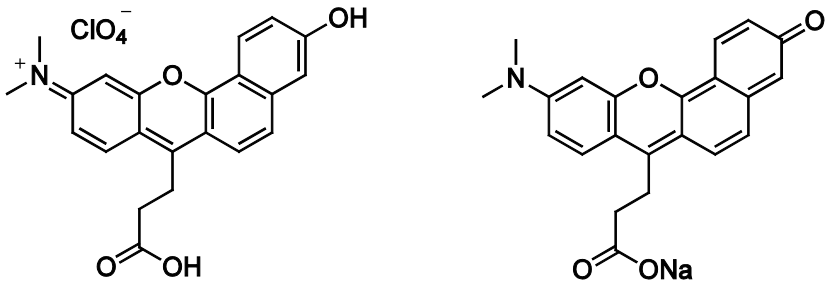
Supporting Information



$C_{22}H_{20}ClNO_8$
Mol. Wt.: 461.8491

Form for storage

$C_{22}H_{18}NNaO_4$
Mol. Wt.: 383.3724

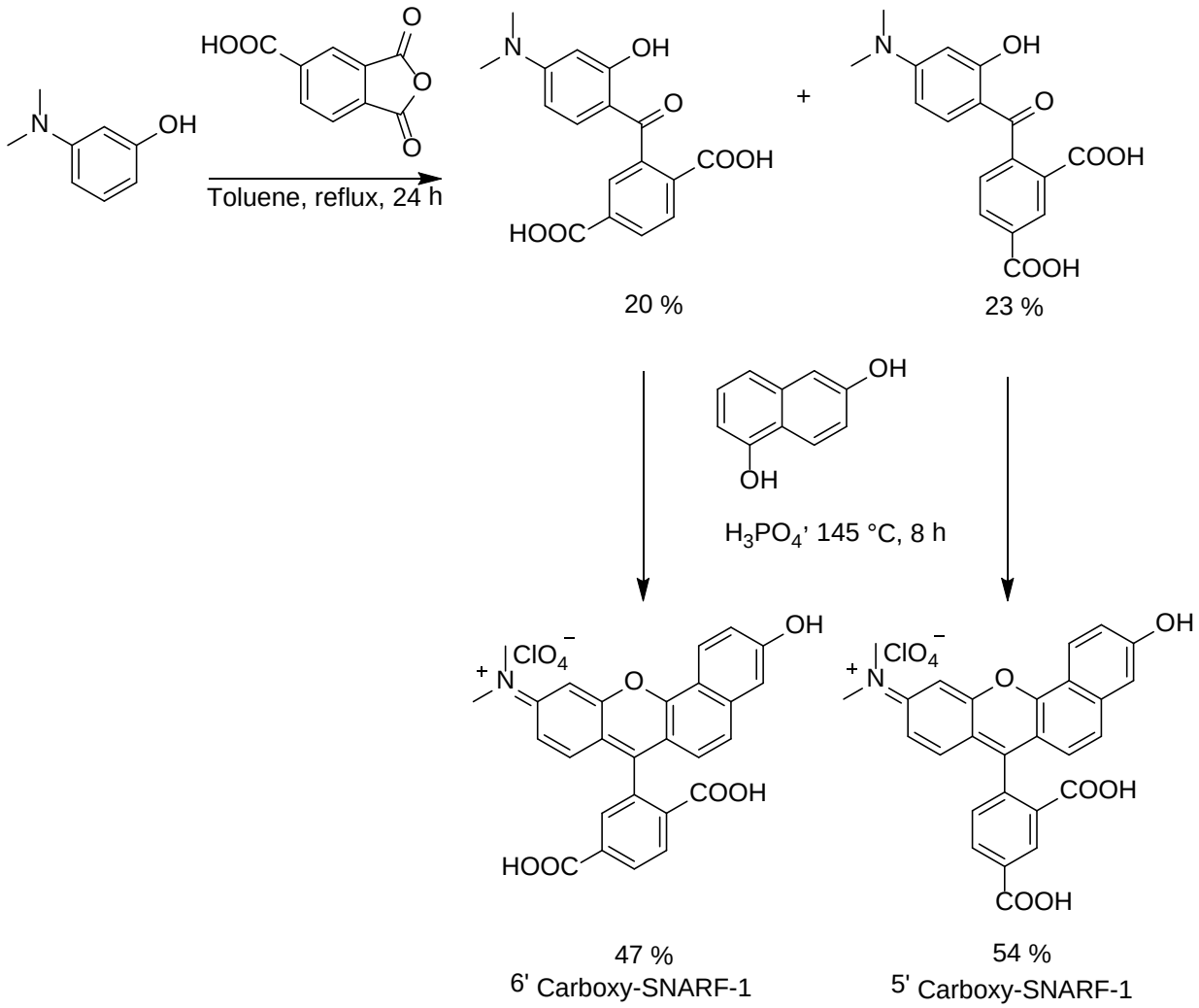
water-soluble form

Scheme S0 Formulations of PAc-SNARF (see below)

S1. Syntheses

S1.1 Synthesis of Carboxy-SNARFs

The synthesis of the Carboxy-SNARF precursor was accomplished by modifying a procedure originally due to *Kvach* and *Stepanova*.[1] Dimethylaminophenol was heated under reflux with trimellic anhydride in toluene, and the resulting mixture of the 5' and 6' isomers were separated by column chromatography in 23 % and 20 % yield respectively. The separated isomers were then stirred with 1,6-dihydroxynaphtalene in ortho-phosphoric acid at 145 °C for 6 hours, following Poronik *et al*.[2] The resulting 5' or 6' Carboxy-SNARF precipitated after treatment with a $LiClO_4$-solution. It was purified through recrystallization from ethanol and subsequently from acetic acid, yielding pure 5' (54 % yield) or 6' (47 % yield) Carboxy-SNARF (see **Scheme S1**). NMR spectra in pH neutral DMSO were difficult to evaluate due to different states of the SNARF. Addition of 2 % DCl to the NMR-solution forced the SNARF into its acidic form, allowing conclusive analysis of the spectra.



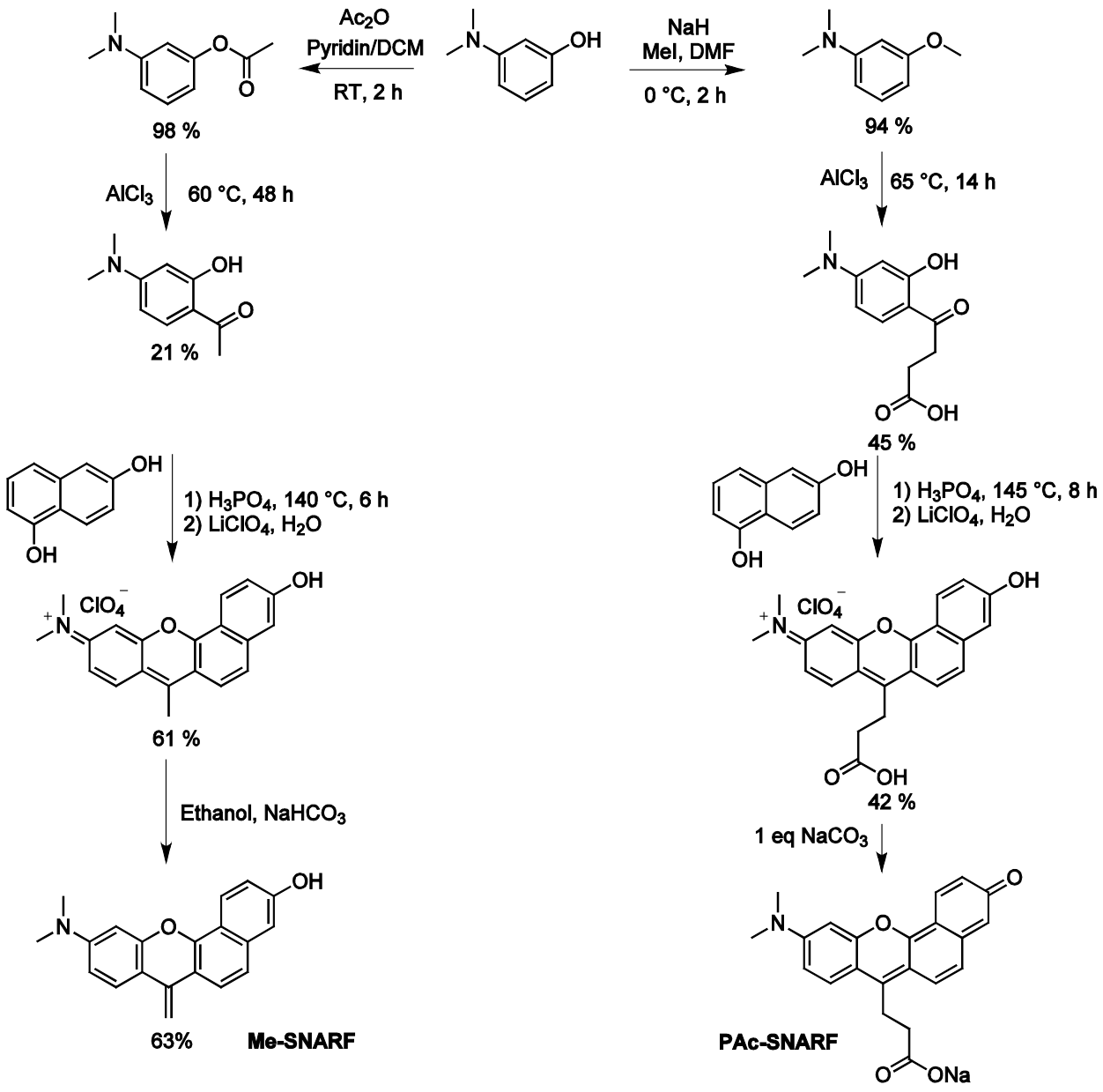
Scheme S1 Synthesis of the 5’ and 6’ separated C-SNARF

S1.2 Synthesis of Me-SNARF and PAc-SNARF

The synthesis of Me-SNARF is based on the methyl-rhodafluor-synthesis of Poronik *et al.*[2] Dimethylaminophenol was O-acylated with Acetic anhydride in DCM/pyridine in 98 % yield, followed by a solvent-free Fries reorder with $AlCl_3$ at 60 °C for 48 h with 21 % yield. Condensation of the precursor with 1,6-dihydroxynaphtalene under the above described conditions resulted in Me-SNARF with 61 % yield (see Scheme **S2**).

Synthesis of the PAc-SNARF precursor was attempted by a Friedel-Crafts-acylation of dimethylaminophenol. To prevent the formation of the ester, 3-dimethylaminophenol was O-

protected with sodium hydride and methyliodide in 98 % yield. Friedel-Crafts-acylations of anisols like 1,3-dimethoxybenzene with $AlCl_3$, $TiCl_4$ and BCl_3 are widely known.[3;4] To create similar conditions as in the Fries-reorder reaction of 3-(Dimethylamino)-phenyl acetate (see above), N,N-dimethylamino-3-methoxybenzene was adsorbed on 10 eq $AlCl_3$, mixed with succinic anhydride, and the batch was heated at 65 °C for 14 h. The reaction resulted in 4-(4-(dimethylamino)-2-hydroxyphenyl)-4-oxobutanoic acid in 45 % yield. Condensation with 1,6-dihydroxynaphtalene under the conditions described above resulted in PAc-SNARF in 42 % yield. The ^{1}H-NMR-spectra were measured in the same fashion as Carboxy-SNARF, since the different states of the SNARF again complicated the evaluation. PAc-SNARF was suspended in methanol and 1 eq of a 0.02 M Na_2CO_3-solution in methanol was added to deprotonate the fluorophore and generate the SNARF sodium salt. The resulting dark purple solution was filtrated, the filtrate dried in vacuum, resolved in water, filtrated again and dried. The residue was washed with a small amount of ethanol and dried in high vacuum.

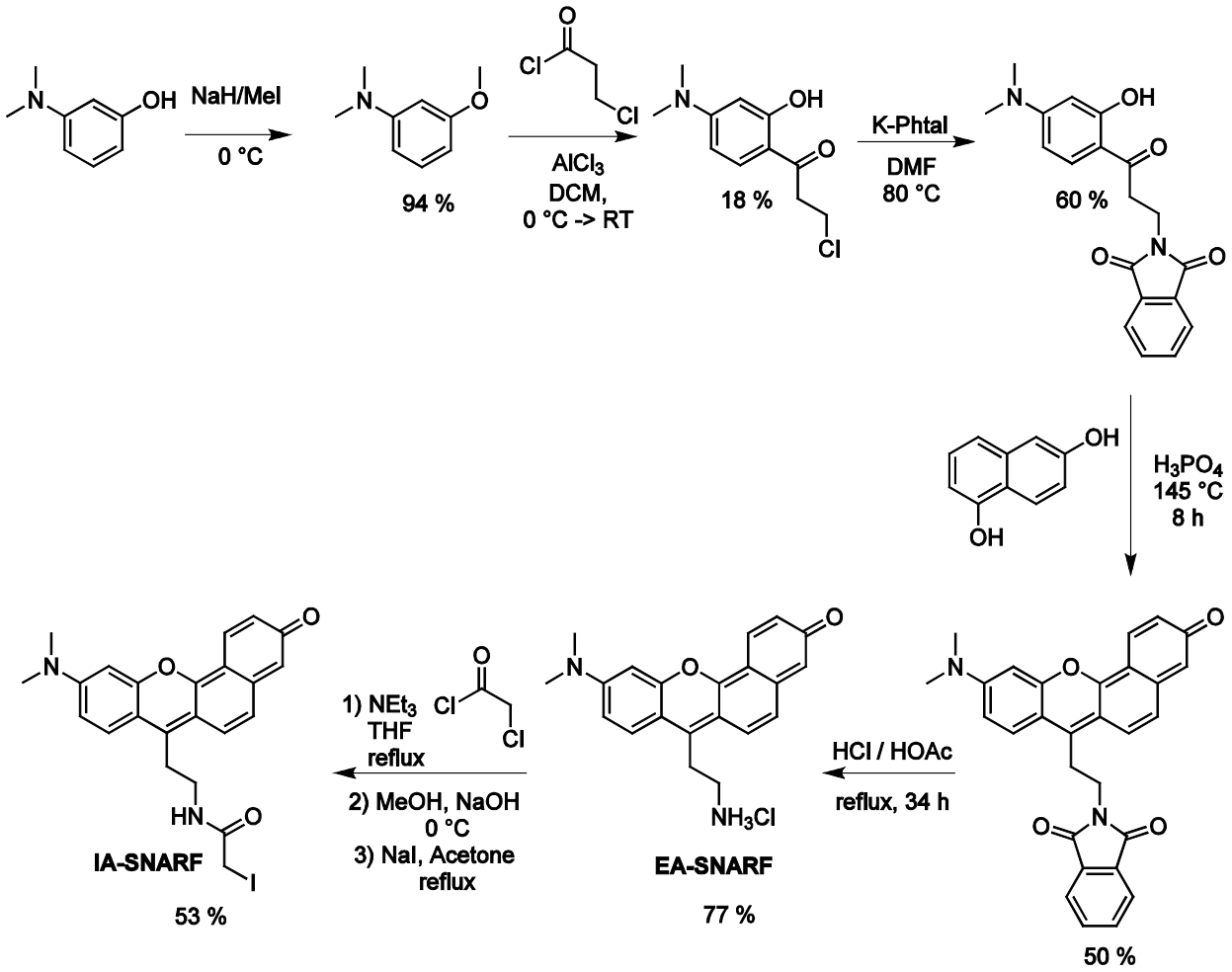


Scheme S2 Synthesis of Me-SNARF (left branch) and of PAc-SNARF (right).

S1.3 Synthesis of IA-SNARF

For investigations near a protein-pump-channel, for example, covalent linkage of the dye to a target site is needed. Cytochrom c oxidase mutants with cysteine-sites near the D-pathway have been developed by Alexiev *et al.*[5] A widely used linker for such cysteines is maleimide; connection of a maleimide linker to the SNARF-system was already described by Srikun *et al.*[6]

But their chain is quite long and correspondingly, the footprint of the tethered dye too large for spatial resolution of the proton wire topology at the protein surface. To overcome this limitation we developed IA-SNARF (see **Scheme S3**) where the fluorescent core bears an ethyl-chain with an iodacetamido-group. This kind of functionality is new to xanthene-based fluorophores. Here we describe the synthesis in detail. The synthetic strategy is shown in **Scheme S3**.

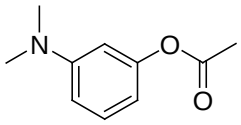


Scheme S3: Synthesis of EA-and IA-SNARF.

The optimized Friedel-Crafts-acylation of N,N-dimethylamino-3-methoxybenzene with 7 eq $AlCl_3$ and 2 eq chloropropionyl chloride afforded 3-chloro-1-(4-(dimethylamino)-2-hydroxyphenyl)propan-1-one in 18 % yield (lower or higher amounts of $AlCl_3$ and the acyl chloride reduced the yield). Gabriel synthesis in DMF at 80 °C gave the respective phtalimide-

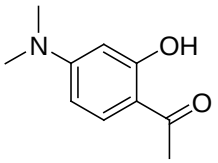
precursor. After standard condensation reaction with 1,6-Dihydroxynaphthalene at 145 °C the SNARF-Phtalimide was obtained. The protective group was cleaved by heating under reflux in a 1:1 mixture of hydrochloric acid and acetic acid for 34 h. EA-SNARF was treated with an excess of chloroacetic chloride and subsequent basic cleavage of O-acylated side product with sodium hydroxide in methanol/water. The raw product was precipitated with 2 N hydrochloric acid, purified by column chromatographie and a Finkelstein reaction was performed by heating under reflux with sodium iodide in acetone to obtain the IA-SNARF in 53 % yield (2 % overall yield after 8 steps).

3-(Dimethylamino)-phenyl acetate[7]



Pyridin (5.4 mL, 66.9 mmol) was dissolved in 35 mL of dry DCM in a 100 mL Schlenk-flask and the solution was cooled down to 0 °C. Acetic anhydride (6.40 mL, 67.7 mmol) was added slowly followed by 3-(dimethylamino)phenol (8.1 g, 59.0 mmol). After stirring for two hours while slowly warming to room temperature, the mixture was poured into 100 mL of water and extracted two times with 100 mL DCM. The extract was dried over magnesium sulfate and the solvent removed under reduced pressure. Purification by column chromatography (PE:EE 8:2) afforded 10.3 g (57.5 mmol, 98 % yield) of 3-(dimethylamino)-phenyl acetate. Spectroscopic data were identical to the literature.

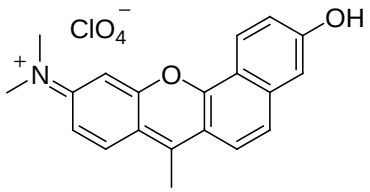
1-(4-(dimethylamino)-2-hydroxyphenyl)ethanone[2]



To 3-(Dimethylamino)-phenyl acetate (10.6 g, 59.0 mmol) was added aluminium chloride (22 g, 165.0 mmol) in a 25 mL Schlenk-flask and the mixture was heated to 60 °C for 48 hours. The resulting solid was given into 300 mL of 1 N hydrochloric acid and extracted five times with 200 mL of DCM. The extract was dried over magnesium sulfate and the solvent removed under

reduced pressure. Purification by column chromatography (PE:EE 9:1 -> 85:15) afforded 1-(4-(dimethylamino)-2-hydroxyphenyl)ethanone (2.18 g, 12.2 mmol, 21 % yield) as a yellow solid. Spectroscopic data were identical to the literature.

N-(3-hydroxy-7-methyl-10H-benzo[c]xanthen-10-ylidene)-N-dimethyliminium perchlorate

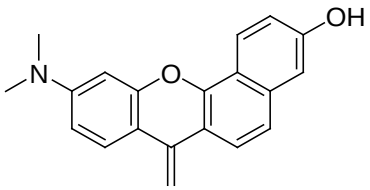


To a mixture of 1-(4-(dimethylamino)-2-hydroxyphenyl)ethanone (2.18 g, 12.2 mmol) and 1,6-dihydroxynaphtalene (2.18 g, 13.6 mmol) were added 13 mL of ortho-phosphoric acid at room temperature and heated at 140 °C for 6 h. Afterwards 50 mL of water were added, followed by addition of a $LiClO_4$-solution (2.5 g $LiClO_4$ solved in 7 mL water) while stirring. The dark red precipitate was filtered off and recrystallized from ethanol and subsequently acetic acid to afford N-(3-hydroxy-7-methyl-10H-benzo[c]xanthen-10-ylidene)-N-dimethyliminium perchlorate (3 g, 7.4 mmol, 61 % yield).

^{1}H NMR (500 MHz, DMSO) δ = 10.99 (s, 1H), 8.44 (d, *J* = 9.0 Hz, 1H), 8.08 (d, *J* = 9.7 Hz, 1H), 7.85 (d, *J* = 9.1 Hz, 1H), 7.66 (d, *J* = 9.1 Hz, 1H), 7.27 (dd, *J* = 9.0, 2.0 Hz, 1H), 7.23 – 7.19 (m, 1H), 7.20 (d, *J* = 2.2 Hz, 1H), 3.29 (s, 3H), 3.25 (s, 3H), 2.92 (s, 3H).
^{13}C NMR (125 MHz, DMSO) δ = 161.3, 159.5, 157.7, 156.3, 152.0, 139.0, 129.9, 125.8, 125.1, 122.1, 119.9, 117.1, 116.4, 115.7, 115.0, 110.7, 95.7, 40.9, 40.6, 15.4.

HRMS calcd. for $C_{20}H_{18}NO_2^+$: 304.1332 found: 304.1333

10-(dimethylamino)-7-methylene-7H-benzo[c]xanthen-3-ol



N-(3-hydroxy-7-methyl-10H-benzo[c]xanthen-10-ylidene)-N-dimethyliminium perchlorate (100 mg, 0.25 mmol) was solved in 4 mL ethanol. Saturated sodiumbicarbonate solution was added

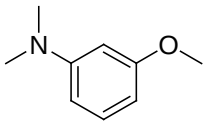
slowly. The resulting precipitate was filtered off, washed with water and dried in vacuum to afford 10-(dimethylamino)-7-methylene-7H-benzo[c]xanthen-3-ol (48 mg, 0.16 mmol, 63 % yield).

^{1}H NMR (500 MHz, DMSO) δ = 10.11 (s, 1H), 8.25 (d, *J* = 8.9 Hz, 1H), 7.78 (d, *J* = 9.0 Hz, 1H), 7.68 (d, *J* = 8.9 Hz, 1H), 7.44 (d, *J* = 8.7 Hz, 1H), 7.18 (dd, *J* = 8.9, 2.4 Hz, 1H), 7.16 (s, *J* = 1.5 Hz, 1H), 6.64 (dd, *J* = 8.9, 2.6 Hz, 1H), 6.60 (d, *J* = 2.6 Hz, 1H), 5.33 (d, *J* = 8.2 Hz, 2H), 2.98 (s, 6H).

^{13}C NMR (125 MHz, DMSO) δ = 156.6, 151.5, 150.8, 145.3, 135.6, 131.6, 124.7, 123.5, 121.8, 121.5, 118.5, 117.8, 112.8, 109.3, 109.0, 98.7, 94.9, 39.9.

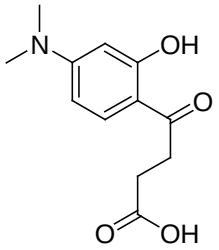
HRMS calcd. for $C_{20}H_{17}NO_2$: [$+H^+$] 304.1332 found: 304.1333

3-methoxy-N,N-dimethylbenzenamine[8]



To 32 mL DMF were added NaH (5.30 g, 131.1 mmo, 60 % dispersion in mineral oil) at 0 °C. A solution of 3-(dimethylamino)phenol (15 g, 107.2 mmol) solved in 108 mL DMF was added slowly. After stirring at 0 °C for one hour, methyliodide (7.5 mL, 120 mmol) was added while stirring. The mixture was stirred for two hours, while slowly warming to room temperature. A saturated ammonium chloride solution (500 mL) was added and extracted 4 times with 250 mL of diethylether. The extract was washed with saturated sodium chloride solution (200 mL), dried over magnesium sulfate and the solvent removed under reduced pressure. Purification by column chromatography (PE:EE 8:2) afforded 3-methoxy-N,N-dimethylbenzenamine (13.2 g, 100.5 mmol, 94 % yield) as a colorless liquid. Spectroscopic data were identical to the literature.

4-(4-(dimethylamino)-2-hydroxyphenyl)-4-oxobutanoic acid



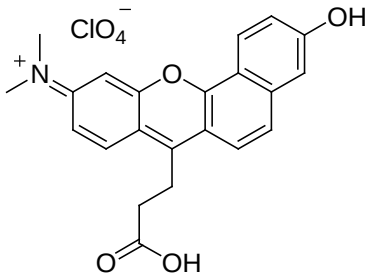
To 3-methoxy-N,N-dimethylbenzenamine (2 g, 13.2 mmol) was added succinic anhydride (1.45 g, 14.5 mmol, 1.1 eq) and aluminium chloride (5.6 g, 42 mmol) at 0 °C. The mixture was heated very carefully to 65 °C, stirred for 14 hours at 65 °C, and poured into 100 mL 1 N hydrochloric acid. The aqueous phase was extracted 5 times with 75 mL EE each, dried over magnesium sulfate and volatile compounds were removed under reduced pressure. Purification by column chromatography (PE:EE 6:4 -> 1:1) afforded 4-(4-(dimethylamino)-2-hydroxyphenyl)-4-oxobutanoic acid (1.4 g, 5.9 mmol, 45 % yield) as a white solid.

^{1}H NMR (300 MHz, DMSO) δ = 12.68 (s, 1H), 12.14 (s, 1H), 7.69 (d, J = 9.2 Hz, 1H), 6.31 (dd, J = 9.2, 2.5 Hz, 1H), 6.02 (d, J = 2.5 Hz, 1H), 3.13 (t, J = 6.4 Hz, 2H), 3.00 (s, 6 H), 2.54 (t, J = 6.4 Hz, 2H).

^{13}C NMR (75 MHz, DMSO) δ = 201.1, 173.9, 163.9, 155.7, 131.9, 109.0, 104.3, 97.0, 39.6, 31.8, 27.9.

HRMS calcd. for $C_{12}H_{15}NO_4$: [$-H^+$] 236.0928 found: $-H^+$ 236.0928

N-(7-(propionic-acid)-3-hydroxy-10H-benzo[c]xanthen-10-ylidene)-N-dimethyliminium perchlorate (PAc-SNARF)



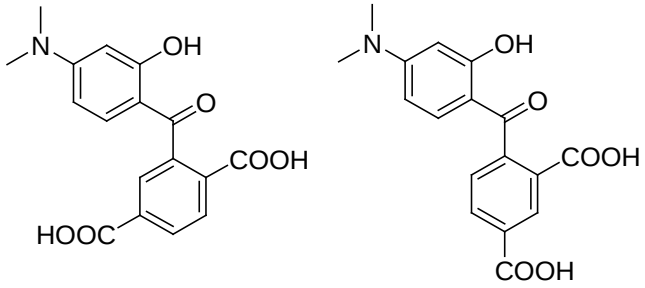
To a mixture of 4-(4-(dimethylamino)-2-hydroxyphenyl)-4-oxobutanoic acid (1.6 g, 6.74 mmol) and 1,6-dihydroxynaphtalene (1.1 g, 6.86 mmol) were added 10 mL of ortho-phosphoric acid at room temperature and heated at 145 °C for 6 h. Afterwards, 350 mL of water were added, followed by addition of a $LiClO_4$-solution (2.3 g $LiClO_4$ solved in 10 mL water) while stirring. The dark red precipitate was filtered off, dried overnight under vacuum and recrystallized from ethanol and subsequently from acetic acid to afford N-(7-(2-propionic-acid)-3-hydroxy-10H-benzo[c]xanthen-10-ylidene)-N-dimethyliminium perchlorate (1.3 g, 2.81 mmol, 42 % yield) as a red solid.

^{1}H NMR (500 MHz, DMSO) δ = 11.04 (s, 1H), 8.49 (d, J = 9.0 Hz, 1H), 8.19 (d, J = 9.8 Hz, 1H), 7.93 (d, J = 9.2 Hz, 1H), 7.74 (d, J = 9.2 Hz, 1H), 7.34 (dd, J = 9.8, 2.4 Hz, 1H), 7.29 (dd, J = 9.0, 2.4 Hz, 1H), 7.25 (d, J = 2.4 Hz, 1H), 7.06 (d, J = 2.4 Hz, 1H), 3.66 (t, J = 7.8 Hz, 2H), 3.36 (s, 3H), 3.31 (s, 3H), 2.64 (t, J = 7.8 Hz, 2H).

^{13}C NMR (125 MHz, DMSO) δ = 172.5, 161.4, 161.0, 157.8, 156.8, 152.7, 139.0, 129.8, 125.9, 125.4, 121.8, 119.9, 117.6, 116.4, 115.3, 115.1, 110.7, 96.1, 39.7, 34.4, 23.3.

HRMS calcd. for $C_{22}H_{20}NO_4^+$: 362.1387 found: 362.1387

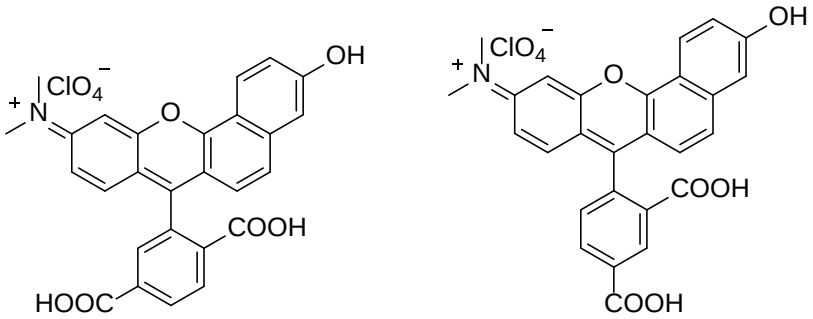
4-Dimethylamino-2-hydroxy-2',5'(4')-dicarboxy-benzophenones[1]



Freshly distilled 3-dimethylaminophenol (8.60 g, 62.7 mol) was dissolved in toluene (200 mL), heated to 60 °C, and pounded trimellitic anhydride (14.4 g, 75.2 mol, 1.2 eq) was added with magnetic stirring. The mixture was refluxed for 24 h and allowed to cool to room temperature. The residue was filtered off, washed with toluene (3×50 mL), dissolved in MeOH (300 mL), and refluxed for 10 min. Then, acetic acid (100 mL) was added and the mixture was evaporated to dryness. Purification through column chromatographie (MeOH:DCM.HCOOH 98:2:0.1)

afforded 4.1 g (12.5 mmol, 20%) of the 5'-acid and 4.7 g (14.3 mmol, 23 %) of the 4'-acid. Spectroscopical data were identical to the literature.

*10-(dimethylamino)-3-hydroxy-3'-oxo-spiro[7*H*-benzo[c]xanthene-7,1'(3'*H*)-isobenzofuran]-4'(5')-carboxylic acid (5C-SNARF and 6C-SNARF)*



To a mixture of 1.00 g (3.04 mmol) of the 6'-precursor or the 5'-precursor and 0.35 g (3.34 mmol) 1,6-dihydroxynaphtalene were added 7 mL of ortho-phosphoric acid at room temperature and heated at 145 °C for 8 h. Afterwards 40 mL of water were added followed by addition of 1.00 g $LiClO_4$ solved in 8 mL water while stirring. The dark violet precipitate was filtered off, dried overnight under vacuum and recrystallized from ethanol and subsequently from acetic acid to afford 0.74 g (1.62 mmol, 47 % yield) 6' Carboxy-SNARF-1 or 0.65 g (1.43 mmol, 54 %) 5' Carboxy-SNARF-1

5C-SNARF

^{1}H NMR (300 MHz, DMSO + 2 % DCl/D_2O) δ = 8.56 (d, *J* = 9.0 Hz, 1H), 8.33 (q, *J* = 8.2 Hz, 2H), 7.93 (s, 1H), 7.59 (d, *J* = 9.0 Hz, 1H), 7.35 (d, *J* = 9.0 Hz, 1H), 7.28 (s, 1H), 7.26 – 7.09 (m, 3H), 6.97 (d, *J* = 8.9 Hz, 1H), 3.27 (s, 6H).

^{13}C NMR (75 MHz, DMSO + 2 % DCl/D_2O) δ = 166.3, 166.2, 161.9, 158.3, 157.7, 153.0, 139.4, 134.8, 134.6, 134.1, 132.1, 131.9, 131.8, 131.1, 126.4, 126.3, 126.0, 123.3, 120.7, 118.7, 117.0, 116.1, 115.7, 111.3, 96.9, 41.5.

HRMS calcd. for $C_{27}H_{19}NO_6$: [$+H^+$] 454.1285 found: 454.1284

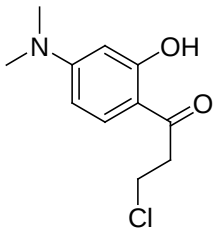
6C-SNARF:

^{1}H NMR (500 MHz, DMSO + 2 % DCl/D_2O) δ = 8.75 (d, *J* = 1.4 Hz, 1H), 8.60 (d, *J* = 9.0 Hz, 1H), 8.40 (dd, *J* = 9.0, 1.4 Hz, 1H), 7.64 (dd, *J* = 8.4, 5.0 Hz, 1H), 7.30 (m, 6H), 6.97 (d, *J* = 9.0 Hz, 1H), 3.30 (s, 6H).

^{13}C NMR (125 MHz, DMSO) δ 172.0, 165.9, 165.5, 161.5, 157.9, 157.2, 152.5, 139.0, 137.6, 133.3, 132.7, 131.6, 131.1, 130.8, 130.7, 125.8, 125.6, 122.8, 120.3, 118.3, 116.3, 115.5, 115.3, 110.9, 96.6, 41.1.

HRMS calcd. for $C_{27}H_{19}NO_6$: [+H^+] 454.1285 measured: 454.1285

3-chloro-1-(4-(dimethylamino)-2-hydroxyphenyl)propan-1-one



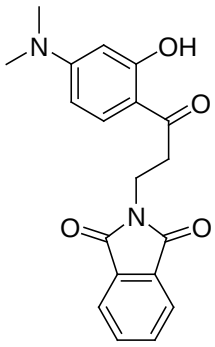
To 21,6 g (162.0 mmol, 6.1 eq) aluminiumchloride suspended in 250 mL dry DCM were added 4 g (26.5 mmol) of 3-methoxy-N,N-dimethylbenzenamine dropwise at 0 °C in a 250 mL flask. After 5 min of stirring, 6.6 g (53 mmol, 2 eq) of 3-chloropropionyl chloride were added dropwise and the mixture stirred for 1 h at 0 °C and additional 23 h at room temperature. The mixture was poured into 1.5 L of ice water, the layers were separated and the aqueous phase extracted six times with 200 mL of DCM. The organic phase was then washed three times with a saturated sodium bicarbonate solution, dried over magnesium sulfate and the volatile compounds removed under reduced pressure. The crude product was purified by column chromatographie (PE:EE 9:1 -> 8:2) to obtain 1.1 g (4.6 mmol, 18 %) of the title compound as a yellow solid.

^{1}H NMR (300 MHz, $CDCl_3$) δ = 12.68 (s, 1H), 7.52 (d, J = 9.1 Hz, 1H), 6.22 (dd, J = 9.1, 2.5 Hz, 1H), 6.09 (d, J = 2.5 Hz, 1H), 3.90 (t, J = 6.9 Hz, 2H), 3.33 (t, J = 6.9 Hz, 2H) 3.05 (s, 6H).

^{13}C NMR (75 MHz, CDCl3) δ = 198.60, 165.10, 156.16, 131.60, 109.81, 104.28, 97.91, 40.10, 39.89, 39.37.

HRMS calcd. for $C_{11}H_{14}ClNO_2$: [+H^+] 228.0786 found: [+H^+] 228.0787

2-(3-(4-(dimethylamino)-2-hydroxyphenyl)-3-oxopropyl)isoindoline-1,3-dione



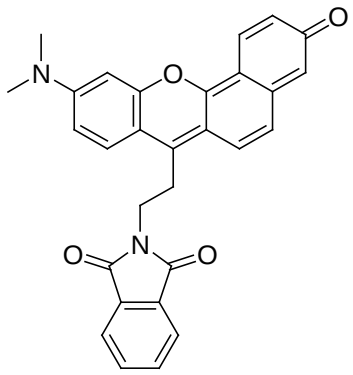
To 100 mg (0.44 mmol) of 3-chloro-1-(4-(dimethylamino)-2-hydroxyphenyl)propan-1-one solved in 5 mL DMF were added 122 mg (0.66 mmol, 1.5 eq) of potassium phtalimide. The mixture was heated over a period of 1 h to 80 °C and then cooled down to room temperature. 25 mL of a saturated ammonium chloride solution and 25 mL EE were added, the layers separated and the aqueous phase extracted three times with 25 mL EE each. The combined organic phases were washed three times with a saturated sodium chloride solution, dried over magnesium sulfate and the solvent removed under reduced pressure. The crude product was recrystallized from methanol to obtain 88 mg (0.26 mmol, 60 %) of the title compound as a pale yellow solid.

^{1}H NMR (300 MHz, DMSO) δ = 12.66 (s, 1H), 7.88 – 7.80 (m, 4H), 7.61 (d, J = 9.2 Hz, 1H), 6.27 (dd, J = 9.2, 2.5 Hz, 1H), 6.00 (d, J = 2.5 Hz, 1H), 3.90 (t, J = 7.2 Hz, 2H), 3.24 (t, J = 7.2 Hz, 2H), 2.99 (s, 6H).

^{13}C NMR (75 MHz, DMSO) δ = 200.5, 168.1, 164.6, 156.2, 134.8, 132.4, 132.1, 123.5, 109.4, 104.8, 97.4, 40.0, 35.7, 34.2.

HRMS calcd. for $C_{19}H_{18}N_2O_4$: [$+H^+$] 339.1339 found: [$+H^+$] 339.1339

10-(dimethylamino)-7-(2-(isoindoline-1,3-dione)ethyl)-3-hydroxy-7H-benzo[c]xanthene

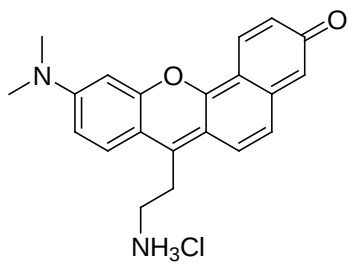


This product was synthesized according to the synthesis of PAc-SNARF (145 °C, 8 h). The recrystallized product was purified by column chromatographie (DCM:MeOH 95:5) to obtain the title compound in 50 % yield as a violet solid.

^{1}H NMR (300 MHz, DMSO/2 % DCl) δ = 8.46 (d, *J* = 9.0 Hz, 1H), 7.96 (d, *J* = 9.8 Hz, 1H), 7.85 (d, *J* = 9.0 Hz, 1H), 7.78 – 7.65 (m, 4H), 7.60 (d, *J* = 9.0 Hz, 1H), 7.29 (dd, *J* = 9.0, 1.9 Hz, 1H), 7.21 (d, *J* = 1.9 Hz, 1H), 7.19 (dd, J = 9.1, 2.0, 1H), 7.06 (s, 1H), 3.84 (d, *J* = 5.4 Hz, 2H), 3.72 (d, *J* = 5.4 Hz, 2H), 3.23 (s, 6H).
^{13}C NMR (75 MHz, DMSO/2 % DCl) δ = 175.8, 168.0, 161.9, 161.6, 159.7, 158.1, 157.3, 156.9, 156.8, 152.9, 152.4, 144.8, 139.4, 135.0, 131.5, 123.5, 123.0, 115.7, 115.5, 111.2, 56.5, 55.3, 39.9.

HRMS calcd. for $C_{29}H_{22}N_2O_4$: [$+H^+$] 463.1652 found: [$+H^+$] 463.1650

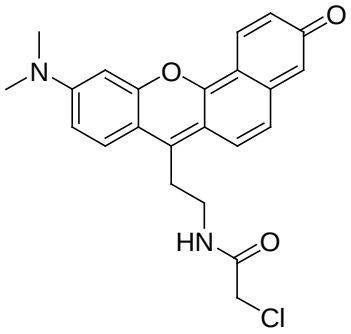
10-(dimethylamino)-7-(2-(amino)ethyl)-3-oxo-3H-benzo[c]xanthene hydrochlorid (EA-SNARF)



To 50 mg (0.11 mmol) of 10-(dimethylamino)-7-(2-(isoindoline-1,3-dione)ethyl)-3-hydroxy-7H-benzo[c]xanthene were added 2 mL of a 1:1 HCl/AcOH solution dropwise and the solution was heated under reflux for 34 hours. After evaporation of volatile compounds the residue was suspended in water, filtrated and washed with water. The water was removed under reduced pressure and the resulting solid washed with diethylether and little amounts of ethanol to obtain the title compound in 77 % yield as a violet solid.

^{1}H NMR (500 MHz, D_2O) δ = 7.42 (d, *J* = 9.1 Hz, 1H), 7.16 (d, *J* = 7.8 Hz, 1H), 7.10 (d, *J* = 8.5 Hz, 1H), 6.89 (d, *J* = 9.1 Hz, 1H), 6.87 (d, *J* = 8.5 Hz, 1H), 6.37 (d, *J* = 7.8 Hz, 1H), 6.15 (s, 1H), 5.61 (s, 1H), 3.17 – 3.00 (m, 4H), 2.99 (s, 6H)
^{13}C NMR (125 MHz, D_2O) δ = 159.1, 157.6, 154.7, 151.7, 150.3, 137.4, 127.8, 125.1, 124.6, 120.1, 119.3, 118.0, 116.3, 114.3, 113.5, 109.5, 95.7, 40.8, 40.4, 38.4, 25.4.
HRMS calcd. for $C_{21}H_{21}N_2O_2^+$: 333.1598 found: 333.1597

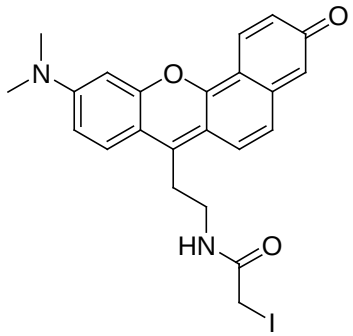
N-(2-(10-(dimethylamino)-3-oxo-3H-benzo[c]xanthen-7-yl)ethyl)-2-chloroacetamide



To 100 mg (0.27 mmol) of EA-SNARF dissolved in 5 mL dry THF were added 110 µL (0.81 mmol, 3 eq) triethylamine at 0 °C for 5 minutes. To this solution were added 215 µL(2.71 mmol, 10 eq) of chloroacetic chloride and the solution heated under reflux for 2 hours. After cooling 5 mL of methanol and 216 mg (5.4 mmol, 20 eq) sodium hydroxide, solved in 3 mL water, were added drop wise at 0 °C and the solution stirred for 2 hours. Volatile organic compounds were removed under reduced pressure and the resulting aqueous solution acidified to pH 3 with 2 N hydrochloric acid. The precipitate was filtered off, washed with water and dried under reduced pressure. The crude product was purified through column chromatographie (DCM:MeOH 95:5 -> 9:1) to obtain 70 mg (0.17 mmol, 64 %) of the title compound as a violet powder.

^{1}H NMR (300 MHz, DMSO + 2 % DCl/D_2O) δ = 8.72 (d, *J* = 9.8 Hz, 1H), 8.28 (d, *J* = 9.8 Hz, 1H), 8.13 (d, *J* = 9.2 Hz, 1H), 7.87 (d, *J* = 9.1 Hz, 1H), 7.51 (dd, *J* = 9.8, 2.5 Hz, 1H), 7.45 (dd, *J* = 6.4, 2.3 Hz, 1H), 7.43 (s, 1H), 7.37 (d, *J* = 2.5 Hz, 1H), 3.92 (s, 2H), 3.52 (s, 2H), 3.41 (s, 6H), 3.15 (s, 2H).
^{13}C NMR (75 MHz, DMSO + 2 % DCl/D_2O) δ = 166.5, 161.7, 160.1, 158.2, 157.2, 139.4, 130.2, 126.2, 125.6, 122.4, 122.2, 120.3, 117.9, 117.7, 116.0, 111.0, 96.5, 55.0, 48.8, 42.5.
HRMS calcd. for $C_{23}H_{21}ClN_2O_3$: [+H^+] 409.1313 found: [+H^+] 409.1311

N-(2-(10-(dimethylamino)-3-oxo-3H-benzo[c]xanthen-7-yl)ethyl)-2-iodoacetamide (IA-SNARF)



To 15.0 mg (0.037 mmol) of N-(2-(10-(dimethylamino)-3-oxo-3H-benzo[c]xanthen-7-yl)ethyl)-2-chloroacetamide solved in 5 mL acetone were added 56.0 mg (0.37 mmol, 10 eq) sodium iodide and the suspension was heated under reflux for 24 h. The solvent was removed under reduced pressure and the crude product was purified through column chromatographie (DCM:MeOH 95:5) to obtain 15.1 mg (0.030 mmol, 82 %) of the title compound as a violet powder.

^{1}H NMR (500 MHz, DMSO+ 2 % DCl) δ = 8.71 (d, *J* = 9.8 Hz, 1H), 8.26 (d, *J* = 9.8 Hz, 1H), 8.12 (d, *J* = 9.2 Hz, 1H), 7.87 (d, *J* = 9.1 Hz, 1H), 7.50 (dd, *J* = 9.7, 2.4 Hz, 1H), 7.42 (dd, *J* = 8.3, 1.9 Hz, 1H), 7.41 (s, 1H), 7.35 (d, *J* = 2.5 Hz, 1H), 3.91 (s, 2H), 3.71 (t, *J* = 6.3 Hz, 2H), 3.51 (d, *J* = 6.3 Hz, 2H), 3.40 (s, 6H).
^{13}C NMR (125 MHz, DMSO + 2 % DCl) δ = 166.6, 166.5, 161.7, 158.3, 157.3, 153.1, 139.4, 130.1, 126.2, 125.6, 122.3, 120.3, 117.9, 117.7, 116.1, 115.7, 111.0, 96.5, 55.0, 48.7, 42.5, 28.5.
HRMS calcd. for $C_{23}H_{21}IN_2O_3$: [+H^+] 501.0670 found: [+H^+] 501.0671

S2. About the lactonic form of SNARF

The NMR spectrum of PAc-SNARF in neutral DMSO can largely be assigned to the fluorescent form having the open ethyl carboxy configuration. However a second state is also observed, amounting to 14 % (see figure). The similar case of Rhodamine 101 was already investigated by Karpiuk *et al.* and Kaneko *et al*.[9-11] where the formation of the lactonic form was observed in basic environments. The corresponding signal disappeared almost completely after addition of 2 % DCl. Let us now treat the solution of PAc-SNARF in DMSO solution in a similar fashion. When treated with basic ion-exchange resin (IRA-410), a colorless solution is obtained. The corresponding product must therefore be the lactonic form. Its ^{1}H-NMR signals are identical to the second component in neutral DMSO. After addition of 2 % DCl, the DMSO-solution turned violet and the ^{1}H-NMR became identical to that of PAc-SNARF in DMSO/2 % DCl. The investigation of 6'-carboxy-SNARF as a reference showed similar results in DMSO and DMSO/2 % DCl, having 25 % lactonic form in pure DMSO (i.e. without acid).

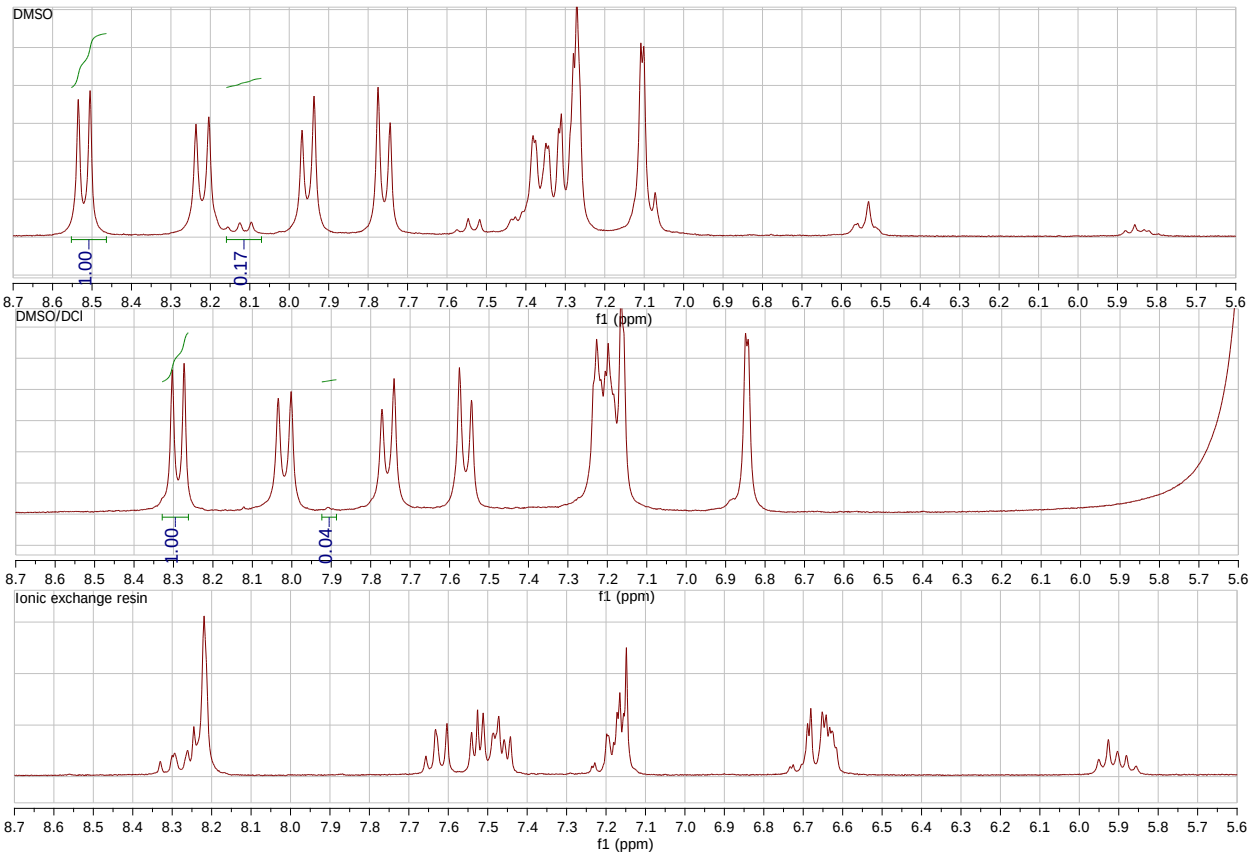


Figure S1. ^{1}H-NMR of PAc-SNARF in DMSO (*top*) , DMSO/2 % DCl (*middle*), and in DMSO after treatment with basic ion exchange resin (*bottom*). Changes in chemical shifts are due to the different pH-values.

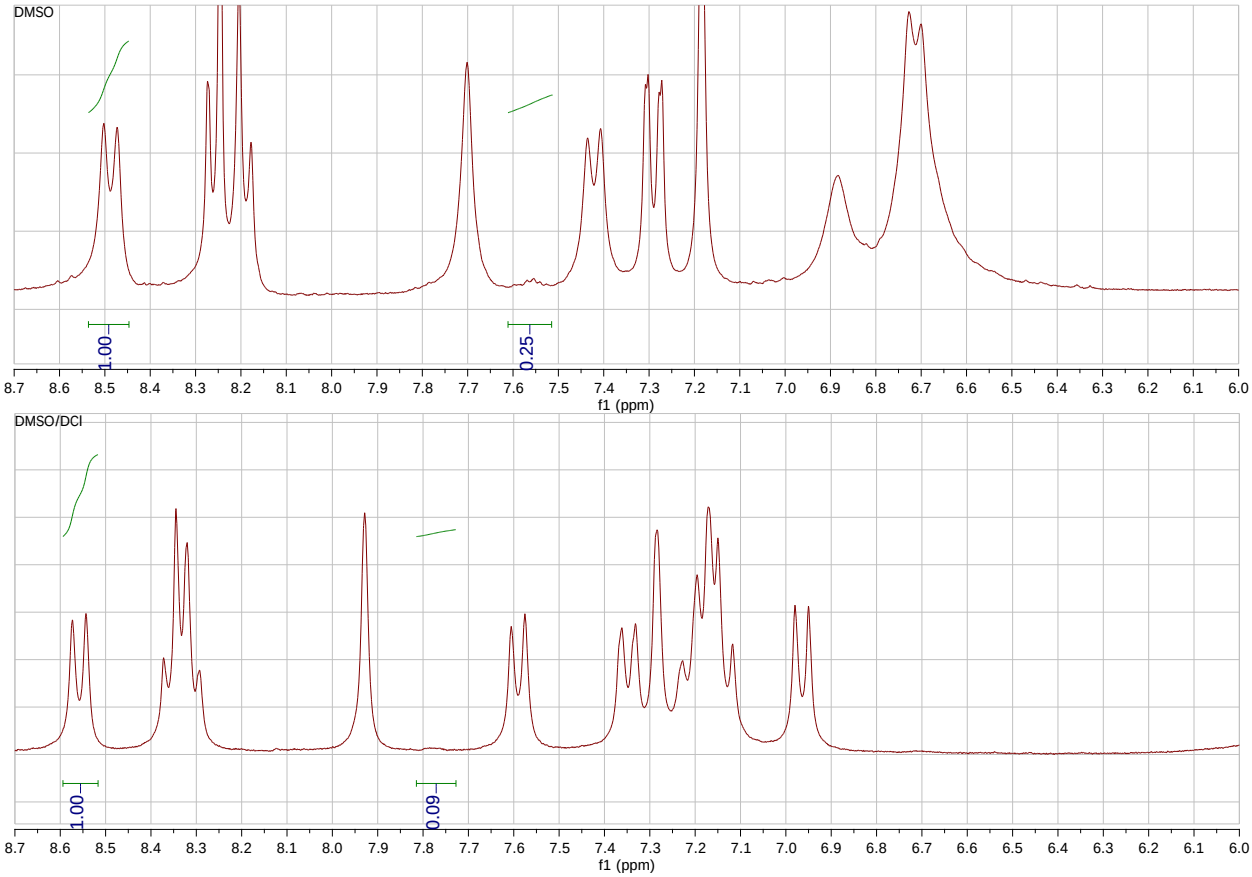


Figure S2. ^{1}H-NMR of 6'-carboxy-SNARF in DMSO (*top*) and in DMSO/2 % DCl (*bottom*).

S3. Absorption and fluorescence spectra of 5´-C and 6´C-SNARF, etc

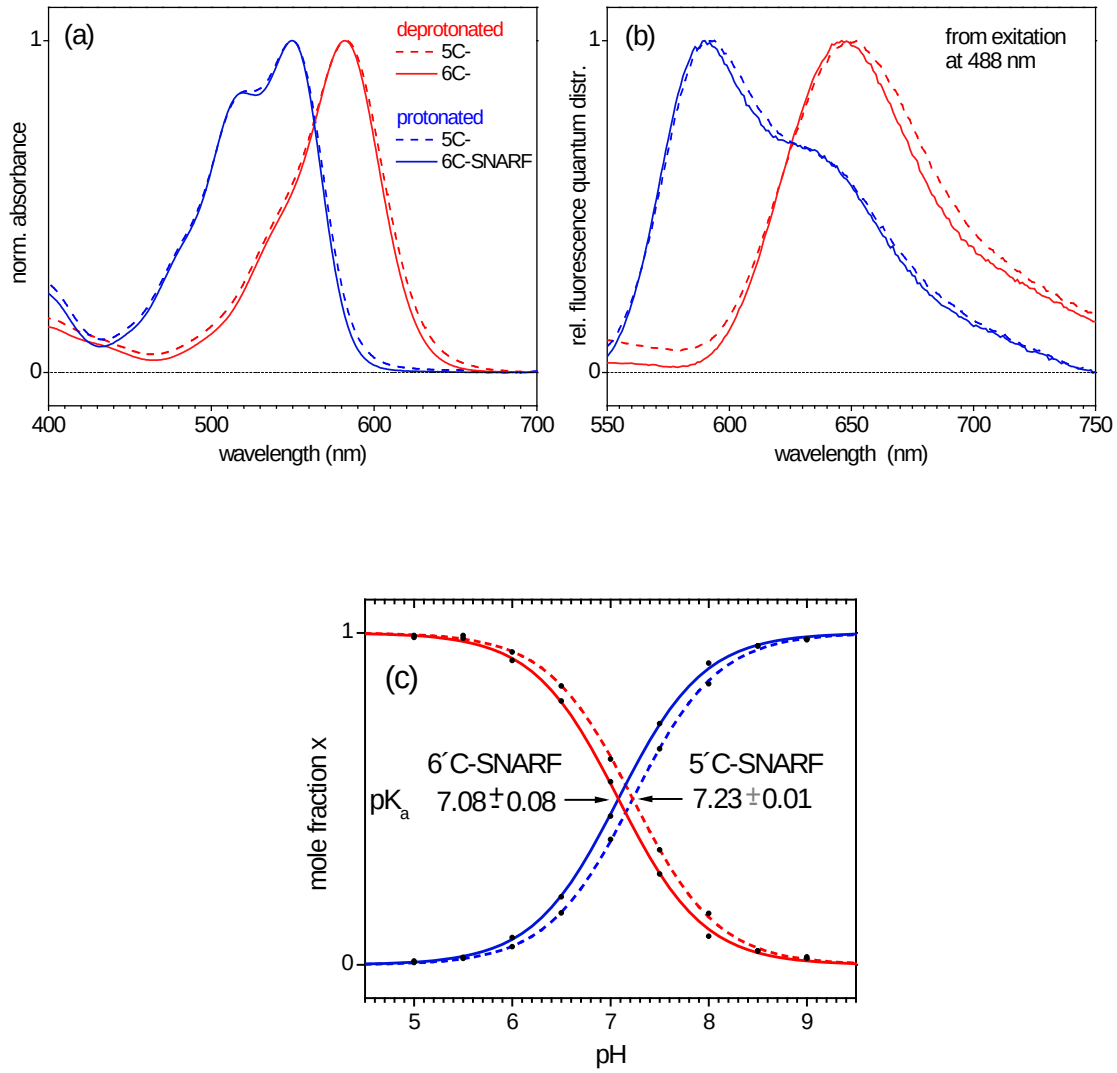


Figure S3. Absorption (a) and fluorescence (b) spectra of 5´C- and 6´C-SNARF and corresponding titration curves (c), in water:dmso 5:1 (vol:vol) at 22°C. A relatively high concentration of cosolvent was used to avoid solubility problems around pH 5.

Table S1: Lognormal description of S_1-S_0 bands of 5'Carboxy-SNARF w:dmso 5:1 (vol:vol).

	$\tilde{\nu}_p$ /cm^{-1}	Δ /cm^{-1}	γ	h
Absorption				
Deprotonated [(a)]	17095 18100 18714	1283 1394 2367	-0.16 0.42 0.86	46252 18369 7037
Protonated [(b)]	18059 19038 20086	1098 1123 1961	-0.15 0.14 0.72	26297 21716 13144
Emission from 488 nm excitation				
Deprotonated[(c)]	15370 13791	1554 1735	-0.11 1.10	1.000 0.290
Protonated[(d)]	17011 16171 14900	1019 1991 1895	-0.06 0.30 -0.47	0.443 0.668 0.370
Emission from 545 nm excitation				
Deprotonated[(e)]	15364 13926	1477 1903	-0.05 0.34	1.000 0.331
Protonated[(f)]	16990 15983 14798	1106 1742 1958	-0.06 0.23 -0.40	0.195 0.205 0.111

[(a)] For 15500 – 21000 cm^{-1}. [(b)] For 16000 – 23000 cm^{-1}.

[(c),(f)] For 13500 – 17500 cm^{-1}. [(d),(e)] For 13500 – 18000 cm^{-1}.

Table S2: Lognormal description of S_1-S_0 bands of 6'-CarboxySNARF w:dmso 5:1 (vol:vol).

	$\tilde{\nu}_p$ /cm^{-1}	Δ /cm^{-1}	γ	h
Absorption				
Deprotonated [(a)]	17198 18186 19302	1256 1300 1889	-0.10 0.17 0.61	45275 21584 6459
Protonated [(b)]	17997 18957 20113	1026 1556 1967	-0.01 -0.18 0.60	17456 20642 12786
Emission from 488 nm excitation				
Deprotonated[(c)]	15457 15457	1408 1896	-0.06 0.34	1.000 0.328
Protonated[(d)]	17049 16073 14848	1027 1919 2001	-0.05 0.32 -0.40	0.424 0.524 0.276
Emission from 545 nm excitation				
Deprotonated[(e)]	15401 13851	1485 1482	-0.06 -0,17	1.000 0.249
Protonated[(f)]	17032 15943 14756	1082 1731 2084	-0.06 0.25 -0.27	0.151 0.146 0.076

[(a)] For 15500 – 21000 cm^{-1}. [(b)] For 16000 – 23000 cm^{-1}.

[(c)] For 13500 – 17500 cm^{-1}. [(d)] For 13500 – 18000 cm^{-1}.

[(e)] For 13500 – 18000 cm^{-1}. [(f)] For 13500 – 17500 cm^{-1}.

Table S3: Lognormal description of S_1-S_0 bands of PAc-SNARF w:dmso 5:1 (vol:vol).

	$\tilde{\nu}_p$ /cm^{-1}	Δ /cm^{-1}	γ	h
Absorption				
Deprotonated [(a)]	17346 18299 19480	1138 1241 2064	-0.24 0.40 0.99	46375 26109 4798
Protonated [(b)]	17997 18957 20113	1026 1556 1967	-0.01 -0.18 0.60	17456 20642 12786
Emission from 488 nm excitation				
Deprotonated[(c)]	15457 14009	1408 1896	-0.06 0.34	1.000 0.328
Protonated[(d)]	17049 16073 14848	1027 1919 2001	-0.05 0.32 -0.40	0.424 0.524 0.276
Emission from 545 nm excitation				
Deprotonated[(e)]	15401 13851	1485 1482	-0.06 -0.17	1.000 0.249
Protonated[(f)]	17032 15944 14756	1082 1731 2084	-0.06 0.25 -0.27	0.152 0.146 0.076

[(a)] For 14000 – 22000 cm^{-1}. [(b)] For 16000 – 23000 cm^{-1}.

[(c)] For 13500 – 17000 cm^{-1}. [(d)] For 13500 – 20000 cm^{-1}.

[(e),(f)] For 13500 – 18000 cm^{-1}.

Table S4: Lognormal description of S_1-S_0 bands of PAc-SNARF w:dmso 10:1 (vol:vol).

	$\tilde{\nu}_p$ /cm^{-1}	Δ /cm^{-1}	γ	h
Absorption				
Deprotonated [(a)]	17247 17795 18513	794 597 1270	-0.52 0.08 0.86	39779 43392 27977
Protonated [(b)]	18359 19457 21291	1072 1688 2073	-0.29 0.67 1.30	28674 25763 2047
Emission from 488 nm excitation				
Deprotonated[(c)]	15830 14337	1461 1626	-0.05 -0.13	1.000 0.265
Protonated[(d)]	17336 16019 14473	1098 1942 1428	-0.01 0.29 -0.38	0.244 0.283 0.089
Emission from 545 nm excitation				
Deprotonated[(e)]	15802 14185	1541 1444	-0.10 -0.18	1.000 0.218
Protonated[(f)]	17263 15893 14234	1207 1627 1507	-0.08 -0.19 -0.44	0.126 0.102 0.020

[(a)] For 14000 – 22000 cm^{-1}. [(b)] For 16000 – 23000 cm^{-1}.

[(c),(d),(e),(f)] For 13500 – 18000 cm^{-1}.

Table S5: Lognormal description of S_1-S_0 emission bands of Me-SNARF w:dmso 5:1 (vol:vol).

	$\tilde{\nu}_p$ /cm^{-1}	Δ /cm^{-1}	γ	h
Emission from 488 nm excitation				
Deprotonated[(a)]	15896 14725	1396 1504	0.23 -0.54	1.000 0.382
Protonated[(b)]	17224 15860 14093	1557 1467 2051	0.16 -0.46 -0.59	0.478 0.325 0.056
Emission from 545 nm excitation				
Deprotonated[(c)]	15885 14286	1581 1499	-0.06 0.26	1.000 0.232
Protonated[(d)]	17333 16031 12971	1250 1745 8843	0.13 -0.46 -1.77	0.104 0.088 0.011

[(a),(b),(c),(d)] For 13500 – 18000 cm^{-1} .

Absorption spectra were not recorded due to low solubility.

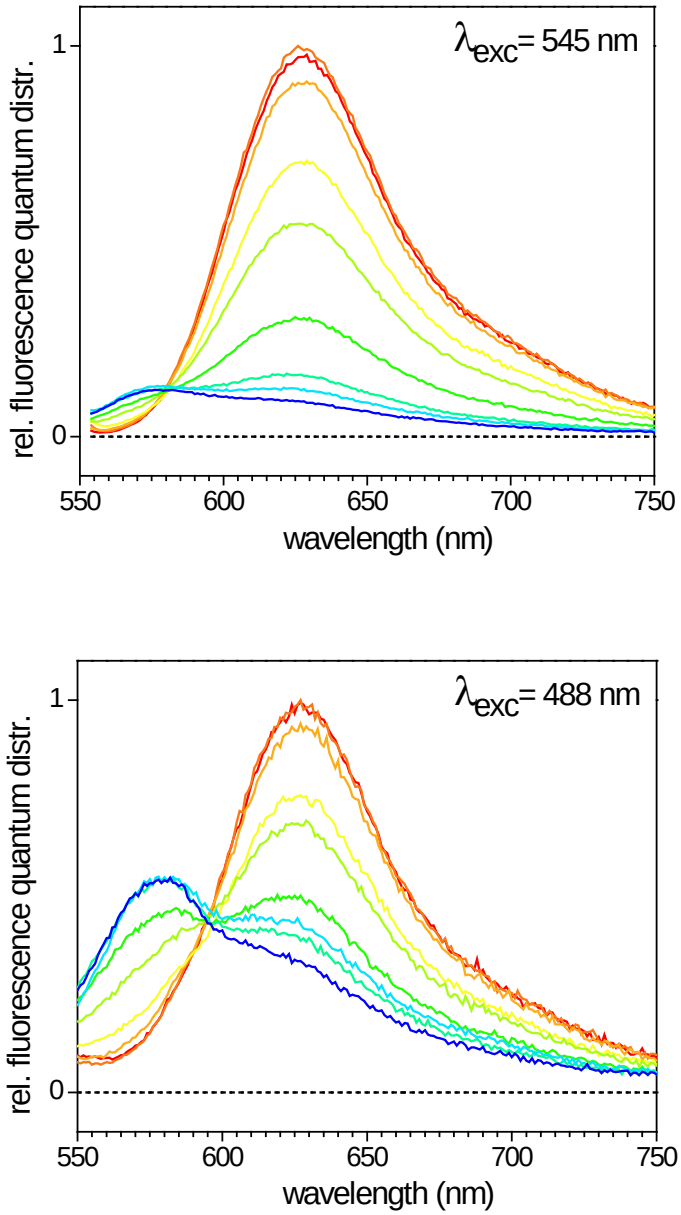


Figure S4. Fluorescence spectra of Me-SNARF with excitation wavelength of (top) 545 nm or (bottom) 488 nm in water:dmso (5:1 by volume, for buffer see text) as function of pH at 22°C. Red and blue curves represent the species-associated spectra (SAS) of the pure deprotonated and protonated species, respectively, from global analysis. In stationary measurements, excitation wavelength λ_{exc}=488 nm was chosen in order to enhance the fluorescence contribution by the protonated species.

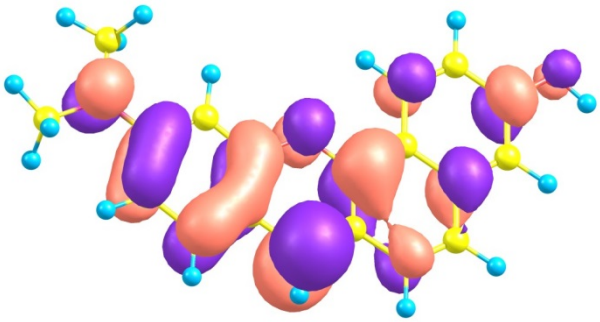
S4. Preparation of SNARF solutions in water:dmso with various pH

Into a beaker on a balance, 30.0 g of Millipore water are given and 3x1000 µl dmso added. The masses of water and dmso are noted (for control), and the mixture is left to cool to room temperature. A dilute NH_4OH solution (1 drop of 10% NH_4OH in 10 ml Millipore water) is then used to adjust the pH to 9.4 - 9.6. To the resulting mixture a small amount of solid SNARF is given, resulting in a peak optical density of 0.7 (for 1 cm path length) relative to the pure water:dmso mixture. This is now the "SNARF stock-solution".

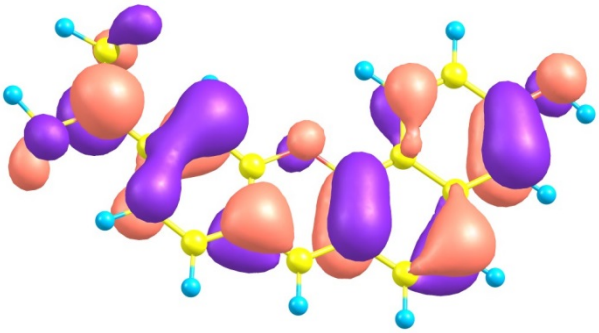
For each pH-value to be investigated, 3x1000 µl of the SNARF stock-solution and 3x1000 µl of pure buffer (from Merck) are pipetted into a 10 ml vessel with a snap-on cap and allowed to mix well. Other rations are achieved correspondingly.

S5. Orbitals of the SNARF core (R=H) in PCM water

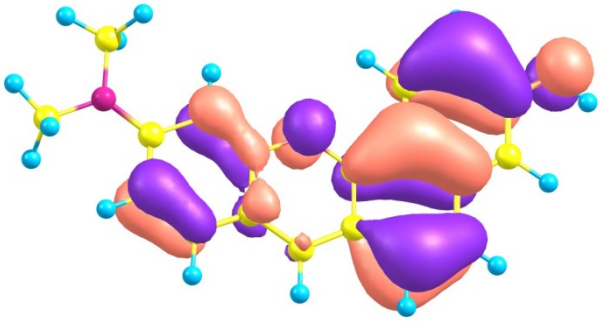
Orbitals of the protonated form:



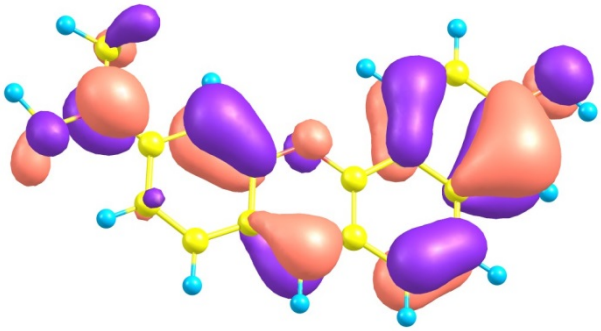
Orb 77 (LUMO)



Orb 76 (HOMO)



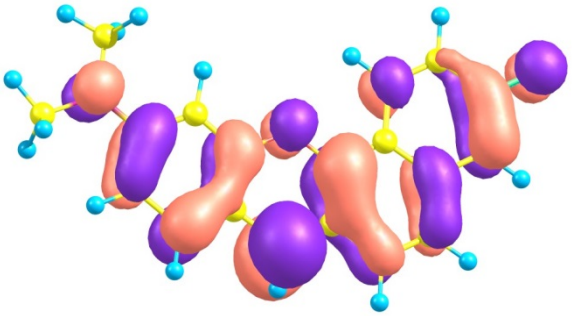
Orb 75



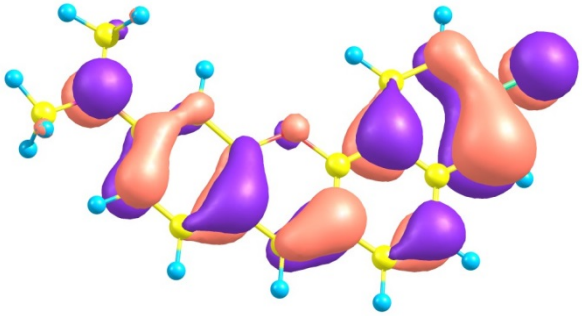
Orb 74

The excited states are 1 = 76 -> 77; 2 = 75 -> 77, 3 = 74 -> 77.

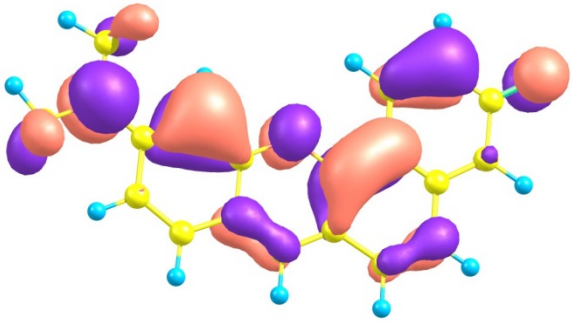
Orbitals of the deprotonated form:



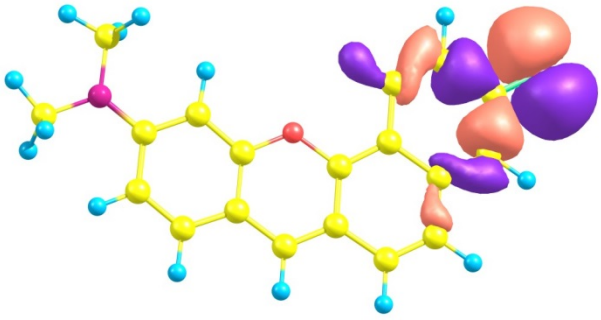
Orb 77 (LUMO)



Orb 76 (HOMO)



Orb 75



Orb 74

The excited states are 1 = 76 -> 77; 2 = 74 -> 77 (strong CT character), 3 = 75 -> 77.

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