DOI: 10.1002/chem.201201168

Red-Emitting Rhodamines with Hydroxylated, Sulfonated, and Phosphorylated Dye Residues and Their Use in Fluorescence Nanoscopy

Kirill Kolmakov,[a] Christian A. Wurm,*[a, b] René Hennig,[c] Erdmann Rapp,[c] Stefan Jakobs,[a, b] Vladimir N. Belov,*[a] and Stefan W. Hell*[a]

Abstract: Fluorescent dyes emitting red light are frequently used in conventional and super-resolution microscopy of biological samples, although the variety of the useful dyes is limited. We describe the synthesis of rhodamine-based fluorescent dyes with absorption and emission maxima in the range of 621–637 and 644–660 nm, respectively and demonstrate their high performance in confocal and stimulated emission depletion (STED) microscopy. New dyes were prepared by means of reliable chemical transformations applied to a rhodamine scaffold with three variable positions. They feature polarity, water solubility, variable net charges, improved stabilities of *N*-hydroxysuccinimidyl (NHS) esters, as well as large fluorescence quantum yields in dye solutions and antibody conjugates. The photophysical and imaging properties of dyes containing three different polar groups, namely primary phosphate, sulfonic acid (in two different positions), and hydroxyl were compared. A dye with two primary phosphate groups was explored as a valuable alternative to dyes with "classical" sulfonic acid groups. Due to the increased net charge of the phosphorylated dye ($q=-4$ at pH 8), it demonstrated a far better electrophoretic mobility compared with analogues with two sulfonic acid groups ($q=-2$). As an example, one fluorescent dye was designed to be especially convenient for practical use. It is characterized by sufficiently high chemical stability of the NHS ester, its simple isolation, handling, and solubility in aqueous buffers, as well as in organic solvents. All these features, accompanied by a zero net charge in conjugates, were accomplished by the introduction of hydrophilic groups of two types: two hydroxyl groups and one sulfonic acid residue.

Keywords: chromophores · conjugation · electrophoresis · fluorescence · microscopy · rhodamines

Introduction

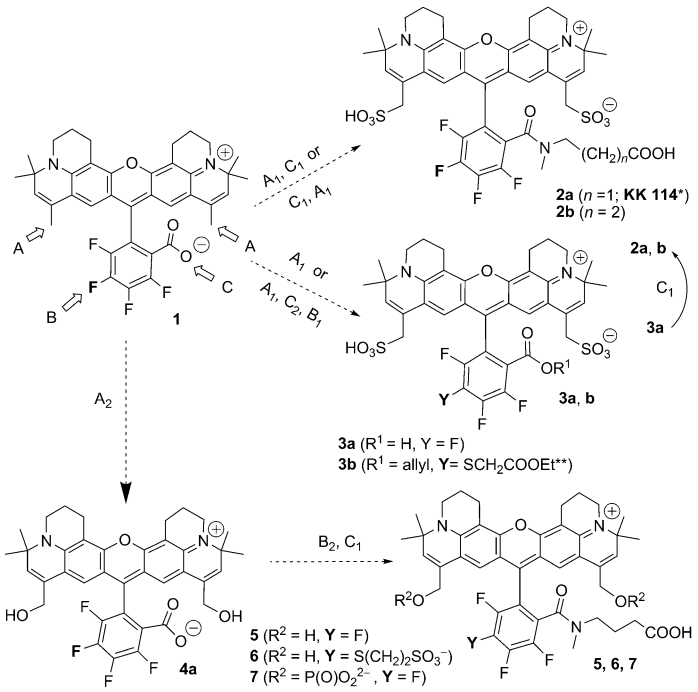
Red-emitting fluorescent dyes are widely used as makers in optical microscopy, biological imaging and sensing, especially in recent years.[1] Good imaging performance in optical microscopy and modern super-resolution techniques[2] demands high absorption coefficients and large fluorescence quantum yields of the markers, their resistance against photobleaching, and low background emission caused by unspecific labeling. However, for practical reasons (e.g., for providing convenient and reliable protocols for labeling), the properties of the reactive marker such as chemical stability, solubility in aqueous media, the possibility to afford high degrees of labeling, and simple handling are also important but not easy to achieve. Due to their good photochemical and photophysical properties, rhodamine dyes are widely used in modern far-field optical microscopy and nanoscopy.[3] Despite their long history and wide use, rhodamine dyes, their preparation and post-synthetic modifications have not been studied in depth,[3,4] particularly in the case of red-emitting dye **1** (Scheme 1) with promising spectral properties. Recently we reported the synthesis and properties of the red-emitting rhodamine dye KK114 (compound **2a** in Scheme 1),[5] which turned out to be a bright and photostable fluorescent marker in various optical microscopy and nanoscopy techniques.[6] In the conjugated state, the residue of KK114 is negatively charged (due to the presence of the two sulfonic acid groups). The negatively charged dye conjugates do not penetrate through the outer plasma membrane of living cells,[6c] and this feature limits the application scope of this dye. An additional negative charge can also strongly influence the properties of lipids labeled with compound **2a**.[5a] It was therefore necessary to further improve dye KK114 in such a way that its net electrical charge in conjugates could be varied, for example, from +1 to −3. To

[a] Dr. K. Kolmakov, Dr. C. A. Wurm, Prof. S. Jakobs, Dr. V. N. Belov, Prof. S. W. Hell
Department of NanoBiophotonics
Max Planck Institute for Biophysical Chemistry
Am Fassberg 11, 37077 Göttingen (Germany)
Fax: (+49) 551 2012505
E-mail: cwurm@gwdg.de
vbelov@gwdg.de
shell@gwdg.de

[b] Dr. C. A. Wurm, Prof. S. Jakobs
Georg-August University of Göttingen
Medical School, Department of Neurology
37073 Göttingen (Germany)

[c] Dipl.-Ing. R. Hennig, Dr. E. Rapp
Max Planck Institute for Dynamics of Complex Technical Systems
Sandtorstrasse 1, 39106 Magdeburg (Germany)
Fax: (+49) 391 6110535

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201201168.



Scheme 1. Red-emitting rhodamine dye **1** as a scaffold with three variable positions: A) methyl groups subjected to sulfonation (A_1) or oxidation with SeO_2 followed by reduction with $NaBH_4$ (A_2); B) aromatic F-atoms subjected to S_N reactions with $HSCH_2CO_2C_2H_5$ (B_1) or $HS(CH_2)_2SO_3Na$ (B_2); C) COOH group subjected to amidation with $CH_3NH(CH_2)_nCOOR$ (C_1), or esterification with alcohols, e.g., allyl alcohol (C_2). For a detailed description of the reagents and conditions, see Scheme 2 and Scheme 3; *) see ref. [5a]; **) see ref. [8].

pursue this goal, we used rhodamine dye **1** (Scheme 1) as a scaffold for derivatization at various positions. This combinatorial approach is illustrated by Scheme 1, in which the three variable positions in compound **1** are indicated as A, B, and C. Proper chemical transformations enabled us to obtain new red-emitting fluorescent dyes with high imaging performance, variable net charge, various electrophoretic mobilities, controlled polarity, desired solubility, and stable *N*-hydroxysuccinimidyl (NHS) esters.

Results and Discussion

Motivation and possible synthesis strategies: Dye KK114, a very bright and photostable near-IR fluorescent marker (compound **2a** in Scheme 1), has repeatedly demonstrated its excellent imaging performance.[5,6] The disadvantages of this compound are the lower photostability under confocal conditions (compared to Atto647N; see below), the negative charge in conjugates (therefore they are not cell-permeable) and the relatively low chemical stability of the NHS ester. Another widely used near-IR emitting dye is Atto647N.[7] Its main drawbacks are the lower fluorescence quantum yield in conjugates with proteins (relative to these of KK114), the lipophilic character, which is reflected in a lower imaging contrast due to unspecific binding, and the presence of two diastereomers. The challenging part of this study was to design new rhodamines without these shortcomings while trying to keep or even surpass the valuable properties of the best near-IR-emitting dyes. The most important parameters that have to be maintained are the positions of absorption and emission maxima (excitation with He–Ne laser at 633 nm or diode laser at 635–640 nm; sufficient optical cross section for stimulated emission at 750–775 nm), high brightness (product of the fluorescence quantum yield and the absorption coefficient), photostability under excitation and stimulated emission depletion (STED) conditions (with excitation light of 633 nm and powerful STED beam of 750–775 nm, respectively), hydrophilic character (which hinders the unspecific binding and provides high imaging contrast due to the absence of the fluorescent background), and stability of an amino reactive derivative (NHS ester). The chemical stability of the reactive markers in the solid state and in aqueous solutions is a significant parameter, considering prolonged storage and/or shipping of the samples and their stock solutions, and relatively long (normally 1 hour) reaction times in aqueous buffers at pH 7–9 at room temperature (typical conditions for the conjugation with proteins and other biomolecules).

As regards the active ester of dye KK114 (**2a** in Scheme 1), we found that its hydrolytic stability can be characterized as medium (see Table 1). The NHS ester can be isolated in the pure state by column chromatography over a normal phase (SiO_2) with eluents that contain acetonitrile and water (due to the high polarity of this compound decorated with two sulfonic acid residues). After direct lyophilization of the fractions (without removal of acetonitrile), the solid residue was analyzed by HPLC, and the area of the peak which corresponds to the NHS ester was determined to be about 85–90% (detection at 636 nm); the starting acid was present in amounts of approximately 10–15% (HPLC area). Polar solvents, especially water, solubilize a noticeable amount of silica gel, which is difficult to remove completely (by centrifugation and filtration). Silica gel, as an impurity in "dried" solids, always retains some water, which is harmful for hydrolytically unstable substances; as a result, the content of the active ester in the solid material after freeze-drying drops down to 60–80% and even further upon storage under argon at −20°C. Therefore, an important part of this study was to improve the stability of the NHS esters, maintaining their solubility in polar aprotic solvents (used for the preparation of the stock solutions), to make the markers easier to handle, taking aliquots, ship and store.

The parent compound—rhodamine **1**[9]—is insoluble in water (we could not even record its absorption spectrum in pure water and had to add 10% (*v*/*v*) of methanol), but its spectroscopic properties are very attractive (see Figure 1 and Table 2). Upon a closer look, the fluorescent dye **1** possess three variable positions (three functional groups; see Scheme 1), which can be easily transformed in a combinatorial chemistry fashion. However, until our recent study, no attempts to further redshift its absorption and emission

Figure 1. Aqueous solutions of the fluorescent dyes (Scheme 1) exposed to daylight. Left: compound **1**; right: compound **2a** (KK114). Note the absence of the red fluorescence in the case of compound **2a.** For spectral and related data, see Table 2.

bands, to attach a convenient linker with reactive groups (for conjugation with proteins and small molecules), and, finally, to improve its solubility of this compound in aqueous solutions have been undertaken.[5a] We extended the direct sulfonation at the allylic position of rhodamine dyes[10] to tertiary amides of **1** and obtained dye KK114 (**2a**; see Scheme 1 and Figure 1) with two sulfonic acid residues (and the net charge of −1 in conjugates).[5a] The structure of this dye has the unique combination of three structural features: the tertiary amide bond, a benzene ring with four fluorine atoms, and two sulfonic acid groups. However, we have not explored any alternatives to the sulfonic acid groups—charged or uncharged polar groups that improve fluorescence quantum yields and hinder the undesired aggregation of the dye molecules in aqueous solutions.

Apart from the "conventional" carboxylic acid function (position C, compound **1** in Scheme 1) and far less exploited "allylic site" (position A), there is another variable position in rhodamine **1**, a fluorine atom, indicated as position B. At the beginning of the work with amides of compound **1**, we observed the undesirable exchange of one fluorine atom to methoxy group under mild basic conditions (with K_2CO_3) in methanol.[5a,11] Later, we explored the substitution reaction of one fluorine atom with ethyl thioglycolate ($HSCH_2CO_2Et$) in the allyl ester of sulfonated rhodamine **3a**.[8] After the substitution with thioglycolate had been performed, it was important to learn that the spectral properties of the product **3b** were almost unaffected. In fact, the absorbance maximum in **3b** shifted only by 1–2 nm to the blue region (compared with the allyl ester of **3a**),[8] and the fluorescent quantum yield did not change much either. Therefore, we considered the site with one fluorine atom (position B in compound **1**, Scheme 1) as an important tool that could help to change and control the polarity and solubility of the resulting dyes (in the case of using bifunctional thiols with polar groups). It might be also important to remove the most reactive fluorine atom from the fluorescent dye, preventing any uncontrolled reactions with thiol groups in cysteine residues of proteins or amino groups in biologically relevant substrates.

As regards the "conventional" position C at the carboxylic group (Scheme 1), we planned some improvements as well. In our previous studies, we used only one linker, the *N*-methyl-β-alanine residue (taken as methyl or *tert*-butyl ester). It would also be interesting to try a longer linker, for example, *N*-methyl-4-aminobutyric acid, expecting better stability of the NHS ester and, as a result, higher degrees of labeling in aqueous media.

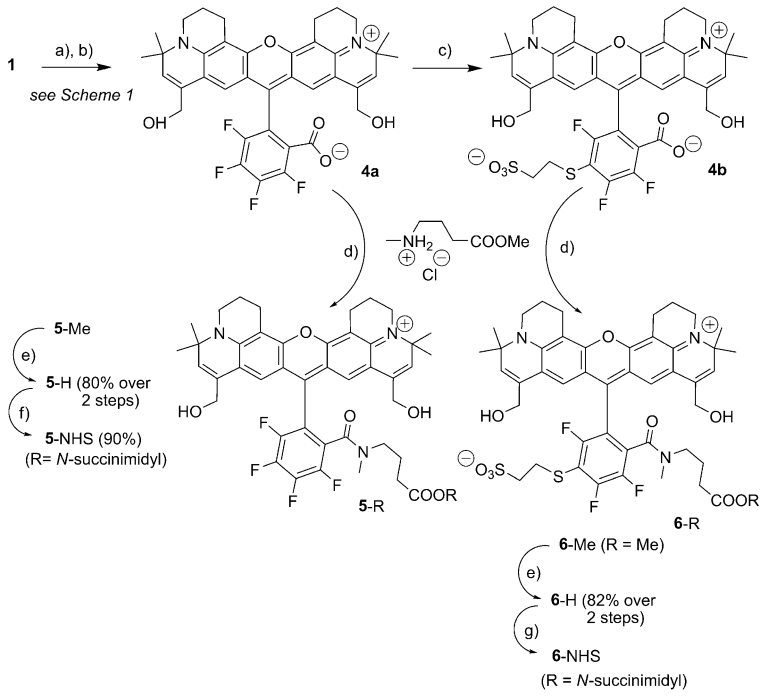
At both allylic sites (positions A in Scheme 1), a direct oxidation to the corresponding diol (**4a**, Scheme 1) could be a good alternative to sulfonation.[12a] Generally, alcohols offer more opportunities for further modifications than any other derivatives.[12b] Hydroxyl (or hydroxyalkyl) groups alone substantially improve hydrophilic properties, increase solubility in water and slightly increase the quantum yield of fluorescence.[12a, 5b] Finally, as regards the general strategy of the synthesis, direct modification of a chromophore-containing intermediate (e.g., rhodamine **1**) is preferable. Condensation is the crucial final step in the synthesis of rhodamines; it proceeds under harsh conditions and always in the presence of acids. The influence of new substituents and functional groups on this reaction might be absolutely prohibiting and/or require a long time to find proper condensation conditions and suitable protective groups for the hydroxyl.

Hydroxyl-substituted red-emitting rhodamine dyes: Selenium dioxide (SeO_2) is one of the softest oxidants, known in preparative organic chemistry.[13] In practice, oxidation of "allylic" CH_3 groups leads to the corresponding aldehydes or mixtures with the alcohols. Subsequent reduction of the aldehydes with sodium borohydride ($NaBH_4$) makes the separation of the two products unnecessary. For the oxidation, 1,4-dioxane (often containing 5–10% water) is the solvent of choice because it dissolves most organic substrates and SeO_2 as well. In our initial experiments with rhodamine **1** in anhydrous dioxane we observed a fast reaction (with heating) that did produce the desired diol **4a** (Scheme 1) and the corresponding aldhehyde(s) with CH=O group(s) at the allylic site(s), as established by the mass spectrometric analysis. However, side products were also formed and the isolation of the desired oxidized compounds proved difficult. As a result, the isolated yields of **4a** were poor. In aqueous dioxane as the solvent (with up to 10% water), fewer unidentified side products were detected, although the reaction required a much longer time, even at reflux (at about 100 °C). This reaction required a large excess of the oxidant. Probably, for kinetic reasons, it is crucial to maintain high concentrations of selenious acid (H_2SeO_3, the hydrated form of SeO_2) in the reaction solution.

Although rhodamine **1** is completely oxidized by SeO_2 in dry dioxane within 30 min at 60–80 °C, the full conversion of the starting material and optimal yield of the oxidation reaction in the presence of water are achieved after heating at reflux temperature overnight. The reaction mixture that contained diol **4a** as a major product and variable amounts of the mono- and di-aldehydes was subjected to an aqueous workup, the dyes extracted, dissolved in ethanol and treated with $NaBH_4$ (to reduce the aldehydes to diol **4a**). Luckily,

this operation did not further reduce the dye **4a** to its colorless leuco-form. Finally, diol **4a** was isolated in a 35–50% yield (several experiments), which can be considered as quite satisfactory regarding that oxidation with SeO_2 has not been reported for rhodamine dyes so far.

We briefly checked if the same reaction sequence was applicable to the preparation of compounds **5**-R straight from the tertiary amides of the parent dye **1**.[15] Unfortunately, the method described above (oxidation followed by reduction; see Scheme 2) did not work for compounds **5**-R. We chose



Scheme 2. Rhodamine dyes, modified with hydroxyl groups: a) SeO_2, aq. 1,4-dioxane, reflux, overnight; b) $NaBH_4$, EtOH, 0°C; c) $HS(CH_2)_2SO_3H$, Et_3N, DMF, RT; d) 2-(1*H*-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HATU), Et_3N, CH_3CN, −5 to 0°C; e) 0.05 M KOH in aq. THF, RT; f) HATU, *N*-hydroxysuccinimide, Et_3N, CH_3CN; g) *O*-(*N*-Succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU), Et_3N, CH_3CN.

ester **5**-*t*Bu[5a] as a model compound. The oxidation step was relatively clean, but the reduction with $NaBH_4$ caused nearly complete discoloration of the reaction mixture and led to a complex mixture of (leuco-) compounds. All attempts to reoxidize the reaction mixture to one major product with soft oxidants, like periodates or hydrogen peroxide, failed. One can explain the selectivity of the oxidation and, especially, of the reduction with the borohydride, assuming that the starting compound **1**, intermediates and diol **4a** are stabilized in their zwitterionic forms, which are less prone to further reduction than the corresponding cationic forms (e.g., amides **5**-R, for which the zwitterionic form is obviously impossible).

Next, the properties of the hydroxylated rhodamine dye, diol **4a**, were studied. In contrast to the parent compound **1**, which already precipitated from a 10 μmoL solution in 10% aq. methanol upon further dilution with water, the solubility of diol **4a** in water was clearly more than 100 μmol L^{-1}. Even more importantly, as seen in Table 2, an increase in the quantum yield of fluorescence was observed in aqueous solutions: from 36% (compound **1**) up to 53–55% (compounds **2a**,**b**, **6**-H, and **7**-H). However, the absorption maximum (621 nm) should be shifted further to the red spectral region for a better match to the emission lines of commercial red lasers (633 nm for a He–Ne laser and 635–640 nm for a diode laser). Amidation of the free carboxyl group is known to provide the required bathochromic shift. Initially, we used *N*-methyl-β-alanine esters as amino components.[5] As mentioned in the introduction, the chemical stability of the dye NHS esters is very important. Expecting to improve it, we used a longer linker, *N*-methyl-4-aminobutyric acid. The 4-aminobutyric acid residue is incorporated into many commercially available fluorescent dyes which form relatively stable NHS esters.[17] Earlier, we had established that NHS esters of the masked near-IR fluorescent dyes with elongated linking bridges demonstrate very good stability as well.[8]

For the preparation of methyl *N*-methyl-4-aminobutyrate (Scheme 2), the commercial *N*-Z-4-aminobutyric acid was subjected to the double (*N*,*O*-) methylation with methyl iodide in the presence of silver oxide,[16] and then benzyloxycarbonyl (Z) protection was cleaved by hydrogenolysis over palladium catalyst. A solution of HCl in 2-propanol was added to the reaction mixture to block the cyclization of the product to *N*-methylpyrrolidone. Methyl *N*-methyl-4-aminobutyrate was isolated as a hydrochloride, which proved to be fairly stable.[14a,b]

For the reaction of this amino ester with the hydroxylated dye **4a** we used HATU, an active coupling reagent (Scheme 2), and triethyl amine. In the presence of the amino ester (a strong nucleophile), the free hydroxyl groups did not react and thus require no protection. At low temperatures (−5 to 0°C), despite noticeable cyclization of the amino ester to *N*-methylpyrrolidone in a basic medium, the coupling reaction was complete. The methyl ester **5**-Me was saponified, and the resulting dye, free acid **5**-H, was isolated in a pure state and explored. The properties of this and other fluorescent dyes are given in Table 2, and the imaging performance is discussed below. A dye with two hydroxyl

groups (**5**-H) demonstrates an intermediate value of the fluorescence quantum yield in aqueous solutions, and its solubility in water is limited. The restricted solubility leads to large deviations from the Beer–Lambert Law when the absorption spectra are measured in aqueous solutions.[14] These non-linearities may be explained by the fact that at high concentrations the dye **5**-H sticks to the glass (quartz) walls of the cuvette. The NHS ester of this dye (**5**-NHS) is easy to prepare, very stable, yet rapidly reacts with amines and thus provides sufficiently high degrees of labeling with antibodies. The resulting images have high contrast with no agglomerates or fluorescent background (see Figure 3).

In a free state, dye **5**-H exists as a zwitterion and in conjugates the dye residue is positively charged. The zwitterionic character of this dye explains why this compound is only slightly soluble in basic aqueous solutions (e.g., aqueous $NaHCO_3$, Et_3N, or PBS buffer). The limited solubility in water might be a problem in some applications, for example, when high concentrations of the marker in aqueous solutions are required. To improve the solubility in aqueous buffers, we decided to modify dye **5**-H with an additional hydrophilic anionic group. A fluorescent marker that bears only one anion-providing group (except the carboxyl group) would be of special interest. In conjugates or in the case of the NHS ester, in which the carboxyl function is occupied, the new dye will have a zero net charge. The resulting dye will be the first one in this series to have hydrophilic groups of two different types.

One fluorine atom in compound **1** and its derivatives (at C-6′) can be selectively substituted with nucleophiles and it therefore offers a very useful second variable position (B; Scheme 1). From all available nucleophiles with additional anionic groups, 2-mercaptoethyl sulfonic acid was chosen as a reagent (see Scheme 2). First attempts to react it directly (under basic conditions) with amide **5**-H led to a mixture of products. Luckily, in diol **4a** the free carboxyl group reacted cleanly, and only one fluorine atom was selectively substituted to form **4b** (in the *para*-position to the carboxyl group, as shown in Scheme 2). This result can be explained if we assume that the aromatic nucleophilic substitution proceeds in the "closed" (lactone) form of the fluorescent dye, in which the carboxyl group forms in fact an ester with an oxygen atom attached to the quaternary carbon (C-9 = C-1′). The ester group is known to be a stronger electron-withdrawing substituent than an amide group or carboxylate anion, and it directs the aromatic nucleophilic substitution preferably into the *para*-position (to C-6′). *ortho*-Substitution (at C-4′) does not take place at all because this reaction center is sterically hindered and has only one vicinal fluorine atom with a strong $-I$ effect, whereas the nucleophilic substitution at C-6′ is additionally activated by two neighboring F atoms. Due to the influence of the negatively charged sulfonic acid group (field effect), the thiol group proved to be not as reactive, as was in the case of alkyl mercaptoacetates.[8] The lower reactivity of 2-mercaptoethyl sulfonic acid is an advantage because there is no need to control the reaction conditions very strictly and the reaction was found to be selective and complete at room temperature in the presence of about four equivalents of the nucleophile. The reaction product **4b** can be extracted with dichloromethane in the course of aqueous workup, which separates the inorganic salts, the unreacted thiol, and the small amount of the di-substituted derivative (with two $(CH_2)_2SO_3^-$ groups).

The linker (methyl *N*-methyl-4-aminobutyrate) was attached to dye **4b** according to the standard procedure (as described above for dye **5**-H) using HATU as a coupling reagent and Et_3N as a base (Scheme 2). Saponification of **6**-Me with diluted aq. NaOH furnished the free acid **6**-H. Isolated as a trifluoroacetate (in the course of the RP chromatography with CF_3COOH in the eluent),[14a] the free acid is only slightly soluble in water. However, the solubility is very good when two equivalents of a base (Et_3N, $NaHCO_3$) are added. Also importantly, the dye readily dissolves in PBS buffer solutions at pH 7.4. Therefore, compound **6**-H can be characterized as "amphiphilic" because of its good solubility in all organic solvents, except alkanes.

Luckily, the preparation and isolation of the corresponding NHS ester (**6**-NHS) did not present much difficulty and its stability proved to be surprisingly high. Particularly, the neat compound does not develop noticeable changes (less than 2% loss in the content of **6**-NHS as established by HPLC) after storage for one day at room temperature in air. Also remarkably, this active ester stays almost unchanged when left for one day in an aqueous PBS buffer solution at pH 7.4 (with dye concentration of 100 μmoL). On the other hand, the marker rapidly reacts with diluted ammonia when added to this solution. In aqueous 0.1 M solutions of $NaHCO_3$, this NHS ester is fairly stable for several hours at room temperature, yet hydrolyzed completely when left overnight. We can assume that at pH values in the range of 7–8, compound **6**-NHS still exists predominantly in its zwitterionic form. The high stability of this NHS ester can be due to zero net charge of its molecule and the absence of the negatively charged groups in the close proximity to the active ester residue, which never occurred before in this series of dyes. Indeed, the NHS esters with sulfonic (**2a,b**) or phosphoric acid groups (**7**) are negatively charged, whereas those without anion-providing groups have a positive net charge (which requires a counter ion, like in compound **1a**,[5a] Figure 2).

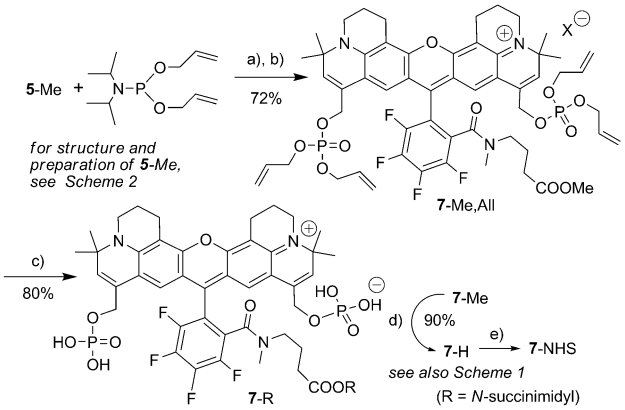
The phosphorylated red emitting rhodamine dye: Our first study on rhodamine **1** showed that the introduction of the sulfonic acid groups drastically improves the solubility in aqueous solutions, fluorescence quantum yield, photostability, imaging contrast, ground-state depletion parameters and other valuable dye properties.[5a] Importantly, the absorption and emission maxima were only slightly shifted. Having obtained diol **4a**, we considered other modifications at OH sites so that the dye would get polar and ionizable functional groups with acidic protons. Free hydroxyl groups offer a variety of opportunities for derivatization (especially if the carboxyl residue can be amidated with the functionally sub-

stituted amines, as described above, in the presence of the unprotected hydroxyls).

It is clear that the dye modified in this way must be an ester of a strong mineral acid. Esters of mineral acids obviously raise the hydrolytic stability concerns because labeling with fluorescent markers is normally done in aqueous $NaHCO_3$ or PBS buffer solutions. Moreover, the final steps of the synthesis involve saponification in alkaline solutions, followed by the preparation of NHS esters (with *N*-hydroxy succinimide) in basic media. Therefore, we had to exclude nitric and sulfonic acid ester groups as candidates. Indeed, esters of these acids are hydrolytically unstable and, in the case of sulfonic acid, are even used as alkylating agents. Phosphoric acid was thus chosen as an alternative functional group. Indeed, this acid has a medium strength, an increased number of acidic protons (relative to sulfuric acid), and most importantly, its primary and secondary alkyl esters are hydrolytically stable at pH 2–12.[18]

Finally, decoration with phosphoric acid residues might be "biologically friendly" (e.g., make the dye less poisonous to living cells). To the best of our knowledge, no attempts have been undertaken so far to attach phosphoric acid residues directly to fluorescent dyes.[19] In this respect, phosphoric acid has an advantage over phosphonic acid,[1h,19b] because it is a much stronger acid. For example, pK_a^1 and pK_a^2 values for primary alkyl phosphates are 1.5–1.9 and 6.3–6.8, respectively,[20a] whereas for primary alkyl phosphonates, the first and second ionization constants were found to be 2.4–2.8 and 7.8–8.9, respectively.[20b] Therefore, at pH 8.0–8.3, which is recommended for electrophoresis of DNA and RNA probes, fluorescent dyes with primary phosphate groups will exist in the fully ionized form and move as single bands (peaks). Hydrolytic stability of primary phosphates is also a benefit. The disadvantage of the primary phosphonates as labels for biomolecules is that at pH 7–9 they are present as mixtures of mono- and di-anions, which may form separate bands in the course of capillary (gel)electrophoresis or HPLC analysis.

In biochemistry, phosphorylation of hydroxyl-substituted substrates (*O*-phosphorylation) is of special importance.[18] One of the best ways of phosphorylation under mild conditions involves the reaction of an alcohol with *O*,*O*′-diallyl-*N*,*N*-diisopropylphosphoramidite, assisted by 1*H*-tetrazole, and followed by a one-pot oxidation of the intermediate to the phosphate.[21] In the final step, the allyl ester is cleaved with a palladium catalyst.[21a] This sequence gives way to a primary phosphoric acid ester with $ROP(O)(OH)_2$ group(s). We applied this phosphorylation protocol to rhodamine **5**-Me and obtained the corresponding bis-diallyl phosphate (**7**-Me,All) with high yields as shown in Scheme 3. The excess of the phosphorylating agent (diallyl *N*,*N*-diisopropylphosphoramidite) and the oxidant (*tert*-butyl hydroperoxide) did not cause any complications.[14] The cleavage of all four allyl groups with $[Pd(PPh_3)_4]$ proceeded quite smoothly. The methyl ester group in phosphorylated rhodamine **7**-Me was saponified in dilute aqueous alkaline solution to the free acid **7**-H under the conditions that were used for other



Scheme 3. Synthesis of rhodamine dyes with phosphorylated CH_2OH sites: a) 1*H*-tetrazole, THF, RT; b) *t*BuOOH, RT; c) $[Pd(PPh_3)_4]$, Et_3N, HCOOH, THF, 40 °C; d) 0.05 M KOH aq. THF, RT; e) TSTU, Et_3N, DMF, RT.

methyl esters in this study. Neither primary phosphoric acid ester groups nor fluorine atoms in the aromatic ring reacted with alkali under these conditions. The final product of the synthesis—dye **7**-H (Scheme 3) with two primary phosphate groups and a free carboxylic acid group—demonstrates high extinction and a large fluorescence quantum yield, which is as high as those of compounds **2a**,**b** and **6**-H. Importantly, dye **7**-H is perfectly soluble in aqueous PBS or $NaHCO_3$ solutions and moderately soluble in pure water. However, the solubility of this dye in water containing an excess of trifluoroacetic acid, which completely suppresses the ionization, is very low. The absorption band is slightly shifted to the red spectral region, relative to compound **6**-H. As regards the NHS ester of the phosphorylated dye **7**-H, under conventional conditions of HPLC analysis (0.1% TFA, pH $\approx$2), it always gave two to three unresolved peaks of variable intensity.[14] This is probably due to the equilibrium between the protonated and deprotonated forms in the substrate under acidic conditions at a pH that corresponds to the pK_a^1 value of the phosphate group. We also noticed that in the course of the chromatographic isolation of the NHS ester (both over regular SiO_2 and RP-SiO_2), trifluoroacetic acid strongly increases the adsorption of this compound so that mobile phases with triethylamine were used for elution.[14] Despite the difficulties with its analysis, the NHS ester of the phosphorylated dye (**7**-NHS) clearly consisted of the required compound predominantly. In the ESI mass spectrum, the peak with the required mass dominates strongly; the material completely reacts with diluted aqueous ammonia (HPLC). An important feature is that the solution of **7**-NHS in aqueous $NaHCO_3$, which is relatively stable alone, reacts with NH_3 very quickly. Most importantly, the amine-reactive compound **7**-NHS provides sufficiently high labeling degrees, when being coupled to antibodies. The phosphorylated dye **7**-H demonstrated good imaging performance, especially in STED microscopy (see Figure 3).

Sulfonated dye with a longer linker and relative stability of NHS esters: In an earlier study, comparing the stability of the NHS esters of the dye with and without sulfonic acid groups (compounds **1a** and **2a** in Figure 2), much better stability of the latter was noticed.[5a] We believe that, together with other benefits, the SO_3H groups in the dye stabilize the NHS ester. This was also in line with the results of our previous study on certain carbopyronine dyes with an elongated linker that contained one sulfonic acid residue.[5b] To evaluate the influence of the linker length on the stability of NHS esters, we obtained the analogue of dye KK114 (**2a**, Scheme 1) with the linker elongated by one methylene group (**2b**, Scheme 1) and explored the stability of its NHS ester. The data is presented in Tables 1 and 2. In fact the NHS ester of the dye without the sulfonic acid groups (**1a**) proved impossible to isolate.[5a] Dye **2b** was synthesized starting from the sulfonated rhodamine **3a**,[8] as shown in Scheme 1. Exploring dye **2b**, which is spectrally identical to dye **2a** (KK114), we did observe better hydrolytic stability, especially in the course of isolation. The latter involves evaporation and freeze-drying of aqueous solutions, which always caused a noticeable hydrolysis in case of **2a**-NHS. The hydrolytic stability of the two compounds (**2a**-NHS and **2b**-NHS) in dilute aqueous solutions is similar, yet the longer-chained homologue (**2b**-NHS) is more stable. The stability of the NHS esters for three dyes (**2a**, **2b**, and **6**) in water and PBS buffer are compared in Table 1. For the dyes with two sulfonic acid groups, PBS buffer strongly accelerates the hydrolysis, compared with pure water. Remarkably, compound **6**-NHS (Scheme 2) is very stable in PBS buffer even at room temperature. It should be noted that all three dyes have sulfonic acid groups, yet their positions and number are different. We noticed that one sulfonic acid group is enough to provide good solubility in water; it also gives zero net charge to the molecule of an (active) ester or to the zwitterionic dye residue in bioconjugates. Probably, the better hydrolytic stabilities of the NHS esters correlate with a zero charge of the molecule (at least in this series of zwitterionic dyes). This is also true for the active ester **6**-NHS and the NHS ester of the carbopyronine dye with one sulfonic acid group described in our previous study (compound **22**-NHS in ref. [5b]). Both of them demonstrated very good hydrolytic stability, whereas the NHS ester of the analogue without sulfonic acid group (compound **1c** in ref. [5b]) is rather unstable in water. In this regard, it is worth mentioning that it was not even possible to isolate the NHS ester of the "lipohilic" rhodamine without sulfonic acid groups (**1a**, Figure 2) in satisfactory yields.[5a] In the present study, we explored the "hydroxylated" analogue **5**-H of compound **1a** (Scheme 2, Figure 2) with the same net charge of +1, the NHS ester of which is fairly stable. One concludes that the hydroxyl groups can stabilize active esters in aqueous solutions. From the three dyes with sulfonic acid groups discussed above (**2a**, **2b**, and **6**-H), dye **6**-H is clearly the best as regards the stability of the NHS ester. To sum up, compound **6**-H combines the following structural features: three polar hydrophilic groups of two types, including one SO_3H moiety that provides a zero net charge to the zwitterionic molecule of the marker and the dye residue in conjugates, and the elongated linker bridge that generally increases the stability of the active esters. As a result, dye **6**-H and its NHS ester, as zwitterions, are soluble in most organic solvents. High stability, purity, and reproducibly high content of **6**-NHS (>95% HPLC area) are advantageous for taking aliquots, followed by evaporation of the solvent in vacuo. An opportunity to take precise amounts of a marker, in turn, is very important when controlled degrees of labeling are required. Also importantly, inorganic salts and silica gel are easy to remove from solutions of **6**-NHS in non-polar organic solvents.

Table 1. Stability of the NHS esters with sulfonic acid groups.[a]

Compound	Number of SO_3H groups	Number of CH_2 groups in the linker	Medium and exposure conditions: water RT 1 d	aqueous PBS buffer, pH 7.4 RT 1 d	aqueous PBS buffer, pH 7.4 +5 °C 1 d
			Content of NHS ester		
2a-NHS	2	2	20	0	30
2b-NHS	2	3	60	0	70
6-NHS	1	3	100	98	100

[a] For structures, see Scheme 1 and Figure 2. Content of NHS ester was measured as HPLC area (%) in the mixture with the corresponding acid after exposure.

Spectral properties and imaging performance of modified near-IR emitting dyes: Table 2 presents the most important photophysical properties of ten dyes. Amidation of the carboxyl group (position C) produces a noticeable bathochromic shift of 10–20 nm (cf. Figure 1). In the series of the five dyes with linkers (**1a**, **2a**, **5**, **6**, and **7** in Figure 2), all absorption maxima lie in the narrow region between 632 and 638 nm. Therefore, all these dyes may be efficiently brought into the first excited state (S_1) with a He–Ne laser (633 nm), or with the red line of a diode laser (635–640 nm). As expected, the ionizable groups (sulfonic acid and primary phosphate residues) increase the quantum yield of fluorescence and the solubility in water, relative to the non-substituted dyes **1**, **1a**, and **4a**. The more intense fluorescence of the sulfonated and phosphorylated dyes is due to the hydrophilic ionizable groups preventing the aggregation of the dye molecules and/or the formation of non-emitting dimers in aqueous solutions.[22] The sulfonation increases the quantum yield of fluorescence more than the hydroxylation (compare the values for compounds **3a** and **4a**, as well as **2a** and **5**-H in the last column of Table 2). In water, compounds **1**, **1a**, and the "hydroxylated" dyes (except **4b**) demonstrate deviations from the Beer–Lambert law. For example, in Table 2 one can see that the "actual" extinction coefficient is lower than anticipated for dyes **4a**, **5**, and **6**. Such deviations are typical for dyes with limited solubility and are due to adsorption (or even precipitation) on the quartz surface of a cuvette. Therefore, these dyes are very likely to form aggregates in water. Nevertheless, for these hydroxyl-

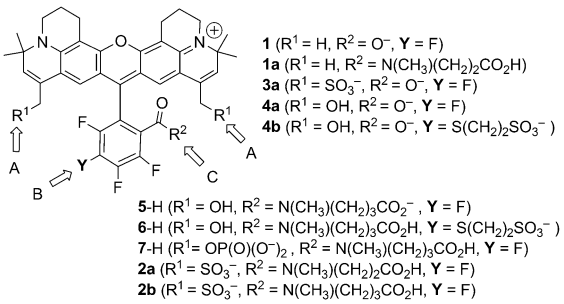


Figure 2. Derivatives of dye **1** with variable positions A, B, and C. For spectral properties, see Table 2.

ated dyes with linkers (**5**-H and **6**-H), the quantum yields of fluorescence in solutions are as good as that of the sulfonated and phosphorylated analogues (**2a**, **2b**, and **7**).[23] One can see that substitution at the allylic sites (position A in compound **1**, Scheme 1 and Figure 2) practically does not shift the absorption and emission maxima (±6 nm). The sulfonic acid group which substitutes one of four fluorine atoms in the aromatic ring (position B in compound **1**, Figure 2) only slightly increases the fluorescence quantum yield (compare dyes **4a** and **4b**, Table 2). This is also true for the carbopyronine dye that had a sulfonic acid group attached to the linker.[5b] Thus, two ionizable groups in the allylic positions of the rhodamine scaffold have a crucial influence on the efficiency of emission.

Remarkably, the replacement of the fluorine with thiol moiety (2-mercaptoethyl sulfonic acid) practically did not shift the absorption and emission bands in the dyes (compare dye **4a** with **4b**, and **5**-H with **6**-H in Table 2).

In conjugates with antibodies, the absorption and emission bands of the fluorescent markers are further shifted to the red region by approximately 5 nm, relative to the starting dye in the form of an acid. This means that the small hypsochromic shift in dyes **5**-H, **6**-H, and **7**-H (2–5 nm; see Table 2) compared with dye KK114 (**2a**), is insignificant regarding their practical use as fluorescent markers. To evaluate the imaging performance of the dyes in conventional and super-resolution microscopy, six dyes were coupled to secondary antibodies and used for immunofluorescence labeling of tubulin. The following dyes were used: four new dyes with linkers (**2b**, **5**, **6**, and **7**), and two reference dyes, KK114 (**2a**)[5] and Atto647N.[7] Several factors influence the performance of a fluorophor in imaging applications: brightness of the fluorescent label (proportional to the absorption coefficient and the fluorescence quantum yield), sufficient degree of labeling, photostability of the dye, solubility of the fluorophor (which does not unspecifically bind with proteins and can be completely removed after coupling), and so on.

It is typical that in conjugates with antibodies the quantum yield of fluorescence decreases.[24] The fluorescence quantum yields ($\Phi_{fl.}$) in conjugates with antibodies often depend on the degree of labeling (DOL):[25] higher quantum yields are observed at lower DOL values (see values with footnote [c] in Table 3). For the new dyes, in antibody conjugates, the fluorescence quantum yields were found to be very high (up to 53% or even 64%; see data in Table 3). These are, in fact, the highest quantum yields of fluorescence that have ever been reported for the near-IR emitting fluorescent dye conjugates.

Table 3. Conjugates of dyes with antibodies (Sheep anti-Mouse in all cases) used for immunolabeling, imaging of tubulin filaments in fixed PtK2 cells, as shown in Figure 3, and evaluation of bleaching rates, as shown in Figure 4.

Conjugated dye[a]	**2a**	**2b**	**5**-H	**6**-H	**7**-H	Atto-647N
Degree of labeling[b]	1	8	5	2.8	2.5	4.6
$\Phi_{fl.}$ [%] in conjugates	53[c]	9	15[c]	46	64	12
DOL × $\Phi_{fl.}$ (normalized to 100%)	25	45	47	81	100	35

[a] For structures see Scheme 1 or Figure 2. [b] DOL: average amount of the dye residues attached to one antibody molecule ($M \approx 150000$). [c] Dye **2a** (KK114) had $\Phi_{fl.} = 40\%$ at DOL = 2.2 and dye **5**-H demonstrated $\Phi_{fl.} = 53\%$ at DOL = 0.7 (see footnote [f] to Table 2).

We found that all new dyes are suitable for immunofluorescence assays. The confocal and STED nanoscopy images were bright, demonstrated good signal-to-noise ratio, and excellent optical resolution.[26] In this respect, the tested new dyes seem advantageous compared to Atto647N (Figure 3).

Table 2. Properties of red-emitting rhodamine dyes (Figure 2).

Dye	Polar groups	Linker	Net charge[a]	Solubility in PBS buffer, pH 7.4	NHS ester stability in H_2O or PBS buffer	Absorption λ_{max} [nm] (H_2O)	Emission λ_{max} [nm] (H_2O)	ε [$M^{-1}cm^{-1}$] $\times 10^{-5}$ (H_2O)	$\Phi_{fl.}$ [%] (H_2O)
1	–	–	+1	insoluble	–	615[b]	646	0.35[c]	36
1a[5a]	–	yes	+1	very low	very low	638	661	0.73[c]	42
3a	2 × SO_3H	–	−1	excellent	–	625	644	0.93	49
4a	2 × OH	–	+1	low	–	621	649	0.50[c]	31
4b	2 × OH, SO_3H	–	0	good[d]	–	623	644	0.95	39
5-H	2 × OH	yes	+1	low	good	632	654	0.87[c]	45[e]
6-H	2 × OH, SO_3H	yes	0	good[d]	excellent	634	654	0.63[c]	55
7-H	2 × $OPO(OH)_2$	yes	−3	good[d]	good	635	655	0.75	55[f]
2a[5a]	2 × SO_3H	yes	−1	excellent	moderate	637	660	0.92	53[f,e]
2b	2 × SO_3H	yes	−1	excellent	good	637	660	0.90	55

[a] In conjugates; in the free acid the charge is one unit more negative. [b] In water with 10% methanol. [c] Deviations from the Beer–Lambert law due to the low polarity/solubility; extinction is lower than anticipated due to adsorption on a quartz surface. [d] Only slightly soluble in pure water, well-soluble in basic media, including aqueous $NaHCO_3$. [e] In conjugates with Goat-anti-Rabbit antibodies, compounds **2a** and **5**-H have $\Phi_{fl.} = 40$ and 53% at degrees of labeling (DOL) 2.2 and 0.7, respectively. [f] Lifetimes of the exited state for **2a** and **7**-H are 3.4 and 3.6 ns, respectively.

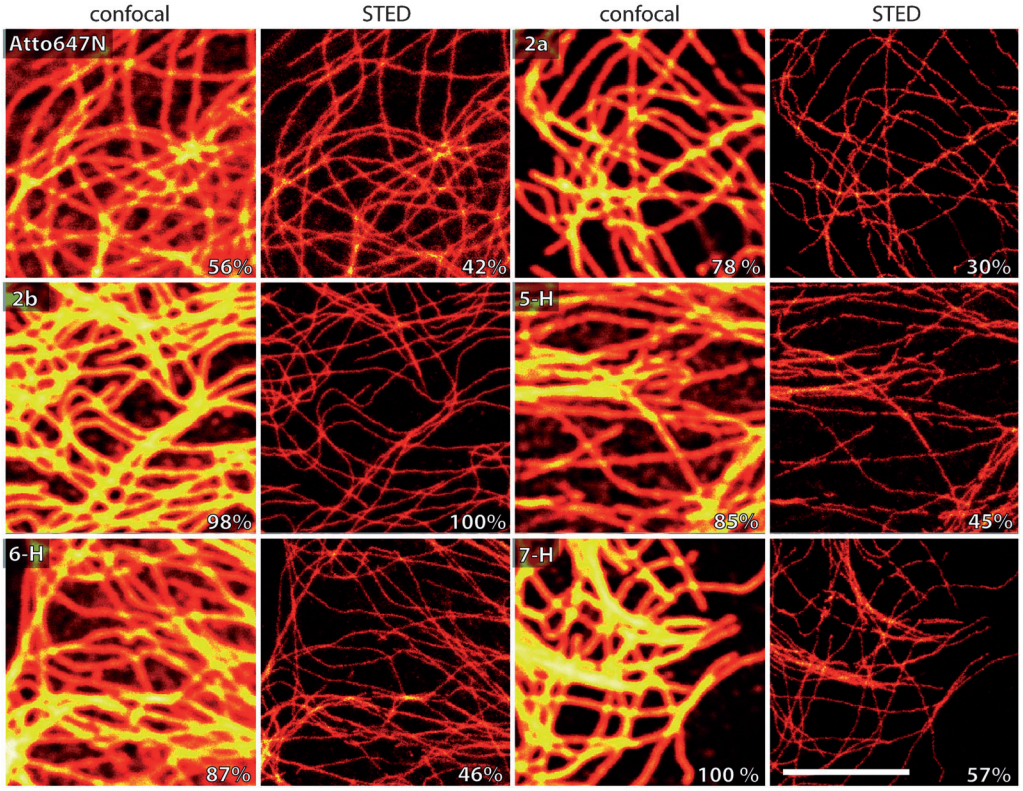


Figure 3. Confocal (left) and STED (right) microscopy images of tubulin filaments in fixed PtK2 cells (raw data). The tubulin cytoskeleton was immunolabeled with the commercial dye Atto647N, and compounds **2a**, **2b**, **5**-H, **6**-H, or **7**-H respectively; excitation with a 640 nm diode laser ($\approx$10 μW). Detection at 670 ± 40 nm, pulsed STED at 760 nm with power of 200 mW at the objectives back aperture; pulse duration 300 ps at 76 MHz repetition rate). The numbers (bottom, right) indicate the relative brightness (%) of the resulting image. The brightness of the individual images is normalized to the brightest pixel in each image (to be able to evaluate contrast and background fluorescence), and the relative brightness of each image is indicated in its lower right corner. The average number of fluorophores attached to one molecule of an antibody (DOL) is not matched for the different images (see Table 3), and that influences the brightness of the images. Scale bar: 5 μm.

If we compare the experimental values for the relative brightness in Figure 3 with the calculated values DOL × $\Phi_{fl.}$ (last line in Table 3), we may conclude that among the hydrophilic rhodamine dyes, the brightness of the images (under confocal conditions) is only very roughly correlated with the labeling degrees and fluorescence quantum yields in conjugates. According to the data in Table 3, dyes **6**-H and **7**-H are expected to provide the brightest images. However, the highest brightness was detected for the phosphorylated dye **7**-H and compound **2b**. Moreover, as expected, dyes **2a** and Atto647N give images that are darker than the images obtained with other dyes.

Another important imaging parameter associated with brightness is the contrast, or the absence of the fluorescent background. In this respect, compounds **2a** and **7**-H are the best because they demonstrate high contrast and very low background (compare the STED images in Figure 3). The imaging performance of the two hydroxylated dyes **5**-H and **6**-H is virtually the same, which is in a good agreement with their properties in aqueous solutions (which are also similar; see Table 2). The sulfonic acid group attached to position B of the scaffold (see Scheme 1 and Figure 2) affects only solubility and the stability of the NHS esters.

To evaluate and quantify the photostability of the new dyes, bleaching curves under confocal and STED conditions (Figure 4) were recorded. For this purpose, the tubulin cytoskeleton of fixed PtK2 cells was labeled using the antibodies described in Table 3. The total fluorescence signal of the same area in the immunolabeled cell samples over the course of several scans was then compared. The photostability of the reference dye Atto647N under confocal conditions is the highest (under irradiation with 640 nm light). That is not surprising, and can be explained by the absence of the C=C double bond in the structure of Atto647N.[5b]

However, under STED conditions (under irradiation with very strong 760 nm light), dyes **5**-H, **6**-H, and **2b** were found to be more photoresistant than Atto647N. Moreover, three new dyes (**5**-H, **6**-H, and **7**-H) appear to be more photostable than KK114 (**2a**). These results indicate that, unexpectedly, the hydroxyl groups protect the fluorescent dyes from photobleaching at least as well as sulfonic or phosphoric acid residues, or even better.

Studies on the intracellular availability of the new fluorescent dyes: Microscopic imaging of protein dynamics and distribution in living cells is one of the most important tasks in

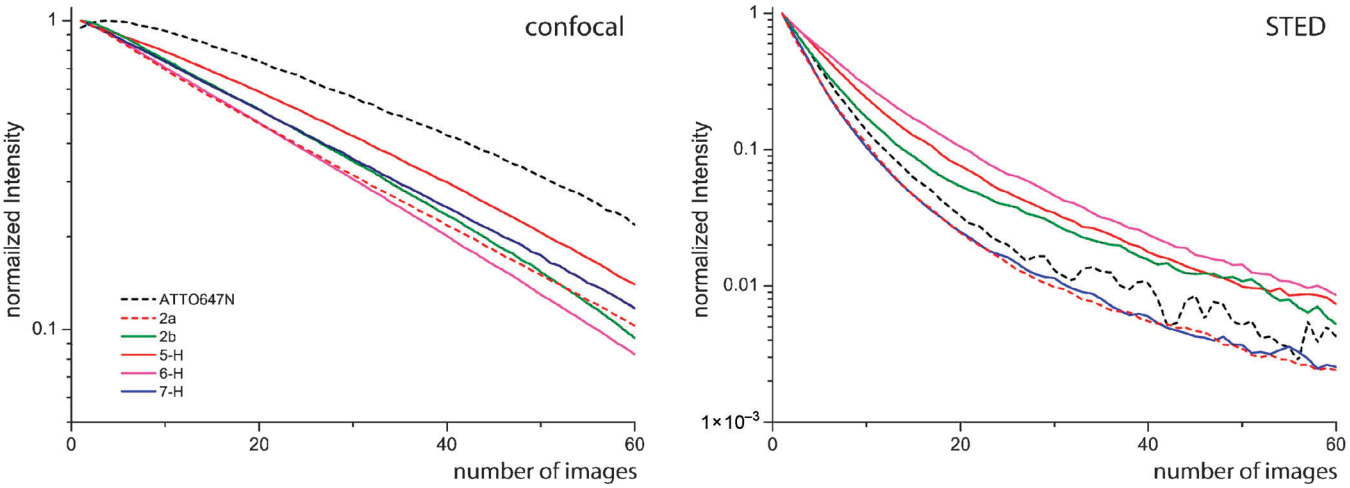


Figure 4. Photostability of dyes **2a**, **2b**, **5**-H, **6**-H, **7**-H, and the commercial dye Atto647N in bioconjugates. Bleaching curves were measured by taking consecutive confocal (left) and STED (right) microscopic images of tubulin filaments in fixed PtK2 cells. To this end, the tubulin cytoskeleton was immunolabeled with the corresponding fluorophores; excitation with 640 nm diode laser (~10 μW). Detection at 670±40 nm; STED at 760 nm with 200 mW at 76 MHz at the objectives back aperture; pulse duration 300 ps). The average number of fluorophores per antibody (DOL) is not matched (see Table 3). This may influence the bleaching rates for different fluorophores.

cell biology. In addition to the available fluorescent fusion proteins, several strategies such as SNAP-, CLIP- or Halo-tags were established in recent years to label proteins in living cells specifically with organic fluorophores.[27] Modified cellular enzymes are applicable for covalent addition of a dye that in this case has to be bound with a certain small molecule. However, only a limited number of dyes are currently available for these labeling strategies. The most important requirement for the dyes used in these labeling strategies is that a sufficient amount of it needs to pass through cellular membrane(s) to reach the target protein. Dyes with positive or zero charged residues are the most promising candidates for testing the cell permeability.[28] Therefore we chose methyl esters **5**-Me (with a positively charged dye residue; $q=+1$) and **6**-Me (with an uncharged moiety; $q=0$) as model compounds imitating the corresponding conjugates of dyes **5**-H and **6**-H. To explore the permeability of the cell membrane, the following protocol was used. The living mammalian cells (PtK2) were grown overnight on cover slips, and were then subjected to 1 μg of a dye in 1 mL of the growth medium for 10 min at room temperature (the amount of dye used for the labeling of SNAP-fusion proteins is generally below 5 μg in 1 mL). After that, the cells were washed with a pure growth medium (without a dye), and imaged using an epifluorescence microscope (Figure 5). We found that both dyes cross the cellular membranes and are incorporated into cellular structures, although to different extents. As visible in Figure 5, the zwitterionic dye **6**-Me can hardly be detected in the cell, whereas the positively charged dye **5**-Me enriches the cellular structures, even if it is applied in high dilution. It may thus be useful for the new labeling strategies in living cells. It is not a trivial result because the dye molecule is large and relatively heavy ($M\approx1000$). In this respect, it is important to note that compound **5**-H gave bright and high

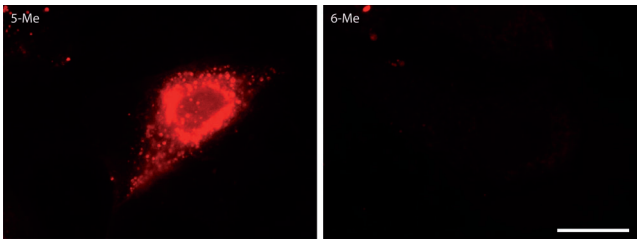


Figure 5. Membrane-permeation experiments of the model rhodamine dyes **5**-Me (left) and **6**-Me (right) (see Figure 2 for the structures of compounds **5**-H and **6**-H). Wide-field fluorescence images of living mammalian PtK2 cells after 10-minute incubation with aqueous buffer solution of the dyes (1 μg mL^{-1}). For comparison, the imaging conditions and color tables are kept constant. Scale bar: 20 μ.

contrast fluorescent images under confocal and STED conditions (Figure 3), so that the method of STED nanoscopy is expected to be applied for example in studying the SNAP-tagged protein distribution using amides prepared from compound **5**-H. The neutral (zwitterionic with a zero net charge) dye **6**-Me was found to be less cell-permeable, as can be seen in Figure 5 (at least at low concentrations).

Electrophoretic mobility of the sulfonated and phosphorylated fluorescent dyes: To estimate the actual negative net charges in dyes **6**-H, **2b**, and **7**-H in aqueous solutions (at pH 8), these compounds were analyzed by capillary gel electrophoresis with laser-induced fluorescence detection (CGE-LIF), utilizing a standard capillary DNA sequencer.[14a,29] The electropherograms of the three dyes **6**-H, **2b**, and **7**-H are presented in Figure 6. Although the main emission wavelength of the excitation source (argon laser (488 nm)) does not match the absorption bands of the dyes, these compounds have sufficient absorption at 514 nm, and can be excited by this weaker line of the argon laser (514.5 nm). The

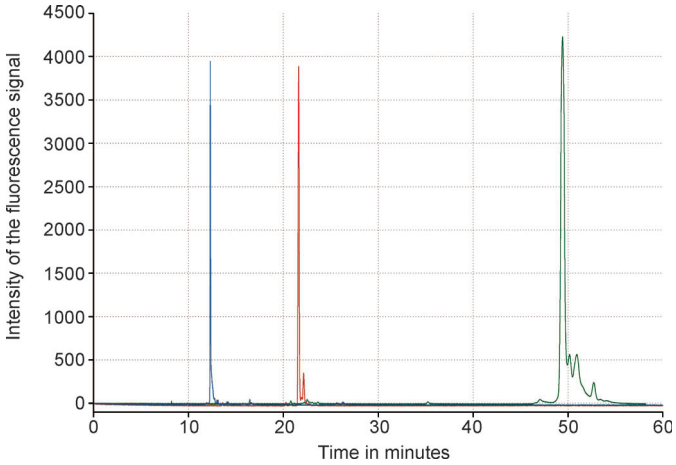


Figure 6. Electrophoretic mobility of sulfonated and phosphorylated fluorescent dyes. From right to left: **6**-H (green curve; $q=-1$), **2b** (red curve; $q=-2$), **7**-H (blue curve, $q=-4$). See text for details.

molecular masses of the compounds **7**-H, **2b**, and **6**-H, their molecular shapes and thus their hydrodynamic radii are similar. Therefore, their relative migration times depend almost exclusively on their negative net charges. Comparing the migration times of the distinct peaks, one can clearly see that the electrophoretic mobility of a dye is directly proportional to its negative charge. Thus, the migration time of the phosphorylated dye **7**-H (with two $OP(OH)_2$ groups and net charge $q=-4$) is two times shorter than that of **2b** (the analogue with two SO_3H groups and $q=-2$), and the migration rate of **2b** is half that of **6**-H (one SO_3H group, $q=-1$). Figure 6 proves that at pH 8, all the anion-providing groups, particularly primary phosphate, are completely ionized. Thus, it was shown that phosphorylation is indeed a new efficient tool to drastically increase the electrophoretic mobility of a dye. One small additional peak near the main peak of **2b** appears probably due to the presence of amide rotamers. The existence of these rotamers was confirmed by NMR spectroscopy (see the Supporting Information), in which certain signals are split.

Conclusion

Three variable positions in a scaffold of a rhodamine dye (**1**, Scheme 1 and Figure 2) were utilized to obtain red-emitting fluorescent dyes with new valuable properties. Particularly, direct oxidation of the two methyl groups attached to the double C=C bonds to the corresponding allylic alcohols was explored. This transformation gives way to a more hydrophilic compound with a considerably increased fluorescence quantum yield (in aqueous solutions), and certain solubility in water, although the starting material is insoluble. Hydroxylation does not introduce an additional negative charge and therefore does not inhibit cell-permeability. Derivatives of compound **5**-H (e.g., methyl ester **5**-Me in Figure 2) with two hydroxyl groups and positively charged dye residue readily cross the cellular membranes, even if applied in high dilution. On the other hand, compound **6**-Me, with a zero net charge, proved to be far less cell-permeable. Importantly, aliphatic tertiary amides (dyes with a linker for conjugation) were prepared from the precursors with free carboxylic acid group without protection of the hydroxyl. The dye with two hydroxyl groups and an amide linker demonstrated very high fluorescence quantum yield as a free acid and in conjugates with antibodies (at low degrees of labeling).

The hydroxymethyl residues in a rhodamine dye were transformed into the primary phosphate groups. This study is the first to describe a fluorescent dye with two primary phosphate residues, which are expected to make the compound more "bio-compatible". The phosphorylated dye (**7**-H in Figure 2) was shown to be an important alternative to widely used sulfonated fluorescent dyes. High polarity and the increased (relative to the sulfonic acid residue) number of acidic protons in the phosphorus-containing dye results in good solubility in neutral and basic aqueous solutions (pH > 7), accompanied by high fluorescence quantum yield. Far better electrophoretic mobility (at pH 8) is the result of the higher net charge ($q=-4$) of the phosphorylated dye.

Moreover, a third variable position was involved to improve the water solubility of the hydroxyl substituted dye. One of the four fluorine atoms in the aromatic ring was selectively exchanged to the residue of 2-mercaptoethyl sulfonic acid—the commercially available bifunctional reagent. This modification did not affect the spectral properties very much and provided an "amphiphilic dye" (**6**-H, Figure 2), which forms an exceptionally stable active NHS ester. Thus, a convenient fluorescent marker was developed, which provides excellent imaging performance, is well-soluble in most organic solvents, aqueous PBS buffer, or $NaHCO_3$ solutions, and is easy to purify and handle. The influence of three polar substituents (hydroxyl, phosphoric, and sulfonic acid) on the spectral properties and the imaging performance of the near-IR-emitting rhodamine dye were explored. In many respects, both hydroxyl and phosphoric acid ester groups proved to be viable alternatives to "classical" sulfonic acid groups. Special attention was paid to the stability and solubility of the corresponding amino-reactive markers (the NHS esters), which are very important and often decisive in many biological applications. Finally, a simple method for preparation of methyl *N*-methyl-4-aminobutyrate hydrochloride was used. This ester represents a versatile linker for the preparation of (amino) reactive markers with improved stability.

The new dyes demonstrated excellent imaging performance in conventional and super-resolution microscopy and perfectly matched the required spectral parameters.

Acknowledgements

The authors are grateful to Dr. Mariano Bossi, Dr. Heiko Schill, Marianne Pulst, Nina Ohm and Jürgen Bienert (MPI BPC Göttingen), Reinhard Machinek, Dr. Holm Frauendorf and their co-workers (Institut für

Organische und Biomolekulare Chemie, Georg-August-Universität Göttingen) for recording spectra. We are indebted to Bundesministerium für Bildung und Forschung (BMBF 513) for financial support in the program Optische Technologien für Biowissenschaften und Gesundheit (FKZ 13N11066), and for support by the DFG-Research Center for Molecular Physiology of the Brain. We appreciate the assistance of E. Rothermel with the cell culture and J. Jethwa for proof-reading the manuscript.

[1] For some recent examples, see: a) 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; b) J. Arden-Jacob, J. Frantzeskos, N. U. Kemnitzer, A. Zilles, K.-H. Drexhage, *Spectrochim. Acta* **2001**, *57A*, 2271–2283; c) C. Jung, B. K. Müller, D. C. Lamb, F. Nolde, K. Müllen, C. Bräuchle, *J. Am. Chem. Soc.* **2006**, *128*, 5283–5291; d) K. Peneva, G. Mihov, F. Nolde, S. Rocha, J. Hotta, K. Braeckmans, J. Hofkens, H. Uji-i, A. Hermann, K. Müllen, *Angew. Chem.* **2008**, *120*, 3420–3423; *Angew. Chem. Int. Ed.* **2008**, *47*, 3372–3375; e) S. L. Niu, G. Ulrich, R. Ziessel, A. Kiss, P.-Y. Renard, A. Romieu, *Org. Lett.* **2009**, *11*, 2049–2052; f) M. Fu, Y. Xiao, X. Qian, D. Zhao, Y. Xu, *Chem. Commun.* **2008**, 1780–1782; g) Z. Lu, N. Liu, S. J. Lord, S. D. Bunge, W. E. Moerner, R. J. Twieg, *Chem. Mater.* **2009**, *21*, 797–810; h) F. Bruyneel, L. D'Auria, O. Payen, P. J. Courtoy, J. Marchand-Brynaert, *ChemBioChem* **2010**, *11*, 1451–1457; i) Y. Koide, Y. Urano, K. Hanaoka, T. Terai, T. Nagano, *ACS Chem. Biol.* **2011**, *6*, 600–608; j) L. Yuan, W. Lin, Y. Yang, H. Chen, *J. Am. Chem. Soc.* **2012**, *134*, 1200–1211; k) M. P. Shandura, V. P. Yakubovskyi, A. O. Gerasov, O. D. Kachkovsky, Y. M. Poronik, Y. P. Kovtun, *Eur. J. Org. Chem.* **2012**, 1825–1834.

[2] For reviews on modern optical nanoscopy methods, see: a) S. W. Hell, in *Single Molecule Spectroscopy in Chemistry, Physics and Biology* (Eds.: A. Gräslund, R. Rigler, J. Widengren), Springer, Berlin, **2010**, pp. 365–398; b) D. Puliti, D. Warther, C. Orange, A. Specht, M. Goeldner, *Bioorg. Med. Chem.* **2011**, *19*, 1023–1029; c) S. van de Linde, S. Wolter, M. Sauer, *Austr. J. Chem.* **2011**, *64*, 503–511; d) T. Müller, C. Schumann, A. Kraegeloh, *ChemPhysChem* **2012**, *13*, 1986–2000; for the most recent references, see: e) G. T. Dempsey, J. C. Vaughan, K. H. Chen, M. Bates, X. Zhuang, *Nat. Methods* **2011**, *8*, 1027–1040; f) M. Bates, G. T. Dempsey, K. H. Chen, X. Zhuang, *ChemPhysChem.* **2012**, *13*, 99–107.

[3] For applications and spectral range of rhodamines as fluorescent dyes, see: a) M. Beija, C. A. M. Afonso, J. M. G. Martinho, *Chem. Soc. Rev.* **2009**, *38*, 2410–2433; b) M. S. T. Gonçalves, *Chem. Rev.* **2009**, *109*, 190–212; c) H. N. Kim, M. H. Lee, H. J. Kim, J. S. Kim, J. Yoon, *Chem. Soc. Rev.* **2008**, *37*, 1465–1472; d) L. D. Patsenker, A. L. Tataretz, Y. A. Povrozin, E. A. Terpetsching, *Bioanal. Rev.* **2011**, *3*, 115–137; e) R. P. Haugland, *A Guide to Fluorescent Probes and labeling Technologies*, Invitrogen, Carlsbad, **2010**, pp. 15–23, 35–41, 47–48, 74–77.

[4] For recent reviews on rhodamine synthesis and derivatization, see: a) S. Murphree in *Progress in Heterocyclic Chemistry, Vol. 22*, (Eds.: G. Gribble, J. A. Joule), Elsevier, Amsterdam, **2011**, pp. 21–58; b) L. M. Wysocki, L. D. Lavis, *Curr. Opin. Chem. Biol.* **2011**, *15*, 752–759; for the most recent references, see: c) T. Peng, D. Yang, *Org. Lett.* **2010**, *12*, 496–499; d) A. Romieu, T. Bruckdorfer, G. Clavé, V. Grandclaude, C. Massif, P.-Y. Renard, *Org. Biomol. Chem.* **2011**, *9*, 5337–5342; e) J. B. Grimm, L. D. Lavis, *Org. Lett.* **2011**, *13*, 6354–6357.

[5] a) K. Kolmakov, V. N. Belov, J. Bierwagen, C. Ringemann, V. Müller, C. Eggeling, S. W. Hell, *Chem. Eur. J.* **2010**, *16*, 158–166; b) K. Kolmakov, V. N. Belov, C. A. Wurm, B. Harke, M. Leutenegger, C. Eggeling, S. W. Hell, *Eur. J. Org. Chem.* **2010**, 3593–3610.

[6] For leading references on applications of red-emitting dye KK114, see: a) R. Schmidt, C. A. Wurm, A. Punge, A. Egner, S. Jakobs, S. W. Hell, *Nano Lett.* **2009**, *9*, 2508–2510; b) J. Bückers, D. Wildanger, G. Vicidomini, L. Kastrup, S. W. Hell, *Opt. Express* **2011**, *19*, 3130–3143; c) P. A. Pellett, X. Sun, T. J. Gould, J. E. Rothman, M.-Q. Xu, I. R. Corrêa Jr., J. Bewersdorf, *Biomed. Opt. Exp.* **2011**, *2*, 2364–2371; d) C. Kukat, C. A. Wurm, H. Spåhr, M. Falkenberg, N.-G. Larsson, S. Jakobs, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 13534–13539; e) C. A. Wurm, D. Neumann, M. A. Lauterbach, B. Harke, A. Egner, S. W. Hell, S. Jakobs, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 13546–13551; f) Y. Hua, R. Sinha, C. S. Thiel, R. Schmidt, J. Hüve, H. Martens, S. W. Hell, A. Egner, J. Klingauf, *Nat. Neurosci.* **2011**, *14*, 833–839.

[7] Atto-Tec GmbH, Siegen, Germany. For the structure of Atto647N, see ref. [5]. This dye is a carbopyronine with a positively charged dye residue

[8] K. Kolmakov, C. A. Wurm, M. V. Sednev, M. L. Bossi, V. N. Belov, S. W. Hell, *Photochem. Photobiol. Sci.* **2012**, *11*, 522–532.

[9] L. G. Lee, R. J. Graham, W. E. Werner, E. Swartzman, L. Lu, Apptera Corp. USA, US Pat. 6372907, **2002**.

[10] a) F. Mao, W.-Y. Leung, R. P. Haugland, Molecular Probes, Inc., Eugene, Oregon, USA, US Pat. 6130101, **2000**; b) A. Zilles, J. Arden-Jacob, K.-H. Drexhage, N. U. Kemnitzer, M. Hammers-Schneider, Atto-Tec GmbH, Siegen, Germany, WO 2005/003086, **2005**.

[11] See: a) Ref. [10a]; b) B. Agnew, K. R. Gee, T. G. Nyberg, Invitrogen, USA, US Pat. 2007/0249014, **2007**; c) S. M. Polyakova, V. N. Belov, S. F. Yan, C. Eggeling, C. Ringemann, G. Schwarzmann, A. de Meijere, S. W. Hell, *Eur. J. Org. Chem.* **2009**, 5162–5177.

[12] a) For an example of improving the solubility in water by replacing ethyl substituents in *N,N,N′,N′*-tetraethyl-3,6-diaminoxanthene fragment by 2-hydroxyethyl groups, see: R. Bandichhor, A. D. Petrescu, A. Vespa, A. B. Kier, F. Schroeder, K. Burgess, *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) except perhaps amino groups; but these are prohibitive in the allylic positions, because in close proximity to the fluorophore, they may quench the emission of light due to the photoinduced electron transfer (PET) from the lone electron pair of the nitrogen atom to the half-occupied HOMO of the fluorophore in the excited state.

[13] N. Rabjohn, *Selenium Dioxide Oxidation, Organic Reactions*, (Ed.: S. E. Denmark, Wiley Online Library, New York, **1976**, pp. 261–416,.

[14] a) See the Supporting Information for details; b) K. Kolmakov, S. Nizamov, V. N. Belov, C. Wurm, K. Willig, S. W. Hell, H. Schill, L. Kastrup, G. Donnert, J. Bückers, Max-Planck Innovation, PCT/EP 2011/005231, **2011**.

[15] The final product, a red emitting dye, needs to have a linker for conjugation with the target molecules. The presence of a tertiary amide (compounds **2a**,**b**) or an ester group (compound **3b**) additionally redshift the absorption and emission bands to 5–10 nm.

[16] See, for example, the available structures of fluorescent dyes produced by Atto-Tec GmbH, Siegen (Germany), www.atto-tec.de.

[17] R. K. Olsen, *J. Org. Chem.* **1970**, *35*, 1912–1915.

[18] a) F. H. Westheimer, *Science* **1987**, *235*, 1173–1178; b) F. H. Westheimer, S. Huang, F. Covitz, *J. Am. Chem. Soc.* **1988**, *110*, 181–185.

[19] See: a) J. Beechem, K. R. Gee, D. C. Hagen, I. D. Johnson, H.-C. Kang, C. Pastula, Invitrogen Corp., Carlsbad, CA, USA, US pat. 7282339, **2007**; b) E. Lukhatov, EPOCH Biosciences, Inc., Washington DC, USA, WO 2009/046165, **2009**.

[20] a) F. Wold, C. E. Ballou, *J. Biol. Chem.* **1957**, *227*, 301–312; b) L. D. Freedman, G. O. Doak, *Chem. Rev.* **1957**, *57*, 479–523.

[21] a) Y. Kagoshima, M. Mori, E. Suzuki, N. Kobayashi, T. Shibayama, M. Kubota, Y. Kamai, T. Konosu, *Chem. Pharm. Bull.* **2010**, *58*, 794–804; b) S. L. Beaucage, *Oligodeoxyribonucleotides Synthesis Phosphoramidite Approach* in *Methods in Molecular Biology: Protocols for Oligonucleotides and Analogs* (Ed.: S. Agrawal), Humana Press Inc., Totowa, **1993**, pp. 33–61.

[22] O. Valdes-Aguilera, D. C. Neckers, *Acc. Chem. Res.* **1989**, *22*, 171–177.

[23] That means that the polarity and solubility in water do not directly correlate with the fluorescence quantum yield in solutions. For an example of the hydrophilic rhodamine derivative with four 2-hy-

droxyethyl groups replacing ethyl substituents in *N,N,N′,N′*-tetraethyl-3,6-diaminoxanthene fragment, see reference [12a].

[24] See: V. P. Boyarskiy, V. N. Belov, R. Medda, B. Hein, M. Bossi, S. W. Hell, *Chem. Eur. J.* **2008**, *14*, 1784–1792.

[25] For the protocols of the labeling reaction between proteins and NHS esters of the fluorescent dyes and estimation of DOL values, see: www.abberior.com.

[26] C. A. Wurm, K. Kolmakov, F. Göttfert, S. Berning, M. L. Bossi, S. Jakobs, G. Donnert, V. N. Belov, S. W. Hell, *Journal of Optical Nanoscopy*, unpublished results.

[27] H. M. O'Hare, K. Johnsson, A. Gautier, *Curr. Opin. Struct. Biol.* **2007**, *17*, 488–494.

[28] The outer part of the plasma membrane is negatively charged, and, due to electrostatic repulsion, the negatively charged ions as a rule do not penetrate through it (if there is no specific transport mechanism for them).

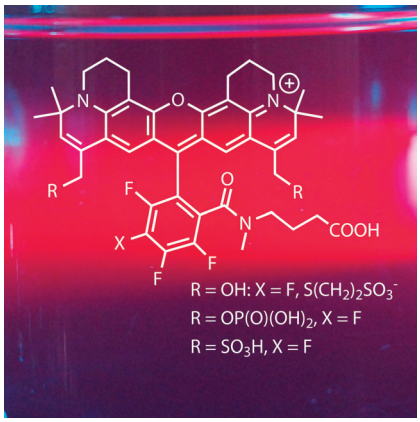
[29] Recorded electropherogram raw data were processed and analyzed using glyXtool V3.3 software: R. Hennig, U. Reichl, E. Rapp, *Glycoconjugate J.* **2011**, *28*, 331.

Received: April 5, 2012
Published online: ■■ ■■, 0000

Fluorescence

*K. Kolmakov, C. A. Wurm,**
R. Hennig, E. Rapp, S. Jakobs,
V. N. Belov, S. W. Hell**.... ■■■■–■■■■

Red-Emitting Rhodamines with Hydroxylated, Sulfonated, and Phosphorylated Dye Residues and Their Use in Fluorescence Nanoscopy



A study in scarlet: Rhodamines that absorb at 621–637 nm and emit at 644–660 nm were prepared and used in fluorescence microscopy and nanoscopy. They are photostable, feature variable polarities and net charges, high stabilities of *N*-hydroxysuccinimidyl (NHS) esters, large fluorescence quantum yields in aqueous solutions, and antibody conjugates.

"A Study in Scarlet"...... by Sir Arthur Conan Doyle was a detective novel introducing Sherlock Holmes (who was fond of chemistry) and the magnifying glass as an investigative contraption. Color, chemistry, and contraption all relate to the picture, which shows one of the best red-emitting rhodamine dyes described by C. A. Wurm, V. N. Belov, S. W. Hell et al. in their Full Paper on pages ■■ ff. The tubular network in a cell was stained with the dye and imaged in a modern light microscope, a refinement of the magnifying glass, as well as by STED microscopy.