Manipulation of Thiocillin Variants by Prepeptide Gene Replacement: Structure, Conformation, and Activity of Heterocycle Substitution Mutants

Albert A. Bowers, Michael G. Acker, Alexander Koglin, and Christopher T. Walsh*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115 and Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM 87544

Supporting Information

Contents:

1. Materials and General Methods

All molecular biology, recombinant DNA manipulation and microbiological assays were performed following the protocols of Sambrook *et al*.[1] Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich. Restriction enzymes and Quick Ligase were purchased from New England Biolabs (Boston, MA). Pfu Turbo DNA Polymerase was purchased from Invitrogen (Carlsbad, CA) and Paq 5000 DNA polymerase from Stratagene (La Jolla, CA).DNA oligonucleotide primers were synthesized by Integrated DNA technologies (Coralville, IA). PCR was performed on a Biorad MyCycler thermal cycler. DNA sequencing was performed by the Molecular Biology Core Facilities at the Dana Farber Cancer Institute (Boston, MA). Top10

chemically competent *E. coli* cells were purchased from Invitrogen. Restriction endonuclease cleanup and gel extraction of DNA fragments were performed with QiaQuick PCR cleanup kit from Qiagen. Recombinant plasmids were isolated using the QiaPrep Spin Miniprep Kit from Qiagen. *B. cereus* ATCC 14579 genomic DNA was isolated from cultures using the DNeasy Kit from Qiagen. Extraction of thiocillins from cell-free media was performed on Sep-Pak C18 cartidges from Waters Corp. (Milford, MA). Analytical RP-HPLC was performed on a Beckman System Gold (Beckman Coulter) instrument using a Phenomenex Luna 5 µm C18(2) 100 Å 250 x 4.6 mm column, monitoring eluent absorption at 220 and 350 nm. Preparative RP-HPLC was performed on a Beckman System Gold (Beckman Coulter) instrument using a Phenomenex Luna 10 µm C18(2) 100 Å 250 x 21.20 mm column. Purification of derivatives from crude extracts was also performed on a Biotage Isolera flash purification system (Biotage) using silica gel columns. ^{1}H NMR spectra were recorded on a Varian 600 MHz spectrometer. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance resulting from incomplete deuteration as the internal standard (CDCl3 δ 7.26, D2O δ 4.79, CD3OD δ 3.31). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants (Hz), and integration. Software and methods used for generation of solution phase structures from NMR data are described in the text.

2. Mutagenesis of *tclE* in pMGA-tclE-KI

As previously described,[2] plasmid pMGA-tclΔE-H was generated from plasmid pKM082[3], containing ampicillin and erythromycin resistance cassettes and employed in excision of copies of the thiocillin structural gene *via* double crossover homologous recombination. Additionally, a rescue plasmid, pMGA-tclE-KI was generated using pLW111[3] which already contains ~1 kb of homology to *tclD*. Mutants of *tclE* were generated by site-directed mutagenesis of plasmid pMGA-tclE-KI using overlapping primer extension. Briefly, homologous primers were designed each containing the mutation of interest flanked by 15-20 bps of homologous plasmid DNA sequence. PCR was performed with Pfu Turbo to extend the primers, generating entire circular plasmid strands, each containing the mutation of interest. Restriction endonuclease DpnI was then added to the reaction. DpnI selectively cleaves the methylated template plasmid, having been purified from bacterial cultures, while leaving intact the unmethylated mutant plasmid generated by PCR. The resulting mixture was transformed into chemically competent *E. coli* TOP10 cells and positive transformants were selected for on LB agar supplemented with 100 µg/mL ampicillin. Plasmid DNA was purified and mutants were confirmed by DNA sequencing. pMGA-tclE mutant plasmids were transformed into B. cereus and selected as described previously.[2]

SI Table 2.1. Oligonucleotides used for cloning and *tclE* mutagenesis

Oligo	Sequence	Role
Primer 193	5'-GCT TGA AAT TAT GGG AGC GGC ATG TAC GAC ATG CGT ATG TAC-3'	S1A mutagenesis
Primer 194	5'-GTA CAT ACG CAT GTC GTA CAT GCC GCT CCC ATA ATT TCA AGC-3'	S1A mutagenesis
Primer 160	5'-GCT TGA AAT TAT GGG AGC GTC AGCTAC GAC ATG CGT ATG TAC-3'	C2A mutagenesis
Primer 161	5'-GTA CAT ACG CAT GTC GTA GCT GAC GCT CCC ATA ATT TCA AGC-3'	C2A mutagenesis
Primer 209	5'-GAA ATT ATG GGA GCG TCA TGT GCG ACA TGC GTA TGT ACA TGC AG-3'	T3A mutagenesis
Primer 210	5'-CTG CAT GTA CAT ACG CAT GTC GCA CAT GAC GCT CCC ATA ATT	T3A mutagenesis

	TC-3’	
Primer 253	5’-ATT ATG GGA GCG TCA TGT ACG GCT TGC GTA TGT ACA TGC AGT TG-3’	T4A mutagenesis
Primer 254	5’-CAA CTG CAT GTA CAT ACG CAA GCC GTA CAT GAC GCT CCC ATA AT-3’	T4A mutagenesis
Primer 243	5'-GGA GCGTCA TGT ACG ACA GCC GTA TGT ACA TGC AGT TGT TG-3’	C5A mutagenesis
Primer 244	5'-CAA CAA CTG CAT GTA CAT ACG GCT GTC GTA CAT GAC GCT CC-3’	C5A mutagenesis
Primer 59	5’-GCG TCA TGT ACG ACA TGC GCT TGT ACA TGC AGT TGT TGT AC-3’	V6A mutagenesis
Primer 60	5’-GTA CAA CAA CTG CAT GTA CAA GCG CAT GTC GTA CAT GAC GC-3’	V6A mutagenesis
Primer 247	5'-CGT CAT GTA CGA CAT GCG TAG CTA CAT GCA GTT GTT GTA CAA C-3'	C7A mutagenesis
Primer 248	5'-GTT GTA CAA CAA CTG CAT GTA GCT ACG CAT GTC GTA CAT GAC G-3'	C7A mutagenesis
Primer 211	5’-CAT GTA CGA CAT GCG TAT GTG CAT GCA GTT GTT GTA CAA CTT G-3’	T8A mutagenesis
Primer 212	5’-CAA GTT GTA CAA CAA CTG CAT GCA CAT ACG CAT GTC GTA CAT G-3’	T8A mutagenesis
Primer 162	5’-CAT GTA CGA CAT GCG TAT GTA CAG CTA GTT GTT GTA CAA CTT G-3’	C9A mutagenesis
Primer 162	5’-CAA GTT GTA CAA CAA CTA GCT GTA CAT ACG CAT GTC GTA CAT G-3’	C9A mutagenesis
Primer 195	5’-GTA CGA CAT GCGTAT GTA CAT GCG CTT GTT GTA CAA CTT GAT TTT TC-3’	S10A mutagenesis
Primer 196	5’-GAA AAA TCA AGT TGT ACA ACA AGC GCA TGT ACA TAC GCATGT CGT AC-3’	S10A mutagenesis
Primer 177	5’-CGA CAT GCG TAT GTA CAT GCA GTG CTT GTA CAA CTT GAT TTT TC-3’	C11A mutagenesis
Primer 178	5’-GAA AAA TCA AGT TGT ACA AGC ACT GCA TGT ACA TAC GCA TGT CG-3’	C11A mutagenesis
Primer 166	5'-CAT GCG TAT GTA CAT GCA GTT GTG CTA CAA CTT GAT TTT TCA AG-3'	C12A mutagenesis
Primer 167	5'-CTT GAA AAA TCA AGT TGT AGC ACA ACT GCA TGT ACA TAC GCA TG-3'	C12A mutagenesis
Primer 189	5’-GTA TGT ACA TGC AGT TGT TGT GCA ACT TGA TTT TTC AAG AAG C-3’	T13A mutagenesis
Primer 190	5’-GCT TCT TGA AAA ATC AAG TTG CAC AAC AAC TGC ATG TAC ATA C-3’	T13A mutagenesis
Primer 255	5’-CAT GCA GTT GTT GTA CAG CTT GAT TTT TCA AGA AGC TTA ATT G-3’	T14A mutagenesis
Primer 256	5’-CAA TTA AGC TTC TTG AAA AAT CAA GCT GTA CAA CAA CTG CAT G-3’	T14A mutagenesis
Primer 207	5’-GCT TGA AAT TAT GGG AGC GTC ATC AAC GAC ATG CGT ATG TAC-3’	C2S mutagenesis
Primer 208	5’-GTA CAT ACG CAT GTC GTT GAT GAC GCT CCC ATA ATT TCA AGC-3’	C2S mutagenesis
Primer 51	5’-ATT ATG GGA GCG TCA TGT AGT ACA TGC GTA TGT ACA TGC-3’	T3S mutagenesis
Primer 52	5’-GCA TGT ACA TAC GCA TGT ACT ACA TGA CGC TCC CAT AAT-3’	T3S mutagenesis
Primer 245	5’-GGA GCG TCA TGT ACG ACA TCC GTA TGT ACA TGC AGT TGT TG-3’	C5S mutagenesis
Primer 246	5’-CAA CAA CTG CAT GTA CAT ACG GAT GTC GTA CAT GAC GCT CC-3’	C5S mutagenesis
Primer 249	5’-CGT CAT GTA CGA CAT GCGTAT CTA CAT GCA GTT GTT GTA CAA C-3’	C7S mutagenesis

Primer 250	5'-GTT GTA CAA CAA CTG CAT GTA GAT ACG CAT GTC GTA CAT GAC G-3'	C7S mutagenesis
Primer 181	5'-CAT GTA CGA CAT GCG TAT GTA CAA GTA GTT GTT GTA CAA CTT G-3'	C9S mutagenesis
Primer 182	5'-CAA GTT GTA CAA CAA CTA CTT GTA CAT ACG CAT GTC GTA CAT G-3'	C9S mutagenesis
Primer 183	5'-CGA CAT GCG TAT GTA CAT GCA GTA GTT GTA CAA CTT GAT TTT TC-3'	C11S mutagenesis
Primer 184	5'-GAA AAA TCA AGT TGT ACA ACT ACT GCA TGT ACA TAC GCA TGT CG-3'	C11S mutagenesis
Primer 185	5'-CAT GCG TAT GTA CAT GCA GTT GTA GTA CAA CTT GAT TTT TCA AG-3'	C12S mutagenesis
Primer 186	5'-CTT GAA AAA TCA AGT TGT ACT ACA ACT GCA TGT ACA TAC GCA TG-3'	C12S mutagenesis

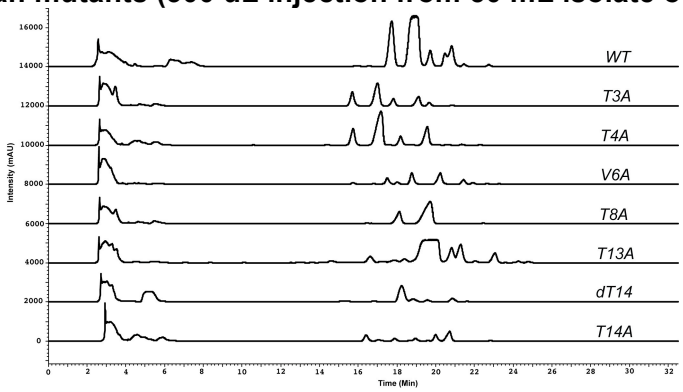
3. Extraction of Thiocillin Compounds

WT or *tclE* mutant *B. cereus* starter cultures (5 mL) were grown in LB for 20 hours at 30 °C. Larger cultures (0.5 L LB in 2 L culture baffles culture flasks) were inoculated with 300 µL of starter culture and grown for 68 hours at 30 °C with shaking at 200 rpm. (*tclE* mutant strains were grown in media supplemented with 1µg/mL erythromycin and 25µg/mL lincomycin.) Cultures were harvested and both the cell pellet and spent media were saved. To the pellet, 50 mL methanol was added along with 15 g sodium sulfate. The mixture was vortexed vigorously and allowed to sit for at least 10 minutes. The mixture was then filtered through Whatman filter paper (no. 1) and the methanol was removed by vacuum. Solid was solubilized in 10 mL 33% acetonitrile in water for HPLC analysis. *tclE* mutants that produced compound at low levels were grown in a 5L fermenter in ECPM1 media lacking glycerol (20 g N-Z amine; 3 g Yeast Extract; 1 g KH_2PO_4; 4 g K_2HPO_4; 1 g NH_4Cl; 2.4g K_2SO_4 in 1 L supplemented with 10 mL 100X Trace Elements (5 g EDTA; 0.5 g $FeCl_3$•$6H_2O$; 0.05 g ZnO; 0.01 g $CuCl_2$•$2H_20$; 0.01 g $Co(NO_3)_2$•6H2O; 0.01 g $(NH_4)_6$ Mo_7O_{24} in 1 L) and 2 mL of 500X Mg/Ca solution (203 g $MgCl_2$; 66.2 g $CaCl_2$ in 1 L). Cells and media were harvested after 24 hours and extraction was performed as detailed above, scaled accordingly.

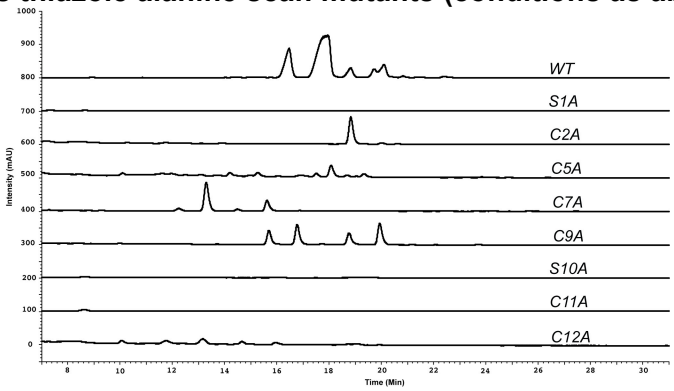
Further purification was accomplished by ethyl acetate extraction. Solvents were removed from the crude compound extracts on a rotary evaporator. The crude residue was then dissolved in 40 mL of 1:1 EtOAc: water. The biphasic solution was transferred to a 60mL separatory funnel, shaken and the organic layer removed. The aqueous layer was washed with a further 20 mL of EtOAc and the combined organics were dried over Na_2SO_4, filtered through a 60 mL coarse fritted glass funnel, and evaporated to dryness. For purposes of assessing the thiocillin content of the individual layers, the residue from the organic layer was redissolved in 10 mL of acetonitrile. 180 µL of the acetonitrile solution was combined with 180 µL of water and 300 µL of this solution was injected onto the analytical HPLC. 300 µL of the aqueous layer was also injected, being careful to avoid the surface organics retained from the extraction.

Additional compound was extracted from the cell free media. The cell free media was passed over a Sep-Pak C18 column (Waters) and material was sequentially eluted with 10 mL of 20%, 50% and 100% acetonitrile in water. Derivatives commonly eluted in 50% acetonitrile. All compounds were finally purified by silica gel chromatography (Biotage, eluate: 95:5% CH_2Cl_2:MeOH) for use in disk diffusion and liquid culture assays. Compounds were eluted as mixtures of the tailored states and used as such. Compounds characterized by NMR were further separated by RP-HPLC.

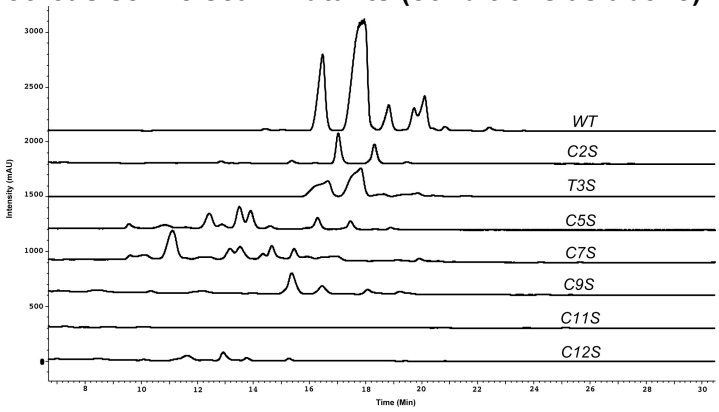
SI Figure 3.1. HPLC traces (350 nm) of methanolic extracts from 3 day growths of *B. cereus* alanine scan mutants (300 uL injection from 50 mL isolate of 500 mL culture).



SI Figure 3.2. HPLC traces (350 nm) of methanolic extracts from 3 day growths of *B. cereus* thiazole alanine scan mutants (conditions as above).



SI Figure 3.3. HPLC traces (350 nm) of methanolic extracts from 3 day growths of *B. cereus* serine scan mutants (conditions as above).



4. LC-MS and MS/MS Analysis.

High-resolution LC-MS data was collected in positive ion mode, on an Agilent 6520 Accurate-Mass Q-TOF Mass Spectrometer fitted with an electrospray ionization (ESI) source. The capillary voltage was set to 3500 kV, and the fragmentor voltage at 250 V. The drying gas temperature was maintained at 350°C with a flow rate of 12 L/min and a nebulizer pressure of 45 psi. Separation was effected on a Gemini-NX C18 reverse phase column (5µm, 110A, 2.0 x 50 mm, Phenomonex) for crude mixtures and a Kinetex C18 reverse phase column (2.6µm, 100A, 2.10 x 50 mm, Phenomonex) for chromatographically pure samples. Compounds were

eluted in a gradient of solvents A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile): 2 min. isocratic 2%B, then increasing to 100%B over 10 min., and finally isocratic at 100%B for 2 min. before returning to 2%B and reequilibrating over 4 min. The order of elution relative to tailored states of the final products was conserved across variants, except where the short gradient created elution overlap. At least two analytical runs were performed for extracts from each mutant: crude extract was used in the first run in order to better search for the presence of trace quantities of all tailored states and purified compounds were examined in a second run to obtain high resolution masses with lower ppm error than those observed in the crude runs. Additional structural analysis was accomplished by targeted CID-MS/MS. For all samples examined, the collision energy was varied between 40 and 65 eV, with optimum fragmentation generally being observed at 45 eV. Representative spectra are illustrated below. Essential diagnostic peaks have been labeled.

5. Determination of Minimum Inhibitory Concentrations (MICs)

Over night cultures of the individual strains (MRSA strains COL and MW2 grown in TSB media and *B. subtilis* strain 168 in LB at 37 °C) were diluted 1000-fold and used to fill 96-well plates (150 μL per well). Serial dilutions of the variant mixtures (1.5 μL of 800-6.25 μg/μL solutions in DMSO) were transferred from library plates to the culture plates. For each variant mixture one adjacent well was treated with 1.5 μL of unadulterated DMSO and one with 1.5 μL of erythromycin (10 μg/μL in 95% ethanol). The plates were incubated at 30 °C for 20 hours. The OD_{600} was read on a Perkin Elmer Envision plate reader. MICs were designated as the lowest concentration that produced an increase of less than 10% in OD over that of the adjacent erythromycin well.

Four individual WT thiocillin compounds isolated from *B. cereus* ATCC 14579 were subjected to MIC analysis against *B. subtilis* and two different strains of methicillin-resistant *Staphylococcus aureus* (MRSA). For each strain, all four compounds inhibited growth with similar MICs.[2]

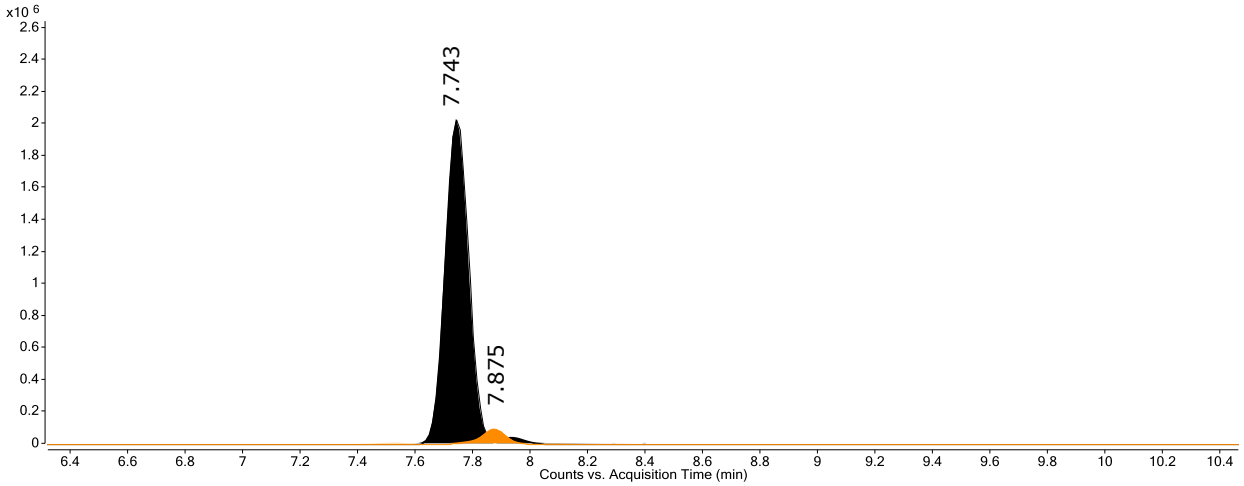
(1) Sambrook, J.; Fitsch, E. F.; Maniatis, T. *Molecular Cloning. A Laboratory Manual. 3rd ed*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2001.

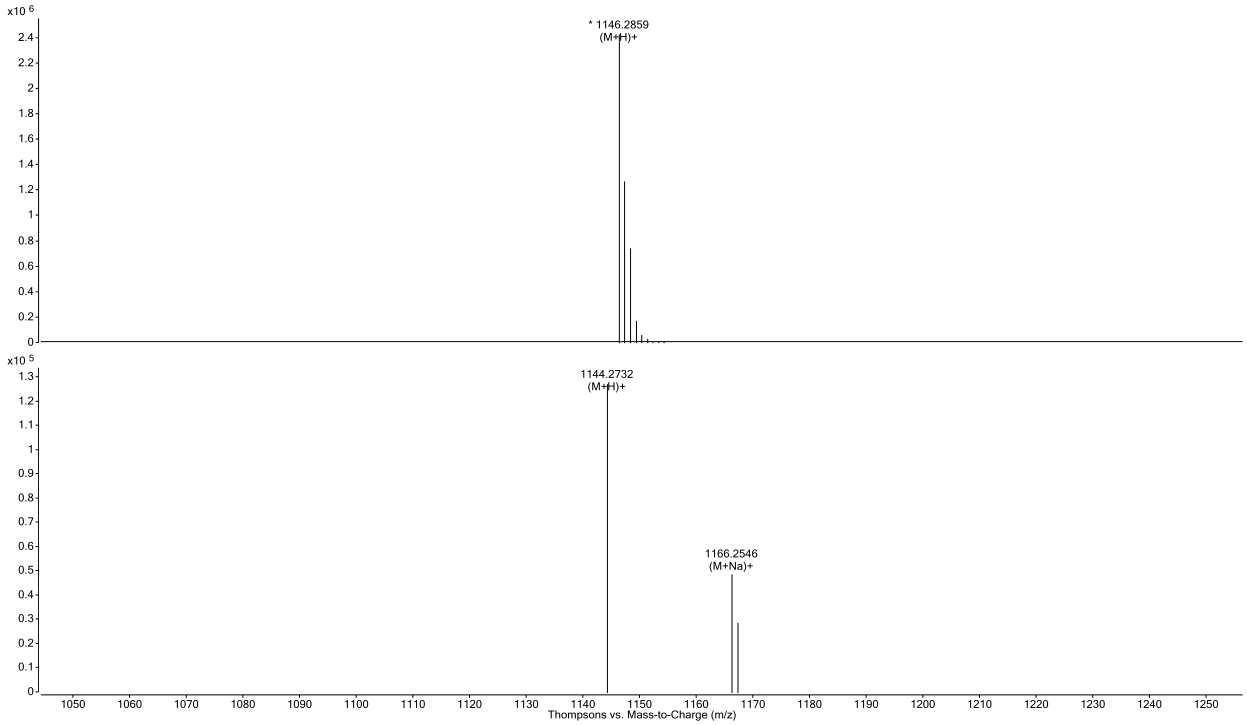
(2) Acker, M. G.; Bowers, A. A.; Walsh, C. T. *J. Am. Chem. Soc.* **2009**, *131*, 17563-17565.

(3) Brown, L. C.; Acker, M. G.; Clardy, J.; Walsh, C. T.; Fischbach, M. A. *Proc Natl Acad Sci U S A* **2009**, *106*, 2549-53.

MS Figure 1: C2A

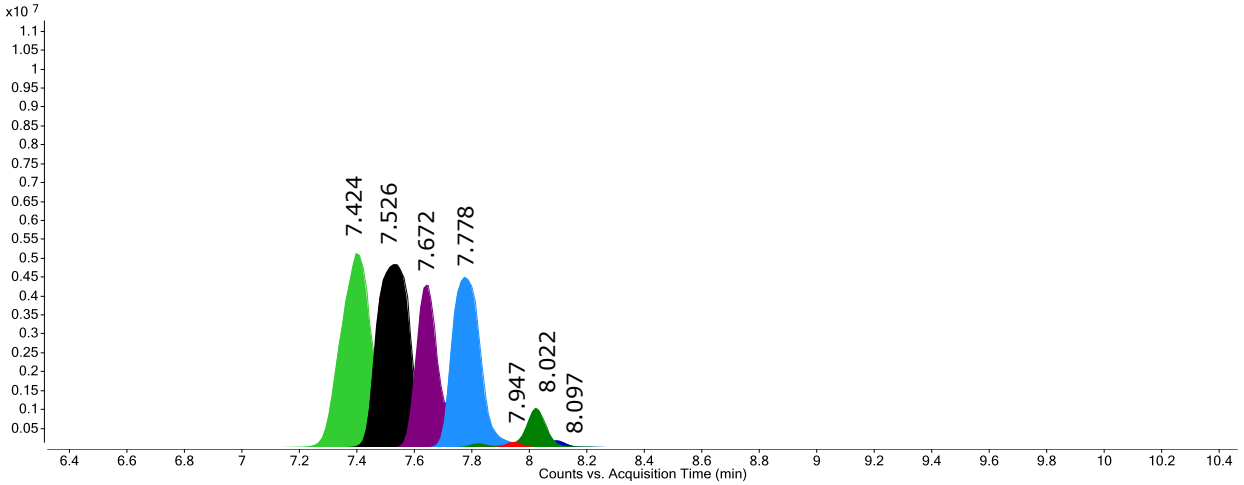
Tailoring (6,8,14)	RT	Molecular Ion	Observed Mass	Expected Mass	Error (ppm)	Area	Vol %
H, CH_3, red	7.743	1146.2859	1145.2787	1145.27984	1.00	2428295	23.81
H, CH_3, ox	7.875	1144.2732	1143.2658	1143.26419	-1.41	127437	0.77

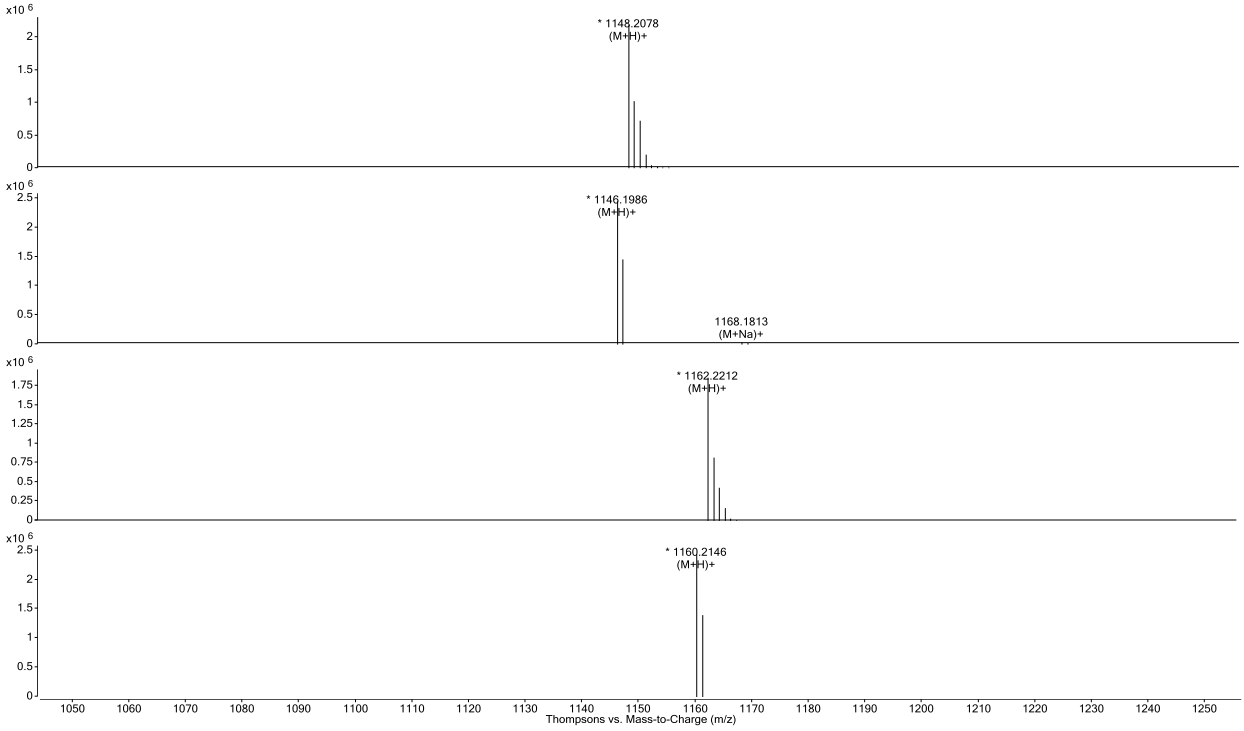




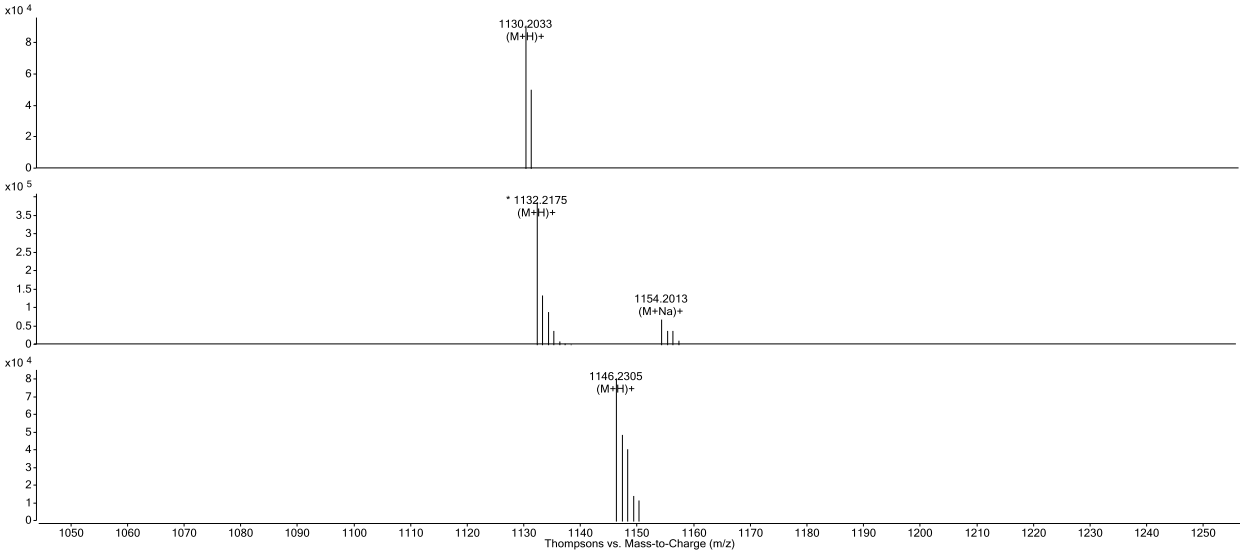
MS Figure 2a: T4A

Tailoring (6,8,14)	RT	Molecular Ion	Observed Mass	Expected Mass	Error (ppm)	Area	Vol %
OH, H, red	7.424	1148.2078	1147.2005	1147.205	3.92	2191662	31.71
OH, H, ox	7.526	1146.1986	1145.1913	1145.1893	-1.75	2456462	22.61
OH, CH_3, red	7.672	1162.2212	1161.214	1161.2206	5.68	1866423	21.06
OH, CH_3, ox	7.778	1160.2146	1159.2073	1159.205	-1.98	2451287	17.81
H, H, ox	7.947	1130.2033	1129.196	1129.1944	-1.42	91346	0.38
H, H, red	8.022	1132.2175	1131.2105	1131.21	-0.44	389584	3.33
H, CH_3, red	8.097	1146.2305	1145.2232	1145.2257	2.18	81256	0.55



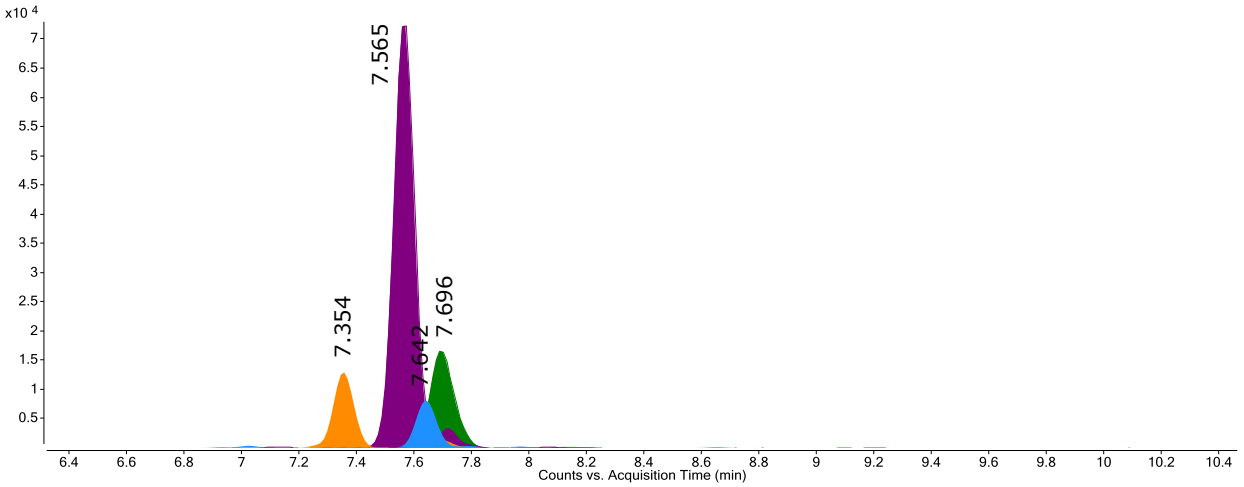


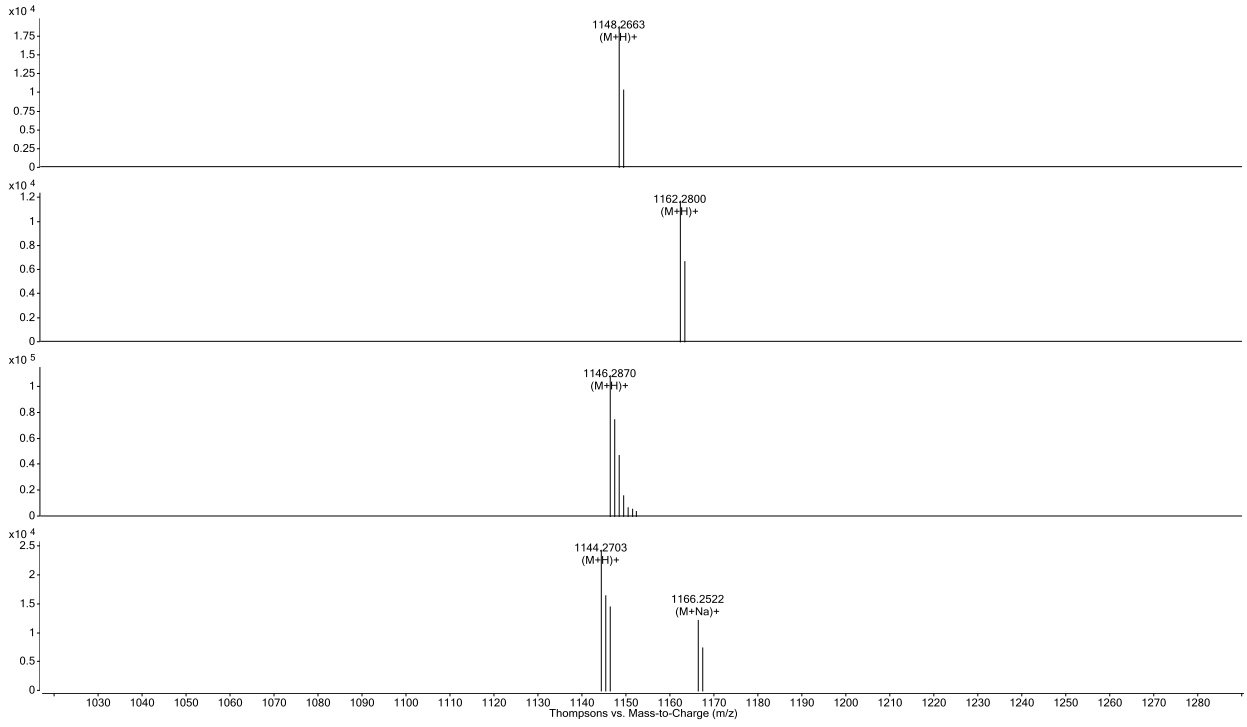
MS Figure 2b: T4A



MS Figure 3: C5A

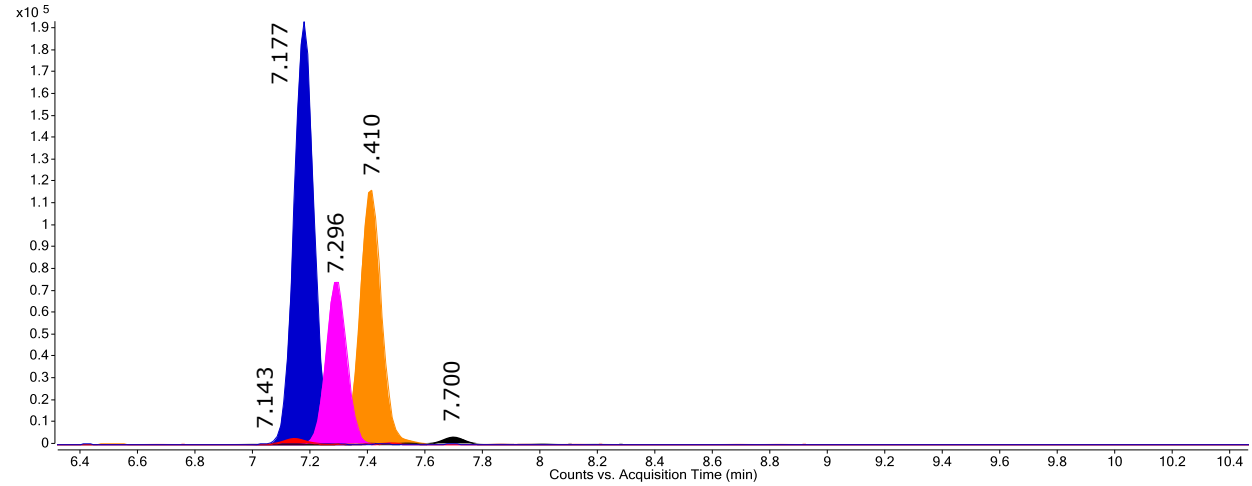
Tailoring (6,8,14)	RT	Molecular Ion	Observed Mass	Expected Mass	Error (ppm)	Area	Vol %
OH, H, red	7.354	1148.2663	1147.258	1147.2591	0.96	18948	4.15
H, CH_3, red	7.565	1146.287	1145.2797	1145.27984	0.12	109434	40.1
OH, CH_3, red	7.642	1162.28	1161.2761	1161.27475	-1.16	11810	2.55
H, CH_3, ox	7.696	1144.2703	1143.263	1143.26419	1.04	24595	15.41

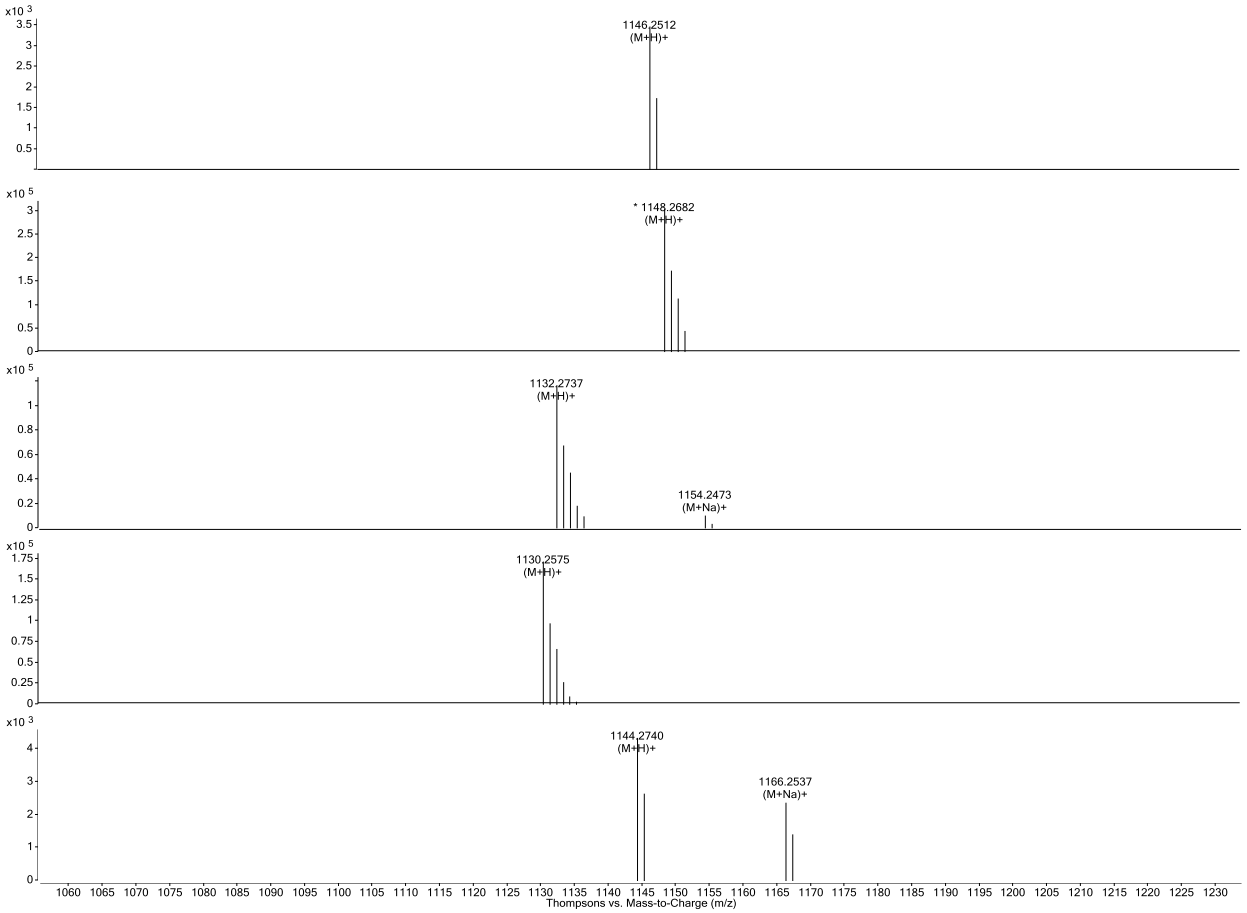




MS Figure 4: C7A

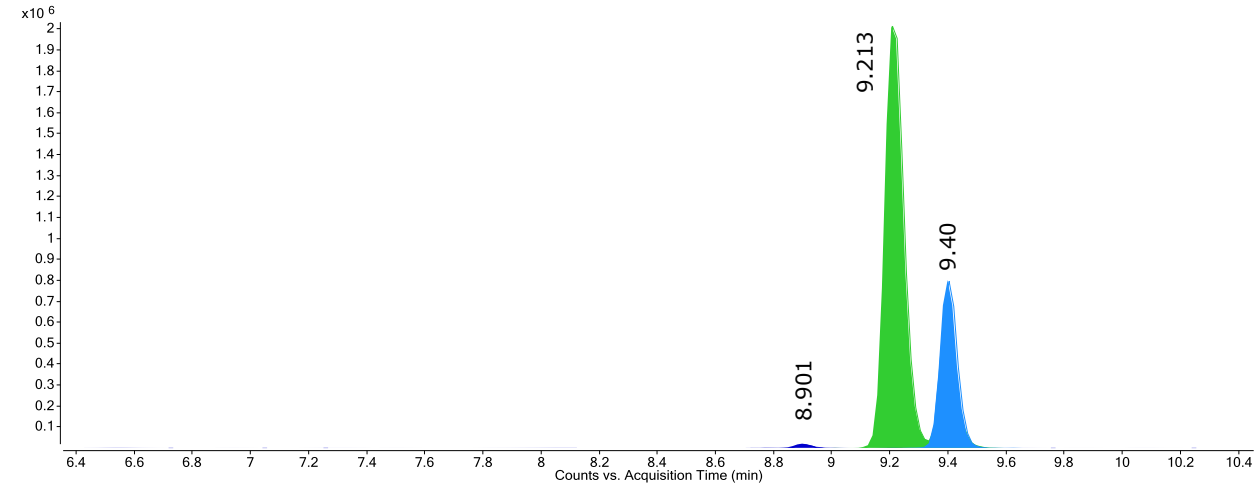
Tailoring (6,8,14)	RT	Molecular Ion	Observed Mass	Expected Mass	Error (ppm)	Area	Vol %
OH, H, ox	7.143	1146.2512	1145.245	1145.24345	-1.35	3480	0.47
OH, H, red	7.177	1148.2682	1147.2609	1147.2591	-1.57	304950	37.41
H, H, red	7.296	1132.2737	1131.2644	1131.26419	-0.19	117311	16.95
H, H, ox	7.41	1130.2575	1129.2494	1129.24854	-0.76	172360	24.49
H, CH_3, ox	7.7	1144.274	1143.2649	1143.26419	-0.62	4364	0.84

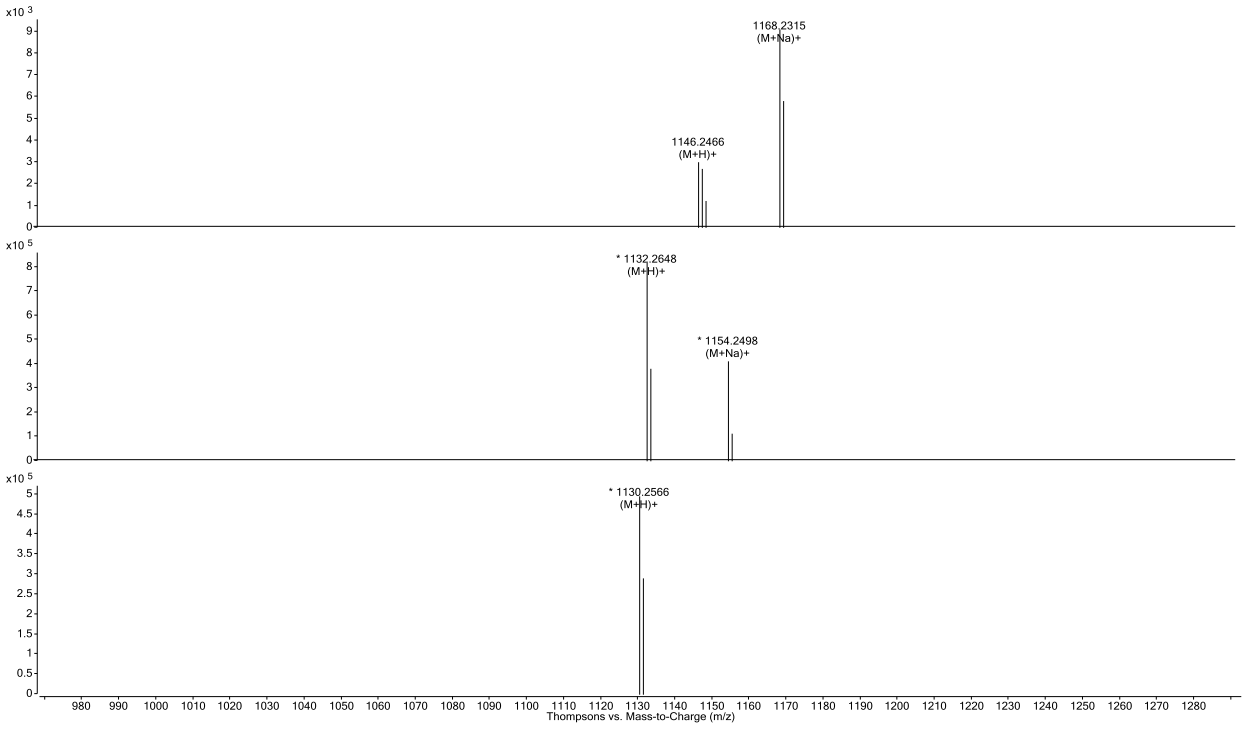




MS Figure 5: C9A

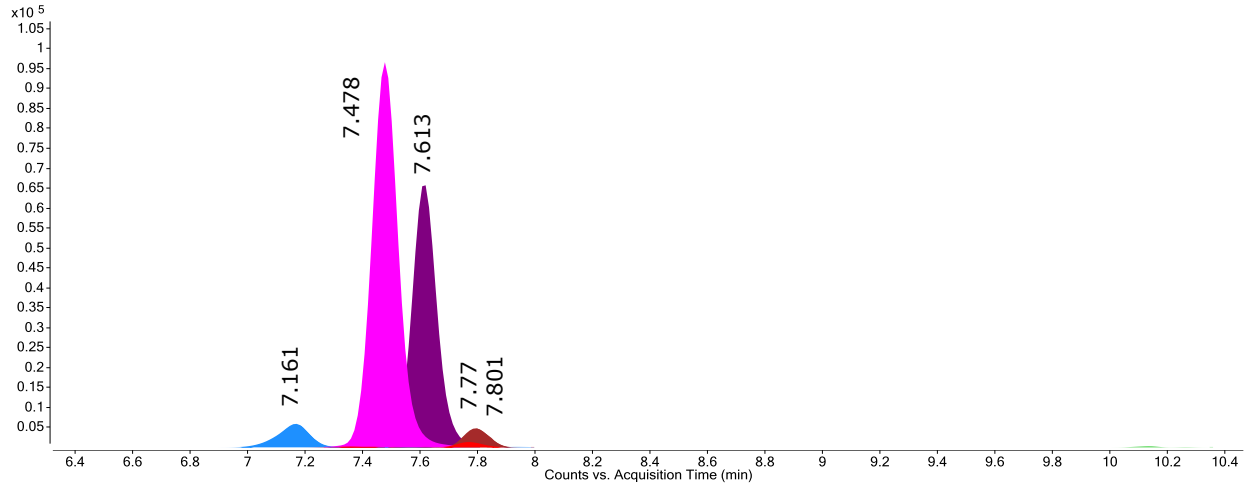
File	RT	Base Peak	Mass	Algorithm	Hits (DB)	Height	Vol %
OH, H, ox	8.901	1168.2315	1145.2418	1145.24345	1.44	9080	0.43
H, H, red	9.213	1132.2648	1131.2605	1131.26419	3.26	816717	51.5
H, H, ox	9.4	1130.2566	1129.2491	1129.24854	-0.50	495469	15.07

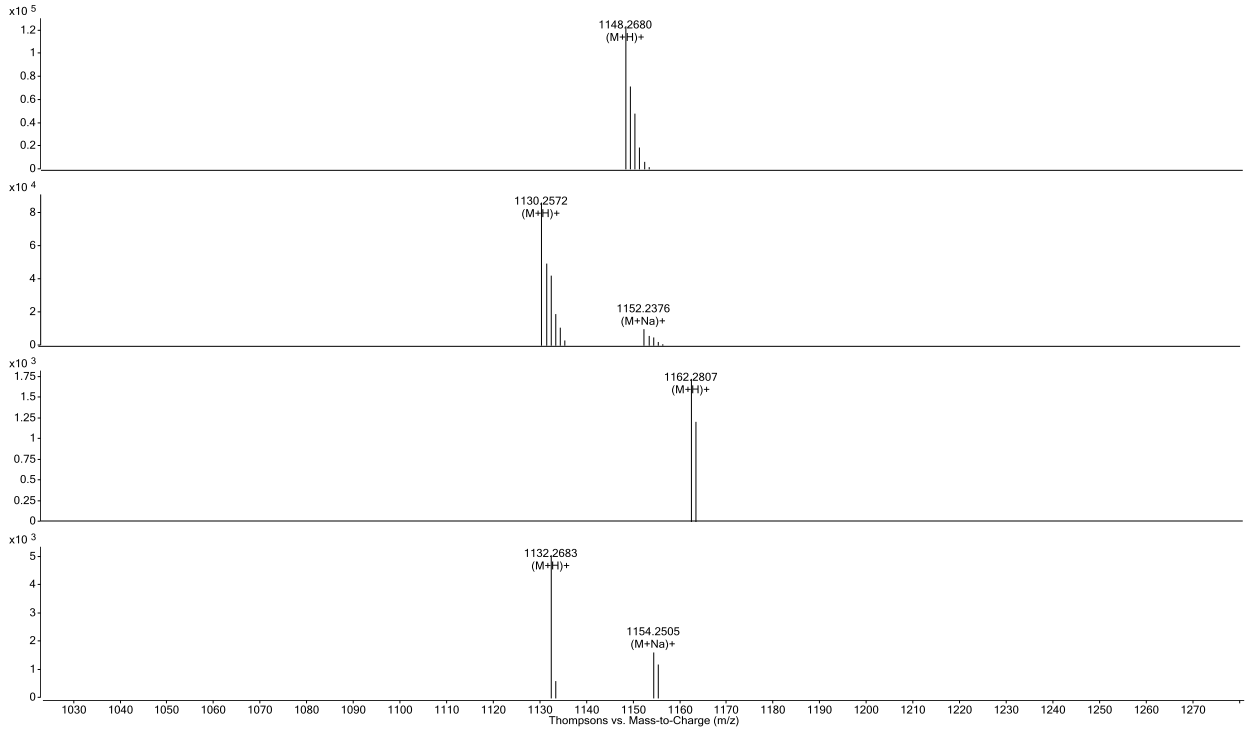




MS Figure 6: C12A

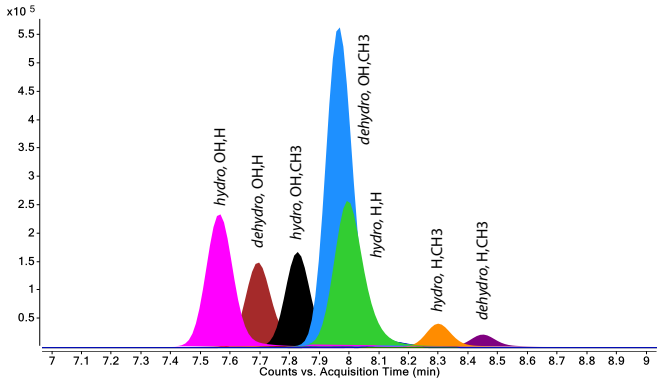
Tailoring (6,8,14)	RT	Molecular Ion	Observed Mass	Expected Mass	Error (ppm)	Area	Vol %
OH, H, ox	7.161	1146.2499	1145.2439	1145.24345	-0.39	7441	4.74
OH, H, red	7.478	1148.268	1147.2598	1147.2591	-0.61	123918	46.87
H, H, ox	7.613	1130.2572	1129.2496	1129.24854	-0.94	86466	38.52
OH, CH_3, red	7.77	1162.2807	1161.2725	1161.27475	1.94	1735	0.55
H, H, red	7.801	1132.2683	1131.2615	1131.26419	2.38	5094	1.18



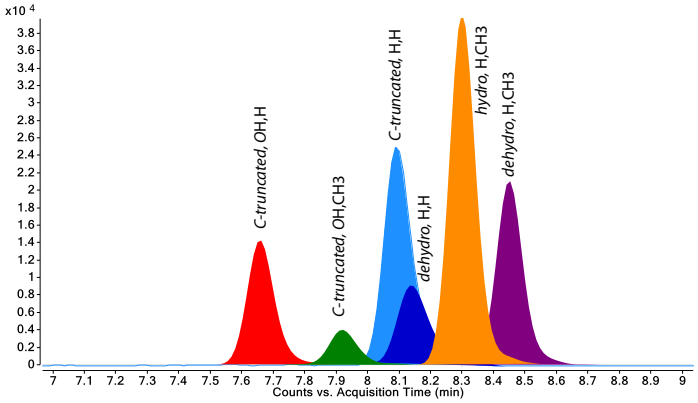


MS Figure 7a: T14A

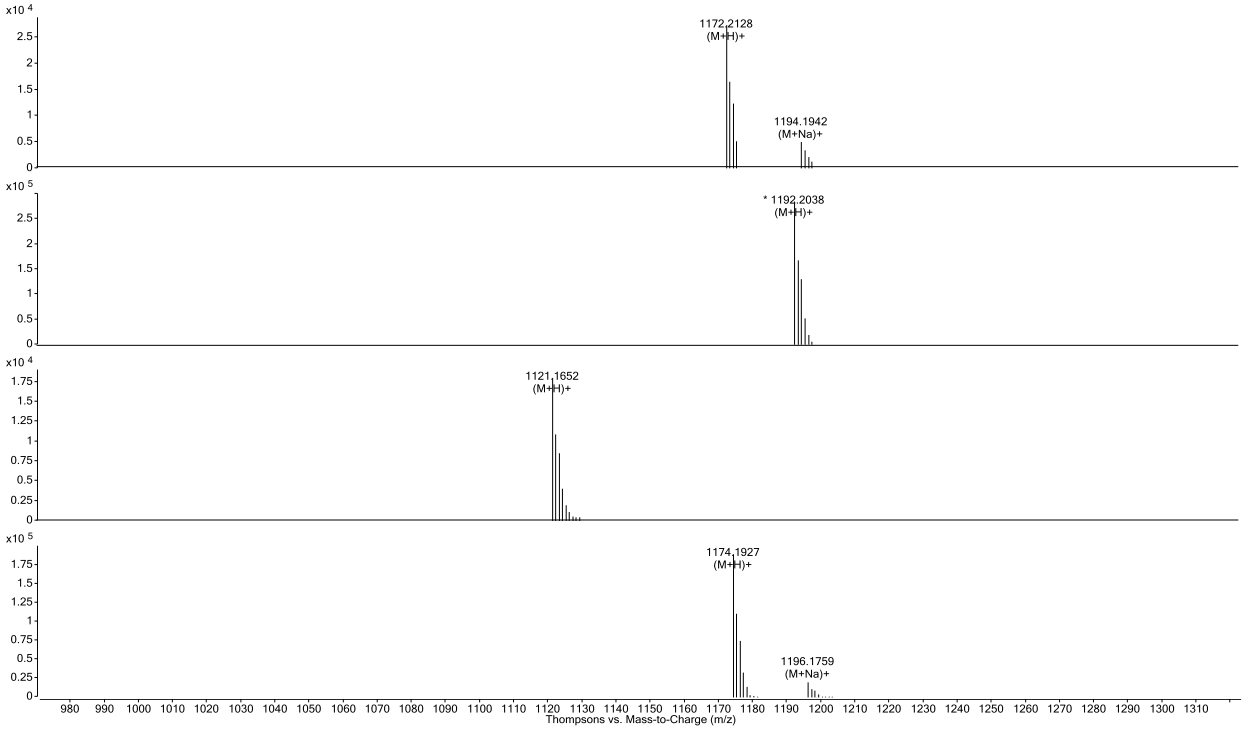
Tailoring (6,8,14)	RT	Molecular Ion	Observed Mass	Expected Mass	Error (ppm)	Area	Vol %
hydro, OH, H	7.564	1192.2038	1191.1966	1191.1948	-1.51	285906	14.86
C-truncated, OH, H	7.66	1121.1652	1120.1579	1120.1577	-0.18	18070	0.97
dehydro, OH, H	7.694	1174.1927	1173.1856	1173.1842	-1.19	190119	10.56
hydro, OH, CH_3	7.827	1206.2183	1205.2111	1205.2104	-0.58	213764	9.39
dehydro, OH, CH_3	7.966	1188.2082	1187.201	1187.1999	-0.93	689332	38.36
hydro, H, H	7.997	1176.2083	1175.201	1175.1999	-0.94	311929	17.85
C-truncated, H, H	8.091	1105.1716	1104.1644	1104.1628	-1.45	32441	1.56
dehydro, H, H	8.143	1158.1959	1157.1886	1157.1893	0.60	10909	0.86
hydro, H, CH_3	8.301	1190.2248	1189.2175	1189.2155	-1.68	50614	2.29
dehydro, H, CH_3	8.45	1172.2128	1171.2055	1171.205	-0.43	27392	1.24



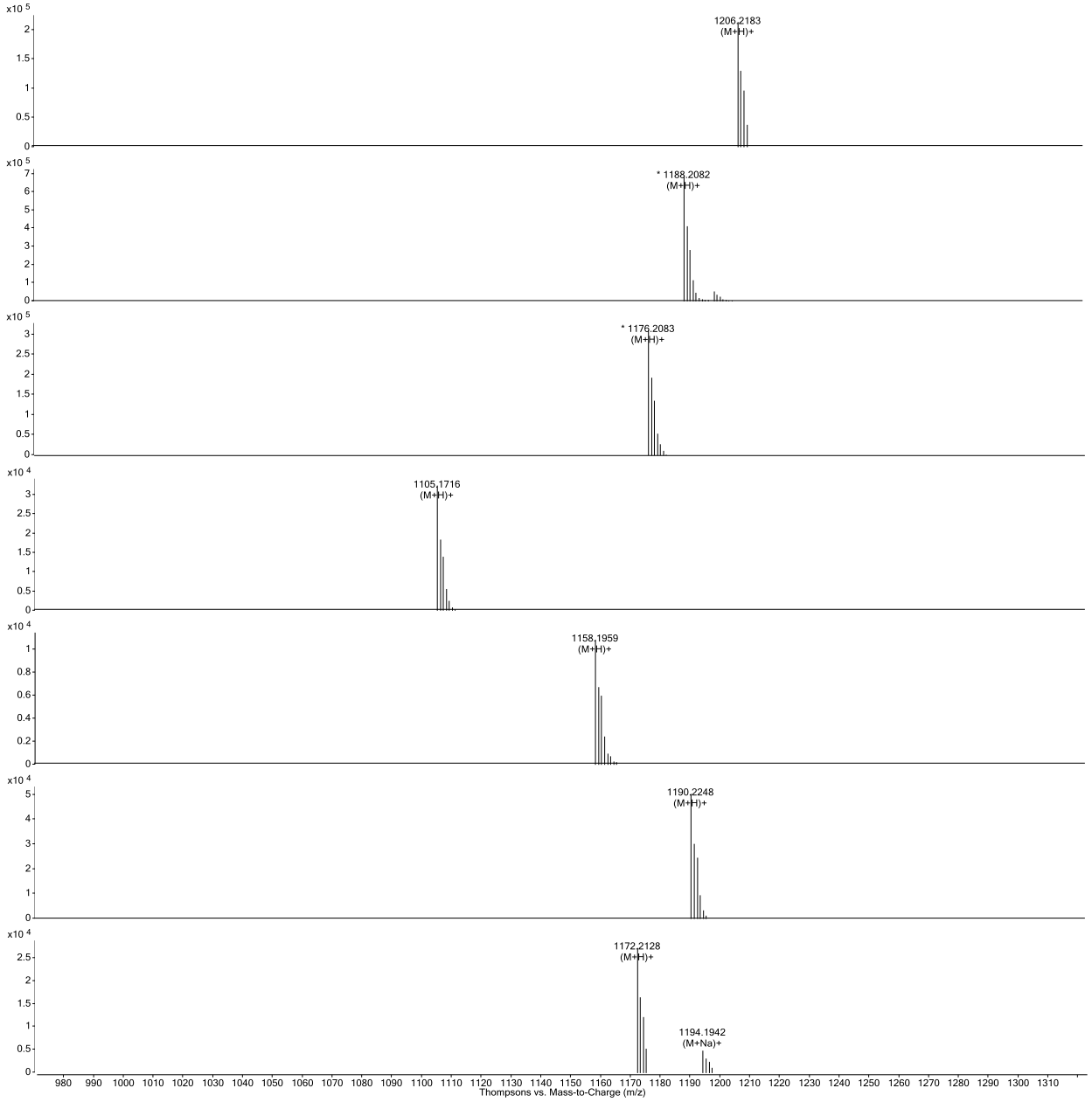
(zoom out)



(zoom in - early peaks removed)

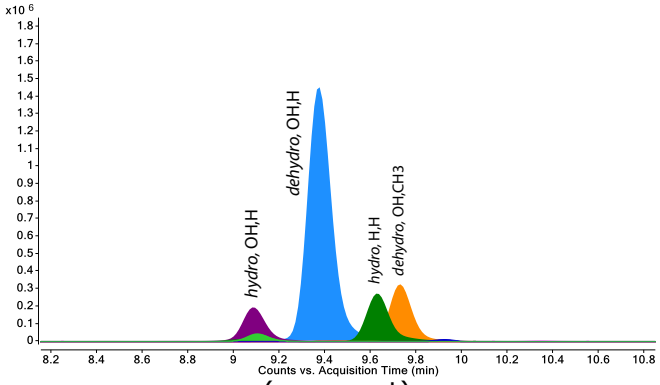


MS Figure 7b: T14A

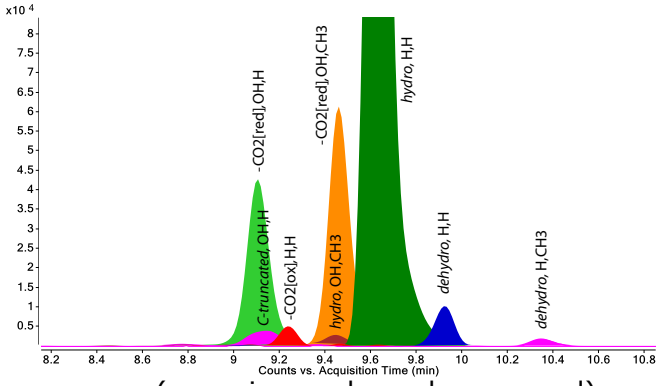


MS Figure 8a: ΔT14

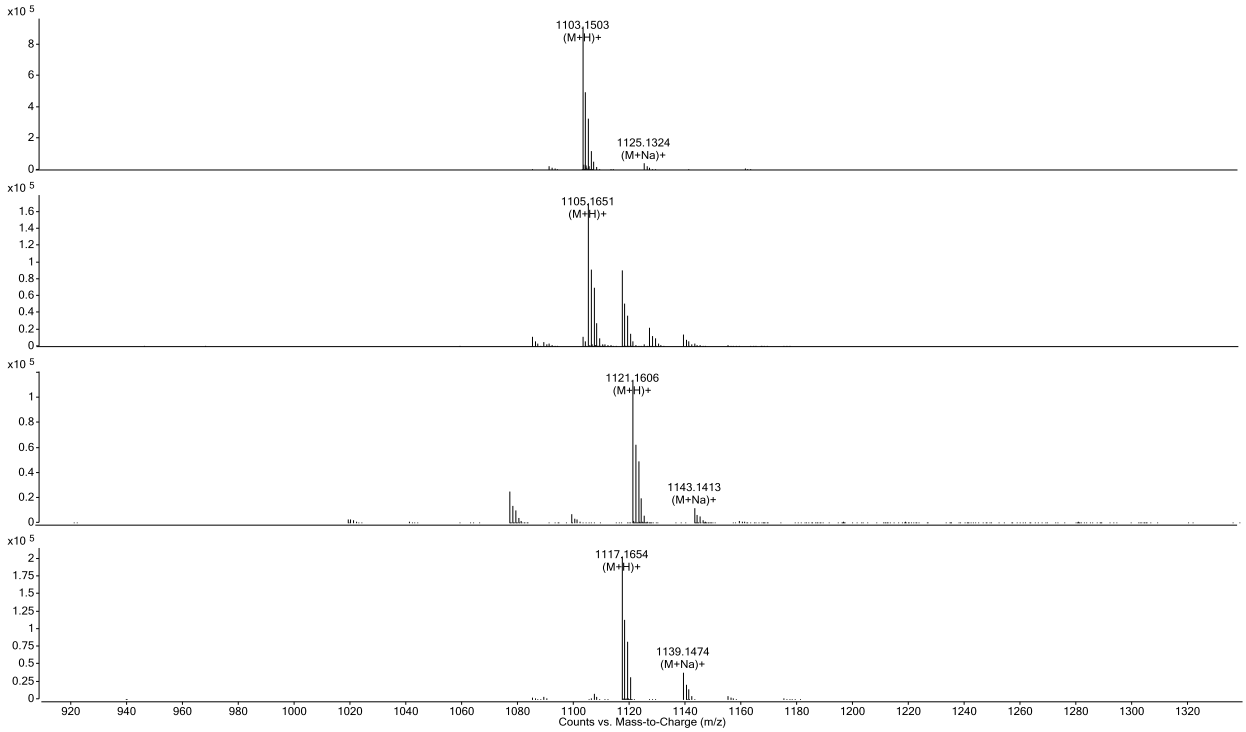
Tailoring (6,8,14)	RT	Molecular Ion	Observed Mass	Expected Mass	Error (ppm)	Area	Vol %
H, CH_3, hydro	8.953	1119.18844	1118.1805	1118.1784	-1.88	2420	0.04
OH, H, hydro	9.083	1121.1606	1120.1544	1120.1577	2.95	490341	8.24
H, CH_3, dehydro	9.083	1101.18024	1100.1723	1100.1678	-4.09	153291	2.58
OH, H, red	9.106	1077.17104	1076.1631	1076.1678	4.37	120802	2.03
H, H, ox	9.236	1059.16614	1058.1582	1058.1573	-0.85	20585	0.35
OH, H, dehydro	9.366	1103.1503	1102.1449	1102.1471	2.00	3354905	56.41
OH, CH_3, hydro	9.449	1135.17684	1134.1689	1134.1733	3.88	7705	0.13
OH, CH_3, red	9.46	1091.18674	1090.1788	1090.1835	4.31	186867	3.14
H, H, hydro	9.626	1105.1651	1104.1578	1104.1628	4.53	712965	11.99
OH, CH_3, dehydro	9.732	1117.1654	1116.1583	1116.1628	4.03	855370	14.38
H, H, dehydro	9.933	1087.15524	1086.1473	1086.1522	4.51	42028	0.71



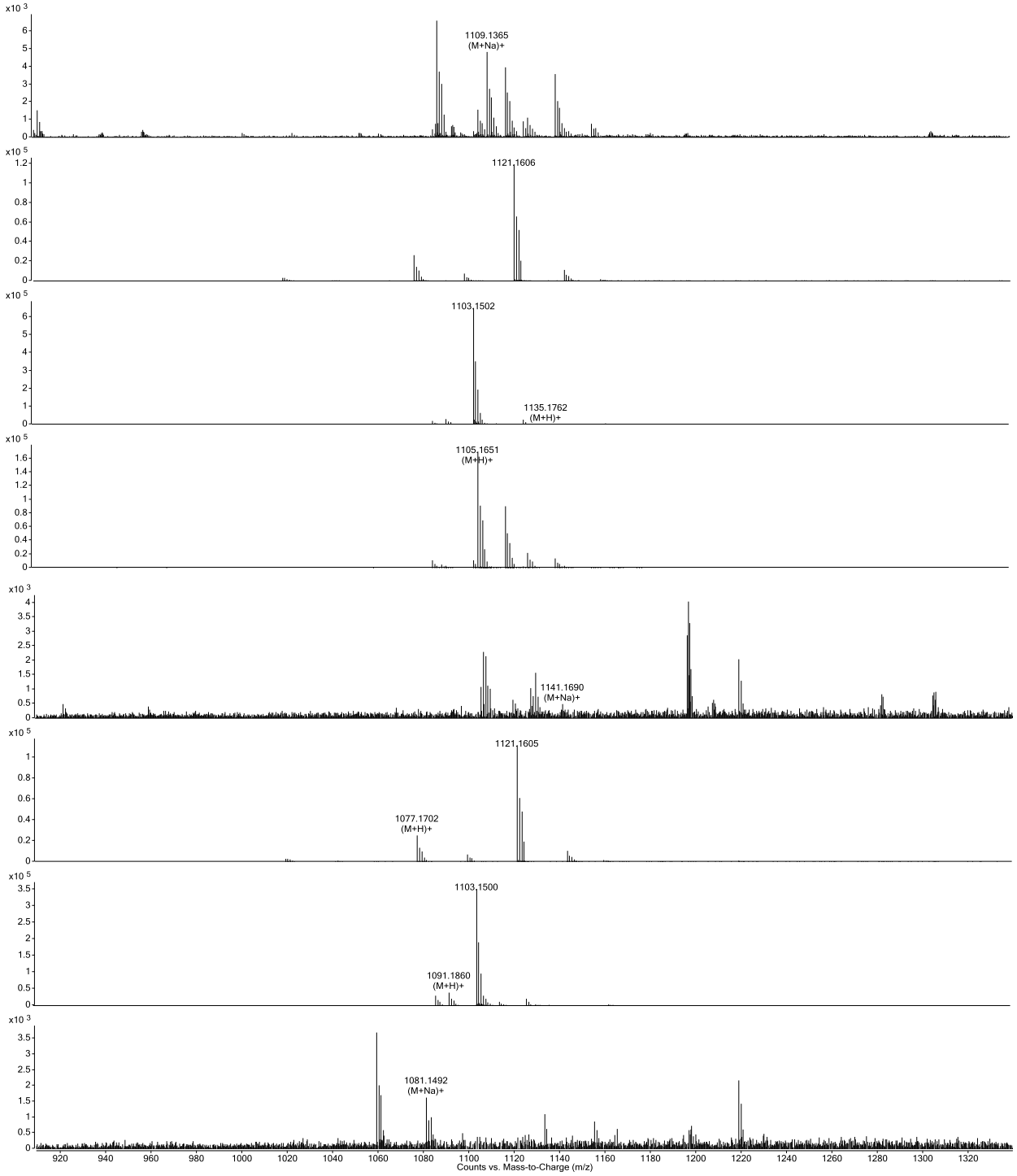
(zoom out)



(zoom in - early peaks removed)

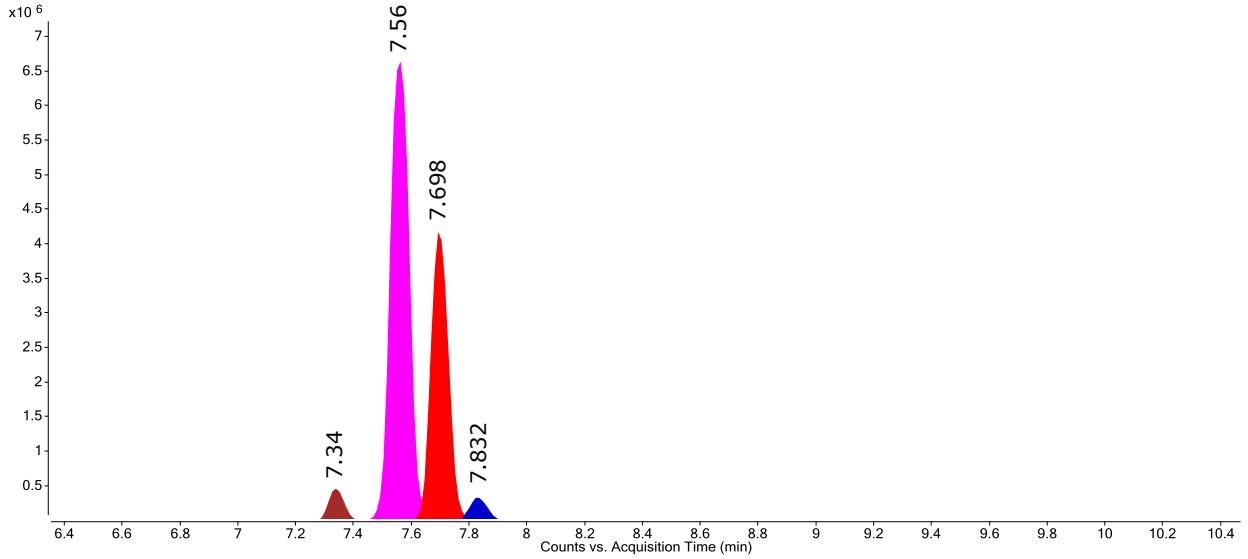


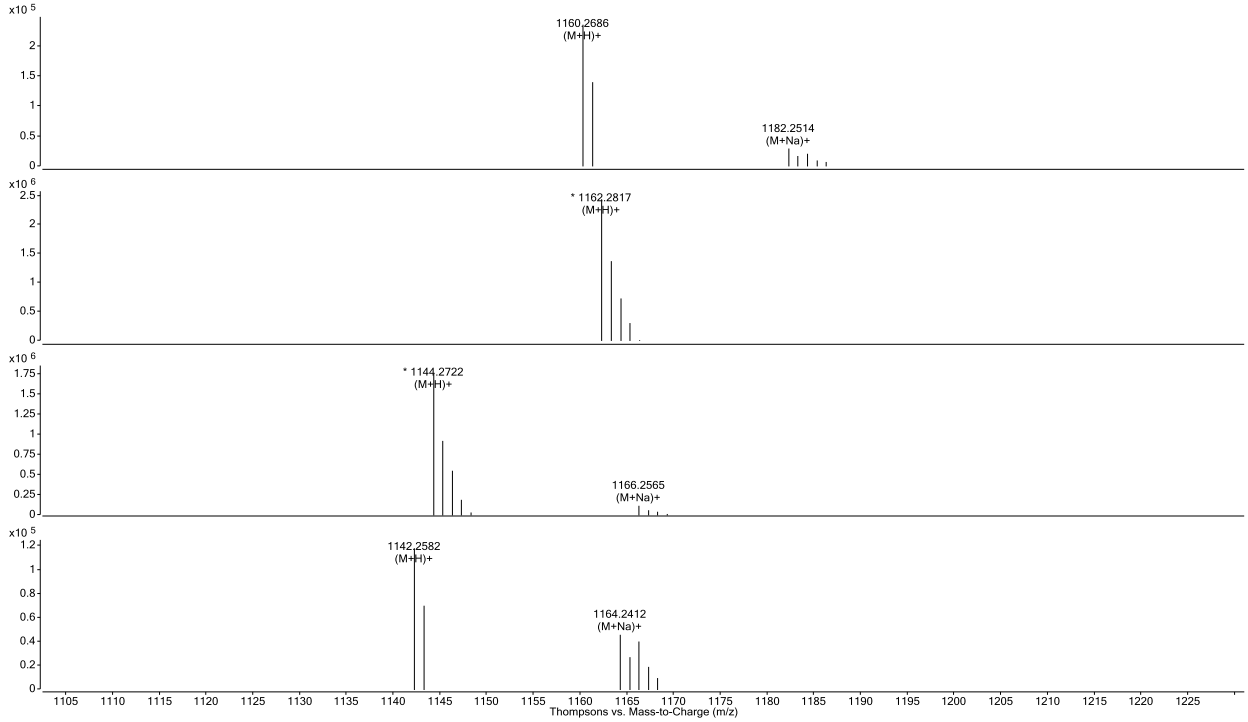
MS Figure 8b: ΔT14



MS Figure 9: C2S

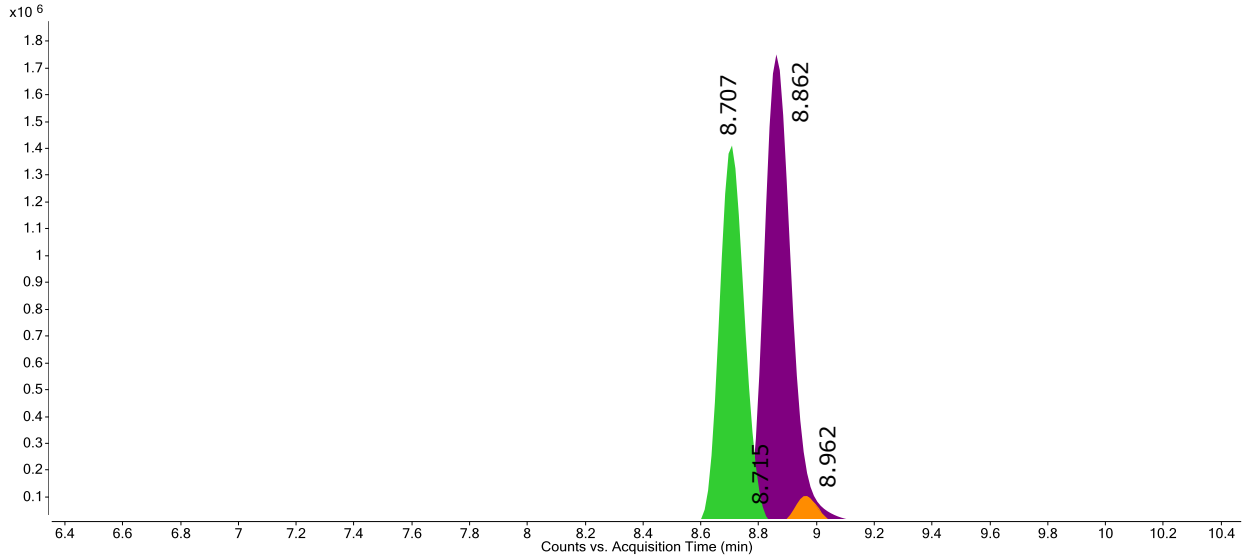
Tailoring (6,8,14)	RT	Molecular Ion	Observed Mass	Expected Mass	Error (ppm)	Area	Vol %
H, CH_3, ox (alcohol)	7.34	1160.2686	1159.2614	1159.2591	-1.98	236280	2.93
H, CH_3, red (alcohol)	7.56	1162.2817	1161.2744	1161.2748	0.34	2449516	57.14
H, CH_3, red (dehydro)	7.698	1144.2722	1143.2651	1143.2642	-0.79	1763434	32.95
H, CH_3, ox (dehydro)	7.832	1142.2582	1141.2513	1141.2485	-2.45	118719	2.39

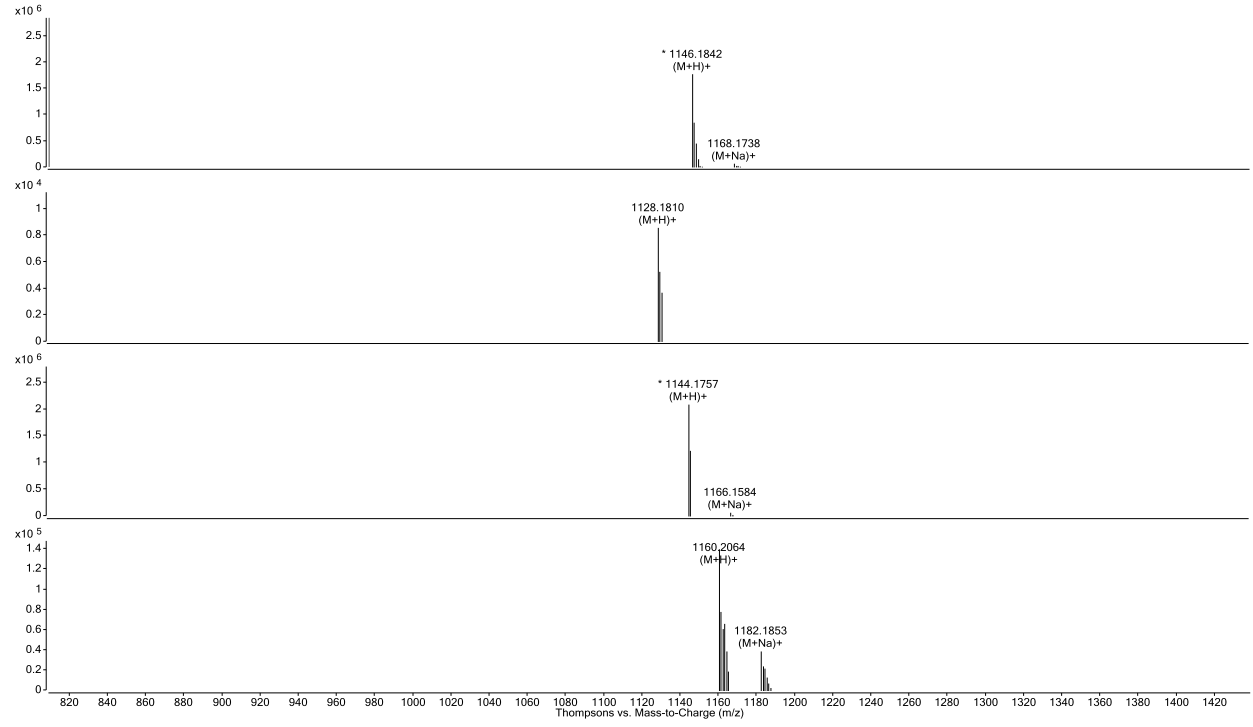




MS Figure 10: T3S

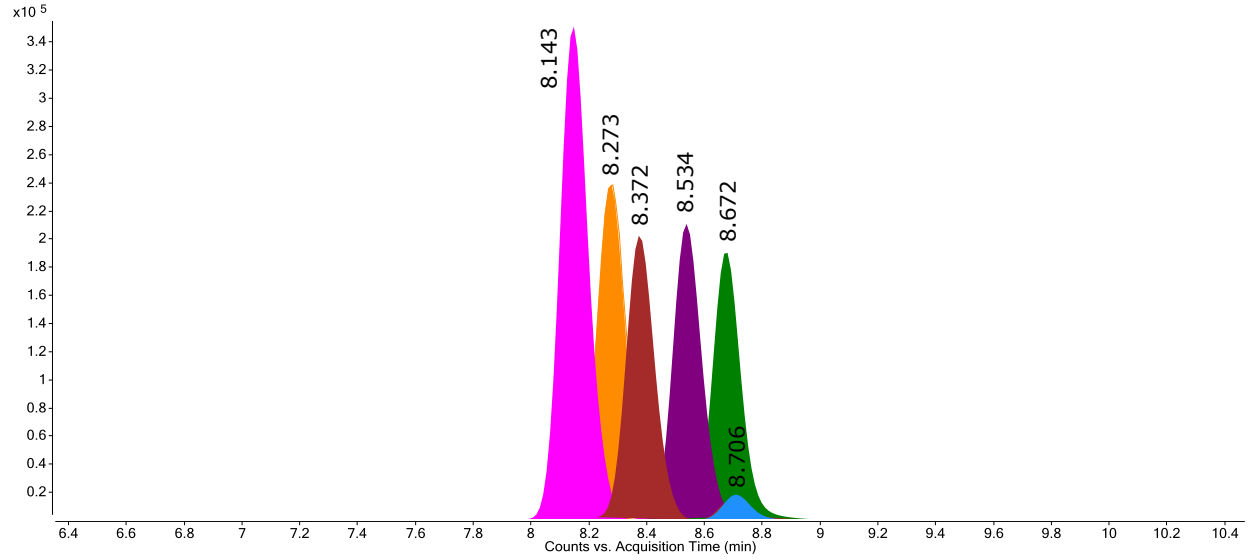
Tailoring (6,8,14)	RT	Molecular Ion	Observed Mass	Expected Mass	Error (ppm)	Area	Vol %
OH, H, red	8.707	1146.1842	1145.1825	1145.18931	5.95	1770807	49.06
H, H, ox	8.715	1128.181	1127.1737	1127.17874	4.47	8587	0.17
OH, H, ox	8.862	1144.1757	1143.1685	1143.17366	4.51	2097506	32.02
OH, CH_3, red	8.962	1160.2064	1159.1984	1159.20496	5.66	140609	3.59

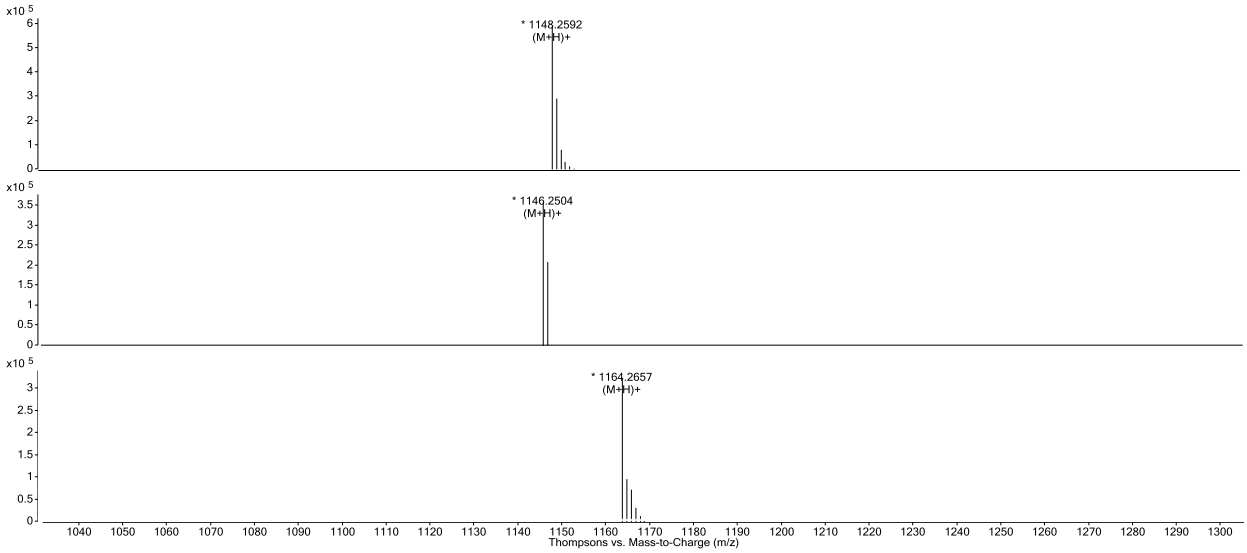




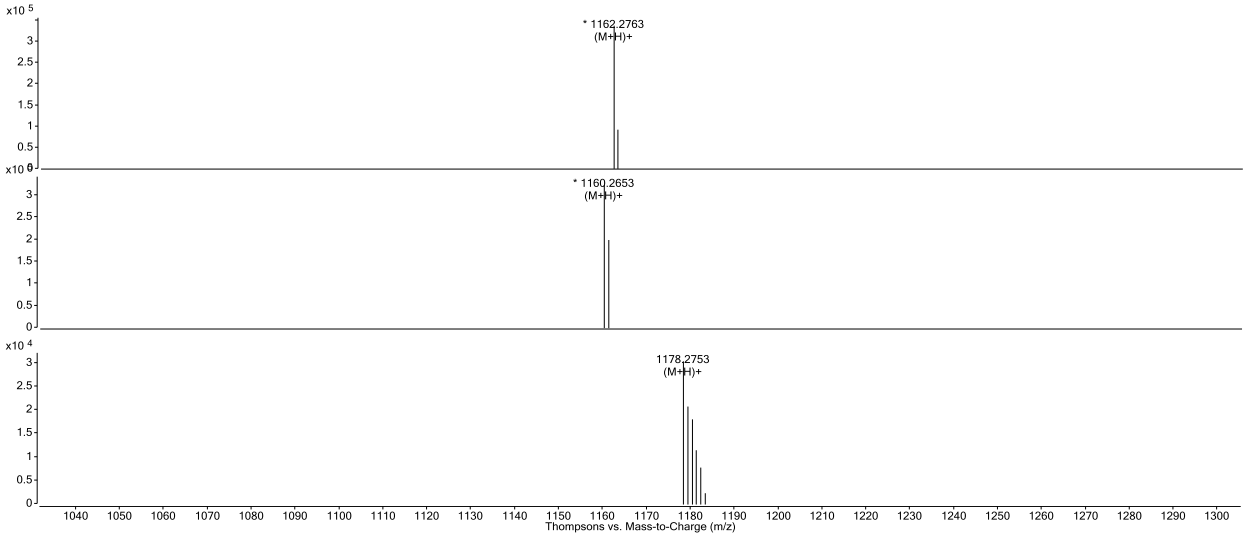
MS Figure 11a: C5S

Tailoring (6,8,14)	RT	Molecular Ion	Observed Mass	Expected Mass	Error (ppm)	Area	Vol %
H, H, red (alcohol)	8.143	1148.2592	1147.2519	1147.2591	6.28	591296	27.02
H, H, ox (alcohol)	8.273	1146.2504	1145.2431	1145.2435	0.35	359247	11.84
OH, H, red (alcohol)	8.372	1164.2657	1163.2584	1163.254	-3.78	322906	17.45
H, CH_3, red (alcohol)	8.534	1162.2763	1161.2691	1161.2748	4.91	338276	12.64
H, CH_3, ox (alcohol)	8.672	1160.2653	1159.258	1159.2591	0.95	324360	9.16
OH, CH_3, red (alcohol)	8.706	1178.2753	1177.268	1177.2697	1.44	30481	1.59



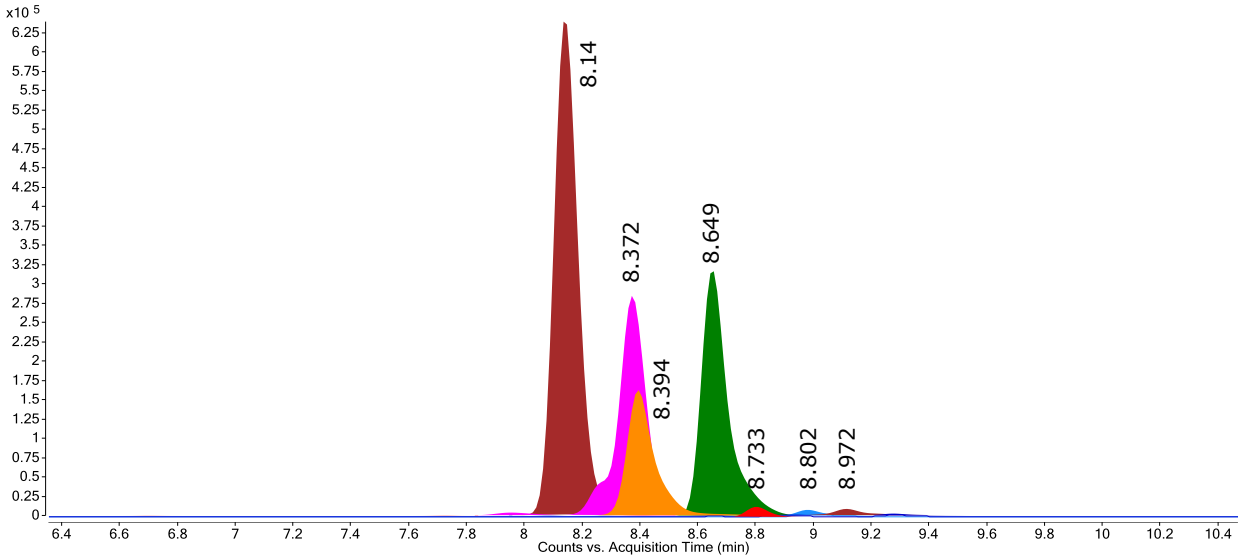


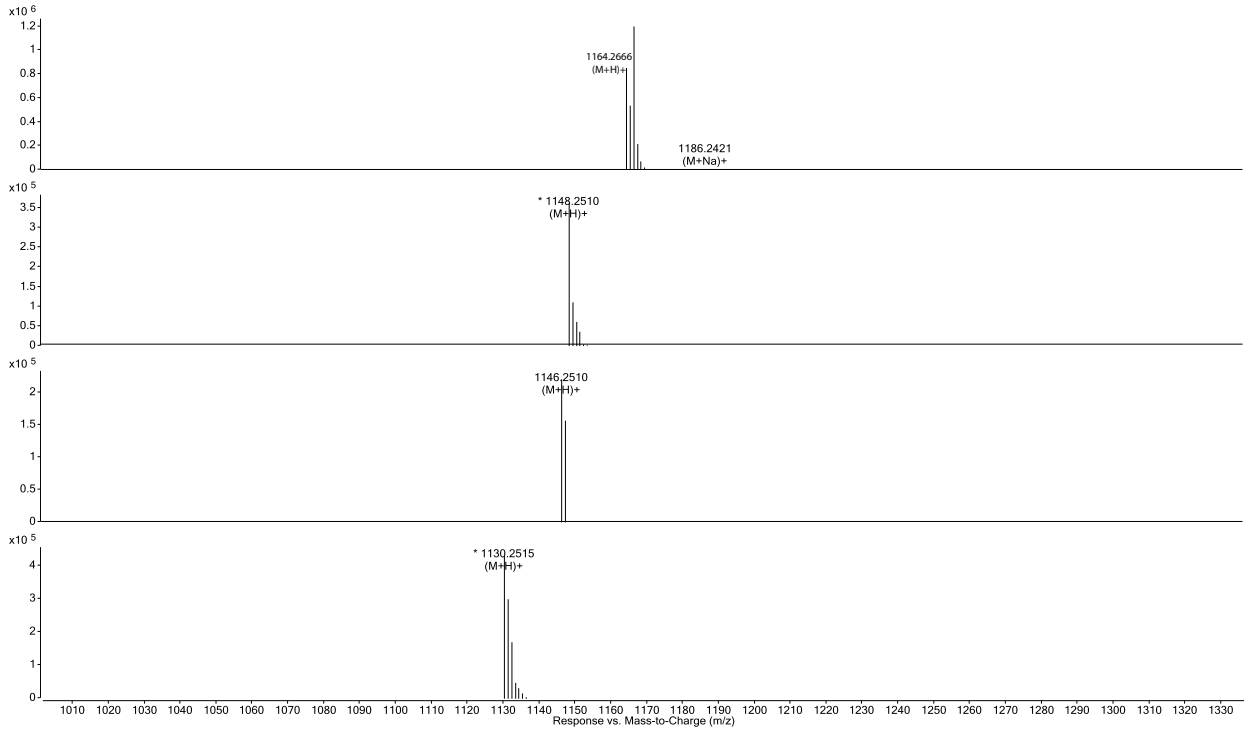
MS Figure 11b: C5S



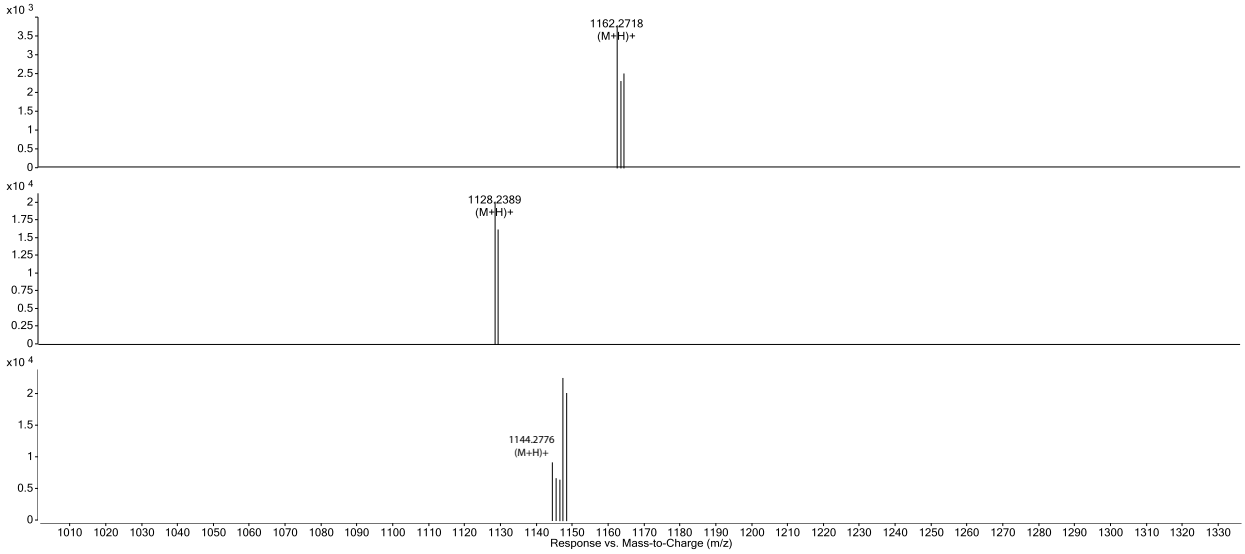
MS Figure 12a: C7S

Tailoring (6,8,14)	RT	Molecular Ion	Observed Mass	Expected Mass	Error (ppm)	Area	Vol %
OH, H, red (alcohol)	8.14	1164.2666	1163.2526	1163.254	1.20	1199988	35.38
H, H, red (alcohol)	8.372	1148.251	1147.2537	1147.2591	4.71	364366	16.13
H, H, ox (alcohol)	8.394	1146.251	1145.2437	1145.2435	-0.17	220933	6.56
H, H, red (dehydro)	8.649	1130.2515	1129.2442	1129.2485	3.81	433087	19.62
H, CH_3, red (alcohol)	8.733	1162.2718	1161.2745	1161.2748	0.26	3810	0.15
H, H, ox (dehydro)	8.802	1128.2389	1127.2316	1127.2329	1.15	20220	0.56
H, CH_3, red (dehydro)	8.972	1144.2776	1143.262	1143.2642	1.92	22655	0.96



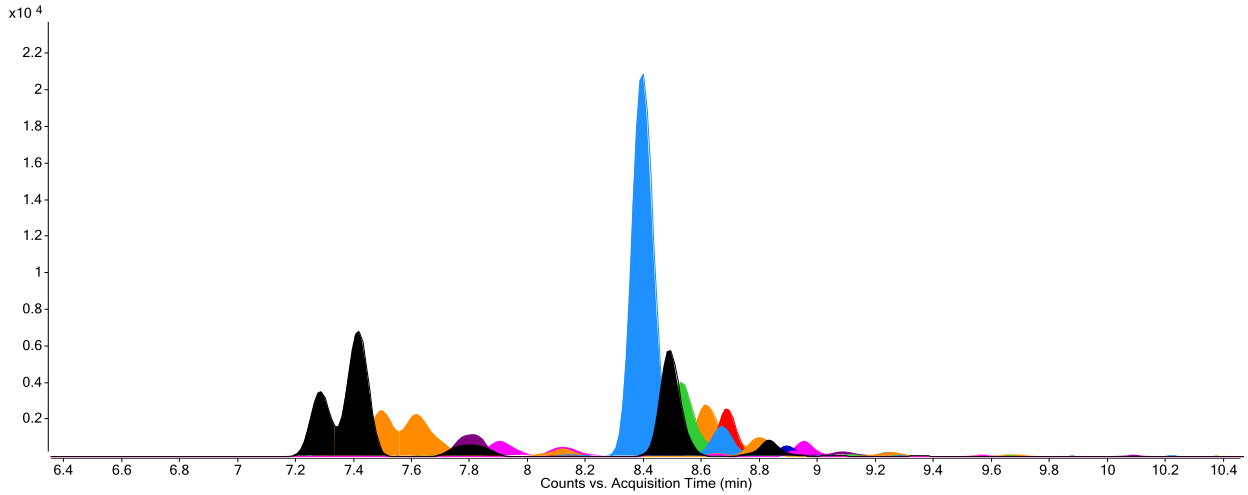


MS Figure 12b: C7S

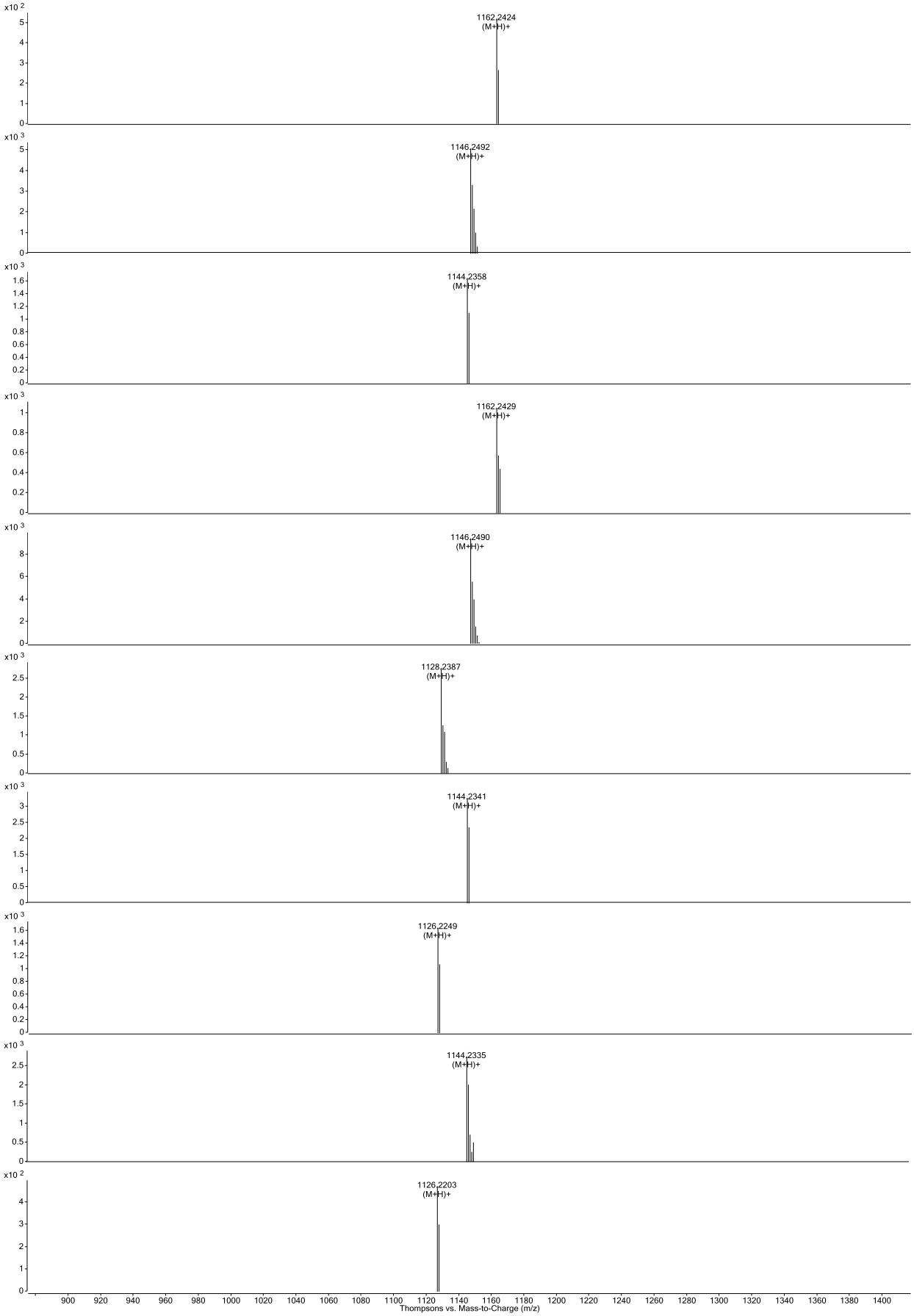


MS Figure 13a: C9S

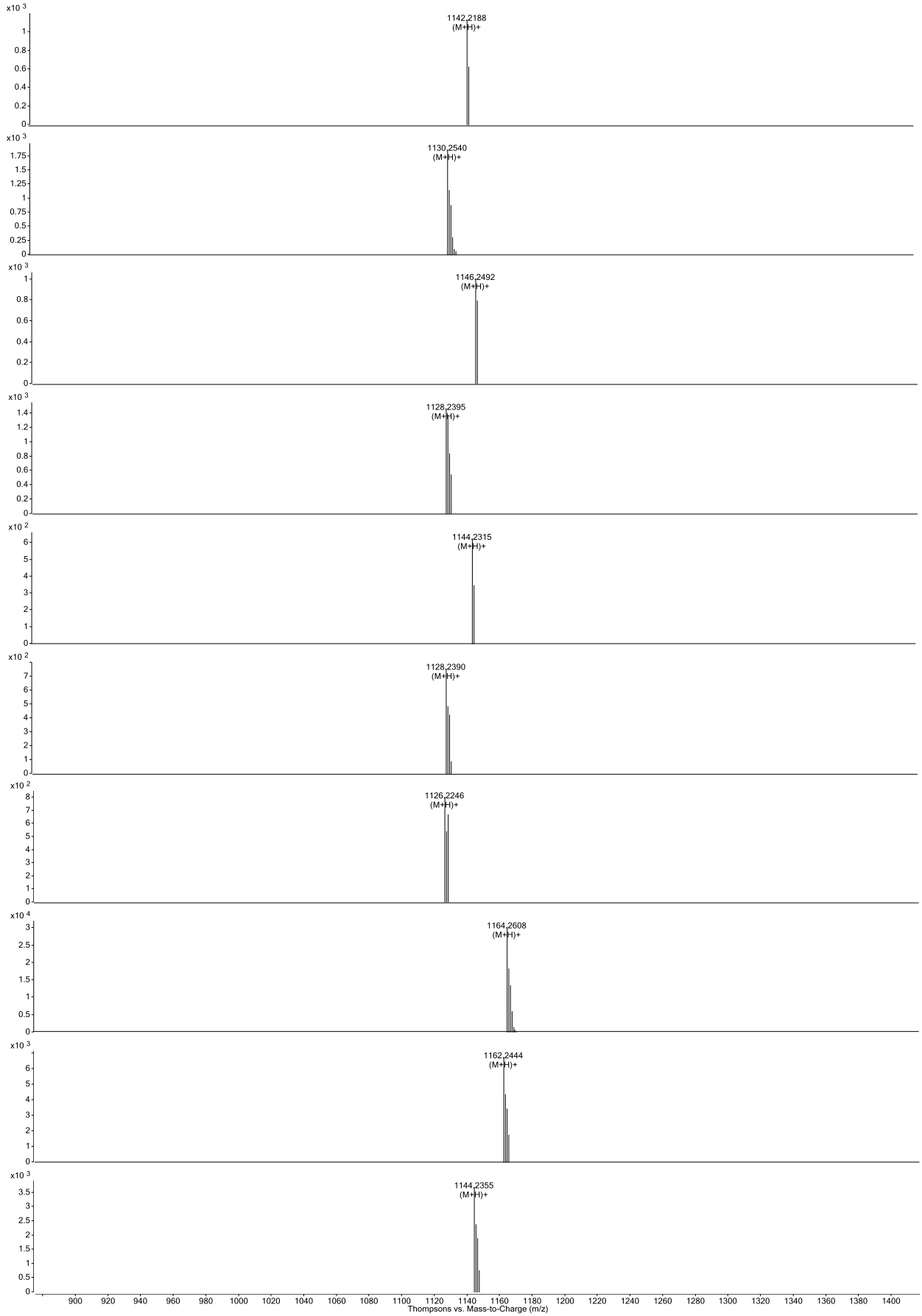
RT	Molecular Ion	Observed Mass	Expected Mass	Error (ppm)	Area	Vol %
7.267	1162.2424	1161.2351	1161.23837	2.82	523	0.28
7.285	1146.2492	1145.2419	1145.24345	1.35	5106	4.24
7.36	1144.2358	1143.2286	1143.2278	-0.70	1660	1.02
7.402	1162.2429	1161.2356	1161.23837	2.39	1057	1.01
7.416	1146.249	1145.2417	1145.24345	1.53	9470	8.72
7.417	1128.2387	1127.2314	1127.23289	1.32	2778	2.7
7.495	1144.2341	1143.2268	1143.2278	0.87	3288	1.86
7.499	1126.2249	1125.2176	1125.21724	-0.32	1654	0.97
7.617	1144.2335	1143.2262	1143.2278	1.40	2760	2.78
7.678	1126.2203	1125.213	1125.21724	3.77	474	0.31
7.693	1142.2188	1141.2115	1141.21215	0.57	1142	0.6
7.82	1130.254	1129.2467	1129.24854	1.63	1879	2.58
7.82	1146.2492	1145.2419	1145.24345	1.35	1012	0.82
7.919	1128.2395	1127.2323	1127.23289	0.52	1475	1.54
8.119	1144.2315	1143.2242	1143.2278	3.15	629	0.35
8.121	1128.239	1127.2317	1127.23289	1.06	762	0.59
8.209	1126.2246	1125.2173	1125.21724	-0.05	806	0.48
8.392	1164.2608	1163.2535	1163.25402	0.45	30468	22.86
8.519	1162.2444	1161.2371	1161.23837	1.09	6803	6.33
8.61	1144.2355	1143.2282	1143.2278	-0.35	3719	3.14
8.616	1176.2536	1175.2463	1175.25402	6.57	782	0.45
8.672	1148.2639	1147.2558	1147.2591	2.88	4299	5.78
8.689	1160.2609	1159.2536	1159.2591	4.74	558	0.3
8.798	1176.2626	1175.2553	1175.25402	-1.09	1741	1.42
8.8	1130.2546	1129.2473	1129.24854	1.10	1690	1.41
8.812	1146.2483	1145.2409	1145.24345	2.23	1832	4.11
8.892	1158.2505	1157.2432	1157.24345	0.22	1288	1.48
8.932	1178.275	1177.2677	1177.26967	1.67	608	0.39
8.941	1128.2375	1127.2315	1127.23289	1.23	1730	2.23
8.998	1159.2549	1157.2399	1157.24345	3.07	504	0.26
9.231	1142.2536	1141.2463	1141.24854	1.96	625	0.42



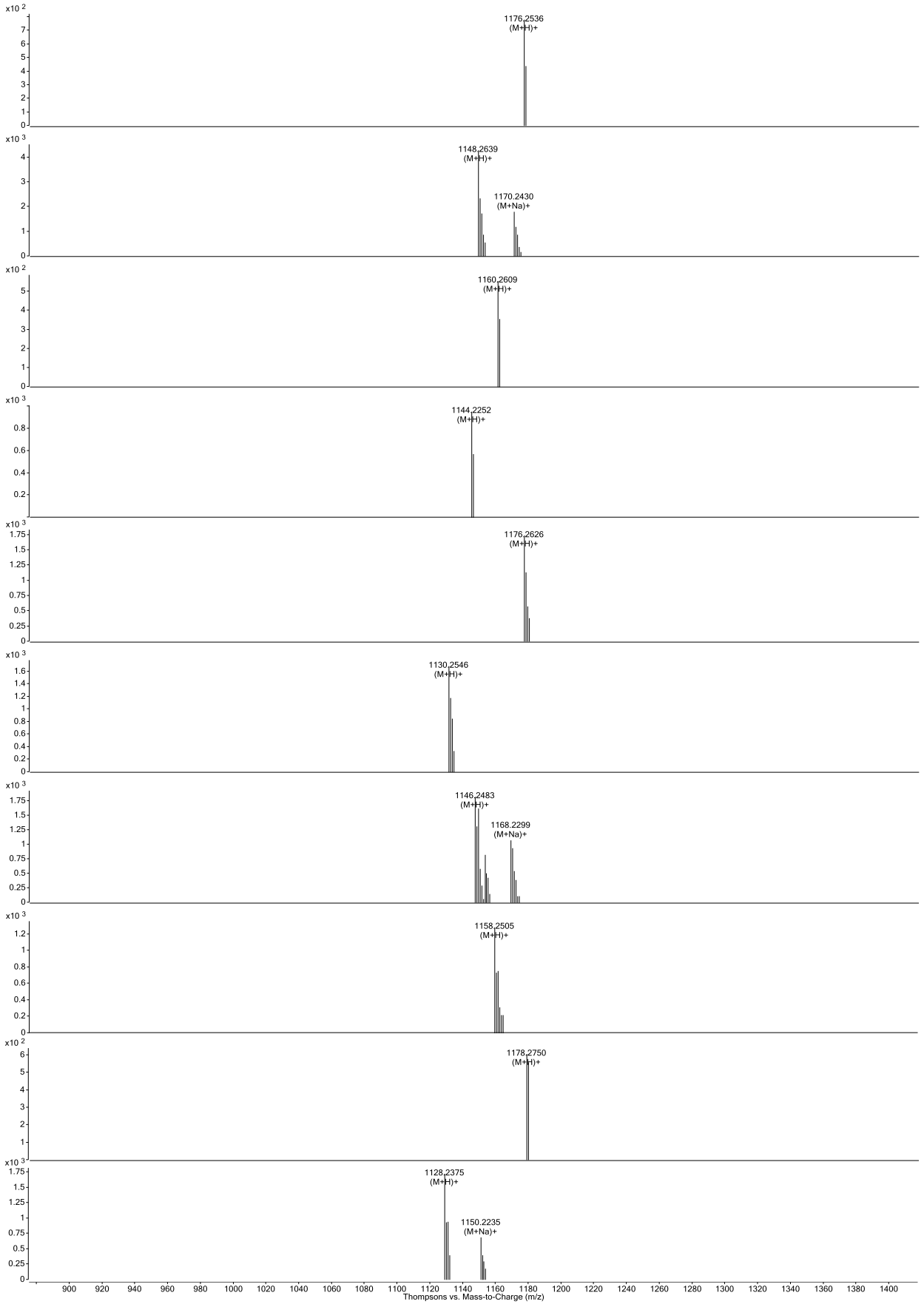
MS Figure 13b: C9S



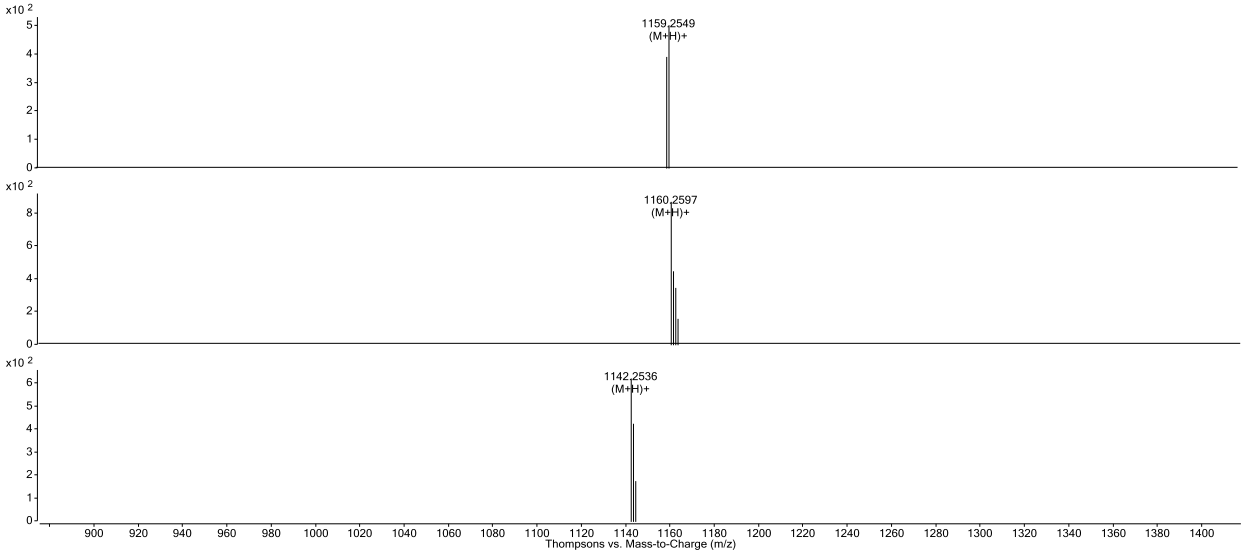
MS Figure 13c: C9S



MS Figure 13d: C9S

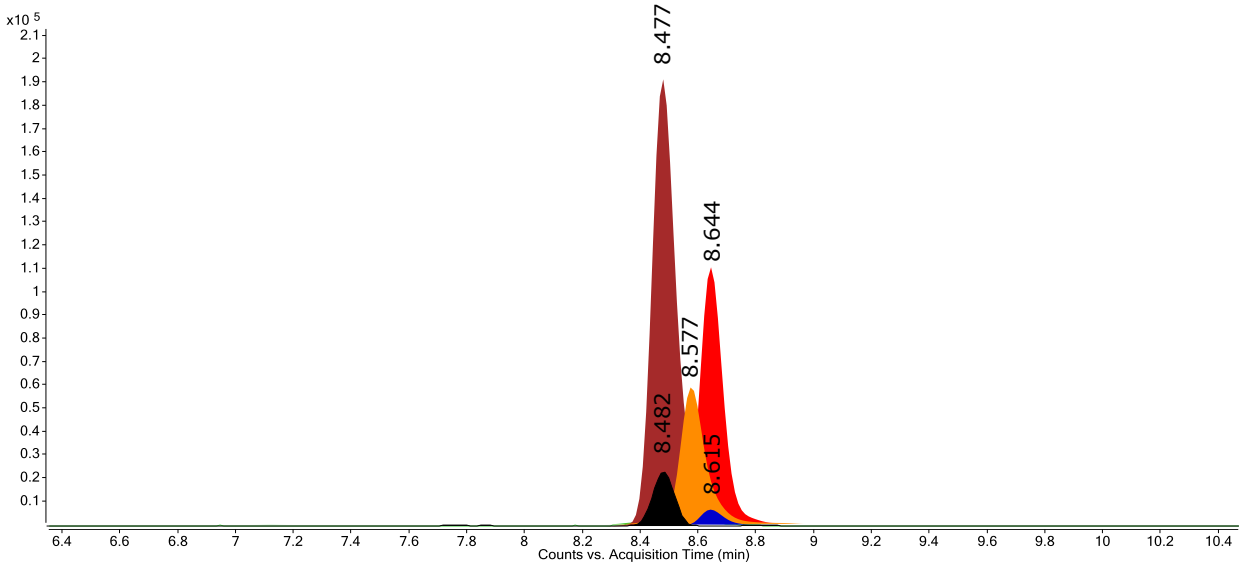


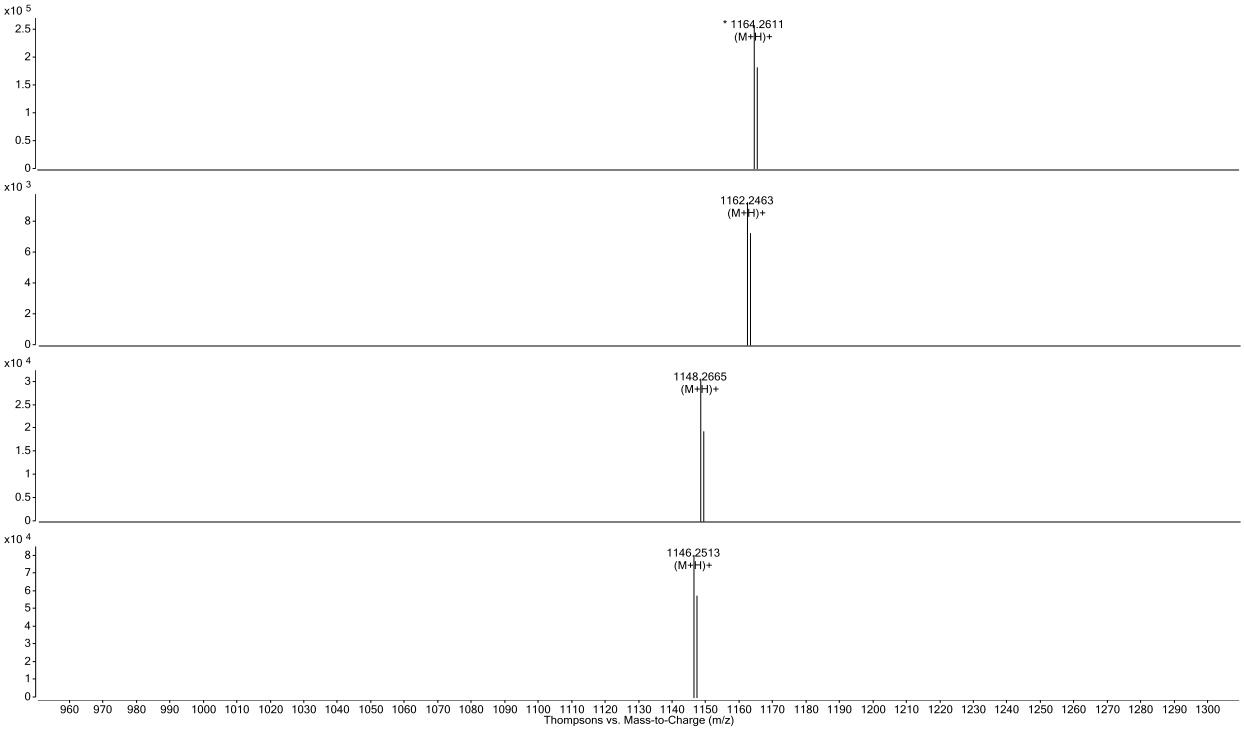
MS Figure 13e: C9S



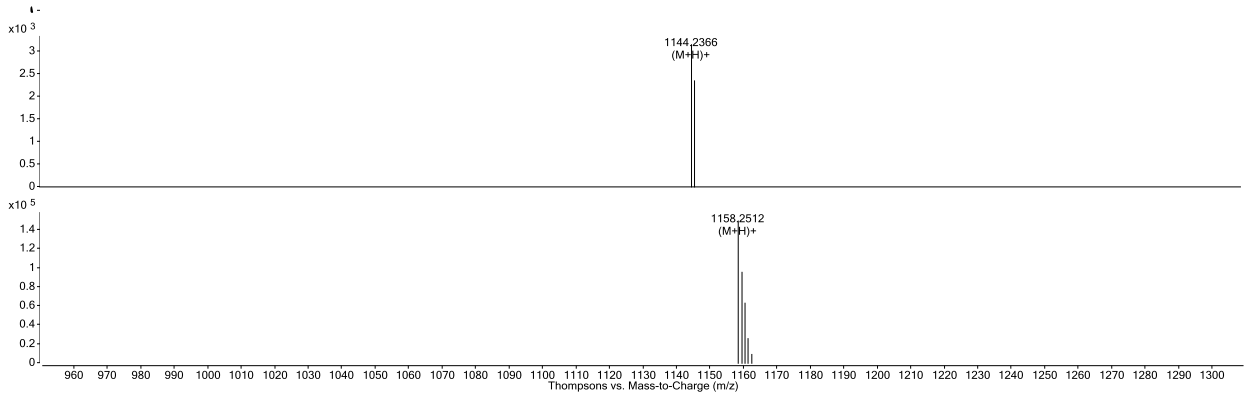
MS Figure 14a: C12S

Tailoring (6,8,14)	RT	Molecular Ion	Observed Mass	Expected Mass	Error (ppm)	Area	Vol %
OH, H, red (alcohol)	8.477	1164.2611	1163.2542	1163.254	-0.17	257693	33.79
OH, H, ox (alcohol)	8.479	1162.2463	1161.2391	1161.2384	-0.60	9312	1.15
H, H, red (alcohol)	8.482	1148.2665	1147.2592	1147.2591	-0.09	30826	3.05
OH, H, red (dehydro)	8.577	1146.2513	1145.2444	1145.2435	-0.79	81032	10.33
OH, H, ox (dehydro)	8.615	1144.2366	1143.2288	1143.2278	-0.87	3173	0.42
OH, CH_3, red(oxazole)	8.644	1158.2512	1157.2433	1157.2435	0.17	150618	24.26

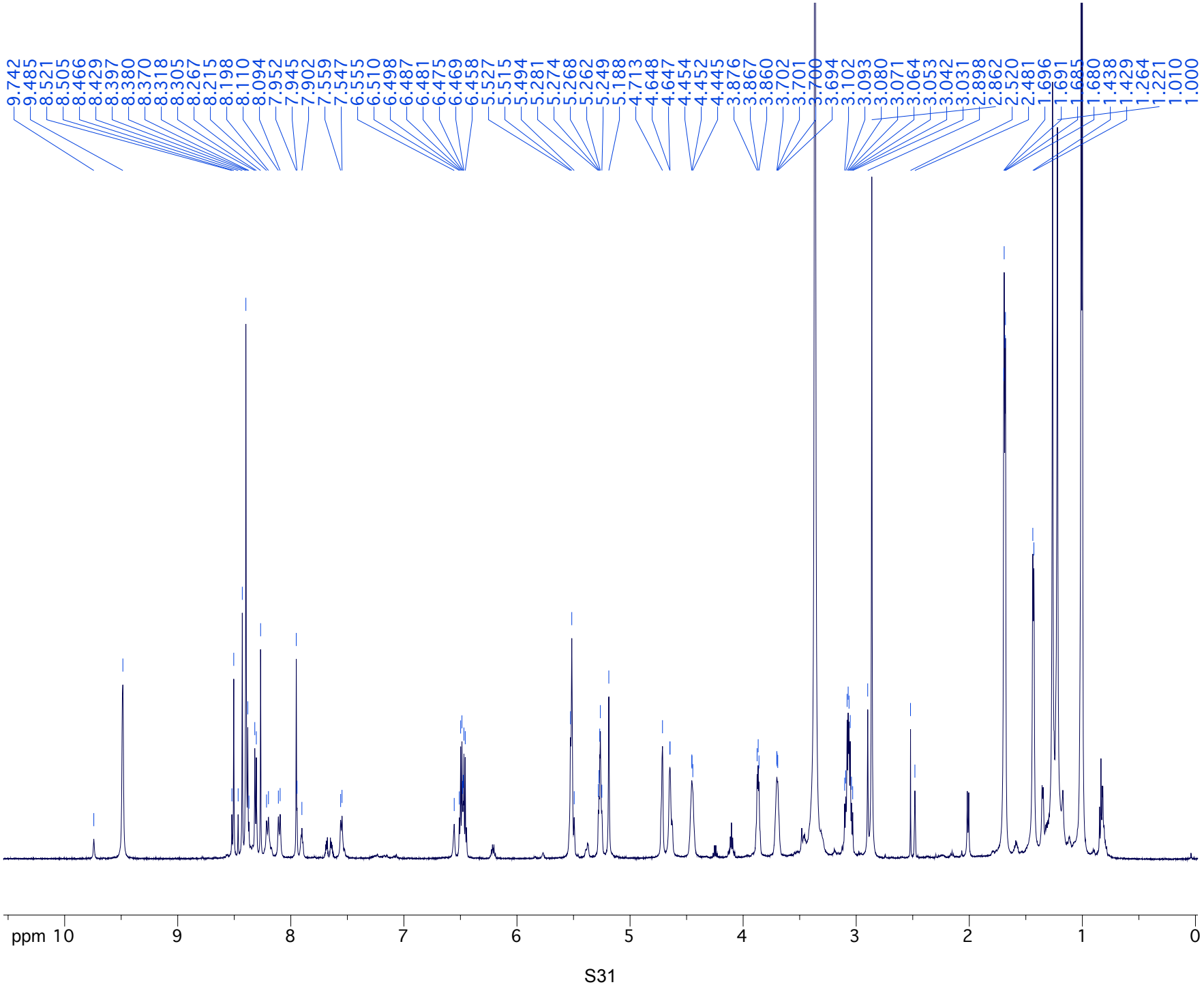




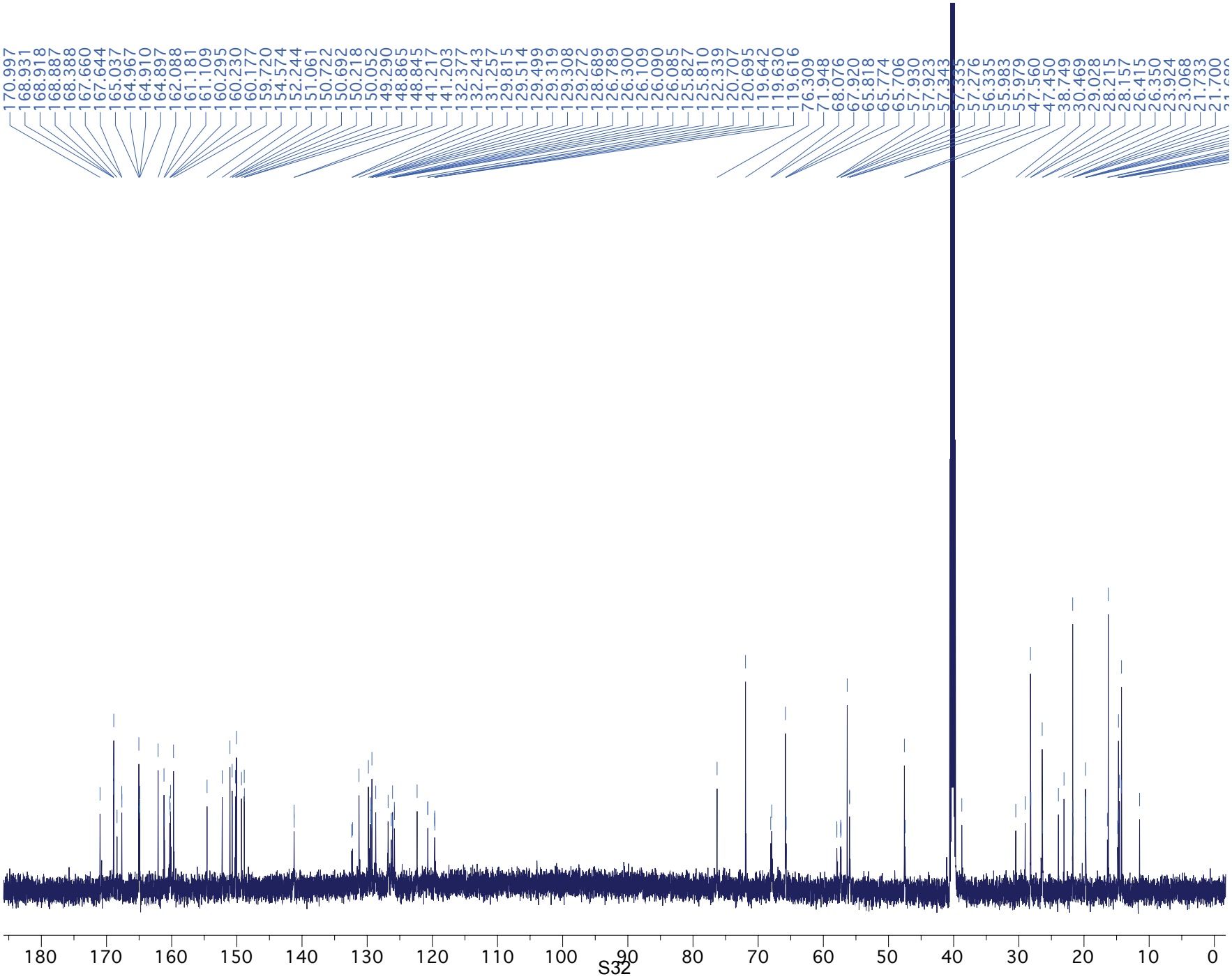
MS Figure 14b: C12S



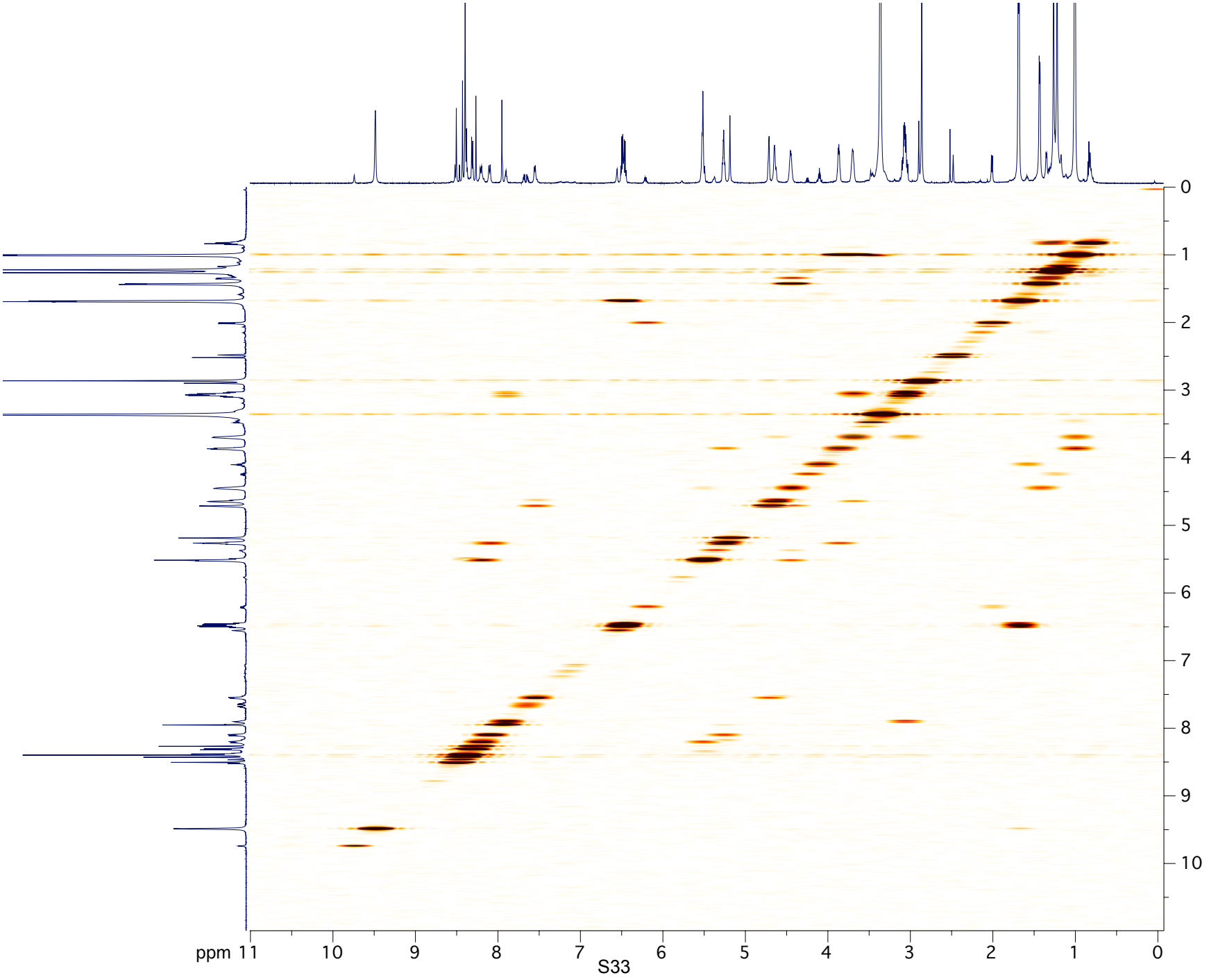
NMR Figure 1: Thiocillin II 1H NMR (600 MHz)



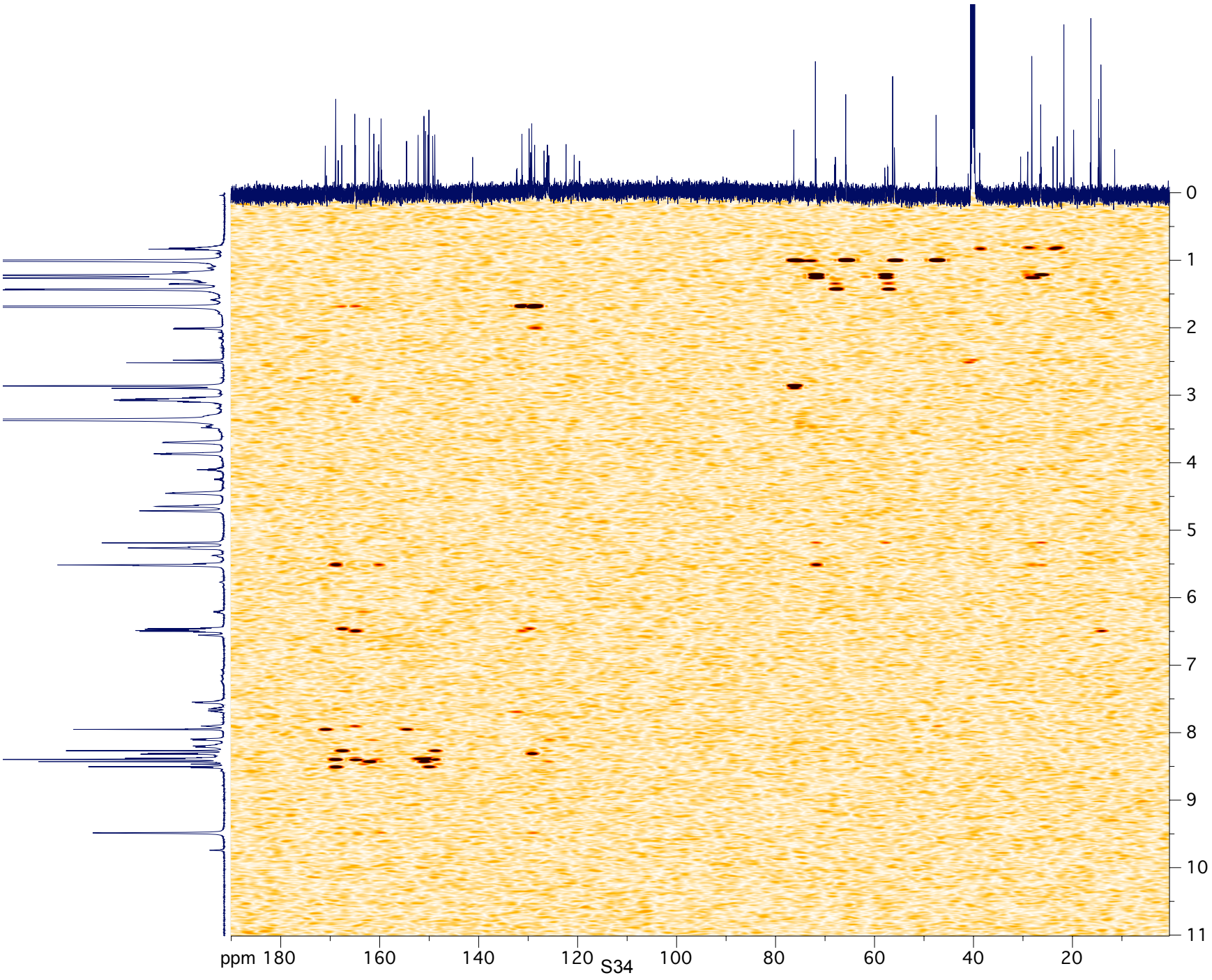
NMR Figure 2: Thiocillin II 13C NMR (150.6 MHz)



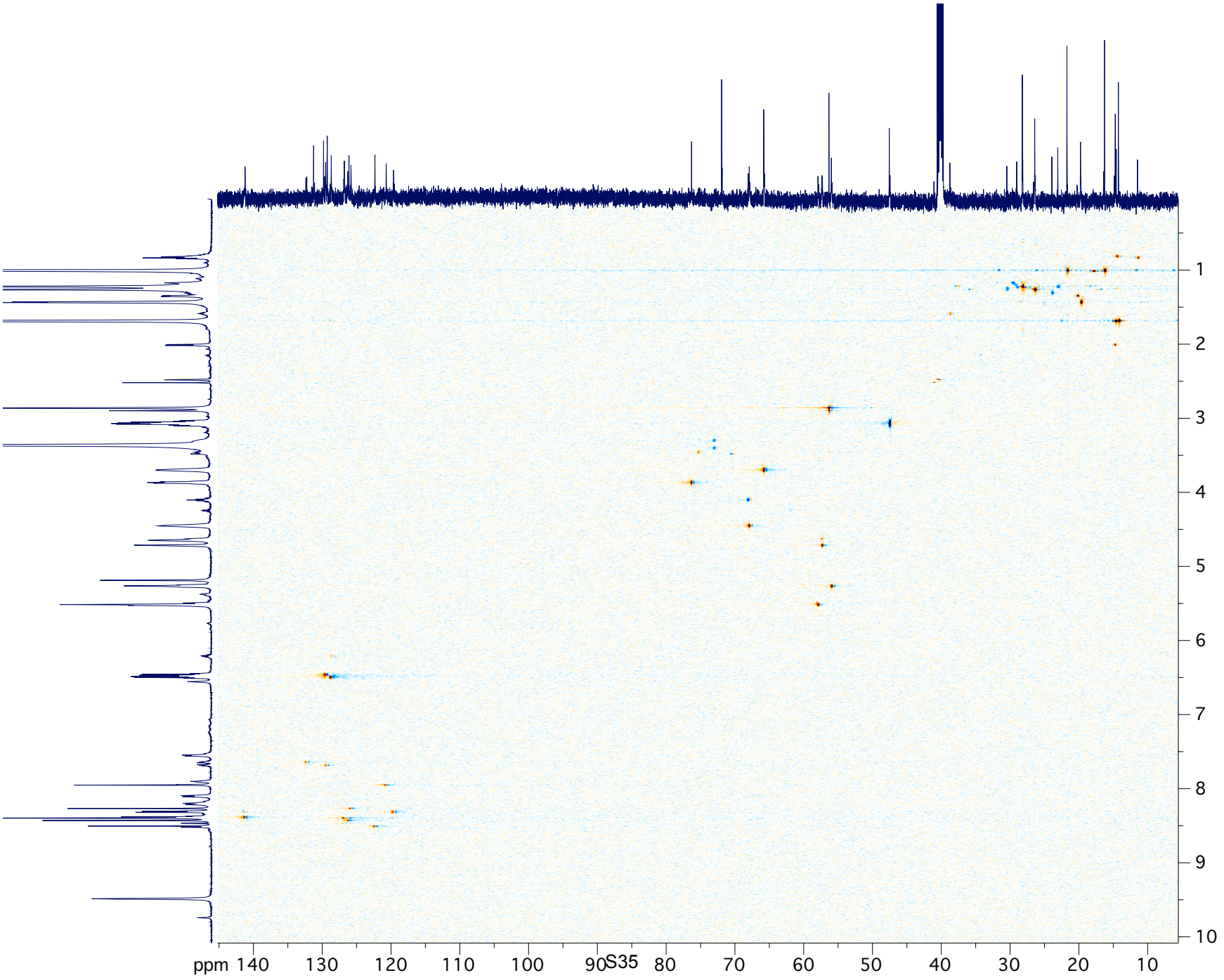
NMR Figure 3: Thiocillin II COSY spectrum (600 MHz)



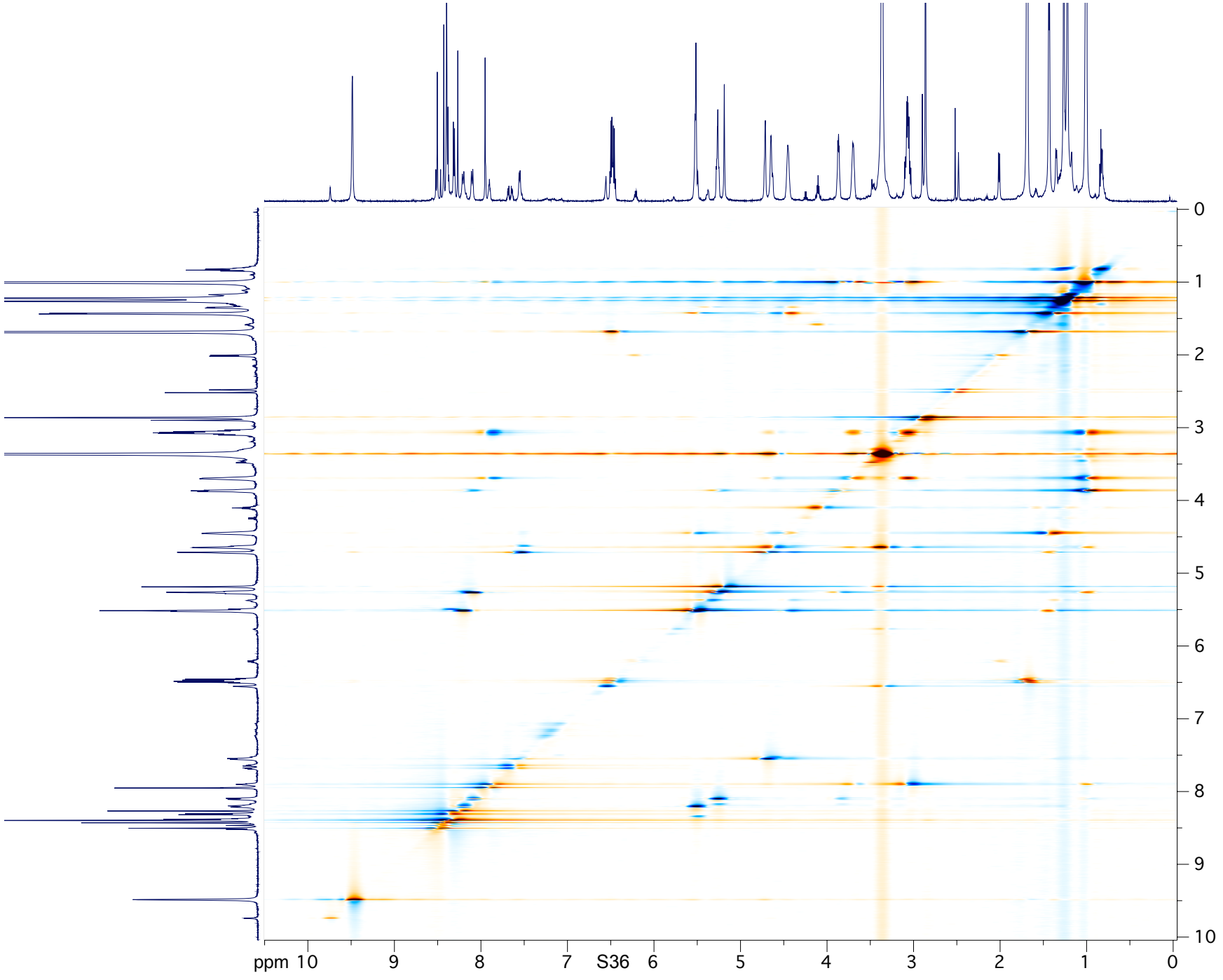
NMR Figure 4: Thiocillin II HMBC spectrum (600 MHz)



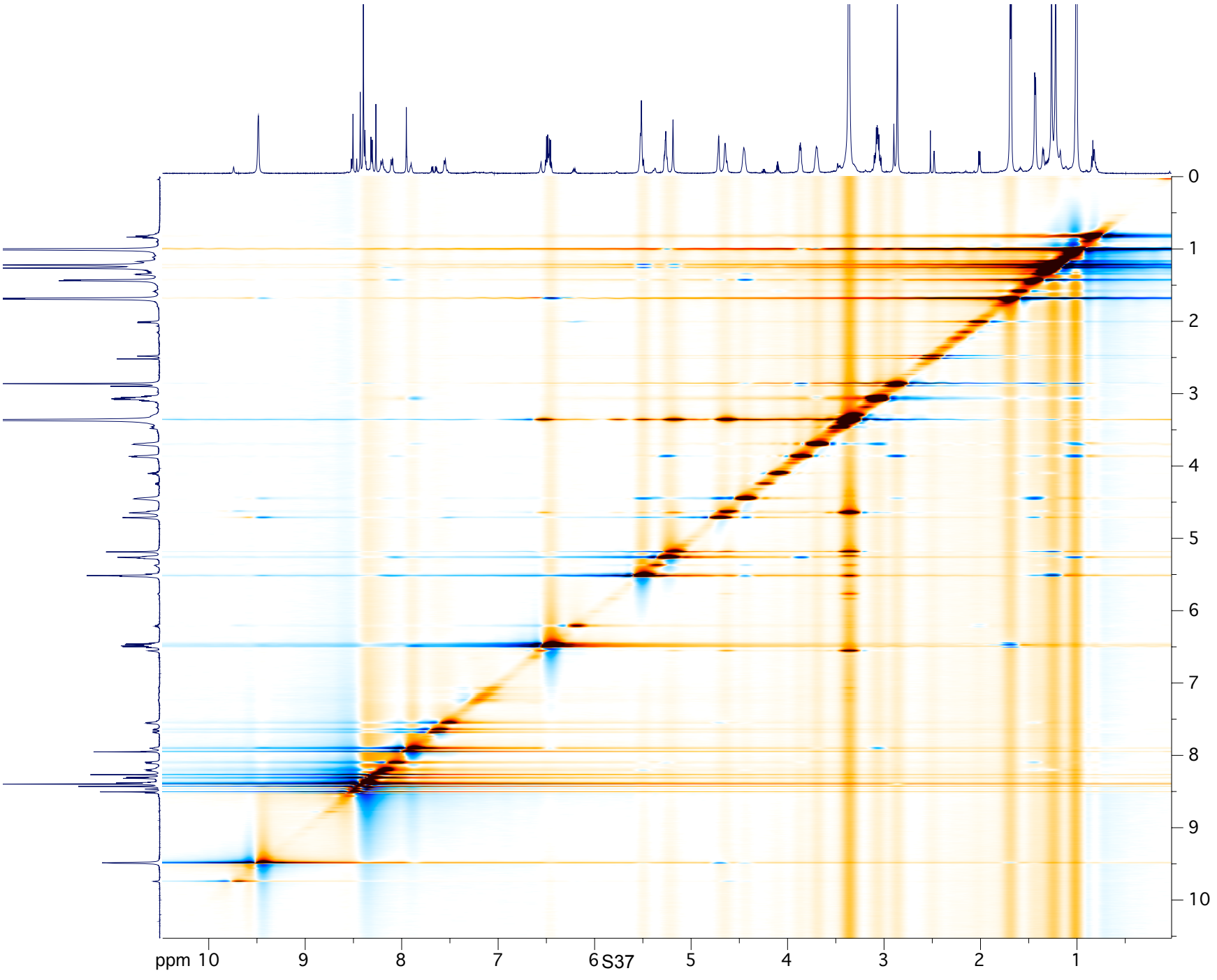
NMR Figure 5: Thiocillin II HSQC spectrum (600 MHz)



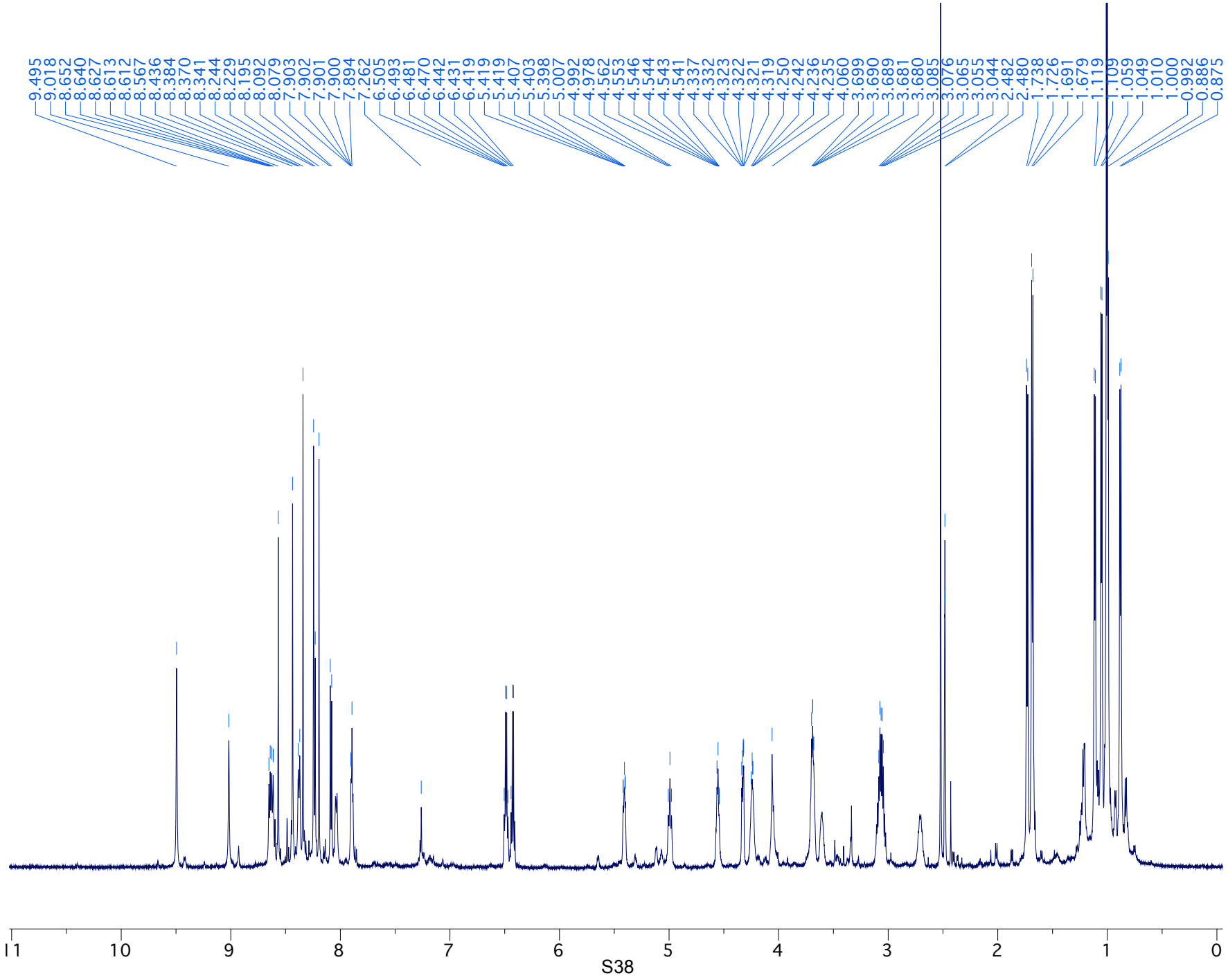
NMR Figure 6: Thiocillin II TOCSY spectrum (600 MHz)



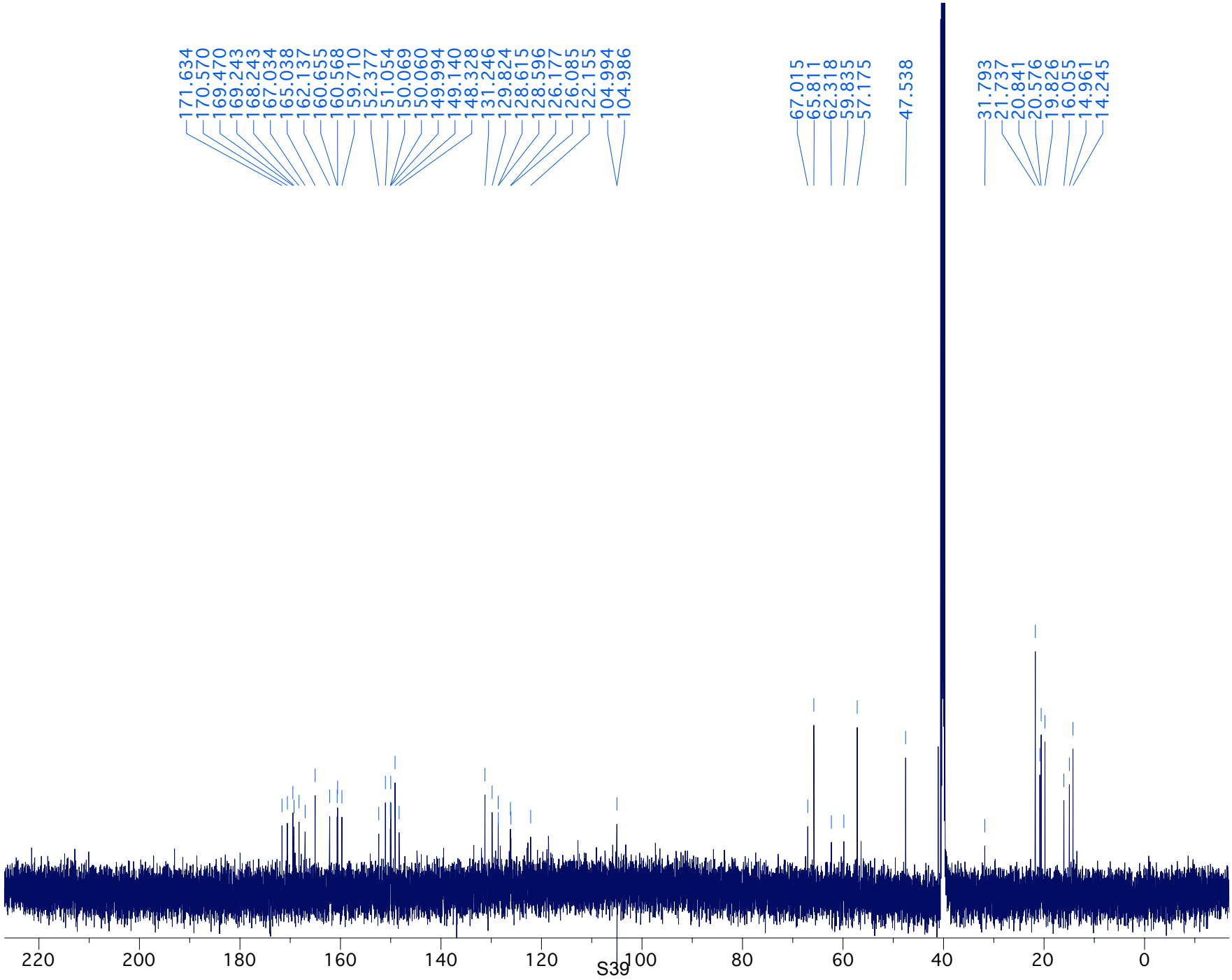
NMR Figure 7: Thiocillin II ROESY spectrum (600 MHz)



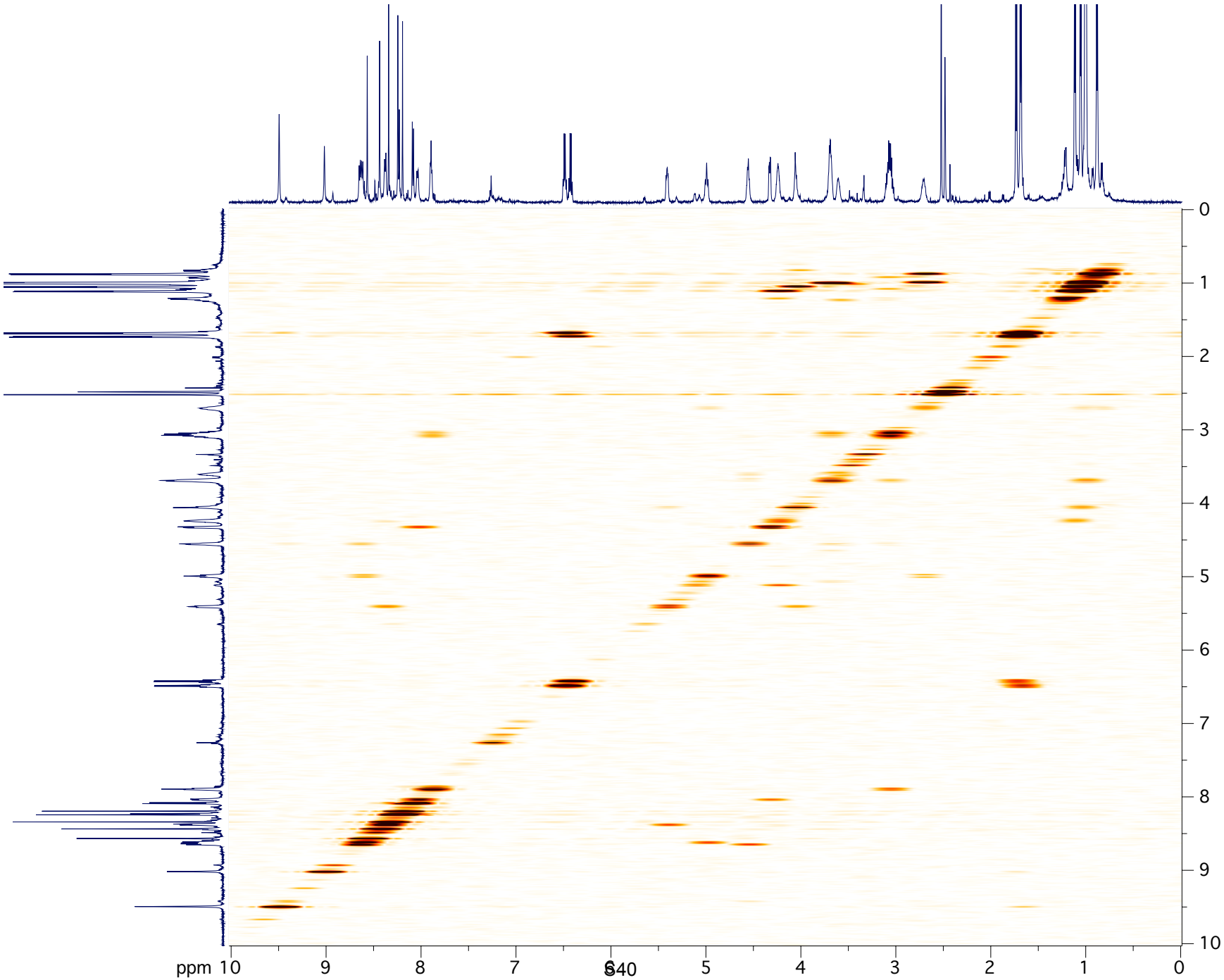
NMR Figure 8: C2S alcohol 1H NMR (600 MHz)



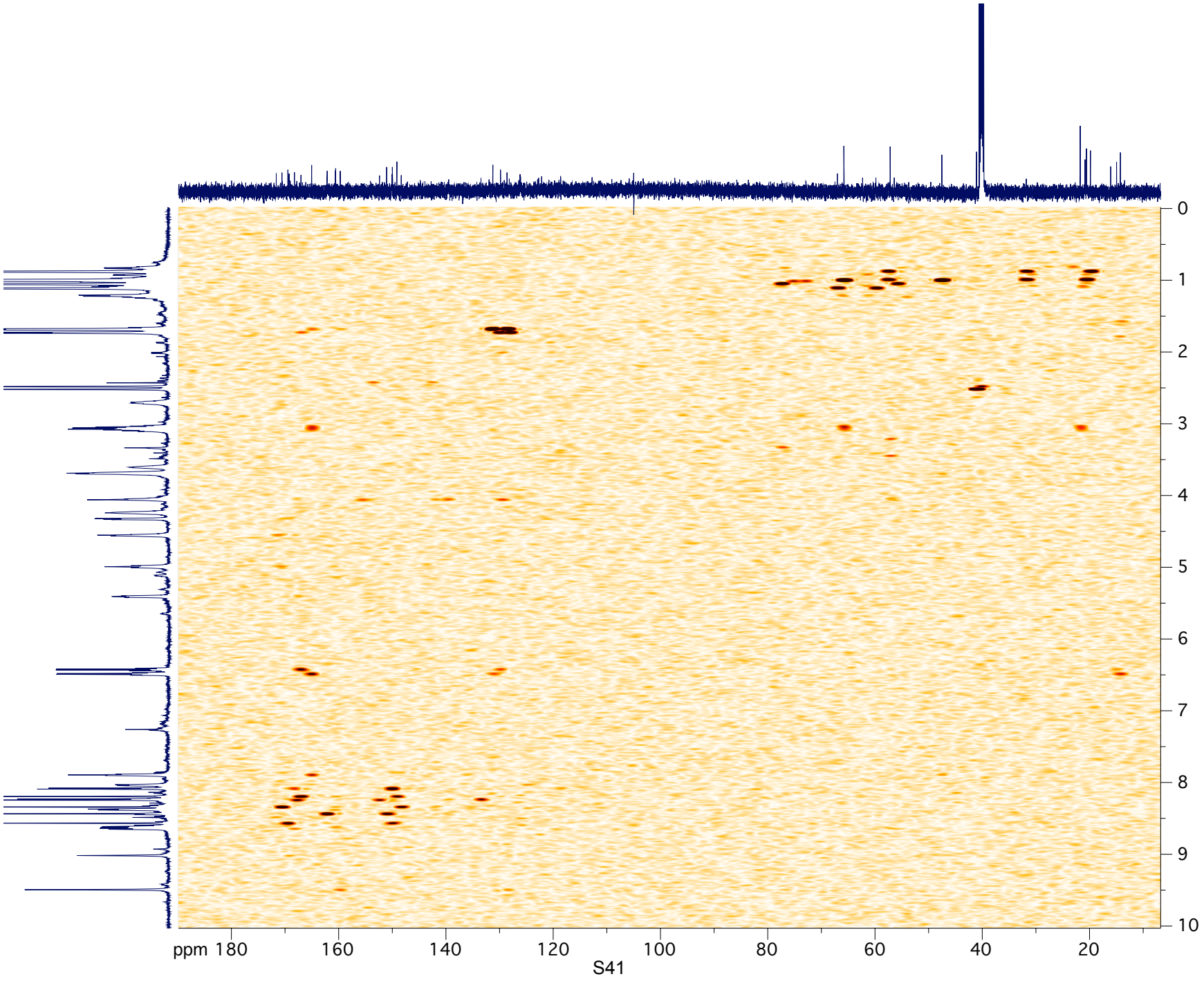
NMR Figure 9: C2S alcohol 13C NMR (150.6 MHz)



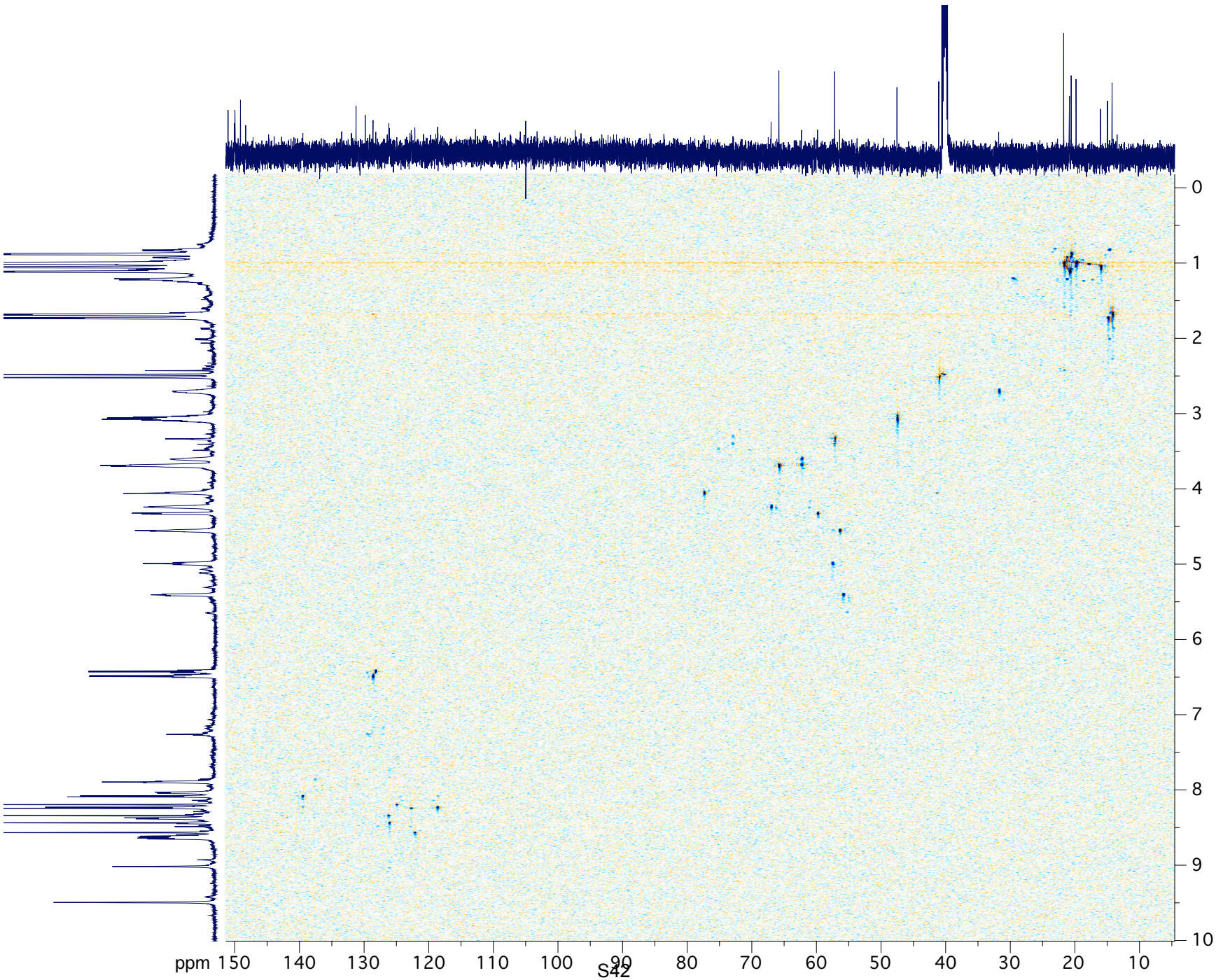
NMR Figure 10: C2S alcohol COSY spectrum (600 MHz)



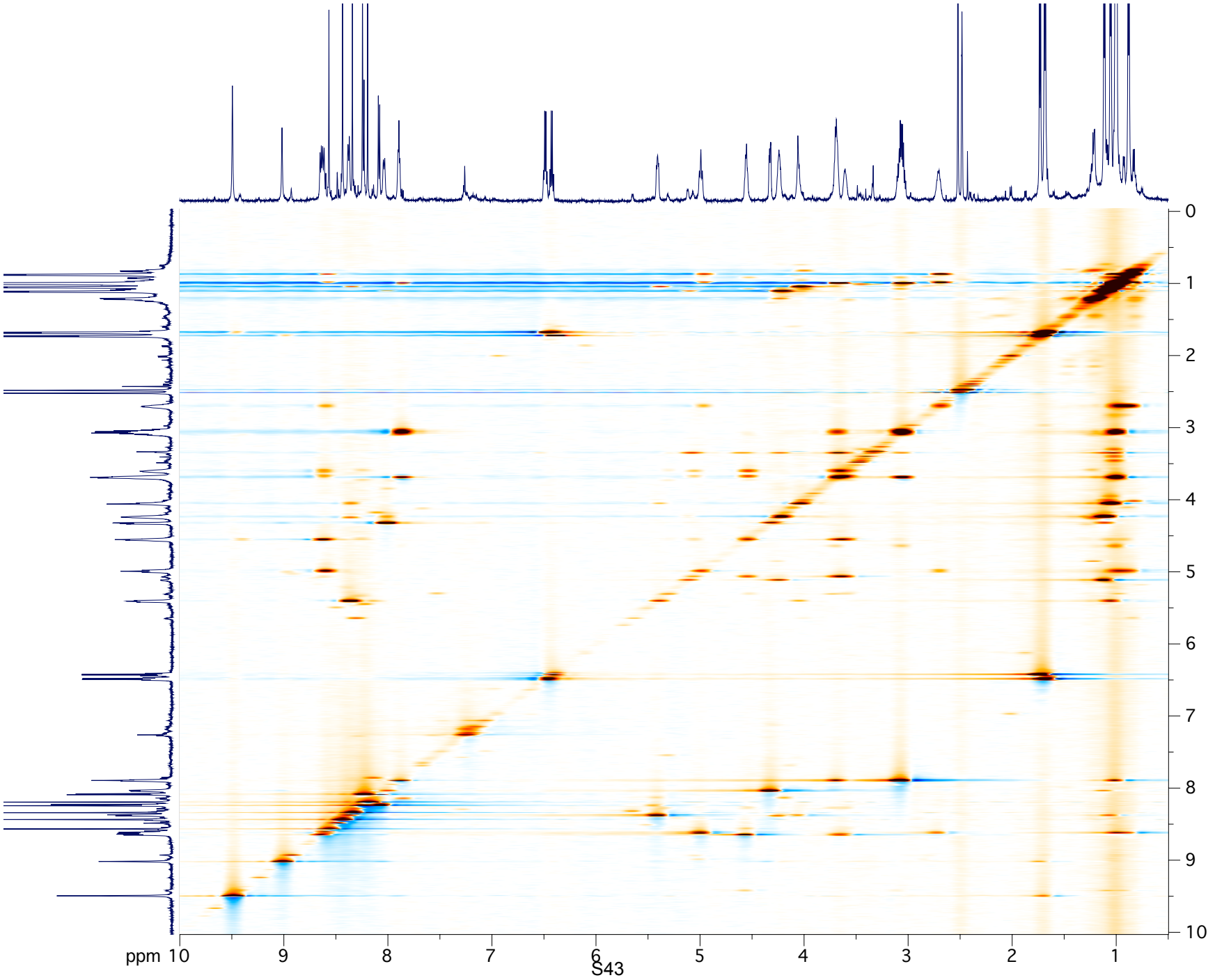
NMR Figure 11: C2S alcohol HMBC spectrum (600 MHz)



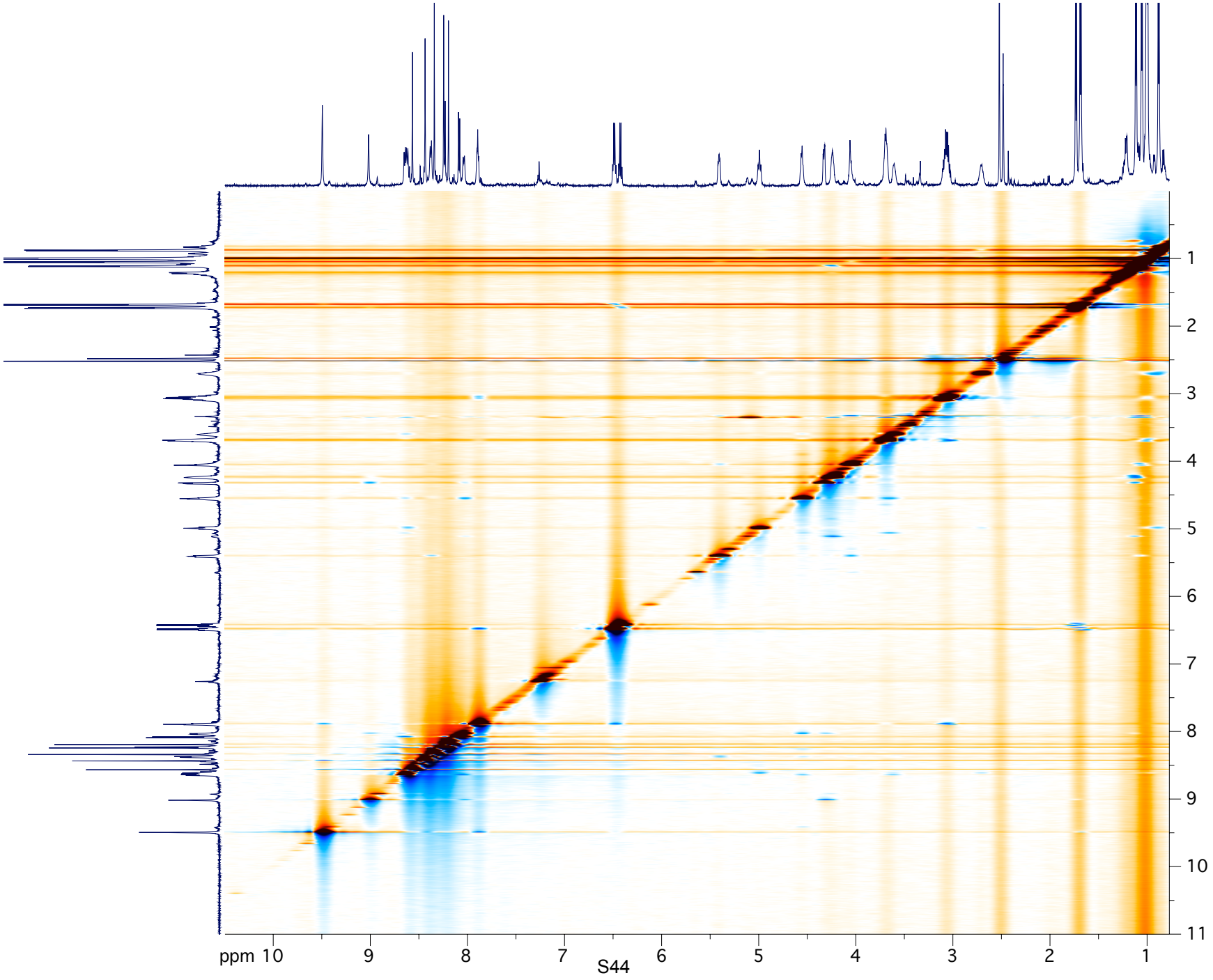
NMR Figure 12: C2S alcohol HSQC spectrum (600 MHz)



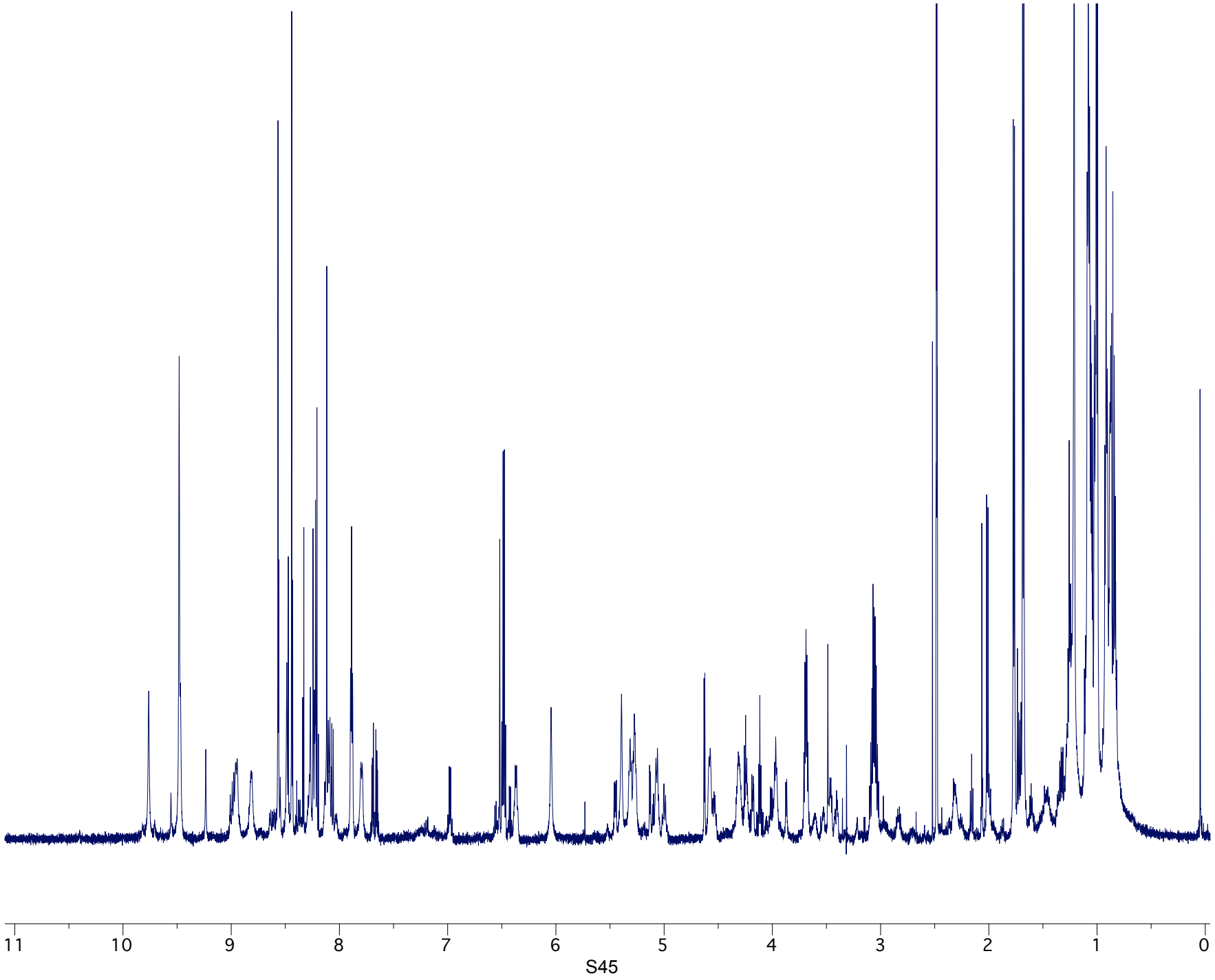
NMR Figure 13: C2S alcohol TOCSY spectrum (600 MHz)



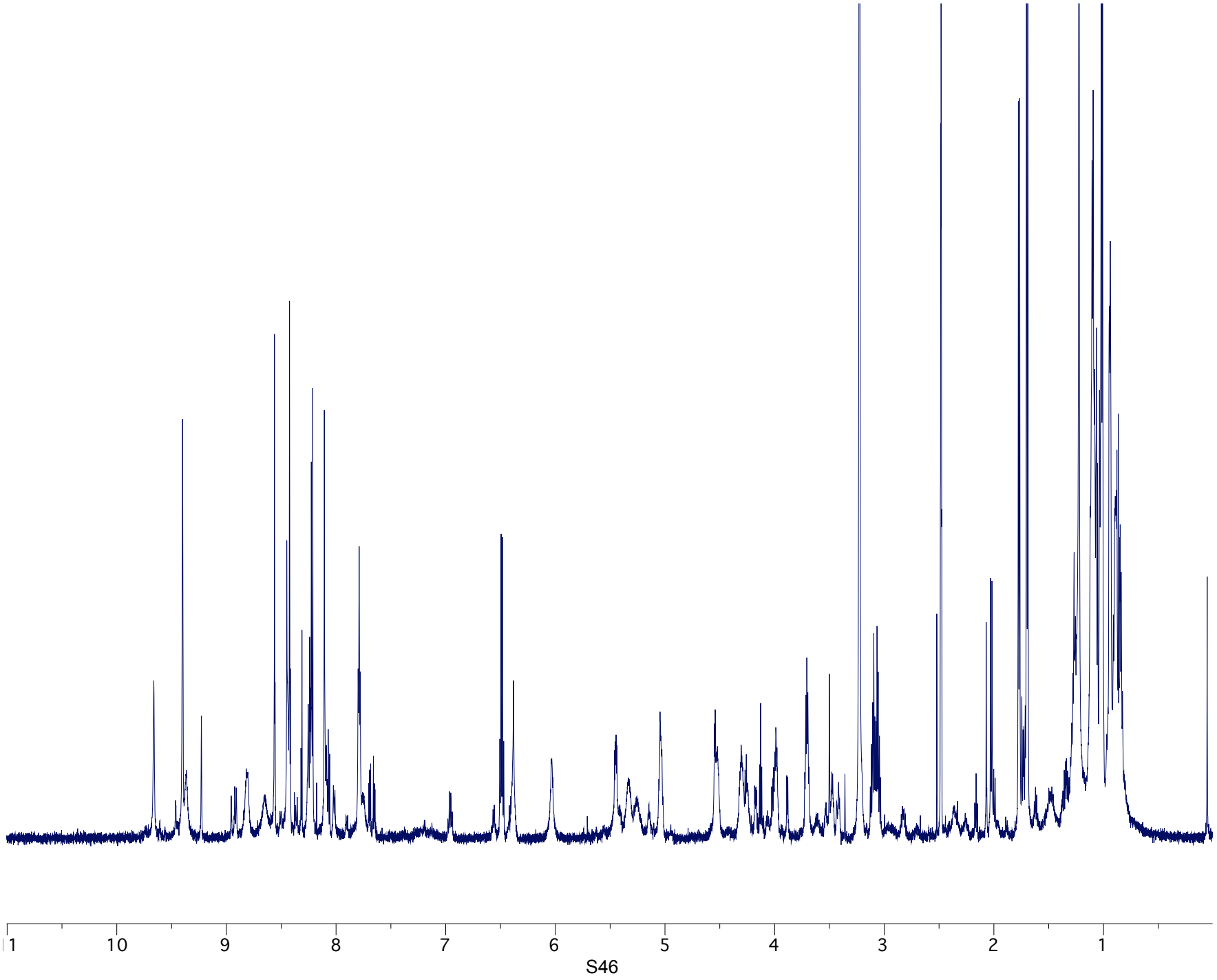
NMR Figure 14: C2S alcohol ROESY spectrum (600 MHz)



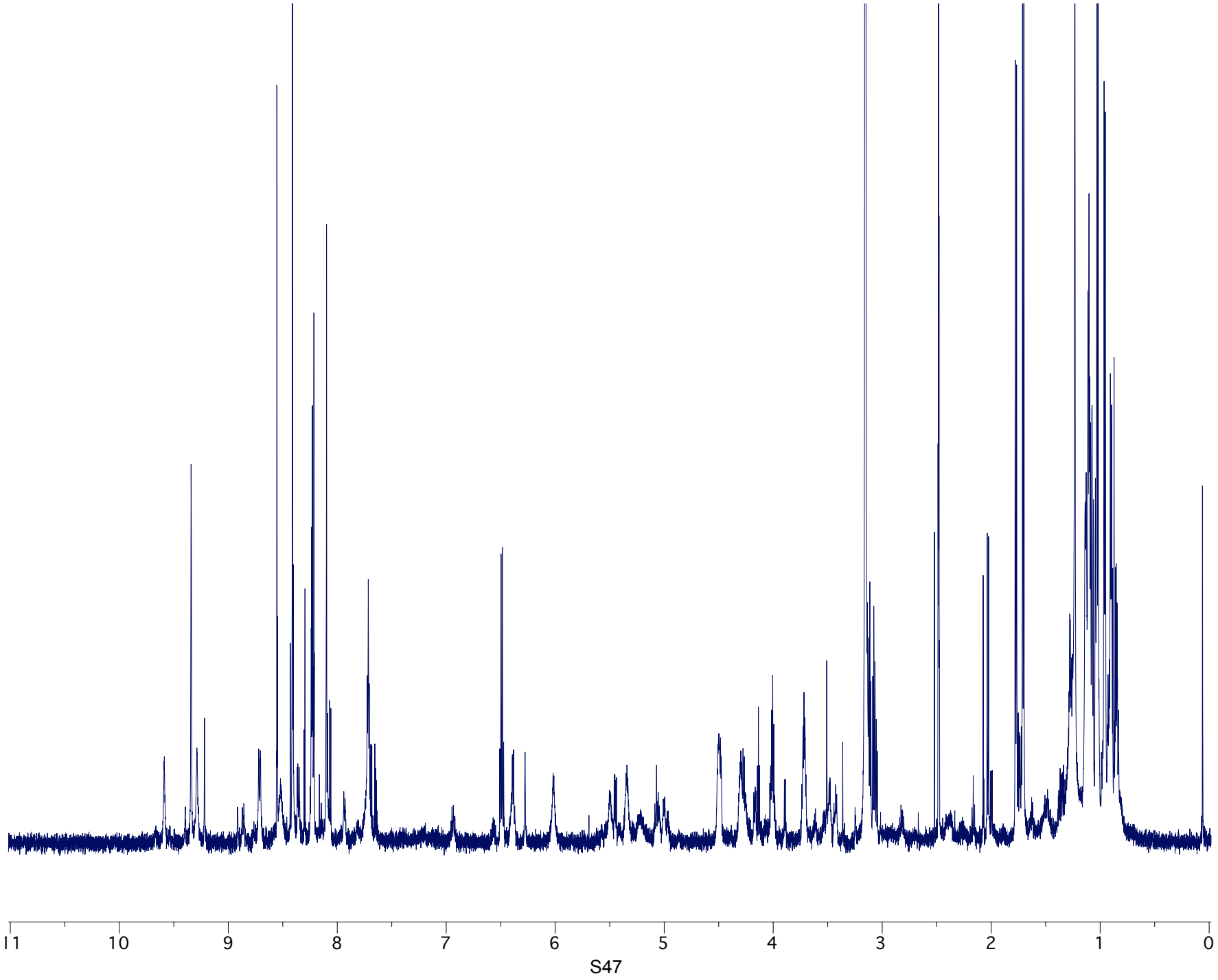
NMR Figure 15: C2S oxazoline 1H NMR at 25C (600 MHz)



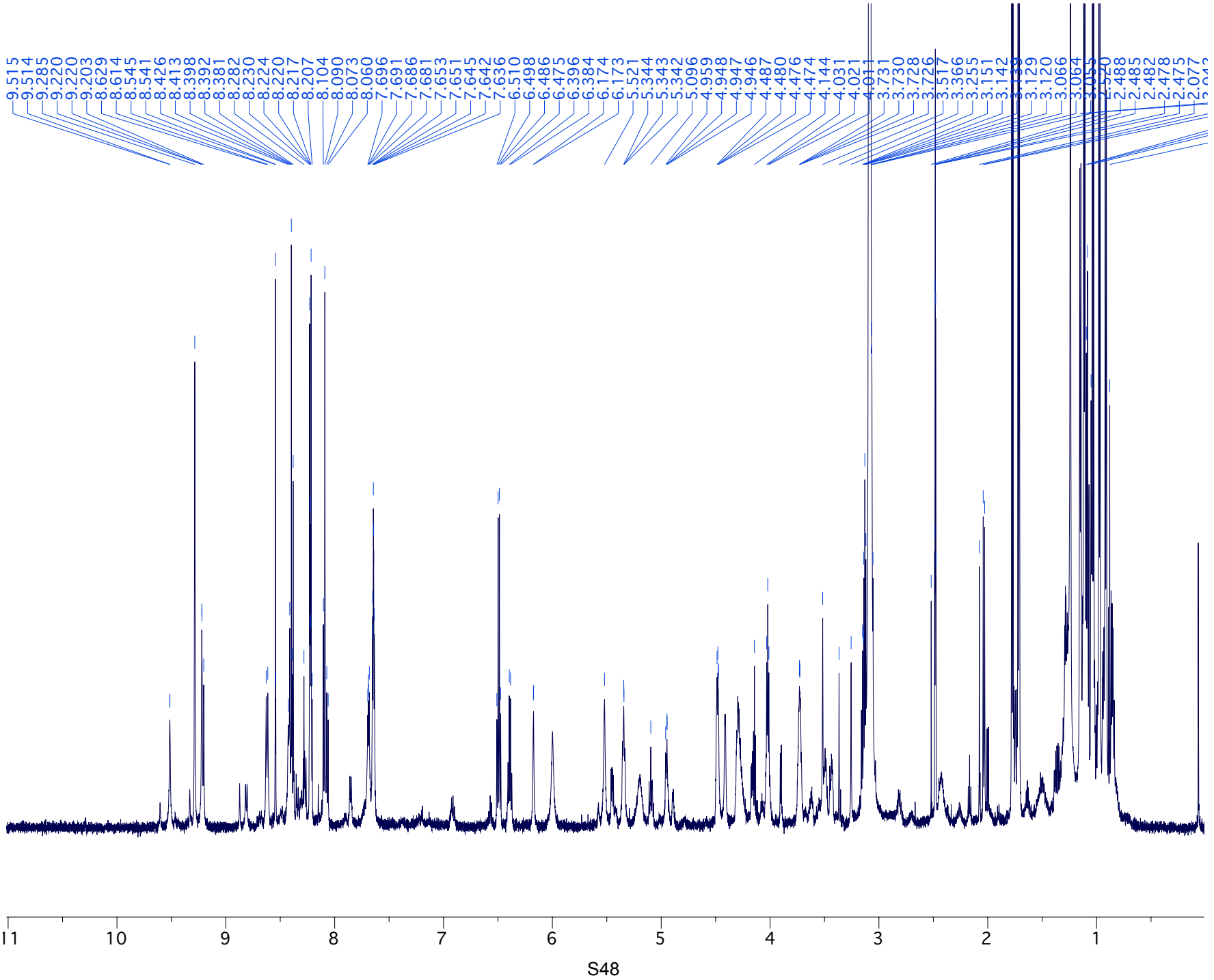
NMR Figure 16: C2S oxazoline 1H NMR at 45C (600 MHz)



NMR Figure 17: C2S oxazoline 1H NMR at 60C (600 MHz)



NMR Figure 18: C2S oxazoline 1H NMR at 75C (600 MHz)



NMR Figure 19: C2S oxazoline COSY spectrum at 75C (600 MHz)