CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200701058

Photostable, Amino Reactive and Water-Soluble Fluorescent Labels Based on Sulfonated Rhodamine with a Rigidized Xanthene Fragment

Vadim P. Boyarskiy,[a, b] Vladimir N. Belov,*[a] Rebecca Medda,[a] Birka Hein,[a] Mariano Bossi,[a] and Stefan W. Hell*[a]

Abstract: Highly water soluble fluorescent dyes were synthesized and transformed into new amino reactive fluorescent labels for biological microscopy. To this end, rhodamine **8** (prepared from 7-hydroxy-1,2,3,4-tetrahydroquinoline (**7**) and phthalic anhydride in 85% aq. H_3PO_4) was sulfonated with 30% SO_3 in H_2SO_4 and afforded the water soluble disulfonic acid **3a** (64%). Amidation of the carboxy group in **3a** with 2-(methylamino)ethanol in the presence of *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium·PF_6^- (HATU) led to alcohol **3b** (66%), which was transformed into the amino reactive mixed carbonate **3d** with di(*N*-succinimidyl)carbonate and Et_3N. Reaction of the carboxy group in **3a** with $MeNH(CH_2)_2CO_2Me$ and *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)-uronium·BF_4^- (TSTU) yielded methyl ester **13**. After saponification of the aliphatic carboxy group in **13**, the compound was converted into NHS-ester **3e** (using HATU and Et_3N). Heating of **7** with trimellitic anhydride in H_3PO_4 gave a mixture of dicarboxylic acids **14** and **15** (1:1). Regioisomer **15** was isolated, sulfonated with 30% SO_3 in H_2SO_4, and disulfonic acid **3f** was used for the synthesis of the mono NHS-ester **3g**, in which the sterically unhindered carboxy group was selectively activated (with *N*-hydroxysuccinimide, HATU, and Et_3N). The sulfonated rhodamines **3b**, **c** and **f** are soluble in water (up to 0.1 M), have excellent photostabilities and large fluorescence quantum yields. Subdiffraction resolution images of tubulin filaments of mammalian cells stained with these dyes illustrate their applicability as labels for stimulated emission depletion microscopy and other fluorescence techniques.

Keywords: conjugation · dyes/pigments · fluorescence · rhodamines

Introduction

The invention of lens-based (far-field) optical nanoscopy spurred a search for novel fluorescent tags which facilitate an optical resolution in the nanometre region.[1] For example, the first viable approach to break the diffraction barrier, stimulated emission depletion (STED) fluorescence microscopy requires fluorophores that are particularly photostable.[1a] Laser dyes are characterized by high resistance towards photobleaching,[2] implying that they may well serve as lead structures for developing novel highly photostable markers. Rhodamines are widely used both as laser dyes and fluorescent compounds for labeling proteins and nucleic acids.[3,4] For instance, the readily available and inexpensive rhodamines 6G, B, and 101 are especially photo- and chemically stable, possess high fluorescence quantum yields ($\Phi_{Fluo} = 0.5$–1.0),[3c,d] and emit at 500–600 nm, where the autofluorescence of cells and proteins is negligible. However, rhodamine 6G, one of the best laser dyes is poorly hydrophilic. Its use in purely aqueous solutions is hampered by the formation of dimers, aggregates,[5a] and unspecific adsorption.[5b] As a laser dye, it is employed in diluted aqueous solutions with detergents or in alcoholic solutions. Water is preferred as a solvent, due to its high heat capacity. Conjugation reactions involving biologically relevant macromolecules (proteins, nucleic acids, carbohydrates) must be performed in water or aqueous buffers, where they are stable. The marker is commonly dissolved in an organic solvent,

[a] Dr. V. P. Boyarskiy, Dr. V. N. Belov, Dipl.-Biol. R. Medda, Dipl.-Chem. B. Hein, Dr. M. Bossi, Prof. Dr. S. W. Hell
Department of NanoBiophotonics
Max Planck Institute for Biophysical Chemistry
Am Fassberg 11, 37077 Göttingen (Germany)
Fax: (+49) 551-201-2506
E-mail: vbelov@gwdg.de
shell@gwdg.de

[b] Dr. V. P. Boyarskiy
Chair of Physical Organic Chemistry
Chemistry Department, St. Petersburg State University
198504 St. Petersburg (Russia)

Supporting information for this article is available on the WWW under http://www.chemeurj.org/ or from the author.

such as DMF or DMSO, and then added to the aqueous solution of the target molecule. An excess of the organic solvent may cause protein denaturation, and hence should be avoided. On the other hand, if the amount of the organic solvent is too low, precipitation of the marker in the reaction mixture is frequently observed, which may result in a low coupling efficiency. Water-soluble fluorescent markers are advantageous in this regard, because they may be used for labeling without the addition of organic solvents. Moreover, hydrophilic labels are less prone to aggregation and to non-specific binding with biological objects.

An efficient and simple way to increase the water solubility is to sulfonate the corresponding reagent.[6a] The sulfonation of the fluorophores is also known to inhibit the fluorescence quenching (reduction in Φ_{Fluo}) commonly observed after conjugation with proteins and increase both the photostability and the insensitivity to pH changes.[6b] Therefore, it is not surprising that some sulfonated xanthene derivatives—analogues of rhodamine 6G—have been developed and commercialized (Figure 1).[4,6b] In all of them, the nitrogen atoms are involved in the five- or six-membered rings condensed with the xanthene system. The rigidification of the rhodamine fragment improves the π-conjugation in the whole fluorophore by blocking the rotation around the C–N bonds. The rigidification also increases the fluorescence quantum yield and makes it independent of the temperature and the environment.[2b]

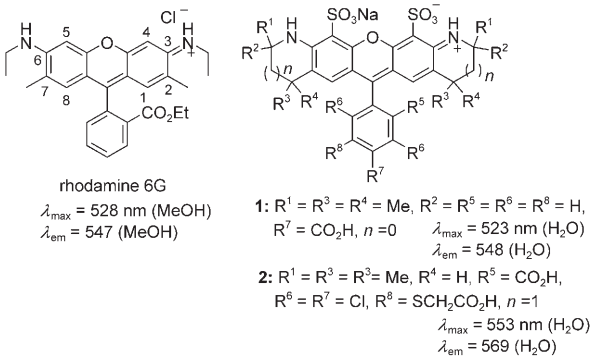


Figure 1. Rhodamine 6G and its sulfonated commercially available analogues with the rigidized xanthene fragment (**1**: Alexa Fluor 532 and **2**: Alexa Fluor 546 from Molecular Probes).

The compounds **1** and **2** were prepared by sulfonation of the corresponding rhodamines. Their further conjugation with goat anti-mouse IgG antibodies was achieved by using the corresponding amino reactive NHS esters (R^7 = CO-NHS (**1**) and R^8 = $SCH_2CONH(CH_2)_5CO$-NHS (**2**)). The conjugates were found to have absorption λ_{max} = 531 nm (**1**)/556 nm (**2**) and λ_{em} = 554 nm (**1**)/575 nm (**2**).[4] Compounds **1** and **2** possess six (!) methyl groups. These complicate their structures, considerably increase their molecular weight and lipophilicity, thereby reducing the solubility in water and creating two asymmetric CH atoms (two diastereomers). Working with diastereomeric mixtures is not convenient, because two sets of signals in the analytical data (HPLC and NMR) complicate the characterization of the intermediates and the final products.

The most straightforward way to obtain water-soluble analogues of rhodamine 6G would be by direct sulfonation. It is well known, for example, that rhodamine 110—a 3,6-diaminoxanthene derivative with unsubstituted amino groups—smoothly reacts with 30% SO_3 in H_2SO_4 giving the corresponding 4,5-disulphonated product regioselectively.[6b] However, our attempts to sulfonate rhodamine 6G under similar conditions left the starting compound intact (only the ethyl ester group was cleaved). Prolonged exposure to 30% SO_3 in H_2SO_4 at room temperature or by heating (40–50 °C) gave a complex mixture of products; the major product was non-fluorescent.

Taking this into consideration, together with the very high prices for synthetically useful amounts of all water-soluble fluorophores (several thousand Euro per 50 mg), and the advantages of rhodamines without structural mobility of alkylamino groups, we have synthesized new simple water-soluble analogues of rhodamine 6G with a rigidized xanthene fragment (**3a**–**c**, **f**). We also transformed them into the amino reactive compounds **3d**, **e**, **g** (Figure 2), conjugated

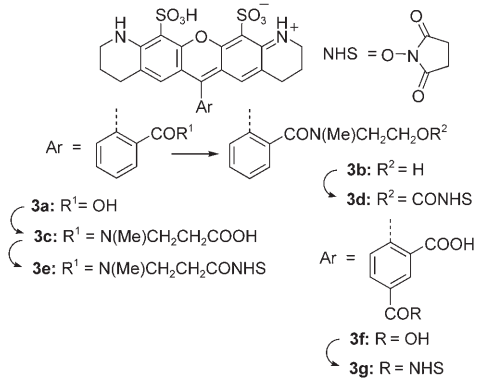


Figure 2. Water-soluble fluorescent labels prepared in this study.

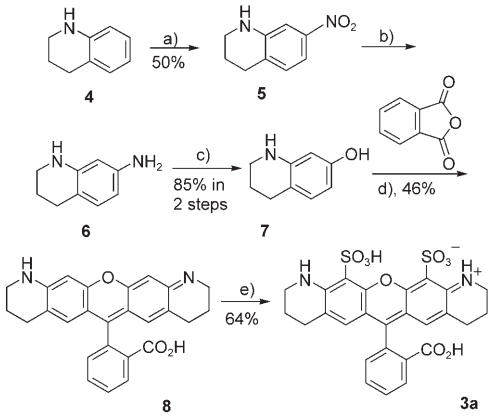
them with antibodies and studied their spectral and staining properties. Special efforts were taken in the synthesis of derivatives with various linkers and reactive groups. Synthetic procedures, isolation, and handling of the highly amino reactive water-soluble derivatives are described in detail herein. Moreover, their use as photostable labels for biological samples in fluorescence microscopy, particularly in subdiffraction resolution STED microscopy,[1,7] is demonstrated.

Results and Discussion

Synthesis of the fluorescent markers 3d, e and g: If the nitrogen atoms of the xanthene fragment are included into the six-membered rings condensed with the aromatic system, the rotation around the C–N bonds is blocked, and the unshared electrons of the nitrogen atoms are effectively conju-

gated with the whole π system (in the ground and in the excited state). The lack of an additional degree of mechanical freedom decreases the undesirable nonradiative deactivation of fluorescence. Derivatives with five-membered rings—corresponding indolines—are more difficult to synthesize. Thus, we have chosen the analogues of rhodamine 6G as target compounds with nitrogen atoms included into the six-membered rings and with the same number of carbon atoms as in the parent dye (Figure 2).

Aminophenol **7** was easily prepared from 1,2,3,4-tetrahydroquinoline via a slightly modified method of Field and Hammond by nitration of the commercially available 1,2,3,4-tetrahydroquinoline (**4**), reduction of nitro compound **5**, and hydrolysis of the crude amino compound **6** with 85% H_3PO_4 (Scheme 1).[8] Rhodamine **8** was synthesized from

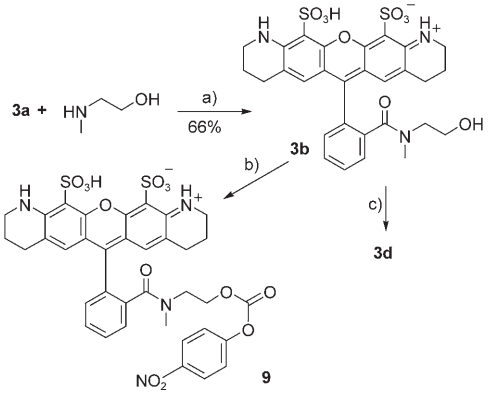


Scheme 1. Sulfonated analogue of rhodamine 6G with a rigidized xanthene fragment: a) 100% HNO_3, conc. H_2SO_4, 5–7°C; b) $N_2H_4{\cdot}H_2O$, Ra/Ni, MeOH, reflux; c) 85% H_3PO_4, 150°C, 24 h; d) 170°C, 3 h, then 85% H_3PO_4, 170°C, 3 h; e) 30% SO_3 in H_2SO_4, 0°C, 12 h.

the phthalic anhydride and compound **7** in 46% yield.[9] The sulfonation of this compound has not yet been reported. The procedure described for rhodamine 110 (sulfonation with 30% SO_3 in H_2SO_4)[6b] afforded the water-soluble derivative **3a** with 64% yield.

The carboxylic group of the 2-substituted benzene ring in rhodamines is not suitable for direct attachment of a dye to the primary amino groups in proteins for several reasons. First, this group is difficult to activate due to steric hindrance of the very bulky xanthene fragment. Second, its primary amides cyclise easily under neutral or basic conditions: the NH group adds to the electron-deficient C^9 atom, giving the corresponding five-membered non-fluorescent and colorless spirolactames with broken π-conjugation.[10] Therefore, it is necessary to use a linker between this carboxylic group and an amino reactive site. 2-Substituted benzoic acid fragment in rhodamines can react with alcohols to form esters, but this ester linkage is unstable. Due to the close proximity of the electron-deficient C^9 atom of the xanthene fragment, 2-substituted alkyl benzoates may be very easily hydrolysed, even under relatively mild conditions (e.g. at pH 8–9, in the course of conjugation with antibodies). The amides are more stable for hydrolysis than esters and, therefore, we used the secondary amide bridge prepared from 2-(methylamino)ethanol or 3-(methylamino)propionic acid for linking the fluorophore and the amino reactive site.

Activated esters or carbonates are among the most widely used amino reactive groups.[3,4,11] Mixed activated carbonates are known to be more stable than the corresponding activated esters (yet enough amino reactive), but not all of them are suitable for bioconjugation. For example, we used 2-(methylamino)ethanol as a linker (Scheme 2), coupled it with the acid **3a** using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) as a very strong activating agent, and then transformed alcohol **3b** into the 4-nitrophenylcarbonate **9**, which was found to be highly reactive and, at the same time, stable enough to be isolated by reversed-phase column chromatography.



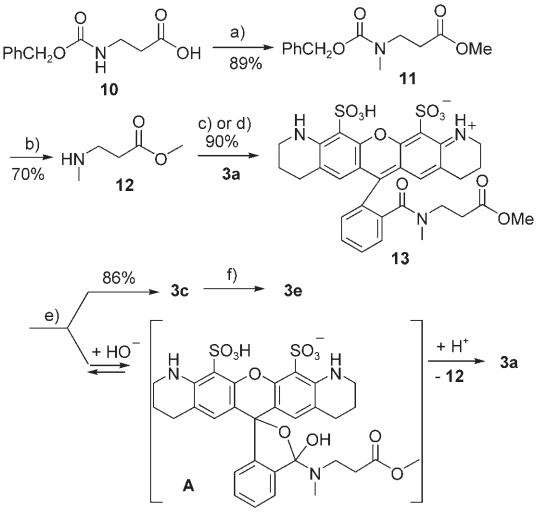
Scheme 2. Synthesis of the amino reactive carbonates **9** and **3b**: a) *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium·PF_6^- (HATU), *N*,*N*-dimethylacetamide (DMAA), RT, 1.5 h; b) bis(4-nitrophenyl)carbonate, Et_3N, DMAA, RT, 22 h; c) di(*N*-succinimidyl)carbonate, Et_3N, DMAA, RT, 1 h.

The fluorescence quantum yield (Φ_{fl}) of the compound **9** was found to be low (15%), probably because the xanthene fragment forms an intramolecular π-stacking complex with the *p*-nitrophenoxy residue, or the resonance energy transfer (RET) between them quenches the fluorescence.[12] After coupling this active carbonate **9** with an aliphatic amine ($MeNH_2$), Φ_{fl} increased up to the value observed for the compound **3b** (94%), and the solution became brightly fluorescent. However, reaction with bovine serum albumine (BSA) did not restore the initial Φ_{fl}, and the conjugate emitted poorly ($\Phi_{fl}=15\%$). This result may be explained by the retaining of the *p*-nitrophenol on a protein nearby the fluorophore, so that its quenching effect remained intact.

Due to this drawback of the mixed carbonate **9**, we prepared another reagent of this type (**3d**) and used it for bioconjugation (which contained *N*-hydroxysuccinimide as a leaving group). The Φ_{fl} value of the adduct with antibodies was found to be high. (Spectral properties and stabilities of

the fluorescent markers and their bioconjugates are discussed in the next section.)

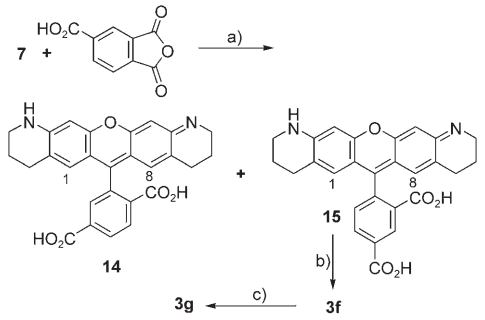
Activated esters represent another type of an amino reactive site. To prepare them from compound **3a**, it was necessary first to synthesize an *N*-alkyl-ω-amino acid as a linker with an appropriate length. The secondary amino group blocks the undesirable spiro-lactonization, and we have chosen the methyl group as the simplest *N*-alkyl substituent. It may be useful to keep the distance between the fluorophore and the active group short enough. Longer alkyl chains not only increase the lipophilicity of the whole reagent, but they also may considerably limit the freedom, if assembling of the RET pairs from the fluorescent markers described in this work (as donors) and variable acceptors will further be used. The total length of the linker between the donor and the acceptor should not exceed the Foerster radius.[12] We used 3-aminopropionic acid as a linker, which provides a distance of about 7–8 Å between the fluorophore (the centre of the xanthene fragment) and the terminal carboxy group. Alkylation of the commercially available 3-(benzyloxycarbonylamino)propionic acid (**10**) with MeI and Ag_2O, followed by catalytic hydrogenation of the crude methylated compound **6** with 10% Pd/C under atmospheric pressure, gave the required methyl 3-(*N*-methylamino)propionate (**12**) (Scheme 3).



Scheme 3. Preparation of the active ester **3e**: a) MeI, Ag_2O, DMF, RT, 28 h; b) 10% Pd/C, H_2, EtOAc, RT, 7 h. c) HATU, Et_3N, DMF, 0°C → RT, 6 h; d) *N,N,N′,N′*-tetramethyl-*O*-(*N*-succinimidyl)uronium·BF_4^- (TSTU), Et_3N, DMF, RT, 2 d; e) 0.23 M aq. NaOH, 0°C, 1 h; f) HATU, *N*-hydroxysuccinimide, Et_3N, DMF, 0°C, 1 h.

The condensation product of amine **12** and rhodamine **3a**—the secondary amide **13**—was obtained in almost quantitative yield, even if the less reactive and cheaper coupling agent TSTU (compared to HATU) was used. Surprisingly, uncontrolled saponification of this ester to the free acid **3c** under usual conditions (1.5–2 fold excess of aq. NaOH at room temperature) gave a mixture of the target compound and the benzoic acid **3a**, which had been formed by hydrolysis of the secondary amide bond. This result may be explained by the activating effect of the electron deficient atom (C^9), which bears a part of the positive charge of the rhodamine fragment and is situated in close proximity to the carbonyl group of the secondary amide. The positively charged centre (C^9) and oxygen atom of the amide [$C_6H_4CON(Me)$] are separated by four chemical bonds, so that the whole geometry and charge distribution of this fragment favor the attack of the hydroxyl ion (OH^-) on the carbonyl group and equilibration with the five-membered spiro-intermediate (**A**), which may irreversibly decompose to give the acid **3a**. Saponification with the diluted aq. NaOH at 0°C and HPLC control allowed reaching about 90% conversion of the ester **13** into the desired acid **3c**, while the content of the rhodamine **3a** did not exceed 2–6%. After that, the cold reaction mixture was carefully neutralized with aq. HCl, and the acid **3c** was isolated by reversed-phase column chromatography.[13]

An alternative way to obtain a rhodamine with an amino reactive group in the benzene ring and to avoid the spiro-lactonization and saponification problems associated with an "amide" linker is to use the trimellitic anhydride at the condensation step. This route always provides a mixture of 2 regioisomers (Scheme 4). Each of them possesses 2 carboxyl groups: a sterically hindered one and an unhindered (remote) one.



Scheme 4. Preparation of sulfonated regioisomer **3f** and its mono NHS ester: a) 170°C, 3 h, then 85% H_3PO_4, 170°C, 3 h; b) 30% SO_3 in H_2SO_4, 0°C, 12 h; c) *N*-hydroxysuccinimide, HATU, Et_3N, DMF, 0°C, 1 h.

We have found that the mixture of the acids **14** and **15** could be separated by recrystallization or column chromatography on silica gel so that compound **15** could easily be isolated in a pure form. The structures of compounds **14** and **15** were unambiguously established with ^1H NMR spectra by using the NOE experiments. Irradiation at the frequency of the H^1/H^8 protons of the xanthene system causes an increase in the intensity of one-proton multiplet. It corresponds to the hydrogen atom in the benzene ring, which is situated in the *ortho* position to the C–C bond between the aromatic rings. In one case (**15**), this signal is a well-resolved doublet ($\delta = 7.5$ ppm) with $J = 7.8$ Hz, and in the other case

(compound **14**)—a badly resolved doublet ($\delta = 7.9$ ppm) with $J = 1.5$ Hz.

The pure regioisomer **15** was isolated in about 10% yield and sulfonated under conditions described for compound **8**. We obtained the target disulfonated compound **3f** with good yield (63%).

Due to the steric hindrance, the carboxyl group nearby the xanthene fragment is much less reactive than the carboxyl at the remote position. Thus, it is possible to obtain the activated ester only from the remote, less sterically shielded carboxyl.[2] We used HATU as the coupling reagent, and isolated the amino reactive NHS-ester **3g** from the reaction mixture by preparative HPLC.

Water solubility of the compounds synthesized: The solubility of rhodamine derivatives **3a–c**, **f** and **8** in water was measured by absorption spectroscopy. An excess of a test compound was mixed with about 1 mL of distilled water, then sonicated for one hour in an ultrasound bath, allowed to rest overnight, and finally centrifuged at 16060 × g (RCF) for 30 min. The supernatant was diluted with water, and the concentration of the solution was estimated by measuring the absorbance (all ε values were measured by us). The results are listed in Table 1. Not unexpectedly, the unsulfonated parent rhodamine—compound **8** with five condensed cycles—was found to be very poorly soluble in water. All sulfonated derivatives (**3a–f**) are 1000–20000 times more soluble than compound **8**. Additional polar groups attached to the benzoic acid residue in compound **3a** further increase the solubility. For example, amidation of acid **3a** with 2-(methylamino)ethanol substantially improved the solubility of product **3b**. Secondary amide **3c** (with an additional free carboxy group) was even more soluble than the corresponding alcohol **3b**. If a second carboxy group was attached to compound **3a** without blocking the first one (**3f**), the solubility also increased, but to a less extent than for acid **3c**. A direct evaluation of the solubility of the corresponding NHS esters was not possible as they rapidly decompose in water. However, it is practically important that all compounds (**3b**, **c** and **f**), which were used for the preparation of the corresponding NHS esters, are very good soluble in water. Thus the solubility of the NHS esters (**3d**, **e** and **g**) is also secured.

Table 1. Spectral properties of rhodamine **8**, its sulfonated derivatives **3a–c**, **f** and their coupling products with antibodies[a] in water.

Compound[b]	λ_{abs}^{max} [nm]	ε [$M^{-1}m^3cm^{-1}$]	λ_{em}^{max} [nm]	Φ_{Fluo}	τ_{Fluo} [ns]	Solubility in water [mM][c]
8	538	8.4×10^4	559	0.95	3.8	0.006
3a	530	9.8×10^4	550	0.98	3.9	6
3b	539	8.8×10^4	560	0.94	4.0	45
3c	539	8.6×10^4	560	0.96	4.1	120
3f	531	7.4×10^4	555	0.99	4.1	37
3b[d]–**AB**[a]	542	–	560	0.70	3.4	–
3c[e]–**AB**[a]	543	–	560	0.74	3.7	–
3f[f]–**AB**[a]	534	–	555	0.82	3.9	–

[a] Goat anti-rabbit IgG (H+L). [b] See Figure 2 for structures. [c] Solubility of rhodamine 6G hydrochloride was measured to be 18 mM. [d] Mixed NHS-carbonate **3d** (obtained from **3b**) was used for coupling. [e] NHS-ester **3e** (obtained from **3c**) was coupled with antibodies. [f] NHS-ester **3g** (obtained from **3f**) was used in the coupling reaction with antibodies.

Photophysical and photochemical properties of water soluble rhodamines: The spectral properties of sulfonated rhodamines (Figure 2) and their coupling products with antibodies were measured in water (Table 1). For comparison, the properties of the unsulfonated parent rhodamine (compound **8**, Scheme 1) were also measured and are given in the same table.

Sulfonation in the positions 4 and 5 of the xanthene chromophore resulted in a hypsochromic effect for both the absorption and the emission maxima (compare **8** and **3a**), and a very slightly improved fluorescence efficiency (Φ_{Fluo}). Changes in the spectroscopic properties of amides **3b** and **c** (compared with the corresponding carboxylic acid **3a**) are in agreement with the regularities found for similarly modified rhodamines.[14] Two clear effects were observed, after the *ortho*-carboxy substituted phenyl ring at the position 9 of the chromophore had been modified: amidation of this group resulted in a red shift of $\approx$10 nm in the absorption and emission bands and also a very small decrease in the emission quantum yield. Addition of a second carboxylate to the *m*-position with respect to the "first" carboxylic group (compound **3f**) only resulted in a red shift of $\approx$5 nm in the emission maximum with no effect in the fluorescence efficiency. Variation in the fluorescence lifetime of all the free dyes in water was marginal and close to the measurement error (0.1 ns).

Antibody labeling resulted in a bathochromic effect in the absorption, while the emission maxima were not significantly shifted. The fluorescence quantum yields and lifetimes of the conjugates decreased moderately. These effects were more pronounced when the linking group was in the *ortho* position of the phenyl group with respect to the chromophore. Nevertheless, Φ_{Fluo} values of the conjugates were found to be very high (0.7–0.8), and therefore, they are perfectly suited for labeling of biological structures.

Photochemical stability is a crucial property if soluble rhodamines are required as fluorescent labels or laser dyes. To evaluate the fatigue resistance of the prepared dyes, water solutions of compounds **3b**, **c** and **f** (3 mL each) were irradiated in a continuously stirred cuvette at 532 nm with a power of 3 W cm^{-2}, and the fluorescence emission was monitored. Rhodamine 6G was used as a reference. The absorption at the irradiation wavelength of all solutions was matched to within 1% error. The results are shown in Figure 3. All the sulfonated rhodamines proved to be more photostable than rhodamine 6G. In particular, the amide substituted compounds **3b**,**c** were considerably more stable than the reference; after two hours of irradiation less than 10% of their fluorescence signal was lost. In the same time, 25% of rhodamine 6G was bleached. Photobleaching of rhodamine 6G involves radicals which are formed from the dye cations (in an excited state) by electron transfer from

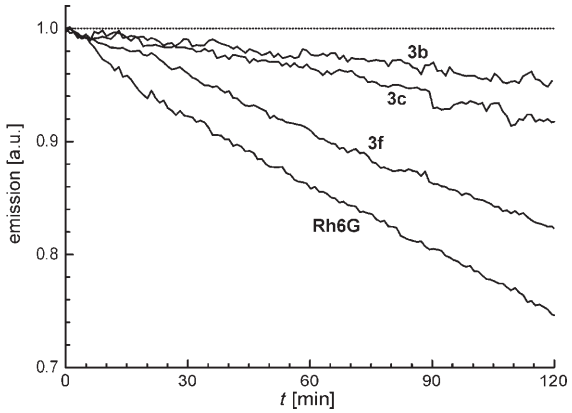


Figure 3. Fluorescence signal upon irradiation of aqueous solutions of compounds **3b**, **c**, **f** and rhodamine 6G with green light (532 nm, laser intensity 3 $W cm^{-2}$).

the solvent, followed by the formation of reduced (colorless) species.[15] Higher photostability of the compounds **3b**,**c**,**f** (compared with rhodamine 6G) may be explained, if we assume that the introduction of the polar charged sulfo group(s) stabilizes the reactant (due to its better solvation in water) and/or destabilizes the intermediately formed anion radical.

Microscopy applications: All labeled antibodies (**3d–AB**, **3e–AB**, and **3g–AB**) were found to have excellent performance in confocal laser scanning microscopy. High brightness and a fairly good fatigue resistance, as well as a minimal nonspecific background staining, allowed good quality pictures to be recorded.[16]

STED microscopy provides a spatial resolution beyond the diffraction barrier through a saturated depopulation of excited molecules residing in the fluorescent first singlet state (S_1). This concept is usually implemented in a beam-scanning microscope, whereby the focal spot of a scanning excitation beam is superimposed by a red-shifted doughnut-shaped focal intensity distribution for the saturated quenching. The doughnut-beam quenches excited molecules throughout the excitation spot except for its very centre. This quenching by stimulated emission (light induced $S_1 \rightarrow S_0$ transition) is performed at the red edge of the emission spectrum of the fluorophore, with intensities $>10\,MW cm^{-2}$. The latter are required to saturate the depopulation and to attain a spatial resolution <30 nm as a result. Therefore, the markers used in STED microscopy should have an outstanding photochemical stability in their excited states. Another important requirement for a STED fluorescent marker is a large cross section for stimulated emission at available laser wavelengths. To evaluate the performance of the new fluorescent dyes, we have compared their properties with those of the commercially available compound ATTO-532 (Atto-Tec GmbH, Siegen, Germany), a well-known and relatively photostable fluorophore routinely used in STED microscopy.[17] The STED efficiencies measured for all dyes and the reference compound are shown in Figure 4. Optimal depletion wavelengths were found to be approximately 630 nm for ATTO-532 and compound **3f**, and 640 nm for compounds **3b** and **c**. All the newly prepared compounds can be depleted with high efficiency, and to a similar extent as the reference compound. More than 90% of the signal can be depleted with intensities of 40 $MW cm^{-2}$ in the focus, a typical value in STED microscopy.

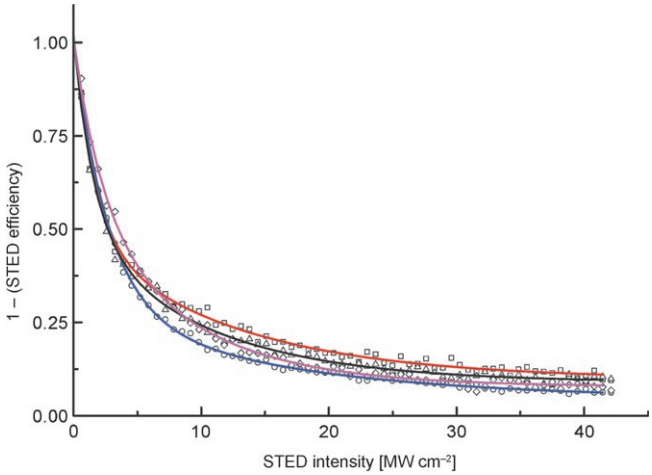


Figure 4. STED efficiency as a function of the depletion intensity for compounds **3b** (——), **c** (——), **3f** (——) and ATTO-532 (——). Excitation was performed with a constant intensity at 532 nm, and depletion was performed at 630 nm for compound **3f** and ATTO-532, and 640 nm for compounds **3b** and **3c**.

The tubular network of mammalian PtK2 cells was immunostained with the prepared conjugates (**3d–AB**, **3e–AB**, and **3g–AB**). These samples were investigated in a STED microscope operating with synchronized 80 MHz pulse trains for excitation and depletion. The excitation wavelength was 532 nm, whereas STED was performed in the range between 630 and 640 nm.[16] An ATTO-532 IgG conjugate was prepared from the amino reactive NHS ester of this dye, by the same procedure as for the sulfonated rhodamines **3d**, **e** and **g**. The depletion wavelengths that were actually used to record the images are specified in Figure 4. All new dyes performed well in STED microscopy; the resulting subdiffraction images possessed similar spatial resolution as the reference dye (ATTO-532) in the setup employed. Figure 5 shows the data obtained for compound **3f–AB** and for the reference. (The images recorded with compounds **3d–AB** and **3g–AB** are given in Figures S2 and S3, Supporting Information).[16] Single tubuline fibres are reproduced with a diameter of $\approx$90 nm,[16] which is slightly larger than the size of the fibre plus the dimensions of a primary and secondary antibodies. As there is no difference in resolution in the images obtained with the reference and the novel dyes, the latter represent a viable alternative to the expensive commercial dyes. The possibility to obtain them in relatively large amounts (up to several grams) is an important advantage, particularly if further chemical modifications of the dyes are desired.

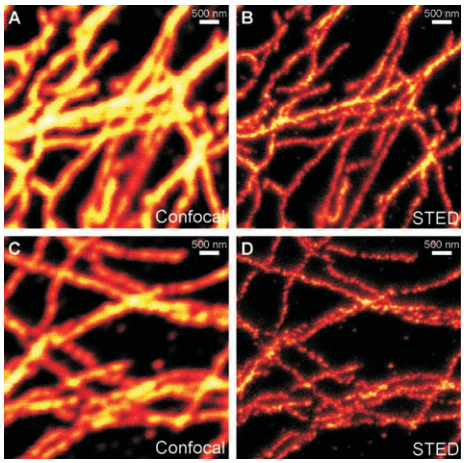


Figure 5. Subdiffraction imaging of the microtubules of a mammalian PtK2 cell immunostained with ATTO-532 (A: confocal; B: STED) and compound **3f–AB** (C: confocal; D: STED); scale bars: 500 nm.

Conclusion

Water-soluble analogues of rhodamine 6G with high photostability (**3b**,**c**,**f**) and very large fluorescence quantum yields (>0.9) were synthesized. They were used as starting materials for preparation of the fluorescent labels **3d**, **e** and **g**, which react with amino groups in purely aqueous solutions without any addition of an organic co-solvent (e. g. DMF or DMSO). Their conjugates with antibodies are highly fluorescent ($\Phi_{Fluo}=0.7$–0.8) and produce bright and stable images in fluorescence microscopy. These conjugates are also suitable for STED microscopy, where images with a spatial resolution below the diffraction limit were obtained. Amino reactive compounds **3d**, **e** and **g** have been prepared in 7–8 steps from commercially available 1,2,3,4-tetrahydroquinoline. Several "pitfalls" were identified in the course of attaching the secondary amide linker to the carboxy group of the rhodamine fragment and by choosing an appropriate amino reactive group. Mixed NHS-carbonate **3d** is the most easy to prepare and to isolate. It is a relatively chemically stable, yet a highly amino reactive compound. The practical procedures of the present study allow the preparation of synthetically useful amounts of photostable water-soluble rhodamines. Besides, they may further be used for linking any sterically hindered aromatic carboxy group with an amino reactive site.

Experimental Section

General remarks: NMR spectra were recorded at ambient temperature with Varian MERCURY 300 and Bruker AM 250 spectrometers at 300 (^{1}H) and 75.5 MHz (^{13}C and APT), as well as at 250 (^{1}H) and 62.9 MHz (^{13}C and DEPT), respectively. ^{1}H NMR spectra were also recorded with Varian INOVA 500 or 600 (600 MHz) instruments. All spectra are referenced to tetramethylsilane as an internal standard ($\delta=0$ ppm) using the signals of the residual protons of $CHCl_3$ (7.26 ppm) in $CDCl_3$, CHD_2OD (3.31 ppm) in CD_3OD, HOD (4.79 ppm) in D_2O or [D_5]DMSO (2.50 ppm) in [D_6]DMSO. Multiplicities of signals are described as follows: s = singlet, brs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Coupling constants (J) are given in Hz. Multiplicities in the ^{13}C NMR spectra were determined by APT (Attached Proton Test) measurements. Low resolution mass spectra (electro spray ionization, ESI) were obtained with LCQ spectrometer. High resolution mass spectra (ESI-HRMS) were obtained on APEX IV spectrometer. HPLC system (Knauer): Smartline pump 1000 (2×), UV detector 2500, column thermostat 4000, mixing chamber, injection valve with 20 and 100 μL loop for the analytical and preparative columns, respectively; 6-port-3-channel switching valve; analytical column: Eurospher-100 C18, 5 μm, 250×4 mm, 1 mL min^{-1}; preparative column: Eurosphere-100 C18, 5 μm, 250×8 mm, 4 mL min^{-1}; solvent A: water + 0.1% v/v trifluoroacetic acid (TFA); solvent B: MeCN + 0.1% v/v TFA; A/B: 80/20 → 50/50 in 25 min, then 50/50 for 5 min; 25 °C, detection at 530 nm. Analytical TLC was performed on MERCK ready-to-use plates with silica gel 60 (F_{254}) and MACHEREY-NAGEL plates for HPTLC with 0.20 mm silica gel 100 C_{18}. Column chromatography: MERCK silica gel, grade 60, 0.04–0.063 mm; fraction collector RETRIEVER II (ISCO). Reversed phase column chromatography: MACHEREY-NAGEL Polygoprep 60–50 C_{18}. Organic solutions were dried over Na_2SO_4. Absorption and fluorescence stationary measurements were carried out with a Varian Cary 4000 UV/Vis spectrophotometer, and with a Varian Cary Eclipse fluorescence spectrophotometer, respectively. ε Values are given in $M^{-1}m^3cm^{-1}$. Emission spectra were corrected for instrument response.

Rhodamine 3a: Compound **8**[9,16] (1.3 g, 3.2 mmol) was added carefully to 30% SO_3 in H_2SO_4 (17 g, obtained by mixing of 20% SO_3 and 65% SO_3 in H_2SO_4) at 0–5 °C. The mixture was stirred at 0 °C for 1 h and kept at 4 °C for 14 h. Then it was poured onto frozen 1,4-dioxane (100 g) and mixed carefully. Diethyl ether (200 mL) was added to the mixture, and the precipitate was filtered off. The precipiate was heated under reflux in *i*PrOH (50 mL) for several minutes. The suspension was cooled and diluted with diethyl ether (150 mL). The title compound (1.2 g, 64%) was filtered off and dried in vacuo. HPLC: $t_R=11.0$ min (area 97%). ^{1}H NMR (300 MHz, [D_6]DMSO): $\delta=1.78$ (brs, 4H; $(CH_2)CH_2(CH_2)$), 2.70 (brs, 4H; $ArCH_2$), 3.56 (brs, 4H; CH_2N), 6.73 (brs, 2H; H-1/8), 7.43 (d, $J=6.6$ Hz, 1H; H-3′), 7.8–7.9 (m, 2H; H-4′/5′), 8.24 (d, $J=6.6$ Hz, 1H; H-6′), 9.43 ppm (brs, 2H; NH); ^{13}C NMR (75.5 MHz, [D_6]DMSO): $\delta=18.7$ ($(CH_2)CH_2(CH_2)$), 27.1 ($ArCH_2$), 42.0 (CH_2N), 110.0 (C), 111.9 (C), 125.4 (C), 128.7 (CH), 130.2 (CH), 130.3 (CH), 130.5 (C), 131.0 (CH), 132.8 (CH), 133.1 (C), 150.9 (C), 152.6 (C), 166.0 ppm (CO); ESI-MS, negative mode: m/z (%): 569 (100) $[M-H]^-$, 591 (16) $[M-2H+Na]^-$; HR-MS (ESI, positive mode): m/z: calcd for $C_{26}H_{22}N_2O_9S_2$: 571.0840; found: 571.0828 $[M+H]^+$, 593.0647 $[M+Na]^+$.

Alcohol 3b: Rhodamine **3a** (98 mg, 0.17 mmol) and HATU (86 mg, 0.23 mmol) were placed into a dry Schlenk flask under N_2. Dry *N*,*N*-dimethylacetamide (DMAA) (3 mL) and 2-(methylamino)ethanol (67 mg, 0.89 mmol) were added through a septum. The mixture was stirred at RT for 1.5 h. Then the solvent was evaporated in vacuo, and the title compound was isolated from the residue by column chromatography (CH_2Cl_2/MeOH 2:1), yield 72 mg (66%). HPLC: $t_R=7.2$ min (area 98%). ^{1}H NMR (300 MHz, [D_6]DMSO, 2 rotamers): $\delta=1.78$ (m, 4H; $(CH_2)CH_2(CH_2)$), 2.57 (s, ≈2.3H; NMe, rotamer 1), 2.65–2.75 (m, 6H; $ArCH_2$ and $NCH_2(CH_2OH)$), 3.00 (s, ≈0.7H; NMe, rotamer 2), 3.45–3.60 (m, 6H; CH_2NAr and $(CH_2)CH_2O$), 6.73–6.77 (m, 2H; H-1/8), 7.36–7.44 (m, 1H; H-3′), 7.6–7.8 (m, 3H; H-4′/5′/6′), 10.2 ppm (brs, 2H; NH); ^{13}C NMR (75.5 MHz, [D_6]DMSO, 2 rotamers): $\delta=19.0$ ($(CH_2)CH_2(CH_2)$), 25.1/27.2 ($ArCH_2$), 31.8/38.0 (NMe), 41.6 (CH_2NAr), 48.8/52.4 ($NCH_2(CH_2OH)$), 58.0/58.1 (OCH_2), 112.2 (C), 112.3 (C), 112.9 (C), 124.0 (C), 127.3 (CH), 127.8 (CH), 128.6 (CH), 128.9 (CH), 129.3 (CH), 130.1 (CH), 130.3 (CH), 131.3 (C), 131.4 (C), 135.9 (C), 136.1 (C), 151.6 (C), 153.5 (C), 154.2 (C), 154.4 (C), 167.2/168.0 ppm (CO); HR-MS (ESI, positive mode): m/z: calcd for: $C_{29}H_{29}N_3O_9S_2$: 628.1418; found: 628.1418 $[M+H]^+$, 650.1235 $[M+Na]^+$, 672.1054 $[M-H+2Na]^+$.

***p*-Nitrophenyl carbonate 9**: Compound **3b** (23 mg, 0.037 mmol) was dissolved in dry DMAA (1 mL), bis(4-nitrophenyl)carbonate (48 mg, 0.16 mmol) and Et_3N (0.10 mL, 0.72 mmol) were added under N_2 with stirring. The reaction mixture was left at RT for 22 h. Then the solvent

and excess of Et_3N were removed in vacuo, and the title product was isolated by reversed-phase column chromatography on Polygoprep (MeCN/H_2O 1:2), yield 28 mg (97%). HPLC: t_R = 18.7 min (area 100%). ^{1}H NMR (300 MHz, [D$_6$]DMSO + [D$_4$]methanol, 2 rotamers): δ = 1.88 (m, 4H; (CH$_2$)*C*H$_2$(CH$_2$)), 2.75 (m, 4H; ArCH$_2$), 3.07 (s, ≈0.5H; NMe, rotamer 1), 3.18 (s, ≈2.5H; NMe, rotamer 2), 3.40–3.65 (m, 6H; *C*H$_2$NAr and NCH$_2$(CH$_2$OCO)), 3.73 (m, 1H; (CH$_2$)*CH*$_A$H$_B$OCO), 4.10 (m, 1H; (CH$_2$)*C*H$_A$*H*$_B$OCO), 6.78–6.96 (m, 4H; H-1/8 and H-2″), 7.36–7.50 (m, 1H; H-3′), 7.68–7.82 (m, 2H; H-4′/5′), 8.06–8.23 ppm (m, 3H; H-6′ and H-3″); UV/Vis (water): λ_{max} (ε) = 540 nm (84000), λ_{em} = 560 nm, Φ_{fl} = 0.15; ESI-MS, negative mode: *m*/*z* (%): 791 (100) [*M*−H]$^-$; HR-MS (ESI, positive mode): *m*/*z*: calcd for $C_{36}H_{32}N_4O_{13}S_2$: 793.1480; found: 793.1478 [*M*+H]$^+$, 815.1297 [*M*+Na]$^+$, 831.1043 [*M*+K]$^+$.

***N*-Hydroxysuccinimidyl carbonate (3d)**: Compound **3b** (20 mg, 0.030 mmol) was dissolved with dry DMAA (1 mL), di(*N*-succinimidyl)-carbonate (76 mg, 0.30 mmol) and Et_3N (0.10 mL, 0.72 mmol) were added under N_2 with stirring. The reaction mixture was left at RT for 1 h. The solvent and excess of Et_3N were removed in vacuo, water (5 mL) was added to the residue, and the title product was isolated by reversed-phase preparative HPLC: t_R = 11.6 min (area 100%). ^{1}H NMR (300 MHz, [D$_6$]DMSO, 2 rotamers): δ = 1.78 (m, 4H; (CH$_2$)*C*H$_2$(CH$_2$)), 2.70 (m, 4H; Ar*C*H$_2$), 2.80 (s, 4H; (OC)*C*H$_2$CH$_2$(CO)), 2.90 (s, 1H; NMe, rotamer 1), 3.00 (s, 2H; NMe, rotamer 2), 3.53 (m, 4H; *C*H$_2$NAr), 4.36 (m, 2H; (CH$_2$)*C*H$_2$OCO), 6.78 (brs, 2H; H-1/8), 7.36–7.48 (m, 1H; H-3′), 7.6–7.8 (m, 3H; H-4′/5′/6′), 10.16 ppm (brs, 2H; NH); HR-MS (ESI, positive mode): *m*/*z*: calcd for $C_{34}H_{33}N_4O_{13}S_2$: 769.1480; found: 769.1479 [*M*+H]$^+$.

Methyl 3-(methylamino)propionate (12):[18] A mixture of 3-(benzyloxycarbonylamino)-propionic acid **10** (FLUKA, 2.23 g, 10 mmol), Ag_2O (7.3 g, 31 mmol), MeI (8.5 g, 60 mmol) and DMF (40 mL) was stirred at room temperature in the dark for 16 h. The solvent and excess of MeI were removed in vacuo at 50°C, the residue was suspended in CH_2Cl_2, and filtered. The filtrate was washed with sat. aq. $NaHCO_3$, brine, dried and evaporated in vacuo to give crude **11** (2.2 g, 89%). It was dissolved in ethyl acetate (20 mL). The solution was added through a septum under N_2 to 0.87 g of 10% Pd/C (Merck), placed into dry Schlenk flask equipped with a reflux condenser. The flask was filled with H_2 and stirred under H_2 at atmospheric pressure and ambient temperature for 7 h. Then the reaction mixture was flashed with N_2, the catalyst was filtered off, and the filtrate was carefully evaporated at reduced pressure (50 mbar) and 30°C (bath). The crude product **12** (1.03 g, 100%, content of the product was about 70 mol%; EtOAc: 30 mol%) was used for next step without further purification. ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.68 (s, 1H; NH), 2.42 (s, 3H; NMe), 2.50 (m, 2H; (CH$_2$)*C*H$_2$(CO$_2$Me)), 2.83 (m, 2H; NCH$_2$(CH$_2$CO$_2$Me)), 3.67 ppm (s, 3H, OMe).

Methyl ester 13: Rhodamine **3a** (0.57 g, 1.0 mmol) and TSTU (0.60 g, 2.0 mmol) were placed into a dry Schlenk flask under N_2. Dry DMF (19 mL) and Et_3N (1 mL, 6.8 mmol) were added through a septum, and the mixture was placed into an ultrasonic bath for 5 minutes and kept at room temperature for 19 h. The solution (1.2 mL; about 0.06 mmol of activated ester of **3a**) was taken with syringe and added through a septum to the crude compound **12** (75 mg, ≈0.5 mmol), placed into a dry flask. The mixture was kept at RT for 24 h. Then the solvent was evaporated in vacuo, and the title product was isolated from the residue by column chromatography (R_f = 0.4, CH_2Cl_2/MeOH 4:1). Pure product (36 mg, 90%) was precipitated by adding ether to the solution in aq. MeOH). HPLC: t_R = 10.4 min (area 100%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 1.79 (m, 4H; (CH$_2$)*C*H$_2$(CH$_2$)), 2.24 (m, 2H; (CH$_2$)*C*H$_2$(CO$_2$CH$_3$)), 2.70 (m, 4H; Ar*C*H$_2$), 2.98 (s, 3H; NMe), 3.39 (m, 2H; NCH$_2$(CH$_2$CO$_2$Me)), 3.56 (m, 7H; *C*H$_2$NAr and OMe), 6.78 (brs, 2H; H-1/8), 7.38–7.42 (m, 1H; H-3′), 7.6–7.8 (m, 3H; H-4′/5′/6′), 10.18 ppm (brs, 2H; NH); ^{13}C NMR (75.5 MHz, [D$_6$]DMSO): δ = 19.0 ((CH$_2$)*C*H$_2$(CH$_2$)), 27.2 (Ar*C*H$_2$), 30.8 ((CH$_2$)*C*H$_2$(CO$_2$Me)), 37.2 (NMe), 41.6 (*C*H$_2$NAr), 42.7 (NCH$_2$(CH$_2$CO$_2$Me)), 51.4 (OMe), 112.2 (C), 112.8 (C), 124.1 (C), 127.3 (CH), 128.6 (CH), 129.4 (CH), 129.5 (CH), 130.4 (CH), 131.3 (C), 135.8 (C), 151.5 (C), 153.5 (C), 154.3 (C), 167.4 (CO), 171.4 ppm (CO); UV/Vis (water): λ_{max} (ε) = 539 nm (92400), λ_{em} = 558 nm, Φ_{fl} = 0.93; ESI-MS, negative mode: *m*/*z* (%): 668 (96) [*M*−H]$^-$, 690 (100) [*M*−2H+Na]$^-$; HR-MS (ESI, positive mode): *m*/*z*: calcd for $C_{31}H_{31}N_3O_{10}S_2$: 670.1524; found: 670.1522 [*M*+H]$^+$, 692.1341 [*M*+Na]$^+$.

Acid 3c: Ester **13** (160 mg, 0.228 mmol) was suspended in H_2O (2 mL) and cooled with an ice bath. Cold aq. NaOH (1 M, 0.6 mL) was added dropwise to the reaction mixture, and it was stirred at 0–4°C for 1 h. After 50 min, HPLC displayed full conversion of the starting compound. Aqueous HCl (1 M, 1.0 mL) was added, and the mixture was applied on top of the column with 100 mL of Polygoprep 60–50 C18 equilibrated with MeCN/H_2O 2:1. The title compound (134 mg, 86%) was eluted with MeCN/H_2O 2:1 → 4:1. Water was removed from the fractions by freeze-drying. HPLC: t_R = 9.4 min (area 94%, ca. 6% of the acid **3a** with higher t_R). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 1.76 (brs, 4H; (CH$_2$)*C*H$_2$-(CH$_2$)), 2.12 (m, 2H; (CH$_2$)*C*H$_2$(CO$_2$H)), 2.68 (m, 4H; Ar*C*H$_2$), 2.96 (s, 3H; NMe), 3.32 (m, 2H; NC*H*$_2$(CH$_2$CO$_2$H)), 3.53 (brs, 4H; *C*H$_2$NAr), 6.75 (brs, 2H; H-1/8), 7.38 (m, 1H; H-3′), 7.60–7.75 (m, 3H; H-4′/5′/6′), 10.18 ppm (brs, 2H; NH); ESI-MS, negative mode: *m*/*z* (%): 654 (100) [*M*−H]$^-$; HR-MS (ESI, positive mode): *m*/*z*: calcd for: $C_{30}H_{29}N_3O_{10}S_2$: 656.1367; found: 656.1365 [*M*+H]$^+$, 678.1185 [*M*+Na]$^+$, 700.1005 [*M*−H+2Na]$^+$.

***N*-Hydroxysuccinimidyl ester 3e**: Acid **3c** (0.13 g, 0.20 mmol) was dissolved in dry DMF (4 mL), TSTU (0.24 g, 0.80 mmol) was added followed by Et_3N (0.7 mL, 10 mmol). The reaction mixture was stirred at room temperature overnight; DMF was evaporated in vacuo, and the title compound was isolated by preparative HPLC. Corresponding fractions were pooled and freeze-dried. The residue was stored at −20°C. HPLC: t_R = 10.4 min (coincides with the methyl ester **13**; area 98%). HR-MS (ESI, positive mode): *m*/*z*: calcd for $C_{34}H_{32}N_4O_{12}S_2$: 753.1531; found: 753.1530 [*M*+H]$^+$, 775.1351 [*M*+Na]$^+$, 797.1170 [*M*−H+2Na]$^+$. Stock-solutions in DMF decompose slowly at room temperature; typically the content of NHS-ester **3e** decreases from 95–97 to 80% in ca. 12–18 h (and the content of the starting acid **3c** increases proportionally).

Rhodamines 14 and 15: A mixture of finely grounded compound **7** (2.7 g, 18 mmol) and trimellitic anhydride (4.7 g, 24 mmol) was heated at 170°C for 3 h. Then an additional portion of **7** (2.8 g, 19 mmol) and 85% aq. H_3PO_4 (7.5 mL) were added to the cooled reaction mixture and heating was continued at 170°C for 3 h. After cooling, the reaction mixture was stirred and refluxed with methanol (40 mL) for several minutes, cooled, and kept at RT to form a precipitate for 10 h. The crude isomer **15** (1.1 g, 13%) was filtered off and purified (as a salt with Et_3N) by column chromatography (CH_2Cl_2/MeOH 1:1, + 1% Et_3N v/v). HPLC: t_R = 23.7 min (area 98%). ^{1}H NMR (600 MHz, [D$_4$]MeOH): δ = 1.30 (t, *J* = 7.5 Hz, 9H; *C*H$_3$(CH$_2$NEt$_2$)), 1.88 (m, 4H; (CH$_2$)*C*H$_2$(CH$_2$)), 2.69 (brt, *J* = 6.0 Hz, 4H; Ar*C*H$_2$), 3.20 (q, *J* = 7.5 Hz, 6H; (CH$_3$)*C*H$_2$NEt$_2$)), 3.44 (t, *J* = 5.7 Hz, 4H; *C*H$_2$N), 6.65 (s, 2H; H-4/5), 6.71 (brs, 2H; H-1/8), 7.48 (d, *J* = 7.8 Hz, 1H; H-5′), 8.42 (dd, *J* = 1.8, 7.8 Hz, 1H; H-6′), 8.90 ppm (d, *J* = 1.8 Hz, 1H; H-2′); HR-MS (ESI, positive mode): *m*/*z*: calcd for $C_{27}H_{22}N_2O_5$: 455.1602; found: 455.1601 [*M*+H]$^+$.

The filtrate was diluted with water and kept at RT for 24 h. A mixture of **14** (major component) and **15** (minor component) precipitated (1.4 g, 17%). Isomer **14** was isolated as a salt with Et_3N by column chromatography (CH_2Cl_2/MeOH 1:1, + 1% NEt_3 v/v). HPLC: t_R = 21.7 min (area 99%). ^{1}H NMR (600 MHz, [D$_4$]MeOH): δ = 1.30 (t, *J* = 7.5 Hz, 3.5H; *C*H$_3$(CH$_2$NEt$_2$)), 1.88 (m, 4H; (CH$_2$)*C*H$_2$(CH$_2$)), 2.69 (m, 4H; Ar*C*H$_2$), 3.20 (q, *J* = 7.5 Hz, 2.4H; (CH$_3$)*C*H$_2$NEt$_2$)), 3.43 (m, 4H; *C*H$_2$N), 6.63 (s, 2H; H-4/5), 6.71 (brs, 2H; H-1/8), 7.87 (brs, 1H; H-2′), 8.34–8.38 (m, 2H; H-5′, H-6′); HR-MS (ESI, positive mode): *m*/*z*: calcd for $C_{27}H_{22}N_2O_5$: 455.1602; found: 455.1601 [*M*+H]$^+$.

Rhodamine 3f: Compound **15** (0.40 g, 0.72 mmol of the adduct with Et_3N) was added carefully to 30% SO_3 in H_2SO_4 (5.9 g, obtained by mixing of 20% SO_3 and 65% SO_3 in H_2SO_4) at 0–5°C. The mixture was stirred at 0°C for 15 min and kept at 4°C for 14 h. Then it was poured onto 40 g of frozen 1,4-dioxane and mixed carefully. Diethyl ether (80 mL) was added, and after 3 h the precipitate was filtered off. The precipitate was heated under reflux in *i*PrOH (20 mL) for several minutes; the suspension was cooled and diluted with diethyl ether (60 mL). The title compound was filtered off, purified by column chromatography (CH_2Cl_2/MeOH 2:1) and dried in vacuo (0.28 g, 63%). HPLC: t_R = 9.7 min (area 100%). ^{1}H NMR (300 MHz, D_2O): δ = 1.85 (brs, 4H;

(CH_2)CH_2(CH_2)), 2.68 (brs, 4H; ArCH_2), 3.53 (brs, 4H; CH_2N), 6.94 (s, 2H; H-1/8), 7.31 (d, J=7.8 Hz, 1H; H-5′), 8.05 (dd, J=1.5, 7.8 Hz, 1H; H-6′), 8.29 ppm (d, J=1.5 Hz, 1H; H-2′); ^{13}C NMR (75.5 MHz, D_2O): δ=19.0 ((CH_2)CH_2(CH_2)), 27.4 (ArCH_2), 42.1 (CH_2N), 110.6 (C), 112.6 (C), 125.5 (C), 129.0 (CH), 129.8 (CH), 130.0 (CH), 130.3 (CH), 134.1 (C), 138.0 (C), 139.6 (C), 151.9 (C), 154.3 (C), 158.5 (C), 174.1 (CO), 174.4 ppm (CO); ESI-MS, negative mode: m/z (%): 613 (10) $[M-H]^-$, 635 (100) $[M-2H+Na]^-$; HR-MS (ESI, positive mode): m/z: calcd for $C_{27}H_{22}N_2O_{11}S_2$: 615.0738; found: 615.0739 $[M+H]^+$.

***N*-Hydroxysuccinimidyl ester 3g**: Compound **3f** (35 mg, 0.055 mmol) and HATU (21 mg, 0.059 mmol) were dissolved with dry DMAA (7 mL), and Et_3N (0.10 mL, 0.72 mmol) was added under N_2 with stirring. After 10 min, a solution of *N*-hydroxysuccinimide (5.8 mg, 0.050 mmol) in dry DMAA (0.58 mL) was added. The mixture was left at RT for 1.5 h. The solvent and excess of Et_3N were removed in vacuo, water (5 mL) was added to the residue, and the title product was isolated by reversed phase preparative HPLC: t_R=10.2 min (area 97%). ^{1}H NMR (300 MHz, [D_6]DMSO): δ=1.74 (m, 4H; (CH_2)CH_2(CH_2)), 2.69 (m, 4H; ArCH_2), 2.93 (s, 4H; (OC)CH_2CH_2(CO)), 3.51 (m, 4H; CH_2NAr), 6.73 (brs, 2H; H-1/8), 7.62 (d, J=7.5 Hz, 1H; H-5′), 8.46 (dd, J=1.8, 7.5 Hz, 1H; H-6′), 8.80 (d, J=1.8 Hz, 1H; H-2′), 10.27 ppm (brs, 2H; NH); HR-MS (ESI, positive mode): m/z: calcd for $C_{31}H_{25}N_3O_{13}S_2$: 712.0902; found: 712.0900 $[M+H]^+$.

Conjugation of secondary antibodies: All antibodies were purchased from Jackson ImmunoResearch Inc. and conjugated according to the Molecular Probes labeling protocol.

Cell culture and immunocytochemistry: The mammalian PtK2 cell line was grown as described previously.[19] The human glioma cell line U373 MG was grown in RPMI Glutamax, high glucose (Gibco) supplemented with 10% FBS, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 1 mM sodium pyruvate. Cells were seeded on standard glass coverslips to a confluency of about 80% and fixed with ice cold methanol (−20 °C) for 4 min followed by an incubation in blocking buffer (PBS containing 1% BSA). Immunostaining of microtubules was performed with anti-α-tubulin rabbit IgG (Abcam), of mitochondria—with anti-Cyclophilin D mouse IgG (Mito Science) and of the nuclear lamina—with anti-lamin B rabbit IgG (Abcam) as primary antibodies followed by **3d**-, **3e**- or **3g**-conjugated sheep anti-mouse IgG and sheep anti-rabbit IgG (Jackson ImmunoResearch Inc.) as secondary antibody respectively. Both antibodies were diluted in blocking buffer and incubated for 1 h each followed by several washes in blocking buffer.

Acknowledgements

This work was supported by the Max-Planck-Gesellschaft, a Bundesministerium für Bildung und Forschung (BMBF) grant (Biophotonics program), and the European Commission through a Marie Curie Fellowship to M.B. The authors are grateful to Mr. R. Machinek and co-workers, Dr. H. Frauendorf, and Mrs. G. Udvarnoki for recording NMR and mass-spectra at the Institut für Organische und Biomolekulare Chemie (Georg-August-Universität Göttingen), and to Mr. J. Jethwa for reading of the manuscript.

[1] a) S. W. Hell, *Nat. Biotechnol.* **2003**, *21*, 1347–1355; b) S. W. Hell, *Science* **2007**, *316*, 1153–1158.
[2] a) F. P. Schäfer, *Top. Appl. Phys.* **1973**, *1*, 3–4; b) K.-H. Drexhage, *Top. Appl. Phys.* **1973**, *1*, 158; c) K.-H. Drexhage, *Top. Appl. Phys.* **1990**, *1*, 175–180; d) K. H. Drexhage, *Top. Appl. Phys.* **1973**, *1*, 155–200.
[3] a) G. T. Hermanson, *Bioconjugate Techniques*, Academic Press, San Diego, **1996**, pp. 316–331; b) R. Y. Tsien, A. Waggener in *Handbook of Biological Confocal Microscopy* (Ed.: J. B. Pawley), Plenum Press, New York, **1995**, pp. 267–279; c) M. Sauer, K.-T. Han, R. Müller, S. Nord, A. Schulz, S. Seeger, J. Wolfrum, J. Arden-Jacob, G. Deltau, N. J. Marx, C. Zander, K.-H. Drexhage, *J. Fluoresc.* **1995**, *5*, 247–261; d) T. Karstens, K. Kobs, *J. Phys. Chem.* **1980**, *84*, 1871–1872.
[4] a) R. P. Haugland, *A Guide to Fluorescent Probes and Labelling Technologies*, Invitrogen, Carlsbad, **2005**, pp. 11–37; b) N. Panchuk-Voloshina, R. P. Haugland, J. Bishop-Stewart, M. K. Bhalgat, P. J. Millard, F. Mao, W-Y. Leung, R. P. Haugland, *J. Histochem. Cytochem.* **1999**, *47*, 1179–1188.
[5] a) O. Valdes-Aguilera, D. C. Neckers, *Acc. Chem. Res.* **1989**, *22*, 171–177; b) C. Eggeling, J. Widengren, R. Rigler, C. A. M. Seidel, *Anal. Chem.* **1998**, *70*, 2651–2659.
[6] a) Alternatively, water-solubility may be improved by replacing ethyl substituents in *N*,*N*,*N*′,*N*′-tetraethyl-3,6-diaminoxanthene fragment by 2-hydroxyethyl groups: R. Bandichher, A. D. Petrescu, A. Vespa, Ann B. Kier, F. Schroeder, K. Bürgess, *Bioconjugate Chem.* **2006**, *17*, 1219–1225 (however, unprotected hydroxyls may not be used in the coupling reactions involving the 2-carboxy group of the rhodamine phenyl ring); b) for sulfonation of rhodamines, see: F. Mao, W.-Y. Leung, R. P. Haugland (Molecular Probes), US Patent 6130101 (10.10.2000)..
[7] a) V. Westphal, S. W. Hell, *Phys. Rev. Lett.* **2005**, *94*, 143903; b) S. W. Hell, S. Jakobs, L. Kastrup, *Appl. Phys. A* **2003**, *77*, 859–860; c) S. W. Hell, *Phys. Lett. A* **2004**, *326*, 140–145.
[8] G. Field, P. R. Hammond (Dept. of Energy, USA), US Patent 5283336 (01.02.1994).
[9] P. R. Hammond (Dept. of Energy, USA), US Patent 4622400 (11.11.1986).
[10] a) K. H. Knauer, R. Gleiter, *Angew. Chem.* **1977**, *89*, 116–117; *Angew. Chem. Int. Ed. Engl.* **1977**, *16*, 113; b) H. Willwohl, S. Wolfrum, R. Gleiter, *Laser Chem.* **1989**, *10*, 63–72; c) M. Adamczyk, J. Grote, *Synth. Commun.* **2001**, *31*, 2681–2690.
[11] M. Reddington, M. Little (Biosearch Technologies), US Patent Appl. 2005/0170363 (04.08.2005).
[12] For definitions, regularities and formalism of RET, see: B. Wieb Wan Der Meer, G. Gorker III, S.-Y. Simon Chen, *Resonance Energy Transfer (Theory and Data)*, VCH, Weinheim, **1991**.
[13] An excess of a strong acid and heating may cause cleavage of the sulfonic acid group. This happened once by heating and drying of the acid **3a**. For this reason, if *tert*-butyl ester is used as a protecting group for the aliphatic carboxyl, its removal might also turn out problematic.
[14] a) T. Nguyen, M. B. Francis, *Org. Lett.* **2003**, *5*, 3245–3248; b) M. Bossi, V. Belov, S. Polyakova, S. W. Hell, *Angew. Chem.* **2006**, *118*, 7623–7627; *Angew. Chem. Int. Ed.* **2006**, *45*, 7462–7465.
[15] For a short review on the photobleaching kinetics of rhodamine 6G, see: a) C. Eggeling, A. Volkmer, C. A. M. Seidel, *ChemPhysChem* **2005**, *6*, 791–804 (and the references therein); b) V. E. Korobov, A. K. Chibisov, *J. Photochem.* **1978**, *9*, 411–424; c) radicals, which were formed by irradiation of rhodamine 6G in solution, were detected by ESR-spectroscopy: R. Zondervan, F. Kulzer, S. B. Orlinskii, M. Orrit, *J. Phys. Chem. A* **2003**, *107*, 6770–6776.
[16] See Supporting Information for details.
[17] The structure of ATTO 532 has not been disclosed; a) R. Kellner, C. Baier, K. Willig, S. Hell, F. Barrantes, *Neuroscience* **2007**, *144*, 135–143; b) K. Willig, S. Rizzoli, V. Westphal, R. Jahn, S. Hell, *Nature* **2006**, *440*, 935–939; c) K. Willig, J. Keller, M. Bossi, S. Hell, *New J. Phys.* **2006**, *8*, 106.
[18] H. Rosenberg, H. Rapport, *J. Org. Chem.* **1985**, *50*, 3979–3982.
[19] K. Weber, T. Bibring, M. Osborn, *Exp. Cell Res.* **1975**, *95*, 111–120.

Received: July 10, 2007
Published online: December 4, 2007

