SUPPORTING INFORMATION

Virginia Orange: A versatile, red-shifted fluorescein scaffold for single- and dual-input fluorogenic probes

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EXPERIMENTAL INFORMATION

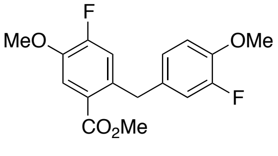
GENERAL EXPERIMENTAL INFORMATION FOR SYNTHESIS

Commercial reagents were obtained from reputable suppliers and used as received. All solvents were purchased in septum-sealed bottles stored under an inert atmosphere. All reactions were sealed with septa through which a nitrogen atmosphere was introduced unless otherwise noted. Reactions were conducted in round-bottomed flasks or septum-capped crimp-top vials containing Teflon-coated magnetic stir bars. Heating of reactions was accomplished with a silicon oil bath or an aluminum reaction block on top of a stirring hotplate equipped with an electronic contact thermometer to maintain the indicated temperatures.

Reactions were monitored by thin layer chromatography (TLC) on precoated TLC glass plates (silica gel 60 F_{254}, 250 µm thickness) or by LC/MS (Phenomenex Kinetex 2.1 mm × 30 mm 2.6 µm C18 column; 5 µL injection; 5–98% $MeCN/H_2O$, linear gradient, with constant 0.1% v/v HCO_2H additive; 6 min run; 0.5 mL/min flow; ESI; positive ion mode). TLC chromatograms were visualized by UV illumination or developed with *p*-anisaldehyde, ceric ammonium molybdate, or $KMnO_4$ stain. Reaction products were purified by flash chromatography on an automated purification system using pre-packed silica gel columns or by preparative HPLC (Phenomenex Gemini–NX 30 × 150 mm 5 µm C18 column). Analytical HPLC analysis was performed with an Agilent Eclipse XDB 4.6 × 150 mm 5 µm C18 column under the indicated conditions. High-resolution mass spectrometry was obtained by the Mass Spectrometry Center in the Department of Medicinal Chemistry at the University of Washington and the High Resolution Mass Spectrometry Facility at the University of Iowa.

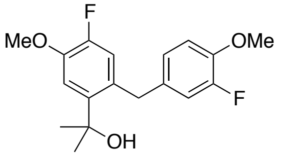
NMR spectra were recorded on a 400 MHz spectrometer. ^1H and ^{13}C chemical shifts (δ) were referenced to TMS or residual solvent peaks,[1] and ^{19}F chemical shifts (δ) were referenced to $CFCl_3$. Data for ^1H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, m = multiplet), coupling constant (Hz), integration. Data for ^{13}C NMR spectra are reported by chemical shift (δ ppm) with hydrogen multiplicity (C, CH, CH_2, CH_3) information obtained from DEPT spectra.

SYNTHESIS OF VIRGINIA ORANGE

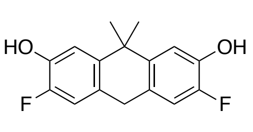


Methyl 4-fluoro-2-(3-fluoro-4-methoxybenzyl)-5-methoxybenzoate (7): A round bottom flask equipped with a reflux condenser was charged with methyl 2-bromo-4-fluoro-5-methoxybenzoate (**5**; 7.44 g, 28.3 mmol), $Pd(OAc)_2$ (319 mg, 1.42 mmol, 0.05 eq), and SPhos (1.16 g, 2.83 mmol, 0.10 eq). The flask was evacuated and backfilled with nitrogen (3×). Degassed THF (85 mL) was added, followed by 3-fluoro-4-methoxybenzylzinc chloride (**6**; 0.5 M in THF, 85.0 mL, 42.5 mmol, 1.5 eq). The reaction was stirred under nitrogen in a 60 °C oil bath for 5 h. It was subsequently cooled to room temperature, quenched with saturated NH_4Cl, diluted with water, and extracted with

EtOAc (2×). The combined organic extracts were washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. Flash chromatography on silica gel (0–30% EtOAc/hexanes, linear gradient; mixed fractions repurified with 0–20% Et_2O/hexanes, linear gradient) afforded 8.68 g (95%) of **7** as a pale yellow solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.56 (d, $^4J_{HF}$ = 8.8 Hz, 1H), 6.91 – 6.81 (m, 4H), 4.23 (s, 2H), 3.91 (s, 3H), 3.85 (s, 3H), 3.85 (s, 3H); ^{19}F NMR ($CDCl_3$, 376 MHz) δ -129.06 (dd, J_{FH} = 12.1, 8.8 Hz, 1F), -135.80 (dd, J_{FH} = 12.2, 7.6 Hz, 1F); ^{13}C NMR ($CDCl_3$, 101 MHz) δ 167.0 (C), 154.5 (d, $^1J_{CF}$ = 253.8 Hz, C), 152.5 (d, $^1J_{CF}$ = 245.6 Hz, C), 146.1 (d, $^2J_{CF}$ = 10.8 Hz, C), 145.9 (d, $^2J_{CF}$ = 10.9 Hz, C), 136.6 (d, $^3J_{CF}$ = 6.7 Hz, C), 133.8 (d, $^3J_{CF}$ = 6.0 Hz, C), 125.5 (d, $^4J_{CF}$ = 3.5 Hz, C), 124.4 (d, $^3J_{CF}$ = 3.5 Hz, CH), 119.1 (d, $^2J_{CF}$ = 18.9 Hz, CH), 116.6 (d, $^2J_{CF}$ = 18.4 Hz, CH), 116.2 (d, $^3J_{CF}$ = 3.3 Hz, CH), 113.6 (d, $^4J_{CF}$ = 2.2 Hz, CH), 56.6 (CH_3), 56.5 (CH_3), 52.2 (CH_3), 38.1 (CH_2); HRMS (EI) calcd for $C_{17}H_{16}F_2O_4$ $[M\cdot]^+$ 322.1017, found 322.1013.

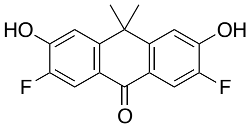


2-(4-Fluoro-2-(3-fluoro-4-methoxybenzyl)-5-methoxyphenyl)propan-2-ol (8): A solution of ester **7** (8.37 g, 26.0 mmol) in THF (120 mL) was cooled to −78 °C under nitrogen. Methylmagnesium chloride (3 M in THF, 26.0 mL, 77.9 mmol, 3 eq) was added; the reaction was allowed to warm to room temperature and stirred overnight. It was subsequently quenched with saturated NH_4Cl, diluted with water, and extracted with EtOAc (2×). The combined organic extracts were washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by silica gel chromatography (0–30% EtOAc/hexanes, linear gradient; mixed fractions repurified with 0–10% EtOAc/toluene, linear gradient) to provide **8** as a pale yellow oil (6.42 g, 77%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.11 (d, $^4J_{HF}$ = 8.9 Hz, 1H), 6.89 – 6.78 (m, 3H), 6.76 (d, $^3J_{HF}$ = 12.6 Hz, 1H), 4.23 (s, 2H), 3.90 (s, 3H), 3.86 (s, 3H), 1.63 (s, 6H); ^{19}F NMR ($CDCl_3$, 376 MHz) δ -135.72 – -135.82 (m, 1F), -138.36 (dd, J_{FH} = 12.6, 8.9 Hz, 1F); ^{13}C NMR ($CDCl_3$, 101 MHz) δ 152.5 (d, $^1J_{CF}$ = 245.6 Hz, C), 151.1 (d, $^1J_{CF}$ = 245.5 Hz, C), 145.9 (d, $^2J_{CF}$ = 10.8 Hz, C), 145.2 (d, $^2J_{CF}$ = 10.3 Hz, C), 142.3 (d, $^4J_{CF}$ = 3.6 Hz, C), 135.3 (d, $^3J_{CF}$ = 5.8 Hz, C), 131.3 (d, $^3J_{CF}$ = 5.4 Hz, C), 124.3 (d, $^3J_{CF}$ = 3.4 Hz, CH), 120.1 (d, $^2J_{CF}$ = 18.0 Hz, CH), 116.6 (d, $^2J_{CF}$ = 18.4 Hz, CH), 113.6 (d, $^3J_{CF}$ = 2.2 Hz, CH), 112.2 (d, $^4J_{CF}$ = 2.2 Hz, CH), 73.6 (C), 56.7 (CH_3), 56.5 (CH_3), 38.0 (CH_2), 32.0 (CH_3); HRMS (EI) calcd for $C_{18}H_{20}F_2O_3$ $[M\cdot]^+$ 322.1381, found 322.1381.

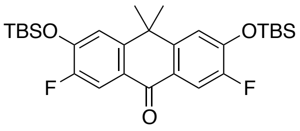


3,6-Difluoro-9,9-dimethyl-9,10-dihydroanthracene-2,7-diol (9): Alcohol **8** (6.17 g, 19.1 mmol) was taken up in CH_2Cl_2 (150 mL) under nitrogen and cooled to 0 °C. BBr_3 (1.0 M in CH_2Cl_2, 68.9 mL, 68.9 mmol, 3.6 eq) was added dropwise. The reaction was warmed to room temperature and stirred for 2 h. It was then carefully quenched with water (~50 mL) and vigorously stirred for 30 min. The mixture was neutralized (to pH ~ 7) with saturated $NaHCO_3$ and extracted with CH_2Cl_2 (2×) and EtOAc (2×). The combined organics were dried over anhydrous

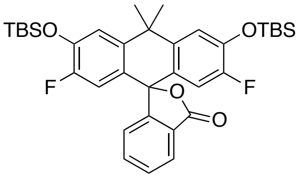
$MgSO_4$, filtered, concentrated *in vacuo*, and deposited onto Celite. Flash chromatography on silica gel (0–50% EtOAc/hexanes, linear gradient; dry load with Celite) yielded 4.60 g (87%) of **9** as an air-sensitive, orange-yellow solid. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.50 (s, 2H), 7.08 (d, $^4J_{HF}$ = 8.9 Hz, 2H), 7.01 (d, $^3J_{HF}$ = 11.6 Hz, 2H), 3.80 (s, 2H), 1.43 (s, 6H); ^{19}F NMR (DMSO-d_6, 376 MHz) δ -140.30 (dd, J_{FH} = 11.3, 9.1 Hz); ^{13}C NMR (DMSO-d_6, 101 MHz) δ 149.0 (d, $^1J_{CF}$ = 239.3 Hz, C), 142.7 (d, $^2J_{CF}$ = 12.1 Hz, C), 140.4 (d, $^4J_{CF}$ = 3.1 Hz, C), 126.5 (d, $^3J_{CF}$ = 6.1 Hz, C), 114.7 (d, $^2J_{CF}$ = 18.0 Hz, CH), 114.0 (d, $^3J_{CF}$ = 3.0 Hz, CH), 38.2 (C), 32.7 (CH_2), 29.0 (CH_3); HRMS (EI) calcd for $C_{16}H_{14}F_2O_2$ $[M\cdot]^+$ 276.0962, found 276.0968.



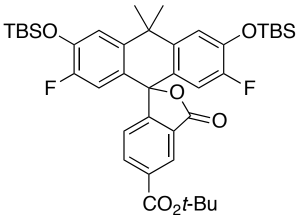
2,7-Difluoro-3,6-dihydroxy-10,10-dimethylanthracen-9(10*H*)-one (10): Phenol **9** (4.79 g, 17.3 mmol) was taken up in a mixture of CH_2Cl_2 (150 mL) and dioxane (75 mL), and water (19 mL) was added. The mixture was cooled to 0 °C, and DDQ (11.80 g, 52.0 mmol, 3 eq) was added. The reaction was warmed to room temperature and stirred overnight. The crude reaction mixture was deposited onto Celite and concentrated to dryness. Flash chromatography (10–100% EtOAc/hexanes, linear gradient; dry load with Celite) afforded **10** (3.19 g, 63%) as an off-white solid. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 10.92 (s, 2H), 7.77 (d, $^3J_{HF}$ = 11.7 Hz, 2H), 7.30 (d, $^4J_{HF}$ = 8.1 Hz, 2H), 1.60 (s, 6H); ^{19}F NMR (DMSO-d_6, 376 MHz) δ -137.16 (dd, J_{FH} = 11.5, 8.2 Hz); ^{13}C NMR (DMSO-d_6, 101 MHz) δ 179.1 (C), 150.3 (d, $^2J_{CF}$ = 13.1 Hz, C), 150.2 (d, $^1J_{CF}$ = 243.5 Hz, C), 148.3 (d, $^4J_{CF}$ = 2.7 Hz, C), 121.9 (d, $^3J_{CF}$ = 4.4 Hz, C), 115.5 (d, $^3J_{CF}$ = 2.6 Hz, CH), 113.1 (d, $^2J_{CF}$ = 18.3 Hz, CH), 37.2 (C), 32.8 (CH_3); HRMS (ESI) calcd for $C_{16}H_{13}F_2O_3$ $[M+H]^+$ 291.0827, found 291.0831.



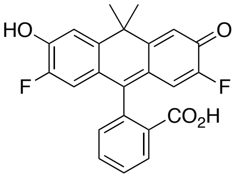
3,6-Bis((*tert*-butyldimethylsilyl)oxy)-2,7-difluoro-10,10-dimethylanthracen-9(10*H*)-one (11): To a solution of **10** (436 mg, 1.50 mmol) in DMF (10 mL) were added imidazole (307 mg, 4.51 mmol, 3 eq) and TBSCl (680 mg, 4.51 mmol, 3 eq). The reaction was stirred at room temperature for 3 h. It was subsequently diluted with water and extracted with EtOAc (2×). The combined organic extracts were washed with water and brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. Silica gel chromatography (0–10% EtOAc/hexanes, linear gradient) afforded 631 mg (81%) of **11** as a colorless solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.99 (d, $^3J_{HF}$ = 11.1 Hz, 2H), 7.11 (d, $^4J_{HF}$ = 7.7 Hz, 2H), 1.64 (s, 6H), 1.03 (s, 18H), 0.27 (s, 6H), 0.26 (s, 6H); ^{19}F NMR ($CDCl_3$, 376 MHz) δ -133.34 – -133.46 (m); ^{13}C NMR ($CDCl_3$, 101 MHz) δ 180.7 (C), 153.3 (d, $^1J_{CF}$ = 246.7 Hz, C), 148.6 (d, $^2J_{CF}$ = 13.4 Hz, C), 147.8 (d, $^4J_{CF}$ = 3.2 Hz, C), 124.9 (d, $^3J_{CF}$ = 5.5 Hz, C), 120.1 (d, $^3J_{CF}$ = 1.7 Hz, CH), 114.7 (d, $^2J_{CF}$ = 19.6 Hz, CH), 37.4 (C), 33.3 (CH_3), 25.7 (CH_3), 18.6 (C), -4.4 (CH_3), -4.5 (CH_3); HRMS (ESI) calcd for $C_{28}H_{41}F_2O_3Si_2$ $[M+H]^+$ 519.2557, found 519.2566.



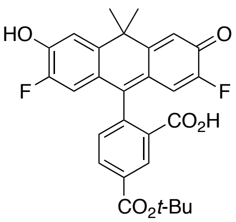
3,6-Bis((*tert*-butyldimethylsilyl)oxy)-2,7-difluoro-10,10-dimethyl-3'*H*,10*H*-spiro[anthracene-9,1'-isobenzofuran]-3'-one (14): A vial was charged with *tert*-butyl 2-bromobenzoate (**12**; 372 mg, 1.45 mmol, 1.5 eq), sealed, and flushed with nitrogen. After dissolving the bromide in THF (2.5 mL) and cooling the reaction to −15 °C, *i*-PrMgCl·LiCl (1.3 M in THF, 1.11 mL, 1.45 mmol, 1.5 eq) was added. The reaction was warmed to −5 °C and stirred for 6 h. Ketone **11** (500 mg, 0.964 mmol) in THF (2.5 mL) was then added dropwise. The reaction mixture was warmed to room temperature and stirred for 30 min. It was subsequently quenched with saturated NH_4Cl, diluted with water, and extracted with EtOAc (2×). The combined organics were washed with brine, dried over anhydrous $MgSO_4$, filtered, and evaporated. Silica gel chromatography (0–20% Et_2O/hexanes, linear gradient) provided 302 mg (50%) of **14** as a colorless foam. ^1H NMR ($CDCl_3$, 400 MHz) δ 8.05 – 8.01 (m, 1H), 7.68 – 7.58 (m, 2H), 7.11 (d, $^4J_{HF}$ = 8.3 Hz, 2H), 7.07 – 7.03 (m, 1H), 6.38 (d, $^3J_{HF}$ = 11.4 Hz, 2H), 1.76 (s, 3H), 1.67 (s, 3H), 1.00 (s, 18H), 0.20 (s, 12H); ^{19}F NMR ($CDCl_3$, 376 MHz) δ -133.46 – -133.54 (m); ^{13}C NMR ($CDCl_3$, 101 MHz) δ 170.1 (C), 154.4 (C), 152.7 (d, $^1J_{CF}$ = 245.5 Hz, C), 144.5 (d, $^2J_{CF}$ = 12.7 Hz, C), 141.7 (d, $^4J_{CF}$ = 3.4 Hz, C), 135.2 (CH), 129.8 (CH), 126.3 (C), 125.6 (CH), 125.2 (d, $^3J_{CF}$ = 5.6 Hz, C), 123.8 (CH), 120.1 (d, $^3J_{CF}$ = 1.9 Hz, CH), 115.1 (d, $^2J_{CF}$ = 20.1 Hz, CH), 85.5 (C), 37.7 (C), 35.3 (CH_3), 33.1 (CH_3), 25.7 (CH_3), 18.5 (C), -4.49 (CH_3), -4.51 (CH_3); HRMS (ESI) calcd for $C_{35}H_{45}F_2O_4Si_2$ $[M+H]^+$ 623.2819, found 623.2827.



***tert*-Butyl 3,6-bis((*tert*-butyldimethylsilyl)oxy)-2,7-difluoro-10,10-dimethyl-3'-oxo-3'*H*,10*H*-spiro[anthracene-9,1'-isobenzofuran]-5'-carboxylate (15):** A vial was charged with di-*tert*-butyl 4-bromoisophthalate[2] (**13**; 1.03 g, 2.89 mmol, 1.5 eq), sealed, and flushed with nitrogen. After dissolving the bromide in THF (5 mL) and cooling the reaction to −15 °C, *i*-PrMgCl·LiCl (1.3 M in THF, 2.22 mL, 2.89 mmol, 1.5 eq) was added. The reaction was warmed to −5 °C and stirred for 5 h. Ketone **11** (1.00 g, 1.93 mmol) in THF (4 mL) was then added dropwise. The reaction mixture was warmed to room temperature and stirred for 2 h. It was subsequently quenched with saturated NH_4Cl, diluted with water, and extracted with EtOAc (2×). The combined organics were washed with brine, dried over anhydrous $MgSO_4$, filtered, and evaporated. Silica gel chromatography (0–10% Et_2O/hexanes, linear gradient) provided 672 mg (48%) of **15** as a colorless foam. ^1H NMR ($CDCl_3$, 400 MHz) δ 8.63 (dd, J = 1.5, 0.7 Hz, 1H), 8.28 (dd, J = 8.0, 1.5 Hz, 1H), 7.13 (d, $^4J_{HF}$ = 8.3 Hz, 2H), 7.09 (dd, J = 8.0, 0.7 Hz, 1H), 6.37 (d, $^3J_{HF}$ = 11.3 Hz, 2H), 1.77 (s, 3H), 1.68 (s, 3H), 1.64 (s, 9H), 1.00 (s, 18H), 0.208 (s, 6H), 0.206 (s, 6H); ^{19}F NMR ($CDCl_3$, 376 MHz) δ -133.11 – -133.18 (m); ^{13}C NMR ($CDCl_3$, 101 MHz) δ 169.3 (C), 164.1 (C), 157.9 (C), 152.8 (d, $^1J_{CF}$ = 246.0 Hz, C), 144.7 (d, $^{2}J_{CF}$ = 12.7 Hz, C), 141.7 (d, $^{4}J_{CF}$ = 3.4 Hz, C), 136.2 (CH), 134.2 (C), 127.0 (CH), 126.5 (C), 124.4 (d, $^{3}J_{CF}$ = 5.5 Hz, C), 123.8 (CH), 120.3 (d, $^{3}J_{CF}$ = 1.8 Hz, CH), 115.0 (d, $^{2}J_{CF}$ = 20.2 Hz, CH), 85.6 (C), 82.5 (C), 37.7 (C), 35.2 (CH_3), 33.3 (CH_3), 28.3 (CH_3), 25.7 (CH_3), 18.5 (C), -4.49 (CH_3), -4.51 (CH_3); HRMS (ESI) calcd for $C_{40}H_{53}F_2O_6Si_2$ $[M+H]^+$ 723.3343, found 723.3349.



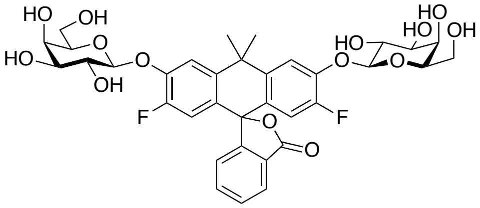
Virginia Orange (“VO,” 4): To a solution of silyl ether **14** (295 mg, 0.474 mmol) was added TBAF (1.0 M in THF, 1.89 mL, 1.89 mmol, 4 eq). The reaction was stirred at room temperature for 30 min. It was subsequently acidified with 1 N HCl, diluted with water, and extracted with EtOAc (2×). The organic extracts were dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. Flash chromatography (20–100% EtOAc/hexanes, linear gradient) yielded **4** (182 mg, 97%) as a pale yellow solid. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 10.23 (s, 2H), 8.03 – 7.97 (m, 1H), 7.75 (td, J = 7.5, 1.3 Hz, 1H), 7.69 (td, J = 7.4, 1.0 Hz, 1H), 7.27 (d, $^{4}J_{HF}$ = 8.8 Hz, 2H), 7.15 – 7.10 (m, 1H), 6.31 (d, $^{3}J_{HF}$ = 12.0 Hz, 2H), 1.72 (s, 3H), 1.62 (s, 3H); ^{19}F NMR (DMSO-d_6, 376 MHz) δ -136.98 (dd, J_{FH} = 11.9, 8.8 Hz); ^{13}C NMR (DMSO-d_6, 101 MHz) δ 169.2 (C), 154.0 (C), 149.7 (d, $^{1}J_{CF}$ = 242.4 Hz, C), 145.9 (d, $^{2}J_{CF}$ = 12.5 Hz, C), 141.6 (d, $^{4}J_{CF}$ = 3.0 Hz, C), 135.7 (CH), 129.9 (CH), 125.14 (C), 125.11 (CH), 123.4 (CH), 122.1 (d, $^{3}J_{CF}$ = 5.2 Hz, C), 115.5 (d, $^{3}J_{CF}$ = 2.8 Hz, CH), 113.7 (d, $^{2}J_{CF}$ = 18.9 Hz, CH), 84.8 (C), 37.2 (C), 34.5 (CH_3), 33.2 (CH_3); Analytical HPLC: t_R = 12.7 min, >99% purity (5 μL injection; 10–95% MeCN/H_2O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; UV detection at 254 nm); HRMS (ESI) calcd for $C_{23}H_{17}F_2O_4$ $[M+H]^+$ 395.1089, found 395.1091.



5-(*tert*-Butoxycarbonyl)-Virginia Orange (16): To a solution of silyl ether **15** (610 mg, 0.844 mmol) in THF (10 mL) was added TBAF (1.0 M in THF, 3.37 mL, 3.37 mmol, 4 eq). The reaction was stirred at room temperature for 45 min. It was subsequently acidified with 1 N HCl, diluted with water, and extracted with EtOAc (2×). The organic extracts were dried over anhydrous $MgSO_4$, filtered, and evaporated. Flash chromatography (0–40% EtOAc/toluene, linear gradient) afforded **16** as a yellow-orange solid (401 mg, 96%). ^{1}H NMR (DMSO-d_6, 400 MHz) δ 10.26 (s, 2H), 8.37 (dd, J = 1.4, 0.6 Hz, 1H), 8.21 (dd, J = 8.1, 1.5 Hz, 1H), 7.28 (d, $^{4}J_{HF}$ = 8.8 Hz, 2H), 7.24 (dd, J = 8.1, 0.5 Hz, 1H), 6.42 (d, $^{3}J_{HF}$ = 11.9 Hz, 2H), 1.73 (s, 3H), 1.62 (s, 3H), 1.59 (s, 9H); ^{19}F NMR (DMSO-d_6, 376 MHz) δ -136.86 (dd, J_{FH} = 11.8, 8.9 Hz); ^{13}C NMR (DMSO-d_6, 101 MHz) δ 168.4 (C), 163.6 (C), 157.5 (C), 149.8 (d, $^{1}J_{CF}$ = 242.5 Hz, C), 146.1 (d, $^{2}J_{CF}$ = 12.5 Hz, C), 141.6 (d, $^{4}J_{CF}$ = 3.0 Hz, C), 135.9 (CH), 133.0 (C), 125.9 (CH), 124.0

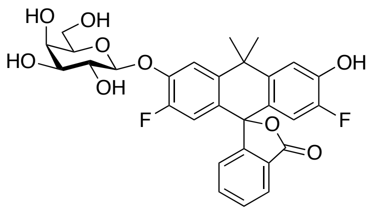
(CH), 121.3 (d, ${}^{3}J_{CF}$ = 5.3 Hz, C), 115.5 (d, ${}^{3}J_{CF}$ = 2.7 Hz, CH), 114.1 (d, ${}^{2}J_{CF}$ = 19.0 Hz, CH), 85.1 (C), 81.8 (C), 37.2 (C), 34.5 (CH_3), 33.2 (CH_3), 27.7 (CH_3); HRMS (ESI) calcd for $C_{28}H_{25}F_2O_6$ $[M+H]^+$ 495.1614, found 495.1595.

SYNTHESIS OF SINGLE-INPUT FLUOROGENIC PROBES



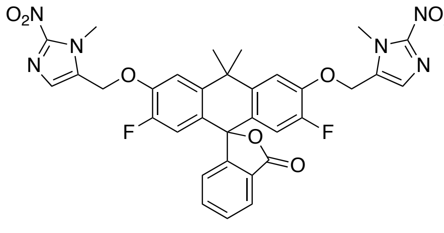
Gal$_2$–VO (20): Virginia Orange (**4**; 50 mg, 0.127 mmol) and acetobromo-α-D-galactose (**17**; 209 mg, 0.507 mmol, 4 eq) were combined in MeCN (3 mL) under nitrogen, and Ag_2O (118 mg, 0.507 mmol, 4 eq) was added. The resulting mixture was stirred at 50 °C for 3 h. The reaction was then cooled to room temperature, filtered through Celite with MeCN, and evaporated. The crude residue was purified by reverse phase HPLC (40–95% MeCN/H_2O, linear gradient, with constant 0.1% v/v TFA additive) to provide 75 mg (56%) of the octaacetate as a white solid.

The octaacetate (75 mg, 71.1 μmol) was subsequently dissolved in 1:1 MeOH/MeCN (5.7 mL), and 1 M Na_2CO_3 (aq, 1.42 mL, 1.42 mmol, 20 eq) was added. The reaction was stirred at room temperature for 2 h. It was then neutralized with 1 N HCl (2.85 mL), and the resulting solution was directly purified by reverse phase HPLC (10–95% MeCN/H_2O, linear gradient, with constant 0.1% v/v TFA additive) to afford **20** (49 mg, 96%) as a white solid. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.04 (ddd, J = 7.3, 1.3, 0.8 Hz, 1H), 7.74 (td, J = 7.4, 1.4 Hz, 1H), 7.69 (td, J = 7.4, 1.1 Hz, 1H), 7.65 (d, ${}^{4}J_{HF}$ = 8.2 Hz, 1H), 7.64 (d, ${}^{4}J_{HF}$ = 8.2 Hz, 1H), 7.05 (dt, J = 7.7, 1.0 Hz, 1H), 6.40 (d, ${}^{3}J_{HF}$ = 11.7 Hz, 1H), 6.39 (d, ${}^{3}J_{HF}$ = 11.8 Hz, 1H), 5.03 (d, J = 7.8 Hz, 1H), 4.99 (d, J = 7.8 Hz, 1H), 3.95 – 3.90 (m, 2H), 3.90 – 3.74 (m, 8H), 3.65 – 3.59 (m, 2H), 1.87 (s, 3H), 1.77 (s, 3H); ^{19}F NMR (CD_3OD, 376 MHz) δ -134.52 (dd, J_{FH} = 11.3, 8.2 Hz, 1F), -134.55 (dd, J_{FH} = 11.5, 8.2 Hz, 1F); ^{13}C NMR (CD_3OD, 101 MHz) δ 171.9 (C), 156.1 (C), 152.62 (d, ${}^{1}J_{CF}$ = 247.4 Hz, C), 152.59 (d, ${}^{1}J_{CF}$ = 247.3 Hz, C), 147.5 (d, ${}^{2}J_{CF}$ = 11.0 Hz, C), 143.1 (d, ${}^{4}J_{CF}$ = 3.5 Hz, C), 143.0 (d, ${}^{4}J_{CF}$ = 3.5 Hz, C), 136.8 (CH), 131.2 (CH), 126.65 (C), 126.61 (d, ${}^{3}J_{CF}$ = 5.7 Hz, C), 126.57 (d, ${}^{3}J_{CF}$ = 5.4 Hz, C), 126.4 (CH), 124.6 (CH), 117.6 (CH), 117.5 (CH), 115.1 (d, ${}^{2}J_{CF}$ = 19.1 Hz, CH), 103.2 (CH), 103.0 (CH), 86.4 (C), 77.5 (CH), 77.4 (CH), 74.9 (CH), 72.1 (CH), 72.0 (CH), 70.3 (CH), 62.5 (CH_2), 39.3 (C), 35.0 (CH_3), 34.0 (CH_3); Analytical HPLC: t_R = 8.0 min, >99% purity (5 μL injection; 10–95% MeCN/H_2O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; UV detection at 254 nm); HRMS (ESI) calcd for $C_{35}H_{36}F_2O_{14}Na$ $[M+Na]^+$ 741.1965, found 741.1928.



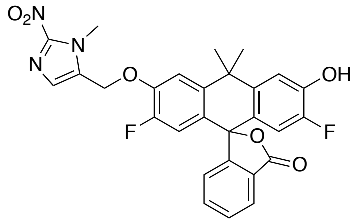
Gal–VO (21): Virginia Orange (**4**; 14 mg, 0.0355 mmol) and acetobromo-α-D-galactose (**17**; 146 mg, 0.355 mmol, 10 eq) were combined in MeCN (4 mL) under nitrogen, and Et_3N (495 µL, 3.55 mmol, 100 eq) was added. The resulting solution was stirred at 50 °C for 24 h. After cooling the reaction to room temperature, it was concentrated *in vacuo* and directly purified by reverse phase HPLC (30–95% $MeCN/H_2O$, linear gradient, with constant 0.1% v/v TFA additive) to give 9.5 mg (37%) of the galactoside tetraacetate as a white solid.

The tetraacetate (9.5 mg, 13.1 µmol) was subsequently dissolved in 1:1 MeOH/MeCN (524 µL), and 1 M Na_2CO_3 (aq, 131 µL, 131 µmol, 10 eq) was added. The reaction was stirred at room temperature for 2 h. It was then diluted with MeOH (1 mL) and neutralized through the addition of 1 N HCl (262 µL). Reverse phase HPLC of the crude reaction (10–95% $MeCN/H_2O$, linear gradient, with constant 0.1% v/v TFA additive) afforded **21** (6.8 mg, 93%, diastereomeric mixture) as a white solid. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.05 – 8.01 (m, 1H), 7.77 – 7.72 (m, 1H), 7.72 – 7.66 (m, 1H), 7.64 (d, J = 8.2 Hz, 0.45H), 7.63 (d, J = 8.2 Hz, 0.55H), 7.24 (d, $^4J_{HF}$ = 8.7 Hz, 1H), 7.10 – 7.05 (m, 1H), 6.373 (d, $^3J_{HF}$ = 11.8 Hz, 0.55H), 6.368 (d, $^3J_{HF}$ = 11.8 Hz, 0.45H), 6.313 (d, $^3J_{HF}$ = 11.9 Hz, 0.55H), 6.306 (d, $^3J_{HF}$ = 11.9 Hz, 0.45H), 5.02 (d, J = 7.8 Hz, 0.55H), 4.98 (d, J = 7.8 Hz, 0.45H), 3.94 – 3.74 (m, 5H), 3.65 – 3.59 (m, 1H), 1.83 (s, 3H), 1.73 (s, 3H); ^{19}F NMR (CD_3OD, 376 MHz) δ δ -134.66 – -134.75 (m, 1F), -138.47 – -138.59 (m, 1F); Analytical HPLC: t_R = 9.8 min, >99% purity (diastereomeric mixture; 5 µL injection; 10–95% $MeCN/H_2O$, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; UV detection at 254 nm); HRMS (ESI) calcd for $C_{29}H_{26}F_2O_9Na$ $[M+Na]^+$ 579.1437, found 579.1433.

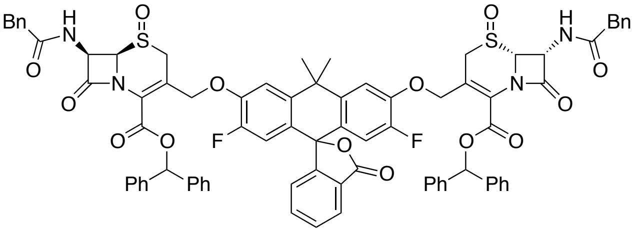


NM_2–VO (22): To a solution of Virginia Orange (**4**; 40 mg, 0.101 mmol) and 5-(bromomethyl)-1-methyl-2-nitro-1*H*-imidazole (**18**; 89 mg, 0.406 mmol, 4 eq) in MeCN (3.5 mL) was added Ag_2O (94 mg, 0.406 mmol, 4 eq), and the resulting mixture was stirred at 60 °C for 90 min. The reaction was then cooled to room temperature, filtered through Celite with MeCN, and evaporated. The crude residue was purified by silica gel chromatography (0–50% $MeCN/CH_2Cl_2$, linear gradient) to afford **22** (64 mg, 94%) as an off-white solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.08 – 8.04 (m, 1H), 7.70 – 7.63 (m, 2H), 7.26 (d, $^4J_{HF}$ = 8.0 Hz, 2H), 7.23 (s, 2H), 7.04 – 7.01 (m, 1H), 6.47 (d, $^3J_{HF}$ = 11.8 Hz, 2H), 5.21 (s, 4H), 4.11 (s, 6H), 1.82 (s, 3H), 1.73 (s, 3H); ^{19}F NMR ($CDCl_3$, 376 MHz) δ -133.81 (dd, J_{FH} = 11.8, 8.0 Hz); ^{13}C NMR ($CDCl_3$, 101 MHz) δ 169.7 (C), 153.8 (C), 151.9 (d, $^1J_{CF}$ = 248.2 Hz, C), 146.7 (C), 146.2 (d, $^2J_{CF}$ = 11.3 Hz, C), 141.7 (d, $^4J_{CF}$ = 3.5 Hz, C), 135.5 (CH), 132.0 (C), 130.2 (CH), 129.5 (CH), 126.5 (d, $^3J_{CF}$ =

5.7 Hz, C), 126.0 (C), 125.9 (CH), 123.6 (CH), 115.6 (d, ${}^{2}J_{CF}$ = 19.5 Hz, CH), 115.0 (d, ${}^{3}J_{CF}$ = 1.1 Hz, CH), 84.6 (C), 61.8 (CH_2), 38.3 (C), 35.3 (CH_3), 34.7 (CH_3), 33.4 (CH_3); HRMS (ESI) calcd for $C_{33}H_{26}F_2N_6O_8Na$ $[M+Na]^+$ 695.1672, found 695.1659.

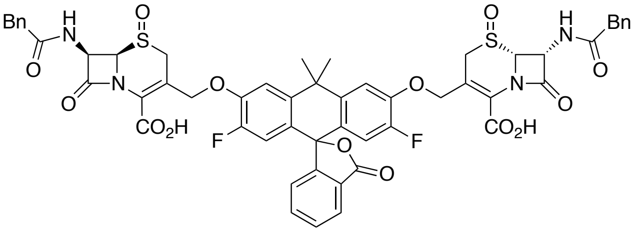


NM–VO (23): To a solution of Virginia Orange (**4**; 140 mg, 0.355 mmol) and 5-(bromomethyl)-1-methyl-2-nitro-1*H*-imidazole (**18**; 78 mg, 0.355 mmol, 1 eq) in MeCN (8 mL) was added Ag_2O (82 mg, 0.355 mmol, 1 eq), and the resulting mixture was stirred at 60 °C for 90 min. After cooling the reaction to room temperature, 1 N HCl (20 mL) and EtOAc (20 mL) were added. The gray suspension was filtered through Celite; the filtrate was diluted with water and extracted with EtOAc (2×). The combined organic extracts were washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. Purification by flash chromatography on silica gel (0–50% EtOAc/CH_2Cl_2, linear gradient) yielded 82 mg (43%) of **23** as a pale yellow solid. ${}^{1}H$ NMR ($CDCl_3$, 400 MHz) δ 8.05 – 8.01 (m, 1H), 7.69 – 7.61 (m, 2H), 7.29 – 7.21 (m, 3H), 7.06 – 7.02 (m, 1H), 6.65 (s, 1H), 6.44 (d, ${}^{3}J_{HF}$ = 11.9 Hz, 1H), 6.38 (d, ${}^{3}J_{HF}$ = 11.4 Hz, 1H), 5.22 (AB quartet, ν_A = 2089.8 Hz, ν_B = 2084.8 Hz, J_{AB} = 13.1 Hz, 2H), 4.10 (s, 3H), 1.77 (s, 3H), 1.64 (s, 3H); ${}^{19}F$ NMR ($CDCl_3$, 376 MHz) δ -134.35 (dd, J_{FH} = 11.8, 8.1 Hz, 1F), -141.70 (dd, J_{FH} = 11.0, 8.9 Hz, 1F); ${}^{13}C$ NMR ($CDCl_3$, 101 MHz) δ 170.3 (C), 154.1 (C), 151.7 (d, ${}^{1}J_{CF}$ = 247.7 Hz, C), 150.0 (d, ${}^{1}J_{CF}$ = 240.3 Hz, C), 146.5 (C), 146.1 (d, ${}^{2}J_{CF}$ = 11.3 Hz, C), 145.2 (d, ${}^{2}J_{CF}$ = 14.2 Hz, C), 142.2 (d, ${}^{4}J_{CF}$ = 3.5 Hz, C), 141.9 (d, ${}^{4}J_{CF}$ = 3.2 Hz, C), 135.5 (CH), 132.1 (C), 130.1 (CH), 129.3 (CH), 126.6 (d, ${}^{3}J_{CF}$ = 5.6 Hz, C), 126.0 (C), 125.7 (CH), 123.7 (CH), 123.4 (d, ${}^{3}J_{CF}$ = 5.3 Hz, C), 115.473 (d, ${}^{3}J_{CF}$ = 2.0 Hz, CH), 115.472 (d, ${}^{2}J_{CF}$ = 19.4 Hz, CH), 114.9 (CH), 114.4 (d, ${}^{2}J_{CF}$ = 19.2 Hz, CH), 85.4 (C), 61.7 (CH_2), 38.1 (C), 35.2 (CH_3), 34.7 (CH_3), 33.2 (CH_3); HRMS (ESI) calcd for $C_{28}H_{21}F_2N_3O_6Na$ $[M+Na]^+$ 556.1291, found 556.1266.



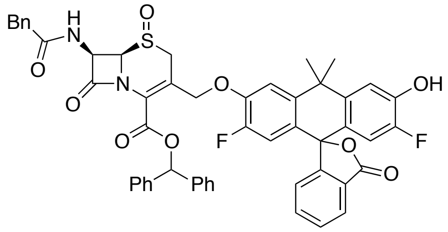
[Ceph(Bzh)]$_2$–VO (S1): Virginia Orange (**4**; 100 mg, 0.254 mmol), (6*R*,7*R*)-benzhydryl 3-(chloromethyl)-8-oxo-7-(2-phenylacetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (**19**; 541 mg, 1.01 mmol, 4 eq), K_2CO_3 (140 mg, 1.01 mmol, 4 eq), and NaI (76 mg, 0.507 mmol, 2 eq) were combined in MeCN (10 mL) and stirred at room temperature for 7 h. The reaction was subsequently diluted with water and extracted with EtOAc (2×). The combined organics were washed with brine, dried over anhydrous $MgSO_4$, filtered, and evaporated. Silica gel chromatography (0–50% EtOAc/toluene, linear gradient) provided the bis-alkylated product as a mixture of isomers (274 mg).

The isomeric mixture (274 mg) was dissolved in CH_2Cl_2 (5 mL) and cooled to 0 °C. *m*-Chloroperoxybenzoic acid (77%, 106 mg, 0.474 mmol, 2.4 eq) was added, and the reaction was stirred at 0 °C for 30 min. It was then diluted with saturated $NaHCO_3$ and extracted with CH_2Cl_2 (2×). The organics were washed with brine, dried over anhydrous $MgSO_4$, filtered, and evaporated. Flash chromatography (0–50% EtOAc/CH_2Cl_2, linear gradient) afforded 125 mg (35%, 2 steps) of **S1** as a pale yellow solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.06 – 7.99 (m, 1H), 7.66 – 7.58 (m, 2H), 7.54 – 7.46 (m, 4H), 7.41 – 7.26 (m, 26H), 7.06 (d, $^4J_{HF}$ = 8.1 Hz, 2H), 6.97 – 6.93 (m, 1H), 6.93 (s, 1H), 6.91 (s, 1H), 6.77 – 6.70 (m, 2H), 6.391 (d, $^3J_{HF}$ = 11.7 Hz, 1H), 6.390 (d, $^3J_{HF}$ = 11.7 Hz, 1H), 6.16 – 6.10 (m, 2H), 5.39 (d, J = 14.0 Hz, 1H), 5.38 (d, J = 13.8 Hz, 1H), 4.91 (d, J = 14.0 Hz, 1H), 4.88 (d, J = 13.8 Hz, 1H), 4.50 – 4.47 (m, 2H), 4.02 (d, J = 19.1 Hz, 2H), 3.63 (AB quartet, ν_A = 1459.1 Hz, ν_B = 1446.0 Hz, J_{AB} = 15.7 Hz, 4H), 3.36 (d, J = 19.1 Hz, 1H), 3.34 (d, J = 19.2 Hz, 1H), 1.62 (s, 3H), 1.53 (s, 3H); ^{19}F NMR ($CDCl_3$, 376 MHz) δ -135.57 (dd, J_{FH} = 11.7, 8.2 Hz, 1F), -135.77 (dd, J_{FH} = 11.6, 8.2 Hz, 1F); ^{13}C NMR ($CDCl_3$, 101 MHz) δ 171.5 (C), 171.4 (C), 169.9 (C), 164.5 (C), 160.13 (C), 160.11 (C), 154.3 (C), 151.32 (d, $^1J_{CF}$ = 247.6, C), 151.26 (d, $^1J_{CF}$ = 247.5 Hz, C), 146.73 (d, $^2J_{CF}$ = 11.3 Hz, C), 146.71 (d, $^2J_{CF}$ = 11.1 Hz, C), 141.7 (d, $^4J_{CF}$ = 3.2 Hz, C), 139.13 (C), 139.07 (C), 139.06 (C), 139.03 (C), 135.4 (CH), 133.8 (C), 130.0 (CH), 129.50 (CH), 129.49 (CH), 129.2 (CH), 128.9 (CH), 128.8 (CH), 128.7 (CH), 128.5 (CH), 128.4 (CH), 127.7 (CH), 127.4 (CH), 127.19 (CH), 127.17 (CH), 125.8 (C), 125.7 (CH), 125.2 (d, $^3J_{CF}$ = 5.4 Hz, C), 125.1 (d, $^3J_{CF}$ = 5.8 Hz, C), 124.5 (C), 124.4 (C), 123.8 (C), 123.5 (CH), 115.0 (d, $^2J_{CF}$ = 19.1 Hz, CH), 113.6 (CH), 113.3 (CH), 84.8 (C), 80.9 (CH), 80.8 (CH), 68.7 (CH_2), 68.5 (CH_2), 67.0 (CH), 66.9 (CH), 59.1 (CH), 45.44 (CH_2), 45.36 (CH_2), 43.49 (CH_2), 43.48 (CH_2), 38.2 (C), 34.8 (CH_3), 33.6 (CH_3); Analytical HPLC: t_R = 11.3 min, 97.1% purity (5 μL injection; 65–95% MeCN/H_2O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; UV detection at 254 nm); HRMS (ESI) calcd for $C_{81}H_{64}F_2N_4O_{14}S_2Na$ $[M+Na]^+$ 1441.3721, found 1441.3735.



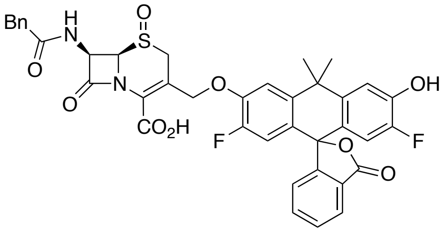
Ceph$_2$–VO (24): Diester **S1** (45 mg, 31.7 μmol) was taken up in CH_2Cl_2 (4 mL) and cooled to 0 °C. Anisole (200 μL) and TFA (400 μL) were added, and the reaction was stirred at 0 °C for 2 h. Toluene (4 mL) was added, and the reaction mixture was concentrated to dryness. Reverse phase HPLC (10–95% MeCN/H_2O, linear gradient, with constant 0.1% v/v TFA additive) afforded **24** (27 mg, 79%) as a white solid. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 13.99 (s, 2H), 8.42 (d, J = 8.7 Hz, 1H), 8.39 (d, J = 8.7 Hz, 1H), 8.05 – 7.97 (m, 1H), 7.73 (td, J = 7.5, 1.5 Hz, 1H), 7.69 (td, J = 7.5, 1.3 Hz, 1H), 7.54 (d, $^4J_{HF}$ = 8.6 Hz, 1H), 7.52 (d, $^4J_{HF}$ = 8.6 Hz, 1H), 7.34 – 7.18 (m, 10H), 7.09 – 7.04 (m, 1H), 6.43 (d, $^3J_{HF}$ = 11.9 Hz, 2H), 5.87 – 5.77 (m, 2H), 5.22 (d, J = 12.1 Hz, 1H), 5.19 (d, J = 11.9 Hz, 1H), 5.06 (d, J = 11.8 Hz, 1H), 5.03 (d, J = 11.8 Hz, 1H), 4.94 – 4.88 (m, 2H), 3.98 (d, J = 18.5 Hz, 2H), 3.70 (d, J = 14.1

Hz, 2H), 3.67 – 3.61 (m, 2H), 3.55 (d, J = 14.0 Hz, 1H), 3.54 (d, J = 14.1 H, 1H), 1.81 (s, 3H), 1.71 (s, 3H); ^{19}F NMR (DMSO-d_6, 376 MHz) δ -135.00 – -135.11 (m); ^{13}C NMR (DMSO-d_6, 101 MHz) δ 171.06 (C), 171.05 (C), 169.2 (C), 164.30 (C), 164.29 (C), 162.3 (C), 154.1 (C), 150.5 (d, $^1J_{CF}$ = 245.7 Hz, C), 146.7 (d, $^2J_{CF}$ = 11.0 Hz, C), 141.6 (C), 135.82 (CH), 135.81 (CH), 130.1 (CH), 129.1 (CH), 128.3 (CH), 126.6 (CH), 126.2 (C), 126.1 (C), 125.4 (CH), 124.8 (C), 123.74 (d, $^3J_{CF}$ = 5.4 Hz, C), 123.72 (d, $^3J_{CF}$ = 5.7 Hz, C), 123.3 (CH), 118.8 (C), 113.9 (CH), 113.8 (CH), 113.6 (d, $^2J_{CF}$ = 20.0 Hz, CH), 84.1 (C), 68.2 (CH_2), 66.3 (CH), 58.3 (CH), 45.2 (CH_2), 41.5 (CH_2), 38.0 (C), 33.8 (CH_3), 33.6 (CH_3); Analytical HPLC: t_R = 14.0 min, >99% purity (5 µL injection; 10–95% MeCN/H_2O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; UV detection at 254 nm); HRMS (ESI) calcd for $C_{55}H_{44}F_2N_4O_{14}S_2Na$ $[M+Na]^+$ 1109.2156, found 1109.2164.



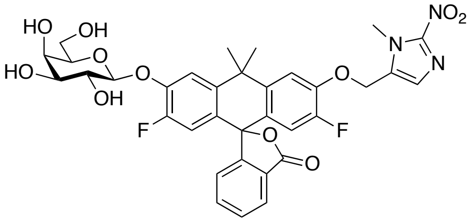
Ceph(Bzh)–VO (S2): Virginia Orange (**4**; 200 mg, 0.507 mmol), (6*R*,7*R*)-benzhydryl 3-(chloromethyl)-8-oxo-7-(2-phenylacetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (**19**; 270 mg, 0.507 mmol, 1 eq), K_2CO_3 (105 mg, 0.761 mmol, 1.5 eq), and NaI (76 mg, 0.507 mmol, 1 eq) were combined in MeCN (7 mL) and stirred at room temperature for 7 h. The reaction was subsequently diluted with water and extracted with EtOAc (2×). The combined organics were washed with brine, dried over anhydrous $MgSO_4$, filtered, and evaporated. Silica gel chromatography (0–50% EtOAc/toluene, linear gradient) provided the mono-alkylated product as a mixture of isomers (323 mg).

The isomeric mixture (323 mg) was dissolved in CH_2Cl_2 (10 mL) and cooled to 0 °C. *m*-Chloroperoxybenzoic acid (77%, 98 mg, 0.363 mmol, 1.2 eq) was added, and the reaction was stirred at 0 °C for 30 min. It was then diluted with saturated $NaHCO_3$ and extracted with CH_2Cl_2 (2×). The organics were washed with brine, dried over anhydrous $MgSO_4$, filtered, and evaporated. Flash chromatography (0–40% EtOAc/CH_2Cl_2, linear gradient) afforded 128 mg (28%, 2 steps, diastereomeric mixture) of **S2** as an off-white solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.06 – 8.01 (m, 1H), 7.68 – 7.59 (m, 2H), 7.53 – 7.46 (m, 2H), 7.40 – 7.27 (m, 13H), 7.21 (d, $^4J_{HF}$ = 8.6 Hz, 0.4H), 7.20 (d, $^4J_{HF}$ = 8.6 Hz, 0.6H), 7.11 – 7.05 (m, 1H), 7.03 – 6.97 (m, 1H), 6.92 (s, 0.6F), 6.90 (s, 0.4F), 6.77 – 6.69 (m, 1H), 6.41 (d, $^3J_{HF}$ = 11.2 Hz, 1.2H), 6.39 (d, $^3J_{HF}$ = 11.7 Hz, 0.8H), 6.17 – 6.09 (m, 1H), 5.57 – 5.52 (m, 1H), 5.40 (d, J = 14.0 Hz, 0.6H), 5.39 (d, J = 13.8 Hz, 0.4H), 4.88 (d, J = 13.9 Hz, 0.6H), 4.85 (d, J = 13.7 Hz, 0.4H), 4.50 – 4.45 (m, 1H), 4.024 (d, J = 19.3 Hz, 0.6H), 4.017 (d, J = 19.2 Hz, 0.4H), 3.70 – 3.58 (m, 2H), 3.36 (d, J = 18.8 Hz, 0.4H), 3.34 (d, J = 19.1 Hz, 0.6H), 1.70 (s, 1.2F), 1.69 (s, 1.8F), 1.62 (s, 1.2F), 1.59 (s, 1.8F); ^{19}F NMR ($CDCl_3$, 376 MHz) δ 135.69 (dd, J_{FH} = 11.9, 8.2 Hz, 0.4F), -135.90 (dd, J_{FH} = 11.7, 8.2 Hz, 0.6F), -141.79 – -141.93 (m, 1F); Analytical HPLC: t_R = 10.7 min, 96.2% purity (diastereomeric mixture; 5 µL injection; 50–95% MeCN/H_2O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; UV detection at 254 nm); HRMS (ESI) calcd for $C_{52}H_{40}F_2N_2O_9SNa$ $[M+Na]^+$ 929.2315, found 929.2323.



Ceph–VO (25): Ester **S2** (29 mg, 32.0 μmol) was taken up in CH_2Cl_2 (3 mL) and cooled to 0 °C. Anisole (150 μL) and TFA (300 μL) were added, and the reaction was stirred at 0 °C for 2 h. Toluene (4 mL) was added, and the reaction mixture was concentrated to dryness. Reverse phase HPLC (10–95% $MeCN/H_2O$, linear gradient, with constant 0.1% v/v TFA additive) afforded **25** (17 mg, 71%, diastereomeric mixture) as a white solid. ^1H NMR (DMSO-d_6, 400 MHz) δ 10.25 (s, 1H), 8.41 (d, J = 8.5 Hz, 0.4H), 8.39 (d, J = 8.4 Hz, 0.6H), 8.02 – 7.98 (m, 1H), 7.77 – 7.72 (m, 1H), 7.72 – 7.66 (m, 1H), 7.51 (d, $^4J_{HF}$ = 8.5 Hz, 0.4H), 7.48 (d, $^4J_{HF}$ = 8.6 Hz, 0.6H), 7.33 – 7.19 (m, 6H), 7.12 – 7.07 (m, 1H), 6.41 (d, $^3J_{HF}$ = 12.0 Hz, 1H), 6.33 (d, $^3J_{HF}$ = 11.9 Hz, 1H), 5.85 – 5.79 (m, 1H), 5.27 – 5.18 (m, 1H), 5.07 – 4.98 (m, 1H), 4.93 – 4.89 (m, 1H), 3.98 (d, J = 18.5 Hz, 1H), 3.69 (d, J = 14.1 Hz, 1H), 3.67 – 3.60 (m, 1H), 3.55 (d, J = 14.1 Hz, 1H), 1.76 (s, 3H), 1.66 (s, 3H); ^{19}F NMR (DMSO-d_6, 376 MHz) δ -135.06 – -135.19 (m, 1F), -136.75 – -136.89 (m, 1F); Analytical HPLC: t_R = 13.4 min, 13.5 min, >99% purity (diastereomeric mixture; 5 μL injection; 10–95% $MeCN/H_2O$, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; UV detection at 254 nm); HRMS (ESI) calcd for $C_{39}H_{31}F_2N_2O_9S$ $[M+H]^+$ 741.1713, found 741.1727.

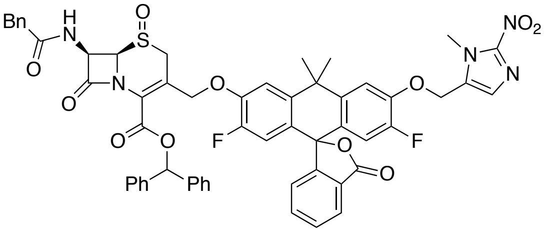
SYNTHESIS OF DUAL-INPUT FLUOROGENIC PROBES



Gal–VO–NM (26): Imidazole **23** (60 mg, 0.112 mmol) and acetobromo-α-D-galactose (**17**; 185 mg, 0.450 mmol, 4 eq) were combined in MeCN (4 mL) under nitrogen, and Ag_2O (104 mg, 0.450 mmol, 4 eq) was added. The resulting mixture was stirred at 50 °C for 2 h. The reaction was then cooled to room temperature, filtered through Celite with MeCN, and concentrated *in vacuo*. The crude residue was purified by flash chromatography on silica gel (0–40% $MeCN/CH_2Cl_2$, linear gradient) to provide 82 mg (84%) of the galactoside tetraacetate as a white solid.

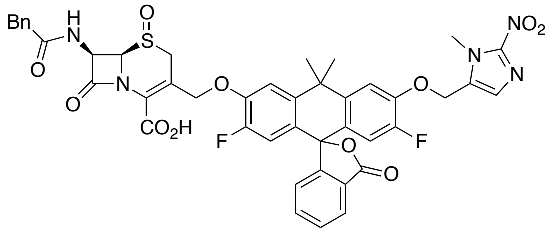
The tetraacetate (82 mg, 0.0949 mmol) was subsequently dissolved in 1:1 MeOH/MeCN (4 mL), and 1 M Na_2CO_3 (aq, 949 μL, 0.949 mmol, 10 eq) was added. The reaction was stirred at room temperature for 2 h. It was then neutralized with 1 N HCl (2 mL), and the resulting solution was directly purified by reverse phase HPLC (10–95% $MeCN/H_2O$, linear gradient, with constant 0.1% v/v TFA additive) to yield 57 mg (86%, diastereomeric mixture) of **26** as a white solid. ^1H NMR (CD_3OD, 400 MHz) δ 8.05 – 8.02 (m, 1H), 7.76 – 7.71 (m, 1H), 7.71 –

7.66 (m, 1H), 7.66 (d, $^4J_{HF}$ = 8.2 Hz, 0.5H), 7.65 (d, $^4J_{HF}$ = 8.2 Hz, 0.5H), 7.57 (d, $^4J_{HF}$ = 8.2 Hz, 1H), 7.30 (s, 1H), 7.06 – 7.02 (m, 1H), 6.42 (d, $^3J_{HF}$ = 11.9 Hz, 0.5H), 6.42 (d, $^3J_{HF}$ = 11.8 Hz, 0.5H), 6.39 (d, $^3J_{HF}$ = 11.8 Hz, 0.5H), 6.39 (d, $^3J_{HF}$ = 11.7 Hz, 0.5H), 5.40 (s, 2H), 5.05 – 4.97 (m, 1H), 4.08 (s, 3H), 3.93 – 3.75 (m, 5H), 3.65 – 3.59 (m, 1H), 1.89 (s, 3H), 1.79 (s, 3H); ^{19}F NMR (CD_3OD, 376 MHz) δ -134.41 (dd, J_{FH} = 11.7, 7.7 Hz, 0.5F), -134.43 (dd, J_{FH} = 11.8, 7.8 Hz, 0.5F), -135.31 (dd, J_{FH} = 11.8, 8.4 Hz, 0.5F), -135.32 (dd, J_{FH} = 11.9, 8.1 Hz, 0.5F); Analytical HPLC: t_R = 11.4 min, >99% purity (diastereomeric mixture; 5 μL injection; 10–95% MeCN/H_2O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; UV detection at 254 nm); HRMS (ESI) calcd for $C_{34}H_{31}F_2N_3O_{11}Na$ $[M+Na]^+$ 718.1819, found 718.1789.

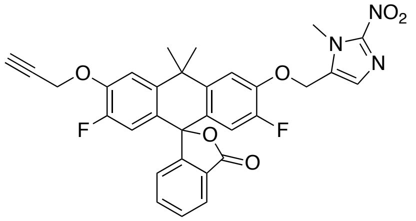


Ceph(Bzh)–VO–NM (S3): Imidazole **23** (161 mg, 0.302 mmol), (6*R*,7*R*)-benzhydryl 3-(chloromethyl)-8-oxo-7-(2-phenylacetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (**19**; 483 mg, 0.905 mmol, 3 eq), K_2CO_3 (125 mg, 0.905 mmol, 3 eq), and NaI (68 mg, 0.453 mmol, 1.5 eq) were combined in MeCN (12 mL) and stirred at room temperature for 7 h. The reaction was subsequently diluted with water and extracted with EtOAc (2×). The combined organics were washed with brine, dried over anhydrous $MgSO_4$, filtered, and evaporated. Silica gel chromatography (0–40% EtOAc/CH_2Cl_2, linear gradient) provided the alkylated product as a mixture of isomers (260 mg).

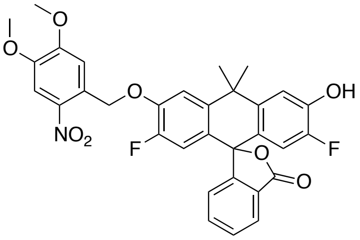
The isomeric mixture (260 mg) was dissolved in CH_2Cl_2 (10 mL) and cooled to 0 °C. *m*-Chloroperoxybenzoic acid (77%, 68 mg, 0.303 mmol, 1.2 eq) was added, and the reaction was stirred at 0 °C for 30 min. It was then diluted with saturated $NaHCO_3$ and extracted with CH_2Cl_2 (2×). The organics were washed with brine, dried over anhydrous $MgSO_4$, filtered, and evaporated. Flash chromatography (0–50% EtOAc/CH_2Cl_2, linear gradient) afforded 181 mg (57%, 2 steps, diastereomeric mixture) of **S3** as a pale yellow solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.07 – 8.02 (m, 1H), 7.69 – 7.60 (m, 2H), 7.52 – 7.46 (m, 2H), 7.40 – 7.27 (m, 13H), 7.233 (d, $^4J_{HF}$ = 8.0 Hz, 0.5H), 7.230 (s, 1H), 7.224 (d, $^4J_{HF}$ = 7.8 Hz, 0.5H), 7.11 (d, $^4J_{HF}$ = 8.1 Hz, 0.5H), 7.10 (d, $^4J_{HF}$ = 8.1 Hz, 0.5H), 7.02 – 6.96 (m, 1H), 6.92 (s, 0.5H), 6.91 (s, 0.5H), 6.75 – 6.67 (m, 1H), 6.46 (d, $^3J_{HF}$ = 11.8 Hz, 1H), 6.41 (d, $^3J_{HF}$ = 11.7 Hz, 0.5H), 6.40 (d, $^3J_{HF}$ = 11.7 Hz, 0.5H), 6.17 – 6.10 (m, 1H), 5.380 (d, J = 14.0 Hz, 0.5H), 5.376 (d, J = 13.7 Hz, 0.5H), 5.19 (s, 2H), 4.90 (d, J = 13.6 Hz, 0.5H), 4.88 (d, J = 13.5 Hz, 0.5H), 4.52 – 4.47 (m, 1H), 4.11 (s, 3H), 4.02 (d, J = 19.1 Hz, 1H), 3.70 – 3.57 (m, 2H), 3.37 (d, J = 19.2 Hz, 0.5H), 3.36 (d, J = 19.3 Hz, 0.5H), 1.74 (s, 1.5H), 1.73 (s, 1.5H), 1.66 (s, 1.5H), 1.63 (s, 1.5H); ^{19}F NMR ($CDCl_3$, 376 MHz) δ -134.09 (dd, J_{FH} = 11.8, 8.0 Hz, 0.5F), -134.10 (dd, J_{FH} = 11.8, 7.9 Hz, 0.5F), -135.26 (dd, J_{FH} = 11.7, 8.1 Hz, 0.5F), -135.47 (dd, J_{FH} = 11.6, 8.1 Hz, 0.5F); Analytical HPLC: t_R = 12.3 min, >99% purity (diastereomeric mixture; 5 μL injection; 50–95% MeCN/H_2O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; UV detection at 254 nm); HRMS (ESI) calcd for $C_{57}H_{45}F_2N_5O_{11}SNa$ $[M+Na]^+$ 1068.2697, found 1068.2709.



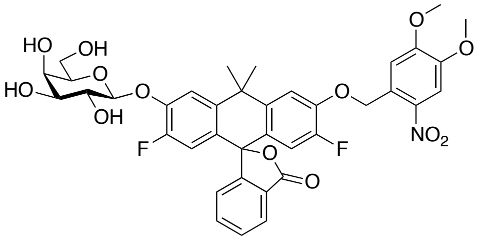
Ceph–VO–NM (27): Ester **S3** (60 mg, 57.4 µmol) was taken up in CH_2Cl_2 (4 mL) and cooled to 0 °C. Anisole (200 µL) and TFA (400 µL) were added, and the reaction was stirred at 0 °C for 3 h. Toluene (5 mL) was added, and the reaction mixture was concentrated to dryness. Reverse phase HPLC (10–95% MeCN/H_2O, linear gradient, with constant 0.1% v/v TFA additive) afforded **27** as a white solid (TFA salt, 38 mg, 67%, diastereomeric mixture). ^1H NMR (DMSO-d_6, 400 MHz) δ 14.01 (s, 1H), 8.44 – 8.37 (m, 1H), 8.03 – 7.98 (m, 1H), 7.77 – 7.65 (m, 3H), 7.56 (d, $^4J_{HF}$ = 8.5 Hz, 0.5H), 7.53 (d, $^4J_{HF}$ = 8.4 Hz, 0.5H), 7.39 (s, 1H), 7.34 – 7.27 (m, 4H), 7.27 – 7.20 (m, 1H), 7.09 – 7.03 (m, 1H), 6.45 (d, $^3J_{HF}$ = 11.9 Hz, 1H), 6.44 (d, $^3J_{HF}$ = 11.9 Hz, 1H), 5.86 – 5.80 (m, 1H), 5.50 (s, 2H), 5.27 – 5.17 (m, 1H), 5.10 – 5.00 (m, 1H), 4.94 – 4.90 (m, 1H), 3.98 (d, J = 18.2 Hz, 1H), 3.97 (s, 3H), 3.70 (d, J = 14.1 Hz, 1H), 3.69 – 3.61 (m, 1H), 3.551 (d, J = 14.0 Hz, 0.5H), 3.548 (d, J = 14.1 Hz, 0.5H), 1.862 (s, 1.5H), 1.857 (s, 1.5H), 1.77 (s, 3H); ^{19}F NMR (DMSO-d_6, 376 MHz) δ -134.95 – -135.07 (m, 1F), -135.24 – -135.34 (m, 1F); Analytical HPLC: t_R = 12.1 min, 12.2 min, >99% purity (diastereomeric mixture; 5 µL injection; 30–95% MeCN/H_2O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; UV detection at 254 nm); HRMS (ESI) calcd for $C_{44}H_{35}F_2N_5O_{11}SNa$ $[M+Na]^+$ 902.1914, found 902.1906.



NM–VO–Prg (28): To a mixture of imidazole **23** (40 mg, 75.0 µmol) and K_2CO_3 (41 mg, 0.300 mmol, 4 eq) in DMF (2 mL) was added propargyl bromide (80% w/w in toluene, 33 µL, 0.300 mmol, 4 eq), and the reaction was stirred at room temperature for 18 h. It was subsequently diluted with water and extracted with EtOAc (2×). The combined organics were washed with brine, dried over anhydrous $MgSO_4$, filtered, and evaporated. Silica gel chromatography (10–100% EtOAc/hexanes, linear gradient, with constant 40% v/v CH_2Cl_2 additive) provided **28** as a yellow solid (39 mg, 91%). ^1H NMR ($CDCl_3$, 400 MHz) δ 8.07 – 8.03 (m, 1H), 7.66 (td, J = 7.3, 1.6 Hz, 1H), 7.63 (td, J = 7.3, 1.3 Hz, 1H), 7.34 (d, $^4J_{HF}$ = 8.1 Hz, 1H), 7.26 (d, $^4J_{HF}$ = 8.0 Hz, 1H), 7.23 (s, 1H), 7.05 – 7.01 (m, 1H), 6.48 (d, $^3J_{HF}$ = 11.9 Hz, 1H), 6.44 (d, $^3J_{HF}$ = 11.8 Hz, 1H), 5.20 (s, 2H), 4.83 (d, J = 2.2 Hz, 2H), 4.11 (s, 3H), 2.60 (t, J = 2.4 Hz, 1H), 1.83 (s, 3H), 1.74 (s, 3H); ^{19}F NMR ($CDCl_3$, 376 MHz) δ -134.11 (dd, J_{FH} = 11.9, 8.0 Hz, 1F), -134.95 (dd, J_{FH} = 11.8, 8.1 Hz, 1F); ^{13}C NMR ($CDCl_3$, 101 MHz) δ 169.8 (C), 154.0 (C), 151.8 (d, $^1J_{CF}$ = 247.9 Hz, C), 151.6 (d, $^1J_{CF}$ = 248.0 Hz, C), 146.7 (C), 146.4 (d, $^2J_{CF}$ = 11.1 Hz, C), 146.1 (d, $^2J_{CF}$ = 11.3 Hz, C), 142.0 (d, $^4J_{CF}$ = 3.5 Hz, C), 141.1 (d, $^4J_{CF}$ = 3.5 Hz, C), 135.4 (CH), 132.0 (C), 130.1 (CH), 129.5 (CH), 126.7 (d, $^3J_{CF}$ = 5.7 Hz, C), 126.0 (C), 125.8 (CH), 125.1 (d, $^3J_{CF}$ = 5.7 Hz, C), 123.6 (CH), 115.58 (d, $^2J_{CF}$ = 19.5 Hz, CH), 115.10 (d, $^3J_{CF}$ = 1.4 Hz, CH), 115.06 (d, $^2J_{CF}$ = 19.4 Hz, CH), 114.17 (d, $^3J_{CF}$ = 1.4 Hz, CH), 84.8 (C), 77.8 (C), 76.9 (CH), 61.8 (d, $^4J_{CF}$ = 1.8 Hz, CH_2), 57.4 (CH_2), 38.2 (C), 35.1 (CH_3), 34.7 (CH_3), 33.4 (CH_3); HRMS (ESI) calcd for $C_{31}H_{24}F_2N_3O_6$ $[M+H]^+$ 572.1628, found 572.1636.



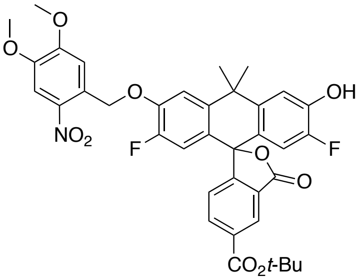
NV–VO (29): To a solution of Virginia Orange (**4**; 300 mg, 0.761 mmol) and 4,5-dimethoxy-2-nitrobenzyl bromide (210 mg, 0.761 mmol, 1 eq) in MeCN (15 mL) was added Ag_2O (176 mg, 0.761 mmol, 1 eq), and the resulting mixture was stirred at 60 °C for 1 h. After cooling the reaction to room temperature, 1 N HCl (40 mL) and EtOAc (40 mL) were added. The gray suspension was filtered through Celite, and the filtrate was extracted with EtOAc (2×). The combined organic extracts were washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. Purification by flash chromatography on silica gel (0–40% EtOAc/hexanes, linear gradient, with constant 40% v/v CH_2Cl_2 additive) yielded 133 mg (30%) of **29** as a pale yellow solid. ^1H NMR ($CDCl_3$, 400 MHz) δ 8.07 – 8.03 (m, 1H), 7.78 (s, 1H), 7.66 (td, J = 7.4, 1.5 Hz, 1H), 7.63 (td, J = 7.4, 1.2 Hz, 1H), 7.45 (s, 1H), 7.28 (d, $^4J_{HF}$ = 8.1 Hz, 1H), 7.26 (d, $^4J_{HF}$ = 8.6 Hz, 1H), 7.06 – 7.02 (m, 1H), 6.47 (d, $^3J_{HF}$ = 11.8 Hz, 1H), 6.43 (d, $^3J_{HF}$ = 11.3 Hz, 1H), 5.60 (s, 2H), 5.41 (d, $^4J_{HF}$ = 3.5 Hz, 1H), 3.98 (s, 3H), 3.98 (s, 3H), 1.81 (s, 3H), 1.70 (s, 3H); ^{19}F NMR ($CDCl_3$, 376 MHz) δ -135.81 (dd, J_{FH} = 11.8, 8.1 Hz, 1F), -142.19 (ddd, J_{FH} = 11.6, 8.7, 3.8 Hz, 1F); ^{13}C NMR ($CDCl_3$, 101 MHz) δ 170.3 (C), 154.4 (C), 154.3 (C), 151.6 (d, $^1J_{CF}$ = 247.2 Hz, C), 149.9 (d, $^1J_{CF}$ = 149.9 Hz, C), 148.2 (C), 147.25 (d, $^2J_{CF}$ = 11.1 Hz, C), 144.84 (d, $^2J_{CF}$ = 14.4 Hz, C), 142.22 (d, $^4J_{CF}$ = 3.3 Hz, C), 142.02 (d, $^4J_{CF}$ = 3.5 Hz, C), 139.1 (C), 135.4 (CH), 130.0 (CH), 128.6 (C), 126.1 (C), 125.7 (CH), 125.29 (d, $^3J_{CF}$ = 5.6 Hz, C), 123.81 (d, $^3J_{CF}$ = 5.4 Hz, C), 123.7 (CH), 115.3 (d, $^3J_{CF}$ = 1.6 Hz, CH), 115.04 (d, $^2J_{CF}$ = 19.2 Hz, CH), 114.32 (d, $^2J_{CF}$ = 19.1 Hz, CH), 113.7 (d, $^3J_{CF}$ = 1.0 Hz, CH), 109.6 (CH), 108.1 (CH), 85.5 (C), 68.5 (CH_2), 56.62 (CH_3), 56.57 (CH_3), 38.2 (C), 35.2 (CH_3), 33.4 (CH_3); HRMS (ESI) calcd for $C_{32}H_{26}F_2NO_8$ $[M+H]^+$ 590.1621, found 590.1630.



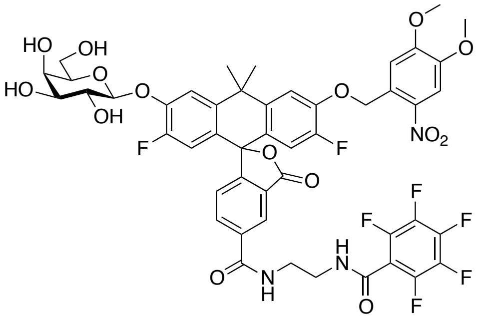
Gal–VO–NV (30): Phenol **29** (50 mg, 84.8 μmol) and acetobromo-α-D-galactose (**17**; 140 mg, 0.339 mmol, 4 eq) were combined in MeCN (4 mL) under nitrogen, and Ag_2O (79 mg, 0.339 mmol, 4 eq) was added. The resulting mixture was stirred at 50 °C for 90 min. The reaction was then cooled to room temperature, filtered through Celite with MeCN, and concentrated *in vacuo*. The crude residue was purified by flash chromatography on silica gel (0–50% EtOAc/hexanes, linear gradient, with constant 40% v/v CH_2Cl_2 additive) to provide 54 mg (69%) of the galactoside tetraacetate as a white solid.

The tetraacetate (54 mg, 58.2 μmol) was subsequently dissolved in 1:1 MeOH/MeCN (2.4 mL), and 1 M Na_2CO_3 (aq, 582 μL, 0.582 mmol, 10 eq) was added. The reaction was stirred at room temperature for 2 h. It was then neutralized with 1 N HCl (1.2 mL), diluted with water, and extracted with CH_2Cl_2 (2×). The combined organic extracts were dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by reverse phase HPLC (10–75% MeCN/H_2O, linear gradient) to yield 27 mg (62%, diastereomeric mixture) of **30** as a white solid. ^1H NMR (CD_3OD, 400 MHz) δ 8.07 – 8.03 (m, 1H), 7.78 (s, 1H), 7.77 – 7.72 (m, 1H), 7.72 – 7.67 (m, 1H), 7.65 (d, $^4J_{HF}$ = 8.2 Hz, 0.6H), 7.64 (d, $^4J_{HF}$ = 8.2 Hz, 0.4H), 7.43 (d, $^4J_{HF}$ = 8.2 Hz, 1H), 7.38 (s, 1H), 7.08 – 7.04 (m, 1H), 6.44 (d, $^3J_{HF}$ = 11.9 Hz, 0.4H), 6.43 (d, $^3J_{HF}$ = 11.9 Hz, 0.6H), 6.40 (d, $^3J_{HF}$ = 11.8 Hz, 0.4H), 6.39 (d, $^3J_{HF}$ = 11.8 Hz, 0.6H), 5.61 (s, 2H), 5.03 (d, J = 7.8 Hz, 0.4H), 4.99 (d, J = 7.8 Hz, 0.6H), 3.93 (s, 3H), 3.91 (s, 3H), 3.94 – 3.74 (m, 5H), 3.65 – 3.59 (m, 1H), 1.85 (s, 3H), 1.74 (s, 3H); ^{19}F NMR (CD_3OD, 376 MHz) δ -134.44 – -134.55 (m, 1F), -135.59 – -135.69 (m, 1F); Analytical HPLC: t_R = 13.1 min, >99% purity (diastereomeric mixture; 5 μL injection; 10–95% MeCN/H_2O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; UV detection at 254 nm); HRMS (ESI) calcd for $C_{38}H_{35}F_2NO_{13}Na$ $[M+Na]^+$ 774.1969, found 774.1983.

SYNTHESIS OF PROBE 33



NV–VO(5-CO$_2$*t*-Bu) (31): To a solution of 5-(*tert*-butoxycarbonyl)-Virginia Orange (**16**; 260 mg, 0.526 mmol) and 4,5-dimethoxy-2-nitrobenzyl bromide (145 mg, 0.526 mmol, 1 eq) in MeCN (10 mL) was added Ag_2O (122 mg, 0.526 mmol, 1 eq), and the resulting mixture was stirred at 60 °C for 90 min. After cooling the reaction to room temperature, 1 N HCl (30 mL) and EtOAc (30 mL) were added. The gray suspension was filtered through Celite; the filtrate was diluted with water and extracted with EtOAc (2×). The combined organic extracts were washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. Silica gel chromatography was performed twice (0–25% EtOAc/toluene, linear gradient; then, 5–50% EtOAc/hexanes, linear gradient) to yield 85 mg (23%) of **31** as a pale yellow solid. ^1H NMR ($CDCl_3$, 400 MHz) δ 8.64 (dd, J = 1.5, 0.7 Hz, 1H), 8.29 (dd, J = 8.0, 1.5 Hz, 1H), 7.78 (s, 1H), 7.45 (s, 1H), 7.29 (d, $^4J_{HF}$ = 8.2 Hz, 1H), 7.27 (d, $^4J_{HF}$ = 8.2 Hz, 1H), 7.08 (dd, J = 8.0, 0.7 Hz, 1H), 6.45 (d, $^3J_{HF}$ = 11.7 Hz, 1H), 6.41 (d, $^3J_{HF}$ = 11.1 Hz, 1H), 5.60 (s, 2H), 5.31 (d, $^4J_{HF}$ = 3.6 Hz, 1H), 3.99 (s, 3H), 3.98 (s, 3H), 1.82 (s, 3H), 1.71 (s, 3H), 1.64 (s, 9H); ^{19}F NMR ($CDCl_3$, 376 MHz) δ -135.52 (dd, J_{FH} = 11.6, 8.1 Hz, 1F), -141.97 (ddd, J_{FH} = 11.5, 8.8, 3.6 Hz, 1F); ^{13}C NMR ($CDCl_3$, 101 MHz) δ 169.4 (C) 164.1 (C), 157.8 (C), 154.3 (C), 151.6 (d, $^1J_{CF}$ = 247.7 Hz, C), 149.9 (d, $^1J_{CF}$ = 239.8 Hz, C), 148.3 (C), 147.4 (d, $^2J_{CF}$ = 11.0 Hz, C), 145.0 (d, $^2J_{CF}$ = 14.5 Hz, C), 142.3 (d, $^4J_{CF}$ = 3.3 Hz, C), 142.0 (d, $^4J_{CF}$ = 3.4 Hz, C), 139.1 (C), 136.4 (CH), 134.4 (C), 128.5 (C), 127.1 (CH), 126.3 (C), 124.55 (d, $^3J_{CF}$ = 5.5 Hz, C), 123.7 (CH), 123.14 (d, $^3J_{CF}$ = 5.2 Hz, C), 115.5 (d, $^3J_{CF}$ = 1.7 Hz, CH), 114.9 (d, $^2J_{CF}$ = 19.3 Hz, CH), 114.2 (d, $^2J_{CF}$ = 19.2 Hz, CH), 113.7 (d, $^3J_{CF}$ = 0.8 Hz, CH), 109.6 (CH), 108.1 (CH), 85.5 (C), 82.7 (C), 68.5 (CH_2), 56.63 (CH_3), 56.57 (CH_3), 38.2 (C), 35.2 (CH_3), 33.5 (CH_3), 28.3 (CH_3); HRMS (ESI) calcd for $C_{37}H_{34}F_2NO_{10}$ $[M+H]^+$ 690.2145, found 690.2153.



Gal–VO(5-PFB)–NV (33): Phenol **31** (68 mg, 98.6 μmol) and acetobromo-α-D-galactose (**17**; 162 mg, 0.394 mmol, 4 eq) were combined in MeCN (5 mL) under nitrogen, and Ag_2O (91 mg, 0.394 mmol, 4 eq) was added. The resulting mixture was stirred at 50 °C for 90 min. The reaction was then cooled to room temperature, filtered through Celite with MeCN, and concentrated *in vacuo*. The crude residue was purified by flash chromatography on silica gel (5–40% EtOAc/toluene, linear gradient) to afford 71 mg (70%) of the galactoside tetraacetate as a white solid.

The tetracetate-*t*-butyl-ester (60 mg, 58.8 μmol) was taken up in CH_2Cl_2 (2.5 mL), and trifluoroacetic acid (0.5 mL) was added. The reaction was stirred at room temperature for 5 h. Toluene (3 mL) was added; the reaction mixture was concentrated to dryness and then azeotroped with MeOH three times to provide the deprotected carboxylic acid as an off-white solid (54 mg, 95%).

The tetraacetate-acid (54 mg, 56.0 μmol) was combined with HATU (43 mg, 0.112 mmol, 2 eq) in DMF (3 mL). After adding DIEA (29 μL, 0.168 mmol, 3 eq), the reaction was stirred at room temperature for 15 min. A solution of *N*-(2-aminoethyl)-2,3,4,5,6-pentafluorobenzamide trifluoroacetate[3] (**32**; 41 mg, 0.112 mmol, 2 eq) in DMF (300 μL) was then added, and the reaction was stirred for an additional 2 h at room temperature. It was subsequently diluted with water and extracted with EtOAc (2×). The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. Flash chromatography on silica gel (5–100% EtOAc/CH_2Cl_2, linear gradient) afforded 54 mg (80%) of the amide as an off-white solid.

The tetraacetate-amide (54 mg, 45.1 μmol) was subsequently dissolved in 1:1 MeOH/MeCN (2 mL), and 1 M Na_2CO_3 (aq, 451 μL, 0.451 mmol, 10 eq) was added. The reaction was stirred at room temperature for 2 h. It was then neutralized with 1 N HCl (910 μL) and directly purified by reverse phase HPLC (30–95% MeCN/H_2O, linear gradient) to yield 35 mg (75%, diastereomeric mixture) of **33** as a white solid. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.47

(dd, J = 1.6, 0.7 Hz, 1H), 8.17 (dt, J = 8.1, 1.4 Hz, 1H), 7.78 (s, 1H), 7.66 (d, $^4J_{HF}$ = 8.1 Hz, 0.5H), 7.65 (d, $^4J_{HF}$ = 8.2 Hz, 0.5H), 7.44 (d, $^4J_{HF}$ = 8.1 Hz, 1H), 7.38 (s, 1H), 7.152 (dd, J = 8.1, 0.7 Hz, 0.5H), 7.146 (dd, J = 8.1, 0.7 Hz, 0.5H), 6.47 (d, $^3J_{HF}$ = 11.8 Hz, 0.5H), 6.46 (d, $^3J_{HF}$ = 11.8 Hz, 0.5H), 6.43 (d, $^3J_{HF}$ = 11.7 Hz, 0.5H), 6.42 (d, $^3J_{HF}$ = 11.7 Hz, 0.5H), 5.61 (s, 2H), 5.03 (d, J = 7.7 Hz, 0.5H), 5.00 (d, J = 7.7 Hz, 0.5H), 3.94 – 3.74 (m, 5H), 3.93 (s, 3H), 3.91 (s, 3H), 3.71 – 3.59 (m, 5H), 1.85 (s, 3H), 1.75 (s, 3H); ^{19}F NMR (CD_3OD, 376 MHz) δ -134.26 – -134.37 (m, 1F), -135.39 – -135.49 (m, 1F), -141.83 – -141.97 (m, 2F), -153.53 (tt, J = 19.7, 2.2 Hz, 1F), -162.01 – -162.19 (m, 2F); Analytical HPLC: t_R = 10.3 min, >99% purity (diastereomeric mixture; 5 μL injection; 30–95% MeCN/H_2O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; UV detection at 254 nm); HRMS (ESI) calcd for $C_{48}H_{41}F_7N_3O_{15}$ $[M+H]^+$ 1032.2420, found 1032.2422.

OPTICAL SPECTROSCOPY AND MICROSCOPY METHODS

General. Fluorescent and fluorogenic molecules were prepared as stock solutions in DMSO and diluted such that the final DMSO concentration did not exceed 1% v/v. Enzymes were obtained from Sigma-Aldrich (β-galactosidase from *Escherichia coli*, G4155; nitroreductase from *Escherichia coli*, N9284) and ThermoFisher (TEM-1 β-lactamase, PV3575).

UV–Vis and Fluorescence Spectroscopy. Spectroscopy was performed using 1-cm path length, 3.5-mL quartz cuvettes from Starna Cells. All measurements were taken at ambient temperature (22 ± 2 °C), and spectra are uncorrected. Absorption spectra were recorded on a Cary Model 100 spectrometer (Varian), and fluorescence spectra were recorded on a Cary Eclipse fluorometer (Varian; slit widths at 5 nm). The spectra, maximum absorption wavelength (λ_{max}), and extinction coefficient (ε) of **4** were taken in 0.1 M NaOH; the reported value for ε is an average (n = 3).

Quantum Yield Determination. Absolute quantum yields (Φ) were measured using a Quantaurus-QY spectrometer (model C11374) from Hamamatsu. This instrument uses an integrating sphere to determine photons absorbed and emitted by a sample. Measurements were carried out using dilute samples ($A < 0.1$) and self-absorption corrections were performed using the instrument software.[4]

p*K*$_a$ Determination (Figure 2d). The pK_a values for compounds **1**, **2** and **4** were determined in buffers containing 150 mM NaCl and 10 mM buffer. The following buffer systems were used: citrate (pH 4.0–6.2); phosphate (pH 5.8–8.0); tris (pH 7.8–9.0); carbonate (pH 9.2–10.0). Buffer solutions containing 250 nM fluorophore were placed in a black, clear-bottom 96-well microplate with a non-binding surface coating (Corning, product #3651). Fluorescence values were read on FlexStation 3 platereader (Molecular Devices) using $\lambda_{ex}/\lambda_{em}$ = 490 nm/520 nm or 550 nm/580 nm (n = 3) and plotted using GraphPad Prism software. The points were fitted to a sigmoidal dose response curve with a variable slope to determine the Hill coefficient (*h*). We observed *h* values of 0.97, 1.65, and 1.46 for compounds **1**, **2** and **4**, respectively.[5]

Enzyme Kinetics (Figure S2). The initial velocity of fluorescence release of mono- and bis-masked VO substrates (eight two-fold dilutions, 10 µM to 0.078 µM) was measured in triplicate on a FlexStation 3 platereader (Molecular Devices) in 10 mM HEPES pH 7.3, 0.1% DMSO (plus 100 µM NADH for nitroreductase reactions). Data were fit using GraphPad Prism software to the Michaelis–Menten equation for nitroreductase (8.3 nM enzyme), or the Michaelis–Menten equation at low substrate concentration ($v_0 = V_{max}[S]/K_M$) for β-galactosidase (17 nM) and TEM-1 β-lactamase (19 nM).

Activation of Fluorogenic Virginia Orange Derivatives. All measurements of fluorescence accumulation upon activation of the fluorogenic probes were determined in quartz cuvettes (1-cm path length, 3.5-mL) with 3.0 mL 10 mM HEPES pH 7.3, 0.1% DMSO (and 100 μM NADH for nitroreductase reactions). The same settings were used for all measurements of fluorescence (λ_{ex} = 555 nm, λ_{em} = 582 nm, 5 nm slit widths, PMT voltage 500 V). The final concentration for each enzyme was kept the same across all assays unless noted otherwise: 17 nM (~1.5 U/mL) for β-galactosidase; 113 nM (~1.5 U/mL) for nitroreductase; and 57 nM for TEM-1 β-lactamase (~1.8 U/mL).

(a) To demonstrate the relative rates of unmasking for the mono- and bis-masked single-enzyme substrates (Figure 3b–d), cuvettes were first charged with buffer (t = 0). Fluorescence was recorded for 10 min, with the addition of probe (1 μM) at t = 0.5 min and enzyme at t = 2 min.

(b) The contrast ratios (fluorescence increase, F_{post}/F_{pre}) for probes **20**–**25** were determined by first measuring the fluorescence of 1 μM probe prior to addition of enzyme (F_{pre}, t = 0 h, n = 3). Enzyme was then added, and the samples were aged until no further increase in fluorescence was seen (4 h). Fluorescence was recorded at 2 h and 4 h (F_{post}, t = 4 h, n = 3) to calculate the contrast values (Figure S1).

(c) The sequential unmasking of **26** (Figure 4b,c) was accomplished by first charging a cuvette with buffer (t = 0). Fluorescence was recorded for 10 min, with the addition of probe (1 μM) at t = 0.5 min, the first enzyme at t = 2 min, and the second enzyme at t = 4 min.

(d) The contrast ratios of the dual-input bis-masked probes (**26**–**28**, **30**; Figure 5) were determined by measuring the accumulated fluorescence after aging/activating each probe (1 μM) with no input ("blank"), each input alone, or both inputs (n = 3 for each condition). For **26** (β-gal/NTR) and **27** (Blac/NTR), the samples were incubated for 20 min; in the case of the two-input samples, both enzymes were added at t = 0 min. The β-gal/hν compound **30** was aged with β-galactosidase alone, irradiated in the absence of enzyme (Luzchem LZC 4V photoreactor, 365 nm lamps), or irradiated in the presence of β-galactosidase (20 min for all conditions). For **28** (NTR/Pd), the single-input samples were incubated with nitroreductase or $[PdCl(allyl)]_2$ (10 μM) for 3 h. The two-input condition was performed by aging the probe with nitroreductase for 20 min, then adding $[PdCl(allyl)_2]$ and recording the fluorescence after 3 h total.

Cell Staining and Imaging. COS-7 cells were grown in DMEM with 10% FBS and Glutamax (Gibco) at 37 °C/10% CO_2. Cells seeded in 35 mm glass bottom dishes (MatTek) were transiently transfected with a β-galactosidase expression vector (pCMVSport-βgal) using Lipofectamine 2000 (Thermo Fisher Scientific) at an efficiency of 65%. After 24 hours of recovery, **33** was added at a final concentration of 20 μM in the presence of 0.2% Pluronic F-127. The dye was allowed to load for 24 hours prior to imaging. Cells were imaged with a Zeiss LSM 710 with a 20×/0.8 NA Plan-Apochromat objective. The sample was excited with a 561 nm source at 10% power, and 566-685 nm BP filter emission from a single plane of 3.5 μm. Activation of the dye was done with a 405 nm diode at 100% power with a single scan at 1024 × 1024p. Fluorescence image analysis was done using the Fiji package.[6]

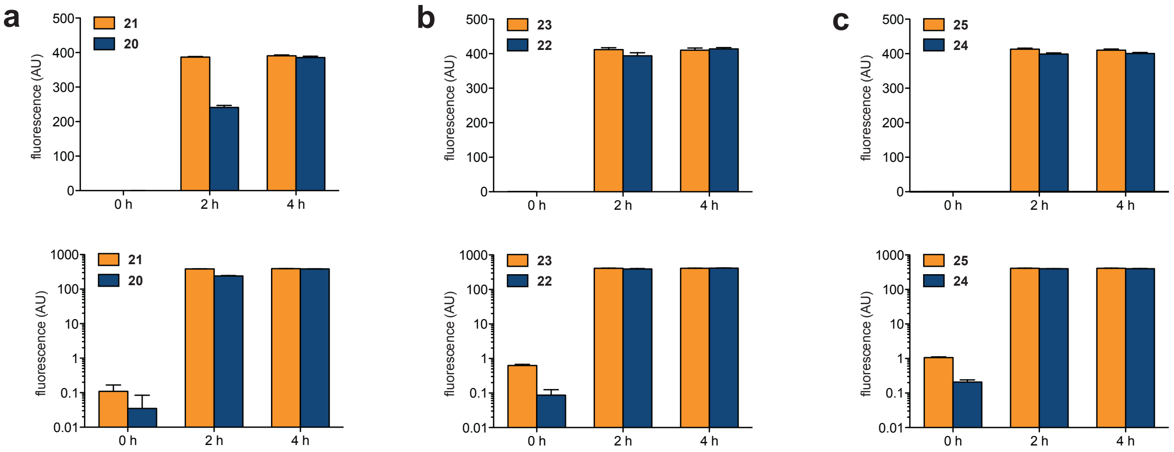


Figure S1. Determination of contrast for Virginia Orange probes by comparison of fluorescence intensity pre-activation (0 h) to fluorescence 2 h and 4 h post-activation (addition of enzyme); linear plots (top panels) and logarithmic plots (bottom panels) shown (mean ± SD, n = 3). (**a**) Pre- and post-activation fluorescence of **20** (Gal_2–VO) and **21** (Gal–VO) when unmasked with β-galactosidase. (**b**) Pre- and post-activation fluorescence of **22** (NM_2–VO) and **23** (NM–VO) when unmasked with nitroreductase. (**c**) Pre- and post-activation fluorescence of **24** ($Ceph_2$–VO) and **25** (Ceph–VO) when unmasked with TEM-1 β-lactamase.

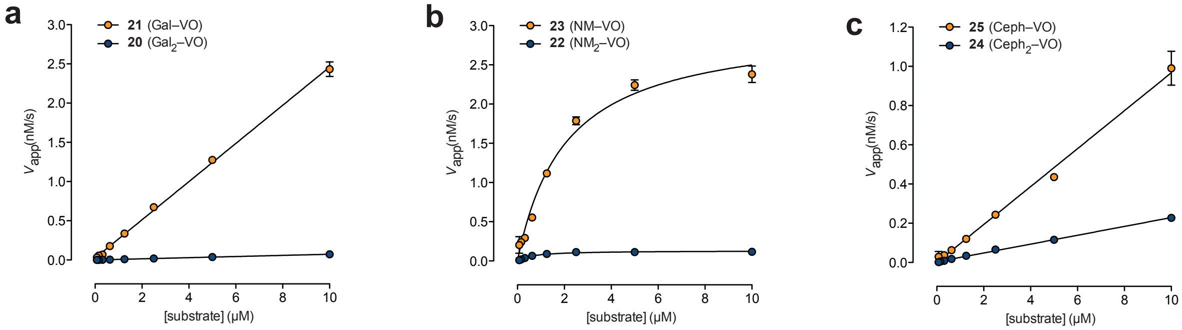


Figure S2. Apparent rate of fluorescence release versus substrate concentration for Virginia Orange enzyme substrates (mean ± SD, n = 3). (**a**) Rate plot for β-galactosidase substrates **20** (Gal_2–VO) and **21** (Gal–VO); saturation was not achieved (high K_M), so the Michaelis-Menten equation at low substrate concentration (linear regression) was used to determine V_{max}/K_M from the slope. (**b**) Rate plot for nitroreductase substrates **22** (NM_2–VO) and **23** (NM–VO); fit with Michaelis–Menten equation. (**c**) Rate plot for TEM-1 β-lactamase substrates **24** ($Ceph_2$–VO) and **25** (Ceph–VO); saturation was not achieved (high K_M), so the Michaelis–Menten equation at low substrate concentration (linear regression) was used to determine V_{max}/K_M from the slope.

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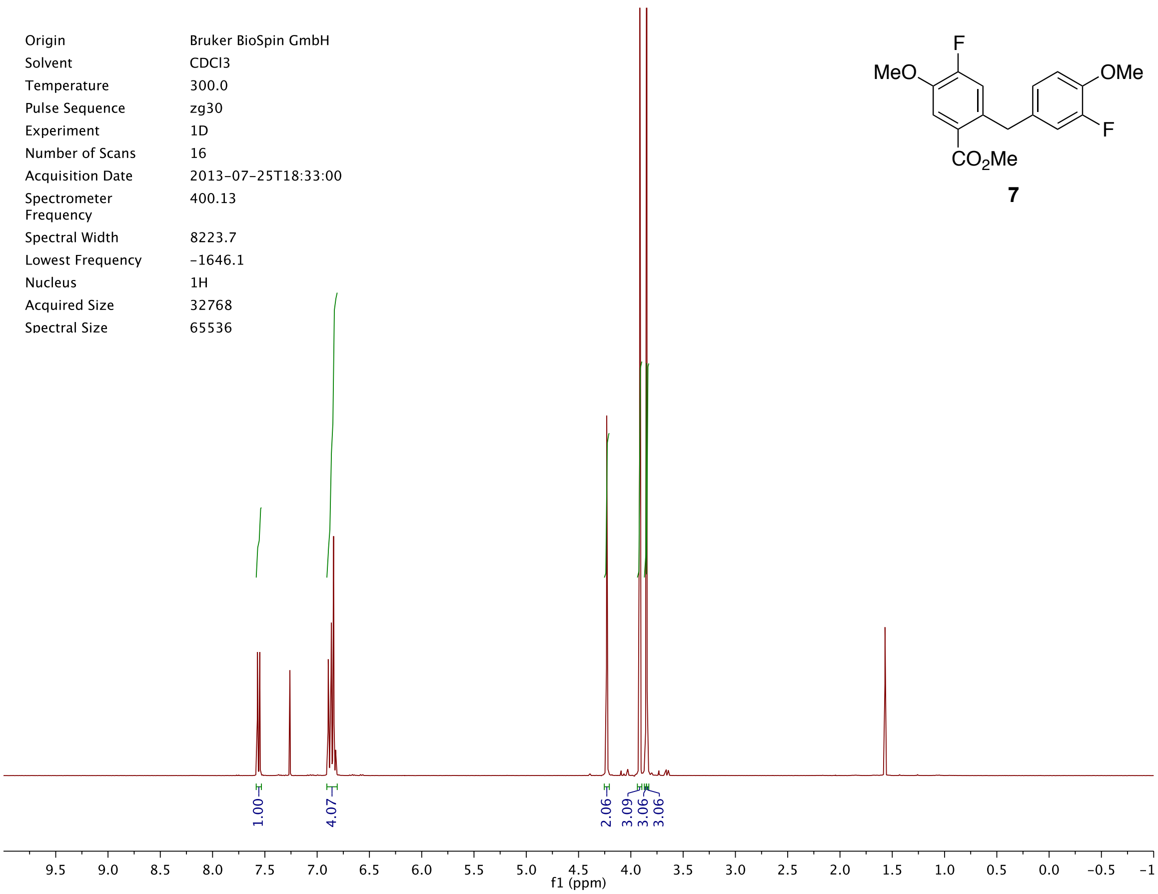

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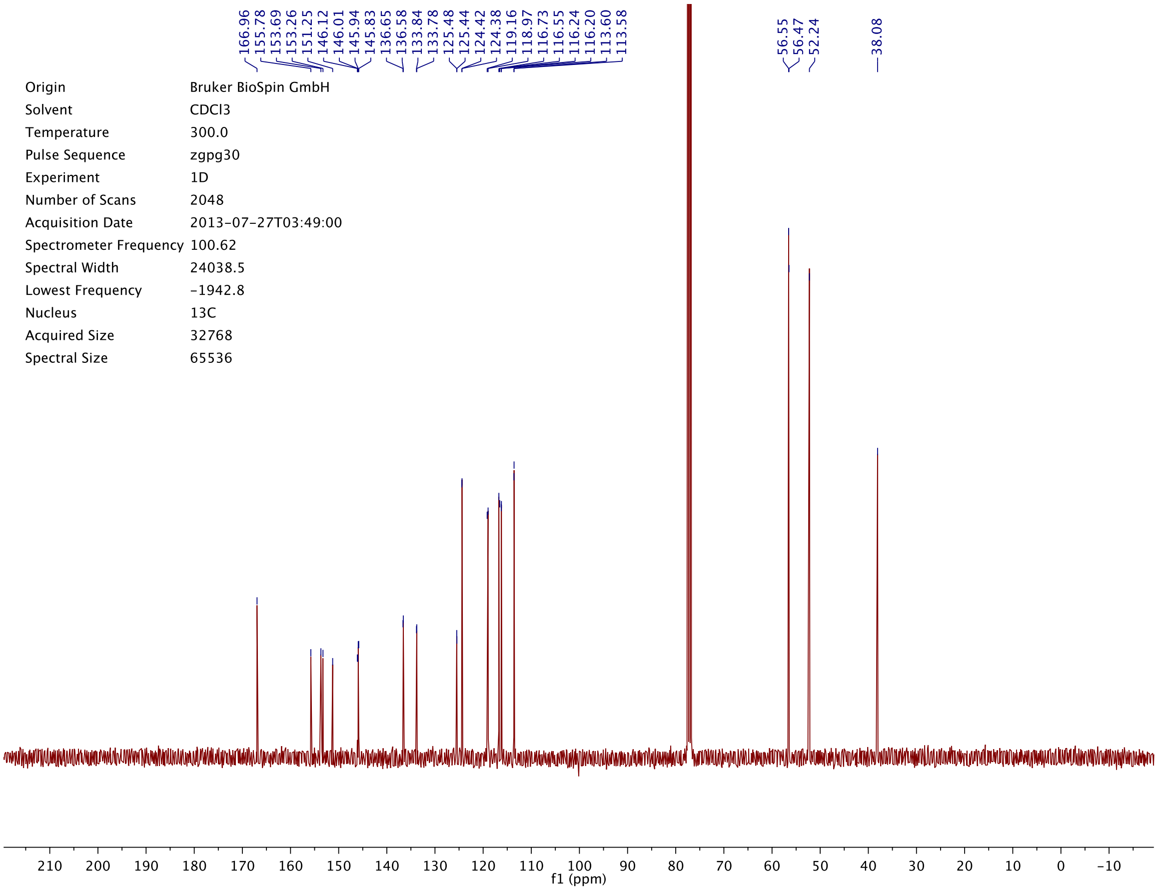

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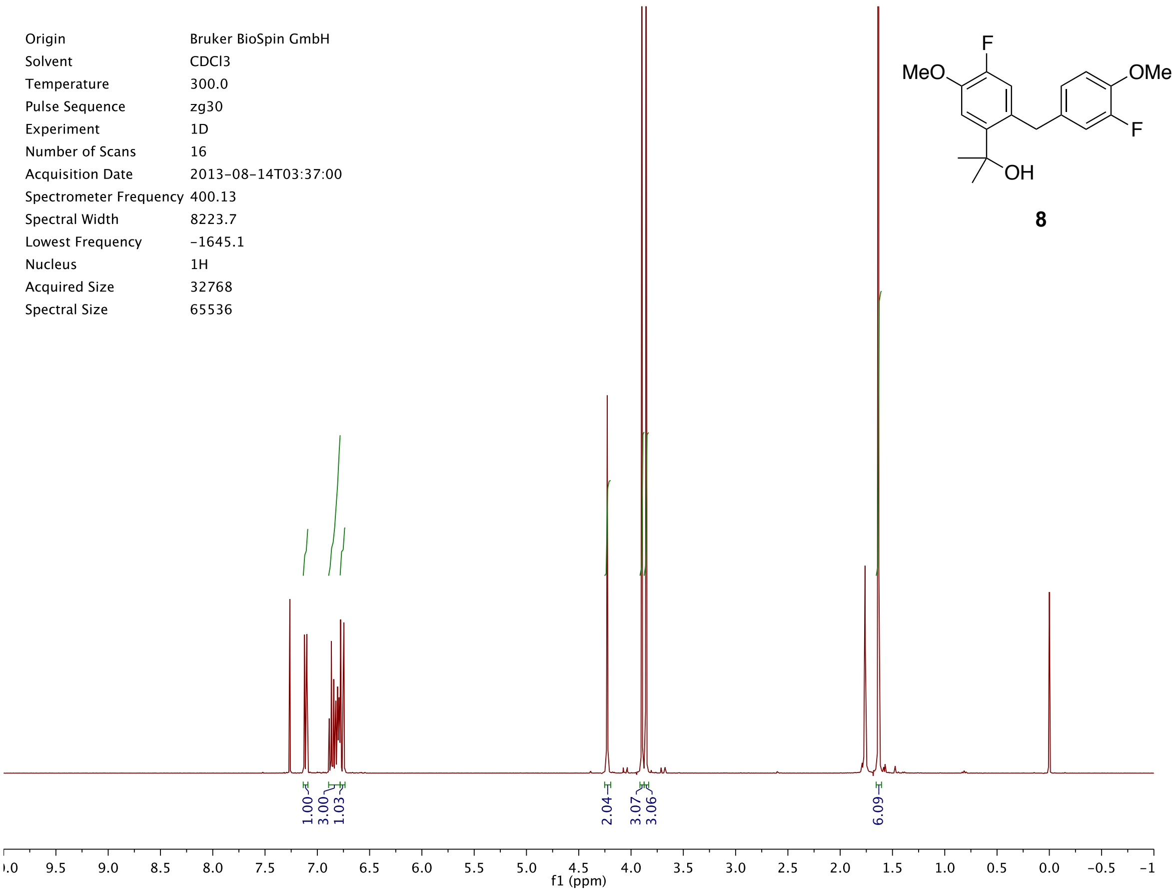

8

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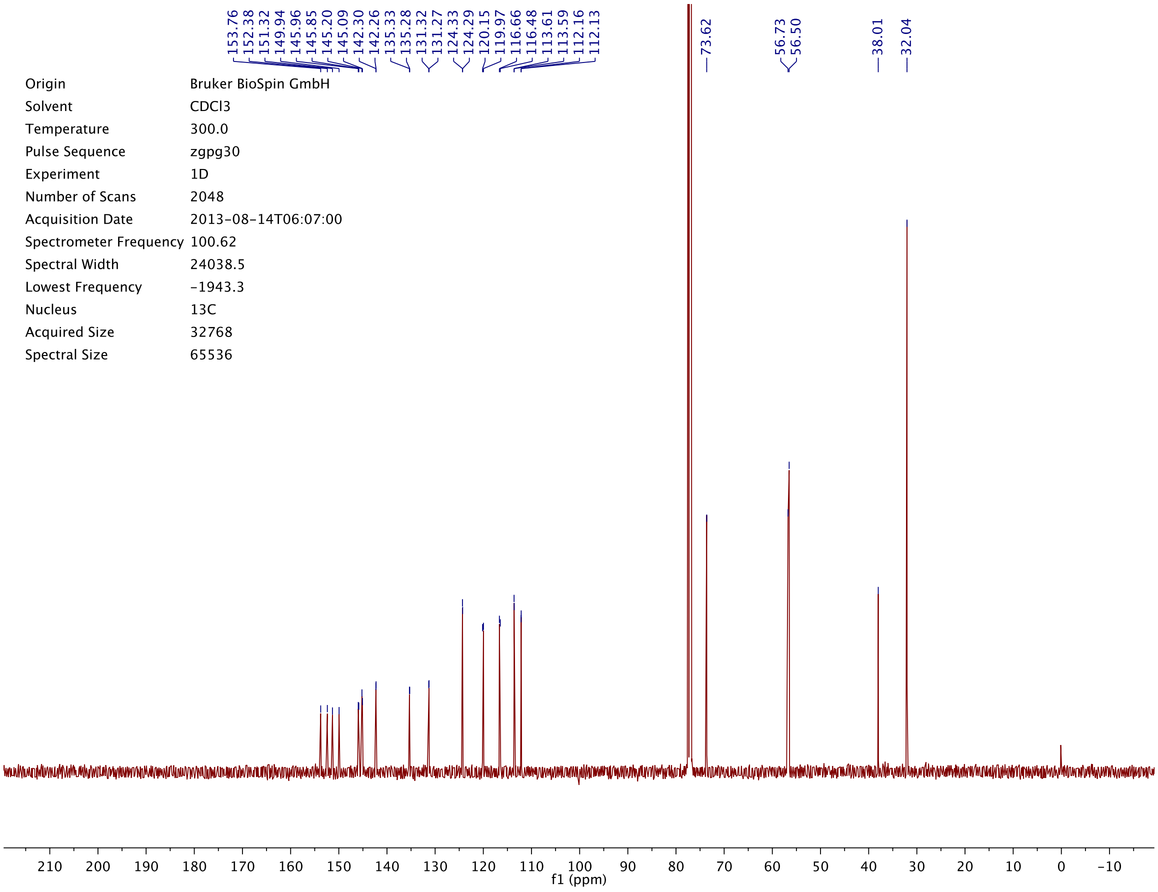

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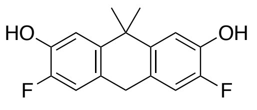
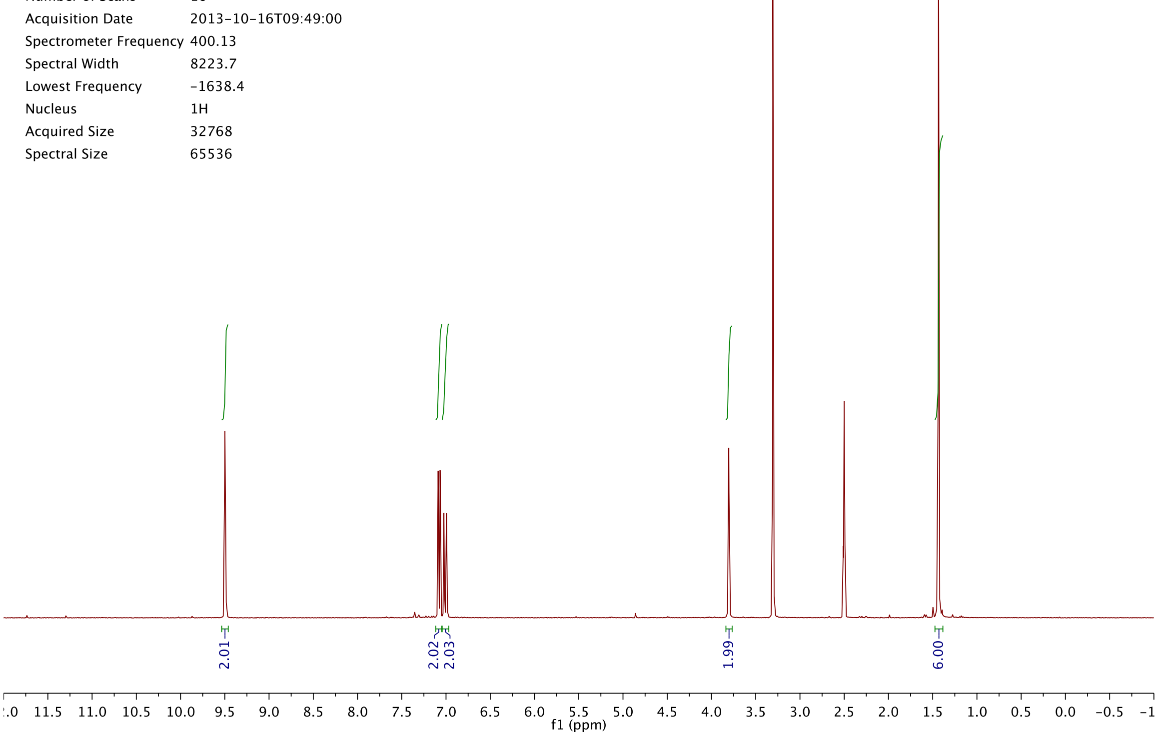


9



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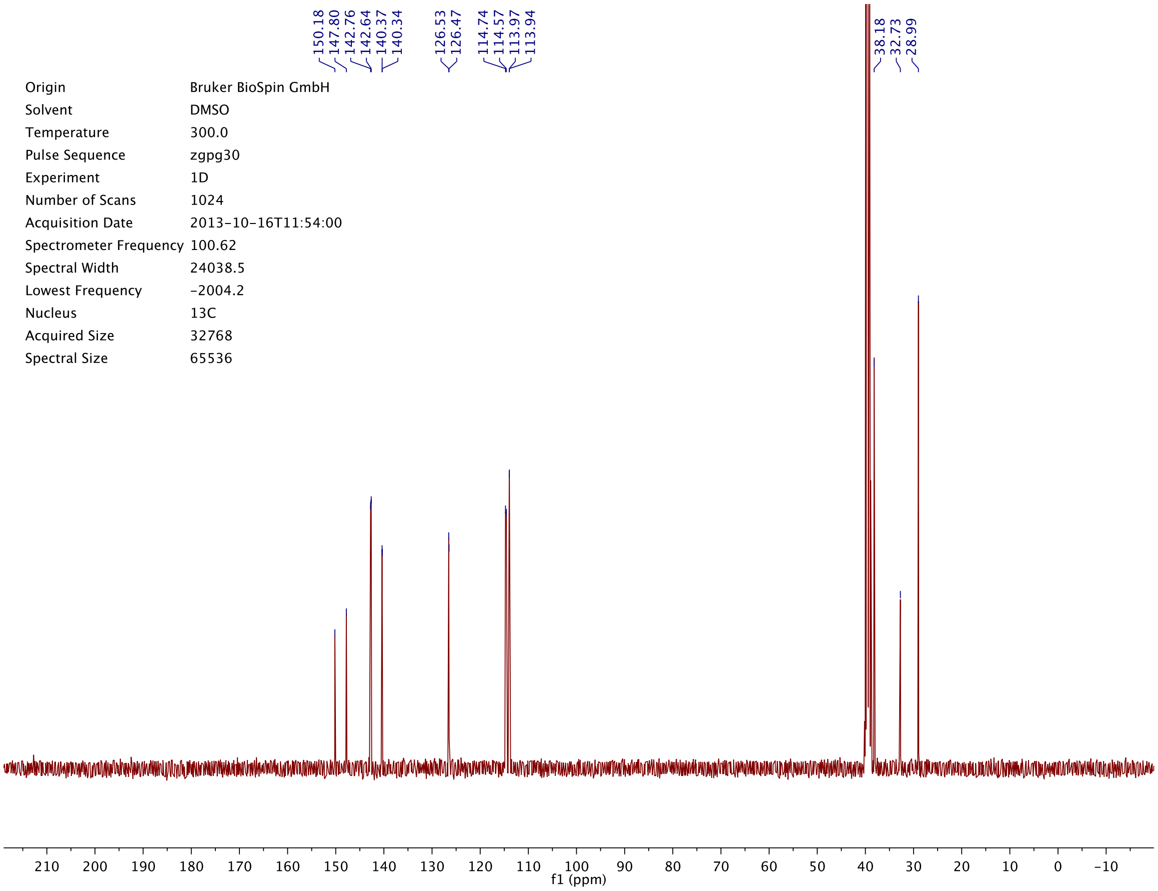

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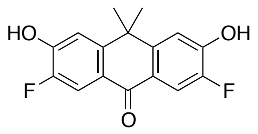

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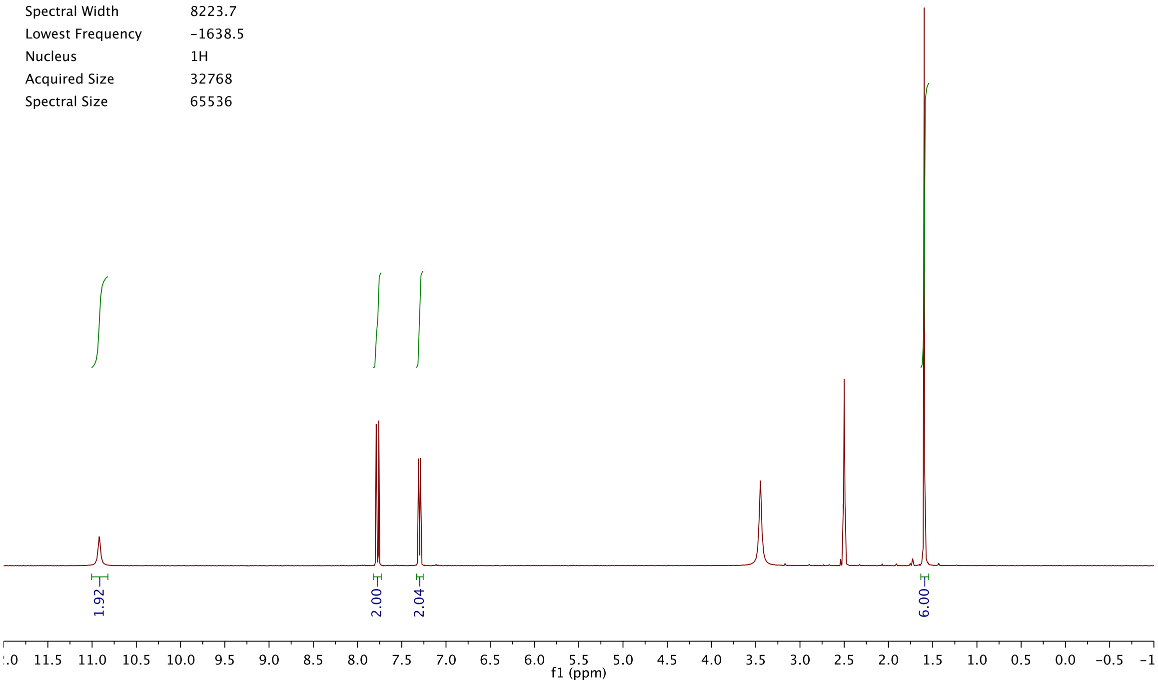

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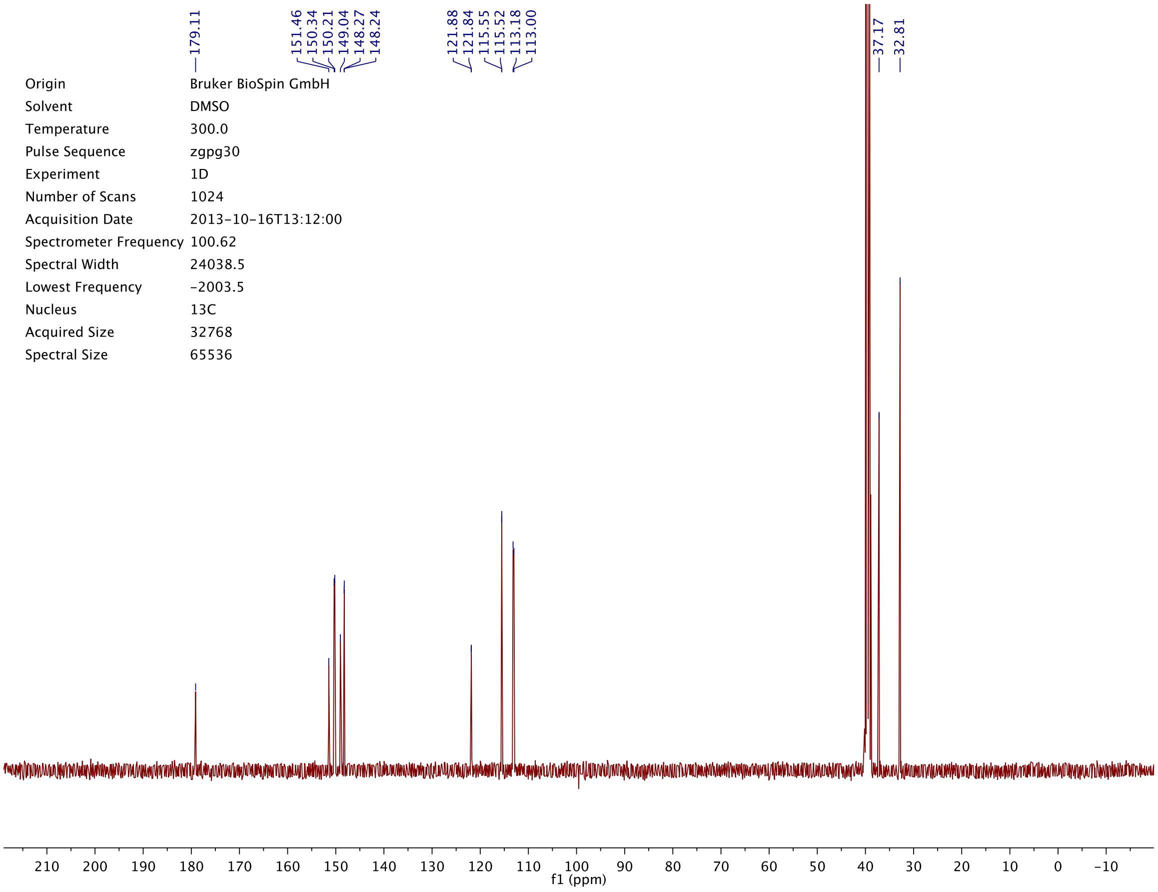

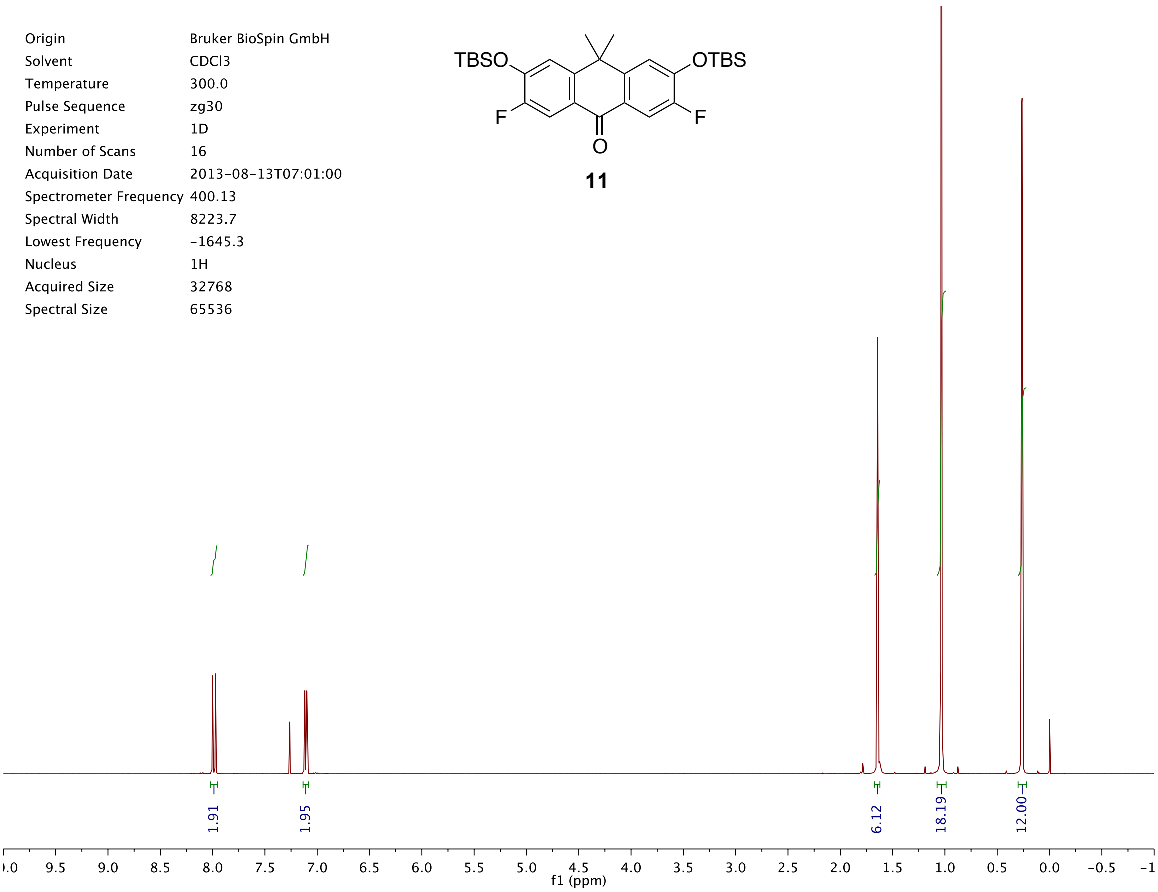

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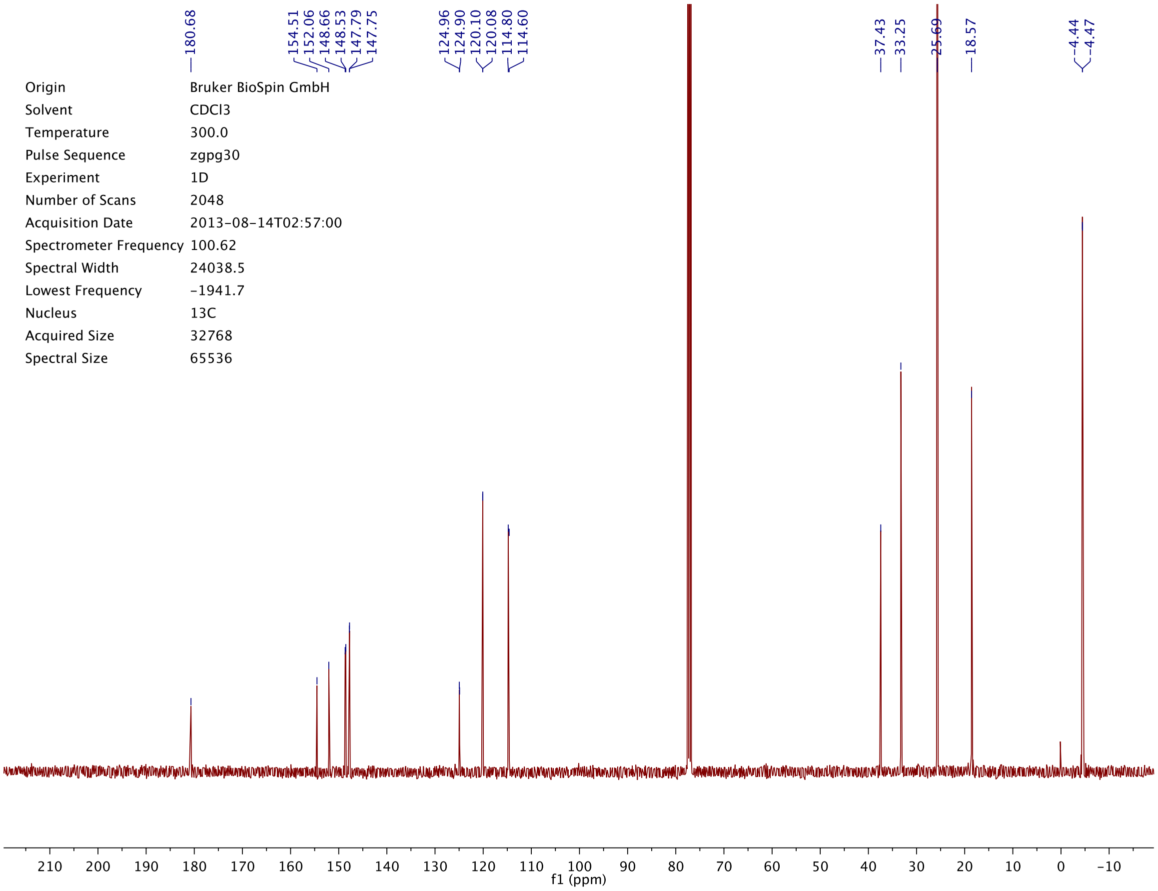

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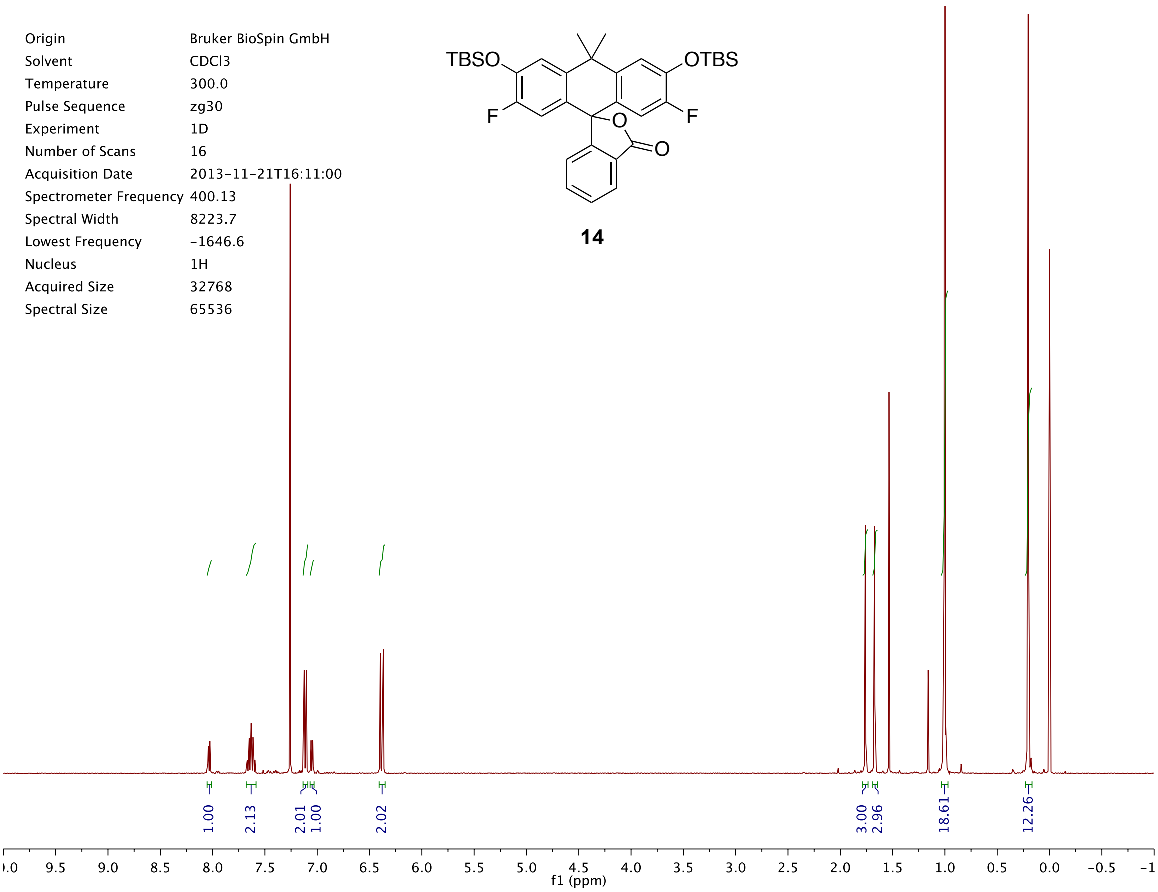

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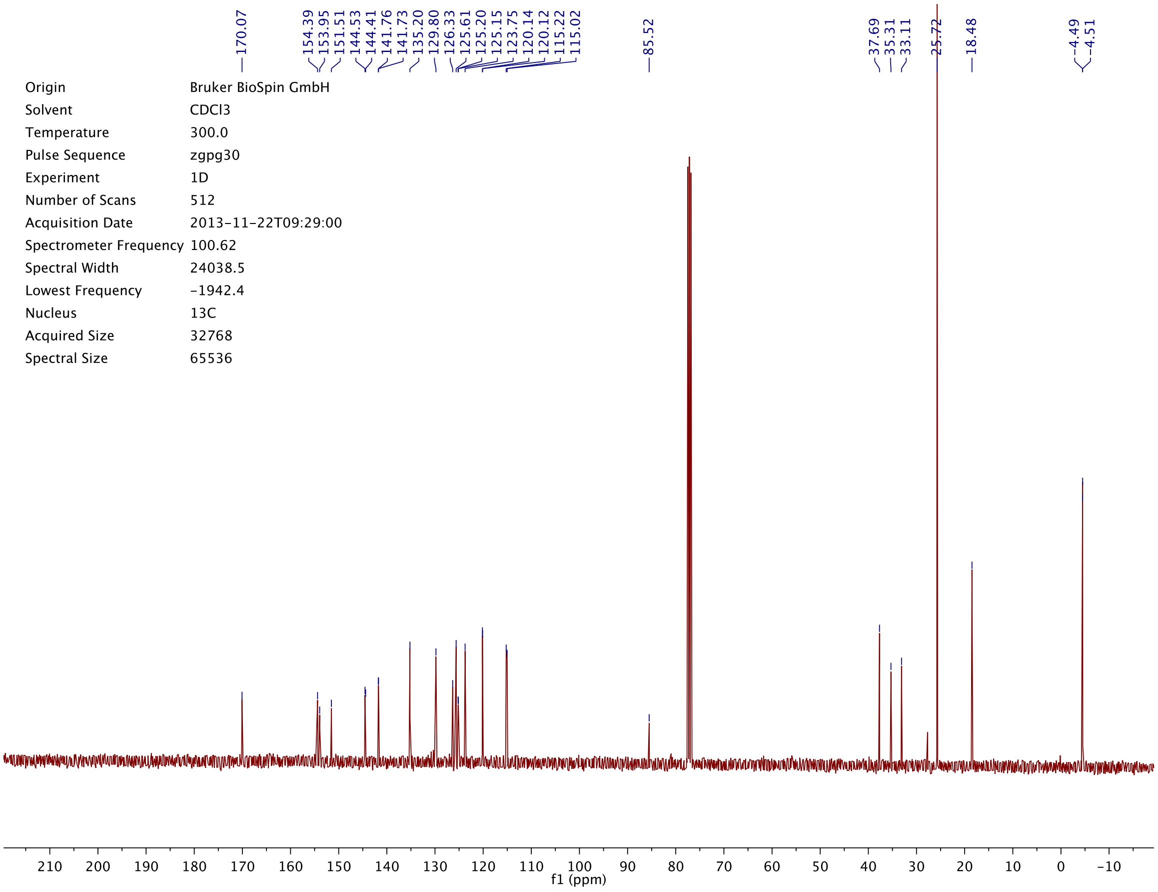

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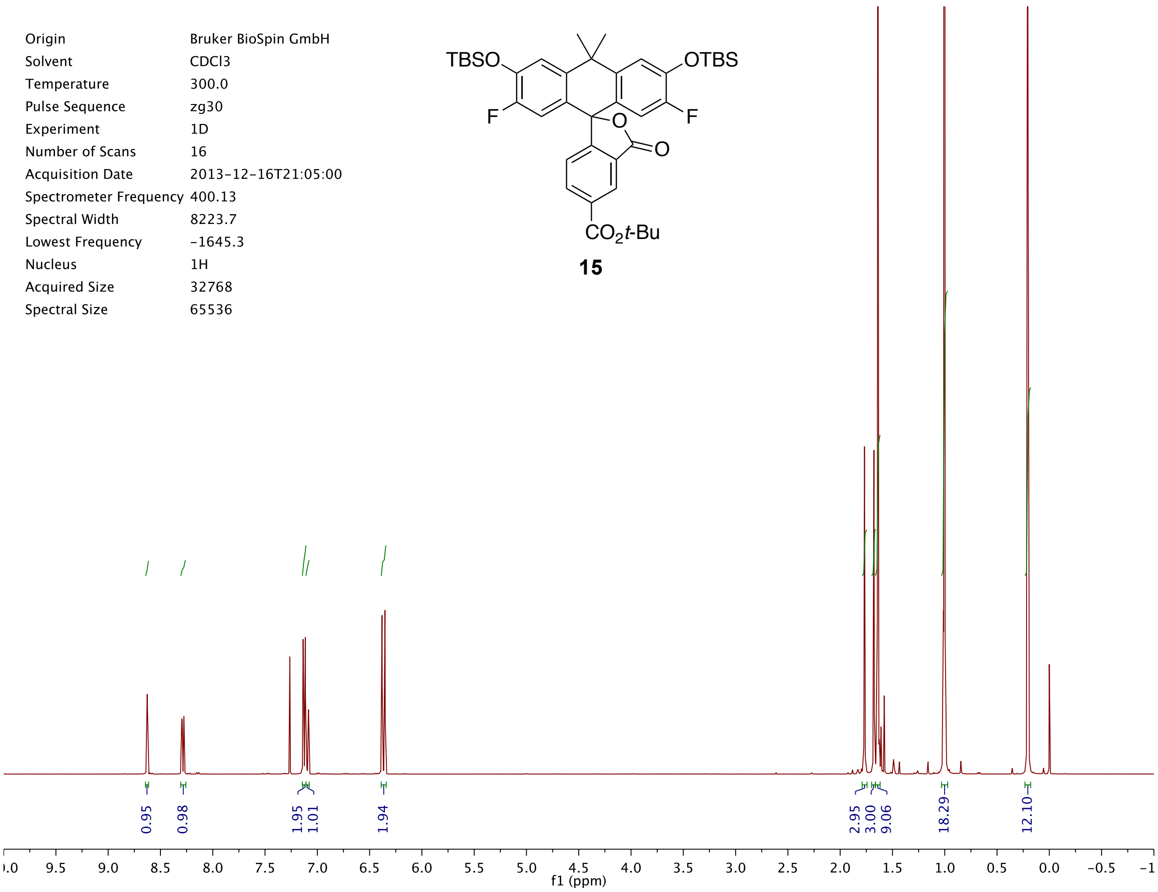
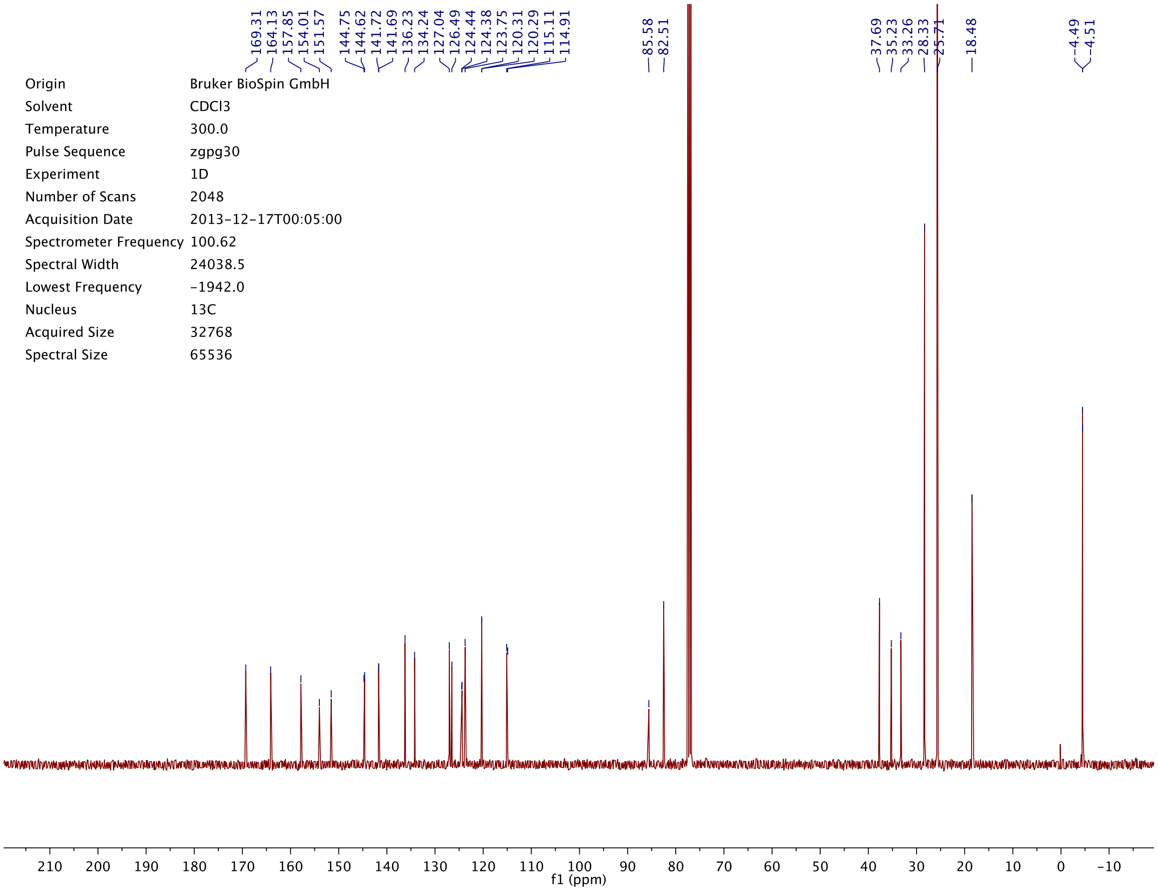
Acquisition Date	2013-11-22T09:29:00
Spectrometer Frequency	100.62
Spectral Width	24038.5
Lowest Frequency	−1942.4
Nucleus	13C
Acquired Size	32768
Spectral Size	65536



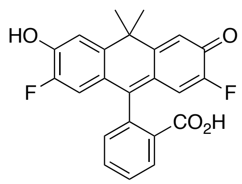
15

Origin	Bruker BioSpin GmbH
Solvent	CDCl3
Temperature	300.0
Pulse Sequence	zg30
Experiment	1D
Number of Scans	16
Acquisition Date	2013-12-16T21:05:00
Spectrometer Frequency	400.13
Spectral Width	8223.7
Lowest Frequency	-1645.3
Nucleus	1H
Acquired Size	32768
Spectral Size	65536

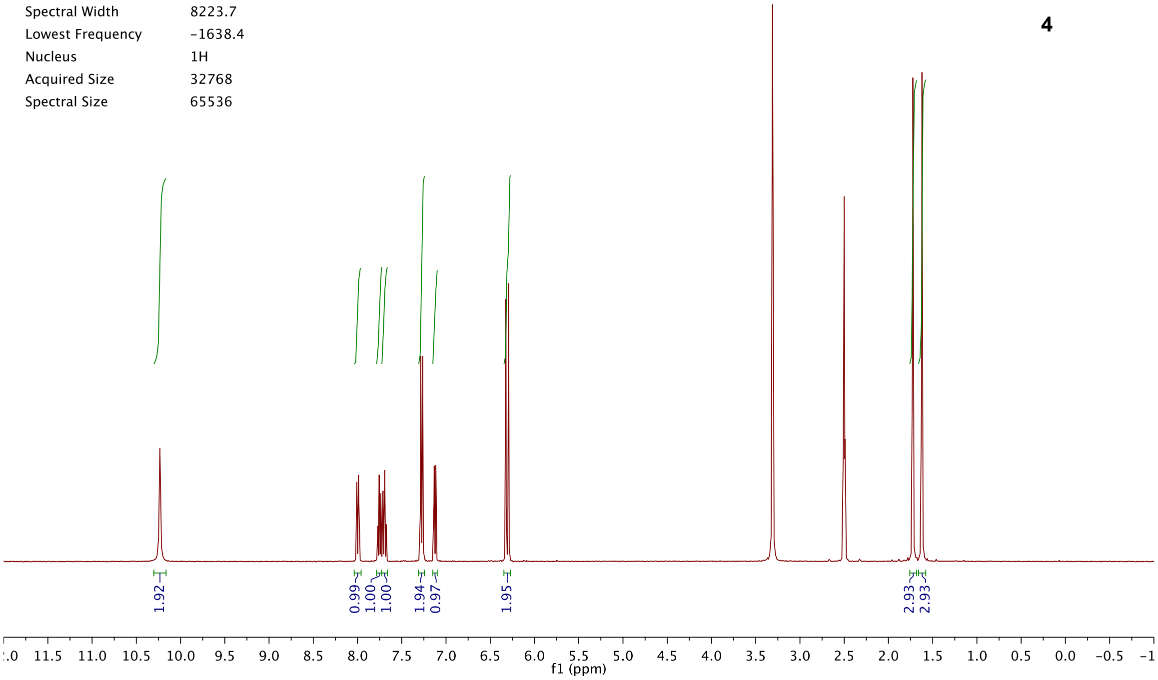


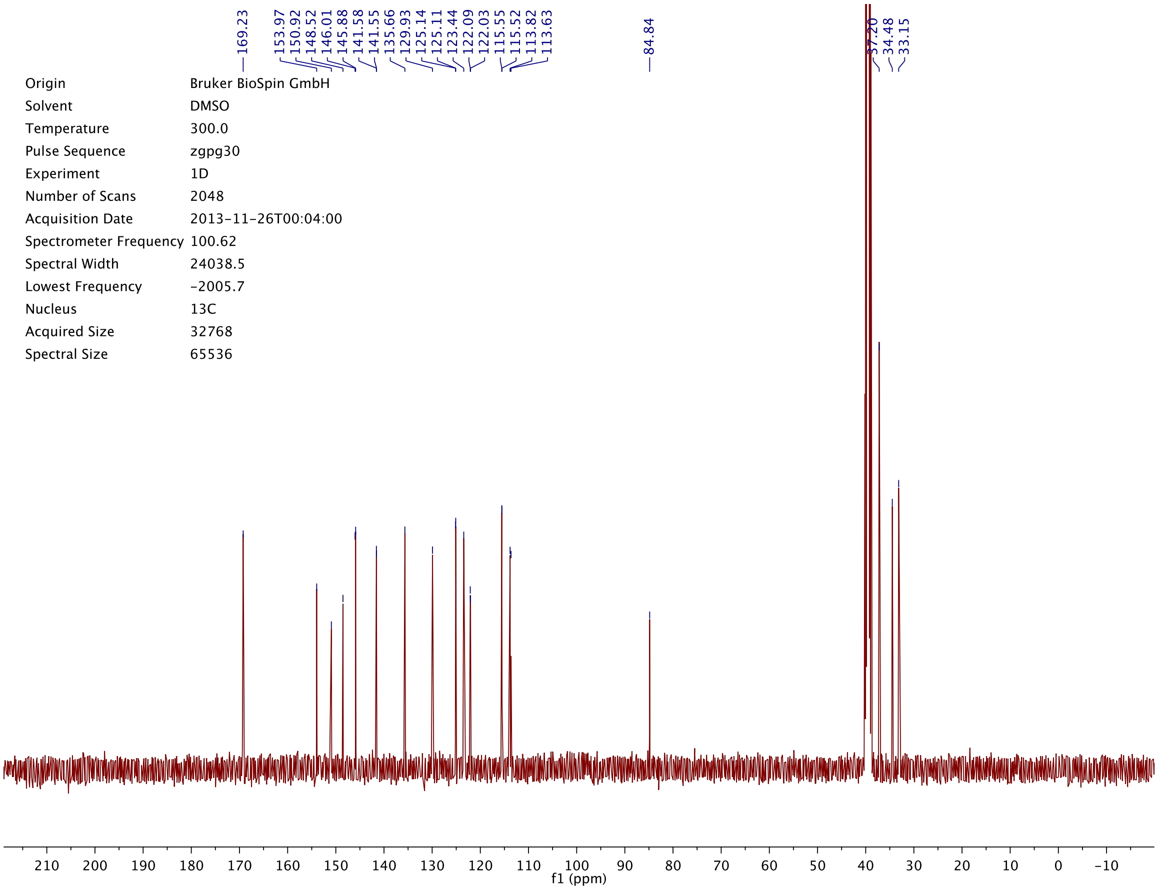
Origin	Bruker BioSpin GmbH
Solvent	CDCl3
Temperature	300.0
Pulse Sequence	zgpg30
Experiment	1D
Number of Scans	2048
Acquisition Date	2013-12-17T00:05:00
Spectrometer Frequency	100.62
Spectral Width	24038.5
Lowest Frequency	-1942.0
Nucleus	13C
Acquired Size	32768
Spectral Size	65536

Origin	Bruker BioSpin GmbH
Solvent	DMSO
Temperature	300.0
Pulse Sequence	zg30
Experiment	1D
Number of Scans	16
Acquisition Date	2013-11-25T18:14:00
Spectrometer Frequency	400.13
Spectral Width	8223.7
Lowest Frequency	-1638.4
Nucleus	1H
Acquired Size	32768
Spectral Size	65536

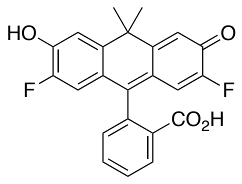


4





Origin	Bruker BioSpin GmbH
Solvent	DMSO
Temperature	300.0
Pulse Sequence	zgpg30
Experiment	1D
Number of Scans	2048
Acquisition Date	2013-11-26T00:04:00
Spectrometer Frequency	100.62
Spectral Width	24038.5
Lowest Frequency	-2005.7
Nucleus	13C
Acquired Size	32768
Spectral Size	65536



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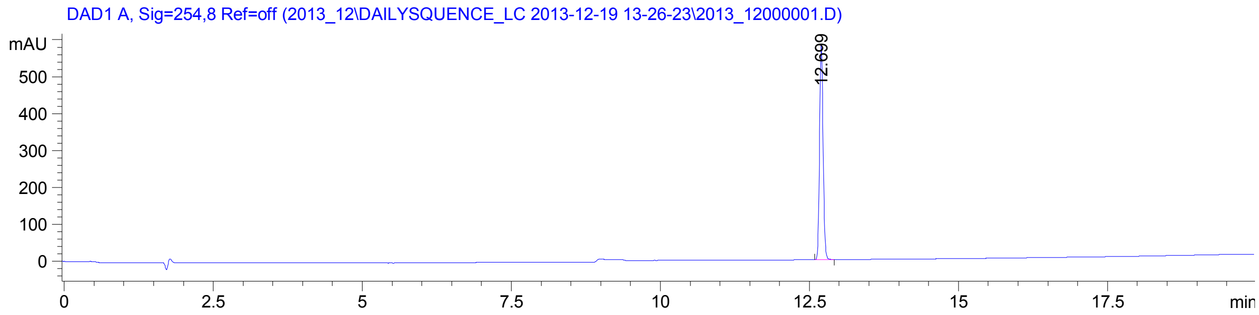
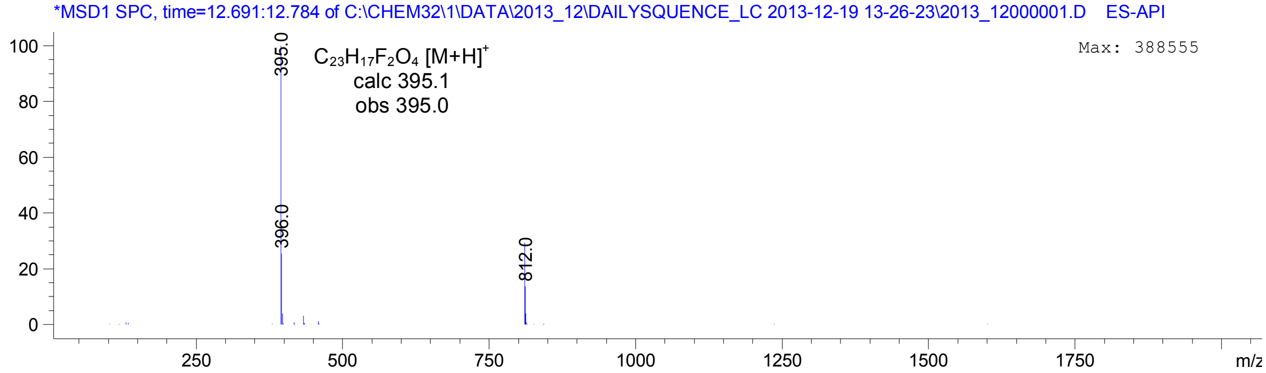

DAD1 A, Sig=254,8 Ref=off (2013_12\DAILYSQUENCE_LC 2013-12-19 13-26-23\2013_12000001.D)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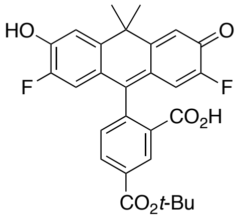
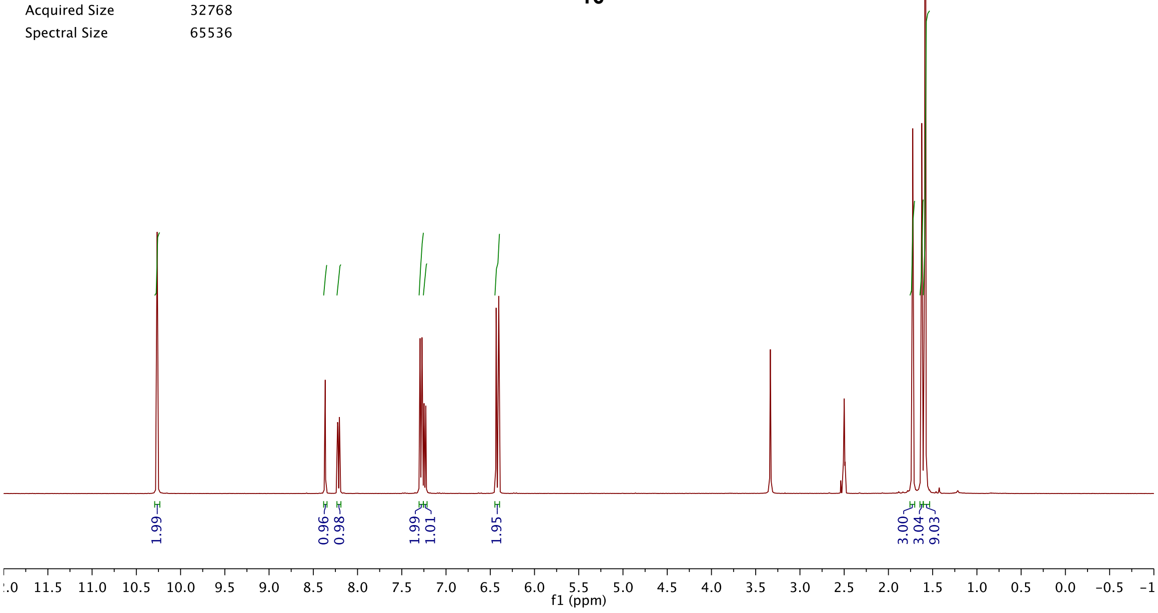
$C_{23}H_{17}F_2O_4$ [M+H]$^+$
calc 395.1
obs 395.0

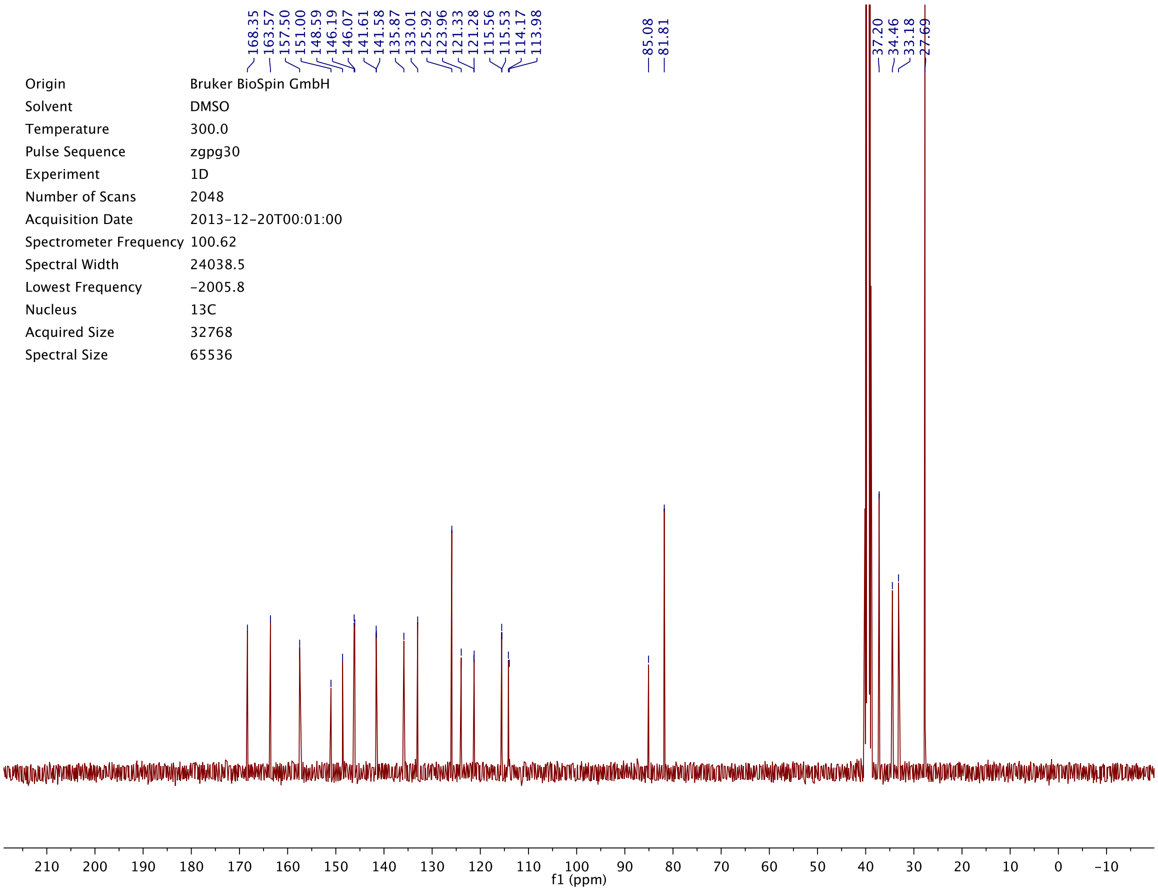
Origin	Bruker BioSpin GmbH
Solvent	DMSO
Temperature	300.0
Pulse Sequence	zg30
Experiment	1D
Number of Scans	16
Acquisition Date	2013-12-19T09:36:00
Spectrometer Frequency	400.13
Spectral Width	8223.7
Lowest Frequency	-1638.6
Nucleus	1H
Acquired Size	32768
Spectral Size	65536

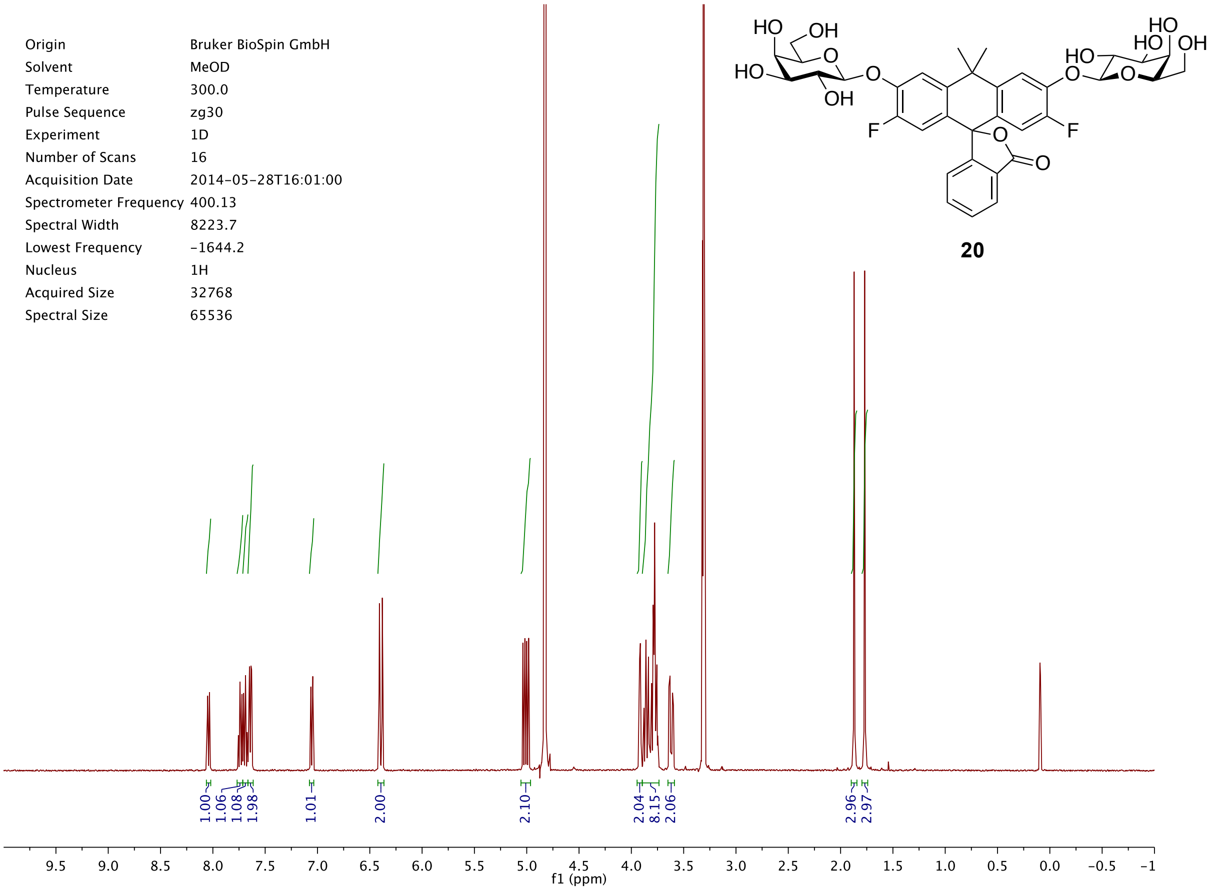


16



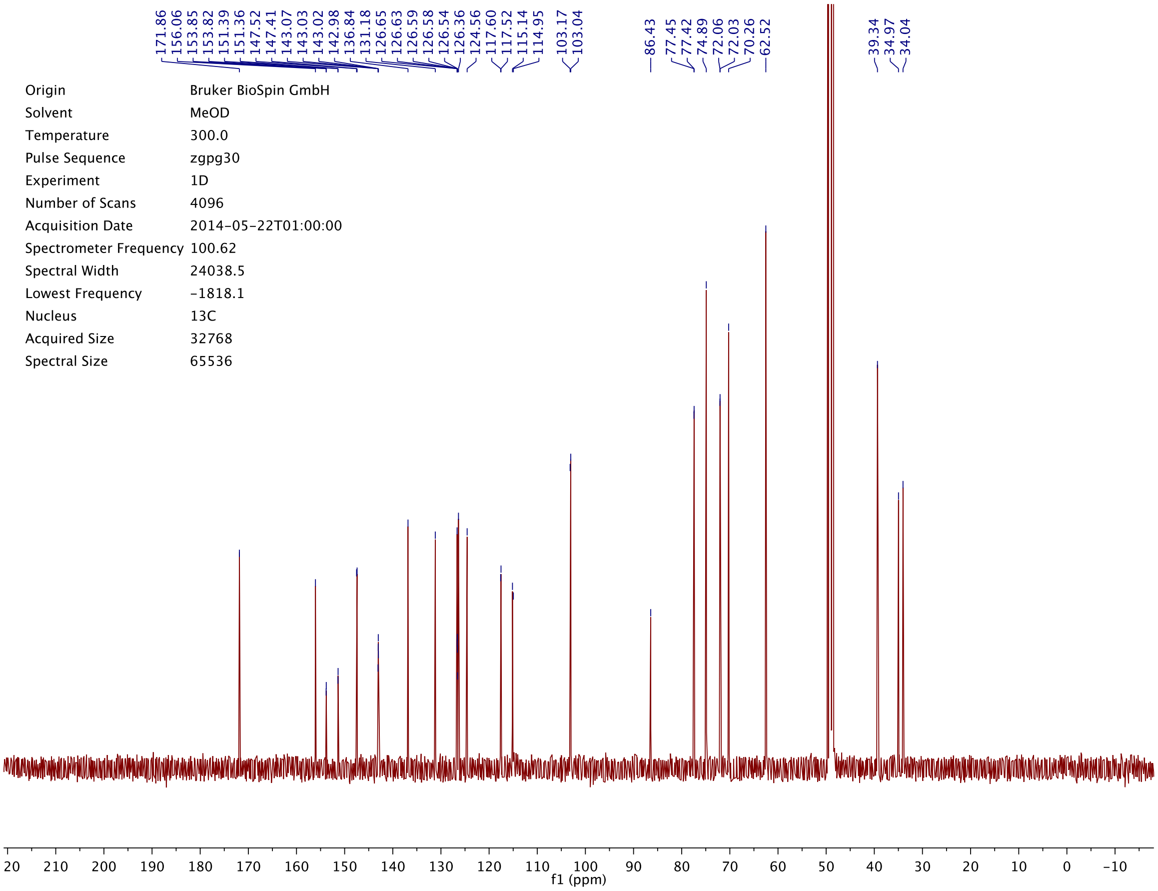
Origin	Bruker BioSpin GmbH
Solvent	DMSO
Temperature	300.0
Pulse Sequence	zgpg30
Experiment	1D
Number of Scans	2048
Acquisition Date	2013-12-20T00:01:00
Spectrometer Frequency	100.62
Spectral Width	24038.5
Lowest Frequency	-2005.8
Nucleus	13C
Acquired Size	32768
Spectral Size	65536



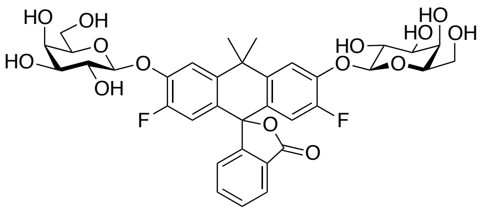


20

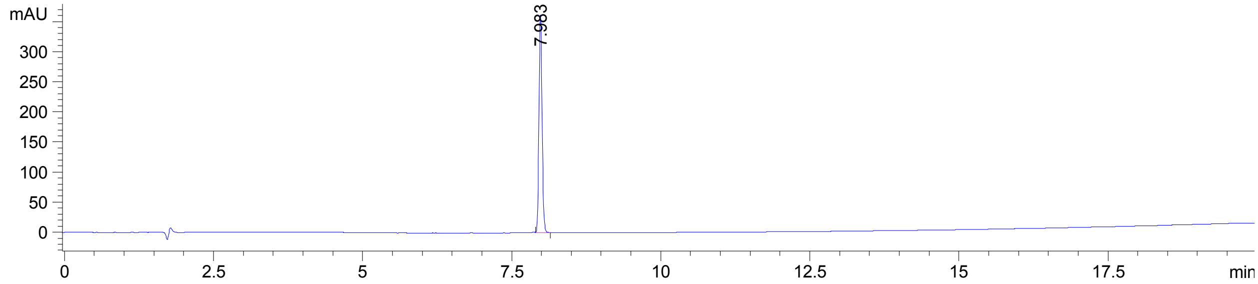
Origin	Bruker BioSpin GmbH
Solvent	MeOD
Temperature	300.0
Pulse Sequence	zg30
Experiment	1D
Number of Scans	16
Acquisition Date	2014-05-28T16:01:00
Spectrometer Frequency	400.13
Spectral Width	8223.7
Lowest Frequency	-1644.2
Nucleus	1H
Acquired Size	32768
Spectral Size	65536



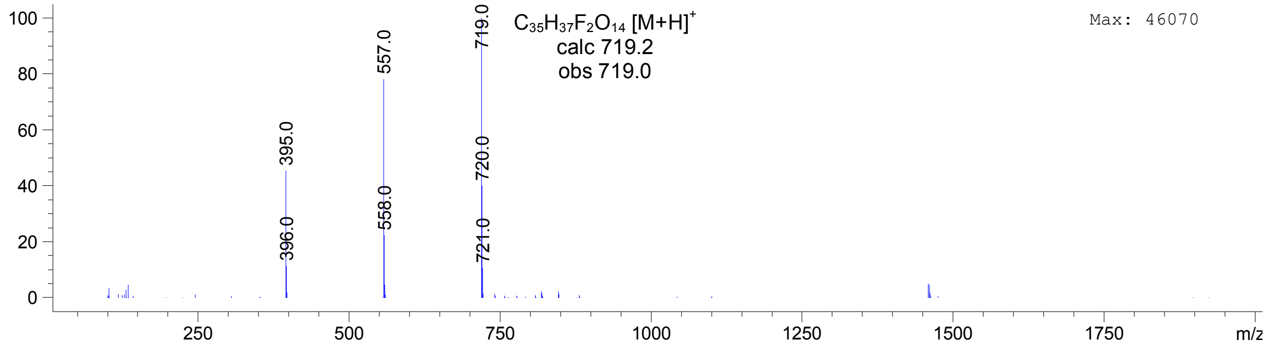
Origin	Bruker BioSpin GmbH
Solvent	MeOD
Temperature	300.0
Pulse Sequence	zgpg30
Experiment	1D
Number of Scans	4096
Acquisition Date	2014-05-22T01:00:00
Spectrometer Frequency	100.62
Spectral Width	24038.5
Lowest Frequency	-1818.1
Nucleus	13C
Acquired Size	32768
Spectral Size	65536



20



DAD1 A, Sig=254,4 Ref=off (2013_12\DAILYSQUENCE_LC 2014-01-24 15-53-14\2013_12000003.D)



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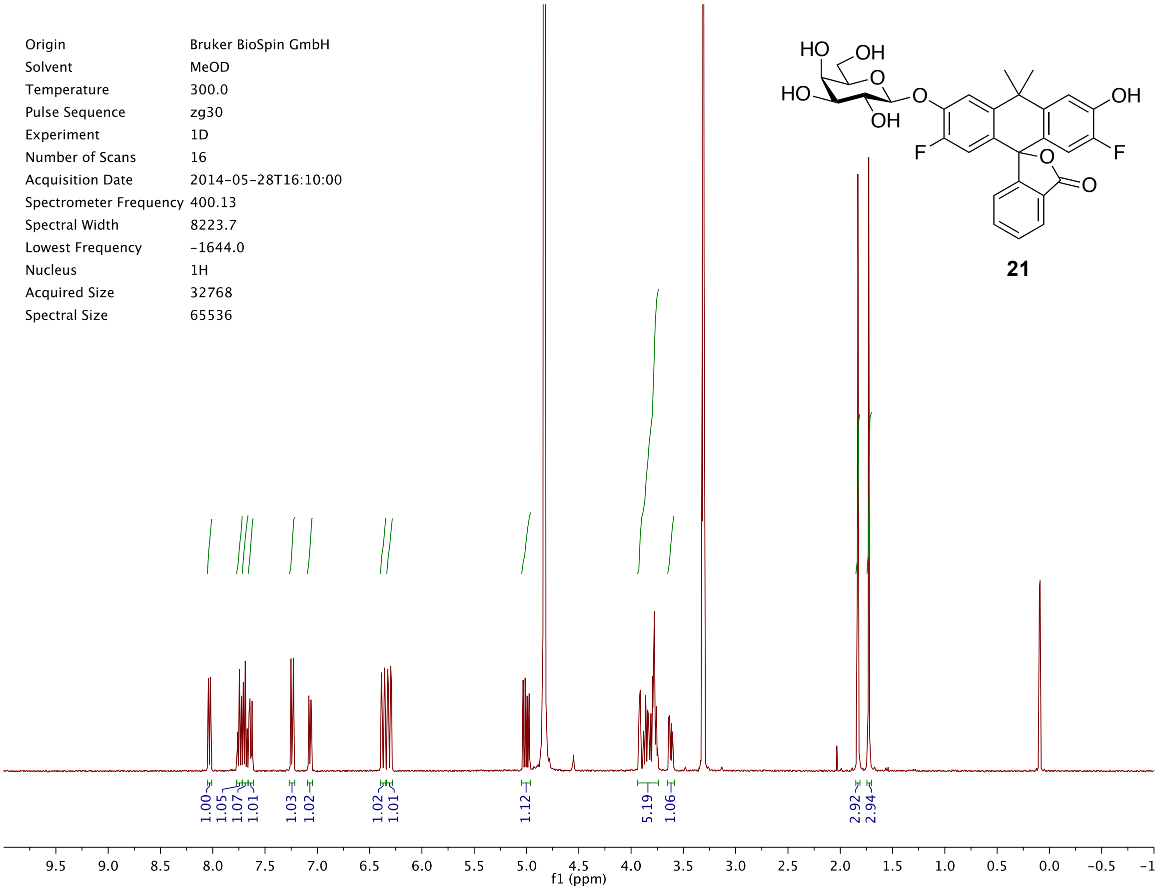
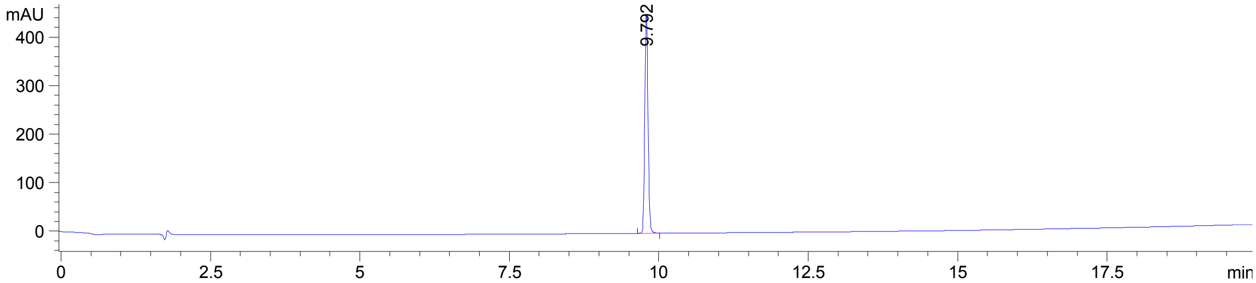
$C_{35}H_{37}F_2O_{14}$ $[M+H]^+$
calc 719.2
obs 719.0



Origin	Bruker BioSpin GmbH
Solvent	MeOD
Temperature	300.0
Pulse Sequence	zg30
Experiment	1D
Number of Scans	16
Acquisition Date	2014-05-28T16:10:00
Spectrometer Frequency	400.13
Spectral Width	8223.7
Lowest Frequency	-1644.0
Nucleus	1H
Acquired Size	32768
Spectral Size	65536

21

DAD1 A, Sig=254,8 Ref=off (2013_12\DAILYSQUENCE_LC 2014-01-17 16-09-24\2013_12000001.D)



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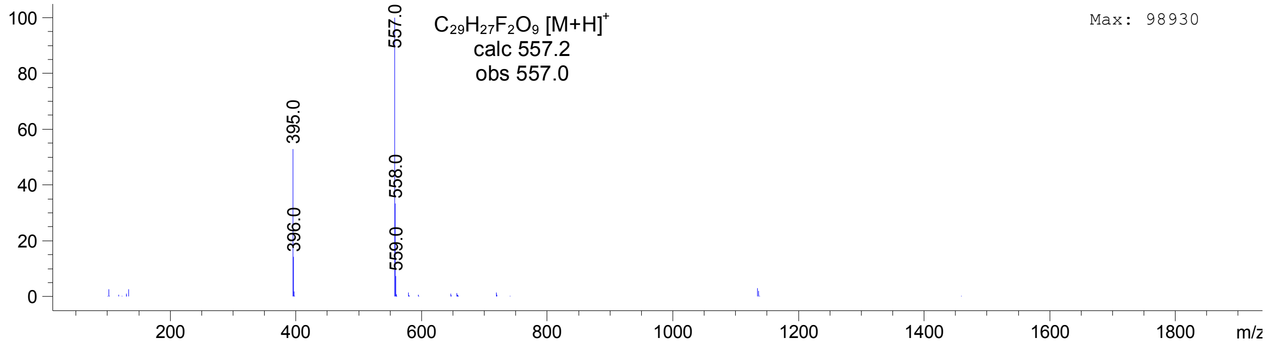
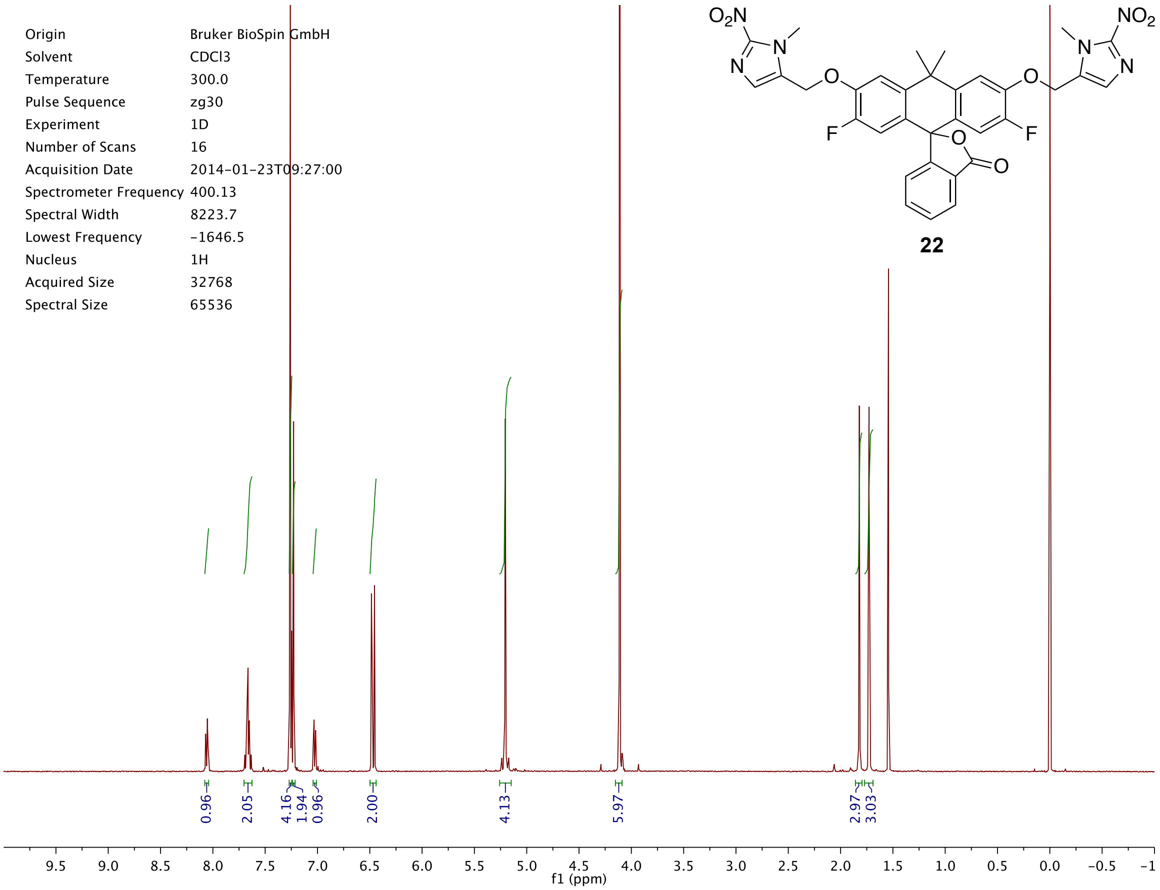
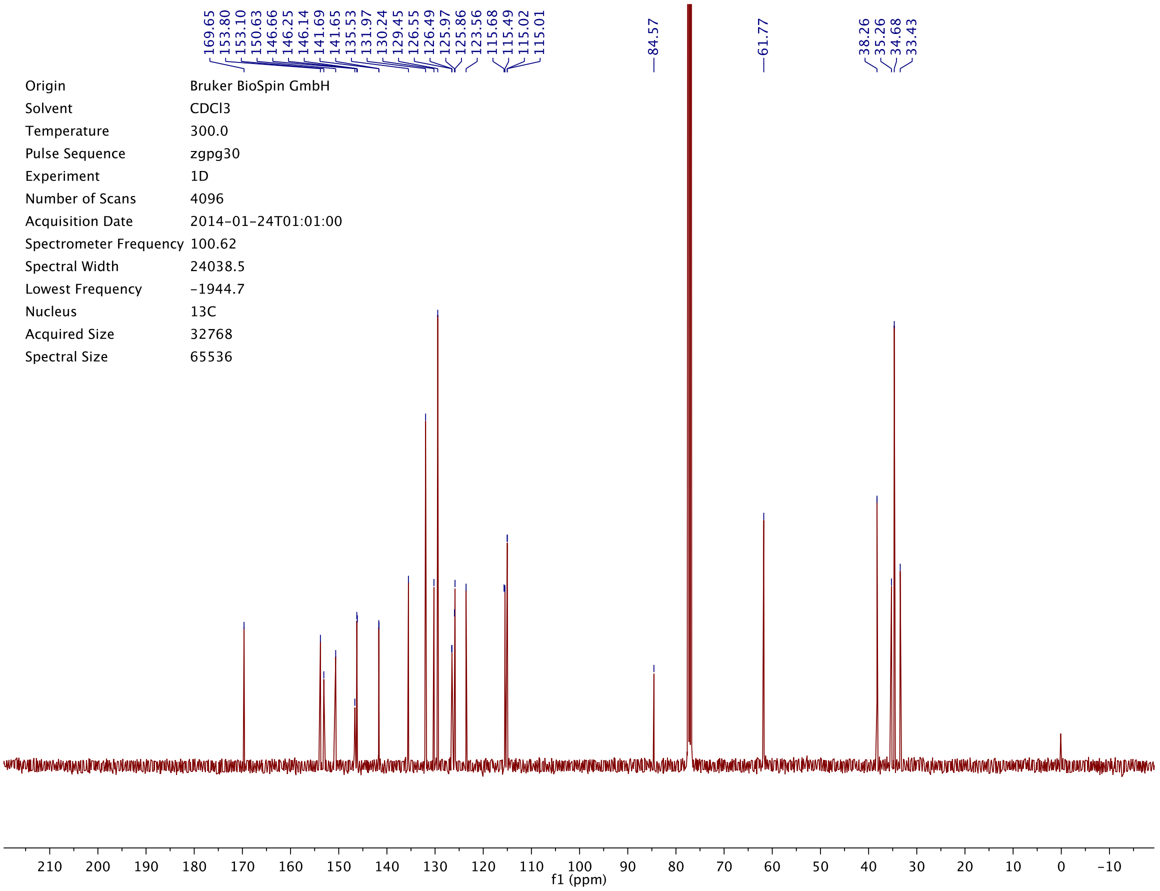


$C_{29}H_{27}F_2O_9$ $[M+H]^+$
calc 557.2
obs 557.0

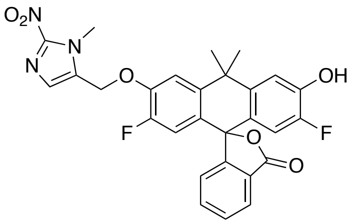


22

Origin	Bruker BioSpin GmbH
Solvent	CDCl3
Temperature	300.0
Pulse Sequence	zg30
Experiment	1D
Number of Scans	16
Acquisition Date	2014-01-23T09:27:00
Spectrometer Frequency	400.13
Spectral Width	8223.7
Lowest Frequency	−1646.5
Nucleus	1H
Acquired Size	32768
Spectral Size	65536

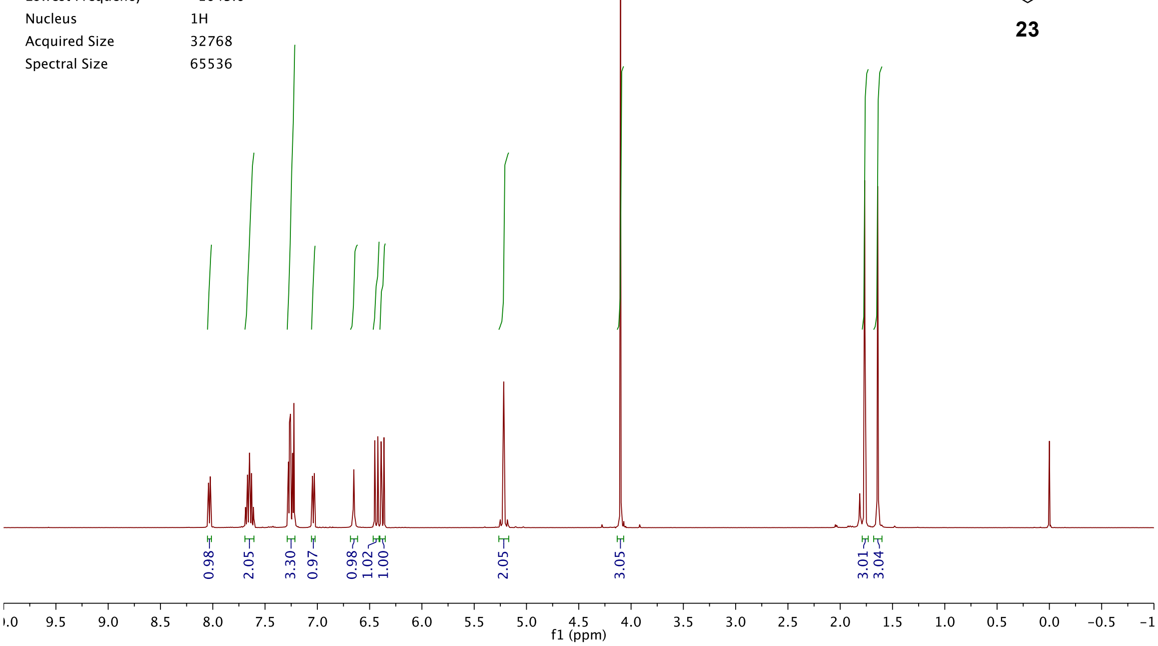


Origin	Bruker BioSpin GmbH
Solvent	CDCl3
Temperature	300.0
Pulse Sequence	zgpg30
Experiment	1D
Number of Scans	4096
Acquisition Date	2014-01-24T01:01:00
Spectrometer Frequency	100.62
Spectral Width	24038.5
Lowest Frequency	−1944.7
Nucleus	13C
Acquired Size	32768
Spectral Size	65536

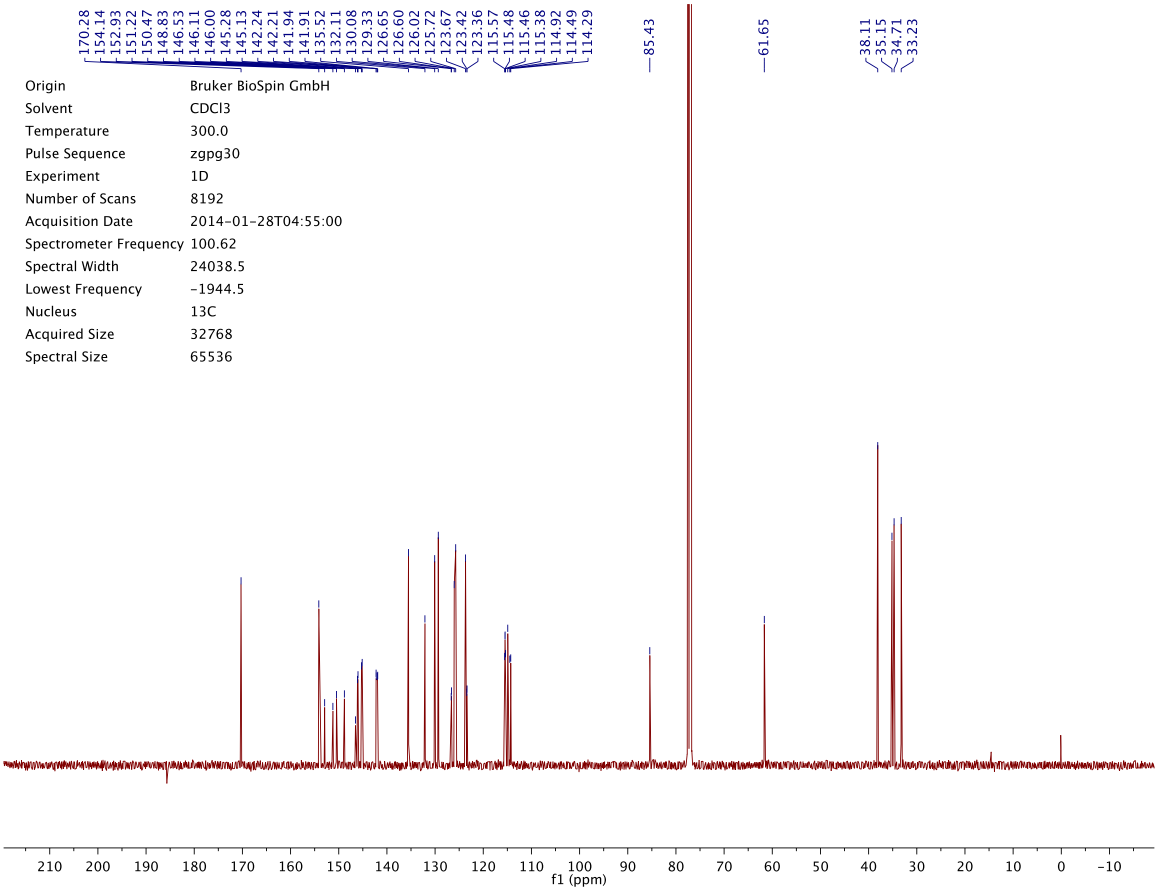


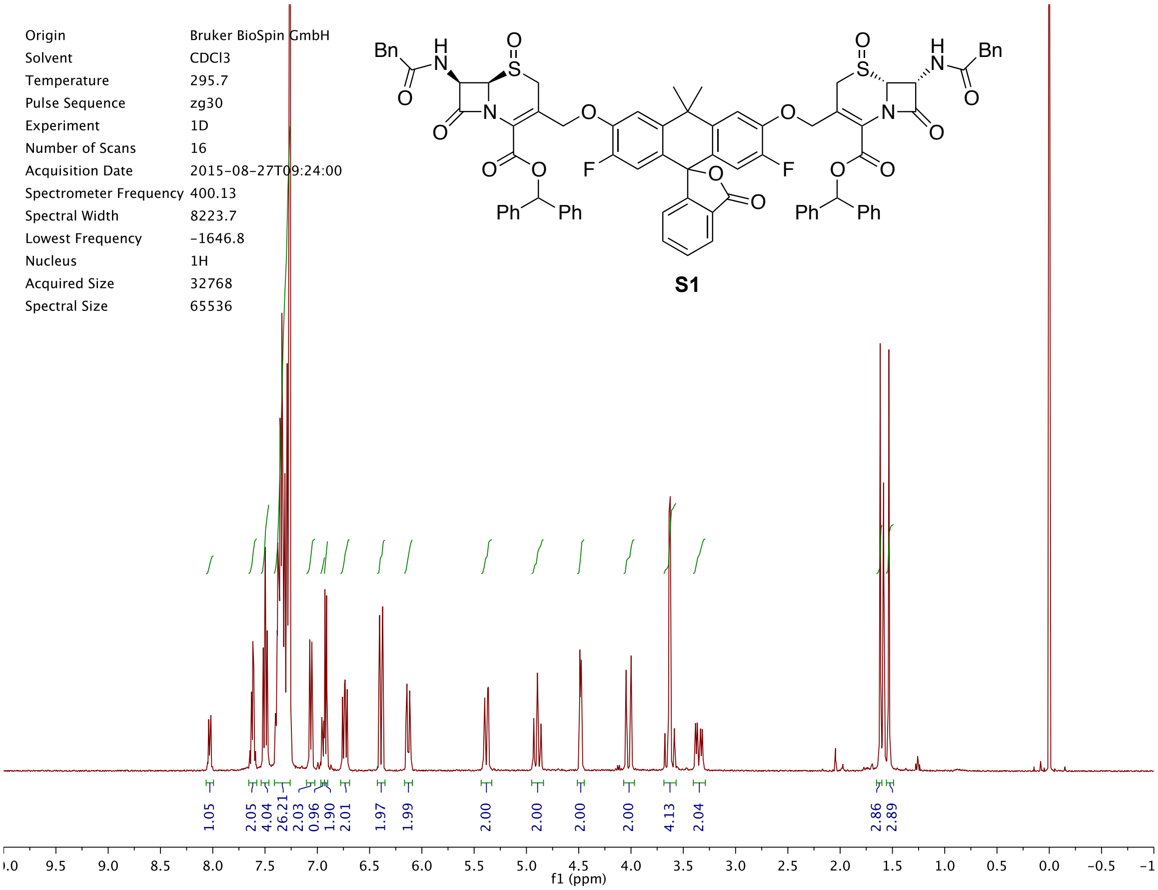
23

Origin	Bruker BioSpin GmbH
Solvent	CDCl3
Temperature	300.0
Pulse Sequence	zg30
Experiment	1D
Number of Scans	16
Acquisition Date	2014-01-28T09:31:00
Spectrometer Frequency	400.13
Spectral Width	8223.7
Lowest Frequency	-1643.6
Nucleus	1H
Acquired Size	32768
Spectral Size	65536



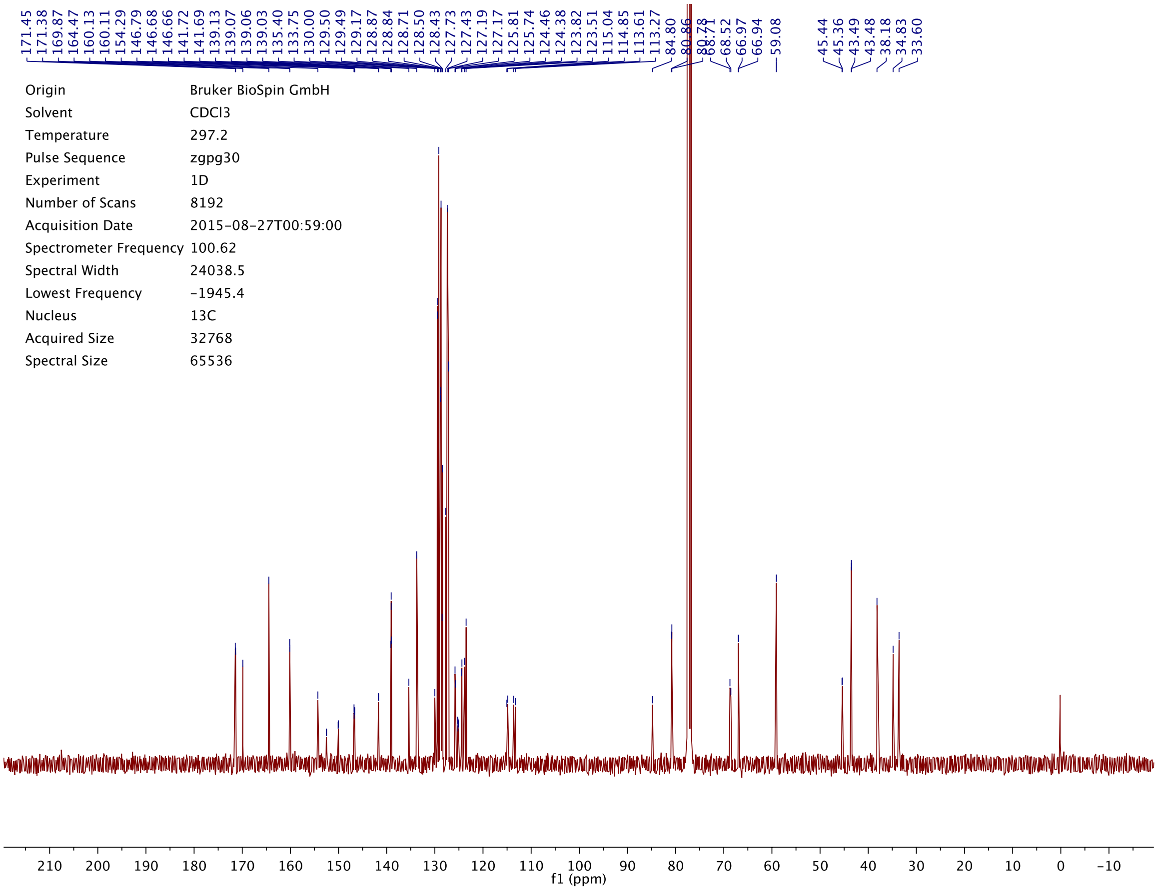
Origin	Bruker BioSpin GmbH
Solvent	CDCl3
Temperature	300.0
Pulse Sequence	zgpg30
Experiment	1D
Number of Scans	8192
Acquisition Date	2014-01-28T04:55:00
Spectrometer Frequency	100.62
Spectral Width	24038.5
Lowest Frequency	-1944.5
Nucleus	13C
Acquired Size	32768
Spectral Size	65536



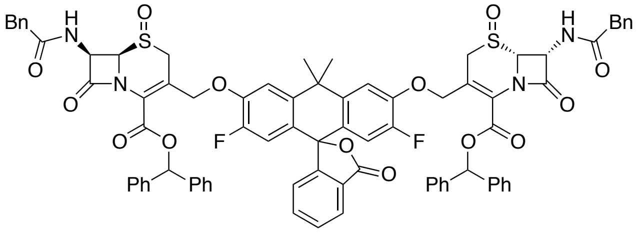


S1

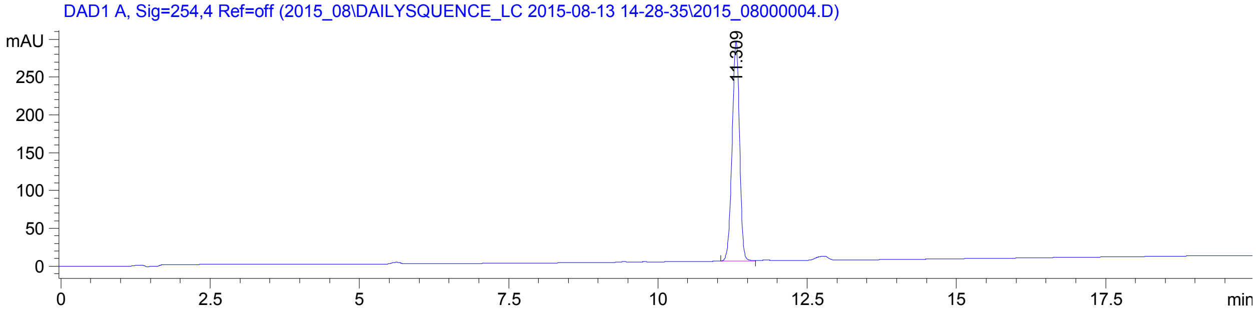
Origin	Bruker BioSpin GmbH
Solvent	CDCl3
Temperature	295.7
Pulse Sequence	zg30
Experiment	1D
Number of Scans	16
Acquisition Date	2015-08-27T09:24:00
Spectrometer Frequency	400.13
Spectral Width	8223.7
Lowest Frequency	-1646.8
Nucleus	1H
Acquired Size	32768
Spectral Size	65536

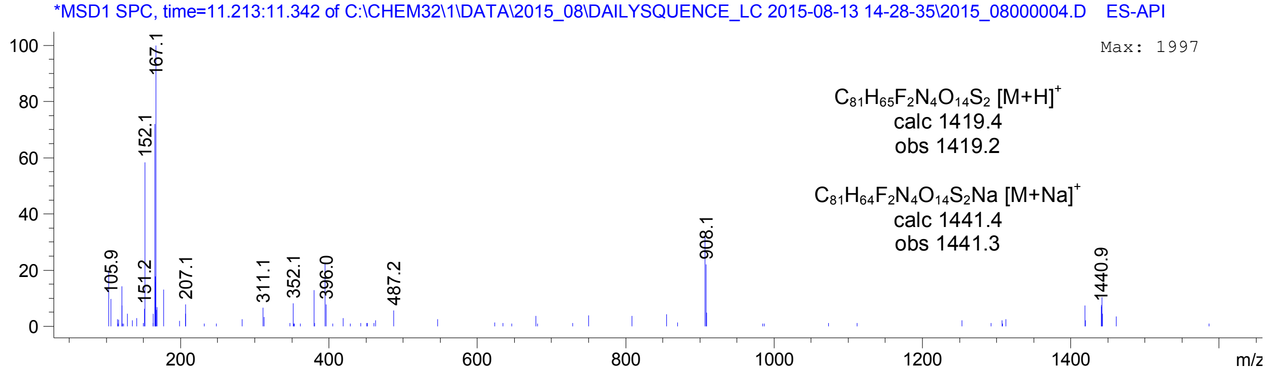


Origin	Bruker BioSpin GmbH
Solvent	CDCl3
Temperature	297.2
Pulse Sequence	zgpg30
Experiment	1D
Number of Scans	8192
Acquisition Date	2015-08-27T00:59:00
Spectrometer Frequency	100.62
Spectral Width	24038.5
Lowest Frequency	-1945.4
Nucleus	13C
Acquired Size	32768
Spectral Size	65536



S1

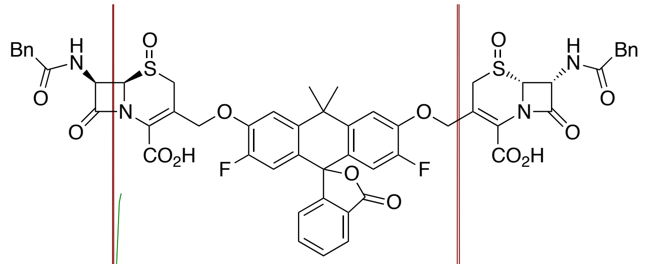




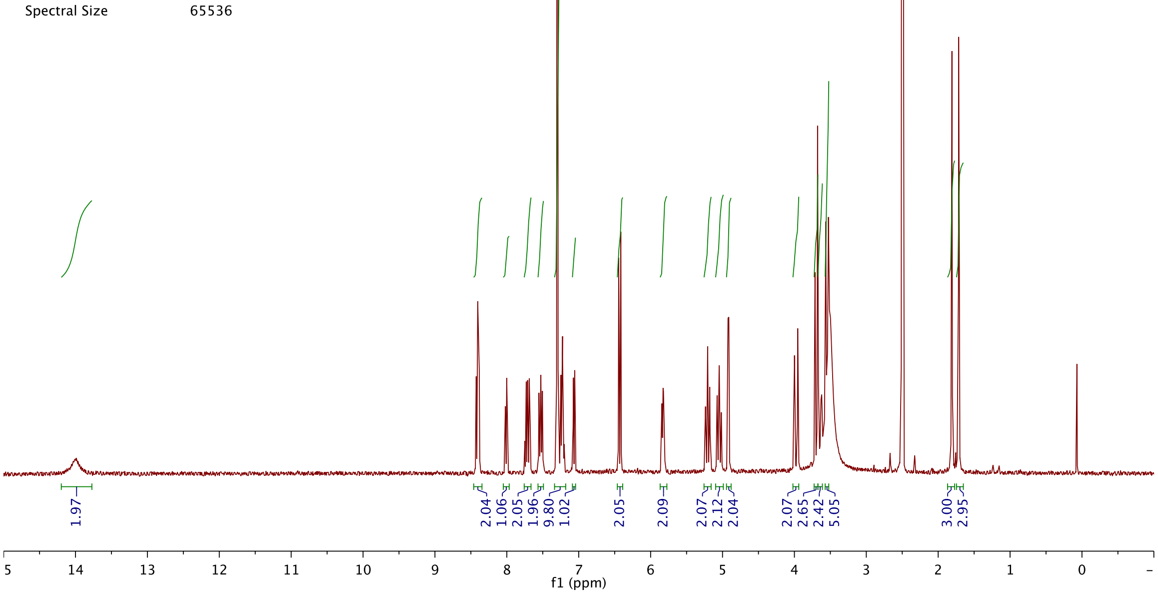
$C_{81}H_{65}F_2N_4O_{14}S_2$ $[M+H]^+$
calc 1419.4
obs 1419.2

$C_{81}H_{64}F_2N_4O_{14}S_2Na$ $[M+Na]^+$
calc 1441.4
obs 1441.3

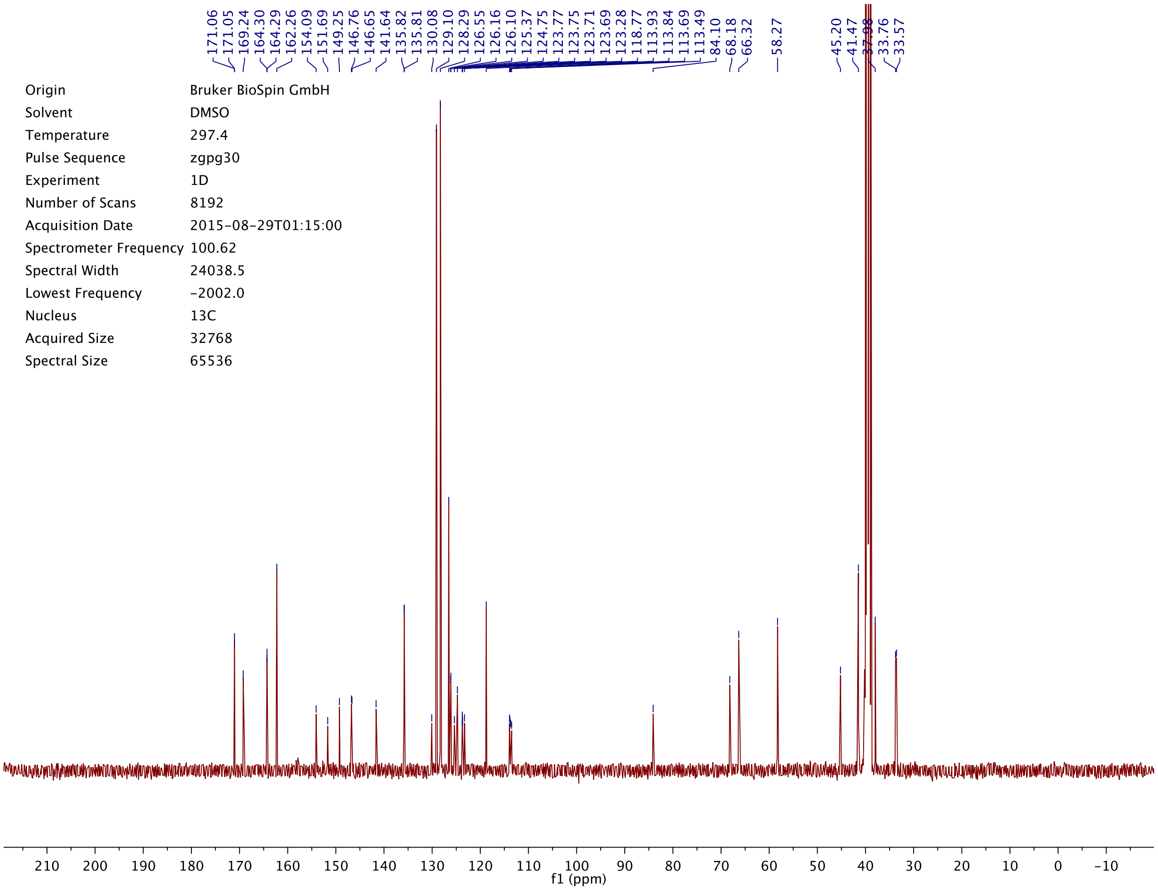
Origin	Bruker BioSpin GmbH
Solvent	DMSO
Temperature	300.0
Pulse Sequence	zg30
Experiment	1D
Number of Scans	16
Acquisition Date	2015-04-06T09:57:00
Spectrometer Frequency	400.13
Spectral Width	8223.7
Lowest Frequency	-1638.6
Nucleus	1H
Acquired Size	32768
Spectral Size	65536

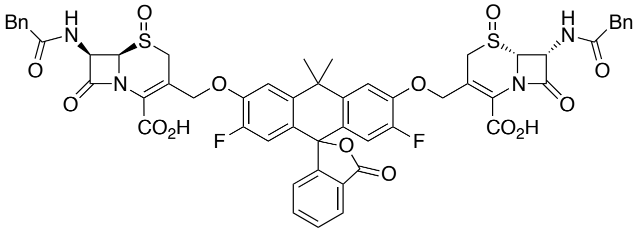


24

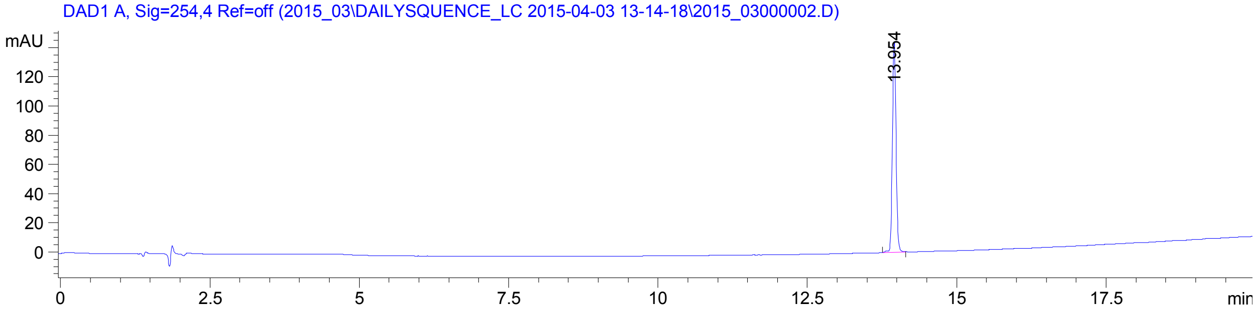


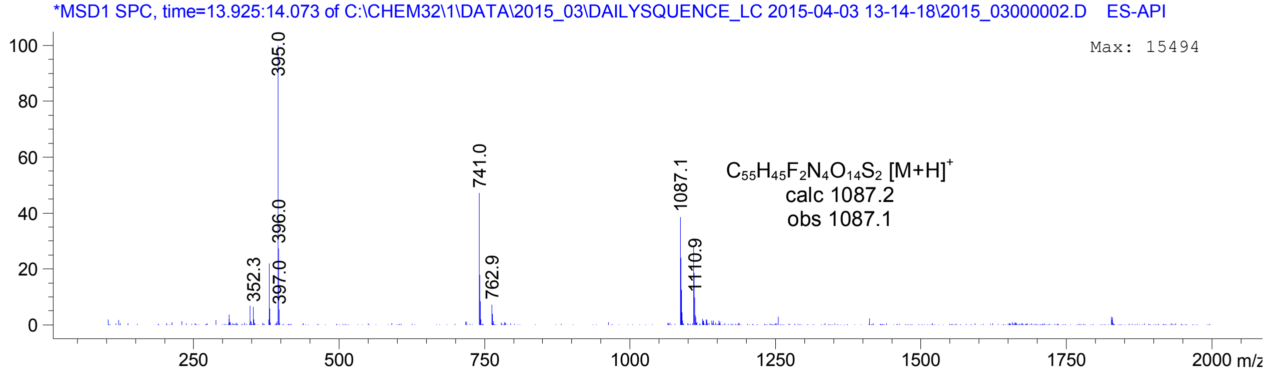
Origin	Bruker BioSpin GmbH
Solvent	DMSO
Temperature	297.4
Pulse Sequence	zgpg30
Experiment	1D
Number of Scans	8192
Acquisition Date	2015-08-29T01:15:00
Spectrometer Frequency	100.62
Spectral Width	24038.5
Lowest Frequency	-2002.0
Nucleus	13C
Acquired Size	32768
Spectral Size	65536



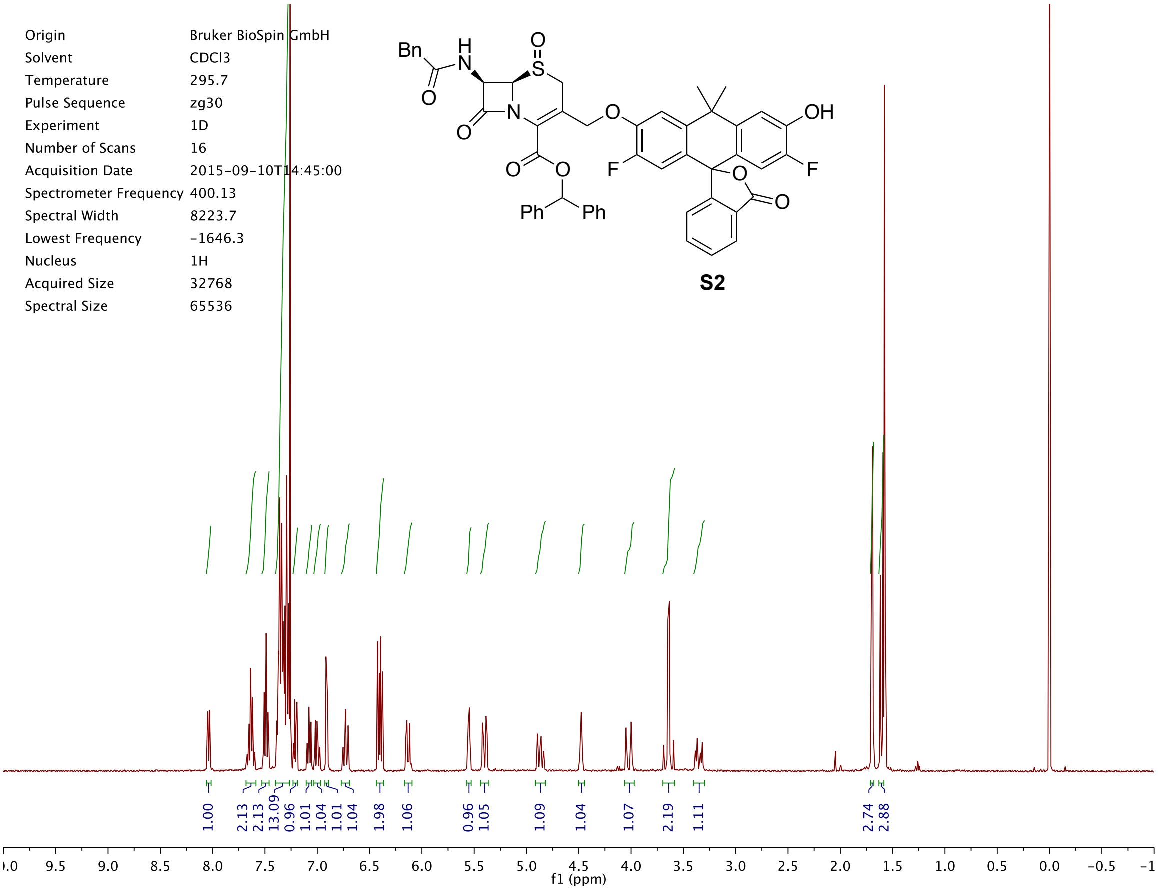


24



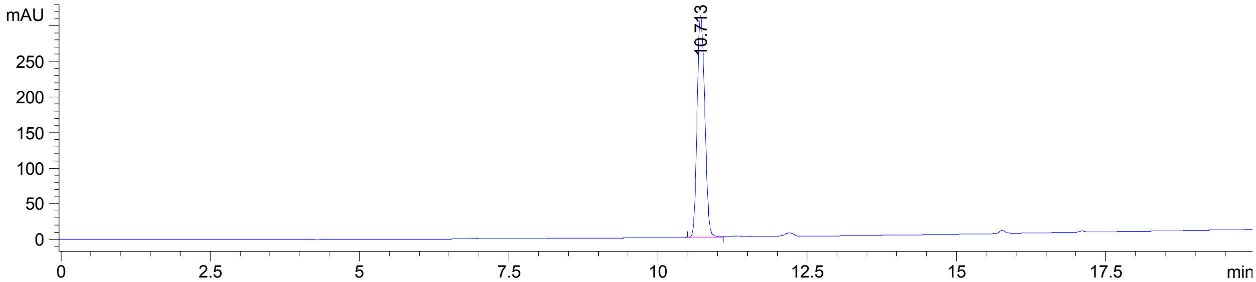


$C_{55}H_{45}F_2N_4O_{14}S_2$ $[M+H]^+$
calc 1087.2
obs 1087.1

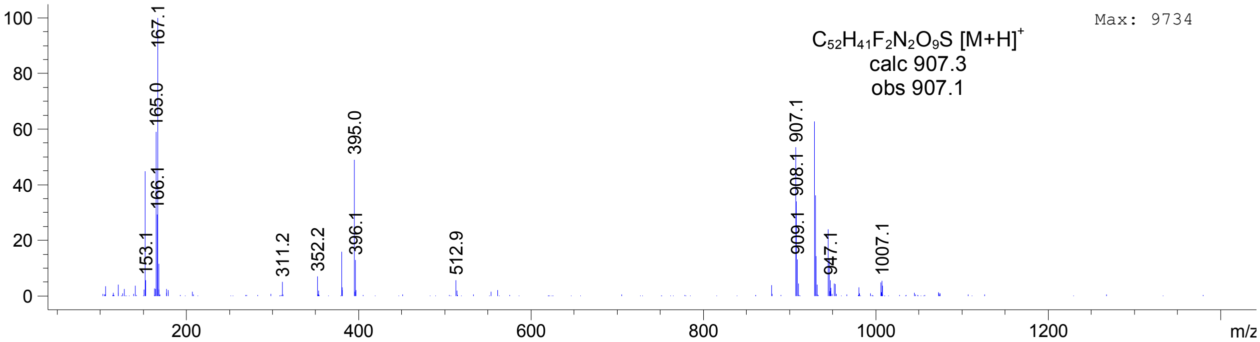


Origin	Bruker BioSpin GmbH
Solvent	CDCl3
Temperature	295.7
Pulse Sequence	zg30
Experiment	1D
Number of Scans	16
Acquisition Date	2015-09-10T14:45:00
Spectrometer Frequency	400.13
Spectral Width	8223.7
Lowest Frequency	-1646.3
Nucleus	1H
Acquired Size	32768
Spectral Size	65536

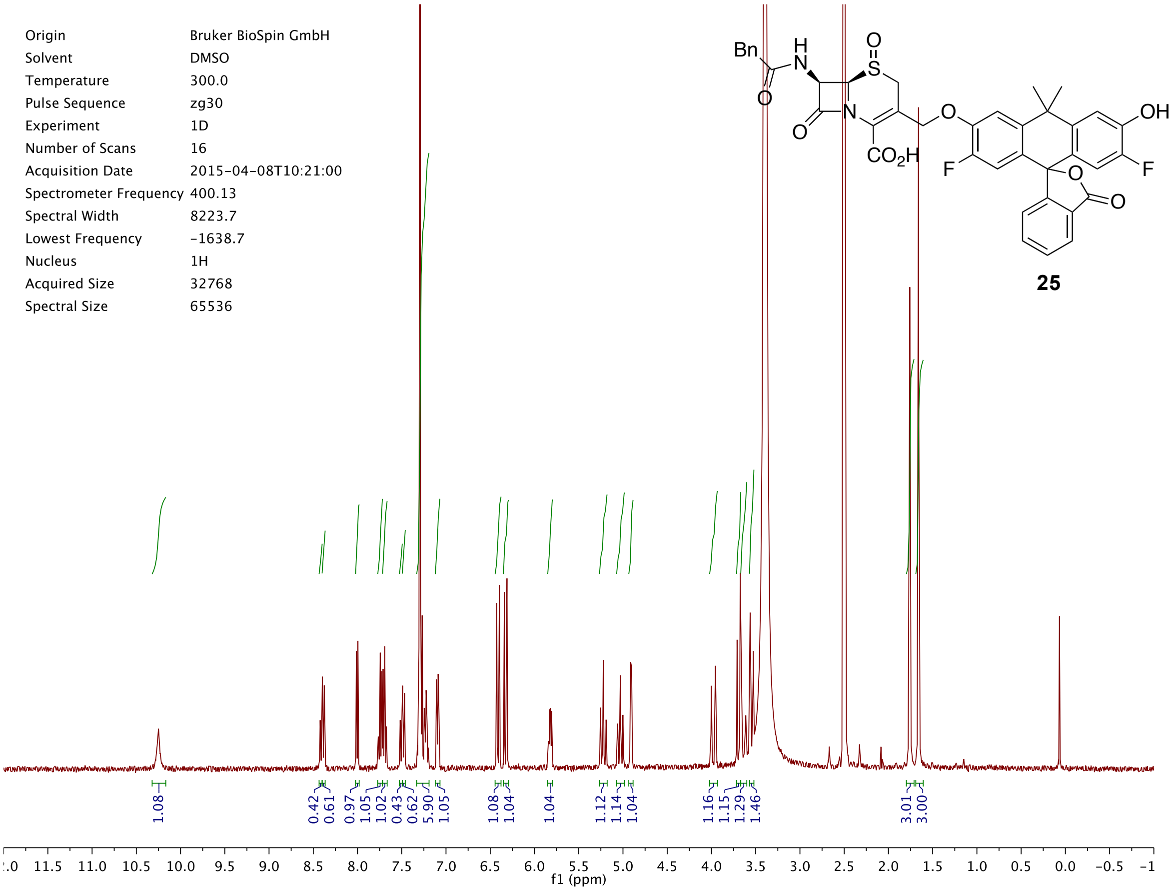
DAD1 A, Sig=254,4 Ref=off (2015_09\DAILYSQUENCE_LC 2015-09-09 16-32-01\2015_09000003.D)



*MSD1 SPC, time=10.637:10.840 of C:\CHEM32\1\DATA\2015_09\DAILYSQUENCE_LC 2015-09-09 16-32-01\2015_09000003.D ES-API



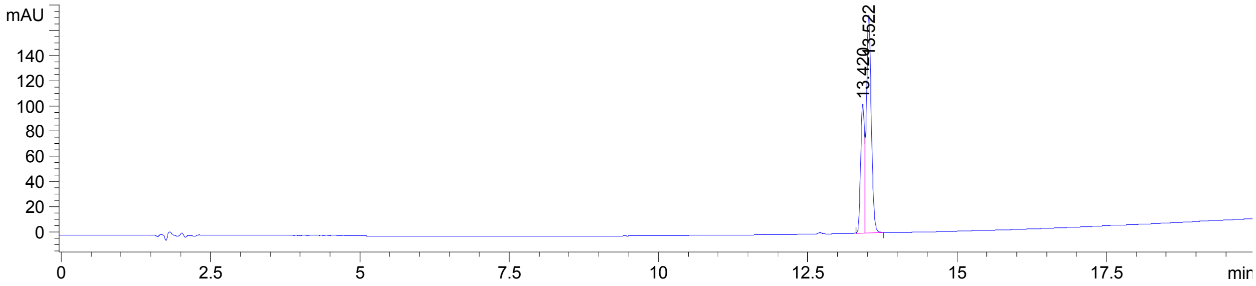
$C_{52}H_{41}F_2N_2O_9S$ $[M+H]^+$
calc 907.3
obs 907.1



Origin	Bruker BioSpin GmbH
Solvent	DMSO
Temperature	300.0
Pulse Sequence	zg30
Experiment	1D
Number of Scans	16
Acquisition Date	2015-04-08T10:21:00
Spectrometer Frequency	400.13
Spectral Width	8223.7
Lowest Frequency	-1638.7
Nucleus	1H
Acquired Size	32768
Spectral Size	65536

25

DAD1 A, Sig=254,4 Ref=off (2015_08\DAILYSQUENCE_LC 2015-08-13 14-28-35\2015_08000002.D)



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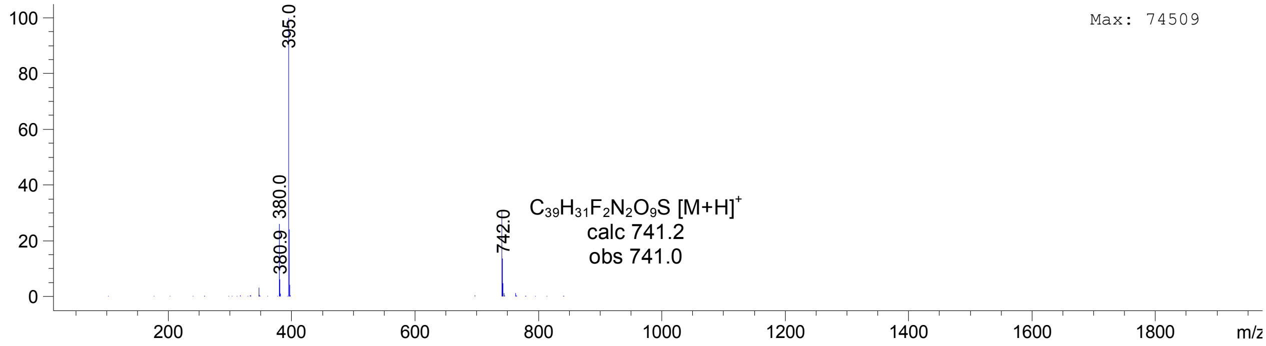
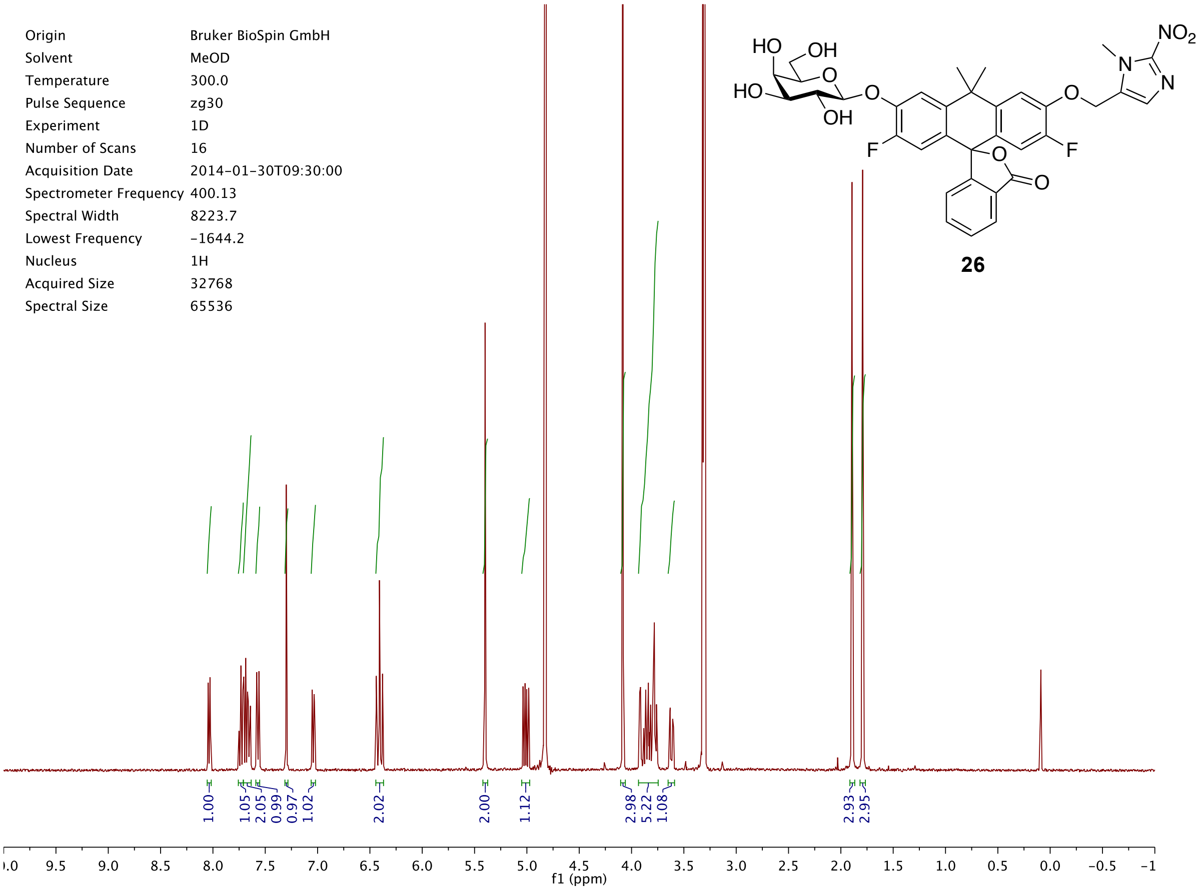


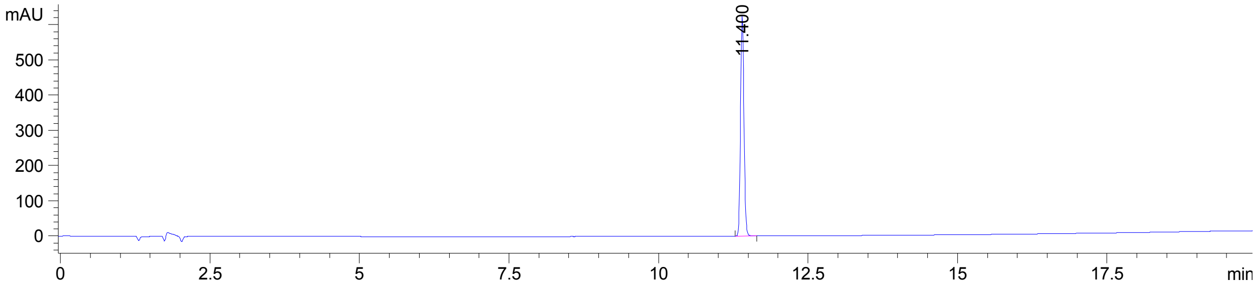
$C_{39}H_{31}F_2N_2O_9S$ $[M+H]^+$
calc 741.2
obs 741.0

Origin	Bruker BioSpin GmbH
Solvent	MeOD
Temperature	300.0
Pulse Sequence	zg30
Experiment	1D
Number of Scans	16
Acquisition Date	2014-01-30T09:30:00
Spectrometer Frequency	400.13
Spectral Width	8223.7
Lowest Frequency	-1644.2
Nucleus	1H
Acquired Size	32768
Spectral Size	65536



26

DAD1 A, Sig=254,8 Ref=off (2013_12\DAILYSQUENCE_LC 2014-01-29 15-26-16\2013_12000004.D)



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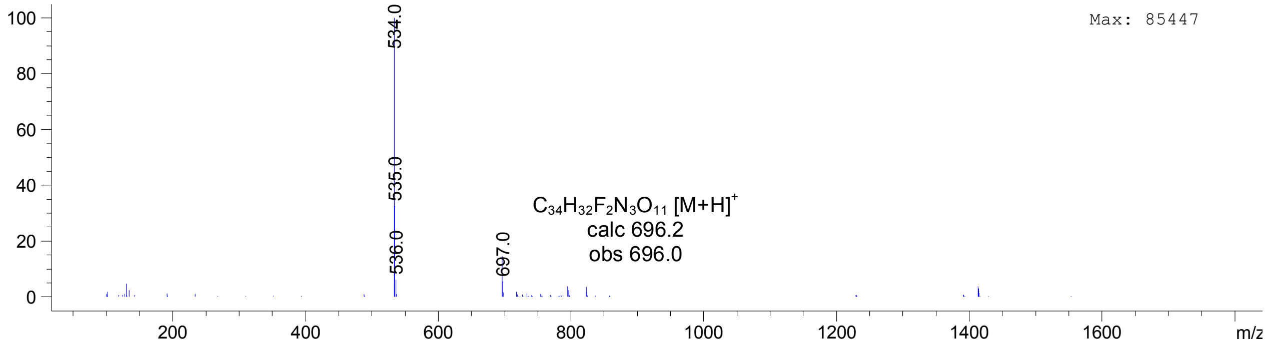
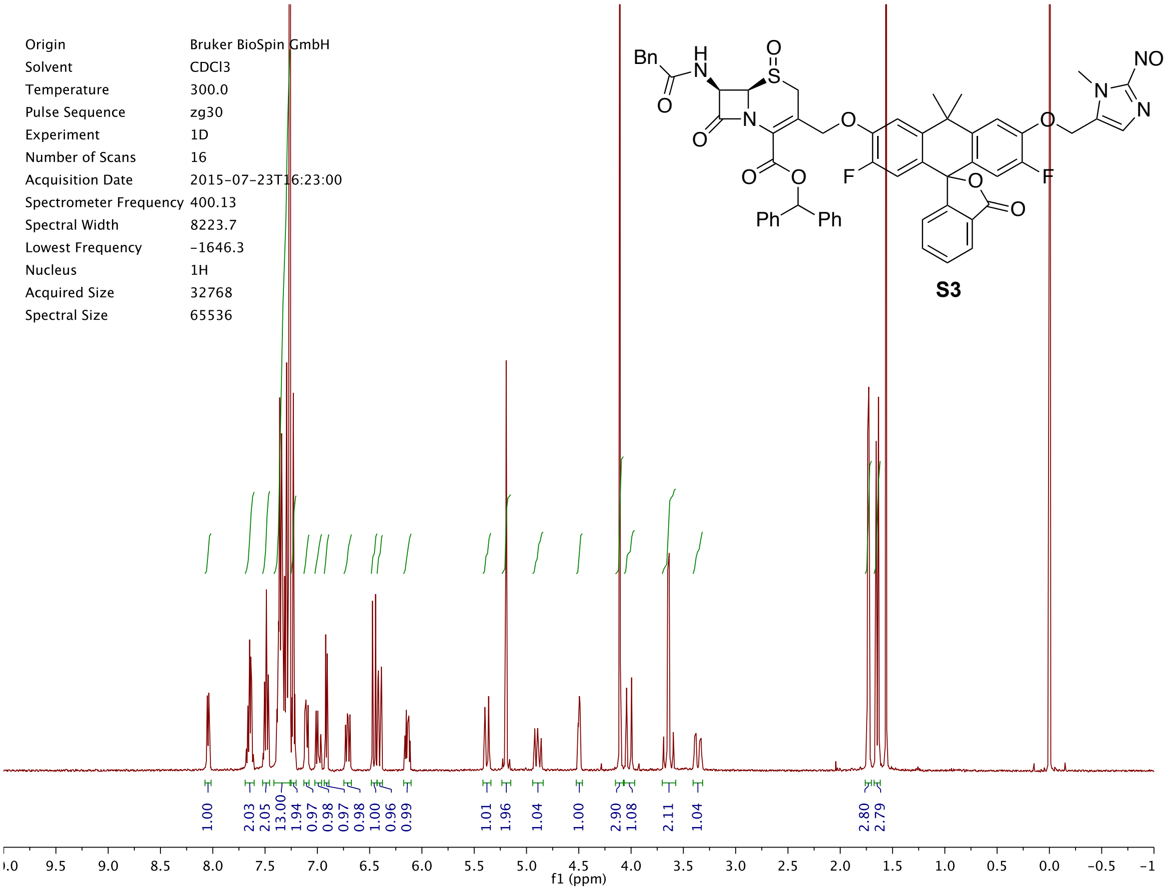


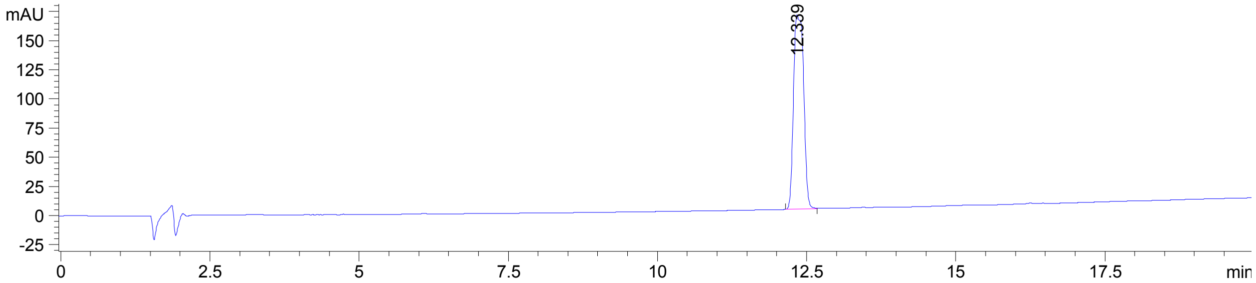
$C_{34}H_{32}F_2N_3O_{11}$ $[M+H]^+$
calc 696.2
obs 696.0



Origin	Bruker BioSpin GmbH
Solvent	CDCl3
Temperature	300.0
Pulse Sequence	zg30
Experiment	1D
Number of Scans	16
Acquisition Date	2015-07-23T16:23:00
Spectrometer Frequency	400.13
Spectral Width	8223.7
Lowest Frequency	-1646.3
Nucleus	1H
Acquired Size	32768
Spectral Size	65536

S3

DAD1 A, Sig=254,4 Ref=off (2015_07\DAILYSQUENCE_LC 2015-07-22 15-46-20\2015_07000002.D)



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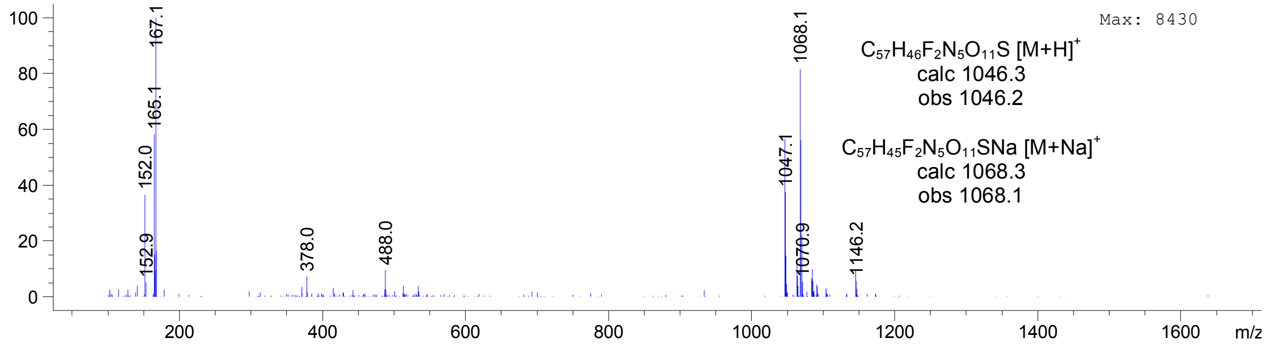
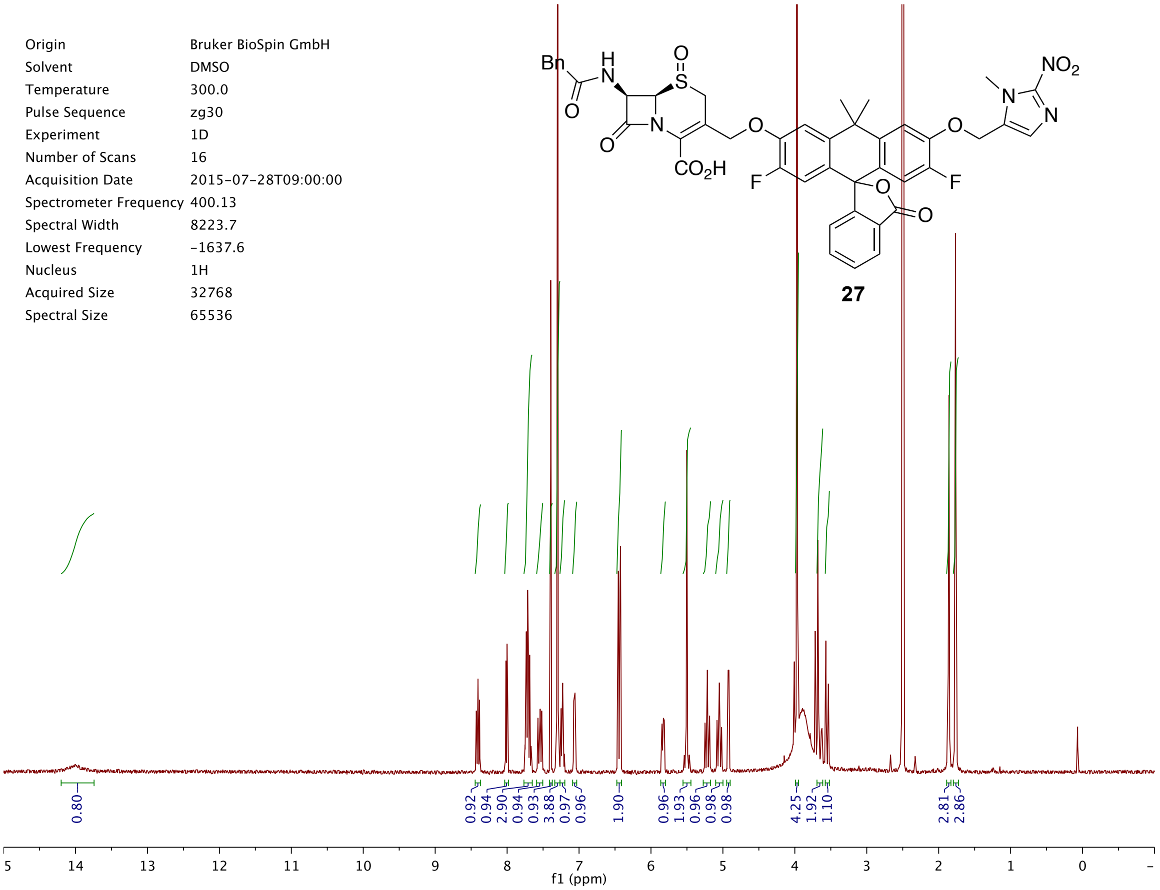


$C_{57}H_{46}F_2N_5O_{11}S$ [M+H]$^+$
calc 1046.3
obs 1046.2

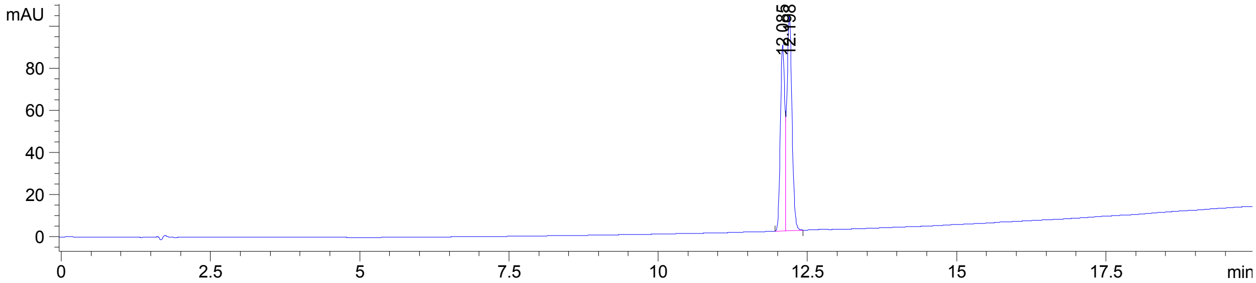
$C_{57}H_{45}F_2N_5O_{11}SNa$ [M+Na]$^+$
calc 1068.3
obs 1068.1



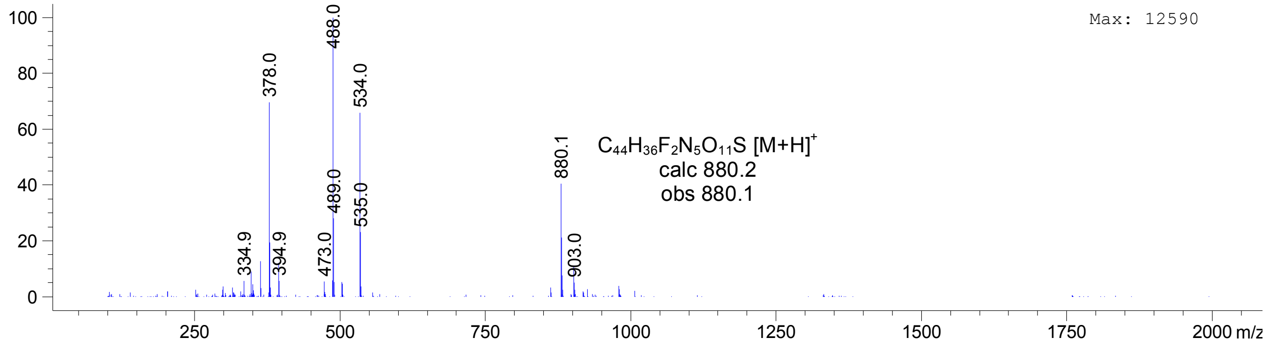
Origin	Bruker BioSpin GmbH
Solvent	DMSO
Temperature	300.0
Pulse Sequence	zg30
Experiment	1D
Number of Scans	16
Acquisition Date	2015-07-28T09:00:00
Spectrometer Frequency	400.13
Spectral Width	8223.7
Lowest Frequency	-1637.6
Nucleus	1H
Acquired Size	32768
Spectral Size	65536

27

DAD1 A, Sig=254,4 Ref=off (2015_07\DAILYSQUENCE_LC 2015-07-24 13-26-16\2015_07000011.D)

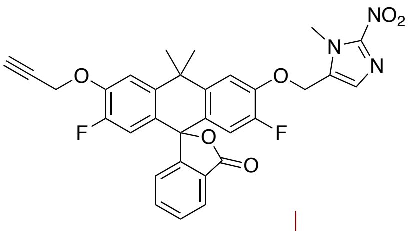


*MSD1 SPC, time=12.022:12.262 of C:\CHEM32\1\DATA\2015_07\DAILYSQUENCE_LC 2015-07-24 13-26-16\2015_07000011.D ES-API

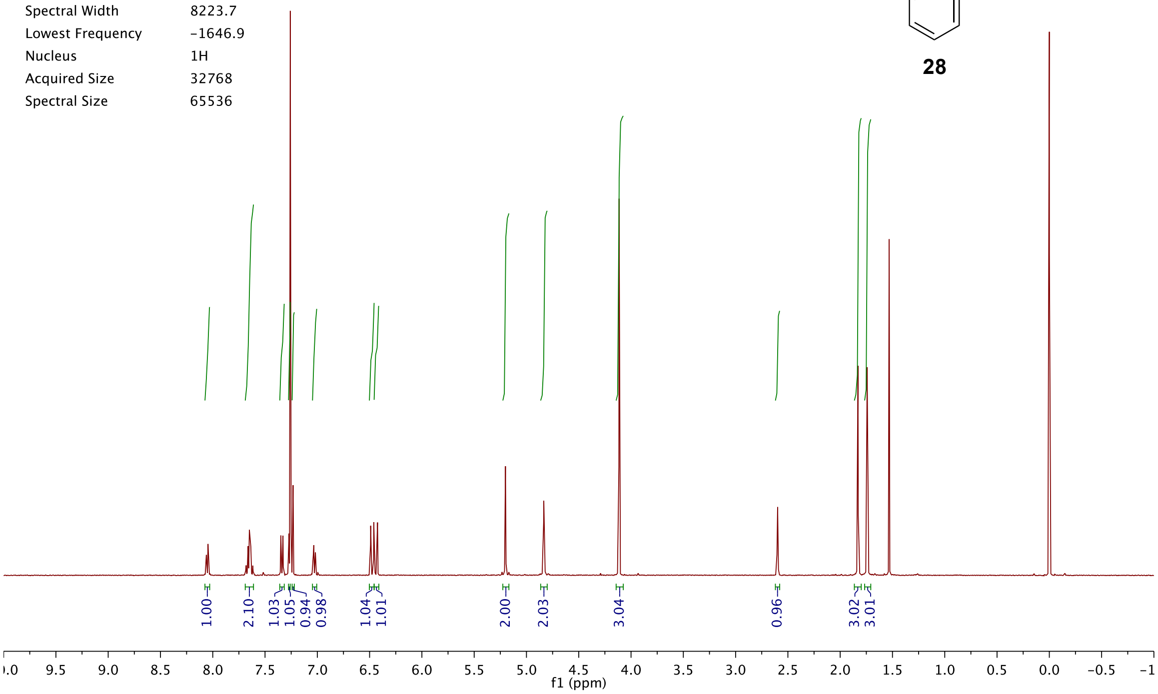


$C_{44}H_{36}F_2N_5O_{11}S$ $[M+H]^+$
calc 880.2
obs 880.1

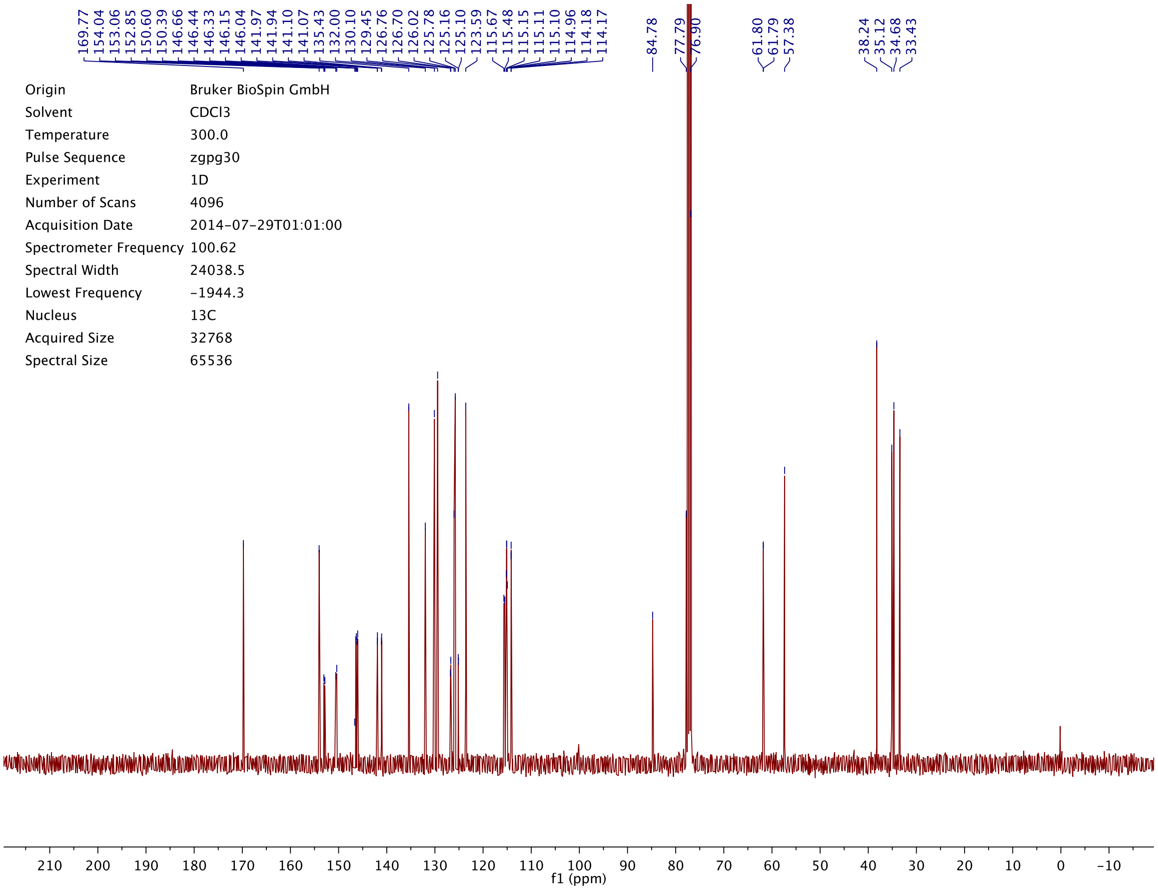
Origin	Bruker BioSpin GmbH
Solvent	CDCl3
Temperature	300.0
Pulse Sequence	zg30
Experiment	1D
Number of Scans	16
Acquisition Date	2014-07-28T09:13:00
Spectrometer Frequency	400.13
Spectral Width	8223.7
Lowest Frequency	-1646.9
Nucleus	1H
Acquired Size	32768
Spectral Size	65536



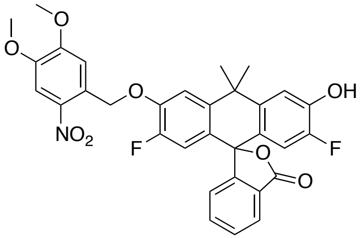
28



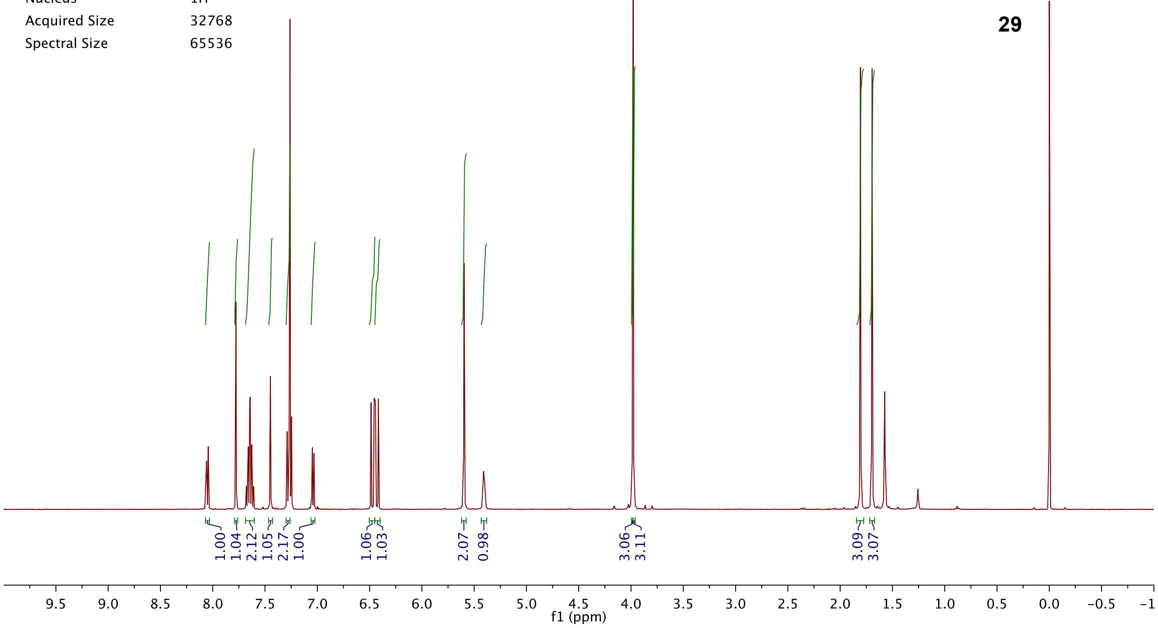
Origin	Bruker BioSpin GmbH
Solvent	CDCl3
Temperature	300.0
Pulse Sequence	zgpg30
Experiment	1D
Number of Scans	4096
Acquisition Date	2014-07-29T01:01:00
Spectrometer Frequency	100.62
Spectral Width	24038.5
Lowest Frequency	-1944.3
Nucleus	13C
Acquired Size	32768
Spectral Size	65536



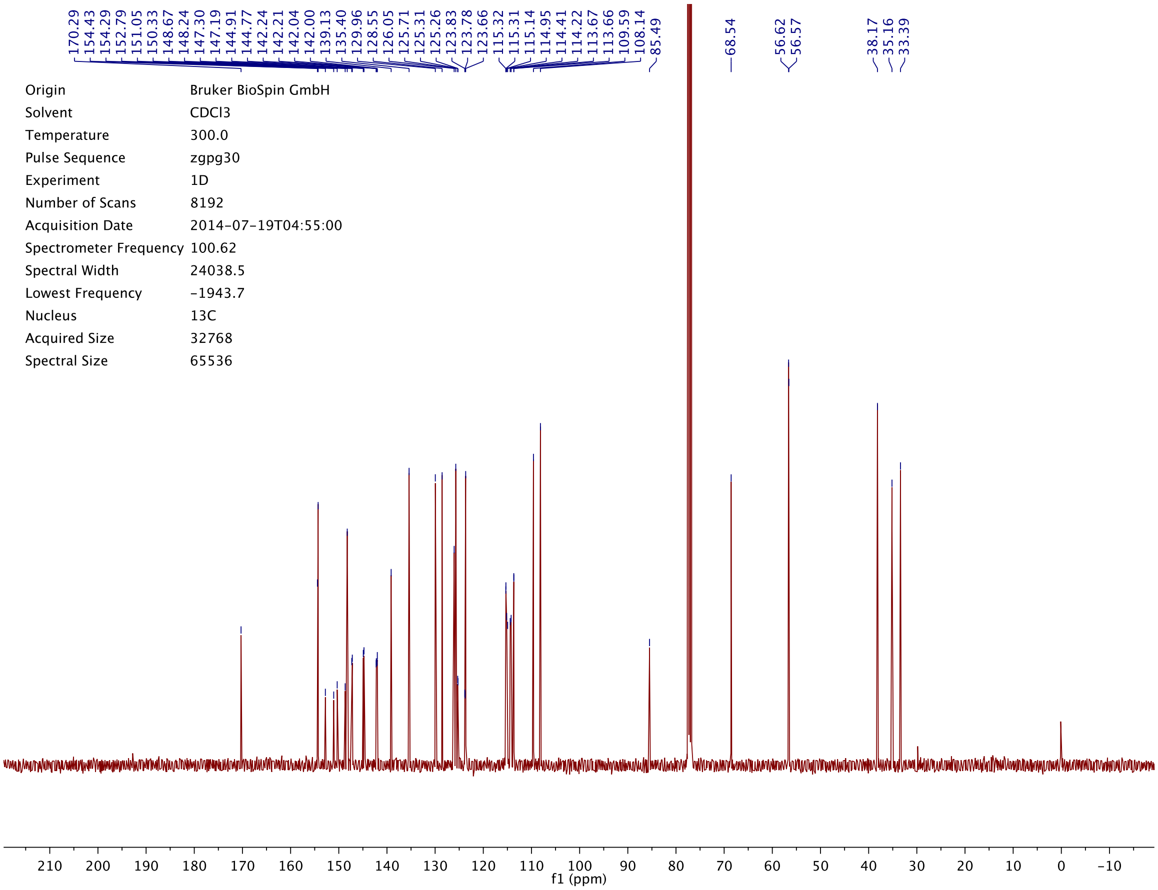
Origin	Bruker BioSpin GmbH
Solvent	CDCl3
Temperature	300.0
Pulse Sequence	zg30
Experiment	1D
Number of Scans	16
Acquisition Date	2014-07-21T09:32:00
Spectrometer Frequency	400.13
Spectral Width	8223.7
Lowest Frequency	-1646.0
Nucleus	1H
Acquired Size	32768
Spectral Size	65536

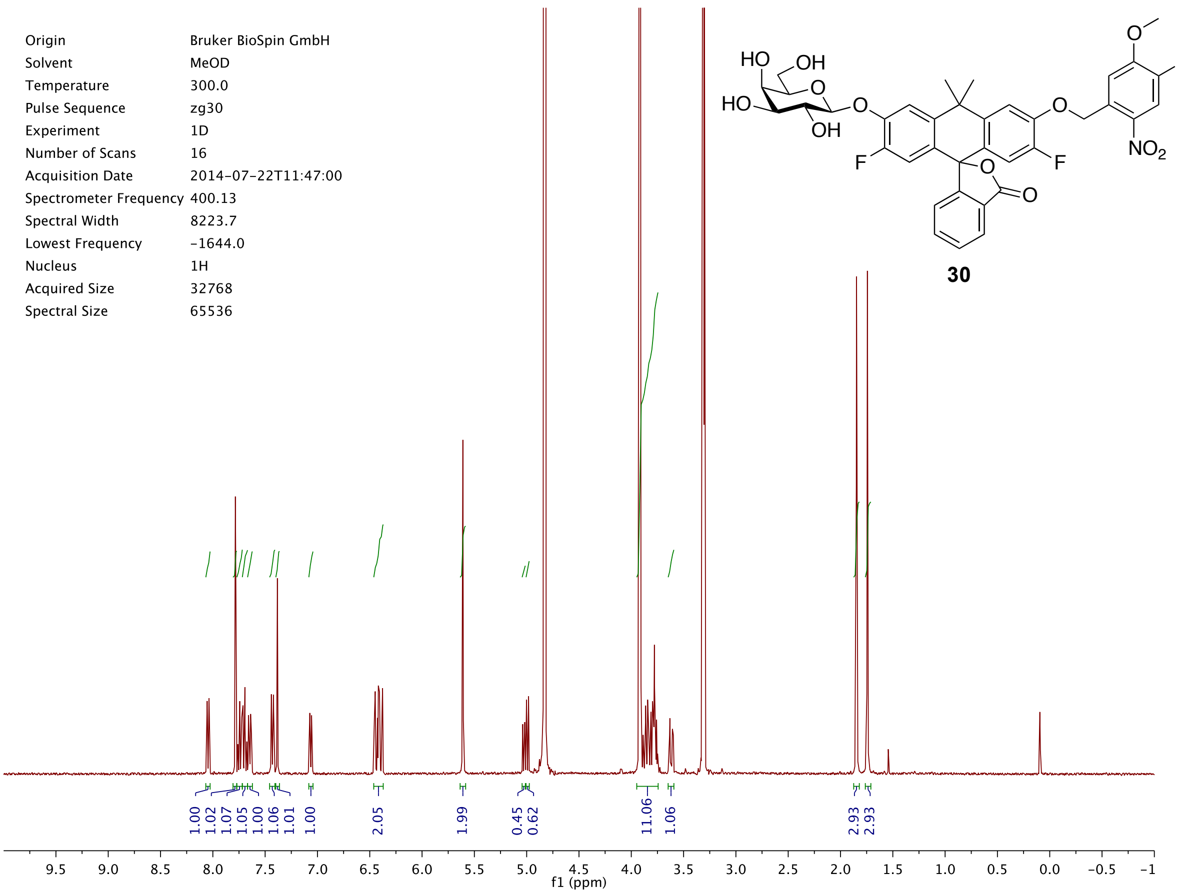


29



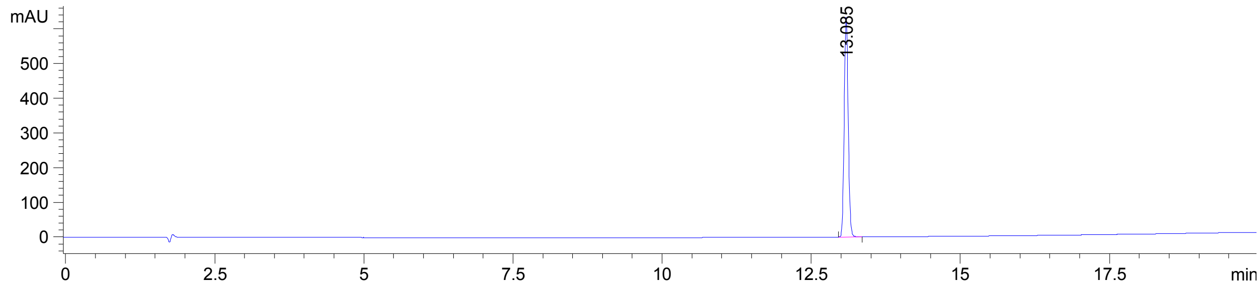
Origin	Bruker BioSpin GmbH
Solvent	CDCl3
Temperature	300.0
Pulse Sequence	zgpg30
Experiment	1D
Number of Scans	8192
Acquisition Date	2014-07-19T04:55:00
Spectrometer Frequency	100.62
Spectral Width	24038.5
Lowest Frequency	-1943.7
Nucleus	13C
Acquired Size	32768
Spectral Size	65536



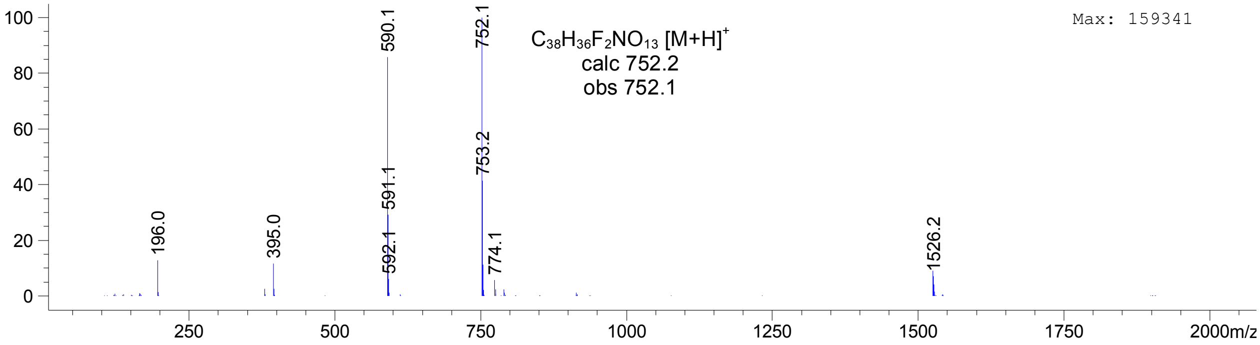


30

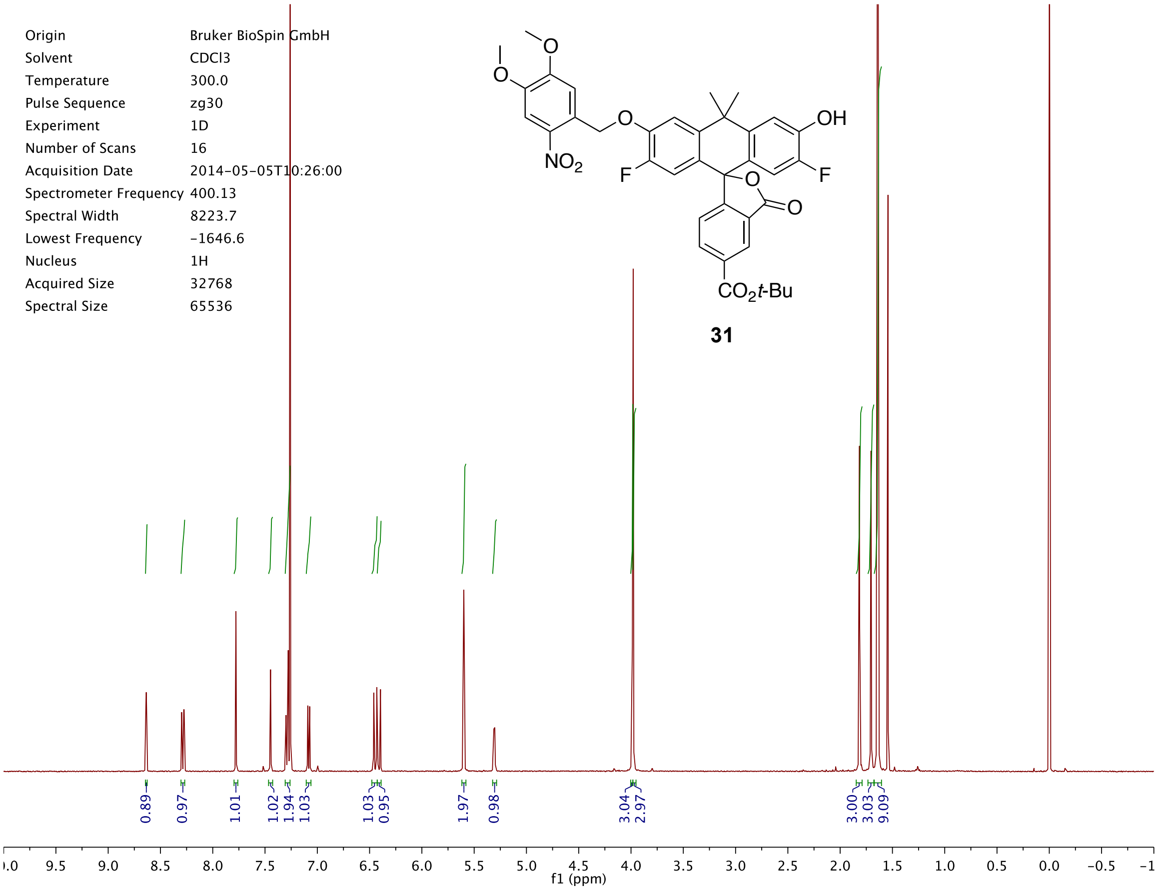
DAD1 A, Sig=254,4 Ref=off (2014_07\DAILYSQUENCE_LC 2014-07-21 10-47-12\2014_07000013.D)



*MSD1 SPC, time=13.061:13.190 of C:\CHEM32\1\DATA\2014_07\DAILYSQUENCE_LC 2014-07-21 10-47-12\2014_07000013.D ES-API

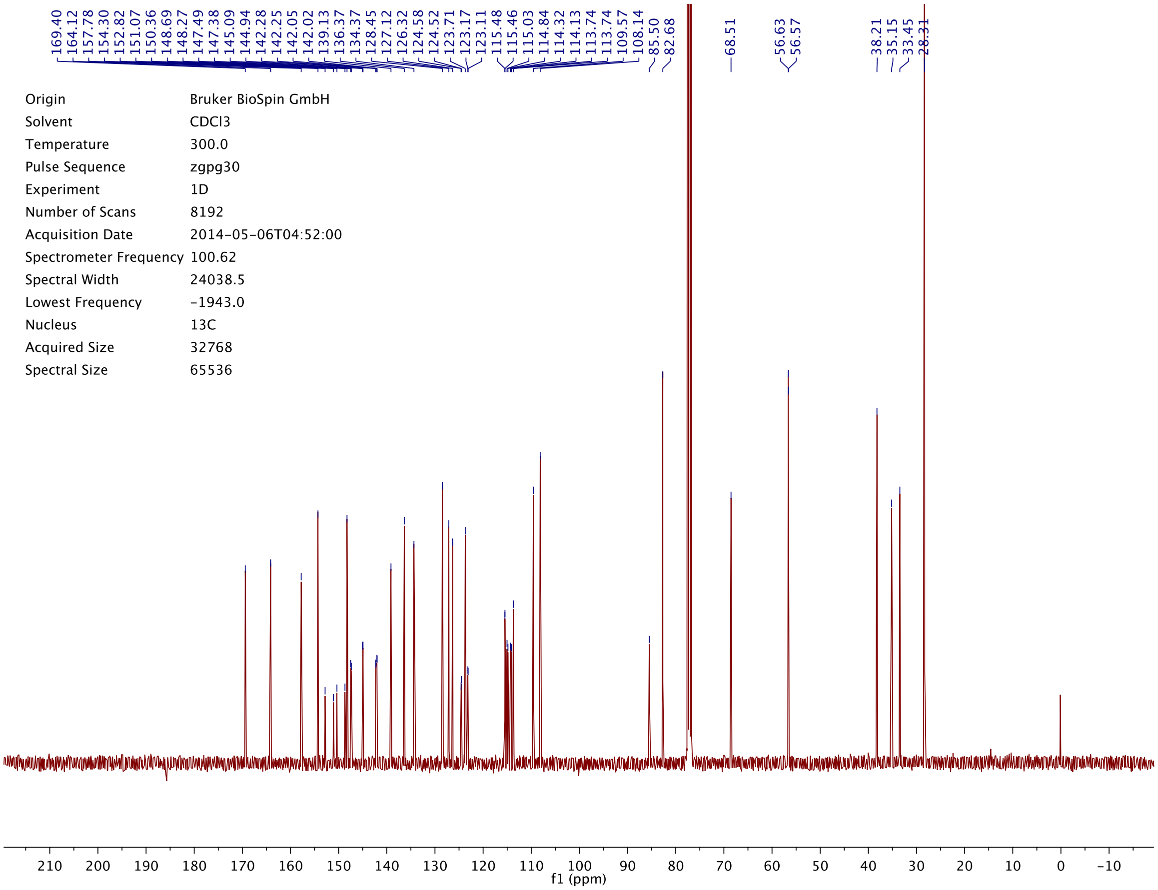


$C_{38}H_{36}F_2NO_{13}$ $[M+H]^+$
calc 752.2
obs 752.1



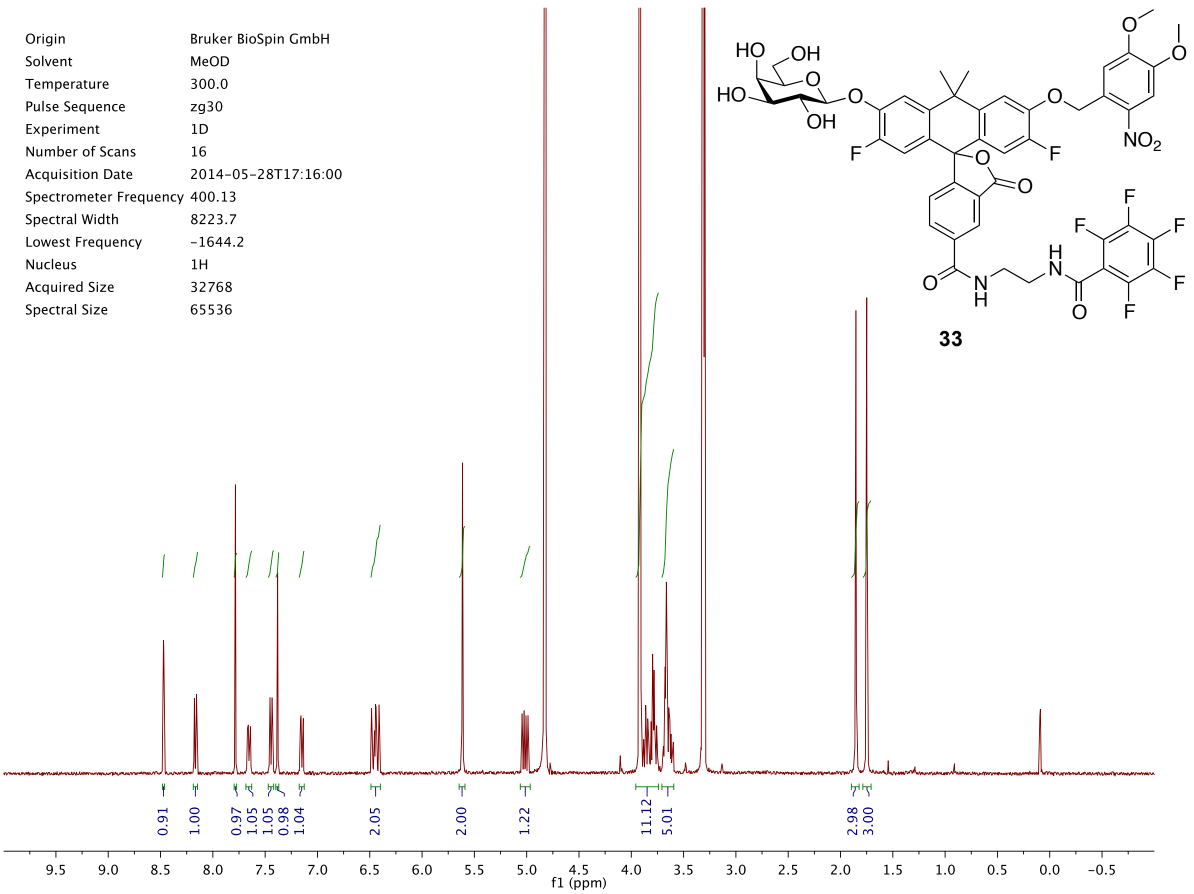
31

Origin	Bruker BioSpin GmbH
Solvent	CDCl3
Temperature	300.0
Pulse Sequence	zg30
Experiment	1D
Number of Scans	16
Acquisition Date	2014-05-05T10:26:00
Spectrometer Frequency	400.13
Spectral Width	8223.7
Lowest Frequency	-1646.6
Nucleus	1H
Acquired Size	32768
Spectral Size	65536



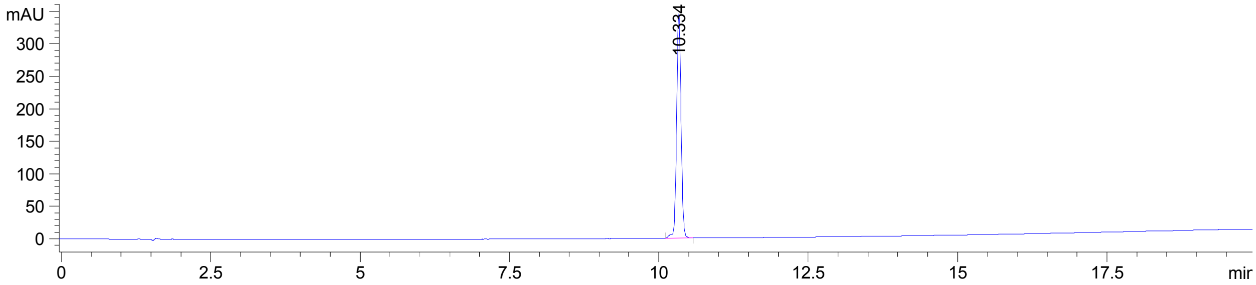
Origin	Bruker BioSpin GmbH
Solvent	CDCl3
Temperature	300.0
Pulse Sequence	zgpg30
Experiment	1D
Number of Scans	8192
Acquisition Date	2014-05-06T04:52:00
Spectrometer Frequency	100.62
Spectral Width	24038.5
Lowest Frequency	-1943.0
Nucleus	13C
Acquired Size	32768
Spectral Size	65536

Origin	Bruker BioSpin GmbH
Solvent	MeOD
Temperature	300.0
Pulse Sequence	zg30
Experiment	1D
Number of Scans	16
Acquisition Date	2014-05-28T17:16:00
Spectrometer Frequency	400.13
Spectral Width	8223.7
Lowest Frequency	-1644.2
Nucleus	1H
Acquired Size	32768
Spectral Size	65536

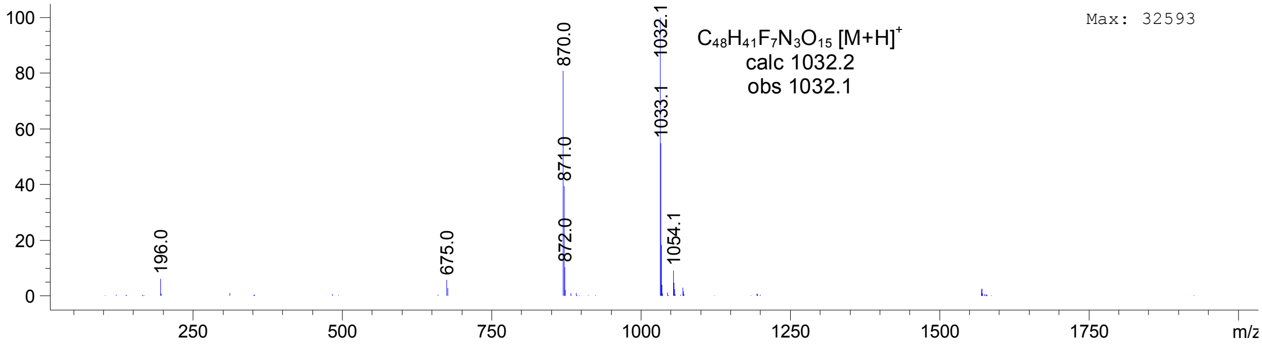


33

DAD1 A, Sig=254,4 Ref=off (C:\CHEM32O...DATA\2014_05\DAILYSQUENCE_LC 2014-05-27 14-24-45\2014_05000004.D)



*MSD1 SPC, time=10.304:10.433 of C:\CHEM32OLD\1\DATA\2014_05\DAILYSQUENCE_LC 2014-05-27 14-24-45\2014_05000004.D ES-



$C_{48}H_{41}F_7N_3O_{15}$ $[M+H]^+$
calc 1032.2
obs 1032.1