Genetic Interception and Structural Characterization of Thiopeptide Cyclization Precursors from *Bacillus cereus*

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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. 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. ^{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. Procedures for Generation of a *tclM* Knockout and Subsequent Reintroduction

In a manner similar to that previously described for plasmid pMGA-tclΔE-H,[2] pMKO was generated from plasmid pKM082[3], containing ampicillin and erythromycin resistance cassettes and employed in excision of *tclM via* double crossover homologous recombination. Two regions of homology were cloned into this plasmid: 1) the 1kb sequence immediately upstream of the *tclM* start codon and 2) the 1 kb sequence beginning 15bp upstream of the *tclN* start codon. A rescue plasmid, pMKI, was further generated from pMKO itself, by excision of the 3'-homology region and subsequent ligation with sequence containing *tclM* and a new 3'-homology region (ABprimer112 and 130).

Removal of *tclM* from the genome of *B. cereus* ATCC 14579 was accomplished through two individual rounds of homologous recombination. In the first round, the entire plasmid was integrated into the chromosome. Transformation was effected into both wild type *B. cereus* ATCC 14579 and our previously reported *tclE-H* knockout strain; the transformation protocol, including conditions for growth, inoculation, and electroporation of competent cells from *B. cereus* ATCC14579 has been previously published.[4] Positive transformants were selected for on MLS LB-agar plates (containing 1µg/ml erythromycin and 25µg/mL lincomycin) for 36 hours at 30 °C. Individual colonies were transferred to LB liquid cultures without antibiotic and incubated for 24 hours at 30 °C. The liquid cultures were diluted 1:1000 in antibiotic free LB and again incubated for 24 hours at 30 °C. The second recombination removes the plasmid, the gene of interest, and the erythromycin resistance cassette together with it. Thus, after 7-9 rounds of dilution and growth, the cultures were diluted 10^{-5} , plated on LB agar and grown up over 24 hours at 30 °C. Colonies were then replica-plated *via* sterile velvet onto MLS LB-agar plates. Both the original, antibiotic-free plates and the fresh, MLS-supplemented plates were grown up over 24 hours at 30 °C. Colonies exhibiting growth on the antibiotic-free plates, but not on the MLS plates were re-struck for verification. Subsequent colony PCR in presence of ABprimer109 and 131 was used to confirm the *tclM* knockout; the knockout strain exhibited a loss of 924 bp in the PCR product, which was further verified by DNA sequencing.

Upon confirmation of the *tclM* knockout, the above procedure was again employed for reconstitution with pMKI. Introduction of a single copy of *tclE* and the mutant *tclE-T3A* into the *E-H,MKO* strain was achieved by our previously reported protocol.[5]

SI Table 2.1. Oligonucleotides used for cloning and *tclM* knockout

Oligo	Sequence	Role
ABprimer109	5'-GAT CGG ATC CGT TTT TTA ATA AAG GAA TGA TTA TAT G-3'	MKO-5'-homology
ABprimer110	5'-GAT CCT CGA GTT AAT CAT CCC TTT CTA CTC TTA TAC-3'	MKO-5'-homology
ABprimer112	5'-GAT CCT CGA GGG ATT TTG GTA AAG GGA GGG ATA ATA-3'	MKO-3'-homology
ABprimer131	5'-GAT CGC ATG CAA TAT TCA AAA AAT CAG ACA AAA AG-3'	MKO-3'-homology
ABprimer130	5'-GAT CCT CGA GAT GGA GCA GTA TCA TAA AAT TG-3'	MKI-3'-homology
MGAprimer 209	5'-GAA ATT ATG GGA GCG TCA TGT GCG ACA TGC GTA TGT ACA TGC AG-3'	T3A mutagenesis
MGAprimer 210	5'-CTG CAT GTA CAT ACG CAT GTC GCA CAT GAC GCT CCC ATA ATT TC-3'	T3A mutagenesis

3. Extraction of Thiocillin Compounds

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 $E_{in}MKO$ and $T3A_{in}MKO$ 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.

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 35 and 50 eV, with optimum fragmentation generally being observed at 45 eV. Representative spectra are illustrated below. Essential diagnostic peaks have been labeled.

(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.

(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.

(4) Turgeon, N.; Laflamme, C.; Ho, J.; Duchaine, C. *J. Microbiol. Methods.* **2006**, 67, 543.

(5) Bowers, A. A.; Acker, M. G.; Koglin, A.; Walsh, C. T. *J. Am. Chem. Soc.* articles ASAP.

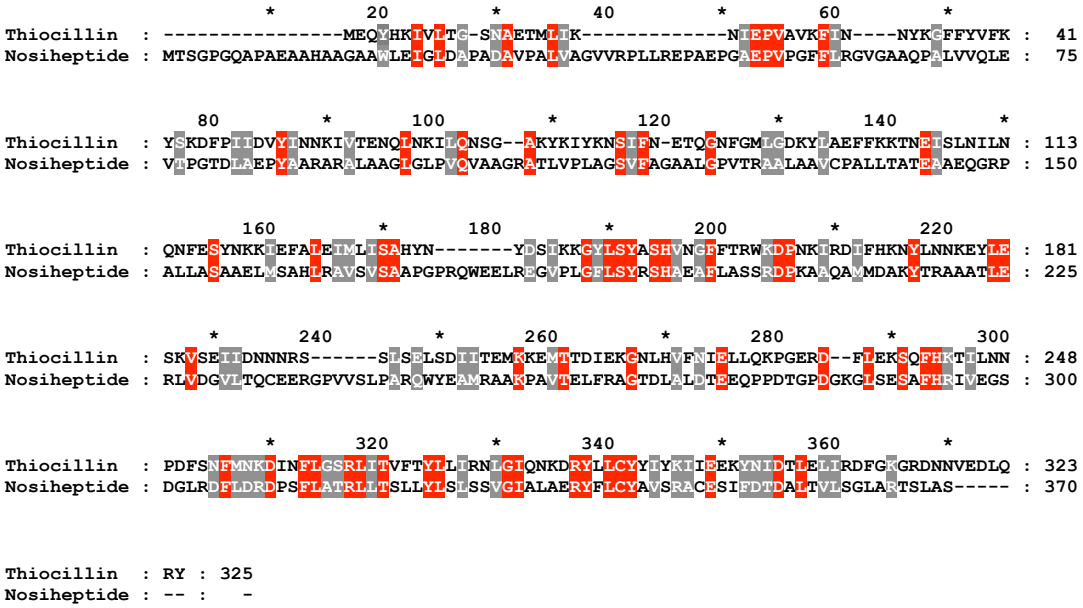
Alignments:
All protein sequences were acquired from GenBank according to published accession numbers or compound-producing species. Protein alignments were performed with ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) using the default parameters. Alignment files were analyzed using GeneDoc software version 2.6.002 for PC (http://www.psc.edu/biomed/genedoc) with similarity groups enabled.

Table 1: Results of protein alignment of TclM with proposed Diels-Alderase enzymes from other thiazolyl peptide natural product gene clusters.

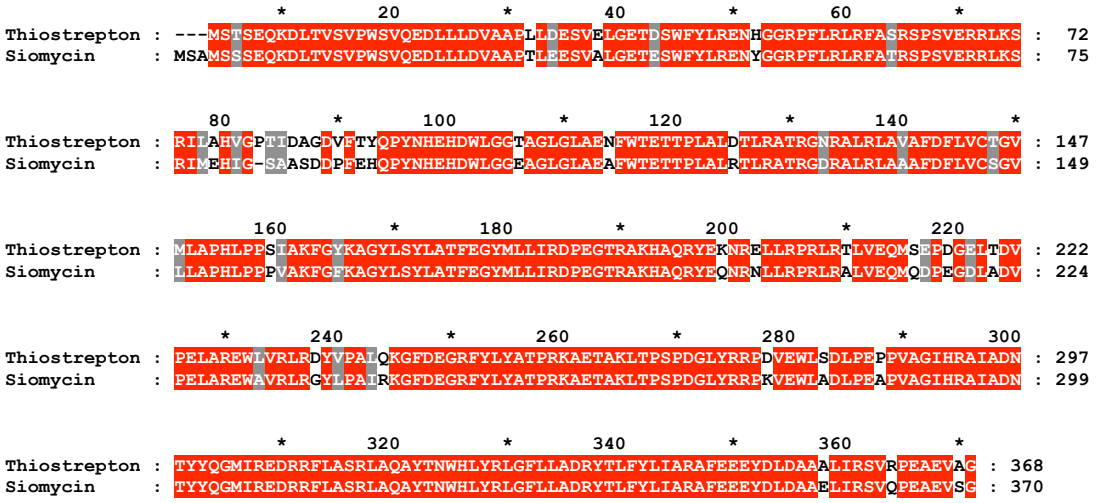
Compound	Producing Organism (Diels-Alderase)	%Identity/Similarity*
Thiocillin	Bacillus cereus ATCC 14579 (TclM)	N/A
Nosiheptide	Streptomyces actuosus (NosO)	13/27
Thiostrepton	Streptomyces laurentii (TsrE)	14/31
Siomycin	Streptomyces sioyaensis (SioL)	14/31
GE2270A	Nonomuraea sp. WU8817 (TpdD)	12/32
Thiomuracin	Nonomuraea sp. Bp3714-39 (TpdD)	11/27
Cyclothiazamycin	Streptomyces hygroscopicus (CltG)	12/29

*%Identity/Similarity compared to the Thiocillin Diels-Alderase, TclM

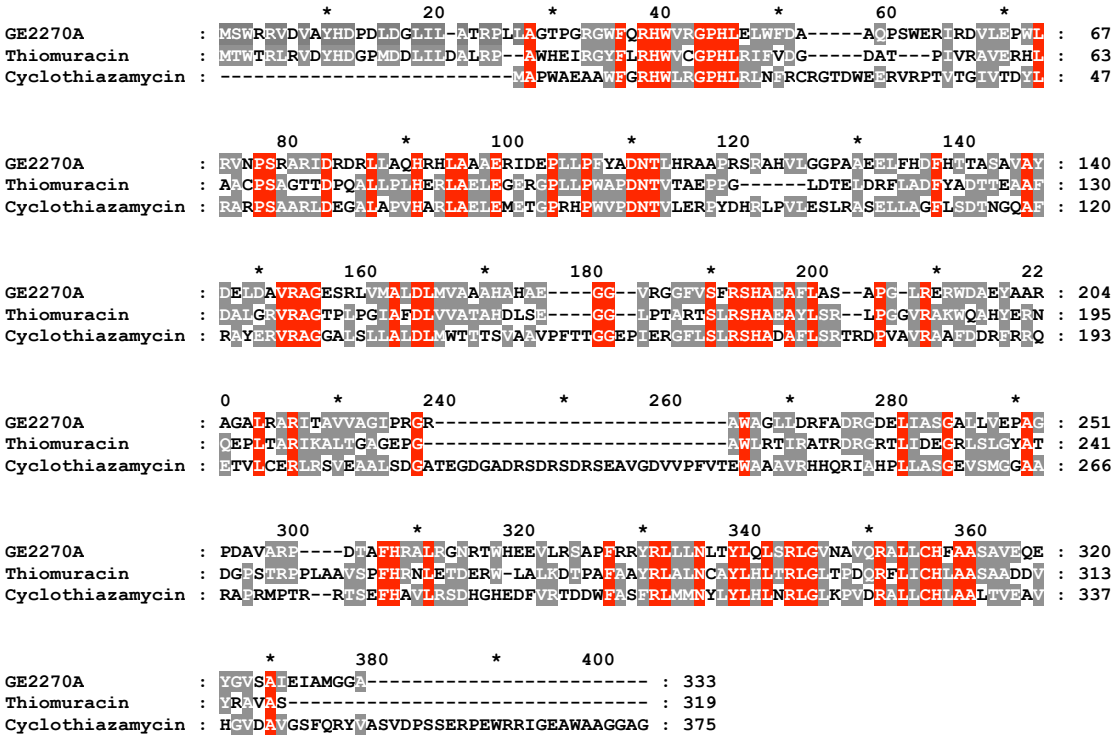
Alignment 1: TclM (Thiocillin) and NosO (Nosiheptide). Identical residues are highlighted in red. Similar residues are highlighted in gray.



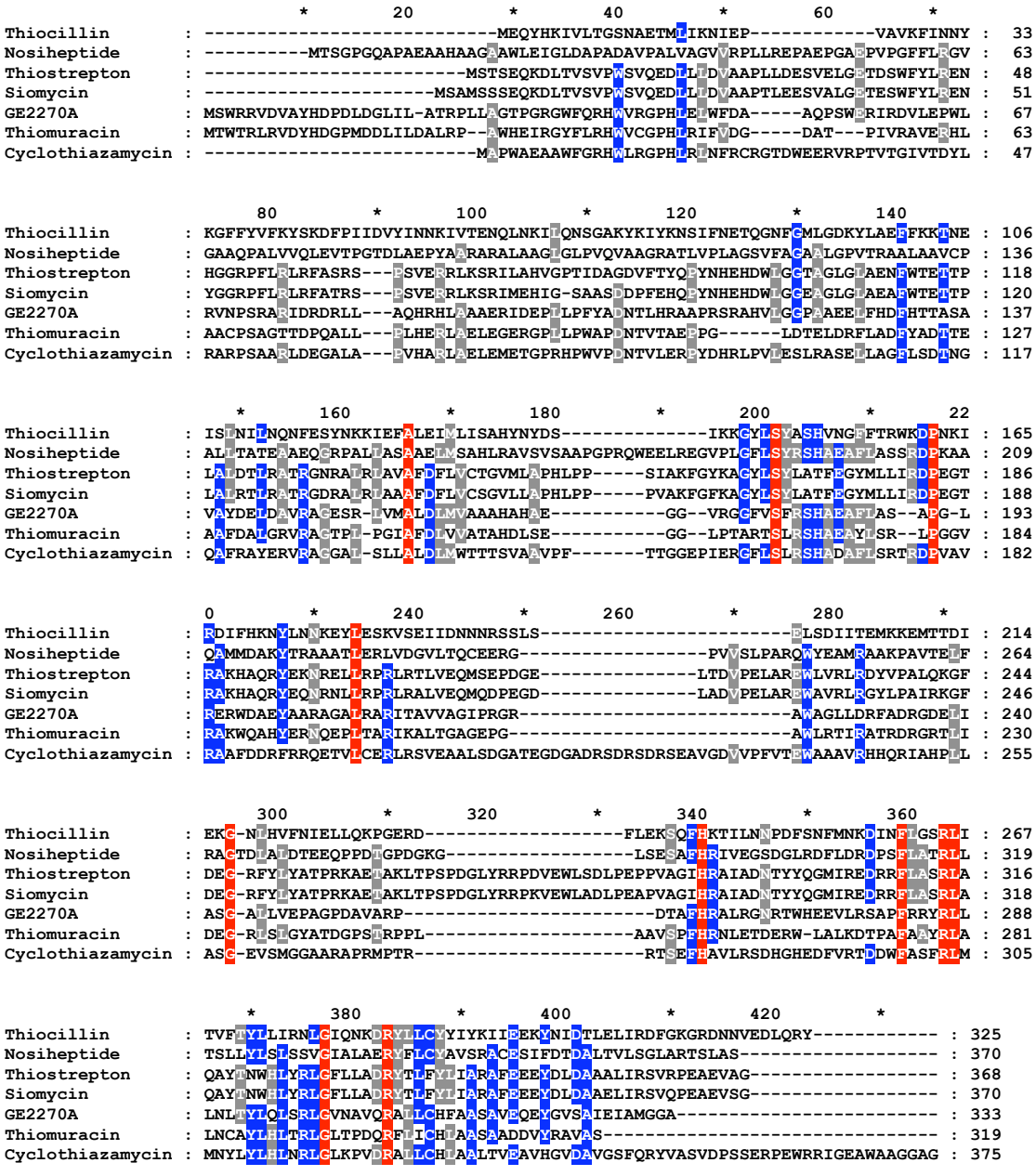
Alignment 2: TsrE (Thiostrepton) and SioL (Siomycin). Identical residues are highlighted in red. Similar residues are highlighted in gray.



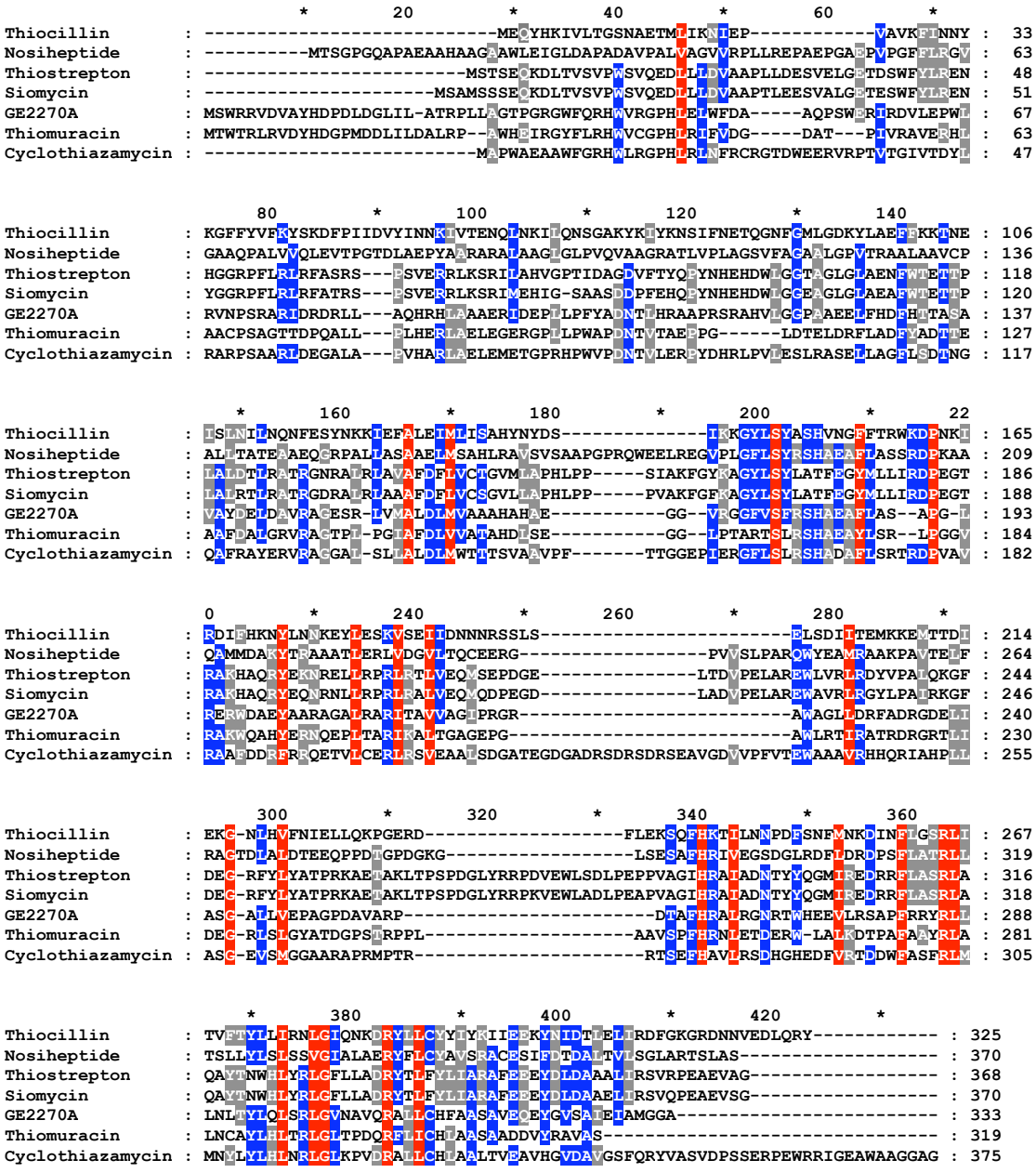
Alignment 3: TpdD (GE2270A), TpdD (Thiomuracin) and CltG (Cyclothiazamicin). Identical residues are highlighted in red. Similar residues (similarity limit = 50%) are highlighted in gray.



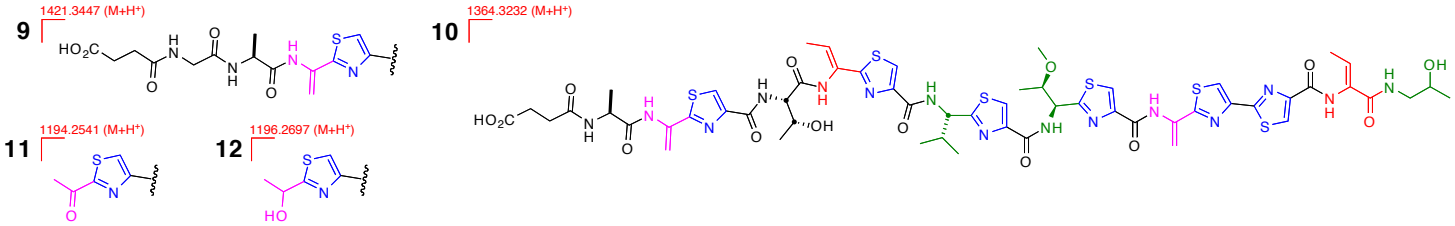
Alignment 4a: Predicted Diels-Alderase enzymes in thiazolyl peptide antibiotic biosynthesis gene clusters, depicting amino acid identity. 100% identity = red; 75% identity = blue; 50% identity = gray.



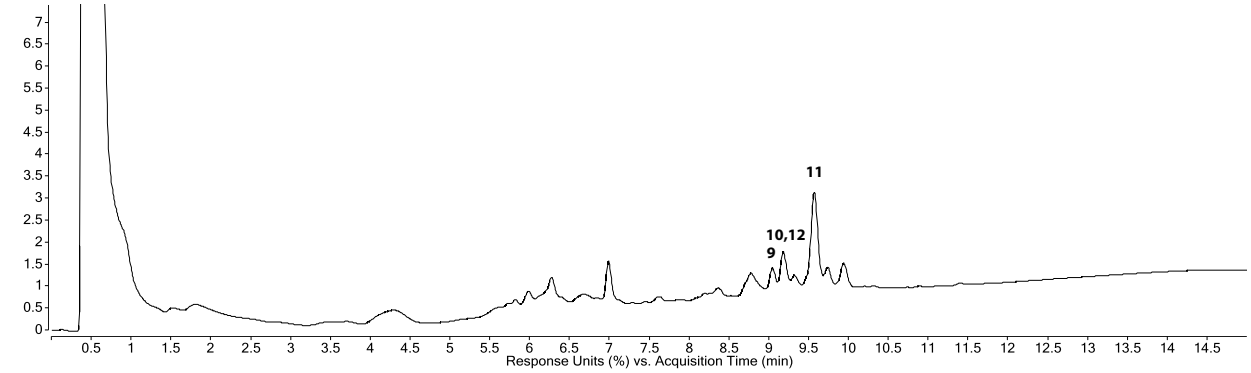
Alignment 4b: Predicted Diels-Alderase enzymes in thiazolyl peptide antibiotic biosynthesis gene clusters, depicting amino acid similarity. 100% similarity = red; 75% similarity = blue; 50% similarity = gray.

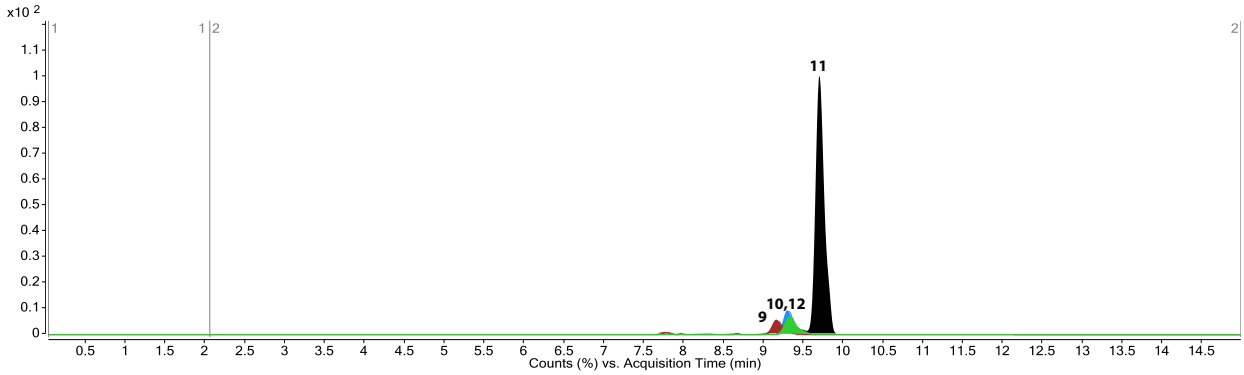


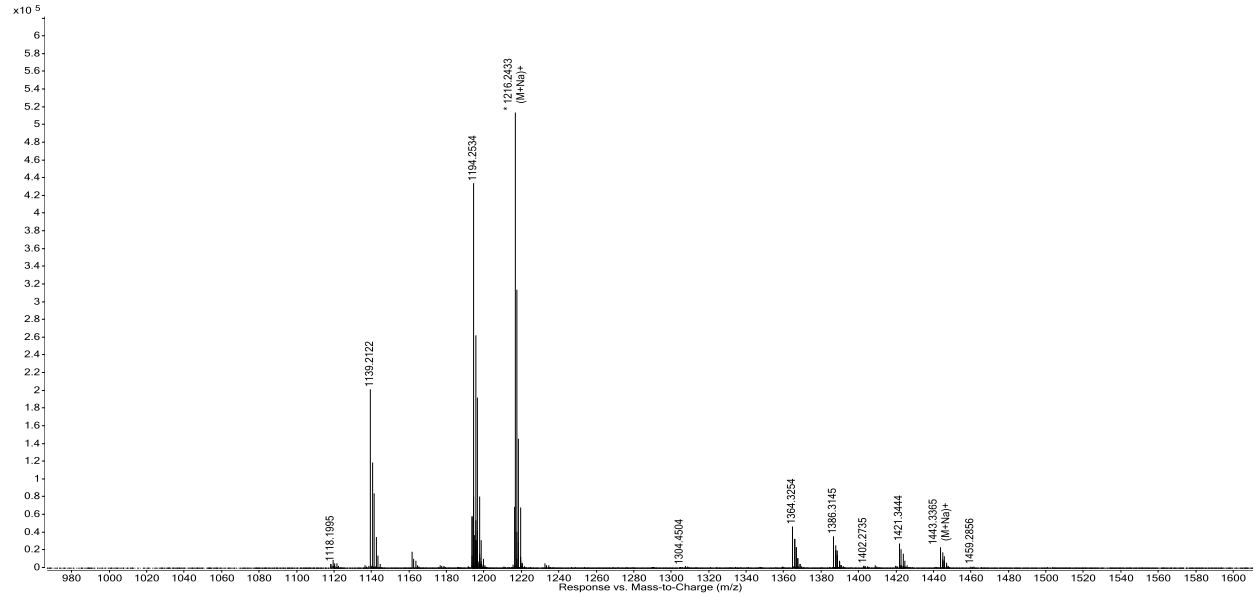
LC/MS Figure 1: Extracts from *MKO* cultures.



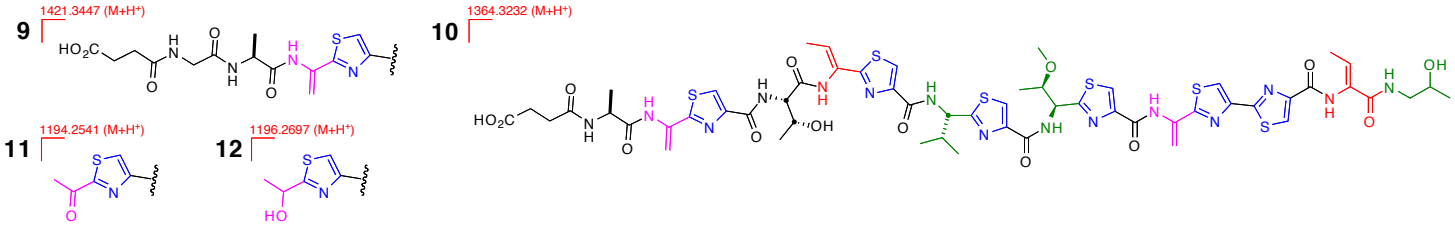
Compound	RT	Expected $(M+H)^+$	Observed $(M+H)^+$	Error (ppm)	Expected $(M+Na)^+$	Observed $(M+Na)^+$	Error (ppm)
9 (*N*-succ-Gly-Ala)	9.03	1421.3447	1421.3444	0.21	1443.3266	1443.3365	-6.86
10 (*N*-succ-Ala)	9.29	1364.3232	1364.3254	1.61	1386.3054	1386.3154	-7.21
11 (*N*-term ketone)	9.64	1194.2541	1194.2534	-0.59	1216.236	1216.2433	-6.00
12 (N-term hydroxyl)	9.38	1196.2697	1196.27	0.25	1218.2516	1218.2445	5.83



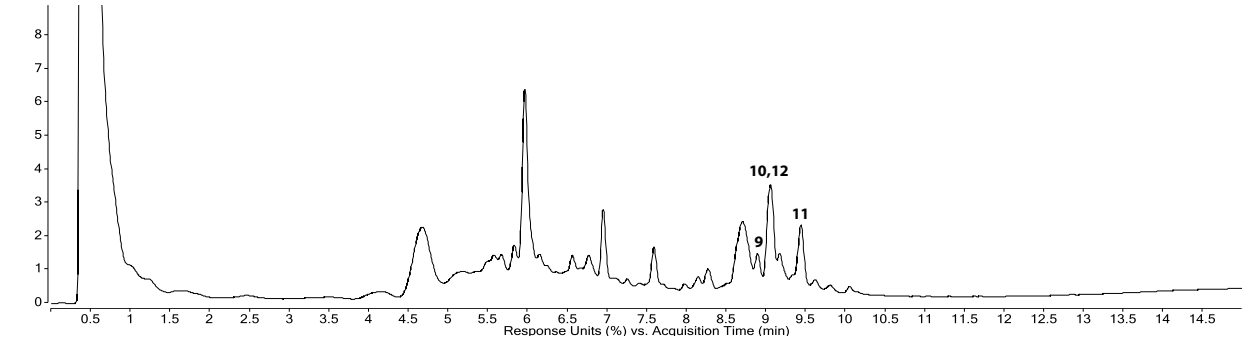


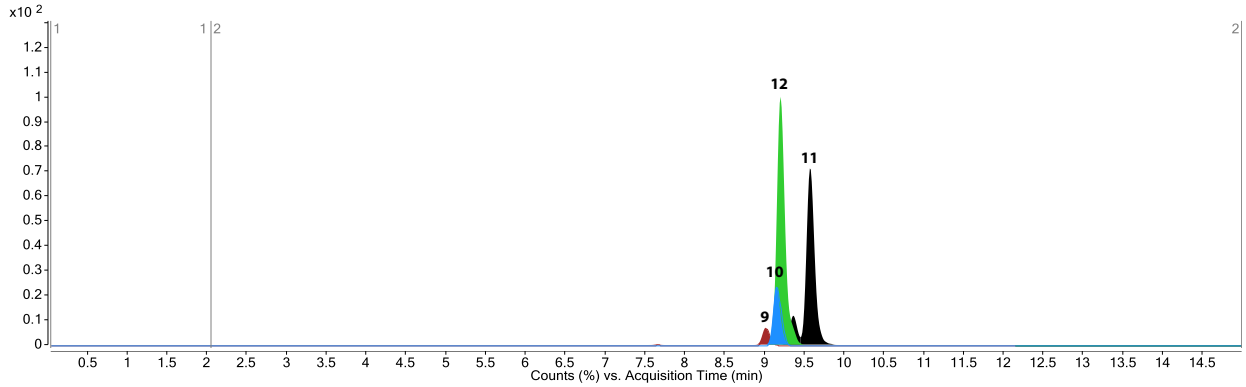


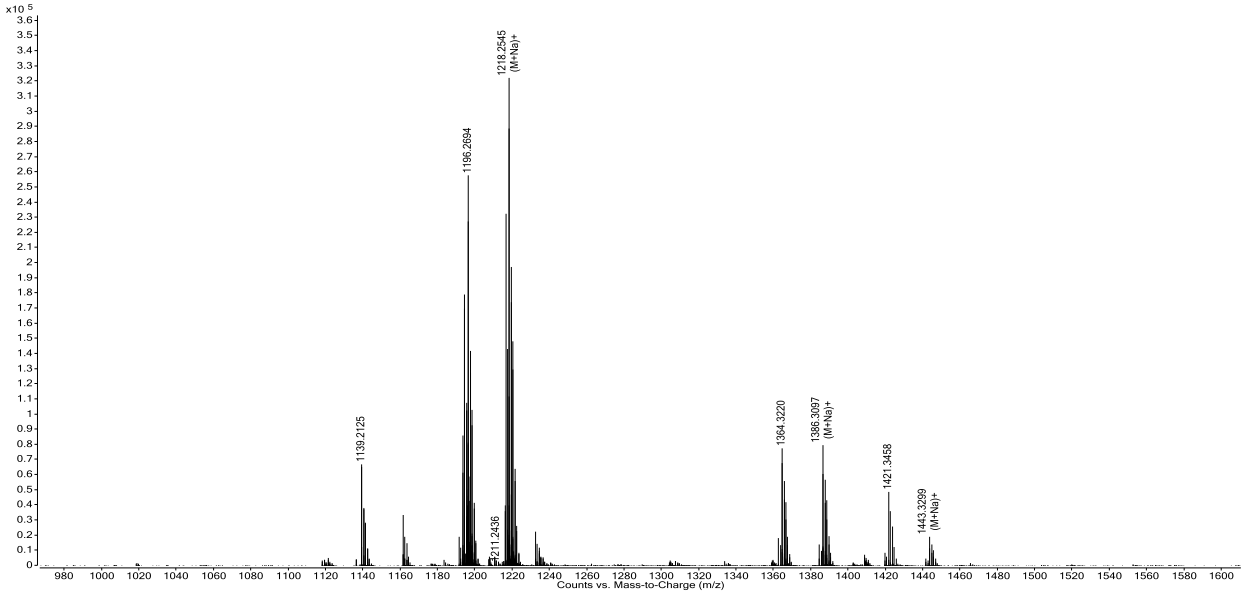
LC/MS Figure 2: Extracts from *EinMKO* cultures.



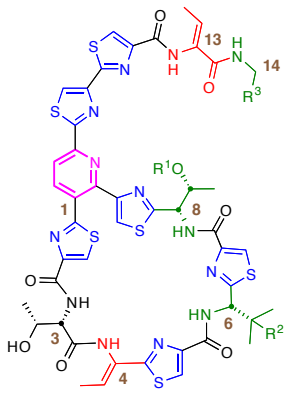
Compound	RT	Expected $(M+H)^+$	Observed $(M+H)^+$	Error (ppm)	Expected $(M+Na)^+$	Observed $(M+Na)^+$	Error (ppm)
9 (*N*-succ-Gly-Ala)	9.03	1421.3447	1421.3458	-0.77	1443.3266	1443.3299	-2.29
10 (*N*-succ-Ala)	9.29	1364.3232	1364.322	-0.88	1386.3054	1386.3097	-3.10
11 (*N*-term ketone)	9.64	1194.2541	1194.2551	0.84	1216.236	1216.238	-1.64
12 (N-term hydroxyl)	9.38	1196.2697	1196.2694	-0.25	1218.2516	1218.2545	-2.38





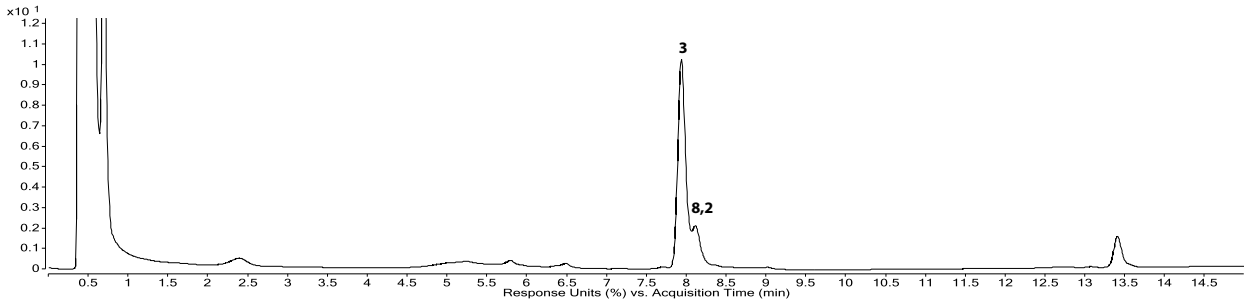


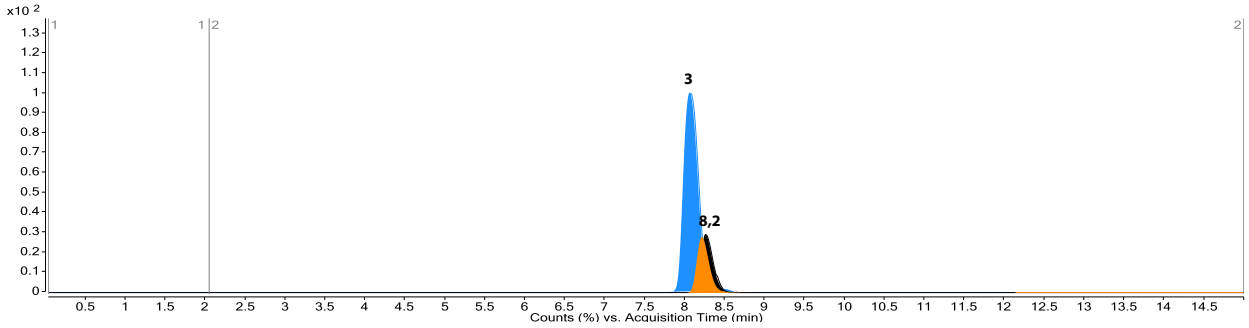
LC/MS Figure 3: Extracts from *MKI* cultures.

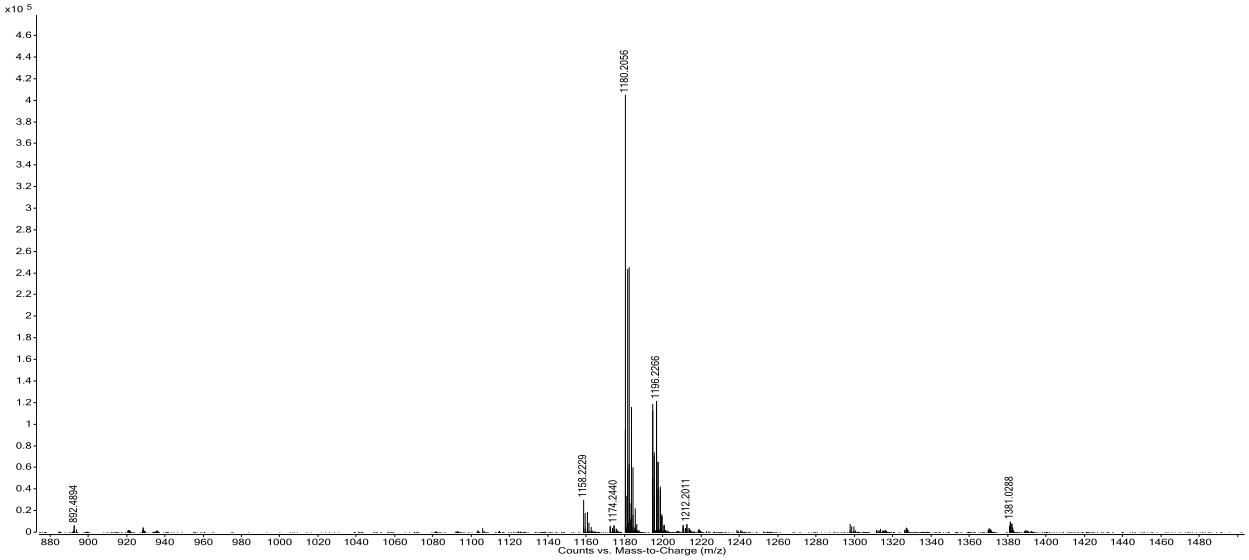


	R_1		R_2		R_3	
	H	CH_3	H	OH	=O	OH
thiocillin I (**1**)	■			■		■
thiocillin II (**2**)		■		■		■
thiocillin III (**3**)		■	■			■
thiocillin IV (**4**)		■	■		■	
micrococcin P1 (**5**)	■		■			■
micrococcin P2 (**6**)	■		■		■	
YM-266183 (**7**)	■			■	■	
YM-266184 (**8**)		■		■	■	

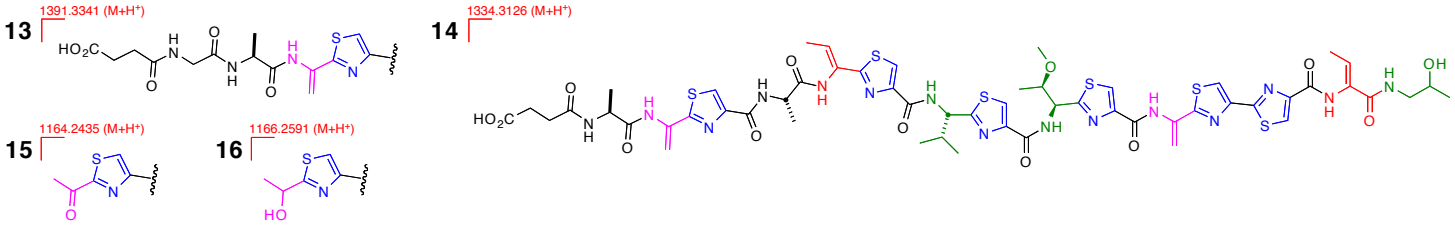
Compound	RT	Expected $(M+H)^+$	Observed $(M+H)^+$	Error (ppm)	Expected $(M+Na)^+$	Observed $(M+Na)^+$	Error (ppm)
2 (thiocillin II)	8.15	1174.2278	1174.244	-13.80	1196.2098	1196.2206	-9.03
3 (thiocillin III)	7.95	1158.2329	1158.2229	-8.63	1180.2149	1180.2056	7.88
8 (YM-266184)	8.10	1172.2122	1172.21	-1.88	1194.1941	1194.2001	-5.02



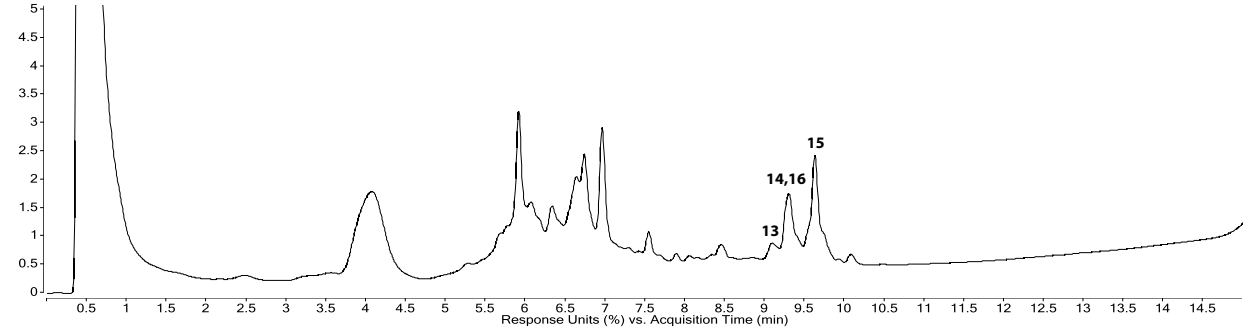


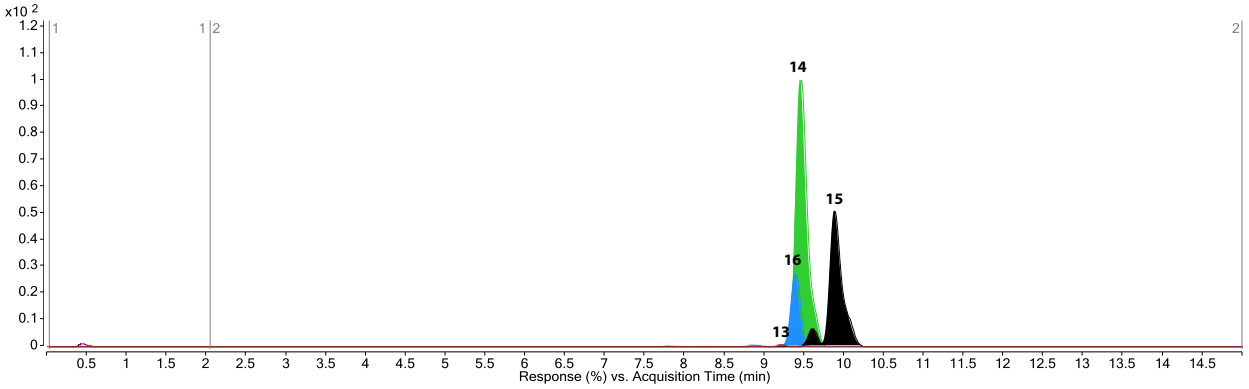


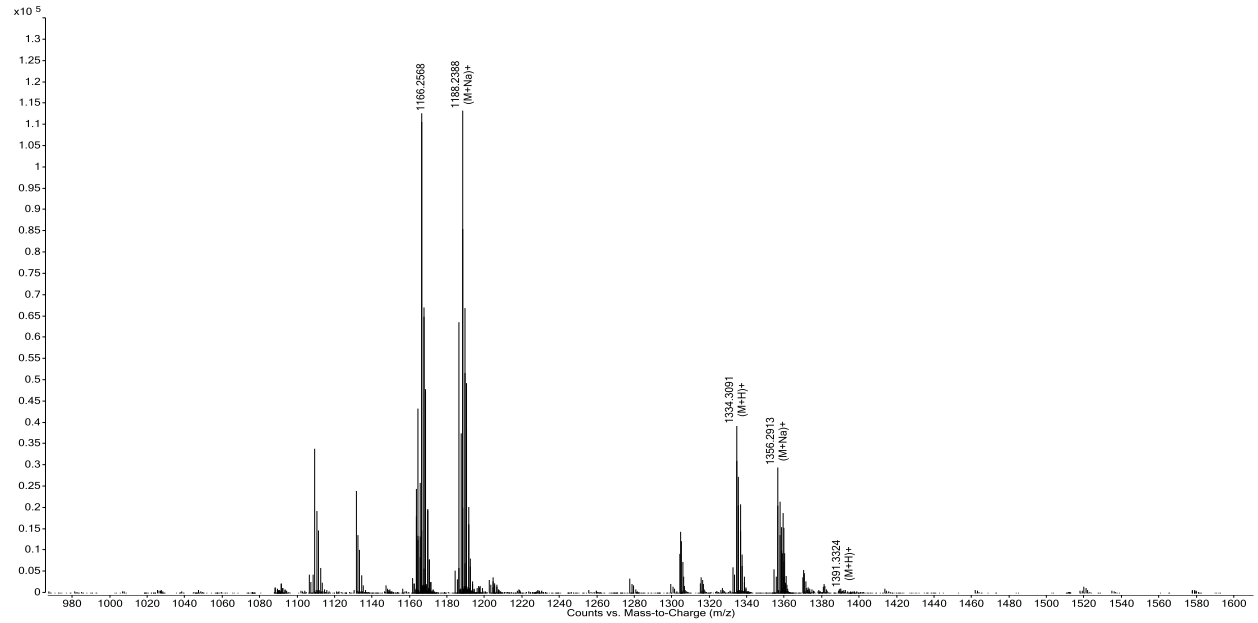
LC/MS Figure 4: Extracts from *T3A*$_{in}$*MKO* cultures.



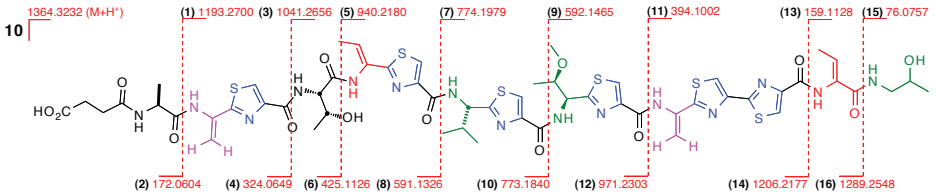
Compound	RT	Expected $(M+H)^+$	Observed $(M+H)^+$	Error (ppm)	Expected $(M+Na)^+$	Observed $(M+Na)^+$	Error (ppm)
13 (*N*-succ-Gly-Ala)	9.26	1391.3341	1391.3322	1.37	1413.316	1413.32	-2.83
14 (*N*-succ-Ala)	9.44	1334.3126	1334.3091	-2.62	1356.2946	1356.2913	2.43
15 (*N*-term ketone)	9.95	1164.2435	1164.244	0.43	1186.2254	1186.2274	-1.69
16 (N-term hydroxyl)	9.48	1166.2591	1166.2568	-1.97	1188.2411	1188.2388	1.94



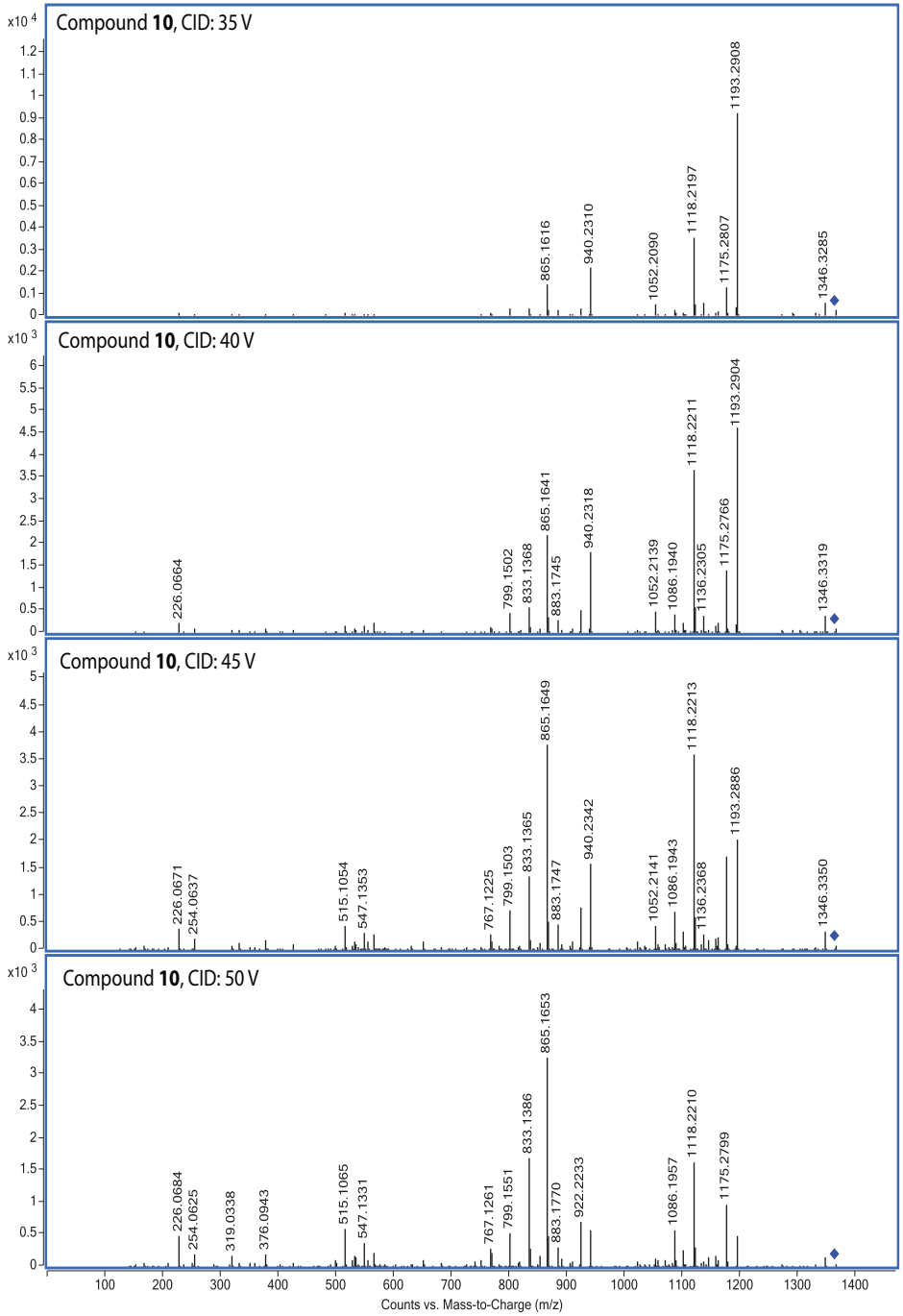




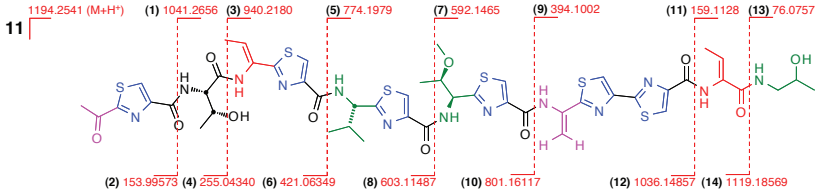
MS/MS Figure 1: Compound **10** (1364.3232).



Fragment	Observed $(M+H)^+$	Expected $(M+H)^+$	Error (ppm)
Parent-H_2O	1346.3285	1346.31268	-11.75
1	1193.2908	1193.27008	-17.36
1-H_2O	1175.2807	1175.25952	-18.02
16-2	1118.2197	1118.20167	-16.12
16-2-CH_3OH	1086.194	1086.17546	-17.07
14-2	1052.209	1052.19111	-17.00
5	940.231	940.21797	-13.86
5-H_2O	922.2233	922.20741	-17.23
14-4	883.1745	883.16012	-16.28
14-4-H_2O	865.1616	865.14956	-13.92
14-4-H_2O-CH_3OH	833.1368	833.12334	-16.16
14-6+NH_2	799.1502	799.13899	-14.03
14-6+NH_2-CH_3OH	767.1225	767.11278	-12.67
12-6	547.1353	547.12504	-18.75
12-6-CH_3OH	515.1054	515.09883	-12.75
11-H_2O	376.0943	376.08964	-12.39
16-12	319.0338	319.03179	-6.30
6-2	254.0625	254.05939	-12.24
6-2-CO	226.0664	226.06447	-8.54



MSMS Figure 2: Compound **11** (1194.2541).



Fragment	Observed $(M+H)^+$	Expected $(M+H)^+$	Error (ppm)
Parent-H_2O	1176.2572	1176.24354	-11.61
Parent-H_2O-CH_3OH	1144.2334	1144.21732	-14.05
14	1119.1996	1119.18569	-12.43
14-CH_3OH	1087.1707	1087.15947	-10.33
12+NH_2	1053.1847	1053.17512	-9.10
3	940.2309	940.21797	-13.75
3-H_2O	922.2191	922.20741	-12.68
12-2-H_2O	865.1602	865.14956	-12.30
12-2-H_2O-CH_3OH	833.1347	833.12334	-13.64
12-4+NH_2	799.147	799.13899	-10.02
12-4+NH_2-CH_3OH	767.1235	767.11278	-13.97
10-4	547.1332	547.12504	-14.91
10-4-CH_3OH	515.1031	515.09883	-8.29
9-H_2O	376.0942	376.08964	-12.12
14-10	319.0349	319.03179	-9.75
4-CO	227.0501	227.04849	-7.09

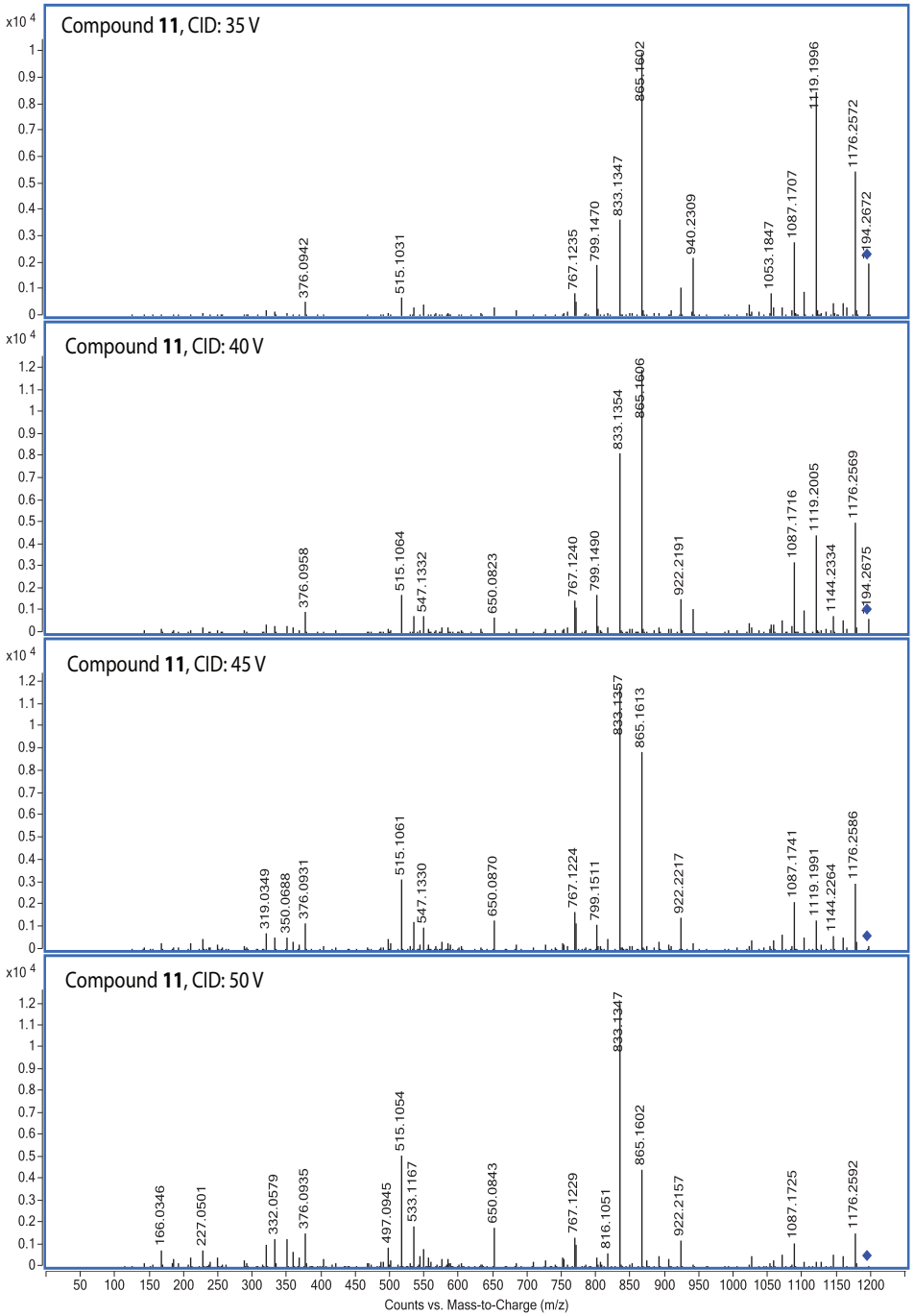
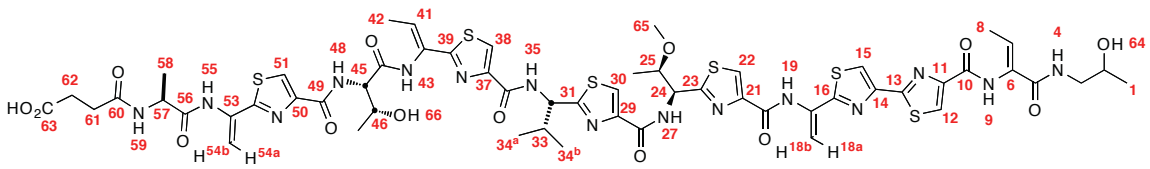


Table of 1H & 13C NMR shifts (ppm) for Compound **10** (1364.3232) (600 MHz, DMSO-*d*6).[a]

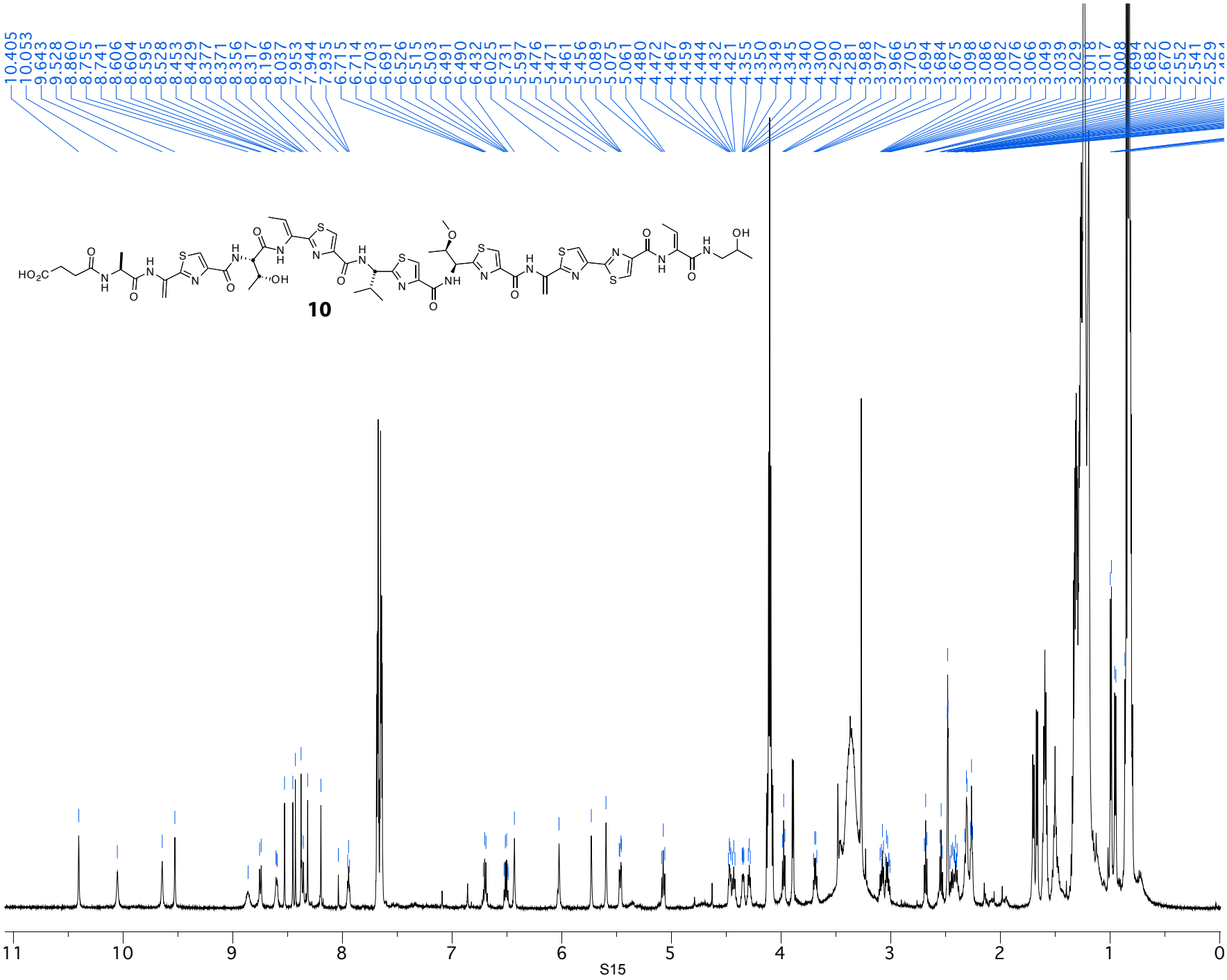


Shift (ppm)	Integration	Proton	HSQC	COSY1	COSY2	TOCSY1	TOCSY2	HMBC1	HMBC2	HMBC3
10.41	1H	**19**	N-H	x	x	6.43	x	166.3	x	x
10.05	1H	**43**	N-H	x	x	6.70	x	131.0	x	x
9.64	1H	**55**	N-H	x	x	6.03	x	173.2	x	x
9.53	1H	**9**	N-H	x	x	1.69	6.51	159.9	x	x
8.86	1H	**48**	N-H	4.46-4.48	x	4.29	x	x	x	x
8.75	1H	**35**	N-H	5.08	x	0.86	0.95	161.2	x	x
8.60	1H	**59**	N-H	4.42-4.44	x	1.29	x	173.3	x	x
8.53	1H	**12** (Thz 12)	126.0	x	x	x	x	162.0	151.2	x
8.45	1H	**15** (Thz 11)	120.4	x	x	x	x	166.4	147.6	x
8.43	1H	**22** (Thz 9)	126.7	x	x	x	x	172.9	149.3	x
8.38	1H	**30** (Thz 7)	126.2	x	x	x	x	173.8	148.3	x
8.36	1H	**27**	N-H	5.47	x	4.34-4.36	x	161.0	56.2	x
8.32	1H	**51** (Thz 2)	125.9	x	x	x	x	165.5	150.0	x
8.20	1H	**38** (Thz 5)	124.9	x	x	x	x	168.6	149.8	x
7.94	1H	**4**	N-H	3.00-3.03	3.07-3.11	0.99	3.68-3.71	165.0	x	x
6.70	1H	**41**	126.9	1.67	x	10.05	x	168.0	131.0	14.2
6.51	1H	**7**	128.7	1.58-1.62	x	9.53	x	165.0	131.5	13.8
6.43	1H	**18a**	104.0	x	x	10.41	x	166.4	134.2	x
6.03	1H	**54a**	105.9	x	x	9.64	x	165.5	134.9	x
5.73	1H	**18b**	104.0	x	x	x	x	166.4	x	x
5.60	1H	**54b**	105.9	x	x	x	x	165.5	x	x
5.47	1H	**24**	56.2	4.34-4.36	8.36	1.28	x	16.4	x	x
5.08	1H	**32**	57.6	2.42-2.46	8.75	0.86	0.95	173.8	x	x
4.46-4.48	1H	**45**	60.8	4.29	8.86	1.22	x	x	x	x
4.42-4.44	1H	**57**	50.1	1.29	8.6	x	x	173.7	18.0	x
4.34-4.36	1H	**25**	77.6	1.28	5.47	8.36	x	x	x	x
4.29	1H	**46**	67.2	1.22	4.46-4.48	8.86	x	x	x	x
3.68-3.71	1H	**2**	65.8	3.00-3.03	3.07-3.11	7.94	x	21.6	x	x
3.07-3.11	1H	**3a,b**	47.6	3.68-3.71	7.94	0.99	x	165.0	x	x
3.00-3.03	1H	**3a,b**	47.6	3.68-3.71	7.94	0.99	x	165.0	x	x
3.27	3H	**65**	57.5	x	x	x	x	x	x	x
2.68	1H	**62a,b**	27.0	2.25-2.29	x	x	x	172.0	34.0	x
2.54	1H	**62a,b**	27.0	2.25-2.29	x	x	x	172.0	34.0	x
2.42-2.46	1H	**33**	32.7	0.86	0.95	x	x	173.6	58.0	40.3
2.25-2.29	2H	**61a,b**	33.9	2.54	2.68	x	x	173.3	27.0	x
1.69	3H	**8**	13.8	6.51	x	x	x	130.5	128.3	x
1.67	3H	**42**	14.2	6.70	x	x	x	68.0	29.9	11.5
1.29	3H	**58**	18.1	4.42-4.44	x	8.60	x	173.2	50.1	x
1.28	3H	**26**	16.4	4.34-4.36	x	5.47	x	77.6	56.2	x
1.22	3H	**47**	21.4	4.29	x	4.46-4.48	x	60.8	67.2	x
0.99	3H	**1**	21.6	3.68-3.71	x	3.00-3.03	3.07-3.11	65.8	47.6	x
0.95	3H	**34a,b**	19.2	2.42-2.46	x	5.08	8.75	57.7	32.7	20.1
0.86	3H	**34a,b**	20.1	2.42-2.46	x	5.08	8.75	57.6	32.9	19.2

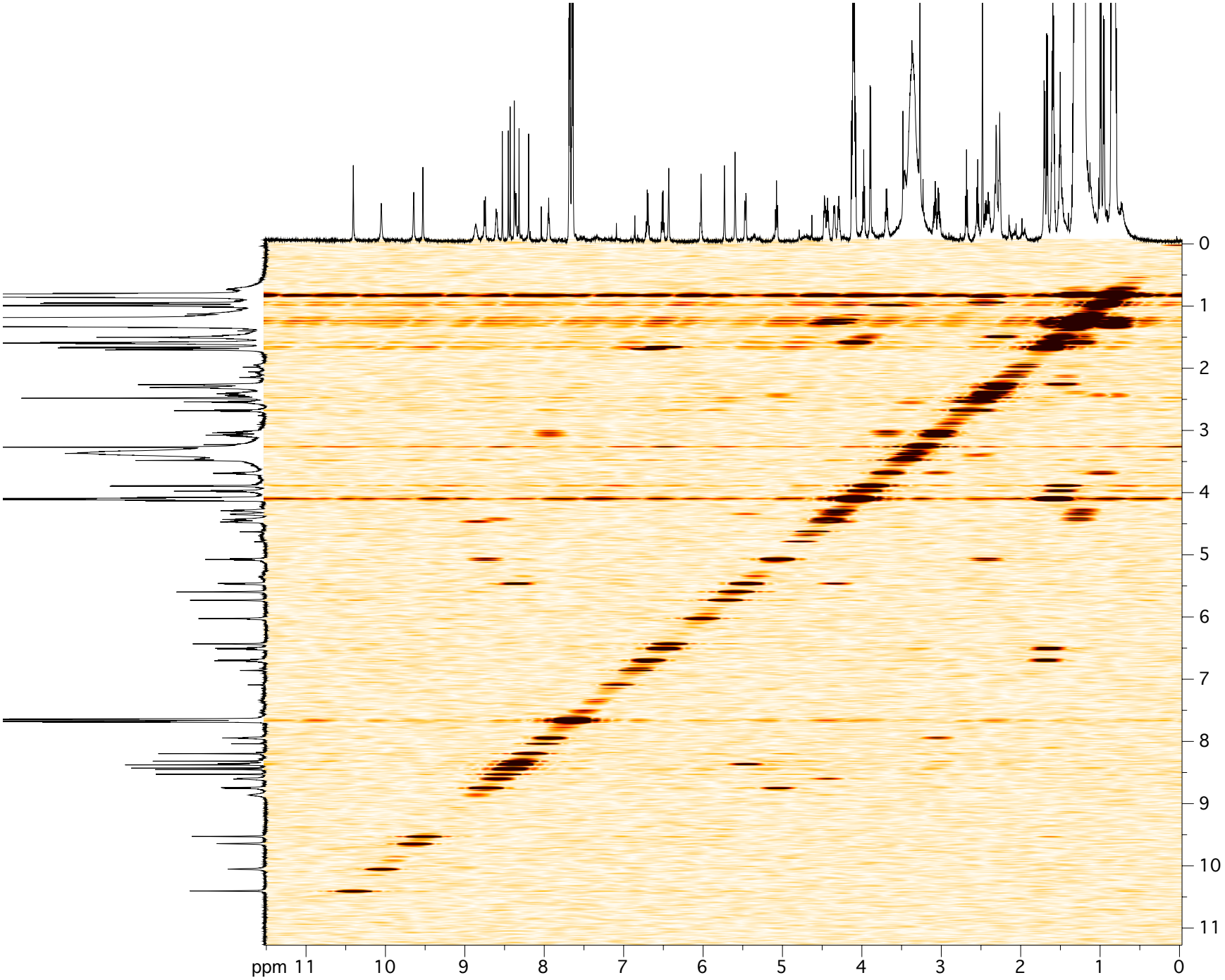
Carbon	Shift (ppm)
1	21.6
2	65.8
3	47.6
5	165.0
6	131.5
7	128.7
8	13.8
10	159.9
11	151.2
12	126.0
13	162.0
14	147.6
15	120.4
16	166.4
17	134.2
18	104.0
20	166.3
21	149.3
22	126.7
23	172.9
24	56.2
25	77.6
26	16.4
28	161.0
29	148.3
30	126.2
31	173.8
32	57.6
33	32.7
34a	19.2
34b	20.1
36	161.2
37	149.8
38	124.9
39	168.6
40	131.0
41	126.9
42	14.2
45	60.8
46	67.2
47	21.4
50	150.0
51	125.9
52	165.5
53	134.9
54	105.9
56	173.2
57	50.1
58	18.1
60	173.3
61	34.0
62	27.0
63	172.0
65	57.5

a Carbon shifts are based on 2D HSQC/HMBC data. We tentatively assign the stereochemistry at carbons 24, 25, 32, 45, 46, and 57 on analogy to that present in Micrococcin P1. Additionally, we note that this is the stereochemistry of the natural amino acids encoded by the structural genes, tclE-H. The strong TOCSY between acetamide N-Hs 9 and 43 with their respective vinylic protons, 7 and 41, indicates a *cis*-double bond geometry at these positions, as illustrated. Due to bond angle constraint, correlations between Dhb/Dha N-Hs and *cis*-vinylic protons do not typically appear in a 2D TOCSY.

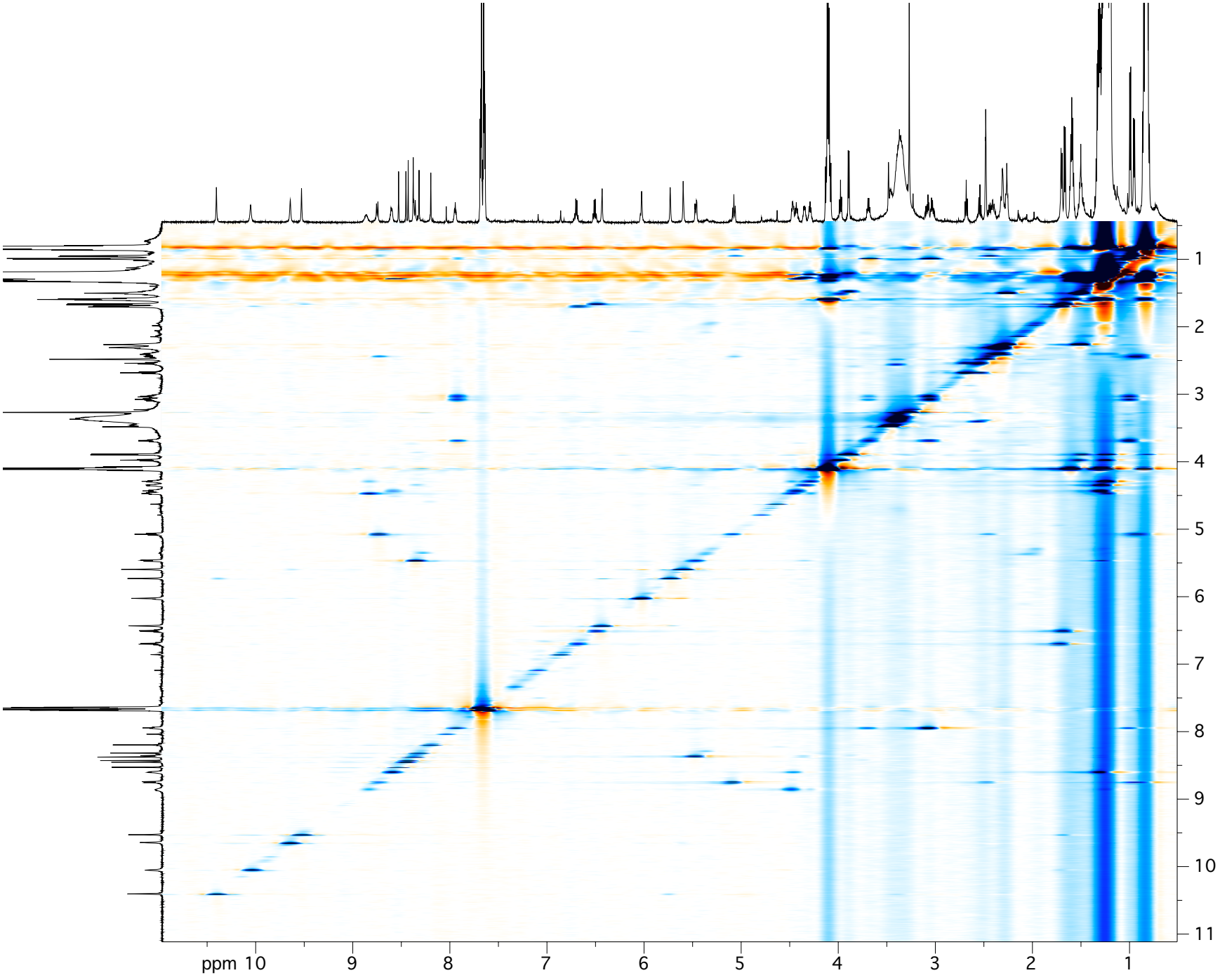
NMR Figure 1: Compound **10** (1364.3232) 1H NMR (600 MHz, DMSO-*d*6)



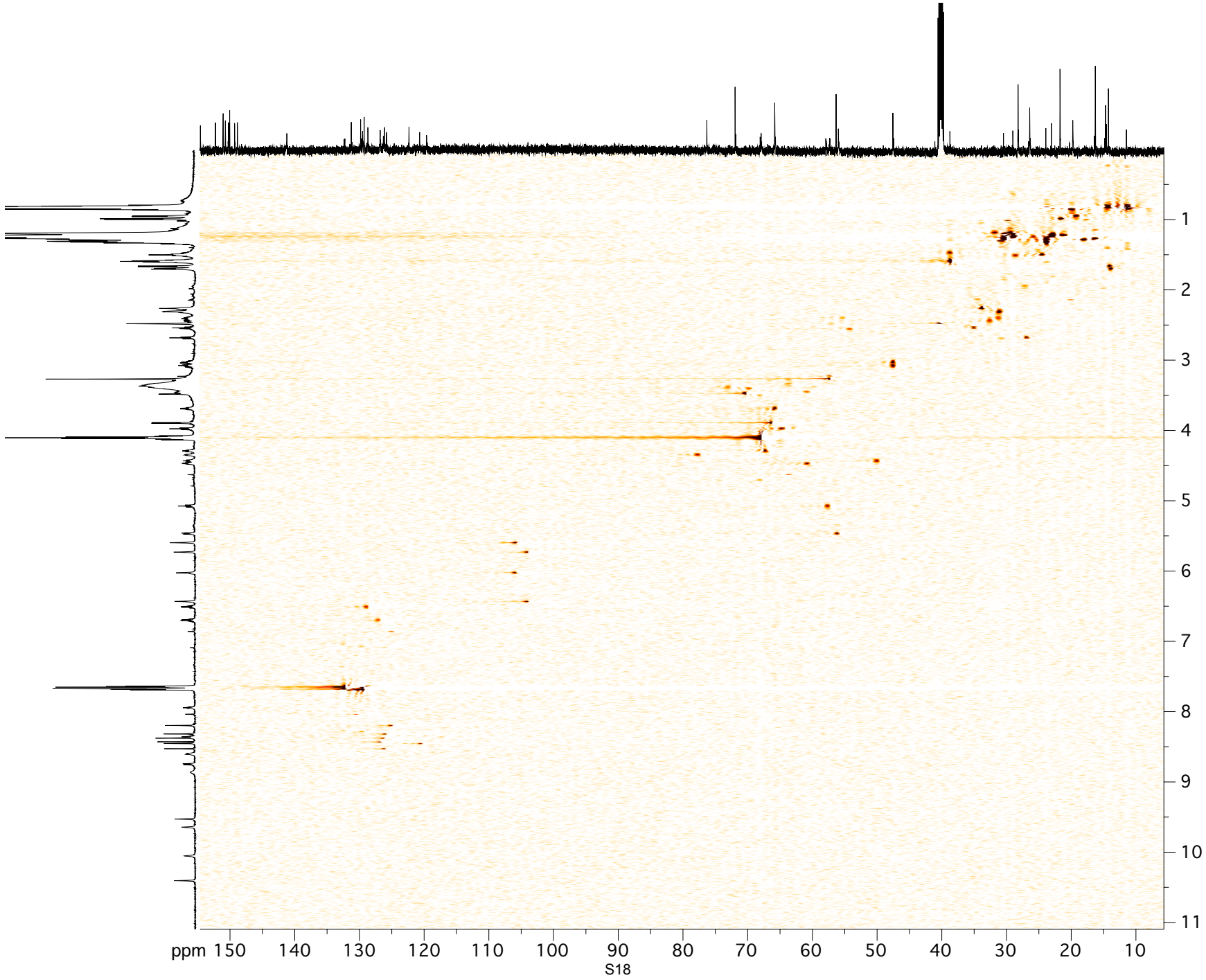
NMR Figure 2: Compound **10** (1364.3232) 1H-COSY (600 MHz, DMSO-*d*6)



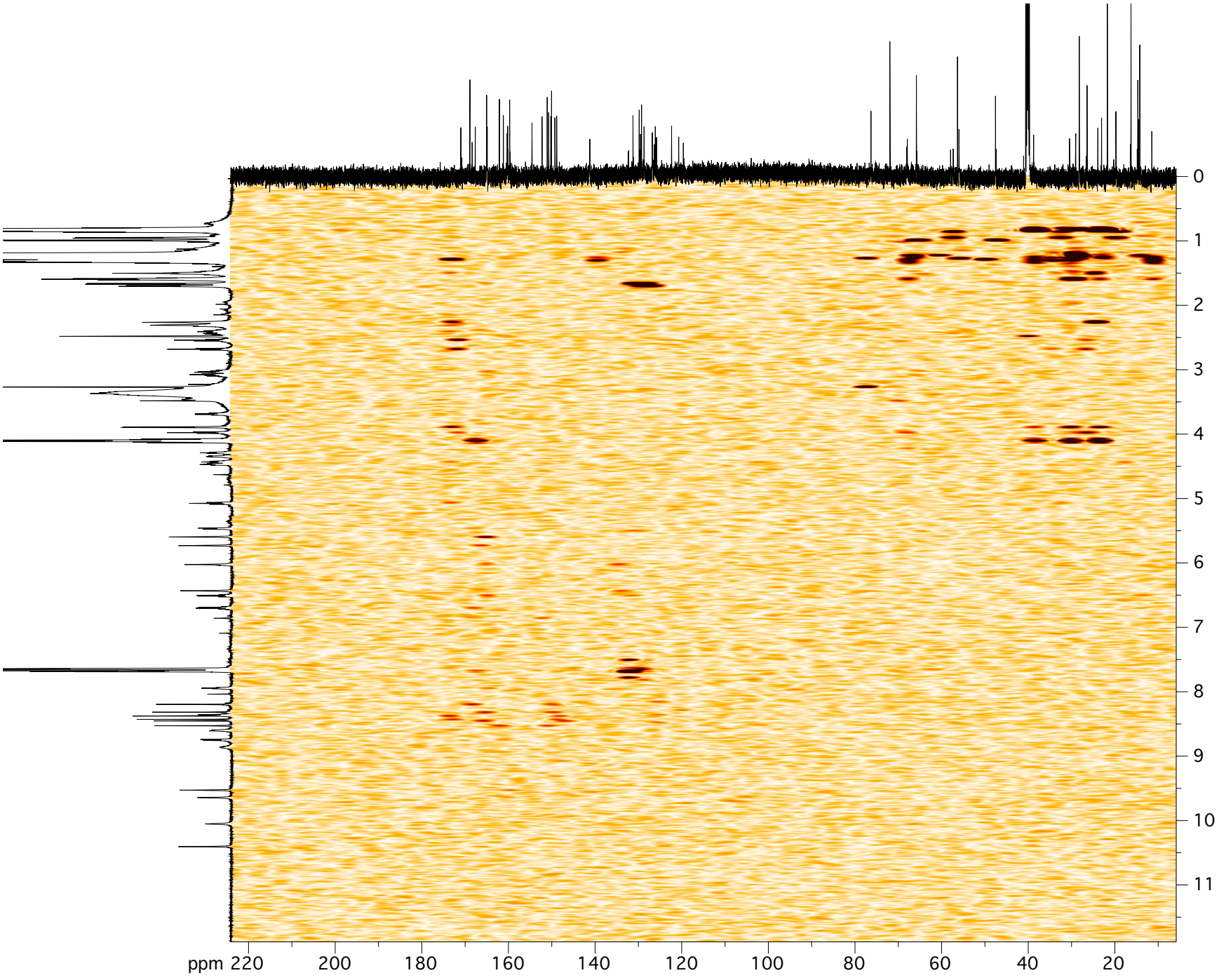
NMR Figure 3: Compound **10** (1364.3232) 1H-TOCSY (600 MHz, DMSO-*d*6)



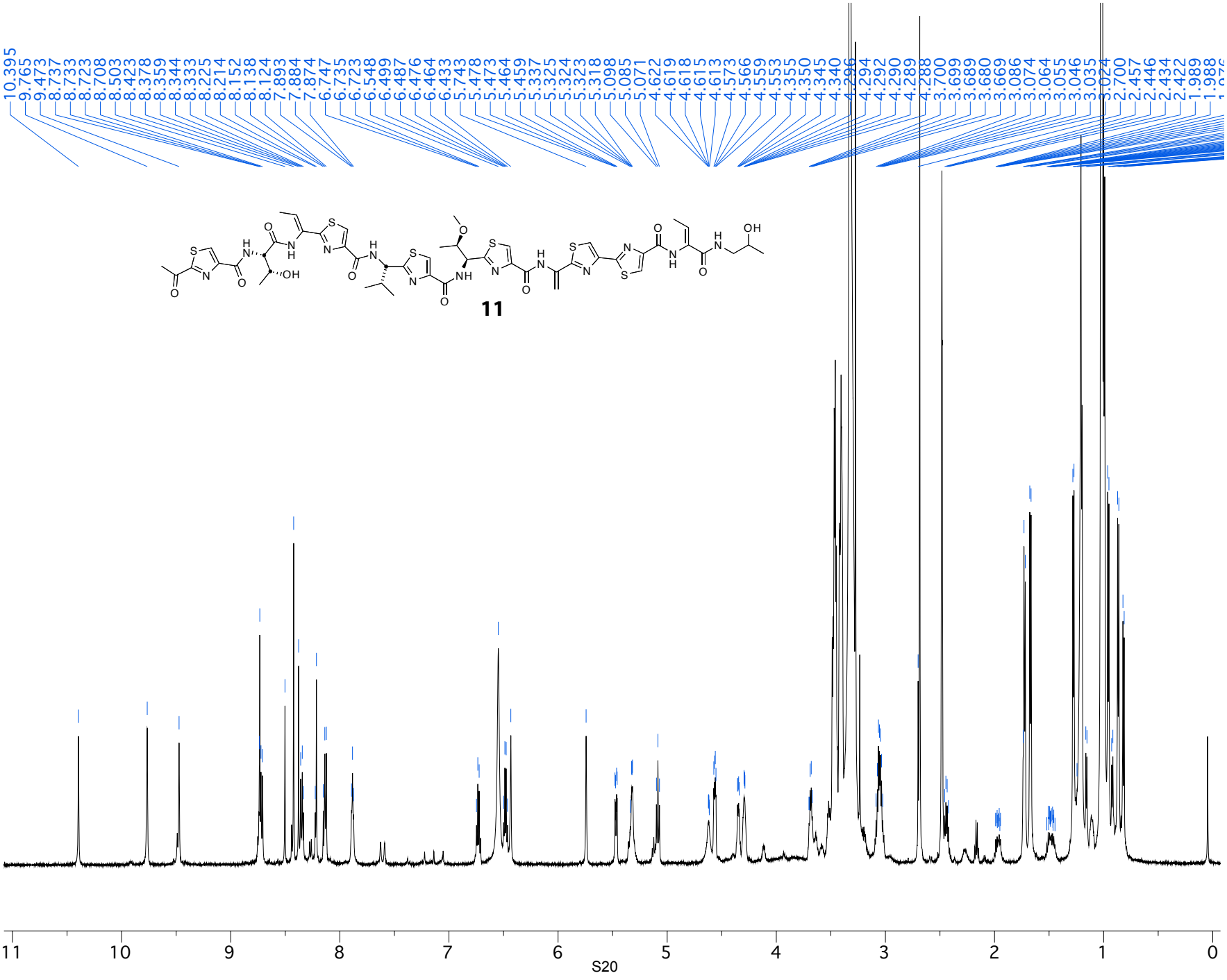
NMR Figure 4: Compound **10** (1364.3232) 1H-13C-gHSQC (600 MHz, DMSO-*d*6)



NMR Figure 5: Compound **10** (1364.3232) 1H-13C-gHMBC (600 MHz, DMSO-*d*6)



NMR Figure 6: Compound **11** (1194.2541) 1H NMR (600 MHz, DMSO-*d*6)



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