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PAPER

Masked red-emitting carbopyronine dyes with photosensitive 2-diazo-1-indanone caging group†‡

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Caged near-IR emitting fluorescent dyes are in high demand in optical microscopy but up to now were unavailable. We discovered that the combination of a carbopyronine dye core and a photosensitive 2-diazo-1-indanone residue leads to masked near-IR emitting fluorescent dyes. Illumination of these caged dyes with either UV or visible light ($\lambda < 420$ nm) efficiently generates fluorescent compounds with absorption and emission at 635 nm and 660 nm, respectively. A high-yielding synthetic route with attractive possibilities for further dye design is described in detail. Good photostability, high contrast, and a large fluorescence quantum yield after uncaging are the most important features of the new compounds for non-invasive imaging in high-resolution optical microscopy. For use in immunolabelling the caged dyes were decorated with a (hydrophilic) linker and an (activated) carboxyl group.

Introduction

Near-IR emitting fluorescent dyes are widely used in far-field optical microscopy.[1] In the long run, for various applications, like protein tracking,[2a,b] multi-colour imaging,[2c] far-field optical nanoscopy,[2d–i] single-molecule photoswitching and spectroscopy, it would be very useful to have a near-IR dye *in a caged (masked) form*. The key element of caged fluorophores is a photosensitive masking group or a 'molecular cage' that can be cleaved non-invasively by irradiation with light.[3] Recently, we reported a new type of caged cationic dyes, Rhodamines NN, where the photosenstive 2-diazoketone group is incorporated into the 9-spiro xanthene fragment.[4a] To develop the colors, these dyes are subjected to illumination with UV or even visible light (<420 nm).[4a] The uncharged 2-diazo-1-indanone derivatives (a case of aryl diazoketones) have been synthesized from the zwitterionic fluorescent dyes. The small size of the caging diazoketone group, accompanied by the absence of the net charge, low polarity and low dipole moment, as well as compact structure of the molecule as a whole, provide several valuable properties which the starting fluorescent dyes do not have (*e.g.* cell permeability). The caging procedure was first applied to *N,N,N′,N′*-tetraalkyl rhodamines.[4a] However, since the modern trends of light microscopy favor the use of red-emitting dyes, we studied

the possibility of incorporating a 2-diazo-1-indanone caging group into the core of a near-IR emitting fluorophore. In this paper, we first present a feasibility study on the caged sulfonated dye **2b** related to rhodamine **4a** (or KK 114 in Scheme 1), as a very bright and photostable hydrophilic near-IR emitting dye which performs very well in stimulated emission depletion (STED) microscopy.[1a,b] Following this way, we investigated the applicability of the caging technique that utilizes spiro diazoketones[4] to fluorinated rhodamines decorated with the sulfonic acid residues. However, compound **2b** turned out to be very difficult in preparation and isolation in the pure state. Therefore, we used another class of red-emitting dyes for caging.

Combining the scaffolds of cationic carbopyronine dyes (another type of xanthene dyes; *e.g.* **3e** in Scheme 1)[1b] with a 2-diazo-1-indanone caging group,[4a] we developed the full synthesis of caged near-IR emitting dyes **1a–d** (Scheme 1). They are photostable, and provide high contrast, as well as large fluorescence quantum yields after the photo-induced transformations to the uncaged fluorescent dyes **3a–d** (and "dark" side-products in Scheme 1).

The present study deals with the scope and limitations of the caging technique that utilizes spirodiazoketones. The syntheses and spectral properties of the masked compounds are discussed in detail, and their use as photoactivable labels in immunofluorescence microscopy is further illustrated.

Feasibility study on a near-IR rhodamine dye with sulfonic acid groups

The carboxylic acid function that is initially present in rhodamines can be transformed into the 2-diazo-1-indanone caging group.[4] Therefore, all practically useful masked rhodamines require an additional carboxyl group as a binding site. Thus,

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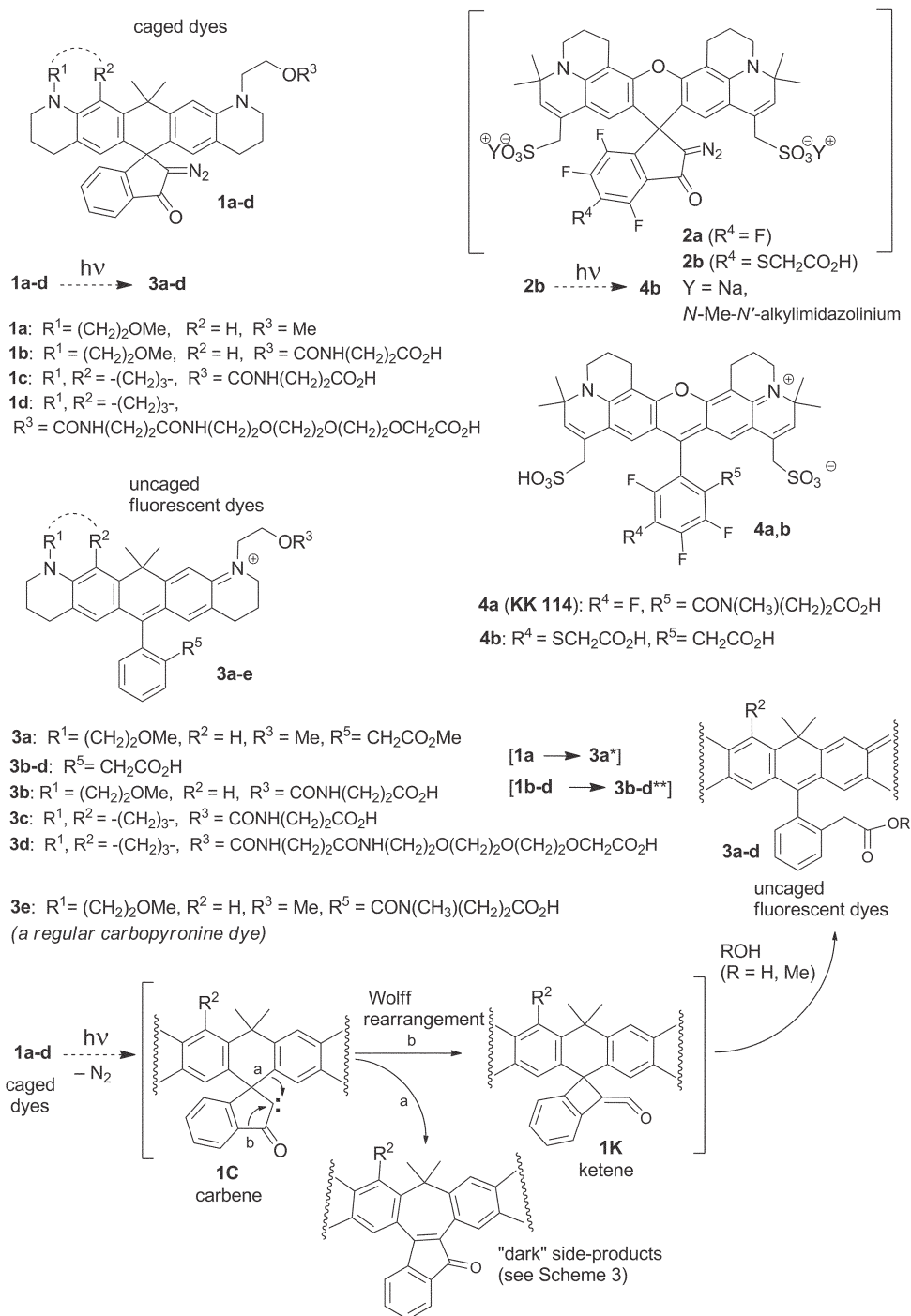
Scheme 1 Near-IR emitting caged fluorescent dyes and related compounds. * Carbopyronine **3a** was obtained by preparative photolysis of model compound **1a** in methanol. ** Dye structures **3b–d** represent the expected fluorescent products, generated by photolysis of the caged dyes **1b–d**.

compound **2b** in Scheme 1 was considered as a synthetic target for a sulfonated red-emitting rhodamine in the caged form. In order to obtain a *sulfonated diazoketone* **2b**, we had to explore the challenging and important problem of selective and orthogonal protection of the sulfonic and carboxylic acids. Our study started with compound **5**-H (Scheme 2) that has both types of functional groups. Despite the wide-spread use of sulfonic and carboxylic acids in dye chemistry (many industrial dyes have

them), their orthogonal protection (in one molecule) has not been described in the literature so far. In our strategy, illustrated in Scheme 2, it was crucial to consecutively protect the carboxylic and sulfonic acid groups in compound **5**-H. After that, we needed to transform the carboxyl group selectively (in order to convert it into the diazoketone), so that the SO_3H groups still remained protected. In fact, it was necessary to orthogonally protect three acidic centers in intermediate compounds **6**-R^1, R^2
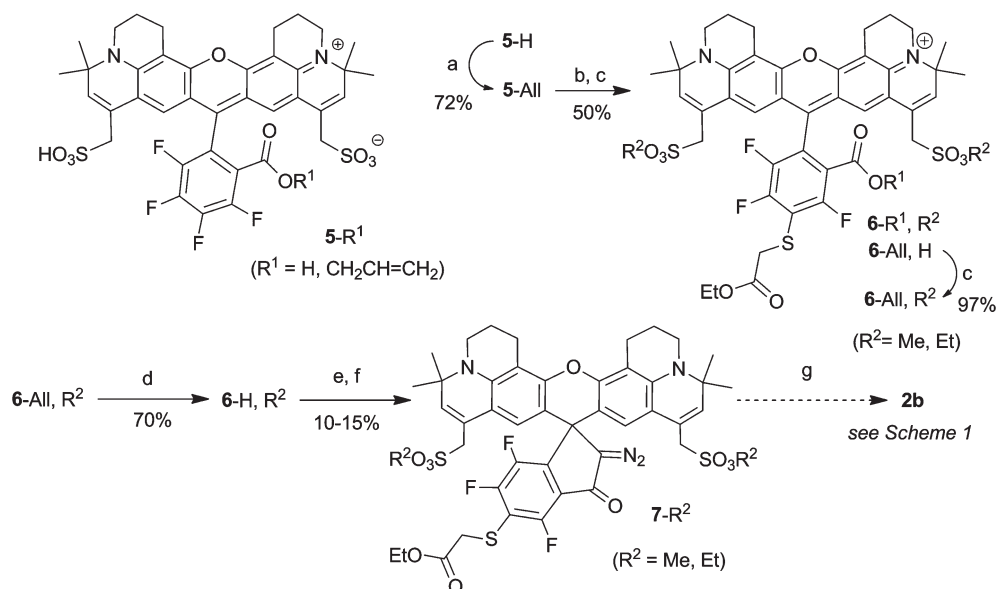
Scheme 2 Feasibility study on a caged near-IR emitting rhodamine dye with sulfonic acid groups (**2b** in Scheme 1). Reagents and conditions: (a) $CH_2{=}CHCH_2OH$, *p*-TsOH, reflux; (b) $HSCH_2COOEt$, Et_3N, MeCN, −5 °C, 30 min.; (c) $R_3^2O(+)\cdot BF_4(-)$; iPr_2NEt, MeCN, r. t.; (d) $Pd(Ph_3P)_4$, HCOOH·NEt_3, THF, r. t., 1 h; (e) $(COCl)_2$, CH_2Cl_2, 0 °C–r. t., 4 h; (f) CH_2N_2, Et_2O (Et_3N), −5°–0 °C, 8–12 h; (g) NaOH in EtOH or *N*-methylimidazole in MeCN, r. t.−50 °C. Only trace amounts of the target compound **2b** were detected, while other products predominated (see ESI‡ for details).

in Scheme 2: two different carboxylic acid groups and two identical sulfonic acid residues.

The carboxylic group in compound **5**-H was protected as allyl ester. An obvious advantage of this approach is that allyl esters, unlike conventional alkyl esters, are smoothly cleaved with palladium catalysts in neutral or acidic media.[5] The alkyl sulfonates and carboxylates, both sensitive to alkali, are thus unaffected. A straightforward way to temporarily protect the sulfonic acid groups is to convert them into methyl or ethyl esters. As established in initial experiments, the esterification of compound **5**-All is best performed with trialkyl oxonium salts, powerful reagents often used in peptide chemistry.[6] After that, one fluorine atom in precursor **5**-All was exchanged to a thioglycolic acid residue ($SCH_2CO_2C_2H_5$), thus providing the protected reactive site (which is necessary for the conjugation). Ethyl mercaptoacetate is a strong nucleophile which is able to cleave alkyl sulfonic esters. Therefore, it was necessary to introduce the second carboxylic acid residue into compound **5**-All, and only then alkylate the sulfonate residues. Also importantly, the nucleophilic substitution of the most reactive fluorine atom with sulfur prevents the undesirable substitution with another nucleophile – diazomethane. Exploring the reaction of allyl ester **5**-All with ethyl thioglycolate ($HSCH_2CO_2Et$), we observed a very high reactivity of the two fluorine substituents towards the thiol. In order to reduce the formation of the disubstituted product, the reaction should be carried out at low temperatures (<0 °C). It is very important that the substitution of one fluorine atom to sulfur does not cause a large shift of the absorption and emission maxima (only 1–2 nm), relative to **5**-All. The high fluorescence quantum yield was also unaffected. Only then was the intermediate with a linker (**6**-All,H) converted to the alkyl sulfonates **6**-All,R^2 (Scheme 2). The allyl carboxylate was cleaved with Pd$(Ph_3P)_4$/HCOOH·NEt_3, and the free acid **6**-H,R^2 converted to the acid chloride, following the previously described recipe.[4a]

Treatment of the acid chloride with an excess of diazomethane afforded the corresponding diazoketones **7**-R^2, isolated in a poor yield (less than 15%). Most likely, in the case of compounds **6**-H,R^2, the cyclization to diazo ketones **7**-R^2 is not favored. The expected Wolff rearrangement products (structure **4b**, Scheme 2) are formed from **7**-R^2 already upon reaction with diazomethane, as established by MS and NMR analyses. Moreover, ethyl sulfonate groups in compound **7**-Et (R^2 = Et) are much more difficult to saponificate than the ethyl carboxylate group. Heating of **7**-Et with NaOH in ethanol led to mixtures of colored products and compounds that were not sensitive to light, as indicated by TLC and HPLC analyses. *N*-Methylimidazole, taken as a nucleophile, did not help much. In view of these disappointing results, we switched to methyl sulfonate **6**-All,Me, expecting the better reactivity towards bases (NaOH) and nucleophiles (*N*-methylimidazole).[7] However, other complications arose on this way. We noticed that, after the allyl ester **6**-All,Me was cleaved, the free acid (**6**-H,Me) proved to be extremely unstable upon isolation. According to HPLC and MS analyses, a disproportionation reaction occurred in the neat compound, even under very mild conditions. As the methyl group is a better leaving group than ethyl, it either "drifts" to the carboxylic acid group, or goes off from the sulfonate residue. In other words, the free carboxylate group in compounds **6**-H,R^2 is much more prone to intramolecular alkylation with methyl sulfonate than with ethyl sulfonate. This stability problem was overcome when high-boiling solvents were used and the solutions of the free acid **6**-H,Me never evaporated to dryness.

However, after the subsequent reaction with diazomethane, only trace amounts of (impure) diazo ketone **7**-Me were isolated. Despite our expectations, even in the case of methyl sulfonate **7**-Me, the reactions with alkali and *N*-methylimidazole were still too slow and did not proceed cleanly. Probably, the remaining fluorine atoms in the aromatic ring also reacted with nucleophiles

(*i.e.*, *N*-methylimidazole or NaOH). In all experiments, the desired diazo ketone **2b** was obtained only in trace amounts, with a low purity, and proved quite unstable (even neat). All these features must be due to the effect of the three fluorine atoms. As regards the potential use of the caged dye **2b** as a marker, it is worth mentioning that its sensitivity to light and "brightness" (absorption and emission intensities) after uncaging proved to be insufficient. This was established by simple tests (exposure to UV and sunlight in solutions and on TLC plates) on the small amounts of the compound we managed to isolate. Therefore, we did not further improve these poor yielding transformations and concentrated on the synthesis of another promising class of the caged near-IR emitting dyes – the carbopyronines.

Synthesis of near-IR emitting carbopyronine dyes

It is known that certain carbopyronine dyes, particularly **3e** (Scheme 1), well match the spectroscopic requirements (positions of the absorption and emission bands, fluorescence quantum yield) for near-IR emitting dyes.[1b] As it was mentioned in the introduction, we expected that the combination of a carbopyronine dye core and a 2-diazo-1-indanone function would be possible, and provide a caged dye soluble enough in water. As regards red-emitting dyes, there is a rule of thumb to distinguish near-IR fluorophores that are suitable for non-invasive imaging with minimal autofluorescence of proteins and DNA. They have blue color in solutions, while their fluorescence is not seen under daylight (observe the blue solution of the uncaged carbopyronine dye in Fig. 1; also see photos of the dye solutions in ESI‡). At the same time, the fluorescence becomes visible under the light from incandescent lamps (with a larger proportion of red and IR-light, as compared to daylight). Other important parameters include the sensitivity to focusable light (>360 nm), sufficient quantum yields of the uncaging reactions, minimal amounts of the dark non-fluorescent by-products formed in the course of Wolff rearrangement[4b] (see Schemes 1 and 3), low sensitivity to green and red light (>500 nm), and high photostability of the uncaged fluorescent dye.
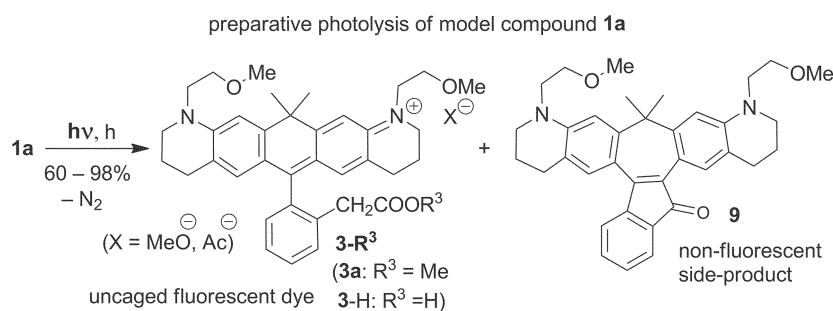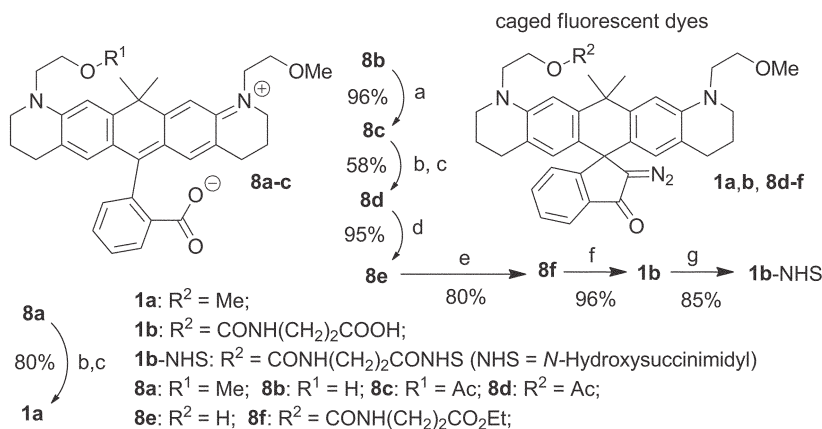
Here we extended the recently developed caging technique (that utilizes spiro-diazoketones[4a]) to carbopyronines[1b] – another type of xanthene dye. The synthesis involved the conversion of carbopyronine-containing precursors with a free carboxylic

group (**8a** and **8c**, Scheme 3) to the corresponding acid chlorides followed by a one-pot reaction with diazomethane leading to the spiro-diazoketones (cyclization products **1a** and **8d** in Scheme 3).

In order to check if the published approach to spiro-diazoketones[4] would work for carbopyronine dyes in general, we started with a simple carbopyronine-containing precursor, that was obtained in the course of our previous study (**8a**, Scheme 3).[1b] Only minor modifications were made to the protocols developed for the rhodamines (see ref. 4 and ESI‡ for details). After we had achieved good yields (up to 80%) of diazoketone **1a**, the most interesting part was to explore the photolysis of this caged dye in methanolic or aqueous media, isolate the reaction products, and examine their spectral properties. The spectroscopic data for the first caged carbopyronine **1a**, the uncaged dye **3**-Me and the non-fluorescent side-product **9** (Scheme 3) are presented in Fig. 2 and Table 1. The starting compound **1a** is pale yellow, with low absorbance in the visible range. Remarkably, in the course of a TLC analysis of the diazo ketone **1a**, a bright blue spot with red fluorescence starts to develop after a minute of exposure to a standard UV-lamp (254 or 365 nm), or when the TLC plate is left under daylight. Thus, the initial tests showed that the "uncaging" of **1a** readily proceeds upon illumination with UV or even daylight. The reaction is witnessed by the appearance of the *intense and clean blue color* and the beautiful emission of red light (fluorescence becomes visible upon illumination with a flash lamp light), as one can see in Fig. 1. Irradiation with a low-pressure mercury lamp in methanol provided the expected main product of the Wolff rearrangement – fluorescent dye **3**-Me (Scheme 3) – with 60% (isolated) yield. The spectral parameters of **3**-Me well match the required criteria: its absorption and emission maxima are above 630 nm, the extinction coefficient is high (ε >100 000 M^{-1} cm^{-1}), and the fluorescence quantum yield is very good (53% in aqueous solutions; see Table 1). All these properties are as good as those for previously obtained regular near-IR emitting carbopyronine dyes.[1b] Due to the poor solubility of compound **1a** in water, photolysis was only possible in very dilute aqueous solutions (1–10 μmol L^{-1}). However, the HPLC monitoring showed that the yields of the free acid (compound **3**-H) were as good as for methyl ester **3**-Me in methanol. Though the presence of the non-emissive side-product **9** could not be detected by the naked eye, it was isolated in the course of the preparative photolysis of the
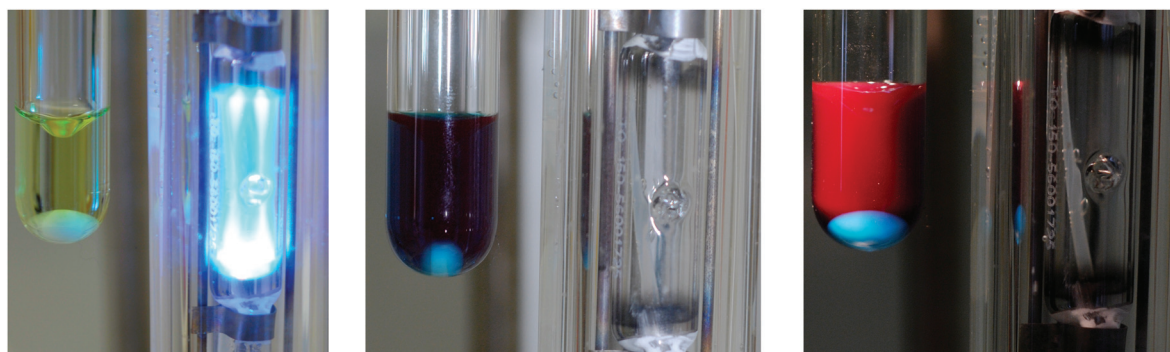
Fig. 1 Photolysis of caged carbopyronine dye **1a** in methanol. Irradiation of the pale yellow, non-fluorescent solution with the medium pressure mercury lamp through a Pyrex filter (left) gives a blue dye (middle) with intense red fluorescence visible only under light from incandescent lamps (right).

caged fluorescent dyes

1a: R² = Me;
1b: R² = CONH(CH₂)₂COOH;
1b-NHS: R² = CONH(CH₂)₂CONHS (NHS = N-Hydroxysuccinimidyl)
8a: R¹ = Me; 8b: R¹ = H; 8c: R¹ = Ac; 8d: R² = Ac;
8e: R² = H; 8f: R² = CONH(CH₂)₂CO₂Et;

preparative photolysis of model compound 1a

Scheme 3 Synthesis and photolysis of the caged red-emitting carbopyronines. Reagents and conditions: (a) Ac₂O, AcOH, HCl, 40 °C, 20 min; (b) (COCl)₂, CH₂Cl₂, THF, r. t., 4 h; (c) CH₂N₂/Et₂O, Et₃N; −5–0 °C, 8–12 h; (d) K₂CO₃, MeOH, r. t., 1 h; (e) O═C═N(CH₂)₂CO₂Et, Et₃N, DMF, 50 °C, 6 h; (f) 0.05 M NaOH in aq. THF (1 : 1), 5 °C; (g) N-hydroxysuccinimide, HATU, Et₃N, MeCN, 0 °C, 30 min; (h) photolysis in MeOH (>350 nm), r. t. (AcOH: 0–5%, v/v).

Table 1 Spectral properties of caged dyes **1a–d** and their fluorescent counterparts

Caged dye (Scheme 1)	Absorption (MeOH)			Fluorescent dyes after uncaging[b] (in aq. PBS buffer)		
	λmax (nm)	ε (M⁻¹ cm⁻¹) at 317 nm	Uncaging quantum yields η (%, MeOH) FP/DP[a]	λmax (nm) absorption [ε (M⁻¹ cm⁻¹)]	λmax (nm) emission	Φfl. (%)[c]
1a	214, 267, 317	14 000	1.3/0.7	637 [1.2 × 10⁵][d]	660	53
1b	213, 268, 316	12 000	0.7/1.4	632 [1.1 × 10⁵][e,f]	653	57
1c	212, 260, 317	7000	2.0/0.7	634 [0.28 × 10⁵][g]	651	43
1d	212, 262, 317	6000	not measured	633 [1.1 × 10⁵][e]	652	53

[a] Quantum yields of the uncaging reaction leading to fluorescent (FP) and dark products (DP) under irradiation with 405 nm light. [b] Measured directly after photolysis of 30 μmol solutions in quartz cuvettes to the full conversion of the starting compounds after irradiation with a medium pressure mercury lamp. [c] Fluorescence quantum yields measured in the reaction mixtures (see note "b" above) are unaffected by the presence of the "dark" side-products. [d] ε was measured for the isolated product 3a (Schemes 1 and 3) formed from compound 1a in the course of the preparative photolysis in MeOH. [e] ε was calculated for the reaction mixture (without isolation of the fluorescent product) obtained after complete photolysis of 30 μmol aqueous solutions of compounds 1b and 1d. [f] Contrast ratio (absorption intensity at 632 nm before and after photolysis) was measured to be about 32. [g] For a 10 μmol solution of compound 1c.

model compound **1a** in methanol (Scheme 3). As in the case of Rhodamines NN, the side product with an extended carbocyclic ring (compound **9**) was formed as a result of an intramolecular carbene attack.[4] The absorption spectrum of the non-emissive compound **9** is given in Fig. 2. In the red spectral region, this compound has a constant and low absorbance, and acts, in fact, as neutral filter. Later we established that this undesired process (intramolecular carbene rearrangement in Scheme 1) is totally suppressed if acetic acid is added to the methanolic or aqueous solutions beforehand. Presumably, acetic acid favors the

formation of the positively charged dye from the short-living carbene intermediate, providing higher concentration of counter anions. The caged dye solutions acidified with HOAc proved absolutely stable in the dark for weeks. Strong mineral acids, on the other hand, are known to cause rapid decomposition of the diazoketones (an acid-assisted Wolff Rearrangement).

Both syntheses and photolysis of model compound **1a** proved to be high-yielding. Therefore, this approach looked a lot more promising than the route to the caged rhodamines **7**-R² and **2b** described above (illustrated by Scheme 2). As potential near-IR
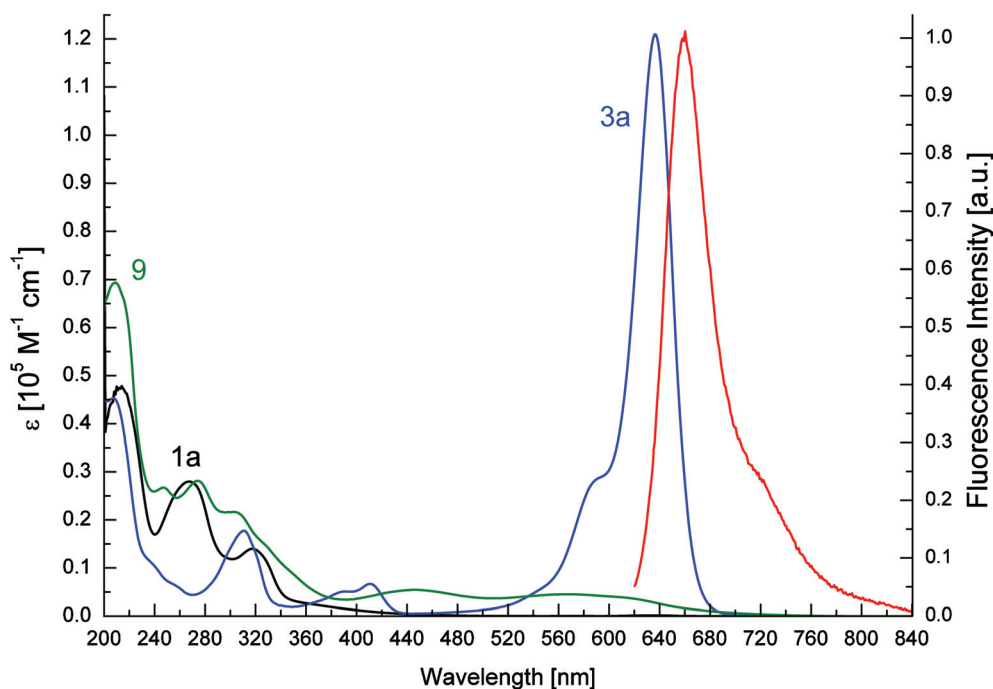
Fig. 2 UV-Vis spectra of caged dye **1a** (black line) and the products of its photolysis: the fluorescent product **3a** (blue line) and the "dark" side-product **9** (green line), as well as the normalized emission spectrum of compound **3a** (red line).

fluorescent *markers*, the successful candidates need to be suitable for conjugation to biological objects. To this end, a hydrolytically stable "bridge" containing the carboxy group is absolutely necessary. The latter is usually converted to amine-reactive derivatives (*e.g.*, *N*-hydroxysuccinimidyl esters). As a precursor for near-IR emitting markers, we utilized a carbopyronine derivative with a free hydroxy group (**8b**, Scheme 3).[1b] 2-Hydroxyethyl and/or 2-methoxyethyl groups are important for dye design, as they not only increase the solubility in water, but also assist in creating a new binding site. The proper reagents for derivatization need to be sufficiently reactive towards OH groups (at the 2-hydroxyethyl site) and possess the protected carboxylic acid residues. On the other hand, they need to form derivatives whose hydrolytic stability is better than that of carbonic acid esters. Otherwise, these derivatives would not withstand the saponification conditions or enzymatic hydrolysis. Therefore, from all available reagents, alkyl isocyanates (R–N═C═O) seemed to be most promising. Indeed, upon reacting with alcohols, they form urethanes, which are difficult to hydrolyze. Ethyl 3-isocyanatopropionate (O═C═N(CH$_2$)$_2$CO$_2$Et), a commercially available product, was chosen for a first try. The synthesis of a carbopyronine dye with an additional binding site (structure **1b**) and its active ester **1b**-NHS is illustrated by Scheme 3. To avoid complications in the preparation of diazoketone **8d**, we had to use an acetyl group for *temporal protection* of the hydroxy group in compound **8b**. In general, the synthesis proved high-yielding, as seen in Scheme 3 (for more details see ESI†). The required zwitterionic compound without a net charge (free acid **1b**) was fully characterized and converted to its active ester (**1b**-NHS). It is possible to generate the fluorescent dye from acid **1b** using UV or even visible light ($\lambda < 430$ nm). The spectroscopic properties of **1b** before and after the uncaging are presented in Table 1. The stability of the active ester **1b**-NHS in

aqueous solutions proved to be high enough to follow the standard labeling protocols. Also importantly, this dye possesses sufficient water solubility and, therefore, an excess of the active ester and the acid are easily separated from the conjugates. The uncaging efficiency and imaging performance of dye **1b** (brightness, contrast) in conjugates with antibodies is illustrated in Fig. 3.

To further improve the performance of the caged carbopyronine-containing fluorescent marker (**1b**), we utilized the julolidine structural fragment and prepared compounds **1c,d** (that originate from compound **14** in Scheme 4). The julolidine fragment with its additional trimethylene bridge, when attached to the nitrogen atom, further "rigidizes" the molecule and increases fluorescence quantum yield and photostability of fluorophores.[1] In our recent research we explored the photostability of the dye Atto 647 N (ATTO-Tec, Siegen, Germany), that contains a julolidine fragment, and compared it with the photostability of a structurally similar carbopyronine dye without julolidine fragment.[1b] As seen from the bleaching curves, in confocal microscopy, the performance of all dyes is similar (excitation with 633 nm laser), while under STED conditions (depletion with very powerful light of 750 nm), Atto 647 N was found to bleach slower. At the same time, dye **4a** (KK114) in Scheme 1, whose STED performance is excellent, also contains two julolidine fragments (yet with double bonds). Considering all these, we decided to synthesize and test a julolidine-containing caged carbopyronine dye using the general method for carbopyronine synthesis developed previously[1b] and the synthetic approach described above. We expected to obtain a caged dye with higher photostability, higher quantum yield, and, most likely, better imaging performance (especially under STED conditions).

The actual synthesis of julolidine-containing dyes is illustrated in Scheme 4. Following the previously developed route,[1b] the
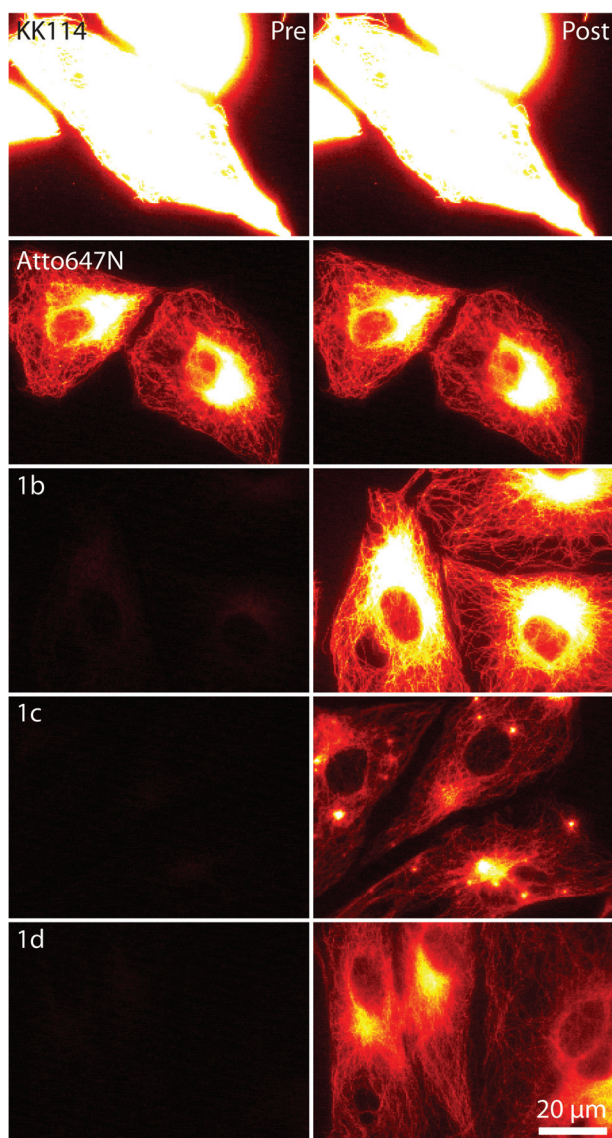
Fig. 3 Immunofluorescence microscopy. Imaging with two *regular* red-emitting dyes, taken as standards: **KK114** (very bright, top), the commercially available **Atto 647N** (below) and *caged* dyes: **1b**, **1c** and **1d** before (left) and after uncaging (right). For imaging with the epifluorescence microscope, the tubulin cytoskeleton of PtK2 cells was indirectly immunolabelled using antibodies coupled to the different fluorophores. Imaging conditions were kept constant between the left and the right image (exc. 630 ± 20 nm; det. 667 ± 30 nm). Photoactivation was performed by illumination with 360 ± 40 nm light. No image processing has been applied to the data.

carbopyronine core was constructed from two building blocks: alkene **13** and julolidine carbinol **14**.[8] The preparation of the alkene started from commercially available 3-(4-bromophenyl) propanoic acid **10**, which was cyclized to indanone **11a**.[9] In the presence of BCl$_3$, oxime **11b** underwent a Beckmann rearrangement to 7-bromo-1,2,3,4-tetrahydroquinoline (**12**).[10] This approach is an alternative to direct bromination of 1,2,3,4-tetrahydroquinoline and it proved better (in respect to overall yield and the absence of the tedious separation of isomeric bromides). Condensation of alkene **13** and carbinol **14**, removal of the

benzyl (Bn) protective group followed by alkylation with 2-methoxyethyl bromide to compound **15c**, and oxidation to the dye **16** went smoothly (see Scheme 4 and ESI for details). Luckily, the isolation procedure could be skipped in each of the four steps. That is very convenient, considering that all three intermediates (**15a–c**) are air-sensitive. Oxidation of compound **15c** with KMnO$_4$ in acetone gave poor yields of the cyclic diaryl ketone, unlike to the previously described analogue.[1b] Moreover, the ketone proved to be quite unstable, especially on silica gel. Therefore, we oxidized compound **15c** with a softer oxidant – periodate – to the deeply coloured cationic dye of structure **16** and tried to directly arylate it with compound **17**. The coupling reaction with aryl lithium compound **17** proceeded more difficult, as compared to that of the structurally related diaryl ketone.[1b] Despite the large excess of the lithium reagent **17** (and TMEDA as a promoter), the conversion of compound **16** was not complete and hardly ever exceeded 80%. Certain difficulties arose while liberating the carboxy group in compound **18** in hydrochloric acid media. In contrast to the previously described analog,[1b] this reaction required longer time. Also unexpectedly, demethylation of **19a** in a one-pot fashion (while heating **18** in conc. aq. HCl) proved too slow and was therefore carried out separately. The best yield of **19b** (compound with free hydroxy group) was achieved with BBr$_3$ in chlorobenzene (see ESI†).

All the subsequent transformations of compound **19b** (Scheme 4) were performed analogously to those of compound **8b** (see Scheme 3). The reactions proceeded with satisfactory yields, yet required longer time. Surprisingly, the presence of the julolidine fragment slowed down the reactions at the remote sites of the molecule (particularly, in the course of demethylation of **19a** and the reaction of **20b** with the isocyanate). It is worth mentioning that all further intermediates, up to the final step, proved rather unstable in a neat state, far less stable than in case of compound **1b**. Therefore, the isolation of neat compounds and additional purification steps were skipped where possible. The julolidine diazo ketones were purified using alumina because they decomposed on silica gel. Apart from light-sensitivity, these compounds were found to decompose upon concentrating of the solutions even at ambient temperature. High-boiling solvents (*e.g.*, chlorobenzene) were used for stabilization. Luckily, the final product – the caged dye with a carboxy group (**1c**) – was stable enough to be isolated in the pure state and obtain good NMR spectra. High crystallinity and low solubility are typical for julolidine derivatives. In the case of dye **1c**, the incorporation of the julolidine moiety also resulted in a lower solubility, as compared to dye **1b**. This might be unacceptable for most bio-conjugation procedures performed in aqueous solutions.

To improve the water solubility of dye **1c**, we attached an additional hydrophilic spacer to it (Scheme 4, bottom). For that, we used an amino ester with the incorporated diethylene glycol fragment.[11] The relatively polar and hydrophilic addition decreases the symmetry of the resulting compound **1d** as whole, without introducing a net charge. The coupling reaction leading to compound **1d**-Me was carried out by the conventional method (utilizing HATU reagent). Alkaline saponification of the coupling product **1d**-Me and the preparation of the NHS ester (**1d**-NHS) were performed precisely as described for **1b**/**1c** and **1b**-NHS/**1c**-NHS, respectively.
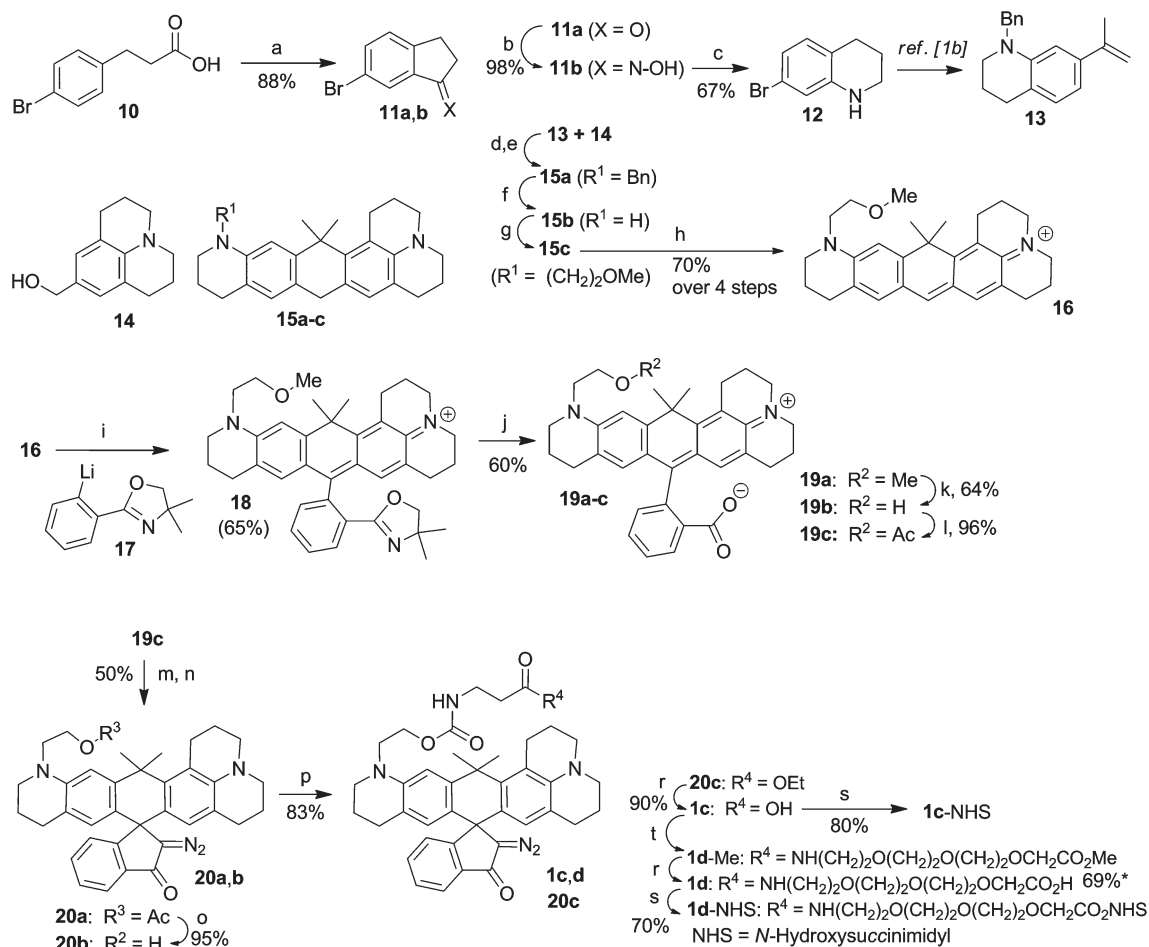
Scheme 4 Synthesis of caged red-emitting carbopyronine dyes with julolidine fragment. Reagents and conditions: (a) ClSO₃H, r. t., 1 h; (b) NH₂OH·HCl, MeOH, reflux, 1 h; (c) DIBAL-H, CH₂Cl₂, 0 °C–r.t., 1 h; (d) BCl₃, CH₂Cl₂, r. t., 12 h; (e) poly(phosphoric acid), 110 °C; (f) Pd/C, HCOONH₄, MeOH, Et₂O, 50 °C, 1 h; (g) Br(CH₂)₂OCH₃, DMF, K₂CO₃, 100 °C, 3h; (h) Bu₄NIO₄, HClO₄, EtOH, r.t., 1 h; (i) THF, –78°C–r.t.; (j) conc. HCl, reflux, 14 h; (k) BBr₃, PhCl, r. t, 1 h; (l) Ac₂O, AcOH, HCl, 40 °C, 1 h; (m) (COCl)₂, CH₂Cl₂, THF, r. t., 3 h; (n) CH₂N₂/Et₂O, Et₃N; −5 °C, 8–10 h; (o) K₂CO₃, MeOH, r. t. 1 h; (p) O═C═N(CH₂)₂CO₂Et, Et₃N, DMAP, 60 °C, 15 h; (r) 0.05 M NaOH in aq. THF (1 : 1), 5 °C, 10 h; (s) N-hydroxysuccinimide, HATU, Et₃N, MeCN, 0 °C, 30 min; (t) H₂N[(CH₂)₂O]₃CH₂CO₂Me, HATU, Et₃N, MeCN, 0 °C, 1 h. *Note*: * yield over 2 steps from compound **1c**.

The spectral properties of caged carbopyronines and their photolysis products

The spectral properties of the caged carbopyronine dyes **1a–d** before and after the uncaging are listed in Table 1. One can see that the spectra of all four dyes in the caged form are almost identical. Although the absorption intensities at 317 nm are low ($\varepsilon = 6000$–14 000), the band edge extends beyond 350–360 nm (up to 400–440 nm), so that the masked dyes are sensitive to daylight. As regards the incorporation of the julolidine fragment in the dye structures, we did not observe any marked influence on the spectral properties. In particularly, it did not shift the absorbance of the caged dyes closer to the visible region. Also, despite our expectations, it did not increase the fluorescence quantum yield in solutions after uncaging. It is worth mentioning that the julolidine fragment in compound **1c** drastically decreases the dye solubility in water, compared to that of **1b**. The preparative photolysis of dye **1a** in methanol gave the fluorescent dye **3a** and a non-fluorescent side-product **9** (in ratio *ca.* 2 : 1).

To evaluate the uncaging quantum yields of the reactions leading to the fluorescent and "dark" products, respectively, we irradiated the solutions of the caged dyes in methanol with a 405 nm laser, and analyzed the reaction mixtures using HPLC with UV detection at the isosbestic point (where both products have equal absorption).[12] The photoisomerization of azobenzene was used as an actinometric system.[13] The photolysis of the caged compounds **1a–d** in methanol is exemplified in Fig. 1: blue dyes with intense red fluorescence, well-seen with the naked eye, were readily generated by irradiation of colourless solutions. All four compounds **1a–d** demonstrated moderate uncaging quantum yields.[3d,14] Apart from Table 1, all these are well-illustrated by Fig. 2 (see below), where three absorption spectra – for caged dye **1a**, its fluorescent uncaging product **3a**, and side-product **9** – are put together on the same scale. The fluorescence spectrum of **3a** is also depicted. That demonstrates the Stokes shift of *ca.* 20 nm, typical for rhodamines and carbopyronines. In fact, all four compounds **1a–d** are spectrally almost identical in both states (masked and unmasked). For compound

1a, the ratio of the uncaging quantum yields of the fluorescent and "dark" products was found to be 2 : 1, which is in a good agreement with the preparative experiment (see ESI†). When measured at 635 nm, the absorption intensities of all fluorescent dyes obtained in the course of the photolysis are at least 20 times higher than these of the "dark" side-products (Fig. 2). The colour of the dye solutions obtained upon irradiation in methanol or water always looked "clean" (from light to dark blue). The absorption and emission spectra also indicated that the side-products of the photolysis do not interfere spectrally. For example, the brown and non-fluorescent compound **9** in Scheme 3 has a very low absorption (ε less than 4000) at 600–650 nm, while the photo-generated fluorescent dye **3a** has an extinction coefficient of 120 000 (at 637 nm). Thus, if the yields of these two products are comparable, the "dark" one acts, in fact, as a neutral filter which does not quench much of the emitted light (see the green curve for compound **9** in Fig. 2). Photolysis under biologically relevant conditions (in aqueous PBS buffer, pH 7.4) also produced clean blue colours, and the dark tones were not visible. All the absorption maxima lie in the narrow range between 632 and 637 nm, and the fluorescence quantum yields were good (0.43–0.57; see Table 1). The positions of absorption and emission bands well match the excitation wavelength of the red He–Ne laser (633 nm) and the STED depletion laser (750 nm), respectively. Aqueous photolysis of dyes **1a–d** was performed at low concentrations of 10–30 µmol. Photolysis of dye **1b** (analogue of **1a** with a linker for conjugation) in methanol was found to produce less of the fluorescent product than of the "dark" product (compared to the parent dye **1a**). However, in aqueous solutions the photolyzed compounds **1b** and **1d** always demonstrated a very high absorption at 632 nm ($\varepsilon = 1.1 \times 10^5$; $c = 15$ µmol), which is attributed to the uncaged acids **3b** and **3d**. Assuming that carbopyronines normally have ε values in the range of 10^5, we may conclude that in water the relative uncaging quantum yield of the reaction leading to the fluorescent product is apparently higher than in methanol. Photolysis of a 10 µmol aqueous solution of compound **1c** (whose solubility is limited) indicated that the apparent ε-value for the uncaged dye is *ca.* 28 000. The lower absorbance may be explained either by photobleaching or the larger proportion of the "closed" (colourless) form with the six-membered lactone ring, or by the adsorption of the lipophilic dyes on the glass surfaces. The latter factor most likely explains why the julolidine-containing dye with an additional hydrophilic spacer (**1d**) demonstrated almost a "normal" absorption (as high as dye **3a**) after photolysis in aqueous PBS buffer (see Table 1). Note that the modified dye **1d** allows concentrations above 200 µmol in water, while **1c** precipitates already at 20–30 µmol (therefore, the photolysis of **1c** was done in a 10 µmolar solution; see Table 1). Even though the photolysis of the caged dyes in solutions always leads to a mixture of the fluorescent and "dark" products, the caged fluorescent dyes proved to be valuable markers for antibodies in the immunofluorescence method. In order to evaluate the uncaging contrast ratio, a 40 µmol solution of caged dye **1a** in aqueous PBS buffer (pH 7.4) was irradiated for 3 min with a medium-pressure mercury lamp (250 W) through a Pyrex glass ($\lambda > 330$ nm) from the distance of 5 cm. The contrast was measured as a ratio of the absorption intensity at 633 nm after and before uncaging. At approx. 50% conversion of **1a** (HPLC), the conditional contrast

ratio was estimated about 46 (for details see ESI†). Meanwhile, the fluorescence of the caged dyes (when the solutions are well-protected from light before irradiation) was impossible to detect. Also, in Fig. 2, it is clearly seen that compound (**1a**) has almost zero absorbance at 600–700 nm.

Application of masked carbopyronines in immunofluorescence microscopy

The imaging performance of three dyes in epifluorescence microscopy is illustrated by Fig. 3. Comparing the images of PtK2 cells stained with the caged compounds **1b–d** and two standard near-IR emitting fluorescent dyes (KK 114 and Atto 647 N), one can see that after photoactivation all three uncaged dyes are as bright as Atto 647N. This dye contains a julolidine fragment, which provides the high photostability under STED conditions and very good performance in light microscopy.[1b] The first standard red-emitting dye was KK 114, which proved to be much brighter. All three caged dyes **1b–d** provided a very low fluorescent background before uncaging (see left images **1b–d** in Fig. 3) and high contrast. In case of activated dye **1c**, one can observe some bright aggregates. Most likely, the low solubility of the unreacted NHS ester and the free acid **1c** (formed in the course of immunolabelling) led to the formation of aggregates which were difficult to remove. Generally, the julolidine-containing dyes **1c** and **1d** do not demonstrate great advantages over dye **1b**. The imaging performance of the dyes and the scope of their applicability depend on photostability, fluorescence quantum yields in conjugates (which is in most cases lower, as compared to free dye solutions), intersystem crossing rates, the excited state lifetimes and other features which should be evaluated for compounds **1b–d**. In case of photosensitive masked dyes, the uncaging parameters (quantum yields at various wavelengths) and the spectral properties of photo-generated side-products are also very important.

An interesting application of the masked fluorescent dyes is illustrated in Fig. 4. In the simplest case, they enable new
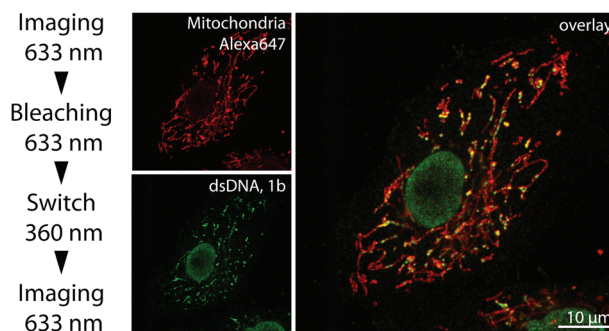
Fig. 4 Monochromatic multilabel imaging (with two labels). Left: imaging scheme. Right: application of the imaging scheme to immunolabeled cultured mammalian (PtK2) cells. Mitochondria labeled with primary antibodies against Tom20 protein and secondary antibodies coupled to Alexa647™ were imaged first. Then, after bleaching Alexa647™ fluorophores and irradiation with 360 ± 40 nm light, the cell's DNA, indirectly immunolabeled with an antibody coupled to compound **1b** was visualized. For convenience, different colours are used in the overview and overlay images. No image processing has been applied to the data.

imaging protocols based on the simultaneous detection of two fluorescent markers using one detection channel, one red excitation laser, and an additional source of UV or violet light for the uncaging (*e.g.* 405 nm diode laser). This approach is very flexible and promising, because the dyes with large Stokes shifts, other caged fluorescent dyes[15] and photoactivable rhodamine spiroamides[16] can easily be involved in a similar multilabel monochromatic imaging scheme.[4a]

Conclusion

Our study provided the *first caged fluorescent dyes that absorb and emit in the red* (at 637 and 660 nm, respectively). The uncaging (switching on) is achieved by illumination with UV or even visible light ($\lambda < 420$ nm) and provides images with high contrast. The introduction of the rigid structural fragment of julolidine (with additional methylene bridges) into the carbopyronine core in some cases improves the performance of the caged dyes, but decreases the water-solubility to a level hardly acceptable for staining in aqueous media. The solubility and the performance can be improved by introduction of a hydrophilic spacer with a diethylene glycol fragment. The syntheses of the dyes are optimized and high-yielding. The protocols make it possible to omit isolation and purification steps for certain precursors. The present study demonstrated the use of *a hydrolytically stable urethane-containing linker, important for dye design*. The syntheses of dyes **1b–d** also rely upon the 2-methoxyethyl group attached to a nitrogen atom in dye cores. Demethylation is followed by reaction with an isocyanate reagent bearing a protected carboxyl group. The activated carboxy group affords the use of the caged dyes as labels in bioconjugation reactions. Application of the caged red-emitting dyes in monochromatic multilabel imaging schemes offers the possibility of simultaneous detection of several fluorescent markers using only one detection channel. Due to the compact structure and the very small size of the caging group, Carbopyronines NN are expected to cross membranes of living cells. In combination with the site specific labeling protocols,[17] realized for the Halo-, CLIP- and SNAP-tags, the novel caged Carbopyronines NN extend the set of live cell labelling strategies based on attaching the red-emitting fluorophores to the genetically encoded protein tags. The spatially restricted photoactivation of Carbopyronines NN followed by tracking of the uncaged molecules may enable measurements of biomolecular dynamics, as well as diffusion parameters or flow velocities in microfluidic devices. The combination of Carbopyronines NN with nanoscopic techniques[2] (*e.g.* STED or single molecule switching and localization[16e–g]) is supposed to provide some additional information on subcellular structure. Moreover, measurements of the FRET signal in the newly uncaged areas (acting as fluorescent acceptors) located in the close proximity to the permanently colored regions (fluorescent donors) are likely to co-localize objects at the distances of 1–10 nm.

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