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 “cроссbred” fluorescent dyes were obtained by the combination of hemicyanine dyes with coumarin6 or benzopyrylim 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 reagents6,7,9−13 and rarely used as fluorescent labels in light microscopy.8 In this respect, the combination of fluorescein and carbopyronine dyes14−20 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 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–carbopyronine 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. 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. 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.

The synthesis of “pure” carbopyronines is a challenging task because in all available publications, except the most recent, preparation procedures were described either incompletely or for simple derivatives only. 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, 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 an 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, CH3CN + 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 13C signals of the methyl (CH3) and methyne (CH) groups are “positive” (+), while the signals of methylene groups (CH2) are “negative” (−). The reagent solutions were introduced via syringes through septa into the Schlenk flasks connected with a vacuum–argon manifold.

**Synthesis.** (2-Bromo-4-methoxyphenyl)(4-nitrophenyl)methanone (2). A dry 50 mL Schlenk flask was charged with AlCl3 (1.73 g; 13 mmol) and CH2Cl2 (13 mL). To this suspension, 3-bromoanisole 1 (2.5 g, 13 mmol) was added.
under vigorous stirring at 0 °C. After warming up to rt, a solution of p-nitrobenzoyl chloride (2.73 g, 15 mmol) in CH2Cl2 (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, and the aqueous phase was extracted with CH2Cl2 (2 × 100 mL), and the combined organic solutions were washed with 1 M NaOH (3 × 50 mL) and dried with Na2SO4. 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 yellow powder. 1H NMR (300 MHz, CDCl3): δ = 3.90 (s, 3 H, OMe), 6.69 (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). 13C NMR (100.7 MHz, CDCl3): δ = 55.8(+), 113.4(+), 119.1(+), 121.5(-), 130.3(+), 131.7(+), 142.0(-), 150.3(-), 162.1(-), 193.8(-). HRMS: found 335.9863; calcd for C14H10NO4Br [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 SnCl2·2H2O (2.64 g, 11.7 mmol) in conc 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 15% NaOH with ice, pH was adjusted to 12 with solid NaOH, and the solution was extracted with CH2Cl2 (2 × 50 mL), AlCl3 (1.7 g, 13 mmol) was added in one portion, and the reaction mixture was stirred for 6 h at 0 °C. After warming up to rt, an excess of satd aq NH4Cl was added, and the mixture was extracted with EtOAc (3 × 50 mL). The combined organic solutions were dried with Na2SO4 and evaporated in vacuo. The title compound was isolated as a colorless oil (453 mg, 95%) by column chromatography (100 g SiO2, hexane/EtOAc, 2:1). 1H NMR (300 MHz, CDCl3): δ = 1.63 (s, 3 H, CMe2), 2.91 (s, 3 H, OMe), 3.81 (s, 3 H, OMe), 4.24 (s, 2 H, CH2), 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). 13C NMR (125.7 MHz, CDCl3): 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 C19H19NO2 [M + Na]+ 322.1778.

(3-Dimethylamino)-6-methoxy-10,10-dimethylanthracene-9(10H)-one (7). Cyclization of compound 6 to 1,2-dihydroanthracene derivative was performed according to the known method,55 and further oxidation to anthracenone 7 was carried out as described.56 To a solution of compound 6 (100 mg, 0.33 mmol) in dry CH2Cl2 (4 mL), AlCl3 (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 CH2Cl2 (3 × 10 mL). Combined organic solutions were dried with Na2SO4 and evaporated in vacuo. The residue was dissolved in acetone (4 mL), the solution was cooled down to −18 °C, and a powder of KMnO4 (110 mg, 0.70 mmol) was added in small portions over 2 h. The reaction mixture was diluted with 2 volumes of CH2Cl2, MnO2 was filtered off and washed with CH2Cl2, and the filtrate was evaporated to dryness. The title compound was isolated by column chromatography (50 g SiO2; hexane/EtOAc, 4:1) as a yellow solid (296 mg, 31%). 1H NMR (300 MHz, CDCl3): δ = 1.71 (s, 6 H, CMe2), 3.12 (s, 6 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). 13C NMR (125.7 MHz, CDCl3): 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 C19H18NO2 [M + H]+ 296.1645.
droxazole (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 SiO2, MeCN/H2O (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 CH2Cl2 and washed with brine and satd aq NH4Cl. 
Evaporation of CH2Cl2 gave 270 mg (73%) of the title 
compound. 1H NMR (300 MHz, CDCl3): 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, 
NH2), 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). 
13C NMR (75.5 MHz, CDCl3): 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−(−), 124.9−(−), 125.9−(−), 129.6+(+), 130.4+(+), 132.5+(+), 132.6+(+), 135.9+(+), 136.8−(−), 139.9−(−), 154.3−(−), 158.8−(−), 160.1−(−), 164.4−(−), 166.0−(−). HRMS: found 471.2633; calc for C30H35N3O3 [M+H] 
471.2642.

2-(3-(Dimethylamino)-6-methoxy-10,10-dimethyl-3,10-di-
hydroanthracen-9-yl)benzoate (10- Me) and 2-(3-(Dimethyl-
aminio)-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 NaHCO3 and extracted with CH2Cl2 (2 × 20 mL). 
Combined organic extracts were dried with Na2SO4 and 
evaporated. The residue was subjected to column chromatography 
(100 g SiO2, hex/EtOAc, 2:1) to furnish 68 mg 
compound. 1H NMR (300 MHz, CDCl3): δ = 1.34 (s, 2 H, CH2), 1.65 
(3 s, 3 H, Me), 1.72−1.82 (m, 2 H, CH2COOME), 1.80 (s, 3 H, Me), 2.86 (s, 3 H, NMe), 3.11−3.23 (m, 2 H, NCH2), 3.44 (s, 6 
H, NMe2), 3.53 (s, 3 H, COOMe), 3.94 (s, 3 H, OMe), 6.93 
(2 H, Ar), 7.17−7.24 (2 H, Ar), 7.27 (m, 1 H, Ar), 7.39 
(1 H, Ar), 7.41 (m, 1 H, Ar), 7.56 (m, 1 H, Ar), 7.66 (m, 2 
H, Ar). 13C NMR (75.5 MHz, CDCl3): δ = 22.5−(−), 31.4−(−), 32.0+(+), 35.0+(+), 37.6+(+), 42.6+(+), 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; calc for C31H30N3O6 [M+H] 
513.2748.

Methyl 4-(2-(6-(Dimethylamino)-10,10-dimethyl-3-oxo-
3,10-dihydroanthracen-9-yl)-N-methylbenzamido)-buteanoate 
(13-Me) and 4-(2-(6-(Dimethylamino)-10,10-dimethyl-3-oxo-
3,10-dihydroanthracen-9-yl)-N-methylbenzamido)-butanoic 
Acid (13-H). Into a Schlenk flask charged with a 
solution of compound 12 (36 mg, 0.065 mmol) in CH2Cl2 (5 
ml), 1 M solution of BuLi in THF (8 mL) was added dropwise at rt. The reaction mixture was stirred for 1 h and 
then charged with satd aq NaHCO3 (15 mL). The organic 
layer was separated, and the aqueous phase was saturated with 
NH4Cl and extracted with CH2Cl2 (5 × 30 mL). Combined 
organic solutions were dried with Na2SO4 and evaporated. 
Column chromatography (40 g SiO2, CH2Cl2/MEOH, 20:1, then 
MeCN/H2O, 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. 1H NMR (300 MHz, CD2CN, only 
the signals of the major isomer are given): δ = 1.46 (m, 2 
H, CH2), 1.55 (s, 3 H, Me), 1.68 (s, 3 H, Me), 1.86−1.94 (m, 2 
H, CH2COOME), 2.78 (s, 3 H, NMe), 3.09 (s, 6 H, NMe2), 3.10− 
3.32 (m, 2 H, NCH2), 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). 13C NMR (75.5 MHz, CD2CN): 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; calc for C33H25N3O4 [M]+ 
499.2591.
Lifetime of the excited state ($\tau$): 2.2 ns (aq PBS buffer). 13-H was an ~1:2:5 mixture of two diastereomers, $^1$H NMR (300 MHz, CD$_3$CN, only signals of the major isomer are given): $\delta = 1.38$ (m, 1 H, CH$_3$), 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$_2$COOCH$_3$), 1.93–2.00 (m, 1 H, CH$_2$COOCH$_3$), 3.09 (s, 3 H, NMe), 3.0–3.23 (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, 2 H, Ar), 7.45 (m, 1 H, Ar), 7.56 (m, 2 H, Ar). $^{13}$C NMR (75.5 MHz, CD$_3$CN): $\delta = 23.0$ (m), 32.0 (s), 32.7 (s), 38.8 (s), 37.9 (s), 40.5 (s), 47.1 (s), 110.4 (s), 111.5 (s), 120.4 (s), 121.5 (s), 123.1 (s), 124.5 (s), 127.8 (s), 129.5 (s), 129.7 (s), 131.4 (s), 135.5 (s), 135.6 (s), 137.3 (s), 140.0 (s), 153.3 (s), 154.6 (s), 158.6 (s), 170.1 (s), 185.7 (s). HRMS: found 582.2605; calcd for C$_{34}$H$_{35}$N$_3$O$_6$ $[M + H]^+$ to 582.2599. The conjugate with goat antirabbit antibodies was an ~1:2:5 mixture of two diastereomers, $^1$H NMR (613 nm, CD$_3$CN, only signals of the major isomer are given): $\delta = 2.41$ (s, 3 H, NMe), 2.76 (m, 2 H, Ar), 3.65 (m, 4 H, Me), 3.89 (s, 2 H, CH$_2$COOH), 8.55 (s, 1 H, COOH). $^{13}$C NMR (100.7 MHz, CD$_2$OD): $\delta = 38.4$ (s), 54.1 (s), 72.9 (s), 73.3 (s), 73.4 (s), 74.2 (s), 180.3 (s).

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. 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$_2$PO$_4$ (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$_2$O, 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, $R = 9.6$ min. The mixture was purified by column chromatography (5 g SiO$_2$, MeCN/H$_2$O, 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, detection at 550 nm, $R = 7.5$ 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 μL, 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$_2$O, 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, detection at 550 nm, $R = 9.6$ 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. 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 0.1% 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 and 2,2'-thiodiethanol containing DABCO.
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(10H)-ones. Therefore, 3-(dimethylamino)-6-methoxy-10,10-dimethylanthracen-9(10H)-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. 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, we easily performed the final transformations depicted in Scheme 1. Aryl lithium compound 8 was generated from the corresponding aryl bromide. 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 POCl₃, 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 be “recycled”. The carboxyl group in 10-Me was activated by heating with POCl₃ in dichloroethane. The subsequent reaction with an excess of amino ester 11 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 BBr₃ was accompanied by the partial cleavage

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

Scheme 2. The Equilibrium between the Colorless “Closed” Forms of Compounds 10-R (R = CH₃, H) and Colored Protonated “Open” Forms Generated in Acidic Solutions

“Deprotonation of compound 10-H leads to the colored anionic form of this dye.
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-hydroxysuccinimidyld ester 13-NHS was obtained in acetonitrile using 2-(7-aza-1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) in the presence of N-hydroxysuccinimide and base (Et3N). 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-hydroxysuccinimydyl 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

<table>
<thead>
<tr>
<th>compd</th>
<th>abs βmax (nm)</th>
<th>em λmax (nm)</th>
<th>ε × 10^4 (M^−1 cm^−1)</th>
<th>ΦB (%)</th>
<th>τ (ns)</th>
<th>DOL^B</th>
</tr>
</thead>
<tbody>
<tr>
<td>13-H MeOH</td>
<td>573</td>
<td>613</td>
<td>0.41</td>
<td>64</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>13-H PBS</td>
<td>586</td>
<td>613</td>
<td>0.59</td>
<td>32</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>13-H* AB</td>
<td>586</td>
<td>613</td>
<td>0.59</td>
<td>39⁵</td>
<td>1.3⁶</td>
<td>1.6⁷</td>
</tr>
<tr>
<td>13-H** AB</td>
<td>636</td>
<td>660</td>
<td>0.9</td>
<td>53</td>
<td>3.6</td>
<td>13²</td>
</tr>
<tr>
<td>KK114</td>
<td>639</td>
<td>660</td>
<td>0.9</td>
<td>40</td>
<td>3.6</td>
<td>2.2²</td>
</tr>
</tbody>
</table>

⁵Lifetime of the excited state (S₁). ⁶Degree of labeling: average amount of the dye residues attached to one antibody molecule with M ~ 150 000. ⁷Sheep-antimouse. ⁸Goat-antirabbit (excitation at 540–560 nm).

### Physical Properties and Imaging Performance of the Hybrid Carborohodol 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).

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 pKₐ 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 (ΦB) 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 ε × DOL × ΦB. 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 ΦB. In this respect, the conjugates of dye 13-H with DOL = 5.2 and ΦB = 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 Cy5, the similar ΦB 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

![Figure 2. Color (a) and emission (b) of the dyes 13-H and KK114 in aqueous solutions photographed with incandescent and luminescent light, respectively.](image-url)
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. 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 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.
“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 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 transformations presented in Scheme S1 in Supporting Information may be useful for the design of the other fluorinated 10,10-dimethylanthracen-9(10H)-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 two-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 carborhodols 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-
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 and (7-azabenzotriazol-1-yl)-N-fluorophosphate; HRMS, high resolution mass-spectrometry; STED, stimulated emission depletion; THF, tetrahydrofuran; TFA, trifluoroacetic acid; ΦF, fluorescence quantum yield; τ, fluorescence lifetime

**REFERENCES**


(8) See the structure of DY-630 (Dyomics GmbH).


(34) Amino acid 14 was kindly provided by Dr. Matthias Bischof (MPI BPC).


(39) By the reaction of compound 7 with HC(OηMe), in the presence of various acids, as well as by transforming benzophenone 7 to the corresponding diaryl dichloromethane (which was successful), followed by the reaction with MeONa (which failed to give the desired dimethyl ketal).


