Supporting Information

Absolute Configuration and Biological Properties of Enantiomers of CFTR Inhibitor BPO-27

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General – NMR spectra (^{1}H at 600 MHz; ^{13}C at 150 MHz) were obtained in methanol (CD_3OD) or dimethyl sulfoxide (DMSO-d_6) using a 600 MHz Varian spectrometer. Chemical shifts are expressed in parts per million relative to the solvent. ^{1}H NMR spectra were acquired at -20 °C due to broadening of the 11-phenyl protons at ambient temperature. ^{13}C NMR spectra were obtained at ambient temperature. High-resolution mass spectra were acquired on a LTQ Orbitrap XL mass spectrometer equipped with an electrospray ionization source (ThermoFisher, San Jose, CA), operating in the negative ion mode. Samples were introduced into the source via loop injection at a flow rate of 200 µL/min, in 1:1 acetonitrile:water (containing 0.1% formic acid). Mass spectra were acquired using Xcalibur, version 2.0.7 SP1 (ThermoFinnigan). Compound purity was > 99% as judged by the peak area percentage of the UV absorbance signal using a Waters LC/MS instrument under operating conditions: electrospray (+) ionization, mass ranging from 100 to 900 Da, 20 V cone voltage; LC, Xterra MS C18 column (2.1 mm x 50 mm x 3.5 µm), 0.2 mL/min water/acetonitrile (containing 0.1% TFA). Flash chromatography was done using EM silica gel (230-400 mesh); thin-layer chromatography was done on EMD silica gel 60 F254 plates (Darmstadt, Germany).

Synthesis Procedures – Racemic (±)-**1** (BPO-27) was synthesized as previously reported.[1]

Separation and optical rotation of enantiomers – The separation of ~1.0 g of (±)-**1** was performed by Averica Discovery Services Inc. (Worchester, MA). Preparative-scale chiral SFC was carried out on a RegisCell 3.0 x 25.0 cm column using an isocratic method: 75% CO_2, and 25% ethanol containing 1% isopropylamine, 80 mL/min, 100 bar, 25 °C. Analysis of the separated enantiomers was carried out on a RegisCell 4.6 x 100 mm column, using an isocratic method: 75% CO_2, 25% ethanol with 0.1% isopropylamine, 4 mL/min, 100 bar, 25 °C. Both processes were monitored for UV absorption at 230 nm. Optical rotation was measured using a Rudolph Research Analytical AUTOPOL IV Polarimeter with a 40T-5.0-100-2-H sample cell (length = 10 cm, volume = 2 mL). Racemic **1** was separated into two enantiopure fractions and isolated as the isopropyl amine carboxylic salt **2**. Fraction 1 corresponds to (+)-**2**, $[\alpha]_D^{22}$ + 73.1 ° (*c* 0.126, CH_3OH), amount = 413 mg (74.5% yield, 99.5% e.e.). Fraction 2 corresponds to (–)-**2** , $[\alpha]_D^{22}$ −72.4 °, (*c* −0.128, CH_3OH), amount = 396 mg **(**71.5% yield, 98.6% e.e.) ^{1}H and ^{13}C NMR for (+)-**2** (fraction 1) and (–)-**2** (fraction 2) were identical. (+)-**2** ^{1}H NMR (600 MHz, methanol-d_4) δ 7.86 (d, *J* = 7.7 Hz, 1H), 7.61 (d, *J* = 8.3 Hz, 1H), 7.60 (dd, *J* = 8.4, 1.9 Hz, 1H), 7.49 (tt, *J* = 7.6, 1.3 Hz, 1H), 7.32 (t, *J* = 7.7 Hz, 1H), 7.29 (d, *J* = 1.8 Hz, 1H), 7.13 (s, 1H), 7.03 (d, *J* = 8.4 Hz, 1H), 7.02 (d, *J* = 7.6 Hz, 1H), 6.26 (d, *J* = 3.4 Hz, 1H), 6.15 (dd, *J* = 3.5, 0.9 Hz, 1H), 3.54 (s, 3H), 3.38 (h, *J* = 6.6 Hz, 1H), 3.29 (s, 3H), 1.28 (d, *J* = 6.6 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 167.43, 158.43, 152.66, 151.05, 146.21, 130.39, 129.67, 129.04, 128.16, 127.64, 124.38, 123.74, 123.15, 121.12, 117.88, 114.40, 112.57, 106.72, 104.78, 67.05, 59.74, 42.38, 40.05, 31.99, 27.54, 21.15, 14.08. HRMS (ES-) (m/z): $[M-1]^-$ calculated for $C_{26}H_{17}BrN_3O_6$, 546.0301, found 546.0266.

Crystal preparation – Inactive (–)-**2** (30 mg, 49 mmol) was placed in a 75 mL separatory funnel filled with very dilute aqueous HCl (20 mL) and ethyl acetate (20 mL). The mixture was shaken

vigorously until all solids were dissolved. The layers were separated and the aqueous layer was extracted two more times with ethyl acetate. The organic extracts were combined and back-extracted with brine and then dried over Na_2SO_4. The ethyl acetate solution was evaporated in a 50 mL recovery flask on a rotory evaporator and then dried under high vacuum to give a white solid (24 mg, 44 mmol, yield 89%). To the recovery flask was added a magnetic stir bar, DMAP (1 mg, 8 mmol), EDAC (9 mg, 47 mmol) and dry dichloromethane (15 mL). Once the stirred solution became homogenous, dry ethanol (10 μL, 7.9 mg, 172 mmol) was added and the mixture was stirred for 24 h in a sealed vial with a rubber septum. The reaction mixture was transferred to a separatory funnel and extracted with dilute citric acid (2 x10 mL). The organic layer was collected in a 50 mL beaker, dried over Na_2SO_4 and evaporated in a 50 mL recovery flask on a rotary evaporator. The residue, which had the same R_f value on TLC as racemic (±)-**3**, was purified by flash chromatography (EtOAc:hexanes, 2:3) to give a clear residue (20 mg, 35 mmol, yield 79%). The residue was dissolved in toluene (0.5 mL) in a 1 dram vial. The vial was placed upright in a jar filled with 3-4 mm of mixed hexanes and then sealed. The setup was placed for 6 d in a quiet, vibration-free room, yielding diffraction-quality crystals.

X-ray crystallography – A colorless crystalline needle of enantiopure **3** derived from inactive (–)-**2** with approximate orthogonal dimensions 0.31 x 0.04 x 0.04 mm^3 was optically centered on a Bruker Duo[2] APEXII CCD system and cooled to -183 °C (90 °K). Indexing of the unit cell used a random set of reflections collected from three series of 0.5° wide ω-scans, 10 s per frame, and 30 frames per series that were well distributed in reciprocal space. Data were collected [CuKα] with 0.5° wide scans, variable time per frame dependent upon detector 2θ angle and varying φ and omega angles such that nearly all unique reflections were collected at least once. The crystal to detector distance was 4.96 cm, providing a complete sphere of data to $2\theta_{max}$=136.52. Crystallographic calculations were performed on an iMac with 2.80 GHz quad core processor and 8 GB of extended memory. A total of 18750 reflections were collected and corrected for Lorentz and polarization effects with SAINT[2] and absorption using crystal faces and Blessing's method as incorporated into the program SADABS[3,4] with 4943 unique for point group 222. The SHELXTL[5] software package was implemented to determine the probable space group and set up the initial files. System symmetry, systematic absences, and intensity statistics indicated the non-centrosymmetric orthorhombic space group $P2_12_12_1$ (no. 19). The structure was determined by direct methods with the successful location of a majority of the main molecule using the program XS.[6] The structure was refined with XL.[6] The data collected were merged for least squares refinement to 4752 unique data [R(int)=0.0925]. A series of least-squares difference-Fourier cycles were required to locate the remaining non-hydrogen atoms and optimize the full occupancy, disordered solvent toluene molecule. All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were idealized throughout the final refinement stages. The final structure was refined to convergence with R(F)=9.09%, wR(F2)=22.64%, GOF=1.128 for all 4752 unique reflections [R(F)=8.86, wR(F2)=22.46% for those 4529 data with Fo > 4σ(Fo)]. The final difference-Fourier map was featureless indicating that the structure is correct and complete. An empirical correction for extinction was also attempted and found to be negative

and therefore not applied. The absolute structure parameters, Flack(x),[7] was refined and found to be -0.08(4) while the Hooft parameter[8] was -0.085(18), indicating reliable determination of absolute configuration as having (S) chirality. Data were submitted to the Cambridge structural database (ID JF2111).

Cell culture – Fischer rat thyroid (FRT) cells expressing human wild type CFTR were cultured in porous Snapwell inserts in Coon`s modified F12 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin.[9]

Short-circuit current measurement – Snapwell inserts containing CFTR-expressing FRT cells were mounted in an Ussing chamber. The hemichambers contained 5 mL of buffer consisting of 75 mM NaCl and 75 mM Na gluconate (apical) and 150 mM NaCl (basolateral) (pH 7.3). The basolateral membrane was permeabilized with 250 μg/mL amphotericin B, as described.[9] Short-circuit current was recorded continuously using a DVC-1000 voltage clamp (World Precision Instruments) using Ag/AgCl electrodes and 3 M KCl agar bridges.

In vitro metabolism in hepatic microsomes – (*R*)-**1** and (*S*)-**1** (each 5 μM) were (separately) incubated for specified times at 37 °C with rat liver microsomes (1 mg protein/ml; Sigma-Aldrich, St. Louis, MO) in potassium phosphate buffer (100 mM) containing 1 mM NADPH. The mixture was then chilled on ice, and 0.5 mL of ice-cold ethyl acetate was added. Samples were centrifuged for 15 min at 3,000 rpm, and the supernatant was evaporated to dryness under nitrogen. The residue was dissolved in 150 μL mobile phase (acetonitrile:water 3:1, containing 0.1% formic acid) for LC/MS analysis. Reverse-phase HPLC separations were carried out using a Waters C18 column (2.1 x 100 mm, 3.5 mm particle size) equipped with a solvent delivery system (Waters model 2690, Milford, MA). The solvent system consisted of a linear gradient from 5 to 95% acetonitrile run over 16 min (0.2 mL/min flow rate). Mass spectra were acquired on an Alliance HT 2790 + ZQ mass spectrometer using negative ion detection.

Mouse pharmacokinetics and renal accumulation – (*R*)-**1** was formulated at 1 mg/mL in 5% DMSO, 2.5% Tween-80 and 2.5% PEG400 in H_2O, based on formulations used for compounds of similar polarity and chemical properties. Male mice in a CD1 genetic background (age 8–10 weeks, 25–35 g) were administered 300 μL of the (*R*)-**1** formulation by intraperitoneal injection. At specified times, blood samples were collected by eye bleed. At 4 h, kidneys were removed following renal arterial perfusion with PBS. Kidneys were weighed, mixed with acetic acid (100 μL per 1 g tissue) and ethyl acetate (10 mL per 1 g tissue), and homogenized. The homogenate was centrifuged at 3,000 rpm for 15 min. Calibration standards were prepared in kidney homogenates from control mice to which known amounts of (*R*)-**1** was added. The ethyl acetate-containing supernatant was dried under nitrogen and the residue was reconstituted in acetonitrile: H_2O (3:1) containing 0.1% formic acid. For analysis of blood, equal volumes of blood and acetonitrile with 0.1% formic acid

were mixed, vortexed and centrifuged at 12000 rpm for 20 min. Calibration standards were prepared by adding known amounts of standards in blood from control mice before extraction. The supernatants were collected and 10 μL was analyzed by LC-MS, as described above.

Hydrogen-deuterium exchange – Compound **1** (5 mg) was incubated with 2 mL of mouse serum:D_2O (1:1) at 37 ºC for 4 h. The aqueous layer was extracted with ethyl acetate (3 x 2 mL). Combined organics were dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude material was purified by flash silica gel chromatography to yield **1**. ^{1}H NMR of starting **1** and isolated final **1** were identical with no reduction in the proton singlet peak (δ = 7.13 ppm) corresponding to the proton on the chiral carbon.

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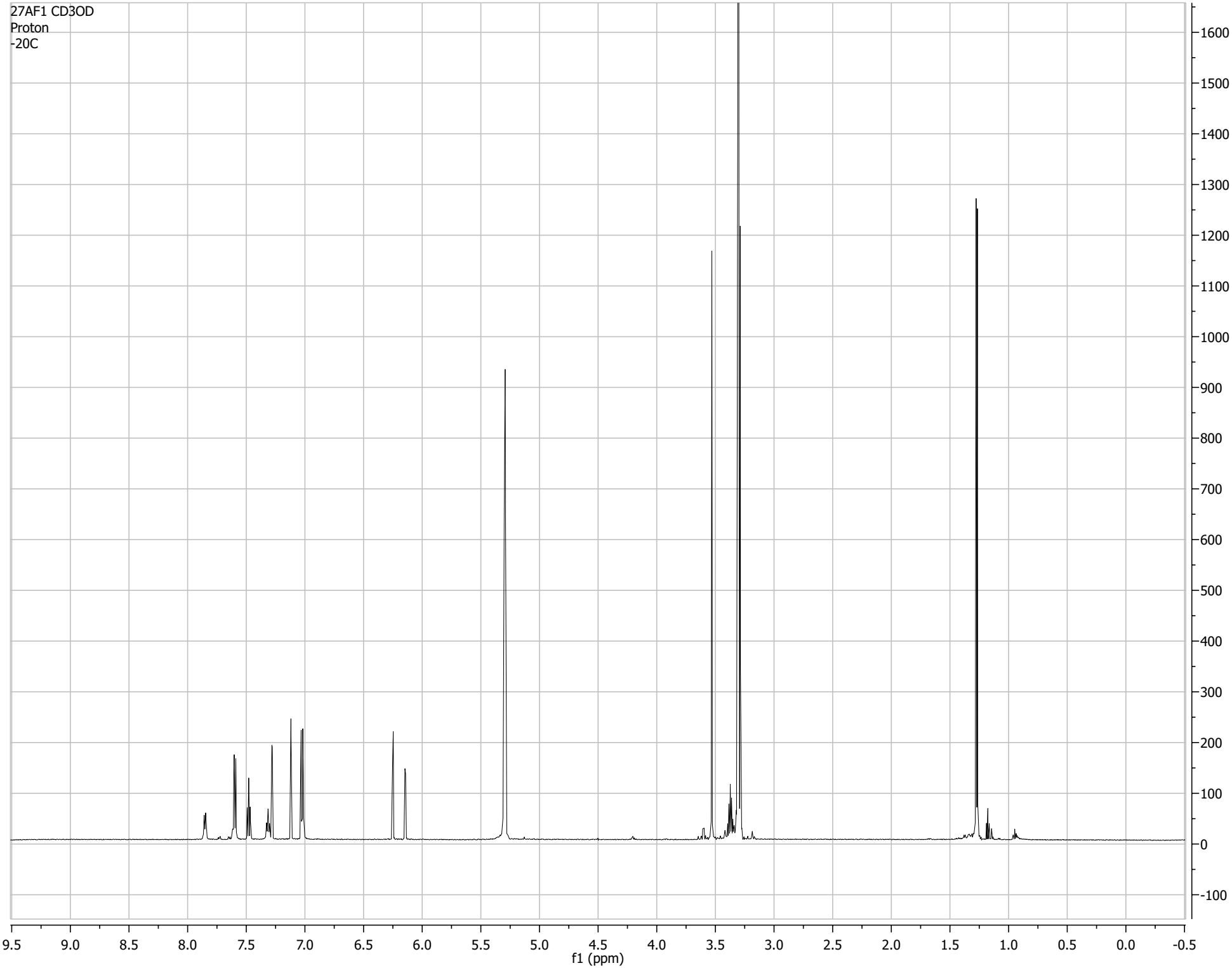
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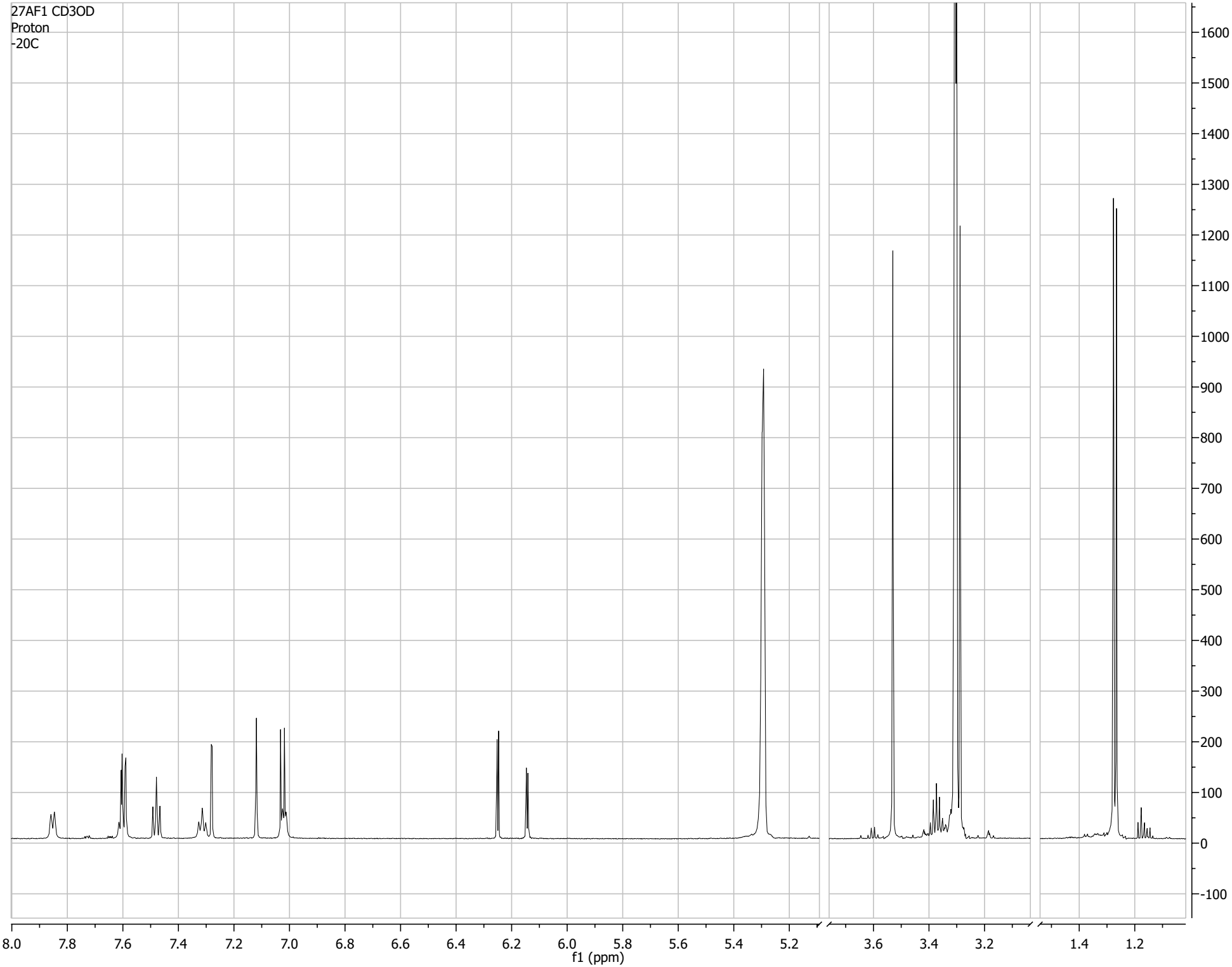
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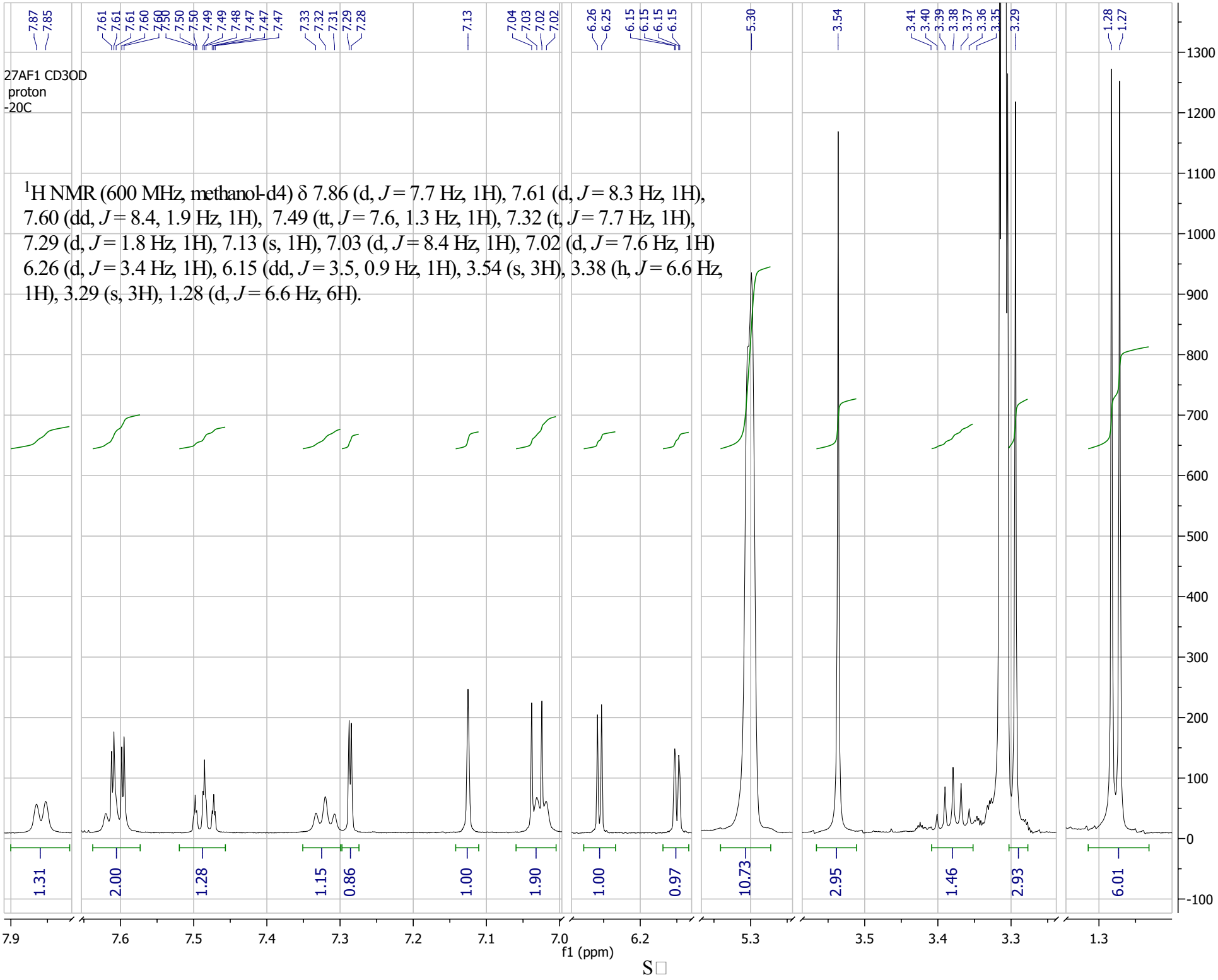

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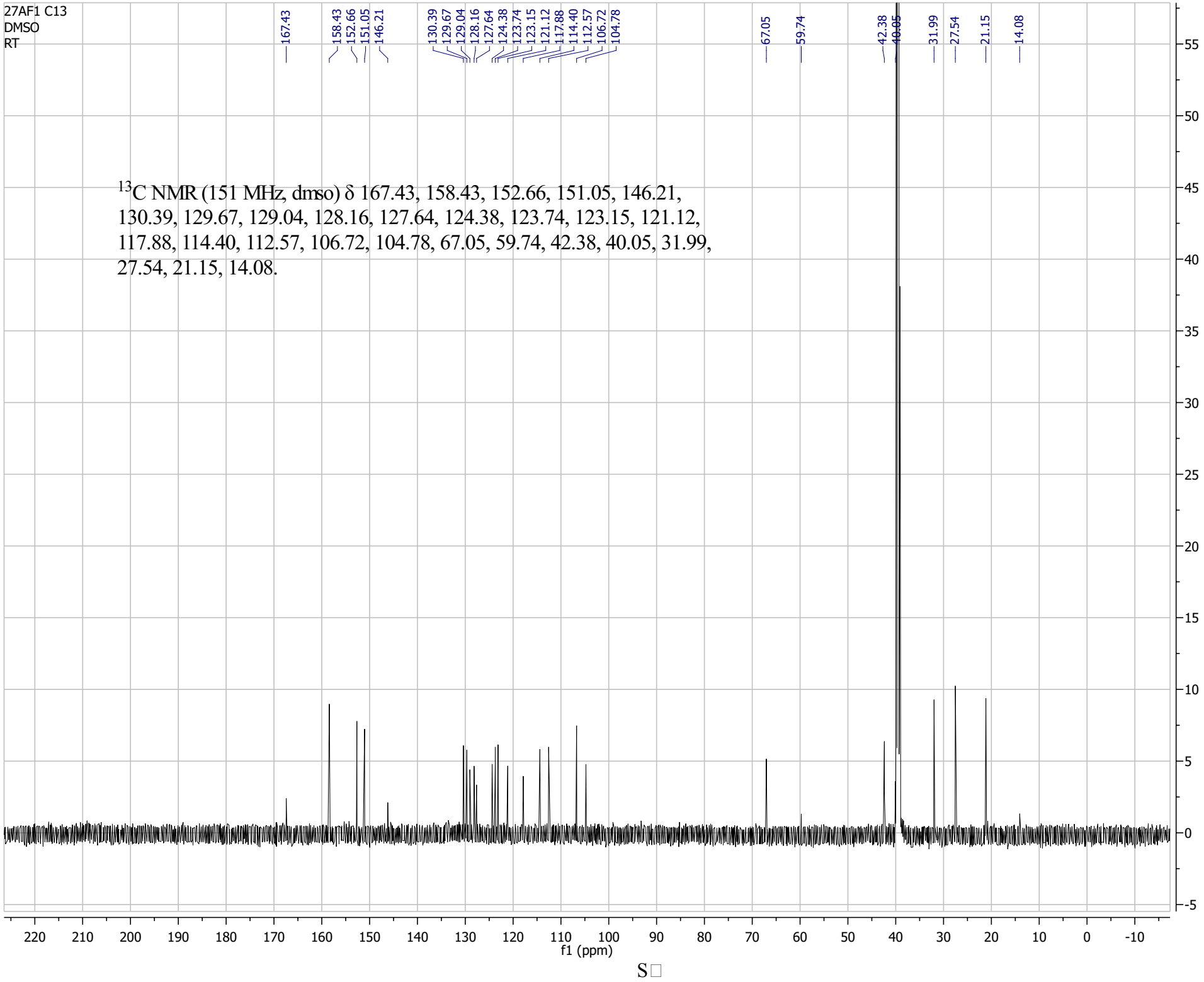

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