Supporting Information

Two-color 810 nm STED Nanoscopy of Living Cells with Endogenous SNAP-tagged Fusion Proteins

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Absorption/emission spectra and fluorescence quantum yield determination

The absorption spectra were recorded on a Varian Cary 4000 UV-Vis spectrophotometer. The emission spectra were recorded on a Varian Cary Eclipse fluorescence spectrophotometer. The fluorescence quantum yields of the dyes *SiR*, *610CP* and *620CP* were previously reported.[S1] The fluorescence quantum yields of the dyes *620SiR* and *680SiR* were recorded in PBS (pH 7.4) solutions on a Quantaurus QY C11347-12 spectrometer[S2] (Hamamatsu Photonics, Shizuoka, Japan) at room temperature.

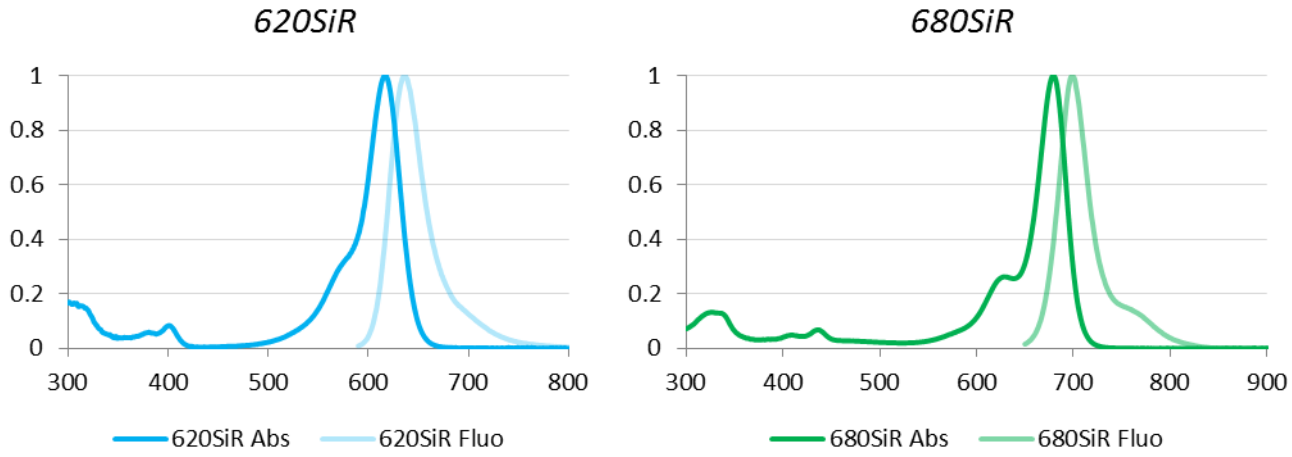


Figure S1. Absorption (Abs) and emission (Fluo) spectra of the dyes *620SiR*, *680SiR*.

The photophysical data are given in Table 1 in the main part of the manuscript.

Response of the dyes *680SiR* and *SiR* to solvent polarity

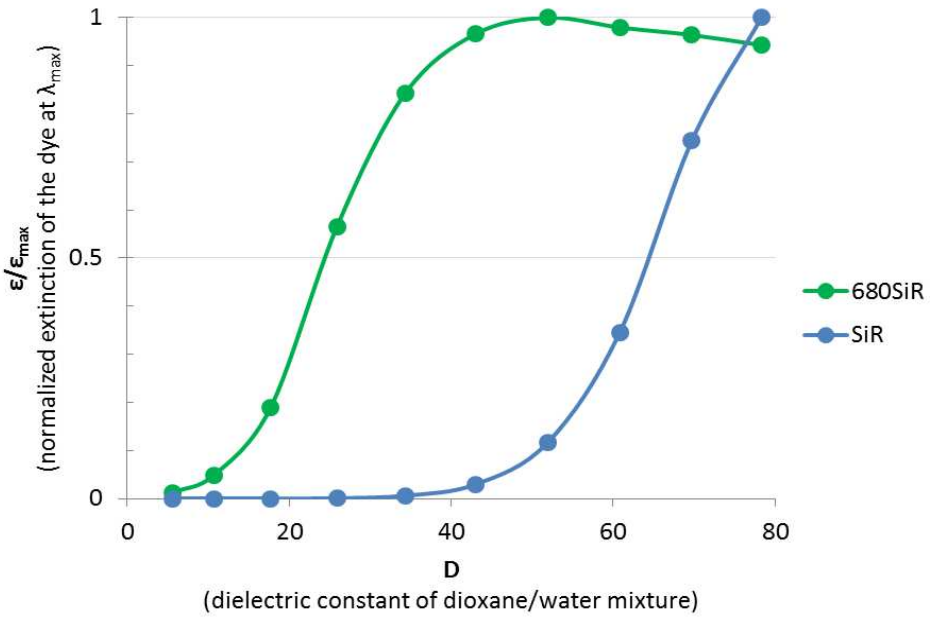


Figure S2. Normalized extinction, $\varepsilon/\varepsilon_{max}$, at λ_{max} of the dyes *680SiR* and *SiR* versus the dielectric constant, D, of dioxane–water mixtures. The $D_{0.5}$ values correspond to the intersection of interpolated graphs with a $\varepsilon/\varepsilon_{max} = 0.5$ line.

Generation of knock-in cell lines

Endogenous tagging of vimentin (VIM) with SNAP or Halo was essentially done as described previously.[S3] Briefly, Cas9 was targeted to the stop codon region of the human VIM locus by a guide RNA (gRNA) with the sequence 5'-GCGCAAGATAGATTTGGAAT that was inserted into the plasmid pX330 via oligo cloning.[S4] The DNA sequence for SNAP or Halo was amplified from a plasmid (pSNAPf: NEB, Ipswich, MA, USA; pHTC: Promega, Mannheim, Germany) using the primer pair 5'-ATATTAGCGGCCGCCGGTAGTGGTTCAGACAAAGACTGCGAAATGAAGCGC/5'-TTTAATTCCA TGGTTAATTAACCTCGAGTTTAAACGCGG or 5'-GATCTGGCGGCCGCGGGTAGTGGTTCAGGA TCCGAAATCGGTACTGGC/5'-GCATGCCACCATGGTTAACCGGAAATCTCCAGAGTAGACAGCC, respectively, followed by cloning into a VIM donor plasmid[S3] via NotI/NcoI restriction digests.

Human U2OS cells (ATCC, Manassas, VA, USA) were co-transfected with the Cas9/gRNA plasmid and the respective donor plasmid using FuGENE HD transfection reagent (Promega, Mannheim, Germany). Ten days post transfection successfully tagged cells were identified by incubating the culture with 5 µM SiR-Halo or SiR-SNAP for 30 min followed by single cell sorting into 96-well plates using a FACSAria II flow cytometer (BD Biosciences, Heidelberg, Germany). Two weeks after sorting the cells were split and transferred into 12-well plates containing glass cover slips and tagging of VIM-SNAP or VIM-Halo was confirmed using an epifluorescence microscope (DM6000B, Leica Microsystems, Wetzlar, Germany) equipped with an oil immersion objective (1.4 NA; 100x; Planapo; Leica) and a S-FRED filter cube (excitation filter: HQ 630/20x; emission filter: HQ 667/30x). Western blotting was carried out as described previously[S3] using the primary antibodies anti-Vimentin (V9; 151000; Santa Cruz Biotechnology, Heidelberg, Germany) and anti-Actin (AC74; 153000, Sigma-Aldrich) as well as HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (Dianova, Hamburg, Germany).

STED setup description

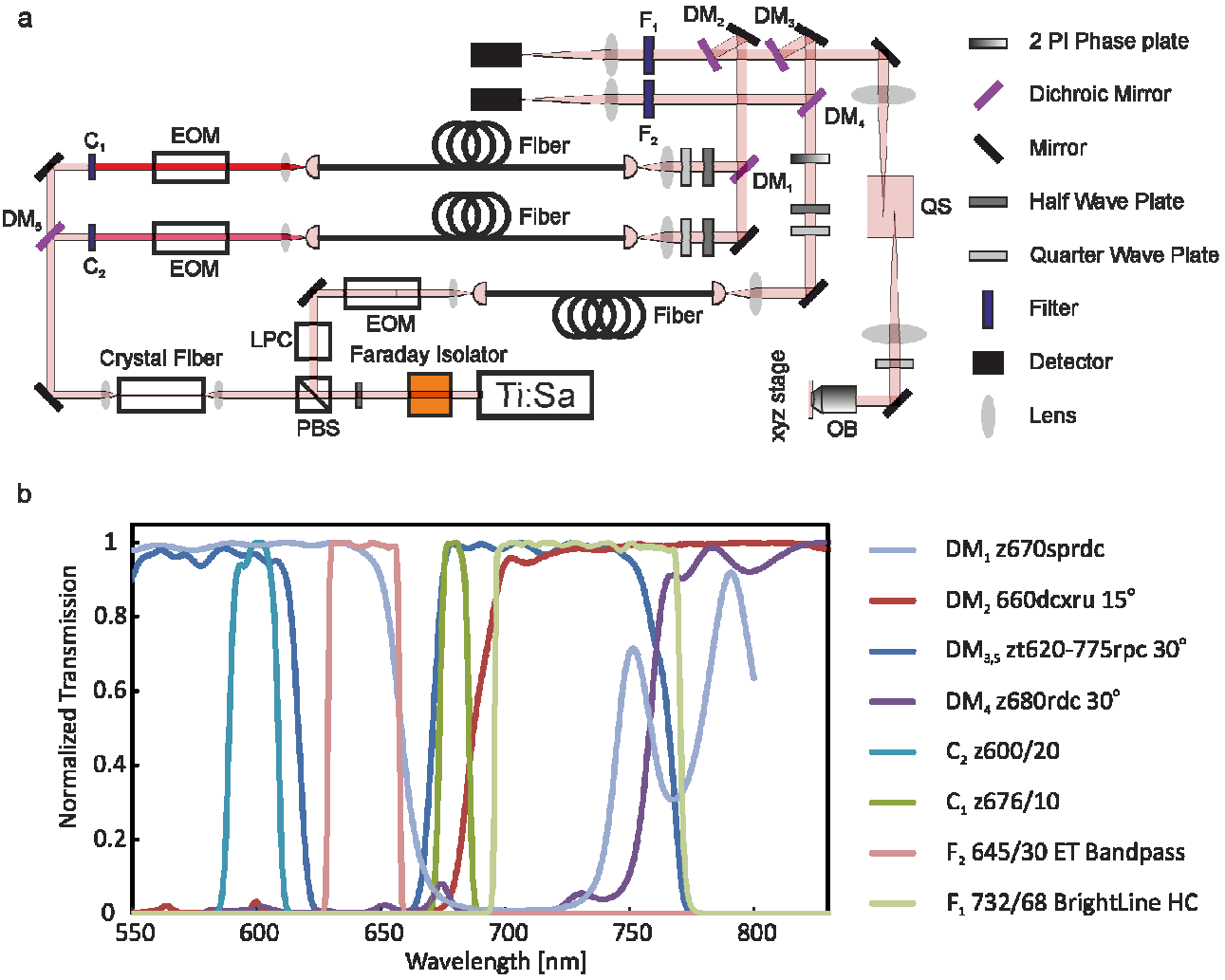


Figure S3. Scheme of the home-built STED setup and filter/dichroic mirror settings. OB: objective; EOM: electro-optical modulator; PBS: polarization beam splitter; LPC: laser power controller; QS: home-built quartz scanner with four scanning mirrors; $DM_{1\sim5}$: dichroic mirrors; $C_{1,2}$: clean up filter; $F_{1,2}$: fluorescence band pass filter.

(a) Scheme of the setup. Both excitation and STED light (620 nm, 676 nm and 806~820 nm) is delivered by a titanium-sapphire laser (Mai Tai - Spectra-Physics, Santa Clara, CA, United States, pulse repetition rate: 80 MHz, pulse duration ~150 fs). The 806~820 fs pulsed laser beam is split in two with a polarization beam splitter: about 120 mW is fed into a crystal fiber (FemtoWHITE 800, NKT Photonics A/S, Birkerød, Denmark) to generate white light and >1 W is used for stimulated emission depletion. The white light is again split with a dichroic mirror to generate two excitation beams by passing through band pass filters (z600/20 and z676/10). Each of the excitation beams can be switched on and off with electro-optical modulators (EOM, LM0202, Qioptiq Photonics) to switch between the two channels for each pixel in raster scans. The excitation beams are fed into two polarization-maintaining single-mode fibers (11 m, PM-S405-XP, Thorlabs GmbH, Dachau/Munich, Germany). STED beam is fed into a long fiber (120 m, PMJ-3U3A-633-4/125-3-120-HP, AMS Technologies AG, Martinsried, Germany) to disperse the pulsed light to ~120 ps.

The output of the STED fiber is guided onto a vortex phase plate (VPP1b or VPP1a, RPC Photonics, Rochester, NY, USA), imprinting a helical phase shift on the beam and thereby creating a doughnut-shaped depletion pattern in the focal plane. λ/2- and λ/4-plates are inserted into the STED beam path to create circular polarization. Dichroic mirrors are purchased either from AHF analysentechnik AG, Tübingen, Germany or Chroma, Bellows Falls, VT, USA. An oil immersion objective lens (HCX-PL-APO 100x/1.4-0.7 OIL CS, Leica Microsystems, Wetzlar, Germany) is used for focusing excitation and STED beams and fluorescence collection in combination with a tube lens (200 mm, Thorlabs) was used. Laser beams are scanned by a home-built scanner ("Quad Scanner") including four galvanometer scan mirrors. Coarse movement of the sample is accomplished by a piezo translator (NanoMax, Thorlabs). Fluorescence is collected by two multimode fibers with a core diameter of 62.5 μm and the fiber cores are acting as a confocal pinhole corresponding to ~1 times the Airy disk at this position. Fluorescence is detected with two avalanche photodiodes (APD) (SPCM-AQRH-13, Excelitas, Waltham, MA, USA). The APD signal is then collected with a time correlated single photon counting data acquisition card (DPC 230, Becker & Hickl GmbH, Berlin, Germany) with additional controlling home-built electronics. For adjustment, a removable pellicle beam splitter (BP145B1, Thorlabs, Newton, NJ, USA) was used in the beam path, and laser light reflected from gold nanospheres was collected with a photomultiplier tube (PMT) (H10723-01, Hamamatsu Photonics). Experimental control and image acquisition were done with home-built MATLAB software. All laser power values refer to the back aperture of the objective lens. Actual power at the sample is lower due to finite transmission of the objective lens.

(b) Transmission spectra of dichroic mirror, clean up filter and fluorescence band pass filters. Two additional short pass filters (F37-774, AHF Analysentechnik AG) are used in the fluorescence detection paths to block the STED light.

Fluorescence intensity changes of substrates *680SiR*-SNAP, *SiR700*-C_8-SNAP and *SiR*-SNAP in the presence of SNAP-tag protein

In 1.5 mL test tubes (Eppendorf), 5 µL of 25 µM stock solution of a dye-ligand conjugate *SiR*-SNAP, *680SiR*-SNAP or *SiR700*-C_8-SNAP in DMSO was added to 250 µL of

1) 1 mM DTT in 1x PBS (pH 7.4),
2) 1 µM SNAP-tag purified protein (M = 20 kDa; available from New England BioLabs, cat. No. P9312S), prepared by dissolution of a 50 µg sample in 2.45 mL of 1 mM DTT in 1x PBS (pH 7.4),
3) 1 mM DTT in 0.1% (1 mg/mL) of BSA (bovine serum albumin) in 1x PBS (pH 7.4),
4) 1 µM SNAP-tag purified protein, prepared by dissolution of a 50 µg sample in 2.45 mL of 1 mM DTT in 0.1% (1 mg/mL) of BSA in 1x PBS (pH 7.4),
5) 0.5% SDS and 1 mM DTT in 0.1% (1 mg/mL) of BSA (bovine serum albumin) in 1x PBS (pH 7.4) *or*
6) 1x PBS (pH 7.4) control

and incubated at 37 °C for 2 h (all samples prepared ×2; protein/dye ratio = 2). The samples were aliquoted (50 µL each, ×4) onto a 96-well microplate, and fluorescence intensity was measured on a Spark 20M (Tecan) microplate reader in 10 min intervals over 1 h (excitation and emission bandwidths were set to 10 nm). The excitation and emission wavelengths were selected as follows:

Dye	*SiR*-SNAP	*680SiR*-SNAP	*SiR700*-C_8-SNAP
Excitation wavelength, nm	635	670	690
Detection wavelength, nm	660	700	720

The last two readings of four samples per dye (8 values total) were averaged and considered obtained in a single experiment. The results (an average of two independent experiments) are presented as fluorescence intensity ratios for:

a) solutions containing SNAP-tag protein(+DTT)/solutions containing DTT in PBS only,
b) solutions containing SNAP-tag protein+BSA(+DTT)/solutions containing DTT in 0.1% BSA/PBS,
c) solutions containing SDS+BSA(+DTT)/solutions containing DTT in 0.1% BSA/PBS.

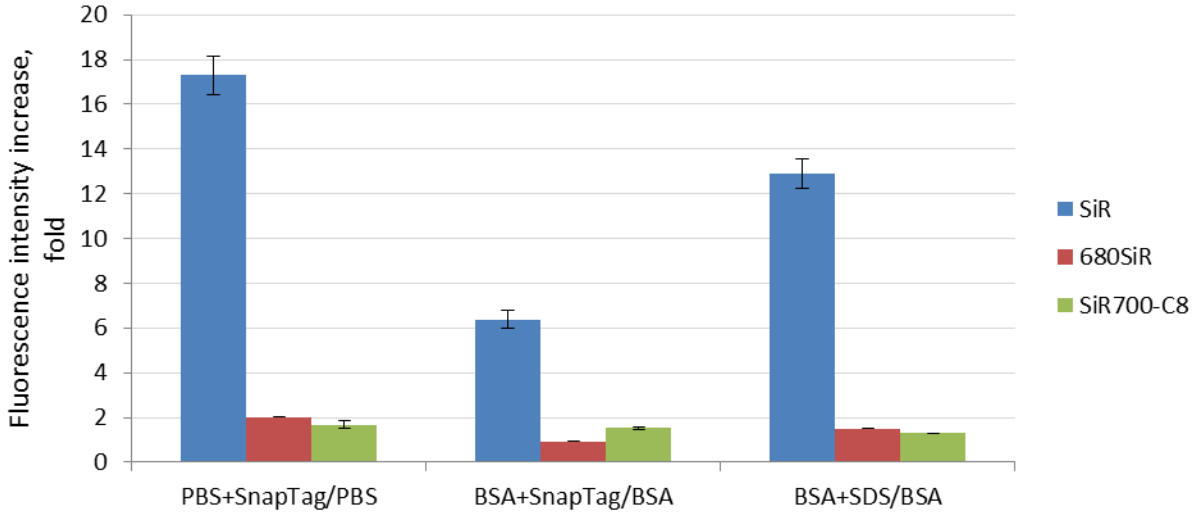


Figure S4. Fluorescence intensity increase of dye-ligand conjugates (*SiR*-SNAP, *680SiR*-SNAP and *SiR700*-C_8-SNAP) a) upon reaction with SNAP-tag protein in 1x PBS 7.4 + 1 mM DTT; b) upon reaction with SNAP-tag protein in 0.1% BSA in PBS 7.4 + 1 mM DTT; c) upon addition of 0.5% SDS in 0.1% BSA in PBS 7.4 + 1 mM DTT.

Live cell labeling

Labeling with *610CP*-Halo, *620CP*-Halo or *620SiR*-Halo (Figure S4): Endogenous vimentin Halo tagged human U2OS cells (Halo-VIM U2OS) were incubated with one of the live cell fluorescent probes (0.2 µM, 1.5 mL in HDMEM) for 30 min and washed for 10~30 minutes with HDMEM at 37 °C.

Labeling with *610CP*-tubulin2 and *680SiR*-SNAP probes for two-color 810 nm STED microscopy (Figure 6): Snap-VIM U2OS cells were incubated with *680SiR*-BG (0.5 µM, 1.5 mL in HDMEM) for 30 min, and then incubated with *610CP*-tubulin2 for 3 hours (1 µM, 1.5 mL in HDMEM) at 37 °C. After the medium was changed to HDMEM buffer, the stained cells were ready for imaging.

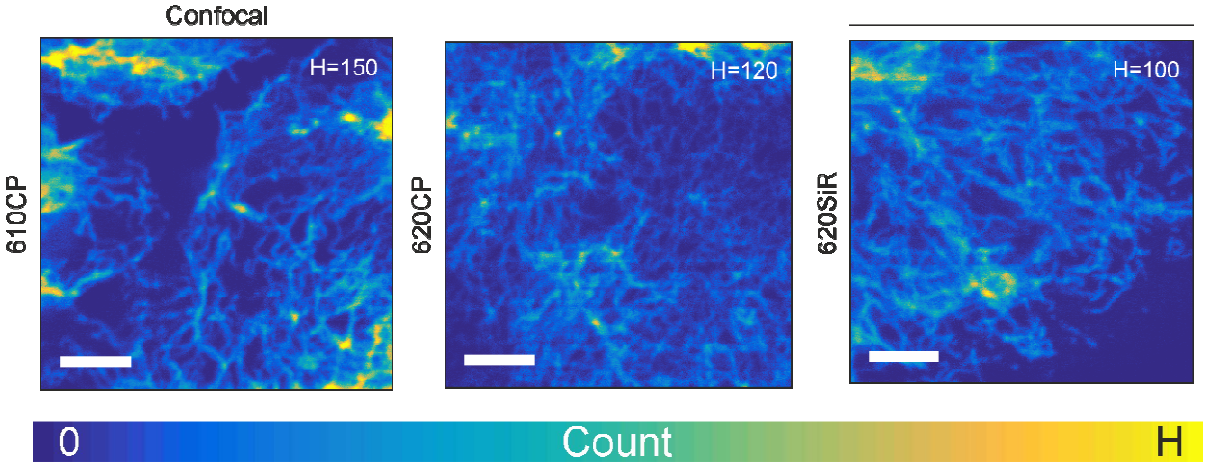


Figure S5. Comparison of the single color stainings with *610CP*-Halo, *620CP*-Halo, *620SiR*-Halo probes.

Halo-VIM U2OS cells were incubated with *610CP*-Halo, *620CP*-Halo or *620SiR*-Halo for 30 minutes (200 nM, 1.5 mL in HDMEM), washed with HDMEM at 37 °C for 10~30 minutes and imaged under confocal conditions. Color encodes the number of photons at each pixel. Pixel size: 30 nm; pixel dwell time: 300 µs; excitation wavelength: 600/20 nm; excitation power: ~ 5 µW; detection wavelength: 645/30; scale bar: 5 µm.

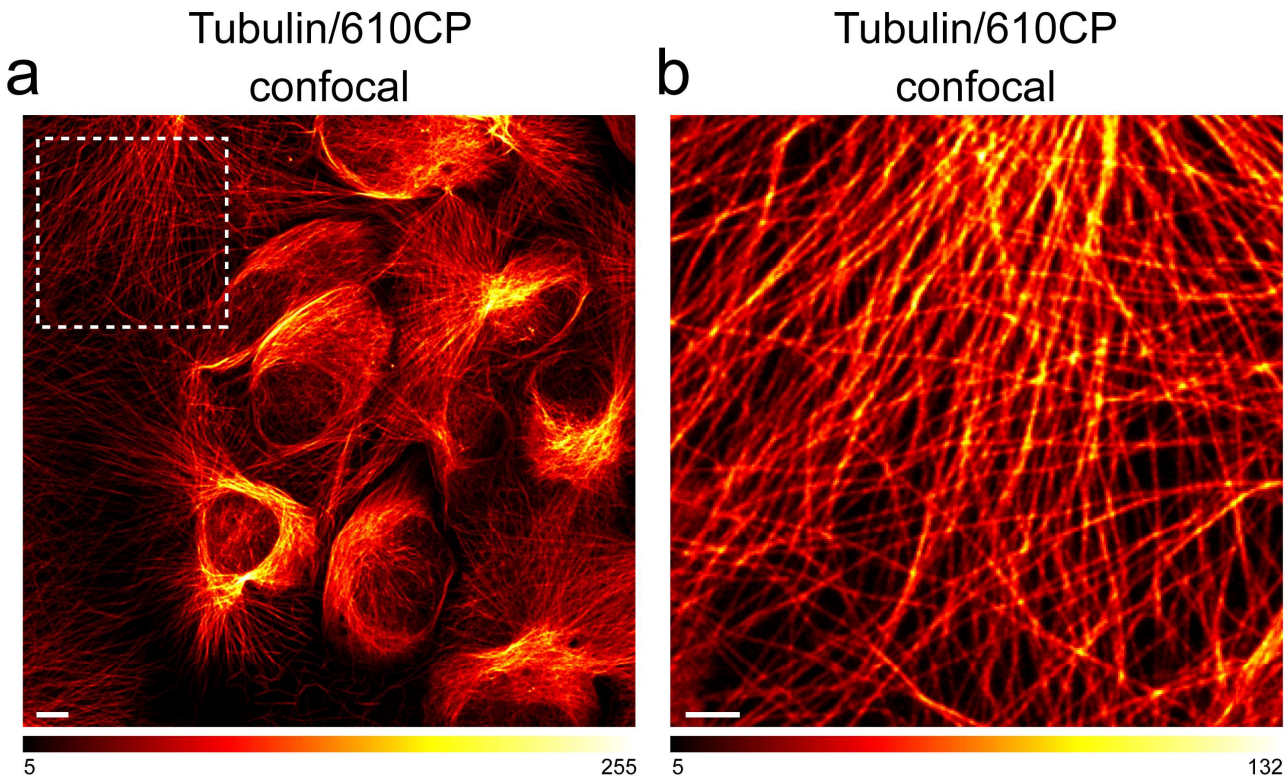


Figure S6. Confocal images of U2OS cells labelled with *610CP*-tubulin2.

U2OS cells were incubated with *610CP*-tubulin2 probe (final concentration 5 μM) for 30 minutes to label tubulin, washed for 30 minutes and finally imaged utilizing a Leica TCS SP8 beam scanning confocal microscope (Leica Microsystems CMS GmbH, Wetzlar Germany) (**a**, **b**); settings: excitation 561 nm 5%, detection: PMT 580-650 nm. Except for contrast stretching and smoothing no further image processing was applied. Scale bars: 10 μm (**a**); 5 μm (**b**).

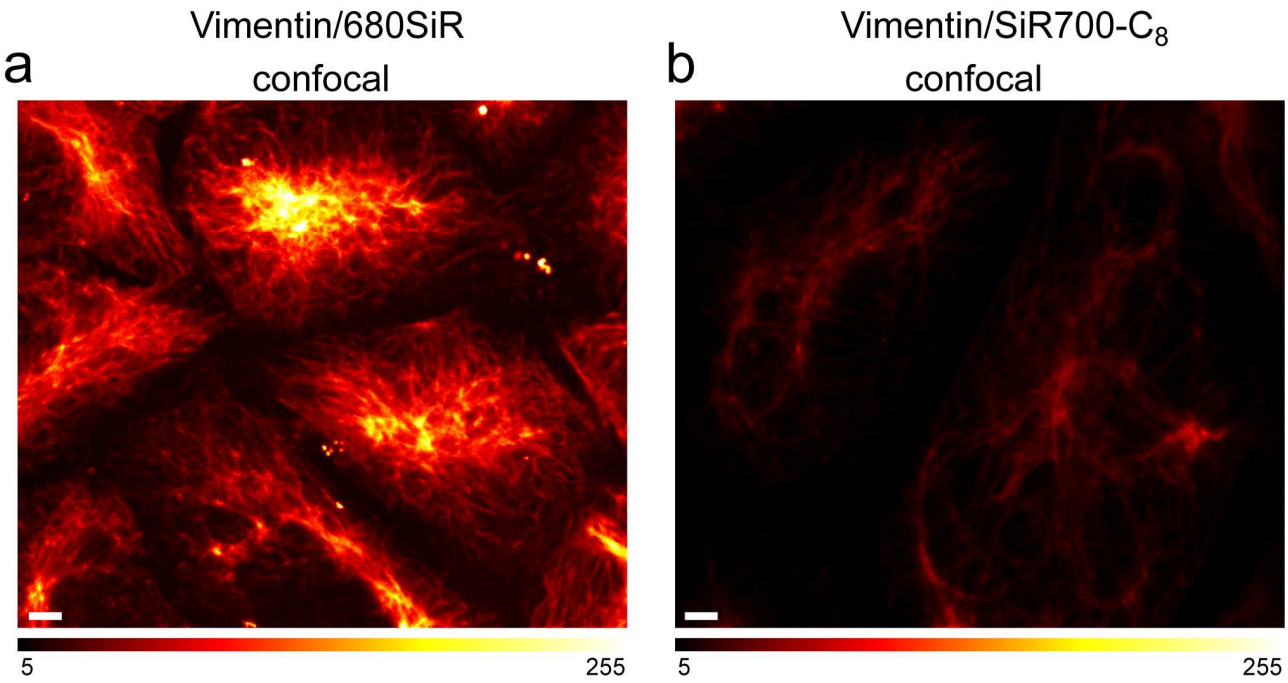


Figure S7. Confocal images of Vim-SNAP knock-in cells labelled with *680SiR*-SNAP (**a**) or *SiR700*-C_8-SNAP (**b**).

U2OS cells were incubated with the probes (final concentration 1 µM) for 60 minutes, washed for 60 minutes and finally imaged utilizing a Leica TCS SP8 beam scanning confocal microscope (Leica Microsystems CMS GmbH, Wetzlar Germany) (**a**, **b**). The same settings (excitation: 633 nm 30%, detection: PMT 660-794 nm, gain for detection: 650 V) were used for both images. A comparison of both images with respect to brightness shows that *680SiR*-SNAP is better suited for applications with the commercial setup utilized in this study. Except for contrast stretching, no further image processing was applied. Scale bars: 5 µm (**a,b**).

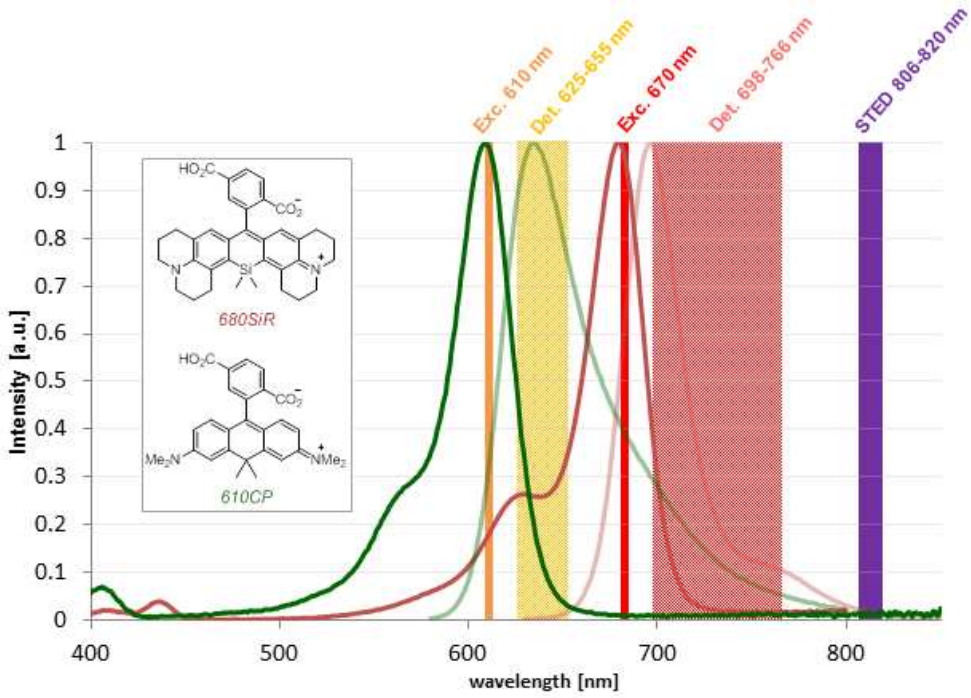


Figure S8. Two-color STED imaging scheme based on the use of *610CP* and *680SiR* dyes providing no crosstalk between observation channels.

For confocal two-color images shown in Figure 4 and obtained on a Leica TCS SP8 beam scanning confocal microscope, the following settings have been used:

610CP: excitation 561 nm 10%, detection PMT1 600-650 nm;

680SiR: excitation 633 nm 100%, detection PMT2 730-794 nm.

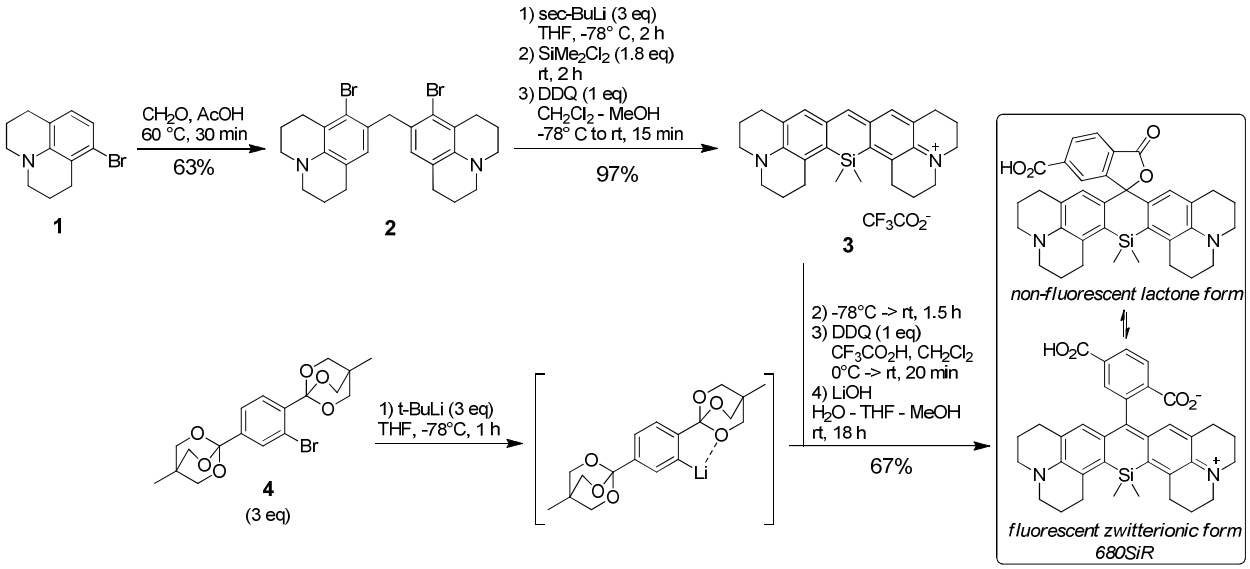
General experimental information and synthesis

NMR spectra were recorded at 25 °C with Agilent 400-MR spectrometer at 400.06 MHz (^{1}H), 376.40 MHz (^{19}F) and 100.60 MHz (^{13}C) and are reported in ppm. All ^{1}H spectra are referenced to tetramethylsilane (δ = 0 ppm) using the signals of the residual protons of $CHCl_3$ (7.26 ppm) in $CDCl_3$, acetone-d_5 (2.05 ppm) in acetone-d_6, CHD_2OD (3.31 ppm) in CD_3OD or DMSO-d_5 (2.50 ppm) in DMSO-d_6. ^{13}C spectra are referenced to tetramethylsilane (δ = 0 ppm) using the signals of the solvent: $CDCl_3$ (77.16 ppm), acetone-d_6 (CD_3, 29.84 ppm), CD_3OD (49.00 ppm) or DMSO-d_6 (39.52 ppm). Multiplicities of signals are described as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet or overlap of non-equivalent resonances; br = broad signal. Coupling constants (J) are given in Hz.

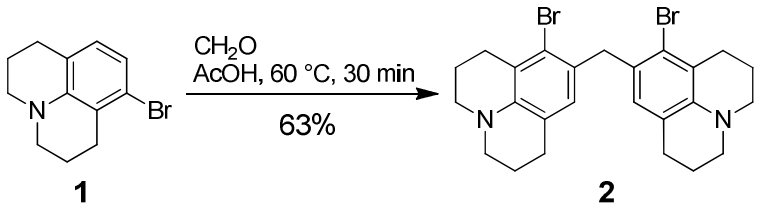
ESI-MS were recorded on a Varian 500-MS spectrometer (Agilent). ESI-HRMS were recorded on a MICROTOF spectrometer (Bruker) equipped with ESI ion source (Apollo) and direct injector with LC autosampler Agilent RR 1200.

Liquid chromatography: HPLC was performed with Knauer Smartline liquid chromatography system: two pumps (1000), with an UV-detector 2500 with the column thermostat 4000, the mixing chamber and the injection valve with 20 and 100 µL loop for the analytical and preparative columns, respectively; 6-port-3-channel switching valve. Analytical column: Eurospher 100 C18, 5 µm, 250×4 mm; preparative column: Eurospher 100 C18, 5 µm, 250×8 mm; solvent A: acetonitrile + 0.1% v/v TFA, solvent B: H_2O + 0.1% v/v TFA; temperature 25 °C. Analytical TLC was performed on Merck Millipore ready-to-use plates with silica gel 60 (F_{254}). Preparative TLC was performed on precoated thin-layer plates with silica gel for high performance TLC (HPTLC Silica gel 60 F_{254} 10×10 cm, with concentrating zone 10 x 2.5 cm), purchased from Merck Millipore (Darmstadt, Germany; Cat. No. 113727). Flash chromatography was performed on Merck Millipore Silica 60 0.04–0.063 mm for column chromatography (Cat. No. 815360) or on Biotage Isolera™ flash purification system using the type of cartridge and solvent gradient indicated. Reversed-phase chromatography was done on Polygoprep 60-50 C_{18} from Macheray-Nagel (Düren, Germany; Cat. No. 711500).

Fluorescent dye *680SiR*



Compound 2



Formaldehyde solution (37 wt.% in water, 0.8 mL) was added to a solution of 8-bromojulolidine[S5] **1** (1.0 g, 4.0 mmol) in acetic acid (6 mL). The resulting emulsion was stirred at 60 °C (bath temperature) under nitrogen atmosphere for 30 min (lots of crystalline precipitate formed within 10 min). The solvent was then evaporated, the residue was redissolved in CH_2Cl_2 (50 mL), the solution was washed with sat. aq. $NaHCO_3$ (30 mL), brine and dried over Na_2SO_4. The product was isolated by flash column chromatography (35 g silica; gradient 0% to 20% ethyl acetate – hexane); fractions containing the product **2** were pooled, evaporated and the residue was recrystallized from CH_2Cl_2 – hexane. Yield 646 mg (63%), white crystals.

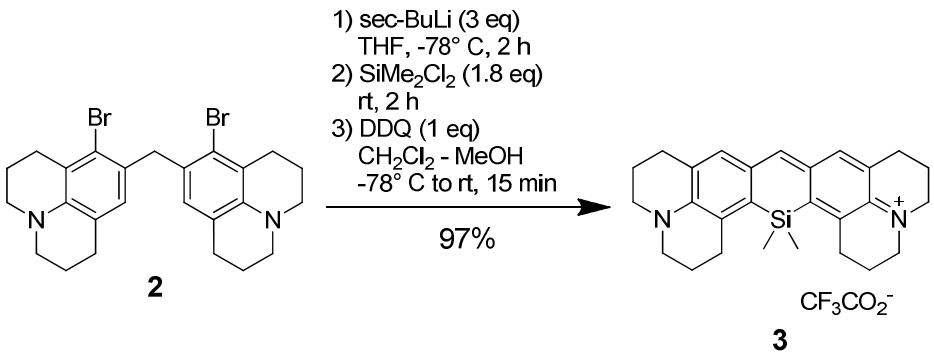
^{1}H NMR (400 MHz, $CDCl_3$): δ 6.48 (s, 2H), 3.96 (s, 2H), 3.13 – 3.05 (m, 8H), 2.84 (t, J = 6.7 Hz, 4H), 2.65 (t, J = 6.4 Hz, 4H), 2.05 – 1.90 (m, 8H).

^{13}C NMR (101 MHz, $CDCl_3$). δ 143.0, 128.6, 127.3, 125.8, 121.3, 120.9, 50.3, 49.7, 42.1, 29.6, 27.6, 22.5, 22.1.

ESI-MS, positive mode: *m/z* (rel. int., %) = 517.2 (100) $[M+H]^+$.

HR-MS (ESI, positive mode): 517.0668 (found), 517.0673 (calculated for $C_{25}H_{29}Br_2N_2$, $M+H^+$).

Compound 3



sec-BuLi (2.6 mL of 1.4 M in cyclohexane, 3.57 mmol, 3 eq) was added quickly dropwise to a cold (-78 °C) degassed solution of **2** (615 mg, 1.19 mmol) in dry THF (30 mL). The resulting clear bright yellow solution was stirred at -78 °C for 2 h. Dichlorodimethylsilane (0.26 mL, 2.14 mmol, 1.8 eq) was then added dropwise, the mixture was allowed to warm up to rt and stirred at rt for 2 h. The resulting solution was cooled in ice-water bath, and trifluoroacetic acid (0.5 mL) was added dropwise (light green suspension formed). The solvents were evaporated, and the residue was redissolved in CH_2Cl_2 (20 mL) and methanol (5 mL). The solution was cooled to -78° C, and DDQ (272 mg, 1.2 mmol, 1 eq), suspended in CH_2Cl_2 (5 mL), was added portionwise. The resulting bright blue solution was allowed to warm up to rt and stirred at rt for 15 min. The solvents were evaporated, and the residue was subjected to flash chromatography on a reversed phase (twice; 10 g RP-C_{18}, gradient 50% to 0% H_2O – acetonitrile + 0.1% trifluoroacetic acid). The fractions containing the product were pooled, acetonitrile was evaporated and the residue was lyophilized. The crude salt was re-purified by flash chromatography on silica (25 g Biotage SNAP Ultra; gradient 0% to 20% {methanol + 1% trifluoroacetic acid} – CH_2Cl_2 over 22 CV); fractions containing the product **3** were evaporated and the residue was lyophilized from aq. dioxane. Dark blue fluffy solid, yield 608 mg (97%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.26 (s, 1H), 7.10 (s, 2H), 3.54 (q, J = 5.6, 5.0 Hz, 8H), 2.88 (dd, J = 7.2, 4.8 Hz, 4H), 2.74 (t, J = 6.1 Hz, 4H), 2.03 (dt, J = 24.0, 5.9 Hz, 8H).

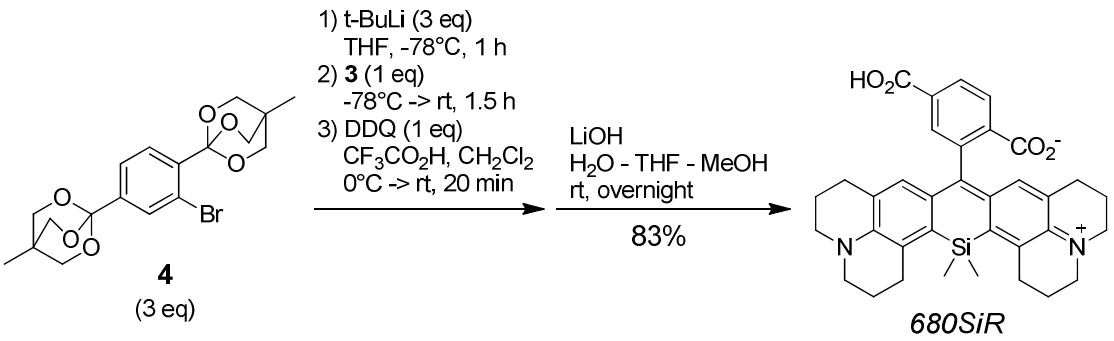
^{13}C NMR (101 MHz, $CDCl_3$): δ 158.4, 151.0, 140.8, 140.3, 131.4, 127.1, 123.8, 52.0, 51.3, 28.6, 27.3, 20.9, 20.6, -1.6.

^{19}F NMR (376 MHz, $CDCl_3$): δ -75.60.

ESI-MS, positive mode: *m/z* (rel. int., %) = 413.5 (100) $[M+H]^+$.

HR-MS (ESI, positive mode): 413.2404 $[M+H]^+$ (found), 413.2408 (calculated for $C_{27}H_{33}N_2Si$, M^+).

Dye *680SiR*



In a 50 mL round-bottom flask, a degassed solution of bis-OBO-ester **4**[S1] (347 mg, 0.84 mmol, 3 eq) in anhydrous THF (15 mL) was cooled to -78 °C (dry ice – acetone bath). *tert*-Butyllithium (0.5 mL of 1.7 M solution in pentane, 0.85 mmol, 3 eq) was added quickly dropwise. Bright yellow-orange solution turned into a light brown thin suspension, which was stirred at -78 °C for 1 h. A solution of **3** (150 mg, 0.29 mmol) in anhydrous THF (5 mL) was then added at -78 °C, the mixture was allowed to warm up to rt (blue mixture turned into a light yellow thin suspension) and stirred at rt for 1.5 h. The reaction mixture was cooled in ice-water bath, and trifluoroacetic acid (1.5 mL) was added followed by DDQ (65 mg dissolved in 5 mL CH_2Cl_2; 0.29 mmol, 1 eq). The resulting blue-green mixture was stirred at rt for 20 min, evaporated and re-evaporated twice with CH_2Cl_2 (2×30 mL) to dryness. The residue was dissolved in THF (20 mL) and methanol (5 mL), a solution of lithium hydroxide (2 g $LiOH \cdot H_2O$ in 20 mL water) was added, and the mixture was stirred overnight at rt. Afterwards, it was cooled in ice-water bath, adjusted to pH 1-2 with 2 N HCl, extracted with CH_2Cl_2 (3×40 mL). The combined organic layers were dried over Na_2SO_4, and the product was isolated by flash column chromatography (35 g silica; gradient 5% to 40% methanol – CH_2Cl_2, then 50% methanol – CH_2Cl_2 + 0.5% trifluoroacetic acid; the dye is mainly eluted in the last fraction).
Fractions containing the product were evaporated, redissolved in dioxane, microfiltered through a 0.2 µm PTFE membrane filter and freeze-dried, providing 137 mg of the crude dye with ~80% purity (HPLC). Analytically pure material was prepared from this sample by preparative HPLC (gradient A:B 30:70 → 100:0 over 20 min, A – acetonitrile, B – water + 0.05% trifluoroacetic acid).

HPLC (gradient A:B 30:70 → 100:0 over 20 min, A – acetonitrile, B – water + 0.05% trifluoroacetic acid, column 4.6×250 mm, 1.2 mL/min, detection at 635 nm): τ = 10.4 min.

^{1}H NMR (400 MHz, CD_3OD): δ 8.23 (br.s,1H), 7.72 (br.s, 1H), 6.46 (br.s, 1H), 5.49 (s, 2H), 3.55 (t, *J* = 6.0 Hz, 4H), 3.49 (t, *J* = 5.8 Hz, 4H), 2.99 (dd, *J* = 7.3, 5.0 Hz, 4H), 2.44 (t, *J* = 6.2 Hz, 4H), 2.11 – 2.02 (m, 4H), 1.92 – 1.83 (m, 4H), 0.73 (s, 3H), 0.71 (s, 3H).

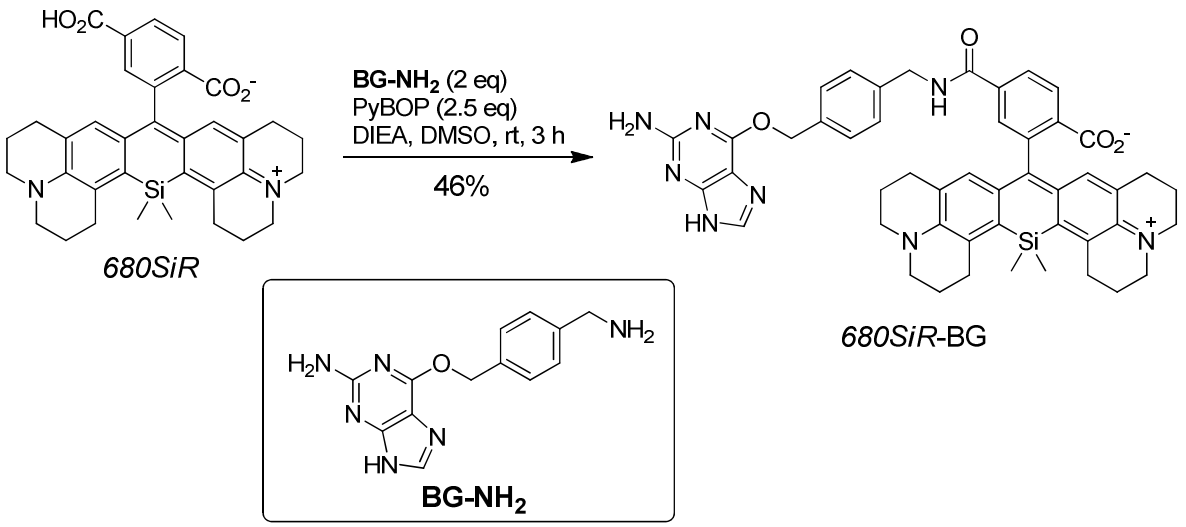
^{19}F NMR (376 MHz, CD_3OD): δ -76.94 ($CF_3CO_2^-$).

^{13}C NMR (101 MHz, CD_3OD): δ 168.6, 151.8, 144.2, 143.0, 139.3, 133.2, 132.8, 132.5, 131.2, 129.1, 125.3, 97.9, 69.5, 55.4, 53.3, 52.7, 30.7, 29.2, 27.1, 24.9, 22.7, 22.4, 0.0, -0.2.

ESI-MS, positive mode: *m/z* (rel. int., %) = 577.5 (100) $[M+H]^+$.

HR-MS (ESI, positive mode): 577.2517 $[M+H]^+$ (found), 577.2517 (calculated for $C_{35}H_{36}N_2O_4Si$, $[M+H]^+$).

680SiR-SNAP probe



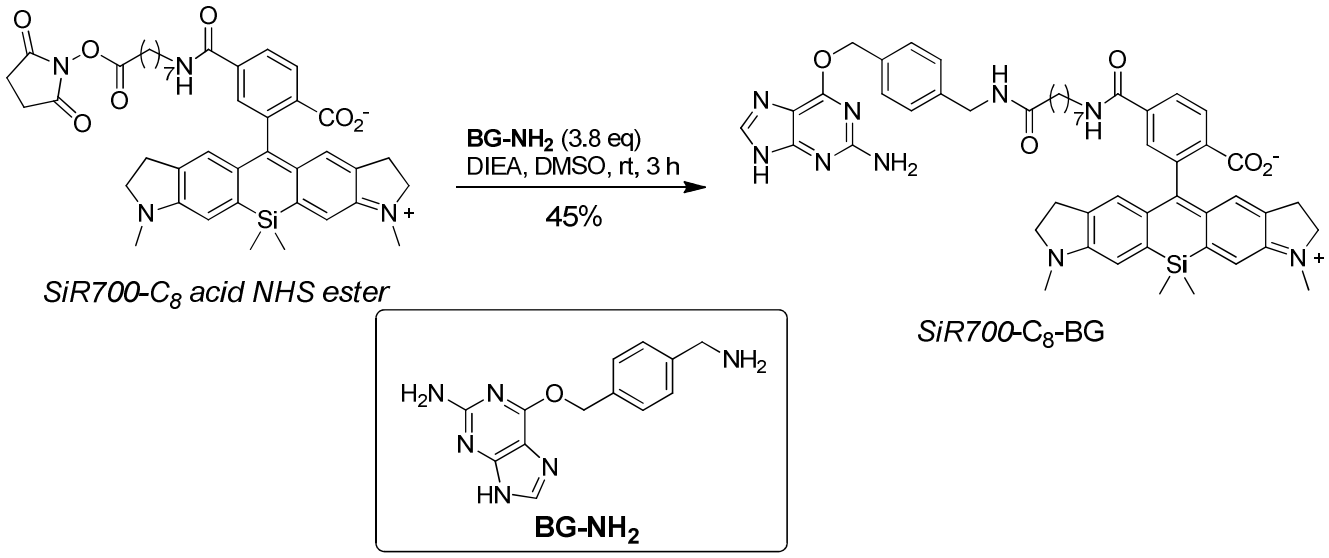
PyBOP (10 µL of 17 mg/50 µL stock solution in DMSO, 6.5 µmol, 2.5 eq) was added to a solution of *680SiR* (1.5 mg, 2.6 µmol), **BG-NH$_2$** (6-[4-(aminomethyl)benzyloxy]-9*H*-purin-2-amine; 1.4 mg, 5.2 µmol, 2 eq) and DIEA (*N*-ethyldiisopropylamine; 15 µL) in DMSO (100 µL). After 3 h, the solvent was evaporated on a lyophilizer, and the product *680SiR*-BG was isolated by preparative TLC (silica, 15% methanol – CH_2Cl_2), giving the material with ~80% purity (HPLC). The pure material was isolated from the crude by preparative HPLC (gradient A:B 20:80 → 100:0 over 20 min, A – acetonitrile, B – 0.05 M triethylammonium hydrogen carbonate in water). Yield 1.0 mg (46%).

HPLC (A:B 20:80 → 100:0 over 20 min, A – acetonitrile, B – 0.05 M triethylammonium hydrogen carbonate in water, column 4.6×250 mm, 1.2 mL/min, detection at 679 nm): τ = 11.7 min.

ESI-MS, positive mode: *m/z* (rel. int., %) = 829.8 (100) $[M+H]^+$.

HR-MS (ESI, positive mode): 829.3639 (found), 829.3641 (calculated for $C_{48}H_{49}N_8O_4Si$, $[M+H]^+$).

SiR700-C_8-SNAP probe



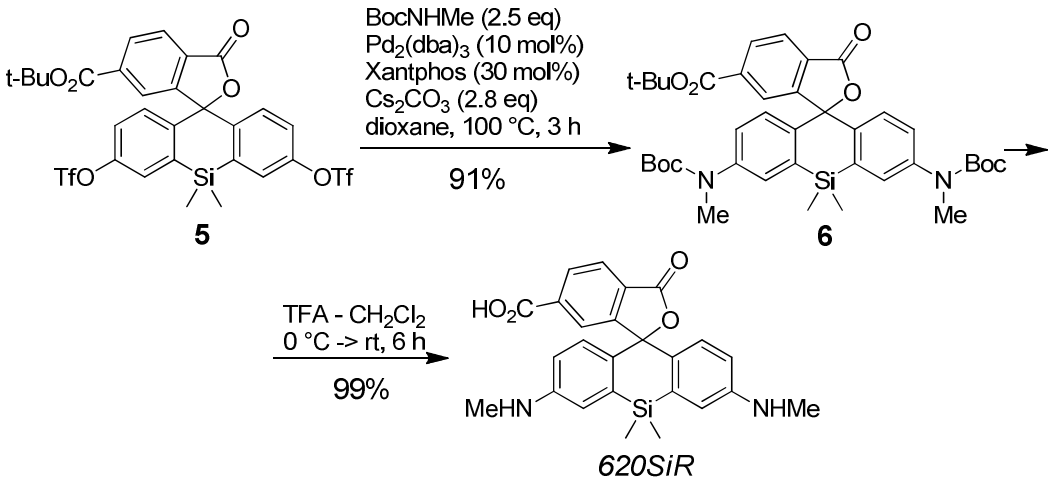
BG-NH_2 (6-[4-(aminomethyl)benzyloxy]-9*H*-purin-2-amine; 20 µL of 3.4 mg/100 µL stock solution in DMSO, 2.53 µmol, 1.9 eq) followed by DIEA (*N*-ethyldiisopropylamine; 5 µL) was added to a stirred solution of *SiR700*-C_8 acid NHS ester[S6] (1 mg, 1.36 µmol) in dry DMSO (50 µL). After 30 min at rt, another portion of BG-NH_2 solution (20 µL, 1.9 eq) and DIEA (5 µL) was added. The reaction mixture was stirred at rt for 3 h, and the solvent were removed *in vacuo* at rt. The product was isolated from the crude residue by preparative HPLC (gradient A:B 20:80 → 100:0 over 20 min, A – acetonitrile, B – 0.05% TFA in water). Yield 0.54 mg (45%).

HPLC (A:B 20:80 → 100:0 over 10 min, A – acetonitrile, B – 0.05% TFA in water, column Kinetex 2.6 µm C18 4.6×75 mm, 1 mL/min, detection at 700 nm): τ = 3.9 min.

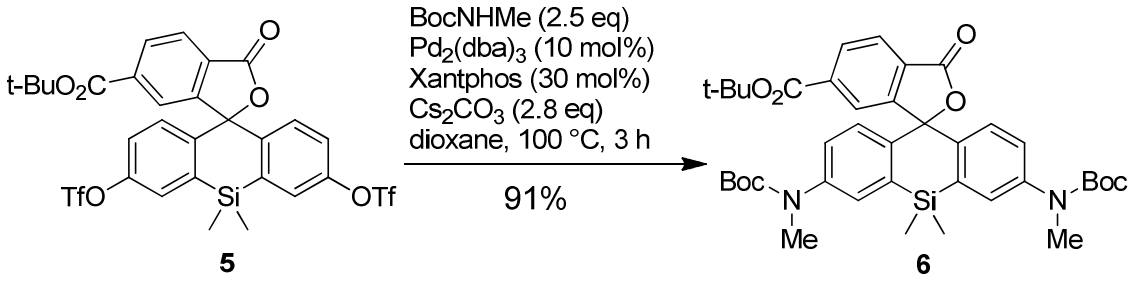
ESI-MS, positive mode: *m/z* (rel. int., %) = 890.8 (100) $[M+H]^+$. ESI-MS, negative mode: *m/z* (rel. int., %) = 889.0 (100) $[M-H]^-$.

HR-MS (ESI, positive mode): 890.4159 (found), 890.4168 (calculated for $C_{55}H_{56}N_9O_5Si$, $[M+H]^+$).

Fluorescent dye *620SiR*



Compound 6



A mixture of silafluorescein *tert*-butyl ester ditriflate **5**[S7] (186 mg, 0.25 mmol), *tert*-butyl *N*-methylcarbamate (83 mg, 0.63 mmol, 2.5 eq), $Pd_2(dba)_3$ (23 mg, 0.025 mmol, 10 mol%), Xantphos (44 mg, 0.076 mmol, 30 mol%) and Cs_2CO_3 (230 mg, 0.71 mmol, 2.8 eq) in dry dioxane (2 mL) was degassed on a Schlenk line and stirred at 100 °C under argon (bath temperature) in a sealed flask for 3 h. TLC control (SiO_2 / 20% ethyl acetate – hexane, stained by heating with aq. NaOH): R_f (product) = 0.55 (blue), R_f (starting material) = 0.32 (pink). Upon cooling, the reaction mixture was diluted with water (30 mL), extracted with ethyl acetate (4×20 mL), the combined organic layers were washed with brine and dried over Na_2SO_4. The product was isolated by flash column chromatography (15 g silica, gradient 0% to 5% ethyl acetate – CH_2Cl_2) and freeze-dried from 1,4-dioxane to yield 161 mg (91%) of **6** as light pink fluffy solid.

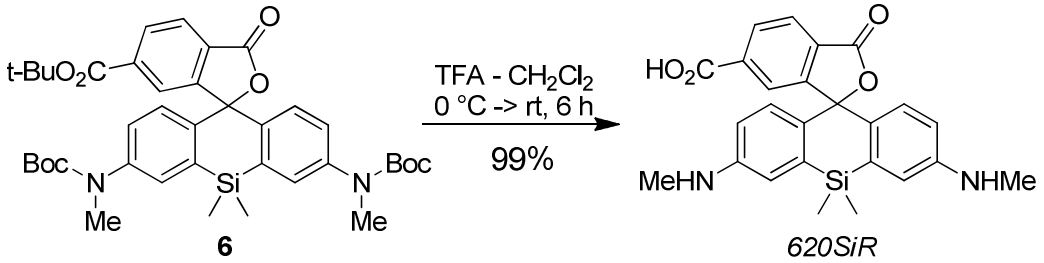
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.11 (dd, *J* = 8.0, 1.3 Hz, 1H), 7.96 (dd, *J* = 8.0, 0.7 Hz, 1H), 7.81 (s, 1H), 6.85 (d, *J* = 8.7 Hz, 2H), 6.69 (d, *J* = 2.6 Hz, 2H), 6.33 (dd, *J* = 8.8, 2.6 Hz, 2H), 4.78 – 4.69 (m, 2H), 4.19 – 4.12 (m, 4H), 3.69 – 3.62 (m, 4H), 1.55 (s, 9H), 0.89 (s, 18H), 0.66 (s, 3H), 0.58 (s, 3H), 0.07 (s, 12H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 170.3, 164.4, 155.4, 150.7, 137.3, 136.1, 132.7, 129.9, 129.0, 127.7, 125.7, 125.1, 116.3, 113.3, 82.3, 62.5, 62.1, 28.2, 25.9, 18.1, 0.2, -0.6, -4.8.

ESI-MS, positive mode: *m/z* (rel. int., %) = 813.4 (100) $[M+H]^+$.

HR-MS (ESI, positive mode): 813.4120 $[M+H]^+$ (found), 813.4145 (calculated for $C_{45}H_{65}N_2O_6Si_3$, $[M+H]^+$).

Dye *620SiR*



6 *620SiR*

Trifluoroacetic acid (3 mL) was added to a solution of **6** (161 mg, 0.23 mmol) in CH_2Cl_2 (15 mL), cooled in ice-water bath. The cooling bath was removed, and the reaction mixture was stirred at rt for 6 h. The solvents were evaporated, and the residue was re-evaporated twice with toluene (2×20 mL) and freeze-dried from 1,4-dioxane, giving 154 mg of crude trifluoroacetate salt of the dye. The product was isolated by chromatography on a reversed phase (RP-C_{18} 10 g; gradient 80% to 30% water – acetonitrile). Fractions containing the product were evaporated and freeze-dried from 1,4-dioxane to give 102 mg (99%) of *620SiR* as blue fluffy solid (free base).

HPLC (10/90−100/0 over 25 min, column 4×250 mm, 1.2 mL/min, detection at 254 nm): τ = 9.98 min.

UV-Vis (PBS 7.4): λ_{max} (ε) = 641 nm (51000 $M^{-1}cm^{-1}$); fluorescence (PBS 7.4): λ_{excit} = 610 nm, λ_{em} = 662 nm; Φ_{fl} = 0.49.

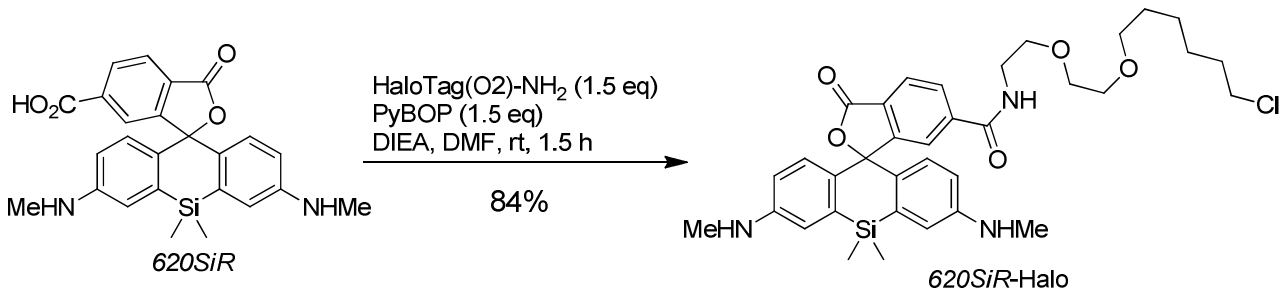
^{1}H NMR (400 MHz, CD_3OD): δ 8.22 (dd, *J* = 7.9, 1.3 Hz, 1H), 8.00 (d, *J* = 8.0 Hz, 1H), 7.83 (d, *J* = 1.0 Hz, 1H), 6.78 (d, *J* = 2.6 Hz, 2H), 6.74 (d, *J* = 8.7 Hz, 2H), 6.39 (dd, *J* = 8.7, 2.7 Hz, 2H), 4.66 (tt, *J* = 6.4, 4.8 Hz, 2H), 4.16 (ddd, *J* = 7.4, 6.4, 1.0 Hz, 4H), 3.66 – 3.60 (m, 4H), 0.64 (s, 3H), 0.54 (s, 3H).

^{13}C NMR (101 MHz, CD_3OD): δ 171.7, 156.3, 152.5, 137.8, 133.5, 131.3, 130.5, 128.9, 126.8, 126.6, 117.5, 114.3, 63.0, 62.5, 0.1, -1.2.

ESI-MS, positive mode: *m/z* (rel. int., %) = 529.2 (100) $[M+H]^+$.

HR-MS (ESI, positive mode): 529.1787 (found), 529.1789 (calculated for $C_{29}H_{29}N_2O_6Si$, $[M+H]^+$).

***620SiR*-Halo probe**



620SiR *620SiR*-Halo

PyBOP (25 µL of 21 mg/100 µL stock solution in DMF, 10.14 µmol, 1.5 eq) was added to a solution of *620SiR* (3 mg, 6.76 µmol), HaloTag Amine (O2) (2-(2-((6-chlorohexyl)oxy)ethoxy)ethanamine; 2.3 mg, 10.14 µmol, 1.5 eq) and DIEA (*N*-ethyldiisopropylamine; 10 µL) in DMF (100 µL). After 1.5 h, the solvent

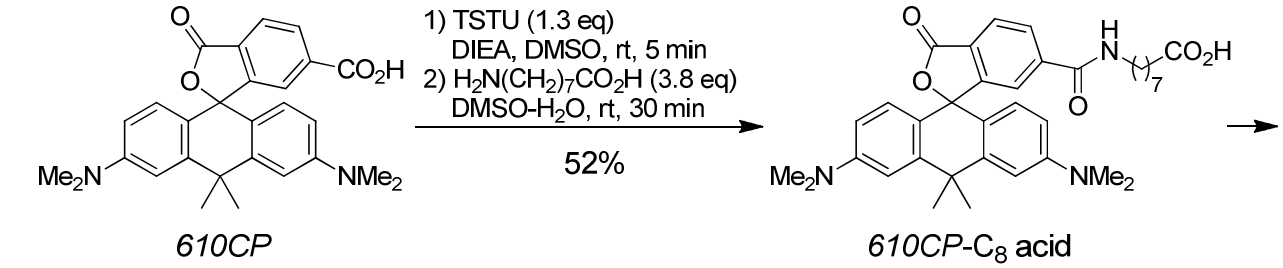
was evaporated *in vacuo* at rt, and the product *620SiR*-Halo was isolated by preparative TLC (silica, 2.5% methanol – CH_2Cl_2 over 2 passes). Yield 3.68 mg (84%), purity (HPLC) ~96%.

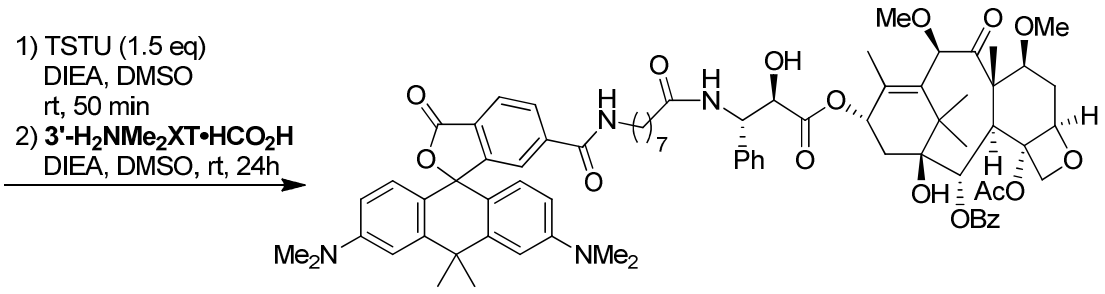
HPLC (30/70−100/0 over 25 min, column 4×250 mm, 1.2 mL/min, detection at 254 nm): τ = 12.15 min.

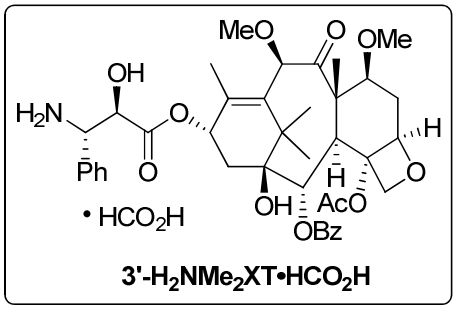
ESI-MS, positive mode: *m/z* (rel. int., %) = 650.3 (100) $[M+H]^+$.

HR-MS (ESI, positive mode): 650.2816 (found), 650.2812 (calculated for $C_{35}H_{45}ClSiN_3O_5$, $[M+H]^+$).

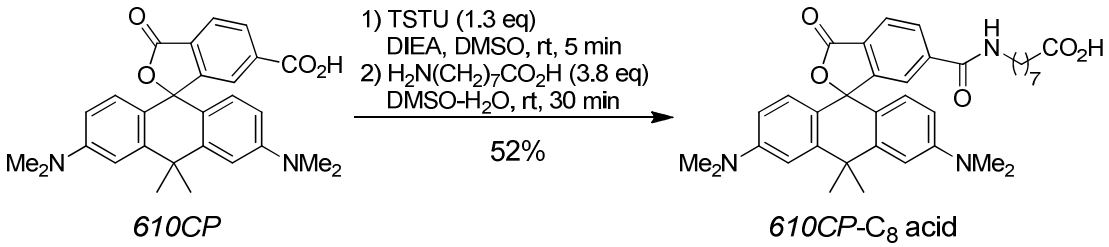
610CP-tubulin2 probe







610CP-C_8 acid



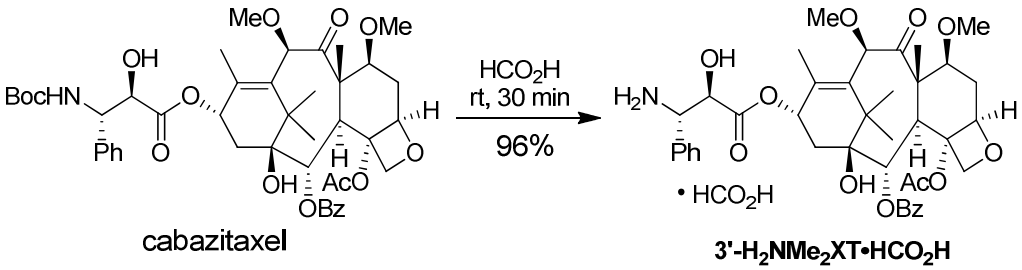
Dye *610CP*[S1] (2.2 mg, 4.8 μmol) was dissolved in dry DMSO (500 μL) under argon. DIEA (*N*-ethyldiisopropylamine; 21.9 μL, 125 μmol) and TSTU (2.0 mg, 6.2 μmol) were added and the mixture was stirred for 5 min. 8-Aminooctanoic acid (2.9 mg, 18.2 μmol) was added, and the reaction mixture was sonicated at room temperature for 15 min. Water (20 μL) was then added, and the resulting solution was stirred for further 15 min. The reaction mixture was then quenched by addition of glacial acetic acid (8.7 μL, 144 μmol) and freeze-dried. The product was isolated by prep. HPLC (Kinetex 5 μm C18 100, 10 mm×250 mm, gradient A:B 30/70 – 70/30, A – acetonitrile, B – 0.05% TFA in water; τ(*610CP*) = 7.8 min, τ(product) = 9.9 min) to give 1.5 mg (52%) of blue solid.

HPLC (Kinetex 2.6 μm C18 100, 4.6 mm×75 mm, gradient A:B 20/80 – 100/0, A – acetonitrile, B – 0.05% TFA in water, detection at 600 nm): τ(*610CP*) = 4.2 min, τ(product) = 5.0 min.

ESI-MS, positive mode: *m/z* (rel. int., %) = 598.4 (100) $[M+H]^+$.

HR-MS (ESI, positive mode): 598.3273 (found), 598.3275 (calculated for $C_{36}H_{44}N_3O_5$, $[M+H]^+$).

3'-$H_2NMe_2XT•HCO_2H$



Cabazitaxel (50 mg, 60 µmol) was dissolved in 99% formic acid (2 mL), and the solution was stirred at rt for 30 min. The solvent was then evaporated (bath temperature 25 °C), the residue was redissolved in water (2 mL) and freeze-dried to give 45 mg (96%) of 3'-$H_2NMe_2XT•HCO_2H$ as white fluffy solid.

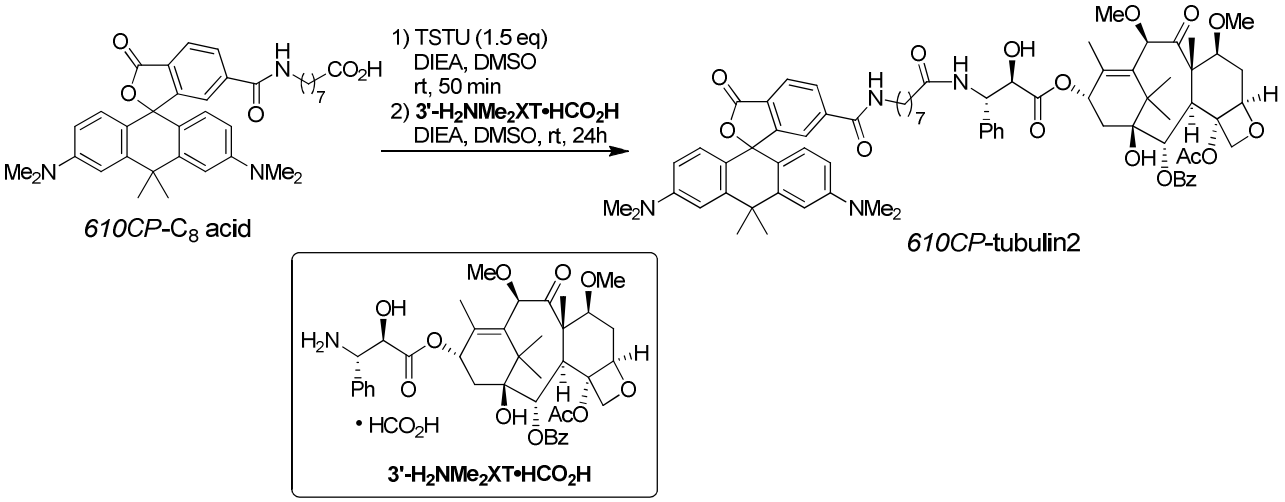
^{1}H NMR (400 MHz, CD_3CN): δ 8.25 (br.s, 2H), 8.06 – 8.01 (m, 2H), 7.73 – 7.66 (m, 1H), 7.62 – 7.55 (m, 2H), 7.46 – 7.38 (m, 4H), 7.32 – 7.25 (m, 1H), 6.04 (td, *J* = 9.1, 1.7 Hz, 1H), 5.50 (d, *J* = 7.1 Hz, 1H), 4.94 (dd, *J* = 9.7, 2.1 Hz, 1H), 4.78 (s, 1H), 4.37 (app.q, *J* = 7.2 Hz, 2H), 4.12 – 4.05 (m, 2H), 3.88 (br.s, 2H), 3.82 (dd, *J* = 10.7, 6.5 Hz, 1H), 3.69 (d, *J* = 7.1 Hz, 1H), 3.36 (s, 3H), 3.24 (s, 3H), 2.68 (ddd, *J* = 14.2, 9.8, 6.5 Hz, 1H), 2.16 (s, 3H), 1.98 (dd, *J* = 15.4, 8.9 Hz, 1H), 1.90 (d, *J* = 1.4 Hz, 3H), 1.73 (dd, *J* = 15.4, 9.1 Hz, 1H), 1.63 – 1.55 (m, 1H), 1.58 (s, 3H), 1.10 (s, 3H), 1.05 (s, 3H).

^{13}C NMR (101 MHz, CD_3CN): δ 206.2, 171.3, 166.8, 140.1, 136.1, 134.5, 131.1, 130.9, 129.63, 129.61, 129.2, 128.5, 84.7, 83.4, 81.9, 81.7, 78.8, 76.9, 75.9, 75.5, 71.6, 59.1, 57.6, 57.41, 57.37, 48.1, 44.1, 36.5, 32.8, 27.3, 23.1, 21.7, 14.9, 11.0.

ESI-MS, positive mode: *m/z* (rel. int., %) = 736.4 (100) $[M+H]^+$.

HR-MS (ESI, positive mode): 736.3332 (found), 736.3328 (calculated for $C_{40}H_{50}NO_{12}$, $[M+H]^+$).

610CP-tubulin2



610CP-C_8 acid (1.5 mg, 2.5 μmol) was dissolved in dry DMSO (400 μL) under argon. DIEA (*N*-ethyldiisopropylamine; 3.5 μL, 20 μmol) and TSTU (10 μL of 5.7 mg/50 μL DMSO stock solution, 3.8 μmol, 1.5 eq) were added and the mixture was stirred at rt for 50 min. The conversion of the acid into NHS ester had been confirmed by HPLC control (Kinetex 2.6 μm C18 100, 4.6 mm×75 mm, gradient A:B 20/80 – 100/0, A – acetonitrile, B – 0.05% TFA in water, detection at 600 nm): τ(*610CP*-C_8 acid) = 4.9 min, τ(*610CP*-C_8 acid NHS ester) = 5.5 min). Another portion of DIEA (3.5 μL, 20 μmol) followed by 3'-$H_2NMe_2XT•HCO_2H$ (2.9 mg, 3.8 μmol) was added, and the reaction mixture was left stirring at rt for 23 h (HPLC control). The entire mixture was freeze-dried, and the product was isolated by prep. HPLC as blue solid (Kinetex 5 μm C18 100, 21 mm×250 mm, gradient A:B 40/60 – 100/0, A – acetonitrile, B – 0.05% TFA in water over 20 min; τ(product) = 8.7 min).

HPLC (Kinetex 2.6 μm C18 100, 4.6 mm×75 mm, gradient A:B 20/80 – 100/0, A – acetonitrile, B – 0.05% TFA in water, detection at 600 nm): τ(product) = 6.4 min.

ESI-MS, positive mode: *m/z* (rel. int., %) = 1315.6 (100) $[M+H]^+$.

HR-MS (ESI, positive mode): 1315.6428 (found), 1315.6425 (calculated for $C_{76}H_{91}N_4O_{16}$, $[M+H]^+$).

References

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[S3] Ratz, M.; Testa, I.; Hell, S. W.; Jakobs, S. *Sci. Rep.* **2015**, *5*, 9592.

[S4] Ran, F. A.; Hsu, P. D.; Wright, J.; Agarwala, V.; Scott, D. A.; Zhang, F. *Nat. Protocols* **2013**, *8*, 2281–2308.

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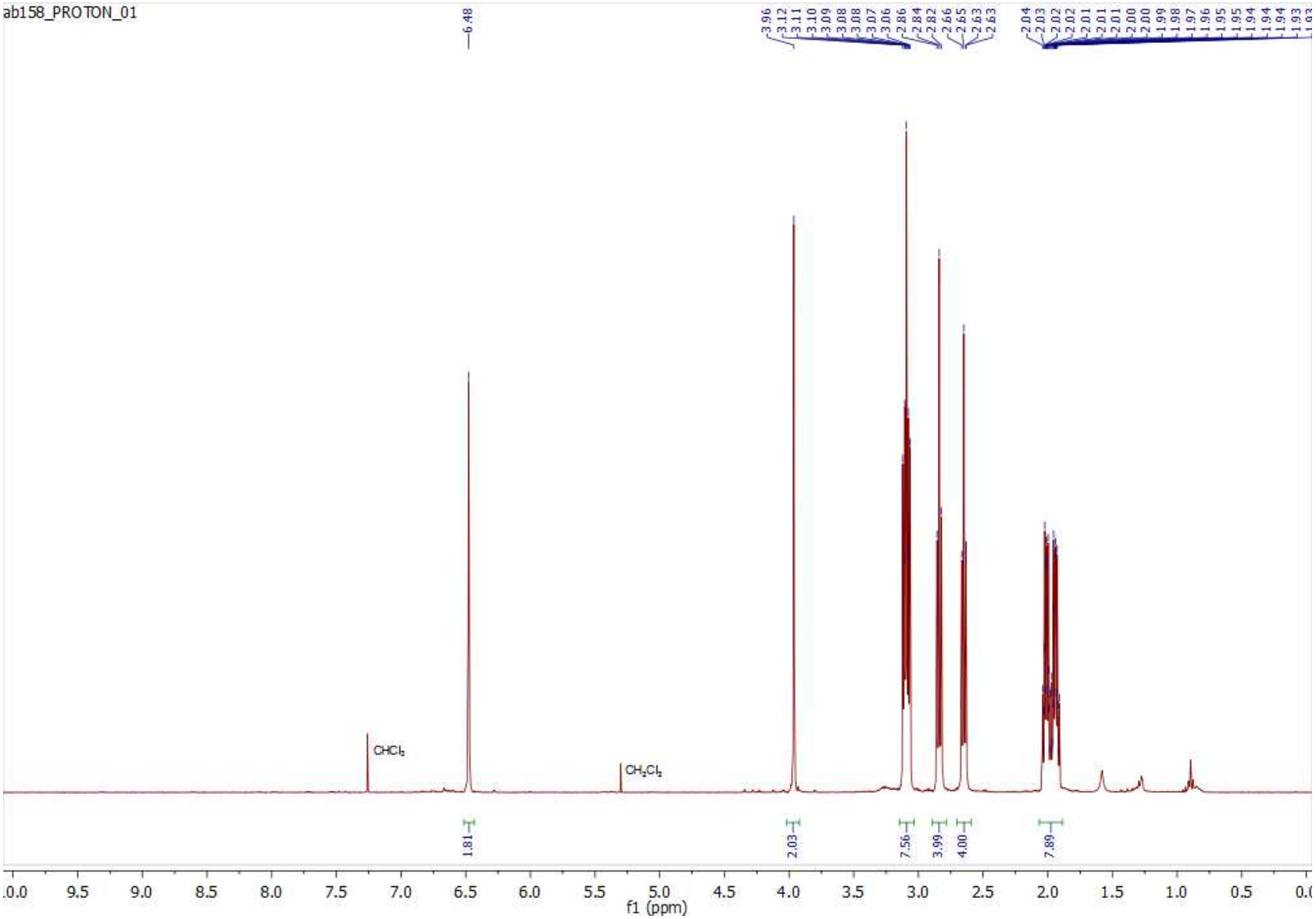
[S6] Lukinavičius, G., Reymond, L., Umezawa, K., Sallin, O., D'Este, E., Göttfert, F., Ta, H., Hell, S. W., Urano, Y., Johnsson, K. *J. Am. Chem. Soc.* **2016**, *138*, 9365–9368.

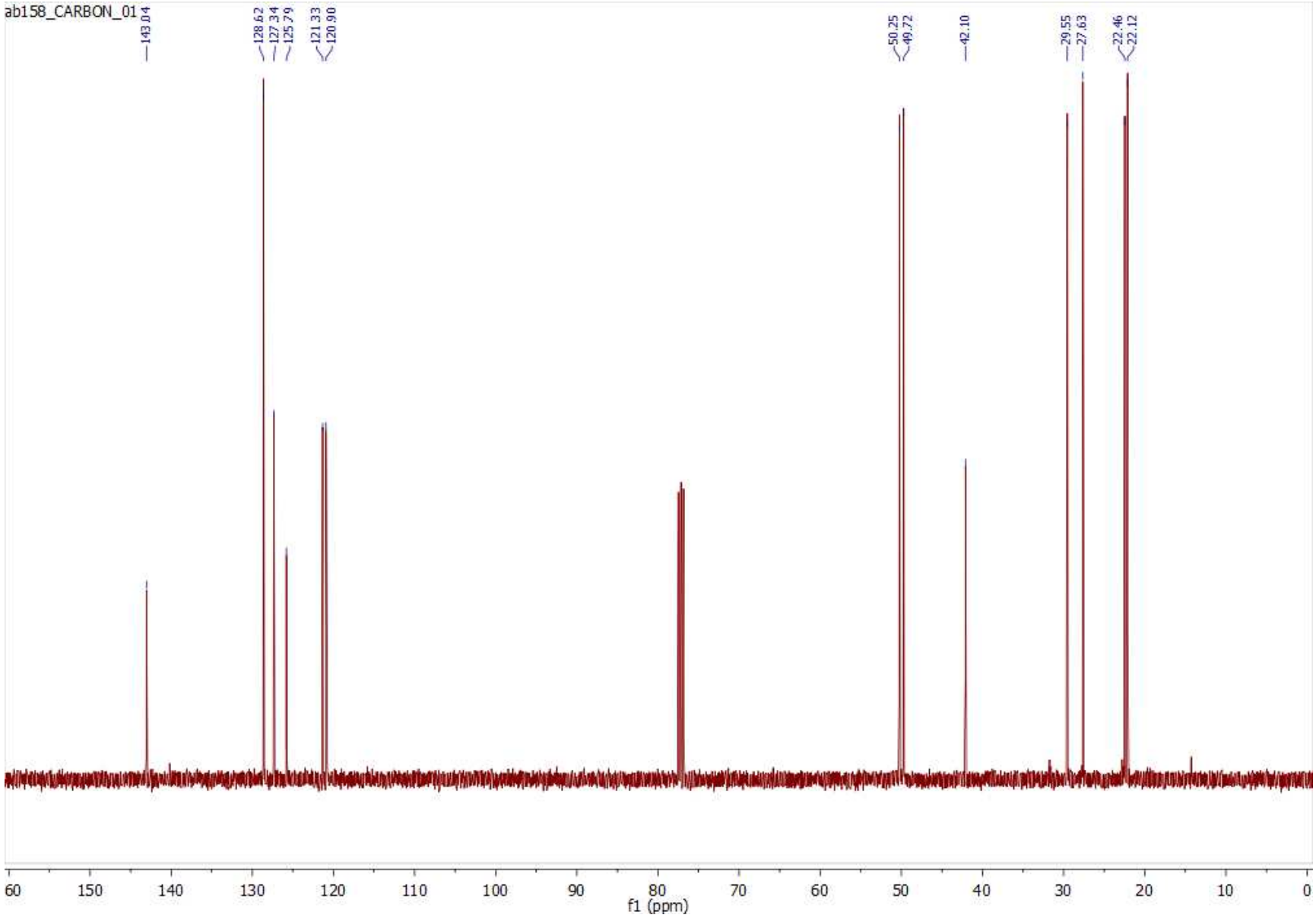
[S7] Butkevich, A. N., Belov, V. N., Kolmakov, K., Sokolov, V. V., Shojaei, H., Sidenstein, S. C., Kamin, D., Matthias, J., Vlijm, R., Engelhardt, J. and Hell, S. W. *Chem. Eur. J.* **2017**, doi:10.1002/chem.201701216

NMR spectra of new compounds

2 (^{1}H)



2 (^{13}C)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20
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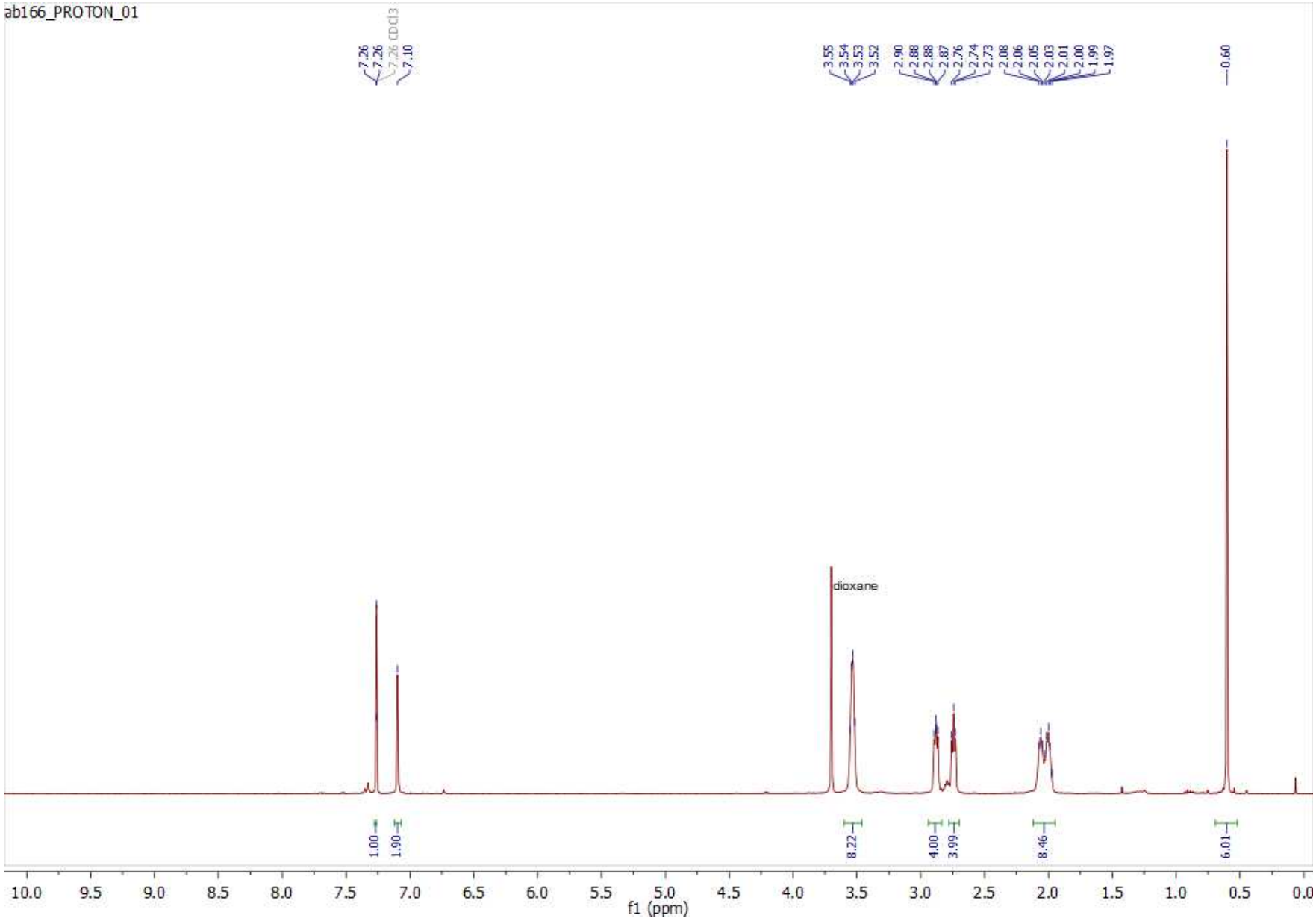
f1 (ppm)

3 (^{1}H)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2.74
2.73
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2.05
2.03
2.01
2.00
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1.97
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dioxane
1.00
1.90
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4.00
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8.46
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8.5
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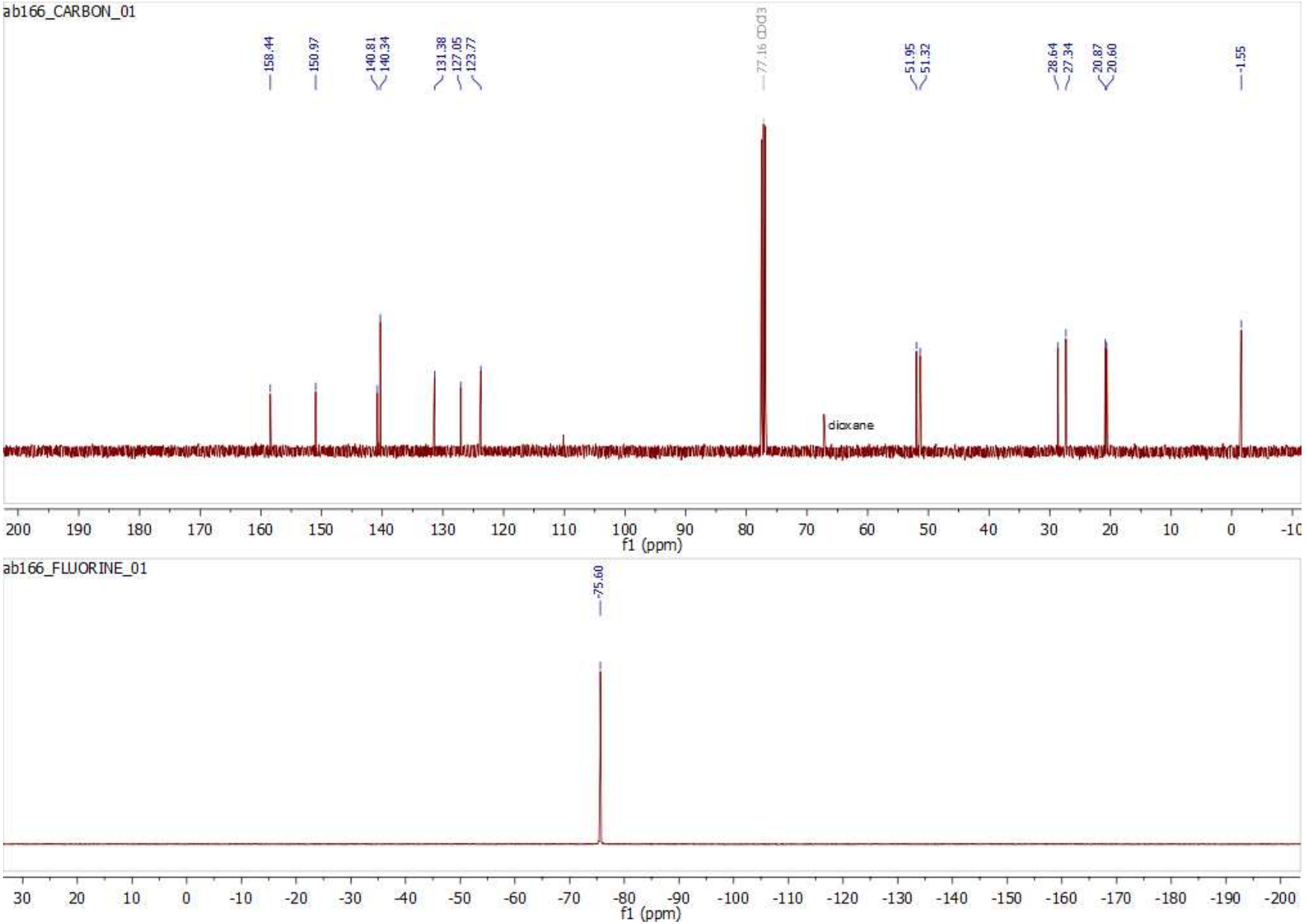
f1 (ppm)

3 (^{13}C, ^{19}F)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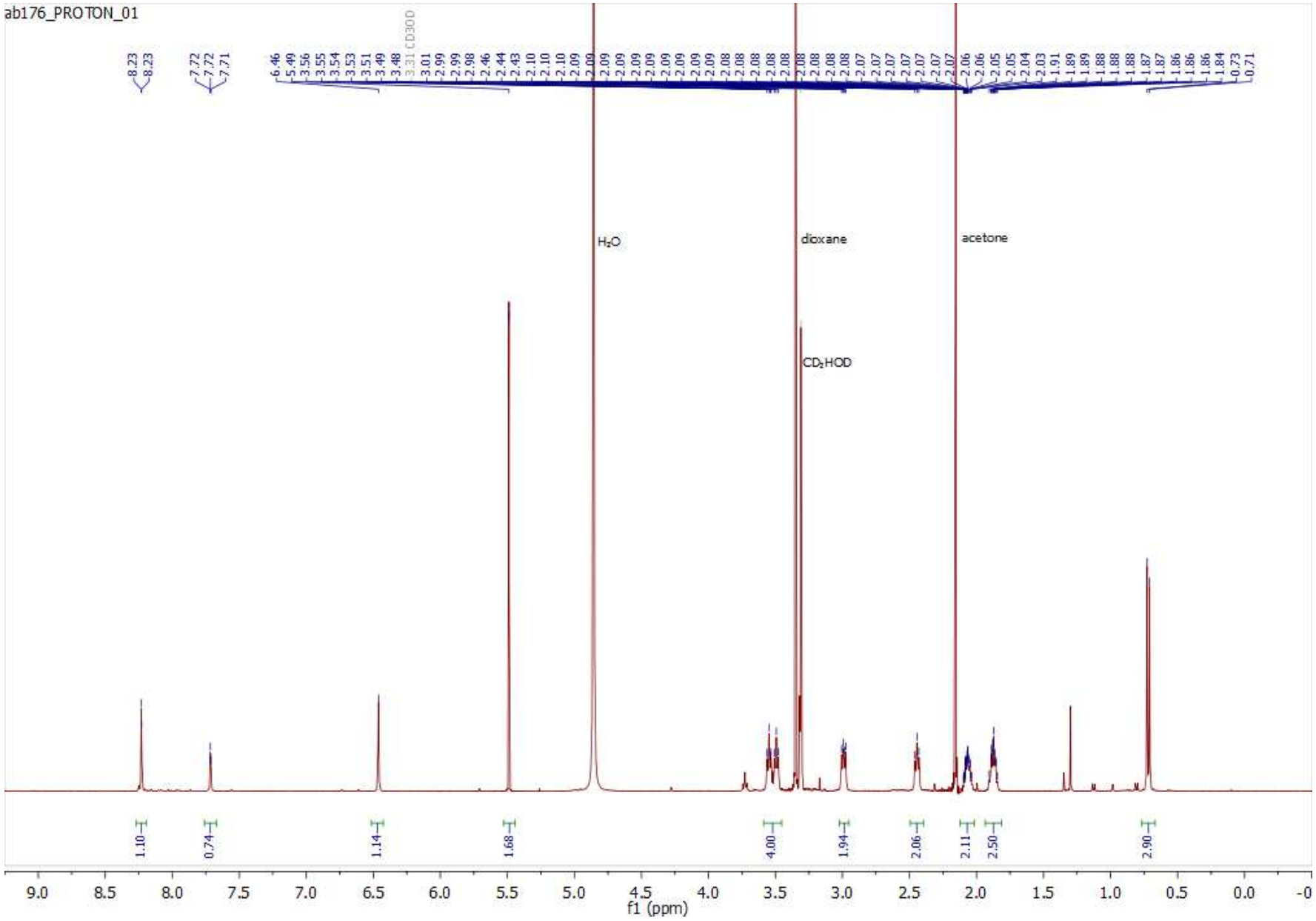
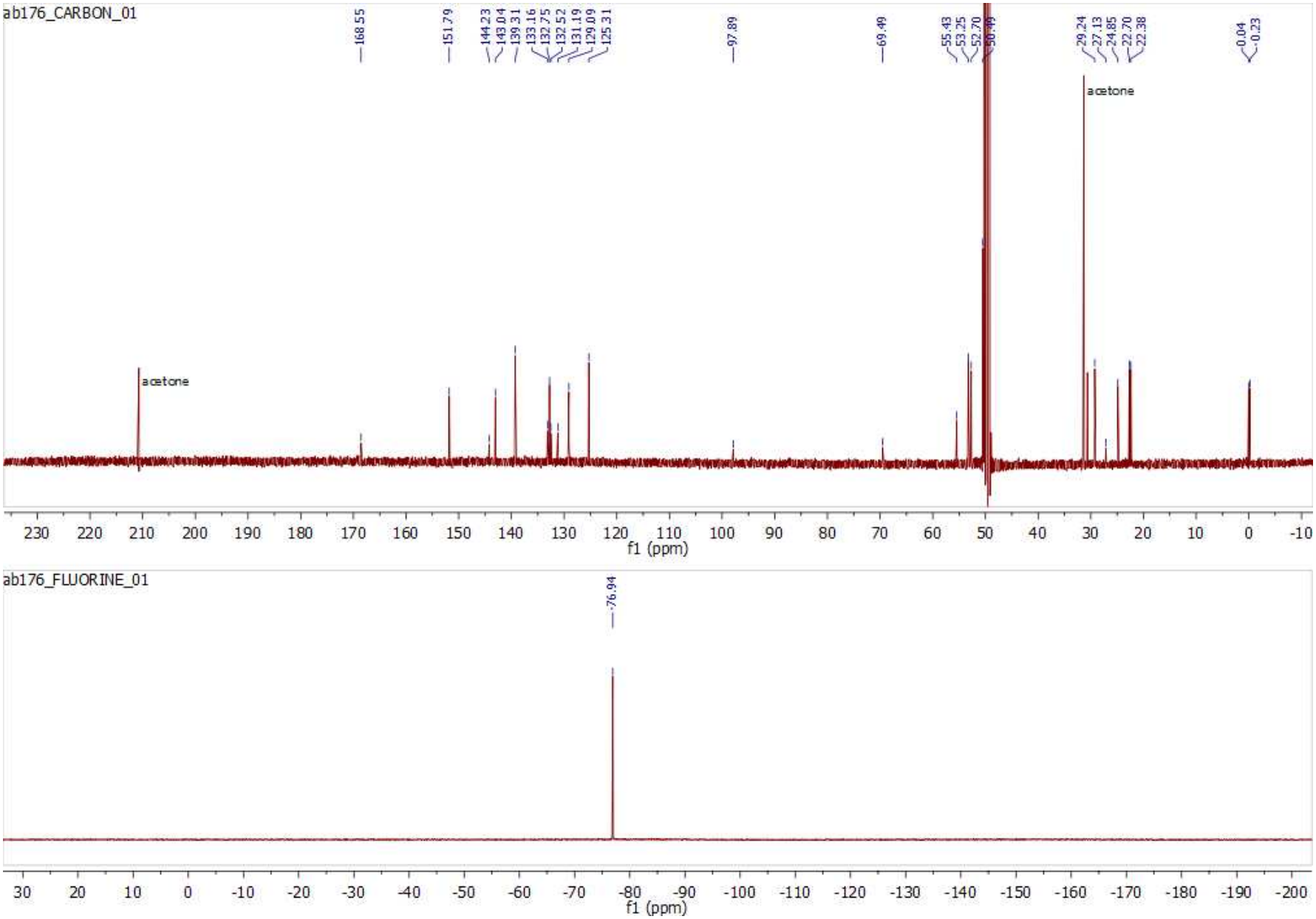
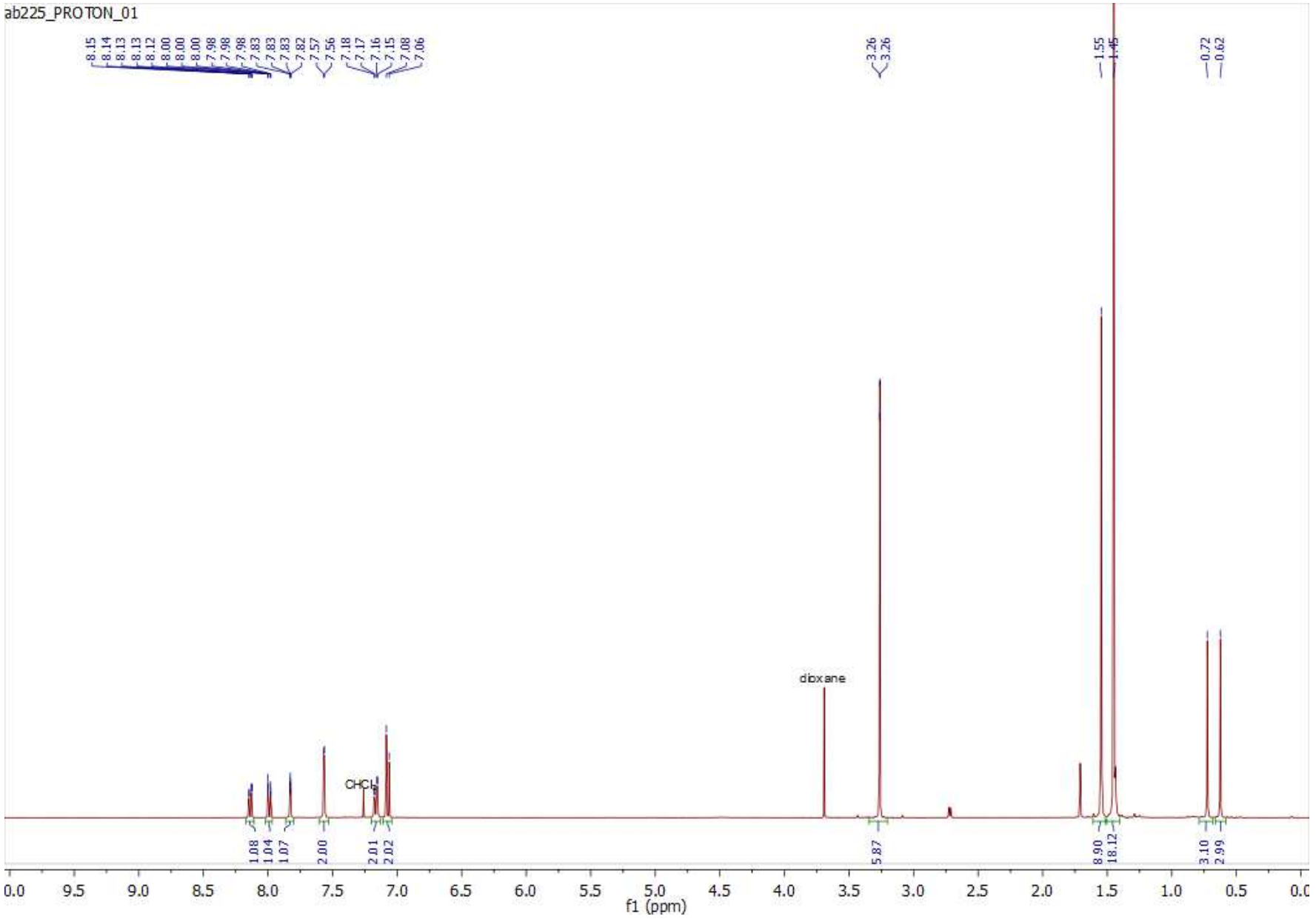
680SiR (^{1}H)



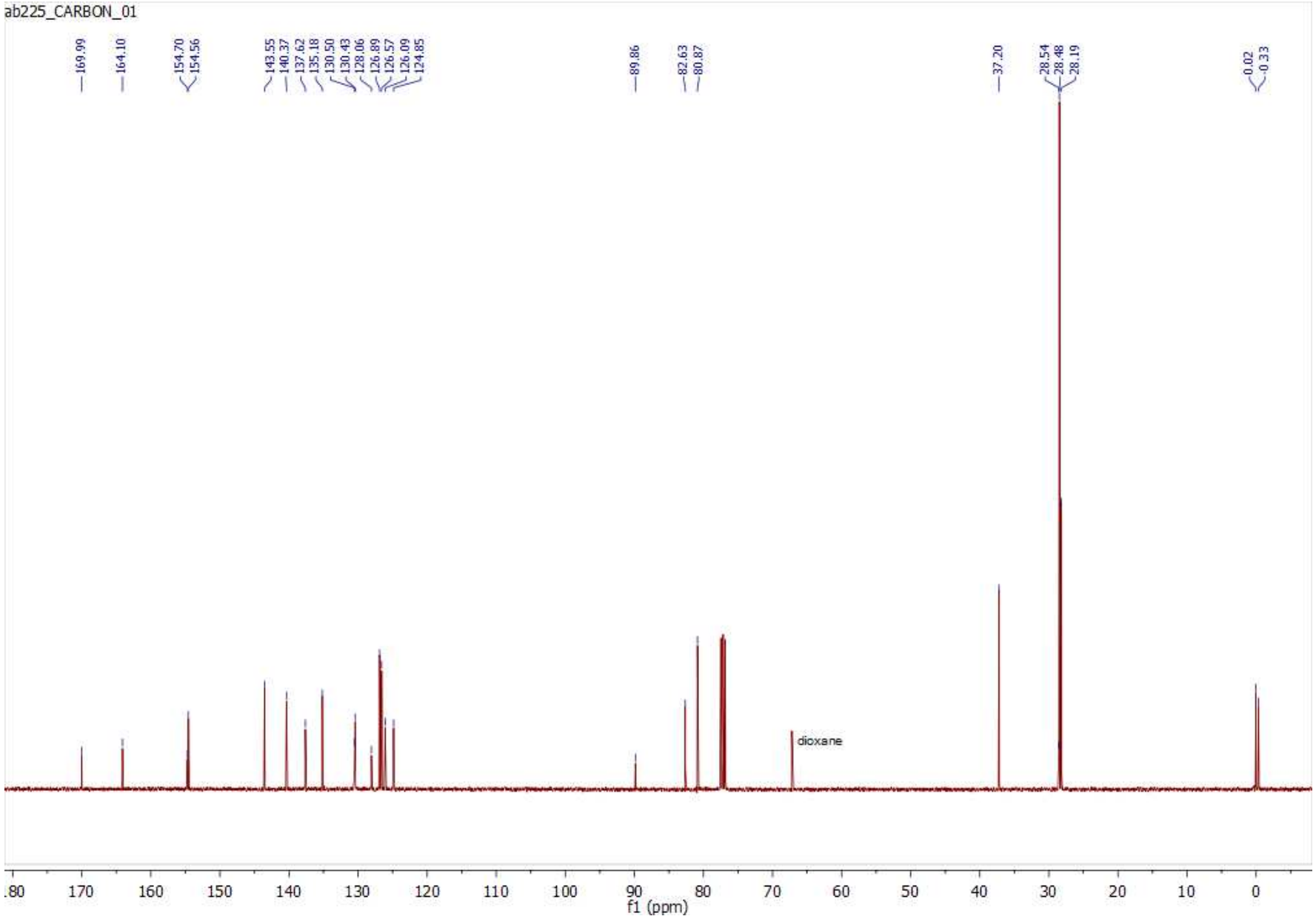
680SiR (^{13}C, ^{19}F)



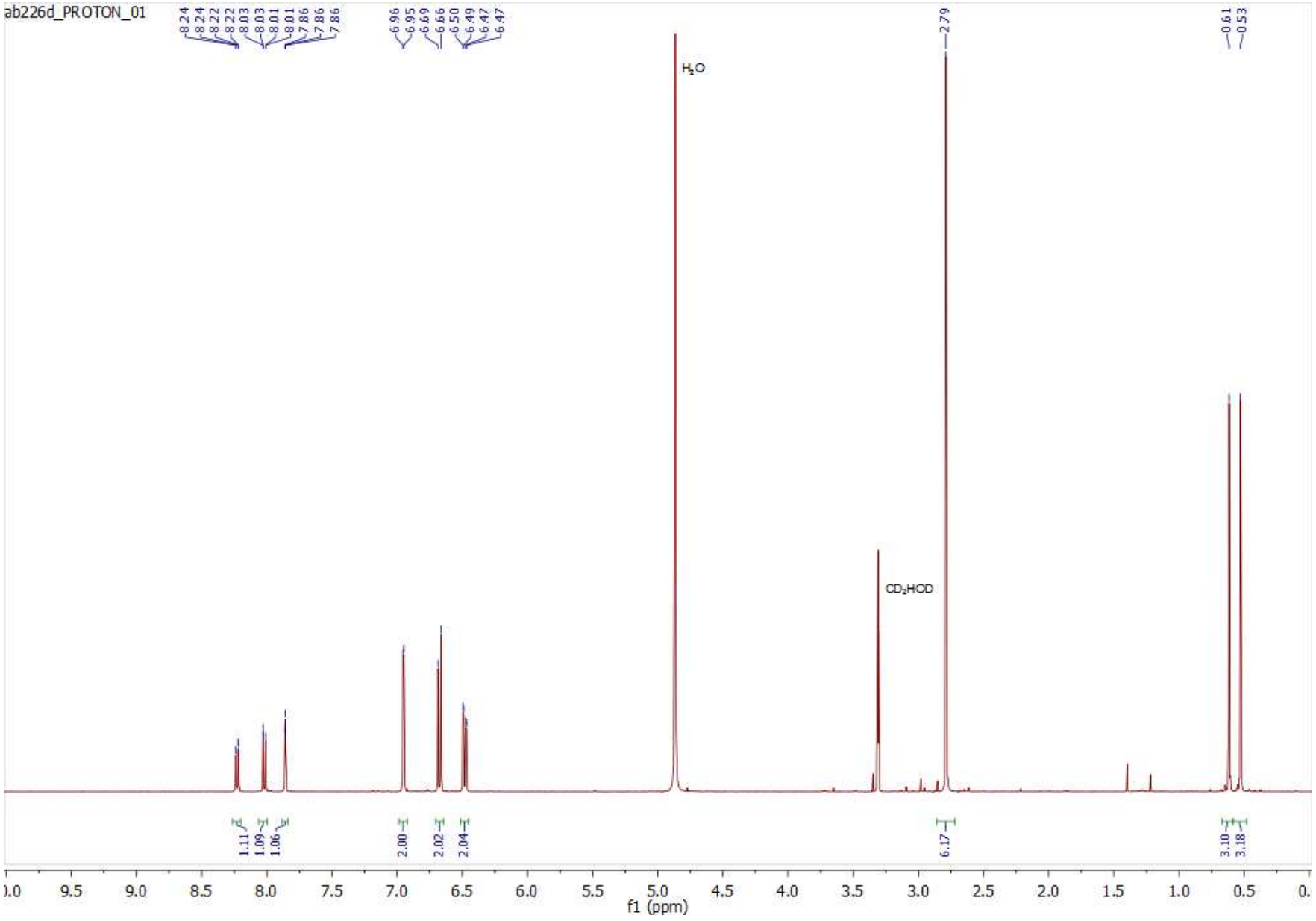
6 (^{1}H)


ab225_PROTON_01
8.15
8.14
8.13
8.13
8.12
8.00
8.00
8.00
7.98
7.98
7.98
7.83
7.83
7.82
7.57
7.56
7.18
7.17
7.16
7.15
7.08
7.06
3.26
3.26
1.55
1.45
0.72
0.62
dioxane
CHCl3
1.08
1.04
1.07
2.00
2.01
2.02
5.87
8.90
18.12
3.10
2.99
0.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

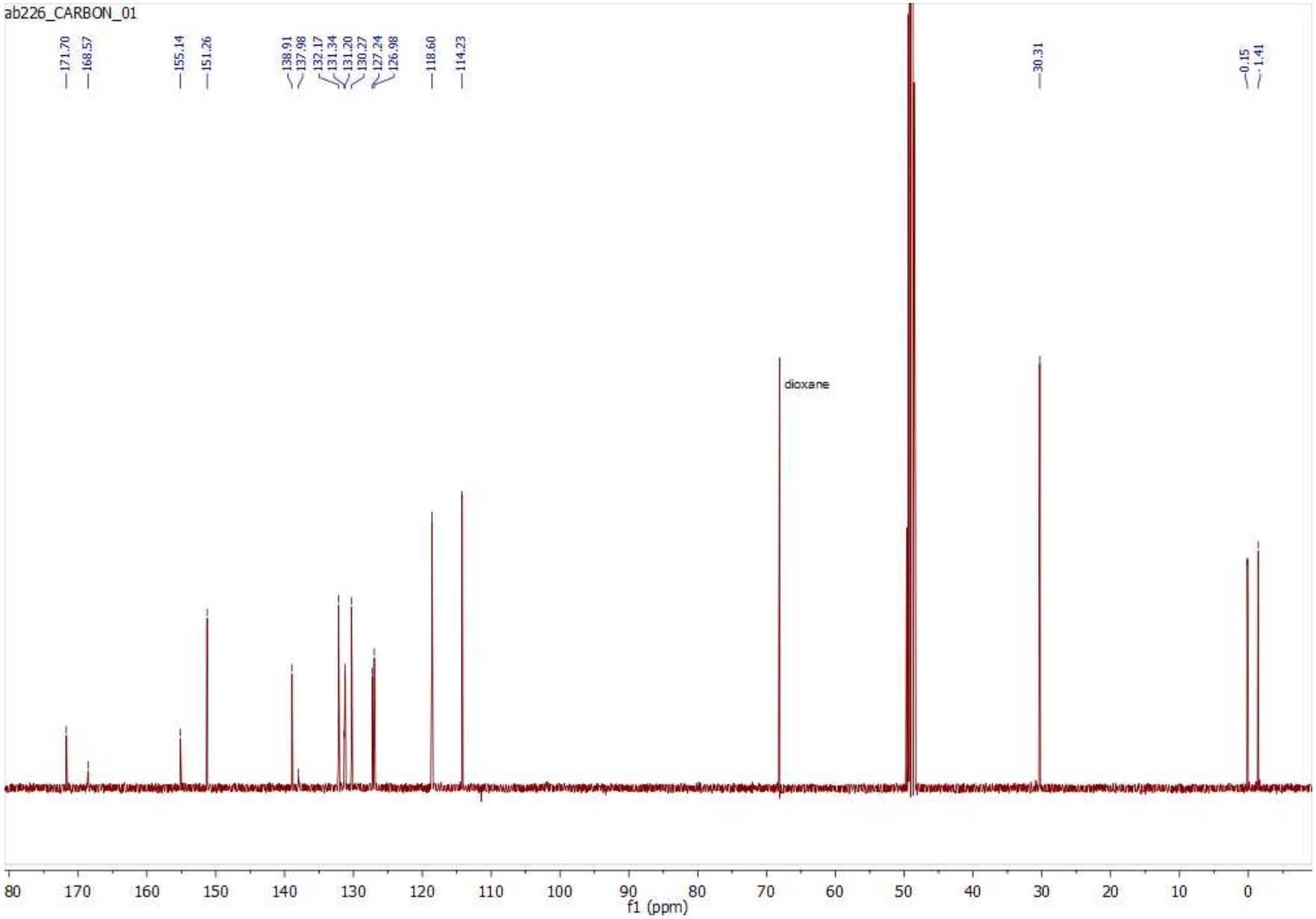
6 (^{13}C)


ab225_CARBON_01
169.99
164.10
154.70
154.56
143.55
140.37
137.62
135.18
130.50
130.43
128.06
126.89
126.57
126.09
124.85
89.86
82.63
80.87
37.20
28.54
28.48
28.19
0.02
-0.33
dioxane
f1 (ppm)

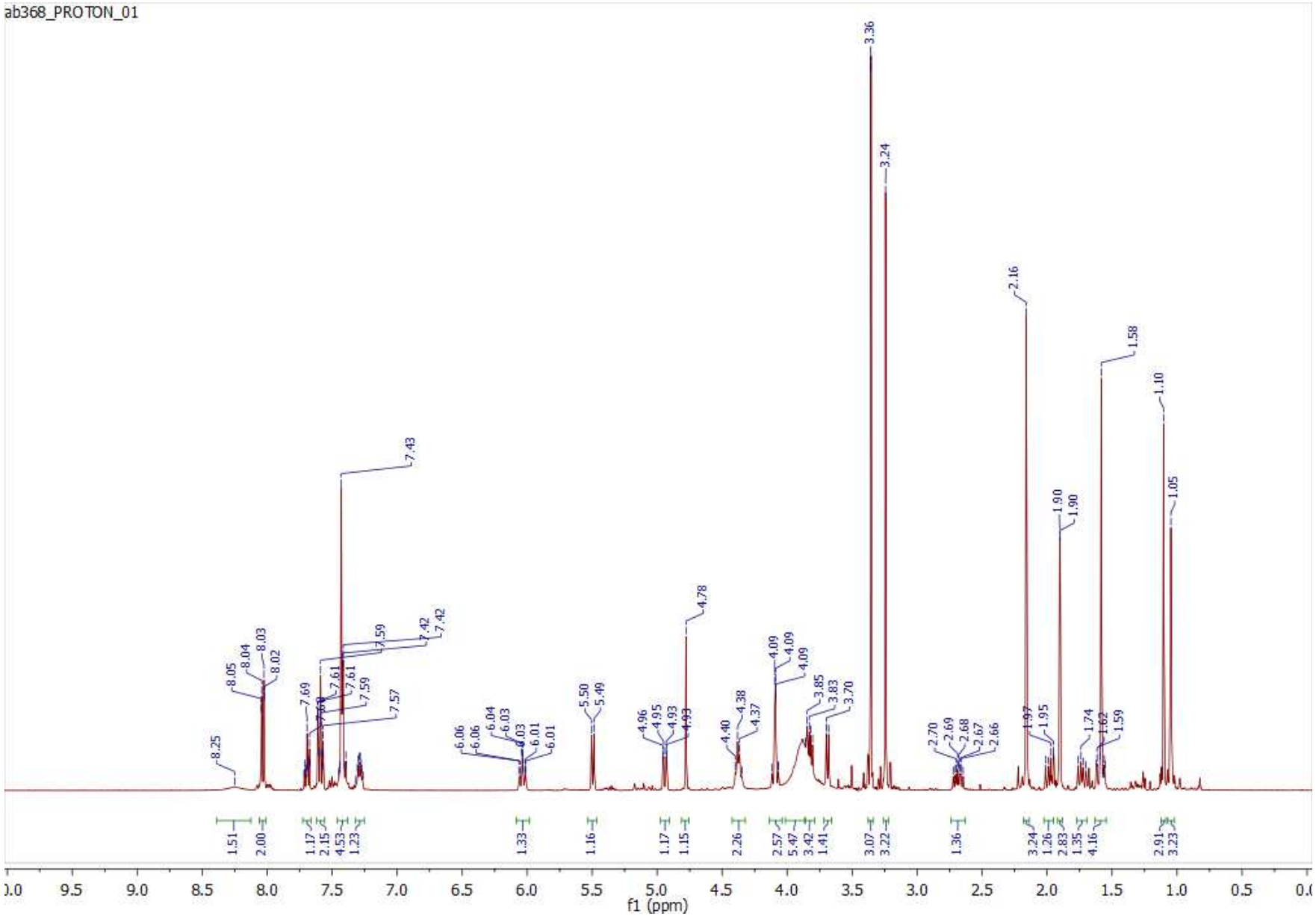
620SiR (^{1}H)


ab226d_PROTON_01
8.24
8.24
8.22
8.22
8.03
8.03
8.01
8.01
7.86
7.86
7.86
6.96
6.95
6.69
6.66
6.50
6.49
6.47
6.47
2.79
0.61
0.53
H2O
CD2HOD
1.11
1.09
1.06
2.00
2.02
2.04
6.17
3.10
3.18
f1 (ppm)

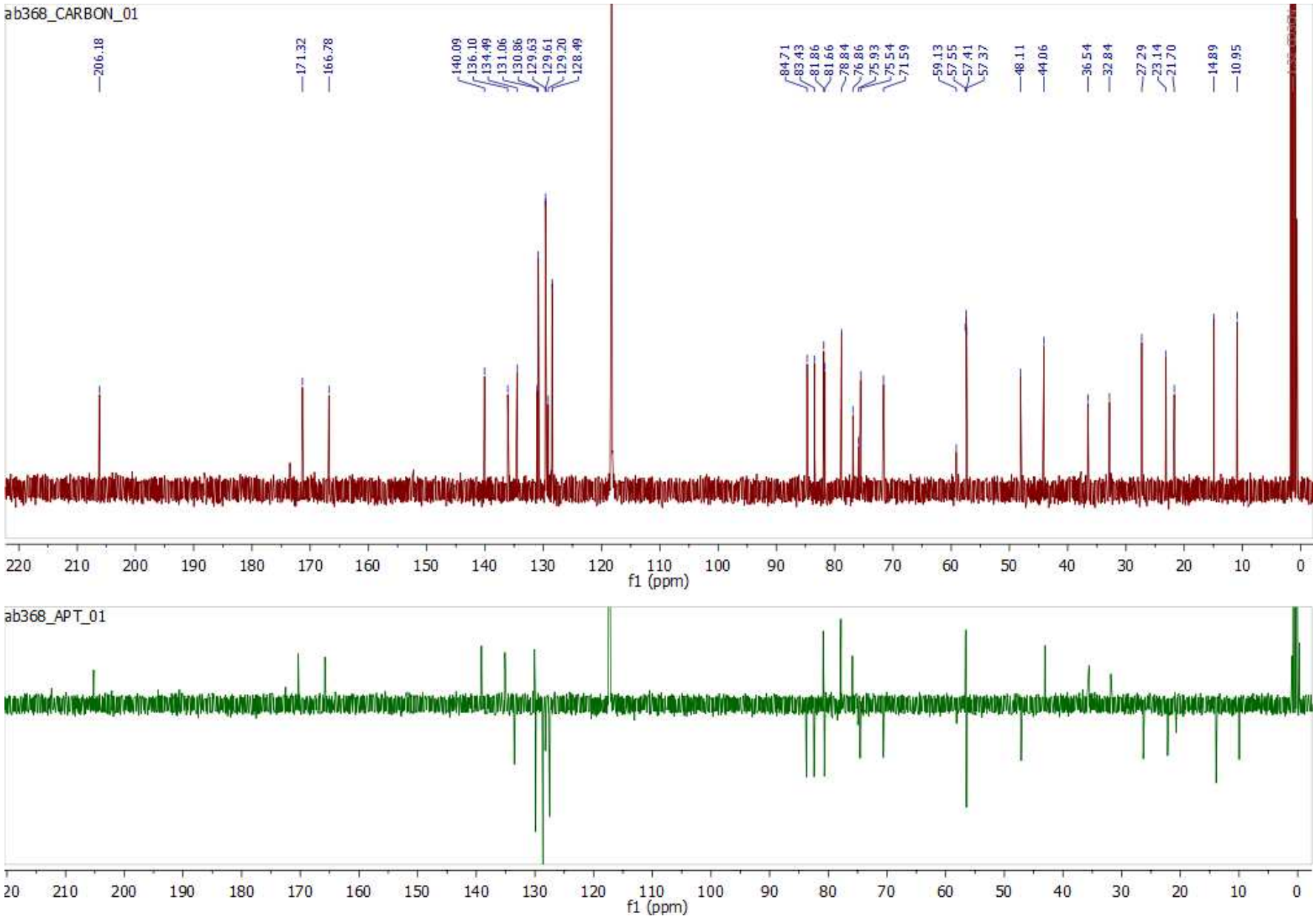
620SiR (^{13}C)


ab226_CARBON_01
171.70
168.57
155.14
151.26
138.91
137.98
132.17
131.34
131.20
130.27
127.24
126.98
118.60
114.23
30.31
0.15
-1.41
dioxane
80
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

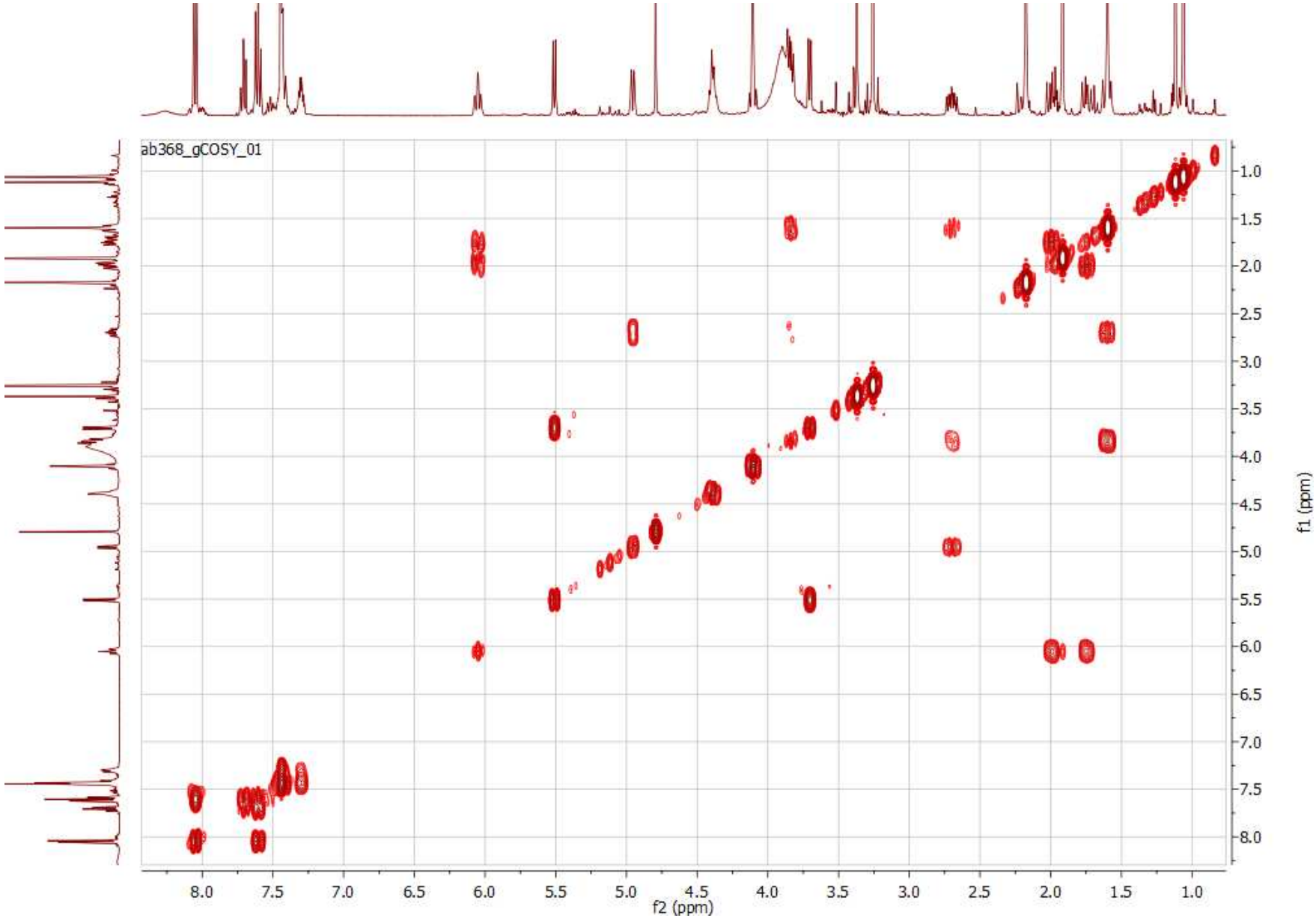
3'-$H_2NMe_2XT•HCO_2H$ (^1H)



3'-$H_2NMe_2XT•HCO_2H$ (^{13}C, APT)



3'-$H_2NMe_2XT•HCO_2H$ (^1H-^1H gCOSY)



3'-$H_2NMe_2XT•HCO_2H$ (^1H-^{13}C ASAPHMQC)