Supporting Information

In Vivo Methylation of OLA1 Revealed by Activity-Based Target Profiling of NTMT1

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1. General information

All chemical and biochemical reagents were purchased at the highest purity grade. Unless otherwise specified, all solvents were anhydrous, and all chemical syntheses were performed under argon atmosphere. Thin layer chromatography (TLC) was performed using 60 mesh silica gel plates and visualization was performed using short wavelength UV light (254 nm) and basic $KMnO_4$ staining. All samples for HPLC and mass spectrometry (MS) were filtered using 0.2 μm PTFE syringe or centrifugal filters to remove particles before injection. HPLC was performed with a Waters Breeze 2 system consisting of a 1525 pump and 2998 photodiode array detector. High-resolution MS (HRMS) was recorded on a Waters Xevo G2-XS QTof mass spectrometer. NMR spectra were recorded on a Varian 400 MHz or Bruker 600 MHz spectrometer. Chemical shifts of proton (^{1}H NMR) and carbon (^{13}C NMR) were reported in ppm relative to the peaks of residual solvents. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was performed with a Waters Acquity H- or M-class UPLC connected to a Waters Xevo G2-XS QTof mass spectrometer, which were controlled by Waters MassLynx V4.1. Mobile phases of A and B for LC-MS were 0.1% formic acid in water and in acetonitrile, respectively. The expanded N-terminal sequences for motif analysis are listed in Table S1. Putative NTMT1 targets based on proteomics and motif analysis are listed in Table S2.

2. Chemical synthesis of Hey-SAM (Scheme S1 and Figure 1A)

*4-((tetrahydro-2H-pyran-2-yl)oxy)but-2-yn-1-ol **4***.[S1-2] A solution of 3, 4-dihydro-2*H*-pyran (DHP, 16.8 g, 1.0 equiv.) in MeCN was added dropwise at room temperature to a suspension of **3** (86.0 g, 5.0 equiv.) and $CuSO_4$•$5H_2O$ (2.5 g, 0.05 equiv.) in MeCN. The mixture was stirred until DHP was completely consumed as determined by TLC. The reaction mixture was filtered by Celite and the filtrate was concentrated under a reduced pressure. The residue was purified by silica gel chromatography (hexane/EtOAc = 3/1) to give **4** as colorless oil (23.8 g, 70%).^{1}H NMR (400 MHz, $CDCl_3$) δ 4.80 (t, *J* = 3.4 Hz, 1H), 4.50–4.10 (m, 4H), 3.91–3.74 (m, 1H), 3.64–3.47 (m, 1H), 1.82–1.47 (m, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 96.86, 84.61, 81.32, 62.01, 54.39, 50.84, 30.18, 25.30, 18.95; HRMS: calcd. for $C_9H_{14}O_3Na^+$ $[M+Na]^+$: 193.0835, found: 193.0847.

*(E)-4-((tetrahydro-2H-pyran-2-yl)oxy)but-2-en-1-ol **5***.[S3-4] To a solution of compound **4** (11.0 g, 1.0 equiv.) in THF (165 mL) was added dropwise at −78 ˚C a solution of 70 wt% sodium bis(2-methoxyethoxy)aluminium hydride (Red-Al) in toluene (37 g, 2.0 equiv.). The mixture was then kept stirring for 2 h at −78 ˚C and another 30 min at 0 ˚C. After the mixture was poured into ice, the precipitates formed were treated with aqueous 50% KOH (140 g, 19 equiv.) to obtain a clear solution. The aqueous layer was separated and then extracted twice with THF. The organic layer and extraction were combined, dried with $MgSO_4$, and concentrated under a reduced pressure. The residue was purified by silica gel chromatography (hexane/EtOAc = 3/1) to give **5** as colorless oil (8.2 g, 74%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.05–5.65 (m, 2H), 4.64 (t, *J* = 3.6 Hz, 1H), 4.24 (dd, *J* = 12.5, 5.3 Hz, 1H), 4.15 (t, *J* = 5.2 Hz, 2H), 3.98 (dd, *J* = 12.6, 5.8 Hz, 1H), 3.89–3.82 (m, 1H), 3.54–3.46 (m, 1H), 1.89–1.43 (m, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 132.17, 127.47, 97.93, 67.06, 62.73, 62.16, 30.55, 25.40, 19.37; HRMS: calcd. for $C_9H_{16}O_3Na^+$ $[M+Na]^+$: 195.0992, Found: 195.1005.

*(E)-4-((tetrahydro-2H-pyran-2-yl)oxy)but-2-en-1-yl methanesulfonate **6***.[S5] To a solution of compound **5** (11.4 g, 2.0 equiv.) and Et_3N (10.0 g, 1.5 equiv.) in CH_2Cl_2 (133 mL) was added methanesulfonyl chloride (9.86 g, 1.3 equiv.) at 0 ˚C. The mixture was kept stirring at 0 ˚C for 5 min and then quenched with saturated $NaHCO_3$ (aq). The organic layer was separated and concentrated under a reduced pressure. The residue was purified by silica gel chromatography (hexane/EtOAc = 3/1) to give **6** as colorless oil (15.6 g, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.02 (dt, *J* = 15.6, 5.1 Hz, 1H), 5.97–5.81 (m, 1H), 4.82–4.69 (m, 2H), 4.68–4.60 (m, 1H), 4.32–4.21 (m, 1H), 4.02 (dd, *J* = 13.8, 5.4 Hz, 1H), 3.90–3.80 (m, 1H), 3.56–3.47 (m, 1H), 3.01 (s, 3H), 1.87–1.51 (m, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 133.96, 123.71, 98.18,

69.74, 66.15, 62.17, 38.02, 30.45, 25.31, 19.31; HRMS: calcd. for $C_{10}H_{18}O_5SNa^+$ $[M+Na]^+$: 273.0767, Found: 273.0776.

(E)-trimethyl(6-((tetrahydro-2H-pyran-2-yl)oxy)hex-4-en-1-yn-1-yl)silane 7.[S6-7] To a flame-dried flask containing NaI (30.0 g, 2.0 equiv.), CuI (19.0 g, 1.0 equiv.), and K_2CO_3 (27.6 g, 2.0 equiv.) was added compound **6** (25 g, 1.0 equiv.), anhydrous DMF (200 mL), and trimethylsilylacetylene (12.1 g, 1.2 equiv.). After the reaction mixture was stirred at room temperature overnight, it was diluted with CH_2Cl_2 (600 mL) and then centrifuged. The solvent/supernatant was concentrated under high vacuum using an oil pump and liquid nitrogen trap. The residue was purified by silica gel chromatography eluted with 100% CH_2Cl_2 to give **7** as colorless oil (6.0 g, 24%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.97–5.78 (m, 1H), 5.69 (dt, *J* = 15.4, 5.3 Hz, 1H), 4.63 (t, *J* = 3.7 Hz, 1H), 4.22 (dd, *J* = 12.4, 5.5 Hz, 1H), 3.97 (dd, *J* = 12.4, 6.4 Hz, 1H), 3.90–3.82 (m, 1H), 3.54–3.45 (m, 1H), 3.00 (d, *J* = 5.5 Hz, 1H), 1.88–1.79 (m, 1H), 1.75–1.69 (m, 1H), 1.62–1.49 (m, 4H), 0.15 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 128.30, 127.36, 103.72, 98.02, 86.90, 67.26, 62.34, 30.73, 25.58, 23.04, 19.62, 0.21; HRMS: calcd for $C_{14}H_{24}O_2SiH^+$ $[M+H]^+$: 253.1618, Found: 253.1627.

*(E)-6-(trimethylsilyl)hex-2-en-5-yn-1-ol **8**.*[S6] To a solution of compound **7** (6.0 g, 1.0 equiv.) in MeOH (20 mL) was added pyridinium p-toluenesulfonate (PPTS, 0.3 g, 0.05 equiv.). The mixture was heated to reflux until compound **7** was completely consumed as determined by TLC. The reaction was quenched with saturated $NaHCO_3$ (aq). The crude product was extracted by CH_2Cl_2. The extractions were combined, dried with anhydrous $MgSO_4$, and concentrated to dryness under a reduced pressure. The residue was purified by silica gel chromatography (EtOAc/CH_2Cl_2 = 1/9) to give **8** as colorless oil (3.5 g, 87%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.91 (dtt, *J* = 15.0, 5.6, 1.7 Hz, 1H), 5.68 (dtt, *J* = 15.0, 5.1, 1.3 Hz, 1H), 4.15 (s, 2H), 3.01 (dt, *J* = 5.4, 1.6 Hz, 2H), 1.44 (br, 1H), 0.16 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 130.95, 126.01, 103.64, 87.00, 63.14, 22.89, 0.18; HRMS: calcd. for $C_9H_{17}OSiH^+$ $[M+H]^+$:169.1043, Found: 169.1035.

*(E)-hex-2-en-5-yn-1-ol **9**.* To a solution of compound **8** (1.30 g, 1.0 equiv.) in THF (15 mL) was added TBAF (1M solution in THF, 9.3 mL, 1.2 equiv.) at 0 ˚C. The mixture was kept stirring until compound **8** was completely consumed as determined by TLC. The reaction was quenched with saturated NH_4Cl (aq). After the THF was removed under reduce pressure, the crude product was extracted by CH_2Cl_2. The extractions were combined, dried with anhydrous $MgSO_4$, and concentrated to dryness under a reduced pressure. The residue was purified by silica gel chromatography (EtOAc/CH_2Cl_2 = 1/9) to give **9** as colorless oil (0.7 g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.94 (dtt, *J* = 15.0, 5.6, 1.8 Hz, 1H), 5.69 (dtt, *J* = 15.2, 5.4, 1.4 Hz, 1H), 4.14 (d, *J* = 5.6 Hz, 2H), 2.97 (ddt, *J* = 6.8, 2.8, 1.3 Hz, 2H), 2.11 (t, *J* = 2.7 Hz, 1H), 1.60 (br, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 131.14, 125.50, 81.31, 70.56, 62.87, 21.38; HRMS: calcd. for $C_6H_9OH^+$ $[M+H]^+$: 97.0648, Found: 97.0640.

*(E)-hex-2-en-5-yn-1-yl methanesulfonate (Hey-OMs) **2**.* The reaction and silica gel chromatography were performed in the dark in the same way as **6** using **9** (0.67 g, 1.0 equiv.), Et_3N (1.40 g, 2.0 equiv.), and methanesulfonyl chloride (1.20 g, 1.5 equiv.) in CH_2Cl_2 (7 mL) to yield **2** as colorless oil (3.5 g, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.12–5.74 (m, 2H), 4.72 (d, *J* = 6.6 Hz, 2H), 3.06–2.95 (m, 5H), 2.15 (t, *J* = 2.6 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 131.66, 124.30, 71.44, 69.73, 38.24, 21.43; HRMS: calcd. for $C_7H_{10}O_3SNa^+$ $[M+Na]^+$: 197.0243, Found: 197.0238.

*((S)-3-amino-3-carboxypropyl)(((2S,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-3,4 dihydroxytetra-hydro-furan-2-yl)methyl)((E)-hex-2-en-5-yn-1-yl)sulfonium methanesulfonate (Hey-SAM) **1**.*[S8] To a solution of *S*-adenosyl-*L*-homocysteine (SAH, 10 mg, 1.0 equiv.) in 88-90% HCOOH (20 μL) was added freshly prepared compound **2** (22.6 mg, 5.0 equiv.) at 0 ˚C. The mixture was kept stirring in the dark at 0 ˚C under Ar protection for 4 h followed by the addition of a second batch of **2** (22.6 mg, 5.0 equiv.). After the mixture was kept stirring for another 16 h under the same condition, water (1.0 mL) was added and then

extracted with CH_2Cl_2 (3.0 mL) for three times. The aqueous layer was analyzed by HPLC using an X-Bridge Shield RP18 column (5.0 μm, 4.6 × 250 mm, Waters) eluted with water containing 0.03% TFA at the flow rate of 1.0 mL/min. A typical HPLC profile is shown in Figure 1B. Since the purity of Hey-SAM was ≥98%, it was used for study without further purification.

Partial separation of the two epimers were performed with a Luna C18(2) column (5 μm, 100 Å, 10 × 250 mm, Phenomenex) eluted with water containing 0.03% TFA at the flow rate of 3.0 mL/min. The fractions were pooled and lyophilized to yield two samples of epimer mixtures, in which ratios of fast- and slow-eluting epimers were at 7:3 and 4:6, respectively, based on NMR analysis.

Fast- and slow-eluting epimers of Hey-SAM at ***7:3 ratio***. ^{1}H NMR (600 MHz, D_2O) δ 8.41–8.39 (m, 2H), 6.11 (m, 1H), 6.06 (dt, *J* = 15.4, 5.1 Hz, 0.7H), 5.91 (dt, *J* = 15.2, 5.2 Hz, 0.3H), 5.77 (dt, *J* = 15.1, 7.1Hz, 0.7H), 5.67 (dt, *J* =15.0, 7.5 Hz, 0.3H), 4.76 (t, *J* = 6.6 Hz, 1H), 4.60–4.56 (m, 1H), 4.51–4.47 (m, 1H), 4.13 (d, *J* = 7.6 Hz, 1.4H), 4.09 (d, *J* = 7.7 Hz, 0.6H), 3.97 (m, 0.3H), 3.92 (m, 0.7H), 3.85–3.81 (m, 1.4H), 3.79–3.76 (m, 0.6H), 3.55–3.47 (m, 1H), 3.46–3.37 (m, 1H), 3.02(d, *J* = 3.0 Hz, 1.4H), 2.94 (d, *J* = 4.8 Hz, 0.6H), 2.49 (s, 0.7H), 2.45 (s, 0.3H), 2.37–2.27 (m, 2H); ^{13}C NMR (151 MHz, D_2O) δ 171.92, 151.27, 149.19, 145.72, 144.83, 140.59, 120.65, 116.93, 91.43, 81.80, 79.89, 74.31, 73.95, 73.62, 52.76, 43.63, 42.43, 36.75, 26.32, 22.33; HRMS: calcd. for $C_{20}H_{27}N_6O_5S^+$ $[M+H]^+$: 463.1758, Found: 463.1776.

Fast- and slow-eluting epimers of Hey-SAM at ***4:6 ratio***. ^{1}H NMR (600 MHz, D_2O) δ 8.41–8.39 (m, 2H), 6.11 (m, 1H), 6.06 (dt, *J* = 15.4, 5.1 Hz, 0.4H), 5.91(dt, *J* = 15.2, 5.2 Hz, 0.6H), 5.77 (dt, *J* = 15.1, 7.1Hz, 0.4H), 5.67 (dt, *J* =15.0, 7.5 Hz, 0.6H), 4.76 (t, *J* = 6.6 Hz, 1H), 4.60–4.56 (m, 1H), 4.51–4.47 (m, 1H), 4.13 (d, *J* = 7.6 Hz, 1.4H), 4.09 (d, *J* = 7.7 Hz, 0.6H), 3.97 (m, 0.6H), 3.92 (m, 0.4H), 3.85–3.81 (m, 1.4H), 3.79–3.76 (m, 0.6H), 3.55–3.47 (m, 1H), 3.46–3.37 (m, 1H), 3.02 (d, *J* = 3.0 Hz, 1.4H), 2.94(d, *J* = 4.8 Hz, 0.6H), 2.49 (s, 0.4H), 2.45 (s, 0.6H), 2.37–2.27 (m, 2H); ^{13}C NMR (151 MHz, D_2O) δ 171.96, 151.31, 149.25, 145.78, 144.74, 140.63, 120.59, 116.68, 91.34, 81.72, 79.92, 74.33, 74.13, 73.49, 52.68, 42.88,-42.03, 36.51, 26.23, 22.29.

3. Peptide synthesis

Peptide analogs of N-terminal RCC1 [SPKRIAKRRS($CONH_2$)], PB1 [GSKRRRATSP($CONH_2$)], SPD2B [PPRRSIVEVK($CONH_2$)], DDX60L [GSKDHAVFFR($CONH_2$)], H2A1B [SGRGKQGGKA($CONH_2$)], FECH [GAKPQVQPQK($CONH_2$)], LAMC2 [TSRREV(COOH)], OLA1 [PPKKGGDGIK($CONH_2$)], and K4Q-OLA1 [PPQKGGDGIK($CONH_2$)] were synthesized on a Rink amid or 2-chlorotrityl chloride resin (Peptides International) using standard Fmoc chemistry.[S9] Peptides were purified by semi-preparative HPLC, and their purity and identities were confirmed by analytical HPLC and HRMS, respectively. Conditions of semi-preparative and analytical HPLC are summarized in Table S3.

4. Human cell culture

Normal and NTMT1 KO HEK293FT cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Corning, 10-013-CV, containing 4.5 g/L glucose, L-glutamine and sodium pyruvate) supplemented with 10% fetal bovine serum (FBS, Gibco, 16140089) and 1% penicillin-streptomycin (Gibco, 30-002-CI) in a humidified atmosphere containing 5% CO_2 at 37 °C.

5. Plasmid construction

Sequences of primers, restriction sites, tags, and vectors used to construct plasmids are summarized in Table S4. Plasmids were constructed following a standard protocol.[S10] The gene corresponding to human NTMT1 was synthesized by GenScript. Human RCC1 gene was amplified from *p*ST50Tr-hRCC1 (a gift from Prof. Song Tan at Penn State)[S11]. Genes corresponding to RS14 and OLA1 were amplified

from *c*DNA of HEK293FT cell line. Mammalian expression vector *p*EGFP-n1 was modified to introduce a FLAG tag at the C terminus of EGFP, yielding a new vector *p*EGFP-n1-FLAG. Plasmids to express OLA1 and K4Q-OLA1 in mammalian cells were constructed using *p*EGFP-n1-FLAG. To generate an NTMT1 KO cell line using CRISPR-Cas9, guide sequences were designed according to an online CRISPR Design Tool (http://tools.genome-engineering.org).[S12] Gene insertions were verified by DNA sequencing.

6. Protein expression and purification

Buffers used for protein purification are listed here. For purification of NTMT1, RCC1 and RS14, buffer A consisted of 300 mM NaCl and 5 mM imidazole in 25 mM HEPES (pH 7.4). Buffers B and C were the same as the buffer A except that the imidazole concentrations were 30 and 250 mM, respectively. Buffer D consisted 150 mM NaCl and 50 mM KCl in 25 mM HEPES (pH 7.4). For OLA1 purification,[S13] buffer A consisted of 700 mM NaCl, 5 mM $MgCl_2$ and 10% glycerol in 50 mM Tris (pH 7.9). Buffer B consisted of 100 mM NaCl, 5 mM $MgCl_2$, 5% glycerol, and 40 mM imidazole in 50 mM Tris (pH 7.9). Buffer C was the same as the buffer B except that the imidazole concentration was 250 mM. Buffer D consisted of 100 mM NaCl in 50 mM Tris (pH 7.4).

All proteins were overexpressed in *Escherichia coli* BL21(DE3) (Lucigen) except that RCC1 was overexpressed in *E. coli* C41(DE3) (Lucigen). Bacteria were grown in 4L of Luria Bertani (LB) media in the presence of 50 μg/mL kanamycin. When A_{600} reached 0.6–0.8, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.4 mM to induce protein expression. Bacteria were grown at 18 ˚C for additional 18 h and then harvested by centrifugation at 5,000 *g* for 20 min at 4˚C. The cell pellets were collected and stored at −80 ˚C until further use.

All the following purification steps were carried out at 4 ˚C. The cell pellets were re-suspended in buffer A and lysed by sonication for 15 min (5-second on and 3-second off) at 40% amplitude. The cell debris was removed by centrifugation at 18,000 *g* for 45 min. The supernatant was decanted and incubated with Ni-NTA resin (Qiagen) that had been pre-equilibrated with buffer A for 30 min. The resin was washed with 30 column volumes (CV) of buffer B and then eluted with 5CV of buffer C. Fractions containing the targeted protein were collected and exchanged into buffer D using a HiPrep™ 26/10 desalting column (GE Healthcare). Purified proteins were concentrated to 5–20 mg/ml, flash-freezed in liquid N_2, and stored in aliquots at −80 ˚C until further use. Protein purity was assessed by 12% tris-tricine SDS-PAGE (Figure S1), and their concentrations were determined by bicinchoninic acid assays (BCA) using a BSA calibration curve.[S14]

7. MS-based assay for RCC1 peptide and protein modifications

RCC1 peptide ($RCC1_p$) concentration was determined at 205 nm using a calculated extinction coefficient of 29,070 M^{-1} cm^{-1}. Modifications of RCC1 at peptide and protein levels by *wt* NTMT1 were investigated using MS in the presence of cofactors. Specifically, 20 μM $RCC1_p$ in 10 mM ammonium bicarbonate (ABC) or RCC1 in 25 mM HEPES (pH 7.4) containing 150 mM NaCl and 50 mM KCl, 200 μM SAM or Hey-SAM, and 3 μM *wt* NTMT1 were incubated at 37 ˚C for 2 h. Negative controls were performed in the absence of *wt* NTMT1. The $RCC1_p$ modification reaction was quenched with an equal volume of water containing 0.1% formic acid and then analyzed by LC-MS using a BEH C18 analytical column (1.7 μm, 130 Å, 2.1 mm × 50 mm, Waters) eluted at a flow rate of 0.4 mL/min. The gradient of B increased from 0 to 90% in 2.5 min. The RCC1 protein modification reaction was not quenched and directly analyzed by intact protein MS, which was performed on a BEH C4 analytical column (1.7 μm, 300 Å, 2.1 mm × 50 mm, Waters) eluted at a flow rate of 0.4 mL/min. The gradient was: 0–1 min, 5% B; 1–3.5 min, 5–100% B; and 3.5–4.7 min, 100% B. Elution from 2–4.7 min was injected into the mass spectrometer. The data were processed by Waters MassLynx and BiopharmaLynx. Results are summarized in Figures 1C-D, S2, and S3A-E.

8. Isothermal titration calorimetry (ITC)

ITC measurements were performed with MicroCal iTC200 (GE Healthcare) at 25 °C. Purified *wt* NTMT1 was diluted to 50 μM using the buffer consisting of 150 mM NaCl and 50 mM KCl in 25 mM HEPES (pH 7.4). Cofactors or RCC1 peptide substrate was dissolved and diluted to 300−500 μM using the same buffer. Measurements were carried out with 18 injections of 2 μL with an interval time of 180 s and a reference power of 7 μcal/s. Experiments were done in triplicate and data were analyzed by Origin software. The results are shown in Figures 2A-C.

9. Steady-state kinetics

SAH release monitored by HPLC was used to determine steady-state kinetics. Adenosine was added as an internal standard to quantify the SAH formation. Experiments were performed by varying concentrations of one substrate in the presence of the other substrate at saturation. Specifically, concentrations of SAM and Hey-SAM varied from 0.1 to 8 μM and from 0.2 to 16 μM, respectively, in the presence of 40 μM $RCC1_p$. Concentration of $RCC1_p$ varied from 0.5 to 16 μM in the presence of 20 μM SAM or Hey-SAM.

Experiments were carried out in the following way. In a final volume of 400 μL, 0.3 μM *wt* NTMT1 was incubated with a cofactor and $RCC1_p$ at 37 °C in the buffer consisting of 50 mM KCl in 25 mM Tris (pH 7.5). Aliquots of 40 μL were withdrawn at selected time intervals (1 to 90 min) and the reactions were quenched with 40 μL of 0.4% TFA solution containing 4.0 μM adenosine. The reaction mixtures were then centrifuged at 18,000 *g* for 2 min. The supernatants were removed and 40 μL were injected into an XBridge Shield RP 18 column (3.5 μm, 130 Å, 4.6 × 150 mm, Waters). The column was eluted at 1 mL/min with an isocratic gradient of 1% methanol in water supplemented with 0.01% TFA. Measurements were done in triplicate. Concentrations of released SAH were determined by A_{260} ratios of the peak areas corresponding to SAH and adenosine. The data obtained were fitted into the Michaelis-Menten equation ($v = \frac{V_{max}[S]}{K_M+[S]}$) using SigmaPlot. Results are shown in Figures 2D-E and summarized in Table 1.

10. Peptide mapping of RCC1 purified from *E. coli*

To map the site of RCC1 modified by Hey-SAM in the presence of *wt* NTMT1, the reaction mixture was separated by SDS-PAGE. The protein band corresponding to RCC1 was cut out and treated with in-gel trypsin digestion following a standard protocol.[S15] Briefly, the RCC1 gel band was cut into 1.0 mm^3 pieces, reduced with 100 mM dithiothreitol (DTT), and then alkylated with 55 mM iodoacetamide. Digestion was implemented at 37 °C for 18 h with 20 ng/μL sequencing-grade trypsin (Promega) in 10 mM ABC containing 10% (vol/vol) acetonitrile. The peptides were extracted with gentle shaking at 37 °C for 1 h using a water acetonitrile mixture (1:1, vol/vol) containing 5% formic acid. The peptide extraction was concentrated by SpeedVac and analyzed by LC-MS/MS.

For LC−MS/MS analysis, the digestion products were re-dissolved in water containing 5% formic acid and then separated on a BEH C18 column (1.7 μm, 130 Å, 2.1 mm × 50 mm, Waters) eluted at 0.2 mL/min. The gradient of mobile phase B increased from 0 to 50% in 50 min. The mass spectrometer was operated in an MS^E mode with a ramp collision energy ranging from 20 to 45 V. Data were analyzed by BiopharmaLynx (Waters), for which the searching parameters were set at peptide mass tolerance of 10 ppm, MS^E mass tolerance of 30 ppm, and no missed cleavages allowed. The fixed modification of cysteine carbamidomethylation and variable modification of N-terminal chain by Hey were also used in the data searching. A precursor ion corresponding to the Hey-alkylated SPK tripeptide (m/z: 409.2451) was detected. Further fragmentation of this precursor ion gave y_2 and y_3 ions, confirming the N-terminus of methionine-removed RCC1 was modified by Hey (Figures S3F).

11. Click chemistry of labelled RCC1 and DBA

In a final volume of 200 µl, 20 µM purified RCC1 was incubated overnight at 4 °C with 50 µM Hey-SAM and 3 µM *wt* NTMT1 in the buffer consisting of 150 mM NaCl and 50 mM KCl in 25 mM HEPES (pH 7.4). Negative controls were obtained either using SAM to substitute Hey-SAM or in the absence of *wt* NTMT1. Proteins were then precipitated out using 1.0 mL cold methanol and the resultant samples were centrifuged at 14,100 *g* for 2 min. After the supernatant was removed, protein pellets were washed with 1.0 mL cold methanol, air-dried for 15 min, and re-dissolved in 200 µL buffer E consisting of 150 mM NaCl and 1% SDS in 25 mM HEPES (pH 7.4). A total volume of 22 µL click chemistry cocktail [one volume of 5 mM diazo biotin azide (DBA) and ten volumes of premixed solution consisting of 1 mM tris(benzyltriazolylmethyl)amine (TBTA), 10 mM $CuSO_4$, and 10 mM tris(2-carboxyethyl)phosphine (TCEP) with the pH adjusted to 7.0 using 1 M NaOH] was added into the samples and the click reaction was performed in the dark for 2 h at room temperature.[S16] The samples were mixed with 1.0 mL methanol and kept overnight at −80 °C. The precipitated proteins were washed with 1.0 mL cold methanol and re-dissolved in 100 µL buffer E. Presence of RCC1 and biotin were confirmed by western blot (WB) as described below in Section 12 using rabbit anti-RCC1 antibody (Abcam) and streptavidin-HRP (SA-HRP, Abcam), respectively. Results are shown in Figures S4A-B.

To further confirm the purified RCC1 had been N-terminally biotinylated using click chemistry, we decided to perform LC-MS/MS analysis. Gel shift assay was employed to enrich the biotinylated RCC1, as only a fraction of recombinant RCC1 purified from *E. coli* could serve as the active NTMT1 substrate. It is of note that RCC1 produced from *E. coli* exists in three formats: full-length, N-terminal methionine removed, and N-terminal methionine and serine removed. Among them, only the RCC1 with the methionine removed is active and can be alkylated by *wt* NTMT1. The amount of active RCC1 varies between 30 and 50% of the total protein. To eliminate the interference of the inactive formats of RCC1 in MS, a gel shift assay using streptavidin (SA) was performed. Briefly, 10 µL of the above re-dissolved protein samples after click chemistry were mixed with 10 µL of 2 × Laemmli buffer and heated at 100 °C for 5 min. After cooling down to room temperature, the samples were incubated with 15 µL of 1 mg/ml SA solution (NEB) at room temperature for 30 min.[S17] Protein mixtures were then separated by SDS-PAGE. Native SA exists as a mixture of tetramer and dimer at 52.8 and 26.4 KDa, respectively (Lane 3 in Figure S4A). After it was added to the controls and reaction (Lanes 7-9 in Figure S4A), only the reaction sample formed two new bands at 75 and 150 KDa (arrows in Figure S4A), corresponding to RCC1+2SA and 2RCC1+4SA, respectively. These two new bands were cut out, treated with trypsin, and then analyzed by LC-MS/MS as described above in Section 10. A precursor ion corresponding to the biotinylated SPK tripeptide (m/z: 1120.5615) was detected. Further fragmentation of the precursor ion gave b_1, y_2, and y_4 ions, confirming the N-terminus of methionine-removed RCC1 was modified by a biotin tag (Figures S4C).

12. Western blots

Equal amounts of cell lysates or protein samples were separated in a 12% SDS-PAGE and then transferred to a supported nitrocellulose membrane (Bio-Rad). Immunoblotting was performed overnight at 4 °C with a rabbit anti-RCC1 antibody (Abcam), rabbit anti-OLA1 antibody (Bethyl Laboratories), rabbit anti-NTMT1 antibody (Abcam), rabbit anti-actin antibody (Sigma), or streptavidin-HRP (Abcam) at 1:1000-1:3000 dilutions. Membrane was washed with TBS buffer (pH 7.4) containing 0.1% Tween 20 (TBS-T). If the primary antibodies did not conjugate with HRP, then they would be recognized by an HRP-conjugated goat anti-rabbit antibody (Abcam, 1:5000 dilutions) by gentle shaking for 1 h at room temperature. After washing membrane with TBS-T buffer, bands were detected using SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Fisher Scientific) following manufacturer's protocol. The bands were visualized using Amersham Imager 600 (GE Healthcare).

13. Pull-down of overexpressed RCC1 from *E. coli* lysates

E. coli C41(DE3) cells transformed with *p*RCC1 were grown and harvested as described above in Section 6. They were re-suspended in a buffer consisting of 300 mM NaCl in 25 mM HEPES (pH 7.4) and sonicated for 15 min (5-second on and 3-second off) at 40% amplitude. After centrifugation at 18,000 *g* for 45 min, 1 mL supernatant was withdrawn and incubated with 50 μM Hey-SAM and 3 μM *wt* NTMT1 overnight at 4 ˚C. Negative controls were performed either using SAM to substitute Hey-SAM or in the absence of *wt* NTMT1. Proteins were precipitated out using 4.0 mL cold methanol, air-dried for 15 min, and re-dissolved in 4.0 mL buffer E (Section 11). Click chemistry was performed with 440 μL click chemistry cocktail (Section 11) in the dark for 2 h at room temperature.[S16] After proteins were precipitated out using 16.0 mL cold methanol and re-dissolved in 1.0 mL PBS (1×, pH 7.4) containing 1% SDS, 60 μL SA beads (GE Healthcare) prewashed with PBS (1×, pH 7.4) were added and incubated with the protein mixtures for 2 h at room temperature with gentle shaking. Samples were centrifuged at 4,000 *g* for 2 min and the unbound proteins were pipetted out. The SA beads were then washed sequentially with PBS (1×, pH 7.4) containing 1% SDS, PBS (1×, pH 7.4) containing 4 M urea, and 100 mM ABC, each for three times. Finally, the biotin labelled proteins were cleaved and eluted from the beads using 60 μL 25 mM $Na_2S_2O_4$ in 20 mM ABC. The elution was mixed with 2 × Laemmli buffer and analyzed by SDS-PAGE (Figure S5A). Presence of RCC1 was detected and quantified by WB as described above in Section 12 using rabbit anti-RCC1 (Abcam) and goat anti-rabbit antibodies (Abcam) as the primary and secondary antibodies, respectively (Figure S5B). To calculate the recovery yield, a standard curve of signal versus protein amount was generated using a series dilution of purified RCC1 from *E. coli* at a known concentration (Figure S5C).

14. Generation of NTMT1 KO cell line using CRISPR-Cas9

NTMT1 knockout (KO) HEK293FT cell line was generated according to the published protocol.[S12] Briefly, plasmids of PX459-NTMT1$_{sgRNA1}$ and PX459-NTMT1$_{sgRNA2}$ (1 μg each) were transfected into HEK293FT cells pre-seeded in a 6-well plate using LipoD293 (SignaGen Laboratories) and Opti-MEM™ I reduced serum medium (ThermoFisher) when cell confluency reached to 50-70%. After 36 h, the transfected cells were treated with 3 μg/mL puromycin for one week. Cells were then dissociated with TrypLE™ express enzyme (ThermoFisher), counted, and serially diluted in DMEM supplemented with 20% FBS to a final cell density of 50-100 cells per 15 mL medium to reduce the possibility of having multiple cells in one well. The diluted cells were plated to two 96-well plates and cultured for additional two weeks. After passaging into a 24-well plate, cells were screened by WBs (Figure S6). For the cells without detection of NTMT1, their genomic DNAs were extracted with a tissue DNA kit (Omega, D3396-01), and the NTMT1 exon 1 was amplified and sequenced. The KO cell lines confirmed by DNA sequencing were frozen in liquid N_2.

15. Activity-based substrate profiling of NTMT1

NTMT1 KO cells were cultured as described in Section 4. At 70%–90% confluence stage, cells (4 of 175 cm^2 flasks) were harvested and lysed by sonication for 8 min (5-second on and 3-second off) at 40% amplitude in 9 mL cold buffer consisting of 50 mM KCl, 10% glycerol, and 1× protease inhibitor (Pierce) in 25 mM HEPES (pH 7.4). Cell lysates were centrifuged at 15,000 *g* for 15 min at 4 ˚C to remove cell debris. The supernatant was incubated with 50 μM Hey-SAM or SAM (negative control) in the presence of 3 μM *wt* NTMT1 for 4 h at 37 ˚C. Four-time volumes of cold methanol were added into each sample and kept at –80 ˚C overnight to precipitate proteins. The samples were centrifuged at 7,197 *g* for 30 min and the supernatant was removed. The protein pellets were air-dried for 15 min and re-dissolved in 4.0 mL buffer E (Section 11). Click chemistry and subsequent treatment were performed in the same way as

described above in Section 13. As a positive control, presence of RCC1 was detected by WB (Figure 3C) as described above in Section 12.

Lanes of samples treated with Hey-SAM and SAM (negative control) were excised and digested with trypsin following the procedures described above in Section 10. For LC-MS/MS analysis, protein digestion products were re-dissolved in water containing 1% formic acid, filtered, and injected into Acquity M-class UPLC coupled with Xevo G2-XS QTof high-resolution mass spectrometer (Waters). The injected peptides were first passed through a Symmetry trap column (C18, 100 Å, 5 µm, 300 µm × 50 mm, Waters), and then separated on an HSS T3 column (C18, 100 Å, 1.8 µm, 300 µm × 100 mm, Waters), which were eluted at 8 µL/min. The gradient of mobile phase B for separation increased from 1% to 40% in 45 min. The MS was obtained under a positive polarity and sensitivity mode. The tandem MS was recorded under an MS^E mode with a ramp collision energy ranging from 20 to 45 V. Raw MS data were analyzed by Progenesis QI for Proteomics (Waters) to give protein candidates, for which they had to pass (1) the Anova p value ≤ 0.05 and (2) max fold change (Hey-SAM treated sample/SAM treated sample) ≥ 2. Experiments were done in triplicate.

16. Motif and signal peptide analyses

NTMT1 was previously reported to methylate proteins with an N-terminal sequence of Xaa (Ala/Pro/Ser)-Pro-Lys after methionine removal.[S18] Additional studies revealed that the 2nd residue Xaa can also be replaced with other residues except for hydrophobic Leu/Ile/Trp and negatively charged Asp/Glu residues.[S19-20] *In vitro* peptide methylation assays indicated that the proline at the 3rd position could be substituted with asparagine, glycine, alanine, methionine, serine, or threonine to some extent.[S19] The 4th residue was demonstrated to be only limited to a positively charged lysine or arginine, as it is critical for the formation of hydrogen bonds with the substrate binding pocket in NTMT1.[S20-21] Based on these expanded N-terminal sequence motifs (Table S1), a search of the human protein database using https://www.genome.jp/tools/motif/MOTIF2.html returned 733 potential NTMT1 targets.

Protein targets identified by Progenesis QI were further analyzed using UniProt online tools (https://www.uniprot.org/uploadlists/) to find proteins that may undergo N-terminal truncation. Signal and transit peptides should be selected for PTM processing in the UniProt. After the proteins were identified and signal sequences were manually removed, the resulting matured proteins were then subject to motif analysis as described above.

17. *In vitro* target validation

As described above in Section 7, MS-based methylation assays were carried out with the N-terminal peptide analogs of PB1, SPD2B, DDX60L, H2A1B, FECH, and LAMC2, as well as the recombinant proteins of RS14 and OLA1. Peptide data and intact protein mass were analyzed by MassLynx (Waters) and BiopharmaLynx (Waters), respectively. Results are shown in Figures S7-15.

18. *In vivo* methylation of OLA1

Normal and NTMT1 KO cells were cultured as described in Section 4. At 70-90% confluence stage, cells were transfected with 20 µg *p*OLA1-EGFP-FLAG or *p*K4Q-OLA1-EGFP-FLAG using LipoD293 (SignaGen Laboratories). After 36 h, cells were harvested, re-suspended in 3 mL PBS (1×, pH 7.4), and lysed by sonication for 4 min (5-second on and 3-second off) at 40% amplitude. The cell debris was removed by centrifugation at 15,000 *g* for 15 min and the supernatant was incubated with 30 µL FLAG-M2 magnetic beads (Sigma) for 4 h at 4 °C. After washing the magnetic beads with PBS buffer (1×, pH 7.4) containing 0.2% Triton X-100 for three times, the FLAG-tagged protein was eluted with 60 µL of 200 mM glycine (pH 2.5). The pH of the elution was adjusted to ~7 using 1 M Tris (pH 7.9). The eluted protein was first reduced by 6 µL buffer consisting of 10% SDS and 20 mM TCEP in 500 mM Tris (pH 7.9) for 10 min at 37 °C and then alkylated with 100 mM Tris buffer (pH 7.9) containing 100 mM

iodoacetamide for 15 min at 37 ˚C.[S22] The resulting sample was concentrated to ~ 10 μL by SpeedVac, mixed with 10 μL of 2 × Laemmli buffer, and heated at 100 ˚C for 5 min before it was loaded onto a gel for SDS-PAGE. After gel staining and de-staining, the band corresponding to OLA1-EGFP-FLAG or K4Q-OLA1-EGFP-FLAG (~73kD) was excised, cut into small pieces, and washed with a mixture of 100 mM ABC and acetonitrile (1:1, vol/vol) at 37 ˚C to completely de-stain the gel. After shrunk and dehydrated with acetonitrile, the gel pieces were incubated with chymotrypsin [Promega, 200 ng diluted in 20 μL buffer consisting of 10 mM $CaCl_2$ in100 mM Tris (pH 7.9)] for 18 h at 25 ˚C. The digested peptides were extracted sequentially with 100 μL of 5% formic acid in water, a mixture of acetonitrile and water (1:1, vol/vol) containing 5% formic acid, and pure acetonitrile. The extracts were combined and concentrated to dryness by SpeedVac. The residue was processed and analyzed by LC-MS/MS in the same way as described in Section 15 except that the gradient of mobile phase B increased from 1% to 40% in 40 min. The data obtained were mapped to the primary structure of OLA1 or K4Q-OLA1 by BiopharmaLynx (Waters). Peptide fragmentation patterns were analyzed manually and results are shown in Figures 4B-C and S16.

19. References

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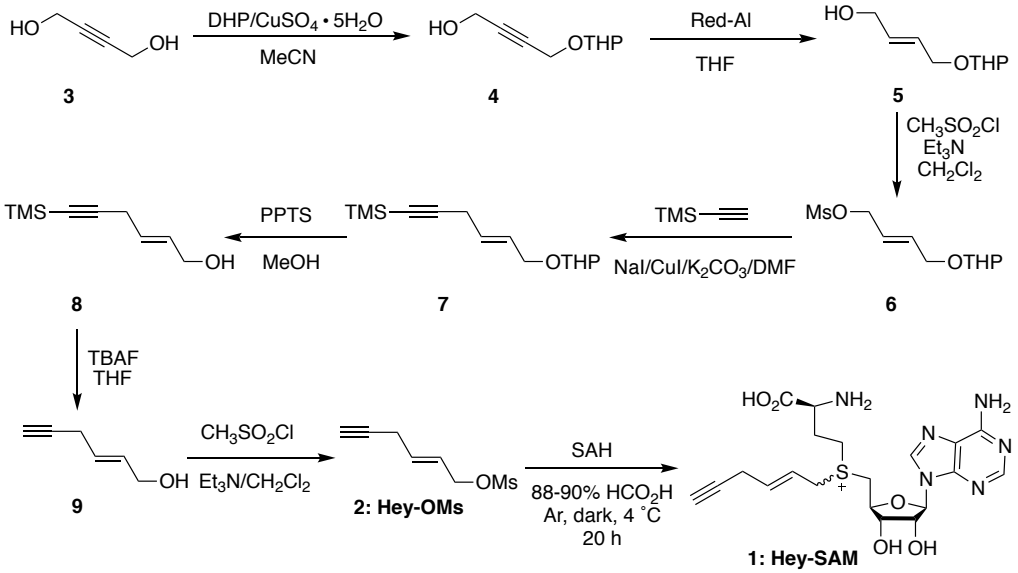


Scheme S1. Chemical synthesis of Hey-SAM **1**

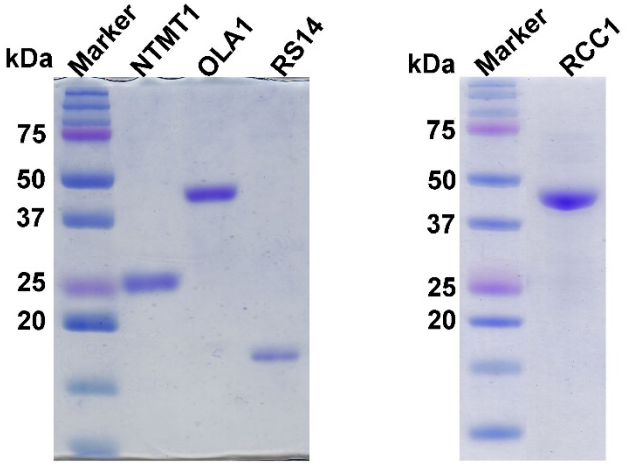


Figure S1. Tris-tricine SDS-PAGE of recombinant proteins from *E. coli*. The calculated MWs for NTMT1, OLA1, RS14, and RCC1 are 27.7, 45.7, 17.7, and 45.9 KDa, respectively.

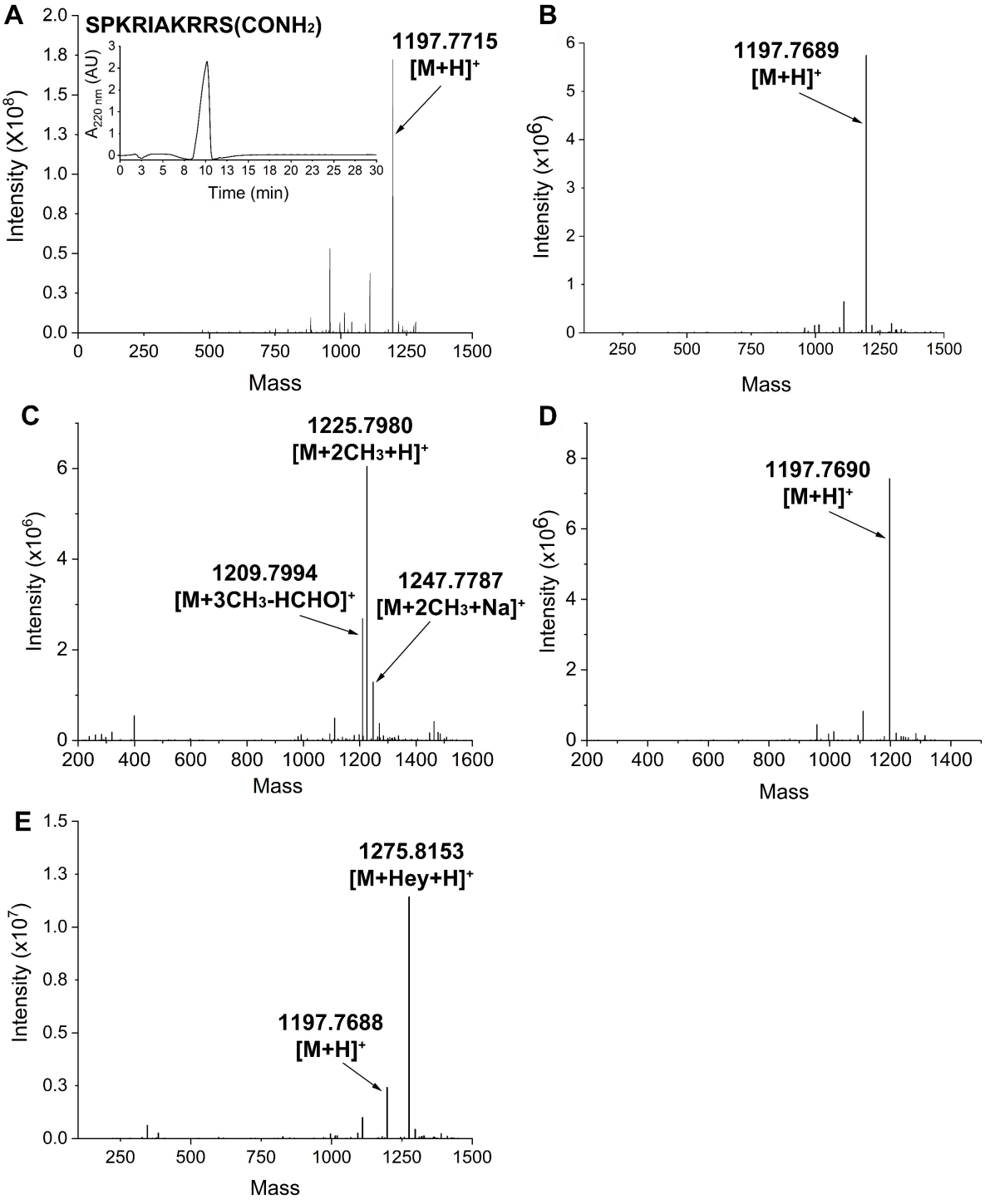


Figure S2. MS analysis of $RCC1_p$ modification by *wt* NTMT1 using SAM or Hey-SAM as the cofactor. All reactions were carried out with 20 μM $RCC1_p$ and 200 μM cofactor for 2 h at 37 °C. (A) MS and HPLC (inset) analyses of the synthesized $RCC1_p$. The calculated exact mass for $[M+H]^+$ is 1197.7656 Da. (B) MS analysis of the reaction between $RCC1_p$ and SAM in the absence of the enzyme. (C) MS analysis of the reaction between $RCC1_p$ and SAM catalyzed by *wt* NTMT1. The calculated exact masses for $[M+2CH_3+H]^+$, $[M+2CH_3+Na]^+$, and $[M+3CH_3–HCHO]^+$ are 1225.7969, 1247.7789, and 1209.8020 Da. (D) MS analysis of the reaction between $RCC1_p$ and Hey-SAM in the absence of the enzyme. (E) MS analysis of the reaction between $RCC1_p$ and Hey-SAM catalyzed by *wt* NTMT1. The calculated exact mass for monoalkylated ion, $[M+Hey+H]^+$, is 1275.8126 Da.

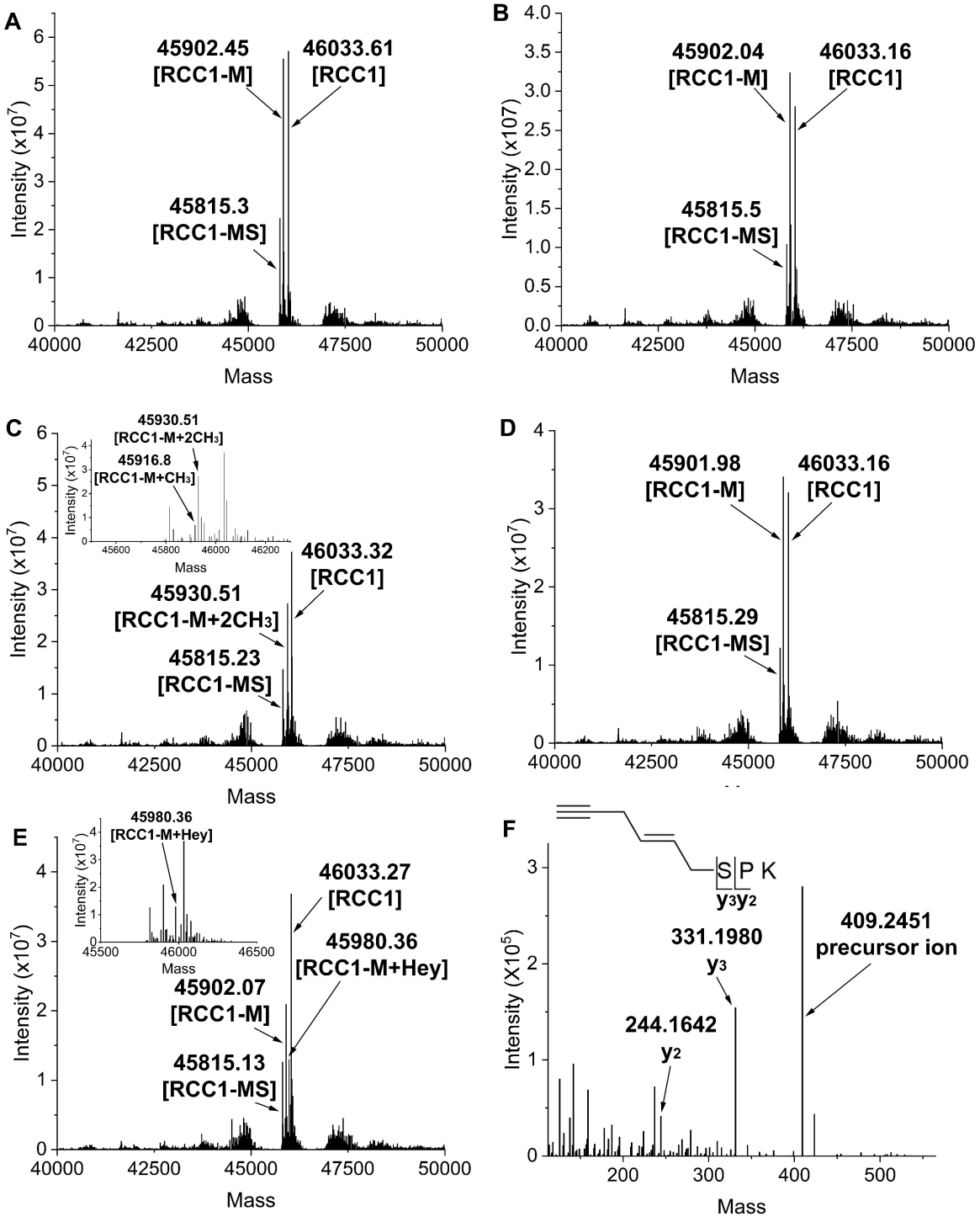


Figure S3. Intact protein MS analysis of RCC1 modification by *wt* NTMT1 using SAM or Hey-SAM as the cofactor. All reactions were carried out with 20 μM RCC1 protein and 200 μM cofactor for 2 h at 37 °C. Calculated MWs of full-length, M removed, and MS removed RCC1 are 46034.14, 45902.95, and 45815.87 Da, respectively. Only the RCC1 with the M removed is the active substrate of *wt* NTMT1. The insets in (C) and (E) are expansions of MWs. (A) RCC1 purified from *E. coli* C41(DE3) strain. (B) RCC1 and SAM in the absence of *wt* NTMT1. (C) RCC1 and SAM in the presence of 3 μM *wt* NTMT1. Calculated MWs of mono- ([RCC1–M+CH_3]) and dimethylated RCC1 ([RCC1–M+$2CH_3$]) are 45916.98 and 45931.00 Da, respectively. (D) RCC1 and Hey-SAM in the absence of *wt* NTMT1. (E) RCC1 and Hey-SAM in the presence of 3 μM *wt* NTMT1. The calculated MW of monoalkylated RCC1 ([RCC1–M+Hey]) is 45981.06 Da. (F) Tandem MS spectrum of mapping the modification site on RCC1.

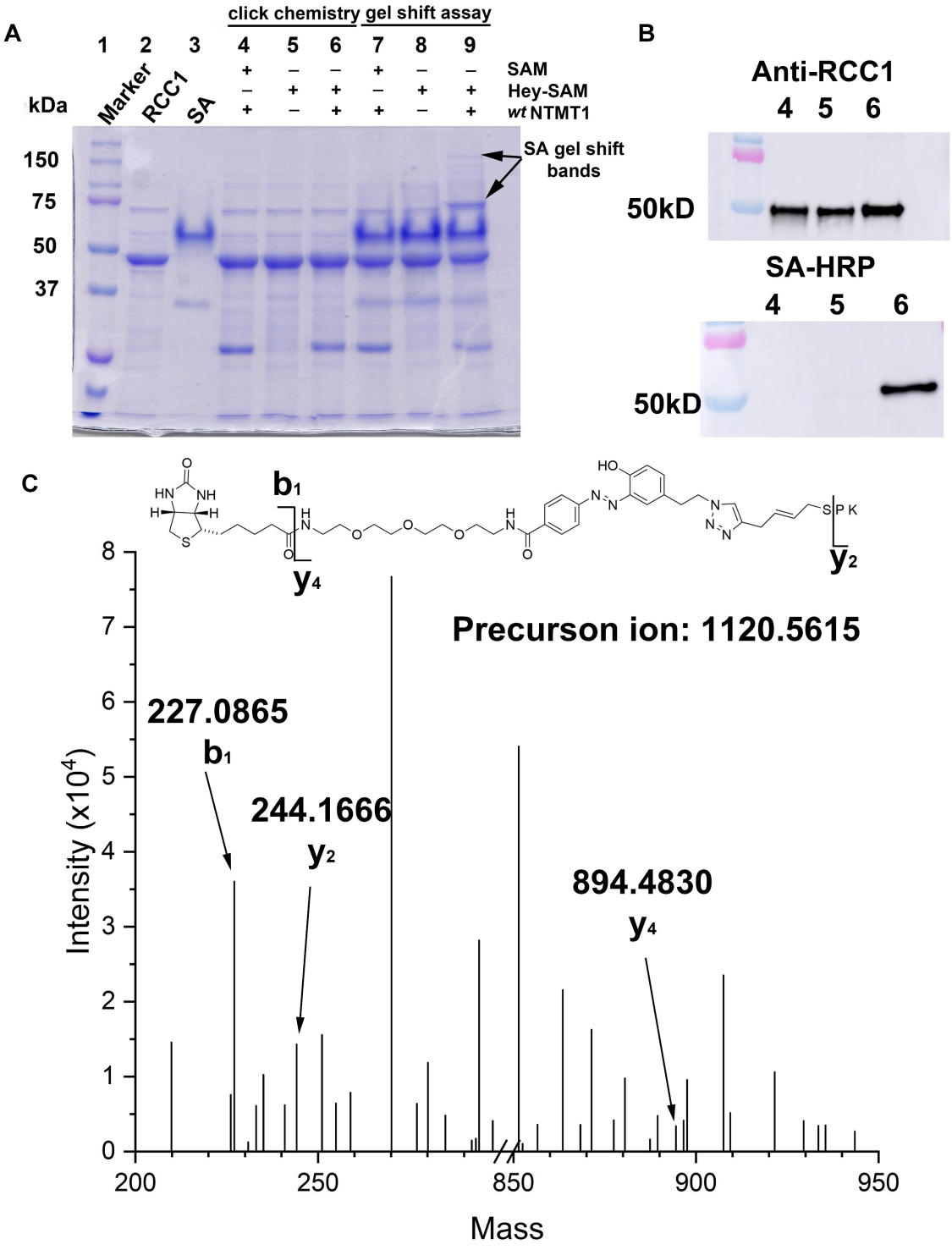


Figure S4. Click chemistry between DBA and purified RCC1 pre-labeled with different cofactors. (A) SDS-PAGE gel. Lanes 1, 2, and 3 are MW markers, purified RCC1, and purchased SA, respectively. Non-denatured SA exists as a mixture of dimer (26.4 KDa) and tetramer (52.8 KDa). Lanes 4, 5, and 6 are click chemistry of purified RCC1 pre-labeled with SAM (control), no NTMT1 (control), and Hey-SAM (Lane 6) using DBA, respectively. Lanes 7-9 are gel shift assays of click reactions shown in Lanes 4-6 with non-denatured SA. (B) WBs to detect RCC1 by rabbit anti-RCC1 antibody (top) and the presence of biotin using SA-HRP in Lanes 4-6 shown in A. (C) Tandem MS spectrum of mapping the biotin tag to the N-terminal serine of RCC1. The exact mass of the calculated parent tripeptide ion ($[M+H]^+$) is 1120.5608 Da.

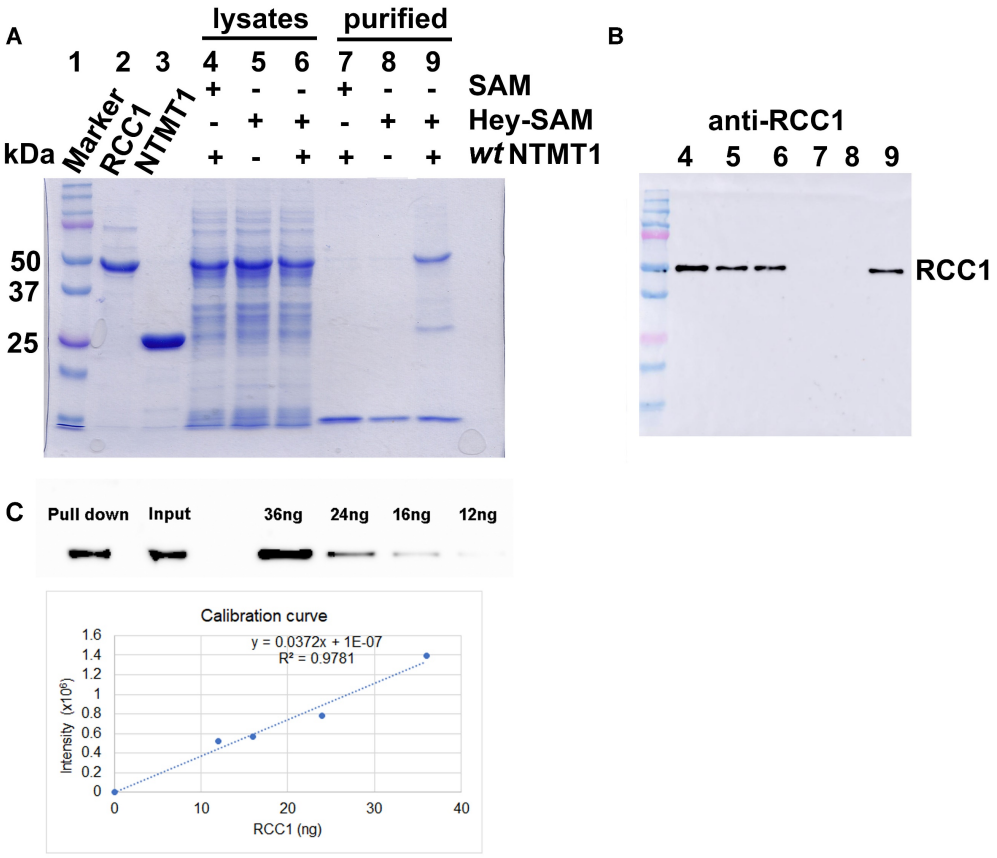


Figure S5. Proof-of-concept to pull-down overexpressed RCC1 from *E. coli* lysates. (A) SDS-PAGE gel. Lanes 1, 2, and 3 are MW markers, RCC1, and NTMT1, respectively. Lanes 4-6 are lysates labelled with SAM or Hey-SAM followed by click chemistry with DBA. Lanes 7-9 are proteins pulled-down from the treated lysates in Lanes 4-6 using SA beads. (B) Detection of RCC1 by WB using rabbit anti-RCC1 antibody. (C) WB standard curve and recovery yield calculated from WB.

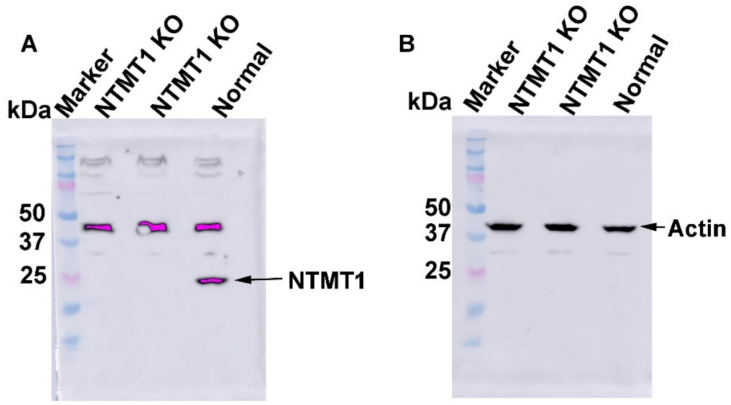


Figure S6. Generation of NTMT1 KO cells using CRISPR-Cas9. (A) WB detection using rabbit anti-NTMT1 antibody. (B) WB detection using rabbit anti-actin antibody. Actin served as a control.

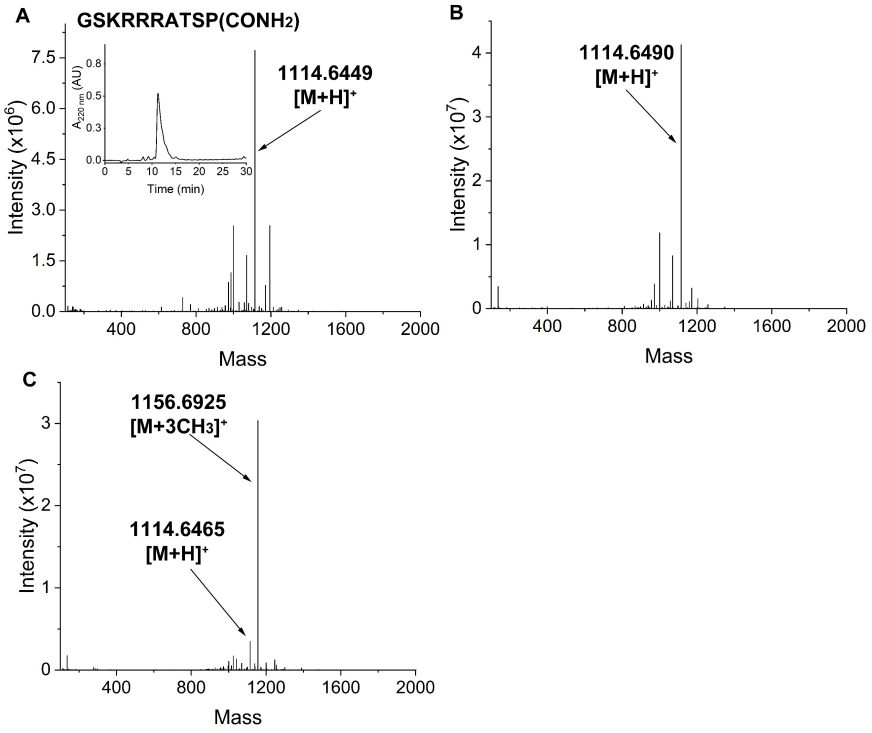


Figure S7. MS analysis of PB1 peptide methylation. **(**A) MS and HPLC (inset) analyses of the synthesized PB1 peptide. The calculated exact mass for $[M+H]^+$ is 1114.6557 Da. (B) MS analysis of the reaction between 20 μM PB1 peptide and 200 μM SAM in the absence of *wt* NTMT1. (C) MS analysis of the reaction between 20 μM PB1 peptide and 200 μM SAM catalyzed by 3 μM *wt* NTMT1. The calculated exact mass for trimethylated product ($[M+3CH_3]^+$) is 1156.7027 Da.

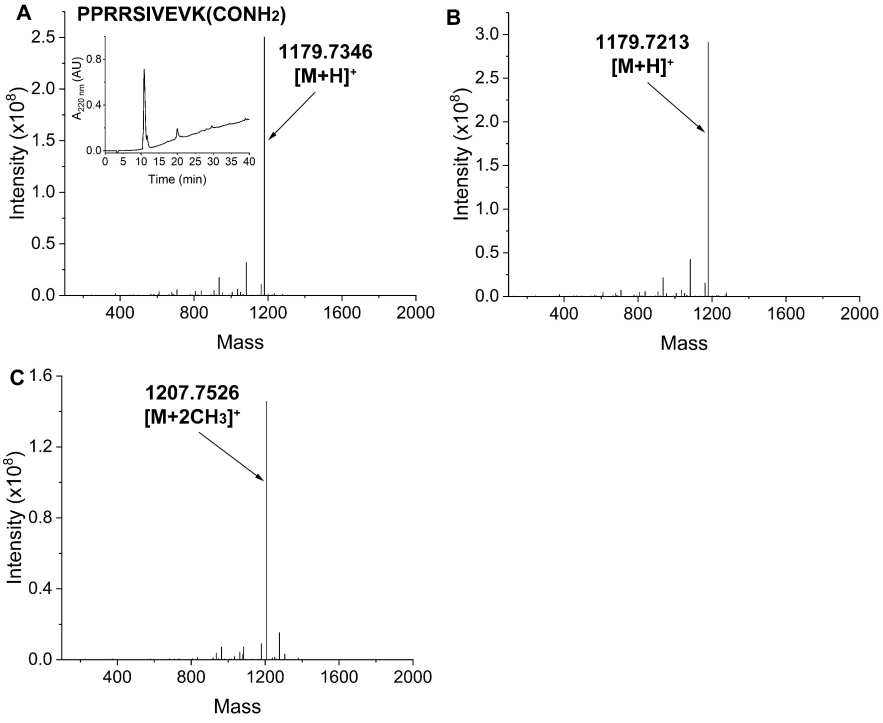


Figure S8. MS analysis of SPD2B peptide methylation. (A) MS and HPLC (inset) analyses of the synthesized SPD2B peptide. The calculated exact mass for $[M+H]^+$ is 1179.7326 Da. (B) MS analysis of the reaction between 20 μM SPD2B peptide and 200 μM SAM in the absence of *wt* NTMT1. (C) MS analysis of the reaction between 20 μM SPD2B peptide and 200 μM SAM catalyzed by 3 μM *wt* NTMT1. The calculated exact mass for dimethylated product ($[M+2CH_3]^+$) is 1207.7639 Da.

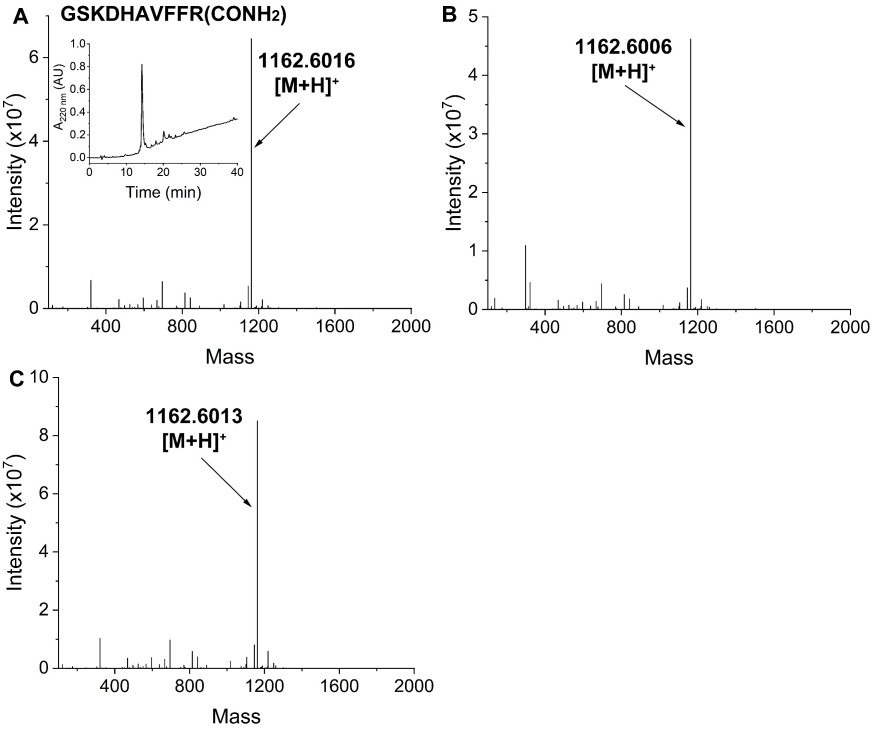


Figure S9. MS analysis of DDX60L peptide methylation. (A) MS and HPLC (inset) analyses of the synthesized DDX60L peptide. The calculated exact mass for $[M+H]^+$ is 1162.6122 Da. (B) MS analysis of the reaction between 20 μM DDX60L peptide and 200 μM SAM in the absence of *wt* NTMT1. (C) MS analysis of the reaction between 20 μM DDX60L peptide and 200 μM SAM catalyzed by 3 μM *wt* NTMT1. The calculated exact mass for trimethylated product ($[M+3CH_3]^+$) is 1204.6591 Da, which was not detected in MS.

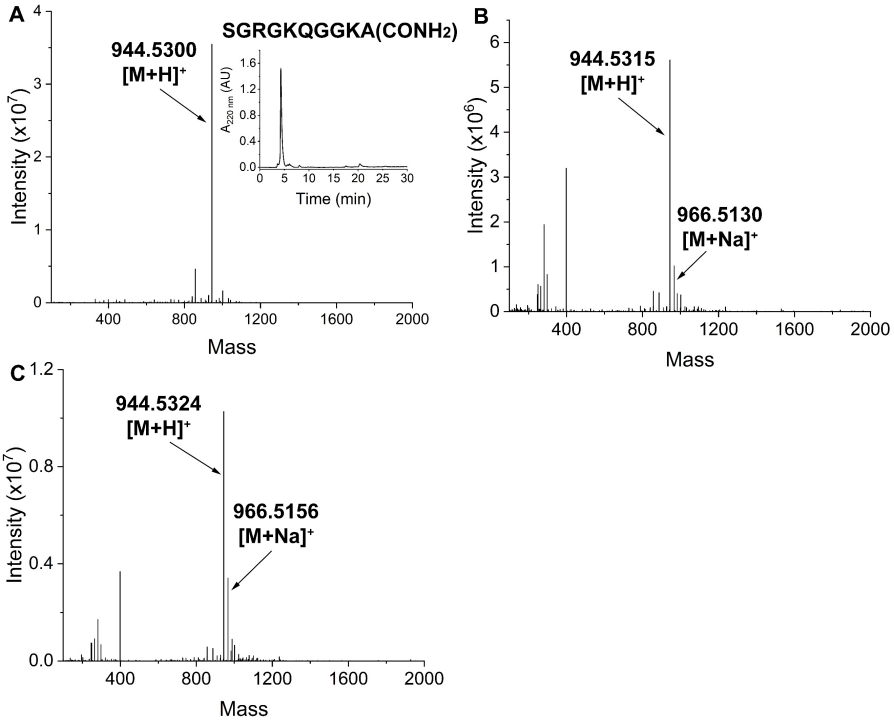


Figure S10. MS analysis of H2A1B peptide methylation. (A) MS and HPLC (inset) analyses of the synthesized H2A1B peptide. The calculated exact mass for $[M+H]^+$ is 944.5390 Da. (B) MS analysis of the reaction between 20 μM H2A1B peptide and 200 μM SAM in the absence of *wt* NTMT1. (C) MS analysis of the reaction between 20 μM H2A1B peptide and 200 μM SAM catalyzed by 3 μM *wt* NTMT1. The calculated exact mass for trimethylated product ($[M+3CH_3]^+$) is 986.5859 Da, which was not detected in MS.

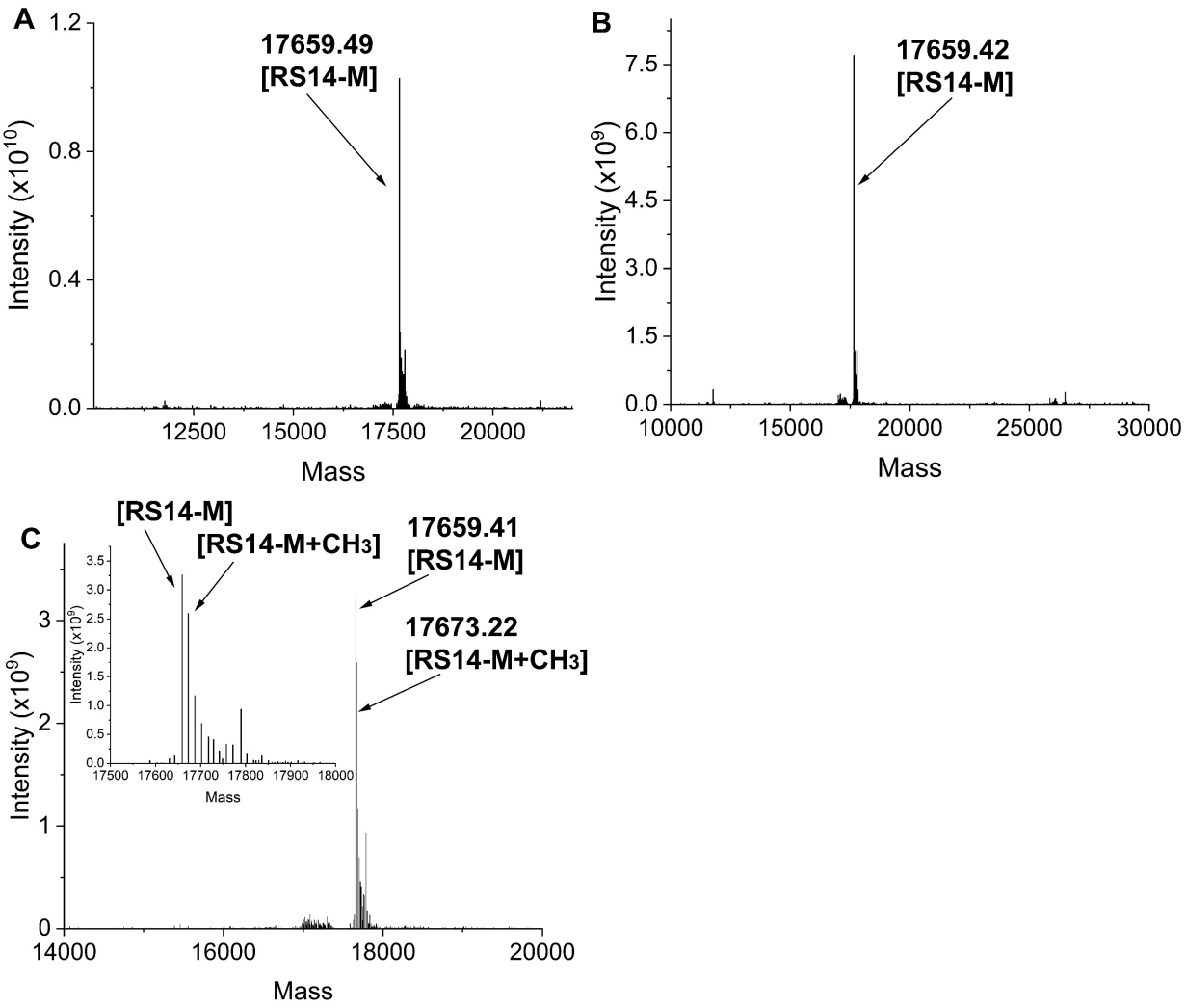


Figure S11. Intact protein MS analysis of RS14 methylation. (A) RS14 purified from *E. coli* BL21(DE3) strain. The calculated MW of RS14 with the initial methionine removal is 17661.2 Da. (B) 20 μM RS14 incubated with 200 μM SAM in the absence of *wt* NTMT1. (C) 20 μM RS14 incubated with 200 μM SAM in the presence of 3 μM *wt* NTMT1. The inset shows the expansion of MWs. The calculated MW of monomethylated product ([RS14–M+CH_3]) is 17675.2 Da.

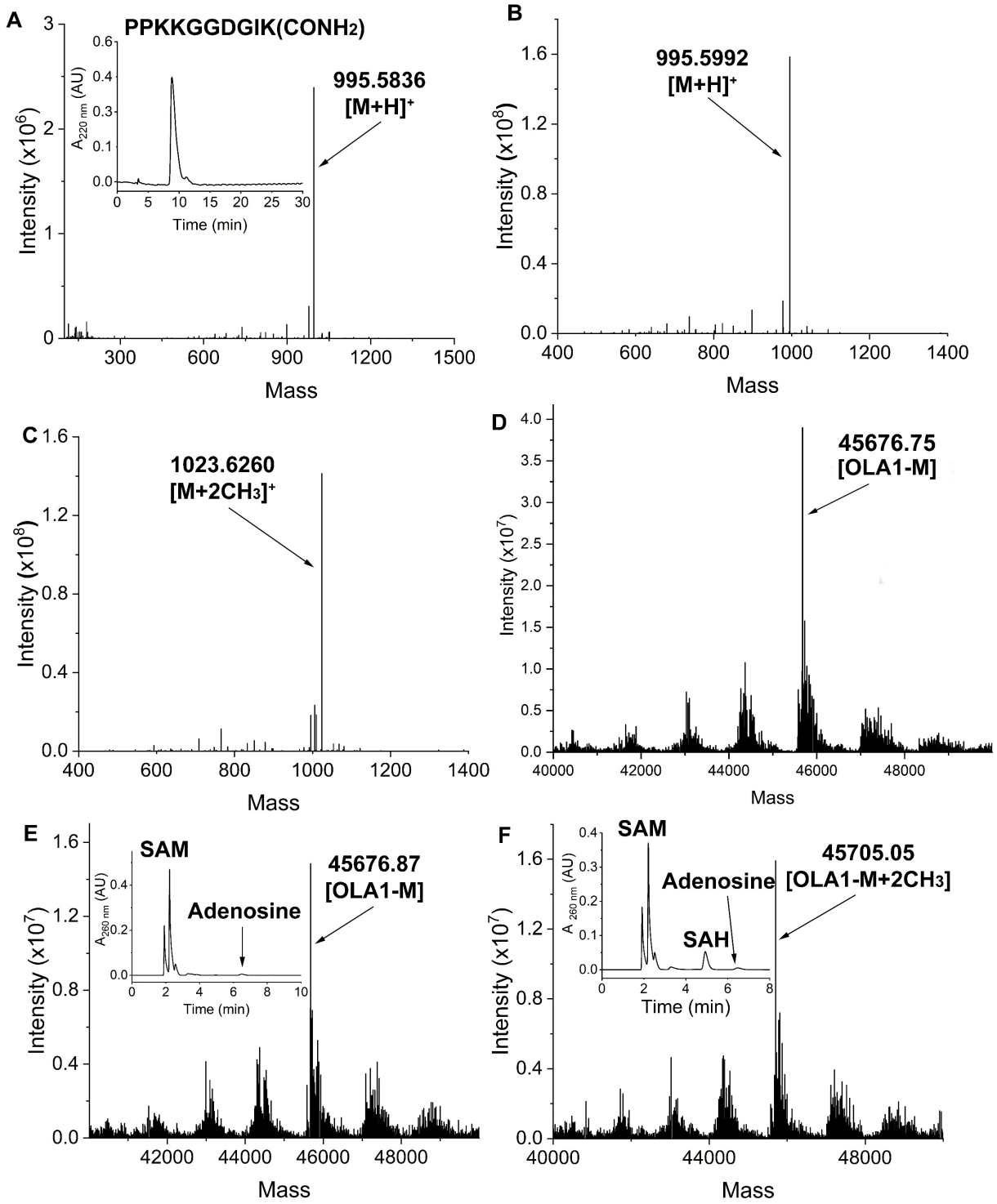


Figure S12. MS analysis of OLA1 methylation *in vitro*. (A) MS and HPLC (inset) analyses of the synthesized OLA1 peptide. The calculated exact mass for $[M+H]^+$ is 995.6002 Da. (B) MS analysis of the reaction between 20 μM OLA1 peptide and 200 μM SAM in the absence of *wt* NTMT1. (C) MS analysis of the reaction between 20 μM OLA1 peptide and 200 μM SAM catalyzed by 1 μM *wt* NTMT1. The calculated exact mass for dimethylated product ($[M+2CH_3]^+$) is 1023.6315 Da. (D) Intact protein mass of OLA1 purified from *E. coli* BL21(DE3) strain. The calculated MW of OLA1 with the initial methionine removal is 45677.5 Da. (E) Intact protein mass of OLA1 (20 μM) after incubating with 200 μM SAM in the absence of *wt* NTMT1. (F) Intact protein mass of OLA1 (20 μM) after incubating with 200 μM SAM in the presence of 1 μM NTMT1. The calculated MW of dimethylated product ($[OLA1–M+2CH_3]$) is 45706.56 Da. The insets in (E) and (F) show SAH release monitored by HPLC at 260 nm.

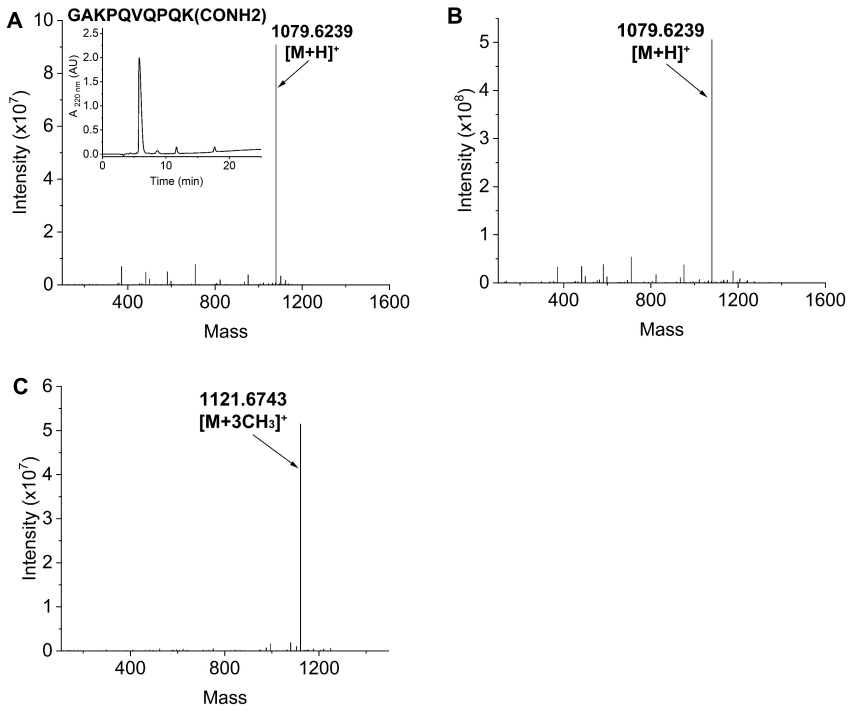


Figure S13. MS analysis of peptide methylation. (A) MS and HPLC (inset) analyses of the synthesized FECH peptide. The calculated exact mass for $[M+H]^+$ is 1079.6326 Da. (B) MS analysis of the reaction between 20 μM FECH peptide and 200 μM SAM in the absence of *wt* NTMT1. (C) MS analysis of the reaction between 20 μM FECH peptide and 200 μM SAM catalyzed by 3 μM *wt* NTMT1. The calculated exact mass for trimethylated product ($[M+3CH_3]^+$) is 1121.6795 Da.

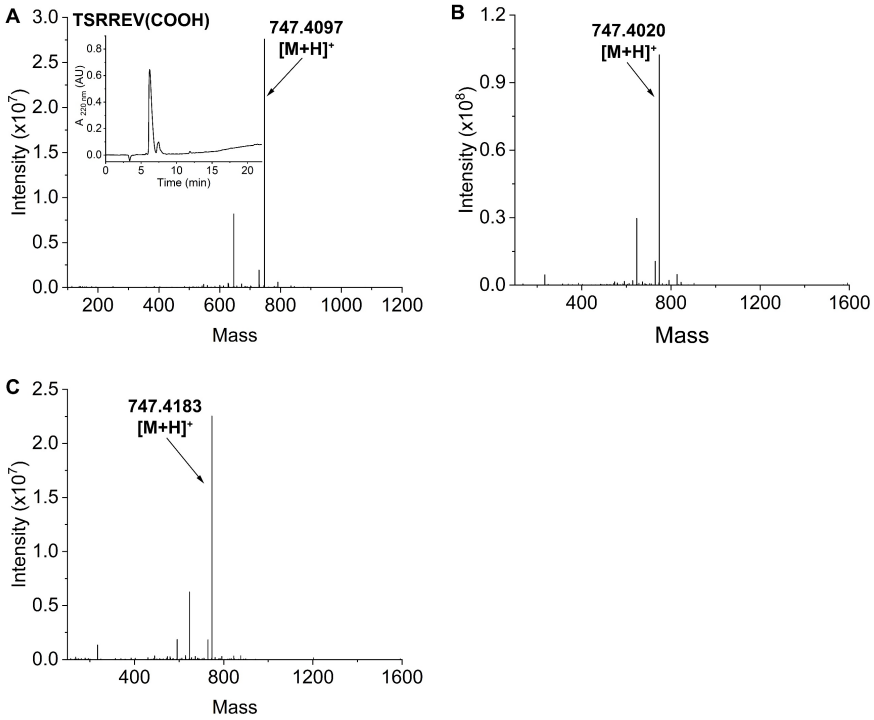


Figure S14. MS analysis of LAMC2 peptide methylation. (A) MS and HPLC (inset) analyses of the synthesized LAMC2 peptide. The calculated exact mass for $[M+H]^+$ is 747.4113 Da. (B) MS analysis of the reaction between 20 μM LAMC2 peptide and 200 μM SAM in the absence of *wt* NTMT1. (C) MS analysis of the reaction between 20 μM LAMC2 peptide and 200 μM SAM catalyzed by 3 μM *wt* NTMT1. The calculated exact mass for trimethylated product ($[M+3CH_3]^+$) is 789.4583 Da, which was not detected in MS.

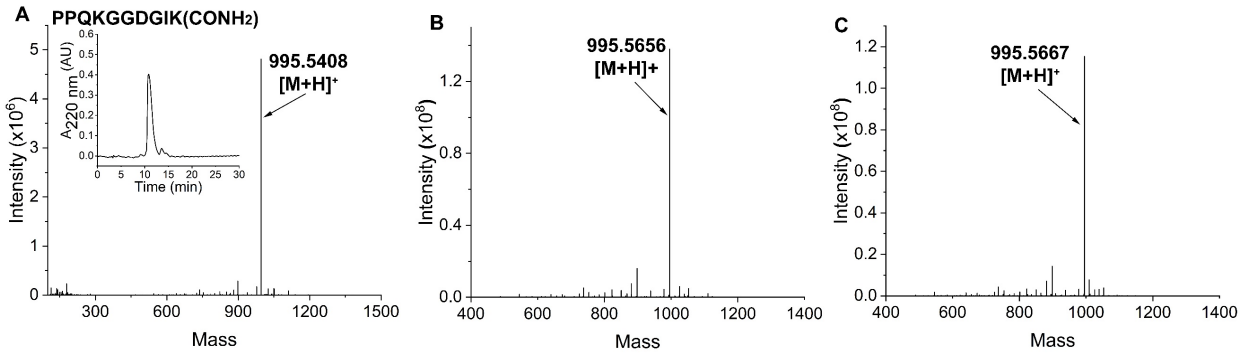


Figure S15. MS analysis of K4Q-OLA1 peptide methylation. (A) MS and HPLC (inset) analyses of the synthesized K4Q-OLA1 peptide. The calculated exact mass for $[M+H]^+$ is 995.5638 Da. (B) MS analysis of the reaction between 20 μM K4Q-OLA1 peptide and 200 μM SAM in the absence of *wt* NTMT1. (C) MS analysis of the reaction between 20 μM K4Q-OLA1 peptide and 200 μM SAM catalyzed by 1 μM *wt* NTMT1. The calculated exact mass for dimethylated product ($[M+2CH_3]^+$) is 1023.5951 Da, which was not detected in MS.

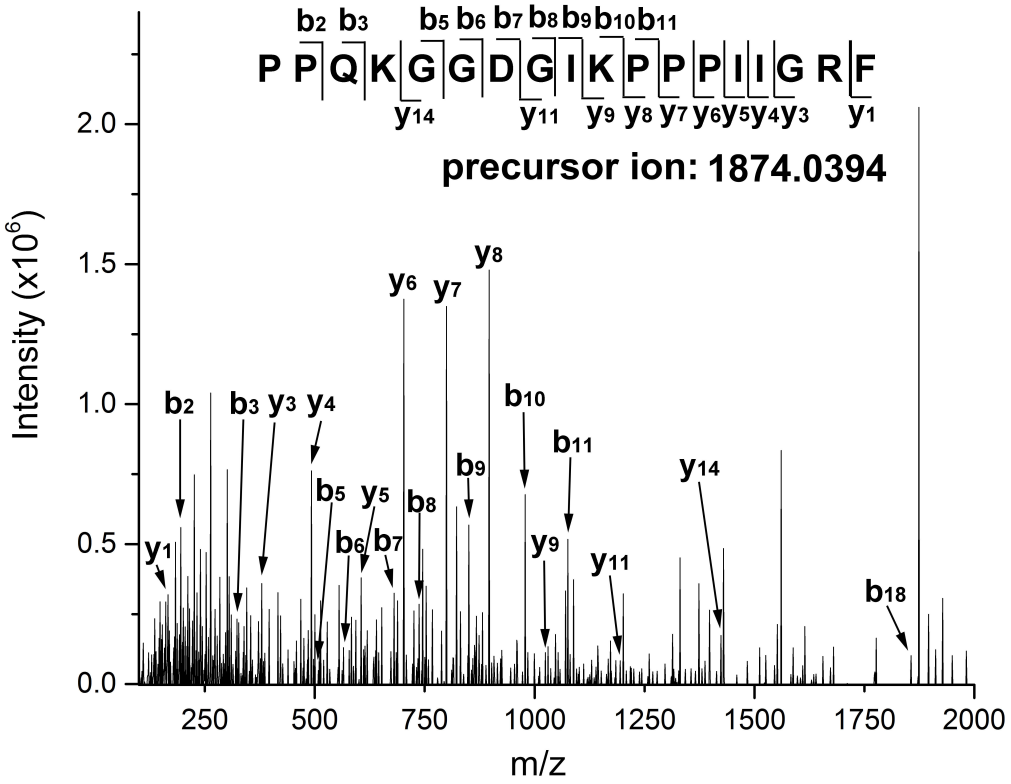


Figure S16. Tandem MS spectrum of nonmethylated peptide fragment obtained from normal HEK293FT cells. This 18-mer peptide fragment corresponds to the first 18 N-terminal residues of K4Q-OLA1 after methionine removal.

Table S1. Expanded N-terminal motifs for NTMT1 substrates

Residues	1	M							
	2	A	G	F	M	P	S	T	R	Y
	3	A	G	M	N	P	S	T		
	4	K	R							

Table S2. Potential protein targets of NTMT1*

UniProt ID	Protein name	Confidence score	Anova (*p*)	Max fold change
P08670	VIME	449.57	7.82E-06	2.88
P53396	ACLY	439.42	9.73E-05	2.95
Q9NR22	ANM8	289.60	1.34E-05	6.37
P54652	HSP72	246.86	0.000118	2.97
P22102	PUR2	173.66	0.000116	2.52
P13667	PDIA4	137.95	5.01E-05	2.40
P04181	OAT	115.58	8.47E-05	2.88
Q5H9U9	DDX60L	92.45	5.94E-05	4.12
Q05BV3	EMAL5	90.08	7.60E-05	3.16
P37802	TAGL2	75.66	0.000104	3.12
P62750	RL23A	70.68	4.02E-05	4.77
Q6GYQ0	RGPA1	68.95	5.95E-05	2.36
Q8N7X0	ADGB	64.13	0.002031	2.91
P15121	ALDR	59.92	2.11E-06	3.15
P42336	PK3CA	58.43	0.000209	4.44
P18754	RCC1	56.32	5.01E-05	3.46
Q9Y6M1	IF2B2	50.81	6.73E-05	2.85
P46776	RL27A	45.86	3.58E-05	3.28
P61158	ARP3	43.57	0.000311	2.70
P35232	PHB	43.24	0.001095	3.05
Q659A1	ICE2	42.68	0.000314	3.08
Q6UB35	C1TM	42.05	0.00011	2.43
O00160	MYO1F	38.42	1.53E-05	7.83
B4DIP2	B4DIP2	38.40	0.000237	2.79
Q9Y4E1	WAC2C	37.10	0.000257	8.23
Q641Q2	WAC2A	37.10	0.000257	8.23
Q8IYD8	FANCM	36.06	0.000281	3.22
Q9Y5S2	MRCKB	34.31	2.07E-05	3.44
P62263	RS14	34.10	0.000113	4.20
Q16576	RBBP7	32.22	0.000883	4.23
Q8NBS9	TXND5	29.80	0.000341	2.77
Q8TF47	ZFP90	29.54	0.000505	2.82
P04908	H2A1B	29.50	0.00172	2.57
P0C0S8	H2A1	29.50	0.00172	2.57
P20671	H2A1D	29.50	0.00172	2.57
Q16777	H2A2C	29.50	0.00172	2.57
Q6FI13	H2A2A	29.50	0.00172	2.57
Q7L7L0	H2A3	29.50	0.00172	2.57
Q93077	H2A1C	29.50	0.00172	2.57
Q96KK5	H2A1H	29.50	0.00172	2.57
Q99878	H2A1J	29.50	0.00172	2.57
Q9BTM1	H2AJ	29.50	0.00172	2.57
P16104	H2AX	29.50	0.00172	2.57
Q8IUE6	H2A2B	29.50	0.00172	2.57
Q96QV6	H2A1A	29.50	0.00172	2.57

Q13017	RHG05	26.46	0.000342	2.05
Q9Y2Z9	COQ6	26.40	7.67E-05	2.74
P62805	H4	26.25	0.000414	2.72
P10606	COX5B	26.03	0.000181	4.29
Q8IWG1	WDR63	23.87	0.000666	3.67
Q8NB90	AFG2H	23.41	7.21E-05	3.09
Q13753	LAMC2	23.13	2.40E-06	4.10
P46459	NSF	21.53	0.000219	2.44
Q01955	CO4A3	20.50	7.26E-06	8.67
Q2PPJ7	RGPA2	20.03	0.000238	3.61
Q5T5U3	RHG21	19.69	1.60E-05	3.01
Q9UQB3	CTND2	17.29	0.004354	2.48
Q9NTK5	OLA1	16.12	0.000527	3.57
Q12965	MYO1E	15.73	0.000128	2.90
Q86U86	PB1	15.47	4.33E-06	7.29
P22830	FECH	15.4	6.27E-05	2.97
Q9BQ39	DDX50	12.22	5.70E-05	2.94
Q86VW1	S22AG	11.67	1.39E-05	3.90
Q6ZRP0	PR23C	11.44	5.58E-06	5.67
Q01105	SET	11.44	8.35E-05	4.14
Q96L34	MARK4	11.40	0.000136	2.85
Q9Y3F4	STRAP	10.70	0.000349	2.99
Q6NSJ2	PHLB3	9.44	0.000226	2.32
Q96GM5	SMRD1	6.01	0.001129	3.81
P42679	MATK	5.76	5.39E-05	2.67
A1X283	SPD2B	5.37	0.000273	2.94
P26358	DNMT1	4.99	3.55E-05	6.33

*Proteins highlighted in magenta are obtained from signal peptide and motif sequence analyses. Proteins highlighted in green are NTMT1 substrates previously confirmed. Proteins highlighted in yellow and magenta were selected for target validation.

Table S3. HPLC conditions for peptide purification and characterization*

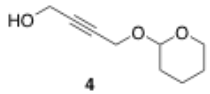
		Semi-preparative HPLC	Analytical HPLC
Column		Phenomenex Luna C18(2), 5 µm, 100 Å, 10 mm × 250 mm	Waters XBridge BEH Shield RP 18, 5 µm, 130 Å, 4.6 mm × 250 mm
Flow Rate (mL/min)		3.0	1.0
Mobile Phases		**A:** 0.1% TFA in H_2O; **B:** 0.1% TFA in MeOH	
Gradient (**B**)	RCC1	0-5-30 min: 5-5-30% (23.7 min)	0-40 min: 5-95% (10.2 min)
	PB1	N/A	0-30 min: 1-15% (11.2 min)
	SPD2B	0-40 min: 10-40% (18.3 min)	0-40 min: 10-90% (10.8 min)
	DDX60L	N/A	0-40 min: 10-90% (14.2 min)
	H2A1B	N/A	0-30 min: 1-15% (4.3 min)
	FECH	N/A	0-40 min: 5-90% (5.8 min)
	LAMC2	N/A	0-40 min: 5-90% (6.2 min)
	OLA1	0–25 min: 5–40% (11.9 min)	0-30 min: 5–15% (9.0 min)
	K4Q-OLA1	0–30–50 min: 5–15–50% (37.0 min)	0-30 min: 5–15% (10.8 min)

*Numbers shown in parentheses are HPLC retention times.

Table S4. Plasmid constructions

Plasmid	Primer sequence (restriction site bolded and underlined)		Vector	Tag
*p*NTMT1	NdeI_Fw	TAGCG**CATATG**ACGAGCGAGGTGATAG	*p*ET28-MHL (Addgene)	His-tag
	HindIII_Rv	TGACG**AAGCTT**CATCTCAGGGCAAAGC		
*p*RCC1	NdeI_Fw	TCGTTC**CATATG**TCACCCAAGCGCATAGC	*p*ET30a (+) (Novagen)	
	XhoI_Rv	AAACTA**CTCGAG**GCTCTGTTCTTTGTCCTTGAC		
*p*RS14	NcoI_Fw	TTTTTA**CCATGG**CACCTCGAAAGGGGAAGG	*p*ET28b (+) (Novagen)	
	HindIII_Rv	TTTTTT**AAGCTT**CAGACGGCGACCACGGCGACC C		
*p*OLA1	NdeI_Fw	TTGATA**CATATG**CCCCCTAAAAAGGGAGGTGATG	*p*ET30a (+) (Novagen)	
	XhoI_Rv	AAACAC**CTCGAG**TTTCTTCTTCGGTTGTTGAG		
*p*K4Q-OLA1	NdeI_Fw	TTGATA**CATATG**CCCCCTCAAAAGGGAGGTGATG	*p*ET30a (+) (Novagen)	
	XhoI_Rv	AAACAC**CTCGAG**TTTCTTCTTCGGTTGTTGAG		
*p*EGFP-n1-FLAG	BamHI_Fw	AAAATT**GGATCC**ACCGGTCGCCACCATGG	*p*EGFP-n1 (Clontech)	FLAG-tag
	NotI_Rv	AA**GCGGCCGC**CTACTTATCGTCATCGTCCTTGTA ATCCTTGTACAG CTCGTCCATGCC		
*p*OLA1-EGFP-FLAG	XhoI_Fw	TTGATA**CTCGAG**GCCACCATGCCCCCTAAAAAGG GAGGTGATG	*p*EGFP-n1-FLAG (this study)	
	BamHI_Rv	AAACAC**GGATCC**ACTTTCTTCTTCGGTTGTTGAG		
*p*K4Q-OLA1-EGFP-FLAG	XhoI_Fw	ATA**CTCGAG**GCCACCATGCCCCCTCAAAAGGGAG GTGATG		
	BamHI_Rv	AAACAC**GGATCC**ACTTTCTTCTTCGGTTGTTGAG		
PX459-NTMT1$_{sgRNA1}$	BbsI_Fw	**CACC**GCTGGAAACAAATCCCACCCA	PX459 (Addgene)	N/A
	BbsI_Rv	**AAAC**TGGGTGGGATTTGTTTCCAGC		
PX459-NTMT1$_{sgRNA2}$	BbsI_Fw	**CACC**GCATGCCGTCCACCGTGGGT		
	BbsI_Rv	**AAAC**ACCCACGGTGGACGGCATGC		
Seq-NTMT1$_{exon1}$	Forward	AAAACCCATATGCTCTGCACTCAGCAGGATGTGT GAC	for sequencing to confirm NTMT1 KO	

^{1}H NMR (400 MHz, $CDCl_3$)



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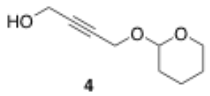
^{1}H NMR, gCOSY (400, MHz, $CDCl_3$)



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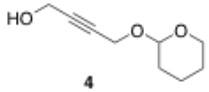

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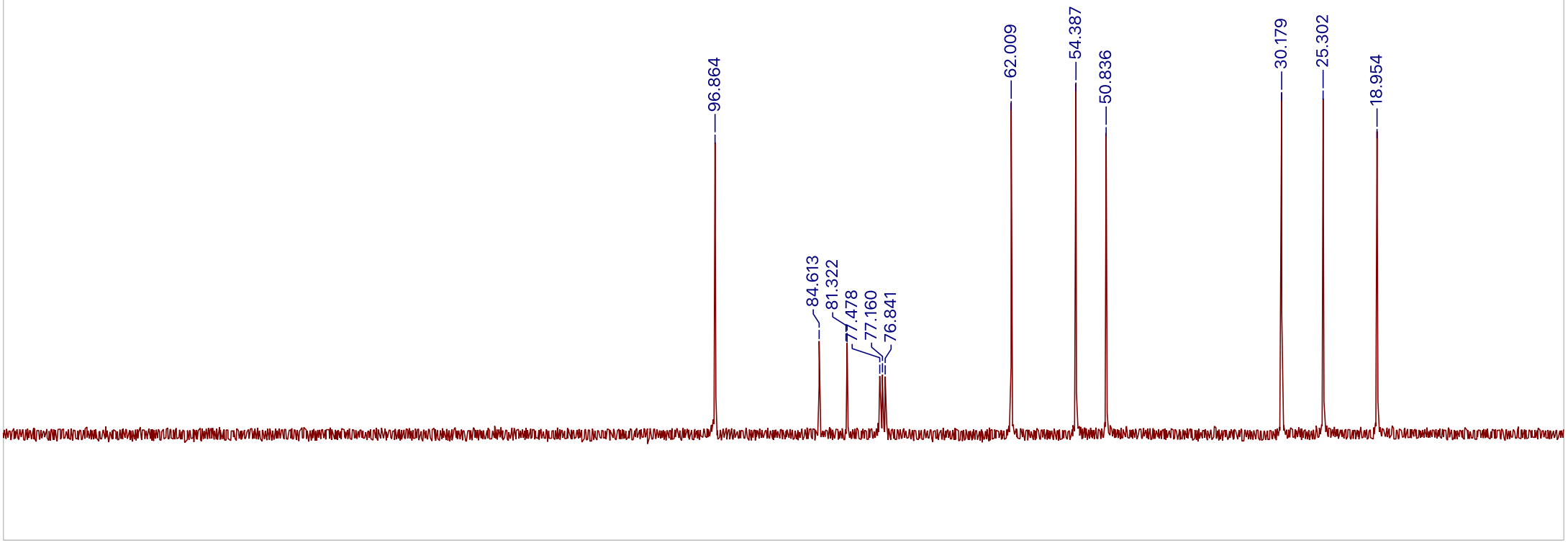

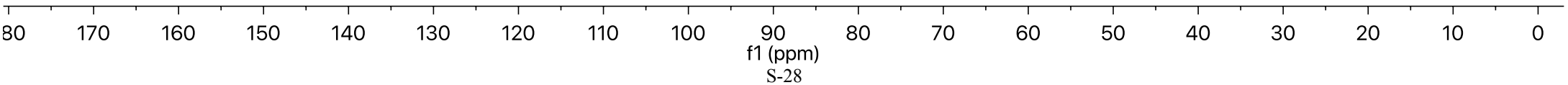

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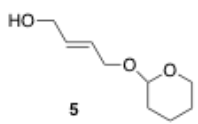

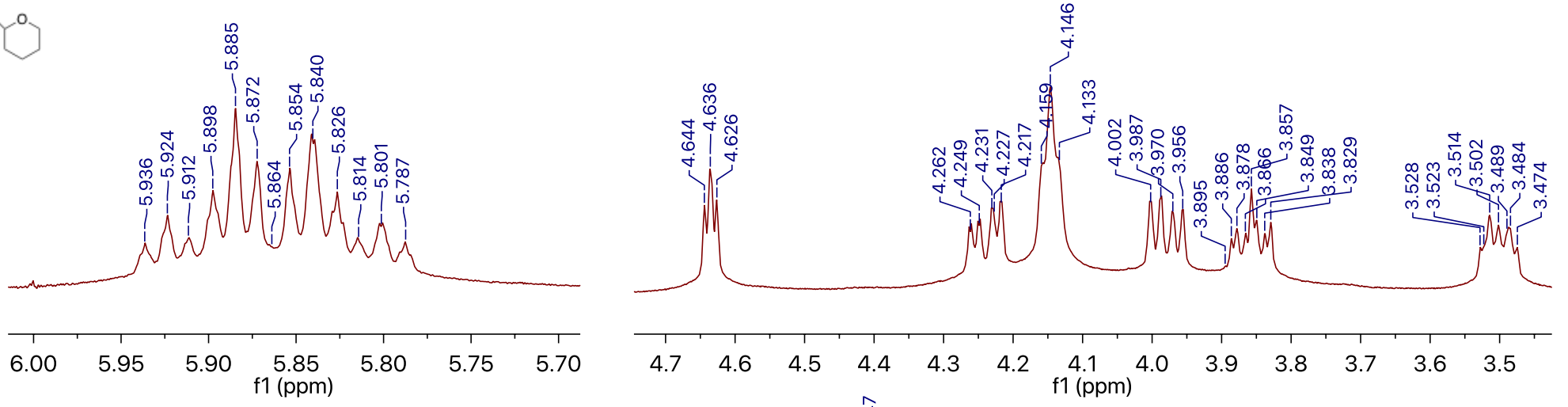

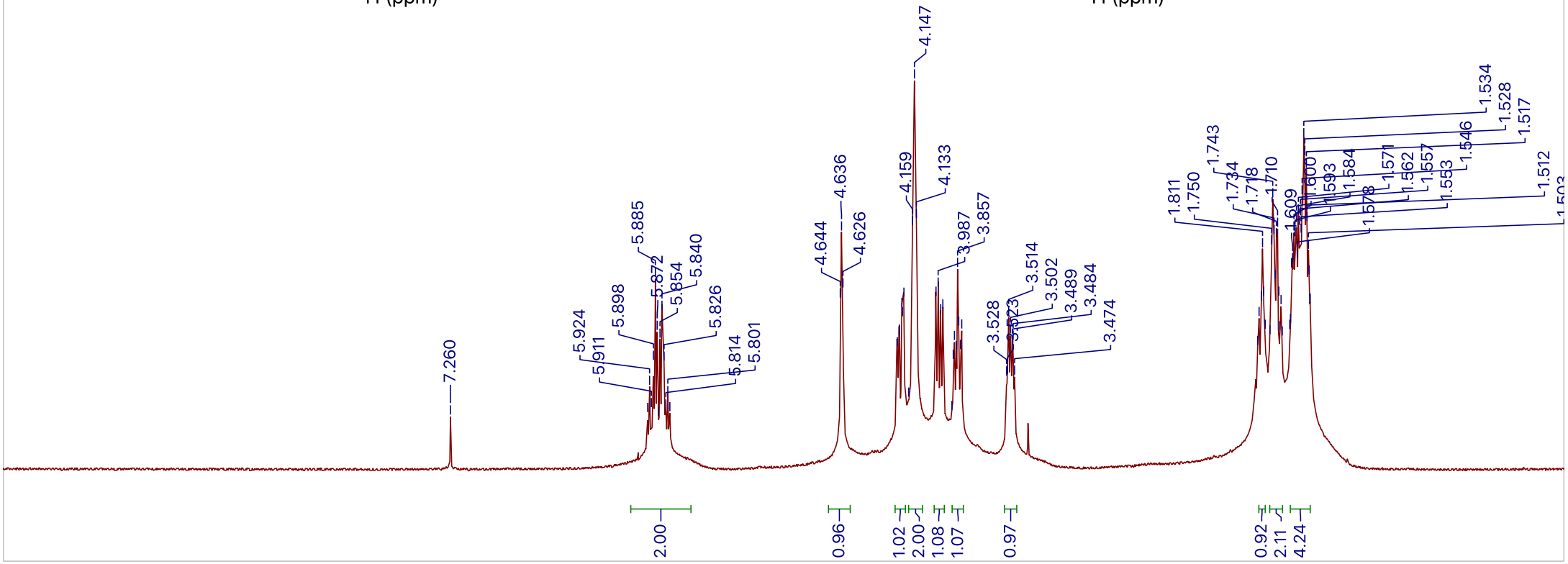

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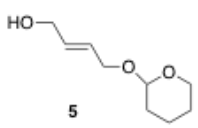

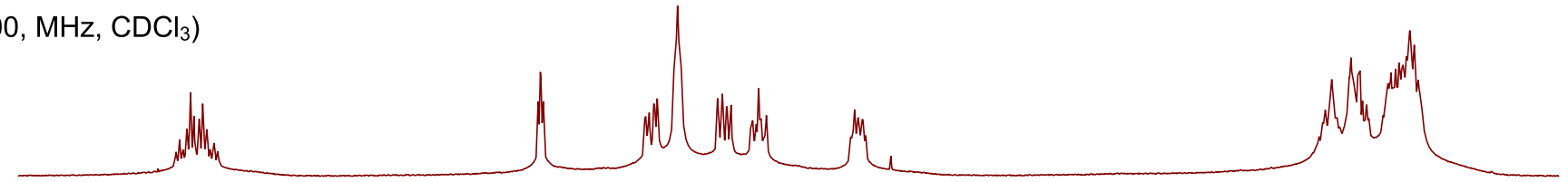

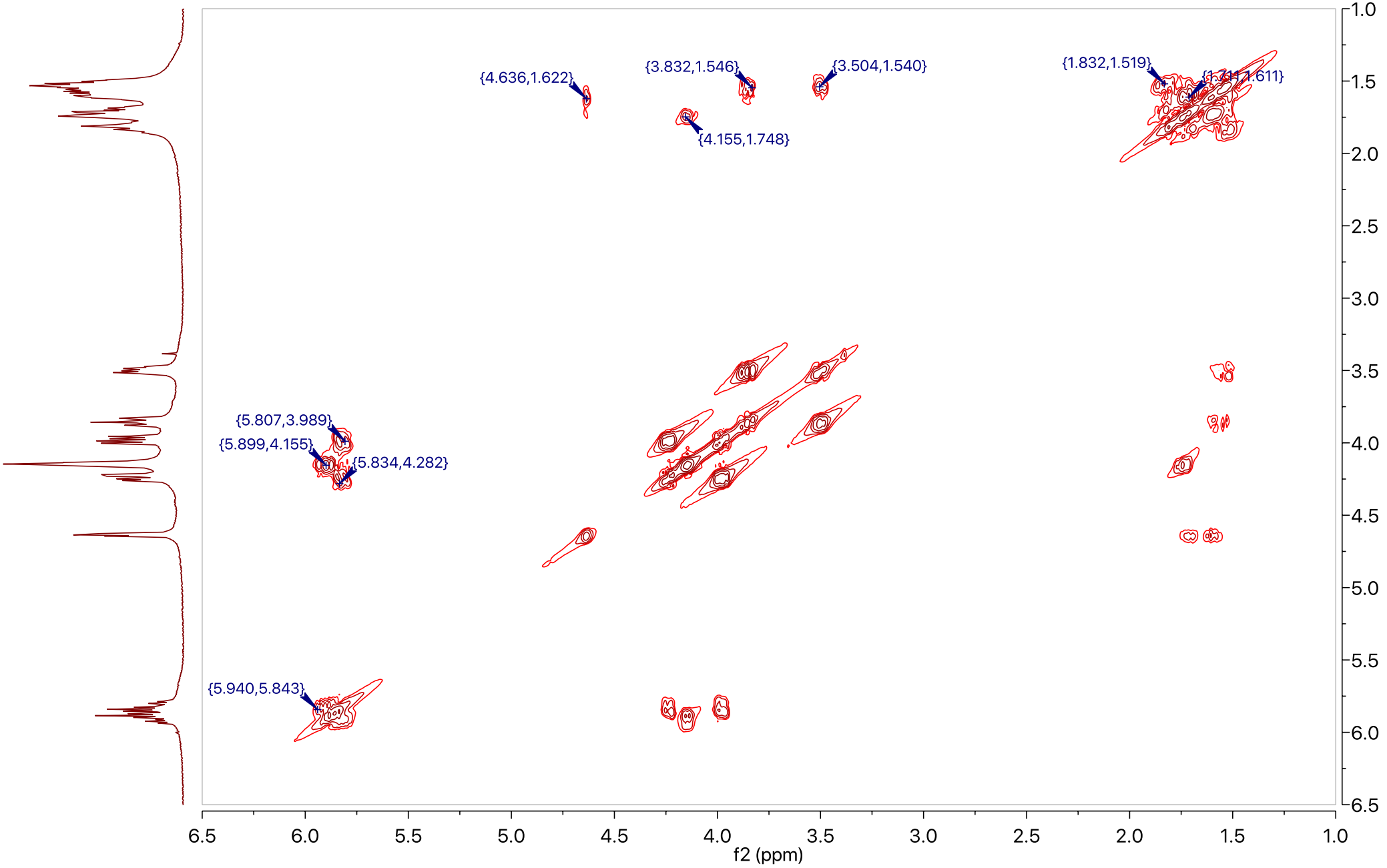

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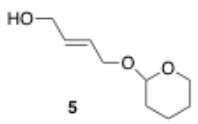

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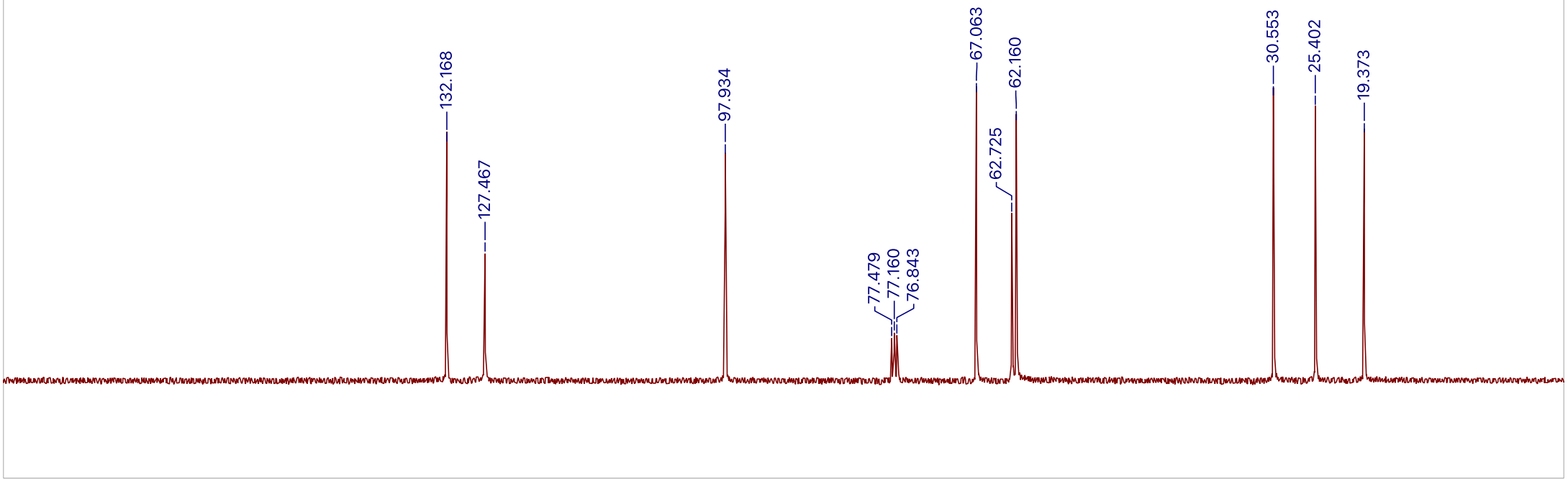

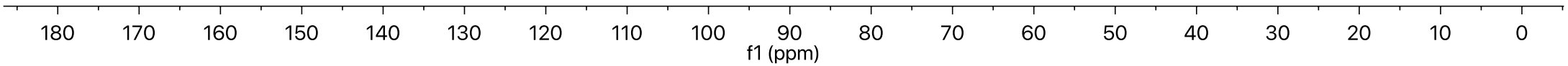

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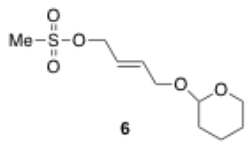
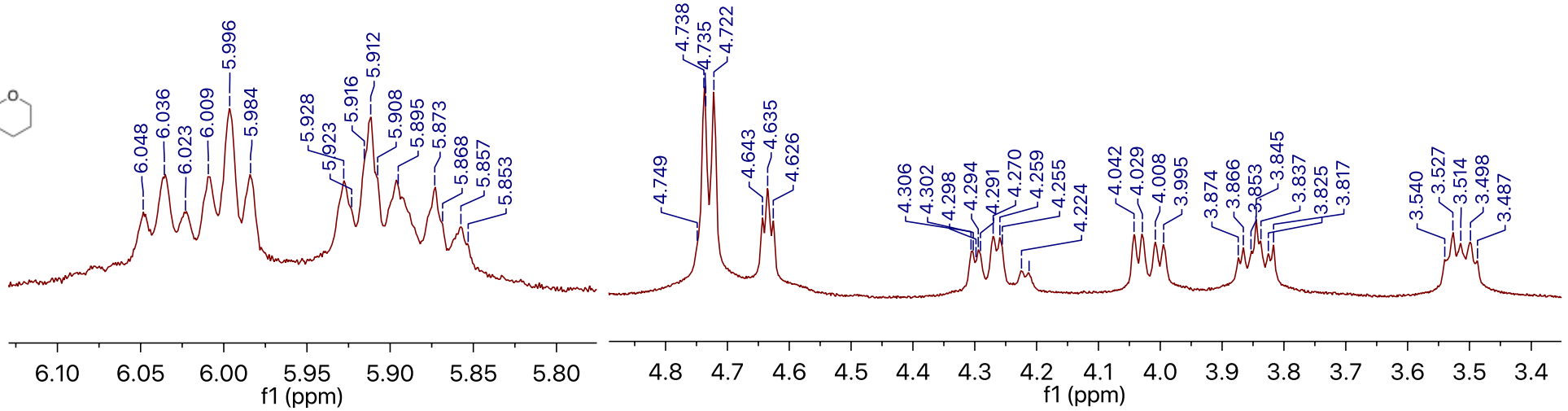
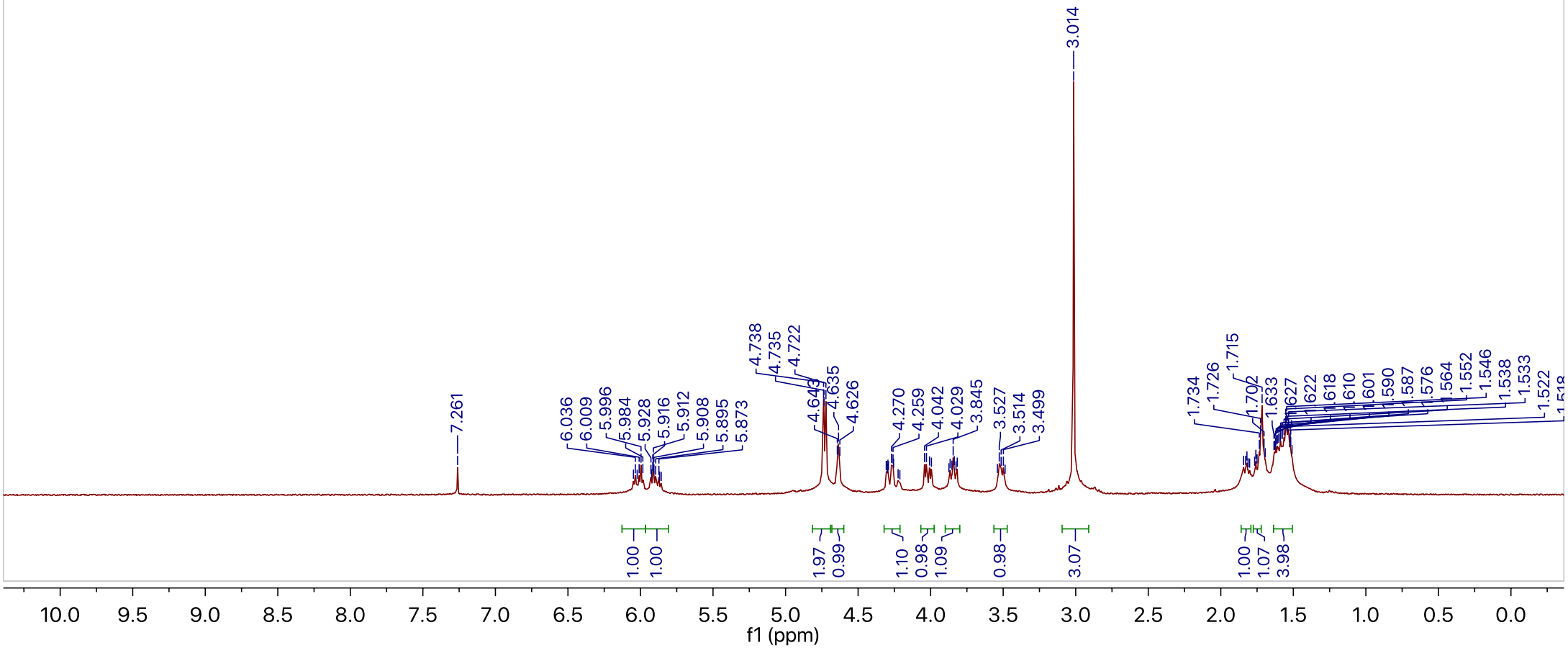
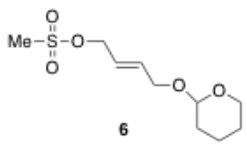






^{1}H NMR, gCOSY (400, MHz, $CDCl_3$)



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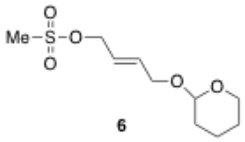

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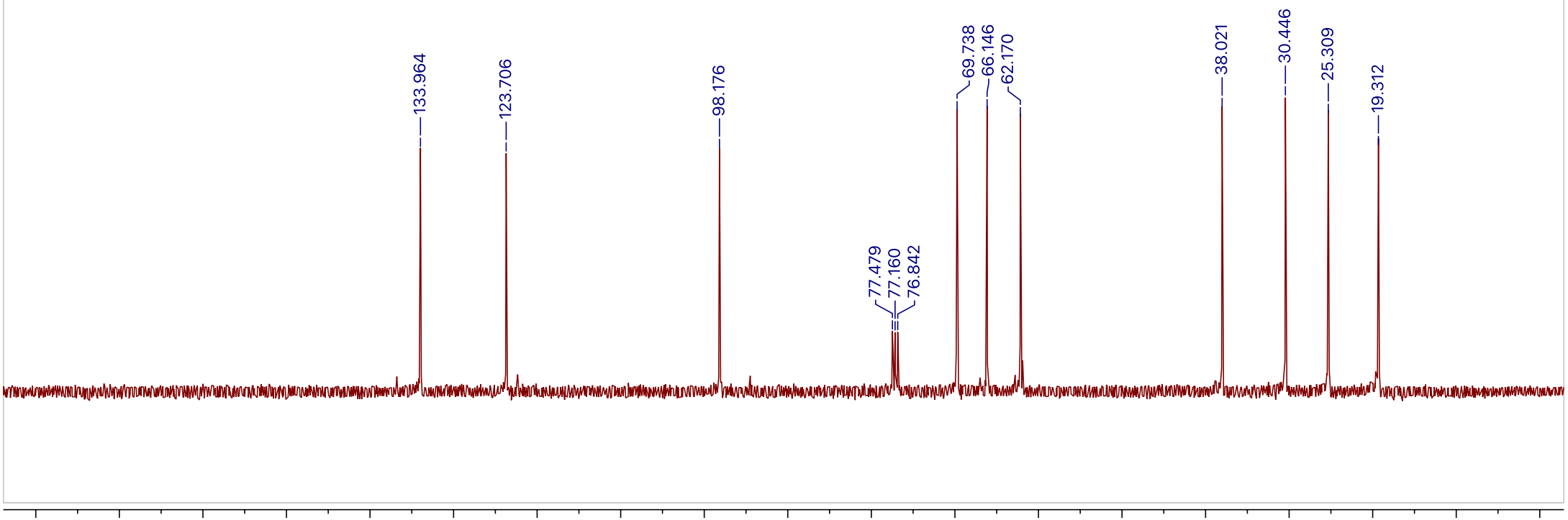
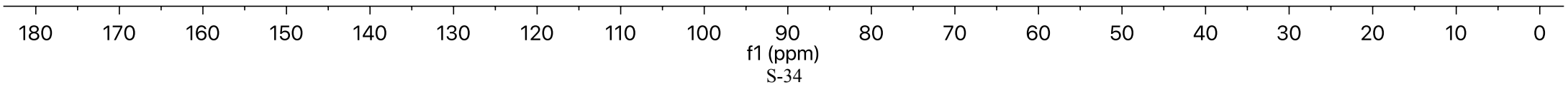
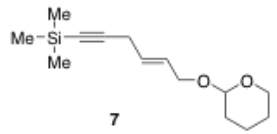


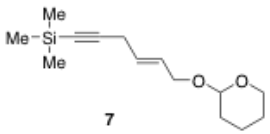


^{1}H NMR (400 MHz, $CDCl_3$)



7

^{1}H NMR, gCOSY (400, MHz, $CDCl_3$)



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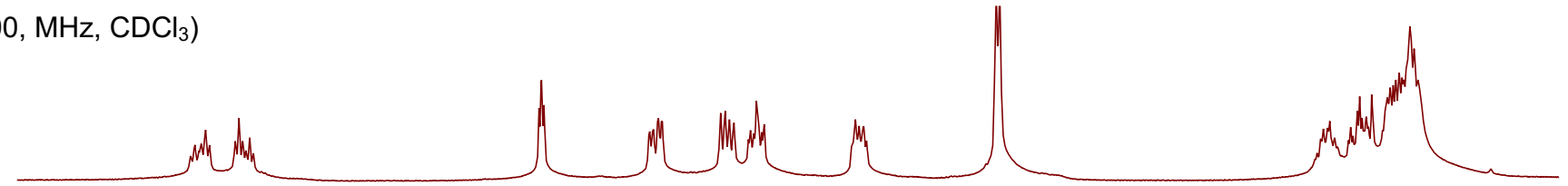
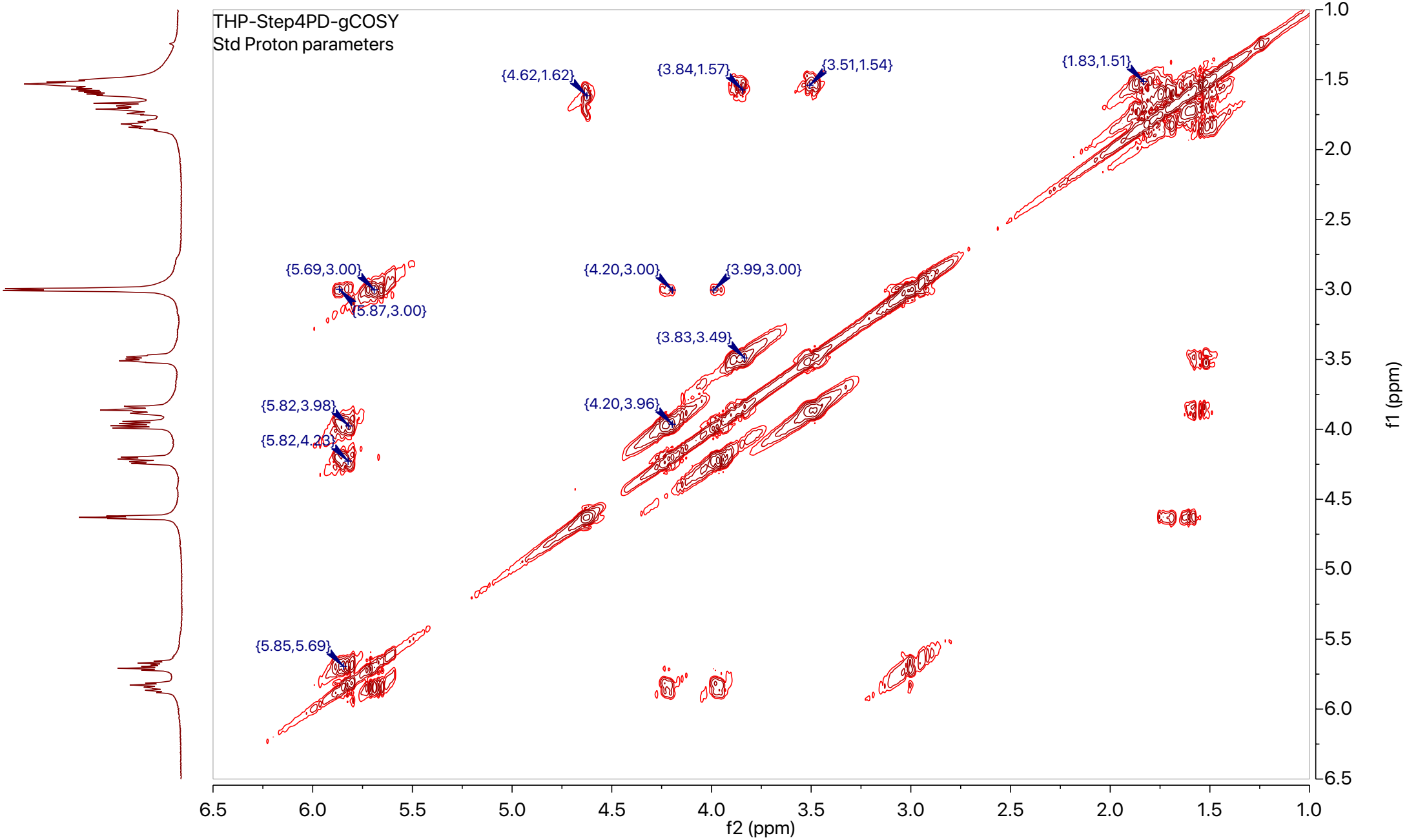
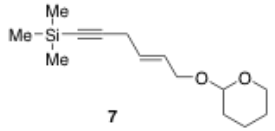




^{13}C NMR (101 MHz, $CDCl_3$)



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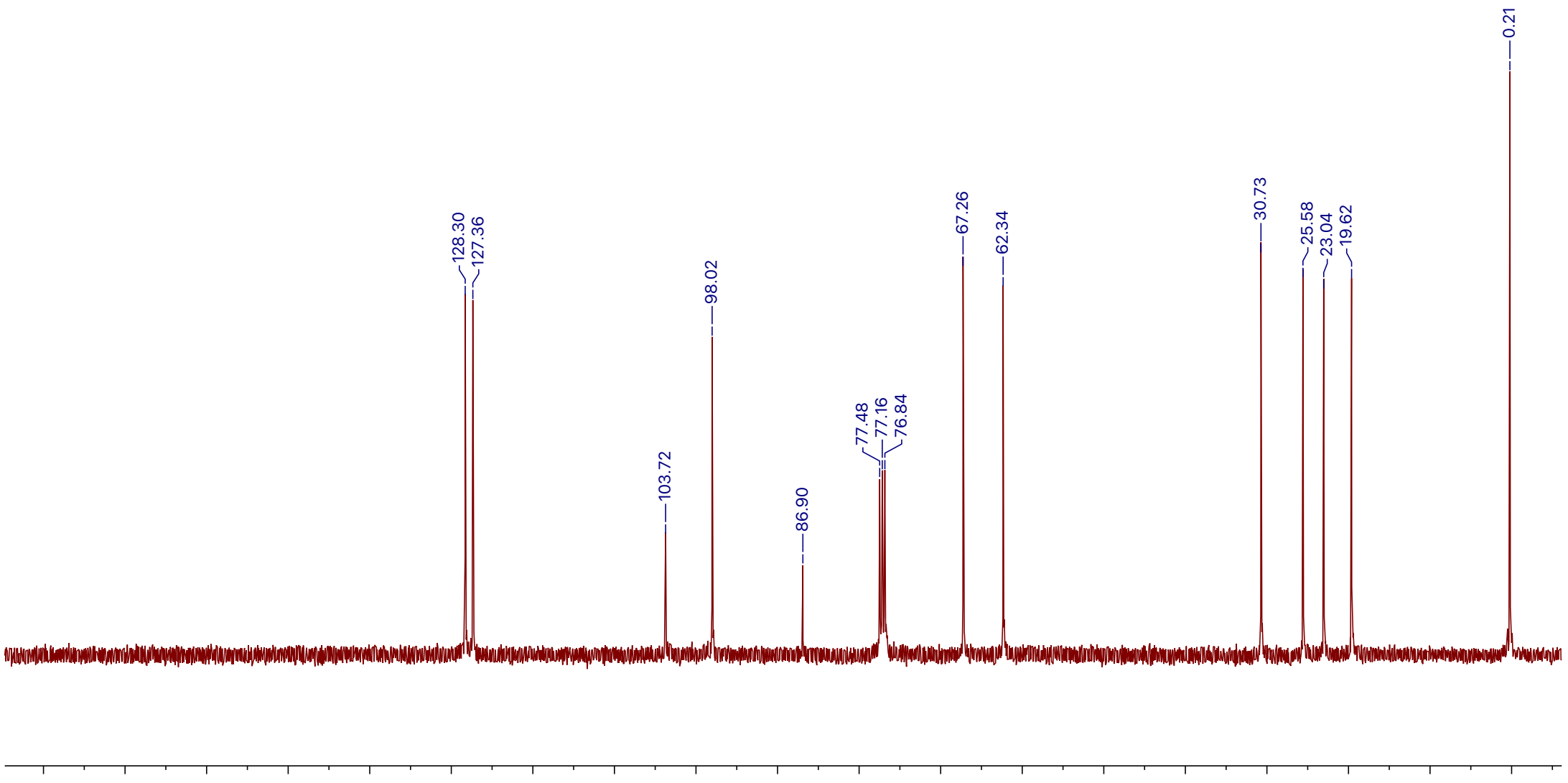
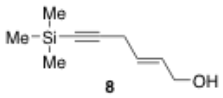
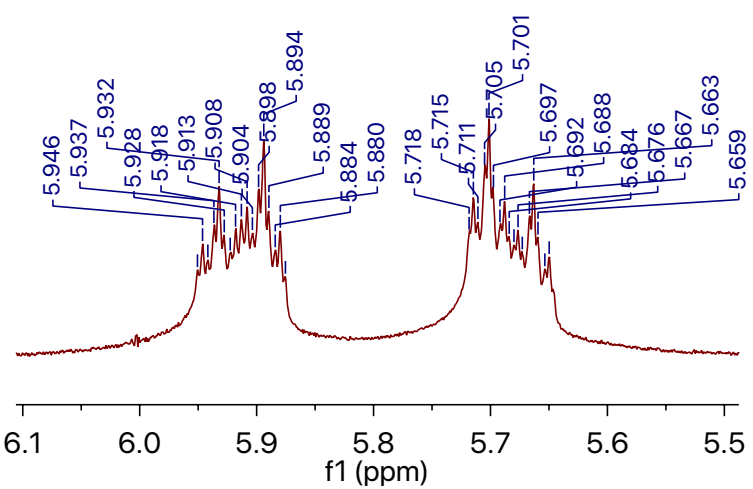
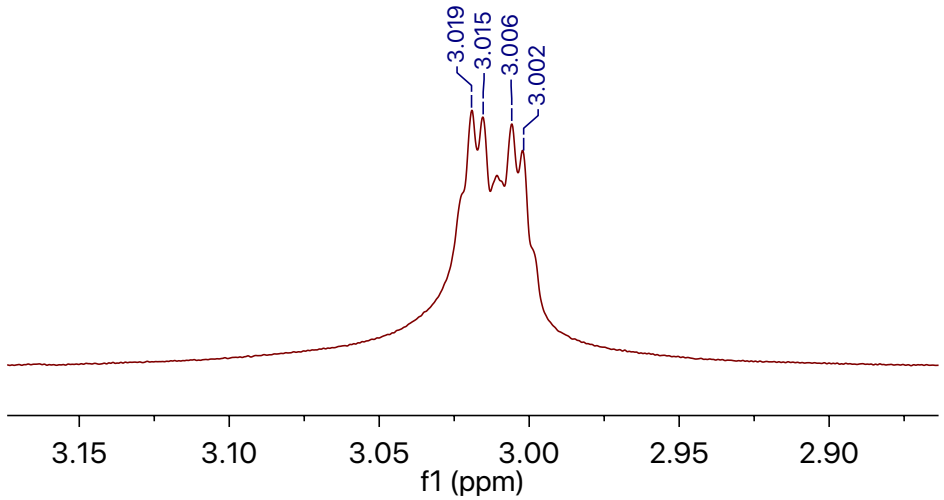


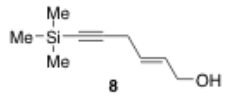
^{1}H NMR (400 MHz, $CDCl_3$)

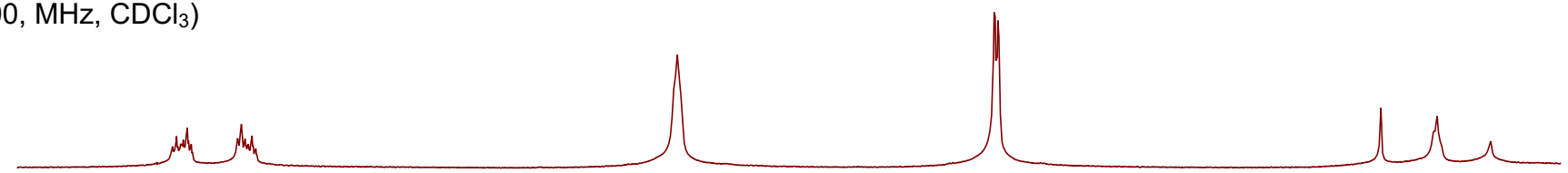


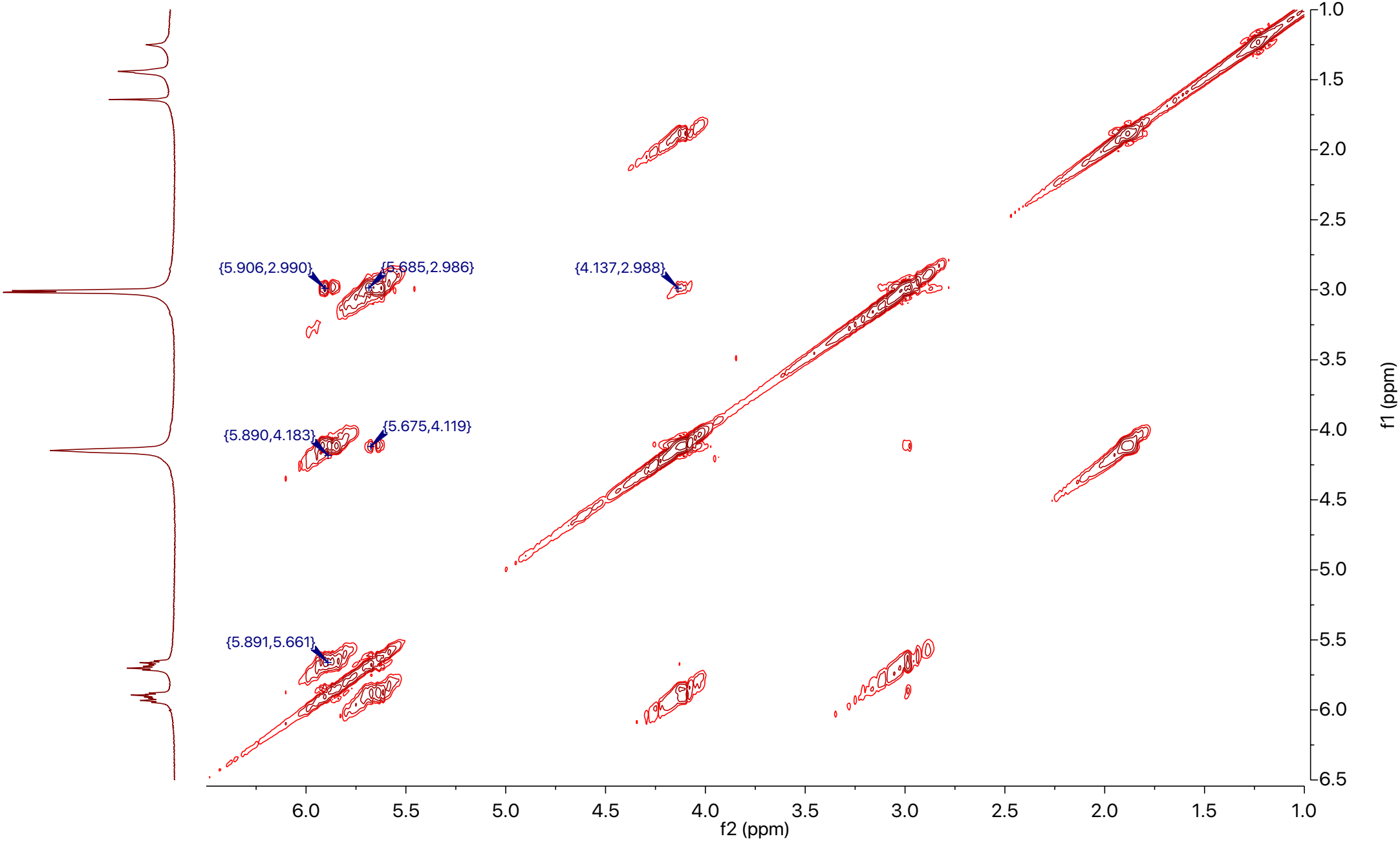




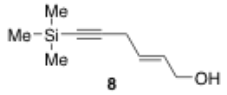
^{1}H NMR, gCOSY (400, MHz, $CDCl_3$)







^{13}C NMR (101 MHz, $CDCl_3$)



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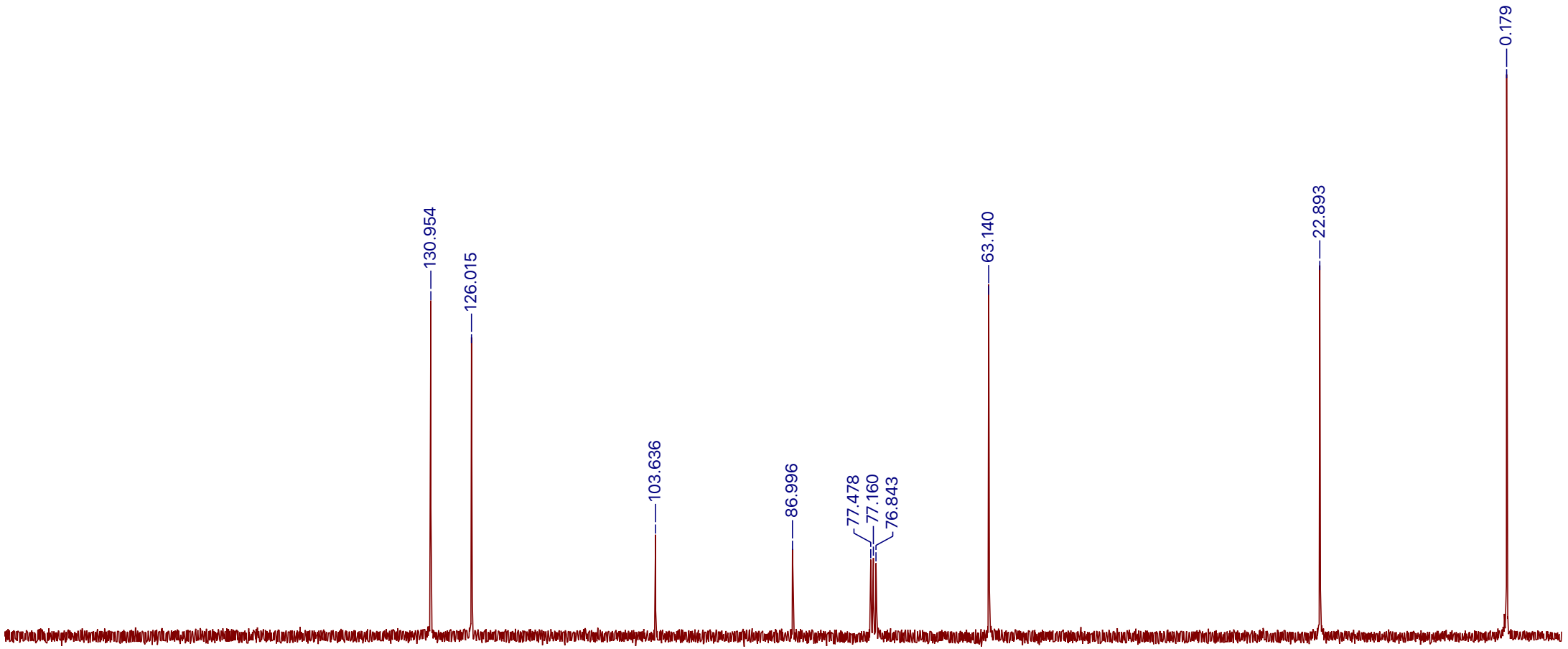
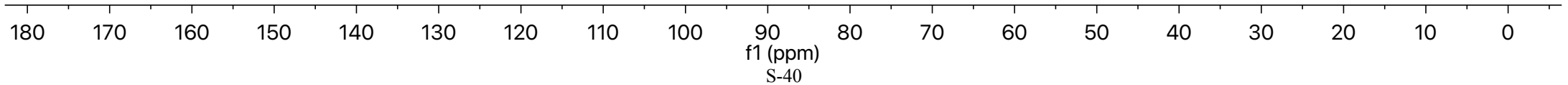
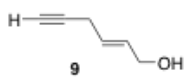
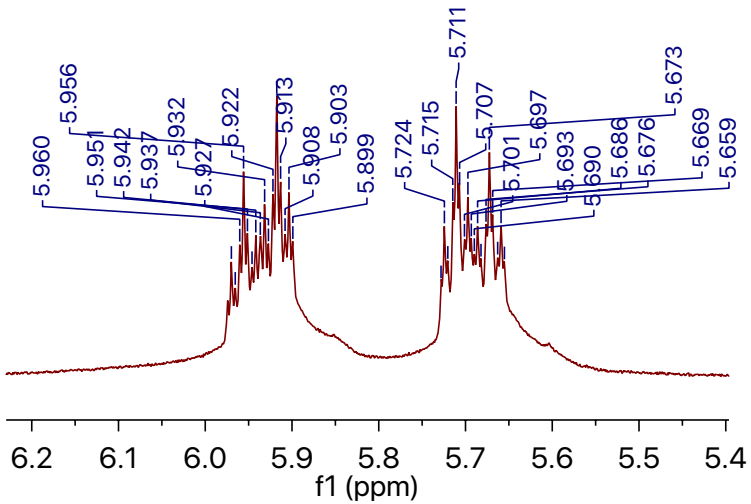
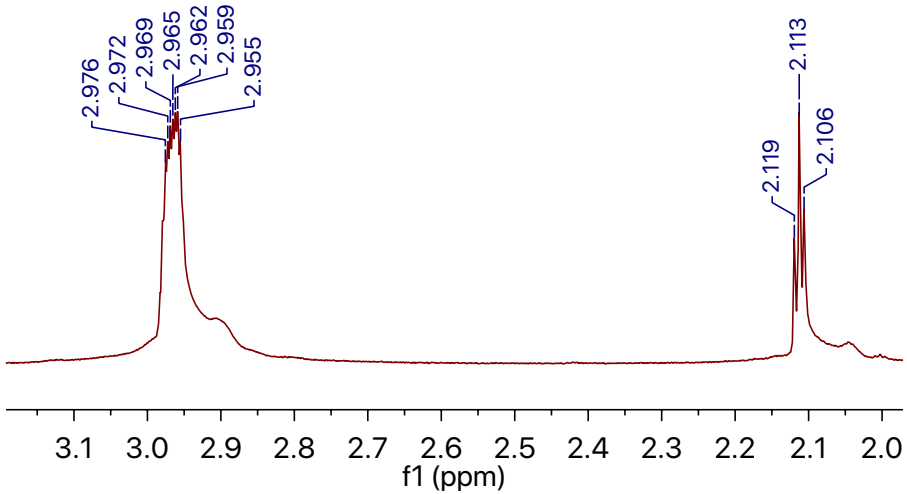


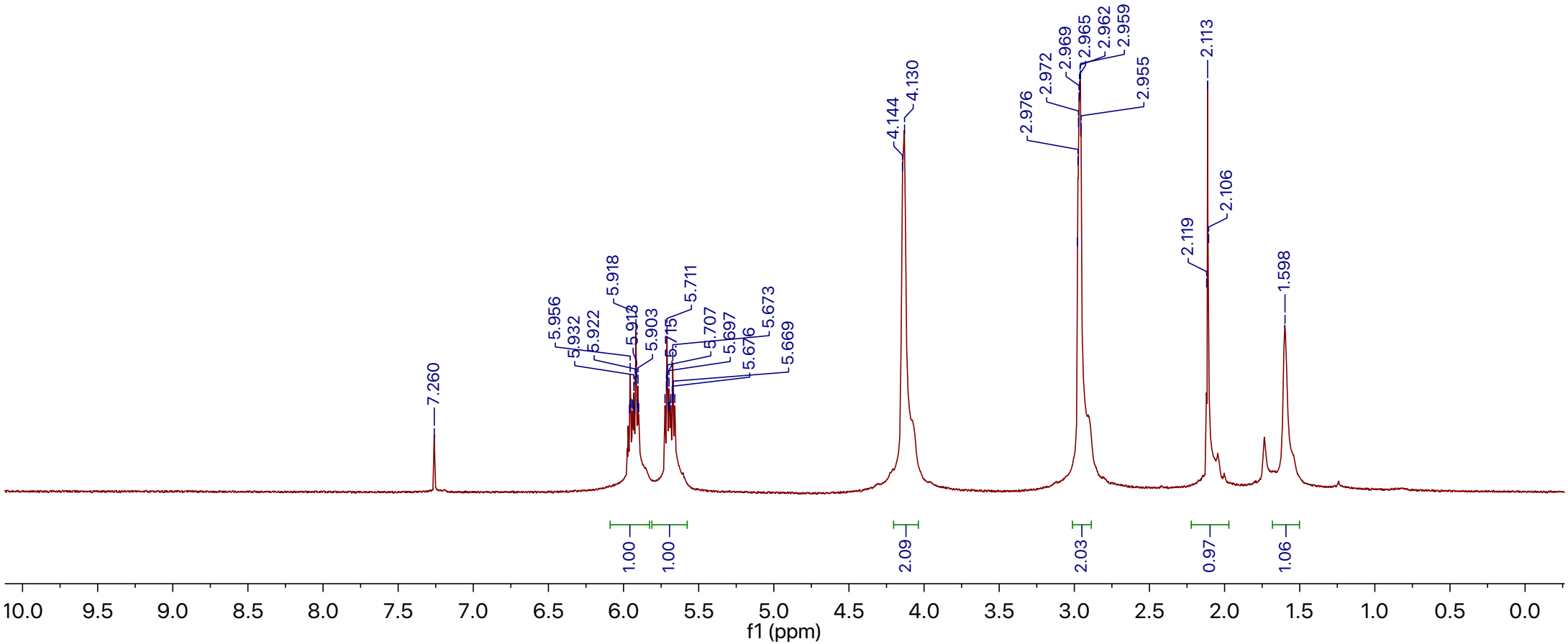


^{1}H NMR (400 MHz, $CDCl_3$)

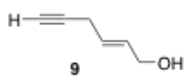


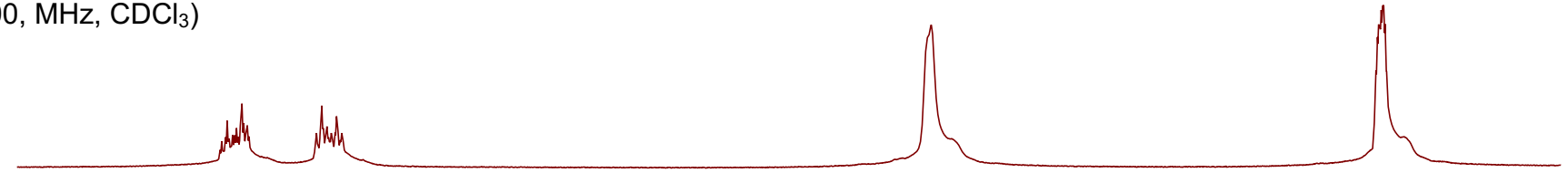


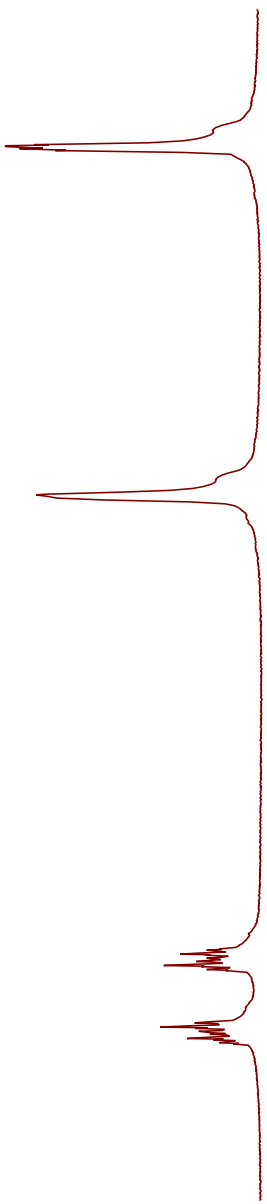


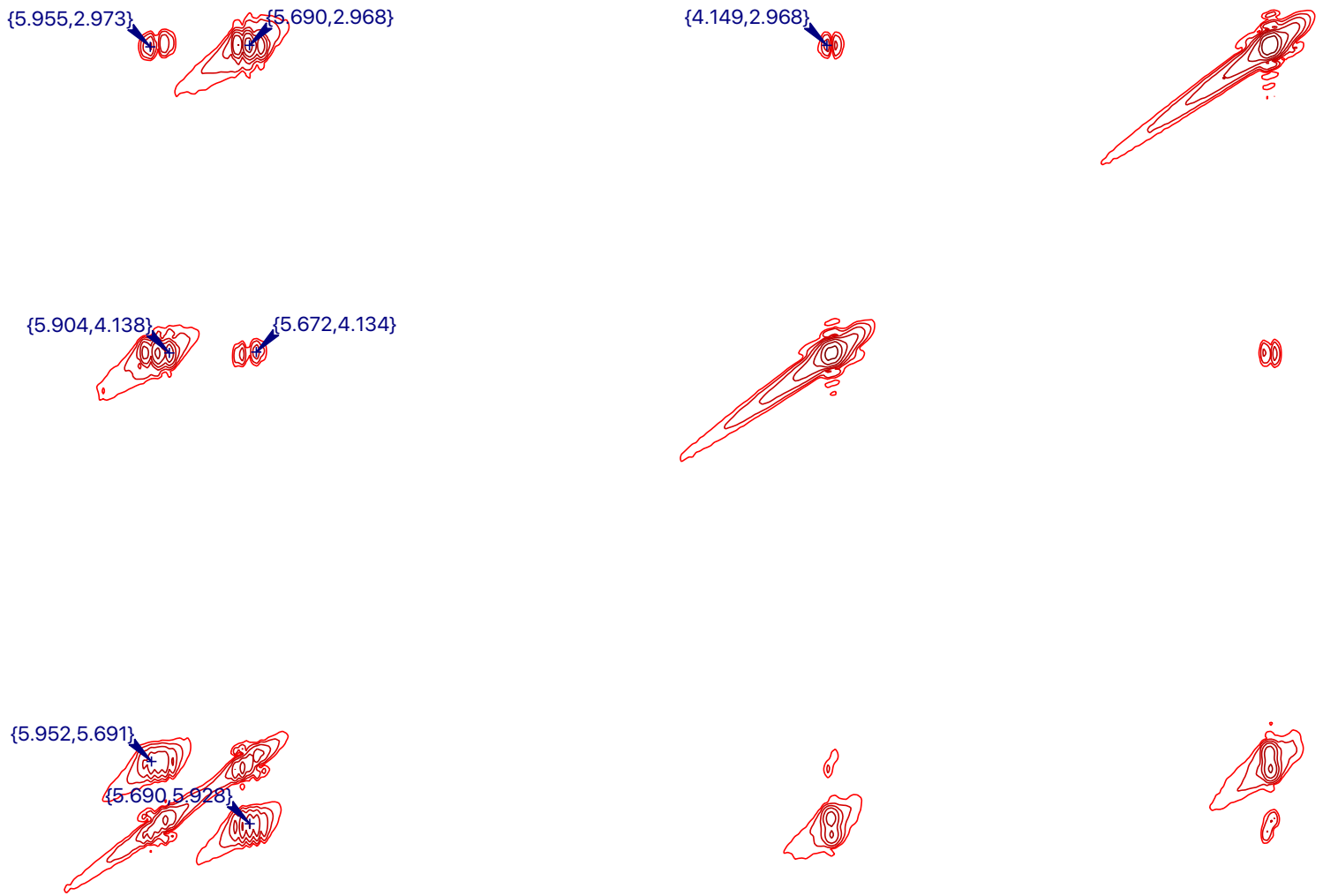


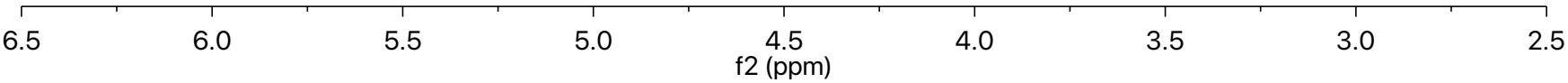
^{1}H NMR, gCOSY (400, MHz, $CDCl_3$)

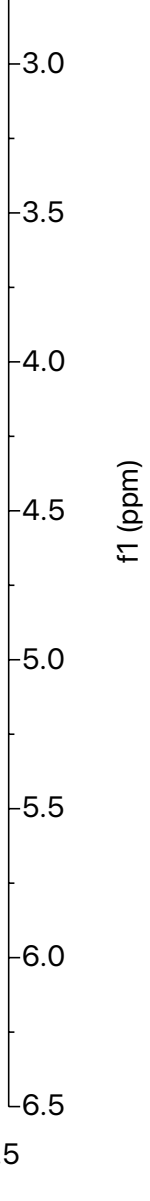




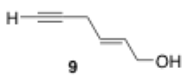


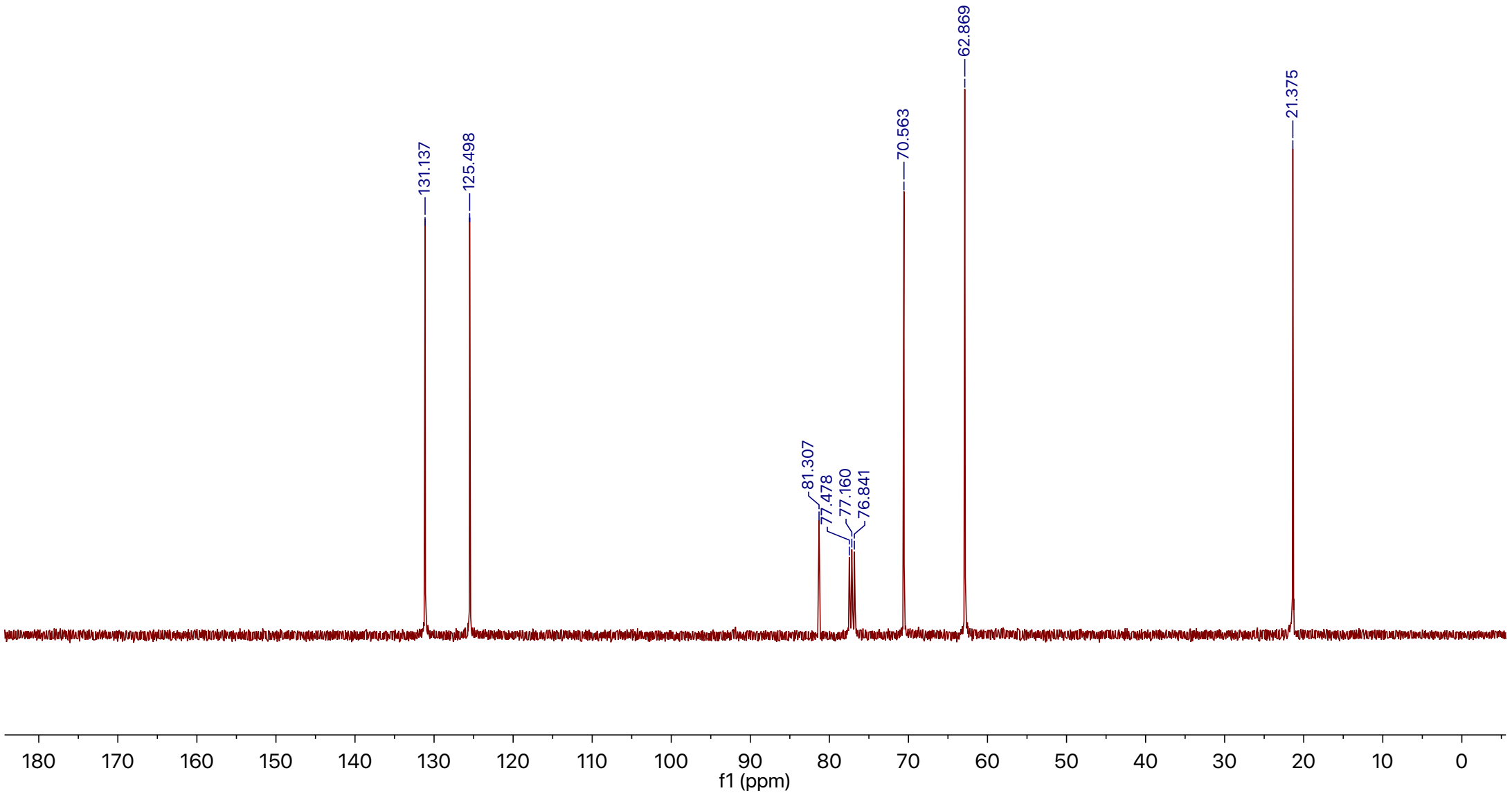




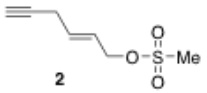


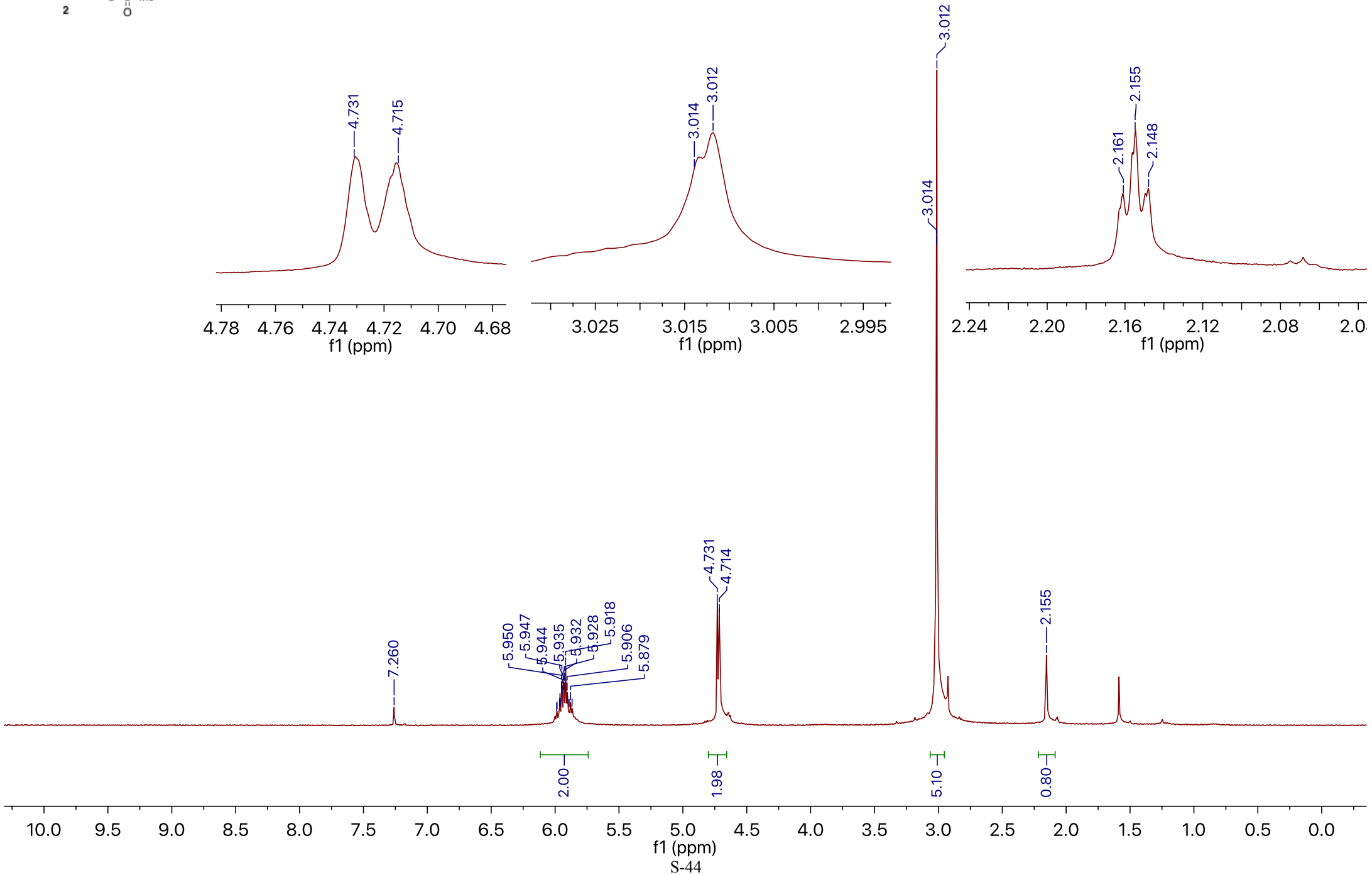
^{13}C NMR (101 MHz, $CDCl_3$)



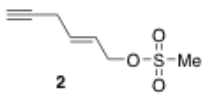


^{1}H NMR (400 MHz, $CDCl_3$)



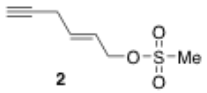


^{1}H NMR, gCOSY (400, MHz, $CDCl_3$)



2

^{13}C NMR (101 MHz, $CDCl_3$)



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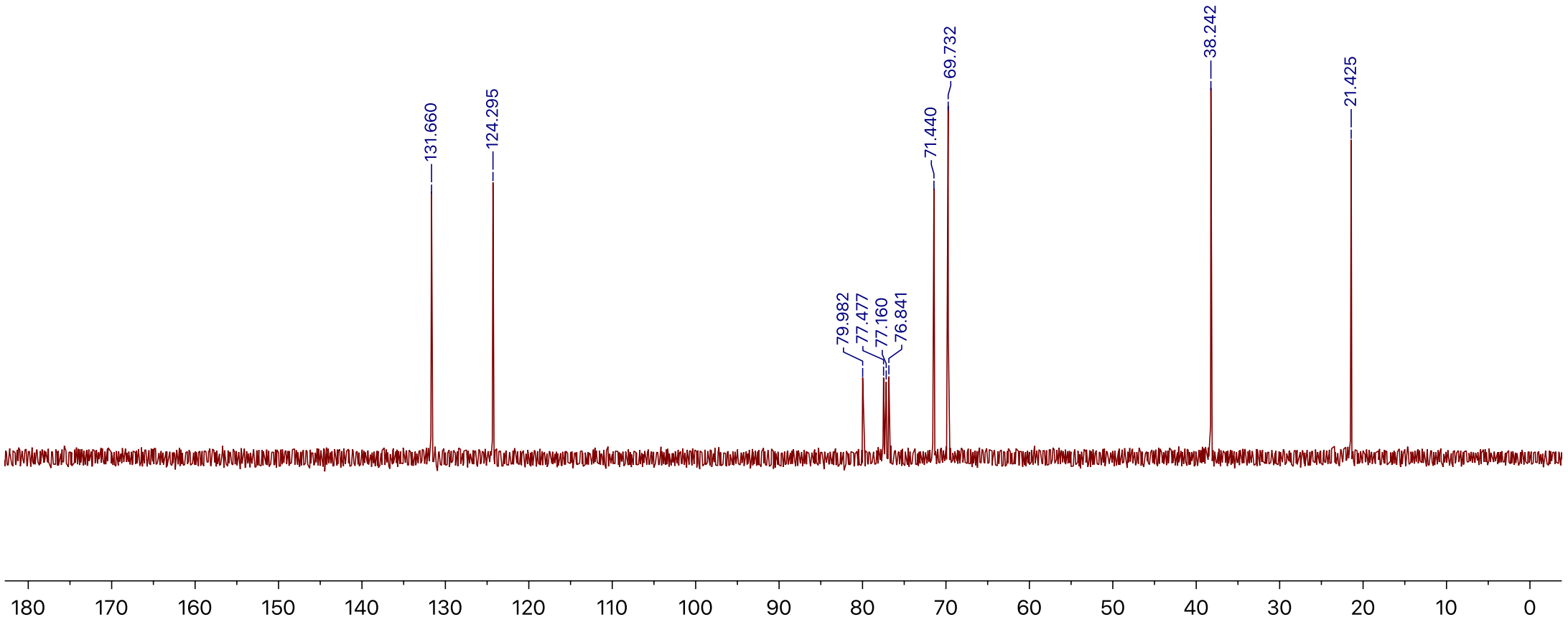
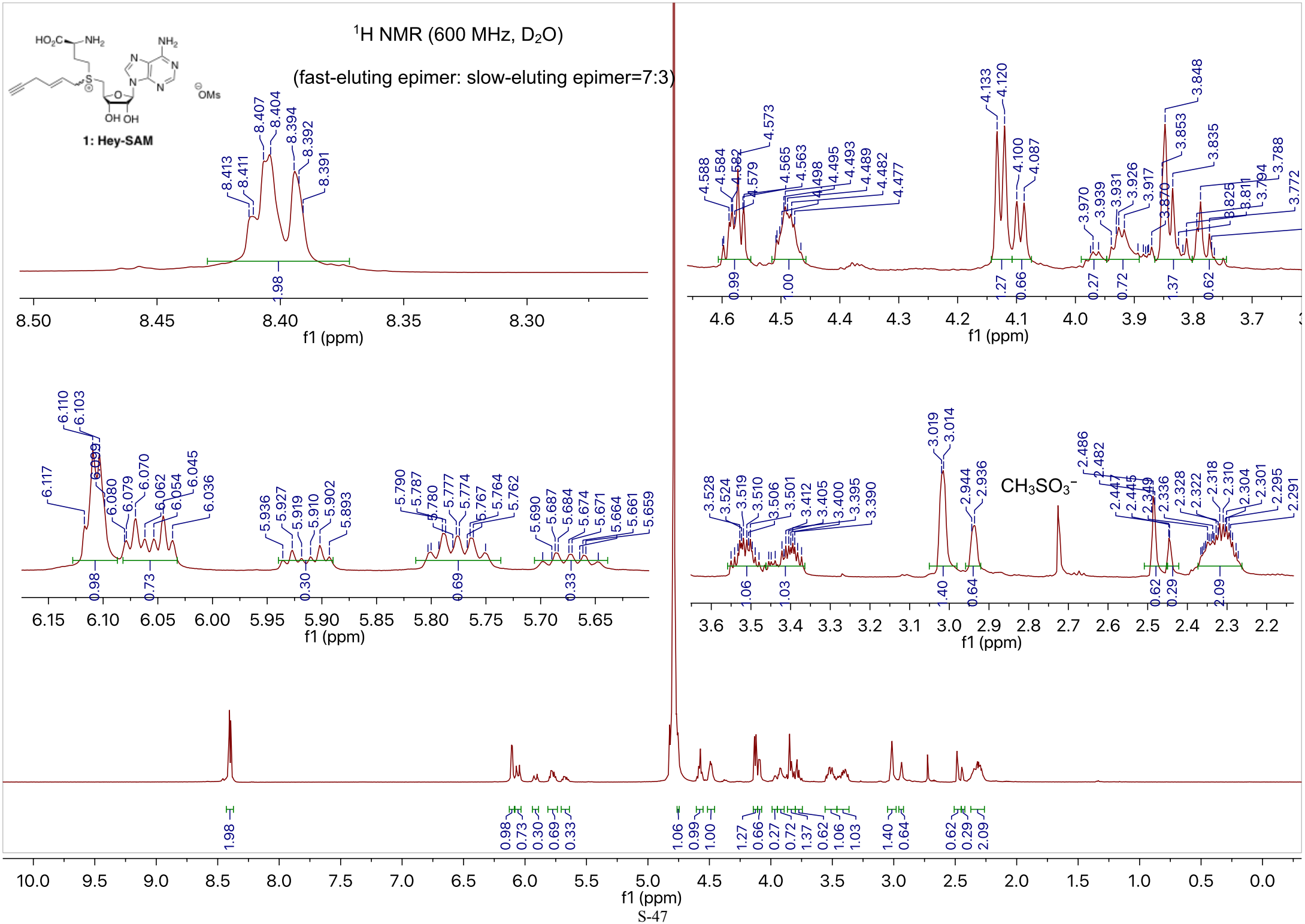
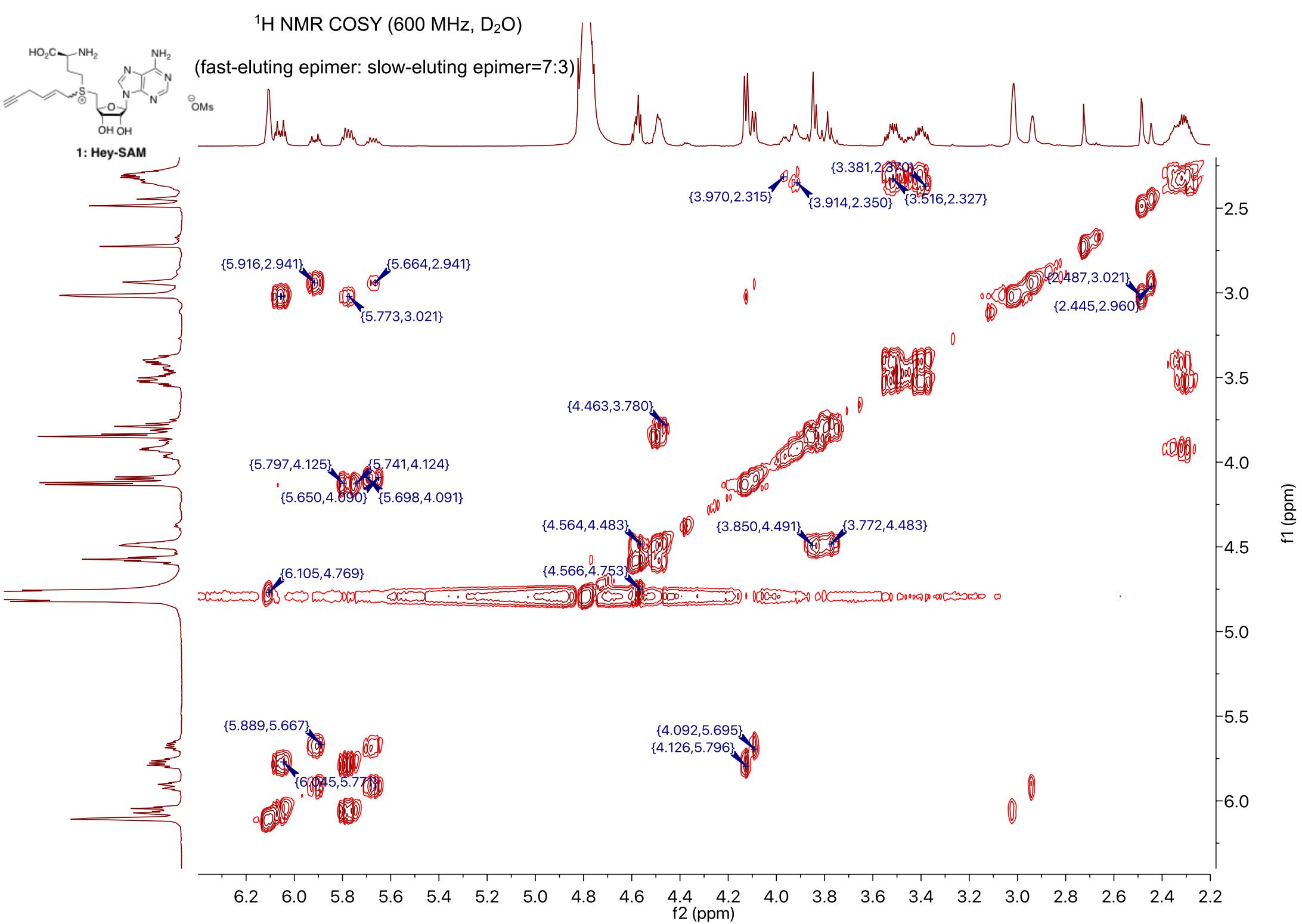
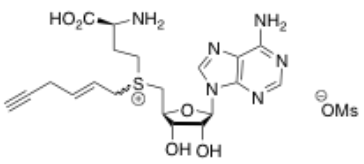


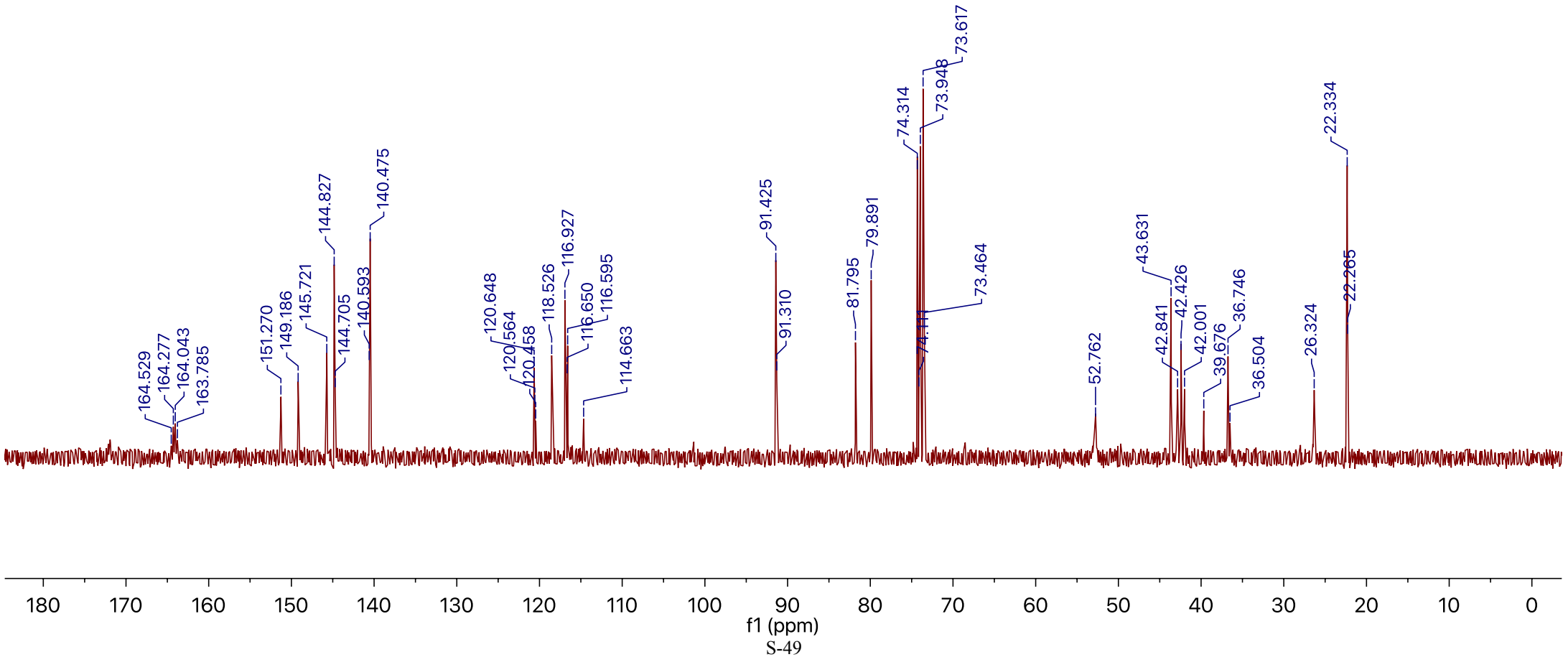

1: Hey-SAM
^{1}H NMR (600 MHz, D_2O)
(fast-eluting epimer: slow-eluting epimer=7:3)
$CH_3SO_3^-$
f1 (ppm)

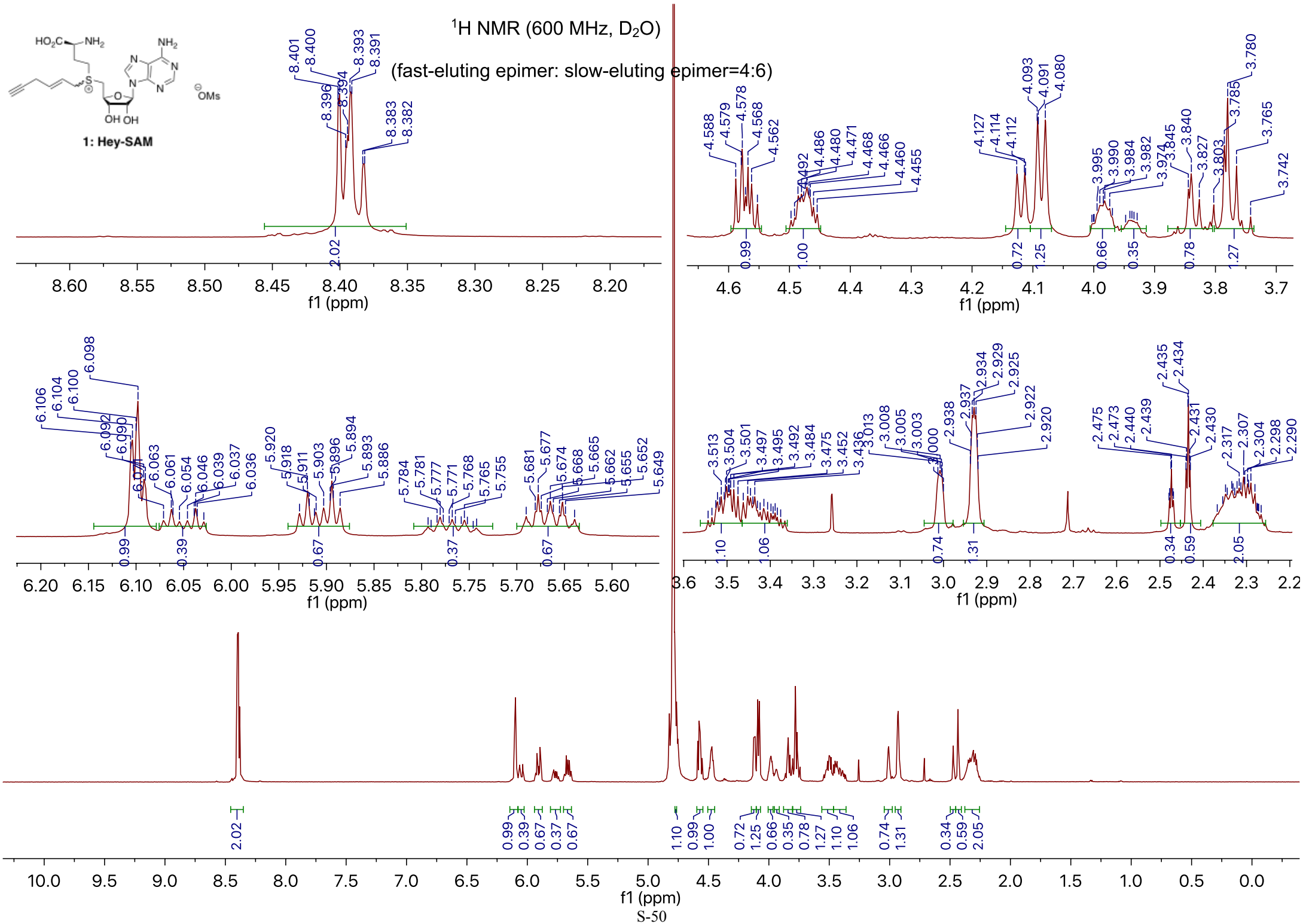

1H NMR COSY (600 MHz, D2O)
(fast-eluting epimer: slow-eluting epimer=7:3)
1: Hey-SAM
{3.970,2.315}
{3.914,2.350}
{3.516,2.327}
{5.916,2.941}
{5.664,2.941}
{5.773,3.021}
{2.487,3.021}
{2.445,2.960}
{4.463,3.780}
{5.797,4.125}
{5.741,4.124}
{5.650,4.090}
{5.698,4.091}
{4.564,4.483}
{3.850,4.491}
{3.772,4.483}
{6.105,4.769}
{4.566,4.753}
{5.889,5.667}
{4.092,5.695}
{4.126,5.796}
f2 (ppm)
f1 (ppm)

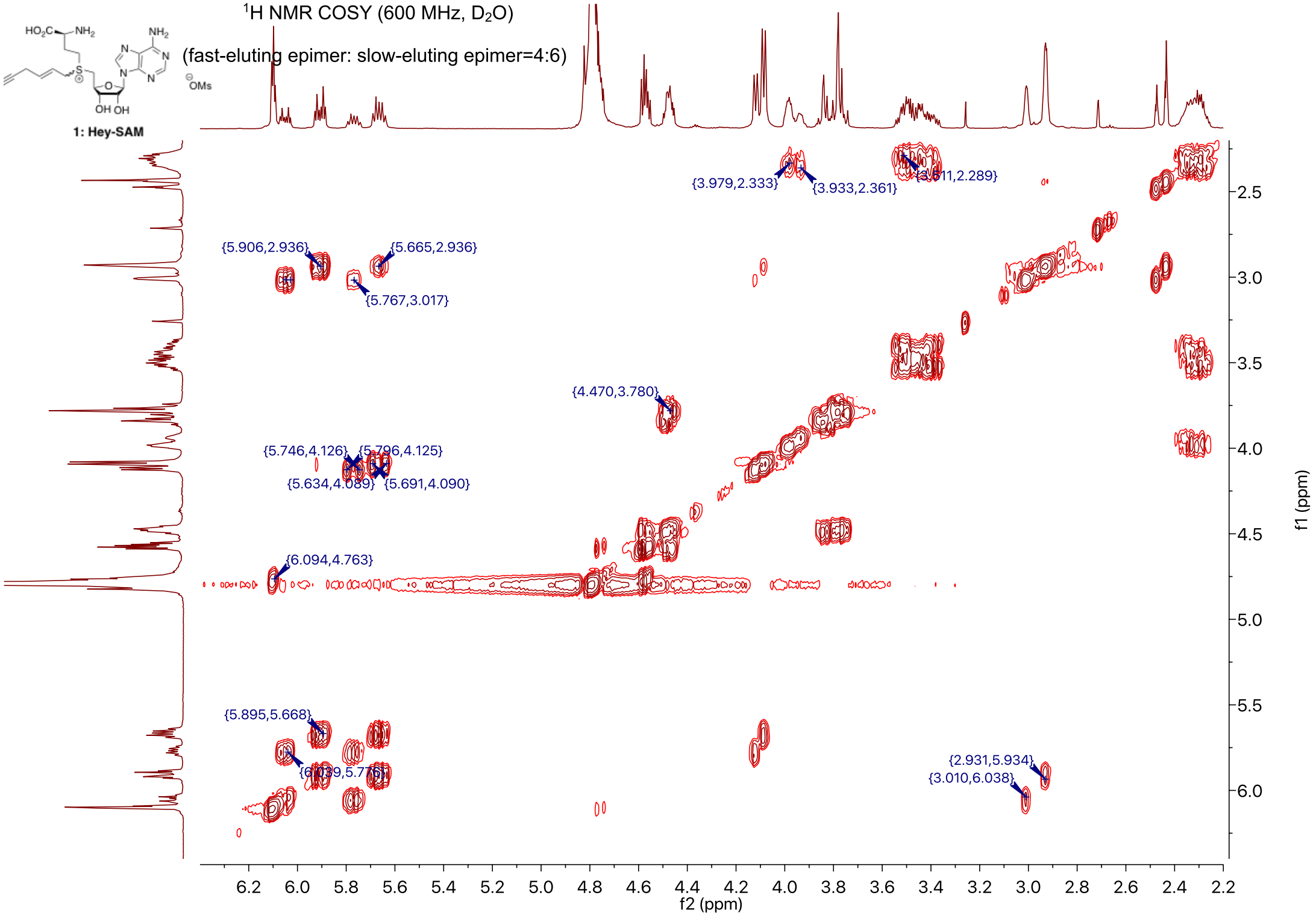


1: Hey-SAM

^{13}C NMR (151 MHz, D_2O) (fast-eluting epimer: slow-eluting epimer=7:3)

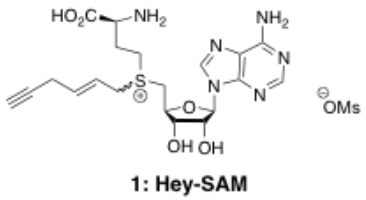



1: Hey-SAM
(fast-eluting epimer: slow-eluting epimer=4:6)
f1 (ppm)



^{1}H NMR COSY (600 MHz, D_2O)

(fast-eluting epimer: slow-eluting epimer=4:6)



1: Hey-SAM

^{13}C NMR (151 MHz, D_2O) (fast-eluting epimer: slow-eluting epimer=4:6)

Figure S2A (TOF transformed)

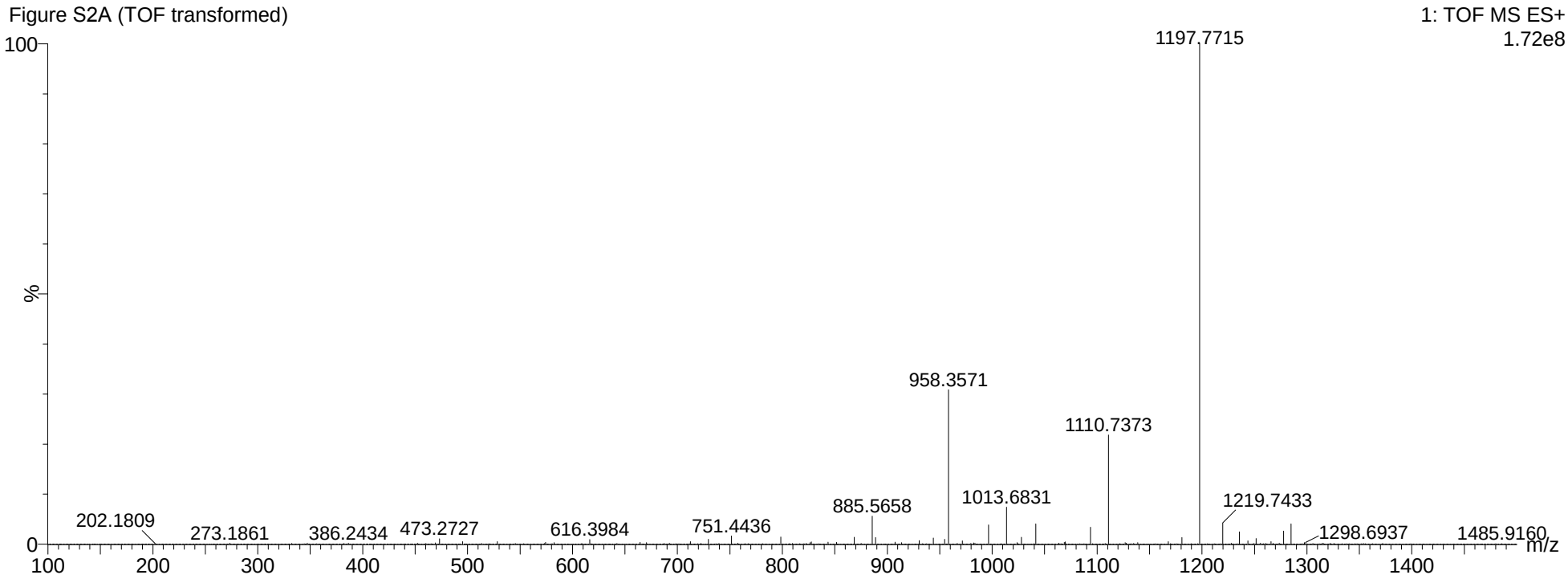


Figure S2A (Lock mass corrected)

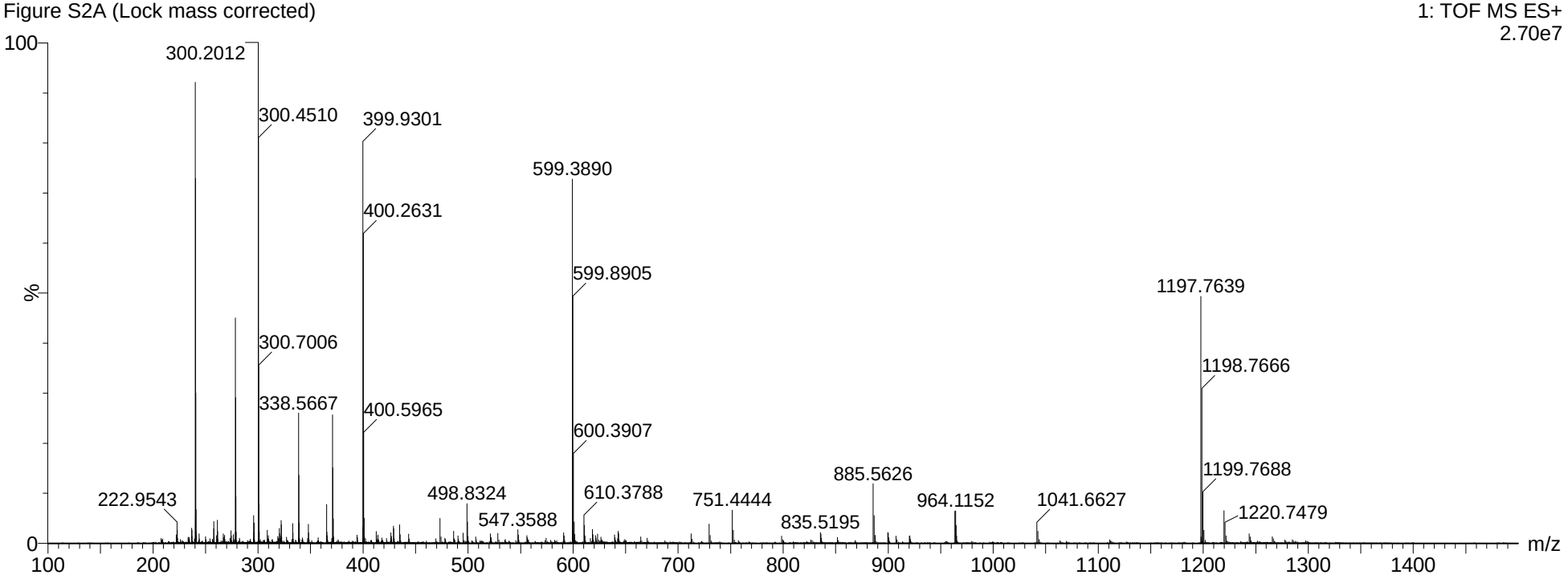


Figure S2B (TOF transformed)

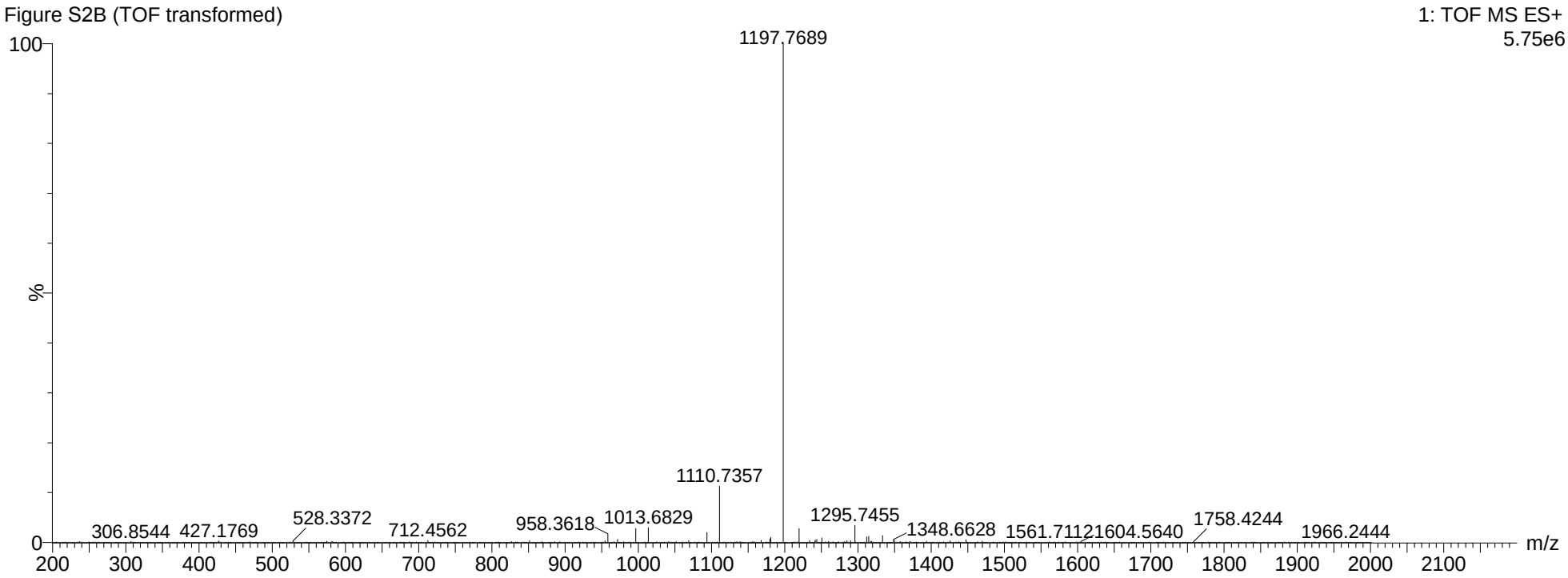


Figure S2B (Lock mass corrected)

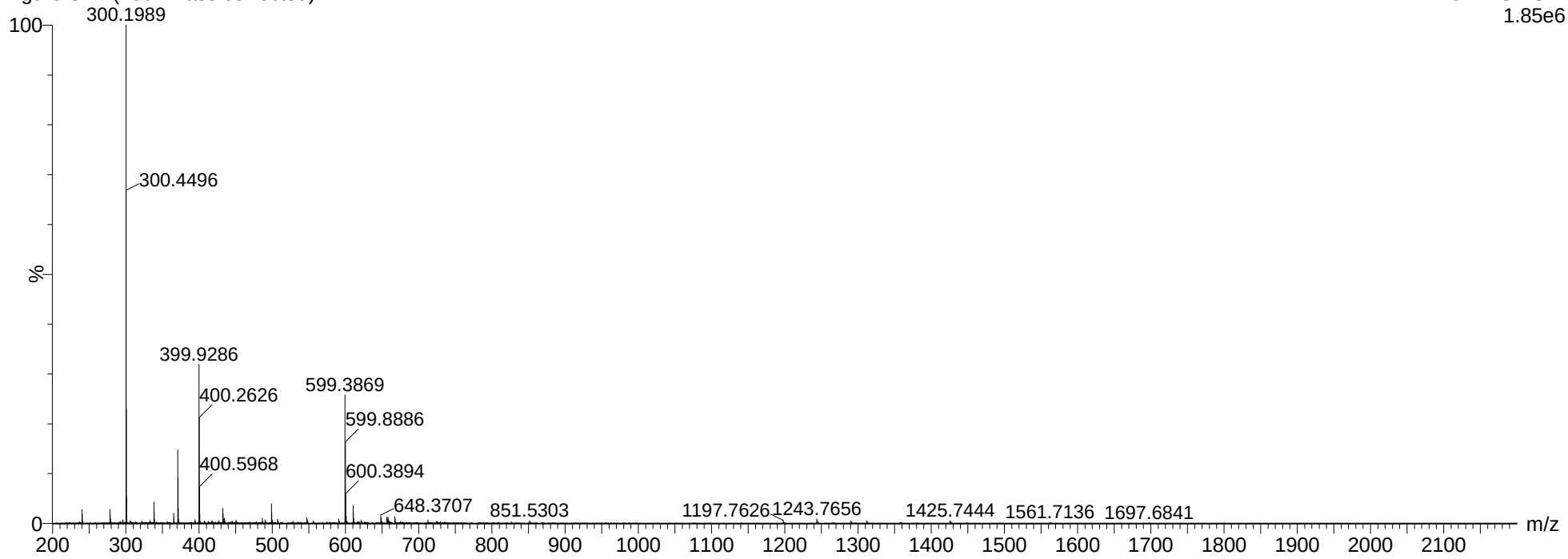


Figure S2C (TOF transformed)

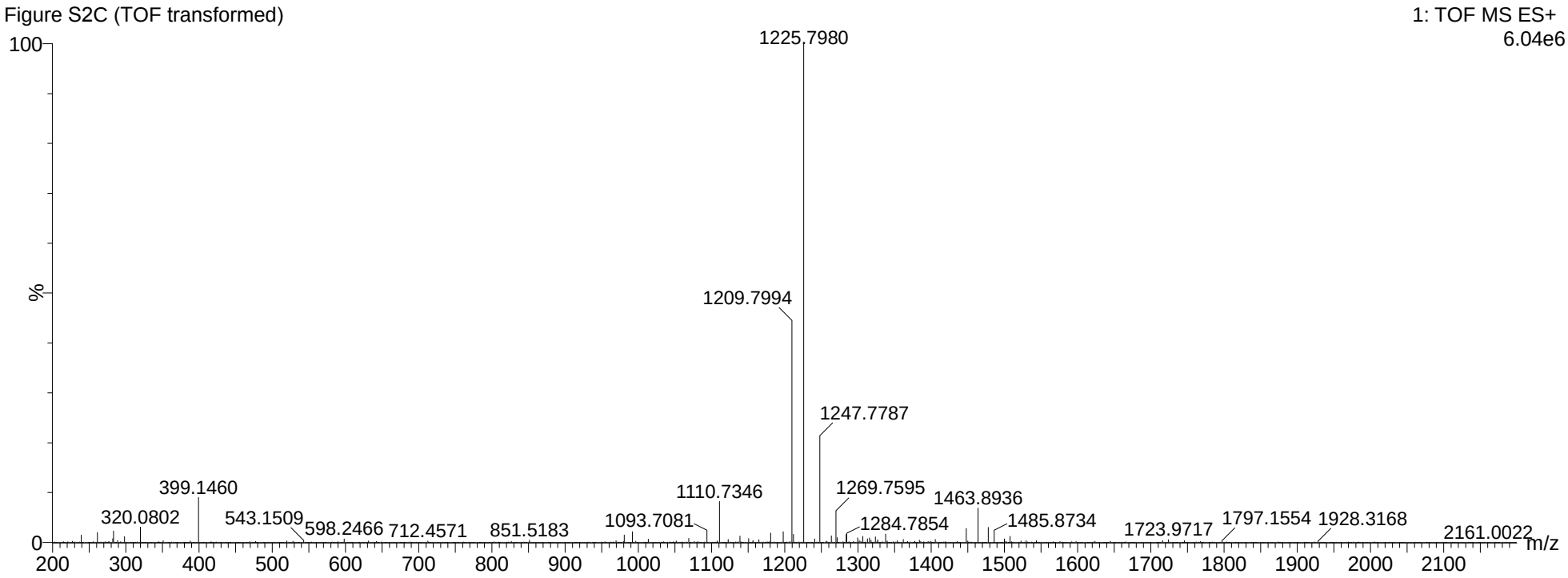


Figure S2C (Lock mass corrected)

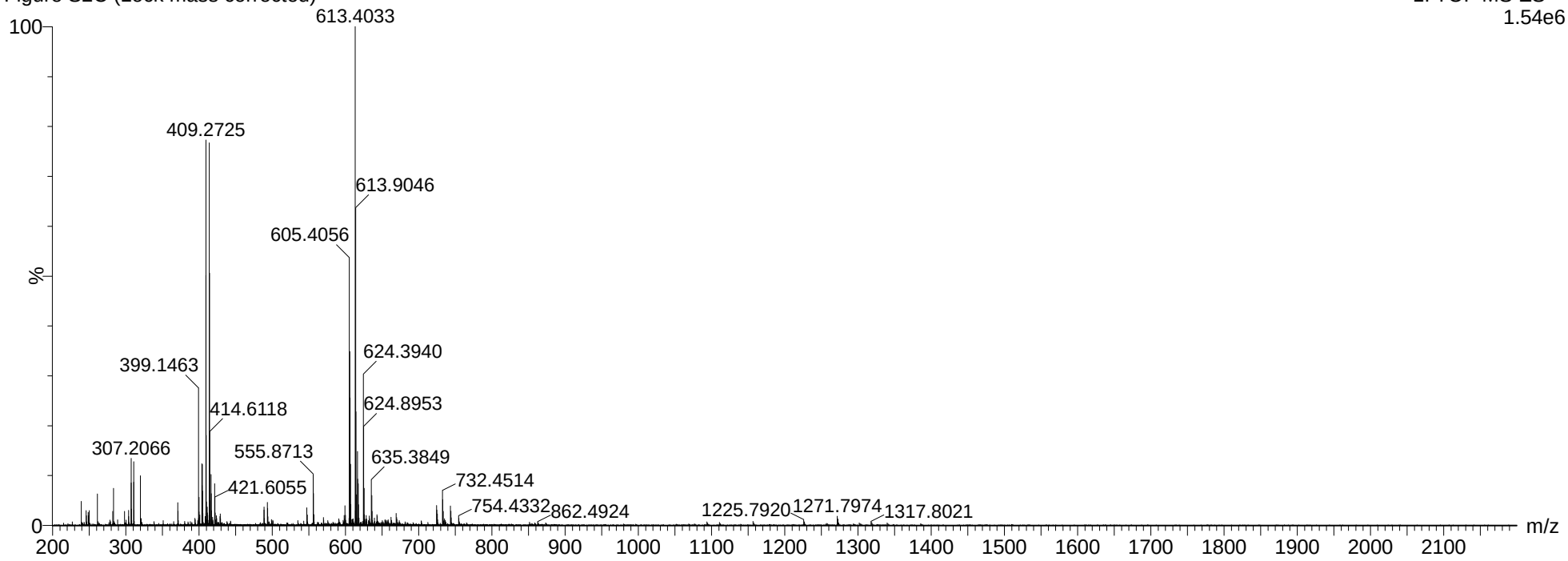


Figure S2D (TOF transformed)

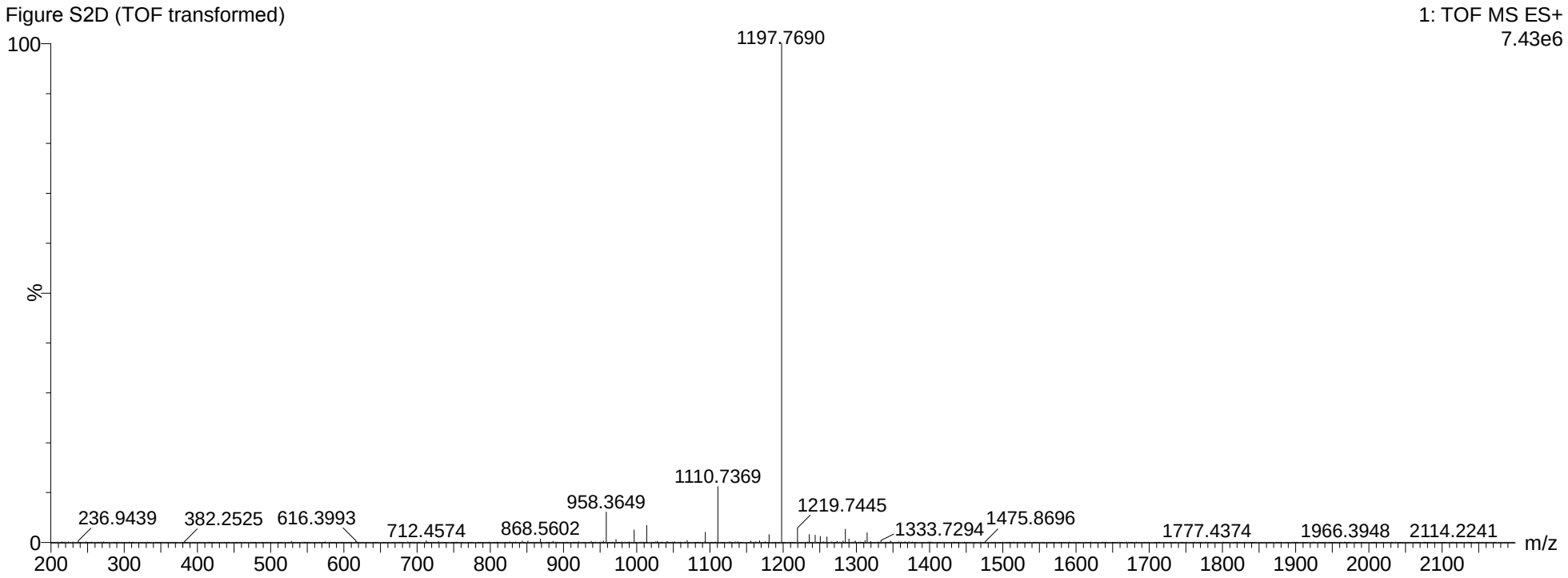


Figure S2D (Lock mass corrected)

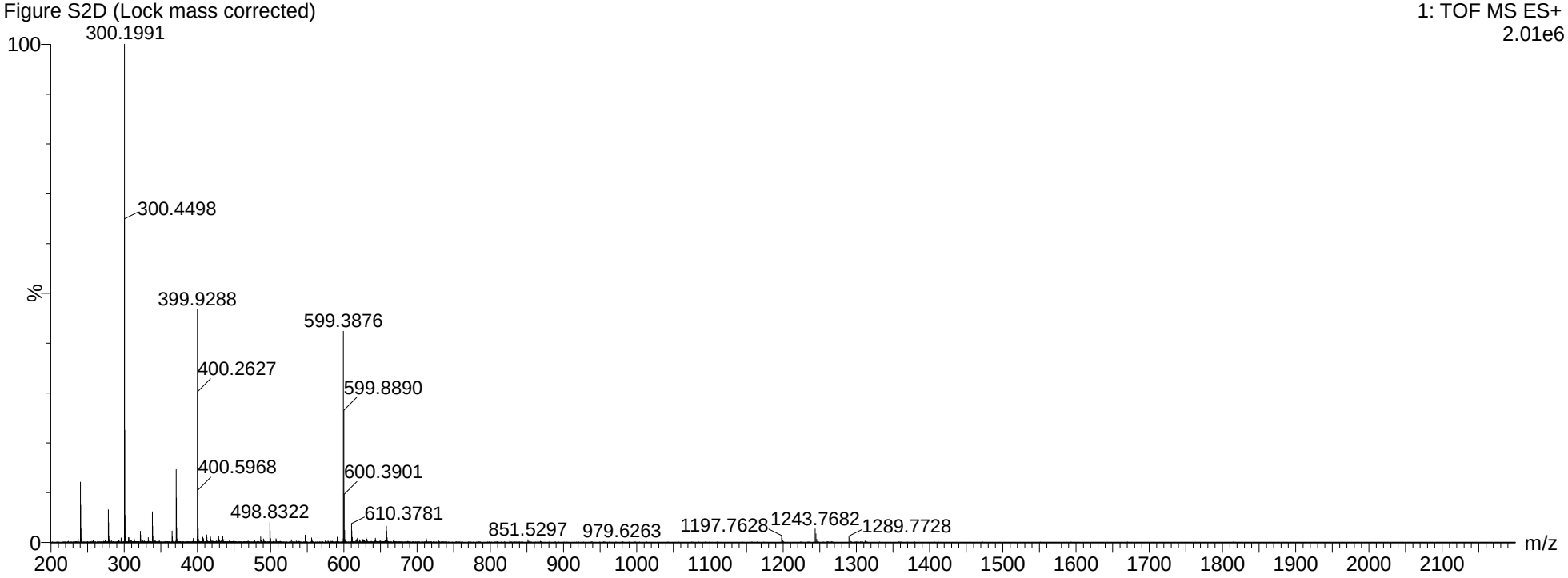


Figure 1C&S2E (TOF transformed)

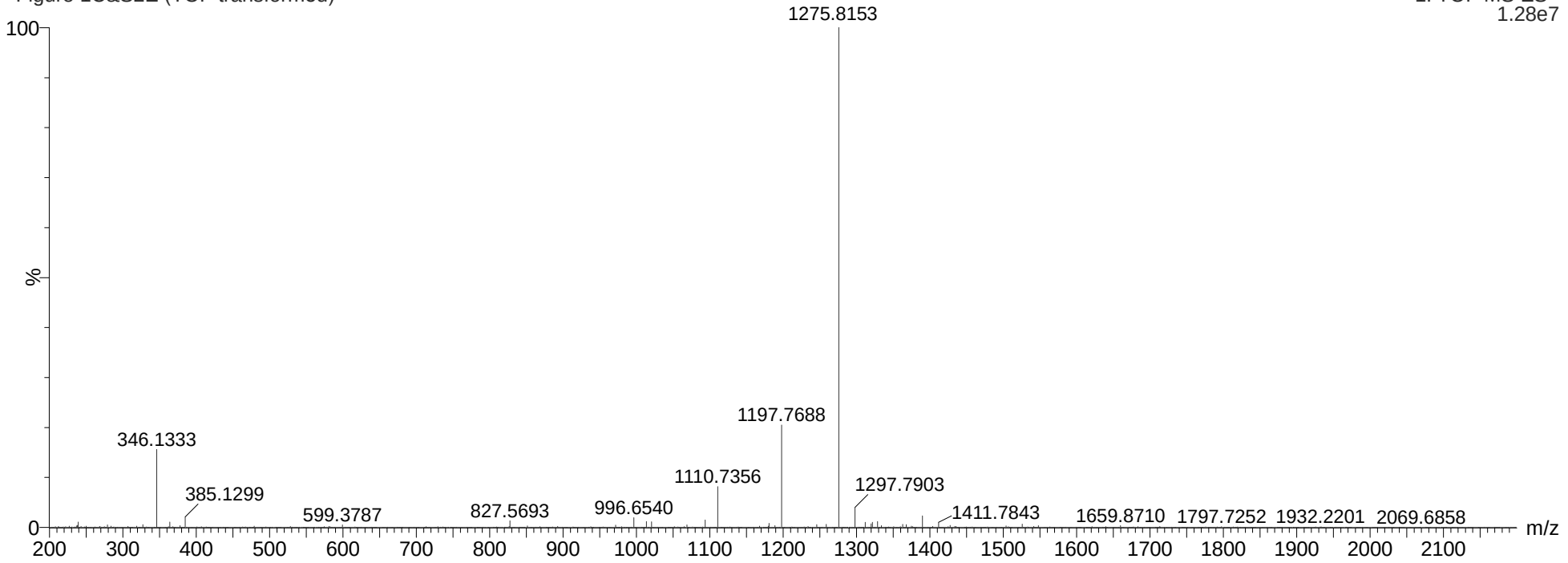


Figure 1C&S2E (Lock mass corrected)

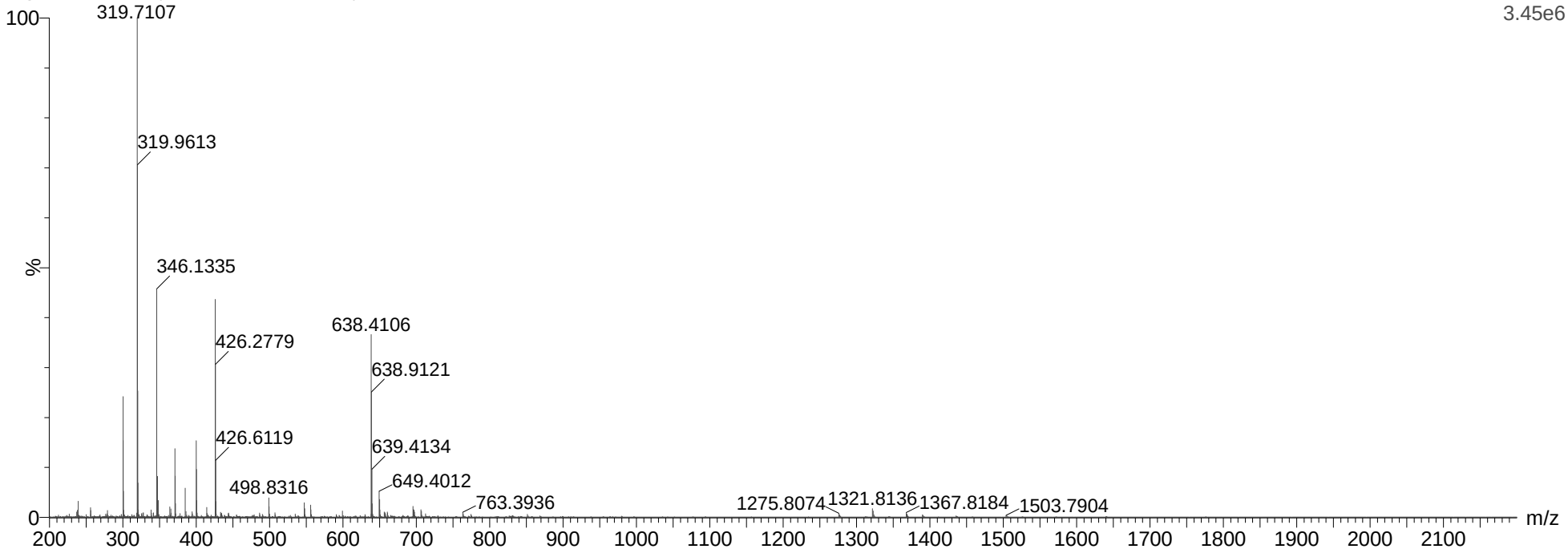


Figure S3F (TOF transformed)

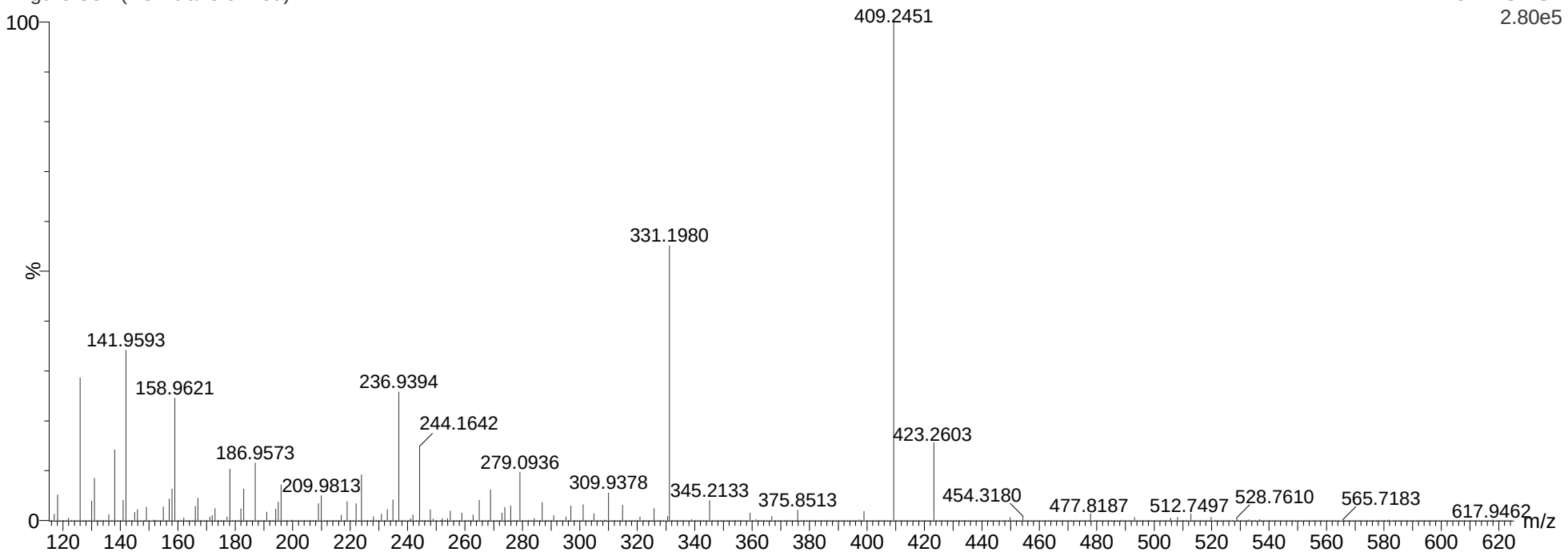


Figure S3F (Lock mass corrected)

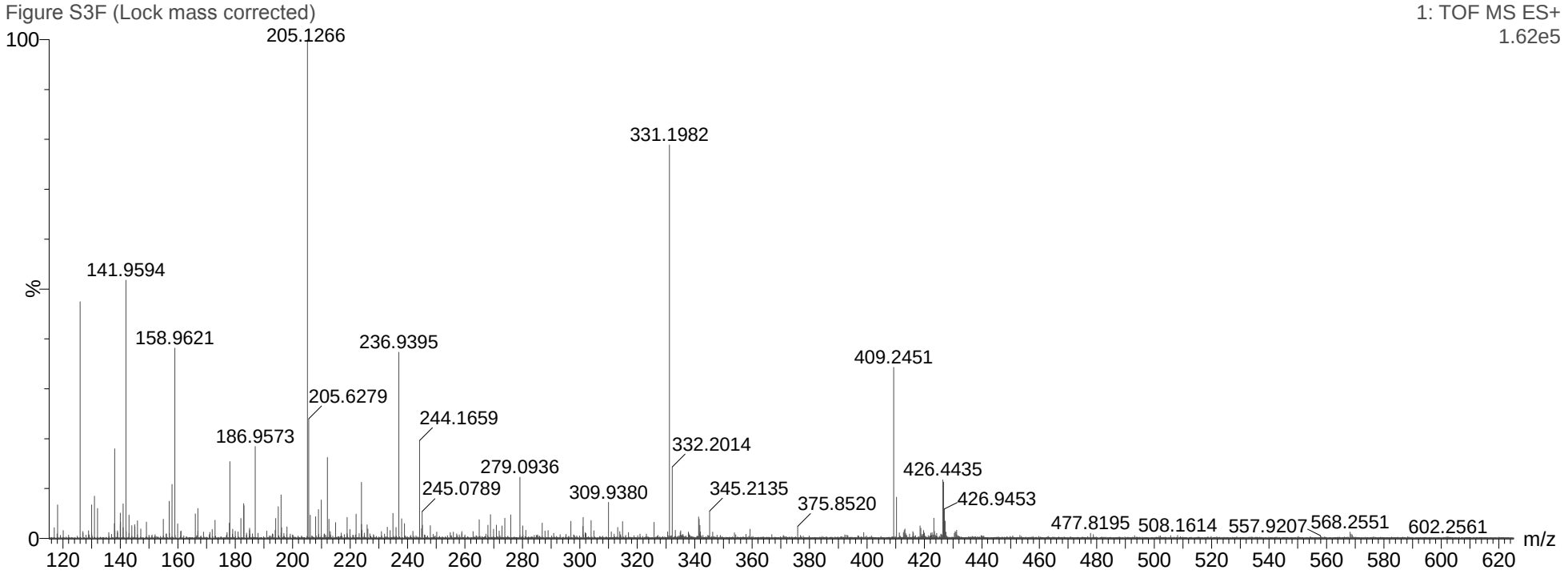


Figure S4C (Precursor, TOF transformed)

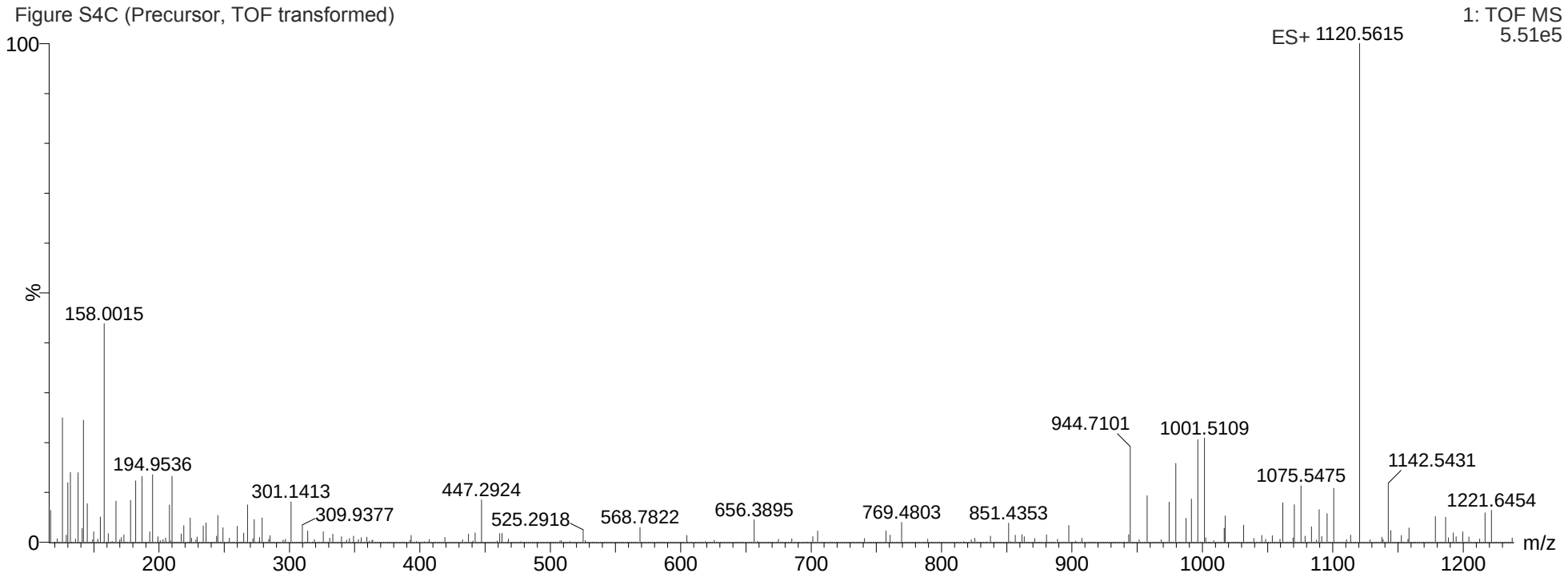


Figure S4C (Precursor, Lock mass corrected)

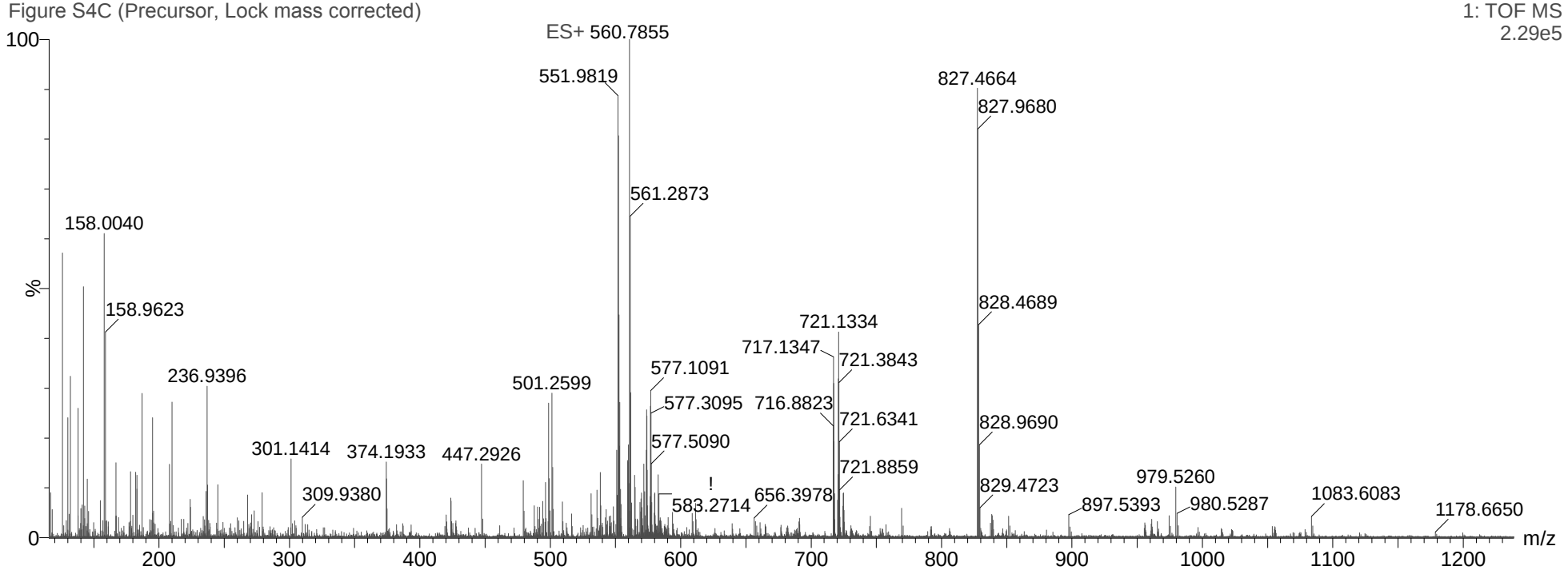


Figure S4C (TOF transformed)

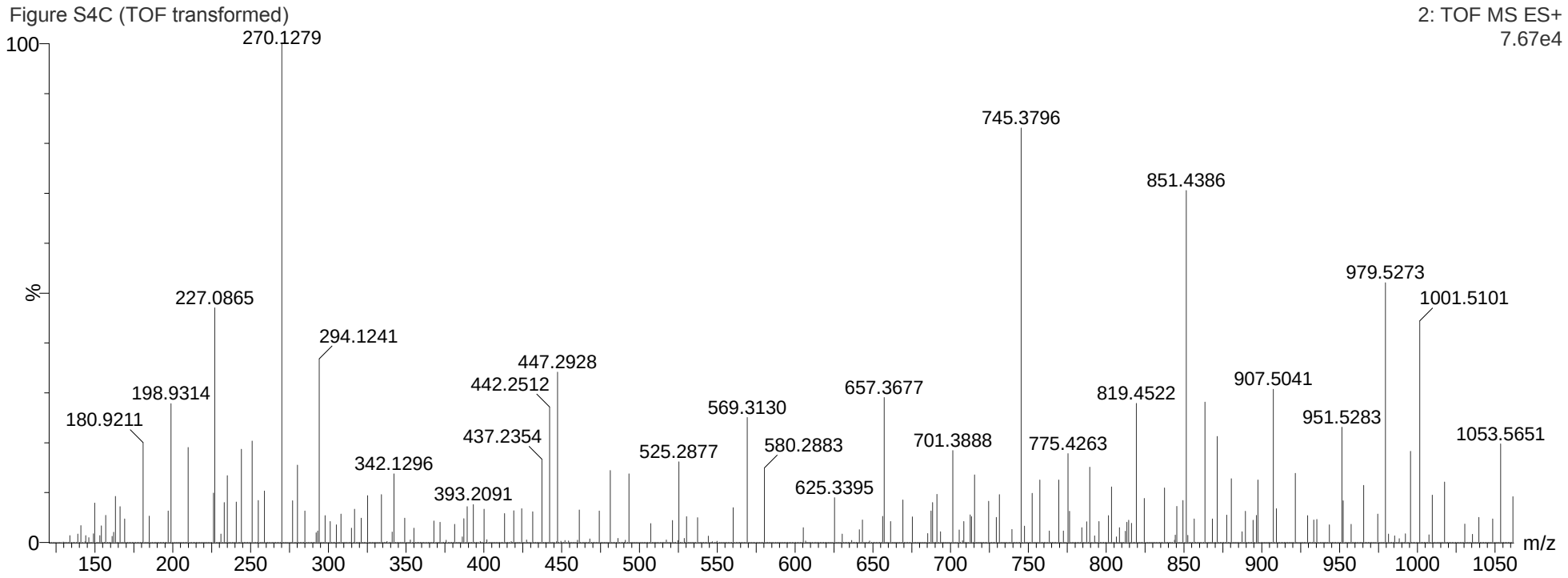


Figure S4C (Lock mass corrected)

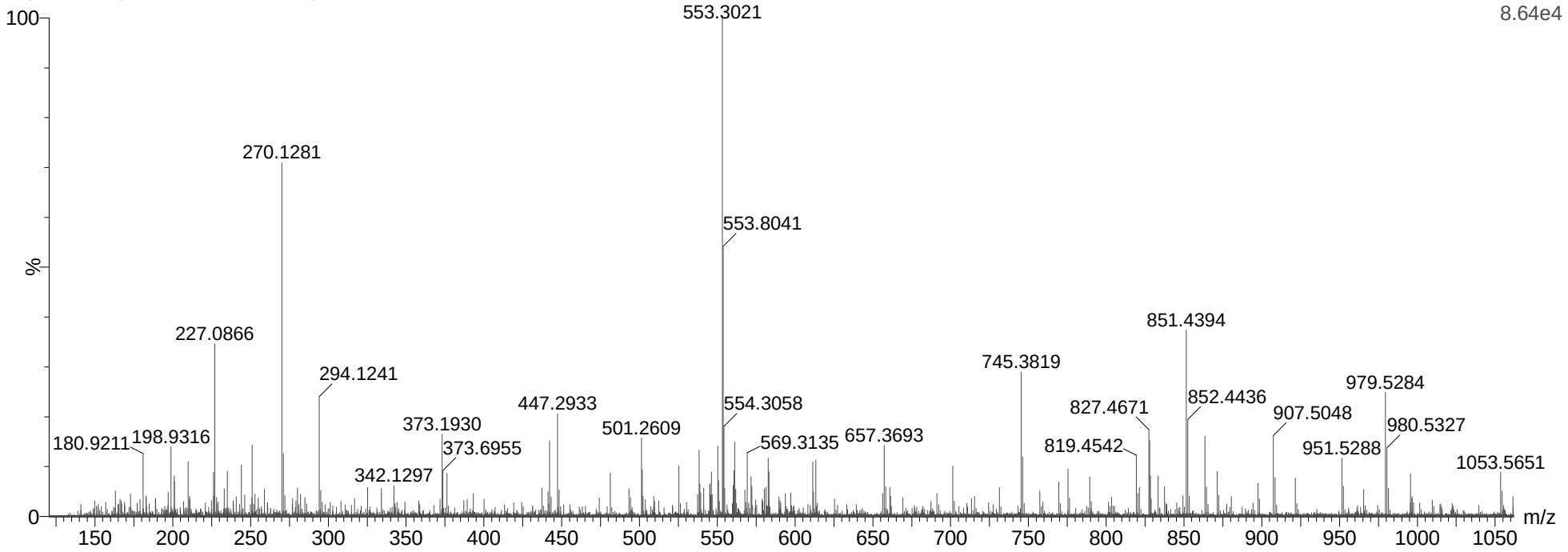


Figure S7A (TOF transformed)

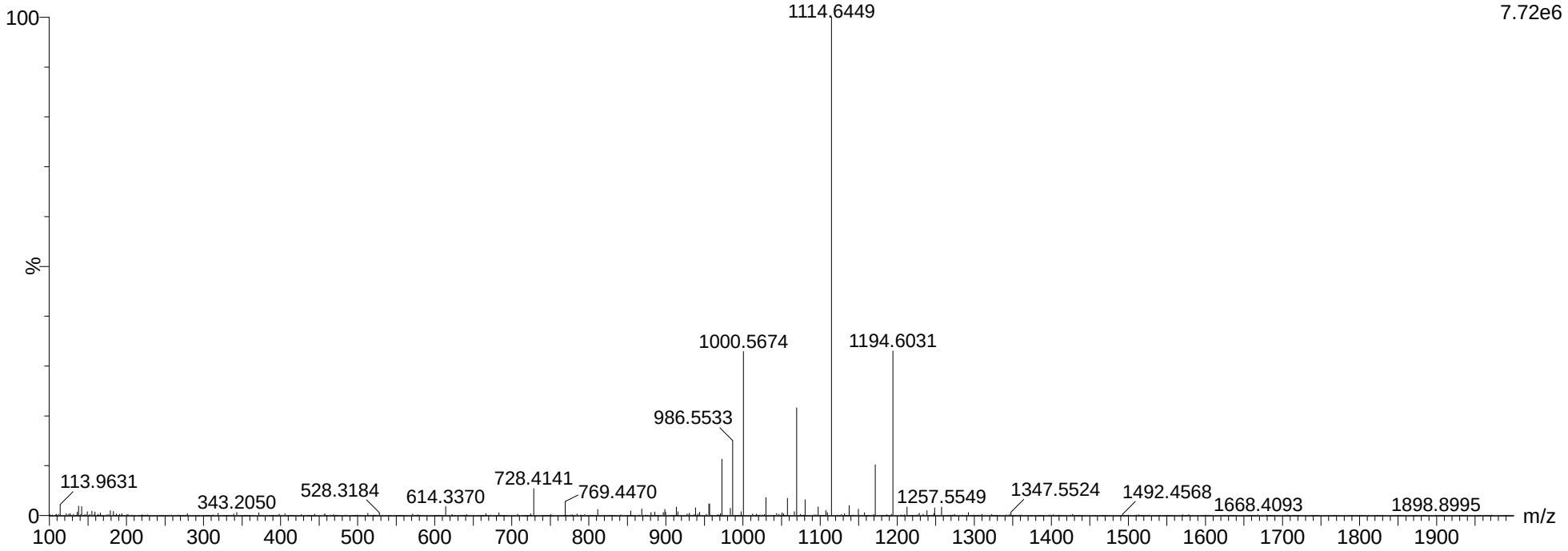


Figure S7A (Lock mass corrected)

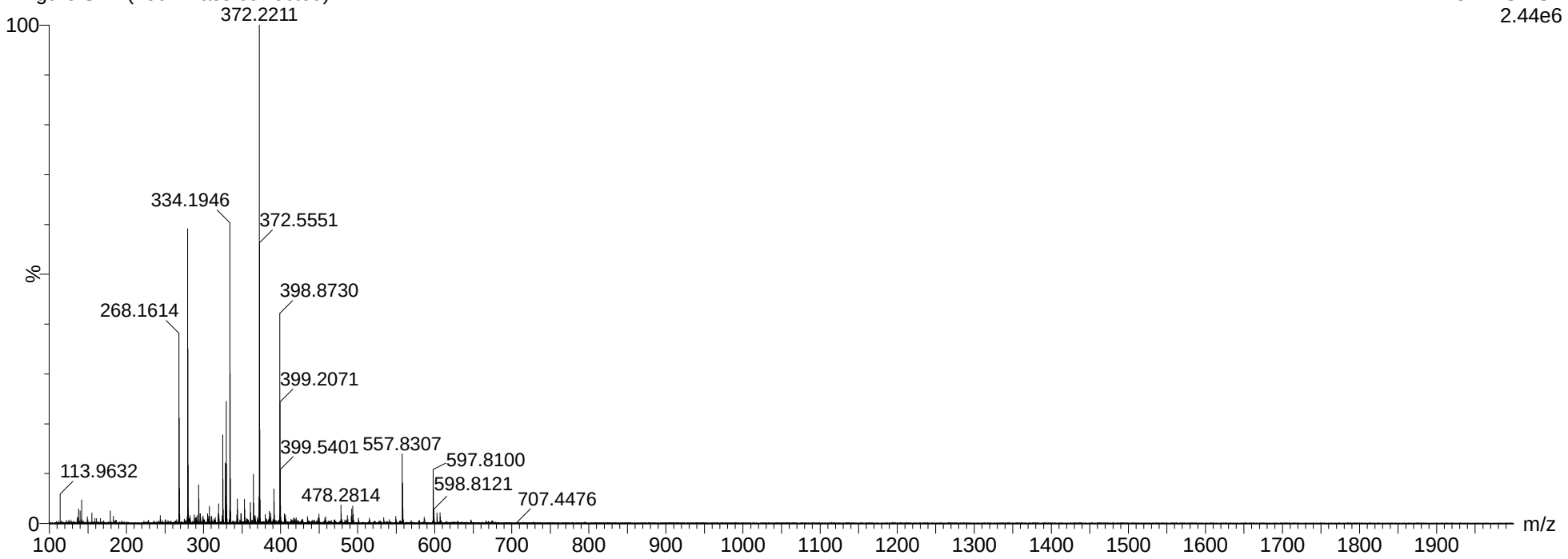


Figure S7B (TOF transformed)

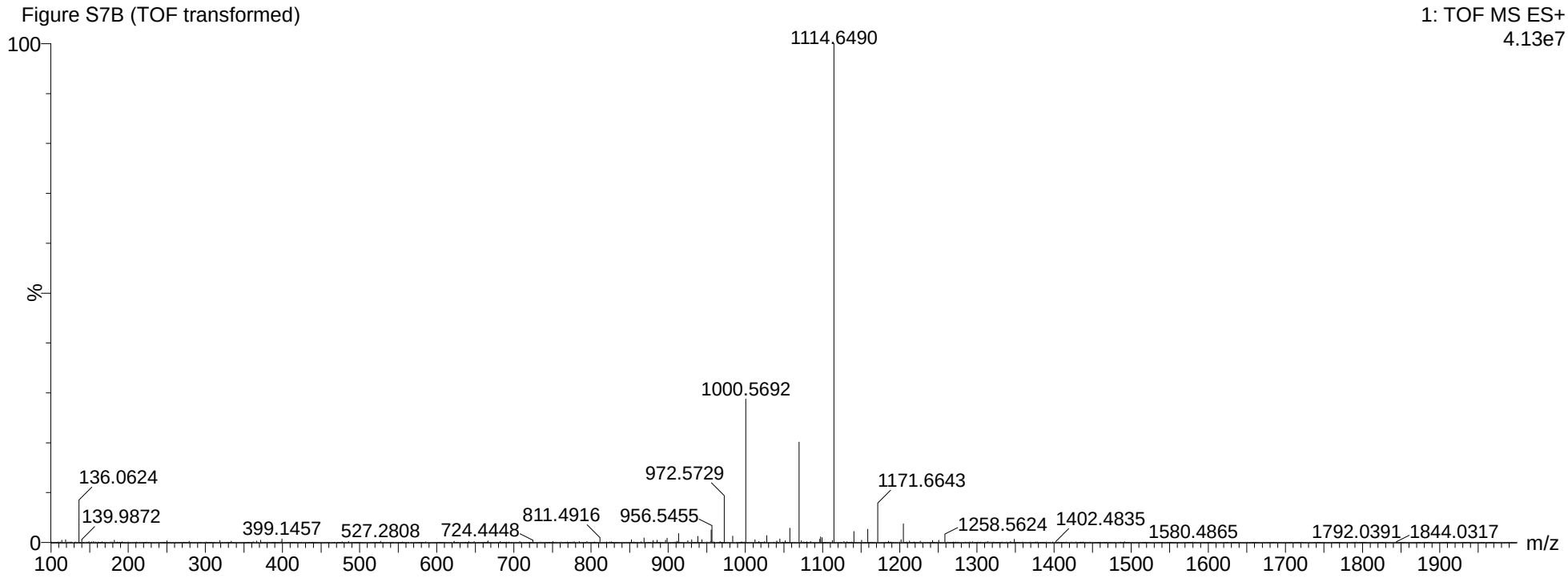


Figure S7B (Lock mass corrected)

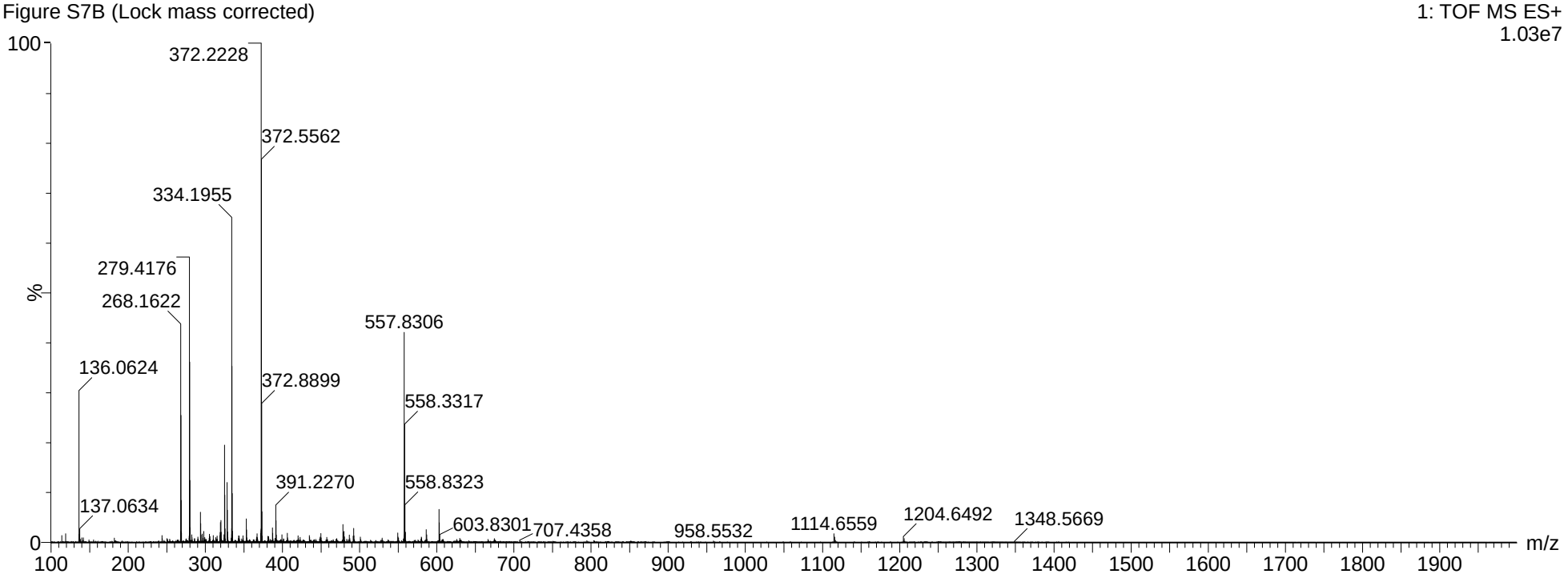


Figure S7C (TOF transformed)

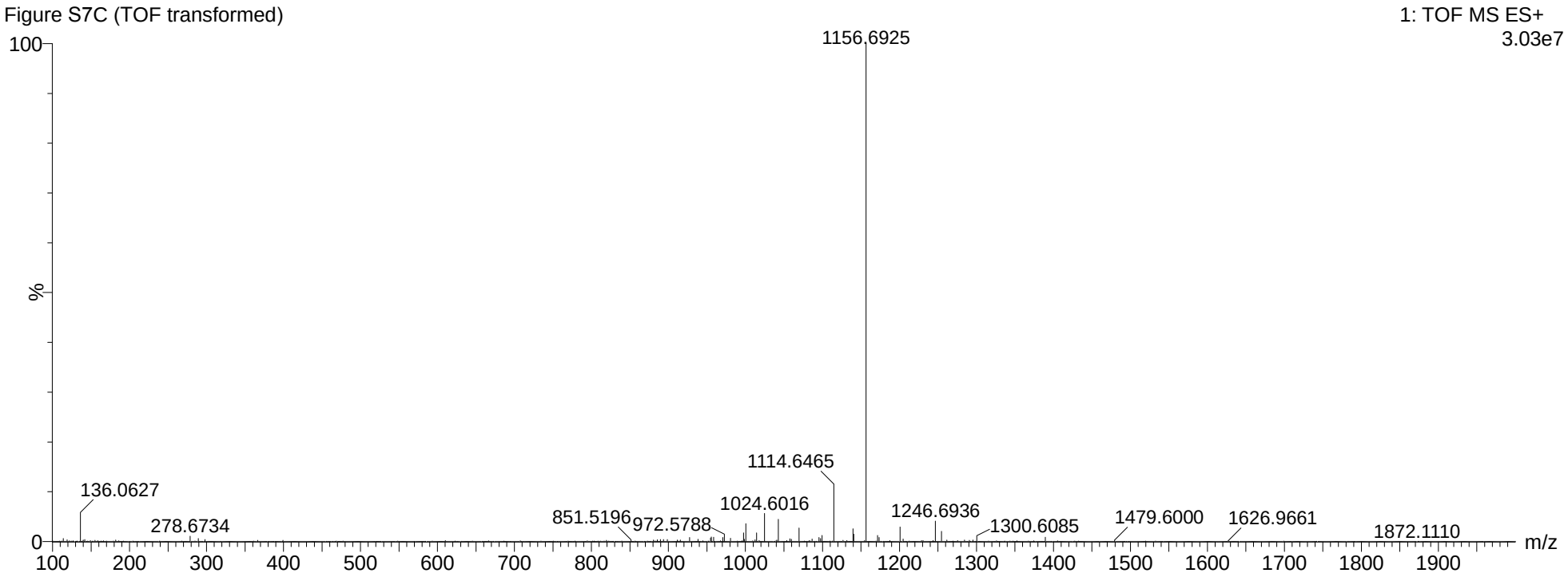


Figure S7C (Lock mass corrected)

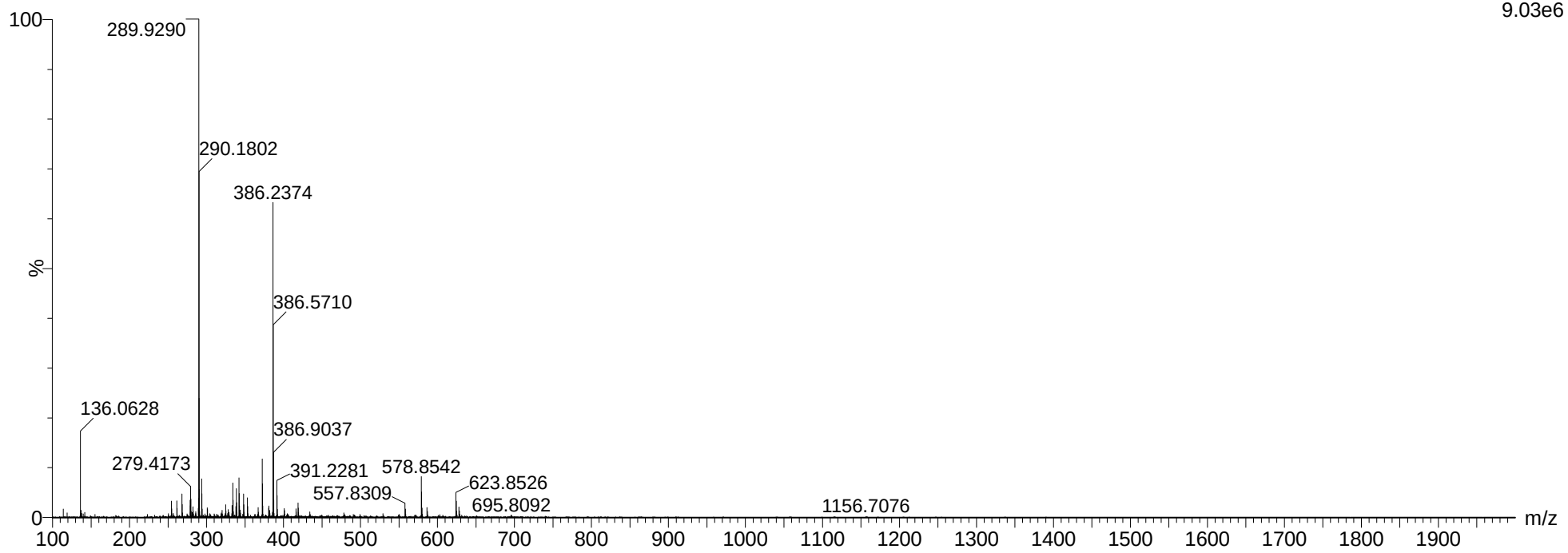


Figure S8A (TOF transformed)

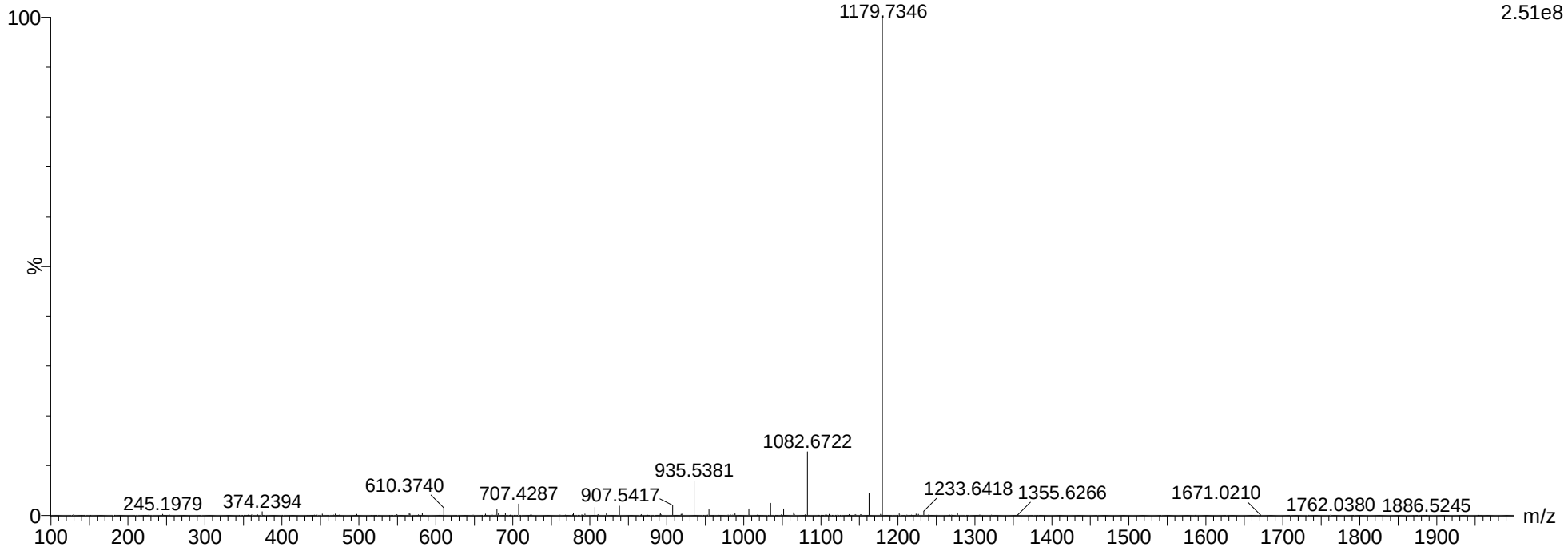


Figure S8A (Lock mass corrected)

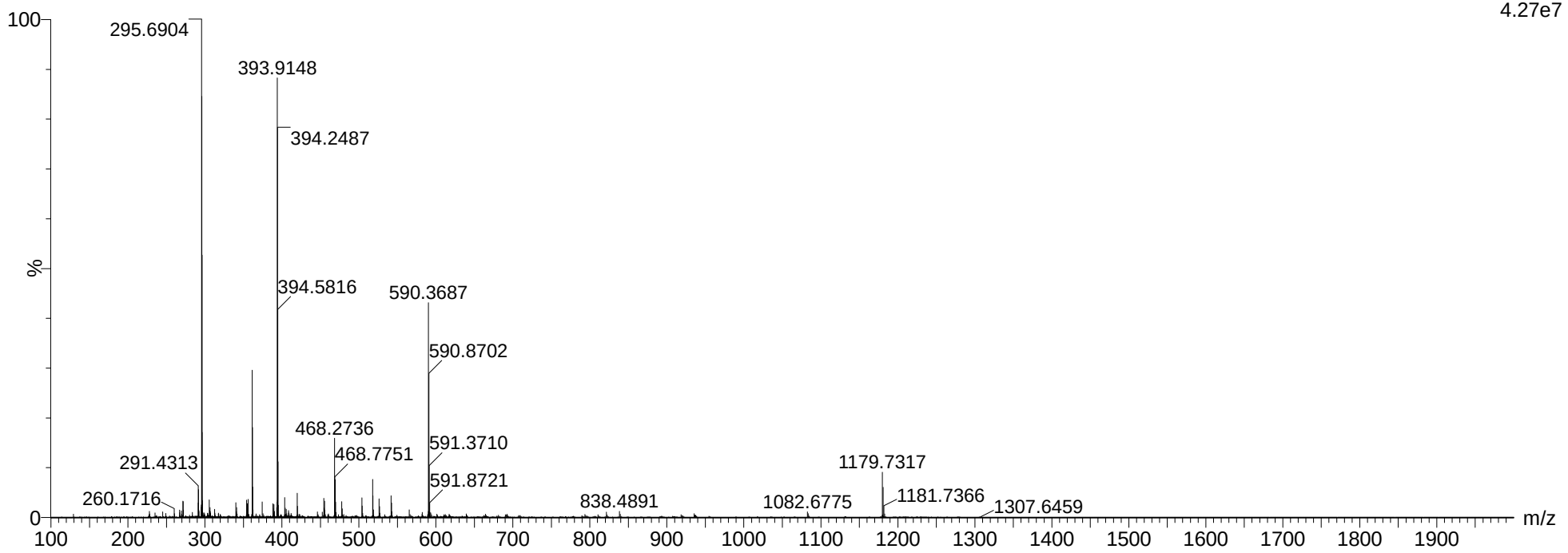


Figure S8B (TOF transformed)

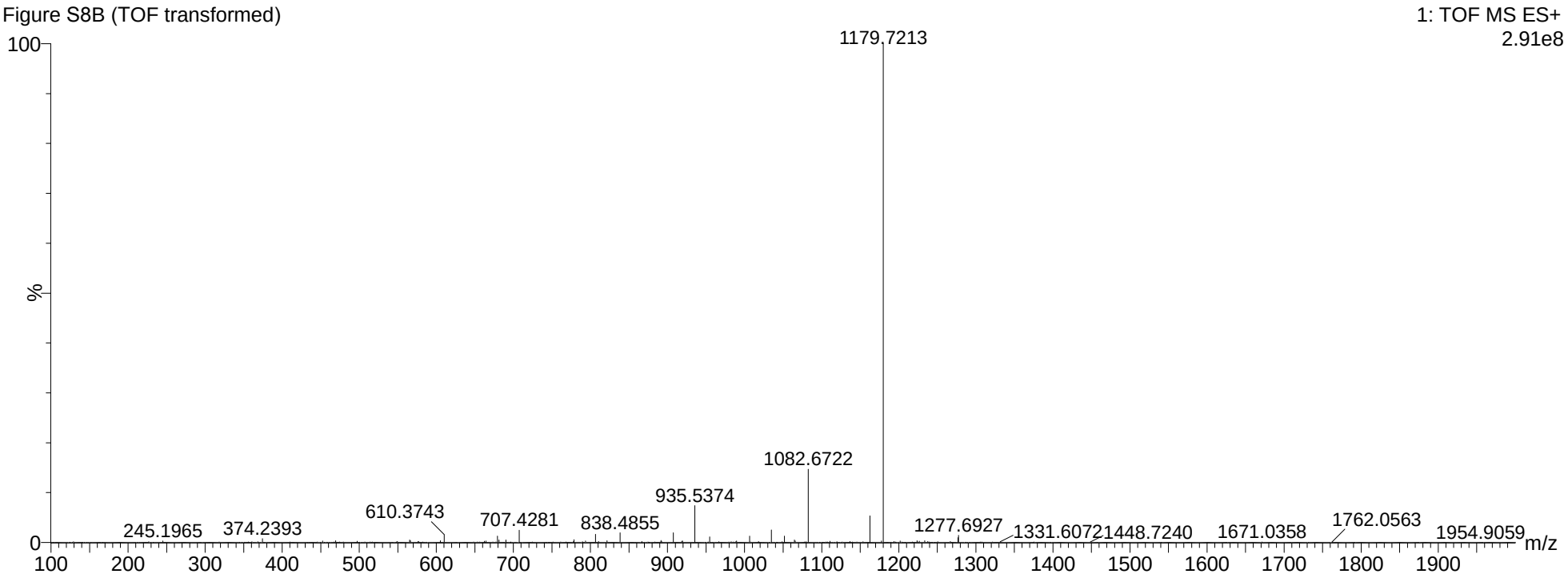


Figure S8B (Lock mass corrected)

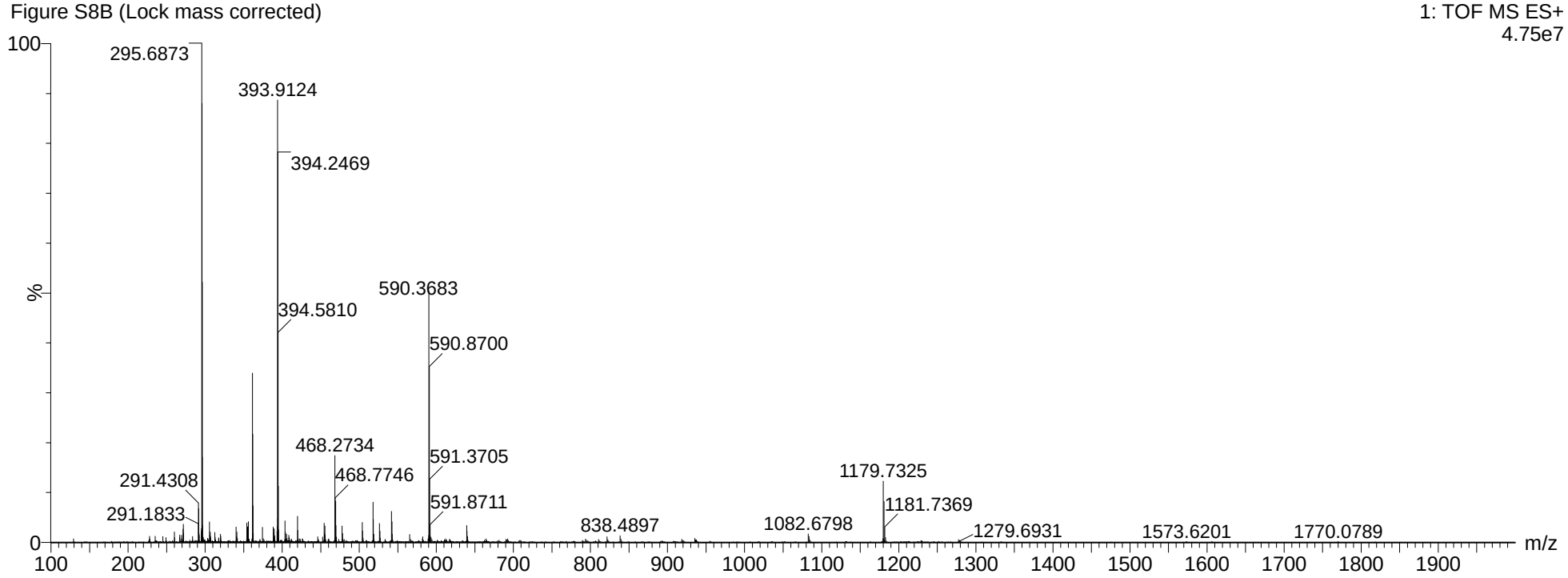


Figure S8C (TOF transformed)

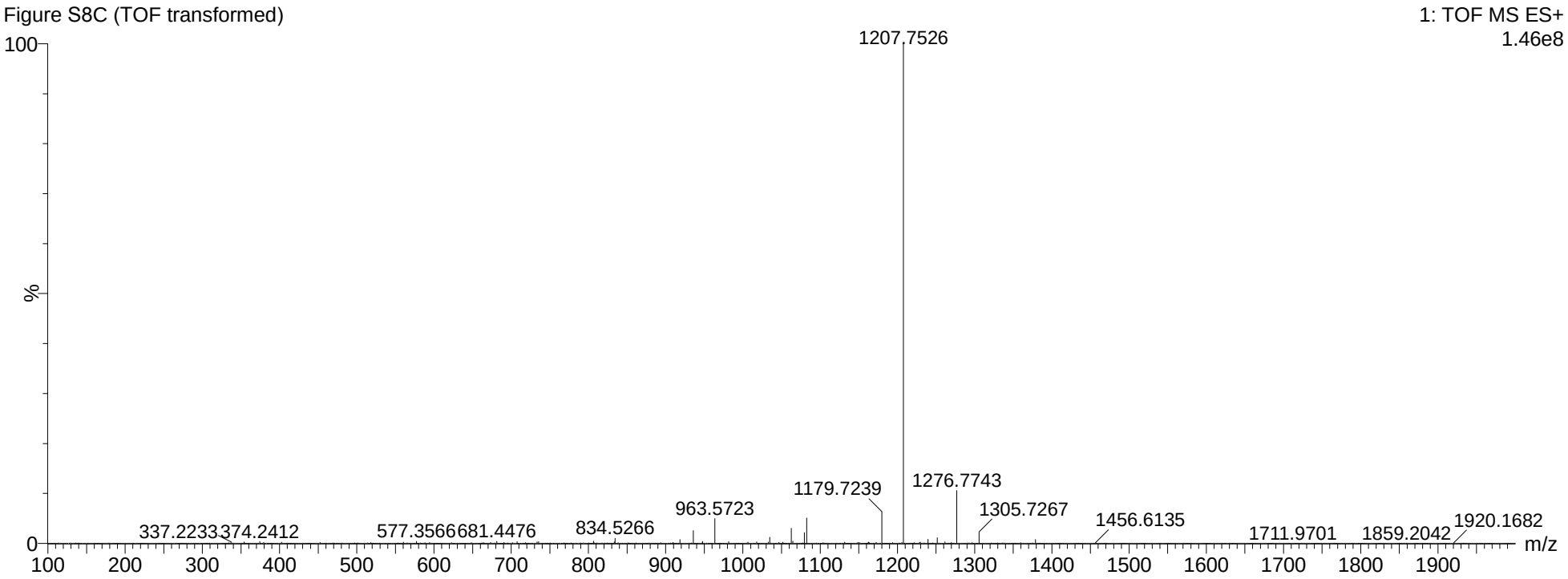


Figure S8C (Lock mass corrected)

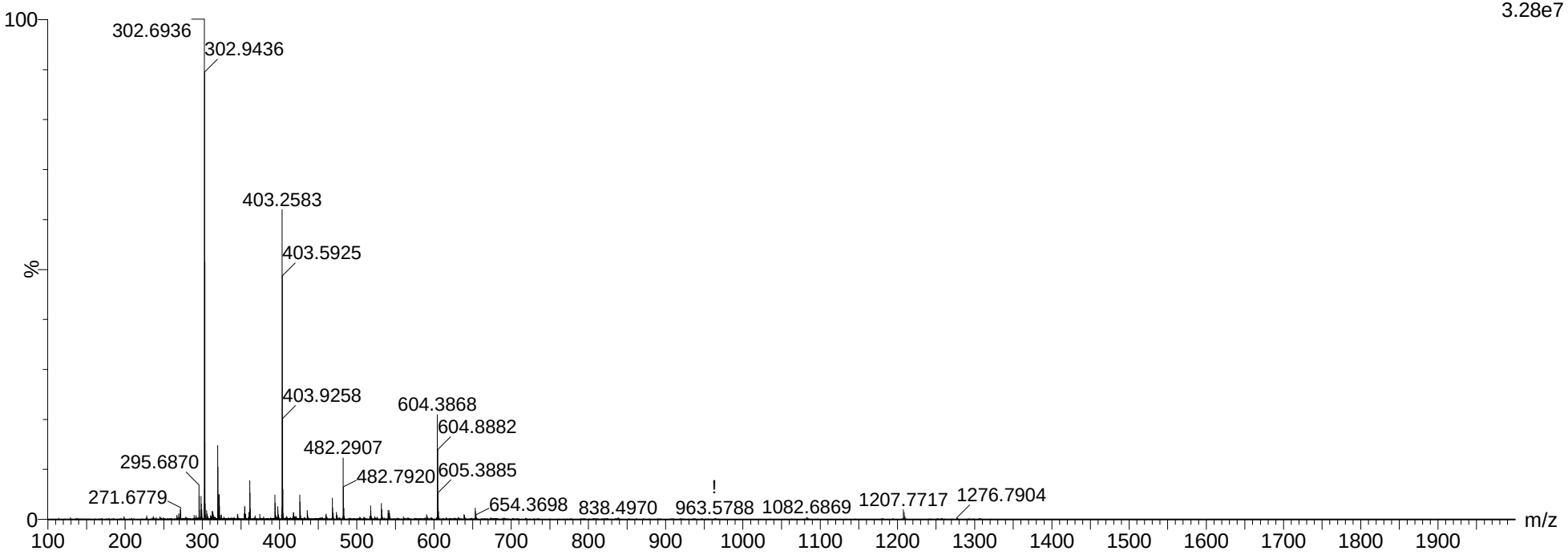


Figure S9A (TOF transformed)

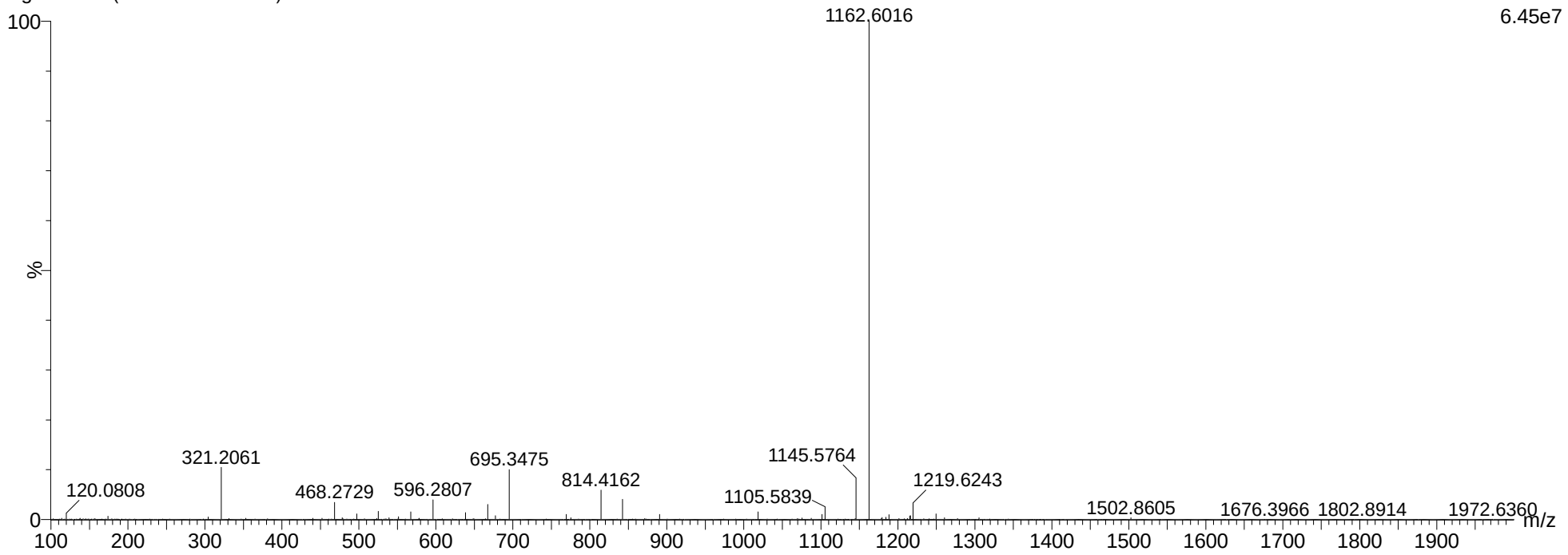


Figure S9A (Lock mass corrected)

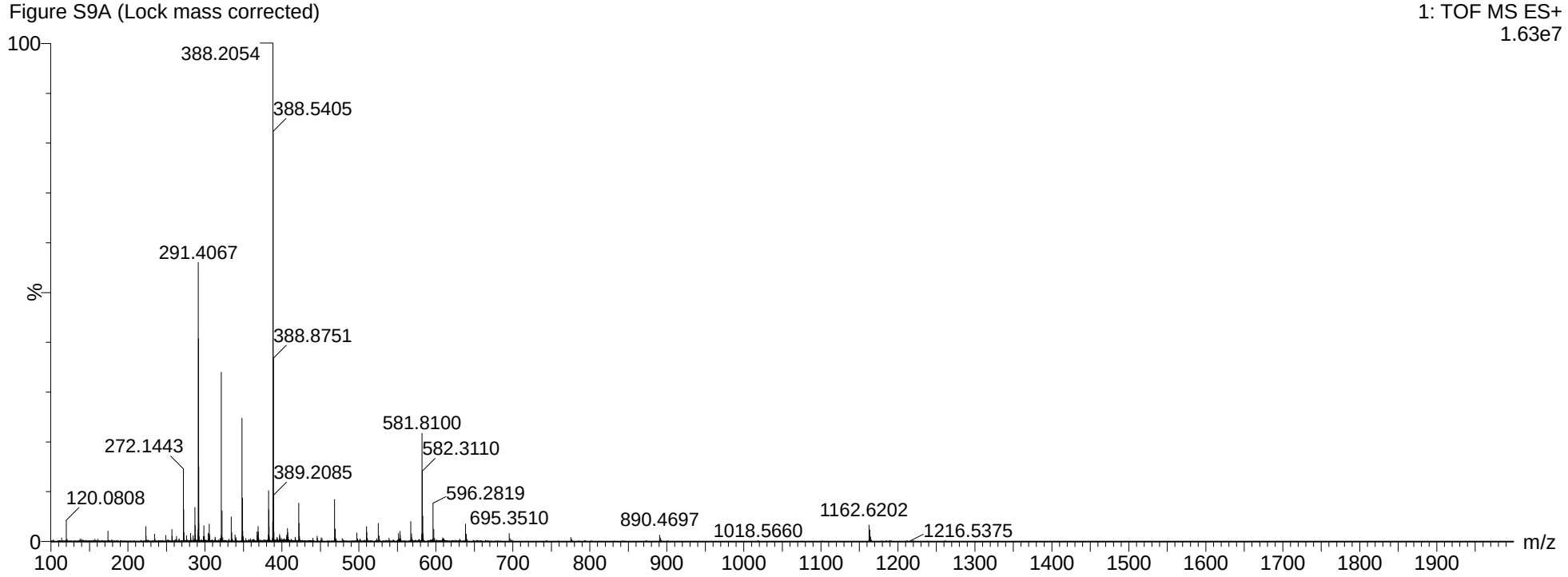


Figure S9B (TOF transformed)

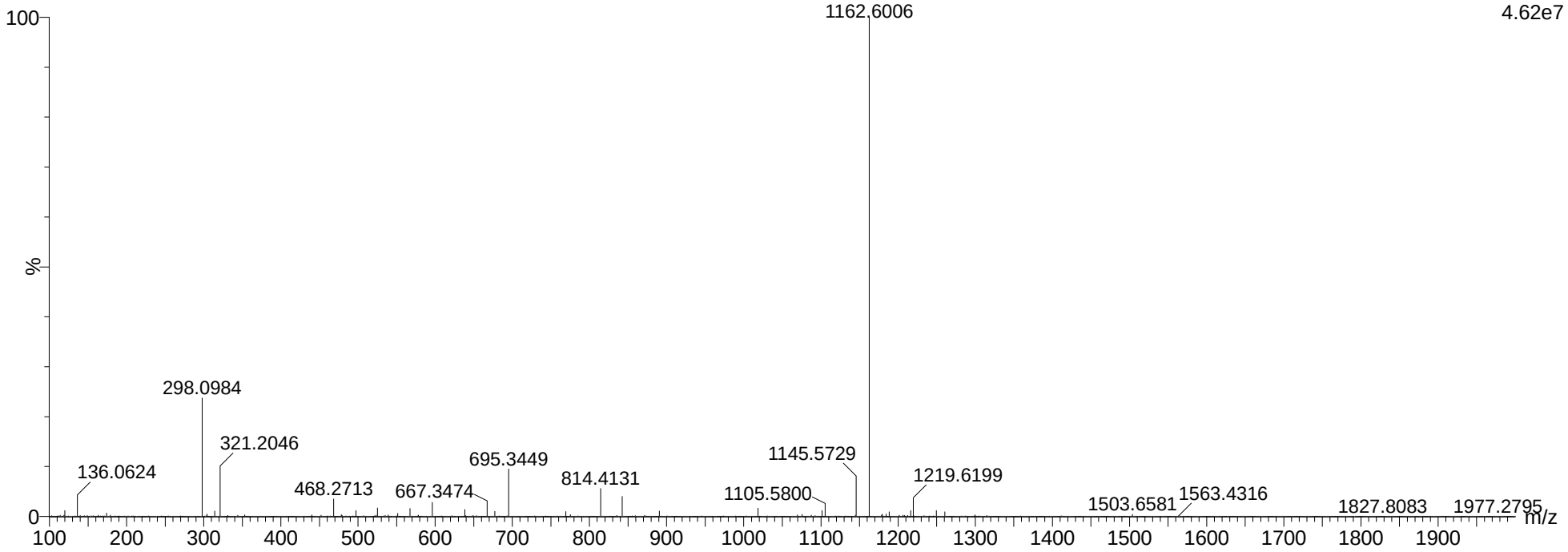


Figure S9B (Lock mass corrected)

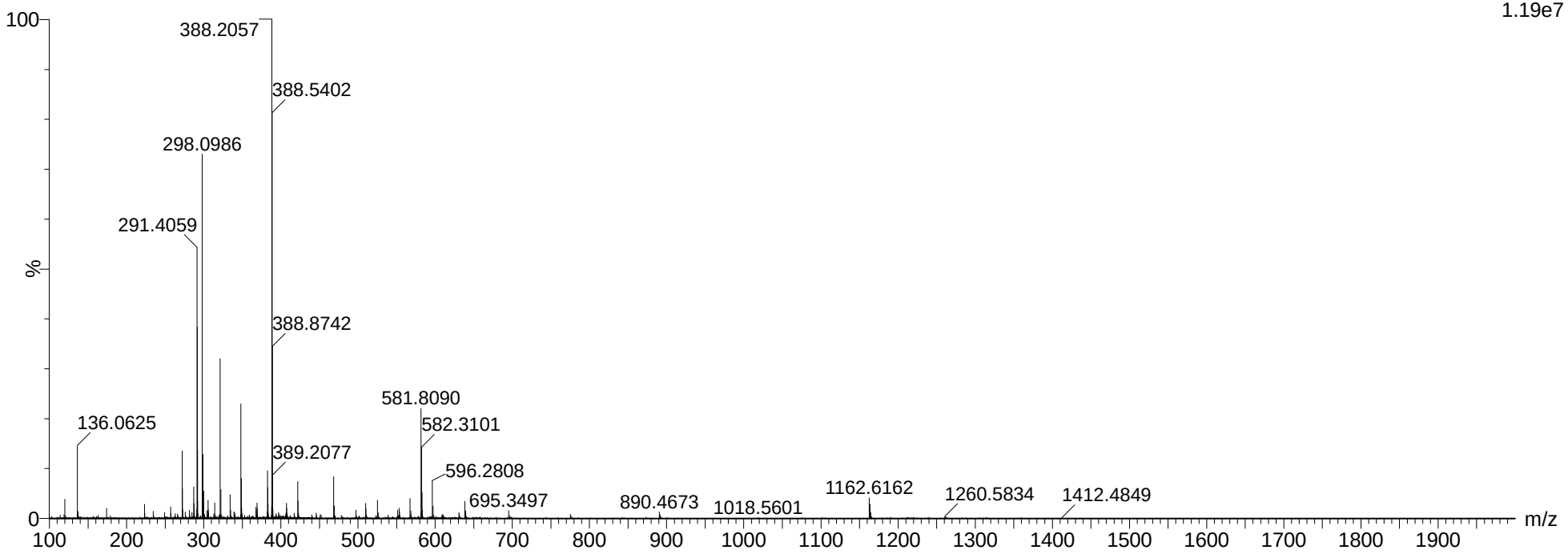


Figure S9C (TOF transformed)

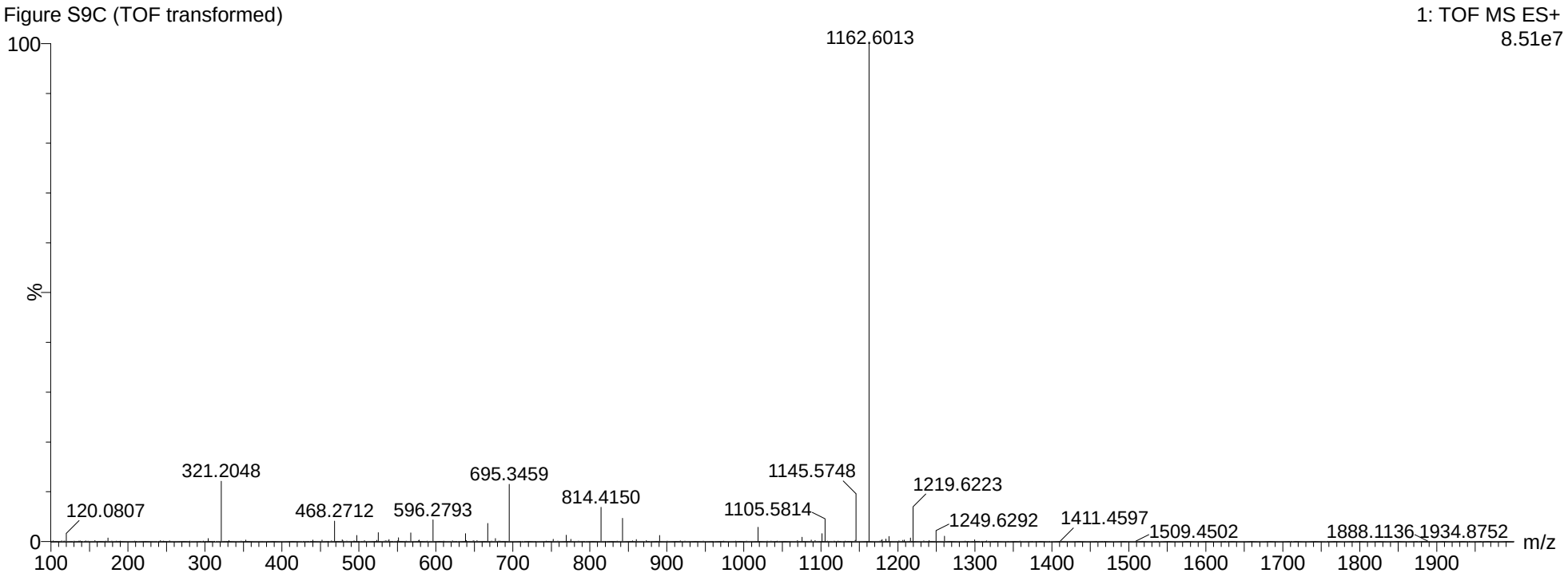


Figure S9C (Lock mass corrected)

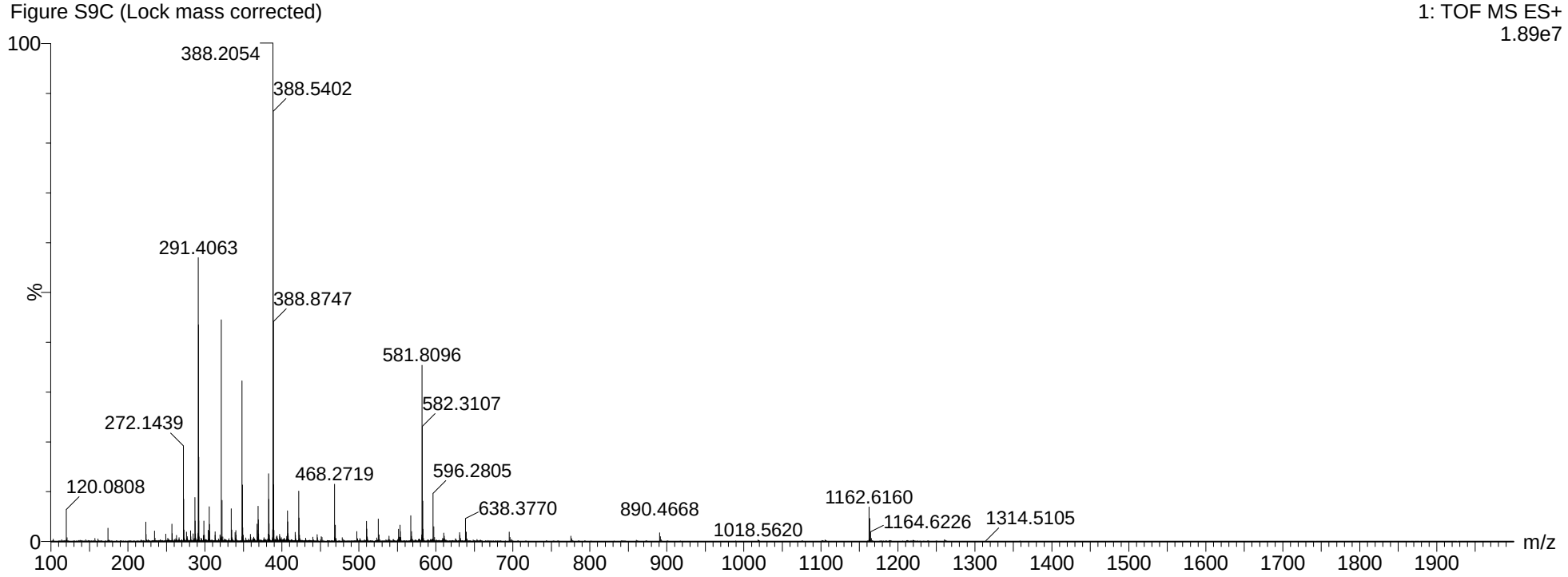


Figure S10A (TOF transformed)

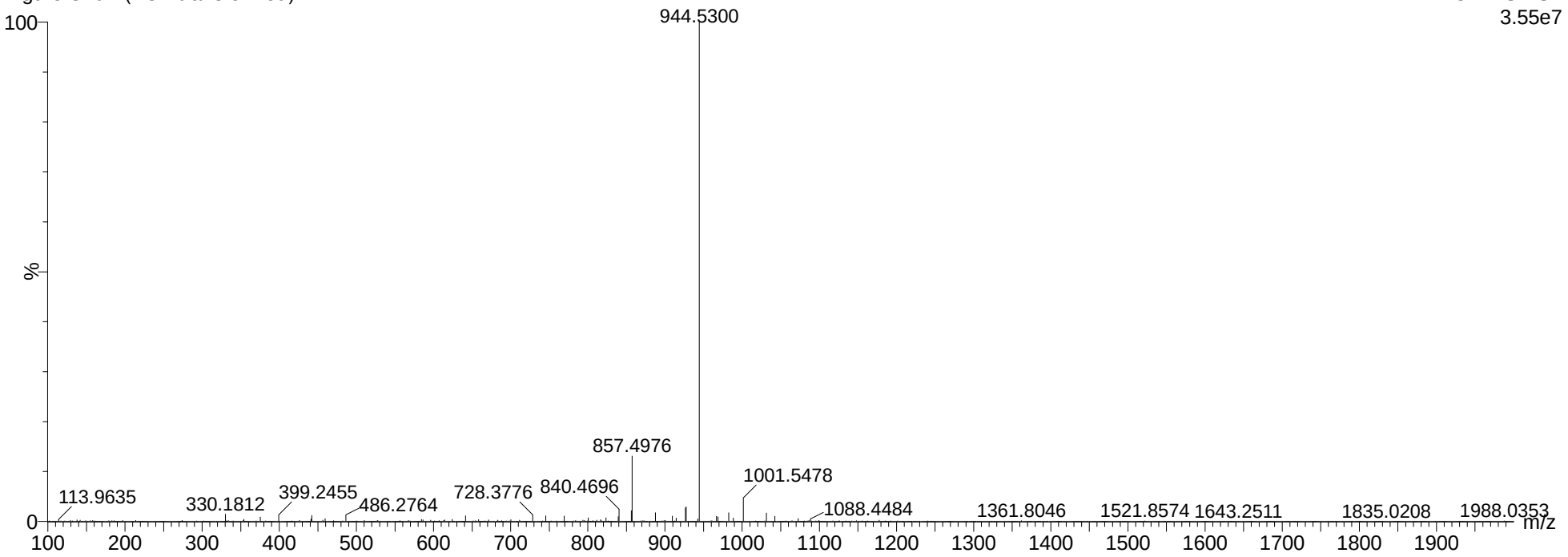


Figure S10A (Lock mass corrected)

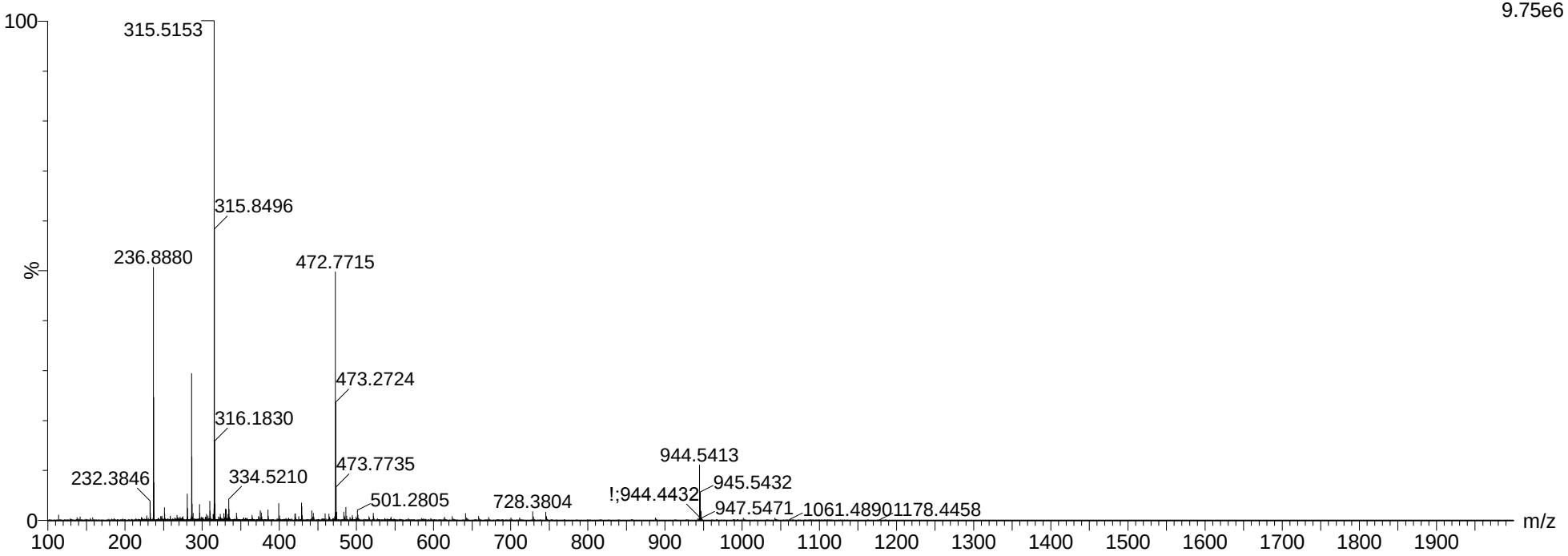


Figure S10B (TOF transformed)

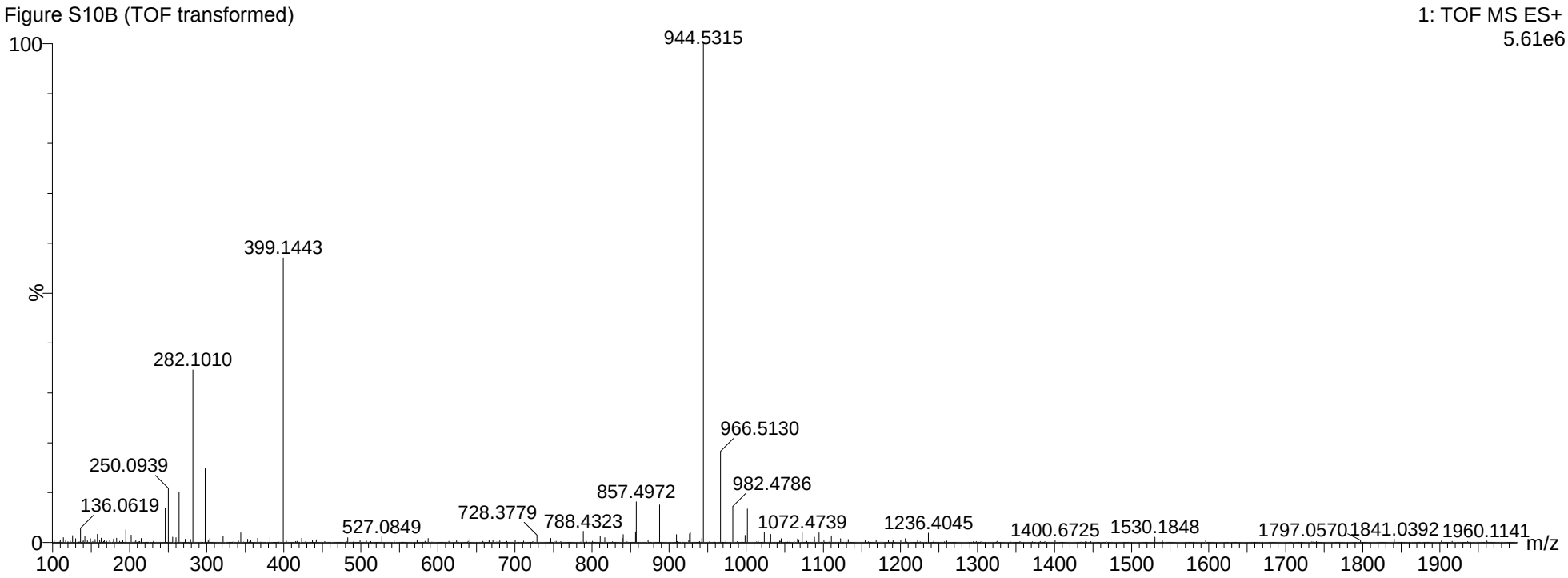


Figure S10B (Lock mass corrected)

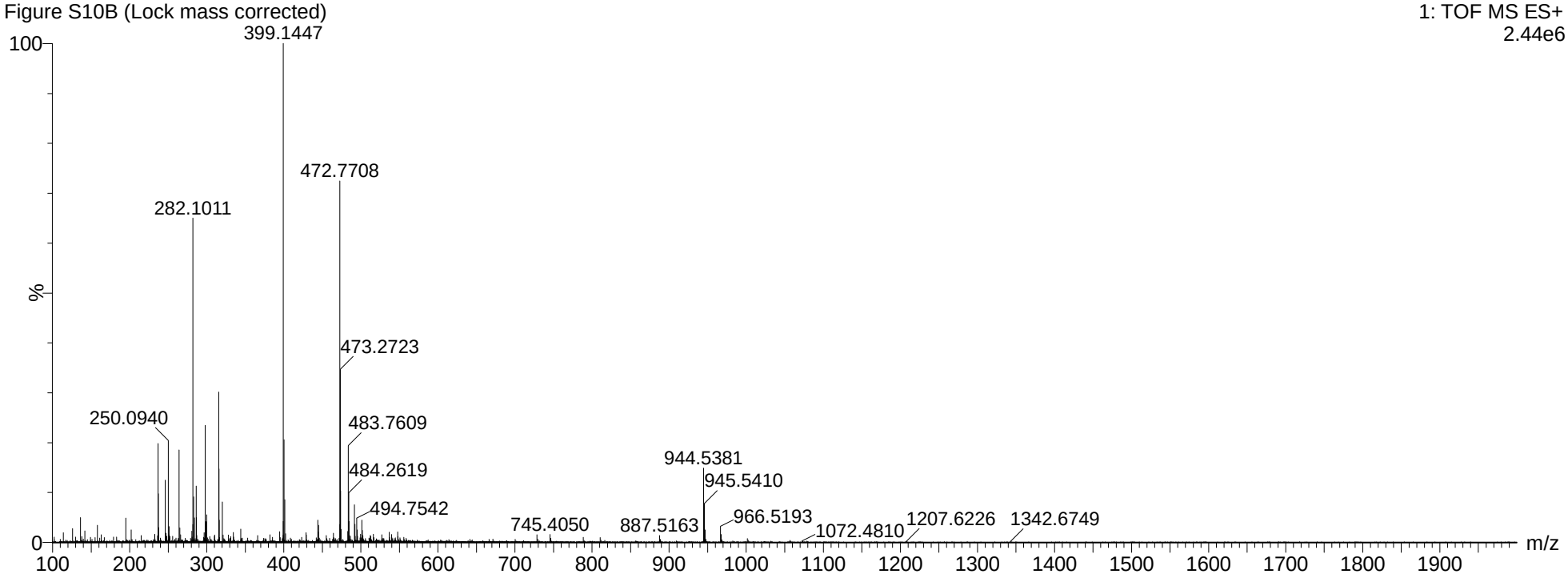


Figure S10C (TOF transformed)

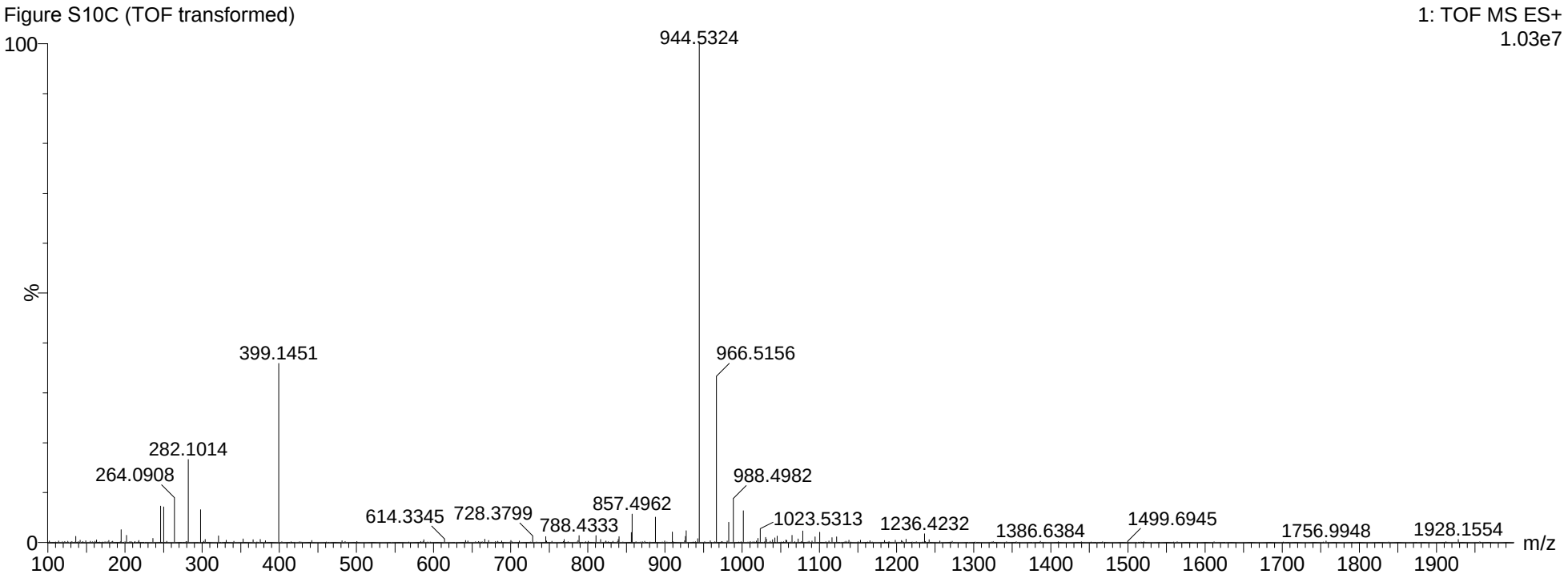


Figure S10C (Lock mass corrected)

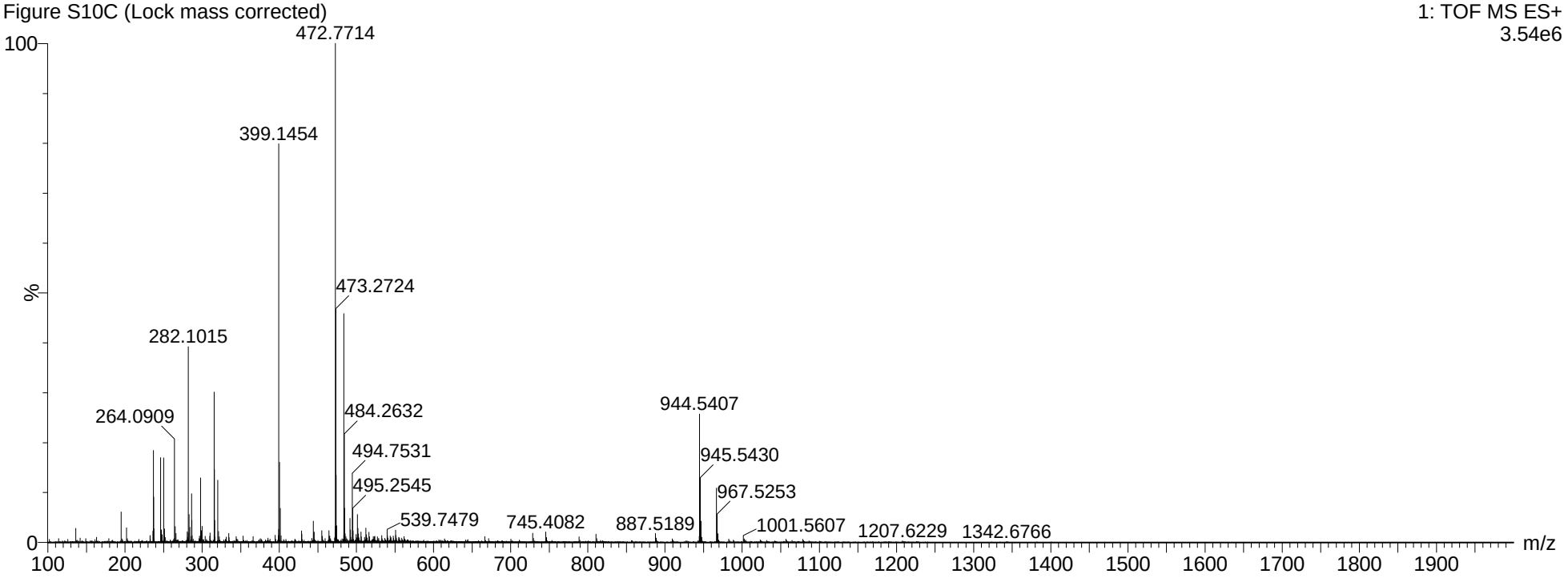


Figure S12A (TOF transformed)

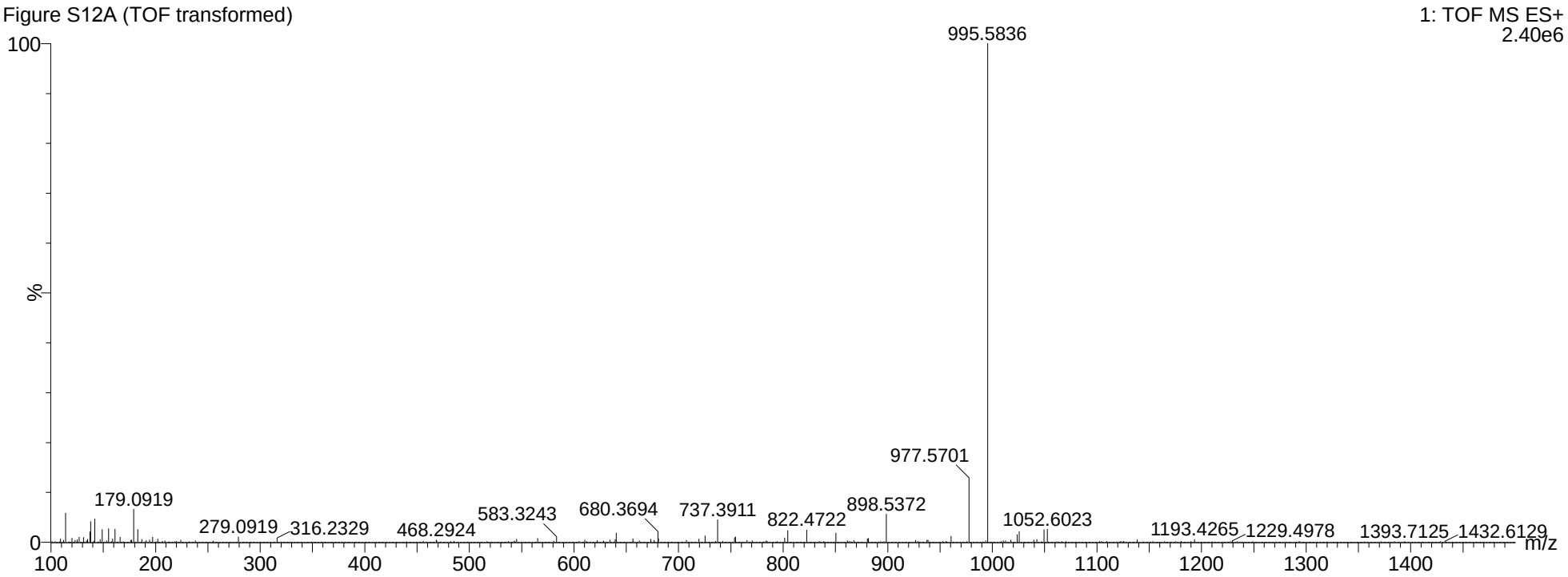


Figure S12A (Lock mass corrected)

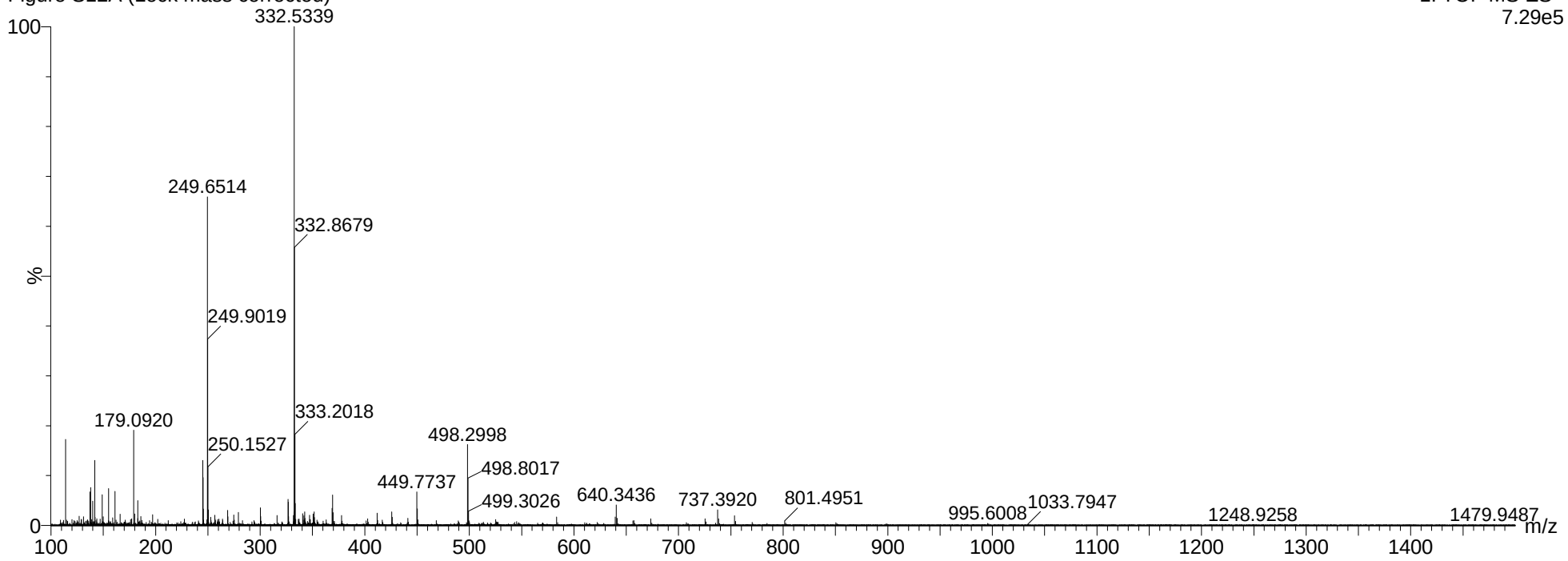


Figure S12B (TOF transformed)

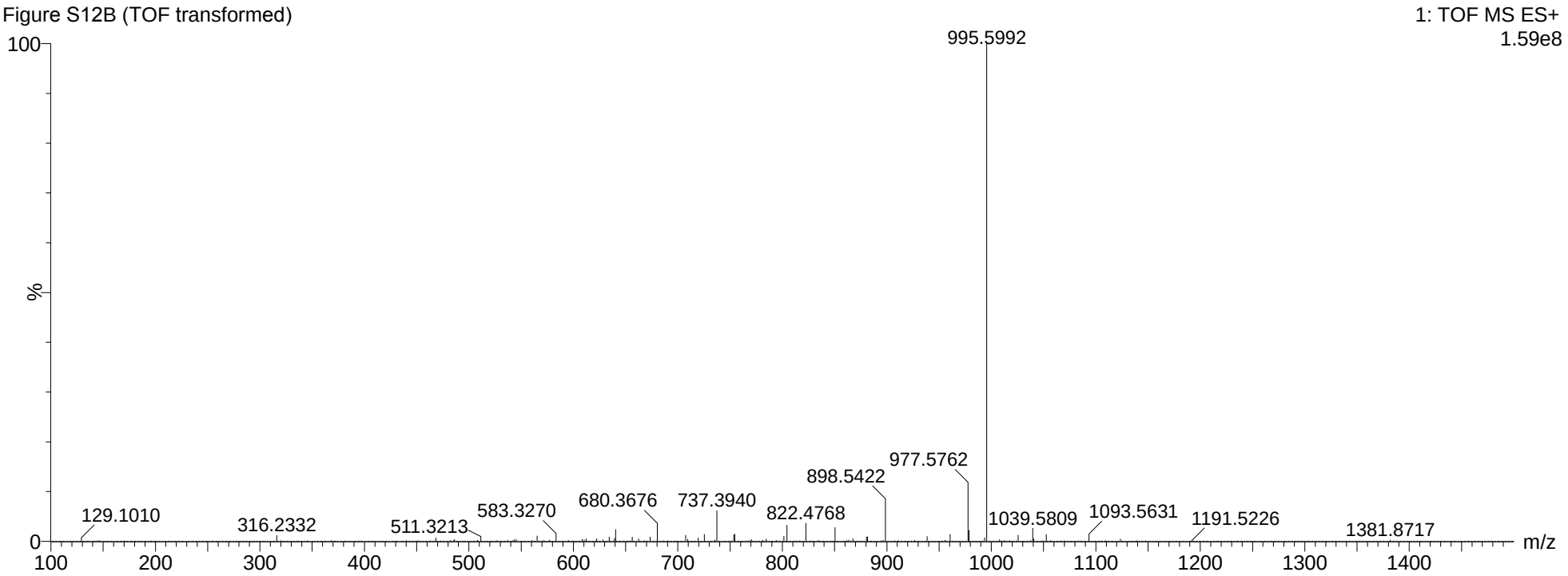


Figure S12B (Lock mass corrected)

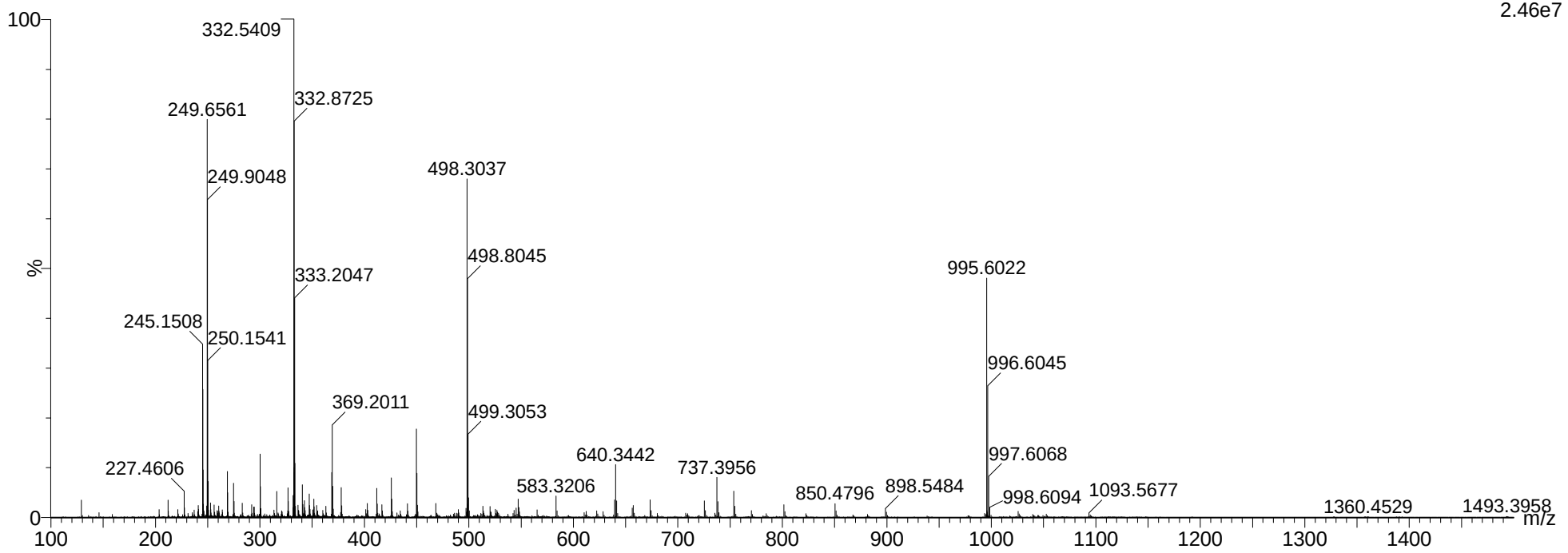


Figure S12C (TOF transformed)

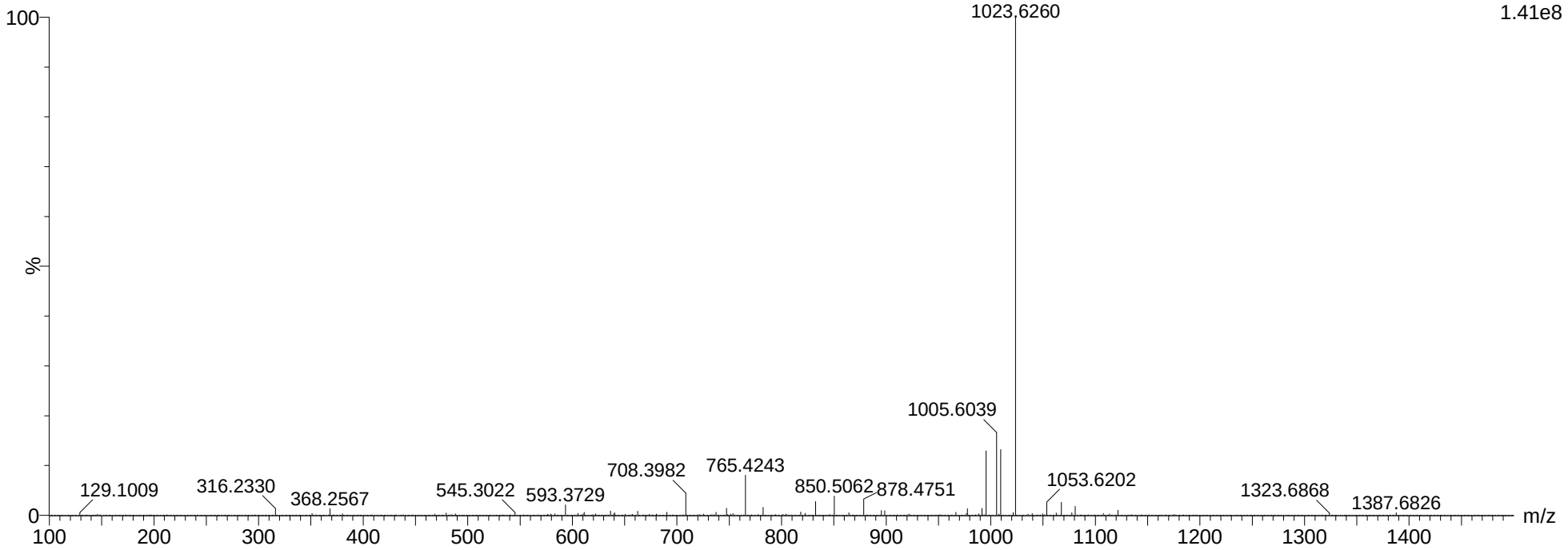


Figure S12C (Lock mass corrected)

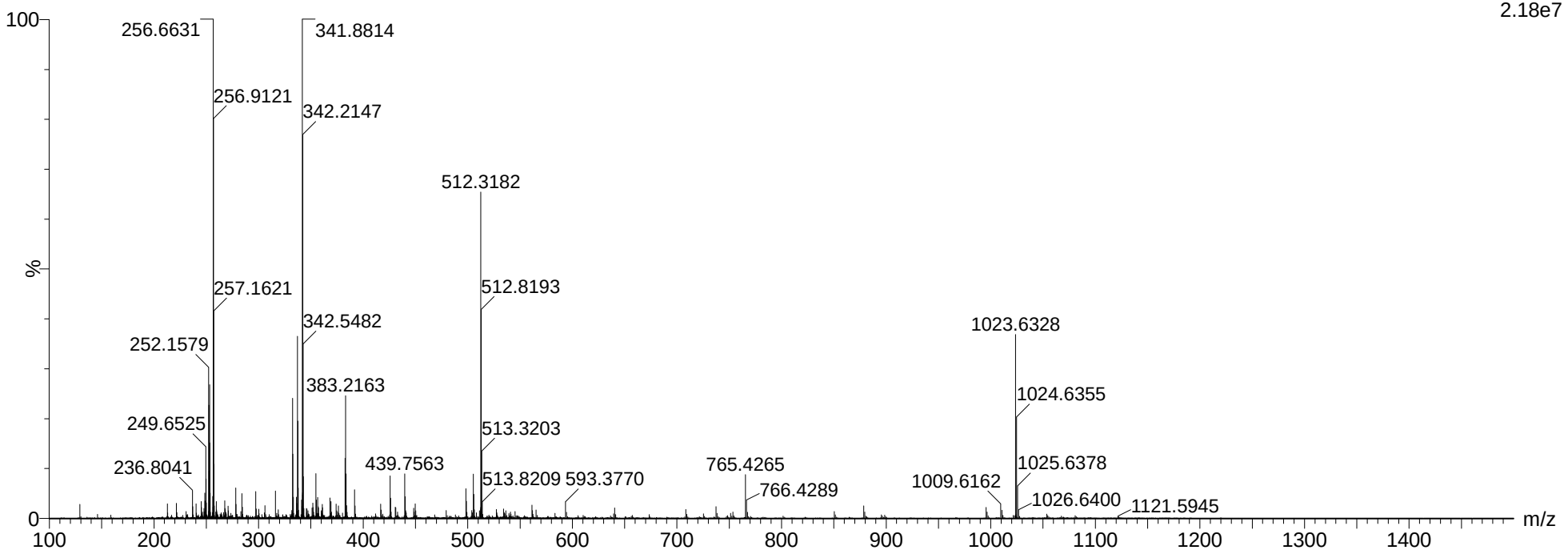


Figure S13A (TOF transformed)

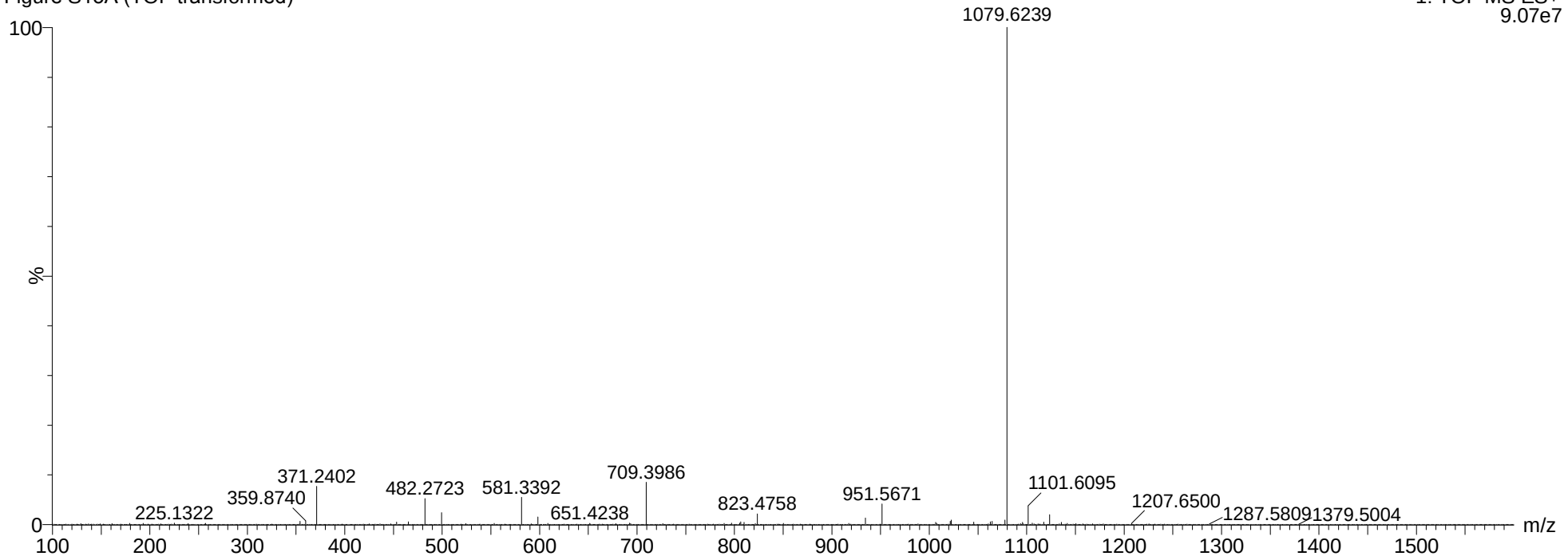


Figure S13A (Lock mass corrected)

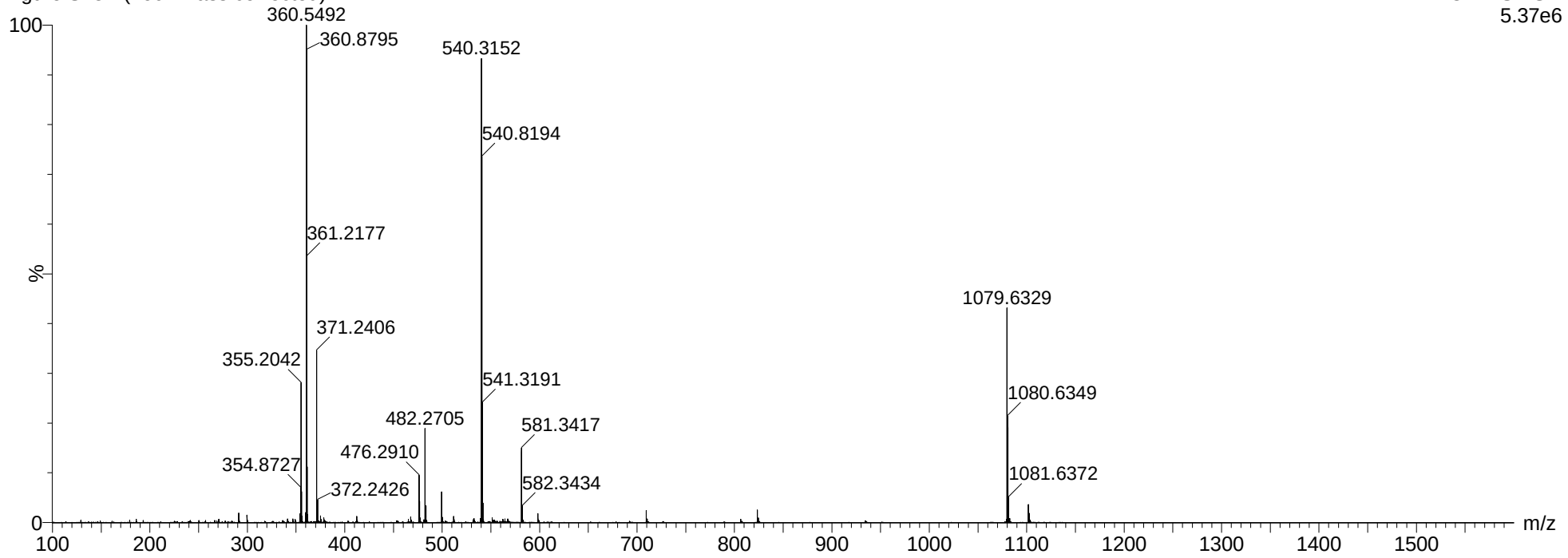


Figure S13B (TOF transformed)

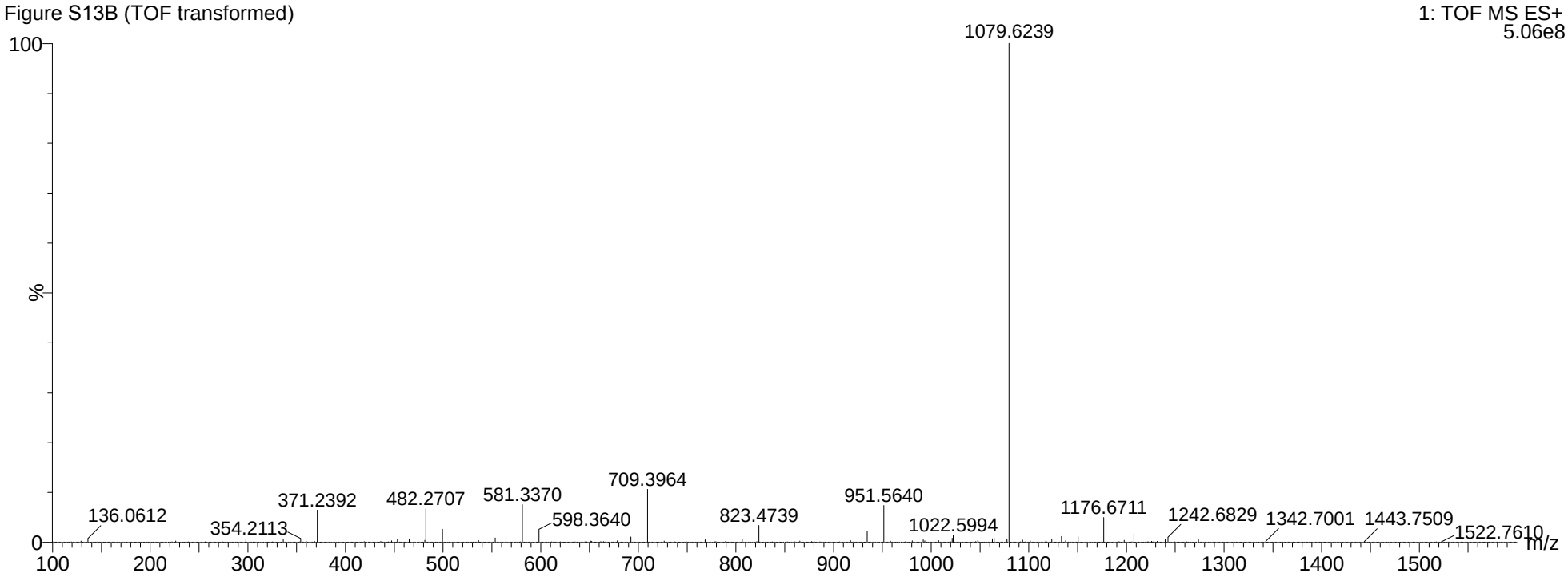


Figure S13B (Lock mass corrected)

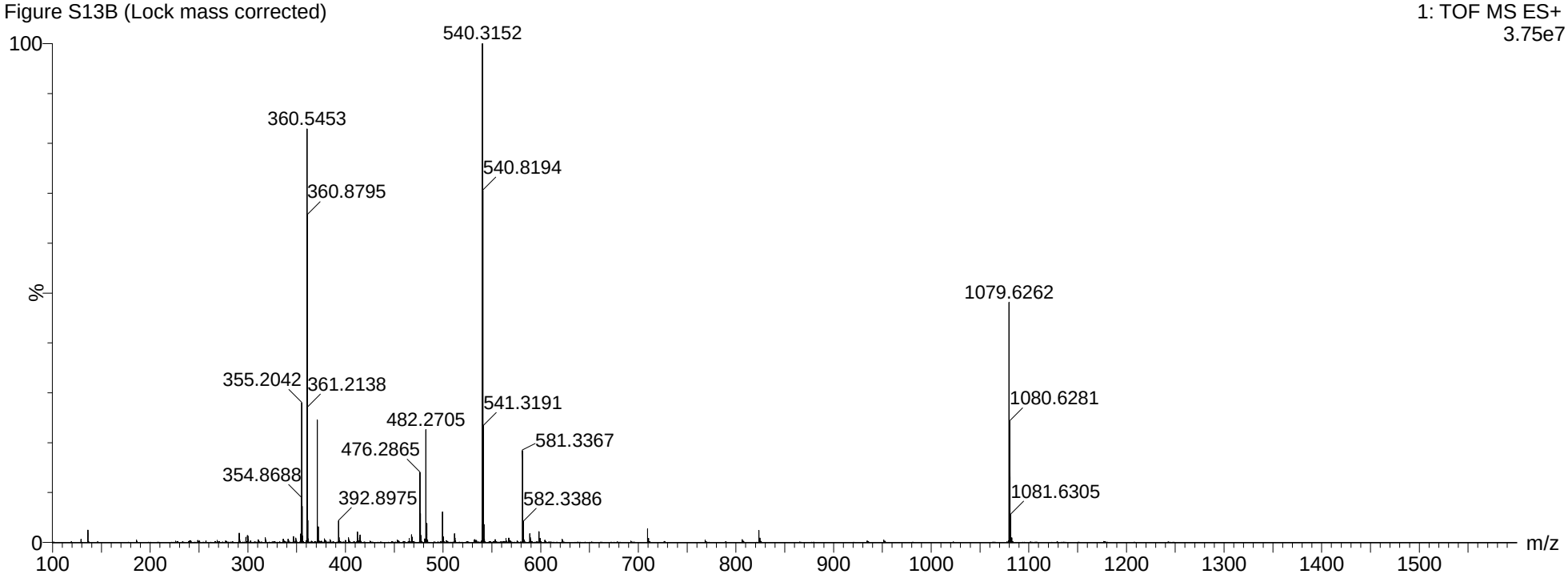


Figure S13C (TOF transformed)

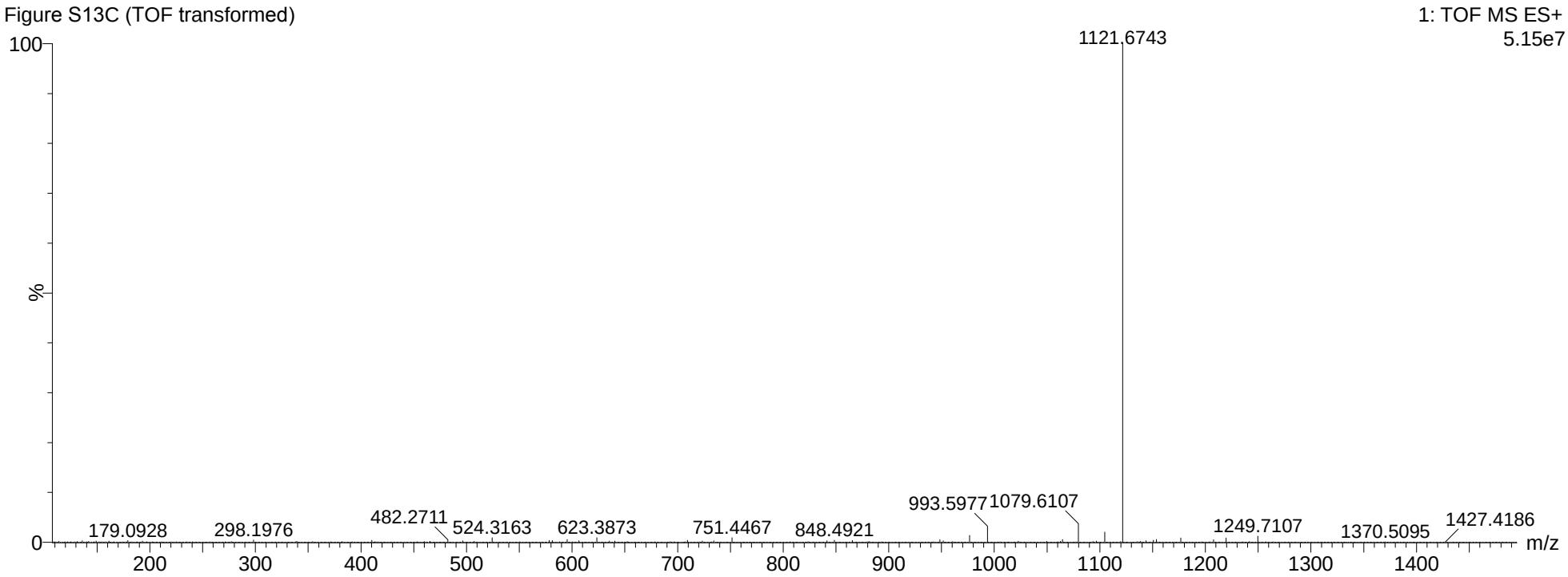


Figure S13C (Lock mass corrected)

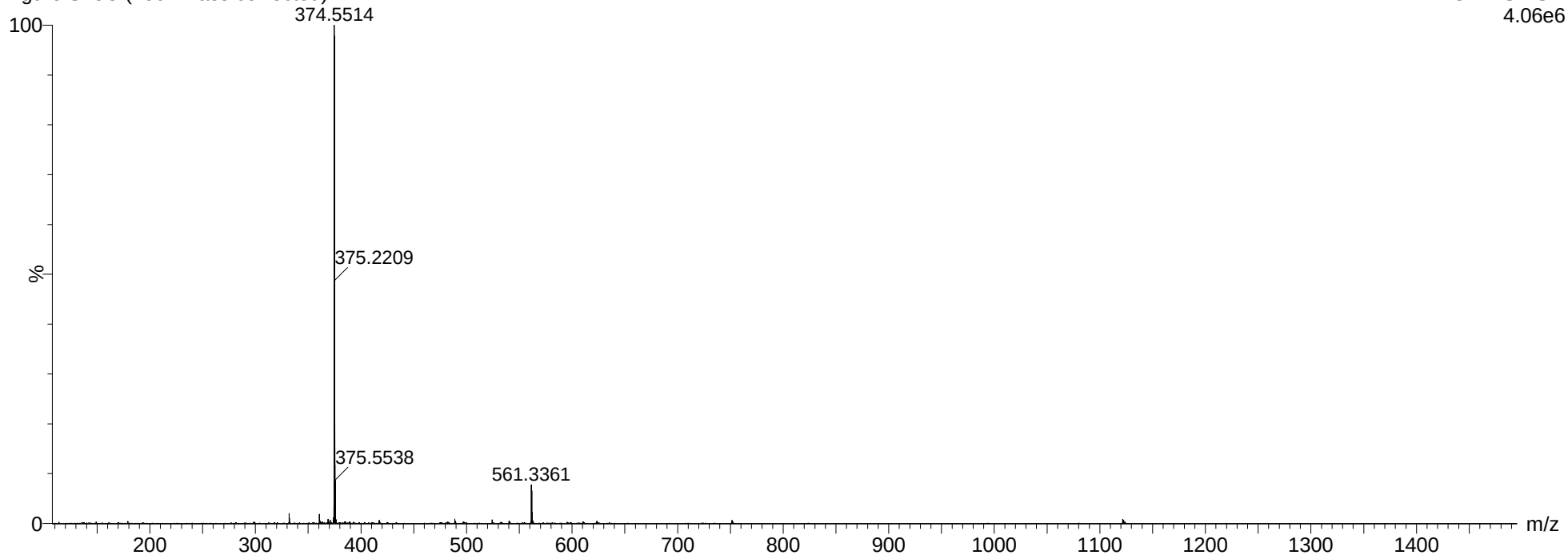


Figure S14A (TOF transformed)

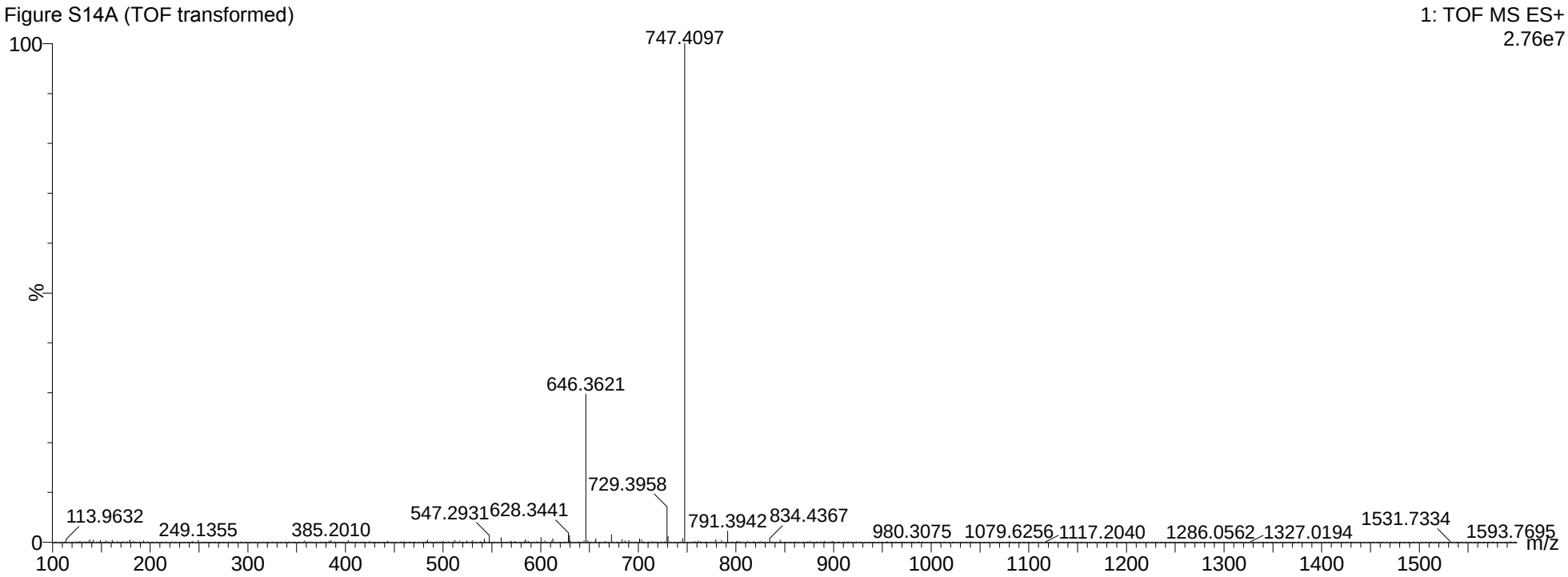


Figure S14A (Lock mass corrected)

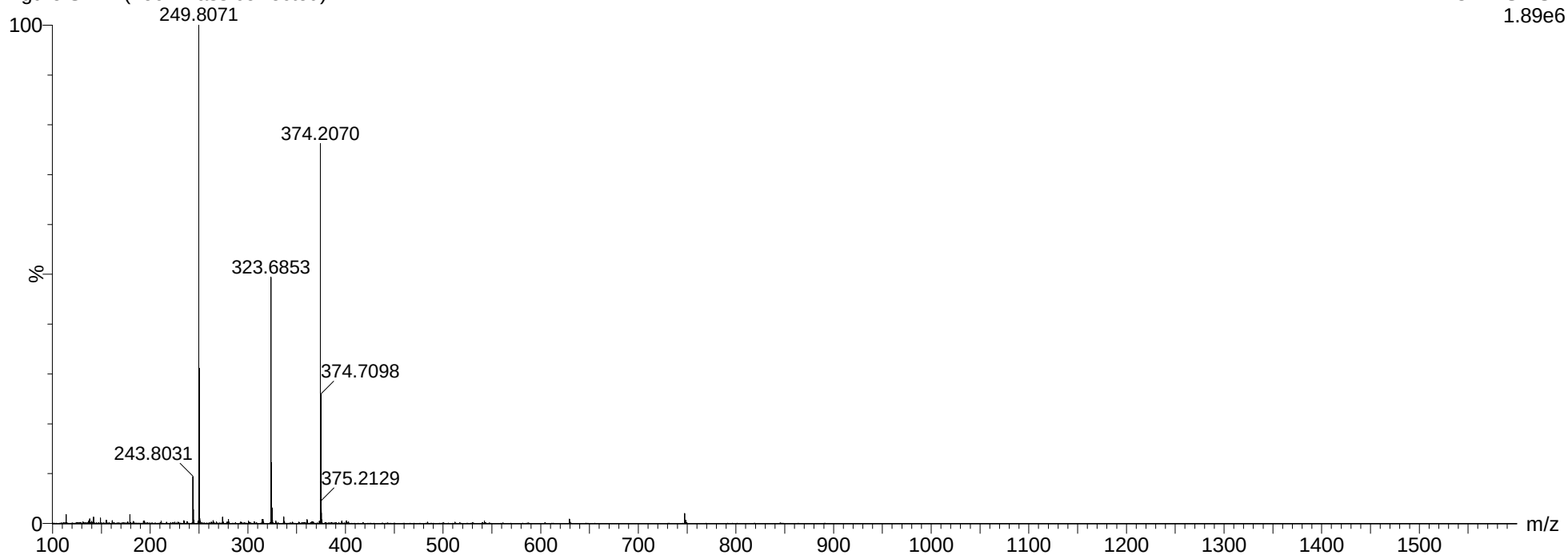


Figure S14B (TOF transformed)

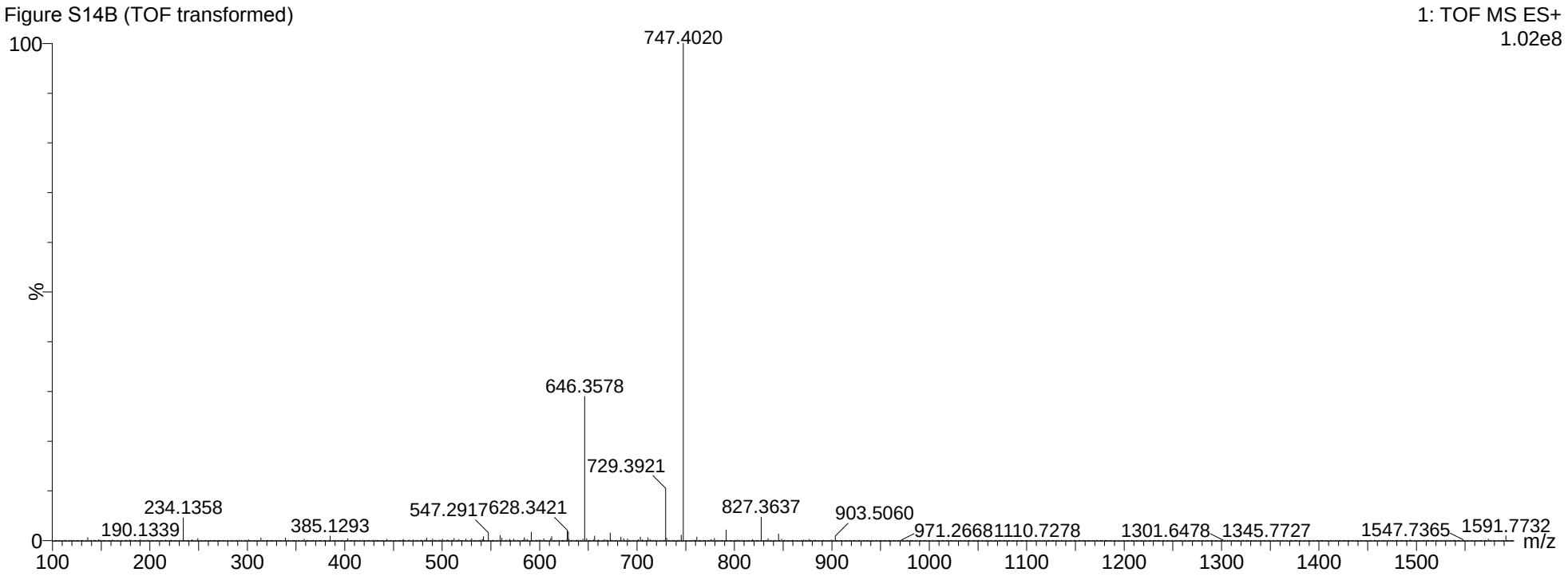


Figure S14B (Lock mass corrected)

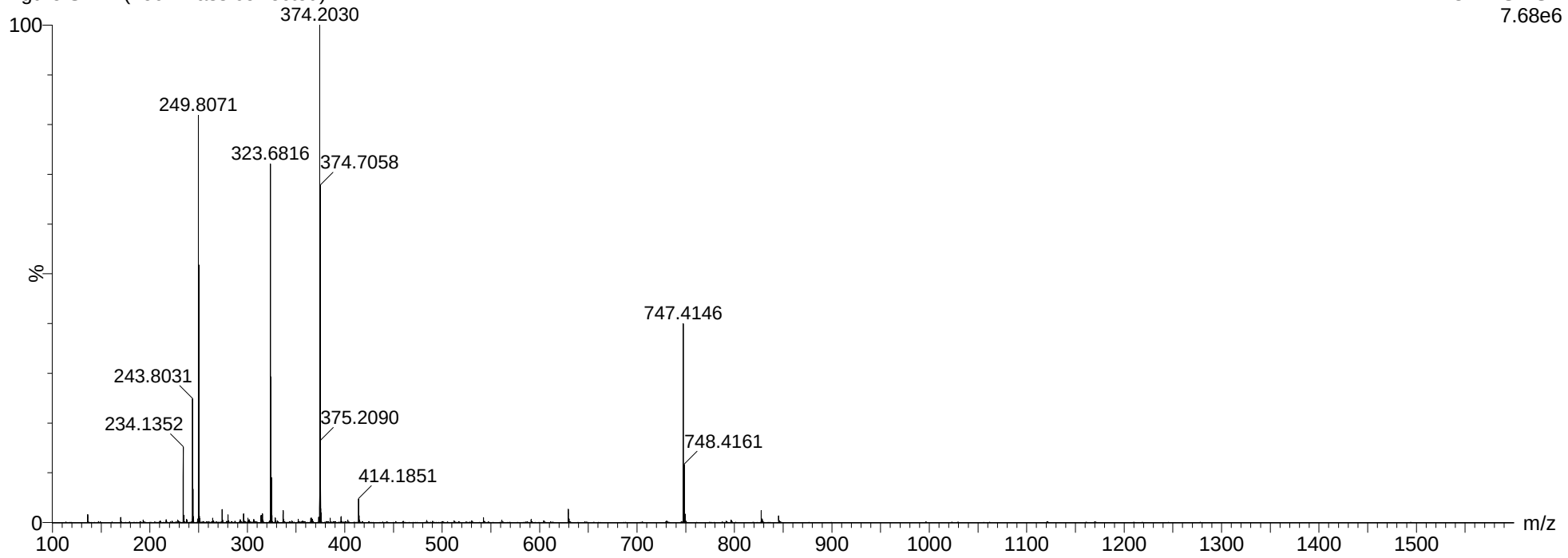


Figure S14C (TOF transformed)

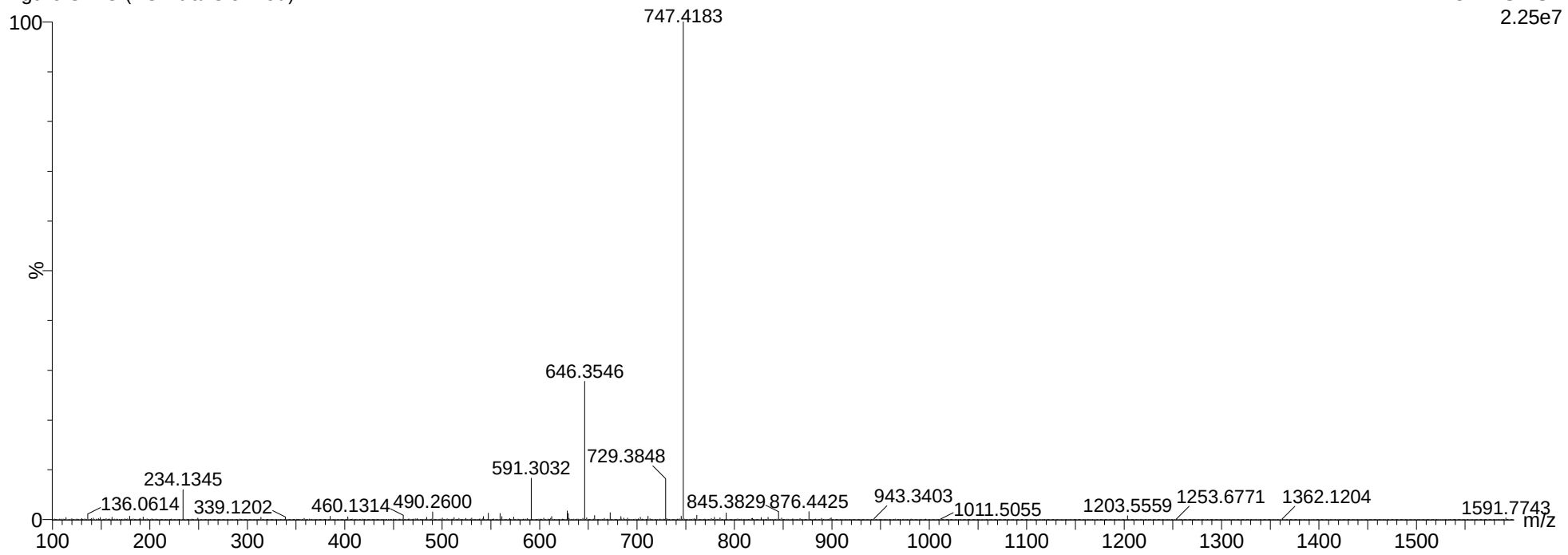


Figure S14C (Lock mass corrected)

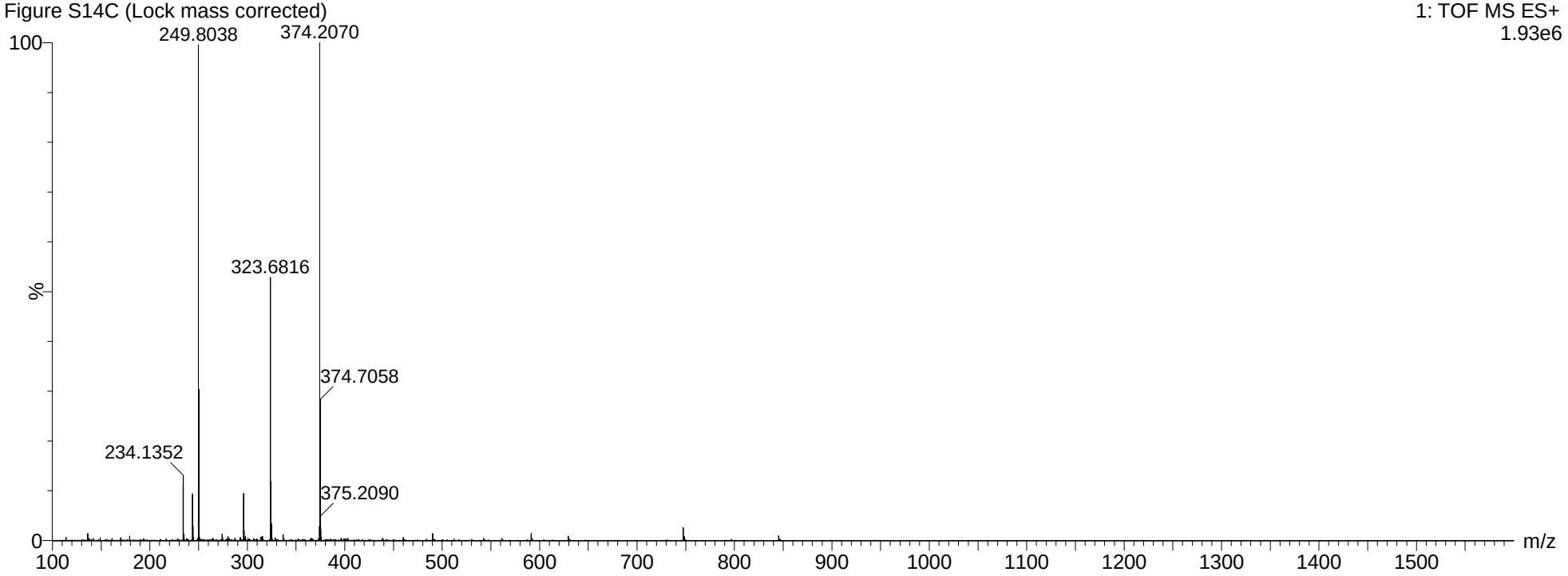


Figure S15A (TOF transformed)

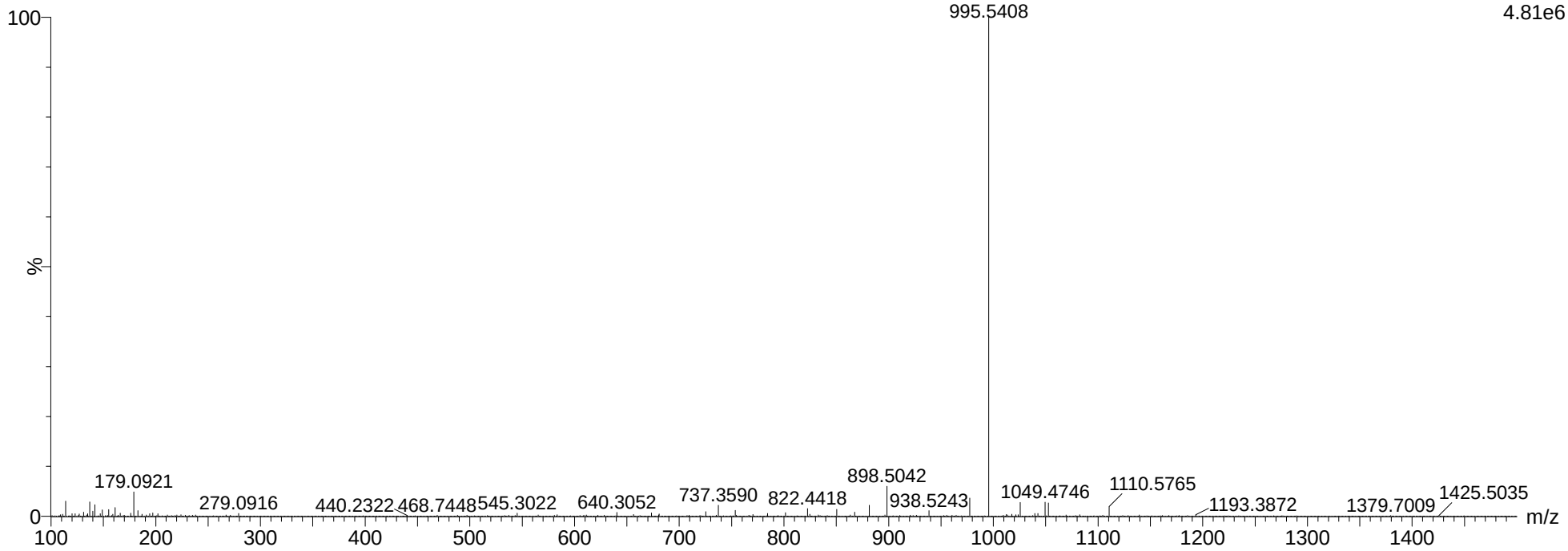


Figure S15A (Lock mass corrected)

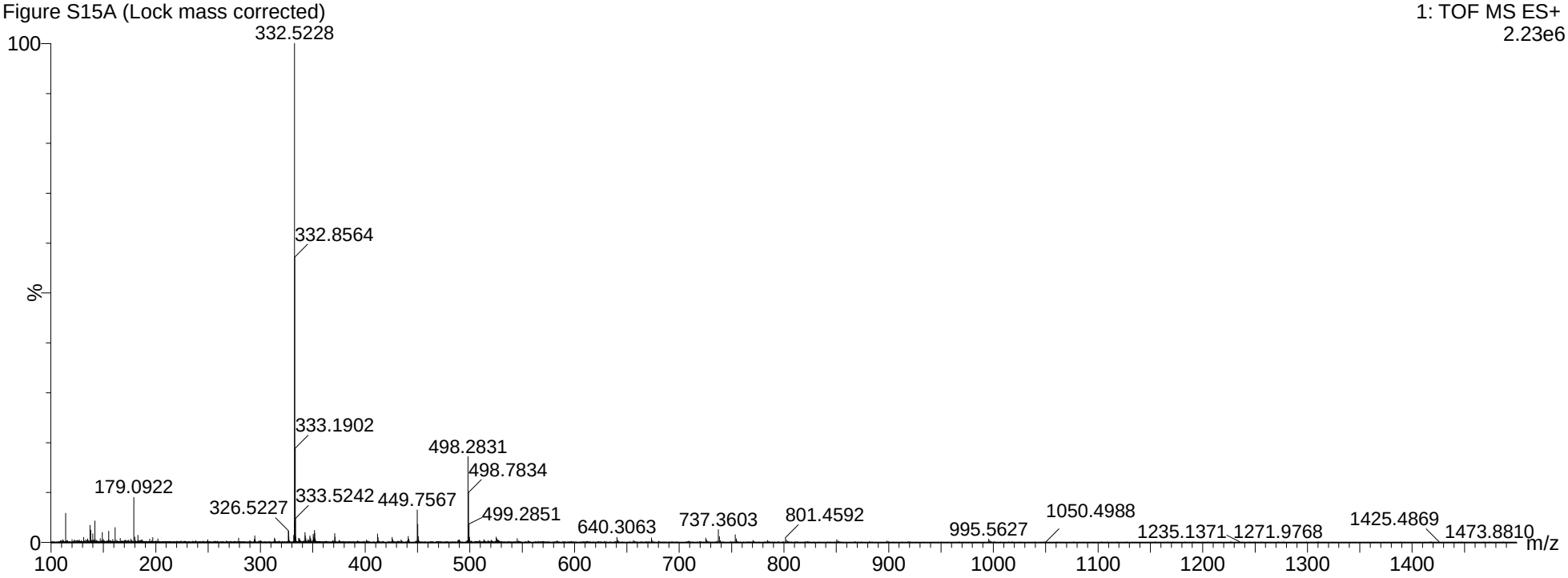


Figure S15B (TOF transformed)

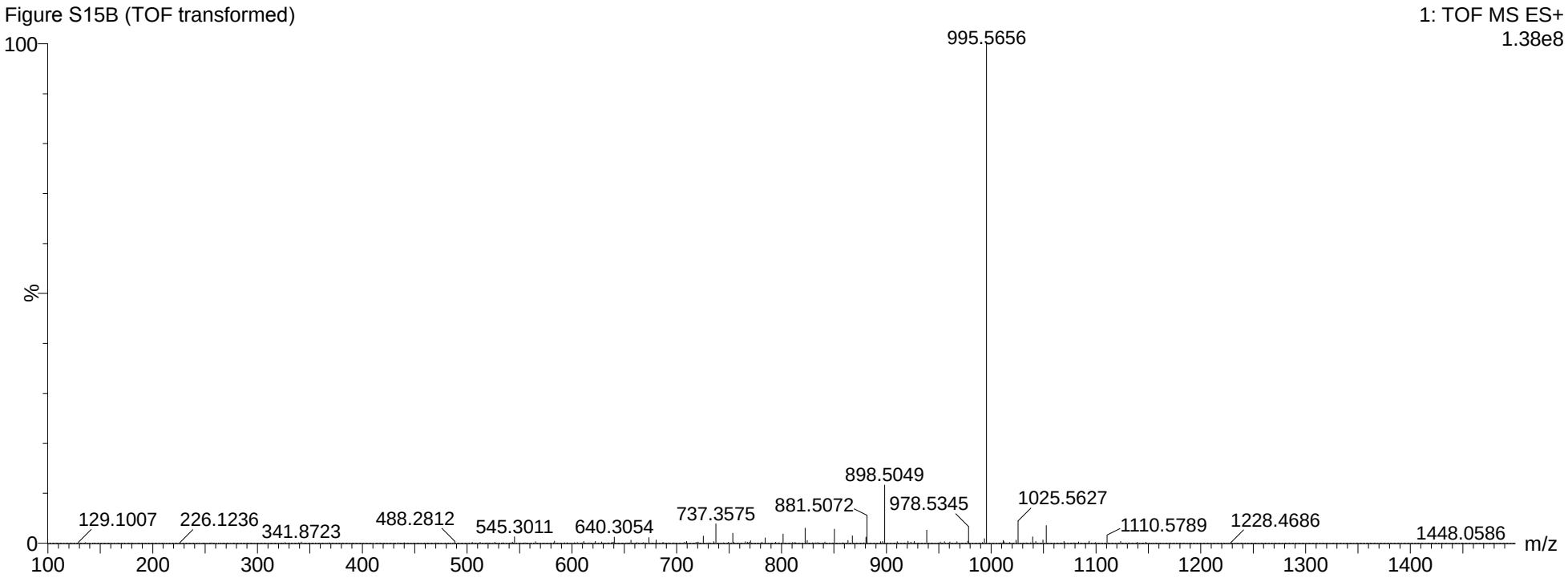


Figure S15B (Lock mass corrected)

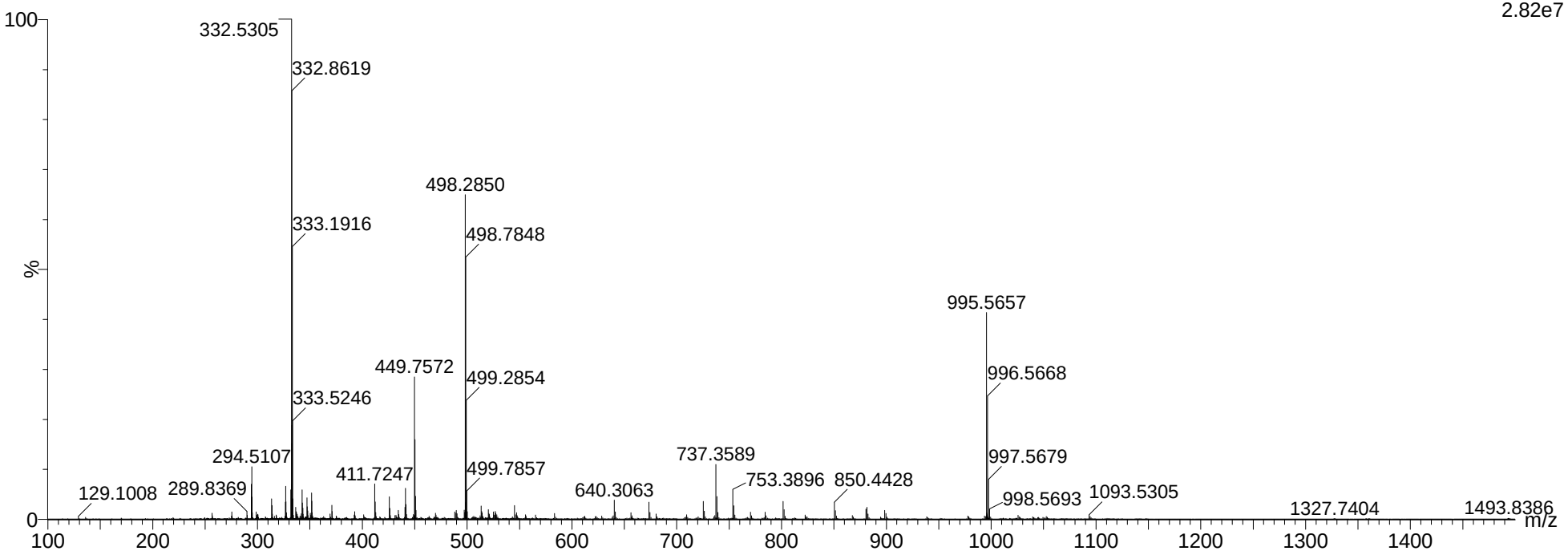


Figure S15C (TOF transformed)

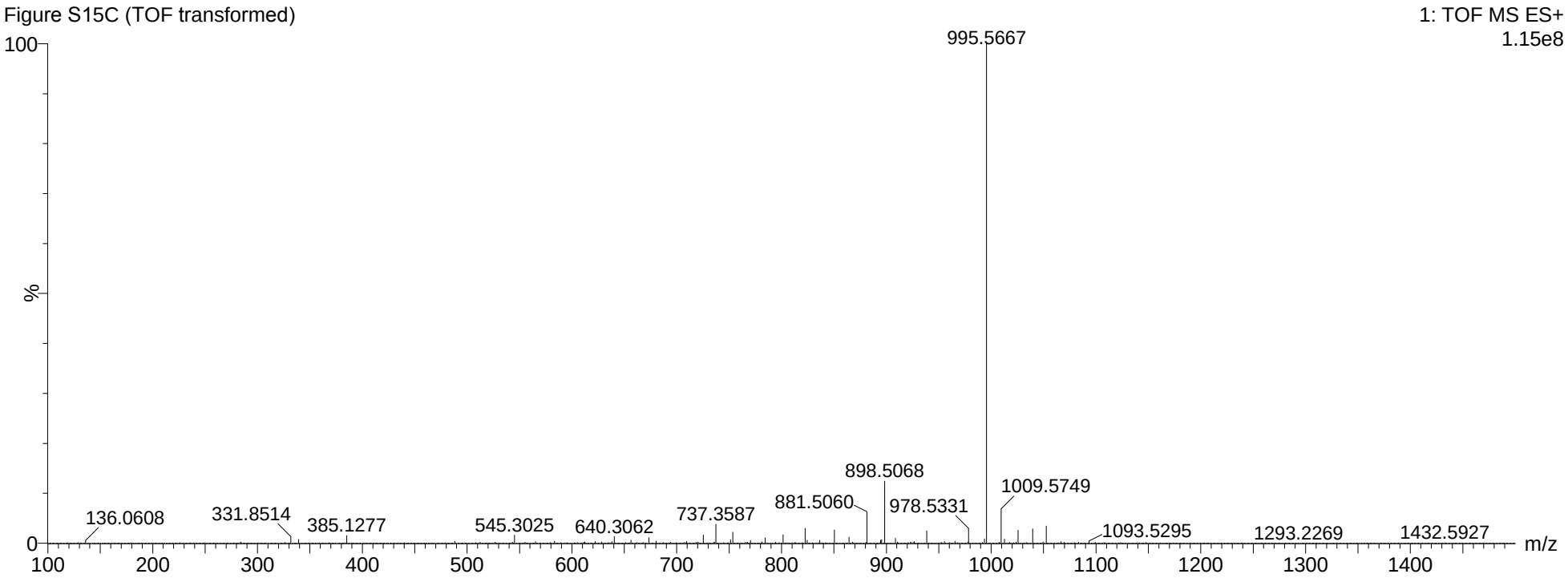


Figure S15C (Lock mass corrected)

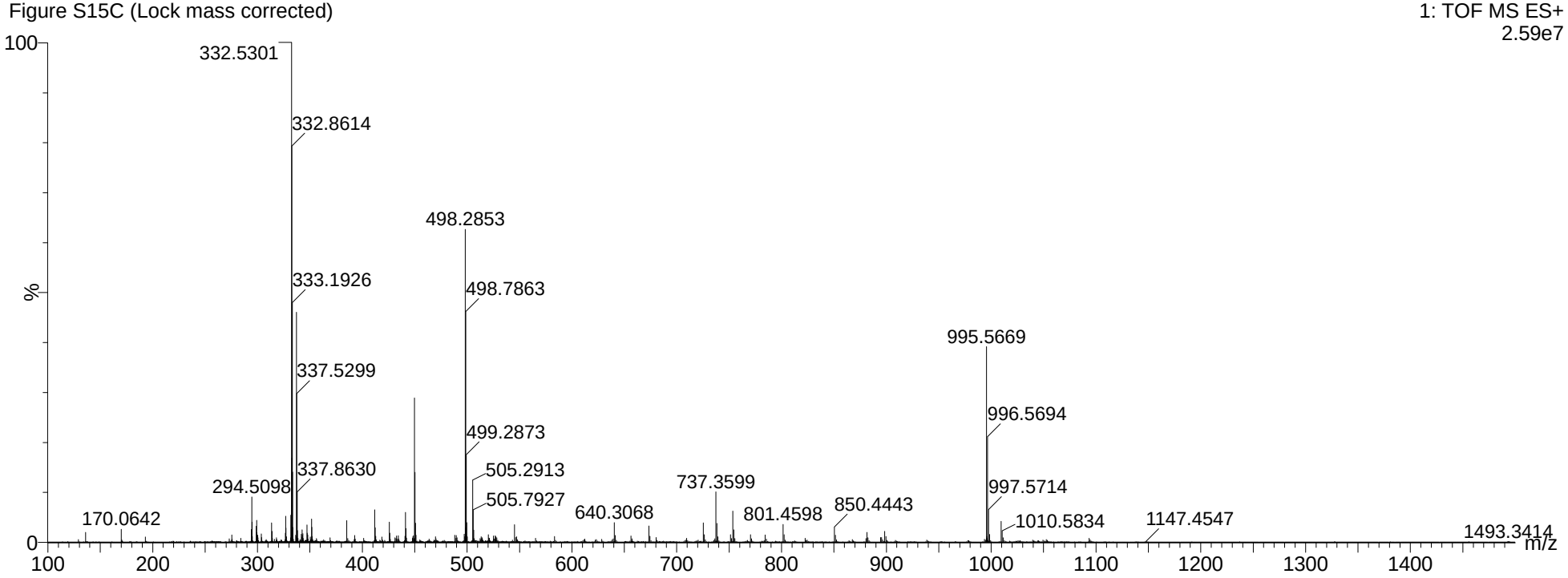


Figure 4B (Precursor, TOF transformed)

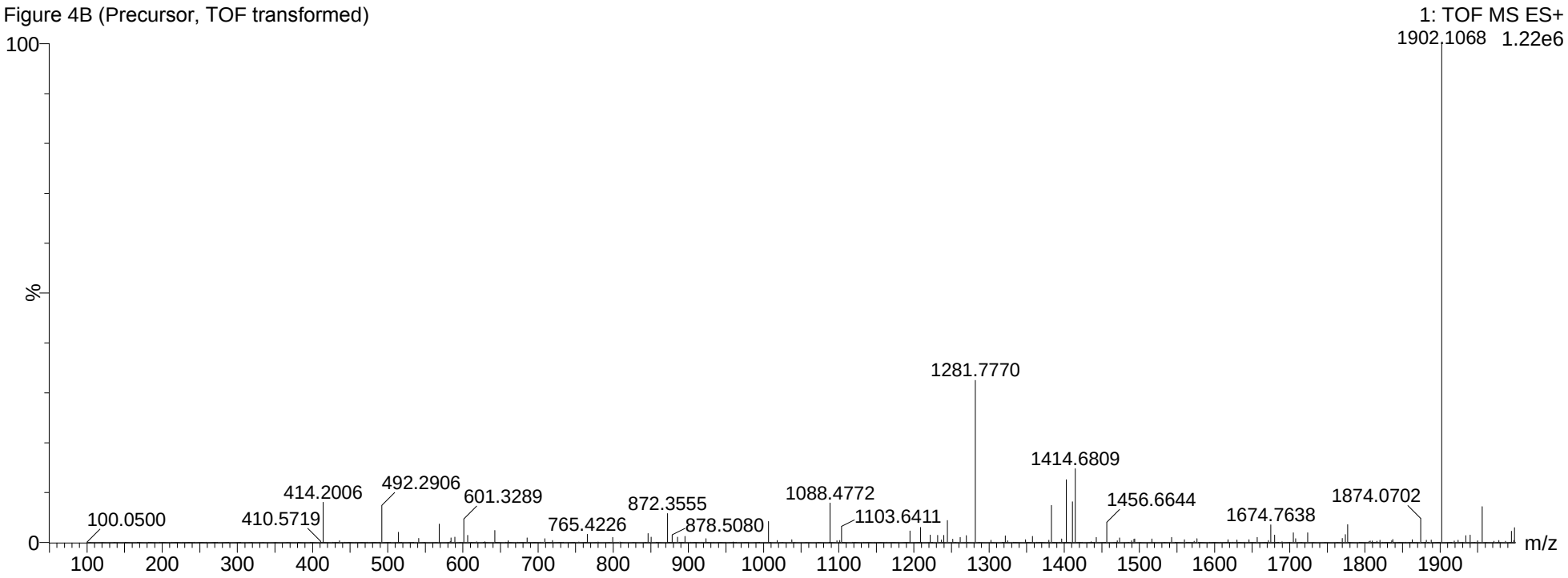


Figure 4B (Precursor, Lock mass corrected)

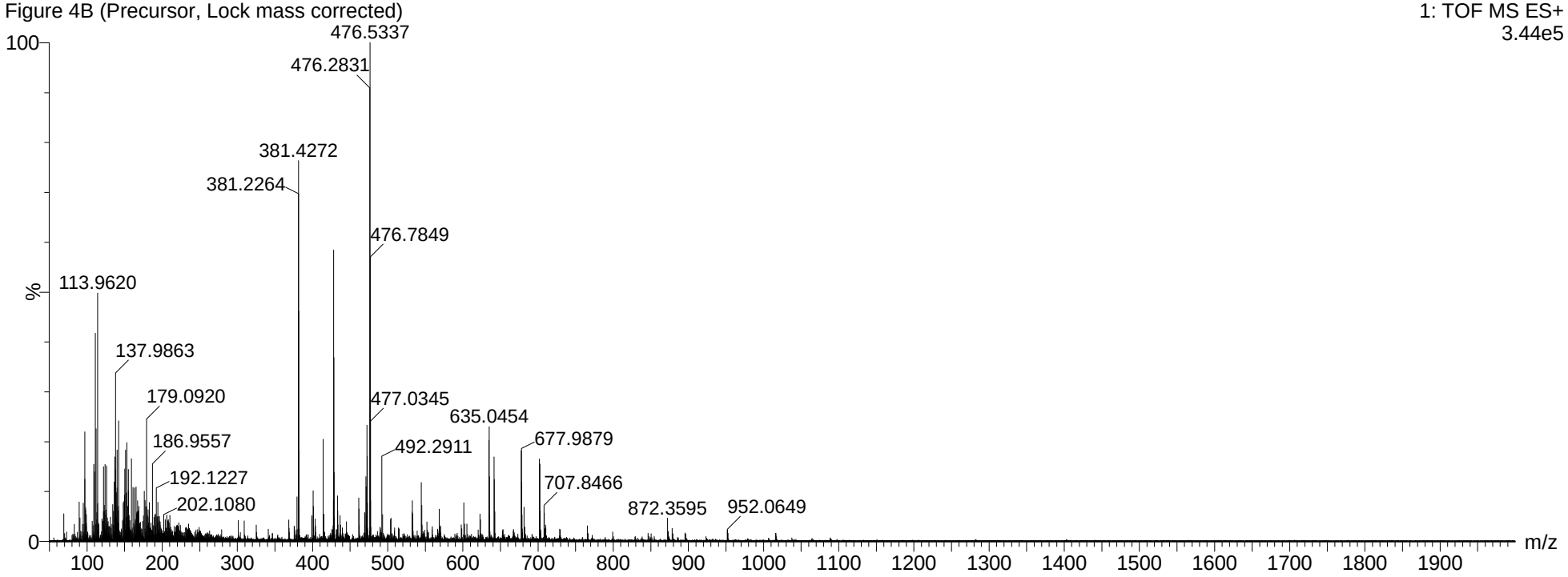


Figure 4B (TOF transformed)

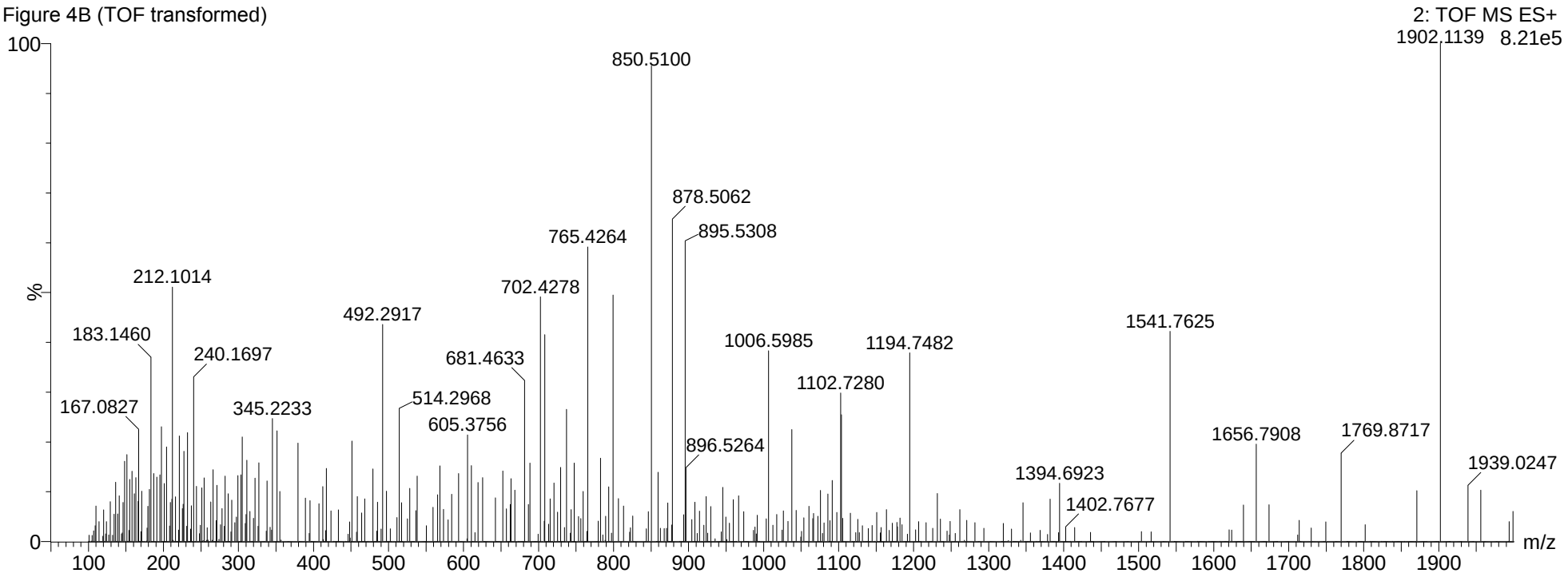


Figure 4B (Lock mass corrected)

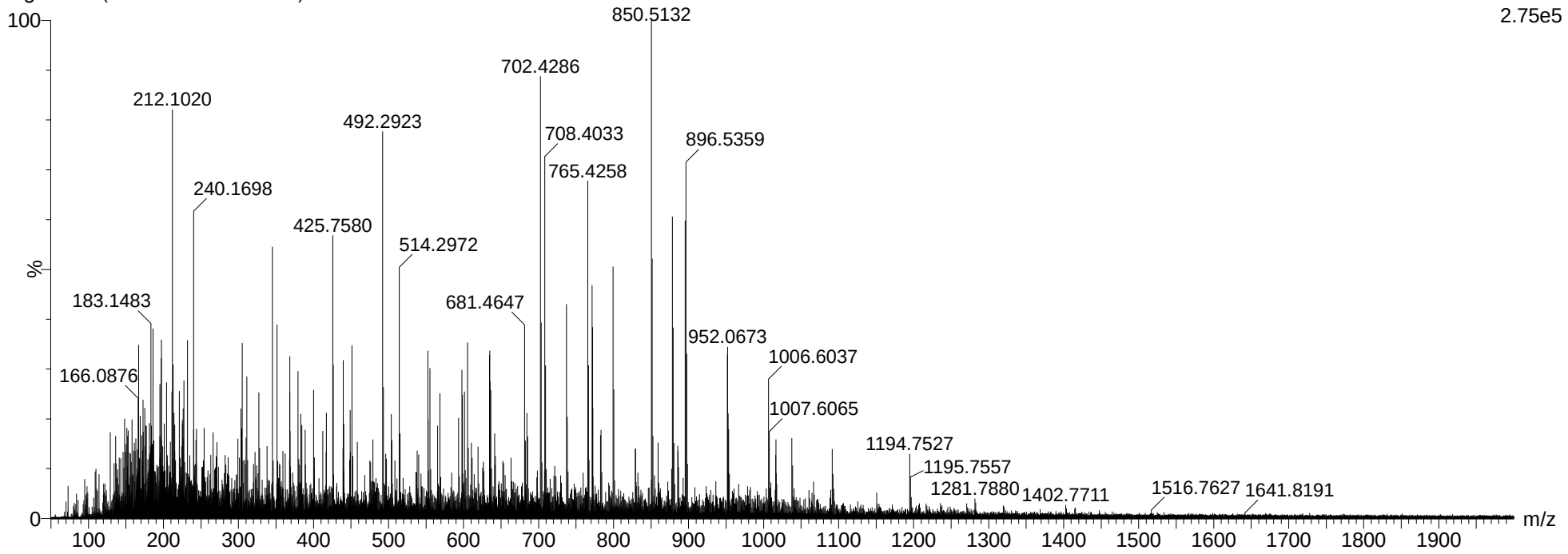


Figure 4C (Precursor, TOF transformed)

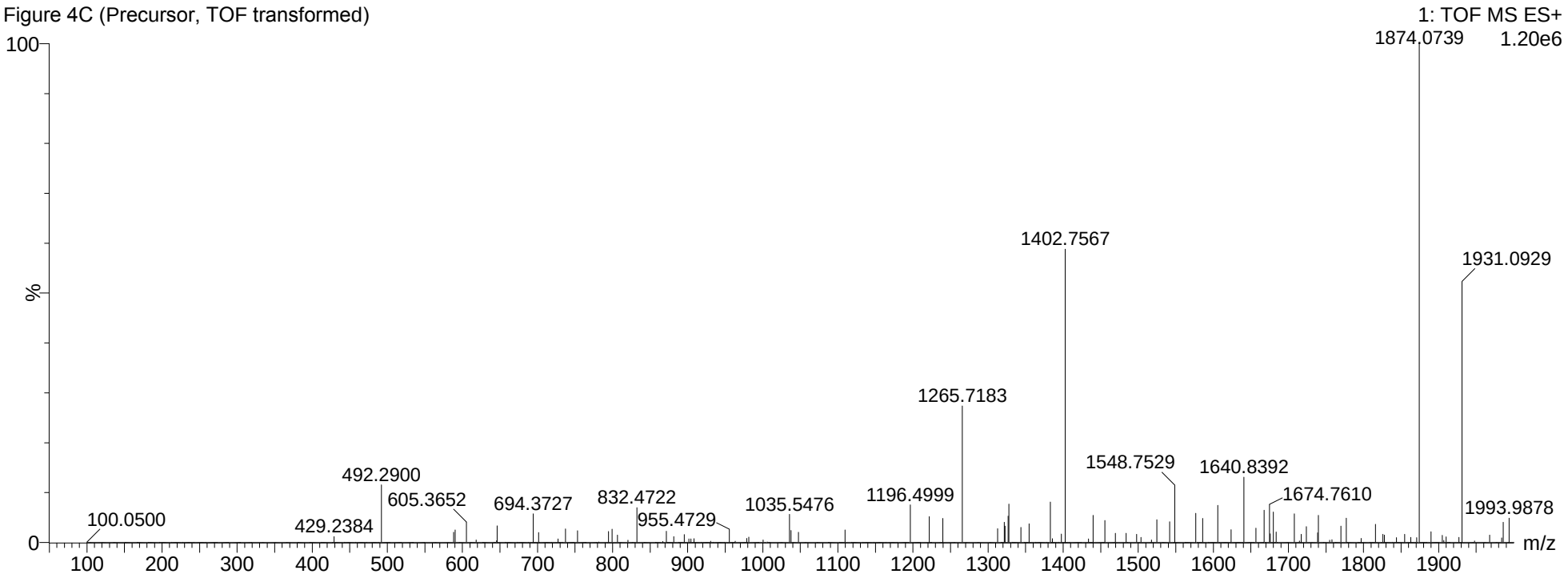


Figure 4C (Precursor, Lock mass corrected)

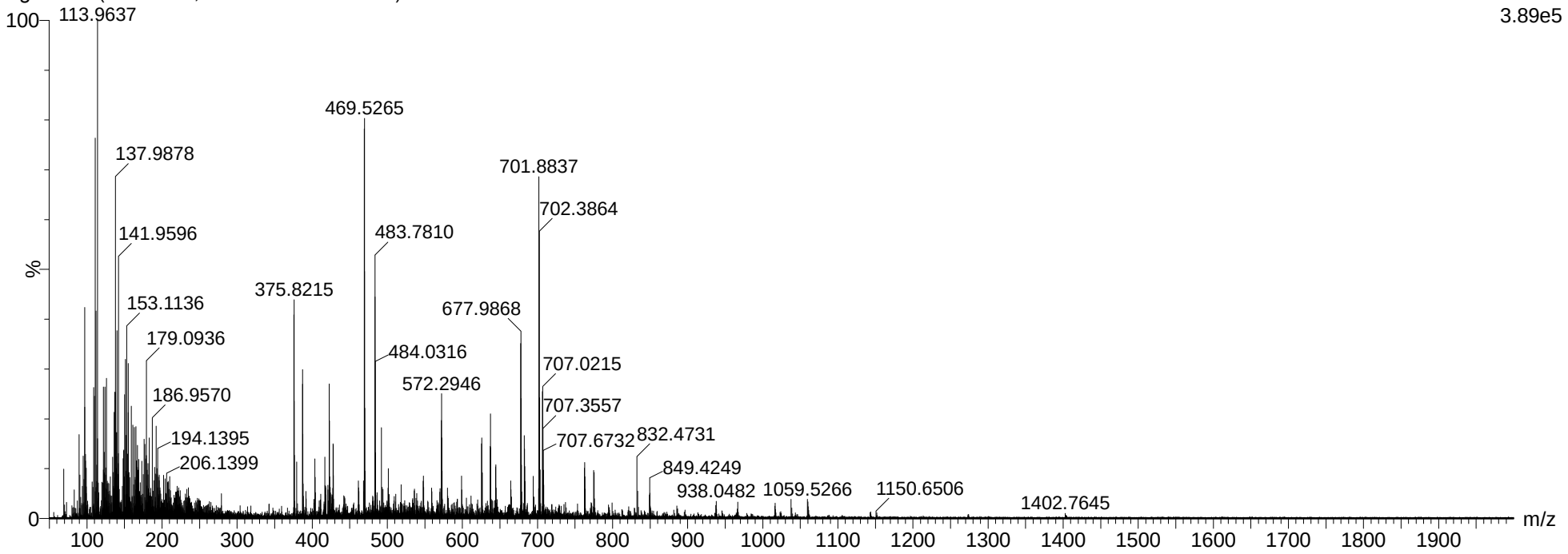


Figure 4C (TOF transformed)

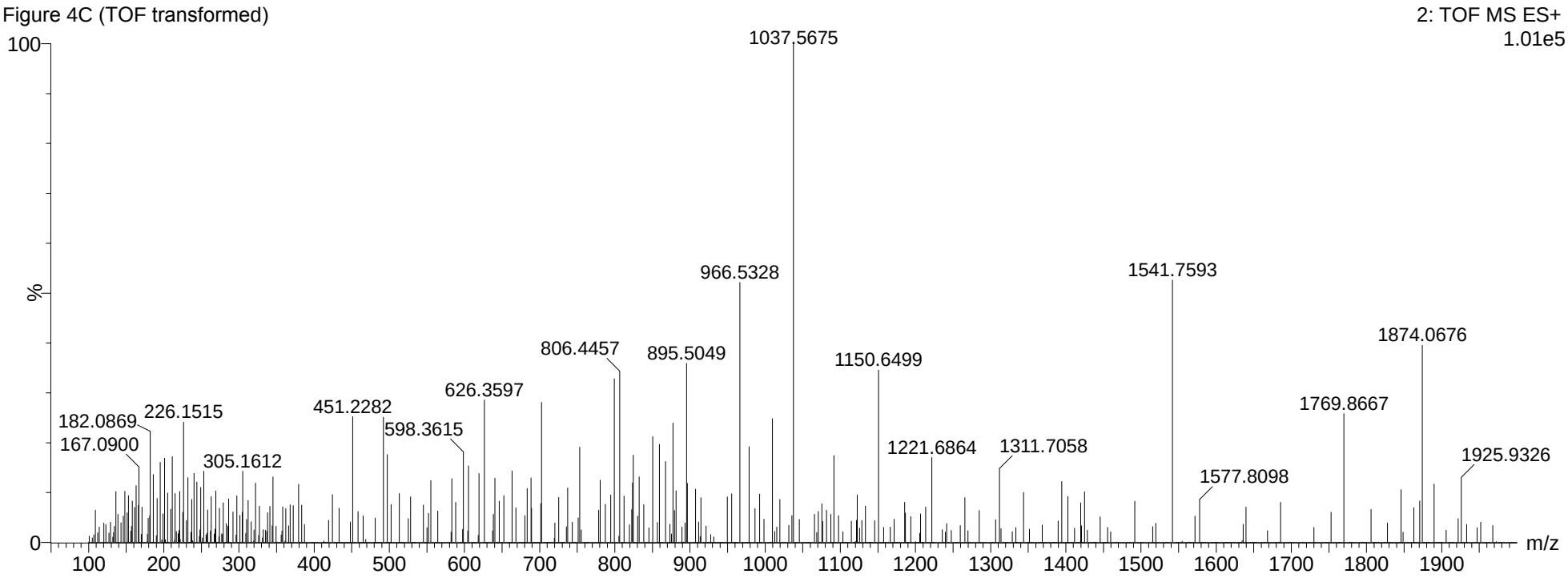


Figure 4C (Lock mass corrected)

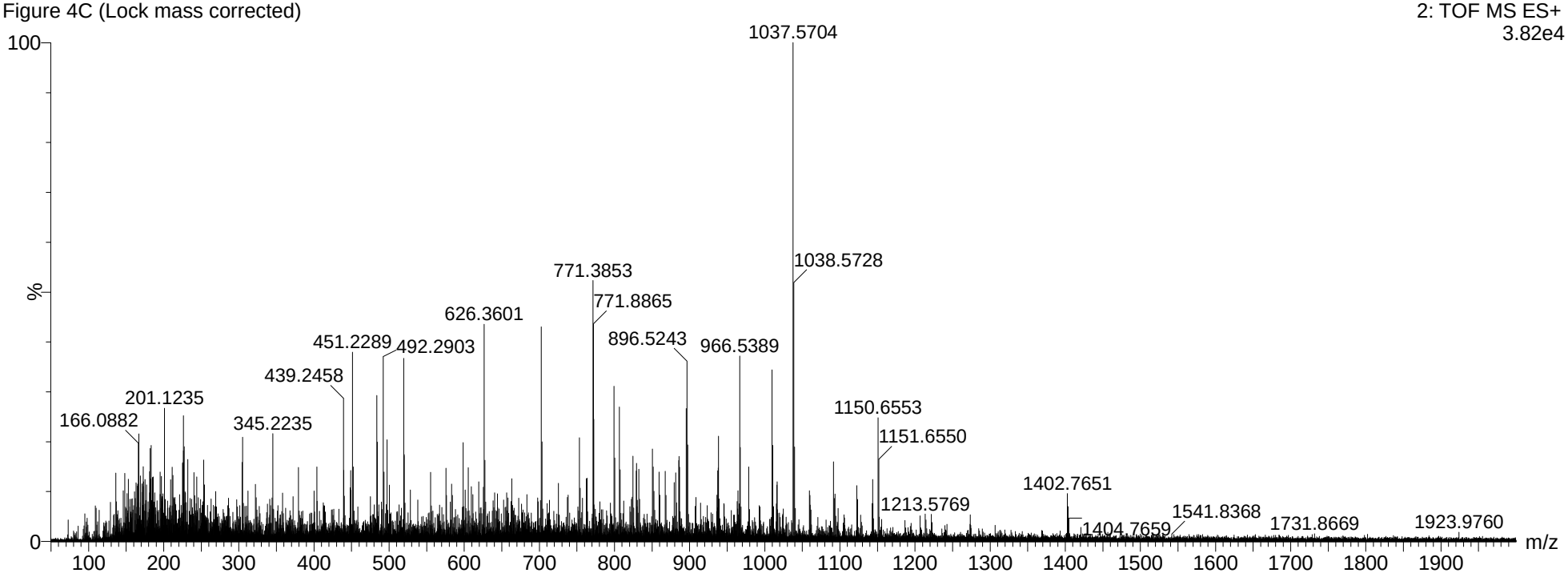


Figure S16 (Precursor, TOF transformed)

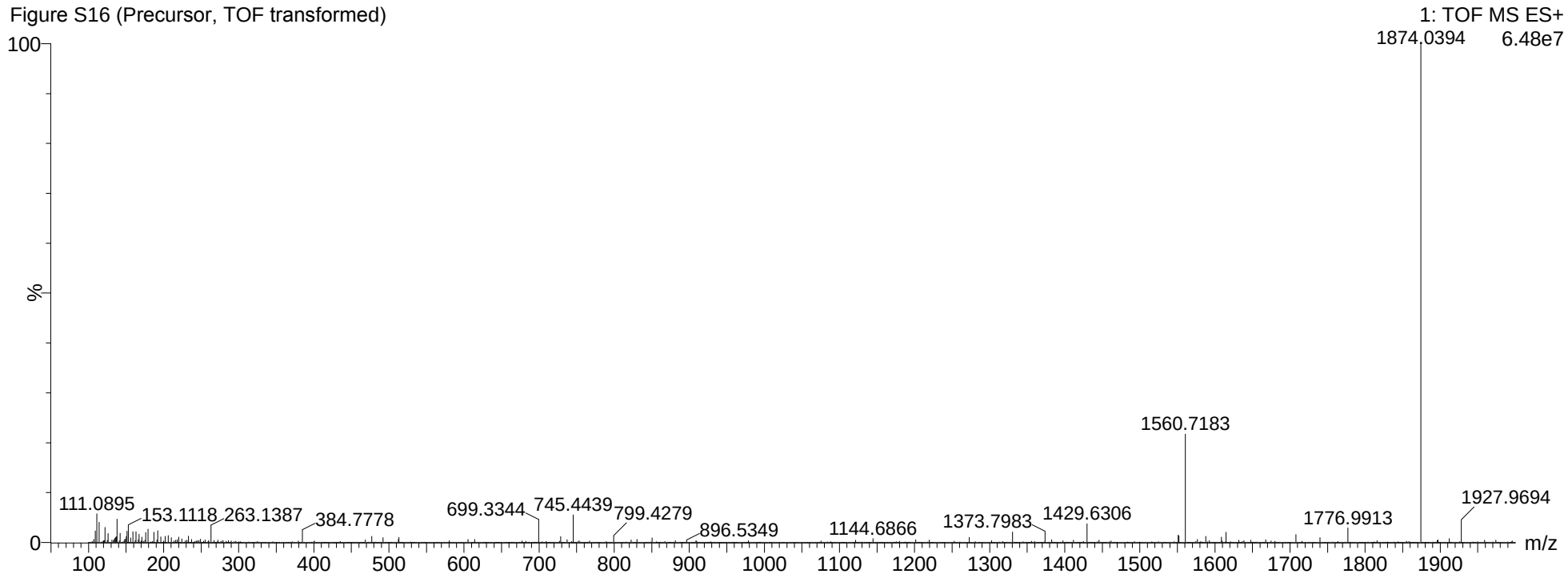


Figure S16 (Precursor, Lock mass corrected)

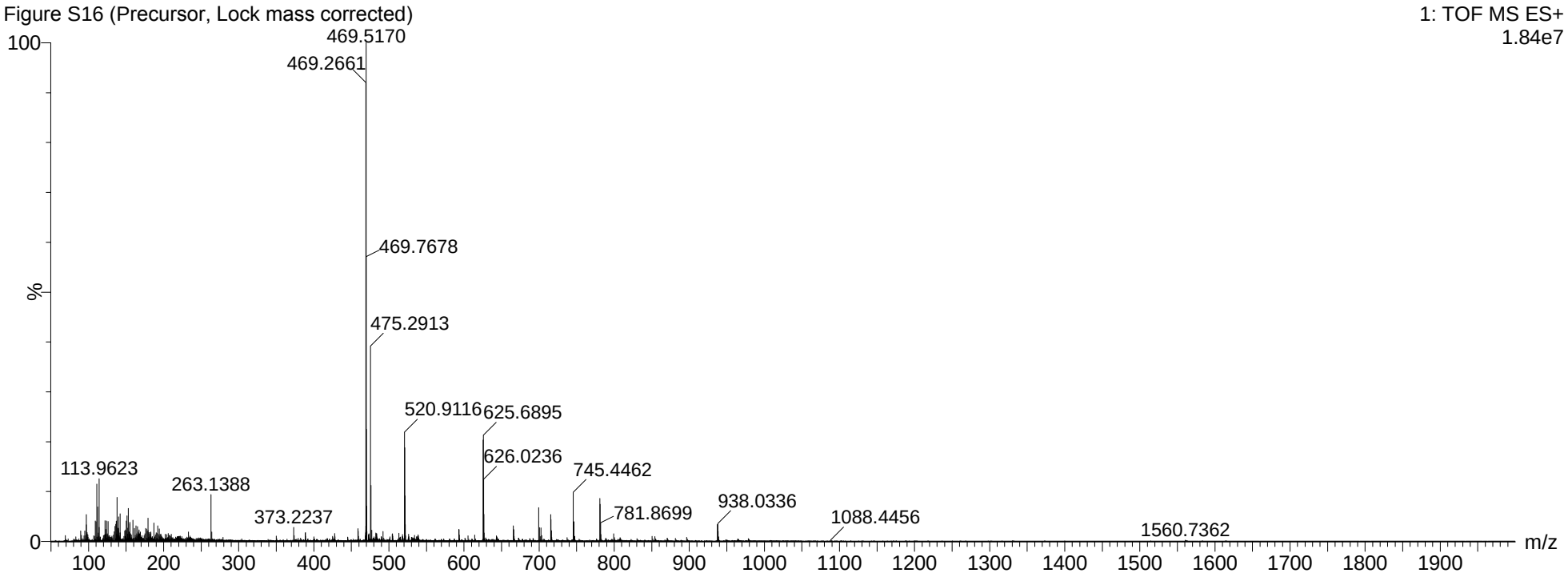


Figure S16 (TOF transformed)

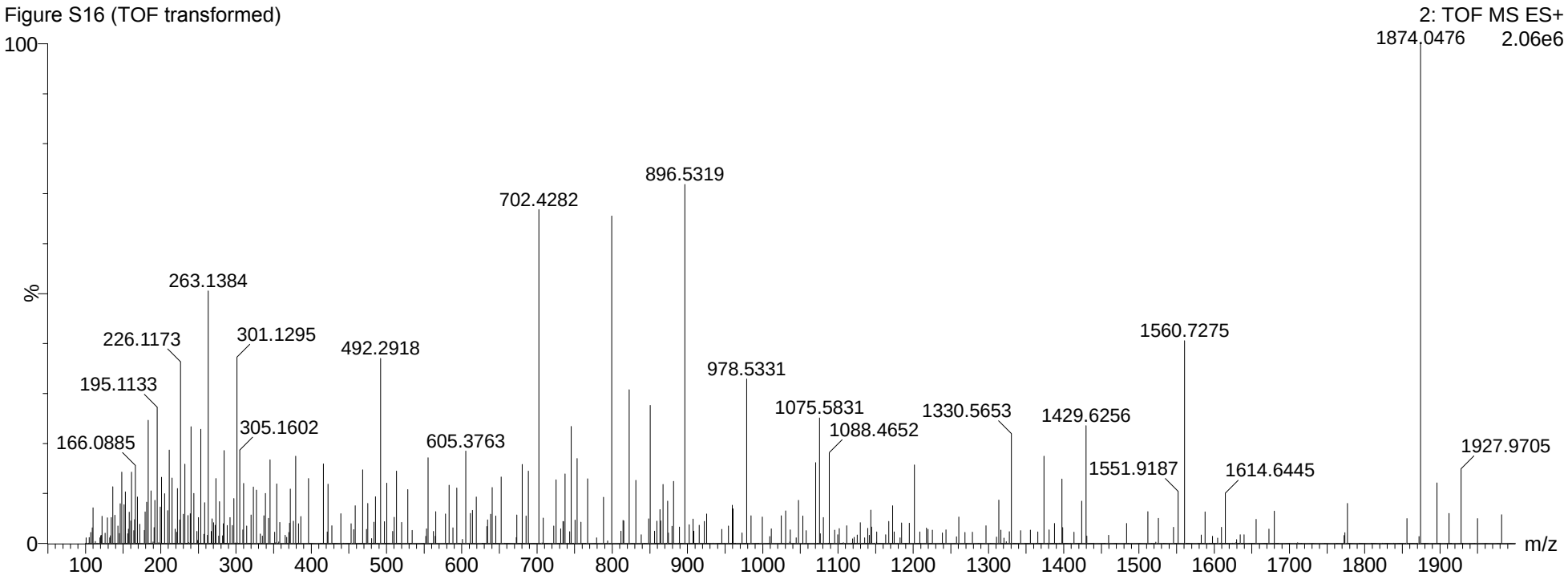


Figure S16 (Lock mass corrected)