Supporting Information

for

An improved synthesis of a fluorophosphonate–polyethyleneglycol–biotin probe and its use against competitive substrates

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Biology experimental details and digital NMR spectra for the synthesized compounds

Biological materials. Enalapril and oseltamivir were purchased from Fisher Scientific. Porcine liver esterase (EC 3.1.1.1), trypsin from bovine pancreas (EC 3.4.21.4), nucleoside phosphorylase from calf spleen (EC 2.4.2.1), and bovine serum albumin were purchased from Sigma. Cell culture reagents were obtained from Invitrogen (Rockville, MD) and cell culture supplies from Corning (Corning, NY) and Falcon (Lincoln Park, NJ).

Cells. The human colon carcinoma cell line, Caco-2, was obtained from the American Type Culture Collection (ATCC HTB37, passage numbers 18-50, Rockville, MD). The cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% nonessential amino acids. The cells were grown in 150 × 100 mm tissue culture dishes in an atmosphere of 5% CO_2 and 90% relative humidity at 37 °C. The cells were grown for 14–16 days postconfluence before harvest.

Cell homogenate preparation. Caco-2 cell homogenate was prepared by homogenizing the Caco-2 cells with Dounce Tissue Homogenizer (Bellco Glass, Inc. Vineland, NJ) in 50 mM Tris buffer containing 0.25 M sucrose (pH 8.0). The homogenate was centrifuged for 20 min at 25,000*g*. The pellet was resuspended in 50 mM Tris buffer (pH 8.0) containing 0.5 M NaCl, and vortexed for 30 min. The resulting sample was centrifuged for 1 h at 25,000*g*. The combined supernatants were stored at −80 °C.

Tissue sample preparation. Rat tissue homogenates were prepared as follows: Immediately after surgery, rat tissues were washed three times with phosphate buffer saline (PBS). Minced samples were suspended in 50 mM Tris buffer (pH 8.0) containing

0.25 M sucrose. Samples were homogenized with Tissue-Tearor (Biospec Products, Inc., Bartlesville, OK) in an ice-water bath, and then sonicated three times on ice, 15 sec each. Each sample was centrifuged at 10,000*g* for 20 min, and the resulting pellet was resuspended in Tris buffer containing 0.5 M NaCl. After being vortexed vigorously, the suspension was sonicated briefly and centrifuged at 10,000*g* for 20 min. The combined supernatants were stored at −80 °C.

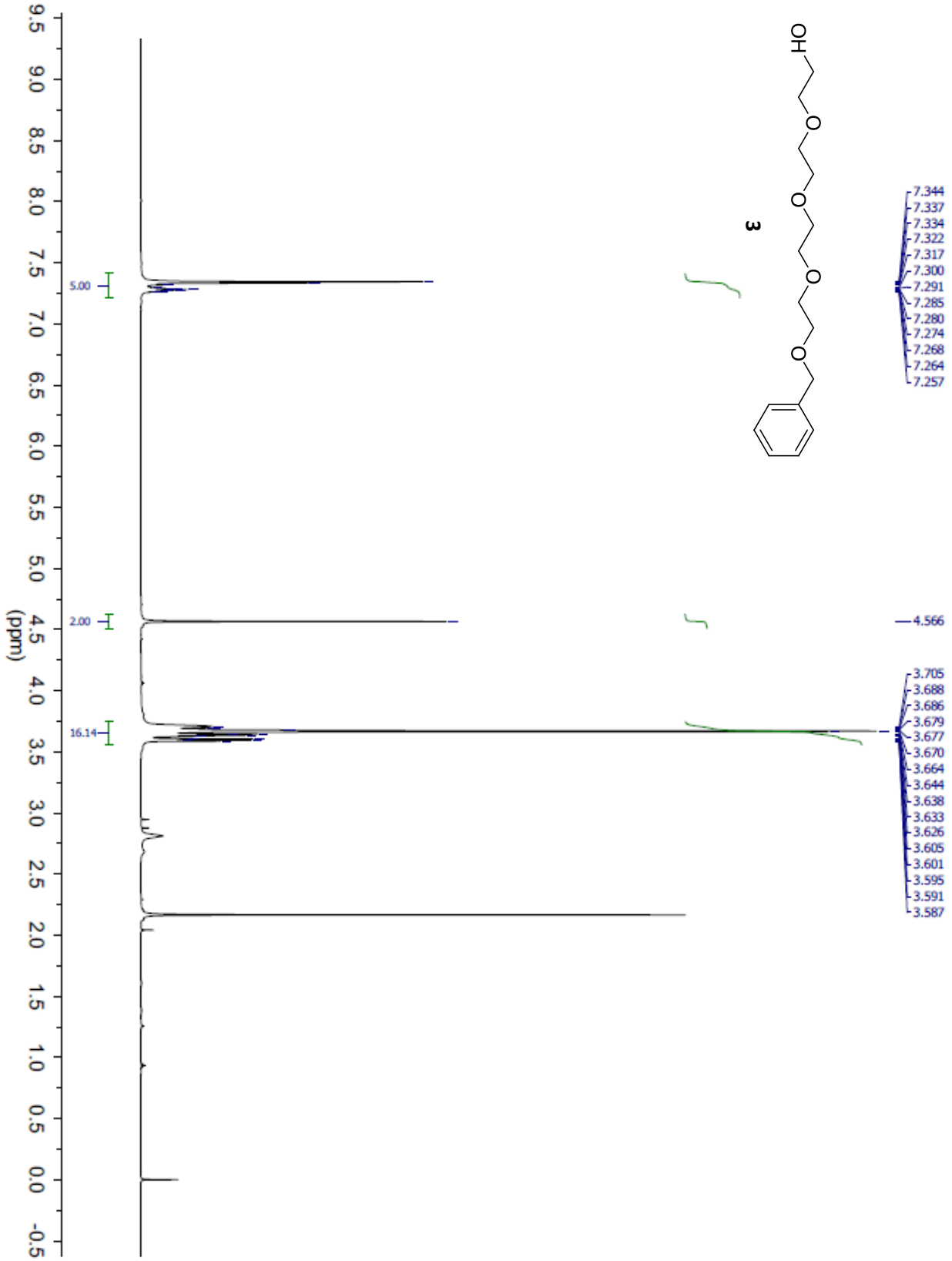
Protein labeling. Protein concentration was determined by the BCA assay and adjusted to a final concentration of 1 mg/mL in 50 mM Tris buffer (pH 8.0). The protein sample was treated with 4 μM FP-PEG-biotin probe. The reaction was incubated at room temperature for 1 min up to 1 h before being terminated by 5× sample loading buffer, and subsequently heated at 85 °C for 5 min. The treated samples were subjected to 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-Page, Invitrogen Corporation). The gel was electrophoretically transferred onto Hybond PVDF membrane (Amersham, UK). The membrane was blocked in Tris buffer saline with 0.1% tween-20 (TBS-T) containing 1% casein for 1 h at room temperature, followed by incubation with streptavidin alkaline phosphatase (1:2500 dilution) (R & D, Minneapolis, MN) in TBS-T at room temperature for 1 h. The membrane was washed with TBS-T three times, 10 min for each washing. The blot was subsequently visualized with ECF substrate (GE Healthcare, NJ) on Typhoon 9200 Variable Mode Imager (GE Healthcare, NJ).

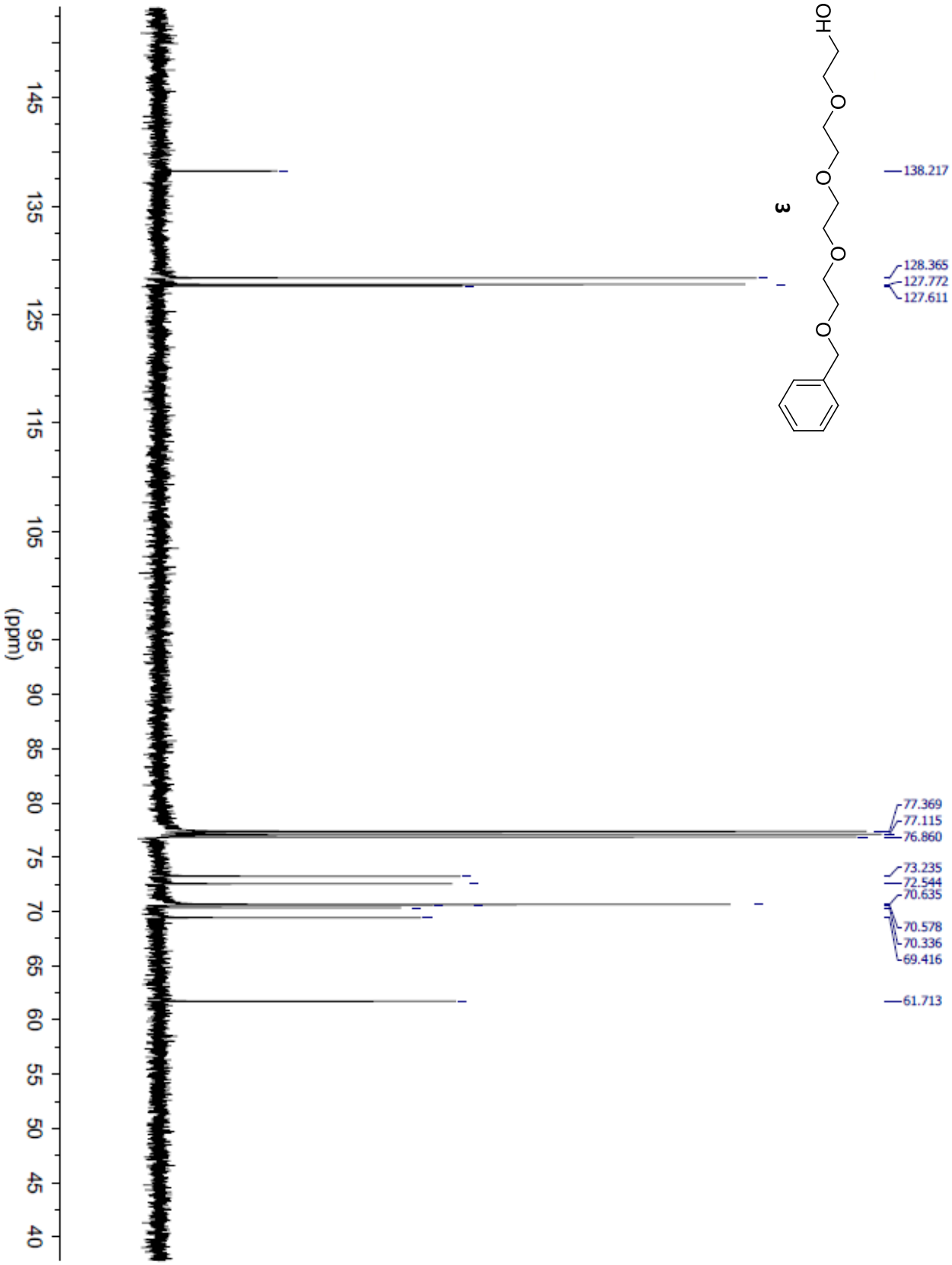
Purification of FP–PEG–biotin labeled proteins. Protein mixture was first incubated with the FP–PEG–biotin probe for 1 h at room temperature with rocking. The sample was then run through a PD-10 column (GE Healthcare, Piscataway, NJ). The eluent

was treated with SDS (0.5%) and heated for 10 min at 95 °C. After rocking with avidin agarose beads for 1 h at room temperature, the mixture was centrifuged for 3 min at 3000*g* and the supernatant was removed. The resin was washed twice each with 50 mM TBS-T buffer, and then 50 mM Tris buffer (pH 8.0). After washing, an equal volume of 2× sample buffer was added to the final remaining resin, and the mixture was heated for 10 min at 95 °C. The mixture was centrifuged at 5,000*g* for 5 min. The supernatant was then subjected to SDS-Page analysis.

Competition assay. Protein sample (1mg/mL) in 50 mM Tris buffer (pH 8.0) was incubated with enalapril (at specified concentration) for 15 min at room temperature. FP–PEG–biotin (4 μM) was then added to the sample and incubated for another 15 min before being quenched with 5× samples loading buffer, and subsequent heating at 85 °C for 5 min. Western blot analysis was conducted as described in the protein labeling section.

Digital NMR spectra

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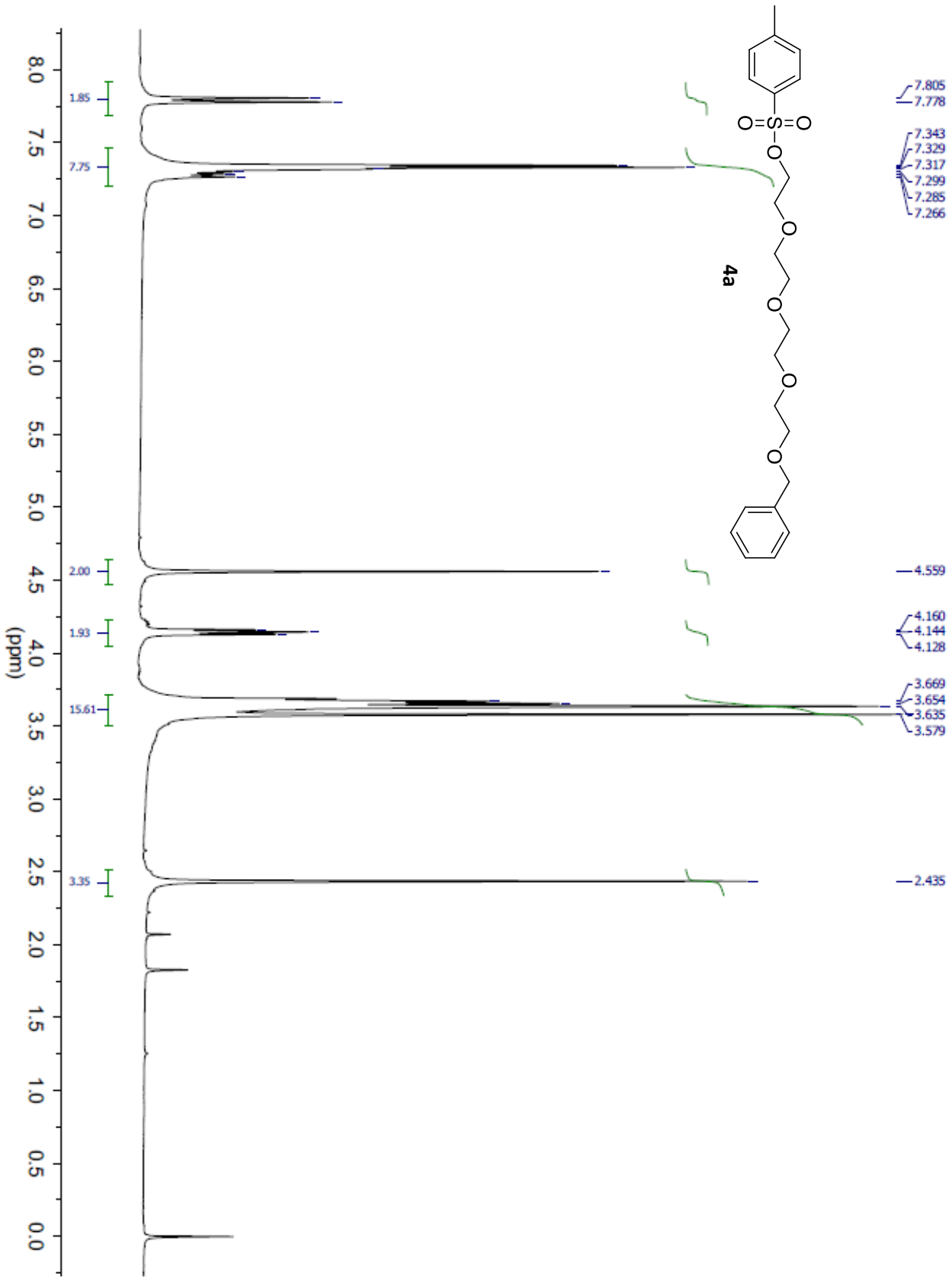
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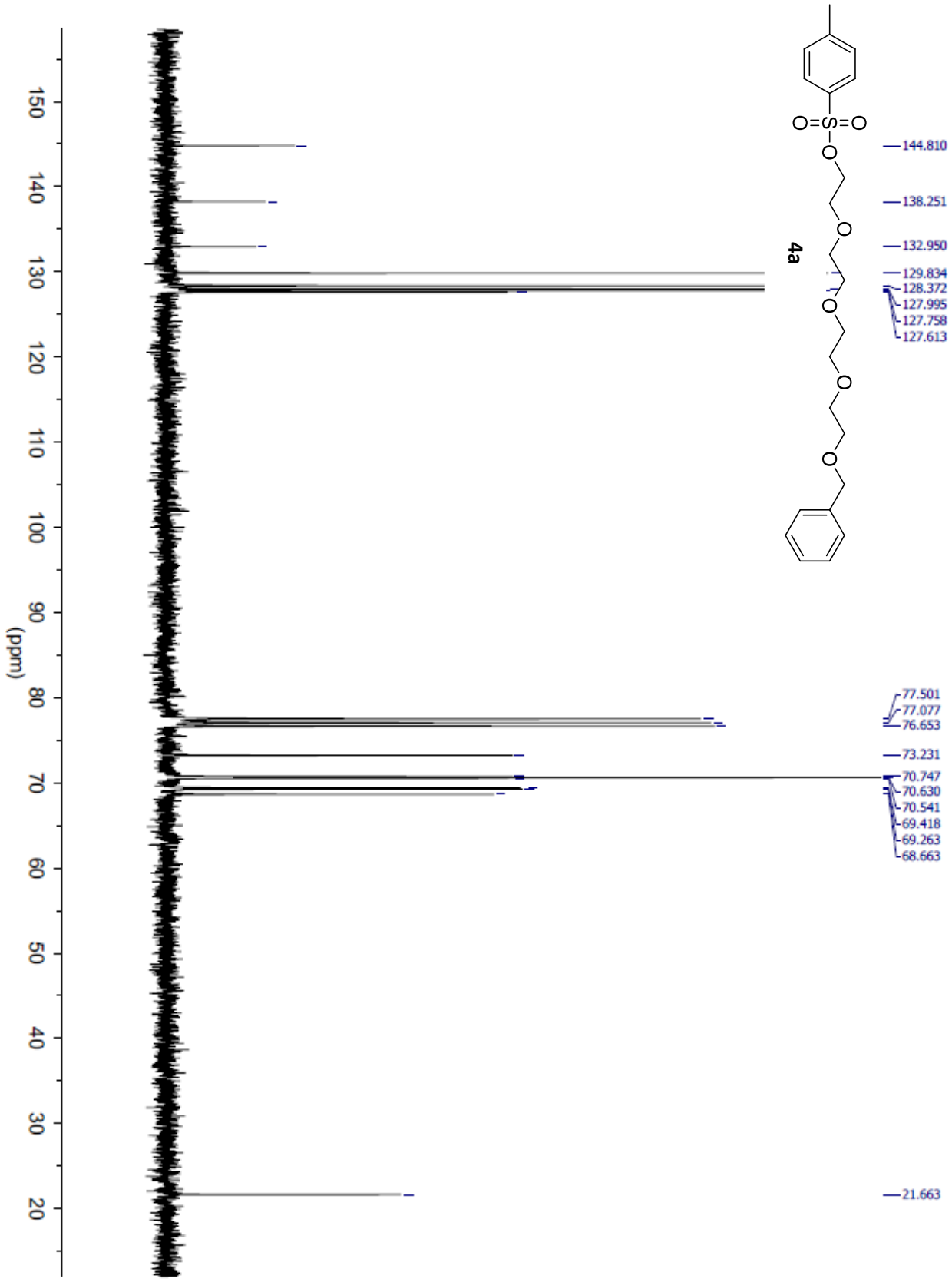
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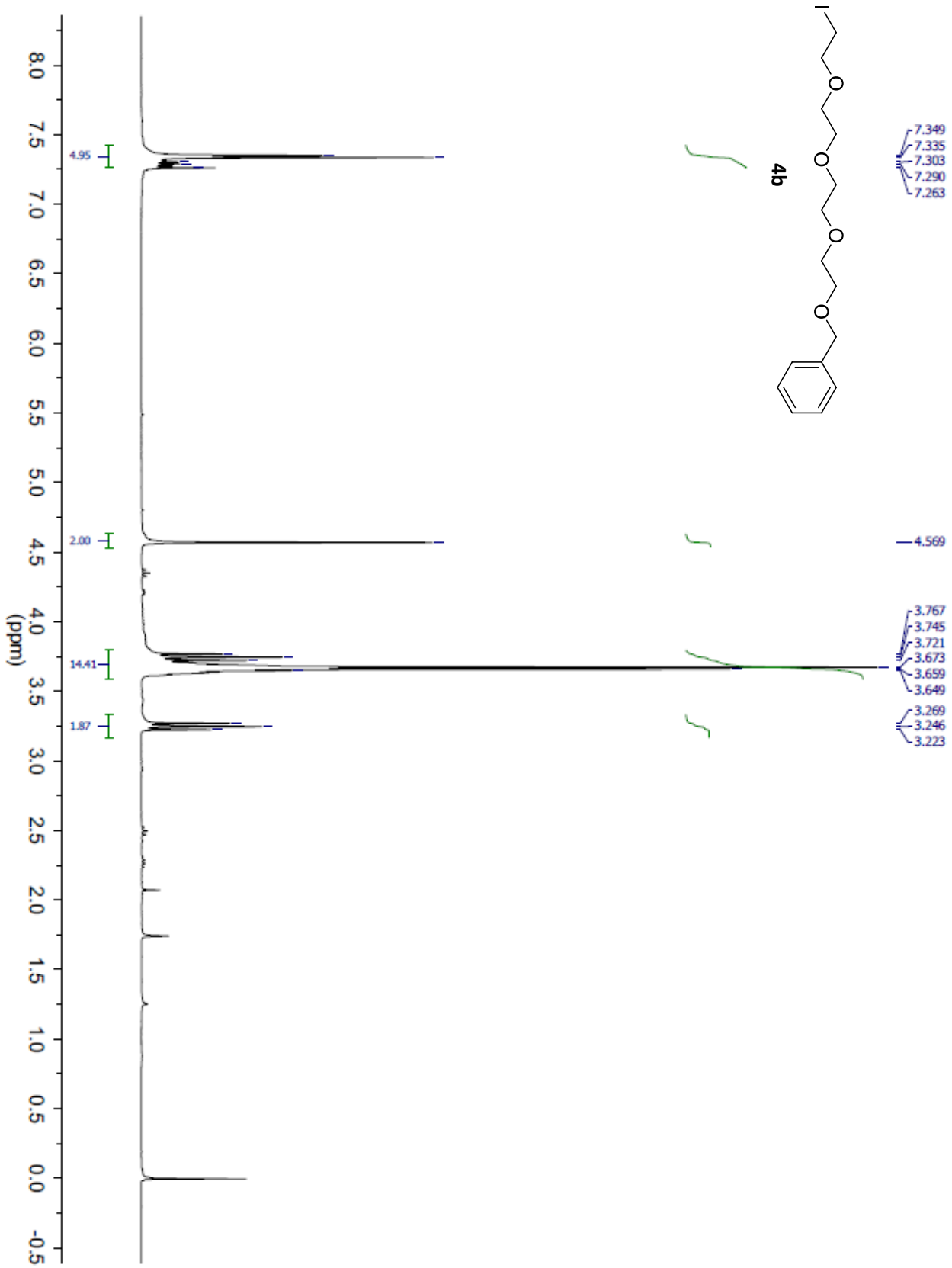
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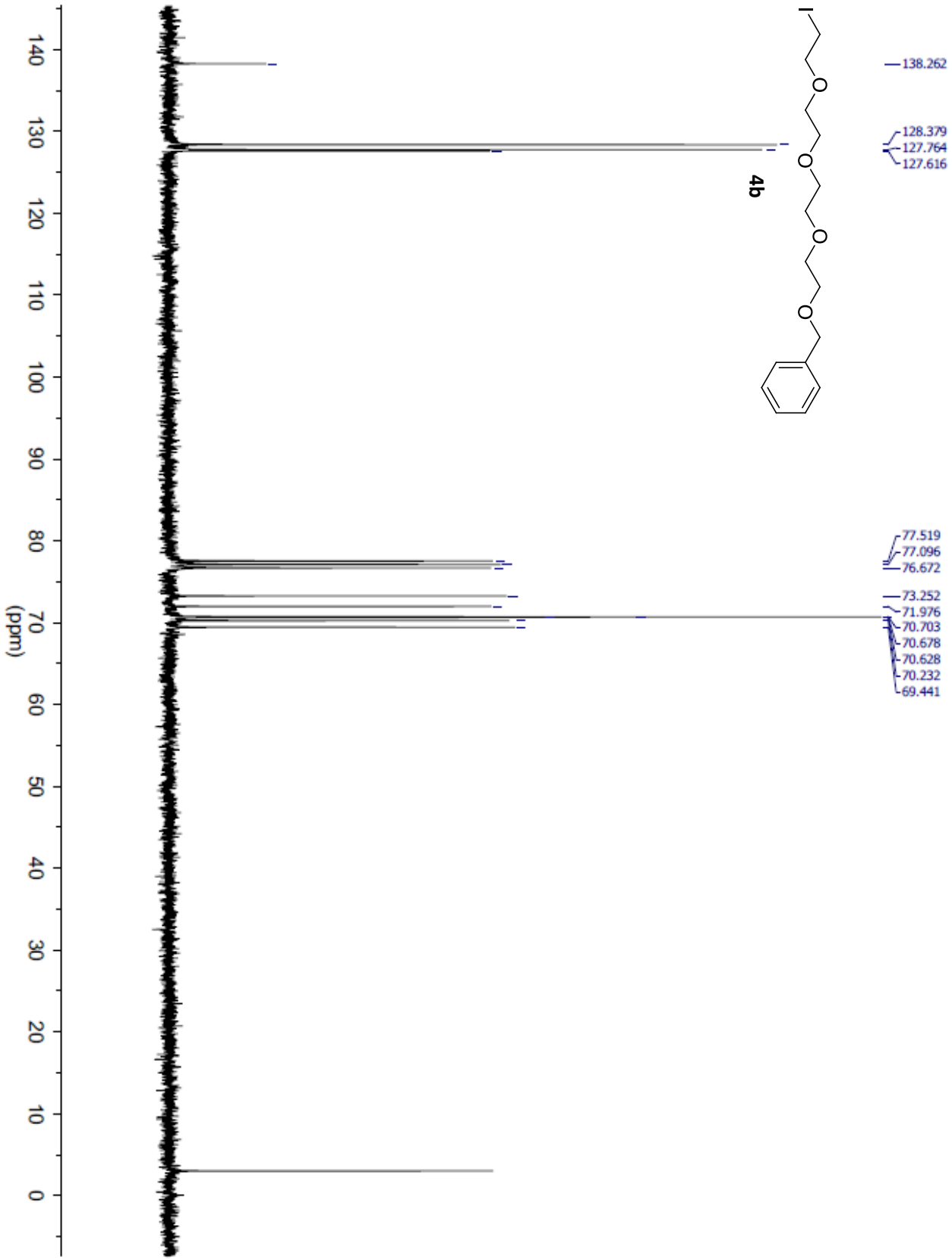
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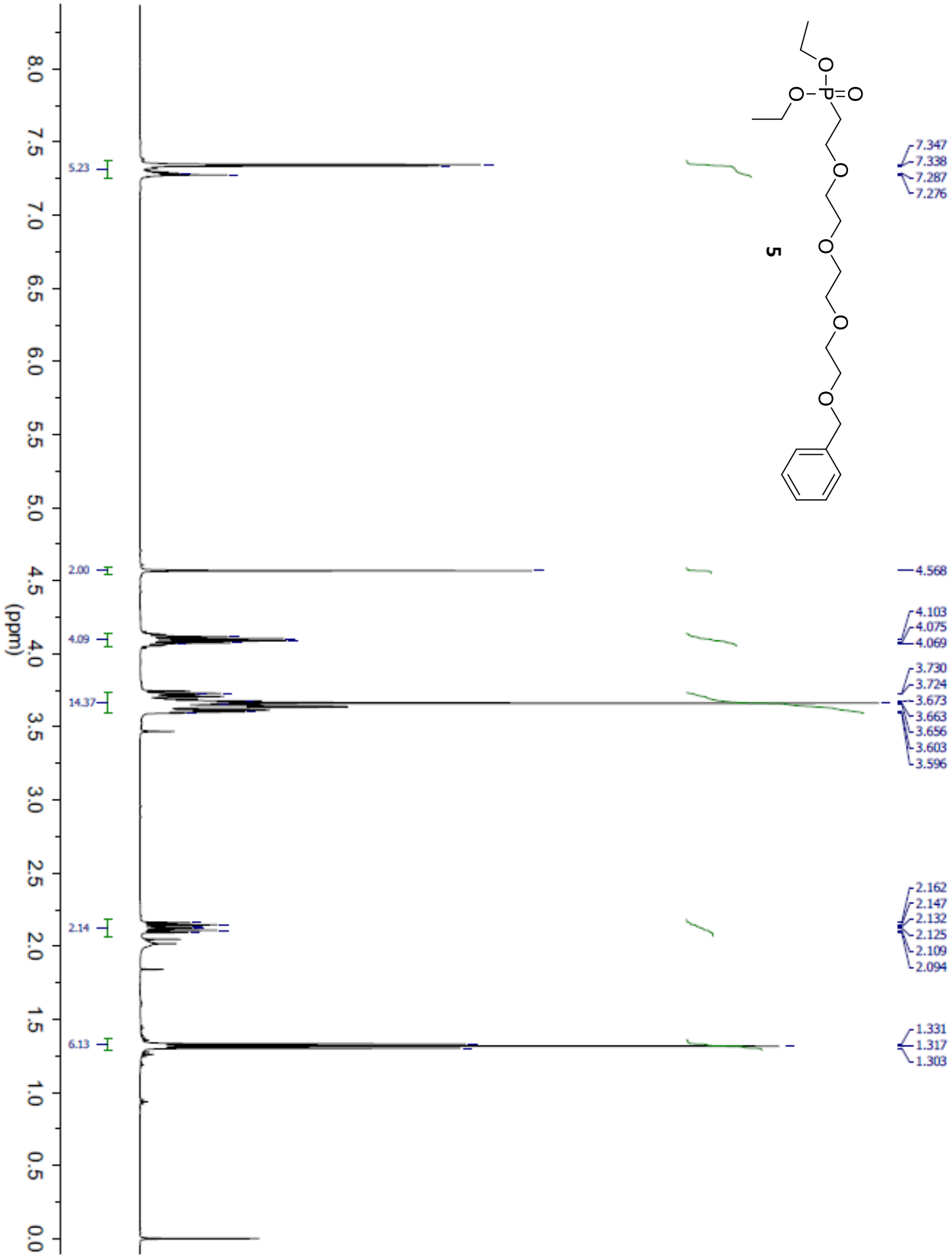
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(ppm)
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1.303
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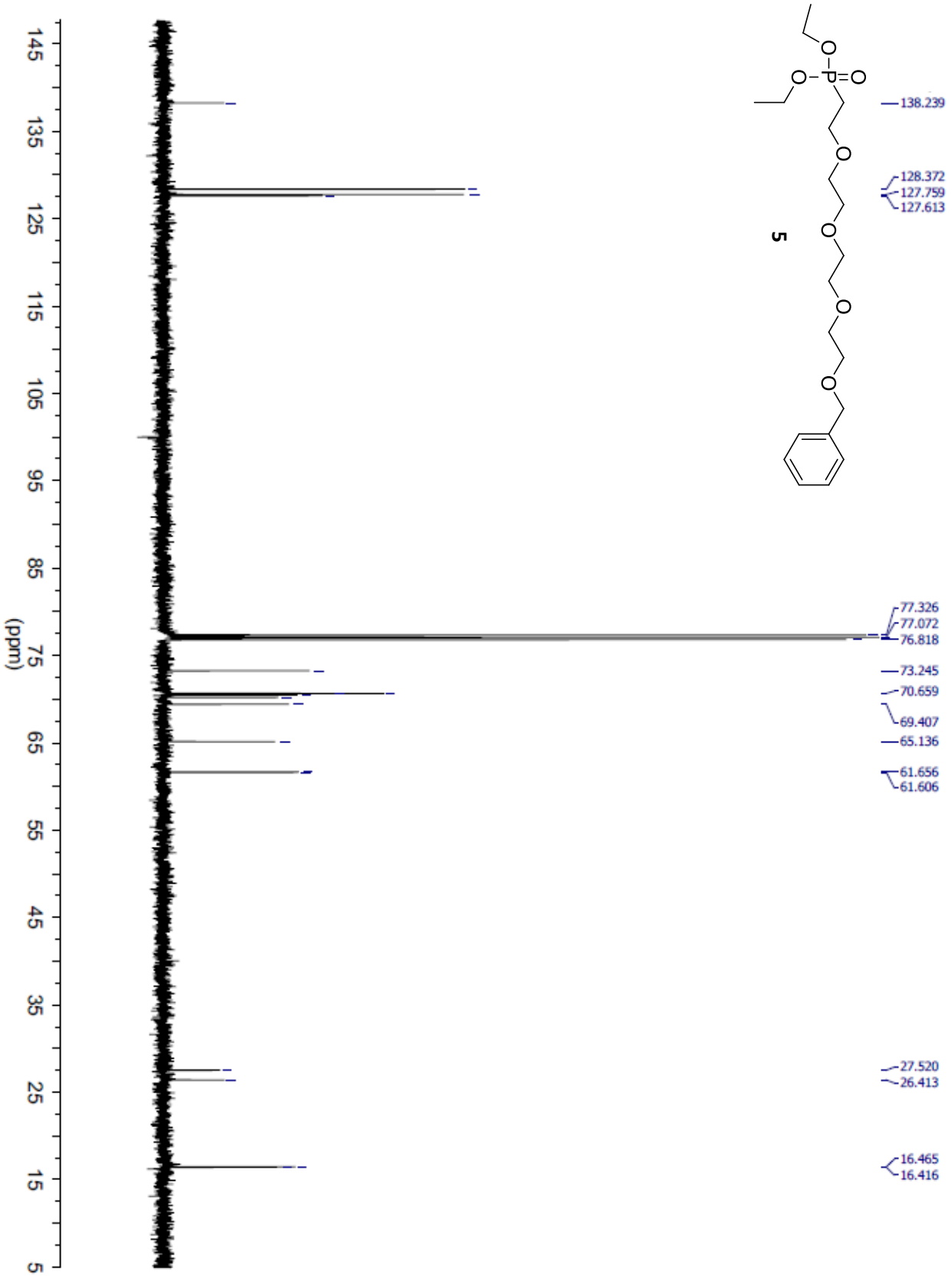
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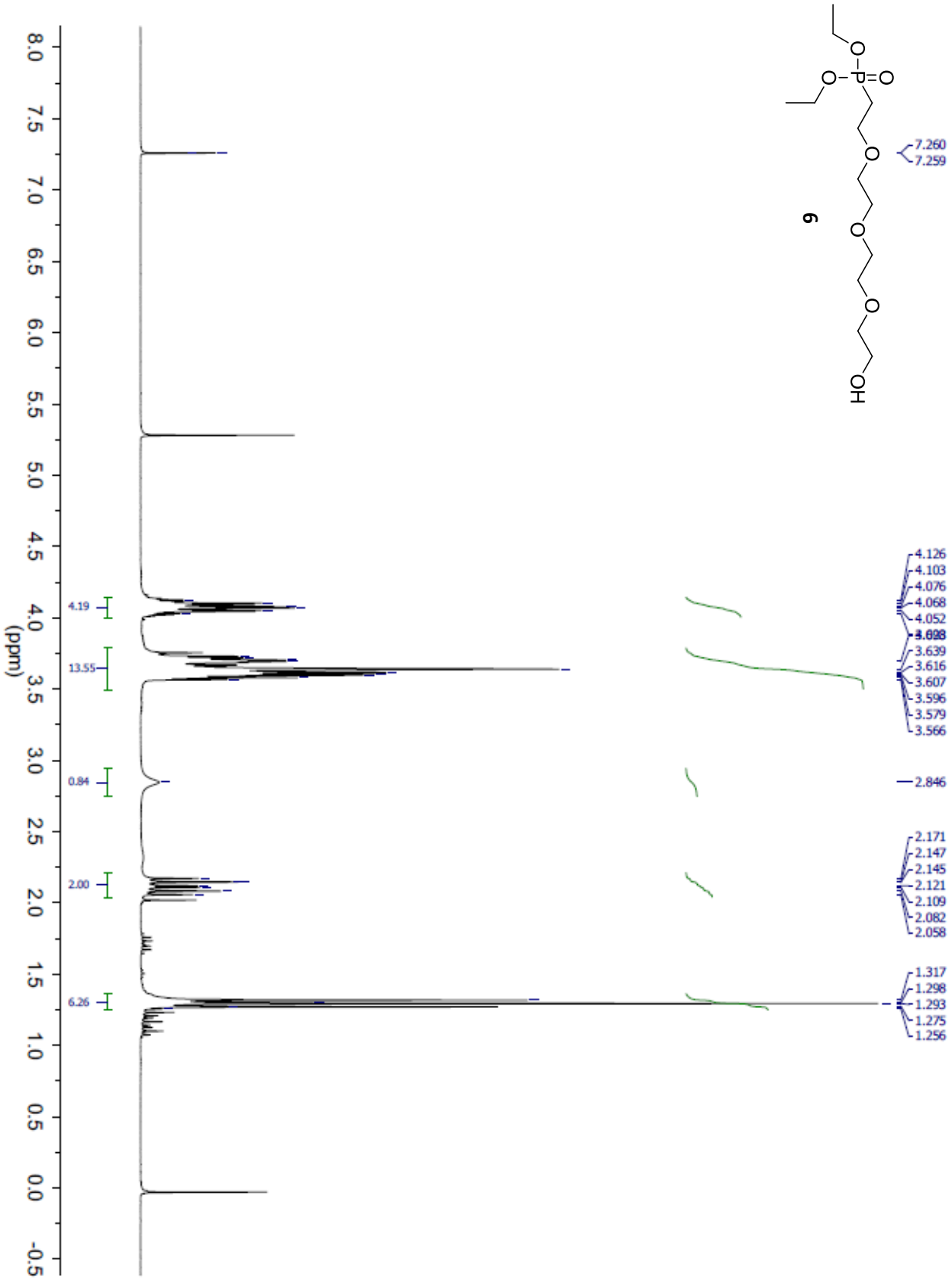
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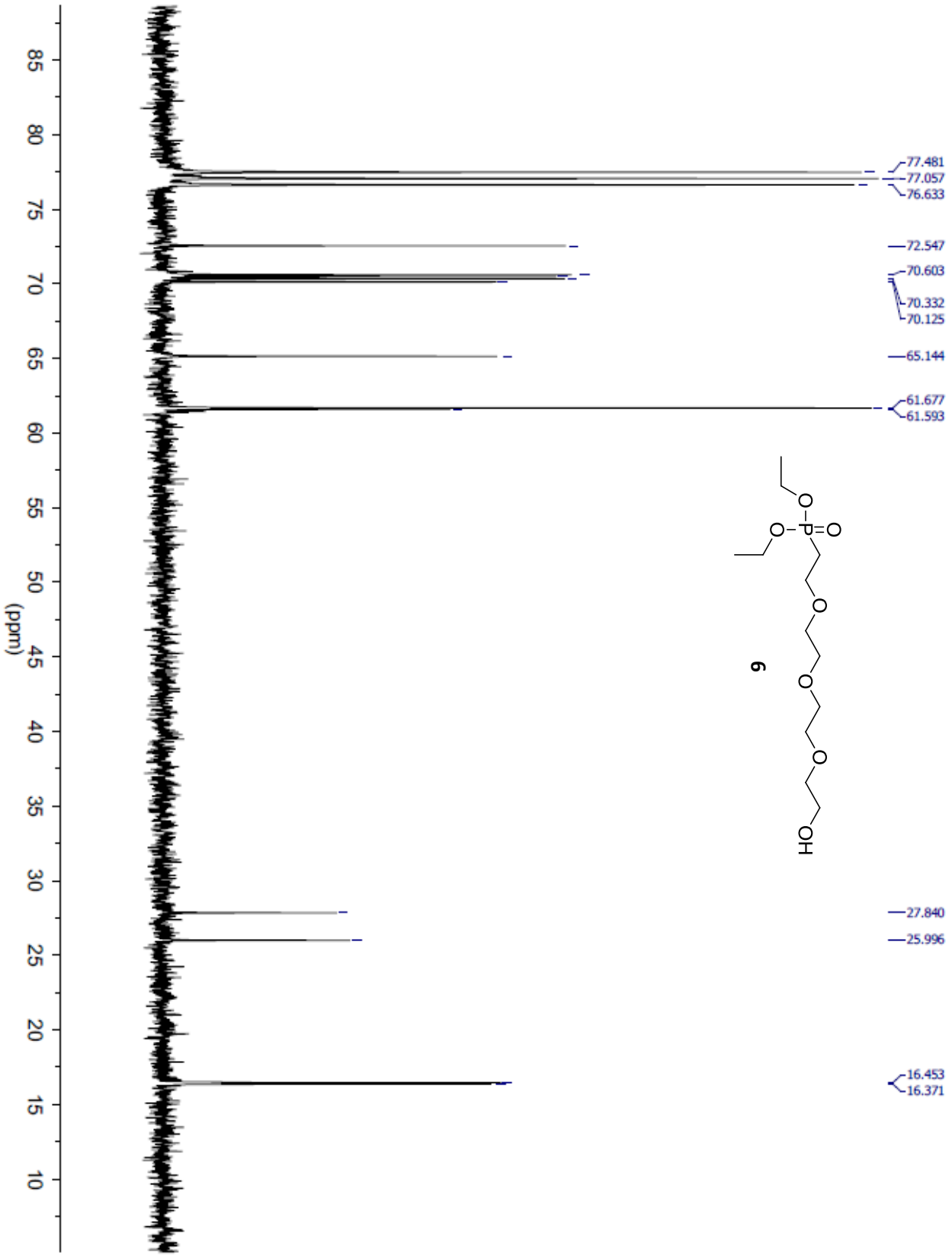
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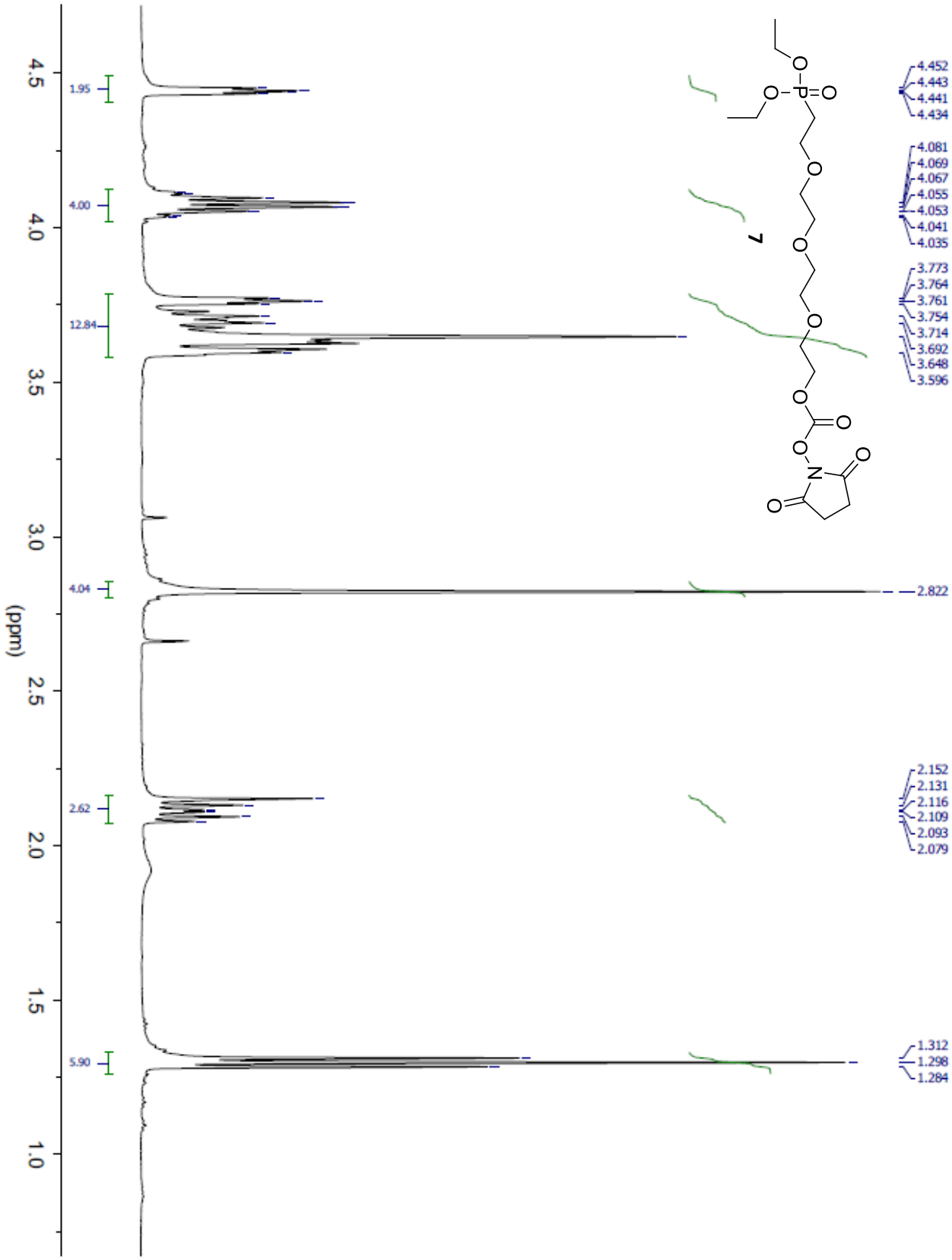
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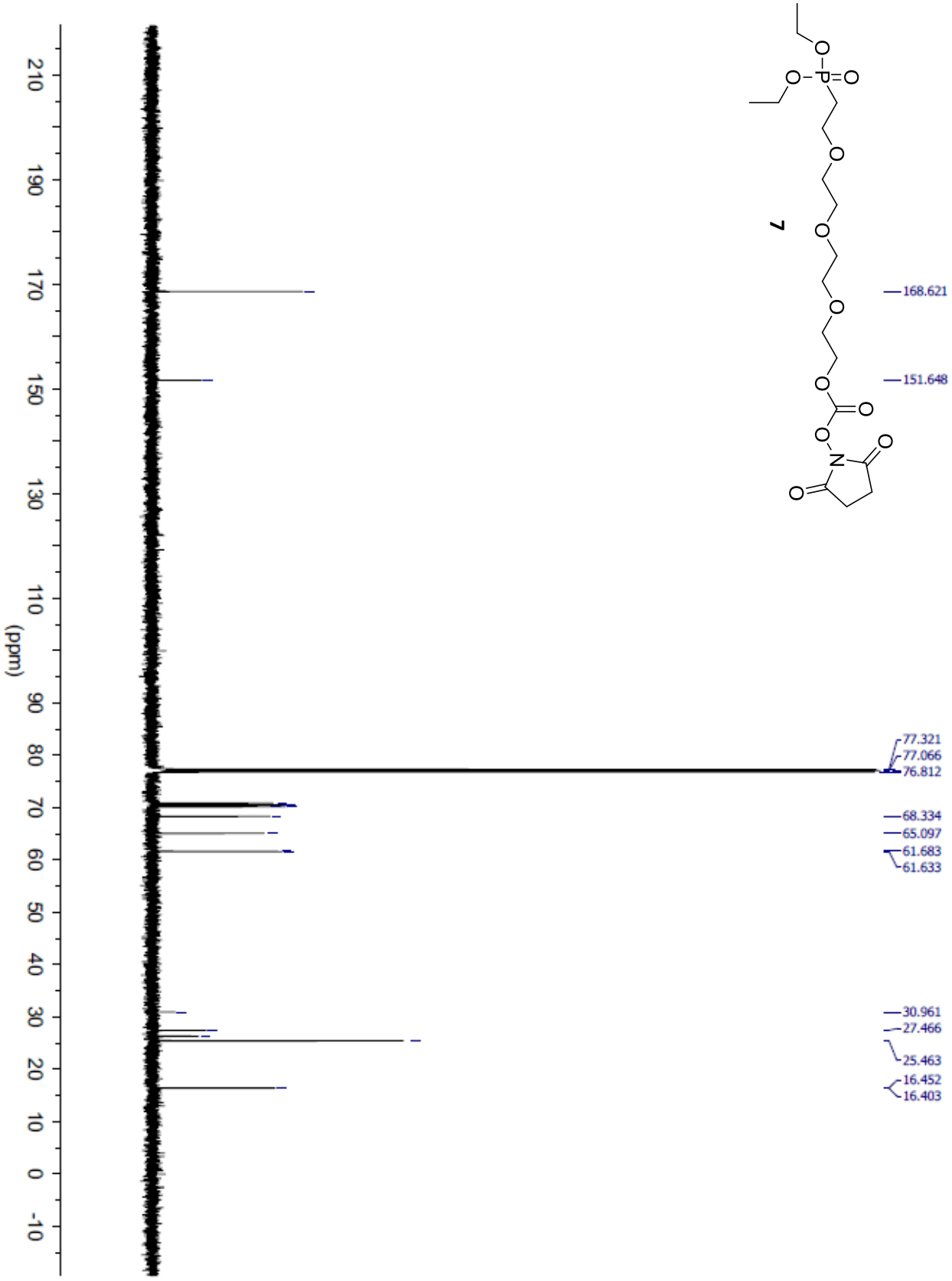
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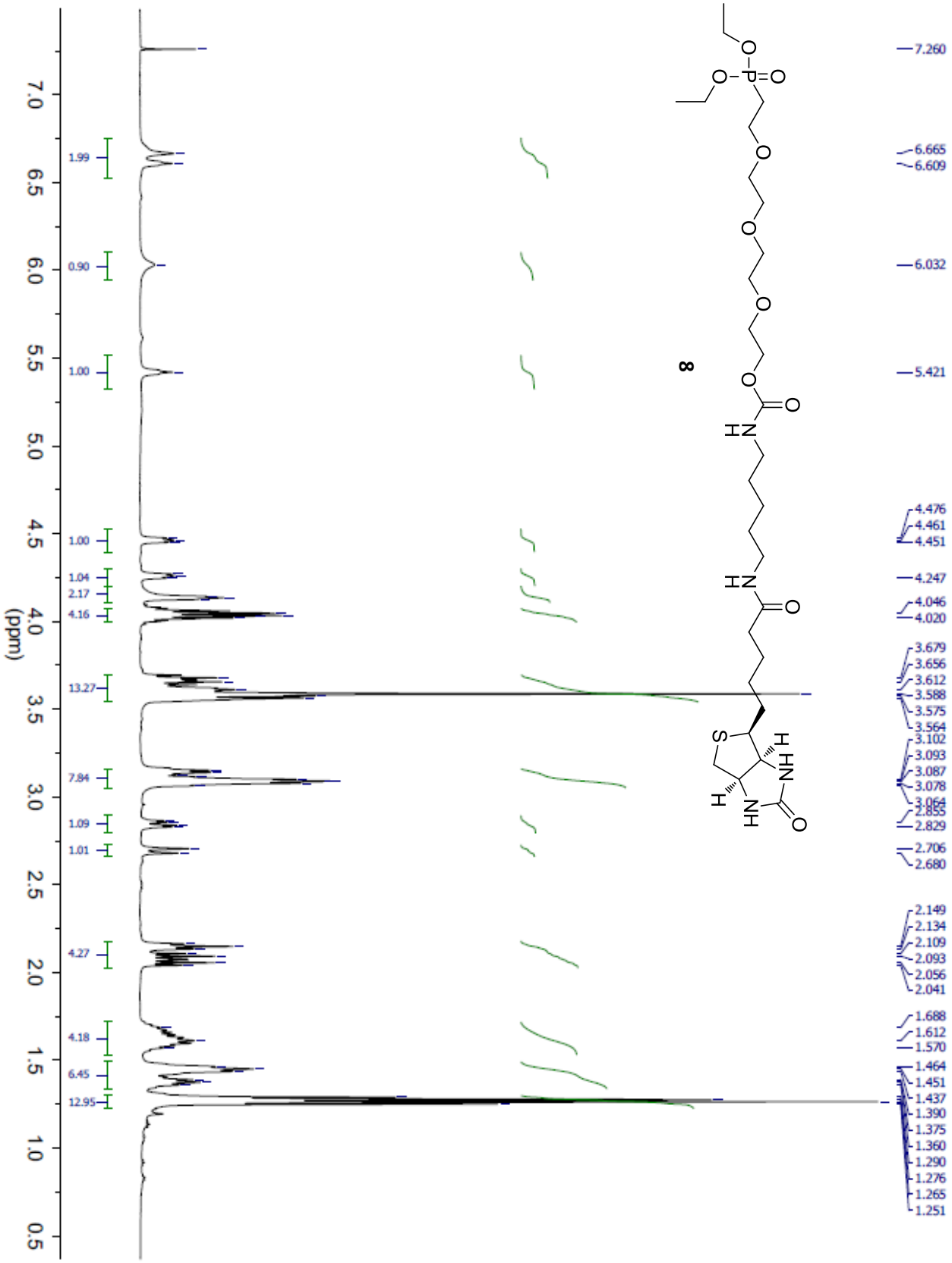
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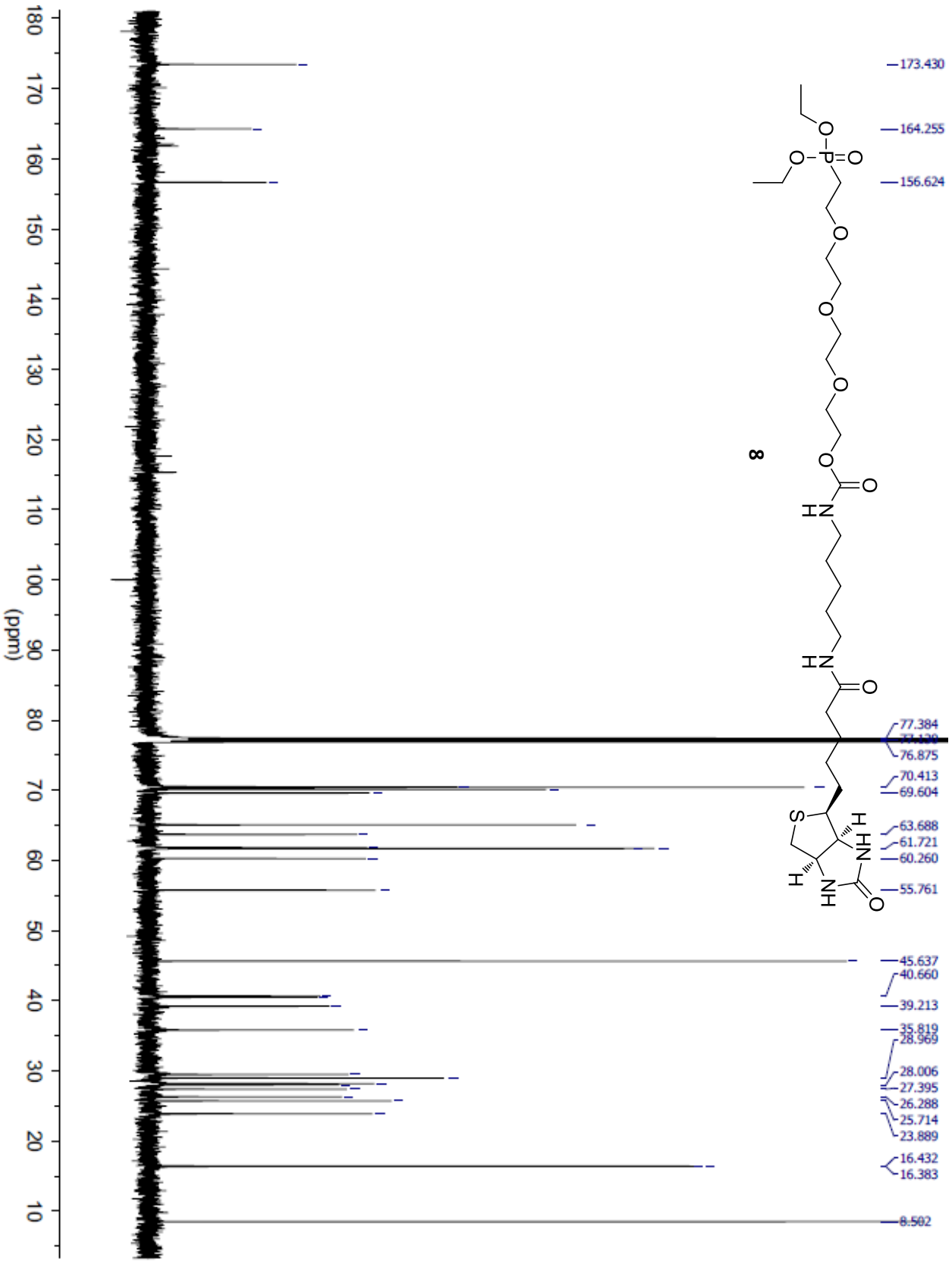
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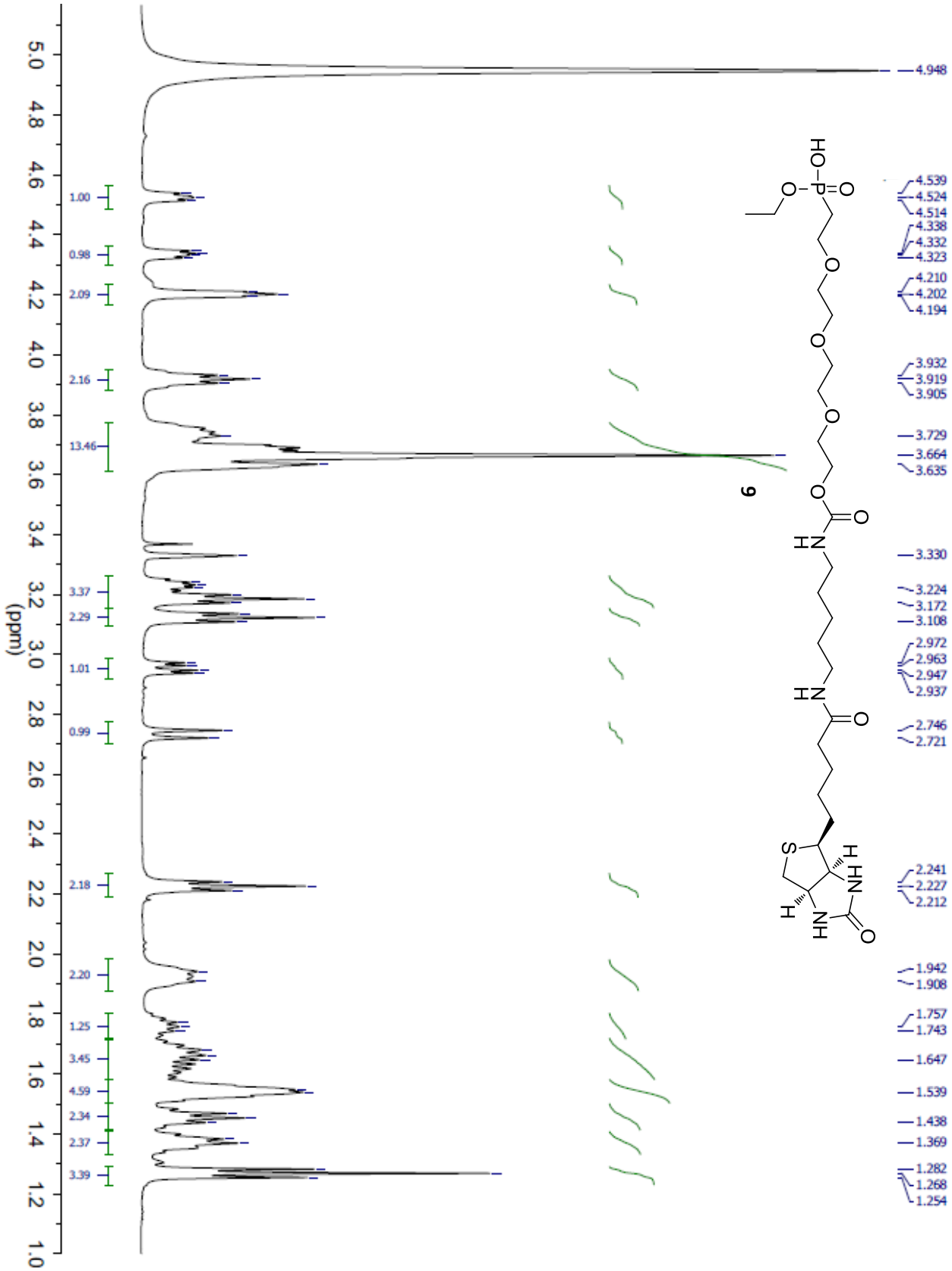
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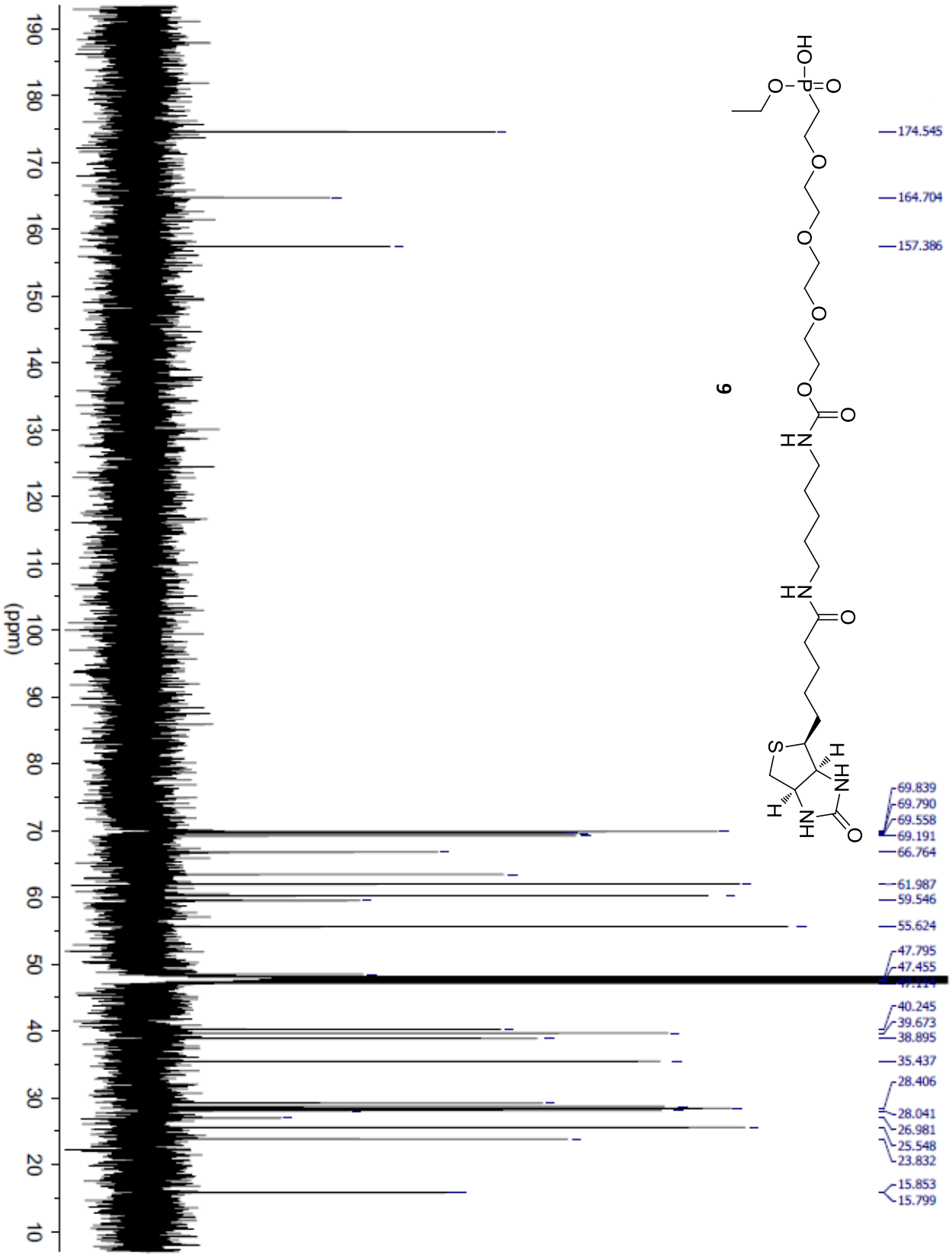
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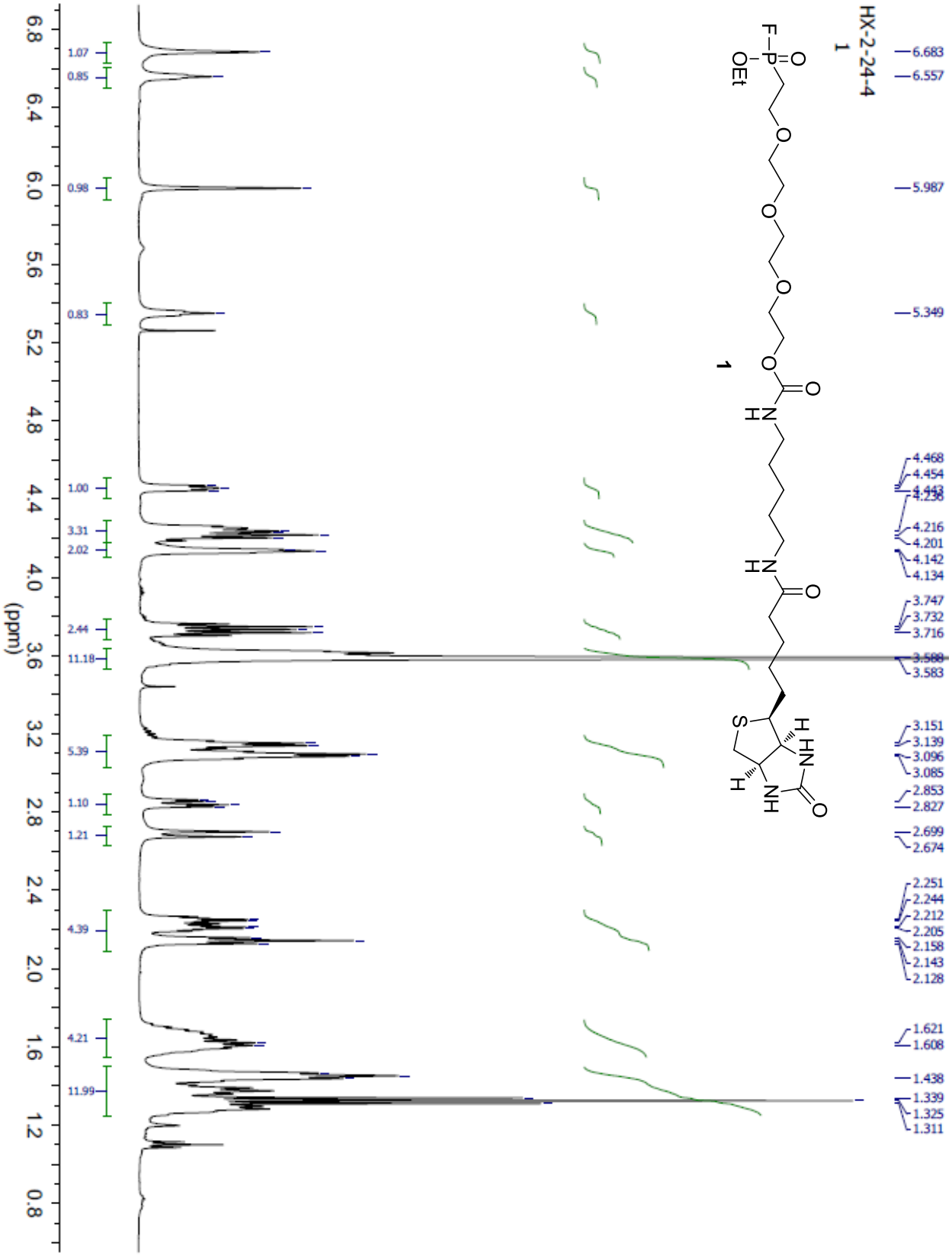
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2.674
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1.339
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0.98
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11.18
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1.10
1.21
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4.21
11.99
(ppm)
6.8
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5.6
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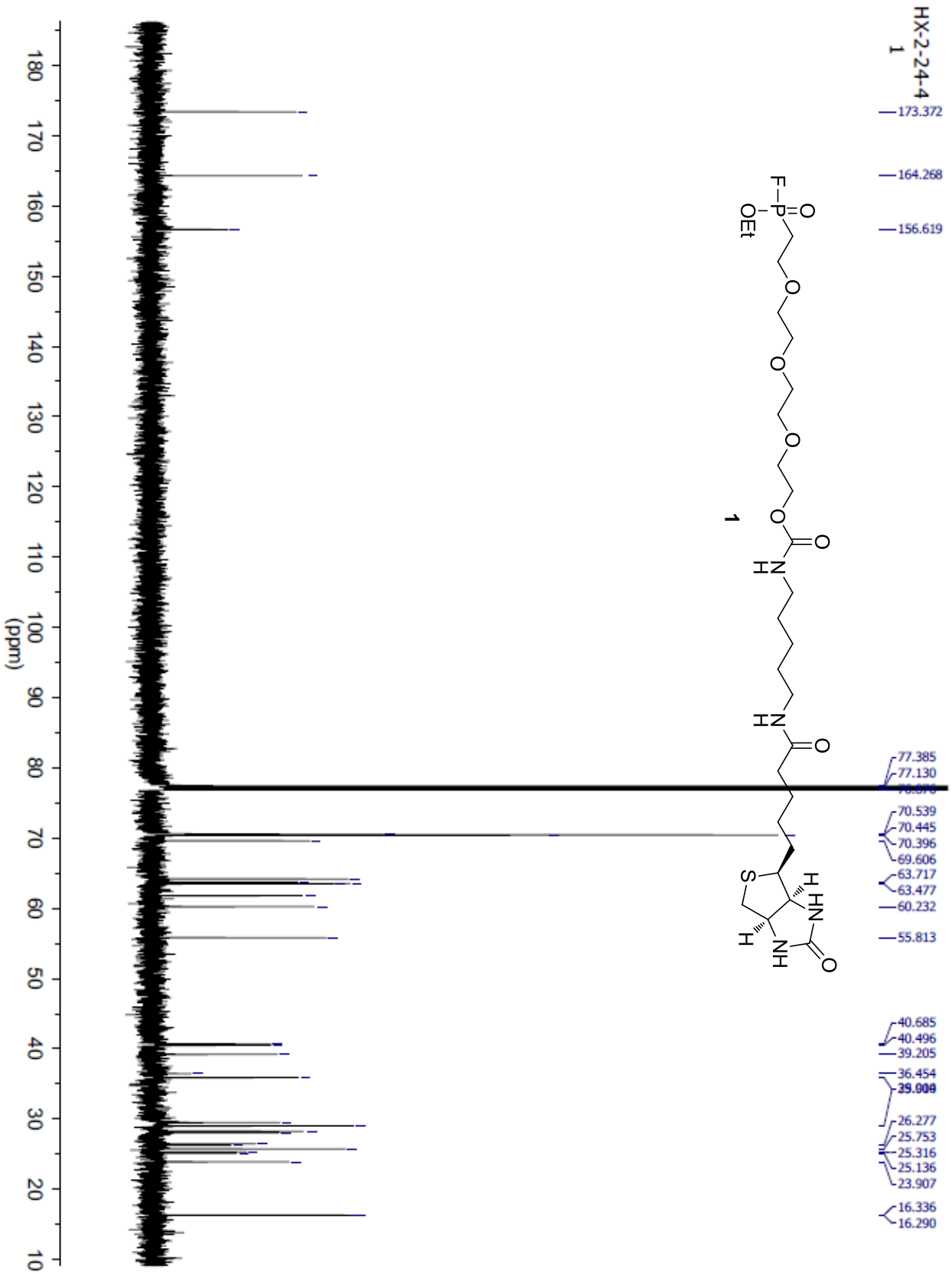
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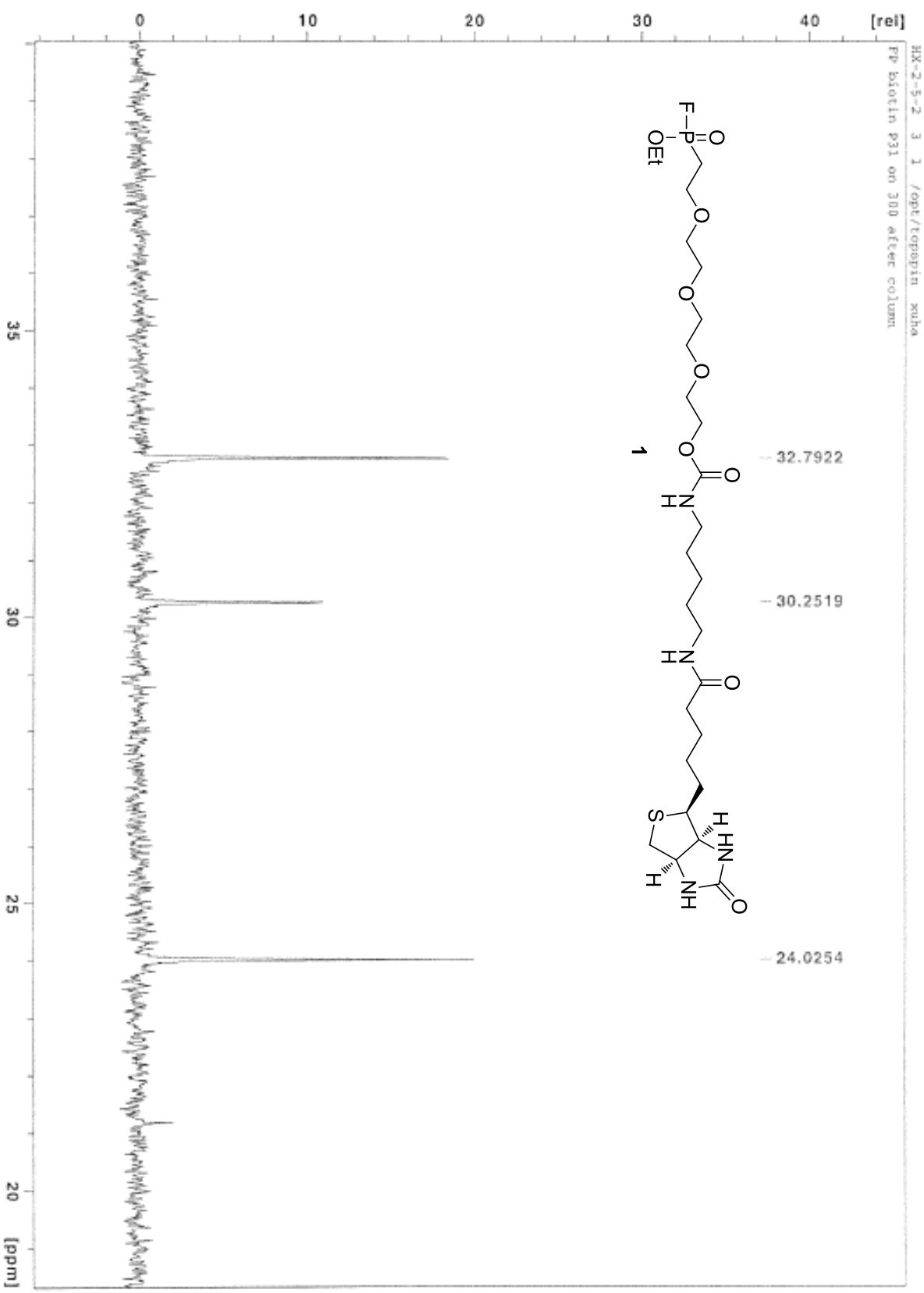
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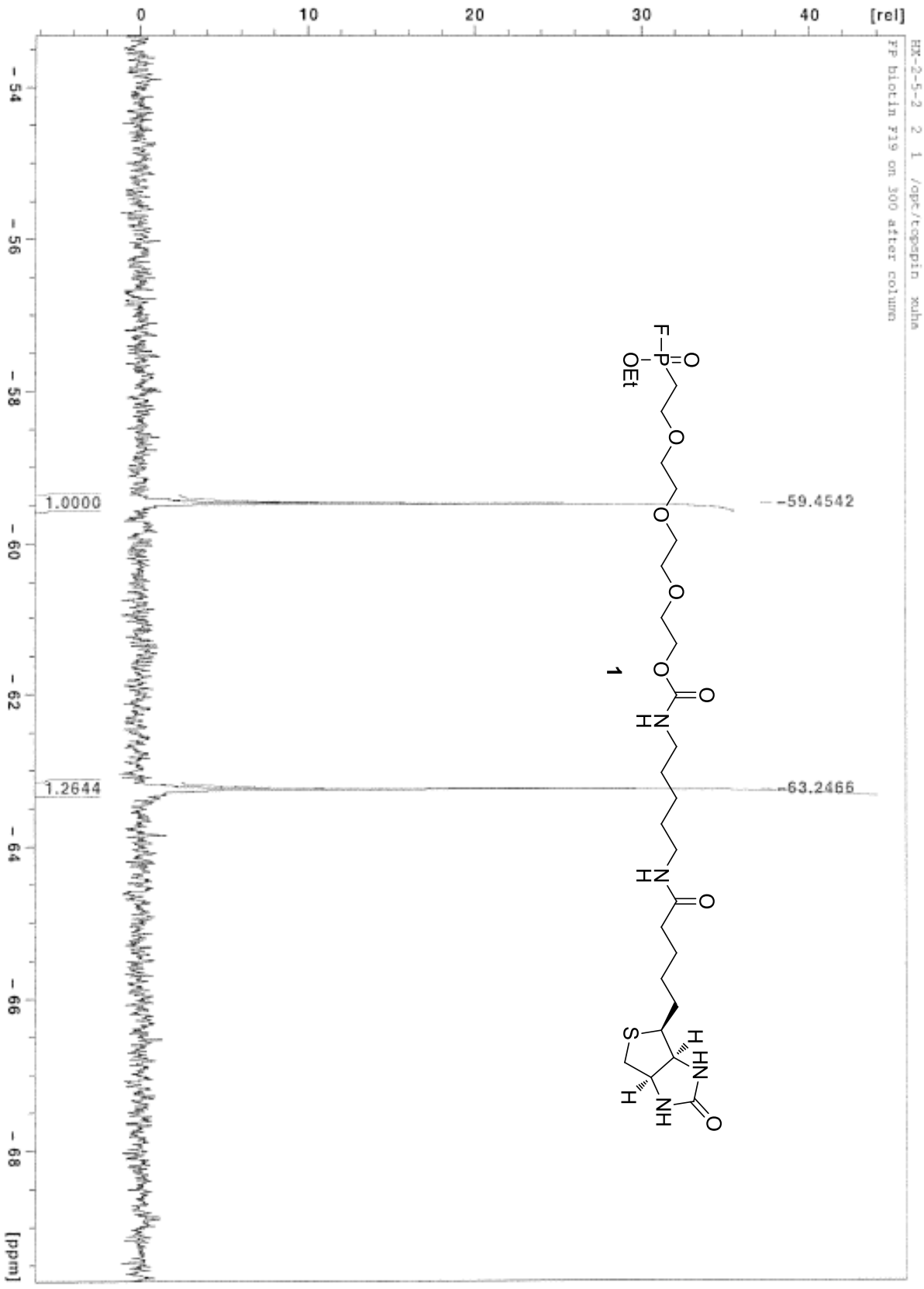
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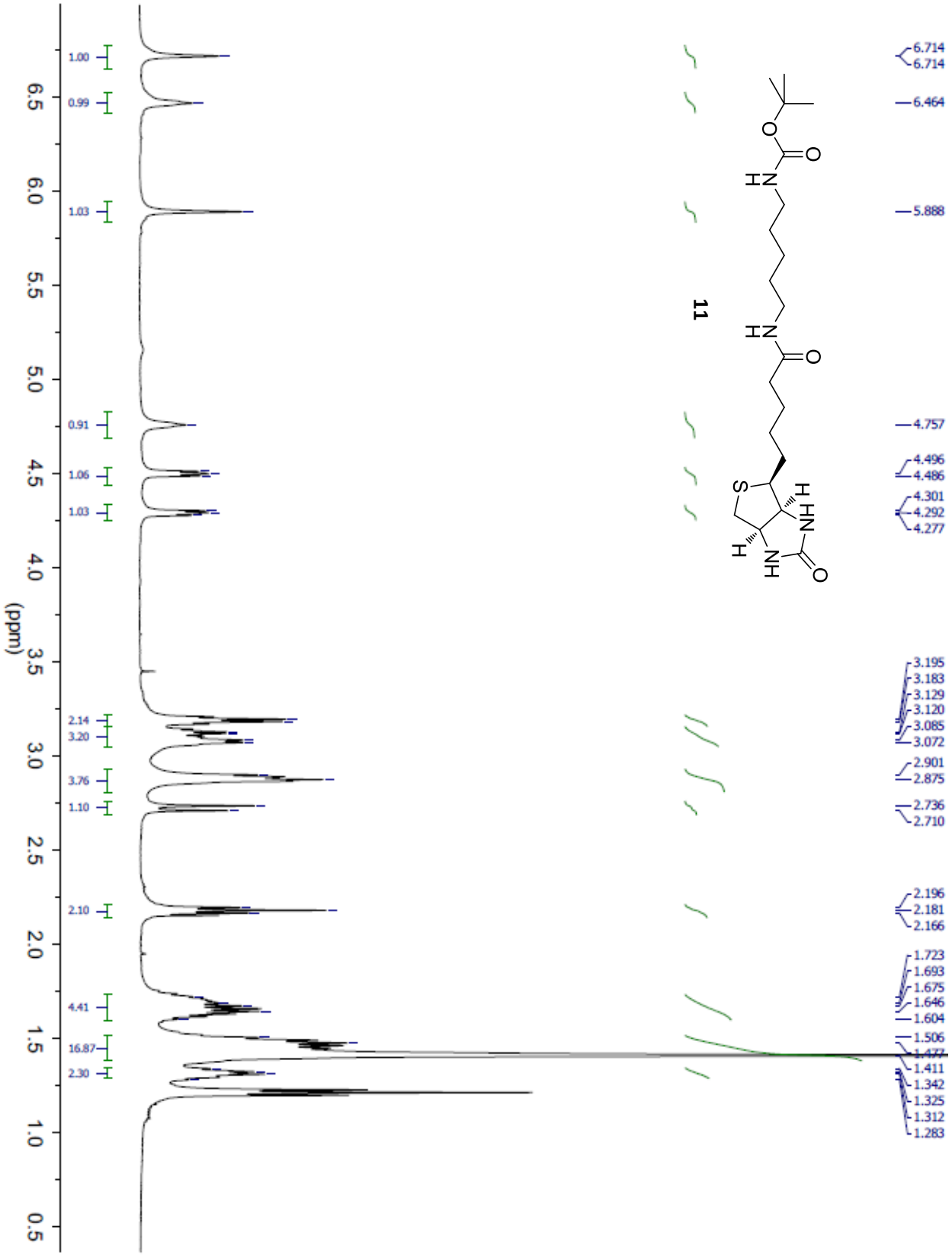
S
H
HX-2-5-2 2 1 /opt/topspin xuha
FP biotin F19 on 300 after column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2.181
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1.604
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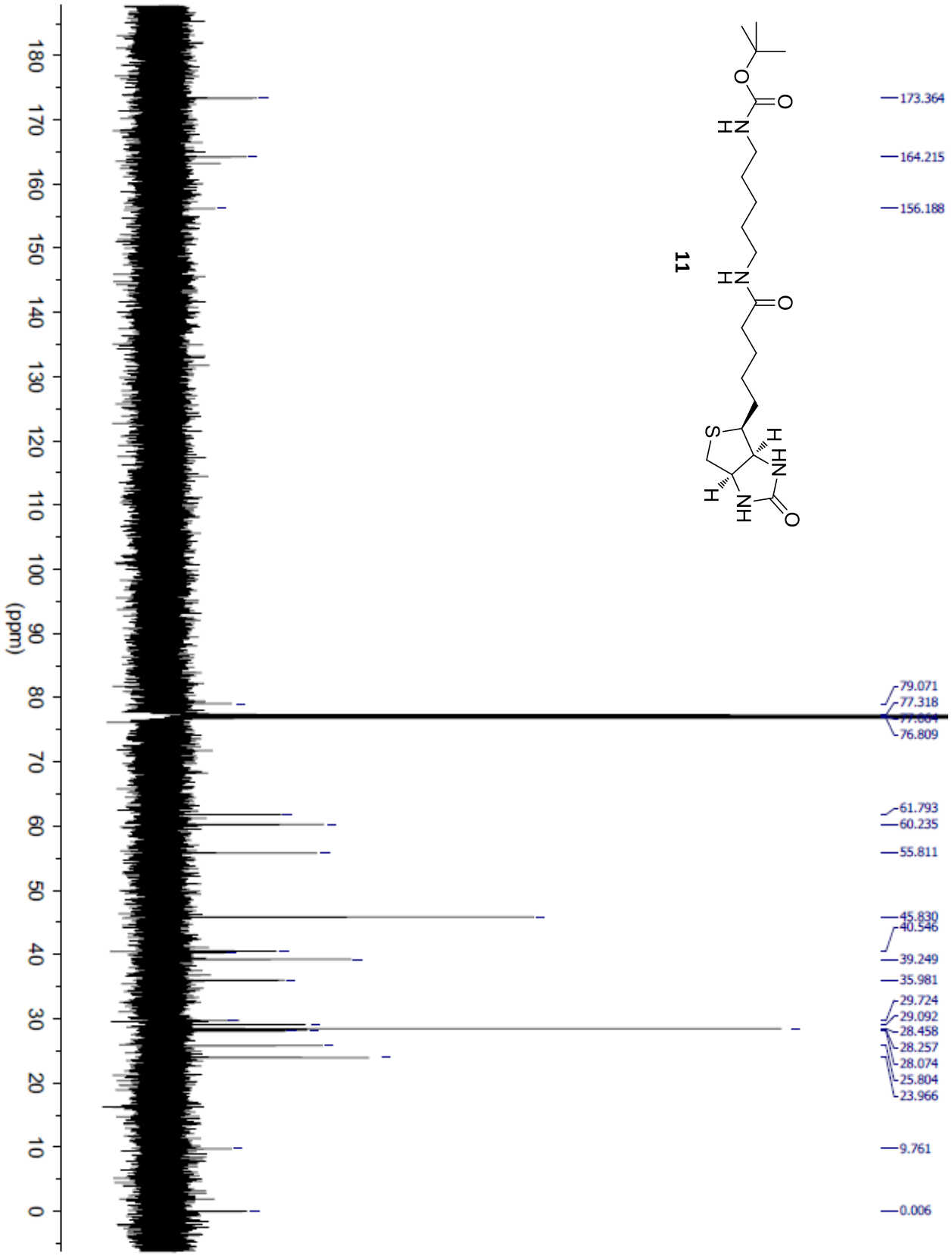
(ppm)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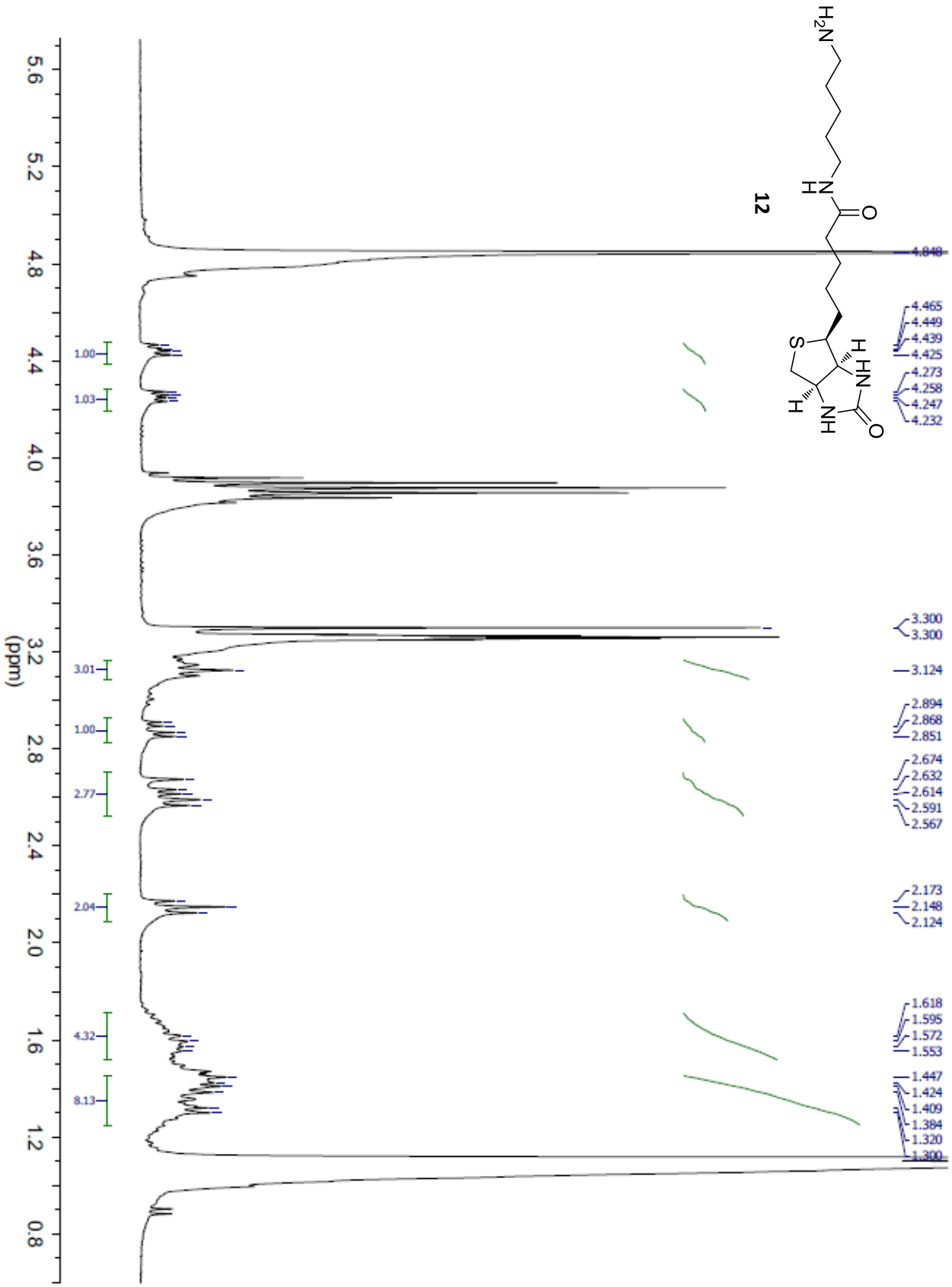
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3.2
2.8
2.4
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1.2
0.8
(ppm)
1.00
1.03
3.01
1.00
2.77
2.04
4.32
8.13
4.848
4.465
4.449
4.439
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2.614
2.591
2.567
2.173
2.148
2.124
1.618
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1.553
1.447
1.424
1.409
1.384
1.320
1.300