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Carborhodol: A New Hybrid Fluorophore Obtained by Combination of Fluorescein and Carbopyronine Dye Cores

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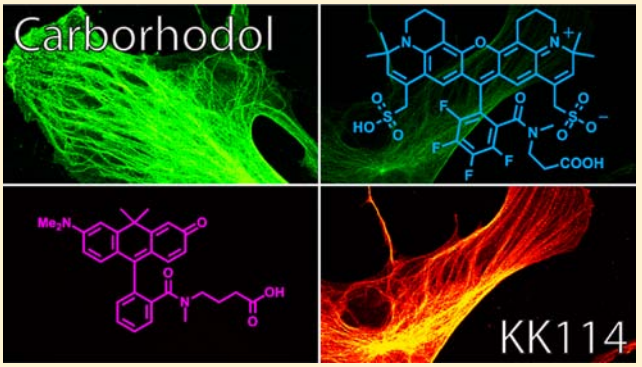
Supporting Information

ABSTRACT: Asymmetric hybrid fluorophores are built from the structural elements of two (or even more) symmetric dyes and can develop valuable new features which their parents do not possess. A new hybrid carborhodol dye was obtained by the combination of fluorescein and carbopyronine fluorophores. The brightly fluorescent hybrid dye with a linker and reactive group was prepared in 12 steps with overall yield of 1.6%. In aqueous solutions, it has absorption and emission maxima at 586 and 613 nm, respectively. Antibodies labeled with a carborhodol dye possess broad absorption and emission bands so that the effective Stokes shift is increased (compared with small Stokes shifts of the parent dyes) and the fluorescence quantum yield of 39% at a degree of labeling of 5.2. Two samples of secondary antibodies labeled with carborhodol and the benchmark red-emitting rhodamine dye (KK114) were used in two-color imaging experiments with excitation at 514−532 (carborhodol dye) and 633−640 nm (KK114). When emitted light was detected above 650 nm, the novel carborhodol dye provided a lower crosstalk than spectrally similar emitters (e. g., Atto594; crosstalk 40−60% with KK114 under the same conditions). The optical resolution of ca. 80 nm was attained using the new dye in stimulated emission depleted (STED) microscopy. The relatively short fluorescence lifetime in conjugates with antibodies (τ = 1.2−1.6 ns) suggests the possibility of dual FLIM with numerous dyes having τ values in the range of 3−5 ns. All of these features make the carborhodol fluorophore a valuable addition to the family of the red-emitting fluorescent dyes.



INTRODUCTION

The structures of hybrid fluorophores are derived from fragments of traditional fluorescent dyes. In most cases, these novel compounds possess asymmetric structures and share the features of both (symmetric) ancestors.

Rhodols (3-amino-6-hydroxyfluorans; Figure 1) represent one of the simplest classes of hybrid fluorophores. Rhodols belong to the family of xanthene dyes and may be considered as hybrids of rhodamines and fluorescein.[1−5] In neutral or basic solutions, the spectral properties of fluorescein and Rhodamine 110 (the simplest unsubstituted rhodamine in Figure 1) are very similar. Their absorption maxima are at 485 and 496 nm, while emission bands have maxima at 514 and 520 nm, respectively. Introduction of alkyl groups to the nitrogen atom in rhodols leads to bathochromic and bathofluoric shifts and, together with further structural changes, allows a very fine tuning of the absorption and emission bands in the relatively narrow region between 503 and 555 nm. The Stokes shifts (separation between the absorption and emission maxima) of rhodamines, fluorescein, and rhodols are small (20−30 nm). Only rhodols with monosubstituted nitrogen atoms were found to be highly fluorescent.[5]

Other "crossbred" fluorescent dyes were obtained by the combination of hemicyanine dyes with coumarin[6] or benzopyrilium fragments.[7,8] The former dyes possess features of both parents, e.g., the large Stokes shifts inherited from coumarins and the far red spectral emission of cyanine dyes.[6]

Until now, hybrid dyes were often designed and applied as sensors and analytical reagents[6,7,9−13] and rarely used as fluorescent labels in light microscopy.[8] In this respect, the combination of fluorescein and carbopyronine dyes[14−20] is particularly interesting because it should enable design of a new and simple fluorophore with asymmetric electron density distribution. In turn, this may lead to a larger Stokes shift and other favorable properties. For applications in imaging and microscopy, the most important characteristics of a free dye and its bioconjugates are: the position, form, and width of the absorption and emission bands, fluorescence quantum yield, and lifetime of the excited state of a free dye and its bioconjugates. In addition, red-emitting dyes represent a particularly useful family of fluorescent labels as they emit light in the spectral region where cellular autofluorescence is negligible.[21−26]

The performance of a dye in live cell experiments depends on the size of a dye molecule, its electrical charge, and the related charge of the bioconjugates. Bulky, negatively charged

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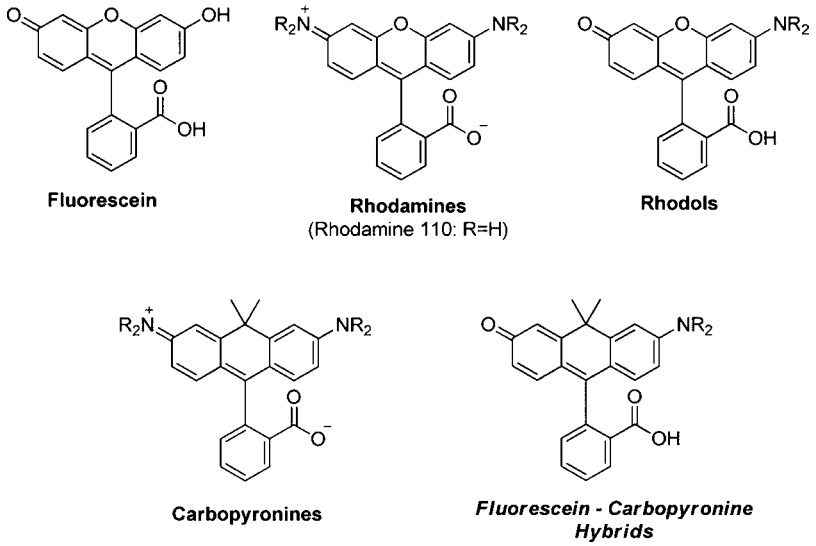

Figure 1. Prototypes for the new fluorescein−carbopyronin hybrids sharing the structural features of carbopyronines and rhodols. Rhodols are, in turn, asymmetric hybrid dyes derived from fluorescein and rhodamines.

dyes and their conjugates cannot penetrate the plasma membrane of living cells. Thus, many fluorescent dyes may only be used for staining fixed cells.[26] In addition, the electrical charge and size of a dye residue can also influence the properties of lipids labeled with such a dye.

In the case of rhodamines and carbopyronines without a second carboxylic acid group (Figure 1), the dye residues in conjugates are positively charged. An additional carboxylic group can provide the zero net charge, but the zwitterionic dye residues are often not preferred because the free carboxylic group close to the positively charged fluorophore may form the colorless lactone form of rhodamines and carbopyronines (see Experimental Procedures). Therefore it is desirable to create small red-emitting fluorophores with uncharged conjugates.

Taking all these pieces of information into account, we considered small fluorescein−carbopyronine hybrids with uncharged dye residues to be our synthetic target. In carbopyronines, the oxygen atom at position 10 of the xanthene fragment is "replaced" with the geminal dimethyl group $>C(CH_3)_2$ (Figure 1). This results in a bathochromic shift of the absorption and emission bands (ca. 50 nm in comparison with the corresponding rhodamines), but the Stokes shift remains small.[14−20]

The synthesis of "pure" carbopyronines is a challenging task because in all available publications,[14−18] except the most recent,[19,20] preparation procedures were described either incompletely or for simple derivatives only.[14−16] The crucial parameters of a new dye (in exception to those mentioned above) are the possibility of excitation with common diode lasers, applicability in two-color imaging, high "brightness" in the free and conjugated states (product of the fluorescence quantum yield and the absorption coefficient), sufficient photostability, solubility in aqueous buffers (hydrophilic dyes devoid of nonspecific binding provide high imaging contrast due to the absence of the fluorescent background), and stability of an amino reactive derivative (NHS ester). Yet another important feature of a fluorescent dye is the imaging performance in light microscopy and, in particular, in super-resolution microscopy, e.g., stimulated emission depletion (STED) microscopy.[27−30] For that, a sufficient optical cross section for stimulated emission (at certain, "fixed" wavelengths, e.g., 600 or 750−775 nm) and the resistance against photobleaching under irradiation with intense STED light are required.

EXPERIMENTAL PROCEDURES

Instruments, Materials and General Remarks. UV−visible absorption spectra were recorded on a Varian Cary 4000 UV−vis spectrophotometer, and the fluorescence spectra on a Varian Cary Eclipse fluorescence spectrophotometer. The fluorescence quantum yields were determined by comparison with the known quantum yields for reference dyes (for a detailed description, see: *A Guide to Recording Fluorescence Quantum Yields*; www.horiba.com/fileadmin/uploads/Scientific/Documents/Fluorescence/quantumyieldstrad.pdf). A MICROTOF spectrometer equipped with ESI ion source Apollo and direct injector with LC autosampler Agilent RR 1200 was used for obtaining high resolution mass spectra (ESI-HRMS). ESI-HRMS were obtained also on an APEX IV spectrometer (Bruker). HPLC system (Knauer): Smartline pump 1000 (2×), UV detector 2500, column thermostat 4000 (25 °C), mixing chamber, injection valve with 20 and 100 μL loop for the analytical and preparative columns, respectively; 6-port−3-channel switching valve; analytical column, Eurospher-100 C18, 5 μm, 250 mm × 4 mm, 1.1 mL/min; solvent A, water + 0.1% v/v trifluoroacetic acid (TFA); solvent B, CH_3CN + 0.1% v/v TFA; detection at 254 nm or as specified. Analytical TLC was performed on MERCK ready-to-use plates with regular silica gel 60 (F254) and UV detector (unless specified otherwise). Preparative column chromatography was performed on silica gel 60 (40−63 μm) from Macherey-Nagel (Germany). NMR device: Varian (Agilent) 400-MR (400 MHz). Coupling constants (J) are given in Hz. In the DEPT mode, the ^{13}C signals of the methyl (CH_3) and methyne (CH) groups are "positive" (+), while the signals of methylene groups (CH_2) are "negative" (−). The reagent solutions were introduced via syringes through septa into the Schlenk flaskes connected with a vacuum−argon manifold.

Synthesis. *(2-Bromo-4-methoxyphenyl)(4-nitrophenyl)-methanone (2).* A dry 50 mL Schlenk flask was charged with $AlCl_3$ (1.73 g; 13 mmol) and CH_2Cl_2 (13 mL). To this suspension, 3-bromoanisole **1** (2.5 g, 13 mmol) was added

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under vigorous stirring at 0 °C. After warming up to rt, a solution of *p*-nitrobenzoyl chloride (2.73 g, 15 mmol) in CH_2Cl_2 (20 mL) was introduced, and the reaction mixture refluxed for 3 h. After cooling down to rt, the reaction mixture was poured into a mixture of ice and 1 M HCl. The organic layer was separated, the aqueous phase was extracted with CH_2Cl_2 (2 × 100 mL), and the combined organic solutions were washed with 1 M NaOH (3 × 50 mL) and dried with Na_2SO_4. Evaporation of volatile solvents in vacuo gave a residue which was recrystallized from ethanol to yield 3.05 g (71%) of the title compound as a yellowish powder. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.90 (s, 3 H, OMe), 6.97 (dd, J = 8.6, 2.4, 1 H, Ar), 7.21 (d, J = 2.4, 1 H, Ar), 7.39 (d, J = 8.6, 1 H, Ar), 7.93 (m, 2 H, Ar), 8.30 (m, 2 H, Ar). ^{13}C NMR (100.7 MHz, $CDCl_3$): δ = 55.8(+), 113.4(+), 119.1(+), 121.5(−), 123.7(+), 130.8(+), 131.2(−), 131.7(+), 142.0(−), 150.3(−), 162.1(−), 193.8(−). HRMS: found 335.9863; calcd for $C_{14}H_{10}NO_4Br$ [M + H]$^+$ 335.9866.

*(4-Aminophenyl)(2-bromo-4-methoxyphenyl)methanone (**3**).* To 1.0 g (3.0 mmol) of compound **2** in a mixture of 1,2-dimethoxyethane and ethanol (9 and 11 mL, respectively), a solution of $SnCl_2{\cdot}2H_2O$ (2.64 g, 11.7 mmol) in concd aq HCl (20 mL) was added at such a rate that the temperature did not exceed 30 °C. After stirring overnight at rt, the reaction mixture was poured into an excess of ice−water and CH_2Cl_2. Then the aqueous layer was neutralized to pH 12 with solid NaOH, the organic layer was separated, and the aqueous solution was extracted with CH_2Cl_2 (50 mL). The combined organic solutions were dried with Na_2SO_4 and evaporated in vacuo to give a residue which was purified by column chromatography (100 g SiO_2, CH_2Cl_2/MeOH, 20:1) to furnish 758 mg (82%) of the title compound. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.85 (s, 3 H, OMe), 6.62 (m, 2 H, Ar), 6.89 (dd, J = 8.5, 2.4, 1 H, Ar), 7.15 (d, J = 2.4, 1 H, Ar), 7.39 (d, J = 8.5, 1 H, Ar), 7.64 (m, 2 H, Ar). ^{13}C NMR (100.7 MHz, $CDCl_3$): δ = 55.6(+), 113.0(+), 113.9(+), 118.4(+), 120.7(−), 127.2(−), 130.3(+), 132.9(+), 133.6(−), 151.2(−), 160.7(−), 193.9(−). HRMS: found 306.0126; calcd for $C_{14}H_{12}NO_2Br$ [M + H]$^+$ 306.0124.

*(2-Bromo-4-methoxyphenyl)(4-(dimethylamino)phenyl)-methanone (**4**) Was Prepared According to the Known Procedure.*[31] To a mixture of compound **3** (2.0 g, 6.5 mmol) and paraformaldehyde (1.96 g, 65 mmol) in AcOH (50 mL), $NaBH_3CN$ (1.97 g, 31.3 mmol) was added in one portion at 0 °C. The reaction mixture was stirred overnight at rt, and then an additional amount of $NaBH_3CN$ (1.00 g, 15.9 mmol) was added. After stirring for 2 h at rt, the reaction mixture was transferred into an excess of 15% NaOH with ice, pH was adjusted to 12 with solid NaOH, and the solution was extracted with CH_2Cl_2 (3 × 100 mL). The combined organic solutions were dried with Na_2SO_4 and evaporated in vacuo to give 2.06 g (83%) of the title compound as a yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.08 (s, 6 H, NMe_2), 3.15 (s, 3 H, OMe), 6.64 (m, 2 H, Ar), 6.90 (dd, J = 8.5, 2.4, 1 H, Ar), 7.16 (d, J = 2.4, 1 H, Ar), 7.26 (d, J = 8.5, 1 H, Ar), 7.71 (m, 2 H, Ar). ^{13}C NMR (100.7 MHz, $CDCl_3$): δ = 40.1(+), 55.6(+), 110.7(+), 113.0(+), 118.2(+), 120.6(−), 124.4(−), 130.1(+), 132.6(+), 134.0(−), 153.6(−), 160.6(−), 193.7(−). HRMS: found 320.0432; calcd for $C_{16}H_{16}NO_2Br$ [M + H]$^+$ 334.0437.

*4-(2-Bromo-4-methoxybenzyl)-N,N-dimethylaniline (**5**).* **5** was prepared according to the published method.[32] To a cooled (0 °C) solution of compound **4** (1.6 g, 4.8 mmol) in THF (45 mL), $AlCl_3$ (1.7 g, 13 mmol) was added in one portion, followed by $NaBH_4$ (890 mg, 23.5 mmol). The mixture was heated with reflux for 2 h, and then water (25 mL) was carefully added dropwise at 0 °C. The reaction mixture was extracted with CH_2Cl_2 (4 × 50 mL) and combined organic solutions were dried with Na_2SO_4 and evaporated in vacuo. Purification on SiO_2 (150 g; hexane/EtOAc, 6:1) furnished 970 mg (65%) of the titled compound. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.93 (s, 6 H, NMe_2), 3.78 (s, 3 H, OMe), 3.96 (s, 2 H, CH_2), 6.71 (m, 2 H, Ar), 6.78 (dd, J = 8.5, 2.6, 1 H, Ar), 7.05 (m, 3 H, Ar), 7.12 (d, J = 2.6, 1 H, Ar). ^{13}C NMR (100.7 MHz, $CDCl_3$): δ = 39.8(−), 40.8(+), 55.5(+), 110.0(−), 113.0(+), 113.6(+), 117.8(+), 124.8(−), 129.5(+), 131.2(+), 133.3(−), 149.1(−), 158.3(−). HRMS: found 320.0648; calcd for $C_{16}H_{18}NOBr$ [M + H]$^+$ 320.0645.

*2-(2-(4-(Dimethylamino)benzyl)-5-methoxyphenyl)-propan-2-ol (**6**).* Into a Schlenk flask charged with a solution of compound **5** (516 mg, 1.6 mmol) in THF (10 mL), 2.2 M BuLi in hexanes (2.5 mL, 5.4 mmol) was added dropwise at −78 °C. After stirring for 40 min at −78 °C, acetone (1.30 g, 22.4 mmol) was slowly added, and the reaction mixture was allowed to warm up to rt An excess of satd aq NH_4Cl was added, and the mixture was extracted with EtOAc (3 × 50 mL). The combined organic solutions were dried with Na_2SO_4 and evaporated in vacuo. The title compound was isolated as a colorless oil (453 mg, 95%) by column chromatography (100 g SiO_2, hexane/EtOAc, 2:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.63 (s, 6 H, CMe_2), 2.91 (s, 6 H, NMe_2), 3.81 (s, 3 H, OMe), 4.24 (s, 2 H, CH_2), 6.69 (m, 2 H, Ar), 6.73 (dd, J = 8.5, 2.7, 1 H, Ar), 6.97 (m, 2 H, Ar), 7.05 (m, 2 H, Ar). ^{13}C NMR (125.7 MHz, $CDCl_3$): δ = 31.6(+), 37.9(−), 40.8(+), 55.2(+), 73.8(−), 111.3(+), 112.3(+), 113.0(+), 129.3(+), 130.9(−), 134.1(+), 147.5(−), 149.0(−), 157.6(−). HRMS: found 322.1778; calcd for $C_{19}H_{25}NO_2$ [M + Na]$^+$ 322.1778.

*3-(Dimethylamino)-6-methoxy-10,10-dimethylanthracen-9(10H)-one (**7**).* Cyclization of compound **6** to 1,2-dihydroanthracene derivative was performed according to the known method,[33] and further oxidation to anthracenone **7** was carried out as described.[19] To a solution of compound **6** (100 mg, 0.33 mmol) in dry CH_2Cl_2 (4 mL), $AlCl_3$ (110 mg, 0.825 mmol) was added at 0 °C. The mixture was stirred for 6 h at 0 °C and for 10 h at rt. After quenching with 1 M aq NaOH (4 mL), the organic layer was separated and the aqueous solution was extracted with CH_2Cl_2 (3 × 10 mL). Combined organic solutions were dried with Na_2SO_4 and evaporated in vacuo. The residue was dissolved in acetone (4 mL), the solution was cooled down to −18 °C, and a powder of $KMnO_4$ (110 mg, 0.70 mmol) was added in small portions over 2 h. The reaction mixture was diluted with 2 volumes of CH_2Cl_2; MnO_2 was filtered off and washed with CH_2Cl_2, and the filtrate was evaporated to dryness. The title compound was isolated by column chromatography (50 g SiO_2; hexane/EtOAc, 4:1) as a yellow solid (30 mg, 31%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.71 (s, 6 H, CMe_2), 3.12 (s, 6 H, NMe_2), 3.91 (s, 3 H, OMe), 6.77 (m, 2 H, Ar), 6.95 (dd, J = 2.5, 8.7, 1 H, Ar), 7.09 (d, J = 2.5, 1 H, Ar), 8.26 (d, J = 8.40, 1 H, Ar), 8.34 (d, J = 8.7, 1 H, Ar). ^{13}C NMR (125.7 MHz, $CDCl_3$): δ = 33.5(+), 38.2(−), 40.2(+), 55.4(+), 107.5(+), 110.9(+), 111.5(+), 112.2(+), 119.4(−), 124.3(−), 129.4(+), 129.5(+), 152.2(−), 152.5(−), 153.2(−), 162.9(−), 181.2(−). HRMS: found 296.1649; calcd for $C_{19}H_{21}NO_2$ [M + H]$^+$ 296.1645.

*N-(10-(2-((2-Amino-2-methylpropoxy)carbonyl)phenyl)-7-methoxy-9,9-dimethylanthracen-2(9H)-ylidene)-N-methyl methanaminium Chloride (**9**).* Into a Schlenk flask charged with a solution of 2-(2-bromophenyl)-4,4-dimethyl-4,5-dihy-

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drooxazole (926 mg, 3.60 mmol) in THF (15 mL), 1.5 M *t*-BuLi in pentane (2.6 mL, 3.9 mmol) was added dropwise at −78 °C to form the organolithium compound **8**. The mixture was stirred for 40 min at −78 °C, and a solution of ketone **7** (215 mg, 0.73 mmol) in THF (8 mL) was added dropwise. The reaction mixture was stirred at −78 °C for 1 h at rt and finally transferred into a cooled (0 °C) mixture of MeOH and AcOH (15 and 2 mL, respectively). The residue after complete evaporation of the reaction mixture was subjected to column chromatography (100 g SiO_2, MeCN/H_2O (both with 0.1% v/v TFA), 10:1 → 10:1). Fractions containing the title compound were combined, evaporated in vacuo, and residue was dissolved in CH_2Cl_2 and washed with brine and satd aq NH_4Cl. Evaporation of CH_2Cl_2 gave 270 mg (73%) of the title compound. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.36 (s, 3 H, Me), 1.43 (s, 3 H, Me), 1.75 (s, 3 H, Me), 1.84 (s, 3 H, Me), 3.43 (s, 3 H, NMe), 3.53 (s, 3 H, NMe), 3.94 (s, 3 H, OMe), 4.05 (d, J = 11.7, 1 H), 4.22 (d, J = 11.7, 1 H), 5.82 (s, broad, NH_2), 6.83 (m, 2 H, Ar), 7.17 (m, 2 H, Ar), 7.29 (d, J = 2.4, 1 H, Ar), 7.72 (m, 2 H, Ar), 8.69 (m, 1 H, Ar), 8.86 (m, 2 H, Ar). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 22.7(−), 22.8(+), 31.6(+), 31.9(−), 35.0(+), 41.9(+), 42.0(−), 53.3(−), 56.1(+), 69.7(−), 112.2(+), 113.6(+), 115.7(+), 122.7(−), 124.3(−), 129.5(−), 129.6(+), 130.4(+), 132.5(+), 132.6(+), 135.9(+), 136.8(−), 139.9(−), 154.3(−), 158.8(−), 160.1(−), 164.4(−), 166.0(−), 168.1(−). HRMS: found 471.2633; calcd for $C_{30}H_{35}N_2O_3$ [M^+] 471.2642.

*2-(3-(Dimethyliminio)-6-methoxy-10,10-dimethyl-3,10-dihydroanthracen-9-yl)benzoate (**10**-Me) and 2-(3-(Dimethyliminio)-6-hydroxy-10,10-dimethyl-3,10-dihydroanthracen-9-yl) Benzoate (**10**-H).* A solution of compound **9** (200 mg, 0.40 mmol) in 20% aq HCl (30 mL) was stirred at 80 °C for 6.5 h. After cooling to rt, the reaction mixture was neutralized with solid $NaHCO_3$ and extracted with CH_2Cl_2 (4 × 20 mL). Combined organic extracts were dried with Na_2SO_4 and evaporated. The residue was subjected to column chromatography (100 g SiO_2, hexane/EtOAc, 2:1) to furnish 68 mg (43%) of **10**-Me and 40 mg (26%) of **10**-H as colorless powders. **10**-Me: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.76 (s, 3 H, Me), 1.87 (s, 3 H, Me), 2.99 (s, 6 H, NMe_2), 3.82 (s, 3 H, OMe), 6.50−6.65 (m, 2 H, Ar), 6.68 (m, 2 H, Ar), 6.92 (m, 1 H, Ar), 7.05 (m, 1 H, Ar), 7.15 (m, 1 H, Ar), 7.58 (m, 2 H, Ar), 8.00 (m, 1 H, Ar). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 32.4(+), 35.5(+), 38.6(−), 40.5(+), 55.3(+), 111.8(+), 112.3(+), 123.8(+), 124.4(−), 124.9(+), 126.9(−), 128.9(+), 129.3(+), 134.5(+), 146.4(−), 147.5(−), 155.3(−), 159.9(−), 170.6(−). HRMS: found 400.1898; calcd for $C_{26}H_{25}NO_3$ [M + H]$^+$ 400.1907. Spectal data in MeOH with 0.1% v/v TFA: λ_{abs}, nm (ε, $M^{-1}cm^{-1}$) = 482 sh (10290), 513 (18250), 549 (18430), λ_{em} = 585 nm, Φ_{fl} = 0.01 (standard: RDC, Φ_{fl} = 0.33 in THF).

10-H: ^{1}H NMR (600 MHz, $CDCl_3$): δ = 1.56 (s, 3 H, Me), 1.73 (s, 3 H, Me), 3.17 (s, 6 H, NMe_2), 6.64 (m, 2 H, Ar), 6.88 (m, 1 H, Ar), 7.03 (m, 2 H, Ar), 7.12 (m, 1 H, Ar), 7.61 (m, 2 H, Ar), 7.68 (m, 1 H, Ar), 8.05 (m, 1 H, Ar). HRMS: found 386.1747, calcd for $C_{25}H_{23}NO_3$ [M + H]$^+$ 386.1751. Spectal data in MeOH: λ_{abs} (ε, $M^{-1}cm^{-1}$) = 561 (620), λ_{em} = 598 nm, Φ_{fl} = 0.85 (standard: rhodamine 630, Φ_{fl} = 0.97 in ethanol). Low absorption in the visible range is explained by the fact that under neutral conditions, the equilibrium between the colorless "closed" form and the colored "open" form is shifted toward the former one (Scheme 2). Spectal data in MeOH with 0.1% v/v TFA: λ_{abs}, nm (ε, $M^{-1}cm^{-1}$) = 483 sh (13500), 515(30000), 553 (37400), λ_{em} = 585 nm (broad), Φ_{fl} = 0.11 (standard: perylene diimide KP174, Φ_{fl} = 0.58 in water). Spectal data in MeOH with 0.1% v/v Et_3N: λ_{abs}, nm (ε, $M^{-1}cm^{-1}$) = 560 (34300), λ_{em} = 598 nm, Φ_{fl} = 0.94 (standard: perylene diimide KP174, Φ_{fl} = 0.58 in water).

*N-(7-Methoxy-10-(2-((4-methoxy-4-oxobutyl)(methyl)-carbamoyl)phenyl)-9,9-dimethylanthracen-2(9H)-ylidene)-N-methylmethanaminium (**12**).* Into a Schlenk flask charged with a solution of compound **10**-Me (34 mg, 0.085 mmol) in dichloroethane (3 mL), $POCl_3$ (390 μL, 4.3 mmol) was injected. After stirring at 80 °C for 2 h, all volatile materials were evaporated in vacuo and the solid residue was dissolved in CH_3CN (5 mL). A solution of methyl 4-(methylamino)-butyrate hydrochloride (**11**) (71 mg, 0.425 mmol) in MeCN (3 mL) was added into the flask, followed by NEt_3 (360 μL, 2.5 mmol). After stirring at rt for 15 min, all volatile materials were removed in vacuo, and the residue was dissolved in CH_2Cl_2 (20 mL) and washed with a satd aq solution of NH_4Cl. The organic layer was separated, dried with Na_2SO_4, and evaporated. The titled compound (36 mg, 76%) was isolated by column chromatography (40 g SiO_2, MeCN/H_2O, 15:1) as a red solid. ^{1}H NMR (300 MHz, CD_3CN): δ = 1.34 (m, 2 H, CH_2), 1.65 (s, 3 H, Me), 1.72−1.82 (m, 2 H, C$\underline{H_2}$COOMe), 1.80 (s, 3 H, Me), 2.86 (s, 3 H, NMe), 3.11−3.23 (m, 2 H, NCH_2), 3.44 (s, 6 H, NMe_2), 3.53 (s, 3 H, COOMe), 3.94 (s, 3 H, OMe), 6.93 (m, 2 H, Ar), 7.17−7.24 (m, 2 H, Ar), 7.27 (m, 1 H, Ar), 7.39 (m, 1 H, Ar), 7.41 (m, 1 H, Ar), 7.56 (m, 1 H, Ar), 7.66 (m, 2 H, Ar). ^{13}C NMR (75.5 MHz, CD_3CN): δ = 22.5(−), 31.4 (−), 32.0(+), 35.0(+), 37.6(+), 42.6(+), 42.8(−), 46.7(−), 51.9(+), 57.0(+), 114.4(+), 114.5(+), 115.8(−), 116.5(+), 123.5(−), 124.2(−), 125.1(−), 128.0(+), 130.0(+), 130.2(+), 130.5(+), 131.1(+), 135.0(−), 137.0(+), 137.4(−), 141.3(+), 155.2(−), 160.0(−), 160.8(−), 166.8(−), 174.1(−). HRMS: found 513.2740; calcd for $C_{32}H_{37}N_2O_4$ [M^+] 513.2748.

*Methyl 4-(2-(6-(Dimethylamino)-10,10-dimethyl-3-oxo-3,10-dihydroanthracen-9-yl)-N-methylbenzamido)-butanoate (**13**-Me) and 4-(2-(6-(Dimethylamino)-10,10-dimethyl-3-oxo-3,10-dihydroanthracen-9-yl)-N-methyl-benzamido)-butanoic Acid (**13**-H).* Into a Schlenk flask charged with a solution of compound **12** (36 mg, 0.065 mmol) in CH_2Cl_2 (5 mL), 1 M solution of BBr_3 in CH_2Cl_2 (400 μL, 0.4 mmol) was added dropwise at rt The reaction mixture was stirred for 1 h and quenched with satd aq $NaHCO_3$ (15 mL). The organic layer was separated, and the aqueous phase was saturated with NH_4Cl and extracted with CH_2Cl_2 (5 × 30 mL). Combined organic solutions were dried with Na_2SO_4 and evaporated. Column chromatography (40 g SiO_2, CH_2Cl_2/MeOH, 20:1, then MeCN/H_2O, 5:1) afforded 16 mg (49%) of **13**-Me and 11 mg (23%) of **13**-H as dark-violet solids. **13**-Me was an ∼1:2.5 mixture of 2 diastereomers. ^{1}H NMR (300 MHz, CD_3CN, only the signals of the major isomer are given): δ = 1.46 (m, 2 H, CH_2), 1.55 (s, 3 H, Me), 1.68 (s, 3 H, Me), 1.86−1.94 (m, 2 H, C$\underline{H_2}$COOMe), 2.78 (s, 3 H, NMe), 3.09 (s, 6 H, NMe_2), 3.10−3.32 (m, 2 H, NCH_2), 3.53 (s, 3 H, COOMe), 6.17 (dd, J = 9.6, 2.0, 1 H, Ar), 6.55 (dd, J = 9.1, 2.6, 1 H, Ar), 6.66 (m, J = 2.0, 1 H, Ar), 6.81 (m, J = 9.1, 1 H, Ar), 6.92 (m, J = 9.6, 1 H, Ar), 6.97 (m, J = 2.6, 1 H, Ar), 7.31 (m, 1 H, Ar), 7.45 (m, 1 H, Ar), 7.56 (m, 2 H, Ar). ^{13}C NMR (75.5 MHz, CD_3CN): δ = 22.6(−), 31.4(−), 31.8(+), 35.5(+), 37.6(+), 40.5(+), 40.9(−), 46.5(−), 51.8(+), 110.4(+), 111.3(+), 120.6(−), 120.9(−), 121.9(−), 123.5(+), 124.9(+), 127.6(+), 129.1(+), 129.5(+), 131.4(+), 134.8(+), 136.1(−), 137.7(−), 139.0(+), 152.1(−), 154.2(−), 157.2(−), 169.4(−), 174.1(−), 184.2(−). HRMS: found 499.2592; calcd for $C_{31}H_{35}N_2O_4$ [M^+] 499.2591.

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Lifetime of the excited state (τ): 2.2 ns (aq PBS buffer). **13**-H was an ~1:2.5 mixture of two diastereomers, ^{1}H NMR (300 MHz, CD_3CN, only signals of the major isomer are given): δ = 1.38 (m, 1 H, CH_2), 1.43−1.57 (m, 1 H, CH_2), 1.53 (s, 3 H, Me), 1.66 (s, 3 H, Me), 1.83 (m, 1 H, CH_2COOMe), 1.93−2.00 (m, 1 H, CH_2COOMe), 3.09 (s, 3 H, NMe), 3.09−3.23 (m, 2 H, NCH_2), 3.27 (s, 6 H, NMe_2), 6.23 (dd, J = 9.6, 2.1, 1 H, Ar), 6.55 (dd, J = 9.1, 2.6, 1 H, Ar), 6.72 (m, 1 H, Ar), 6.81 (m, J = 9.1, 1 H, Ar), 6.95−6.99 (m, 2 H, Ar), 7.30 (m, 1 H, Ar), 7.45 (m, 1 H, Ar), 7.56 (m, 2 H, Ar). ^{13}C NMR (75.5 MHz, CD_3CN): δ = 23.0(−), 32.0(+), 32.7(−), 35.8(+), 37.9(+), 40.5(+), 47.1(−), 110.4(+), 111.5(+), 120.4(−), 121.5(−), 123.1(+), 124.5(+), 127.8(+), 129.5(+), 129.7(+), 131.4(+), 135.5(+), 135.6(−), 137.3(−), 140.0(+), 153.3(−), 154.6(−), 158.6(−), 170.1(−), 185.7(−). HRMS: found 485.2433; calcd for $C_{30}H_{32}N_2O_4$ $[M + H]^+$ 485.2435.

*Preparation of **13**-H from **13**-Me.* Compound **13**-Me (4 mg; 8 μmol) was stirred for 1 h at rt in a 10:15:0.7 mixture of H_2O, THF, and 1 M aq NaOH (2.5 mL). Then AcOH (1 mL) was added, and the reaction mixture was evaporated to dryness. Purification by flash chromatography (15 g SiO_2, MeCN/H_2O, 5:1) followed by evaporation, dissolution in CH_2Cl_2, and filtration through a fine glass filter afforded 4 mg (quantitative yield) of the solid title compound. HPLC: B/A = 30/70 to 100/0 in 25 min, detection at 550 nm, t_R = 7.6 min (100%). Data in methanol: λ_{abs} (ε, $M^{-1}cm^{-1}$) = 573 nm (41000), λ_{em} = 613 nm, Φ_{fl} = 0.64, τ = 3.95 ns. Excitation spectrum in methanol: emission at 625 nm was monitored and found to be maximal at λ_{excit} = 588 nm. Data in PBS buffer at pH 7.4: λ_{abs} = 586 nm; ε = 58600 M^{-1} cm^{-1}, λ_{em} = 613 nm, Φ_{fl} = 0.32 (standard: perylene diimide, Φ_{fl} = 0.58 in H_2O), τ = 2.54 ns.

*2,5-Dioxopyrrolidin-1-yl 4-(2-(6-(Dimethylamino)-10,10-dimethyl-3-oxo-3,10-dihydroanthracen-9-yl)-N-methyl benzamido)butanoate (**13**-NHS).* To a stirred solution of compound **13**-H (3 mg, 6.2 μmol) and *N*-hydroxysuccinimide (11 mg, 93 μmol) in MeCN/DMF mixture (2:1, 2 mL), HATU (10 mg, 25 μmol) was added followed by NEt_3 (16 μL, 112 μmol). The reaction mixture was stirred overnight and evaporated in vacuo. The residue was subjected to flash chromatography on SiO_2 (20 g, MeCN/H_2O, 10:1). The main fraction was filtered through a fine glass filter and lyophilized to give 3.1 mg (86%) of the title compound. HPLC: B/A = 30/70 to 100/0 in 25 min, detection at 550 nm, t_R = 9.7 min (100%). HRMS: found 582.2605; calcd for $C_{34}H_{35}N_3O_6$ $[M + H]^+$ 582.2599. The conjugate with goat antirabbit antibodies (AB256): λ_{abs} = 585 nm, λ_{em} = 613 nm, DOL = 10.9, Φ_{fl} = 0.04 in PBS buffer at pH 7.4 (standard: rhodamine 101, Φ_{fl} = 1 in EtOH). The conjugate with goat antirabbit antibodies (AB257): λ_{abs} = 586 nm, λ_{em} = 614 nm, DOL = 12.4, Φ_{fl} = 0.04 in PBS buffer at pH 7.4 (standard: perylene diimide KP174, Φ_{fl} = 0.58 in H_2O, excitation at 550 nm).

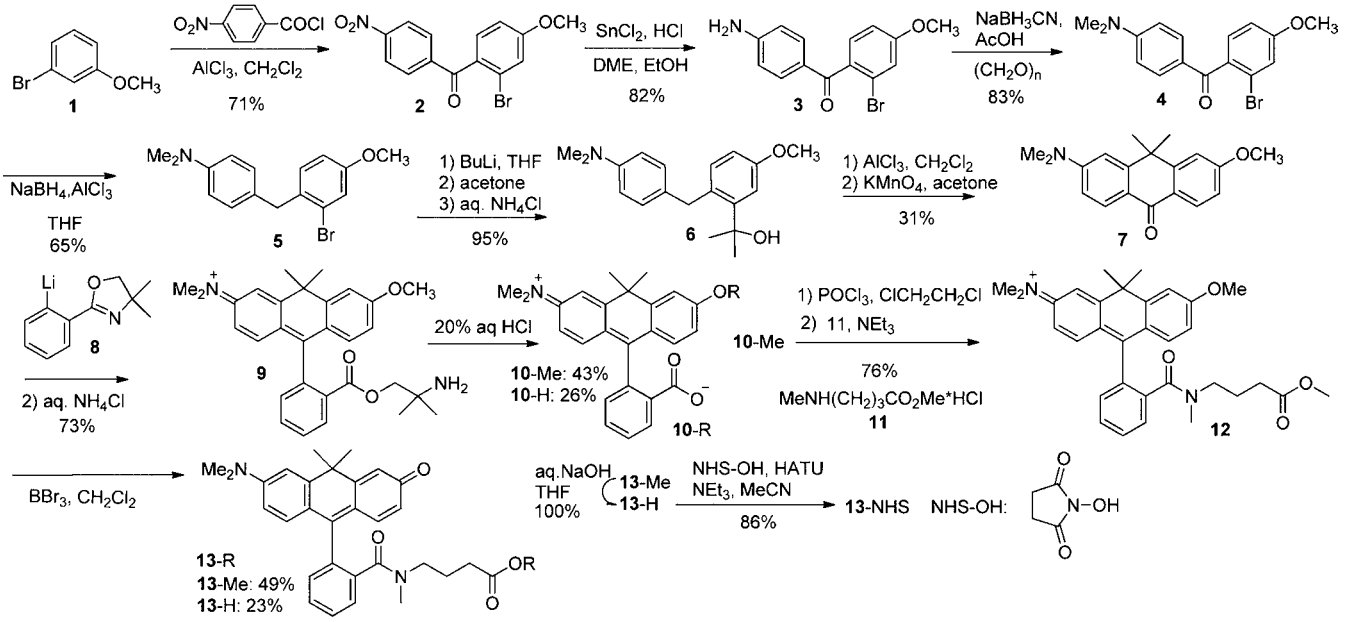
*2-(2-(2-(Methylamino)ethoxy)ethoxy)acetic Acid Hydrochloride (**15**-H×HCl).* One-pot reductive *N*-methylation of compound **14**[34] was accomplished according to the known method.[35] To a stirred solution of compound **14** (100 mg, 0.26 mmol) in a 1:1 mixture of TFA and $CHCl_3$ (3.5 mL), formaline (37% aqueous solution, 250 μL, 3.1 mmol) was added at +4 °C. After stirring at rt for 30 min, Et_3SiH (600 mg, 840 μL, 5.2 mmol) was added and the reaction mixture was stirred for additional 30 min. All volatiles were evaporated in vacuo, and the residue was subjected to column chromatography (50 g SiO_2, CH_2Cl_2/MeOH, 5:1 → 3:1) to yield 100 mg (96%) of compound **15**-Fmoc. It was dissolved in 3 mL of 30% piperidine in DMF, and the mixture was stirred for 30 min at rt. Next, an excess of 1 M aq HCl was added, and the mixture was extracted with Et_2O (3 × 20 mL). The aqueous solution was evaporated to dryness; the residue was dissolved in H_2O and freeze-dried. The title compound (20 mg, 74%) was isolated by column chromatography (25 g SiO_2, $CHCl_3$/MeOH/H_2O/HCOOH, 75:25:5:1) as a white powder. ^{1}H NMR (400 MHz, CD_3OD): δ = 2.41 (s, 3 H, NMe), 2.76 (m, 2 H), 3.60 (m, 2 H), 3.65 (m, 4 H), 3.89 (s, 2 H, CH_2COOH), 8.55 (s, 1 H, COOH). ^{13}C NMR (100.7 MHz, CD_3OD): δ = 38.4(+), 54.1(−), 72.9(−), 73.3(−), 73.4(−), 74.2(−), 180.3(−).

*Compounds **16**-H and **16**-NHS.* Amidation of the carboxyl group in compound **10**-H with amino acid **15**-H was performed similarly to the published procedure.[36] In a stirred solution of compound **10**-H (5 mg, 13 μmol) in dichloroethane (3 mL), $POCl_3$ was introduced (99 mg, 59 μL, 0.65 mmol). After stirring at 80 °C for 1.5 h, all volatile materials were evaporated in vacuo, THF (5 mL), **15**-H×HCl (3.4 mg, 19 μmol), and K_3PO_4 (6.9 mg, 32.5 μmol) were added into the flask. After 15 min of vigorous stirring, NEt_3 (50 μL, 0.35 mmol) was injected, and the reaction mixture was stirred overnight. All volatiles were evaporated, and the residue was subjected to column chromatography (6 g SiO_2, MeCN/H_2O, 7:1) to yield 3 mg (43%) of compound **16**-H. HPLC: B/A = 30/70 to 100/0 in 25 min, detection at 550 nm, t_R = 7.5 min (100%). The mixture of acid **16**-H (3 mg, 5.5 μmol), *N*-hydroxysuccinimide (0.8 mg, 6.6 μmol), HATU (3.1 mg, 8.3 μmol), and NEt_3 (5 μLl, 33 μmol) in MeCN (2 mL) was stirred overnight at rt. The reaction mixture was evaporated to dryness, and the residue was purified by column chromatography (5 g SiO_2, MeCN/H_2O, 10:1). The main fraction was freeze-dried to give 4 mg of **16**-NHS as violet solid. HPLC analysis indicated that the content of the title compound was ca. 45% (HPLC: B/A = 30/70 to 100/0 in 25 min, 550 nm, t_R = 9.6 min). This material was used directly in immunolabeling experiments without further purification.

Sample Preparation for Confocal and STED Microscopy. The cultivation of cells, antibody and immunolabeling was performed as described.[37] In brief, for immunolabeling, cultured mammalian cells were seeded on coverslips one day before the experiment. Fixation was performed using anhydrous methanol (−20 °C/5 min) or formaldehyde (4%/ RT/5 min). After extraction with Triton X100 in PBS (0.5%) and blocking in 5% bovine serum albumin, the cells were incubated in primary antibodies directed against the proteins ATP-Synthase (Abcam, Cambridge, UK), PMP70 (Abcam, Cambridge, UK), α-tubulin (Sigma-Aldrich, USA), or Vimentin (Sigma-Aldrich, USA). The detection of these primary antibodies was performed using secondary antibodies (Dianova) custom labeled with the dyes indicated. Finally, the samples were mounted in mowiol or 2,2′-thiodiethanol containing DABCO.

STED and Confocal Imaging. For the generation of crosstalk images, a confocal microscope (TCS SP5 Leica Microsystems, Mannheim, Germany) equipped with a argon ion laser (488 nm; 514 nm) and a HeNe Laser (633 nm) was used. For STED microscopy and the corresponding confocal microscopy, a beam scanning STED microscope was used.[38] In brief, the fluorophores were excited with a pulsed laser diode emitting 70 ps pulses at 532 nm (PicoQuant, Berlin, Germany). STED was performed using a femtosecond modelocked titanium sapphire laser (MIRA900, Coherent, USA) operating at a repetition rate of 76 MHz at 760 nm center wavelength. The STED pulses of originally ~100 fs duration were stretched

Scheme 1. General Approach to Carborhodols (Fluorescein−Carbopyronin Hybrids)

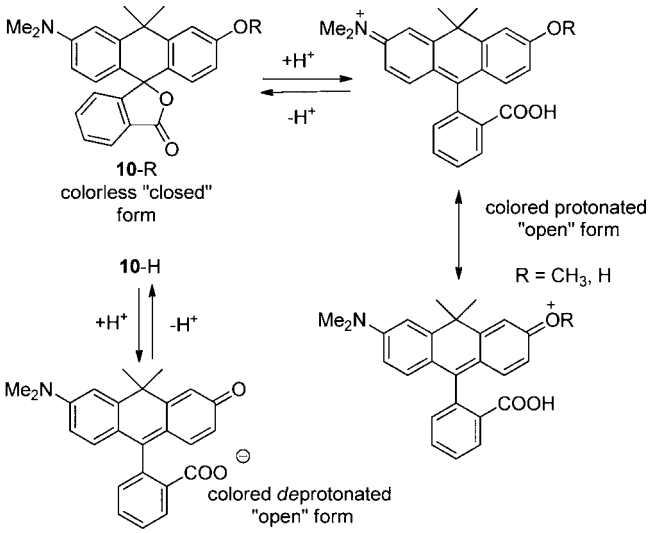


to about 300 ps by guiding the light through 100 m of a single-mode polarization-maintaining fiber. The optical delay between the excitation, and STED pulses was realized electronically. The STED beam was converted into a doughnut shape by passing the light through a polymeric phase plate (Vortex pattern, RPC Photonics, Rochester, NY, USA) and subsequently overlaid with the excitation beam. The excitation and the STED beams were coupled onto an oil immersion objective (NA 1.4 PL APO, 100×, Leica Microsystems, Wetzlar, Germany). The fluorescence signal was collected by the same lens and confocally detected between 650 and 690 nm using a counting avalanche photodiode (SPCM-AQRH13, Perkin-Elmer).

RESULTS AND DISCUSSION

Synthesis and Chemical Properties of Carborhodol Hybrids 13-H and 16-H. The most convenient and flexible approach to carbopyronines makes use of substituted 10,10-dimethylanthracen-9(10*H*)-ones.[19,20] Therefore, 3-(dimethylamino)-6-methoxy-10,10-dimethylanthracen-9(10*H*)-one (**7**) in Scheme 1 represents the simplest intermediate for the synthesis of the required fluorescein−carbopyronin hybrids. Compound **7** was obtained in 7 steps starting from *m*-bromoanisole (**1**) and *p*-nitrobenzoyl chloride. The most interesting feature of these transformations is the necessity to fully reduce the benzophenone keto group (in order to be able to perform bromine−lithium exchange by transforming compound **5** to compound **6**) and then to restore the keto group by oxidation of the intermediate 9,10-dihydro-10,10-dimethylanthracen derivative (not shown in Scheme 1). We failed to protect this keto group by transforming it to the corresponding dimethyl ketal.[39] On the way from bromide **1** to ketone **7**, the yields on all steps, except the last two (cyclization and oxidation), were very good. Having diaryl ketone **7** at hand and using the known methodology,[19,20] we easily performed the final transformations depicted in Scheme 1. Aryl lithium compound **8** was generated from the corresponding aryl bromide.[19,20] The ring-opening of the oxazolidinone cycle and formation of ester **9** occurred during the workup procedure, and the deprotection of the carboxylic group was completed by heating with 20% aq HCl. These harsh conditions caused the partial cleavage of the aryl methyl ether, and a mixture of two products was formed (**10**-Me and **10**-H). For the reaction with $POCl_3$, it was not necessary to keep the phenolic hydroxyl in compound **10**-H protected (to prevent its substitution with a chlorine atom and formation of the cyclic five-membered lactone ring, as in Scheme 2). Therefore, compound **10**-H was useful and could

Scheme 2. The Equilibrium between the Colorless "Closed" Forms of Compounds 10-R (R = CH_3, H) and Colored Protonated "Open" Forms Generated in Acidic Solutions[a]



[a]Deprotonation of compound **10**-H leads to the colored anionic form of this dye.

be "recycled". The carboxyl group in **10**-Me was activated by heating with $POCl_3$ in dichloroethane. The subsequent reaction with an excess of amino ester **11**[40] afforded amide **12** (along with *N*-methyl pyrrolidone which formed under basic conditions in the course of cyclization of **11**). Demethylation of amide **12** with BBr_3 was accompanied by the partial cleavage

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of both methyl groups and formation of the mixture **13**-H and **13**-Me. In this case, methyl ester **13**-Me could easily be transformed into the required acid **13**-H by saponification of the ester group (Scheme 1). The amino reactive *N*-hydroxysuccinimidyl ester **13**-NHS was obtained in acetonitrile 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). It was found to be fairly stable: upon storage of the sample at −20 °C under argon for one year, the content of the active ester was still about 70%.

We also prepared the similar hybrid dye **16**-H with a longer and more hydrophilic linker (Scheme 3). The motivation was to improve the hydrophilic properties of the dye and its imaging performance by reducing aggregation of the dye residues in aqueous solutions. Unfortunately, *N*-hydroxysuccinimidyl ester of **16**-NHS was less stable than compound **11**-NHS, and the quality of the images obtained with its bioconjugates was worse than in the case of ester **11**-NHS.

As a result of the chemical synthesis and evaluation of the stabilities of NHS esters, the hybrid fluorescein−carbopyronine dye **11**-H with a linker and reactive group was prepared from the readily available starting materials in 12 steps with overall yield of 1.6%.

Table 1. Properties of Compound 13-H, Dye KK114, and Their Conjugates with Antibodies (AB) in PBS Buffer at pH 7.4 and Room Temperature

compd	abs λ_{max} (nm)	em λ_{max} (nm)	$\varepsilon \times 10^{-5}$ (M^{-1} cm^{-1})	Φ_{fl} (%)	τ (ns)[a]	DOL[b]
13-H MeOH	573 broad	613	0.41	64	4.0	
13-H PBS	586	613	0.59	32	2.5	
13-H* AB	586 broad	613		39[c] 9[d]	1.3[c] 1.6[d]	5.2[c] 13[d]
KK114	636	660	0.9	53	3.6	
KK114* AB	639	660		40	3.6	2.2

[a]Lifetime of the excited state (S_1). [b]Degree of labeling: average amount of the dye residues attached to one antibody molecule with M ∼ 150 000. [c]Sheep-antimouse. [d]Goat-antirabbit (excitation at 540−560 nm).

Physical Properties and Imaging Performance of the Hybrid Carborhodol Dye 13-H. Table 1 presents the most important photophysical properties of the new dye **13**-H, its conjugate with antibodies, and the related data for compound KK114,[40] which was used together with **13**-H in two-color imaging. The fluorescence quantum yields were determined by comparison with reference dyes with known emission efficiencies (see Experimental Procedures for details).

The aqueous solutions of compound **13**-H are red-violet (absorption from 500 to 650 nm). The color is similar to that of Atto594, Alexa Fluor 594, or Abberior Star580 dyes. The color of the emitted light is red (580−750 nm). Interestingly, in methanol, dye **13**-H displays a broader absorption band with a blue-shifted maximum so that the solutions have a raspberry color. In methanol, the extinction coefficient is lower than in aqueous PBS buffer, but due to the broader band, the oscillator strength is approximately the same. The broader absorption band in methanol may be explained by the presence of the neutral and zwitterionic forms of this dye (Scheme 4). In aqueous solutions, the zwitterionic form is stabilized by hydrogen bonds, and the equilibrium in Scheme 4 is shifted to the right so that only one zwitterionic form dominates. This results in a narrower absorption band. Remarkably, in aqueous solutions, the absorption band of the antibody conjugates is broadened as well (Figure 2).

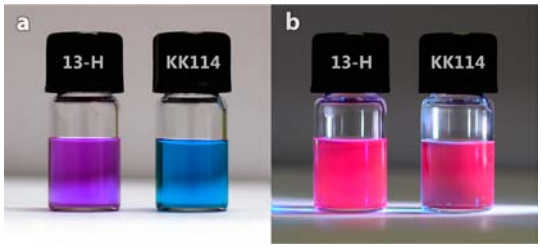


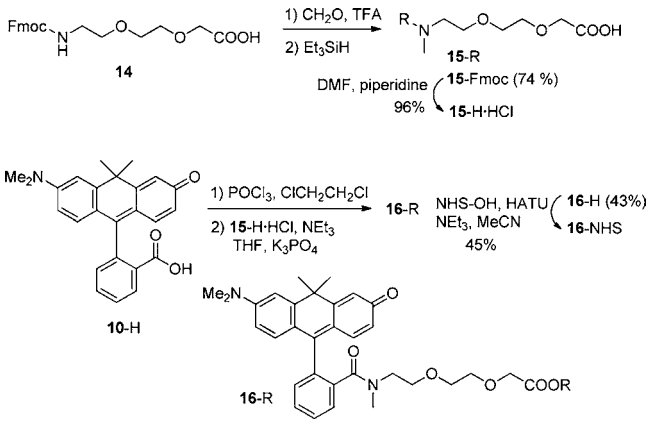
Figure 2. Color (a) and emission (b) of the dyes **13**-H and KK114 in aqueous solutions photographed with incandescent and luminescent light, respectively.

This effect may be explained by the presence of the amide bond which makes the zwitterionic form unattainable, and therefore, the protonation of these fluorophores results in the formation of ion pairs. The pK_a value of fluorescein is about 6.4, and we may expect a similar (somewhat higher) value for the amides derived from compound **13**-H. Therefore, under neutral conditions, the fluorophores of compound **13**-amide (Scheme 4) are expected to be in equilibrium between the protonated (charged) and neutral (uncharged) forms, and the actual absorption band may correspond to the superposition of the absorption curves of both species. An important feature of compound **13**-H is that it contains a *secondary* amide group which cannot participate in the formation of a colorless and nonfluorescent five-membered lactam (or lactone) ring (Scheme 2). Therefore, dye **13**-H is always fluorescent, even in unpolar and aprotic solvents, where carboxylic acids **10**-R (Scheme 2) give almost colorless and nonfluorescent solutions.

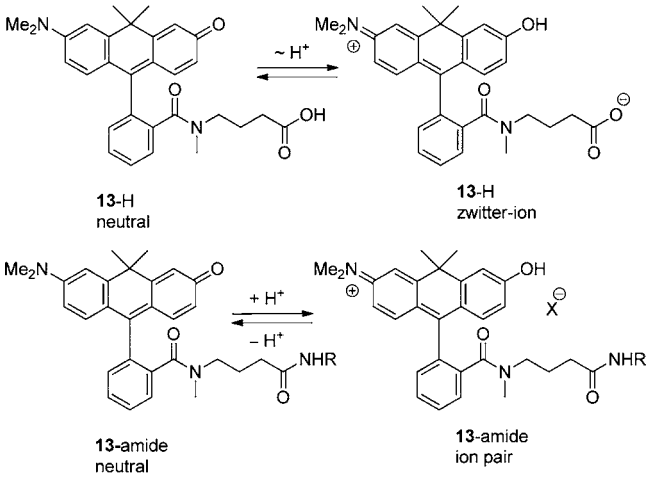
The fluorescence quantum yield (Φ_{fl}) of the free dye **13**-H in PBS buffer (0.32) was found to be lower than in methanol (0.64). For the antibody conjugates with a relatively high degree of labeling (DOL = 5.2), a quantum yield of 39% in aqueous PBS buffer was found. This is a relatively high value for a red-emitting fluorescent dye without ionic groups. The fluorescence quantum yields in conjugates with proteins often depend on the DOL values: higher quantum yields are observed at lower DOL values (see Table 1). On the other hand, for getting the highest imaging brightness, it is necessary to maximize the values of $\varepsilon \times \mathrm{DOL} \times \Phi_{fl}$. If we assume that the extinction coefficient (ε) is the same for the dye in the free state and in conjugates, then it will be necessary to optimize the product of DOL and Φ_{fl}. In this respect, the conjugates of dye **13**-H with DOL = 5.2 and Φ_{fl} = 39% are advantageous. The quantum yield decreased to 9% as DOL increased to 13, and further decreased to 4%, when the DOL value reached 15. However, even for some of the benchmark dyes, like Cy3, the similar Φ_{fl} value (4%) was reported (for the solutions in PBS buffer).[41]

An other important feature of the new hybrid dye is associated with the shortened fluorescence lifetimes (τ) when it is conjugated with antibodies (Table 1). The lifetimes were measured to be 1.2−1.6 ns, while KK114 dye and its bioconjugates invariably displayed constant τ values in the range of 3.3−3.7 ns.[42] Therefore, proteins labeled with the new hybrid dye **13**-H and KK114 may be discriminated by multilifetime STED imaging as previously described.[42]

Scheme 3. Synthesis of the Hybrid Fluorescein–Carbopyronin Dye 16-H with a Hydrophilic Linker



Scheme 4. Possible Equilibrium between the Neutral and Charged Forms of Dye 13-H and Its Amides



The Stokes shift of **13**-H (measured as the difference between the absorption and emission maxima) was found to be relatively small: ~28 nm. However, due to the broad absorption and emission bands, the "effective" value is higher (Figure 3). In other words, the hybrid compound **13**-H can be efficiently excited with green light (e.g., with a 514 nm line of an argon laser), while the emitted light is red (Figure 3). This is a distinguished and useful feature which is not typical for the parent dyes, rhodamines and carbopyronines. 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, two or more dyes emitting in a similar wavelength region with well separated absorption bands may be used for multicolor labeling and detection of colocalization between different biological targets. An advantage of this approach is that only one detection channel is used. The crosstalk observed in the course of the excitation with different light sources has to be low. Indeed, the spectral properties of compound **13**-H enabled its use in two-color imaging.

As a second dye, we used a very bright and photostable near-IR emitting fluorescent marker KK114 (for structure, see ref 40), which has repeatedly demonstrated its excellent imaging performance.[42] Its main spectral parameters are given in Table 1 and Figure 3. Conjugates of KK114 with antibodies demonstrated the same band widths in the absorption and emission spectra as the free dye, aside a very small red-shift of the absorption band in antibody conjugates of KK114 (see Figure 3 and Table 1).

The novel dye **13**-H provides an option for use with the benchmark dye KK114 in two-color imaging with the same detection channel (Figure 4). To evaluate the crosstalk between the channels, mammalian tubulin was labeled with compound **13**-H or Atto594 (Atto-tec GmbH) and KK114 dyes. Imaging was performed in a confocal microscope with two excitation lasers (514 and 633 nm), and emission was collected above 650 nm. Mowiol was used as an embedding medium. Only low level crosstalk was visible in the "KK114 channel" (10–15%). Atto594 displayed a high level of crosstalk (up to 60%) in the

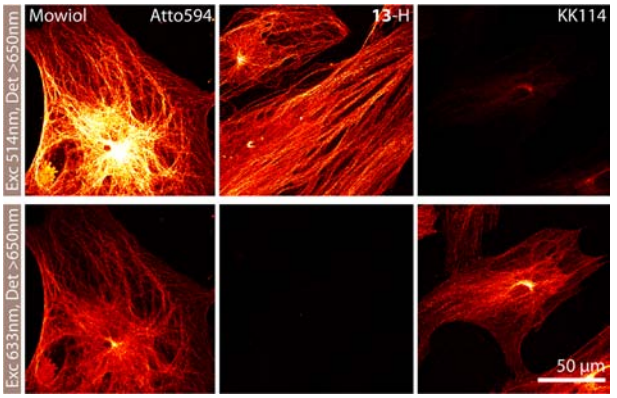


Figure 4. Immunofluorescence imaging and comparison of the crosstalk observed between compound **13**-H or Atto594 (Atto-tec GmbH), and KK114 dyes using mowiol as embedding medium, two excitation sources (514 and 633 nm), and one detection channel (650–750 nm); see text for details.

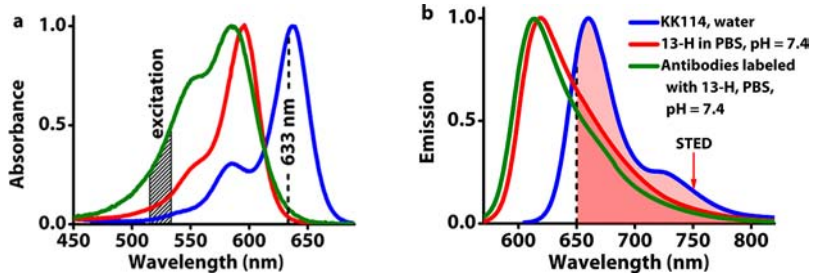


Figure 3. Normalized absorption (a) and emission (b) spectra of compound **13**-H, its conjugate with goat-anti rabbit antibody, and the reference dye KK114. Excitation regions (514–532 nm and 633–640 nm, respectively), detection area (650–800 nm), and a STED wavelength of 750 nm are shown.

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"KK114 channel", while **13**-H proved to have negligible crosstalk, as predicted.

As visible in Figures 4 and Figure S2 (in Supporting Information), the dye pair **13**-H/KK114 always provides much lower crosstalk than Atto594/KK114 pair (irrespective of the embedding medium). Therefore, these dyes may be advantageously used in two-color imaging and colocalization studies, even without linear unmixing or other image processing techniques. For example, Figure 5 shows fluorescent images

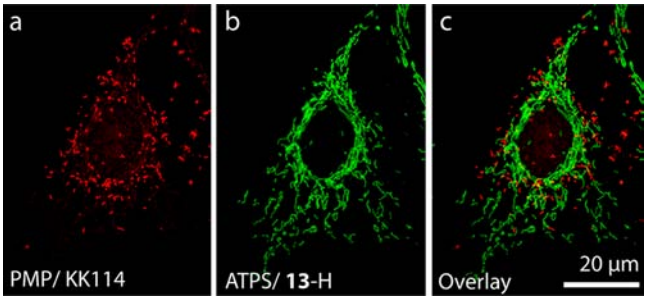

Figure 5. Dual color imaging with dyes KK114 and **13**-H. A subunit of the mitochondrial ATP synthase and the peroxysomal protein PMP70 were labeled in Vero cells using dyes **13**-H and KK114, respectively. Imaging was performed in a custom-made microscope; excitation with laser light at 640 nm (a) and 532 nm (b), respectively; confocal detection between 650 and 690 nm. (c) Overlay of (a) and (b). Please note that the "real" colors of the images are similar. To discriminate the structures, the pictures in the "KK114 channel" and "**13**-H channel" are pseudocolored in red and green, respectively.

of cells labeled with two dyes, **13**-H (mitochondria) and KK114 (peroxysomes), and embedded in Mowiol. Although some crosstalk is visible in the "KK114 channel", both objects are well discernible.

To demonstrate the applicability of compound **13**-H in super-resolution STED microscopy, we immunolabeled the vimentin cytoskeleton in Vero cells using compound **13**-H. Imaging was performed in a custom-built STED microscope with excitation wavelength of 532 nm (~40 μW) and a STED wavelength of 760 nm (~200 mW). The confocal detection was performed between 650 and 690 nm. As visible in Figure 6, compound **13**-H functions well in STED microscopy, even when the STED wavelength is shifted more than 140 nm to the red from the emission maximum of the dye. The "best" resolution of this STED microscope (using different dyes) is between 40 and 50 nm; the achieved optical resolution using compound **13**-H was in the range of 80 nm. The reason for this might be photobleaching. Indeed, the structure of the carborhodol dye contains "half" of fluorescein molecule, and fluorescein is known to be a poorly photostable xanthene dye. The photostabilities of compounds **13**-H and KK114 have been compared under STED conditions (see Figure S1 in Supporting Information), and it was shown that compound **13**-H bleaches significantly faster than KK114, which was shown to be one of the best STED dyes.[19] Trying to improve the photostability of hybrid carborhodol dyes, we planned to replace three hydrogen atoms in the fluorescein region with fluorine substituents. The similar (but much simpler) structural modification converts fluorescein to the Oregon Green dye and considerably increases its resistance against photobleaching. Detailed descriptions of the several synthetic routes are given in Scheme S1 (see Supporting Information). Although we failed to prepare the fluorinated analogue of compound **8**, the

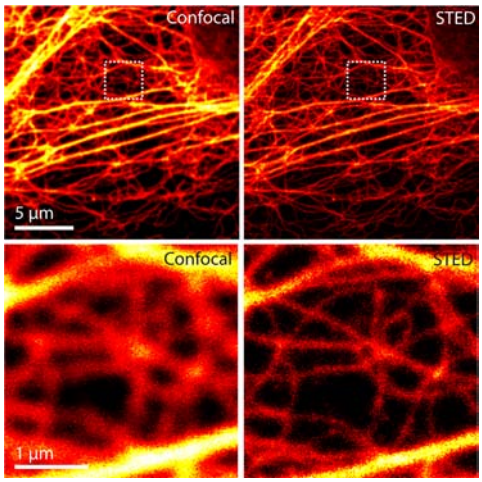

Figure 6. STED imaging with compound **13**-H. The vimentin cytoskeleton of mammalian cells was labeled using antibodies labeled with compound **13**-H. Top: confocal and STED images of the same region of the sample. Bottom: close-up of the boxed region in the upper panels.

transformations presented in Scheme S1 in Supporting Information may be useful for the design of the other fluorinated 10,10-dimethylanthracen-9(10*H*)-ones.

In total, these results show that the novel carborhodol dye **13**-H can be well used in multicolor imaging and super resolution STED microscopy. In the future it may be applied in dual-color super-resolution imaging together with conventional red-emitting fluorophores like ATTO647N, KK114, or Abberior Star635(P). It is important to note that even without the presence of the polar and/or ionizable groups, carborhodol dye **13**-H provides images with high contrast and without visible fluorescence background (Figures 4–6). On the contrary, in order to suppress the unspecific binding, most red-emitting fluorescent dyes like Atto594, Alexa Fluor 594, Alexa Fluor 633, KK114, Abberior Star635(P), have two negatively charged sulfonic (phosphoric) acid residues which not only increase the size of these markers and their molecular mass but also inhibit the penetration of their derivatives into the interior of cellular organelles.

■ CONCLUSION AND OUTLOOK

A new hybrid dye was prepared by "crossbreeding" carbopyronines with rhodols. Rhodols are, in turn, asymmetric hybrid dyes derived from fluorescein and rhodamines, so that the true ancestors of caborhodols are fluorescein and carbopyronines. A general approach to the synthesis of the amino reactive carborhodols was developed. The new hybrid dye provides bright protein conjugates and low crosstalk in two-color imaging when used with the established red-emitting fluorescent dyes, such as KK114 (and spectrally similar dyes Atto647N, Abberior Star635, Alexa Fluor 647, Cy5, etc). Another remarkable feature is the relatively short fluorescence lifetime in conjugates with antibodies (τ = 1.2–1.6 ns). Therefore, they can be used in microscopy schemes based on multilifetime discrimination together with other dyes that possess longer lifetimes.[43]

The synthetic route to fluorescein–carbopyronin hybrids given in Scheme 1 can be applied for the preparation of structurally diverse dyes with extended conjugation (e. g., compounds with 1,2-dihydro-1,2,2,4-tetramethylquinoline frag-

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ments). However, the crucial aspect of this approach, the necessity of the halogen−lithium exchange at the two key steps, restricts the synthetic freedom. The use of butyl lithium requires special protecting groups which are stable against this reagent.

Another prospective piece of research is the future evaluation of the cell permeability of carborhodol **13**-H and its derivatives.

■ ASSOCIATED CONTENT

Supporting Information

Comparison of photostabilities of compounds **13**-H and KK114; attempts to obtain carborhodol precursors with three fluorine substituents in the fluorescein fragment; crosstalk between Atto594, **13**-H, and STAR635P dyes in TDE (2,2′-thiodiethanol) embedding medium. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AcOH, acetic acid; ACN, acetonitrile; AB, antibodies; (*t*)BuLi, (*tert*-)butyl lithium; DME, 1,2-dimethoxyethane; DMF, *N*,*N*-dimethylformamide; DOL, degree of labeling; ESI, electrospray ionization; Et_3N, triethylamine; EtOH, ethanol; FLIM, fluorescence lifetime imaging microscopy; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluoro phosphate; HRMS, high resolution mass-spectrometry; NHS-OH, *N*-hydroxysuccinimide; MeOH, methanol; STED, stimulated emission depletion; THF, tetrahydrofurane; TFA, trifluoroacetic acid; Φ_{fl}, fluorescence quantum yield; τ, fluorescence lifetime

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