Supporting Information for

Thiostrepton Variants Containing a Contracted Quinaldic Acid Macrocycle Result from Mutagenesis of the Second Residue

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Supplemental Methods

General Materials and Methods. Unless otherwise indicated, all chemicals and solvents were purchased from common vendors and used as received. Enzymes and buffers were acquired from New England Biolabs. The Wizard® Genomic DNA Purification Kit was used to isolate *Streptomyces* genomic DNA. Isolation of plasmids and fosmids from *E. coli* strains were performed using the QIAprep Spin Miniprep Kit and DirectPrep 96 MiniPrep Kit from Qiagen. Polymerase chain reaction (PCR) products were cloned using the StrataClone Blunt PCR Cloning Kit (Agilent Technologies). 96-well tissue culture plates and 96-well black with clear flat bottom plates were obtained from Becton Dickinson and Corning, respectively. 384-well black plates were purchased from PerkinElmer. A BioTek H4 Multi-Mode Microplate Reader was used for optical density, fluorescence, and luminescence measurements. All oligonucleotides were synthesized by Integrated DNA Technologies. DNA sequencing was performed by Eurofins MWG Operon, and sequence analysis was completed using the VectorNTI software package from Invitrogen.

High performance liquid chromatography (HPLC) analysis was performed on a Beckman Coulter System Gold instrument. High-resolution matrix-assisted laser desorption/ionization mass spectrometry (HR-MALDI-MS), MALDI-MS/MS and HPLC-MS were conducted with an Applied Biosystems 4700 Proteomics Analyzer and a Micromass Quattro LC at the Georgia Institute of Technology Bioanalytical Mass Spectrometry Facility. High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) was performed on a Thermo LTQ-FTMS at the Emory University Mass Spectrometry Center. For HPLC-MS, a Phenomenex Synergi RP column (250 mm x 2 mm, 4 μm) (Torrance, CA) was developed at 0.25 mL min^{-1} with 20% solvent B in solvent A for 8 min followed by a gradient from 20 to 100% solvent B over 35 min (Solvent A: 5% acetonitrile and 0.1% formic acid in water; solvent B: 5% water and 0.1% formic acid in acetonitrile). NMR experiments were completed on a Bruker 500 MHz spectrometer according to standard pulse sequences supplied with the instrument at the Georgia Institute of Technology School of Chemistry and Biochemistry.

Bacterial Strains, Plasmids, and Growth Media. *Streptomyces laurentii* ATCC 31255 (*S. laurentii*), methicillin-resistant *Staphylococcus aureus* ATCC 10537 (MRSA), vancomycin-resistant *Enterococcus faecium* ATCC 12952 (VRE), *Bacillus* sp. ATCC 27859 (*Bacillus*), and *Escherichia coli* (*E. coli*) ATCC 27856 were all purchased from American Type Culture Collection (ATCC). *E. coli* EPI300 was obtained from Epicentre®. All strains, plasmids, and fosmids are itemized in Table S8, and primers are listed in Table S9. All *E. coli* strains were grown in Luria-Bertani liquid or solid medium with the appropriate antibiotic(s). For the selective growth of *E. coli* or *Streptomyces*, the following antibiotics and concentrations were used: apramycin (50 μg mL^{-1}), kanamycin (50 μg mL^{-1}), ampicillin (100 μg mL^{-1}), nalidixic acid (25 μg mL^{-1}), and chloramphenicol (12.5 μg mL^{-1}). MS agar and ISP3 agar were used for the intergeneric conjugation of *S. laurentii* NDS1 and sporulation of *S. laurentii* strains, respectively.[1, 2]

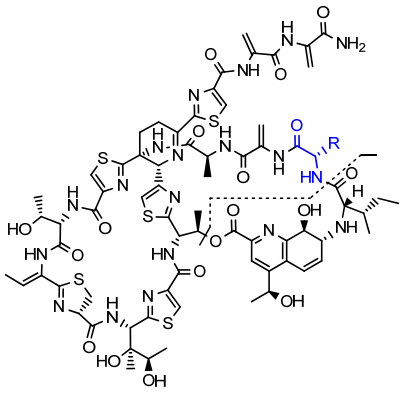
Purification and Mass Spectrometry Analyses of Thiostrepton Ala2 Analogues. *S. laurentii* variant culture extracts were first precipitated as previously described.[3] The solid residue of the culture extract was dissolved in a minimum volume of chloroform and mixed with 10 volumes of *n*-hexane followed by centrifugation for 5 min at 4000 rpm (2559 x *g*) and 25 °C. The precipitate was then dissolved in a minimum volume of dichloromethane:ethanol (4:1). Diethyl ether (5 volumes) was added to the sample and the mixture was centrifuged at 4000 rpm (2559 x

g) for 5 min at 25 °C. The solid residue was dissolved in chloroform:methanol (4:1) and further purified by semi-preparative HPLC using a Phenomenex Luna C18(2) column (250 x 10 mm, 5 μm) with water and acetonitrile, as described below, at 4.3 mL min^{-1} while monitoring absorbance at 254 nm.

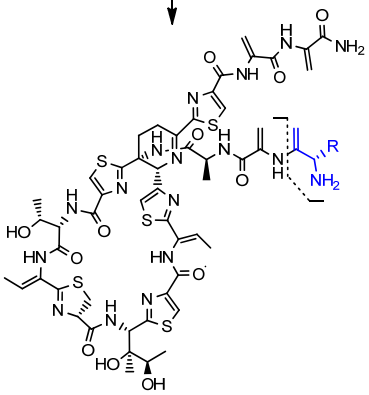
Purification of thiostreptons Ala2Dha, Ala2Dhb, Ala2Met, Ala2Phe, Ala2Tyr, and Ala2Ile-ΔIle1, and Ala2Val-ΔIle1 all utilized the same gradient: acetonitrile was increased from 0 to 100% in water over 30 min. Thiostrepton Ala2Ile-ΔIle1 and Ala2Val-ΔIle1 were subjected to a second round of semi-preparative HPLC purification by holding the mobile phase constant at 40% acetonitrile in water for 10 min. Purified samples were analyzed by HPLC-MS, MALDI-MS/MS, either HR-ESI-MS or HR-MALDI-MS, and stored under argon at -80 °C. Thiostreptons Ala2Dha, Ala2Dhb, Ala2Ile-ΔIle1, and Ala2Val-ΔIle1 were further analyzed by one- and two-dimensional NMR.

Kinetic solubilities of the thiostreptons were determined using a previously described protocol.[4, 5]

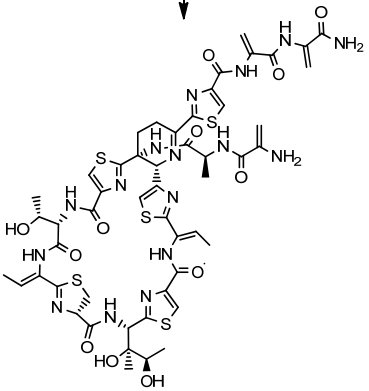
Figure S1. Two diagnostic daughter ions from the MALDI MS/MS of thiostrepton A and its analogues used to confirm the identity of the second residue. Fragment **A** corresponds to the loss of the quinaldic acid (QA) moiety and Ile1 from the parent ion, and fragment **B** additionally lacks the second residue (Xxx2).



Parent Ion: $[M+H]^+$



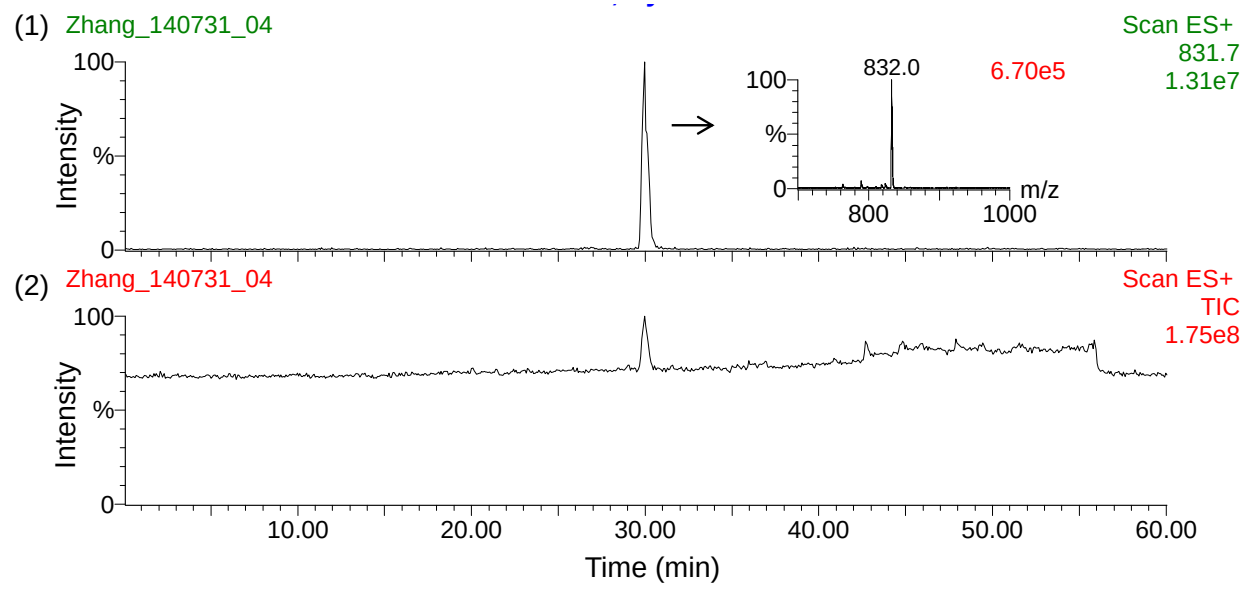
Fragment **A**:
$[M\text{-}QA\text{-}Ile1+H]^+$



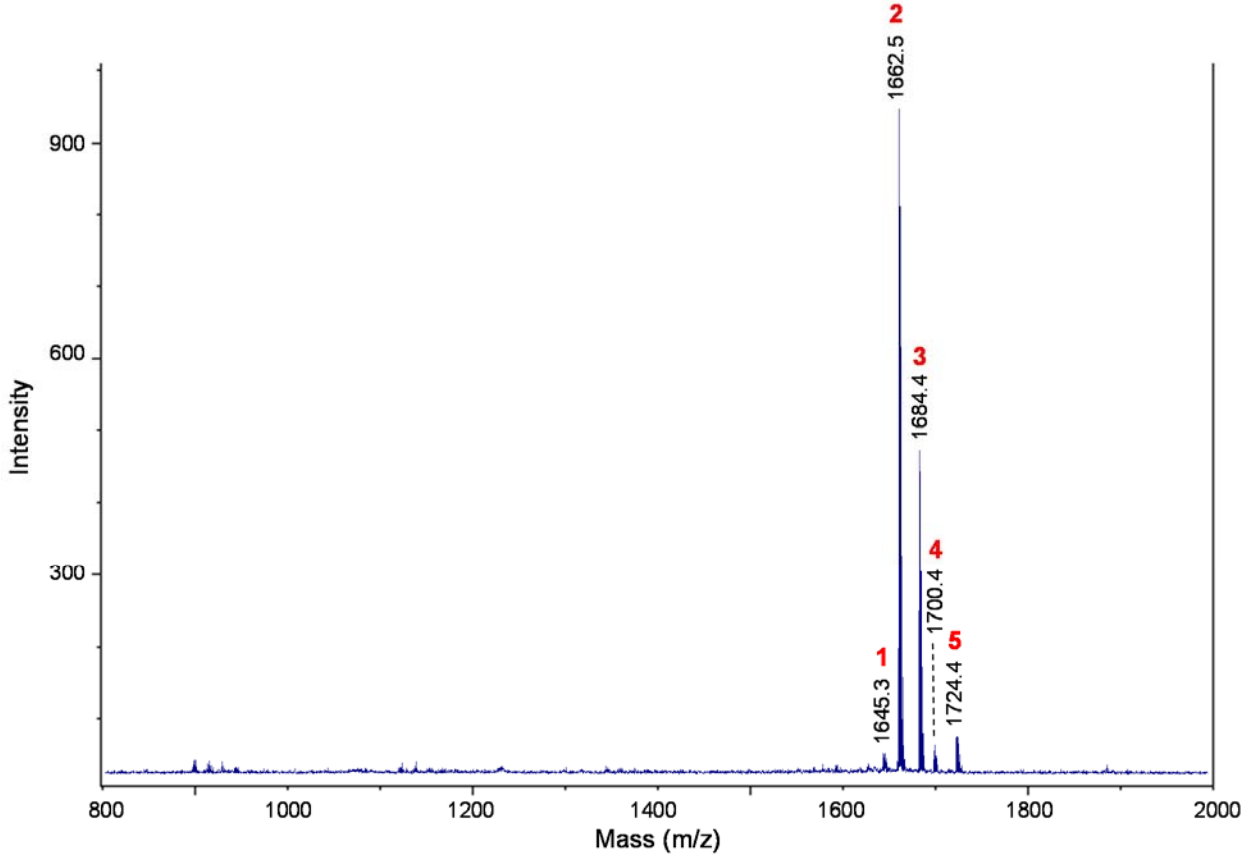
Fragment **B**:
$[M\text{-}QA\text{-}Ile1\text{-}Xxx2+H]^+$

Figure S2. MS analysis of thiostrepton Ala2Dha isolated from *S. laurentii* NDS1/int-A2S.

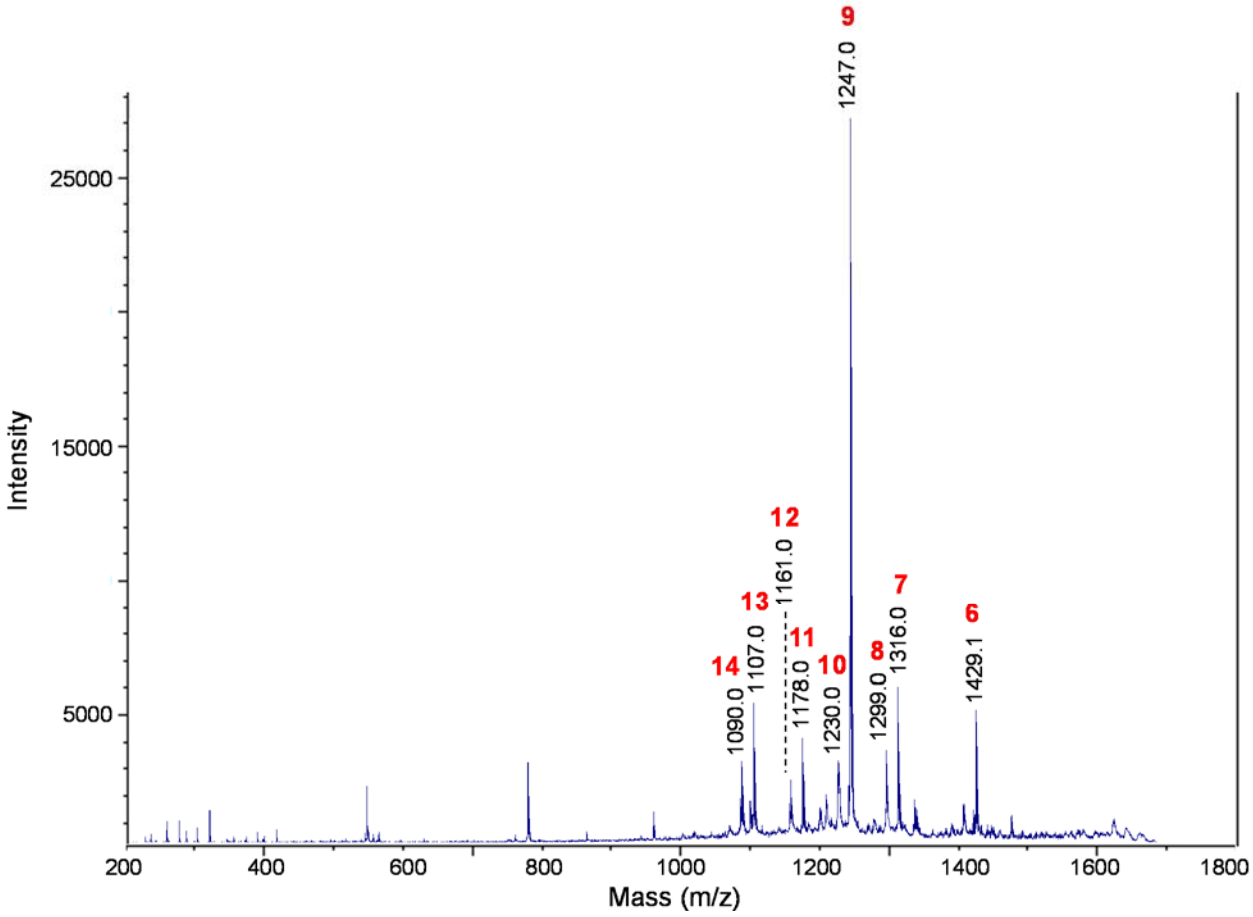
(**A**) HPLC-MS analysis. (1) Chromatogram extracted for *m*/*z* 831.7, the calculated $[M+2H]^{2+}$ ion of thiostrepton Ala2Dha. (2) Total ion chromatogram.



(**B**) MALDI MS spectrum of thiostrepton Ala2Dha.



(**C**) MALDI MS/MS of parent ion *m/z* 1662.5.



(**D**) Table and structure showing key ions and fragments in the MALDI MS and MS/MS of thiostrepton Ala2Dha.

Fragment	Expected	Observed
1. M-OH+H$^+$	1645.5	1645.3
2. M+H$^+$ (Parent ion)	1662.5	1662.5
3. M+Na$^+$	1684.5	1684.4
4. M+K$^+$	1700.4	1700.4
5. M+Cu$^+$	1724.4	1724.4
6. M-QA+H$^+$	1429.4	1429.1
7. M-QA-Ile1+H$^+$	1316.3	1316.0
8. M-QA-Ile1-OH+H$^+$	1299.3	1299.0
9. M-QA-Ile1-Dha2+H$^+$	1247.3	1247.0
10. M-QA-Ile1-Dha2-OH+H$^+$	1230.3	1230.0
11. M-QA-Ile1-Dha2-Dha3+H$^+$	1178.3	1178.0
12. M-QA-Ile1-Dha2-Dha3-OH+H$^+$	1161.3	1161.0
13. M-QA-Ile1-Dha2-Dha3-Ala4+H$^+$	1107.3	1107.0
14. M-QA-Ile1-Dha2-Dha3-Ala4-OH+H$^+$	1090.2	1090.0

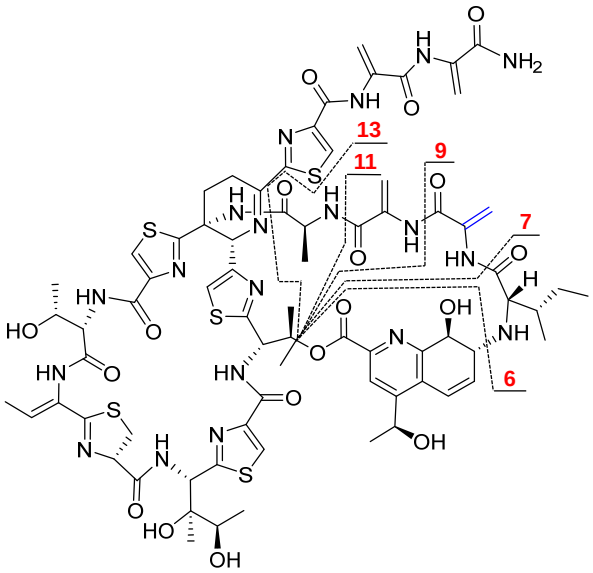
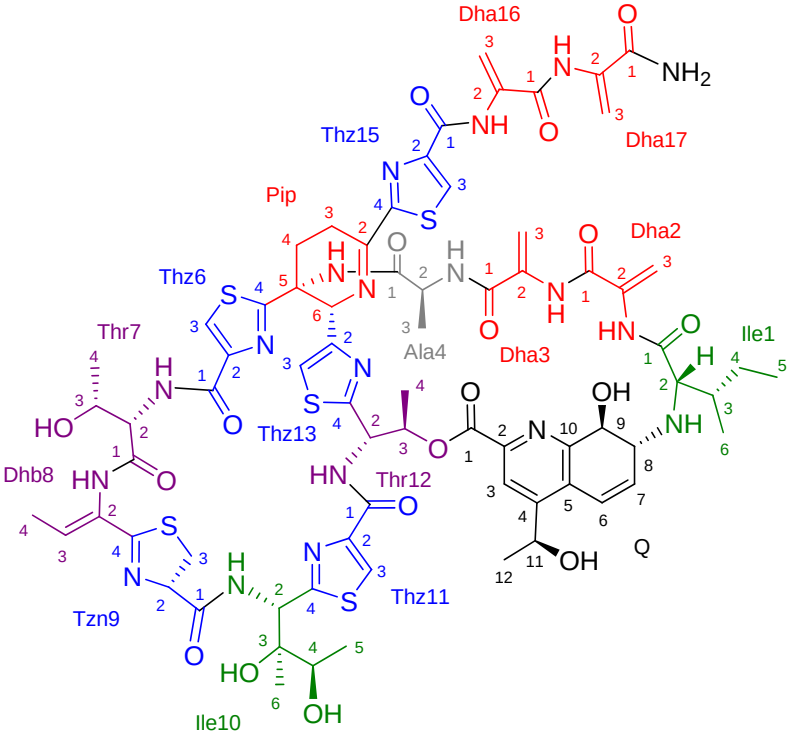


Figure S3. Structure and numbering system used for thiostrepton Ala2Dha.



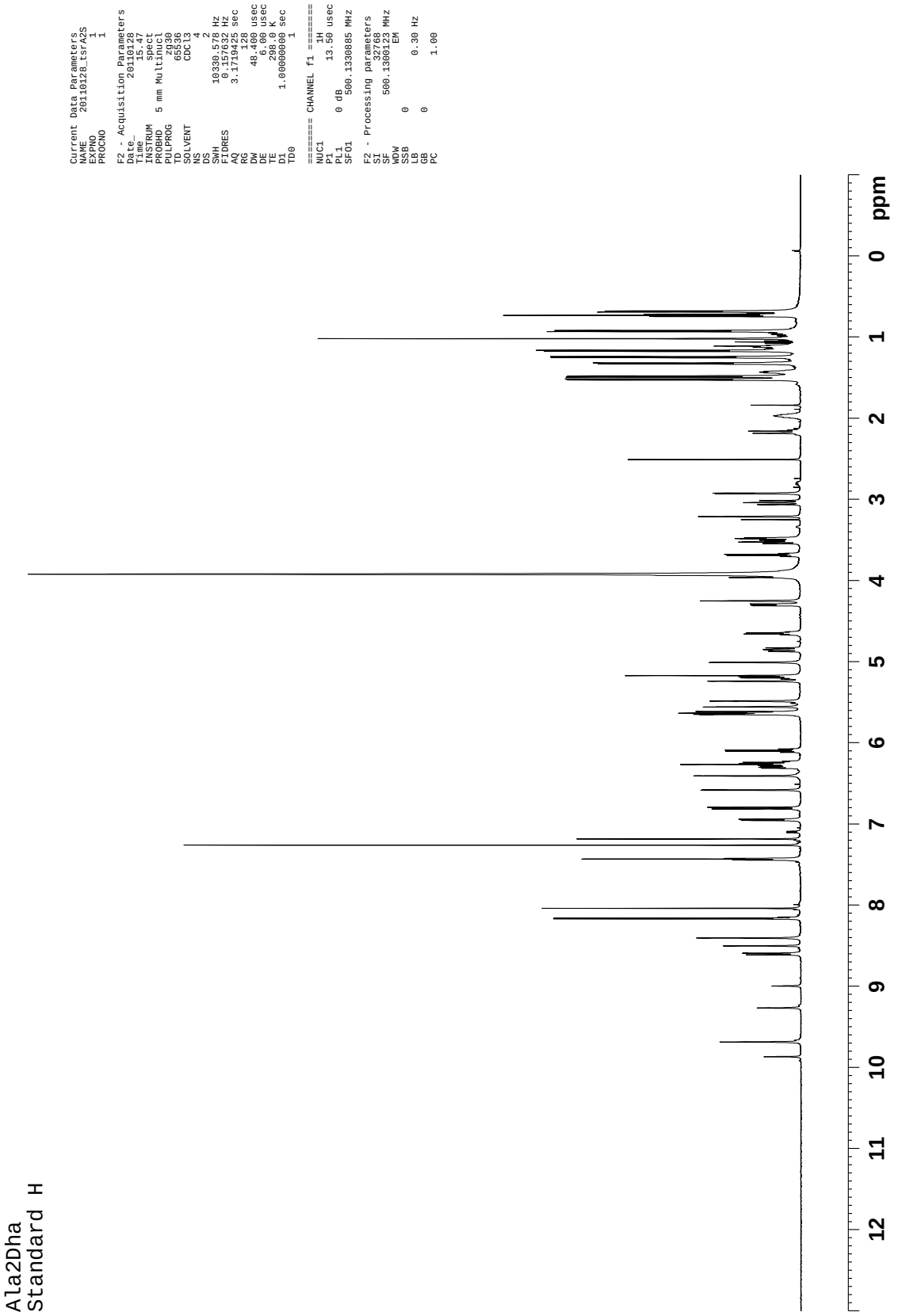


Figure S4. ^{1}H NMR spectrum of thiostrepton Ala2Dha (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

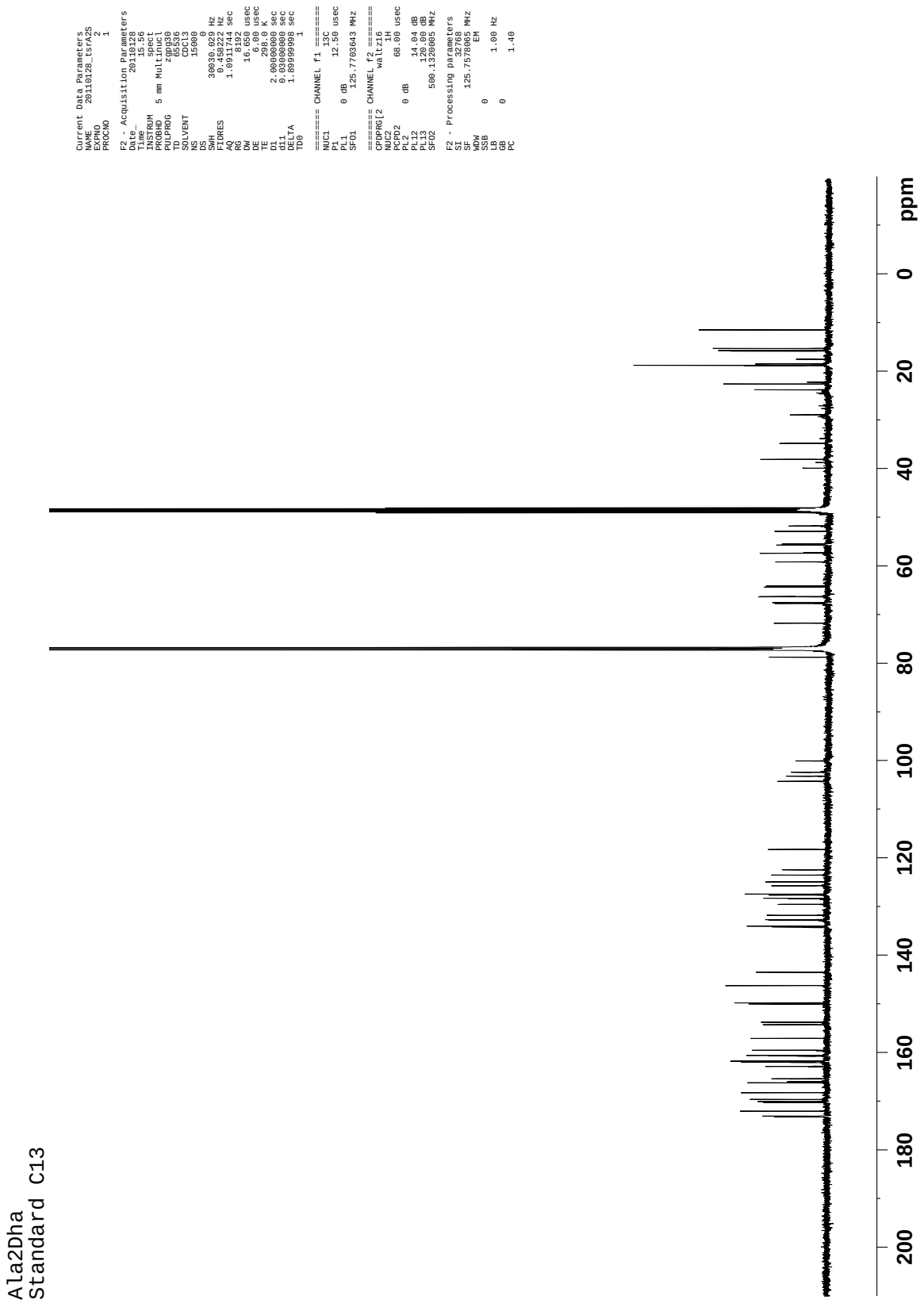


Figure S5. ^{13}C NMR spectrum of thiostrepton Ala2Dha (125 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

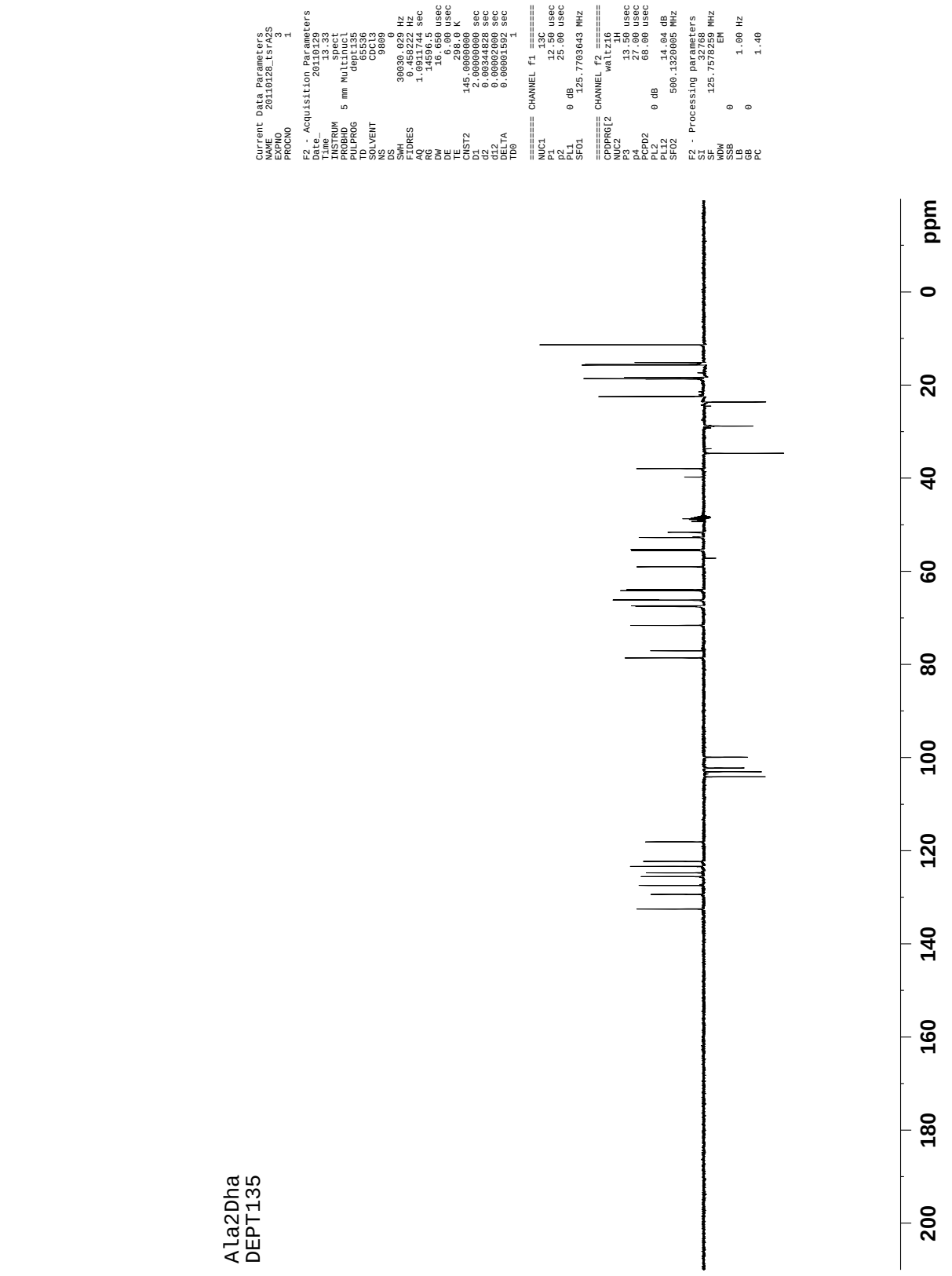


Figure S6. DEPT-135 NMR spectrum of thiostrepton Ala2Dha (125 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

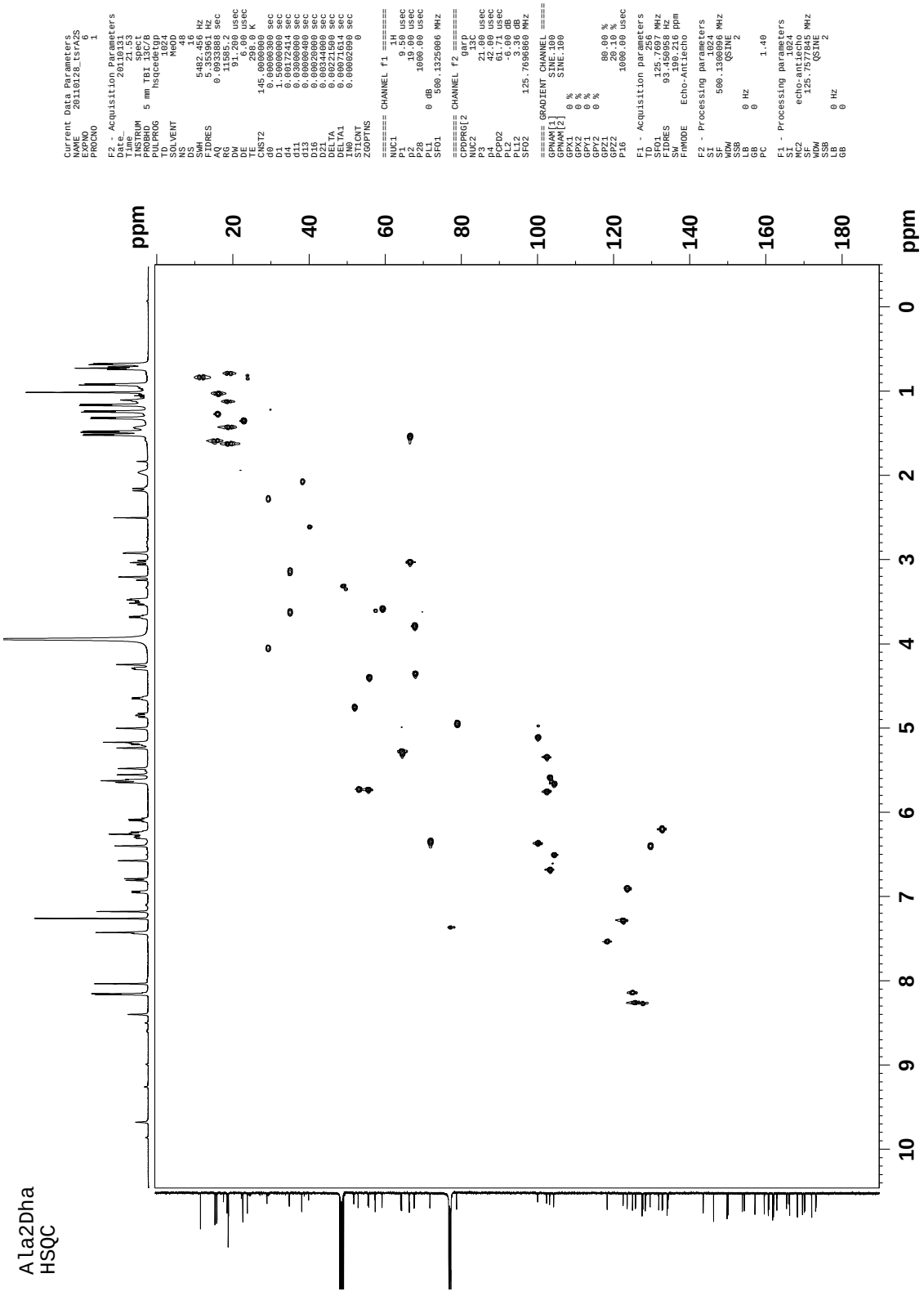


Figure S7. gHSQC spectrum of thiostrepton Ala2Dha (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

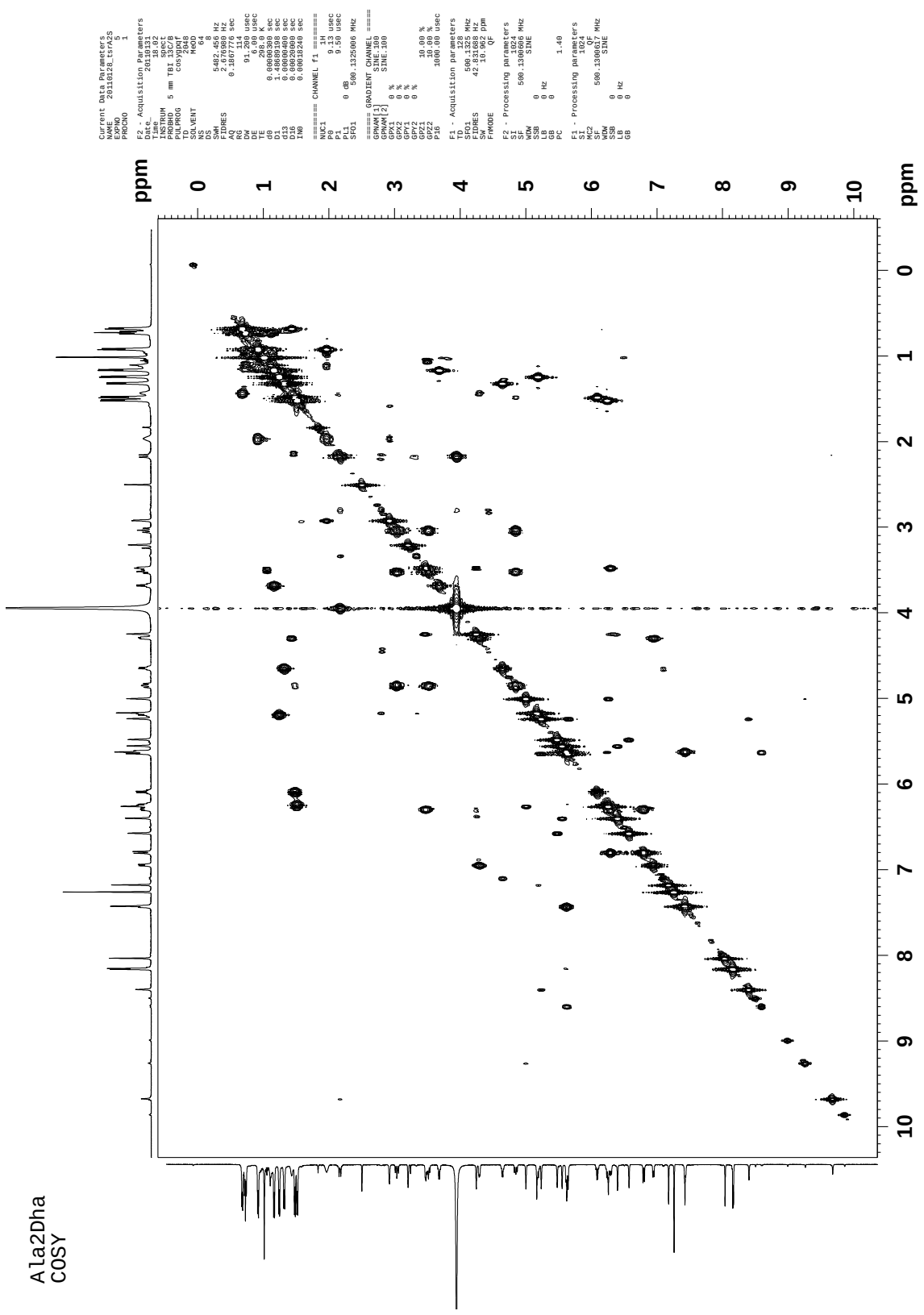


Figure S8. gCOSY spectrum of thiostrepton Ala2Dha (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

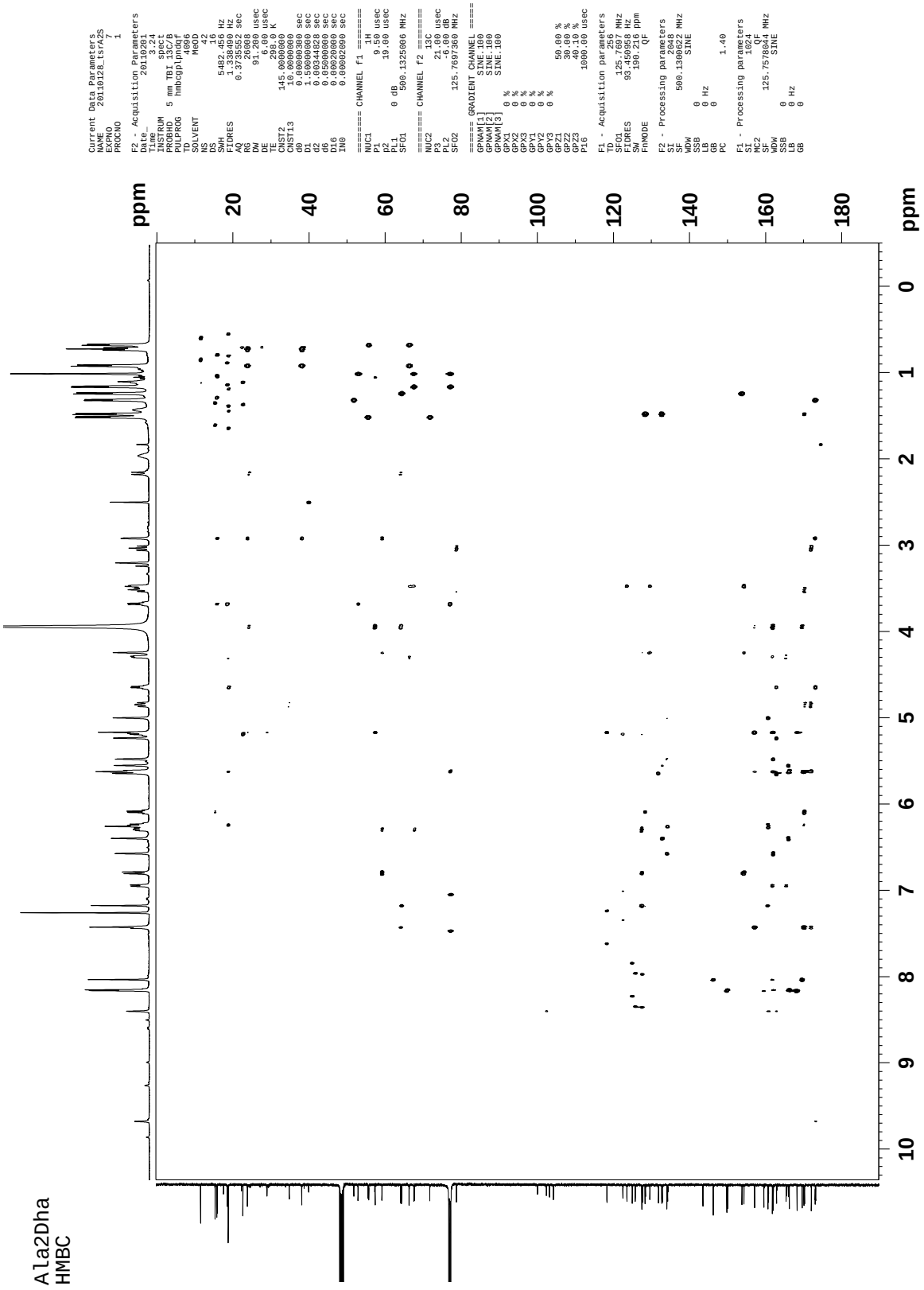


Figure S9. gHMBC spectrum of thiostrepton Ala2Dha (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

Table S1. ^{1}H and ^{13}C NMR assignments of thiostrepton Ala2Dha

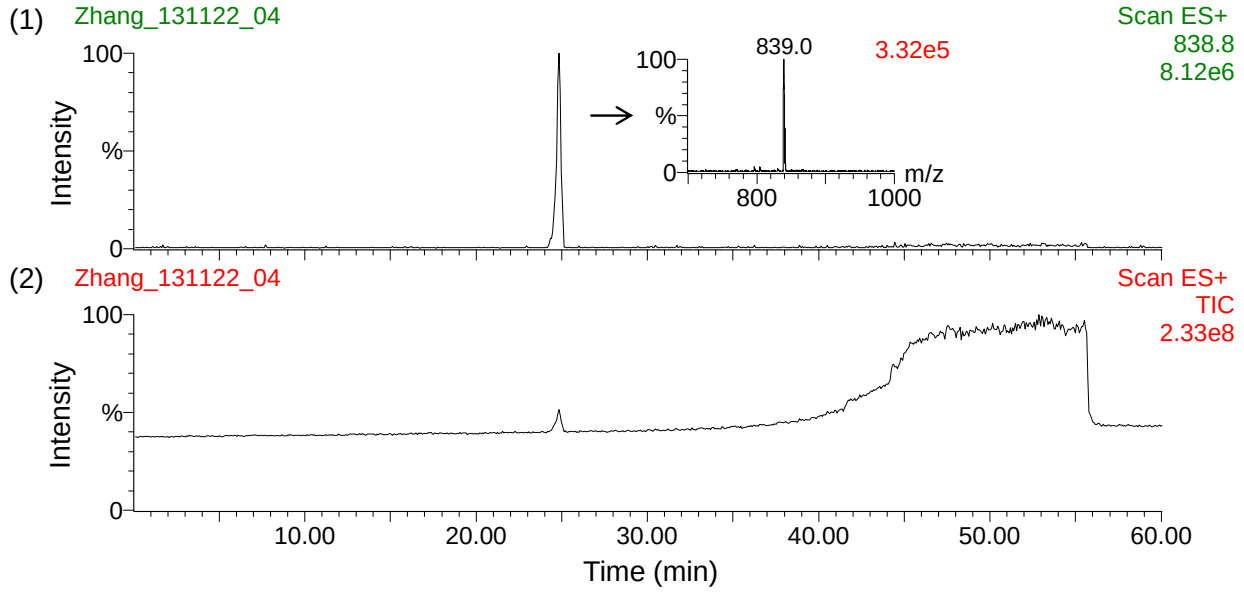
Position	δ_C [ppm]; mult	δ_H [ppm]; (mult, J in Hz)	HMBC[a]	COSY[b]
Ile1				
Ile1-1	173.0; C q			
Ile1-2	66.3; CH	2.93 (d, 3.8)	Ile1-1; Ile1-3; Ile-4; Ile1-6; Q8	Ile1-3
Ile1-3	38.1; CH	2.00-1.94 (m)		Ile1-2; Ile1-4-H_A; Ile1-4-H_B; Ile1-6
Ile1-4	23.8; CH_2	H_A: 1.14-1.10 (m)	Ile1-5	Ile1-3; Ile1-4-H_B; Ile1-5
		H_B: 0.99-0.94 (m)		Ile1-3, Ile1-4-H_A; Ile1-5
Ile1-5	11.5; CH_3	0.73 (t, 7.1)	Ile1-3; Ile1-4	Ile1-4-H_A; Ile1-4-H_B
Ile1-6	15.7; CH_3	0.92 (d, 6.9)	Ile1-2; Ile1-3; Ile1-4	Ile1-3
Dha2				
Dha2-1	160.7; C q			
Dha2-2	134.2; C q			
Dha2-3	100.1; CH_2	H_A: 6.27 (br s)	Dha2-1; Dha2-2	Dha2-3-H_B
		H_B: 5.01 (d, 1.4)	Dha2-1; Dha2-2	Dha2-3-H_A
Dha3				
Dha3-1	162.8; C q			
Dha3-2	131.8; C q			
Dha3-3	102.4; CH_2	H_A: 5.65 (d, 2.2)	Dha3-1; Dha3-2	Dha3-3-H_B
		H_B: 5.24 (br s)	Dha3-1	Dha3-3-H_A; Dha3-NH
Dha3-NH[d]		8.40 (s)	Dha2-1; Dha3-1; Dha3-3	Dha3-3-H_B
Ala4				
Ala4-1	173.1; C q			
Ala4-2	51.8; CH	4.65 (q, 6.6)	Dha3-1; Ala4-1; Ala4-3	Ala4-3; Ala4-NH
Ala4-3	18.8; CH_3	1.32 (d, 6.7)	Ala4-1; Ala4-2	Ala4-2
Ala4-NH[d]		7.10 (d, 8.2)		Ala4-2
Pip				
Pip-2	161.9; C q			
Pip-3	24.5; CH_2	H_A: 3.36-3.32 (m)		Pip-4-H_B
		H_B: 2.83-2.77 (m)		Pip-4-H_A; Pip-4-H_B
Pip-4	28.9; CH_2	H_A: 3.95 (m)	Pip-2; Pip-3; Pip-5; Pip-6; Thz6-4; Thz13-2	Pip-4-H_B
		H_B: 2.17 (m)	Pip-3; Pip-6	Pip-3-H_A; Pip-4-H_A; Pip-3-H_B
Pip-5	57.3; C q			
Pip-6	64.1; CH	5.17 (s)	Pip-2; Pip-4; Pip-5; Thz13-2; Thz13-3; Thz15-4	Pip-3-H_A; Pip-3-H_B
Thz6				
Thz6-1	161.7; C q			
Thz6-2	146.2; C q			
Thz6-3	124.9; CH	8.04 (s)	Thz6-1; Thz6-2; Thz6-4	
Thz6-4	169.6; C q			
Thr7				
Thr7-1	165.4; C q			
Thr7-2	55.7; CH	4.30 (dd, 7.7, 3.6)	Thz6-1; Thr7-1; Thr7-3; Thr7-4	Thr7-3; Thr7-NH
Thr7-3	66.3; CH	1.46-1.40 (m)	Thr7-4	Thr7-2; Thr7-4
Thr7-4	18.8; CH_3	0.68 (d, 6.3)	Thr7-2; Thr7-3	Thr7-3
Thr7-NH[d]		6.95 (d, 7.6)	Thz6-1; Thr7-1	Thr7-2
Dhb8				
Dhb8-2	128.3; C q			
Dhb8-3	132.7; CH	6.10 (q, 7.0)	Dhb8-2; Dhb8-4; Tzn9-4	Dhb8-4
Dhb8-4	15.3; CH_3	1.48 (d, 7.3)	Dhb8-2; Dhb8-3; Tzn9-4	Dhb8-3
Tzn9				
Tzn9-1	172.0; C q			
Tzn9-2	78.8; CH	4.85 (dd, 12.8, 9.0)	Tzn9-1; Tzn9-3; Tzn9-4	Tzn9-3-H_A; Tzn9-3-H_B
Tzn9-3	34.8; CH_2	H_A: 3.52 (dd, 11.2, 9.0)	Tzn9-2; Tzn9-4	Tzn9-2; Tzn9-3-H_B
		H_B: 3.04 (dd, 12.8, 11.5)	Tzn9-1; Tzn9-2	Tzn9-2; Tzn9-3-H_A
Tzn9-4	170.2; C q			
Ile10				
Ile10-2	52.9; CH	5.62 (d, 5.1)	Tzn9-1; Ile10-3; Thz11-4	Ile10-NH
Ile10-3	77.1[c]; C q			
Ile10-4	67.6; CH	3.68 (q, 6.4)	Ile10-2; Ile10-3; Ile10-5; Ile10-6	Ile10-5
Ile10-5	15.8; CH_3	1.17 (d, 6.3)	Ile10-3; Ile10-4	Ile10-4
Ile10-6	18.5; CH_3	1.02 (s)	Ile10-2; Ile10-3; Ile10-4	
Ile10-NH[d]		7.43 (d, 9.8)	Tzn9-1	Ile10-2

Position	δ_C [ppm]; mult	δ_H [ppm]; (mult, J in Hz)	HMBC[a]	COSY[b]
Thz11				
Thz11-1	162.0; C q			
Thz11-2	150.0; C q			
Thz11-3	125.7; CH	8.16 (s)	Thz11-1; Thz11-2; Thz11-4	
Thz11-4	166.2; C q			
Thr12				
Thr12-2	55.5; CH	5.64 (d, 4.1)	Thz11-1; Thr12-4; Thz13-2; Thz13-4	Thr12-3, Thr12-NH
Thr12-3	71.8; CH	6.25 (q, 6.7)	Thr12-4; Thz13-4; Q-1	Thr12-2, Thr12-4
Thr12-4	18.8; CH_3	1.52 (d, 6.3)	Thr12-2; Thr12-3	Thr12-3
Thr12-NH[d]		8.60 (d, 8.9)		Thr12-2
Thz13				
Thz13-2	157.1; C q			
Thz13-3	118.3; CH	7.43 (s)	Pip-6; Thz13-2; Thz13-4	
Thz13-4	170.0; C q			
Thz15				
Thz15-1	159.5; C q			
Thz15-2	149.8; C q			
Thz15-3	127.6; CH	8.17 (s)	Thz15-1; Thz15-2; Thz15-4	
Thz15-4	168.2; C q			
Dha16				
Dha16-1	162.0; C q			
Dha16-2	134.0; C q			
Dha16-3	103.2; CH_2	H_A: 6.58 (d, 2.2)	Dha16-1; Dha16-2	Dha16-3-H_B
		H_B: 5.48 (d, 2.2)	Dha16-1; Dha16-2	Dha16-3-H_A
Dha17				
Dha17-1	166.0; C q			
Dha17-2	132.8; C q			
Dha17-3	104.3; CH_2	H_A: 6.41 (d, 1.2)	Dha17-1; Dha17-2	Dha17-3-H_B
		H_B: 5.56 (d, 1.6)	Dha17-1; Dha17-2	Dha17-3-H_A
Q				
Q-1	160.6; C q			
Q-2	143.5; C q			
Q-3	122.5; CH	7.18 (s)	Q-1; Q-5; Q-11	
Q-4	153.8; C q			
Q-5	127.4; C q			
Q-6	123.5; CH	6.80 (d, 10.1)	Q-5; Q-8; Q-10	Q-7
Q-7	129.5; CH	6.30 (dd, 9.8, 5.7)	Q-5; Q-8; Q-9	Q-6; Q-8; Q-9
Q-8	59.2; CH	3.48 (dd, 5.9, 1.8)	Q-6; Q-7; Q-9; Q-10; Ile1-2	Q-7; Q-9
Q-9	67.7; CH	4.25 (s)	Q-5; Q-7; Q-8; Q-10	Q-7; Q-8
Q-10	154.3; C q			
Q-11	64.3; CH	5.19 (q, 6.6)	Q-3; Q-5; Q-12	Q-12
Q-12	22.6; CH_3	1.25 (d, 6.6)	Q-4; Q-11	Q-11

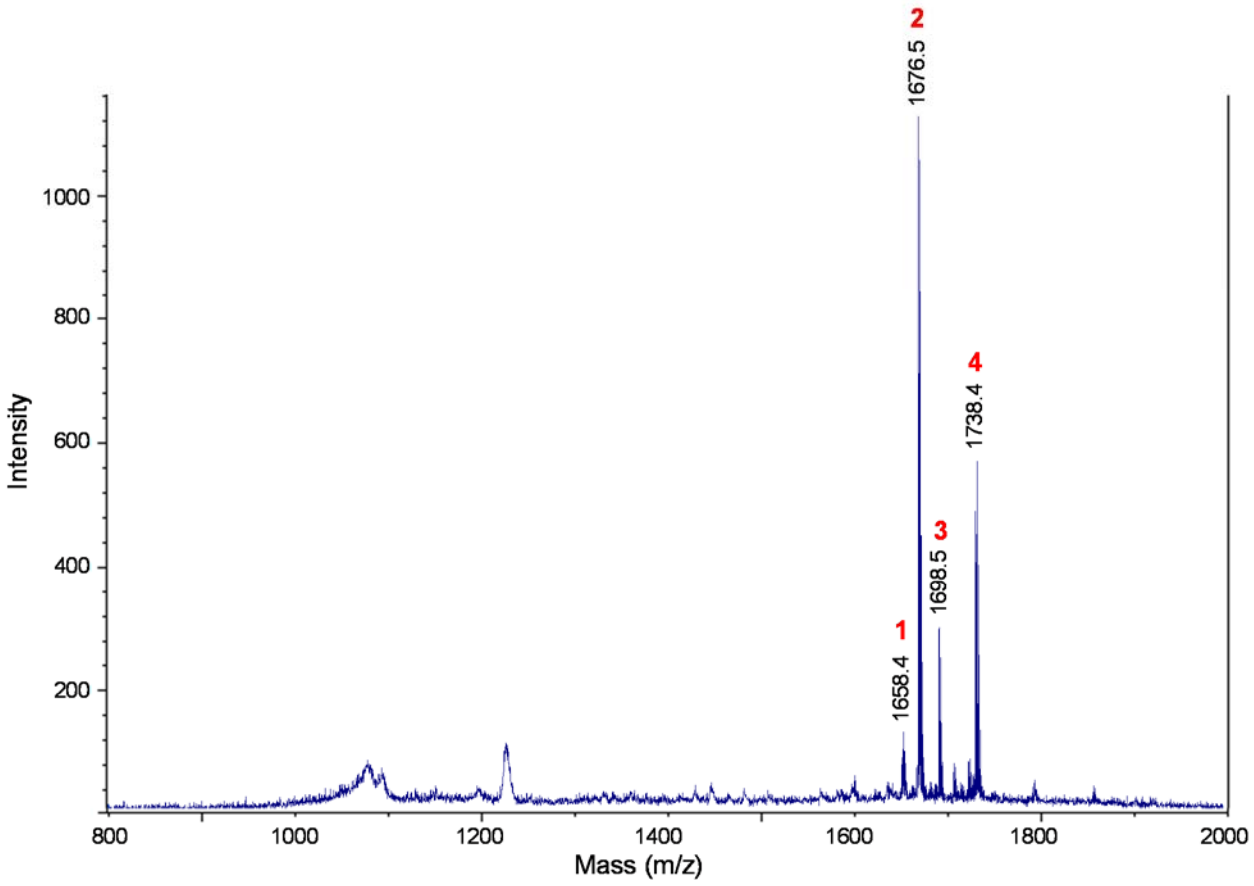
[a] HMBC correlations are from the proton to the indicated carbon.
[b] COSY correlations are from the proton to the proton attached to the indicated position.
[c] The δ of this resonance was determined by HMBC due to overlap with the $CDCl_3$ resonance.
[d] Only those amide resonances demonstrating HMBC or COSY correlations to neighboring carbons or protons, respectively, were assigned.

Figure S10. MS analysis of thiostrepton Ala2Dhb isolated from *S. laurentii* NDS1/int-A2T.

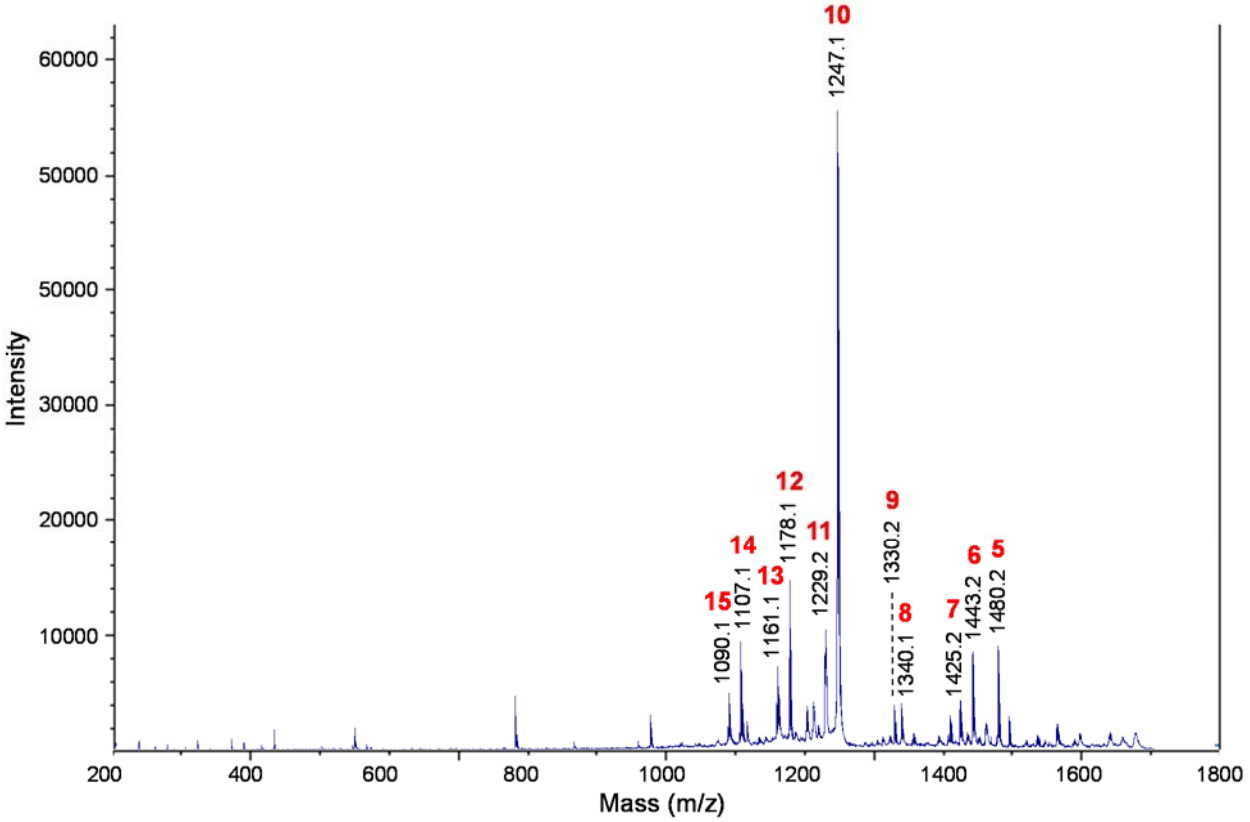
(**A**) HPLC-MS analysis. (1) Chromatogram extracted for *m*/*z* 838.8, the calculated $[M+2H]^{2+}$ ion of thiostrepton Ala2Dhb. (2) Total ion chromatogram.



(**B**) MALDI MS spectrum of thiostrepton Ala2Dhb.



(**C**) MALDI MS/MS of parent ion *m/z* 1676.5.



(**D**) Table and structure showing key ions and fragments in the MALDI MS and MS/MS of thiostrepton Ala2Dhb.

Fragment	Expected	Observed
1. M-H_2O+H^+	1658.5	1658.4
2. M+H^+ (Parent ion)	1676.5	1676.5
3. M+Na^+	1698.5	1698.5
4. M+Cu^+	1738.4	1738.4
5. M-Ile1-Dhb2+H^+	1480.4	1480.2
6. M-QA+H^+	1443.4	1443.2
7. M-QA-H_2O+H^+	1425.4	1425.2
8. M-Ile1-Dhb2-Dha3-Ala4+H^+	1340.3	1340.1
9. M-QA-Ile1+H^+	1330.3	1330.2
10. M-QA-Ile1-Dhb2+H^+	1247.3	1247.1
11. M-QA-Ile1-Dhb2-H_2O+H^+	1229.3	1229.2
12. M-QA-Ile1-Dhb2-Dha3+H^+	1178.3	1178.1
13. M-QA-Ile1-Dhb2-Dha3-OH+H^+	1161.3	1161.1
14. M-QA-Ile1-Dhb2-Dha3-Ala4+H^+	1107.3	1107.1
15. M-QA-Ile1-Dhb2-Dha3-Ala4-OH+H^+	1090.2	1090.1

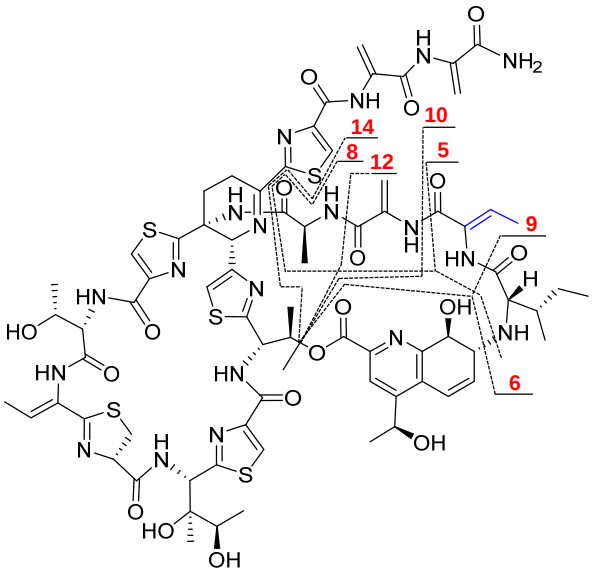
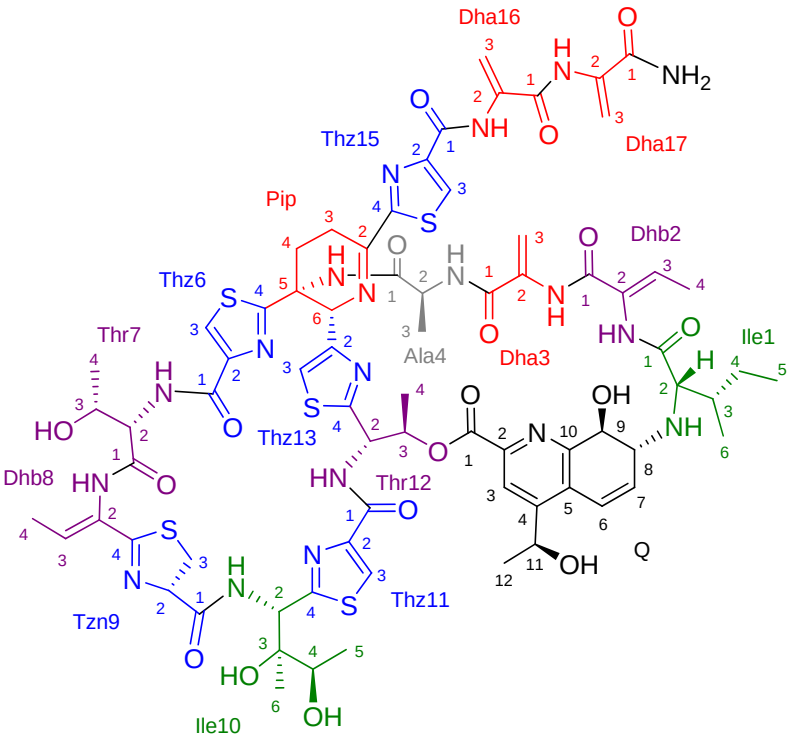


Figure S11. Structure and numbering system used for thiostrepton Ala2Dhb.



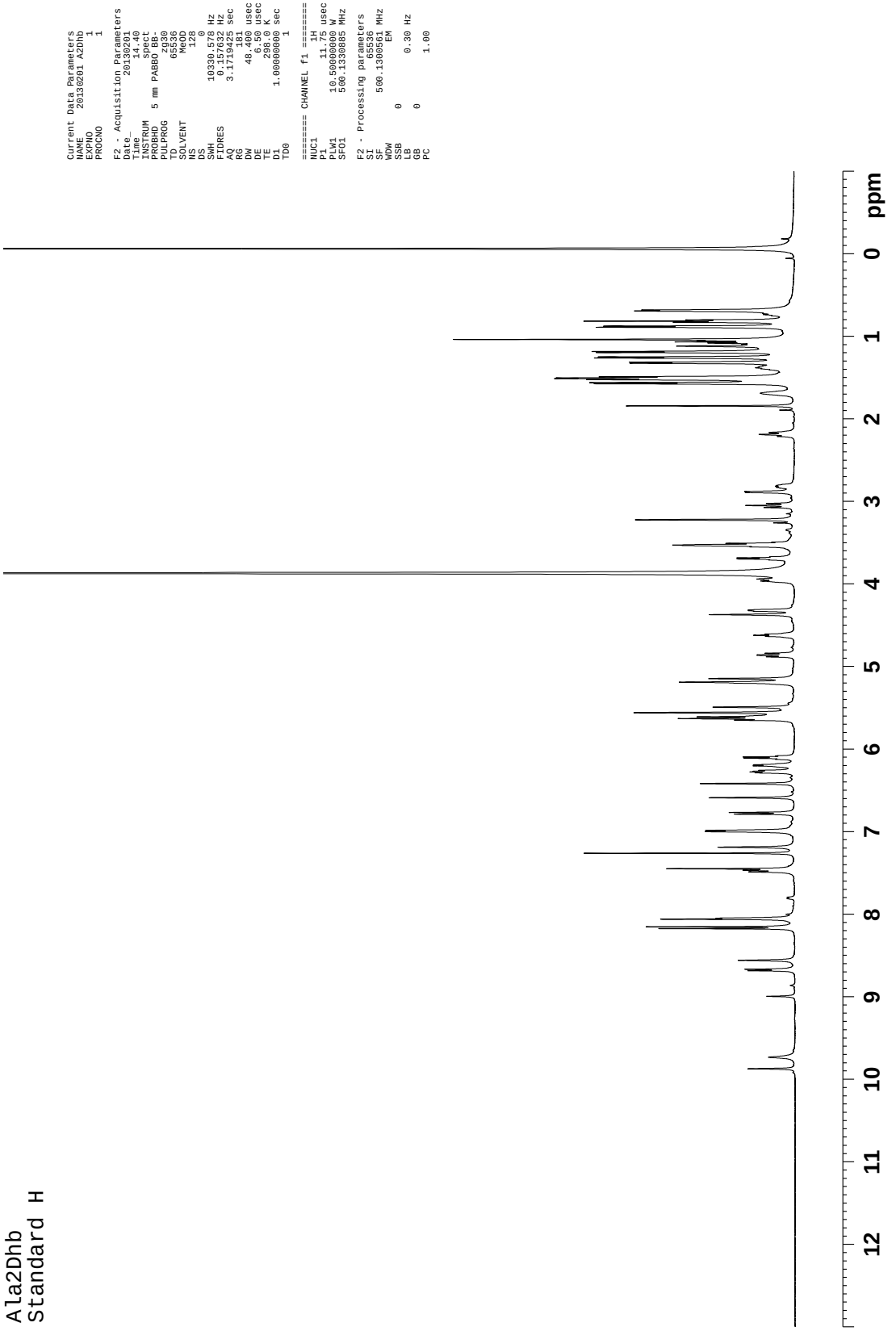


Figure S12. ^{1}H NMR spectrum of thiostrepton Ala2Dhb (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

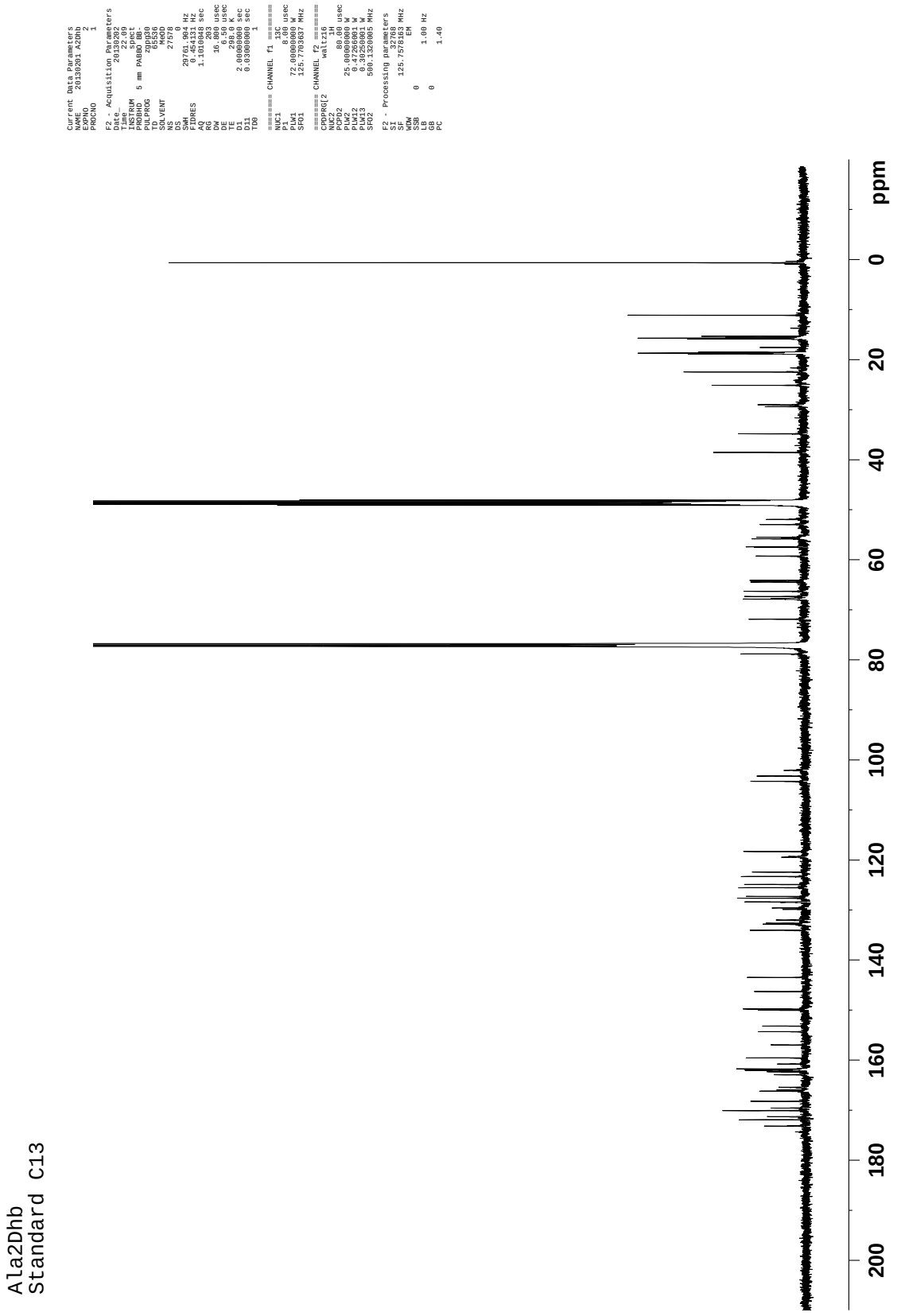


Figure S13. ^{13}C NMR spectrum of thiostrepton Ala2Dhb (125 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

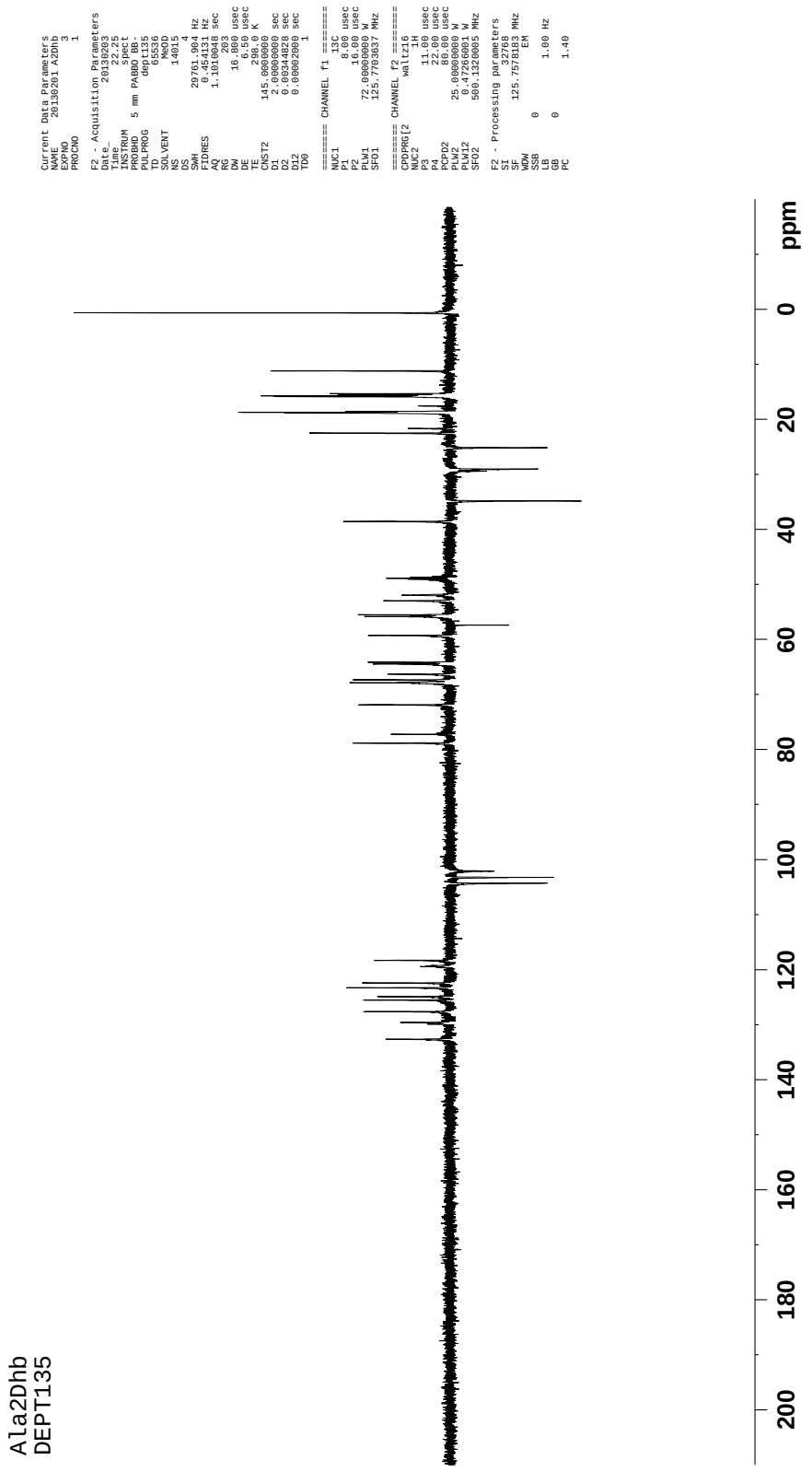


Figure S14. DEPT-135 NMR spectrum of thiostrepton Ala2Dhb (125 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

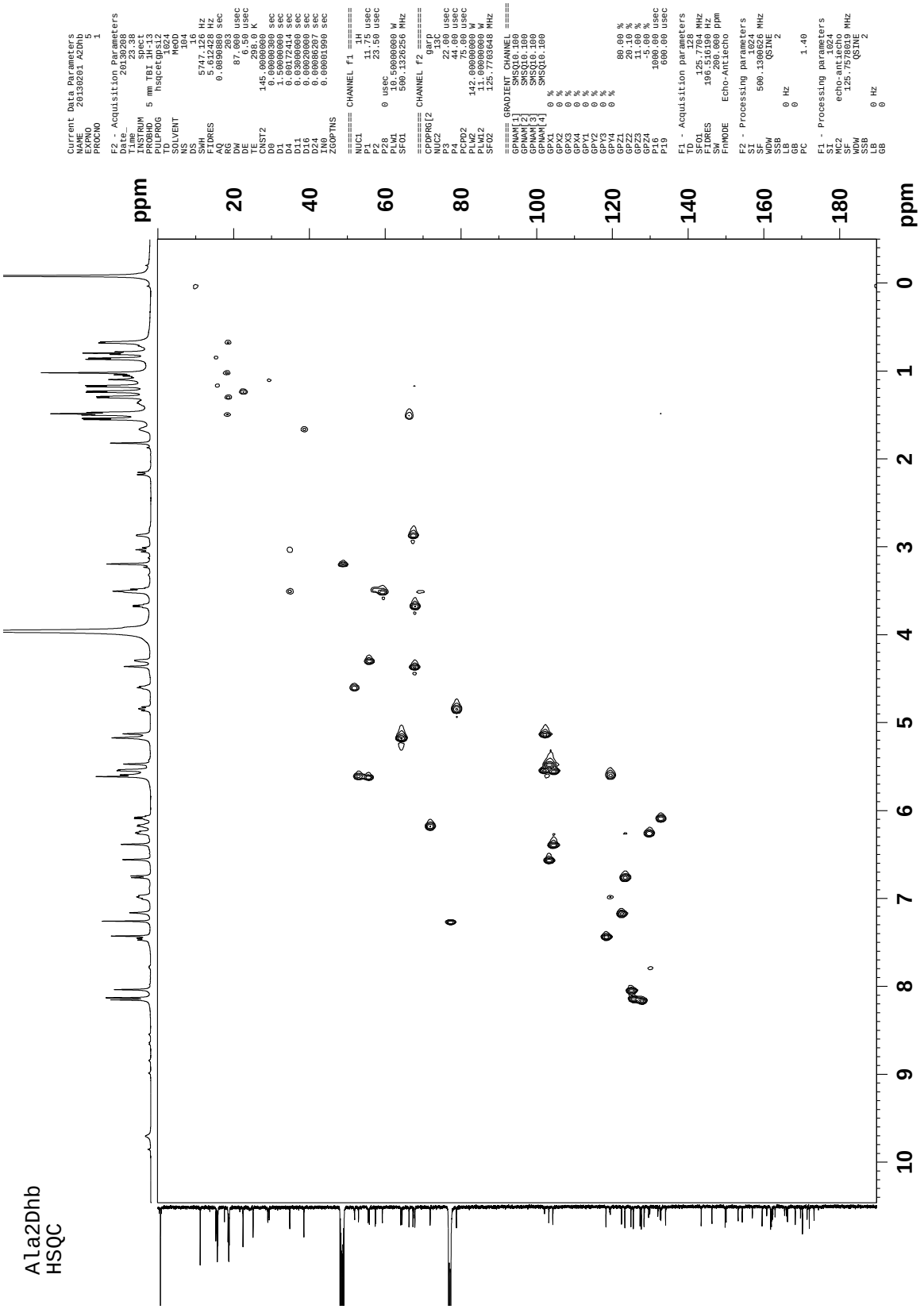


Figure S15. gHSQC spectrum of thiostrepton Ala2Dhb (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

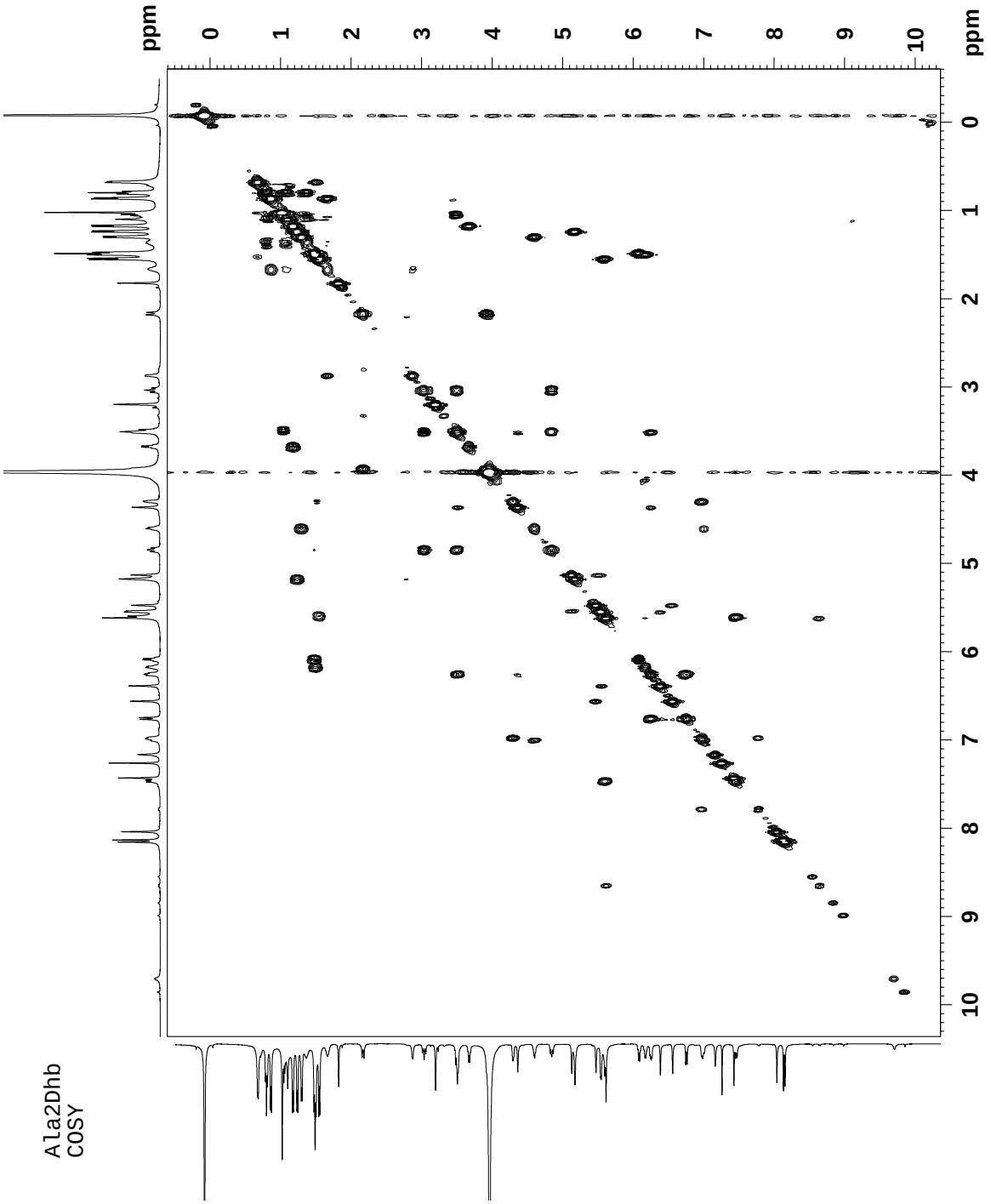


Figure S16. gCOSY spectrum of thiostrepton Ala2Dhb (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

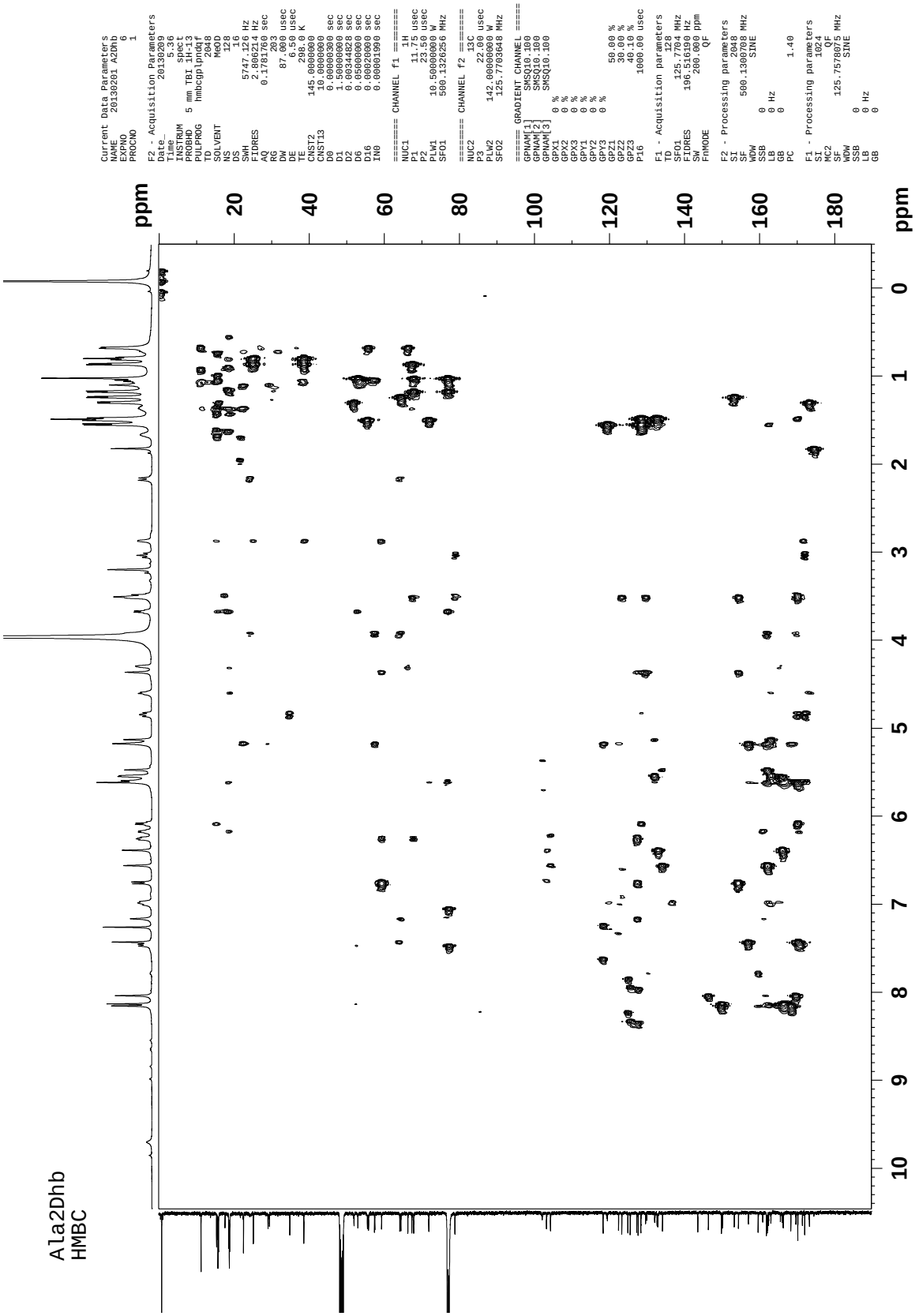


Figure S17. gHMBC spectrum of thiostrepton Ala2Dhb (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

Table S2. ^{1}H and ^{13}C NMR assignments of thiostrepton Ala2Dhb

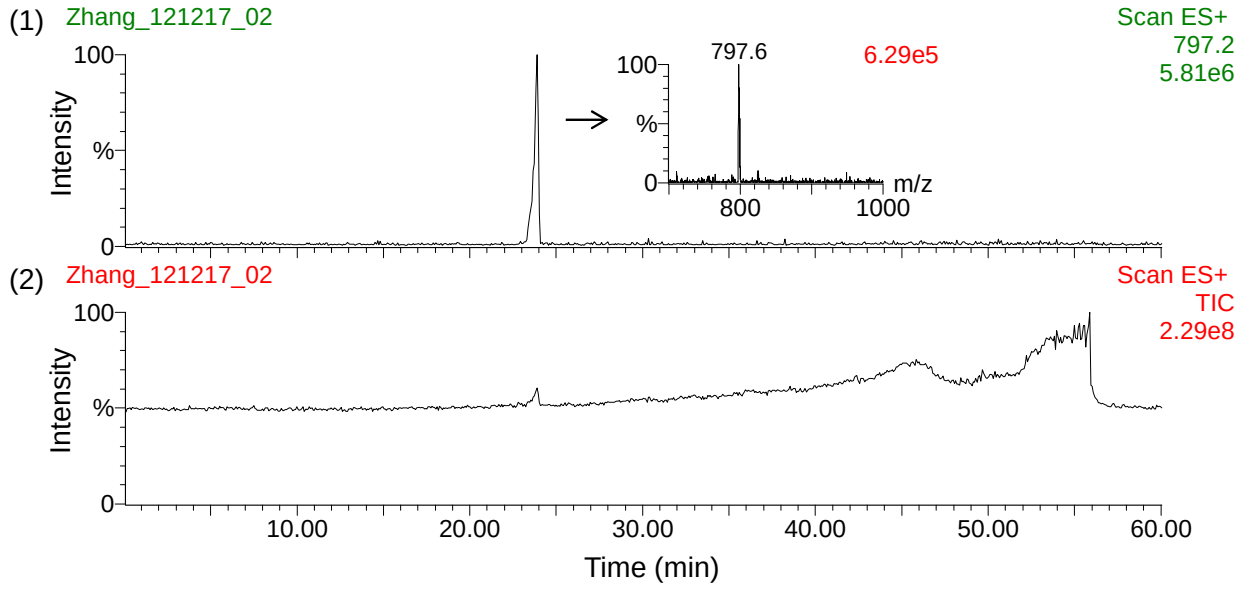
Position	δ_C [ppm]; mult	δ_H [ppm]; (mult, J in Hz)	HMBC[a]	COSY[b]
Ile1				
Ile1-1	171.3; C q			
Ile1-2	67.3; CH	2.87 (d, 4.1)	Ile1-1; Ile1-3; Ile1-4; Ile1-6; Q-8	Ile1-3
Ile1-3	38.5; CH	1.70-1.63 (m)	Ile1-6	Ile1-2; Ile1-4-H_A; Ile1-4-H_B; Ile1-6
Ile1-4	25.1; CH_2	H_A: 1.41-1.33 (m)	Ile1-2; Ile1-5; Ile1-6	Ile1-3; Ile1-4-H_B; Ile1-5
		H_B: 1.12-1.07 (m)	Ile1-2; Ile1-3; Ile1-5; Ile1-6	Ile1-3; Ile1-4-H_A; Ile1-5
Ile1-5	11.2; CH_3	0.80 (t, 6.9)	Ile1-3; Ile1-4	Ile1-4-H_A; Ile1-4-H_B
Ile1-6	15.3; CH_3	0.86 (d, 6.7)	Ile1-2; Ile1-3; Ile1-4	Ile1-3
Dhb2				
Dhb2-1	162.1; C q			
Dhb2-2	128.4; C q			
Dhb2-3	119.4; CH	5.64-5.60 (m)	Dhb2-1; Dhb2-4	Dhb2-4
Dhb2-4	18.5; CH_3	1.55 (d, 7.0)	Dhb2-1; Dhb2-2; Dhb2-3	Dhb2-3
Dhb2-NH[d]		7.02-6.94 (m)	Dha2-1; Dhb2-3	
Dha3				
Dha3-1	163.0; C q			
Dha3-2	132.0; C q			
Dha3-3	102.1; CH_2	H_A: 5.54 (s)	Dha3-1; Dha3-2	Dha3-3-H_B
		H_B: 5.13 (s)	Dha3-1; Dha3-2	Dha3-3-H_A
Ala4				
Ala4-1	173.2; C q			
Ala4-2	51.9; CH	4.60 (q, 6.0)	Dha3-1; Ala4-1; Ala4-3	Ala4-3; Ala4-NH
Ala4-3	18.8; CH_3	1.30 (d, 6.2)	Ala4-1; Ala4-2	Ala4-2
Ala4-NH[d]		7.02-6.94 (m)		Ala4-2
Pip				
Pip-2	161.9; C q			
Pip-3	24.1; CH_2	H_A: 3.34-3.30 (m)		Pip-4-H_B
		H_B: 2.83-2.76 (m)		Pip-4-H_A; Pip-4-H_B
Pip-4	29.0; CH_2	H_A: 3.94-3.87 (m)	Pip-2; Pip-3; Pip-5; Pip-6; Thz6-4	Pip-4-H_B
		H_B: 2.21-2.12 (m)	Pip-3; Pip-6	Pip-3-H_A; Pip-3-H_B; Pip-4-H_A
Pip-5	57.5; C q			
Pip-6	64.1; CH	5.17 (br s)	Pip-2; Pip-4; Pip-5; Thz13-2; Thz13-3; Thz15-4	Pip-3-H_B
Thz6				
Thz6-1	161.8; C q			
Thz6-2	146.3; C q			
Thz6-3	124.9; CH	7.98 (s)	Thz6-1; Thz6-2; Thz6-4	
Thz6-4	169.6; C q			
Thr7				
Thr7-1	165.5; C q			
Thr7-2	55.8; CH	4.32-4.27 (m)	Thr7-3; Thr7-4	Thr7-3; Thr7-NH
Thr7-3	66.3; CH	1.52-1.45 (m)	Thr7-2	Thr7-4
Thr7-4	18.7; CH_3	0.68 (d, 5.5)	Thr7-2; Thr7-3	Thr7-3
Thr7-NH[d]		7.02-6.94 (m)	Thr7-1	Thr7-2
Dhb8				
Dhb8-2	128.4; C q			
Dhb8-3	132.6; CH	6.08 (q, 6.6)	Dhb8-2; Dhb8-4; Tzn9-4	Dhb8-4
Dhb8-4	15.3; CH_3	1.48 (d, 7.8)	Dhb8-2; Dhb8-3; Tzn9-4	Dhb8-3
Tzn9				
Tzn9-1	172.0; C q			
Tzn9-2	78.8; CH	4.84 (dd, 11.8, 9.8)	Dhb8-2; Tzn9-1; Tzn9-3; Tzn9-4	Tzn9-3-H_A; Tzn9-3-H_B
Tzn9-3	34.8; CH_2	H_A: 3.54-3.46 (m)	Tzn9-2; Tzn9-4	Tzn9-2; Tzn9-3-H_B
		H_B: 3.03 (t, 12.1)	Tzn9-1; Tzn9-2	Tzn9-2; Tzn9-3-H_A
Tzn9-4	170.1; C q			
Ile10				
Ile10-2	52.9; CH	5.59 (s)	Ile10-3; Thz11-4	Ile10-NH
Ile10-3	77.2[c]; C q			
Ile10-4	67.8; CH	3.67 (q, 6.3)	Ile10-2; Ile10-3; Ile10-5; Ile10-6	Ile10-5
Ile10-5	15.7; CH_3	1.17 (d, 6.2)	Ile10-3; Ile10-4; Ile10-6	Ile10-4
Ile10-6	18.5; CH_3	1.02 (s)	Ile10-2; Ile10-3; Ile10-4	
Ile10-NH[d]		7.46 (d, 9.8)		Ile10-2

Position	δ_C [ppm]; mult	δ_H [ppm]; (mult, J in Hz)	HMBC[a]	COSY[b]
Thz11				
Thz11-1	162.4; C q			
Thz11-2	150.0; C q			
Thz11-3	125.5; CH	8.13 (s)	Thz11-1; Thz11-2; Thz11-4	
Thz11-4	166.2; C q			
Thr12				
Thr12-2	55.5; CH	5.62 (s)	Thr12-3; Thr12-4; Thz13-2; Thz13-4	Thr12-3; Thr12-NH
Thr12-3	71.9; CH	6.17 (q, 6.2)	Thr12-4; Thz13-4; Q-1	Thr12-4
Thr12-4	18.7; CH_3	1.49 (d, 7.4)	Thr12-2; Thr12-3	Thr12-3
Thr12-NH[d]		8.65 (d, 9.1)		Thr12-2
Thz13				
Thz13-2	157.0; C q			
Thz13-3	118.3; CH	7.43 (s)	Pip-6; Thz13-2; Thz13-4	
Thz13-4	170.1; C q			
Thz15				
Thz15-1	159.6; C q			
Thz15-2	149.8; C q			
Thz15-3	127.6; CH	8.15 (s)	Thz15-1; Thz15-2; Thz15-4	
Thz15-4	168.3; C q			
Dha16				
Dha16-1	162.0; C q			
Dha16-2	134.0; C q			
Dha16-3	103.2; CH_2	H_A: 6.56 (s)	Dha16-1; Dha16-2	Dha16-3-H_B
		H_B: 5.47 (s)	Dha16-1; Dha16-2	Dha16-3-H_A
Dha17				
Dha17-1	166.0; C q			
Dha17-2	132.8; C q			
Dha17-3	104.3; CH_2	H_A: 6.39 (s)	Dha17-1; Dha17-2	Dha17-3-H_B
		H_B: 5.55 (s)	Dha17-1; Dha17-2	Dha17-3-H_A
Q				
Q-1	160.8; C q			
Q-2	143.5; C q			
Q-3	122.4; CH	7.16 (s)	Q-1; Q-5; Q-11	
Q-4	153.2; C q			
Q-5	127.3; C q			
Q-6	123.3; CH	6.76 (d, 9.9)	Q-5; Q-8; Q-10	Q-7
Q-7	129.6; CH	6.25 (dd, 9.0, 5.8)	Q-5; Q-8; Q-9	Q-6; Q-8; Q-9
Q-8	59.2; CH	3.54-3.46 (m)	Ile1-2; Q-6; Q-7; Q-9; Q-10	Q-7; Q-9
Q-9	67.7; CH	4.36 (s)	Q-5; Q-7; Q-8; Q-10	Q-7; Q-8
Q-10	154.3; C q			
Q-11	64.4; CH	5.17 (br s)	Q-3; Q-12	Q-12
Q-12	22.5; CH_3	1.24 (d, 6.4)	Q-4; Q-11	Q-11

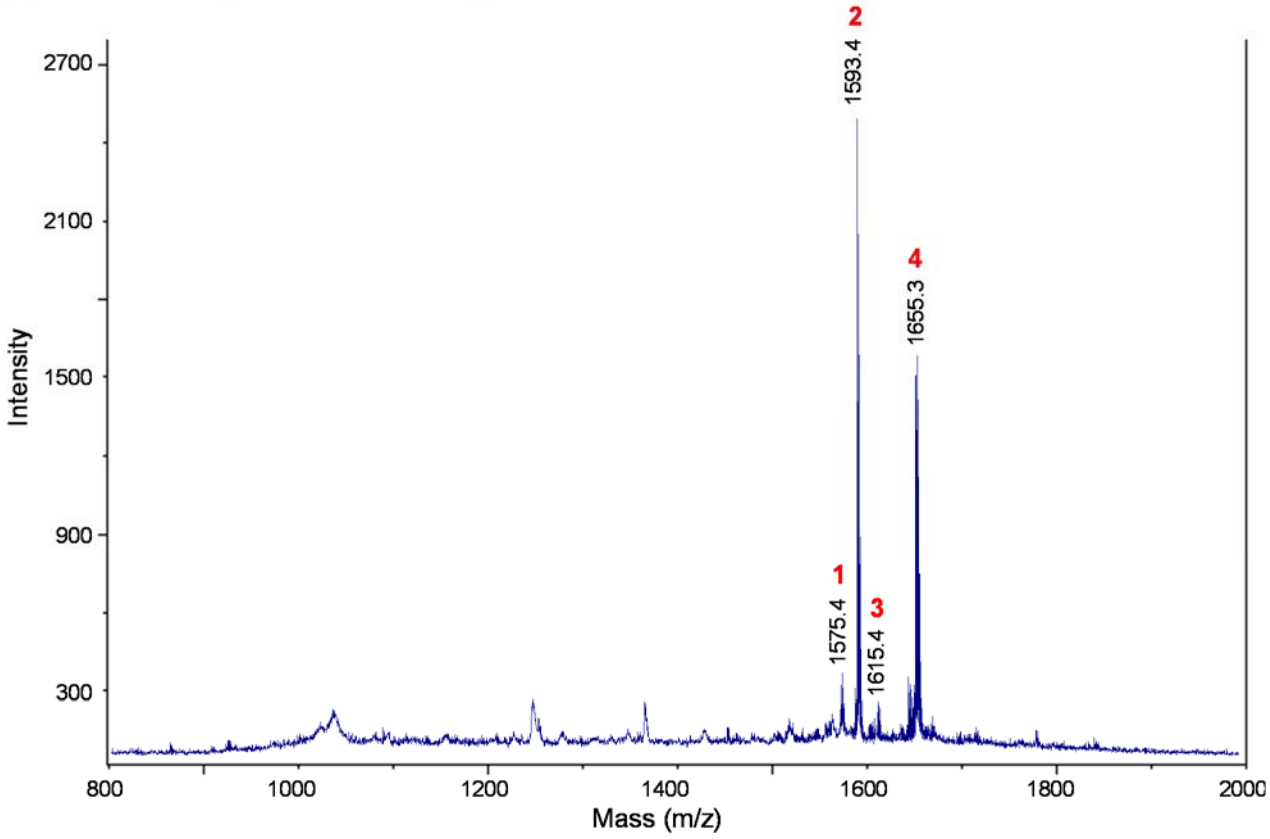
[a] HMBC correlations are from the proton to the indicated carbon.
[b] COSY correlations are from the proton to the proton attached to the indicated position.
[c] The δ of this resonance was determined by HMBC due to overlap with the $CDCl_3$ resonance.
[d] Only those amide resonances demonstrating either HMBC or COSY correlations to neighboring carbons or protons, respectively, were assigned.

Figure S18. MS analysis of thiostrepton Ala2Ile-ΔIle1 isolated from *S. laurentii* NDS1/int-A2I.

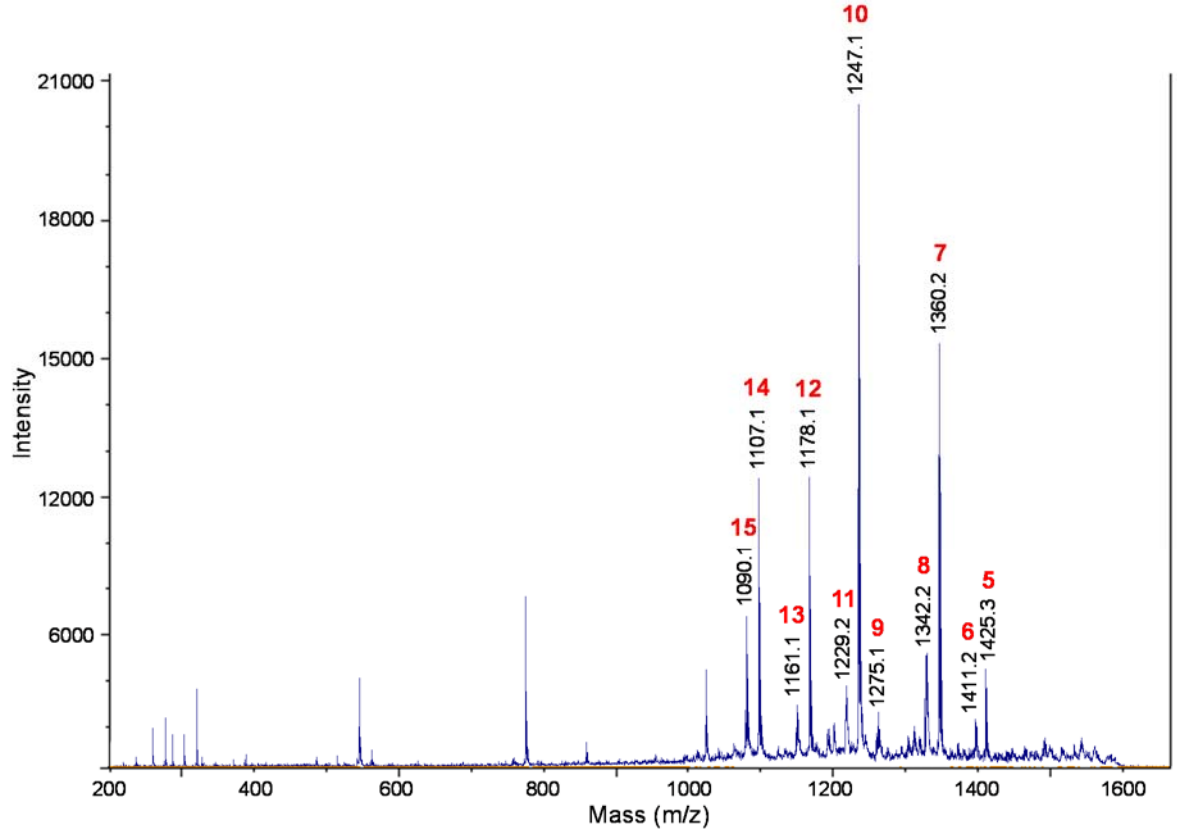
(**A**) HPLC-MS analysis. (1) Chromatogram extracted for *m*/*z* 797.2, the calculated $[M+2H]^{2+}$ ion of thiostrepton Ala2Ile-ΔIle1. (2) Total ion chromatogram.



(**B**) MALDI MS spectrum of thiostrepton Ala2Ile-ΔIle1.



(**C**) MALDI MS/MS of parent ion *m/z* 1593.4.



(**D**) Table and structure showing key ions and fragments in the MALDI MS and MS/MS of thiostrepton Ala2Ile-ΔIle1.

Fragment	Expected	Observed
1. M-H_2O+H^+	1575.5	1575.4
2. M+H^+ (Parent ion)	1593.5	1593.4
3. M+Na^+	1615.4	1615.4
4. M+Cu^+	1655.4	1655.3
5. M-Dhb8-Tzn9+H^+	1425.4	1425.3
6. M-Ile2-Dha3+H^+	1411.4	1411.2
7. M-QA+H^+	1360.4	1360.2
8. M-QA-H_2O+H^+	1342.4	1342.2
9. M-QA-(Ile2-CO)+H^+	1275.3	1275.1
10. M-QA-Ile2+H^+	1247.3	1247.1
11. M-QA-Ile2-H_2O+H^+	1229.3	1229.2
12. M-QA-Ile2-Dha3+H^+	1178.3	1178.1
13. M-QA-Ile2-Dha3-OH+H^+	1161.3	1161.1
14. M-QA-Ile2-Dha3-Ala4+H^+	1107.3	1107.1
15. M-QA-Ile2-Dha3-Ala4-OH+H^+	1090.2	1090.1

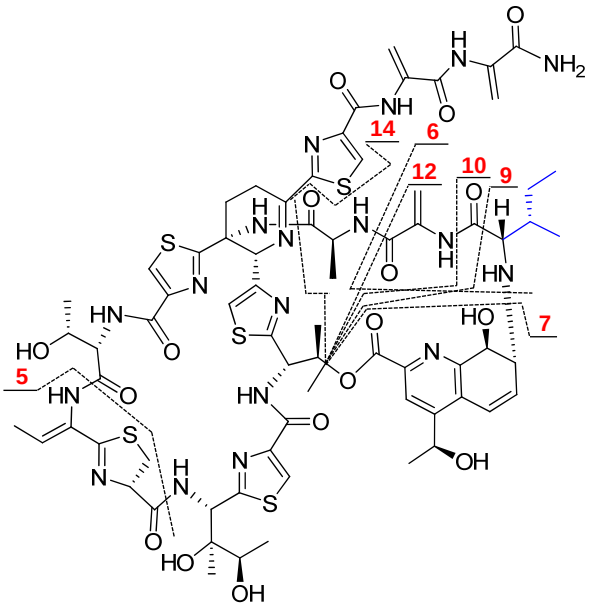
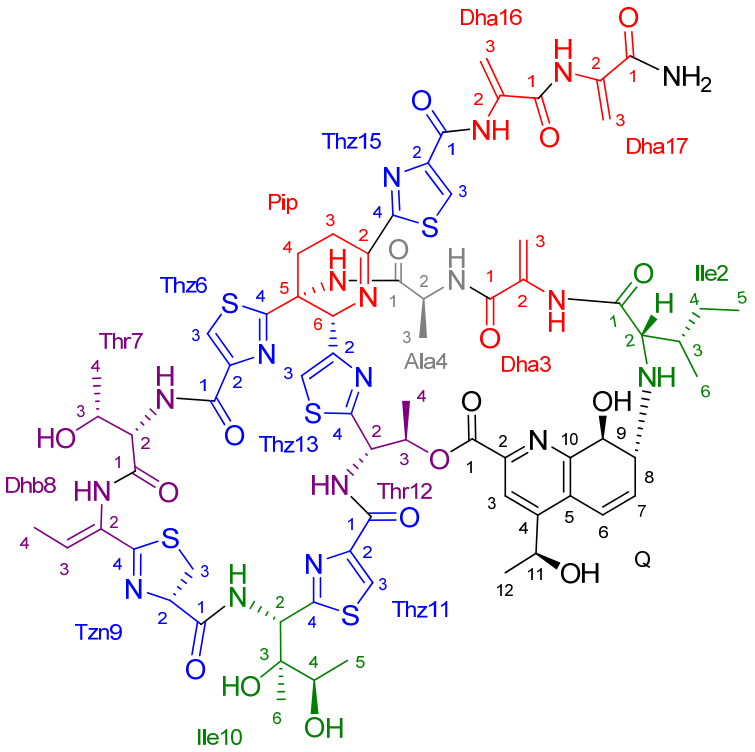


Figure S19. Structure and numbering system used for thiostrepton Ala2Ile-ΔIle1.



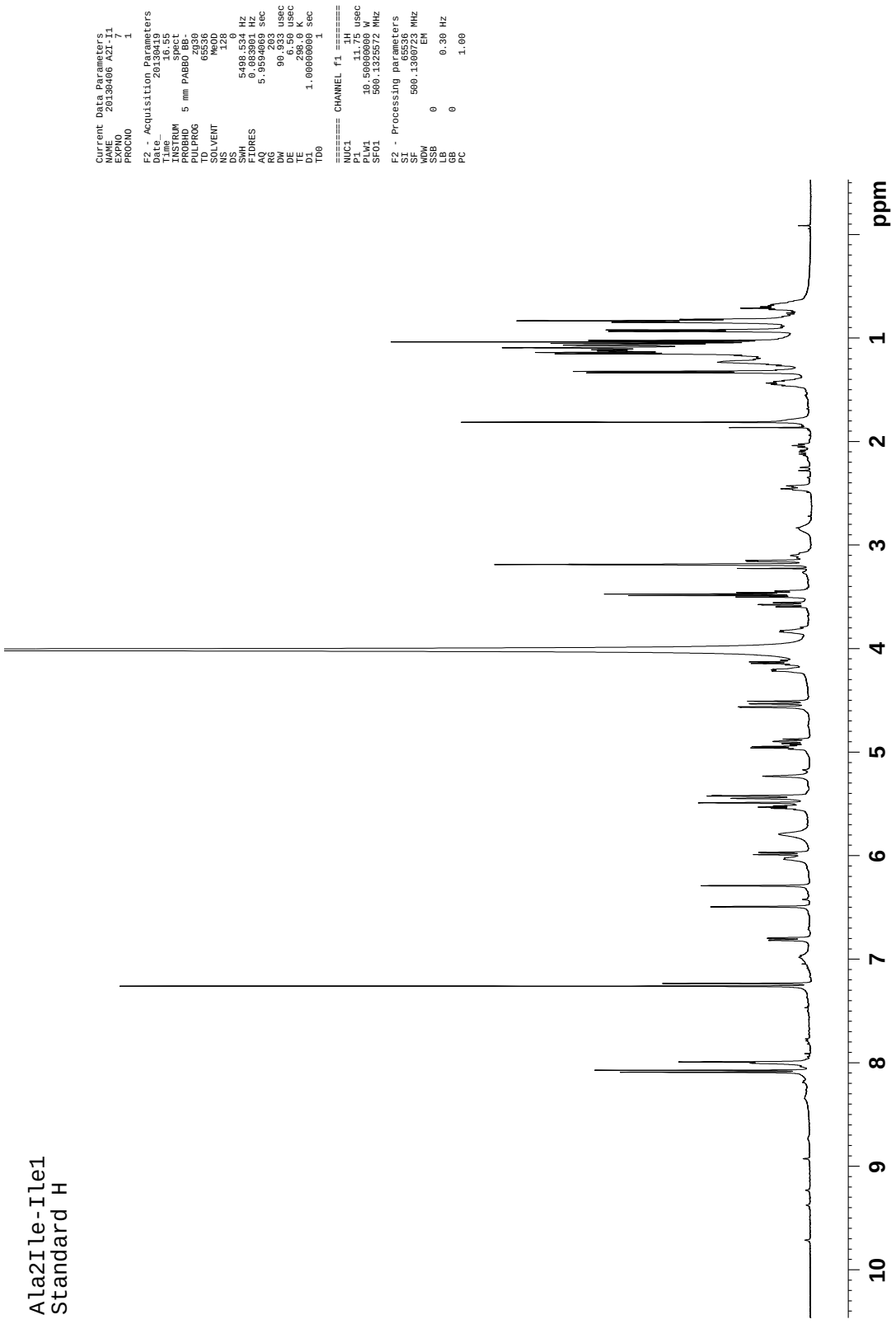


Figure S20. ^{1}H NMR spectrum of thiostrepton Ala2Ile-ΔIle1 (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

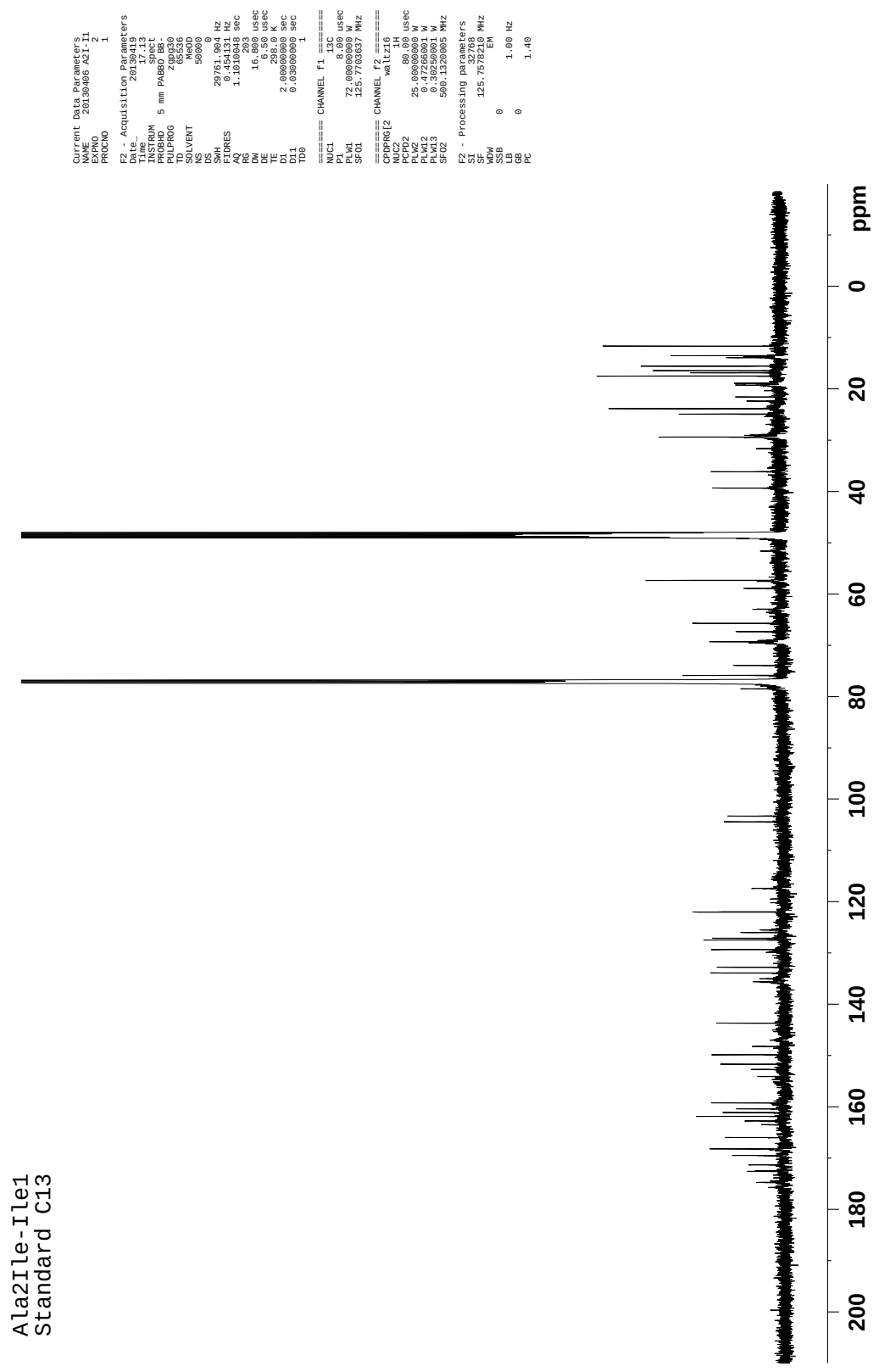


Figure S21. ^{13}C NMR spectrum of thiostrepton Ala2Ile-ΔIle1 (125 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

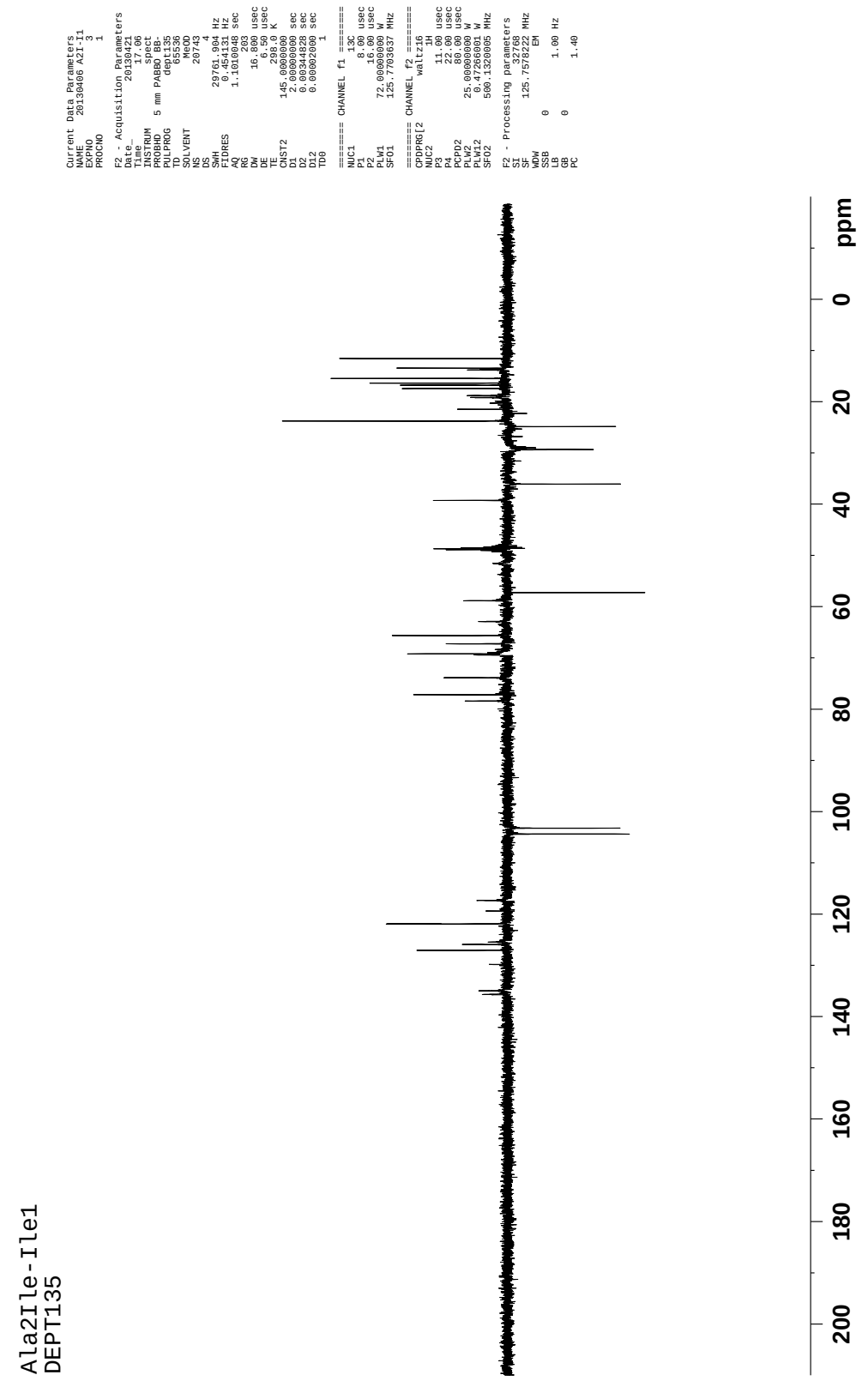


Figure S22. DEPT-135 NMR spectrum of thiostrepton Ala2Ile-ΔIle1 (125 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

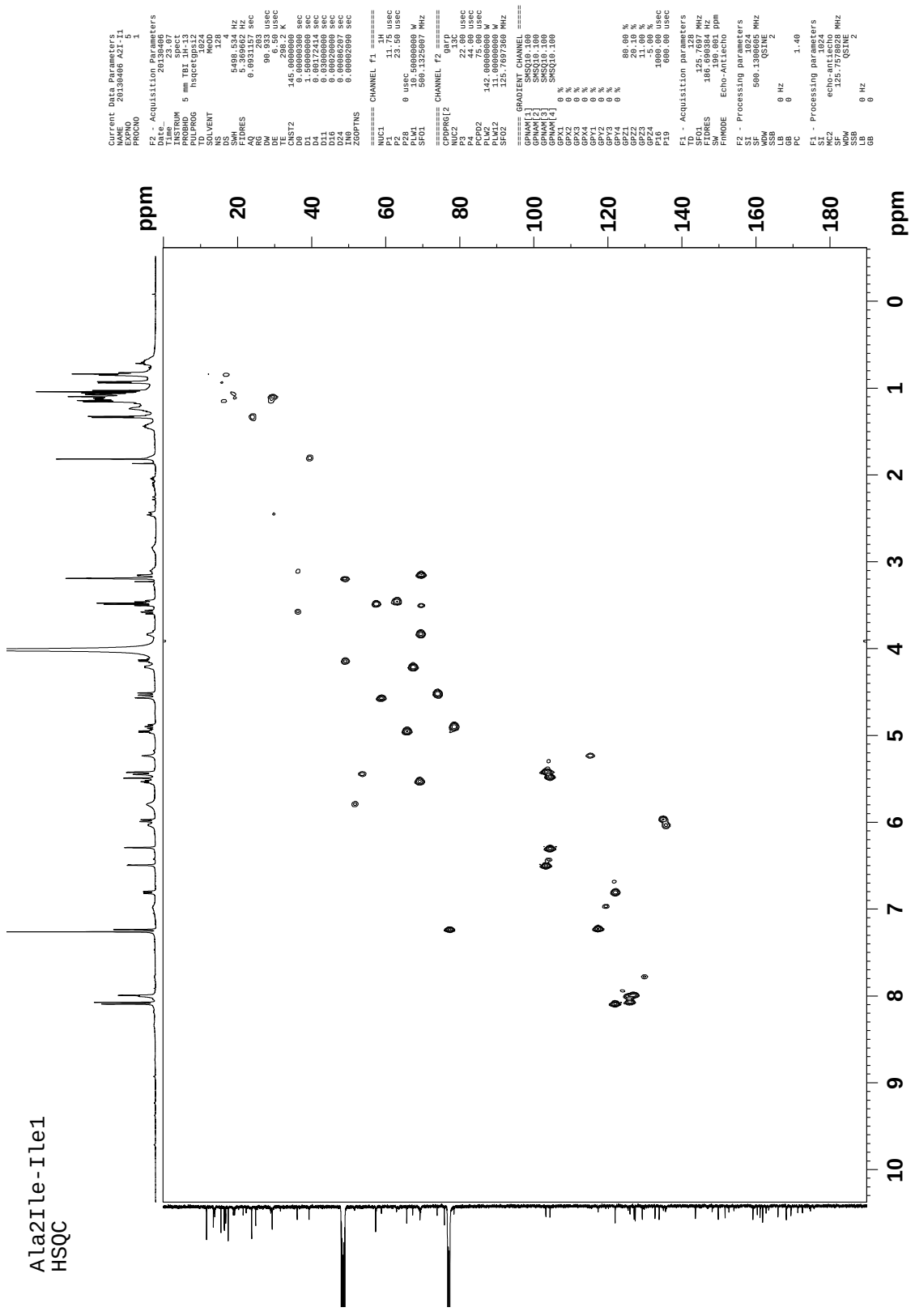


Figure S23. gHSQC NMR spectrum of thiostrepton Ala2Ile-ΔIle1 (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

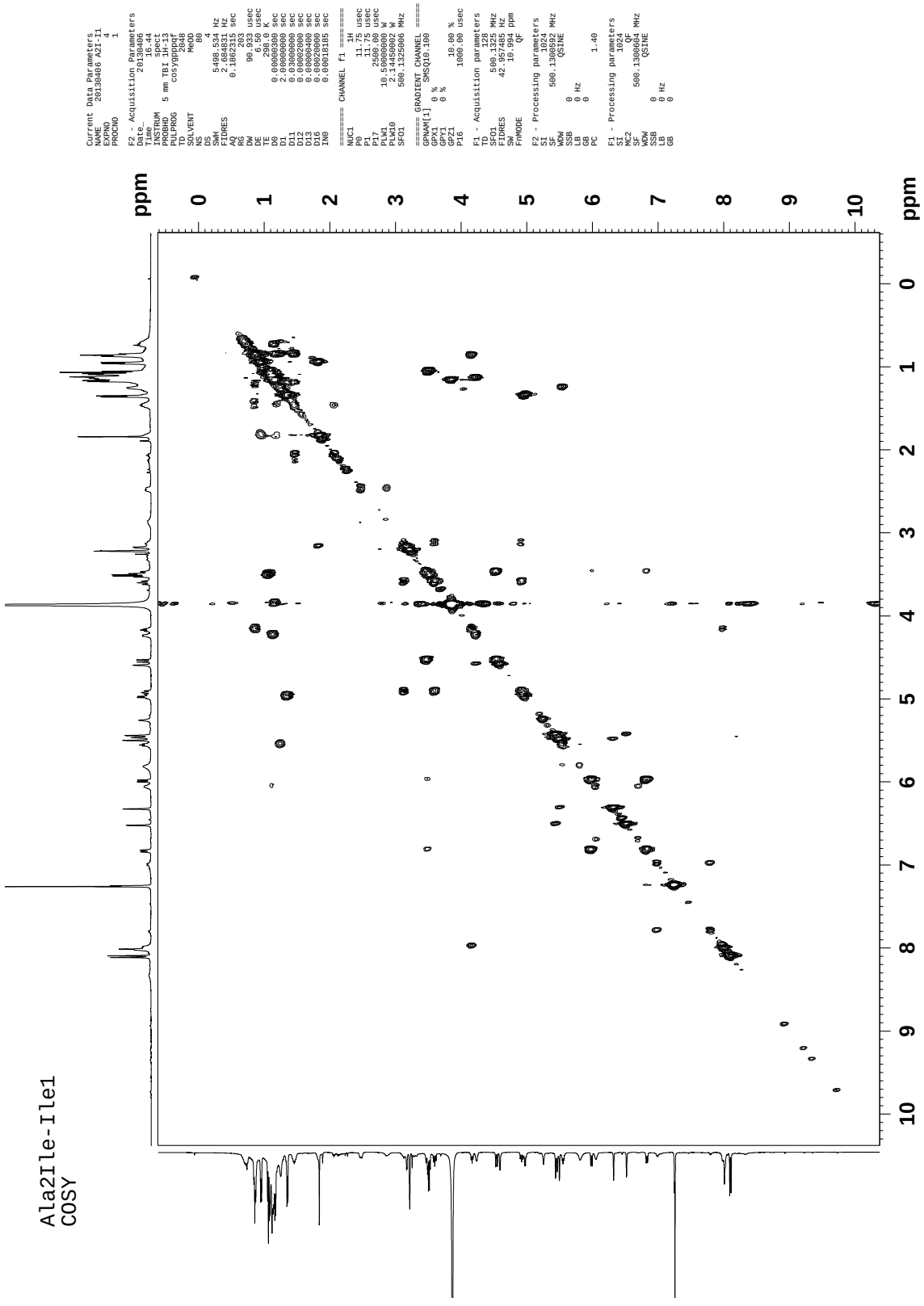


Figure S24. gCOSY NMR spectrum of thiostrepton Ala2Ile-ΔIle1 (125 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

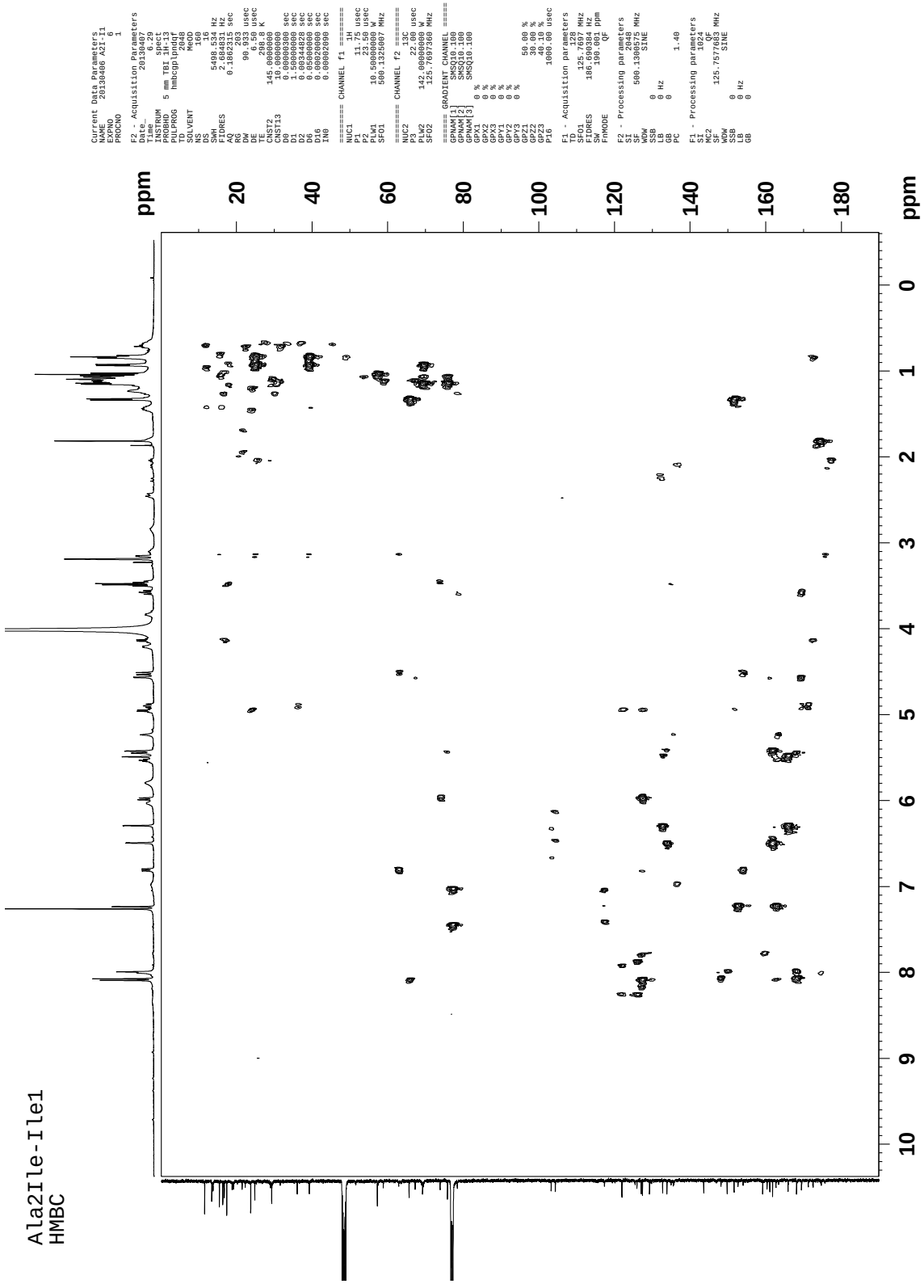


Figure S25. gHMBC NMR spectrum of thiostrepton Ala2Ile-ΔIle1 (125 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

Table S3. ^{1}H and ^{13}C NMR assignments of thiostrepton Ala2Ile-ΔIle1

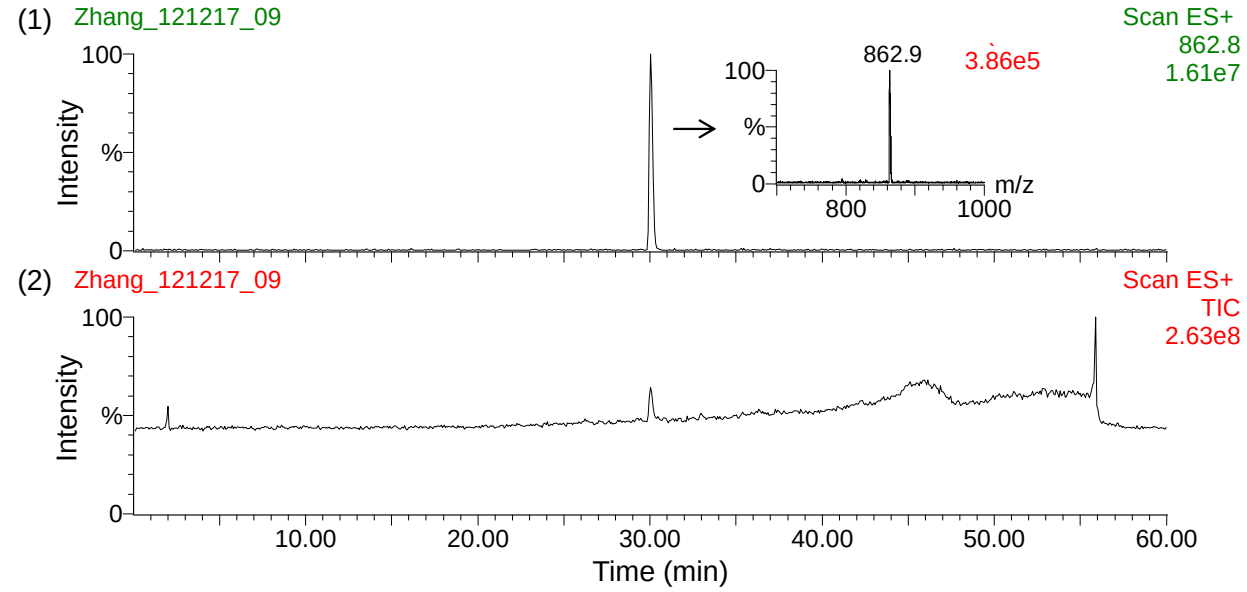
Position	δ_C [ppm]; mult	δ_H [ppm]; (mult, J in Hz)	HMBC[a]	COSY[b]
Ile2				
Ile2-1	174.7; C q			
Ile2-2	69.4; CH	3.15 (d, 3.8)	Ile2-3; Ile2-4; Ile2-6	Ile2-3
Ile2-3	39.3; CH	1.82-1.78 (m)	Ile2-1	Ile2-2; Ile2-4-H_A; Ile2-4-H_B; Ile2-6
Ile2-4	24.9; CH_2	H_A: 1.48-1.39 (m)	Ile2-3; Ile2-5; Ile2-6	Ile2-3; Ile2-4-H_B; Ile2-5
		H_B: 1.21-1.16 (m)	Ile2-2	Ile2-3; Ile2-4-H_A; Ile2-5
Ile2-5	11.6; CH_3	0.83 (t, 7.3)	Ile2-3; Ile2-4; Ile2-6	Ile2-4-H_A; Ile2-4-H_B
Ile2-6	15.5; CH_3	0.93 (d, 6.9)	Ile2-2; Ile2-3; Ile2-4; Ile2-5	Ile2-3
Dha3				
Dha3-1	163.4; C q			
Dha3-2	135.6; C q			
Dha3-3	115.2; CH_2	H_A: 5.79 (br s)		
		H_B: 5.23 (br s)	Dha3-1; Dha3-2	
Ala4				
Ala4-1	172.5; C q			
Ala4-2	49.3; CH	4.14 (q, 7.4)	Ala4-1; Ala4-3	Ala4-3
Ala4-3	16.8; CH_3	0.84 (d, 7.3)	Ala4-1; Ala4-2	Ala4-2
Pip				
Pip-2	159.2; C q			
Pip-3	22.3; CH_2	H_A: 3.50-3.40 (m)		Pip-3-H_B; Pip-4-H_B
		H_B: 2.89-2.80 (m)		Pip-4-H_B
Pip-4	29.3; CH_2	H_A: 3.86-3.80 (m)		
		H_B: 2.48-2.42 (m)		Pip-3-H_A; Pip-3-H_B
Pip-5	57.5; C q			
Pip-6	63.6; CH	5.23 (br s)		
Thz6				
Thz6-1	161.1; C q			
Thz6-2	149.8; C q			
Thz6-3	127.1; CH	7.99 (s)	Thz6-2; Thz6-4	
Thz6-4	168.2; C q			
Thr7				
Thr7-1	169.5; C q			
Thr7-2	58.8; CH	4.56 (d, 2.0)	Thz6-1; Thr7-1; Thr7-3	Thr7-3
Thr7-3	67.3; CH	4.23-4.18 (m)		Thr7-2; Thr7-4
Thr7-4	19.2; CH_3	1.12 (d, 6.0)	Thr7-2; Thr7-3	Thr7-3
Dhb8				
Dhb8-2	127.4; C q			
Dhb8-3	135.7; CH	6.08-6.03 (m)	Dhb8-2	Dhb8-4
Dhb8-4	19.2; CH_3	1.13-1.09 (m)		
Tzn9				
Tzn9-1	171.3; C q			
Tzn9-2	78.4; CH	4.90 (dd, 11.4, 9.8)	Tzn9-1; Tzn9-3; Tzn9-4	Tzn9-3-H_A; Tzn9-3-H_B
Tzn9-3	36.1; CH_2	H_A: 3.58 (dd, 11.1, 9.2)	Tzn9-2; Tzn9-4	Tzn9-2; Tzn9-3-H_B
		H_B: 3.10 (t, 12.0)		Tzn9-2; Tzn9-3-H_A
Tzn9-4	169.5; C q			
Ile10				
Ile10-2	53.7; CH	5.45 (s)	Ile10-3; Thz11-4	
Ile10-3	75.8; C q			
Ile10-4	69.3; CH	3.86-3.80 (m)		Ile10-5
Ile10-5	16.4; CH_3	1.15 (d, 6.5)	Ile10-3; Ile10-4	Ile10-4
Ile10-6	18.8; CH_3	1.07 (s)	Ile10-2; Ile10-3; Ile10-4; Ile10-5	
Thz11				
Thz11-1	162.7; C q			
Thz11-2	149.8; C q			
Thz11-3	121.9; CH	8.09 (s)	Thz11-1; Thz11-4	
Thz11-4	168.3; C q			
Thr12				
Thr12-2	51.6; CH	5.79 (br s)		Thr12-3
Thr12-3	69.3; CH	5.53 (pentet, 6.1)	Q-1	Thr12-2; Thr12-4
Thr12-4	13.8; CH_3	1.26-1.21 (m)		Thr12-3

Position	δ_C [ppm]; mult	δ_H [ppm]; (mult, J in Hz)	HMBC[a]	COSY[b]
Thz13				
Thz13-2	159.2; C q			
Thz13-3	125.5; CH	8.00 (s)	Thz13-4	
Thz13-4	168.2; C q			
Thz15				
Thz15-1	160.4; C q			
Thz15-2	148.2; C q			
Thz15-3	126.0; CH	8.07 (s)	Thz15-2; Thz15-4	
Thz15-4	168.3; C q			
Dha16				
Dha16-1	161.8; C q			
Dha16-2	133.9; C q			
Dha16-3	103.2; CH_2	H_A: 6.49 (d, 2.2)	Dha16-1; Dha16-2	Dha16-3-H_B
		H_B: 5.42 (d, 2.1)	Dha16-1; Dha16-2	Dha16-3-H_A
Dha17				
Dha17-1	165.9; C q			
Dha17-2	132.7; C q			
Dha17-3	104.4; CH_2	H_A: 6.29 (d, 1.4)	Dha17-1; Dha17-2	Dha17-3-H_B
		H_B: 5.49 (d, 1.3)	Dha17-1; Dha17-2	Dha17-3-H_A
Q				
Q-1	162.7; C q			
Q-2	152.7; C q			
Q-3	117.4; CH	7.23 (s)	Q-1; Q-2; Q-10	
Q-4	151.7; C q			
Q-5	127.4; C q			
Q-6	122.0; CH	6.81 (dd, 10.1, 2.3)	Q-5; Q-8; Q-10	Q-7; Q-8
Q-7	135.0 CH	5.98 (m)	Q-5; Q-9	Q-6; Q-8
Q-8	62.9; CH	3.50-3.40 (m)	Q-7; Q-9	Q-6; Q-7; Q-9
Q-9	73.9; CH	4.52 (d, 12.4)	Q-8; Q-10	Q-8
Q-10	154.0; C q			
Q-11	65.7; CH	4.95 (q, 6.5)	Q-4; Q-5; Q-6; Q-12	Q-12
Q-12	23.8; CH_3	1.33 (d, 6.6)	Q-4; Q-11	Q-11

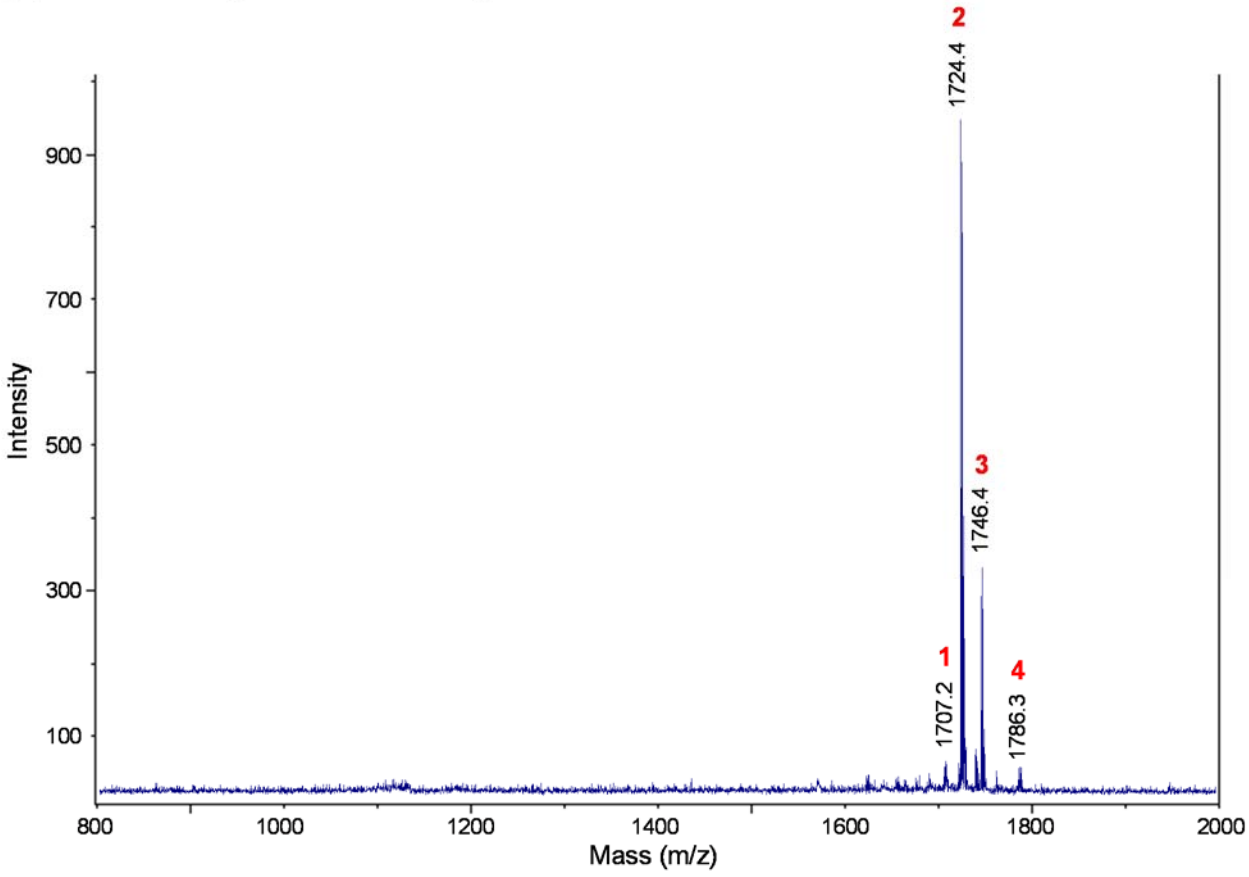
[a] HMBC correlations are from the proton to the indicated carbon.
[b] COSY correlations are from the proton to the proton attached to the indicated position.

Figure S26. MS analysis of thiostrepton Ala2Met isolated from *S. laurentii* NDS1/int-A2M.

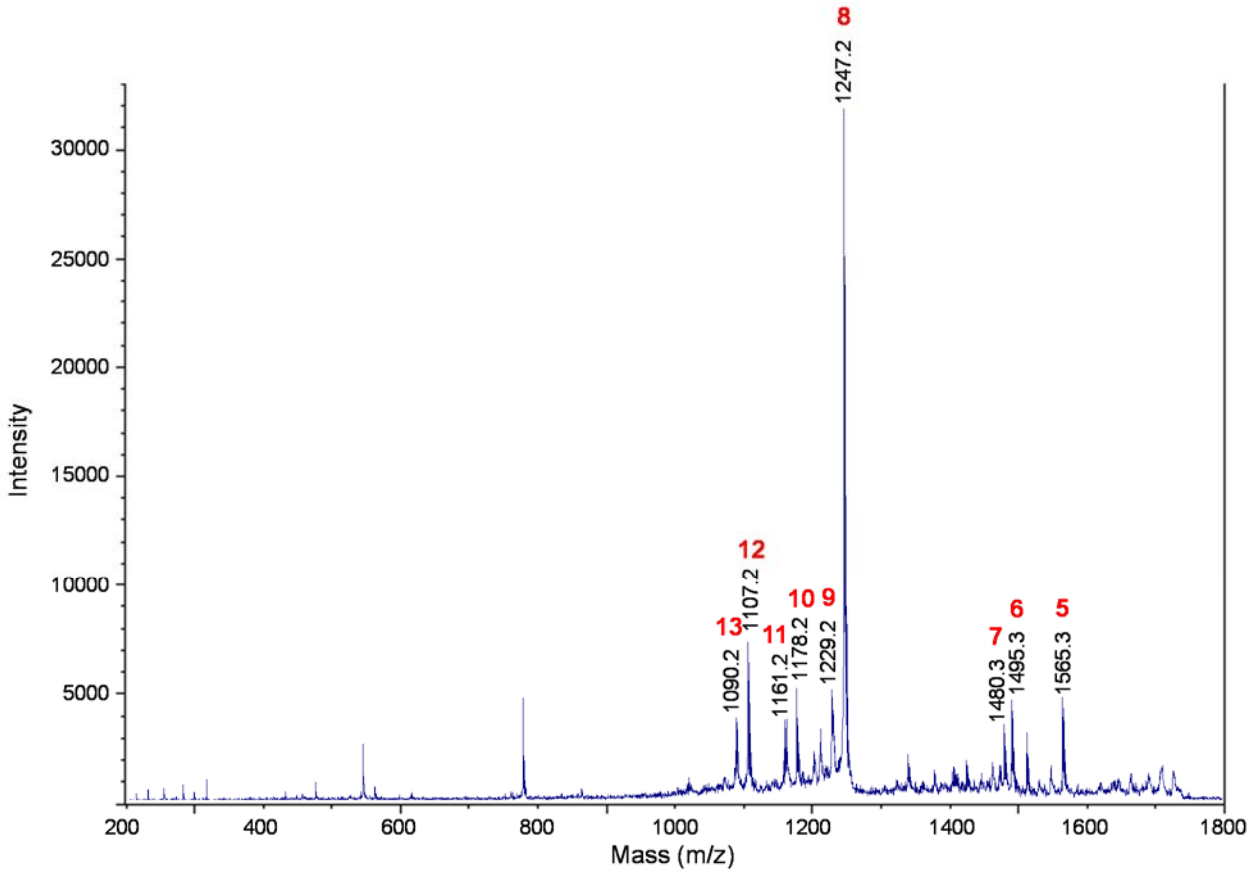
(**A**) HPLC-MS analysis. (1) Chromatogram extracted for *m*/*z* 862.8, the calculated $[M+2H]^{2+}$ ion of thiostrepton Ala2Met. (2) Total ion chromatogram.



(**B**) MALDI MS spectrum of thiostrepton Ala2Met.



(**C**) MALDI MS/MS of parent ion *m/z* 1724.4.



(**D**) Table and structure showing key ions and fragments in the MALDI MS and MS/MS of thiostrepton Ala2Met.

Fragment	Expected	Observed
1. M-OH+H$^+$	1707.5	1707.2
2. M+H$^+$ (Parent ion)	1724.5	1724.4
3. M+Na$^+$	1746.5	1746.4
4. M+Cu$^+$	1786.4	1786.3
5. M-Met2-CO+H$^+$	1565.5	1565.3
6. M-(Ile1-NH)-Met2+H$^+$	1495.4	1495.3
7. M-Ile1-Met2+H$^+$	1480.4	1480.3
8. M-QA-Ile1-Met2+H$^+$	1247.3	1247.2
9. M-QA-Ile1-Met2-H_2O+H$^+$	1229.3	1229.2
10. M-QA-Ile1-Met2-Dha3+H$^+$	1178.3	1178.2
11. M-QA-Ile1-Met2-Dha3-OH+H$^+$	1161.3	1161.2
12. M-QA-Ile1-Met2-Dha3-Ala4+H$^+$	1107.3	1107.2
13. M-QA-Ile1-Met2-Dha3-Ala4-OH+H$^+$	1090.2	1090.2

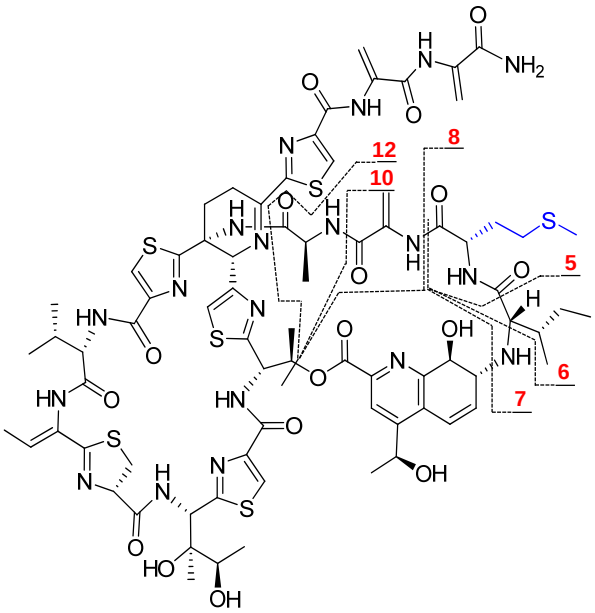
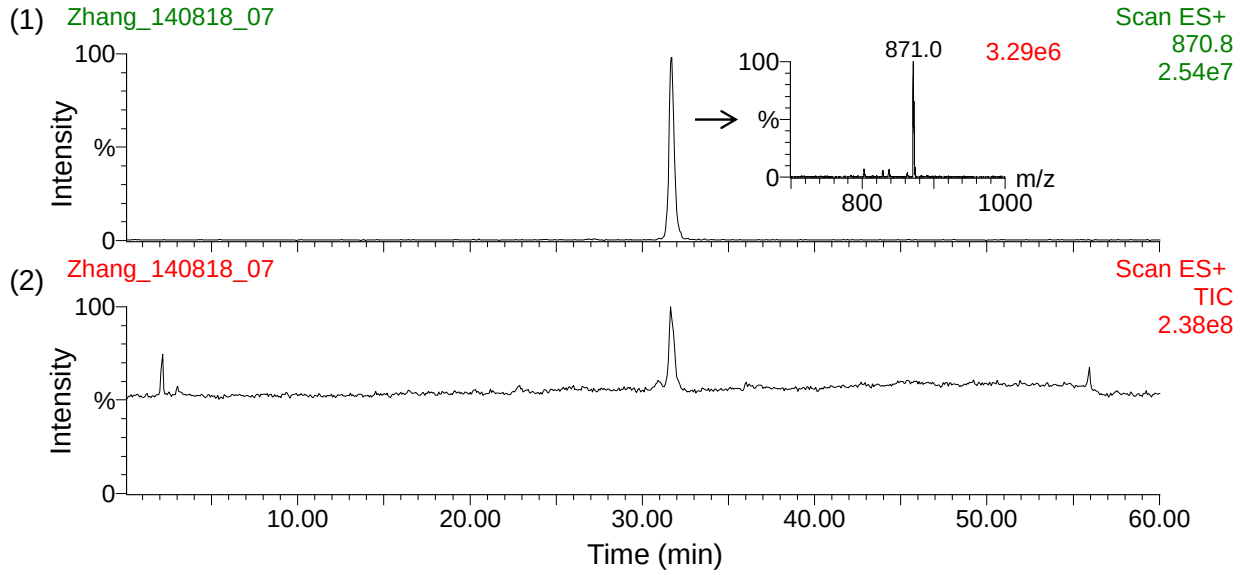
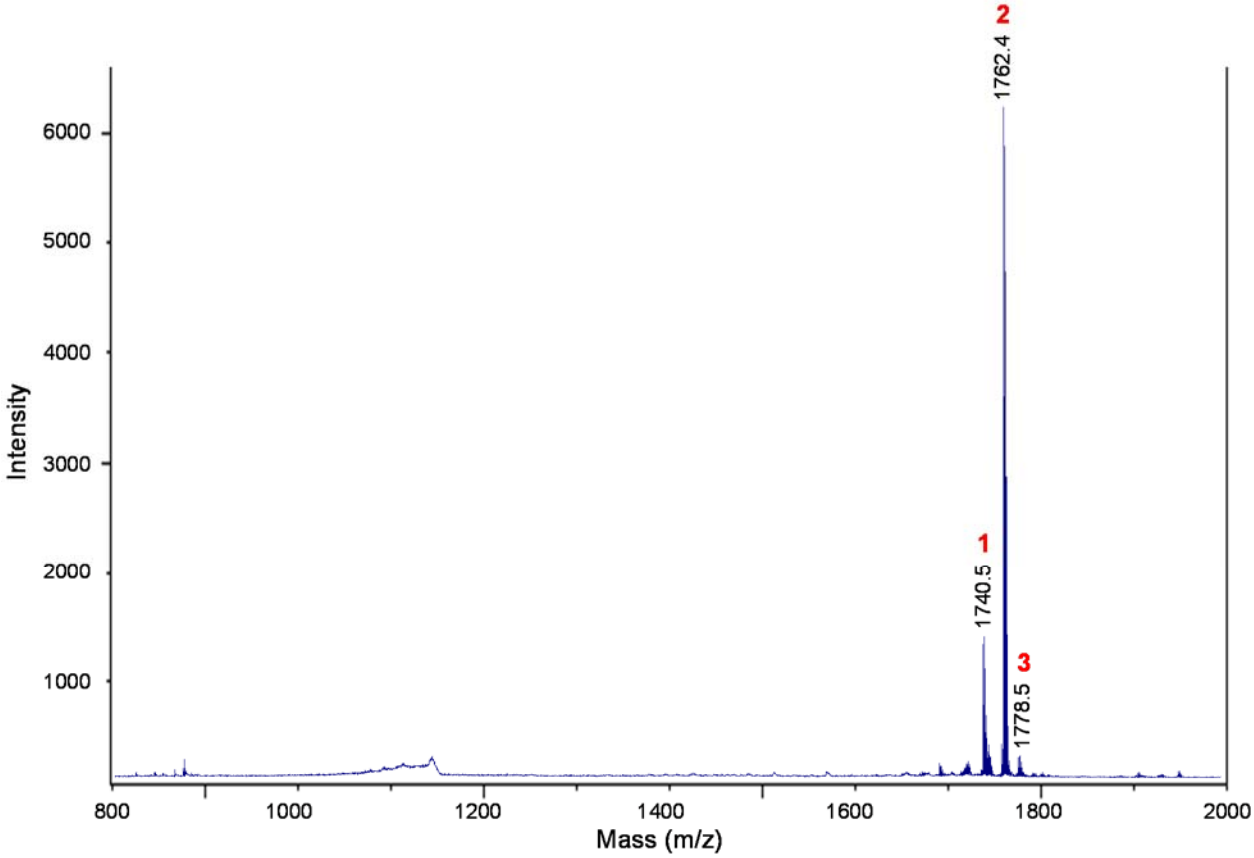


Figure S27. MS analysis of thiostrepton Ala2Phe isolated from *S. laurentii* NDS1/int-A2F.

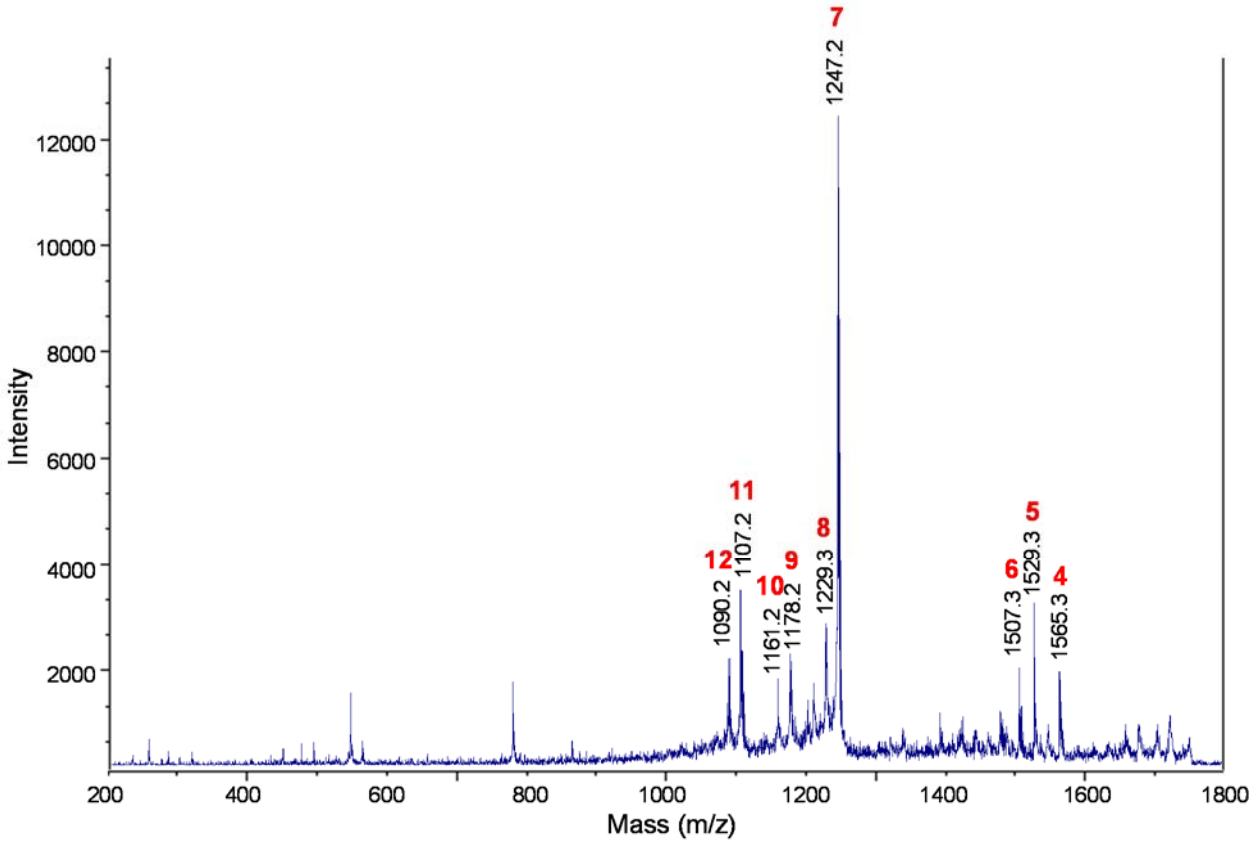
(**A**) HPLC-MS analysis. (1) Chromatogram extracted for *m/z* 870.8, the calculated $[M+2H]^{2+}$ ion of thiostrepton Ala2Phe. (2) Total ion chromatogram.



(**B**) MALDI MS spectrum of thiostrepton Ala2Phe.



(**C**) MALDI MS/MS of parent ion *m/z* 1740.5.



(**D**) Table and structure showing key ions and fragments in the MALDI MS and MS/MS of thiostrepton Ala2Phe.

Fragment	Expected	Observed
1. $M+H^+$ (Parent ion)	1740.5	1740.5
2. $M+Na^+$	1762.5	1762.4
3. $M+K^+$	1778.5	1778.5
4. M-Phe2-CO+H^+	1565.5	1565.3
5. M-Dhb8-Tzn9-CO-NH+H^+	1529.5	1529.3
6. M-QA+H^+	1507.5	1507.3
7. M-QA-Ile1-Phe2+H^+	1247.3	1247.2
8. M-QA-Ile1-Phe2-H_2O+H^+	1229.3	1229.3
9. M-QA-Ile1-Phe2-Dha3+H^+	1178.3	1178.2
10. M-QA-Ile1-Phe2-Dha3-OH+H^+	1161.3	1161.2
11. M-QA-Ile1-Phe2-Dha3-Ala4+H^+	1107.3	1107.2
12. M-QA-Ile1-Phe2-Dha3-Ala4-OH+H^+	1090.2	1090.2

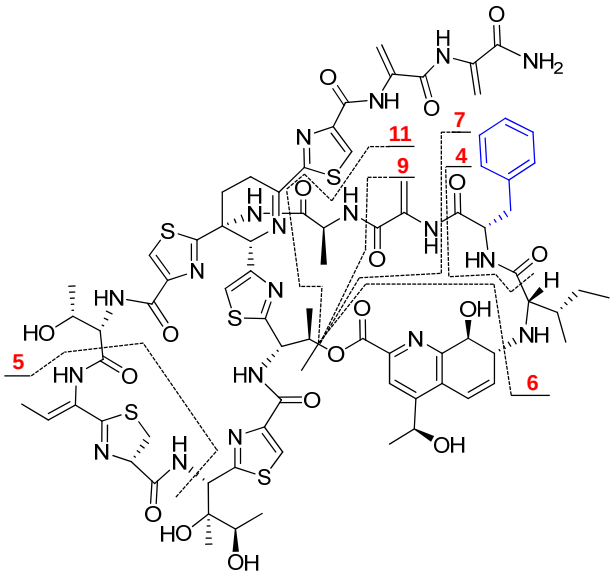
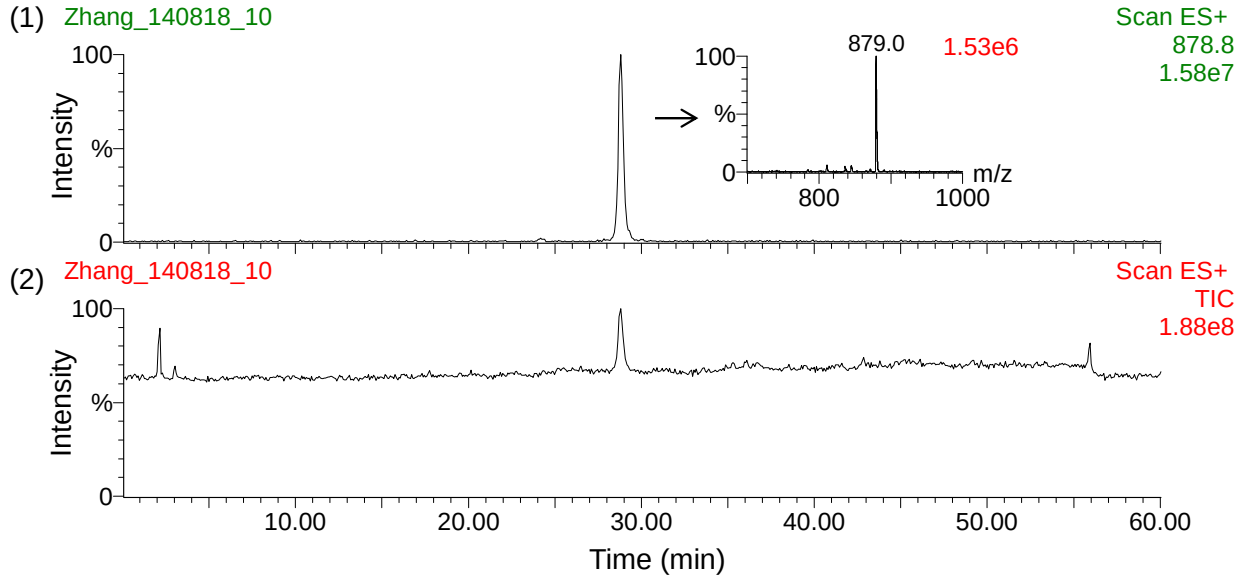
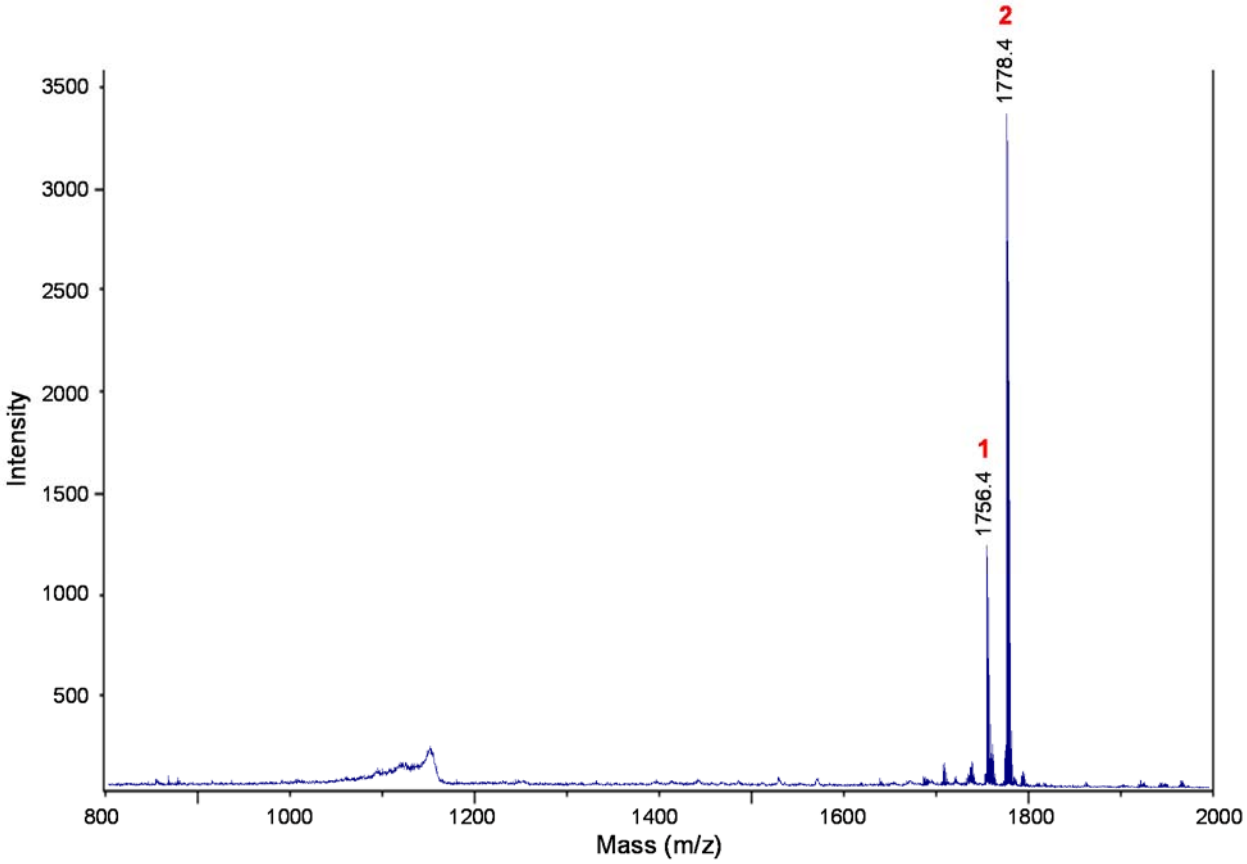


Figure S28. MS analysis of thiostrepton Ala2Tyr isolated from *S. laurentii* NDS1/int-A2Y.

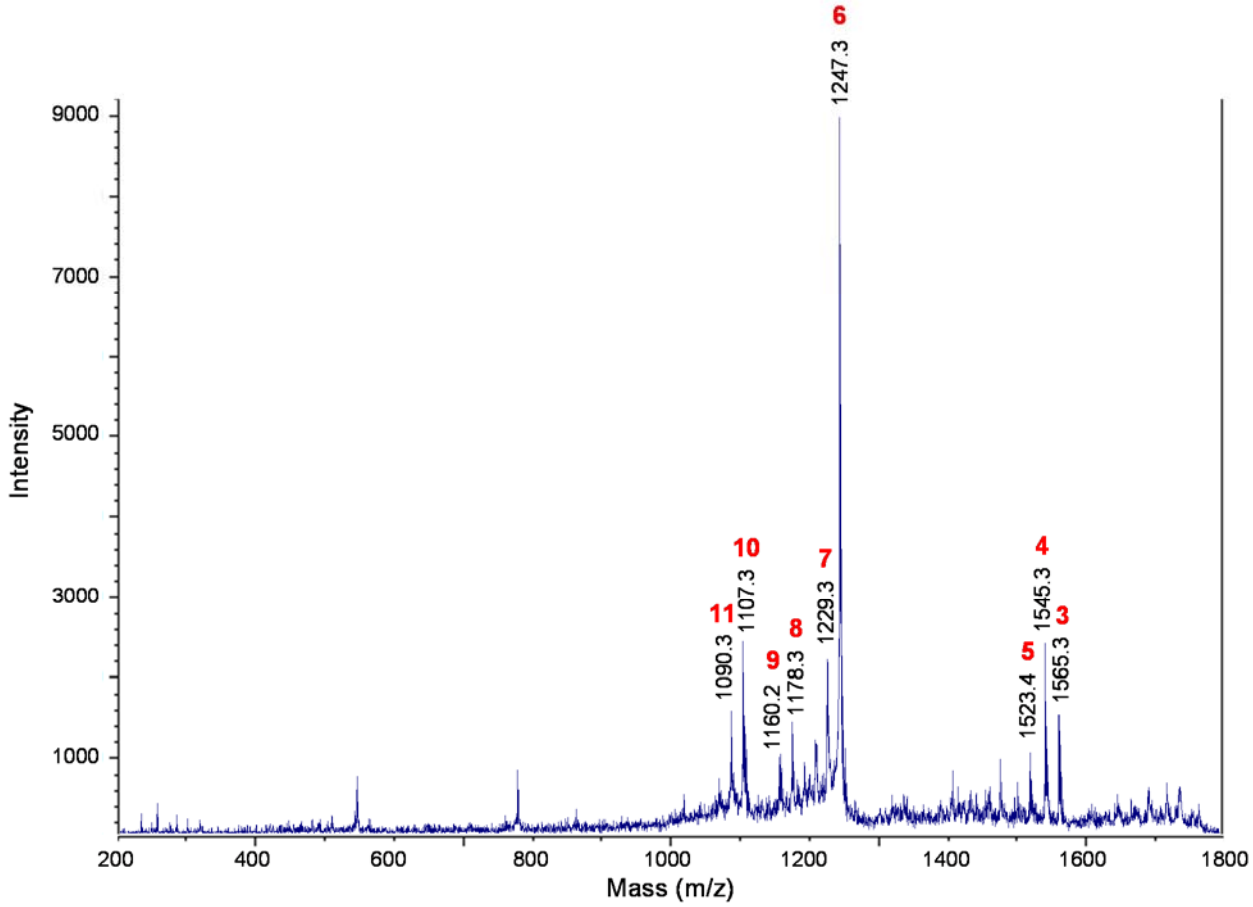
(**A**) HPLC-MS analysis. (1) Chromatogram extracted for *m*/*z* 878.8, the calculated $[M+2H]^{2+}$ ion of thiostrepton Ala2Tyr. (2) Total ion chromatogram.



(**B**) MALDI MS spectrum of thiostrepton Ala2Tyr.



(**C**) MALDI MS/MS of parent ion *m/z* 1756.4.



(**D**) Table and structure showing key ions and fragments in the MALDI MS and MS/MS of thiostrepton Ala2Tyr.

Fragment	Expected	Observed
1. $M+H^+$ (Parent ion)	1756.5	1756.4
2. $M+Na^+$	1778.5	1778.4
3. M-Tyr2-CO+H^+	1565.5	1565.3
4. M-Dhb8-Tzn9-CO-NH+H^+	1545.5	1545.3
5. M-QA+H^+	1523.5	1523.4
6. M-QA-Ile1-Tyr2+H^+	1247.3	1247.3
7. M-QA-Ile1-Tyr2-H_2O+H^+	1229.3	1229.3
8. M-QA-Ile1-Tyr2-Dha3+H^+	1178.3	1178.3
9. M-QA-Ile1-Tyr2-Dha3-H_2O+H^+	1160.3	1160.2
10. M-QA-Ile1-Tyr2-Dha3-Ala4+H^+	1107.3	1107.3
11. M-QA-Ile1-Tyr2-Dha3-Ala4-OH+H^+	1090.2	1090.3

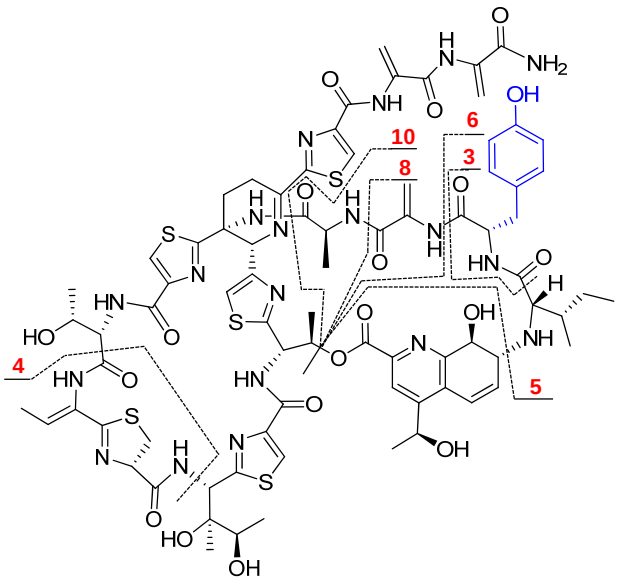
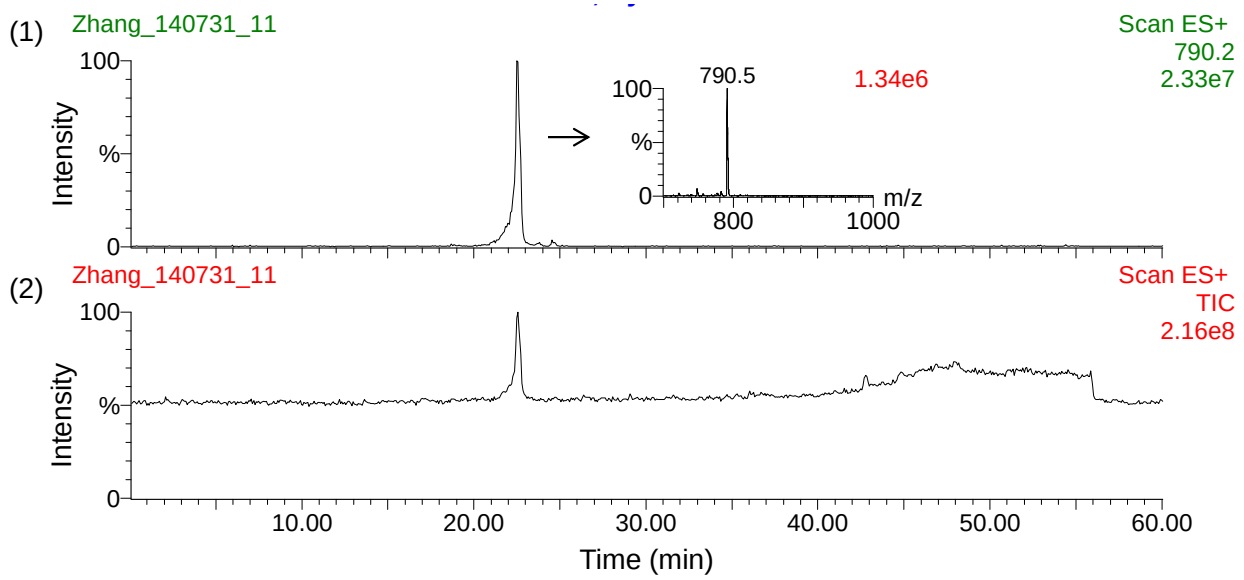
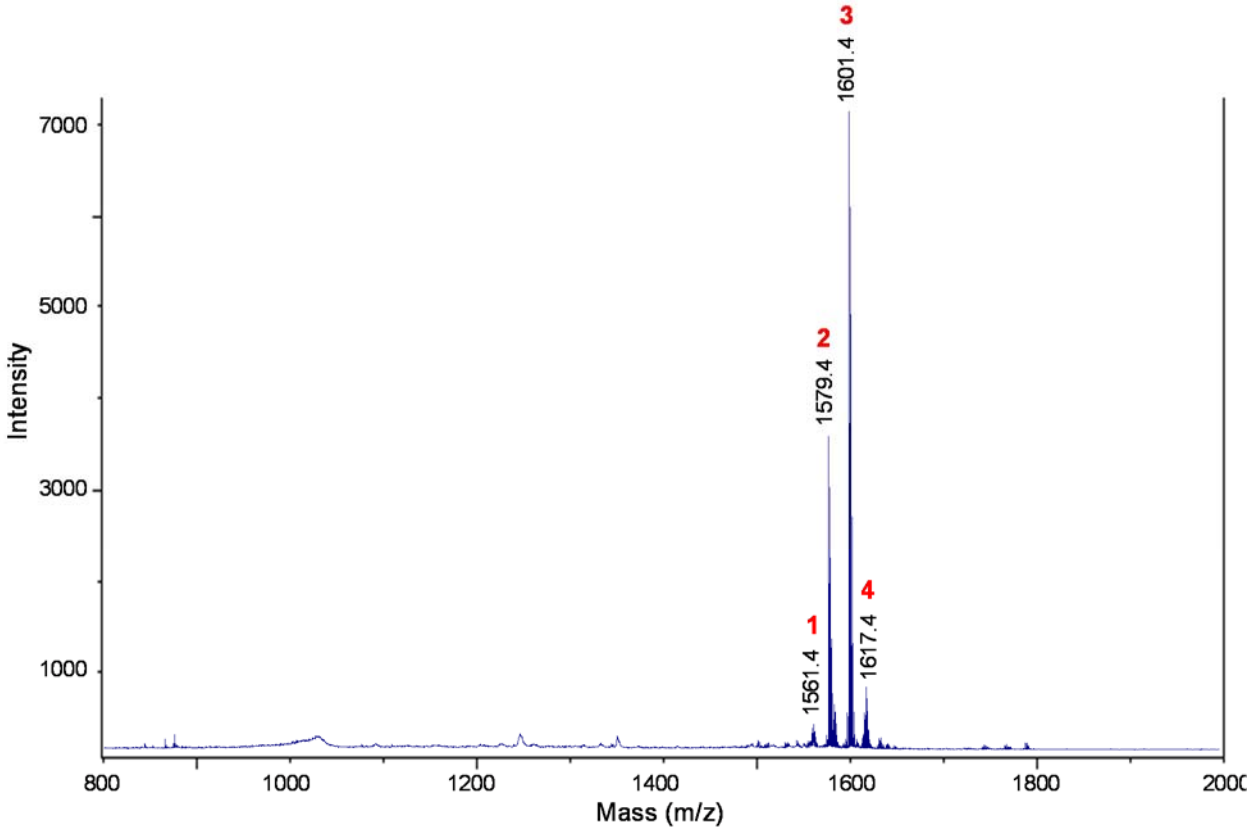


Figure S29. MS analysis of thiostrepton Ala2Val-ΔIle1 isolated from *S. laurentii* NDS1/int-A2V.

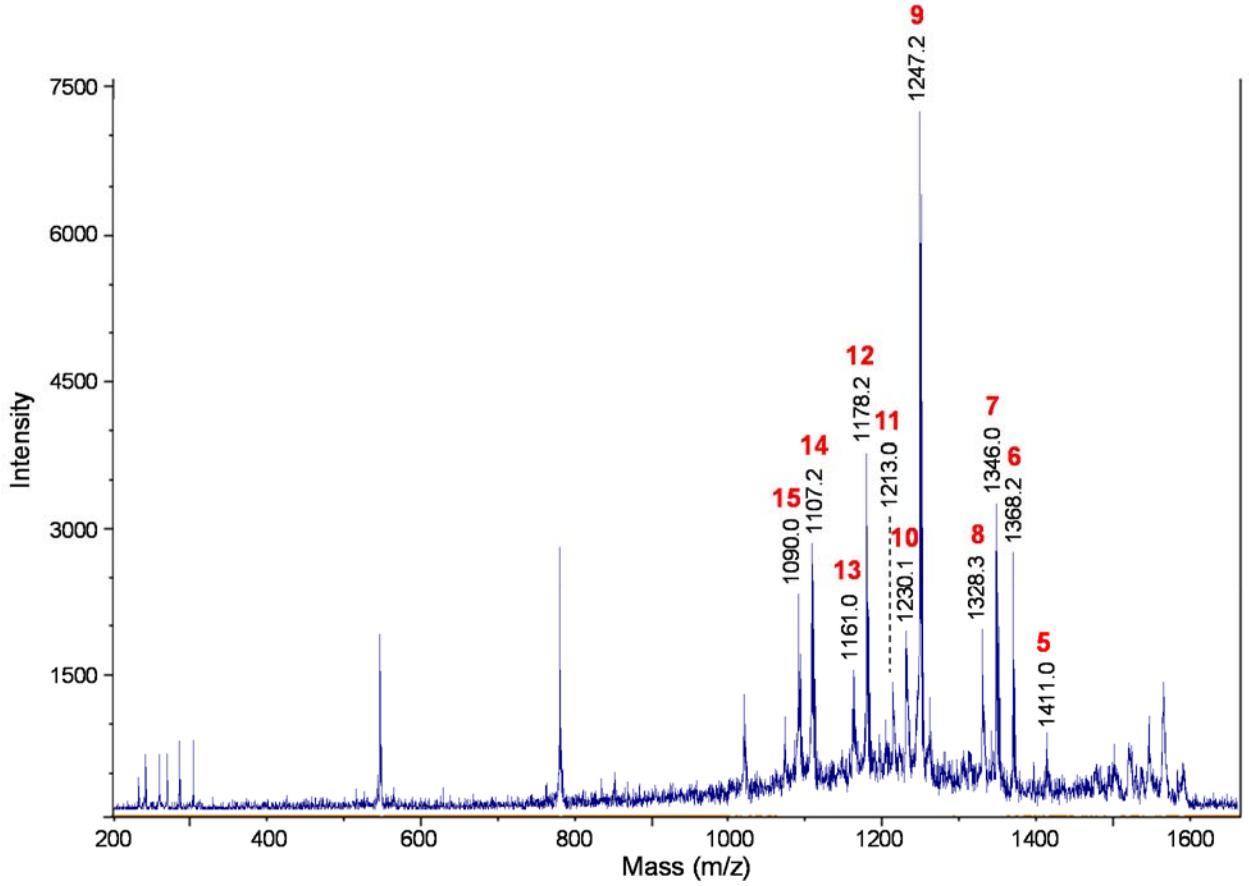
(**A**) HPLC-MS analysis. (1) Chromatogram extracted for *m*/*z* 790.2, the calculated $[M+2H]^{2+}$ ion of thiostrepton Ala2Val-ΔIle1. (2) Total ion chromatogram.



(**B**) MALDI MS spectrum of thiostrepton Ala2Val-ΔIle1.



(**C**) MALDI MS/MS of parent ion *m/z* 1579.4.



(**D**) Table and structure showing key ions and fragments in the MALDI MS and MS/MS of thiostrepton Ala2Val-ΔIle1.

Fragment	Expected	Observed
1. M-H_2O+H^+	1561.4	1561.4
2. M+H^+ (Parent ion)	1579.4	1579.4
3. M+Na^+	1601.4	1601.4
4. M+K^+	1617.4	1617.4
5. M-Dhb8-Tzn9+H^+	1411.4	1411.0
6. M-Val2-Dha3-(Ala4-CO)+H^+	1368.3	1368.2
7. M-QA+H^+	1346.4	1346.3
8. M-QA-H_2O+H^+	1328.4	1328.3
9. M-QA-Val2+H^+	1247.3	1247.2
10. M-QA-Val2-OH+H^+	1230.3	1230.1
11. M-QA-Val2-OH-OH+H^+	1213.3	1213.0
12. M-QA-Val2-Dha3+H^+	1178.3	1178.2
13. M-QA-Val2-Dha3-OH+H^+	1161.3	1161.0
14. M-QA-Val2-Dha3-Ala4+H^+	1107.3	1107.2
15. M-QA-Val2-Dha3-Ala4-OH+H^+	1090.2	1090.0

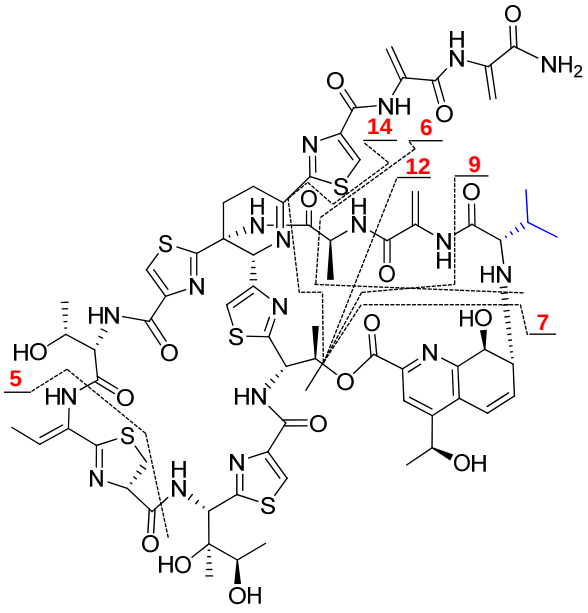
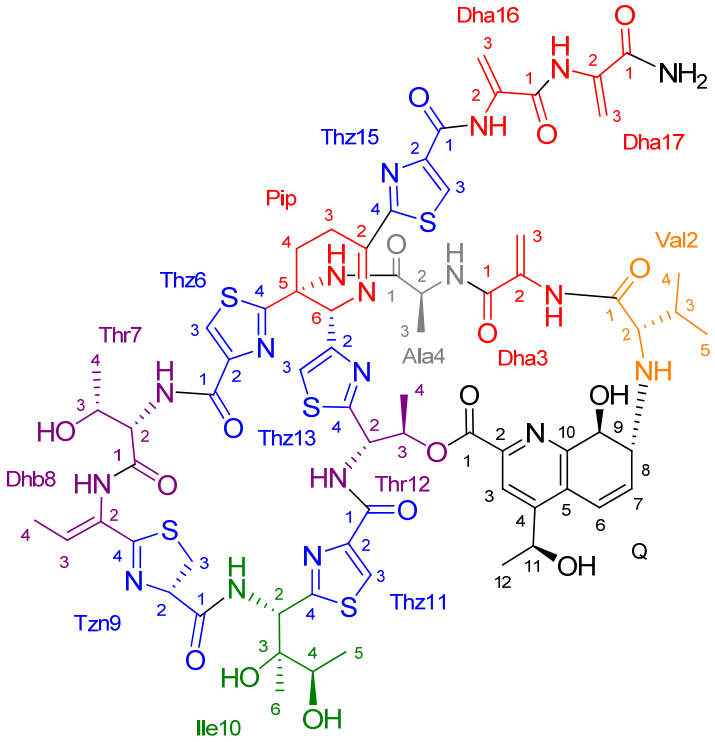


Figure S30. Structure and numbering system used for thiostrepton Ala2Val-ΔIle1.



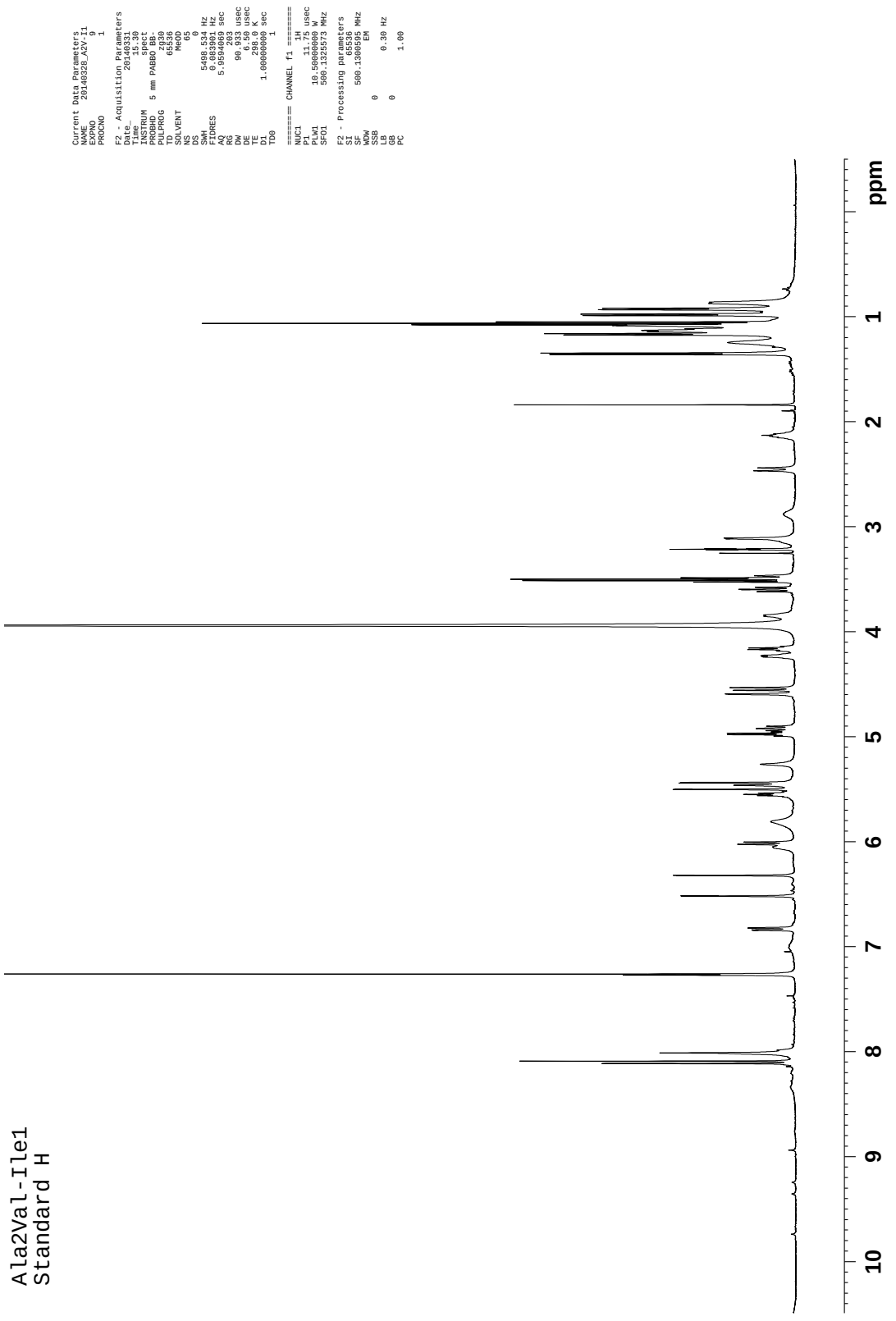


Figure S31. ^{1}H NMR spectrum of thiostrepton Ala2Val-ΔIle1 (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

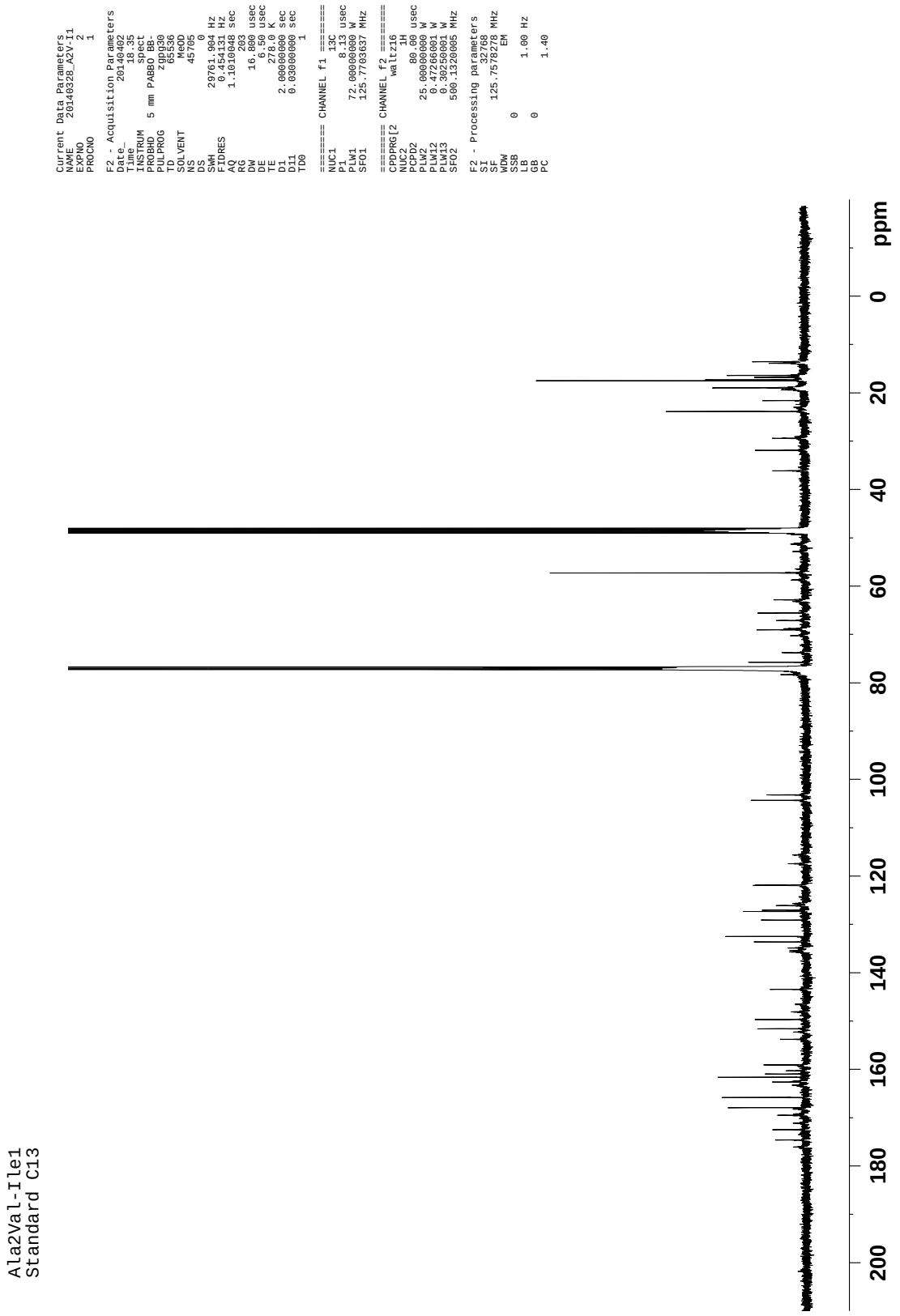


Figure S32. ^{13}C NMR spectrum of thiostrepton Ala2Val-ΔIle1 (125 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

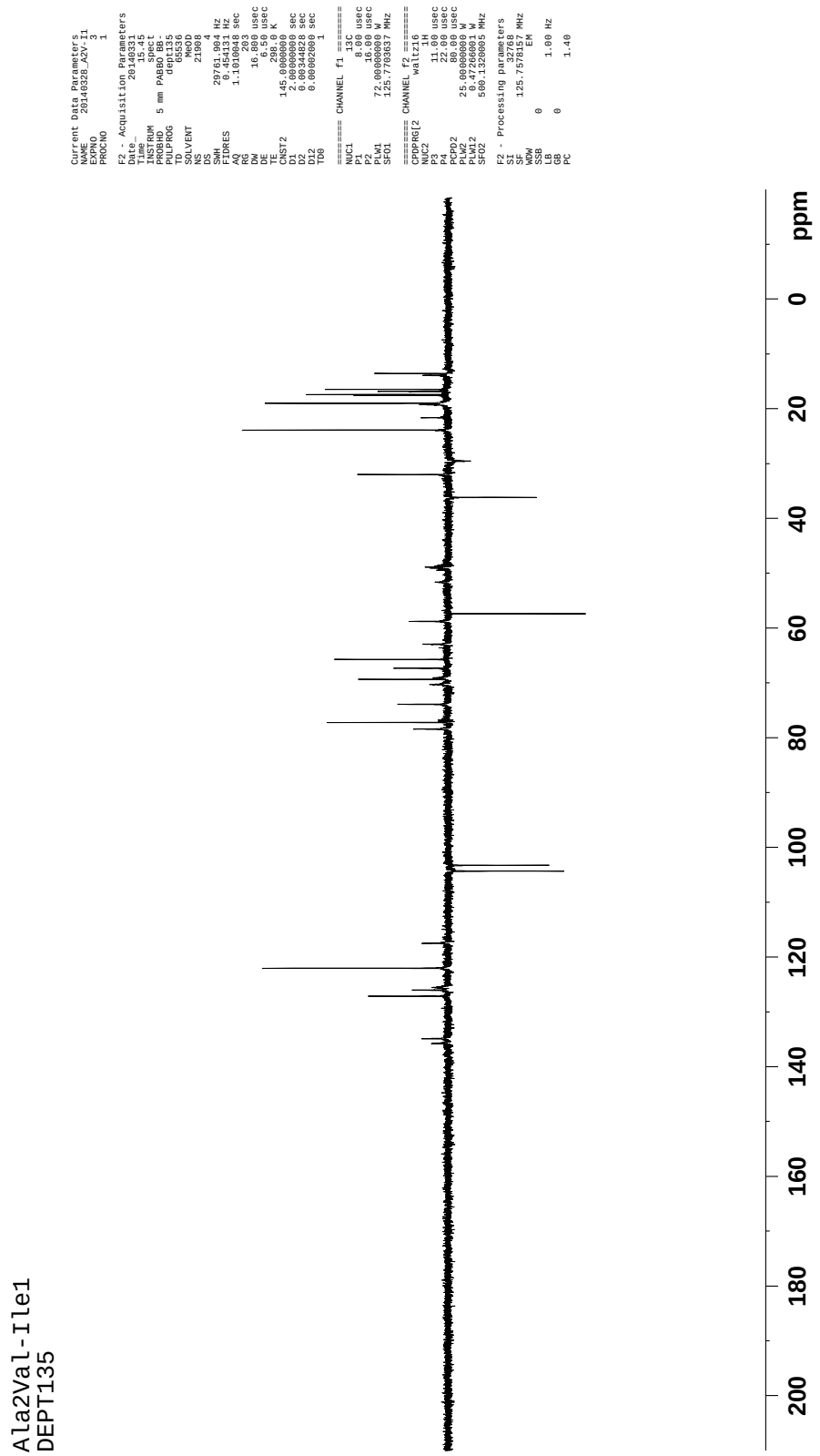


Figure S33. DEPT-135 NMR spectrum of thiostrepton Ala2Val-ΔIle1 (125 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

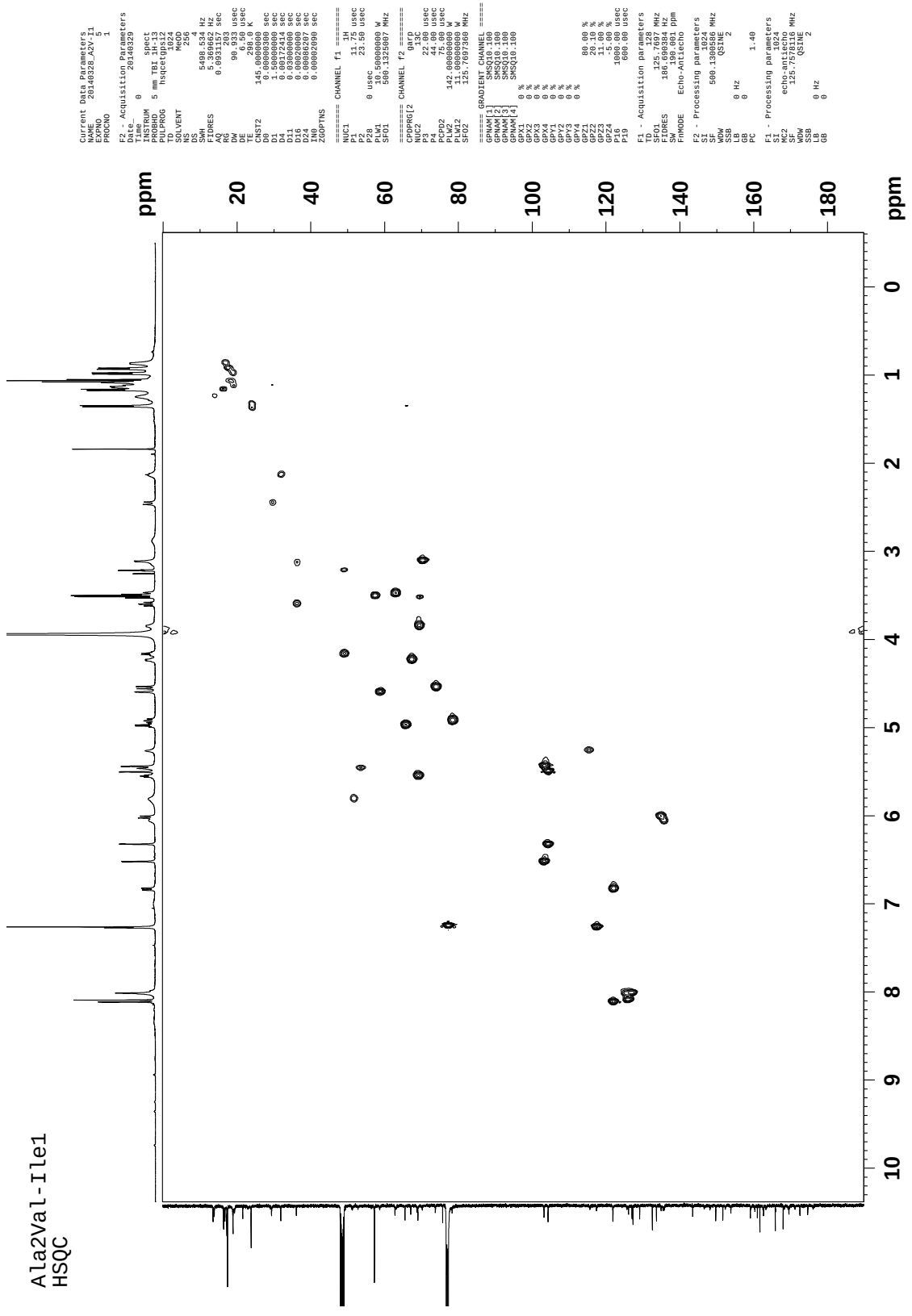


Figure S34. gHSQC NMR spectrum of thiostrepton Ala2Val-ΔIle1 (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

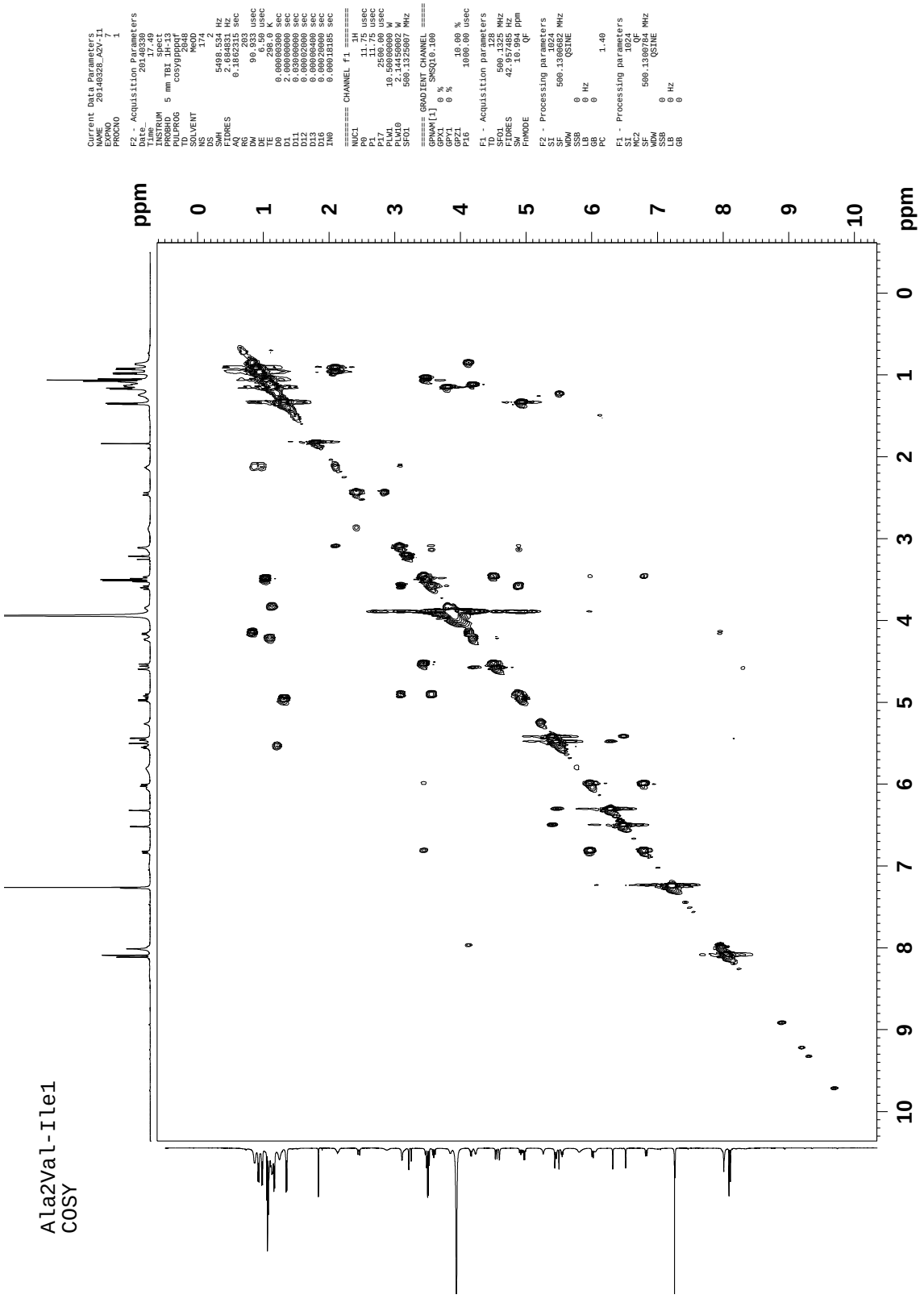


Figure S35. gCOSY NMR spectrum of thiostrepton Ala2Val-ΔIle1 (125 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

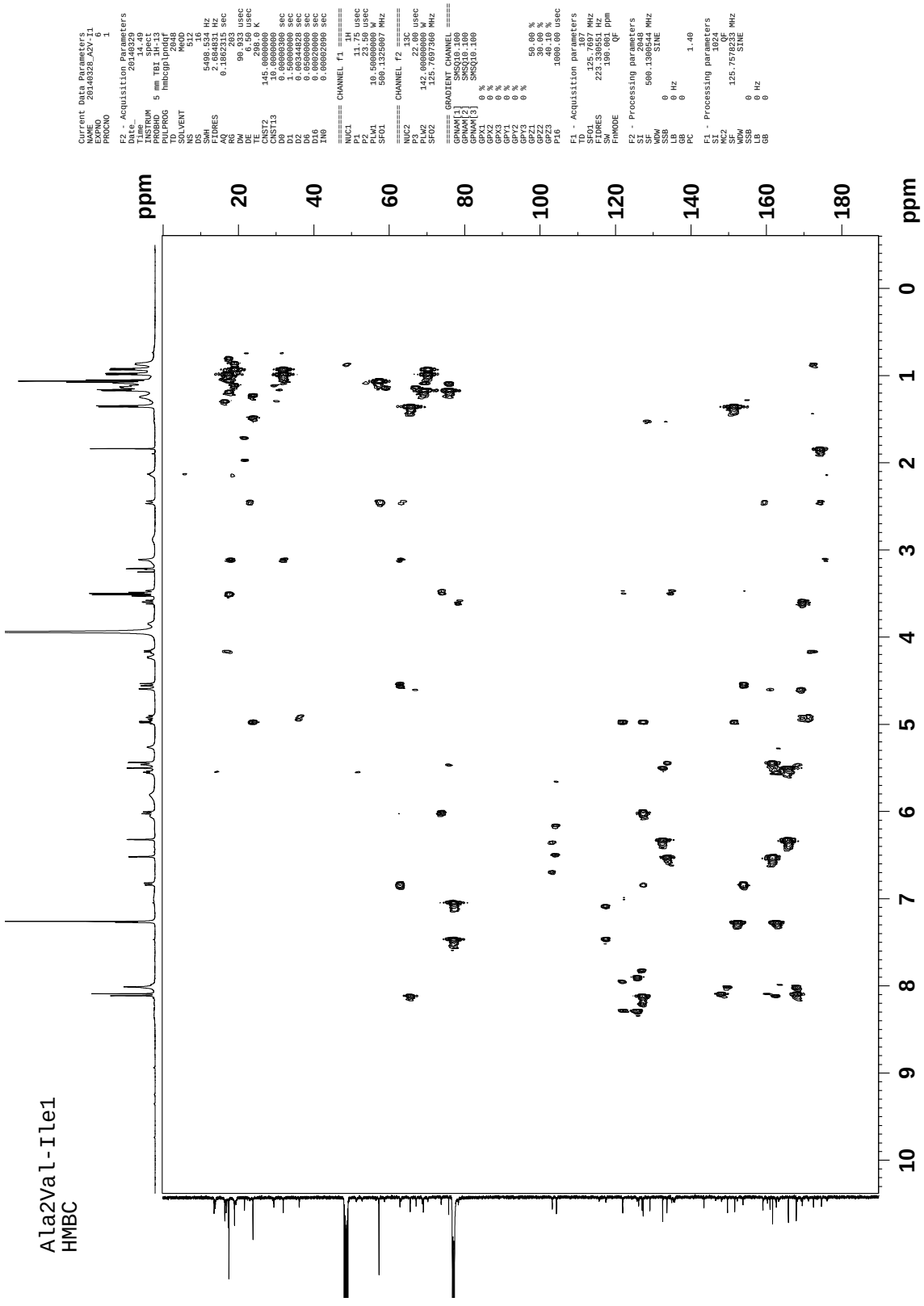


Figure S36. gHMBC NMR spectrum of thiostrepton Ala2Val-ΔIle1 (125 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

Table S4. ^{1}H and ^{13}C NMR assignments of thiostrepton Ala2Val-ΔIle1

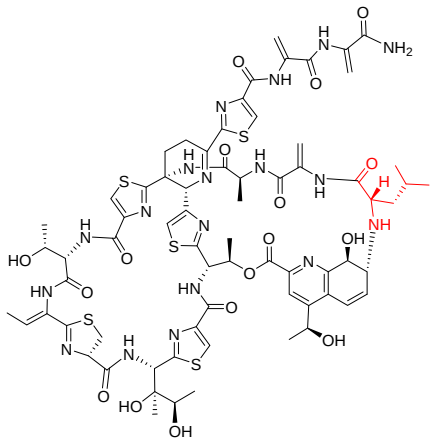
Position	δ_C [ppm]; mult	δ_H [ppm]; (mult, J in Hz)	HMBC[a]	COSY[b]
Val2				
Val2-1	176.1; C q			
Val2-2	70.2; CH	3.11 (d, 3.6)	Val2-1; Val2-3; Val2-4_B; Q-8	Val2-3
Val2-3	31.9; CH	2.17-2.09 (m)	Val2-1	Val2-2; Val2-4_A; Val2-4_B
Val2-4_A	19.3; CH_3	0.98 (d, 6.9)	Val2-2; Val2-3; Val2-4_B	Val2-3
Val2-4_B	17.5; CH_3	0.92 (d, 6.9)	Val2-2; Val2-3; Val2-4_A	Val2-3
Dha3				
Dha3-1	163.3; C q			
Dha3-2	135.4; C q			
Dha3-3	115.6; CH_2	H_A: 5.81 (br s)		
		H_B: 5.26 (br s)	Dha3-1	
Ala4				
Ala4-1	172.5; C q			
Ala4-2	49.4; CH	4.16 (q, 7.3)	Ala4-1; Ala4-3	Ala4-3
Ala4-3	16.8; CH_3	0.87 (d, 5.8)	Ala4-1; Ala4-2	Ala4-2
Pip				
Pip-2	159.1; C q			
Pip-3	23.0; CH_2	H_A: 3.53-3.48 (m)		
		H_B: 2.92-2.83 (m)		Pip-4-H_B
Pip-4	29.4; CH_2	H_A: 3.88-3.81 (m)		
		H_B: 2.47-2.42 (m)	Pip-2; Pip-3; Pip-5; Pip-6	Pip-3-H_B
Pip-5	57.3; C q			
Pip-6	63.1; CH	5.26 (br s)		
Thz6				
Thz6-1	161.0; C q			
Thz6-2	149.7; C q			
Thz6-3	127.1; CH	8.01 (s)	Thz6-2; Thz6-4	
Thz6-4	167.9; C q			
Thr7				
Thr7-1	169.5; C q			
Thr7-2	58.7; CH	4.59 (d, 2.0)	Thz6-1; Thr7-1; Thr7-3	Thr7-3
Thr7-3	67.1; CH	4.26-4.20 (m)		Thr7-2; Thr7-4
Thr7-4	19.3; CH_3	1.13 (d, 5.5)	Thr7-2; Thr7-3	Thr7-3
Dhb8				
Dhb8-2	127.3; C q			
Dhb8-3	135.7; CH	6.08-6.03 (m)	Dhb8-2	Dhb8-4
Dhb8-4	19.0; CH_3	1.13-1.09 (m)		
Tzn9				
Tzn9-1	171.2; C q			
Tzn9-2	78.3; CH	4.92 (dd, 11.5, 9.6)	Tzn9-1; Tzn9-3; Tzn9-4	Tzn9-3-H_A; Tzn9-3-H_B
Tzn9-3	36.1; CH_2	H_A: 3.60 (dd, 11.2, 9.1)	Tzn9-1; Tzn9-2; Tzn9-4	Tzn9-2; Tzn9-3-H_B
		H_B: 3.17-3.12 (m)		Tzn9-2; Tzn9-3-H_A
Tzn9-4	169.5; C q			
Ile10				
Ile10-2	52.8; CH	5.46 (s)	Ile10-3; Thz11-4	
Ile10-3	75.8; C q			
Ile10-4	69.0; CH	3.88-3.81 (m)		Ile10-5
Ile10-5	16.4; CH_3	1.17 (d, 6.5)	Ile10-3; Ile10-4	Ile10-4
Ile10-6	19.0; CH_3	1.08 (s)	Ile10-3; Ile10-4	
Thz11				
Thz11-1	162.6; C q			
Thz11-2	149.7; C q			
Thz11-3	121.9; CH	8.11 (s)	Thz11-1; Thz11-2; Thz11-4	
Thz11-4	168.2; C q			
Thr12				
Thr12-2	51.3; CH	5.84-5.77 (m)		Thr12-3
Thr12-3	69.0; CH	5.55 (pentet, 6.0)	Thz11-1; Thr12-2; Thr12-4; Q-1	Thr12-2; Thr12-4
Thr12-4	13.9; CH_3	1.28-1.21 (m)		Thr12-3

Position	δ_C [ppm]; mult	δ_H [ppm]; (mult, J in Hz)	HMBC[a]	COSY[b]
Thz13				
Thz13-2	159.1; C q			
Thz13-3	125.7; CH	8.01 (s)	Thz13-4	
Thz13-4	167.9; C q			
Thz15				
Thz15-1	160.3; C q			
Thz15-2	148.1; C q			
Thz15-3	126.1; CH	8.09 (s)	Thz15-1; Thz15-2; Thz15-4	
Thz15-4	168.2; C q			
Dha16				
Dha16-1	161.6; C q			
Dha16-2	133.6; C q			
Dha16-3	103.2; CH_2	H_A: 6.52 (d, 2.3)	Dha16-1; Dha16-2	Dha16-3-H_B
		H_B: 5.44 (d, 2.3)	Dha16-1; Dha16-2	Dha16-3-H_A
Dha17				
Dha17-1	165.8; C q			
Dha17-2	132.5; C q			
Dha17-3	104.3; CH_2	H_A: 6.32 (d, 1.7)	Dha17-1; Dha17-2	Dha17-3-H_B
		H_B: 5.50 (d, 1.7)	Dha17-1; Dha17-2	Dha17-3-H_A
Q				
Q-1	162.6; C q			
Q-2	151.6; C q			
Q-3	117.4; CH	7.27 (s)	Q-1; Q-2; Q-4; Q-10	
Q-4	152.3; C q			
Q-5	127.3; C q			
Q-6	121.9; CH	6.83 (dd, 10.2, 2.5)	Q-5; Q-8; Q-10	Q-7; Q-8
Q-7	134.9; CH	6.01 (m)	Q-5; Q-8; Q-9	Q-6; Q-8
Q-8	62.8; CH	3.50-3.45 (m)	Q-6; Q-7; Q-9; Q-10	Q-6; Q-7; Q-9
Q-9	73.8; CH	4.54 (d, 12.3)	Q-8; Q-10	Q-8
Q-10	153.8; C q			
Q-11	65.6; CH	4.97 (q, 6.5)	Q-2; Q-5; Q-6; Q-12	Q-12
Q-12	23.9; CH_3	1.35 (d, 6.6)	Q-4; Q-11	Q-11

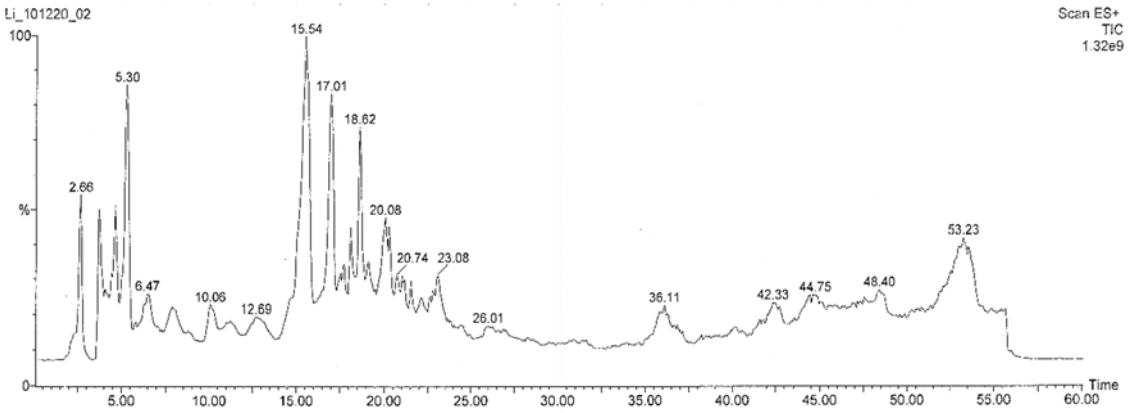
[a] HMBC correlations are from the proton to the indicated carbon.
[b] COSY correlations are from the proton to the proton attached to the indicated position.

Figure S37. HPLC-MS analysis of a crude culture extract from *S. laurentii* NDS1/int-A2L.

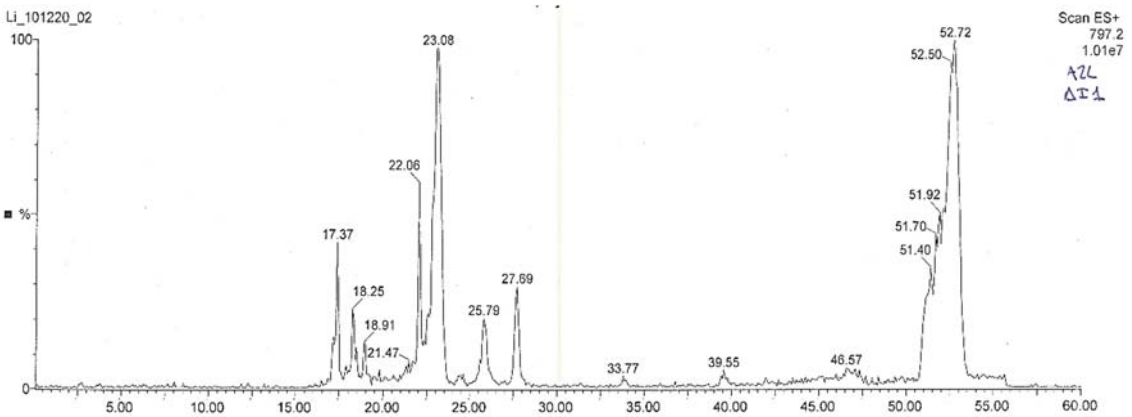
(**A**) Structure of thiostretpon Ala2Leu-ΔIle1. The expected $[M+H]^+$ *m/z* is 1593.5 and the expected $[M+2H]^{2+}$ *m/z* is 797.2.



(**B**) Total ion chromatogram of *S. laurentii* NDS1/int-A2L crude extract.



(**C**) Chromatogram extracted for *m/z* 797.2, the calculated $[M+2H]^{2+}$ ion of thiostrepton Ala2Leu-ΔIle1.



(**D**) Mass spectrum of the thiostrepton Ala2Leu-ΔIle1 extract eluting at t_R = 25.79, which contains a +2 ion, the ionization state typically observed for thiostrepton metabolites in ESI-MS analysis, at about 797.2 and consistent with that expected for the ring-contracted thiostrepton metabolite. Another +2 ion at about 797.2 was detected at t_R of 22.06.

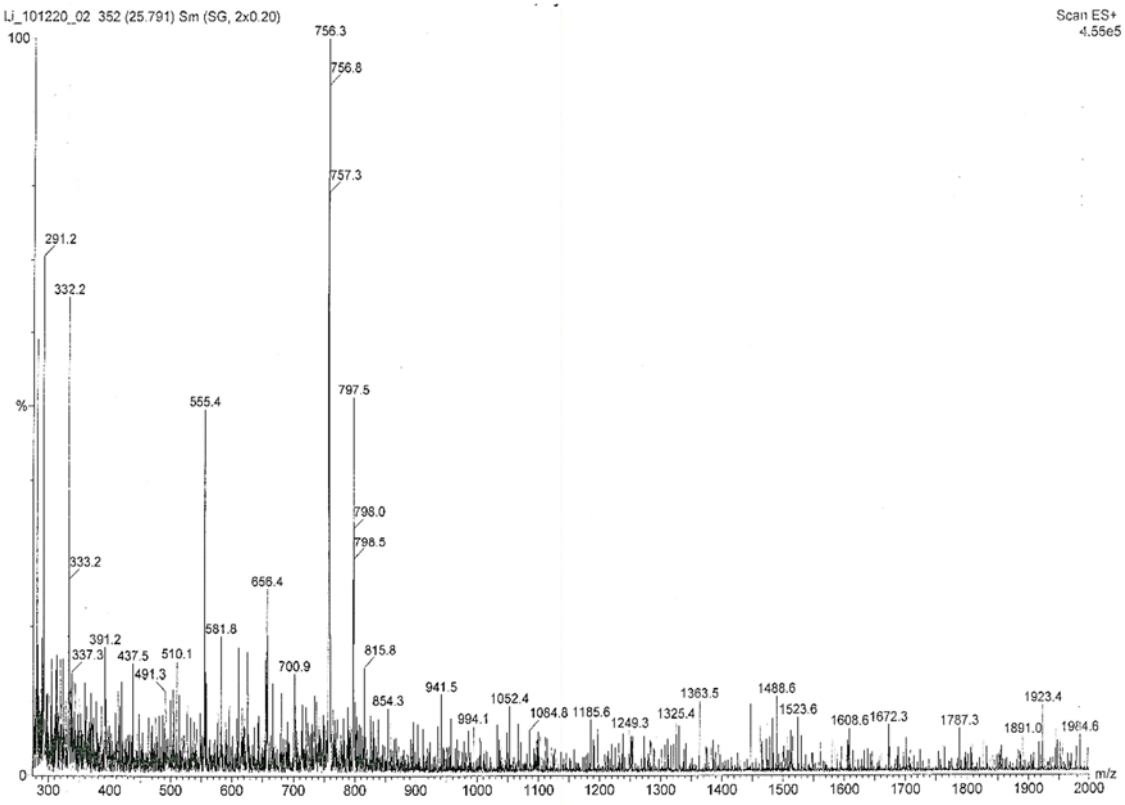
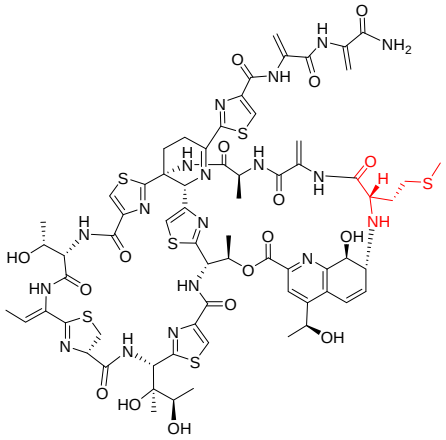
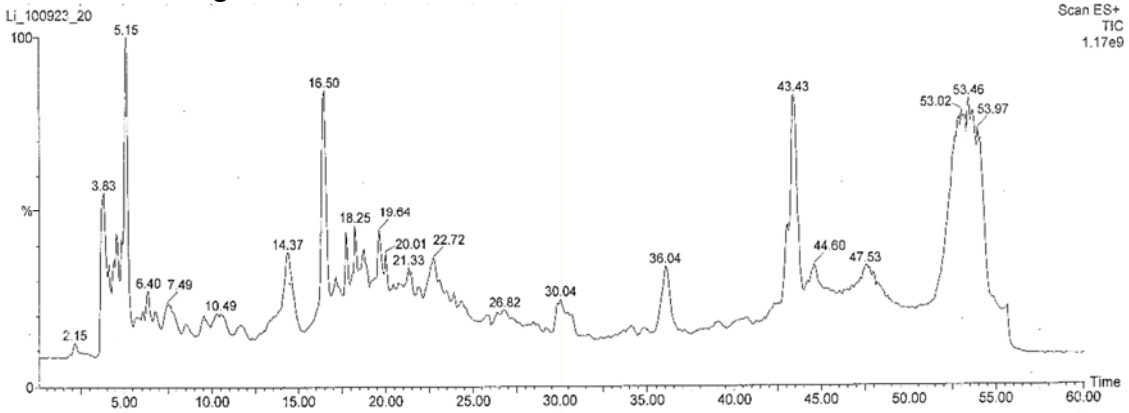


Figure S38. HPLC-MS analysis of a crude culture extract from *S. laurentii* NDS1/int-A2M.

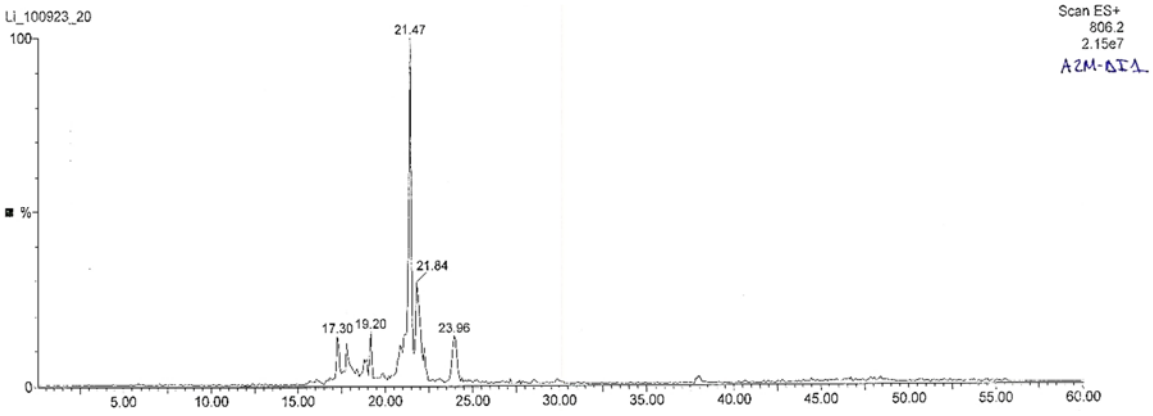
(**A**) Structure of thiostretpon Ala2Met-ΔIle1. The expected $[M+H]^+$ *m/z* is 1611.4 and the expected $[M+2H]^{2+}$ *m/z* is 806.2.



(**B**) Total ion chromatogram of *S. laurentii* NDS1/int-A2M crude extract.



(**C**) Chromatogram extracted for *m/z* 806.2, the calculated $[M+2H]^{2+}$ ion of thiostrepton Ala2Met-ΔIle1.



(**D**) Mass spectrum of the thiostrepton Ala2Met-ΔIle1 extract eluting at t_R = 21.47, which contains a +2 ion, the ionization state typically observed for thiostrepton metabolites in ESI-MS analysis, at about 806.2 and consistent with the mass expected for the ring-contracted thiostrepton metabolite. Other +2 ions at about 806.2 were detected at t_R's of 21.84 and 23.96.

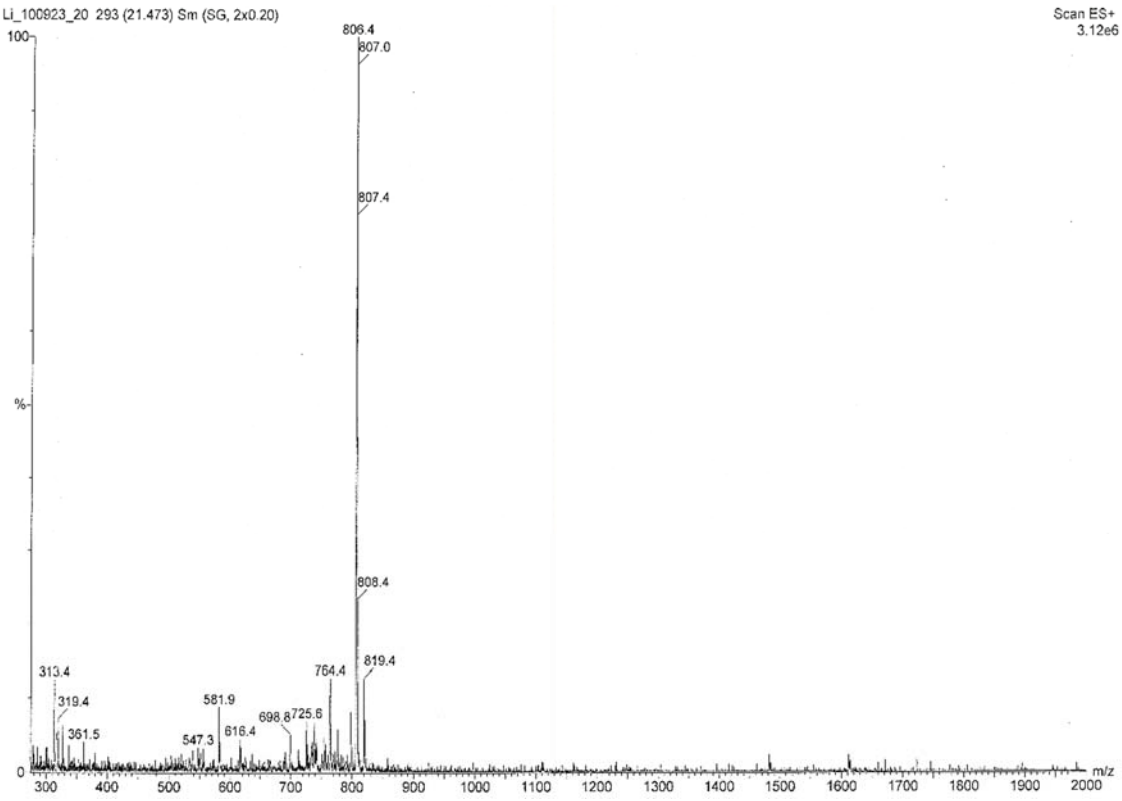
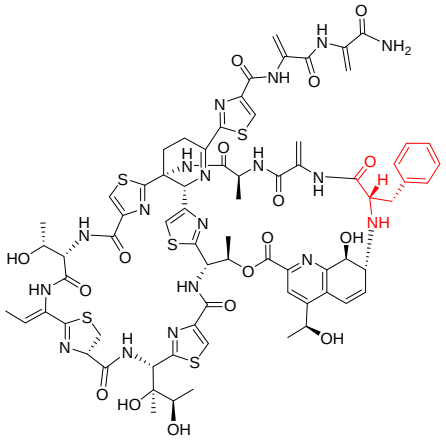
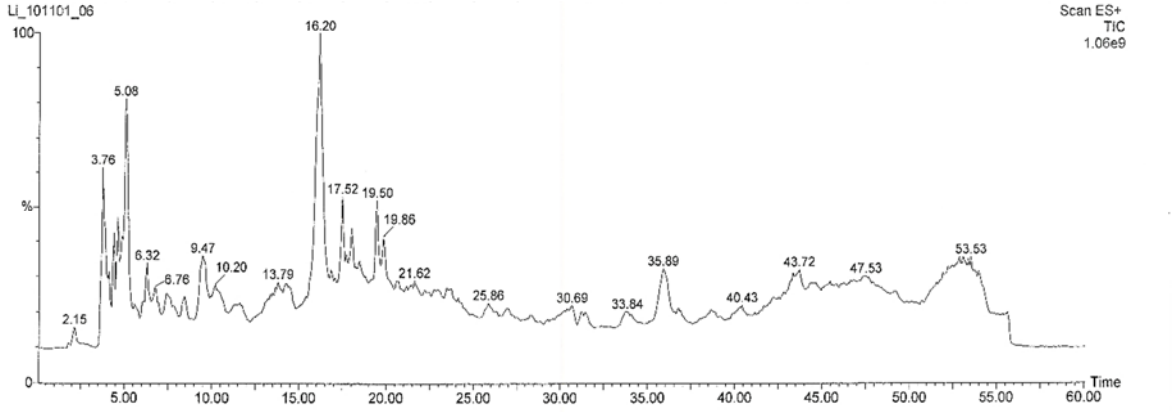


Figure S39. HPLC-MS analysis of a crude culture extract from *S. laurentii* NDS1/int-A2F.

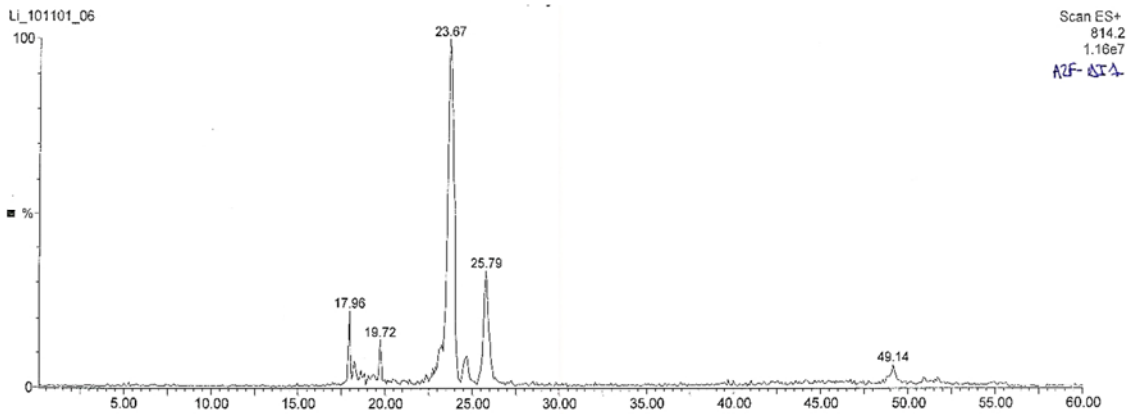
(**A**) Structure of thiostretpon Ala2Phe-ΔIle1. The expected $[M+H]^+$ *m/z* is 1627.4 and the expected $[M+2H]^{2+}$ *m/z* is 814.2.



(**B**) Total ion chromatogram of *S. laurentii* NDS1/int-A2F crude extract.



(**C**) Chromatogram extracted for *m/z* 814.2, the calculated $[M+2H]^{2+}$ ion of thiostrepton Ala2Phe-ΔIle1.



(**D**) Mass spectrum of the thiostrepton Ala2Phe-ΔIle1 extract eluting at t_R = 23.67, which contains a +2 ion, the ionization state typically observed for thiostrepton metabolites in ESI-MS analysis, at about 814.2 and a mass consistent with that expected for the ring-contracted thiostrepton metabolite. Another +2 ion at about 814.2 was detected at a t_R of 25.79.

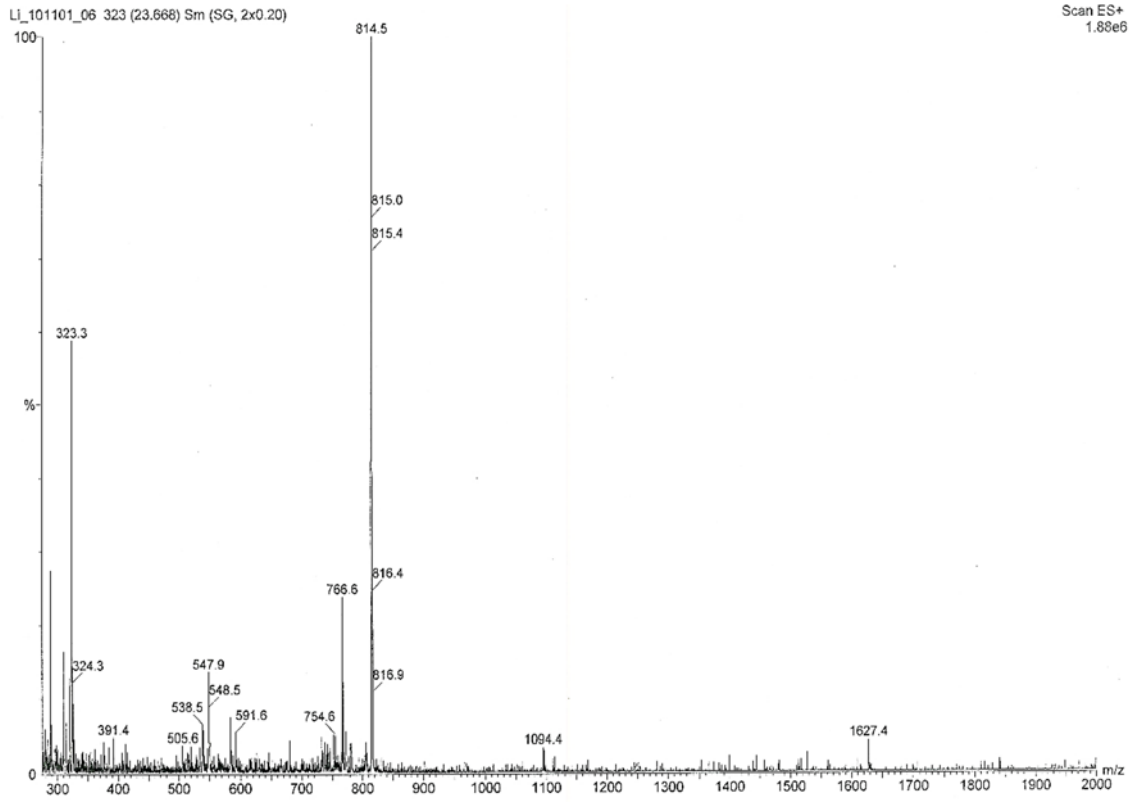
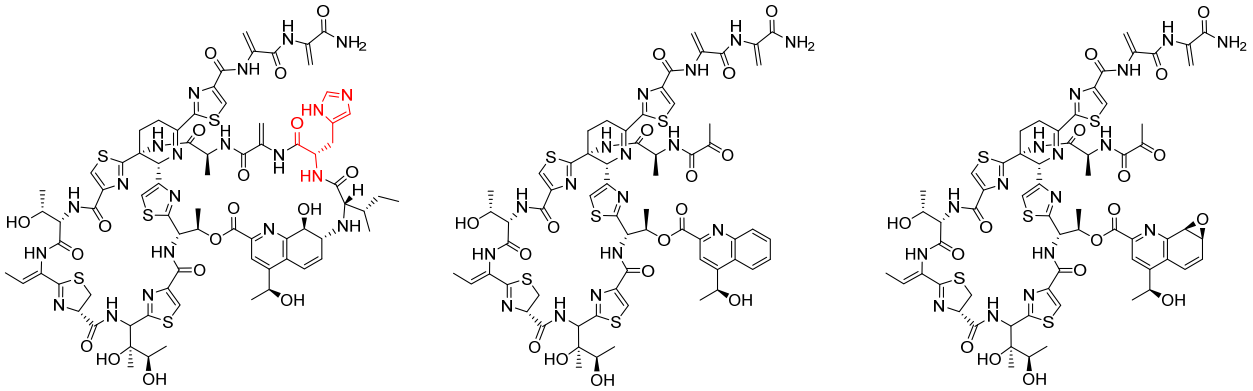


Figure S40. A representative MS analysis revealing the presence of shunt metabolites of thiostrepton biosynthesis. MS analysis of a crude culture extract from *S. laurentii* NDS1/int-A2H is shown.

(**A**) Structures of thiostrepton Ala2His and the two predicted shunt metabolites SL-1 (the wild-type analog to SL105-1 reported for the TsrA Thr7Ala variant) and SL-2 (a shunt metabolite bearing an epoxidized quinaldic acid moiety). Thiostreptons have typically been observed as the +2 ion by our HPLC-MS analysis and as the +1 ion by our MALDI-MS analysis. The expected thiostrepton Ala2His $[M+2H]^{2+}$ *m/z* is 865.8 and the $[M+H]^{+}$ *m/z* is 1730.5; the expected SL-1 $[M+2H]^{2+}$ *m/z* is 733.2 and $[M+H]^{+}$ *m/z* is 1465.4; and the expected SL-2 $[M+2H]^{2+}$ *m/z* is 741.2 and $[M+H]^{+}$ *m/z* is 1481.4.

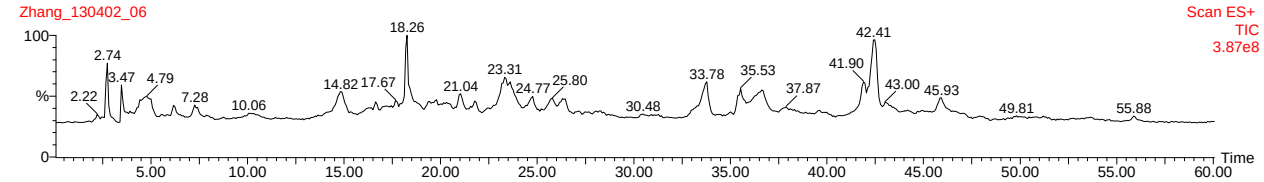


Thiostrepton Ala2His
$[M+H]^{+}$ *m/z* 1730.5
$[M+2H]^{2+}$ *m/z* 865.8

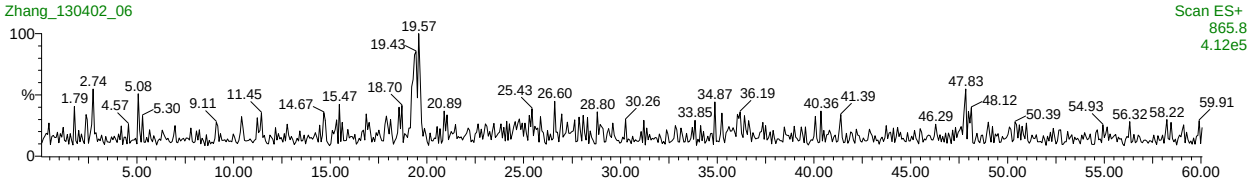
SL-1
$[M+H]^{+}$ *m/z* 1465.4
$[M+2H]^{2+}$ *m/z* 733.2

SL-2
$[M+H]^{+}$ *m/z* 1481.4
$[M+2H]^{2+}$ *m/z* 741.2

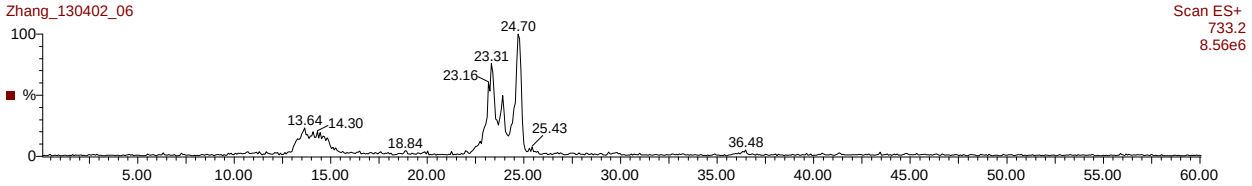
(**B**) Total ion chromatogram of *S. laurentii* NDS1/int-A2H crude extract.



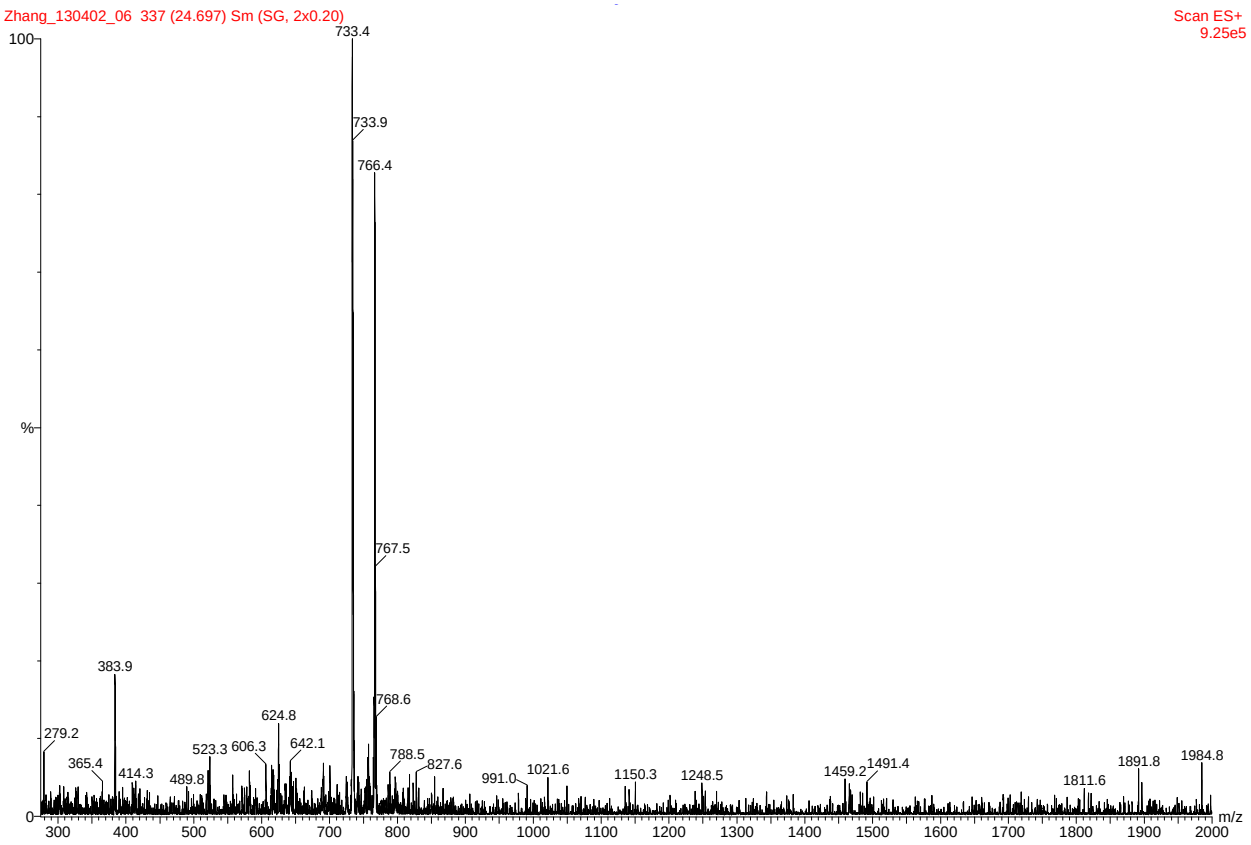
(**C**) Chromatogram extracted for *m/z* 865.8, the calculated $[M+2H]^{2+}$ ion of thiostrepton Ala2His.



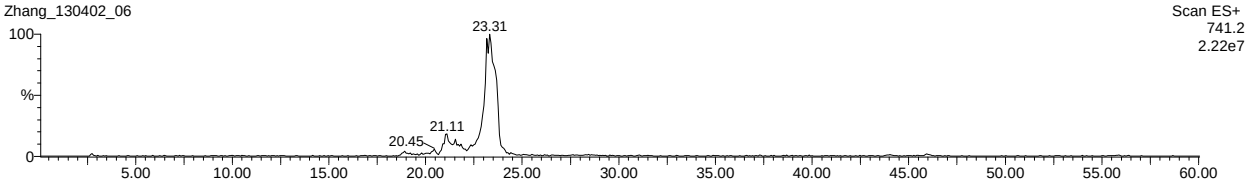
(**D**) Chromatogram extracted for *m/z* 733.2, the calculated $[M+2H]^{2+}$ ion of SL-1.



(**E**) Mass spectrum of the thiostrepton Ala2His extract eluting at t_R = 24.70, which contains a +2 ion at *m/z* 733.4, consistent with that predicted for the shunt metabolite SL-1. HR-MALDI-MS analysis of partially purified SL-1 gave $[M+H]^+$ *m/z* 1465.3564 (calculated 1465.3681, -8.0 ppm).



(**F**) Chromatogram extracted for *m/z* 741.2, the calculated $[M+2H]^{2+}$ ion of SL-2.



(**G**) Mass spectrum of the thiostrepton Ala2His extract eluting at t_R = 23.31, which contains a +2 ion at *m/z* 741.4, consistent with that predicted for the shunt metabolite SL-2. HR-MALDI-MS analysis of partially purified SL-2 gave $[M+H]^+$ *m/z* 1481.3560 (calculated 1481.3630, -4.7 ppm).

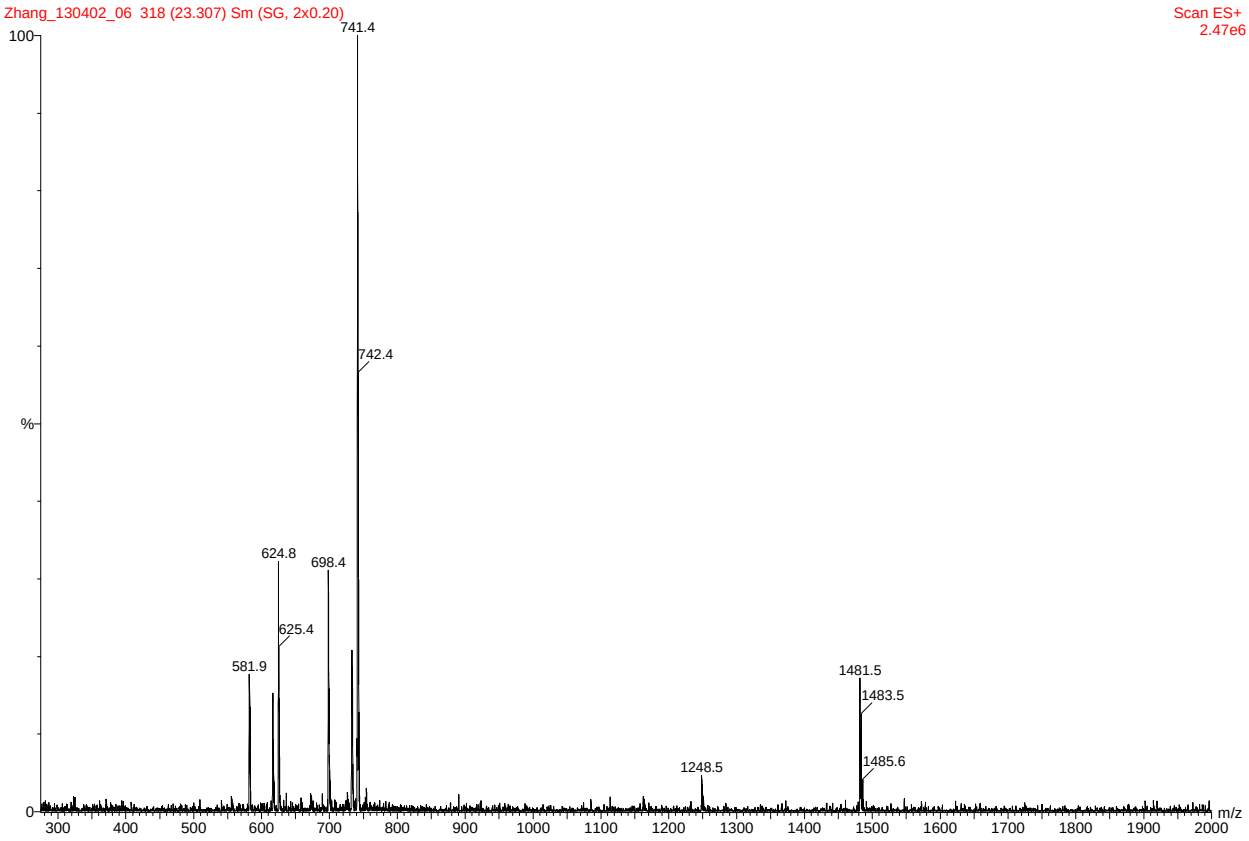


Table S5. Summary of HR-MS results of thiostrepton Ala2 analogues

Thiostrepton variant	Analysis	Molecular Formula	Calculated $[M+Na]^+$	Observed $[M+Na]^+$	ppm Error
Thiostrepton Ala2Dha	HR-ESI-MS	$C_{72}H_{83}N_{19}O_{18}S_5$	1684.4665	1684.4718	3.1

Thiostrepton variant	Analysis	Molecular formula	Calculated $[M+H]^+$	Observed $[M+H]^+$	
Thiostrepton Ala2Dhb	HR-MALDI-MS	$C_{73}H_{85}N_{19}O_{18}S_5$	1676.5002	1676.5043	2.4
Thiostrepton Ala2Ile-ΔIle1	HR-MALDI-MS	$C_{69}H_{80}N_{18}O_{17}S_5$	1593.4631	1593.4673	2.6
Thiostrepton Ala2Met	HR-MALDI-MS	$C_{74}H_{89}N_{19}O_{18}S_6$	1724.5036	1724.4999	-2.1
Thiostrepton Ala2Phe	HR-MALDI-MS	$C_{78}H_{89}N_{19}O_{18}S_5$	1740.5315	1740.5244	-4.1
Thiostrepton Ala2Tyr	HR-MALDI-MS	$C_{78}H_{89}N_{19}O_{19}S_5$	1756.5264	1756.5267	0.2
Thiostrepton Ala2Val-ΔIle1	HR-MALDI-MS	$C_{68}H_{78}N_{18}O_{17}S_5$	1579.4474	1579.4519	2.8

Table S6. Titers of the thiostrepton Ala2 analogues

Fermentation of each *S. laurentii* NDS1/int-A2X variant was carried out in two sets of triplicate and crude chloroform extracts were prepared. The yields of all thiostrepton analogues were quantified by HPLC against a standard calibration curve of thiostrepton A assuming similar spectral properties for each derivative.

Compound	Titer (mg/L)
Thiostrepton A	115 ± 35
Ala2Dha	301 ± 7
Ala2Dhb	39 ± 9
Ala2Gly	19 ± 3
Ala2Ile-ΔIle1	34 ± 4
Ala2Met	3.1 ± 0.2
Ala2Phe	2.5 ± 0.6
Ala2Tyr	1.7 ± 0.6
Ala2Val-ΔIle1	51 ± 16

Table S7. Aqueous solubilities of thiostrepton Ala2 analogues

The kinetic aqueous solubilities of thiostrepton A and its analogues were measured as described previously.[4] 1 mM DMSO stock solutions of thiostrepton A and its analogues were quantified by their absorbance at 280 nm using an extinction coefficient of 0.027 $cm^{-1}\mu M^{-1}$, assuming similar spectral properties for the compounds.[6] The solution was diluted with buffer (10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.0, 5 mM $MgCl_2$, 200 mM KCl, and 5% DMSO) to a nominal concentration of 20 μM. The resulting mixture was incubated at 25 °C for 2 h, then the sample was centrifuged at 14,000 rpm (18,000 x *g*) at 25 °C for 10 min. The absorbance of the supernatant at 280 nm was used to define the solubility of a thiostrepton analogue. Each analysis was performed at least in triplicate.

Compound	Water solubility (μM)
Thiostrepton A	8.10 ± 0.24
Ala2Dha	4.14 ± 0.28
Ala2Dhb	11.10 ± 0.27
Ala2Gly	16.79 ± 0.60
Ala2Ile-ΔIle1	10.18 ± 0.36
Ala2Met	13.62 ± 0.38
Ala2Phe	4.71 ± 0.13
Ala2Tyr	0.92 ± 0.10
Ala2Val-ΔIle1	3.28 ± 0.22

Table S8. Strains and plasmids used in this study

Strain/Plasmid	Description	Reference or source
Streptomyces strains		
S. laurentii ATCC 31255	Wild-type, thiostrepton A producer	ATCC
S. laurentii NDS1	*S. laurentii* containing an in-frame deletion of *tsrA*	[2]
S. laurentii NDS1/int-3A101	*S. laurentii* NDS1 containing int-3A101 (A2G)	[2]
S. laurentii NDS1/int-A2C	*S. laurentii* NDS1 containing int-A2C	This study
S. laurentii NDS1/int-A2D	*S. laurentii* NDS1 containing int-A2D	This study
S. laurentii NDS1/int-A2E	*S. laurentii* NDS1 containing int-A2E	This study
S. laurentii NDS1/int-A2F	*S. laurentii* NDS1 containing int-A2F	This study
S. laurentii NDS1/int-A2H	*S. laurentii* NDS1 containing int-A2H	This study
S. laurentii NDS1/int-A2I	*S. laurentii* NDS1 containing int-A2I	This study
S. laurentii NDS1/int-A2K	*S. laurentii* NDS1 containing int-A2K	This study
S. laurentii NDS1/int-A2L	*S. laurentii* NDS1 containing int-A2L	This study
S. laurentii NDS1/int-A2M	*S. laurentii* NDS1 containing int-A2M	This study
S. laurentii NDS1/int-A2N	*S. laurentii* NDS1 containing int-A2N	This study
S. laurentii NDS1/int-A2P	*S. laurentii* NDS1 containing int-A2P	This study
S. laurentii NDS1/int-A2Q	*S. laurentii* NDS1 containing int-A2Q	This study
S. laurentii NDS1/int-A2R	*S. laurentii* NDS1 containing int-A2R	This study
S. laurentii NDS1/int-A2S	*S. laurentii* NDS1 containing int-A2S	This study
S. laurentii NDS1/int-A2T	*S. laurentii* NDS1 containing int-A2T	This study
S. laurentii NDS1/int-A2V	*S. laurentii* NDS1 containing int-A2V	This study
S. laurentii NDS1/int-A2W	*S. laurentii* NDS1 containing int-A2W	This study
S. laurentii NDS1/int-A2Y	*S. laurentii* NDS1 containing int-A2Y	This study
E. coli strains		
EPI 300	Host for inducing high copy number of a fosmid	Epicentre®
BW25113/pKD46	Host for PCR-targeted disruption of a gene from a fosmid or plasmid	[7]
ET12567/pUZ8002	Host for conjugation with *Streptomyces* species	[1]
Strains used for antimicrobial assays		
Bacillus sp. ATCC 27859	Wild-type	ATCC
Escherichia coli ATCC 27856	Wild-type	ATCC
Staphylococcus aureus ATCC 10537	Methicillin-resistant	ATCC
Enterococcus faecium ATCC 12952	Vancomycin-resistant	ATCC
Plasmids		

pSC-B-amp/kan	A routine vector from StrataClone Blunt PCR Cloning Kit, for cloning blunt-end PCR product	Agilent Technologies
int-3A10	A fosmid containing the entire *tsr* gene cluster and all essential genes for the conjugal transfer and integration into a streptomycete chromosome	[2]
int-3A100	Derived from int-3A10, *tsrA* is replaced by *chl*^{R}-*sacB* cassette	[2]
pCL61	pSC-B-amp/kan containing the *tsrA* variant encoding the Ala2Gly mutation	[2]
int-3A101	Derived from int-3A100. The *chl*^{R}-*sacB* cassette is replaced by the *tsrA* variant encoding the Ala2Gly mutation	[2]
pA2C	pSC-B-amp/kan containing the *tsrA* variant encoding the Ala2Cys mutation	This study
pA2D	pSC-B-amp/kan containing the *tsrA* variant encoding the Ala2Asp mutation	This study
pA2E	pSC-B-amp/kan containing the *tsrA* variant encoding the Ala2Glu mutation	This study
pA2F	pSC-B-amp/kan containing the *tsrA* variant encoding the Ala2Phe mutation	This study
pA2H	pSC-B-amp/kan containing the *tsrA* variant encoding the Ala2His mutation	This study
pA2I	pSC-B-amp/kan containing the *tsrA* variant encoding the Ala2Ile mutation	This study
pA2K	pSC-B-amp/kan containing the *tsrA* variant encoding the Ala2Lys mutation	This study
pA2L	pSC-B-amp/kan containing the *tsrA* variant encoding the Ala2Leu mutation	This study
pA2M	pSC-B-amp/kan containing the *tsrA* variant encoding the Ala2Met mutation	This study
pA2N	pSC-B-amp/kan containing the *tsrA* variant encoding the Ala2Asn mutation	This study
pA2P	pSC-B-amp/kan containing the *tsrA* variant encoding the Ala2Pro mutation	This study
pA2Q	pSC-B-amp/kan containing the *tsrA* variant encoding the Ala2Gln mutation	This study
pA2R	pSC-B-amp/kan containing the *tsrA* variant encoding the Ala2Arg mutation	This study
pA2S	pSC-B-amp/kan containing the *tsrA* variant encoding the Ala2Ser mutation	This study
pA2T	pSC-B-amp/kan containing the *tsrA* variant encoding the Ala2Thr mutation	This study
pA2V	pSC-B-amp/kan containing the *tsrA* variant encoding the Ala2Val mutation	This study
pA2W	pSC-B-amp/kan containing the *tsrA* variant encoding the Ala2Trp mutation	This study
pA2Y	pSC-B-amp/kan containing the *tsrA* variant encoding the Ala2Tyr mutation	This study
int-A2C	Derived from int-3A100. The *chl*^{R}-*sacB* cassette is replaced by the *tsrA* variant encoding the Ala2Cys mutation	This study
int-A2D	Derived from int-3A100. The *chl*^{R}-*sacB* cassette is replaced by the *tsrA* variant encoding the Ala2Asp mutation	This study
int-A2E	Derived from int-3A100. The *chl*^{R}-*sacB* cassette is replaced by the *tsrA* variant encoding the Ala2Glu mutation	This study
int-A2F	Derived from int-3A100. The *chl*^{R}-*sacB* cassette is replaced by the *tsrA* variant encoding the Ala2Phe mutation	This study
int-A2H	Derived from int-3A100. The *chl*^{R}-*sacB* cassette is replaced by the *tsrA* variant encoding the Ala2His mutation	This study
int-A2I	Derived from int-3A100. The *chl*^{R}-*sacB* cassette is replaced by the *tsrA* variant encoding the Ala2Ile mutation	This study
int-A2K	Derived from int-3A100. The *chl*^{R}-*sacB* cassette is replaced by the *tsrA* variant encoding the Ala2Lys mutation	This study
int-A2L	Derived from int-3A100. The *chl*^{R}-*sacB* cassette is replaced by the *tsrA* variant encoding the Ala2Leu mutation	This study
int-A2M	Derived from int-3A100. The *chl*^{R}-*sacB* cassette is replaced by the *tsrA* variant encoding the Ala2Met mutation	This study
int-A2N	Derived from int-3A100. The *chl*^{R}-*sacB* cassette is replaced by the *tsrA* variant encoding the Ala2Asn mutation	This study
int-A2P	Derived from int-3A100. The *chl*^{R}-*sacB* cassette is replaced by the *tsrA* variant encoding the Ala2Pro mutation	This study

int-A2Q	Derived from int-3A100. The *chl*^{R}-*sacB* cassette is replaced by the *tsrA* variant encoding the Ala2Gln mutation	This study
int-A2R	Derived from int-3A100. The *chl*^{R}-*sacB* cassette is replaced by the *tsrA* variant encoding the Ala2Arg mutation	This study
int-A2S	Derived from int-3A100. The *chl*^{R}-*sacB* cassette is replaced by the *tsrA* variant encoding the Ala2Ser mutation	This study
int-A2T	Derived from int-3A100. The *chl*^{R}-*sacB* cassette is replaced by the *tsrA* variant encoding the Ala2Thr mutation	This study
int-A2V	Derived from int-3A100. The *chl*^{R}-*sacB* cassette is replaced by the *tsrA* variant encoding the Ala2Val mutation	This study
int-A2W	Derived from int-3A100. The *chl*^{R}-*sacB* cassette is replaced by the *tsrA* variant encoding the Ala2Trp mutation	This study
int-A2Y	Derived from int-3A100. The *chl*^{R}-*sacB* cassette is replaced by the *tsrA* variant encoding the Ala2Tyr mutation	This study

Table S9. Primers used in this study

Primer	Sequence	Description
A2X	5'-ACTACATGGACGAGACGCTGCTCGACGGTGAGGA CCTGACCGTCACGATGATCNNSTCCGCCTCCTGCAC CACCTGCATCTGCACCTGCAGCTGCAGCTCCTGAGG TAACACCCGGCGCGGAGGACTGTTCCTCCCCGCCCG CCGGACCTG-3'	Chemically synthesized ultramer to generate TsrA Ala2 mutants. Underlined degenerate codon encodes the amino acid at the 2nd position of the TsrA core peptide.
Amp-TsrA-SP-F	5'-GAGATCAGCGACTACATGGACGAGA-3'	Primers used in the amplification of *tsrA* Ala2 variants.
Amp-TsrA-SP-R	5'-CTGCAACGGTCAGGTCCGGCGGGCG-3'	
SD3-F	5'-ATCGTGTTGGGCTTGACG-3'	Primers used in PCR and DNA sequencing to confirm Ala2 variant fosmids.[2]
SD3-R	5'-CGCGGTGCAATAGGACAT-3'	

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