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Supporting Information

“Reduced” Coumarin Dyes with an O-Phosphorylated 2,2-Dimethyl-4-(hydroxymethyl)-1,2,3,4-tetrahydroquinoline Fragment: Synthesis, Spectra, and STED Microscopy**

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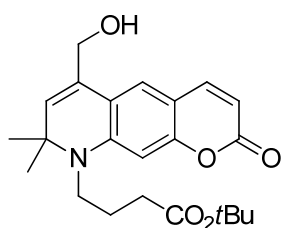
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Supporting Information

General remarks

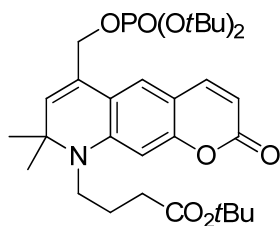
Reactions were carried out upon magnetic stirring in Schlenk flasks equipped with septa or reflux condensers with bubble-counters under argon using a standard manifold with vacuum and argon lines. Organic solutions were dried with anhydrous Na_2SO_4 , filtered, and the solvents were evaporated *in vacuo* using rotary evaporator. The microTOF mass spectrometer (Bruker) equipped with an ESI ion source and direct injection using an LC autosampler (Agilent RR 1200) and a binary pump (Agilent RR 1200) was applied for obtaining high resolution mass spectra (ESI-HRMS). 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. ESI-HRMS were obtained also on a maXis QTOF instrument (Bruker). MALDI-TOF measurements were performed using an autoflex speed mass spectrometer (Bruker) and sinapinic acid as a matrix. HPLC system (Knauer): Smartline pump 1000 (2 \times), 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 \times 4 mm, 1.2 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). Freeze-drying of the dye solutions in aqueous acetonitrile was performed with an ALPHA 2- 4 LD plus device with the cooler maintained at $-80\text{ }^\circ\text{C}$ (Martin Christ, Germany). Coupling constants (J) are given in Hz. In the APT and DEPT modes the ^{13}C signals of the methyl (CH_3) and methyne (CH) groups are “positive” (+), while the signals of methylene groups (CH_2) and quaternary carbons are negative (–).

Synthetic procedures



***tert*-Butyl 4-(6-(hydroxymethyl)-8,8-dimethyl-2-oxo-2*H*-pyrano[3,2-*g*]quinolin-9(8*H*)-yl)butanoate (2):**

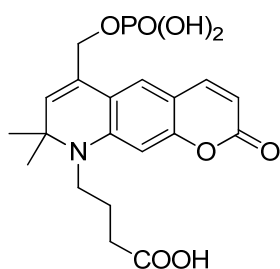
CeCl₃ (61 mg; 0.25 mmol) was added to a stirred and ice-cooled solution of compound **1** (100 mg; 0.25 mmol) in a THF/MeOH mixture (5 ml, 1:1). After complete dissolution, NaBH₄ (11 mg; 0.25 mmol) was added in one portion. The reaction mixture was stirred for 5 min, acetone (5 ml) was added, and the mixture was allowed to warm up to r. t. All volatile materials were evaporated *in vacuo*, the residue was taken up in water (10 ml) and extracted with CHCl₃ (3×10 ml). The combined organic solutions were dried and concentrated *in vacuo* to give a crude product. The title compound was isolated by column chromatography (25 g of SiO₂, CH₂Cl₂/MeOH, 25:1) as a yellow amorphous solid (94 mg, 94%). ¹H NMR (400 MHz, CDCl₃): δ = 1.39 (s, 6 H, 2×Me), 1.47 (s, 9 H, *t*Bu), 1.86 (m, 2 H, CH₂), 2.31 (t, *J* = 7.0 Hz, 2 H, CH₂), 3.28 (m, 2 H, CH₂), 4.45 (s, 2 H, OCH₂), 5.52 (s, 1 H), 6.01 (d, *J* = 9.3 Hz, 1 H), 6.34 (s, 1 H), 7.16 (s, 1 H), 7.49 (d, *J* = 9.3 Hz, 1 H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 22.9(–), 28.1(+), 29.0(+), 32.6(–), 43.9(–), 57.7(–), 63.0(–), 80.8(–), 97.5(+), 108.4(–), 109.5(+), 117.3(–), 122.2(+), 129.7(–), 129.8(+), 143.8(+), 147.6(–), 156.5(–), 162.1(–), 172.1(–) ppm. HRMS (C₂₃H₂₉NO₅): *m/z* (positive mode) = 422.1936 (found [M+Na]⁺), 422.1938 (calcd).



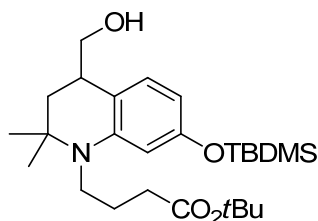
***tert*-Butyl 4-(6-((di-*tert*-butoxyphosphoryloxy)methyl)-8,8-dimethyl-2-oxo-2*H*-pyrano[3,2-*g*]quinolin-9(8*H*)-yl)butanoate (3):**

Di-*tert*-butyl *N,N*-diisopropylphosphoramidite (264 mg; 0.96 mmol) and 1*H*-tetrazole (71 mg; 1.02 mmol of the solid material) were added to a stirred and preheated (40 °C) solution of compound **2** (130 mg; 0.32 mmol) in CH₂Cl₂ (10 ml) in two equal portions at an interval of 20 min under Ar. After stirring for further 20 min, the reaction mixture was cooled with an ice bath (0 °C), and a solution of *m*CPBA (235 mg, content 70%, 0.96 mmol) in CH₂Cl₂ (5 ml) was added. After stirring for additional 30 min, aqueous solutions of Na₂SO₃ (4 ml; 10%) and NaHCO₃ (5 ml; saturated) were added, and the reaction mixture was allowed to warm up to r. t. The organic layer was separated and the aqueous phase was extracted with CH₂Cl₂ (3×20 ml). The combined organic extracts were dried, the solvents were evaporated, and the titled compound was isolated by column chromatography (30 g of SiO₂, hexane/EtOAc, 1:1) as a yellow amorphous solid (170 mg; 90%). ¹H NMR (400 MHz, CDCl₃): δ = 1.39 (s, 6 H, 2×Me), 1.47 (s, 9 H, *t*Bu), 1.48 (s, 18 H, 2×*t*Bu), 1.80–1.92 (m, 2 H, CH₂), 2.31 (t, *J* = 6.9 Hz, 2 H,

CH₂), 3.29 (m, 2 H, CH₂), 4.73 (d, J_{HP} = 6.9 Hz, 2 H, OCH₂), 5.58 (s, 1 H), 6.03 (d, J = 9.3 Hz, 1 H), 1.70 (s, 1 H), 7.15 (s, 1 H), 7.49 (d, J = 9.3 Hz, 1 H), 8.61 (s, 1 H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 22.9(-), 28.1(+), 29.9(+, J_{CP} = 4.3 Hz), 32.6(-), 43.9(-), 57.8(-), 66.1 (-, d, J_{CP} = 5.5 Hz), 80.8(-), 82.7(-, J_{CP} = 7.3 Hz), 97.5(+), 108.4(-), 109.7(+), 116.9(-), 122.4(+), 131.7(+), 143.6(+), 147.4(-), 156.6(-), 162.0(-), 172.1(-) ppm. ³¹P NMR (162 MHz, CDCl₃): δ = -9.63 ppm. HRMS (C₃₁H₄₆NO₈P): m/z (positive mode) = 614.2853 (found [M+Na]⁺), 614.2856 (calcd).

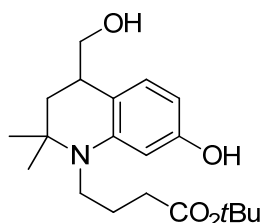


4-(8,8-Dimethyl-2-oxo-6-(phosphonooxymethyl)-2H-pyrano[3,2-g]quinolin-9(8H)-yl)butanoic acid (410-H): A solution of compound **3** (150 mg; 0.25 mmol) in TFA (1 ml) was stirred for 30 min at r. t. Afterwards, all volatiles were removed *in vacuo*. The residue was subjected to reverse-phase column chromatography (20 g of RP C₁₈ SiO₂ from Macherey Nagel, MeCN/H₂O, 1:10 with 0.05–0.1 M NEt₃·H₂CO₃ buffer with pH 8). The homogeneous fractions containing the title product were pooled and lyophilized. The solid residue was re-dissolved in water, and the solution was filtered through a Rotilabo syringe filter (0.20 μ m; diameter 13mm). The filtrate was freeze-dried to give 137 mg (88%) of the title product as a yellow powder (**410-H** * ~2NEt₃). ¹H NMR (500 MHz, CD₃OD): δ = 1.28 (t, J = 7.3 Hz, CH₃ in NEt₃), 1.42 (s, 6 H, 2×Me), 1.85–1.93 (m, 2 H, CH₂), 2.30 (t, J = 2.3 Hz, 2 H, CH₂), 3.15 (q, J = 7.3 Hz, CH₂ in NEt₃), 3.38 (m, 2 H, CH₂), 4.66 (d, J_{CP} = 4.7 Hz, 2 H, OCH₂), 5.69 (s, 1 H), 5.99 (d, J = 9.3 Hz, 1 H), 6.56 (s, 1 H), 7.39 (s, 1 H), 7.77 (d, J = 9.3 Hz, 1 H) ppm. ³¹P NMR (162 MHz, CD₃OD): δ = 0.92 ppm. ¹³C NMR (125 MHz, CD₃OD): δ = 9.2(+), 22.6(+), 25.3(-), 29.1(+), 35.0(-), 45.5(-), 47.6(-), 65.8(-, d, J_{CP} = 4.6 Hz), 98.1(+), 109.1(+), 109.7(-), 119.1(-), 124.1(+), 132.4(+), 146.4(+), 149.5(-), 157.8(-), 164.5(-), 176.4(-) ppm. HRMS (C₁₉H₂₂NO₈P): m/z (negative mode) = 422.1007 (found [M-H]⁻), 422.1010 (calcd).



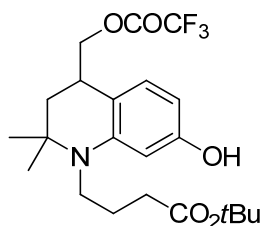
tert-Butyl**4-(7-(tert-butyldimethylsilyloxy)-4-(hydroxymethyl)-2,2-dimethyl-1,2,3,4-**

tetrahydroquinolin-1-yl)butanoate (5): Finely powdered SeO₂ (29.6 g; 0.266 mol) was added to a hot (70 °C) solution of compound **4** (79.0 g; 0.177 mol) in dioxane (600 ml). The dark brown reaction mixture was stirred at ~70 °C for 15 min and then cooled down to 0 °C. A mixture of THF and MeOH (60 and 20 ml, respectively) and NaBH₄ (16.8 g; 0.444 mol) was added thereto, and the reaction mixture was stirred for 2 days at r. t. After that, it was again cooled down to 0 °C, and the residual amount of NaBH₄ was quenched by careful addition of a MeOH-acetone mixture. When the gas evolution ceased (~2 h), the reaction mixture was filtered through a plug of SiO₂ (~100 g) eluting with EtOAc. The volatiles were evaporated *in vacuo*, and the residue was purified by column chromatography (300 g of SiO₂, Et₂O in *n*-hexane, 0%→50%) to afford 18.1 g of the title compound and ~15 g of an impure fraction which was re-purified using the same chromatography procedure. Overall yield: 26.4 g (33%). ¹H NMR (400 MHz, CDCl₃): δ = 0.21 (s, 6 H, 2×Me), 0.99 (s, 9 H, *t*Bu), 1.14 (s, 3 H, Me), 1.34 (s, 3 H, Me), 1.50 (s, 9 H, *t*Bu), 1.78–1.92 (m, 2 H, CH₂), 1.93–2.00 (m, 2 H, CH₂), 2.28 (t, *J* = 7.2 Hz, 2 H, CH₂), 2.98–3.06 (m, 2 H, CH₂), 3.25–3.36 (m, 1 H), 3.95–4.05 (m, 1 H, OCH₂), 4.37–4.47 (m, 1 H, OCH₂), 6.09–6.17 (m, 2 H), 6.97 (d, *J* = 8.2 Hz, 1 H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = -4.30(+), -4.32(+), 18.2(-), 24.2(+), 24.5(-), 25.7(+), 28.1(+), 29.7(+), 33.0(-), 34.9(+), 41.2(-), 44.6(-), 54.0(-), 65.6(-), 80.3(-), 104.2(+), 107.2(+), 115.2(-), 126.2(+), 145.0(-), 155.3(-), 172.5(-) ppm. HRMS (C₂₆H₄₅NO₄Si): *m/z* (positive mode) = 486.3015 (found [M+Na]⁺), 486.3010 (calcd).

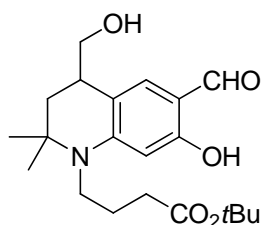
**tert-Butyl (7-hydroxy-4-hydroxymethyl-2,2-dimethyl-1,2,3,4-tetrahydroquinolin-1-yl)butanoate (6):**

TBAF·3H₂O (136 mg; 0.43 mmol) was added to a solution of compound **5** (200 mg; 0.43 mmol) in THF (10 ml) at 0 °C. After stirring for 5 min at 0 °C, TLC indicated that the starting material could not be detected in the reaction mixture. The reaction mixture was diluted with water (~20 ml) and extracted with EtOAc (3×). Combined organic extracts were dried with Na₂SO₄ and evaporated to dryness. The residue was subjected to flash chromatography using Isolera™ purification system: column SNAP Ultra 25 g, flow rate 50 ml/min, elution with EtOAc in *n*-hexane, gradient 12%→100%. Yield: 148 mg (99%) of the title compound as a brown solid. ¹H NMR (400 MHz, CDCl₃): δ = 1.12 (s, 3 H, Me), 1.33 (s, 3 H, Me), 1.47 (s, 9 H, *t*Bu), 1.75–1.85 (m, 2 H, CH₂), 1.87–2.00 (m, 2 H, CH₂), 2.28 (m, 2 H, CH₂), 2.90–3.06 (m, 2 H, CH₂),

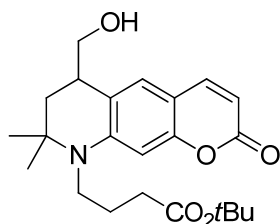
3.23–3.34 (m, 1 H), 3.92–3.97 (m, 2 H, OCH₂), 5.78 (br. s, 1 H, OH), 6.14 (dd, *J* = 8.1 and 2.3 Hz, 1 H), 6.17 (d, *J* = 2.2 Hz, 1 H), 7.00 (d, *J* = 8.1 Hz, 1 H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 24.1(+), 24.4(-), 28.1(+), 29.7(+), 32.9(-), 34.7(+), 41.1(-), 44.7(-), 54.0(-), 65.7(-), 80.8(-), 99.3(+), 102.6(+), 114.2(-), 126.6(+), 147.2(-), 155.7(-), 173.1(-) ppm. HRMS (C₂₀H₃₁NO₄): *m/z* (positive mode) = 350.2329 (found [M+H]⁺), 350.2326 (calcd).



tert-Butyl 4-(7-hydroxy-2,2-dimethyl-4-((2,2,2-trifluoroacetoxy)methyl)-1,2,3,4-tetrahydroquinolin-1-yl)butanoate (7): TFAA (70 mg; 0.34 mmol) and BF₃·Et₂O (18 mg; 0.13 mmol) were injected to a solution of compound **6** (50 mg; 0.14 mmol) in dry THF (5 ml) kept at 0 °C under Ar. After stirring for 2 h at 0 °C, the reaction mixture was poured into a cold 5% aq. solution of NaHCO₃. The resulting slurry was extracted with EtOAc (3×); the combined organic extracts were dried with Na₂SO₄ and evaporated. The residue was subjected to flash chromatography on Isolera™ purification system: column SNAP KP Sil 10 g, flow rate 25 ml/min, eluent EtOAc in *n*-hexane, gradient 5%→40%. Yield: 61 mg (98%) of the title compound as a yellow oil. ¹H NMR (400 MHz, CDCl₃): δ = 1.13 (s, 3 H, Me), 1.33 (s, 3 H, Me), 1.46 (s, 9 H, *t*Bu), 1.57–1.65 (m, 1 H), 1.72–1.97 (m, 3 H), 2.28 (t, *J* = 7.1 Hz, 2 H, CH₂), 3.03 (m, 1 H), 3.14–3.23 (m, 1 H), 3.24–3.33 (m, 1 H), 4.37 (dd, *J* = 10.7 and 8.0 Hz, 1 H), 4.76 (dd, *J* = 10.7 and 5.3 Hz, 1 H), 6.15 (dd, *J* = 8.1 and 2.3 Hz, 1 H), 6.18 (d, *J* = 2.2 Hz, 1 H), 6.56 (br. s, 1 H, OH), 6.89 (d, *J* = 8.1 Hz, 1 H) ppm. ¹⁹F NMR (376 MHz, CDCl₃): δ = -74.98 ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 24.3(-), 24.4(+), 28.1(+), 29.4(+), 31.9(+), 32.9(-), 41.2(-), 44.6(-), 53.9(-), 71.5(-), 80.5(-), 99.6(+), 102.9(+), 112.8(-), 126.7(+), 146.4(-), 156.4(-), 172.8(-) ppm. HRMS (C₂₂H₃₀F₃NO₅): *m/z* (positive mode) = 446.2148 (found [M+H]⁺), 446.2149 (calcd).

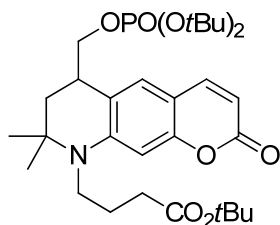


tert-Butyl 4-(6-formyl-7-hydroxy-4-(hydroxymethyl)-2,2-dimethyl-1,2,3,4-tetrahydroquinolin-1-yl)butanoate (8): POCl₃ (291 mg; 1.89 mmol) was added dropwise to DMF (2 ml) at 0 °C under Ar. This solution was allowed to warm up to r. t., and then again was cooled down to 0 °C. A solution of compound **7** (281 mg; 0.63 mmol) was added dropwise thereto and the reaction mixture was stirred at 0 °C for 2.5 h. After that, the reaction mixture was poured into ice mixed with sat. aq. NaHCO₃ solution and the obtained slurry was extracted with EtOAc (3×). Combined organic extracts were dried with Na₂SO₄ and evaporated *in vacuo*. The oily residue was subjected to column chromatography (30 g of SiO₂, *n*-hexane/EtOAc, 3:1 → 1:1) to afford 110 mg (46%) of the title product as a bluish oil. ¹H NMR (400 MHz, CDCl₃): δ = 1.23 (s, 3 H, Me), 1.42 (s, 3 H, Me), 1.47 (s, 9 H, *t*Bu), 1.68–1.77 (m, 1 H), 1.80–1.99 (m, 3 H), 2.30 (m, 2 H, CH₂), 2.92 (m, 1 H), 3.18 (s, 1 H), 3.41 (s, 1 H), 3.92–4.04 (m, 2 H, OCH₂), 6.03 (s, 1 H), 7.27 (m, 1 H), 9.5 (s, 1 H, CHO), 11.5 (s, 1 H, OH) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 23.7(–), 25.4(+), 28.1(+), 29.5(+), 32.8(–), 34.1(+), 40.2(–), 44.9(–), 55.5(–), 65.4(–), 80.8(–), 97.6(+), 111.2(–), 115.6(–), 130.8(+), 152.8(–), 163.0(–), 172.1(–), 192.1(+) ppm. HRMS (C₂₁H₃₁NO₅): *m/z* (positive mode) = 378.2273 (found [M+H]⁺), 378.2275 (calcd).

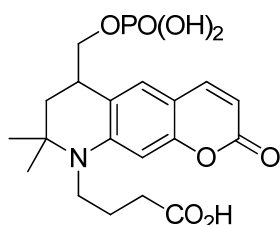


tert-Butyl 4-(6-(hydroxymethyl)-8,8-dimethyl-2-oxo-7,8-dihydro-2H-pyrano[3,2-*g*]quinolin-9(6H)-yl)butanoate (9): A solution of compound **8** (48 mg; 0.13 mmol) and triphenylcarbethoxymethylenephosphorane (51 mg; 0.15 mmol) in xylene (5 ml) was refluxed for 3 h. TLC indicated that the reaction mixture still contained significant amounts of the uncyclized intermediate. All volatile materials were removed *in vacuo*, the residue was dissolved in 1,2-dichlorobenzene (3 ml), and the solution was refluxed for additional 1.5 h. After cooling, the reaction mixture was subjected to column chromatography (30 g of SiO₂, CH₂Cl₂/Et₂O, 4:1) to afford 42 mg (82%) of the title compound as a yellow solid. ¹H NMR (400 MHz, CDCl₃): δ = 1.22 (s, 3 H, Me), 1.41 (s, 3 H, Me), 1.48 (s, 9 H, *t*Bu), 1.71–1.80 (m, 1 H), 1.82–1.93 (m, 3 H), 2.28–2.34 (m, 2 H, CH₂), 2.99 (m, 1 H), 3.14 (m, 1 H), 3.43 (m, 1 H), 3.95–4.06 (m, 2 H), 6.04 (d, *J* = 9.3 Hz, 1 H), 6.44 (s, 1 H), 7.27–7.29 (m, 1 H), 7.52 (d, *J* = 9.3 Hz, 1 H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 23.5(–), 25.3(+), 28.1(+), 29.5(+), 32.8(–), 34.7(+), 40.3(–), 44.8(–), 55.0(–), 65.4(–), 80.7(–), 98.3(+), 108.3(–), 109.6(+), 120.6(–), 124.7(–), 143.6(+),

149.0(-), 155.3(-), 162.3(-), 172.2(-) ppm. HRMS ($C_{23}H_{31}NO_5$): m/z (positive mode) = 402.2271 (found $[M+H]^+$), 402.2275 (calcd).

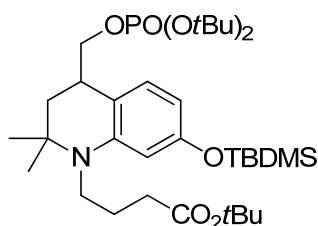


tert-Butyl 4-(6-((di-tert-butoxyphosphoryloxy)methyl)-8,8-dimethyl-2-oxo-7,8-dihydro-2H-pyrano[3,2-g]quinolin-9(6H)-yl)butanoate (10): 1H-Tetrazole (8.4 mg; 0.12 mmol) was added to a boiling solution of compound **9** (42 mg; 0.10 mmol) and $iPr_2NP(OtBu)_2$ (35 mg; 0.12 mmol) in CH_2Cl_2 (5 ml). After refluxing for 15 min, the second portion of $iPr_2NP(OtBu)_2$ (35 mg; 0.12 mmol) and 1H-tetrazole (8.4 mg; 0.12 mmol) were added. After refluxing for further 15 min, TLC indicated that the starting material had been consumed. The reaction mixture was cooled to 0 °C, and a solution of *m*CPBA (content 70%, 42 mg; 0.24 mmol) in CH_2Cl_2 (1 ml) was added. After stirring at 0 °C for 15 min, sat. aq. solutions of $Na_2S_2O_3$ (1 ml) and $NaHCO_3$ (1 ml) were added. The organic layer was separated and the aq. layer was extracted with CH_2Cl_2 (3×). Combined organic solutions were dried with Na_2SO_4 and evaporated. The residue was subjected to column chromatography (30 g of SiO_2 , CH_2Cl_2/Et_2O , 4:1) to afford 50 mg (85%) of the title product as a yellow oil. 1H NMR (400 MHz, $CDCl_3$): δ = 1.40 (s, 3 H, Me), 1.48 (s, 9 H, *t*Bu), 1.48–1.49 (m, 18 H, $2 \times tBu$), 1.51 (s, 3 H, Me), 1.67–1.77 (m, 3 H), 1.82–1.92 (m, 2 H, CH_2), 2.32 (t, J = 7.0 Hz, 2 H, CH_2), 3.30 (m, 2 H, CH_2), 4.73 (dd, J = 6.9 and 0.8 Hz, 2 H, OCH_2), 6.04 (d, J = 9.3 Hz, 1 H), 6.36 (s, 1 H), 7.16 (s, 1 H), 7.50 (d, J = 9.3 Hz, 1 H) ppm. ^{31}P NMR (162 MHz, $CDCl_3$): δ = -9.62 ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 22.9, 28.1, 28.8, 29.9 (d, J_{CP} = 4.3 Hz), 32.6, 43.9, 57.8, 66.1 (J_{CP} = 5.6 Hz), 80.8, 97.5, 108.4, 109.8, 117.0, 122.4, 126.3, 126.4, 131.7, 143.6, 147.4, 156.6, 162.0, 172.1 ppm. HRMS ($C_{31}H_{48}NO_8P$): m/z (positive mode) = 594.3191 (found $[M+H]^+$), 594.3190 (calcd).



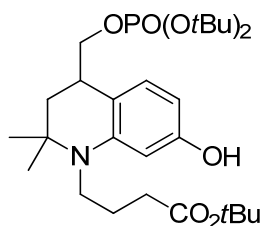
4-(8,8-Dimethyl-2-oxo-6-(phosphonooxymethyl)-7,8-dihydro-2H-pyrano[3,2-g]quinolin-9(6H)-yl)butanoic acid (400-H): A solution of compound **10** (203 mg; 0.34 mmol) in TFA (1 ml) was stirred for

30 min at r. t. All volatile materials were removed *in vacuo*, and the residue was subjected to reverse-phase column chromatography (15 g of RP C₁₈-SiO₂, Macherey-Nagel, MeCN/H₂O, 1:5 with 0.05 M NEt₃·H₂CO₃ buffer with pH 8). The homogeneous fractions containing the title product were pooled and freeze-dried. The solid residue was re-dissolved in water, and the solution was filtered through a Rotilabo syringe filter (0.20 µm; diameter 13 mm). The filtrate was freeze-dried to give 120 mg (83%) of the title product as a yellow powder. ¹H NMR (400 MHz, DMSO-*d*₆): δ = 1.24 (s, 3 H, Me), 1.43 (s, 3 H, Me), 1.66–1.77 (m, 1 H), 1.79–2.01 (m, 3 H), 2.22–2.31 (m, 2 H, CH₂), 3.15–3.21 (m, 2 H), 3.43–3.54 (m, 1 H), 4.13–4.21 (m, 1 H), 4.23–4.30 (m, 1 H), 5.97 (d, *J* = 9.2 Hz, 1 H), 6.64 (s, 1 H), 7.51 (s, 1 H), 7.77 (d, *J* = 9.3 Hz, 1 H) ppm. ³¹P NMR (162 MHz, DMSO-*d*₆): δ = 1.21 ppm. ¹³C NMR (100 MHz, DMSO-*d*₆): δ = 24.0(+), 24.7(-), 28.4(+), 33.3(+, d, *J*_{CP} = 8.4 Hz), 34.2(-), 40.2(-), 45.1(-), 54.9(-), 67.1(-, d, *J*_{CP} = 5.3 Hz), 97.4(+), 107.6(+), 108.1(-), 121.4(-), 125.4(+), 145.0(+), 149.5(-), 155.1(-), 163.2(-), 179.9(-) ppm. HRMS (C₁₉H₂₄NO₈P): *m/z* (negative mode) = 424.1167 (found [M-H]⁻), 424.1167 (calcd).

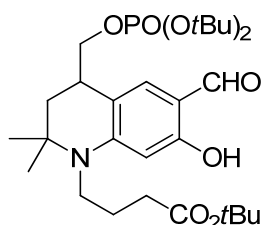


tert-Butyl 4-(7-(tert-butyldimethylsilyloxy)-4-((di-tert-butoxyphosphoryloxy)methyl)-2,2-dimethyl-1,2,3,4-tetrahydroquinolin-1-yl)butanoate (14): 1*H*-Tetrazole (3.73 g; 53.3 mmol) was added to a preheated (~40 °C) solution of compound **5** (13.70 g; 29.6 mmol) and *i*Pr₂NP(OTBu)₂ (12.3 g; 44.4 mmol) in CH₂Cl₂ (150 ml). The reaction mixture was stirred at 40 °C for 20 min, and then cooled down to 0 °C with an ice bath. A solution of *m*CPBA (9.6 g, content 70%; 44.4 mmol) in CH₂Cl₂ (100 ml) was added slowly, and after 2 min stirring, the reaction was “quenched” by addition of aq. solutions of NaHSO₃ (2%, 100 ml) and NaHCO₃ (sat., 150 ml). The organic phase was separated, the aq. solution was extracted with CH₂Cl₂ (2×), the combined organic solutions were dried over Na₂SO₄, and passed through a plug of SiO₂ (eluting with Et₂O). After evaporation of the solvents, the residue was subjected to column chromatography on SiO₂ (*n*-hexane/Et₂O, 3:1→1:1) to give 12.9 g (66%) of the title compound as a yellowish oil. ¹H NMR (400 MHz, CDCl₃): δ = 0.19 (s, 6 H, 2×Me, TBDMS), 0.97 (s, 9 H, *t*Bu, TBDMS), 1.12 (s, 3 H, Me), 1.32 (s, 3 H, Me), 1.45 (s, 9 H, *t*Bu), 1.48 (m, 18 H, 2×*t*Bu), 1.61 (t, *J* = 12.5 Hz, 1 H), 1.75–1.90 (m, 2 H, CH₂), 1.95 (dd, *J* = 13.1 and 5.2 Hz, 1 H), 2.26 (t, *J* = 7.2 Hz, 2 H, CH₂), 2.96–3.13 (m, 2 H, CH₂), 3.29 (m, 1 H, CH₂), 3.97 (m, 1 H, OCH₂), 4.41 (m, 1 H, OCH₂), 6.08–6.14 (m, 2 H), 6.95 (d, *J* = 8.1 Hz,

1 H) ppm. ^{31}P NMR (162 MHz, CDCl_3): $\delta = -9.67$ ppm. ^{13}C NMR (100 MHz, CDCl_3): $\delta = -4.4(+)$, $-4.3(+)$, $14.1(+)$, $18.2(-)$, $22.6(-)$, $24.4(+)$, $24.5(-)$, $25.7(+)$, $28.1(+)$, $29.5(+)$, $29.9(+)$, d, $J_{\text{CP}} = 4.2$ Hz), $33.0(-)$, $41.4(-)$, $44.5(-)$, $54.0(-)$, $70.1(-)$, d, $J_{\text{CP}} = 6.6$ Hz), $80.2(-)$, $82.1(-)$, $J_{\text{CP}} = 7.3$ Hz), $104.0(+)$, $107.1(+)$, $115.4(-)$, $126.5(+)$, $146.3(-)$, $155.2(-)$, $172.4(-)$ ppm. HRMS ($\text{C}_{34}\text{H}_{62}\text{NO}_7\text{PSi}$): m/z (positive mode) = 656.4107 (found $[\text{M}+\text{H}]^+$), 656.4106 (calcd).

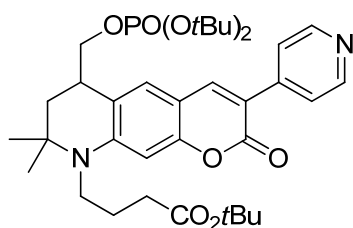


tert-Butyl 4-(4-((di-tert-butoxyphosphoryloxy)methyl)-7-hydroxy-2,2-dimethyl-1,2,3,4-tetrahydroquinolin-1-yl)butanoate (15): A solution of TBAF \cdot 3H $_2$ O (660 mg; 1.01 mmol) in THF (3 ml) was added to a cooled (0 °C) solution of compound **14** (662 mg; 1.01 mmol) in THF (3 ml). The reaction mixture was stirred for 5 min at 0 °C, and then sat. aq. NaCl was added. The organic phase was separated, and the aq. layer was extracted once with Et $_2$ O. The combined organic solutions were dried with Na $_2$ SO $_4$ and evaporated *in vacuo*. The residue was purified by column chromatography (25 g of SiO $_2$, Et $_2$ O in *n*-hexane, 0%→83%) to afford 490 mg (90%) of a bluish amorphous solid. ^1H NMR (400 MHz, CDCl_3): $\delta = 1.14$ (s, 3 H, Me), 1.34 (s, 3 H, Me), 1.48 (s, 9 H, *t*Bu), 1.50 (s, 9 H, *t*Bu), 1.53 (s, 9 H, *t*Bu), 1.77 – 2.00 (m, 4 H, 2 \times Me), 2.29 (t, $J = 7.0$ Hz, 2 H, CH $_2$), 2.99 – 3.16 (m, 2 H, CH $_2$), 3.25 – 3.35 (m, 1 H), 3.92 – 4.00 (m, 1 H, OCH $_2$), 4.44 – 4.51 (m, 1 H, OCH $_2$), 6.16 – 6.21 (m, 2 H), 6.98 (d, $J = 7.6$ Hz, 1 H) ppm. ^{31}P NMR (162 MHz, CDCl_3): $\delta = -10.07$ ppm. ^{13}C NMR (100 MHz, CDCl_3): $\delta = 14.0$, 22.5 , 24.2 , 24.4 , 28.0 , 29.4 , 29.7 (d, $J_{\text{CP}} = 4.2$ Hz), 29.8 (d, $J_{\text{CP}} = 4.2$ Hz), 31.4 , 32.9 , 41.5 , 44.4 , 53.8 , 70.6 (d, $J_{\text{CP}} = 6.4$ Hz), 80.3 , 82.5 (d, $J_{\text{CP}} = 2.4$ Hz), 82.6 (d, $J_{\text{CP}} = 2.3$ Hz), 99.3 , 102.9 , 113.1 , 126.6 , 146.3 , 156.7 , 172.8 ppm. HRMS ($\text{C}_{28}\text{H}_{48}\text{NO}_7\text{P}$): m/z (positive mode) = 542.3244 (found $[\text{M}+\text{H}]^+$), 542.3241 (calcd).



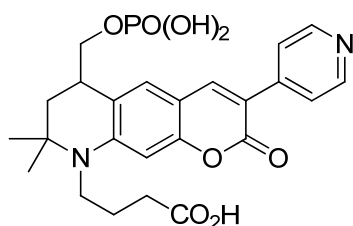
tert-Butyl 4-(4-((di-tert-butoxyphosphoryloxy)methyl)-6-formyl-7-hydroxy-2,2-dimethyl-1,2,3,4-tetrahydroquinolin-1-yl)butanoate (16): POCl $_3$ (194 mg; 1.26 mmol) was added to a cold (0–5 °C) DMF

(2 ml). The reagent solution was stirred for 5 min under cooling and for 15 min at r. t. Then it was cooled down again with an ice bath, and a solution of compound **15** (456 mg; 0.84 mmol) in DMF (2 ml) was added. The yellow reaction mixture was stirred for 5 min at 0 °C and for 4 h at r. t., and then was poured into a mixture of ice with sat. aq. NaHCO₃. The slurry was stirred until its color became pale yellow and then extracted with an EtOAc/Et₂O mixture (1:1, 3×). The combined organic solutions were dried with Na₂SO₄ and evaporated *in vacuo*. The residue was purified by column chromatography (50 g of SiO₂, Et₂O/*n*-hexane, 1:1→3:1) to afford 242 mg (50%) of a colorless viscous oil which gradually became bluish upon contact with air. ¹H NMR (400 MHz, CDCl₃): δ = 1.20 (s, 3 H, Me), 1.39 (s, 3 H, Me), 1.45 (s, 18 H, 2×*t*Bu), 1.46 (s, 9 H, *t*Bu), 1.59–1.67 (m, 1 H), 1.78–1.90 (m, 2 H, CH₂), 1.90–1.96 (m, 1 H), 2.27 (t, *J* = 7.0 Hz, 2 H, CH₂), 3.04 (m, 1 H), 3.16 (m, 1 H), 3.38 (m, 1 H), 4.09 (m, 1 H, OCH₂), 4.35 (m, 1 H, OCH₂), 6.01 (s, 1 H), 7.21 (d, *J* = 1.2 Hz, 1 H), 9.45 (s, 1 H, CHO), 11.45 (s, 1 H, OH) ppm. ³¹P NMR (162 MHz, CDCl₃): δ = –9.53 ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 23.6, 25.5, 28.1, 29.4, 29.9 (dd, *J*_{CP} = 4.3 and 1.0 Hz), 32.3 (d, *J*_{CP} = 7.9 Hz), 32.7, 40.4, 44.8, 55.5, 69.1 (d, *J* = 6.2 Hz), 80.7, 82.4 (d, *J*_{CP} = 7.4 Hz), 97.6, 111.1, 115.1, 131.1, 152.4, 163.0, 172.0, 192.1 ppm. HRMS (C₂₉H₄₈NO₈P): *m/z* (positive mode) = 592.3008 (found [M+Na]⁺), 592.3010 (calcd).

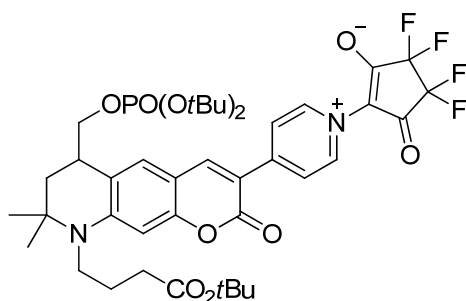


tert-Butyl 4-(6-((di-tert-butoxyphosphoryloxy)methyl)-8,8-dimethyl-2-oxo-3-(pyridin-4-yl)-7,8-dihydro-2H-pyrano[3,2-g]quinolin-9(6H)-yl)butanoate (17): NEt₃ (199 mg; 1.97 mmol), 4-pyridylacetic acid hydrochloride (116 mg; 0.67 mmol), DCC (211 mg; 1.02 mmol) and DMAP (5 mg; 0.04 mmol) were added to a solution of compound **16** (224 mg; 0.39 mmol) in CH₂Cl₂ (10 ml). The reaction mixture was stirred for 14 h at r. t., and then filtered through a glass filter. The filtrate was evaporated and passed through a plug of SiO₂ using a CH₂Cl₂/EtOAc/MeOH mixture (5:5:1) as an eluent. Afterwards, the volatile materials were removed *in vacuo*, and the residue was dissolved in acetone. The precipitated *N,N'*-dicyclohexylurea was removed by filtration, the filtrate was evaporated, and the residue was passed through a plug of SiO₂ (25 g of SiO₂) eluting with an acetone/Et₂O mixture (3:1). The eluate was evaporated to give 232 mg (89%) of the title product as a yellow oil. ¹H NMR (400 MHz, CDCl₃): δ = 1.28 (s, 3 H, Me), 1.45 (s, 3 H, Me), 1.49 (s, 9 H, *t*Bu), 1.51 (s, 9 H, *t*Bu), 1.53 (s, 9 H, *t*Bu), 1.73 (t, *J* = 12.9 Hz, 2

H, CH₂), 1.86–1.96 (m, 1 H), 2.01 (dd, *J* = 13.3 and 4.8 Hz, 1 H), 2.35 (m, 2 H, CH₂), 3.13–3.26 (m, 1 H), 3.42–3.53 (m, 1 H), 4.17–4.25 (m, 1 H, OCH₂), 4.38–4.46 (m, 1 H, OCH₂), 6.50 (s, 1 H), 7.37 (d, *J* = 1.1 Hz, 1 H), 7.69 (m, 2 H), 7.86 (s, 1 H), 8.65 (m, 2 H) ppm. ³¹P NMR (162 MHz, CDCl₃): δ = –9.41 ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 25.4, 25.5, 28.1, 29.3, 29.8 (d, *J*_{CP} = 3.2 Hz), 29.9 (d, *J*_{CP} = 3.1 Hz), 32.6, 33.1 (d, *J*_{CP} = 7.8 Hz), 40.3, 44.8, 55.3, 68.8 (d, *J*_{CP} = 6.2 Hz), 80.7, 82.4 (d, *J*_{CP} = 7.4 Hz), 82.5 (d, *J*_{CP} = 7.3 Hz), 97.6, 108.6, 117.4, 120.7, 122.2, 125.6, 141.8, 143.3, 149.4, 149.7, 155.3, 160.7, 172.0 ppm. HRMS (C₃₆H₅₁N₂O₈P): *m/z* (positive mode) = 671.3457 (found [M+H]⁺), 671.3456 (calcd).

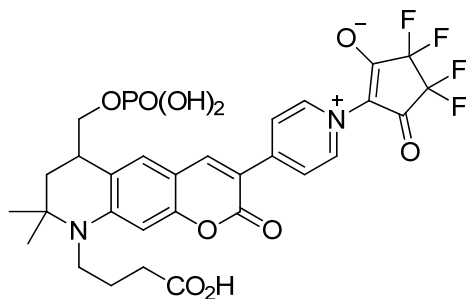


4-(8,8-Dimethyl-2-oxo-6-(phosphonooxymethyl)-3-(pyridin-4-yl)-7,8-dihydro-2H-pyrano[3,2-g]quinolin-9(6H)-yl)butanoic acid (430-py): Compound **17** (20 mg, 0.030 mmol) was dissolved in TFA (0.5 mL). After stirring for 4 h, TFA was evaporated *in vacuo* and the residue was triturated with ether. Decantation and drying afforded 16 mg (85%) of dye **430-py** as a brown solid. ¹H NMR (300 MHz, DMSO-*d*₆): δ = 1.10 (s, 3 H, Me), 1.27 (s, 3 H, Me), 1.34–1.52 (m, 1 H), 1.60–1.90 (m, 3 H), 2.17–2.35 (m, 2 H), 2.80–2.96 (m, 1 H), 3.06–3.22 (m, 1 H), 3.30–3.46 (m, 1 H), 3.74–3.90 (m, 1 H, OCH₂), 4.08–4.23 (m, 1 H, OCH₂), 6.53 (s, 1 H), 7.60 (s, 1 H), 7.76 (m, 2 H), 8.29 (s, 1 H), 8.51 (m, 2 H) ppm. ¹³C NMR (125 MHz, DMSO-*d*₆): δ = 23.2(–), 25.1(+), 31.7(–), 32.7(+), 44.2(–), 55.1(–), 66.5 (–, br. s.), 96.4(+), 108.0(–), 114.6(–), 121.8(+), 122.4(–), 126.2(+), 142.8(–), 143.0(+), 149.3(+), 149.4(–), 154.7(–), 159.8(–), 174.9(–) ppm. HRMS (C₂₄H₂₇N₂O₈P): *m/z* (positive mode) = 525.1378 (found [M+Na]⁺), 525.1397 (calcd).



2-(4-(9-(4-tert-Butoxy-4-oxobutyl)-6-((di-tert-butoxyphosphoryloxy)methyl)-8,8-dimethyl-2-oxo-6,7,8,9-tetrahydro-2H-pyrano[3,2-g]quinolin-3-yl)pyridinium-1-yl)-4,4,5,5-tetrafluoro-3-oxocyclopent-

1-enolate (18): In a screw-cap tube, perfluorocyclopentene (~1 ml) and a drop of AcOH were added to a cooled solution (-5 °C) of compound **17** (170 mg; 0.25 mmol) in *i*PrOH (10 ml). The resulting reaction mixture was stirred for 4 h at 80 °C. After cooling to r. t. and adding sat. aq. NaHCO₃ (1 ml), all volatile materials were evaporated, and the residue was subjected to column chromatography on SiO₂ (CH₂Cl₂/*i*Pr₂O, 3:1→CH₂Cl₂/*i*Pr₂O/acetone, 6:2:1). The residue obtained after evaporation of the eluent was triturated with *n*-hexane to give 130 mg (61%) of the title compound. ¹H NMR (400 MHz, CDCl₃): δ = 1.30 (s, 3 H, Me), 1.44 (s, 9 H, *t*Bu), 1.47 (s, 3 H, Me), 1.48 (s, 9 H, *t*Bu), 1.49 (s, 9 H, *t*Bu), 1.75 (t, *J* = 13.1 Hz, 2 H, CH₂), 1.83–1.95 (m, 2 H, CH₂), 2.00 (dd, *J* = 13.4 and 4.3 Hz, 1 H), 2.34 (t, *J* = 6.8 Hz, 2 H, CH₂), 3.15 (m, 1 H), 3.22–3.35 (m, 1 H), 3.44–3.57 (m, 1 H), 4.21–4.30 (m, 1 H, OCH₂), 4.32–4.41 (m, 1 H, OCH₂), 6.51 (s, 1 H), 7.47 (s, 1 H), 8.30 (s, 1 H), 8.48 (d, *J* = 7.4 Hz, 2 H), 9.48 (d, *J* = 7.4 Hz, 2 H) ppm. ¹⁹F NMR (376 MHz, CDCl₃): δ = -126.8 ppm. ³¹P NMR (162 MHz, CDCl₃): δ = -9.39 ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 23.3(-), 25.8(+), 28.1(+), 29.3(+), 29.9 (+, m, *J*_{CP} = 4.1 Hz), 32.5(-), 32.9 (+, d, *J*_{CP} = 7.7 Hz), 39.8(-), 45.2(-), 56.4(-), 68.3 (-, d, *J*_{CP} = 6.1 Hz), 81.0(-), 82.8 (-, m, *J*_{CP} = 7.2 Hz), 97.5(+), 108.9(-), 109.2 (-, tt, *J*_{CF} = 263.5 and 21.3 Hz), 110.8(-), 114.2(-, m, *J* = 5.3 Hz), 122.1(-), 123.0(+), 127.3(+), 138.9(+), 145.0(+), 149.7(-), 152.1(-), 156.8(-), 159.7(-), 171.9(-), 173.8(-, m, *J*_{CF} = 25.2 Hz) ppm. HRMS (C₄₁H₅₁N₂O₁₀F₄P): *m/z* (positive mode) = 861.3106 (found [M+Na]⁺), 861.3110 (calcd).



2-(4-(9-(3-Carboxypropyl)-8,8-dimethyl-2-oxo-6-(phosphonoxyethyl)-6,7,8,9-tetrahydro-2H-pyrano[3,2-*g*]quinolin-3-yl)pyridinium-1-yl)-4,4,5,5-tetrafluoro-3-oxocyclopent-1-enolate (510-bet): Compound **18** (6 mg, 7.1 μmol) was dissolved in TFA (0.5 mL). After stirring for 16 h at r. t., TFA was evaporated *in vacuo*, and the residue was triturated with ether to afford 4.3 mg (90%) of the title compound as a red solid.

¹H NMR (400 MHz, DMSO-*d*₆): δ = 1.24 (s, 3 H, Me), 1.41 (s, 3 H, Me), 1.63 (t, *J* = 13.1 Hz, 1 H), 1.68–1.85 (m, 2 H, CH₂), 1.96 (dd, *J* = 13.3 and 4.6 Hz, 1 H), 2.41 (t, *J* = 7.0 Hz, 2 H, CH₂), 3.10 (m, 1 H), 3.27 (m, 1 H), 3.53 (m, 1 H), 4.14 (m, 1 H, OCH₂), 4.31 (m, 1 H, OCH₂), 6.76 (s, 1 H), 7.57 (s, 1 H), 8.60 (d, *J* = 7.3 Hz, 2 H),

8.82 (s, 1 H), 9.10 (d, $J = 7.3$ Hz, 2 H) ppm. ^{19}F NMR (376 MHz, DMSO- d_6): $\delta = -126.04$ ppm. ^{31}P NMR (162 MHz, DMSO- d_6): $\delta = -1.08$ ppm. ^{13}C NMR (100 MHz, DMSO- d_6): $\delta = 23.3(-)$, $25.6(+)$, $29.1(+)$, $30.9(-)$, $32.8(+)$, d, $J_{\text{CP}} = 7.8$ Hz), $45.0(-)$, $56.4(-)$, $67.4(-)$, $J_{\text{CP}} = 4.5$ Hz), $97.3(+)$, $109.0(-)$, $110.0(-)$, tt, $J_{\text{CF}} = 262.7$ and 21.0 Hz), $110.6(-)$, 116.6 (-, m), $121.9(-)$, $123.2(+)$, $127.7(+)$, $141.5(+)$, $146.9(+)$, $150.8(-)$, $152.1(-)$, $156.6(-)$, $159.7(-)$, 172.1 (m, $J_{\text{CF}} = 26.6$ Hz), $174.8(-)$ ppm. HRMS ($\text{C}_{29}\text{H}_{27}\text{N}_2\text{O}_{10}\text{PF}_4$): m/z (negative mode) = 669.1279 (found $[\text{M}-\text{H}]^-$), 669.1267 (calcd).

General procedure for the preparation of NHS-esters

Stock solutions of *N*-hydroxysuccinimide (NHS-OH; ca 10 mg in 0.1 mL), Et_3N (ca 10 mg in 0.10 mL) and HATU (38 mg in 0.10 mL) in dry DMF were prepared. To a solution of the dye (ca. 5 mg) in dry DMF (0.3–1.0 mL depending on the solubility of the dye), the stock solutions of NHS-OH (1.5 eq.) and NEt_3 (3 eq.) were added, followed by a stock solution of HATU (1.2 eq.). All operations were performed under argon. It is advantageous to use as less DMF as possible, because the reaction proceeds faster in concentrated solutions. After the starting material was consumed (~30 min, TLC or HPLC control), all volatiles were removed *in vacuo*, and the solid residue was separated by reversed-phase chromatography (HPLC or flash chromatography with aqueous acetonitrile and TFA). Collecting, pooling and freeze-drying of the homogeneous fractions containing the NHS-ester afforded products with preparative yields ranging from 50 to 80%.

Supplementary Figures

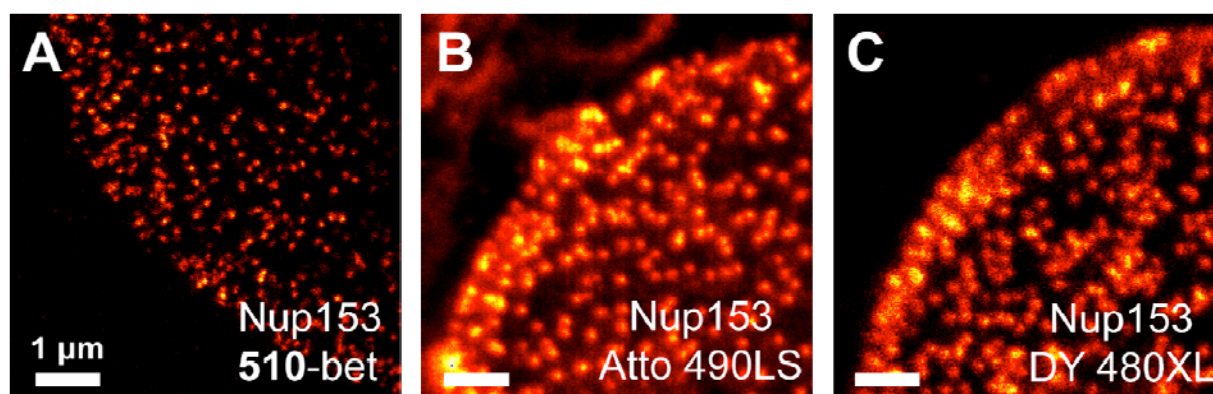


Figure S1 Comparative STED images of the immunolabeled nuclear pore complex protein Nup153 in mammalian cells (Vero cell line). Nup153 was labeled with the fluorescent dyes **510-bet**, Atto 490LS, and DY-480XL, respectively. Super-resolution images were acquired using an excitation and depletion beam at 532 nm and 750 nm, respectively. Fluorescence was collected within the detection window of a bandpass filter at (675 ± 30) nm for the dye compounds **510-bet** and Atto 490LS, and within the detection window of a bandpass filter at (630 ± 30) nm

for the dye DY 480XL. Image acquisition settings were optimized for each dye within a range of parameters that is described in detail further below in this *Supporting Information*. All images show smoothed data with normalized pixel brightness. Image smoothing was performed using a low-pass Gaussian filter with a width of 1.5 times the pixel size (corresponding to 30 nm). The scale bar is 1 μm in each image. Image resolution was determined from fitting a Lorentzian shaped curve to summed-up line profiles of Nup153 signal structures in the samples as is described further below in this *Supporting Information*. **A:** STED super-resolution image of Nup153 labeled with the dye compound **510**-bet. Excitation power and STED power in a backfocal plane of the objective: 40 μW and 280 mW, respectively. Image resolution: 50 nm. **B:** STED super-resolution image of Nup153 labeled with the dye Atto 490LS (ATTO-TEC, Siegen, Germany). Excitation power and STED power in a backfocal plane of the objective: 5 μW and 310 mW, respectively. Image resolution: 150 nm. Atto 490LS shows an unspecific background signal of unknown origin. **C:** STED super-resolution image of Nup153 labeled with the dye DY-480XL (Dyomics, Jena, Germany). Excitation power and STED power in a backfocal plane of the objective: 1 μW and 300 mW, respectively. Image resolution: 100 nm.

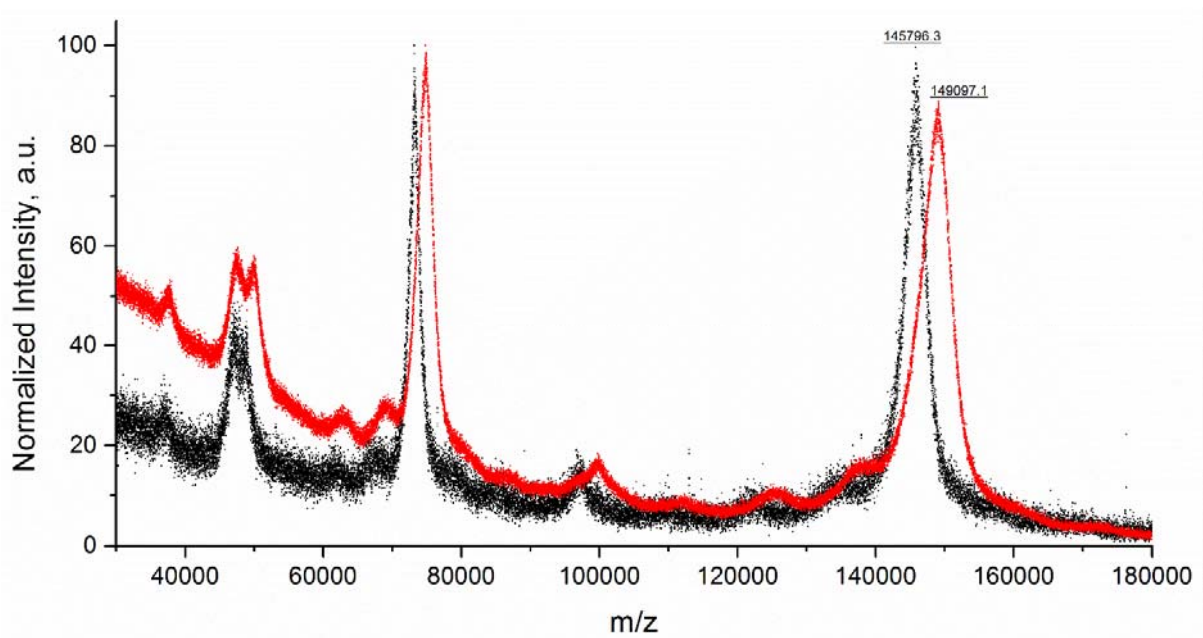


Figure S2 Mass spectra of the unlabeled immunoglobulin IgG (black) and its conjugate with dye **400**-H (red) with a relatively high DOL. The average molecular mass difference ($\Delta M \approx 3300$) allowed the determination of the $\text{DOL}_M \approx 8$ which was found to be in good agreement with the value DOL_S calculated from the measurement of the optical density. For that, the optical density of the bioconjugate solution was measured at two wavelengths – the absorption maxima of the dye (394 nm) and protein (280 nm). The dye correction factor ($A_{280}/A_{394} = 0.129$) is valid, strictly speaking, only for the free dye. If this value was used for calculation, the DOL was found to be 9.8.

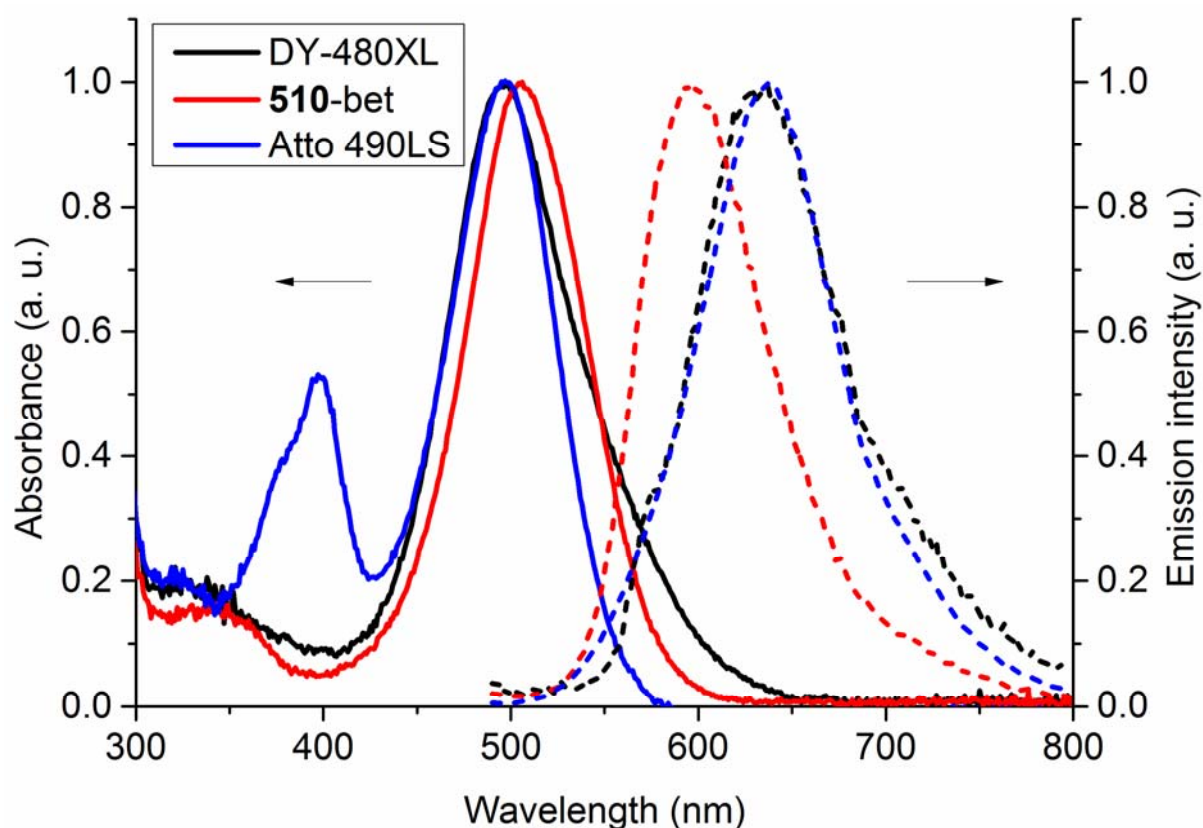


Figure S3 Normalized absorption spectra (solid lines) and emission spectra (dashed lines) of DY-480XL (black), 510-bet (red) and Atto 490LS (blue) attached to antibodies in aq. PBS buffer at pH 7.4.

Immunofluorescence: Sample Preparation

For immunolabeling, Vero cells were grown on cover slips and then fixed with anhydrous methanol for 5 min at -20°C and blocked with 5% (w/v) BSA in PBS. Then the cells were incubated with a monoclonal mouse antibody directed against α -tubulin (Sigma-Aldrich, St. Louis, MO, USA) or nuclear pore complex subunits (in the central channel of the complex) (NUP153, Abcam, Cambridge, UK). The primary antibodies were detected with secondary antibodies (sheep anti-mouse; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) custom-labeled with the appropriate fluorescent dyes. For that, the required N-hydroxysuccinimidyl ester of the dye (0.2 mg) was dissolved in 20–40 μL of dry DMF, and this solution was slowly added to the stirred solution of the secondary antibody (ca. 1 mg of the protein in 1 mL of the buffer with pH = 8–8.5) at ambient temperature. The dye-labeled antibodies were isolated by gel filtration (see ref.^[1] for an example). Sample preparation was carried out according to the standard protocols described in detail by C. A. Wurm and coworkers.^[2] In brief, after several washing steps with PBS buffer, the cell samples were mounted in Mowiol (an embedding medium).

For the preparation of double-immunolabeled murine cardiomyocyte cell samples, myocytes were obtained and prepared as previously described.^[3] After permeabilization (0.2 % Triton X-100, 10 % bovine calf serum in PBS) cells were incubated overnight with a primary monoclonal mouse antibody directed against α -Tubulin (Sigma-Aldrich T6074; at a concentration of 1:500) and a primary polyclonal rabbit antibody directed against Caveolin-3 (Abcam ab2912; at a concentration of 1:500) and then washed three times in PBS. Cells were then incubated for 3 h with the respective secondary antibodies: sheep anti-mouse KK114 (*cf.* Abberior STAR RED, 2-0002-011-2, Abberior Inc., Goettingen, Germany; at a concentration of 1:1000) and goat anti-rabbit **510**-bet (at a concentration of 1:1000).

STED image acquisition, image processing and image evaluation

STED super-resolution images of single- and double-immunolabeled fixed mammalian cells were obtained with a custom built STED microscopy setup which is described in more detail elsewhere.^[4] All lasers employed in this study were operated in the pulsed mode and their pulse trains synchronized. Excitation laser beams at wavelengths of 640 nm, 532 nm, and 490 nm were provided by triggerable laser diodes with pulse durations of 120 ps (PicoQuant, Berlin, Germany). The STED laser pulses had a pulse duration of 300 ps and stemmed from a tunable Ti:sapphire laser with a repetition rate of 80 MHz and a 120 fs pulse duration (Coherent Inc., Santa Clara, CA, USA) that was up-chirped.

The three excitation laser lines at 640 nm, 532 nm, and 490 nm are very commonly used in commercial setups and were therefore chosen for the two-color STED images in this study, as well as the comparison of the dye compounds **510**-bet, Atto 490LS, and DY 480XL regarding their suitability for STED microscopy. For the dye compounds **520**-bet, **510**-bet, Atto 490LS, and DY 480XL the excitation at 532 nm led to better STED images than the excitation at 490 nm. The center wavelength of the STED laser was set to 750 nm after testing STED wavelengths ranging from 750 nm to 790 nm and finding that the 750 nm STED laser wavelength yielded the best images for all investigated dye compounds.

Both excitation and STED laser powers passing the objective lens (NA = 1.4 oil immersion, HCX PL AP, Leica) were optimized to yield the best images. All laser powers were measured in a backfocal plane of the objective. For the optimization of the STED image quality we tested different excitation laser powers ranging from 0.5 μ W to 50 μ W and different STED laser powers ranging from 50 mW to 310 mW. The most well-suited laser power settings differed for each dye compound and ranged from 1 μ W to 40 μ W for the excitation laser beams and from 280 mW to 310 mW for the STED laser beam. These laser powers are explicitly stated in the figure captions of each displayed exemplary STED image in this study.

Fluorescence was detected using two avalanche photo diodes (APDs, SPCM-AQR13, Perkin Elmer, Fremont, CA, USA). The one APD was provided with a (675 ± 30) nm bandpass filter (AHF, Tübingen, Germany) and was used for the detection of the fluorescence of the dye compounds **510**-bet, **520**-bet, Atto 490LS, as well as KK 114. The other APD was provided with a (630 ± 30) nm bandpass filter (AHF, Tübingen, Germany) and was used for the detection of the fluorescence of the dye compound DY 480XL. The used bandpass filters are standardly commercially available components that provided the best STED images for each respective dye compound. Other bandpass filters were also tested, among which were a (610 ± 75) nm, a (620 ± 60) nm, and a (660 ± 52) nm bandpass filter (all from (AHF, Tübingen, Germany) but they yielded STED images of a lower quality.

Image acquisition was performed by stage-scanning in one lateral direction using a piezo stage (733-3DD and E-710, Physik Instrumente GmbH, Karlsruhe, Germany) and beam-scanning in the other lateral direction using a 16 kHz resonant mirror (SC-30, EOPC, Glendale, NY, USA). All samples were scanned within a region of interest of $(9 \times 9) \mu\text{m}^2$. The image pixel size was set to 20 nm and the pixel dwell time was 50 μs . We used the software ImSpector^[5] for hardware control, data acquisition and data analysis.

Samples of single-immunolabeled fixed Vero cells stained for Nup153 and Tubulin were excited with the laser line at 532 nm, fluorescence was precluded with the STED laser at 750 nm and detected with the (675 ± 30) nm bandpass filter in front of the APD. All figures of single-labeled Vero cells within this manuscript show smoothed data normalized to the respective pixel brightness of each image. Image smoothing was performed using a low-pass Gaussian filter with a width of 1.5 times the pixel size (corresponding to 30 nm). Images of the double-immunolabeled fixed mouse cardiomyocytes stained for Tubulin (with the dye KK114) and Caveolin-3 (with the dye compound **510**-bet) were acquired in two consecutive sample scans. Both scans employed the two carefully aligned excitation laser lines at 640 nm to excite the dye KK114 and at 532 nm to excite the dye compound **510**-bet. The same fluorescence detection window of (675 ± 30) nm and the same STED laser beam at 750 nm for excited state depletion of both dyes were used. Due to the efficient separation of the dye emission signals by different excitation wavelengths, cross-talk between observation channels was negligible ($<5\%$ of the respective signal brightness). Raw image data from each color channel was then processed using the deconvolution algorithm of the software ImSpector. The data was deconvolved with a measured point spread function of 40 nm (at full width at half maximum) using two iterations of a 2D Richardson-Lucy deconvolution. Images were then corrected for background signal with the software Fiji^[6] using a rolling ball radius of 30 px, image brightness was adjusted and false color maps were chosen for representation.

For the determination of image resolution we followed a protocol similar to the one we employed and described elsewhere.^[7] For the circular sample structures Nup153 and Caveolin-3 that were stained in this study a box of between (200 x 200) nm² and (400 x 400) nm² was drawn around a single structure in the raw data image. The signal intensity was added up along the X-axis of this box and then graphically displayed against the x-position of the boxed region. This plotting procedure was performed with the software Imspector. The signal intensity graphs were then exported to the software Origin (OriginLab, Northampton, MA). The signal intensity was normalized and the Origin built-in Lorentzian function was fit to the data. Resolution was extracted from these fits as the width of the Lorentzian curve. The stated resolutions of images of labeled Nup153 and Caveolin-3 were obtained from the arithmetic mean of ten such Lorentzian curve fits per displayed image. For the elongated sample structure Tubulin a line of a width of 5 pixels (corresponding to 100 nm) was drawn to perpendicularly cross a selected Tubulin filament in the raw data image. The signal intensity was added up along this line and graphically displayed against the position along the line. This plotting procedure was performed with the software Imspector. The signal intensity graphs were then exported to the software Origin, normalized and again, a Lorentzian function was fit to the data. Resolution was again extracted as the width of the Lorentzian curve fit; all stated resolutions stem from the arithmetic mean of ten such evaluated line profiles per displayed image.

References to Supporting Information

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