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Supporting Information

3D-Non-destructive Imaging through Heavy-Metal Eosin Salt Contrast Agents

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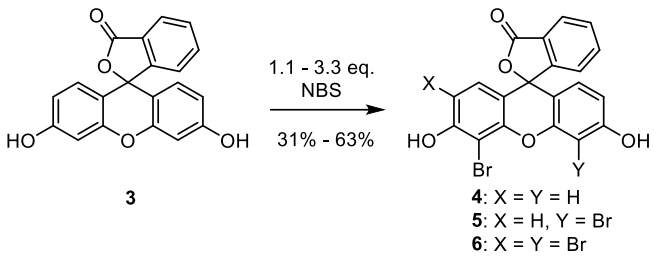
Content

1. General Information

Solvents used in reactions were p.A. grade. *N*-Bromosuccinimide (NBS) was purchased from Carbolution Chemicals and recrystallized from water. Fluorescein and Eosin y were purchased from Sigma-Aldrich, respectively, and used as received. Barium hydroxide octahydrate was purchased from Alfa Aesar. Other reagents were purchased at the highest commercial quality and used without further purification. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials, unless otherwise stated. NMR spectra were recorded on Bruker AV500-cryo spectrometer. The spectra were calibrated using residual undeuterated solvent as an internal reference (^{1}H NMR: Acetone-d_6 at 2.05, DMSO-d_6 at 2.50 ppm; ^{13}C NMR: Acetone-d_6 at 29.84, DMSO-d_6 at 39.52 ppm). The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, dt = doublet of triplets, td = triplet of doublets, m = multiplet, br = broad. Melting points were measured on a Büchi 510 and are not calibrated. IR spectra were recorded on a JASCO FT-IR-4100 (ATR) and are reported in terms of frequency of absorption (cm^{-1}). Mass spectrometry was conducted on a Thermo Scientific LTQ-FT ultra and ThermoFisher Scientific LTQ Orbitrap XL spectrometer (ESI HRMS). Combustion analysis was performed on a ELEMENTAR vario EL. High performance liquid chromatography (HPLC) analysis was performed on a HITACHI Chromaster employing the following conditions: 150 x 4 mm Eurospher II (KNAUER®), 100 Å, 5 µm, C18, 25 °C, flow rate: 1.0 mL/min, water (+0.01% TFA)/ acetonitrile (+0.01% TFA), method: [water (A)/acetonitrile (B), gradient: 0 min 90% A, 35 min 100% B]. Medium pressure liquid chromatography (MPLC) purification was performed on a Reverelis X2 (GRACE®) employing the following conditions: flow rate: 40 mL/min, water (+0.01% TFA)/acetonitrile (+0.01% TFA), method: [water (A)/acetonitrile (B), gradient: 0-10 min 65% A, 25 min 100% B]. 13 x 3 cm Reverelis (GRACE®), 40 µm, C18, 25 °C UV/Vis spectra were recorded on a BioTek® Eon spectrophotometer.

2. Synthetic Procedures

2.1 Synthesis of Brominated Fluorescein Derivatives 4 – 6

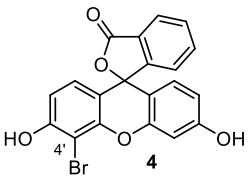


3

4: X = Y = H
5: X = H, Y = Br
6: X = Y = Br

4'-Bromofluorescein (4)

Fluorescein (**3**, 1.00 g, 3.01 mmol, 1.00 eq.) was suspended in abs. methanol (0.03 M) and the mixture was cooled to 0 °C. NBS (589 mg, 3.31 mmol, 1.10 eq.) was added in one portion and the resulting reaction mixture was stirred at room temperature for 2 h. The reaction was terminated by addition of NaOH (0.1 M, 3 mL) and methanol was subsequently removed in vacuo. The crude product was diluted with water until a clear solution was obtained and then filtered. The solution was than cooled to 0 °C and HCl (1 M) was added until reaching pH 2, causing precipitation of a red solid. The resulting suspension was stirred for 12 h at room temperature, filtered and washed with water (3 × 10 mL). The crude product was purified using MPLC to yield the monobrominated fluorescein **4** as an amorphous solid (396 mg, 0.96 mmol, 32%).

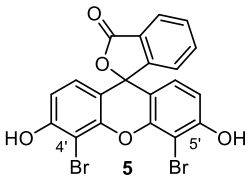


4

m.p. > 230 °C (MeCN); **HPLC:** t_R = 7.28 min (RP-C_{18}); **^{1}H NMR** (500 MHz, DMSO-d_6) δ 10.9 (br, 1H, OH), 10.2 (br, 1H, OH), 8.00 (dt, *J* = 7.7, 1.0 Hz, 1H), 7.80 (td, *J* = 7.5, 1.2 Hz, 1H), 7.72 (td, *J* = 7.5, 1.0 Hz, 1H), 7.31 (dt, *J* = 7.7, 1.2 Hz, 1H), 6.74 (m, 2H), 6.58 (m, 3H) ppm; **^{13}C NMR** (126 MHz, DMSO-d_6) δ 168.6, 159.7, 156.6, 152.3, 151.6, 148.7, 135.8, 130.3, 129.1, 127.6, 126.1, 124.7, 124.2, 113.3, 112.2, 111.0, 109.4, 102.3, 97.36, 82.82 ppm; **IR** (ATR) $\tilde{\nu}_{max}$ = 3078, 1753, 1603, 1433, 1115, 1023, 760 cm^{-1}; **HRMS** (ESI) calcd. for $C_{20}H_{12}BrO_5^+$ $[M+H]^+$ 410.9863, found 410.9861.

4', 5'-Dibromofluorescein (5)

Fluorescein (**3**, 1.00 g, 3.01 mmol, 1.00 eq.) was suspended in abs. methanol (0.03 M) and the mixture was cooled to 0 °C. NBS (1.18 g, 6.62 mmol, 2.20 eq.) was added in one portion and the resulting reaction mixture was stirred at room temperature for 2 h. The reaction was terminated by addition of NaOH (0.1 M, 3 mL) and methanol was subsequently removed in vacuo. The crude product was diluted with water until a clear solution was obtained and then filtered. The solution was then cooled to 0 °C and HCl (1 M) was added until reaching pH 2, causing precipitation of a red solid. The resulting suspension was stirred for 12 h at room temperature, filtered and washed with water (3 × 10 mL). The crude product was purified using MPLC to yield the dibrominated fluorescein **5** as an amorphous solid (929 mg, 1.90 mmol, 63%).



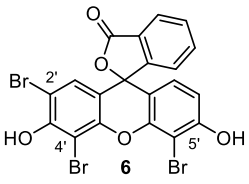
5

m.p. > 230 °C (MeCN); **HPLC:** t_R = 8.23 min (RP-C_{18}); **^{1}H NMR** (500 MHz, Acetone-d_6) δ 9.61 (br, 2H, OH), 8.00 (dt, *J* = 7.6, 1.0 Hz, 1H), 7.83 (td, *J* = 7.5, 1.2 Hz, 1H), 7.76 (td, *J* = 7.5, 1.0 Hz, 1H), 7.43 (dt, *J* = 7.7, 0.9 Hz, 1H), 6.84 (d, *J* = 8.7, 2H), 6.69 (d, *J* = 8.8, 2H) ppm; **^{13}C NMR** (126 MHz, Acetone-d_6) δ 169.2, 157.4, 153.3, 150.1,136.3, 131.1, 128.4, 127.6, 125.5, 125.2, 113.5, 113.0, 98.88, 83.28 ppm; **IR** (ATR) $\tilde{\nu}_{max}$ = 3077, 1657, 1574, 1497, 1455, 1241, 1112, 947, 764 cm^{-1}; **HRMS** (ESI) calcd. for $C_{20}H_{11}Br_2O_5^+$ $[M+H]^+$ 488.8968, found 488.8964.

2', 4',5'-Tribromofluorescein (6)

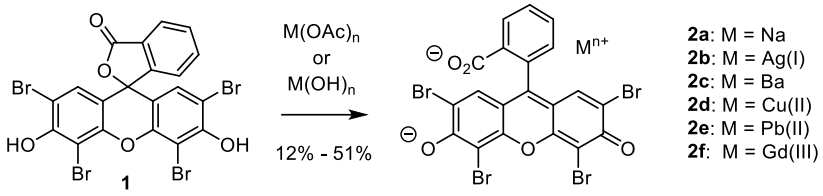
Fluorescein (**3**, 1.00 g, 3.01 mmol, 1.00 eq.) was suspended in abs. methanol (0.03 M) and the mixture was cooled to 0 °C. NBS (1.77 g, 9.93 mmol, 3.30 eq.) was added in one portion and the resulting reaction mixture was stirred at room temperature for 2 h. The reaction was terminated by addition of NaOH (0.1 M, 3 mL) and methanol was subsequently removed in vacuo. The crude product was diluted with water until a clear solution was obtained and then filtered. The solution was than cooled to 0 °C and HCl (1 M) was added until reaching pH 2, causing precipitation of a red solid. The resulting suspension was stirred for 12 h at room temperature, filtered and washed

with water (3 × 10 mL). The crude product was purified using MPLC to yield the tribrominated fluorescein **6** as an amorphous solid (529 mg, 0.93 mmol, 31%).



m.p. > 230 °C (MeCN); **HPLC:** t_R = 9.74 min (RP-C_{18}); **^{1}H NMR** (500 MHz, DMSO-d_6) δ 11.1 (br, 1H, OH), 10.8 (br, 1H, OH), 8.02 (d, *J* = 7.6, 1H), 7.82 (td, *J* = 7.5, 1.2 Hz, 1H), 7.75 (td, *J* = 7.5, 1.0 Hz, 1H), 7.43 (d, *J* = 7.6, 1H), 6.91 (s, 1H), 6.79 (d, *J* = 8.8 Hz, 1H), 6.60 (d, *J* = 8.8 Hz, 1H) ppm; **^{13}C NMR** (126 MHz, DMSO-d_6) δ 168.3, 156.9, 153.2, 151.5 148.4, 148.0,136.0, 130.7, 129.8, 127.5, 125.9, 125.1, 124.3, 113.1, 113.0, 110.7, 106.9, 100.7, 97.55, 81.82 ppm; **IR** (ATR) $\tilde{\nu}_{max}$ = 3055, 1690, 1560, 1515, 1448, 1301, 970 cm^{-1}; **HRMS** (ESI) calcd. for $C_{20}H_{10}Br_3O_5^+$ $[M+H]^+$ 566.8073, found 566.8067

2.2 Synthesis of Eosin y Heavy Metal Salts 2b – 2f



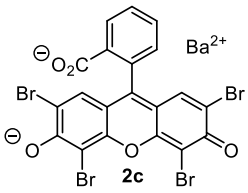
Eosin y silver (I) salt (2b)

After sonication of a freshly prepared suspension of eosin y (**1**, 2.00 g, 3.09 mmol, 1.00 eq.) in water (6.00 mM) for 10 min at room temperature, AgOAc (1.03 g, 6.18 mmol, 2.00 eq.) was added to the suspension in one portion under vigorous stirring. After stirring for 12 h at room temperature the reaction mixture was filtered and the filtrate dried in vacuum to yield the eosin y silver (I) salt as a crystalline purple solid (852 mg, 989 µmol, 32%).

Eosin y barium salt (2c)

After sonication of a freshly prepared suspension of eosin y (**1**, 500 mg, 772 µmol, 1.00 eq.) in water (6.00 mM) for 10 min at room temperature, $Ba(OH)_2{\cdot}8H_2O$ (244 mg, 772 µmol, 1.00 eq.) was added to the suspension in one portion under vigorous stirring. The suspension deepened its

color immediately and turned into a clear solution over time. After stirring for 6 h at room temperature the reaction mixture was filtered and the filtrate dried in vacuum to yield the eosin y barium salt (**2c**) as a crystalline deep red solid (310 mg, 396 µmol, 51%).



m.p. > 230 °C (H_2O); **^{1}H NMR** (500 MHz, DMSO-d_6) δ 8.18 (d, J = 7.0 Hz, 1H), 7.66-7.55 (m, 2H), 7.23 (dd, J = 7.0, 1.9 Hz, 1H), 6.97 (s, 2H) ppm; **^{13}C NMR** (126 MHz, DMSO-d_6) δ 171.3, 168.1, 156.1, 153.0, 139.2, 132.8, 130.5, 130.1, 129.4, 129.2, 128.9, 117.9, 109.7, 99.03 ppm; **IR** (ATR) $\tilde{\nu}_{max}$ = 1545, 1406, 1340, 1018, 977, 927 cm^{-1}; **Combustion analysis** (%) calcd. for $C_{20}H_6BaBr_4O_5 \cdot 4H_2O$: C 28.09, H 1.65; found: C 27.77, H 1.58.

Eosin y copper(II) salt (2d)

After sonication of a freshly prepared suspension of eosin y (**1**, 1.00 g, 1.54 mmol, 1.00 eq.) in water (5.00 mM) for 10 min at room temperature, $Cu(OAc)_2 \cdot H_2O$ (307 mg, 1.54 mmol, 1.00 eq.) was added to the suspension in one portion under vigorous stirring. The suspension deepened its color immediately. After stirring for 12 h at room temperature the reaction mixture was filtered and the filtrate dried in vacuum to yield the eosin y copper (II) salt (**2d**) as crystalline deep blue solid (412 mg, 581 µmol, 38%).

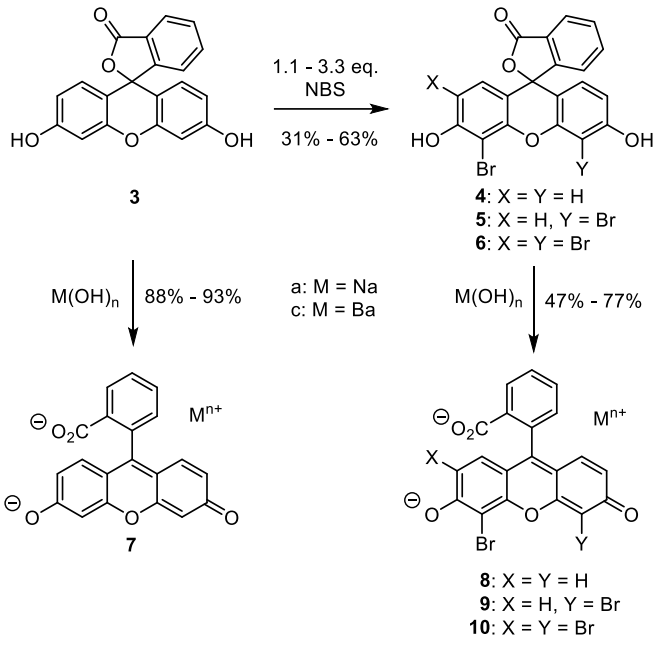
Eosin y lead(II) salt (2e)

After sonication of a freshly prepared suspension of eosin y (**1**, 2.00 g, 3.09 mmol, 1.00 eq.) in water (6.00 mM) for 10 min at room temperature, $Pb(OAc)_2$ (1.01 g, 3.09 mmol, 1.00 eq.) was added to the suspension in one portion under vigorous stirring. The suspension deepened its color immediately. After stirring for 12 h at room temperature the reaction mixture was filtered and the filtrate dried in vacuum to yield the eosin y lead (II) salt (**2e**)[1] as crystalline deep pink solid (422 mg, 495 µmol, 16%).

Eosin y gadolinium (III) salt (2f)

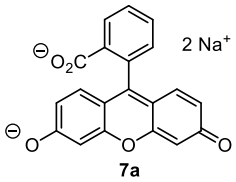
After sonication of a freshly prepared suspension of eosin y (**1**, 2.00 g, 3.09 mmol, 1.00 eq.) in water (6.00 mM) for 10 min at room temperature, $Gd(OAc)_3 \cdot 3H_2O$ (726 mg, 2.06 mmol, 0.67 eq.) was added to the suspension in one portion under vigorous stirring. The suspension deepened its color immediately. After stirring for 12 h at room temperature the reaction mixture was filtered and the filtrate dried in vacuum to yield the eosin y gadolinium (III) salt (**2f**) as crystalline deep red solid (835 mg, 371 µmol, 12%).

2.3 Synthesis of (Brominated) Fluorescein Salts 7 and 9



Fluorescein disodium salt (7a)

After sonication of a freshly prepared suspension of fluorescein (**3**, 442 mg, 1.33 mmol, 1.00 eq.) in water (5.00 mM) for 10 min at room temperature, NaOH (106 mg, 2.66 mmol, 2.00 eq.) was added to the suspension in one portion under vigorous stirring. After stirring for 6 h at room temperature the reaction mixture was filtered and the filtrate dried in vacuum to yield the fluorescein disodium salt (**7a**) as a crystalline red solid (440 mg, 1.17 mmol, 88%).

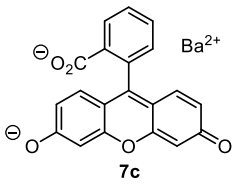


^{1}H NMR (500 MHz, DMSO-d_6) δ 8.00 (d, *J* = 6.8 Hz, 1H), 7.48-7.36 (m, 2H), 7.02 (d, *J* = 6.8 Hz, 1H), 6.53 (d, *J* = 9.5, 2H), 5.98 (d, *J* = 9.5, 2H), 5.90 (s, 2H) ppm.

The spectroscopic and physical data was in accordance to that reported in the literature.[2]

Fluorescein barium salt (7c)

After sonication of a freshly prepared suspension of fluorescein (**3**, 442 mg, 1.33 mmol, 1.00 eq.) in water (5.10 mM) for 10 min at room temperature, $Ba(OH)_2 \cdot 8H_2O$ (420 mg, 1.33 mmol, 1.00 eq.) was added to the suspension in one portion under vigorous stirring. After stirring for 6 h at room temperature the reaction mixture was filtered and the filtrate dried in vacuum to yield the fluorescein barium salt (**7c**) as crystalline dark red solid (581 mg, 1.24 mmol, 93%).

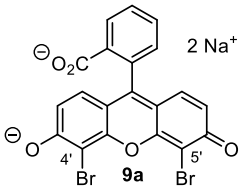


m.p. > 230 °C (H_2O); **^{1}H NMR** (500 MHz, DMSO-d_6) δ 8.13-8.07 (m, 1H), 7.52-7.45 (m, 2H), 7.10-7.04 (m, 1H), 6.52 (d, *J* = 9.3 Hz, 2H), 6.01 (dd, *J* = 9.3, 2.1 Hz, 2H), 5.92 (d, *J* = 2.1 Hz, 2H) ppm; **^{13}C NMR** (126 MHz, DMSO-d_6) δ 180.6, 172.2, 157.8, 155.2, 139.7, 134.4, 130.2, 129.5, 128.8, 127.8, 123.0, 108.5, 102.6 ppm; **IR** (ATR) $\tilde{\nu}_{max}$ = 1634, 1559, 1457, 1384, 1293, 1102, 916, 842, 753 cm^{-1}; **Combustion analysis** (%) calcd. for $C_{20}H_{10}BaO_5 \cdot 4H_2O$: C 40.51, H 3.36; found: C 42.98, H 3.01.

4', 5'-Dibromo fluorescein disodium salt (9a)

After sonication of a freshly prepared suspension of dibromo fluorescein (**5**, 243 mg, 496 µmol, 1.00 eq.) in water (3.00 mM) for 10 min at room temperature, NaOH (39.7 mg, 992 µmol, 2.00 eq.) was added to the suspension in one portion under vigorous stirring. After stirring for 6 h at room temperature the reaction mixture was filtered and the filtrate dried in vacuum to yield

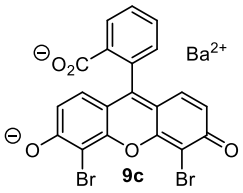
the dibromo fluorescein disodium salt (**9a**) as a dark red, crystalline solid (125 mg, 234 µmol, 47%).



m.p. > 230 °C (H_2O); **^{1}H NMR** (500 MHz, DMSO-d_6) δ 8.11-8.06 (m, 1H), 7.53-7.42 (m, 2H), 7.15-7.06 (m, 1H), 6.60 (d, *J* = 9.3 Hz, 2H), 6.23 (d, *J* = 9.3, 2H) ppm; **^{13}C NMR** (126 MHz, DMSO-d_6) δ 174.3, 169.7, 156.6, 153.7, 140.3, 133.8, 130.3, 129.0, 128.4, 128.1, 122.0, 109.3, 99.79 ppm; **IR** (ATR) $\tilde{\nu}_{max}$ = 1612, 1550, 1449, 1344, 1237, 974, 880, 844, 759, 713 cm^{-1}; **Combustion analysis** (%) calcd. for $C_{20}H_8Br_2Na_2O_5 \cdot 3H_2O$: C 40.85, H 2.40; found: C 41.41, H 2.41.

4', 5'-Dibromo fluorescein barium salt (9c)

After sonication of a freshly prepared suspension of dibromo fluorescein (**5**, 520 mg, 1.06 mmol, 1.00 eq.) in water (3.00 mM) for 10 min at room temperature, $Ba(OH)_2 \cdot 8H_2O$ (334 mg, 1.06 mmol, 1.00 eq.) was added to the suspension in one portion under vigorous stirring. After stirring for 6 h at room temperature the reaction mixture was filtered and the filtrate dried in vacuum to yield the dibromo fluorescein barium salt (**9c**) as crystalline dark red solid (512 mg, 819 µmol, 77%).



m.p. > 230 °C (H_2O); **^{1}H NMR** (500 MHz, DMSO-d_6) δ 8.22-8.12 (m, 1H), 7.56-7.45 (m, 2H), 7.17-7.10 (m, 1H), 6.57 (d, *J* = 9.4 Hz, 2H), 6.23 (d, *J* = 9.4, 2H) ppm; **^{13}C NMR** (126 MHz, DMSO-d_6) δ 174.4, 171.9, 155.9, 153.7, 139.0, 134.0, 130.7, 129.3, 129.0, 128.3, 122.2, 109.3, 99.90 ppm; **IR** (ATR) $\tilde{\nu}_{max}$ = 3244, 1540, 1407, 1337, 1017, 928, 648 cm^{-1}; **Combustion analysis** (%) calcd. for $C_{20}H_8BaBr_2O_5 \cdot 4H_2O$: C 34.44, H 2.31; found: C 34.40, H 1.97.

3. Determination of the Partition Coefficient logP

1 mg of each compound was dissolved in 1 mL water and sonicated for 30 min. The resulting clear solution was mixed with 9 mL PBS buffer giving the stock solution. 2 mL of the stock solution was mixed with 100 µL *n*-octanol, which was saturated with PBS buffer before use, and then shaken for 30 min at room temperature. After centrifugation for 15 min at 3000 *rpm* the *n*-octanol phase of each sample was analyzed by HPLC-UV. The compound concentrations W_1 of the *n*-octanol phase were determined using the calibration curve prepared by a dilution series of the stock solution before. The integration of the area of the HPLC signals was done at 234 nm for each compound. The partition coefficient logP was calculated using the following equation.

$$logP = log\frac{(W_0 - W_1)V_{aq}}{W_1 V_{oct}}$$

W_0 = initial concentration of compounds **1**, **2a**, **3** – **6**, **7a** and **9a**.

W_1 = concentration of compounds **1**, **2a**, **3** – **6**, **7a** and **9a** in *n*-octanol

V_{aq} = volume of the aqueous phase

V_{oct} = volume of the *n*-octanol phase

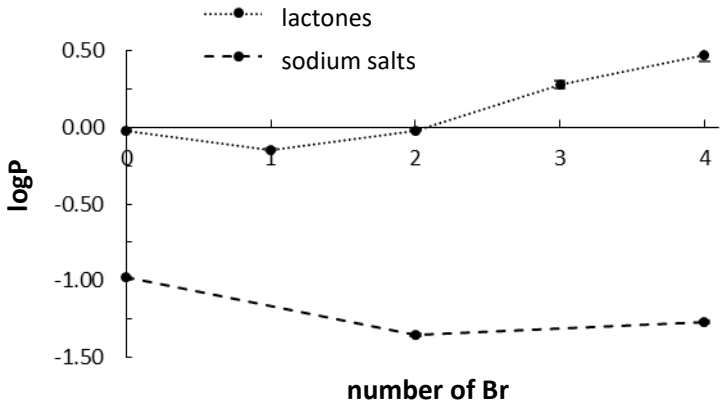


Figure S1. Partition coefficient (logP) of lactones **1**, **3** – **6** and disodium salts **2a**, **7a** and **9a**. The logP values were determined in triplicates.

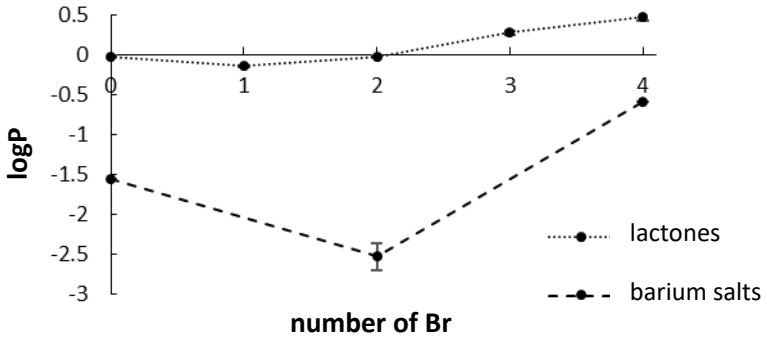


Figure S2. Partition coefficient (logP) of the lactones **1** and **3** – **6** and the barium derivatives **2c**, **7c** and **9c**. The logP values were determined in triplicates.

4. Maximum Solubility of Eosin y 2a – 2f and Dibromo Fluorescein Salts 9a and 9c

compound	concentration [mg/mL]
Eosin y disodium salt (**2a**)[3]	300 mg/mL
Eosin y silver(I) salt (**2b**)	< 10 mg/mL
Eosin y barium salt (**2c**)	25 mg/mL
Eosin y copper(II) salt (**2d**)	66 mg/mL
Eosin y lead(II) salt (**2f**)	50 mg/mL
Eosin y gadolinium(III) salt (**2f**)	< 10 mg/mL
Dibromo disodium salt (**9a**)	549 mg/mL
Dibromo barium salt (**9c**)	45 mg/mL

5. Animal Tissue Samples

The fresh turkey livers were purchased from a butcher and fixated as described in the section below. All procedures were in accordance with relevant guidelines and regulations. All laboratories are inspected for accordance with the OECD principles of good laboratory practice. We prepared a turkey liver piece (ca. 27 mm^3) using the final version of the eosin staining / barium-eosin staining procedure. The soft-tissue sample was then used to evaluate structural preservation and to assess stain quality, identify morphological structures, compare with conventional histological methods and evaluate for further histological staining.

6. Sample Screening

Whole turkey livers were fixated and preserved under conditions described below. Cuboidal soft-tissue samples from turkey liver (1 mm edge length) were used for stain development and optimization. The small cuboidal tissue samples were cut with a scalpel (Aesculap). Temperature was controlled by placing samples in a refrigerator (4°C) or in ambient conditions of the laboratory. lncubations were done in sample holders with a flat bottom, which were replaced after each step but not after rinse or dehydration steps. For stain development and optimization several parameters such as fixative, concentration of fixative or staining agent, incubation time or pH of fixative or staining agents were tested. The stained soft-tissue samples were investigated on the phoenix vltomelx s 240 CT scanner with typical settings of 50kV peak voltage, 6.0W and with 1601 projections distributed over 360°. The low-resolution CT data were acquired with an exposure time of 1s per projection with an effective pixel size of *ca.* 30µm. The microCT data were reconstructed with the integrated phoenix datos x CT software and analyzed for (i) completeness of staining, (ii) appearance of diffusion rings, (iii) contrast enhancement, (iv) appearance of CT artifacts as streaks and (v) homogeneity of the staining.

7. Staining Protocols

7.1 For Eosin y Disodium Salt (2a)

The fixated turkey liver piece was acidified prior to use. Therefore, it was placed in a 50-mL Falcon Centrifuge Tube (neolab), which was filled with a fixative solution containing 9.5 mL of 4% (v/v) formaldehyde solution (FA, derived from a 37% acid free FA solution stabilized with ca. 10% methanol from Carl Roth; further dilution with DPBS without calcium and magnesium) and 0.5 glacial acetic acid (AA, Alfa Aesar). The sample was refrigerated for 24-72h and then washed with phosphate saline buffer solution for 1 h (DPBS without calcium and magnesium). The turkey liver was placed in the staining solution of **2a** (c = 31.9 mM) in dist. water; Sigma-Aldrich, product number: E4382, stain certified by the Biological Stain Commission.). The soft-tissue sample was stained with 1 mL of staining solution for 24h (the soft-tissue sample was moving freely within the sample container). During the incubation time the soft-tissue sample was kept on a horizontal shaking plate allowing for a smooth rocking of 60 rpm. After staining, the soft-tissue sample was carefully removed from the sample container and access of staining agent **2a** was softly patted with a cellulose tissue paper. The soft-tissue sample was stored in an Eppendorf tube above an ethanol vapor phase (the Eppendorf tube contained a few drops of 70% (v/v) ethanol at the bottom of the tube).

7.2 For Eosin y Barium Salt (2c)

The fixated turkey liver piece was acidified prior to use. Therefore, it was placed in a 50-mL Falcon Centrifuge Tube (neolab), which was filled with a fixative solution containing 9.5 mL of 4% (v/v) formaldehyde solution (FA, derived from a 37% acid free FA solution stabilized with ca. 10% methanol from Carl Roth; further dilution with DPBS without calcium and magnesium) and 0.5 mL glacial acetic acid (AA, Alfa Aesar). The sample was refrigerated for 24-72h and then washed with phosphate saline buffer solution for 1 h (DPBS without calcium and magnesium). The soft-tissue sample was transferred into a sample container with 1 mL of a staining solution comprising a barium-eosin Y salt (**2c**) in water (25 mg/mL; c = 31.9 mM). The soft-tissue sample was stained for 72 h while placed on a shaker (horizontal shaking with 60 rpm). After staining with the staining

solution comprising **2c**, the turkey liver was removed, and the remaining staining solution **2c** was carefully padded off the soft-tissue sample with a cellulose tissue paper. The soft tissue sample was stored over 70 vol/vol-% ethanol vapor prior to CT measurement.

7.3 For Dibromo Fluorescein Sodium Salt (9a)

The fixated turkey liver piece was acidified prior to use. Therefore, it was placed in a 50-mL Falcon Centrifuge Tube (neolab), which was filled with a fixative solution containing 9.5 mL of 4% (v/v) formaldehyde solution (FA, derived from a 37% acid free FA solution stabilized with ca. 10% methanol from Carl Roth; further dilution with DPBS without calcium and magnesium) and 0.5 mL glacial acetic acid (AA, Alfa Aesar). The sample was refrigerated for 24-72h and then washed with phosphate saline buffer solution for 1 h (DPBS without calcium and magnesium). The soft-tissue sample was transferred into a sample container with 1 mL of a staining solution comprising the dibromo fluorescein sodium salt (**9a**) in water (45 mg/mL; c = 76.5 mM). The soft-tissue sample was stained for 72 h while placed on a shaker (horizontal shaking with 60 rpm). After staining with the staining solution comprising **9a**, the turkey liver was removed, and the remaining staining solution **9a** was carefully padded off the soft-tissue sample with a cellulose tissue paper. The soft tissue sample was stored over 70 vol/vol-% ethanol vapor prior to CT measurement.

7.4 For Dibromo Fluorescein Barium Salt (9c)

The fixated turkey liver piece was acidified prior to use. Therefore, it was placed in a 50-mL Falcon Centrifuge Tube (neolab), which was filled with a fixative solution containing 9.5 mL of 4% (v/v) formaldehyde solution (FA, derived from a 37% acid free FA solution stabilized with ca. 10% methanol from Carl Roth; further dilution with DPBS without calcium and magnesium) and 0.5 mL glacial acetic acid (AA, Alfa Aesar). The sample was refrigerated for 24-72h and then washed with phosphate saline buffer solution for 1 h (DPBS without calcium and magnesium). The soft-tissue sample was transferred into a sample container with 1 mL of a staining solution comprising the dibromo fluorescein barium salt (**9c**) in water (45 mg/mL; c = 72.0 mM). The soft-tissue sample

was stained for 72 h while placed on a shaker (horizontal shaking with 60 rpm). After staining with the staining solution comprising **9c**, the turkey liver was removed, and the remaining staining solution **9c** was carefully padded off the soft-tissue sample with a cellulose tissue paper. The soft tissue sample was stored over 70 vol/vol-% ethanol vapor prior to CT measurement.

8. Macroscopic Imaging of stained Turkey Liver Samples

Before the stained turkey liver pieces were investigated by µCT, a macroscopic investigation of the soft-tissue samples was performed by halving the stained specimen. The results are listed in Table S2.

Table S2. Macroscopic investigation of the studied tissues stained under different conditions.

entry	Stain	acidified	Incubation time [h]	macroscopic image
1	**2a**	X	24	
2	**2c**	X	24	
3	**2a**		24	
4	**2c**		24	
5	**2a**	X	48	
6	**2c**	X	48	
7	**2a**		48	
8	**2c**		48	
9	**2a**	X	72	
10	**2c**	X	72	
11	**2a**		72	

12	**2c**		72	
13	**2d**	X	144	
14	**2e**	X	144	

The tissue samples were treated with a 4% formaldehyde solution and with or without 0.5 mL glacial acetic acid prior to staining. Staining was performed using a 31.9 mM aq. solution of eosin y salt **2a** or **2c** – **2e**. For each condition, an individual number of three turkey liver pieces was stained to ensure reproducibility; one of these pieces was investigated macroscopically.

9. Line Plot Analysis of Turkey Liver Tissue Samples treated with 2c and 9c

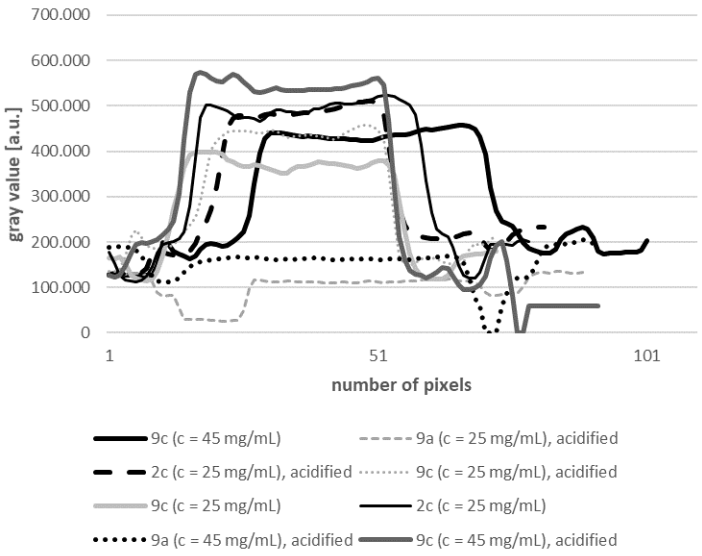


Figure S3. The line plots of the dibromo sodium **9a**, dibromo barium **9c** and tetrabromo barium fluorescein **2c** derivatives are displayed highlighting the influence of the different degree of halogenation of the xanthene core, aspect of acidification and concentrations on the contrast enhancement of the soft tissue sample.

10. X-Ray µCT Imaging of Turkey Liver

The stained turkey liver pieces (acidified and non-acidified) as well as a control sample (fixated and acidified, but not stained) were transferred to a sample holder, which allows the anchorage of the soft-tissue pieces above 70 % (v/v) ethanol vapor. The X-ray microCT measurements were performed with the phoenix vltomelx s 240 CT scanner. All shown images were acquired at 50kV peak voltage, 5.5W and with 1601 projections distributed over 360°. The low-resolution CT data were acquired with an exposure time of 2s per projection with an effective voxel size of *ca.* 40µm.

11. High-Resolution µCT of Mouse Kidney

A stained mouse kidney piece of approximately 5 mm edge length (with the barium eosin y salt (**9c**) using the staining protocol described in section 7.4) was mounted to a sample holder above 70 % (v/v) ethanol vapor. The high-resolution µCT data was acquired with the Zeiss Xradia Versa 500 CT scanner using the following parameters: 50 kV peak Voltage, 3.5 W with 1601 projections distributed over 360° using the 0.39x objective. An exposure time of 10 s per projection was used with an effective voxel size of approximately 3.3 µm. The integrated software, Scout and Scan 11.1.8043.19515, was used to reconstruct the data. The volume renderings of the µCT data shown in Figure 6 were processed with Avizo Fire 8.1 (ThermoFisher Scientific).

12. Histological Analysis of Turkey Liver Samples stained with 2c

The stained turkey liver pieces were dehydrated and embedded in paraffin according to standard procedures prior to histological sectioning and further histochemical counter staining. The samples were dehydrated in 70% and 96% ethanol twice for one hour and cleared in xylol twice for one hour. Subsequently, the sample was incubated overnight in paraffin wax at 50°C to infiltrate the complete sample with wax, embedded in paraffin wax and sections of 7µm thickness were cut using a microtome (Leica). Sections were rehydrated and either directly embedded (Eukitt, Merck) or counterstained for 6min with Mayer's sour hematoxylin (Morphisto) according

to the manufacturer's protocol. Histological analysis was performed using an Axio Imager 2 microscope and AxioVision Software (Zeiss).

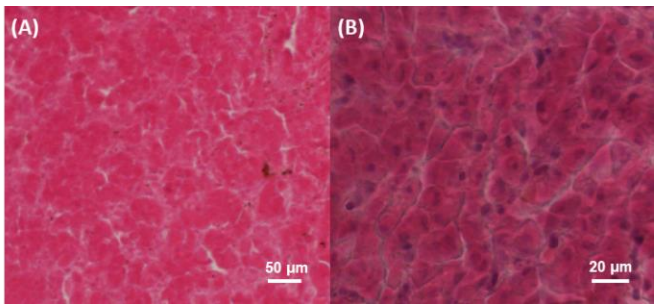
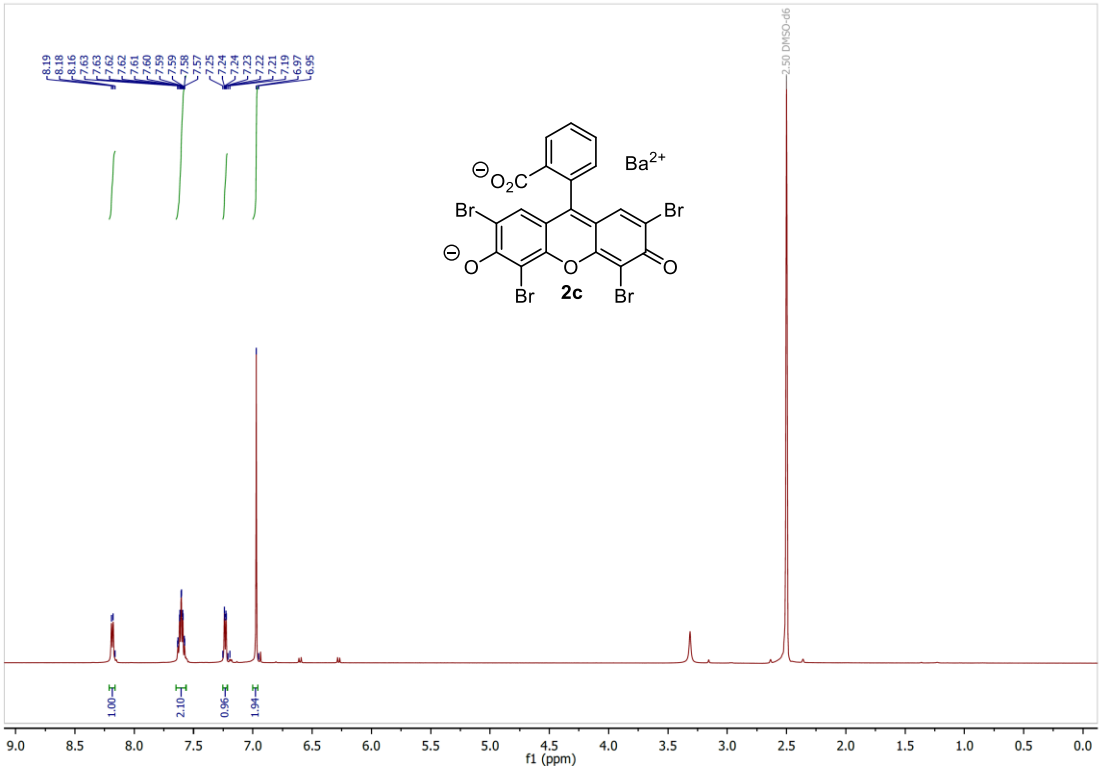
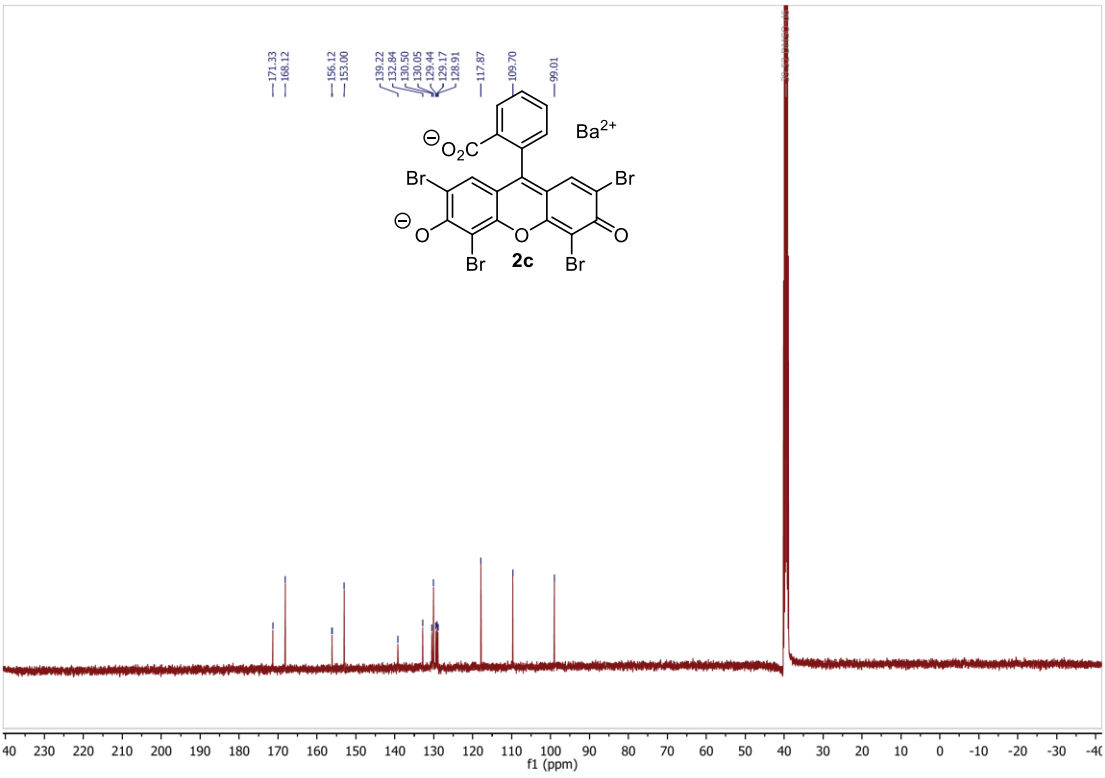
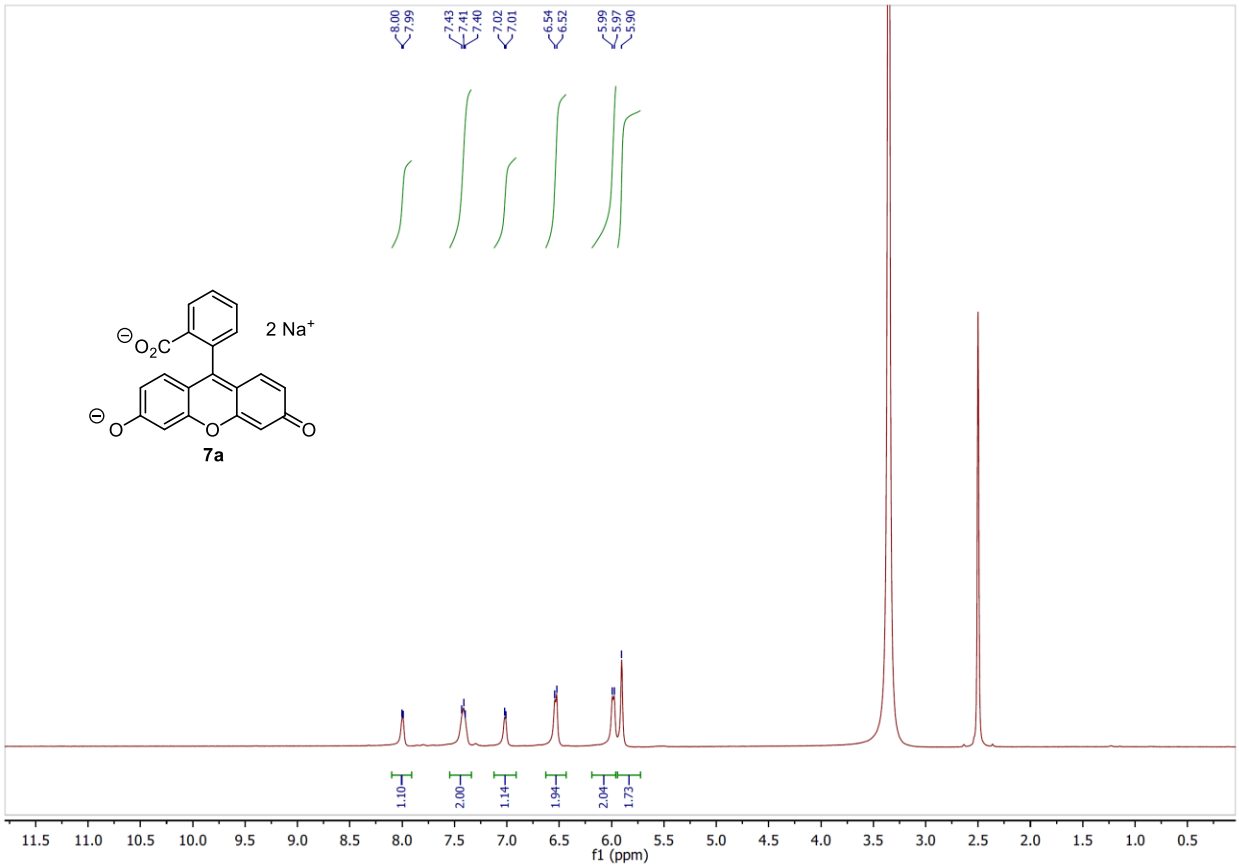


Figure S4. Histological microscopic slide obtained from a turkey liver stained with barium eosin y salt (**2c**) for 72 h (c = 25 mg/mL). The microscopic slide was directly obtained after staining and CT investigation. (**A**) No further treatments by the histologist were performed. (**B**) Hematoxylin was applied as counter stain to **2c**. All histological analyses were performed using an Axio Imager 2 microscope and AxioVision Software (Zeiss). Image in (**A**) was produced with 20x magnification. Image in (**B**) was produced with 40x magnification.

13. NMR Spectra of Compounds 2, 4-7, and 9



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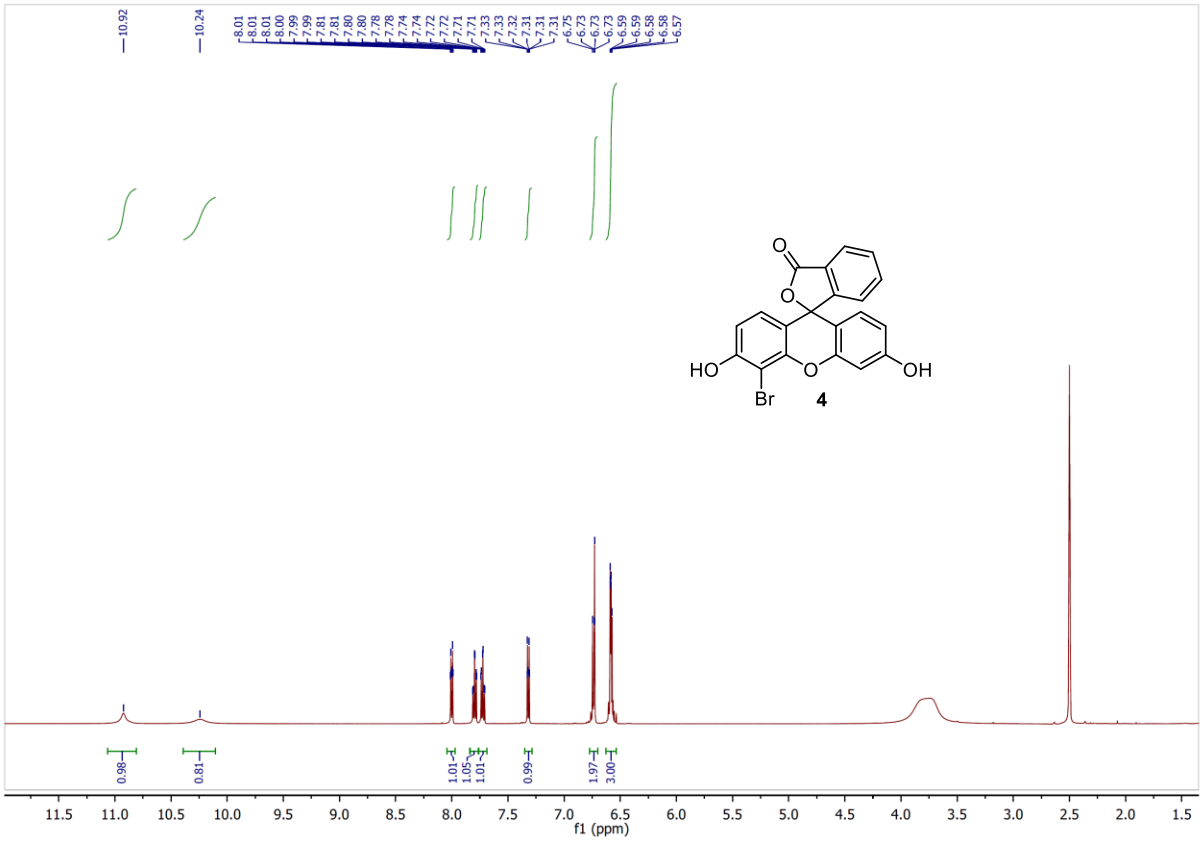
7.43
7.41
7.40
7.02
7.01
6.54
6.52
5.99
5.97
5.90
2 Na+
7a
1.10
2.00
1.14
1.94
2.04
1.73
11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)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7.31
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6.59
6.59
6.58
6.58
6.57
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OH
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1.01
0.99
1.97
3.00
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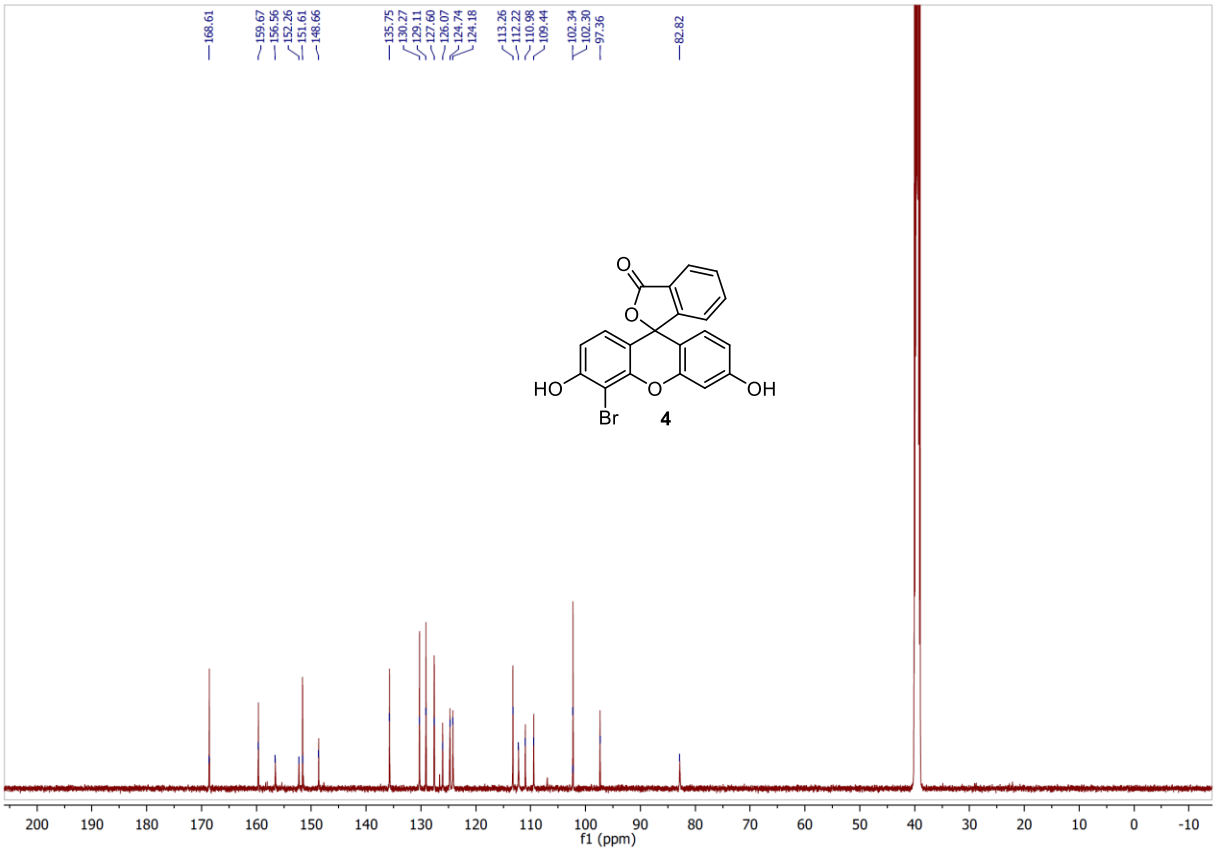
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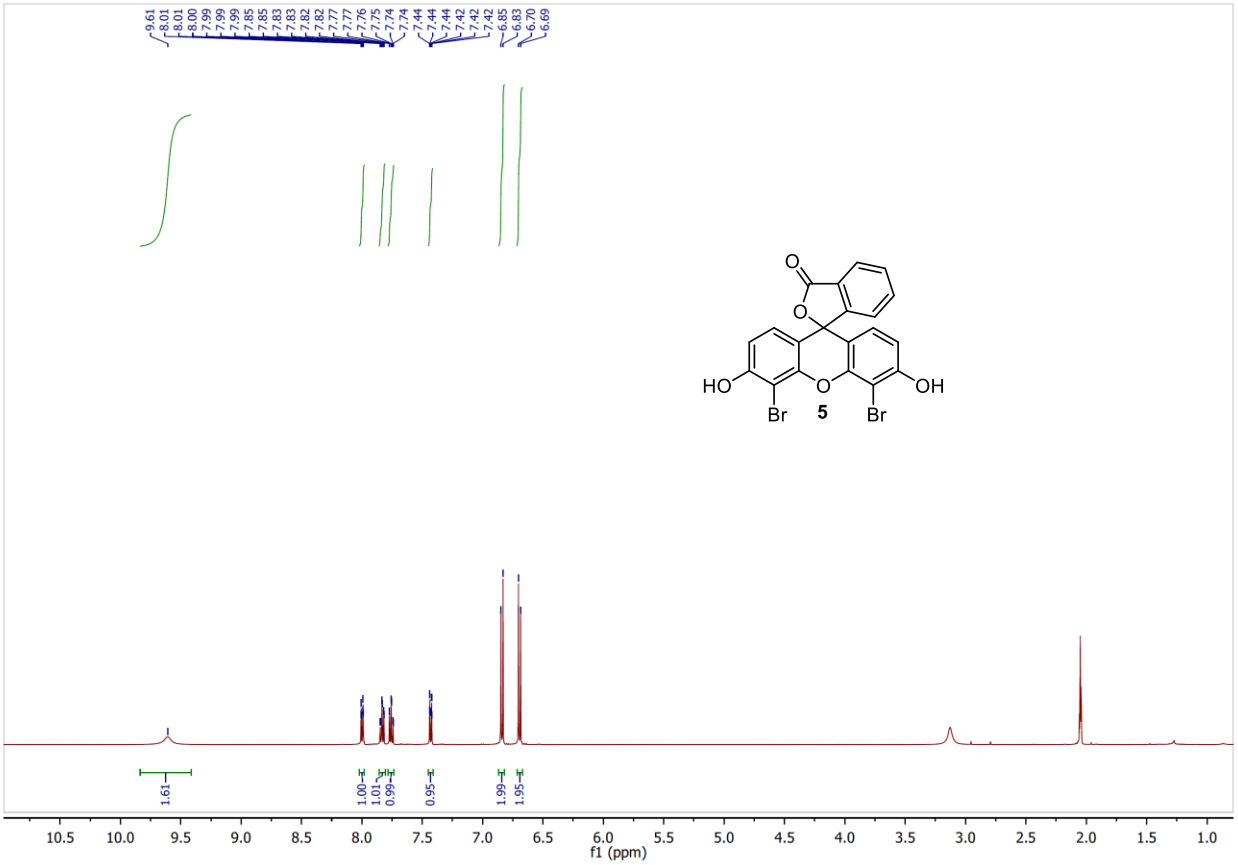
OH
Br
4
f1 (ppm)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7.44
7.44
7.42
7.42
7.42
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6.83
6.70
6.69
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1.01
0.99
0.95
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10.5
10.0
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8.0
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5.0
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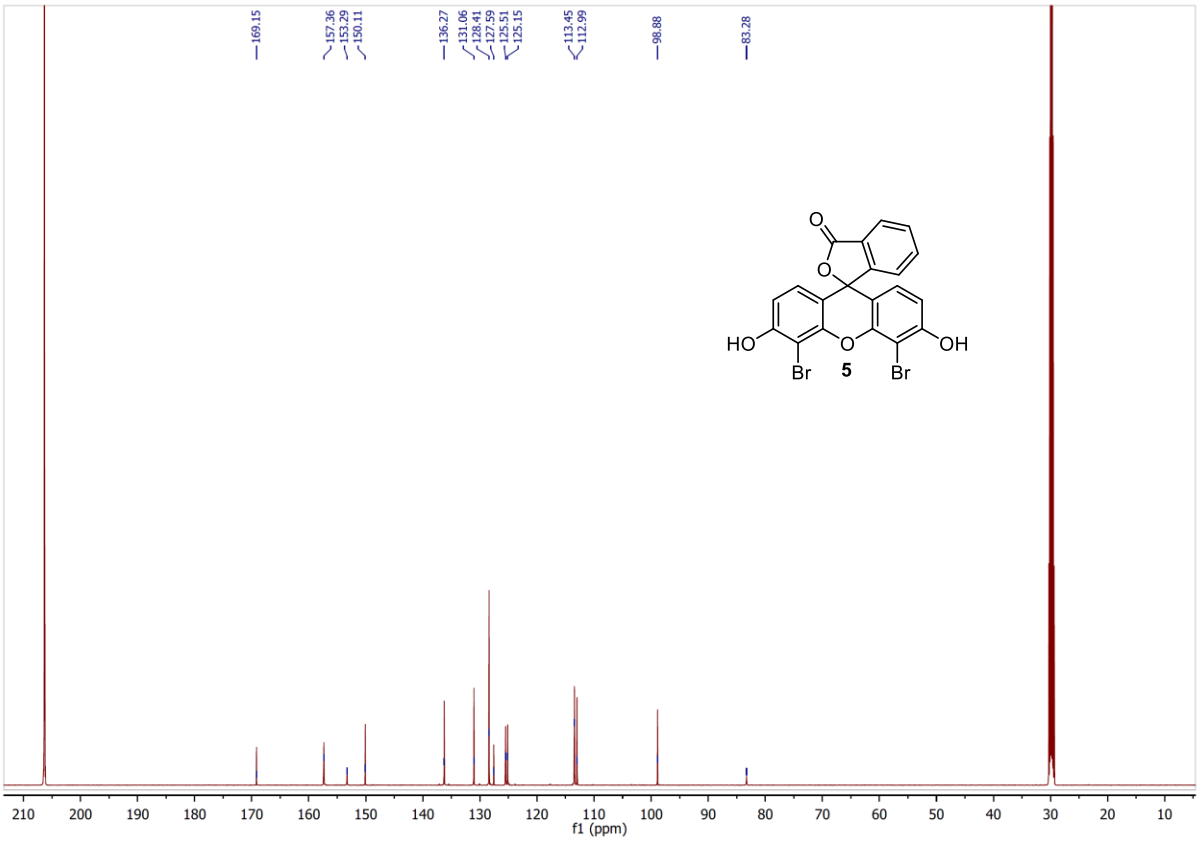
f1 (ppm)
HO
OH
Br
Br
5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150
140
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f1 (ppm)
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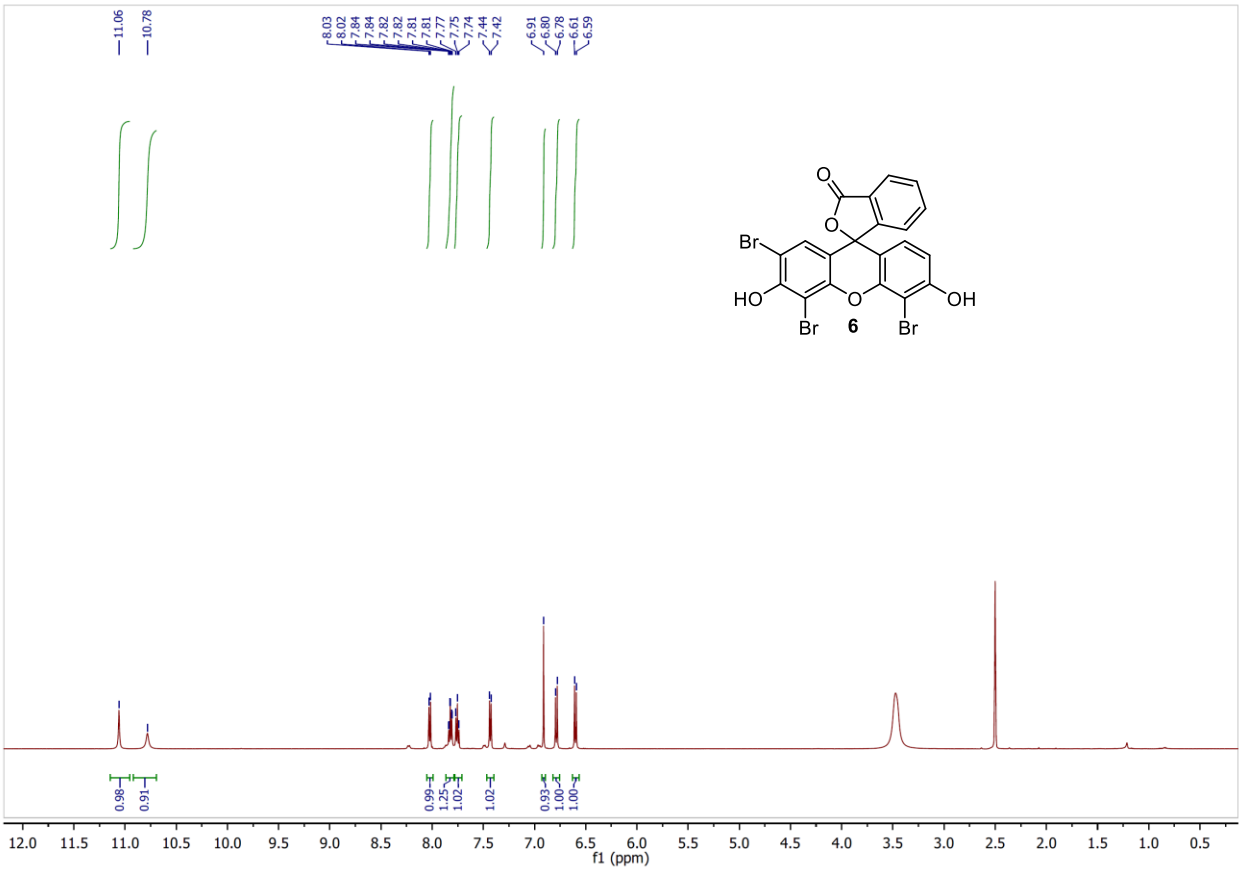
OH
Br
Br
5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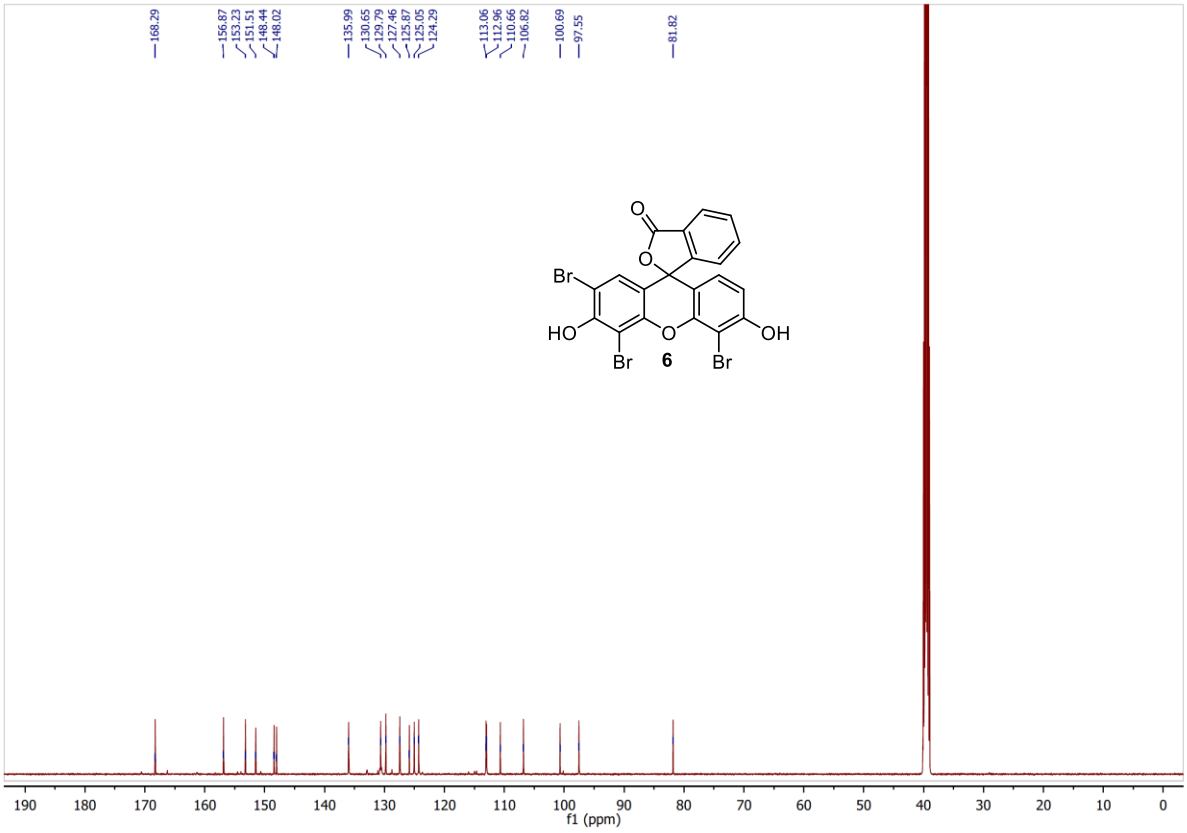
7.42
6.91
6.80
6.78
6.61
6.59
O
O
Br
HO
O
OH
Br
6
Br
0.98
0.91
0.99
1.25
1.02
1.02
0.93
1.00
1.00
12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)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113.06
112.96
110.66
106.82
100.69
97.55
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Br
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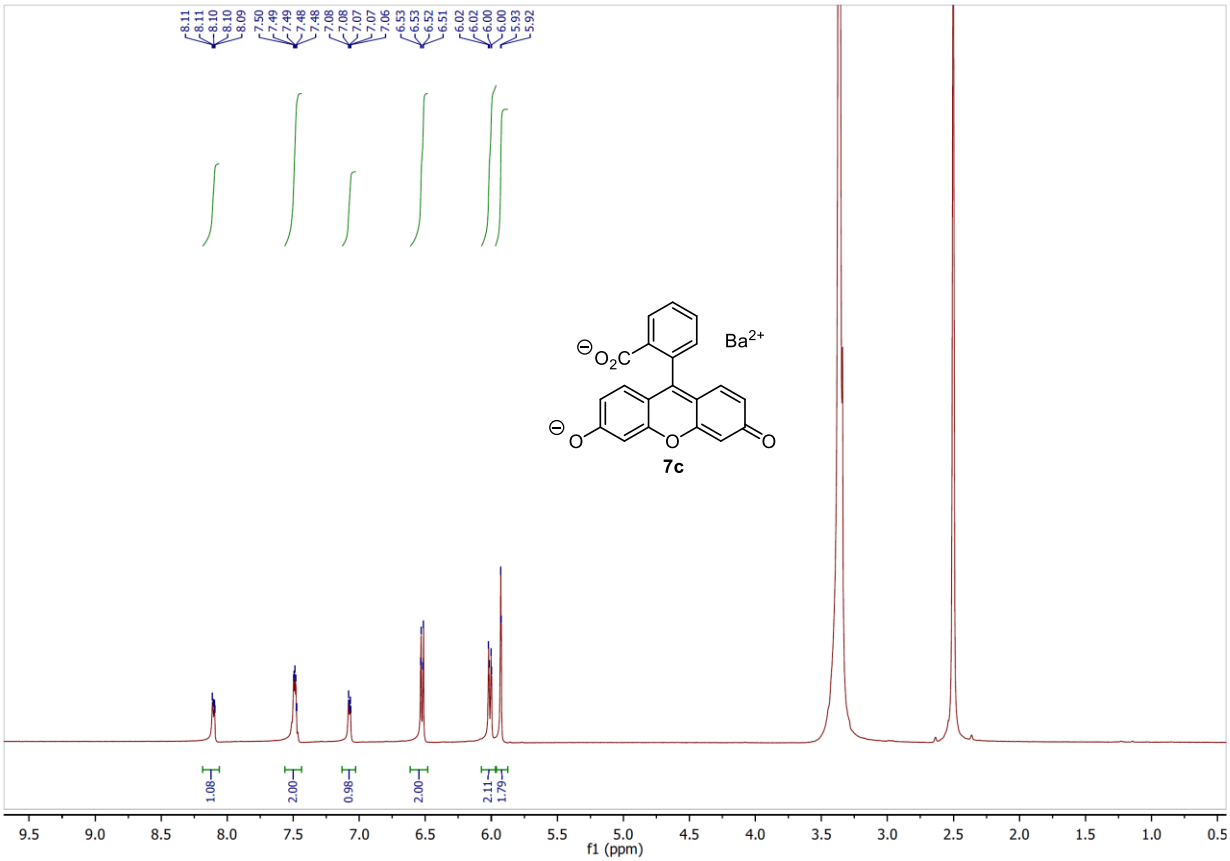
f1 (ppm)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7c
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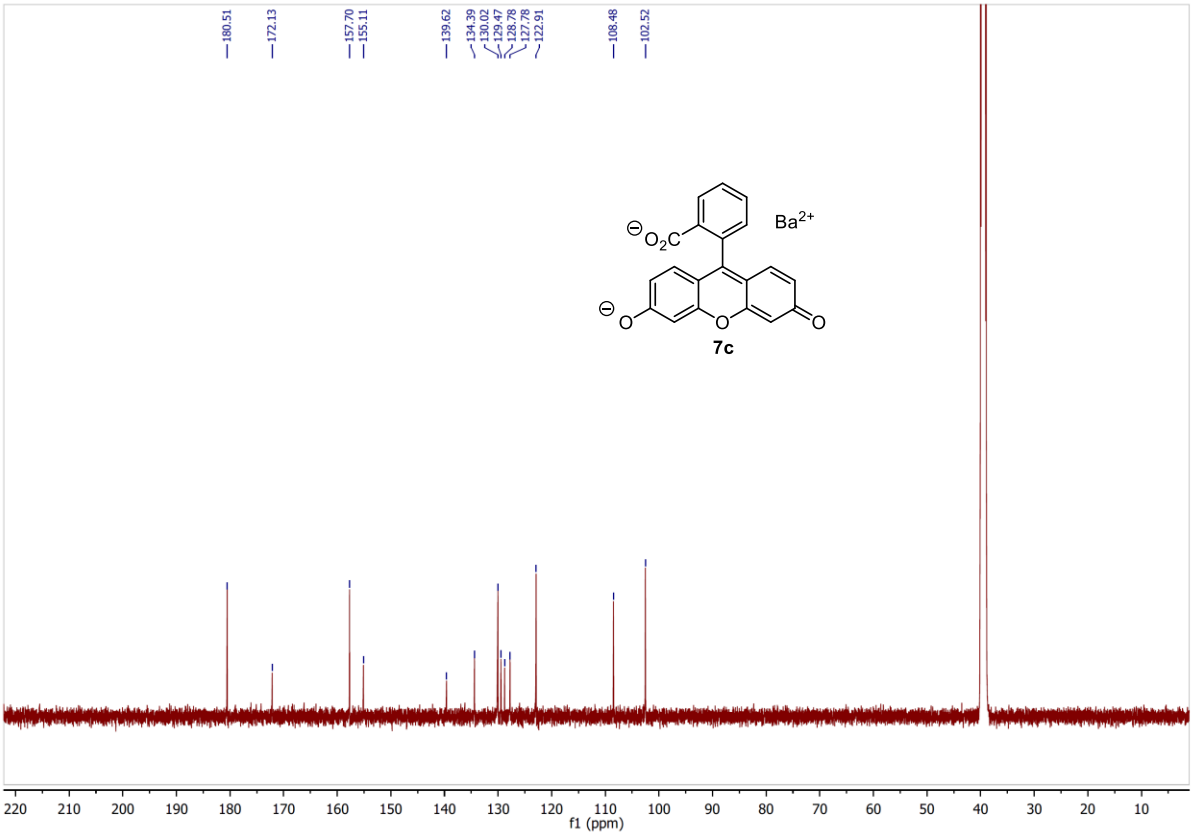
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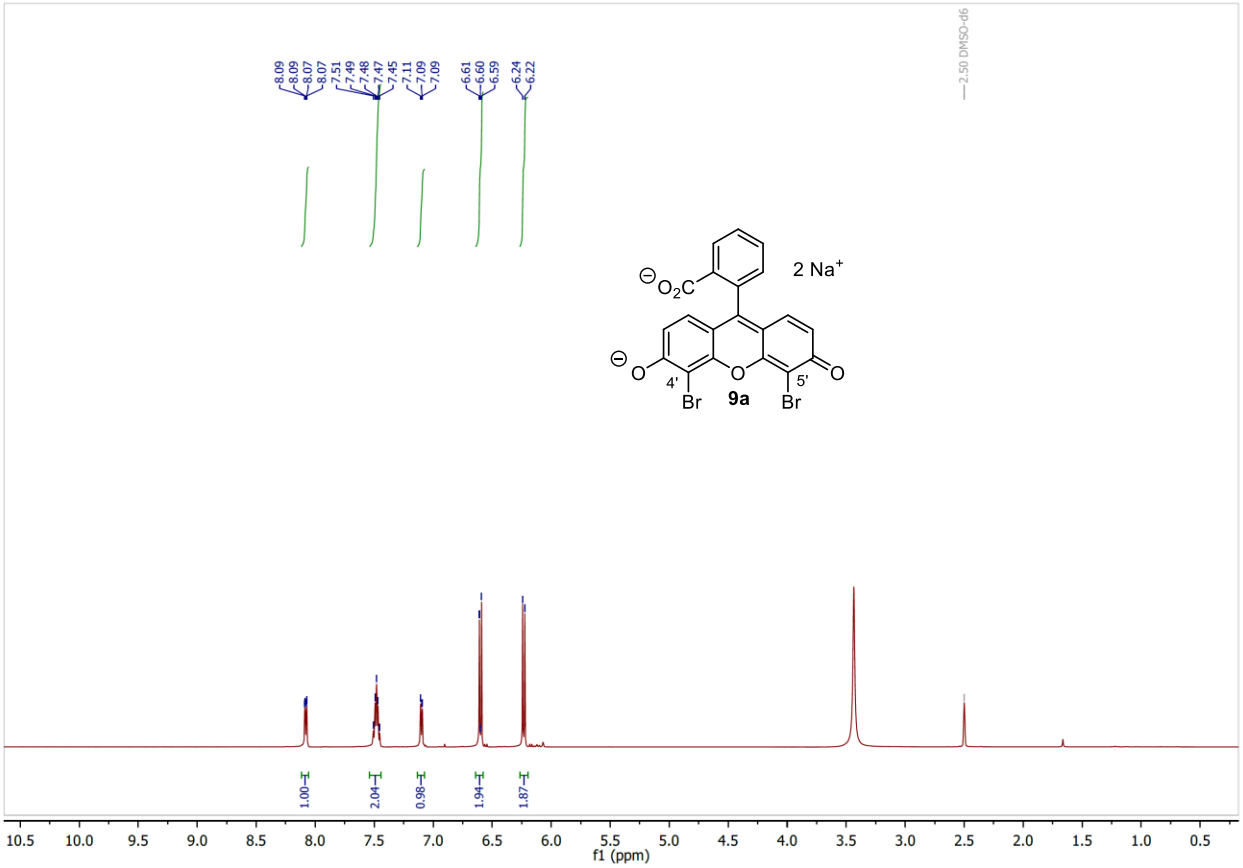
Ba2+
7c
f1 (ppm)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6.22
2.50 DMSO-d6
2 Na+
O2C
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Br
9a
5'
Br
O
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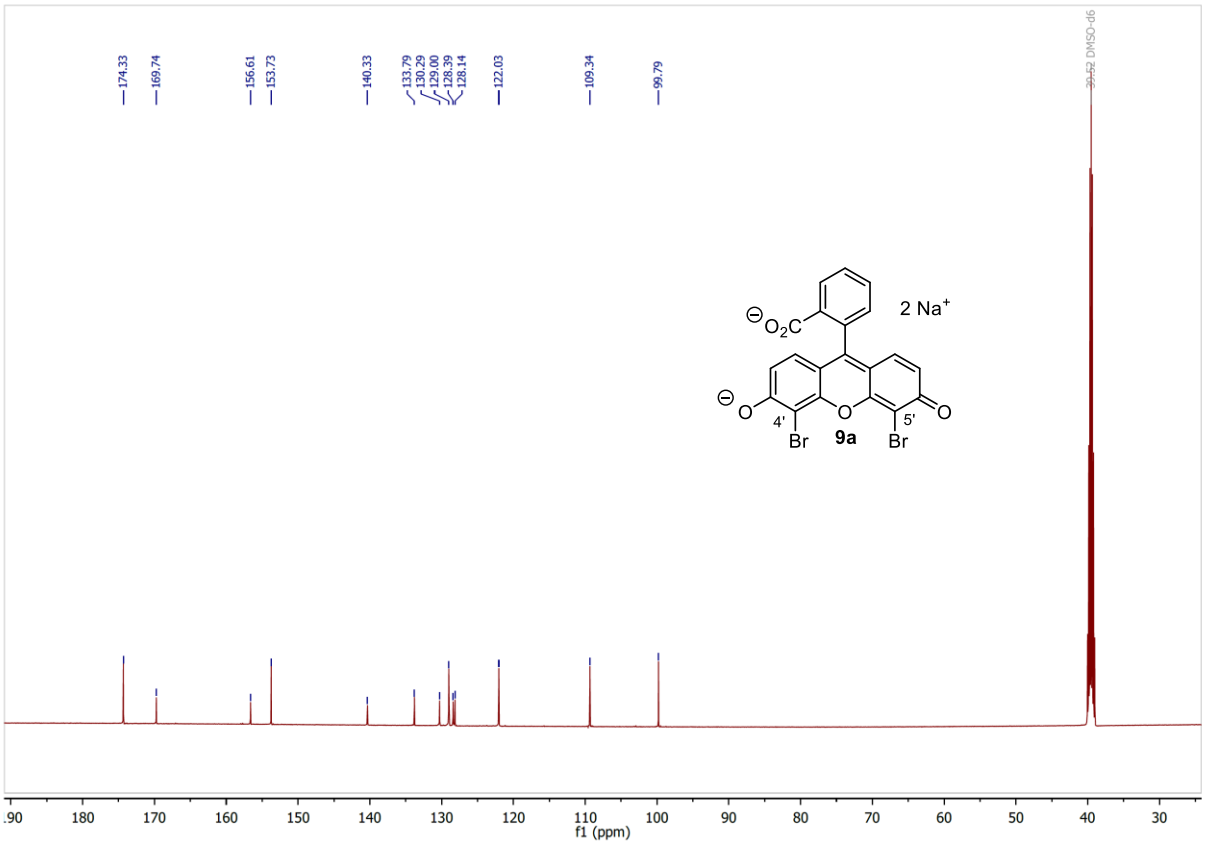
0.98
1.94
1.87
f1 (ppm)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Br
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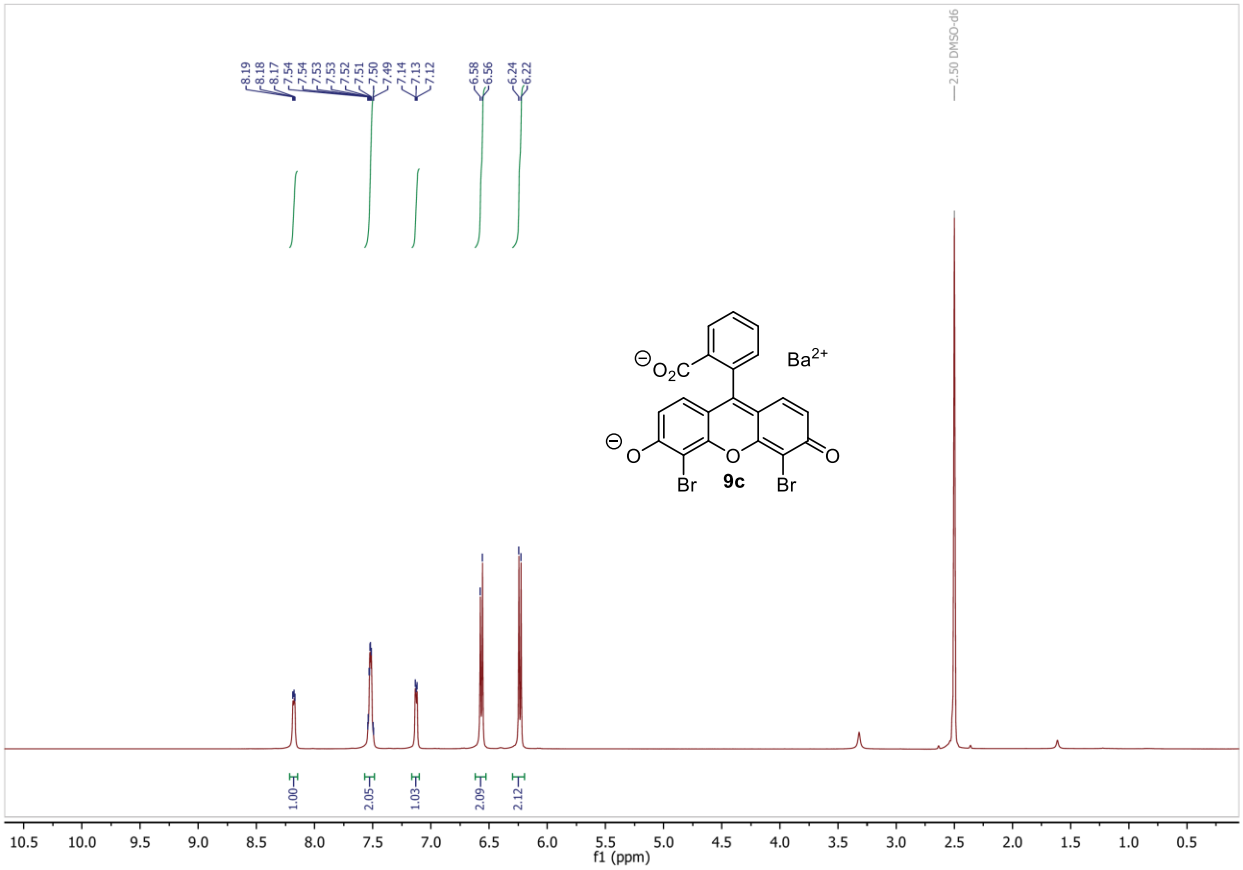
f1 (ppm)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6.22
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9c
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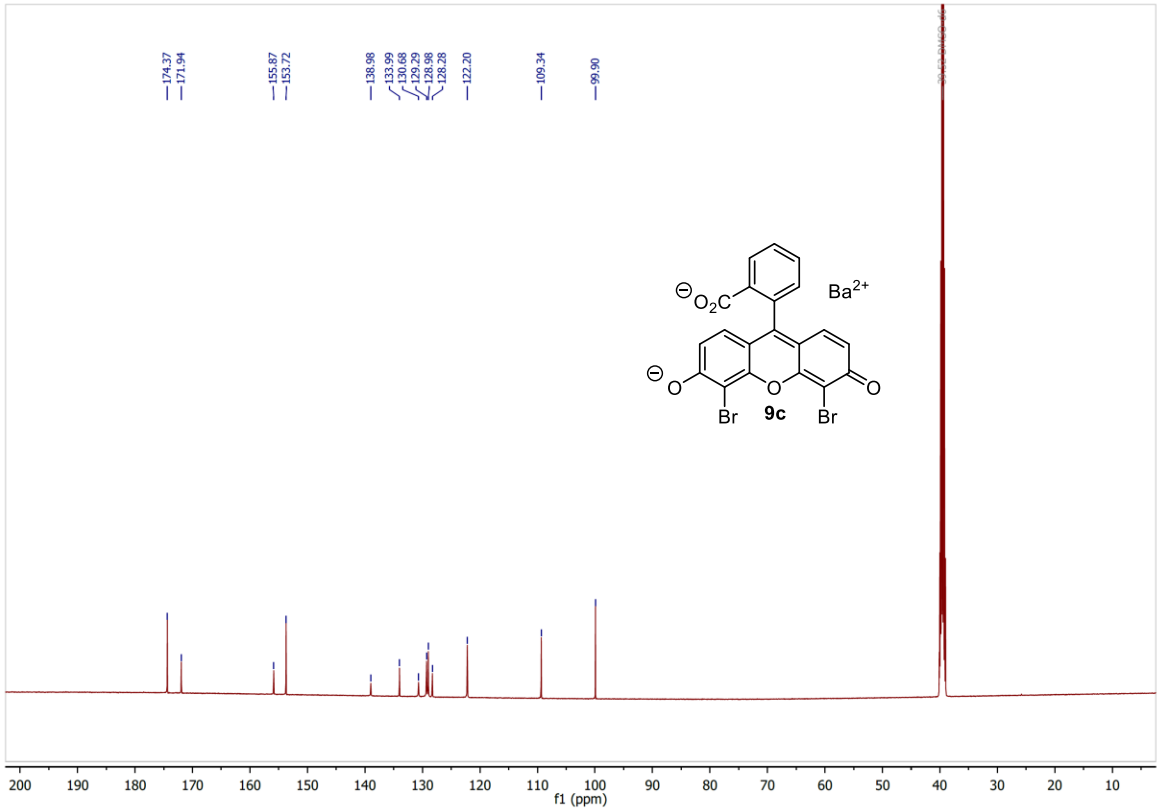
f1 (ppm)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Br
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9c
f1 (ppm)

11. Literature

[1] C. Anselmi, D. Capitani, A. Tintaru, B. Doherty, A. Sgamellotti, C. Miliani, *Dyes Pigments* **2017**, *140*, 297-311.

[2] Q. Xu, K.-A. Lee, S. Lee, K. M. Lee, W.-J. Lee, J. Yoon, *J. Am. Chem. Soc.* **2013**, *135*, 9944–9949.

[3] M. Busse, M. Müller, M. A. Kimm, S. Ferstl, S. Allner, K. Achterhold, J. Herzen, F. Pfeiffer, *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115*, 2293-2298.