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Photoactivatable Rhodamine Spiroamides and Diazoketones Decorated with "Universal Hydrophilizer" or Hydroxyl Groups

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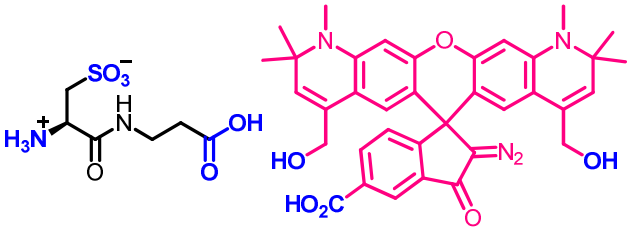
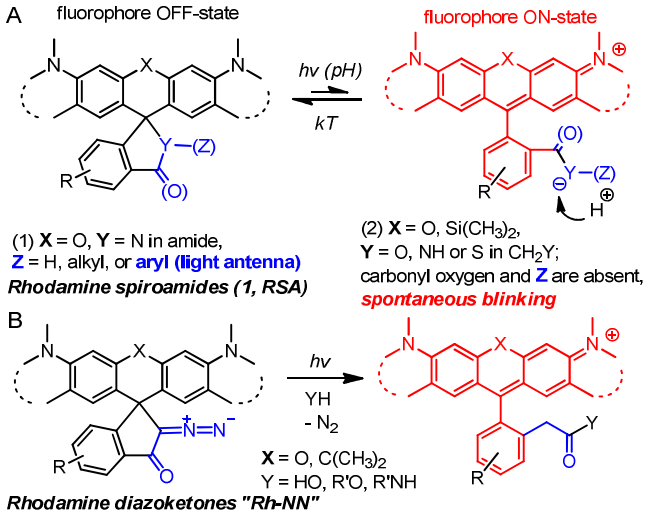


ABSTRACT: Photoactivatable rhodamine spiroamides and spirocyclic diazoketones emerged recently as synthetic markers applicable in multicolor superresolution microscopy. However, their applicability in single molecule localization microscopy (SMLM) is often limited by aggregation, unspecific adhesion and low reactivity caused by insufficient solubility and precipitation from aqueous solutions. We report here two synthetic modifications increasing the polarity of compact polycyclic and hydrophobic labels decorated with a reactive group: attachment of 3-sulfo-L-alanyl – beta-alanine dipeptide (a "universal hydrophilizer") or allylic hydroxylation in photosensitive rhodamine diazoketones (and spiroamides). The superresolution images of tubulin and keratin filaments in fixed and living cells exemplify the performance of "blinking" spiroamides derived from *N,N,N′,N′*-tetramethyl rhodamine.

INTRODUCTION

Colorless and non-fluorescent spiroamides[1,2] and spirocyclic diazoketones[3-5] (Scheme 1) are easily prepared from fluorescent rhodamine and carbopyronine dyes. Rhodamine spiroamides can be transformed by light into a colored and fluorescent open-ring isomer (Scheme 1A, 1) which cyclizes spontaneously (in the dark) back into the colorless and non-fluorescent form. Photolysis of spirocyclic diazoketones obtained from rhodamines (so called Rh-NN)[3,4] and carbopyronines[5] is carried out in the presence of nucleophiles and gives fluorescent homologs (accompanied by non-fluorescent by-products). These transformations may be induced by UV-light (313–380 nm, Scheme 1A, 1), UV- and violet light (405-440 nm, Scheme 1B), or with powerful red light in a two-photon mode (650–800 nm, Scheme 1A, 1 and 1B). Another effect – spontaneous blinking[6-8] – was observed with spiroamides prepared from aliphatic amines,[7] as well as rhodamines and silicon-rhodamines having spiro-cyclic ether, thioether and amine residues.[8] Along with bis-*N,N′*-(*o*-nitrobenzyloxy)-carbonylated *Si*-analog of *Q*-rhodamine,[9] rhodamine spiroamides[2,7] and diazoketones[3-5] were applied as photoactivatable labels in stochastic optical reconstruction microscopy (STORM)[10,11] and related methods of optical superresolution.[12,13] For example, *N*-aryl derivatives of Rhodamine B (Scheme 1A) were used as small labels on live cell surfaces in three-dimensional superresolution imaging.[14] An amide obtained from Rhodamine B and 4-aminophthalimide bearing a reactive group at the imide nitrogen is non-soluble in water.[2] It was attached to cell-penetration peptides and applied in STORM microscopy of actin in living BSC-1 cells.[15]

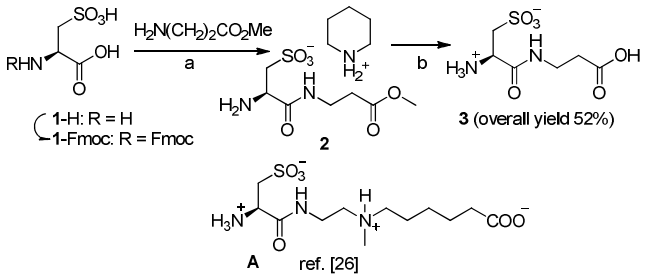
Scheme 1. Photoactivation of rhodamine spiroamides (**A**, 1), spontaneous blinking of rhodamine and silicon-rhodamine based spiro-cyclic ethers, thioethers and amines (**A**, 2). A Wolff rearrangement of rhodamine and carbopyronine diazoketones results in fluorescent homologs (**B**, the "dark" by-product is not shown).



Interestingly, blinking inside the cells was observed without activation with UV-light; only 561 nm laser was used for irradiation and excitation.[15] Prepared from Rhodamine B, spirocyclic *N*-alkylamides have a shoulder at 320 nm ($\varepsilon \sim 5600\ M^{-1}\ cm^{-1}$). They do not absorb beyond 350 nm and require very drastic activation with UV light of 312 nm or 302 nm.[16,17] Due to inefficient light activation, spirocyclic *N*-alkylamides prepared from rhodamines (with exceptions of spontaneously blinking ones[6,7]) have not yet been applied in biology-related single molecule localization microscopy. Photoactivatable Rh-NN[3-4] (prepared from carboxylic acid chlorides and diazomethane) were attached to primary antibodies and used as single molecules in superresolution imaging of asymmetric G-protein coupled receptor oligomers.[18] Hydrophobic Rh-NN were applied for imaging nanostructures by single-molecule localization microscopy in organic solvents (with addition of protic solvents);[19] e.g., for the in situ visualization of block copolymers' self-assembly.[20]

Most of photoactivatable rhodamine spiroamides and spirocyclic diazoketones are lipophilic and non-water-soluble.[1-5,7,8,14,15,18-20] Their imaging performance is limited by aggregation, unspecific adhesion and low reactivity caused by insufficient solubility and precipitation from aqueous buffers. In order to increase polarity of these promising fluorescent markers and stabilize their bioconjugates, we synthesized cysteic acid – beta-alanine dipeptide ("universal hydrophylizer"; Scheme 2) and attached it to a spiroamide obtained from *N,N,N′,N′*-tetramethylrhodamine (TMR) (**5**-H) and two spirocyclic diazoketones prepared from green- and red-emitting rhodamines (**8**-H and **9**-H). Besides that, we used allylic hydroxylation as an "electrically neutral" tool for increasing polarity[21] and applied it to the red-emitting Rh-NN with a compact polycyclic and hydrophobic core decorated with a reactive group.

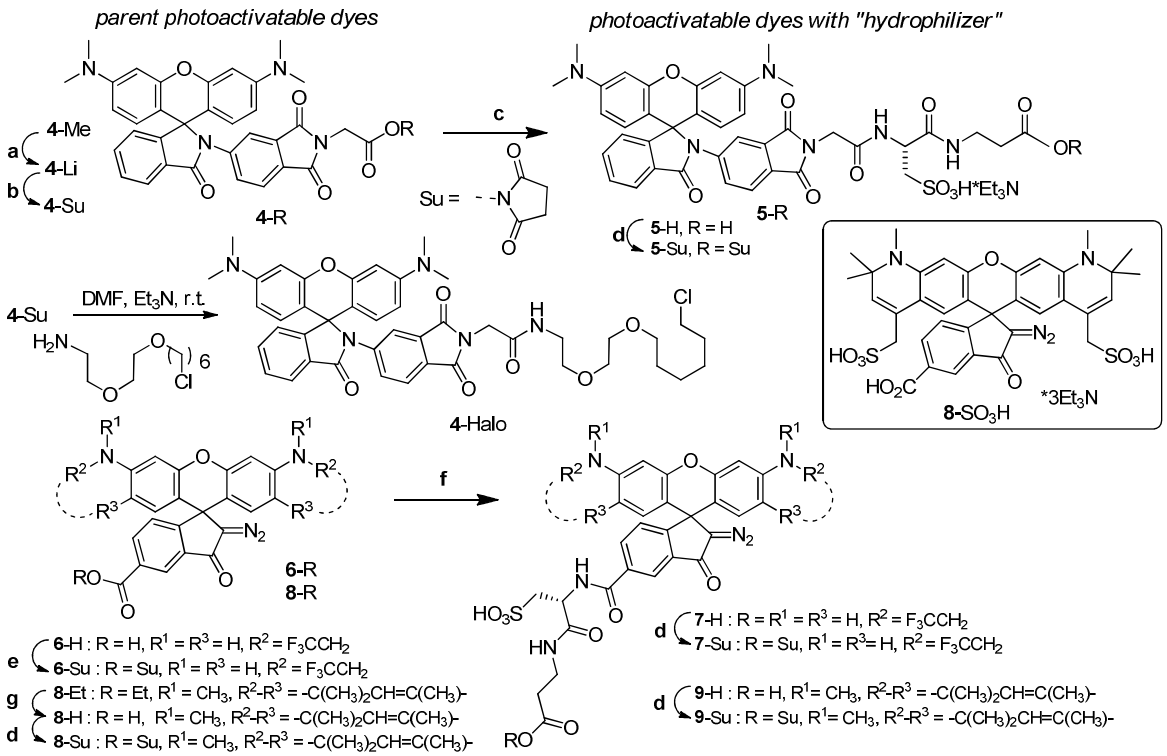
Scheme 2. Cysteic acid – beta-alanine dipeptide **3** as a "universal hydrophylizer": a) CDI (1.1 equiv.), DMF, r.t., 1 h; Et_3N (3 equiv.), r.t., 2 h; piperidine, r.t., 1 h; b) aq. NaOH (1 M, 3.3 equiv), H_2O, 0 °C, 1 h; Amberlite IR-120 (H^+-form), H_2O.



RESULTS AND DISCUSSION

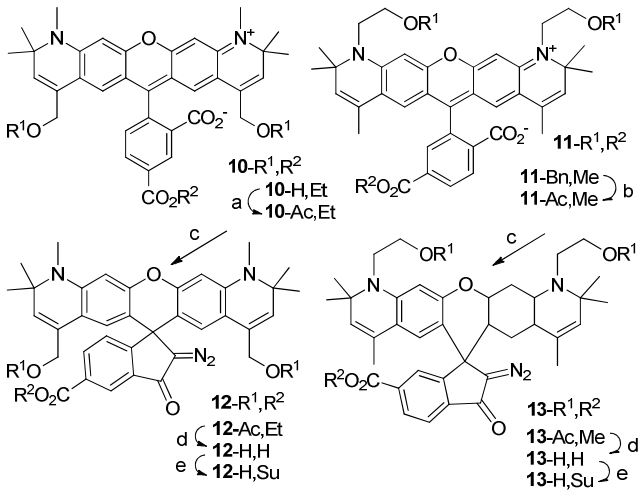
Synthesis. Hydrophilic dipeptide **3** (3-sulfo-L-alanyl-beta-alanine, Scheme 2) was obtained from commercially available L-cysteic acid (**1**-H). The amino group in acid **1** was protected, and urethane **1**-Fmoc converted into amide **2** in a one-pot fashion. For that, the carboxylic acid group in **1**-Fmoc was activated with 1,1′-carbonyldiimidazole (CDI), coupled with methyl 3-aminopropionate, and then the protecting group removed with piperidine. Saponification of methyl ester with aqueous NaOH followed by treating with a strong ion-exchange resin (H^+-form) afforded the hydrophilic dipeptide **3** in an overall yield of 52%. Chromatography (on regular SiO_2) was applied only once: for isolation of piperidinium salt **2**. To demonstrate the utility of compound **3** as an efficient polar linker, we first attached it to a compact rhodamine spiroamide derived from TMR (Scheme 3). Ester **4**-Me in Scheme 3 has the same "light antenna" (based on 4-aminophthalimide) as spiroamides of Rhodamine B applied in earlier studies.[2,7,15] TMR has a somewhat higher fluorescence quantum yield than Rhodamine B (0.41[22] vs. 0.31[23] in aqueous solutions). Additionally, four carbon atoms can be "saved" by using TMR instead of Rhodamine B. Using the published method,[2] we prepared ester **4**-Me, cleaved-off the methyl group, activated the carboxylic acid group in **4**-Li and coupled it with an amino group in dipeptide **3** (Scheme 3). Saponification of ester **4**-Me is accompanied with a ring opening of the phthalimide cycle (it readily occurs already at pH 9). Fortunately, excess of LiI in refluxing THF cleanly cleaves the methyl ester group in **4**-Me within 2 or 3 days. Although this reaction is more rapid in ethyl acetate,[2] the solvent also reacts with lithium iodide (lithium acetate is formed), and the isolation of the target salt **4**-Li is complicated. Conversion of **4**-Li to **4**-Su was straightforward (Scheme 3). The reaction of an active ester **4**-Su with amino acid **3** (1.4 equiv.) took place in DMF in the presence of triethylamine and afforded the coupling product **5**-H (as triethylammonium salt) in moderate yield (29%). The greater excess of dipeptide **3** (3-5 fold) provides a better conversion to the product (>50%), but the chromatographic separation becomes more difficult. The anionic centers in dipeptide **3** remained unprotected, and this provided the shortest synthetic path. Finally, the relatively stable *N*-hydroxysuccinimidyl ester **5**-Su was prepared from carboxylic acid **5**-H. If cysteic acid **1**-H is used as hydrophilizer without attaching β-alanine "extender", the stability of active esters becomes low. In one of the recent applications, a 3D single-molecule based superresolution method (single-objective selective-plane illumination microscopy) was realized with ester **5**-Su as a photoactivatable marker conjugated with secondary antibodies.[25] Structure **3** relates to a similar, yet more complex, compound **A** in Scheme 2 prepared by P. A. Grieco et al. in 9 steps starting from *N*-methylcarprolactame.[24] Being bound on both sides with amide bonds, linker **A** is zwitterionic with a zero net charge, while linker **3** is negatively charged. The negative charge provided by hydrophilizer **3** in conjugates was intended to neutralize the positive charge emerging upon photoactivation of rhodamine spiroamides or diazoketones (Scheme 1).

Scheme 3. Photoactivatable rhodamine spiroamide and diazoketones decorated with a hydrophilic peptide **3**: a) LiI, THF, reflux, 2 – 3 d; b) TSTU (1.7 equiv.), Et_3N, DMF, rt, 4 h; c) **3** (2 equiv.), Et_3N, (3 equiv.), DMF, rt, 24 h; d) NHS (1.2 equiv.), HATU (3.3 equiv.), Et_3N (5.0 equiv.), DMF, rt, 16-19 h; e)



TSTU (40 equiv.), Et_3N (70 equiv.), CH_3CN, rt, 1 h; f) **3** (2 equiv.), Et_3N (5 of 10 equiv), DMF, rt; g) 1 M aq. NaOH (5 equiv.), THF/H_2O, rt, 23 h.

Scheme 4. Hydroxylated photoactivatable *Rh590-NN*: a) Ac_2O, pyridine (1:4, v/v), rt, 4 h; b) Ac_2O, TMSOTf (9 equiv.), DCM, 0 °C, 1 h; c) 1. $(COCl)_2$, CH_2Cl_2, 0 °C - rt, 6 h, 2. CH_2N_2, ether, CH_2Cl_2, 0 °C, overnight; d) aq. NaOH, MeOH, 0 °C, overnight; e) NHS, EDC, CH_2Cl_2, rt, overnight.



Irreversibly photoactivatable rhodamines and carbopyronines with a light-sensitive 3-spiro-2-diazoindanone fragment (Scheme 1B)[3-5] are structurally similar to rhodamine spiroamides,[1,2,6,7] "blinking" spiro-cyclic ethers, thioethers and amines.[8] All of them are polycyclic aromatic compounds with only a few polar groups. For "hydrophilization", we used a masked green-emitting dye **6**-H[4] (Abberior Cage 500)[18,20] and diazoketone **8**-H (Scheme 3) prepared from the rhodamine emitting at about 610–620 nm,[4,21] in a color channel complimentary to emission centered at 650 nm. The standard filter settings in optical microscopy resolve these emission bands with minimal cross-talk.[21] A very powerful 775 nm laser used in stimulation emission depletion microscopyphotoactivates dyes **8**-H and **8**-SO_3H in a two photon mode. In an earlier report, the polycyclic hydrophobic framework **8**-H had been decorated with two sulfonic acid groups (compound **8**-SO_3H in Scheme 3).[4] However, it is difficult to isolate compound **8**-SO_3H in a pure state, free from fluorescent impurities which accumulate in the course of multistep synthesis and manipulation with protecting groups on unstable intermediates. Therefore, a robust photoactivatable marker with the emission maximum in the range of 610–620 nm, complementary to "caged" carbopyronines[5] and a *Si*-analog of *Q*-rhodamine (all emitting at about 655 nm),[9] would be an important addition to a set of photoactivatable "masked" fluorescent dyes.[2] As starting materials for "hydrophilization", we used *N*-hydroxysuccinimidyl esters **6**-Su and **8**-Su (Scheme 3). They reacted with peptide **3** and gave coupling products **7**-H and **9**-H decorated with polar groups (yields 84% and 32%, respectively). Activation of the carboxylic acid residues in diazo-

ketones **7**-H and **9**-H led to amino-reactive *N*-hydroxysuccinimidyl esters **7**-Su and **9**-Su.

Recently, hydroxylated rhodamines, carbopyronines, silicon- and germanium-containing rhodamines have been prepared and applied as cell-permeant fluorescent markers in live-cell STED microscopy with optical resolution below 60 nm.[21,27] As alternatives to negatively charged "masked" rhodamines **8**-SO_3H and **9**-H (Scheme 3), we prepared new hydroxylated diazoketones (**12**-H,H and **13**-H,H in Scheme 4) and their *N*-hydroxysuccinimidyl esters **12**-H,Su and **13**-H,Su. The spiro-diazoketone group was introduced by activation of the free carboxylates in dyes **10**-Ac,Et and **11**-Ac,Me with oxalyl chloride followed by reaction with diazomethane (39 and 47% yields).[3-5] The hydroxyl groups were protected as acetates, and the "remote" carboxyl group – as an ester. These groups are stable against HCl evolving in the course of the reaction with oxalyl chloride. On the other hand, diazoketones are stable in aqueous alkaline solutions. Therefore, all ester groups in the intermediates **12**-Ac,Et and **13**-Ac,Me could be hydrolyzed with aqueous NaOH in ethanol, and the desired "masked" rhodamines **12**-H,H and **13**-H,H obtained (Scheme 4). Prior to introduction of the diazoketone fragment, but after formation of the dye core and separation of isomers, dye **10**-H,Et (precursor of **12**-Ac,Et) had been decorated with hydroxyl groups upon oxidation of allylic methyl group with selenium dioxide.[28] To obtain dye **13**-H,H with *N*-(2-hydroxyethyl) residues, we used benzyl 2-bromoethyl ether for *N*-alkylation of an aminophenol precursor[29] (see Supporting Information for details). The relatively harsh conditions of the alkylation reaction required robust protection of the hydroxyl groups as benzyl ethers. After formation of the rhodamine fluorophore,[30] the benzyl groups were replaced with acetyls, because cleavage of benzyl groups requires strongly acidic or reducing conditions, incompatible with diazoketone functionality. When compound **11**-Bn,Me was treated with BCl_3 or BBr_3 in CH_2Cl_2, the desired alcohols reacted further to the corresponding chlorides or bromides. By using a large excess of TMS triflate (9 equiv.) in acetic anhydride (a reagent and solvent) at 0 °C for 1 h, we managed to obtain dye **11**-Ac,Me directly and with 48% yield.

PHOTOPHYSICAL PROPERTIES

Rhodamine spiroamides (RSA) 4-H and 5-H. The fluorescent form of rhodamine spiroamides can be generated by irradiation with UV-light (photoinduced activation). In addition, they are also halochromic: the protonation of the colorless "closed" form (CF) transform it to an "open" (colored and fluorescent) form (OF; Scheme 1, Y = NH). The pK_a value of such equilibrium depends on the substituents attached to the "central" chromophore and to the lactam ring (R, and Z in Scheme 1).[31] Therefore, the pH range in which the RSAs can be used as single molecule markers in localization-based superresolution microscopies, is limited. Indeed, such markers are applicable only in the pH range where the equilibrium is shifted towards the "dark" closed-ring isomer. To this end, compounds **4**-H (generated from **4**-Li in situ) and **5**-H were studied in aqueous buffered solutions in the range of pH from 1 to 9. Absorption and emission changes for compounds **5**-H and **4**-H are given in Figures 1 and S1, respectively. We found that three species are necessary to explain the observed behavior and a bell-shaped curve. In addition to two species shown in Scheme 1, a third (protonated) form accounts for decoloration at low pH:

$$CF \underset{-H^+}{\overset{+H^+}{\rightleftarrows}} OF \underset{-H^+}{\overset{+H^+}{\rightleftarrows}} OFH^+$$

This protonated form (OFH^+) does not absorb light at 550 nm. The protonation site is yet undefined. Assuming that only the OF absorbs and emits in the green/yellow spectral region, the two equilibrium constants were calculated, in addition to the absorption coefficient of the emissive OF (note that there is no pH at which this form is present in more than 95% of the total concentration). The parameters extracted from the best fits (insets in Figures 1 and S1) are presented in Table 1.

Figure 1. Absorption and emission of compound **5**-H in buffered solutions (excitation with 530 nm light). The absorption intensity at the maximum (558 nm) is plotted *vs* pH of the solution in the inset; data was fit with a two-equilibrium model. For the data related to compound **4**-H / **4**-Li, see Figure S1.

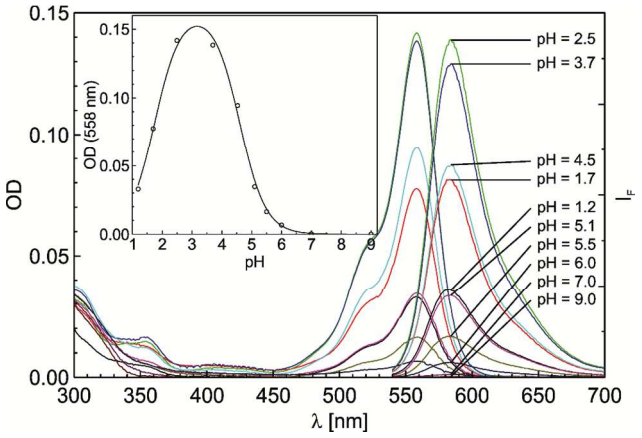


Table 1. Photophysical properties of spiroamides **4**-H / **4**-Li and **5**-H in aqueous solutions: Ka_1 *and* Ka_2 – acidity constants, Φ_{Fl} – emission efficiency, τ_{Fl} – fluorescence lifetime.

	4-H / **4**-Li	**5**-H
λ^{MAX} – Abs (OF), nm	558	558
λ^{MAX} – Em (OF), nm	583	583
$\varepsilon^{558\ nm}$ (OF), $M^{-1}\ cm^{-1}$	40 000	51 000
$Ka_1 * 10^2$	2.3±0.3	1.8±0.2

Ka_2*10^5	3.0±0.4	2.6±0.3
Φ_{Fl}	0.21	0.22
τ_{Fl}, ns	0.9	0.9

Equilibrium constants are consistent with the values obtained previously for similar RSAs derived from 4-aminophthalimide,[2,7] with absorption coefficients being 40-50% lower than the extinction of the parent tetramethyl rhodamine, a tendency observed for (some) tertiary amides prepared from rhodamines.[32] The emission quantum efficiencies of the fluorescent forms are equal in compounds **4**-H and **5**-H.These results indicate that at pH > 6 (two units over pK_{a2}), both compounds are in the "closed" forms and can be used as fluorescent photoactivable probes at biologically relevant neutral pH (6–8). However, at higher pH (>8), the phthalimide unit (light antenna) hydrolyses rapidly forming monoamides of the phthalic acid, and the efficiency of photoactivation decreases strongly. Incorporation of the hydrophilizer has no significant effect on the photophysical properties of RSAs. The improved polarity of compound **5**-H has a side effect: amides obtained from this carboxylic acid are cell impermeable (see next section with imaging results).

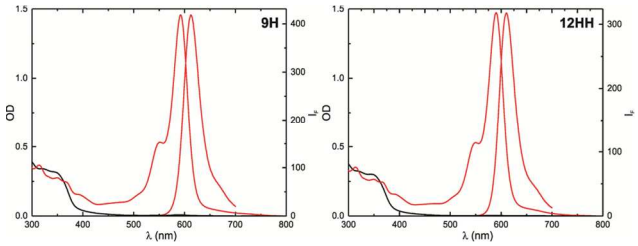
Spiro-cyclic diazoketones 8-H, 9-H, 12-H,H. While RSAs can be reversibly photoactivated by irradiation with UV light, spiro-cyclic diazoketons in Schemes 3 and 4 are masked fluorescent dyes (rhodamines) that can be photoactivated with UV and visible light (λ< 440 nm), or in a 2-photon mode with very powerful red and near IR light (e.g., 775 nm laser).[4] Under these conditions, an irreversible extrusion of dinitrogen is followed by Wolff rearrangement (carbene to ketene)[33] and addition of methanol, water or another nucleophile.[3] To determine the key parameters of this transformation – the quantum efficiency of the photolysis, product distribution and the properties of the fluorescent dye – we irradiated solutions of compounds **8**-H, **9**-H and **12**-H,H in methanol with 405 nm light (~100 mW/cm^2; Figure 2). This wavelength is available as an illumination source in commercial microscopes. To calculate photoactivation efficiencies, the light intensity was measured with a chemical actinometer (azobenzene in MeOH) having the same geometry, as for photolysis. The progress of the reaction was monitored by HPLC under conditions, where the starting compound, the photoproduct (new fluorescent dye) and a "dark" byproduct were clearly separated (Figure S2; for structures of the fluorescent and "dark" products, see refs. 3 and 4). The amount of fluorescent product was estimated assuming all dyes (**8**-H, **9**-H and **12**-H,H) have the same absorption coefficient (61000 M^{-1}cm^{-1} at the maximum).[4] The amount of "dark" product was calculated from the absorption at 660 nm, where the fluorescent product has negligible absorption.[3-5] The HPLC analyses of irradiated solutions were performed using diode array detection, and the absorption spectra of the separate peaks confirmed this assumption. Fluorescence quantum yields and emission lifetimes were calculated from solutions irradiated at low conversions (OD_{MAX} < 0.1).

Table 2. Photolysis of diazoketones **8**-H, **9**-H and **12**-H,H in methanol: FP – fluorescent product; DP –"dark" product, ϕ - quantum yields of the photochemical reactions; for other definitions, see Table 1. Fluorescence quantum yields and lifetimes correspond to FP.

	8-H	**9**-H	**12**-H,H
λ^{MAX} – Abs (FP), nm	594	595	590
λ^{MAX} – Em (FP), nm	617	618	610
ϕ_{FP}*10^3	4.8	5.7	4.6
ϕ_{DP}*10^3	1.3	1.2	0.8
ϕ_{FP}/ϕ_{DP}	3.6	4.9	5.5
Φ_{Fl}	0.82	0.86	0.85
τ_{Fl}, ns	4.0	4.1	4.0

The photo-physical properties are given in Table 2. We found no significant differences among all compounds in Table 3. All of them show high emission quantum yields and relatively long lifetimes. Activation efficiencies are below 1% and allow manipulations with dyes (isolation by flash chromatography, preparation of derivatives, antibody staining and immunolabeling) without rigorous exclusion of light. However, uncaging quantum yields are high enough for efficient activation using relatively low light intensities, even with 405 nm light.

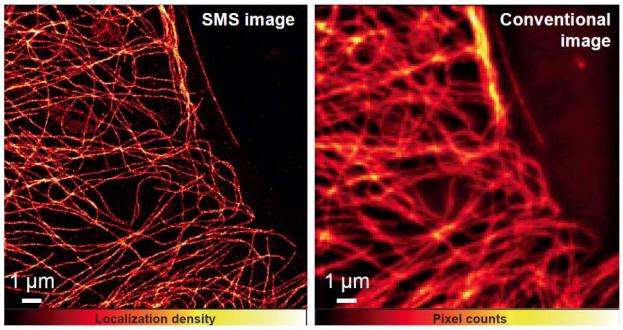
Figure 2. Photoactivation of compounds **9**-H and **12**-H,H dissolved methanol with 405 nm light. Black curves – initial state (diazoketones); red curves – absorption and emission spectra (excitation with 530 nm light) after photoactivation.



Superresolution imaging. Rhodamine spiroamides prepared from Rhodamine B[2,7,14,15] were applied as photoactivatable labels in STORM[10,11] and related methods of optical superresolution[12,13] based on single molecule switching (SMS). Addition of solubilizing groups to the hydrophobic core structure (rhodamine + light antenna) provided higher solubility and sufficient concentration of the reactive marker in aqueous solutions (in this study). In previous experiments with polycyclic spiroamides prepared from Rhodamine B and similar dyes, we often applied *N*-hydroxysulfosuccinimdyl

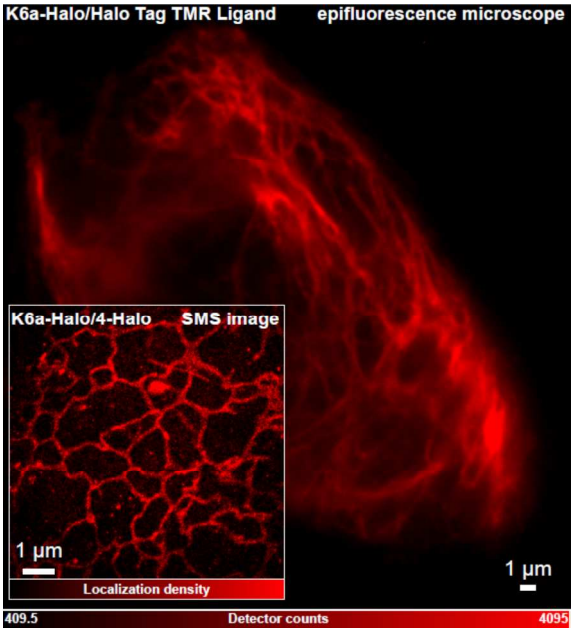
esters in immunolabeling.[2,7] These reagents have a negatively charged group and do not precipitate from aqueous buffers so easily as more lipophilic *N*-hydroxysuccinimidyl (NHS) esters do. Unfortunately, *N*-hydroxysulfosuccinimdyl esters are less stable than their neutral analogs and have to be generated in situ. In this respect, compound **5**-Su is advantageous: it is based on TMR instead of Rhodamine B (see *Synthesis*) and represents a relatively stable NHS ester which can be isolated and used in bioconjugation experiments in a wide concentration range. Higher degrees of labeling (up to 3-5 and even more) can be achieved by using ester **5**-Su, without excessive precipitation of the labeled protein or aggregation of the unspecifically labeled objects. Figure 3 shows super-resolution and conventional images of α-tubulin in a Ptk2 cell labeled by ester **5**-Su conjugated with a secondary antibody. The super-resolution image was taken in a custom-built setup (modified, ref. 36) implementing the principle of SMS in optical nanoscopy.

Figure 3. Super-resolution (left) and conventional (right) images of **5**-Su labeled α-tubulin in a Ptk2 cell. The sample was mounted in Mowiol. Reconstruction from a sequence of 40 000 single frames, each recorded with a camera exposure time of 20 ms. A 532 nm laser was used for wide-field excitation with an average intensity of 2 kW/cm^2 in the sample plane. For activation, a 375 nm laser was kept constant at an average intensity of 0.1 W/cm^2 during the acquisition. The SMS image contains ca. 2.3*10^6 localized positions; each plotted as a 2D Gaussian with full width at half maximum (FHWM) of 20 nm. The conventional image is the sum of the background-corrected single camera frames. Scale bars: 1 µm.



The left image in Figure 3 was acquired with superesolution; it is bright and contrast-rich. Some diffuse background is seen in the upper left corner, but the tubulin filaments are "smooth" and do not have the explicit dot-like pattern (often observed with hydrophobic markers). The presence of the negatively charged groups in spiroamides **5**-R makes them cell-impermeable. Therefore, they are generally not applicable inside living cells. On the other hand, spiroamides **4**-R are uncharged and "small" enough to penetrate through the outer plasma membranes of live cells. For specific labeling of the intracellular targets in living cells, we used a well-established and robust procedure based on HaloTag® fusion proteins.[35] In this technology, the protein of interest is genetically fused (tagged) with an engineered enzyme (modified *Rhodococcus rhodochrous dehalogenase),* which is able to selectively and rapidly form a covalent bond with the substrate. The images of keratin in living Ptk2 cells were obtained with compounds **4**-Halo (Scheme 3) and TMR-Halo (commercially available from Promega Corp. as amide obtained from 6-carboxy-TMR and amine in Scheme 3). The overview image (Figure 4) was obtained using a Leica DM 6000B epifluorescence microscope (Leica Microsystems, Germany), and the super-resolution image was taken in the same setup as in Figure 3.[34] Only in one case (Figure 3), a laser emitting at 375 nm was used for the photoactivation. For imaging of keratin (Figure 4), UV light was not required, because the blinking density was high enough. Thus, the keratin filaments are resolved in the superresolution mode, and their morphology revealed. However, due to highly unpolar nature of the probe **4**-Halo, unspecific background is present in the SMS image (Figure 4).

Figure 4. In vivo labelling of Keratin 6A-Halo fusion proteins with Halo Tag TMR Ligand and 4-Halo probes. Transiently transfected Ptk2 cells expressing *KRT6A-Halo* under the control of a *CMV* promotor were incubated with Halo Tag TMR ligand (Promega Corporation, USA; 5 µM) or **4**-Halo (10 µM) for 30 min followed by a washing step (30 min); pH = 7.2. For excitation, the sample plane was illuminated with an average intensity of 1 kW/cm^2 (532 nm laser). No UV light was used for activation. Except for contrast stretching, no further image processing was applied. The super-resolution image (inset) was reconstructed from a sequence of 20 000 single frames, each recorded with a camera exposure time of 10 ms. The SMS image contains ca.140 000 localized positions; each plotted as a 2D Gaussian with FHWM of 30 nm. Scale bars: 1 µm.



Conclusion and outlook. Compounds **4**-R and **5**-R are introduced as cell-permeate and cell-impermeable photosensitive rhodamine spiroamides, respectively. The structure **5**-R incorporates an amino sulfonic acid residue as a solubilizing unit.[36] Spiro-amides **4**-R and **5**-R were shown to be applicable

in superresolution microscopy based on single molecule switching. The intracellular performance of the probes **4**-R can be improved by attaching neutral solubilizing groups; e. g. oligo(ethylene glycol) residues. In this context, the use of neutral polar groups is advantageous, because the negatively charged residues are known to inhibit cell-permeability, and the positively charged – facilitate non-specific binding of the probes with cell organelles. Photoactivatable dye **5**-H enabled to realize single-objective selective-plane illumination microscopy.[25] The optical setup was constructed on the basis of a conventional Nikon Ti-E microscope, and photoactivation was achieved with 405 nm laser (focusable light),[25] highlighting the importance of a light-absorbing "antenna" incorporated into the spiro-xanthene fragment and its sensitivity not only to 375 nm light (this report) but also to irradiation at 405 nm. Diazoketones **12**-H,R and **13**-H,R are intended for further use in two- and multicolor superresolution imaging based on stimulated emission depletion of SMS modalities. Taking into account the published data,[4] we expect them to be photoactivatable by STED laser (775 nm) in a two-photon mode. Importantly, neither of these probes requires the use of "blinking buffers" essential, for example, for cyanine dyes applied in SMS experiments.[11]

EXPERIMENTAL SECTION

General Remarks. Flash column chromatography was performed using regular silica gel 60 (40-63 μm) from Macherey-Nagel, or cartridges from Interchim (PF-SIHC, 15 μM, 25 g or 40 g SiO_2) or Teledyne Isco (RediSep®Rf, 35 μM, 24 g or 40 g SiO_2). Filtration through BGB or Rotilabo syringe filters (0.22 and 0.45 μm) removed silica gel from the products, when acetonitrile – water mixtures were used as eluents in column chromatography on regular SiO_2. Analytical TLC was performed on Merck Millipore ready-to-use plates with silica gel 60 (F_{254}). The spots were visualized by illumination with a UV lamp (λ = 254 and 365 nm) and/or staining with aq. $KMnO_4$ solution. Anhydrous DMF and HPLC grade acetonitrile were purchased from Sigma-Aldrich; DMF was stored over molecular sieves (4 Å). Water for HPLC was distilled in an ELGA system. The following starting material were synthesized according to published procedures: 2-formylphthalic acid 4-methyl ester,[37] 7-(*tert*-butyldimethylsilyl)oxy-1,2-dihydro-2,2,4-trimethyl-quinoline,[29] 4-amino-*N*-(methoxycarbonylmethyl)-phthalimide,[2] **6**-Su and **8**-Et (compounds **2a**-CONHS and **2a**-CO_2Et in ref. [4]), and **10**-H,Et.[28] *Instruments and methods:* UV/Vis absorption and fluorescence spectra were recorded in closed quartz cuvettes with 1 cm path length on a Varian Cary 4000 UV/Vis and Eclipse spectrophotometers, respectively. ^{1}H and ^{13}C NMR spectra were recorded at 25 °C on Varian Mercury 300, Agilent 400-MR and Inova 500 (^{13}C) spectrometers. Chemical shifts are given in parts per million (ppm) using the residual solvent peak(s) as references. Multiplicities of the signals are described as follows: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet or overlap of non-equivalent resonances. *J* values are given in Hz. Mass-spectra with electro-spray ionization (ESI-MS) were recorded on a Varian 500-MS spectrometer (Agilent). ESI-HRMS were recorded on a MICROTOF spectrometer (Bruker) equipped with an *Apollo* ion source and a direct injector with an LC-autosampler Agilent RR 1200. Analytical and preparative RP-HPLC were carried out with a Knauer Smartline system equipped with a Dionex Ultimate 3000 detector or a UV-VIS detector 2500. Automated flash chromatography on regular silica gel and reversed phase (RP-C18) cartridges was performed with a Biotage Isolera One device. *HPLC systems: System A*: HPLC (Kinetec C_{18} 100 column, 5 μm, 4.6 × 250 mm) with CH_3CN and water (+0.05% v/v TFA) as eluents; linear gradient from 30 to 100% of CH_3CN in 20 min, flow rate 1.2 mL/min; detection at 260 nm. *System B:* HPLC (Eurospher-100 C18, 5 μm, 250 × 4 mm) with CH_3CN and water (+0.05% v/v TFA) as eluents; linear gradient from 30 to 100% of CH_3CN in 25 min, flow rate of 1.0 mL/min; detection at 254 nm. *System C:* HPLC (Eurospher-100 C18, 5 μm, 250 × 4 mm with CH_3CN and water (+0.05% v/v TFA) as eluents; linear gradient from 50 to 100% of CH_3CN in 25 min, flow rate of 1.0 mL/min; detection at 254 nm. *System D:* automated flash purification device Biotage Isolera One (ISO-1EW); cartridge PF-C_{18}-HC, 30 μM, with 20 g of RP silica gel; eluent: H_2O / CH_3CN (linear gradient from 40 % to 60% in 10 column volumes), flow rate of 20 mL/min; detection at 254 nm. *System E:* HPLC (Kinetec C_{18} 100 column, 2.6 μm, 4.6 × 75 mm) with CH_3CN and water with 0.05% v/v TFA as eluents; linear gradient from 20% to 100% in 10 min; flow rate of 1 mL/min; detection at 254 nm.

General procedures A, B (GP A, B) for the preparation of *N*-hydroxysuccinimidyl (NHS) esters. To a solution of "masked" fluorescent dye (1 equiv.) in DMF (1 mL), NHS (1.2 equiv.), HATU (3.3 equiv. [**A**] or 2.1 equiv. [**B**]) and NEt_3 (5.0 equiv.) were added and the mixture was stirred at r.t. for 16-19 h. After removal of volatile materials in vacuo, the product was isolated by column chromatography on silica gel.

Methyl *N*-(3-sulfo-L-alanyl)-3-aminopropanoate piperidinium salt (2). To a solution of L-cysteic acid **1** (1.00 g, 5.91 mmol) in water (5 mL) and dioxane (5 mL), $NaHCO_3$ (1.99 g, 23.7 mmol) was added followed by Fmoc-Cl (1.68 g, 6.49 mmol), and the reaction mixture stirred at r.t. for 2 h. Water (20 mL) was added, the aqueous phase was separated, washed with EtOAc (20 mL), acidified to pH 1 with 1 M aq. HCl and lyophilized to give the *N*-Fmoc protected cysteic acid and NaCl (total amount 3.31 g) as a colorless solid. This material was suspended in DMF (20 mL), CDI (1.05 g, 6.50 mmol,) was added and the mixture was stirred at r.t. for 1 h. Afterwards methyl 3-aminopropanoate hydrochloride (866 mg, 6.21 mmol) and NEt_3 (2.47 mL, 17.7 mmol) were added,

and the mixture was stirred at r.t. for 2 h. Finally, piperidine (1 mL, 10 mmol) was added, and the mixture was stirred at r.t. for 1 h. After evaporation of the solvent and volatile materials under reduced pressure, the residue was subjected to column chromatography on regular silica gel ($MeCN/H_2O$; a linear gradient from 10:1 to 5:1). The product was detected on TLC with a ninhydrin stain; R_f = 0.33 ($MeCN/H_2O$, 5:1). Ester **2** was isolated as a colorless solid (1.74 g, 77%). ^{1}H-NMR (300 MHz, D_2O): δ = 1.78 (m, ~3H, CH_2 in 1.5×$C_5H_{11}N$), 1.90 (m, ~6H, CH_2 in 1.5×$C_5H_{11}N$), 2.77 (t, J = 6.5 Hz, 2H, CH_2CO_2Me), 3.28 (m, ~6H, CH_2 in 1.5×$C_5H_{11}N$), 3.34 (dd, J = 14.8 and 8.3 Hz, 1H, $C\underline{H}^AH^BSO_3$), 3.45 (dd, J = 14.8 and 4.5 Hz, 1H, $CH^A\underline{H}^BSO_3$), 3.58 (m, 2H, CH_2N), 3.77 (s, 3 H, CO_2Me), 4.31 (dd, J = 8.2 and 4.5 Hz, 1H). ^{13}C-NMR (125 MHz, D_2O): δ = 24.1, 24.9, 35.8, 38.0, 47.2 (all CH_2), 52.9 (CH/CH_3), 53.7 (CH_3/CH), 171.3, 177.2 (CO). HRMS (ESI, Q-TOF) *m/z*: [M-H]– 253.0500, calcd for $C_7H_{13}N_2O_6S$; found, 253.0493.

***N*-(3-Sulfo-L-alanyl)-3-aminopropanoic acid (3)**. Ester **2** (1.66 g, 4.36 mmol) was dissolved in H_2O (15 mL), the solution was cooled to 0 °C, 1 M aq. NaOH (15 mL) was added, and the solution was stirred at 0°C for 1 h. The solution was passed slowly through an Amberlite IR-120 ion-exchange resin (82 g, H^+-form). Washing of the resin with water (3 × 50 mL) and lyophilization afforded compound **3** as a colorless solid (709 mg, 67%). R_f = 0.18 ($MeCN/H_2O$, 5:1, ninhydrin stain). Decomposition point: 238–240 °C (sealed capillary): $[\alpha]_D^{24}$ = +12.0° (c = 5.0, H_2O). ^{1}H-NMR (300 MHz, D_2O): δ = 2.69 (t, J = 6.5 Hz, 2H, CH_2CO_2Me), 3.40 (dd, J = 14.9 and 8.4 Hz, 1H, $C\underline{H}^AH^BSO_3$), 3.49 (dd, J = 14.9 and 4.4 Hz, 1H, $CH^A\underline{H}^BSO_3$), 3.57 (m, 2H, CH_2N), 4.43 (dd, J = 8.4 and 4.4 Hz, 1H). ^{13}C-NMR (125 MHz, D_2O): δ = 35.7, 38.1, 52.6, 52.9, 170.0, 178.5. HRMS (ESI, Q-TOF) *m/z*: [M-H]– 239.0343, calcd for $C_6H_{11}N_2O_6S$; found, 239.0345.

Methyl ester 4-Me. Dry 1,2-dichloroethane (20 mL) was added via syringe into a dry Schlenk flask charged with TMR (1.09 g, 2.82 mmol), and the suspension was stirred under Ar. $POCl_3$ (2.0 mL, 22 mmol, 7.8 equiv.) was added, and the reaction mixture was refluxed (bath temp. ~ 110 ˚C) for 5 h under Ar. After distilling off the solvent and excess of $POCl_3$ in vacuo, the residue was dissolved in a mixture of dry CH_3CN and toluene (10 mL, 1:1) under Ar, 4-amino-*N*-[(methoxycarbonyl)methyl]-phthalimide[1] (690 mg, 3.15 mmol, 1.1 equiv.) added, and the reaction mixture stirred at 80 ˚C overnight. After concentration under reduced pressure, the residue was dissolved in $CHCl_3$ (100 mL), washed with water (4 × 20 mL), dried over Na_2SO_4 and evaporated in vacuo. Column chromatography on silica gel (100 g, DCM/hexane/1,4-dioxane, 4:3:1) gave compound **4-Me** as a yellow solid (983 mg, 58%) with m. p. 291–293°C (sealed capillary). ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 2.95 (s, 12 H, 2×NMe_2), 3.73 (s, 3 H, OCH_3), 4.35 (s, 2 H, CH_2N), 6.36 (m, 4 H, H-2,4,5,7*)*, 6.64 (d, J = 8.1, 2 H, H-1,8), 7.09 (m, 1 H, H-7′), 7.29 (dd, J = 8.2, 1.9, 1 H, H-5′′), 7.50 (m, 2 H, H-5′,6′), 7.59 (dd, J = 8.2 and 0.6, 1 H, H-6′′), 7.71 (dd, J = 1.9 and 0.6, 1 H, H-3′′), 8.02 (m, 1 H, H-4′). ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 38.9, 40.3, 52.8, 98.9, 106.6, 109.2, 121.0, 123.8, 124.0, 124.1, 128.3, 128.5, 128.6, 129.4, 130.1, 132.7, 133.9, 143.5, 151.6, 152.6, 153.8, 167.0, 167.1, 167.9, 168.3. HRMS (ESI, Q-TOF) *m/z*: [M+H]+ calcd for $C_{35}H_{31}N_4O_6$, 603.2238 ; found, 603.2223.

Lithium salt 4-Li. Ester **4-Me** (966 mg, 1.60 mmol) was dissolved in dry THF (8 mL) under Ar in a screw-cap test-tube, LiI (649 mg, 4.28 mmol, 2.7 equiv.) added, and the mixture refluxed at 90 ˚C (bath temp.) for 48 h. Then, Et_2O (6 mL) was added, and the reaction mixture stirred for 5 min. The precipitate was filtered off, washed with Et_2O and dried in vacuo to give **4-Li** as a light yellow solid (stored in a "dark" flask; 945 mg, 99%). ^{1}H-NMR (400 MHz, CD_3OD): δ = 2.89 (s, 12 H), 3.77 (s, 2 H), 6.42 (m, 4 H, H-2,4,5,7), 6.64 (d, J = 7.8 Hz, 2 H, H-1,8), 7.02 (m, 1 H, H-7′), 7.40 (dd, J = 8.2 and 1.9, 1 H, H-5′), 7.57 (m, 3 H, H-5′/6′/6′′), 7.66 (d, J = 8.2, 1 H, H-3′), 7.93 (m, 1 H, H-4′). ^{13}C-NMR (100 MHz, CD_3OD): δ = 40.6, 42.4, 70.0, 100.1, 107.6, 110.6, 122.0, 124.4, 124.5, 125.4, 129.4, 130.1, 131.0, 131.4, 132.2, 134.5, 135.3, 143.8, 153.4, 154.2, 155.1, 168.9, 169.0, 170.0, 174.3. HRMS (ESI, Q-TOF) *m/z*: [M]– 587.1936, calcd for $C_{34}H_{27}N_4O_6$; found, 587.1937.

***N*-Hydroxysuccinimidyl ester 4-Su**. To a solution of **4**-Li (400 mg, 0.67 mmol) in DMF (3 mL), TSTU (344 mg 1.14 mmol) and NEt_3 (101 mg, 1.0 mmol) were added, and the reaction mixture was stirred at r.t. for 4 h. All volatile materials were removed in vacuum; the residue was taken-up in DCM and applied onto a plug of silica gel. Rapid elution with EtOAc provided ester **4**-Su as a yellow solid (160 mg, 35%). R_f = 0.67 (EtOAc). HR-MS (ESI, positive mode): 708.2046 (found $[M+Na]^+$), 708.2065 (calculated for $C_{38}H_{31}N_5NaO_8^+$).

Amide 4-Halo. To 5.0 mg (7.3 μmol) of *N*-hydroxysuccinimidyl ester **4**-Su in 0.2 mL DMF, 1.7 mg (7.7 μmol) of $H_2N(CH_2)_2O(CH_2)_2O(CH_2)_6Cl$ and of 10 μL Et_3N were added, and the reaction mixture was stirred overnight at rt. The product was isolated by chromatography on SiO_2 (13 g) using hexane – ethyl acetate (1:2) mixture as eluent. Yield – 3.0 mg (51%) of yellowish solid. HRMS (ESI, Q-TOF) *m/z*: [M+H]+ 794.3315, calcd for $C_{44}H_{49}N_5O_7Cl$; found, 794.3291.

Spiroamide 5-H. To a solution of ester **4**-Su (150 mg, 0.22 mmol) in DMF (3 mL), NEt_3 (92 μL, 0.66 mmol) and peptide **3** (74 mg, 0.31 mmol) were added, and the reaction mixture was stirred at r.t. for 24 h. After evaporation of the solvent under reduced pressure, the residue was subjected to column chromatography on regular silica gel ($MeCN/H_2O$, 10:1). The fractions containing spiroamide **5**-H were pooled and lyophilized to give a pink solid (52 mg, 29% yield). R_f = 0.11

(MeCN/H_2O, 10:1). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 2.26 (t, J = 7.2 Hz, 2H, CH_2CO_2H), 2.67 (dd, J = 13.8, 6.4Hz, 1H, $CH^AH^BSO_3H$), 2.79 (dd, J = 13.8, 5.9 Hz, 1H, $CH^AH^BSO_3H$), 2.90 (s, 12H, 4×NMe), 3.20 (m, 2H, $NHCH_2$, masked by water peak in DMSO), 4.08 (d, J = 16.5 Hz, 1H, NCH^CH^D), 4.19 (d, J = 16.5 Hz, 1H, NCH^CH^D), 4.32 (q, J = 6.4 Hz, 1H, CH), 6.42 (m, 4H, 2/4/5/7-H), 6.64 (d, J = 9.5 Hz, 2H, 1/8-H), 7.01 (dd, J = 6.2 and 1.6 Hz, 1H, 7′-H), 7.48 (dd, J = 8.2, 1.9 Hz, 1H, 5′′-H), 7.58 (m, 3 H, 5′/6′/3′′-H), 7.72 (d, J = 8.2 Hz, 1H, 6′′-H), 7.79 (t, J = 6.0 Hz, 1H, $CONHCH_2$), 7.94 (dd, J = 6.6, 2.3 Hz, 1H, 4′-H), 8.33 (d, J = 6.4 Hz, 1H, CONHCH). ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 34.7, 35.4, 50.8, 52.3, 66.7, 98.3, 98.5, 106.0, 109.2, 109.3, 118.3, 123.3, 123.4, 123.6, 123.8, 127.8, 128.0, 128.3, 128.7, 132.3, 134.1, 142.8, 151.2, 151.6, 153.8, 165.5, 166.7, 166.8, 167.5, 170.1. HRMS (ESI, Q-TOF) *m/z*: [M-H]- 809.2247, calcd for $C_{40}H_{37}N_6O_{11}S$; found, 809.2245. HPLC (*system A*): t_R= 11.5 min, peak area 92% (260 nm).

***N*-Hydroxysuccinimidyl ester 5-Su.** Compound **5**-Su was synthesized from **5**-H (15.0 mg, 18.5 μmol) according to GP A, and purified by column chromatography on silica gel (MeCN/H_2O, with a gradient from 25:1 to 10:1). The pure fractions were lyophilized to give compound **5**-Su as a pink solid (4.0 mg, 24%). R_f = 0.57 (MeCN/H_2O, 10:1). Isolation with prep. HPLC provided higher yields (7–8 mg) of **5**-Su as a red solid. (Eurospher-100 C18, 5 μm, 250 × 16 mm, CH_3CN and water (+0.05% v/v TFA) as eluents; linear gradient from 30 to 100% of CH_3CN in 20 min, flow rate of 10 mL/min; detection at 254 nm. Analytical HPLC (*system B*): t_R = 8.8 min, HPLC area = 97% (254 nm). HRMS (ESI, Q-TOF) *m/z*: [M+Na]+ 930.2375, calcd for $C_{44}H_{41}N_7NaO_{13}S$; found, 930.2378.

Compound 7-H. To a solution of compound **6**-Su[4] (10.0 mg, 15.2 μmol) in DMF (0.5 mL) were added NEt_3 (21 μL, 0.15 mmol) and peptide **3** (7.3 mg, 31 μmol), and the mixture was stirred at r.t. for 16 h. The solvent was evaporated under reduced pressure, and the residue was subjected to column chromatography on silica gel. Elution with a mixture of MeCN/H_2O (10:1) followed by lyophilization afforded compound **7**-H as a slightly brown solid (10 mg, 84%). R_f = 0.20 (MeCN/H_2O, 10:1). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 1.02 (t, J = 7.2, "1H", Et_3N), 2.33 (t, J = 7.0 Hz, 2H CH_2CO_2H), 2.66 (q, "0.8H", J = 7.2 Hz, Et_3N), 2.90 (m, 2H, CH_2SO_3), 3.25 (t, J = 6.6 Hz, 2H, CH_2N), 3.94 (m, J = 10.0 and 7.2 Hz, 4H, CH_2CF_3), 4.49 (m, 1 H, J = 5.3 Hz, CH), 6.46 (dd, J = 8.6, 2.4 Hz, 2H), 6.54 (m, 4H), 6.64 (dd, J = 8.6 and 4.3 Hz, 2H), 7.15 (d, J = 8.1 Hz, 1H, 7′-H), 7.91 (t, J = 5.4 Hz, 1H, $CONHCH_2$), 8.04 (dd, J = 8.1, 1.6 Hz, 1H, 6′-H), 8.23 (d, J = 1.6 Hz, 1H, 4′-H), 8.88 (d, J = 5.7 Hz, 1H, CONHCH. HRMS (ESI, Q-TOF) *m/z*: [M-H]– 783.1313, calcd for $C_{32}H_{26}F_6N_6O_9S$; found, 783.1317.

***N*-Hydroxysuccinimidyl ester 7-Su**. Compound **7-Su** was synthesized from **7**-H (10 mg, 14 μmol) according to GP B, and purified by column chromatography on silica gel (MeCN/H_2O, 25:1 to 10:1). Lyophilization afforded compound **7**-Su as a slightly brown solid (3 mg, 27% yield). R_f = 0.65 (MeCN/H_2O, 10:1). HRMS (ESI, Q-TOF) *m/z*: [M-H]– 880.1477, calcd for $C_{36}H_{29}F_6N_7O_{11}S$; found, 880.1482.

Carboxylic acid 8-H. To a solution of ester **8**-Et[4] (10 mg, 16 μmol) in a mixture of THF/H_2O (0.3 mL, 2:1, v/v), 1 M aq. NaOH (82 μL, 5.0 eq.) was added, and the mixture was stirred at r.t. for 23 h. After completion of the reaction (TLC control), the solution was cooled in an ice bath and acidified carefully to pH = 2 – 3 with 1 M aqueous $KHSO_4$. The reaction mixture was extracted with CH_2Cl_2 (2×10 mL), organic solutions were dried over Na_2SO_4 and concentrated in vacuum to give acid **8**-H as a green solid (9 mg, 94%). R_f = 0.52 (EtOAc). ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.26 (s, 6H, CH_3), 1.29 (s, 6H, CH_3), 1.70 (q, J = 1.8 Hz, 6H, CH_3C=), 2.81 (s, 6H, NCH_3), 5.18 (d, J = 2.0 Hz, 2H, CH=), 6.26 (d, J = 1.8 Hz, 2H), 6.40 (d, J = 1.8 Hz, 2H), 7.18 (d, J = 8.1, 1.8 Hz, 1 H, 7′-H), 8.17 (dd, J = 8.1, 1.6 Hz, 1 H, 6′-H), 8.56 (d, 1H, J = 1.8 Hz, 4′-H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 18.7, 27.9, 28.0, 31.1, 56.7, 98.1, 106.8, 120.3, 122.0, 124.6, 125.8, 127.1, 129.2, 129.3, 130.3, 134.9, 136.0, 146.4, 152.0, 160.8, 170.1, 186.3. HRMS (ESI, Q-TOF) *m/z*: [M-H]– 585.2507, calcd for $C_{36}H_{33}N_4O_4$; found, 585.2503.

***N*-Hydroxysuccinimidyl ester 8-Su**. Compound **8**-Su was prepared from **8**-H (8.0 mg, 14 μmol) according to GP B, and isolated by column chromatography on silica gel (pentane/EtOAc, 2:1) as a green solid (8.0 mg, 86%). R_f = 0.14 (pentane/EtOAc, 2:1). HRMS (ESI, Q-TOF) *m/z*: [M+Na]+ 706.2636, calcd for $C_{40}H_{37}NaN_5O_6$; found, 706.2630.

Compound 9-H. To a solution of compound **8**-Su (8.0 mg, 12 μmol) in DMF (0.5 mL), NEt_3 (8.0 μL, 59 μmol) was added followed by peptide **3** (6.2 mg, 26 μmol), and the mixture was stirred at r.t. for 5 days. After removal of volatile materials in vacuo, the residue was subjected to column chromatography on silica gel (MeCN/H_2O, 10:1), and compound **9**-H was isolated as a green solid (3.0 mg, 32%). R_f = 0.64 (MeCN/H_2O, 10:1). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 1.24 (s, 6H, CH_3), 1.28 (s, 6H, CH_3), 1.63 (s, 6H, CH_3), 2.31 (t, J = 7.2 Hz, 2H, CH_2CO_2H), 2.78 (s, 6H, NCH_3), 2.90 (m, 1H, $CH^AH^BSO_3H$), 3.24 (m, 3H, NCH_2+$CH^AH^BSO_3H$), 4.48 (m, 1H, CH), 5.27 (s, 2H, CH=), 6.25 (s, 2H), 6.34 (s, 2H), 7.16 (d, J = 8.1 Hz, 1H, 7′-H), 7.95 (t, 1H, $CONHCH_2$), 8.05 (dd, J = 8.1, 1.5 Hz, 1H, 6′-H), 8.23 (d, J = 1.5 Hz, 1H, 4′-H), 8.91 (d, J = 5.6 Hz, 1H, CONHCH). ^{13}C-NMR (125 MHz, DMSO-d_6; due to the presence of the chiral center, "right and "left" parts of the masked fluorophore are inequivalent): δ = 17.9, 25.2, 27.66/27.71, 27.94/27.96, 30.8. 35.8, 45.7, 48.7, 51.5/51.8, 56.3, 74.6, 97.3, 106.1, 119.3, 120.7, 121.0, 125.3,

125.58/125.61, 129.4, 133.6, 134.4, 134.7, 145.8, 151.3, 158.1, 164.7, 170.3, 185.3. HRMS (ESI, Q-TOF) *m/z*: [M-H]– 807.2818, calcd for $C_{42}H_{43}N_6O_9S$; found, 807.2885. HPLC (*system C*): t_R= 7.1 min, peak area 91% (254 nm).

Compound 9-Su. Compound **9**-Su was synthesized from **9**-H (3.0 mg, 3.7 µmol) according to GP B, and purified by column chromatography on silica gel (MeCN/H_2O, 25:1 to 10:1). The pure fractions were lyophilized to afford ester **9**-Su as a slightly green solid (1.3 mg, 39%). R_f = 0.77 (MeCN/H_2O, 10:1). MS (ESI, negative mode) *m/z*: [M-H]– 904.3, calcd for $C_{46}H_{47}N_7O_{11}S$; found, 904.9. HPLC (*system C*): t_R= 8.7 min, peak area = 96% (254 nm).

Triester 10-Ac,Et. To a cold solution of compound **10**-H,Et[28] (74 mg, 0.12 mmol; see Scheme 4) in dry pyridine (2 mL), Ac_2O (0.5 mL) was added at 0°C, and the reaction mixture was stirred at r.t. for 4 h. Volatile materials were removed in vacuo, the residue was dissolved in DCM (30 mL), washed successively with aq. HCl (0.1 M, 10 mL) and sat. aq. $NaHCO_3$ (20 mL), dried over Na_2SO_4 and concentrated in vacuo. Column chromatography on silica gel (40 g, 15 µM, DCM/MeOH, with a linear gradient from 100:0 to 80:20) afforded compound **10**-Ac,Et as a blue solid (49 mg, 58%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.35 (s, 6 H), 1.37 (s, 6 H), 1.45 (t, J = 7.1, 3 H, C$\underline{H}_3$CH$_2$), 1.83 (s, 6 H, 2×CH_3CO), 2.87 (s, 6 H, CH_3N), 4.45 (q, J = 7.1, 2 H, CH_3C$\underline{H}_2$O), 4.55 (s, 4 H, CH_2OAc), 5.46 (s, 2 H, 2×CH=), 6.33 (s, 2 H, H-1,12), 6.35 (s, 2 H, H-6,7), 7.24 (d, J = 8.7, 1 H, H-7′), 8.29 (dd, J = 8.0 and 1.5, 1 H, H-6′), 8,72 (d, J = 1.6, 1 H, H-3′). ^{13}C-NMR (101 MHz, $CDCl_3$): δ = 14.4, 20.8, 27.7, 28.2, 31.3, 57.1, 61.8, 64.0, 97.7, 106.3, 117.3, 122.7, 124.6, 126.4, 127.2, 132.4, 132.7, 135.2, 148.1, 153.4, 165.3, 168.5, 170.5. HRMS (ESI, Q-TOF) *m/z*: [M+H]+ 707.2963, calcd for $C_{41}H_{43}N_2O_9$; found, 707.2957.

***N*-[2-(Benzyloxy)ethyl]-1,2-dihydro-7-hydroxy-2,2,4-trimethylquinoline.** In a sealed tube, 7-(*tert*-butyldimethylsilyl)oxy-1,2-dihydro-2,2,4-trimethylquinoline[29] (1.0 g, 3.3 mmol), DIPEA (1.15 mL, 6.60 mmol,) and benzyl 2-bromoethyl ether (1.04 mL, 6.60 mmol) were combined and stirred at 110 °C for 3 days. More 2-bromoethyl ether (0.5 mL, 3.30 mmol) was added, and the reaction mixture was further stirred at 110 °C for 1 day. After cooling, it was diluted with diethyl ether, washed with brine, dried over Na_2SO_4, evaporated and purified by flash chromatography on silica gel (*n*-hexane/EtOAc, with a step gradient from 100:0 to 90:0) to obtain a colorless oil (mixture of the product and starting material). It was used in TBDMS deprotection without further purification. The oil was dissolved in THF (10 mL) and added to a stirred solution of TBAF×3H_2O (828 mg, 2.62 mmol) in THF (10 mL) at -5 °C. After stirring for 10 min, the reaction mixture was diluted with $CHCl_3$ (20 mL), washed with brine, dried over Na_2SO_4, and evaporated. Flash chromatography on silica gel (*n*-hexane/EtOAc, with a step gradient from 100:0 to 70:30) gave an orange oil (787 mg, 74%). R_f (*n*-hexane/EtOAc, 70:30) = 0.29. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.26 (s, 6H), 1,92 (d, J = 1.5, 3 H, CH_3C=), 3.45 (t, J = 7.2, 2 H, CH_2N), 3.60 (t, J = 7.8, 2 H, CH_2O), 4.55 (s, 2 H, CH_2Ph), 4.84 (br. s, 1 H, OH), 5.09 (q, J = 1.5, 1 H, CH=), 5.95 (d, J = 2.4, 1 H), 6.07 (dd, J = 8.1 and 2.3, 1 H), 6.90 (d, J = 8.1, 1 H), 7.41-7.26 (m, 5 H). ^{13}C-NMR (101 MHz, $CDCl_3$): δ = 18.9, 28.6, 43.7, 56.8, 67.8, 73.4, 98.0, 102.4, 116.6, 125.0, 126.8, 127.6, 127.9, 128.0, 128.6, 138.4, 145.5, 156.6. ESI-MS, positive mode, *m/z*(rel. int., %): 324.2 (100) $[M+H]^+$, 324.4 ($C_{21}H_{26}NO_2$).

Rhodamine 11-Bn,Me. In a sealed tube, 2-formylphthalic acid 4-methyl ester[37] (91 mg, 0.41 mmol), *N*-[2-(benzyloxy)ethyl]-1,2-dihydro-2,2,4-trimethylquinolin-7-ol (370 mg, 1.15 mmol) and *p*-TsOH (14.1 mg, 0.08 mmol) were dissolved in propionic acid (1.6 mL), and the resulting mixture was heated at 80 °C overnight. Then, tetrachloro-1,4-benzoquinone (100 mg, 0.41 mmol) was added, and the reaction mixture was stirred at 80 °C for 2 h. After cooling down to rt, concentration under reduced pressure and purification by flash chromatography on silica gel (DCM/MeOH; step gradient from 100:0 to 90:0), rhodamine **11**-Bn,Me was isolated as a blue solid (99 mg, 30%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.34 (s, 12 H), 1.64 (s, 6 H), 3.62 (t, J = 7.5, 4 H), 3.70 (t, J = 7.9, 4 H), 3.89 (s, 3 H, OCH_3), 4.59 (s, 4 H, OCH_2Ph), 5,23 (s, 2 H, H-3,10), 6.43 (s, 2 H, H-1,12), 6.47 (s, 2 H, H-6,7), 7.45-7.26 (m, 10 H), 7.81 (s, 1 H, H-7′), 8.23 (m, 2 H, H-4′,5′). ^{13}C-NMR (101 MHz, $CDCl_3$): δ = 18.4, 29.2, 29.3, 44.5, 52.6, 58.1, 67.4, 73.6, 96.9, 121.4, 122.7, 126.4, 127.1, 127.8, 127.9, 128.6, 130.1, 130.7, 134.3, 138.0, 148.6, 154.7.0, 166.0, 168.6. HRMS (ESI, Q-TOF) *m/z*: [M+H]+ 817.3847, calcd for $C_{52}H_{52}N_2O_7$; found, 817.3850.

Rhodamine 11-Ac,Me. To a solution of rhodamine **11**-Bn,Me (90 mg, 0,11 mmol) in acetic anhydride (2 mL), a freshly prepared solution of TMSOTf in DCM (500 µL, 50% v/v, 0.96 mmol) was carefully added at 0 °C, and the reaction mixture was stirred for 1 h at 0°C. Then, the reaction mixture was transferred into a saturated aq. solution of $NaHCO_3$ (25 mL) and extracted with DCM (2×25 mL). Organic solutions were dried over Na_2SO_4, evaporated, and the residue was separated by column chromatography on silica gel (DCM/MeOH, with a linear gradient from 100:0 to 80:20). Compound **11**-Ac,Me was isolated as a blue solid (37 mg, 48%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.32 (s, 6 H), 1.33 (s, 6 H), 1.62 (d, J = 1.4 Hz, 6 H, CH_3C=CH), 2.12 (s, 6 H, O$\underline{Ac}$), 3.58 (t, J = 7.2, 4 H), 3.87 (s, 3 H, OCH_3), 4.24 (t, J = 7.0, 4 H), 5.20 (s, 2 H, H-3,10), 6.35 (s, 2 H, H-1,12), 6.49 (s, 2 H, H-6,7), 7.80 (dd, J = 1.4 and 0.7 Hz, 1 H, H-7′), 8.13 (dd, J = 8.0 and 0.7 Hz, 1 H, H-5′), 8.24 (dd, J = 8.0 and 1.4 Hz, 1 H, H-4′). ^{13}C-NMR (101 MHz, $CDCl_3$): δ = 18.5, 21.0, 28.8, 29.1, 42.8, 52.7, 57.8, 61.1, 97.2, 107.5, 121.0, 122.8, 126.5, 129.6, 130.7, 132.9, 135.1, 147.4, 153.8, 165.9, 168.7, 171.1.

HRMS (ESI, Q-TOF) *m/z*: [M+H]+ 721.3120, calcd for $C_{42}H_{44}N_2O_9$; found, 721.3120.

Diazoketone 12-Ac,Et. To a solution of diacetate **10**-Ac,Et (49 mg, 0.069 mmol) in dry DCM (5 mL), oxalyl chloride (625 µL) was added dropwise at 0 °C, followed by 1 small drop of dry DMF. The reaction mixture was stirred for 6 h at r.t. All volatile materials were distilled in vacuo into a trap cooled with dry ice/acetone mixture, the residue was flushed with Ar, dissolved in dry DCM (8 mL) at 0 °C, and a solution of diazomethane in Et_2O (0.2 M, 5 mL) was added. The reaction mixture was stirred overnight at 0 °C. The color of the solution became less intense; a clear pink solution was formed. Solvents were evaporated in vacuo, and the residue was subjected to chromatography on silica gel (20 g, 15 µM, *n*-hexane/EtOAc, step gradient from 90:10 to 50:50). Diazoketone **12**-Ac,Et was isolated as a green oil (24 mg, 47%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.32 (s, 6 H), 1.33 (s, 6 H), 1.40 (t, J = 7.2, 3 H, C<u>H</u>$_3$CH$_2$), 1.80 (s, 6 H, 2×Ac), 2.83 (s, 6 H, 2×NCH$_3$), 4.38 (q, J = 7.1, 2 H), 4.60 (AB-q, J = 12.6, 4 H, CH$_2$OAc), 5.42 (s, 2 H, H-3,10), 6.27 (s, 2 H, H-1,12), 6.44 (s, 2 H, H-6,7), 7.15 (dd, J = 8.0 and 0.7, 1 H, H-7′), 8.14 (dd, J = 8.1 and 1.7, 1 H, H-6′), 8.51 (dd, J = 1.6 and 0.7 H, 1 H, H-3′). ^{13}C-NMR (101 MHz, $CDCl_3$): δ = 14.4, 20.7, 27.6, 27.7, 31.1, 49.2, 56.7, 61.5, 64.0, 98.5, 107.3, 117.3, 122.7, 123.9, 125.7, 126.7, 131.1, 132.3, 135.1, 135.4, 146.5, 152.2, 159.9, 165.7, 170.4, 185.8. HRMS (ESI, Q-TOF) *m/z*: [M+H]+ 731.3075, calcd for $C_{42}H_{43}N_4O_8$; found, 731.3059.

Compound 12-H,H. To a solution of compound **12**-Ac,Et (24 mg, 33 µmol) in EtOH (20 mL) at 0 °C, a solution of aq. NaOH (1 M, 2.8 mL) was added, and the mixture was keep in the dark and stirred at 0 °C overnight. Then, the reaction mixture was concentrated in vacuum, cooled down to 0 °C and acidified carefully to pH = 2 – 3 with a 5% aq. citric acid. After that, the mixture was extracted with CH_2Cl_2 (2×15 mL), and the green organic solutions dried over Na_2SO_4 and concentrated in vacuo. The residue was subjected to an automated flash chromatography on a RP-C18 cartridge with H_2O/CH_3CN (system D) as eluent. The fractions containing the product were lyophilized to give compound **8**-H as a green amorphous solid (9.0 mg, 45%). ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 1.24 (s, 6H), 1.30 (s, 6H), 2.77 (s, 6H), 3.89 (dd, J = 13.2 and 1.3 Hz, C<u>H</u>^{A}H^{B}O, 2H), 3.97 (dd, J = 13.2 and 1.3 Hz, CH^A<u>H</u>^{B}O, 2H), 5.43 (s, CH=, 2H), 6.27 (s, 2 H), 6.54 (s, 2 H), 7.14 (d, J = 7.4 Hz, 1 H, 7′H), 8.11 (dd, J = 8.1, 1.7 Hz, 1 H, 6′H), 8.18 (d, J = 1.7 Hz, 1 H, 3′H). ^{13}C-NMR (101 MHz, DMSO-d_6): δ = 27.5, 27.9, 30.8, 48.9, 56.2, 60.5, 73.9, 97.6, 106.4, 117.4, 121.0, 122.7, 125.5, 128.2, 129.9, 131.3, 133.8, 135.7, 146.0, 151.2, 159.5, 166.4, 185.1. HRMS (ESI, Q-TOF) *m/z*: [M-H]– 617.2406, calcd for $C_{36}H_{33}N_4O_6$; found, 617.2398. HPLC (*system E*): t_R= 7.1 min, peak area = 87% (254 nm).

***N*-Hydroxysuccinimidyl ester 12-H,Su**. To a solution of compound **12**-H,H (2.0 mg, 3.2 µmol) in anhydrous CH_2Cl_2 (500 µL), *N*-hydroxysuccinimide (4.0 mg, 35 µmol) was added followed by a stock solution of *N,N′*-dicyclohexyl carbodiimide (DCC, 80 mg in 1.0 mL) which was added at 0…+5°C in 10 µL portions. After addition of each portion and stirring in the dark at 0…+5°C for several hours, HPLC analysis was performed. For that, dichloromethane was evaporated from an aliquote of several µL, the residue was dissolved in acetonitrile (+0.1% TFA v/v) and immediately after that injected into a HPLC column. Addition of four 10 µL aliquotes of DCC (15.6 µmol) at 0°C in 2 days provided the full conversion of the starting material. No macrocyclic lactone was detected. After concentration of the reaction mixture in vacuum, the residue was applied onto a column with regular silica gel. Elution with a mixture of *n*-hexane and EtOAc (80:20 to 0:100) afforded ester **12**-H,Su as green oil (1.9 mg, 82%). HRMS (ESI, Q-TOF) *m/z*: [M-H]– 714.2569, calcd for $C_{40}H_{37}N_5O_8$; found, 714.2541. HPLC (*system E*): t_R= 7.8 min, peak area = 82% (254 nm).

Diazoketone 13-Ac, Me. To a cold solution of **11**-Ac,Me (40 mg, 0.055 mmol) in dry DCM (5 mL), oxalyl chloride (625 µL) was added dropwise at 0 °C, followed by 1 drop of dry DMF. The reaction mixture was stirred for 6 h at rt. After concentration in vacuo (volatile materials were condensed into a trap cooled with a mixture of dry ice with acetone), the residue was flushed with Ar, dissolved in dry DCM (8 mL) at 0 °C, and a solution of diazomethane in Et_2O (0.2 M, 5 mL) was added. Upon stirring overnight at 0 °C, the color of the reaction mixture vanished. The reaction mixture was evaporated in vacuo, and the residue was purified by column chromatography on silica gel (20 g, 15 µM, *n*-hexane/EtOAc, with a step gradient from 90:10 to 70:30). Compound **13**-Ac,Me was isolated as a green oil (16 mg, 39%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.29 (s, 6 H), 1.31 (s, 6 H), 1.68 (d, J = 1.4, 3 H, CH_3C=CH), 2.13 (s, 6 H, O<u>Ac</u>), 3.52 (t, J = 7.0 Hz, 4 H, CH_2N), 3.84 (s, 3 H, OCH_3), 4.23 (t, J = 7.2 Hz, 4 H, CH_2O), 5.15 (q, J = 1.5, 2 H, C<u>H</u>=CMe), 6.36 (s, 2 H, H-1,12), 6.39 (s, 2 H, H-6,7), 7.72 (dd, J = 1.2 and 0.5, 1 H, H-7′), 7.88 (d, J = 8.0, 1 H, H-4′), 8.08 (dd, J = 8.0, 1.4 Hz, 1 H, H-5′). ^{13}C-NMR (101 MHz, $CDCl_3$): δ = 18.7, 21.1, 28.8, 42.6, 49.4, 52.6, 57.1, 61.6, 98.2, 107.3, 120.3, 122.3, 122.8, 126.9, 127.0, 128.8, 129.8, 135.7, 138.3, 144.8, 152.0, 156.1, 166.3, 171.2, 186.2. HRMS (ESI, Q-TOF) *m/z*: [M+H]+ 745.3232, calcd for $C_{43}H_{44}N_4O_8$; found, 745.3232.

Carboxylic acid 13-H,H. To a solution of compound **13**-Ac,Me (24 mg, 32 µmol) in MeOH (10 mL), aq. NaOH (1 M, 2.8 mL) was added at 0 °C, and the mixture was stirred in the dark at 0 °C overnight. The reaction mixture was concentrated in vacuum, cooled down to 0 °C and acidified carefully to pH = 2–3 with 5% aq. citric acid. The mixture was extracted with CH_2Cl_2 (2×15 mL), and the green organic solutions were dried over Na_2SO_4. The solvent was evaporated in vacuum, and the residue subjected to an automated flash chromatography on a RP-C18 cartridge with H_2O/CH_3CN mixture (sys-

tem D) as eluent. The fractions containing acid **13**-H,H were lyophilized to give a green amorphous solid (8.0 mg, 39% yield). ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 1.22 (s, 6H), 1.26 (s, 6H), 1.62 (s, 6H), 3.37 (m, 4H, masked by water peak in DMSO), 3.49 (t, *J* = 7.2 Hz, 4H), 5.24 (s, 2H, CH=), 6.37 (s, 2H), 6.38 (s, 2H), 7.47 (dd, *J* = 1.4, 0.6 Hz, 1 H, 7′H), 7.87 (d, *J* = 8.0, 0.5 Hz, 1H, 4′H), 8.02 (dd, *J* = 8.0, 1.4 Hz, 1H, 5′H). ^{13}C-NMR (101 MHz, DMSO-d_6): δ = 185.1, 166.3, 155.9, 151.4, 144.8, 136.8, 136.5, 129.7, 129.1, 125.5, 125.4, 122.5, 121.5, 119.3, 106.1, 97.4, 74.8, 58.2, 56.5, 48.7, 46.0. 28.8, 28.6, 18.0. HRMS (ESI, Q-TOF) *m/z*: [M-H]– 645.2719, calcd for $C_{38}H_{37}N_4O_6$; found, 645.2710. HPLC (*system E*): t_R= 7.1 min, peak area = 97% (254 nm).

Ester 13-H,Su was prepared from carboxylic acid **13**-H,H (2.0 mg, 3.2 µmol) in dry CH_2Cl_2 (100 µL) according to the method given for compound **12**-H,Su. Column chromatography on silica gel (n-hexane/EtOAc, 80:20 to 0:100) afforded **13**-H,Su as green oil (1.9 mg, 83% yield). HRMS (ESI, Q-TOF) *m/z*: [M+Na]+ 766.2847, calcd for $C_{42}H_{41}N_5NaO_8$; found, 766.2828.

ASSOCIATED CONTENT

Supporting information

The supporting information is available free of charge on the ACS Publications website at DOI: 101021/acs.joc. Supporting information contains absorption and emission spectra of compound **4**-H / **4**-Li in buffered solutions (Figure S1), details of the photolysis experiments with compounds **8**-H, **9**-H and **12**-H,H (Figures S2 and S3), data on cloning, cell culture and lasers, as well as copies of NMR spectra for all new compounds.

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Notes

VNB and SWH own shares in Abberior GmbH which produces compounds **5**-Su and **12**-H,Su as photoactivatable dyes FLIP 565 and Cage 590, respectively.

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