

Supporting information

Development of Bifunctional Fluorescent Probes and their Application to a-Helix Labelling

Laszlo Kondacs^{1,2}, David R. Trentham², Thomas Kampourakis^{*,3} and Alexander J. A. Cobb^{*,1}

¹Department of Chemistry, King's College London, 7 Trinity Street, London SE1 1DB, UK

²Randall Centre for Cell and Molecular Biophysics and British Heart Foundation Centre of Research Excellence,

³College of Medicine, University of Kentucky, 900 S. Limestone Street, William R. Willard Medical Education Building, MN 150, Lexington KY, USA 40536-0298

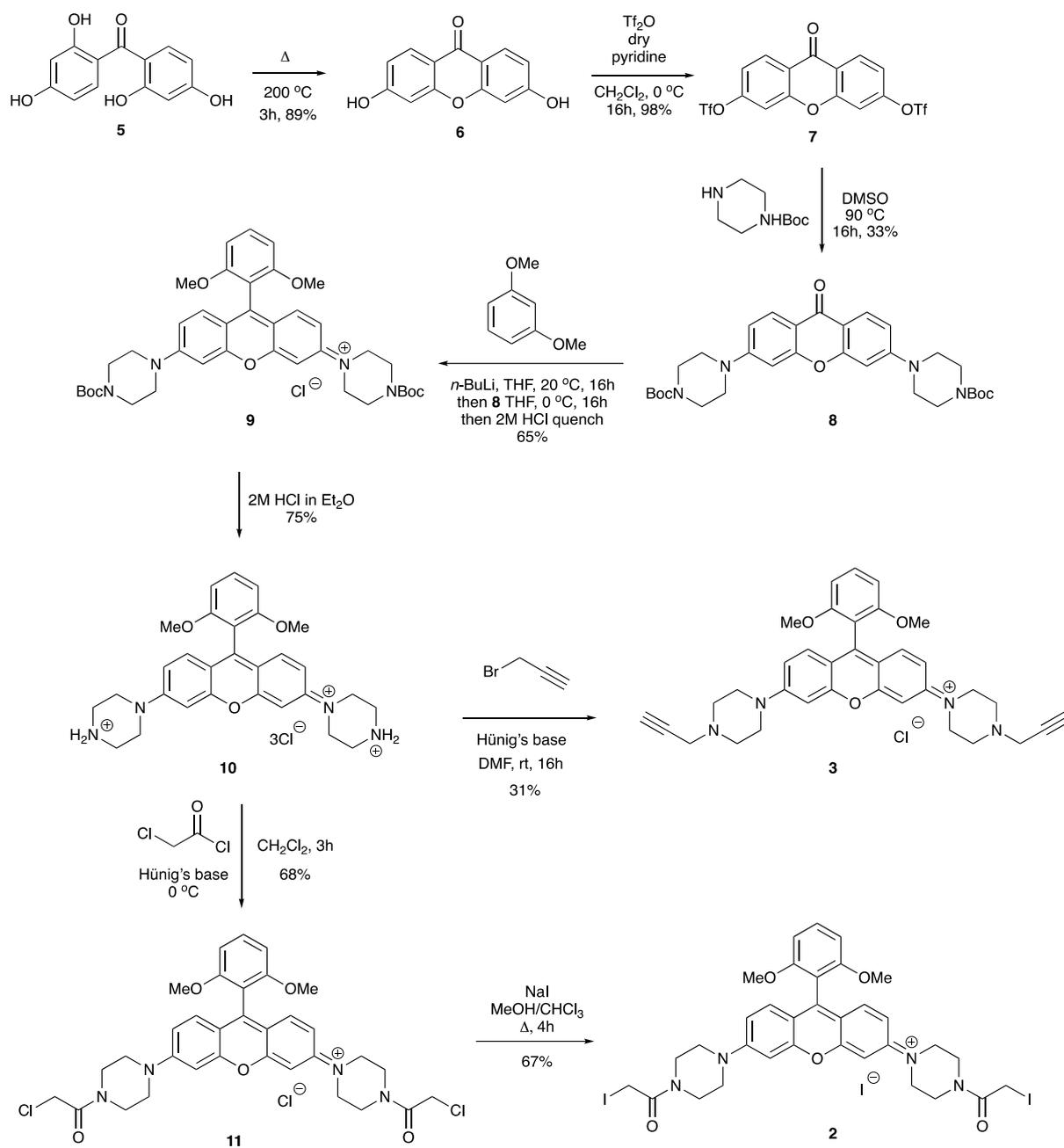
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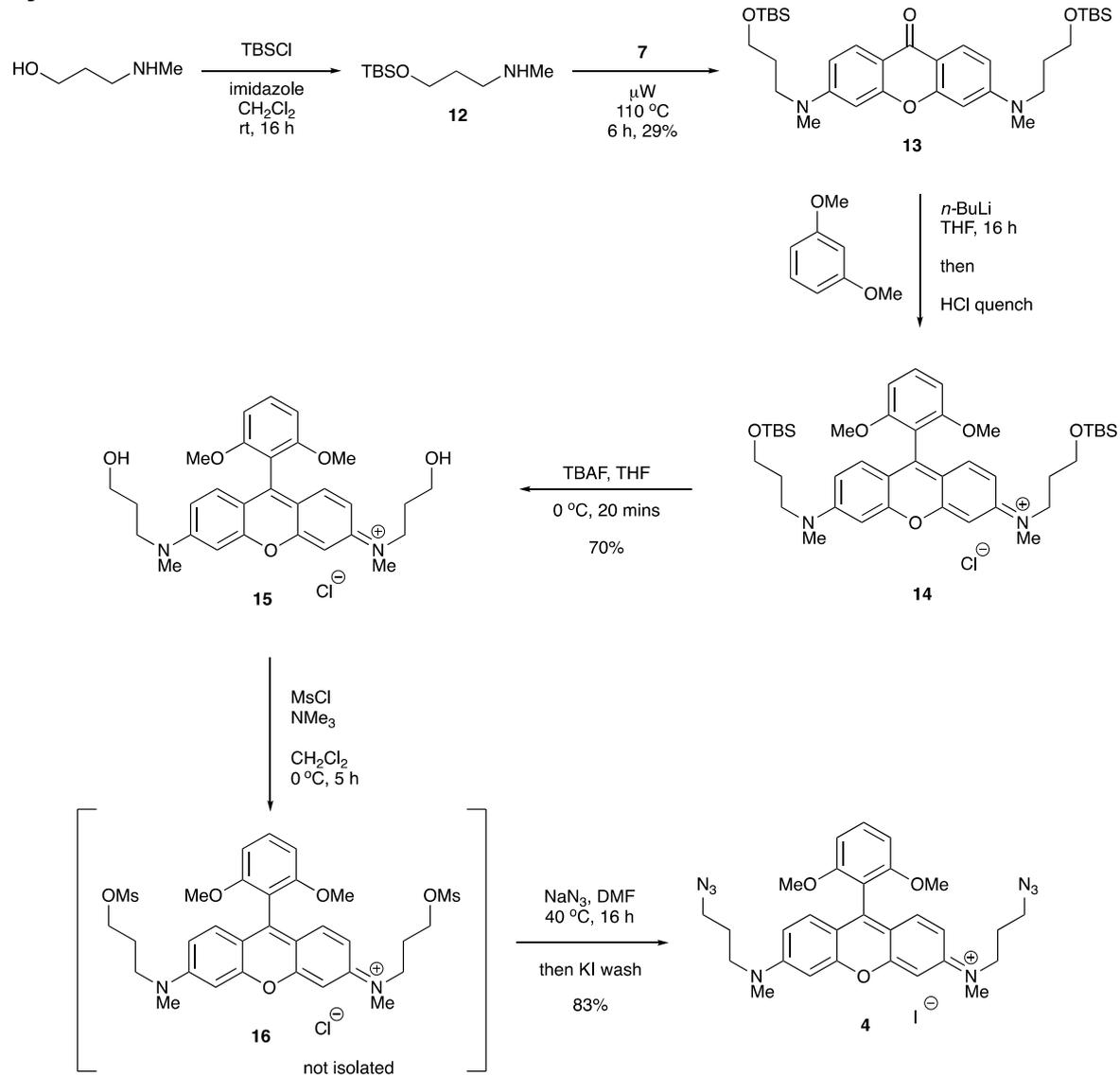
Chemical Synthesis

All commercially available reagents and solvents were obtained from Sigma-Aldrich, Apollo Scientific, Alfa Aesar, Fluorochem, VWR or Fischer Scientific and were used without further purification. Melting points were recorded on Stuart SMP30 melting point apparatus and are uncorrected. Infrared spectra were recorded in the range 4000 – 600 cm⁻¹ using a Shimadzu IRAffinity-1S FTIR Spectrophotometer as a thin film. Bruker Ascend 400 at 400 MHz for ¹H spectra or at 100 MHz for ¹³C spectra. Chemical shifts (δ) are quoted in parts per million (ppm) using the abbreviations: s: singlet; d: doublet; dd: double of dublets; t: triplet; quart: quartet; quint: quintet. Signals that could not be interpreted were designated multiplets (m) or broad (br). Coupling constants *J* are quoted in Hz. High resolution accurate mass measurements were collected by King's College London mass spec service with an Acquity UPLC coupled to a Xevo G2-XS QTOF. UV – VIS measurements were carried out by PerkinElmer LAMBDA 465 UV/Vis Spectrophotometer in quartz cuvettes. Fluorescence was measured with an Agilent Cary Eclipse Fluorescence Spectrophotometer in quartz cuvettes. VWR silica gel 60 (40-63 micron) was used for column chromatography; the samples were pre-absorbed onto silica 60 (40-63 micron). Teledyne ISCO CombiFlash Rf+ was used for flash chromatography. Air and moisture sensitive reactions were carried out in oven dried glassware under a nitrogen atmosphere. Microwave reactions were carried out in a Biotage Initiator+ microwave synthesizer. Thin layer chromatography was performed on VWR silica gel 60 F254. Thin layer chromatography results were analysed by UV lamp (254 nm), ninhydrin stain (amine, amide content) and/or ceric ammonium molybdate stain.

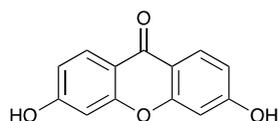
Synthetic Scheme for Rosamine Probes 2 and 3



Synthetic Scheme for Rhodamine Probe 4

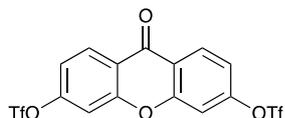


3,6-dihydroxy-9H-xanthen-9-one **6**¹



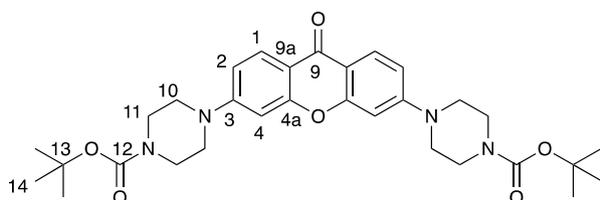
A suspension of 2,2',4,4'-tetrahydroxybenzophenonein **5** (1.60 g, 6.80 mmol) in distilled water (20 mL) was stirred and heated for 3 h at 200 °C in a microwave reactor. After the reaction mixture was cooled down to room temperature, it was filtered and washed with hot water (80 - 90 °C) to give the pure product as a peach-coloured fluffy solid (1.38 g, 89%). Mp: 358 °C (decomposed); ¹H NMR (400 MHz, DMSO-d₆) δ_H 10.85 (2H, br s, 3-OH), 7.99 (2H, d, *J* 8.8, 4-H), 6.86 (2H, dd, *J* 8.8, 2.4, 2-H), 6.82 (2H, d, *J* 2.0, 1-H); ¹³C NMR (101 MHz, DMSO-d₆) δ_C 174.4 (9-C), 163.8 (3-C), 157.9 (4a-C), 128.2 (1-C), 114.5 (9a-C), 114.1 (2-C), 102.6 (4-C); IR : ν_{max}/cm⁻¹ 3127 (O-H), 1612 (C=O), 1247 (C-O); HRMS : Found (M+H)⁺ 229.0515. Calcd. for C₅H₁₁ON₆: (M+H)⁺ 228.0495.

9-oxo-9H-xanthene-3,6-diyl bis(trifluoromethanesulfonate) **7**¹



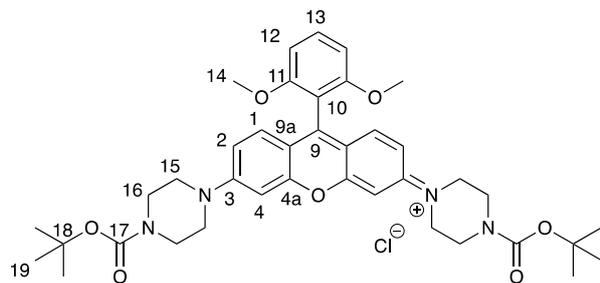
To a solution of 3,6-dihydroxy-9H-xanthen-9-one **6** (3.32 g, 14.6 mmol) in dichloromethane (60 mL) at 0 °C was added pyridine (146.0 mmol, 11.8 mL) and the mixture was stirred for 15 minutes. Triflic anhydride (5.88 mL, 35.0 mmol) was added dropwise to the reaction mixture at 0 °C, and the resulting solution stirred overnight at room temperature. After the reaction was complete, it was quenched with 1M aqueous HCl solution (30 mL). The organic phase was washed with brine (2 x 40 mL), dried over MgSO₄, and evaporated under reduced pressure to give the product as butter-coloured crystals (7.06 g, 98%). Mp: 113 - 115 °C). ¹H NMR (400 MHz, CDCl₃) : δ_H 8.47 (2H, d, *J* 8.8 Hz, 1-H), 7.52 (2H, d, *J* 2.4 Hz, 4-H), 7.38 (2H, dd, *J*=8.8 Hz, *J*=2.4 Hz, 2-H); ¹³C NMR (101 MHz, CDCl₃) δ_C 174.5 (9-C), 156.6 (4a-C), 153.4 (3-C), 129.6 (1-C), 121.4 (9a-C), 118.7 (*J*_{C-F}=322.2, 12-C), 118.2 (2-C), 111.4 (4-C); ¹⁹F NMR (376 MHz, CDCl₃) δ_F 72.5 (12-F); ν_{max}/cm⁻¹ 1664 (C=O), 1611 (C=O), 1420 (S=O), 1206 (C-O), 1134 (S=O), 1119 (C-F); HRMS: Found (M+H)⁺ 492.9470. Calcd. for C₁₅H₆F₆O₈S₂: (M+H)⁺ 492.9487.

Di-tert-butyl 4,4'-(9-oxo-9H-xanthene-3,6-diyl)bis(piperazine-1-carboxylate) **8**²



To the solution of 9-oxo-9H-xanthene-3,6-diyl bis(trifluoromethanesulfonate) **7** (3.00 g, 6.1 mmol) in DMSO (30 mL), was added Boc-piperazine (11.35 g 61.0 mmol) in one portion and the resulting mixture heated overnight at 90 °C. After the reaction was complete, it was cooled to room temperature and quenched with saturated aqueous Na₂CO₃ solution (30 mL). The precipitate was filtered, washed with water and dried to give the crude product as yellow solid (8.00 g). The crude was purified by recrystallization from ethyl acetate and hexane solvent mixture to give the title product as yellow crystals (1.15 g, 33%). Mp: 217 - 219 °C; ¹H NMR (400 MHz, CDCl₃) δ_H 8.16 (2H, d, *J* 8.8, 1-H), 6.89 (2H, dd, *J* 8.8, 2.0, 2-H), 6.69 (2H, *J* 2.0, 4-H), 3.63 (8H, t, *J* 4.8, 11-H), 3.40 (8H, t, *J* 5.2, 10-H), 1.51 (18H, s, 14-H). ¹³C NMR (101 MHz, CDCl₃) δ_C 175.1 (9-C), 158.1 (4a-C), 155.0 (3-C), 154.6 (12-C), 127.8 (1-C), 114.0 (9a-C), 111.6 (2-C), 100.3 (4-C), 80.3 (13-C), 47.4 (10-C), 43.0 (br s, 11-C), 28.4 (14-C); ν_{max}/cm⁻¹ 1676 (C=O), 1606 (C=O), 1233 (C-O); HRMS (Found (M+H)⁺ 565.3098. Calcd. for C₃₁H₄₁O₆N₄: (M+H)⁺ 565.3026.).

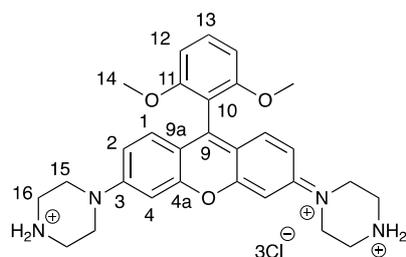
4-(tert-butoxycarbonyl)-1-(6-(4-(tert-butoxycarbonyl)piperazin-1-yl)-9-(2,6-dimethoxyphenyl)-3H-xanthen-3-ylidene)piperazin-1-ium chloride **9**²



To the solution of 1,3-dimethoxybenzene (0.49 g, 3.50 mmol) in dry tetrahydrofuran (5 mL) at 0 °C under a nitrogen atmosphere, was added dropwise 2.5 M solution of *n*-butyl lithium in tetrahydrofuran (1.4 mL, 3.50 mmol), and the resulting solution stirred overnight at room temperature to give suspension A, which was then added to a

solution of di-tert-butyl 4,4'-(9-oxo-9H-xanthene-3,6-diyl)bis(piperazine-1-carboxylate) **8** (0.40 g, 0.71 mmol) in dry tetrahydrofuran (10 mL) at 0 °C under nitrogen atmosphere with a canule. The orange solution generated was stirred overnight at room temperature. After the reaction was complete, it was quenched with 2 M HCl (1.8 mL), and dichloromethane added (30 mL). The organic layer was washed with water (30 mL), brine (30 mL), dried over MgSO₄, filtered and the solvent evaporated under reduced pressure to give the crude product as a dark purple solid (0.87 g). This crude compound was purified by column chromatography (eluent: dichloromethane – methanol, 0% - 8%) to give the pure product as a dark solid with a golden shine (0.33 g, 65%). Mp: 215 °C (decomposed); ¹H NMR (400 MHz, CDCl₃) δ_H 7.50 (1H, t, *J* 8.4, 13-H), 7.31 (2H, d, *J* 9.6, 1-H), 7.23 (2H, dd, *J* 9.6, 2.4 Hz, 2-H), 7.14 (2H, d, *J*=2.4 Hz, 4-H), 6.73 (2H, d, *J* 8.8, 12-H), 3.81 (8H, br m, 15-H), 3.66 (8H, m, 16-H), 3.64 (6H, s, 14-H), 1.47 (18H, s, 19-H); ¹³C NMR (101 MHz, CDCl₃) δ_C 158.4 (3-C), 157.5 (11-C), 157.1 (4a-C), 155.4 (9-C), 154.5 (17-C), 132.6 (13-C), 132.1 (1-C), 115.6 (9a-C), 115.1(2-C), 108.5 (10-C), 104.2 (10-C), 97.9 (4-C), 80.7 (18-C), 56.0 (14-C), 47.1 (15-C), 43.1 (16-C), 28.4 (19-C); ν_{max}/cm⁻¹ 1701 (C=O), 1342 (C-N), 1220 (C-O); HRMS (Found (M+H)⁺ 686.3690. Calcd. for C₃₉H₅₀O₇N₄: (M+H)⁺ 686.3669.).

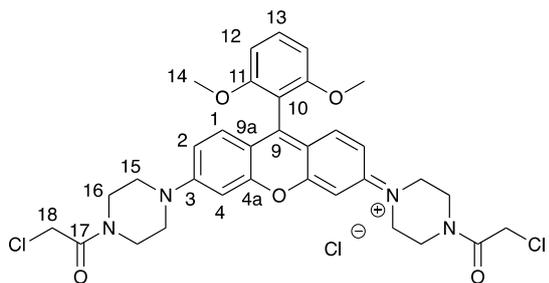
1-(9-(2,6-dimethoxyphenyl)-6-(piperazin-1-ium-1-yl)-3H-xanthen-3-ylidene)piperazine-1,4-diiium chloride **10**



4-(tert-butoxycarbonyl)-1-(6-(4-(tert-butoxycarbonyl)piperazin-1-yl)-9-(2,6-dimethoxyphenyl)-3H-xanthen-3-ylidene)piperazin-1-ium chloride **9** (0.34, 0.47 mmol) was dissolved in a solution of 2M HCl in diethyl ether (10 mL), and stirred overnight. After the reaction was complete, the precipitate was collected by filtration, and washed with diethyl ether (20 mL) to give the product as a red solid (0.21 g, 75%). Due to its slight hygroscopic nature

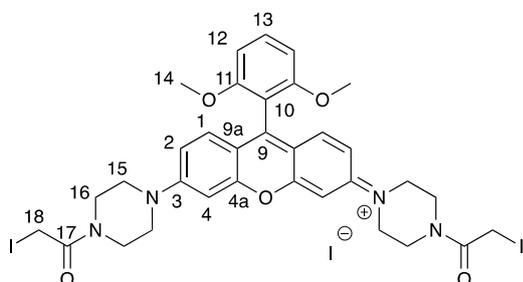
it was dissolved in water, and freeze dried for easier handling. Mp: 259 °C (melted and decomposed); ¹H NMR (400 MHz, MeOD-*d*₄) δ_H 7.68 (1H, t, *J* 8.8, 13-H), 7.47 (2H, d, *J* 10.0, 2-H), 7.40 (2H, m, 1-H), 7.38 (2H, m, 4-H), 6.97 (2H, d, 8.8, 12-H), 4.11 (8H, t, *J* 5.2, 15-H), 3.70 (6H, s, 14-H), 3.49 (8H, t, *J* 5.2, 16-H); ¹³C NMR (101 MHz, MeOD-*d*₄) δ_C 159.8 (3-C), 158.8 (11-C), 158.5 (4a-C), 134.1 (13-C), 133.2 (4-C), 117.0 (9a-C), 116.5 (2-C), 109.3 (10-C), 105.3 (12-C), 99.2 (1-C), 56.4 (14-C), 45.0 (15-C), 43.9 (16-C); ν_{max}/cm⁻¹ 3365 (N-H), 1580 (N-H), 1240 (C-O), 1180 (C-N); HRMS: Found (M+H)²⁺ 243.1334. Calcd. for C₂₉H₃₄O₃N₄: (M+H)²⁺ 243.1310; Found (M)⁺ 485.2576. Calcd. For C₂₉H₃₃O₃N₄: (M)⁺ 485.2547.

4-(2-chloroacetyl)-1-(6-(4-(2-bromo/chloroacetyl)piperazin-1-yl)-9-(2,6-dimethoxyphenyl)-3H-xanthen-3-ylidene)piperazin-1-ium chloride **11**



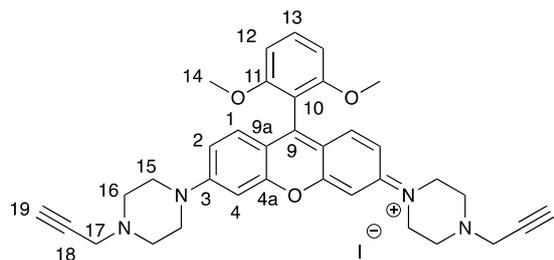
To a solution of compound **10** (0.20 g, 0.36 mmol) in dichloromethane (15 mL) and diisopropylethylamine (0.49 mL, 2.9 mmol) at 0 °C, was added chloroacetyl chloride (0.12 mL, 0.14 mmol) dropwise. The resulting mixture was stirred for 3 hours whilst warming up to room temperature. After the reaction was over, a mixture of chloroform and methanol (9:1 ratio) (45 mL) and 0.5 M HCl (45 mL) were added to the reaction mixture. The aqueous phase was extracted with a further mixture of chloroform and methanol (9:1 ratio) (2 x 45 mL). The combined organic layers were washed with saturated NaHCO₃ solution (2 x 30 mL), brine (2 x 30 mL), dried over MgSO₄, filtered and concentrated under reduced pressure. The crude material (0.64 g) was purified by flash chromatography (eluent: dichloromethane – methanol 0 – 20%) to give the title compound as a dark purple solid (164 mg, 68%). Mp: 123 °C (melted and decomposed); ¹H NMR (400 MHz, CDCl₃) δ_H 7.54 (1H, t, *J* 8.4, 13-H), 7.33 (2H, d, *J* 9.6, 1-H), 7.22 (2H, dd, *J* 2.4, *J* 9.2, 2-H), 7.15 (2H, d, *J* 2.4, 4-H), 6.74 (2H, d, *J* 7.6, 12-H), 4.24 (4H, s, 18-H), 4.04 (4H, m, 15a-H), 3.92 (4H, m, 16a-H), 3.83 (4H, m, 16b-H), 3.80 (4H, m, 15b-H), 3.65 (6H, s, 14-H). ¹³C NMR (101 MHz, CDCl₃) δ_C 165.9 (17-C), 158.4 (4a-C), 157.5 (11-C), 157.1 (3-C), 156.0 (9-C), 132.7 (13-C), 132.2 (1-C), 115.7 (9a-C), 115.1 (2-C), 108.3 (10-C), 104.2 (12-C), 98.0 (4-C), 56.0 (14-C), 47.2 (15a-C), 46.4 (15b-C), 45.2 (16a-C), 41.5 (16b-C), 41.2 (18-C); ν_{max}/cm⁻¹ 1643 (C=O), 1015 (C-O), 779 (C-Cl); HRMS: Found (M)⁺ 637.1987. Calcd. For C₃₃H₃₅O₅N₄Cl₂: (M)⁺ 637.1979.

1-(9-(2,6-dimethoxyphenyl)-6-(4-(2-iodoacetyl)piperazin-1-yl)-3H-xanthen-3-ylidene)-4-(2-iodoacetyl)piperazin-1-ium iodide **2**



To the solution of compound **20** (0.14 g, 0.21 mmol) in methanol (4 mL) and chloroform (9 mL) mixture sodium iodide (1.56 g, 10.4 mmol) was added and the mixture was heated at reflux temperature for 4 hours under nitrogen atmosphere. After the reaction was complete, it was diluted with chloroform and methanol mixture (100 mL, ratio: 4:1). The solution was washed with 5% sodium ascorbate (30 mL), 1 M potassium phosphate buffer (3 x 30 mL, pH=7), and after the addition of methanol (1.5 mL) once more with 1 M potassium phosphate buffer (30 mL). The organic phase was dried over Na₂SO₄ and the solvent was removed under reduced pressure. The residue was triturated with diethyl ether to give the pure product as metallic purple solid (0.13 g, 67%). Mp: 190 °C (melted and decomposed); ¹H NMR (400 MHz, CDCl₃) δ_H 7.49 (1H, t, *J* 10.8, 13-H), 7.28 (2H, d, *J* 9.2, 1-H), 7.12 (2H, dd, *J* 2.4, 9.2, 2-H), 7.09 (2H, d, *J* 2.0 Hz, 4-H), 6.70 (2H, d, *J* 8.8, 12-H), 3.98 (4H, m, 15a-H), 3.82 (4H, s, 18-H), 3.80 (8H, m, 16-H), 3.73 (4H, m, 15b-H), 3.60 (6H, s, 14-H); ¹³C NMR (101 MHz, CDCl₃) δ_C 167.3 (17-C), 158.4 (4a-C), 157.5 (11-C), 157.0 (3-C), 156.1 (9-C), 132.8 (13-C), 132.2 (1-C), 115.7 (9a-C), 115.1 (2-C), 108.3 (10-C), 104.3 (12-C), 98.1 (4-C), 56.1 (14-C), 46.9 (15a-C), 46.4 (15b-C), 46.2 (16a-C), 41.5 (16b-C), -3.44 (18-C); ν_{max}/cm⁻¹ 1636 (C=O), 1224 (C-O); HRMS: Found (M)⁺ 821.0749. Calcd. for C₃₃H₃₅I₂N₄O₅: (M)⁺ 821.0691; UV/VIS (10.7 μM in water and 1 % DMSO): λ_{max} = 553 nm, A₂ = 0.97; Fluorescence (1.07 μM in water and 0.1% DMSO): λ_{ex} = 554 nm, Intensity_{ex} = 191 a.u.; λ_{em} = 580 nm, Intensity_{em} = 197 a.u.

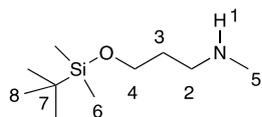
1-(9-(2,6-dimethoxyphenyl)-6-(4-(prop-2-yn-1-yl)piperazin-1-yl)-3H-xanthen-3-ylidene)-4-(prop-2-yn-1-yl)piperazin-1-ium chloride **3**



To the solution of compound **10** (0.25 g, 0.44 mmol) in DMF (5 mL) was added N,N-diisopropylethylamine (0.43 mL, 2.49 mmol) and the resulting solution stirred at room temperature for 20 minutes. Propargyl bromide (0.12 mL, 1.07 mmol) was then added and the mixture stirred overnight at room temperature. After the reaction was complete, ethyl acetate (20 mL) and

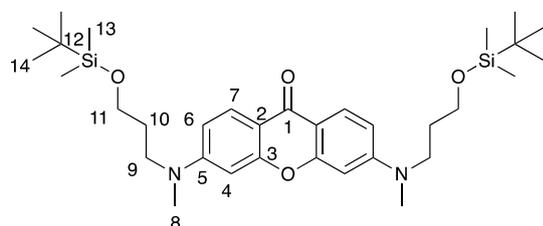
saturated NaHCO₃ solution (20 mL) were added, and the aqueous phase washed with a mixture of isopropanol and dichloromethane (5 – 15 mL x 2). The combined organic phases were dried over MgSO₄, filtered and evaporated under reduced pressure. The crude product was purified by flash chromatography (eluent: dichloromethane – methanol, 0 – 20 %) to give the pure product as a metallic purple solid (83 mg, 31%). Mp: 189 - 191°C; ¹H NMR (400 MHz, CDCl₃) δ_H 7.52 (1H, t, *J* 8.4, 13-H), 7.28 (2H, d, *J* 9.2, 1-H), 7.21 (2H, dd, *J* 2.4, 2-H), 7.08 (2H, d, *J* 2.4, 4-H), 6.74 (2H, d, *J* 8.4, 12-H), 3.82 (8H, t, *J* 4.8, 15-H), 3.65 (6H, s, 14-H), 3.38 (4H, d, *J* 2.4, 17-H), 2.76 (8H, t, *J* 4.8, 16-H), 2.26 (2H, t, *J* 2.0, 19-H); ¹³C NMR (101 MHz, CDCl₃) δ_C 158.5 (4a-C), 157.6 (11-C), 157.1 (3-C), 155.1 (9-C), 132.7 (13-C), 132.1 (1-C), 115.5 (9a-C), 115.2 (2-C), 108.6 (10-C), 104.3 (12-C), 97.9 (4-C), 78.0 (18-C), 74.1 (19-C), 56.2 (14-C), 51.5 (16-C), 47.5 (15-C), 46.7 (17-C); ν_{max}/cm⁻¹ 3380 (C-H_{alkyne}), 1096 (C-O); HRMS: Found (M)⁺ 561.2916. Calcd. for C₃₅H₃₇N₄O₃⁺: (M)⁺ 561.2860. UV/VIS (10.7 μM in water and 1 % DMSO): λ_{max} = 555 nm, A₂ = 0.46; Fluorescence (1.07 μM in water and 0.1% DMSO): λ_{ex} = 555 nm, Intensity_{ex} = 70 a.u.; λ_{em} = 581 nm, Intensity_{em} = 71 a.u.

3-((*tert*-butyldimethylsilyl)oxy)-*N*-methylpropan-1-amine **12**



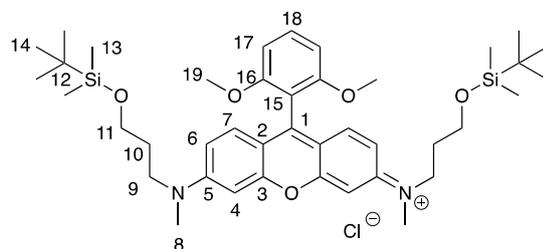
To the solution of 3-(methylamino)propan-1-ol (2.14 mL, 22.0 mmol) in dichloromethane (30 mL) was added TBDMS-chloride (3.73 g, 22.0 mmol) and imidazole (2.09 g, 30.8 mmol), and the resulting solution stirred overnight at room temperature. After this time, the reaction was quenched with water (30 mL), and the aqueous layer was extracted with dichloromethane (3 x 30 mL). The combined organic layers were washed with saturated NaHCO₃ solution (30 mL), brine (30 mL), dried over MgSO₄, filtered and the solvent removed under reduced pressure. The crude material was purified by column chromatography (eluent: ethyl acetate – methanol 10 – 20 % – saturated aqueous NH₃ 1%) to give the pure product as a colourless oil (3.23 g, 72%). ¹H NMR (400 MHz, CDCl₃) δ_H 3.67 (2H t, *J* 6.0, 4-H), 2.66 (2H, t, *J* 7.2, 2-H), 2.42 (3H, s, 5-H), 1.85 (1H, br s, 1-H), 1.70 (2H, quint, *J* 6.8, 3-H), 0.88 (9H, s, 8-H), 0.04 (6-H); ¹³C NMR (101 MHz, CDCl₃) δ_C 61.8 (4-C), 49.4 (2-C), 36.4 (5-C), 32.6 (3-C), 25.9 (8-C), 18.3 (7-C), -5.4 (6-C); ν_{max}/cm⁻¹ 3387 (N-H), 1095 (Si-O); HRMS. Found (M+H)⁺ 204.1824. Calcd. for C₁₀H₂₆NOSi: (M+H)⁺ 204.1778.

3,6-bis((3-((*tert*-butyldimethylsilyl)oxy)propyl)(methyl)amino)-9H-xanthen-9-one **13**



To the solution of 9-oxo-9H-xanthene-3,6-diyl bis(trifluoromethanesulfonate) **7** (1.36 g, 2.8 mmol) in DMSO (14 mL) was added 3-((*tert*-butyldimethylsilyl)oxy)-*N*-methylpropan-1-amine **12** (2.23 g, 11.0 mmol). The solution was stirred and heated at 110 °C in a microwave reactor for 6 hours. After the reaction was complete, water (30 mL) and dichloromethane (40 mL) were added to the mixture, and the aqueous phase extracted with dichloromethane (2 x 40 mL). The combined organic layers were washed with saturated Na₂CO₃ solution (40 mL), brine (40 mL), dried over MgSO₄, filtered and the solvent removed under reduced pressure. The crude was purified by column chromatography (eluent: hexane - ethyl acetate 20%) to give the pure product as pale yellow oil (0.48 g, 29%). ¹H NMR (400 MHz, CDCl₃) δ_H 8.10 (2H, d, *J* 8.8, 7-H), 6.70 (2H, dd, *J* 8.8, 2.4 Hz, 6-H), 6.49 (2H, d, *J* 2.4, 4-H), 3.68 (4H, t, *J* 5.6, 11-H), 3.55 (4H, t, *J* 7.2, 9-H), 3.06 (6H, s, 8-H), 1.82 (4H, quint, *J* 6.0, 10-H), 0.94 (18H, s, 14-H), 0.08 (12H, s, 13-H); ¹³C NMR (101 MHz, CDCl₃) δ_C 175.1 (1-C), 158.3 (2-C), 153.5 (5-C), 127.6 (7-C), 111.8 (3-C), 108.9 (6-C), 96.8 (4-C), 60.1 (11-C), 49.2 (9-C), 38.6 (8-C), 30.1 (10-C), 26.0 (14-C), 18.3 (12-C), -5.4 (13-C); ν_{max}/cm⁻¹ 1601 (C=O), 1119 (Si-O); HRMS: Found (M+H)⁺ 599.3696. Calcd. for C₃₃H₅₄N₂O₄Si₂: (M+H)⁺ 599.3695.

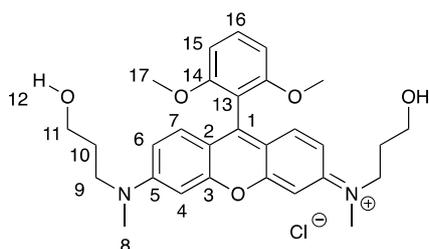
(*E*)-3-((*tert*-butyldimethylsilyl)oxy)-*N*-(6-((3-((*tert*-butyldimethylsilyl)oxy)propyl)(methyl)amino)-9-(2,6-dimethoxyphenyl)-3H-xanthen-3-ylidene)-*N*-methylpropan-1-aminium chloride **14**



To the solution of 1,3-dimethoxybenzene (1.04 g, 7.51 mmol) in dry tetrahydrofuran (10 mL) at 0 °C under nitrogen atmosphere, was added dropwise a 2.5 M solution of *n*-butyl lithium in tetrahydrofuran (3.0 mL, 7.51 mmol). The resulting solution was stirred overnight at room temperature to give a suspension which was added dropwise at 0 °C to a solution of compound **13** (0.90 g, 1.50 mmol) in dry tetrahydrofuran (20 mL) under a nitrogen atmosphere with a cannula. The resulting orange solution was stirred overnight at r.t.. After this time, the reaction was quenched with 2 M HCl (3.9 mL), and dichloromethane added (30 mL). The organic layer was washed with water (60 mL), brine (60 mL), dried over MgSO₄, filtered and the solvent was evaporated under reduced pressure to

give the crude product as dark purple oil (1.97 g). The crude material was purified by column chromatography (eluent: dichloromethane – methanol 2 - 6%) to give the pure product as dark solid with golden shine (0.81 g, 71%). Mp: 128 – 130 °C; ¹H NMR (400 MHz, CDCl₃) δ_H 7.53 (1H, t, *J* 8.4, 18-H), 7.22 (2H, d, *J* 9.6, 7-H), 7.00 (2H, dd, *J* 9.6, 2.4, 6-H), 6.85 (2H, m, 4-H), 6.74 (2H, d, *J* 8.4, 17-H), 3.73 (4H, t, *J* 6.8, 9-H), 3.68 (4H, t, *J* 5.2, 11-H), 3.65 (6H, s, 19-H), 3.31 (6H, s, 8-H), 1.89 (4H, quint, *J* 6.4, 10-H), 0.91 (18H, s, 14-H), 0.06 (12H, s, 13-H); ¹³C NMR (101 MHz, CDCl₃) δ_C 158.0 (3-C), 157.6 (16-C), 156.9 (5-C), 154.7 (1-C), 132.5 (18-C), 131.6 (7-C), 114.5 (2-C), 114.3 (6-C), 108.6 (15-C), 104.2 (17-C), 96.4 (4-C), 59.6 (11-C), 56.1 (19-C), 50.1 (9-C), 39.7 (8-C), 30.2 (10-C), 25.9 (14-C), 18.2 (12-C), -5.4 (13-C); ν_{max}/cm⁻¹ 1096 (C-O), 1068 (Si-O); HRMS: Found (M)⁺ 719.3998. Calcd. for C₄₁H₆₃N₂O₅Si₂⁺: (M)⁺ 719.4270.

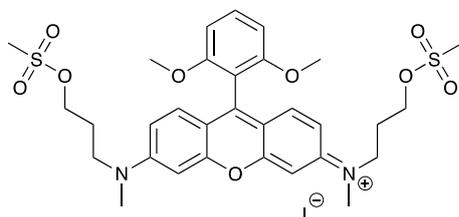
(E)-N-(9-(2,6-dimethoxyphenyl)-6-((3-hydroxypropyl)(methyl)amino)-3H-xanthen-3-ylidene)-3-hydroxy-N-methylpropan-1-aminium chloride **15**



To the solution of (*E*)-3-((*tert*-butyldimethylsilyloxy)-*N*-(6-((3-((*tert*-butyldimethylsilyloxy)propyl)(methyl)amino)-9-(2,6-dimethoxyphenyl)-3H-xanthen-3-ylidene)-*N*-methylpropan-1-aminium chloride **14** (0.80 g, 1.06 mmol) in THF (10 mL) at 0 °C was added a solution of TBAF in THF (1.11 mL, 1M, 1.11 mmol), and the resulting solution stirred for 20 minutes at that temperature. After the reaction

was complete, dichloromethane (10 mL) was added and the resulting organic layer washed with saturated aqueous NaHCO₃ (3 x 10 mL). The aqueous phase was freeze dried to give a crude product that was purified by column chromatography (eluent: dichloromethane – methanol 10 - 50% then ethanol – water 0 - 40%) and purified by reversed phase flash chromatography (column: C18, eluent: water – methanol 0 - 100% and 0.1 % TFA) to give the product as purple solid (0.45 g, 70%, containing 23 mol% TBAF). Mp: 193 – 194 °C; ¹H NMR (400 MHz, MeOD-*d*₄) δ_H 7.62 (1H, t, *J* 8.8, 16-H), 7.27 (2H, d, *J* 9.2, 7-H), 7.10 (2H, dd, *J* 9.6, 2.4, 6-H), 6.98 (2H, d, *J* 2.0, 4-H), 6.91 (2H, d, *J* 8.4, 15-H), 3.77 (4H, t, *J* 7.2, 9-H), 3.70 (6H, s, 17-H), 3.66 (4H, t, *J* 5.6, 11-H), 3.30 (6H, s, 8-H), 1.92 (4H, quint, *J* 6.0, 10-H); ¹³C NMR (101 MHz, MeOD-*d*₄) δ_C 157.9 (3-C), 157.7 (14-C), 157.1 (5-C), 154.7 (1-C), 132.3 (16-C), 131.2 (7-C), 114.1 (2-C), 114.0 (6-C), 108.6 (13-C), 104.0 (15-C), 95.9 (4-C), 58.3 (11-C), 55.1 (17-C), 49.5 (9-C), 38.2 (8-C), 29.6 (10-C); ν_{max}/cm⁻¹ 3372 (O-H), 1099 (C-O); HRMS: Found (M)⁺ 491.2380. Calcd. for C₂₉H₃₅N₂O₅: (M)⁺ 491.2540.

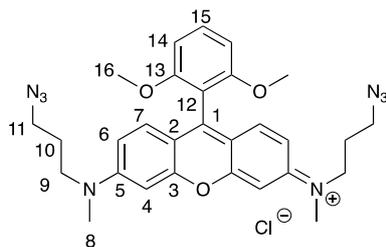
(E)-N-(9-(2,6-dimethoxyphenyl)-6-(methyl(3-((methylsulfonyl)oxy)propyl)amino)-3H-xanthen-3-ylidene)-N-methyl-3-((methylsulfonyl)oxy)propan-1-aminium iodide **16**



To the solution of compound **15** (0.20 g, 0.38 mmol) in anhydrous dichloromethane (6.0 mL) was added trimethylamine (0.32 mL, 2.28 mmol). After the solution was cooled down to 0 °C, methanesulfonylchloride (0.18 mL, 2.28 mmol) was added dropwise, and the resulting solution stirred at this temperature for 5 hours. After this time, the reaction was quenched with saturated aqueous

ammonium chloride solution (10 mL), and the aqueous phase was extracted with dichloromethane (3 x 10 mL). The combined organic layers were washed with aqueous saturated KI solution (10 mL), dried over MgSO₄, filtered and concentrated under reduced pressure. The resulting crude product was used in the next step without further purification and characterization.

(*E*)-3-azido-*N*-(6-((3-azidopropyl)(methyl)amino)-9-(2,6-dimethoxyphenyl)-3*H*-xanthen-3-ylidene)-*N*-methylpropan-1-aminium iodide **4**



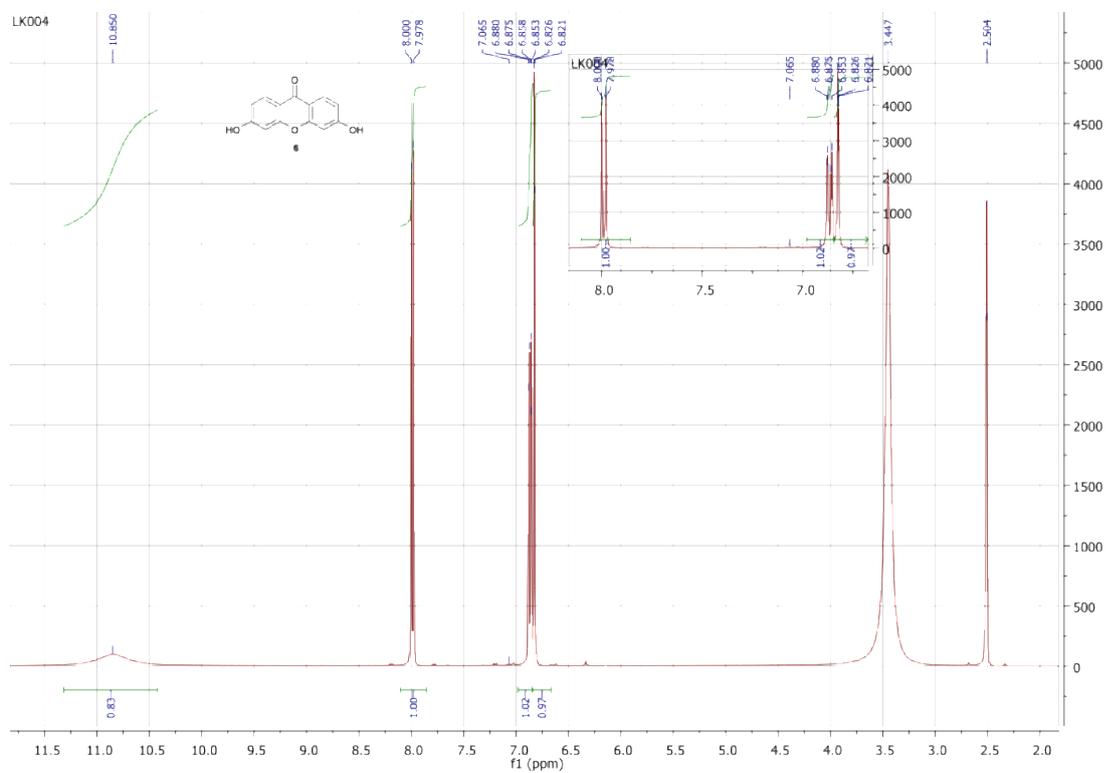
To the solution of crude compound **16** (0.38 mmol) in dry dimethylformamide (6.0 mL) behind a blast shield, was added NaN₃ (0.49 g, 7.60 mmol) using a plastic spatula portion-wise. The resulting mixture was stirred at 40 °C for 16h. After this time, the solvent was removed under reduced pressure and the residue dissolved in dichloromethane (10 ml), which was washed with aqueous saturated KI solution (10 mL), dried over

MgSO₄, filtered and then removed under reduced pressure. The crude product was purified by column chromatography (eluent: dichloromethane methanol (0 – 5%)), and again by reversed phase flash chromatography (C18, eluent: water – methanol – TFA (0.1%)) to give the product as metallic purple solid (0.15 g, 83%). Mp: 88 – 89 °C; ¹H NMR (400 MHz, CDCl₃) δ_H 7.54 (1H, t, *J* 8.4, 15-H), 7.26 (2H, d, *J* 9.6, 7-H), 6.92 (2H, dd, *J* 9.6, 2.4, 6-H), 6.88 (2H, d, *J* 2.4, 4-H), 6.75 (2H, d, *J* 8.4, 14-H), 3.71 (4H, t, *J* 7.2, 9-H), 3.66 (6H, s, 16-H), 3.47 (4H, t, *J* 6.0, 11-H), 3.27 (6H, s, 8-H), 1.96 (4H, quint, *J* 6.4, 10-H). ¹³C NMR (101 MHz, CDCl₃) δ_C 158.1 (3-C), 157.6 (13-C), 156.8 (5-C), 155.5 (1-C), 132.6 (15-C), 131.9 (7-C), 114.8 (2-C), 113.9 (6-C), 108.5 (12-C), 104.1 (14-C), 96.7 (4-C), 56.0 (16-C), 50.3 (9-C), 48.6 (11-C), 39.4 (8-C), 26.6 (10-C); ν_{max}/cm⁻¹ 2095 (N=N-N), 1099 (C-O); HRMS: Found (M)⁺ 541.2747. Calcd. for C₂₉H₃₃N₈O₃: (M)⁺ 541.2670. UV/VIS (10.7 μM in water and 1 % DMSO): λ_{max}= 558 nm, A₂= 0.76; Fluorescence (107 nM in water and 0.01% DMSO): λ_{ex}= 557 nm, Intensity_{ex}= 127 a.u.; λ_{em}= 580 nm, Intensity_{em}= 127 a.u.

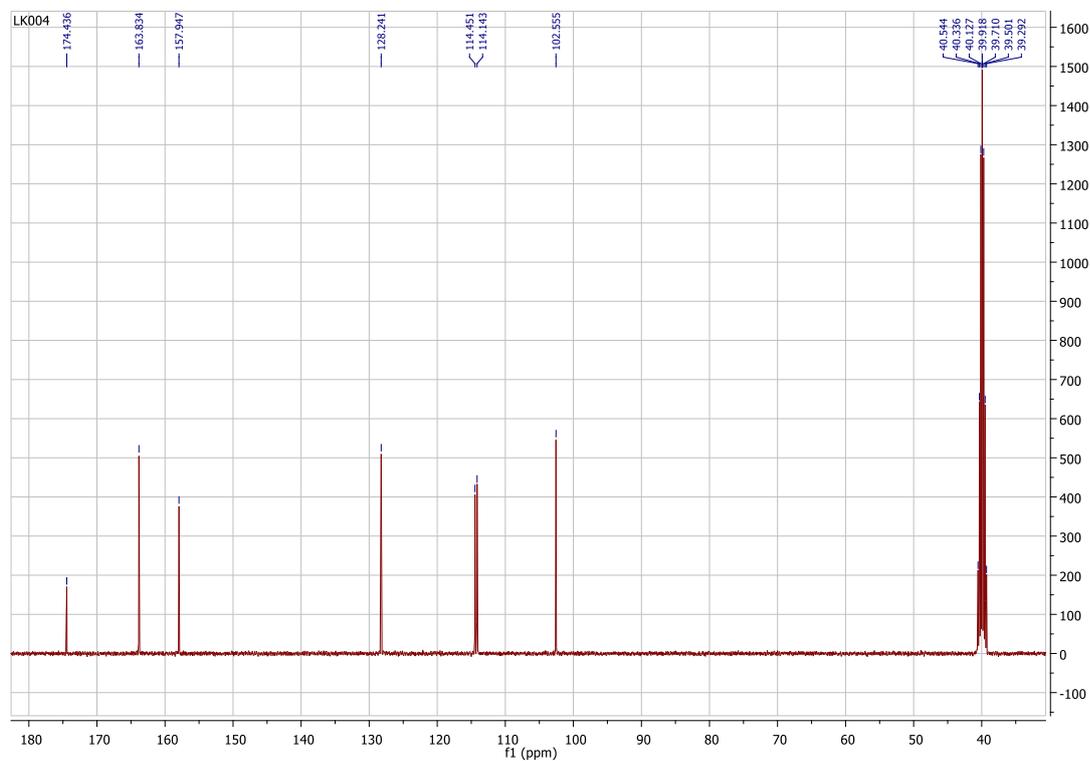
NMR Spectra, Mass Spec and IR Data

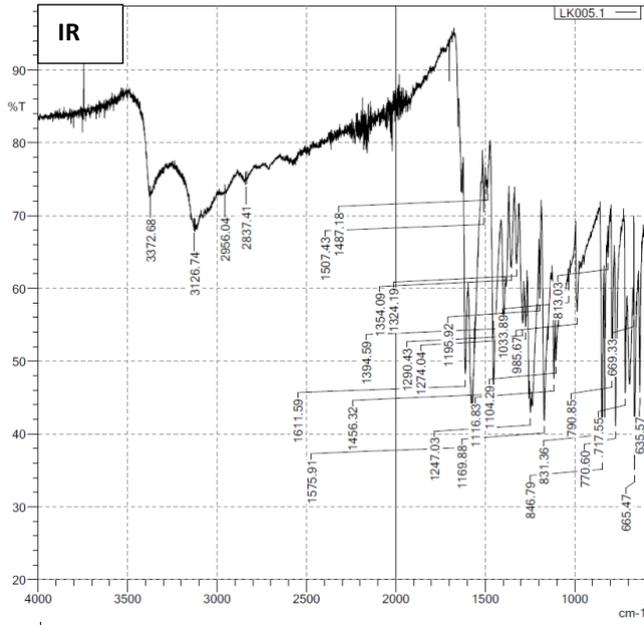
3,6-dihydroxy-9H-xanthen-9-one **6**

¹H NMR

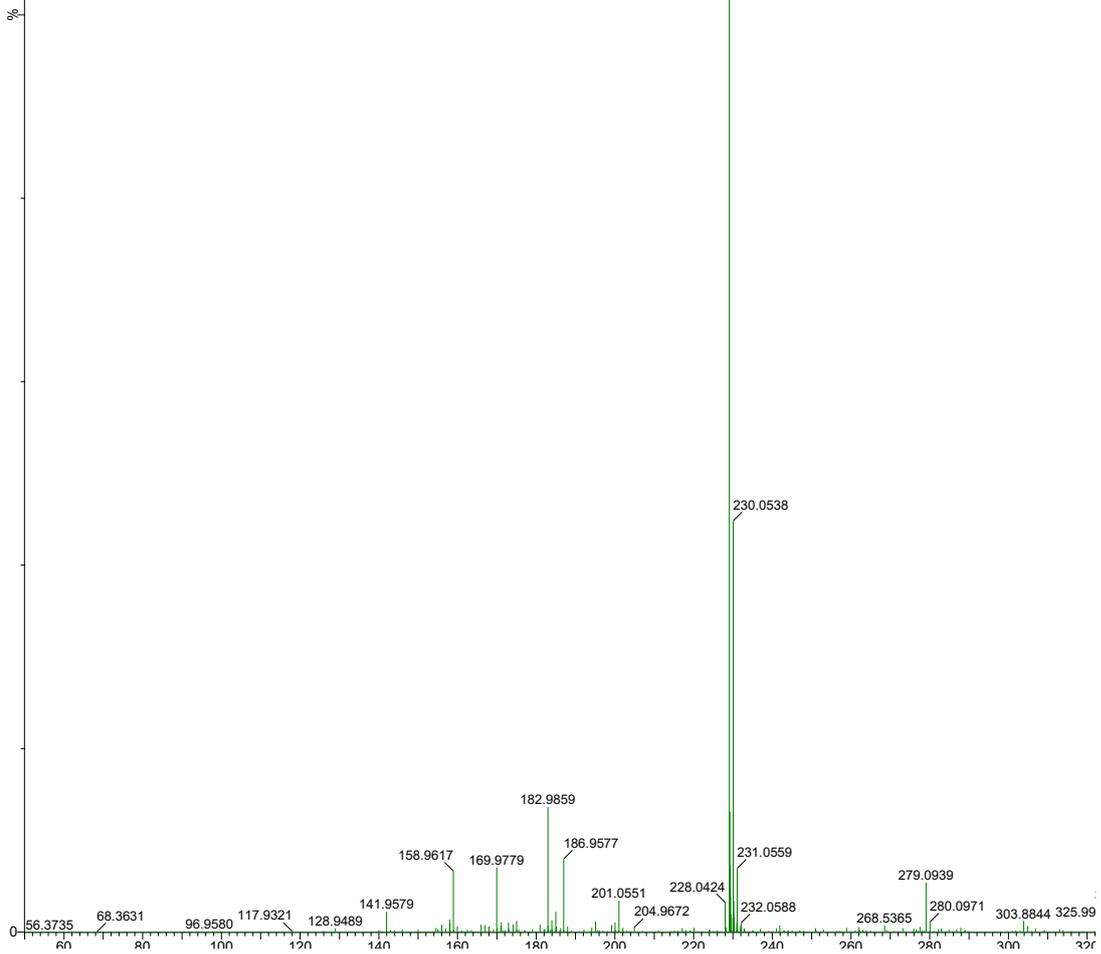


¹³C NMR



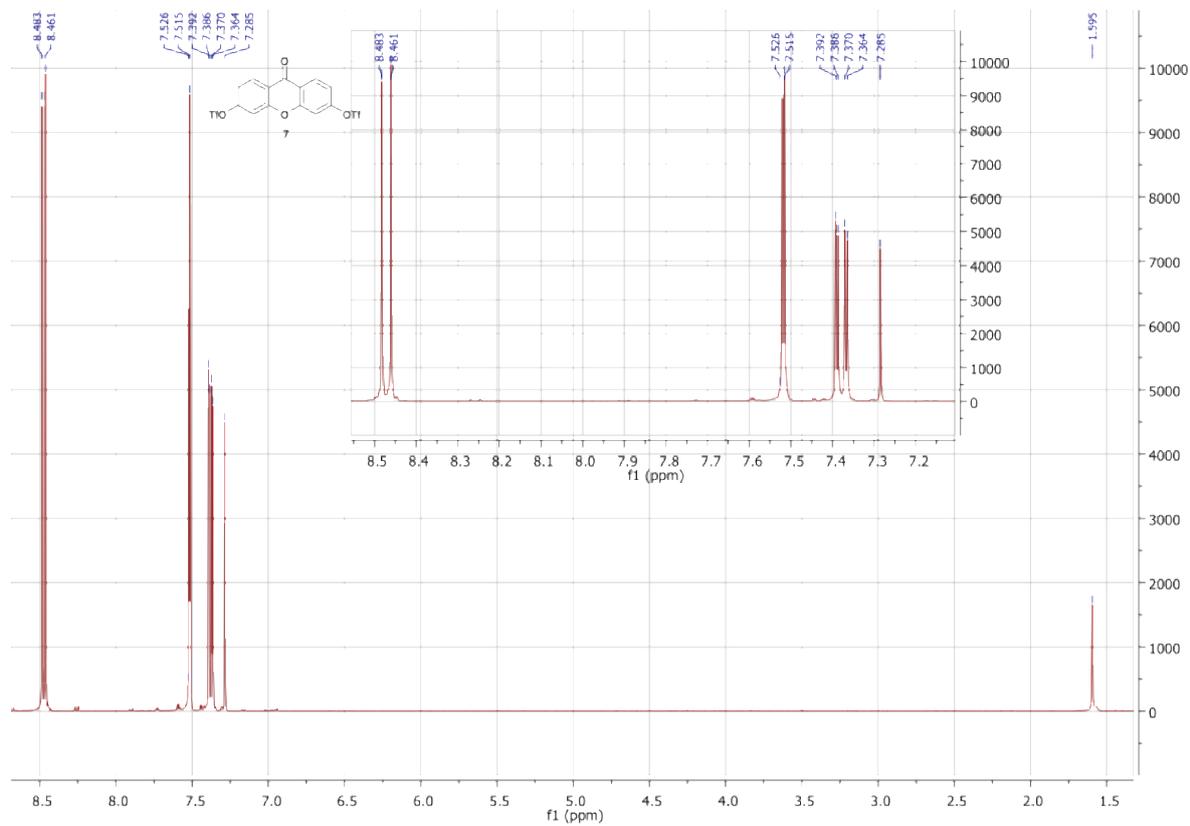


MS

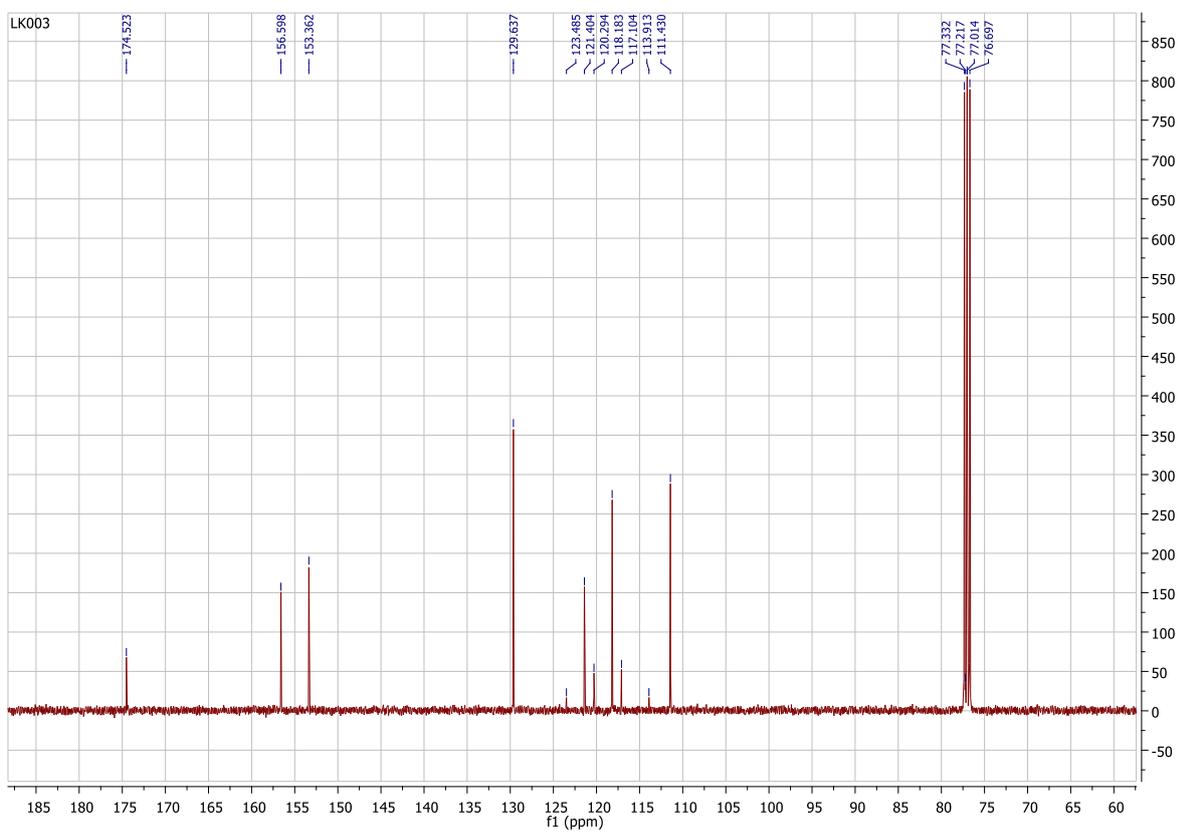


9-oxo-9H-xanthene-3,6-diyl bis(trifluoromethanesulfonate) **7**

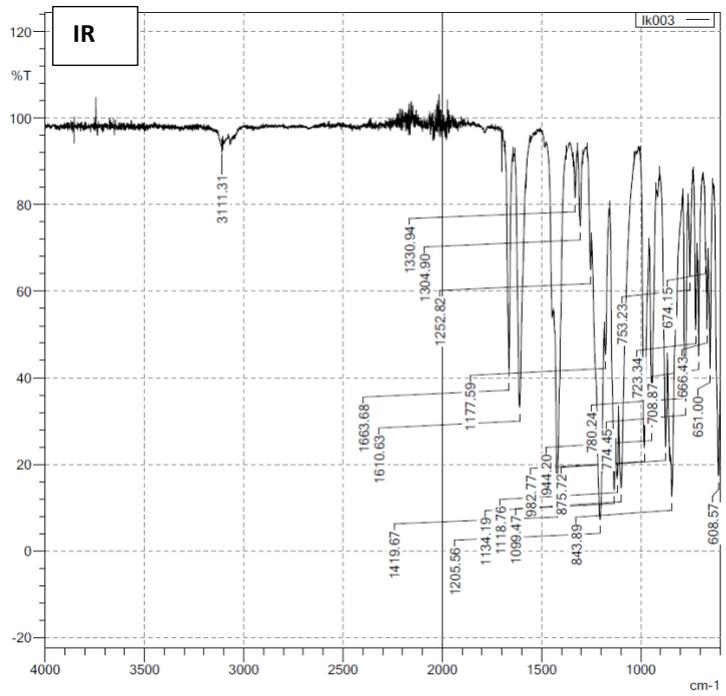
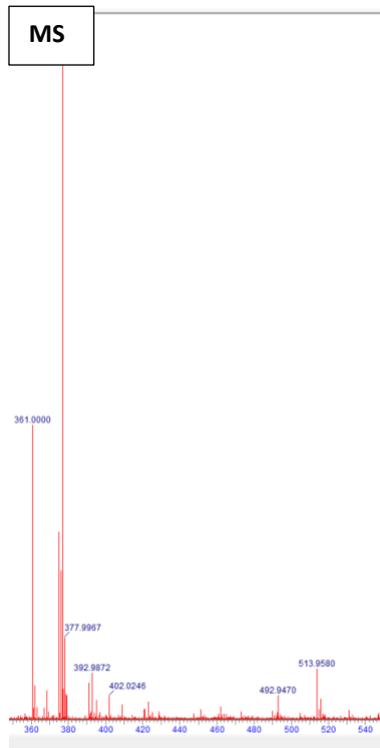
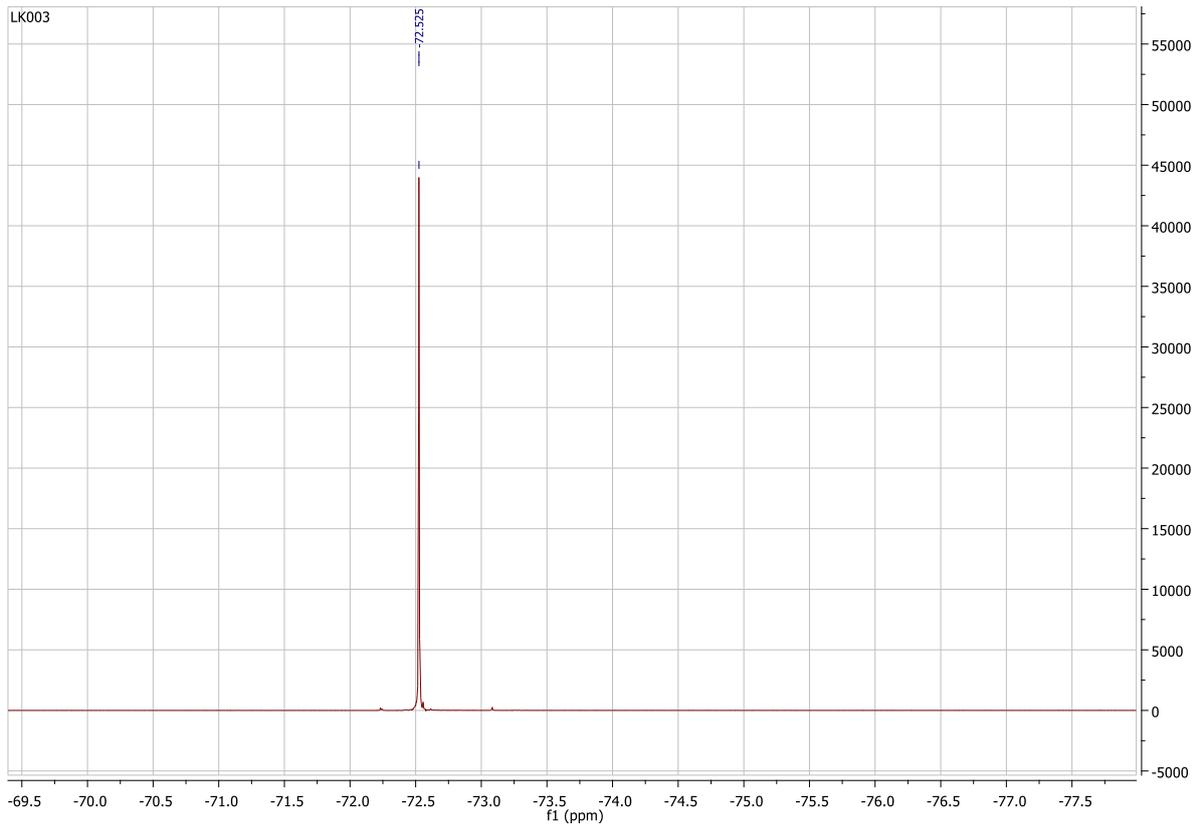
¹H NMR



¹³C NMR

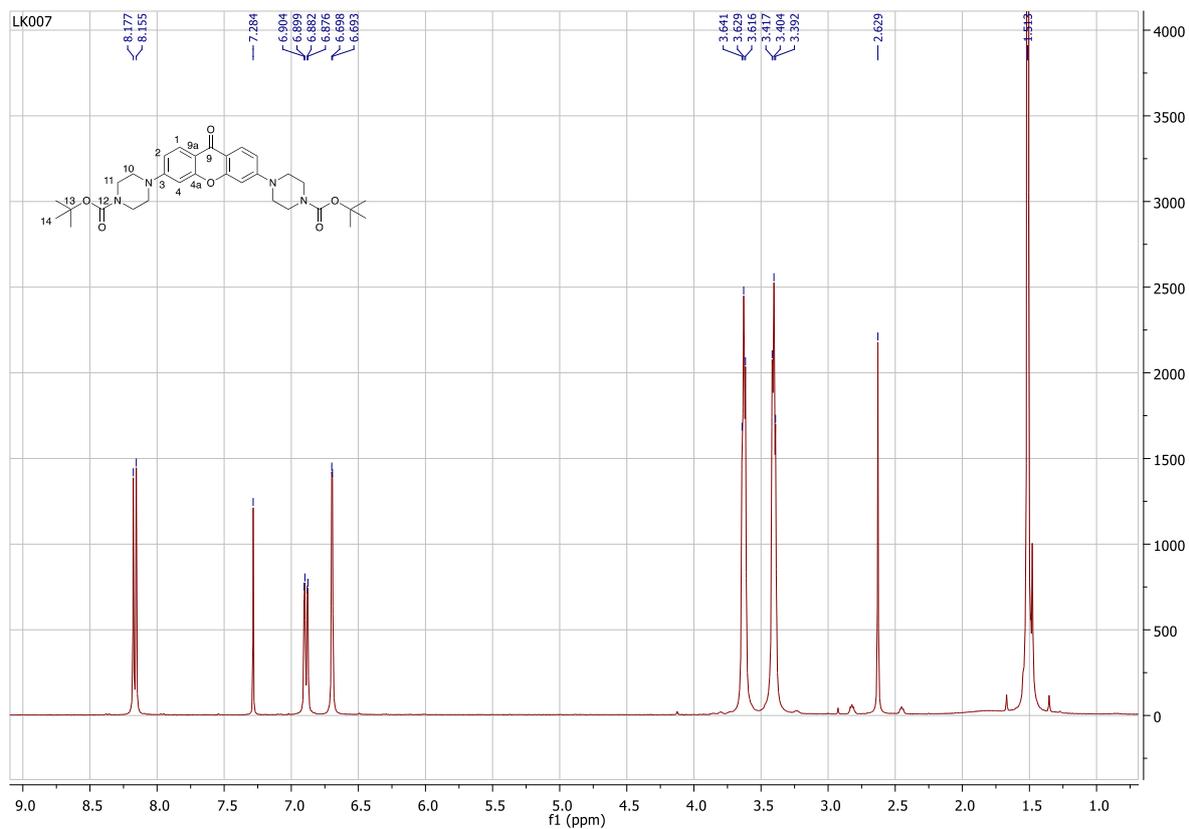


19F NMR

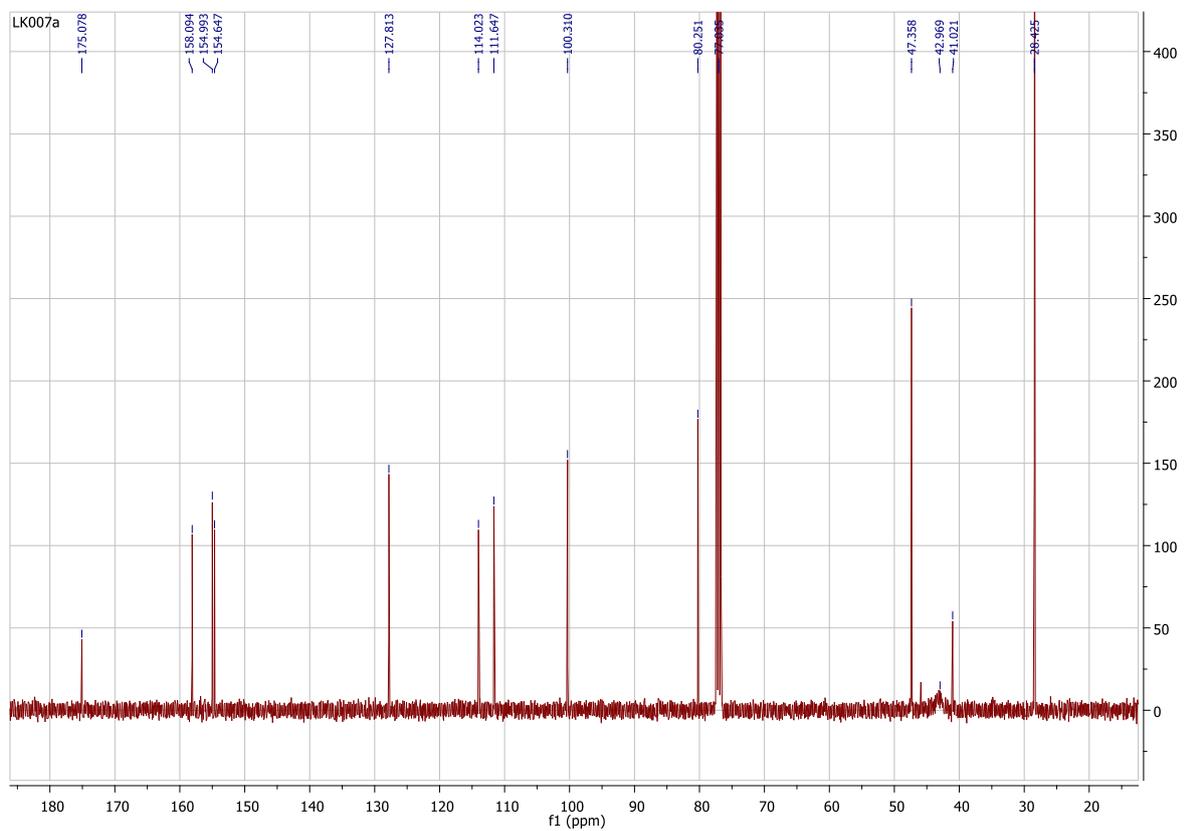


Di-tert-butyl 4,4'-(9-oxo-9H-xanthene-3,6-diyl)bis(piperazine-1-carboxylate) **8**

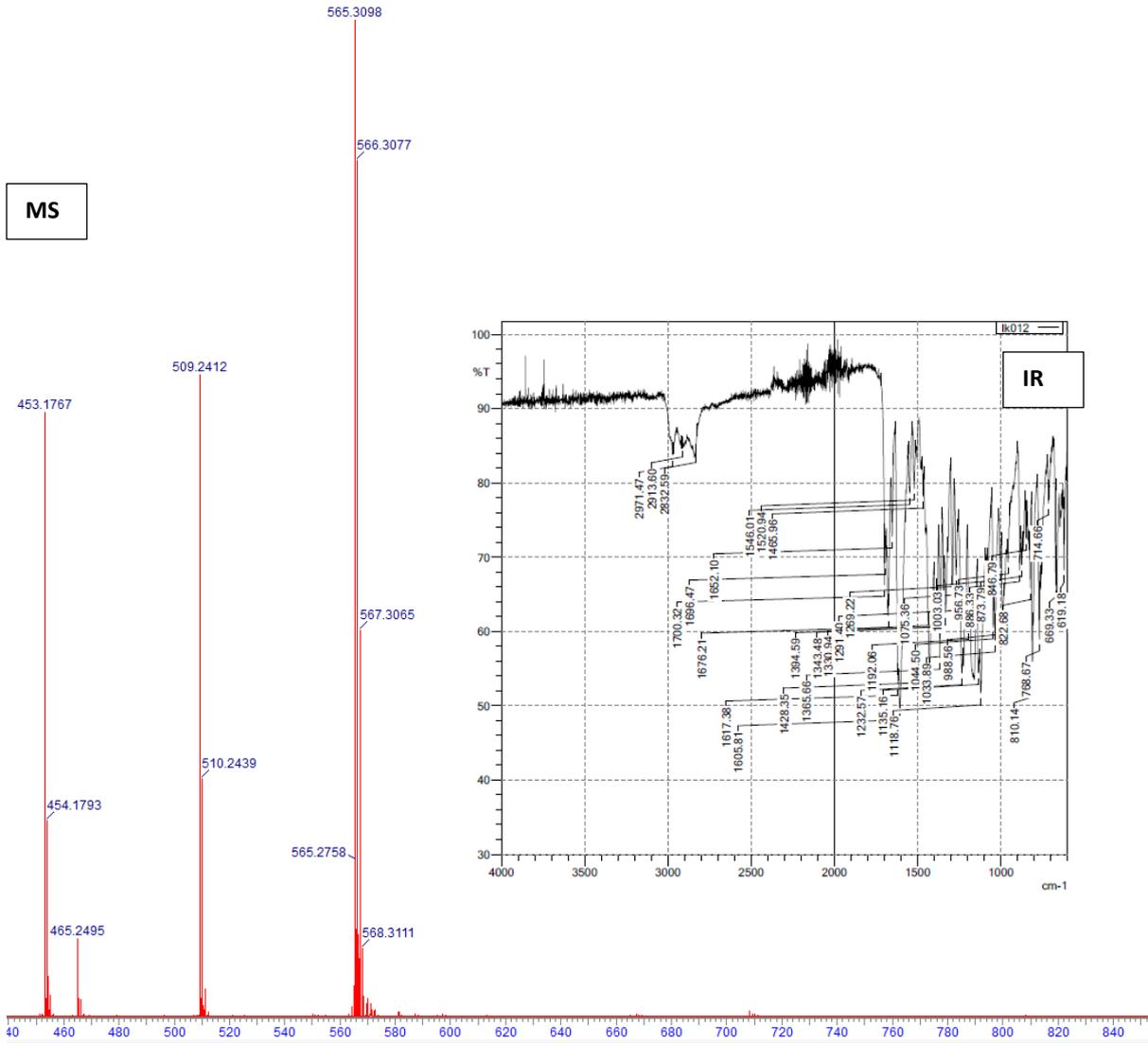
¹H NMR



¹³C NMR

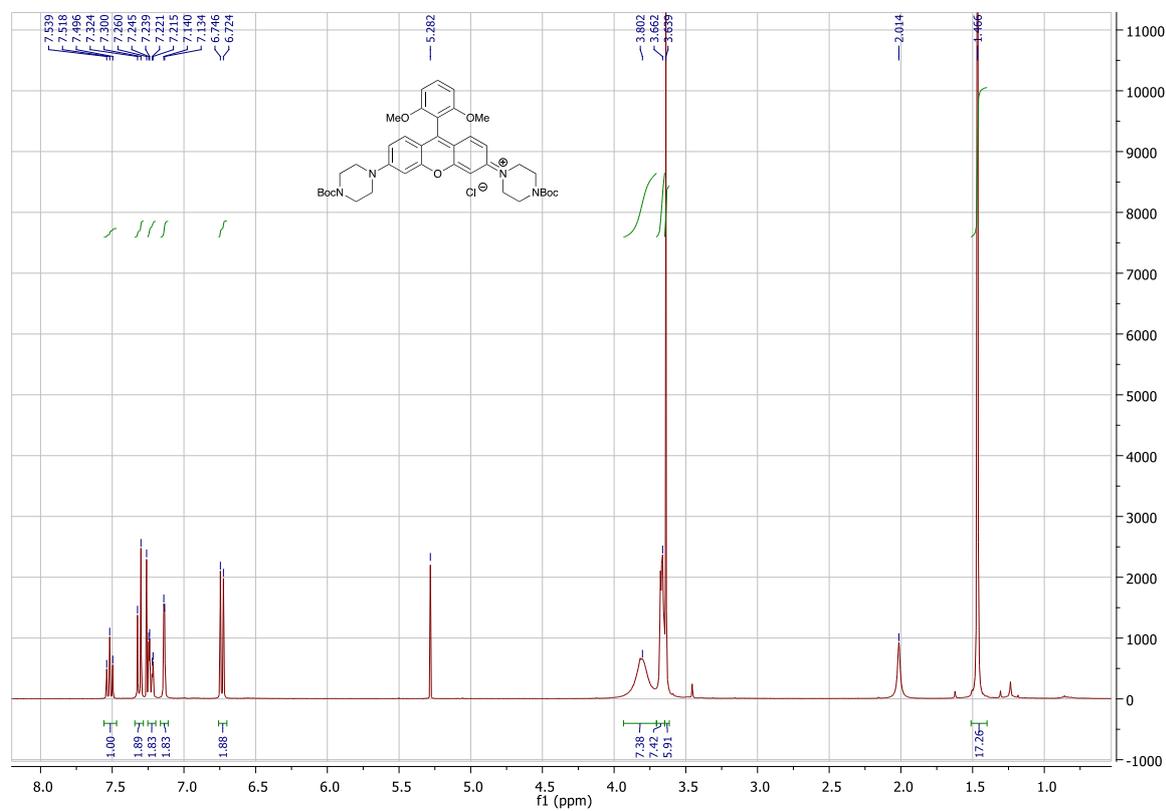


MS

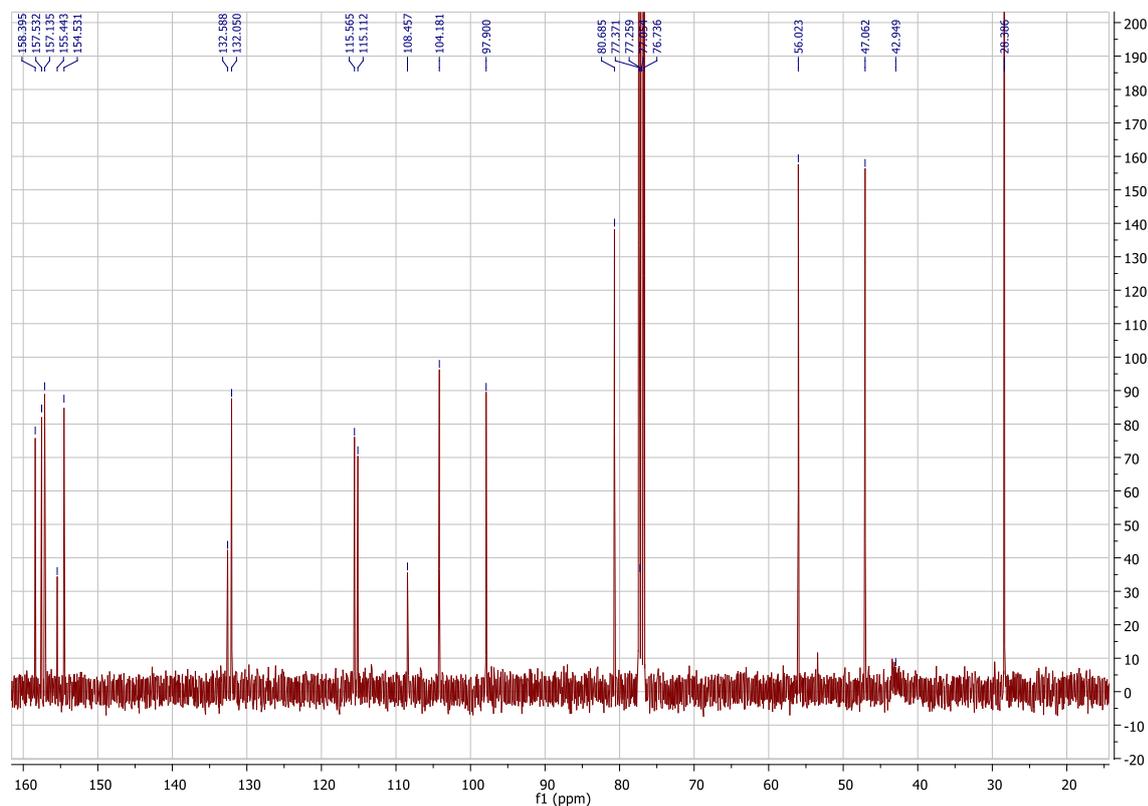


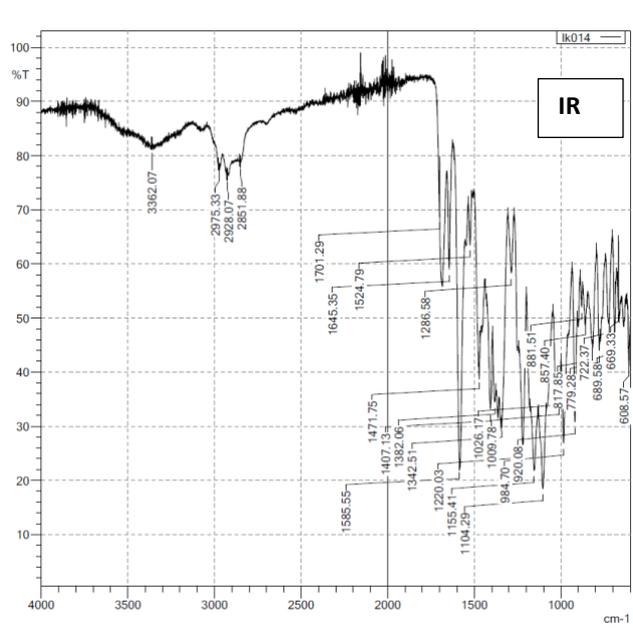
4-(tert-butoxycarbonyl)-1-(6-(4-(tert-butoxycarbonyl)piperazin-1-yl)-9-(2,6-dimethoxyphenyl)-3H-xanthen-3-ylidene)piperazin-1-ium chloride 9

¹H NMR

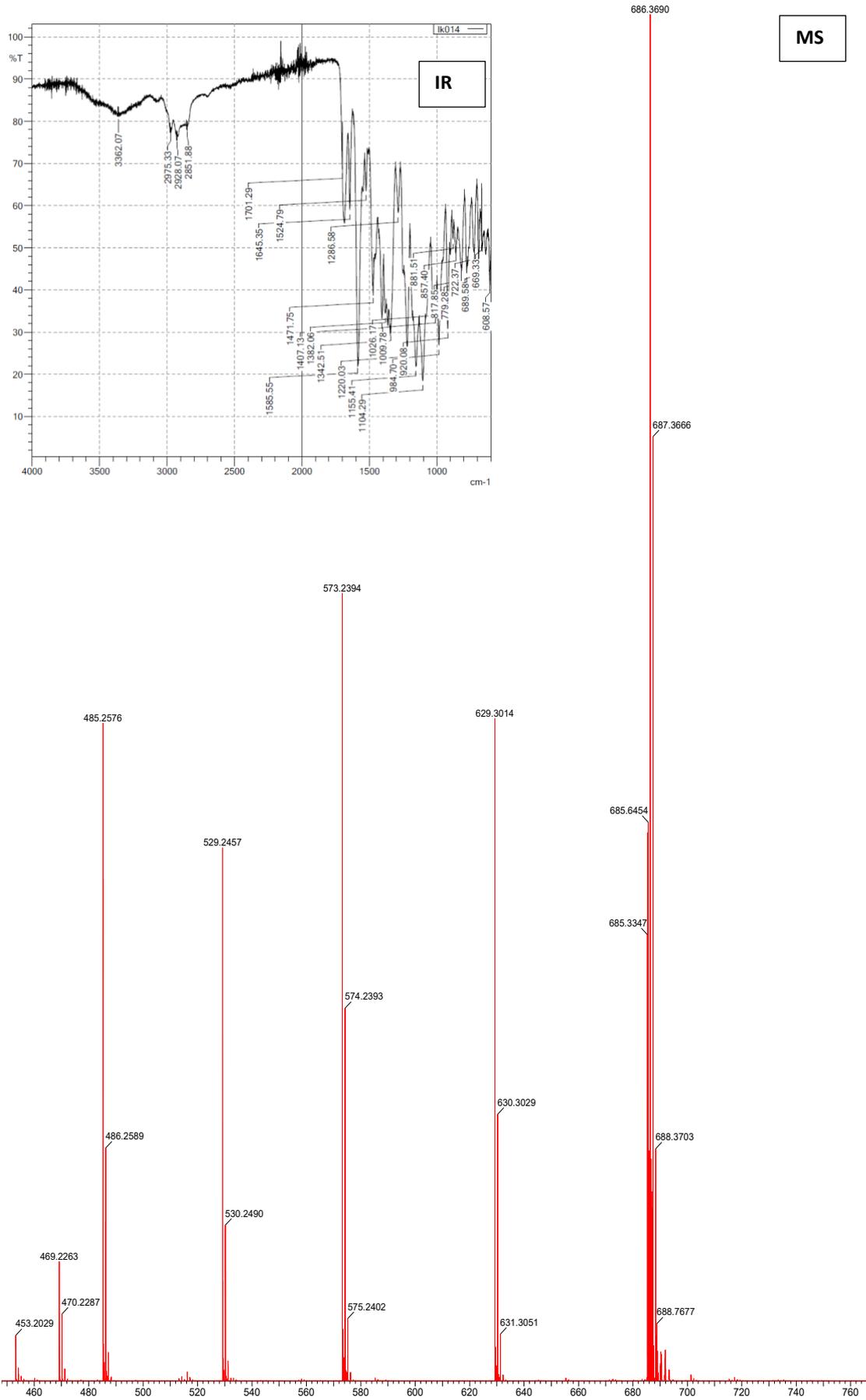


¹³C NMR



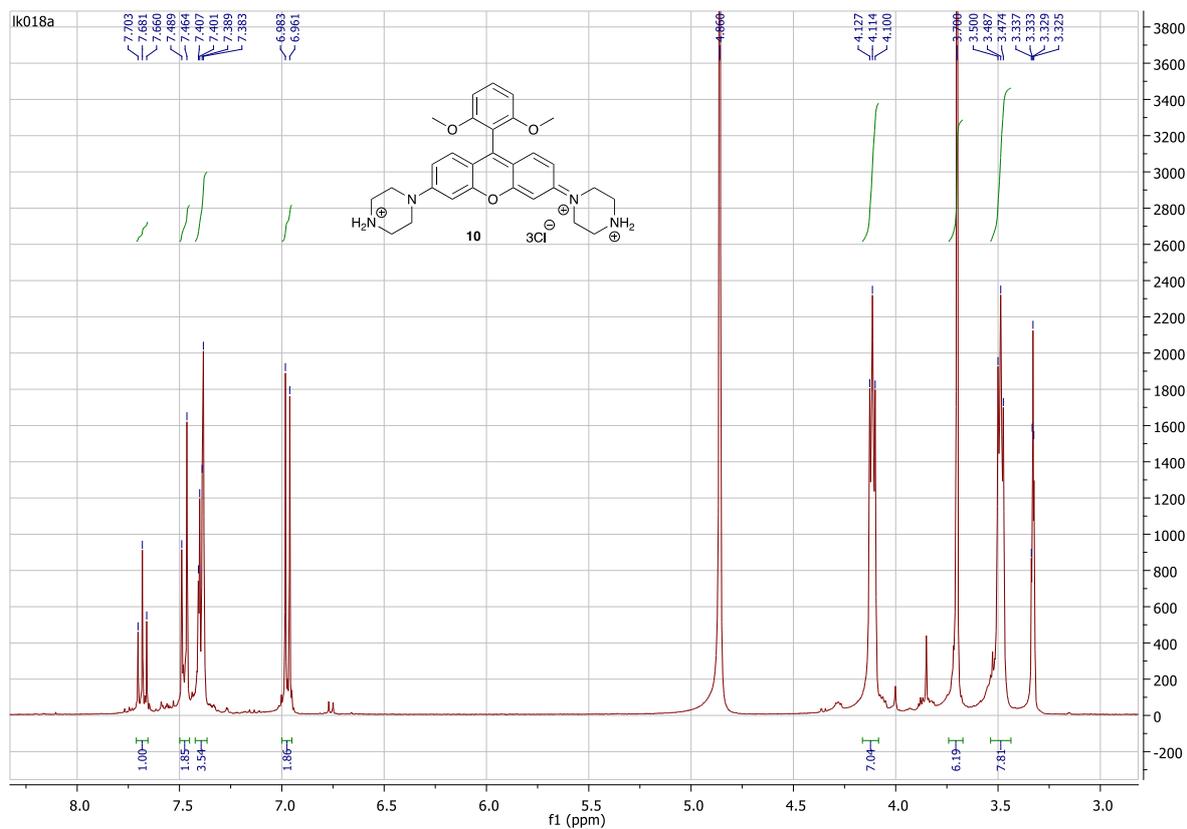


MS

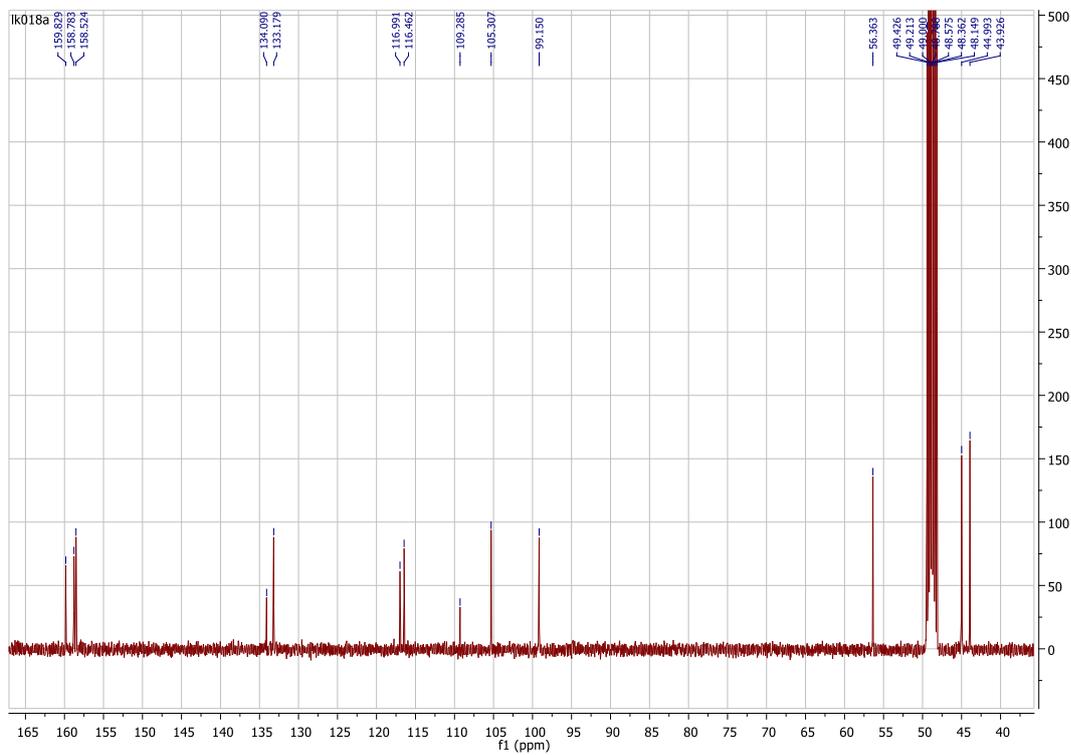


1-(9-(2,6-dimethoxyphenyl)-6-(piperazin-1-ium-1-yl)-3H-xanthen-3-ylidene)piperazine-1,4-dium chloride **10**

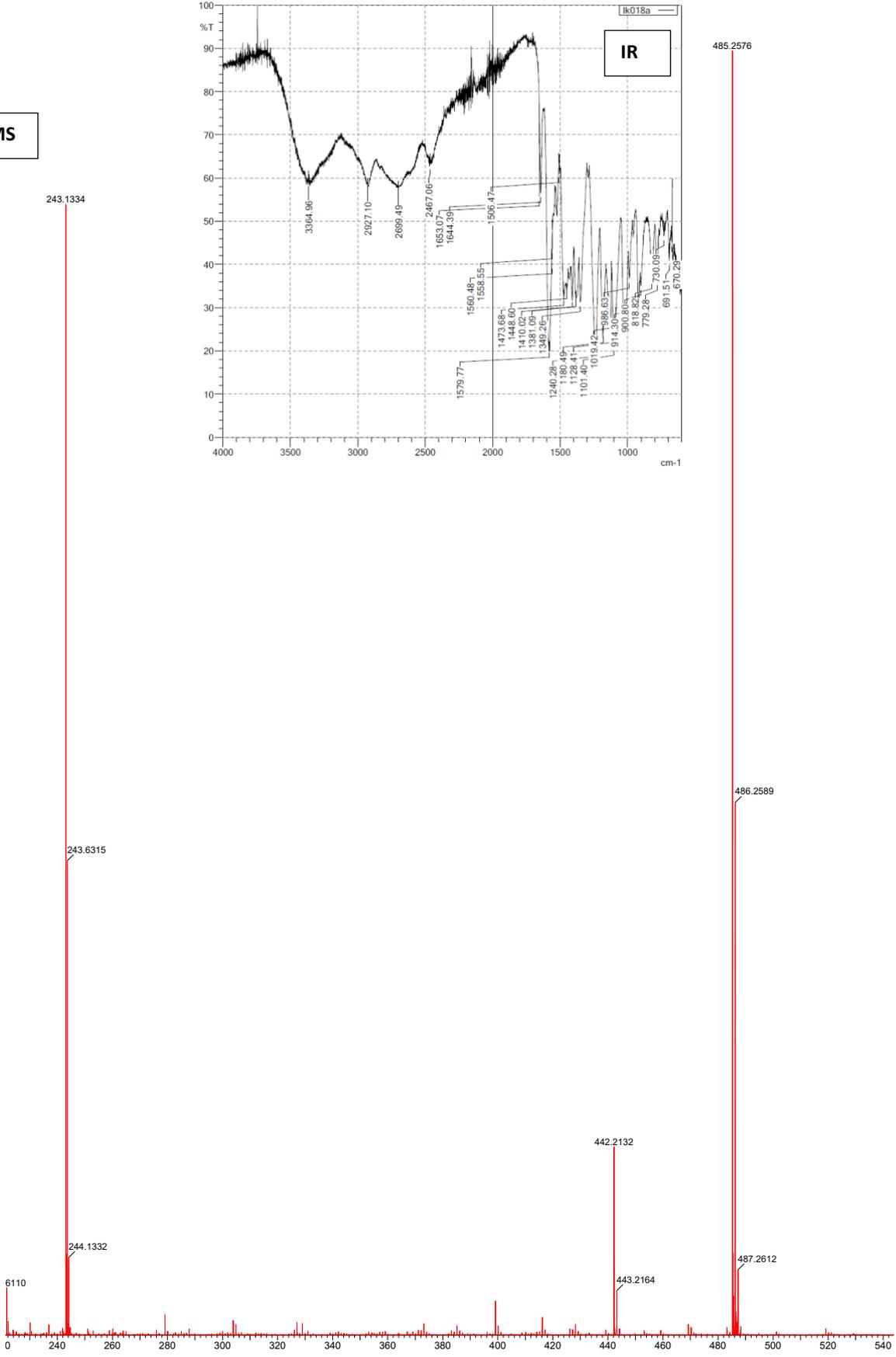
¹H NMR



¹³C NMR

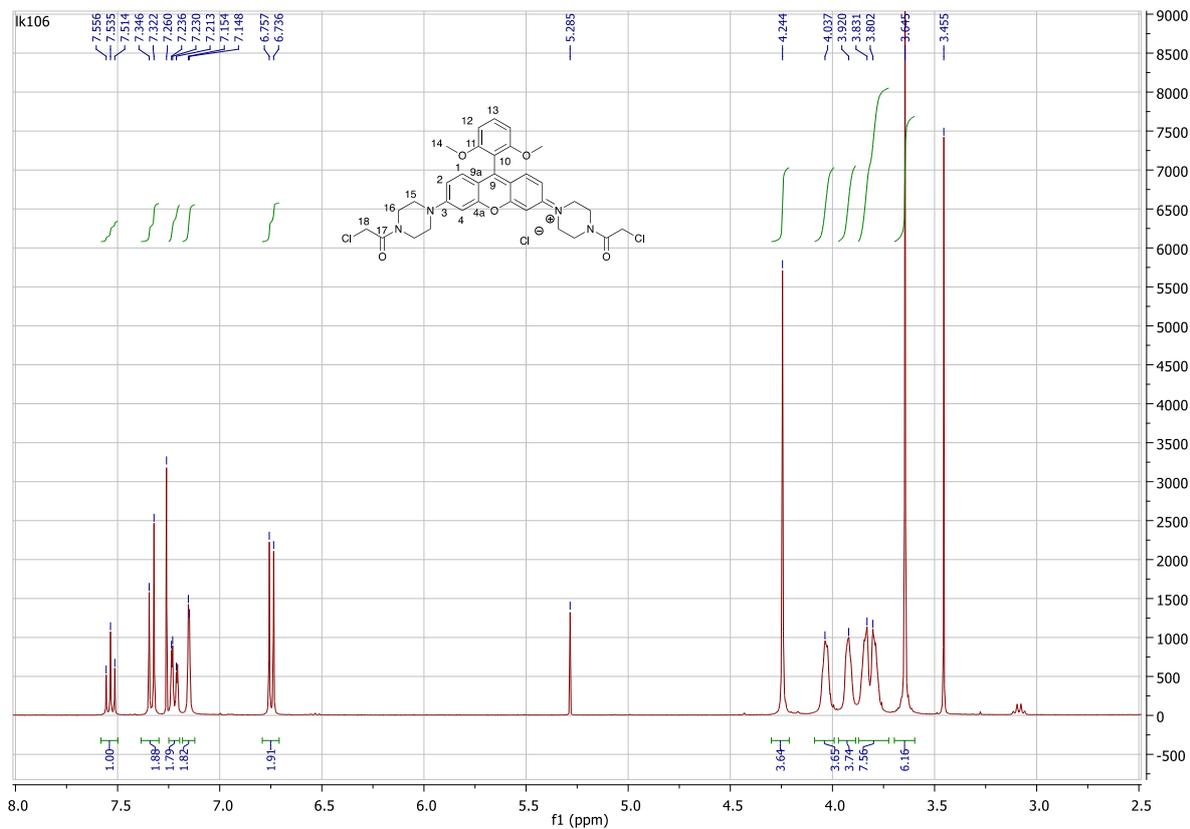


MS

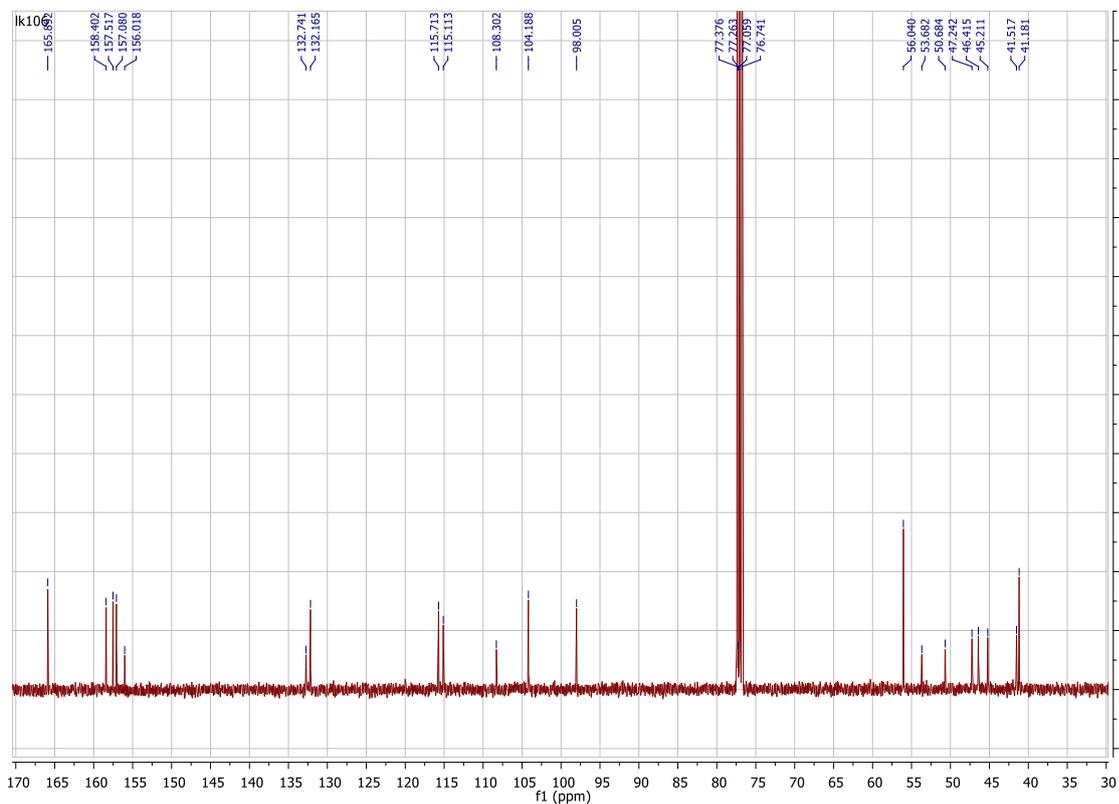


4-(2-chloroacetyl)-1-(6-(4-(2-bromo/chloroacetyl)piperazin-1-yl)-9-(2,6-dimethoxyphenyl)-3H-xanthen-3-ylidene)piperazin-1-ium chloride **11**

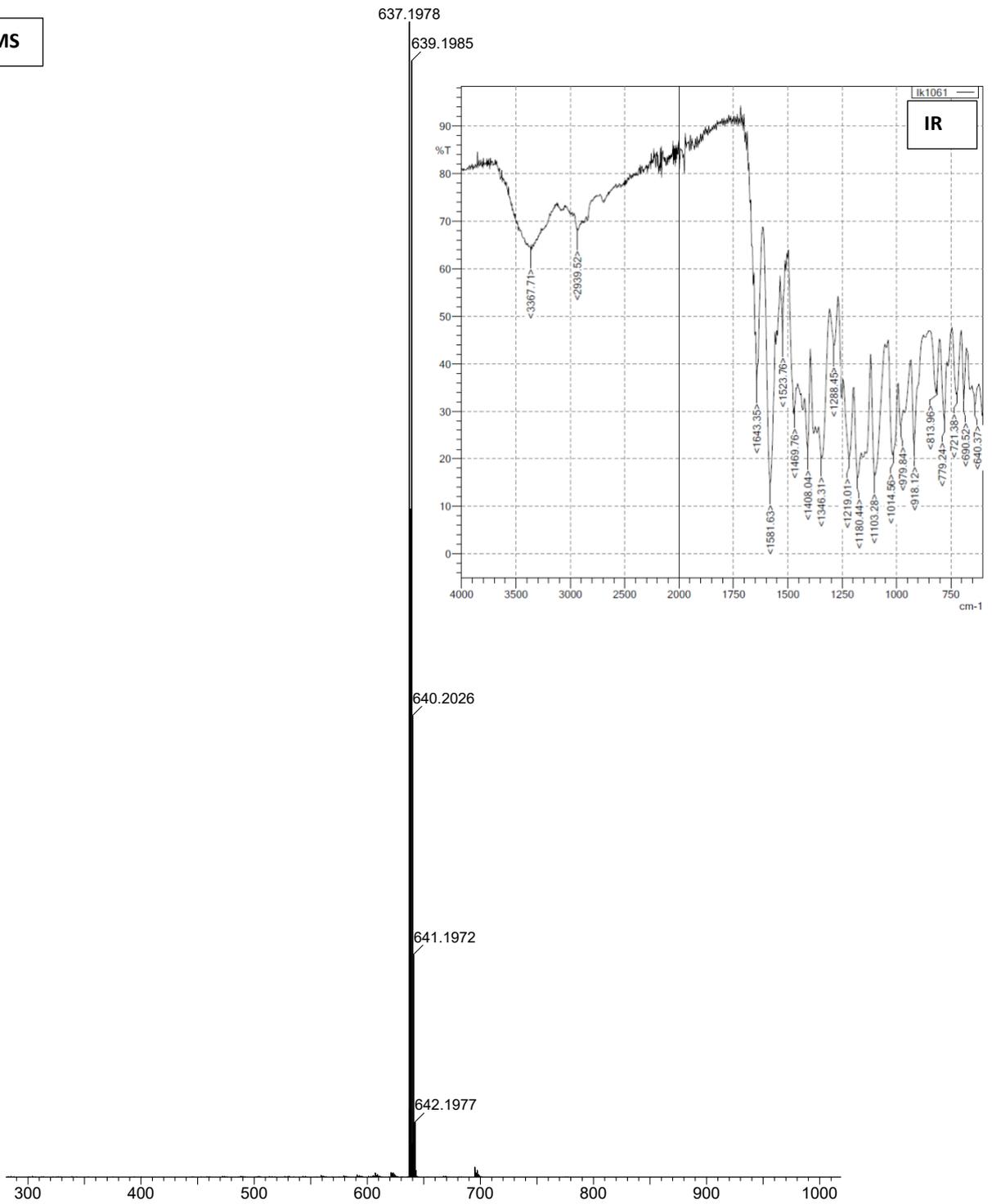
¹H NMR



¹³C NMR

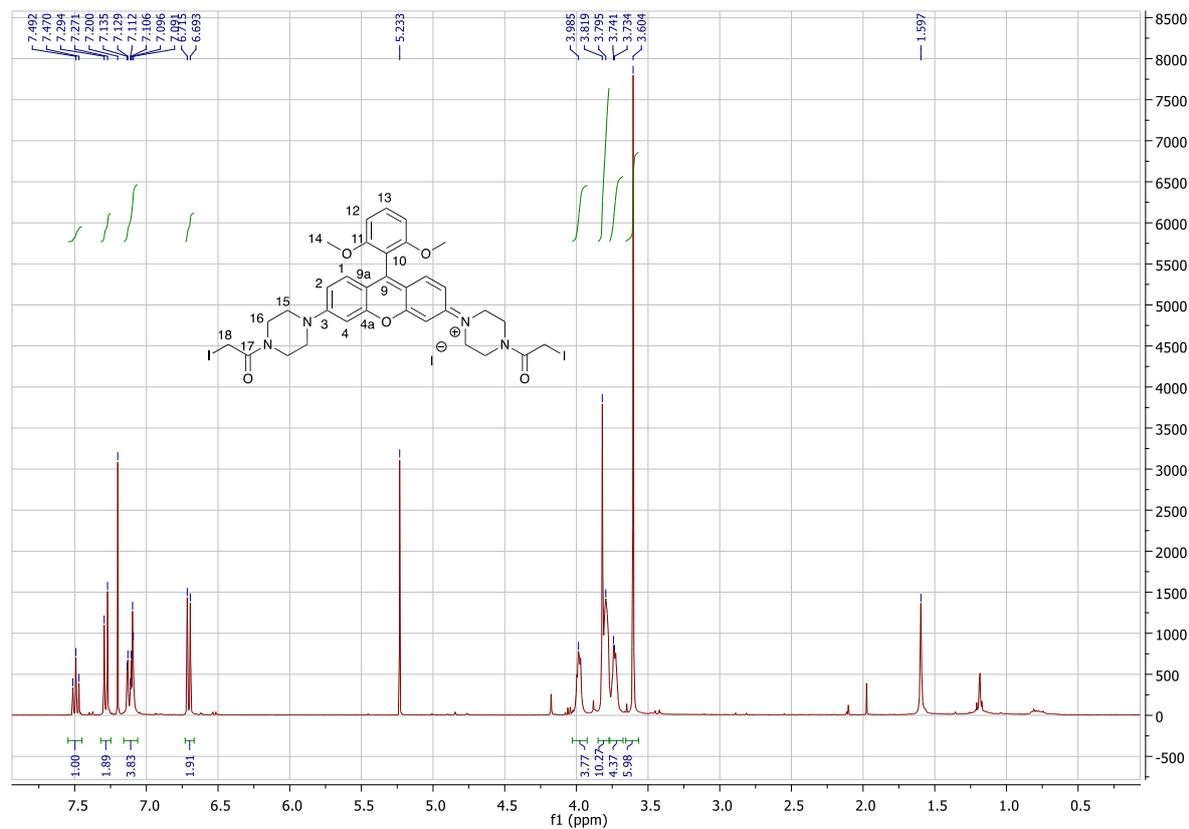


MS

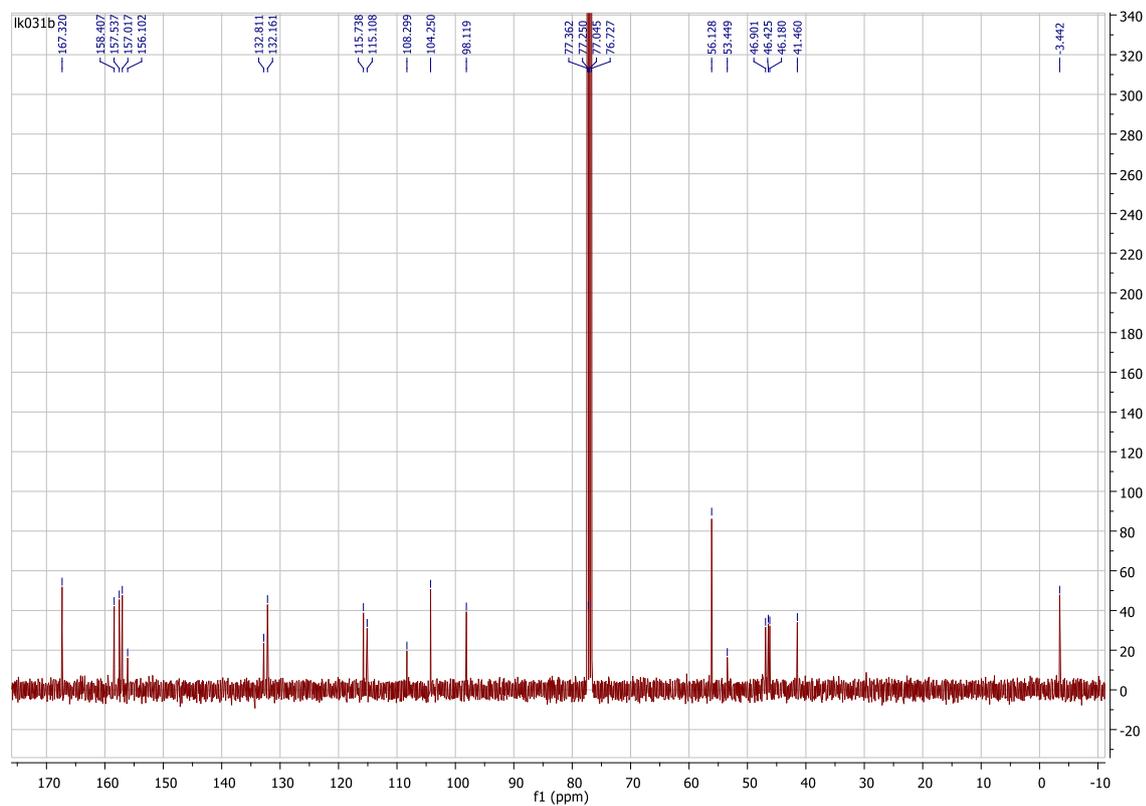


1-(9-(2,6-dimethoxyphenyl)-6-(4-(2-iodoacetyl)piperazin-1-yl)-3H-xanthen-3-ylidene)-4-(2-iodoacetyl)piperazin-1-ium iodide **2**

¹H NMR

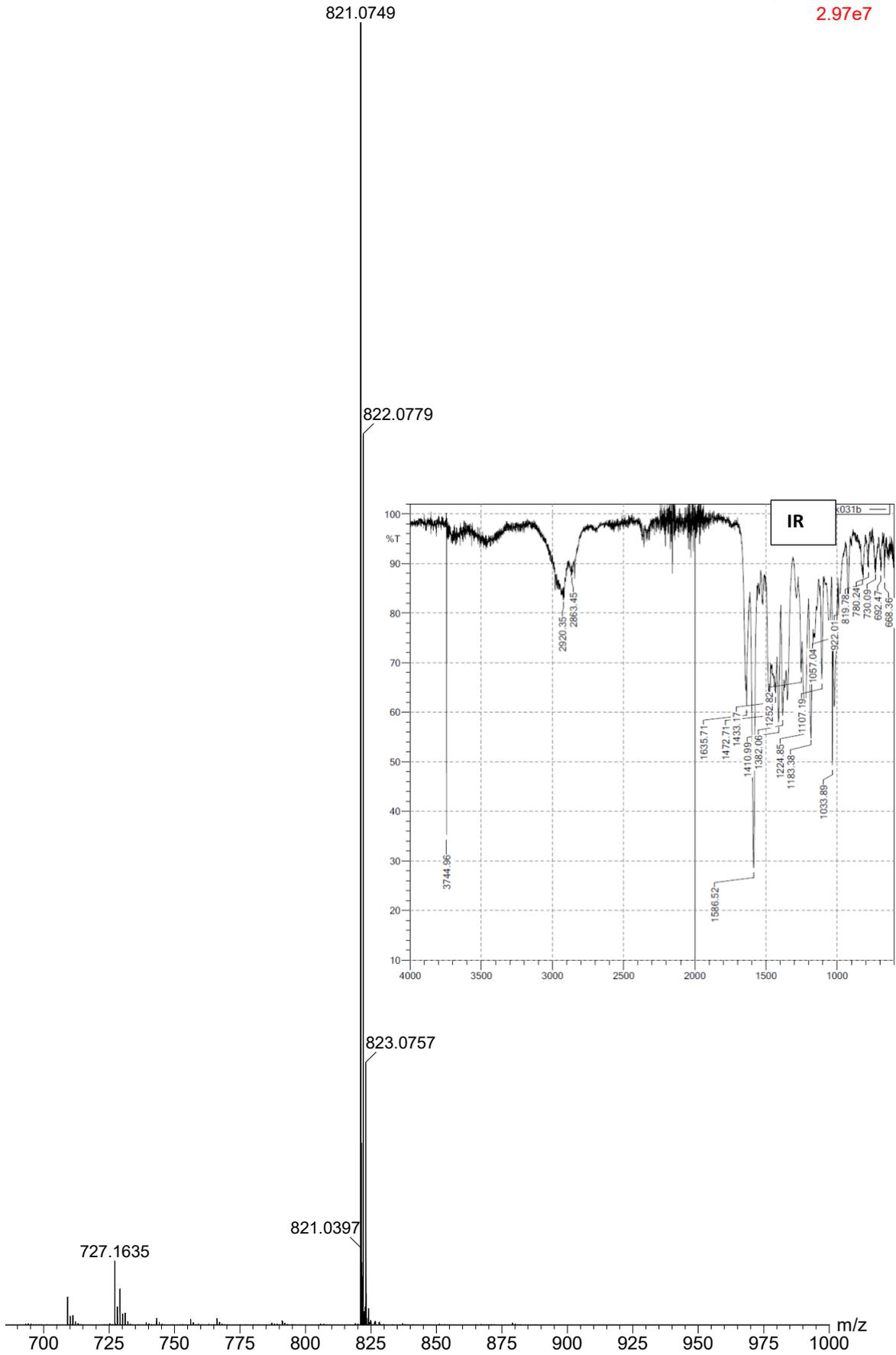


¹³C NMR

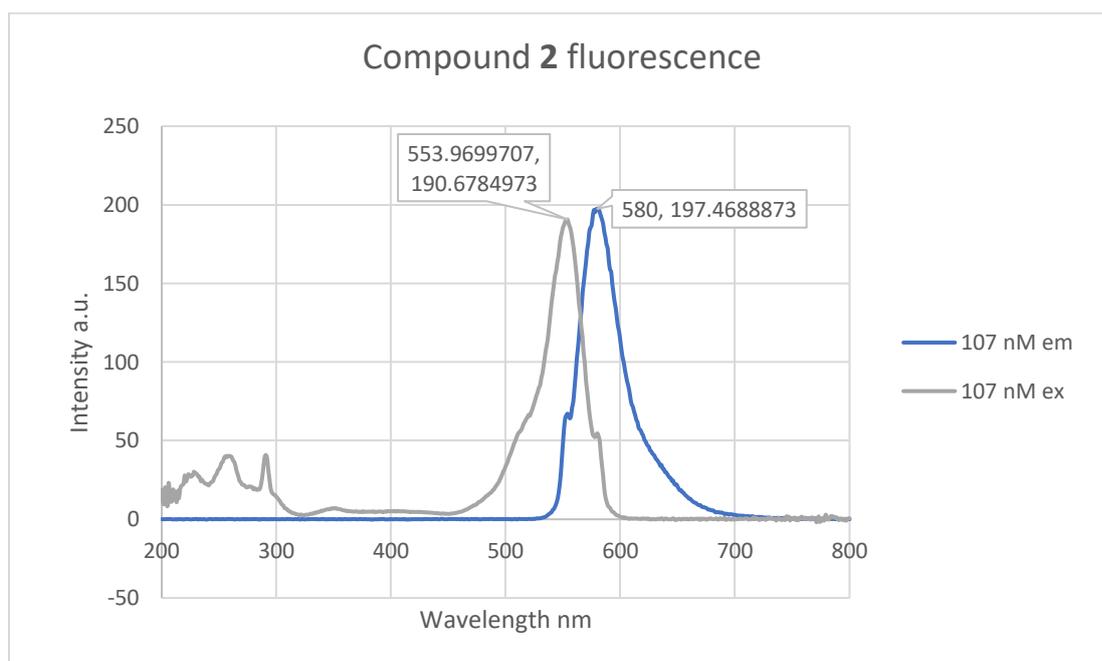
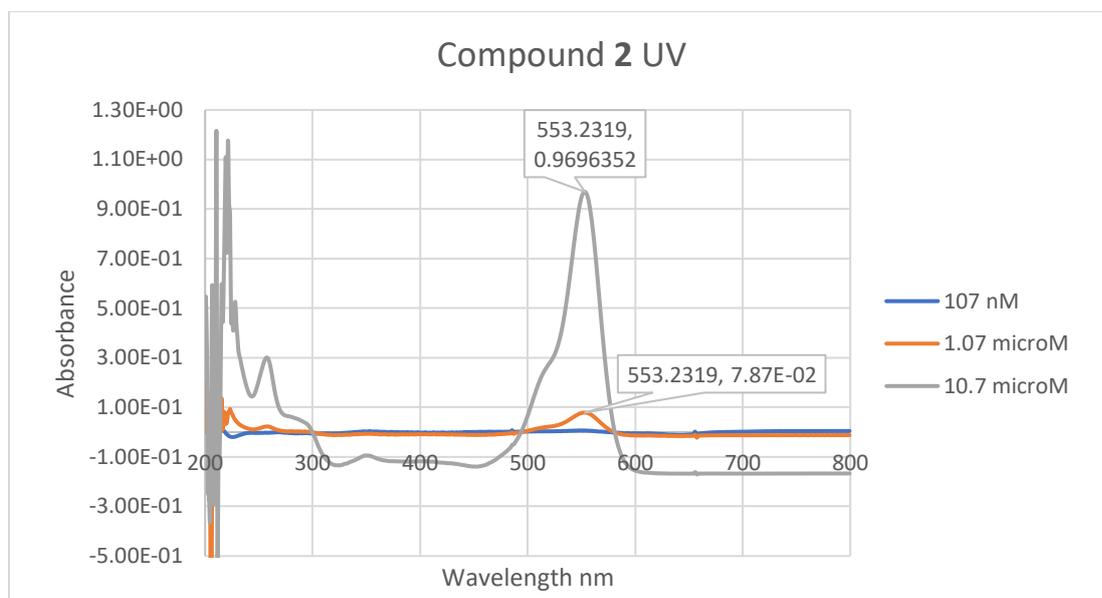


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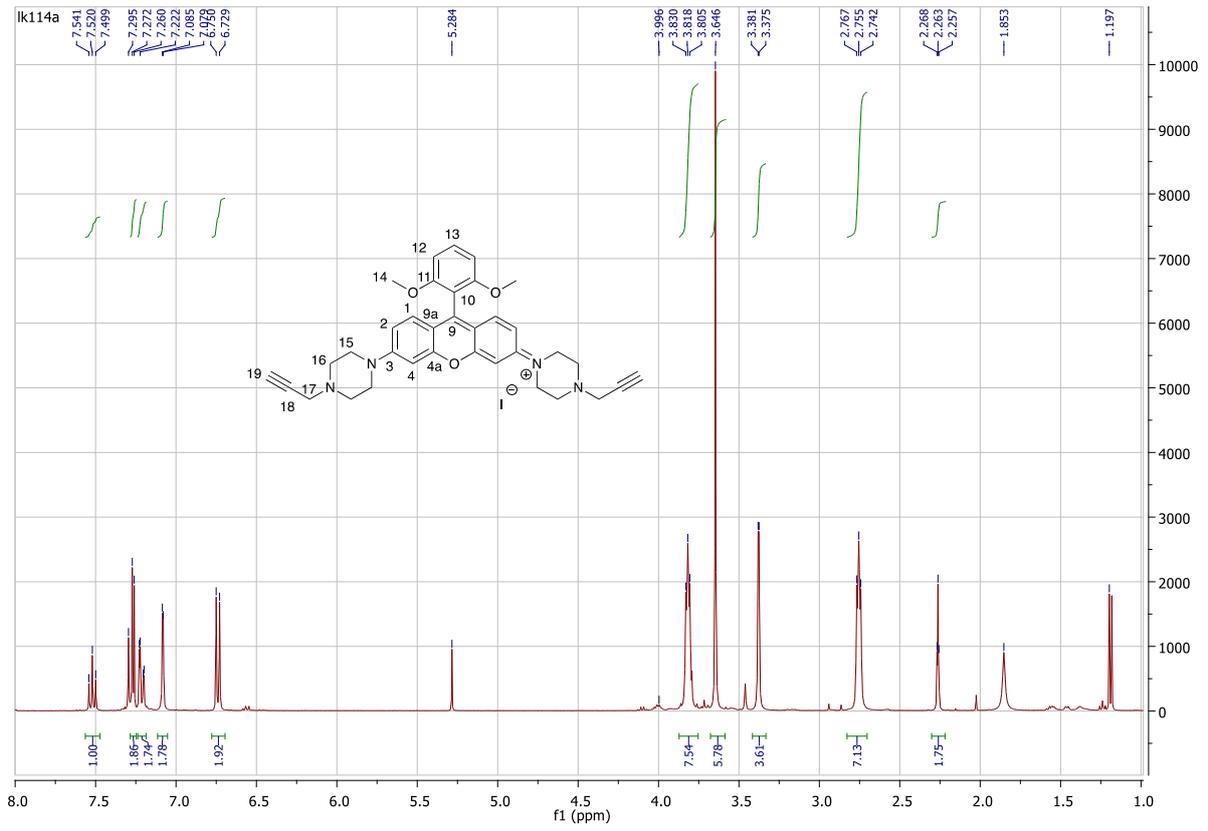
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2.97e7



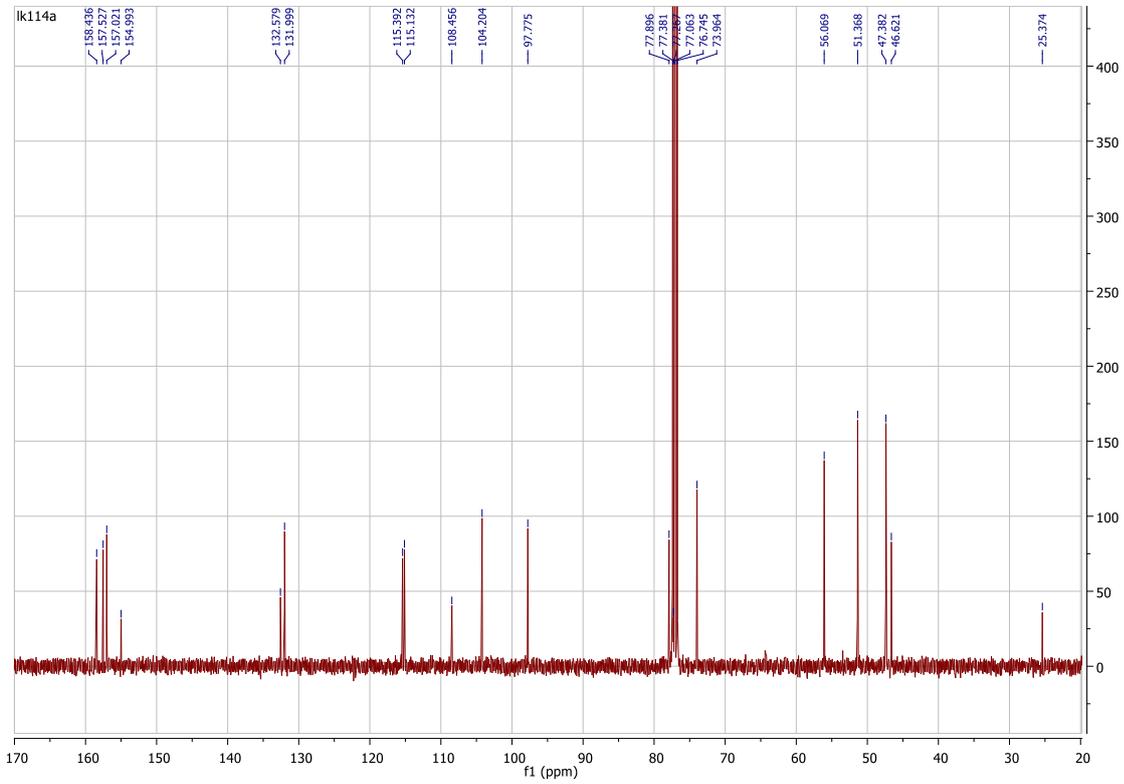
Spectral Data 2



1H NMR

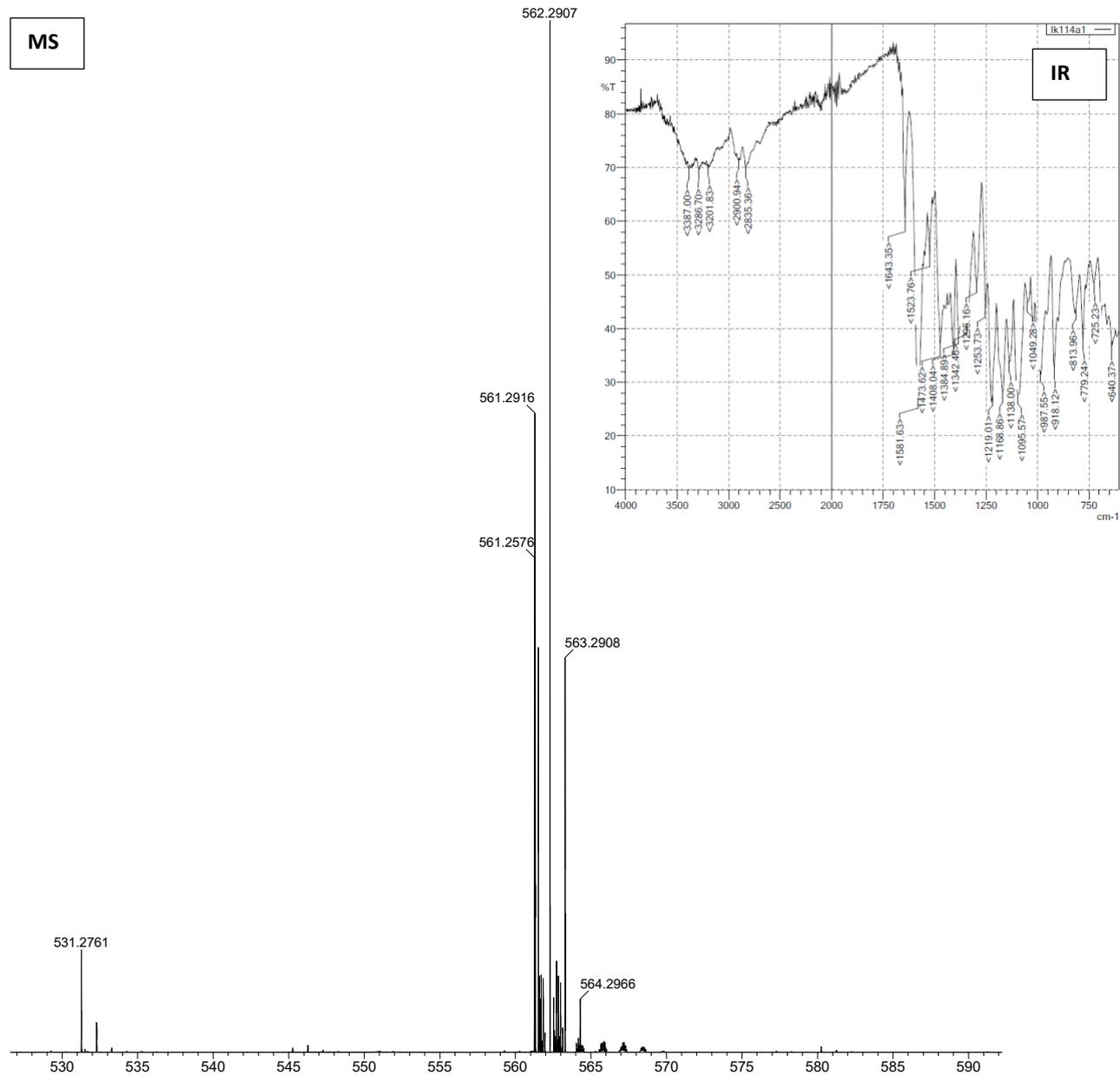


13C NMR



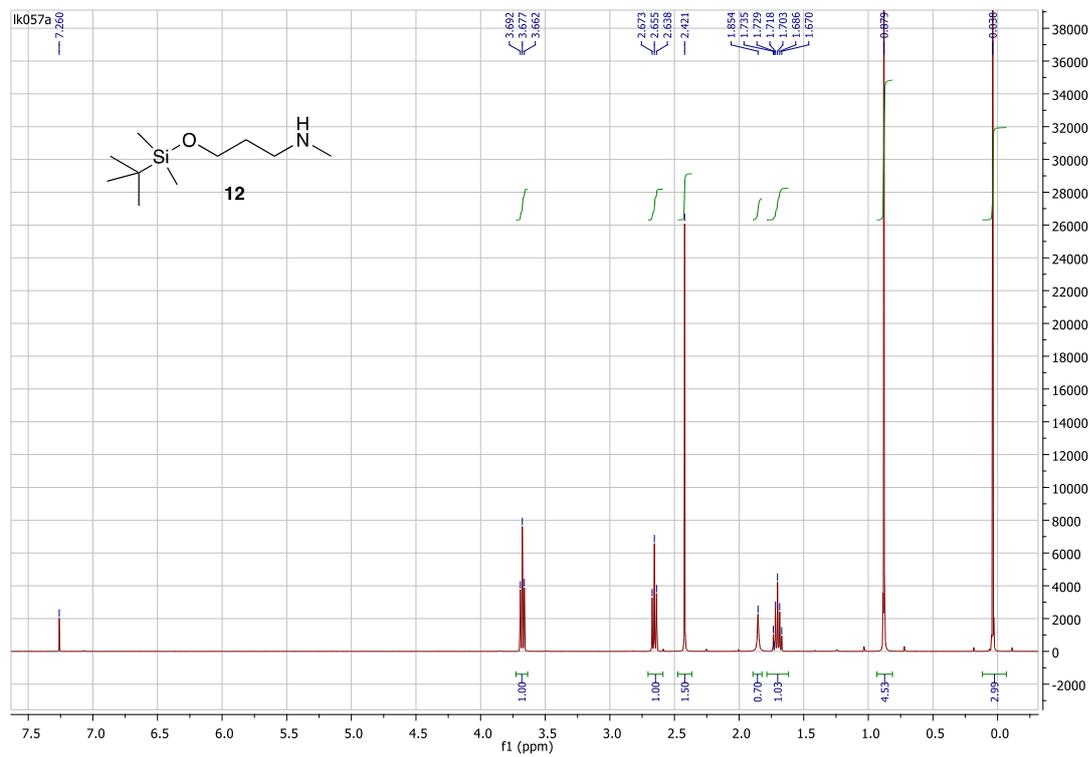
101

MS

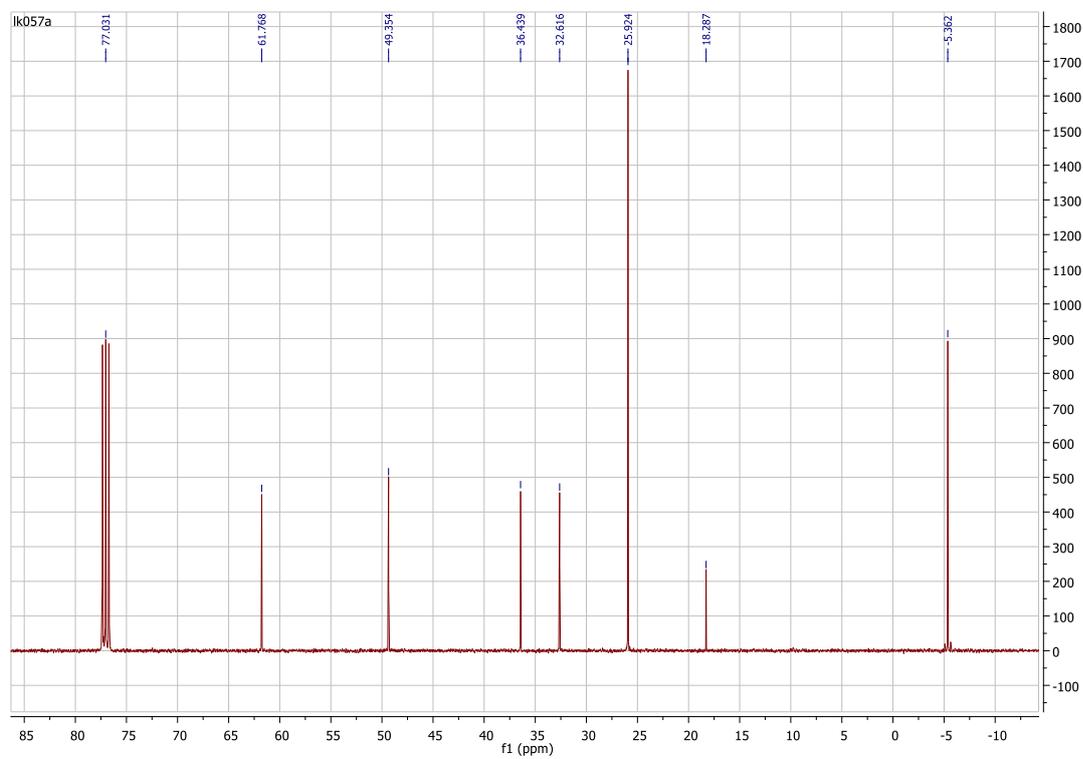


3-((tert-butylidimethylsilyl)oxy)-N-methylpropan-1-amine 12

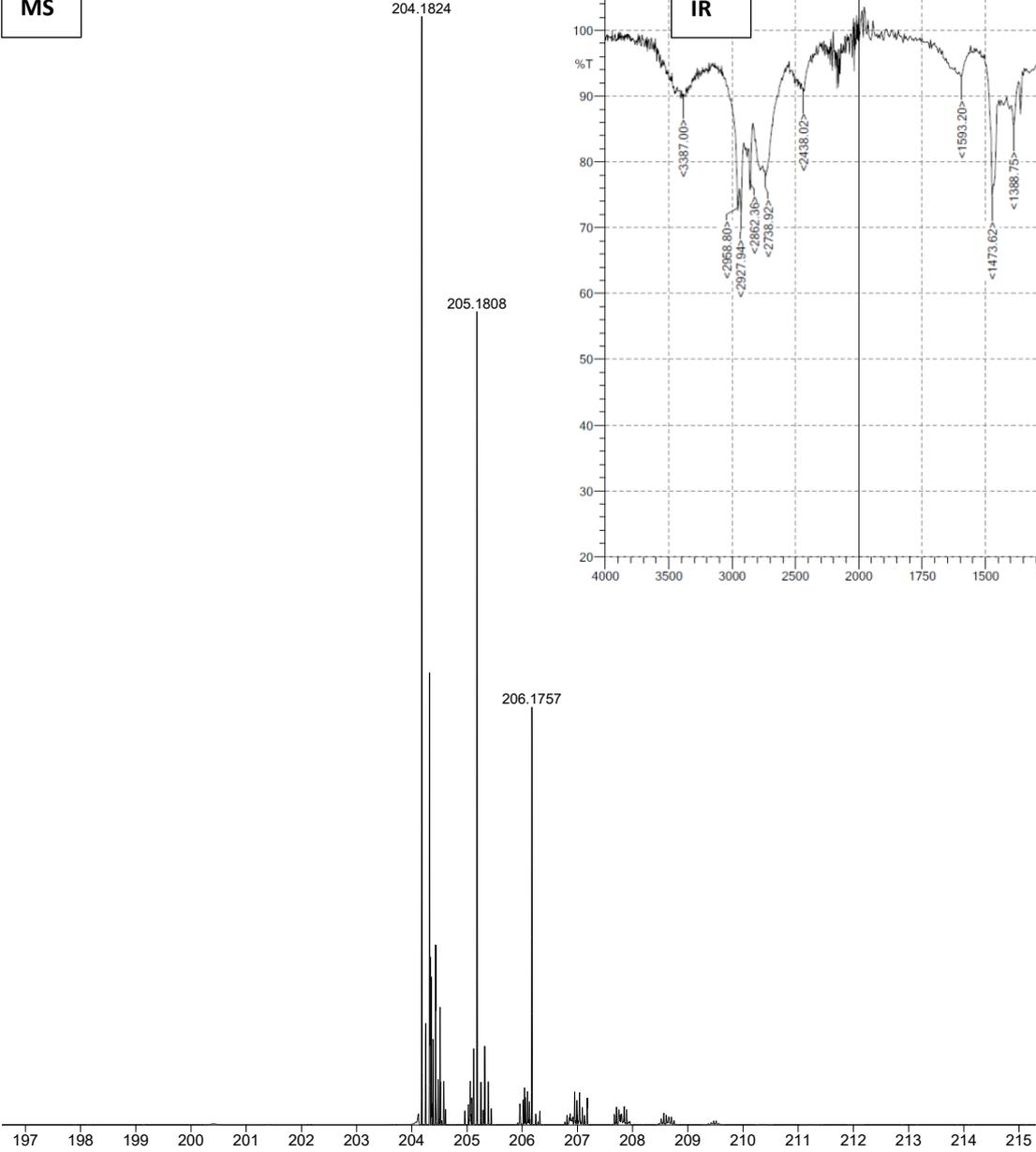
1H NMR



13C NMR

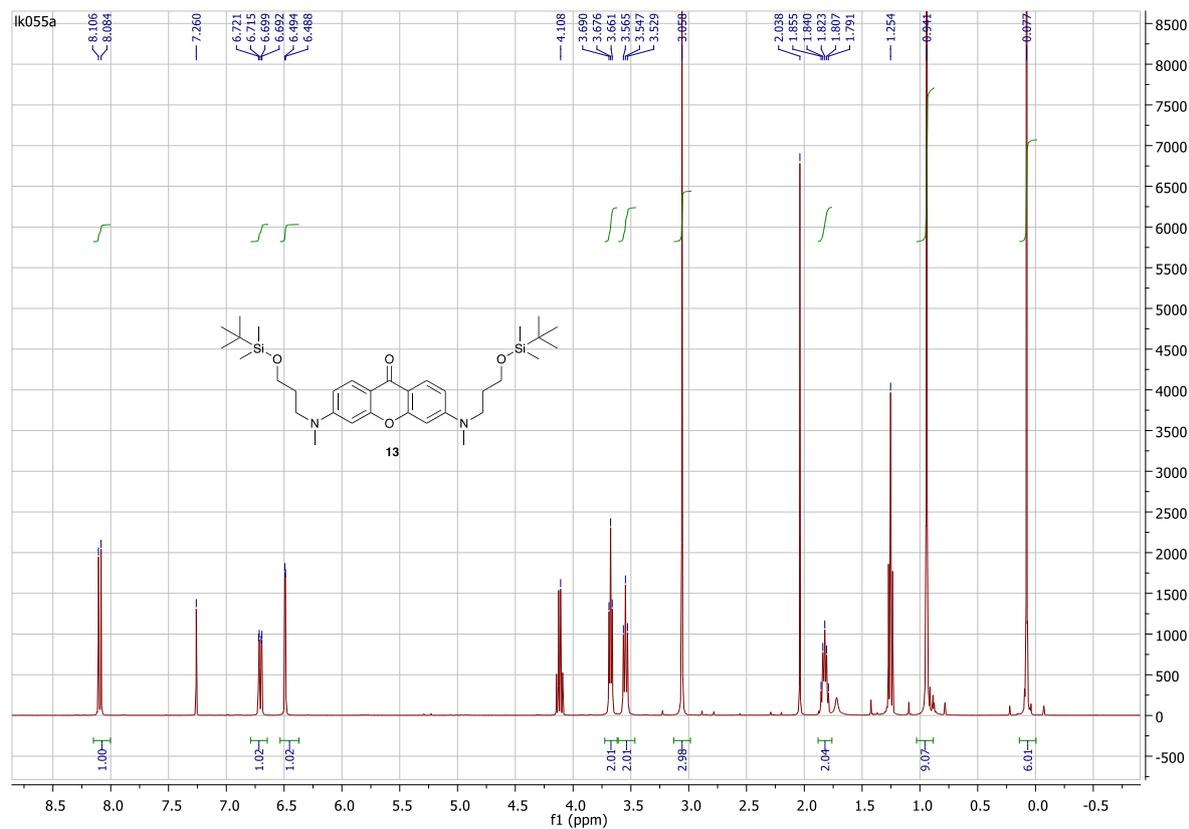


MS

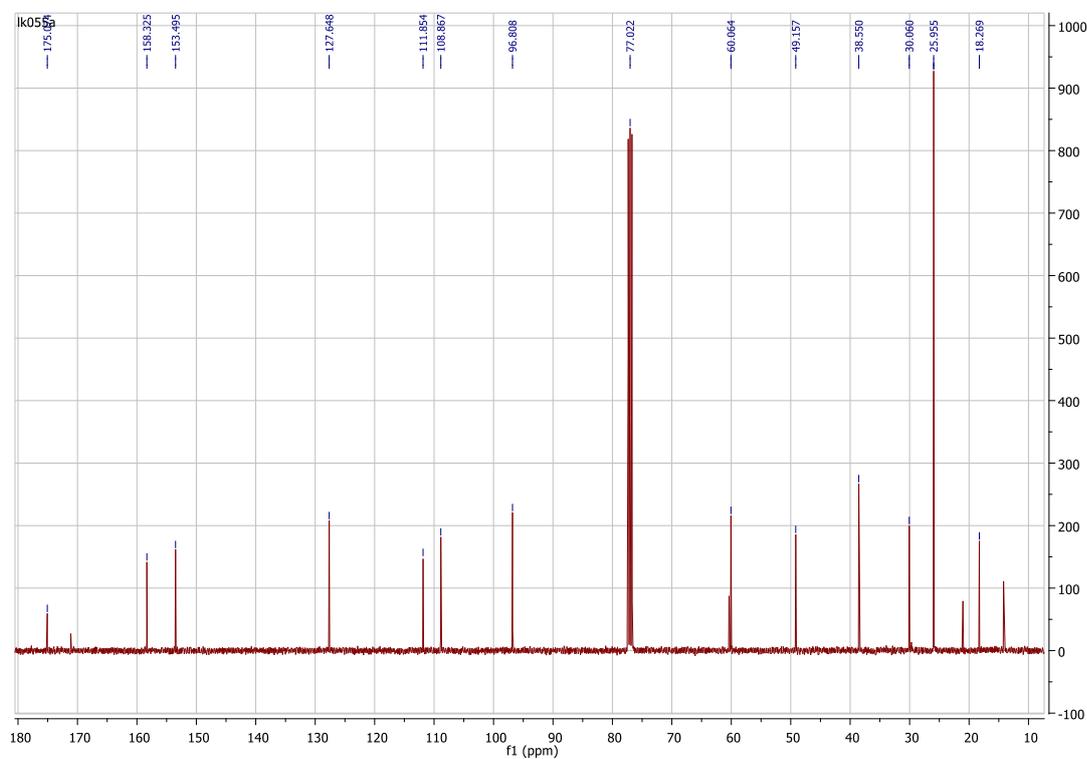


3,6-bis((tert-butylidimethylsilyloxy)propyl)(methylamino)-9H-xanthen-9-one **13**

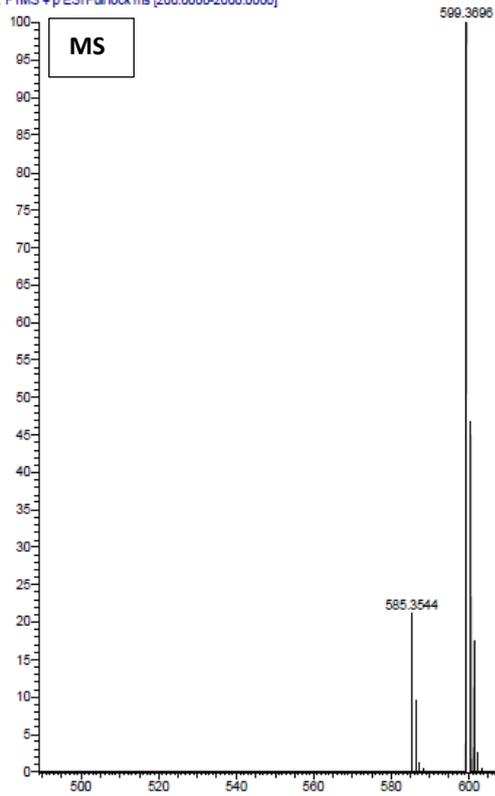
¹H NMR



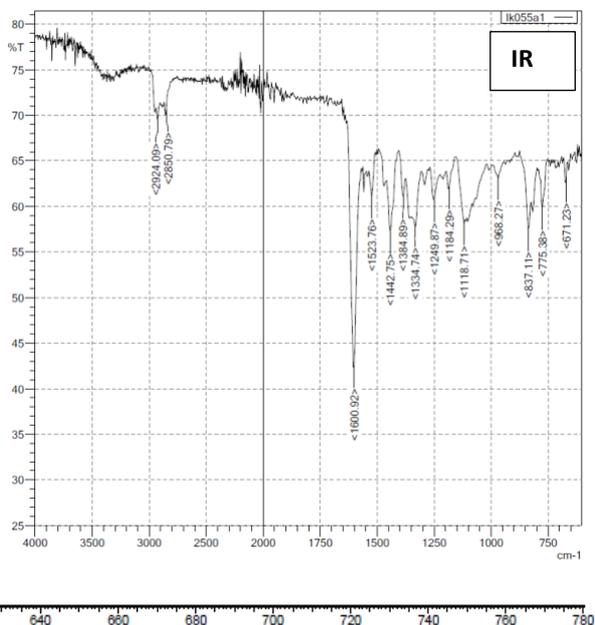
¹³C NMR



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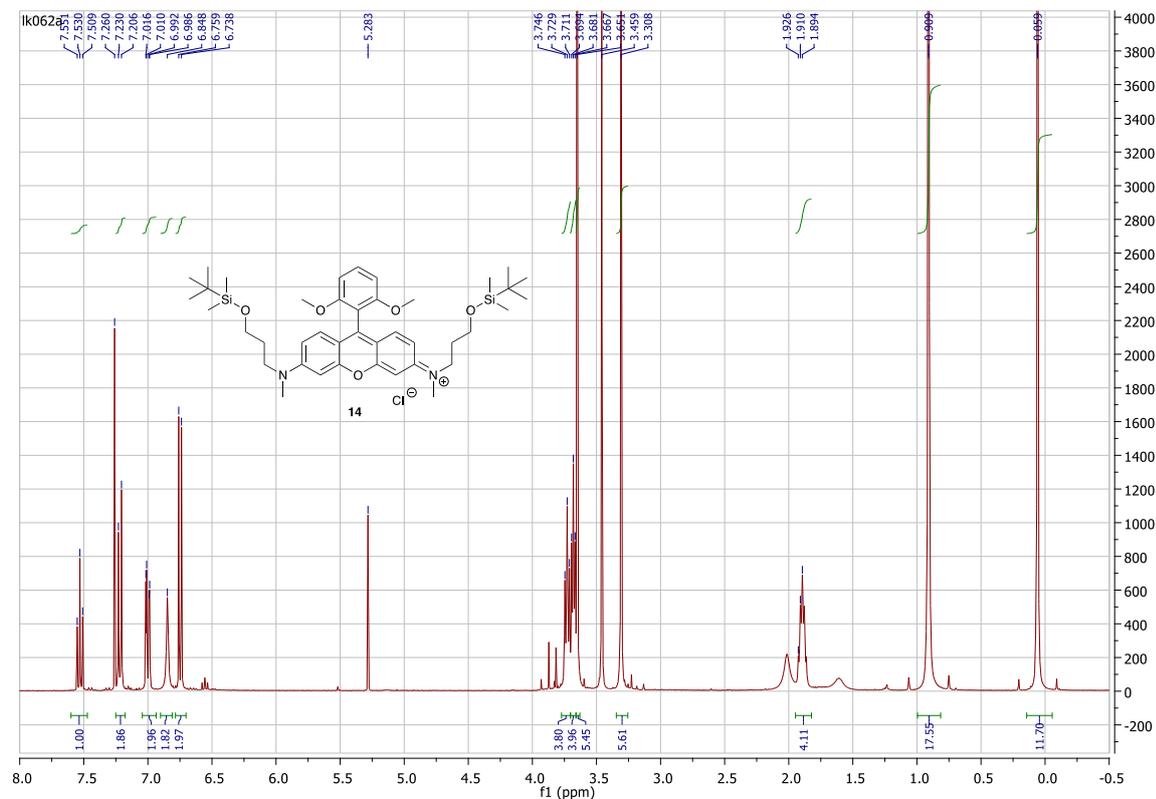


Instrument Resolution: 35,000
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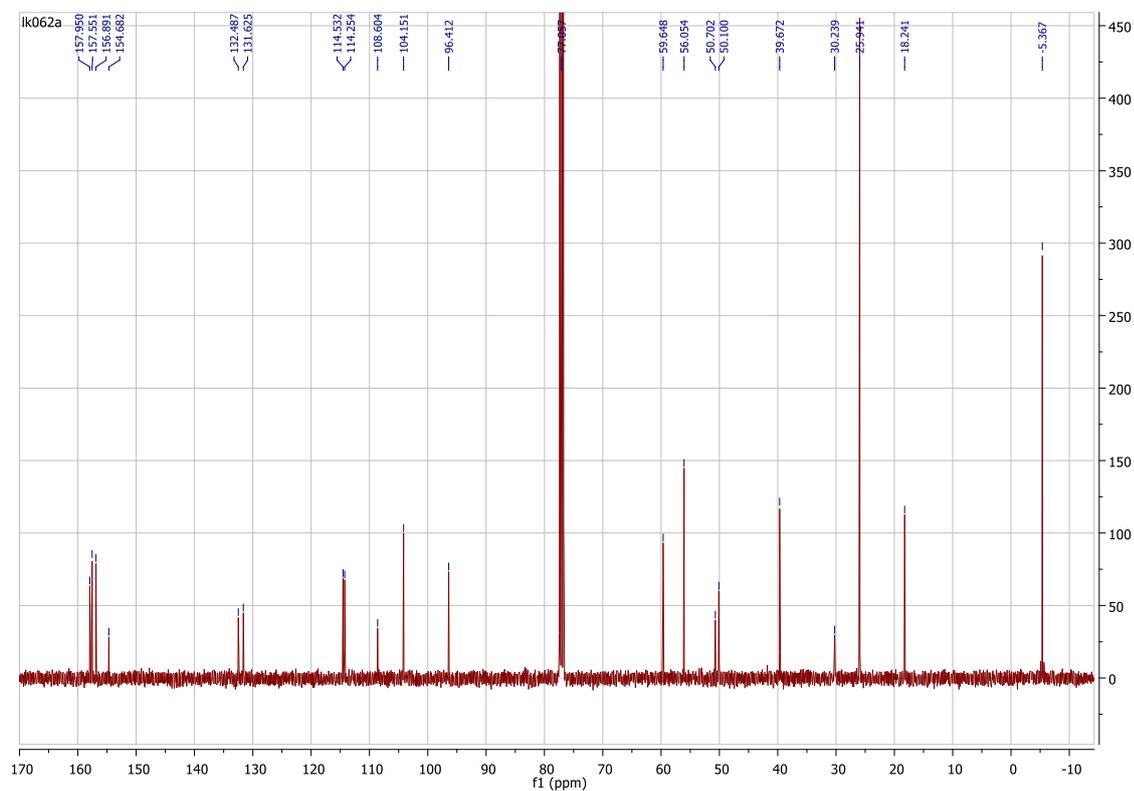


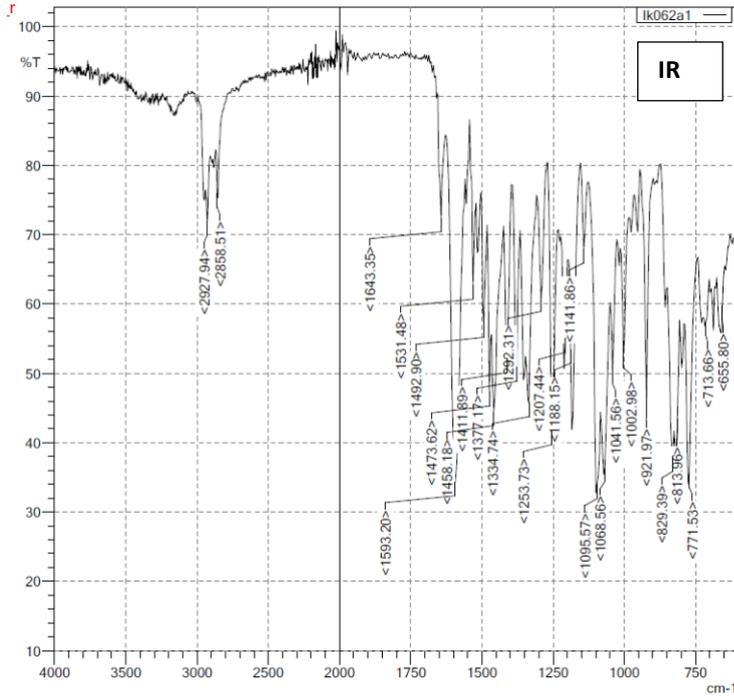
(E)-3-((*tert*-butyldimethylsilyloxy)-*N*-(6-((3-((*tert*-butyldimethylsilyloxy)propyl)(methyl)amino)-9-(2,6-dimethoxyphenyl)-3H-xanthen-3-ylidene)-*N*-methylpropan-1-aminium chloride **14**

¹H NMR

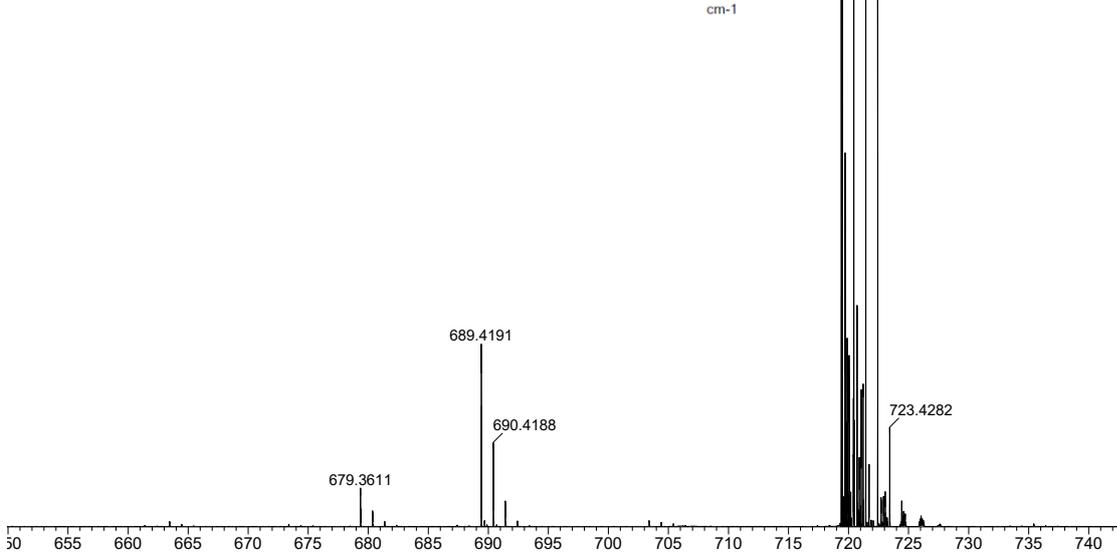


¹³C NMR



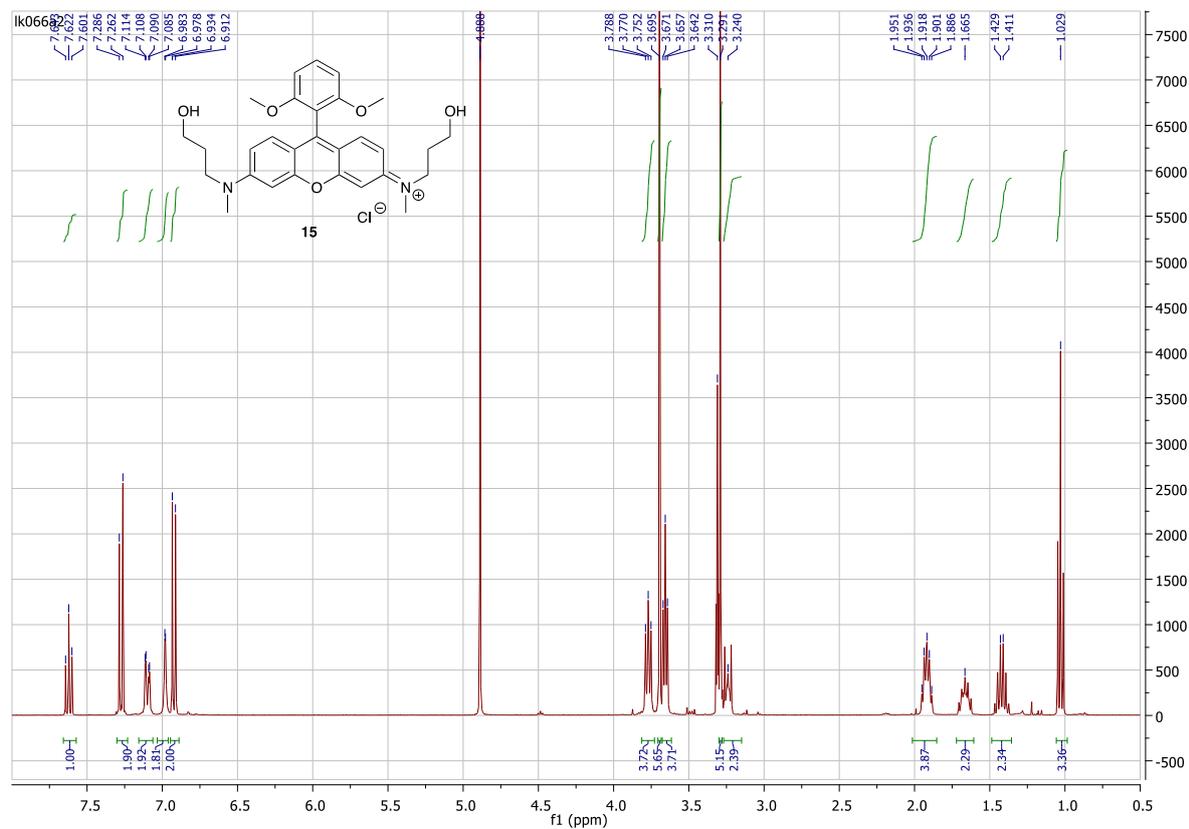


MS

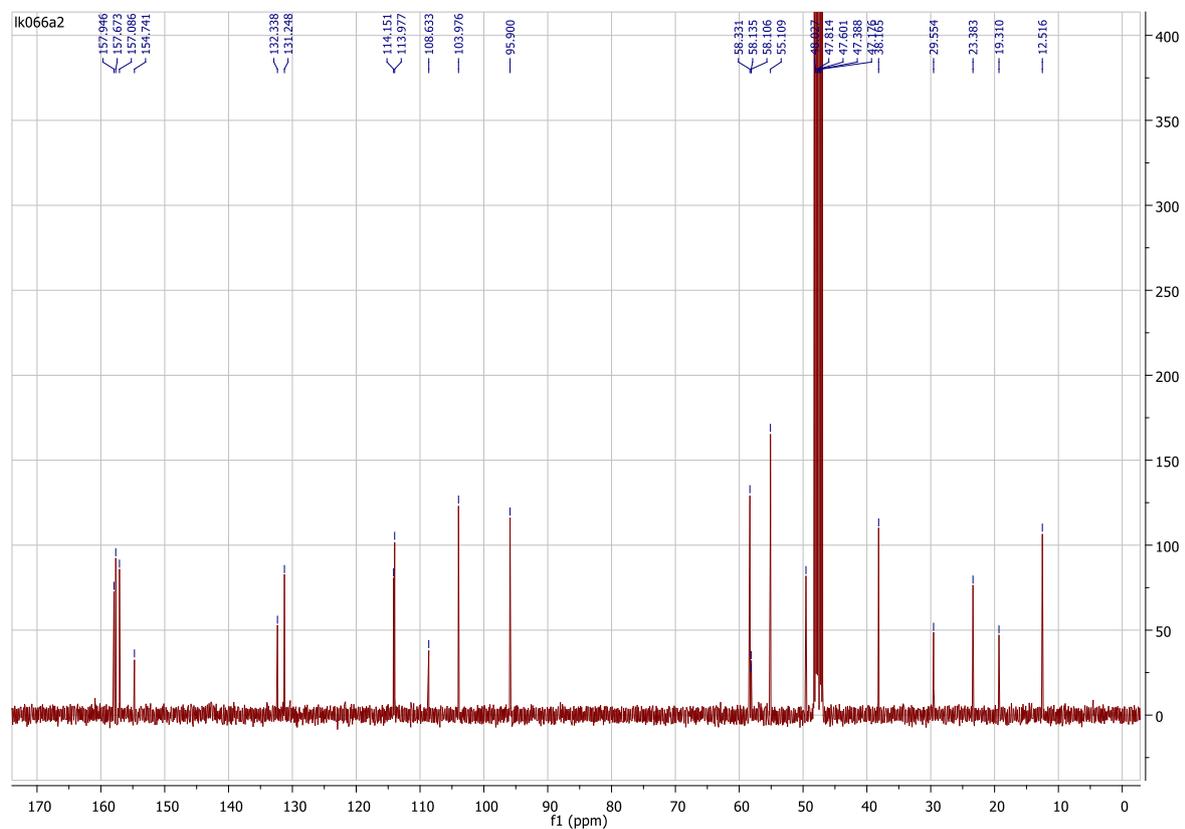


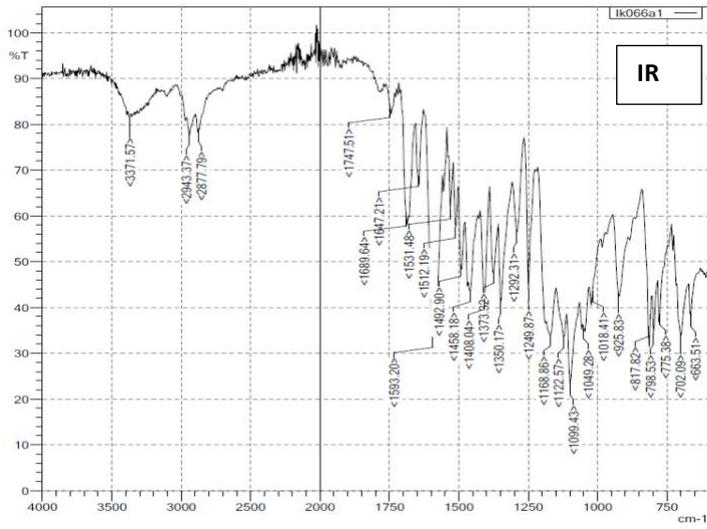
(E)-N-(9-(2,6-dimethoxyphenyl)-6-((3-hydroxypropyl)(methyl)amino)-3H-xanthen-3-ylidene)-3-hydroxy-N-methylpropan-1-aminium chloride **15**

¹H NMR

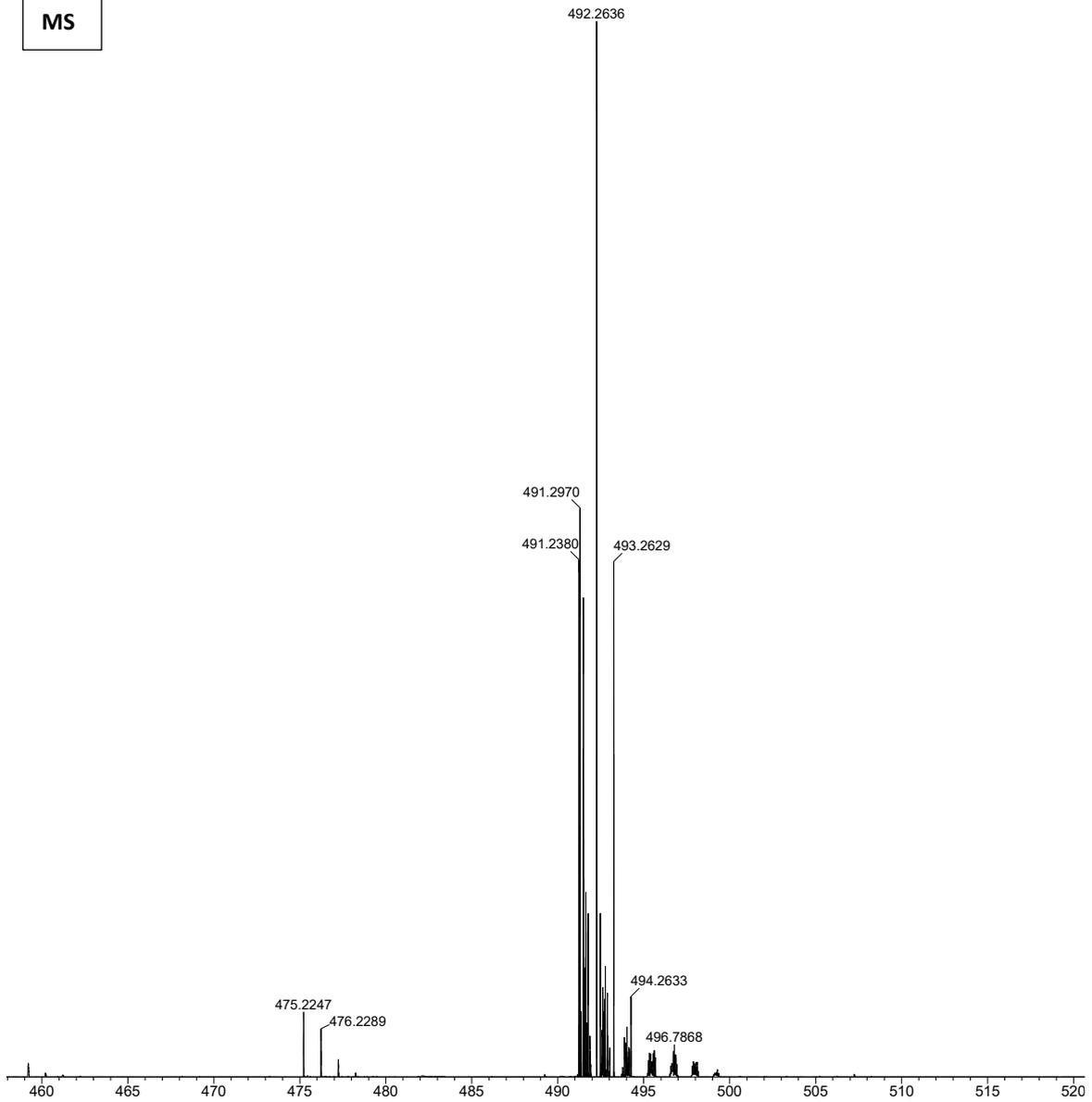


¹³C NMR



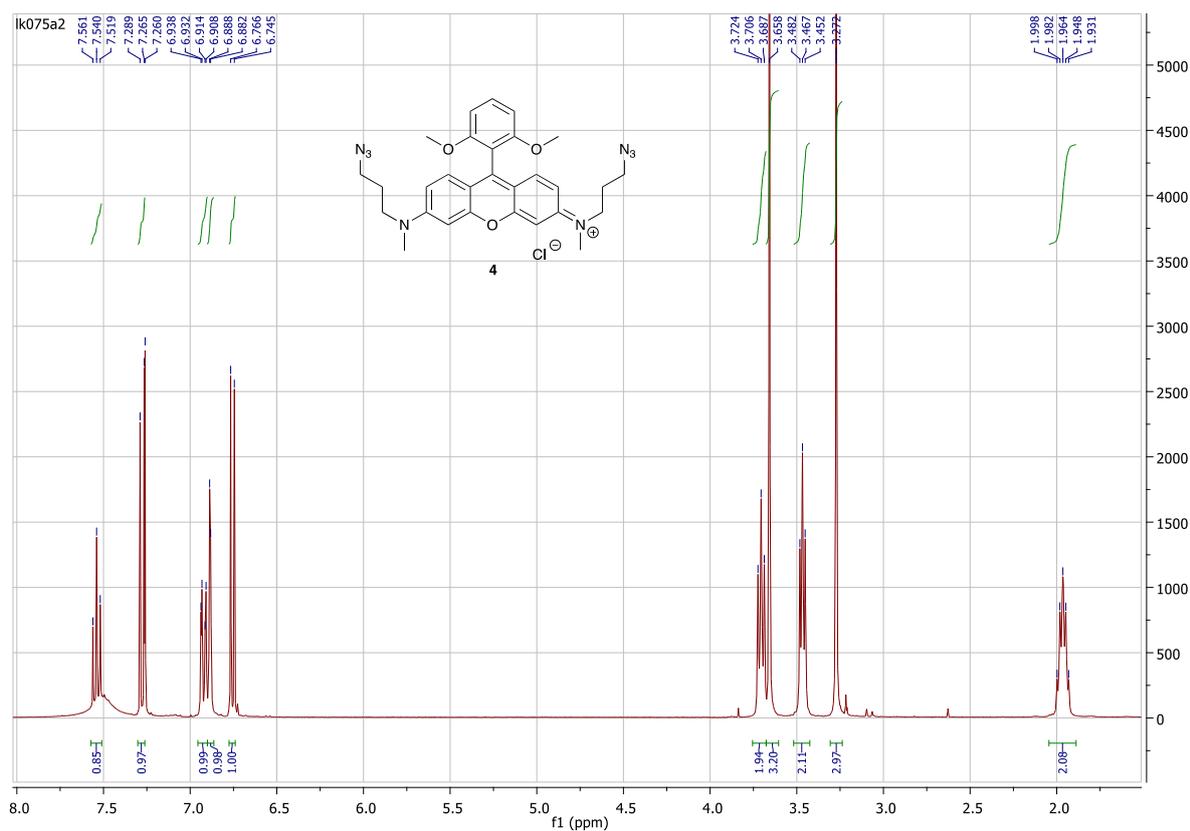


MS

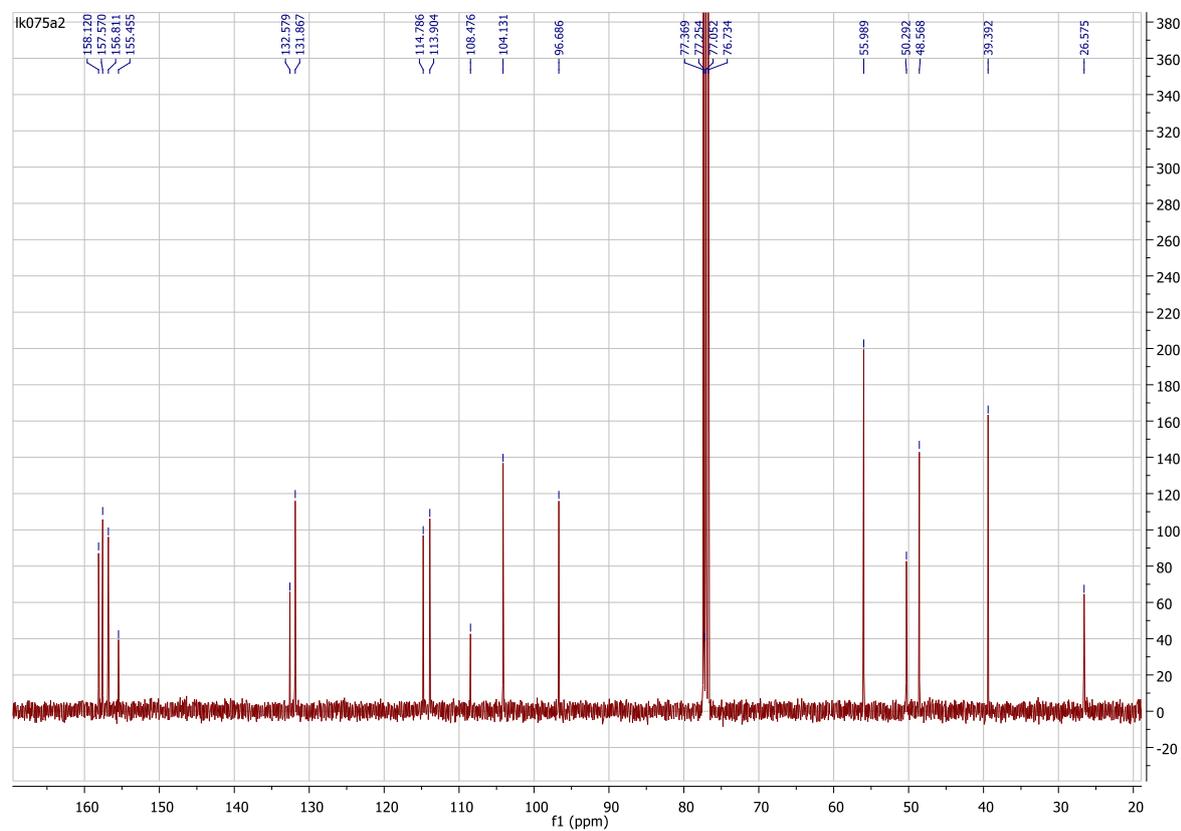


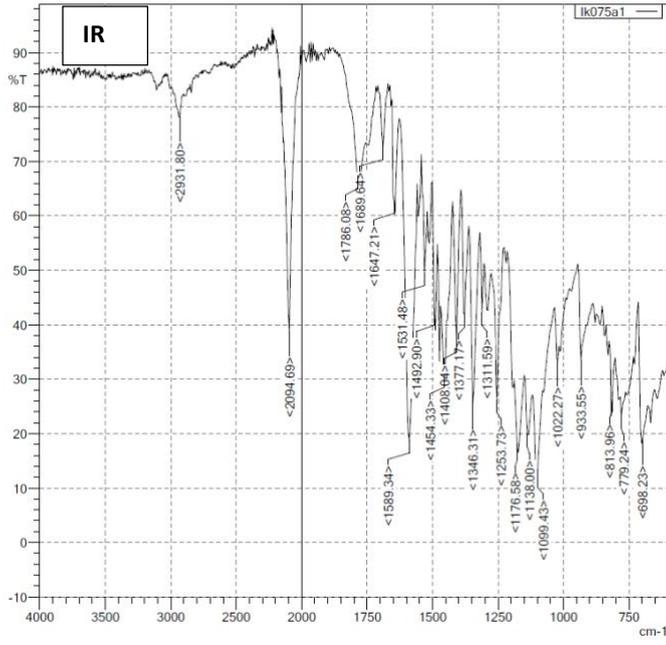
(E)-3-azido-N-(6-((3-azidopropyl)(methyl)amino)-9-(2,6-dimethoxyphenyl)-3H-xanthen-3-ylidene)-N-methylpropan-1-aminium iodide **4**

1H NMR

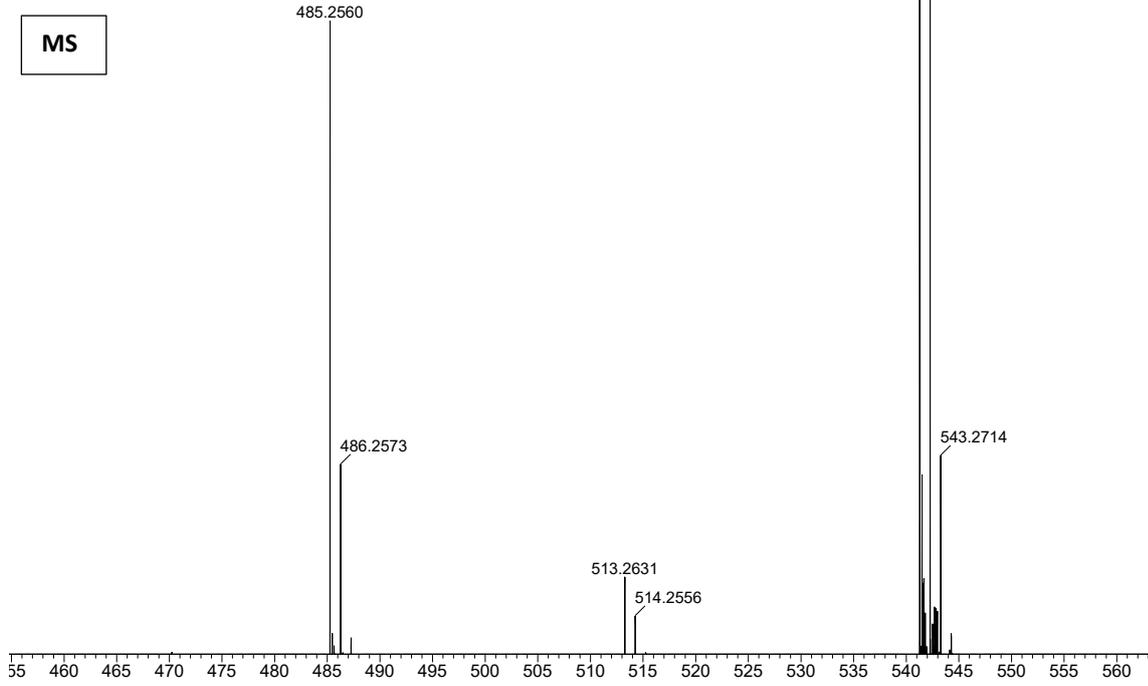


13C NMR





MS



Supporting Information Biology

Peptide Labelling

Peptides corresponding to the C-helix of cardiac troponin C were custom-synthesized by ThermoFisher (>95% purity, TFA removal; **peptide I** sequence: NPTPC_{ELQEMICEV}DEDGS, **peptide II** sequence: NPTP[G]ELQEMI[G]EVDEDGS, **peptide III** sequence: NPTP[A]ELQEMI[A]EVDEDGS. ([G] – L-propargyl-glycine, [A] – L-azido-alanine,).

For thiol-reactive labelling, peptide 1 was solubilized in reaction buffer (50 mmol/L HEPES pH 7.5, 100 mmol/L NaCl and 50 mmol/L tris(2-carboxyethyl)phosphine) to a final concentration of 5 mmol/L. The peptide was further diluted to 50 μ mol/L in the same buffer and bifunctional rhodamine (BSR) or bifunctional rosamine (BRos) were added to a final concentration of 50 μ mol/L from a 50 mmol/L stock solution in DMSO. The reaction was incubated at room temperature for 60 mins and subsequently analyzed by reverse-phase chromatography on a C18 RP column using an Agilent 1100 HPLC system with detection wavelengths of 215 nm and 530 nm for the peptide backbone and fluorophore, respectively. Peptides were eluted with a linear gradient of 5% (v/v) to 100% (v/v) acetonitrile in 0.1% (v/v) trifluoro acetic acid.

For click reactions, peptides 2 and 3 were solubilized in 100 mmol/L potassium phosphate buffer pH 7, diluted to 50 mmol/L and equimolar amounts of either Azido-BRos or Propargyl-BRos added. The reaction was started by adding a pre-mixed solution of CuSO₄ and tris(3-hydroxypropyltriazolyl-methyl)amine (THPTA) with final concentrations of 0.1 mmol/L and 0.5 mmol/L, respectively, and a sodium ascorbate to final concentration of 5 mmol/L. Reactions were incubated for 60 mins at room temperature in the dark and analyzed by Reverse phase - HPLC as described above.

Samples were further analyzed by mass spectrometry on an Agilent 6130B Single Quadrupole Mass Spec w/ Agilent 1200 MS/LC System with electron spray ionization (ESI) (Agilent Technologies). Peptides were eluted with a linear gradient of 5% (v/v) to 100% (v/v) acetonitrile in 0.1% (v/v) formic acid.

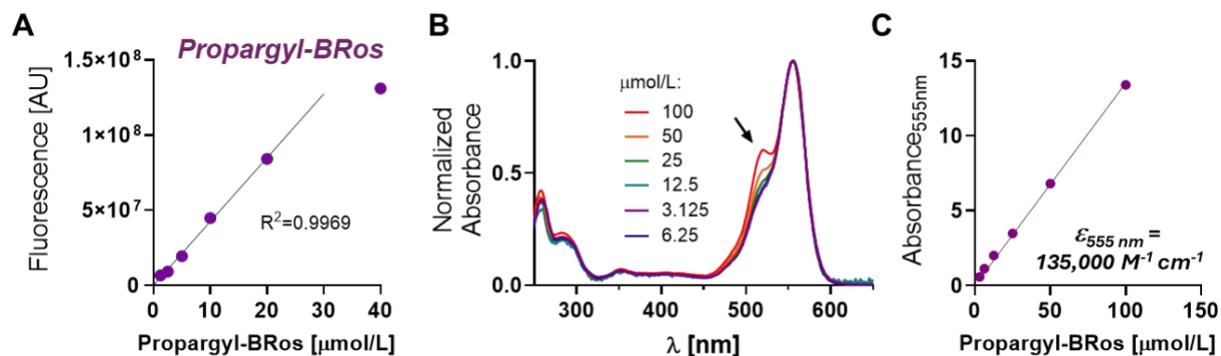
Absorbance and fluorescence spectroscopy

Absorbance spectra of fluorophores were collected in 10 mmol/L Tris-HCl pH 7 in a Mettler Toledo UV-VIS Easy photo-spectrometer in quartz cuvettes after background correction. For probe concentrations >20 μ mol/L, the absorbance spectra were recorded on a ThermoFisher Nanodrop. Fluorescence spectra were collected in DMSO, methanol or 10 mmol/L Tris-HCl pH 7 a SpectraMax i3x plate reader using black 96-well plates with a resolution of 1 nm and bandwidth of 5 nm.

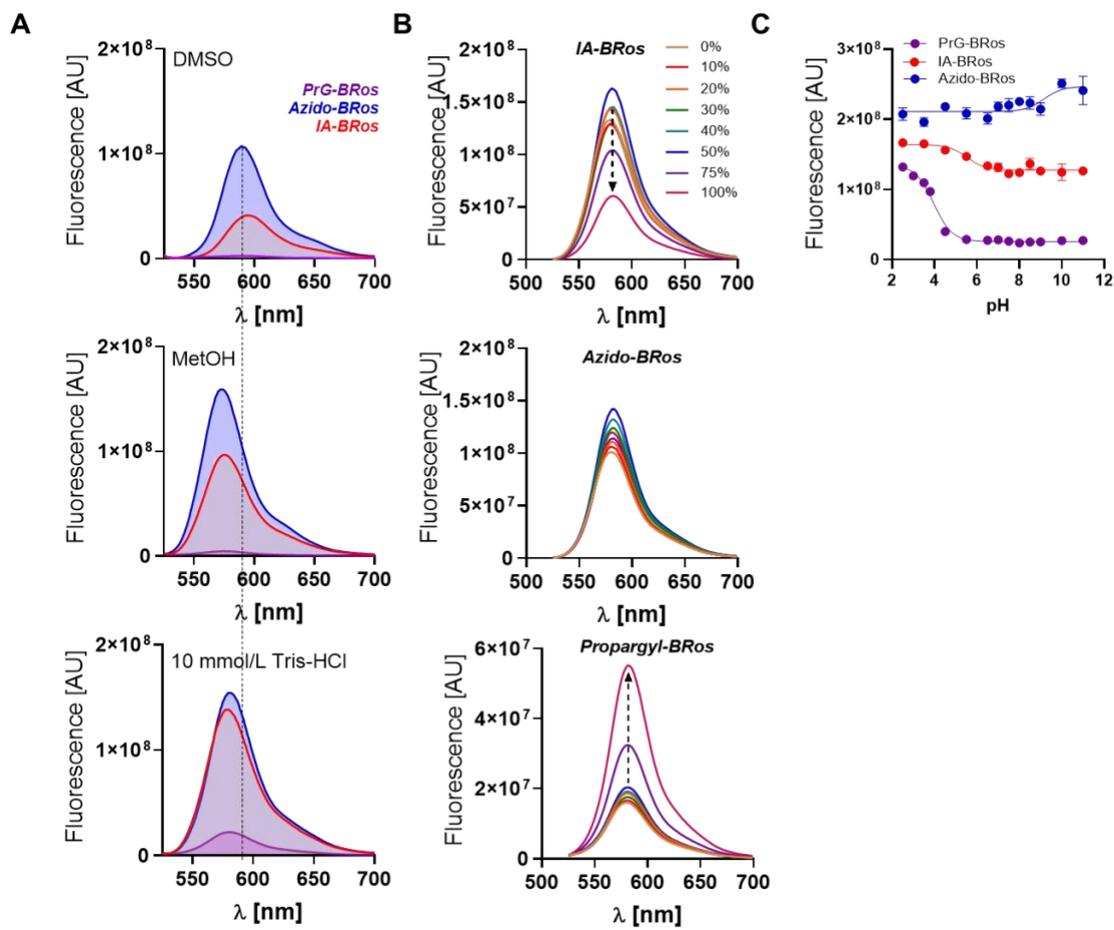
Molecular Modelling

Fluorophore structures were prepared in ChemDraw Suite 23 and energy minimized using the built-in MM2 force field calculations. Peptide structures were built in PyMol and linked to the fluorophore using built-in modelling functions. Conformers of the peptide-fluorophore complex were randomly sampled using the MMFF94 force field.

Supporting Information Figures



Supplemental Information Figure S1. (A) Concentration dependence of PrG-BROs fluorescence (exc 500 nm; em 580 nm). Linear regression to data points for $<40 \mu\text{mol/L}$ is shown as a black continuous line (B) Normalized absorbance spectrum of PrG-BROs at different concentrations. The characteristic shoulder at 520 nm indicating fluorophore stacking is indicated by a black arrow. (C) Concentration-dependence of PrG-BROs absorbance at 555 nm and linear regression to data points (continuous line).



Supplemental Information Figure S2. (A) Emission spectrum of PrG-BRos (purple), Azido-BRos (blue) and IA-BRos (red) in DMSO, methanol and 10 mmol/L Tris HCl pH 7. (B) Emission spectrum of fluorophors at different glycerol concentrations % (v/v). (C) pH-dependence of fluorophore emission (exc 500 nm; em 580 nm).

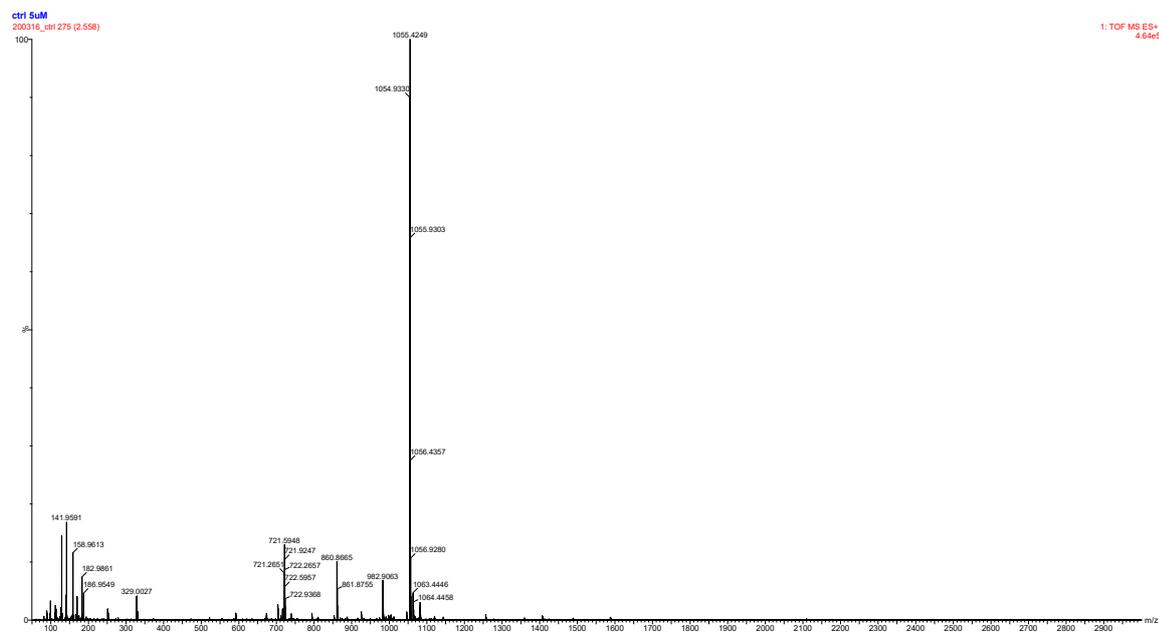
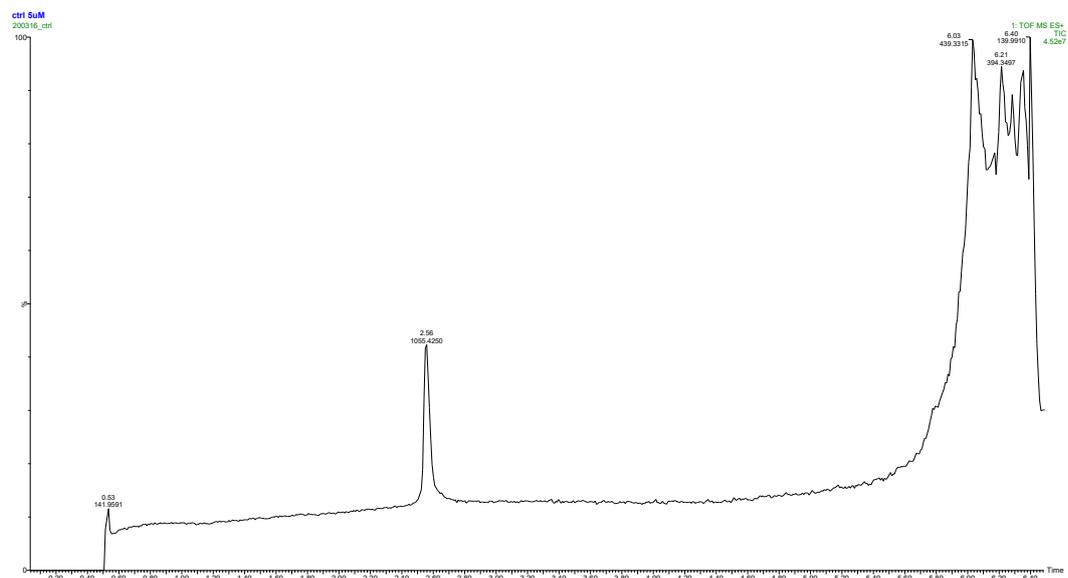
Supporting Information Tables

Supporting Information Table S1. Summary of mass spectrometry analysis of peptides before and after labelling with IA-Bros, Azido-Bros and PrG β BRos.

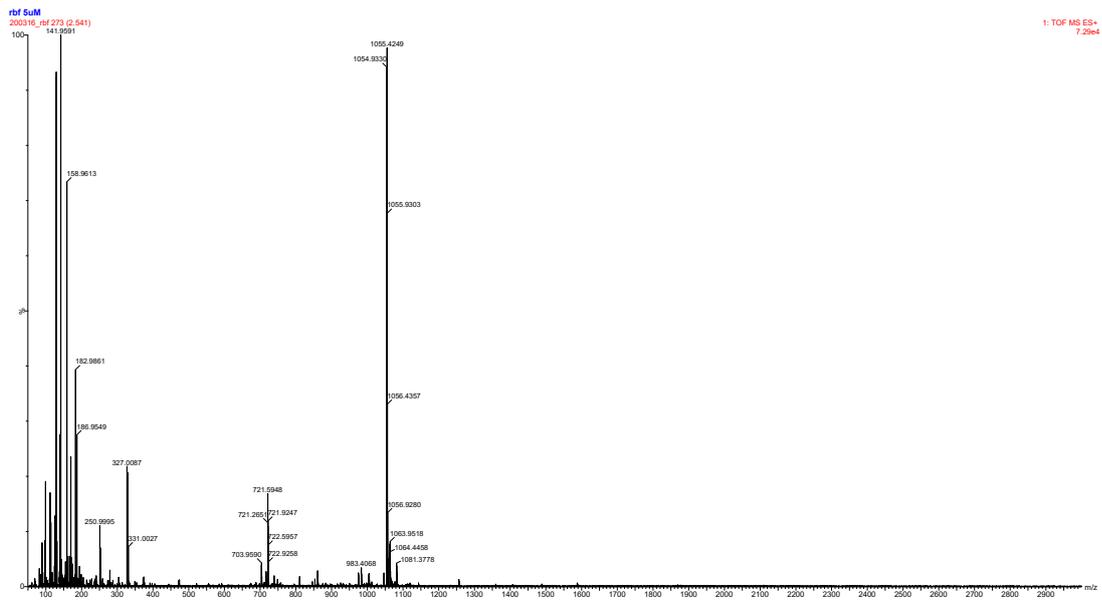
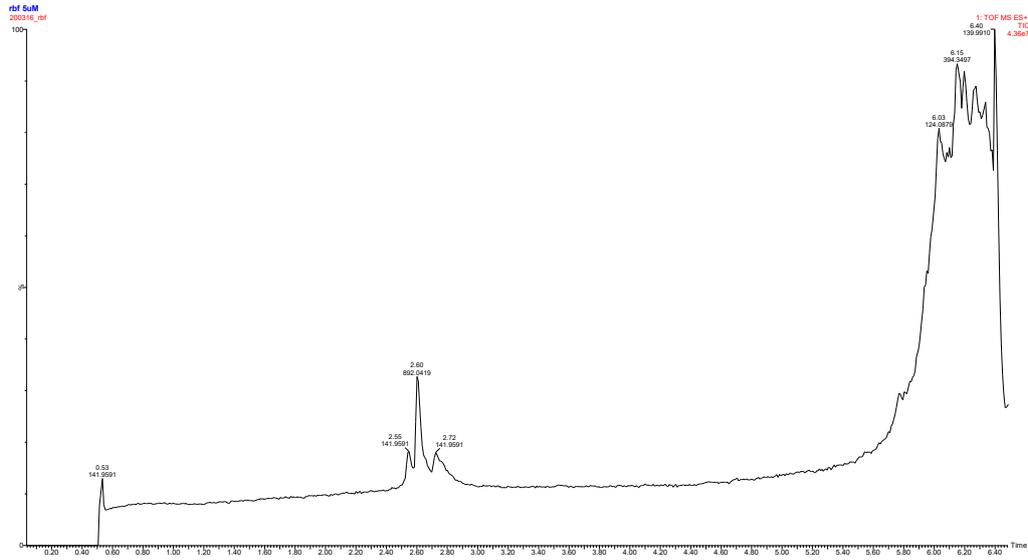
| | calculated mass [Da] | m/z found | species and calculated mass [Da] |
|--|-------------------------|-----------|--|
| unlabelled (cys) ₂ - peptide | 2109.3 | 1055.4 | [M+2H ⁺] 1054.9 |
| peptide crosslinked with IA-BRos | 2674.5 | 892.0 | [M ⁺ +2H ⁺] 892.2 |
| unlabelled (PrG) ₂ - peptide | 2093.2 | 1048.6 | [M+2H ⁺] 1047.6 |
| peptide crosslinked with Azido-BRos | 2634.5 | 880.0 | [M ⁺ +2H ⁺] 878.8 |
| unlabelled (Azido) ₂ - peptide | 2127.2 | 1066.0 | [M+2H ⁺] 1064.6 |
| peptide crosslinked with PrG-BRos | 2688.5 | 897.8 | [M ⁺ +2H ⁺] 896.8 |

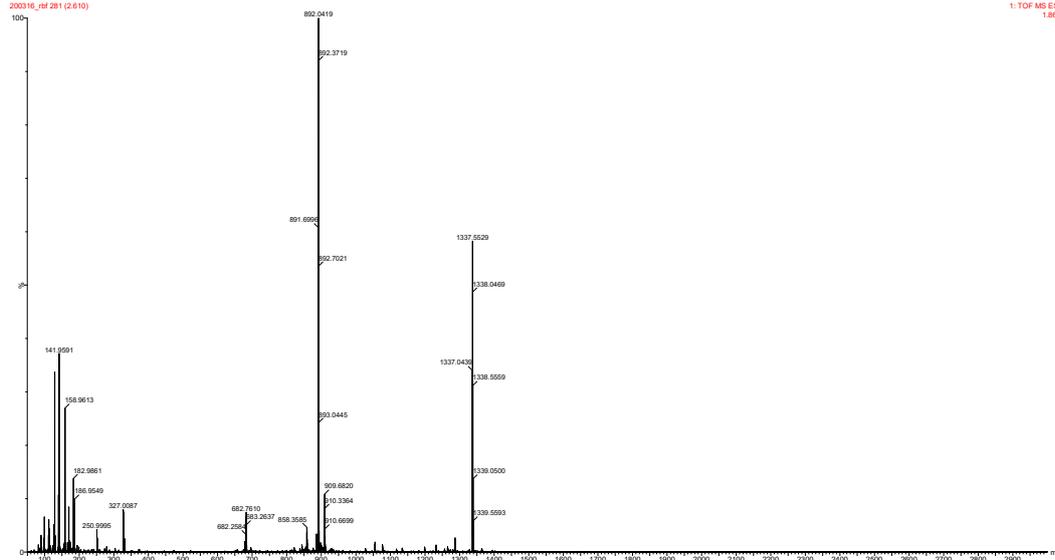
ESI of (a) unbound Peptide I and (b) bound to Bros-IA

(a)



(b)





Supporting Information References

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