Electronic Supplementary Information (ESI) for:

Everlasting rhodamine dyes and true deciding factors in their STED microscopy performance

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Instruments for compound identification and purification

Initially a Knauer **HPLC** system was used (MPI-BPC Göttingen), which had the following configuration: Smartline pump 1000 (2×), UV detector 2500, mixing chamber, injection valve with 20 and 100 µL loop for the analytical and preparative columns, respectively; 6-port-3-channel switching valve; solid (reversed) phase: Eurosphere-100 C18, 5 µm; analytical column: 250 × 4 mm; preparative column: 250 × 8 mm. Later, a Shimadzu analytical HPLC system (BioBeagle, St. Petersburg, Russia) involved: pump LC-20AD with a 4-channel low-pressure gradient valve unit 228-45040-58, degassing unit DGU-10A5R and a diode-array detector SPD-M20A. Columns and phases are specified for each case. Shimadzu preparative HPLC system (Institute of Bioorganic Chemistry, Moscow, Russia) involved: pump LC-20AP with a 4-channel low-pressure gradient valve unit FCV-10 AL, detector SPD-20AV and a fraction collector FRC-10A. Columns and phases are specified for each case. Analytical thin layer chromatography (**TLC**) was performed on MERCK ready-to-use plates with regular silica gel 60 (F_{254}). Preparative column chromatography performed on regular silica gel with the particle size 40 - 63 µm, unless otherwise stated. **ESI-MS** were obtained also on a Varian 500 MS spectrometer (Agilent). High resolution mass spectra (ESI-HRMS) were obtained on a Bruker micro TOF (ESI-TOF-MS) spectrometer. **NMR** spectra were recorded with conventional Agilent 400-MR and a Bruker Avance Neo 600 spectrometer. See also p. 22 for additional instruments.

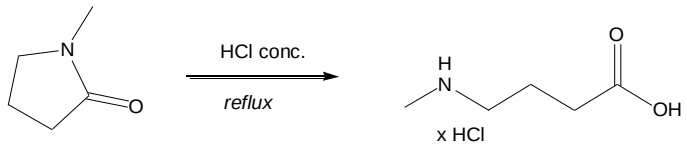
General notes for chromatographic separations

In preparative column chromatography with hand-made columns and the rhodamine dyes of this study, a HILIC effect was utilized (see reviews [4a,b]). That involves a polar solid phase (usually, the inexpensive regular SiO_2) and a polar liquid phase containing water, acetonitrile (MeCN) and, sometimes, also chloroform or dichloromethane (DCM). With the rhodamine dyes of this particular family (prepared from precursor Compound **1a**; see structure below) this technique provides a much better and easier separations. The same works for TLC analyses, as well. The only drawback is, that silica gel is partly solubilized and cannot be separated by filtration, especially in polar solvents. Also, after filtration, the solid can retain a lot of material (as seen by its color). The problem is solved by washing of a dilute (ca. 1 – 2 wt. %) dye solution in a suitable organic solvent with an equal volume of water or brine. The organic layer is separated, the aqueous layer extracted one more time, if necessary (judging by the color), the organic solution dried with a solid drying agent (usually Na_2SO_4) and filtered through a coarse filter or paper. For the sulfonated dyes (not extractable with organic solvents) one can adsorb the compound from water in a small column on a reversed-phase SiO_2 (*e. g.*, Polygoprep™ 60-130 C18, with large particles that have smaller flow resistance and easy to filter off), elute the column with water (≥ 2 column volumes) and then wash the dye off with a suitable solvent (MeCN or MeOH) containing a volatile base or an acid (if the H-form of the dye is preferable). The authors also recommend to wash the RP-SiO_2 before use with 80 vol. % MeCN containing 0.1 vol. % TFA and then with water to remove basic and/or nucleophilic impurities. For preparative separations with regular SiO_2 and a polar liquid phase it is essential to load the substrate as a dilute (0.1 – 1%) solution in the same liquid phase that is used for the run to avoid jumps in the polarity or the formation of a second phase (DCM or H_2O). It is also important to dissolve the dye completely, which is usually difficult to check because the solutions are not transparent (very deeply colored). Therefore, sufficient volumes of the solvent and sonication are recommended. Below described are examples of actual separations. While using MeCN/H_2O mixtures and regulars silica gel, it is recommended first to elute the empty column with one column volume of the mobile phase to wash off the “dust” and possible traces of acids or bases in the material.Due to the high reactivity of the aromatic fluorine atom in this family of dyes some precautions are to be taken. One should avoid heating such substances in basic solutions above 35°C, particularly while evaporating the chromatographic fractions containing Et_3N or TEAB buffer. Otherwise, noticeable amounts if OH- or MeO- derivatives (in water or in methanol, respectively) are formed, which are difficult to separate even by means of HPLC (satellite peaks). Also, the substitution of F with OH or OMe causes an undesirable blue shift. The use of the slightly acidic TEAA (Et_3N×HOAc) buffer or neutralization of Et_3N with excess of HOAc before evaporation solves this problem. However, repeated freeze-drying is required to completely remove the triethylammonium acetate from a sample. All dyes and dye precursors described above should be protected from direct sunlight and stored at +5°C.

Synthesis of the dyes and precursors

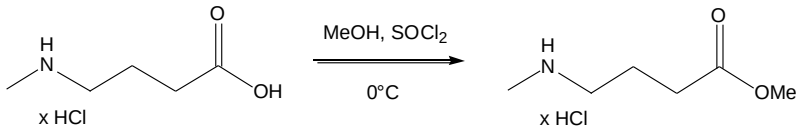
***N*-methylaminobutyric acid hydrochloride** (4-(Methylamino) methyl butyrate, HCl-salt). *The bifunctional linker for dye conjugation (a simplified recipe).*

This bifunctional linker (as an acid) was prepared from *N*-methylpyrrolidone (now a cheap commercial solvent known as NMP), as it was described by Mc Elvain and Vozza in 1947 [1].



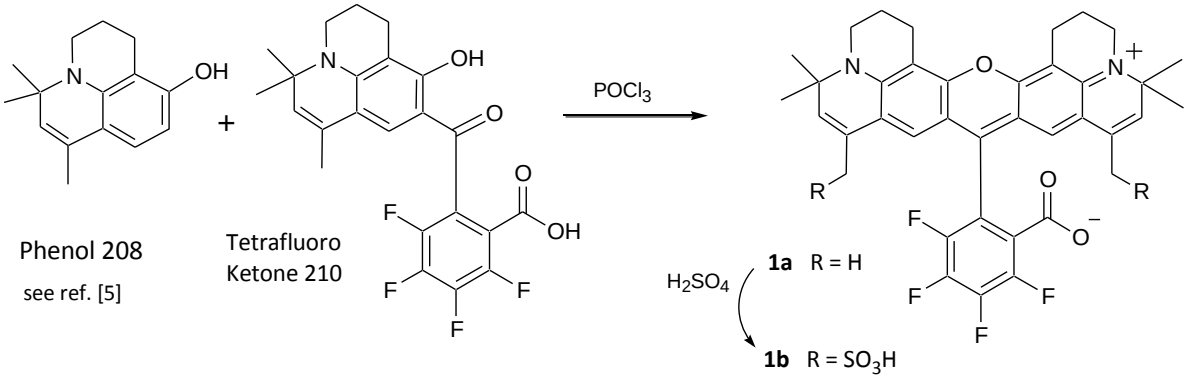
In a 250-mL round-bottom flask a concentrated hydrochloric acid (40 mL) was added within 10 minutes to *N*-methylpyrrolidone (24.8 g, 0.25 mol) under magnetic stirring (caution: exothermic reaction!), and the mixture was refluxed for 17 h. The resulting liquid was concentrated to the weight of ca. 40 g. on a rotary evaporator at 70°C. As the yellowish liquid started to crystallize, acetone (70 mL) was added and the mixture thoroughly stirred. The fine white crystals were filtered off, washed with acetone (3 × 50 mL) and dried in a vacuum desiccator with P_2O_5 to yield 32.9 g (86%) of a product whose NMR spectrum was consistent with the required structure and the melting point (122.5 – 125.5°C) agreed well with reported. NMR spectrum (400 MHz, DMSO-d6): 1.83 (2H, "pseudo pentet", *J* = 7.4 Hz), 2.34 (2H, t, *J* = 7.4 Hz), 2.48 (3H, t, *J* = 5.4 Hz), 2.85 (2H, tt, *J* = 7.4, 5.4 Hz), 9.12 (2H, broad s), 12.22 (1H, broad s).

***N*-methylaminobutyric acid methyl ester hydrochloride** (4-(Methylamino) methyl butyrate*HCl) was obtained using a classical recipe for esterification of amino acids, which utilizes thionyl chloride in methanol [2].



To a chilled solution of *N*-methylaminobutyric acid hydrochloride (3.07 g, 20 mmol) in methanol (20 mL) thionyl chloride (2.1 mL, 28 mmol, 1.4 equiv) was added dropwise at the temperature not exceeding +2°C upon stirring. The stirring was continued at RT for 4 h and the solution was evaporated at room temperature as completely as possible on a rotary evaporator. The pale yellow liquid residue was mixed up with dry diethyl ether, chilled down to 0°C, and the crystallization was initiated by scratching with a glass stick. The flask with the solution was well-shaken, sealed and left at –20°C overnight. A Schott filter was flushed with a pre-cooled ether (–20°C, 20 mL), the solid from the flask was immediately filtered off and flushed three more times with the same pre-cooled solvent (3 × 20 mL). The product was transferred as soon as possible to a small flask and dried in a high vacuum (≤ 1 mbar) overnight to yield a colorless hygroscopic crystalline solid (3.15 g., 94%) with a melting point of 68°C. NMR spectrum (400 MHz, CD_3OD): 1.98 (2H, tt, *J* = 7.4, 7.1 Hz), 2.50 (2H, t, *J* 7.1 Hz), 2.71 (3H, br. s), 3.05 (2H, br. t, *J* = 7.7 Hz), 3.69 (3H, s). These MNR data are also consistent with those reported by Hammler and co-workers [3] who independently obtained this product by a similar recipe. No changes in the NMR spectrum were observed after 2 weeks storage of a methanolic (CD_3OD) sample solution at RT. On the other hand, in a basified solution, the product fast cyclizes "back" into *N*-methylpyrrolidone in the course of an intramolecular amidation. The title compound should be stored and used as a hydrochloride, better in a nitrogen-flushed flask, at –20°C.

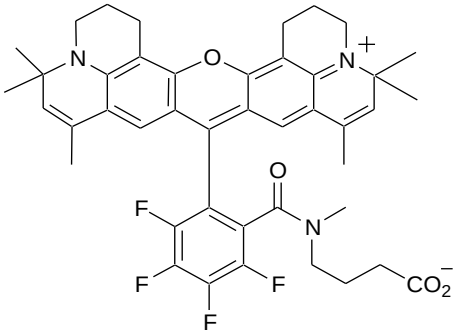
Rhodamine dye **1a** *(the precursor of the whole dye family).*



The recipe for the preparation of rhodamine dye precursor **1a** is based on the one described in [5]. Chloroform is replaced by 1,2-dichloroethane, which has a higher boiling point and no ethanol as stabilizer. Also, the product isolation is much improved. In a typical experiment, a solution of the phenol (0.71 g, 3.1 mmol, "aminophenol 208", see ref. [5] and [6] for preparation and the structure above) in 1.2-dichloroethane (25 mL) was charged into a 100mL flask containing the ketone ("Tetrafluoro Ketone 210" 0.96 g, 2.1 mmol, see structure above and ref. [5] and [6] for preparation). The flask was sealed with a septum, flushed with argon, $POCl_3$ (0.9 mL, 9.6 mmol) was introduced via syringe, and the mixture stirred for 15 min at RT. Further, the homogeneous solution was heated to 60°C upon stirring, while the excessive pressure was being released through a thick cannula. The cannula was removed and the stirring was continued for 8 h at this temperature. The dark blue solution was diluted with approx. an equal volume of DCM and stirred for 2 h with 50 mL of water in a bigger flask. The organic layer was separated, shaken one more time with 50 mL of water, separated again and evaporated by means of a rotary evaporator at t ≤ 35°C. The isolation of the dye was carried out in one run over regular silica gel with MeCN/DCM/H_2O (10:1:1) as the mobile phase (see general notes above). The crude product was dissolved in 100 mL of the mobile phase with sonication and loaded onto a column containing 300g of regular SiO_2. Pure fractions (TLC control with silica gel plates and the same liquid phase) were combined and evaporated to the volume of 10 – 20 mL at t ≤ 40°C. To remove the solubilized SiO_2, the residue was mixed with EtOAc (250 mL), DCM (50 mL) and water (150mL). The mixture was well-shaken, the organic layer separated, and the aqueous layer extracted with EtOAc (50 – 100 mL) until it becomes almost colorless. The combined organic solution was dried with Na_2SO_4, decanted, filtered through a syringe Teflon filter (0.45 – 1.0 µm) or a paper filter of a similar pore size, and evaporated at t ≤ 40°C to give 0.77 g (57%) of rhodamine **1a** (for properties see see [6]). The authors assume that the yield of the rhodamine could be even further improved by using a condensation recipe with trimethylsilyl polyphosphate (TMS-PPA) in DMF [7a,b].

The sulfonation of precursor **1a** was performed as described in [8]. The product (**1b**) was used as is with no additional separation of SiO_2 (see General Notes above). The recipe from ref. [6], which utilizes an RP-SiO_2, can be used alternatively. The sulfonated compound **1b** can be additionally purified by means of preparative HPLC, e.g., on a Kinetex EVO C-18 solid phase with A as an aqueous solution of Et_3N (0.3 vol. %) and MeCN as B with a gradient 0 – 40B in 30 min. Temperatures above 35°C should be avoided while concentrating the fractions (see general comments above). The extra pure product is isolated as a 2×Et_3N salt

Dye **KK 1517** *(the rhodamine dye with a linker and no extra functional group).*



KK 1517 $C_{43}H_{43}F_4N_3O_4$
Exact Mass: 741,32

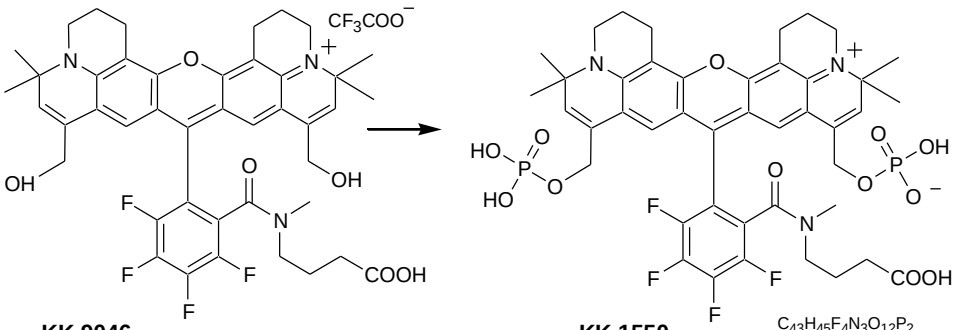
Rhodamine **1a** (15 mg, 0.023 mmol) was dissolved in 1.2-dichloroethane (6mL) in a dry Schlenck flask, the flask was flushed with argon, oxalyl chloride* (100µL, 1.17 mmol, 50 equiv) was added, and the solution was stirred for 3h at RT. A distilling adapter with a long arm and a Schlenck flask as a receiver were connected. The receiver flask was placed in a dry ice bath, the solution carefully evaporated upon magnetic stirring at 1 – 10 mbar, and, in the end, the vacuum (membrane pump) was maintained for 30 min. The residue was dissolved in DCM (6mL), the flask flushed with argon, chilled to +5°C (ice bath), and a solution of *N*-methyl-4-aminobutyrate hydrochloride (23 mg, 0.14 mmol, 6 equiv, for preparation see above) in MeCN (2 mL) was added upon stirring, which was followed by Et_3N (50 µL, 0.35 mmol, 15 equiv). The solution was stirred for 30 more min in an ice bath, diluted with DCM (50 mL), shaken with an equal volume of water, and the organic layer evaporated at t ≤ 35°C. The residue was dissolved in a mixture of THF (7 mL) and water (15 mL). The solution was chilled to +5°C, a 1 mol/L NaOH solution (0.6 mL, 0.6 mmol, 25 equiv) was added in one portion, the mixture swirled and left overnight at this temperature in a fridge. To this mixture DCM (30mL) and water (30mL) were added and, after thorough shaking, the organic layer was separated and evaporated at t ≤ 35°C. The residue was subjected to column chromatography over a column with regular silica gel (20g) and DCM/MeOH (10:1 – 3:1) + 0.1 vol. % Et_3N as the mobile phase. To the combined pure fractions (TLC analysis on silica gel plates with DCM/MeOH 8:1) approximately equal volume of water containing 0.2% TFA and approximately equal volume of DCM were added. The mixture was well-shaken and the organic deeply colored layer separated. The aqueous layer (pH should be acidic) was extracted one more time with a small portion of DCM (20 – 30 mL), combined organic extracts dried with Na_2SO_4, decanted, filtered through a syringe Teflon filter (0.45 µm) and evaporated at t ≤ 40°C to give 11 mg (56%) of the dye **KK 1517** as a TFA salt (M = 741+114). λ max. abs. 639 nm, λ max fl. 663 nm (PBS buffer), ε = 96 020 $M^{-1}cm^{-1}$.

The homolog with a shorter linker (*N*-methyl-β-alanine) was obtained previously by a similar procedure 6].

HPLC: t_R = 21 min (Kinetex C-18, 100 Å, 5 µm, 4.6 × 100 mm), gradient 10 – 80% B in 20 min, with A as an aq. Et_3N (0.3 vol. %) and MeCN as B. TLC: R_f = 0.40 (regular silica gel, MeCN/DCM/H_2O, 10:10:1). MS (ESI): *m/z* (positive mode, %) = 742 (100) $[M+H]^+$; HRMS ($C_{43}H_{44}F_4N_3O_4$): 742.3241 (found M+H), 742.3268 (calc.).

^{1}H NMR (600 MHz, CD_3CN) **. δ = 1.20 – 1.35 (m, CH_2, 4 H), 1.42 – 1.50 (m, 12 H, CH_3), 1.63 – 1.73 (m, CH_2, ~2H), 1.80/1.89 (s × 2, 6 H, CH_3), 1.92 -- 2.10 (m, ~4 H, CH_2), 2.65, 2.74 (s × 2, 3 H, NCH_3), 2.75 – 2.83 (m, ~1 H, CH_2CO), 2.84 – 3.10 (m, 4 H, CH_2), 3.26 — 3,70 (m × 3, 6 H, NCH_2), 5.62 (m, 2 H), 6.64/6.73/6.81 (s × 3, 2 H) ppm; ^{19}F NMR (565 MHz, CD_3CN): δ = –151.8/–151.6 (m, 1 F), –149.2/–149.1 (m, 1 F), –137.1/–136.9 (m, 1 F), –135.7/135.5 (m, 1 F), 73.2 (s, 3 F, CF_3COH) ppm. * -- HATU reagent can be used for amidation instead of oxalyl chloride, as exemplified below for the dye **KK 1103**. ** -- compound is a mixture of amide rotamers with diastereotopic groups, which makes NMR spectra complicated (**see Figs. S27 – S35**).

Dye **KK 1550** *(the dye with two phosphate groups and a linker, "*Abberior® *STAR 635P" according to [9] *). A simplified phosphorylation recipe.*



KK 9046 **KK 1550** $C_{43}H_{45}F_4N_3O_{12}P_2$ 933,24

The hydroxy-substituted dye with a linker **KK 9046** (8 mg, 0.01 mmol, as a TFA salt, see ref. [8] for preparation) in 1,2-dichloroethane (0.7 mL) was added in 1 min to a precooled (0°C, under an argon atmosphere) mixture of $POCl_3$ (0.7 mL) and THF (0.7 mL) upon vigorous stirring. The reaction was continued for 30 min at this temperature and then for 2 h at RT. The solution was diluted with an equal volume of chlorobenzene and evaporated to dryness at t ≤ 25°C). Chlorobenzene (5 mL) was added again, the evaporation repeated, and the residue exposed for 1h to a higher (≤ 1 mbar) vacuum. The solid was dissolved in a mixture of MeCN (1 mL), water (3 mL), and Et_3N (0.1 mL). The solution was left overnight at 5 °C to hydrolyze the intermediate phosphoric dichloride, mixed with an equal volume of DCM and well-shaken. The aqueous deeply-colored solution was evaporated at ≤ 30°C ** as complete as possible, re-dissolved in water (5 mL) containing HCOOH (0.1 mL) and loaded onto a column with reverse-phase silica gel (Polygoprep™ 60-50 C 18, Macherey-Nagel, 10 g). The column was eluted first with water containing 0.1% vol. HCOOH and then with a MeCN/H_2O mixture (2:1 + 0.2 vol. % HCOOH). Pure fractions were pooled, filtered through a syringe filter (0.45 μm), and evaporated at t ≤ 30 °C. That furnished 6.5 mg (70%) of the dye **KK 1550** in the acid form.

λ max. abs. 637 nm, λ max fl. 661 nm (PBS buffer), ε = 116 700 $M^{-1}cm^{-1}$ (consistent with the data from [8,9])

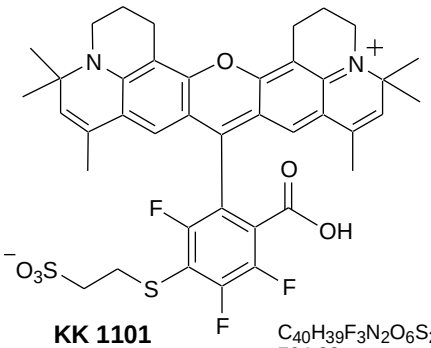
For fluorescence quantum yield estimations, the product was purified by means of a preparative HPLC. Typically, it was performed with a gradient 0 – 30% B (30 min) on a Kinetex EVO C-18 solid phase (column 21 × 250 mm) with A as an aq. Et_3N (0.3 vol. %) and MeCN as B . The extra pure product was isolated as a 2 × Et_3N salt (M = 1135). The mass spectrum was consistent with previously reported [8]. HPLC: t_R = 9 min (Kinetex C-18, 100 Å, 5 μm, 4.6 × 100 mm), gradient (0 – 60% B in 20 min) with A as an aq. Et_3N (0.3 vol. %) and MeCN as B. TLC: R_f = 0.20 (regular silica gel, MeCN/H_2O, 5:1).

^{1}H NMR (600 MHz, D_2O): δ = 1.10-1.16 (m, CH_2, 1 H), 1.18-1.24 (m, CH_2, 1 H), 1.27 (t, J_{HH} = 7.3 Hz, ~18 H, CH_3, Et_3N), 1.38-1.41 (overlapped m, CH_2, 1 H), 1.40 (s, 3 H, CH_3), 1.44 (s, 3 H, CH_3), 1.45-1.48 (overlapped m, 1 H, CH_2), 1.50 (s, 3 H, CH_3), 1.51 (s, 3 H, CH_3), 1.72-1.77 (m, 1 H, CH_2), 1.77-1.82 (m, 1 H, CH_2), 1.82-1.90 (m, 2 H, CH_2), 2.25-2.32 (m, 2 H, CH_2), 2.70 (dt, J_{HH} = 12.9, 6.1 Hz, 1 H, CH_2), 2.79 (s, 3 H, NCH_3), 2.79-2.86 (overlapped m, 2 H, NCH_2), 3.19 (q, J_{HH} = 7.3 Hz, ~12 H, CH_2, Et_3N), 3.30-3.36 (m, 2 H, CH_2), 3.47-3.52 (m, 1 H, CH_2), 3.53-3.59 (m, 1 H, CH_2), 3.71 (dt, J_{HH} = 14.2, 7.4 Hz, 1 H, CH_2), 4.48 (m, J_{HH} = 12.5 Hz, J_{HP} = 5.5 Hz, 3 H, OCH_2), 4.58 (dd, J_{HH} = 12.5 Hz, J_{HP} = 5.5 Hz, 1 H, OCH_2), 6.01 (s, 1 H), 6.03 (s, 1 H), 6.78 (s, 1 H_{ar}), 6.87 (s, 1 H_{ar}) ppm. ^{19}F NMR (565 MHz, D_2O): δ = -137.1 (m, 1 F), -138.9 (m, 1 F), -149.9 (m, $^3J_{FF}$ = 20 Hz, 1 F), -150.1 (m, $^3J_{FF}$ = 20 Hz, 1 F) ppm. ^{31}P NMR (243 MHz, D_2O): δ = -0.2 (t, $^3J_{PH}$ = 5 Hz, 1 P), -0.4 (t, $^3J_{PH}$ = 4 Hz, 1 P) ppm.

* -- in ref. [9] (C. Wurm et al, 2012), this compound is "Abberior® STAR 635P". However, it is very likely that the commercial product with this name (and Φ_f reported to be 90% [20]) has a different structure and might comprise one of phosphorylated NHS carbonates

described in [12]. As seen in **Table1** in the main text, the measured Φ_f of compound **KK 1550** is far lower and is, at least, no higher than that of the benchmark dye **KK 114**. Different to the recipe described in [8], compound **KK 1550** was obtained by a simple direct phosphorylation with $POCl_3$. Other recipes for phosphorylation with $POCl_3$ known form the literature and other solvents can be used, as well. However, it is essential to install the linker *before phosphorylation*, as phosphate groups react with coupling reagents, like HATU. Alternatively, the phosphate groups can be temporarily protected (see [12]). ** -- higher temperatures should be avoided, especially in basic aqueous media (see General Notes above).

Dye **KK 1101** *(dye precursor with -S(CH₂)₂SO₃H as an extra functional group). Halogen exchange reaction in rhodamine **1a** with a thiol.*

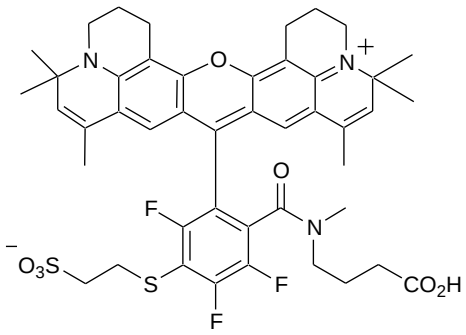


KK 1101 $C_{40}H_{39}F_3N_2O_6S_2$
764,22

In a 1.5 mL vial rhodamine **1a** (20 mg, 0.031 mmol) was dissolved in a mixture of DMF (0.60 mL), water (0.12 mL) and Et_3N (30 µL, 2.1 mmol, 7 equiv). Sodium 2-mercaptoethyl sulfonate (14 mg, 0.09 mmol, 3 equiv) was added, the vial sealed, and the solution was left stirring at RT overnight. The completion of the reaction was witnessed by TLC on regular SiO_2 plates with MeCN/DCM/H_2O (10:10:1) + 0.2 vol. % Et_3N as the mobile phase, and the reaction mixture was acidified with acetic acid (0.3 mL, 5 mmol) and well-shaken with a mixture of DCM (100 mL), water (20 mL), and brine (20 mL). The organic layer was separated, the aqueous layer extracted with DCM (2 × 20 mL), and the combine organic solution (containing DMF too) was evaporated in a rotary evaporator at $t \leq 40$°C. The residue was chromatographed on 30 g of regular SiO_2 with MeCN/DCM/H_2O (10:10:1) + 0.2 vol. % Et_3N as the mobile phase. Pure fractions were combined, filtered through a syringe Teflon filter (0.45 µm) and evaporated at t ≤ 35°C to give 21 mg (74%) of rhodamine **KK 1101** as a Et_3N salt (M = 865). HPLC: t_R = 5 min (Column Kinetex Pheny-hexyl, 100 Å, 5 µm, 4.6 × 100 mm), isocratic mode, 0.5 mL/min, 0.05 M aq. TEAB (A) + 50% MeCN (B), det. at 254 and 635 nm, area 94%; MS (ESI): *m/z* (negative mode, %) = 763 (100) $[M–H]^–$; HRMS ($C_{40}H_{38}F_3N_2O_6S_2$): 763.2106 (found M–H), 763.2123 (calc.).

NMR (400 MHz, CD_3OD) *: δ = 1.26 (t, J = 7 Hz, 9 H, CH_3, Et_3N), 1.45/1.49 (s×2, 12 H, CH_3), 1.90 (m, 6 H, CH_3), 2.05 (m, 4 H, $2CH_2$), 3.01 (br. m, 6 H, CH_2S + $2CH_2$), 3.15 (q, J = 7 Hz, 6 H, Et_3N), 3.28 (m, 2 H, CH_2SO_3), 3.63 (m, 4H, $2CH_2$,), 5.81 (s, 2 H), 6.80 (s, 2 H) ppm; ^{13}C NMR* (100.6 MHz, CD_3OD): 9.4 (CH_2), 18.4 (CH_2), 19.9 (CH_2), 21.2/21.6 (CH_3×2), 44.5 (CH_3), 44.7 (CH_3), 47.8 (CH_3), 53.1 (CH_3), 58.9 (C), 61.0 (C), 59.3 (C), 106.7 (CH), 115.0 (CH), 122.5 (C), 124.0 (CH), 127.3 (CH), 133.5 (CH), 134.3 (C), 151.7 (CH), 155.4 (CH), 167.3 (CO) ppm; ^{19}F NMR (376.4 MHz, CD_3OD): δ = –143.9 (m, 1 F), –125.7 (m, 1 F), –110.3 (m, 1 F) ppm. * -- *due to very low intensities and splitting, the signals of C-F carbon atoms were not recorded.*

Dye **KK 1103** *(the dye with -S(CH₂)₂SO₃H as an extra functional group and a linker). Amidation followed by hydrolysis of the Me-ester group.*



KK 1103 $C_{45}H_{48}F_3N_3O_7S_2$
863,29

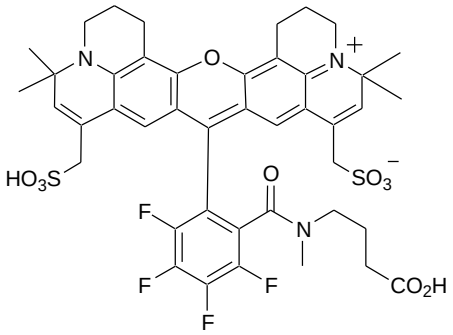
In a 25 mL Schlenck Flask rhodamine **KK 1101** (20 mg, 0.023 mmol) was dissolved in DMF (2mL) containing Et_3N (80 µL, 0.58 mmol, 25 equiv), the flask sealed with a septum, flushed with nitrogen, chilled to +5°C (ice bath) and a solution of HATU reagent (60 mg, 0.16 mmol, 7 equiv) in DMF (1 mL) was added. A slow nitrogen purge and stirring was maintained for 5 -- 10 min., and a freshly-prepared solution of *N*-methylaminobutyric acid methyl ester hydrochloride (30 mg, 0.18 mmol, 8 equiv, preparation see above) in DMF (1 mL) was added at this temperature, which was maintained for 1 hour. The completion of the reaction was witnessed by TLC on regular SiO_2 plates with MeCN/DCM/H_2O (10:10:1) + 0.2 vol. % Et_3N as the mobile phase. The solution was transferred to a bigger flask, the rest of the solution flushed off with few mL DMF, and the solvent evaporated in a rotary evaporator at $t \leq 45$°C almost to dryness. The residue was dissolved in a mixture of DCM (60 mL) and MeCN (20 mL). After that water (50mL), saturated brine (25 mL) and Et_3N (50 µL) were added. The mixture was well-shaken, the organic layer washed with water (50mL), separated, and the solvent removed in a rotary evaporator at $t \leq 45$°C. The residue was dissolved in a mixture of THF (15 mL) and water (7 mL), a 1M aq. solution of NaOH (1 mL, 1 mmol) was added, and the mixture left overnight. The intermediate Me-ester was completely hydrolyzed, as confirmed by HPLC analysis (see below). The solution was neutralized with an excess of HOAc (0.1 mL, 1.7 mmoL) and evaporated to the volume of 10 – 15 mL. Then 15 mL of MeCN/H_2O 1:1 mixture was added, and the flask was sonicated till the precipitate had completely dissolved. The solution was loaded onto a column with reverse-phase silica gel (Polygoprep 60-50, C-18, 25 g) and water as a mobile phase. The column was eluted first with water (100 mL), then with a mixture MeCN/H_2O, to which 1/10 vol. 1M TEAB solution was added. The proportion of MeCN was being gradually increased from 25 to 50%. Pure fractions were pooled, filtered through syringe filters (0.45 µm) and evaporated to give 17 mg (77%) of **KK 1103** as a Et_3N salt (M = 964). The compound is a dark blue crystalline solid, very slightly soluble in pure water, well-soluble in aq. $NaHCO_3$, alkali, Et_3N and most organic solvents. λ max. abs. 635 nm, λ max fl. 660 nm (PBS buffer), ε = 97 100 $M^{-1}cm^{-1}$.

Analytical data: t_R = 6 min (Column Kinetex Pheny-hexyl, 100 Å, 5 µm, 4.6 × 100 mm), isocratic mode, 0.5 mL/min, 0.05 M aq. TEAB (A) + 50% MeCN (B), det. at 254 and 635 nm, area 98%; the intermediate Me-esther has t_R = 23 min under these conditions). TLC: R_f = 0.2 (regular silica gel MeCN/DCM/H_2O, 10:1:1 + 0.2 vol. % Et_3N); MS (ESI): *m/z* (negative mode, %) = 862 (100) $[M–H]^–$; HRMS ($C_{45}H_{47}F_3N_3O_7S_2$): 862.2824 (found M–H), 862.2808 (calc.).

NMR (400 MHz, CD_3OD) *: δ = 1.27 (t, J = 7 Hz, 18 H, 6CH_3, Et_3N), 1.38 (m, CH_2, 2 H), 1.51–1.53 (br. m, 12 H CH_3), 1.80–1.90 (m, 6 H, CH_3), 2.04 (m, 4 H, CH_2), 2.84 (s, 3H, NCH_3) 2.90 (m, 4 H, CH_2), 3.02–3.09 (br. m, 4 H, CH_2S + CH_2), 3.14 (q, J = 7 Hz, 6CH_2, 12 H, Et_3N), 3.30 (m, 2 H CH_2SO_3), 3.32–3.40 (m, 4H, CH_2), 3.66 (m, 4H, 2CH_2), 5.64–5.70 (m, 2 H), 6.70–6.90 (br. m, 2 H) ppm; ^{13}C NMR* (100.6 MHz, CD_3OD): 9.3 (CH_2), 18.6 (CH_2), 18.9 (CH_2), 19.1 (CH_2), 22.0 (CH_3), 21.2/21.6 (CH_3×2), 44.6 (CH_3), 44.7 (CH_3), 47.8 (CH_3), 52.0 (CH_3), 53.1 (C), 61.2 (C), 59.3 (C), 106.7 (CH), 115.0 (CH), 120.1 (C), 122.5 (C), 124.0 (CH), 120.2 (CH), 127.4 (CH), 133.2 (C), 134.3 (C), 151.7

(CH), 155.4 (CH), 176.4 (CO) ppm; ^{19}F NMR (376.4 MHz, CD_3OD): δ = –142.5 (m, 1 F), –1235.8 (m, 1 F), –108.9 (m, 1 F) ppm. * -- compound is a mixture of two amide conformers; due to very low intensities and splitting, the signals of C-F carbon atoms were not properly recorded.

Dye **KK 114L** *(Compound **2b** in Scheme 2, main text), the sulfonated benchmark dye with an elongated linker. Amidation followed by hydrolysis of the Me-ester group. An improved recipe (see [8] and [9]).*



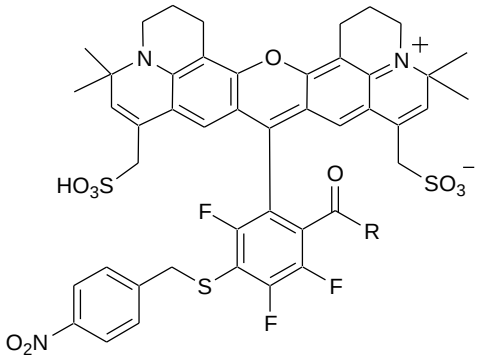
2b (KK 114L)

$C_{43}H_{43}F_4N_3O_{10}S_2$
901,23

The amidation of the sulfonated dye precursor **1b** was carried out as follows: compound **1b** (25 mg, acid form, 0.03 mmol) was dissolved in dry DMF (1.5 mL) containing Et_3N (0.10 mL, 0.70 mmol, 23 equiv) in an argon-flushed Schlenck flask with a magnetic bar and septum. The solution was chilled down to +5°C (ice bath) and HATU reagent (70 mg, 0.18 mmol, 6 equiv) in DMF (0.5 mL) and methyl *N*-methyl-4-aminobutyrate hydrochloride (35 mg, 0.21 mmol, 7 equiv, for preparation see above) in DMF (0.5 mL) were consecutively introduced through syringes upon stirring. The solution was stirred for 1h at this temperature, and then left overnight at RT. The crude methyl ester of **2b** was isolated over a column with a regular SiO_2 (60 g) and DCM/MeOH (10:1 – 4:1) as the mobile phase. The reaction solution was quenched with ca. 10 mL of the eluent and loaded straight onto a column. Pure fractions were pooled, filtered through a syringe Teflon filter (0.45 µm) and evaporated at t ≤ 35°C. The residue was treated with a mixture of THF (10 mL) and H_2O (20 mL) containing KOH (0.5 mL 1M solution, 0.50 mmol). The solution was acidified with CF_3COOH (0.16 mL, 2 mmol), evaporated to the volume of ca. 10 mL and loaded onto a column with reverse-phase silica gel (Polygoprep 60-50 C18, 10 g) and water as the mobile phase. The column was first eluted with water (100 mL) to wash out all the inorganic and small-molecule compounds, then the elution was continued with MeCN/H_2O (1:4 – 1:1) containing 0.1 % TFA. Filtration and freeze-drying of the pure fractions furnished 19 mg (70 %) of the dye **KK 114L** (Compound **2b**, see Scheme 2, main text).

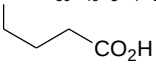
The analytical data was consistent with the previously reported [8]. For fluorescence quantum yield estimations, the product was purified by means of a preparative HPLC. Typically, it was performed with a gradient 0 – 30% B (30 min) on a Kinetex EVO C-18 solid phase (column 21 × 250 mm) with A as an aq. Et_3N (0.3 vol. %) and MeCN as B. The extra pure product was isolated as a 2 × Et_3N salt with MW = 1103. The same procedure can be used for the product isolation instead of hand-made columns. However, one should take in account a considerable saponification of the intermediate Me-ester already with Et_3N upon standing and concentrating its solutions. Also, it is held as obvious that the intermediate Me-esters can be cleaved with an excess of an aq. Et_3N solution overnight at RT, as described for the dye **KK 1555** below. Higher temperatures should be avoided (see General Remarks).

Dye **KK 1555** *(Compound **8** in Scheme 2, main text) the sulfonated dye precursor with a linker and one photostabilizing group (4-nitrobenzyl)thio, NBT))*.



7 R = H $C_{45}H_{40}F_3N_3O_{11}S_3$ 951.17

8 R =  $C_{50}H_{49}F_3N_4O_{12}S_3$ 1050,24

(KK 1555) 

*Halogen exchange reaction in rhodamine **1b** with (4-nitrobenzyl)mercaptan.*

The sulfonated rhodamine precursor **1b** (8 mg, 0.01 mmol, acid form, see structure above) was dissolved in a mixture of DMF (0.80 mL), water (0.1 mL) and Et_3N (15 µL, 0.1 mmol, 10 equiv). Solid (4-nitrobenzyl)mercaptan (3 mg, 0.18 mmol, 1.8 equiv) was added, the vial sealed, and the solution was left stirring at RT overnight. The completion of the reaction was witnessed by TLC (see below), the reaction mixture was diluted with 8 mL of the same liquid and chromatographed over a column with regular SiO_2 (12 g). Pure fractions were combined, filtered through a syringe Teflon filter (0.45 µm) and evaporated at t ≤ 45°C to furnish 10 mg (87%) of 4-nitrobenzylthio-substitstuted rhodamine (Compound **7**, see also Scheme 2 in the main text) as a 2×Et_3N salt (MW = 1153) whose purity and identity was confirmed by HPLC analysis and HRMS. MS (ESI): *m/z* (negative mode, %) = 950 (90) $[M–H]^–$. HRMS ($C_{45}H_{40}F_3N_3O_{11}S_3$): 950.1687 (found M–H), 950.1699 (calc.). TLC: R_f = 0.5 (silica gel, MeCN/H_2O 5:1 + 0.3 vol. % TEAA + 0.3 .3 vol. % HOAc); HPLC: t_R = 8 min (Kinetex C-18, 100 Å, 5 µm, 4.6 × 100 mm), gradient 0 – 80%B in 20 min, 0.5 mL/min, with an aq. Et_3N (0.3 vol. %) as A and MeCN as B.

Amidation with a bifunctional linker followed by hydrolysis of the Me-ester group.

The amidation of Compound **7** (10 mg, 0.009 mmol) was performed exactly as described for **KK 1103** (see above) in DMF (ca. 2.5 mL) with, Et_3N (30 µL, 0.20 mmol, 20 equiv) HATU reagent (19 mg, 0.05 mmol, 5 equiv) and *N*-methylaminobutyric acid methyl ester hydrochloride (10 mg, 0.06 mmol, 6 equiv). The completion of the reaction was witnessed by TLC (see below). The solution was chromatographed over a column with regular SiO_2 (20 g). The column was first eluted with DCM/MeOH (10:1) mixture to remove DMF and salts, then with DCM/MeOH (4:1) containing 0.2 vol. % Et_3N. The colored fractions were combined, evaporated, and the residue dissolved in a pre-cooled (0°C) mixture of water (6mL), THF (3 mL) and 1M NaOH (0.2 mL, 0.2 mmol). After 2h at RT the hydrolysis of the intermediate Me-ester was complete, as seen by TLC and HPLC: t_R changes from 14.5 to 12.5 min (Kinetex C-18, 100 Å, 5 µm, 4.6 × 100 mm), gradient (0 – 80 % B in 20 min), 0.5 mL/min, with A as an aq. Et_3N (0.3 vol. %) and MeCN as B. The solution was neutralized with HOAc (50 µL, 0.8 mmol), evaporated and chromatographed over a column with regular SiO_2 (6g) using MeCN/H_2O (5:1 – 4:1) + 0.2 vol. HCOOH as the mobile phase. Pure fractions were combined, re-dissolved in DCM/MeOH (10:1) mixture, filtered through a

syringe Teflon filter (0.45 µm) and evaporated at t ≤ 45°C to give 5.5 mg (58%) of rhodamine **KK 1555** (Compound **8** in Scheme 2, main text) in the acid form (M = 1050). λ max. abs. 636 nm, λ max fl. 659 nm (PBS buffer), ε = 88 700 $M^{-1}cm^{-1}$.

A larger scale synthesis was performed as follows:

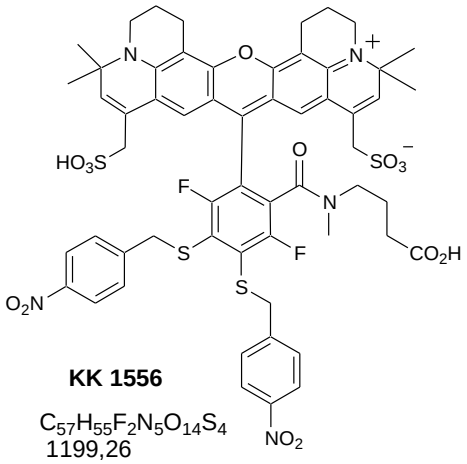
The sulfonated rhodamine precursor **1b** (24 mg, 0.024 mmol, 2xEt_3N salt with MW = 1004) was dissolved in a mixture of DMF (1.4 mL), water (150 µL) and Et_3N (75 µL, 0.52 mmol, 22 equiv). Solid (4-nitrobenzyl)mercaptan (14 mg, 0.08 mmol, 3.3 equiv) was added, the vial sealed, and the solution was left stirring at RT overnight. The completion of the reaction was checked by TLC, the solution was diluted with water (30 mL) and extracted with EtOAc (30 mL). The aqueous solution was filtered through a syringe Teflon filter (0.45 µm), evaporated at t ≤ 40°C to dryness. The residue was divided in two portions and subjected to a gradient (0 – 60% B in 30 min) preparative HPLC on a Kinetex EVO C-18 solid phase (column 21 × 250 mm) with A as aqueous 0.03 M TEAB buffer and MeCN as B. The pure fractions were evaporated to give 24 mg (86%) of Compound **7** as a 2×Et_3N salt (MW = 1153).

The solution of the intermediate Compound **7** (24 mg, 0.02 mmol, 2xEt_3N salt with MW = 1153) in a mixture of dry DMF (4mL), DCM (2 mL) and Et_3N (80 µL, 0.55 mmol, 27 equiv) was purged with nitrogen in a Schlenck flask and chilled down to +5°C (ice bath). HATU reagent (46 mg, 0.12 mmol, 6 equiv) in DMF (~0.5 mL) and methyl *N*-methyl-4-aminobutyrate hydrochloride (23 mg, 0.14 mmol, 7 equiv, for preparation see above) in DMF (~0.5 mL) were consecutively introduced through syringes upon stirring. The solution was stirred for 2h at this temperature and diluted with an ice cold water containing 90 µL(1.5 mmol) HOAc. The solution was extracted with a mixture of EtOAc (60 mL) and cyclohexane (15 mL). The aqueous phase was basified with a 1M TEAB solution (1 mL, 1 mmol), filtered through a syringe Teflon filter (0.45 µm) and concentrated at t ≤ 40°C to the volume of 3 – 5 mL. The product was divided in two portions and subjected to a gradient (0 – 80% B in 30 min) preparative HPLC on a Kinetex EVO C-18 solid phase (column 21 × 250 mm) with A as aqueous 0.03 M TEAB buffer and MeCN as B. The pure fractions were evaporated to dryness, re-dissolved in a mixture of water (5mL) and Et_3N (0.2mL), and the solution stirred overnight at RT. The saponification of the intermediate Me ester was witnessed by HPLC (see retention data above) and TLC (see below). The solution was evaporated and the free acid chromatographed (gradient HPLC) as described above. This gave 22 mg (88%) of the dye **KK 1555** as a 2x Et_3N salt.

Analytical data: t_R = 12.5 min (Kinetex C-18, 100 Å, 5 µm, 4.6 × 100 mm), gradient (0 – 80 % B in 20 min), 0.5 mL/min, with A as an aq. Et_3N (0.3 vol. %) and MeCN as B. TLC: TLC: R_f = 0.5 (silica gel, MeCN/H_2O 5:1 + 0.3 vol. % Et_3N). MS (ESI): *m/z* (negative mode, %) = 1049 (100) $[M–H]^–$; HRMS ($C_{50}H_{48}F_3N_4O_{12}S_3$): 1049.2379 (found M–H), 1049.2383 (calc.).

^{1}H NMR (500 MHz, DMSO-d6) *: δ = 1.27 (t, *J* = 7 Hz, ~18 H, 6CH_3, Et_3N), 1.33 (m, 2 H, $CH_2C\underline{H}_2CH_2$), 1.51 (m, 2 H, CH_2), 1.70/1.74/1.78 (s×3, 12 H, CH_3), 1.96 (m, 2CH_2, 4 H), 2.70/2.81 (s×2, NCH_3, 3 H), 2.81 (q, *J* = 7 Hz, 3CH_2, 12 H, Et_3N), 2.93 (m, CH_2CO, 2 H), 3.10 (m, 2 H, CH_2), 3.66 (m, 4 H, 2CH_2N), 3.77 (m, 2 H, CH_2N), 4.25/4.40 (m, m, 4 H, 2CH_2SO_3), 5.78 (s, SCH_2, 2H), 7.40 – 7.55 (m, 4 H), 7.77 (d, *J* = 8 Hz, 2H, 4-$PhNO_2$), 8.19 (d, *J* = 8 Hz, 2H, 4-$PhNO_2$) ppm; ^{19}F NMR (282.4 MHz, DMSO-d6): δ = –143.1 (m, 1 F), –142.4 (m, 1 F, ≤ 0.15H), –123.8 (m, 1 F), –105.8 (m, 1 F) ppm. * -- compound is a mixture of amide conformers.

Dye **KK 1556** *(Compound **6** in Scheme 2, main text) the sulfonated dye precursor with a linker and two photostabilizing groups (4-nitrobenzyl)thio, NBT))*.



KK 1556

$C_{57}H_{55}F_2N_5O_{14}S_4$
1199,26

To the solution of the dye **KK 114L*** (2×Et_3N salt with M = 1103, 11 mg, 0.01 mmol, for preparation see above) and Et_3N (40 µL, 027 mmol, 27 equiv) in DMF (0.5 mL) solid (4-nitrobenzyl)mercaptan (40 mg, 0.24 mmol, 24 equiv) was added, the vial sealed and left stirring for 10 days** at RT. The solution was transferred into a flask containing water (25 mL), EtOAc (50 mL), DCM (10 mL) and HOAc (90 µL), the vial flushed with water (1 mL), and the mixture well-shaken. The deeply colored aqueous layer was extracted one more time with a mixture of EtOAc (20 mL) and DCM (5 mL) and evaporated at t ≤ 45°C. The dye was dissolved in MeCN (5mL) and purified over a column with regular SiO_2 (12g) and MeCN/DCM/H_2O (10:10:1) as the mobile phase. Pure fractions were pooled, concentrated to the volume of 10-15 mL, and MeCN (10 mL) was added to completely dissolve the precipitate. DCM (60 mL), aqueous NH_4Cl (20 wt. %, 20 mL) and TFA (60 µL) were added, and the solution was well shaken. The aqueous layer was extracted two more times with DCM (20 mL), and the combined organic layer washed with brine (15 mL) and water (5 mL). The organic extract was dried (Na_2SO_4), filtered through a syringe Teflon filter (0.45 µm) and evaporated at t ≤ 40°C. The solid residue was sonicated for few min with EtOAc (5 mL) to remove colorless impurities, centrifuged and dried *in vacuo* to give 7 mg (52%) of rhodamine **KK 1556** (Compound **6** in **Scheme 2**, main text) with the purity ≥ 95%. The compound was recovered as a TFA salt in the acid form (M = 1313), free from SiO_2, starting material (**2b**) and from the mono-substituted product (**8**, see above) which all remain in the aqueous phase.

For photophysical measurements the dye **KK 1556** was additionally purified by means of a gradient (0 – 60% B in 30 min) preparative HPLC on a Kinetex EVO C-18 solid phase (column 21 × 250 mm) with A as aqueous TEAA buffer (0.02 M Et_3N × HOAc + 0.06 M HOAc) and MeCN as B with a flow rate of 12 mL/min. Pure fractions were pooled, concentrated to ca. 1/4 of the initial volume and acidified with TFA (~ 2% vol.). The dye was extracted with a mixture EtOAc/DCM (5:1) till the aqueous phase had become almost colorless. The extract was dried, filtered and evaporated. The solid was sonicated for 10 min with EtOAc (~ 1 mL/mg) to remove salts and most of the TFA. The suspension centrifuged, and the very fine precipitate carefully dried in air and then *in vacuo* (~1 mbar).

* -- prepared from **1b** using HATU reagent, as described in [8] or using an improved recipe above; dye **KK 114L** (**2b**) has a linker which is by one CH_2 unit longer than in **KK 114** (see [6], [8], [9] and **Scheme 2**, main text) . This linker elongation provides much better stability of the NHS ester (used for labeling).

** -- almost the same yield of **KK 1556** was achieved when the reaction proceeded for 3 days at RT and then 1 day at 45°C. On the other hand, elevated temperatures (50 – 60°C), when being maintained from the very start, resulted in much lower yields.

λ max. abs. 638 nm, λ max fl. 662 nm (PBS buffer), ε = 62 200 $M^{-1}cm^{-1}$.

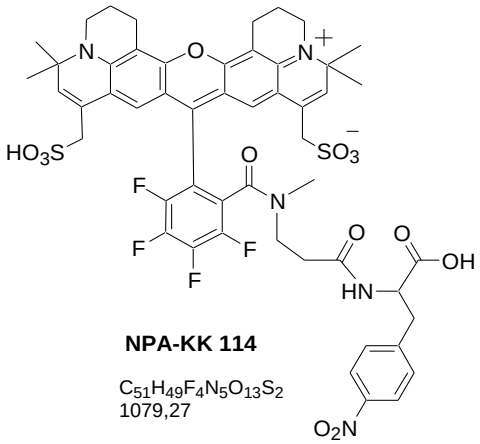
Analytical data: TLC: R_f = 0.6 (regular silica gel, MeCN/H_2O, 5:1). t_R = 11 min (Kinetex Phenyl-hexyl, 100 Å, 5 µm, 4.6 × 100 mm), isocratic mode, 0.5 mL/min, 0.05 M aq. TEAB (A) + 40% MeCN (B), det. at 254 and 635 nm, area 98%). Alternatively, t_R = 11.5 min (Kinetex C-18, 100 Å, 5 µm, 4.6 × 100 mm), gradient 0 – 80% B in 20 min, 0.5 mL/min, with an aq. Et_3N (0.3 vol. %) as A and MeCN as B. MS (ESI): *m/z* (negative mode, %) = 1198 (100) $[M–H]^–$; HRMS ($C_{57}H_{54}F_2N_5O_{14}S_4$): 1198.2509 (found M–H), 1198.2518 (calc.).

^{1}H NMR (400 MHz, DMSO-d6) ***: δ = 1.22 (m, 2 H, $CH_2CH_2CH_2$), 1.48 (m, 2 H, CH_2), 1.70/1.74/1.78 (s×3, 12 H, CH_3), 1.95 (m, 4 H, $2CH_2$), 2.90 (m, NCH_3, 3 H), 2.93 (m, 2 H, CH_2CO), 3.30 (m, 2 H, CH_2), 3.44 (m, 4 H, $2CH_2N$), 3.59 (m, 2 H, CH_2N), 4.20/4.46 (m, m, 4 H, $2CH_2SO_3$), 5.78 (m, 4H, $2SCH_2$), 7.36 (s, 2 H), 7.48 (m, 2 H), 7.63 – 7.73 (m, 4H, 4-$PhNO_2$), 8.14 – 8.19 (m, 4H, 4-$PhNO_2$,) ppm;

^{19}F NMR (376.5 MHz, DMSO-d6): δ = –109.4 (d, *J* = 14 Hz, 1 F), –104.7 (d, *J* = 14 Hz, 1 F), –73,9 (s, 3F, CF_3, TFA) ppm.

*** -- compound is a mixture of amide conformers.

Conjugate **NPA-KK 114** *(4-nitro-L-phenylalanine as photostabilizer). An improved larger-scale recipe, as compared to described in ref. [10] *.*



NPA-KK 114

$C_{51}H_{49}F_4N_5O_{13}S_2$
1079,27

In a 10 mL Schlenck Flask dye **KK 114** (**2a**, see ref. [6] for preparation) (9 mg, 0.01 mmol) was dissolved in DMF (1mL) containing Et_3N (30 µL, 0.2 mmol, 20 equiv). Under a slow nitrogen purge the flask was chilled to +5°C (ice bath) and, under magnetic stirring, HATU reagent (15 mg, 0.04 mmol, 4 equiv) in DMF (0.2 mL) and solid 4-nitro-L-phenylalanine methyl ester hydrochloride (CAS 17193-40-7, 13 mg, 0.05 mmol, 5 equiv) were added. The stirring was maintained for 15 min at this temperature, then for 1h at RT. The completion of the reaction was witnessed by TLC on regular SiO_2 plates with MeCN/H_2O (5:1) as the mobile phase. The solution was, neutralized with HOAc (30 µL), diluted with 5 mL of the mobile phase and loaded onto a column with regular SiO_2 (15g) and MeCN/DCM/H_2O (10:10:1, neutral). After elution, homogeneous fractions were pooled and evaporated at t ≤ 40°. The residue was dissolved in a mixture of water (6mL), THF (3 mL) and 1M NaOH (0.2 mL, 0.2 mmol). After 30 min at RT the hydrolysis of the intermediate Me-ester (not isolated in the pure state) was complete** (as seen by TLC and HPLC), the solution was neutralized with HOAc (100 µL, 1.7 mmol), concentrated to the volume of ca. 10 mL and chromatographed over a column with regular RP-SiO_2 (Polygoprep™ 60-50 C 18, Macherey-Nagel, 5g) using MeCN/H_2O (1:5 – 1:2) + 0.2 vol. % HCOOH as the mobile phase. Pure fractions were combined, filtered through a syringe Teflon filter (0.45 µm) and evaporated at t ≤ 40°C to give 9 mg (83%) of **NPA-KK 114** (in the acid form with M = 1079). Alternatively, the intermediate Me ester and the free acid can be isolated by means of preparative HPLC, better under acidic conditions described for **KK 1553** (the dye with additional linker) below.

TLC: R_f = 0.50 (regular silica gel, MeCN/H_2O, 5:1). HPLC: t_R = 8.4 min (Kinetex Phenyl-hexyl, 100 Å, 5 µm, 4.6 × 100 mm), isocratic mode, 0.05 M aq. TEAB (A) + 35% MeCN (B), det. at 254 and 635 nm, area 98%); the starting compound **KK 114** (**2a**) has t_R = 3.2 min,

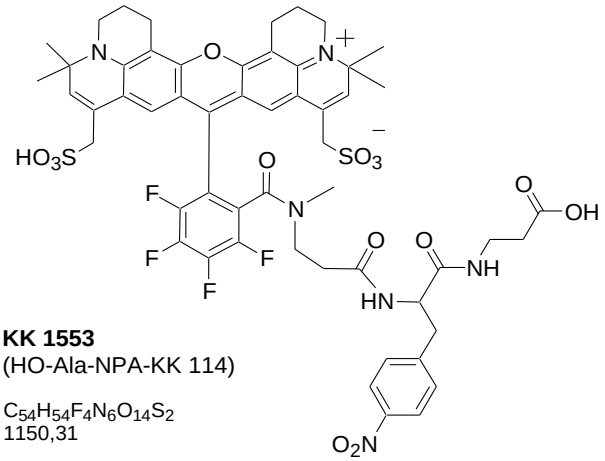
the intermediate Me-ester – 16 min. See also ref. [10] for properties and analytical data. However, that recipe utilized 4-nitro-L-phenylalanine as a free acid and was performed on a small (2 mg) scale.

A simplified one-pot procedure, where the saponification step was skipped, gave a lower yield. It was performed as follows: dye **KK 114** (**2a**) (30 mg, 0.034 mmol) was reacted with 4-nitro-L-phenylalanine methyl ester hydrochloride (45 mg, 0.17 mmol, 6 equiv) in presence of HATU reagent (60 mg, 0.16 mmol, 5 equiv) and Et_3N (100 µL, 0.7 mmol, 20 equiv) in DMF (ca. 3 mL) exactly as described above. The reaction mixture was transferred into a flask containing a pre-cooled to +10°C solution of Et_3N (100 µL) in water (30 mL), left at RT for 4h and evaporated almost to dryness at t ≤ 35°C. TLC and HPLC control (see above) showed a complete hydrolysis of the intermediate Me-ester. The residue was subjected to a gradient (0 – 40% B in 30 min) preparative HPLC on a Kinetex EVO C-18 solid phase (column 21 × 250 mm) with aqueous 0.3 vol. % Et_3N (A) and MeCN (B). Pure fractions were combined and evaporated at t ≤ 35°C to give 24 mg (56%) of **NPA-KK 114** as a 2×Et_3N salt (M = 1281). The HRMS spectrum was consistent with previously reported [10].

^{1}H NMR (400 MHz D_2O) ***: δ = 1.21 (t, J = 7 Hz, 18 H, CH_3, 2Et_3N), 1.32/1.34/1.38/1.42 (s×4, CH_3, 12 H), 1.63 (m, 4 H, CH_2), 2.07 (m, 2 H, CH_2), 2.30 (m, 4 H, 2CH_2), 2.80 (m, 2 H, CH_2CO), 2.83/2.85 (s×2, 3 H, NCH_3), 3.12 (q, J = 7 Hz, 12 H, CH_2, 2Et_3N), 3.30 (m, 4 H, 2CH_2), 3.50, 3.90 (m, m, 4H, 2CH_2SO_3), 3.68 (m, 2 H, CH_2), 4.05, 4.29 (m, m, 2H, CH_2Ar), 4.43 (m, 1H, CHCOO), 5.85 (m, 2H), 7.04 (m, 2H), 7.17 (d, 2H, 4-PhNO_2), 7.63/7.76 (m, m 2H) ppm; ^{19}F NMR (376.5 MHz, D_2O): δ = –151.7 (m, 1 F), –149.4 (m, 1 F), –149.0 (m, ~0.1 F), –139.4 (m, 1 F), –138.30 (m, ~0.1 F), –135.8 (m, 1 F), –134.6 (m, ~0.1 F) ppm.

* -- the previous recipe [10] utilized the unprotected 4-nitro-L-phenylalanine (acid form), and the yield was considerably lower. ** -- the hydrolysis of the L-phenylalanine Me-ester conjugate proceeds with Et_3N even at RT. Also, in basic media, especially at temperatures above 30°C, the substitution of one fluorine atom with OH (or with OMe in methanol) is possible. This produces a side-peak in the HPLC analyses (see General Notes above). *** -- compound is a mixture of two amide conformers.

Dye **KK 1553** *(HO-Ala-NPA-KK 114) with an additional β–alanine linker (see also ref. [10] *).*



KK 1553
(HO-Ala-NPA-KK 114)

$C_{54}H_{54}F_4N_6O_{14}S_2$
1150,31

An extra linker (β-alanine) was installed via a peptide-type coupling, as described for **NPA-KK 114** above. The reagents were combined at +5°C under an N_2 purge, the solution was slowly warmed up to RT and left stirring for 1h. In a typical experiment, **NPA-KK 114** (as a 2×Et_3N salt, 17 mg, 0.013 mmol) was reacted with β-alanine methyl ester hydrochloride (10 mg, 0.07 mmol, 7 equiv) in presence of HATU reagent (21 mg, 0.055 mmol, 5 equiv) and Et_3N (35 µL, 0.24 mmol, 24 equiv) in DMF (1.5 mL). The reaction mixture was diluted with a cold (+5°C) water (8 mL) containing HOAc (200 µL) and divided in two portions (2 × 5mL). Each portion was subjected to a gradient (0 – 60% B in 30 min) preparative HPLC on a Kinetex EVO C-18 solid phase (column 21 × 250 mm) with A as aqueous TEAA

buffer (0.02 M Et_3N × HOAc + 0.06 M HOAc) and MeCN as B with a flow rate of 12 mL/min. Pure fractions were pooled, evaporated at t ≤ 35°C and freeze-dried from water to give the intermediate Me ester as a $2Et_3N$ salt (15 mg, 90%). The product was hydrolyzed with a dilute alkali solution prepared form 1M NaOH (0.2 mL, 0.2 mmol) and water (10 mL). As the HPLC analysis showed the completion of the reaction after 1h at RT, the solution was acidified with HOAc (0.1 mL), and concentrated to the volume of 5 mL. The residue was subjected to a gradient preparative HPLC as described above, pure fractions evaporated at t ≤ 35°C and freeze-dried from water (2 times) to give 14 mg (80%) of **KK 1553** (HO-β-Ala-NPA-KK 114) as a 2×Et_3N salt (M = 1352).

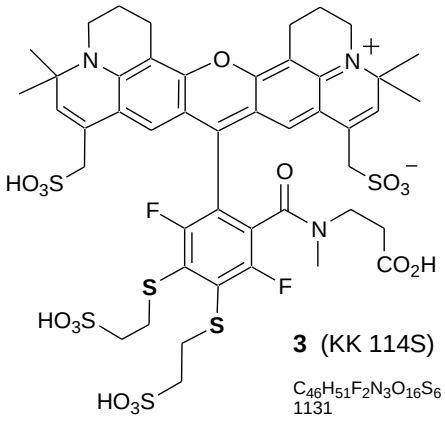
Analytical data: t_R = 6 min (Kinetex Phenyl-hexyl, 100 Å, 5 μm, 4.6 × 100 mm), isocratic mode, 0.5 mL/min, 0.05 M aq. TEAB (A) + 35% MeCN (B), det. at 254 and 635 nm, area 98%); the starting compound **NPA-KK 114** has t_R = 8.4 min, the intermediate Me-ester – 18 min. TLC: R_f = 0.40 (regular silica , $MeCN/H_2O$, 5:1). Alternatively, the dye **KK 1553** shows t_R = 13 min (Kinetex C-18, 100 Å, 5 μm, 4.6 × 100 mm), gradient 0 – 60% B in 30 min, 0.5 mL/min, with an aq. Et_3N (0.3 vol. %) as A and MeCN as B. The MS-specta consistent with previously reported [10]. See below for actual HPLC analyses. λ max. abs. 637 nm, λ max fl. 660 nm (PBS buffer), ε = 88 460 $M^{-1}cm^{-1}$.

* -- as explained in ref. [10], the NHS ester of the unmodified **NPA-KK 114** is unstable and, therefore, unsuitable for labelling.

^{1}H NMR (600 MHz DMSO-d6) **: δ = 1.09 (t, J = 7 Hz, 18 H, CH_3, Et_3N), 1.38 – 1.52 (s×4, 12 H CH_3), 1.89 – 1.95 (m, 4 H, CH_2), 2.28 – 2.30 (m, 4 H, $2CH_2$), 2.37 (m, 2 H, CH_2CO), 2.75/2.79 (s×2, 3 H, NCH_3), 2.05 (m, CH_2, 12 H, Et_3N), 3.12 – 3.68 (m, 12 H), 4.37 (t, 2H, CH_2Ar), 4.44 (m, 1H, CHAla), 5.72 (s, 1H), 5.78 (m, 1H), 7.32 – 7.52 (m, 2H), 7.96 – 8.30 (m, 1H) ppm;

^{19}F NMR (564.7 MHz, DMSO-d6): δ = –153.8 – 153.3 (m, ~1.6 F), –141.7 (m, ~0.2 F), –140.8/–140.4 (m, 2 x ~0.1 F), –135.6 (~0.1F) – 135.4 (m, 1 F) ppm. ** -- compound is a mixture of amide conformers.

*Dye **KK 114S** (Compound **3** in Scheme 2, main text) with four sulfonic acid groups. Modification of the dye **KK 114** "as it is").*



3 (KK 114S)

$C_{46}H_{51}F_2N_3O_{16}S_6$
1131

The substitution of two fluorine atoms in the "ready" dye KK 114 with 2-mercaptoethyl sulfonate groups was typically performed as follows: to a solution of dye **KK 114*** (30 mg, 0.033 mmol) in a mixture of DMF (1.5 mL) and water (75 μL, 1/20 vol.) solid sodium 2-mercaptoethyl sulfonate (110 mg, 0.67 mmol, 20 equiv) was added and heated in a sealed 2.5 mL vial for 8 – 10 h at 90°C. The solution was evaporated almost to dryness at t ≤ 45°C, diluted with water (10 mL) and divided in two portions (2 × 5mL). Each portion was subjected to a gradient (0 – 15% B in 45 min) prep. HPLC on a Kinetex EVO C-18 solid phase (column 21 × 250 mm) with A as an aqueous solution of Et_3N (0.3 vol. %) and MeCN as B with a flow rate of 12 mL/min. Pure fractions were pooled, evaporated at t ≤ 45°C and freeze-dried from water to give 28 mg (55%) of the dye **KK 114S*** as a 3 × Et_3N salt (M = 1434).

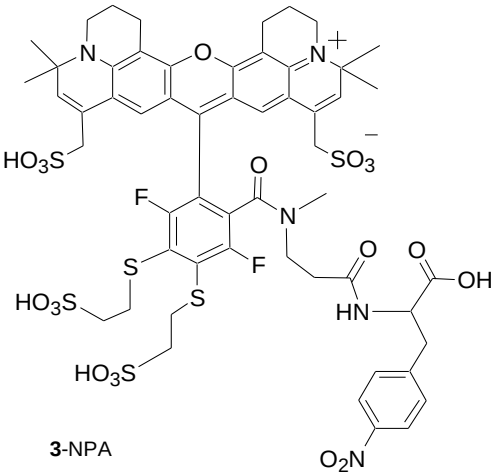
* -- it is held as obvious that exactly same reaction can be performed also with the dye **KK 114L** (longer linker) with no complications.

λ max. abs (PBS buffer) = 635 nm, ε = 104 830 $M^{-1}cm^{-1}$. Analytical data: t_R = 3.2 min (Kinetex Phenyl-hexyl, 100 Å, 5 µm, 4.6 × 100 mm), isocratic mode 0.5 mL/min, 0.05 M aq. TEAB (A) + 30% MeCN (B), det. at 254 and 635 nm, area 97%). Alternatively, t_R = 8 min (Kinetex C-18, 100 Å, 5 µm, 4.6 × 100 mm), gradient 0 – 60% B in 20 min, 0.5 mL/min, with an aq. Et_3N (0.3 vol. %) as A and MeCN as B. TLC: R_f = 0.10 (regular silica gel, MeCN/H_2O, 5:1). MS (ESI): *m/z* (negative mode, %) = 1130 (100) $[M–H]^–$; HRMS ($C_{46}H_{50}F_2N_3O_{16}S_6$): 1130.1479 (found M–H), 1130.1483 (calc.).

^{1}H NMR (400 MHz D_2O) of **KK 114S**: δ = 1.20 (t, *J* = 7 Hz, 27 H, CH_3, Et_3N), 1.40/1.44 (s × 2, 6 H, CH_3), 1.50/1.53 (s × 2, 6 H, CH_3), 1.65 (t, 2 H, CH_2), 1.67 – 1.87 (m, 4 H, CH_2), 2.12 (m, 2 H, CH_2), 2.57 (s, 3 H, CH_3), 2.77 – 2.98 (m, 4 H, CH_2), 2.88 – 2,95 (m, 2 H, ½ CH_2), 3.03 – 3.07 (m, 2 H, ½ CH_2), 3.13 (q, *J* = 7 Hz, 18 H, CH_2, Et_3N), 3.23 – 3.40 (m, 5H, CH_2+ ½CH_2N), 3.45 –3 .57 (m, 6H, CH_2 + CH_2N), 3.63 – 3.68 (m, 1 H, ½ CH_2N), 3.73 (d, *J* = 14.6 Hz, 1 H, CH_2SO_3), 3.79 (d, *J* = 14.4 Hz, 1 H, CH_2SO_3), 3.86 (d, *J* = 14.4 Hz, 1 H, CH_2SO_3), 4.10 (d, *J* = 14.6 Hz, 1 H, CH_2SO_3), 4.03, 5.88 (d x 2, *J* = 8 Hz, 2H), 7.07 (m, 2H) ppm; ^{13}C NMR (150.9 MHz, D_2O): 9.6 (CH_3), 20.6/21.2 (CH_2×2), 29.0 (m, CH_3), 31.1/31.5 (CH_2×2), 38.9 (CH_3), 43.6 (CH_2), 45.9 (CH_2), 48.1 (CH_2), 52.6 (CH_2), 54.3 (CH_2), 55.0 (CH_2), 61.3 (m, CH_2), 107.8 (C), 108.5 (C), 113.5 (C), 114.1 (C), 119.7 (m, C), 121.6 (C), 123.5 (CH), 125.7 (m, C), 131.6 (m, C), 132.3 (m, C), 139.2 (CH), 140.1 (CH), 143.6 (C), 151.6 (C), 152.2 (C), 154.4 (C), 165.0 (CO) ppm;

^{19}F NMR (564.7 MHz, D_2O): δ = –106.6 (d, *J* = 14 Hz, 1 F), –109.6 (d, *J* = 14 Hz, 1 F) ppm.

Dye conjugate **NPA-KK 114S** *(Compound **3-NPA** in Scheme 2, main text)*.



3-NPA

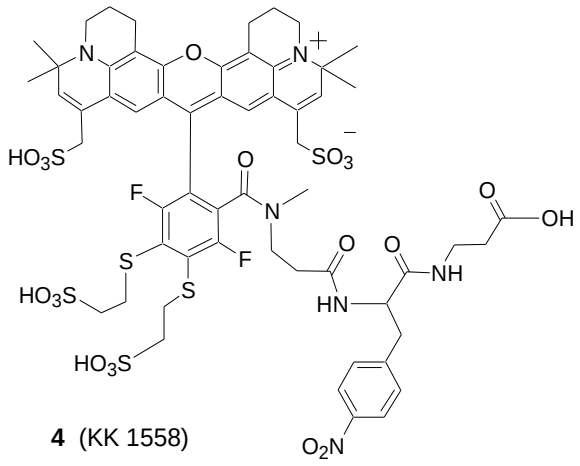
$C_{55}H_{59}F_2N_5O_{19}S_6$
1323,21

The NPA moiety was installed via a peptide-type coupling, as described for **KK 114** above. The reagents were combined at +5°C under a N_2 purge, the solution was slowly warmed up to RT and left stirring for 1 – 2h. In a typical experiment, the dye **KK 114S** (Compound **3** as a 4×Et_3N salt, 27 mg, 0.017 mmol) was reacted with 4-nitro-L-phenylalanine methyl ester hydrochloride (CAS 17193-40-7, 23 mg, 0.09 mmol, 5 equiv) in presence of HATU reagent (32 mg, 0.09 mmol, 5 equiv) and Et_3N (50 µL, 0.35 mmol, 20 equiv) in DMF (2.5 mL). The reaction mixture was diluted with water (20 mL) containing Et_3N (200 µL) to hydrolyze the intermediate Me ester, and after 1h the solution was evaporated t ≤ 45°C. The residue was subjected to a gradient (0 – 35% B in 30 min) prep. HPLC on a Kinetex EVO C-18 solid phase (column 31 × 150 mm) with A as an aq. Et_3N (0.3 vol. %) and MeCN as B with a flow rate of 10 mL/min. Pure fractions were pooled, evaporated at t ≤ 45°C and freeze-dried from water to give 21 mg (72%) of **NPA-KK 114S** as a 4×Et_3N salt (MW = 1727).

Analytical data: t_R = 5.6 min (Kinetex Phenyl-hexyl, 100 Å, 5 µm, 4.6 × 100 mm), isocratic mode 0.5 mL/min, 0.05 M aq. TEAB (A) + 30% MeCN (B), det. at 254 and 635 nm, area 97%. TLC: R_f = 0.12 (regular silica gel, MeCN/H_2O, 5:1). MS (ESI): *m/z* (negative mode, %) = 1322 (100) $[M–H]^–$; HRMS ($C_{55}H_{58}F_2N_5O_{19}S_6$): 1322.2009 (found M–H), 1322.2018 (calc.).

^{1}H NMR (600 MHz D_2O): δ = 1.20 (t, *J* = 7 Hz, 36 H, CH_3, Et_3N), 1.29 – 1.50 (m, CH_3, 6 H), 1.57– 1.78 (m, CH_3, 6 H), 1.84 (m, 2 H, CH_2), 1.98 (m, 2 H, CH_2), 2.08–2.42 (m, 4 H, CH_2), 2.61/2.81 (s×2, 3H, NCH_3), 2.84–3.07 (m, 2 H, CH_2), 3.12 (q, *J* = 7 Hz, 24 H, CH_2, Et_3N), 3.18–3.32 (m, 4H, CH_2 + CH_2N), 3.36–3.49 (m, 4H, CH_2 + CH_2N), 3.55 (m, 1 H, CH_2N), 3.63–3.80 (m, 4 H, CH_2 + CH_2SO_3), 4.08 – 4.29 (m, 1 H, CH_2SO_3 + 2H, CH_2Ar), 4.32 (m, 1H, CHCOO), 5.84–5.94 (m, 2H), 7.05–7.17 (m, 3 H), 7.28 (m, 1H), 7.87 (m, 1H), 7.97 (m, 1H), ppm; ^{19}F NMR (564.7 MHz, D_2O): δ = –110.4/–110.5 (d×2, *J* = 14 Hz, 1 F), –106.6 (d, *J* = 14 Hz, 1 F) ppm.

Dye **KK 1558** *(Compound **4** in Scheme 2, main text) A dye with four SO_3H groups, a 4-nitro-L-phenylalanine (NPA) moiety and an additional linker for conjugation.*



4 (KK 1558)

$C_{58}H_{64}F_2N_6O_{20}S_6$
1394,25

The extra β-alanine linker was installed via a peptide-type coupling, as described for **NPA-KK 114*** above. The reagents were combined at +5°C under a N_2 purge, the solution was slowly warmed up to RT and left stirring for 1 – 2h. In a typical experiment, **NPA-KK 114S** (as a 4×Et_3N salt, 19 mg, 0.011 mmol) was reacted with β-alanine methyl ester hydrochloride (8.5 mg, 0.06 mmol, 6 equiv) in presence of HATU reagent (19 mg, 0.05 mmol, 5 equiv) and Et_3N (35 µL, 0.24 mmol, 22 equiv) in DMF (1.5 mL). After the starting material had completely reacted, the reaction mixture was diluted with dry DMF (5 mL) containing HOAc (40 µL) and evaporated almost to dryness at t ≤ 35°C. The residue was diluted with water (4 mL), TEAB (0.4 mL 1M aq. solution) was added, and the intermediate Me ester was subjected to a gradient (0 – 60% B in 30 min) prep. HPLC on a Kinetex EVO C-18 solid phase (column 31 × 150 mm) with A as an aqueous TEAB (0.05M) and MeCN as B with a flow rate of 10 mL/min. Pure fractions were pooled, evaporated at 45°C, the residue re-dissolved in a fresh water and evaporated again to decompose the TEAB**.** The solid was dissolved in water (30 mL), alkali (3 mL 1M aq. NaOH) was added, and the solution left overnight at +5°C. As the hydrolysis of the Me ester was complete (as seen by HPLC), the solution was neutralized with HOAc (200 µL), basified with an excess of Et_3N (0.3 mL) and concentrated at t ≤ 45°C to the volume of 5 mL. The product was subjected to a gradient (0 – 35% B in 30 min) preparative HPLC on a Kinetex EVO C-18 solid phase (column 31 × 150 mm) with A as an aqueous Et_3N (0.3 vol. %) and MeCN as B with a flow rate of 10 mL/min. The purification was repeated with a 0.05 M aq. TEAB buffer (A) on the same column under the same gradient conditions (after the column was properly washed and equilibrated in this buffer). This afforded 14 mg (70%) of Compound **4** (**KK 1558**)* as a 4×Et_3N salt (M = 1798).

λ max. abs. 634 nm, λ max fl. 658 nm (PBS buffer), ε = 89.230 $M^{-1}cm^{-1}$.

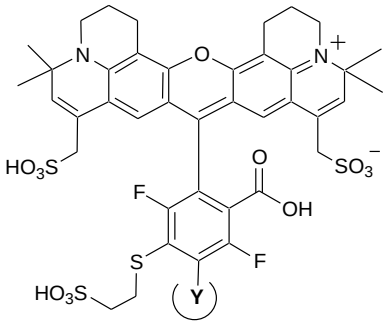
Analytical data: TLC: R_f = 0.10 (regular silica gel, MeCN/H_2O, 5:1). t_R = 5.8 min (Kinetex Phenyl-hexyl, 100 Å, 5 µm, 4.6 × 100 mm), isocratic mode 0.5 mL/min, 0.05 M aq. TEAB (A) + 30% MeCN (B), det. at 254 and 635 nm, area 98%). Alternatively, t_R = 10.5 min (Kinetex C-18, 100 Å, 5 µm, 4.6 × 100 mm), gradient 0 – 60% B in 20 min, 0.5 mL/min, with an aq. Et_3N (0.3 vol. %) as A and MeCN as B. MS (ESI): *m/z* (negative mode, %) = 1393 (100) $[M–H]^–$; HRMS ($C_{58}H_{63}F_2N_6O_{20}S_6$): 1393.2379 (found M–H), 1393.2389 (calc.).

^{1}H NMR (600 MHz, D_2O): δ = 1.08 (t, *J* = 7 Hz, ~36 H, CH_3, Et_3N), 1.12 – 1.24 (m, 4 H, CH_2), 1.33 – 1.59 (m, 12 H, CH_3), 1.85 – 2.05 (m, 8 H, CH_2), 2.20/2.21 (s × 2, 3 H, NCH_3,), 2.20 – 2.41 (m, CH_2, 2 H), 2.78 (m, 24 H, CH_2, Et_3N), 2.85–2.99 (m, 8 H, CH_2), 3.12–3.74 (m, 12 H, CH_2, CH_2 + CH_2SO_3), 4.08 (t, 1H, CHAla), 5.85/5.92 (2m, 2H), 5.97 (m, 2H, CH_2Ar), 7.09 /7.18/7.36 (3d, 4H, *J* = 12 Hz), 7.91/8.03/8.09 (3d, *J* = 12 Hz, 2H) ppm;

^{19}F NMR (564.7 MHz, D_2O): δ= –112.5 (d, *J* = 15 Hz, 1F), –112.4 (d, *J* = 15 Hz, 1F), –111.5 (d, *J* = 13 Hz, 1F), –107.2 (d, *J* = 15 Hz, 1F), –107.1 (d, *J* = 15 Hz, 1F), –107.0 (d, *J* = 13 Hz, 1F) ppm.

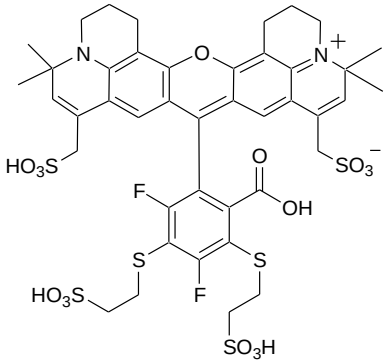
* -- it is held as obvious that exactly same reaction sequence (exchange of the F atoms to $S(CH_2)_2SO_3H$ followed by consecutive amidations) described above for the dye **KK 114**, can be also performed with **KK 114L** (longer linker) with no complications.

Rhodamine dye precursors **5a** and **5b** with four and three SO_3H groups, respectively (see also **Scheme 2**, main text).



5a **Y** = -$S(CH_2)_2SO_3H$ $C_{42}H_{44}F_2N_2O_{15}S_6$ 1046,10

5b **Y** = F $C_{40}H_{39}F_3N_2O_{12}S_4$ 924,13



5c $C_{42}H_{44}F_2N_2O_{15}S_6$ 1046,10

The sulfonated rhodamine **1b** (25 mg, 0.025 mmol, as a 4×Et_3N salt with M = 1004, for preparation see [8]) was dissolved in a mixture of DMF (1,5 mL), water (75 µL mL, 1/20 vol.) and Et_3N (80 µL, 0.55 mmol, 22 equiv). Solid sodium 2-mercaptoethyl sulfonate (70 mg, 0.43 mmol, 17 equiv) was added, the vial was sealed, and the solution was stirred overnight (16h) at 85°C. The reaction mixture was evaporated and subjected to a prep. HPLC on a Kinetex EVO C-18 solid phase (column 31 × 150 mm) with A as an aqueous Et_3N (0.3 vol. %) and MeCN as B with a flow rate of 10 mL/min. That afforded ca. 5mg of the pure mono-substituted product (Compound **5c** as a 3×Et_3N salt) and 24 mg of an isomeric mixture (**5a** and **5c**, see structure above), as suggested by NMR and mass-spectra (single peak in the mass-spectrum and "satellite" signals in the NMR spectrum). Two-times repeated isocratic HPLC with 0.05M aq. TEAB buffer (A) and 30% B (MeCN) on the same solid phase afforded 16 mg (44%) of pure Compound **5a** (as a 4×Et_3N salt with M = 1450). The mixed fractions presumably contained a minor di-substitution product -- isomer **5c**, which was not isolated in the pure state.

Analytical data for **5a** (*di*-substituted thoether): λ max abs. 426 nm, λ max fl. 645 nm (aq. 01 M TEAB Buffer); t_R = 3.6 min (Kinetex Phenyl-hexyl, 100 Å, 5 µm, 4.6 × 100 mm), isocratic mode 0.5 mL/min, 0.05 M aq. TEAB (A) + 30% MeCN (B), det. at 254 and 635 nm,

area 97%). TLC: R_f = 0.25 (regular silica gel, MeCN/H_2O, 5:1 + 0.2% Et_3N). Under the same conditions, the mono-substituted Compound **5b** has the t_R = 5 min and R_f = 0.40, respectively. MS (ESI): *m/z* (negative mode, %) = 1045 (100) $[M–H]^–$; HRMS ($C_{42}H_{43}F_2N_2O_{15}S_6$): 1045.0950 (found M–H), 1045.0956 (calc.).

^1H NMR (600 MHz, D_2O) of **5a** (*di*-substituted thoether)*: δ = 1.17 (t, *J* = 7 Hz, 36 H, CH_3, Et_3N), 1.36/1.44 (s×2, 12 H, CH_3), 1.68–1.76 (m, 4 H, CH_2), 2.23–2.28 (m, 2 H, CH_2), 2.95–2.96 (m, 2 H, CH_2), 3.06 (q, *J* = 7 Hz, 24 H, CH_2, $4Et_3N$), 3.23–3.31 (m, 6H, $2CH_2 + CH_2N$), 3.37–3.45 (m, 6H, $2CH_2 + CH_2N$), 3.65 (m, 2 H, CH_2SO_3), 3.93 (m, 2 H, CH_2SO_3), 5.86 (s, 2 H), 7.09 (s, 2 H) ppm; ^{13}C NMR (150.9 MHz, D_2O): 9.7 (CH_3), 20.7/21.3 (CH_2×2), 28.6/28.9 (CH_3×2), 31.2/31.7 (CH_2×2), 43.7 (CH_2), 44.7 (CH_2), 53.1 (CH_2), 54.9 (CH_2), 61.0 (CH_2), 107.9 (C), 114.4 (C), 122.0 (C), 123.1 (CH), 123.6 (C), 139.3 (CH), 147.0 (C), 151.9 (C), 154.5 (C), 169.8 (CO) ppm; ^{19}F NMR (564.7 MHz, D_2O): δ = –107.0 (d, *J* = 14 Hz, 1 F), –110.3 (d, *J* = 14 Hz, 1 F) ppm. * -- due to the very low intensities and splitting, the signals of C-F carbon atoms were not properly recorded.

Apart from the target compound **5a**, 5.3 mg (16%) of the *mono*-substituted thoether **5b** was isolated (as a $3×Et_3N$ salt with M = 1227). This compound was later obtained in a high yield by reacting sulfonated rhodamine **1b** with sodium 2-mercaptoethyl sulfonate at RT, analogously to the dye **KK 1103** (see above). Compound **5b** is considered as a valuable precursor, as the spectral properties are identical to the non-substituted compounds, but the reactivity/instability is eliminated because the substitution of a fluorine atom is not possible anymore. TLC: R_f = 0.40 (regular silica gel, MeCN/H_2O, 5:1 + 0.2% Et_3N). MS (ESI): *m/z* (negative mode, %) = 923 (100) $[M–H]^–$; HRMS ($C_{40}H_{38}F_3N_2O_{12}S_4$): 923.1257 (found M–H), 923.1260 (calc.).

NMR (600 MHz, D_2O) of **5b** * (*mono*-substituted thoether): δ = 1.21 (t, *J* = 7 Hz, 27 H, CH_3, $3Et_3N$), 1.35/1.42 (s×2, 12 H, CH_3), 1.71–1.76 (m, 4 H, $2CH_2$), 2.32–2.37 (m, 2 H, CH_2), 2.63–2.68 (m, 2 H, CH_2), 2.88–3.10 (m, 2 H, CH_2), 3.06 (q, *J* = 7 Hz, 18 H, CH_2, $3Et_3N$), 3.23–3.27 (m, 4H, $CH_2 + CH_2N$), 3.34–3.40 (m, 4H, $CH_2 + CH_2N$), 3.62 (m, 2 H, CH_2SO_3), 3.94 (m, 2 H, CH_2SO_3), 5.83 (s, 2 H), 7.07 (s, 2 H) ppm; ^{13}C NMR (150.9 MHz, D_2O): 9.6 (CH_3), 20.7/21.3 (CH_2×2), 28.7/28.9 (CH_3×2), 31.1/31.6 (CH_2×2), 43.6 (CH_2), 44.6 (CH_2), 52.7 (CH_2), 54.5 (CH_2), 71.0 (CH_2), 107.9 (C), 114.6 (C), 121.9 (C), 123.1 (CH), 123.6 (C), 139.2 (CH), 146.7 (C), 152.0 (C), 154.7 (C), 169.1 (CO) ppm; ^{19}F NMR (564.7 MHz, D_2O): δ = –145.11 (m, 1 F), –125.7 (m, 1 F), –110.3 (m, 1 F) ppm. * -- due to very low intensities and splitting, the signals of C-F carbon atoms were not properly recorded.

Considering the moderate isolated yield of Compound **5a** and the tedious resolution of the isomers, the synthetic route to the dye **KK 1558** (Compound **4 Scheme 2**, main text) that utilizes Compound **5a** as precursor is clearly inferior to the one described above. The latter involves a direct substitution of fluorine atoms in the "ready" dye **KK 114** with a linker. Presumably, the substitution of the F atom in the *ortho*-position to the linker is somewhat sterically hindered, so the formation of the second isomer is avoided.

Even more interesting dye modification could have been the oxidation of thioethers (*i. e.*, **KK 114S** with two additional SO_3H groups, **Scheme 2**) to the corresponding sulfones. The latter would be even more polar and even further shifted to the red spectral area. We tried out hydrogen peroxide with and without a catalyst (Na_2WO_4), *m*-chloroperoxybenzoic acid, permanganate and periodate. Unfortunately, a recipe for a mild oxidation was not found. Even at room temperature and in dilute solutions the dye color is lost in few minutes, which indicates a destructive reaction. Interestingly, the oxidation was so deep that no tetrafluorobenzene derivatives were found among the reaction products. Such reactivity towards hydrogen peroxide and oxidants is quite disappointing. It also seems unexpected, considering the well-known photostability in this dye family under aerobic conditions (see the photolysis experiments and discussion below). We can assume that the extra double bonds in these red-emitting rhodamines become a "weak point of the dye" only with oxidative reagents. However, similarly to the photolysis in solution, one detects colorless products of deep oxidation (or destruction) only. On the other hand, neither selenium dioxide nor *t*-butyl peroxide (both are known to be milder oxidants) worked in the mild oxidation of Compound **3** (**KK 114S**) to the sulfone. Hydrogen peroxide without catalyst did neither.

Preparation of dye NHS esters for bioconjugation

The NHS esters of poly-sulfonated hydrophilic dyes were prepared as follows:

A sulfonated dye (as an acid or as a Et_3N salt) in amount of 10 mg (0.060 — 0.011 mmol) is dissolved in dry DMF (200 µL) containing 7 equiv Et_3N. This solution is transferred to a 0.5 —1.5 mL vial or vessel containing 5 equiv of freshly weighted DSC reagent (*N,N*'-disuccinimidyl carbonate, Alfa Aesar, technical grade, 85%) and a magnetic stirring bar. The vial is sealed, the solution stirred for 30 min at RT, and the completion of the reaction is checked by TLC. That utilizes regular silica gel, with MeCN/H_2O 5:1 + 0.2% Et_3N as the mobile phase. The reaction product shows a 20-30% higher R_f, compared to the starting dye in the acid form. Alternatively, an HPLC analysis on C-18 phase, preferably with acidic buffers or TFA, can be used. The reaction solution is divided in two (or more, with larger amounts of the dye, respectively) equal parts which are placed in two 2.5 mL Eppendorf vials. The magnetic bar should be removed beforehand. To evaporate the solvent, a pin hole (< 1mm) is pierced in the caps, the vials fixed in upright position in a flask, vessel or a freeze-dryer and kept under vacuum (1 -- 2 hours or overnight) until the content becomes solid. The *N*-hydroxysuccinimide (formed by hydrolysis of DSC reagent in air) was removed from the product as follows: dry EtOAc (2 mL) is added to the solid residue, and the vials are sonicated in a water bath for 5 -- 10 min, whereas the caps are changed to new ones to protect the content from the bath water. The vials are then centrifuged (10 min) and the colorless liquid is carefully removed from the fine dark precipitate by means of a Pasteur pipette. The treatment with EtOAc should be repeated one or two more times to obtain a product that is completely free from *N*-hydroxysuccinimide, as established by HPLC. The residual solvent (EtOAc) evaporates in 15 min at RT under the fumehood from opened vials. The solid is re-dissolved in DMF, aliquoted to small portions (e.g., 0.2 or 1.0 mg), and the solvent removed under vacuum as described above. The NHS esters are relatively stable at RT, especially in a nitrogen-flushed (e.g., using the so-called "back-filling technique") vials. For prolonged storage a freezer (–20°C) is recommended. The vials should be defrosted with due precautions (e. g., using a desiccator with a drying agent) to avoid moisture condensation inside. We established that, contrary to our expectations, small amounts of acids (TFA, HCOOH or HOAc while aliquoting) do not improve the stability of the material. However, the stability of the NHS esters prepared as described above is quite high and allows a week of transportation at ambient temperature. In some experiments DMF was successfully replaced by DMSO, which is more compatible with freeze-drying apparatus, does not "spit" under high vacuum and is less toxic. Analytical data of an exemplary NHS ester **KK 1556** see in **Figs. S20**, **21**.

*The NHS esters of non-sulfonated "lipophilic dyes" (**KK 1517** and **KK 1103***) were prepared as follows:*

To a solution of a dye (1 mg, 1.0 – 1.3 µmol) in a mixture of dry MeCN and DCM (1:1 vol., 200 -- 500 µL), 20 equiv Et_3N and 15 equiv DSC reagent are added. The solution is magnetically stirred for 0.5 – 1h in a small sealed vessel and the completion of the reaction is established by TLC (regular silica and MeCN/DCM/H_2O 10:10:1). The solution is diluted with DCM (5 mL) and shaken for a short time with water (10 mL) containing TFA (5 µL) and then with brine (5 mL). The colored organic solution is dried for 15 min. (Na_2SO_4), filtered and evaporated, preferably, in a purge of an inert gas. The solid residue is kept under high vacuum (≤ 1 mbar) for few minutes and then, if necessary, aliquoted from DMF as described above for the sulfonated dyes. The lipophilic NHS esters should also be stored at –20°C. This recipe is somewhat similar to the one described by A. Brunet and co-workers for TAMRA rhodamine dyes [11]. We found, however, that, at least in this particular case, quenching of the reaction mixture with TFA is preferable, as it is not being extracted by organic solvents. Also, for the chromatographic isolation of NHS esters acids should be avoided, as they get concentrated while evaporation of the liquid phase. That, in turn, promotes the decomposition of NHS esters.

The NHS ester of **KK 1115** (the dye with two OH groups, which can react with DSC; see **Scheme 1** in the main text) was obtained as described previously in ref. [8]. The analytical data was consistent with reported (see also the HPLC data below). * -- the single SO_3H

group in the dyes **KK 1103** and **KK 1115** is “occupied” to form the zwitter-ion, so the NHS esters have a zero net charge. Therefore, they are extractable with organic solvents from water. The latter retains all polar impurities (NHS, salts, acids, bases and SiO_2 particles).

*The NHS ester of the phosphorylated dye **KK 1550** (*“Abberior® STAR 635P"*, according to ref. [9], see also **Fig. 22a,b**).*

Despite the merits, primary phosphate groups (-$OP(O)(OH)_2$) pose a selectivity problem if a carboxyl group also presents in a dye. Particularly, the phosphate group can undergo esterification and, especially, amidation too, depending on the coupling reagent and the conditions used. Also, the NHS ester moiety can even migrate from -COOH to -$OP(O)(OH)_2$. This problem was discussed in ref. [12]. Therefore, the NHS ester of KK 1550 was prepared by a special recipe that utilized TSTU, a “milder reagent”, which is taken in a reasonable excess. The reaction was performed under a strict HPLC control. Typically, to a 1 – 2 % wt. DMF solution of the dye Et_3N (5 equiv) and TSTU (1.5 – 2 equiv, as a freshly-prepared 10 % wt. solution in DMF) was added, and the solution was stirred in a small sealed vial (0.5 – 1h) until the starting dye acid reacts completely (HPLC control; for conditions see above for the dye KK 1550). The reaction solution was evaporated, treated with EtOAc* and aliquoted, as described above for the sulfonated dyes. Usually, the NHS ester had a 90% purity, the rest was the starting acid which, however, causes no problem in the conjugation reaction to e.g., antibodies.

* -- a small amount of HCOOH (1:1000 vol.) was added to the EtOAC while sonicating, which, in this particular case, stabilized the product upon handling and storage. Otherwise, migration of the NHS moiety to the phosphate group was observed to a noticeable extent (see above).

Photophysical properties measurements

Institute of Analytical Chemistry and Food Chemistry, Graz

Relative quantum yields of fluorescence were determined on FluoroLog 3 spectrofluorometer from Horiba Scientific equipped with a R2658 photomultiplier from Hamamatsu. Excitation wavelength of 585 nm was used. The quantum yields were determined using a solution of Oxazine 170 in ethanol as a reference dye (Φ_f = 0.579) [15]. Three measurements at different concentrations were taken for each dye sample. Absorption at the absorbance maxima was below 0.05 in all cases to avoid an inner filter effect. The evaluations were performed according to routine methods described in [15] and [16] with the accuracy ≤ 4%.

Molar absorption coefficients were determined from the absorption spectra acquired by means of a VARIAN CARY 50 Conc UV-VIS Photometer and Hellma Analytics (www.hellma-analytics.com) optical glass 10 mm precision cuvettes.

Measurements conditions: reference dye oxazine 170 in EtOH, all the dyes measured in PBS pH 7.4 (10 mmol/L NaH_2PO_4 + 100 mmol/L NaCl, pH adjusted with NaOH). For the separate comparison experiments with the phosphorylated dye **KK 1550** a PBS buffer and a 10 mM CHES buffer (pH 9.0, also containing 100 mmol/L NaCl) were used. Temperature 23 °C.

Fluorescence lifetimes were obtained via time-correlated single photon counting on a FluoroLog 3 spectrofluorometer equipped with a DeltaHub module controlling a NanoLED (635 nm) laser diode and using DAS-6 Analysis software for data analysis.

Institute of Physics of Georg August University, Göttingen

Determination of fluorescence lifetimes. The epi-fluorescence setup is described in ref. [18]. Earlier, the Φ_f of the reference dye ATTO 647N was measured independently by an alternative method, as mentioned above.

Max Planck Institute for Biophysical Chemistry (MPI-BPC), Göttingen

For absolute quantum yield estimations a Quantaurus-QY Absolute PL quantum yield spectrophotometer C11347 Hamamatsu was used. **UV-visible absorption spectra** were recorded on a Varian Cary 4000 UV-Vis spectrophotometer, and fluorescence spectra and relative quantum yields of fluorescence on a Varian Cary Eclipse fluorescence spectrophotometer with the accuracy ≤ 6%. In the evaluations with ATTO 647N as reference, its Φ_f was taken for 60%. This value is the average from what was obtained by a nanocavity-based method (62%, absolute value, see ref. [17]) and what was obtained by the conventional method (59%) with Oxazine 170 as reference (see [15], and [16]).

STED microscopy: material and methods, setup and sample preparation details (MPI-BPC), Göttingen

Optical set-up. All measurements were performed on a custom-built STED set-up [13]. The excitation wavelength is provided by a super continuum source (model EXWB-6, NKT Photonics, Birkerød, DK) together with an acouto-optical beam splitter (AOBS). The STED beam has a wavelength of 775 nm (Katana08-HP, Onefive, Zürich, CH). Both are synchronized at a repetition rate of 40 MHz. Pulse duration 700 ± 200 ps. Scanning of the sample is achieved by a quad scanner, a beam scanning device. The fluorescence from 640 – 675 nm is collected by an avalanche photo diode (APD) (model SPCM-ARQH-13, Excelitas Technologies Corp., Waltham, US).

All images shown here were recorded with an excitation wavelength of 612 nm, a power of ~5 µW and a STED power of ~ 120 mW (measured in the back focal plane). Detection channel "APD-6" (640 – 675 nm).

Imaging scheme. Data acquisition was done in two line steps. Each line of the 10 x 10 µm area was scanned twice with a total pixel dwell time of 5 µs for each 20 nm pixel. The first scan recorded the signal when excitation and STED beam were switched on, the second scan was done with the STED beam only. In this configuration 15 consecutive frames were taken. Colorbars are the legend to a color-coded image. A specific color corresponds to a certain number of counts. They show how bright every single spot is. One can tell from the individual frames with relatively low counts that the background noise is low and that there is a very little unspecific staining.

Analysis. The STED-only excitation data of the second line step was subtracted from the data of the first line step before further analysis. Any negative counts were set to zero.

Bleaching curves were calculated by adding all the counts in the individual frames and normalizing them to the brightest of the 15 frames. The bleaching curves were recorded at an enhanced STED power of ~ 350 mW, keeping the usual excitation power (see above), except the "STED only" experiment depicted in **Fig S5**.

Signal-to-noise-ratio (SNR) was obtained by adding up all the individual frames to create a sum image. From this image the maximum number of "c" units was divided by the maximum count of the background to yield the SNR.
Resolution determination was done also on the sum image. A Gaussian was fit to the individual nuclear pore complexes (nup153) in x and y directions. The full-width-at-half-maximum (FWHM) of at least 50 pores were averaged and the standard deviation and the standard error were calculated.

Sample preparation. All measurements were performed on MeOH fixed Vero cells where the nuclear pore complex was stained with primary antibody Nup153 (Abcam, Cambridge, UK, ab24700, 1:100). The secondary sheep anti-mouse antibody was coupled to the desired dyes (cat. no. 515-005-003, Dianova GmbH, Hamburg, DE).

Mowiol-based medium. The samples were then mounted in Mowiol containing DABCO (2.5 % (w/v)) Glycerol (9 % (w/v)), 0.1 M Tris (0.1 % (w/v), adjusted to pH 8.5).

GSDIM-buffer (GOXy, GOC). A GSDIM-buffer solution (similar to the one described in [19a,b] was prepared shortly before experiments from the following three stock solutions: №1 (glucose-containing buffer) -- 250 µl, №2 (oxygen scavenger) -- 2.5 µl, and №3 (MEA solution) -- 2.5 µl. Stock solution composition: №1 – TN buffer containing 10 mmol/L TRIS (pH 8.0) and 10 mmol/L NaCl with 10 % (w/v) glucose added, №2 -- 10 mg glucose oxidase powder 100 000 units/g in a mixture of 80 µl catalase solution (2 mg/mL, 2 000 – 5 000 units/mg) and 170 µl TN buffer (see above), №3) -- commercial 1M MEA (β-mercaptoethylamine, also known as cysteamine). All components were purchased from Sigma-Aldrich (Merck).

Figs. 3 and **S5** show the bleaching curves averaged over the 3 measurements taken for each data point. The error of the mean is the standard deviation of the individual measurement divided by sqrt(n) which is 3 in our case. We assume the individual measurements to be normally distributed and add a factor of 4.3 to the error of the individual measurement. Thus the insufficient statistics (just 3 measurements) is taken into account (student t function for 95% confident level with 3 data points). Thus one comes to an error of the mean value of

$$\bar{\sigma} = 4{,}3\sqrt{\frac{\sum_i (x_i - \bar{x})^2}{N \cdot (N-1)}}$$

σ is the error of the mean, x_i is the value of the measurement $\bar{x}$ is the mean and N is the number of measurements (in our case 3) and 4,3 is the correction factor. Estimating the standard deviation we obtain an error of ca. 0.01 error of the mean without correction factor and ca. 0.04 with correction factor. The latter, being considered as the worst possible case, is depicted as error bars. With single rounds of measurements the error (based just on the error of detecting a photon and the noise generated by photon detection) could not have exceeded 0.001 [13].

Image quality analysis was performed via determination of the signal-to-noise-ratio (SNR) in a sum of individual frames with selected STED dyes at approximately same degrees of labeling. Signal and noise are given in counts and SNR in a. u., respectively

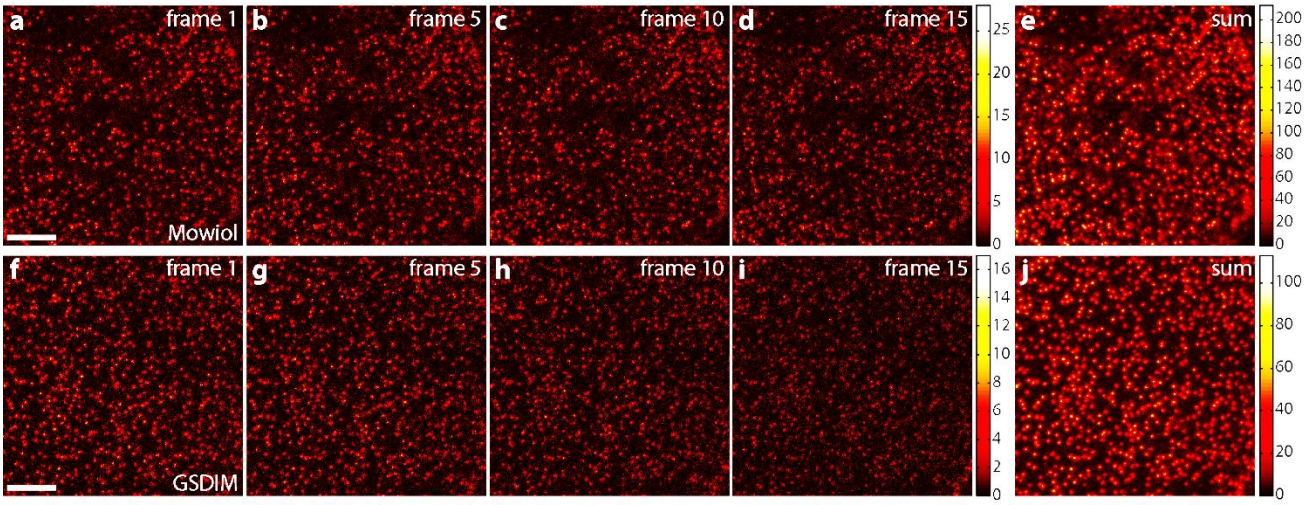
		Mowiol			GSDIM		
Dye	Φ_f **, %	signal	noise	SNR	Signal	noise	SNR
KK114L	70	314	9	35	237	6	40
KK1115	69	205	9	23	113	7	16
KK1555*	33	191	12	16	75	7	11
KK1553*	14	223	12	19	133	12	11
KK1556*	14	85	6	14	40	5	8

Table S1. Signal-to-noise-ratios (SNR) in Mowiol and GSDIM for selected dyes. * – dyes modified with photostabilizing groups (PSG), ** – relative fluorescence quantum yield with ATTO 647N as reference dye (see **Table 1** for more data and **Fig. S8** for fluorescence spectra of the dyes).

	Mowiol							GSDIM						
		X			y				x			y		
Dye	#	FWHM	Std	ste	FWHM	std	ste	#	FWHM	std	ste	FWHM	std	ste
KK114L	65	83	11	.17	76	10	.15	60	95	12	.20	95	12	.20
KK1115	60	92	12	.20	86	11	.18	60	98	11	.18	87	10	.17
KK1553*	50	111	12	.24	116	15	.30	50	110	13	.26	106	14	.28
KK1555*	60	91	12	.20	85	11	.18	50	121	14	.28	140	17	.34
KK1556*	50	90	13	.26	84	13	.26	50	105	20	.40	105	20	.40

Table S2. Resolution analysis in Mowiol and GSDIM for selected dyes. The size of the nuclear pore complex (nup153). In the image consisting of the sum of the individual frames a Gaussian was fit in x and y directions to at least 50 nuclear pores (#). Then the average of the full-width-at-half-maximum (FWHM) was calculated together with the standard deviation (std) and the standard error (ste) in nm. * – dyes with PSG.

Reference dye 1115



Nitrobenzyl-modified model dye KK 1556

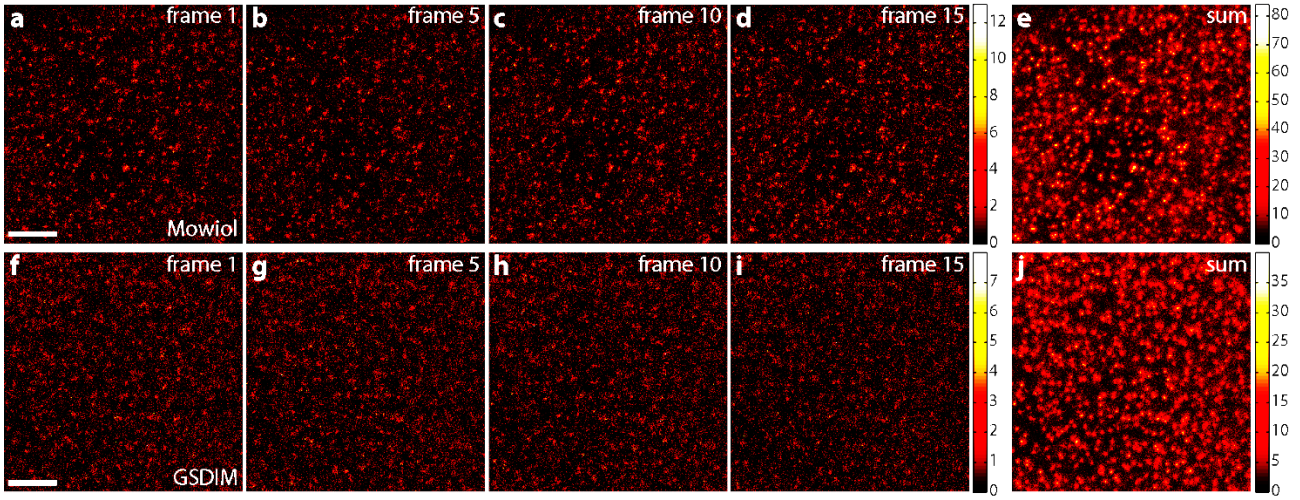


Figure S1. STED image series of 15 frames. *Above:* reference dye **KK 1115** (Abberior® STAR 635, ref. [9])**.** (**a** – **d**) Individual frames (1 – 15) in Mowiol (see also orange bleaching curve in **Fig. 3**, left). Colorbar of (**d**) applies for (**a** – **d**). (**e**) Add-up of 15 frames in Mowiol. (**f** – **i**) Individual frames (1, 5, 10, and 15) in GSDIM buffer. Colorbar of (**i**) applies for (**f** – **i**). (**j**) Add-up of 15 frames. Scale bars of 2 µm; compare with the images in **Fig 4**, main text. *Below:* exactly same series in the same media with the model dye **KK 1556** (contains two lipophilic PSG and has the Φ_f of only 14 – 15%; see also blue bleaching curve in **Fig. 3**).

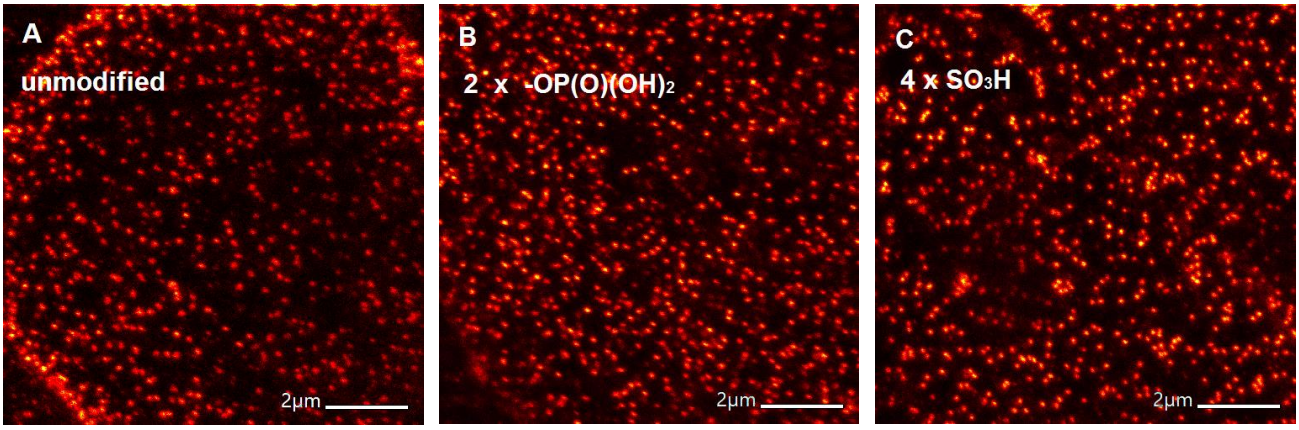


Figure S2. STED images with the unmodified (control) dye KK 1517 (A), the phosphorylated dye KK 1550 (B) and the photostabilized dye KK 1558 (C) with four sulfonic acid groups. Presented are add-ups of 15 frames. Regarding the background and sharpness, see also images in **Fig. 1**, main text. For imaging details see the text above.

Immunofluorescence: labeling, preparation and mounting the samples

The procedures were carried out according to the standard protocols, described by *Wurm et al.* [14]**.** For the preparation of cell samples Vero (or PtK2) cells were grown on cover slips. Cells were 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 antibodies directed against alpha-tubulin (Sigma-Aldrich, St. Louis, MO, USA), vimentin (Sigma-Aldrich) 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 fluorescent dyes. Usually, the corresponding *N*-hydroxysuccinimidyl dye esters (0.2 mg) are dissolved in ca. 20–40 µl of dry DMF and slowly added to the stirred solution of secondary antibodies (containing ca. 1 mg of a protein) in 1 mL of the buffer solution (pH = 8 – 8.5) at ambient temperature. Dye-labelled antibodies were isolated by gel filtration chromatography (see ref. [14] for the standard labeling protocol). After several washing steps with PBS buffer the samples were mounted in Mowiol (for STED microscopy), PBS (for GSDIM microscopy), or GSDIM buffer.

The degree of labeling (DOL) estimations were performed according to the standard protocols recommended by Abberior GmbH [20]. DOL is an average number of dye molecules coupled to a protein molecule (*e. g.,* an antibody). In case of red-emitting dyes the extinction coefficient of the free dye at the maximum absorbance is equal to the value of the dye-antibody conjugate at its maximum absorbance. Luckily, with the amounts of antibodies and dye NHS esters given above, the standard labelling protocol lead to reproducible and almost equal DOL values: 3.6 – 4.8.

The tubulin skeleton was immunolabeled with compounds KK 1517 and KK 114 (Scheme 1 main text and **Fig 1**) taken as NHS esters. The STED imaging was performed using the setup described in ref. [9] and (in more detail) in [21] under the same conditions as described in [8]. That involved excitation with 640 nm diode laser with a power of ~10 µW. Detection in the channel "APD-6" at 640 -- 670 nm, accompanied by a pulsed STED beam at 750 nm (power ~200 mW).

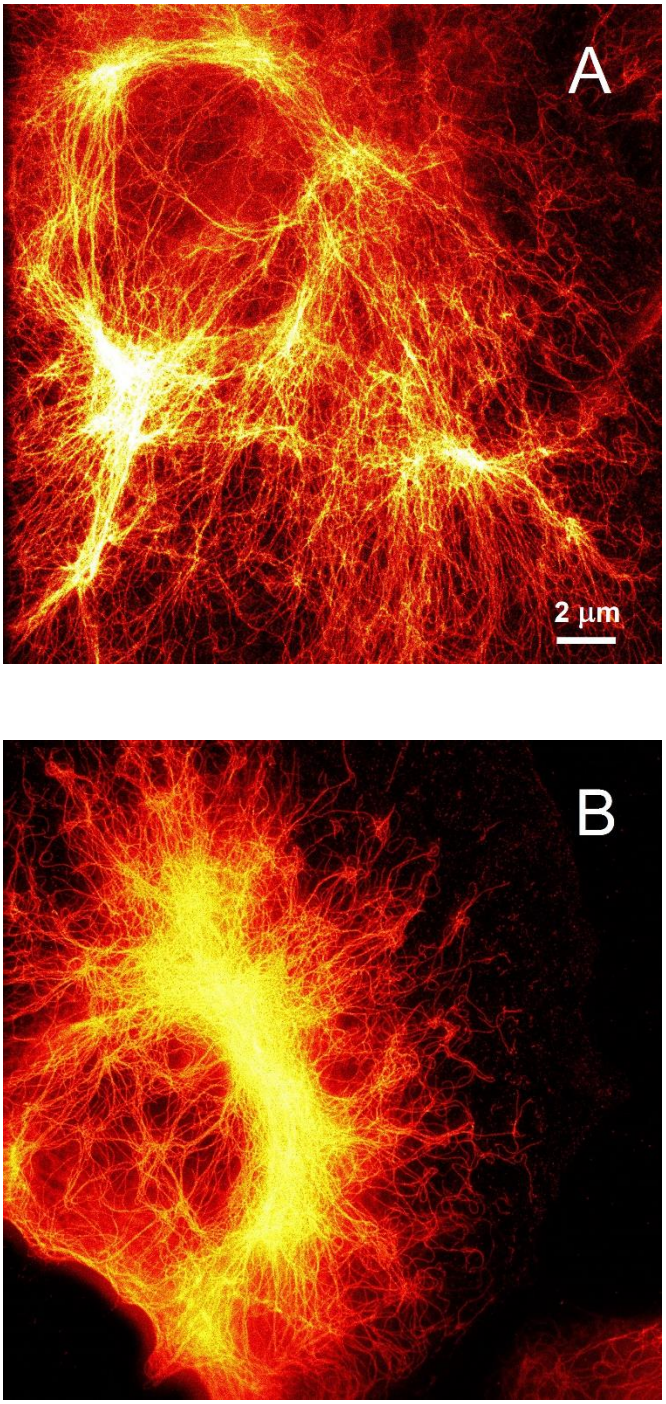


Figure S3. STED images with a red-emitting rhodamine with and without additional polar groups, full scale.

The "control" dye KK 1517 (unmodified, no extra functional groups) -- **A**, above and the well-established dye with two sulfonic acid groups KK 114L -- **B**, below (see **Scheme 1** for structures and **Table 1** for photophysical properties). Full size, raw data. Images were taken with an immune-labelled tubulin cytoskeleton sample. See above for the labeling, imaging and setup details.

STED imaging with Mic60 and PMP70 as substrates

STED microscopy was performed using a Abberior STED 775 QUAD scanning nanoscope (Abberior Instruments) equipped with a Katana-08 HP laser (Onfive GmbH, Regensdorf, Switzerland). In brief, the fluorophores were excited at 640 nm and STED was performed at 775 nm. The STED and the corresponding confocal images were acquired line sequentially and the time series were acquired continuously. All images are raw data.

Samples were essentially prepared as described in [14]. Particularly, HeLa cells were fixed with 4% (w/v) formaldehyde in PBS (137 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, pH 7.4) for 10 min at 37°C, extracted with 0.5% (v/v) Triton X-100 in PBS, blocked with 10% (w/v) bovine serum albumin (BSA) in PBS, and incubated with diluted primary polyclonal antibodies against Mic60 (Abcam, Cambridge, England) or PMP70 (Abcam, Cambridge, England) for 1 h. After three washing steps in PBS, incubation in 0.5% (v/v) Triton X-100 in PBS, and blocking in 10% (w/v) BSA in PBS, the primary antibodies were detected with custom-labeled secondary antibodies against rabbit (goat anti-rabbit antibodies, Jackson Immuno Research Laboratories, West Grove, PA, USA) for 1 h.

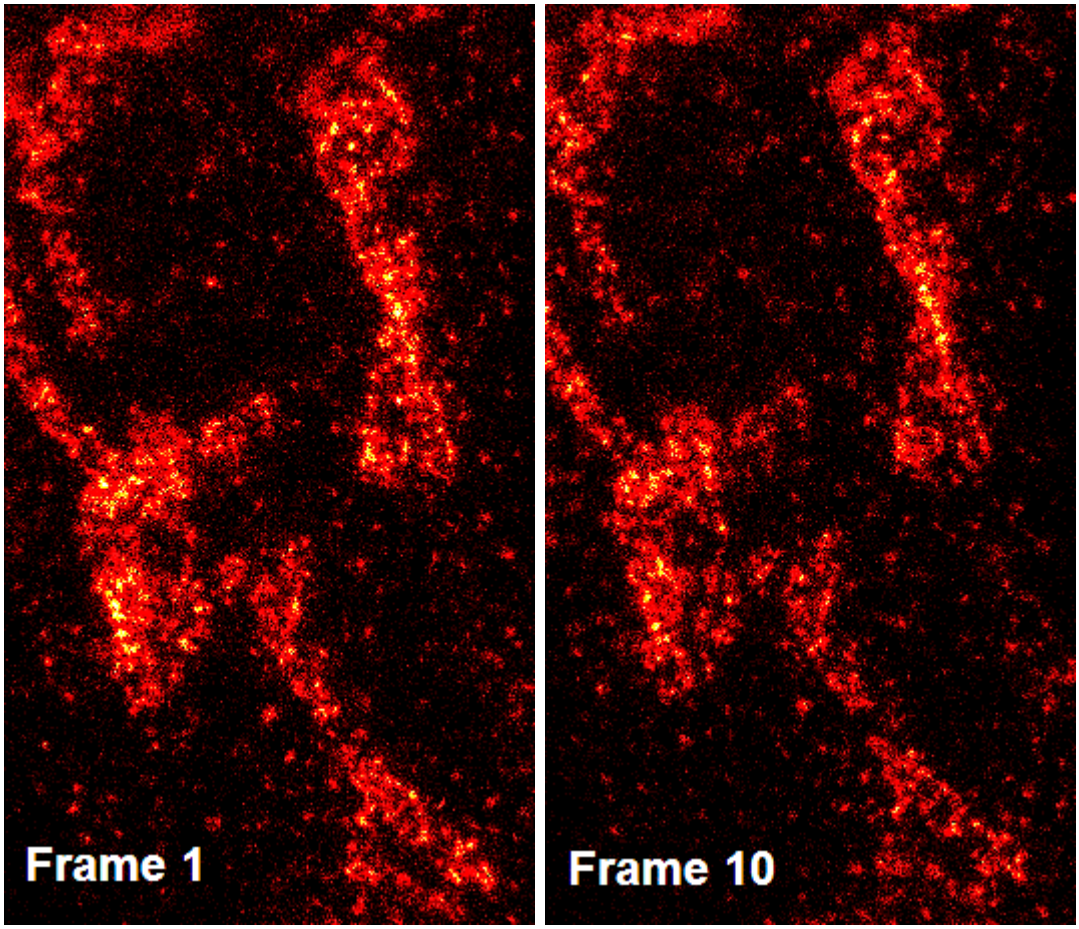


Figure S4. STED frames (#1 and #10) of a mitochondrial inner membrane Mic60 with secondary antibodies custom-labelled with a new "everlasting" NBT-modified dye KK 1555. No fading is observed. See also **Fig.5** (main text).

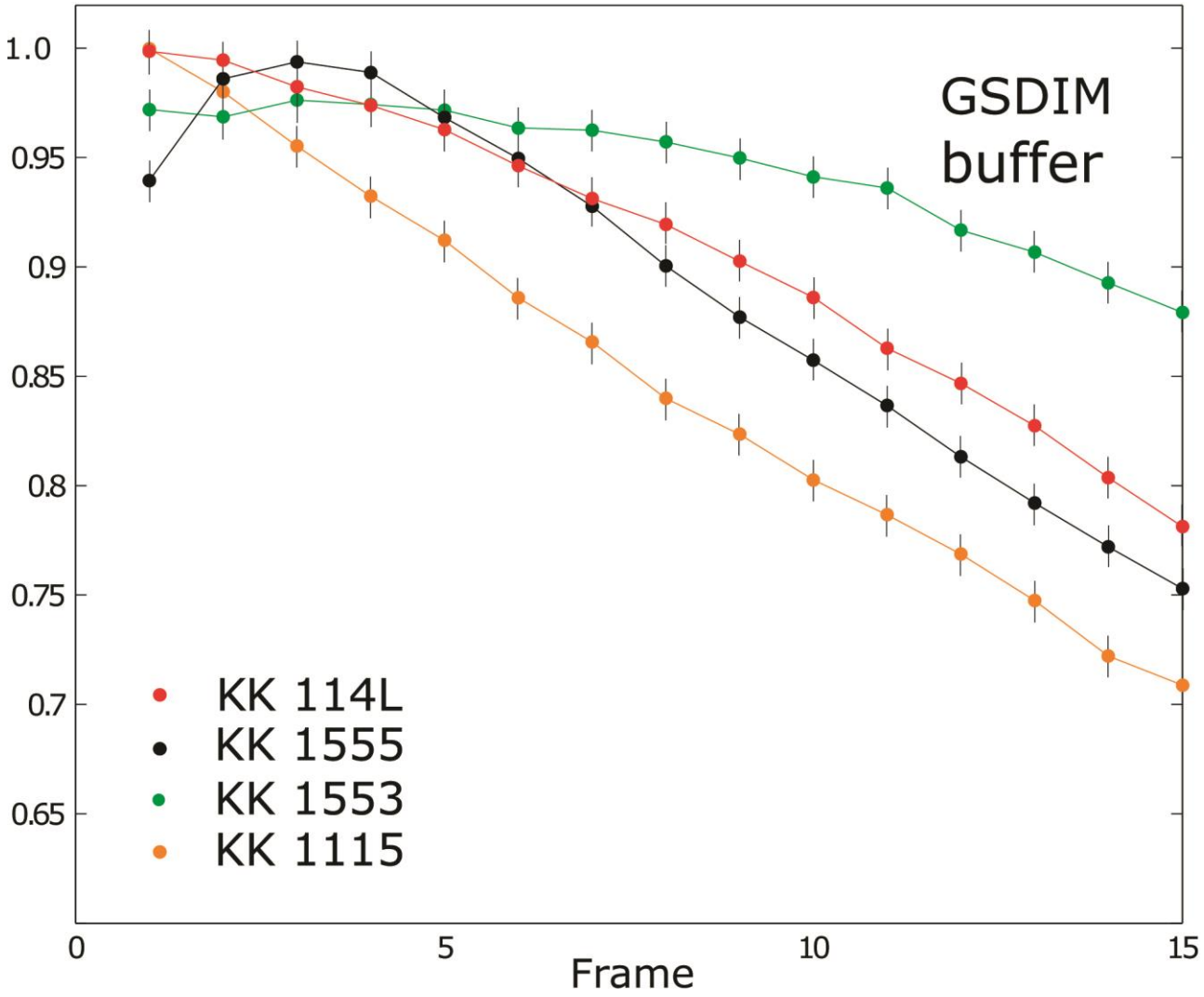


Figure S5. Bleaching curves in GSDIM buffer for selected dyes with the same chromophore: <u>STED beam only</u>, no excitation.

Normalized intensity distribution (Y axis) of the fluorescence signal over 15 frames recorded in in **GSDIM buffer** (anaerobic conditions, freshly mounted). Recorded at STED power of 355 mW. See details above and **Fig 3** in the main text (where both STED and excitation beams were utilized).

<u>Photobleaching of the dyes in bulk aqueous solutions</u>

Light source: Brennenstuhl H 500 Baustrahler 400 with a cylindrical 11-cm long halogen lamp Glühlampe 400W 8 1178560400. Cuvettes: Alftec (glass, $\lambda \geq 350$ nm) 12.5 x 12.5 x 45 mm, 3.5 ml, with polyurethane caps (see **Fig. S3**). In the experiments presented in **Table S3** (below) the following **a**bsorptive (long-pass) optical filter plates were used: IR filter SCHOTT KG 3 (50 x 50 x 2 mm, $\lambda \leq$ 900nm) and UV filter SCHOTT GG 420 (50 x 50 x 2 mm, $\lambda \geq 420$ nm). In the experiments presented in **Table 2** (main text) an IR filter SCHOTT KG 1 ($\lambda \leq 1200$nm) and an UV filter GG 395 ($\lambda \geq 395$ nm) were used. Filter plates were purchased from Edmund Optics.

It was established that the full spectrum of the halogen lamp causes a lot more bleaching. This is probably due to that halogen lamps emit some very small amount of a near-UV light (350 – 400 nm) too. The filtration was performed by using absorptive optical glass filters (see above). A magnetic stirrer and Teflon-coated magnetic stirring bars (7 × 2 mm) were used for stirring the dye solutions. The

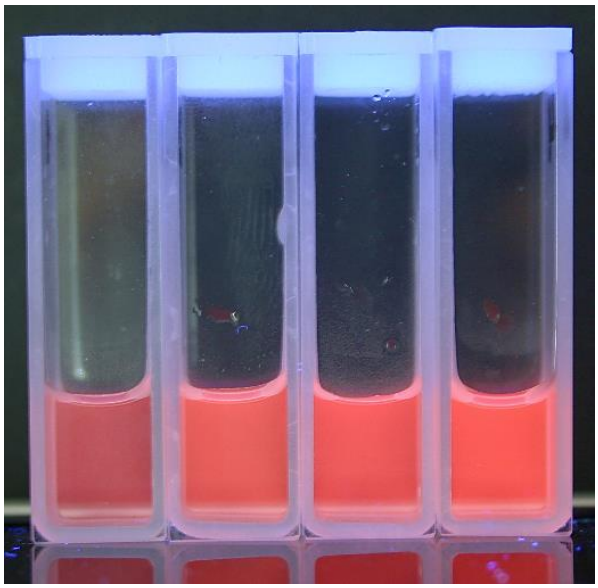
cuvettes were just sealed with caps to protect the solutions from evaporating. A sufficient free volume (ca. 1/2 -- 2/3) was left to provide an excess of atmospheric oxygen (see **Fig. S3**). Usually, the illumination lasted 20 hours in a 25 cm distance from a 400W halogen lamp. In preliminary experiments, however, it was performed for a longer time with less powerful lamps at a distance adjusted accordingly. To cut off the IR irradiation that leads to unnecessary heating, a special heat-absorbing glass plate was used. As a result, the temperature of the solutions did not exceed 35°C, except separate experiments with "harsher conditions". Two identical filter plates were used to cover 8 cuvettes in one round of illumination. The cuvette caps (all together) were covered with aluminum foil to protect them from light and heating. The "harsh conditions" in separate experiments involved quartz cuvettes (see **Fig. S3 A**) and the full spectrum of a halogen lamp, off which only IR was filtered (SCHOTT KG 1).

The pH value is very much supposed to affect the course of the photolysis and redox reactions in general, whereas the dyes were used both in acid form and as Et_3N-salts. The dyes might contain small amounts of residual acids (CF_3COOH, HCOOH), amine (Et_3N) and their salts from the HPLC mobile phases. Occasional fluctuations of pH in a deionized water also need to be excluded. Therefore, to keep on the safe side, we used the conventional PBS buffer with pH 7.4 as the photolysis medium, yet with dilution 1:10 (which comes to the concentration of 10 mmol/L NaCl and ca. 1 mmol/L NaH_2PO_4/Na_2HPO_4). Also, PBS buffer is usually added (in small amounts) to TDE (thiodiethanol or thiodiglycol, another widely used embedding medium in STED microscopy [14]). The concentration of sodium hydrogen phosphates, the crucial ingredients, even at this dilution (1 mmol), was approximately 100 times higher than the concentration of the dyes (10 μmol/L). Preliminary tests showed no significant influence of the buffer taken at this concentration in comparison to pure deionized water. On the other hand, PBS buffer of the normal concentration (10 mmol/L NaH_2PO_4 + 100 mmol/L NaCl) showed a slight negative influence. The extinction and fluorescence losses were by some 10% bigger, as compared to those in pure water under photolysis. The photobleaching experiments were performed with 10 μmol/L solutions because this concentration affords direct absorbance and fluorescence measurements with a good linearity. Importantly, at this concentration, no self-quenching effects are observed anymore. Vigorous stirring eliminates concentration/temperature gradients and provides a good aeration, as well. For convenient and relevant comparison, we set the conditions (illumination time, distance and light source power) such that that the reference dye KK 114L loses a half of its extinction in this concentration and in one round of illumination. Usually it lasted 15 – 20 hours in a 25 cm distance from a 400W halogen lamp with a heat-absorbing glass plate. In this family of dyes, the loss in extinction varied from 25 to 80%, whereas the change in the fluorescence signal also lied in a convenient range, i. e., from –76 up to +20%, as seen in **Tables 2** (main text**)** and **S3**.

The changes in color density and in fluorescence signal were recorded in three channels by means of a multimode microplate reader Tecan™ Infinite 200Pro. Greiner 96 Flat Bottom Transparent Polystyrene multi-well plates were used. The instrument had the following pre-installed settings: excitation bandwidth 9 nm, emission bandwidth 20 nm. The changes in absorbance (color density) and in fluorescence signal were recorded consecutively in three channels in the "fluorescence top reading" mode. For all dyes the sample volume was 0.3 mL and the initial concentration 10 μmol/L (in water). The excitation and emission wavelengths were set accordingly.

The excitation/emission wavelength settings (nm) were the following: 620/660 (Red Channel), 510/580 (Orange Channel) and 500/560 (Yellow Channel). Initially, all the dyes possess a very weak fluorescence in the orange and yellow areas. After illumination the signal in these channels dramatically increases (up to 25 times), as the photolysis product appear. This phenomenon (bluing) occurs to a far smaller extent in the PSG-stabilized dyes. Usually the photolysis was performed until the "weakest" dye loses 50-60% of its extinction, as measured by the microplate reader.

A



B

Figure S6. Red-emittig dyes with different functional groups after a long illumination in stirred 10 µmol/L solutions in water under the same conditions. **A** – **KK 1553** (NPA-KK 114, left) and **KK 114** (unmodified dye, right) taken as 10 µmol/L solutions after 25 hours illumination with a full spectrum of a halogen lamp at 35°C (see above for details), which destroyed more than 80% of the reference dye (**KK 114**). See also **Fig. 6**(**C**) in the main text.

B – Bleaching of non-polar and moderately polar dyes in 10 µmol/L solutions (with almost equal extinction) after 15 hours of illumination; left to right: **KK 1517** (control dye, unmodified), **KK 1103** (+SO_3H), **KK 9046** (+2OH), **KK 1115** (+SO_3H +2OH, or "Abberior® STAR 635", according to ref. [9]). The latter dye proves to be the most photostable from these four. See also **Table S3** above and **Table 2** in the main text. Picture taken under additional illumination with a UV lamp (for TLC monitoring, channel 365 nm).

Dye	Loss in extinction at 635 nm., %	Fluorescence intensity change in three detection channels relative to initial values		
		Red 660 nm., - loss / + gain, %	Orange 580 nm., gain	Yellow 560 nm., gain
Illumination **20 h** in pure water				
KK 1517	-64	-52	x 16	x 20
KK 1103	-43	-36	x 24	x 32
KK 9046	-42	-20	x 19	x 18
KK 1115	-32	-14	x 22	x 12
KK 114L	-48	-22	x 15	x 18
KK 1550	-51	-30	x 11	x 10
KK 1553*	-25	+70	x 4	x 3
KK 1558*	-28	+90	x 3	x 3
Illumination **12 h** with and without additives				
KK 114L	-35	-16	x 5	x 6
KK 114L + DABCO	-20	-8	x 2	x 5
KK 114L + DABCO + TRIS	-52	-18	x 3	x 4
KK 1558	-20	+34	x 2	x 2
KK 1558 + DABCO + TRIS	-32	+6	x 1.1	x 1.1
Illumination **20 h** with and without additives				
KK 114L**	-52	-20	x 12	x 15
KK 114L + DABCO	-58	-36	x 6	x 5
KK 114L + DABCO + TRIS	-74	-52	x 6	x 10
KK 114L + TRIS	-70	-34	x 6	x 9
KK 1553**	-20	+55	x 3	x 4
KK 1553 + DABCO	-80	+11	x 2	x 3
KK 1553 + DABCO + TRIS	-62	+22	x 2	x 3
KK 1553 + TRIS	-51	+30	x 2	x 3

Table S3. Photolysis in dilute bulk solutions with a filtered visible light of a halogen lamp (420 – 900 nm).

Additional experiments (see also **Table 2**, main text). The changes in color density and in fluorescence signal after illumination were recorded in three channels by means of a multimode microplate reader (see above for details) * -- before irradiation the absolute value of the fluorescence signal for those NPA-sabilized dyes was ~ 6 times lower than that of **KK 114L**, after irradiation – only 2 times as low. ** -- water contained 0.01 M $NaHCO_3$ adjusted to pH 8.5. Additives (stabilizers): DABCO (a two-acid base) base was taken as a 0.1 M solution and buffered to pH 8.5 with ~ 1 equiv of HOAc (acetic acid) or, in another experiment series, with ~ 0.5 equiv of TRIS × HCl. TRIS base was also buffered with ~ 0.5 equiv of TRIS × HCl to the same pH value.

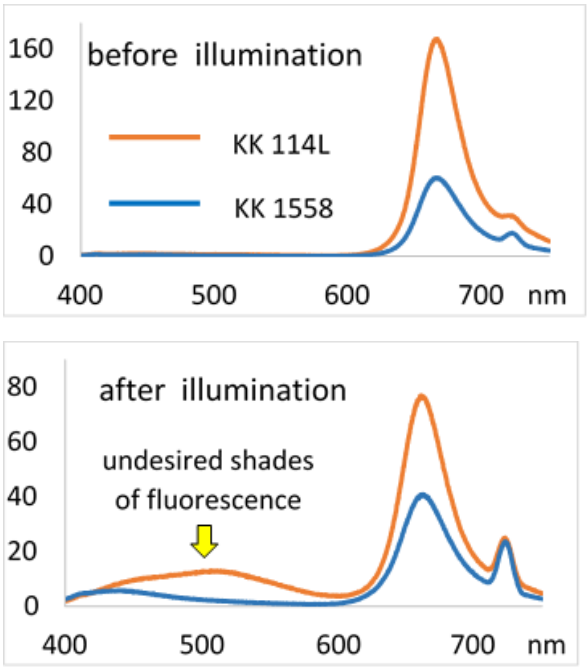


Figure S7a. Fluorescence spectra of the dye solutions before and after illumination.

The reference dye **KK 114L** and the NPA-stabilized dye **KK 1558** as 10 µmol/L aq. solutions after 10 hours illumination with a halogen lamp through an IR heat-absorbing filter (λ ≤ 1200 nm) and a UV filter (λ ≥ 395 nm, see above for details). X axis – λ, nm, Y axis – signal intensity, a.u. The changes in case of KK 114L are consistent with the data in **Table S3** (above), **Table 2** and with what is seen in **Fig. 6** (main text). Fluorimeter Shimadzu RF 5301, excitation at 365 nm. The second maximum at ~ 720 nm is a monochromator-related artefact (not observed in the spectra below recorded at a higher excitation wavelength).

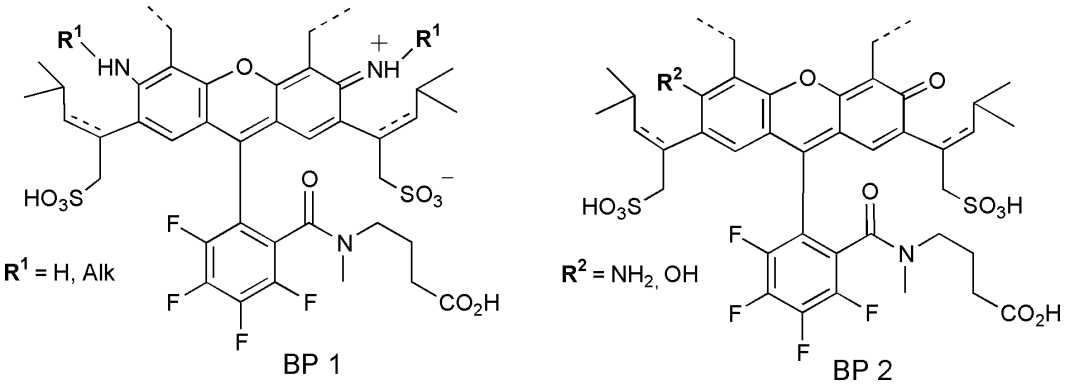


Figure S7b. Possible structures of the bluing products (BP 1 and BP 2) generated during illumination in aqueous dye solutions (see discussion in the main text). Conventional fluorescein has the λ max. fl. of 512 nm. As seen in **Fig. S7a** above**,** the fluorescence of the bluing products is detected between 420 and 580 nm with a "maximum" at ca. 530 nm. The latter probably belongs to fluorescein dyes modified with four fluorine atoms in the *meso*- ring (~ +20 nm to the red) and with an amide group (~ +10 nm to the red). Photo-induced dealkylation and deamination of rhodamine dyes were studied in [22].

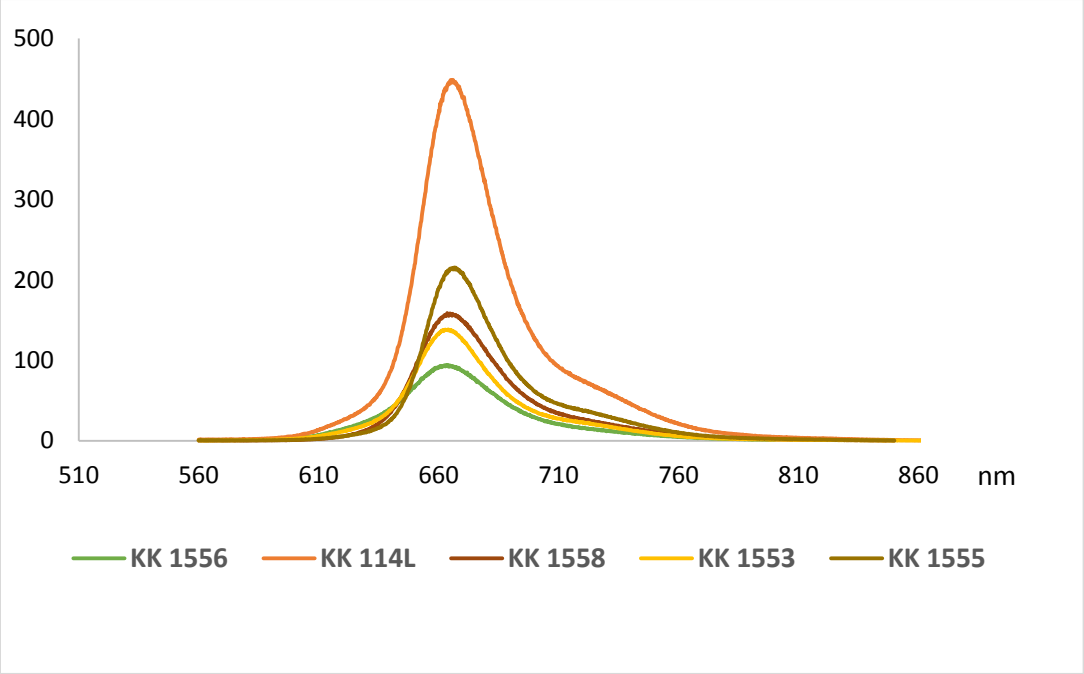


Figure S8. Fluorescence spectra of the nitrobenzyl-modified dyes and the reference dye KK 114L.

Dyes taken as 10 µmol/L aq. solutions. Fluorimeter Shimadzu RF 5301, excitation at 530 nm. See **Table 1** (main text) for the Φ_f values of the dyes. X axis – λ, nm, Y axis – signal intensity, a.u. The signal intensity well correlates with the Φ_f values in **Table 1** (main text), while the maxima and the shapes of the spectra are virtually identical.

Chromatographic analyses and mass-spectrometric data on the dyes.

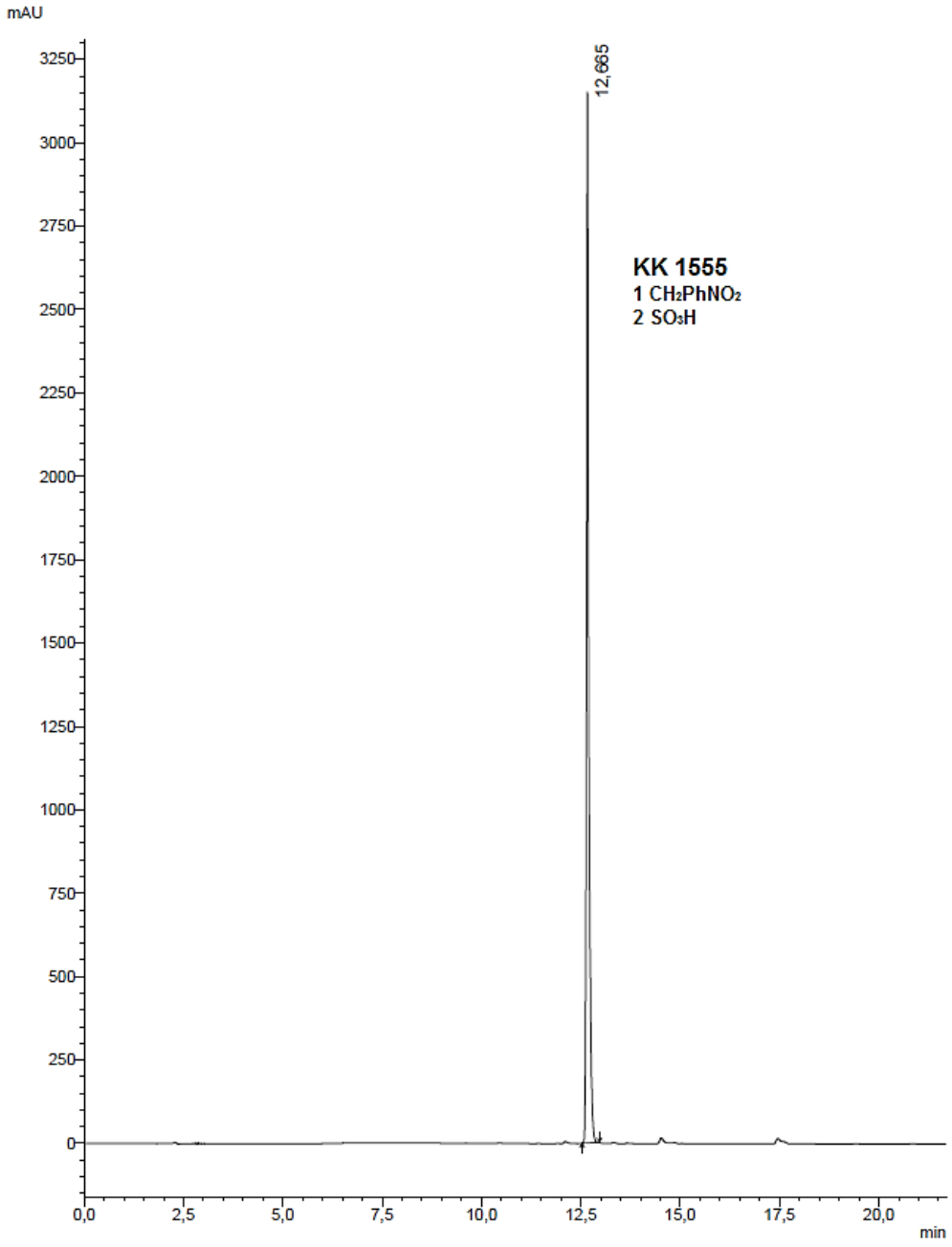


Figure S9. Chromatogram of the dye KK 1555. Column Kinetex C-18, 100 Å, 5 µm, 4.6 × 100 mm, grad. 0 – 80% B in 20 min, 0.5 mL/min, with aq. Et_3N (0.3 vol. %) as A and MeCN as B. See also comparative HPLC analyses of dye mixtures below. Detection channel 635nm (for addirional chromatograms with a two-channel detection 254/635nm see below).

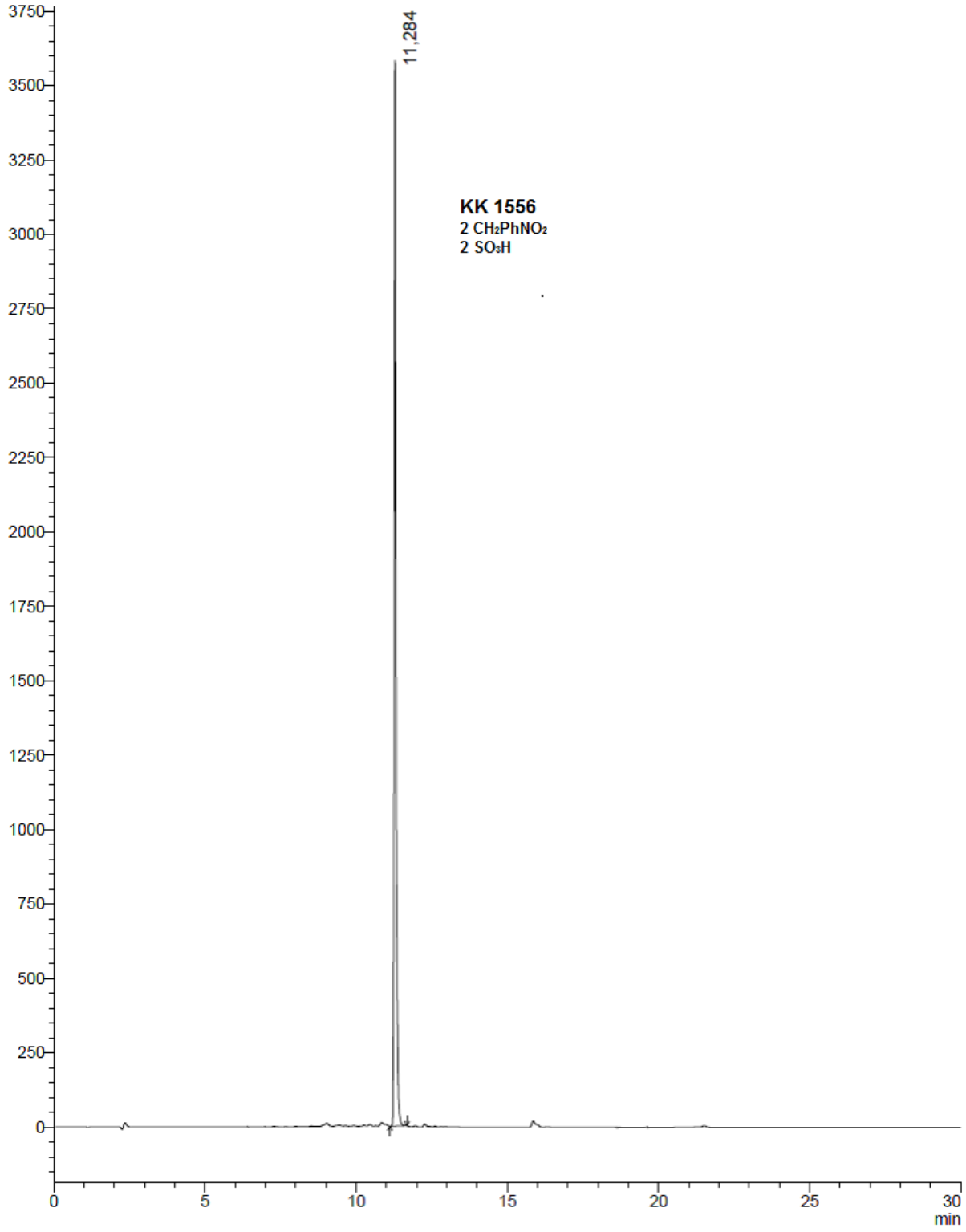


Figure S10. Chromatogram of the dye KK 1556 (free acid, additionally purified by HPLC).

Kinetex C-18, 100 Å, 5 µm, 4.6 × 100 mm, grad. 10 – 80% B in 20 min, 0.5 mL/min, with aq. Et_3N (0.3 vol. %) as A and MeCN as B. See comparative HPLC analyses below. Detection channel 635nm.

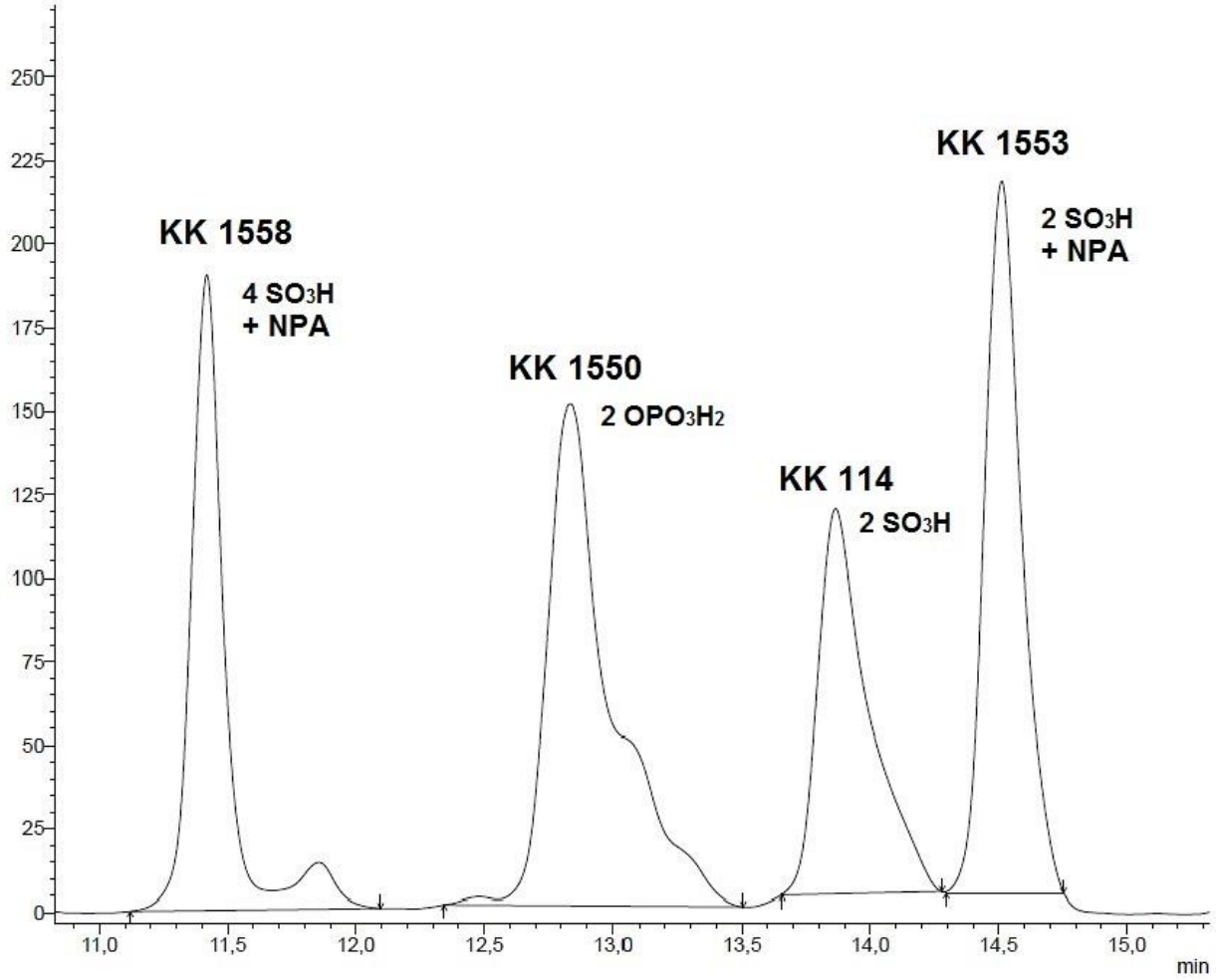


Figure S11. Chromatograms of the dyes with multiple polar groups (NPA – 4-nitrophenylalanine; see compound description part and Scheme 1 in the main text for structures).

Column Kinetex with a *phenyl-hexyl phase*, 100 Å, 5 µm, 4.6 × 100 mm, 0 – 60% B in 20 min, flow 0.5 mL/min with 0.05 M aq. TEAB buffer as A and MeCN as B. The lipophilic and "amphiphilic" dyes (*i. e.*, KK 1517, 1103, 1115 and 1556) demonstrate retention times of more than 45 min under these conditions. As seen in other examples below, a Kinetex C-18 column allows better resolution and better peak shapes for most of the dyes studied here.

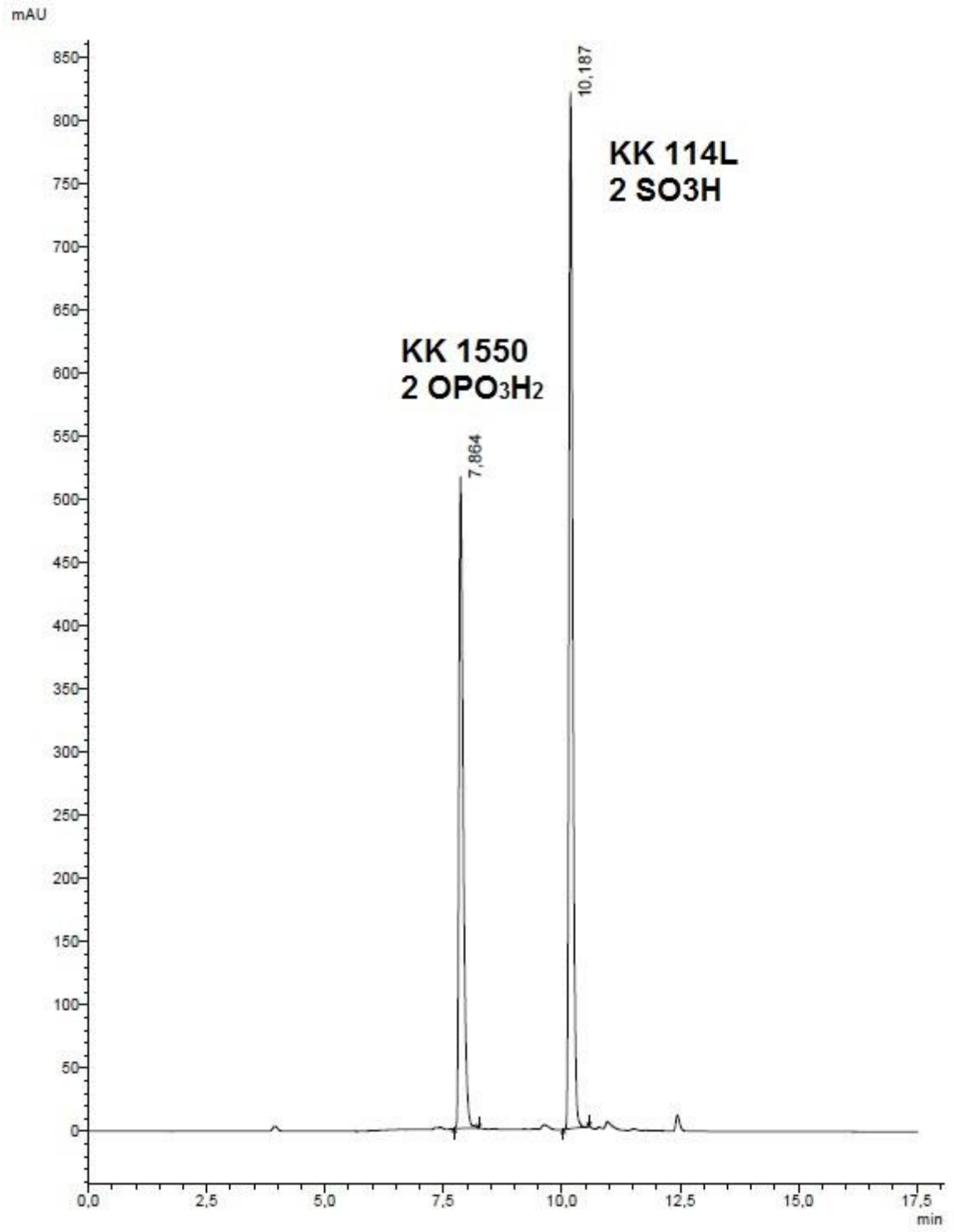


Figure S12. Chromatogram of the sulfonated dye KK 114L and the phosphorylated dye KK 1550 (see compound description part and Scheme 1 in the main text for structures).

Column Kinetex C-18, 100 Å, 5 µm, 4.6 × 100 mm, grad. 0 – 80% B in 20 min, 0.5 mL/min, t = 40°C, with aq. Et_3N (0.3 vol. %) as A and MeCN as B. Detection channel 635nm.

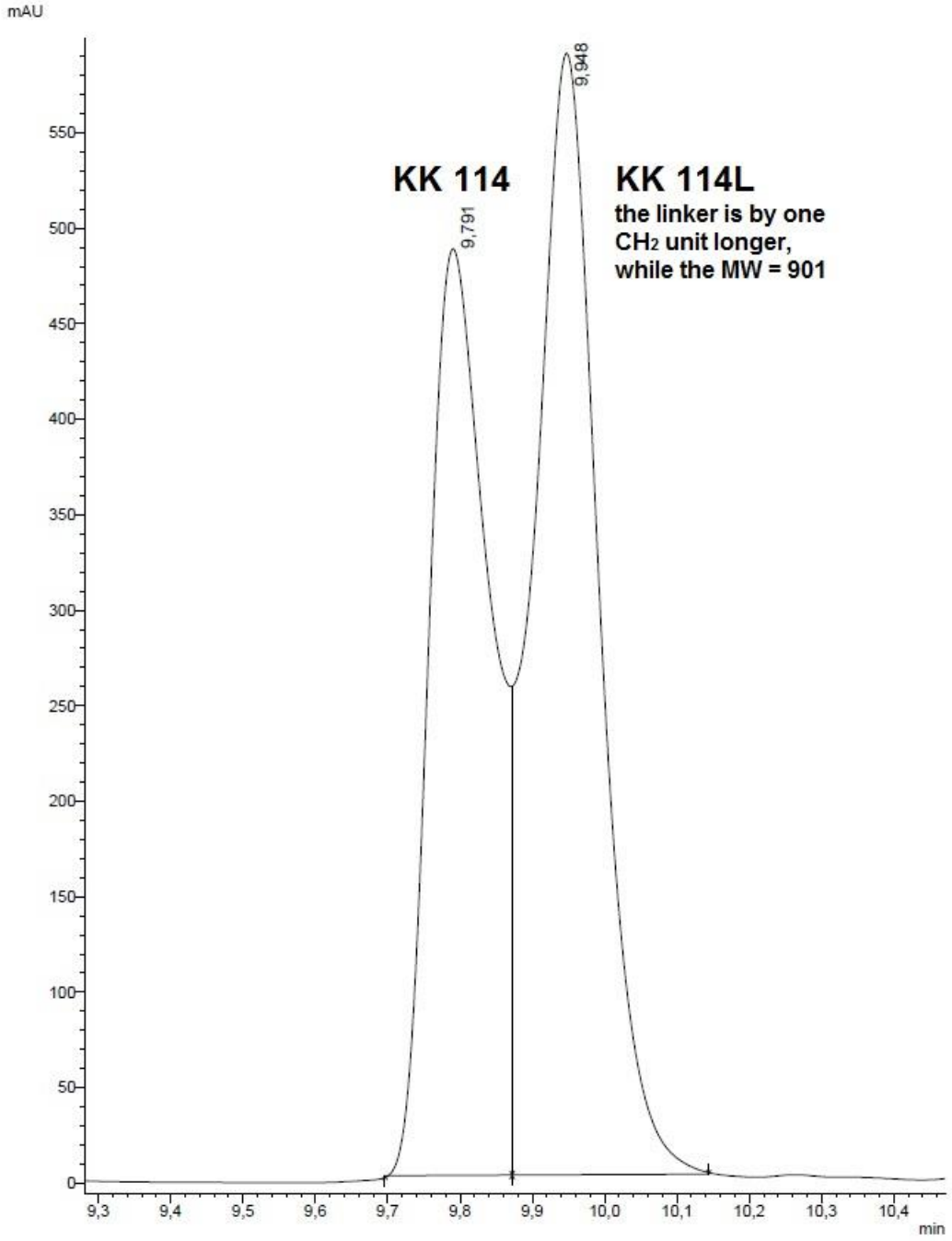


Figure S13. Chromatogram of the sulfonated dyes KK 114 and KK 114L (see compound description part and Scheme 1 in the main text for structures).

Although the difference in polarity is very subtle, one can resolve these dyes under proper conditions. For immunolabelling the dye KK 114L was used, as its NHS ester is much more stable (see the introductory part of the main text). Kinetex C-18, 100 Å, 5 µm, 4.6 × 100 mm, t = 25°C, grad. 0 – 60% B in 20 min, 0.5 mL/min, with aq. Et_3N (0.3 vol. %) as A and MeCN as B. Detection channel 635nm.

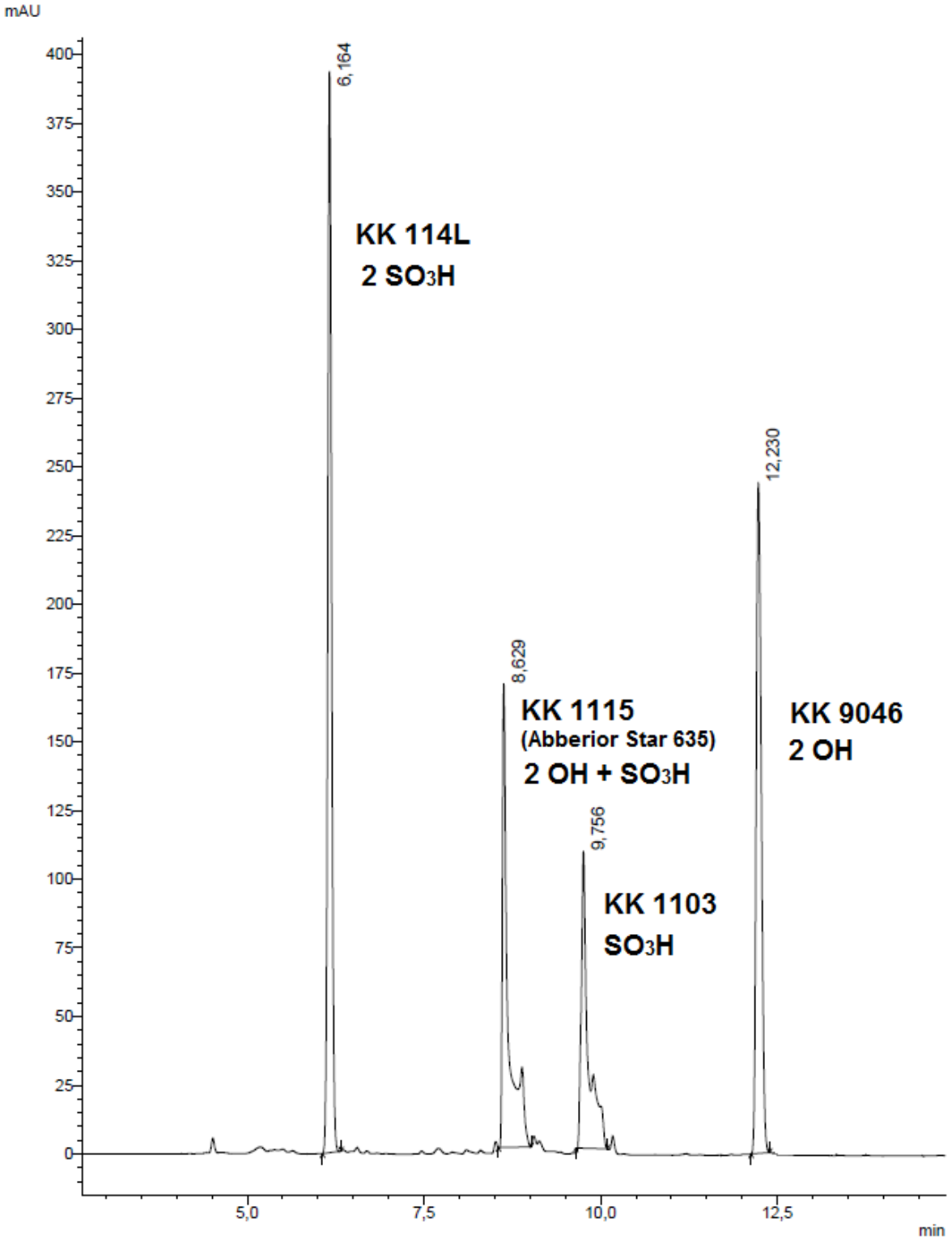


Figure S14a. Chromatogram of moderately polar (amphiphilic) dyes and the reference dye KK 114L.

Column Kinetex C-18, 100 Å, 5 µm, 4.6 × 100 mm, grad. 0 – 90% B in 20 min, 1.2 mL/min, t = 40°C, with aq. Et_3N (0.3 vol. %) as A and MeCN as B. The dyes with just one SO_3H group develop "multiplets" probably due to different degrees of deprotonating, whose proportion is being changed on the solid phase. This does not occur for other dyes. See also **Fig. S14b** below. Detection channel 254 nm.

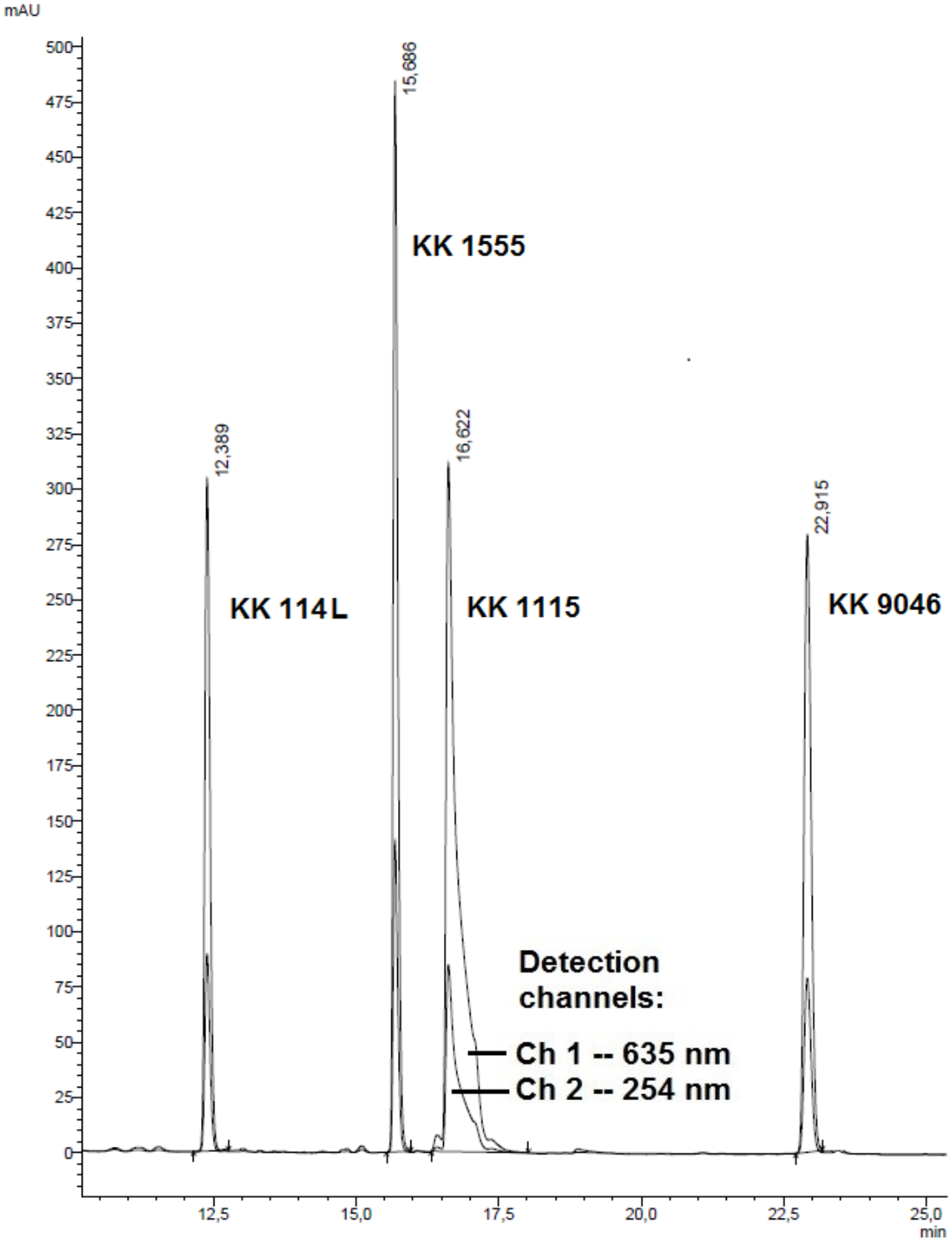


Figure S14b. Resolution of two moderately polar (amphiphilic) dyes KK 1555 and KK 1115 with very similar polarity and retention parameters in comparison to a much more polar dye (KK 114L) and a far less polar dye (KK 9046).

Column Kinetex C-18, 100 Å, 5 µm, 4.6 × 100 mm, grad. 0 – 80% B in 30 min, 0.5 mL/min, t = 40°C, with aq. Et_3N (0.3 vol. %) as A and MeCN as B. See also **Fig. S14a** above. Detection was performed also at 254 nm to see possible colourless impurities.

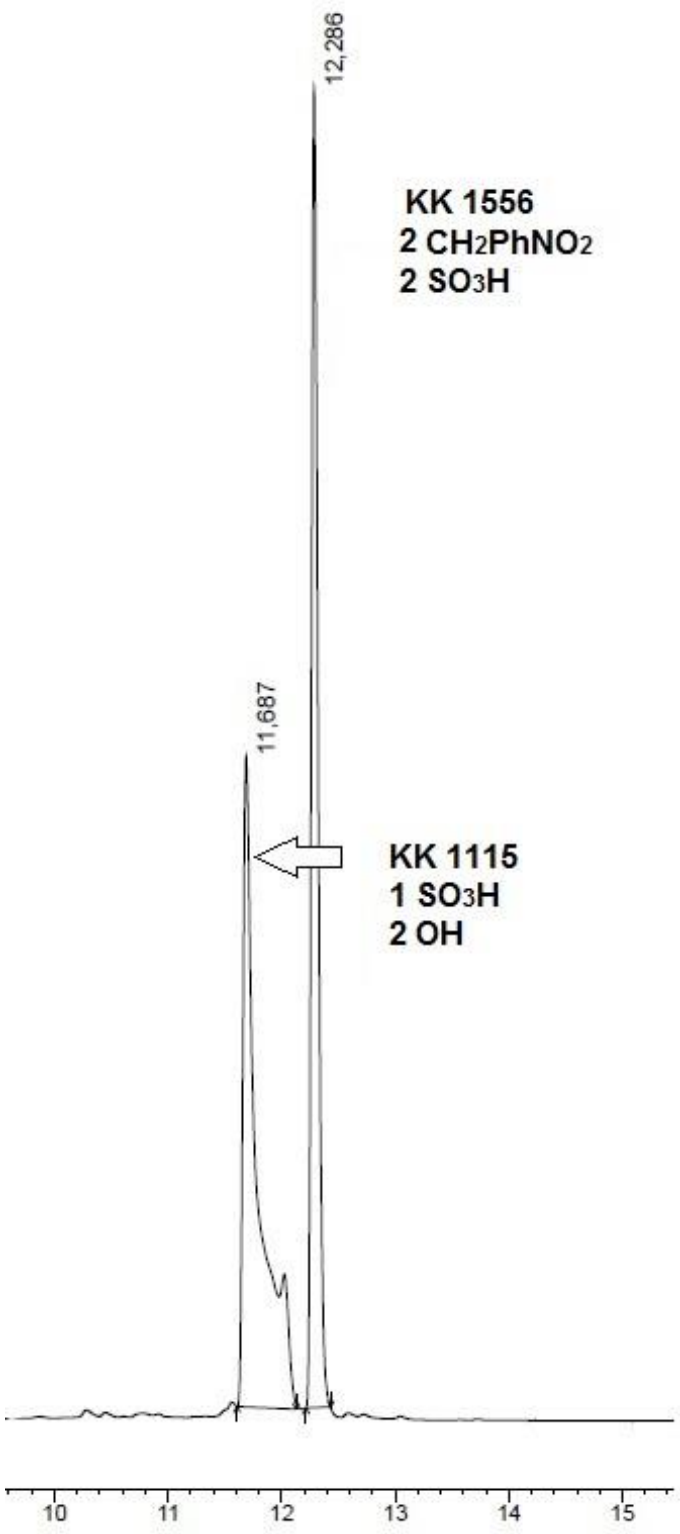


Figure S14c. Resolution of moderately polar (amphiphilic) dyes KK 1115 and KK 1556 with similar polarity and retention parameters.

Kinetex C-18, 100 Å, 5 µm, 4.6 × 100 mm, grad. 0 – 80% B in 20 min, 1.2 mL/min, t = 40°C, with aq. Et_3N (0.3 vol. %) as A and MeCN as B. Detection channel 635nm.

Despite the higher net charge, the dye KK 1556 has a longer retention time due to the additional aromatic ring (4-nitrobenzylthio group) an a higher logD value, respectively (see **Table S4**). If the dyes KK 1115, KK 1555 and KK 1556 are injected all together, a distinct resolution of all three is hardly possible. Whatever the conditions are, the "order of appearance", also listed in **Table S4**, remains the same. This holds true for C-18 phase and basic media (TEAB, Et_3N).

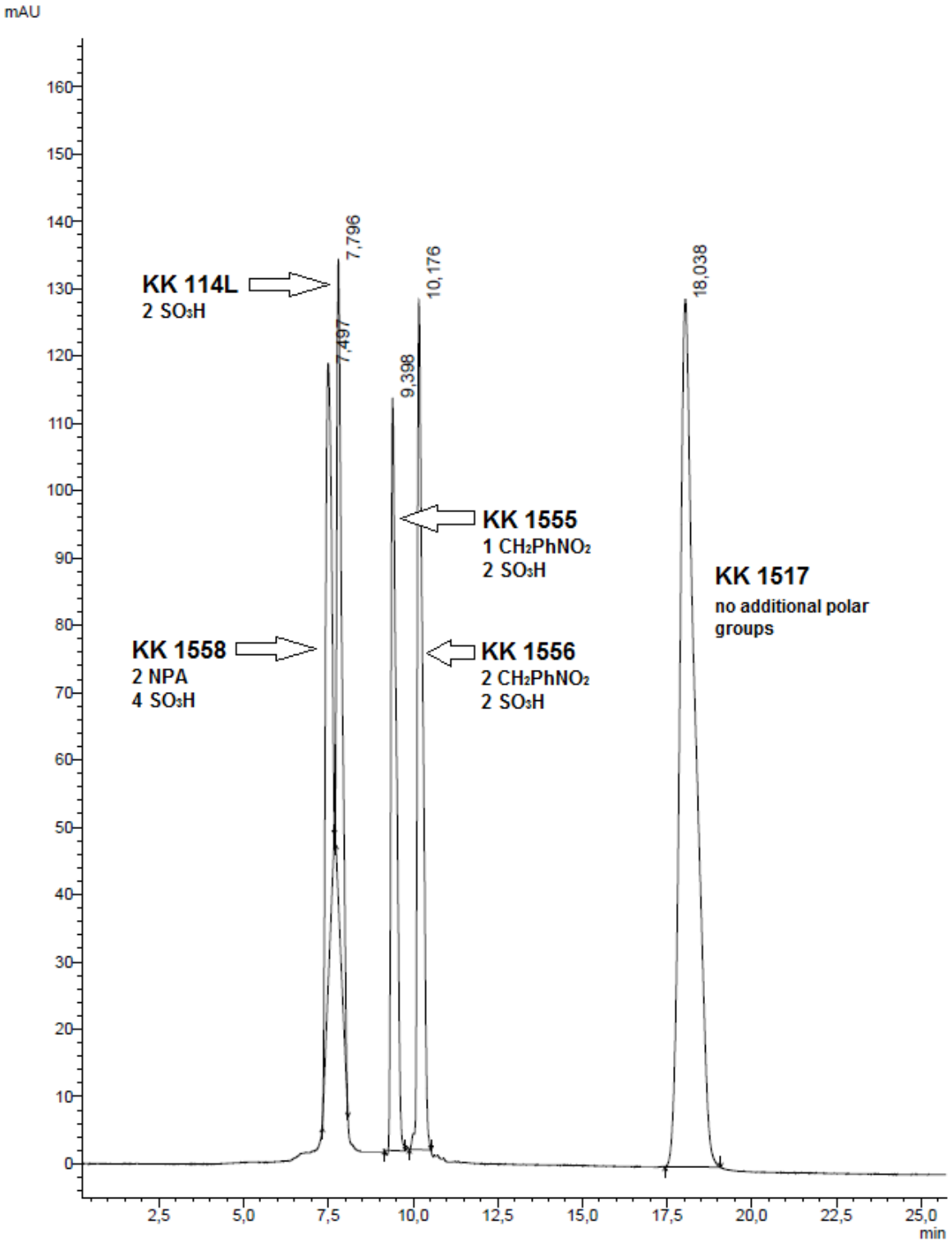


Figure S15. Chromatogram of dyes with different functional groups in comparison to the reference dye KK 114L (NPA – 4-nitrophenylalanine).

Column Kinetex C-18, 100 Å, 5 µm, 4.6 × 100 mm, grad. 0 – 90% B in 20 min, 0.5 mL/min, t = 40°C, with aq. Et_3N (0.3 vol. %) as A and MeCN as B. Detection channel 254nm.

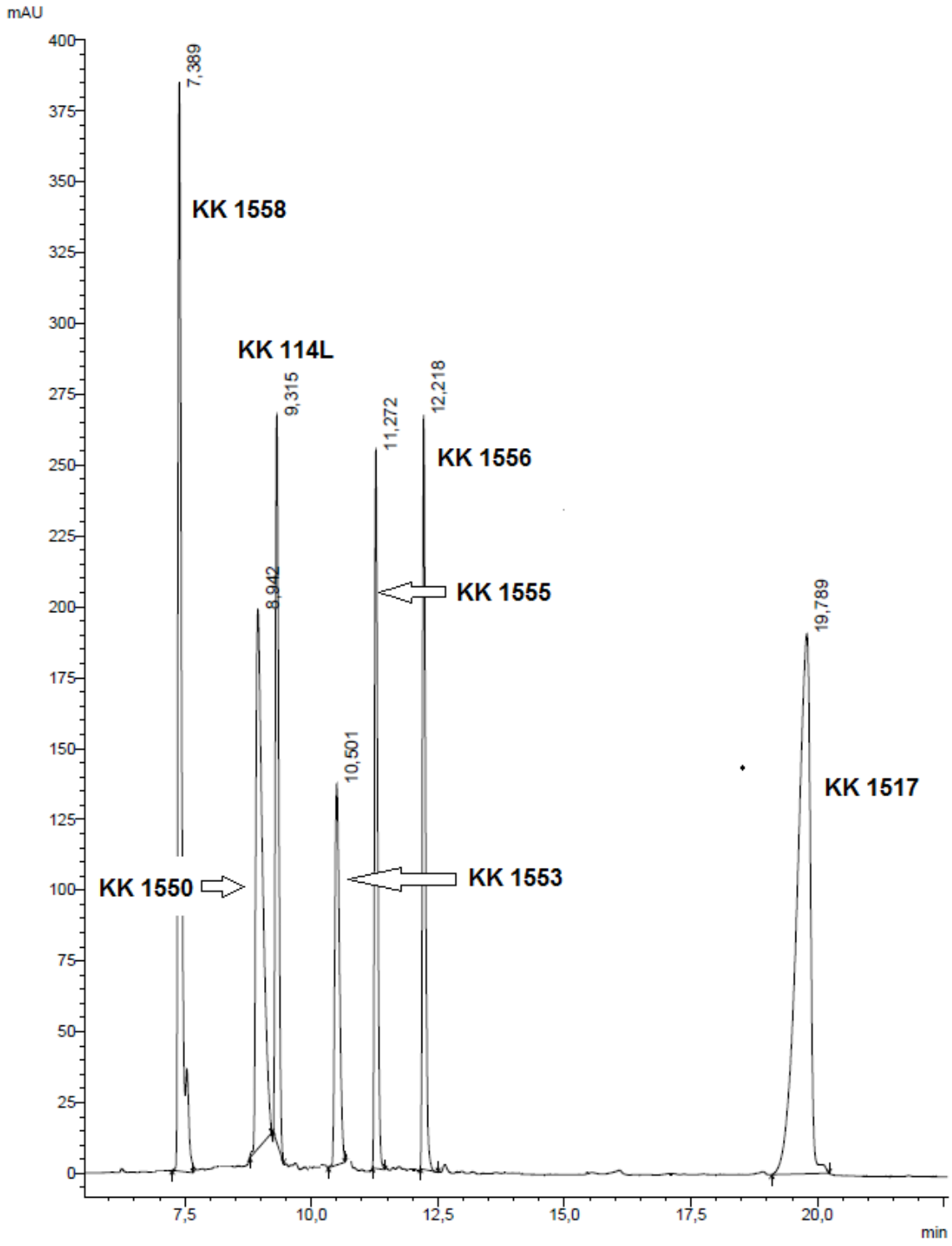


Figure S16. A "set" of 7 new dyes with different functional groups in comparison to the reference dyes KK 114L and KK 1517. See also HPLC analyses above and compound description part for structures. Column Kinetex C-18, 100 Å, 5 µm, 4.6 × 100 mm, grad. 0 – 90% B in 20 min, 0.5 mL/min, t = 25°C, with aq. Et_3N (0.3 vol. %) as A and MeCN as B. Detection channel 635nm.

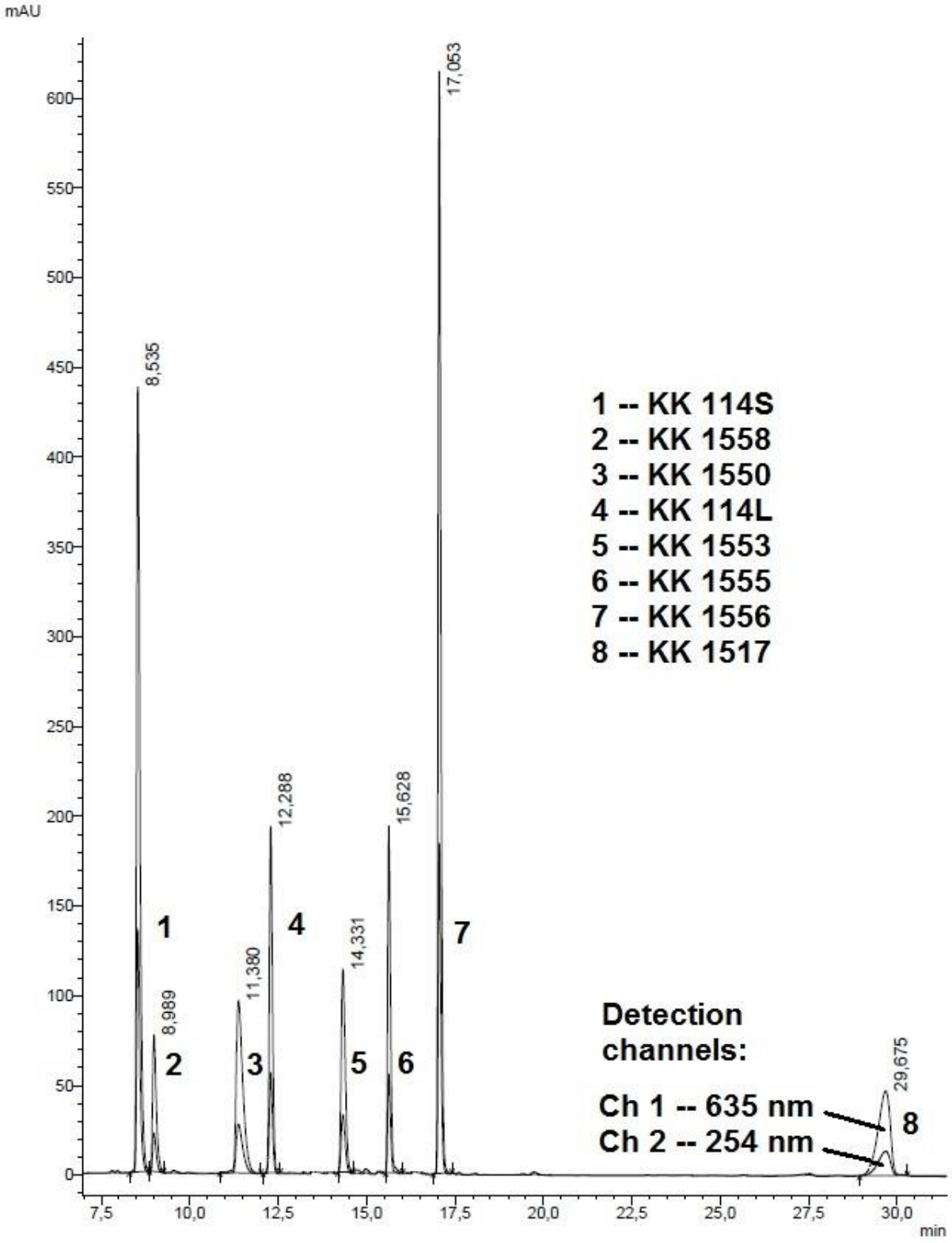


Figure S17. A "set" of all 8 new dyes with different functional groups in comparison to the reference dyes KK 114L and KK 1517. See also HPLC analyses above and compound description part for structures. Detection was performed also at 254 nm to see possible colourless impurities. Column Kinetex C-18, 100 Å, 5 µm, 4.6 × 100 mm, grad. 0 – 80% B in 30 min, 0.5 mL/min, t = 40°C, with aq. Et_3N (0.3 vol. %) as A and MeCN as B.

Operator: J. Bienert Timebase:HPLC Sequence:TRENNUNG_ULTIMATE

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Max-Planck-Institut für biophysikalische Chemie - Göttingen
Facility für synthetische Chemie

Probe : **KK1551a**

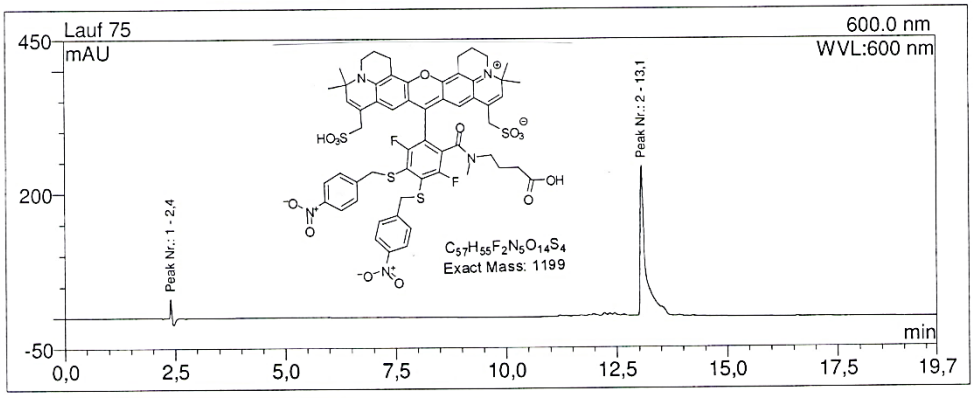
Lösungsmittel : H2O/MeCN **Konzentration :** ? %
Aufgabeart : Handaufgabe **Aufgabemenge :** 1.0 µl

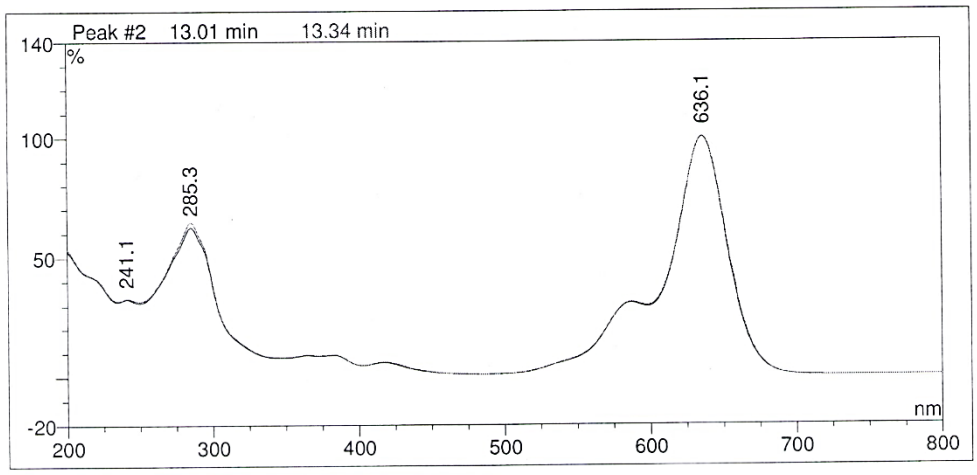
Säule : Kinetex 5µm C18 100
Länge : 25 cm **Innendurchmesser :** 4.6 mm

Fluß (ml / Min) : 1,2 **Temperatur :** 20 °C
Detektor : UltiMate 3000

Laufmittel : A = Acetonitril B = Wasser 0.05% TFA

Gradient : A% = 10 B% = 90 ----> A% = 100 B% = 0 T = 20 Min.





Jürgen Bienert Tel.: 0551 2011251 juergen.bienert@mpibpc.mpg.de

Figure S18. Exemplary HPLC analysis of the dye KK 1556 (here 1551a, free acid) on C-18 with an acidic mobile phase (TFA). (MPI-BPC 2015).

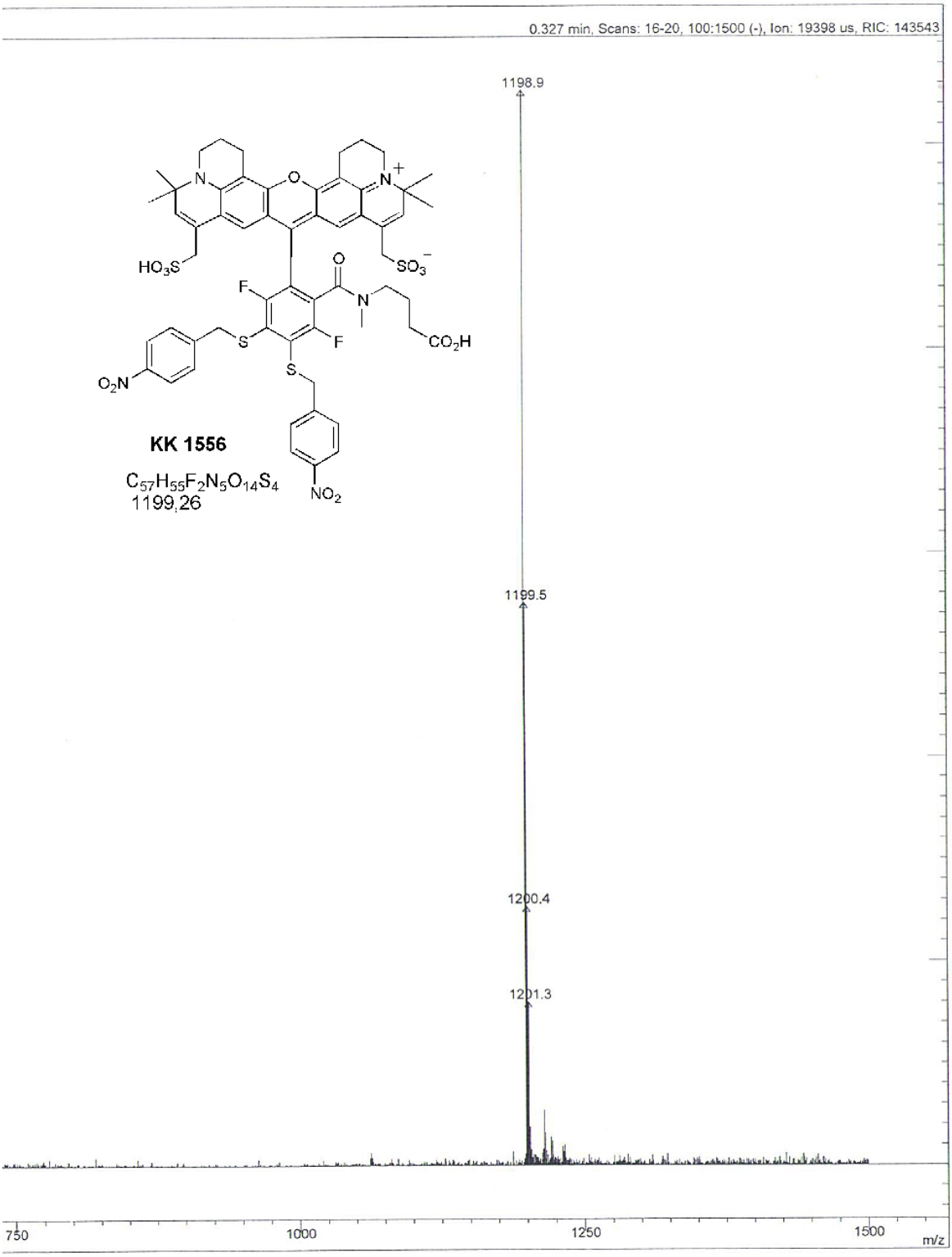


Figure S19. Actual mass-spectrum of the dye KK 1556 as a free acid. For more data see compound description section.

Operator: J. Bienert Timebase:HPLC Sequence:TRENNUNG_ULTIMATE Page 72-1
22.10.2015 2:17 PM

Max-Planck-Institut für biophysikalische Chemie - Göttingen
Facility für synthetische Chemie

Probe : KK1556nhs

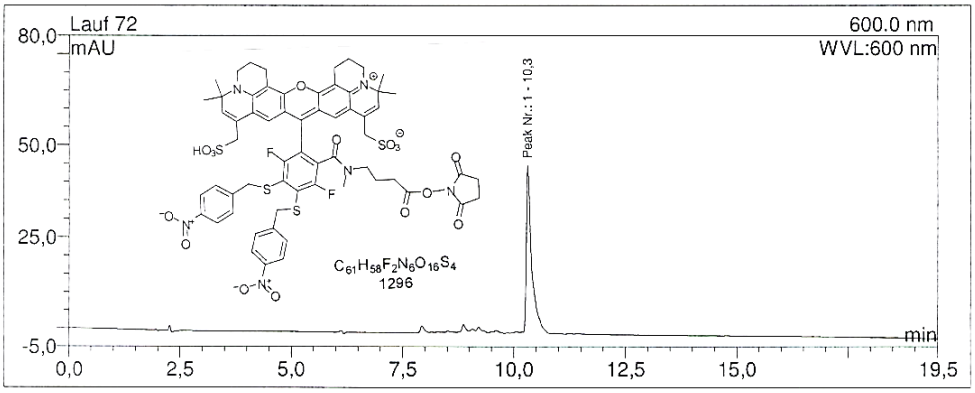
Lösungsmittel : ? **Konzentration :** ? %
Aufgabeart : Handaufgabe **Aufgabemenge :** 1.0 µl

Säule : Kinetex 5µm C18 100
Länge : 25 cm **Innendurchmesser :** 4.6 mm

Fluß (ml / Min) : 1,2 **Temperatur :** 20 °C
Detektor : UltiMate 3000

Laufmittel : A = Acetonitril B = Wasser 0.05% TFA

Gradient : A% = 30 B% = 70 ----> A% = 100 B% = 0 T = 20 Min.



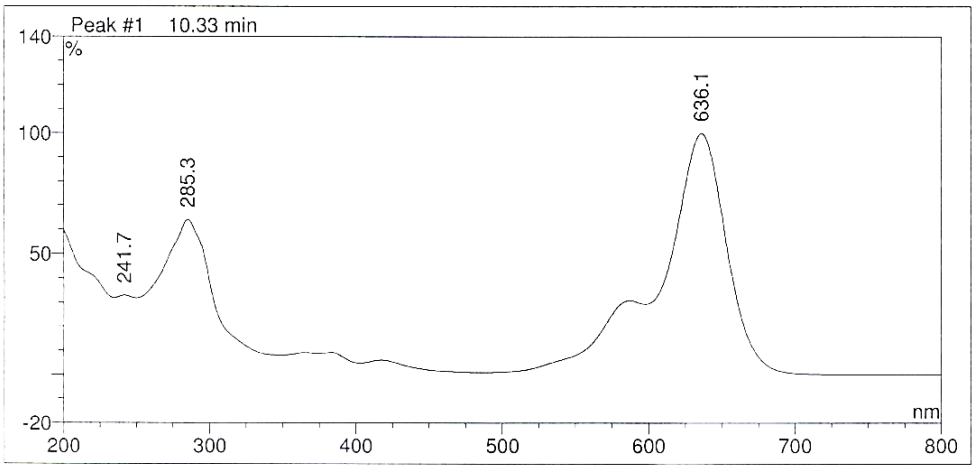


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Figure S20. Exemplary HPLC analysis of the dye KK 1556-NHS (active ester for conjugation) on C-18 with an acidic mobile phase (TFA). (MPI-BPC 2015).

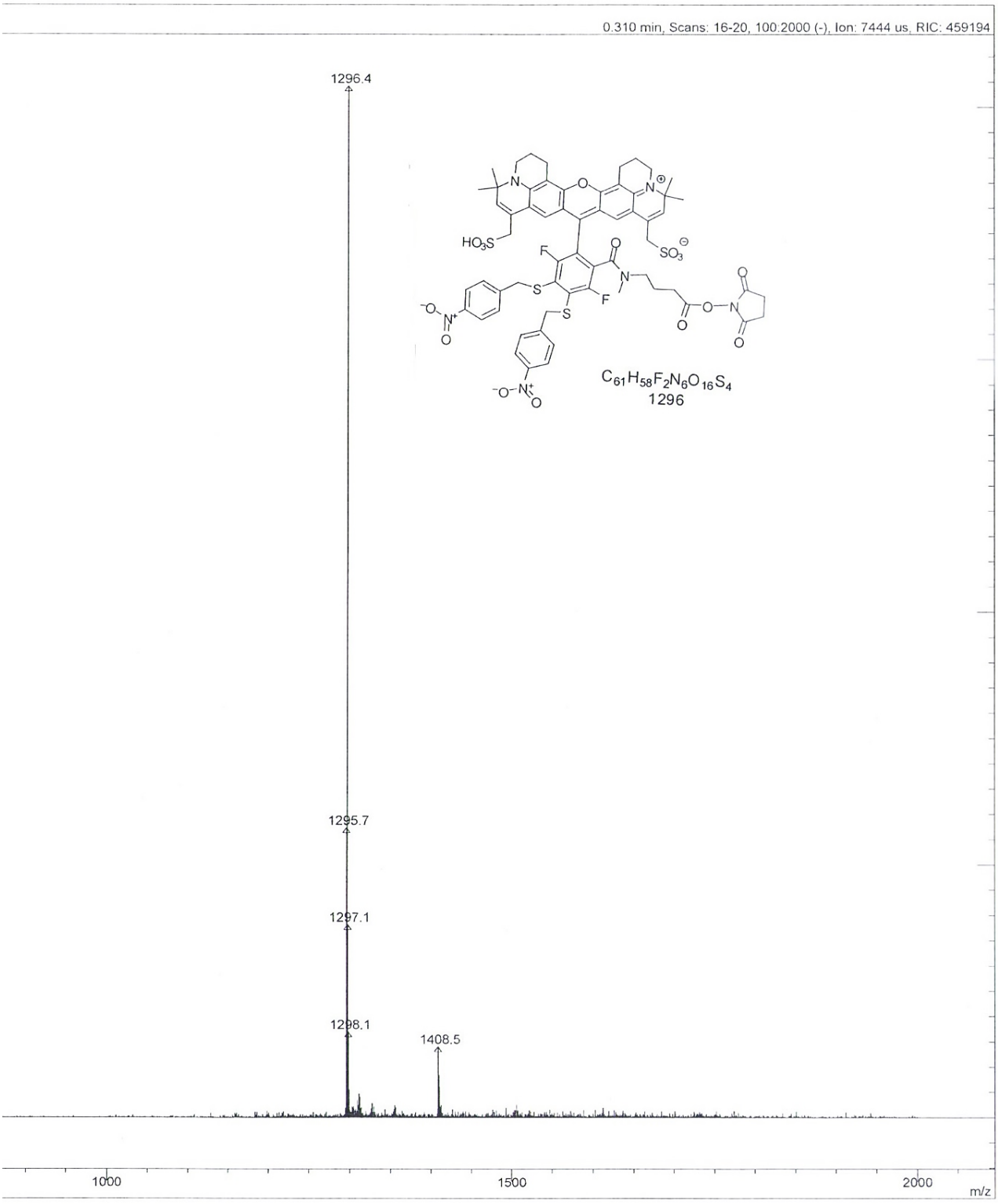


Figure S21. Actual mass-spectrum of the dye KK 1556-NHS (active ester for conjugation).

For more data and preparations see compound description section.

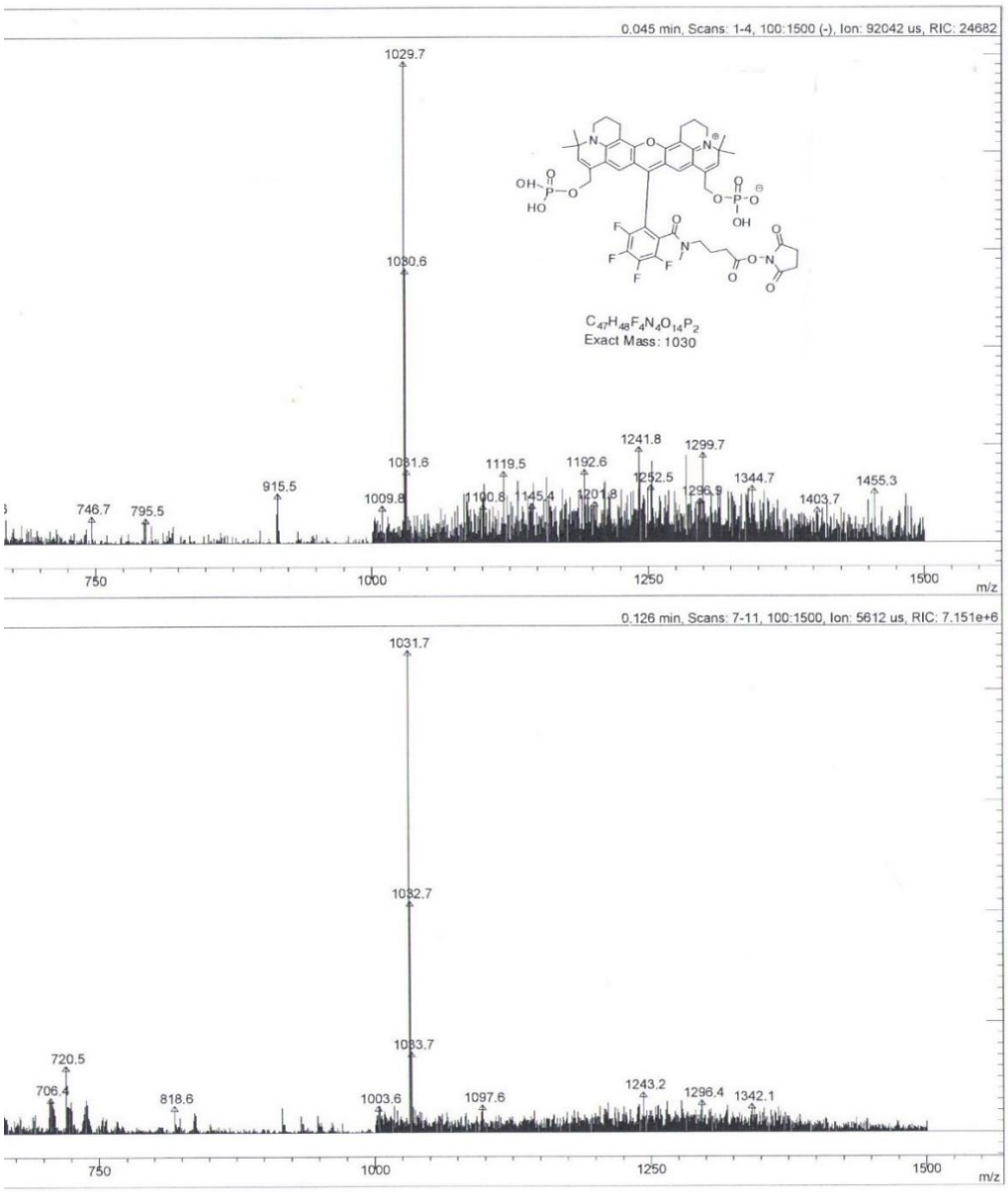


Figure S22a. Actual mass-spectrum of the dye KK 1550 NHS ester (MPI-BPC 2015).

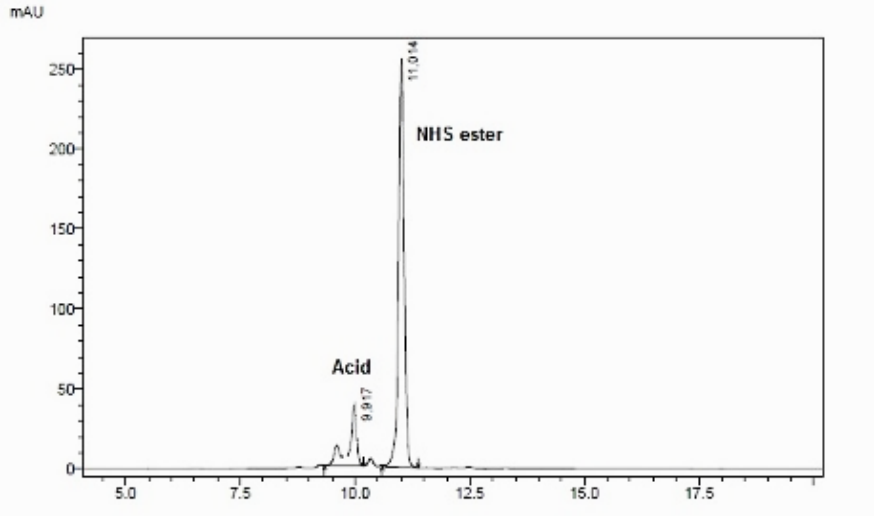


Figure S22b. Chromatogram of a freshly-prepared KK 1550 NHS ester (see structure above).

Kinetex C-18, 100 Å, 5 µm, 4.6 × 100 mm, grad. 10 – 80% B in 20 min, 0.5 mL/min, aq. 0.03 M TEAA (triethylammonium acetate) + 0.03 M HOAc as A and MeCN as B. Detection channel 635nm.

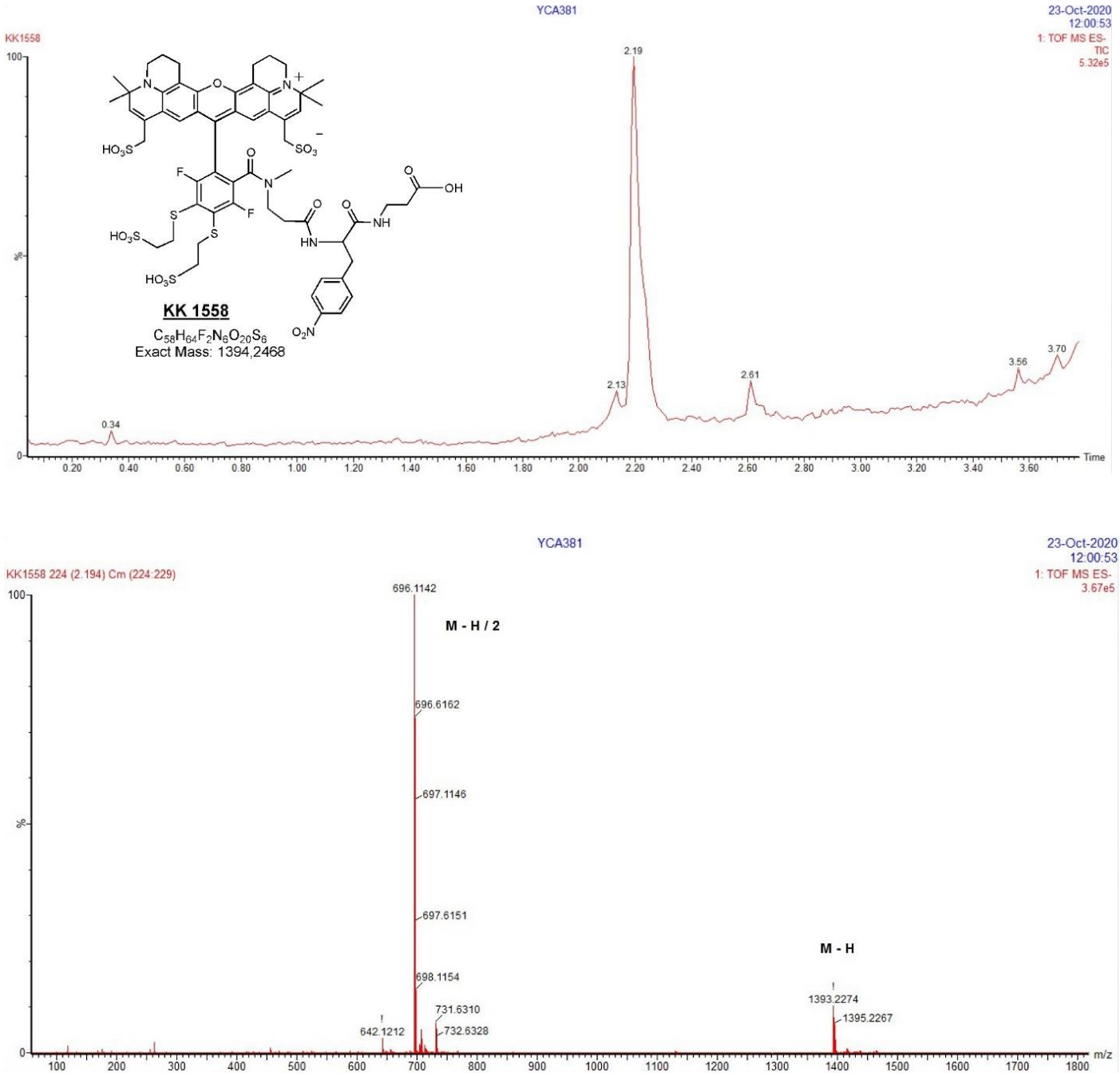


Figure S23. Actual LC/MS analysis and mass-spectrum of the dye KK 1558

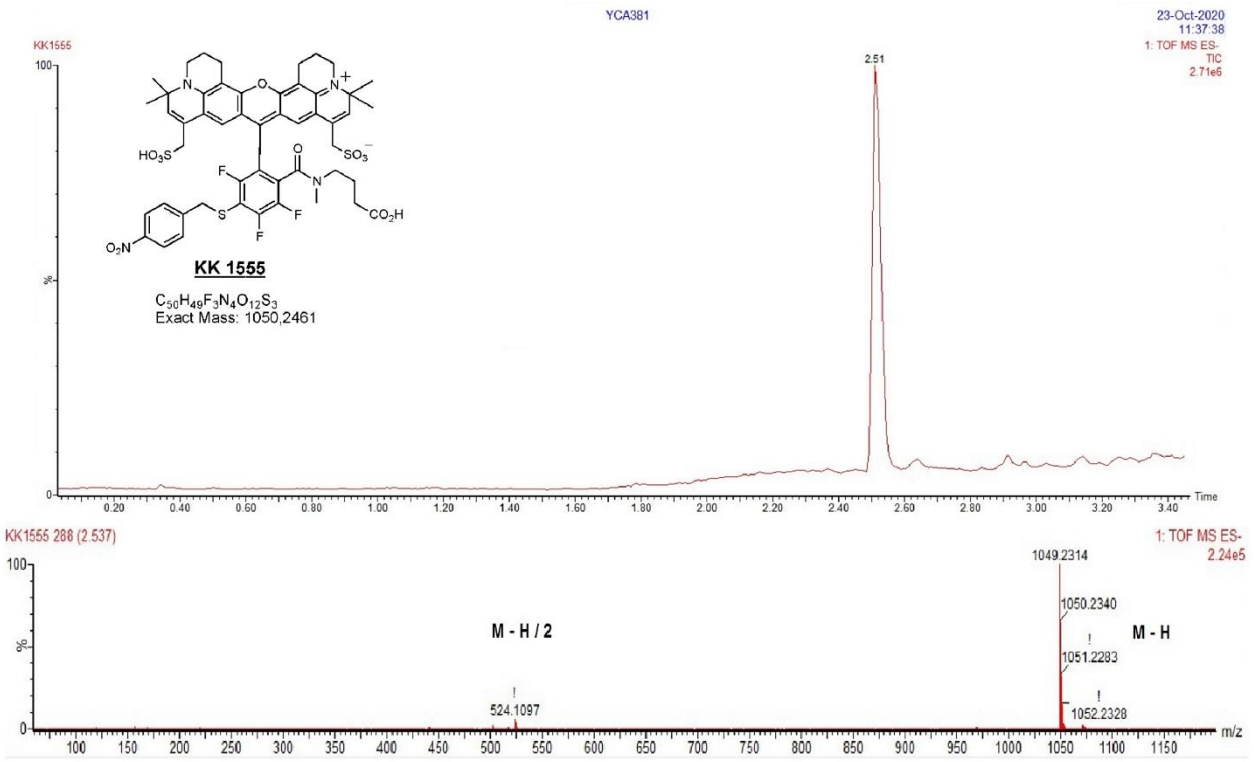


Figure S24. Actual LC/MS analysis and mass-spectrum of the dye KK 1555.

Polarity and hydrophilic/lipophilic properties of the fluorophores

Dye See **Scheme 1** For structures	Additional functional groups	Calculated distribution constant (lipophilicity descriptor) **LogD**	Order of elution in HPLC analyses on C-18 phase See **Figs. S 13 -- 17**	Net charge of the molecule **q**
KK 1517	--	6.40	12	0
KK 9046	2×OH	3.84	11	0
KK 1103	SO_3H	3.35	10	-1
KK 1556	2×SO_3H, 2×4-NO_2PhCH_2	2.76	9	-2
KK 1115	2×OH, SO_3H	0,79	8	-1
KK 1555	2×SO_3H, 4-NO_2PhCH_2	0.64	7	-2
KK 114	2×SO_3H	-1.77	4	-2
KK 1553	2×SO_3H, 4-NO_2PhAla	-1.56	6	-2
KK 114L	2×SO_3H	-1.49	5	-2
KK 1550	2×$OP(O)(OH)_2$	-1.97	3	-4
KK 1558	4×SO_3H, 4-NO_2PhAla	-7.82	2	-4
KK 114S	4×SO_3H	-7.86	1	-4

Table S4. Lipophilicity descriptor LogD for dyes with various functional groups in comparison to their actual HPLC elution/retention parameters. The distribution constant (lipophilicity descriptor for charged molecules) was calculated using the *LogD Predictor* from ChemAxon. The structures were imported as mol.- files. See https://disco.chemaxon.com/calculators/demo/plugins/logd/
For the analyses conditions see chromatograms above. See example in **Figure S25** below.

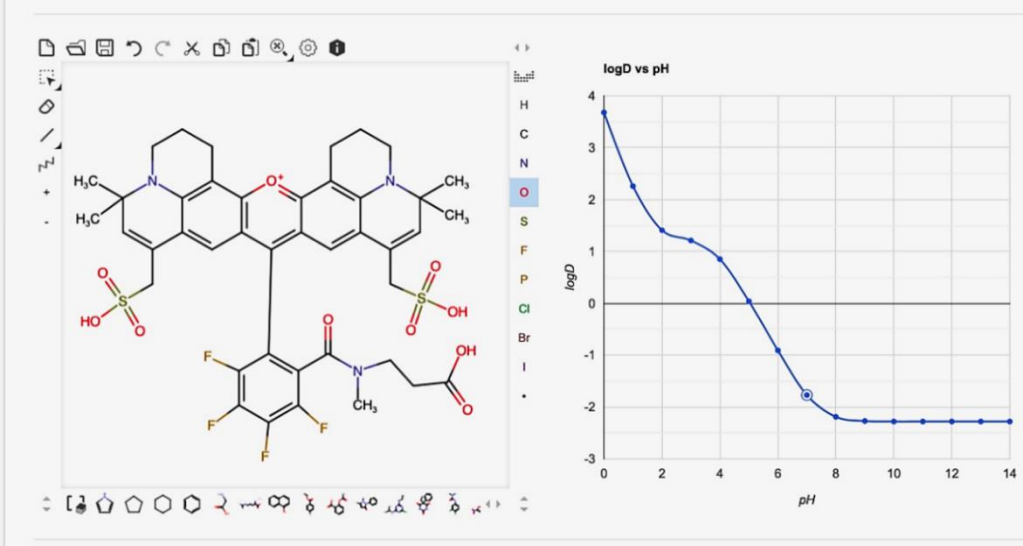


Figure S25. Exemplary calculation of the LogD for the dye KK 114. The structure depicted according to ref. [23]. This way, in our opinion, suits zwitter-ionic rhodamine dyes best.

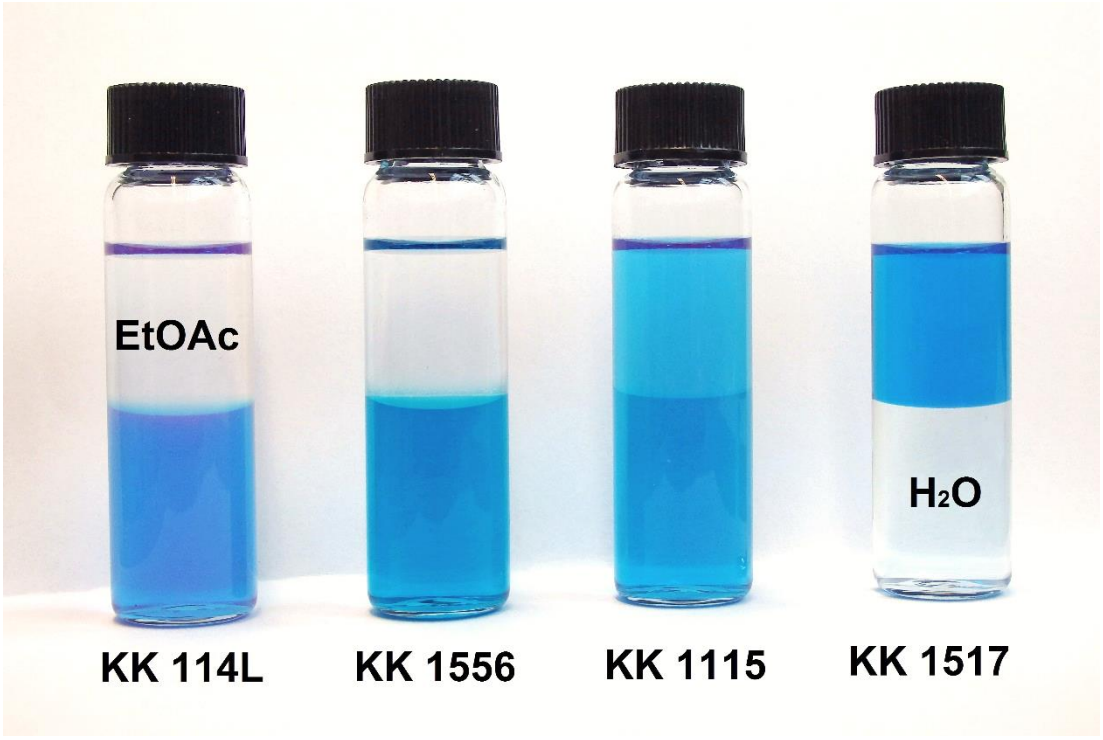


Figure S26. Equilibrial distribution of the dyes between neutral water (lower phase) and ethyl acetate as a lipophilic organic solvent (upper phase). Four illustrative examples are presented.

All the dyes with two SO_3H groups and the phosphorylated dye KK 1550 stay exclusively in the aqueous phase, even in acidic media and even despite the lipophilic NPA or NBT moieties (NPA – 4-nitrophenylalanine, NBT – 4-nitrobenzylthio; see compound description part above and **Scheme 1** in the main text for structures). The lipophilic dye KK 1517 (no additional polar groups, exists as a zwitter-ion) stays exclusively in the organic phase, as expected. On the other hand, the dyes with just one additional SO_3H group (*e. g.*, KK 1115, known as Abberior STAR 635, according to ref. [9]) are amphiphilic, and their distribution strongly depends on the pH. For lipophilic properties and actual chromatographic data see **Table S4** and **Figs. S 13 – 17** above.

Exemplary NMR spectra of the fluorophores.

For full signal assignments and instrument details see compound descriptions above. The dyes of this study comprise mixtures of amide rotamers (analogously to DMF as the simplest example) with diastereotopic groups, which make NMR spectra (also fluorine ^{19}F) complicated. It is even more so for the dyes with long peptide-type linkers and bulky substituents (*i. e.*, 4-nitrobenzyl).

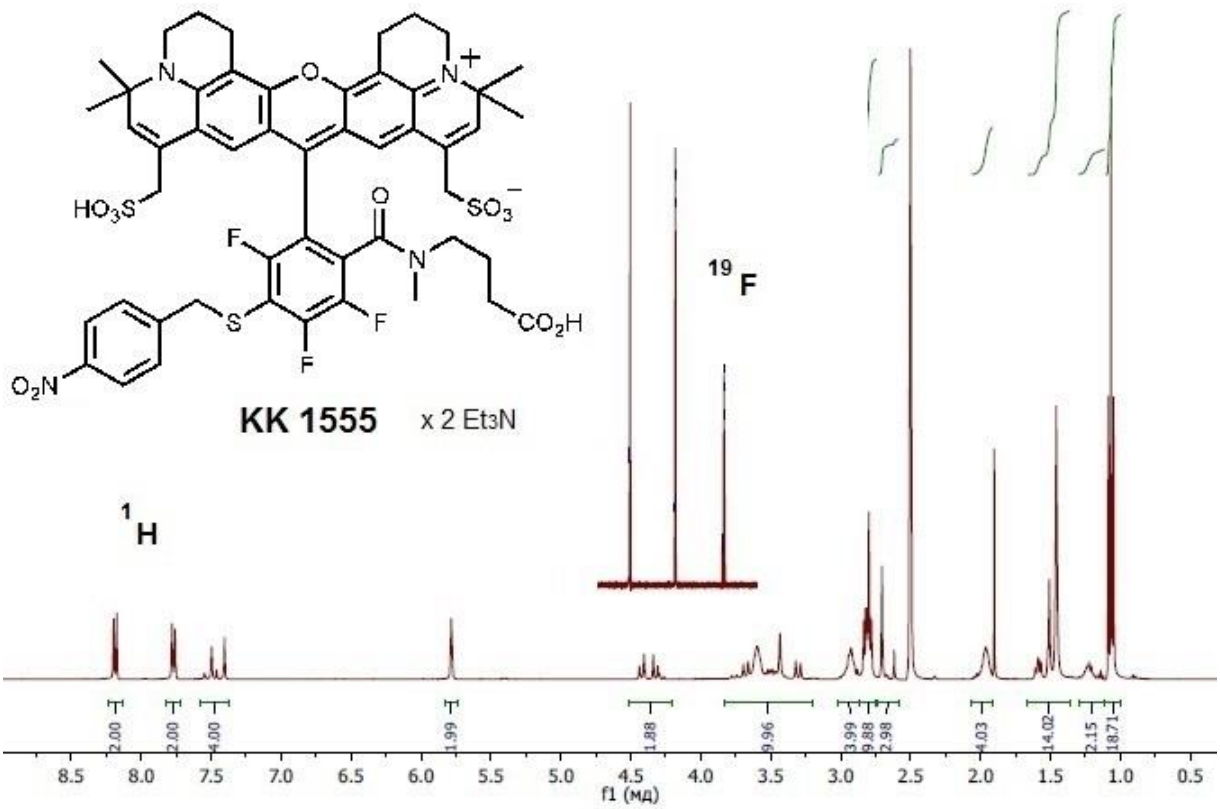


Figure S27. Proton NMR spectrum of the dye KK 1555 (DMSO-d6)

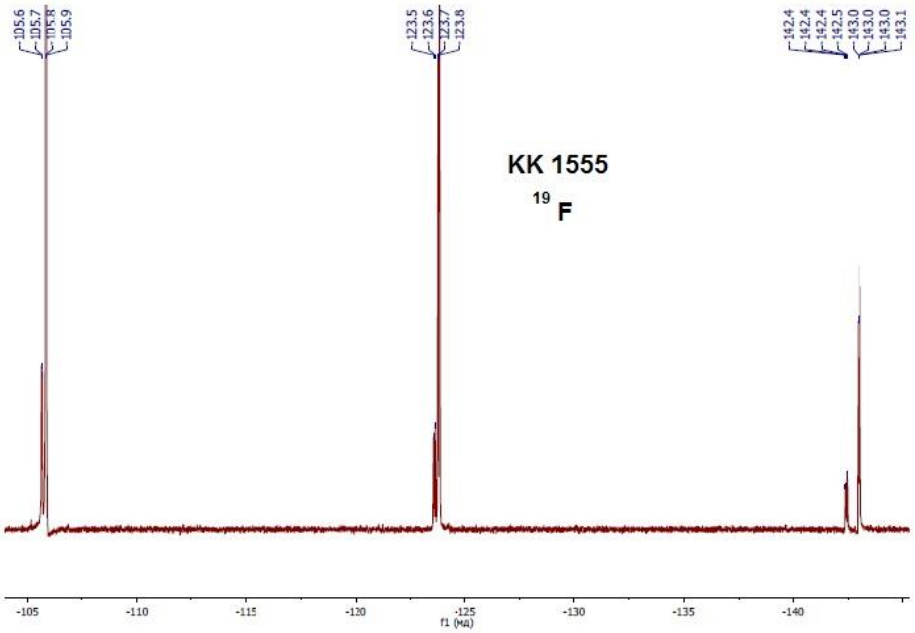


Figure S28. Fluorine NMR spectrum of the dye KK 1555 (DMSO-d6)

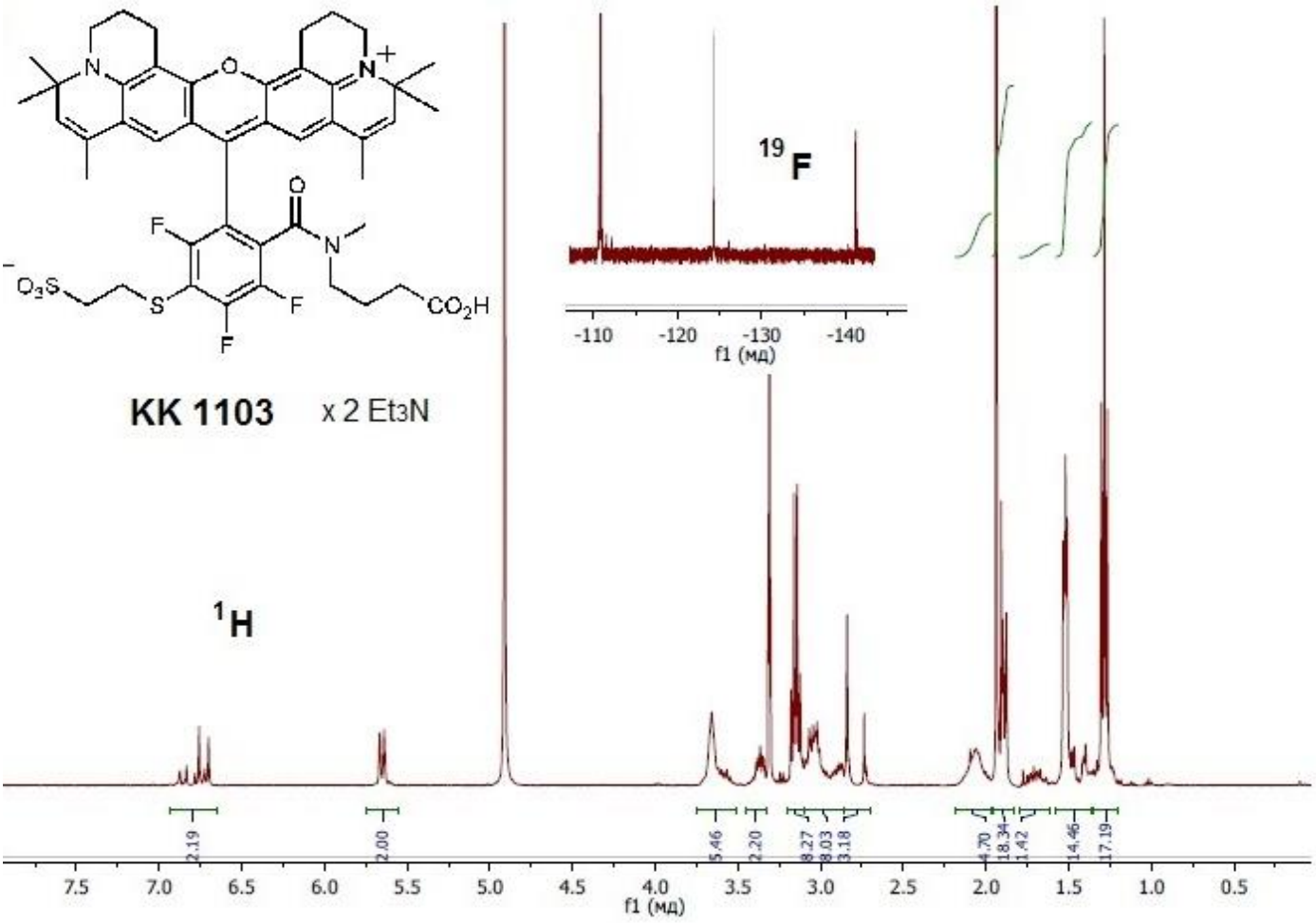


Figure S29a. Proton and fluorine NMR spectra of the dye KK 1103 (CD_3OD)

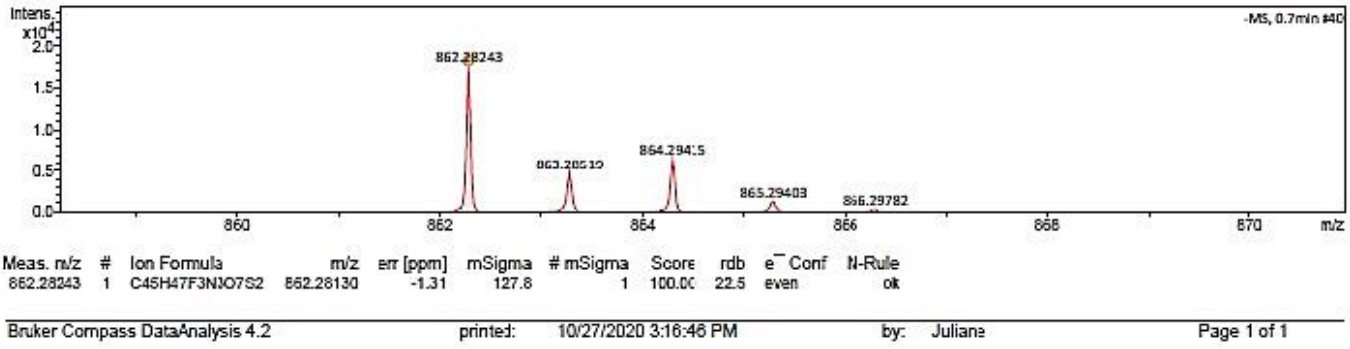


Figure S29b. Actual HRMS analysis of the dye KK 1103. See also HPLC in **Fig. 14a.**

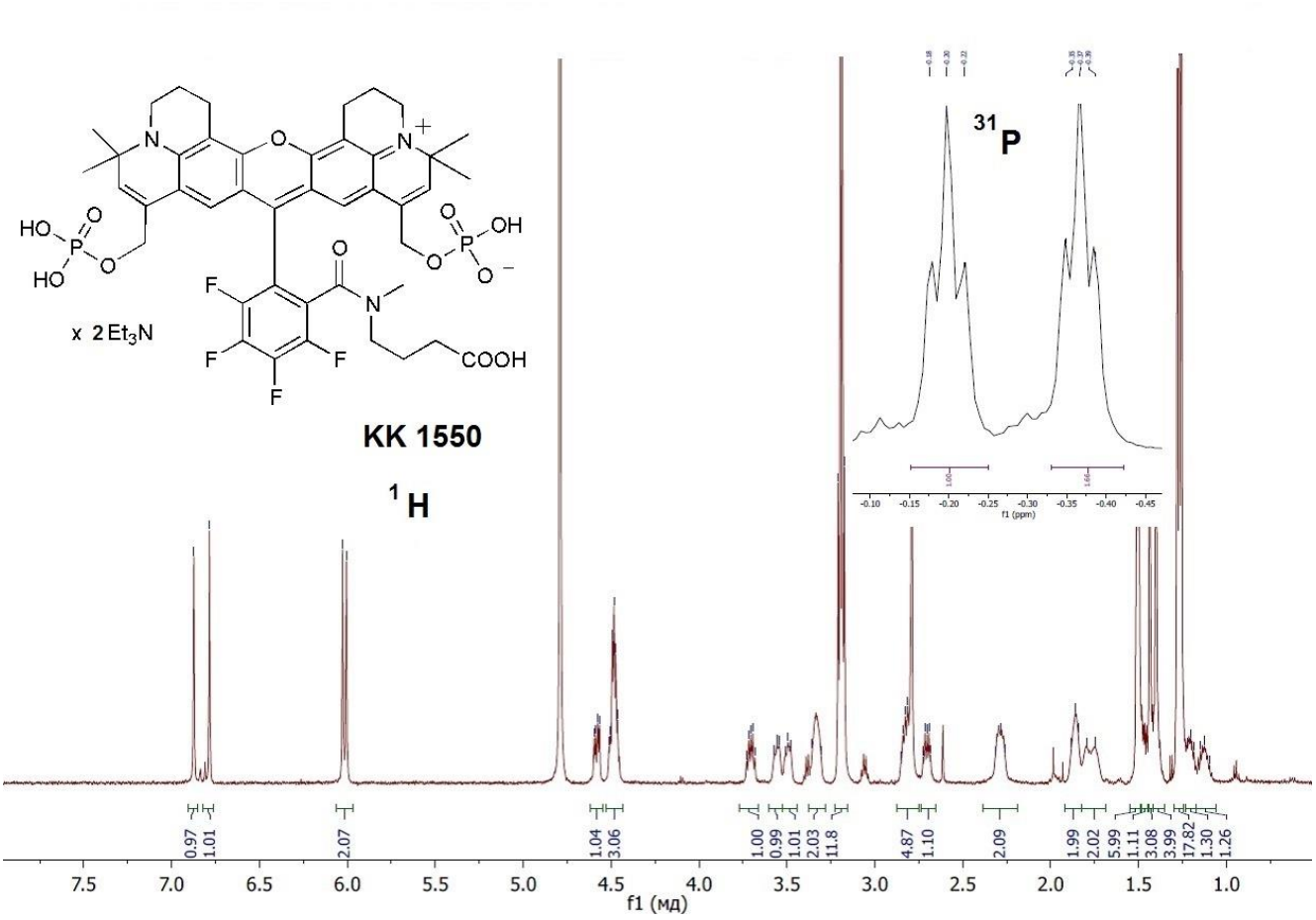


Figure S30a. Proton and phosphorus NMR spectra of the dye KK 1550 (D_2O)

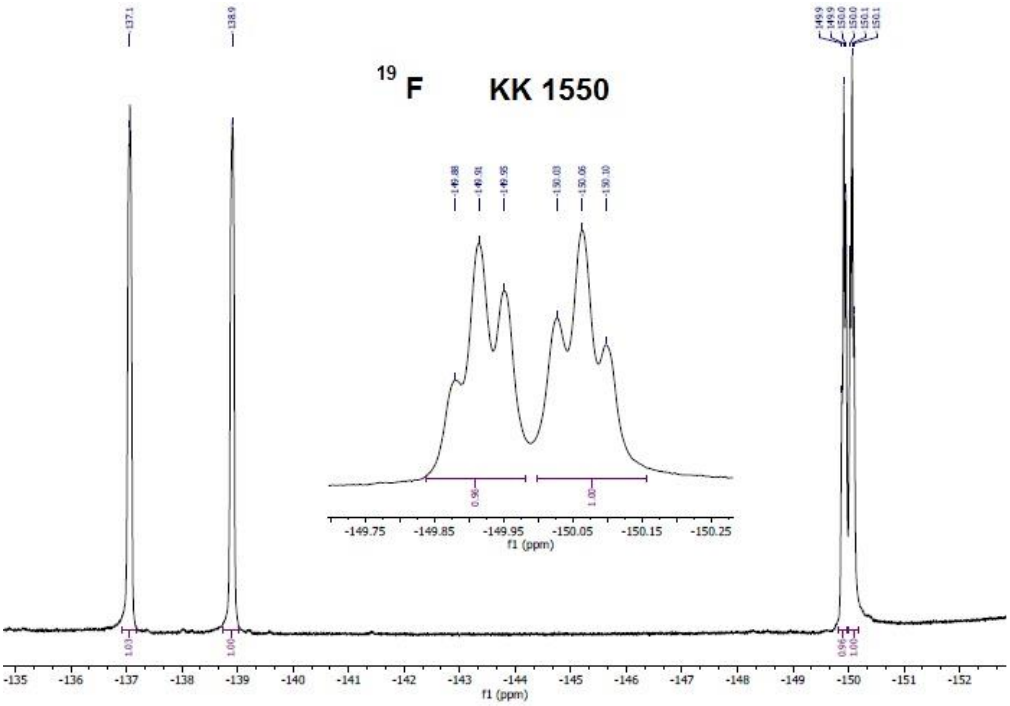


Figure S30b. Fluorine NMR spectra of the dye KK 1550 (D_2O)

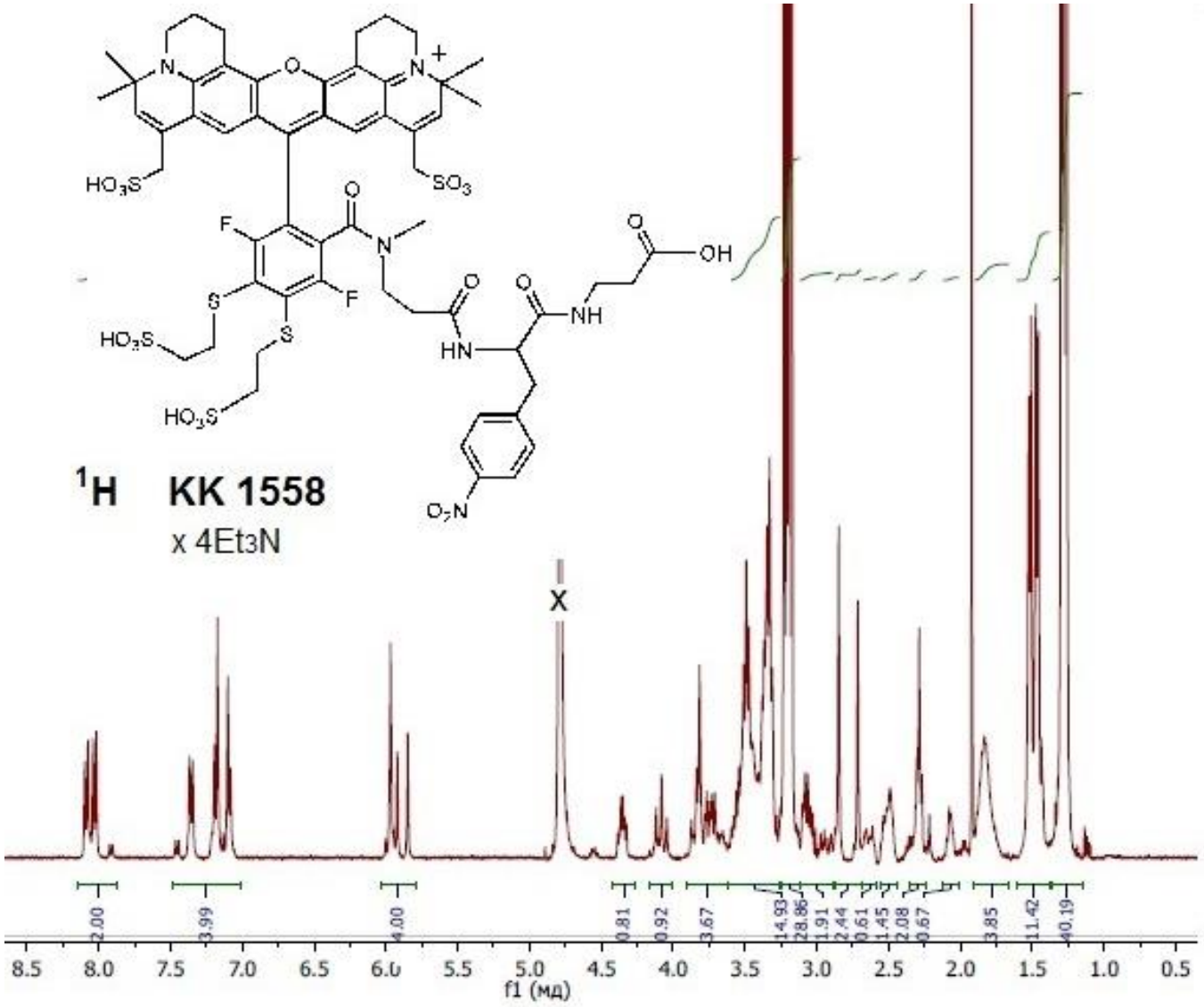


Figure S31a. Proton NMR spectrum of the dye KK 1558 (D_2O).

See HPLC data in **Figs. S 13 -- 17** and LC/MS in **Fig. 23**.

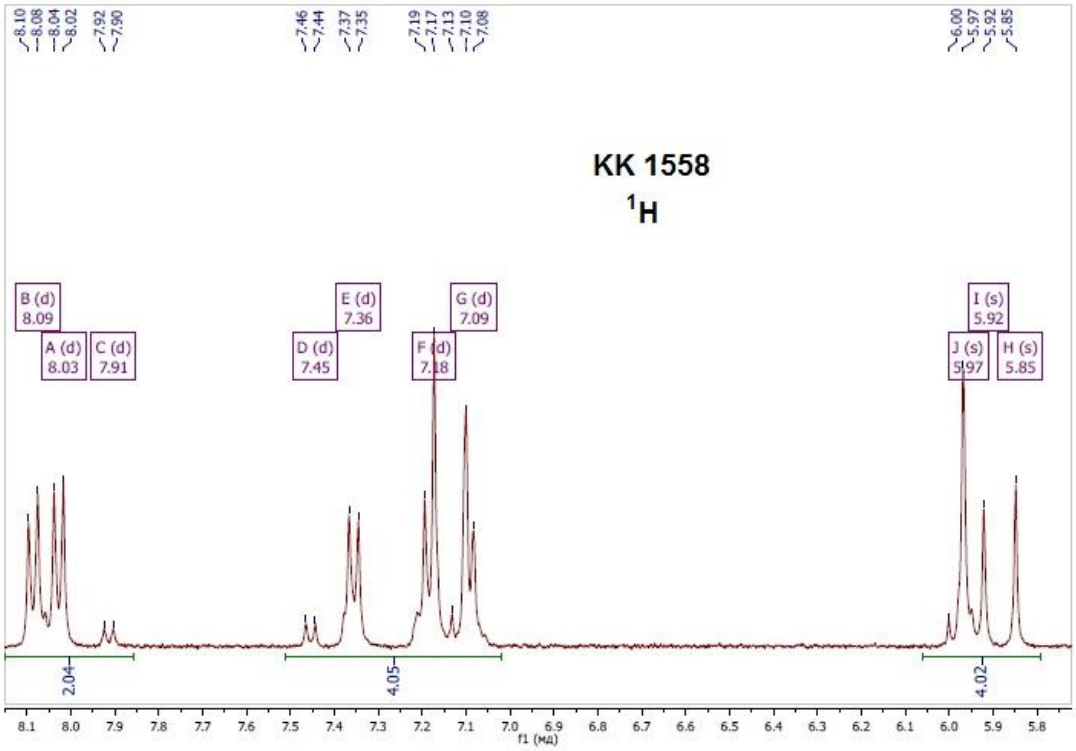


Figure S31b. Proton NMR spectrum of the dye KK 1558 (D_2O, aromatic area + CH_2PhNO_2)

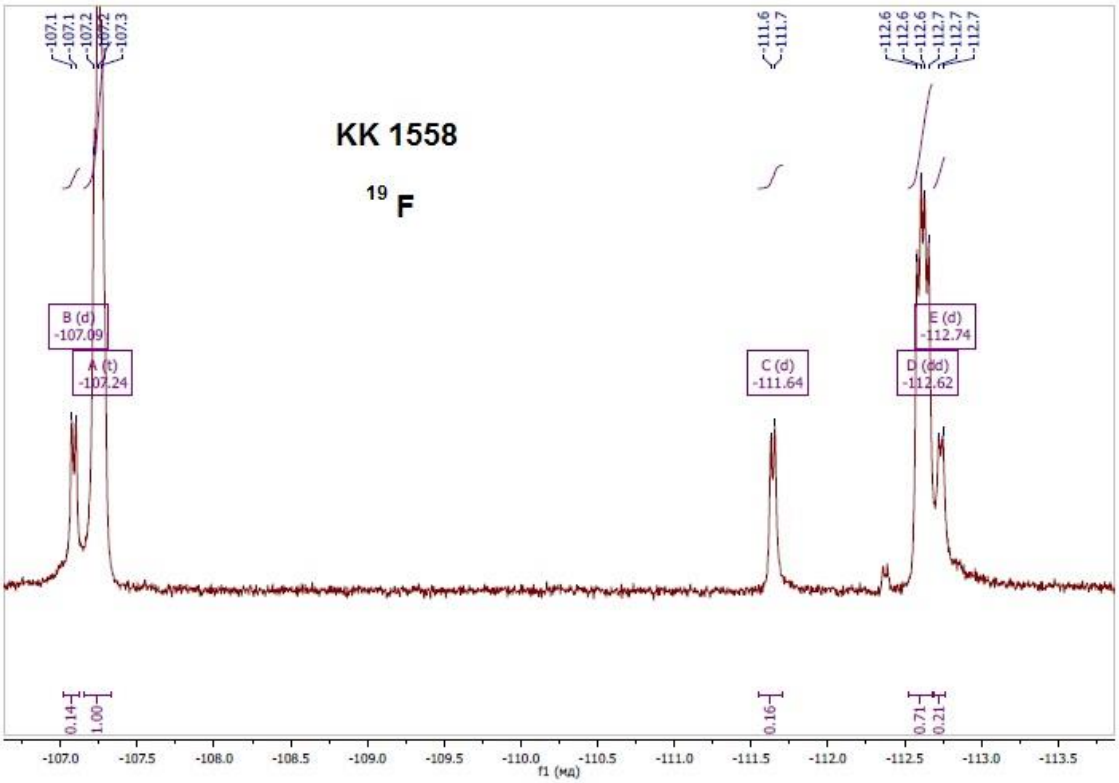


Figure S31c. Fluorine NMR spectra of the dye KK 1558 (D_2O)

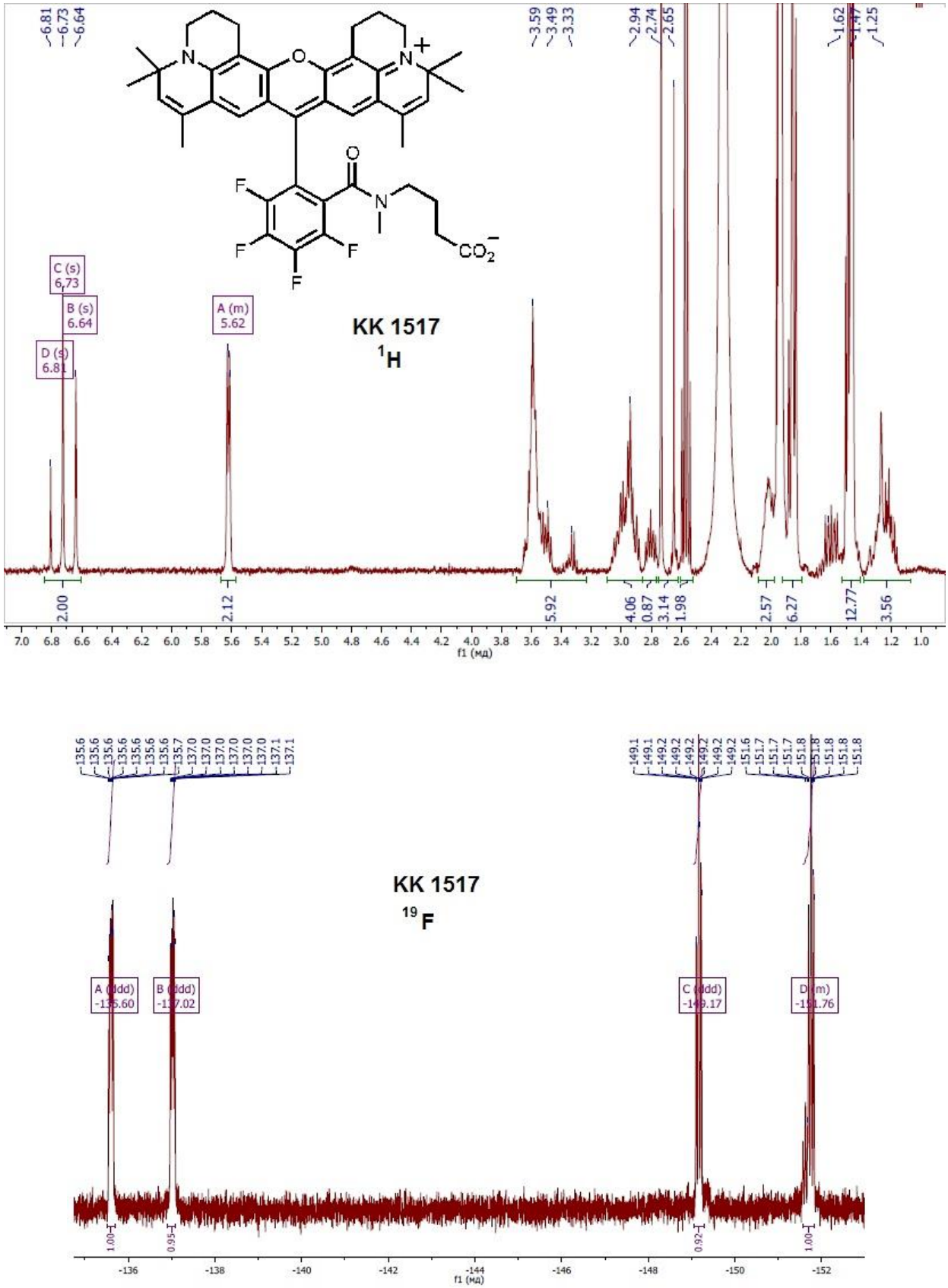


Figure S32. Proton and fluorine NMR spectra of the dye KK 1517 (CD_3CN)

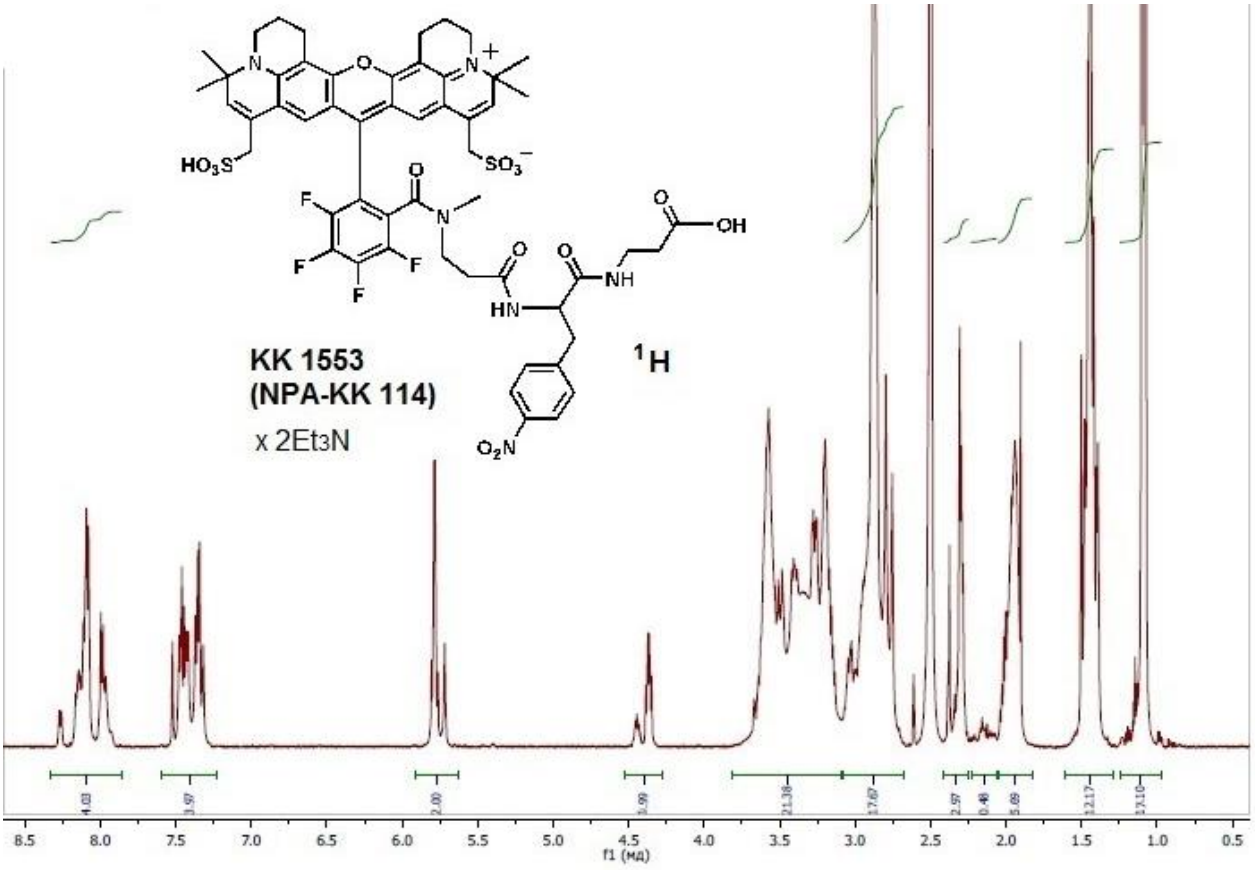


Figure S33a. Proton NMR spectrum of the dye KK 1553 (DMSO-d6)

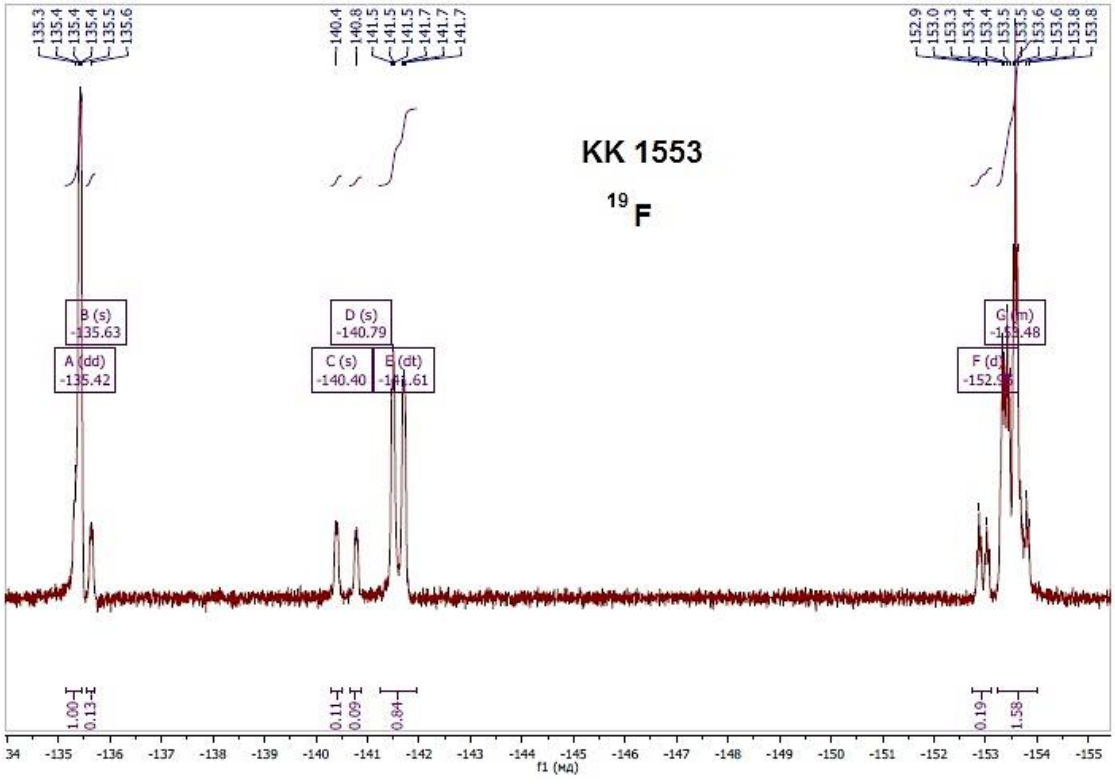
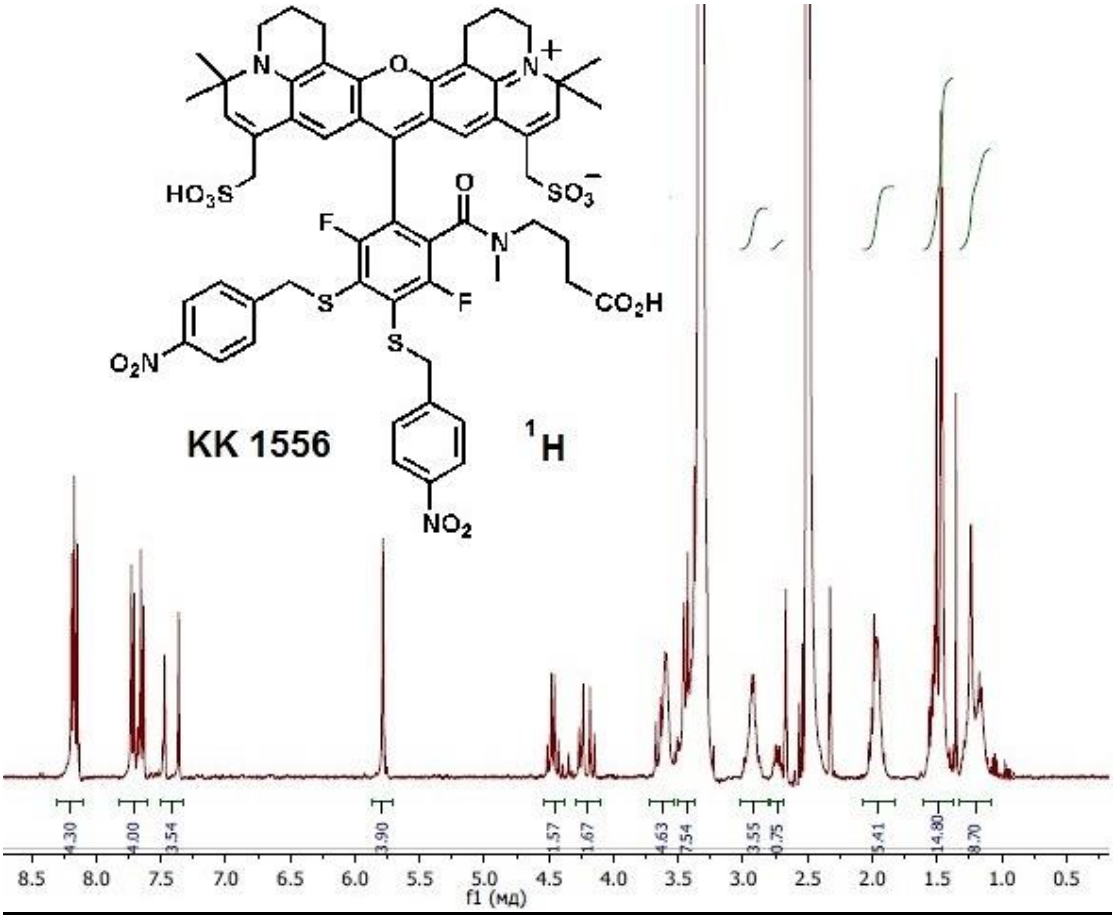


Figure S33b. Fluorine NMR spectrum of the dye KK 1553 (DMSO-d6)



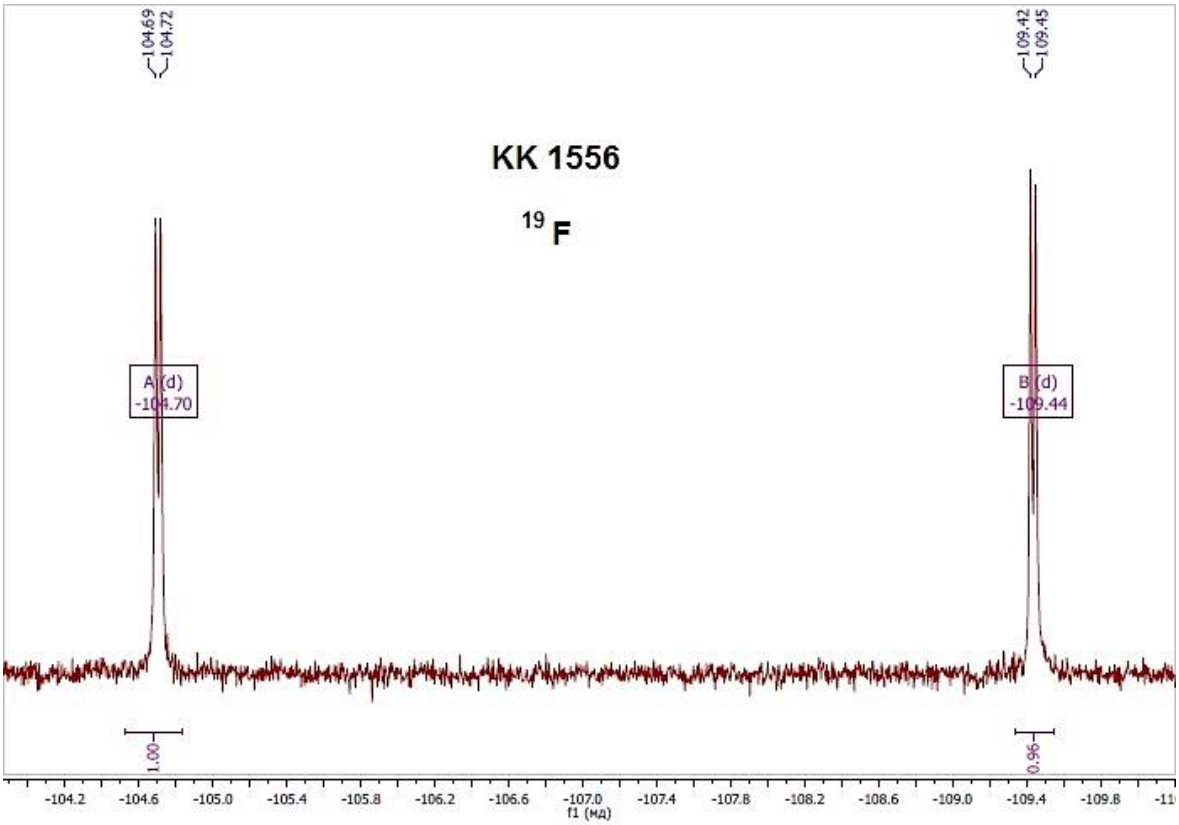


Figure S34. Proton and fluorine NMR spectra of the dye KK 1556 (DMSO-d6)

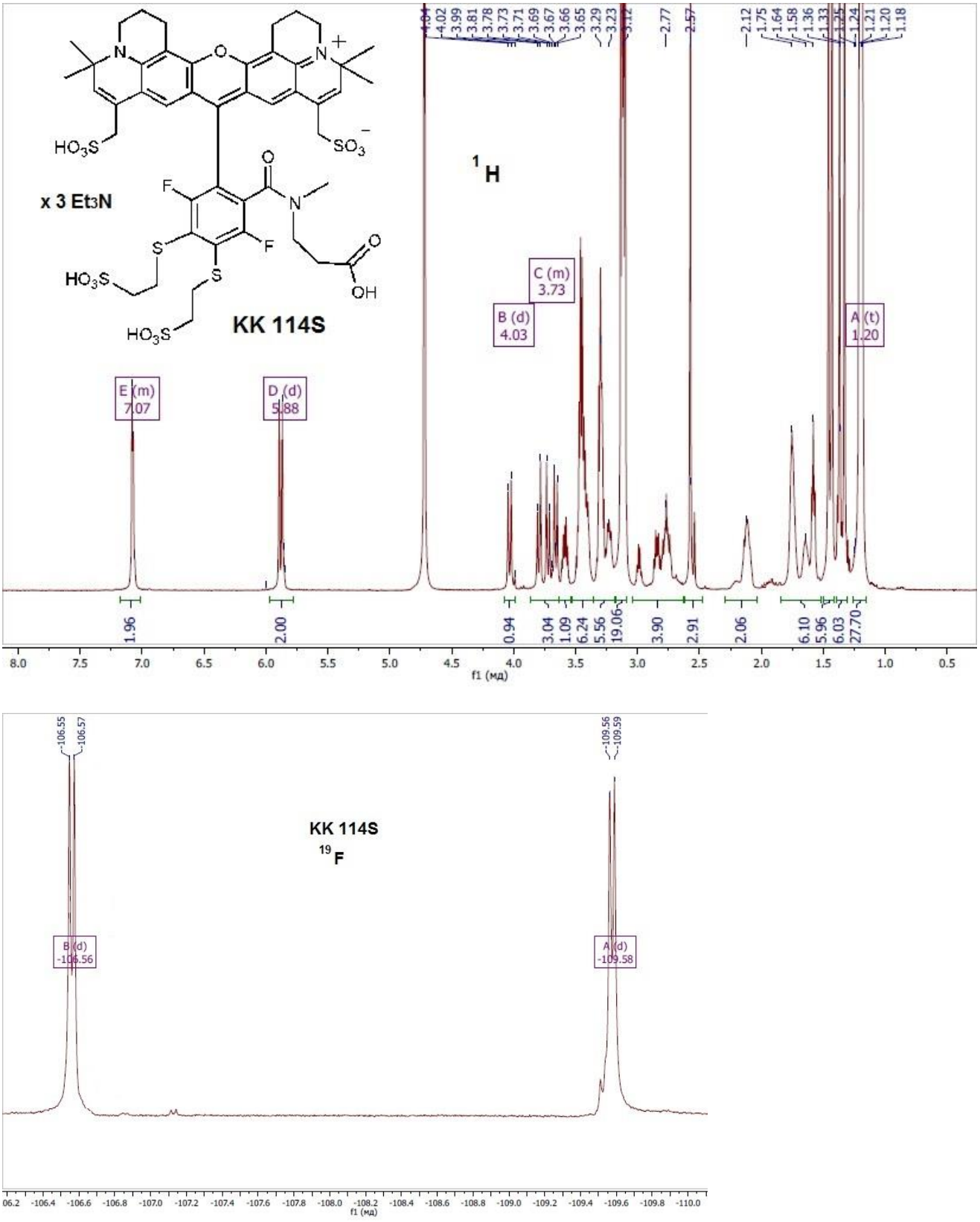


Figure S35. Proton and fluorine NMR spectra of the dye KK 114S (D_2O).

Precursor for the dye **KK 1558** and the most polar fluorophore of this study (see **Figs. S31a**,**b**, analytical HPLC data in **Fig. S17**, data on the lipophilicity and syntheses descriptions above).

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[23] see *Supporting Materials* for the publication of Z. Zhang, D. Yomo and C. Gradinaru in *Biochim. Biophys. Acta (BBA) – Biomembranes,* **2017**, *1859*, 1242–1253 and related discussion in the main text.