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SUPPORTING INFORMATION

Title: A Versatile Route to Red-Emitting Carbopyronine Dyes for Optical Microscopy and Nanoscopy
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Ref. No.: O201000343

Synthesis of the early precursors and some side-products

7-Nitro-1,2,3,4-tetrahydroquinoline (5a).[1] 1,2,3,4-Tetrahydroquinoline (**4**) was nitrated in sulfuric acid solution according to the known procedure.[13] To obtain the highest possible purity of the required isomer (**5a**), CH_2Cl_2/hexane (1:5) mixtures were used for recrystallization (at –20°C). Compound **5a** was isolated in 40% yield.

***N*-Acetyl-7-nitro-1,2,3,4-tetrahydroquinoline (5-Ac, NO_2).** Compound **5a** (6.6 g, 37 mmol) was acetylated with acetic anhydride (25 mL) and pyridine (1.5 mL) as described by *Y. Yamada et al.* for 6-nitroindoline.[2] The reaction mixture was evaporated to dryness (70°C, rotary evaporator), the residue dissolved in CH_2Cl_2 (50 mL), and precipitated with warm (50°C) hexane to furnish 6.65 g (82%) of a pale-yellow crystalline product with m. p. 103–104°C, which was used without further purification in the next step. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.98 (quint, J = 6 Hz, 2 H), 2.12 (s, 3 H, $COCH_3$), 2.82 (t, J = 6 Hz, 2 H), 3.78 (t, J = 6 Hz, 2 H), 7.24 (d, J = 8 Hz, 1 H), 7.84 (d,d, J = 8 Hz, 1 H), 8.40 (br. m, 1 H) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 23.2 (CH_2), 23.3 ($CO\underline{CH_3}$), 27.3 (CH_2), 44 (br. s, CH_2), 119.1, 112.2, 128.9, 139.7, 139.8, 144.4, 169.9 (C=O) ppm. MS (ESI): *m*/*z* (positive mode, %) = 243 (100) $[M+Na]^+$; HRMS ($C_{11}H_{12}N_2O_3$) 243.0741 (found M+Na) 243.0740 (calc.).

***N*-acetyl-7-amino-1,2,3,4-tetrahydroquinoline (5-Ac, NH_2)**. Following the recipe[2] with minor modifications, the nitro compound **5-Ac, NO_2** was reduced to the corresponding amine: compound **5-Ac, NO_2** (6.60 g, 30 mmol) in 150 mL of MeOH was introduced with a syringe into a 1L-Schlenk flask, which was beforehand loaded with 1.00 g of Pd/C (10% Pd, oxidized form, VWR International) suspended in methanol (100 mL) and flushed with H_2 gas with vigorous stirring (thus the catalyst was prereduced). The reaction mixture was vigorously stirred overnight under H_2, diluted with $CHCl_3$ (100 mL), filtered through Celite® (CAUTION: the Pd/C catalyst may cause ignition!), and the solvents removed *in vacuo*. The residue solidified on standing to give 5.60 g (98%) of a colourless material with m.p. 76–78 C and clean ^{1}H NMR spectrum (TLC control with EtOAc/hexane 1:1 as mobile phase). The product was used without purification in the next step. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.82 (quint, J = 6 Hz, 2 H), 2.19 (s, 3 H, $COCH_3$), 2.58 (t, J = 6 Hz, 2 H), 3.66 (t, J = 6 Hz, 2 H), 3.63 (br.s, NH_2, 2 H), 6.42 (dd, J = 8 Hz, 2 H), 6.82 (d, J = 8 Hz, 1 H) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 23.2 ($CO\underline{CH_3}$), 23.9 (CH_2), 25.8 (CH_2), 111.2, 112.3, 129.1, 139.2, 146.1, 169.9 (C=O) ppm. MS (ESI): *m*/*z* (positive mode, %) = 213 (100) $[M+Na]^+$; HRMS ($C_{11}H_{14}N_2O$) 191.1179 (found M+H) 191.1178 (calc.).

***N*-acetyl-7-iodo-1,2,3,4-tetrahydroquinoline (5-Ac, I).** A Sandmeier Reaction was utilized to convert amino derivative **5-Ac, NH_2** to the corresponding iodide, analogously to *N*-acetyl-6-aminoindoline.[2] In a typical experiment compound **5-Ac, NH_2** (5.48 g, 29 mmol) in 300 mL of 80% aq. acetic acid was diazotated with $NaNO_2$ (2.07 g, 30 mmol) in 10 mL H_2O at 0°C under an argon atmosphere with stirring, and 10 min later a solution of KI (5.81 g, 35 mmol) in 10 mL of H_2O was added. After stirring at 0°C (external ice bath) for 25 h, 0.40 g of Na_2SO_3 was added as 10% aqueous solution, until the color of I_2 had disappeared. The solution was evaporated almost to dryness at 40–45°C (rotary evaporator), the residue dissolved in EtOAc (300 mL), and washed with H_2O (100 mL). The aqueous layer was extracted with EtOAc (50 mL), and the combined organic solutions washed with sat. aq. $NaHCO_3$ (70 mL), 0.5 M aq. HCl (50 mL), and brine (50 mL). The extract, dried with Na_2SO_4, was evaporated *in vacuo*, and the residue subjected to column chromatography (100 g SiO_2) with EtOAc/hexane (3:2) as mobile phase, to furnish 5.65 g (65%) of **5-Ac, I** as pale yellow oil (photo-sensitive!). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.84 (quint, J = 6 Hz, 2 H), 2.20 (s, 3 H, $COCH_3$), 2.62 (t, J = 6 Hz, 2 H), 3.76 (t, J = 6 Hz, 2 H), 6.82 (d, J = 8 Hz, 1 H), 7.39 (dd J = 8 Hz, 1 H), 7.60 (br. m, 1 H) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 23.0 ($CO\underline{CH_3}$), 23.2 (CH_2), 23.8 (CH_2), 43 (br. s, CH_2), 89.8 (CI), 130.0 (2×), 133.1, 133.8, 146.1, 169.7 (C=O) ppm. MS (ESI): *m*/*z* (positive mode, %) = 324 (100) $[M+Na]^+$; HRMS ($C_{11}H_{12}INO$) 323.9859 (found M+Na) 323.9856 (calc.).

7-Iodo-1,2,3,4-tetrahydroquinoline (5-H, I). Unlikely to *N*-acetyl-6-iodoindoline,[2] compound **5-Ac, I** required much more time to completely remove the acetyl group under basic conditions. Thus, 5.60 g (18.6 mmol) of **5-Ac, I** was refluxed with stirring in a mixture of 30% aq. NaOH (7.5 g. 5.60 mmol) and MeOH (50 mL) for 27 h. The solution was evaporated *in vacuo* almost to dryness, the residue extracted with ether (3×60 mL, decanting from the inorganic salts), and dried with anhydrous K_2CO_3. The pure amine **5-H, I** was precipitated as a hydrochloride, adding the 5 M HCl solution in isopropanol (4 mL, ACROS Organics) to the dry extract. A heavy colorless

powder was filtered-off, washed with ether and air-dried (not hygroscopic) to give 4.45 g (79%, M = 295) of the product, which was used as such for the further step. This salt is very slightly soluble in MeOH, MeCN, insoluble in ether and $CHCl_3$, decomposes at about 166–168°C. To obtain the MNR spectra of **5-H, I**, a small amount (20 mg) of the hydrochloride was treated with sat. aq. $NaHCO_3$ solution, extracted with $CHCl_3$, the extract dried, evaporated, and the residue (colorless solid, m.p. 73–74 °C) dissolved in $CDCl_3$. TLC control with EtOAc/hexane (1:5) revealed no impurities. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.84 (m, 2 H), 2.63 (m, 2 H), 3.22 (m, 2 H), 3.80 (br.s, NH, 1 H), 6.62 (d, J = 8 Hz, 1 H), 6.78 (d, 1 H), 6.83 (dd, J = 8 Hz, 1 H) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 21.6 (CH_2), 26.6 (CH_2), 41.6 (CH_2), 91.2 (CI), 120.8, 122.2, 125.4, 131.0, 146.1 ppm. MS (ESI): *m*/*z* (positive mode, %) = 259 (100) $[M]^+$; HRMS ($C_9H_{10}IN$) 259.9930 (found M+H) 259.9931 (calc.).

***N*-Benzyl-1,2,3,4-tetrahydroquinoline (6)**. 1,2,3,4-Tetrahydroquinoline (**4**) was benzylated as follows: 10 g (75 mmol) of it was added to a solution containing KOAc (7 g, 71 mmol), HOAc (7 mL, 0.12 mol), benzyl chloride (7.6 g, 0.06 mol), and H_2O (10 mL) in 40 mL of DMF. The mixture was stirred overnight at RT and for additional 2 h at 40 °C, then diluted with H_2O (100 mL) and 1 M aq. HCl (145 mL). The compound was extracted with CH_2Cl_2 (2×60 mL), the extract washed with 0.3 M aq. HCl (100 mL), then with H_2O (50 mL). The evaporation of the solvents furnished 13.0 g (97%) of a colourless oil. TLC control: hexane – EtOAc (8:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.02 (quint, J = 6 Hz , 2 H), 2.82 (t, J = 6 Hz, 2 H), 3.39 (t, J = 6 Hz, 2 H), 4.24 (s, 2 H, CH_2Ph), 6.50–6.60 (m, 2 H), 6.80–7.00 (m, 2 H), 7.20–7.40 (m, 5 H, Ph) ppm.

***N*-Benzyl-1,2,3,4-tetrahydroquinoline-6-carbaldehyde (9).** *N*-Benzy-1,2,3,4-tetrahydroquinoline (**6**) was formylated with a Vilsmeier reagent, following the known recipe[3] to furnish aldehyde **9** in a 63% yield (after recrystallization from hexane/CH_2Cl_2); m.p. = 80–81 °C (lit.[3] 74–75 °C).

1-Benzyl-1,2,3,4-tetrahydroquinolin-6-yl-methanol (**10**). Aldehyde **9** was reduced to the corresponding carbinol with $NaBH_4$, according to the routine procedure. Thus, $NaBH_4$ (1.00 g, 26 mmol) was added in several portions to a stirred solution of **10** (5.02 g, 0.02 mol) in absolute EtOH (170 mL) at RT. According to the TLC analysis (EtOAc/hexane, 1:1), the reaction was complete in 1 h. The reaction mixture was acidified with AcOH (2.5 mL in 10 ml of EtOH) and evaporated to dryness *in vacuo* (t <40°C). Water (20 mL) and CH_2Cl_2 (100 mL) were added to the residue, the aqueous layer extracted with CH_2Cl_2 (40 mL), the combined organic extract washed with sat. aq. $NaHCO_3$ solution (20 mL) and dried (Na_2SO_4). The evaporation furnished 3.90 g (78%) of a title compound as a colorless solid, m.p. = 49–51°C, photo-sensitive, soluble in most organic solvents, unstable in $CHCl_3$ and CCl_4 solutions even in the dark, stable in other solvents (e.g., acetone, MeCN, MeOH). ^{1}H NMR (300 MHz, CD_3CN): δ = 1.96 (m, 2 H), 2.77 (t, J = 6 Hz, 1 H, OH), 2.81 (t, J = 6 Hz, 2 H), 3.38 (t, J = 6 Hz, 2 H), 4.28 (d, 2 H, CH_2O), 4.42 (s, 2 H, CH_2Ph), 6.40 (d, J = 8 Hz, 1 H), 6.82 (dd, J = 8 Hz, 1 H), 6.85 (s, 1 H) 7.20–7.40 (m, 5 H, Ph) ppm; ^{13}C NMR (75.5 MHz, CD_3CN): δ = 23.1 (CH_2), 28.8 (CH_2), 50.9 (CH_2), 55.5 (CH_2N), 64.8 (CH_2OH), 108.4 (CH), 111.5 (C), 123.1 (C), 127.1 (CH), 127.5 (2×CH), 127.6 (CH), 129.3 (CH), 129.4 (2×CH), 130.0 (C), 140.3 (C), 145.3 (C) ppm. MS (ESI): *m*/*z* (positive mode, %) = 236 $[M–OH]^+$; HRMS ($C_{17}H_{19}NO$) 276.1366 (found M+Na) 276.1359 (calc.).

Benzhydrol derivative 23. Aldehyde **9** was reacted with aryl lithium reagent **15** prepared *in situ* (see Scheme 5) as follows: 2-(2-Bromophenyl)-4,4-dimethyl-2-oxazoline (267 mg, 1.05 mmol) in THF (4 mL) was lithiated with 1.5 M *t*BuLi in pentane (0.81 mL, 1.20 mmol) at –78 °C under argon. The mixture was stirred for 2 h at –78 °C, and aldenyde **9** (251 mg, 1.00 mmol) in THF (1 mL) was introduced in one portion. The reaction mixture was stirred overnight at 0°C, poured onto ice-cold aqueous NH_4Cl solution (20% w/w, 40 mL), and extracted with EtOAc (3×20 mL). The evaporation of the dried extracts, followed by column chromatography over SiO_2 (12 g), using hexane/EtOAc (2:1→1:2) as mobile phase, afforded 303 mg (70%) of compound **23** as a pale yellow crystalline solid with m.p. = 134–136 °C (MeOH); sparingly soluble in acetone, MeOH, and MeCN, unstable in $CHCl_3$ solutions. ^{1}H NMR (300 MHz, [D_6]DMSO): δ = 1.22 (s, 3 H, CH_3), 1.40 (s, 3 H, CH_3), 1.84 (m, 2 H, CH_2), 2.71 (m, 2 H, C), 3.32 (overlap: H_2O + m, 2 H, CH_2), 3.40, 3.90 (m×2, 2 H, CH_2O), 4.43 (s, 2 H, CH_2Ph), 4.82 (m, 1 H, CH), 6.79 (s, 1 H, OH), 6.42 (d, J = 7 Hz, 1 H), 6.80 (m, 1 H), 7.11 (d, J = 7 Hz, 1 H), 7.20–7.45 (m, 5 H, Ph), 7.62 (d, J = 7 Hz, 1 H); ^{13}C NMR (75.5 MHz, [D_6]DMSO) δ = 21.6 (CH_2), 23.2 (CH_3), 23.5 (CH_3), 27.5 (CH_2), 38.7 (CH_2), 39.0 (CH_2), 40.0 (CH_2), 40.3 (CH_2), 49.3 (CH_2), 54.3 (CH_2), 59.0 (CH_2), 64.3 (CH), 66.0 (C), 110.7, 122.1, 122.6, 126.5, 127.0,

127.3, 128.4, 131.2, 138.9, 144.6, 147.9, 168.8 (C=N). *m/z* (positive mode, %) = 427 (100) $[M+H]^+$; HRMS ($C_{28}H_{30}N_2O_2$) 427.2379 (found M+H) 427.2380 (calc.).

Spiroamide 17 (the base-assisted cyclization product). Compound **16a** (30 mg, 36 μmol) in THF (3 mL) was stirred with K_2CO_3 (140 mg, 1.0 mmol) for 1.5 h at RT. TLC analysis (MeCN/H_2O 1:10) showed that the starting material had completely reacted to form the colorless compound, that soon turns blue on a plate, and a very polar blue compound (the open form, colored). About 16 mg (74%) of compound **17** was isolated by flash chromatography on SiO_2 (7 g) with MeCN (100%), as a pale-yellow amorphous material. The compound is fairly unstable at RT, especially in solutions; it turns blue on TLC plates soon after a chromatogram is run. Its ^{1}H NMR and mass spectra (as well as the absence of the blue colour) suggested the structure **17**. HPLC: t_R = 14.9 min (A/B 20/80 – 0/100 in 25 min); HPLC area 92%. ^{1}H NMR (300 MHz, CD_3CN): δ = 1.06 (s, 6 H, 2×CH_3), 1.64, (s, 3 H, CH_3), 1.77 (s, 3 H, CH_3), 1.80 (m, 4 H, 2×CH_2), 2.52 (m, 4 H, 2×CH_2), 3.30 (m, 4 H, 2×CH_2), 3.36 (s, 6 H, 2×OCH_3), 3.46 (m, 4 H, 2×CH_2N), 3.54–3.62 (m, 4 H, CH_2O, m, 2 H, CH_2OH), 4.18 (t, *J* = 9 Hz, 1 H, OH), 6.38 (s, 2 H), 6.72 (s, 2 H), 7.14 (m, 1 H), 7.78 (m, 2 H), 7.98 (d, *J* = 9 Hz, 1 H) ppm; MS (ESI): *m/z* (positive mode, %) = 624 (100) $[M]^+$; HRMS ($C_{39}H_{50}N_3O_4$) 624.3798 (found M^+) 624.3796 (calc.).

Oxazoline derivative 19 (the acid-assisted recyclization product). Spiroamide **17** (17 mg, 27 μmol) was stirred at 70 ^{o}C in 1,2-dichloroethane (3 mL) containing $POCl_3$ (0.1 mL, 1.1 mmol) for 1.5 h in a Schlenk flask, the reaction mixture evaporated *in vacuo*, and the residue dissolved in CH_2Cl_2 (20 mL). Et_3N (0.05 mL, 0.35 mmol) was added, the solution washed with H_2O (10 mL), dried (Na_2SO_4), and evaporated. Compound **19** was isolated in ca. 75% yield (dark blue amorphous solid, 9.0 mg) by flash chromatography on SiO_2 (5 g) with MeCN/H_2O (1:5) as mobile phase. HPLC: t_R = 10.9 min, A/B 20/80 – 0/100 in 25 min. The same compound was detected as a single product (100%, HPLC) when **16a** was heated (80 ^{o}C, 2 h) in glacial HOAc containing 2% (w/w) H_2SO_4. Acid-assisted hydrolysis of **19** (14 h, 80 ^{o}C, 22% aq. HCl; see above) cleanly leads to **1a** (80%, HPLC); compound **16a** was detected as an intermediate. ^{1}H NMR (300 MHz, CD_3CN): δ = 0.94 (s, 6 H, 2×CH_3), 1.64, (s, 3 H, CH_3), 1.80 (s, 3 H, CH_3), 1.84 (m, 4 H, 2×CH_2), 2.50 (m, 4 H, 2 CH_2), 3.32 (6 H, 2×OCH_3), 3.63 (m, 4 H, 2×CH_2N), 3.72 (s, 2 H, CH_2), 3.80 (m, 4 H, 2×CH_2O), 6.67 (s, 2 H), 7.18 (s, 2 H), 7.30 (m, 1 H), 7.64 (m, 2 H), 7.98 (m, 1 H) ppm; ^{13}C NMR (75.5 MHz, CD_3CN): δ = 21.7 (CH_2), 22.9 (CH_2), 27.8 (CH_3), 33.1 (CH_3), 34.8 (CH_3), 42.2 (CH_2), 52.2 (CH_2), 52.8 (CH_2), 59.4 (OCH_3), 69.4 (C), 70.9 (C), 110.3, 112.0, 114.1, 117.9, 121.6, 121.7, 124.9, 130.4, 131.8, 132.5, 134.3, 134.8, 154.6, 156.6, 157.8, 158.3, 158.9, 159.4. ppm; MS (ESI): *m/z* (positive mode, %) = 606 (100) $[M]^+$, HRMS ($C_{39}H_{48}N_3O_3$) 606.3693 (found M^+) 606.3690 (calc.).

References:

[1] G. Field, P.R. Hammond (Dept. of Energy, USA), US Pat. 5283336 (01.12.1994).

[2] Y. Yamada, A. Akiba, S. Arima, C. Okada, K. Yoshida, F. Itou, T. Kai, T. Satou, K. Takeda, Y. Harigaya, *Chem. Pharm. Bull.* 2005, *53,* 1277–1290.

[3] J. F. Eggler, J. F. Holland, M. R. Johnson, R. A. Volkmann, (Pfizer Inc.) US Pat. 4738972 (19.04.1988).

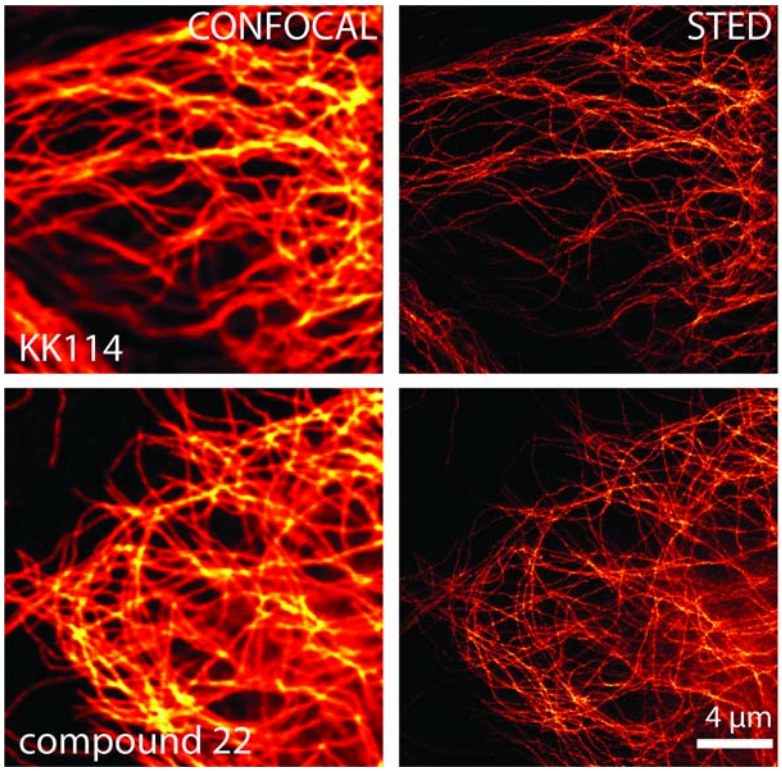

Figure 1S. STED nanoscopy with compound **22** and reference dye KK 114. To evaluate the performance of the new dyes, the tubulin cytoskeleton of cultured mammalian cells was labelled by immunofluorescence (see above for details), and the samples were imaged by STED nanoscopy (see the main text for details).

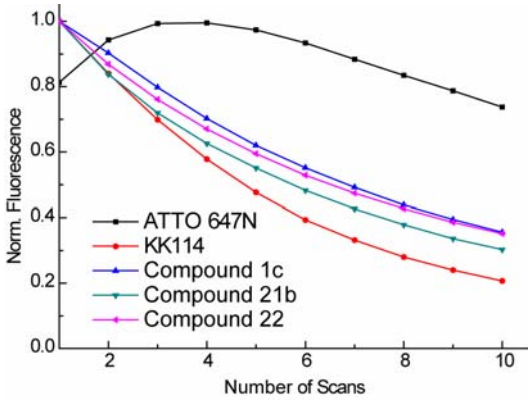

Figure 2S. Bleaching curves for the bioconjugated dyes ATTO 647N, KK114, **1c**, **21b** and **22**. Photostability of different compounds in microtubule under confocal conditions was evaluated *without STED beam*. A higher excitation power was applied (6.6 µW at 640 nm) which, after certain number of scans, led to the moderate bleaching of dye ATTO 647N (compare with Fig 3a, in the main text).

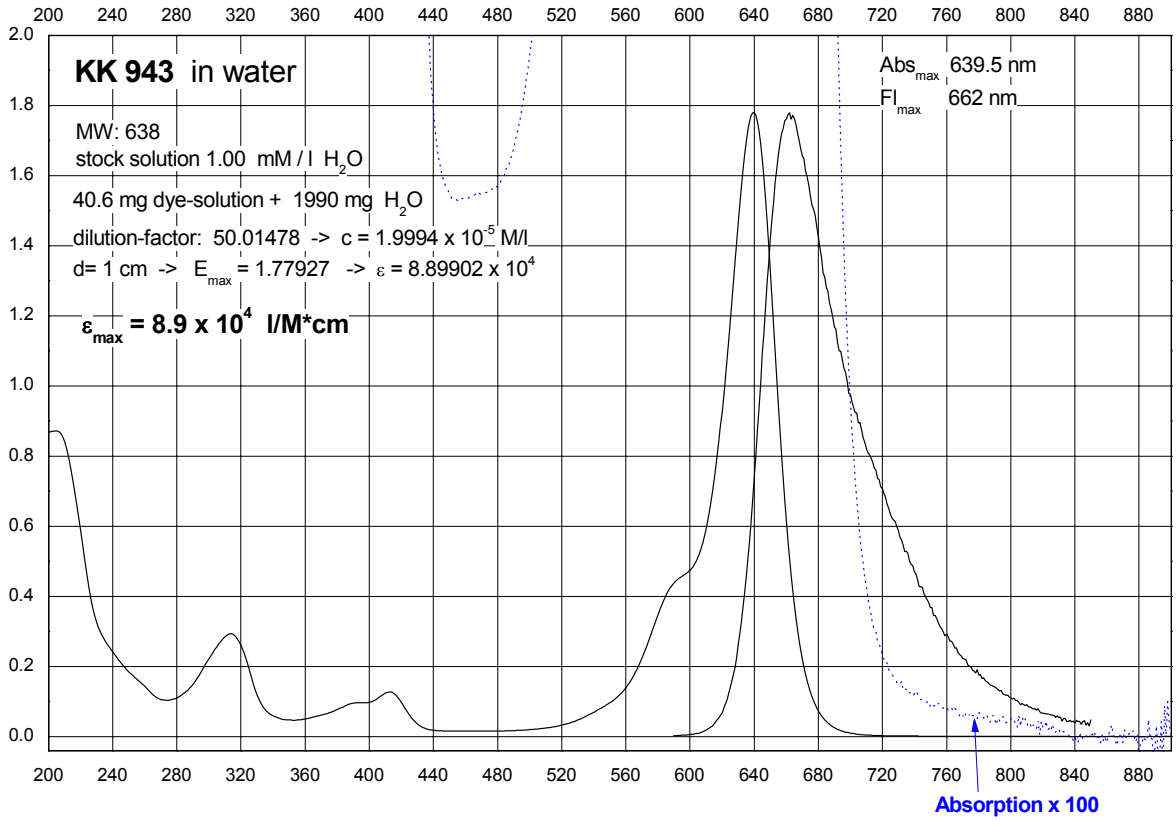

Figure 3S. A typical absorption (UV/vis) and fluorescence spectrum of a carbopyronine dye in water. Compound KK 943 is taken as an example (KK 943 ≡ **1a** in Scheme 3 of the main text). Other new carbopyronine dyes have virtually identical spectra (see Table 1, the main text).

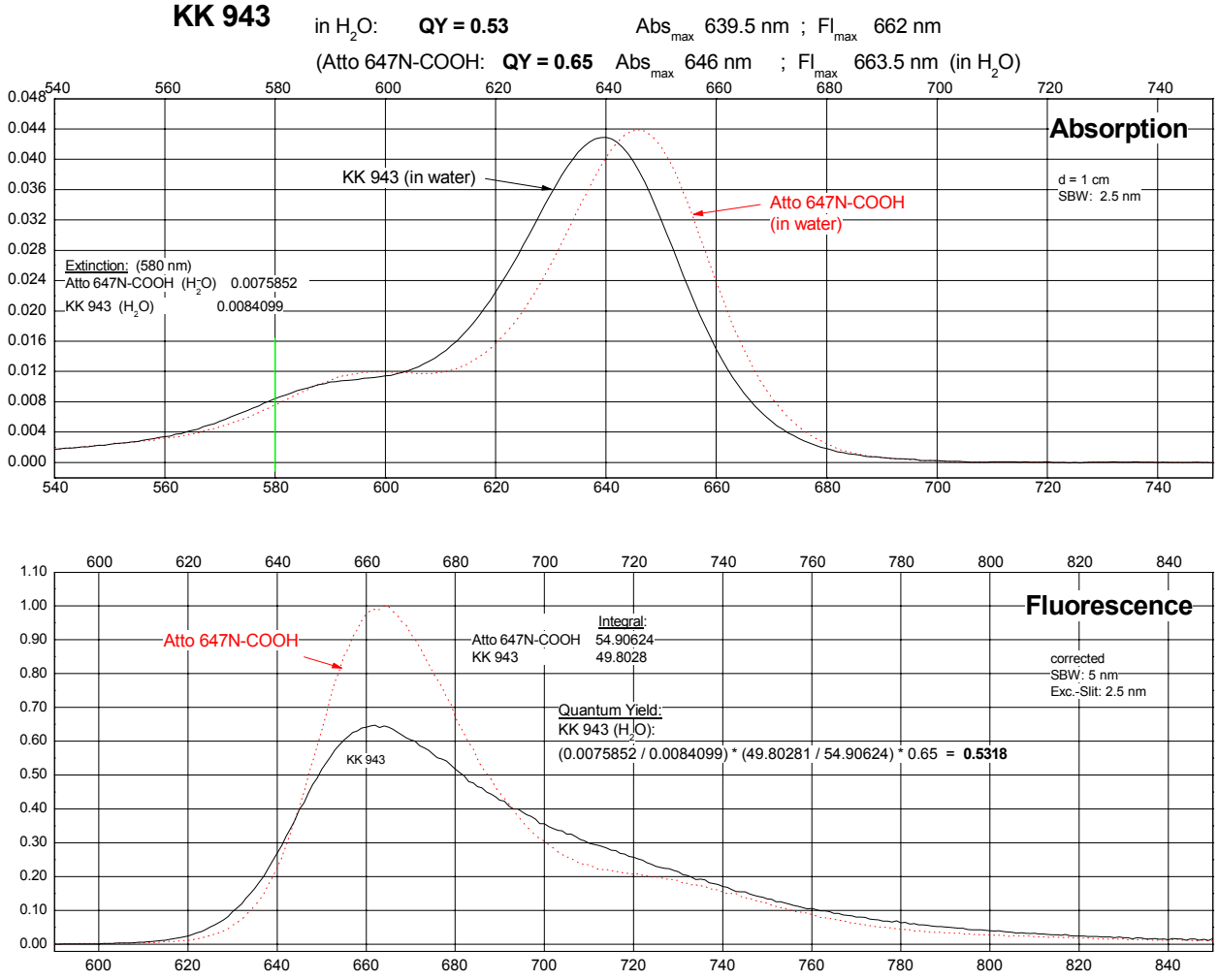

Figure 4S. Main absorption and emission bands of compound KK 943 (**1a**) in water (black lines); KK 943 ≡ **1a** in Scheme 3 of the main text. Absorption and emission bands of the reference compound (ATTO 647N-COOH) in aqueous buffer solution are drawn in red dotted line. ATTO 647N-COOH was used as a reference for the fluorescence quantum yield calculation. For the spectroscopic data of the new dyes see Table 1 in the main text.