Supplemental Information for:

N-Methyl deuterated rhodamines for protein labelling in sensitive fluorescence microscopy

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1. General

All chemical reagents and anhydrous solvents for synthesis were purchased from commercial suppliers (Sigma-Aldrich, Fluka, Acros, Fluorochem, TCI) and were used without further purification if not stated otherwise. Commercial coumarin 461 and methylene blue were HPLC purified before concentration assessment and measuring photophysical properties to ensure similar purity and composition as their synthesizes, deuterated counterparts. BG-TMR and BG-SiR were described before.[1]

NMR spectra were recorded at 300 K in deuterated solvents on a Bruker AVANCE III HD 400 equipped with a CryoProbe or on Bruker AV-III spectrometers using either a cryogenically cooled 5 mm TCI-triple resonance probe equipped with one-axis self-shielded gradients or room-temperature 5 mm broadband probe and calibrated to residual solvent peaks ($^{1}H/^{13}C$ in ppm): DMSO-d_6 (2.50/39.52), MeOD-d_4 (3.31/49.00). Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, h = heptet, br = broad, m = multiplet. Coupling constants *J* are reported in Hz. Spectra are reported based on appearance, not on theoretical multiplicities derived from structural information. This also concerns the report on ^{13}C NMR, where not all signals were obtained even after long-time recording (presumably quaternary carbons since they do not experience an NOE and relaxation is slower), which is annotated for the respective molecules below.

UPLC-UV/Vis for purity assessment was performed on a Waters H-class instrument equipped with a quaternary solvent manager, a Waters autosampler, a Waters TUV detector and a Waters Acquity QDa detector with an Acquity UPLC BEH C18 1.7 µm, 2.1 × 50 mm RP column (Waters Corp., USA). Buffer A: 0.1% TFA in H_2O Buffer B: 0.1% TFA in MeCN. The typical gradient was from 5% B for 0.5 min; gradient to 95% B over 3.0 min; 95% B for 0.9 min; gradient to 5% B over 1.1 min with 0.6 mL/min flow. LC-MS was performed on i) a Shimadzu MS2020 connected to a Nexera UHPLC system equipped with a Waters ACQUITY UPLC BEH C18 (1.7 µm, 50 × 2.1 mm). Buffer A: 0.1% FA in H_2O Buffer B: acetonitrile. The typical gradient was from 10% B for 0.5 min → gradient to 90% B over 4.5 min → 90% B for 0.5 min → gradient to 99% B over 0.5 min with 1 mL/min flow, or ii) an Agilent 1260 Infinity II LC System equipped with Agilent SB-C18 column (1.8 µm, 2.1 × 50 mm). Buffer A: 0.1% FA in H_2O Buffer B: 0.1% FA acetonitrile. The typical gradient was from 10% B for 0.5 min → gradient to 95% B over 5 min → 95% B for 0.5 min → gradient to 99% B over 1 min with 0.8 mL/min flow. Retention times (t_R) are given in minutes (min). Chromatograms were imported into Graphpad Prism8 and purity was determined by calculating AUC ratios.

Preparative or semi-preparative HPLC was performed on different instruments. i) A Shimadzu Prominence 20A system or a Shimadzu Prominence 8A system (both Shimadzu Corporation, Kyoto, Japan) equipped with columns as followed: preparative column – Nucleodur C18 HTec, 5 µm, 250x32 mm; semi-preparative column – Nucleodur C18 HTec, 5 µm, 250x21 mm (all columns purchased from Macherey-Nagel, GmbH & Co. KG, Düren, Germany). Eluents A (0.1% TFA in H_2O) and B (0.1% TFA in MeCN) were applied as a linear gradient. Peak detection was performed at maximal absorbance wavelength. ii) An Agilent 1260 Infinity II LC System equipped with columns as followed: preparative column –Reprospher 100 C18 columns (10 µm: 50 x 30 mm at 20 mL/min flow rate; semi-preparative column – 5 µm: 250 x 10 mm at 4 mL/min flow rate. Eluents A (0.1% TFA in H_2O) and B (0.1% TFA in MeCN) were applied as a linear gradient. Peak detection was performed at maximal absorbance wavelength. iii) A Waters e2695 system on a Supelco Ascentis® C18 HPLC Column (5 µm, 250 × 21.2 mm at 8 mL/min). Eluents A (0.1% TFA in H_2O) and B (0.1% TFA in MeCN) were applied as a linear gradient.

High resolution mass spectrometry was performed on different instruments. i) A Bruker maXis II ETD hyphenated with a Shimadzu Nexera system. The instruments were controlled via Brukers otofControl 4.1 and Hystar 4.1 SR2 (4.1.31.1) software. The acquisition rate was set to 3 Hz and the following source parameters were used for positive mode electrospray ionization: End plate offset = 500 V; capillary voltage = 3800 V; nebulizer gas pressure = 45 psi; dry gas flow = 10 L/min; dry temperature = 250 °C. Transfer, quadrupole and collision cell settings are mass range dependent and were fine-adjusted with consideration of the respective analyte's molecular weight. For internal calibration sodium format clusters were used. Samples were desalted via fast liquid chromatography. A Supelco TitanTM C18 UHPLC Column, 1.9 µm, 80 Å pore size, 20 × 2.1 mm and a 2 min gradient from 10 to 98% aqueous MeCN with 0.1% FA (H_2O: Carl Roth GmbH + Co. KG ROTISOLV® Ultra LC-MS; MeCN: Merck KGaA LiChrosolv® Acetonitrile hypergrade for LC-MS; FA - Merck KGaA LiChropur® Formic acid 98%–100% for LC-MS) was used for separation. Sample dilution in 10% aqueous MeCN (hyper grade) and injection volumes were chosen dependent of the analyte's ionization efficiency. Hence, on-column loadings resulted between 0.25–5.0 ng. Automated internal re-calibration and data analysis of the recorded spectra were performed with Bruker's DataAnalysis 4.4 SR1 software. ii) An Agilent Technologies 6230 series accurate mass TOF LC-MS linked to an Agilent Technologies 1290 Infinity Series machine with a Thermo Accucore™ RP-MS column, 2.6 µm pore size, 30 × 2.1 mm, and a 3 min gradient from 5 to 99% aqueous MeCN with 0.1% TFA and MeCN with 0.1% TFA. flow rate: 0.8 mL/min; UV-detection: 220 nm, 254 nm, 300 nm.

Intact proteins were analyzed using a Waters H-class instrument equipped with a quaternary Solvent manager, a Waters sample manager-FTN, a Waters PDA detector and a Waters column manager with an Acquity UPLC protein BEH C4 column (300 Å, 1.7 µm, 2.1 mm x 50 mm). Proteins were eluted with a flow rate of 0.3 mL/min at a column temperature of 80 °C. The following gradient was used: A: 0.01% FA in H_2O; B: 0.01% FA in MeCN. gradient 5-95% B from 0-6 min. Mass analysis was conducted with a Waters XEVO G2-XS QTof analyzer. Proteins were ionized in positive ion mode applying a cone voltage of 40 kV. Raw data was analyzed with MaxEnt 1. After deconvolution of the crude spectra, no single or non-labelled SNAP-Halo construct was observed, indicating complete reaction.

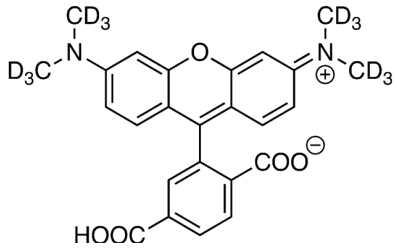
Flash column chromatography (FCC) was performed on a Biotage Isolera One with pre-packed silica columns (0.040–0.063 mm, 230-400 mesh, Silicycle). Reactions and chromatography fractions were monitored by thin layer chromatography (TLC) on Merck silica gel 60 F254 glass plates. The spots were visualized either under UV light at 254 nm and/or 366 nm or with appropriate staining method (iodine, *para*-anisaldehyde, $KMnO_4$) followed by heating.

2. Synthesis

2.1. General procedure A for fluorophore coupling

In an Eppendorf tube, 1.0 equiv. of deuterated carboxy-dye was dissolved in 100 µL/mg DMSO and 8.0 equiv. of DIPEA. Upon addition of 1.5 equiv. TSTU (from a 10 mg/mL stock in DMSO) the reaction mixture was vortexed and allowed to incubate for 10 min, before 1.5 equiv. of amine (BG-NH_2, CA-NH_2 or Mal-NH_2 (*N*-(2-Aminoethyl)-maleinimid-trifluoracetat; Aldrich: #56591) were added. The mixture was vortexed again and allowed to incubate for 60 min before it was quenched by addition of 20 equiv. of acetic acid and 25vol% of water. HPLC (MeCN:H_2O+0.1% TFA = 10:90 to 90:10 over 60 minutes) provided the desired compound, which was aliquoted to 5 nmol and obtained as a colorful powders after lyophilization.

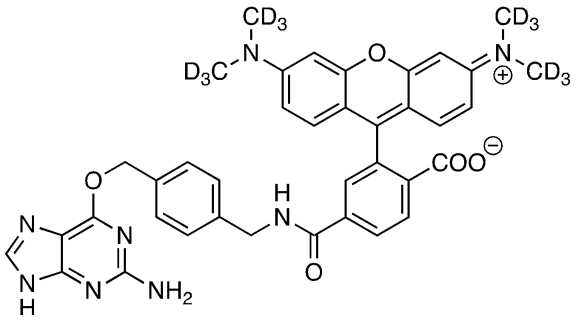
2.2. 2-(6-(Bis(methyl-d_3)amino)-3-(bis(methyl-d_3)iminio)-3*H*-xanthen-9-yl)-4-carboxybenzoate (2)



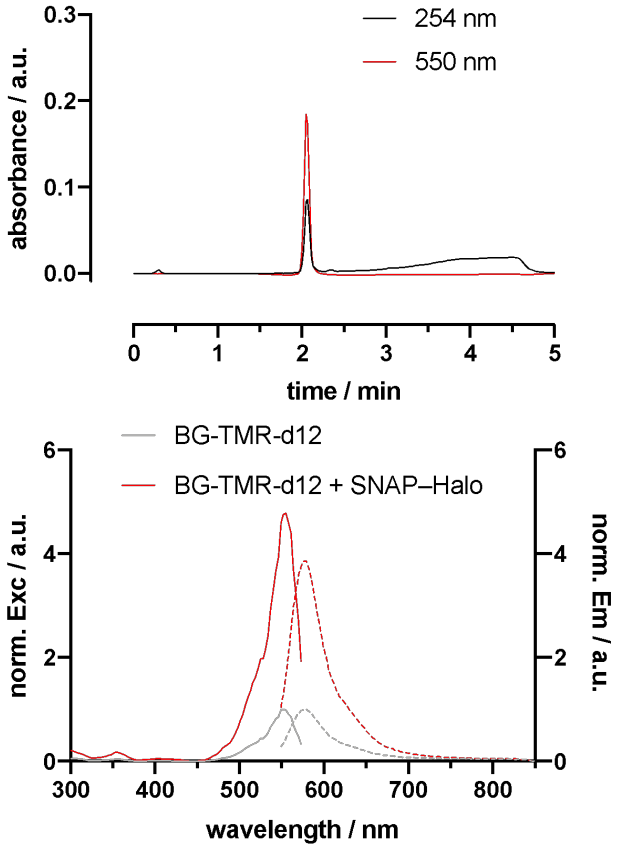
A Schlenk flask was charged under an argon atmosphere with 50.0 mg (71.8 µmol, 1.0 equiv.) of *tert*-butyl 3-oxo-3',6'-bis(((trifluoromethyl)sulfonyl)oxy)-3*H*-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylate[2] (**1**), 13.2 mg (14.4 µmol, 0.2 equiv.) of tris(dibenzylideneacetone)-dipalladium(0) (Pd_2dba_3), 10.3 mg (21.5 µmol, 0.3 equiv.) of 2-(dicyclohexylphosphino)-2',4',6'-tri*iso*propylbiphenyl (XPhos), 112 mg (344 µmol, 4.8 equiv.) of Cs_2CO_3 and 18.9 mg (215 µmol, 3.0 equiv.) of $(D_3C)_2NH$ x HCl), before 2 mL of dry 1,4-dioxane were added via syringe. The reaction mixture was heated to 100 °C over night before it was cooled to room temperature and all volatiles were removed *in vacuo*. 10% TFA in DCM was added to the crude and the deprotection step was allowed to incubate at r.t. over 4 hours. After removal of all volatiles and reuptake of the crude in MeCN:H_2O = 1:1, HPLC (MeCN:H_2O+0.1% TFA = 10:90 to 90:10 over 60 minutes) provided 22.0 mg (49.7 µmol) of the desired compound as a red powder in 69% yield.

^{1}H NMR (600 MHz, MeOD-d_4): δ [ppm] = 8.42–8.37 (m, 2H), 7.97 (d, J = 1.2 Hz, 1H), 7.14 (d, J = 9.5 Hz, 2H), 7.04 (dd, J = 9.5, 2.5 Hz, 2H), 6.96 (d, J = 2.4 Hz, 2H).
^{13}C NMR (150 MHz, MeOD-d_4): δ [ppm] = 167.7, 167.4, 160.4, 159.1, 136.1, 135.9, 135.4, 132.8, 132.3, 132.0, 115.6, 114.9, 97.4. Two carbon signals missing.
HRMS (ESI): calc. for $C_{25}H_{11}D_{12}N_2O_5$ $[M+H]^+$: 443.2355, found: 443.2352.

2.3. 4-((4-(((2-Amino-9*H*-purin-6-yl)oxy)methyl)benzyl)carbamoyl)-2-(6-(bis(methyl-d_3)amino)-3-(bis(methyl-d_3)iminio)-3*H*-xanthen-9-yl)benzoate (BG-TMR-d12)

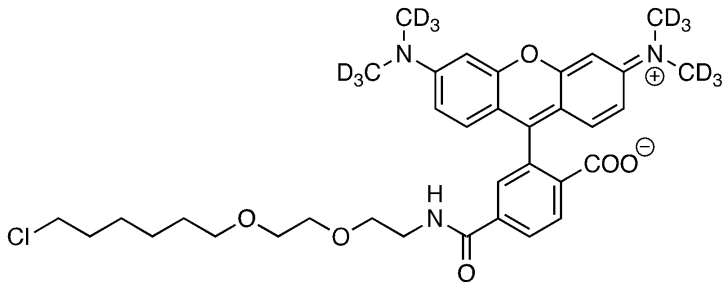


BG–TMR–d12 was prepared according to general procedure A.

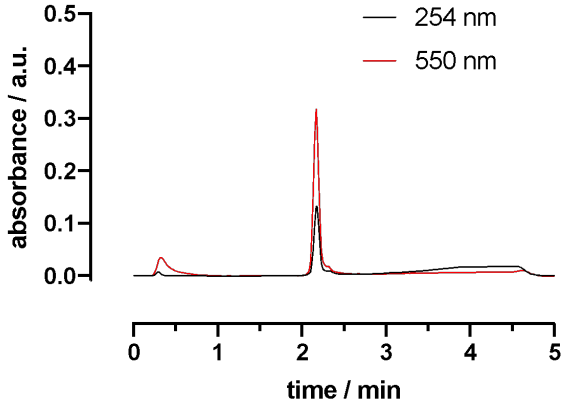


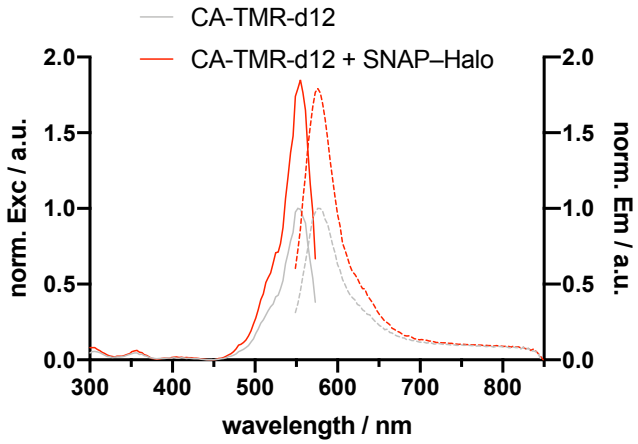
^{1}H NMR (600 MHz, MeOD): δ [ppm] = 8.41 (d, J = 8.3 Hz, 1H), 8.22 (d, J = 8.3, 1H), 8.20 (s, 1H), 7.85 (s, 1H), 7.51 (d, J = 7.9, 2H), 7.40 (d, J = 7.9 Hz, 2H), 7.14 (d, J = 9.5 Hz, 2H), 7.04 (dd, J = 9.5, 2.2 Hz, 2H), 6.98 (d, J = 2.2 Hz, 2H) 5.60 (s, 2H), 4.60 (s, 2H).
^{13}C NMR (150 MHz, MeOD): δ [ppm] = 167.8, 167.3, 161.1, 160.5, 159.1, 159.0, 154.4, 142.5, 140.4, 139.2, 135.9, 135.6, 135.0, 132.8, 132.0, 130.3, 130.0, 129.9, 128.9, 115.5, 114.8, 97.3, 70.2, 44.4. Two carbon signals missing.
HRMS (ESI): calc. for $C_{38}H_{24}D_{12}N_8O_5$ $[M+2H]^{2+}$: 348.1776, found: 348.1770.

2.4. 2-(6-(Bis(methyl-d$_3$)amino)-3-(bis(methyl-d$_3$)iminio)-3*H*-xanthen-9-yl)-4-((2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamoyl)benzoate (CA-TMR-d12)



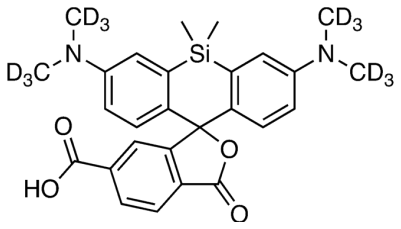
CA–TMR–d12 was prepared according to general procedure A.





^{1}H NMR (600 MHz, MeOD-d$_4$): δ [ppm] = 8.79 (t, J = 5.1 Hz, 1H), 8.41 (d, J = 8.2 Hz, 1H) 8.22 (dd, J = 8.3, 1.6 Hz, 1H), 7.83 (d, J = 1.4 Hz, 1H), 7.16 (d, J = 9.5 Hz, 2H) 7.06 (dd, J = 9.5, 2.4 Hz, 2H), 6.98 (d, J = 2.4 Hz, 2H), 3.66 (t, J = 5.3 Hz, 2H), 3.64–3.58 (m, 4H) 3.55 (m, 2H), 3.53 (t, J = 6.63 Hz, 2H), 3.43 (t, J = 6.5 Hz, 2H), 1.71 (quint, J = 7.1 Hz, 2H), 1.50 (quint, J = 7.0 Hz, 2H) 1.40 (quint, J = 7.5 Hz, 2H), 1.32 (quint, J = 5.1 Hz, 2H).
^{13}C NMR (150 MHz, MeOD-d$_4$): δ [ppm] = 168.0, 167.3, 160.6, 159.1, 159.1, 139.4, 135.5, 134.8, 132.8, 132.1, 130.3, 130.1, 115.5, 114.9, 97.4, 72.1, 71.2, 71.2, 70.4, 45.7, 41.2, 33.7, 30.5, 27.7, 26.4.
HRMS (ESI): calc. for $C_{35}H_{31}D_{12}ClN_3O_6$ $[M+H]^+$: 648.3588, found: 648.3587.

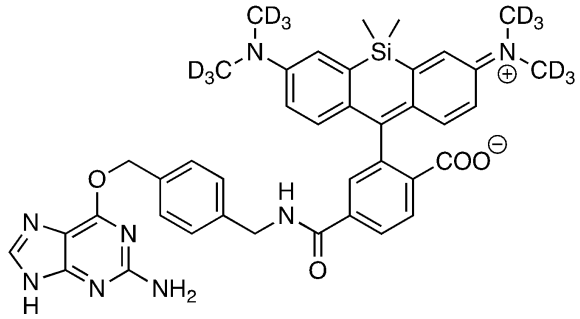
2.5. 3,7-Bis(bis(methyl-d$_3$)amino)-5,5-dimethyl-3'-oxo-3'*H*,5*H*-spiro[dibenzo[*b,e*]siline-10,1'-isobenzofuran]-6'-carboxylic acid (4)



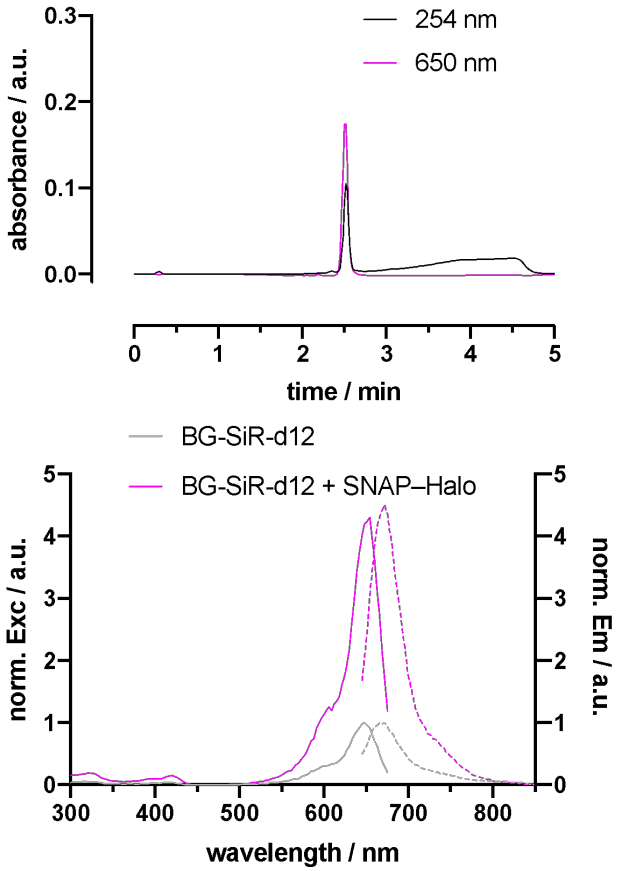
A Schlenk flask was charged under an argon atmosphere with 50.0 mg (67.7 µmol, 1.0 equiv.) of *tert*-butyl 5,5-dimethyl-3'-oxo-3,7-bis(((trifluoromethyl)sulfonyl)oxy)-3'*H*,5*H*-spiro[dibenzo[*b,e*]-siline-10,1'-isobenzo-furan]-6'-carboxylate[2] (**3**), 12.4 mg (13.5 µmol, 0.2 equiv.) of tris(dibenzylideneacetone)dipalladium(0) (Pd_2dba_3), 9.7 mg (20.3 µmol, 0.3 equiv.) of 2-(dicyclohexylphosphino)-2',4',6'-tri*iso*propylbiphenyl (XPhos), 106 mg (325 µmol, 4.8 equiv.) of Cs_2CO_3 and 17.8 mg (203 µmol, 3.0 equiv.) of $(D_3C)_2NH$ x HCl), before 2 mL of dry 1,4-dioxane were added via syringe. The reaction mixture was heated to 100 °C over night before it was cooled to room temperature and all volatiles were removed *in vacuo*. 10% TFA in DCM was added to the crude and the deprotection step was allowed to incubate at r.t. over 4 hours. After removal of all volatiles and reuptake of the crude in MeCN:H_2O = 1:1, HPLC (MeCN:H_2O+0.1% TFA = 10:90 to 90:10 over 60 minutes) provided 8.2 mg (16.9 µmol) of the desired compound as a purple-blue powder in 25% yield.

^{1}H NMR (600 MHz, MeOD-d$_4$): δ [ppm] = 8.34 (m, 2H), 7.83 (s, 1H), 7.35 (d, J = 2.75, 2H), 6.96 (d, J = 9.5 Hz, 2H), 6.77 (dd, J = 9.5, 2.4 Hz, 2H), 0.66 (s, 3H), 0.58 (s, 3H).
^{13}C NMR (150 MHz, MeOD-d$_4$): δ [ppm] = 168.1, 168.0, 154.8, 147.7, 144.3, 140.0, 135.6, 135.2, 131.7, 131.6, 131.1, 130.0, 121.5, 115.3, -0.7, -1.8. One carbon signal missing.
HRMS (ESI): calc. for $C_{27}H_{17}D_{12}N_2O_4Si$ $[M+H]^+$: 485.2644, found: 485.2645.

2.6. 4-((4-(((2-Amino-9*H*-purin-6-yl)oxy)methyl)benzyl)carbamoyl)-2-(7-(bis(methyl-d_3)amino)-3-(bis(methyl-d_3)iminio)-5,5-dimethyl-3,5-dihydrodibenzo[*b*,*e*]silin-10-yl)benzoate (BG-SiR-d12)

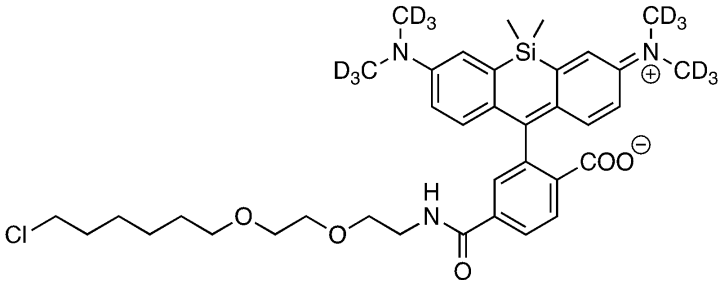


BG–SiR–d12 was prepared according to general procedure A.

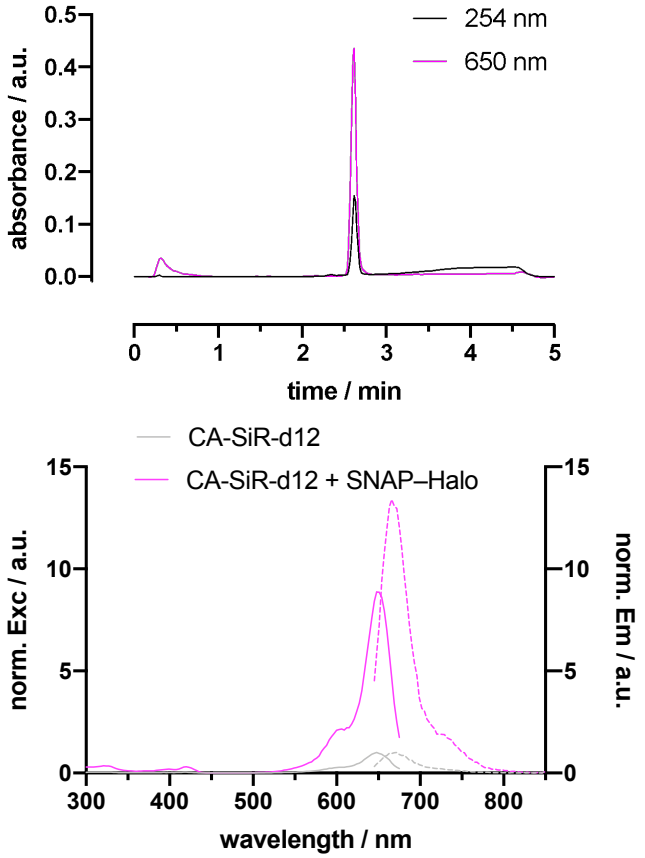


^{1}H NMR (600 MHz, MeOD-d_4): δ [ppm] = 8.33 (s, 1H), 8.30 (d, J = 8.2 Hz, 1H) 8.13 (d, J = 3.3 Hz, 1H), 7.72 (d, J = 1.1 Hz, 1H) 7.74 (d, J = 1.4 Hz, 1H), 7.50 (d, J = 8.0 Hz, 2H), 7.32 (d, J = 2.8 Hz, 2H), 6.95 (d, J = 9.5 Hz, 2H) 6.73 (dd, J = 9.5, 2.8 Hz, 2H) 5.62 (s, 2H), 4.58 (s, 2H), 0.64 (s, 3H), 0.58 (s, 3H).
^{13}C NMR (150 MHz, MeOD-d_4): δ [ppm] = 168.1, 167.9, 161.1, 158.0, 154.9, 153.4, 148.2, 144.1, 143.8, 140.7, 138.9, 135.4, 134.3, 131.9, 130.2, 129.9, 129.8, 129.0, 128.9, 121.5, 118.6, 116.7, 115.2, 70.9, 44.5, -0.8, -1.7. One carbon signal missing.
HRMS (ESI): calc. for $C_{40}H_{30}D_{12}N_8O_4Si$ $[M+2H]^{2+}$: 369.1920, found: 369.1917.

2.7. 2-(7-(Bis(methyl-d$_3$)amino)-3-(bis(methyl-d$_3$)iminio)-5,5-dimethyl-3,5-dihydrodibenzo[*b*,*e*]silin-10-yl)-4-((2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamoyl)benzoate (CA-SiR-d12)

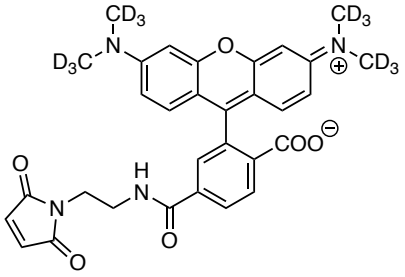


CA–SiR–d12 was prepared according to general procedure A.



^{1}H NMR (600 MHz, MeOD-d$_4$): δ [ppm] = 8.13 (d, J = 7.9 Hz, 1H), 8.12 (d, J = 8.2 Hz, 1H), 7.72 (s, 1H), 7.32 (d, J = 2.6 Hz, 2H), 6.98 (d, J = 9.5 Hz, 2), 6.75 (dd, J = 9.5, 2.4 Hz, 2H), 3.65 (t, J = 5.3 Hz, 2H), 3.63–3.60 (m, 2H), 3.60–3.54 (m, 4H), 3.51 (t, J = 6.6 Hz, 2H), 3.43 (t, J = 6.5 Hz, 2H), 1.71 (quint, J = 7.0 Hz, 2H), 1.51 (quint, J = 7.0 Hz, 2H), 1.40 (quint, J = 7.5 Hz, 2H), 1.32 (quint, J = 8.1 Hz, 2H), 0.64 (s, 3H), 0.59 (s, 3H).
^{13}C NMR (150 MHz, MeOD-d$_4$): δ [ppm] = 168.1 167.8, 155.1, 148.5, 140.9, 139.0, 134.4, 132.0, 129.8, 121.5, 115.1, 72.1, 71.1, 70.4 45.7, 41.1 33.7, 30.7, 30.4, 27.6, 26.4, -0.88, -1.68. Four carbon signals missing.
HRMS (ESI): calc. for $C_{37}H_{37}D_{12}ClN_3O_5Si$ $[M+H]^+$: 690.3878, found: 690.3874.

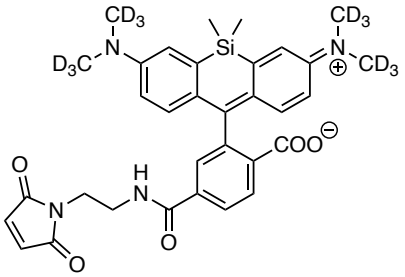
2.8. 2-(6-(Bis(methyl-d_3)amino)-3-(bis(methyl-d_3)iminio)-3*H*-xanthen-9-yl)-4-((2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)ethyl)carbamoyl)benzoate (Mal-TMR-d12)



Mal–TMR–d12 was prepared according to general procedure A.

^{1}H NMR (600 MHz, DMSO-d_6): δ [ppm] = 8.79 (t, J = 4.8 Hz, 1H), 8.50 (br s, 1H), 8.05–8.03 (m, 2H), 7.51 (s, 1H), 6.95 (s, 1H), 6.52–6.48 (m, 6H), 3.52 (t, J = 4.9 Hz, 2H), 3.35–3.32 (m, 2H).
^{13}C NMR (150 MHz, DMSO-d_6): δ [ppm] = 170.9, 168.1, 165.0, 152.8, 152.1, 152.0, 140.4, 134.5, 128.9, 128.4, 124.7, 122.3, 116.4, 109.0, 105.5, 97.9, 84.7, 37.8, 36.9.
HRMS (ESI): calc. for $C_{31}H_{17}D_{12}N_4O_6$ $[M+H]^+$: 565.2835, found: 565.2879.

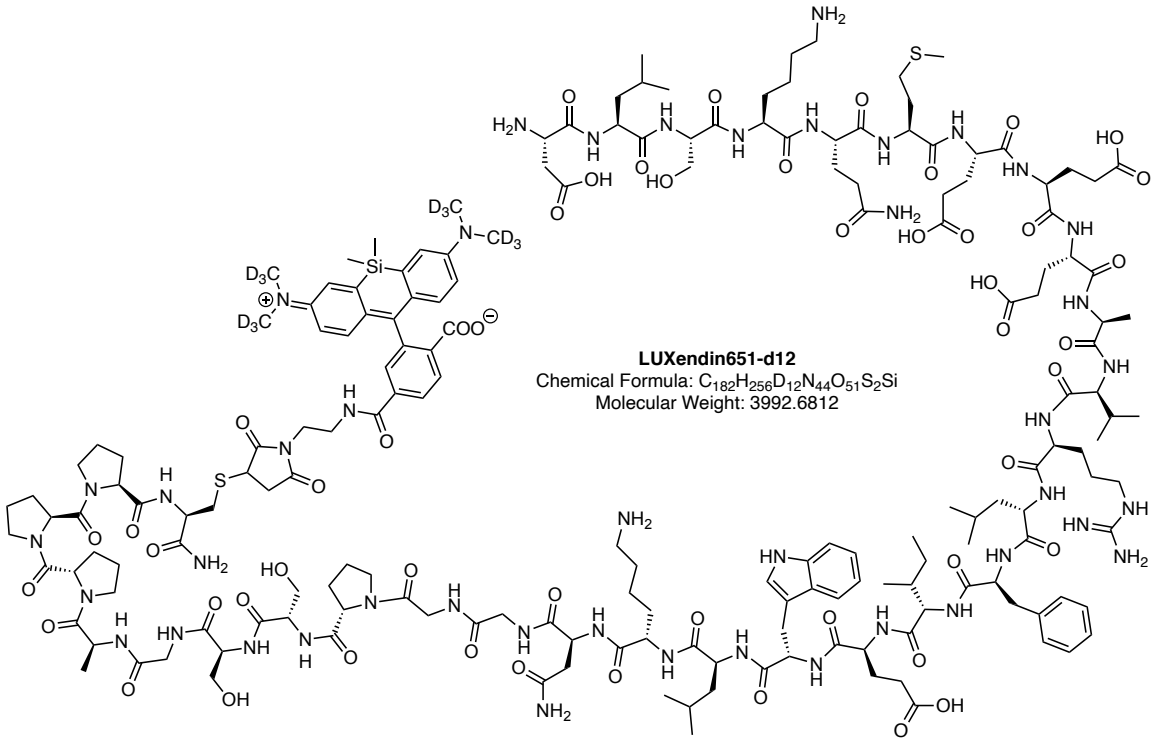
2.9. 2-(7-(Bis(methyl-d3)amino)-3-(bis(methyl-d3)iminio)-5,5-dimethyl-3,5-dihydrodibenzo[b,e]silin-10-yl)-4-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)carbamoyl)benzoate (Mal-SiR-d12)



Mal–SiR–d12 was prepared according to general procedure A.

^{1}H NMR (600 MHz, DMSO-d_6): δ [ppm] = 8.91 (t, J = 5.6 Hz, 0.5H), 8.82 (t, J = 6.0 Hz, 0.7H), 8.09–8.04 (m, 1.2 H), 7.96 (d, J = 8.0 Hz, 0.6H), 7.68 (s, 0.4H), 7.58 (s, 0.6H), 7.09 (br s, 1.5H), 6.96 (s, 0.7H), 6.71–6.65 (m, 3.0H), 3.56–3.50 (m, 2.1H), 3.37 (q, J = 5.7 Hz, 1.3H), 2.86 (q, J = 7.3 Hz, 1.8H), 2.58 (t, J = 7.0 Hz, 0.9H), 0.64 (s, 3H), 0.53–0.52 (m, 3H). Two isomers.
^{13}C NMR (150 MHz, DMSO-d_6): δ [ppm] = 172.6, 171.0, 165.2, 164.9, 158.5, 158.3, 158.1, 157.8, 149.3, 139.6, 134.5, 130.6, 128.2, 128.0, 125.6, 122.7, 114.6, 113.9, 38.8, 37.8, 36.9, 36.7, 33.6, 33.0, -0.1, -1.3, -1.4. Two isomers, some carbons missing.
HRMS (ESI): calc. for $C_{31}H_{22}D_{12}N_4O_5Si$ $[M+H]^+$: 607.3124, found: 607.3192.

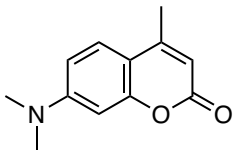
2.10. LUXendin651-d12



LUXendin651-d12 was prepared as described previously by using Mal-SiR-d_{12} instead of Mal-SiR.[3] Briefly, 400 nmol Ex4(9-39)_S39C obtained from solid-phase peptide synthesis[3] was incubated with 500 nmol SiR-d12-Mal in PBS and allowed to incubate at room temperature o.n. before the reaction was submitted to RP-HPLC to obtain 75 nmol of LUXendin651-d12 in 19% yield. LCMS trace is shown in Supporting Figure 2.

MS (ESI): calc. for $C_{182}H_{259}D_{12}N_{44}O_{51}S_2Si$ $[M+3H]^{3+}$: 1331.3315, found: 1331.9871.

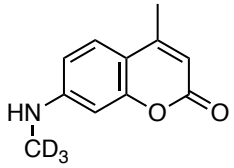
2.11. 7-(Dimethylamino)-4-methyl-2*H*-chromen-2-one (Coumarin 461)



Preparative RP-HPLC provided the desired compound as a faint yellow powder after lyophilization.

^{1}H NMR (600 MHz, DMSO-d_6): δ [ppm] = 7.52 (d, J = 8.9 Hz, 1H), 6.73 (dd, J = 8.9, 2.5 Hz, 1H), 6.53 (d, J = 2.5 Hz, 1H), 5.96 (d, J = 0.8 Hz, 1H), 3.02 (s, 3H), 2.34 (d, J = 0.8 Hz, 3H).
^{13}C NMR (150 MHz, DMSO-d_6): δ [ppm] = 161.1, 155.7, 154.0, 153.3, 126.4, 109.4, 109.2, 108.6, 97.9, 40.2, 18.4.
HRMS (ESI): calc. for $C_{12}H_{14}NO_2$ $[M+H]^+$: 204.1019, found: 204.1018.

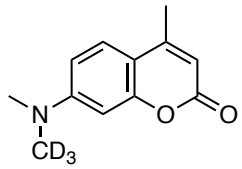
2.12. 4-Methyl-7-((methyl-d3)amino)-2*H*-chromen-2-one (6)



A round bottom flask was charged with 65.0 mg (371 µmol, 1.0 equiv.) of 7-amino-4-methyl-2*H*-chromen-2-one (**5**) and dissolved in 3 mL of MeCN before 102 mg (742.0 µmol, 2.0 equiv.) of K_2CO_3 and 135 mg (928 µmol, 57.8 µL, 2.5 equiv.) iodomethane-d_3 were added. The reaction mixture was heated to 80 °C for 3 h before it was cooled to room temperature and all volatiles were removed *in vacuo*. HPLC (MeCN:H_2O+0.1% TFA = 10:90 to 90:10 over 60 minutes) provided 12.5 mg (65.1 µmol) of the desired compound as a beige powdered side product in 18% yield. The d6 congener was isolated in 27% yield (*vide infra*).

^{1}H NMR (600 MHz, DMSO-d_6): δ [ppm] = 7.44 (d, J = 8.7 Hz, 1H), 6.63 (s, 1H), 6.58 (dd, J = 8.7, 2.3 Hz, 1H), 6.35 (d, J = 2.3 Hz, 1H), 5.91 (d, J = 0.9 Hz, 1H), 2.31 (d, J = 0.8 Hz, 3H).
^{13}C NMR (150 MHz, DMSO-d_6): δ [ppm] = 161.2, 156.2, 154.2, 153.9, 126.3, 110.5, 109.1, 107.8, 96.3, 18.5.
HRMS (ESI): calc. for $C_{11}H_9D_3NO_2$ $[M+H]^+$: 193.1051, found: 193.1051.

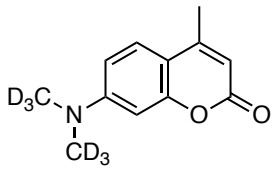
2.13. 4-Methyl-7-(methyl(methyl-d3)amino)-2*H*-chromen-2-one (Coumarin 461-d3)



A round bottom flask was charged with 12.0 mg (62.5 µmol, 1.0 equiv.) of 4-Methyl-7-((methyl-d3)amino)-2*H*-chromen-2-one (**6**) and dissolved in 3 mL of MeCN before 100 mg (725.0 µmol, 11.6 equiv.) of K_2CO_3 and 135 mg (2.4 mmol, 150 µL, 38 equiv.) iodomethane were added. The reaction mixture was heated to 80 °C for 12 h before it was cooled to room temperature and all volatiles were removed *in vacuo*. HPLC (MeCN:H_2O+0.1% TFA = 10:90 to 90:10 over 60 minutes) provided 10.0 mg (48.3 µmol) of the desired compound as a beige powdered side product in 77% yield.

^{1}H NMR (600 MHz, DMSO-d_6): δ [ppm] = 7.52 (d, J = 8.9 Hz, 1H), 6.72 (dd, J = 8.9, 2.6 Hz, 1H), 6.53 (d, J = 2.6 Hz, 1H), 5.96 (d, J = 1.1 Hz, 1H), 3.01 (s, 3H), 2.33 (d, J = 1.1 Hz, 3H).
^{13}C NMR (150 MHz, DMSO-d_6): δ [ppm] = 161.2, 155.7, 154.1, 153.4, 126.4, 109.4, 109.2, 108.6, 97.8, 18.4. One carbon masked by DMSO-d_6.
HRMS (ESI): calc. for $C_{12}H_{11}D_3NO_2$ $[M+H]^+$: 207.1207, found: 207.1205.

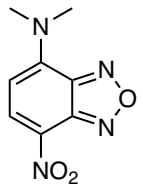
2.14. 7-(Bis(methyl-d3)amino)-4-methyl-2*H*-chromen-2-one (Coumarin 461-d6)



A round bottom flask was charged with 65.0 mg (371 µmol, 1.0 equiv.) of 7-amino-4-methyl-2*H*-chromen-2-one (**5**) and dissolved in 3 mL of MeCN before 102 mg (742.0 µmol, 2.0 equiv.) of K_2CO_3 and 135 mg (928 µmol, 57.8 µL2.5 equiv.) iodomethane-d_3 were added. The reaction mixture was heated to 80 °C for 3 h before it was cooled to room temperature and all volatiles were removed *in vacuo*. HPLC (MeCN:H_2O+0.1% TFA = 10:90 to 90:10 over 60 minutes) provided 21.2 mg (101.4 µmol) of the desired compound as a beige powder in 27% yield.

^{1}H NMR (600 MHz, DMSO-d_6): δ [ppm] = 7.52 (d, J = 8.9 Hz, 1H), 6.72 (dd, J = 8.9, 2.6 Hz, 1H), 6.53 (d, J = 2.5 Hz, 1H), 5.96 (d, J = 1.1 Hz, 1H), 2.33 (d, J = 1.1 Hz, 3H).
^{13}C NMR (150 MHz, DMSO-d_6): δ [ppm] = 161.2, 155.7, 154.0, 153.4, 126.3, 109.4, 108.5, 97.8, 18.4.
HRMS (ESI): calc. for $C_{12}H_8D_6NO_2$ $[M+H]^+$: 210.1394, found: 210.1396.

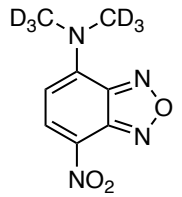
2.15. *N*,*N*-Dimethyl-7-nitrobenzo[*c*][1,2,5]oxadiazol-4-amine (NBD)



A round bottom flask was charged with 30.0 mg (150.3 µmol, 1.0 equiv.) of 4-chloro-7-nitro-2,1,3-benzoxadiazole (**7**) and dissolved in 3 mL of anhydrous EtOH before 24.3 mg (301 µmol, 2.0 equiv.) dimethylamine hydrochloride and 60 µL triethylamine (431 µmol, 3.0 equiv.) were added. The reaction mixture was stirred for 4 h before all volatiles were removed *in vacuo*. HPLC (MeCN:H_2O+0.1% TFA = 10:90 to 90:10 over 60 minutes) provided 18.6 mg (89.4 µmol) of the desired compound as a bright orange powder in 59% yield. Crystals suitable for X-ray crystallography were obtained as fine needles by allowing a 5 mg/mL solution in DMSO stand open to the atmosphere for one week.

^{1}H NMR (600 MHz, DMSO-d_6): δ [ppm] = 8.46 (d, J = 9.2 Hz, 1H), 6.36 (d, J = 9.2 Hz, 1H), 3.60 (br s, 6H).
^{13}C NMR (150 MHz, DMSO-d_6): δ [ppm] =146.6, 144.9, 144.8, 136.2, 119.7, 102.2, 43.5 (br).
HRMS (ESI): calc. for $C_8H_9N_4O_3$ $[M+H]^+$: 209.0669, found: 209.0673.

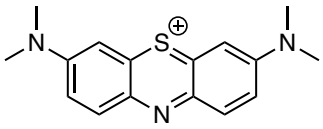
2.16. *N*,*N*-Bis(methyl-d3)-7-nitrobenzo[*c*][1,2,5]oxadiazol-4-amine (NBD-d6)



A round bottom flask was charged with 30.0 mg (150.3 µmol, 1.0 equiv.) of 4-chloro-7-nitro-2,1,3-benzoxadiazole (**7**) and dissolved in 3 mL of anhydrous EtOH before 26.3 mg (301 µmol, 2.0 equiv.) dimethyl-d6-amine hydrochloride and 60 µL triethylamine (431 µmol, 3.0 equiv.) were added. The reaction mixture was stirred for 4 h before all volatiles were removed *in vacuo*. HPLC (MeCN:H_2O+0.1% TFA = 10:90 to 90:10 over 60 minutes) provided 29.2 mg (136.4 µmol) of the desired compound as a bright orange powder in 91% yield. Crystals suitable for X-ray crystallography were obtained as fine needles by allowing a 5 mg/mL solution in DMSO stand open to the atmosphere for one week.

^{1}H NMR (600 MHz, DMSO-d_6): δ [ppm] = 8.46 (d, J = 9.2 Hz, 1H), 6.36 (d, J = 9.2 Hz, 1H).
^{13}C NMR (150 MHz, DMSO-d_6): δ [ppm] = 147.2, 145.3, 145.3, 136.7, 120.2, 102.6.
HRMS (ESI): calc. for $C_8H_3D_6N_4O_3$ $[M+H]^+$: 215.1046, found: 215.1046.

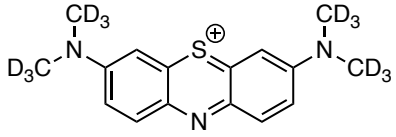
2.17. 3,7-Bis(dimethylamino)phenothiazin-5-ium (Methylene Blue)



Preparative RP-HPLC provided the desired compound as a blue powder after lyophilization.

^{1}H NMR (600 MHz, DMSO-d_6): δ [ppm] = 7.92 (d, J = 9.2 Hz, 2H), 7.51–7.48 (m, 4H), 3.37 (s, 12H).
^{13}C NMR (150 MHz, DMSO-d_6): δ [ppm] = 153.9, 137.8, 135.0, 133.5, 119.0, 106.8, 41.0.
HRMS (ESI): calc. for $C_{16}H_{18}N_3S$ $[M]^+$: 296.1969, found: 296.1972.

2.18. 3,7-Bis(bis(methyl-d3)amino)phenothiazin-5-ium (Methylene Blue-d12)

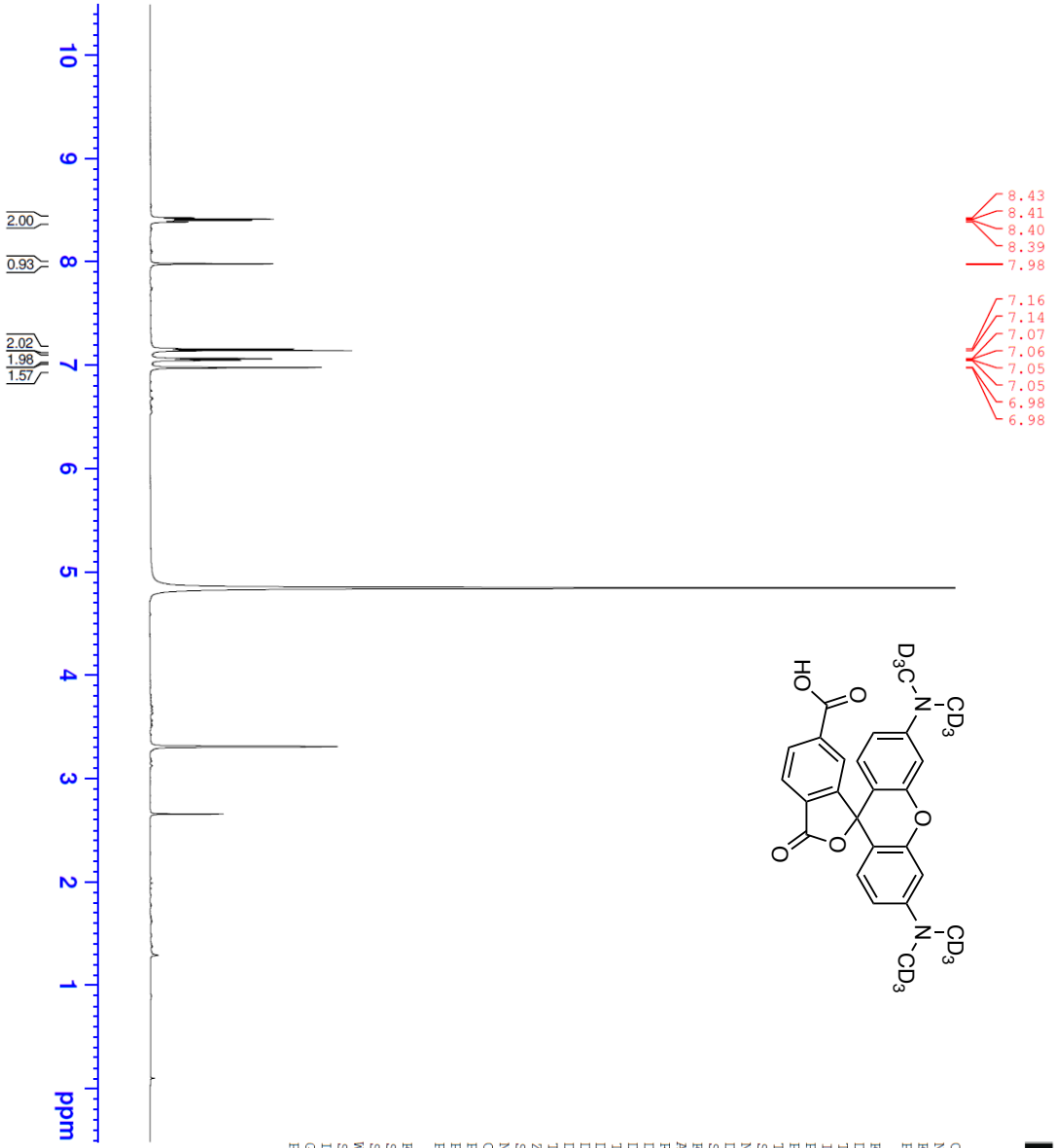


A round bottom flask was charged with 20 mg (69.6 µmol, 1.0 equiv.) of thionine acetate (**8**) and dissolved in 5 mL of MeCN before 96 mg (696 µmol, 10.0 equiv.) of K_2CO_3 and 101 mg (696 µmol, 44.3 µL, 10.0 equiv.) iodomethane-d_3 were added. The reaction mixture was heated to 80 °C for 3 h before it was cooled to room temperature and all volatiles were removed *in vacuo*. Preparative RP-HPLC provided 14 mg (34.2 µmol as TFA salt) of the desired compound as a blue powder after lyophilization in 49% yield.

^{1}H NMR (600 MHz, DMSO-d_6): δ [ppm] = 7.86 (d, J = 9.0 Hz, 2H), 7.45–7.42 (m, 4H).
^{13}C NMR (150 MHz, DMSO-d_6): δ [ppm] = 154.0, 137.9, 135.0, 133.6, 119.0, 106.7.
HRMS (ESI): calc. for $C_{16}H_6D_{12}N_3S$ $[M]^+$: 284.1216, found: 284.1216.

3. NMR spectra

3.1. 2-(6-(Bis(methyl-d_3)amino)-3-(bis(methyl-d_3)iminio)-3*H*-xanthen-9-yl)-4-carboxybenzoate (2)



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PROCNO                 1

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SOLVENT         MeOD
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AQ            1.6384000 sec
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DE                6.50 usec
TE              300.0 K
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D12             0.00002000 sec
TD0                    1
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CNST1          1.0000000
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PLW1          10.00000000 W

F2 - Processing parameters
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PC                 1.00
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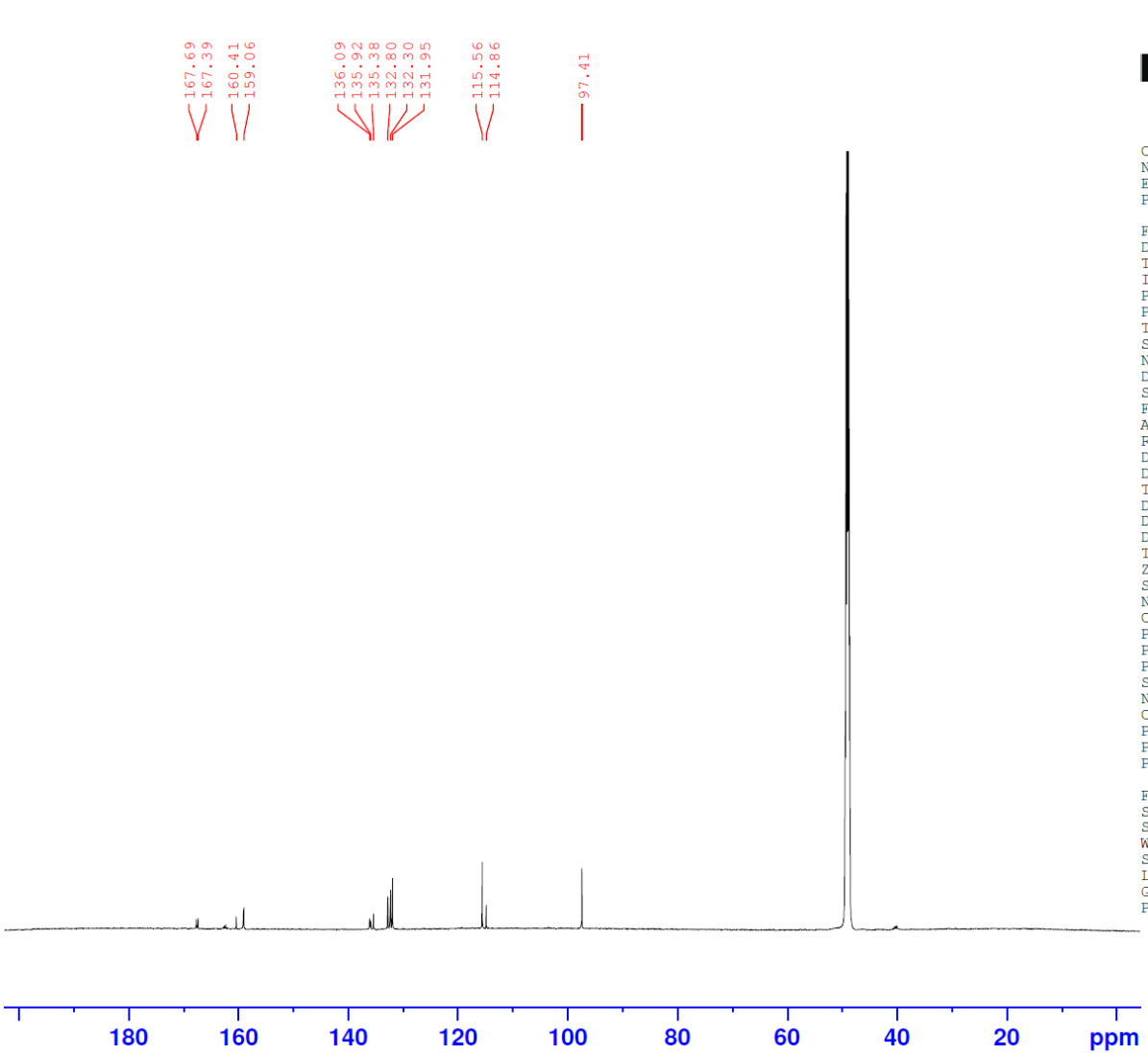

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D12 0.00002000 sec
TD0 1
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NUC1 13C
CNST1 1.0000000
P0 12.00 usec
P3 12.00 usec
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PCPD2 80.00 usec
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PLW19 0.08100000 W

F2 - Processing parameters
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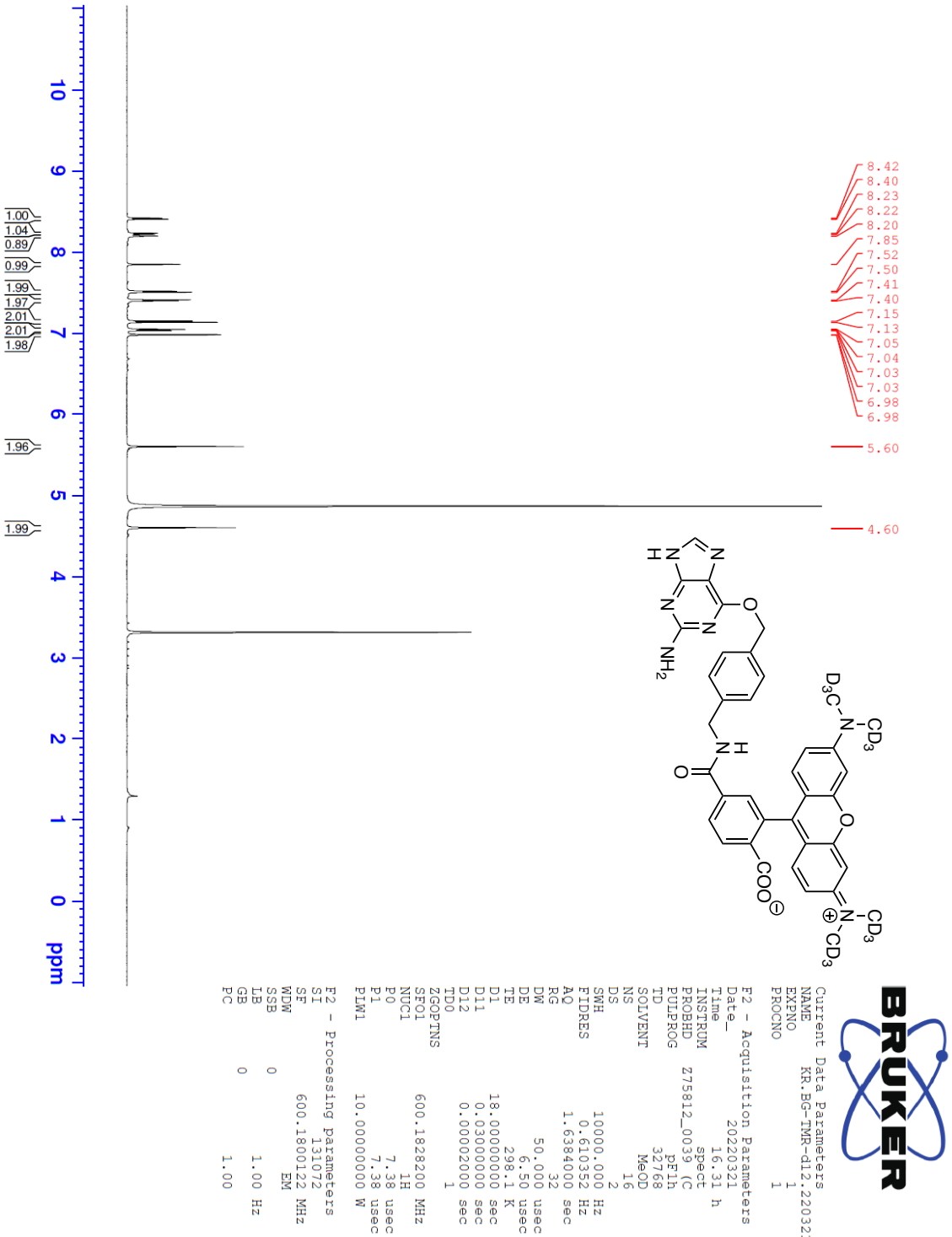
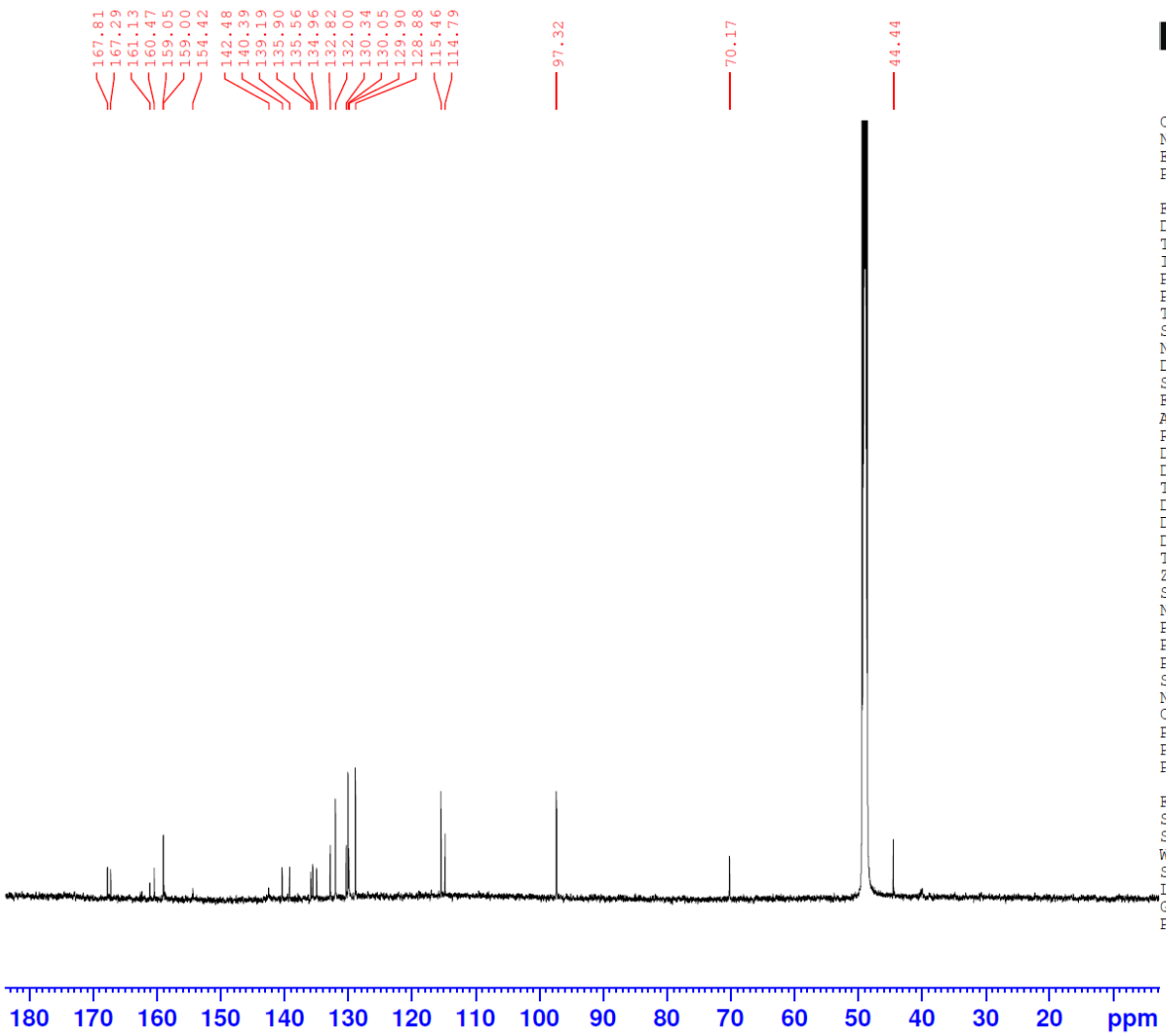
SSB 0
LB 5.00 Hz
GB 0
PC 1.00

3.2. 4-((4-(((2-Amino-9*H*-purin-6-yl)oxy)methyl)benzyl)carbamoyl)-2-(6-(bis(methyl-d_3)amino)-3-(bis(methyl-d_3)iminio)-3*H*-xanthen-9-yl)benzoate (BG-TMR-d12)





BRUKER

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PROCNO                 1

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SOLVENT            MeOD
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RG                  256
DW               16.000 usec
DE               20.00 usec
TE               298.1 K
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D12           0.00002000 sec
TD0                   1
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NUC1               13C
P0               11.43 usec
P3               11.43 usec
PLW1        120.23000336 W
SFO2         600.1828200 MHz
NUC2               1H
CPDPRG[2         waltz16
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PLW2         10.00000000 W
PLW19         0.07620790 W

F2 - Processing parameters
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GB        0
PC                1.40
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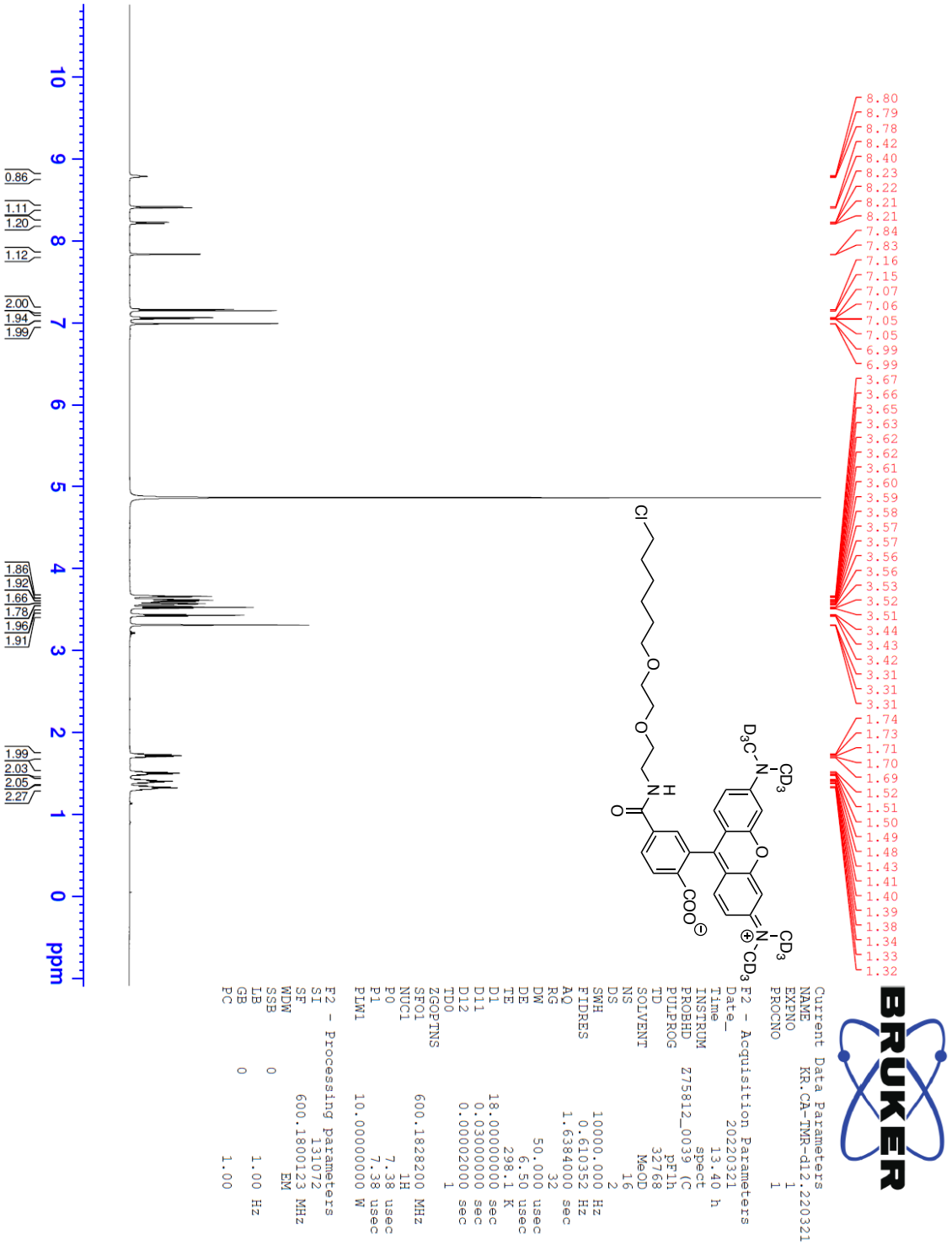
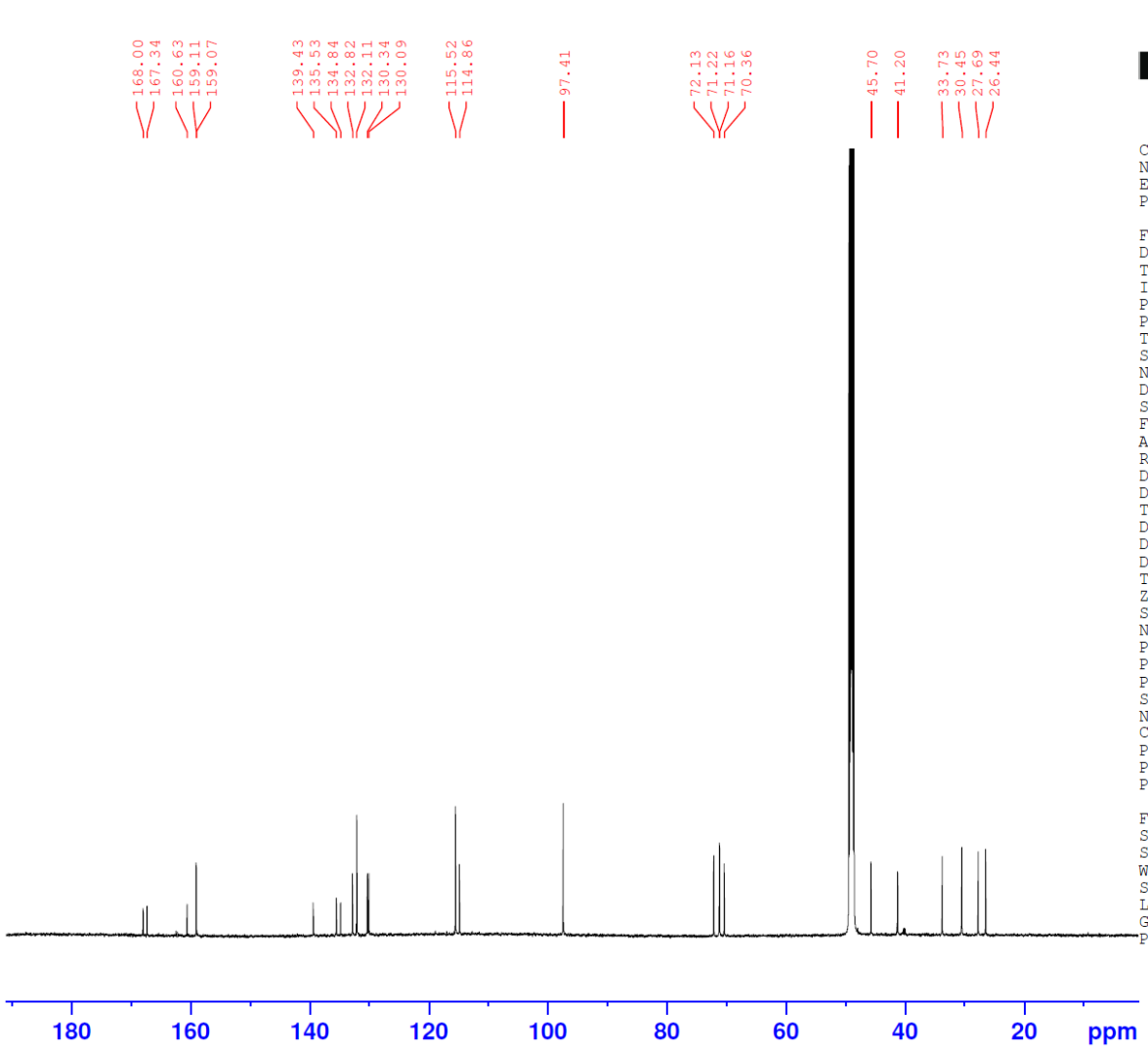
3.3. 2-(6-(Bis(methyl-d_3)amino)-3-(bis(methyl-d_3)iminio)-3*H*-xanthen-9-yl)-4-((2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamoyl)benzoate (CA-TMR-d12)





BRUKER

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PROCNO                      1

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TE                298.1 K
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D12            0.00002000 sec
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P3                11.43 usec
PLW1        120.23000336 W
SFO2         600.1828200 MHz
NUC2               1H
CPDPRG[2         waltz16
PCPD2             80.00 usec
PLW2         10.00000000 W
PLW19         0.07620790 W

F2 - Processing parameters
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WDW                 EM
SSB       0
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GB        0
PC                 1.40
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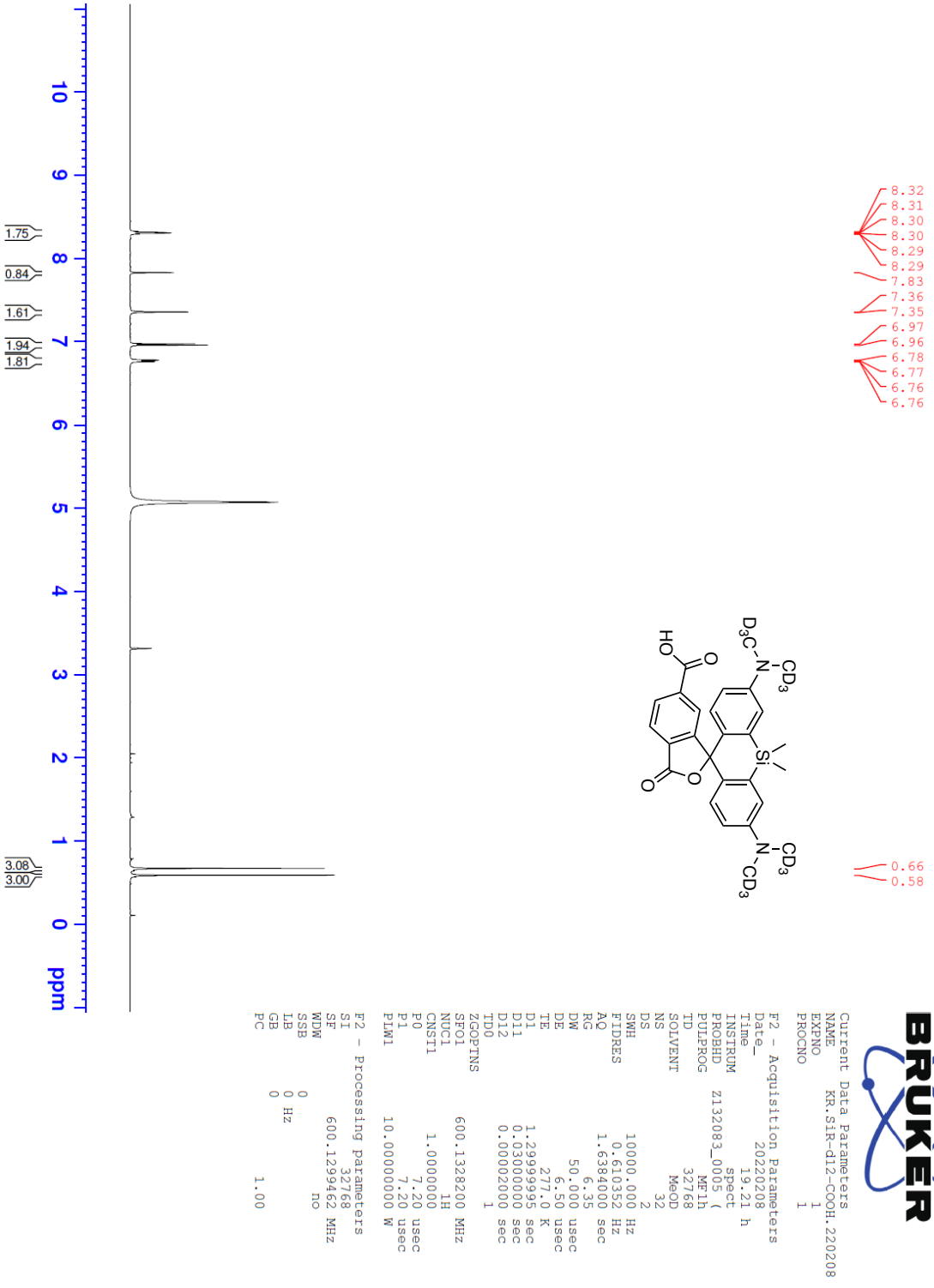
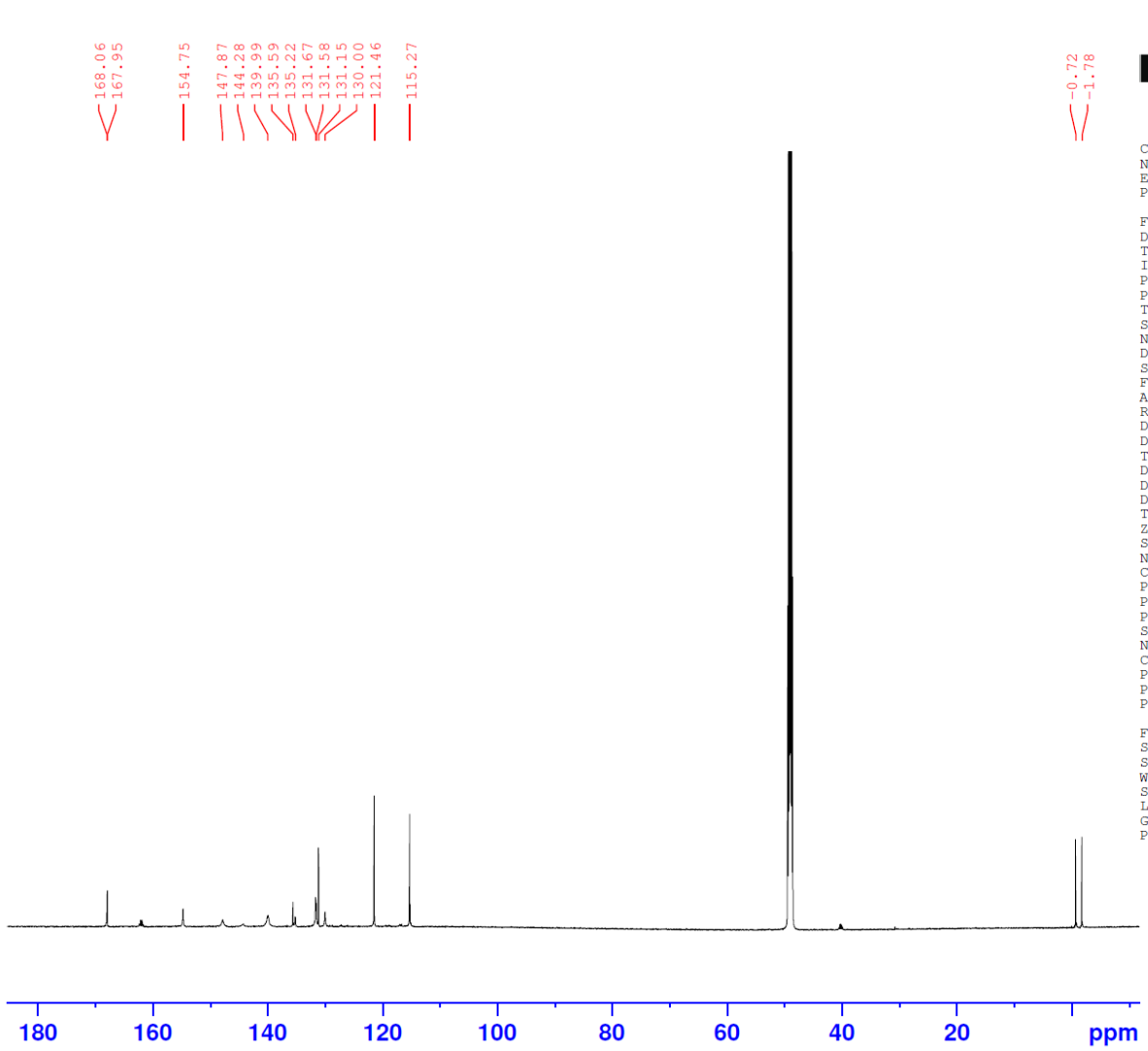
3.4. 3,7-Bis(bis(methyl-d$_3$)amino)-5,5-dimethyl-3'-oxo-3'*H*,5*H*-spiro[dibenzo[*b*,*e*]siline-10,1'-isobenzofuran]-6'-carboxylic acid (4)





BRUKER

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PROCNO                 1

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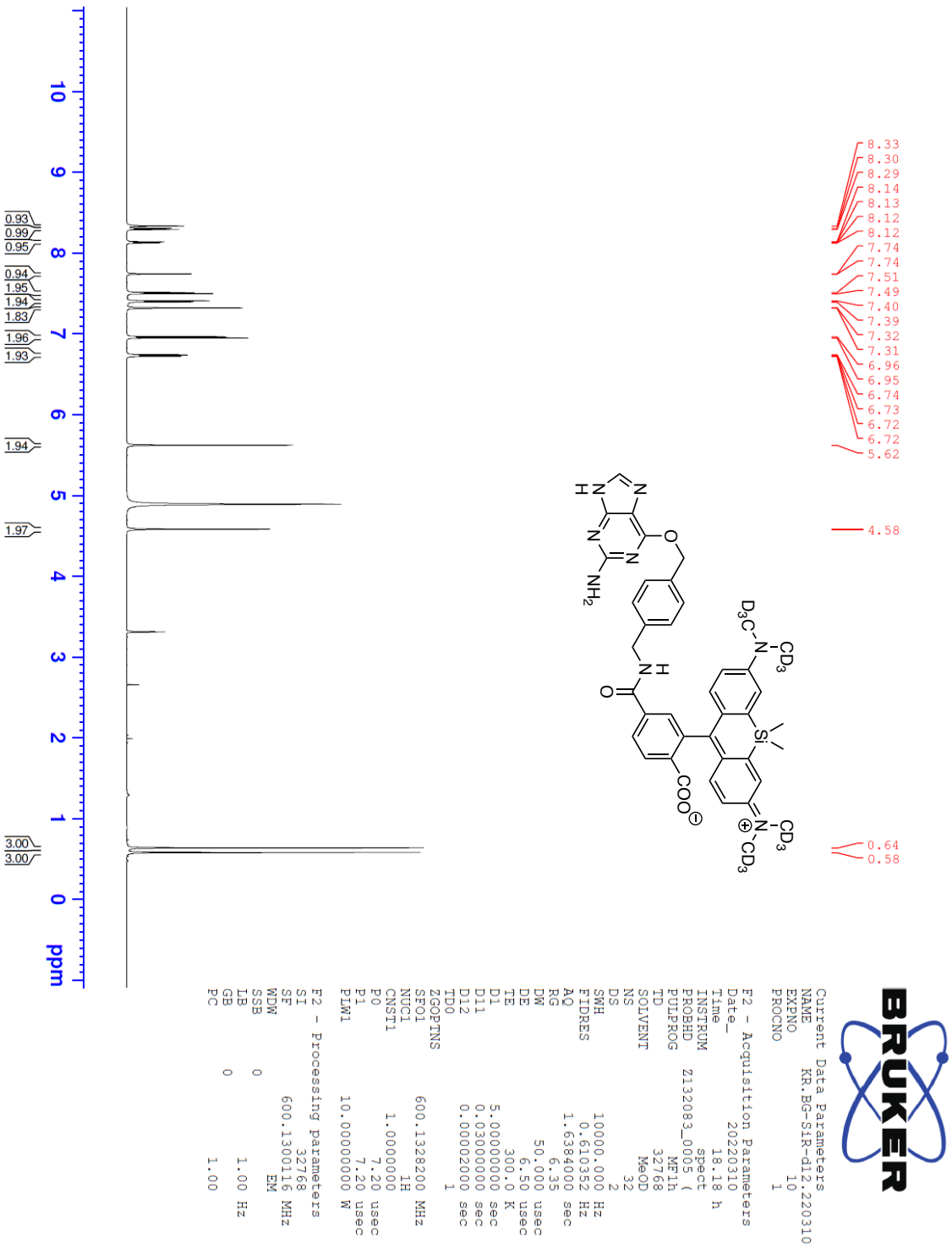
AQ                 1.8087935 sec
RG                   256
DW                  13.800 usec
DE                  20.00 usec
TE                  300.0 K
D1             2.50000000 sec
D11            0.03000000 sec
D12            0.00002000 sec
TD0                    3
ZGOPTNS       -Ddc -Dnoe
SFO1          150.9178090 MHz
NUC1                 13C
CNST1           1.0000000
P0                  12.25 usec
P3                  12.25 usec
PLW1         100.00000000 W
SFO2          600.1328200 MHz
NUC2                 1H
CPDPRG[1          waltz16
PCPD2               80.00 usec
PLW2          10.00000000 W
PLW19          0.08035262 W

F2 - Processing parameters
SI                131072
SF            150.9026383 MHz
WDW                  EM
SSB       0
LB                  3.00 Hz
GB        0
PC                  1.00
```

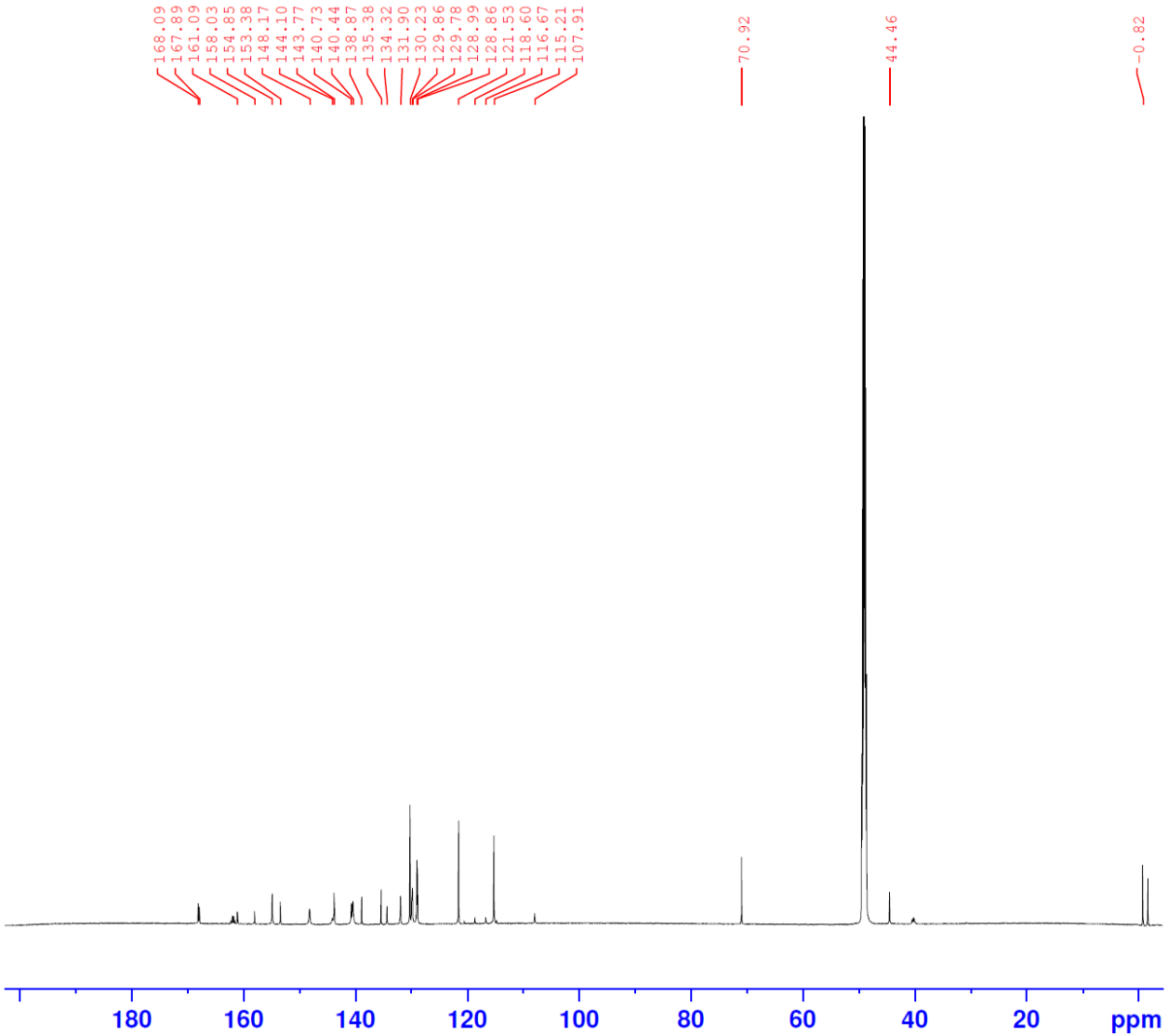
3.5. 4-((4-(((2-Amino-9*H*-purin-6-yl)oxy)methyl)benzyl)carbamoyl)-2-(7-(bis(methyl-d_3)amino)-3-(bis(methyl-d_3)iminio)-5,5-dimethyl-3,5-dihydrodibenzo[*b*,*e*]silin-10-yl)benzoate (BG-SiR-d12)



Current Data Parameters
NAME KR.BG-SiR-d12.220310
EXPNO 10
PROCNO 1

F2 - Acquisition Parameters
Date_ 20220310
Time 18.18 h
INSTRUM spect
PROBHD Z132083_0005 (
PULPROG MF1h
TD 32768
SOLVENT MeOD
NS 32
DS 2
SWH 10000.000 Hz
FIDRES 0.610352 Hz
AQ 1.6384000 sec
RG 6.35
DW 50.000 usec
DE 6.50 usec
TE 300.0 K
D1 5.00000000 sec
D11 0.03000000 sec
D12 0.00002000 sec
TD0 1
ZGOPTNS
SFO1 600.1328200 MHz
NUC1 1H
CNST1 1.0000000
P0 7.20 usec
P1 7.20 usec
PLW1 10.00000000 W

F2 - Processing parameters
SI 32768
SF 600.1300116 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.00



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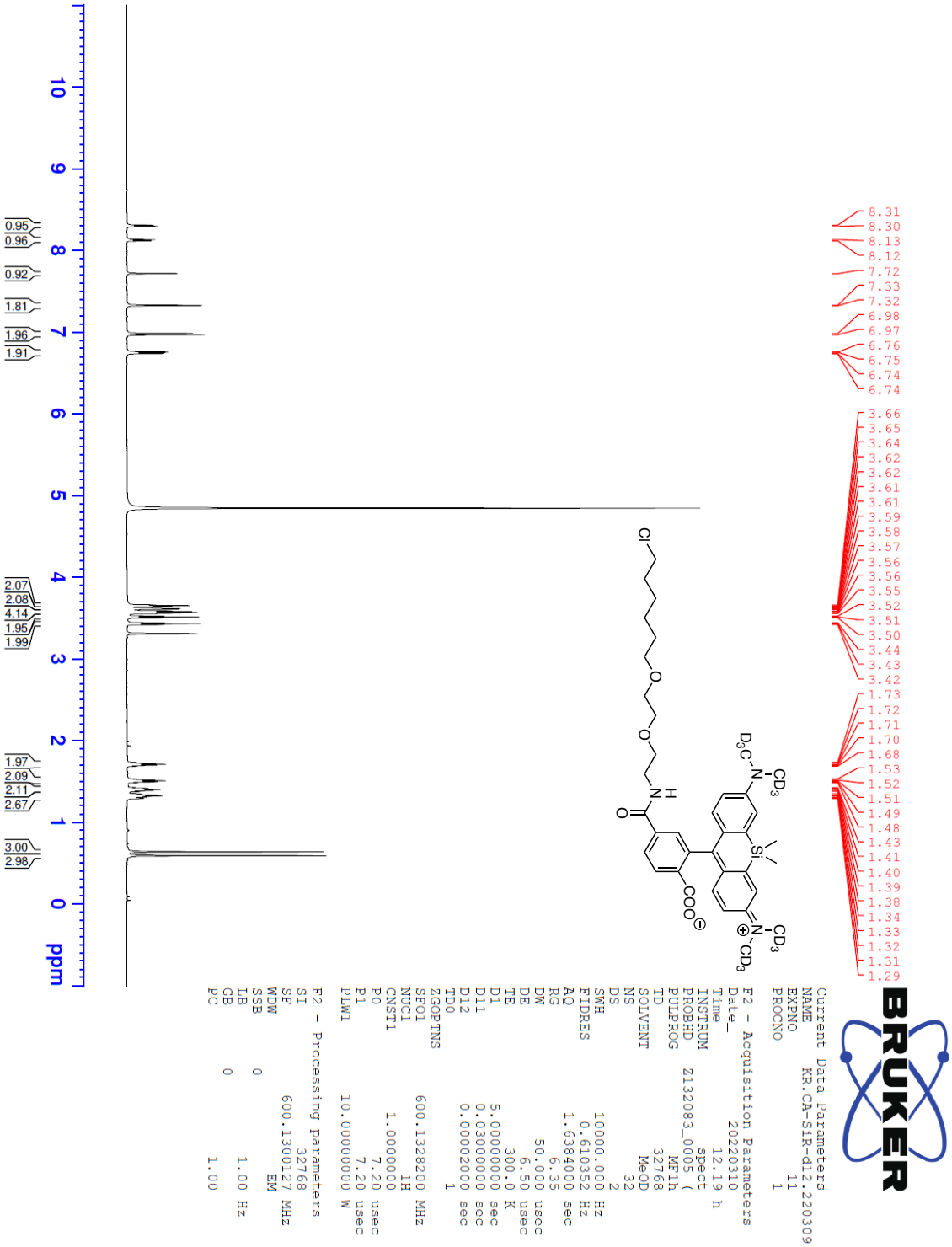
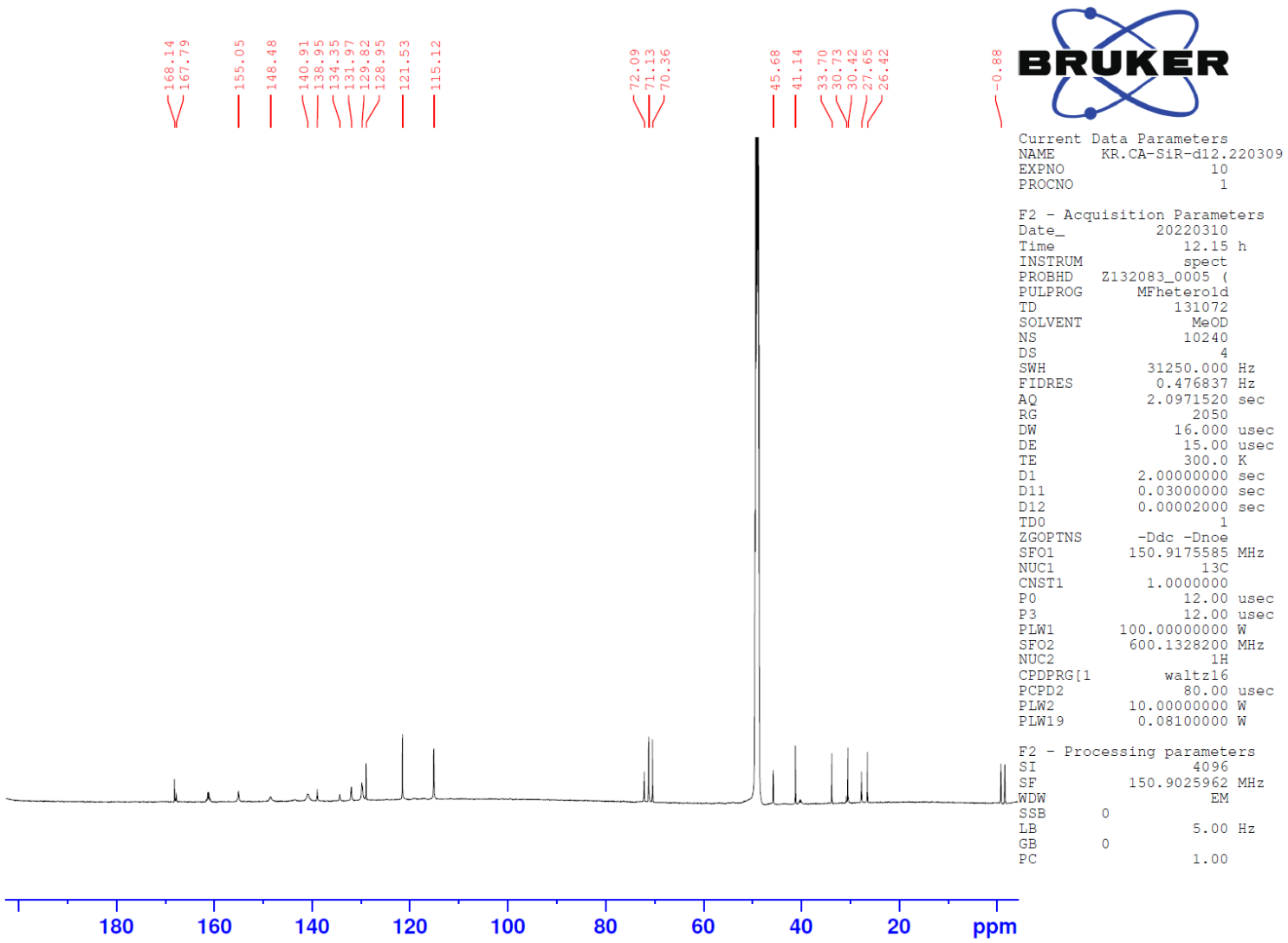
```
Current Data Parameters
NAME      KR.BG-SiR-d12.220310
EXPNO                   11
PROCNO                   1

F2 - Acquisition Parameters
Date_           20220311
Time                 0.14 h
INSTRUM             spect
PROBHD   Z132083_0005 (
PULPROG       MFhetero1d
TD                 131072
SOLVENT              MeOD
NS                   5120
DS                      4
SWH             31250.000 Hz
FIDRES          0.476837 Hz
AQ              2.0971520 sec
RG                   1820
DW                 16.000 usec
DE                 15.00 usec
TE                 300.0 K
D1              2.00000000 sec
D11             0.03000000 sec
D12             0.00002000 sec
TD0                     1
ZGOPTNS        -Ddc -Dnoe
SFO1          150.9175585 MHz
NUC1                  13C
CNST1           1.0000000
P0                   12.00 usec
P3                   12.00 usec
PLW1         100.00000000 W
SFO2          600.1328200 MHz
NUC2                  1H
CPDPRG[1          waltz16
PCPD2                80.00 usec
PLW2          10.00000000 W
PLW19          0.08100000 W

F2 - Processing parameters
SI                   4096
SF            150.9025954 MHz
WDW                    EM
SSB       0
LB                   5.00 Hz
GB        0
PC                   1.00
```

3.6. 2-(7-(Bis(methyl-d_3)amino)-3-(bis(methyl-d_3)iminio)-5,5-dimethyl-3,5-dihydrodibenzo[*b*,*e*]silin-10-yl)-4-((2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamoyl)benzoate (CA-SiR-d12)





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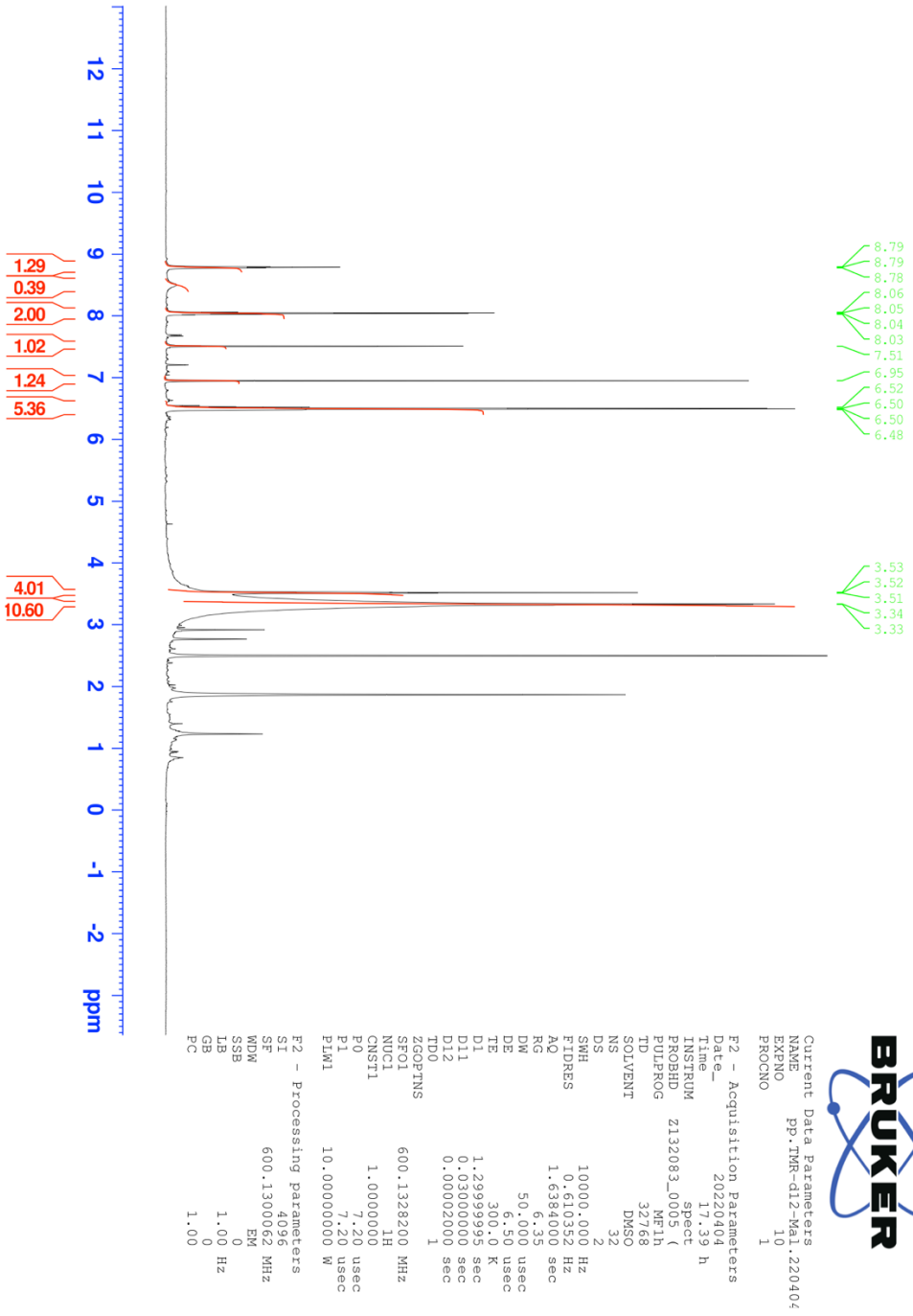
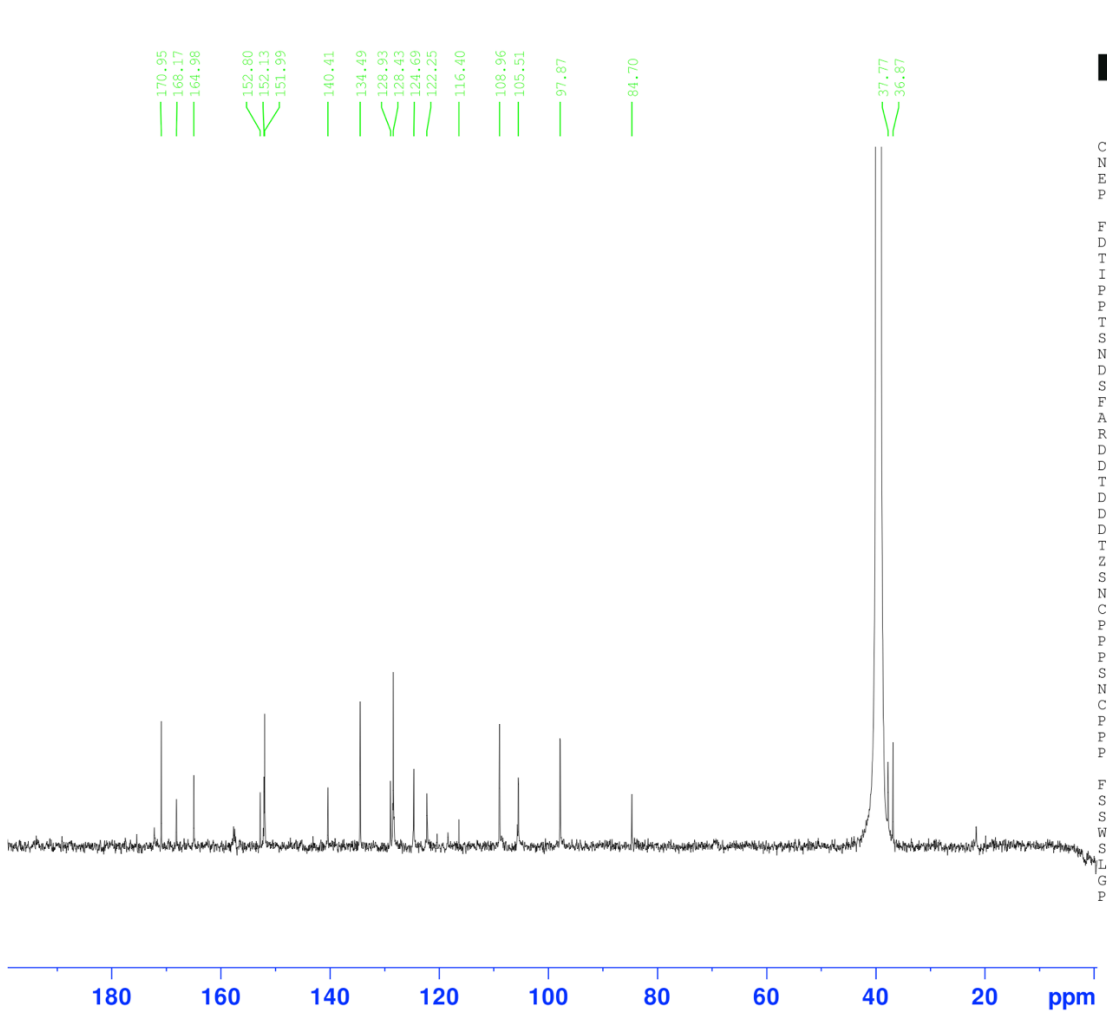
```
Current Data Parameters
NAME      KR.CA-SiR-d12.220309
EXPNO                   10
PROCNO                   1

F2 - Acquisition Parameters
Date_           20220310
Time               12.15 h
INSTRUM             spect
PROBHD    Z132083_0005 (
PULPROG       MFhetero1d
TD                131072
SOLVENT             MeOD
NS                 10240
DS                     4
SWH            31250.000 Hz
FIDRES          0.476837 Hz
AQ             2.0971520 sec
RG                  2050
DW                16.000 usec
DE                15.00 usec
TE                300.0 K
D1             2.00000000 sec
D11            0.03000000 sec
D12            0.00002000 sec
TD0                    1
ZGOPTNS       -Ddc -Dnoe
SFO1         150.9175585 MHz
NUC1                13C
CNST1          1.0000000
P0                12.00 usec
P3                12.00 usec
PLW1        100.00000000 W
SFO2         600.1328200 MHz
NUC2                1H
CPDPRG[1         waltz16
PCPD2             80.00 usec
PLW2         10.00000000 W
PLW19         0.08100000 W

F2 - Processing parameters
SI                  4096
SF           150.9025962 MHz
WDW                   EM
SSB       0
LB                 5.00 Hz
GB        0
PC                 1.00
```

3.7. 2-(6-(Bis(methyl-d_3)amino)-3-(bis(methyl-d_3)iminio)-3*H*-xanthen-9-yl)-4-((2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)ethyl)carbamoyl)benzoate (Mal-TMR-d12)



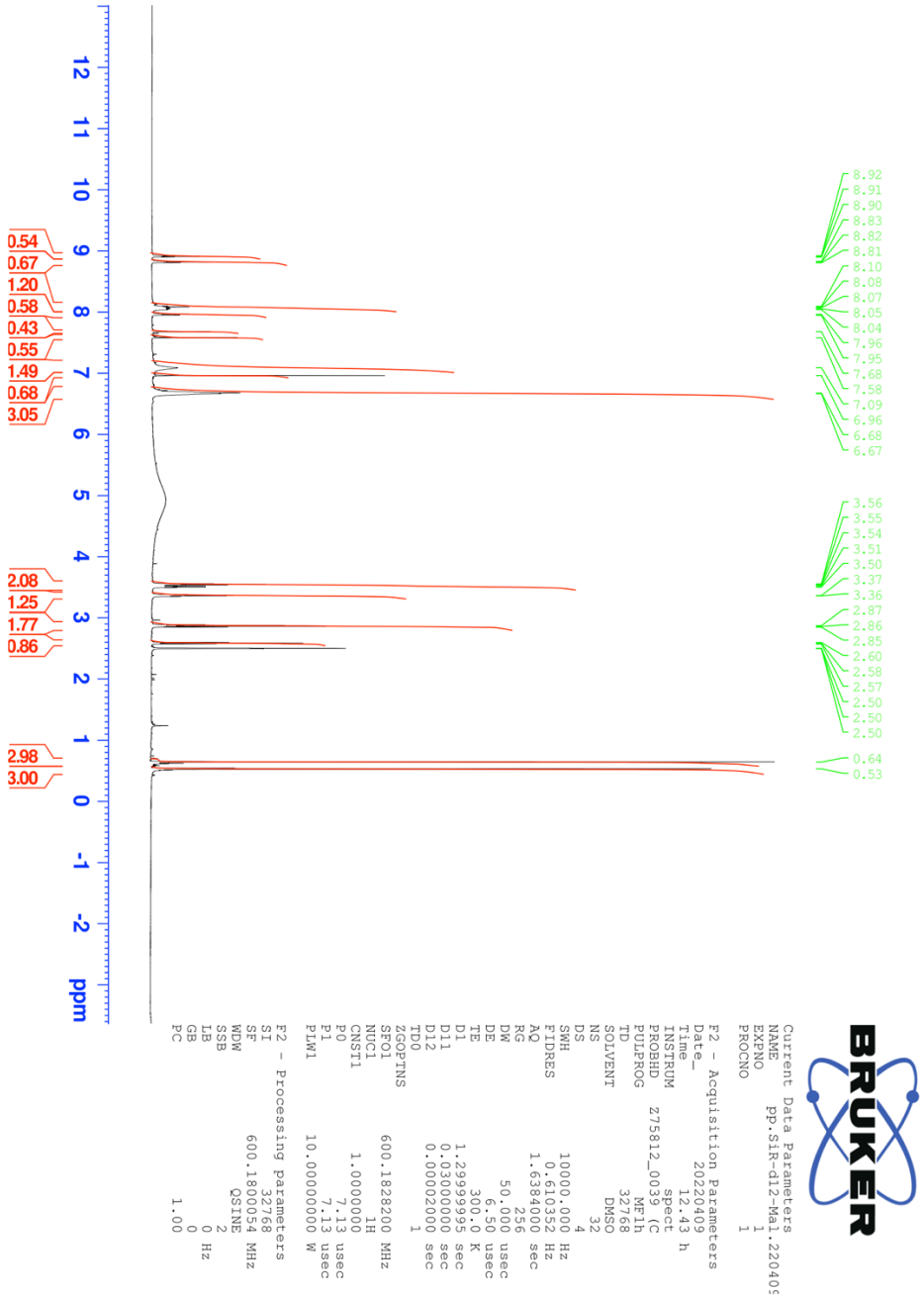


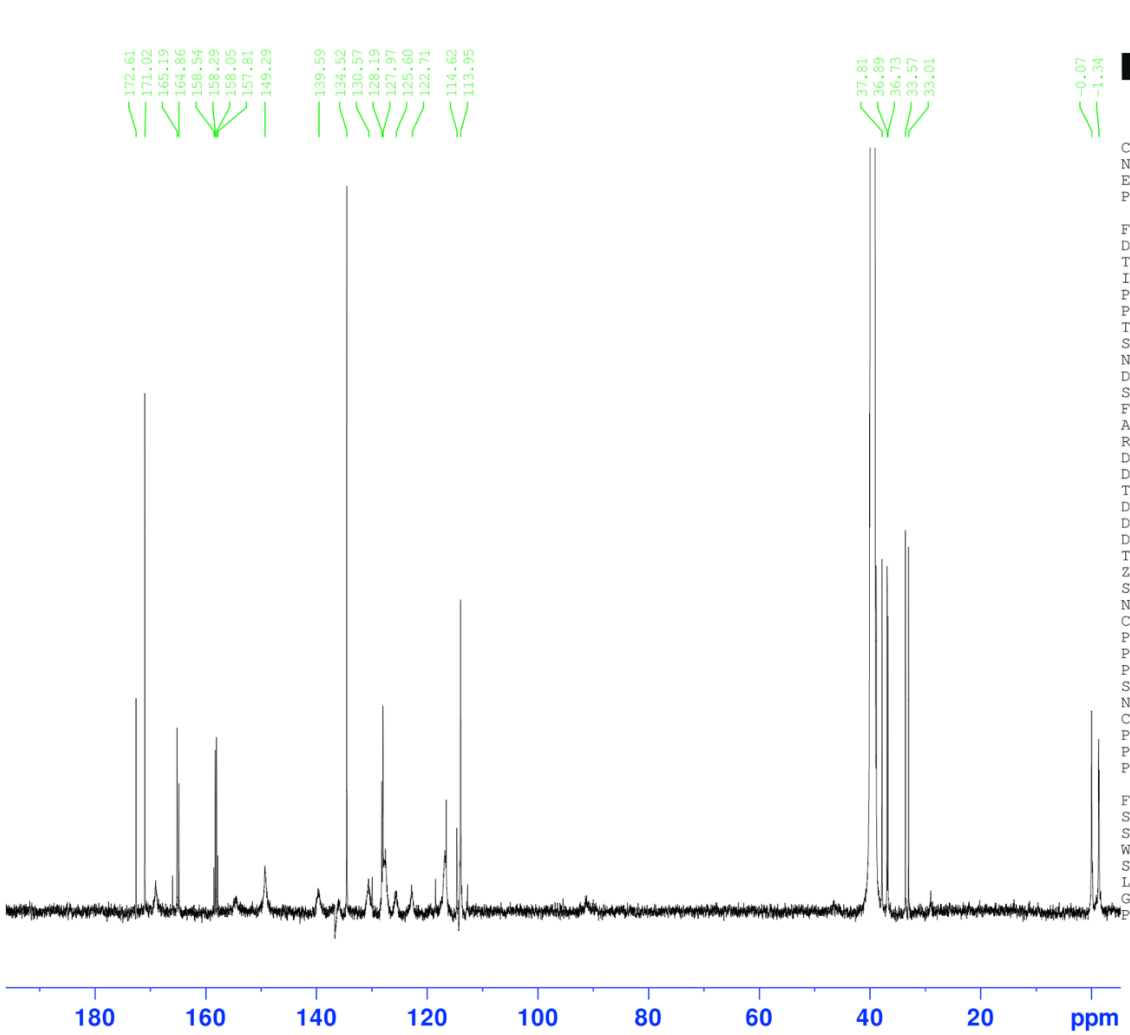

```
Current Data Parameters
NAME      pp.TMR-d12-Mal.220404
EXPNO                   11
PROCNO                   1

F2 - Acquisition Parameters
Date_           20220405
Time                6.56 h
INSTRUM            spect
PROBHD   Z132083_0005 (
PULPROG       MFheteroId
TD                 131072
SOLVENT              DMSO
NS                   10240
DS                       8
SWH             31250.000 Hz
FIDRES          0.476837 Hz
AQ              2.0971520 sec
RG                    2050
DW                 16.000 usec
DE                 20.00 usec
TE                 300.0 K
D1             2.50000000 sec
D11            0.03000000 sec
D12            0.00002000 sec
TD0                      1
ZGOPTNS       -Ddc -Dnoe
SFO1          150.9175585 MHz
NUC1                 13C
CNST1           0.3000000
P0                  3.60 usec
P3                 12.00 usec
PLW1         100.00000000 W
SFO2          600.1328200 MHz
NUC2                 1H
CPDPRG[1          waltz16
PCPD2              80.00 usec
PLW2          10.00000000 W
PLW19          0.08100000 W

F2 - Processing parameters
SI                    4096
SF            150.9028818 MHz
WDW                     EM
SSB                      0
LB                  5.00 Hz
GB                       0
PC                    1.00
```

3.8. 2-(7-(bis(methyl-d3)amino)-3-(bis(methyl-d3)iminio)-5,5-dimethyl-3,5-dihydrodibenzo[b,e]silin-10-yl)-4-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)carbamoyl)benzoate (Mal-SiR-d12)





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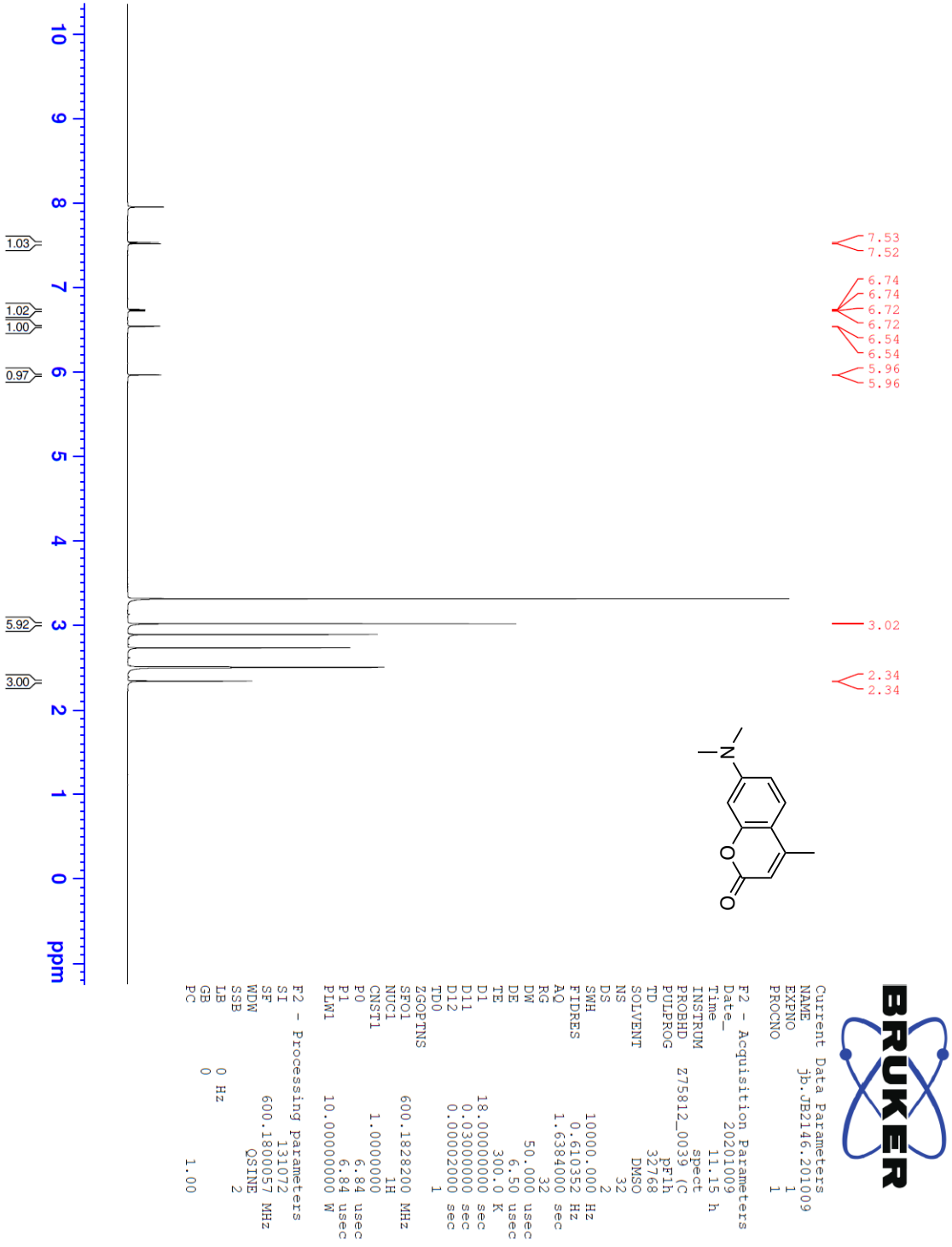
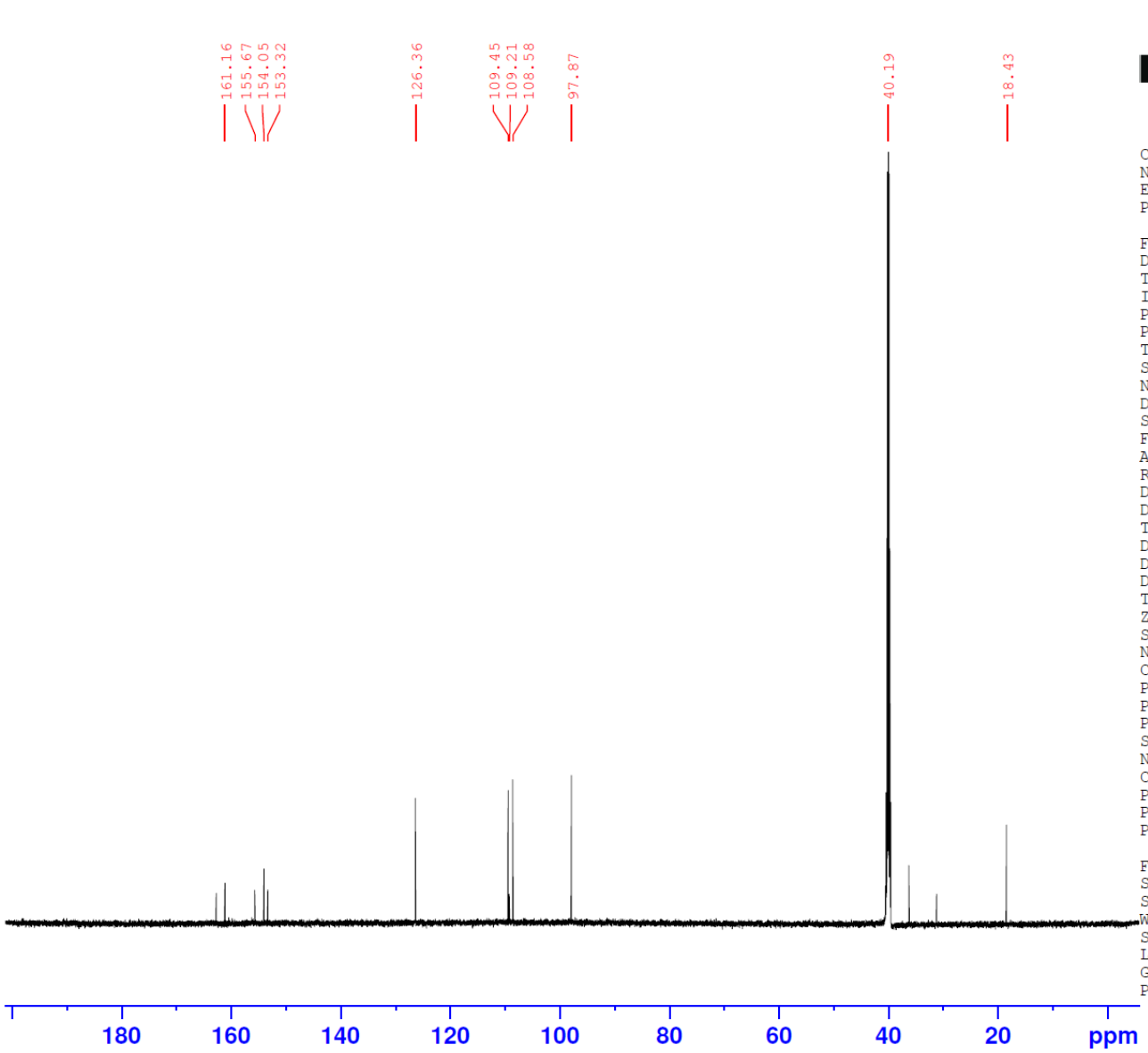
```
Current Data Parameters
NAME      pp.SiR-d12-Mal.220409
EXPNO                        2
PROCNO                       1

F2 - Acquisition Parameters
Date_              20220410
Time                  13.47 h
INSTRUM               spect
PROBHD    Z75812_0039 (C
PULPROG         MFheterold
TD                    131072
SOLVENT                DMSO
NS                    20480
DS                        4
SWH               35714.285 Hz
FIDRES             0.544957 Hz
AQ                 1.8350080 sec
RG                      256
DW                   14.000 usec
DE                   20.00 usec
TE                    300.0 K
D1                2.50000000 sec
D11               0.03000000 sec
D12               0.00002000 sec
TD0                      20
ZGOPTNS         -Ddc -Dnoe
SFO1            150.9303810 MHz
NUC1                    13C
CNST1              1.0000000
P0                   13.20 usec
P3                   13.20 usec
PLW1           100.00000000 W
SFO2            600.1828200 MHz
NUC2                    1H
CPDPRG[2             waltz16
PCPD2                80.00 usec
PLW2           10.00000000 W
PLW19           0.07079457 W

F2 - Processing parameters
SI                    131072
SF              150.9154540 MHz
WDW                     EM
SSB                      0
LB                    3.00 Hz
GB                       0
PC                    1.40
```

3.9. 7-(Dimethylamino)-4-methyl-2*H*-chromen-2-one (Coumarin 461)



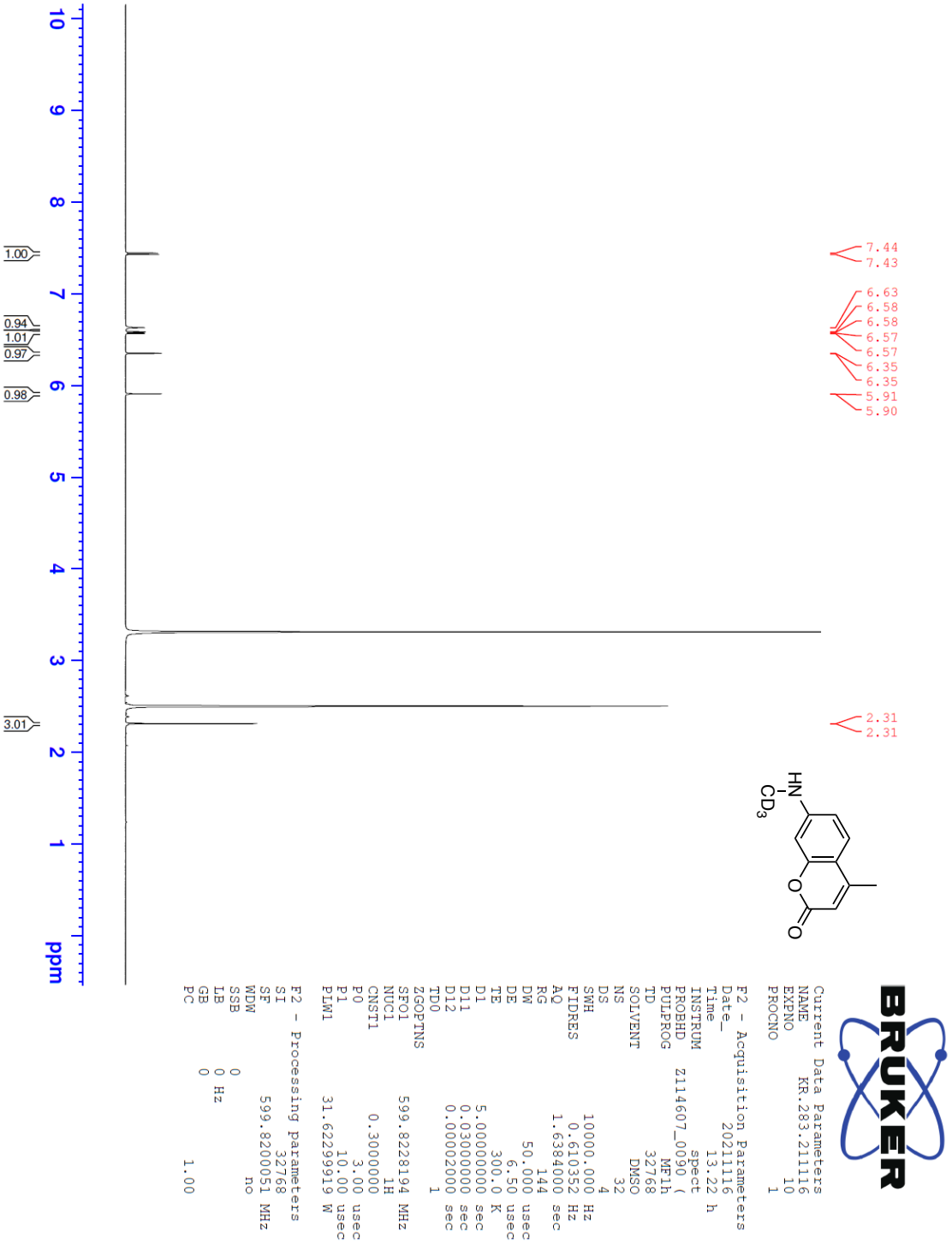


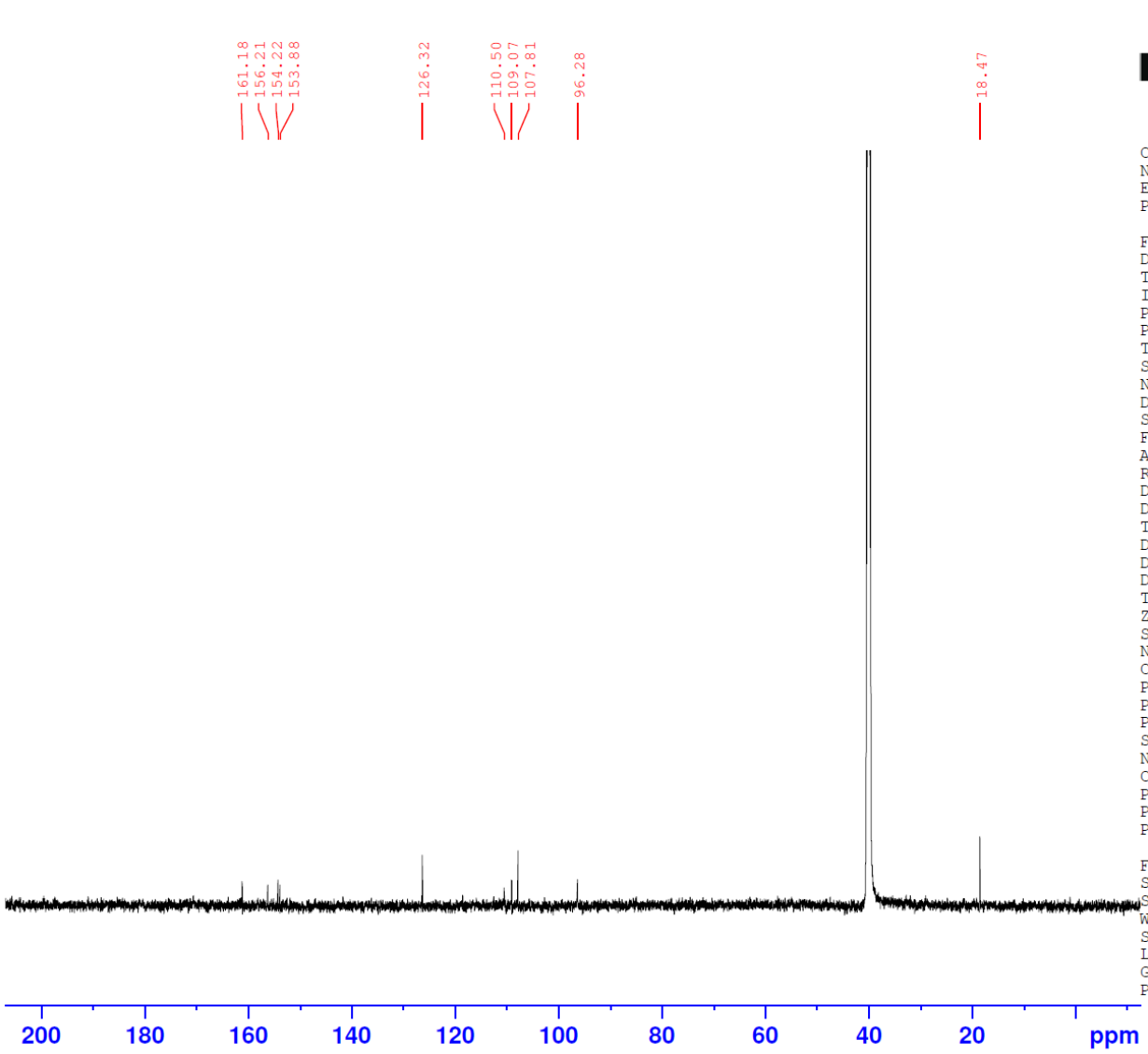
Current Data Parameters
NAME jb.JB2146.201009
EXPNO 2
PROCNO 1

F2 - Acquisition Parameters
Date_ 20201009
Time 11.28 h
INSTRUM spect
PROBHD Z75812_0039 (C
PULPROG MFhetero1d
TD 65536
SOLVENT DMSO
NS 128
DS 0
SWH 31250.000 Hz
FIDRES 0.953674 Hz
AQ 1.0485760 sec
RG 256
DW 16.000 usec
DE 20.00 usec
TE 300.0 K
D1 2.00000000 sec
D11 0.03000000 sec
D12 0.00002000 sec
TD0 1
ZGOPTNS -Ddc -Dnoe
SFO1 150.9301310 MHz
NUC1 13C
CNST1 1.0000000
P0 11.43 usec
P3 11.43 usec
PLW1 120.23000336 W
SFO2 600.1828200 MHz
NUC2 1H
CPDPRG[1 waltz16
PCPD2 80.00 usec
PLW2 10.00000000 W
PLW19 0.09751600 W

F2 - Processing parameters
SI 32768
SF 150.9153833 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40

3.10. 4-Methyl-7-((methyl-d3)amino)-2*H*-chromen-2-one (6)



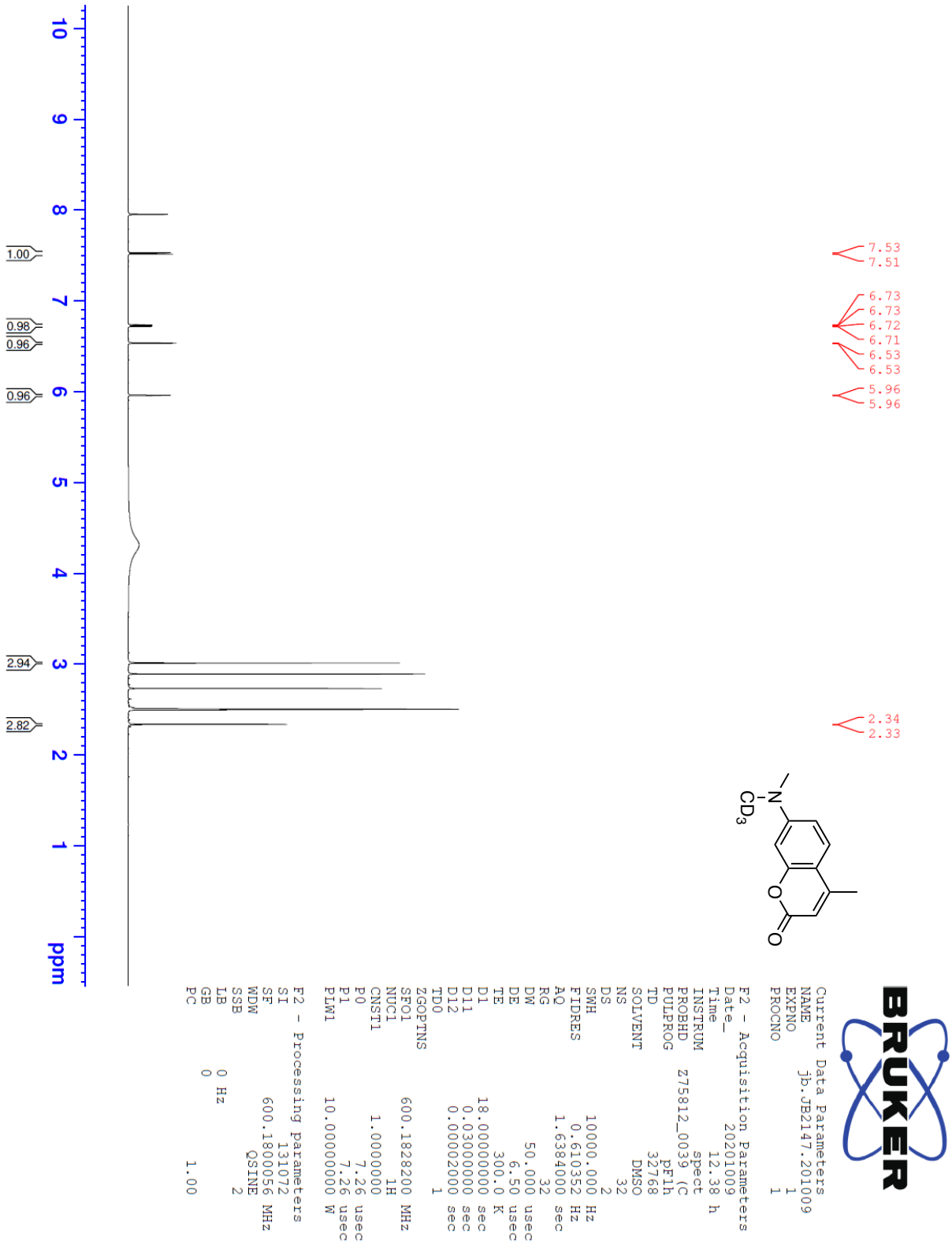


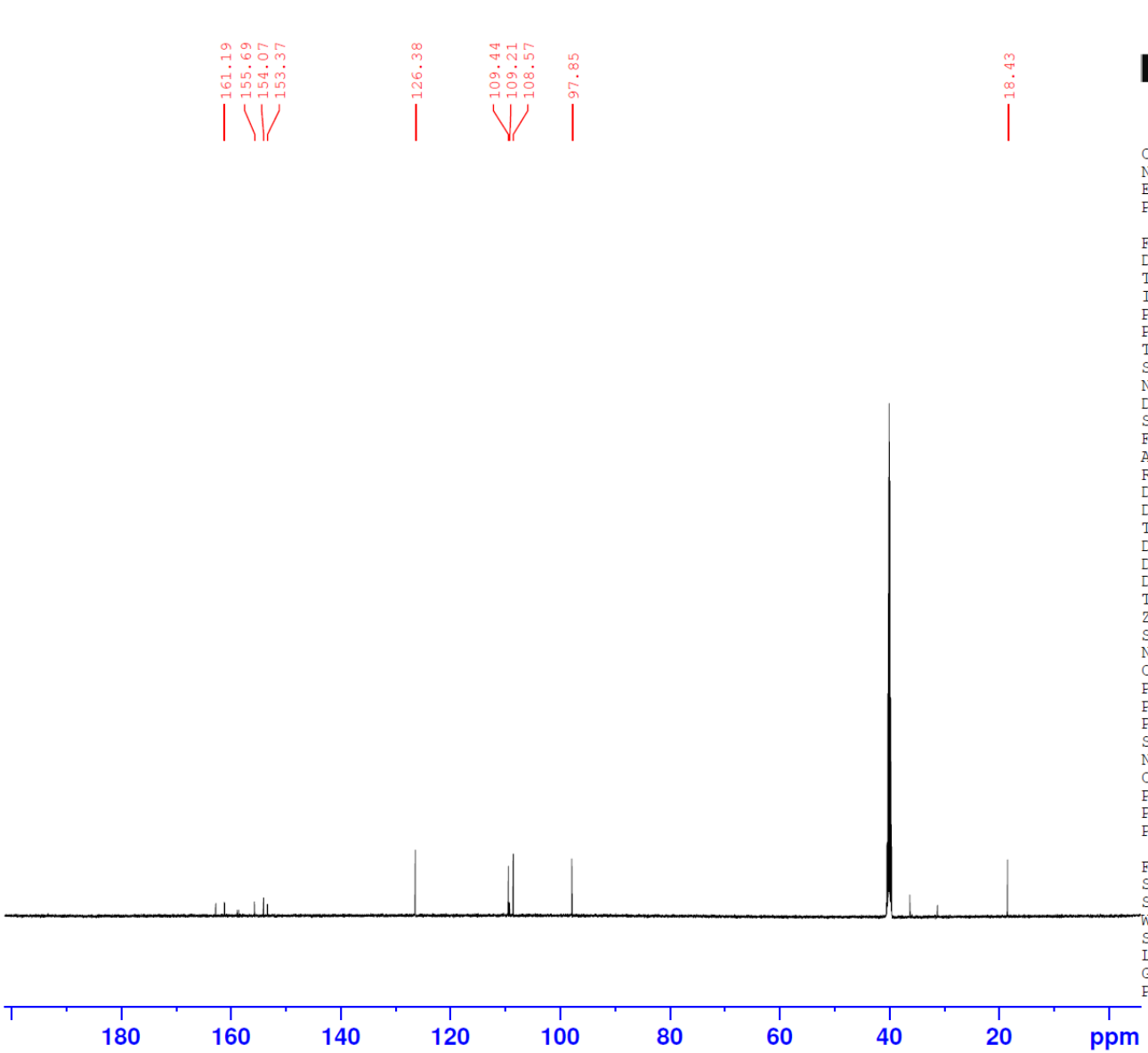

```
Current Data Parameters
NAME      KR.283.211116
EXPNO                11
PROCNO                1

F2 - Acquisition Parameters
Date_          20211116
Time              23.57 h
INSTRUM            spect
PROBHD   Z114607_0090 (
PULPROG      MFheterold
TD               131072
SOLVENT             DMSO
NS                  4096
DS                     4
SWH            45454.547 Hz
FIDRES         0.693581 Hz
AQ             1.4417920 sec
RG                  2050
DW                11.000 usec
DE                 6.50 usec
TE                300.0 K
D1           2.50000000 sec
D11          0.03000000 sec
D12          0.00002000 sec
TD0                    1
ZGOPTNS       -Ddc -Dnoe
SFO1         150.8398557 MHz
NUC1                13C
CNST1          0.3000000
P0                 2.58 usec
P3                 8.60 usec
PLW1        158.49000549 W
SFO2         599.8228182 MHz
NUC2                1H
CPDPRG[1         waltz16
PCPD2             80.00 usec
PLW2         31.62299919 W
PLW19         0.49410999 W

F2 - Processing parameters
SI               131072
SF           150.8248634 MHz
WDW                  EM
SSB       0
LB                3.00 Hz
GB        0
PC                1.00
```

3.11. 4-Methyl-7-(methyl(methyl-d3)amino)-2*H*-chromen-2-one (Coumarin 461-d3)





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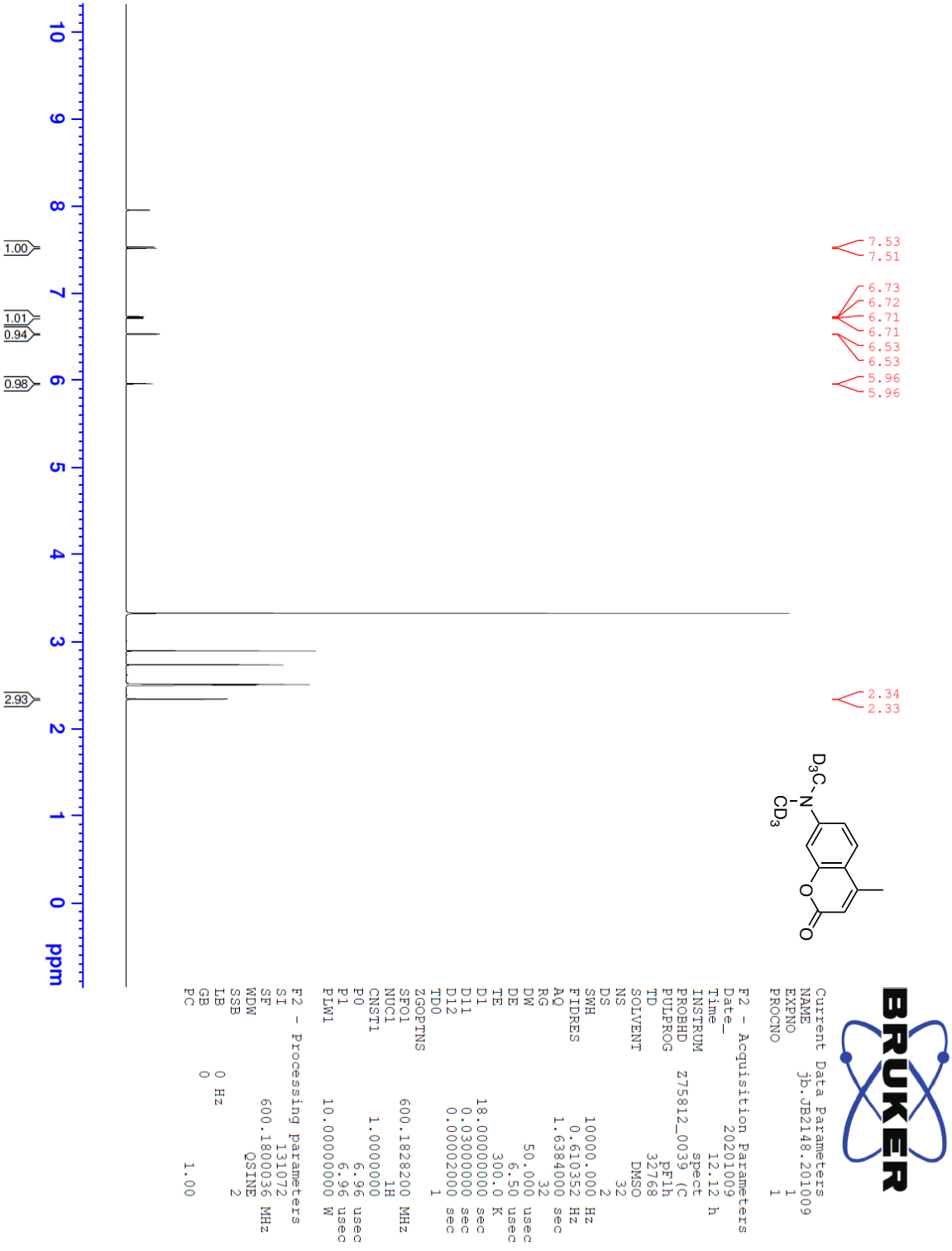
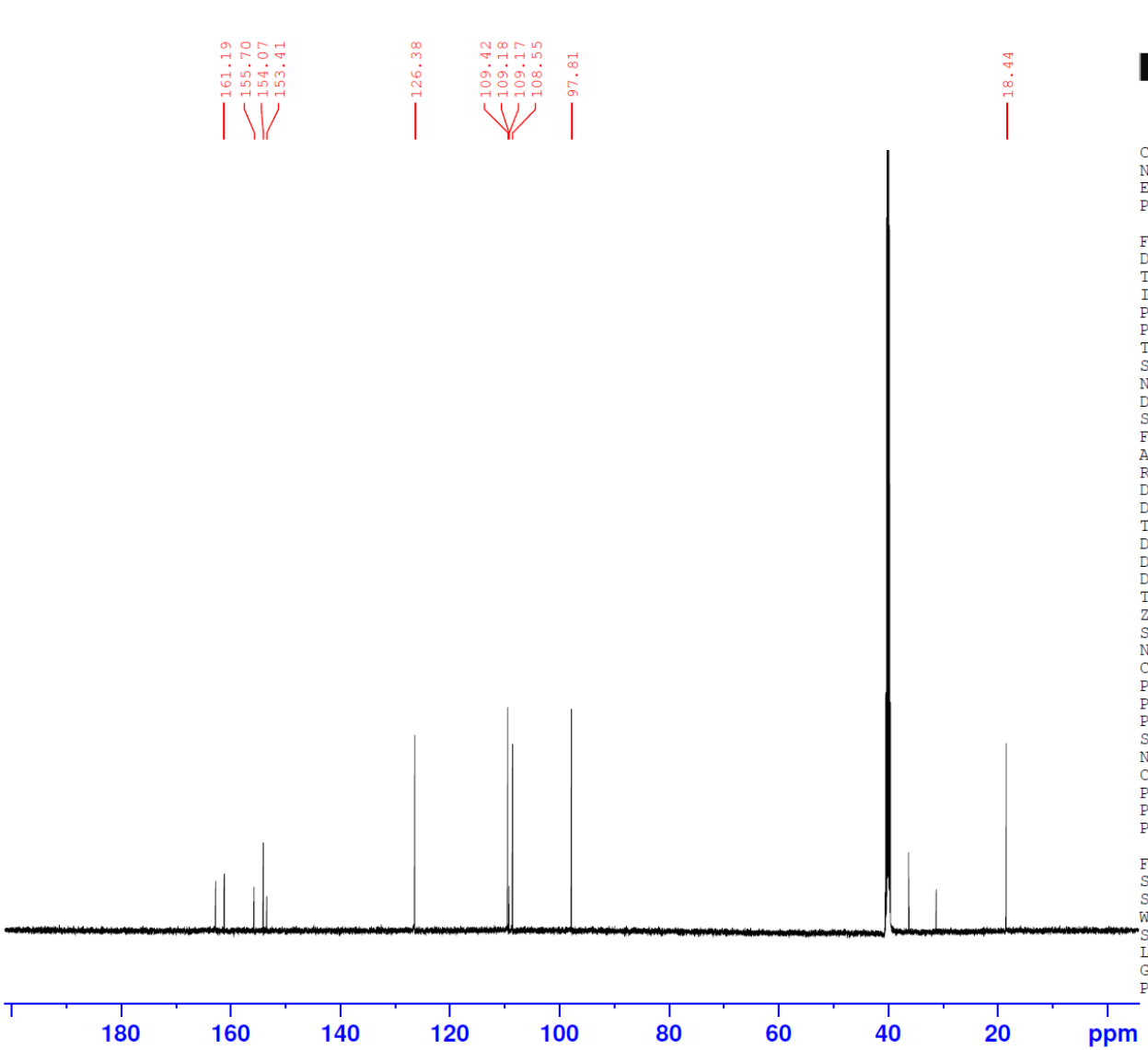
```
Current Data Parameters
NAME      jb.JB2147.201009
EXPNO                    2
PROCNO                   1

F2 - Acquisition Parameters
Date_           20201009
Time              12.45 h
INSTRUM             spect
PROBHD   Z75812_0039 (C
PULPROG       MFhetero1d
TD                 65536
SOLVENT             DMSO
NS                   128
DS                     0
SWH            31250.000 Hz
FIDRES          0.953674 Hz
AQ             1.0485760 sec
RG                   256
DW                16.000 usec
DE                20.00 usec
TE                300.0 K
D1             2.00000000 sec
D11            0.03000000 sec
D12            0.00002000 sec
TD0                    1
ZGOPTNS       -Ddc -Dnoe
SFO1         150.9301310 MHz
NUC1                13C
CNST1          1.0000000
P0                 11.43 usec
P3                 11.43 usec
PLW1        120.23000336 W
SFO2         600.1828200 MHz
NUC2                1H
CPDPRG[1         waltz16
PCPD2              80.00 usec
PLW2         10.00000000 W
PLW19         0.09751600 W

F2 - Processing parameters
SI                 32768
SF           150.9153810 MHz
WDW                   EM
SSB       0
LB                 1.00 Hz
GB        0
PC                 1.40
```

3.12. 7-(Bis(methyl-d3)amino)-4-methyl-2*H*-chromen-2-one (Coumarin 461-d6)





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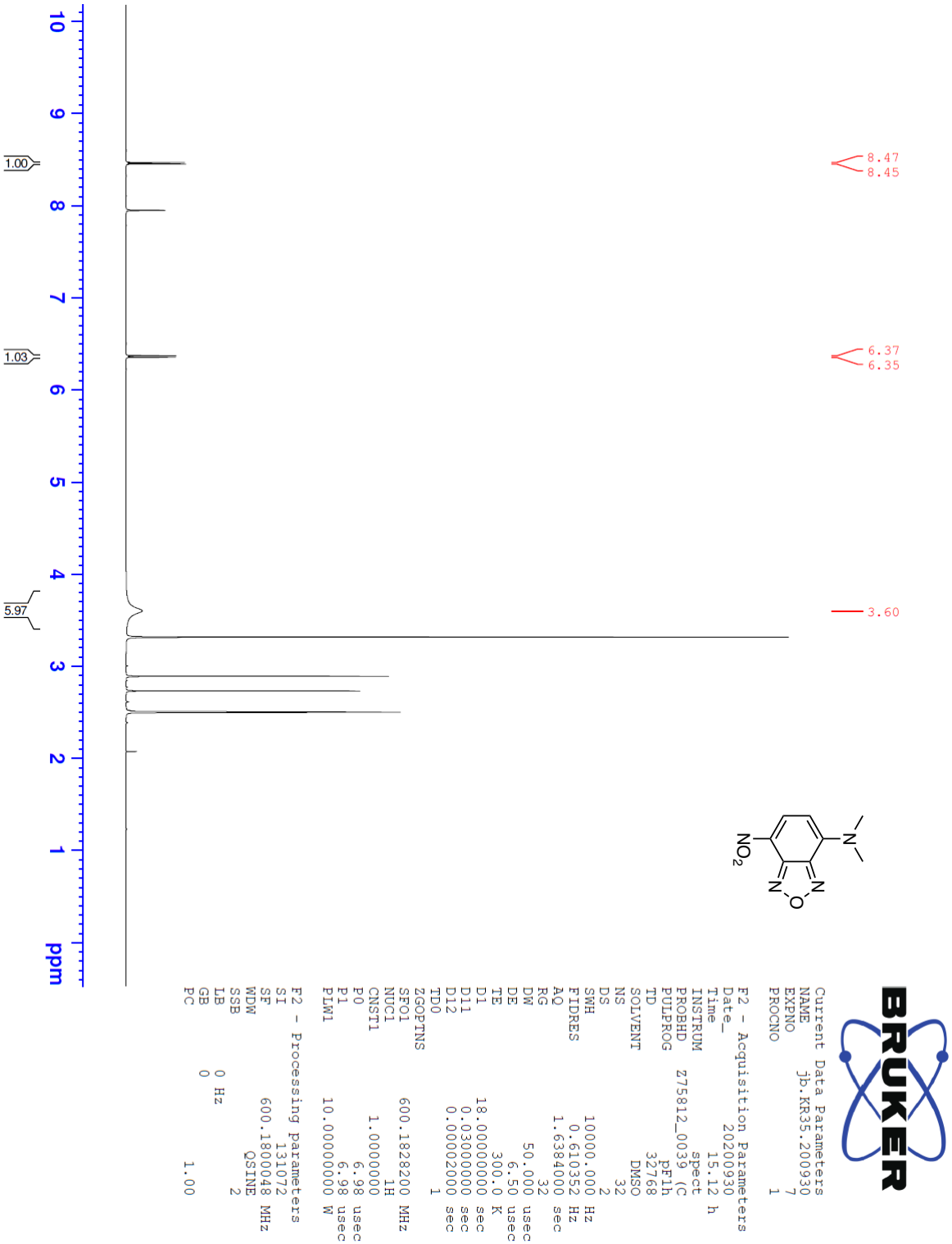
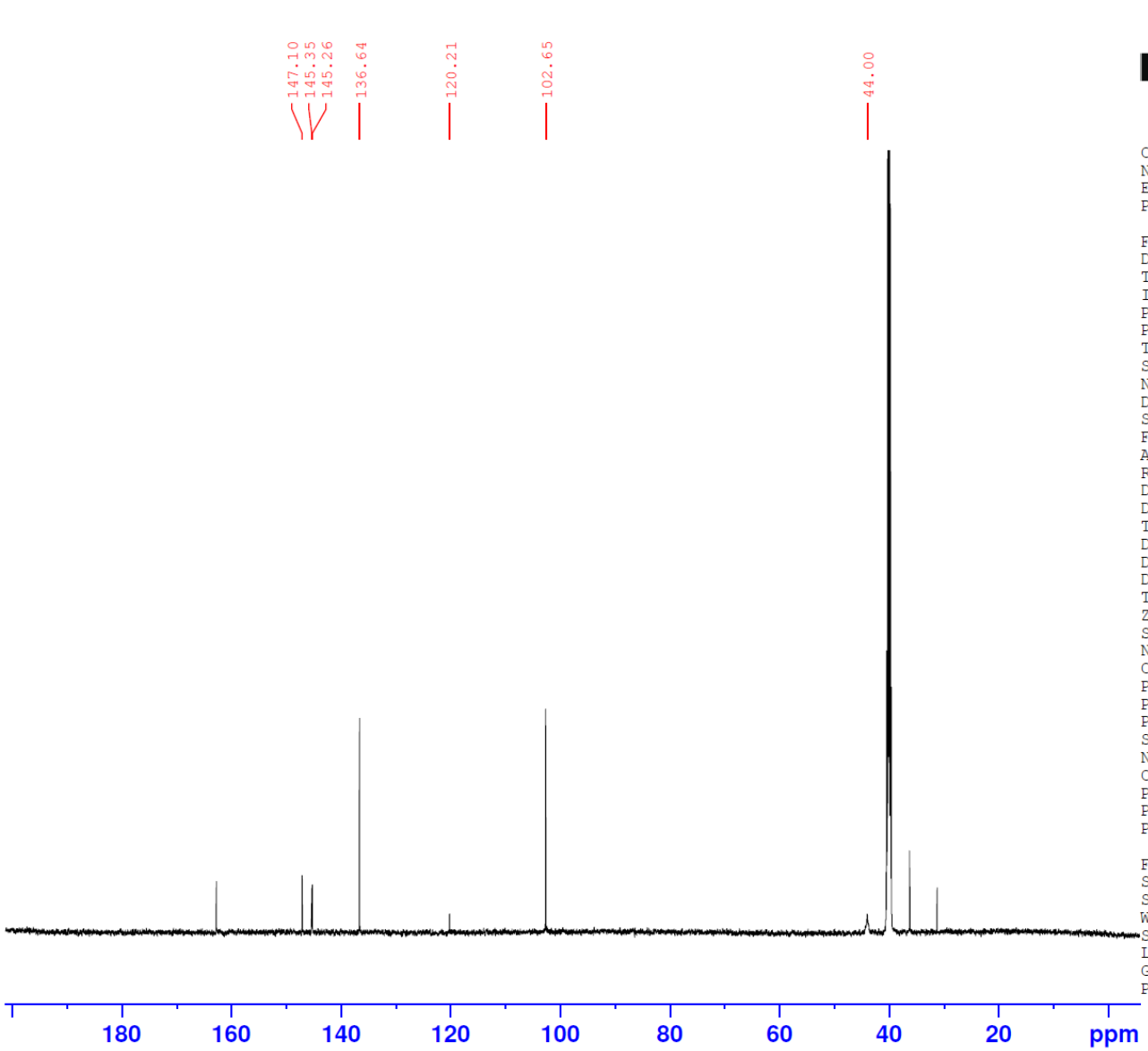
```
Current Data Parameters
NAME      jb.JB2148.201009
EXPNO                    2
PROCNO                   1

F2 - Acquisition Parameters
Date_           20201009
Time               12.20 h
INSTRUM             spect
PROBHD    Z75812_0039 (C
PULPROG       MFheterold
TD                  65536
SOLVENT              DMSO
NS                     128
DS                       0
SWH              31250.000 Hz
FIDRES           0.953674 Hz
AQ               1.0485760 sec
RG                     256
DW                16.000 usec
DE                20.00 usec
TE                 300.0 K
D1            2.00000000 sec
D11           0.03000000 sec
D12           0.00002000 sec
TD0                      1
ZGOPTNS       -Ddc -Dnoe
SFO1         150.9301310 MHz
NUC1                  13C
CNST1          1.0000000
P0                 11.43 usec
P3                 11.43 usec
PLW1        120.23000336 W
SFO2         600.1828200 MHz
NUC2                  1H
CPDPRG[1         waltz16
PCPD2              80.00 usec
PLW2         10.00000000 W
PLW19         0.09751600 W

F2 - Processing parameters
SI                   32768
SF           150.9153810 MHz
WDW                     EM
SSB       0
LB                  1.00 Hz
GB        0
PC                  1.40
```

3.13. *N,N*-Dimethyl-7-nitrobenzo[*c*][1,2,5]oxadiazol-4-amine (NBD)



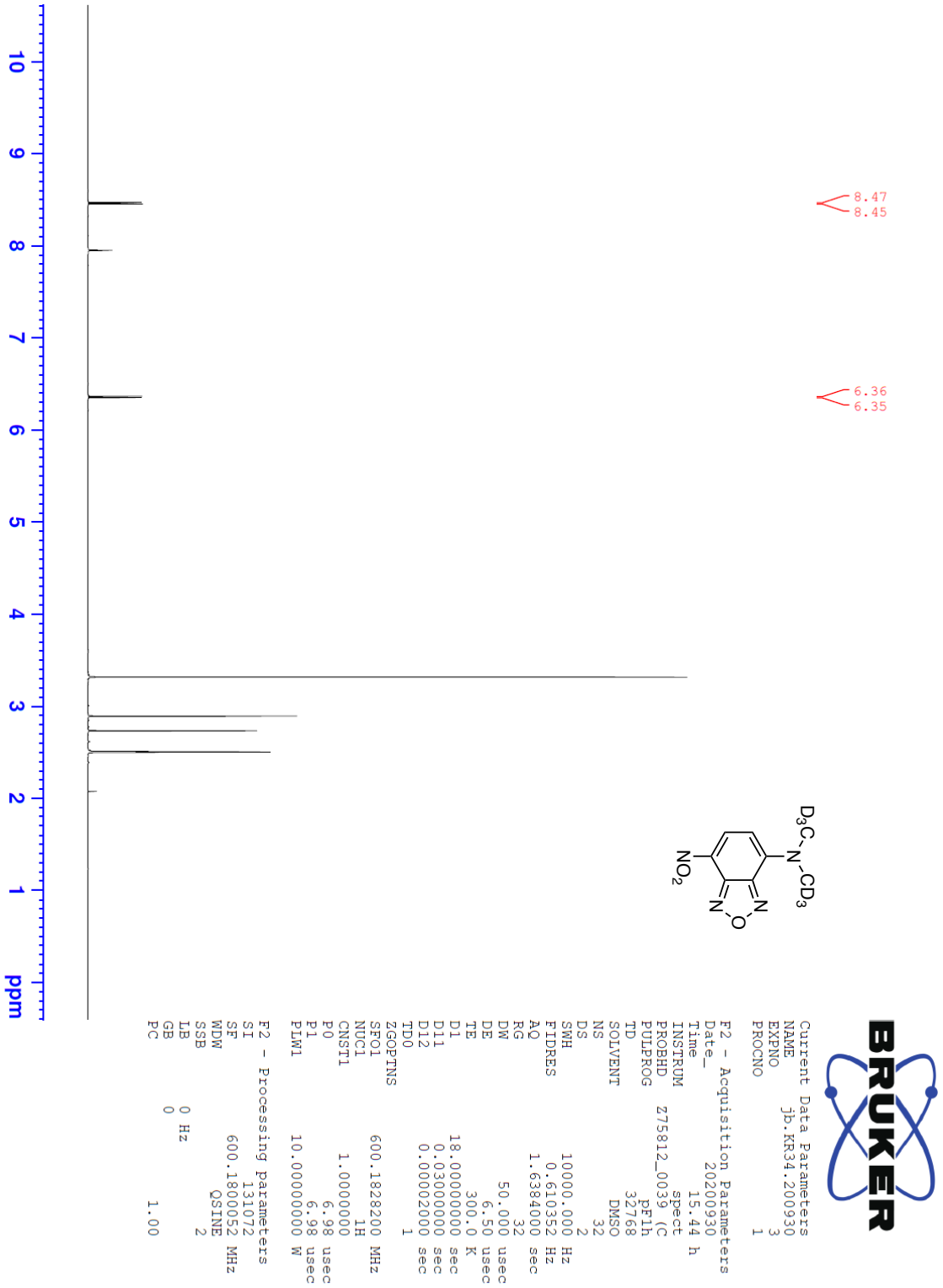


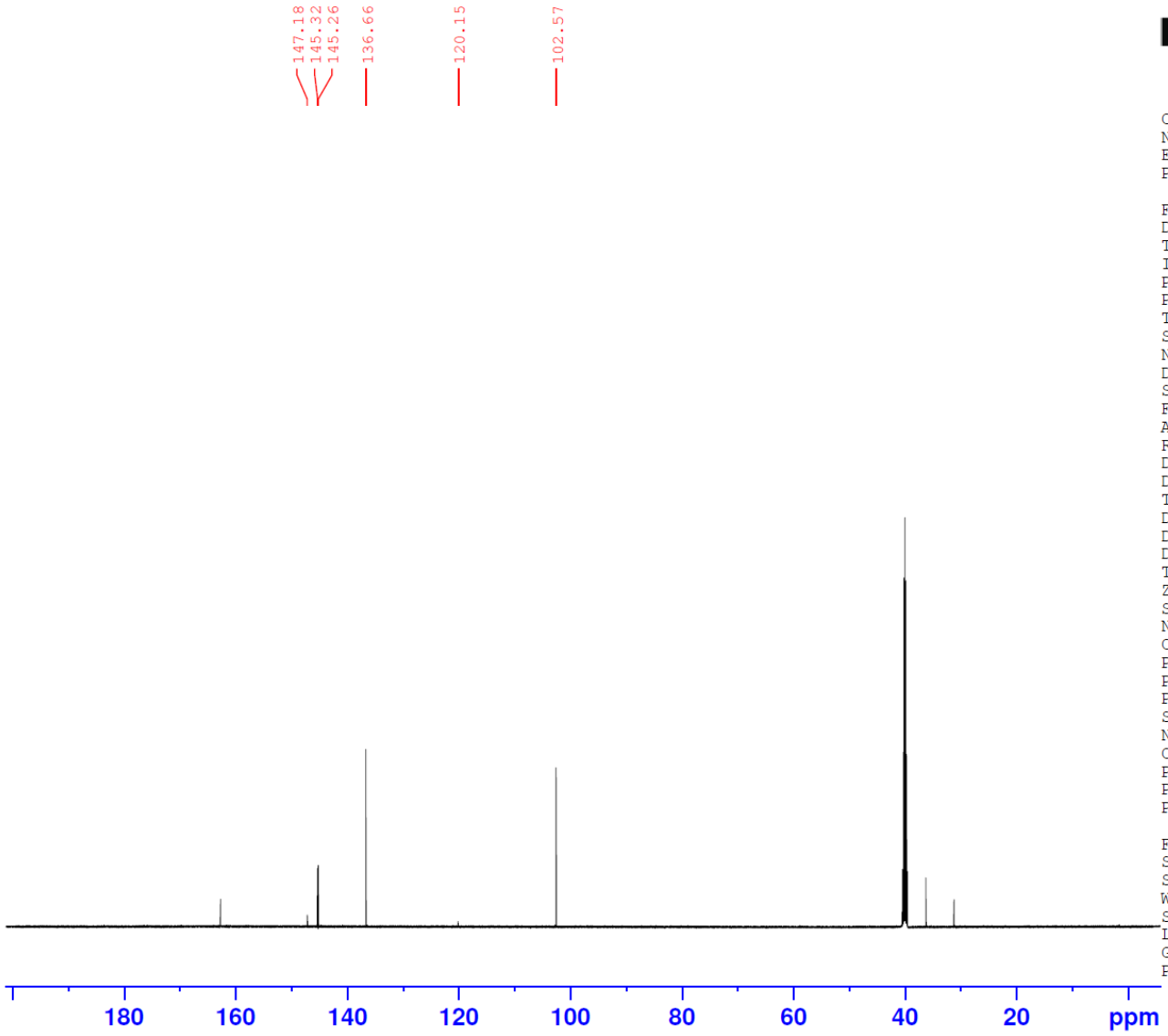

```
Current Data Parameters
NAME      jb.KR35.200930
EXPNO                  8
PROCNO                 1

F2 - Acquisition Parameters
Date_           20200930
Time               15.22 h
INSTRUM             spect
PROBHD    Z75812_0039 (C
PULPROG       MFheterold
TD                131072
SOLVENT              DMSO
NS                    128
DS                      2
SWH             31250.000 Hz
FIDRES          0.476837 Hz
AQ              2.0971520 sec
RG                    256
DW                16.000 usec
DE                20.00 usec
TE                300.0 K
D1             2.00000000 sec
D11            0.03000000 sec
D12            0.00002000 sec
TD0                    1
ZGOPTNS       -Ddc -Dnoe
SFO1          150.9301310 MHz
NUC1                 13C
CNST1          1.0000000
P0                11.43 usec
P3                11.43 usec
PLW1         120.23000336 W
SFO2          600.1828200 MHz
NUC2                 1H
CPDPRG[1         waltz16
PCPD2             80.00 usec
PLW2          10.00000000 W
PLW19          0.07620790 W

F2 - Processing parameters
SI                131072
SF            150.9153819 MHz
WDW                   EM
SSB       0
LB                 3.00 Hz
GB        0
PC                 1.40
```

3.14. *N*,*N*-Bis(methyl-d3)-7-nitrobenzo[*c*][1,2,5]oxadiazol-4-amine (NBD-d6)



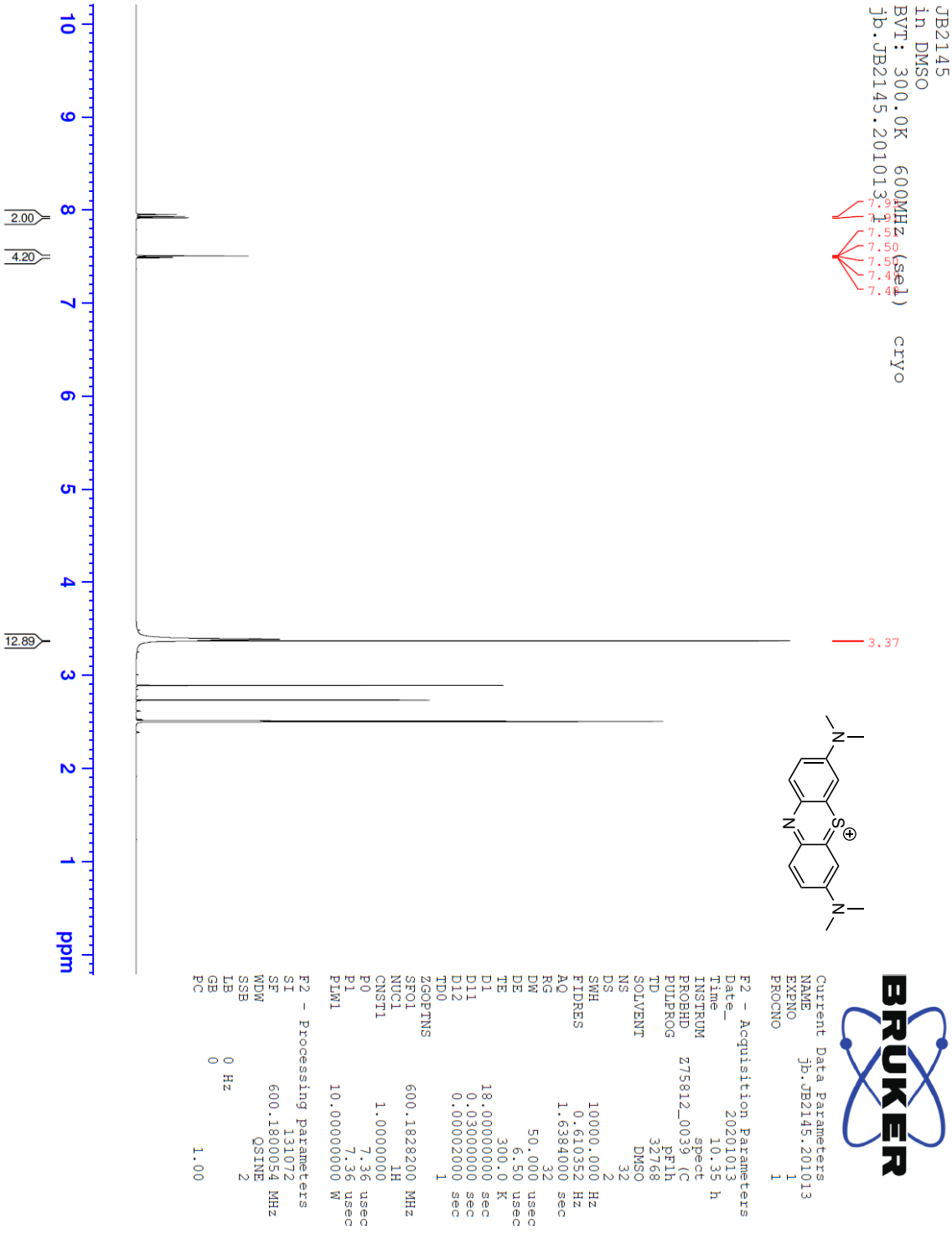


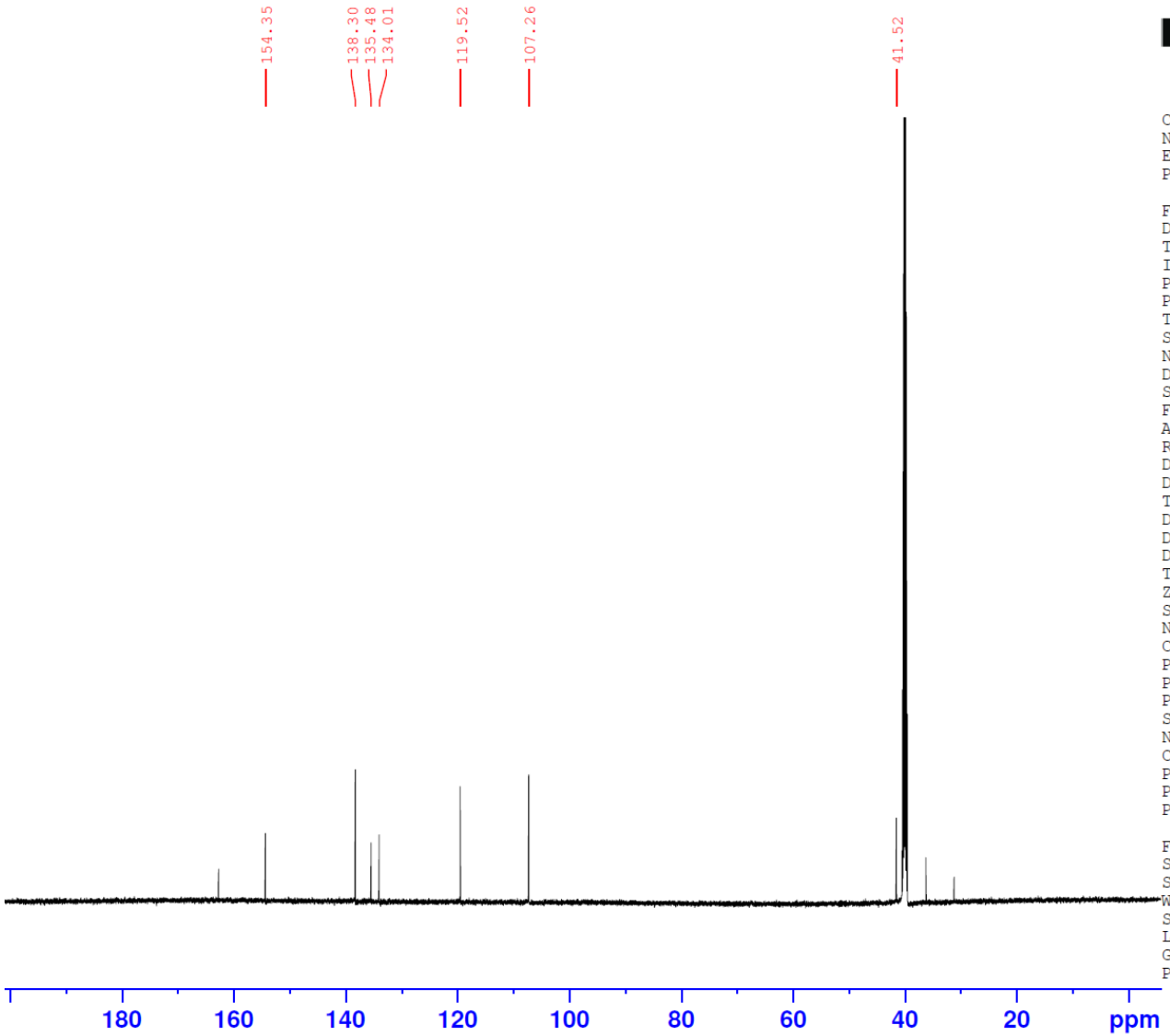
Current Data Parameters
NAME jb.KR34.200930
EXPNO 4
PROCNO 1

F2 - Acquisition Parameters
Date_ 20200930
Time 17.06 h
INSTRUM spect
PROBHD Z75812_0039 (C
PULPROG MFhetero1d
TD 131072
SOLVENT DMSO
NS 1024
DS 2
SWH 31250.000 Hz
FIDRES 0.476837 Hz
AQ 2.0971520 sec
RG 256
DW 16.000 usec
DE 20.00 usec
TE 300.0 K
D1 2.00000000 sec
D11 0.03000000 sec
D12 0.00002000 sec
TD0 1
ZGOPTNS -Ddc -Dnoe
SFO1 150.9301310 MHz
NUC1 13C
CNST1 1.0000000
P0 11.43 usec
P3 11.43 usec
PLW1 120.23000336 W
SFO2 600.1828200 MHz
NUC2 1H
CPDPRG[1 waltz16
PCPD2 80.00 usec
PLW2 10.00000000 W
PLW19 0.07620790 W

F2 - Processing parameters
SI 131072
SF 150.9153830 MHz
WDW no
SSB 0
LB 0 Hz
GB 0
PC 1.40

3.15. 3,7-Bis(dimethylamino)phenothiazin-5-ium (Methylene Blue)





Current Data Parameters
NAME jb.JB2145.201013
EXPNO 2
PROCNO 1

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PULPROG MFhetero1d
TD 65536
SOLVENT DMSO
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DS 0
SWH 31250.000 Hz
FIDRES 0.953674 Hz
AQ 1.0485760 sec
RG 256
DW 16.000 usec
DE 20.00 usec
TE 300.0 K
D1 2.00000000 sec
D11 0.03000000 sec
D12 0.00002000 sec
TD0 1
ZGOPTNS -Ddc -Dnoe
SFO1 150.9301310 MHz
NUC1 13C
CNST1 1.0000000
P0 11.43 usec
P3 11.43 usec
PLW1 120.23000336 W
SFO2 600.1828200 MHz
NUC2 1H
CPDPRG[1 waltz16
PCPD2 80.00 usec
PLW2 10.00000000 W
PLW19 0.09751600 W

F2 - Processing parameters
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SF 150.9153833 MHz
WDW EM
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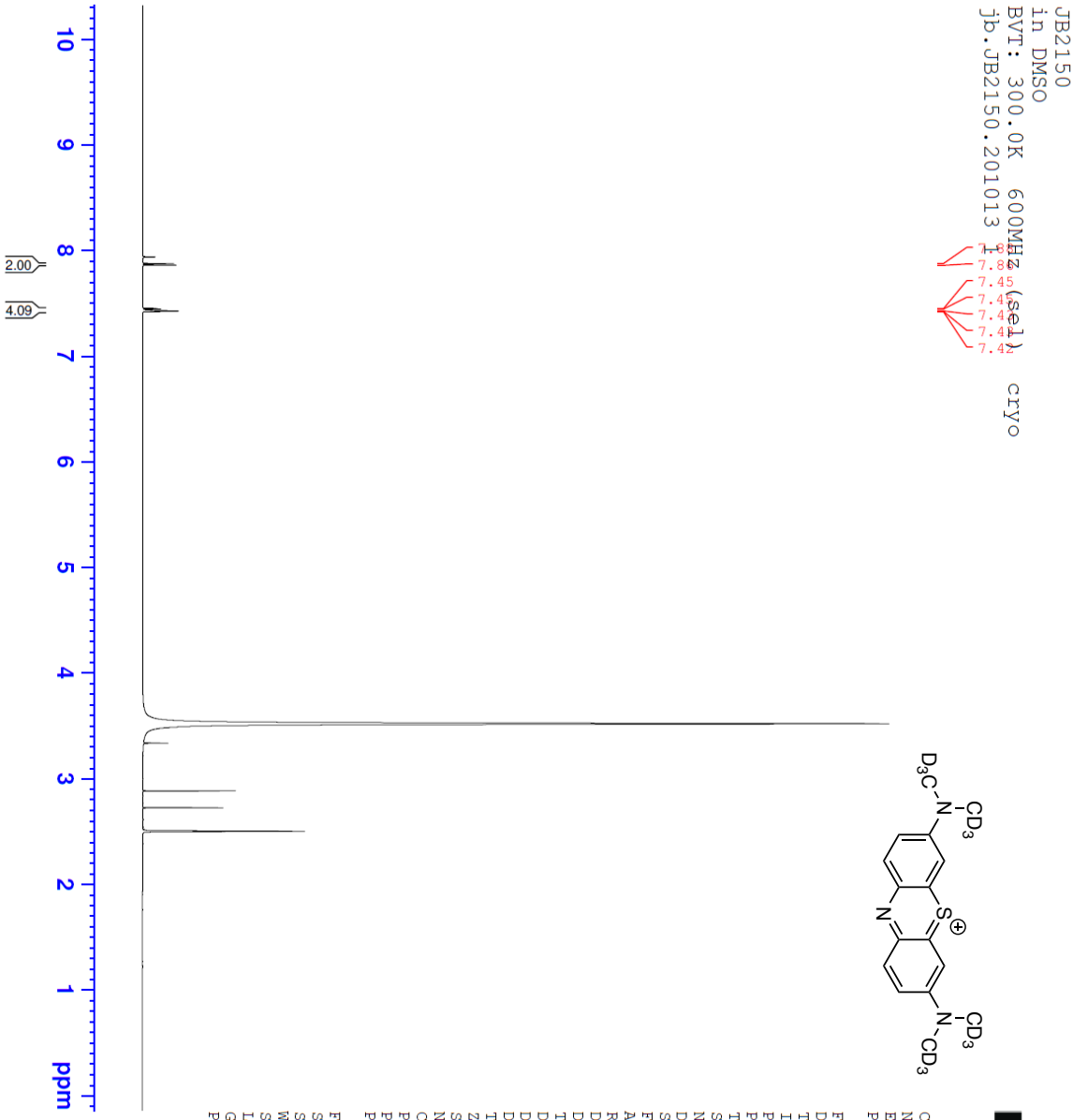
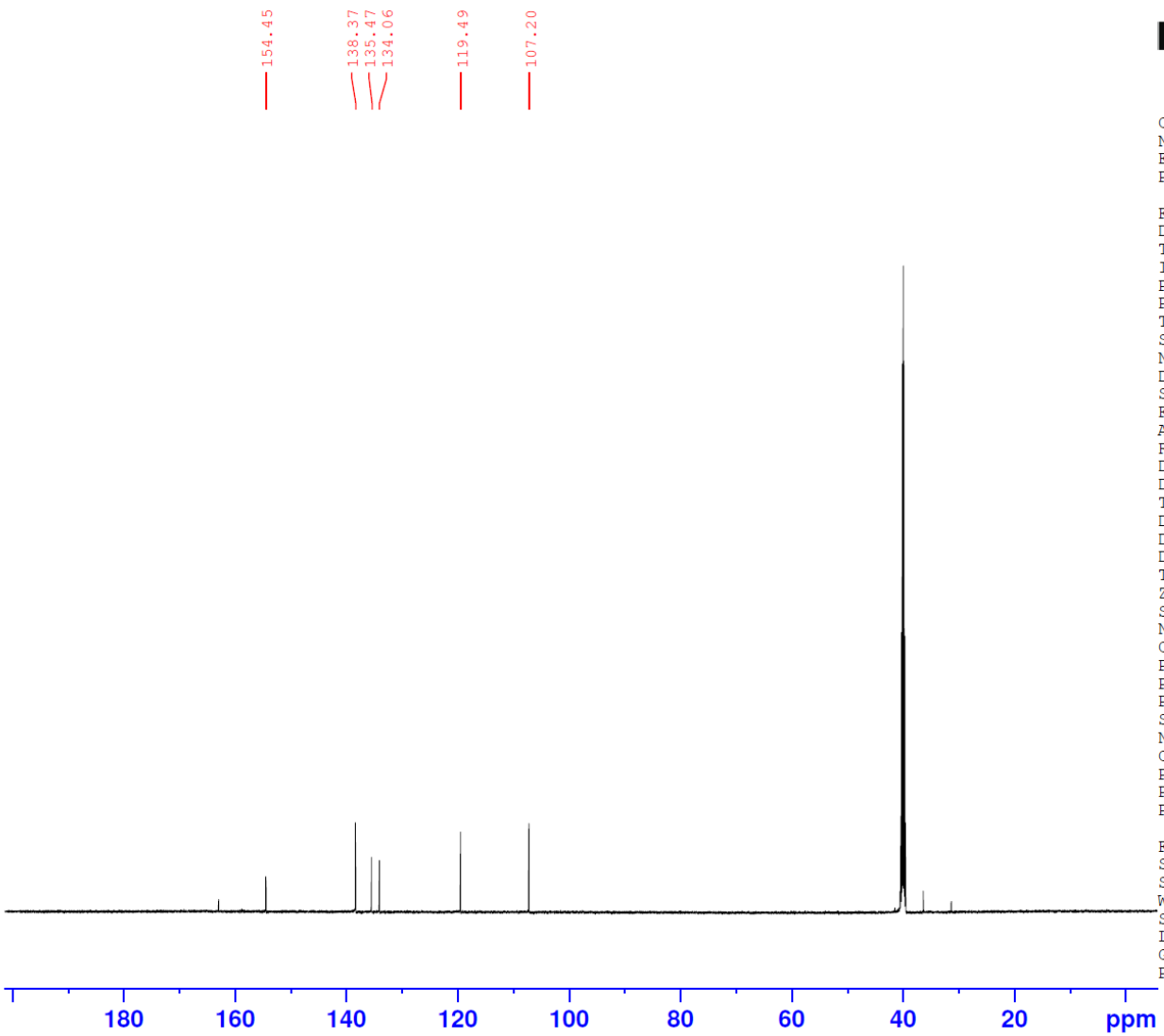
3.16. 3,7-Bis(bis(methyl-d3)amino)phenothiazin-5-ium (Methylene Blue-d12)



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DE                20.00 usec
TE                300.0 K
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TD0                     1
ZGOPTNS       -Ddc -Dnoe
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NUC1                 13C
CNST1          1.0000000
P0                11.43 usec
P3                11.43 usec
PLW1        120.23000336 W
SFO2         600.1828200 MHz
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CPDPRG[1         waltz16
PCPD2             80.00 usec
PLW2         10.00000000 W
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F2 - Processing parameters
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GB        0
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```

4. $SNAP_f$ construct

$SNAP_f$ sequence:

MASWSHPQFE KGADDDDKVP HMDKDCEMKR TTLDSPLGKL ELSGCEQGLH RIIFLGKGTS
AADAVEVPAP AAVLGGPEPL MQATAWLNAY FHQPEAIEEF PVPALHHPVF QQESFTRQVL
WKLLKVVKFG EVISYSHLAA LAGNPAATAA VKTALSGNPV PILIPCHRVV QGDLDVGGYE
GGLAVKEWLL AHEGHRLGKP GLGAPGFSSI SAHHHHHHHHHH

Strep-Tag II, Enterokinase-site, $SNAP_f$, His-Tag

5. SNAP–Halo construct and mass spectrometry

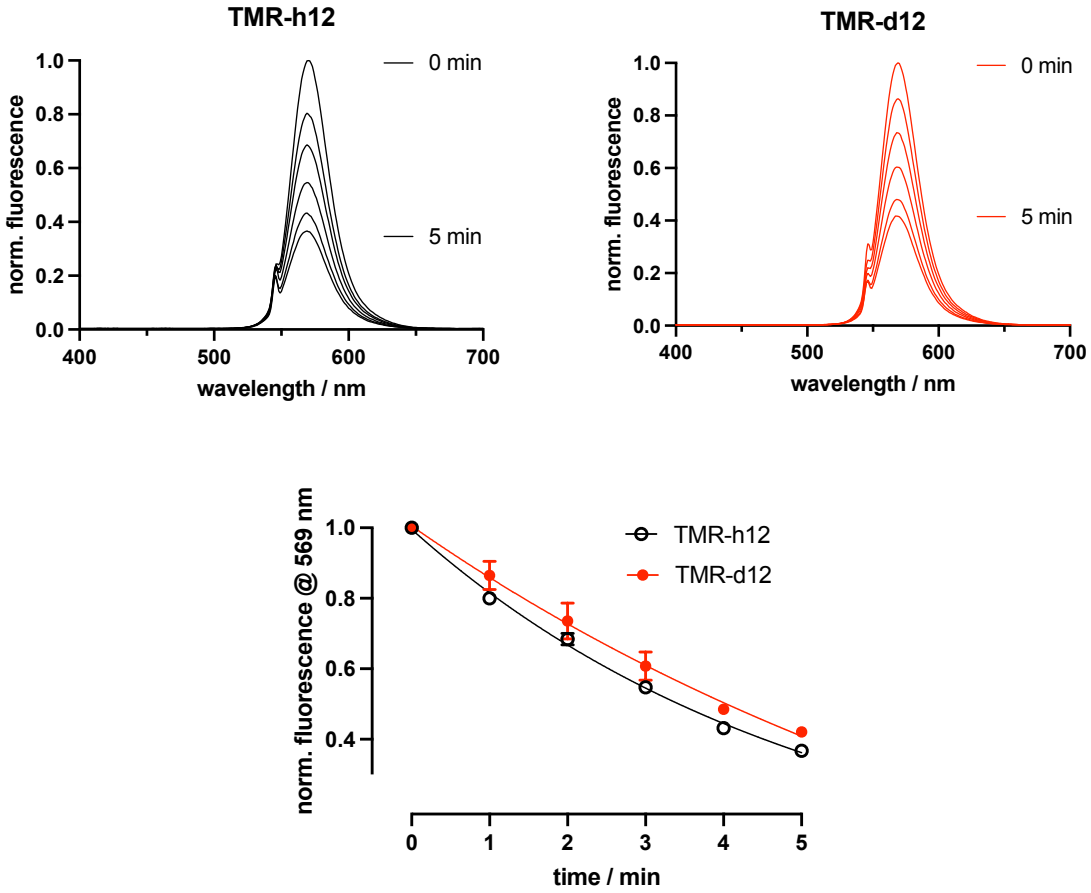
SNAP–Halo sequence:

MASWSHPQFE KGADDDDKVP HMDKDCEMKR TTLDSPLGKL ELSGCEQGLH EIIFLGKGTS
AADAVEVPAP AAVLGGPEPL MQATAWLNAY FHQPEAIEEF PVPALHHPVF QQESFTRQVL
WKLLKVVKFG EVISYSHLAA LAGNPAATAA VKTALSGNPV PILIPCHRVV QGDLDVGGYE
GGLAVKEWLL AHEGHRLGKP GLGGRLEVLF QGPKAFLEGS EIGTGFPFDP HYVEVLGERM
HYVDVGPRDG TPVLFLHGNP TSSYVWRNII PHVAPTHRCI APDLIGMGKS DKPDLGYFFD
DHVRFMDAFI EALGLEEVVL VIHDWGSALG FHWAKRNPER VKGIAFMEFI RPIPTWDEWP
EFARETFQAF RTTDVGRKLI IDQNVFIEGT LPMGVVRPLT EVEMDHYREP FLNPVDREPL
WRFPNELPIA GEPANIVALV EEYMDWLHQS PVPKLLFWGT PGVLIPPAEA ARLAKSLPNC
KAVDIGPGLN LLQEDNPDLI GSEIARWLST LEISGAPGFS SISAHHHHHH HHHH*

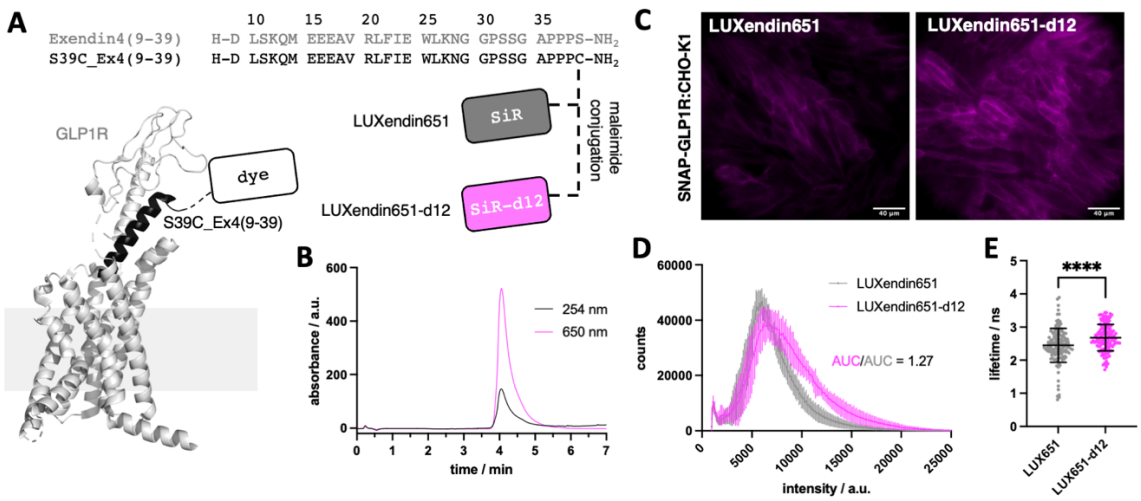
Strep-Tag II, Enterokinase-site, SNAP, Precission Sequence, Halo, His-Tag

Condition	calc.	found
SNAP–Halo	59072	59069
SNAP–Halo:BG-TMR+CA-SiR	60244	60245
SNAP–Halo:BG-SiR+CA-TMR	60244	60241
SNAP–Halo:BG-TMR-d12+CA-SiR-d12	60268	60269
SNAP–Halo:BG-SiR-d12+CA-TMR-d12	60268	60267
SNAP–Halo:BG-TMR	59603	59602
SNAP–Halo:BG-SiR	59645	59642
SNAP–Halo:CA-TMR	59671	59669
SNAP–Halo:CA-SiR	59713	59711
SNAP–Halo:BG-TMR-d12	59612	59615
SNAP–Halo:BG-SiR-d12	59657	59656
SNAP–Halo:CA-TMR-d12	59683	59682
SNAP–Halo:CA-SiR-d12	59725	59724

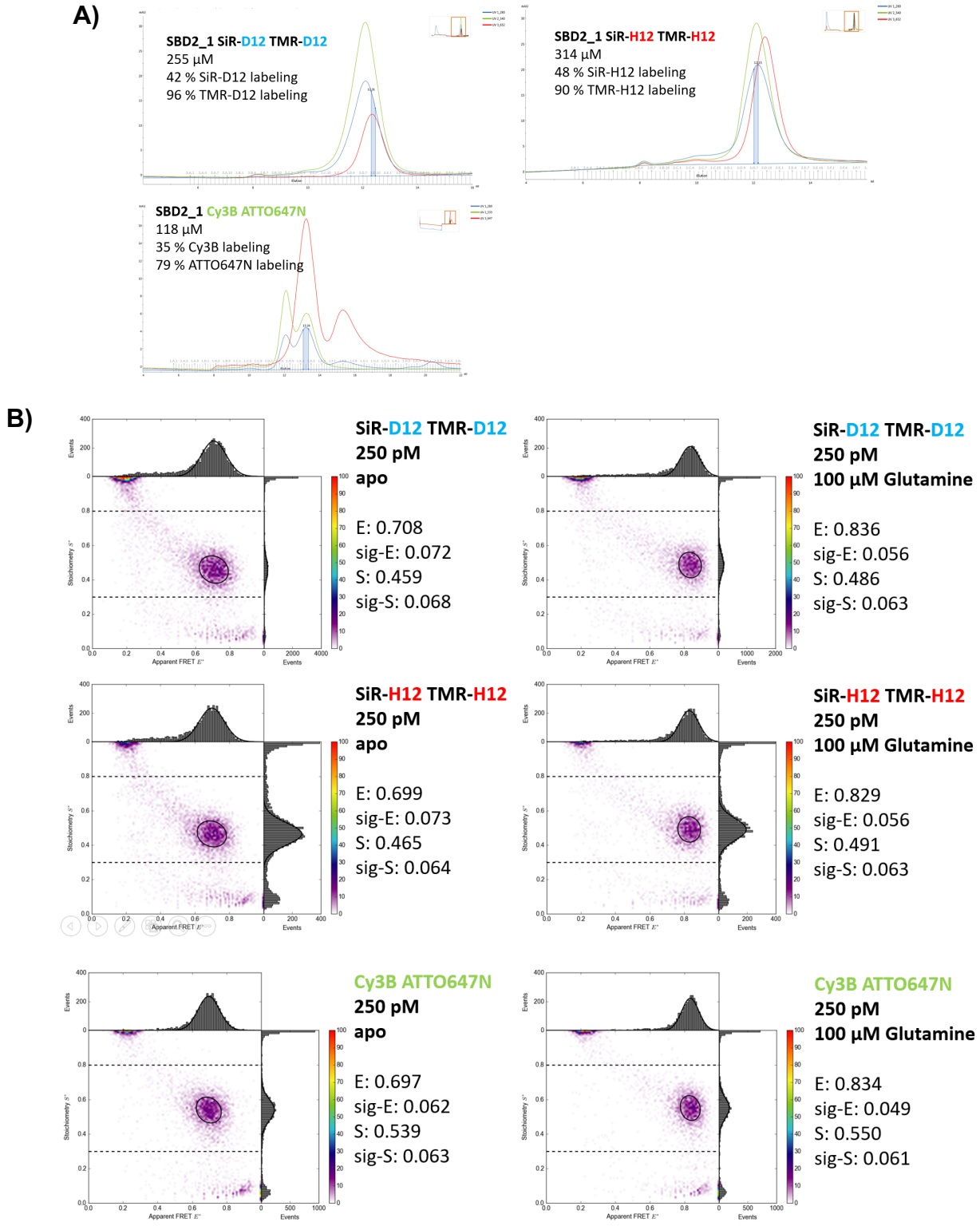
6. Supplementary Figures



Supporting Figure 1: In vitro bleaching of TMR and TMR-d12 with white light. Following fluorescence emission after 0, 1, 2, 3, 4, and 5 minutes of irradiation (top). Normalized fluorescence at 569 nm plotted over time (bottom). Mean±S.D.

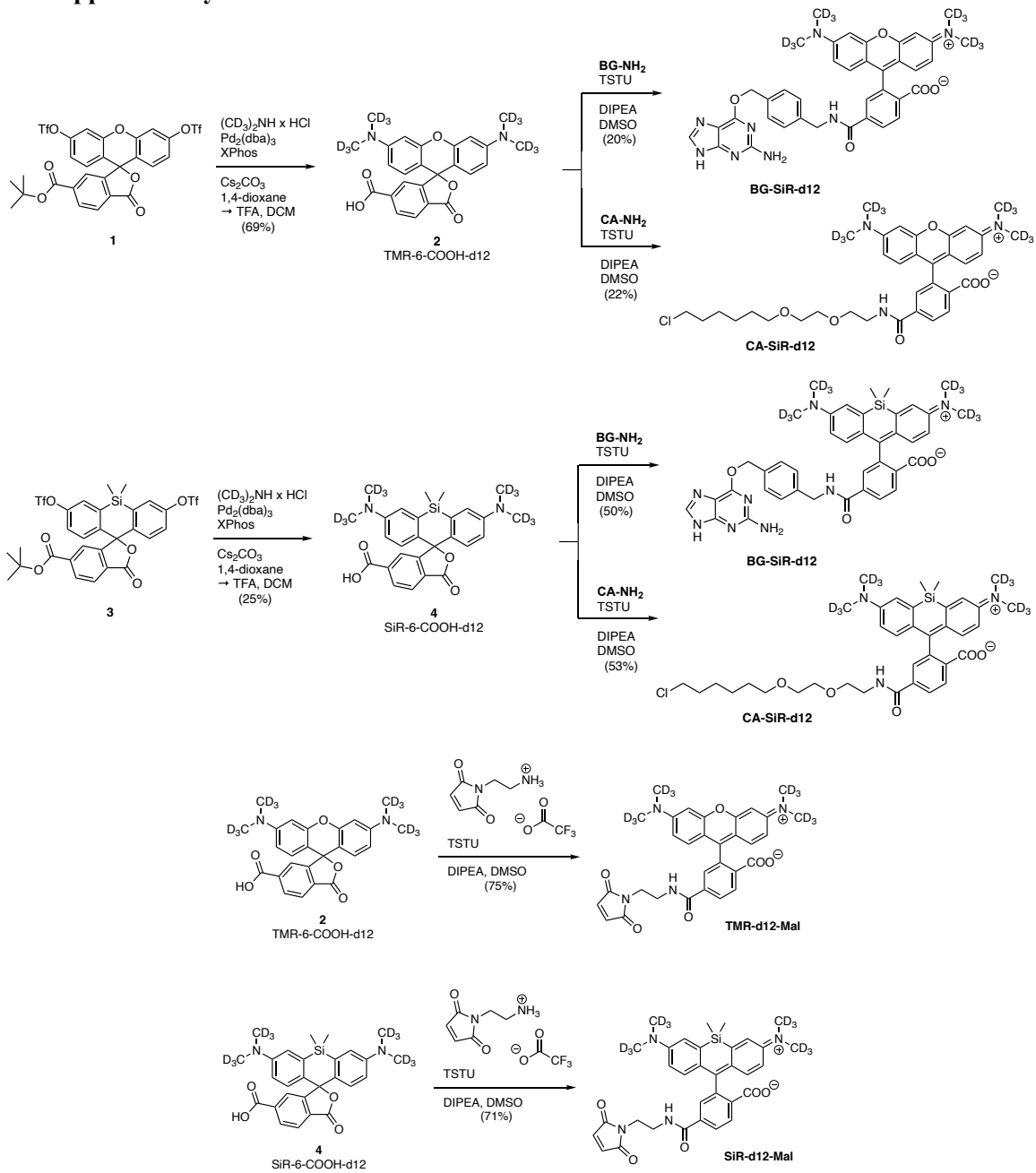


Supporting Figure 2: LUXendin651 versus LUXendin651-d12 in fixed SNAP-GLP1R:CHO-K1 cells. A) S39C_Ex4(9-39) is derived from Exendin4(9-39) with a C-terminal cysteine handle for maleimide bioconjugation wit SiR or SiR-d12, yielding LUXendin651 and LUXendin651-d12, respectively, which are high affinity and selective antagonists towards the GLP1R. B) LCMS trace of LUXendin651-d12. C) Labelling of SNAP-GLP1R in fixed CHO-K1 cells with n = 2 biological replicates. Scale bar = 40 µm. D) Pooled raw histograms from five images as represented in (C) show increased brightness of LUXendin651-d12 versus LUXendin651. E) Fluorescence lifetimes of LUXendin651-d12 versus LUXendin651 in fixed SNAP-GLP1R:CHO-K1 cells. n>200 ROIs, **** indicates statistical significance (unpaired t-test, p<E-26). Mean±SD.

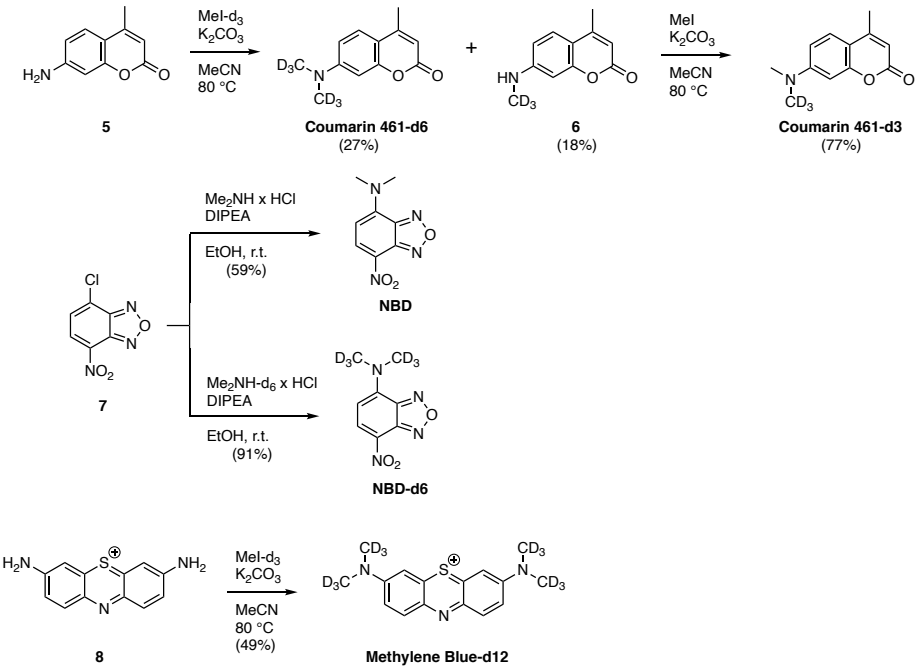


Supporting Figure 3: Summary of SEC labelling characterization (A) and smFRET data for different fluorophore pairs (B) for stochastic labelling of SBD2.

7. Supplementary Schemes



Supporting Scheme 1: Chemical synthesis of rhodamine d12 probes.



Supporting Scheme 2: Chemical synthesis of (deuterated) coumarin, NBD and methylene blue.

8. Supplementary Tables

Table S1: Photophysical parameters from Figure 1 with error margins.

	TMR	TMR-d12	SiR	SiR-d12
Ext. coefficient [M^{-1} cm^{-1}]	78,000±200	90,000±600	141,000±1,000	156,000±1,600
Quantum yield (integrating sphere) [%]	43.1±5.2	51.3±5.0	35.3±4.8	46.1±3.0
Lifetime [ns]	2.287±0.392	2.588±0.495	2.898±0.383	3.534±0.164

Table S2: Photophysical parameters for LUXendin651(-d12).

	LUXendin651	LUXendin651-d12
Lifetime [ns]	2.448±0.513	2.682±0.400

Table S3: Photophysical parameters from Figure 4 with error margins.

	Coumarin461 Coumarin461-d6	NBD NBD-d6	Methylene blue Methylene blue-d12
Ext. coefficient [M^{-1} cm^{-1}]	28,100±400 27,900±2,000	16,300±700 16,000±1,000	45,500±2,500 49,800±1,800
Quantum yield (platreader) [%]	19±0.2 27±2.4	55±7.0 59±1.8	1.0±0.05 1.3±0.02

Table S4: Temperature-dependent lifetimes from Figure 5.

Lifetime [ns]	Halo:SiR	Halo:SiR-d12	SNAP:SiR	SNAP:SiR-d12
20 °C	3.53±0.05	4.02±0.04	3.26±0.27	3.75±0.28
30 °C	3.39±0.04	3.89±0.03	3.03±0.02	3.51±0.05
40 °C	3.16±0.04	3.68±0.03	2.84±0.02	3.37±0.03

Table S5: Normalized values from Table S3.

Lifetime [%]	Halo:SiR	Halo:SiR-d12	SNAP:SiR	SNAP:SiR-d12
20 °C	100	100	100	100
30 °C	96.2±0.7	96.8±0.5	93.5±7.0	93.9±5.3
40 °C	89.6±0.2	91.5±0.6	87.8±7.5	90.3±6.7

9. References

1. Farrants, H. *et al.* SNAP-Tagged Nanobodies Enable Reversible Optical Control of a G Protein-Coupled Receptor *via* a Remotely Tethered Photoswitchable Ligand. *ACS Chem. Biol.* **13**, 2682–2688 (2018).
2. Grimm, J. B. *et al.* A general method to improve fluorophores for live-cell and single-molecule microscopy. *Nat. Methods* **12**, 244–250 (2015).
3. Ast, J. *et al.* Super-resolution microscopy compatible fluorescent probes reveal endogenous glucagon-like peptide-1 receptor distribution and dynamics. *Nat. Commun.* **11**, 467 (2020).