Supplementary Information

For

Mutagenesis of the thiostrepton precursor peptide at Thr7 impacts both biosynthesis and function

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Materials and Methods

General. Unless specified, common chemicals, solvents, restriction enzymes, DNA ligase and other materials were purchased from standard commercial sources and used as provided. Oligonucleotides and ultramer DNA were purchased from Integrated DNA Technologies. All DNA sequence analysis was performed at Eurofins MWG Operon. The QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) was used for the isolation of plasmids and fosmids from *E. coli* strains. *Streptomyces* genomic DNA was isolated using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer's recommendations. High performance liquid chromatography (HPLC) analysis was performed on a Beckman Coulter System Gold instrument. HPLC-MS was performed at the Georgia Institute of Technology Bioanalytical Mass Spectrometry Facility with a Phenomenex Syngeri RP column (250 mm × 2 mm, 4 μm) (Torrance, CA) and developed with 20% Buffer B in Buffer A for 8 min followed by a gradient from 20-100% Buffer B over 35 min at 0.25 mL min^{-1} (Buffer A: 5% acetonitrile and 0.1% formic acid; Buffer B: 95% acetonitrile and 0.1% formic acid). High-resolution matrix-assisted laser desorption/ionization mass spectrometry (HR-MALDI-MS), and MALDI-MS/MS were also performed at the Georgia Institute of Technology Bioanalytical Mass Spectrometry Facility. High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) was performed at the Emory University Mass Spectrometry Center. Proton and carbon NMR spectra were recorded on a Bruker 500 MHz spectrometer. All NMR experiments were performed according to standard pulse sequences supplied with the instrument.

Bacterial strains plasmids and growth medium. *Streptomyces laurentii* ATCC 31255 was obtained from American Type Culture Collection (ATCC). *S. laurentii* NDS1 was previously generated in our lab.[1] All strains and plasmids are listed in Table S4, and primers are listed in Table S5. All *Escherichia coli* (*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: kanamycin (50 μg mL^{-1}), apramycin (50 μg mL^{-1}), ampicillin (100 μg mL^{-1}), nalidixic acid (25 μg mL^{-1}), and chloramphenicol (30 μg mL^{-1}). MS agar was used for the intergeneric conjugation of *S. laurentii* NDS1.[2]

Site-directed mutagenesis of *tsrA*. Site-directed mutagenesis of TsrA to yield Thr7Ser and Thr7Ala followed the procedure described previously.[1] Two pairs of primers were used: T7S-F and T7S-R for the Thr7Ser mutation and T7A-F and T7A-R for the Thr7Ala mutation (Table S5). Mutant plasmids were amplified from pJP11 by adapting the QuikChange site-directed mutagenesis strategy (Agilent Technologies, La Jolla, CA). DNA sequence analysis confirmed successful mutagenesis in each case, including the absence of any undesired mutations. The resulting plasmids were designated as pCL64 for TsrA Thr7Ser and pCL65 for TsrA Thr7Ala (Table S4).

The following procedure was used to generate the Thr7Val mutant of TsrA. The single-strand DNA ultramer T7V was amplified by polymerase chain reaction using the primers T7V-F and T7V-R (Table S5). The PCR product was cloned into pSC-B-amp/kan using the StrataClone Blunt PCR Cloning Kit (Agilent Technologies, La Jolla, CA). The plasmid encoding the expected Thr7Val mutation was confirmed by DNA sequencing and designated as pCL66 (Table S4).

The fosmid int-3A10 is an integrative fosmid harboring the entire *tsr* biosynthetic gene cluster.[1] The fosmid int-3A100 is a derivative of int-3A10, in which the region of *tsrA* encoding the 17 amino acid structural peptide of TsrA is replaced by a dual-marker disruption cassette

consisting of *chl*^{R}, a chloramphenicol resistance gene, and a levansucrase-encoding *sacB* gene.[1] The *tsrA* T7S and T7A mutants were amplified from pCL64 and pCL65 by PCR with primers SD2-F and SD2-R. The *tsrA* T7V mutant was amplified from pCL66 with primers T7V-F and T7V-R. The resulting PCR products were then used to replace the dual-marker cassette in int-3A100 by PCR-targeted gene replacement,[2,3] yielding three sucrose-tolerant fosmids: int-3A104, int-3A105 and int-3A106 (Table S4). The incorporated mutations in int-3A104 to int-3A106 were confirmed by DNA sequence analysis. Fosmids int-3A10 (encoding wild-type TsrA), int-3A104 (for TsrA Thr7Ser), int-3A105 (for TsrA Thr7Ala) and int-3A106 (for TsrA Thr7Val) were first transformed into *E. coli* ET12567/pUZ8002, and then introduced into *S. laurentii* NDS1 through intergeneric conjugation. Colonies resistant to apramycin were selected for fermentation analysis.

Evaluation of thiostrepton analog production by *S. laurentii* NDS1/int-3A104, *S. laurentii* NDS1/int-3A105 and *S. laurentii* NDS1/int-3A106. Growth of *S. laurentii* was performed as described previously.[4] After fermentation, the whole cell culture was extracted twice with an equal volume of chloroform. The chloroform layers were pooled and the solvent removed *in vacuo*. The solid residue from 100 mL of culture was dissolved in 4 mL of chloroform. Samples were analyzed by HPLC with a Phenomenex Luna C18(2) column (250 × 4.6 mm, 5 µm). The column was developed using a gradient of 0-100% acetonitrile in water over 30 min at a flow rate of 1 mL min^{-1}. Absorbance was monitored at 254 nm. Under these conditions, thiostrepton A elutes with a t_R of about 23 min.

Purification and structural determination of thiostrepton Thr7Ala 3 and SL105-1 4. Crude extract from *S. laurentii* NDS1/int-3A105 (10 L) culture was purified by HPLC with a Phenomenex Luna C18(2) RP-Aqueous semi-preparative column (250 x 10 mm, 5 µm) while monitoring absorbance at 254 nm. The column was first developed using a gradient of 0 to 70% acetonitrile in water over 30 min at a flow rate of 4.7 mL min^{-1}, and fractions containing thiostrepton Thr7Ala **3** and SL105-1 **4** were collected separately for each fraction. Solvent was removed *in vacuo* for the two fractions. The solid residues were dissolved in chloroform for further HPLC purification. For purification of thiostrepton Thr7Ala **3**, the column was developed with a gradient of acetonitrile in water at a flow rate of 4.7 mL min^{-1} as follows: 10 to 40% acetonitrile over 5 min, 40% acetonitrile for 5 min, 40 to 48% acetonitrile over 5 min, 48% acetonitrile for 5 min, 48 to 100% acetonitrile over 5 min, and finally 100% acetonitrile for 5 min. For the purification of SL105-1 **4**, the column was developed with a gradient of acetonitrile in water at a flow rate of 4.7 mL min^{-1} as follows: 10 to 35% acetonitrile over 5 min, 35% acetonitrile for 21 min, 35 to 100% acetonitrile over 2 min, and finally 100% acetonitrile for 5 min. Purified samples were analyzed by HPLC-MS, HR-MALDI-MS, MALDI-MS/MS and NMR. HR-MALDI-MS of thiostrepton Thr7Ala **3**: $C_{71}H_{84}N_{19}O_{17}S_5$, *m/z* 1634.4906 $[M+H]^+$ (calculated 1634.4896). HR-MALDI-MS of SL105-1 **4**: $C_{62}H_{67}N_{16}O_{15}S_5$, *m/z* 1435.3618 $[M+H]^+$ (calculated 1435.3575). NMR data is included in supplementary figures and tables.

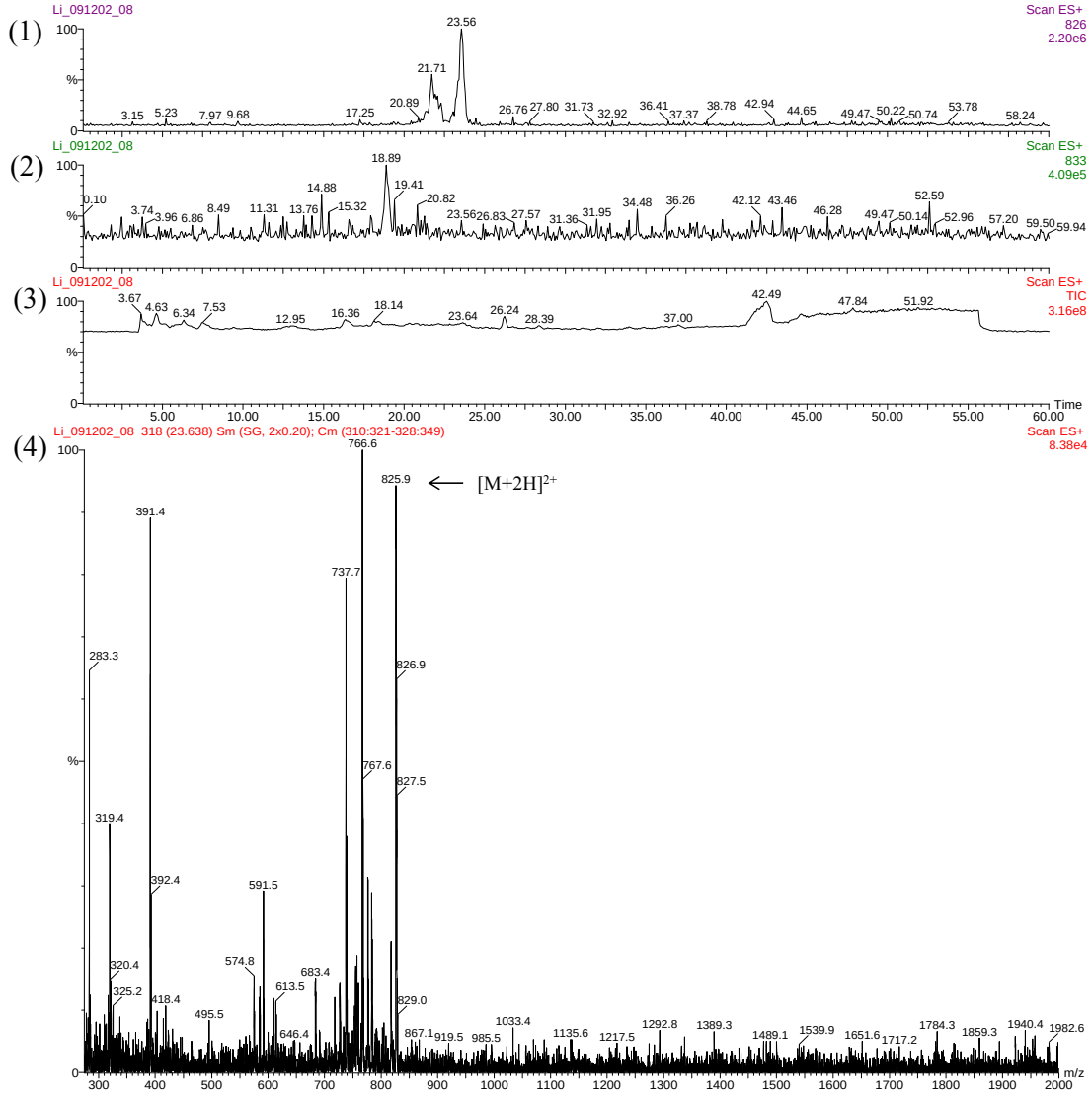
Purification of characterization of thiostrepton Thr7Val 5 and SL106-1 6. Crude extract from *S. laurentii* NDS1/int-3A106 (4 L) culture was purified by HPLC with a Phenomenex Luna C18(2) RP-Aqueous semi-preparative column (250 x 10 mm, 5 µm) while monitoring absorbance at 254 nm. The column was first developed using a gradient of 0 to 60% acetonitrile in water over 30 min at a flow rate of 4 mL min^{-1}, and fractions containing thiostrepton Thr7Val **5** and SL106-1 **6** were collected separately. Solvent was removed *in vacuo*

for each fraction. The solid residues were dissolved in chloroform for further HPLC purification. The next column was developed with a gradient of acetonitrile in water at a flow rate of 4.3 mL min^{-1} as follows: 0 to 100% acetonitrile over 30 min, and finally 100% acetonitrile for 5 min. Purified samples were analyzed by HPLC-MS, HR-MALDI-MS/HR-ESI-MS, and MALDI-MS/MS. HR-ESI-MS of thiostrepton Thr7Val **5**: $C_{73}H_{87}N_{19}O_{17}S_5Na$, *m/z* 1684.5087 $[M+Na]^+$ (calculated 1684.5029). HR-MALDI-MS of SL106-1 **6**: $C_{64}H_{71}N_{16}O_{15}S_5$, *m/z* 1463.3890 $[M+H]^+$ (calculated 1463.3888).

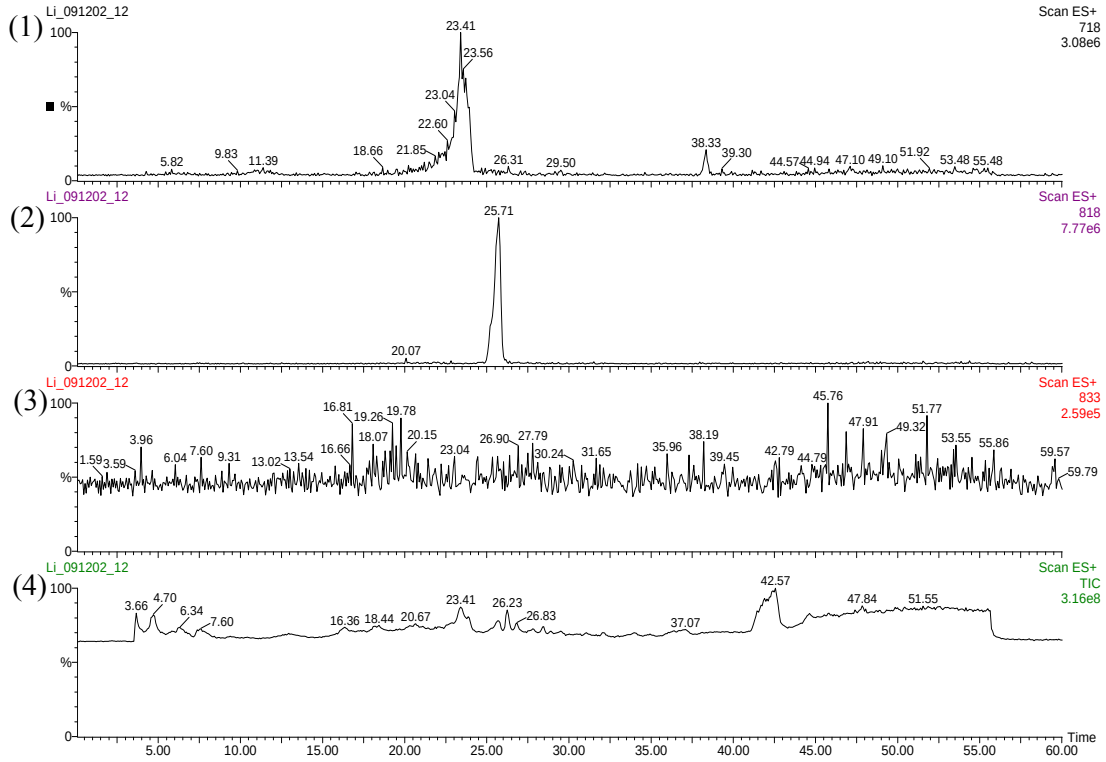
Antibacterial activity of thiostrepton analogs. Minimum inhibitory concentrations (MICs) of thiostrepton analogs against indicator strains were determined following the liquid microdilution method described previously.[1] The indicator strains used in this study were methicillin-resistant *Staphylococcus aureus* ATCC 10537 (MRSA), vancomycin-resistant *Enterococcus faecium* ATCC 12952 (VRE), *Bacillus* sp. ATCC 27859 (*Bacillus*) and *Escherichia coli* ATCC 27856 (*E. coli*). Thiostrepton A **1**, thiostrepton Thr7Ala **3** and thiostrepton Thr7Val **5** were prepared in DMSO and quantified by UV spectroscopy using an extinction coefficient at 280 nm of 0.027 cm^{-1} μM^{-1}.[5] SL105-1 **4** was first quantified by NMR against a tetramethylsilane internal standard and then used to generate a calibration curve by HPLC, and the stock solution was finally prepared in DMSO. SL106-1 **6** was quantified by HPLC against the SL105-1 **5** standard and prepared in DMSO. The positive antibiotic control used for *Bacillus*, and VRE was chloramphenicol, whereas the positive control for MRSA was vancomycin. DMSO was used as the negative control in all assays. Cell growth was monitored by comparing the optical density at 600 nm at the time of treatment and after 18 h incubation at 37 °C. A difference in OD_{600} was considered growth and the lowest concentration causing complete suppression of visible bacterial growth defined the MIC.

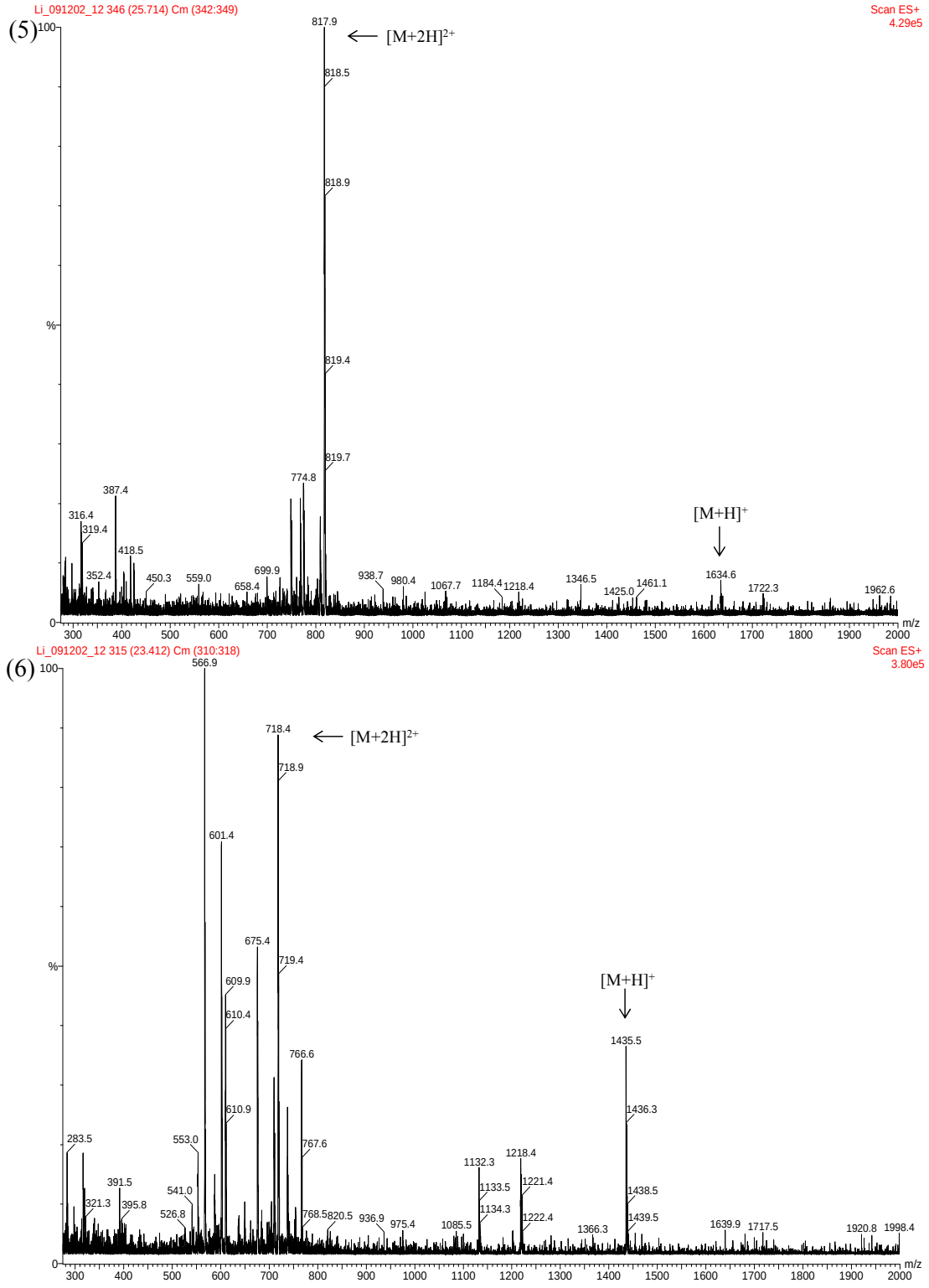
Figure S1. HPLC-MS analysis of culture extracts from *S. laurentii tsrA* mutant strains.

(**A**) HPLC-MS chromatogram of the *S. laurentii* NDS1 int-3A104 (TsrA Thr7Ser) culture extract. (*1*) Chromatogram extracted for *m*/*z* 826. (*2*) Chromatogram extracted for *m*/*z* 833 (the calculated $[M+2H]^{2+}$ *m*/*z* 832.7 for wild-type thiostrepton A **1**, which ordinarily elutes at about 28 min.[1]). (*3*) Total ion chromatogram. (*4*) Mass spectrum of thiostrepton Thr7Ser **2** from *S. laurentii* NDS1 int-3A104 extract eluting at t_R = 23.6 min (calculated *m*/*z* 825.8 $[M+2H]^{2+}$, observed *m*/*z* 825.9 $[M+2H]^{2+}$).



(**B**) HPLC-MS chromatogram of the *S. laurentii* NDS1 int-3A105 (TsrA Thr7Ala) culture extract. (*1*) Chromatogram extracted for *m*/*z* 718. (*2*) Chromatogram extracted for *m*/*z* 818. (*3*) Chromatogram extracted for *m*/*z* 833. (*4*) Total ion chromatogram. (*5*) Mass spectrum of thiostrepton Thr7Ala **3** from *S. laurentii* NDS1 int-3A105 extract eluting at t_R = 25.7 min (calculated *m/z* 817.8 $[M+2H]^{2+}$, observed *m/z* 817.9 $[M+2H]^{2+}$; calculated *m/z* 1634.5 $[M+H]^{+}$, observed *m/z* 1634.6 $[M+H]^{+}$). (*6*) Mass spectrum of SL105-1 **4** from *S. laurentii* NDS1 int-3A105 extract eluting at t_R = 23.4 min (calculated *m/z* 718.2 $[M+2H]^{2+}$, observed *m/z* 718.4 $[M+2H]^{2+}$; calculated *m/z* 1435.4 $[M+H]^{+}$, observed *m/z* 1435.5 $[M+H]^{+}$).




(5)
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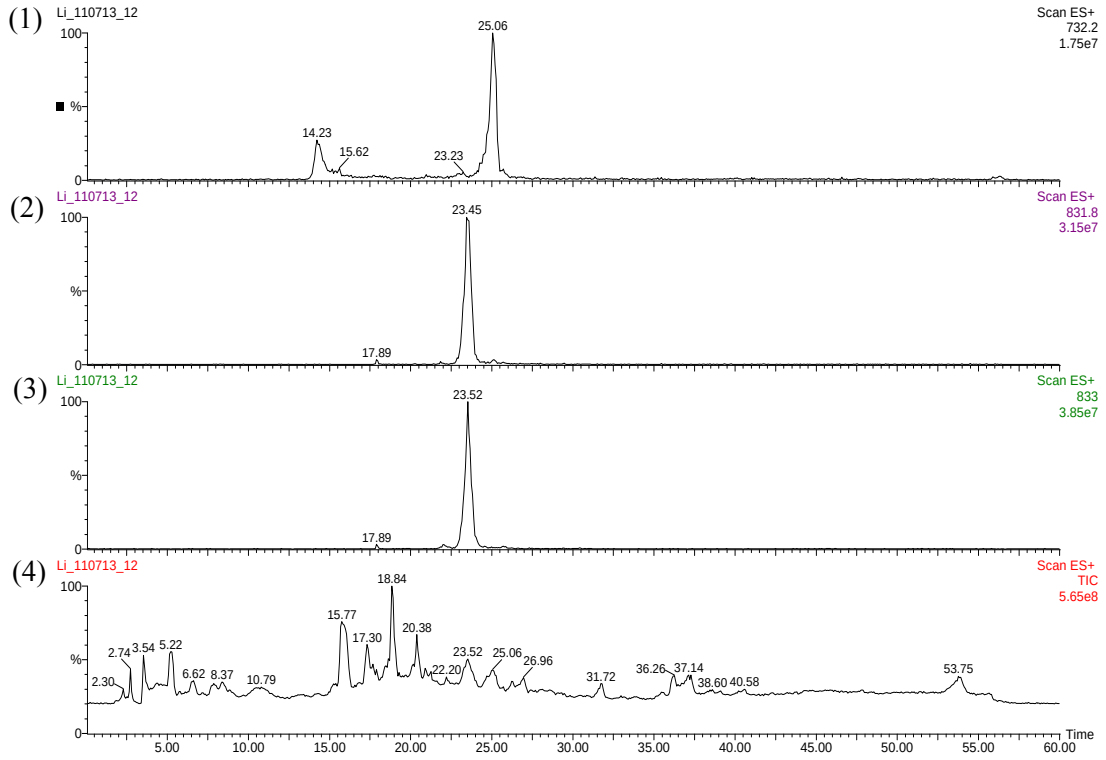
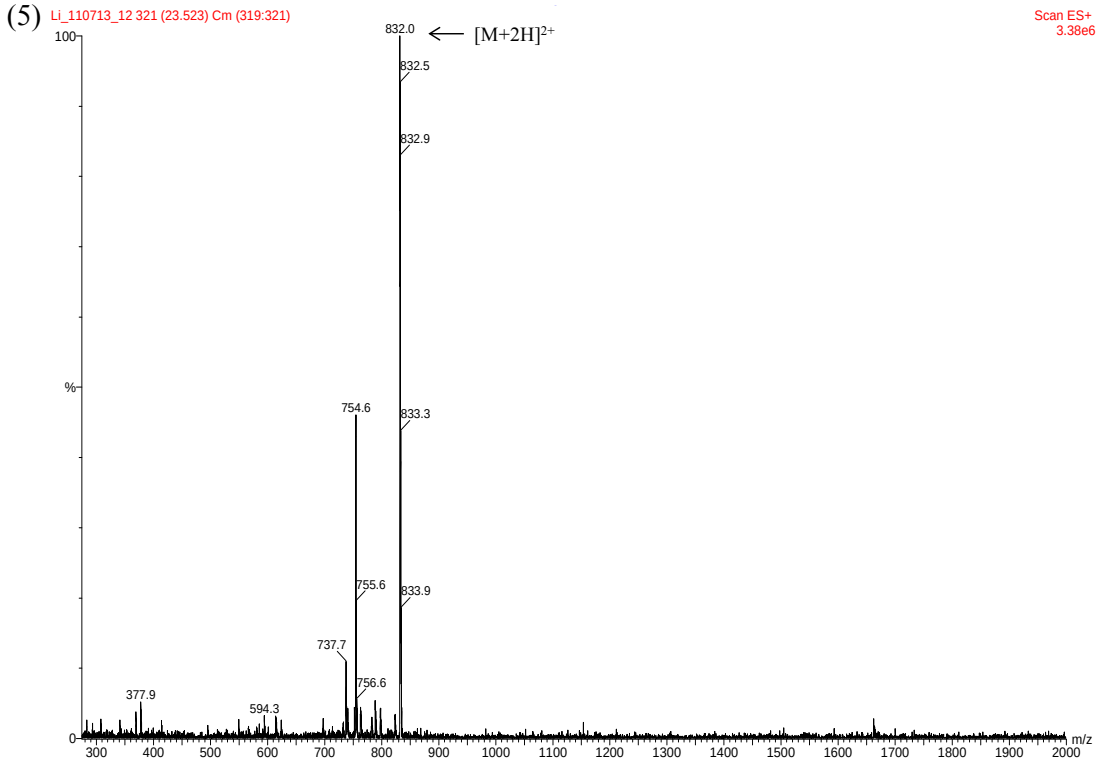
Scan ES+
4.29e5
[M+2H]2+
[M+H]+
(6)
Li_091202_12 315 (23.412) Cm (310:318)
Scan ES+
3.80e5
[M+2H]2+
[M+H]+
m/z

(**C**) HPLC-MS chromatogram of the *S. laurentii* NDS1 int-3A106 (Tsr Thr7Val) culture extract. (*1*) Chromatogram extracted for *m*/*z* 732.2. (*2*) Chromatogram extracted for *m*/*z* 831.8. (*3*) Chromatogram extracted for *m*/*z* 833. (*4*) Total ion chromatogram. (*5*) Mass spectrum of thiostrepton Thr7Val **5** from *S. laurentii* NDS1 int-3A106 extract eluting at t_R = 23.5 min (calculated *m/z* 831.8 $[M+2H]^{2+}$, observed *m/z* 832.0 $[M+2H]^{2+}$). (*6*) Mass spectrum of SL106-1 **6** from *S. laurentii* NDS1 int-3A106 extract eluting at t_R = 25.1 min (calculated *m/z* 732.2 $[M+2H]^{2+}$, observed *m/z* 732.5 $[M+2H]^{2+}$)



(5)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755.6
833.9
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594.3
100
%
0
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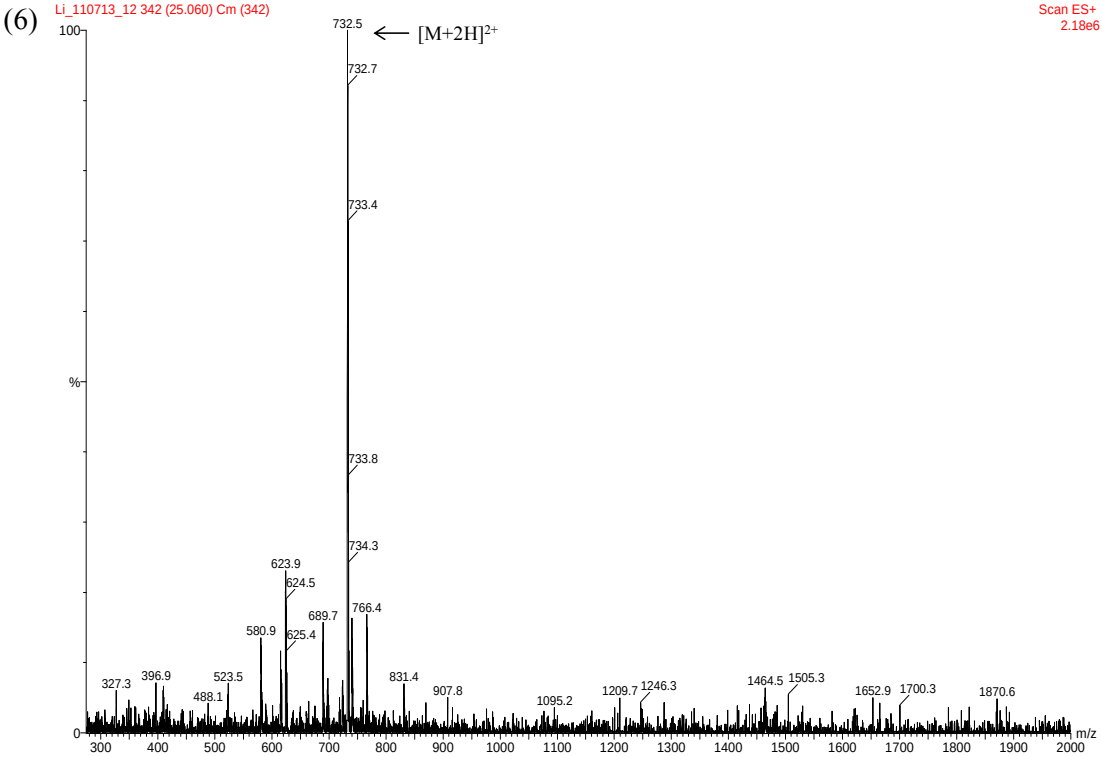
m/z

(6)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[M+2H]2+
732.7
733.4
733.8
734.3
623.9
624.5
689.7
766.4
580.9
625.4
327.3
396.9
523.5
488.1
831.4
907.8
1095.2
1209.7
1246.3
1464.5
1505.3
1652.9
1700.3
1870.6
100
%
0
300 400 500 600 700 800 900 1000 1100 1200 1300 1400 1500 1600 1700 1800 1900 2000
m/z

Figure S2. HPLC and UV-Vis absorption spectra from the TsrA T7A-expressing fermentation extract. (**A**) HPLC analysis of *S. laurentii* NDS1 int-3A105 (TsrA Thr7Ala) culture extract. (**B**) UV-Vis absorption spectrum of SL105-1 **4** (t_R = 16.9 min). (**C**) UV-Vis absorption spectrum of thiostrepton Thr7Ala **3** (t_R = 18.3 min).

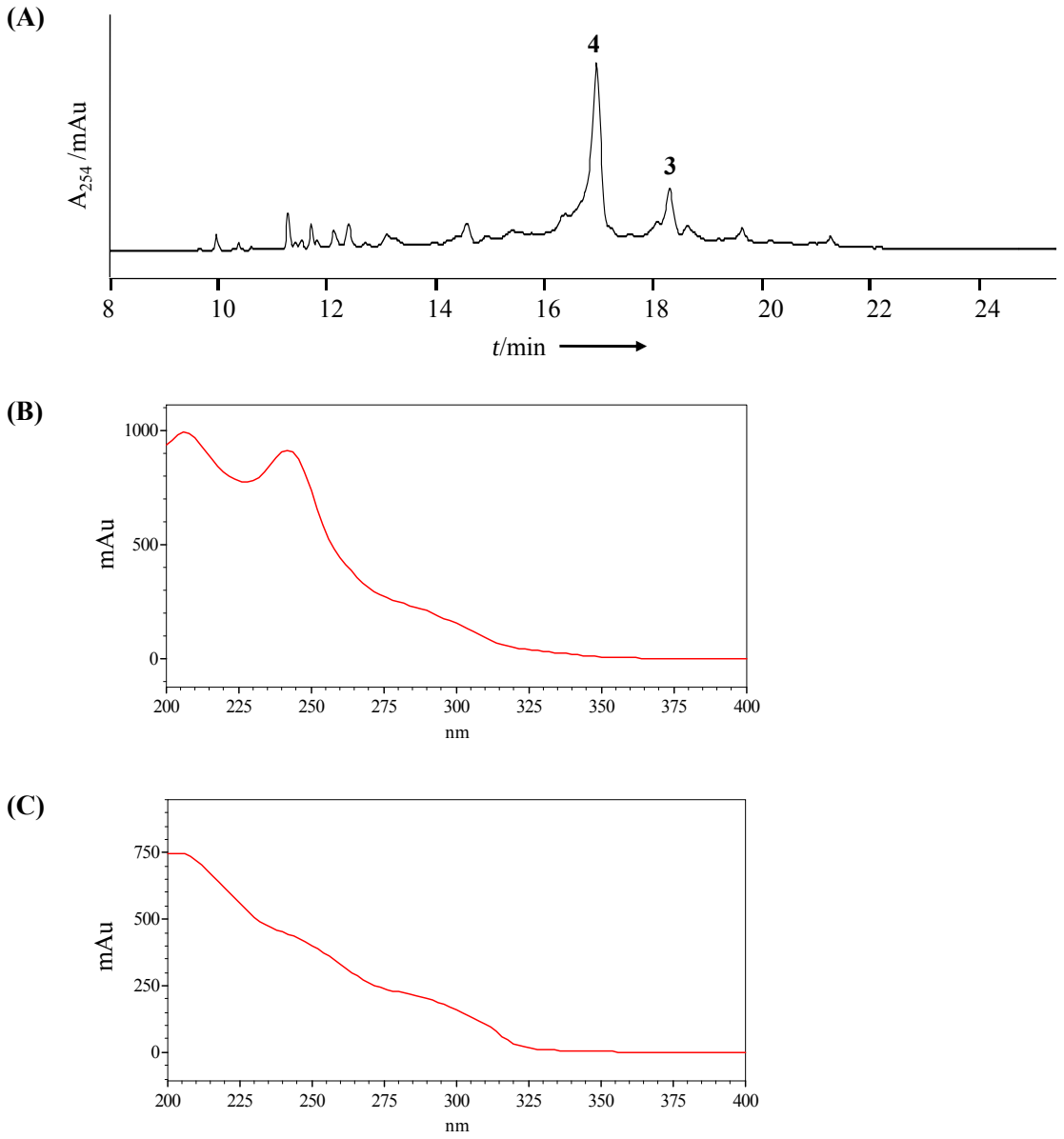
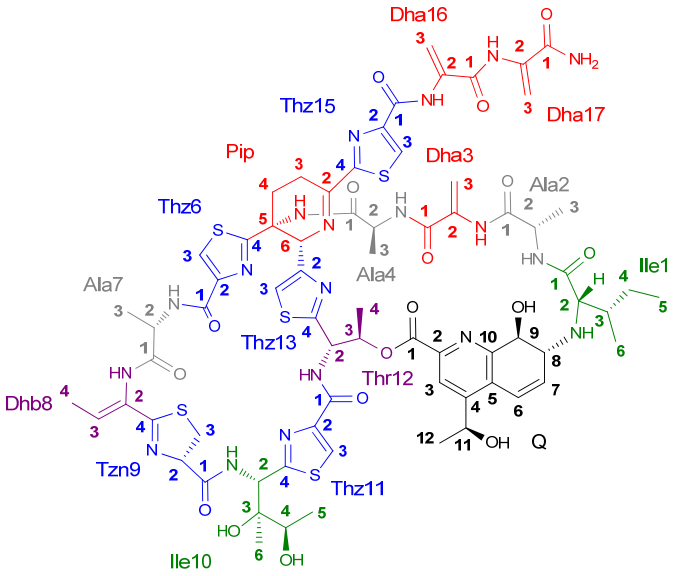


Figure S3. Structures of and numbering systems used for (**A**) thiostrepton Thr7Ala **3** and (**B**) SL105-1 **4**.

(**A**) Thiostrepton Thr7Ala **3**



(**B**) SL105-1 **4**

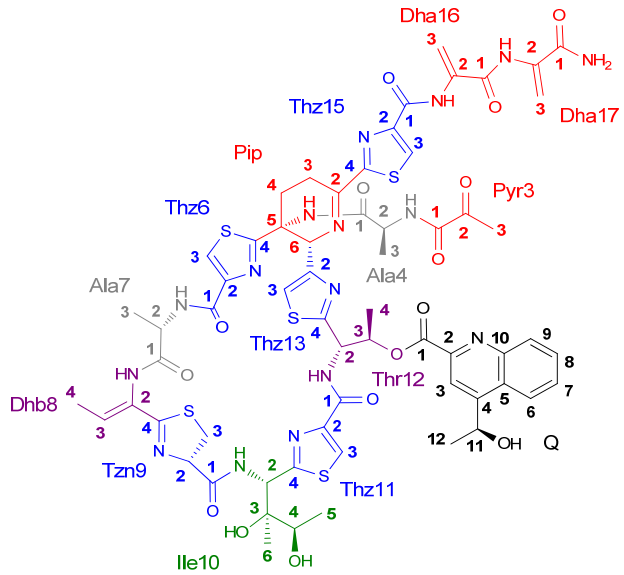


Figure S4. HPLC-MS analysis of thiostrepton Thr7Ala **3** isolated from *S. laurentii* NDS1 int-3A105 (TsrA Thr7Ala). (**A**) Total ion chromatogram. (**B**) Chromatogram extracted for *m*/*z* 818 of thiostrepton Thr7Ala **3**. (**C**) Mass spectrum of thiostrepton Thr7Ala **3** eluting at t_R = 26.3 min.

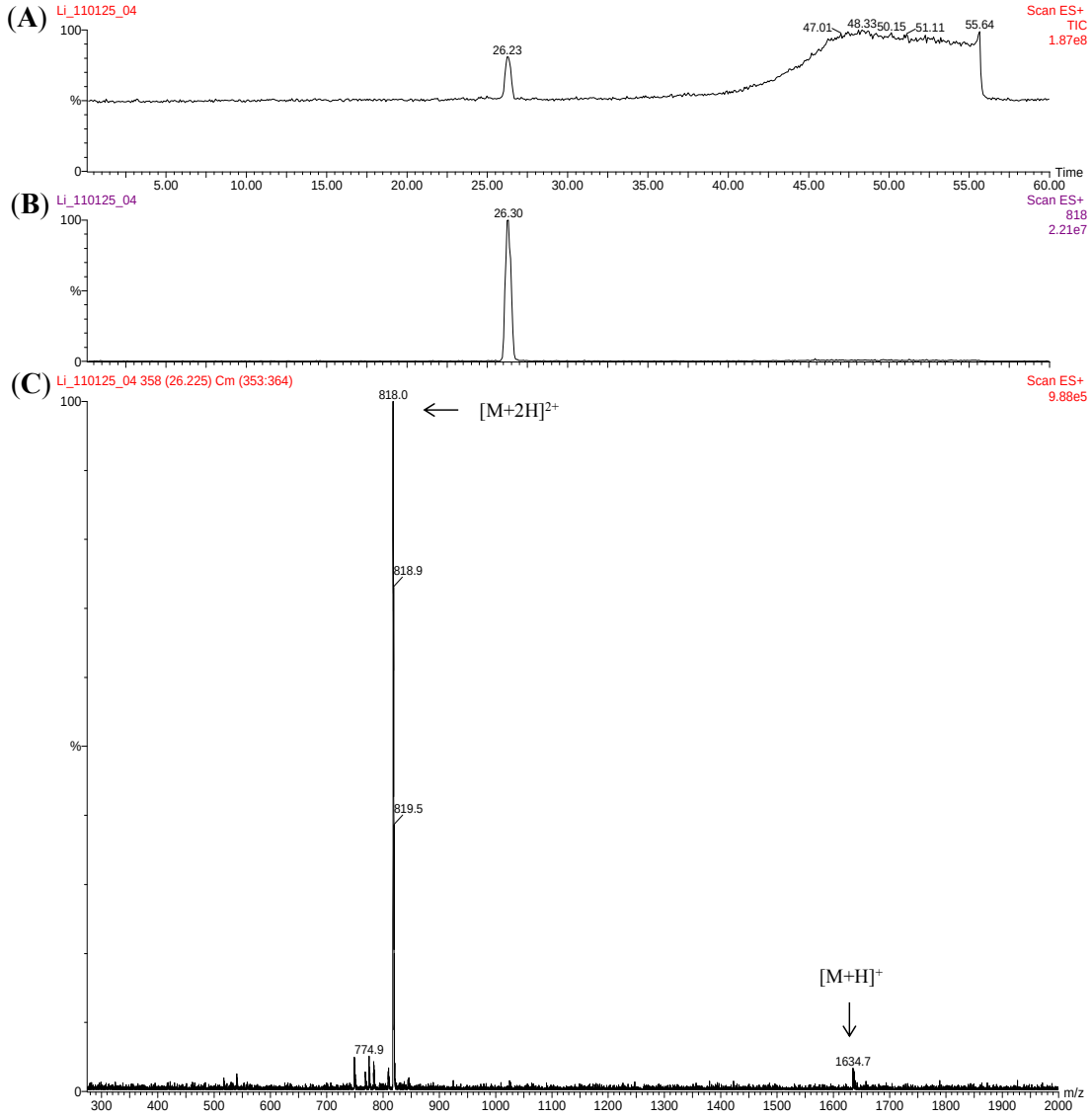
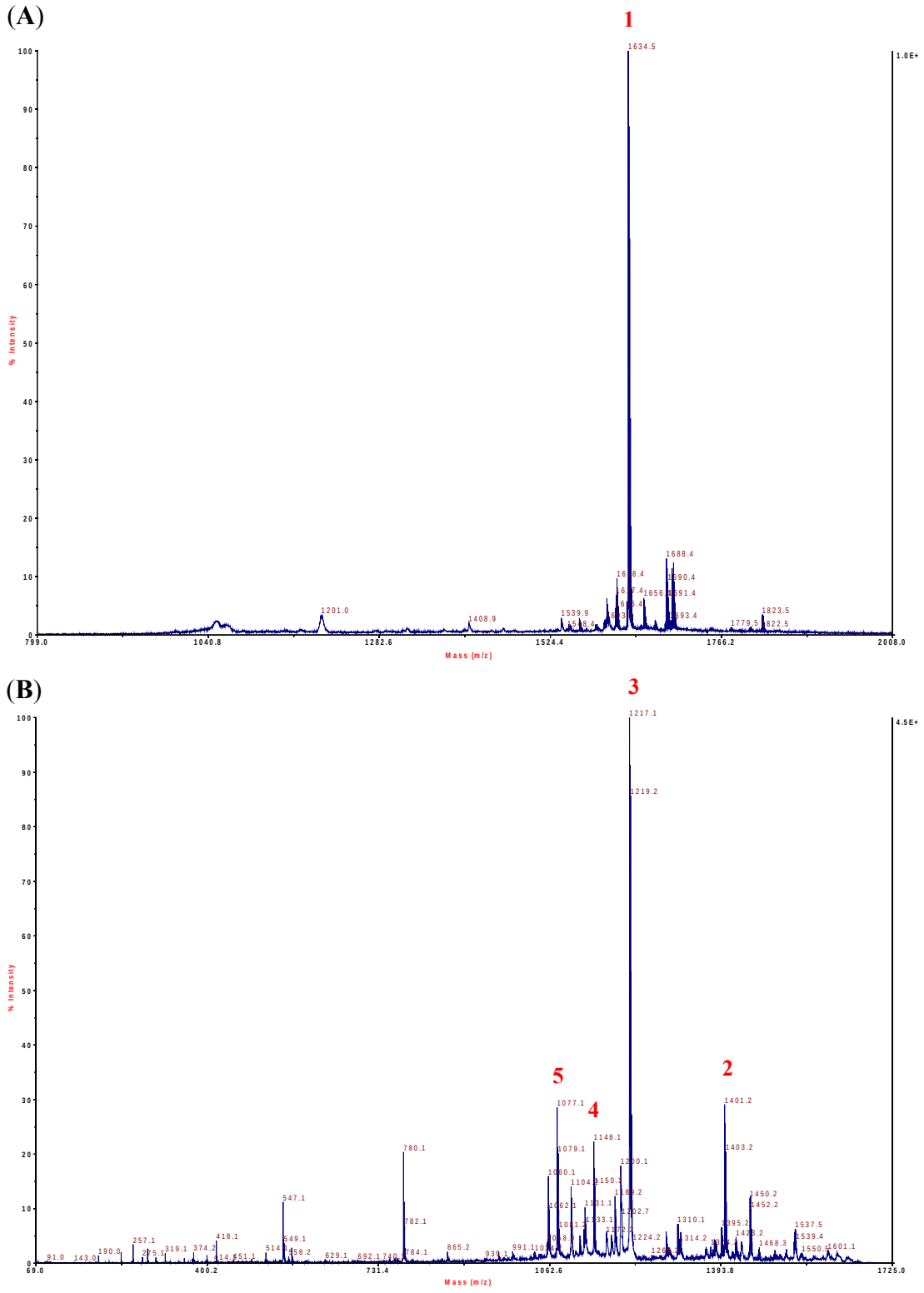
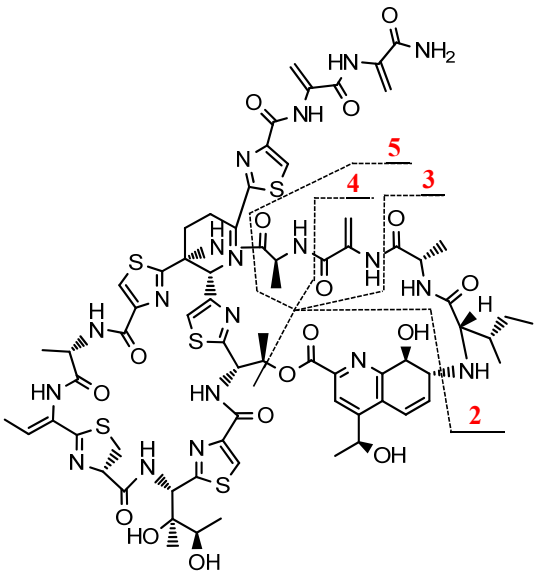


Figure S5. MALDI MS and MS/MS analysis of thiostrepton Thr7Ala **3**. (**A**) Full spectrum of MALDI MS of thiostrepton Thr7Ala **3**. (**B**) MALDI MS/MS of parent ion *m/z* 1634.5. (**C**) Table and structure showing fragments in MALDI MS/MS of thiostrepton Thr7Ala **3**.



(C)

Fragment	Expected	Observed
1. $M+H^+$ (Parent ion)	1634.5	1634.5
2. $M-QA+H^+$	1401.4	1401.2
3. $M-QA-Ile1-Ala2+H^+$	1217.3	1217.1
4. $M-QA-Ile1-Ala2-Dha3+H^+$	1148.3	1148.1
5. $M-QA-Ile1-Ala2-Dha3-Ala4+H^+$	1077.2	1077.1



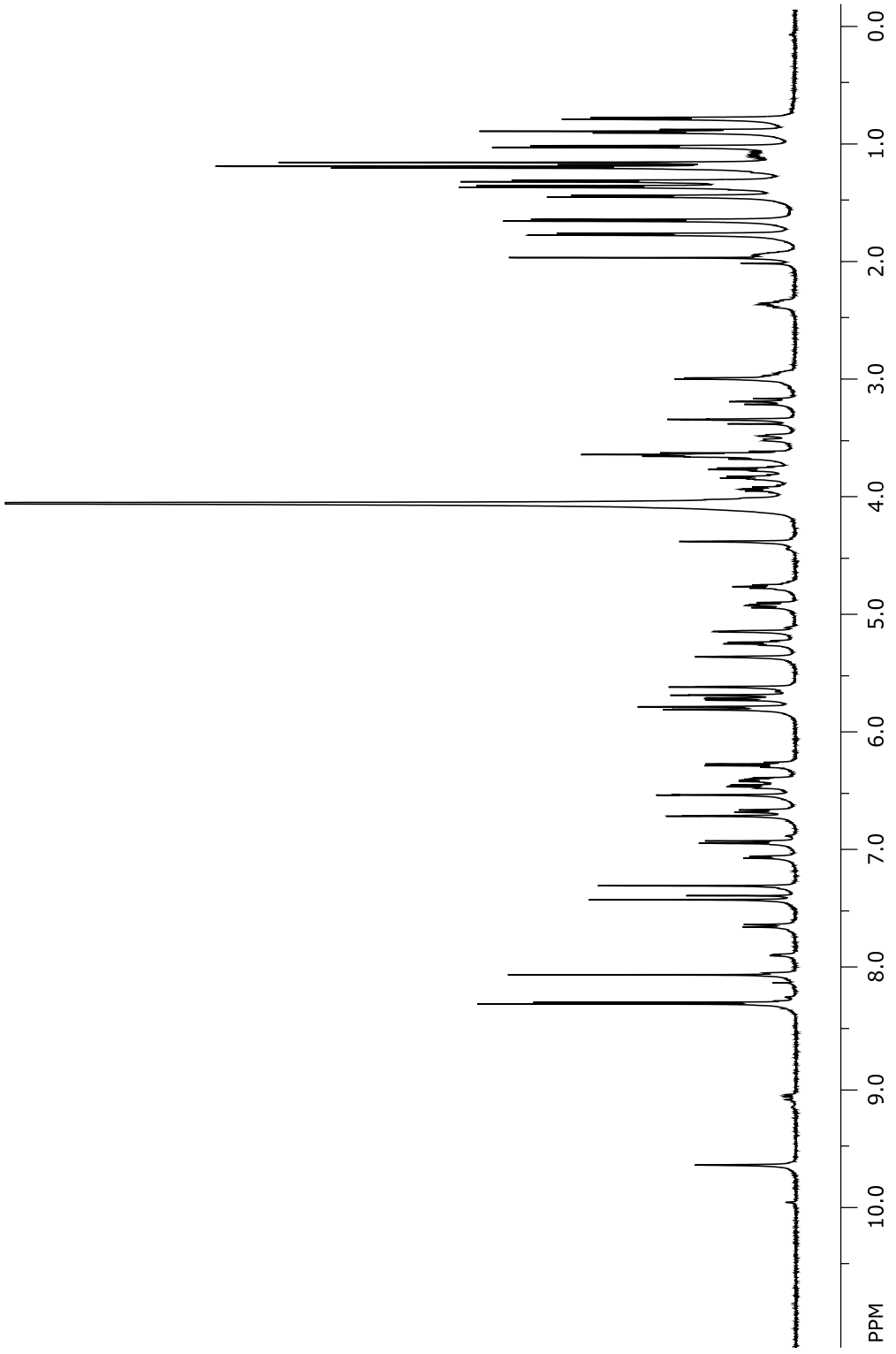


Figure S6. ^{1}H NMR spectrum of thiostrepton Thr7Ala **3** (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

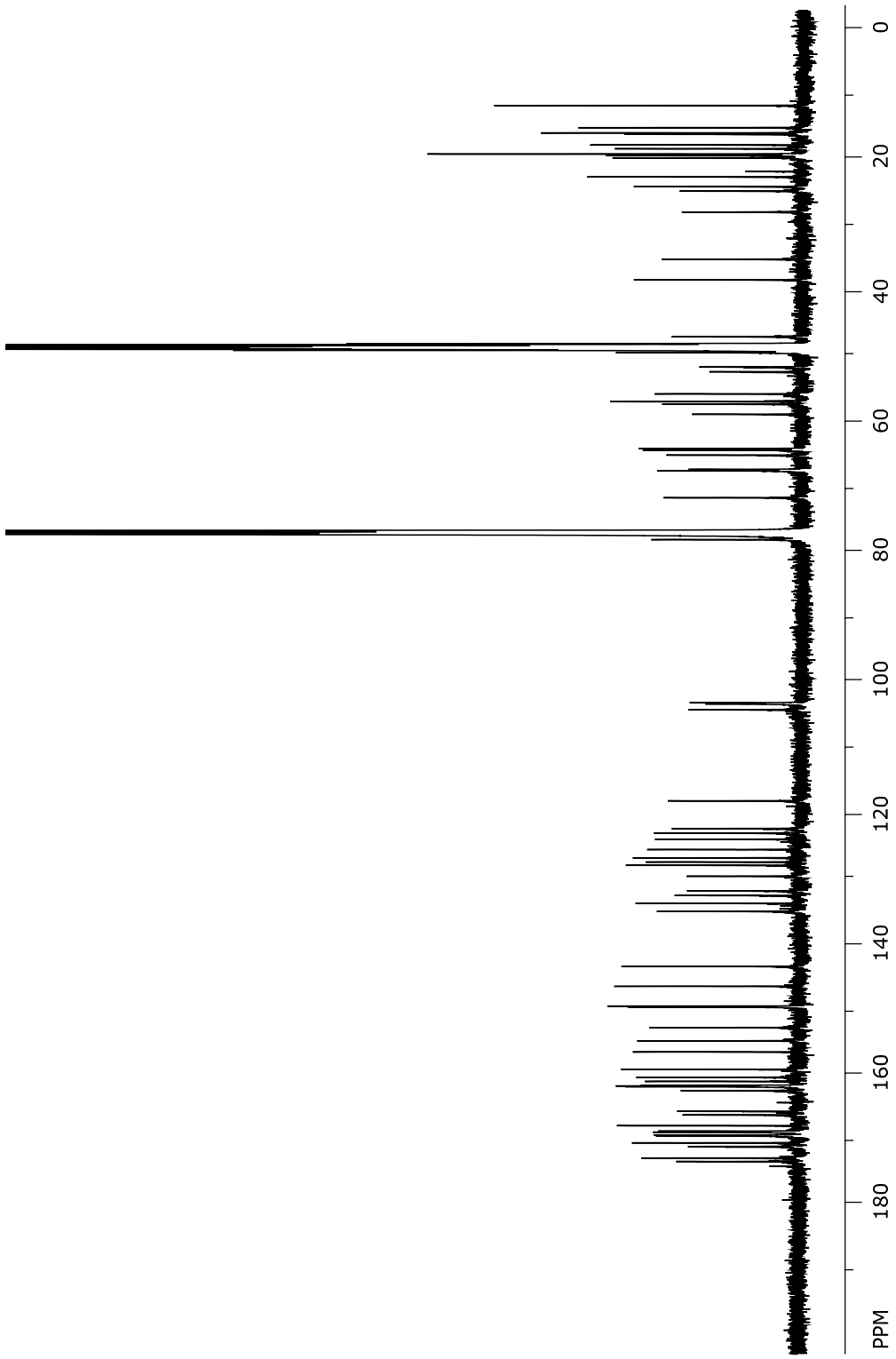


Figure S7. ^{13}C NMR spectrum of thiostrepton Thr7Ala **3** (125 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

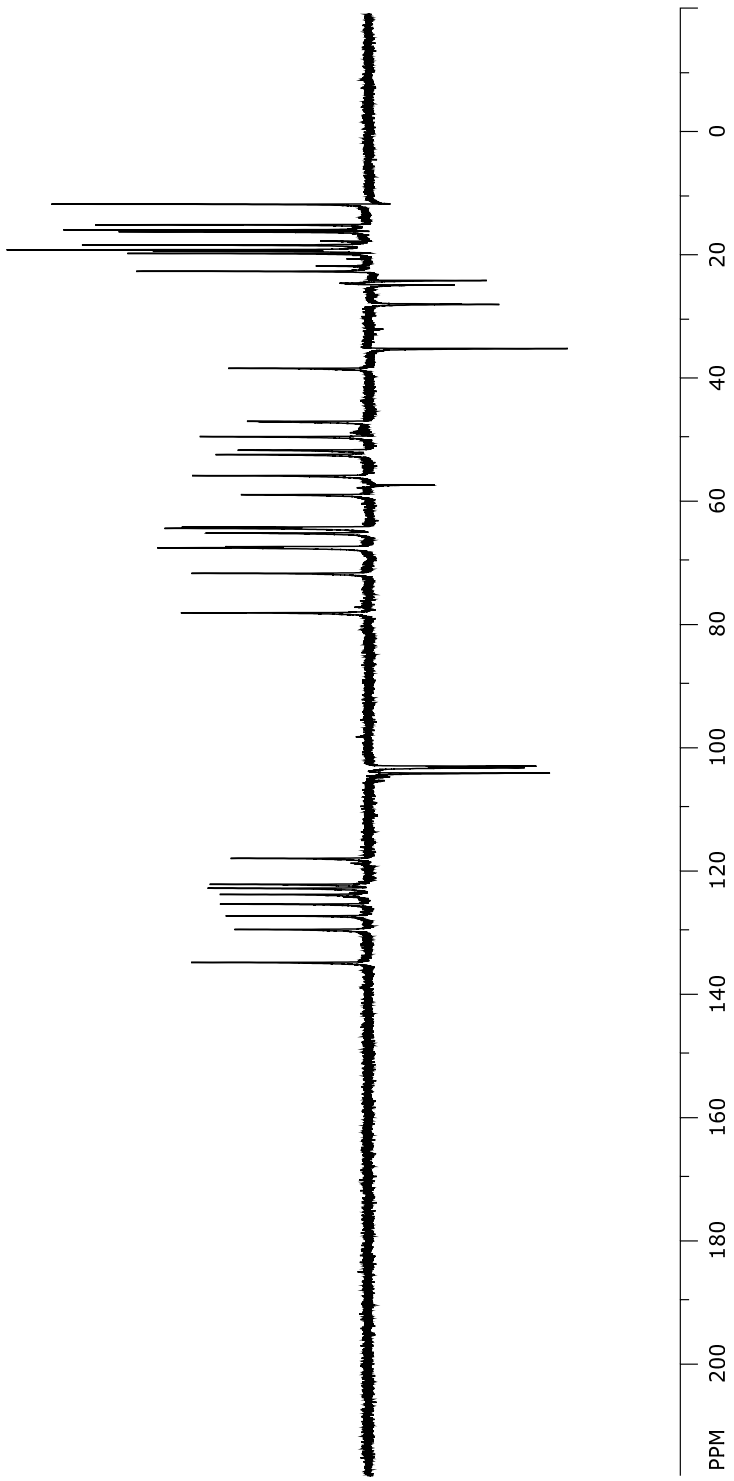


Figure S8. DEPT-135 NMR spectrum of thiostrepton Thr7Ala **3** (125 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

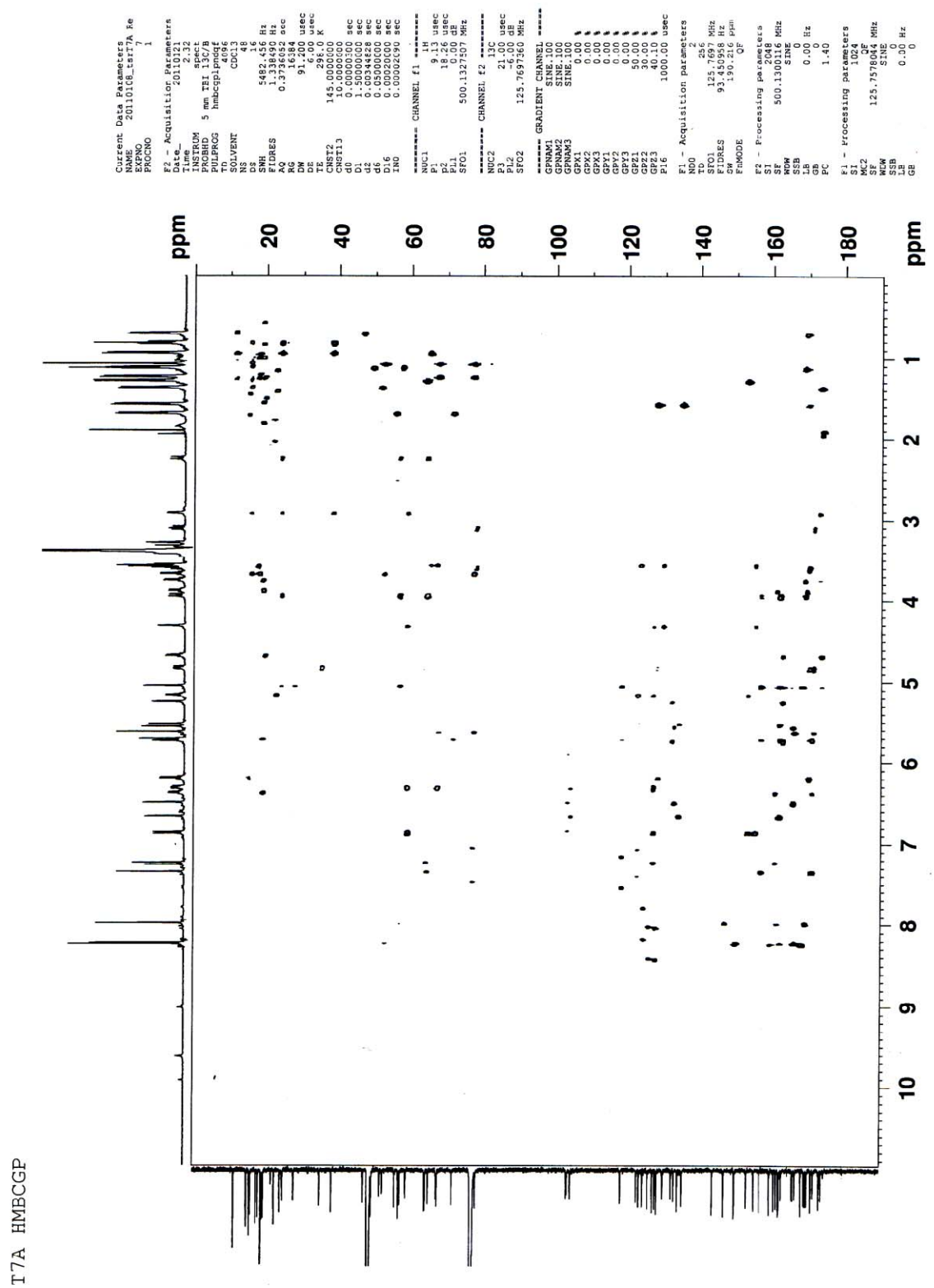


Figure S9. gHMBC spectrum of thiostrepton Thr7Ala **3** (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

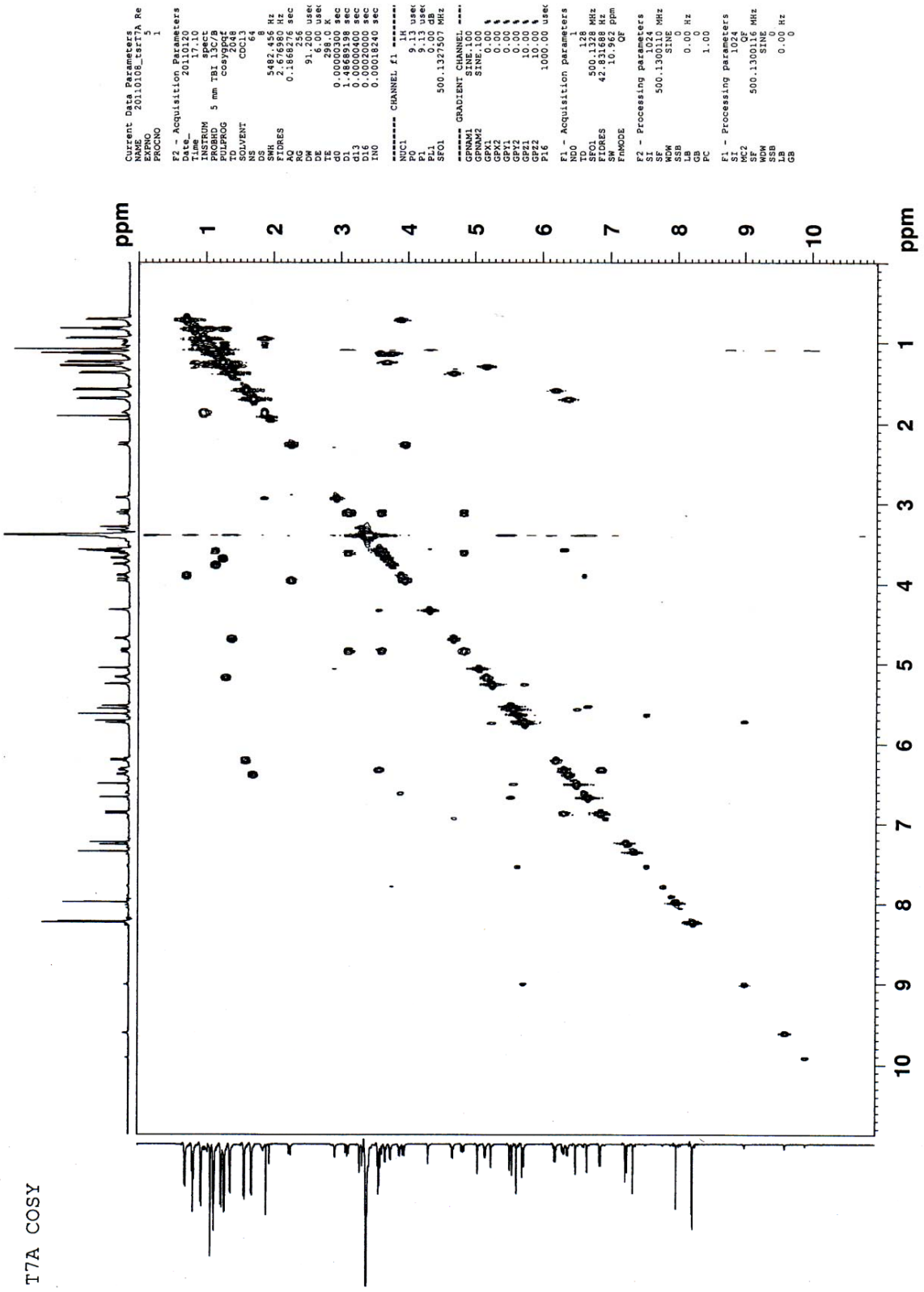


Figure S10. gCOSY spectrum of thiostrepton Thr7Ala **3** (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

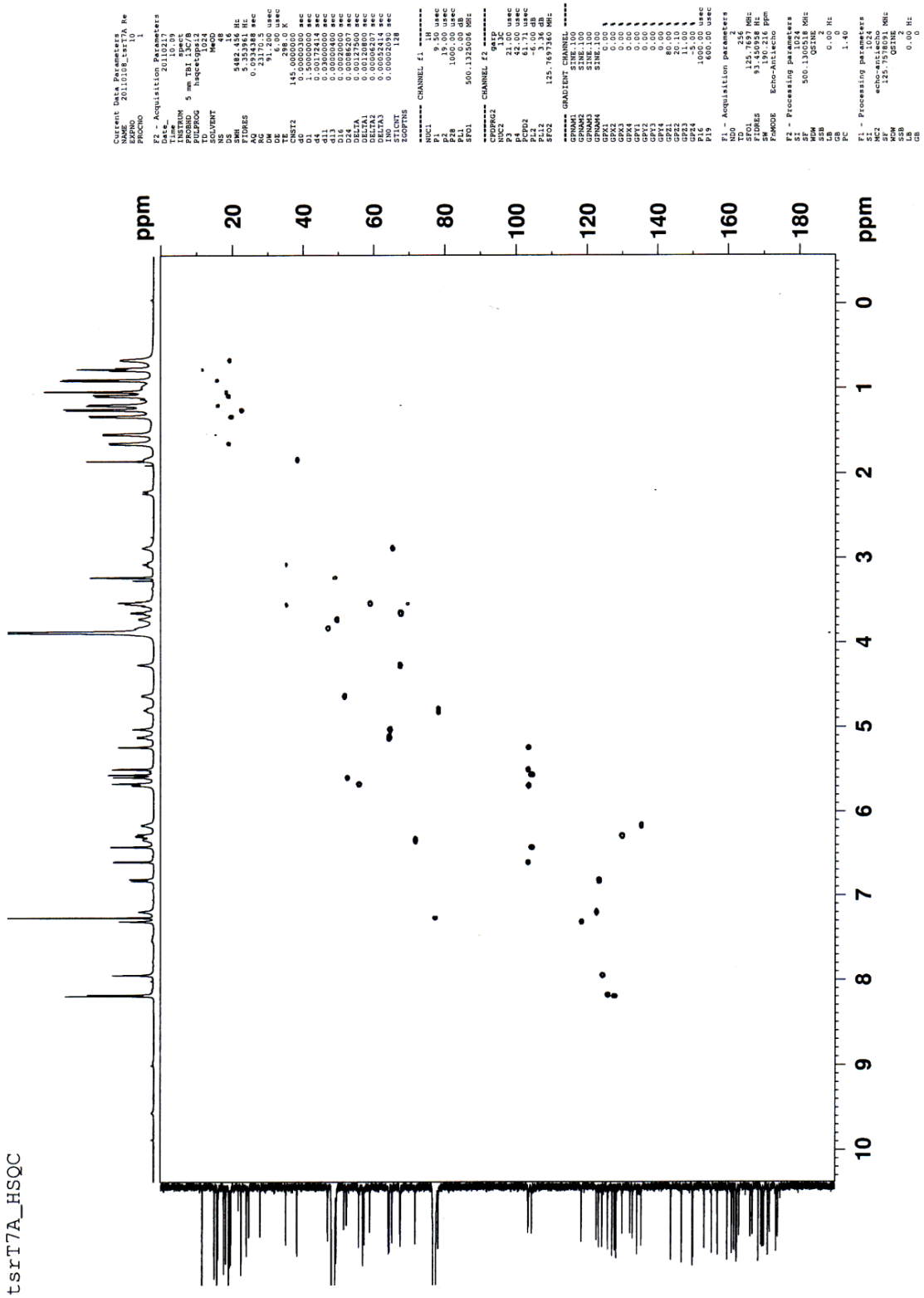


Figure S11. gHSQC spectrum of thiostrepton Thr7Ala **3** (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

Figure S12. HPLC-MS analysis of SL105-1 **4** isolated from *S. laurentii* NDS1 int-3A105 (TsrA Thr7Ala). (**A**) Total ion chromatogram. (**B**) Chromatogram extracted for *m*/*z* 718 of SL105-1 **4**. (**C**) Mass spectrum of SL105-1 **4** eluting at t_R = 23.7 min.

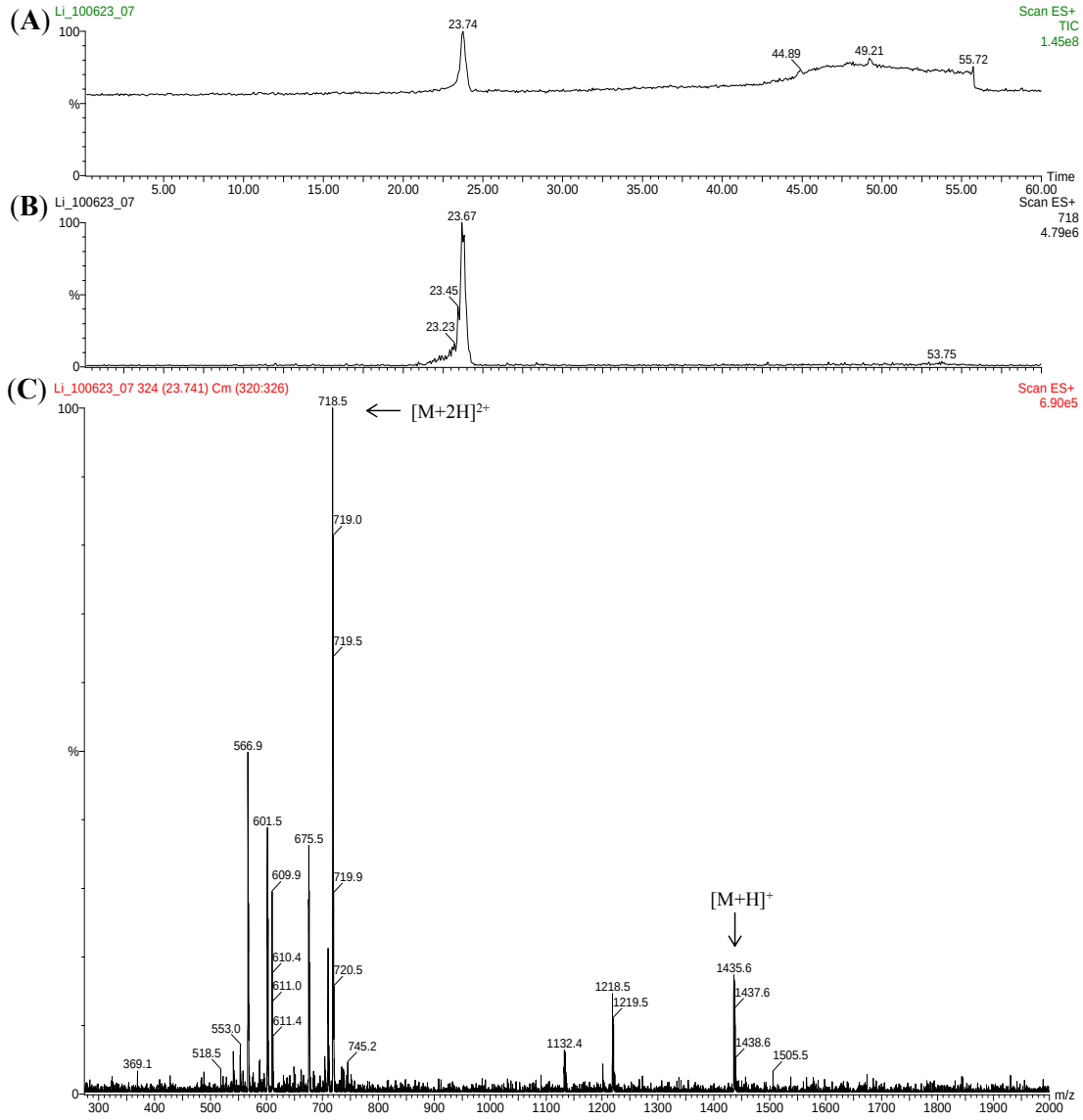
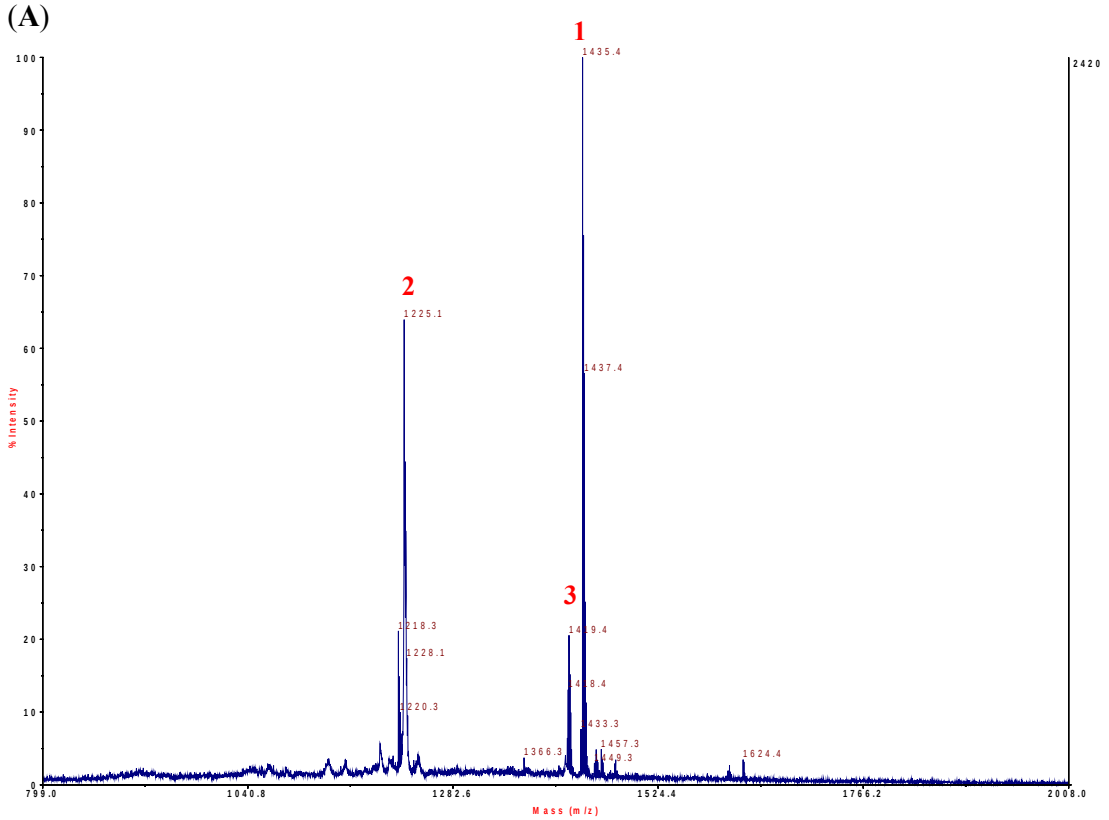
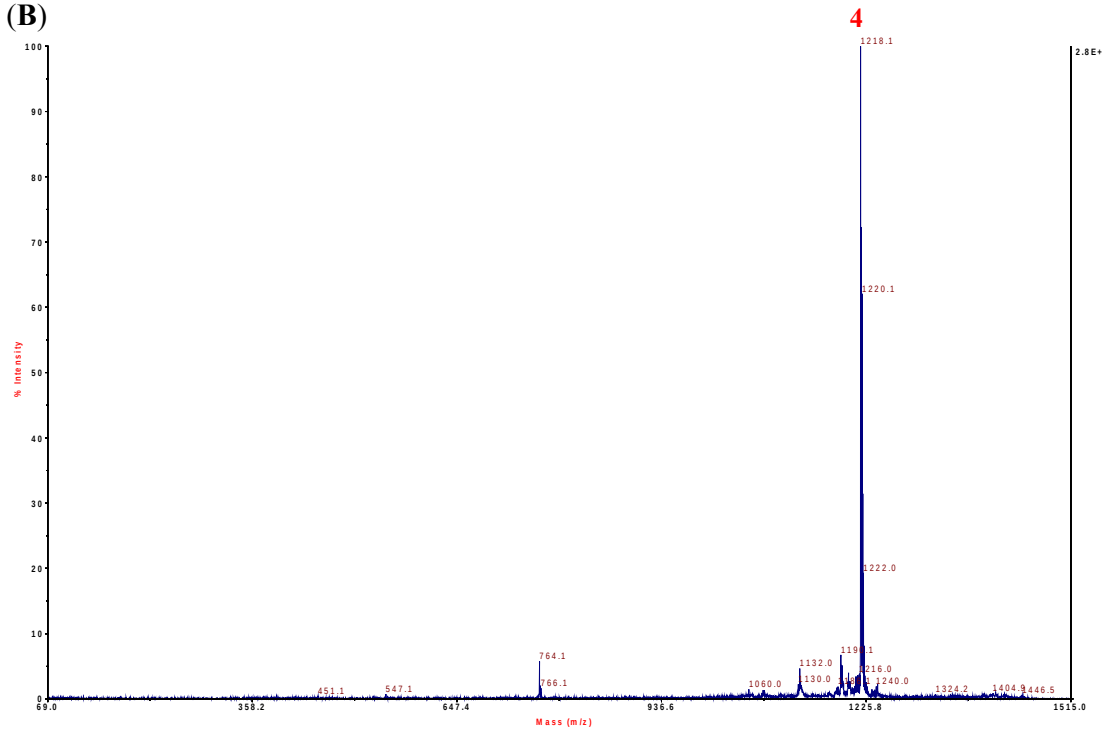


Figure S13. MALDI MS and MS/MS analysis of SL105-1 **4**. (**A**) Full spectrum of MALDI MS of SL105-1 **4**. (**B**) MALDI MS/MS of parent ion *m/z* 1435.4. (**C**) Table and structure showing fragments in MALDI MS/MS of SL105-1 **4**.

(**A**)

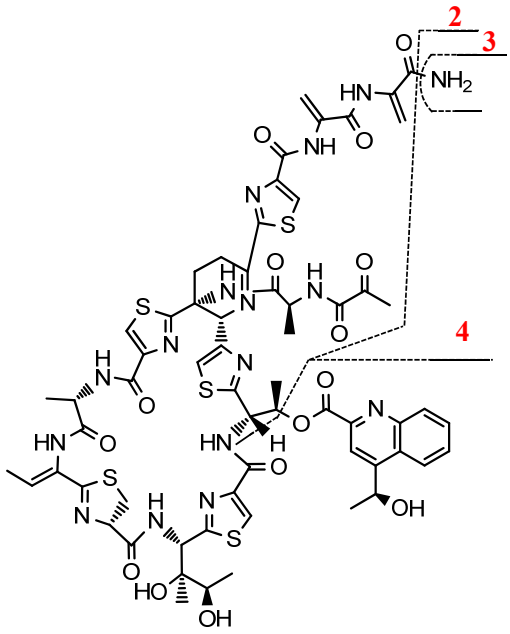


(**B**)



(C)

Fragment	Expected	Observed
1. $M+H^+$ (parent ion)	1435.3	1435.4
2. $M-NH_2-QA+Na^+$	1225.3	1225.1
3. $M-NH_2+H^+$	1419.3	1419.4
4. $M-QA+H^+$	1218.3	1218.1



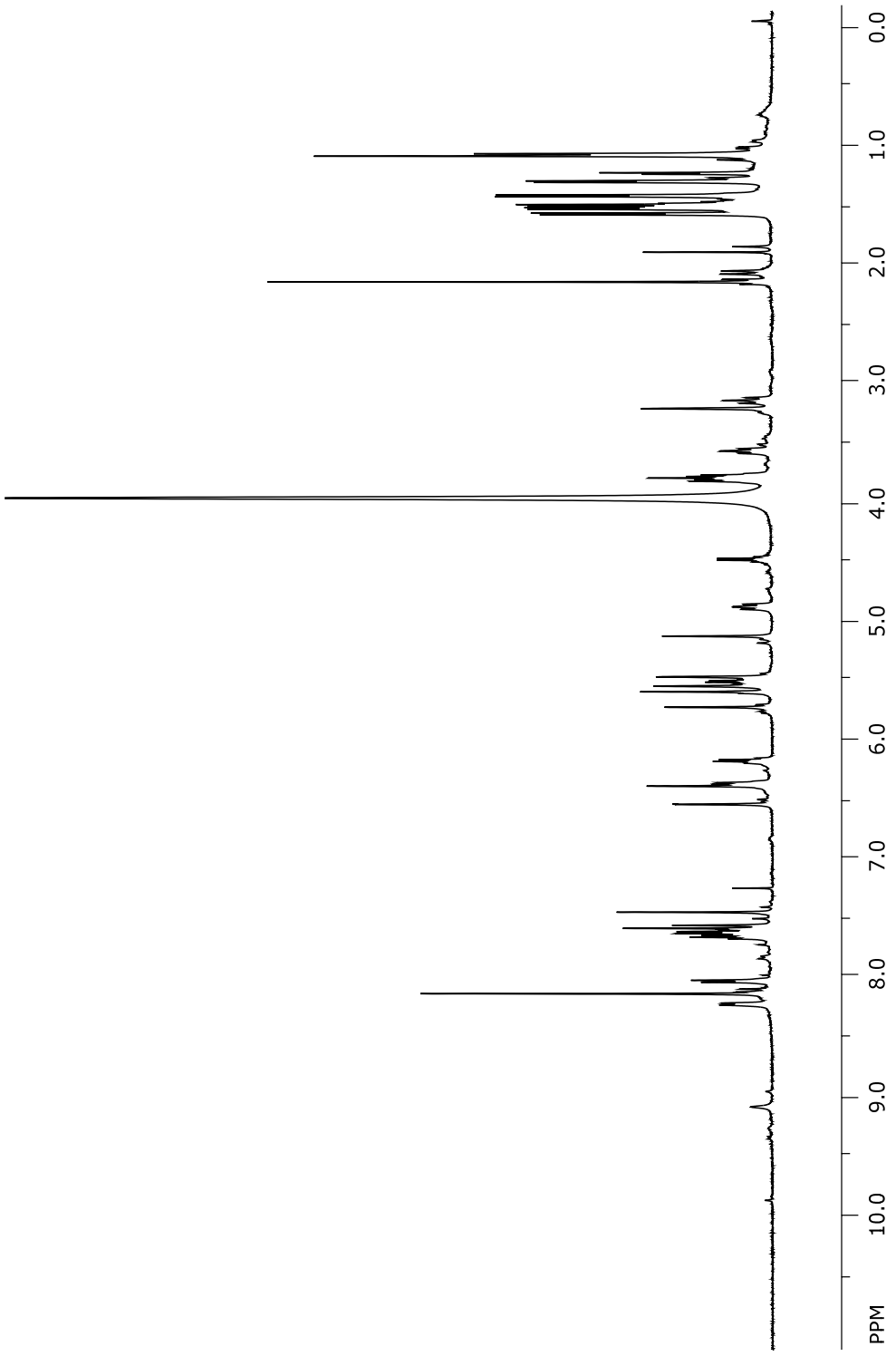


Figure S14. ^{1}H NMR spectrum of SL105-1 **4** (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

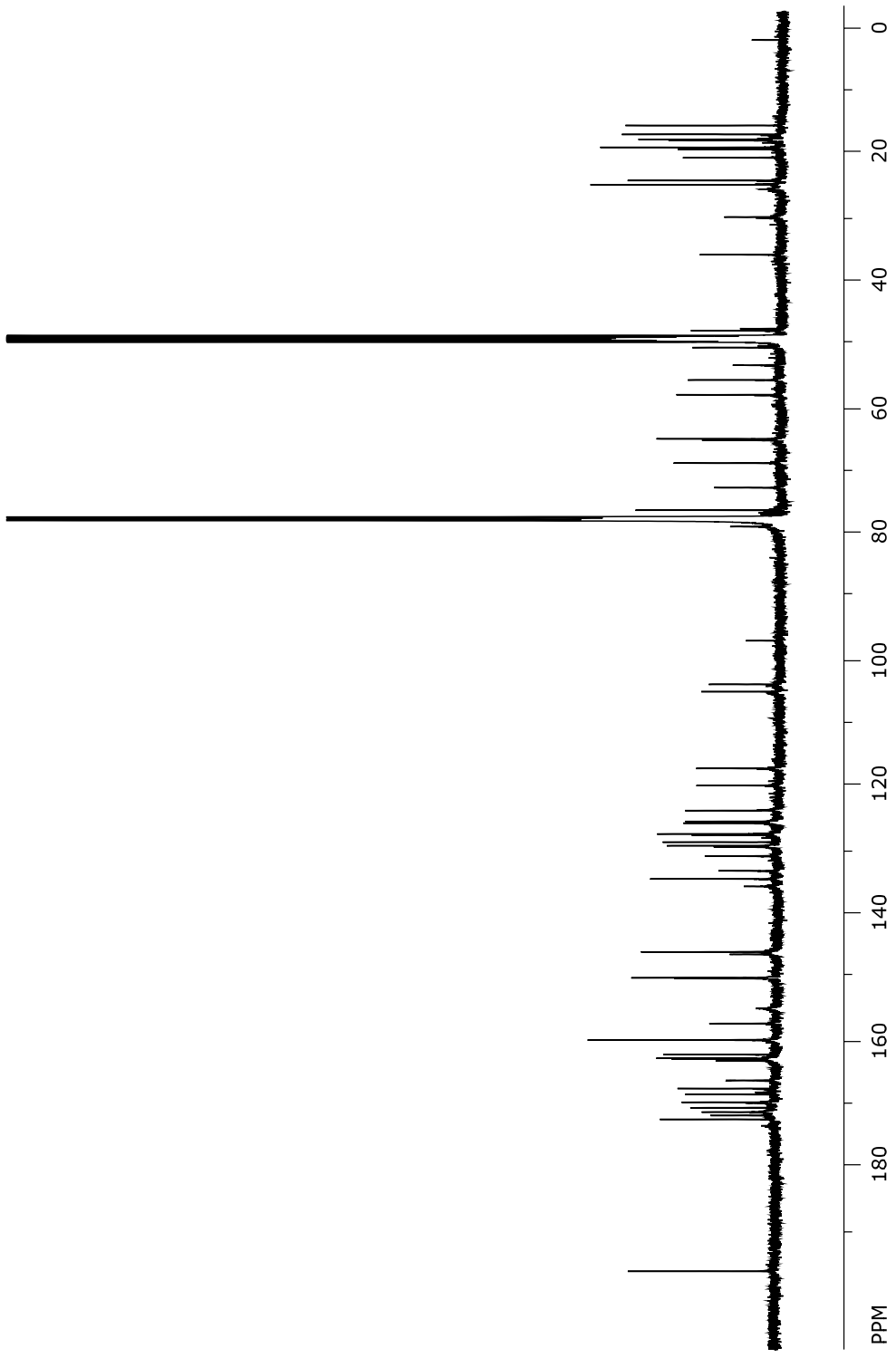


Figure S15. ^{13}C NMR spectrum of SL105-1 **4** (125 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

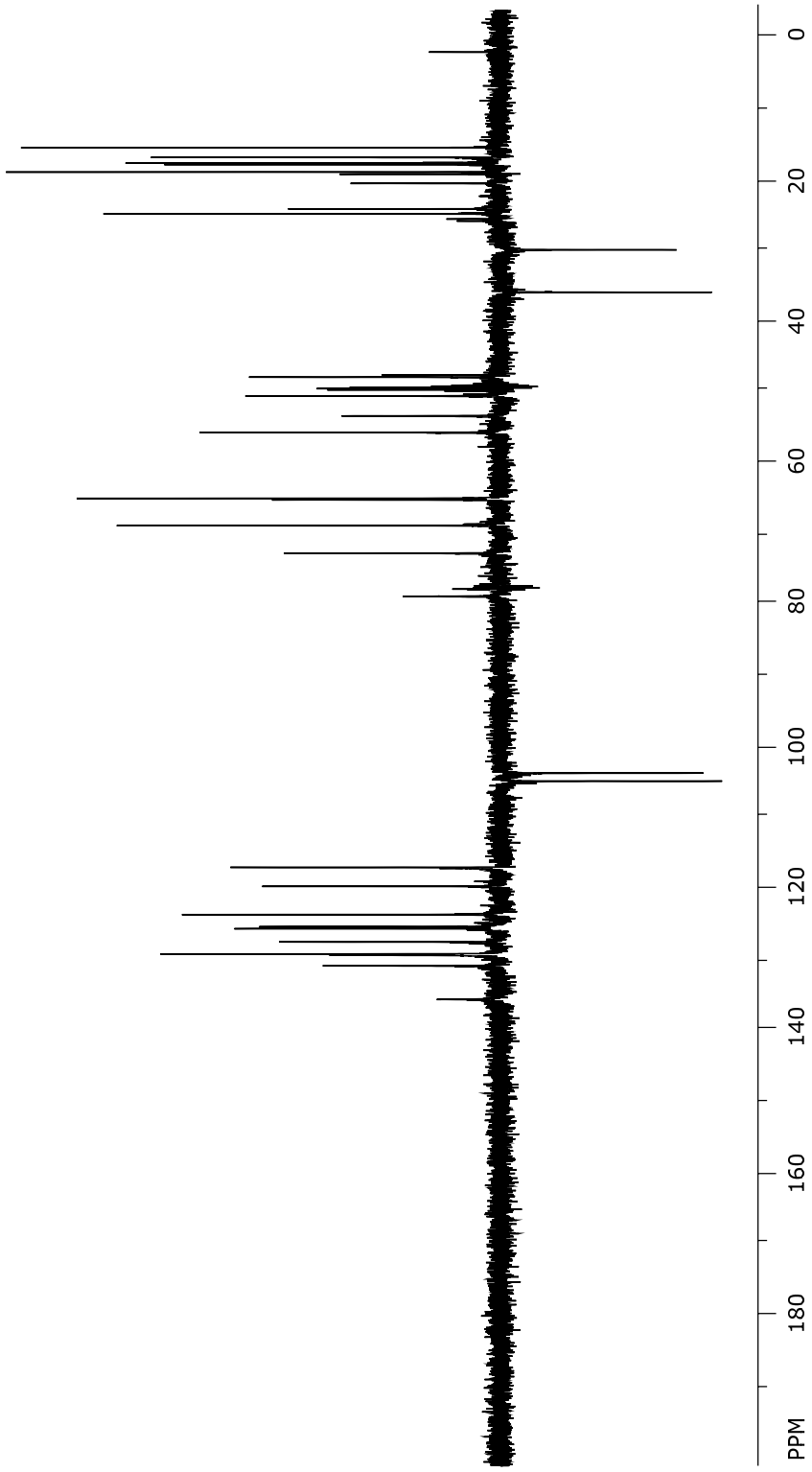


Figure S16. DEPT-135 NMR spectrum of SL105-1 **4** (125 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

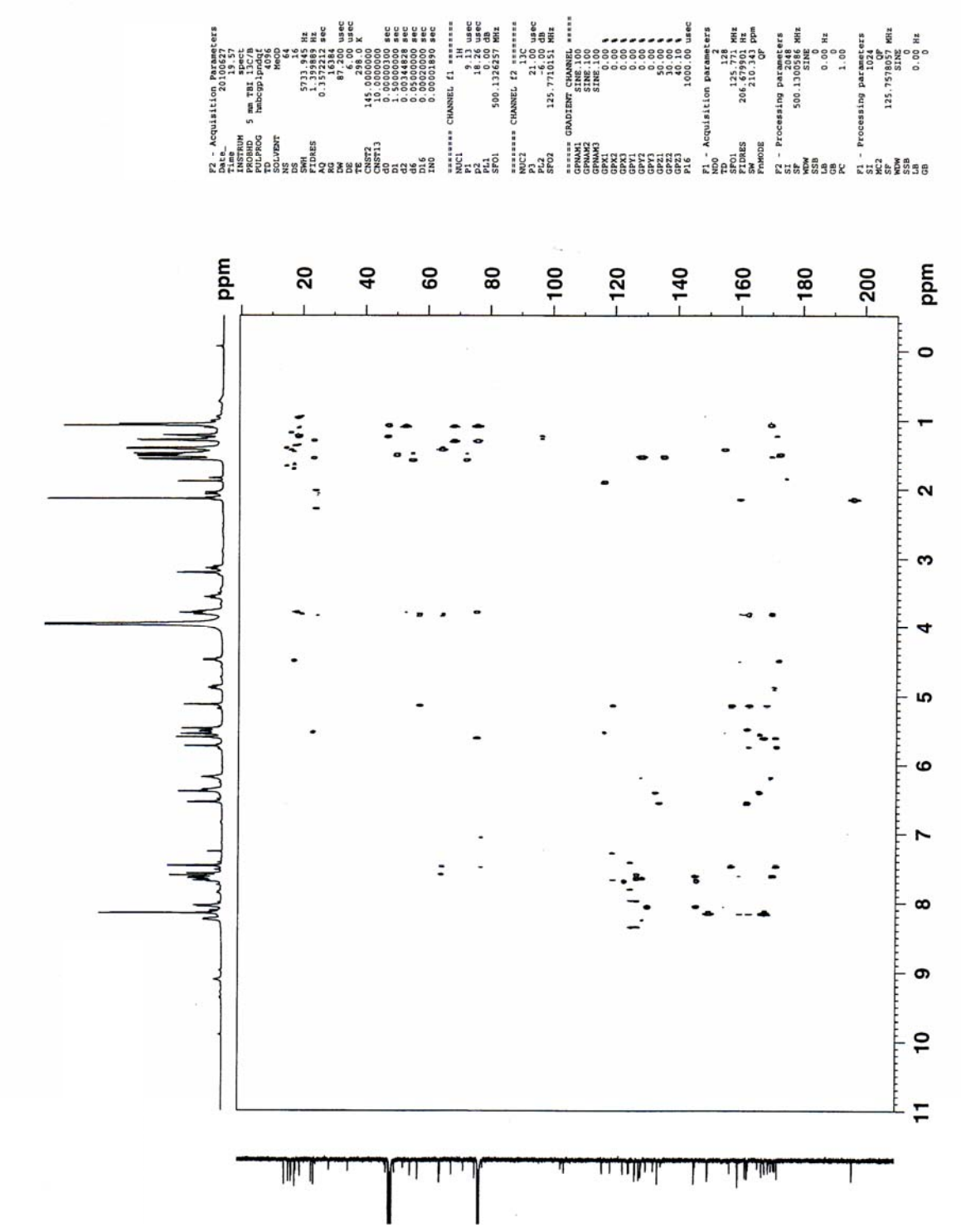


Figure S17. gHMBC spectrum of SL105-1 **4** (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

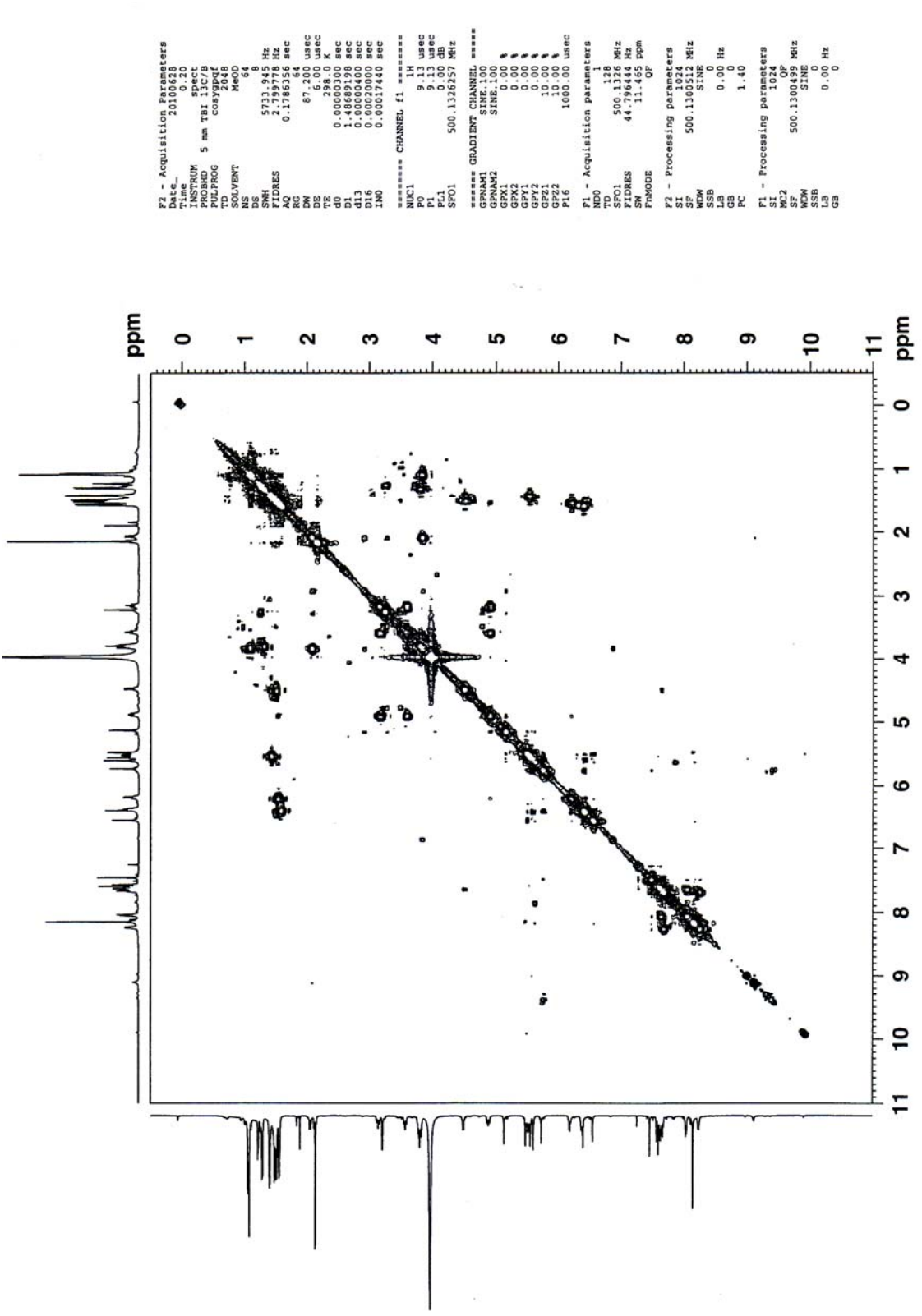


Figure S18. gCOSY spectrum of SL105-1 **4** (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

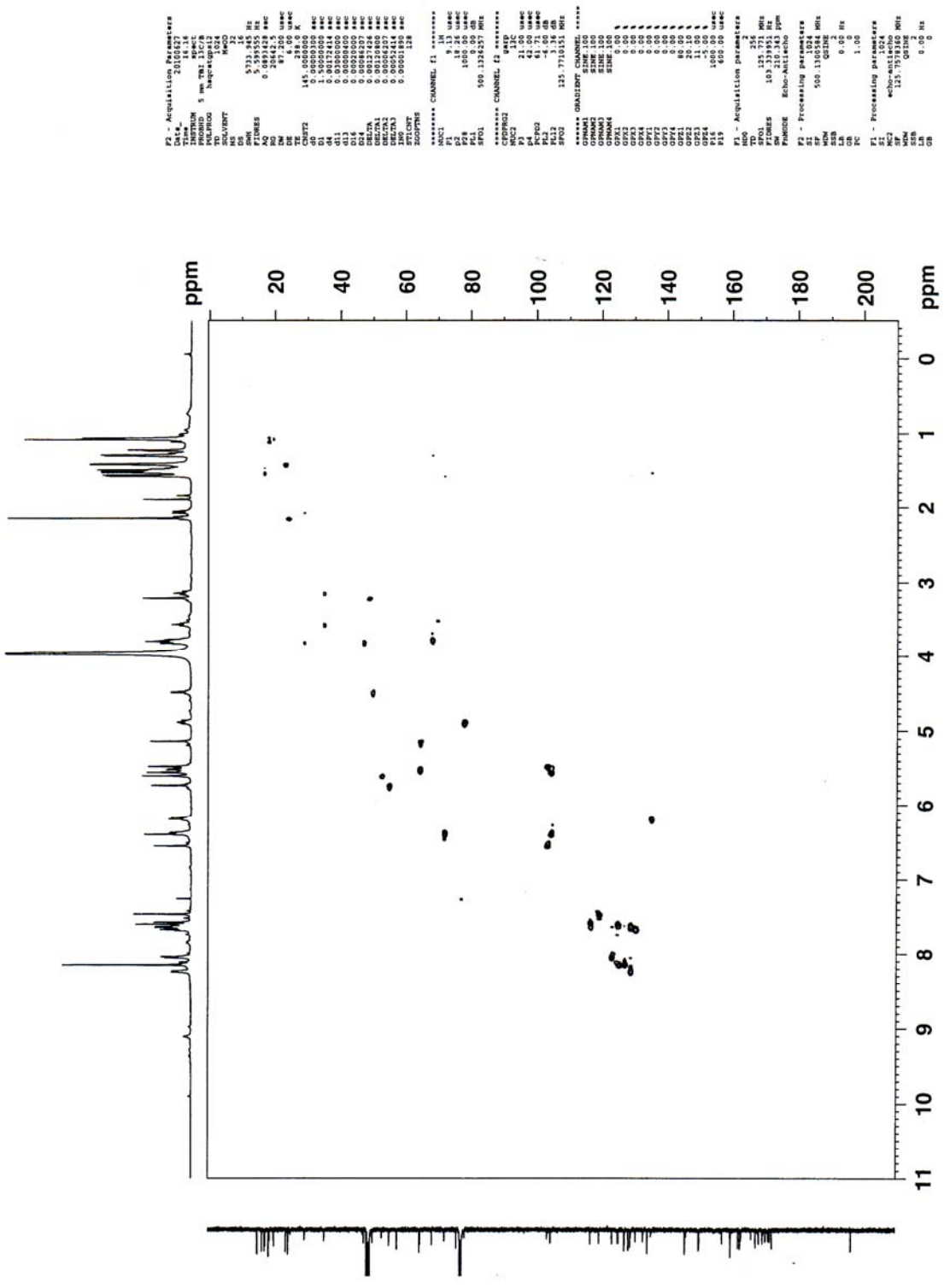


Figure S19. gHSQC spectrum of SL105-1 **4** (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C).

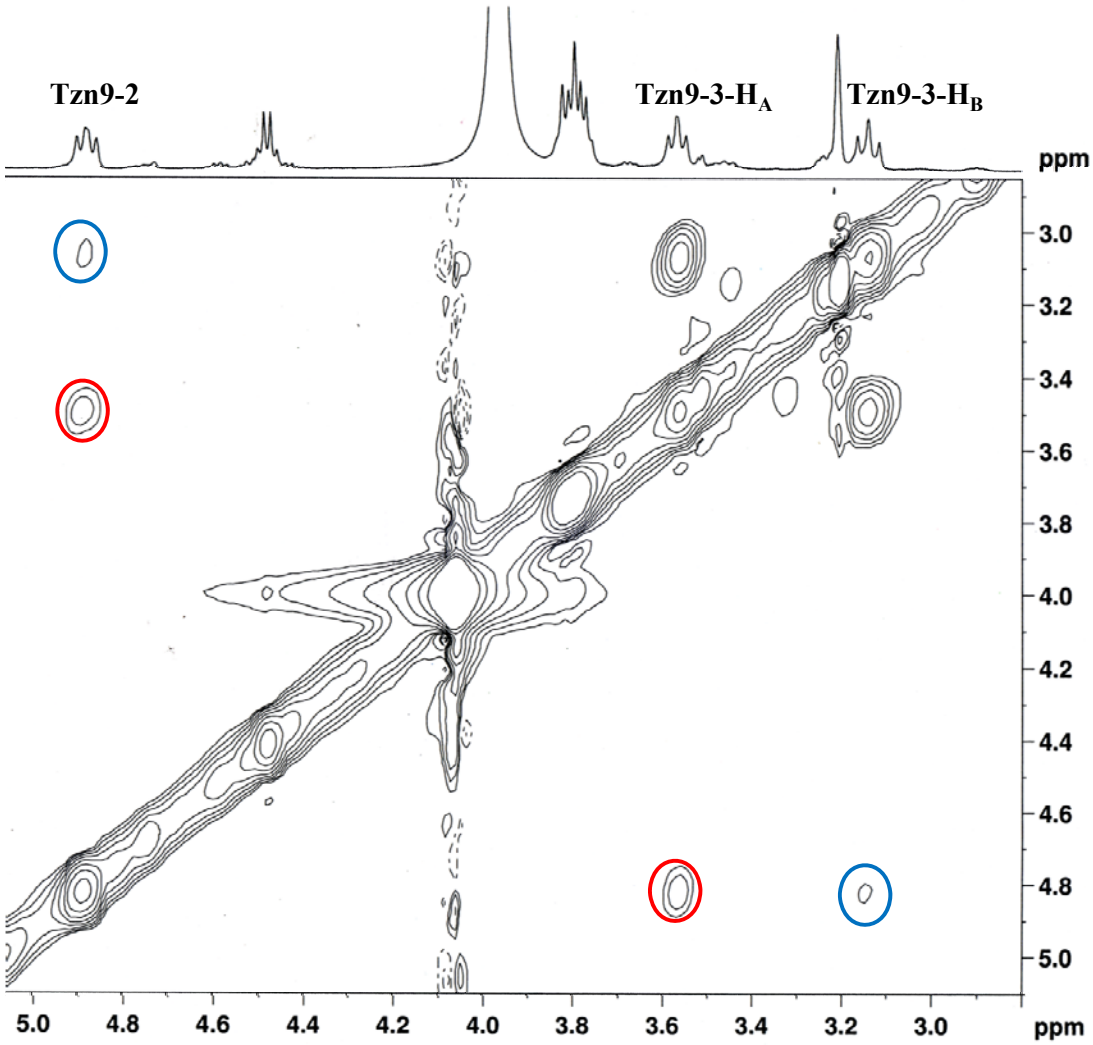


Figure S20. NOESY cross peaks of the thiazoline region of SL105-1 **4** (500 MHz, $CDCl_3$-CD_3OD 4:1, 25 °C). The cross peaks of Tzn9-2 and Tzn9-3-H_A protons are circled in red. The cross peaks of Tzn9-2 and Tzn9-3-H_B protons are circled in blue. Schoof and Arndt observed that the NOE signals between the Tzn9-2 and Tzn9-3-H_A protons in **1** were of greater intensity than the signals for the Tzn9-2 and Tzn9-3-H_B protons.[7]

Figure S21. HPLC-MS analysis of thiostrepton Thr7Val **5** isolated from *S. laurentii* NDS1 int-3A106 (TsrA Thr7Val). (**A**) Total ion chromatogram. (**B**) Chromatogram extracted for *m*/*z* 831.8 of thiostrepton Thr7Val **5**. (**C**) Mass spectrum of thiostrepton Thr7Val **5** eluting at t_R = 23.6 min.

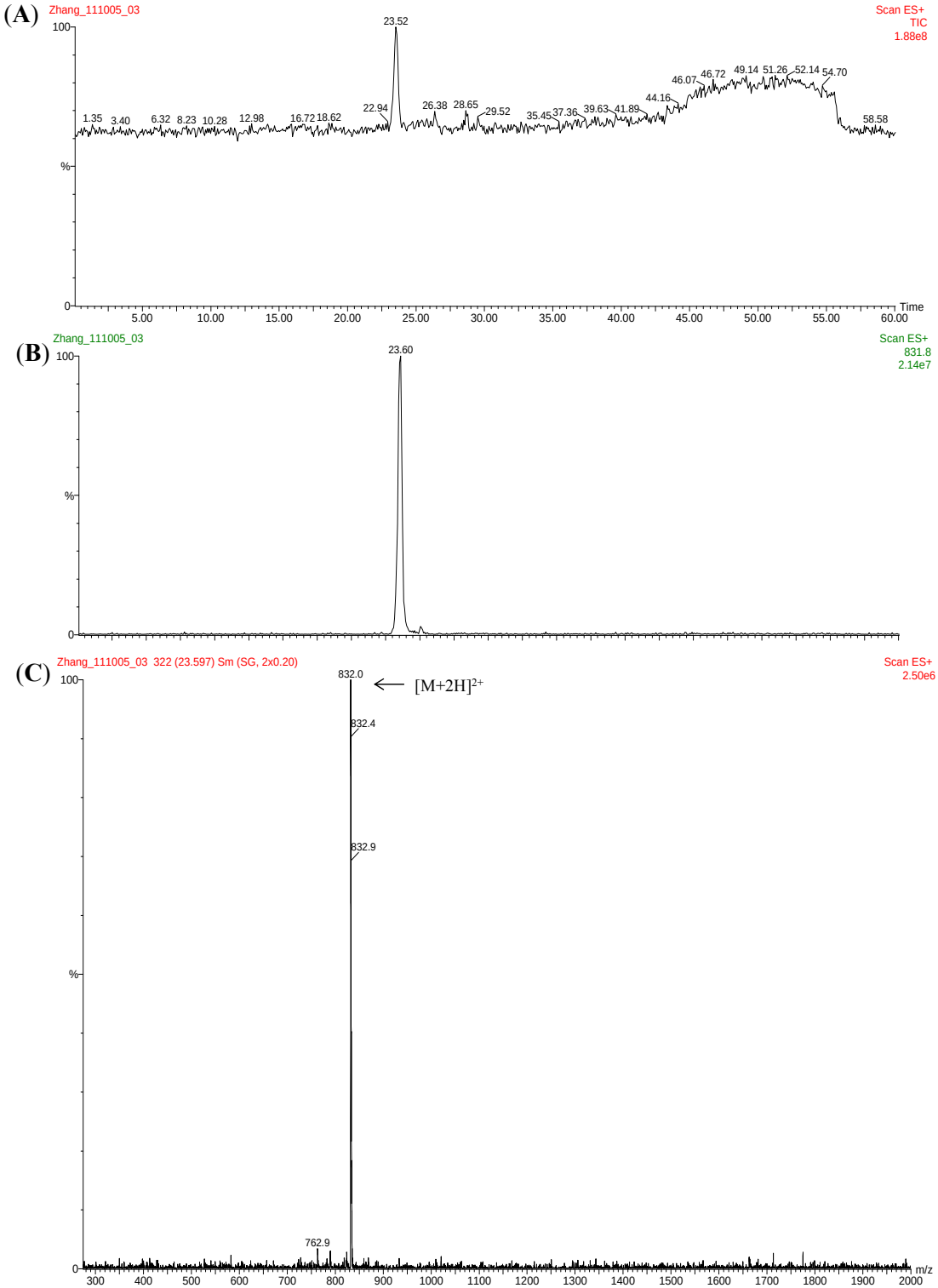
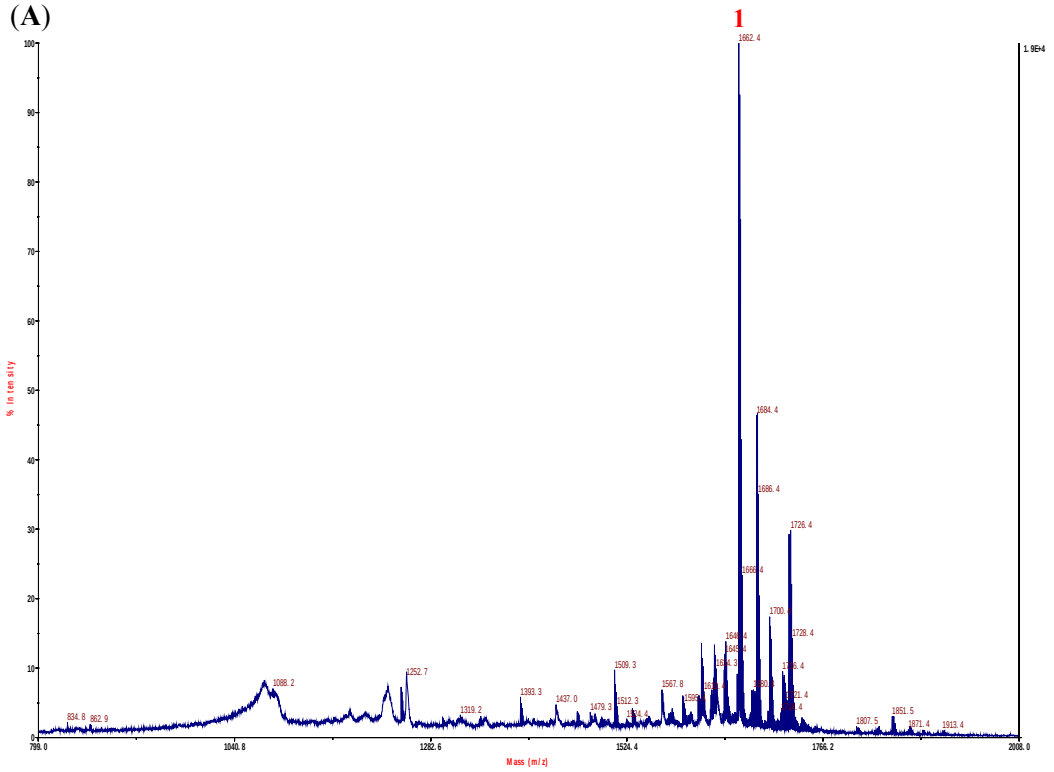
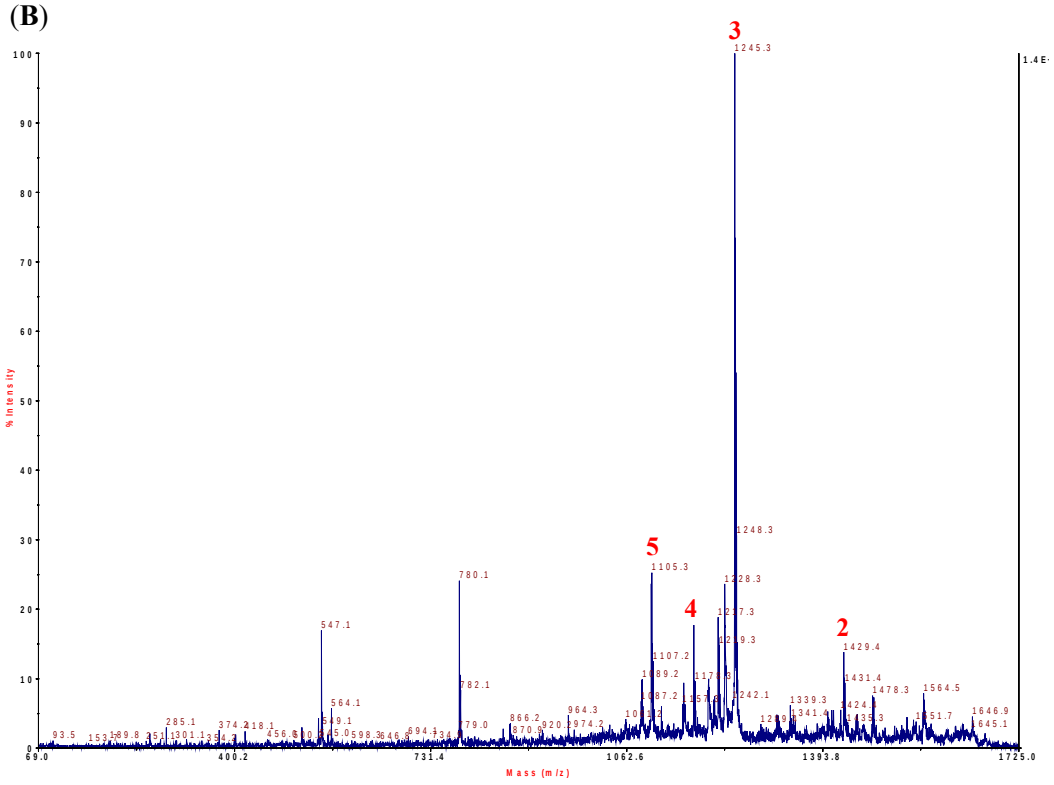


Figure S22. MALDI MS and MS/MS analysis of thiostrepton Thr7Val **5**. (**A**) Full spectrum of MALDI MS of thiostrepton Thr7Val **5**. (**B**) MALDI MS/MS of parent ion *m/z* 1662.4. (**C**) Table and structure showing fragments in MALDI MS/MS of thiostrepton Thr7Val **5**.

(A)



(B)



(C)

Fragment	Expected	Observed
1. $M+H^+$ (Parent ion)	1662.5	1662.4
2. $M-QA+H^+$	1429.4	1429.4
3. $M-QA-Ile1-Ala2+H^+$	1245.3	1245.3
4. $M-QA-Ile1-Ala2-Dha3+H^+$	1176.3	1176.3
5. $M-QA-Ile1-Ala2-Dha3-Ala4+H^+$	1105.2	1105.3

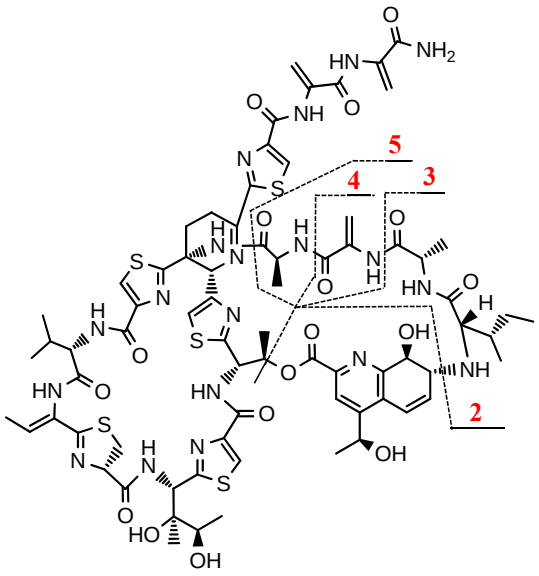


Figure S23. HPLC-MS analysis of SL106-1 **6** isolated from *S. laurentii* NDS1 int-3A106 (TsrA Thr7Val). (**A**) Total ion chromatogram. (**B**) Chromatogram extracted for *m*/*z* 732.2 of SL106-1 **6**. (**C**) Mass spectrum of SL106-1 **6** eluting at t_R = 25.1 min.

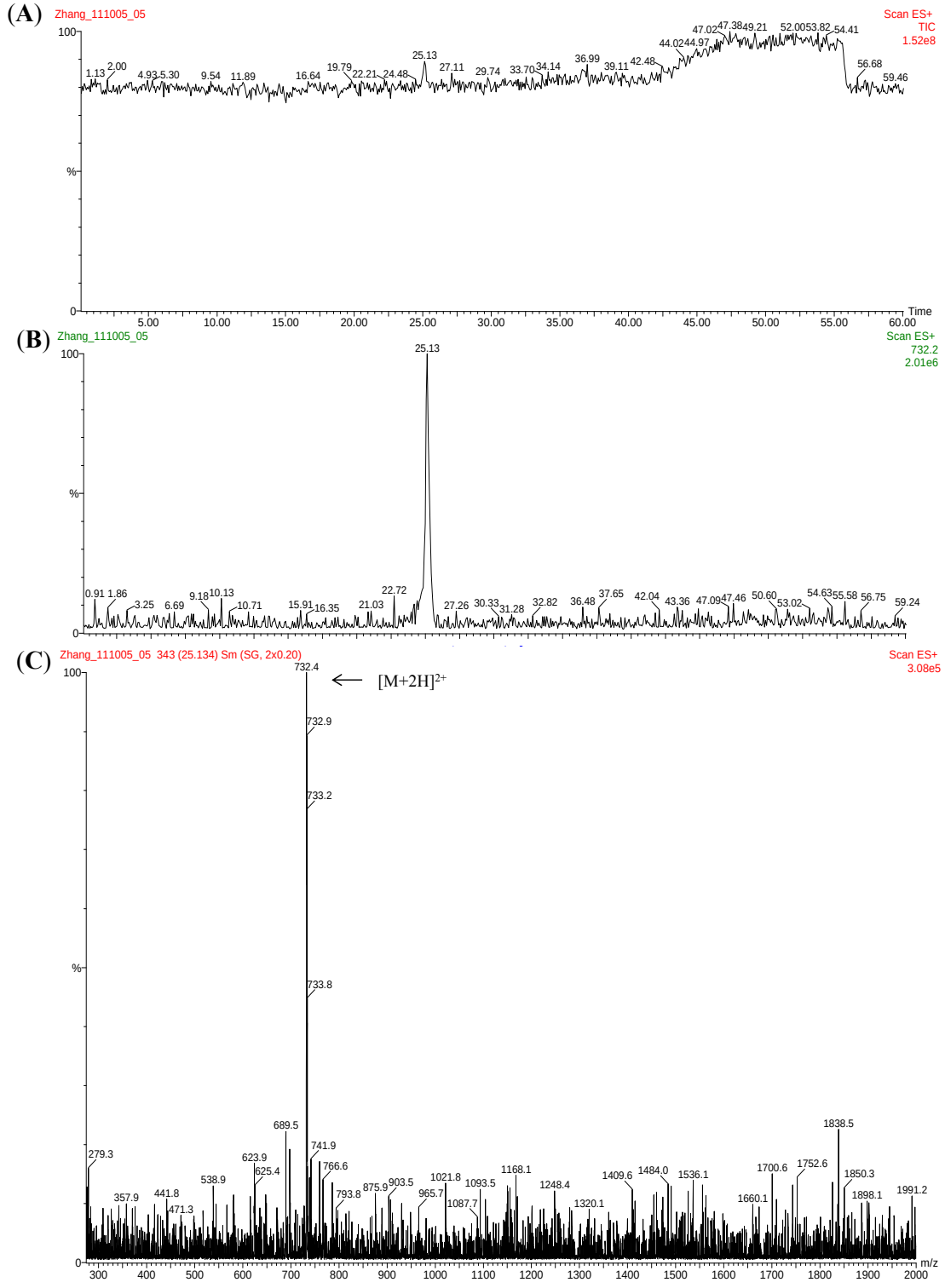
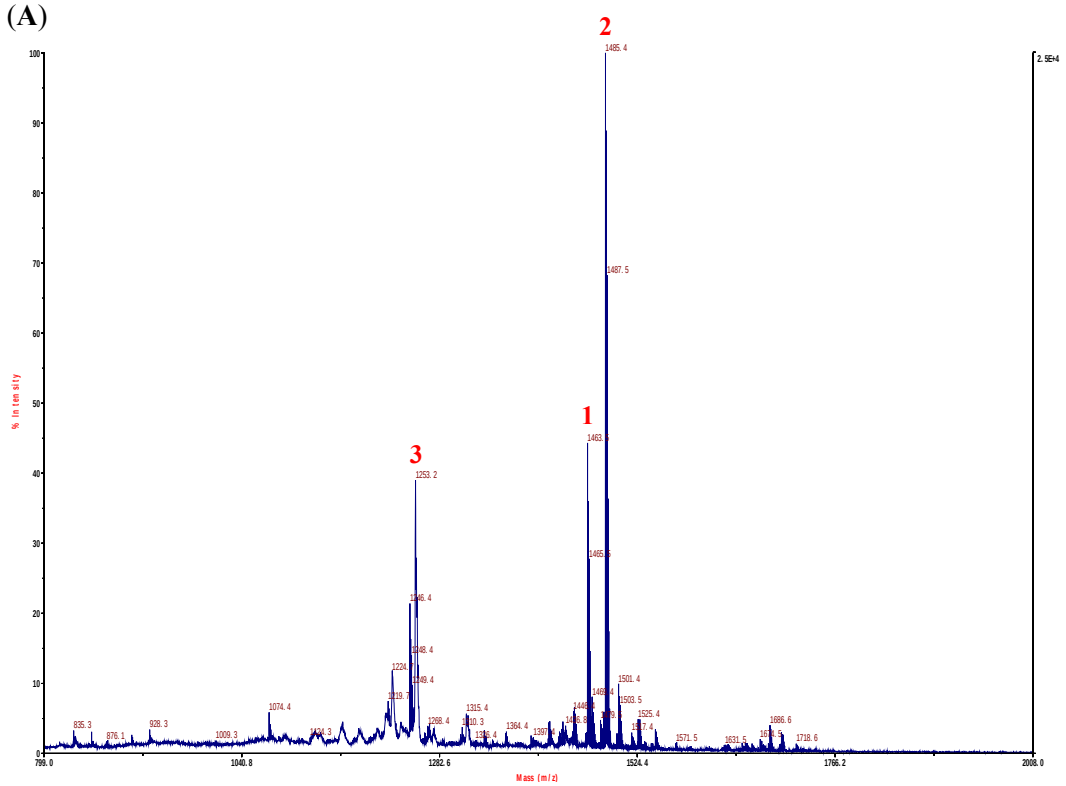
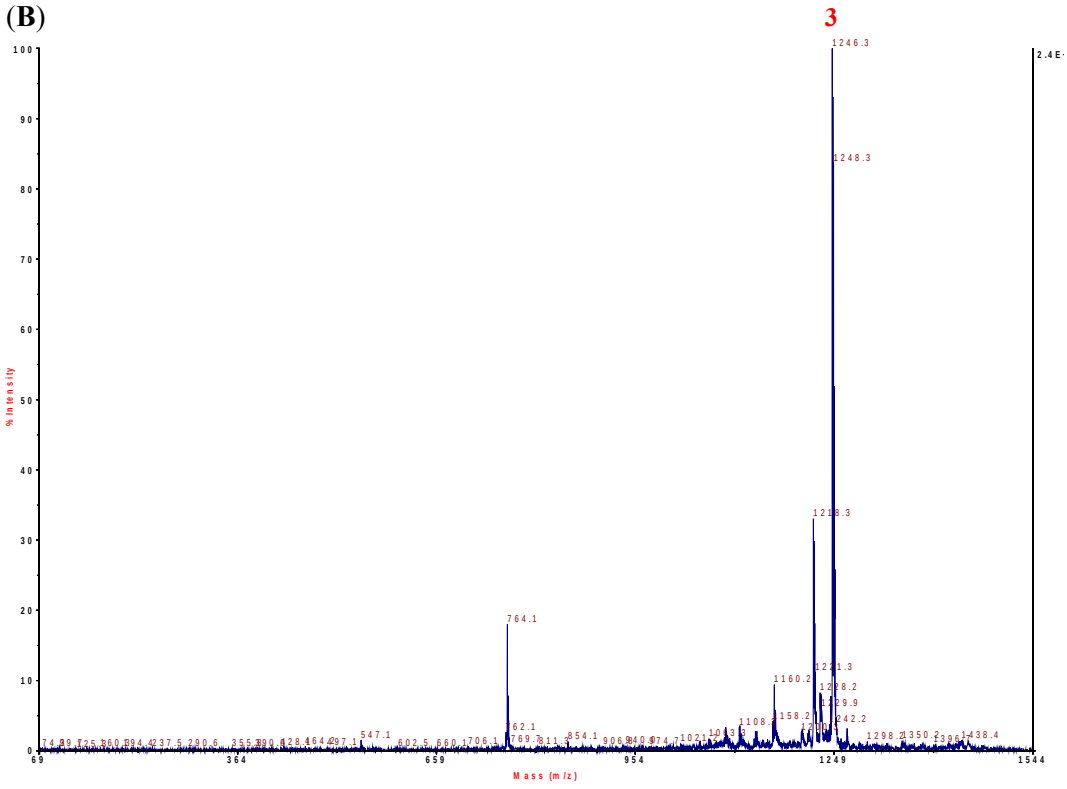


Figure S24. MALDI MS and MS/MS analysis of SL106-1 **6**. (**A**) Full spectrum of MALDI MS of SL106-1 **6**. (**B**) MALDI MS/MS of parent ion *m/z* 1463.5. (**C**) Table and structure showing fragments in MALDI MS/MS of SL106-1 **6**.

(**A**)



(**B**)



(C)

Fragment	Expected	Observed
1. $M+H^+$ (parent ion)	1463.4	1463.5
2. $M+Na^+$	1485.4	1485.4
3. $M-NH_2$-QA+Na^+	1253.3	1253.2
4. M-QA+H^+	1246.3	1246.3

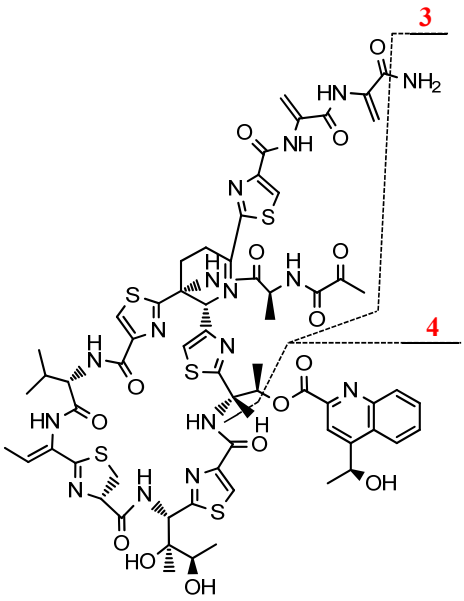


Table S1. ^{1}H and ^{13}C NMR assignments of thiostrepton Thr7Ala **3**

Position	δ_C [ppm]; mult	δ_H [ppm]; (mult, J in Hz)	HMBC[a]	COSY[b]
Ile1				
Ile1-1	173.1; C q			
Ile1-2	65.2; CH	2.92 (d, 4.0)	Ile1-1, Ile1-3, Ile1-4, Ile1-6, Q8	Ile1-3
Ile1-3	38.2; CH	1.88-1.82 (m)		Ile1-4-H_B, Ile1-6
Ile1-4	23.9; CH_2	H_A: 1.32-1.26 (m)	Ile1-5, Ile1-6	Ile1-4-H_B, Ile1-5
		H_B: 1.04-0.98 (m)	Ile-5, Ile1-6	Ile1-3, Ile1-4-H_A, Ile1-5
Ile1-5	11.4; CH_3	0.82 (t, 7.3)	Ile1-3, Ile1-4	Ile1-4-H_A, Ile1-4-H_B
Ile1-6	15.7; CH_3	0.94 (d, 6.9)	Ile1-2, Ile1-3, Ile1-4	Ile1-3
Ala2				
Ala2-1	169.0; C q			
Ala2-2	49.4; CH	3.75 (q, 6.8)	Ile1-1, Ala2-1, Ala2-3	Ala2-3
Ala2-3	18.9; CH_3	1.13 (d, 6.7)	Ala2-1, Ala2-2	Ala2-2
Ala2-NH[d]		7.79 (d, 5.9)		Ala2-2
Dha3				
Dha3-1	162.8; C q			
Dha3-2	132.1; C q			
Dha3-3	103.4; CH_2	H_A: 5.74 (d, 2.0)	Dha3-1, Dha3-2	Dha3-3-H_B
		H_B: 5.25 (d, 2.0)	Dha3-1, Dha3-2	Dha3-3-H_A
Ala4				
Ala4-1	173.7; C q			
Ala4-2	51.6; CH	4.68 (q, 6.6)	Dha3-1Ala4-1, Ala4-3	Ala4-3
Ala4-3	19.5; CH_3	1.37 (d, 6.7)	Ala4-1, Ala4-2	Ala4-2
Ala4-NH[d]		6.93 (d, 7.6)		Ala4-2
Pip				
Pip-2	162.2; C q			
Pip-3	23.9; CH_2	H_A: 3.63-3.55 (m)		
		H_B: 2.89-2.85 (m)		Pip-4-H_B
Pip-4	27.8; 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: 2.25 (m)	Pip-3, Pip-5, Pip-6	Pip-4-H_A, Pip-3-H_B
Pip-5	56.9; C q			
Pip-6	64.4; CH	5.05 (s)	Ala4-1, Pip-2, Pip-3, Pip-4, Pip-5, Thz13-2, Thz13-3, Thz15-4	
Thz6				
Thz6-1	161.4; C q			
Thz6-2	146.8; C q			
Thz6-3	124.2; CH	7.99 (s)	Thz6-1, Thz6-2, Thz6-4	
Thz6-4	169.2; C q			
Ala7				
Ala7-1	169.6; C q			
Ala7-2	47.0; CH	3.89 (q, 7.0)	Thz6-1, Ala7-1, Ala7-3	Ala7-3, Ala7-NH
Ala7-3	19.1; CH_3	0.70 (d, 7.2)	Ala7-1, Ala7-2	Ala7-2
Ala7-NH[d]		6.61 (d, 9.5)		Ala7-2
Dhb8				
Dhb8-2	128.2; C q			
Dhb8-3	135.2; CH	6.20 (q, 7.0)	Dhb8-2, Dhb8-4, Tzn9-4	Dhb8-4
Dhb8-4	14.9; CH_3	1.58 (d, 7.0)	Dhb8-2, Dhb8-3, Tzn9-4	Dhb8-3
Tzn9				
Tzn9-1	171.4; C q			
Tzn9-2	78.1; CH	4.83 (dd, 13.1, 8.9)	Dhb8-2, Tzn9-1, Tzn9-3, Tzn9-4	Tzn9-3-H_A, Tzn9-3-H_B
Tzn9-3	35.1; CH_2	H_A: 3.63-3.55 (m)	Tzn9-2, Tzn9-4	Tzn9-2, Tzn9-3-H_B
		H_B: 3.11 (dd, 13.1, 11.6)	Tzn9-1, Tzn9-2	Tzn9-2, Tzn9-3-H_A
Tzn9-4	169.8; C q			
Ile10				
Ile10-2	52.4; CH	5.63 (s)	Tzn9-1, Ile10-3, Ile10-4, Thz11-4	Ile10-NH
Ile10-3	77.0[c]			
Ile10-4	67.6; CH	3.68 (q, 6.4)	Ile10-2, Ile10-3, Ile10-5, Ile10-6	Ile10-5
Ile10-5	15.9; CH_3	1.24 (d, 6.4)	Ile10-3, Ile10-4, Ile10-6	Ile10-4
Ile10-6	18.1; CH_3	1.08 (s)	Ile10-2, Ile10-3, Ile10-4	
Ile10-NH[d]		7.54 (d, 9.6)		Ile10-2

Position	δ_C [ppm]; mult	δ_H [ppm]; (mult, J in Hz)	HMBC[a]	COSY[b]
Thz11				
Thz11-1	162.1; C q			
Thz11-2	150.0; C q			
Thz11-3	125.8; CH	8.22 (s)	Ile10-2, Thz11-1, Thz11-2, Thz11-4	
Thz11-4	166.5; C q			
Thr12				
Thr12-2	55.8; CH	5.72 (s)	Thz11-1, Thr12-3, Thr12-4, Thz13-2, Thz13-3, Thz13-4	Thr12-NH
Thr12-3	71.7; CH	6.38 (q, 6.6)	Thr12-4, Thz13-4, Q-1	Thr12-4
Thr12-4	18.9; CH_3	1.69 (d, 6.6)	Thr12-2, Thr12-3	Thr12-3
Thr12-NH[d]		9.01 (d, 7.6)		Thr12-2
Thz13				
Thz13-2	156.8; C q			
Thz13-3	118.3; CH	7.35 (s)	Pip-6, Thz13-2, Thz13-4	
Thz13-4	170.8; C q			
Thz15				
Thz15-1	159.5; C q			
Thz15-2	149.8; C q			
Thz15-3	127.7; CH	8.25 (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: 6.67 (d, 2.3)	Dha16-1, Dha16-2	Dha16-3-H_B
		H_B: 5.53 (d, 2.4)	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: 6.50 (d, 1.9)	Dha17-1, Dha17-2	Dha17-3-H_B
		H_B: 5.57 (d, 1.9)	Dha17-1, Dha17-2	Dha17-3-H_A
Q				
Q-1	160.8; C q			
Q-2	143.7; C q			
Q-3	122.5; CH	7.22 (s)	Q-1, Q-5, Q-11	
Q-4	153.1; C q			
Q-5	127.0; C q			
Q-6	123.2; CH	6.86 (d, 10.1)	Q-4, Q-5, Q-8, Q-10	Q-7
Q-7	129.9; CH	6.32 (dd, 9.7, 5.5)	Q-5, Q-8, Q-9	Q-6, Q-8
Q-8	58.9; CH	3.63-3.55 (m)	Q-6, Q-7, Q-9, Q-10	Q-7, Q-9
Q-9	67.4; CH	4.32 (s)	Q-5, Q-7, Q-8, Q-10	Q-8
Q-10	155.1; C q			
Q-11	64.2; CH	5.17 (q, 6.5)	Q-3, Q-4, Q-5, Q-12	Q-12
Q-12	22.4; CH_3	1.29 (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 $CDCl_3$ peak.
[d] Only those amide resonances demonstrating COSY correlations to neighboring protons were assigned.

Table S2. ^{1}H and ^{13}C NMR assignments of SL105-1 **4**

Position	δ_C [ppm]; mult	δ_H [ppm]; (mult, J in Hz)	HMBC[a]	COSY[b]
Pyr3				
Pyr3-1	159.6; C q			
Pyr3-2	196.3; C q			
Pyr3-3	24.0; CH_3	2.14 (s)	Pyr3-1, Pyr3-2	
Ala4				
Ala4-1	172.2; C q			
Ala4-2	49.8; CH	4.48 (q, 7.2)	Ala4-1, Ala4-3	Ala4-3
Ala4-3	17.0; CH_3	1.49 (d, 7.2)	Ala4-1, Ala4-2	Ala4-2
Pip				
Pip-2	162.7; C q			
Pip-3	24.8[c]	H_A: 3.29 (m)		Pip-4-H_B
		H_B: 2.90 (m)		Pip-4-H_A, Pip-4-H_B
Pip-4	27.8; CH_2	H_A: 3.82-3.77 (m)	Pip-2, Pip-3, Pip-5, Pip-6	Pip-3-H_B, Pip-4-H_B
		H_B: 2.10-2.03 (m)	Pip-3	Pip-3-H_A, Pip-3-H_B, Pip-4-H_A
Pip-5	57.3; C q			
Pip-6	64.5; CH	5.13 (s)	Pip-2, Pip-5, Thz13-2, Thz13-3, Thz15-4	
Thz6				
Thz6-1	159.6; C q			
Thz6-2	145.7; C q			
Thz6-3	125.0; CH	7.60 (s)	Thz6-1, Thz6-2, Thz6-4	
Thz6-4	170.4; C q			
Ala7				
Ala7-1	169.5; C q			
Ala7-2	47.2; CH	3.82-3.77 (m)	Ala7-1, Ala7-3	
Ala7-3	19.8; CH_3	1.06 (d, 7.0)	Ala7-1, Ala7-2	
Ala7-NH[d]		6.84 (d, 7.5)		
Dhb8				
Dhb8-2	128.3; C q			
Dhb8-3	135.3; CH	6.18 (q, 6.9)	Dhb8-2, Tzn9-4	Dhb8-4
Dhb8-4	14.7; CH_3	1.52 (d, 6.9)	Dhb8-2, Dhb8-3, Tzn9-4	Dhb8-3
Tzn9				
Tzn9-1	171.4; C q			
Tzn9-2	78.1; CH	4.83 (dd, 12.0, 9.7)	Tzn9-1	Tzn9-3-H_A, Tzn9-3-H_B
Tzn9-3	35.1; CH_2	H_A: 3.57 (dd, 10.7, 9.2)		Tzn9-2, Tzn9-3-H_B
		H_B: 3.14 (t, 12.1)		Tzn9-2, Tzn9-3-H_A
Tzn9-4	169.6; C q			
Ile10				
Ile10-2	52.7; CH	5.60 (s)	Tzn9-1, Ile10-3, Thz11-4	Ile10-NH
Ile10-3	75.7[d]; C q			
Ile10-4	68.2; CH	3.82-3.77 (m)	Ile10-2, Ile10-3, Ile10-6	Ile10-5
Ile10-5	16.1; CH_3	1.29 (d, 6.3)	Ile10-3, Ile10-4	Ile10-4
Ile10-6	18.1; CH_3	1.08 (s)	Ile10-2, Ile10-3, Ile10-4	
Ile10-NH[d]		7.84 (d, 10.3)		Ile10-2
Thz11				
Thz11-1	162.5; C q			
Thz11-2	149.9; C q			
Thz11-3	125.3; CH	8.15 (s)	Thz11-1, Thz11-2, Thz11-4	
Thz11-4	167.3; C q			
Thr12				
Thr12-2	55.0; CH	5.73 (brs)	Thz11-1, Thz13-4	Thr12-NH, Thr12-3
Thr12-3	72.1; CH	6.41-6.35 (m)		Thr12-2, Thr12-4
Thr12-4	16.8; CH_3	1.57 (d, 6.6)	Thr12-2, Thr12-3	Thr12-3
Thr12-NH[d]		9.37 (d, 8.9)		
Thz13				
Thz13-2	157.0; C q			
Thz13-3	119.3; CH	7.46 (s)	Pip-5, Thz13-2, Thz13-4	
Thz13-4	171.6; C q			
Thz15				
Thz15-1	159.6; C q			
Thz15-2	149.8; C q			
Thz15-3	127.1; CH	8.15 (s)	Thz15-1, Thz15-2, Thz15-4	
Thz15-4	168.3; C q			

Position	δ_C [ppm]; mult	δ_H [ppm]; (mult, J in Hz)	HMBC[a]	COSY[b]
Dha16				
Dha16-1	161.9; C q			
Dha16-2	134.1; C q			
Dha16-3	103.2; CH_2	H_A: 6.55 (d, 1.5)	Dha16-1, Dha16-2	Dha16-3-H_B
		H_B: 5.48 (brs)	Dha16-1	Dha16-3-H_A
Dha17				
Dha17-1	166.0; C q			
Dha17-2	132.8; C q			
Dha17-3	104.4; CH_2	H_A: 6.40 (s)	Dha17-1, Dha17-2	Dha17-3-H_B
		H_B: 5.55 (s)	Dha17-1	Dha17-3-H_A
Q				
Q-1	163.0; C q			
Q-2	[f]			
Q-3	116.6; CH	7.57 (s)	Q-5, Q-11	
Q-4	154.7; C q			
Q-5	127.0; C q			
Q-6	123.3; CH	8.05 (d, 8.1)	Q-4, Q-8, Q-10	Q-7
Q-7	128.8; CH	7.68-7.61 (m)	Q-5, Q-9	Q-6
Q-8	130.5; CH	7.68-7.61 (m)	Q-6, Q-10	Q-9
Q-9	129.0; CH	8.24 (d, 8.1)	Q-7	Q-8
Q-10	146.1; C q			
Q-11	64.3; CH	5.51 (q, 6.5)	Q-3, Q-12	Q-12
Q-12	23.4; CH_3	1.41 (d, 6.5)	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] Multiplicity of this resonance was unassigned due to weak signal.
[d] The δ of this resonance was determined by HMBC due to overlap with the methanol-d_6 peak.
[e] Only those amide resonances demonstrating COSY correlations to neighboring protons were assigned.
[f] Unable to assign the resonance for this carbon

Table S3. Antibacterial activity of thiostrepton A **1** and its analogs

Compound	MIC[a] (μg/mL)		
	MRSA[b]	VRE[c]	*Bacillus*[d]
Thiostrepton A **1**	0.012	0.012	0.025
Thiostrepton Thr7Ala **3**	>3.4	>3.4	>3.4
SL105-1 **4**	>3.4	>3.4	>3.4
Thiostrepton Thr7Val **5**	>3.4	>3.4	>3.4
SL106-1 **6**	>3.4	>3.4	>3.4
Vancomycin	0.39	ND[e]	ND
Chloramphenicol	ND	3.9	0.98

[a] Minimum inhibitory concentration.
[b] *Staphylococcus aureus* ATCC 10537.
[c] *Enterococcus faecium* ATCC 12952.
[d] *Bacillus* sp. ATCC 27859.
[e] Not determined.

Table S4. Strains and plasmids used in this study

Strain/Plasmid	Description	Reference or source
Streptomyces strains		
S. laurentii ATCC 31255	Wild-type, thiostrepton producer	ATCC
S. laurentii NDS1	*S. laurentii* containing an in-frame deletion of *tsrA*	[1]
S. laurentii NDS1/int-3A104	*S. laurentii* NDS1 containing int-3A104	This study
S. laurentii NDS1/int-3A105	*S. laurentii* NDS1 containing int-3A105	This study
S. laurentii NDS1/int-3A106	*S. laurentii* NDS1 containing int-3A106	This study
E. coli strains		
BW25113/pKD46	Host for PCR-targeted disruption of a gene from a fosmid or plasmid	[3]
ET12567/pUZ8002	Host for conjugation with *Streptomyces* species	[6]
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
pJP11	pCR4-Blunt vector harboring a 1.8 kb fragment PCR amplified from *S. laurentii* genomic DNA, which contains the 0.2 kb *tsrA* gene and its two flanking 0.8 kb regions	[4]
pCL64	Plasmid containing the *tsrA* variant encoding the Thr7Ser mutation	This study
pCL65	Plasmid containing the *tsrA* variant encoding the Thr7Ala mutation	This study
pCL66	Plasmid containing the *tsrA* variant encoding the Thr7Val mutation	This study
int-3A100	Derived from int-3A10, *tsrA* is replaced by chl^R–*sacB* cassette	[1]
int-3A104	Derived from int-3A100. The chl^R–*sacB* cassette is replaced by the *tsrA* variant encoding the Thr7Ser mutation	This study
int-3A105	Derived from int-3A100. The chl^R–*sacB* cassette is replaced by the *tsrA* variant encoding the Thr7Ala mutation	This study
int-3A106	Derived from int-3A100. The chl^R–*sacB* cassette is replaced by the *tsrA* variant encoding the Thr7Val mutation	This study

Table S5. Primers and ultramer used in this study

Primer	Sequence	Description
T7S-F T7S-R	5'-GTCCGCCTCCTGCTCCACCTGCATCTGC-3' 5'-GCAGATGCAGGTGGAGCAGGAGGCGGAC-3'	Primers to generate TsrA Thr7Ser.
T7A-F T7A-R	5'-GTCCGCC TCCTGCGGCACCTGCATCTGC-3' 5'-GCAGATGCAGGTGCCGCAGGA GGCGGAC-3'	Primers to generate TsrA Thr7Ala.
T7V	5'-ACTACATGGACGAGACGCTGCTCGACGGTGA GGACCTGACCGTCACGATGATCGCGTCCGCCTC CTGCGTCACCTGCATCTGCACCTGCAGCTGCAG CTCCTGAGGTAACACCCGGCGCGGAGGACTGTT CCTCCCCGCCCGCCGGACCTG-3'	Chemically synthesized ultramer to generate TsrA Thr7Val.
T7V-F T7V-R	5'-ACTACATGGACGAGACGCTGCTC-3' 5'-CAGGTCCGGCGGGCGGGGA-3'	Primers for amplification of chemically synthesized ultramer TsrA T7V.
SD2-F SD2-R	5'-GCGCGATCGACGCGACCGCAG-3' 5'-GGCGGGGAGGAACAGTCCTCC-3'	Primers used in the amplification of *tsrA* variants from pCL64 and pCL65.
SD3-F SD3-R	5'-ATCGTGTTGGGCTTGACG-3' 5'-CGCGGTGCAATAGGACAT-3'	Primers used in DNA sequencing to confirm int-3A104, int-3A105, and int-3A106.

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