Fully Automated Sequential Solid Phase Approach Towards Viral RNA-Nucleopeptides

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Supporting Information

General: 3'-O-DMT-2'-O-TBDMS-N^9-benzoyladenosine-5'-(N,N-di-isopropylamino-2-cyanoethoxy) phosporamidite, 3'-O-DMT-2'-O-TBDMS-uridine-5'-(N,N-di-isopropylamino-2-cyanoethoxy) phosporamidite, -O-DMT-2'-O-TBDMS – N^4-acetylcytidine-5'-(N,N-di-isopropylamino-2-cyanoethoxy) phosporamidite and 3'-O-DMT-2'-O-TBDMS – N^2-isobutyrylguanosine-5'-(N,N-di-isopropylamino-2-cyanoethoxy) phosporamidite were purchased from ChemGenes Corporation, Wilmington, USA and used as received. HCP Custom Primer Support™ Amino 200 resin was purchased from GE Healthcare.

LC/MS analysis was performed on **A)** Jasco HPLC system (UV detection simultaneously at 214 and 254 nm) coupled to a PE/SCIEX API 165 single quadruple mass spectrometer (Perkin-Elmer) using an analytical Gemini C_{18} column (Phenomex, 50 x 4.60 mm, 3 micron) in combination with eluents A: H_2O; B: MeCN and C: 0.1 M aq. NH_4OAc **B)** Thermo Finnigan LCQ Advantage MAX ion-trap mass spectrometer with an electrospray ion source coupled to Surveyor HPLC system (Thermo Finnegan) using an analytical Gemini C_{18} column (Phenomex, 50 x 4.60 mm, 3 micron) in combination with eluents A: H_2O; B: MeCN and C: 1% aq. TFA as the solvent system.

High resolution mass spectra spectra were recorded by direct injection (2 µL of a 2 µM solution in water/acetonitrile; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60000 at m/z 400 (mass range m/z = 150-2000) and dioctylpthalate (m/z = 391.2842) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan).

HCP-HMBA resin

Amino HCP resin (1 g, 200 µmol) was treated with 4-hydroxymethylbenzoic acid (HMBA) (92 mg, 600 µmol), BOP (266 mg, 600 µmol), HOBt (82 mg, 600 µmol) and DiPEA (200 µl, 1.2 mmol) in 10 ml DMF for 5 hours at room temperature. After washing with DCM three times, the unreacted amines were capped using 230 µl acetic anhydride, 270 µl DiPEA, 10 mg HOBt in 5 mL NMP for 5 minutes. The resin was then washed three times with DMF, DCM and subsequently air dried.

Fmoc-Gly-Ala-Tyr-Thr-Gly-HMBA-HCP resin (3)

Starting from HCP-HMBA resin (500 mg, 100 µmol) Fmoc-Gly-OH (150 mg, 500 µmol) was coupled using DIC (101 µL, 500 µmol) and catalytic DMAP in DCM (5 mL) for 4 hours at room temperature. After washing with DCM the unreacted resin was capped using the procedure used for oligonucleotide capping as described below. The resin was transferred to an automated peptide synthesizer (ABI-433A, Applied Biosystems, Perkin-Elmer) and the peptide was elongated with Fmoc-Thr(OTBDMS)-OH, Fmoc-Tyr-OH, Fmoc-Ala-OH and Fmoc-Gly-OH using the following repetitive steps:

- Fmoc cleavage using 20% piperidine in DMF.
- Coupling of the appropriate amino acid applying a five-fold excess, activation by 5 eq. HCTU in NMP (0.25M) and 12.5 eq. DiPEA in NMP (1.25 M) for 1 hour.

An analytical sample of resin 3 (ca. 5 µmol) was treated with a saturated solution of NH_3 in trifluoroethanol (1 mL) for 16 hours at room temperature after which the reaction mixture was filtrated and concentrated. LCMS (0 – 90 % MeCN, conditions **B**, 15

min run) Rt = 6.40. analysis indicated formation of one product corresponding to the partially protected peptide carboxamide H-Gly-Ala-Tyr-Thr(TBDMS)-Gly-NH_2.

Procedure for solid phase oligonucleotide synthesis:

The oligoribonucleotides were prepared on a 10 µmol scale using an Akta Oligo Pilot (GE) automated nucleic acid synthesizer starting from peptidyl-HMBA-HCP resin 3. Couplings were performed using 5 eq. of commercially available amidites at 0.1 M concentration. 5-benzylmercaptotetrazole (BMT, 0.3 M) was used as the activating agent in 15 minute coupling cycles. Oxidation was performed by means of standard procedure using I_2 (50 mM) in pyridine/H_2O (90:10, v/v). Capping was performed using a standard protocol employing cap A (40% N-methylimidazole in MeCN) and cap B (20% Ac_2O, 20% lutidine in MeCN) 1:1 (v/v). Cleavage of the DMT group was accomplished using 5% DCA in toluene.

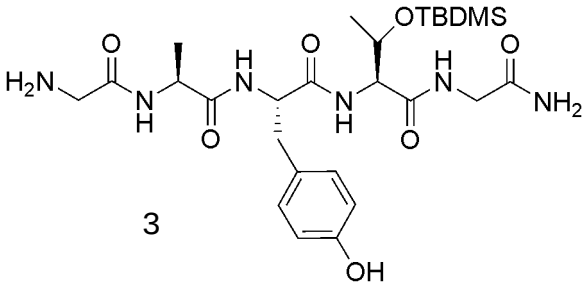
H-Gly-Ala-Tyr(pUpUpApA)-Thr-Gly-NH_2 (1)

The RNA-fragment was assembled on pentapeptide resin 3 using the automated oligonucleotide synthesis protocol as described above on 10 µmol scale. The fully protected product was treated with sat. NH_3 in trifluoroethanol (10 mL) at room temperature for 16 hours to cleave the peptidylated oligonucleotide off the resin as well as removed all base labile protective groups. The resin was washed with 1 mL H_2O/EtOH, 1:1, v/v and the filtrate was concentrated. The crude peptidylated oligonucleotide was dissolved in DMSO (500 µL) and treated with TEA.HF (3 mL) for 16 hours at room temperature followed by precipitation in cold nBuOH, centrifugation and collection of the precipitate. The crude nucleopeptide was purified by ion-exchange chromatography using a Source-Q column (16 x 100 mm, GE-Healthcare) and a gradient of 0 – 50 % 20 mM NaOAc/1 M NaCl in 20 mM NaOAc/ 20 mM NaCl with a flow of 5 mL/min. Subsequent desalting on a Sephadex G25 column (26 x 200 mm, GE Healthcare) using 0.15 M NH_4HCO_3 with a flow of 5 mL/min resulted in the title compound 1 as ammonium salt (4.2 mg, 2.3 µmol, 23%). LC-MS (0 - 20 % MeCN, conditions **A**, 15 min run), Rt = 6.20. ESI-MS m/z: 1737.8 $[M + H]^+$, 869.6 $[M + 2H]^{2+}$. ^{31}P-NMR (161 MHz, D_2O); δ -0.32, -0.39, -0.42, -4.40. ^{1}H-NMR (600 MHz, D_2O); δ 8.27 (s, 1H, H-8 A), 8.21 (s, 1H, H-8 A), 8.09 (s, H-2 A), 7.88 (s, 1H, H-2 A), 7.75 (d, 1H, H-5 U), 7.55 (d, 1H, H-5 U), 7.07 (d, 1H, H_{arom} Tyr), 6.95 (d, 1H, H_{arom} Tyr), 5.92 (d, 1H, H-1'), 5.83 – 5.7 (m, 3H, 3x H-1'), 5.58 (d, 1H, H-6 U), 5.57 (d, 1H, H-6 U), 4.62 – 4.15 (m, 22H, 20x CH-furanose, 2x α amino acid), 3.85 – 3.83 (m, 2H, α Gly), 3.74 (s, 2H, α Gly), 3.60 – 3.59 (m, 1H, α amino acid), 3.04 – 3.01 (m, 1H, β Tyr), 2.92 – 2.88 (m, 1H, β Tyr), 1.23 – 1.19, 1.14 – 1.11 (2 m, 2 x 3H, 2 x CH_3, Ala + Thr). HRMS $[C_{58}H_{76}N_{20}O_{35}P_4 + H]^+$: found 1737.3841, calc. 1737.3850, $[C_{58}H_{76}N_{20}O_{35}P_4 + Na]^+$: found 1759.3611, calc. 1759.3624

H-Gly-Ala-Tyr(pUpUpApApApApCpApG)-Thr-Gly-NH_2 (2)

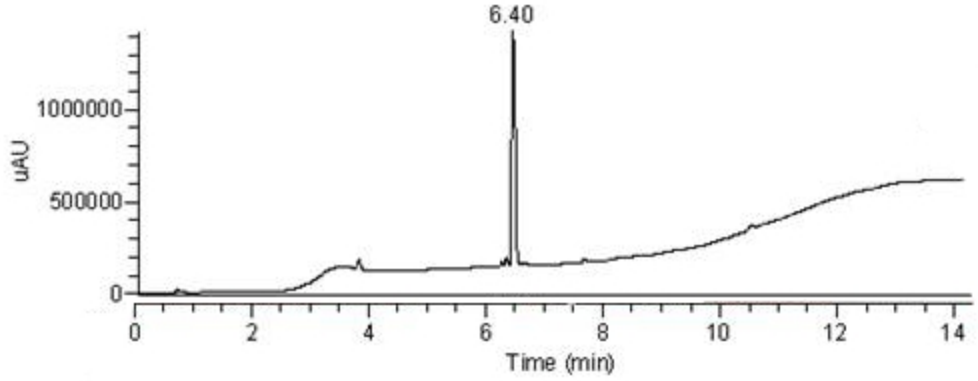
The RNA-fragment was assembled on pentapeptide 3 using the automated oligonucleotide synthesis protocol as described above on 10 µmol scale. The fully protected product was treated with sat. NH_3 in trifluoroethanol (10 mL) at a temperature of 40 ^{0}C for 16 hours to cleave the peptidylated oligonucleotide off the resin as well as removed all the base labile protective groups. The resin was washed with 1 mL H_2O/EtOH, 1:1, v/v and the filtrate was concentrated. The crude peptidylated oligonucleotide was dissolved in DMSO (500 µL) and treated with TEA.HF (3 mL) for 16 hours at room temperature followed by precipitation in cold nBuOH, centrifugation and collection of the precipitate. The crude nucleopeptide was purified by ion-exchange chromatography using a Source-Q column (16 x 100 mm, GE-Healthcare) and a gradient of 0 – 50 % 20 mM NaOAc/1 M NaCl in 20 mM NaOAc/ 20 mM NaCl with a flow of 5 mL/min. Subsequent desalting on a Sephadex G25 column (26 x 200 mm, GE Healthcare) using 0.15 M NH_4HCO_3 with a flow of 5 mL/min resulted in the title compound 2 as ammonium salt (5.1 mg, 1.4 µmol, 14 %). LC-MS (0 - 25 % MeCN, conditions **A**, 15 min run), Rt = 5.70. ESI-MS m/z: 1126.1 $[M + H]^{3+}$. ^{31}P-NMR (161 MHz, D_2O); δ -0.46, -0.51, -0.65, -0.69, -0.85, -4.42. ^{1}H-NMR (600 MHz, D_2O); δ 7.98 – 7.45 (m, 12 H, 5x H-2 A, 5x H-8 A, H-8 G, H-5 C), 7.24 (d, 1H, H-5 U), 7.10 (d, 1H, H-5 U), 6.83 (d, 1 H, H_{arom} Tyr), 6.70 (d, 1 H, H_{arom} Tyr), 5.59 – 5.25 (m, 10H, 7x H-1' furanose, 2 x H-6 U, H-6 C), 4.97 – 4.70 (m, 2H, 2x H-1'furanose), 4.53 – 3.73 (m, 49H, 45x CH furanose, 3x CH α, Ala, Thr, Tyr, 1 x β Thr) 2.79 – 2.76 (m, 1H, β Tyr), 2.65 – 2.62 (m, 1H, β Tyr), 1.02 – 0.97, 0.89 – 087 (2 m, 2 x 3H, 2 x CH_3, Ala + Thr). HRMS $[C_{107}H_{136}N_{43}O_{67}P_9 + H]^+$: found 3374.6152, calc. 3374.6267, $[C_{107}H_{136}N_{43}O_{67}P_9 + Na]^+$: found 3396.6006, calc. 3396.6087, $[C_{107}H_{136}N_{43}O_{67}P_9 + 2H]^{2+}$: found 1687.8194, calc. 1687.8174, $[C_{107}H_{136}N_{43}O_{67}P_9 + Na + H]^{2+}$: found 1698.8079, calc. 1698.8080, $[C_{107}H_{136}N_{43}O_{67}P_9 + 3H]^{3+}$: found 1125.5483, calc. 1125.5471, $[C_{107}H_{136}N_{43}O_{67}P_9 + Na + 2H]^{3+}$: found 1132.8757, calc. 1125.5471.

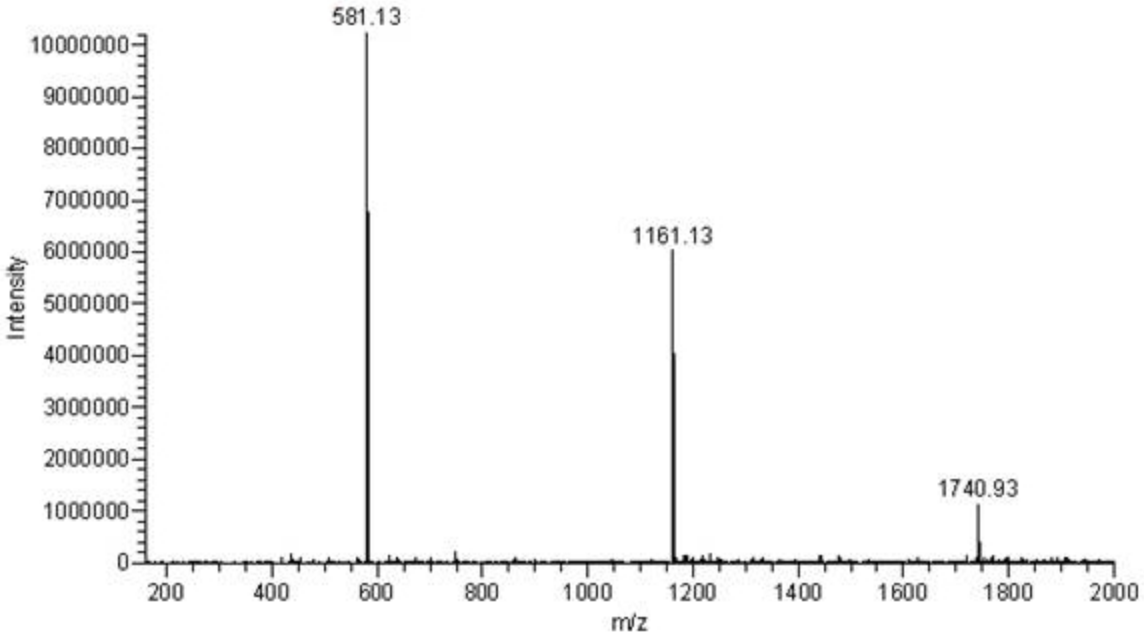
LC-MS analysis of crude 3.



3

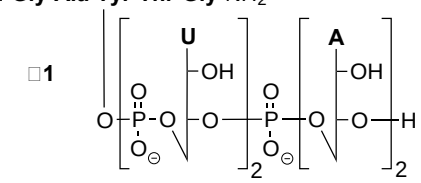
LCMS conditions B

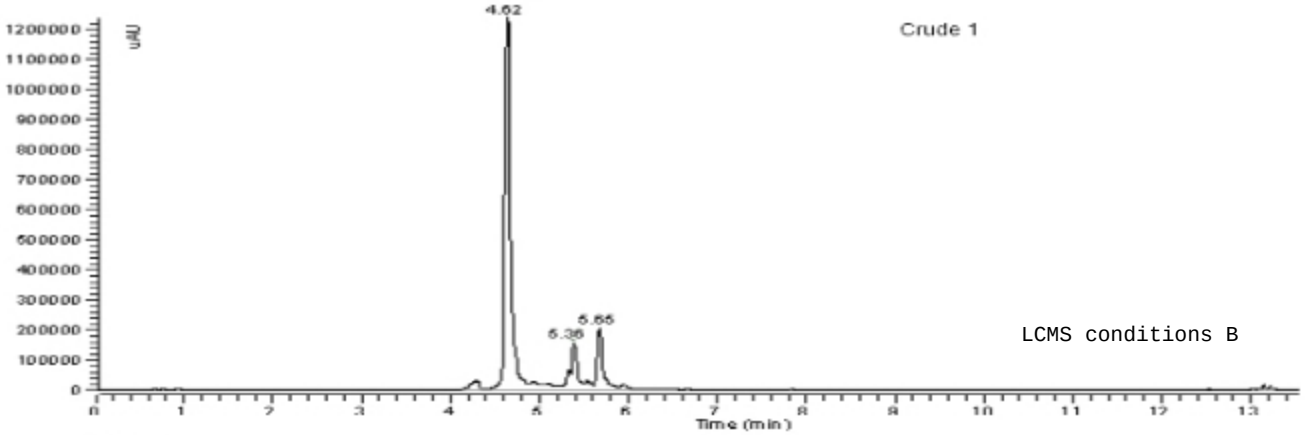


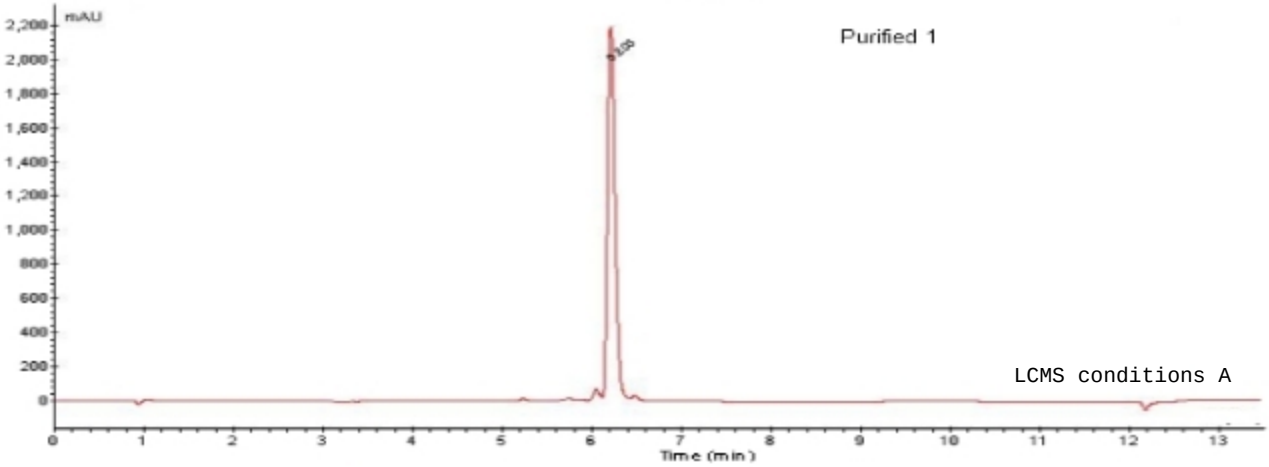


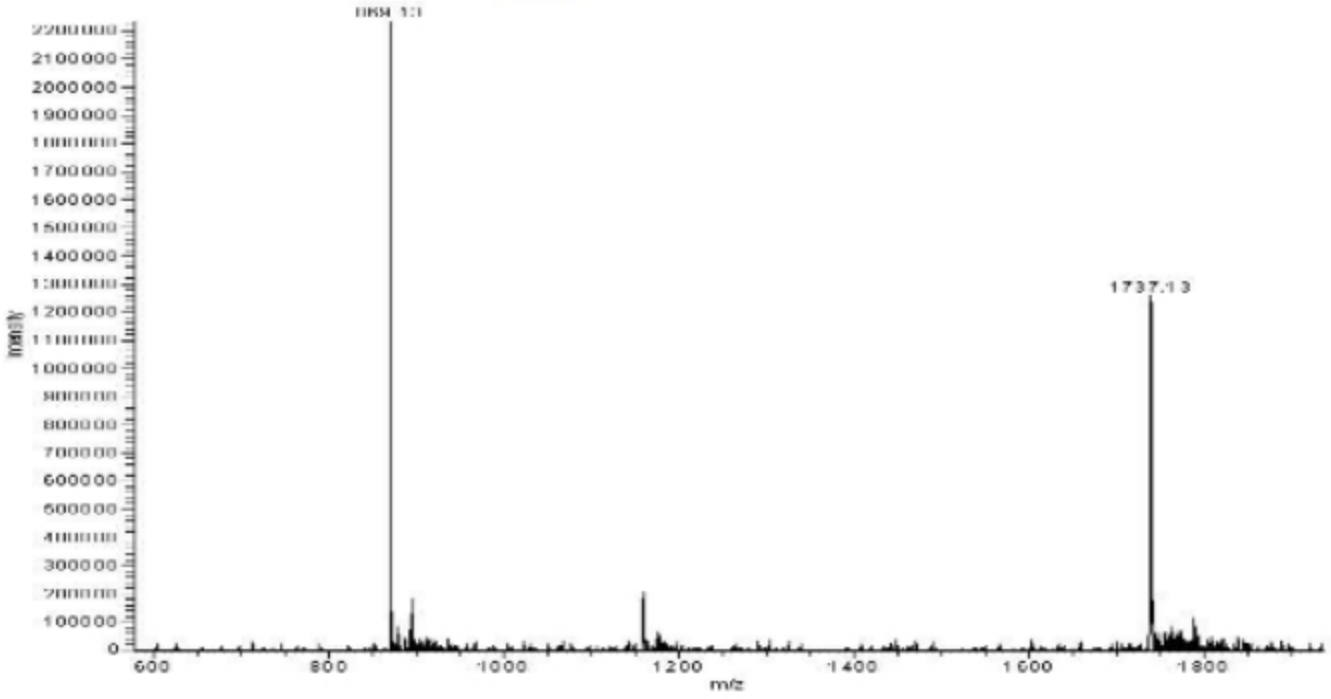
LC-MS analysis of compound 1

H-**Gly-Ala-Tyr-Thr-Gly**-NH_2

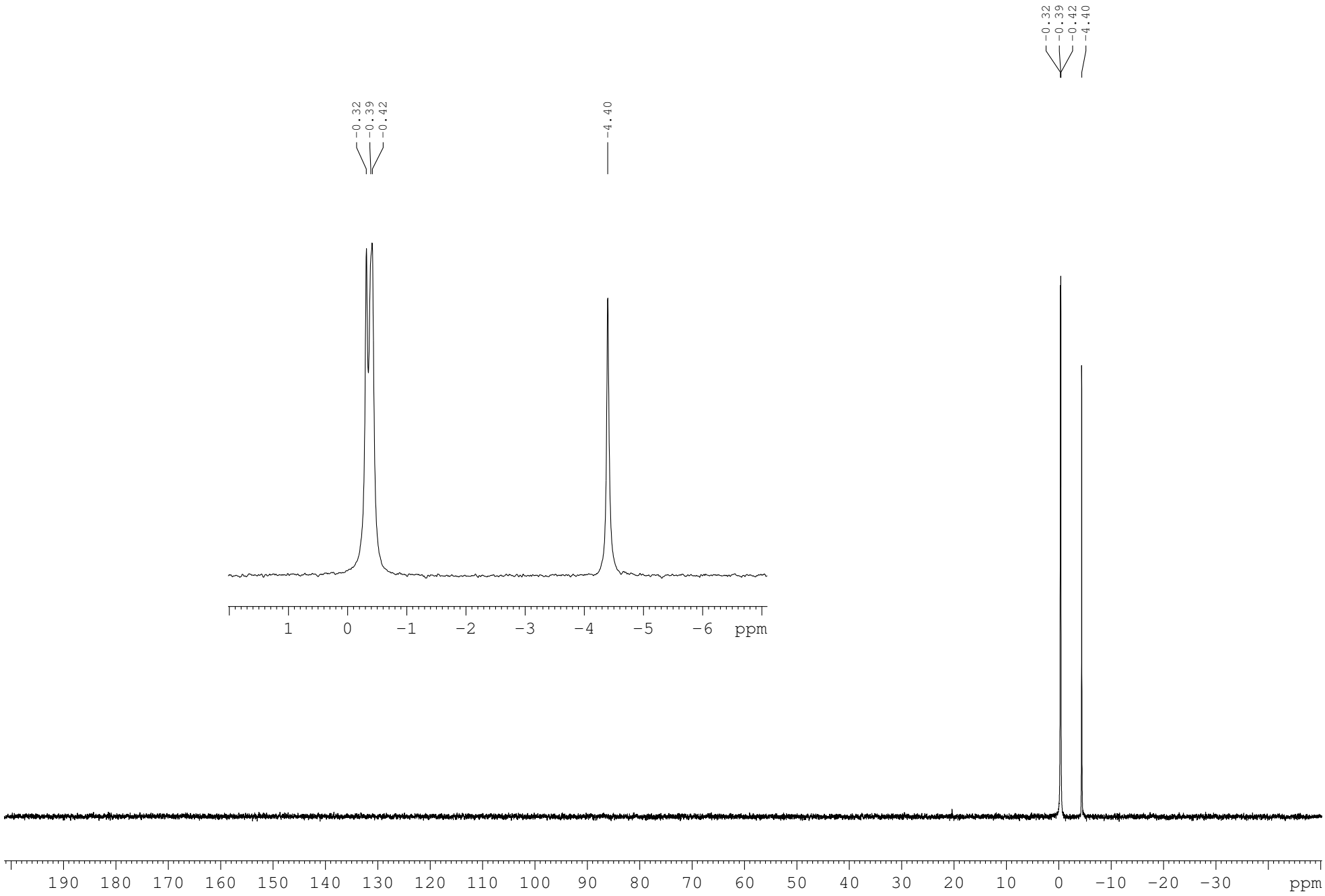


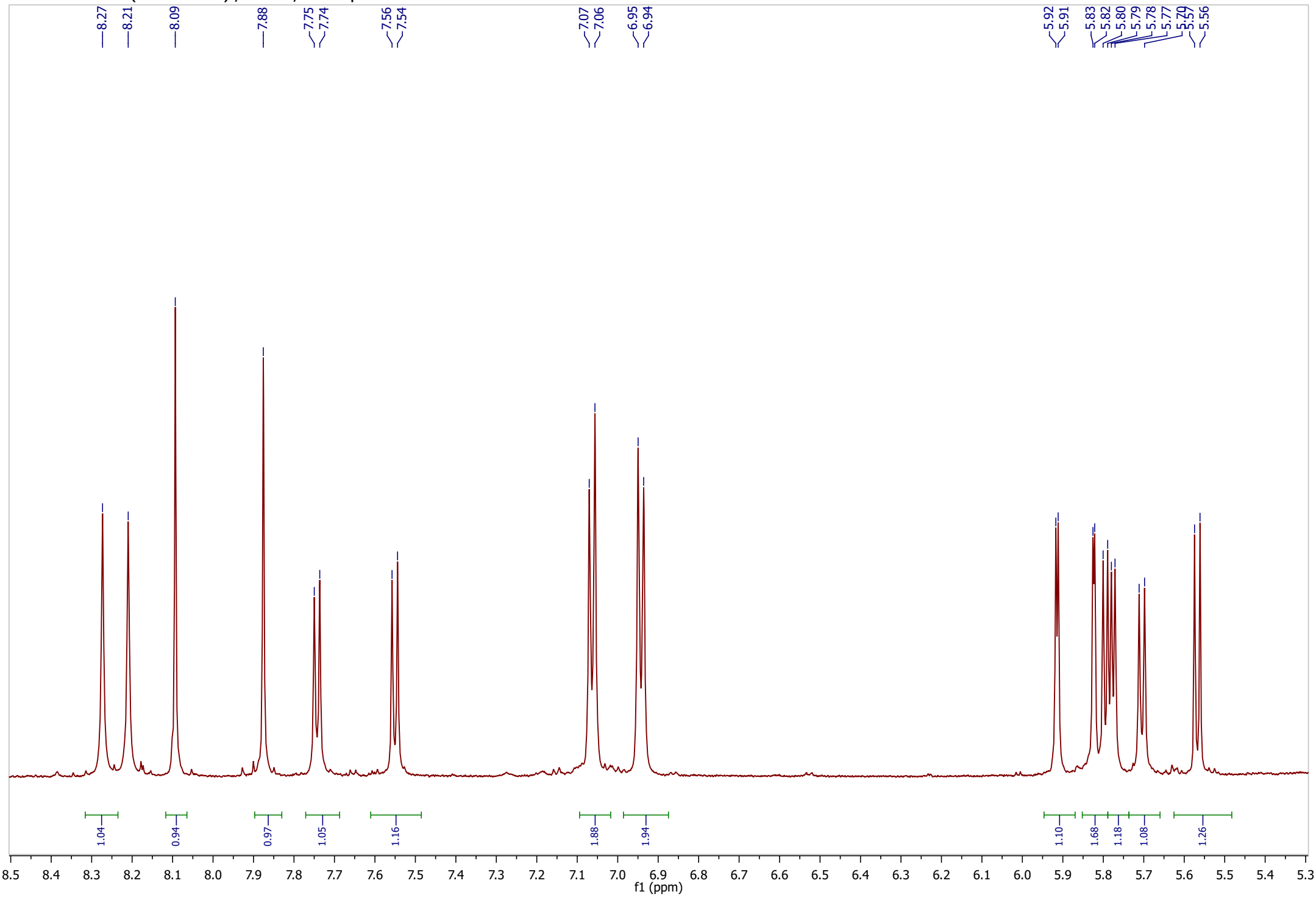


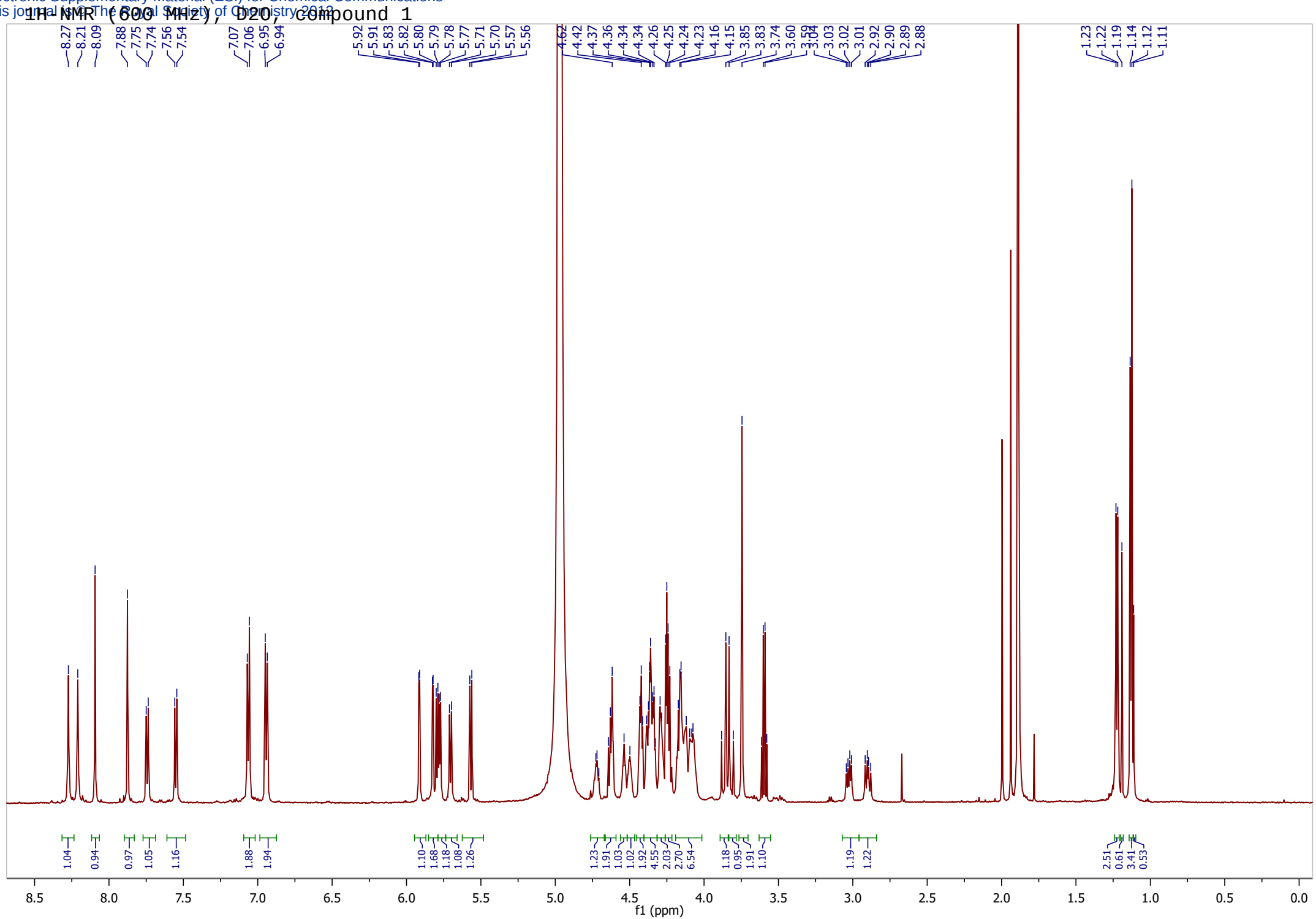




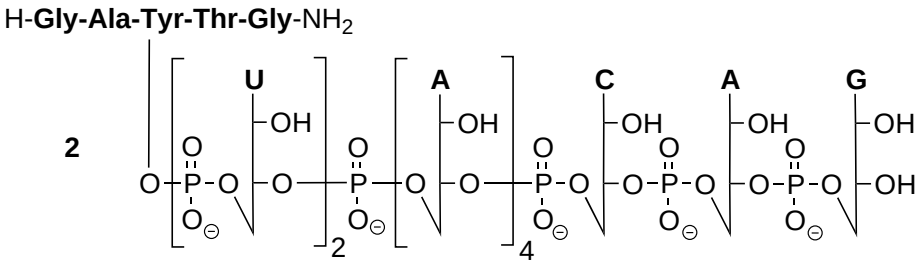
31P-NMR (161 MHz), D2O, compound 1

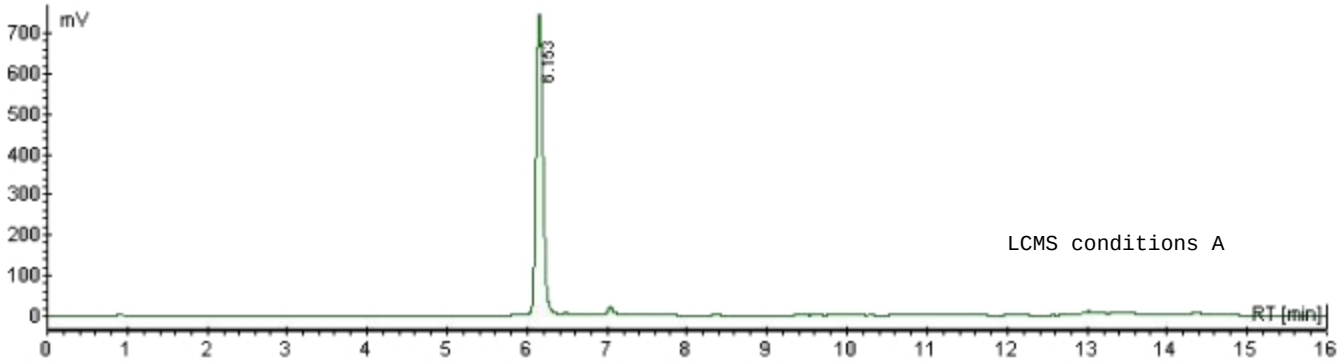


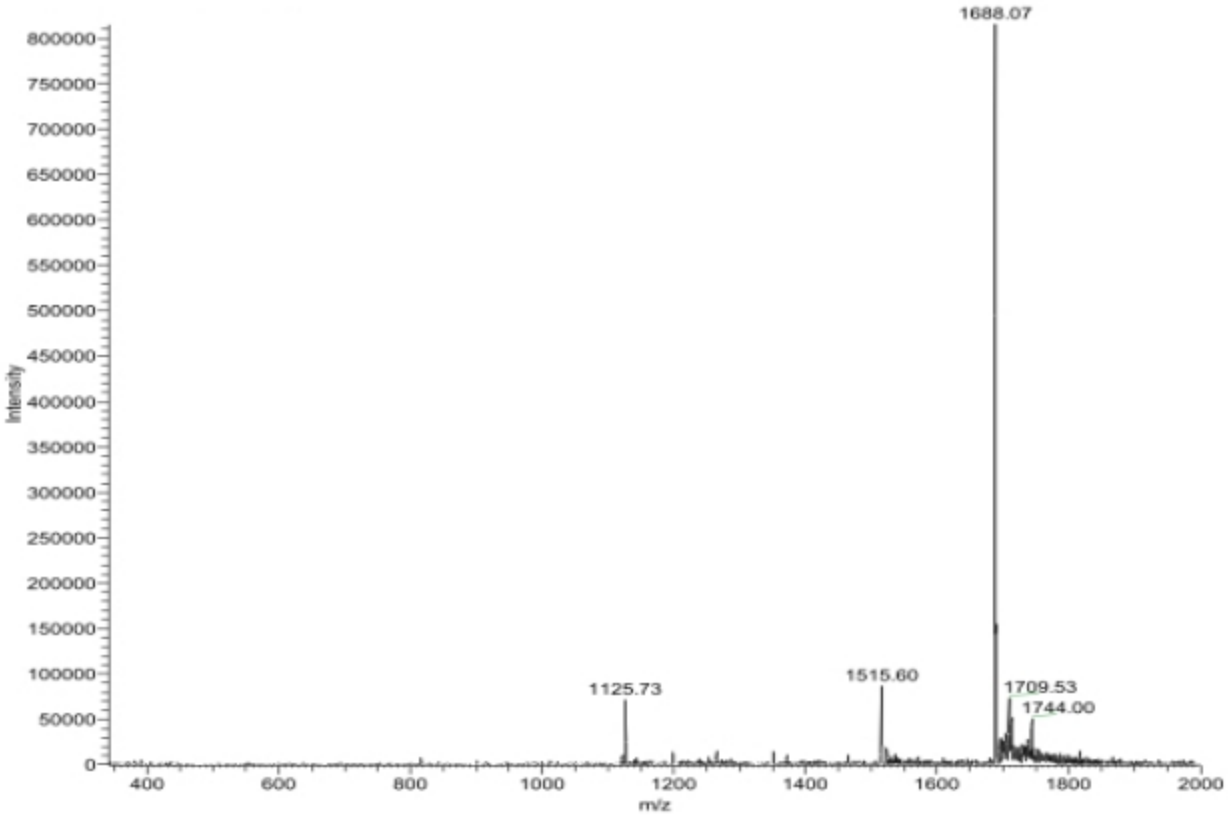

1H-NMR (600 MHz), D2O, compound 1
8.27
8.21
8.09
7.88
7.75
7.74
7.56
7.54
7.07
7.06
6.95
6.94
5.92
5.91
5.83
5.82
5.80
5.79
5.78
5.77
5.70
5.57
5.56
1.04
0.94
0.97
1.05
1.16
1.88
1.94
1.10
1.68
1.18
1.08
1.26
f1 (ppm)


1H-NMR (600 MHz), D2O, compound 1
8.27
8.21
8.09
7.88
7.75
7.74
7.56
7.54
7.07
7.06
6.95
6.94
5.92
5.91
5.83
5.82
5.80
5.79
5.78
5.77
5.71
5.70
5.57
5.56
4.62
4.42
4.37
4.36
4.34
4.34
4.26
4.25
4.24
4.23
4.16
4.15
3.85
3.83
3.74
3.60
3.59
3.04
3.03
3.02
3.01
2.92
2.90
2.89
2.88
1.23
1.22
1.19
1.14
1.12
1.11
1.04
0.94
0.97
1.05
1.16
1.88
1.94
1.10
1.68
1.18
1.08
1.26
1.23
1.91
1.03
1.02
1.92
4.55
2.03
2.70
6.54
1.18
0.95
1.91
1.10
1.19
1.22
2.51
0.61
3.41
0.53
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)

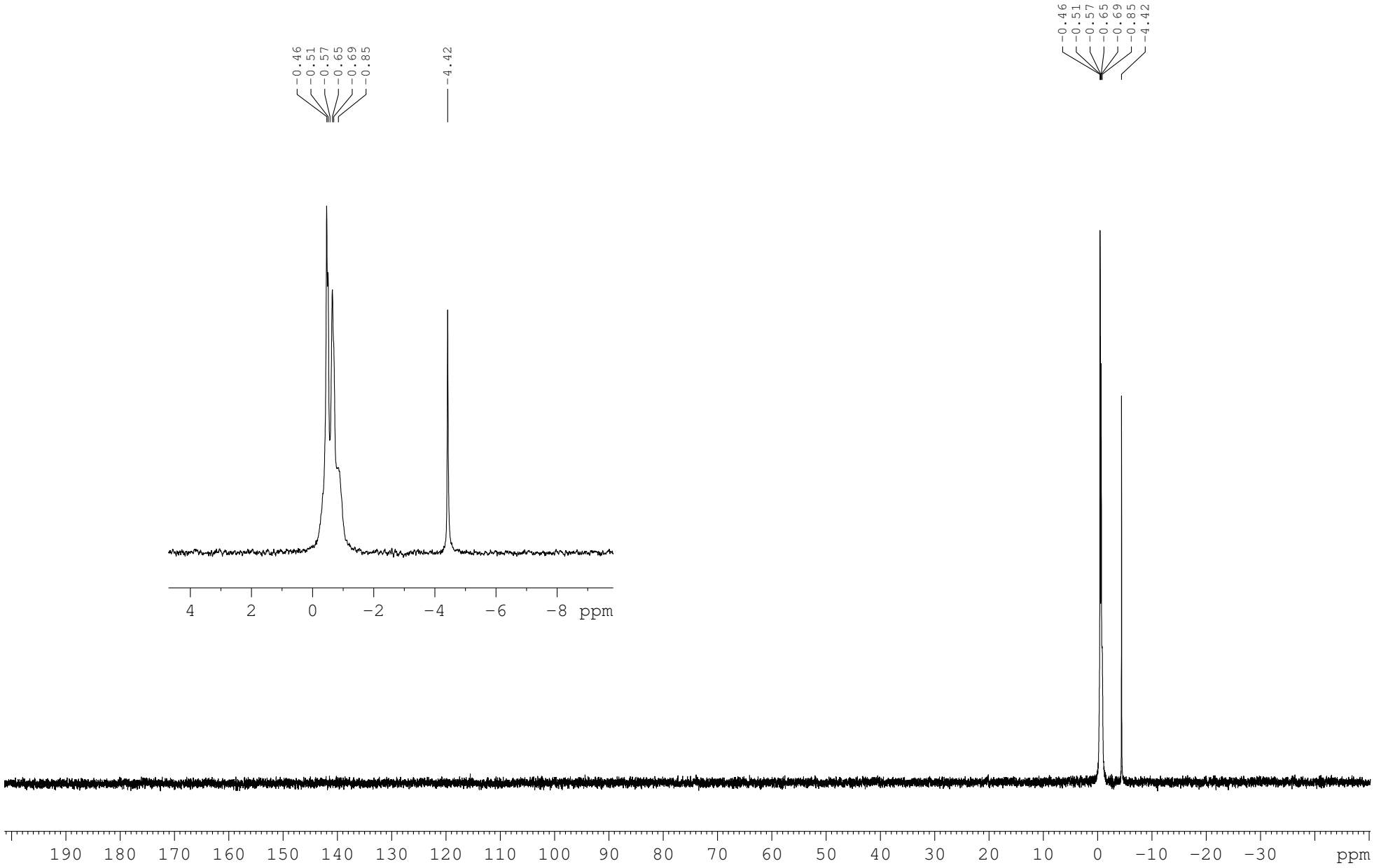
LC-MS analysis of purified compound 2



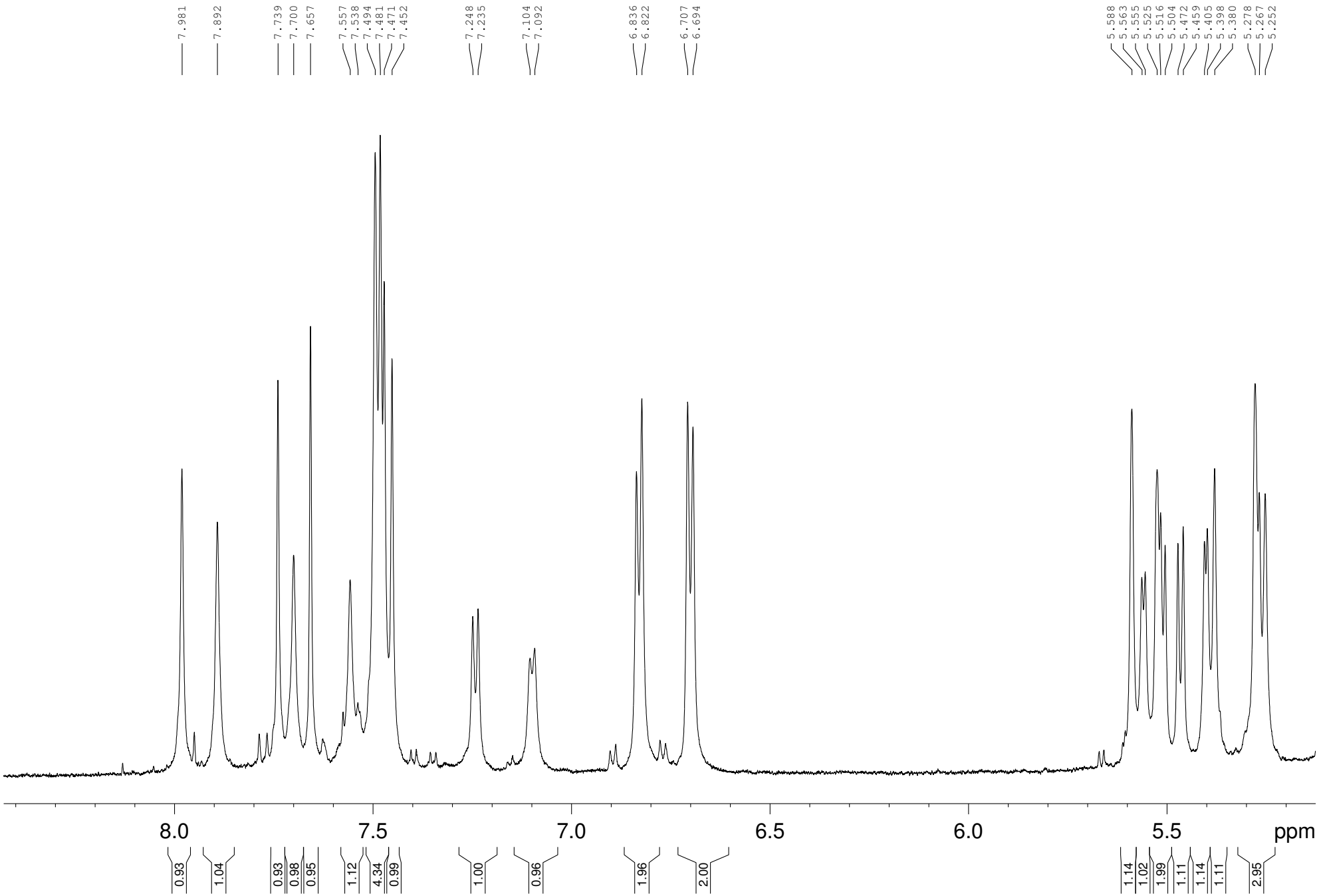




31P-NMR (161 MHz), D2O, compound 2



1H-NMR (600 MHz), D2O, compound 2



1H-NMR (600 MHz), D2O, compound 2