Supporting Information

Design, Analysis and Application of Bifunctional Phosphatidylinositol (3,4,5)-Trisphosphate Activity Probes for Labeling and Proteomic Characterization of Protein Binding Partners

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1. Supplementary Data: Color Gel Images

Lane	1	2	3	4	5
Akt-PH (Heat Denatured)	+	+	+	+	+
Fluorescein Probe **1** (25 uM)	+	+	+	-	-
Alkyne Probe **2** (25 uM)	-	-	-	+	+
Latent Click Labeling with Rh-azide **11**	-	-	-	+	+
hv	-	+	+	+	+

Figure S1. Fluorescence gel images of labeling studies using purified Akt-PH (color version of Figure 2 from manuscript). On the gel, protein bands labeled with probe **1** (green) and **2** (red) exhibit different colors since different fluorescent dyes are used for labeling (fluorescein and rhodamine, respectively). Studies indicated successful labeling of Akt-PH protein by both fluorescein-probe **1** (lane 2) and alkyne-probe **2** (lane 4, after click chemistry post-derivatization) during studies. Additionally, control studies involving no photo-cross-linking (lane 1), or heat denaturation of the protein prior to probe incubation (lanes 3 and 5) yielded no fluorescence, indicating the absence of non-specific labeling.

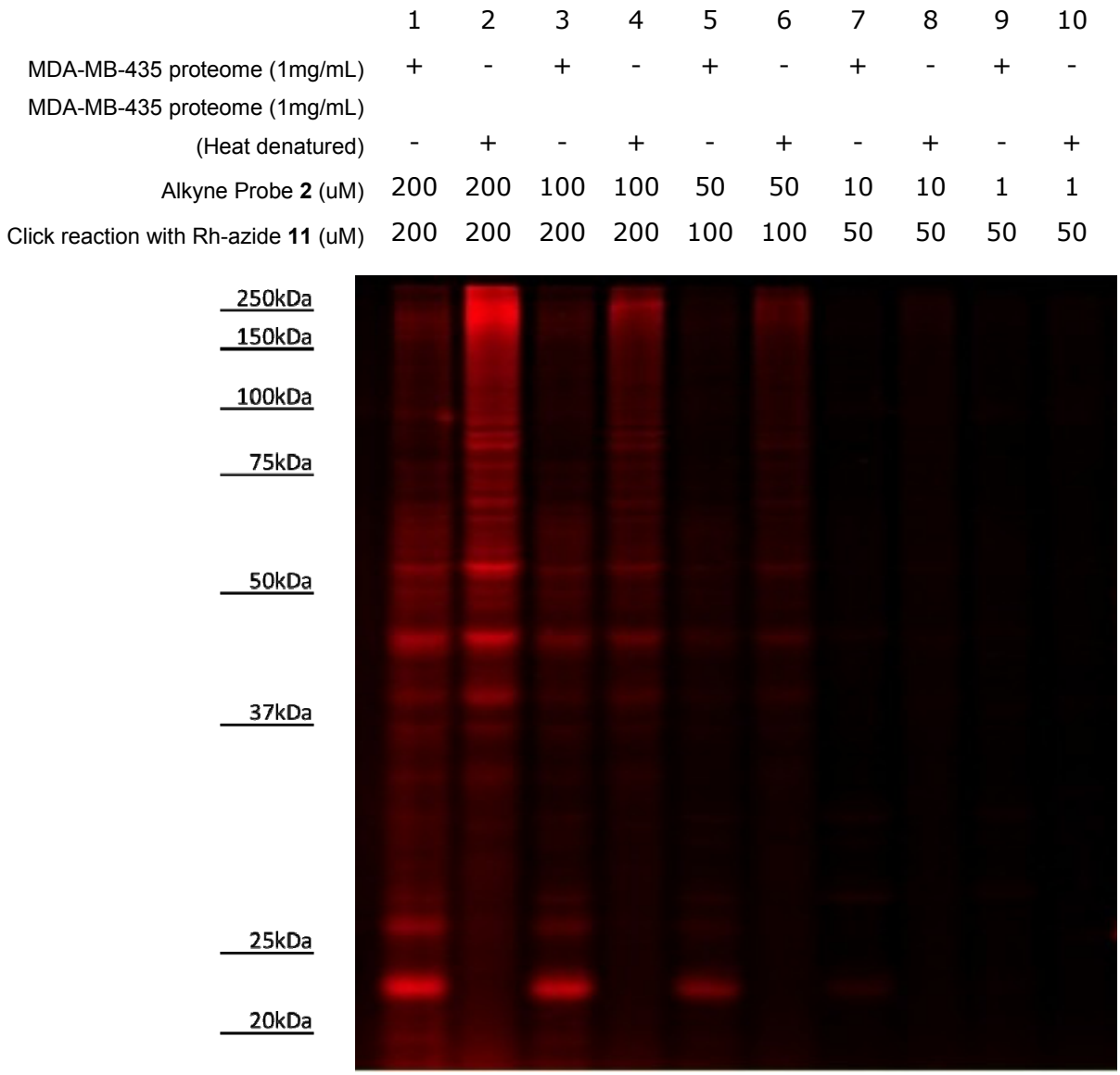


Figure S2. Labeling studies to ascertain effective probe concentrations (color version of Figure 6 from manuscript). Probe concentrations were screened using MDA-MB-435 cancer cell extracts (soluble fraction) to identify concentrations that were effective for studies. Concentrations ranging from 200 nM to 1nM were used to label the same amount of the cell extracts with heat-denatured controls performed for each protein concentration. From fluorescence gel imaging results, probe concentrations of approximately 50 μM (lanes 5 and 6) were selected for other labeling studies.

	1	2	3	4	5	6	7	8	9
MDA-MB-435 proteome (1mg/mL)	+	-	+	+	-	+	+	+	+
MDA-MB-435 proteome (1mg/mL) (Heat denatured)	-	+	-	-	+	-	-	-	-
Alkyne Probe **2** (25 uM)	-	-	-	+	+	-	+	+	+
Fluorescein Probe **1** (25 uM)	+	+	+	-	-	-	-	-	-
Click rxn with Rh-azide (50uM)	-	-	-	+	+	+	+	+	+
hv	+	+	-	+	+	+	+	-	+
ligand TBTA	+	+	+	+	+	+	+	+	-
ligand THPTA	-	-	-	-	-	-	-	-	+

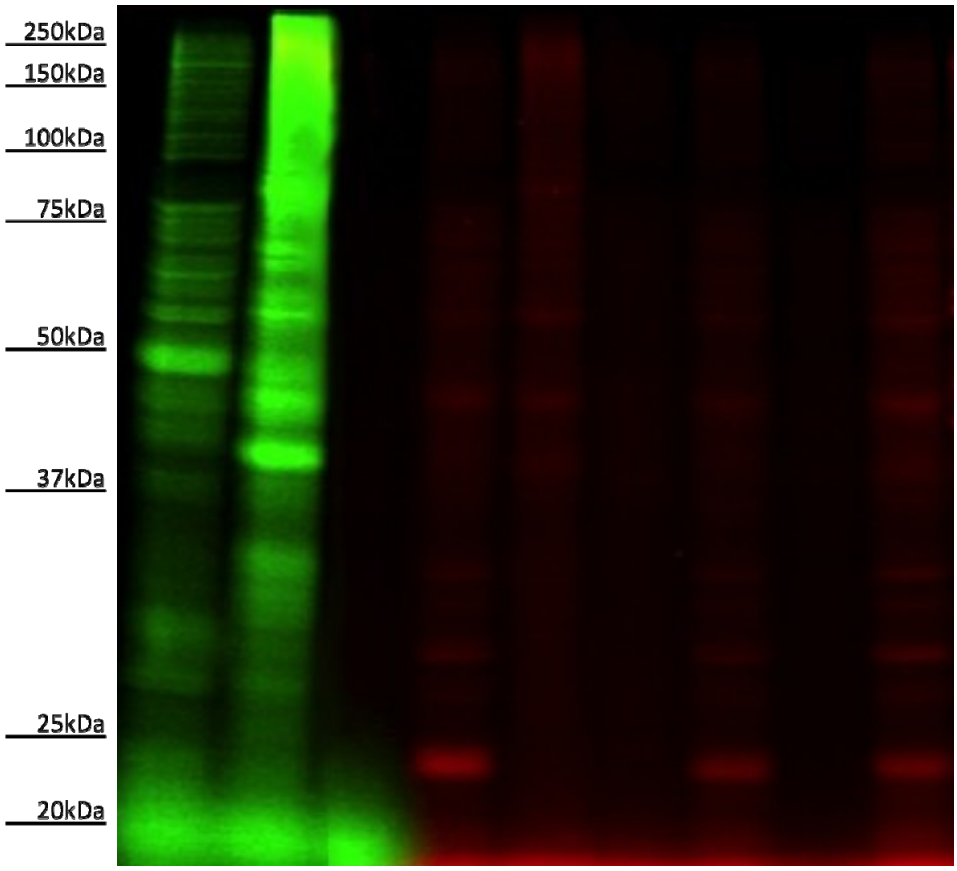


Figure S3. Labeling of MDA-MB-435 soluble proteome using various conditions (color version of Figure 7 from manuscript). Studies using fluorescein-probe **1** resulted in significantly stronger labeling in the heat denatured control (lane 2) compared to the normal study (lane 1), indicating that the presence of the fluorophore during labeling is problematic. Studies using probe **2** and post-labeling led to diminished labeling in heat-the denatured control (lane 5, when compared to lane 4). No probe (lane 6) and no light (lane 8) controls negated labeling, and click chemistry ligands TBTA (lane 7) and THPTA (lane 9) yielded similar results.

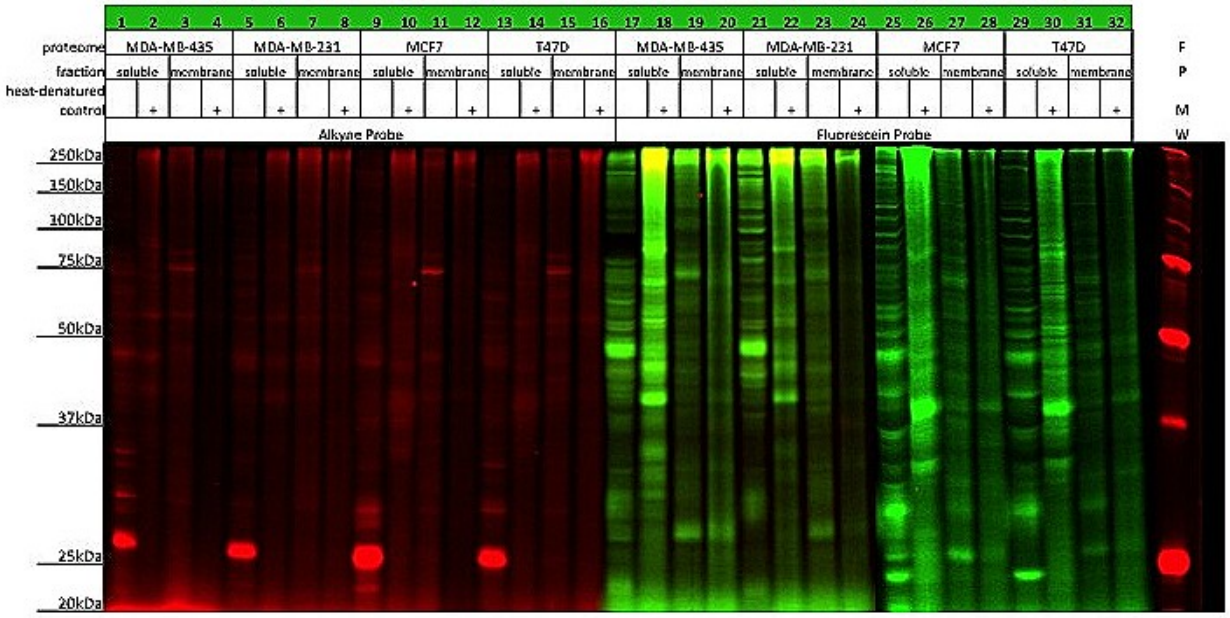
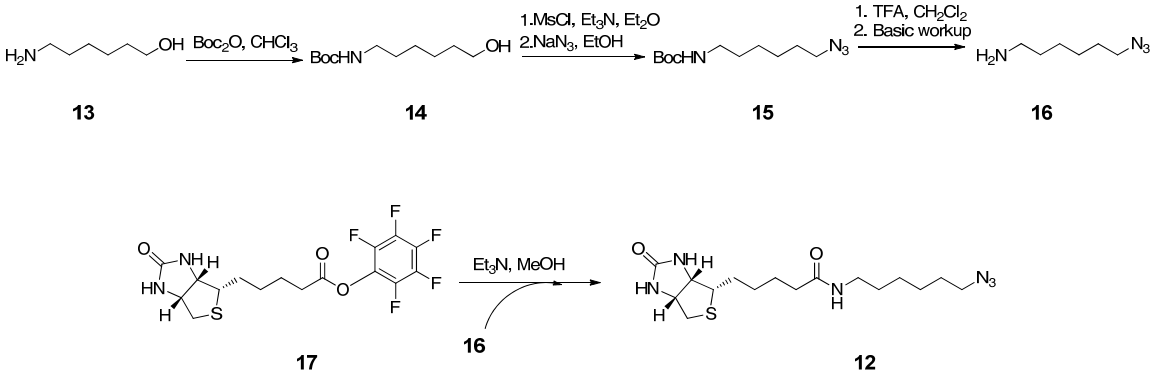


Figure S4. Labeling studies employing multiple cancer cell proteomes (fluorescent gel image). Cell lines MDA-MB-435, MDA-MB-231, MCF7 and T47D were each analyzed using both alkyne-probe **2** (lanes 1-16) and fluorescein-probe **1** (lanes 17-32) with soluble and membrane preparations. Probe **1** again yielded stronger labeling in heat denatured controls, proving problematic. Probe **2** yielded better results with less proteins labeled in heat denatured controls. Enhanced labeling was seen in soluble fractions.

2. Supplementary Procdures: Synthesis of Azido-biotin reagent 12

Azido-biotin **12** was synthesized as described in Scheme S1. The amino group of 6-Aminohexanol (**13**) was first protected using di-*tert*-butyl dicarbonate (Boc_2O) to produced Boc-protected product **14**.(Mattingly, 1990) Next, the hydroxyl group of **14** was converted into a mesylate group,(Mattingly, 1990) which was subsequently substituted with azide to generate **15**. Deprotection of **15** was then followed by a basic extraction to yield **16**, which was coupled to biotin-pentafluorophenyl ester **17**(Kessler, et al., 2009) to form azido-biotin **12**.(Inverarity, et al., 2007)



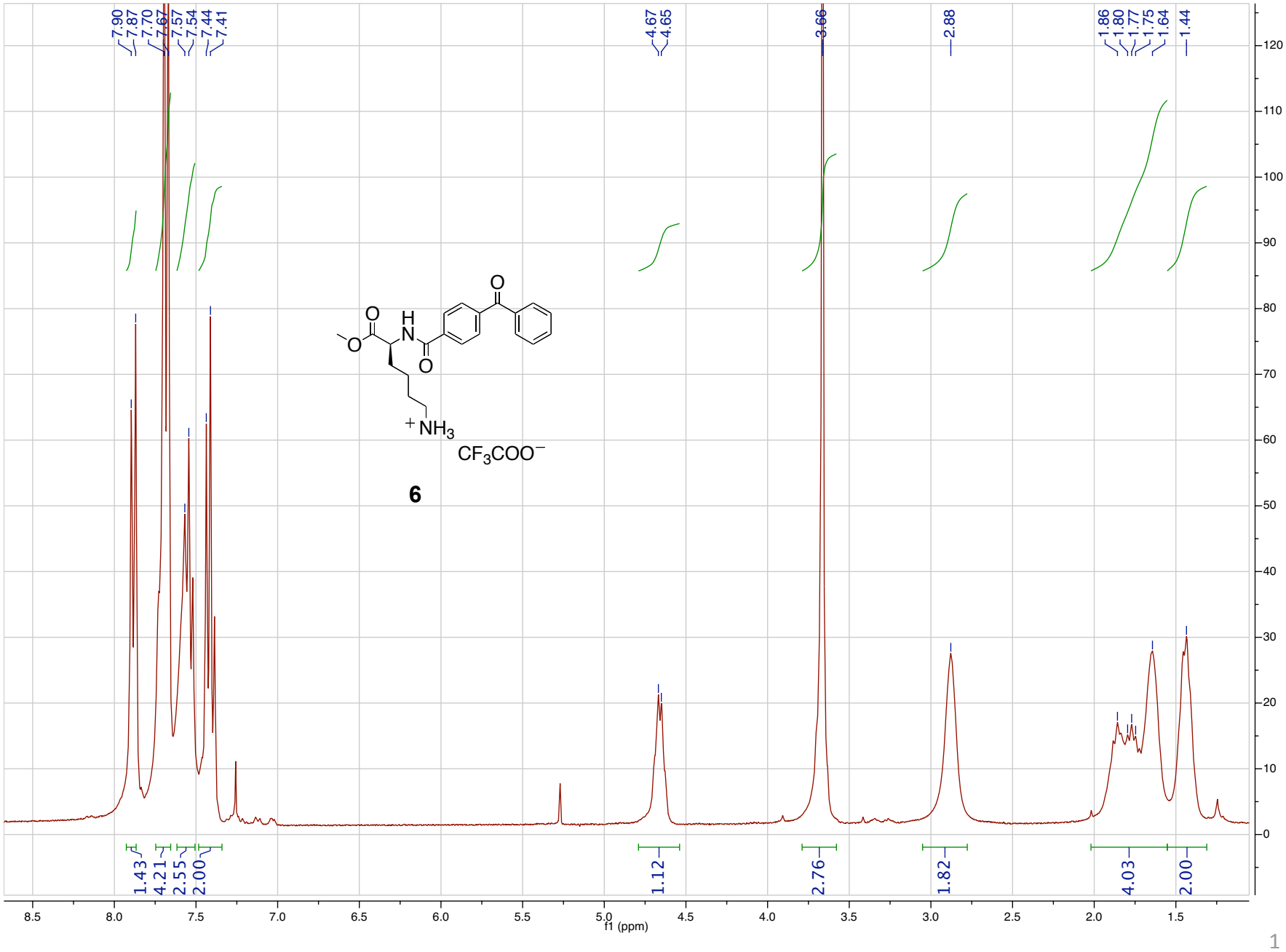
Scheme S1. Synthesis of azido-biotin 12

6-(*N*-*tert*-Butoxycarbonylamino)-1-hexanol (14). 6-Aminohexanol (**13**, 0.80 g, 6.83 mmol) was dissolved in 30 mL of chloroform at 0°C. To this stirred solution was added dropwise a solution of di-*tert*-butyl dicarbonate (1.49 g, 6.83 mmol) in 5 mL of chloroform. The reaction was then stirred at rt overnight. Next, the solvent was removed under reduced pressure and the residue was purified by column chromatography with elution of 50% ethyl acetate/hexanes to yield **14** as a colorless oil (1.484 g, 100%). Characterization data matched those that were previously reported.(Mattingly, 1990)
^{1}H NMR (300MHz, $CDCl_3$) δ 4.51 (bs, 1H), 3.64 (q, *J* = 6 Hz, 2H), 3.12 (q, *J* = 6 Hz, 2H), 1.58-1.36 (m, 17H).

1-Azido-6-(*tert*-butoxycarbonylamino)hexane (15). To a stirred solution of compound **14** (1.484 g, 6.83 mmol) in 20 mL of dry ethyl ether was added triethylamine (2 mL) at 0°C under nitrogen. Next, methanesulfonyl chloride (0.635 mL, 8.20 mmol) was added dropwise over a period of 1 h, and the solution was then concentrated. The resulting residue was next combined with sodium azide (1.33 g, 20.5 mmol) and dissolved in 20 mL of ethanol. The reaction was stirred at reflux for 24 h, followed by removal of the solvent under reduced pressure. The resulting crude was extracted from water (100 mL) with dichloromethane (2 x 80 mL), and the organic layers were combined, dried with magnesium sulfate, filtered and then concentrated. The crude product was purified by column chromatography with 50% ethyl acetate/hexanes to generate compound **15** as a colorless oil (1.610 g, 97%). Characterization data matched those that were previously reported.(Mattingly, 1990)
^{1}H NMR (300MHz, $CDCl_3$) δ 4.60 (bs, 1H), 3.26 (t, *J* = 6 Hz, 2H), 3.11 (q, *J* = 6 Hz, 2H), 1.60-1.34 (m, 17H).

6-azidohexan-1-amine (16). Compound **15** (1.610 g, 6.64 mmol) was combined with trifluoroacetic acid (3 mL) in 10 mL of dichloromethane. The solution was stirred at rt for 2 h followed by extraction from 1 M sodium hydroxide aqueous solution (60 mL) with dichloromethane (2 x 60 mL). The organic layers were then combined, dried with magnesium sulfate, filtered and concentrated to yield compound **16** as a yellowish oil (0.860 g, 91 %). Characterization data matched those that were previously reported.(Inverarity, et al., 2007)
^{1}H NMR (300MHz, $CDCl_3$) δ 3.27 (t, *J* = 6 Hz, 2H), 2.69 (t, *J* = 6 Hz, 2H), 1.64-1.59 (m, 2H), 1.48-1.35 (m, 6H).

***N*-Biotinyl-6-azidohexanamide (12).** Biotin pentafluorophenyl ester **17** (750 mg, 1.83 mmol, prepared using a literature procedure(Kessler, et al., 2009)) and **16** (390 mg, 2.74 mmol) were combined in 10 mL of methanol, to which was added triethylamine (1.27 mL, 9.15 mmol). The solution was stirred at rt overnight, and the solvent was then removed under reduced pressure. The crude was purified by column chromatography with a gradient solvent system of 1-10% methanol/ dichloromethane to yield compound **12** as a light yellowish solid (527 mg, 78%). Characterization data matched those that were previously reported.(Inverarity, et al., 2007)
^{1}H NMR (300MHz, $CDCl_3$) δ 6.19-6.18 (m, 1H), 5.94-5.92 (m, 1H), 5.31-5.30 (m, 1H), 4.54-4.50 (m, 1H), 4.34-4.30 (m, 1H), 3.29-3.15 (m, 3H), 2.92 (dd, J_1 = 12Hz, J_2 = 6 Hz, 1H), 2.74 (d, *J* = 12 Hz, 1H), 2.21 (t, *J* = 6 Hz, 2H), 1.72-1.58 (m, 8H), 1.51-1.36 (m, 8H). MALDI-HRMS $[M + Na]^+$ calcd for 391.1887; found 391.1825.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1.64
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O
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N
O
O
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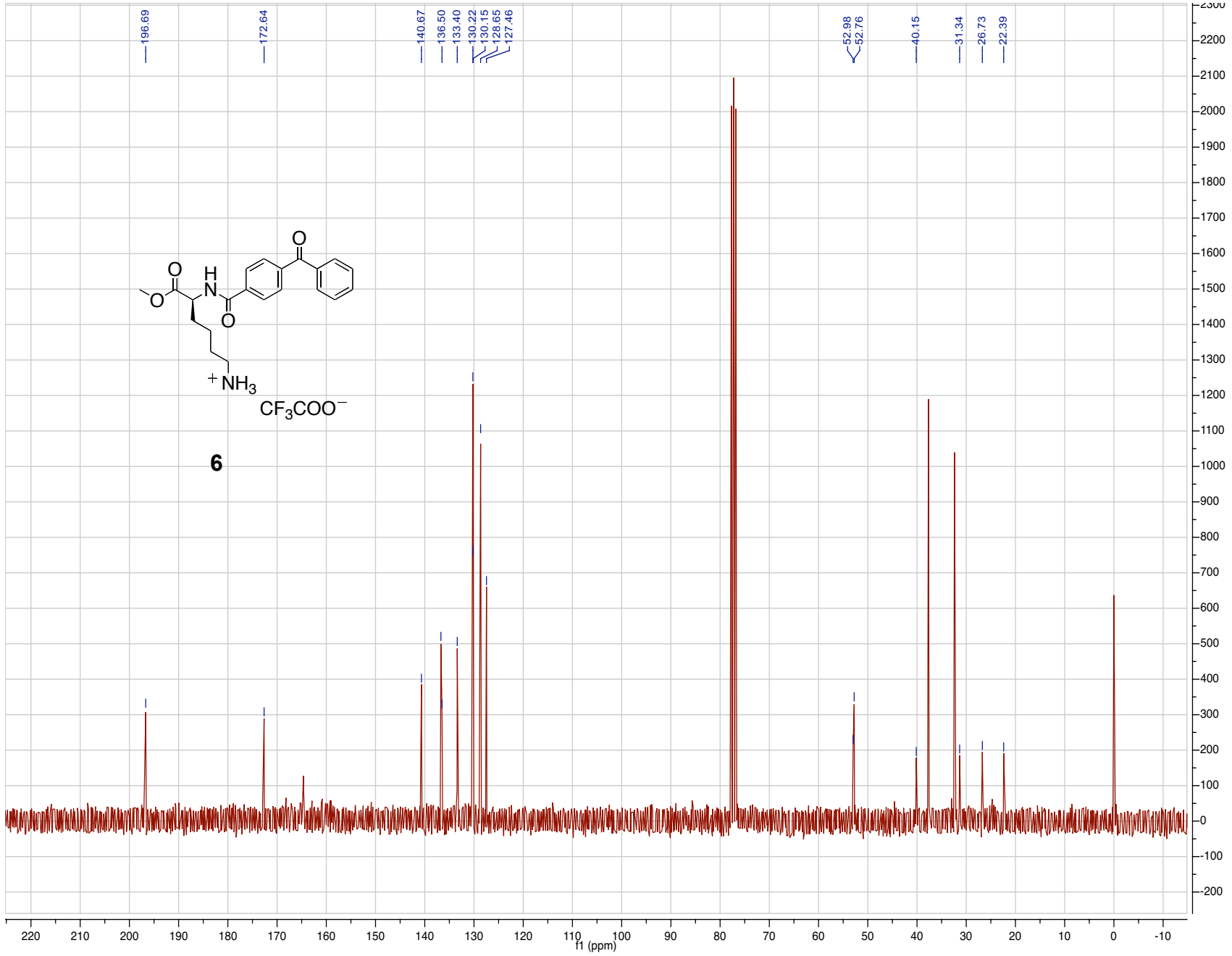
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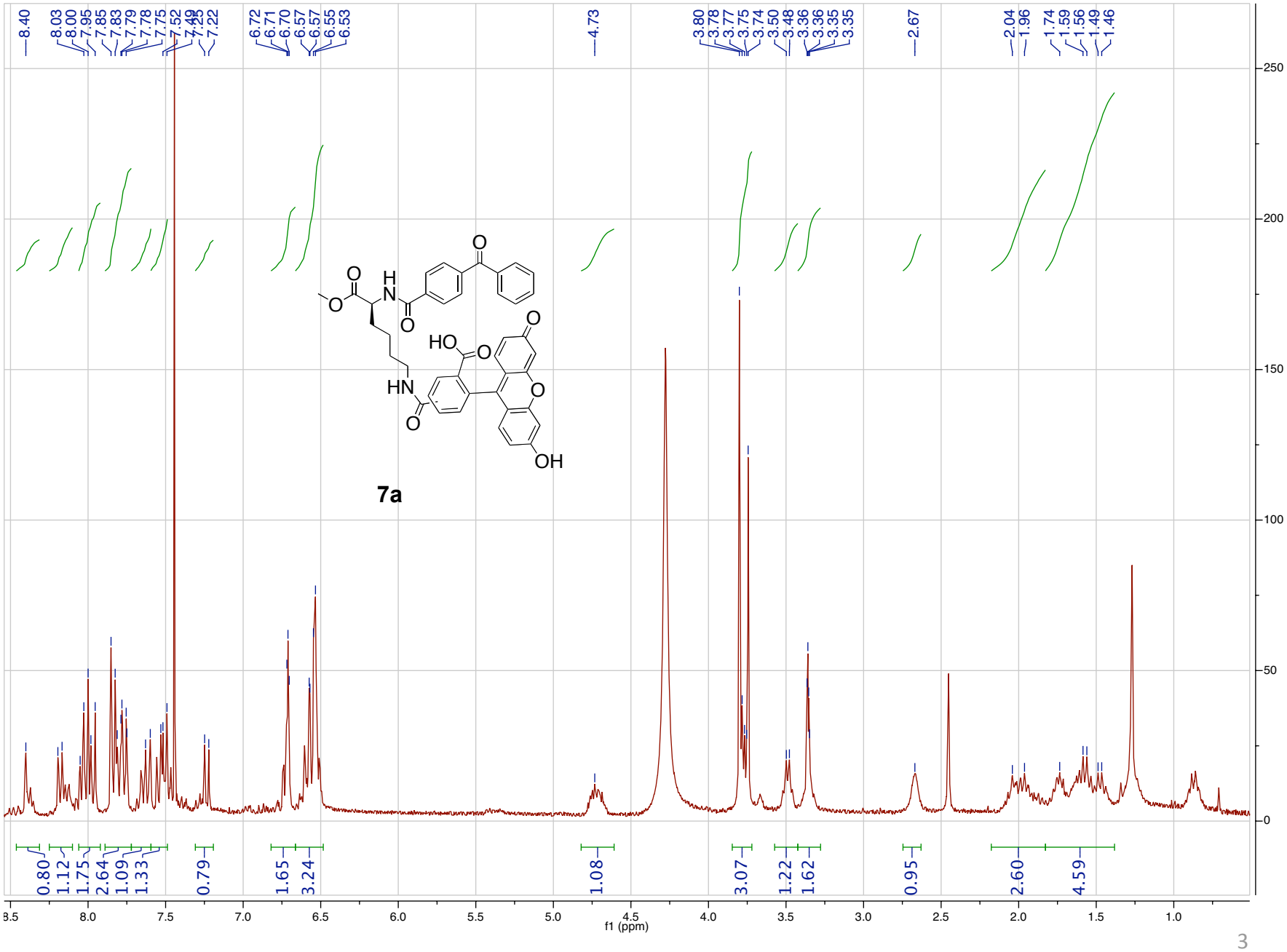
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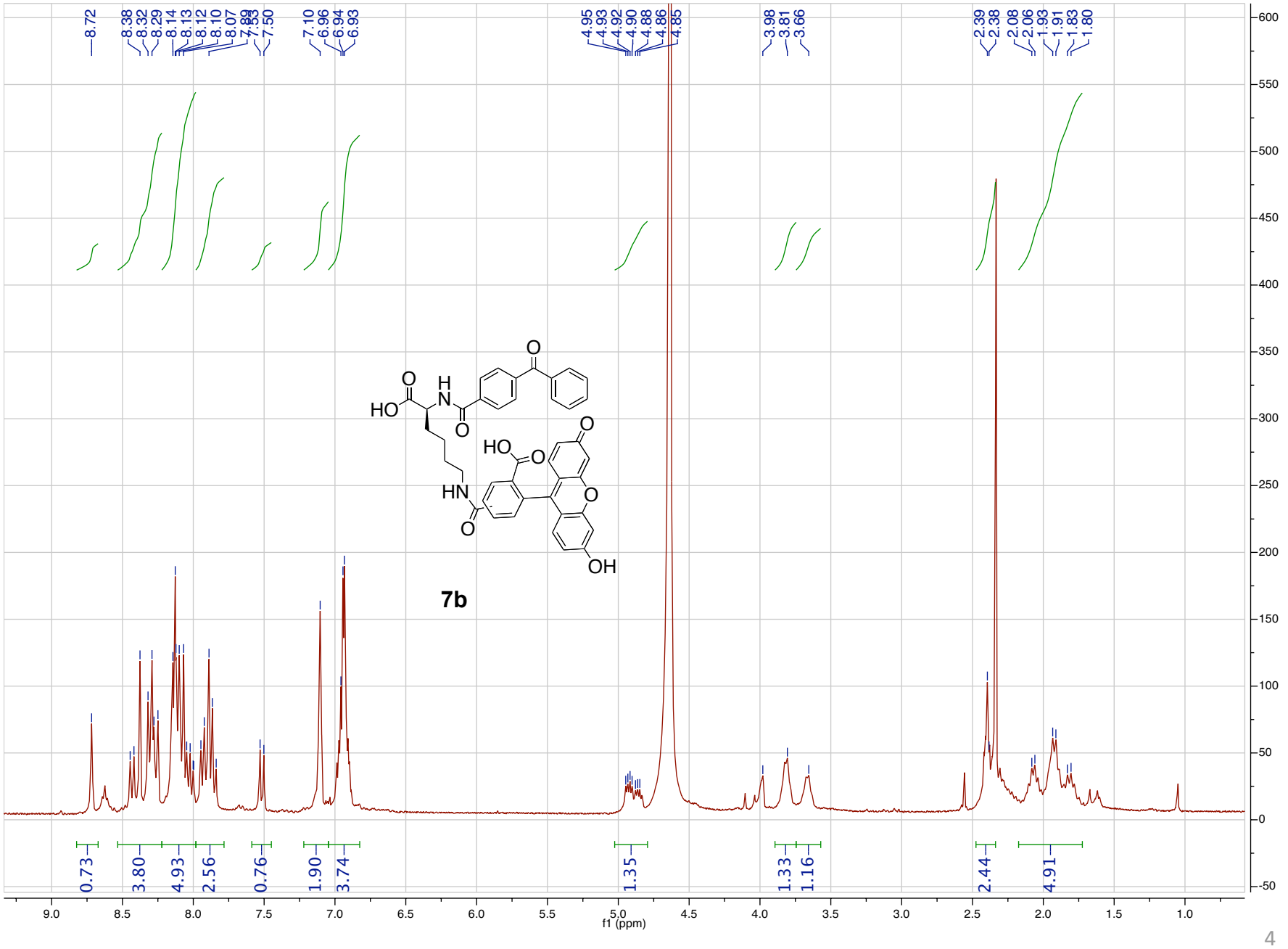
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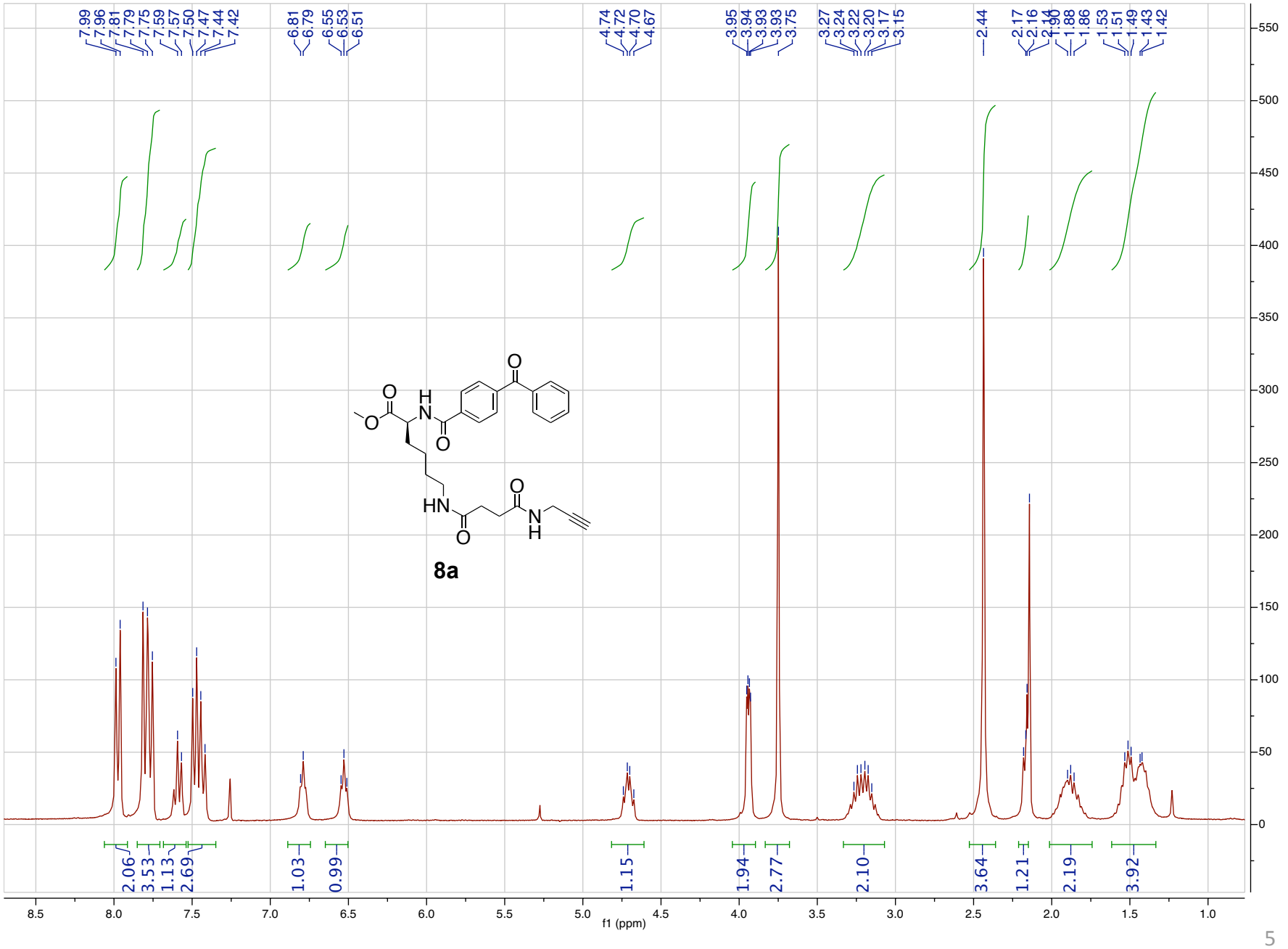
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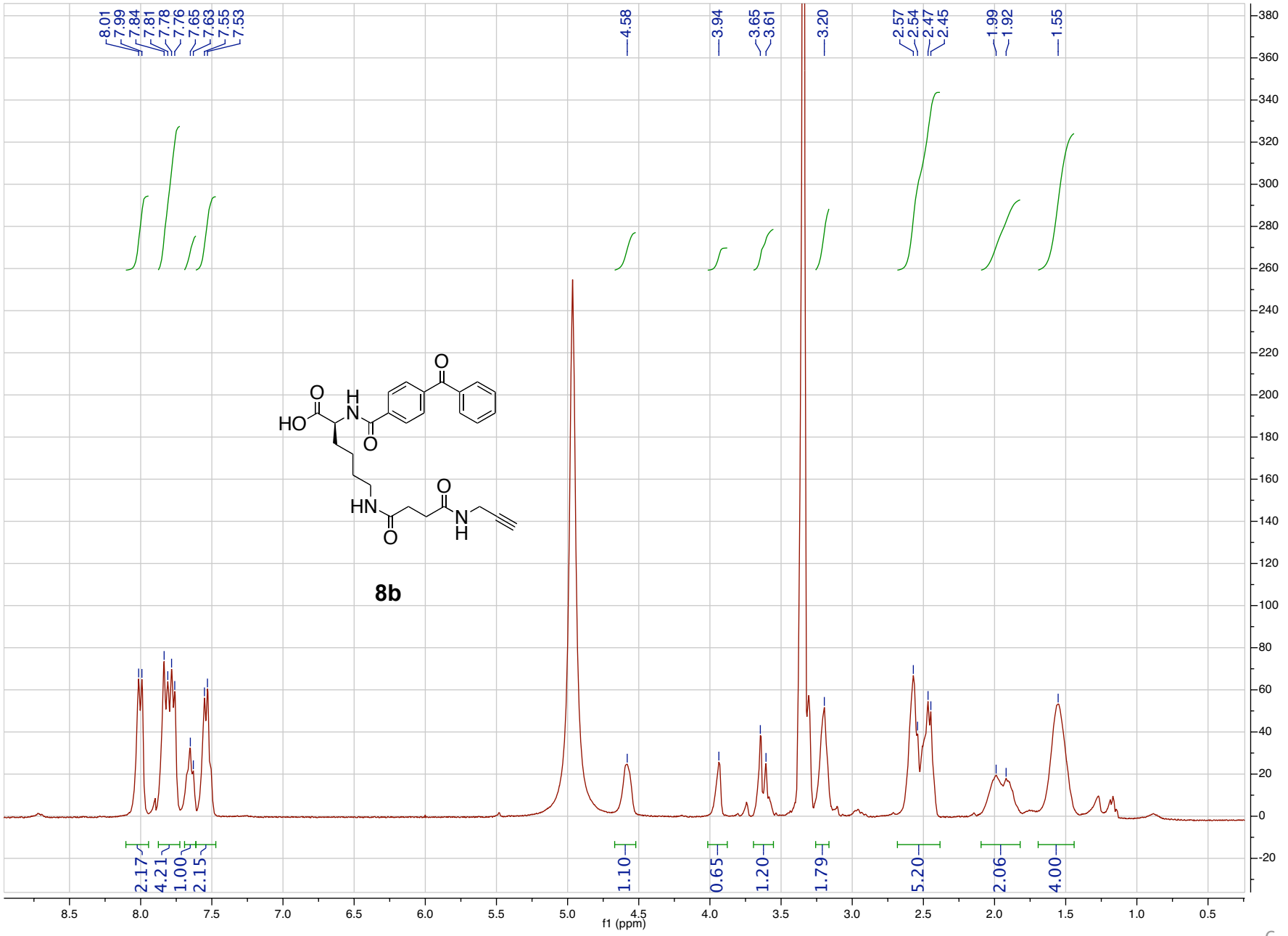
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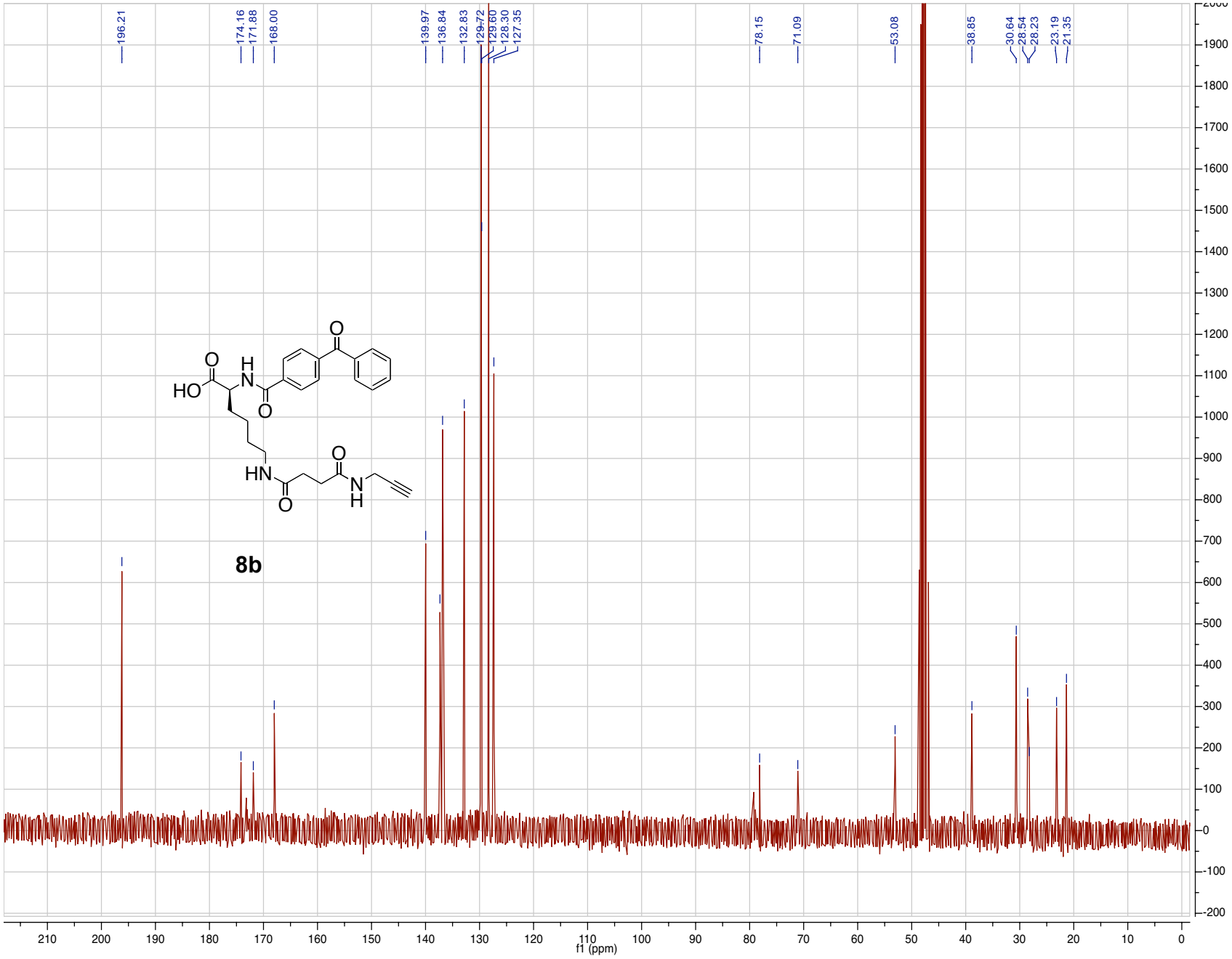
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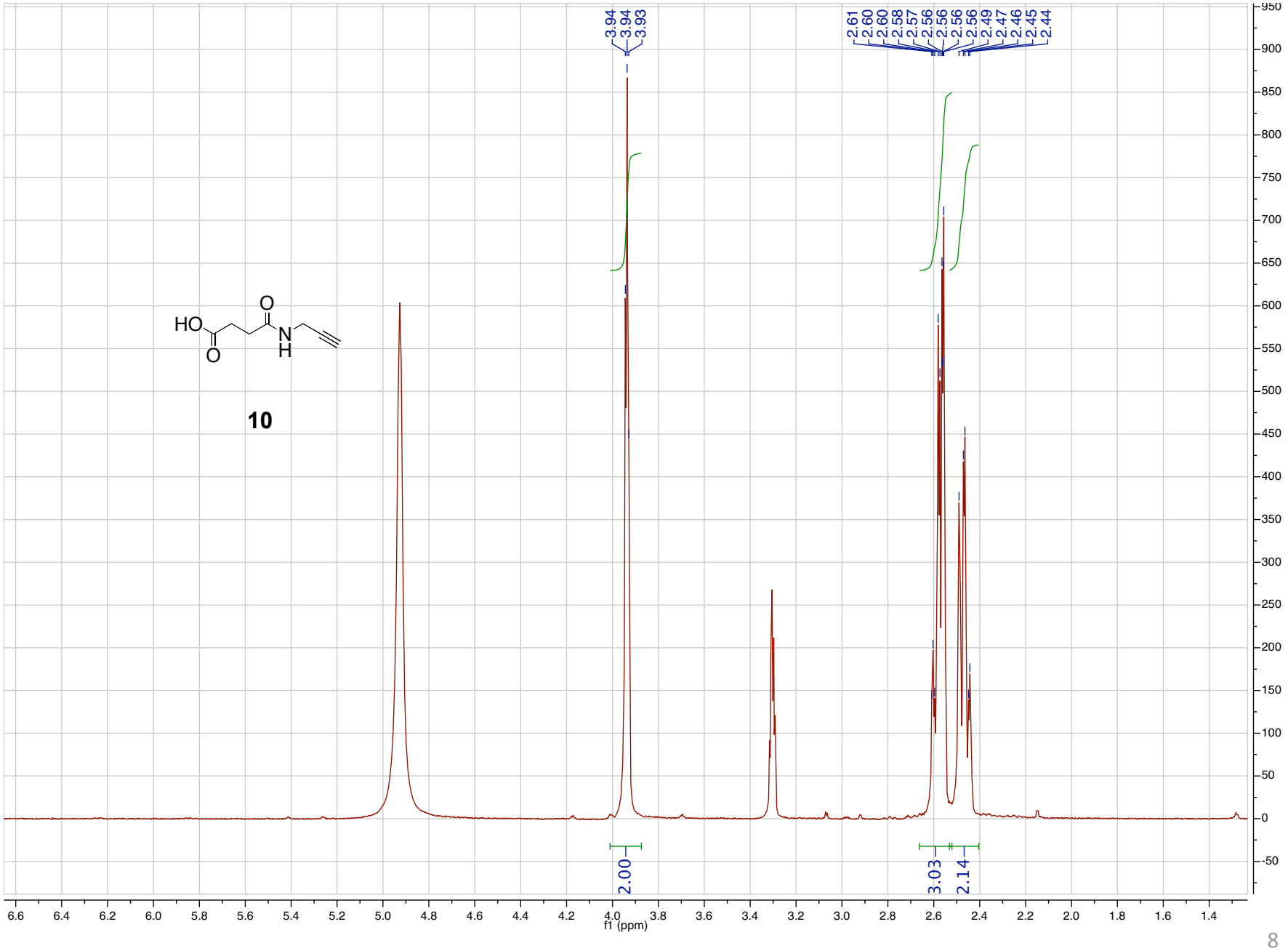
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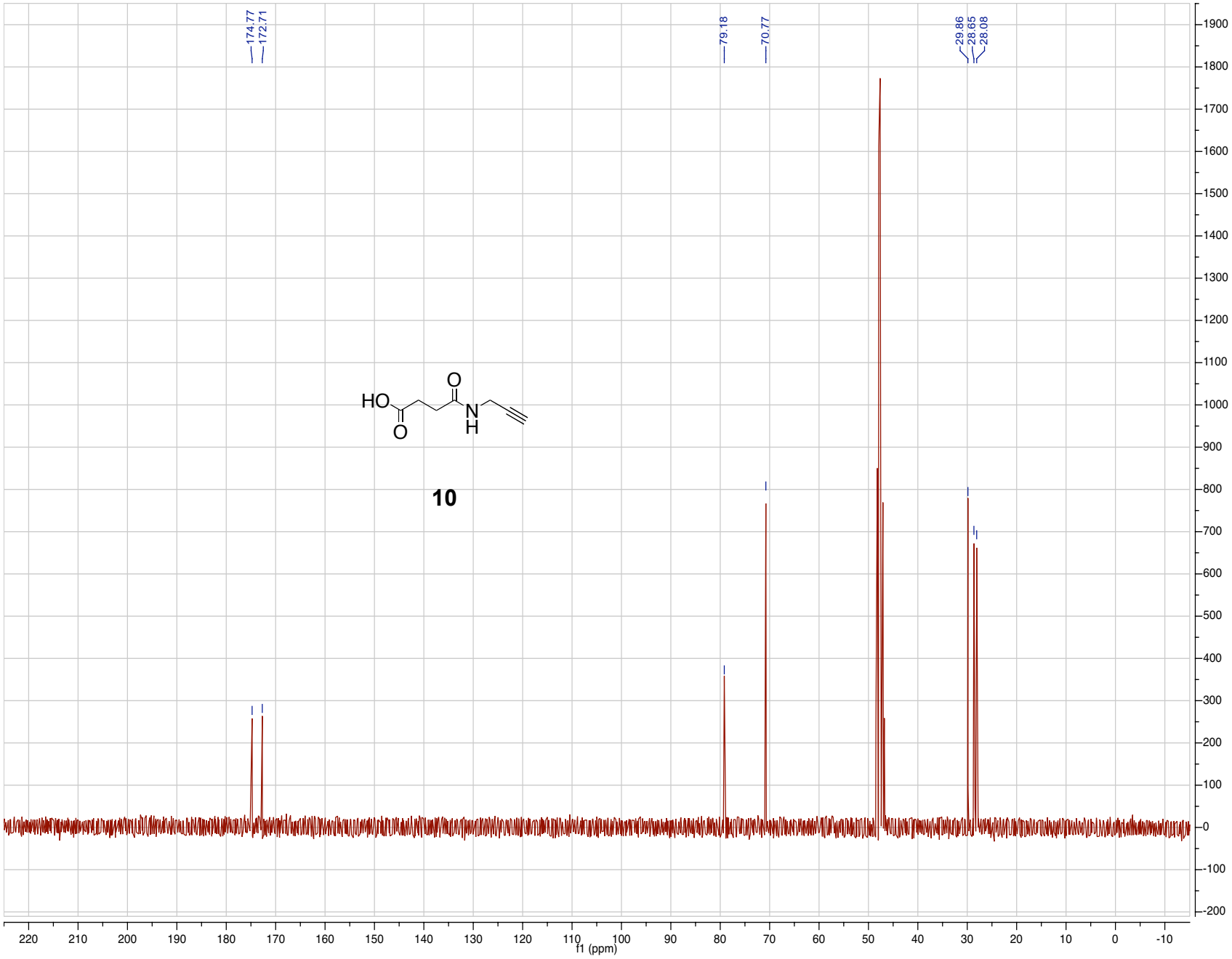
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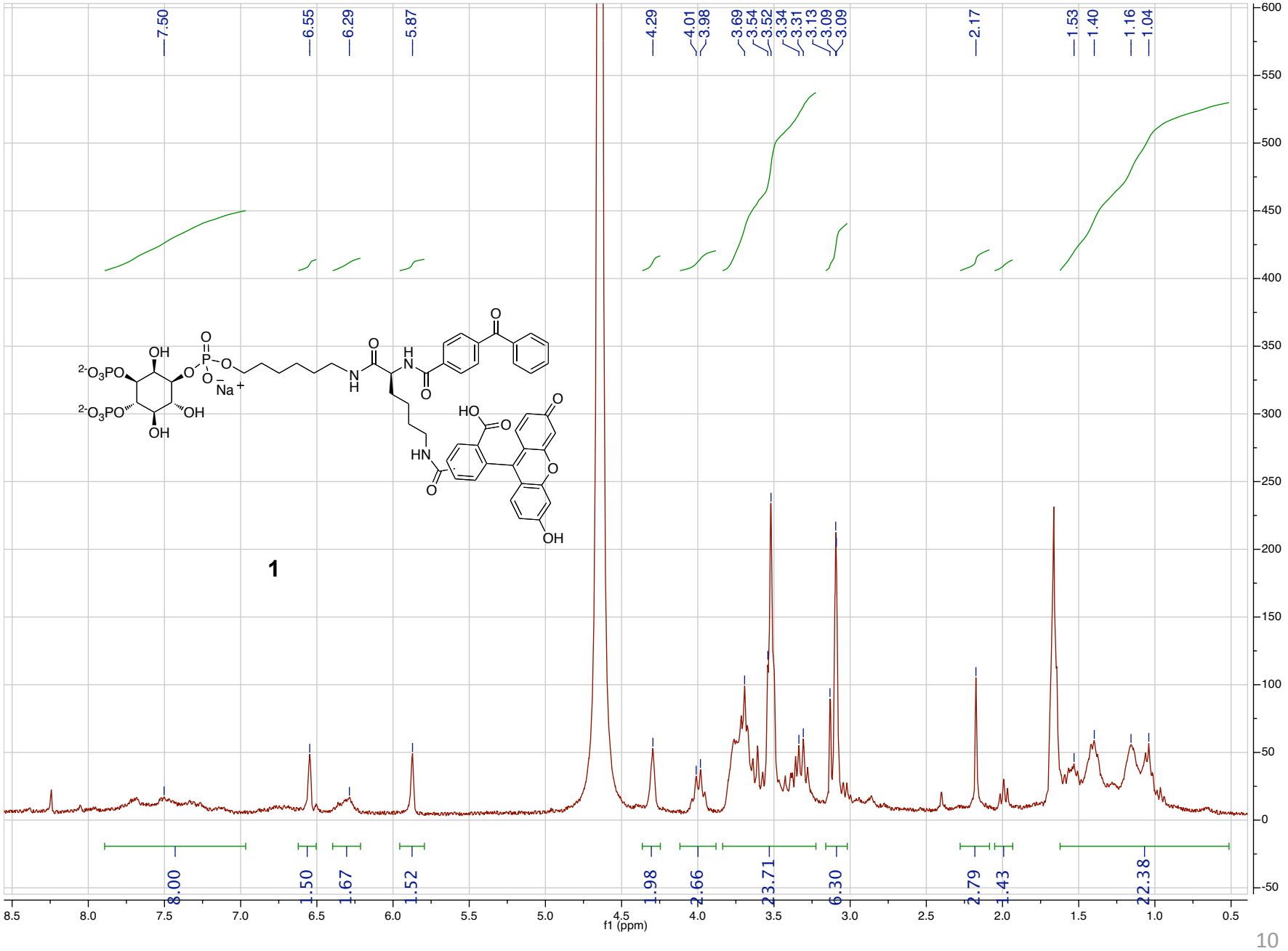
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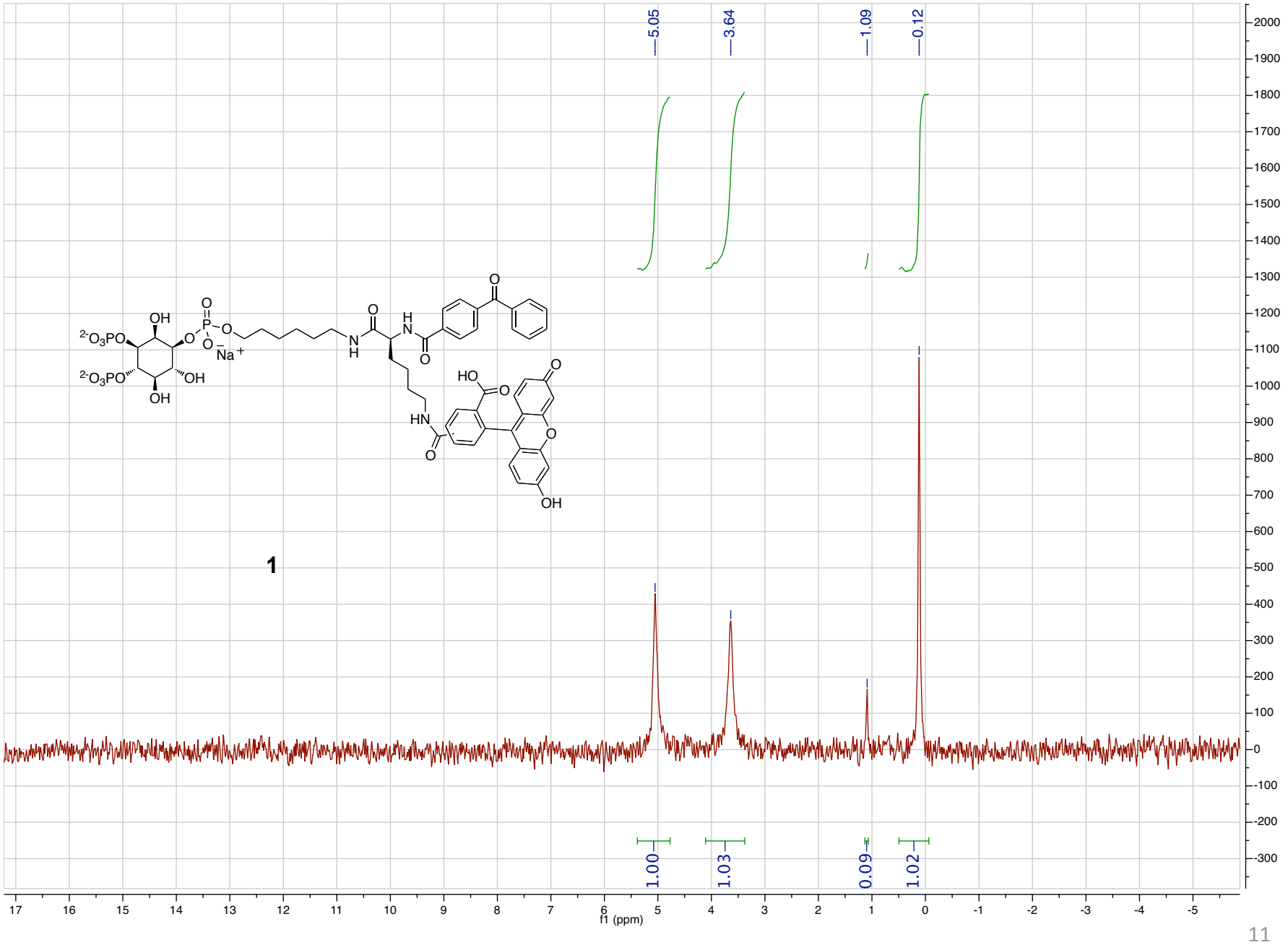
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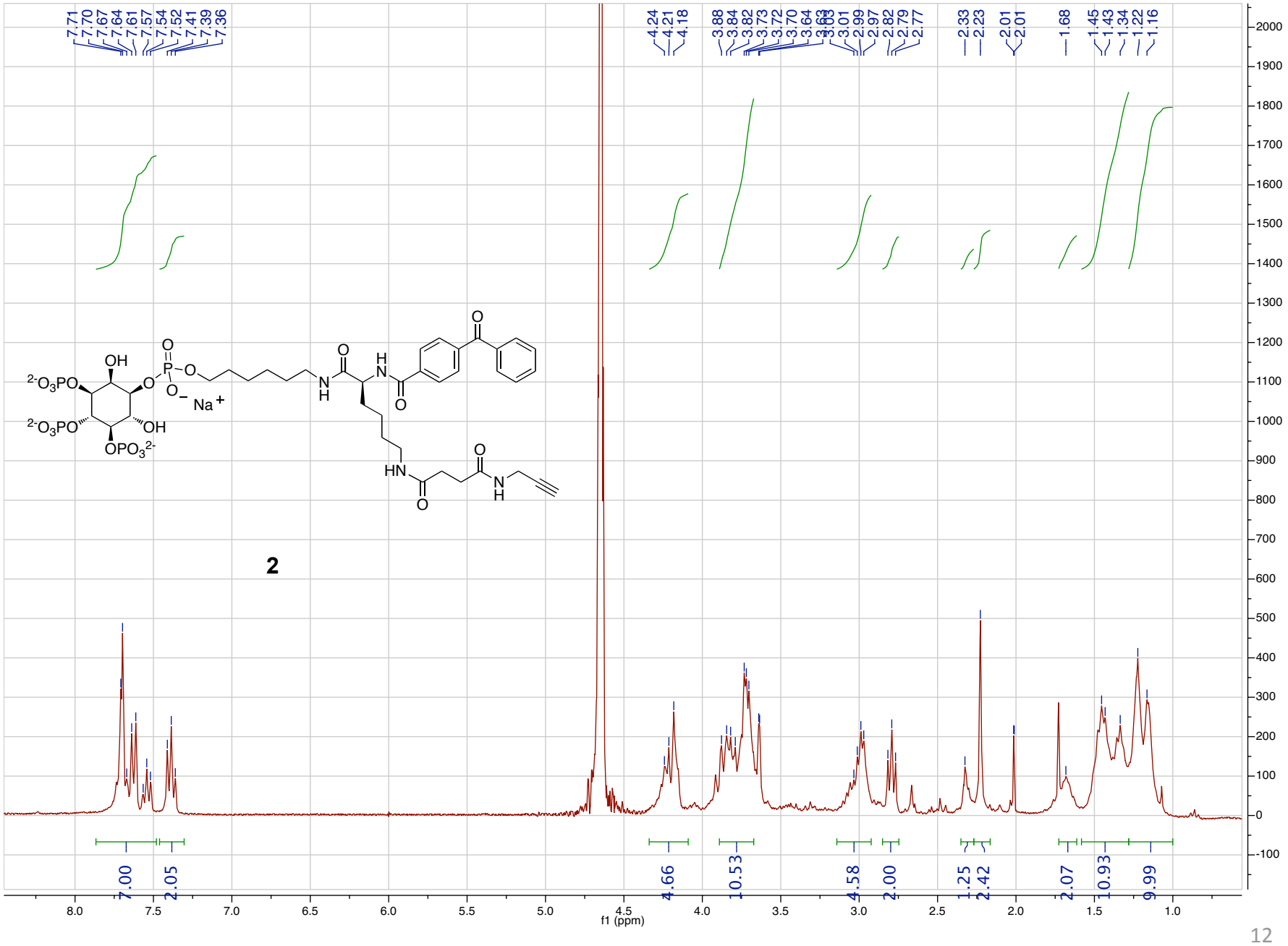
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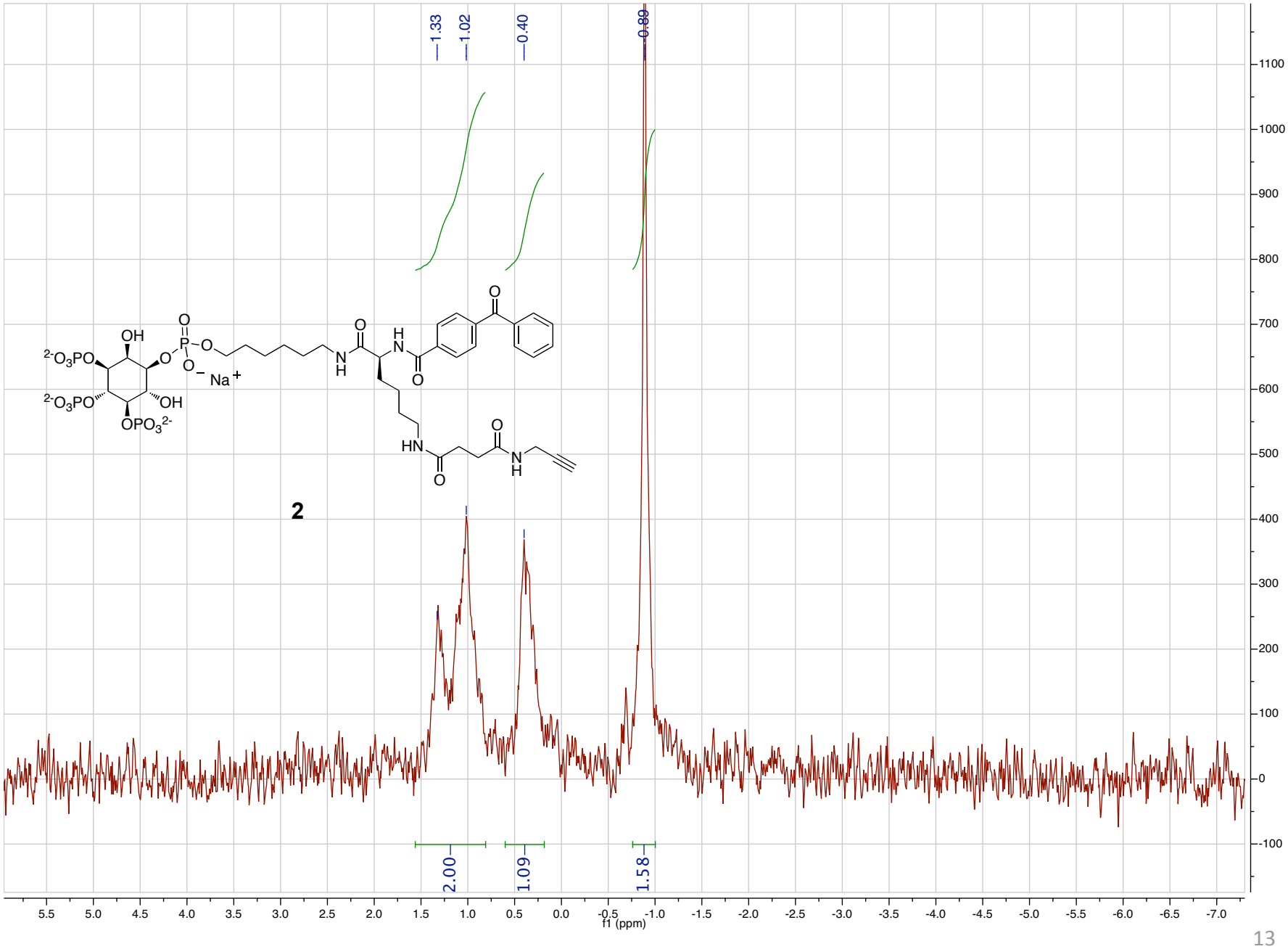
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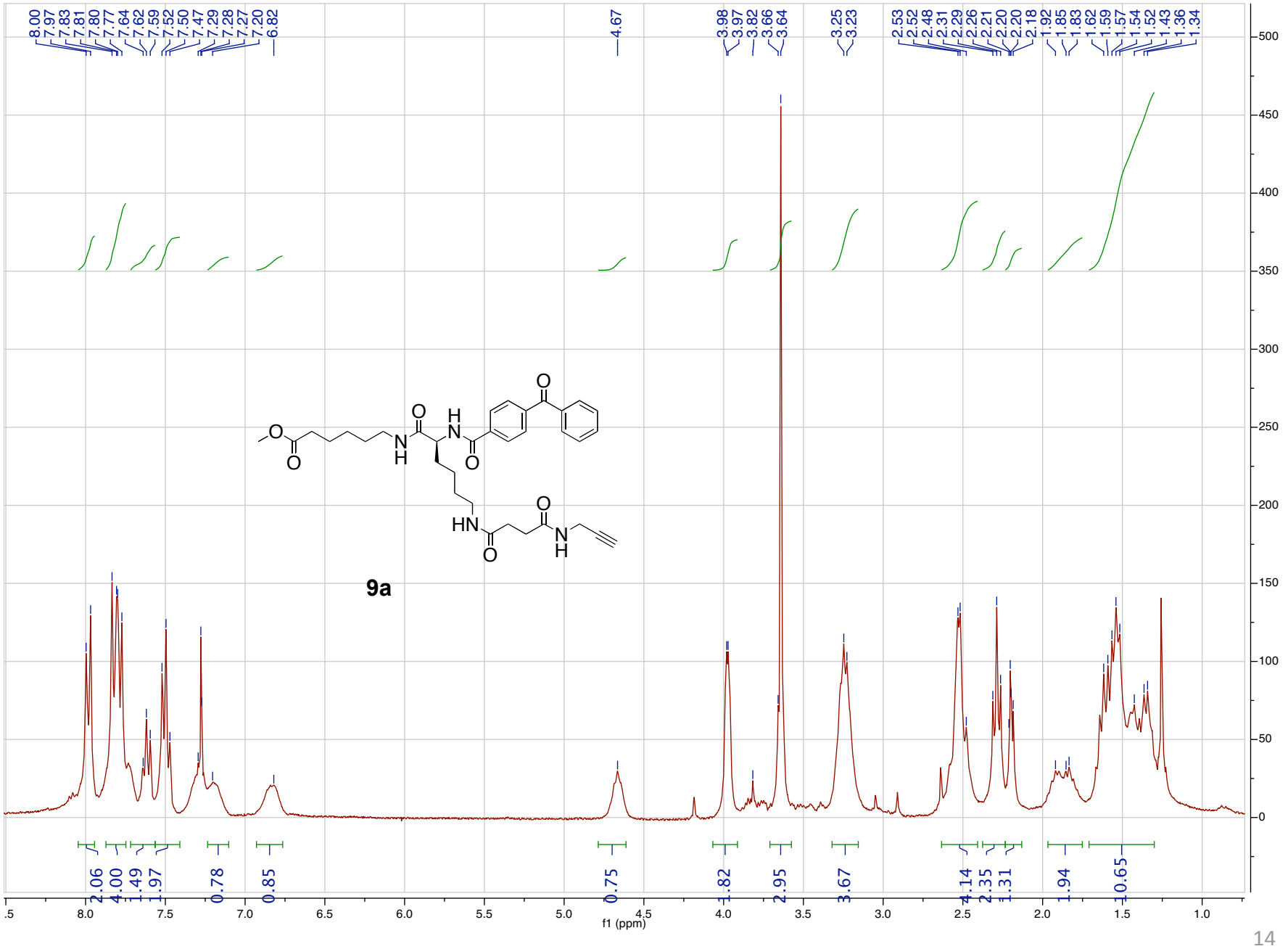
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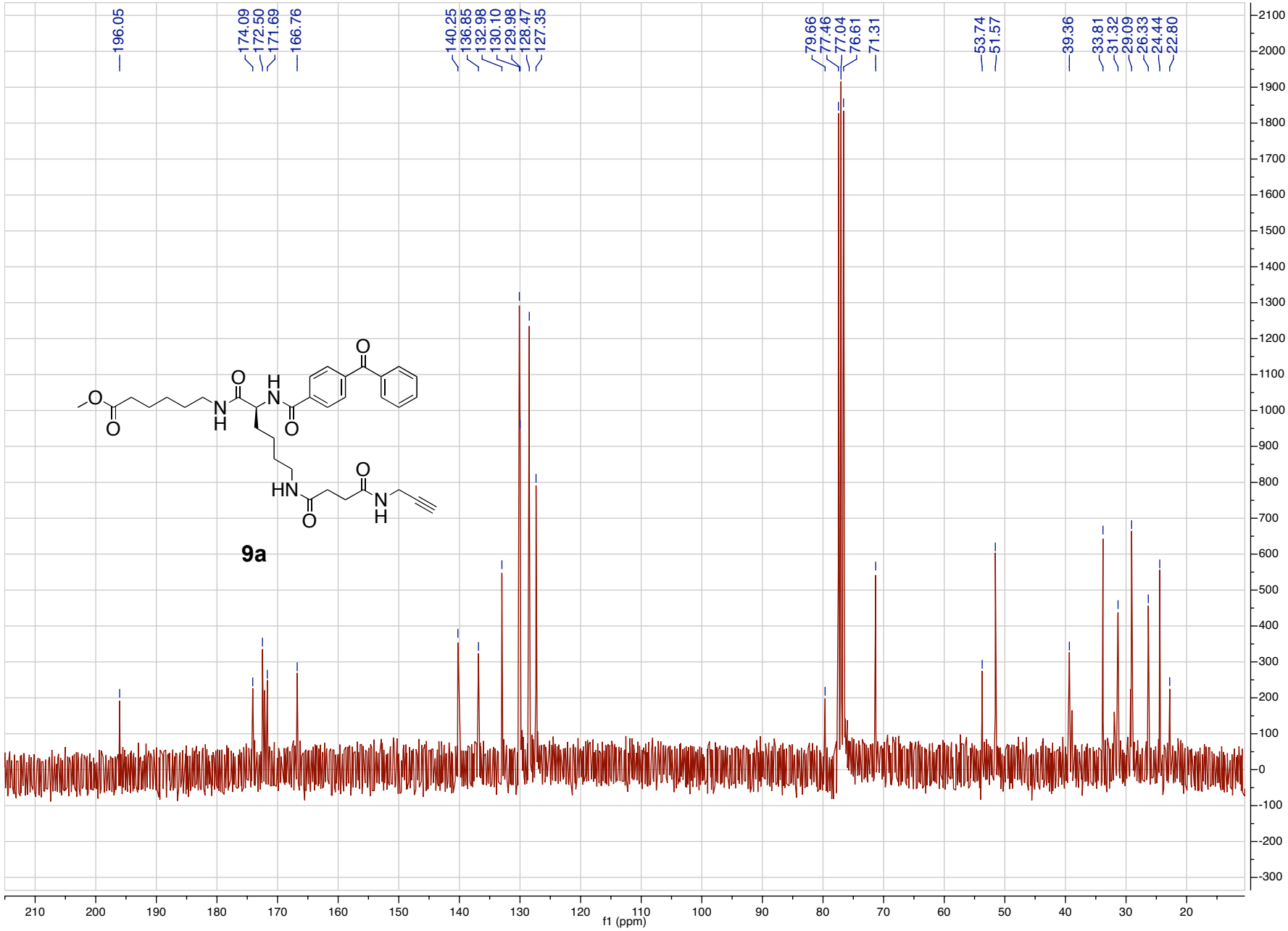
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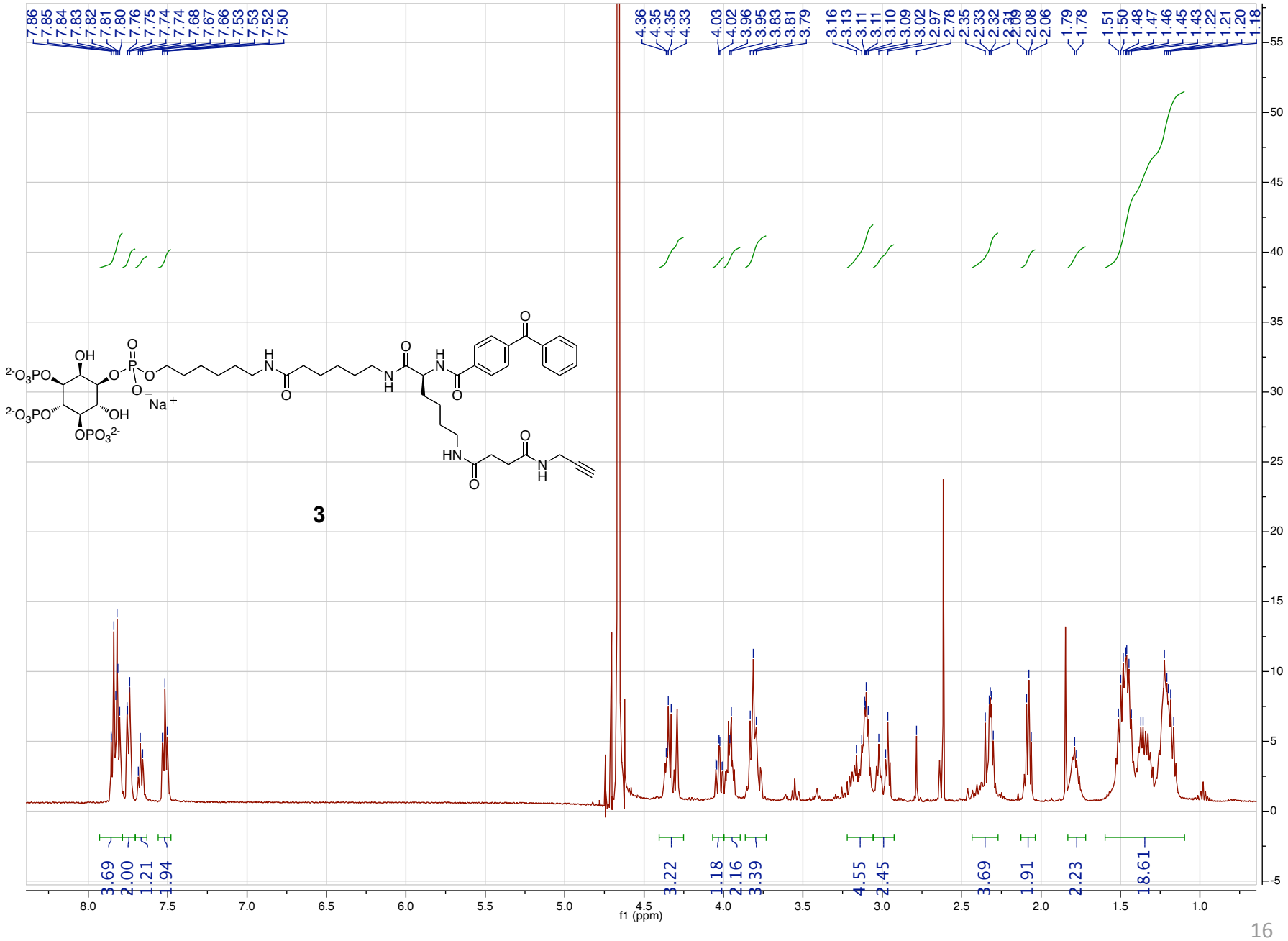
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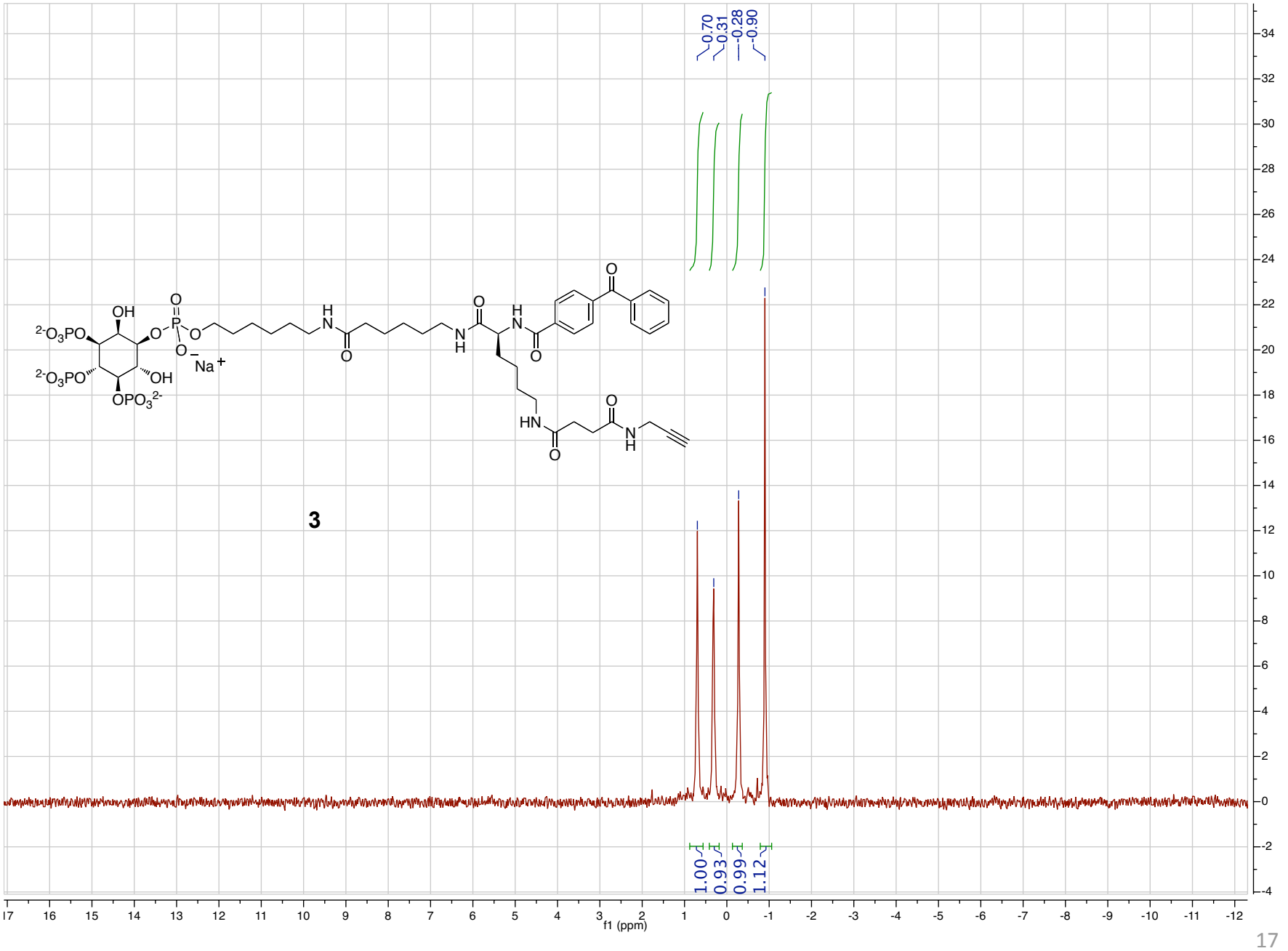
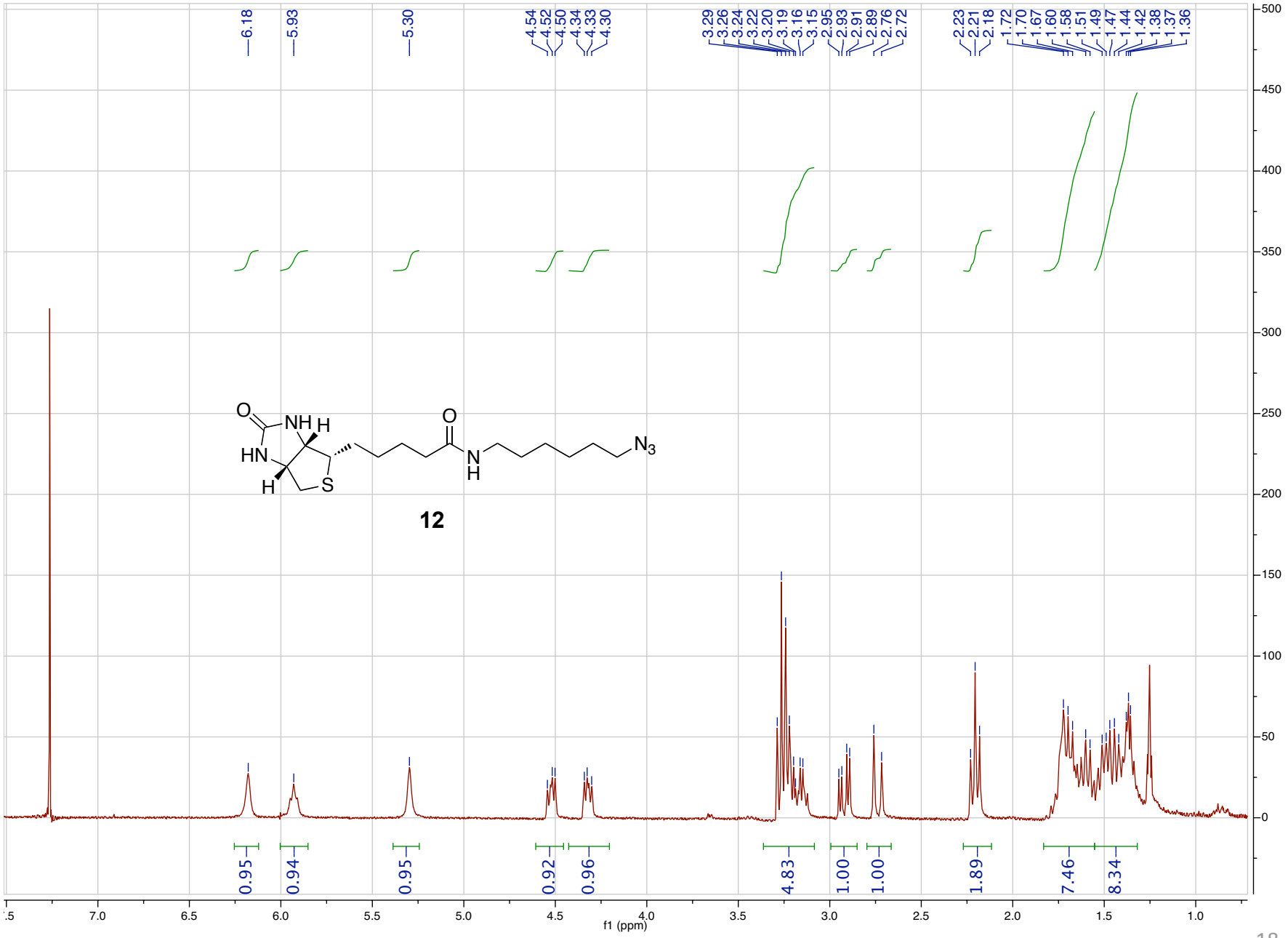
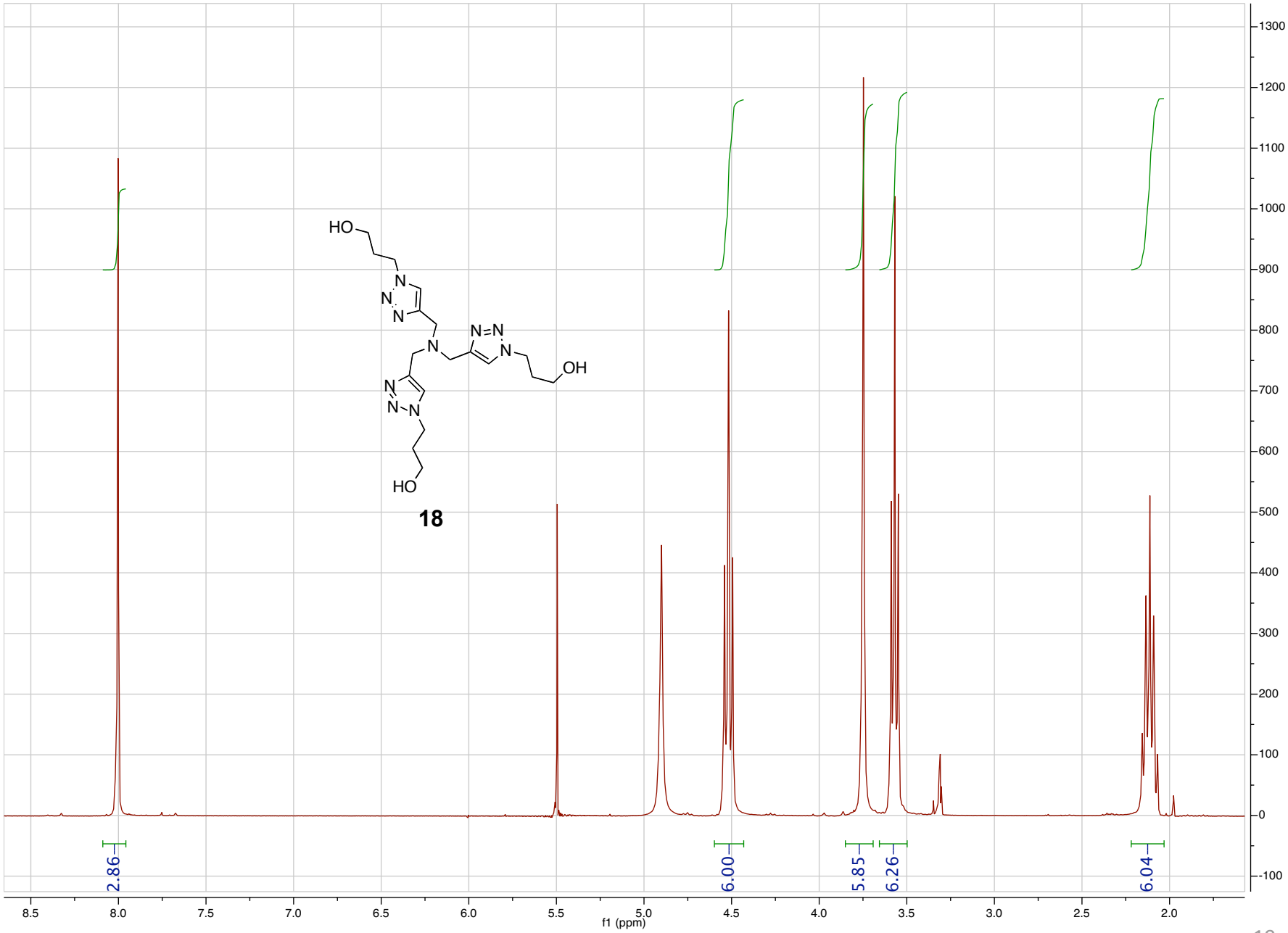
f1 (ppm)
3


-0.70
0.31
-0.28
-0.90
3
1.00
0.93
0.99
1.12
f1 (ppm)


6.18
5.93
5.30
4.54
4.52
4.50
4.34
4.33
4.30
3.29
3.26
3.24
3.22
3.20
3.19
3.16
3.15
2.95
2.93
2.91
2.89
2.76
2.72
2.23
2.21
2.18
1.72
1.70
1.67
1.60
1.58
1.51
1.49
1.47
1.44
1.42
1.38
1.37
1.36
O
NH
H
HN
H
S
O
N
H
N3
12
0.95
0.94
0.95
0.92
0.96
4.83
1.00
1.00
1.89
7.46
8.34
f1 (ppm)


HO
N
N
N
N
N=N
N
OH
N
N
N
HO
18
2.86
6.00
5.85
6.26
6.04
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
f1 (ppm)
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
-100

19

Supplementary References

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