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Synthesis of CID-cleavable protein crosslinking agents containing quaternary amines for structural mass spectrometry

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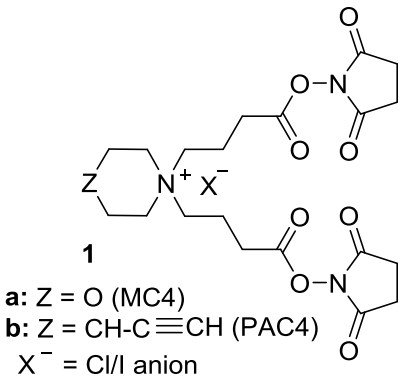

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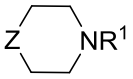
a: Z = O (MC4)
b: Z = CH-C≡CH (PAC4)
X^- = Cl/I anion



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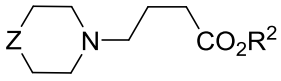

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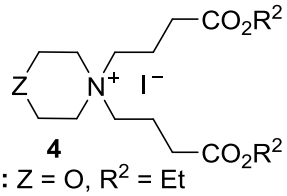

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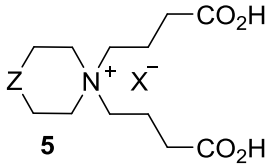

5

a: Z = O
b: Z = CH-C≡CH
X^- = Cl/I anion

Chemistry. *Materials and Methods*. All starting monomers were obtained from commercial suppliers and were used without further purification. Routine ^{1}H NMR spectra were recorded at 400 or 500 MHz on a Varian 400 or 500 instrument, respectively, with $CDCl_3$,,CD_3OD, or DMSO-*d*$_6$ as solvent. ^{13}C NMR were recorded in $CDCl_3$ or DMSO-*d*$_6$ at 126 MHz on a Varian 400 instrument. Chemical shift values are recorded in δ units (ppm). Mass spectra were recorded on a Micromass TofSpec-2E Matrix-Assisted, Laser-Desorption, Time-of-Flight Mass Spectrometer in a positive ESI mode (TOFES$^+$). High resolution mass spectrometry (HRMS) analysis was performed on an Agilent Q-TOF system. Thin-layer chromatography (TLC) was performed on silica gel GHLF plates (250 µm) purchased from Analtech, and developed in an iodine chamber. Column chromatography was carried out in the flash mode utilizing silica gel (220−240 mesh) purchased from Silicycle. Extraction solutions were dried over anhydrous sodium sulfate or magnesium sulfate prior to concentration. Combustion analyses were carried out by Robertson Microlit Laboratories, Ledgewood, NZ.

Ethyl 4-morpholinobutanoate (3a). This compound was made by a variation of a patent procedure[1]. A stirred solution of ethyl 4-bromobutanoate (2.2 mL, 15.4 mmol) and morpholine (**2a**; 5.4 mL, 61.5 mmol) in toluene (15 mL) was heated at 120 °C for 2 h. The mixture was cooled to room temperature and the precipitated solids were collected and washed with ether. The filtrate was washed with water (3x), dried (Na_2SO_4), and concentrated to give **3a** (2g, 65%) as a yellow oil. ^{1}H NMR (400 MHz, chloroform-*d*) δ 4.10 (q, *J* = 7.1 Hz, 2H), 3.67 – 3.65 (t, 4H), 2.45 – 2.35 (m, 4H), 2.31 (td, *J* = 7.3, 4.3 Hz, 4H), 1.78 (p, *J* = 7.4 Hz, 2H), 1.22 (t, *J* = 7.1 Hz, 3H).

***tert*-Butyl 4-(4-ethynylpiperidin-1-yl)butanoate (3b)**

A slurry of crude 4-ethynylpiperidine hydrochloride[2] (**2c**; 0.61 g, 4.2 mmol) in acetonitrile (40 mL) was treated successively with K_2CO_3 (3.5 g, 25 mmol) and *tert*-butyl 4-iodobutanoate[3] (1.20 g, 4.4 mmol). The mixture was stirred at room temperature for 3 d during which a heavy precipitate formed. The suspension was concentrated and partitioned between water and DCM. The aqueous phase was further extracted with DCM (3 x 40 mL), and the combined organic extracts were dried ($MgSO_4$) and concentrated to a yellow oil that was purified by flash silica gel chromatography (elution with 100:0 and then 95 : 5 DCM : MeOH) to give **3b** (0.84 g; 80% yield) as a pale yellow oil. ^{1}H NMR (400 MHz, chloroform-*d*) δ 2.69 (br s, 2H), 2.45 – 2.26 (m, 2H), 2.21 (t, *J* = 7.5 Hz, 2H), 2.12 (br s, 3H), 2.05 (d, *J* = 2.4 Hz, 1H), 1.89 – 1.79 (m, 2H), 1.79 – 1.59 (m, 4H), 1.42 (s, 9H).

4,4-Bis(4-ethoxy-4-oxobutyl)morpholin-4-ium iodide (4a). A mixture of **3a** (2.44 g, 12.12 mmol) and neat ethyl 4-iodobutanoate[4] (5.21 g, 21.52 mmol) was stirred overnight at 115 °C. The mixture was cooled to room temperature leaving a viscous oil, which was diluted with ether. The mixture was stirred for 30 min, the ether decanted and the process of ether dilution was repeated twice more. The resulting pale yellow solid was collected, washed with ether and dried to give **4a** (5.08 g, 95% yield). ^{1}H NMR (500 MHz, chloroform-*d*) δ 4.16 (dt, *J* = 13.3, 6.4 Hz, 8H), 3.69 (s, 8H), 2.64 (d, *J* = 7.5 Hz, 4H), 2.17 (m, 4H), 1.29 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (126 MHz, chloroform-*d*) δ 172.15,

61.19, 60.62, 59.07, 58.56, 29.93, 17.12, 14.20. MS TOFES$^+$ *m/z* (%): 316.2 (M^+, 100%). MW of iodide salt = 443.32.

1,1-Bis(4-(*tert*-butoxy)-4-oxobutyl)-4-ethynylpiperidin-1-ium iodide (4b). A solution of **3b** (0.79 g, 3.1 mmol) in minimal acetonitrile (~3 mL) was treated with a solution of *tert*-butyl-4-iodobutanoate[3] (1.5 g, 5.7 mmol) in acetonitrile (1 mL) and the mixture was stirred at room temperature for 13 d. The thick solution was concentrated *in vacuo* to leave a yellow paste that was triturated in ether, collected and dried to give **4b** (0.82 g, 50%) as an off-white solid, mp: 93-95 °C. ^{1}H NMR (400 MHz, chloroform-*d*) δ 4.05 - 3.85 (m, 1H), 3.77 (t, *J* = 8.3 Hz, 2H), 3.53 (q, *J* = 15.0, 12.2 Hz, 5H), 3.12 (s, 1H), 2.52 - 2.26 (m, 7H), 2.22 (s, 1H, alkyne H), 2.15 - 1.75 (m, 5H), 1.43 (s, 18H). HRMS TOFES$^+$ *m/z*: calc., 394.2952; found, 394.2960 (M^+, 100%). MW of iodide salt = 521.48.

4,4-Bis(3-carboxypropyl)morpholin-4-ium, chloride and iodide salt (5a). To a solution of diester salt **4a** (3 g, 6.77 mmol) in glacial acetic acid (40 mL) was added dropwise conc. HCl (40 mL). The mixture was heated at 100° for 2 d, cooled to room temperature and concentrated to a dark red oil that was triturated in acetone. The white precipitate that formed was collected, washed with acetone, and dried to give **5a** (1.92 g (91%), mp: 199-200 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.38 (s, 2H), 3.97 – 3.83 (m, 4H), 3.53 – 3.38 (m, 8H), 2.36 (t, *J* = 6.9 Hz, 4H), 1.87 (p, *J* = 7.0 Hz, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 173.32, 59.58, 57.71, 56.70, 30.06, 16.29. MS TOFES$^+$ *m/z* (%): 260.0 (M^+, 100%). *Anal*. Calcd. for $C_{12}H_{22}NO_5^+ \cdot 0.80\ Cl^- \cdot 0.17\ I^- \cdot 0.1\ H_2O$ (MW = 312.05): C, 46.19; H, 7.17; N, 4.49; Cl^-, 9.09; I^-, 6.91%. Found: C, 46.08; H, 7.09; N, 4.55; Cl^-, 8.97; I^-, 6.84%.

1,1-Bis(3-carboxypropyl)-4-ethynylpiperidin-1-ium, chloride and iodide salt (**5b)**. A room temperature solution of salt **4b** (0.22 g, 0.42 mmol) in dichloromethane (5 mL) was treated dropwise with anhydrous HCl in *p*-dioxane (1.05 mL of 4M solution). The mixture was stirred for 18 h at room temperature, treated with more HCl (0.2 mL), and stirred additionally overnight. The resulting suspension was concentrated to a residue

that was triturated in ethyl acetate, collected, washed successively with ethyl acetate and ether, and dried to give **5b** (0.11 g, 67%) as an off-white solid, mp: shrinks at 168 °C, melts at 186-188 °C. ^{1}H NMR (400 MHz, methanol-d_4) δ 3.60 (dt, J = 9.0, 4.3 Hz, 2H), 3.41 (ddd, J = 21.6, 10.6, 6.3 Hz, 6H), 2.85 (s, 1H, piperidine-C$_4$H-alkyne), 2.64 (d, J = 2.3 Hz, 1H, alkyne H), 2.48 (q, J = 6.6 Hz, 4H), 2.17 (s, 2H), 2.01 (d, J = 11.8 Hz, 6H). HRMS TOFES$^+$ *m/z*: calc., 282.1700; found, 282.1699 (M$^+$, 100%). *Anal*. Calcd. for $C_{15}H_{24}NO_4^+ \cdot 0.82\ Cl^- \cdot 0.18\ I^- \cdot 3\ H_2O$ (MW = 388.32): C, 46.40; H, 7.79; N, 3.61; Cl$^-$, 7.49; I$^-$, 5.88%. Found: C, 46.04; H, 6.45; N, 3.54; Cl$^-$, 7.65; I$^-$, 5.80%.

4,4-Bis(4-((2,5-dioxopyrrolidin-1-yl)oxy)-4-oxobutyl)morpholin-4-ium, chloride and iodide salt (1a; MC4). To a room temperature suspension of diacid **5a** (50 mg, 0.16 mmol) in dry acetonitrile (5 mL) were added *N*,*N'*-disuccinimidyl carbonate (102.5 mg, 0.4 mmol) and pyridine (32 µL, 0.4 mmol). The mixture was stirred at 50 °C overnight, cooled, and then filtered. The collected solids were washed with acetonitrile and the combined filtrates were concentrated to a residue that was re-dissolved in a minimal volume of acetonitrile. The acetonitrile solution was added dropwise to stirring cold ethyl acetate. The resulting precipitate was collected, washed with ethyl acetate and dried to give **1a** (65 mg, 56%) as a pale white hygroscopic solid, mp: shrinks at 45 °C, decomposes at 98 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.89 (d, J = 4.9 Hz, 4H), 3.57 – 3.40 (m, 8H), 2.88 – 2.77 (m, 12H), 2.58 (s, 6H, 2.04 (q, J = 8.7, 8.0 Hz, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 170.77, 168.67, 60.20, 58.43, 58.29, 27.40, 25.67, 16.51. MS TOFES$^+$ *m/z* (%): 454.0 (M$^+$, 100%). *Anal*. Calcd. for $C_{20}H_{28}N_3O_9^+ \cdot 0.89\ Cl^- \cdot 0.11\ I^- \cdot 1.3\ C_4H_5NO_3 \cdot 0.2$ EtOAc $\cdot 0.1\ C_{12}H_{22}NO5_9^+ \cdot 2.0\ H_2O$ (MW = 729.27): C, 44.80; H, 5.85; N, 8.45; Cl$^-$, 4.33; I$^-$, 1.91%. Found: C, 44.70; H, 5.45; N, 8.22; Cl$^-$, 4.54; I$^-$, 2.04%.

1,1-Bis(4-((2,5-dioxopyrrolidin-1-yl)oxy)-4-oxobutyl)-4-ethynylpiperidin-1-ium, chloride and iodide salt (1b; PAC4). A room temperature suspension of diacid **5b** (88 mg, 0.23 mmol) in acetonitrile (15 mL) was treated with *N*,*N'*-disuccinimidyl carbonate (0.17 g, 0.65 mmol) and pyridine (52 µL, 0.65 mmol). The resulting milky suspension was stirred at 65 °C over the weekend resulting in a clear yellow solution. The cooled

mixture was filtered and the filtrate was concentrated *in vacuo* to leave a viscous yellow-brown oil that was dissolved in acetonitrile (~1 mL). The solution was added dropwise, with stirring, to ~25 mL of ice-cold ethyl acetate. An off-white solid formed that was collected, washed successively with ethyl acetate and ether, and dried to leave crude **1b**. Purification was effected by sonicating the solids in 2-propanol for around 15 min, and then filtering the suspension. The collected solids were washed copiously with 2-propanol. The combined filtrate and washings were concentrated to leave a sticky foam (~90% recovery of crude solids) that was dissolved in a small volume of acetonitrile (<1 mL). The solution was added dropwise with stirring to 25 mL of ether. The precipitated solids were collected, washed with ether, and dried *in vacuo* to give **1b** (51 mg, 37%) as a hygroscopic white powder. mp: shrinks at 65 °C, melts 131-133 °C. ^{1}H NMR (400 MHz, methanol-d_4) δ 3.74 – 3.33 (m, 8H), 2.86 (t, J = 3.0 Hz, 8H), 2.65 (d, J = 3.1 Hz, 1H, piperidine H-4), 2.46 (d, J = 6.4 Hz, 1H, alkyne H), 2.19 – 2.10 (m, 4H), 2.05 – 1.95 (m, 4H). HRMS TOFES$^+$ *m/z*: calc., 476.2027; found, 476.2028 (M^+, 95%). *Anal*. Calcd. for $C_{23}H_{30}N_3O_8^+ \cdot 0.95\ Cl^- \cdot 0.05\ I^- \cdot 0.3\ C_4H_5NO_3 \cdot 0.2\ C_4H_{10}O \cdot 1.8\ H_2O$ (MW = 598.31): C, 50.19; H, 6.25; N, 7.73; Cl^-, 5.63; I^-, 1.06%. Found: C, 50.34; H, 6.24; N, 7.43; Cl^-, 5.94; I^-, 1.16%.

Biology. Materials and Methods. Rabbit muscle aldolase from Sigma Aldrich (Cat # A2714) was used without further purification. N-terminally acetylated peptide: Ac-PIRANNLINKERAGLINKER, synthesized by the Biomedical Research Core Facility at the University of Michigan Medical School using standard Fmoc-based methods, a PTI Symphony synthesizer and purification by reversed-phase HPLC. The endoproteinases Trypsin and GluC were purchased from Promega (Cat # V5280) and Protea (Cat # PE-102-5), respectively. NuPAGE Bis-Tris gel (4%-12%) and NuPAGE LDS sample buffer (4x) were purchased from Novex (ThermoFisher Scientific, Inc.; San Jose, CA, USA). All other reagents and solvents were proteomics grade.

Crosslinking Reaction. A freshly prepared 10 µM solution of aldolase in 100 mM triethylammonium bicarbonate (TEAB), pH 8.5 was crosslinked for 15 min at room temperature using an increasing molar ratio of crosslinker to total lysine residues: **1a**

and **1b**. The reactions were quenched with ammonium bicarbonate at a concentration 20-fold higher than the crosslinker and ¼ volume of NuPAGE® LDS sample buffer (106 mM Tris HCl; 141 mM Tris Base; 2% LDS, 10% glycerol; 0.51 mM EDTA; 0.22 mM SERVA blue G250; 0.175 mM phenol red; pH 8.5) was added. The samples were incubated at 70 °C for 10 min and approximately 7 µg of crosslinked aldolase was loaded on the Bis-Tris gradient gel. For the LCMSMS sample, a newly prepared 10 µM solution of aldolase in 100 mM HEPES pH 7.5 was crosslinked for 30 min at room temperature using a 1:5 molar ratio of lysine residues to **1a** crosslinker. The reaction was quenched with 10% formic acid (FA) and neutralized using NaOH. The peptide crosslink sample was prepared by reacting 200 µM acetylated standard peptide (0.237 µg/µL solution) in 100 mM TEAB pH 8.5 with a 10-fold molar ratio of **1b** crosslinker for 15 min at room temperature. The reaction was quenched with ammonium bicarbonate at a concentration 20-fold higher than the crosslinker. The stock solution of 4 mM **1b** in 100 mM TEAB, pH 8.5, was prepared immediately before use.

Mass Spectrometry. Prior to mass spectrometry, crosslinked aldolase was digested with trypsin at a 20:1 substrate to enzyme ratio overnight at 37 °C, then endoproteinase GluC was added at the same ratio and the digestion was continued for other 6 h at 37 °C. The digest was dried using a vacuum centrifuge, reconstituted in 0.1% trifluoroacetic acid, and desalted on Millipore C18 ZipTips according to the manufacturer's instructions.

The crosslinked aldolase peptides were analyzed on a hybrid linear quadrupole ion trap-Orbitrap mass spectrometer (ThermoFisher Scientific, Inc. model LTQ-Orbitrap XL; San Jose, CA, USA). An Eksigent Nano 2D LC was used with a C18 RP 100Å 15 cm column (CVC MicroTech, Fontana, CA) to separate the peptides. Solvent A was 5:0.1:94.9 acetonitrile : formic acid : water, while Solvent B was 90:0.1:9.9 acetonitrile : formic acid : water. The flow rate was set to 0.55 µL/min with the following binary gradient: 0 min, 9.8% B; 5 min, 10.2% B; 60 min, 40% B; 75 min, 100% B; 80 min, 100% B. The sample was introduced using a TriVersa Nanomate nanospray ionization source from Advion BioSciences (Ithaca, NY, USA). Monoisotopic precursor ions were selected for

multistage tandem mass spectrometric acquisition using an isolation window of 2.0 (m/z units) and excitation energy settings of 35. The MS2 CID spectra were collected in IT (ion trap) mode.

The crosslinked synthetic peptide was digested using trypsin at a ratio 100:1 substrate to enzyme for 2 h at 37 °C to generate two separate peptides crosslinked at the single Lys residues. The digest was dried using a vacuum centrifuge, reconstituted in 0.1% trifluoroacetic acid and desalted using Millipore C18 ZipTips according to the manufacturer's instructions. The digested peptide was introduced by infusion using a syringe system at 3 ul/min flow and analyzed with a tribrid mass spectrometer (ThermoFisher Scientific, Inc. model Orbitrap Fusion Lumos; San Jose, CA, USA). The applied spray voltage was 3.5 kV and the heated capillary temperature was set at 275 °C. Monoisotopic precursor ion was manually selected for fragmentation using an isolation window of 2.0 (m/z units) and excitation energy settings of 35, the CID spectra were collected in FT (Orbitrap) mode and peak resolution of 60,000.

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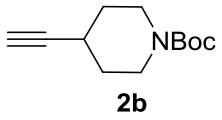
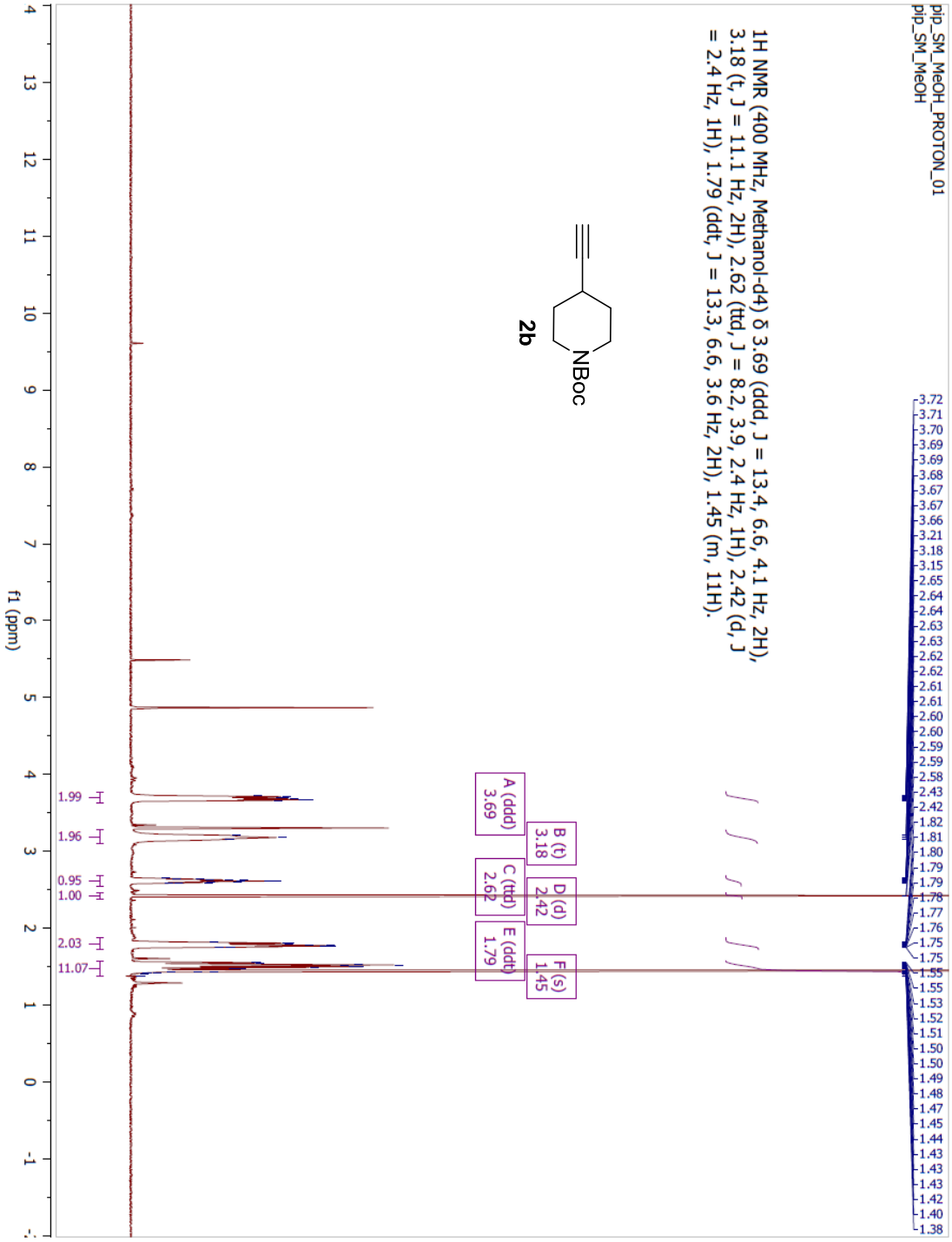
1H NMR (400 MHz, Chloroform-d) δ 3.77 – 3.62 (m, 2H), 3.17 (ddd, J = 13.5, 8.5, 3.5 Hz, 2H), 2.57 (dtd, J = 8.3, 4.2, 2.5 Hz, 1H), 2.09 (d, J = 2.4 Hz, 1H), 1.77 (ddd, J = 13.9, 7.6, 3.5 Hz, 2H), 1.57 (dtd, J = 12.5, 8.2, 3.7 Hz, 2H), 1.44 (s, 9H).



2b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2b
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C (ttd) 2.62
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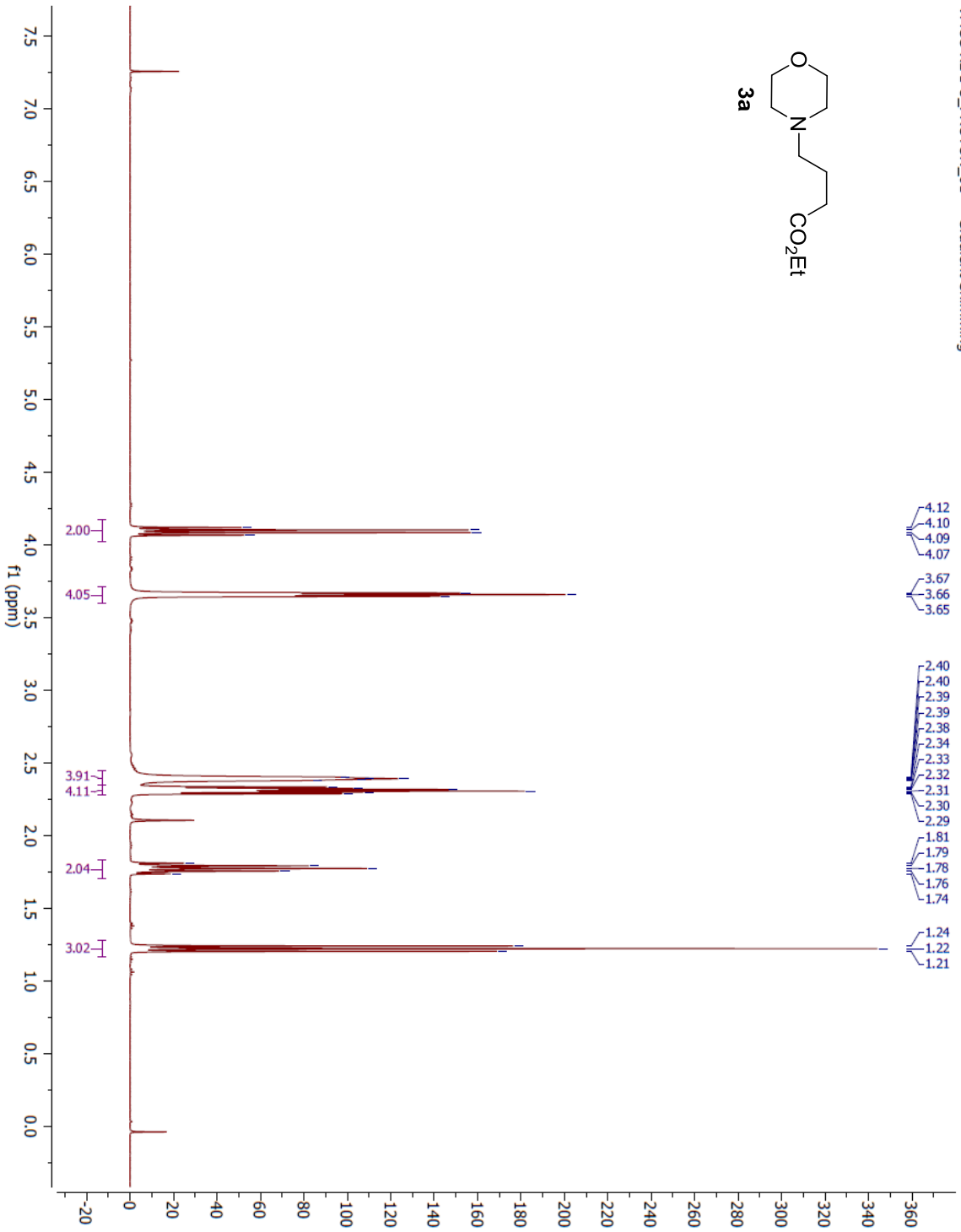
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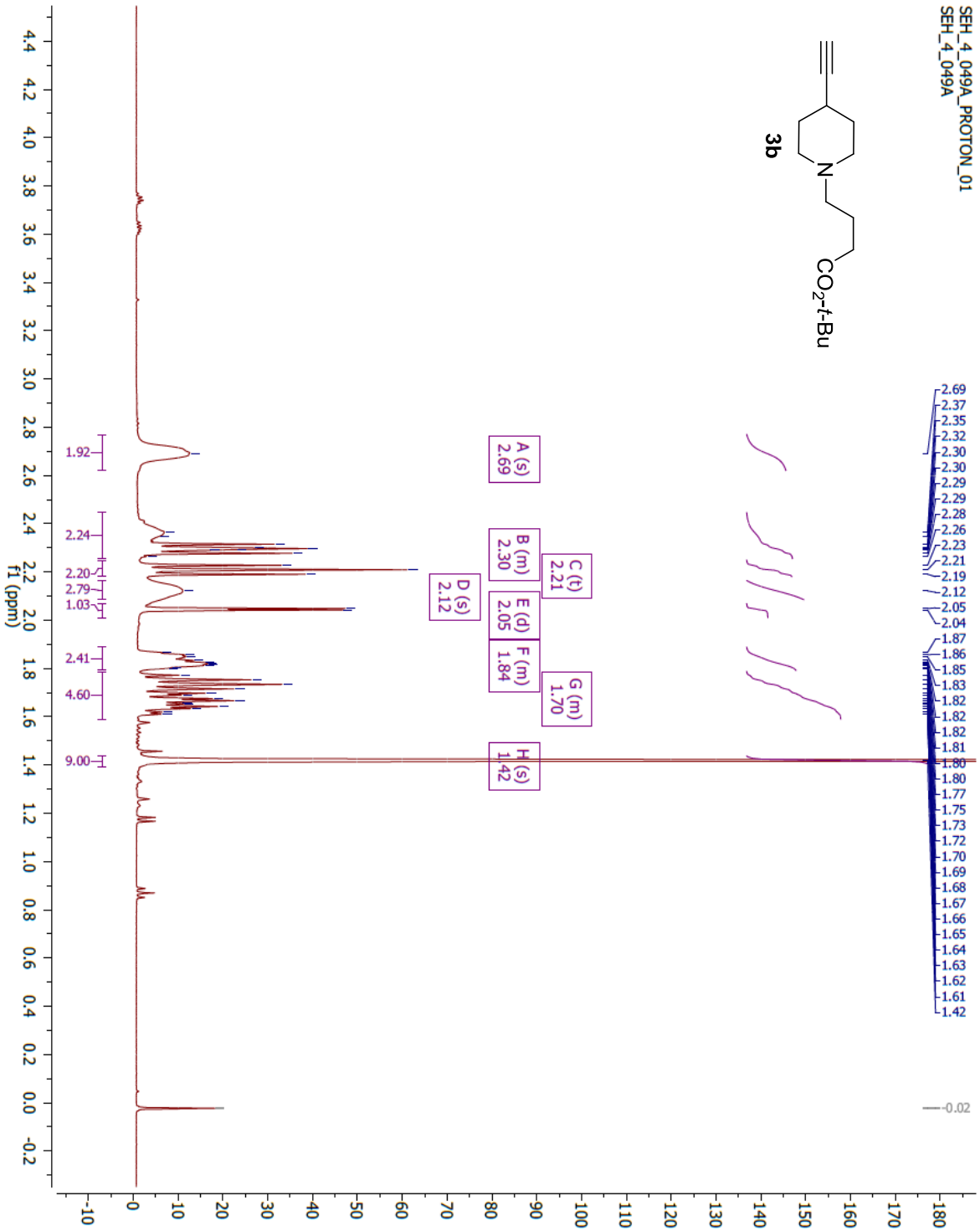
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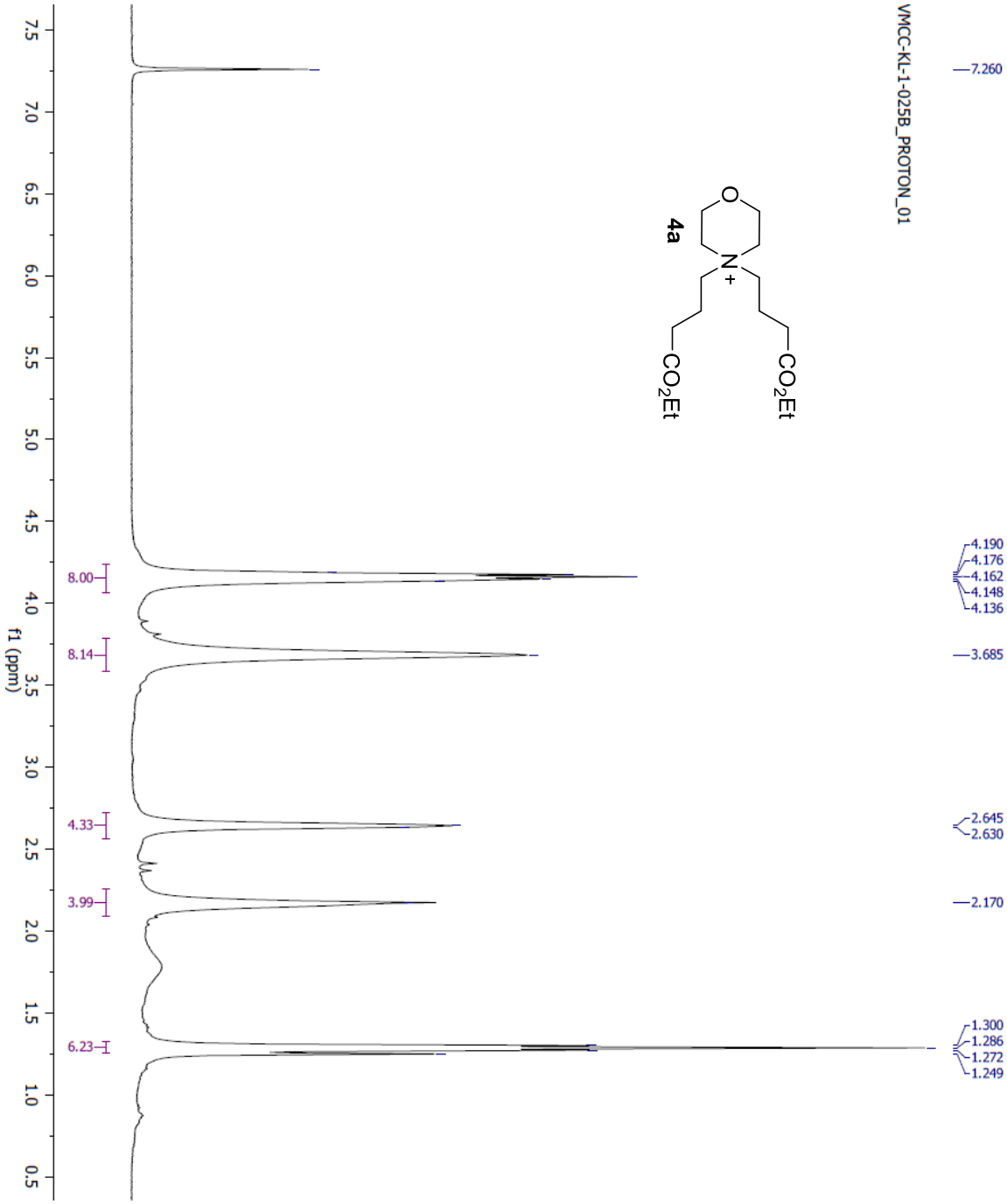
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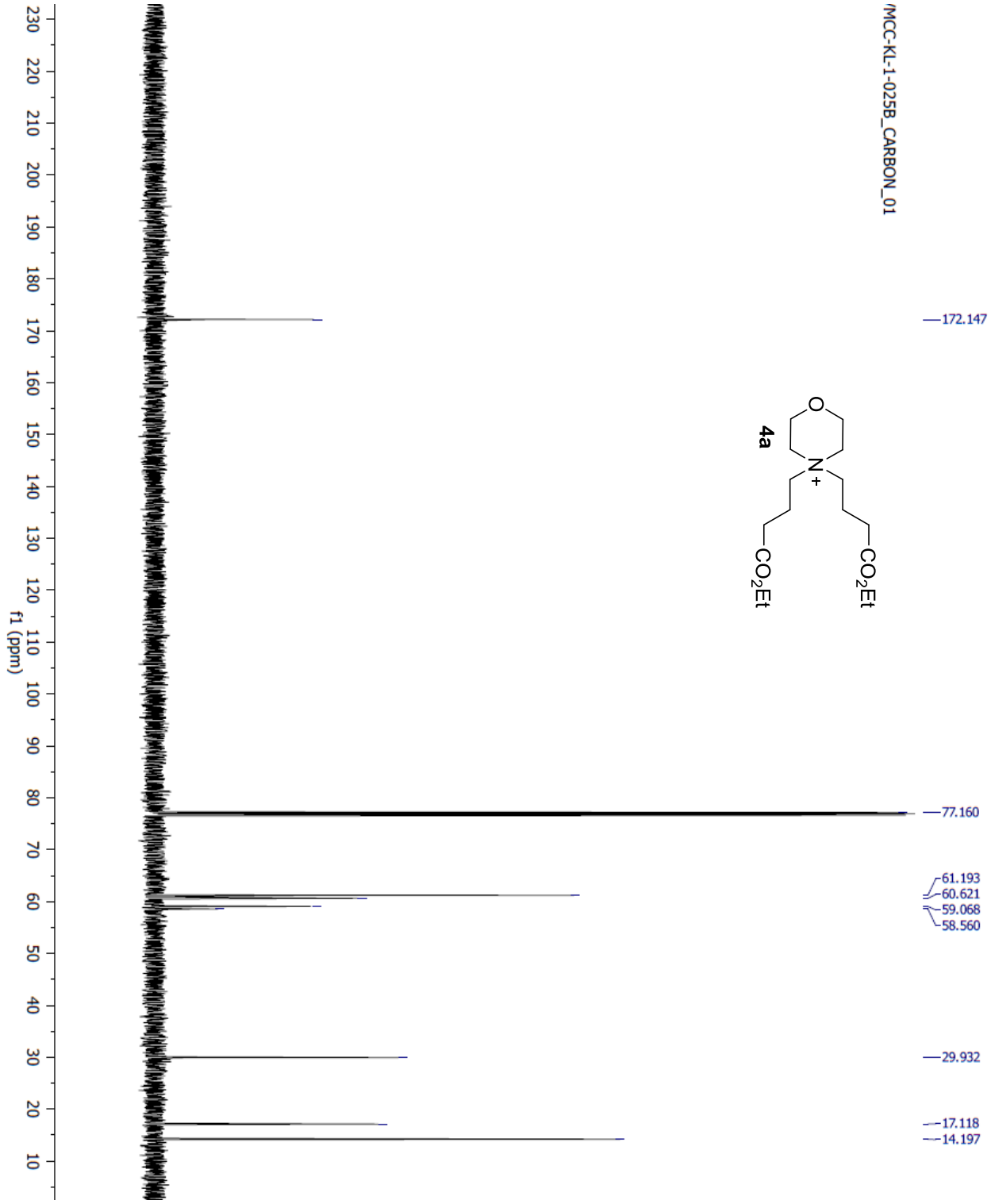
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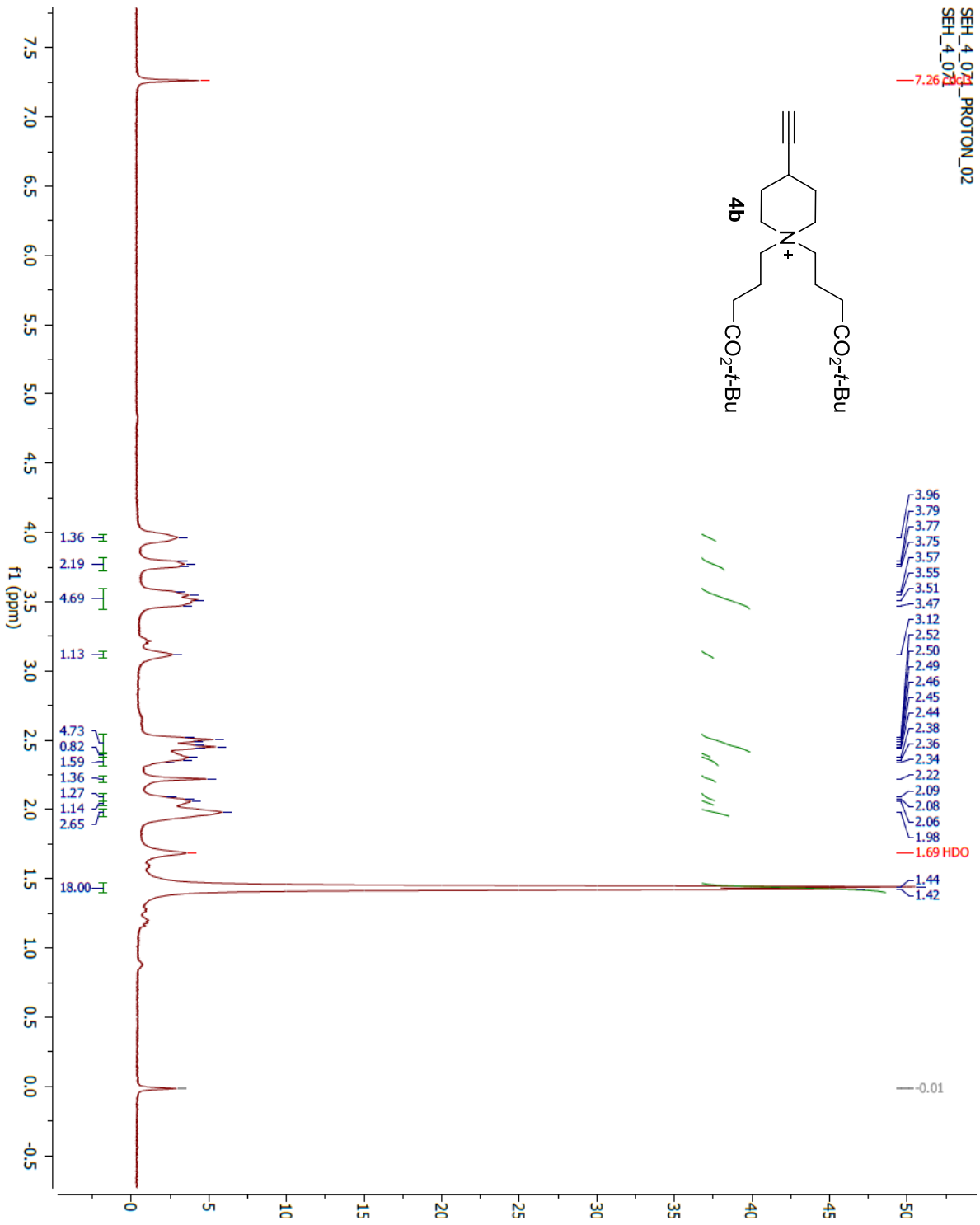
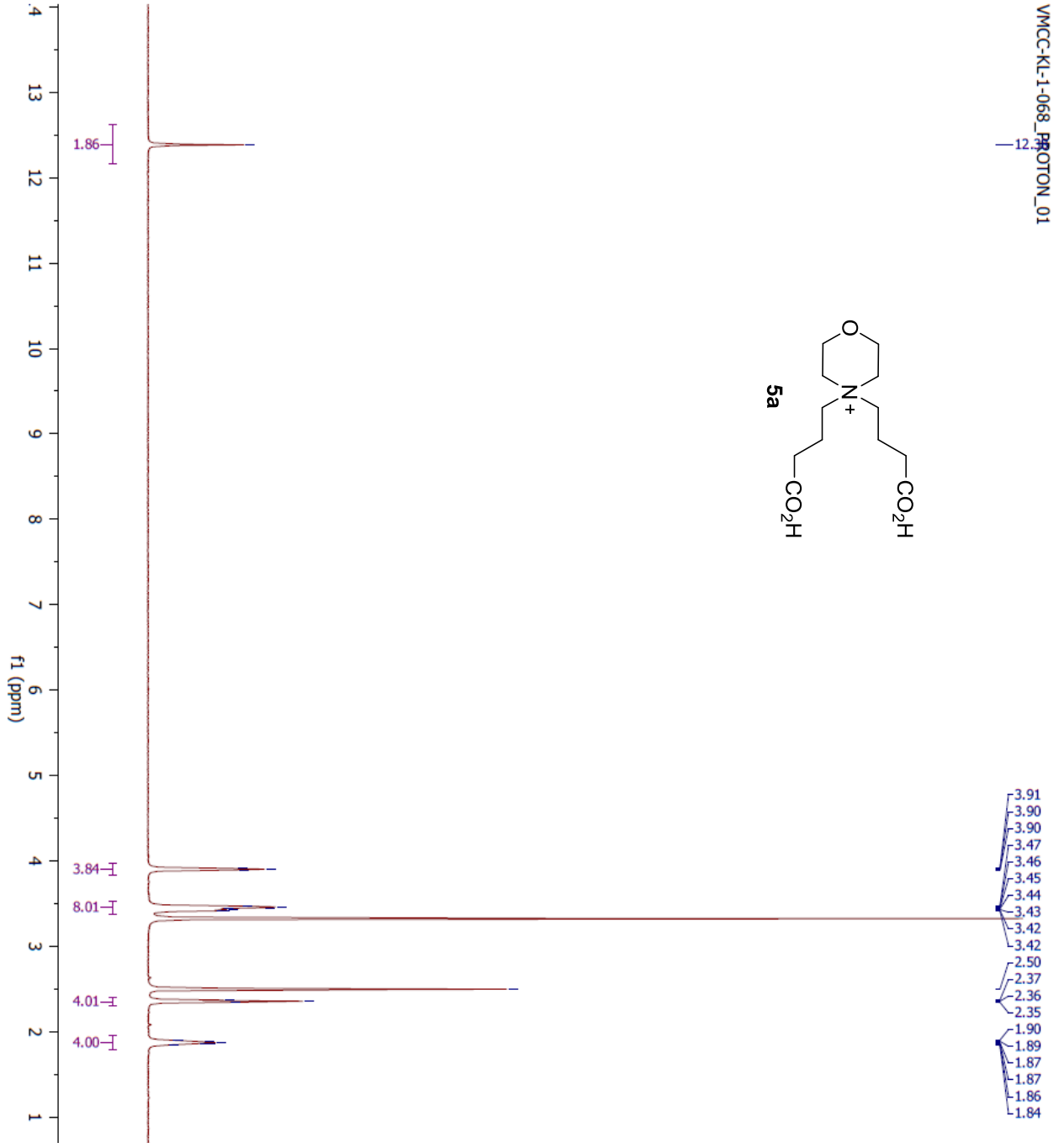
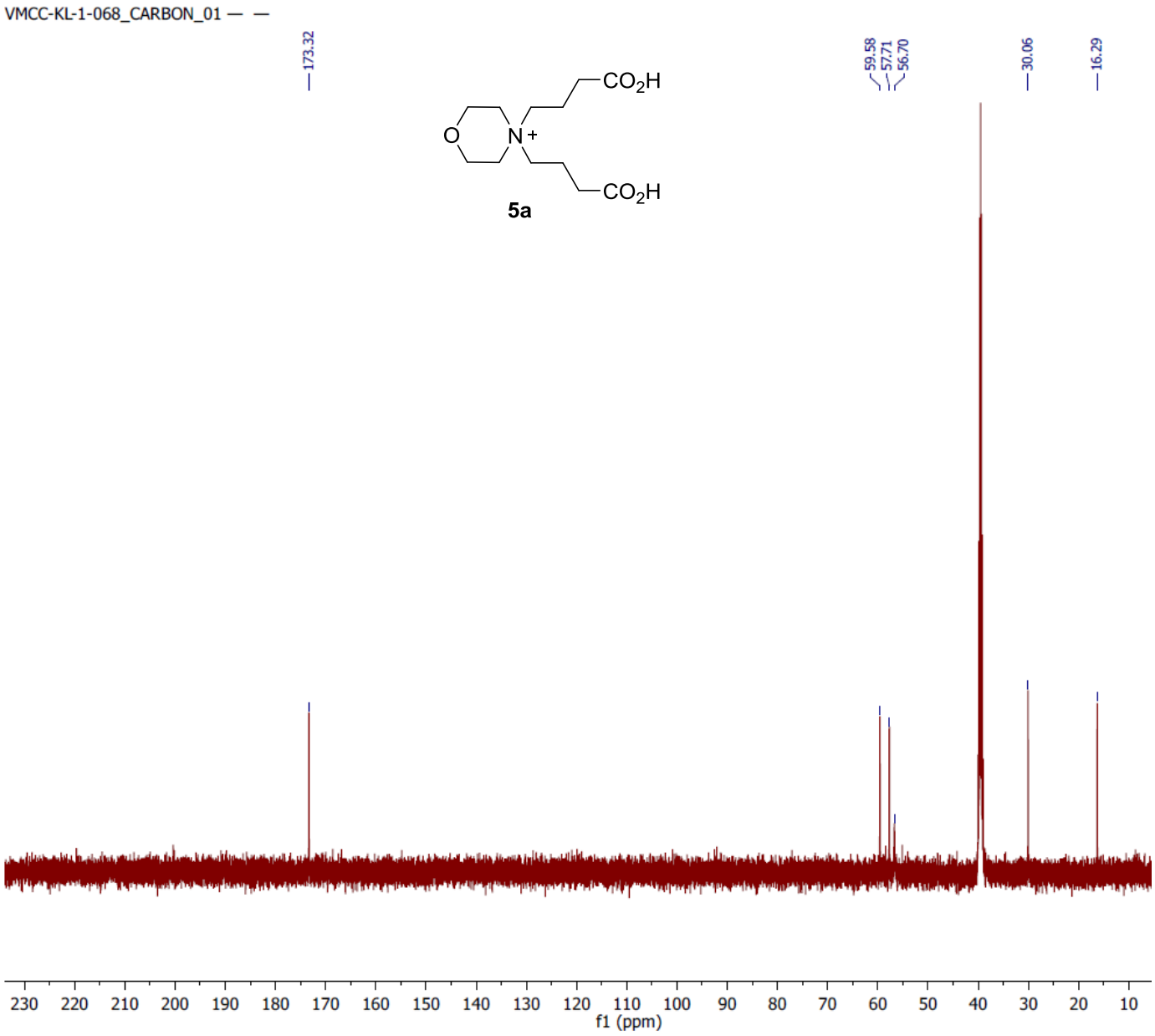
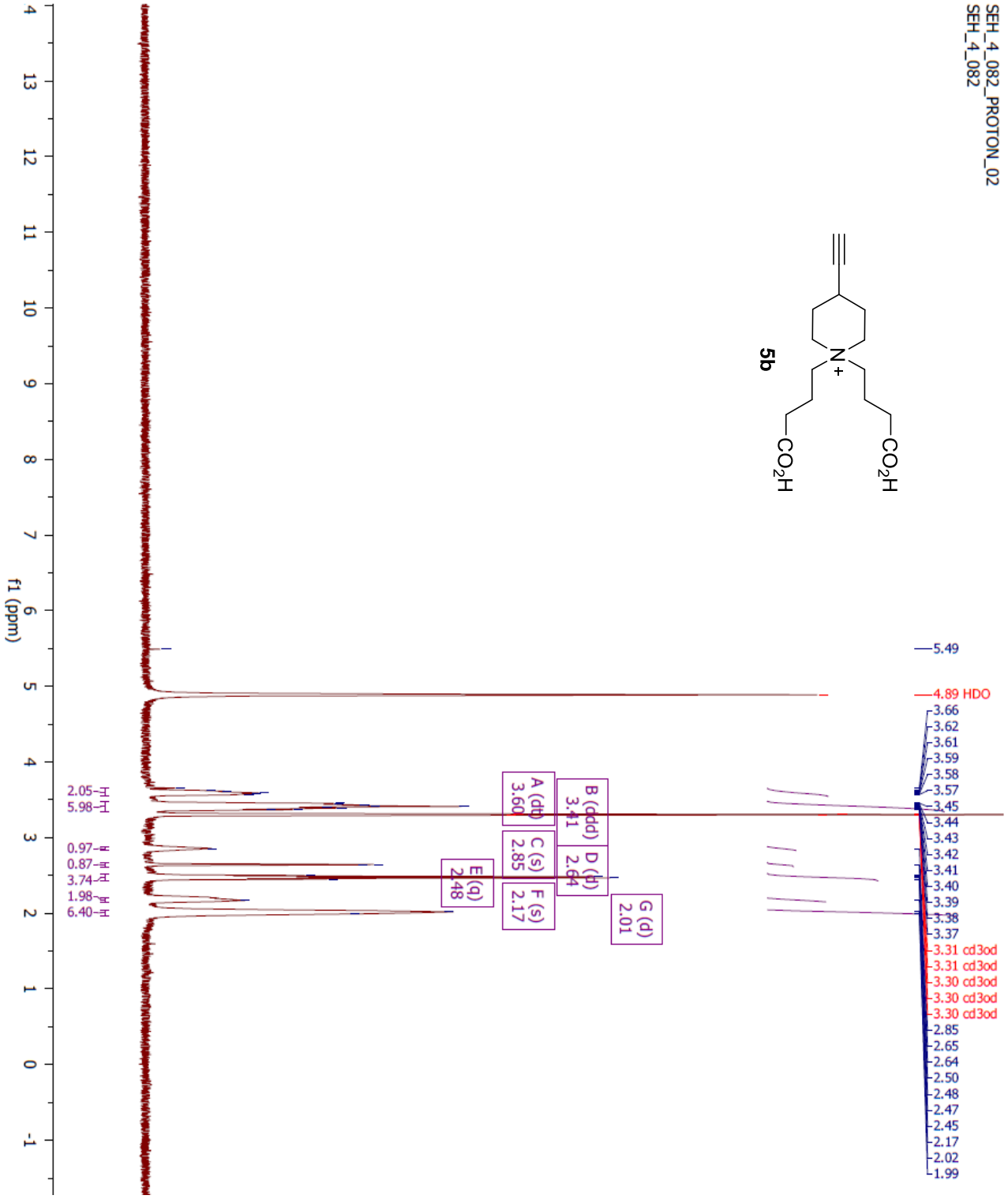
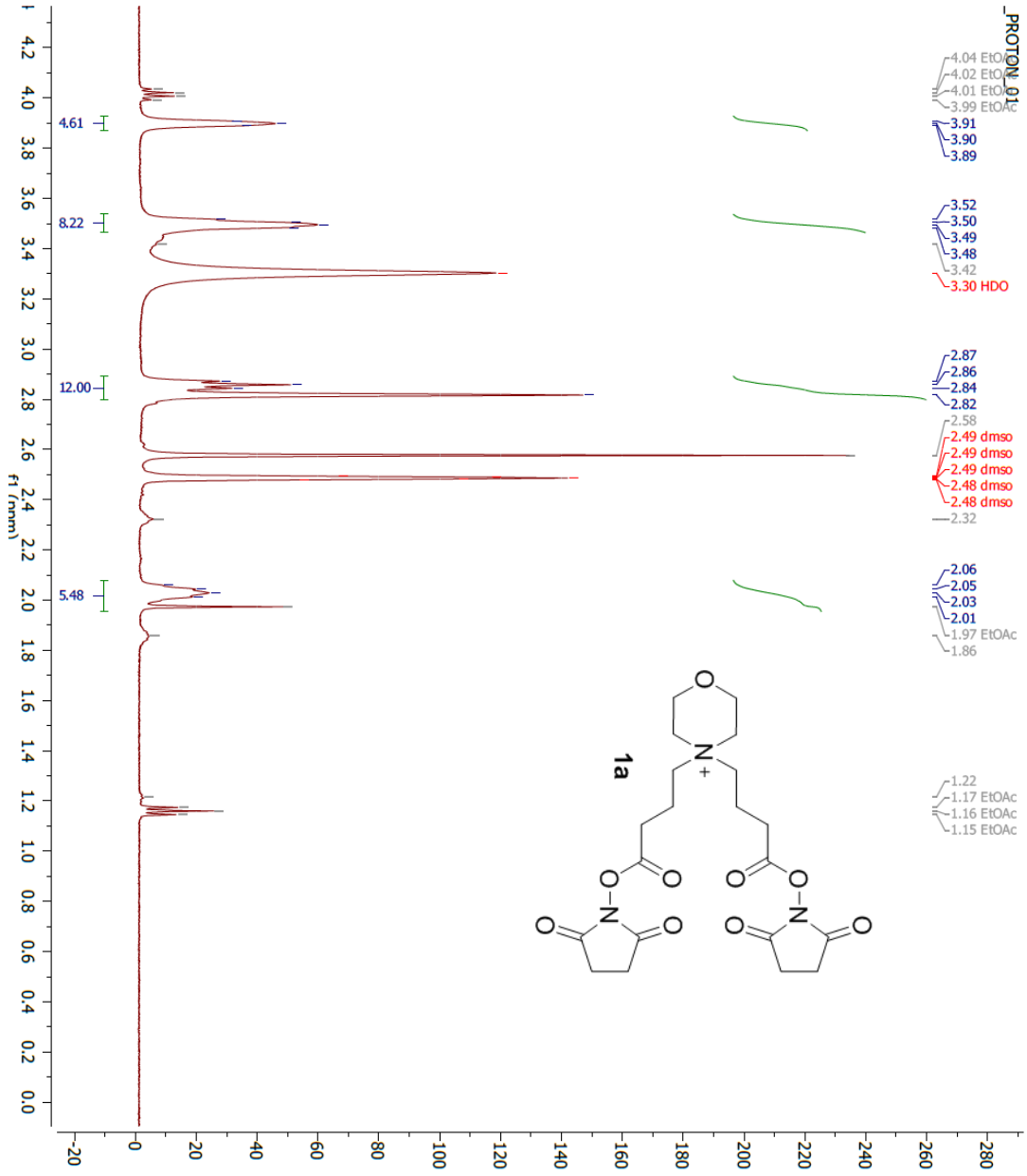
14.197
f1 (ppm)

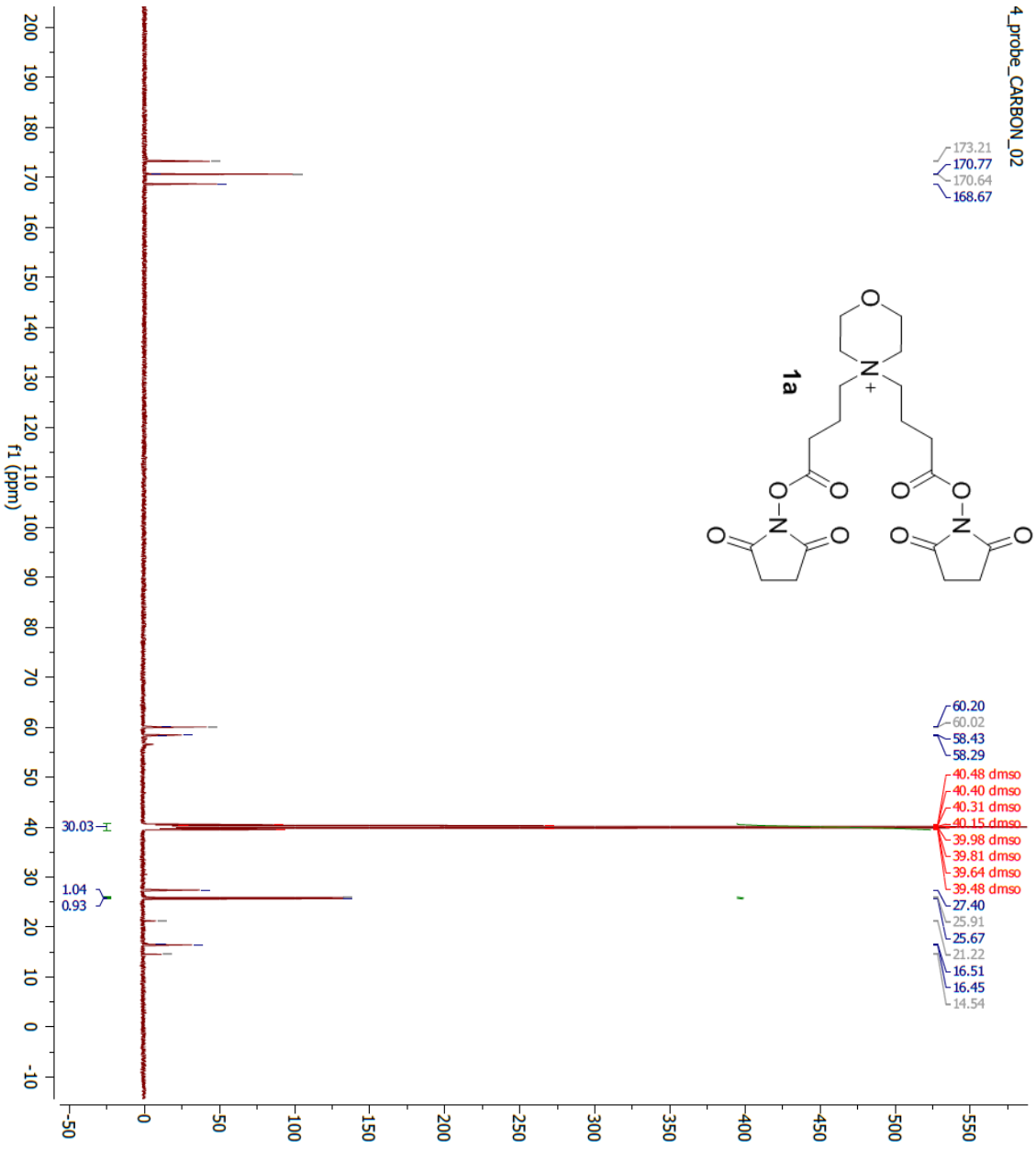

SEH_4_071_PROTON_02
SEH_4_071
7.26
4b
CO2-t-Bu
CO2-t-Bu
3.96
3.79
3.77
3.75
3.57
3.55
3.51
3.47
3.12
2.52
2.50
2.49
2.46
2.45
2.44
2.38
2.36
2.34
2.22
2.09
2.08
2.06
1.98
1.69 HDO
1.44
1.42
-0.01
1.36
2.19
4.69
1.13
4.73
0.82
1.59
1.36
1.27
1.14
2.65
18.00
f1 (ppm)

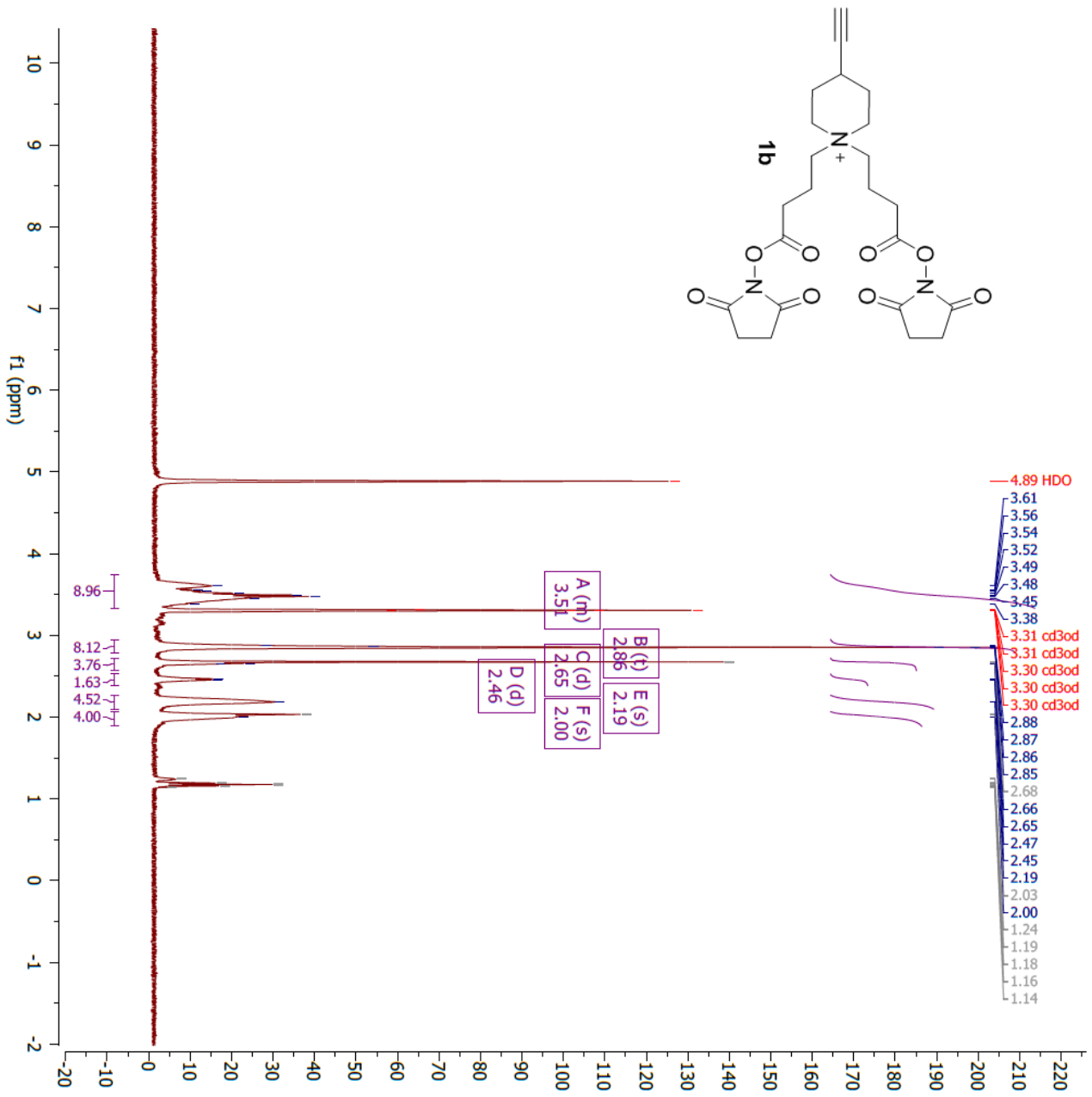

VMCC-KL-1-068_PROTON_01
12.33
5a
1.86
3.84
8.01
4.01
4.00
f1 (ppm)
3.91
3.90
3.90
3.47
3.46
3.45
3.44
3.43
3.42
3.42
2.50
2.37
2.36
2.35
1.90
1.89
1.87
1.87
1.86
1.84

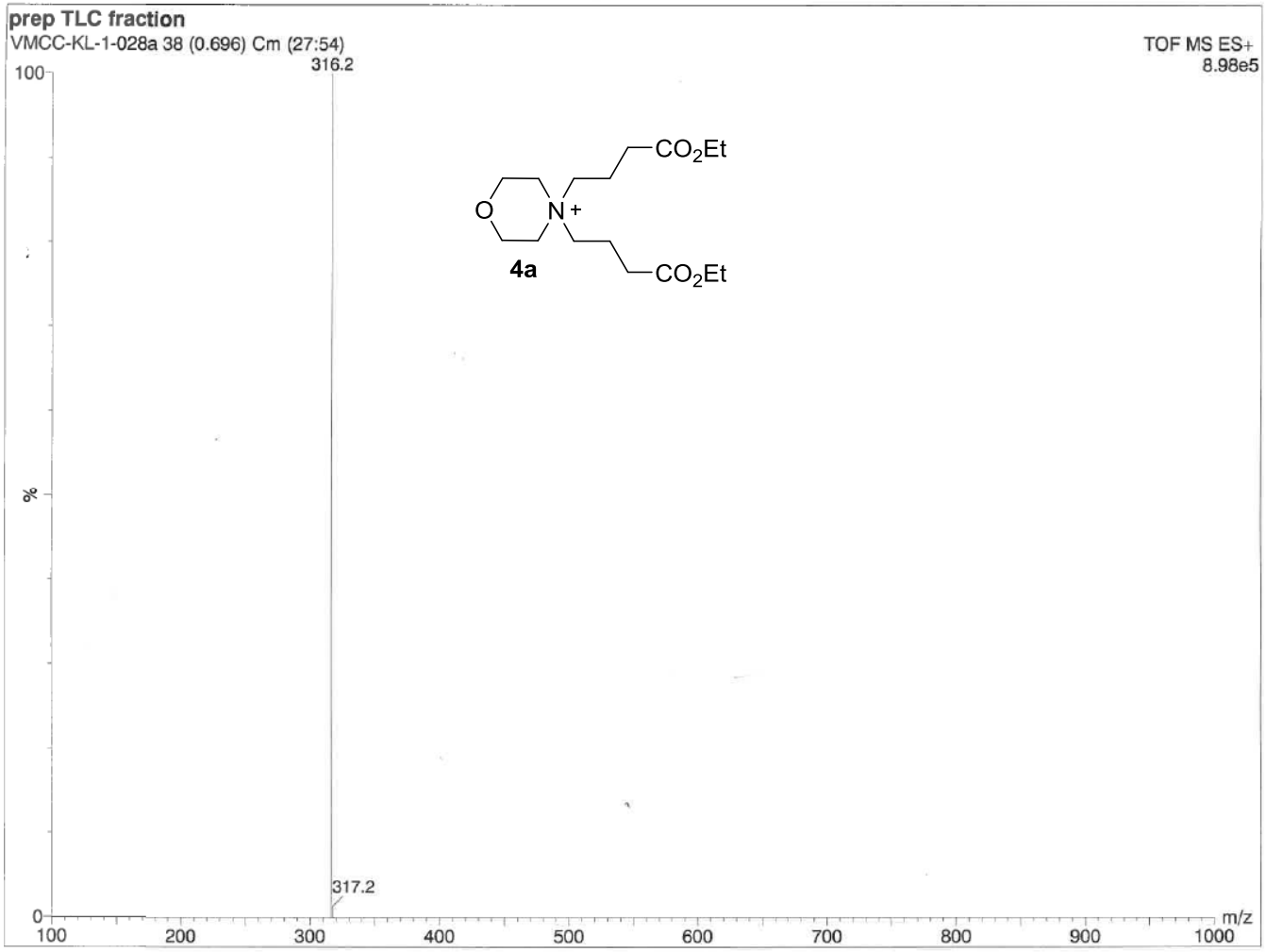

VMCC-KL-1-068_CARBON_01
173.32
59.58
57.71
56.70
30.06
16.29
CO2H
O
N+
CO2H
5a
230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
f1 (ppm)

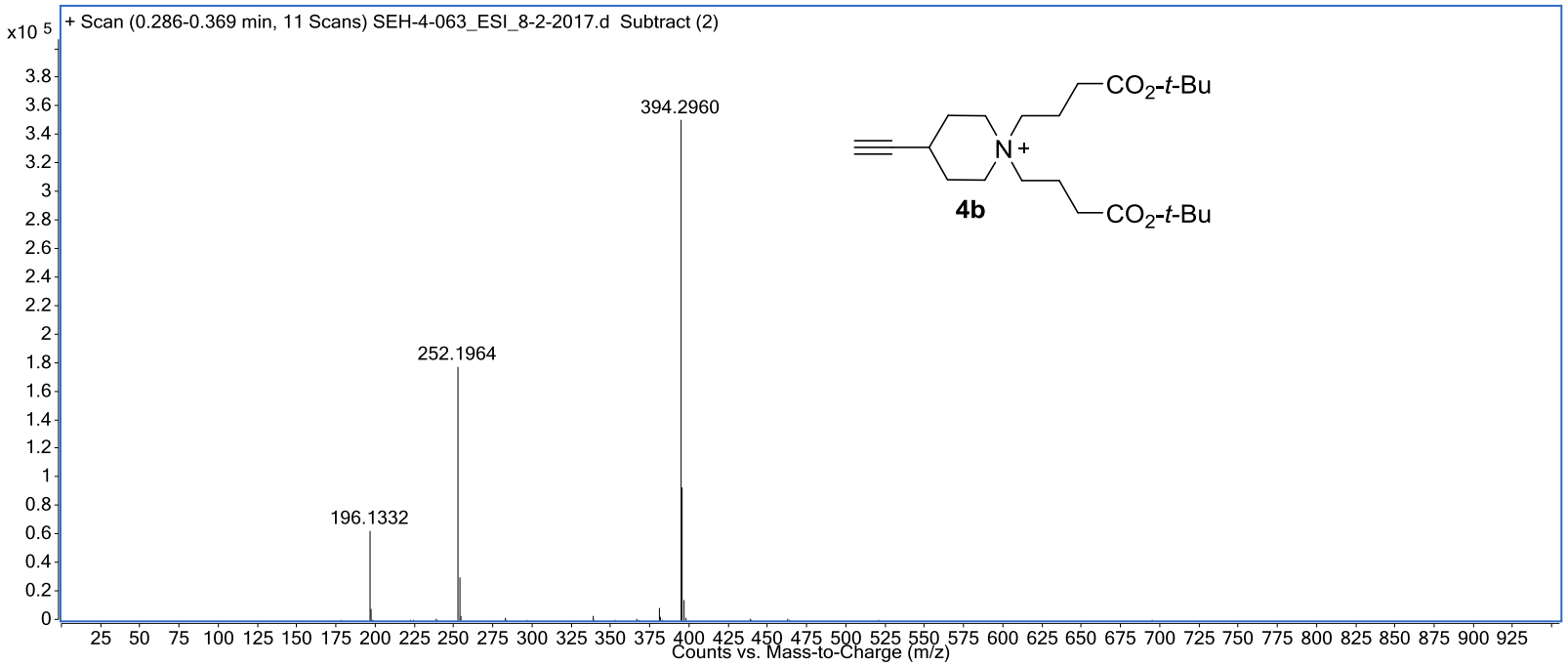

SEH_4_082_PROTON_02
SEH_4_082
5b
CO2H
CO2H
N+
f1 (ppm)
A (dt) 3.60
B (ddd) 3.41
C (s) 2.85
D (t) 2.64
E (q) 2.48
F (s) 2.17
G (d) 2.01
2.05
5.98
0.97
0.87
3.74
1.98
6.40
5.49
4.89 HDO
3.66
3.62
3.61
3.59
3.58
3.57
3.45
3.44
3.43
3.42
3.41
3.40
3.39
3.38
3.37
3.31 cd3od
3.31 cd3od
3.30 cd3od
3.30 cd3od
3.30 cd3od
2.85
2.65
2.64
2.50
2.48
2.47
2.45
2.17
2.02
1.99

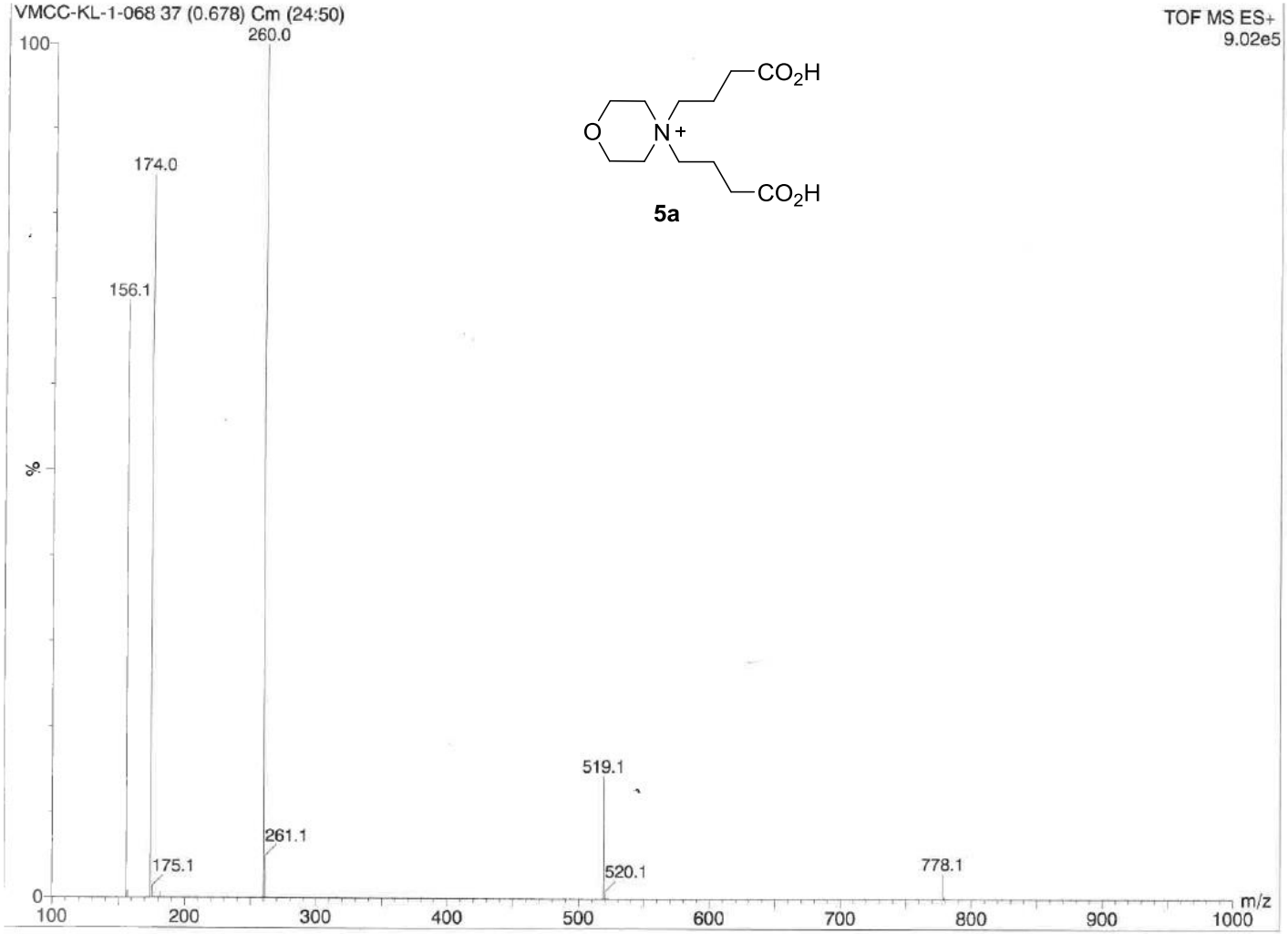

_PROTON_01
4.61
8.22
12.00
5.48
f1 (ppm)
4.04 EtOAc
4.02 EtOAc
4.01 EtOAc
3.99 EtOAc
3.91
3.90
3.89
3.52
3.50
3.49
3.48
3.42
3.30 HDO
2.87
2.86
2.84
2.82
2.58
2.49 dmso
2.49 dmso
2.49 dmso
2.48 dmso
2.48 dmso
2.32
2.06
2.05
2.03
2.01
1.97 EtOAc
1.86
1.22
1.17 EtOAc
1.16 EtOAc
1.15 EtOAc
1a


4_probe_CARBON_02
173.21
170.77
170.64
168.67
1a
60.20
60.02
58.43
58.29
40.48 dmso
40.40 dmso
40.31 dmso
40.15 dmso
39.98 dmso
39.81 dmso
39.64 dmso
39.48 dmso
27.40
25.91
25.67
21.22
16.51
16.45
14.54
30.03
1.04
0.93
f1 (ppm)


1b
f1 (ppm)
8.96
8.12
3.76
1.63
4.52
4.00
A (m) 3.51
B (t) 2.86
C (d) 2.65
D (d) 2.46
E (s) 2.19
F (s) 2.00
4.89 HDO
3.61
3.56
3.54
3.52
3.49
3.48
3.45
3.38
3.31 cd3od
3.31 cd3od
3.30 cd3od
3.30 cd3od
3.30 cd3od
2.88
2.87
2.86
2.85
2.68
2.66
2.65
2.47
2.45
2.19
2.03
2.00
1.24
1.19
1.18
1.16
1.14

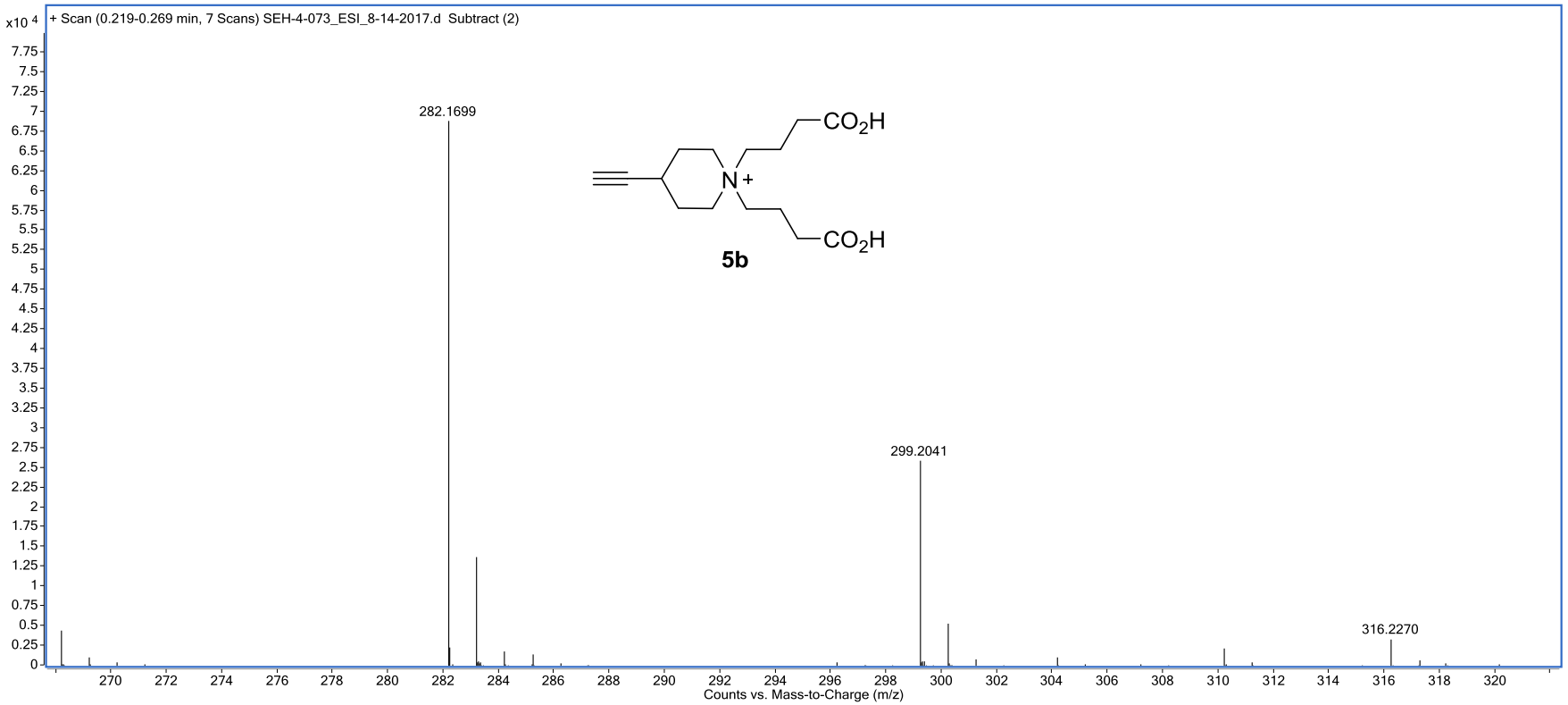

prep TLC fraction
VMCC-KL-1-028a 38 (0.696) Cm (27:54)
TOF MS ES+
8.98e5
316.2
317.2
100
%
0
100
200
300
400
500
600
700
800
900
1000
m/z
O
N+
CO2Et
CO2Et
4a

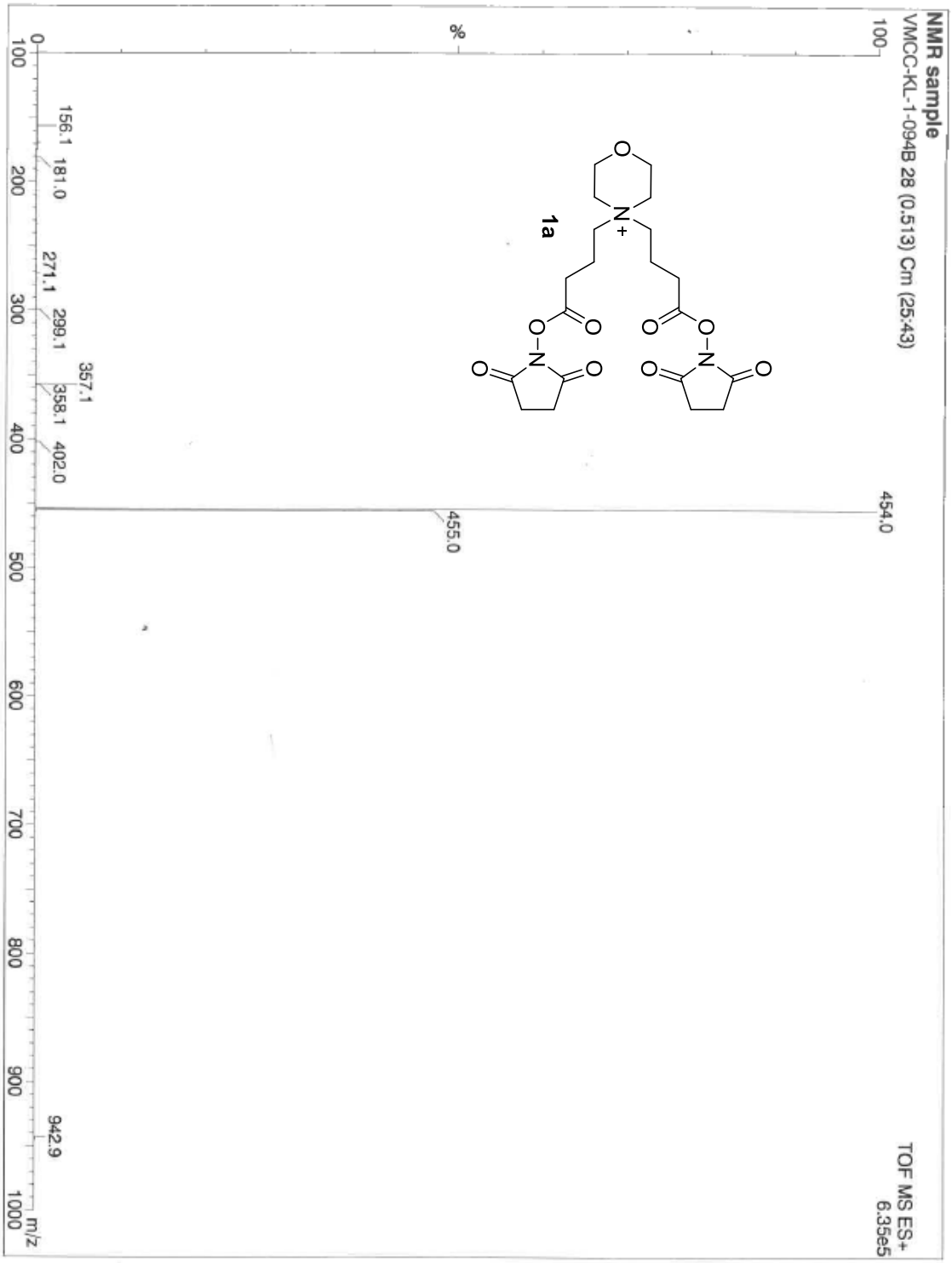

+ Scan (0.286-0.369 min, 11 Scans) SEH-4-063_ESI_8-2-2017.d Subtract (2)
x10 5
394.2960
252.1964
196.1332
CO2-t-Bu
N+
CO2-t-Bu
4b
Counts vs. Mass-to-Charge (m/z)

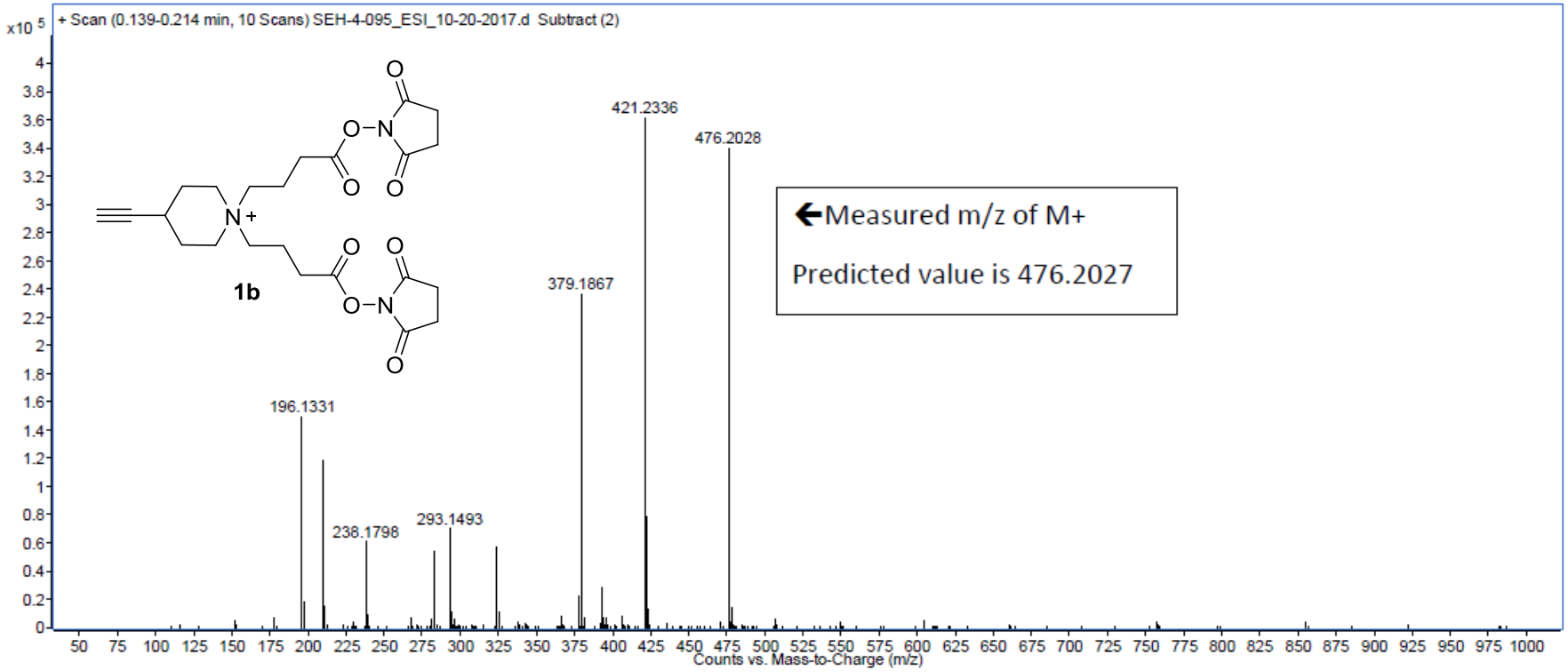

VMCC-KL-1-068 37 (0.678) Cm (24:50)
TOF MS ES+
9.02e5
100
%
0
260.0
174.0
156.1
519.1
261.1
175.1
520.1
778.1
100
200
300
400
500
600
700
800
900
1000
m/z

CO2H
O
N+
CO2H

5a


x10 4
+ Scan (0.219-0.269 min, 7 Scans) SEH-4-073_ESI_8-14-2017.d Subtract (2)
282.1699
299.2041
316.2270
CO2H
N+
CO2H
5b
Counts vs. Mass-to-Charge (m/z)


NMR sample
VMCC-KL-1-094B 28 (0.513) Cm (25:43)
TOF MS ES+
6.35e5
%
m/z
1a
454.0
455.0
156.1
181.0
271.1
299.1
357.1
358.1
402.0
942.9


+ Scan (0.139-0.214 min, 10 Scans) SEH-4-095_ESI_10-20-2017.d Subtract (2)
x10 5
1b
421.2336
476.2028
379.1867
196.1331
238.1798
293.1493
Measured m/z of M+
Predicted value is 476.2027
Counts vs. Mass-to-Charge (m/z)