Masked Rhodamine Dyes of Five Principal Colors Revealed by Photolysis of a 2-Diazo-1-Indanone Caging Group: Synthesis, Photophysics, and Light Microscopy Applications

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Dedicated to Armin de Meijere on the occasion of his 75th birthday

Abstract: Caged rhodamine dyes (Rhodamines NN) of five basic colors were synthesized and used as "hidden" markers in subdiffractional and conventional light microscopy. These masked fluorophores with a 2-diazo-1-indanone group can be irreversibly photoactivated, either by irradiation with UV- or violet light (one-photon process), or by exposure to intense red light ($\lambda \sim 750$ nm; two-photon mode). All dyes possess a very small 2-diazoketone caging group incorporated into the 2-diazo-1-indanone residue with a quaternary carbon atom (C-3) and a spiro-9*H*-xanthene fragment. Initially they are non-colored (pale yellow), non-fluorescent, and absorb at $\lambda = 330$–350 nm (molar extinction coefficient (ε) $\approx 10^4\,M^{-1}cm^{-1}$) with a band edge that extends to about $\lambda =$ 440 nm. The absorption and emission bands of the uncaged derivatives are tunable over a wide range ($\lambda = 511$–633 and 525–653 nm, respectively). The unmasked dyes are highly colored and fluorescent ($\varepsilon =$ 3–$8\times10^4\,M^{-1}cm^{-1}$ and fluorescence quantum yields (ϕ) = 40–85% in the unbound state and in methanol). By stepwise and orthogonal protection of carboxylic and sulfonic acid groups a highly water-soluble caged red-emitting dye with two sulfonic acid residues was prepared. Rhodamines NN were decorated with amino-reactive *N*-hydroxysuccinimidyl ester groups, applied in aqueous buffers, easily conjugated with proteins, and readily photoactivated (uncaged) with $\lambda = 375$–420 nm light or intense red light ($\lambda = 775$ nm). Protein conjugates with optimal degrees of labeling (3–6) were prepared and uncaged with $\lambda =$ 405 nm light in aqueous buffer solutions ($\phi = 20$–38%). The photochemical cleavage of the masking group generates only molecular nitrogen. Some 10–40% of the non-fluorescent (dark) byproducts are also formed. However, they have low absorbance and do not quench the fluorescence of the uncaged dyes. Photoactivation of the individual molecules of Rhodamines NN (e.g., due to reversible or irreversible transition to a "dark" non-emitting state or photobleaching) provides multicolor images with subdiffractional optical resolution. The applicability of these novel caged fluorophores in super-resolution optical microscopy is exemplified.

Introduction

Fluorescent dyes that can be converted into a non-fluorescent state by incorporation of a photosensitive masking group represent unconventional and indispensable markers applicable in chemistry, life- and materials sciences, as well as in optical microscopy. During the course of the masking procedure, the initial strong color of a fluorescent dye disappears and it becomes practically colorless or pale yellow. Irradiation with UV light removes or transforms the molecular "cage" and creates the colored, fluorescent form of a dye with the same (or slightly modified) structure. Small photosensitive groups incorporated into the masked fluorescent dyes are advantageous because they enable production of compact, cell-permeable labeling reagents for biological imaging. In particular, there are many promising, but yet unexplored, applications of caged fluorescent dyes in optical microscopy. They are expected to perform extremely well in super-resolution microscopy based on photoswitching of single molecules, (co)localization of various

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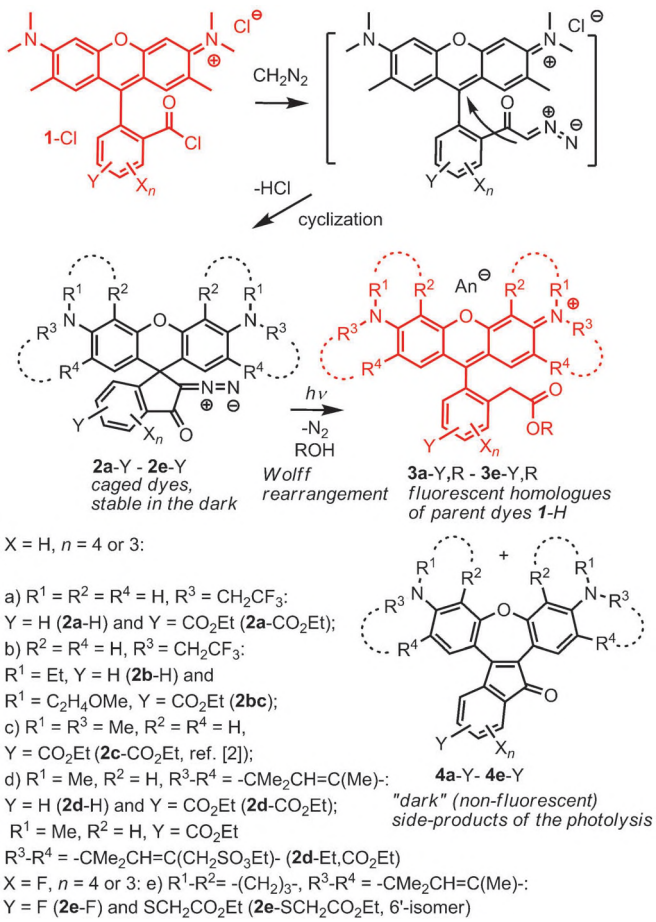
biological objects, tracking the dynamic processes in living cells, and multicolor experiments.[1] Recently, new types of caged fluorescent dyes, Rhodamines NN and Carbopyronines NN, have been reported.[2] They bear a 2-diazoketone (COCNN) caging group attached to either a spiro-9*H*-xanthene fragment in *N*,*N*,*N*′,*N*′-tetraalkylrhodamines[2a] or a 9,10-dihydro-10,10-dimethylanthracene residue in carbopyronines with fully alkylated nitrogen atoms.[2b] In both cases, the photosensitive assembly contains a 2-diazo-1-indanone residue with a quaternary carbon atom (C-3) (Scheme 1). In this respect, the structure of these compounds represents the novel "caging" approach, which is quite dissimilar to the common strategies based on the use of bulky masking groups.[1a,3] Most traditional caged compounds contain a 2-nitrobenzyl residue as a photocleavable unit (or its derivatives with an alkyl or carboxyl group attached to the benzylic carbon atom and/or one or two methoxy groups in the aromatic ring).[1a,3a–f] The synthesis of the masked rhodamines with substituted 2-nitrobenzyl caging groups is rather complex, their structures are large and the photolysis produces highly reactive, toxic, and colored 2-nitrosobenzaldehyde or 2-nitrosobenzophenone derivatives.[3a–c,f] Other modern photocleavable groups that provide the required absorption in the near-UV region are also bulky, rather lipophilic, and the procedures for their synthesis and introduction are often difficult. For example, 2-(*N*,*N*-dimethylamino)-5-nitrophenol was reported to give photoremovable phenyl esters.[4] 7-Diethylamino-4-(hydroxymethyl)-2*H*-chromen-2-one is known to form esters which may easily be cleaved by irradiation at $\lambda = 412$ nm.[5] Other heterocycles, 8-bromo-7-hydroxyquinolines[6] and 6-bromo-7-hydroxycoumarins,[7] have been proposed as light-sensitive protecting groups. The irradiation of the caged compounds containing these photosensitive groups also generates light-absorbing byproducts and it is not yet clear if these groups may be widely applied for masking of the fluorescent dyes. In contrast, the reduced size of the 2-diazoketone caging group in Rhodamines NN and Carbopyronines NN results in compact structures of the caged dyes and their photoactivation conveniently generates a molecule of innocuous dinitrogen as a leaving group or byproduct (Scheme 1).



Scheme 1. General synthetic route to the masked (colorless) rhodamine derivatives **2a**-Y–**2e**-Y that contain a photosensitive spiro-diazoketone fragment. Irradiation of compounds **2a**-Y–**2e**-Y with UV or violet light affords brightly fluorescent dyes of various colors. For the mechanism of the formation of the "bright" and "dark" products see reference [2].

All conventional caging groups are directly attached to the fluorogenic unit as a bulky residue. For example, 2-nitrobenzyl groups participate in the formation of the two urethane moieties, which involves both nitrogen atoms in rhodamines, and provide the presence of the colorless (closed) lactone form.[1a,3a–g] A similar approach was also used in the masking of coumarins[8] and fluorescein,[9] which were also prepared as non-fluorescent derivatives.[10] Urethane or acetyl caging groups limit the structural variety of rhodamines, carbopyronines, and carborhodols[11] that can be masked because they occupy one position at the nitrogen atom and preclude the possibility to add an additional group to this atom of the fluorogenic unit (to tune the photophysical properties of the dye). It is well-known that the alkylation of the nitrogen atom in xanthene dyes (and coumarins) provides relatively strong bathochromic and bathofluoric shifts (+25 nm) and represents an important tool in dye design.[12] However, for the reason mentioned above, the former masking strategy was limited to $-NH_2$ and N-monosubstituted rhodamines and carbopyronines.[13]

In the present report we show that the new approach to caged rhodamines with a 2-diazoindanone unit enables masking of lipophilic *N*,*N*′-bis-(2,2,2-trifluoroethyl)rhodamines, hydrophilic rhodamines with sulfonic acid residues, as well as red-emitting tetrafluorophenyl-substituted rhodamines. Rhodamines NN may be decorated with an additional carboxyl group and hydrophilic residues. The amino-reactive *N*-hydroxysuccinimidyl (NHS) esters can be easily synthesized and conjugated with proteins. These masked fluorescent dyes can be readily photoactivated using standard irradiation sources—lasers or lamps ($\lambda \leq 440$ nm, or under two-photon conditions)—and form highly fluorescent derivatives of various colors. They may be applied in aqueous buffers, as well as in various embedding media used in optical microscopy and nanoscopy, for obtaining images with subdiffractional optical resolution (e.g., by photoactivation of single molecules).[1b–k,3f] As examples, caged rhodamine dyes of five basic colors were synthesized and utilized as fluorescent markers in subdiffrac-

tional and conventional microscopy in a study on the scope and limitations of an approach that utilizes 2-diazoketone as a caging group. The whole spectral area, including the red region, can be operated over because all principal colors are available. The latter is especially important in biology and optical microscopy because red light is less invasive and does not interfere with autofluorescence of cells and their components. Importantly, all five dyes bear linkers for conjugation to proteins. The selective and sequential orthogonal protection/deprotection sequence applicable to carboxylic and sulfonic acid groups has been developed as a challenging and useful tool in dye design. As a result, the first example of a caged dye decorated with two sulfonic acid groups is reported. An alternative way to make a caged dye highly water soluble is to attach a solubilizing moiety. In an illustrative example a dipeptide with a sulfocysteine residue was introduced.

Our synthesis is applicable only to *N*,*N*′-bis-(2,2,2-trifluoroethyl)rhodamines or xanthenes with disubstituted nitrogen atoms. In this respect it is complimentary to the synthetic strategy mentioned above (based on the attachment of the caging groups to the fluorogenic nitrogen atoms).

Results and Discussion

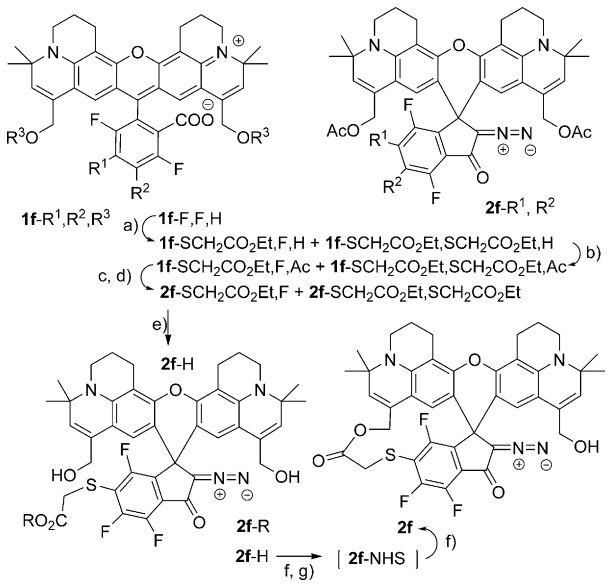
Synthetic strategies: scope and limitations

Before photoactivation, Rhodamines NN (**2a**-Y–**2e**-Y, Scheme 1) are essentially non-fluorescent. Preparation of the chemically stable non-fluorescent compounds **2a**-Y–**2e**-Y is only possible if the initial fluorescent dye can form a stable acid chloride **1** (or mixed anhydride), which reacts further with diazomethane.[2] This requirement poses some limitations on the substitution pattern at the nitrogen atoms in the parent rhodamine, from which the acid chloride or mixed anhydride has to be prepared. The primary amino groups (e.g., in Rhodamine 110) or secondary amino groups (e.g., in Rhodamine 6G) are unacceptable, as they readily react with activating agents used in the preparation of acyl chlorides **1**-Cl, as well as with mixed anhydrides or active esters generated from the carboxyl group. We established that oxalyl chloride, (chloromethylene)dimethyliminium chloride, or $POCl_3$ (in the case of the more robust compounds) provide acceptable yields of the required spiro diazoketones in a subsequent reaction with diazomethane. The latter should be introduced after complete transformation of the free carboxyl group into the acid chloride and removal of the activating agent. All attempts to activate the carboxyl group by the formation of mixed anhydrides ($ClCO_2R$ and Et_3N in THF) resulted in low conversions and low yields of spiro diazoketones. Under these conditions, the corresponding esters (with COOR groups) were found to be the major products. Presumably, they were formed in the course of decarboxylation of the intermediate mixed anhydrides.

Another characteristic feature of Rhodamines NN is the inherent lipophilic character of their core and, as a result, their poor solubility in aqueous solutions. These compounds possess a highly symmetric, rigid, and compact photosensitive unit based on a 2-diazo-2,3-dihydro-1*H*-indene-spiro[1,9′][9*H*]xanthen-3-one fragment (Scheme 1). Without additional polar groups, their solubility in aqueous buffers is negligible. All bioconjugates of the fluorescent markers are applied in aqueous solutions, and the labeling reagent is dissolved in a minimal amount of organic solvent miscible with water (e.g., DMF or DMSO). Rhodamines NN, designed as labeling reagents for biomolecules, need polar groups to enhance their hydrophilic properties to be able to achieve the recommended labeling efficiencies and minimize the change in the properties of the formed bioadducts. In this respect, it is advantageous that upon photoactivation the initial caged dyes turn into more hydrophilic zwitterionic structures, which are less prone to aggregation or precipitation from the aqueous buffers. On the other hand, the polar groups required to provide hydrophilic properties must be compatible (at least, in their protected form) with activation agents (e.g., oxalyl chloride or $POCl_3$) and other reagents required during the course of the synthesis.

Compounds **2a**-CO_2Et, **2bc**, **2c**-CO_2Et,[2a] **2d**-Et,CO_2Et, and **2e**-SCH_2CO_2Et (Scheme 1) have an ethyl ester group, which, after deprotection, liberates the free carboxylic acid residue and can be used for bioconjugation. Ethyl esters **2a**-CO_2Et and **2c**-CO_2Et[2a] were saponified, transformed into the corresponding NHS esters (see Scheme 3) and used in the reaction with secondary antibodies without further structural modifications (without additional polar groups). The model compound **2b**-H is very lipophilic, and the corresponding NHS ester derived from it (by using rhodamine **1bb** as a starting material; Scheme 3) was expected to be insoluble in water. Therefore, we prepared a more hydrophilic version of rhodamine **1bb**, compound **1bc**. Conversion of dye **1bc** into diazoketone **2bc** then activation resulted in NHS ester **2bc**-CONHS, which was applicable for conjugation with proteins under the standard conditions (see Scheme 3). Aside from the straightforward addition of the methoxy groups, we also used other ways of increasing the hydrophilic properties of Rhodamines NN. For example, we synthesized compound **2d**-Et,CO_2Et with ethyl sulfonate and ethyl ester groups. Free sulfonic acid residues are very efficient solubilizing groups, but they require adequate protection (e.g., as alkyl sulfonates) before activation of the carboxyl group and its conversion into the spiro-diazoketone cage (see Scheme 4). This strategy was successful for the red-emitting dye **2db**, but did not work in the case of tetrafluororhodamine **2e**-SCH_2CO_2Et (6′ isomer) decorated with two sulfonic acid residues.[2b]

As an alternative, we tried to use the same fluorophore as in compound **2e**-SCH_2CO_2Et with two hydroxyl groups as polar residues (Scheme 2). The building block **1f**-F,F,H is used in the synthesis of several bright fluorescent dyes that emit red light ($\lambda = 650$–750 nm) and is available in quantities of several-hundred milligrams.[12m–n,14] Hydroxyl groups considerably increase the polarity of organic compounds. In particular, the caged dye **2f**-H with two hydroxyl groups (Scheme 2) is much more soluble in aqueous solutions (at pH > 7) than the less polar analogue **9**-H (see Scheme 5) and the reversed-phase HPLC mobility of the carboxylic acid **2f**-H is much higher than that of **9**-H. First, tetrafluoride **1f**-F,F,H reacted with ethyl thioglycolate to give an inseparable mixture of mono- and di-substituted

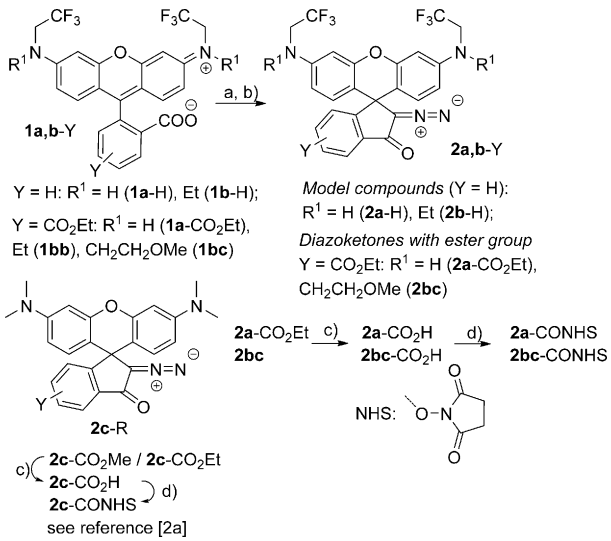


Scheme 2. Synthesis of the caged red-emitting rhodamines **2 f**-R with free hydroxyl and carboxyl groups and their macrolactonization. a) $HSCH_2CO_2Et$, MeCN or DMF, Et_3N, −12–18 °C; b) $(CH_3CO)_2O$, pyridine, 0 °C–RT, overnight; c) $(COCl)_2$, CH_2Cl_2, DMF, 0 °C–RT, 2–6 h; d) CH_2N_2, Et_2O (Et_3N),[a] 0 °C–RT, 11–18 h; e) 1 M NaOH (aq), THF/H_2O, 0 °C– RT; f) *N*-hydroxysuccinimide, HATU, Et_3N, DMF, RT; g) *N*-hydroxysuccinimide, CH_2Cl_2, *N*,*N*′-dicyclohexylcarbodiimide (DCC), RT. [a] The residual acidic impuriries react with diazomethane and have to be removed completely. If so, the presence of triethyl amine is not necessary (but it may be added to neutralize the acidic compounds).

products in ratio of approximately 2:1. Then, the hydroxyl groups in compounds **1 f**-SCH_2CO_2Et,F,H and **1 f**-SCH_2CO_2Et,SCH_2CO_2Et,H were protected by acetylation and the mixture of dyes **1 f**-SCH_2CO_2Et,F,Ac, and **1 f**-SCH_2CO_2Et,SCH_2CO_2Et,Ac was subjected to the standard caging procedure (Scheme 2). The free carboxyl group was converted into an acid chloride and the latter reacted with diazomethane to give the yellow spiro diazoketones **2 f**-SCH_2CO_2Et,F and **2 f**-SCH_2CO_2Et,SCH_2CO_2Et. This is the decisive step of the whole sequence. The relatively non-polar esters **2 f**-R^1,R^2 are easily separated by column chromatography. Deacetylation and saponification of **2 f**-SCH_2CO_2Et,F afforded the required dihydroxyacid **2 f**-H. However, the active NHS ester **2 f**-NHS was unstable and could not be isolated in a pure state. If the reaction of acid **2 f**-H with *N*-hydroxysucccinimide was performed in the presence of (7-azabenzotriazol-1-yl)tetramethyluronium hexafluorophosphate (HATU) and base, the macrocyclic lactone **2 f** was formed. This interesting result proved unequivocally (and for the first time) the position of the sulfur atom (C-6′) in all monosubstituted derivatives **2 e** (Scheme 1), and **1 f**-SCH_2CO_2Et,F,H and **2 f** (Scheme 2), as well as in other commercial fluorescent dyes with trichloro- or difluoro-phenyl rings.[15] For steric reasons, the macrolactonization is only possible if the fluorine atom at the opposite position to the carbonyl group is substituted. If the reaction of *N*-hydroxysuccinimide with acid **2 f**-H was performed in the absence of base the active ester **2 f**-NHS was formed. Unfortunately, after isolation by column chromatography or preparative HPLC, **2 f**-H decomposed upon concentration or lyophilization of the fractions. These results forced us to look for another strategy to attach the hydrophilic groups to spiro diazoketone **2 e**.

Preparation of Rhodamines NN: Masked dyes with a 2-diazo-2,3-dihydro-1*H*-indene-spiro[1,9′][9*H*]xanthen-3-one fragment

During the course of caging, rhodamines are transformed into the corresponding acid chlorides, which further react with diazomethane or trimethylsilyl diazomethane.[2a] Rhodamines that emit green or orange light (Rhodamine 110 or Rhodamine 19) contain primary or secondary amino groups, which are incompatible with all reagents required for the strong activation of the carboxylic acid residue (oxalyl or thionyl chlorides, $POCl_3$, or (chloromethylene)dimethyliminium chloride). However, if a 2,2,2-trifluoroethyl group replaces one hydrogen atom in an amino group of any rhodamine dye (e.g., Rhodamine 110), a derivative with the same absorption and emission spectra is obtained, the carboxylic residue of which can be converted to an ester, or even an acid chloride.[12l,16] Thus we used rhodamines **1 a**-H and **1 a**-CO_2Et as starting compounds to prepare masked yellow-green dyes, diazoketones **2 a**-H and **2 a**-CO_2Et, respectively (Scheme 3). The ethyl ester group in compound **2 a**-CO_2Et can be saponified and used for further transformations. The orange dyes with green fluorescence (**1 b**-H and **1 bc**) were masked by a similar strategy (Scheme 3). Dye **1 bc** is slightly more hydrophilic than rhodamine **1 bb**, and its derivatives with *N*-(2-methoxyethyl) residues (**2 bc**, **2 bc**-CO_2H, and **2 bc**-CONHS) were prepared rather than the corresponding *N*-ethyl counterparts. Though the

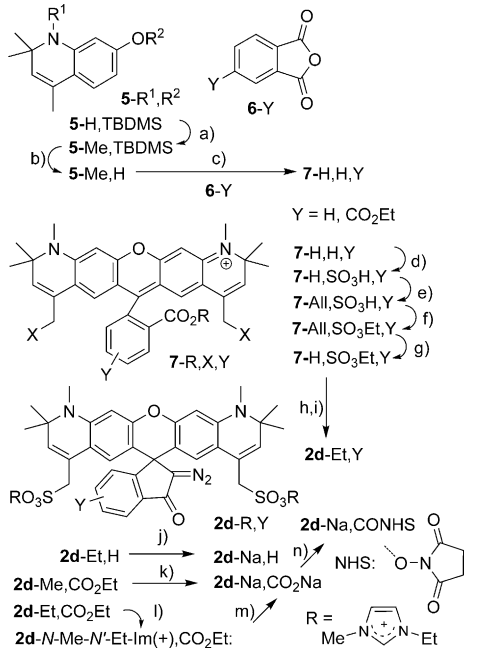


Scheme 3. Synthesis of the caged green- and orange-fluorescent Rhodamines NN. Conversion of the yellow (**1 a**-H/CO_2Et) and orange (**1 b**-H and **1 bc**) rhodamines into diazoketones **2 a**-H and **2 b**-H (model compounds), ethyl esters **2 a**-CO_2Et and **2 bc**, carboxylic acids **2 a**-CO_2H and **2 bc**-CO_2H, and the corresponding amino-reactive NHS esters. a) $POCl_3$ in 1,2-dichloroethane, 70 °C, 2 h or $(COCl)_2$ in CH_2Cl_2, DMF, 0 °C–RT, 2–4 h; b) CH_2N_2 in Et_2O (Et_3N),[a] CH_2Cl_2 or MeCN, 0 °C–RT, 1.5–18 h; c) 1 M NaOH (aq), EtOH or THF, RT, 16 h; d) TSTU or, alternatively, *N*-hydroxysuccinimide with HATU, Et_3N, MeCN or CH_2Cl_2, RT. [a] See note to Scheme 2.

masked compound **2bc**-CONHS does not have highly polar groups, it was successfully used for labeling of bovine serum albumin (BSA) and antibodies under standard conditions: **2bc**-CONHS (0.2 mg) in DMF (40 μL) was added to an aqueous solution of protein (1–2 mg in 1–2 mL of an aqueous buffer with pH ca. 8.5; see Supporting Information for details). However, it was very difficult to couple the relatively lipophilic amino-reactive compounds **2bc**-CONHS or **2c**-CONHS (prepared from the masked 5′/6′-carboxy tetramethylrhodamines)[2a] with very polar 5-(3-aminoallyl)uridine-5′-*O*-triphosphate (AA-UTP). For an acceptable conversion, this reaction required a great excess of these markers, which were dissolved in DMF and added to an aqueous solution of AA-UTP that contained DMF. However, even under optimized conditions, the yield was low and the product could only be isolated by preparative HPLC.

These results indicate that the masked red-emitting rhodamines **2d**-CO_2Et and **2e**-SCH_2CO_2Et, which possess extended and bulky fluorophores (and an ethyl ester group; see Scheme 1), require hydrophilic groups to facilitate their use in aqueous solution and in various bioconjugation procedures. These groups may be attached in the course of post-synthetic modifications of the masked dye core or they may be present in the initial fluorescent dye before the caging procedure is applied.

In this respect, red-emitting and water-soluble rhodamines with sulfonic acid residues (**7**-H,SO_3H,Y, Scheme 4) represent interesting and promising candidates to select and test the protecting groups for the sulfonic acid sites. The appropriate protecting groups should be compatible with the conditions of the diazoketone synthesis. Moreover, the deprotection of these acidic centers should not affect the spiro-diazoketone group. Another challenge was to provide additional protection for at least one of the two different carboxyl groups in the presence of the sulfonic acid residue(s) in such a way that these reaction centers may be addressed independently and in a certain reaction sequence. This issue is of general importance and there is still no universal solution for this problem for many popular fluorescent dyes, for example, Alexa Fluor 594.[17]



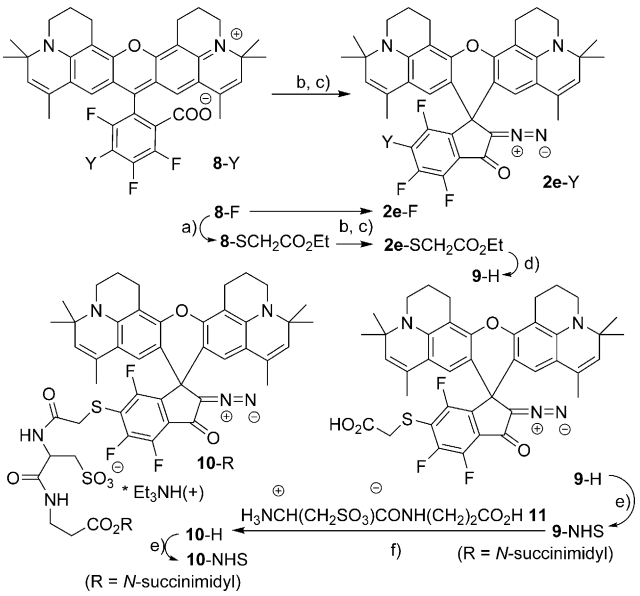
Scheme 4. Synthesis of caged hydrophilic rhodamines **2d** decorated with sulfonic and (activated) carboxylic acid residues. a) MeI, Cs_2CO_3, DMF, RT, overnight; b) $Bu_4NF{\cdot}3H_2O$, THF, 0–4 °C, overnight; c) *o*-dichlorobenzene, 170–190 °C, 8 h; d) concentrated H_2SO_4, RT; e) $CH_2{=}CHCH_2OH$, DCC, 4-dimethylaminopyridine (DMAP), CH_2Cl_2, RT; f) Et_3OBF_4, *i*Pr_2NEt, MeCN, RT; g) $Pd(Ph_3P)_4$, HCOOH, *i*Pr_2NEt, MeCN, RT; h) $(COCl)_2$, CH_2Cl_2, 0 °C–RT, 2–4 h; i) CH_2N_2, Et_2O (Et_3N),[a] 0 °C–RT, 11–18 h; j) 1 M NaOH (aq), THF, 50–60 °C, 8–16 h; k) 0.17 M NaOH in MeOH/H_2O, 50 °C, 7–8 h; l) *N*-methylimidazole, EtOH, 70 °C, 16 h; m) 0.1 M NaOH (aq), 0 °C–RT, 2 h; n) *N*-hydroxysuccinimide, HATU, Et_3N, DMF, RT. [a] See note to Scheme 2.

Compounds **7**-H,SO_3H,Y are readily available by sulfonation of the corresponding precursors **7**-H,H,Y (Y = H, CO_2Et), which, in turn, can be easily synthesized from phenol **5**-H,TBDMS[18] (Scheme 4; TBDMS = *tert*-butyldimethylsilyl). The condensation reaction of **5**-Me,H[16] with 4-ethoxycarbonyl phthalic anhydride (**6**-CO_2Et) was performed in boiling dichlorobenzene and afforded two diastereomers of rhodamine **7**-H,H,CO_2Et with intact ethyl ester groups. This result is very important because the protection of one carboxyl group has been provided automatically (by selection of the appropriate starting material). The synthetic strategy was first elaborated for model compound **7**-H,SO_3H,H (without the second carboxyl group), but the same approach is applicable to all sulfonated rhodamines **7**-H,SO_3H,Y (Y = H, CO_2Et) shown in Scheme 4 and, for example, Alexa Fluor 594 diastereomers (with 5′- and 6′-carboxyl groups).[17] First, the sterically hindered free carboxyl group CO_2R (R = H) in compound **7**-R,X,Y was protected as an allyl (All) ester, then the sulfonic acid residues were alkylated with Meerwein salt [triethyloxonium tetrafluoroborate (Et_3OBF_4)] in the presence of a base. Subsequently, the allyl ester was cleaved with $Pd(PPh_3)_4$ and HCOOH (Scheme 4). The crucial intermediate, **7**-H,SO_3Et,H, was not particularly stable; upon drying (and in the course of further transformations) it partially rearranged into the corresponding ethyl carboxylate with one SO_3Et and one SO_3H group. However, **7**-H,SO_3Et,H was successfully used for the synthesis of 2-diazoketone **2d**-Et,H (by the standard protocol with oxalyl chloride and diazomethane). The same methodology was used for **7**-H,SO_3Et,CO_2Et, with an additional ethyl carboxylate group. As we established, the migration of an alkyl residue from the sulfonic acid to the carboxylic acid site took place upon concentration of the solutions and drying. Therefore, to prevent this, we added small amounts of chlorobenzene to solutions of compounds **7**-H,SO_3Et,H or **7**-H,SO_3Et,CO_2Et and did not evaporate them to dryness. Rhodamine **7**-H,SO_3Et,CO_2Et was successfully used for the preparation of diazoketone **2d**-Et,CO_2Et. The corresponding methyl sulfonates **2d**-Me,Y (Y = H, CO_2Et) were readily obtained when more reactive Me_3OBF_4 was used. However, the intermediates **7**-H,SO_3Me,Y (not shown in Scheme 4) were found to be even less stable than the corresponding ethyl sulfonates **7**-H,SO_3Et,Y because the methyl groups migrated to the neighboring, anionic carboxylic acid groups more easily than ethyl groups.

In alkaline solution, methyl sulfonates are more susceptible to hydrolysis than ethyl sulfonates. However, both are much more resistant to sodium hydroxide than ethyl carboxylates

(due to different mechanisms of the cleavage reactions: S_N2 for alkyl sulfonates and nucleophilic addition for methyl or ethyl esters). The alkyl sulfonate groups in compounds **2d**-Et,H and **2d**-Me,CO_2Et may be deprotected by gentle heating in dilute aqueous NaOH solution without affecting the spiro-diazoketone group. Ethyl sulfonate **2d**-Et,CO_2Et was the most reliable and stable intermediate for the successful completion of the whole reaction sequence. However, it could only be cleaved without decomposition of the diazoketone residue by heating with *N*-ethyl imidazole (a strong nucleophile). Saponification of the ethyl ester at the final step afforded the water-soluble salt **2d**-Na,CO_2Na, which could be converted into the very hydrophilic NHS ester **2d**-Na,CONHS (Scheme 4).

Finally, we prepared masked red-emitting rhodamine **2e**-F as a model compound and used its derivative **2e**-SCH_2CO_2Et for the synthesis of hydrophilic analogues (Scheme 5). These caged rhodamines are non-polar, lipophilic compounds, but they contain the same fluorophore as the bright, photostable, hydrophilic fluorescent dye KK114 with two sulfonic acid residues in allylic positions (in this respect, comparable to compounds **2d** in Scheme 4).[12m–o] Dye KK114 could not be caged by conversion into the corresponding spiro diazoketone without modification of the structure.[2b] Two polar groups are essential to provide the required hydrophilic properties. We introduced the second carboxyl group, replaced the sulfonic acid residues in KK114 with hydroxyl groups, and obtained the required diazoketone (**2f**-H, Scheme 2), but, at the very end, we failed to obtain the stable *N*-hydroxysuccinimidyl ester (**2f**-NHS). As we established, the primary (allylic) hydroxyl groups are incompatible with the presence of an activated carboxyl group in close proximity to one of them (Scheme 2). Therefore, we used another approach based on transformations of ethyl ester **2e**-SCH_2CO_2Et (Scheme 5). The conditions for aromatic nucleophilic substitution of one fluorine atom in rhodamine **8**-F have been optimized previously (Scheme 2). The selectivity was slightly better for compound **8**-F (Scheme 5) than for rhodamine **1**-F,F,H (Scheme 2), and the product of disubstitution was formed to a lesser extent. Further transformations were similar to those shown in Scheme 2. The active ester **9**-NHS (Scheme 5) was obtained and smoothly reacted with the "universal hydrophylizer" **11**, a very hydrophilic dipeptide prepared from sulfocysteine and β-alanine amino acids.[19] Importantly, the amidation reaction between **9**-NHS and **11** proceeded smoothly in DMF in the presence of Et_3N, and no protection of the anionic centers in **11** was required to form acid **10**-H, which represented the caged red-emitting rhodamine decorated with sulfonic and carboxylic acid residues. The selective activation of the carboxylic acid site in the presence of sulfonic acid residue in compound **10**-H was achieved under standard conditions (see Scheme 5) to afford the amino-reactive caged dye **10**-NHS.



Scheme 5. Synthesis of the caged red-emitting rhodamines **2e**-F and **2e**-SCH_2CO_2Et, and their hydrophilic derivatives (**10**-R) decorated with an amino-reactive group. a) $HSCH_2CO_2Et$, MeCN, Et_3N, −12–18 °C; b) $(COCl)_2$, CH_2Cl_2, DMF, 0 °C–RT, 2–6 h; c) CH_2N_2, Et_2O (Et_3N),[a] 0 °C–RT, 11–18 h; d) 1 M NaOH (aq), THF/H_2O, 0 °C–RT; e) *N*-hydroxysuccinimide, HATU, Et_3N, DMF, RT; f) Et_3N, DMF, RT. [a] See note to Scheme 2.

Photophysical properties of Rhodamines NN

In the solid state and in relatively concentrated solutions, all pure Rhodamines NN are pale yellow. The absorption spectra of all five dyes in the caged form show similar features (see Figure S1 in the Supporting Information). Spectra recorded in solution in methanol or water contain a long-wavelength band with a maximum or shoulder at about $\lambda = 330$–350 nm (absorption coefficient (ε) = 4–15 × 10^3 $M^{-1}cm^{-1}$). Although the absorption intensities in the near-UV region are relatively low, the band edge extends beyond $\lambda = 400$ nm, up to about $\lambda =$ 450 nm. As a result, uncaging can be performed, not only with UV light (for example, with a $\lambda = 375$ nm laser), but also with visible light; in fact, the masked dyes are even sensitive to daylight. The photolysis of these model masked rhodamines in methanol is exemplified in Figure 1. Dyes of various colors (yellow, orange, red, magenta, and blue) with intense fluorescence, clear to the naked eye, were readily generated by irradiation of colorless solutions (dye concentration ≈ 10 μM). To evaluate the uncaging quantum yields (Φ) of the reactions leading to the fluorescent ($\Phi_{CF\rightarrow FP}$) and "dark" products ($\Phi_{CF\rightarrow DP}$), respectively, we irradiated solutions ($c = 40$–50 μM) of the model caged dyes (**2a**-H, **2b**-H, **2c**-CO_2Et, **2d**-CO_2Et, and **2e**-F) in methanol with a $\lambda = 405$ nm monochromatic light source of ≈ 20 mW (a continious wave (CW) diode laser). During the course of the irradiation, we recorded the UV/Vis spectra (Figure 2), calculated the amount of fluorescent product (FP) formed and, simultaneously, performed HPLC with UV detection to evaluate the amount of the photolyzed caged compound. The amount of FP formed was calculated from the absorption in the main band in the visible region, neglecting the contribution from the dark product (DP) because the absorption coefficients of DPs are at least ten-times lower than the absorption coefficients of FPs (and the amount of DP formed is lower). The quantity of DP was calculated from the difference between the photolyzed Rhodamine NN (HPLC) and

Figure 1. Irradiation of stirred solutions of Rhodamines NN in ethanol (from left to right in each picture: **2a**-H, **2b**-H, **2c**-CO_2Et, **2d**-CO_2Et, and **2e**-F) with UV light from a middle-pressure mercury lamp (150 W) equipped with Pyrex filter ($\lambda > 330$ nm) provides highly fluorescent dyes of five principal colors. a) Starting solutions are colorless or light yellow; b) the lamp is ON; c) a daylight picture taken after complete photolysis (several minutes) demonstrates the various colors of the solutions; d) a picture of the same solutions in c) taken with a torch light demonstrates the various emission colors.

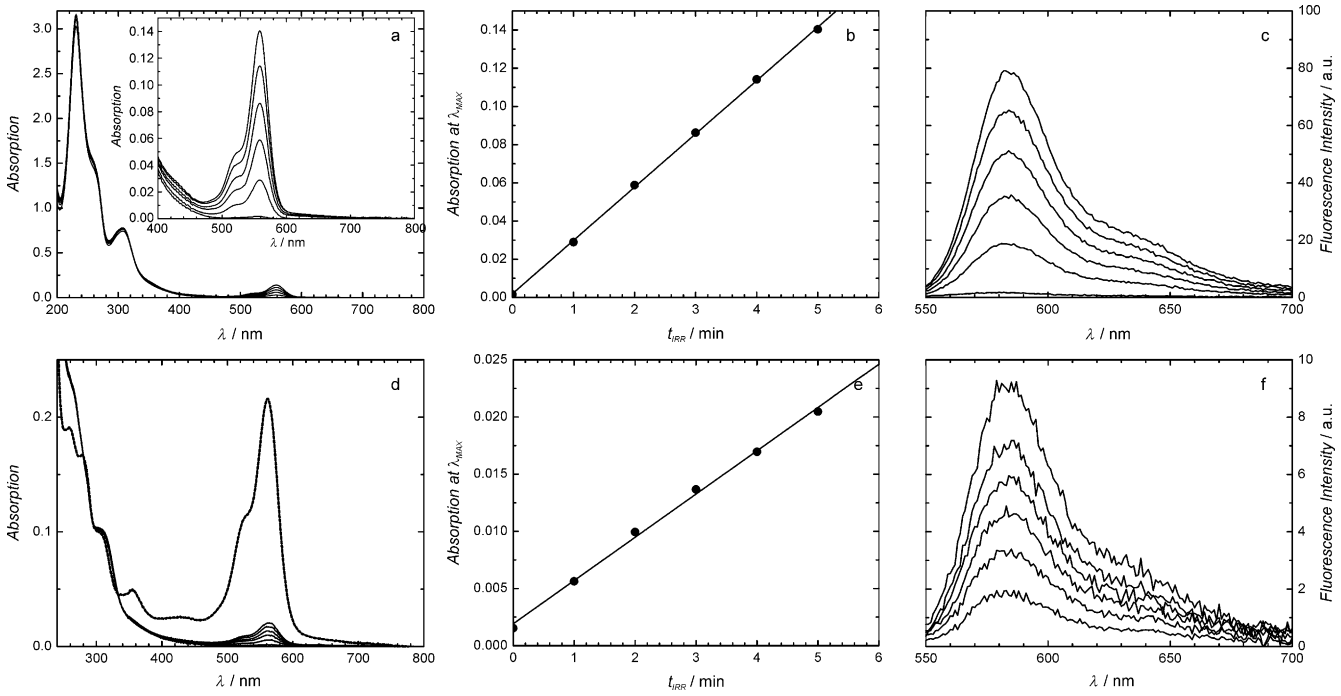


Figure 2. a)–c) Irradiation of the model compound **2c**-CO_2Et in methanol and d)–f) conjugate of **2c**-CONHS (Abberior Cage 552) with BSA in PBS at pH 7.4. a), d) Absorption at the maximum of the FP (see Table 1) as a function of time; b), e) a linear fit; c), f) emission spectra. The absorption and emission spectra were recorded after the irradiation times indicated (1–5 min), with the exception of the spectrum represented by the broken line in d), which was recorded after complete photolysis.

the quantity of the generated FP, assuming that no other by-products were produced during the course of the photolysis.[2b,20] Uncaging quantum yields were calculated at lower conversions (below 10%), for which a linear regime was observed (Figure 2b and c; see also Figures S3 and S4 in the Supporting Information). The photoisomerization of azobenzene in solution in methanol was used as a reference actinometric system to determine the exact irradiation intensity under the experimental conditions.[21] The main photophysical properties of Rhodamines NN are listed in Table 1.

All compounds demonstrated moderate uncaging quantum yields[22] ($\Phi = 0.15$–1%). In fact, moderate or even low uncaging quantum yields may be advantageous because they allow the markers and their conjugates to be handled under weak light rather than in complete darkness. The power of UV lasers may be easily varied and adjusted to provide quick photoactivation and minimal photobleaching of the activated dye. The FP, that is the unmasked fluorescent dyes, demonstrate Stokes shifts of approximately 20 nm (typical for rhodamines) and a small redshift (5–10 nm) of the absorption and emission bands relative to the parent free rhodamine (starting dye), due to conversion of the free carboxyl group COOH to the homologue CH_2COOH group or the corresponding ester. The absorption coefficients at the peak of the band of the FP are at least ten-times higher than those of the DPs at the same wavelengths. The grey and non-fluorescent compounds **4a**-Y–**4e**-Y (Scheme 1) have a low absorption ($\varepsilon < 3000\ M^{-1}cm^{-1}$) at wavelengths that correspond to the absorption maxima of the photogenerated fluorescent dyes **3a**-Y–**4e**-Y ($\varepsilon = 50\,000\ M^{-1}cm^{-1}$; see Table 1 and Figure S2 in the Supporting Information). In addition, the quantum yield for the photogeneration of the DP ($\Phi = 0.05$–0.3%) is 3–12 times smaller than the quantum yield

Table 1. Photophysical properties of the model Rhodamines NN in methanol, including absorption and emission maxima for initial caged dyes (CF), fluorescent products of their photolysis (FP), fluorescence quantum yields of the latter (ϕ_{fl}), uncaging quantum yields of the photoreactions leading to FP ($\Phi_{CF\rightarrow FP}$) and DP ($\Phi_{CF\rightarrow DP}$).

	Closed form (CF)		Fluorescent product (FP)[a]					Dark product (DP)[a]			
Caged dye	Abs max [nm]	$\varepsilon/10^3$ [$M^{-1}cm^{-1}$]	Abs max [nm]	$\varepsilon/10^3$ [$M^{-1}cm^{-1}$]	Em max [nm]	ϕ_{fl}[b]	$\Phi_{CF\rightarrow FP}\times100$[f]	Abs max [nm]	$\varepsilon/10^3$ [$M^{-1}cm^{-1}$]	$\Phi_{CF\rightarrow DP}\times100$[f]	$\Phi_{CF\rightarrow FP}/\Phi_{CF\rightarrow DP}$
2a-H	230, 299, 340	74, 17, 5.1	511	50	525	0.85[c]	0.97	n.d.[g]	n.d.[g]	0.27	4
2b-H	237, 302, 350	62, 16, 3.8	533	82	541	0.84[c]	0.77	365, 405, 551	14, 12, 4.5	0.06	12
2c-CO_2Et	231, 308, 350	74, 18, 4.0	559	43	574	0.40[d]	0.85	n.d.[g]	n.d.[g]	0.11	8
2d-CO_2Et	261, 296, 329	83, 17, 15	597	62	609	0.87[d]	0.49	315, 476, 609	25, 8.1, 5.2	0.15	3
2e-F	272, 300, 348	61, 16, 9.5	633	31	653	0.54[e]	0.15	315, 498, 636	7.3, 2.4, 2.0	0.12	1.3

[a] Obtained upon photolysis with $\lambda > 330$ nm in methanol (lifetimes of the excited states vary in the range of 3.3–4 ns). [b] ϕ_{fl} measured in the reaction mixtures after photolysis. Refererence values: [c] Atto 532 ($\phi = 0.9$);[23c] [d] Rhodamine 101 ($\phi = 1$);[23d] [e] KK114 ($\phi = 0.53$).[12n] [f] Calculated from the optical density of the main absorption band and HPLC analysis in irradiation experiments at $\lambda = 405$ nm. [g] The DP was not isolated in sufficient amount.

for the generation of the FP (with the exception of **2e**-F, for which both products are generated with similar efficiency). Therefore, the color of the dye solutions obtained upon irradiation in methanol or water always looked "clean" (from yellow to dark blue, depending on the dye). The presence of the DP in irradiated solutions was observed as a weak band or a shoulder, appearing at longer wavelengths than the main absorption band of the FPs (Figure 2b and e; see also Figures S3 and S4 in the Supporting Information). Even though the photolysis of the caged dyes in solution always leads to a mixture of FP and DP, the caged fluorescent dyes proved to be valuable markers for biomolecules, such as antibodies in the immunofluorescence method. To investigate the possibility to obtain conjugates with proteins, evaluate the uncaging parameters, and the effect of aqueous solutions on the binding and photolysis of the marker, we first prepared conjugates of NHS esters **2a**-CONHS, **2bc**-CONHS, **2c**-CONHS (Scheme 3), **2d**-Na-CONHS (Scheme 4), and **10**-NHS (Scheme 5) with BSA as a model protein. The conjugates were prepared by standard labeling protocols.[23a,b] Briefly, NHS ester (0.1 mg) was dissolved in DMF (50 μL) [compound **2d**-Na,CONHS was dissolved in phosphate buffered saline (PBS)], then added to a stirred solution of BSA (1 mg) in PBS (0.5 mL), which was buffered at pH ≈8.5. After stirring in the dark at room temperature for 1 h, the protein fraction was separated from the unreacted marker by size-exclusion chromatography on a Sephadex G-25 column (PD-10 desalting column, GE Healthcare). Labeling efficiencies (degrees of labeling (DOL) = average number of markers attached to one molecule of BSA with $M = 66.5$ kDa) were determined by absorption measurements, assuming $\varepsilon = 43\,800\ M^{-1}cm^{-1}$ at $\lambda = 280$ nm for BSA.

The data are given in Table 2. All obtained DOL values are in the range of 3–6, as expected from the protocol used, which demonstrates that the amino-reactive derivatives of our caged fluorescent dyes can be used in the same way as common commercially available markers. The variation in the values obtained do not seem related to the hydrophilicity of the marker, and may reflect either the amount of the reacted dye or the inaccuracy due to overlapping of the BSA and marker bands (Table 1). Dilute solutions of the conjugates (BSA concentration ≈3 μM) in PBS at pH 7.4 were subjected to irradiation with $\lambda =$ 405 nm light under conditions similar to those used for the model caged dyes in methanol (Figure 2). Photolysis of the BSA conjugates under biologically relevant conditions (in aqueous PBS buffer, pH 7.4) also produced clean colors, and the dark tones were not visible. Upon photoactivation, the fluorescence quantum yields of the newly formed dyes were good ($\phi = 0.2$–0.4). The uncaging quantum yields for the FPs were also calculated from absorption measurements ($\Phi_{CF\rightarrow FP} = 0.1$–0.8; Table 2). Importantly, these values comprise at least 60% of uncaging quantum yields found for the free model Rhodamines NN dissolved in methanol. In these cases, the quantum yields for conversion to the DP were not calculated because HPLC measurements were not possible. Solutions of BSA conjugates in PBS were also irradiated until complete photoactivation occurred (spectra are shown in Figure 2d and Figure S4 in the Supporting Information) and the amount of FP formed was determined (% FP formed, Table 2). These values may be interpreted as an additional factor to evaluate the effective DOL (useful labeling efficiencies). A particular case was observed for the conjugate with compound **2bc** (Abberior Cage 532), for which the amount of FP formed seemed too small. Considering the absorption of the solution at $\lambda =$ 600 nm ($\varepsilon_{FP} \ll \Phi_{DP}$), we estimate that 11% of the initial cage compound was converted to the DP, a value in agreement with that observed for the free dye (see Table 1) if other minor photolysis products are neglected. This discrepancy may be explained by photobleaching or aggregation of the uncaged dye residues attached to BSA. Indeed, the absorption spectra at full conversion show that the shoulder at short wavelengths practically disappeared (compare with the spectra at low conversions). This phenomenon indicated the full conversion of the initial caged compounds, which is in line with the previous speculation. The amount of DP formed at complete photolysis for compounds **2d**-BSA and **10**-BSA is in agreement with the calculated quantity of the FP, within a reasonable error ($\Sigma =$ 100%, within 10% error). The absorption spectrum of **2a**-BSA at full photoactivation also differs from the absorption spectra obtained at low conversions, despite the fact that the degree of conversion to the FP is similar to the value observed for the free dye. Nevertheless, these two compounds displayed higher labeling efficiencies and in practical applications it may be

Table 2. Degrees of labelling (DOL), fluorescence quantum yields (ϕ_{fl}) and uncaging efficiencies ($\Phi_{CF\rightarrow FP}$) of conjugates obtained from NHS esters **2a**-CONHS, **2bc**-CONHS, **2c**-CONHS (Scheme 3), **2d**-Na,CONHS (Scheme 4), and **10**-NHS (Scheme 5) and BSA in PBS solutions. Values for the free dyes were used to calculate ratios $\phi_{fl}/\phi_{fl\,free}$ and $\Phi_{CF\rightarrow FP}/\Phi_{CF\rightarrow FPfree}$.

Dye–BSA	DOL[a]	ϕ_{fl}[b]	$\phi_{fl}/\phi_{fl\,free}$	$\Phi_{CF\rightarrow FP}\times 100$[c]	$\Phi_{CF\rightarrow FP}/\Phi_{CF\rightarrow FPfree}$	FP formed [%][d]
2a	6.6	0.24	0.29	0.54	0.56	60
2bc	4.8	0.38	0.45	0.45	0.59	22 (89)[e]
2c	3.4	0.20	0.50	0.77	0.91	87
2d-Na	3.0	0.36	0.42	0.39	0.81	77
10	2.9	0.20	0.37	0.09	0.59	65

[a] DOL = average number of markers attached to one protein molecule. [b] fluorescence quantum yields (ϕ_{fl}) measured in the reaction mixtures after photolysis, neglecting the absorption of the DP (for reference compounds see Table 1). [c] calculated from absorption measurements in irradiation experiments with $\lambda = 405$ nm light, assuming that the absorption coefficient of the FP in the conjugate is the same as that of the free dye in methanol. [d] amount of FP formed in fully irradiated solutions ($t_{IRR}\rightarrow\infty$) calculated from absorption experiments. [e] difference between the amount of starting dye and the quantity of DP formed, estimated from the absorption at $\lambda = 600$ nm, at which the FP does not absorb (see Figure S4 in the Supporting Information).

better to maintain the values between 3 and 4 (and carefully control the irradiation intensity to reduce bleaching of the uncaged dyes).

Light microscopy applications of caged dyes as photoactivatable fluorescent labels

Photoactivation (switching on) of the single molecules of any masked fluorescent dye followed by off-switching (e.g., due to reversible or irreversible transition to a "dark" non-emitting state or photobleaching) allows acquisition of images with subdiffractional optical resolution.[1] Caged dyes with a photosensitive 2-diazo-1-indanone group can be readily activated with UV light (e.g., $\lambda = 375$ or 405 nm lasers) or with very powerful red light (in two-photon mode, e.g., under stimulated emission depletion (STED) conditions; see below). In the single-molecule-switching approach, we used antibody conjugates prepared from the NHS esters of **2a**, **2bc**, **2c**, **2d**-Na, and from **10**-CONHS and caged carbopyronines.[2b] For example, the masked markers of compound **2a** were uncaged stochastically in time and space with $\lambda = 375$ nm light in such a way that only one marker stayed unmasked per camera exposure time in a diffraction-limited volume. The cycle of excitation at $\lambda = 532$ nm, detection of the emitted fluorescence, and bleaching of the uncaged markers was repeated 20000 times. Localization of the markers yielded the super-resolved position histogram with 35 nm FWHM (full width at half maximum) average localization precision (Figure 3).

The caged dyes (and Rhodamines NN, in particular) offer the advantage of a choice between two modes of single-marker switching. They can be imaged in ground state depletion with intramolecular return (GSDIM) mode,[24] that is, a sufficient fraction of all masked molecules is uncaged at first. These may be used for taking a conventional wide-field reference picture. Then, the singlet state of the already uncaged dye molecules is depleted by shelving them to a dark (triplet) state (GSDIM mode). After that, individual molecules return to the ground state (spontaneously or by triggering with UV light). These single markers may be localized by using a charge coupled device (CCD) camera and collecting the photons emitted by each of them. All these events occur within micro- or milli-seconds and can be repeated at different time points during the course of an experiment until the pool of masked dyes becomes empty. This algorithm allows the gradual acquisition of a position histogram with a high spatial resolution (several tens of nm).

Alternatively, it is possible to employ not only the transition between singlet and triplet states, but also to directly use the uncaging reaction to switch between dark and fluorescent states. Irreversible switching may be advantageous for the following reason: in this (ideal) case, each marker contributes to the final high-resolution image only once and is then bleached (under the assumption that the population of long-living "dark" triplet states from which the marker can come back into the "fluorescence-giving" singlet state is negligible). Therefore, the number of "signal-giving" (active) markers is proportional to the number of detected fluorescence bursts. This represents a tool for indirect estimation of the number of labeled objects or their density in clusters. Further illustrations to exemplify the applicability of Rhodamines NN in imaging methods based on single-molecule-switching methodologies have been reported.[25]

This approach may be applied to several Rhodamines NN attached to various biological targets and integrated into the same image. Thus, multicolor optical nanoscopy methods may be implemented.

Additionally, the photoactivation (uncaging) of Rhodamines NN or structurally related caged carbopyronines[2b] may be combined with STED microscopy. After photoactivation, caged rhodamines **2d**-Na and **10**, as well as caged carbopyronines[2b] emit red light and their fluorescent state may be depleted with standard lasers operating at $\lambda = 750$–780 nm. Therefore, red-emitting caged dyes are of special importance. Their emission does not interfere with autofluorescence of

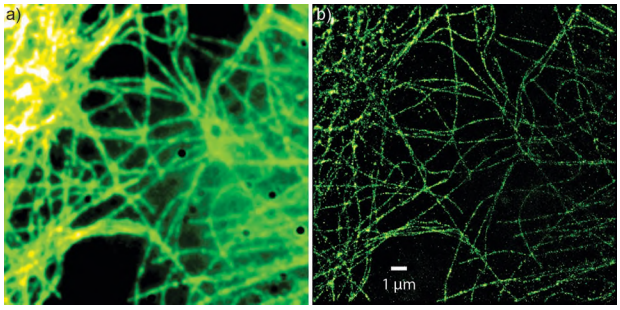


Figure 3. a) A conventional image of the cellular microtubule cytoskeleton labeled with compound **2a**-CONHS (background-corrected sum of all frames) and b) its sub-diffraction representation obtained by uncaging of the single molecules with $\lambda = 375$ nm laser light and subsequent localization. See text for details.

cells, and their excited state may be depleted with the less-invasive red STED beam. Caged carbopyronines are structurally similar to Rhodamines NN: the oxygen atom in the xanthene core is replaced by a tertiary carbon atom (see Figure 4a for structures and reference [2b] for the preparation and properties of caged carbopyronines). Here, we describe the results obtained with caged carbopyronine KK1012 (Figure 4a), the best-performing dye under standard STED conditions. In particular, we explored two modes of its uncaging (photoactivation).

The custom-built setup for STED microscopy utilized an excitation wavelength of $\lambda = 638$ nm, STED wavelength of $\lambda = 750$ nm, and the possibility of unmasking the dye with a bright-field UV-illumination of $\lambda = 375$ nm (see the Supporting Information for further details and an additional example).[26] We showed that unmasking of the dye is possible by either one-photon photoactivation with light of $\lambda = 375$ nm (Figure 4a) or a two-photon process[7a] that uses the high-intensity infrared ($\lambda = 750$ nm) illumination of the STED laser (Figure 4b). The second case offers the valuable possibility to skip the UV-uncaging step. Simultaneous photoactivation and depletion with a STED laser directly provides super-resolved images. This is especially useful for applications in biological samples because the absence of a UV laser reduces the photodamage to the biological specimen. Moreover, the two-photon uncaging conditions with a STED beam ($\lambda = 750$–800 nm) are available for all commercially available STED microscopes produced by LEICA Microsystems and Abberior Instruments. Both photoactivation modes lead to images with subdiffractional optical resolution of about 50 nm. This value is five-times better than the diffraction-limited resolution of $\lambda = 250$ nm (Figure 4a and d). The images obtained after an additional uncaging step at $\lambda = 375$ nm are brighter (by a factor of 1.5) than the images for which the caged dye was photoactivated by the $\lambda = 750$ nm depletion laser. This can be explained by incomplete photoactivation in the latter case.

Conclusion

We have shown that the caging procedure based on incorporation of a 2-diazoketone fragment can be applied to virtually to all *N*,*N*,*N*′,*N*′-tetraalkylrhodamines (this work) and carbopyronines.[2b] The compact structure and very small size of the caging group facilitate the ability of Rhodamines NN and Carbopyronines NN (deprived of the charged groups) to cross the membranes of living cells (see Figure S5 in the Supporting Information). In combination with the site-specific labeling protocols,[27] realized for the Halo-, SNAP-, and CLIP-tags, the availability of the novel cell-permeable, caged fluorescent dyes may extend the set of live-cell labeling strategies based on binding the genetically encoded protein tags with organic fluorophores. Spatially restricted photoactivation followed by tracking of the uncaged molecules may enable measurements of

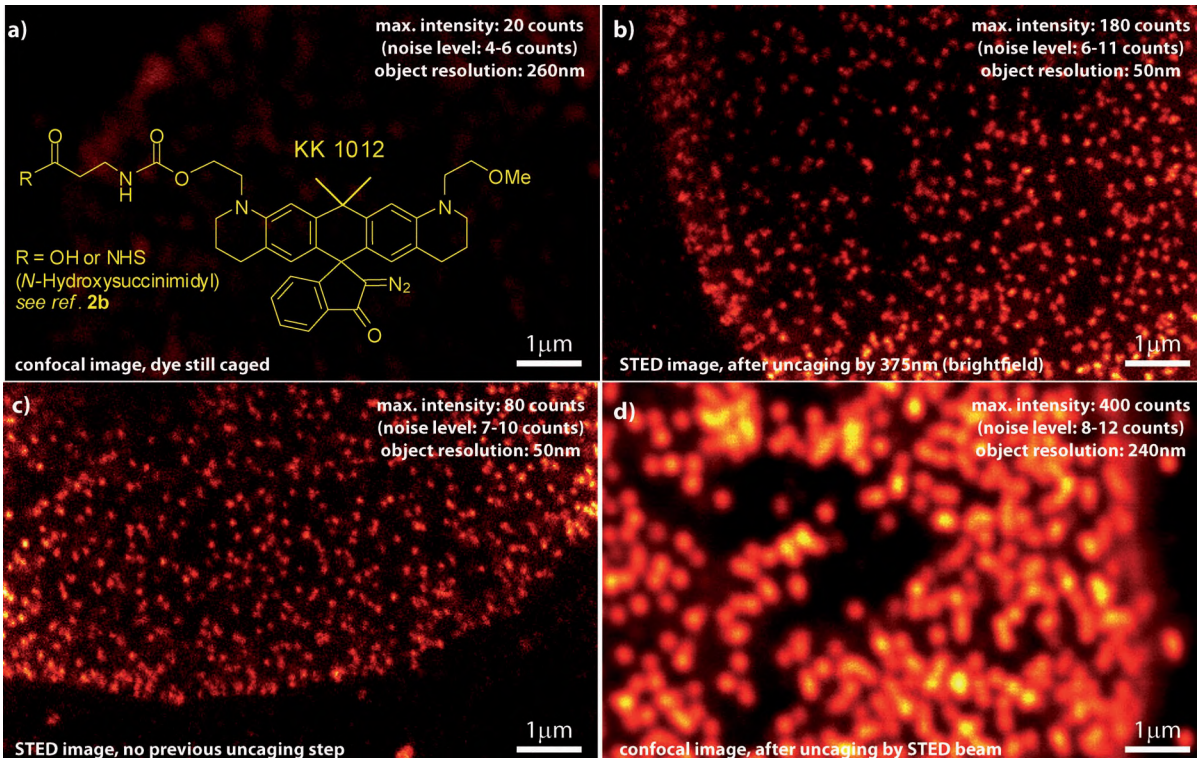


Figure 4. Conventional confocal and STED microscopy images of the nuclear pore complex protein Nup153 in mammalian cells labeled with the caged carbopyronine KK1012. a) Confocal image of Nup153 before uncaging of the dye. b) Super-resolution image of Nup153 after uncaging the dye by bright-field UV-illumination at $\lambda = 375$ nm and subsequent STED microscopic recording. c) Super-resolution image of Nup153 obtained by simultaneously uncaging and depleting the dye during the STED microscopic recording. d) Confocal image of Nup153 obtained after acquisition of a STED image with the same parameters, as described for c). b, c) The STED power in the back focal plane was 200 mW. All images show unprocessed (raw) data. The given resolutions denote the FWHM of exemplarily chosen, Gaussian-fitted object signals (see Supporting Information for details).

molecular dynamics (e.g., diffusion parameters or flow velocities). The combination of Rhodamine NN with nanoscopic techniques (e.g., single-molecule switching followed by localization[24] or STED microscopy) is expected to provide additional information on subcellular structure.[12e,28] Other cationic dyes are also expected to undergo the caging procedure described in this work. In particular, caged fluorescence quenchers represent a very interesting class of photoactivatable dyes. They can be used as masked acceptors in fluorescence resonance energy transfer (FRET) experiments. A caged quencher (masked FRET acceptor) can be combined with a common fluorescent dye (FRET donor) and used in colocalization studies of objects situated very close to each other (at distances comparable with a Förster radius of this pair). In this case, the differential image of the donor emission areas before and after uncaging of the quencher (acceptor dye) can indicate the areas situated in close proximity to each other (at the relatively short distances of 1–10 nm, which correspond to Förster radii of the common dye pairs).

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Keywords: bioconjugation · diazo compounds · fluorescence · photolysis · rhodamines

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