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Fluorescent Probes

Polar Red-Emitting Rhodamine Dyes with Reactive Groups: Synthesis, Photophysical Properties, and Two-Color STED Nanoscopy Applications**

Kirill Kolmakov,[a] Christian A. Wurm,*[a] Dirk N. H. Meineke,[a] Fabian Göttfert,[a] Vadim P. Boyarskiy,[b] Vladimir N. Belov,*[a] and Stefan W. Hell*[a]

Abstract: The synthesis, reactivity, and photophysical properties of new rhodamines with intense red fluorescence, two polar residues (hydroxyls, primary phosphates, or sulfonic acid groups), and improved hydrolytic stability of the amino-reactive sites (NHS esters or mixed *N*-succinimidyl carbonates) are reported. All fluorophores contain an *N*-alkyl-1,2-dihydro-2,2,4-trimethylquinoline fragment, and most of them bear a fully substituted tetrafluoro phenyl ring with a secondary carboxamide group. The absorption and emission maxima in water are in the range of 635–639 and 655–659 nm, respectively. A vastly simplified approach to red-emitting rhodamines with two phosphate groups that are compatible with diverse functional linkers was developed. As an example, a phosphorylated dye with an azide residue was prepared and was used in a click reaction with a strained alkyne bearing an *N*-hydroxysuccinimid (NHS) ester group. This method bypasses the undesired activation of phosphate groups, and gives an amphiphilic amino-reactive dye, the solubility and distribution of which between aqueous and organic phases can be controlled by varying the pH. The presence of two hydroxyl groups and a phenyl ring with two carboxyl residues in the dyes with another substitution pattern is sufficient for providing the hydrophilic properties. Selective formation of a mono-*N*-hydroxysuccinimidyl ester from 5-carboxy isomer of this rhodamine is reported. The fluorescence quantum yields varied from 58 to 92% for free fluorophores, and amounted to 18–64% for antibody conjugates in aqueous buffers. The brightness and photostability of these fluorophores facilitated two-color stimulated emission depletion (STED) fluorescence nanoscopy of biological samples with high contrast and minimal background. Selecting a pair of fluorophores with absorption/emission bands at 579/609 and 635/655 nm enabled two-color channels with low cross-talk and negligible background at approximately 40 nm resolution.

Introduction

Synthetic fluorescence dyes with a reactive group are indispensable as versatile reagents and fluorescent molecular probes, having wide use in chemistry, life sciences, and, particularly, in fluorescence microscopy. Resolution is the most important property of a microscope. While far-field (light-focusing) fluorescence microscopy was limited in optical resolution to about half the wavelength of light (200–350 nm) throughout the 20th century, the development of stimulated emission depletion (STED) microscopy (optical "nanoscopy") at the turn of this century showed that it is fundamentally possible to overcome the diffraction barrier.[1] The best optical resolution reported so far in biological STED imaging ($\approx$20 nm) has been obtained using photostable red-emitting fluorophores with polar groups providing water-solubility and improved labeling.[1l] Fluorophores used for STED should have above-average photostability and large fluorescence quantum yields at fluorescent state lifetimes of about a nanosecond. Moreover, different fluorophores should afford separation through their excitation or emission properties. Fluorophores emitting in the red or near-infrared (near-IR) range have good starting attributes and are promising candidates for achieving many of these properties.[2] Therefore, in our previous studies, we described functionally substituted rhodamine[3a–c] and carbopyronine dyes[3d] that absorb at about 635 nm and emit around 660 nm (see Figure 1). The polarity, solubility and stability of *N*-hydroxysuccinimidyl esters of these dyes were regulated by the incorporation of various polar residues.[3a] As a result, a set of fluorescent markers was obtained, featuring excellent imaging performance and providing convenience in practical use.

[a] Dr. K. Kolmakov, Dr. C. A. Wurm, M. Sc. D. N. H. Meineke, Dipl.-Biol. F. Göttfert, Dr. V. N. Belov, Prof. S. W. Hell
Department of NanoBiophotonics
Max Planck Institute for Biophysical Chemistry
Am Fassberg 11, 37077 Göttingen (Germany)
E-mail: cwurm@gwdg.de
vbelov@gwdg.de
shell@gwdg.de

[b] Prof. V. P. Boyarskiy
Chemistry Department, St. Petersburg State University
Universitetskiy Pr. 26, Petrodvorets, St. Petersburg, 198504 (Russia)

[**] STED = stimulated emission depletion.

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The labeling specificity in bioconjugation reactions frequently depends on the polarity of the fluorophore and on related parameters, such as the presence of charged groups and solubility in aqueous buffer. The solubility of fluorescent dyes in water and aqueous buffers is improved by attaching polar residues. For this purpose, sulfonic acid groups (SO_3H) are mainly introduced, because they are the least reactive and remain intact in many post-synthetic dye modifications. In our previous studies, we decorated rhodamines[3a] and coumarins[4] with primary phosphate groups ($OP(O)(OH)_2$), which were shown to be a good alternative to the more customary sulfonic acid groups used in most organic dyes. Recently, A. Romieu et al. performed a systematic study on a BODIPY dye scaffold with diverse solubilizing residues bearing different net charges.[5a] Interestingly, in this study, the highest fluorescence quantum yield was reported for a phosphonylated dye.

The stability of the amino reactive site towards hydrolysis is a very important feature of any fluorescent label. The hydrolytic stability of the NHS ester of the phosphorylated dye **1a** (**1a**-NHS in Figure 1) in solid samples or aqueous solutions was shown to be modest.[3a] Moreover, while preparing NHS esters of compound **1a** and other phosphorylated rhodamines, we faced a selectivity problem: along with the carboxylic acid, the primary phosphate group was also involved in esterification. All these complications are in line with a report on cyanine and squarene dyes decorated with phosphonic acid groups ($P(O)(OH)_2$).[6] Better selectivity with phosphates ($R\text{-}OP(O)(OH)_2$), compared to phosphonates ($R\text{-}P(O)(OH)_2$), however, could be expected. Indeed, primary phosphates are known to be stronger acids[3a] and, therefore, are very likely to be weaker nucleophiles which are known to be less readily activated. Luckily, in bifunctional coumarin dyes with only one primary phosphate group,[4] the selectivity towards the carboxyl group is good; NHS esters are stable, and can easily be isolated. In amidation reactions, utilizing conventional coupling reagents TSTU and HATU (TSTU = *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; HATU = 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate), the selectivity between C and P sites proved even worse. For this reason, the preparation of phosphorylated dye **1a** by direct amidation of phosphorylated precursor **2a** is impossible, so we had to start the synthesis with the hydroxylated compound **2b** instead.[3a] The competition between two reaction sites never occurred in dyes with sulfonic acid groups (e.g., **1b**). Sulfonates (RSO_3H) are stronger acids and far weaker nucleophiles than phosphates. Thus, dye **1b** ($n = 2$; KK114)[3a–c] selectively forms the NHS ester at its CO_2H site, and therefore, can be obtained directly from precursor **2c** by amidation. According to literature survey, it is not possible to selectively esterify or amidate carboxyl groups leaving the unprotected phosphates intact. Moreover, it is known that secondary NHS phosphates (prepared from the corresponding primary esters) can react with amines.[7] Therefore, in a broader sense, exploring the feasibility of the creation of amino-reactive sites in the presence of free $OP(O)(OH)_2$ groups is important for preparative organic chemistry, considering the abundance of both groups in nature and in many pharmacologically potent compounds.

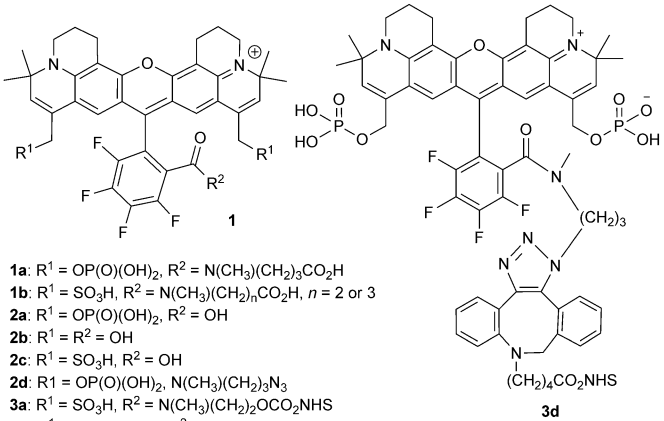
Herein, we report a vastly simplified synthesis of a red-emitting rhodamine building block with two (protected) primary phosphate groups. This approach enabled combination of the phosphorylated dye core with diverse linkers, for example, omega-(alkylamino) azides. As a result, "click chemistry" on substrates with other reactive groups may be used. Thus, a click reaction between an azide-derivatized dye **2d** with primary phosphate residues and an alkyne reagent, already bearing an amino-reactive moiety, was applied to avoid undesired activation of the phosphate groups. Quite recently, certain click and Sonogashira reactions broadened the scope of post-synthetic dye modifications.[5b] We report a synthesis that bypassed the selectivity problem and led to a phosphorylated "amphiphilic" dye **3d** (Figure 1) in the form of an NHS ester. Its solubility, polarity, and partition between aqueous and organic phases could be controlled by varying the pH.

Mixed *N*-succinimidyl carbonates (NHS carbonates, ROCOOSu) of the fluorescent dyes—viable alternatives to conventional *N*-hydroxysuccinimidyl esters (NHS esters)—are shown to be sufficiently reactive toward amines and more hydrolytically stable than NHS esters (even in basic aqueous solutions), and were easier to prepare, purify, and handle at ambient temperature. Excellent hydrolytic stability is featured by two dyes (**3a**,**c**) that combine NHS carbonate function with sulfonate and phosphate, respectively. Additionally, the selective activation of other dyes emitting at about 620 nm and bearing two carboxyl and/or two hydroxyl groups as solubilizing moieties is reported in this work.

Results and Discussion

Motivation and synthetic strategies

The outstanding performance of the sulfonated dye KK114 (**1b**, $n = 2$)[3b–d] and its phosphorylated analogue Abberior Star 635P (**1a**)[3a,b] was the reason to investigate the possibility of obtaining their amino-reactive derivatives with even higher hydrolytic stability (Figure 1).



1a: $R^1 = OP(O)(OH)_2$, $R^2 = N(CH_3)(CH_2)_3CO_2H$
1b: $R^1 = SO_3H$, $R^2 = N(CH_3)(CH_2)_nCO_2H$, $n = 2$ or 3
2a: $R^1 = OP(O)(OH)_2$, $R^2 = OH$
2b: $R^1 = R^2 = OH$
2c: $R^1 = SO_3H$, $R^2 = OH$
2d: $R1 = OP(O)(OH)_2$, $N(CH_3)(CH_2)_3N_3$
3a: $R^1 = SO_3H$, $R^2 = N(CH_3)(CH_2)_2OCO_2NHS$
3b: $R^1 = OP(O)(OH)_2$, $R^2 = N(CH_3)(CH_2)_4OCO_2NHS$
3c: $R^1 = OP(O)(OH)(ONHS)$, $R^2 = N(CH_3)(CH_2)_4OCO_2NHS$
NHS = *N*-succinimidyl

Figure 1. Red-emitting rhodamine dyes (**1a**,**b**), hydrolytically stable reactive markers (**3a–d**) and their precursors (**2a–d**).

Table 1. Properties of red-emitting rhodamines with polar groups and their conjugates with antibodies. For structures see Figure 1 and Scheme 1–6.

Dye	Polar groups[a]	Linker	q[b]	NHS ester stability in H_2O, % left after 24 h at RT[c]	Abs. λ_{max} [nm] (H_2O)	Em. λ_{max} [nm] (H_2O)	ε [$M^{-1}cm^{-1}$] $\times 10^{-5}$ (H_2O)	$\Phi_{fl.}$ [%] in H_2O	$\Phi_{fl.}$ [%] in conjugates with antibodies[e]
1a	$2\times OPO(OH)_2$	amide + carboxyl	−3	moderate,[f] 5	634	655	0.89[d]	82[g]	64
3a	$2\times SO_3H$	amide + NHS carbonate	−1	excellent, 60	636	659	1.0	66	39
3b	$2\times OPO(OH)_2$	amide + NHS carbonate	−3	good, 40	633	653	0.91	89	50
3c	$2\times OPO(OH)(NHS)$	amide + NHS carbonate	−1	excellent, 60	633	653	0.75	92	60
10	$2\times OPO(OH)_2$	amide + triazole + carboxyl	−3	good, 30	639	658	0.88	64[g]	47
14-H	$2\times OH$, CO_2H	no linker	0	excellent, 80	579	609	0.60	42[g]	18
17-H	CO_2H	SCH_2CO_2H	0	good, 60	606	629	0.58	75[g]	–

[a] In addition to the sterically hindered CO_2H group in the benzene ring of rhodamines; [b] net charge of the dye residue in conjugates or NHS esters; [c] the hydrolytic stability was determined for 0.1 mM aqueous solutions by HPLC; [d] due to a new and improved synthesis with extractive isolation and purification steps, the extinction coefficient for **1a** is by about 20% higher than the previously reported value;[3a] [e] sheep anti-mouse antibodies with degrees of labeling (DOL) in the range of 4–6; [f] as compared to other reactive markers in this table, this parameter for **1a** is considered moderate; however, when compared to less stable NHS esters (e.g., NHS ester of **1b** with $n=2$ in Figure 1)[3a] it can be characterized as good; [g] measured for dyes as free acids; stable mixed NHS carbonates (**3a**–**d**) taken as such.

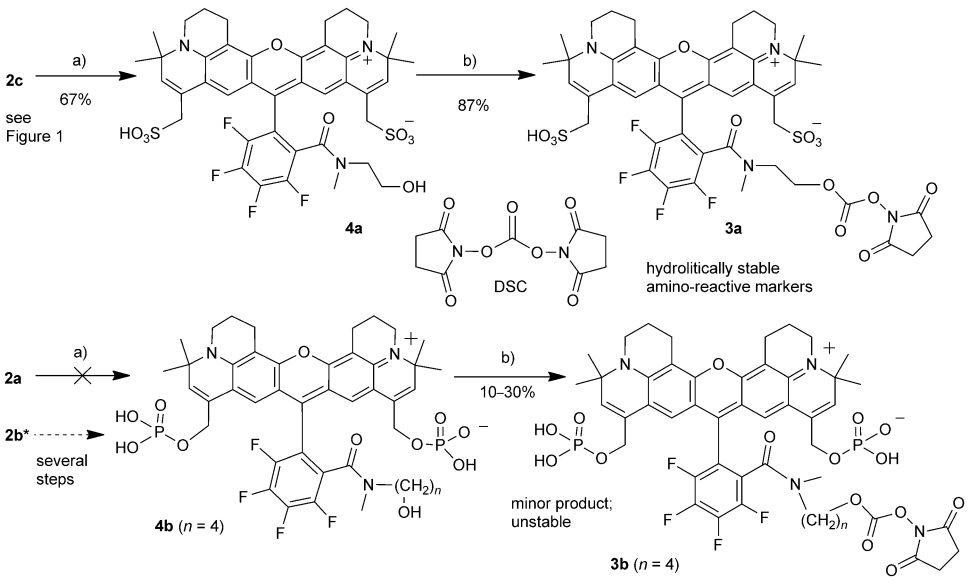
We observed that the stability of NHS esters correlates with the net charge of a dye molecule. The stability proved to be lower for dyes with multiple anion-providing groups (sulfonate or phosphate),[3a] compared to the analogues with noncharged groups (e.g., hydroxyl; see also Table 1). Mixed (asymmetric) *N*-succinimidyl carbonates with improved hydrolytic stability could be an alternative to classical NHS esters.[1j] It is well-known that only carbonates with very good leaving groups readily react with amines to form urethanes. Urethanes prepared from the hydroxylated fluorescent dyes are hydrolytically stable.[8b] Therefore, we applied the same methodology, that is, preparation of mixed NHS carbonates, to other dyes that bear two primary phosphate or two sulfonic acid groups (**3a**,**b**, Figure 1).

In line with this task, there was a more important and general challenge, namely to explore the possibility of selective activation and further reaction of a carboxyl or hydroxyl group in the presence of two free primary phosphate groups. One option to solve this problem could be the use of protective groups on phosphates (e.g., *tert*-butyl esters) the cleavage of which leaves NHS esters or carbonates intact. Another option would be to obtain and use a dye with free phosphate groups and an azide residue, which can undergo a click reaction with an alkyne bearing an activated group. Besides that, our first synthesis of dye **1a** from precursor **2b** was not very simple and left much to be improved. In particular, a palladium-free procedure for this synthesis would be highly desirable.

Furthermore, we intended to address the selective activation of a single carboxyl group (from two) in 5- and 6-carboxyrhodamines with two unprotected hydroxyl groups (compounds **14**-H and **15**-H, Scheme 5). The hydroxyl groups in these compounds provide the required hydrophilic properties without creating the net negative charge, which is undesired in many cases, because it is known to inhibit the permeability of dye molecules through the outer plasma membrane of living cells.

Synthesis

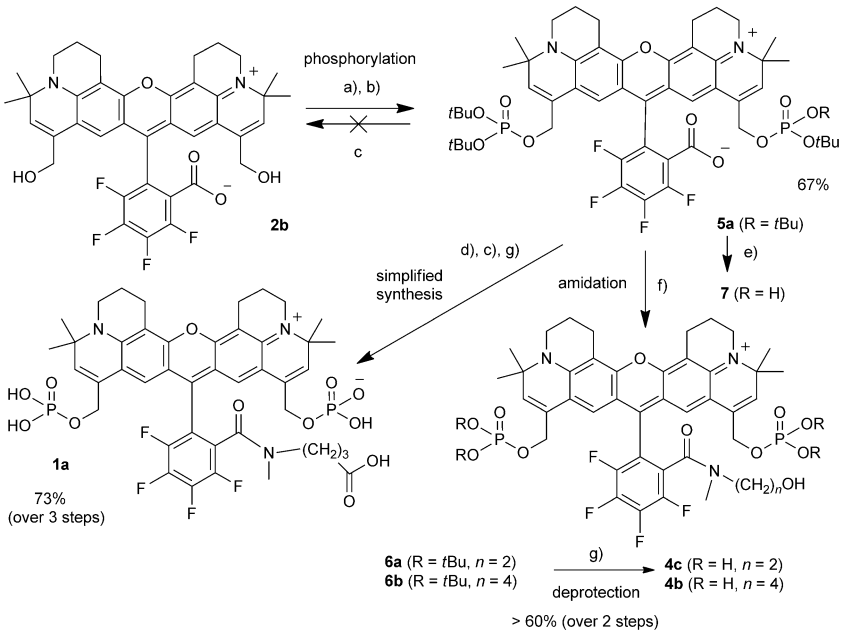
Mixed carbonates as alternatives to conventional NHS esters: Amino-reactive forms of fluorescent dyes are among the most important reagents for obtaining conjugates with biological molecules. As already mentioned, we planned to prepare NHS carbonates **3a**,**b** (Figure 1) as more stable, yet amino-reactive alternatives to NHS esters obtained from dyes **1a**,**b** (Scheme 1). The synthesis of sulfonated NHS carbonate



Scheme 1. Mixed *N*-succinimidyl carbonates containing free sulfonate and phosphate groups: a) $CH_3NHCH_2CH_2OH$, HATU, Et_3N, MeCN; b) *O*,*O*′-di(*N*-succinimidyl)carbonate (DSC), Et_3N, MeCN. *: phosphate groups need to be protected before amidation; see Schemes 2 and 3 for the full synthesis.

3a smoothly proceeded under mild conditions according to Scheme 1. At the first step, this transformation utilized 2-methylamino ethanol and the peptide coupling reagent HATU (both taken in large excess). As it was reported earlier, one fluorine atom in the aromatic ring of tetrafluororhodamines may react with amines.[8a] Also, the dehydration of the amido alcohol **4a** could have been expected. Luckily, none of these side-reactions occurred, yet the activation reaction was rather sluggish at ambient temperature due to the steric hindrance in the rhodamine substrate, which expectedly slowed down the formation of the secondary amide bond. The final asymmetric *N*-succinimidyl carbonate **3a** was isolated as a triethylammonium salt (on phosphate groups). It proved to be exceptionally stable in water, even in the presence of free triethyl amine or sodium bicarbonate (pH 8–9), and in methanol, as well (as established by HPLC; see Table 1 and the Supporting Information). In particular, the new dye marker (**3a**) is not even half-hydrolyzed after 5 h in a 0.1 M aqueous solution of $NaHCO_3$, whereas *N*-hydroxysuccinimidyl (NHS) esters of compounds **1a** and **1b** (Figure 1) and other fluorescent dyes are completely hydrolyzed under these conditions (including NHS esters with phosphonic acid groups).[6] On the other hand, the test reaction of **3a** with aqueous ammonia was rapid (the starting compound vanished in few minutes). Most importantly, the conjugation of **3a** to antibodies, when performed according to the standard protocol in aqueous medium, provided sufficiently high degree of labeling (DOL ~ 4; Table 1). The excellent hydrolytic stability is accompanied by a large quantum yield of fluorescence measured for a free dye and its conjugates with proteins (Table 1), high extinction coefficient and excellent imaging performance.

However, exactly the same approach (as for **3a**) did not work for the phosphorylated analogue **3b**. We established already that unprotected primary phosphate groups react with activating agents (HATU, TSTU, etc.) under basic conditions. In the presence of *N*-hydroxysuccinimide, phosphates and phosphonates form NHS esters at $[O]P(O)(OH)_2$ sites.[6,7] As mentioned above, the steric hindrance at the carboxyl site in rhodamines slows down the amidation, and a large excess of the strong coupling or activation reagents (HATU or TSTU) and long exposure are required. These circumstances make the side reaction at the phosphate sites, which are not sterically hindered, very likely to prevail over C-amidation. Therefore, we started from the hydroxyl derivative **2b** and followed an alternative path, in which the phosphate residues were temporarily protected by *tert*-butyl groups (Scheme 2). Further syntheses utilized phosphorylated precursor **5a**. Usually, *O*-phosphorylation under mild conditions involves the reaction of alcohols with *O,O′*-disubstituted *N,N*-diisopropyl phosphoramidites, assisted by 1*H*-tetrazole and followed by a one-pot oxidation of the intermediate to the *O,O′*-diprotected phosphate. In the final step, two identical phosphoric ester groups (e.g., allyl or *tert*-butyl) are cleaved,[3a,4] and this cleavage liberates free $ROP(O)(OH)_2$ groups. In the rhodamine series, the use of allyl protective groups was not optimal, because their complete removal from the tetra-*O,O′,O″,O‴*-allyl precursor of the phosphorylated dye **1a** required the equimolar amount of palladium catalyst $[Pd(Ph_3P)_4]$. An alternative protecting group, *tert*-butyl (*t*Bu), was used by Nizamov et al. in the synthesis of phosphorylated coumarin dyes, where $(tBuO)_2PNiPr_2$ was applied as a phosphorylating agent.[4] The cleavage at the final step of the synthesis is straightforward, as it requires only trifluoroacetic acid (without trialkyl silane as a scavenger, which in this case might cause the reduction of the positively charged xanthene fragment to the colorless leuco form of the dye).

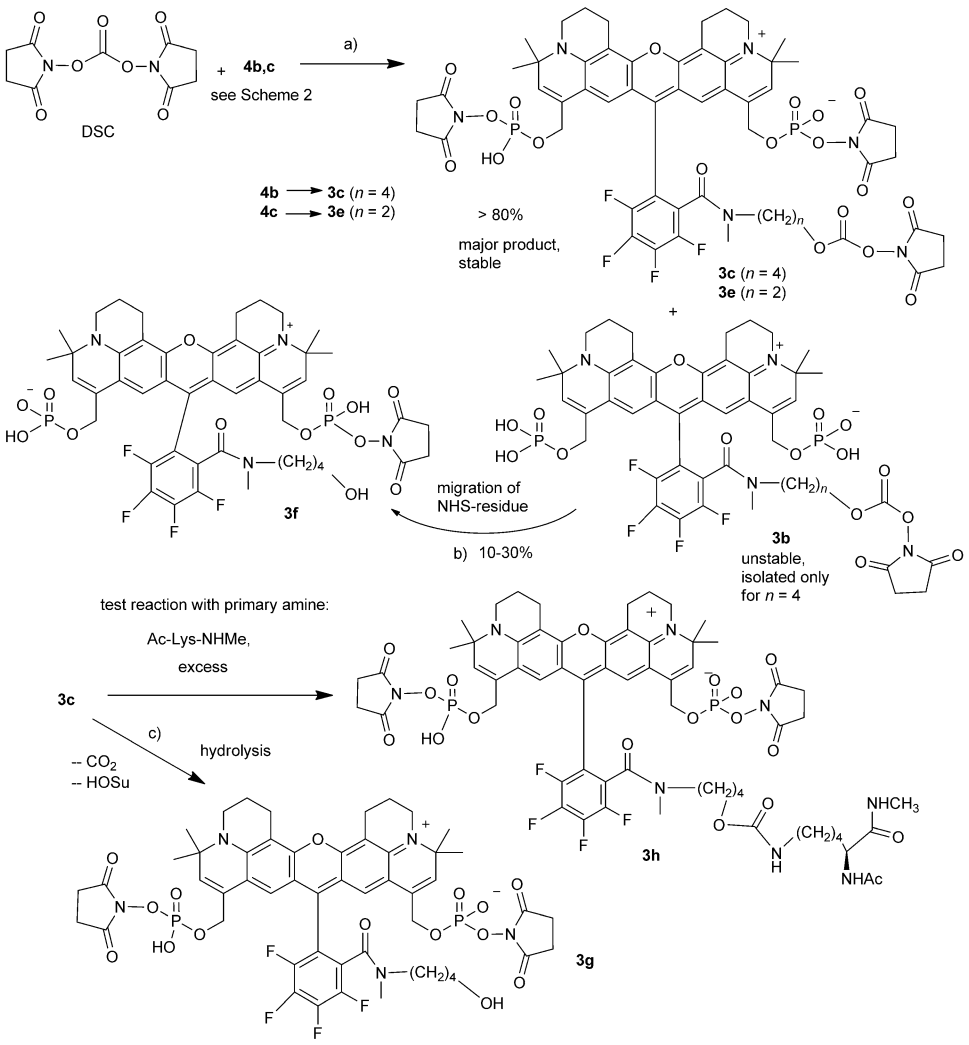


Scheme 2. Synthesis of dyes **1a**, **4b,c** and important phosphorylated precursors **5a** and **6a,b**: a) $(tBuO)_2PN(iPr)_2$, 1*H*-tetrazole/CH_2Cl_2, reflux; b) MCPBA/CH_2Cl_2, 0 °C; c) NaOH (0.05 M) in aq. THF; d) $HCl{\cdot}CH_3NH(CH_2)_3CO_2CH_3$, HATU, Et_3N; e) SiO_2 or weak/diluted acids; f) $CH_3NH(CH_2)_nOH$, HATU, Et_3N; g) 20–30% (v/v) aq. CF_3COOH.

This protocol allows to avoid tedious purification in the last step if the substrates **6a,b** (Scheme 2) are sufficiently pure. Moreover, di-*tert*-butyl phosphates are lipophilic and, therefore, soluble in nonpolar solvents. That offers even more advantages: the chromatographic isolation turned to be easy or even unnecessary, and the residual silica gel or inorganic salts were much easier to remove from nonaqueous solutions. Phosphorylated building block **5a** was obtained in good yield

(Scheme 2). It presents only one difficulty while handling and concentrating: it is sensitive to acids and even undergoes partial deprotection (to form compound **7**) on silica gel in course of the chromatographic isolation. The driving force of this cleavage must be the formation of the zwitterion in compound **7** with a stronger acid (secondary phosphate) as a counter ion.

The undesired reaction is completely avoided when triethyl amine is added to the liquid phase while performing chromatography. Remarkably, compound **5a** is sufficiently stable towards dilute alkali (for several hours in 0.1 M aq. NaOH). Under these conditions, this dye does not lose the phosphate moieties and does not form compound **2b**.[9] The amidation of building block **5a** with 2-methylamino ethanol followed by deprotection with aqueous trifluoroacetic acid (20–30 vol.%) proceeded smoothly. The complications, however, occurred at the final step. The reaction of **4c** with DSC proved to be very slow, considerably slower than in the case of sulfonated analogue **4a** (Scheme 1). The major product was compound **3e**, where both phosphate groups formed the NHS esters as well (Scheme 3). At the same time, the desired mono-NHS carbonate was not formed at all. To exclude all the effects caused by steric hindrance (of the bulky xanthene fragment with phosphate groups), we elongated the chain of the linker, utilized 4-(methylamino)butanol-1 at the amidation step and prepared alcohol **4b**.



Scheme 3. Treatment of *O*,*O*′-di(*N*-succinimidyl)carbonate (DSC) with hydroxyl-substituted phosphorylated dyes **4b**,**c** and further product transformations: a) Et_3N in MeCN; b) dilute aq. Et_3N; c) H_2O or aq. $NaHCO_3$ or aq. Et_3N. Compound **3f** is formed from **3b** while freeze-drying of aqueous solutions or upon storage.

Further results demonstrated that the reactivity of the hydroxyl group in compound **4b** was improved indeed (relative to **4c**), but the elongation of the linker did not prevent the reaction of the phosphate groups. In all experiments with **4b**, along with compound **3b** ($n=4$), we detected compound **3c** (NHS phosphate ester) as the major product. When the starting material (alcohol **4b**) reacted completely, the yield of **3c** was about 80%. Further, while handling and freeze-drying solutions of dye **3b**, we observed the migration of the NHS function to the phosphate site from the mixed carbonate, instead of normal hydrolysis (as illustrated in Scheme 3). This transformation was established by HPLC analysis and mass spectrometry. This intramolecular esterification (with loss of CO_2) is accelerated by bases (triethylamine) and inhibited by small amounts of acids (CF_3COOH).

The alternative synthesis of **3b** that would involve the preparation of the mixed NHS carbonate from **6b** (Scheme 2) followed by deprotection (cleavage of *tert*-butyl groups) at the phosphate sites proved to be unfeasible. The reason was the instability of NHS carbonates in acidic media. The cleavage of all four *tert*-butyl groups requires 30–50% (v/v) aqueous solutions of CF_3COOH and long exposure times. Under these conditions, NHS carbonates, particularly **3a** and **3b** (Scheme 1 and Scheme 3), decomposed in few minutes to furnish complex mixtures of products.

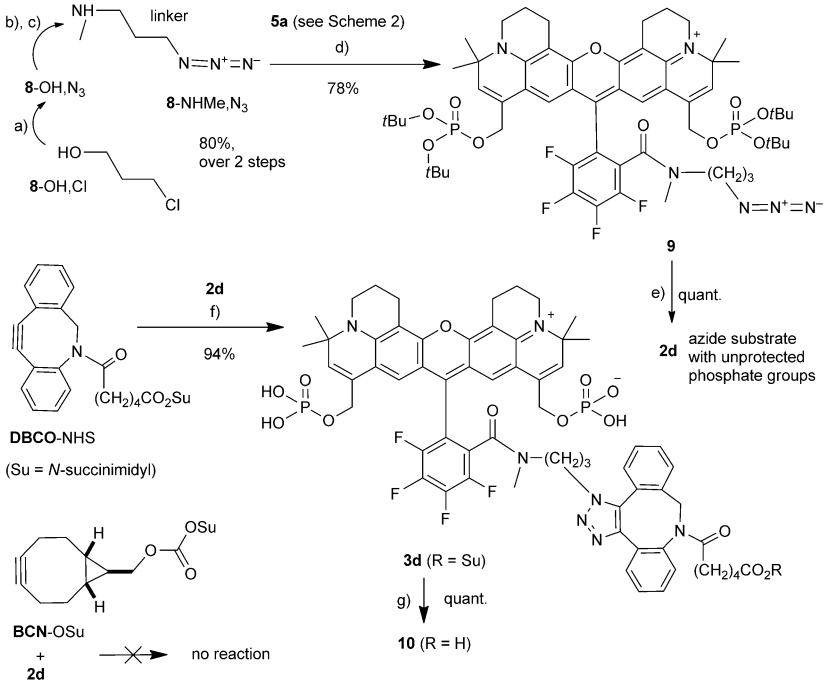
The chemically stable dye, **3c**, demonstrated excellent imaging performance (see below). All important dye properties are summarized in Table 1. With regard to immunolabeling, it was important to establish whether mono-*N*-hydroxysuccinimidyl phosphates react with amines or not, in particular under conditions relevant to conjugation with proteins. In this case, conventional NHS esters react predominantly with terminal amino

groups of lysine residues. Therefore, as a model substrate, we chose a simple and commercially available lysine derivative, Ac-Lys-NHMe. We established that at ambient temperature in aqueous acetonitrile with triethyl amine/carbonate buffer (pH 7.5), only the carbonate site in **3c** reacts with amine, despite the large excess of Ac-Lys-NHMe (>12 equiv) and long exposure times of several hours. Additionally, in aqueous media, alcohol **3f** was detected as a product of hydrolysis. Importantly, mono-*N*-hydroxysuccinimidyl phosphates do not react with amines under these conditions. Therefore, it is unlikely that compound **3c** would cause a cross-link of antibodies in the course of the coupling reaction.

The phosphorylated building block **5a** (Scheme 2) is very useful. In particular, we applied it for a vastly simplified synthesis of dye **1a**.[3b] The procedure is straightforward and purification at the final step is unnecessary (see the Supporting Information). The final product is completely free from silica gel and inorganic salts (as established by measurements of the extinction coefficient). In the course of HPLC, compound **1a** gave a single peak with detection at 254 nm, and was in all respects identical to previously obtained samples, which required tedious purification.[3a] One of the main advantages in these syntheses is that precursors **5a** and **6a**,**b** (Scheme 2) are all lipophilic, soluble in nonpolar organic solvents (DCM), and therefore can be extracted from aqueous solutions and separated from salts and silica gel, which forms suspensions in water, simply by drying the organic extracts followed by filtration.

Click chemistry on a fluorescent azide with free phosphate groups: Another option to obtain the amino reactive fluorescent label with free phosphate groups would be to use a precursor dye with free phosphate groups and an azide or alkyne residue, which can undergo a click reaction with an alkyne or azide, respectively, bearing an amino reactive site. Click reactions, particularly [3+2] azide–alkyne Huisgen cycloaddition, are widely used in chemistry and biochemistry, and certain fluorescent azides, copper catalysts and related reagents are commercially available. Azide–alkyne click reactions feature excellent selectivity, compatibility with a broad range of functional groups and product stability.[10] They proceed under mild conditions in various solvents, and the reaction rate may further be improved by using nitrile oxides and other 1,3-dipoles, instead of the azides.[11] Our preliminary experiments showed that copper ions (required as a catalytic additive) strongly coordinate with rhodamine dyes containing an alkyne moiety to form insoluble precipitates. In this respect, the so-called strain-promoted alkyne reagents are advantageous because in this case copper [Cu^{I} and Cu^{II}] catalysts are not required.[10,11] With regard to the main target—a stable amino-reactive compound bearing free primary phosphate groups—a better and more versatile approach may involve an azide-containing fluorescent dye and an alkyne reagent bearing an amino reactive group. Azide **2d**, which is also useful for other click reactions, was prepared starting from phosphorylated building block **5a** (Scheme 4). The synthesis of the required linker **8**-NHMe,N_3 with an azide function was straightforward; the last two steps were performed in a one-pot fashion. Compound **8**-NHMe,N_3 was not isolated in the neat state and was used in solution. This eliminated safety concerns related to the explosive properties of alkyl azides, but did not affect the amidation step. In the long term, the amino azide compound **8**-NHMe,N_3 and azide **2d** may become very useful for other syntheses and labeling procedures.

The mixed carbonate BCN–OSu (Scheme 4) was the first alkyne substrate we tried for a cooper-free click reaction.[10d,11] Surprisingly, it did not react with azide **2d**, even in the presence of Cu^{I} as catalyst.[10b,h] Therefore, we decided to try a more reactive and commercially available alkyne reagent: DBCO-NHS (dibenzocyclooctyne-NHS ester, Scheme 4). The reaction between compounds **2d** and DBCO-NHS was very fast, clean and complete in 1–2 h at ambient temperature. Its rate depends on the solvent, as established by HPLC analysis. The dyes with phosphate groups are very polar and insoluble in neutral aprotic solvents like acetonitrile, THF, and acetone (alcohols react with the NHS esters and are unacceptable sol-



Scheme 4. Click reaction on a dye with free phosphate groups: a) NaN_3, MeOH; b) CH_3SO_2Cl, Et_3N, CH_2Cl_2; c) 40% aq. $MeNH_2$; d) HATU, Et_3N; e) 30% (v/v) aq. CF_3COOH; f) DMF or aq. THF; g) aq. Et_3N or $NaHCO_3$. Two diastereomeric 1,2,3-triazoles **3d** and **10** are formed (only one is shown in Scheme 4).[10j]

vents in this case). Remarkably, the click reaction between **2d** and DBCO-NHS can be performed in aqueous solutions, and in the presence of water, it is even faster than in DMF. Some amount of organic solvents (e.g., up to 20 vol.% THF), however, should be added to dissolve DBCO-NHS. This click reaction is advantageous, not only because of its kinetics and selectivity, but also due to the simple product isolation. With a reasonable excess of the alkyne, the starting material (**2d**) is completely consumed and its separation is thus avoided. Having two polar anion-providing groups, the reaction product (**3d**) is well-soluble in water, especially at high pH values. That allowed its extraction with water and removal of DBCO-NHS reagent by re-extraction with dichloromethane. Freeze-drying of the solution furnished the NHS ester **3d** containing 15–30% of the corresponding acid, as a result of the hydrolysis. Triethyl amine and sodium bicarbonate in dilute aqueous solutions completely hydrolyze **3d** in 1 h at room temperature. The new NHS ester (**3d**) and the corresponding carboxylic acid **10** are remarkable for being amphipilic. Indeed, this compound (**10**) has the unique combination of a polar ionizable groups ($OP(O)(OH)_2$), and a relatively unpolar (hetero)aromatic core (fluorophore and two benzene rings fused with a 1,2,3-triazole moiety). As expected, its net charge and distribution between organic solvents and water depend on pH. This behavior is well-illustrated by Figure 2. One can see dye **10** distributed between dichloromethane and water with the pH gradually increasing from 2 to 7.4. Bases ($NaHCO_3$, Et_3N) and even PBS buffer (pH 7.4) ionize this compound and keep it predominantly in the aqueous phase (the upper layer in Figure 2). In the acetate buffer or free acetic acid, comparable amounts of the dye are present in both phases (the lower the pH, the more lipophilic the dye becomes). Further, addition of a stronger acid (5–10 equiv of CF_3CO_2H in dilute solutions) fully suppresses the ionization of **10** in water and makes it completely extractable by dichloromethane (the lower layer in Figure 2). Importantly, the hydrolysis of NHS ester **3d** is suppressed already in slightly acidic media. Dye **3c** (Figure 1 and Scheme 3), in contrast, does not have a bulky lipophilic linker and remains almost exclusively in aqueous phase even when acidified with trifluoroacetic acid to pH 2 (in 0.1–0.2 M solution). It is noteworthy that the bulky and complex linker that comprises more than 30% of the molecular mass of **10**, does not affect the imaging performance of this dye (Figure 4). According to the data in Table 1, despite the huge linker in dye **10**, its fluorescence quantum yields (in the free and conjugated states) are only slightly lower than these of the analogues (**1a**, **3b**,**c**). Another interesting feature of dye **10** is that the triazole cycle conjugated with two benzene rings could act as a built-in triplet quencher and increase the photostability. The presence of triazole cycle conjugated with two benzene rings did not significantly change the lifetimes of the excited state found for the free dye **10** and its conjugates with antibodies (relative to dye **1b** ($n=2$) and its conjugates).[3d] For free dyes **10** and **1b** in solution, the lifetimes were found to be 3.9 and 3.6 ns, respectively. Conjugates with secondary antibodies on vimentin displayed values of 2.4 and 3.6 ns for **10** and **1b**, respectively.

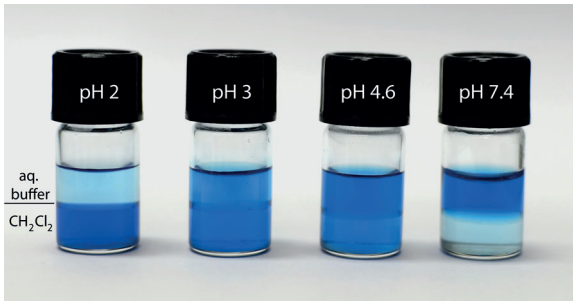
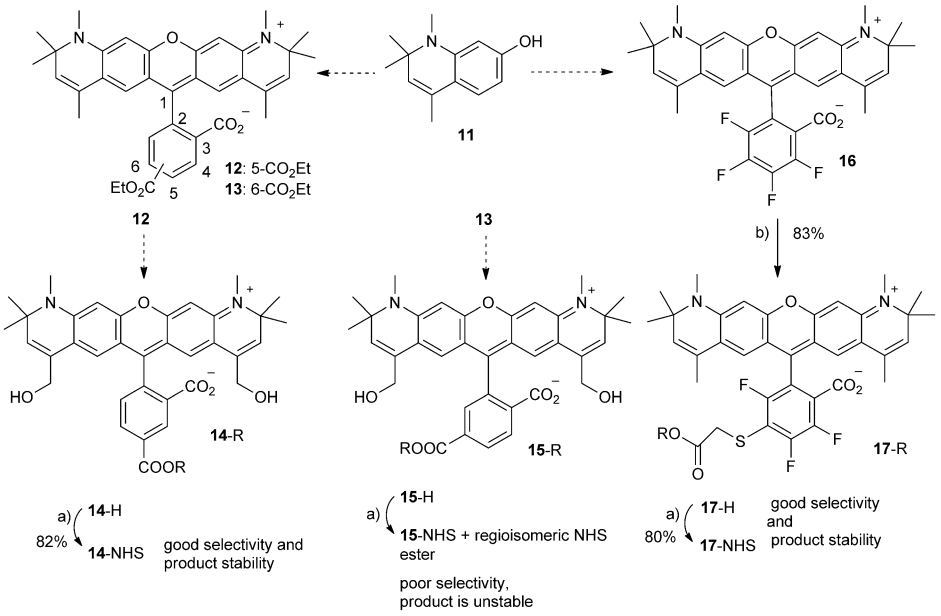


Figure 2. Distribution of the amphiphilic dye **10** (Scheme 4) between an aqueous phase (upper layer) and an organic solvent (dichloromethane, lower phase) as a function of pH. The picture was taken in an equilibrium between equal volumes (1 mL) of a 0.1 mM solution of dye **10** and 0.1 M solutions of the following compounds (left to right): trifluoroacetic acid (pH 2), acetic acid (pH 3), acetate buffer (pH 4.6), and PBS buffer (1:10; pH 7.4). Lipophilic properties increase at lower pH values.

The same type of click reaction can be extended to a wide variety of other dyes. The hydrolytic stability of any click compound of this type can be improved by modifying the amino reactive site; particularly, by the use of mixed NHS carbonates (see above). Using the same structural features, the click reaction product can be isolated in the pure state by simple extraction, without chromatography.

Selective activation of the remote carboxyl group in dicarboxylated rhodamines with two hydroxyl groups: In our previous studies, various rhodamines with multiple and/or diverse functional groups were explored.[1j,k, 3a, 8a] Scheme 5 illustrates two approaches to obtaining rhodamine dyes that have two carboxyl groups. One route utilizes trimellitic anhydride (or its esters) in the condensation step, whereas the other involves tetrafluorophthalic anhydride followed by the aromatic nucleophilic substitution of one fluorine atom in compound **16** with a thiol (or amine) bearing a carboxyl group. Both types of functional groups (carboxyl and hydroxyl) increase solubility in water and polarity to a comparable extent. Relative to non-modified lipophilic dyes, hydrophilic derivatives display higher brightness, contrast and absence of fluorescent background, which may be observed due to unspecific labeling.[3b] Compounds with two hydroxyl and two carboxyl groups are particularly interesting for several reasons. The remote, sterically unhindered carboxyl group at C-5 or C-6 (Scheme 5) is required for bioconjugation, whereas other groups improve hydrophilic properties as well as prevent aggregation of the dye residues and formation of nonemitting dimers. Compared with the negatively charged carboxylic, sulfonic or phosphoric acid residues, hydroxyl groups are advantageous with regard to cell permeability, because anionic residues are known to inhibit the penetration of dyes and their conjugates through the outer plasma membrane of living cells. However, we had first to test if it was possible to selectively activate one of the two carboxyl functions in the presence of free hydroxyl groups, or if it would cause complications. As a rule, a rhodamine dye with two CO_2H groups in the aromatic ring can be selectively converted to a mono-NHS ester.[1j,k] The selectivity is due not only to the steric hindrance at position-2 in the aromatic ring, but also to



Scheme 5. Red-emitting rhodamine dyes with two carboxylic acid residues and two hydroxyl groups: a) HATU, *N*-hydroxysuccinimide (HOSu), Et_3N, MeCN; b) $HSCH_2COOH$, Et_3N, MeCN. For complete set of reagents and conditions see the Supporting Information.

the presence of the closed lactone form of the dye, which has only one carboxyl group and may react faster than the zwitterionic open form.

As illustrated by Scheme 5, one precursor (**11**) gave rise to two types of rhodamine dyes with various positions of absorbance and emission maxima (Table 1). Each of the three dyes (**14**-H, **15**-H, and **17**-H) has two carboxyl groups, and diastereomers **14**-H and **15**-H additionally have two hydroxyl groups. This combination of the functional groups (two OH and two CO_2H groups) in fluorescent dyes has not been reported. The objective of this part of the work was to prepare amino reactive forms of dyes **14**-H and/or **15**-H, obtain bioconjugates and evaluate their applicability in immunofluorescence and STED microscopy.

The precursors to hydroxylated rhodamines **14**-H/**15**-H, dyes **12** and **13**, were obtained as a 1:1 mixture of two diastereomers upon condensation of phenol **11** with 4-ethoxycarbonyl phthalic anhydride. The hydroxylation of the dyes at the allylic sites was achieved analogously to dye precursor **2b** (Figure 1).[3a] The syntheses involved oxidation of methyl groups with SeO_2, followed by one-pot reduction of the aldehydes (formed as byproducts) with sodium borohydride. However, unlike compound **2b**, in which the benzene ring has four fluorine substituents, dyes **14**-H and **15**-H were completely reduced to their leuco forms, which had to be reoxidized (see the Supporting Information). Hydroxyl-substituted dyes **14**-H and **15**-H are well-soluble in water and, if necessary, can be phosphorylated.[4] Isomeric acids **14**-H and **15**-H behave differently in the activation reaction. Only 5-isomer (**14**-H) reacted quite selectively with *N*-hydroxysuccimide in the presence of HATU to furnish monosubstituted NHS ester **14**-NHS. However, disubstituted NHS ester was detected as side product. The active ester **14**-NHS proved to be stable in aqueous media and thus can be used for preparing bioconjugates. On the other hand, 6-isomer (**15**-H) forms significant amounts of the disubstituted NHS ester, even when 1 equivalent of HATU or TSTU is added in small portions at low temperature. Moreover, the pure monosubstituted NHS ester (**15**-NHS) isolated by column chromatography undergoes disproportionation upon concentration of the fractions (even under mild conditions). Thus, compound **15**-NHS turned out to be unsuitable for practical use. Fortunately, both diastereomers **14**-H and **15**-H can be cleanly amidated using conventional peptide coupling reagents (e.g., HATU or EDC).[9]

Compound **17**-H represents another rhodamine with two carboxyl groups. Recently, we reported on the reactivity at the fluorine sites in tetrafluorophenyl substituted rhodamines.[3a,8a] Using this approach, we managed to exchange one fluorine atom in the fluorinated dye **16** to a thioglycolic acid residue (SCH_2COOH). In this case, the target compound was a dye with an absorption maximum at around 610 nm; *between* the absorption maxima of **1a/1b/10** (637 nm) and **14**-H/**15**-H (594 nm; Figure 3). This feature was

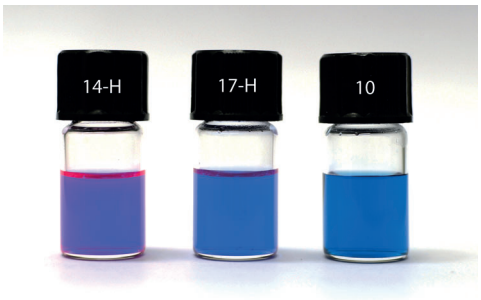
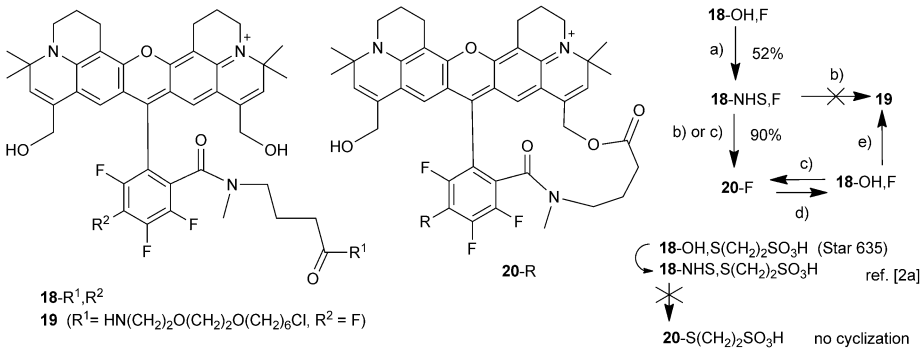


Figure 3. Dyes **14**-H, **17**-H, and **10** as 0.1 mM aqueous solutions. The absorption and fluorescence maxima are gradually shifted to the red spectral region (with an increment of ca. 25 nm; see Table 1). The emission of dye **10** is not seen in daylight (see also Figures 1 and 2). Dye **14**-H was applied in two-color STED nanoscopy (with dye **3c**; see text).

supposed to provide a third color dye that might also be used in multicolor STED nanoscopy.[13] In DMF, the acid **17**-H exists predominantly in the closed (lactone) form, and that probably explains why the mono-NHS ester **17**-NHS is formed selectively, despite the excess of *N*-hydroxysuccinimide and HATU reagent. The product stability is also quite satisfactory. Another observation also confirms the enhanced reactivity of the remote carboxyl group: it easily undergoes esterification already upon

standing in methanolic solutions (to form compound **17**-Me, as established by HPLC and mass spectrometry).

The properties of these dyes are given in Table 1. Importantly, none of the two NHS esters **14**-NHS and **17**-NHS changed upon storage for more than one month at $+5\,^{\circ}C$. Compound **14**-NHS has *free (unprotected) hydroxyl groups*, which may undergo intermolecular esterification, and this was not the case. However, *intramolecular* cyclization was observed for compounds **18**-OH,F and **18**-NHS,F (Scheme 6).[3a] The active ester



Scheme 6. Reactivity of a dye with a linker and free hydroxyalkyl groups (**18**-OH,F): a) HOSu, Et_3N, HATU, MeCN; b) Et_3N, HaloTag® amine [$H_2N(CH_2)_2O(CH_2)_2O(CH_2)_6Cl$]; c) RT, chromatography; d) diluted aq. NaOH; e) $H_2N(CH_2)_2O(CH_2)_2O(CH_2)_6Cl$, HATU, Et_3N.

18-NHS,F is relatively stable after chromatographic isolation, and was used for labeling.[3a] During column chromatography and especially upon concentrating the solutions, we unexpectedly detected the formation of an impurity which turned out to be macrocyclic ester **20**-F. The same product was formed from carboxylic acid **18**-OH,F upon prolonged storage in solutions or upon heating. However, diluted alkalis rapidly hydrolyzed lactone **20**-F back to the starting material **18**-OH,F (without affecting aromatic fluorine substituents).

Also unexpectedly, using NHS ester **18**-NHS,F we failed to obtain amide **19** (from HaloTag® amine). The intramolecular macrocyclization prevailed, and we had to use free acid **18**-OH,F, coupling reagent (HATU), the corresponding amine and a base. The slow addition of HATU to the reaction mixture furnished the desired amide **19**. In our previous study, dye **18**-OH,$S(CH_2)_2SO_3H$ (Abberior STAR 635) was reported, in which one fluorine atom in the benzene ring is substituted by 2-mercaptoethyl sulfonic acid residue [$S(CH_2)_2SO_3H$].[3a] This substitution pattern makes rhodamine dyes water soluble and provides a zero net charge in the dye conjugates. Remarkably, dye **18**-OH,$S(CH_2)_2SO_3H$ and its NHS ester did not cyclize at all (though they possess free hydroxyl groups): coupling with amines always proceeds smoothly, whereas the amino reactive marker **18**-NHS,$S(CH_2)_2SO_3H$ is exceptionally stable and easy to prepare. It is not clear, why the anion-providing substituent ($S(CH_2)_2SO_3H$) makes the cyclization unfavorable. Finally, it is worth mentioning that compound **14**-NHS (Scheme 5), also having two hydroxyl groups but without any linker, smoothly reacts with amines, and free acids **14**-H and **15**-H (both diastereomers) to undergo normal amidation reactions with good selectivity (in favor of the remote carboxyl group).[9] Remarkably, we did not observe the formation of macrocyclic lactones from compounds **14**-H and **15**-H (for steric reasons, it is possible only for compound **15**-H).

In Table 1 one can see that the hydrolitic stability of conventional NHS esters and NHS carbonates correlates with the net charge of the dye, as it was observed previously.[3a] The stability in PBS buffer with pH 7.4 is the same as in water. In aqueous $NaHCO_3$ of Et_3N solutions at pH 8–9, the stability of amino reactive derivatives prepared from dyes **1a**, **3b**,**c** and **10** is lower than the stability of amino reactive compounds obtained from dyes **3c**, **14**-H and **17**-H. Dye probes with a zero net charge prove to be most stable. This is consistent with the data on other dye derivatives. For example, apart from the dye Abberior Star 635 (Scheme 6), fairly good hydrolytic stability was observed for the NHS ester of a carbopyronine dye with one sulfonic acid group[3d] and for a carborhodole hybrid dye with a neutral (uncharged) linker.[14] Having different dye cores, linkers, and/or solubilizing residues, all three NHS esters have only one common feature, namely a zero net charge.

Single- and two-color STED nanoscopy

The suitability of the dyes for imaging and the scope of application as fluorescent markers depends on their excitation and emission wavelengths, photostability under excitation and STED light, fluorescence quantum yields, and degrees of labeling (DOL) in conjugates. Of similar importance are the intersystem crossing rates, the excited state lifetimes, and the percentage of unspecific labeling. For two-color imaging, it is also necessary to have at least two dyes that can be distinguished by their absorption and/or emission spectra, or fluorescence lifetime. STED nanoscopy with red-emitting dyes typically utilizes laser radiation between 750 and 775 nm that prohibits the occupation of the dye's fluorescent state by instant de-excitation through stimulated emission. Therefore, the dyes need to have a sizeable cross-section for stimulated emission at 750–775 nm. For xanthenes, carbopyronines and oxazines, this condition is fulfilled because the red edge of their emission band is broad. In fact, all dyes from Table 1, have a substantial probability of undergoing stimulated emission when exposed to 750–775 nm light, as well as STED when the light is intense enough. Therefore, other parameters, such brightness (product of the fluorescence quantum yield and DOL) and the fluorescence background level (due to unspecific staining) can become equally or even more important.

First, we tested dyes from Table 1 in single-color STED nanoscopy. To this end, we labeled the nuclear pore complex (NPC) and the tubulin cytoskeleton of mammalian cells by indirect

immunofluorescence using primary antibodies against some central NPC subunits and β-tubulin, respectively. These primary antibodies were visualized using secondary antibodies that had been coupled to compounds **3a**–**d** and the spectrally similar reference dyes Atto647N, KK114, and Abberior Star 635P. The images obtained under STED conditions are shown in Figure 4 and Figure S1 in the Supporting Information. All tested compounds performed very well in terms of staining specificity, low background, photostability, and resolution.

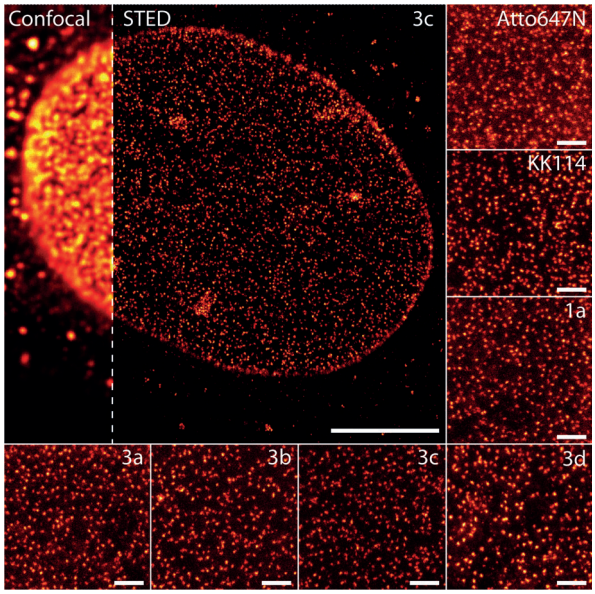


Figure 4. Single-color STED nanoscopy. The performance of the different compounds for imaging was tested by indirect immunolabeling of the central subunits of the nuclear pore complex in fixed HeLa cells and single-color channel STED nanoscopy was performed. Scale bars: 5 μm (overview), 1000 nm (close up).

For dual-color STED nanoscopy we chose compound **14**-H, because its absorption and emission properties promised spectral separation (Table 1). To test the performance of this compound, the central NPC subunits were labeled by indirect immunofluorescence using secondary antibodies coupled with dye **14**-H and the reference dyes (Abberior Star 580, Atto590, AlexaFluor 594 and Atto594). In confocal and STED nanoscopy (Figure S2 in the Supporting Information), the performance of compound **14**-H was similar to the commercial rhodamine derivatives used for comparison. However, its advantage over these compounds is that the dye residue in **14**-H has no negatively charged groups (like sulfonated dyes AlexaFluor 594 and Atto594), but at the same time, rhodamine **14**-H is by far more hydrophilic than Abberior Star580 and Atto590 dyes. The dyes **14**-H (zero charge in conjugates), Star 580 and Atto590 (positive charge in conjugates) are cell-permeable. However, due to their lower hydrophilicity, Abberior Star 580 and Atto590 dyes are less versatile as fluorescent markers than compound **14**-H, because their suitability for microscopy may be compromised if they bind to cellular structures (proteins or certain compartments). In this respect, the hydrophilic compounds are advantageous. They possess polar (ionic) groups, which provide high solubility in aqueous buffers and hence better bioavailability. In particular, they prevent aggregation and self-quenching of the dye residues, reduce the unspecific binding, and increase the fluorescence quantum yields.[3–6] Next we tested whether the photophysical properties of the selected dyes in biological samples fit to the parameters of the two-color STED microscope, which we planned to use.[1l] Therefore, the cells were labeled with a single fluorophore and imaged using the two-color STED setup (Figure S3 in the Supporting Information). The excitation and detection wavelength pairs for the two channels were 640 and 670(±20) nm, as well as 595 and 620(±20) nm.

STED was performed with a single wavelength at 775 nm (Figure 5a). We found that all dyes met the wavelength conditions of the used setup. Fortunately, spectral crosstalk was low.

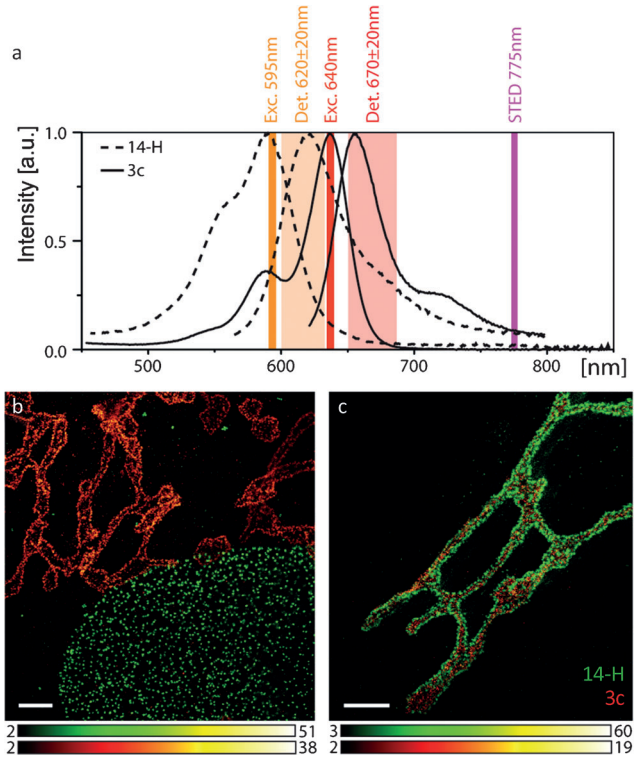


Figure 5. Two-color STED nanoscopy. a) Spectral rationale of the setup used, overlaid with the spectra of dyes **14**-H and **3c**. b) Cellular nanoscopy of giantin (**3c**) and NPC (**14**-H). c) Cellular nanoscopy of giantin (**14**-H) and GM130 (**3c**). Single-color channels (for b and c) are shown in Figure S4 in the Supporting Information. Scale bars: 2 μm.

These parameters enabled direct separation of two color channels. We note that the detection windows were kept narrow. Nevertheless, the high brightness of the fluorescent labels afforded bright images in both channels. The absence of crosstalk is particularly important because it provides an additional degree of freedom and enables two-color imaging and co-localization of biological objects without requiring mathematical unmixing of the acquired raw data. Unmixing typically requires supplementary experiments, complicating the imaging process.

We chose the compounds **3c** and **14**-H (Table 1) for two-color STED nanoscopy (Figure 5 and Figure S4 in the Supporting Information). To this end cellular structures were labeled using these dyes. In a first example, the Golgi membrane protein giantin and the NPC were labeled by indirect immunofluorescence. In a second example two markers at the Golgi apparatus were labeled. The imaging results are illustrated in Figure 5 presenting images of the nuclear pore complex and the Golgi apparatus in human fibroblast cells. As can be seen, the protein giantin is localized in clusters at the membrane of the Golgi apparatus (Figure 5b and c, red and green channels, respectively). The second Golgi marker, the matrix protein GM130, is localized in the interior of the giantin labeled structure (Figure 5c, red channel). The imaging results obtained with dye **3c** are comparable to the imaging performance of **14**-H: both dyes are bright and provide an excellent signal-to-noise ratio with low spectral crosstalk or background staining (Figure 5 and Figure S4 in the Supporting Information).

Under these conditions, an optical resolution of 36 nm (**14**-H) and 44 nm (**3c**) was achieved (Figure S5 in the Supporting Information). Taking into account that dye **3c** has the same fluorophore as reference dyes KK114 or Abberior Star635, which are widely used in immunofluorescence assays,[3] we may conclude that the new marker **14**-H represents a valuable addition to the well-established red-emitting fluorophores applicable to low-cross-talk multicolor confocal and STED nanoscopy.

Conclusion and Outlook

The problem of selective activation of a fluorescent dye with unprotected phosphate groups can be solved by subjecting a dye precursor with an azide linker to a click reaction with a proper alkyne reagent that bears a reactive group. Here we showed that the phosphate groups in a dye are unaffected. This approach may be of general importance for the chemistry of organic dyes and most likely for other labeling reagents with solubilizing groups. If the whole molecule bears a bulky linker and/or additional aromatic rings, the presence of two phosphate groups results in an amphiphilic compound. However, it was also shown that phosphate groups are able to efficiently solubilize very lipophilic and heavy dye cores with aliphatic and aromatic linkers, in which case the photophysical and imaging properties remain unaffected. Remarkably, the distribution of these dyes between organic solvents and water can be controlled by pH, and this property makes isolation/purification of the reactive dye marker very simple.

An alternative way to introduce an amino-reactive group in the presence of unprotected phosphate was studied. It involved the preparation of mixed NHS carbonates. First of all, we prepared the mixed NHS carbonate from a dye with two sulfonic acid groups. The resulting amino-reactive compound (**3a**, Scheme 1) is perfectly soluble in water, stable thermally and hydrolytically, even under basic conditions. The marker has all other useful properties of the known fluorescent dye KK114 (**1b**, Figure 1), the NHS ester of which is only moderately stable. We can assume that in the long run, the mixed NHS carbonates with good hydrolytic stability, yet sufficient amino reactivity, can replace unstable NHS esters of popular fluorescent dyes (e.g., AlexaFluor 488). Mixed NHS carbonates are easily prepared from hydroxyl substituted dye derivatives (alcohols). However, this approach has limitations in the case of dyes with primary phosphate groups. The latter proved to be good enough as nucleophiles in the reaction with *N,N'*-disuccinimidyl carbonate (DSC). In the presence of tertiary amines, primary phosphates react with DSC to form mono-NHS esters of alkyl phosphoric acids. In line with this observation, we found that the base-assisted migration of the NHS function to the phosphate residue from the carboxyl site occurs in the case of NHS ester (**3b**, Scheme 3). The product of P-esterification, a dye bearing two NHS residues at the phosphate sites and at the carboxyl group (**3c**), was isolated and explored. Remarkably, its imaging performance and photophysical properties proved to be as good as those of the red-emitting dyes with free primary phosphate or sulfonic acid groups (e.g., KK114 or dye **1a** (Abberior Star 635P) with two free primary phosphate groups). Interestingly, having a lower net charge than **3b**, the amino reactive marker **3c** is perfectly hydrolytically stable. The NHS moiety at the phosphate sites was shown not to react with amines under mild conditions, which diminishes the undesired possibility of cross-linking (e.g., in proteins). The alternative synthesis of **3b**, involving the preparation of the mixed NHS carbonate with *tert*-butyl phosphate groups followed by cleavage of the protecting groups under acidic conditions proved to be unfeasible due to the instability of NHS carbonates in acidic media (concentrated aqueous solutions of CF_3COOH were required in the final step).

In the course of this study, we developed an improved and short synthesis of a red-emitting rhodamine with two primary phosphate groups (**1a**) which was obtained previously. The new synthesis utilizes a phosphorylated precursor with phosphate groups protected by formation of *tert*-butyl esters. This precursor is stable in basic media and can be converted to other useful dyes with azide, hydroxyl, and NHS carbonate functions. The new synthesis is high-yielding, avoids purification or isolation of most intermediates, and furnishes a phosphorylated dye free from silica gel, mineral salts and heavy metals.

Selected examples of other dyes with various polar functional groups were explored (**14**-H and **17**-H, Scheme 5). They always contained two carboxyl groups and (sometimes) two additional hydroxyl groups. Both groups increase polarity, enhance hydrophilic properties, and are important for dye chemistry with regard to further modifications and imaging performance. We demonstrated that it is possible selectively to convert the remote carboxyl group to NHS ester, which turned out to be stable under these conditions and was used in bioconjugation, followed by imaging; there was no need to use mixed carbonates, as in the case of phosphates. Hydroxy groups are advantageous in providing the hydrophilic properties, because they do not affect the net charge of a dye. Some limitations of this activation procedure were encountered with substrates containing additional CO_2H and/or OH groups: we observed the formation of disubstituted NHS esters, the dis-

proportion of the monosubstituted esters, and the cyclization to a macrocyclic lactone (**20**-F, Scheme 6).

This approach allowed us to obtain bright and photostable red-emitting rhodamines for two-color STED nanoscopy of biological samples. The fluorescence images with high contrast and minimal background were obtained for a pair of new dyes with absorption/emission bands at 579/609 and 635/655 nm. Subdiffraction far-field optical resolution of approximately 40 nm and a relatively cross-talk-free color separation into two observation channels with negligible background signal was obtained, even in the case of relatively broad spectral bands with emission and absorption maxima separated by 45 and 55 nm, respectively. In fact, these results should enable three-color STED microscopy, which involves a pair of dyes with small Stokes shifts (from the present study) and an additional marker with a large Stokes shift, for example, a red-emitting coumarin dye.[4]

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