Development and evaluation of new cyclooctynes for cell surface glycan imaging in cancer cells

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Supplementary information

Cell culture

Lewis Lung Carcinoma (LLC, from ATCC, Teddington, UK) cells were grown in DMEM (Dulbecco's Modified Eagle Medium (Invitrogen, Paisley, UK); 4.5 g L^{-1} glucose, L-glutamine, pyruvate) supplemented with 10% FBS (fetal bovine serum, PAA laboratories, Yeovil, UK) and maintained in a 5% CO_2, water-saturated atmosphere at 37 °C.

Cell surface azidoglycan labeling and detection by flow cytometry

LLC cells were incubated for 24 h in media containing 50 μM Ac_4GalNAz. Control cells were grown in the absence of Ac_4GalNAz. The following procedure was followed for both sugar-pulsed and non-pulsed sets of cells. The medium was removed from the flasks and cells were washed with warm PBS (phosphate buffered saline; water, NaCl, KCl, Na_2HPO_4, KH_2PO_4; Fisher Scientific, Loughborough, UK). Trypsin-EDTA (0.25% trypsin, 1 mM EDTA, Invitrogen) was added to the flask and the cells incubated at 37 °C for 4.5 min before warm complete DMEM was added, for trypsin inactivation. The contents of each flask were transferred to a centrifuge tube, centrifuged (500 g, 4 °C, 4 min), resuspended in cold FACS buffer (1% FBS in 20 mM HEPES, 150 mM NaCl, pH 7.4) and transferred to 1.5 mL Eppendorf tubes. Cells were centrifuged and resuspended in 100 μL labeling buffer (30 μM TMDIBO-Alexa Fluor 647, DIFO3-Alexa Fluor 647 or PHOS-Alexa Fluor 647 in FACS buffer containing 50 nm SYTOX Green, Invitrogen) or as a control 100 μL 50 nM SYTOX Green in FACS buffer. The Eppendorf tubes were incubated in a hot block with orbital shaking (450 rpm, 37 °C, 15 min). After the indicated incubation times, the cells were washed three times with 700 μL ice cold FACS buffer and then filtered through a 50 μm cut-off membrane into flow cytometry tubes and kept on ice. Each sample was analysed by a flow cytometer (model LSRII, BD Oxford, UK) using 20,000 events.

Data analysis was performed using FlowJo flow cytometry analysis software (Tree Star, Ashland, OR). The viable cell population (population of interest) was determined by gating cells to exclude those with a low NADH auto-fluorescence and those with high levels of SYTOX Green (cell death marker). The far-red median fluorescence intensity (MFI, Alexa Fluor 647 fluorophore) of the viable cell population was then assessed (Figure S1).† Data points were collected in triplicate.

Supplementary Material (ESI) for Chemical Science

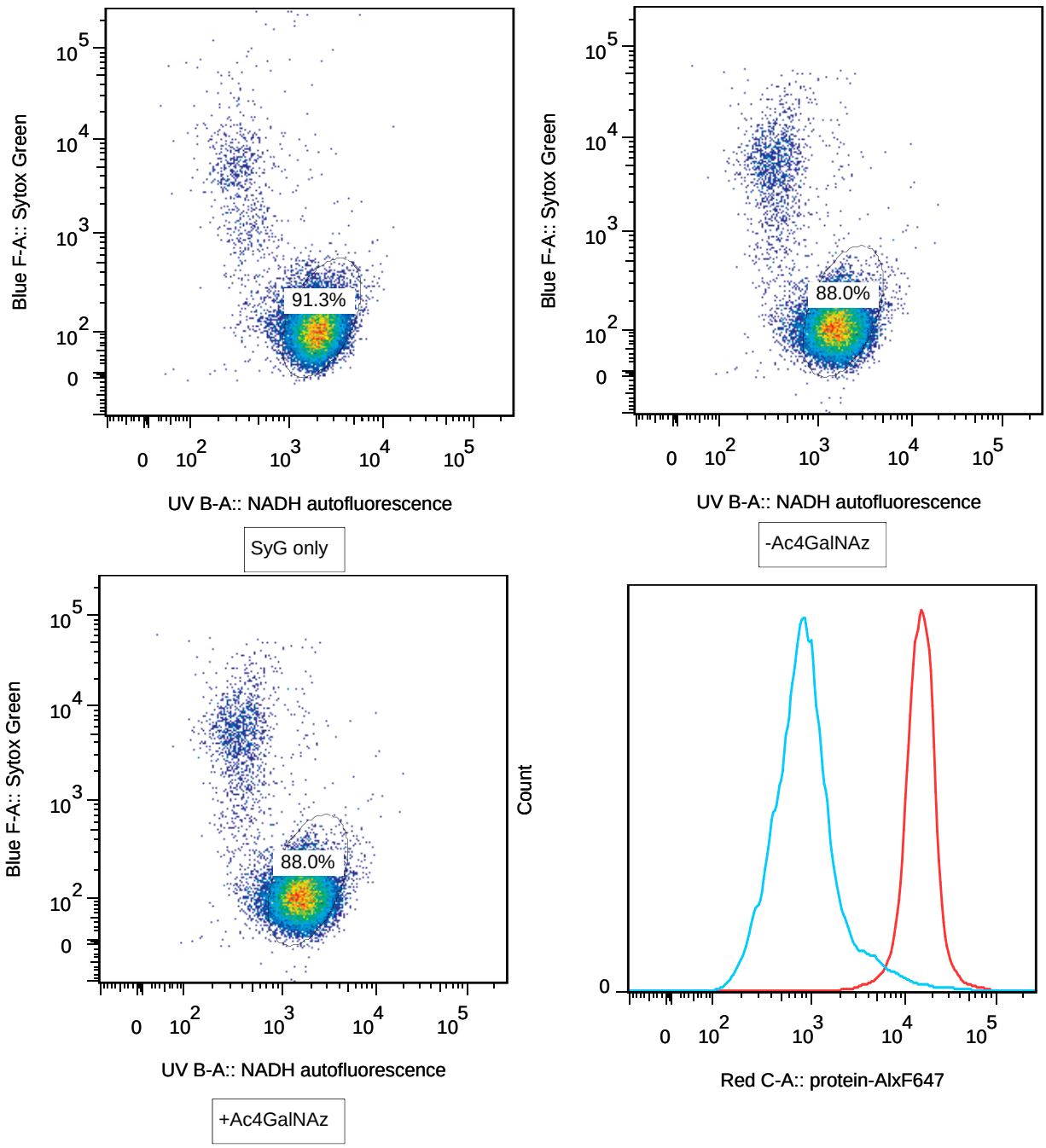


Figure S1 Representative sample showing gating for cells treated with: SYTOX Green only (top left), and azido-sugar-pulsed cells (top right) and non-pulsed cells (bottom left), treated with SYTOX Green and TMDIBO-AF_{647} for 30 min. The viable population of cells (population of interest, grey circle) was determined by gating cells to exclude those with a low NADH auto-fluorescence and those with high levels of the cell death marker SYTOX green. No significant toxicity of either the azido-sugar or the TMDIBO-AF_{647} was observed. Bottom right: the resulting AF_{647}-fluorescence intensities of the viable populations of pulsed (red) and non-pulsed cells (blue).

Cell surface azidoglycan labeling and quantitation with fluorescence microscopy imaging

LLC cells were seeded onto 4-well coverslip chambers (Lab-Tek™ borosylicate, Nunc, Roskilde, Denmark) at 2×10^{-4}/cm^2 and allowed to adhere to the plate surface for 6 h. After adhesion, cells were pulsed with either (+) Ac_4GalNAz (50 μM) or vehicle (-) for 24 hours. Cells were then washed 3 times in ice cold FACS buffer before being incubated (15 min, 37 °C) *in situ* in 200 μL of FACS buffer containing 5 μM TMDIBO-Alexa Fluor 647, DIFO3-Alexa Fluor 647 or PHOS-Alexa Fluor 647 and 300 nM DAPI, Invitrogen). After being washed as above, cells were fixed in PBS containing 4% formaline (RT, 15 min, 37 °C) and washed again twice in cold PBS. The chambers were scanned on an iCys Research Imaging Cytometer (CompuCyte, Westwood, MA, U.S.A.) using 405 nm and 633 nm lasers. A 60x objective was used with 0.5 μm X-step size, giving a field size of 500 μm x 132 μm. A total of 300 fields were scanned for each chamber well. Watershed filters were also included in the protocol to ensure separate contouring on closely spaced cells. Images were analysed using primary and peripheral contours to sample cytoplasmic and membrane staining per cell respectively. The primary contour was set on the blue (DAPI) channel with an integration contour of 8 pixels to include cytoplasmic staining. The peripheral contour was set to a width of 8 pixels measuring from the outer edge of the integration contour. A sub contour was set with a threshold on the long red channel to measure total Alexa Fluor 647 staining per cell.

MSA-binding assay

Mouse serum albumin (Sigma-Aldrich; 10 g/l, 145.6 μM) was incubated with TMDIBO-Alexa Fluor 647, DIFO3-Alexa Fluor 647 or PHOS-Alexa Fluor 647 (4 μM) for 1 h at 37 °C (HBS buffer, 20 mM HEPES, 150 mM NaCl, pH 7.4). After the reaction the samples were cooled to 4 °C, diluted in native gel loading buffer (Expedeon, Harston, UK) and loaded onto native gels (10% acrylamide, Expedeon). Samples of MSA, pre-reacted with iodoacetamide (80-fold molar excess, HBS buffer, 37 °C, 1 h) were incubated with the different probes at similar molar excess, and loaded onto the same gel for comparison. Any unbound fluorophore eluted off the end of the gel during the electrophoresis. Gels were imaged for fluorescence (excitation, λ: 630 nm; emission, λ: 680 nm) on a Typhoon™ Trio scanner (GE Healthcare, Little Chalfont, UK); later stained with Instant Blue™ gel stain (Expedeon) and re-imaged on a bright-field scanner (ImageScanner III™; GE Healthcare). Densitometry analysis of fluorescence-imaged gels was performed using ImageJ™ software (National Institutes of Health, Bethesda, MD, USA). 100 μg of MSA were loaded per gel lane. Native gel sample loading buffer showed some autofluorescence at 647 nm (lanes 1 and 2 in Figure S2A), which was used to standardize the total lane fluorescence in Figure S2A (result shown in Figure S2C).

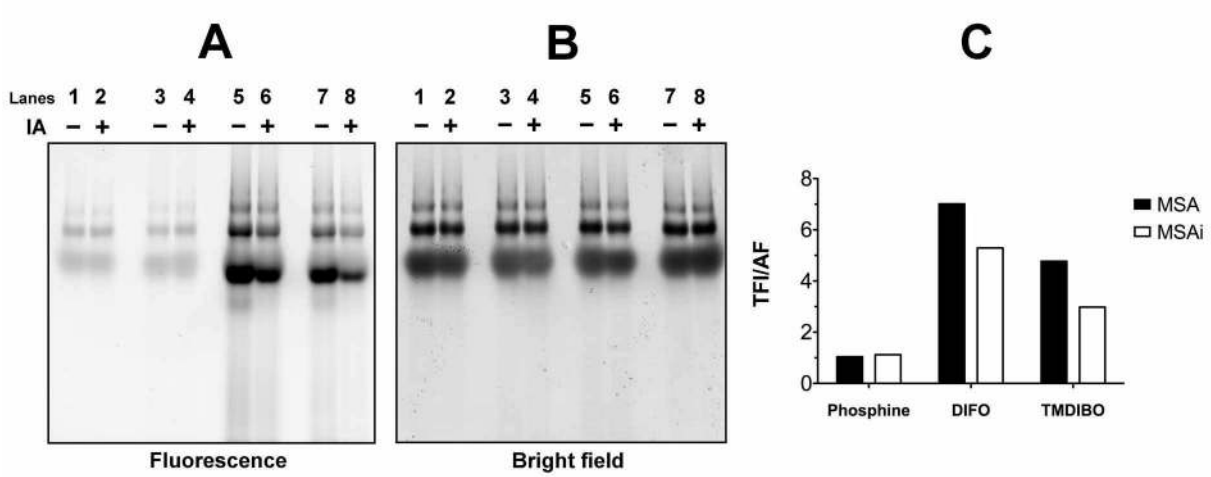


Figure S2 Probe binding to mouse serum albumin (MSA). MSA was incubated with no probe (lanes 1-2), PHOS-AF_{647} (lanes 3-4), DIFO3-AF_{647} (lanes 5-6) or TMDIBO-AF_{647} (lanes 7-8) for 1 h at 37 °C, pH 7.4. The resulting reaction mixtures were resolved in a native gel and imaged with 647-nm fluorescent light (A) or bright field light (B), after Coomassie blue staining. Equivalent iodoacetamide-treated MSA samples were also incubated with each of the probes. Gel densitometry was used to quantitate (C) the total fluorescence intensity per lane in (A). MSA shows a characteristic ladder of oligomers (monomers, dimers and trimers) under native conditions (A and B). 100 μg of MSA were loaded per gel lane. IA(+/-) – Iodoacetamide pretreatment status of MSA; TFI – total lane fluorescence intensity; AF – MSA autofluorescence at 647 nm, due to native gel sample loading buffer. MSAi – Iodoacetamide-treated MSA.

Kinetic evaluation of [3+2] cycloaddition of DIFO and TMDIBO with benzyl azide

DIFO or TMDIBO was mixed in a 1:1 molar ratio (18 or 20 mM final concentration) with benzyl azide in $CDCl_3$ and the reaction was monitored by ^{1}H-NMR (500 MHz) at 300 K probe temperature. The second-order rate constants were determined by plotting $1/[azide]$ versus time. The second-order rate constant ($M^{-1}s^{-1}$) corresponds to the determined slope (Figure S3 and S4).

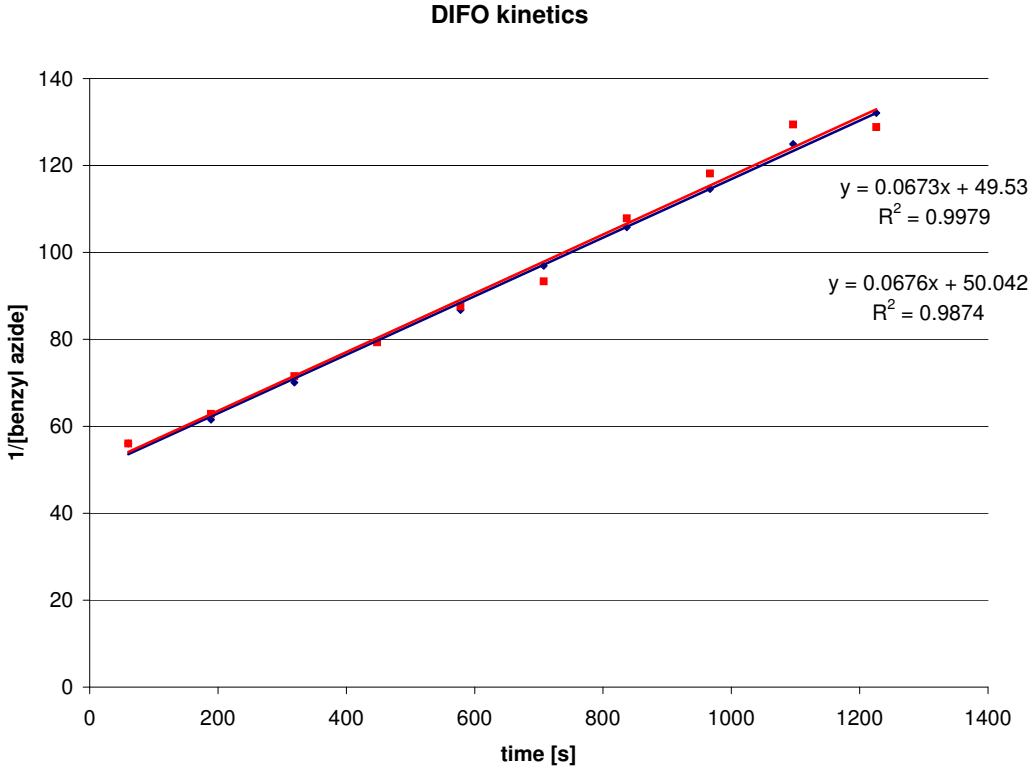


Figure S3 Kinetic evaluation of [3+2] cycloaddition of DIFO and TMDIBO with benzyl azide.

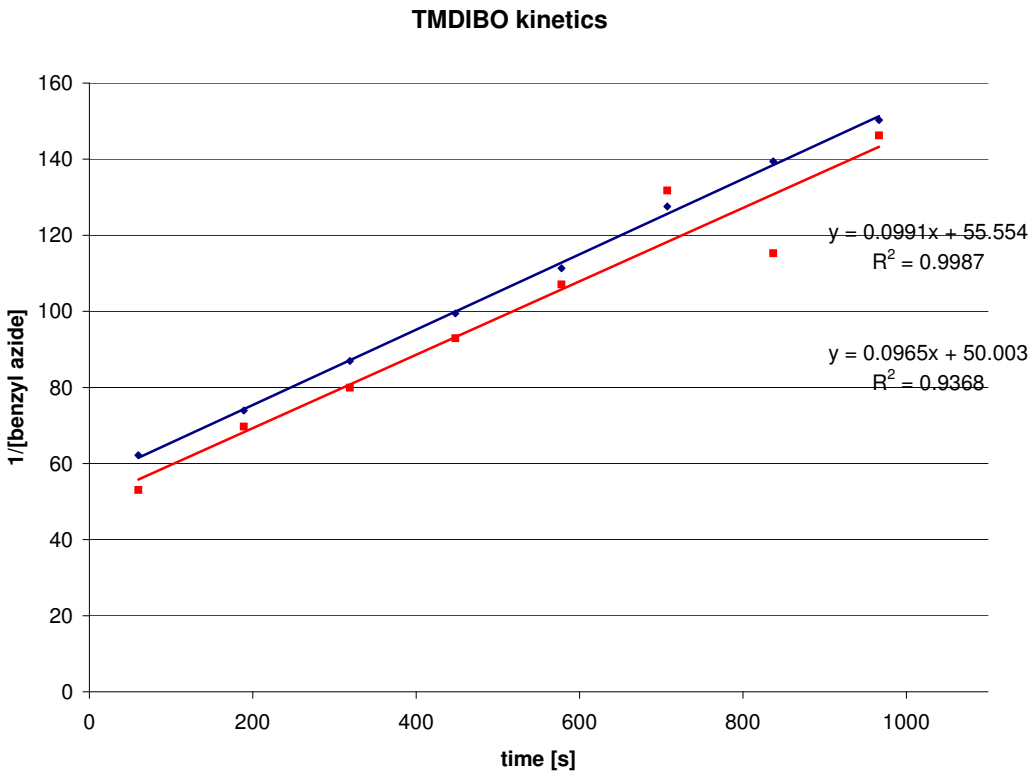


Figure S4 Kinetic evaluation of [3+2] cycloaddition of DIFO and TMDIBO with benzyl azide.

General methods and materials

NMR spectra were recorded on the following instruments: Bruker DRX500, Bruker Avance BB, Bruker Avance TCI, Bruker AM400 and Bruker DRX400. All chemical shifts are quoted in ppm, relative to tetramethylsilane, using the residual solvent peak as a reference standard. All coupling constants are quoted in Hz. Infrared spectra were recorded on a Perkin Elmer Spectrum One (FT-IR) spectrophotometer. High resolution mass spectra (HRMS) were recorded on a Waters LCT Premier TOF mass spectrometer with electrospray and modular Lockspray interface. Analytical thin layer chromatography (TLC) was carried out on Merck Kieselgel 60 F254 plates with visualisation by ultra violet light (254 nm), potassium permanganate and/or phosphomolybdic acid /$Ce(SO_4)_2$ dip. Flash column chromatography was carried out using Merck Kieselgel 60 (230-400 mesh). HPLC was carried out on a Varian ProStar system, UV detection at 254 nm, Phenomenex Jupiter C18 column, particle size 5 μm. LC-MS analysis was performed on a Waters 2795 system, UV detection at 254 nm, Supelco ABZ+plus column, 3.3cm x 4.6mm, particle size 3 μm. All solvent mixtures are reported as % vol/vol unless otherwise stated. Reagents and solvents were purified using standard means. All other chemicals were used as received unless noted otherwise. Extractive procedures were performed using distilled solvents and evaporation of solvents was performed under reduced pressure. All aqueous solutions used were saturated. Unless otherwise stated, all non-aqueous reactions were carried out under an argon atmosphere using anhydrous conditions and oven-dried glassware. Standard techniques were employed for handling air-sensitive materials.

cLogP values were calculated using ChemBioOffice 12.0 as follows: DIFO3 **9** 2.7; TMDIBO **8** 3.2; DIBO **5** 3.9; the amide of PHOS **1** (R = H) 4.5.

Synthetic procedures

2,2-Difluoro-3-hydroxycyclooctanone 25

(*Z*)-3-(Benzyloxy)-2,2-difluorocyclooct-4-enone[1] **24** (2.5 g, 9.39 mmol) was dissolved in EtOH (188 ml). The reaction flask was purged with Argon and Pd/C (1.7 g, 1.597 mmol) was added. The reaction flask was pump-purged with hydrogen, and the reaction was stirred under hydrogen at room temperature for 30 min. The solution was filtered through a pad of celite, and the filtrate was concentrated under reduced pressure to yield the alcohol **25** as a clear oil (1.66 g, 99%).

R_f (30% Et_2O in hexane) 0.18; δ_H(500 MHz, $CDCl_3$) 4.07-3.92 (1H, m, C*H*OH), 2.78-2.67 (1H, m), 2.63-2.53 (1H, m), 2.51-2.44 (1H, m), 2.14-2.00 (1H, m), 1.99-1.85 (2H, m), 1.84-1.71 (1H, m), 1.69-1.52 (3H, m), 1.31-1.17 (1H, m); δ_C(125 MHz, $CDCl_3$) 204.3 (dd, *J* 28.0, 24.6), 117.3, (dd, *J* 257.1, 251.4), 73.2 (dd, *J* 25.5, 22.5), 39.6, 27.3, 27.1 (dd, *J* 3.6, 2.8), 23.0 (m), 19.3; δ_F(376 MHz, $CDCl_3$) -112.2 (1F, d, *J* 246.5), -125.4 (1F, dd, *J* 246.5, 9.5); ν_{max}(film)/cm^{-1} 3428.61, 2940.31, 1725.74; *m/z* (EI) 201.0703 ($M+Na^+$. $C_8H_{12}F_2O_2Na$ requires 201.0698).

3-(*tert*-Butyldimethylsilyloxy)-2,2-difluorocyclooctanone 26

2,2-Difluoro-3-hydroxycyclooctanone (1.6 g, 8.98 mmol), *N*-methylimidazole (4.42 ml, 26.9 mmol), and iodine (6.82 g, 26.9 mmol) were dissolved in CH_2Cl_2 (29.9 ml). *tert*-Butyldimethylsilyl chloride (50% in CH_2Cl_2; 3.75 ml, 10.78 mmol) was added and the reaction mixture was stirred at r.t. until the complete disappearance of the starting material (ca. 1 h). The solvent was evaporated, the residue dissolved in EtOAc and washed with saturated aqueous $Na_2S_2O_3$. The aqueous phase was back extracted with EtOAc after dilution with water, whereupon the cloudy aqueous phase becomes clear. The organic phase was dried over Na_2SO_4 and the solvent evaporated. The product was purified by flash-column chromatography (gradient elution, 0%→10% Et_2O in petroleum ether) to yield silyl ether **26** as a clear oil (2.495 g, 95%).

R_f (5% Et_2O in hexane) 0.38; δ_H(500 MHz, $CDCl_3$) 4.01-3.89 (1H, m, C*H*OSi), 2.75 - 2.65 (1H, m), 2.60 - 2.49 (1H, m), 2.09-1.97 (1H, m), 1.97 - 1.86 (1H, m), 1.83 - 1.74 (2H, m), 1.70 - 1.48 (3H, m), 1.29 - 1.16 (1H, m), 0.89 (9H, s, $SiC(CH_3)_3$), 0.11 (3H, s, $SiCH_3$), 0.07 (3H, s, $SiCH_3$); δ_C(125 MHz, $CDCl_3$) 204.2 (dd, *J* 28.0, 25.4), 117.2 (dd, *J* 258.8, 251.6), 73.7 (dd, *J* 26.7, 21.7), 39.6, 28.8 (t, *J* 3.3), 27.4, 25.6, 23.1 (t, *J* 2.3), 19.2, 18.1, -5.0, -5.2; δ_F(376 MHz, $CDCl_3$) -110.9 (1F, d, *J* 247.6), -122.6 (1F, d, *J* 247.6); ν_{max}(film)/cm^{-1} 2931.02, 2858.37, 1731.86; *m/z* (EI) 315.1588 ($M+Na^+$. $C_{14}H_{26}F_2O_2SiNa$ requires 315.1568).

(*E*)-7-(*tert*-Butyldimethylsilyloxy)-8,8-difluorocyclooct-1-en-1-yl trifluoromethanesulfonate 27

To a round-bottom flask was added THF (82 ml) followed by a solution of NaHMDS (1 M in THF; 9.85 ml, 9.85 mmol). The reaction mixture was cooled to -78 °C with stirring, and silyl ether **26** (2.4 g, 8.21 mmol) in THF (41.0 ml) was added dropwise over 5 min. The reaction mixture was stirred for 60 min, and then a solution of *N*-(5-chloro-2-pyridyl)bis(trifluoromethanesulfonimide) (3.87 g, 9.85 mmol) in THF (41.0 ml) was added via syringe. The reaction mixture was stirred for 2 h at -78 °C and then quenched with 50% saturated NH_4Cl (10 mL). The mixture was diluted with Et_2O and the aqueous phase extracted with Et_2O. The combined organic phases were washed with brine, dried over Na_2SO_4 and the solvent evaporated. The

residue was purified by flash chromatography (gradient elution, 1%→5% Et_2O in petroleum ether) to give the enol triflate **27** as a light-yellow oil (3.03 g, 87%).
R_f (5% Et_2O in hexane) 0.42; δ_H(500 MHz, $CDCl_3$) 6.09 - 6.00 (m, 1H, CH_2-C*H*=C), 4.21 - 4.12 (1H, m, C*H*OSi), 2.62-2.45 (1H, m), 2.30 - 2.16 (1H, m), 1.95 - 1.79 (2H, m), 1.78 - 1.61 (3H, m), 1.49 - 1.36 (1H, m), 0.89 (9H, s, $SiC(CH_3)_3$), 0.10 (3H, s, $SiCH_3$), 0.08 (3H, s, $SiCH_3$); δ_C(125 MHz, $CDCl_3$) 142.4 (t, *J* 29.7), 127.3 (d, *J* 4.9), 119.9 (t, *J* 320.1), 116.8 (t, *J* 250.3), 74.2 (dd, *J* 35.0, 25.7), 28.0 (d, *J* 6.7), 27.8 (s), 25.6 (s), 21.1 (d, *J* 6.1), 18.2 (s), 16.1 (s), -5.1 (br s). δ_F(376 MHz, $CDCl_3$) -74.6 (3F, d, *J* 5.4), -103.19 (1F, d, *J* 268.6), -106.27 (1F, d, *J* 268.6); ν_{max}(film)/cm^{-1} 2931.71, 2861.02, 1673.58; *m/z* (EI) 447.1055 ($M+Na^+$. $C_{15}H_{25}F_5O_4SSiNa$ requires 447.1059).

2,2-Difluorocyclooct-3-ynol (DIFO3) 9
Enol triflate **27** (1.9 g, 4.48 mmol) was dissolved in THF (90 ml) and the mixture was cooled to -20 °C with stirring. Freshly prepared LDA (0.5 M in THF; 11.64 ml, 5.82 mmol) was added dropwise at the same temperature. The reaction mixture was allowed to warm to r.t. over 20 min, quenched with aqueous NH_4Cl and extracted with Et_2O. The organic phase was washed with brine, dried over $MgSO_4$ and the solvents evaporated. The residue was dissolved in THF (90 ml) and TBAF (1 M in THF; 5.82 ml, 5.82 mmol) was added dropwise at 0 °C. The mixture was allowed to warm to r.t. and stirred for 30 min, then quenched with aqueous $NaHCO_3$ and extracted with Et_2O. The organic phase was washed with brine, dried over $MgSO_4$ and the solvents evaporated. Purification by flash chromatography (gradient elution, 0%→4% Et_2O in CH_2Cl_2) gave DIFO3 **9** as a white solid (0.61 g, 85%), mp 41 °C.
R_f (1% Et_2O in CH_2Cl_2) 0.23; δ_H(500 MHz, $CDCl_3$) 4.09 - 4.00 (1H, m, C*H*OH), 2.39 - 2.30 (2H, m), 2.30 - 2.26 (1H, m), 2.22 - 2.12 (m, 1H), 2.07 - 1.98 (1H, m), 1.98 - 1.88 (2H, m), 1.82 - 1.70 (1H, m), 1.47 - 1.36 (1H, m). δ_C(125 MHz, $CDCl_3$) 117.6 (dd, *J* 240.0, 237.8), 110.1 (t, *J* 11.5), 83.1 (dd, *J* 45.6, 42.2), 80.8 (dd, *J* 28.3, 24.4), 34.6 (d, *J* 3.5), 31.0 (d, *J* 1.8), 24.3, 20.5. δ_F(376 MHz, $CDCl_3$) -102.1 (1F, d, *J* 257.8), -111.3 (1F, s, *J* 257.8). ν_{max}(solid)/cm^{-1} 3314.52, 2925.53, 2857.89, 2213.71; *m/z* (EI) 183.0591 ($M+Na^+$. $C_8H_{10}F_2ONa$ requires 183.0592).

2,2-Difluorocyclooct-3-yn-1-yl (4-nitrophenyl) carbonate 33
2,2-Difluorocyclooct-3-ynol **9** (0.1 g, 0.624 mmol) was dissolved in CH_2Cl_2 (12.49 ml) with stirring at r.t., and then pyridine (0.252 ml, 3.12 mmol) and 4-nitrophenyl chloroformate (0.126 g, 0.624 mmol) were added at r.t. The reaction was followed by TLC and when the starting material had disappeared (ca. 1 h), the mixture was diluted with CH_2Cl_2 and washed with brine (2x). The organic layer was dried over $MgSO_4$ and the solvent evaporated. Purification by flash chromatography (gradient elution, 30%→40% CH_2Cl_2 in petroleum ether) gave the carbonate **33** was isolated as a white solid (0.194 g, 96%), mp 102 °C.
R_f (100% CH_2Cl_2) 0.54; δ_H(500 MHz, $CDCl_3$) 8.29 (2H, d, *J* 9.2, Ar-H), 7.41 (2H, d, *J* 9.2, Ar-H), 5.18 - 5.09 (1H, m, C*H*OCO_2Ar), 2.46 - 2.36 (2H, m), 2.31 - 2.19 (1H, m), 2.18 - 2.03 (3H, m), 1.86 - 1.72 (1H, m), 1.37 - 1.23 (1H, m); δ_C(125 MHz, $CDCl_3$) 155.5 (s), 151.9 (s), 145.7 (s), 125.5 (s), 121.9 (s), 115.6 (dd, *J* 244.6, 240.1), 111.9 (t, *J* 11.4), 84.5 (dd, *J* 28.5, 22.5), 82.1 (dd, *J* 45.5, 40.9), 32.7 (s), 30.5 (s), 24.7 (s), 20.3 (s); δ_F(376 MHz, $CDCl_3$) -101.6 (1F, d, *J* 259.4), -108.2 (1F, ddt, *J* 259.4, 13.7, 6.7); ν_{max}(solid)/cm^{-1} 2941.86, 2860.90, 2216.70, 1765.86, 1616.30, 1593.69, 1523.53; *m/z* (EI) 348.0653 ($M+Na^+$. $C_{15}H_{13}F_2O_5NNa$ requires 348.0654).

3-*tert*-Butyldimethylsilyloxy-2',3',2",3"-tetramethoxy-1,2:5,6-dibenzocyclocta-1,5,7-triene 14

tert-Butyldimethylsilyl chloride (12.65 ml, 73.0 mmol) was added to a stirred solution of 3-hydroxy-2',3',2",3"-tetramethoxy-1,2:5,6-dibenzocyclocta-1,5,7-triene[2] **13** (10 g, 29.2 mmol) and pyridine (14.17 ml, 175 mmol) in CH_2Cl_2 (29.2 ml). The reaction mixture was stirred at r.t. for 12 h, diluted with water and extracted with CH_2Cl_2. The combined organic extracts were washed with water and brine, dried over Na_2SO_4 and the solvent evaporated. The residue was purified by flash column chromatography (Alumina, gradient elution 20%→40% EtOAc in petroleum ether) to give silyl ether **14** as a clear oil (10.94 g, 82%).
R_f (50% EtOAc in hexane) 0.50; δ_H(400 MHz, $CDCl_3$) 7.09 (1H, s, Ar-H), 6.73 (1H, d, *J* 12.2, C*H*=CH), 6.61 (1H, d, *J* 12.2, CH=C*H*C), 6.57 (1H, s, Ar-H), 6.56 (1H, s, Ar-H), 6.55 (1H, s, Ar-H), 5.39 (1H, dd, *J* 10.2, 5.4, C*H*OSi), 3.88 (3H, s, OMe), 3.81 (3H, s, OMe), 3.81 (3H, s, OMe), 3.80 (3H, s, OMe), 3.40 (1H, dd, *J* 15.3, 5.4, CH_AH_B), 3.11 (1H, dd, *J* 15.3, 10.2, CH_AH_B), 0.90 (9H, s, SiC$(CH_3)_3$), 0.00 (3H, s, $SiCH_3$), -0.05 (3H, s, $SiCH_3$). δ_C(100 MHz, $CDCl_3$) 148.4, 147.8, 147.2, 146.7, 136.3, 132.6, 129.2, 128.8, 128.2, 126.8, 113.5, 113.2, 110.9, 109.0, 71.7, 55.9, 55.8, 46.9, 26.0, 25.8, 18.4, -4.6, -4.7. ν_{max}(solid)/cm^{-1} 2930.31, 1604.30, 1509.75; *m/z* (EI) 479.2238 ($M+Na^+$. $C_{26}H_{36}O_5SiNa$ requires 479.2230).

3-Hydroxy-2',3',2",3"-tetramethoxy-7,8-didehydro-1,2:5,6-dibenzocyclocta-1,5,7-triene 8
Bromine (1.128 ml, 21.90 mmol) was added dropwise, with stirring, to olefin **14** (10 g, 21.90 mmol) in CH_2Cl_2 (189 ml) at -40 °C until the brown colour persisted. The solvent was evaporated and the residue was dissolved in THF (189 ml). *N*-methylpiperazine (32.9 g, 328 mmol) was added, followed by the addition of KO^{t}Bu (9.83 g, 88 mmol). The suspension was stirred overnight, poured into ice/water and extracted with EtOAc. The combined organic layers were evaporated and the residue was dissolved in THF (189 ml). TBAF (1M in THF; 28.5 ml, 28.5 mmol) was then added dropwise at 0 °C. The mixture was allowed to warm to r.t. and stirred for 30 min. The reaction was quenched with aqueous $NaHCO_3$ and extracted with EtOAc. The combined organic layers were washed with brine and dried over $MgSO_4$. The solvent was slowly evaporated until crystallization commenced. The crystals were filtered off and TMDIBO **8** was obtained as an off-white solid (5.66 g, 76%), mp 232 °C.
R_f (50% EtOAc in hexane) 0.32; δ_H(400 MHz, DMSO-d_6) 7.37 (1H, s, Ar-H), 7.13 (1H, s, Ar-H), 6.96 (1H, s, Ar-H), 6.96 (1H, s, Ar-H), 5.78 (1H, d, *J* 5.0, CHO*H*), 4.28 (1H, app dd, *J* 5.0, 2.1, C*H*OH), 3.86 (6H, s, 2x OMe), 3.81 (6H, s, 2x OMe), 3.06 (1H, dd, *J* 14.2, 2.1, CH_AH_B), 2.64 (1H, dd, *J* 14.2, 3.6, CH_AH_B); δ_C(100 MHz, DMSO-d_6) 151.0, 149.1, 148.8, 147.7, 147.4, 145.7, 115.2, 114.7, 112.4, 112.2, 110.5, 109.6, 109.5, 109.5, 74.5, 56.2, 56.2, 56.0, 55.9, 49.5; ν_{max}(solid)/cm^{-1} 3452.30, 2831.21, 2140.89, 1600.91, 1559.39, 1501.12; *m/z* (EI) 341.1398 ($M+H^+$. $C_{20}H_{21}O_5$ requires 341.1389).

Carbonic acid-2',3',2",3"-tetramethoxy-7,8-didehydro-1,2:5,6-dibenzocyclocta-1,5,7-triene-3-yl 4-nitrophenyl ester 17
TMDIBO **8** (50 mg, 0.147 mmol) was dissolved in CH_2Cl_2 (4.9 mL), followed by the addition of pyridine (0.059 ml, 0.734 mmol) and 4-nitrophenyl chloroformate (59.2 mg, 0.294 mmol). After 1 h stirring at r.t., when the reaction had gone to completion, it was diluted with CH_2Cl_2 and washed with brine (2x). The organic layer was dried over $MgSO_4$ and the solvent evaporated. The residue was recrystallized from EtOAc to give carbonate **17** as a white solid (69 mg, 93%), mp 204-205 °C.
R_f (2.5% Et_2O in CH_2Cl_2) 0.26; major rotamer: δ_H(500 MHz, $CDCl_3$) 8.28 (2H, d, *J* 9.3, Ar-H), 7.42 (2H, d, *J* 9.3, Ar-H), 7.13 (1H, s, Ar-H), 6.90 (1H, s, Ar-H), 6.86 (1H, s, Ar-H), 6.83 (1H, s, Ar-H), 5.51 (1H, dd, *J* 3.7, 1.9, C*H*OCO_2Ar), 3.95 (3H, s, OMe), 3.91 (3H, s, OMe), 3.90 (3H, s, OMe), 3.89 (3H, s, OMe), 3.23 (1H, dd, *J* 15.4, 1.9, CH_AH_B), 2.97 (1H, dd, *J* 15.4, 3.7, CH_AH_B); minor rotamer: δ_H(500 MHz, $CDCl_3$)

8.14 (2H, d, *J* 9.2, Ar-H), 7.13 (1H, s, Ar-H), 6.90 (1H, s, Ar-H), 6.88 (1H, s, Ar-H), 6.87 (2H, d, *J* 9.2, Ar-H), 6.84 (1H, s, Ar-H), 6.14 (1H, dd, *J* 9.8, 2.0, C*H*OCO_2Ar), 3.94 (3H, s, OMe), 3.93 (3H, s, OMe), 3.91 (3H, s, OMe), 3.88 (3H, s, OMe), 3.72 (1H, dd, *J* 14.0, 9.8, CH_AH$_B$), 2.90 (1H, dd, *J* 14.0, 2.0, CH$_A$$H_B$); major+minor rotamer: δ_C(125 MHz, $CDCl_3$) 155.4, 155.3, 152.1, 151.5, 149.6, 149.2, 148.9, 148.2, 148.1, 148.1, 145.4, 145.3, 143.5, 143.0, 140.3, 139.4, 125.3, 125.1, 121.8, 121.5, 118.2, 115.6, 115.6, 115.6, 115.3, 113.9, 113.2, 112.7, 110.4, 109.5, 109.3, 109.2, 108.8, 108.7, 108.2, 107.7, 82.1, 78.5, 56.1, 56.1, 56.1, 56.1, 46.2, 39.3; ν_{max}(solid)/cm^{-1} 2949.19, 2337.71, 1752.09, 1602.48, 1561.66, 1505.46, 1323.20, 1264.41; *m/z* (EI) 506.1462 (M+H^+. $C_{27}H_{24}NO_9$ requires 506.1451).

Conjugation with Alexa Fluor 647 cadaverine, general procedure
The respective carbonates **17**, **33** or Phosphine-PFP (2-diphenylphosphanyl-terephthalic acid 1-methyl ester 4-pentafluorophenyl ester) (0.758 μmol, 1.5 eq.) were dissolved in dry DMF (50.6 μL) (dry, degassed DMF for Phosphine-PFP). Alexa Fluor 647 cadaverine (0.5 mg, 0.506 μmol, 1.0 eq.) and Hünig's base (0.1 molar, 15.17 μl, 1.517 μmol, 3.0 eq.) were added with stirring in the dark. Upon reaction completion (ca. 1 h, confirmed by LC-MS), the solvent was evaporated. The blue residue was purified by reverse-phase HPLC (gradient elution, 20% MeCN/H_2O/0.1% formic acid → 100% MeCN/0.1% formic acid, 20 min).
DIFO3-Alexa Fluor 647 *m/z* (EI) 1129.3503 (M+H^+), 1151.3311 (M+Na^+).
PHOS-Alexa Fluor 647 *m/z* (EI) 1289.3778 (M+H^+), 1311.3607 (M+Na^+).
TMDIBO-Alexa Fluor 647 *m/z* (EI) 1310.4229 (M+H^+), 1332.4126 (M+Na^+).

Stability tests

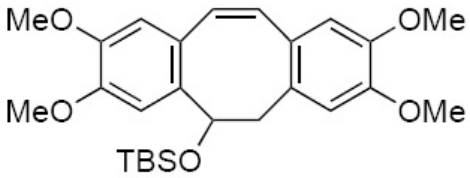
TMDIBO (50 mg, 0.147 mmol) was dissolved in MeCN (0.7 mL), then H_2O (0.7 mL) and K_2CO_3 (60.9 mg, 0.441 mmol, 3.0 eq.) were added. The mixture was heated to 110 °C for 4 h. The solvent was removed *in vacuo* and the residue extracted with EtOAc (3x). The organic phases were dried over $MgSO_4$, the solvent removed *in vacuo*. TMDIBO was isolated as a white solid (50 mg, 100%). The ^{1}H-NMR spectrum was identical to that reported above.
TMDIBO (50 mg, 0.147 mmol) was dissolved in a mixture of MeOH, THF and HCl (3 M) (1:1:1, 1.0 mL). The mixture was heated to 70 °C with stirring, the reaction being monitored by LC-MS. After 12 h (ca. 50% conversion), the solvent was removed *in vacuo*, the residue taken up in H_2O and extracted with EtOAc (3x). The organic phases were dried over $MgSO_4$. The solvent was removed *in vacuo* and the product purified by flash chromatography (gradient elution 30%-60% EtOAc/hexane). The product was isolated as a white solid (24.5 mg, 49%). The ^{1}H-NMR spectrum was identical to that reported above.

References

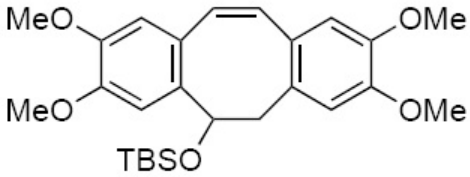
[1] J. A. L. Miles, L. Mitchell, J. M. Percy, K. Singh and E. Uneyama, *J. Org. Chem.*, 2007, **72**, 1575–1587.

[2] M. E. Jung and S. J. Miller, *J. Am. Chem. Soc.*, 1981, **103**, 1984–1992.

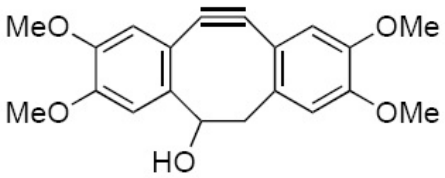
1H NMR
400.13 MHz
CDCl3



13C NMR
100.61 MHz
CDCl3



1H NMR
400.13 MHz
DMSO

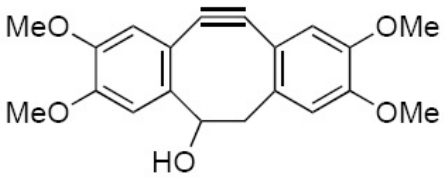


7.13
6.96
6.96
5.79
5.78
3.86
3.81
3.81
3.35
3.08
3.08
3.05
3.04
2.66
2.65
2.63
2.62
2.55
2.55
2.54
2.54
2.53
0.00

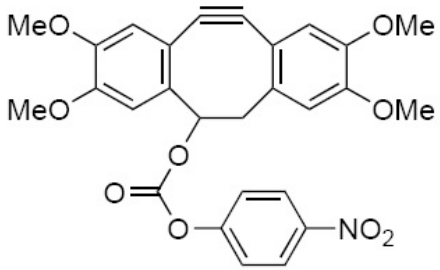
1.00
0.93
2.22
1.01
1.34
6.65
6.84
0.90
0.90

f1 (ppm)

13C NMR
100.61 MHz
DMSO

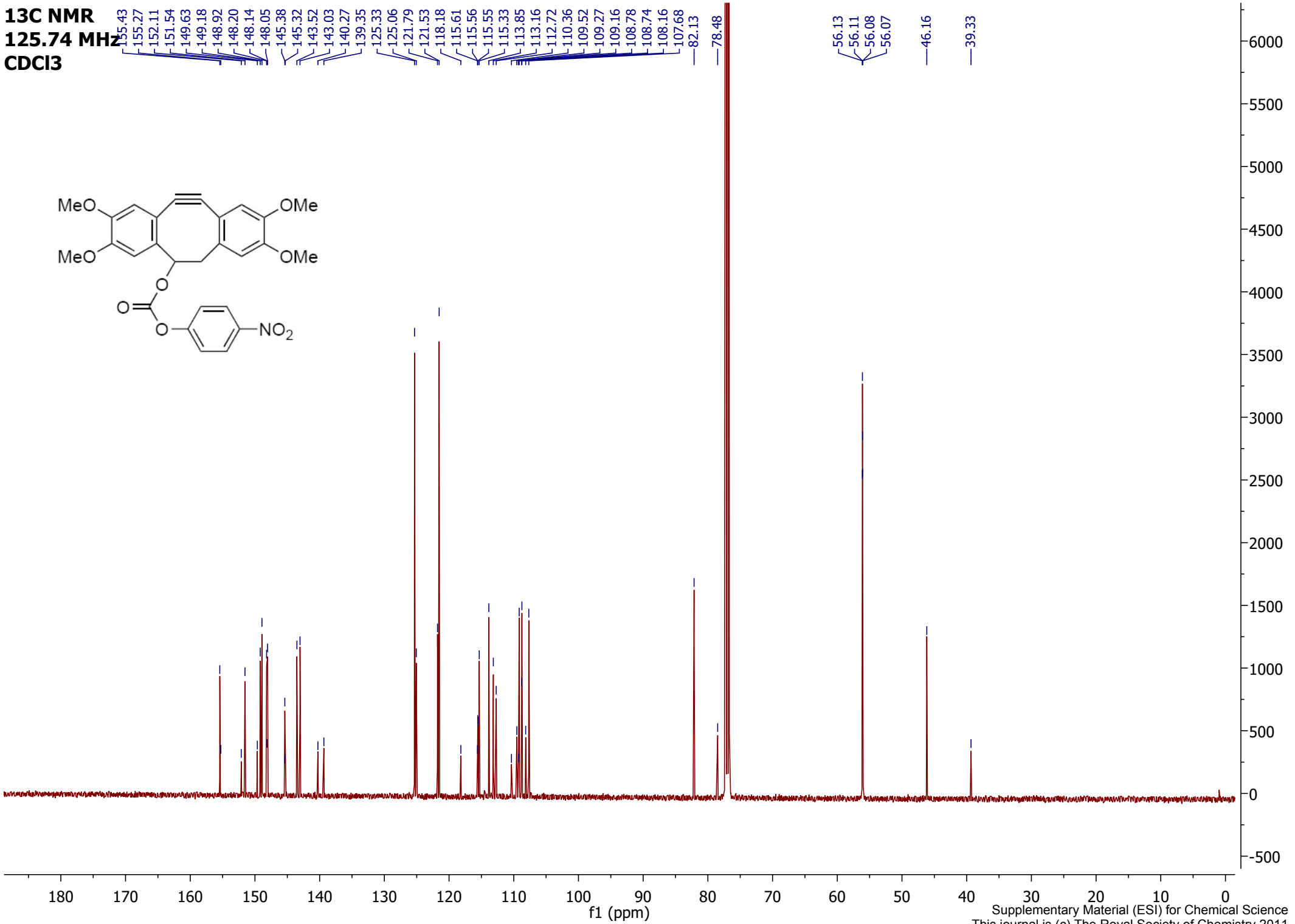


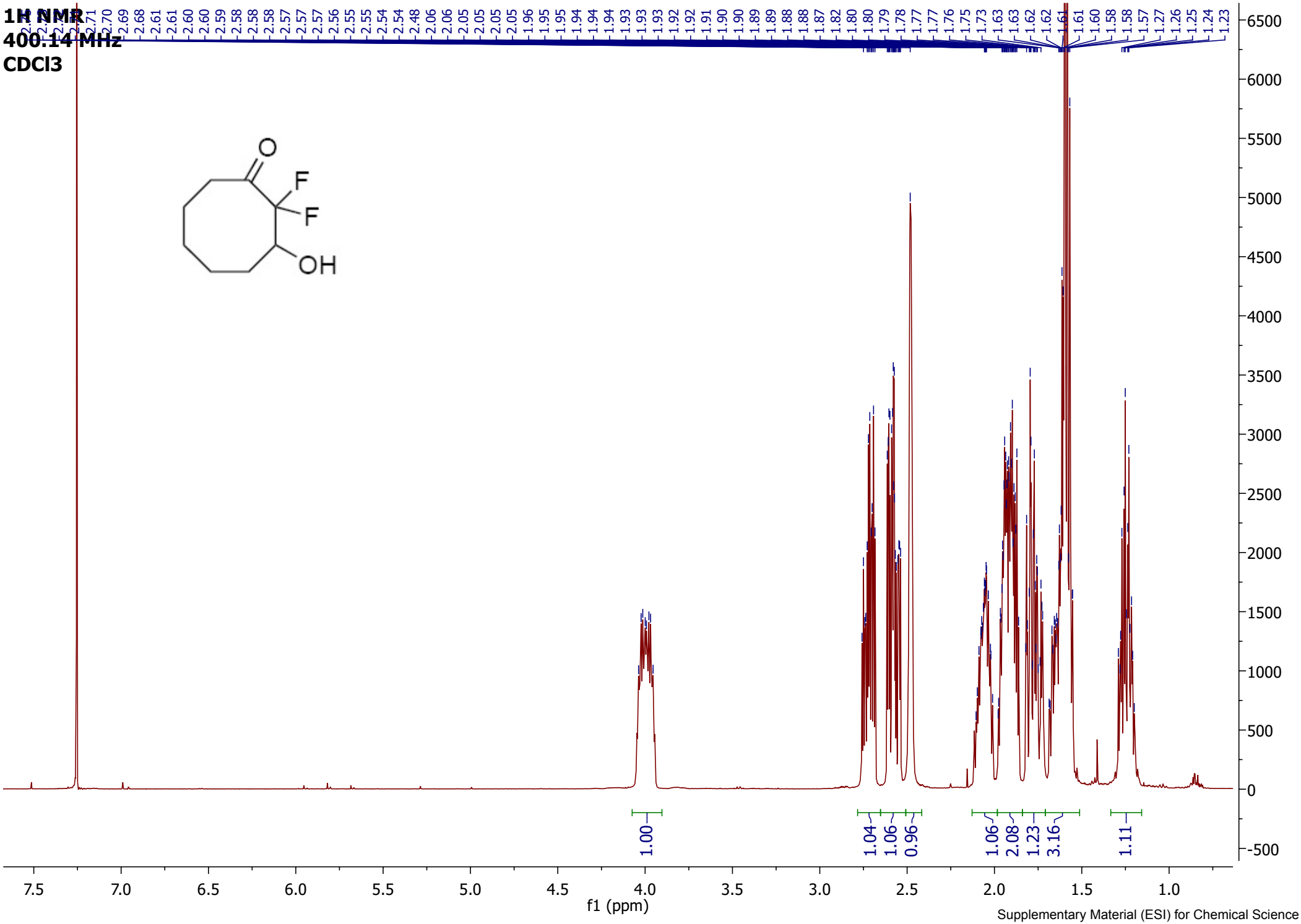
1H NMR
500.05 MHz
CDCl3



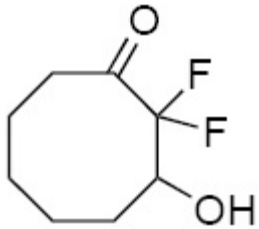
f1 (ppm)

13C NMR
125.74 MHz
CDCl3

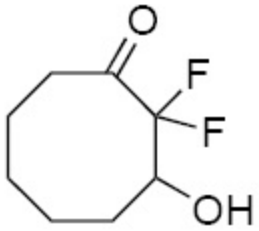




13C NMR
125.75 MHz
CDCl3



19F NMR
376.45 MHz
CDCl3

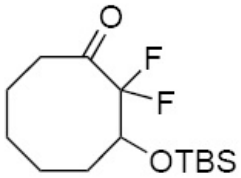


-111.84
-112.50
-125.09
-125.11
-125.73
-125.76
-125.76

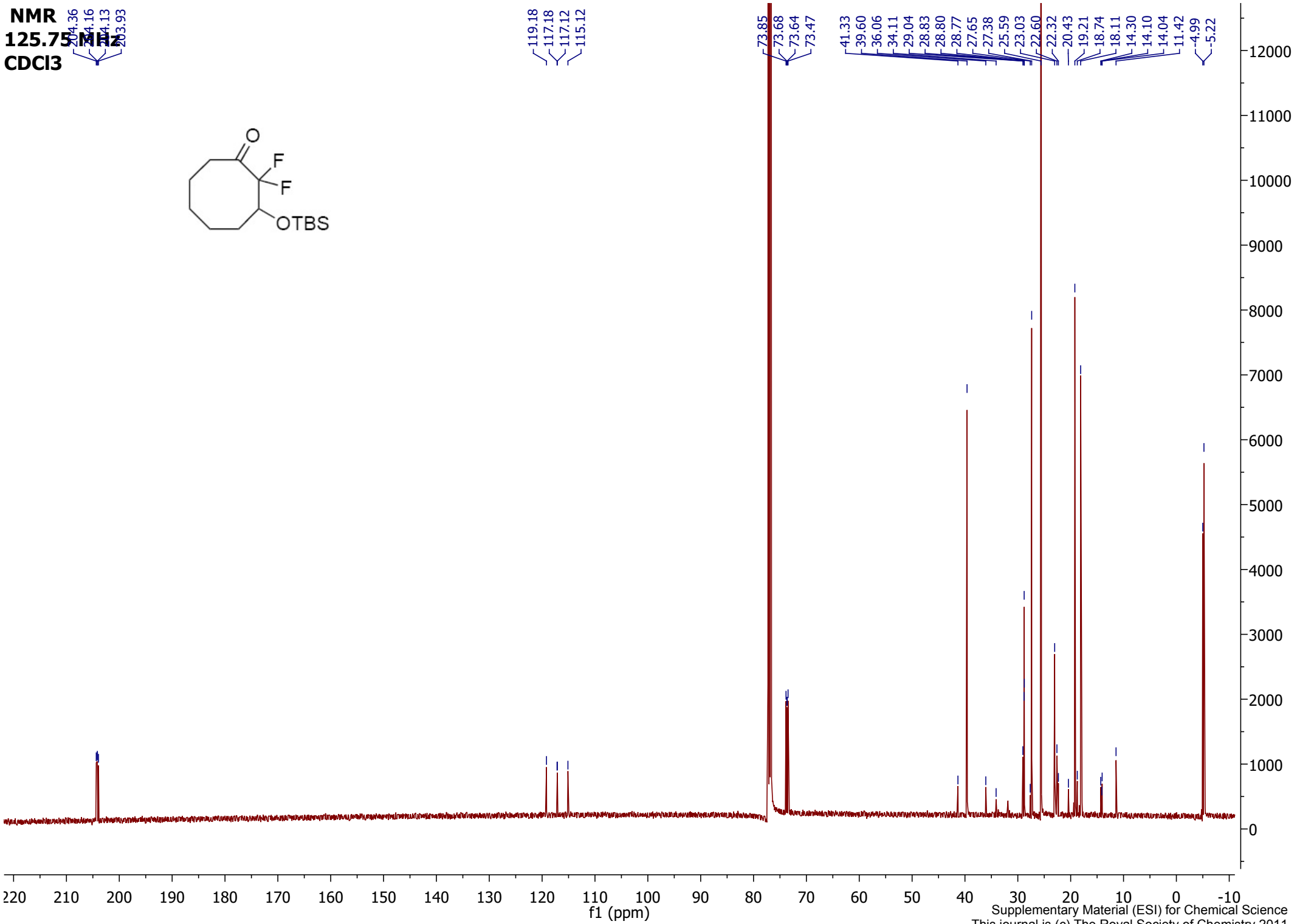
0.93
1.00

f1 (ppm)

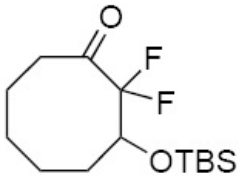
1H NMR
400.13 MHz
CDCl3



f1 (ppm)



19F NMR
376.50 MHz
CDCl3

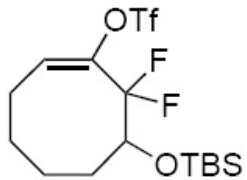


-110.61
-111.26
-122.17
-122.82

1.00
0.97

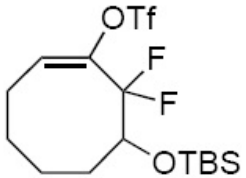
f1 (ppm)

1H NMR
400.13 MHz
CDCl3



f1 (ppm)

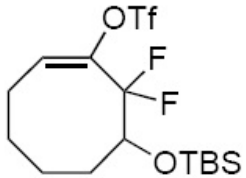
13C NMR
125.74 MHz
CDCl3

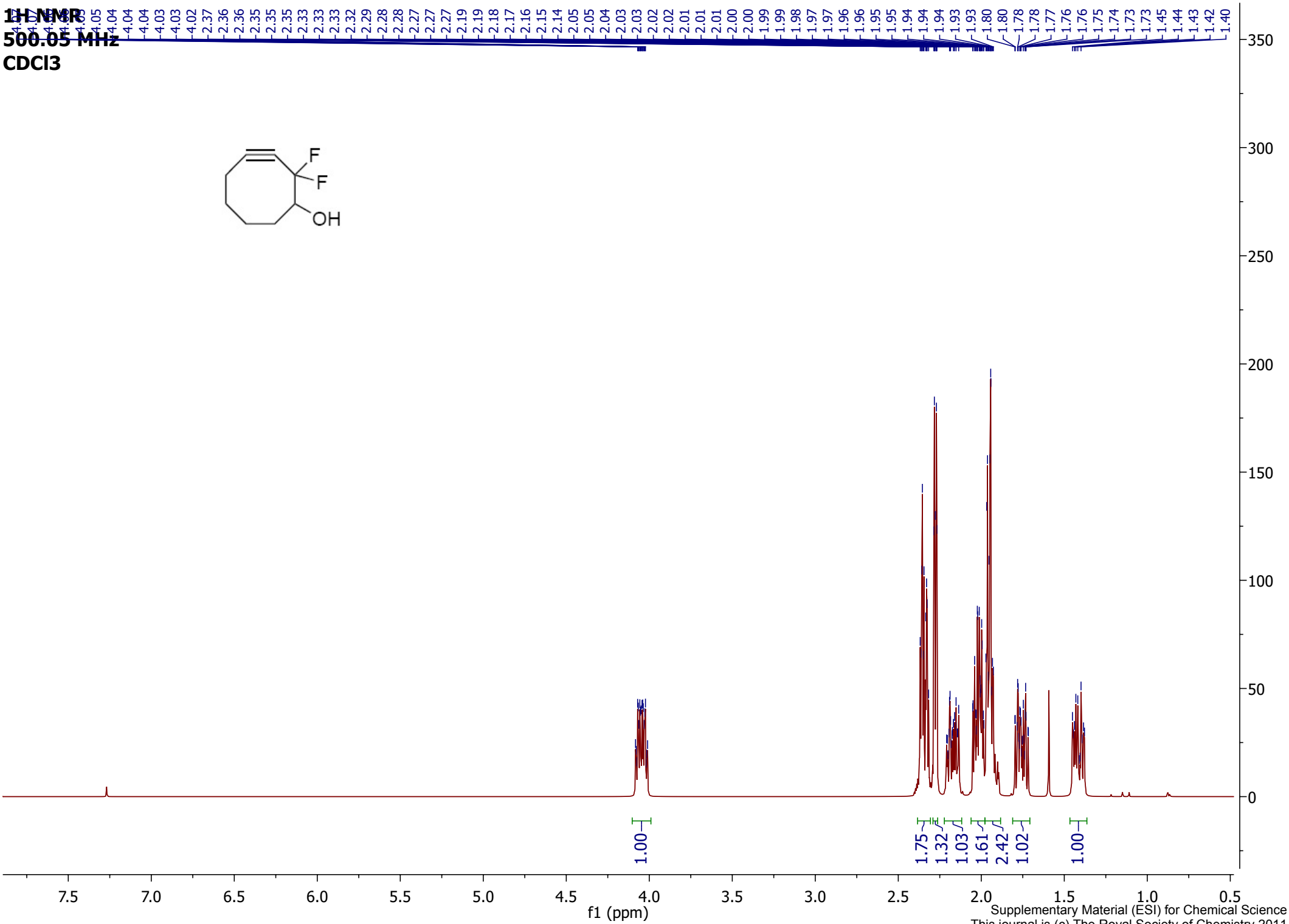


142.66
142.42
142.19
127.29
127.25
122.43
119.89
118.91
117.34
116.92
114.93
114.79
74.46
74.25
74.18
73.97
28.05
28.00
27.76
25.64
21.14
21.09
18.16
16.08
-5.07

f1 (ppm)

19F NMR
376.50 MHz
CDCl3





13C NMR
125.74 MHz
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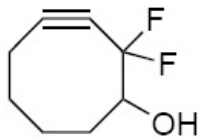
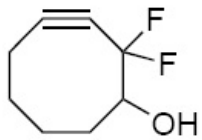


19F NMR
376.50 MHz
CDCl3

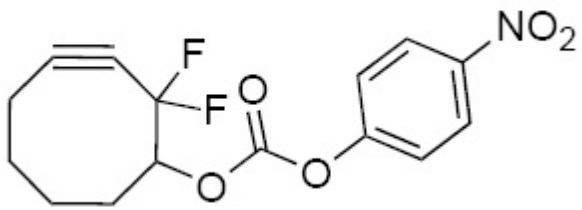


-101.79
-102.47
-110.94
-110.96
-110.97
-110.99
-111.01
-111.62
-111.64
-111.66
-111.68
-111.69

1.00
1.00

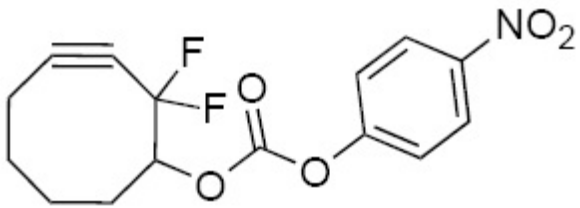
f1 (ppm)

1H NMR
400.13 MHz
CDCl3



f1 (ppm)

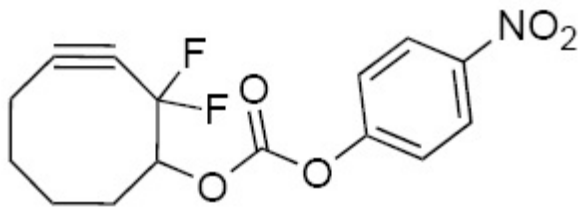
13C NMR
100.61 MHz
CDCl3

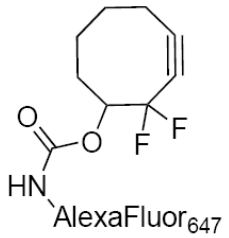


155.47
151.86
145.70
125.48
121.85
117.96
115.57
115.53
113.14
111.96
111.85
111.73
84.72
84.50
84.44
84.21
82.55
82.14
82.10
81.69
32.65
30.49
24.73
20.33

f1 (ppm)

NMR
376.45 MHz
CDCl3





Title :
Run File : C:\Star\data\Henning\difo647.run
Method File : C:\Star\Methods\Henning\Analysis2.mth
Sample ID : difo647

Injection Date: 29/04/10 09:34 Calculation Date: 29/04/10 10:02

Operator : Henning Detector Type: ProStar/Dynamax (2 Volts)
Workstation: Bus Address : 25
Instrument : Instrument #1 Sample Rate : 20.00 Hz
Channel : 1 = UV Run Time : 27.888 min

** Star Chromatography Workstation Version 5.52 ** 01689-3360-243-0711 **

Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: Percent

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
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Totals:		100.0000		0.000	30976			

Total Unidentified Counts : 30976 counts

Detected Peaks: 1 Rejected Peaks: 0 Identified Peaks: 0

Multiplier: 1 Divisor: 1 Unidentified Peak Factor: 0

Baseline Offset: 8577 microVolts

Noise (used): 36 microVolts - monitored before this run

Vial: 1 Injection Number: 1 ul Pickup Volume: 20 ul

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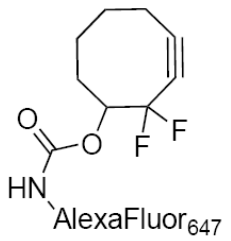
Title :
Run File : C:\Star\data\Henning\difo647.run
Method File : C:\Star\Methods\Henning\Analysis2.mth
Sample ID : difo647



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Operator : Henning
Workstation:
Instrument : Instrument #1
Channel : 1 = UV

Detector Type: ProStar/Dynamax (2 Volts)
Bus Address : 25
Sample Rate : 20.00 Hz
Run Time : 27.888 min

** Star Chromatography Workstation Version 5.52 ** 01689-3360-243-0711 **

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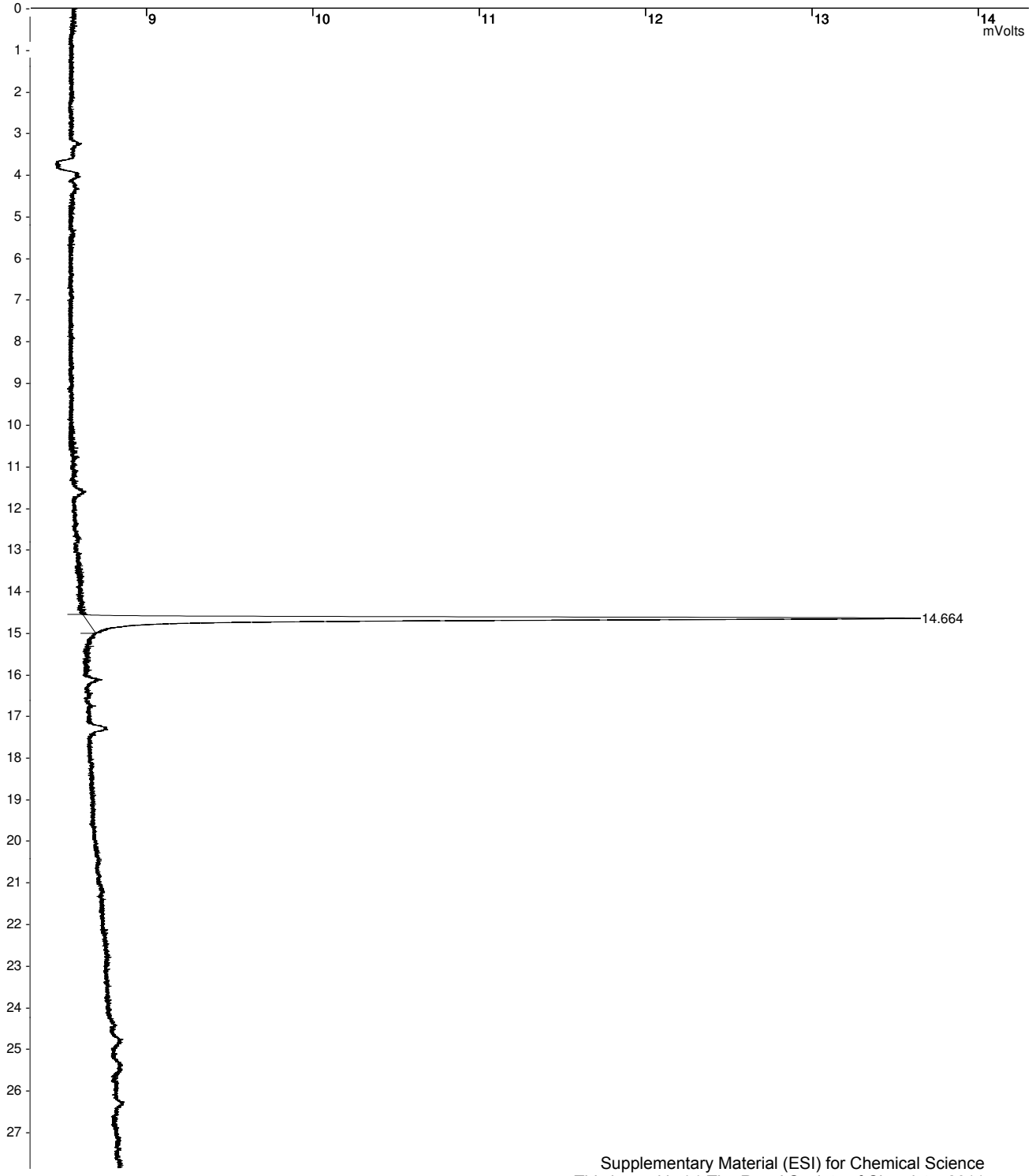
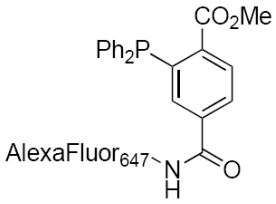




Title :
Run File : C:\Star\data\Henning\phos647.run
Method File : C:\Star\Methods\Henning\Analysis2.mth
Sample ID : PHOS647

Injection Date: 30/04/10 15:20 Calculation Date: 30/04/10 15:48

Operator : Henning
Workstation:
Instrument : Instrument #1
Channel : 1 = UV
Detector Type: ProStar/Dynamax (2 Volts)
Bus Address : 25
Sample Rate : 20.00 Hz
Run Time : 27.992 min

** Star Chromatography Workstation Version 5.52 ** 01689-3360-243-0711 **

Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: Percent

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
1		2.7862	14.556	0.000	8004	BB	5.3	
2		97.2138	17.784	0.000	279272	BB	6.6	
Totals:		100.0000		0.000	287276			

Total Unidentified Counts : 287276 counts

Detected Peaks: 4 Rejected Peaks: 2 Identified Peaks: 0

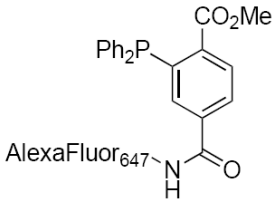
Multiplier: 1 Divisor: 1 Unidentified Peak Factor: 0

Baseline Offset: -3652 microVolts

Noise (used): 65 microVolts - monitored before this run

Vial: 1 Injection Number: 1 ul Pickup Volume: 20 ul

****

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Title       :
Run File    : C:\Star\data\Henning\phos647.run
Method File : C:\Star\Methods\Henning\Analysis2.mth
Sample ID   : PHOS647

Injection Date: 30/04/10 15:20    Calculation Date: 30/04/10 15:48

Operator    : Henning                Detector Type: ProStar/Dynamax (2 Volts)
Workstation:                         Bus Address  : 25
Instrument : Instrument #1           Sample Rate  : 20.00 Hz
Channel    : 1 = UV                  Run Time     : 27.992 min

** Star Chromatography Workstation Version 5.52 ** 01689-3360-243-0711 **

Chart Speed =    0.70 cm/min   Attenuation = 68            Zero Offset = 11%
Start Time  =   0.000   min     End Time    = 27.992  min   Min / Tick  = 1.00
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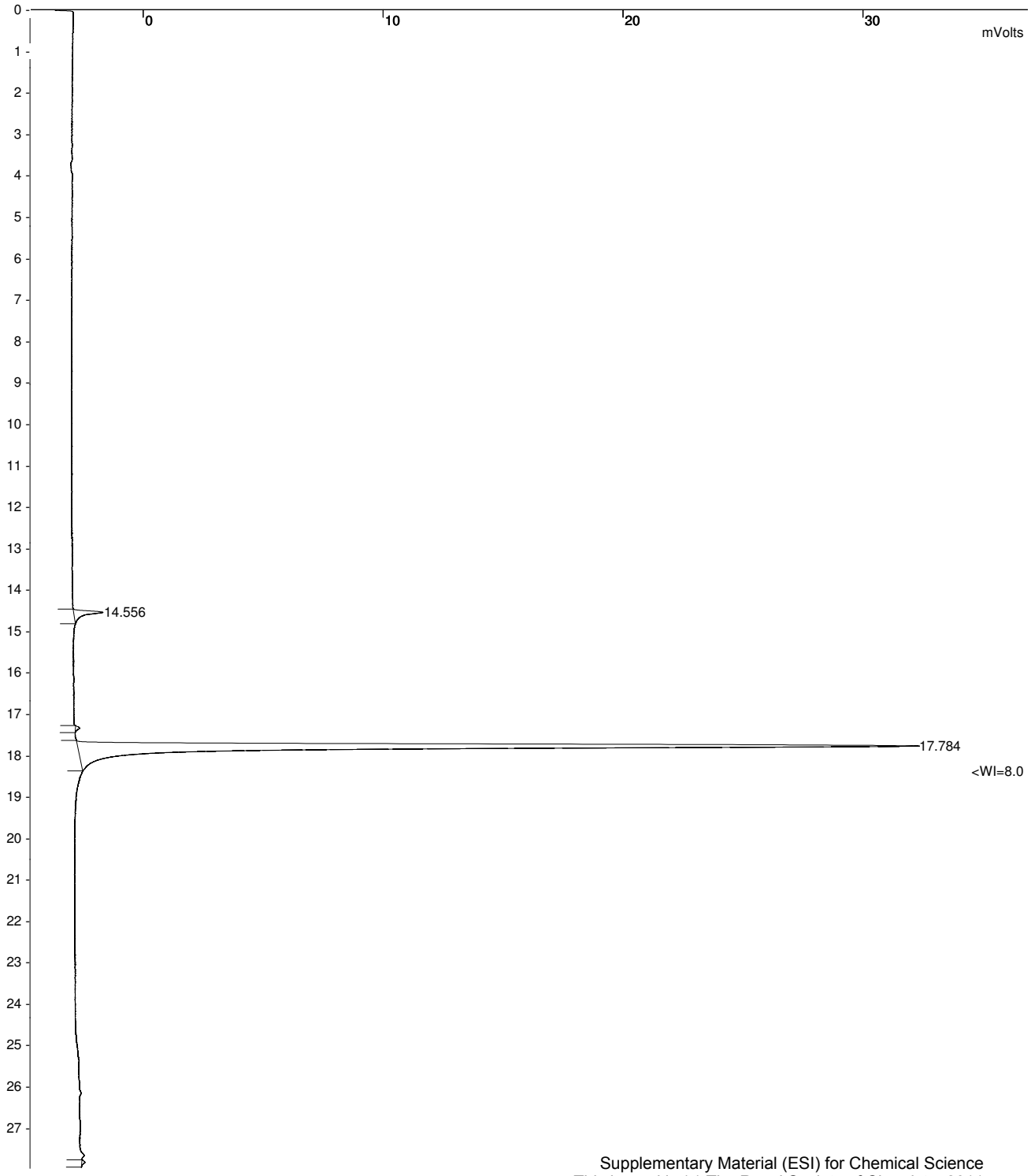
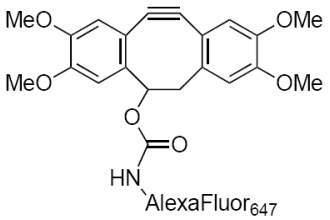




Print Date: Sat May 01 09:50:10 2010 Page 1 of 1

Title :
Run File : C:\Star\data\Henning\tmdibo-647.run
Method File : C:\Star\Methods\Henning\Analysis2.mth
Sample ID : TMDIBO647

Injection Date: 30/04/10 18:05 Calculation Date: 30/04/10 18:33

Operator : Henning Detector Type: ProStar/Dynamax (2 Volts)
Workstation: Bus Address : 25
Instrument : Instrument #1 Sample Rate : 20.00 Hz
Channel : 1 = UV Run Time : 27.987 min

** Star Chromatography Workstation Version 5.52 ** 01689-3360-243-0711 **

Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: Percent

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
1		100.0000	16.806	0.000	22861	PB	7.6	
Totals:		100.0000		0.000	22861			

Total Unidentified Counts : 22861 counts

Detected Peaks: 31 Rejected Peaks: 30 Identified Peaks: 0

Multiplier: 1 Divisor: 1 Unidentified Peak Factor: 0

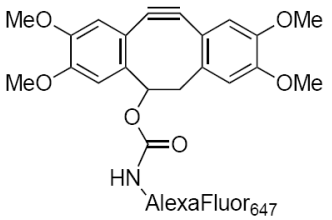
Baseline Offset: -3000 microVolts

Noise (used): 31 microVolts - monitored before this run

Vial: 1 Injection Number: 1 ul Pickup Volume: 15 ul

****

Title :
Run File : C:\Star\data\Henning\tmdibo-647.run
Method File : C:\Star\Methods\Henning\Analysis2.mth
Sample ID : TMDIBO647

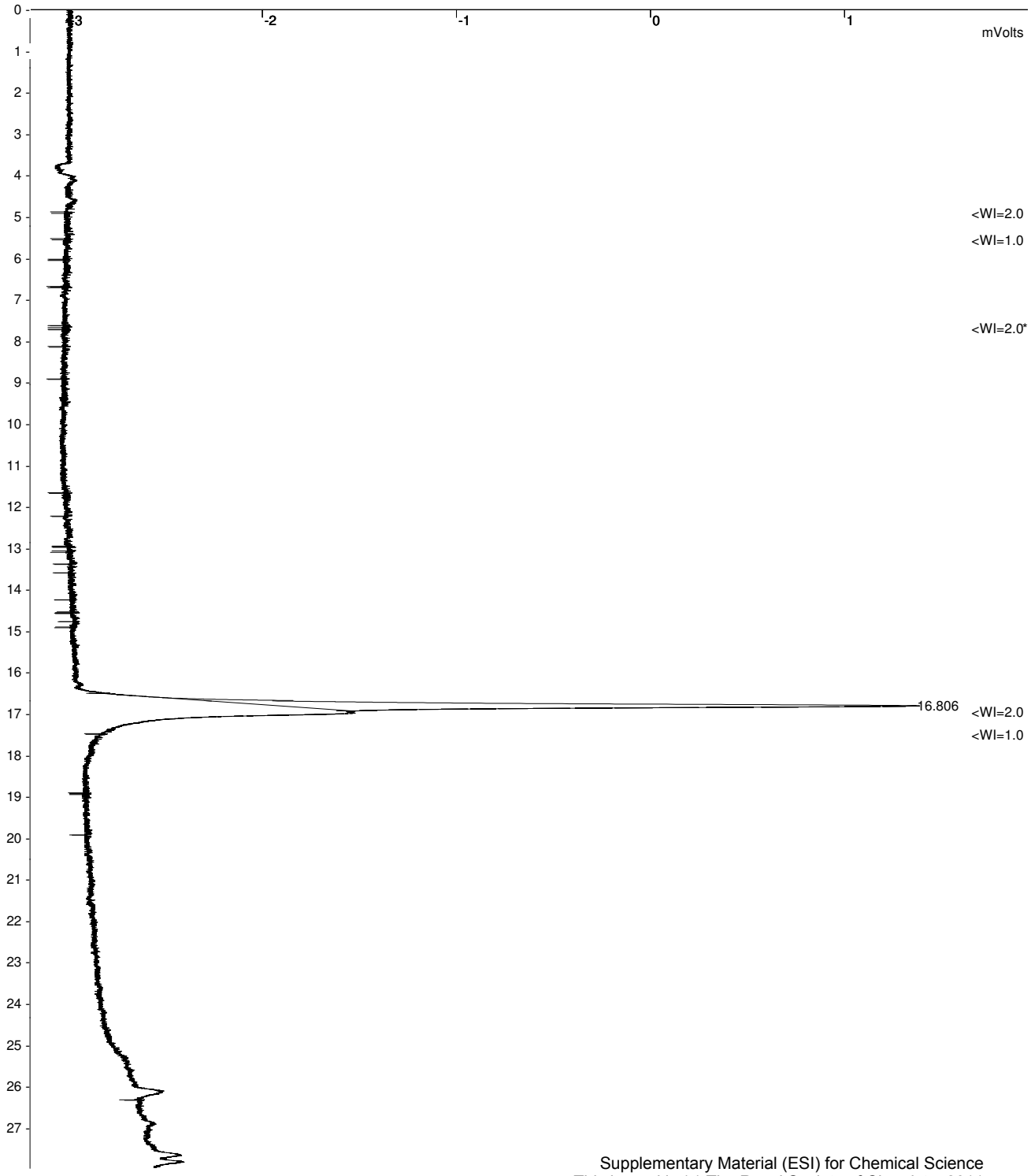


Injection Date: 30/04/10 18:05 Calculation Date: 30/04/10 18:33

Operator : Henning
Workstation:
Instrument : Instrument #1
Channel : 1 = UV
Detector Type: ProStar/Dynamax (2 Volts)
Bus Address : 25
Sample Rate : 20.00 Hz
Run Time : 27.987 min

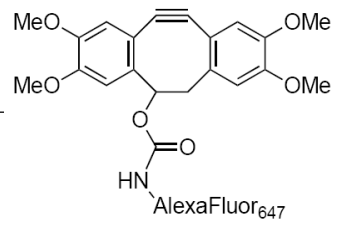
** Star Chromatography Workstation Version 5.52 ** 01689-3360-243-0711 **

Chart Speed = 0.70 cm/min Attenuation = 8 Zero Offset = 65%
Start Time = 0.000 min End Time = 27.987 min Min / Tick = 1.00



Sample: 1 Vial Number:4:24 ID:F_Leeper2197-1
File Name:F_Leeper2197-1 Date:29-Apr-2010 Time:18:36:00
Description:HS-TMDIBO647 Method:C:\Masslynx\650nm.olp

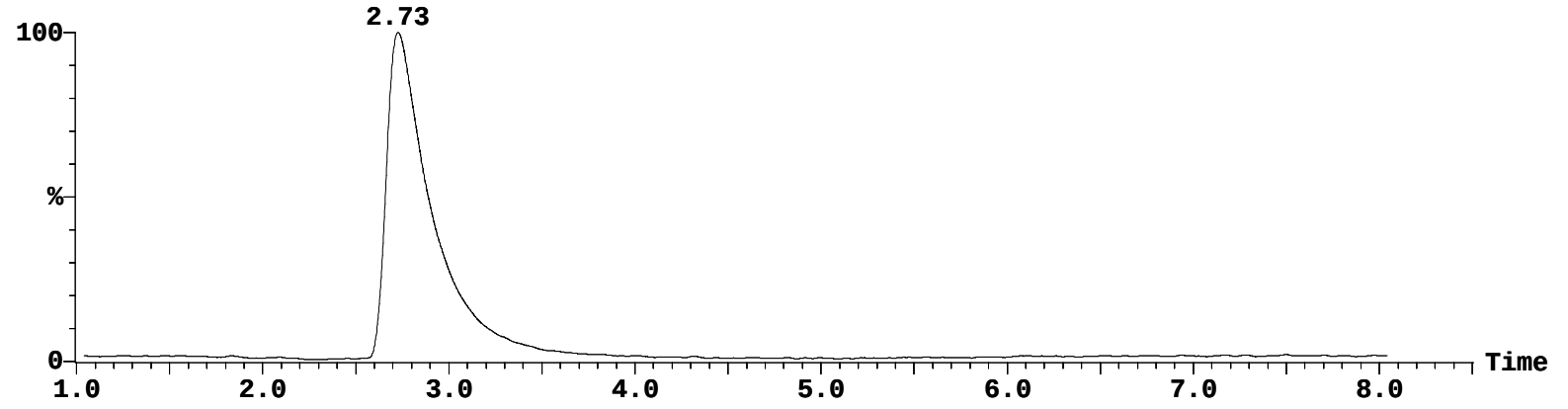
Printed: Tue Jun 01 16:46:46 2010



Sample Report:

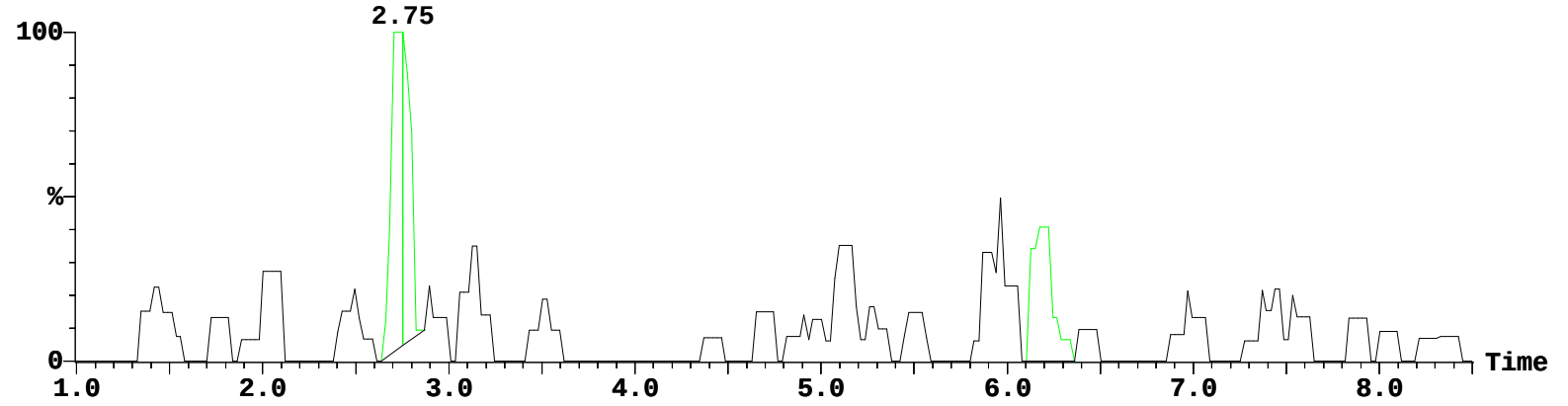
Sample 1 Vial 4:24 ID F_Leeper2197-1 File F_Leeper2197-1 Date 29-Apr-2010 Time 18:36:00 Description HS-TMDIBO647 Job

DAD: TIC **5.7e+005**



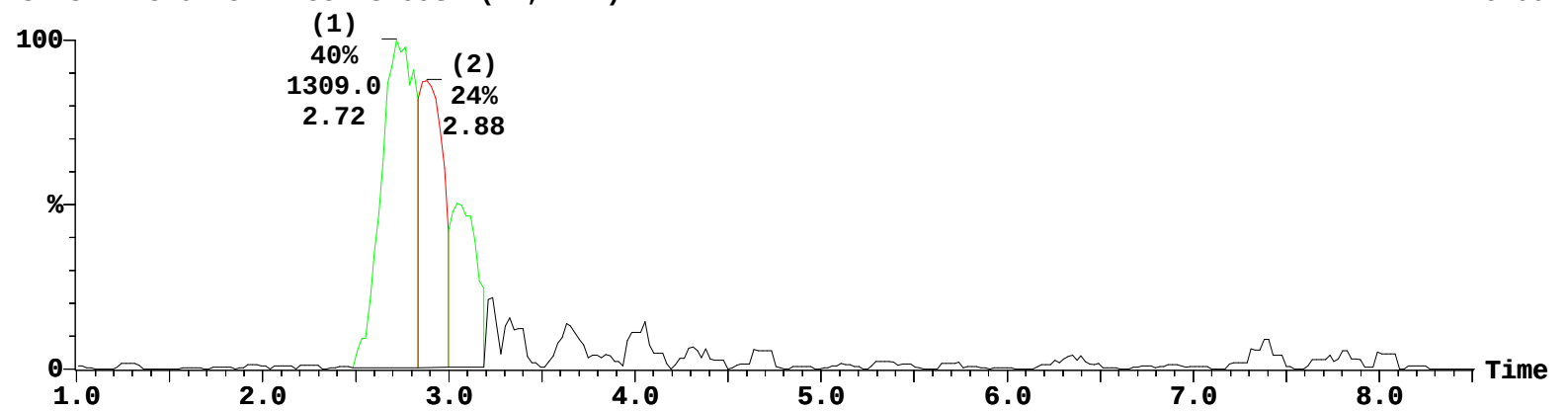
Peak Number	Time	AreaAbs	Area %Total	Height	Mass Found

MS ES- :1308+1354 Smooth (Mn, 1x2) **2.4e+003**



Peak Number	Retention Time	Peak Height	Mass Found	% Area	AreaAbs
1	2.71	2e+003		38.87	1.6650e+002
1	2.75	2e+003		26.09	1.1180e+002
4	6.17	1e+003	1309.00	31.72	1.3590e+002

MS ES+ :1310+1327+1332 Smooth (Mn, 1x2) **4.7e+004**



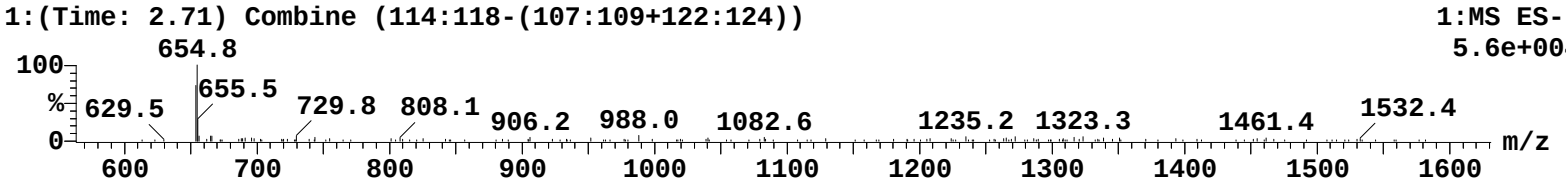
Peak Number	Retention Time	Peak Height	Mass Found	% Area	AreaAbs
1	2.72	5e+004		40.16	9.7735e+003
2	2.88	4e+004		24.49	5.9596e+003
3	3.05	2e+004	1309.00	15.37	3.7402e+003

Sample: 1 Vial Number:4:24 ID:F_Leeper2197-1
File Name:F_Leeper2197-1 Date:29-Apr-2010 Time:18:36:00
Description:HS-TMDIBO647 Method:C:\Masslynx\650nm.olp

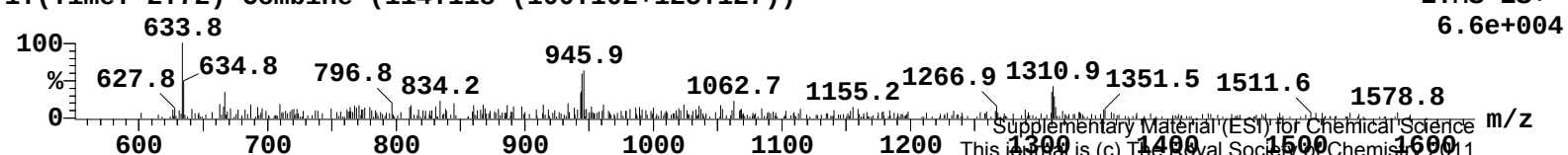
Printed: Tue Jun 01 16:46:46 2010

Sample Report (continued):

Peak Number	Retention Time	Mass Found
1	2.71	

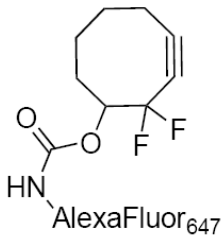


Peak Number	Retention Time	Mass Found
1	2.71	1309.00



Sample: 1 Vial Number:3:15 ID:F_Leeper2187-1
File Name:F_Leeper2187-1 Date:27-Apr-2010 Time:10:33:15
Description:hs-difo647 Method:C:\Masslynx\650nm.olp

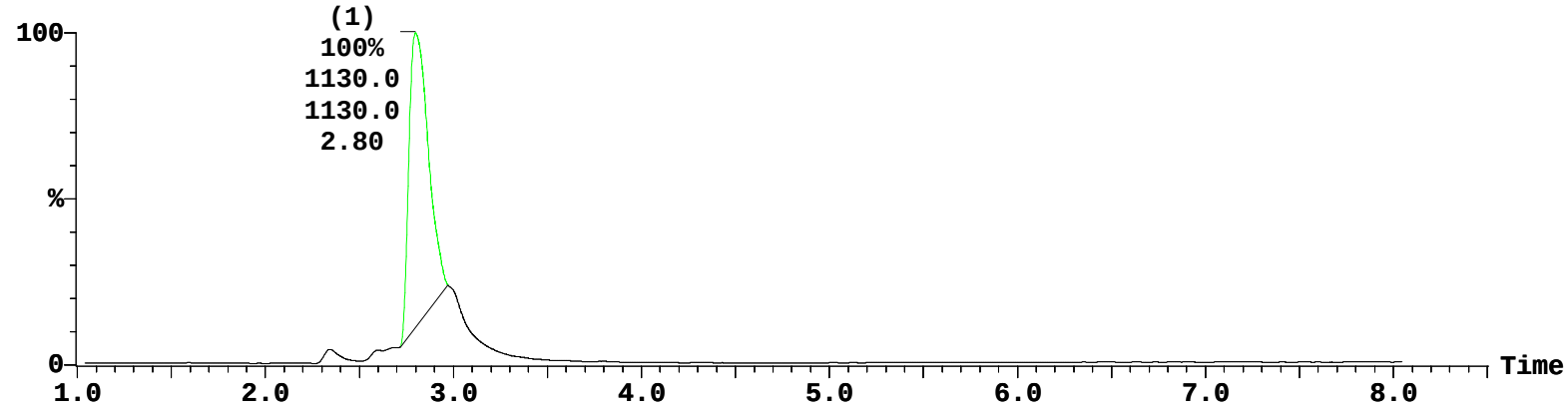
Printed: Tue Jun 01 16:48:13 2010



Sample Report:

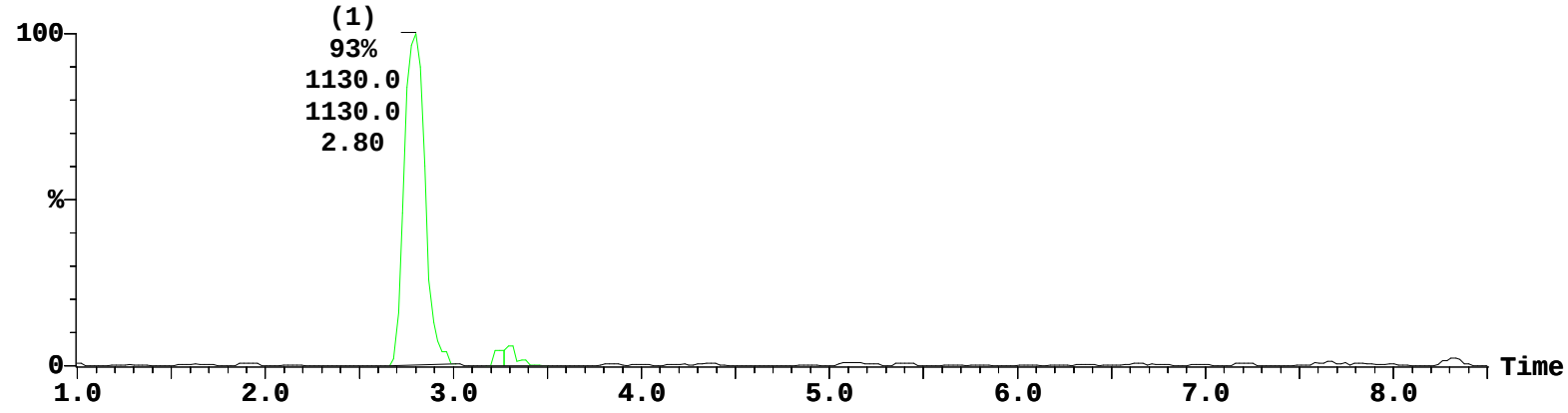
Sample 1 Vial 3:15 ID F_Leeper2187-1 File F_Leeper2187-1 Date 27-Apr-2010 Time 10:33:15 Description hs-difo647 Job Code

DAD: TIC **2.3e+006**



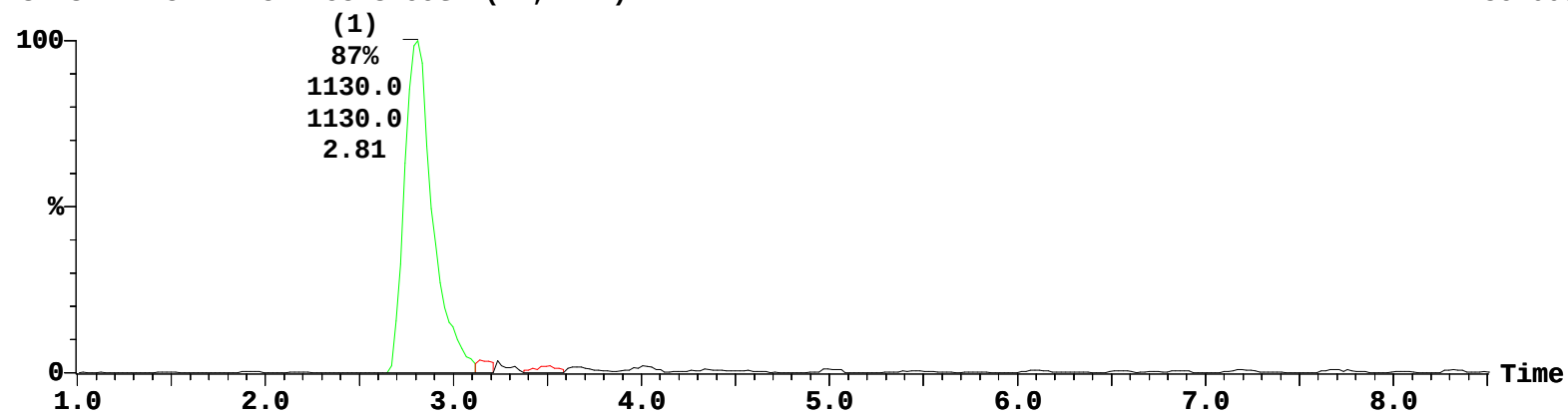
Peak Number	Time	AreaAbs	Area %Total	Height	Mass Found
1	2.80	2.4440e+005	100.00	2e+006	1130.00

MS ES- :1129+1175 Smooth (Mn, 1x2) **7.4e+004**



Peak Number	Retention Time	Peak Height	Mass Found	% Area	AreaAbs
1	2.80	7e+004	1130.00	92.59	9.5204e+003
3	3.22	3e+003	1130.00	1.93	1.9820e+002
4	3.29	4e+003	1130.00	3.34	3.4380e+002

MS ES+ :1131+1148+1153 Smooth (Mn, 1x2) **4.8e+005**



Peak Number	Retention Time	Peak Height	Mass Found	% Area	AreaAbs
1	2.81	5e+005	1130.00	86.61	8.4444e+004
2	3.14	2e+004		1.56	1.5171e+003
5	3.52	1e+004		1.42	1.3890e+003

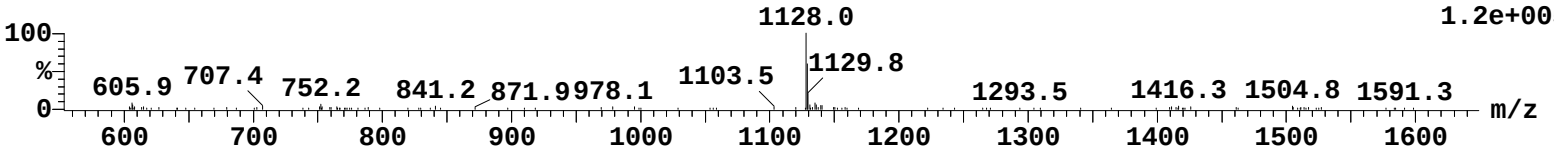
Sample: 1 Vial Number:3:15 ID:F_Leeper2187-1
File Name:F_Leeper2187-1 Date:27-Apr-2010 Time:10:33:15
Description:hs-difo647 Method:C:\Masslynx\650nm.olp

Printed: Tue Jun 01 16:48:13 2010

Sample Report (continued):

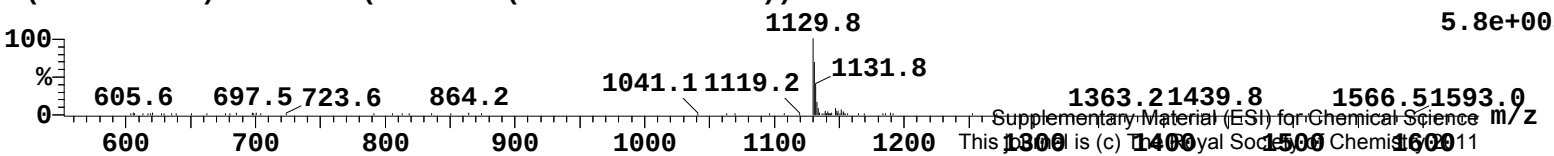
Peak Number	Retention Time	Mass Found
1	2.80	1130.00

1:(Time: 2.80) Combine (118:122-(110:112+132:134)) **1:MS ES-**



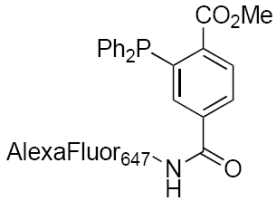
Peak Number	Retention Time	Mass Found
1	2.80	1130.00

1:(Time: 2.80) Combine (117:122-(110:112+131:133)) **2:MS ES+**



Sample: 1 Vial Number:4:38 ID:F_Leeper2202-1
File Name:F_Leeper2202-1 Date:01-May-2010 Time:08:26:
Description:HS-PHOS647 Method:C:\Masslynx\ABZ-MID.olp

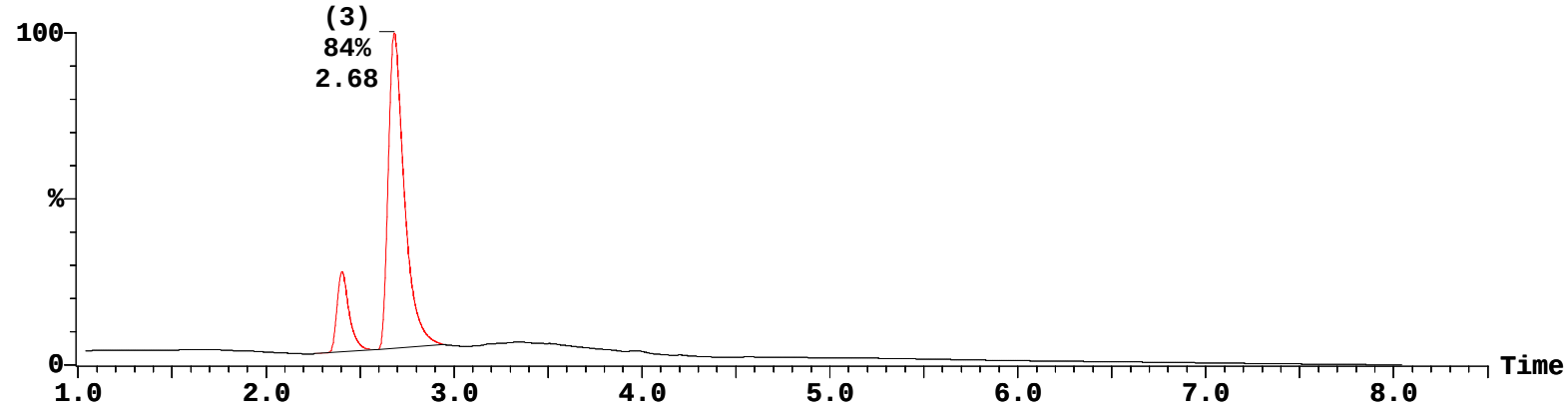
Printed: Tue Jun 01 16:49:01 2010



Sample Report:

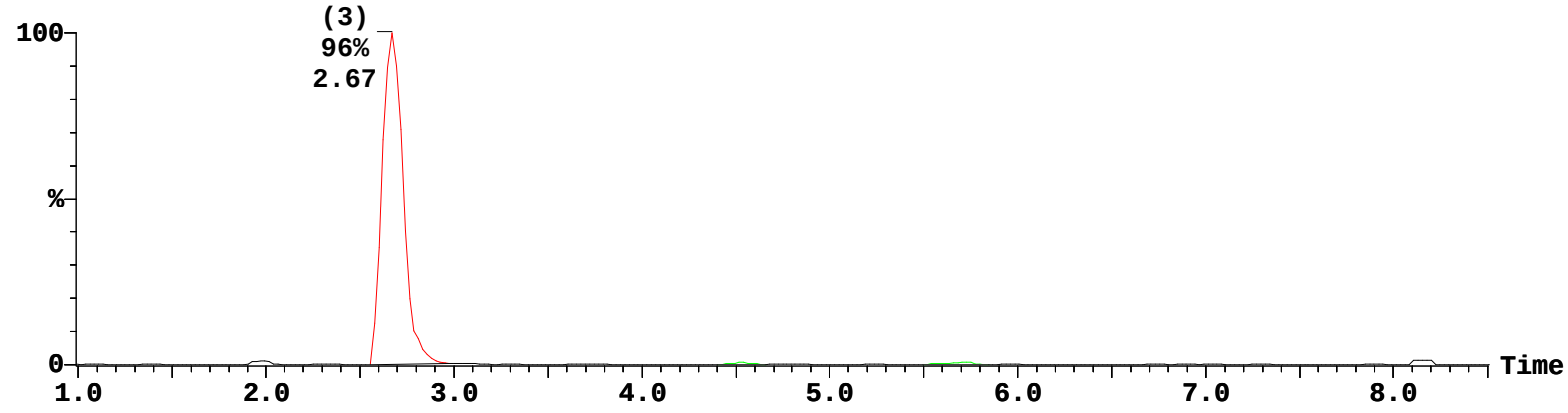
Sample 1 Vial 4:38 ID F_Leeper2202-1 File F_Leeper2202-1 Date 01-May-2010 Time 08:26:27 Description HS-PHOS647 Job Co

DAD: TIC **3.7e+007**



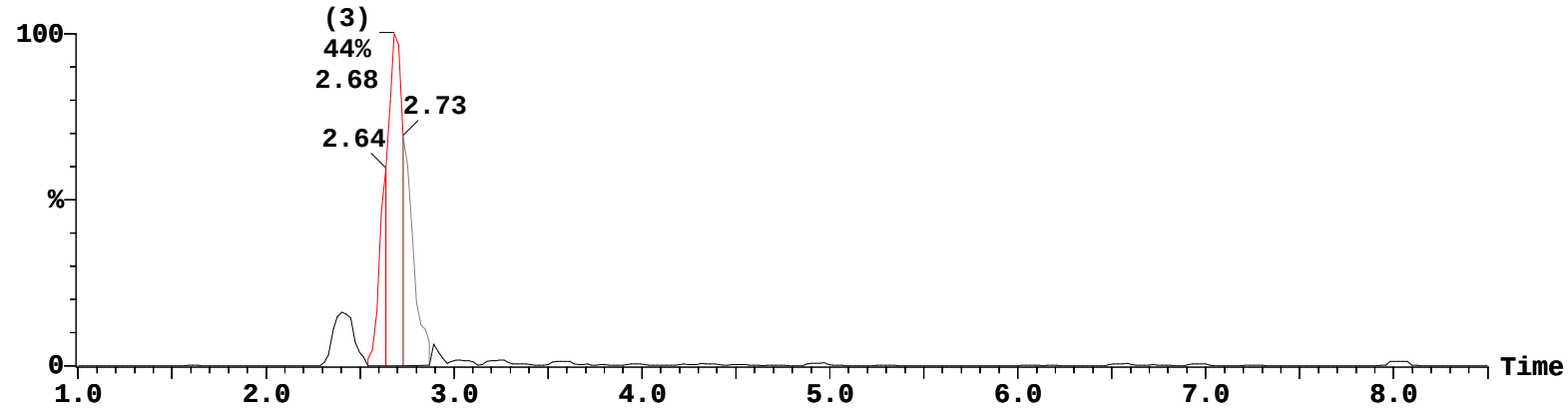
Peak Number	Time	AreaAbs	Area %Total	Height	Mass Found
1	2.40	6.7503e+005	16.13	9e+006	
3	2.68	3.5106e+006	83.87	4e+007	

MS ES- :1288+1334 Smooth (Mn, 1x2) **1.6e+005**



Peak Number	Retention Time	Peak Height	Mass Found	% Area	AreaAbs
3	2.67	2e+005		96.34	2.1134e+004
5	4.51	1e+003	1289.00	0.60	1.3180e+002
6	5.70	1e+003	1289.00	1.00	2.2040e+002

MS ES+ :1290+1307+1312 Smooth (Mn, 1x2) **6.9e+005**



Peak Number	Retention Time	Peak Height	Mass Found	% Area	AreaAbs
2	2.64	4e+005		12.68	1.5824e+004
3	2.68	7e+005		43.97	5.4862e+004
4	2.73	5e+005		23.42	2.9219e+004

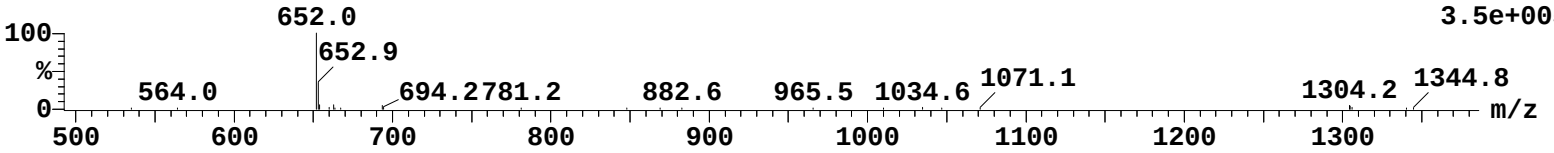
Sample: 1 Vial Number:4:38 ID:F_Leeper2202-1
File Name:F_Leeper2202-1 Date:01-May-2010 Time:08:26:27
Description:HS-PHOS647 Method:C:\Masslynx\ABZ-MID.olp

Printed: Tue Jun 01 16:49:01 2010

Sample Report (continued):

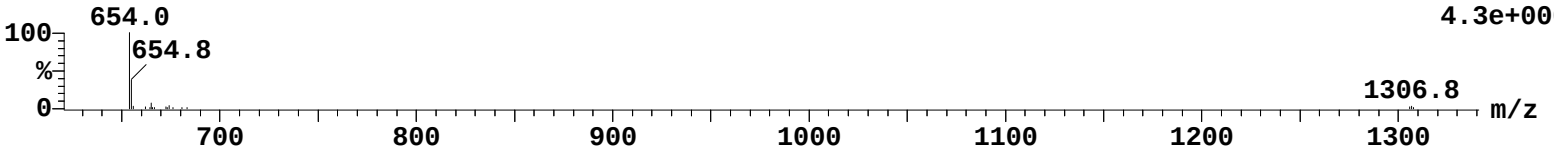
Peak Number	Retention Time	Mass Found
1	2.40	

1:(Time: 2.40) Combine (101:106-(91:93+115:118)) **1:MS ES- 3.5e+005**



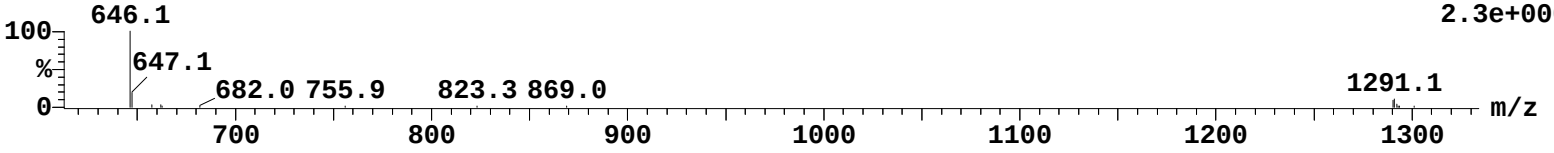
Peak Number	Retention Time	Mass Found
1	2.40	

1:(Time: 2.40) Combine (101:105-(91:93+115:117)) **2:MS ES+ 4.3e+006**



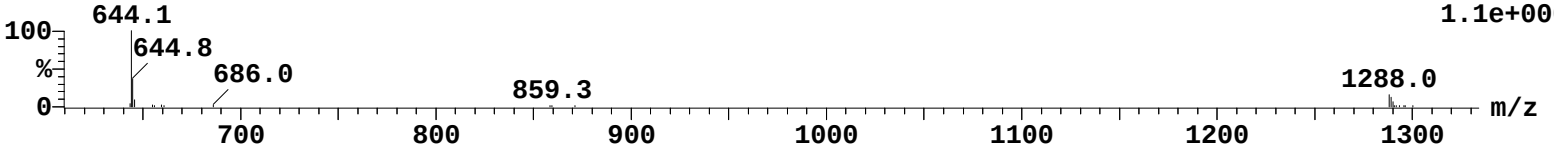
Peak Number	Retention Time	Mass Found
2	2.64	

2:(Time: 2.64) Combine (111:115-(103:105+117:119)) **2:MS ES+ 2.3e+006**



Peak Number	Retention Time	Mass Found
3	2.67	

3:(Time: 2.67) Combine (113:117-(104:106+132:134)) **1:MS ES- 1.1e+006**



Peak Number	Retention Time	Mass Found
3	2.67	

3:(Time: 2.68) Combine (113:117-(105:107+131:133)) **2:MS ES+ 6.4e+006**