Phosphorylated 3-Heteroarylcoumarins and Their Use in Fluorescence Microscopy and Nanoscopy

Shamil Nizamov, Katrin I. Willig, Maksim V. Sednev, Vladimir N. Belov,* and Stefan W. Hell*[a]

Abstract: Photostable and bright fluorescent dyes with large Stokes shifts are widely used as markers in far-field optical microscopy, but the variety of useful dyes is limited. The present study introduces new 3-heteroaryl coumarins decorated with a primary phosphate group ($OP(O)(OH)_2$) attached to C-4 in 2,2,4-trimethyl-1,2-dihydroquinoline fragment fused with the coumarin fluorophore. The general synthetic route is based on the Suzuki reaction of 3-bromocoumarines with hetarylboronic acids followed by oxidation of the methyl group at the C=C bond with SeO_2 (to an aldehyde), reduction with $NaBH_4$ (to an alcohol), and conversion into a primary phosphate. The 4 position in the coumarin system may be unsubstituted or bear a methyl group. Phosphorylated coumarins were found to have high fluorescence quantum yields in the free state and after conjugation with proteins (in aqueous buffers). In super-resolution light microscopy with stimulated emission depletion (STED), the new coumarin dyes provide an optical resolution of 40–60 nm with a low background signal. Due to their large Stokes shifts and high photostability, phosphorylated coumarins enable to combine multilabel imaging (using one detector and several excitation sources) with diffraction unlimited optical resolution.

Keywords: bioconjugation · coumarins · dyes/pigments · fluorescence · synthetic methods

Introduction

Photostable and bright fluorescent dyes with large Stokes shifts are rare and only a few are commercially available. Nearly all these dyes contain the coumarin fragment as the fluorophore. For example, "Mega Stokes" dyes from the Dyomics company are coumarins absorbing at about 500–520 nm, and emitting in the region of 590–670 nm (in ethanol). Another practically useful coumarin dye is AlexaFluor 430 with absorption and emission maxima at 434 and 539 nm, respectively (Figure 1). Many other suppliers (e. g., Invitrogen, BD Biosciences, Kodak, etc.) offer fluorescent dyes and their conjugates with large Stokes shifts, but they do not publish their structures. The lack of essential structural data hinders the proper choice of the fluorescent dye for a particular biochemical application (for example, due to the indefinite hydrophilicity, unknown net electrical charge of the molecule, and potential (photo)toxicity). Unavailable structures of the existing dyes also hamper the progress in basic research aimed at the creation of new fluorophores. This is particularly true for the coumarins, whose common features are moderate photostability and relatively low fluorescence quantum yields in polar solvents. The lack of brightness and low photoresistance are the main drawbacks that preclude their wide use in modern methods of optical "nanoscopy".[1]

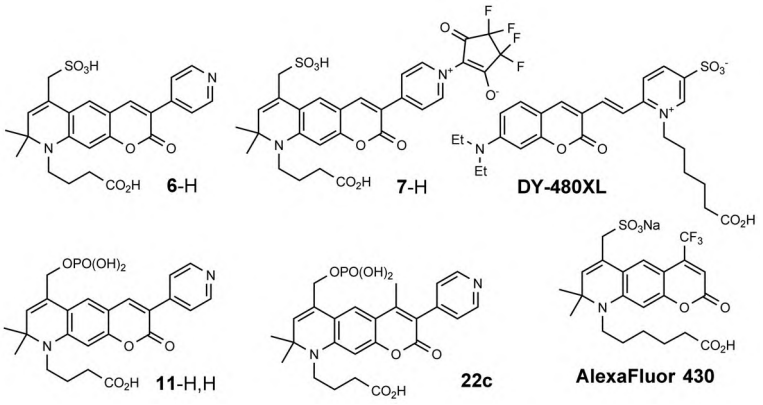

Figure 1. Examples of the new compounds and commercially available coumarin dyes. For complete data on the spectral properties of all coumarin dyes of the present study, see Table 1.

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These new super-resolution methods of the far-field optical microscopy require fluorescent dyes which demonstrate all advantages and full scope of the new physical concepts. Therefore, the synthesis of coumarins emitting in the green and red spectral regions and the optimization of their photostabilty, quantum yields, solubility in aqueous buffers, and chemical reactivity with various biologically relevant targets is an important research field.[2]

For example, chemoselective labeling became possible with "clickable" 3-{2-[*N*-(3-azidopropyl)-2-sulfopyrid-4-yl]-ethenyl}-7-diethylamino coumarins,[2d,e] presenting an important addition to red-emitting fluorescent dyes with large Stokes shifts possessing the carboxylic acid group as a reactive site (e.g., to DY-480XL in Figure 1). The aldehyde group at C3 in coumarin enables the formation of hybrids between coumarin and cyanine dyes.[2f] These new far-red-emitting fluorophores with large Stokes shifts are based on 7-hydroxycoumarins. They were decorated with hydrophilic residues and quenching groups (attached to the phenolic hydroxyl) which may be cleaved-off by hydrolytic enzymes, thus probing the activity of these highly efficient bio-catalysts. A similar approach was used in the course of creating the fluorimetric sensors for other enzymes or physiologically active thiols, which trigger the (cascade) reactions and liberate the free hydroxyl group at C7 (responsible for fluorescence).[2g–j]

Results and Discussion

Motivation of the work and key structures of the new coumarins with large Stokes shifts: A very important feature of a fluorescent dye is the Stokes shift (separation between the absorption and emission maxima measured in nm or cm^{-1}). Fluorescent dyes with large Stokes shifts can be used either alone, or together with emitters possessing small Stokes shifts in various imaging techniques. For example, a pair of dyes emitting approximately at the same wavelength with well separated absorption bands may be used for labeling, detection and co-localization of two different (biological) targets. A great advantage of this approach is that only one detection channel is used. The "cross-talk" observed in the course of the excitation with two different light sources (lasers) has to be low. As mentioned, the chemical structures of many commercially available fluorescent dyes with large Stokes shifts are unknown. For example, the structures of Pacific Orange (abs. 390 nm, emission 540 nm; Invitrogen) and BD Horizon V500 (abs. 415 nm, emission 500 nm; BD Biosciences) have not been disclosed. Despite the very long history and wide use of coumarin dyes, their structure–property relationships have not been studied in depth.[2,3] Large Stokes shifts in coumarins are provided by using intramolecular charge transfer (ICT) and excited state intramolecular proton transfer (ESIPT) mechanisms.[4] In the present study, we relied on the first mechanism. High fluorescence quantum yields and photoresistance were provided by using planar and rigid molecular frameworks with additional substituents at the "sensitive" positions susceptible to (photo) oxidation.

Water is the preferred solvent for operating with the reactive fluorescent dyes, because the conjugation reactions involving biologically relevant macromolecules (e. g. proteins, nucleic acids, carbohydrates) have to be performed in water or aqueous buffers. A chemically reactive marker is usually dissolved in an organic solvent, such as DMF or DMSO, and then added to the aqueous solution of the substrate. High concentrations of an organic solvent may cause protein denaturation, and should be avoided. On the other hand, a low coupling efficiency and/or unspecific binding with a target may be observed if the amount of the organic solvent is too low, and the dye precipitates from the solution. Water-soluble fluorescent markers are advantageous in this regard, because they do not require any organic solvents at all. Moreover, hydrophilic labels are less prone to aggregation and to non-specific binding with biological objects, especially membranes.

The synthesis and properties of hydrophilic coumarins decorated with sulfonic acid groups have been disclosed.[5a,b] An introduction of the sulfonic acid residue into 4-methyl group of 2,2,4-trimethyl-1,2-dihydroquinolines (precursors to hydrophilic coumarins with large Stokes shifts) is performed in concentrated sulfuric acid. Although these harsh conditions provide the required hydrophilic compounds (e.g., AlexaFluor 430 in Figure 1), they are incompatible with several important functional groups that do not tolerate concentrated sulfuric acid (e.g., *tert*-butyl esters, diazo groups, etc.) and therefore seriously limit the "synthesis freedom". In this respect, the synthesis route to the phosphorylated coumarins presented here is advantageous, because it provides the hydrophilic dyes prepared under mild conditions.

Phosphoric acid represents a viable alternative to sulfonic acid as a source of negatively charged groups preventing the aggregation of the dye residues, self-quenching of the fluorescence signal and unspecific binding with liphophilic substrates. Indeed, this acid has medium strength, an increased number of acidic protons (relative to sulfuric acid), and its primary and secondary alkyl esters are hydrolytically stable at pH 2–12.[5c] The BODIPY dyes with two dialkyl phosphate residues have been reported.[5d] Several fluorescent dyes decorated with phosphonic acid residues were prepared.[5e–i] As ionisable groups, primary phosphates have an advantage over primary phosphonates because they are derived from a 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,[5j] 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.[5k] Therefore, at physiological pH of 7.3–7.4, fluorescent dyes with primary phosphate groups will be ionized much better (and their aggregation will be suppressed more efficiently). If they will be analyzed by HPLC or gel electrophoresis, in buffer solutions at pH $>$ 8 they are expected to move as single bands or peaks.

Examples of the commercially available coumarin dyes and new compounds of the present study are given in

Figure 1. They all belong to the family of 7-aminocoumarins, with variable substituents in positions 3 and 4. The electron-acceptor group at position 3 with an extended π-system (e.g., *N*-alkyl-2-vinylpyridinium salt with a negative inductive ($-M$) effect in compound DY-480XL) strongly shifts the absorption and emission bands to the red spectral region and increases the distance between their maxima. A very strong acceptor at position 4 (e.g., CF_3-group in AlexaFluor 430) with a $-M$ effect also increases the charge separation and the "push–pull" character of the chromophore, and provides similar spectral shifts. Incorporation of the 7-amino group into the 2,2,4-trimethyl-1,2-dihydroquinoline ring provides an additional small redshift of 5–10 nm and creates two variable positions: a methyl group at the double bond and the disubstituted nitrogen atom. These variable positions were used to introduce the sulfonic or phosphoric acid residues and ω-carboxyalkyl groups, respectively. The said transformations do not change the positions of the absorption and emission bands, but they rather provide more hydrophilic compounds with considerably increased fluorescence quantum yields (in the free state and in bioconjugates) and an additional functional group (COOH) that is necessary for conjugation reactions. The structures of dyes **6**-H and **11**-H,H differ in only one respect: compound **6**-H bears a sulfonic acid moiety, whereas **11**-H,H has a primary phosphate group. It was interesting to compare the spectral properties, fluorescence quantum yields (also in bioconjugates) and performance of these dyes in optical microscopy. The betain fragment in the structurally very similar dye **7**-H provides a bathochromic shift of absorption and emission bands, and further increases the Stokes shift, but the fluorescence quantum yield in aqueous solutions decreases drastically (see Table 1). A variation of the substituent at C4 in the coumarin system is a more delicate tool that enables a fine "tuning" of the positions of the absorption bands and further reduce absorption at 488 nm and, as a result, a cross-talk between the dyes excited at this wavelength and with 405 nm laser light.

A detailed discussion and comparison of the spectral properties of all new coumarin dyes prepared in this study is given in a separate section below.

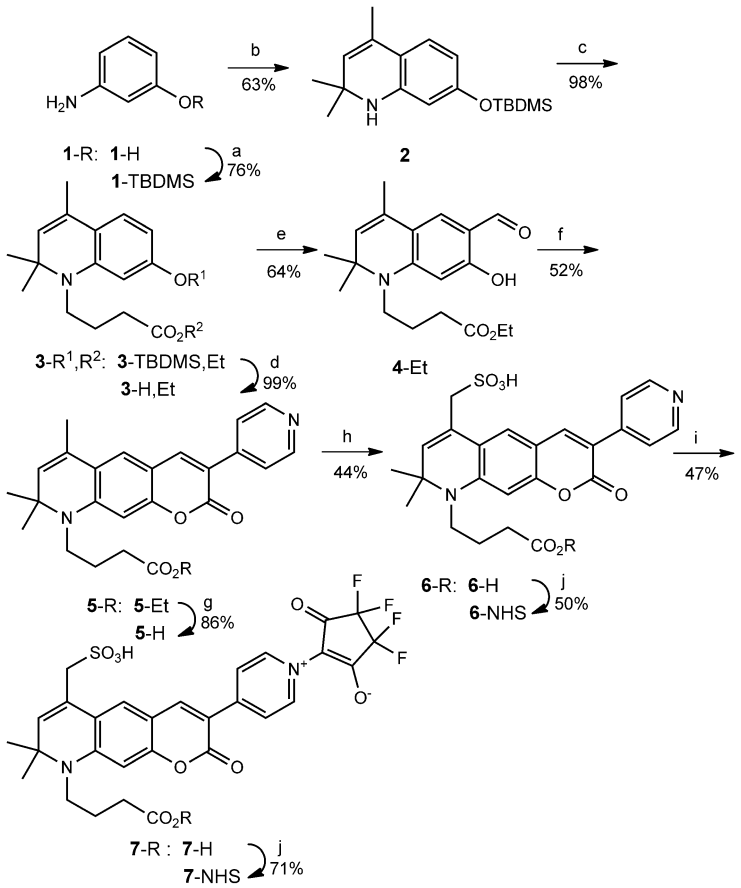
Synthesis of universal precursors: construction of 3-heteroarylcoumarins with hydroxymethyl and primary phosphate groups: The preparation of the starting building blocks **2**–**4** for the synthesis of various coumarines dyes (emitting in the green- and red spectral regions) is given in Scheme 1 and Scheme 2. Selective protection of the hydroxy-group in *m*-aminophenol (**1**-H) was carried out as described in the literature[6] and led to compound **1**-TBDMS. The Skraup–Doebner von Miller reaction of **1**-TBDMS and acetone was performed in the presence of ytterbium(III) triflate[7a] and provided precursor **2** with the 1,2-dihydroquinoline moiety in good yield. At room temperature, this reaction proceeded smoothly and with good regioselectivity. Alkylation of compound **2** with ethyl or *tert*-butyl 3-iodobutyrates in the presence of Hünig's base afforded esters **3**. Compound **3**-TBDMS,Et was used for the preparation of the hydrophilic green-emitting coumarin dye **6**-H decorated with sulfonic acid residue (Scheme 1). Firstly, the hydroxyl group in **3**-TBDMS,Et was deprotected using tetrabutylammonium fluoride, and then the obtained phenol **3**-H,Et was formylated according to Vilsmeier–Haack to give salicylic aldehyde **4**-Et in 64% yield.[7b,c] Next, a coumarin ring was formed by the sequential esterification and Knoevenagel reactions, and the desired compound **5**-Et was obtained in 52% yield.[7d,e] The saponificaton of ethyl ester followed by sulfonation of 4-methyl group in the 2,2,4-trimetyl-1,2-dihydroquinoline fragment provided coumarin **6**-H,[7f] which readily reacted with perfluorocyclopentene and afforded red-emitting betain **7**-H.[8] Overall yields of water-soluble coumarins **6**-H and **7**-H were approximately 12 and 6%, respectively.

The phosphorylated analogues of coumarins **5**-R were prepared as depicted in Scheme 2. Although the N-alkylation of compound **2** with ethyl 4-iodobutanoate proceed very smoothly, under the same conditions, *tert*-butyl 4-iodobutanoate and amine **2** afforded ester **4**-TBDMS,*t*Bu at a lower yield of 60%. The oxidation of the methyl group at the C=C-bond in 1,2-dihydroquinolines **3** (to aldehydes **8**) was achieved using selenium dioxide, but even after optimization, the yields were low (Scheme 2). The low yields of aldehydes **8** may be explained by the susceptibility of C(6)-H

Table 1. Properties of the hydrophilic coumarin dyes in PBS buffer at pH 7.4 and room temperature.

Dye	Absorption λ_{max} [nm]	Emission λ_{max} [nm]	$\varepsilon \times 10^{-5}$ [$M^{-1}cm^{-1}$]	Φ_{fl} [%]	Lifetime τ [ns]	Solubility [mM]	Stability of NHS-ester	DOL[a,b]	Φ_{fl} [%] in conjugates[b]
Alexa Fluor 430[c]	434	541	0.16	55	–	no data	–	3–7[d]	–
6-H[e,f]	432, 444	506, 514	0.1, 0.07[g]	61, 50	–	not measured	–	–	–
6-H	436[h]	515[h]	0.31	57	–	25	good	2.7	17
11-H,H	436[i]	515[i]	0.23	68	3.3[I,b]	20	good	2.6 (6.3)[j]	30 (27)[i]
21c	432	512	0.20	81	3.2	good	good	–	-
22c	410	503	0.14	44	2.7[k]	7	good	2.8	27
7-H	520	639	0.40	0.6	–	low	good	–	-
DY-480XL	504	631	0.50	–	–	no data	–	–	-

[a] Degree of labeling: average amount of the dye residues attached to one antibody molecule with molecular weight (M_w)≈150000. [b] Sheep anti-Mouse secondary antibodies. [c] Data from the web database: www.fluorophores.org. [d] Recommended DOL-values. [e] In MeOH. [f] In H_2O. [g] Deviations from Beer–Lambert law due to the low solubility; extinction is lower than anticipated due to adsorption of the unpolar substance on quartz surface. [h] Conjugates with Sheep anti-Mouse antibodies have absorption and emission maxima at 437 nm and 512 nm, respectively. [i] Conjugates with antibodies have τ = 2.9–3.1 ns; their absorption and emission maxima are at 439 and 513 nm, respectively. [j] Goat anti-Rabbit antibodies. [k] Compound **22a**; 2.2 ns for compound **22d** in antibody conjugates.

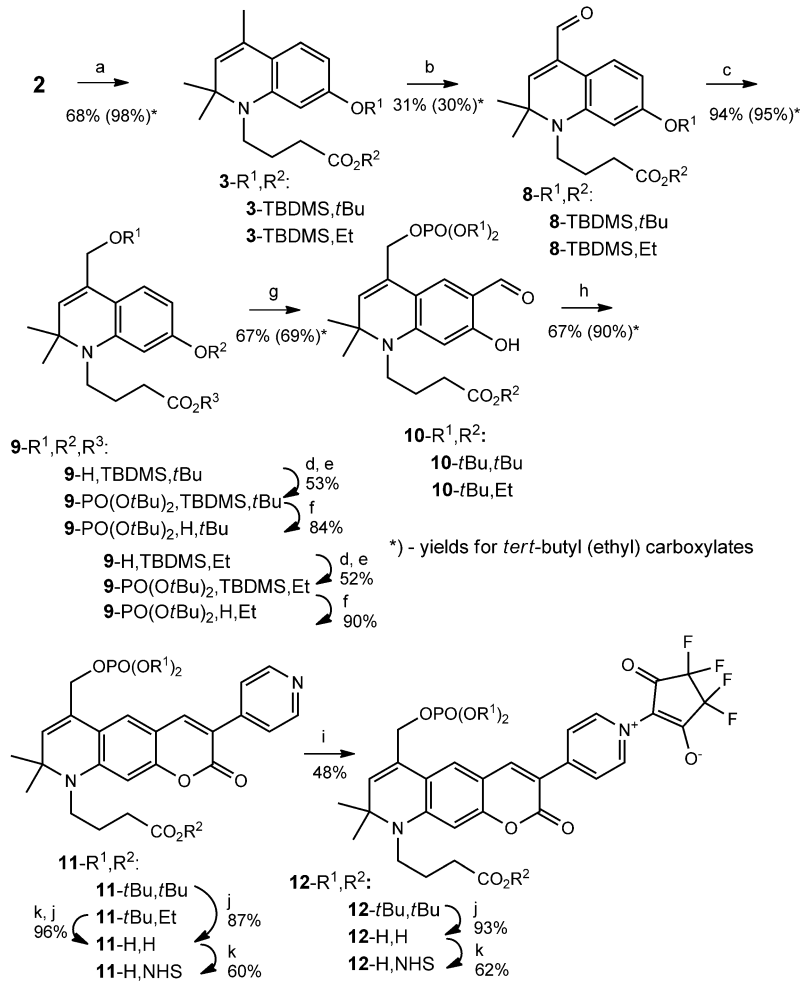


Scheme 1. Synthesis of model green-emitting dye **6**-H and red-emitting coumarin **7**-H decorated with sulfonic acid residue: a) $ClSiMe_2tBu$ (TBDMSCl), imidazole, DMF, 0–55 °C, 2 h; b) acetone, $Yb(OTf)_3$, RT, 16 h; c) $ICH_2CH_2CH_2CO_2Et$, DIEA, 110 °C, 48 h; d) TBAF·$3H_2O$/THF, 0 °C, 5 min; e) $POCl_3$, DMF, 60 °C, 1.5 h; f) 4-pyridylacetic acid hydrochloride, NEt_3, DMAP, CH_2Cl_2, RT, 40 h; g) 1 M aq. NaOH, THF, MeOH, RT, 16 h; h) H_2SO_4, 35 °C, 2 days; i) perfluorocyclopentene, EtOH, aq. AcOH, 70 °C, 2 h; j) *N*-hydroxysuccinimide (HOSu), 2-(7-aza-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), NEt_3, DMF, RT, 16 h.

to oxidation (followed by destruction); higher yields of aldehydes **14**-H,R were obtained under more drastic conditions from coumarines **13**-H,R, which are obviously more resistant to oxidative destruction caused by SeO_2 (see later; Scheme 4). The alcohols **9**-H were obtained by Luche reduction[9a] of aldehydes **8** with $NaBH_4/CeCl_3$. Phosphorylation of alcohols **9**-H,TBDMS,R^3 was performed as described in the literature.[9b,c] Formylation of phenols **9**-PO(O*t*Bu)$_2$,H,R^3 proceeded smoothly and resulted in the target aldehydes **10** with ethyl or *tert*-butyl ester groups (Scheme 2). According to the general approach exemplified in Scheme 1 and 2, the condensation reaction of aromatic aldehydes **4**-Et and **10** with the α-substituted acetic acid derivatives directly provides coumarins.[10] In particular, the reaction of aldehydes **10** with 4-pyridyl acetic acid was performed under mild conditions and gave the phosphorylated coumarins **11** with two protecting groups (Scheme 2). The uniform protection of all acidic centers as *tert*-butyl esters saved one synthetic step later in the synthesis and simplified the isolation of the final dye **11**-H,H (Scheme 2). The overall yields of the phosphorylated coumarins **11** and **12** obtained by this route were 4–8 %.

The compounds with a free carboxylic acid group (e.g., **11**-H,H and **12**-H,H) can be used in various (bio)conjugation procedures. The final activation step, which involved the carboxyl group and produced amino-reactive *N*-hydroxysuccinimidyl esters **7**-NHS (Scheme 1), **11**-H,NHS, and **12**-H,NHS (Scheme 2), as well as **21d** and **22d** (Scheme 4), was performed in DMF using 2-(7-aza-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) in the presence of *N*-hydroxysuccinimide and base (Et_3N). These conditions provided higher yields of the target active esters than the use of other coupling agents (e.g., *O*-(*N*-succinimidyl)-*N,N,N′,N′*-tetramethyl uronium tetrafluoroborate, which gave yields of less than 20 %). Under these conditions, the carboxylic group was activated first, whereas the phosphoric acid residue was kept free (in the absence of the excess of the coupling reagent and *N*-hydroxysuccinimide). At higher conversions, partial activation of the phosphate group was also observed, and di(*N*-succinimidyl) esters were often detected in the reaction mixtures by TLC and HPLC. The isolation and purification of the polar phosphorylated dyes (as carboxylic acids or their *N*-hydroxysuccinimidyl esters) was uniformly performed on silica gel 60 (40–63 μ) using acetonitrile-water mixture as an eluent (e.g., 8:1–4:1), followed by direct lyophylization of the pooled fractions, dissolution of the solid residues in minimal amounts of water or aqueous acetonitrile, filtration from the silica gel, and lyophilization. In the case of carboxylic acids, triethyl amine may be added to the solvent mixture. Methanol has to be avoided, because it may react with *N*-hydroxysuccinimydyl esters, and does not form a solid during freeze-drying (temperature of the cooler is −80 °C).

If the reaction of alcohols **9**-H,R^2,R^3 with (iPr)$_2$NP(O*t*Bu)$_2$ and 1*H*-tetrazole in CH_2Cl_2 was carried out for a long time (20 h at 40 °C), a byproduct (hydrogenphosphonate **9**-PHO(O*t*Bu),TBDMS) was isolated (Scheme 3).[11a,b] Although the phosphonate functionality may be formed using these reagents by various routes,[11b] we consider the

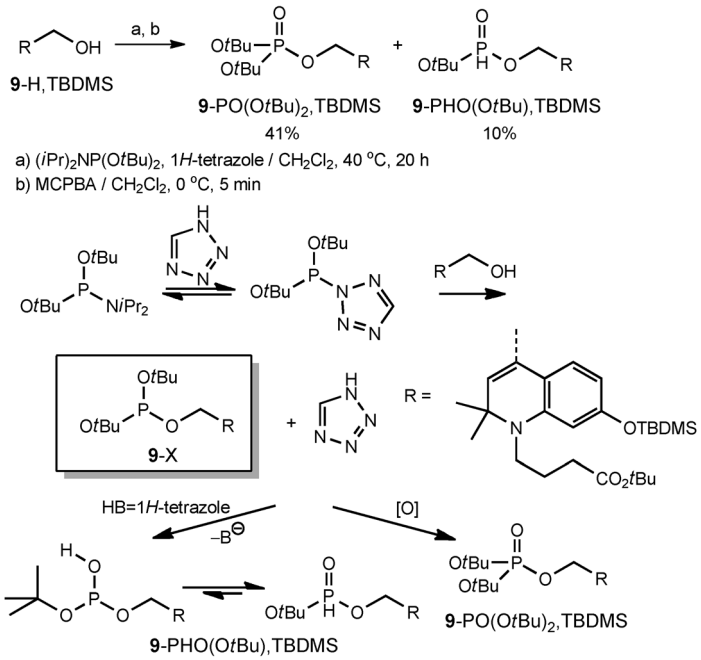


Scheme 2. Synthesis of the hydroxylated precursor **9**-H,TBDMS of phosphorylated coumarins **11** and **12**: a) $ICH_2CH_2CH_2CO_2tBu$, iPr_2NEt, 110 °C, 48 h; b) SeO_2, dioxane, 65 °C, 0.5 h; c) $NaBH_4$, THF, MeOH, 0 °C, 15 min; d) $iPr_2NP(OtBu)_2$, 1*H*-tetrazole, CH_2Cl_2, 40 °C, 1 h; e) *m*CPBA, CH_2Cl_2, 0 °C, 15 min; f) TBAF·$3H_2O$, THF, 0 °C, 5 min; g) $POCl_3$, DMF, 60 °C, 1.5 h; h) 4-pyridylacetic acid hydrochloride, NEt_3, DMAP, CH_2Cl_2, RT, 40 h; i) perfluorocyclopentene/EtOH, aq. AcOH, 70 °C, 2 h; j) TFA, RT, 16 h; k) HOSu, HATU, NEt_3/DMF, RT, 16 h.

acid-catalyzed mono-dealkylation reaction to be the most plausible one (Scheme 3). If the two reagents (1*H*-tetrazole and phosphoroamidite) are used in excess, the first step of this reaction proceeds quickly. Intercepting the trialkylphosphite intermediate **9**-X with an oxidizing agent provides the stable phosphate, whereas the phosphonate is obtained in negligible amount. The acidity of 1*H*-tetrazole ($pK_a = 4.9$) is similar to that of the acetic acid ($pK_a = 4.75$), and, probably, this is the reason why one of the two acid sensitive *tert*-butyl groups is cleaved in the presence of 1*H*-tetrazole.

Using the readily available phenolic compound with 1,2-dihydroquinoline fragment (**3**-H,Et in Scheme 1) as a starting material, we studied various routes leading to 3-heteroaryl coumarins **20** (Scheme 4). They differ according to the method used for creating the coumarin system. The first approach includes the formation of the coumarin ring in the course of the sequential Wittig–Horner and cyclization reactions followed by oxidation of compounds **13**-X,R with SeO_2, bromination of coumarin **14**-X,R with a formyl group, conversion of the corresponding bromides **14**-Br,R into the desired 3-heteroarylcoumarins **18**–**19** by using a Pd catalyst and postsynthetic modifications. The second method is based on the esterification and Knoevenagel condensation reactions using hetaryl acetic acids followed by conversion of the methyl group in 1,2-dihydroquinoline moiety (compounds **5**-Et and **16** and **17**) into the phosphoryl group. The formation of the coumarin systems by a well-known Pechmann condensation is straightforward, albeit low-yielding; it is short and preferable for the synthesis of 4-methylcoumarin derivatives (**13**-X,Me). The highest overall yields were achieved in the following reaction sequence: **3**-H,Et→**4**-Et→**5**-Et/**16**-R/**17**-H→**18**-R/**19**-H→**20**. At the key step, hetaryl acetic acids acylate the phenolic group in compound **4**-Et. The intermediates, 2-formylphenyl acetates, undergo the ring-closure in Knoevenagel reaction producing the coumarin structure. Unfortunately, many hetaryl acetic acids are unstable and lose carbon dioxide.[12a] Therefore, the usefulness of this approach is limited only to commercially available 2-pyridyl and 4-pyridyl derivatives.

We also prepared and isolated pyrazine acetic acid,[12b] which was found to be stable at pH > 3 at room temperature, and was successfully used for the preparation of compound **17**-H. Thus, an alternative general approach to 3-heteroaryl coumarins was developed. It employs a Suzuki reaction of 3-halo coumarins **13**–**15** with hetaryl boronic acids (or a Stille reaction with hetaryl stannanes; see Scheme 4). A wide range of the commercially available hetaryl boronic acids makes this route to be an attractive option. However, if the required aryl- or hetarylacetic acid is stable and can be readily obtained (e.g., by lithiation of the corresponding methyl (het)arene followed by carboxylation), then the first method is superior. The overall yields of the target coumarins obtained by these routes vary in range of 5–35%.



Scheme 3. Monodealkylation of the intermediate trialkylphosphite **9**-X leads to hydrogenphosphonate **9**-PHO(O*t*Bu),TBDMS.

Spectral properties and imaging performance of the phosphorylated coumarin dyes: Table 1 presents the most important photophysical properties of the new coumarin dyes, as well as compounds Alexa Fluor 430 (Invitrogen Inc) and DY-480XL (Dyomics GmbH**)** taken as benchmarks. The fluorescence quantum yields were determined by comparison with reference dyes with known emission efficiencies (see the Supporting Information for details).[13a] As expected, the polar groups, that is sulfonic acid (in compound **6**-H) and primary phosphate residues (in compounds **11-**H,H, **21c** and **22c**), provide large fluorescence quantum yields and solubility in aqueous PBS buffer (at pH 7.4, these values varied in the range of 7–25 mM, or several milligrams in 1 mL). The intense fluorescence of these hydrophilic dyes is due to the ionizable groups preventing the aggregation of the dye molecules and/or the formation of non-emitting dimers in aqueous solutions.[13b]

The solutions of the first five compounds in Table 1 are green with a very slight blue shade. The color is similar to that of Fluorescein, Rhodamine 110, Oregon Green 488 and Alexa Fluor 488 dyes, with the exception of the blue tone. The color of the emitted light is green. This is true for Alexa Fluor 430 and the new dyes **6**-H, **11**-H,H, **21c** and **22c**. The last two compounds in Table 1 emit red light, but nevertheless, they may be excited with the 488 nm line of an argon laser.

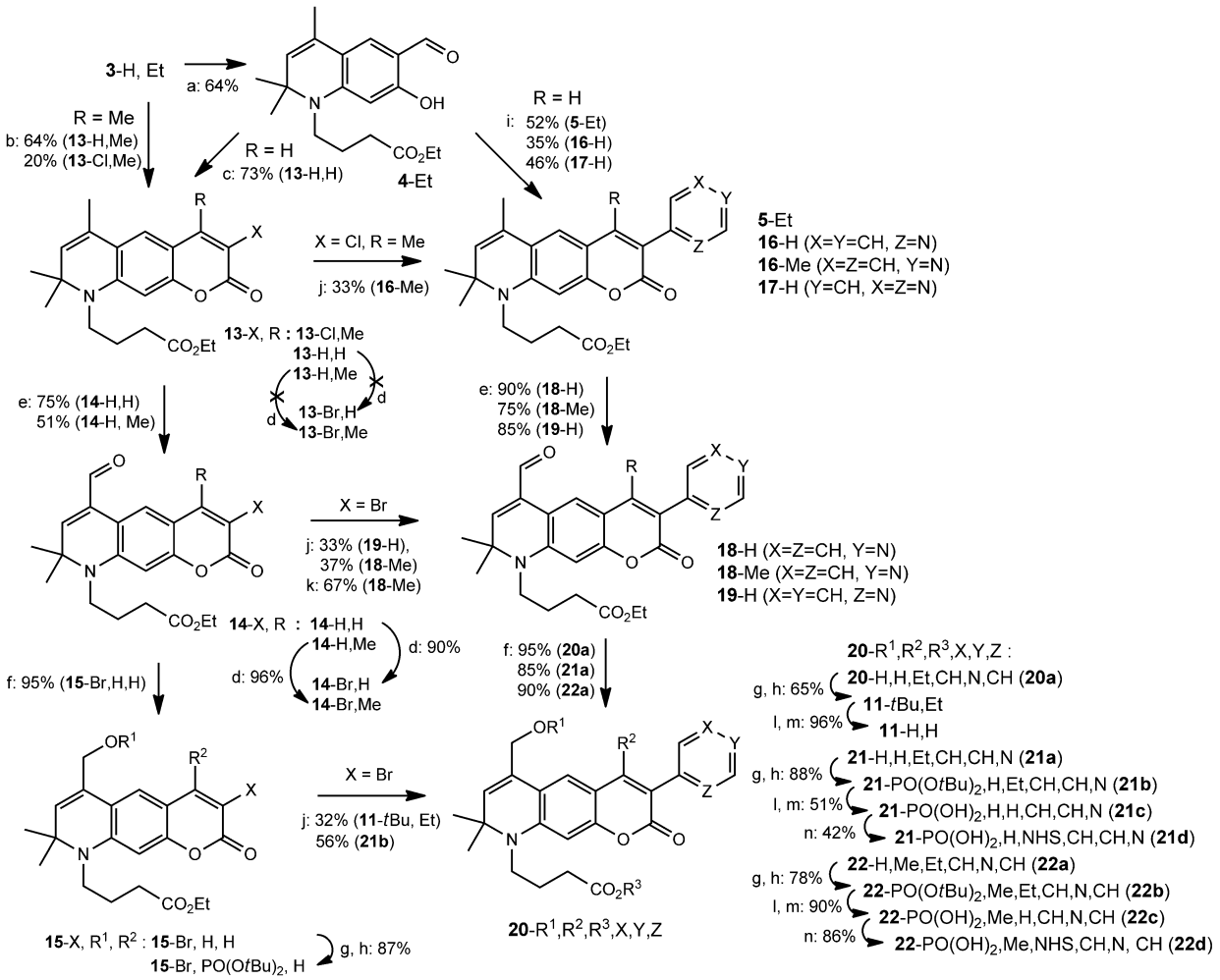
The Stokes shifts of the green-emitting coumarin dyes **5**-Et**, 6**-H**,** **11**-H,H, **21c** and **22c** were found to be approximately 70–80 nm. For example, the Stokes shift of the phosphorylated hydrophilic coumarin dye **11**-H,H is 79 nm; its absorption band matches the excitation line of the violet diode laser (405 nm). Therefore, this dye can be efficiently used in microscopy together with numerous fluorescent labels with smaller Stokes shifts (e.g., Alexa Fluor 488, Oregon Green, or Abberior Star 488), which also emit green light, but absorb strongly at about 480–500 nm and can be excited with 488 nm light. To compare the spectral properties of the phosphorylated coumarin dyes with those of sulfonated ones, we prepared the hydrophilic coumarin dye **6**-H with a sulfonic acid residue attached to the same position at which compound **11**-H,H has the primary phosphate group (Figure 1). The emission and absorption spectra of dye **11**-H,H and its analogue **6**-H are very similar (Table 1). However, after attaching to antibodies, the fluorescence quantum yields of the conjugates obtained from the phosphorylated coumarin dye **11**-H,H were found to be significantly higher (27–30%) than that of the conjugates prepared from the sulfonated dye **6**-H (17–18%).

The alteration in the fluorescence quantum yield upon bioconjugation (compared to that of a free dye in the same solvent) is a very important issue. As a rule, the fluorescence quantum yield of a (hydrophilic) fluorescent dye decreases upon biocojugation.[14] In addition, the fluorescence quantum yields (Φ_{fl}) in conjugates with antibodies often depend on the degree of labeling (DOL): higher quantum yields are observed at lower DOL values (see values in last two columns in Table 1). For dye **11**-H,H, in antibody conjugates, the fluorescence quantum yields were found to not depend on DOL values: for example, $\Phi_{fl}=30$ and 27% at DOL = 2.6 and 6.3, respectively (see data in Table 1).

The 2-pyridyl derivative **21c** was found to have nearly the same properties, as 4-pyridyl analogue **11**-H,H (see Table 1).

The absorption and emission maxima of compound **22**c with the methyl group at C4 in the coumarin fluorophore are shifted to the blue spectral region ($\Delta=16$ and 12 nm, respectively). This hypsochromic shift may be explained by the electron-donating effect of the methyl group (+*I*-effect), which diminishes the electron-acceptor properties of the 4-pyridyl substituent and reduces the overall charge separation in the push–pull 7-amino-4-methyl-3-(4-pyridyl) fluorophore (compared with the 4-unsubstituted compound). Thus, the broad absorption band of compound **22c** (410 nm) perfectly matches the violet line of the 405 nm laser, and the absorption at 488 nm is lower than that in the case of dye **11**-H,H. Therefore, the cross-talk associated with compound **22c** is even less than the cross-talk found for coumarin **11**-H,H, when they are used together with the fluorescent markers possessing small Stokes shifts and excitable with Argon laser at 488 nm (e.g., Abberior Star 488).

Compound **17**-H with a pyrazine substituent (instead of 2- or 4-pyridyl residues) was found to be a poor emitter: its Φ_{fl} value is only 8%. The decreased fluorescence quantum yield may be explained by the excited state photoinduced electron transfer (PeT)[4] from the lone pair of electrons belonging to the nitrogen atom (N4 in pyrazin-2-yl) that is not directly conjugated with 7-amino substituent in coumarin. This lone electron-pair is orthogonal to the π-electron system of the fluorophore and not involved in the charge



Scheme 4. A general approach to phosphorylated coumarins: a) $POCl_3$/DMF, 60 °C, 1.5 h; b) ethyl acetoacetate or ethyl 2-chloroacetoacetate, $ZnCl_2$/EtOH, 90 °C, 40 h; c) $Ph_3P{=}CH\text{-}CO_2Et$/xylene, 140 °C, 3 h; d) Br_2/AcOH, RT, 15 min; e) SeO_2/dioxane, heat to reflux, 2 h; f) $NaBH_4$, $CeCl_3$/THF, MeOH, 0 °C, 15 min; g) *i*$Pr_2NP(OtBu)_2$, 1*H*-tetrazole/CH_2Cl_2, 40 °C, 1 h; h) *m*CPBA/CH_2Cl_2, 0 °C, 15 min; i) Hetaryl-CH_2COOH, EDC, DMAP/CH_2Cl_2, 30 °C, 20 h; j) Hetaryl-$SnBu_3$, $Pd_2(dba)_3$, P*t*Bu_3, toluene or dioxane, 100–110 °C, 2–24 h; k) 4-pyridyl boronic acid, $[Pd(PPh_3)_4]$, 2 M aq. Na_2CO_3/toluene, EtOH, +110 °C, 20 h; l) 1 M aq. NaOH/MeOH, THF, RT, 16 h; m) TFA, RT, 16 h; n) HOSu, HATU, NEt_3/DMF, RT, 16 h.

separation. In fact, unlike the lone pair at N1, it was found to be a nonessential part of the fluorophore. Therefore, if the molecular orbital associated with this lone pair has higher energy than the half-occupied HOMO of the excited state, one of its electrons may easily fill the vacant place in the HOMO (created in the excited state) and quench the emission of light. In spite of this plausible explanation given a posteriori, the spectral properties of compound **17**-H are far from trivial (especially the redshifted absorption and emission bands). Probably, the more nucleophilic nitrogen atom (N4 in pyrazin-2-yl) may be selectively alkylated, and thus a new brightly fluorescent compound with a positively charged nitrogen atom may be obtained.

Compounds **7**-H and **12**-H,H possess betain structures. Unfortunately, the fluorescence quantum yield of compound **7**-H is very low (0.6 % in the free state), and in this respect it cannot be favorably compared to the commercial dye DY-480XL.

The performance of the new coumarins in optical microscopy is illustrated in Figures 2–4. Figure 2 shows the images obtained with the hydrophilic coumarin dye **11**-H,H in multicolor fluorescence microscopy with sub-diffraction resolution (stimulated emission depletion (STED) nanoscopy). Imaging of two cytoskeletal structures, microtubule and vimentin, (stained with dye **11**-H,H and Oregon Green 488, respectively) was achieved using one detection channel, one STED beam (590 nm), and two excitation sources (405 and 488 nm lasers). Under STED conditions, the new dye **11**-H,H (with 79 nm Stokes shift) provides an optical resolution of approximately 50 nm (Figure 3). Compounds **6**-H and **21c** provided similar imaging results as given in Supporting Information (Figures S2 and S3). This section also contains the images (Figure S1) obtained with BD Horizon V500 dye (abs. 415 nm, emission 500 nm; BD Biosciences) under the same conditions as given in Figure 2. They look very similar to the results presented in Figure 2. The drawback of V500

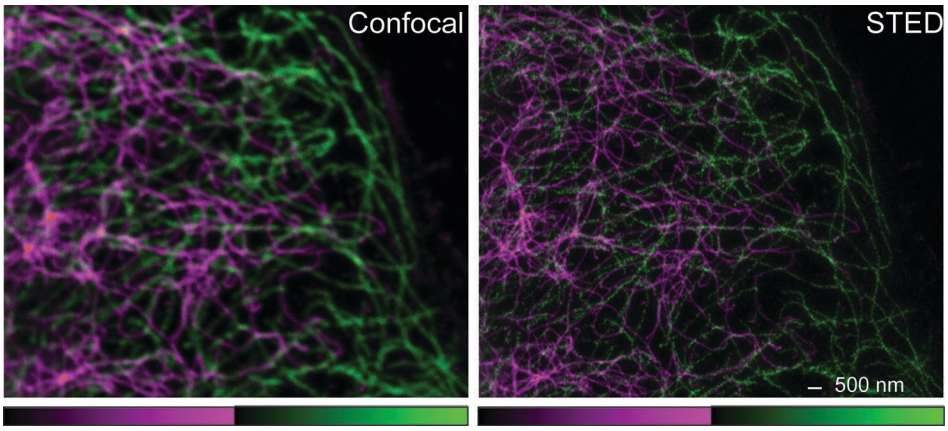


Figure 2. Confocal and STED microscopy images of vimentin (magenta) and microtubule (green), two components of the cytoskeleton in a fixed mammalian PtK2 (Potorous tridactylus, kidney) cell. Vimentin was immunolabeled with NHS ester **11**-H,NHS (dye **11**-H,H, Figure 1 and Table 1); excitation with 405 nm. Tubulin was immunolabeled with Oregon Green 488; excitation with 488 nm. The excitation of both dyes was alternated line-by-line to separate the fluorescence in time. Detection at 510–560 nm for both dyes; STED at 590 nm for both dyes (STED power 84 mW at the back focal plane).

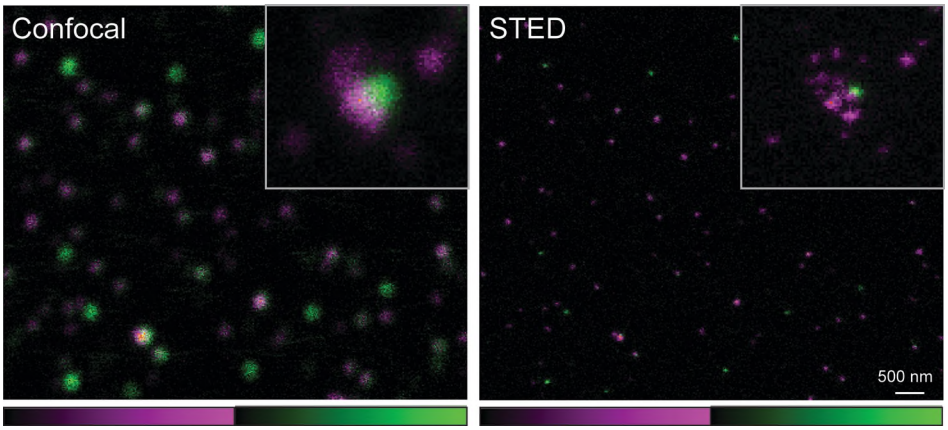


Figure 3. Confocal and STED microscopy images of single (primary + secondary) antibodies in the background to determine the resolution of Figure 2. Confocal resolution ≈200 nm, STED resolution ≈50 nm in both channels (magenta: **11**-H,NHS; green: Oregon Green 488). The boxed area shows a group of antibodies that are close together; the STED microscopy image resolves separate antibodies that appear as a cloud in the corresponding confocal image.

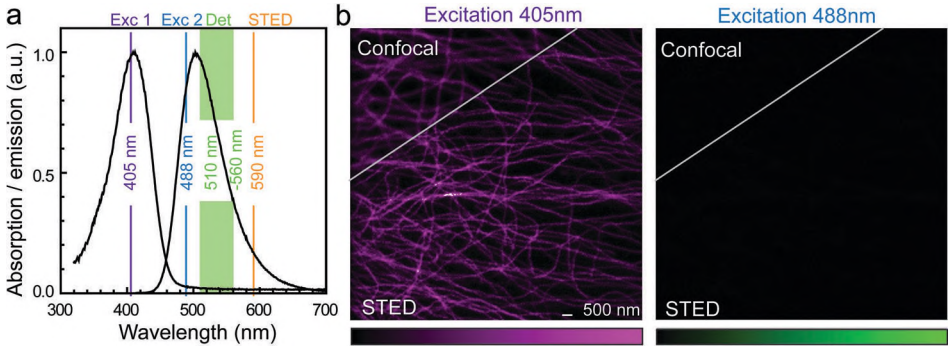


Figure 4. Confocal and STED microscopy imaging without noticeable cross-talk. A) Absorption and emission spectrum of compound **22c** with 93 nm Stokes shift. The violet 405 nm laser line is almost in the absorption maximum whereas the "standard" 488 nm light cannot excite compound **22c**. B) Microtubules visualized through immunolabeling with compound **22c** were irradiated with 405 and 488 nm lasers in an alternating manner with an emission cross-talk less than 1 %; images were recorded in confocal and STED modes.

dye (which is considered to be a benchmark) is that it is not available as NHS ester or other reactive derivative, and its structure is unknown.

In the course of further studies, we prepared compound **22c** with an even larger Stokes shift of 93 nm. This dye has a very low absorption at 488 nm and therefore, it was expected to provide virtually zero cross-talk when combined with all standard "green" dyes (e. g., Alexa Fluor 488 or Oregon Green) and excited with the widely used 488 nm laser line. Figure 4a illustrates the negligible cross-talk recorded for an immunolabeled sample stained with compound **22c** and irradiated at 405 nm and at 488 nm. For comparison, with the same settings, compound **11**-H shows a cross-talk of 19 % when excited with 488 nm. The emission maximum of compound **22c** is at 503 nm and very close to the emission of these "standard" dyes, and indeed, both markers (**22c** and a "green dye") can be detected simultaneously without significant loss. Figure 4 shows that compound **22c** can be used for immunolabeling and in STED microscopy, as well; although it is somewhat "darker" (due to lower absorption coefficient) and the STED resolution of approximately 70 nm is less than that of compound **11**-H.

Conclusion and Outlook

Several synthetic routes to new 3-heteroaryl coumarins with a polar group attached to the C4 position in 2,2,4-trimethyl-1,2-dihydroquinoline fragment fused with the coumarin fluorophore were developed, optimized and compared. As a result, new coumarin dyes **6**-H, **11**-H,H, **21c** and **22c** with Stokes shifts of 80–90 nm suita-

ble for immunofluorescence assays and two-color imaging techniques were synthesized. The primary phosphate group ($OP(O)(OH)_2$) as a polar substituent is advantageous, as it provides higher fluorescence quantum yields in bioconjugates, than the sulfonic acid residue.[15] The confocal and super-resolution STED images demonstrated good signal-to-noise ratio and very good optical resolution. Detection and co-localization of various biological objects can be achieved by staining with these dyes and a standard "green" marker with a small Stokes shift (for example, Oregon Green 488), using one detection channel and two excitation sources (405 and 488 nm lasers, respectively). Under STED conditions, all new dyes provide an optical resolution below the diffraction limit (50–75 nm depending on the actual implementation of the STED microscope and the power of the STED laser). Brighter dyes were obtained if position 4 in the coumarin system was unsubstituted. The presence of two variable positions (3 and 4) in the coumarin scaffold affords flexible structural variations in further design of new coumarin dyes with "tailor-made" properties.

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