000$), and



Scheme 3. Synthesis of the caged TMR-NN derivative **9-R**. Conversion of 5(6)-alkoxycarbonyl *N,N,N',N'*-tetramethylrhodamine **8-R** into diazoketone **9-R**: a) $(\text{COCl})_2$ in CH_2Cl_2 , RT, 2–4 h; 2 M $\text{Me}_3\text{SiCH}=\text{N}=\text{N}$ in diethyl ether, CH_2Cl_2 , Et_3N , 0°C –RT, 11–18 h; b) 1 M aq NaOH, EtOH, THF, RT, 16 h; c) *N,N,N',N'*-tetramethyl-*O*-(*N*-succinimidyl)isourea tetrafluoroborate, DMF, *iPr}_2\text{NEt}, RT, 2 h.*

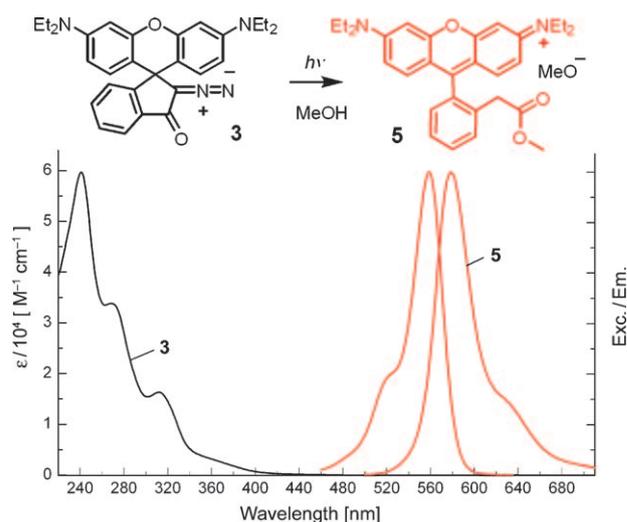


Figure 1. Absorption of rhodamine BNN (**3**; black line; left axis) in acetonitrile; absorption and emission spectra of the uncaged compound **5** in methanol (red lines; right axis).

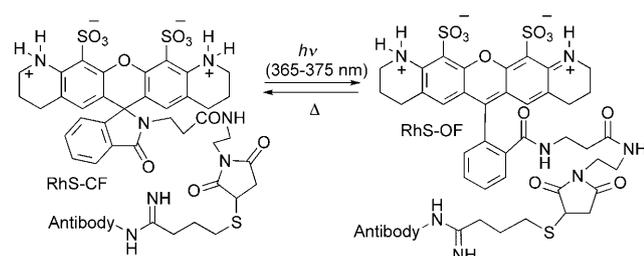
312 nm ($\epsilon = 17000$). α -Benzoyl diazoalkanes are known to absorb at about 320 nm with $\epsilon \approx 12000$. Thus, the maximum at 312 nm corresponds to the electronic transition centered at the benzoyl diazomethane chromophore, although the 9*H*-xanthene fragment is probably also involved. However, the weakest bond in the whole molecule is broken in the course of the photolysis through efficient energy transfer, which results in elimination of dinitrogen. The surrounding solvent, as present in typical biological embedding media, was found to exhibit only a minor influence on the uncaging reaction (Figure S1 in the Supporting Information).

In fact, the most prominent and unusual feature of the absorption spectra of **3** and **9-R** is the shoulder that extends from 375 nm up to 420 nm. It is therefore not surprising that an efficient photoactivation (uncaging) reaction is not only possible by irradiation at 360–375 nm, but also at 405–420 nm (Figure S2 in the Supporting Information).

The absorption and emission maxima of rhodamine derivative **5** isolated after photolysis of diazoketone **3** occur at 559 nm ($\epsilon \approx 66000$) and 579 nm, respectively (Figure 1). The fluorescence quantum yield was found to be 0.37.^[17] After uncaging, the spectroscopic properties of the initial fluores-

cent dye do not change considerably (Table S1 in the Supporting Information). The Wolff rearrangement results in an elongation of the carbon chain by an additional methylene group between the *o*-substituted phenyl residue and the methoxycarbonyl fragment in **3**. This elongation diminishes the steric hindrance and facilitates rotation about the single C9–C2' bond (compared with the situation with a 2-carboxyphenyl group attached to C9). The free rotation of molecular groups in the excited state may dissipate the energy and reduce the probability of the radiative $S_1 \rightarrow S_0$ transition, thereby slightly decreasing the fluorescence quantum yield. We reasoned that the observed fluorescence quantum yields (Table S1 in the Supporting Information) of the uncaged dyes would be sufficient to provide bright fluorescence images, and that the traces of the side products **4** would not interfere with the imaging.

In conventional multicolor microscopy, the fluorophores are distinguished by their absorption and emission spectra, which is prone to producing artifacts that arise from chromatic aberrations.^[3,18] To circumvent this problem and reduce the number of excitation sources and detection channels, we combined three fluorophors with similar absorption and emission spectra: 5/6-carboxy-*N,N,N',N'*-tetramethylrhodamine (TMR **8-H**), TMR-NN derivatives (**9-R**, see Scheme 3), and the spiroamide of rhodamine S^[2e] (RhS, see Scheme 4). To discriminate between these three dyes, the



Scheme 4. Photoactivation of the bioconjugates obtained from the colorless “closed” form (CF) of spiroamide (RhS-CF) and antibodies produces the brightly fluorescent “open” form (RhS-OF), which returns to the initial RhS-CF in the dark.^[15]

respective fluorescent forms of which have highly similar absorption and excitation spectra (see Table S1 in the Supporting Information), their individual switching and uncaging characteristics were exploited: TMR is not switchable, TMR-NN can be uncaged by illumination with UV light ($\lambda \approx 405$ nm), and the spiroamide of RhS can be photoactivated with UV light ($\lambda \leq 375$ nm).

For imaging, we labeled three cellular structures within cultivated mammalian Ptk2 cells by indirect immunofluorescence: The actin cytoskeleton, the microtubule cytoskeleton, and the peroxysomes. Phalloidin labeled with TMR was used for the visualization of actin, whereas two different secondary antibodies coupled with caged TMR (TMR-NN) or RhS were utilized for the other structures. The labeled cells were imaged according to the scheme shown in Figure 2a: First, phalloidin-TMR was imaged (excitation 546/12 nm, emission 600/40 nm; ca. 40 W cm⁻²). All the TMR fluorophores were then irreversibly bleached by intensive irradiation with green

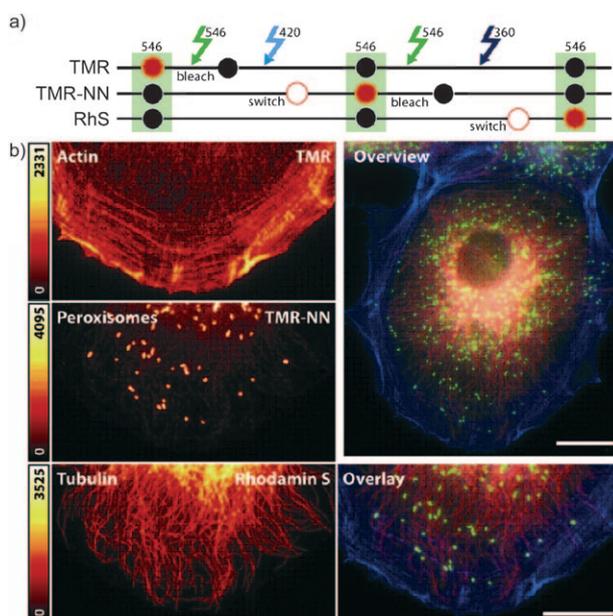


Figure 2. Monochromatic multilabel imaging. a) Imaging scheme (see text for details). b) Application of this imaging scheme to immunolabeled cultured mammalian (PtK2) cells. First, the actin cytoskeleton labeled with TMR-phalloidin, then the peroxysomes, indirectly immunostained with TMR-NN, and finally the microtubule cytoskeleton, indirectly immunolabeled with RhS, were visualized. For clarity, different colors are used in the overview and overlay images. Note that no image processing has been applied on the data. Scale bars: 10 μ m.

light (546 nm). Since neither uncaged TMR-NN nor the “closed” RhS absorb green light, this irradiation step did not influence these dyes. The TMR-NN was then uncaged by irradiation with UV light (420/30 nm; ca. 35 W cm⁻²; see Figure S3 in the Supporting Information). Importantly, despite the absorption maximum of RhS-CF being at 331 nm (in water),^[2e] it is insensitive to light with $\lambda = 420$ nm at the intensities used in this experiment. The peroxysomes were then imaged by exciting the fluorescence of the uncaged TMR-NN with green light. Subsequently, the uncaged fluorophores were irreversibly bleached with green light. Finally, the microtubule cytoskeleton labeled with RhS was imaged. Thus, the closed form was photoactivated with UV light (360/40 nm; ca. 10 W cm⁻²), which resulted in the brightly fluorescent open-form species (Scheme 4 and Figure S4 in the Supporting Information), and imaged using green light.

This imaging scheme is monochromatic, as it uses only green light for excitation and a single detection channel. Although some cross-talk was evident,^[15] the visualization of individual channels without further image processing was possible. Hence this approach may be used for (co)localization studies with three dyes by using just one detector channel.

The described caging procedure can be applied to all *N,N,N',N'*-tetraalkylrhodamines, carbopyronines, and, probably, other cationic dyes (for example, triarylmethyl derivatives). Therefore, the present approach can be extended to a larger set of (fluorescent) dyes (and excitation sources). Moreover, the compact structure and the very small size of

the caging group facilitate the ability of rhodamine NN to cross membranes of living cells.^[19] In combination with site-specific labeling protocols^[20] realized for the Halo and SNAP tags, the availability of the novel cell-permeable and caged rhodamines may extend the set of live cell labeling strategies based on binding genetically encoded protein tags with organic fluorophores. The spatially restricted photoactivation of rhodamine NN followed by tracking of the uncaged molecules may allow measurements of molecular dynamics (for example, determination of diffusion parameters or flow velocities). The combination of rhodamine NN with nanoscopic techniques^[21] (for example, stimulated emission (STED) or single-molecule switching followed by localization^[2e]) is expected to provide additional information on subcellular structure. Moreover, measurements of the FRET signal of the newly uncaged fluorescent areas situated in proximity to the “permanent” colored regions are likely to reveal additional structural details over these relatively short distances (1–10 nm).

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