Supporting Information

Genome Mining, Isolation, Chemical Synthesis and Biological Evaluation of a Novel Lanthipeptide, Tikitericin and *N*-Truncated Analogues, from the Extremophilic Microorganism *Thermogemmatispora* Strain T81

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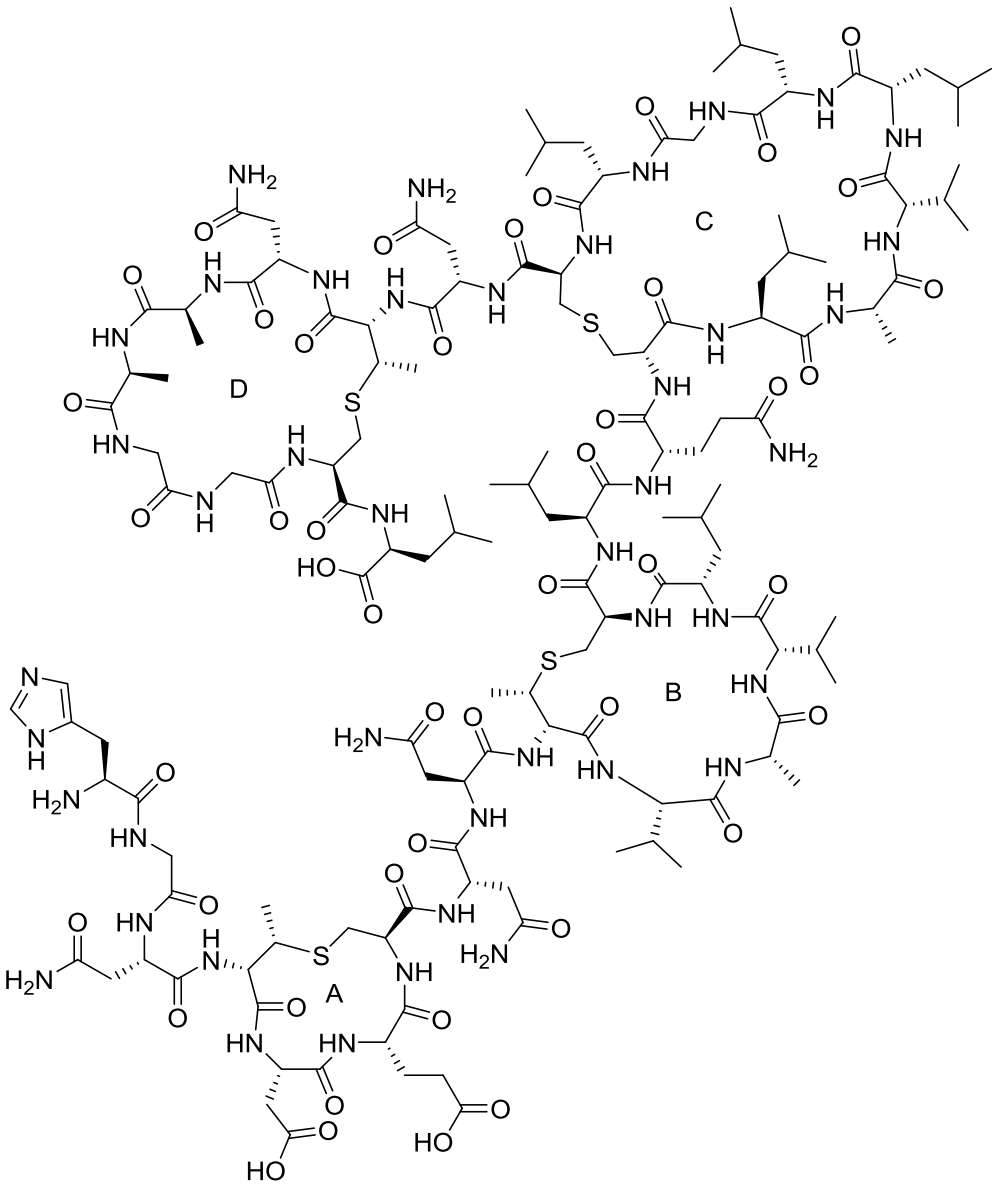

Tikitericin

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1. Isolation of tikitericin 1

1.1 *Thermogemmatispora* strain T81:

Thermogemmatispora strain T81 is an aerobic, Gram-positive, filamentous spore-forming bacterium isolated from steam-affected geothermal soil (pH 4.5, 55°C) at Hell's Gate, Tikitere, New Zealand.[1] Based on near-full length 16S rRNA gene sequence similarity, *Thermogemmatispora* strain T81 belongs to the phylum Chloroflexi (class Ktedonobacteria) and is most closely related to *Thermogemmatispora onikobensis* (98.1%) and *Thermogemmatispora foliorum* (96.7%).

Thermogemmatispora strain T81 has the following identifying characteristics: Gram-positive, filamentous bacteria with branching hyphae and cells of 0.2 – 0.4 µm in width and >30 µm in length. It has white vegetative mycelia with yellow under-layer when grown on solid medium. It is obligately aerobic, a pH growth range 4.0 – 7.5, and a temperature range of 40°C – 75°C. It can grow on a variety of carbohydrate and proteinaceous substrates including gellan/phytagel™, agar, carboxymethylcellulose, xylan, pectin, xanthan, Avicel® (crystalline cellulose), sodium alginate, R2A, yeast extract and nutrient broth.[1]

Thermogemmatispora strain T81 was recovered on plates of AOM1 solid medium[1] incubated aerobically at 60°C. Colonies were isolated through successive rounds of colony picking and plating, and isolate purity was confirmed by 16S rRNA gene sequencing using the universal primers 9F and 1492R. *Thermogemmatispora* strain T81 has been deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, MascheroderWeg 1b, D-38124, Braunschweig, Germany, Accession No. DSM 21103 (deposited 30 January 2008, patent deposit).

The genome of *Thermogemmatispora* strain T81 was sequenced after DNA extraction via a paired-end 454 pyrosequencing and Illumina HiSeq 2000 paired-end technologies runs. Illumina reads were pre-processed by adaptor clipping, quality trimming with the FASTX-Toolkit[2] and random subset data selection. Multiple assembly pipelines using Illumina data were evaluated using Newbler v.2.6. Based on statistics metrics, Newbler v2.6 generated the longest contigs with the highest N50 and the resulting assembly was composed of six scaffolds (scaffold1: 2537 bp, scaffold2: 5961682 bp, scaffold3: 2087 bp, scaffold4: 3112 bp, scaffold5: 2405 bp, scaffold6: 2126 bp) with 25.9-fold coverage. Genome annotation was performed using NCBI Prokaryotic Genome Annotation Pipeline with best-placed reference protein annotation method 3.3 (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/). The genome is approximately 5.9 MB and has a %G+C content of ~60.84%. NCBI; Accession number: MCIF00000000.

An analysis of a gene cluster encoding for genes putatively encoding for lanthionine synthetase and associated lanthipeptide biosynthesis genes (Figure S1) was identified during an assessment of the complete genome. The lanthipeptide precursor peptide (TikA) consists of a 47 amino acid-long leader peptide and a 35 amino acid-long core peptide (Figure S2). The precursor peptide contains a C-terminal leader peptide cleavage site. Four cysteine and hydroxyl-containing residue pairs (Cys, Thr and Ser) show the position of the three (Me)Lan-linked macrocycles and single Lan-linked macrocycle in the mature peptide.

The leader and core peptide sequences of known lanthipeptides were aligned with TikA using Clustal Omega (Figure S3). Very little sequence similarity is evident in the core peptide of TikA. Alignment of the leader peptide shows that with 43.5% homology, mersacidin (class II) is the most closely related of the characterised lanthipeptides.

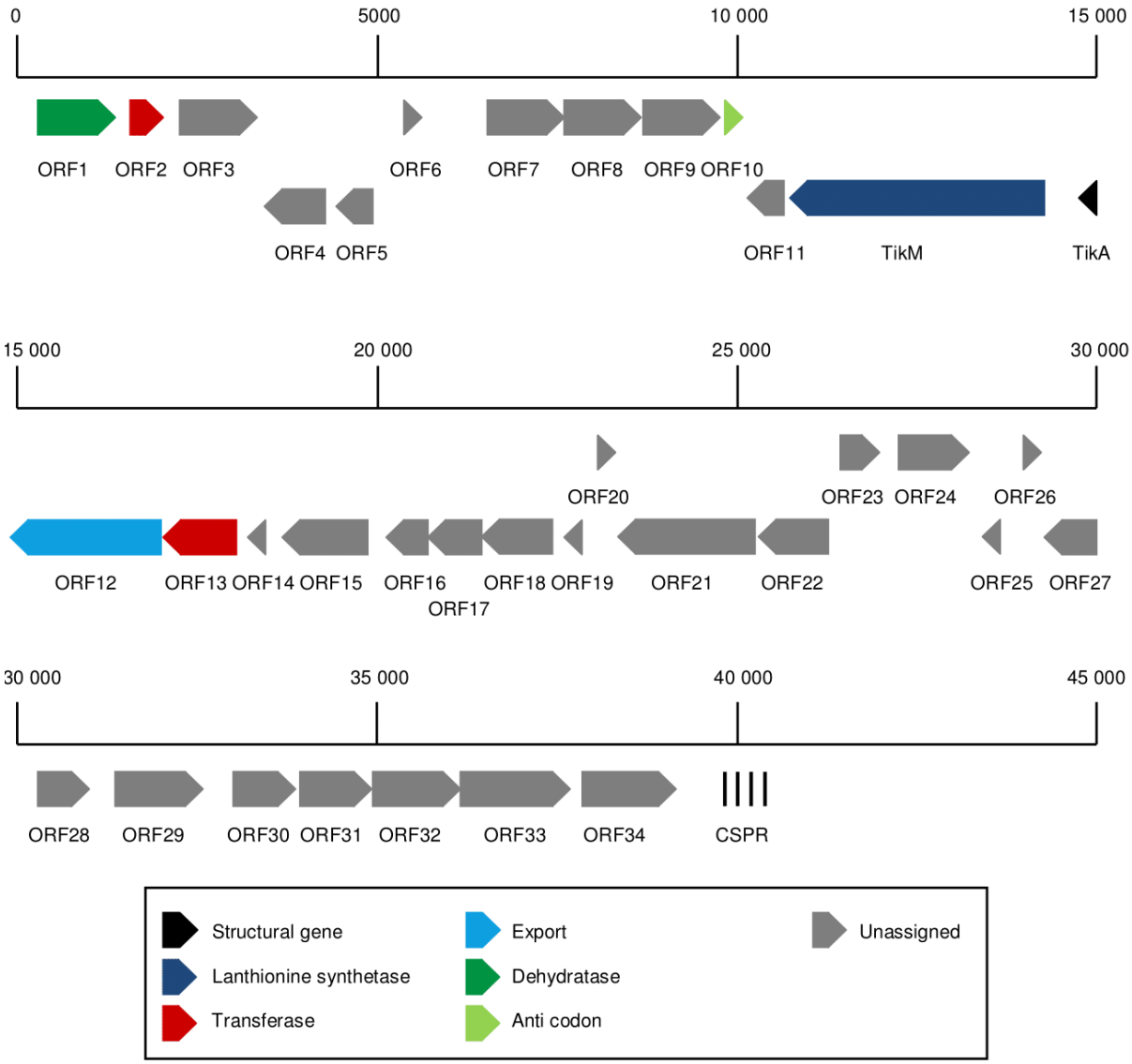


Figure S1. Tikitericin **1** gene cluster. Each putative gene is shown to scale with its position marked in kb. TikA denotes the lanthipeptide precursor peptide. Posttranslational modifications of the precursor peptide are executed by a bifunctional lanthionine synthetase protein, TikM, which is present immediately downstream of TikA.

Figure S2. Tikitericin **1** precursor peptide. The C-terminal leader peptide cleavage site is shaded in red. Posttranslational modifications occur at the Cys, Thr and Ser residues (shaded in blue) forming three (Me)Lan bridges and one Lan bridge in the mature peptide.

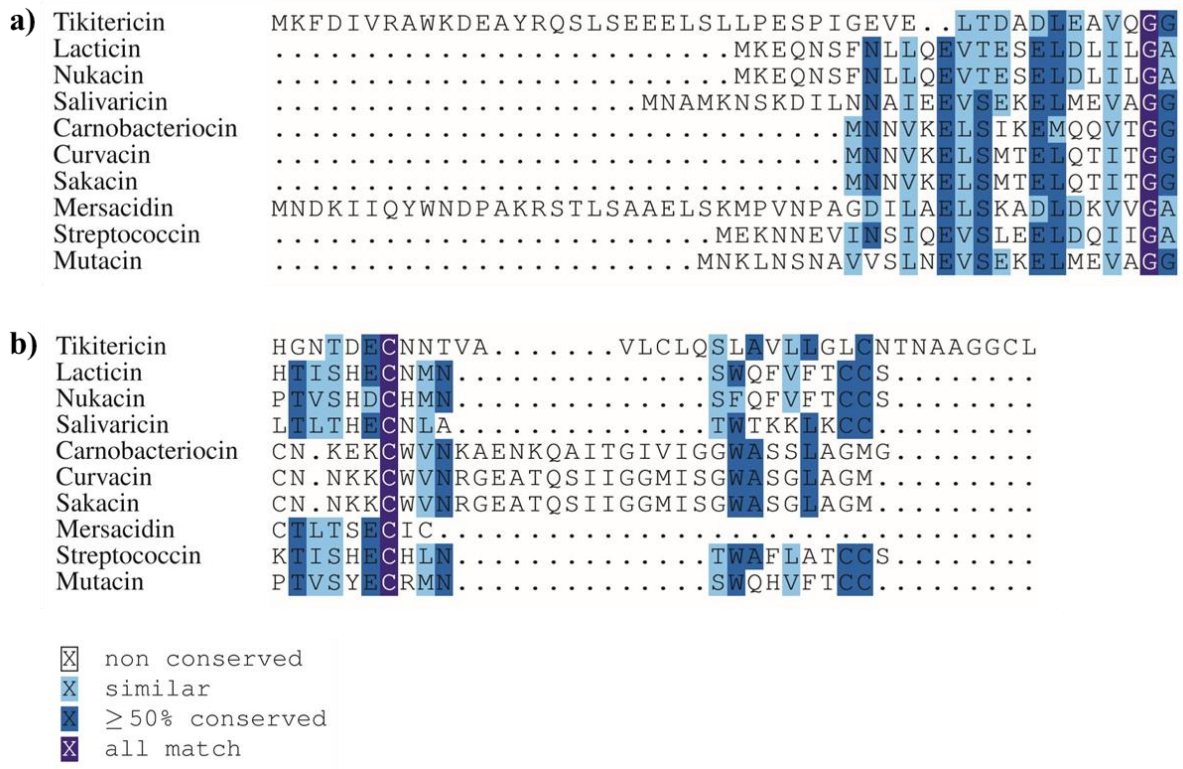


Figure S3. Sequence alignment of the precursor peptides of tikitericin **1** and nine known lanthipeptides. Sequence alignment is colour coded according to amino acids with similar structure and physical properties: a) sequence alignment of the leader peptides; b) sequence alignment of the core peptides.

1.2 General experimental details

Reversed-phase chromatography was performed using poly(styrene-divinyl benzene) copolymer resins (HP-20, HP-20ss) obtained from Supelco® (Sigma Aldrich), while size exclusion chromatography was performed using Sephadex® LH-20 (General Electric). Chromatography solvents (acetone, MeOH and MeCN) were HPLC grade (Fisher Scientific) and water was distilled prior to use. MS-grade solvents were used for LC-MS analysis. Reagents for chemical derivatizations were obtained from Sigma Aldrich and were used without further purification. Millipore C18 ZipTips (10, 100 µL; Merck) were used for desalting proteolytic digestion and linearization reactions.

HPLC purification of tikitericin **1** utilised an Agilent 1260 HPLC system equipped with a photodiode array detector and coupled to a Quicksplit® flow splitter valve (Analytical Scientific Instruments) directing 95% to collection and 5% to an Agilent 380 evaporative light scattering detector (evaporator temp, 80°C; nebuliser temp, 70°C; gas flow, 1.6 SLM; LED intensity, 100%.).

MALDI samples were mixed with aliquots of matrix solution (5, 10, and 20 _L, 10 mg/mL 2,5-dihydroxybenzoic acid (DHB) solubilised in 50% 96 MeCN/0.1% trifluoroacetic acid (TFA), v/v in H_2O). Aliquots (1 µL) were spotted onto a stainless steel MALDI MS target plate and dried with hot air.

MALDI-TOF MS was performed using an AB-Sciex 5800 TOF/TOF MS (Applied Biosystems) equipped with a diode pulse laser (355 nm, 1 kHz). MALDI parameters were as follows: operation mode, MS reflector positive; CID, off; mass range, m/z 700 – 4000; total shots/spectrum, 5000; stage motion, random continuous 600 µm/s; average laser intensity, 7000; pulse rate, 400 Hz. Mass spectra were taken from the edge of the crystals and the laser fluence was optimised to achieve the highest analyte S/N ratios. Mass spectra were internally calibrated using CalMix2 (Applied Biosystems), with peak matching performed against the monoisotopic peaks of bradykinnin fragment 1-7 (m/z 757.4000), angiotensin II (m/z 1046.5420), P14R (m/z 1533.8580), ACTH fragment 18-39 (m/z 2465.1990) and insulin oxidised B chain (m/z 3494.6510). Peak matching criteria for calibration were as follows: S/N ratio, ≥200; mass tolerance, ± m/z 0.05; matched peaks, 4; outlier error, ≤ 3 ppm. Data Explorer (Applied Biosystems) was used for the analysis of the MS spectra using the following parameters: baseline correction; Gaussian smooth, 5; S/N ratio, ≥ 20; mass peak filter, monoisotopic peaks; outlier error, ≤ m/z 0.8.

MALDI MSMS parameters were as follows: operating mode, MSMS 1 kV positive; CID, on; precursor width range, ± 5 Da; metastable suppressor, on; total shots/spectrum, 5000; stage motion, random continuous 600 µm/s; laser intensity, 6000; pulse rate, 1 kHz. The monoisotopic angiotensin 1 peak (m/z 1296.800) was selected as the precursor ion for calibration; MSMS spectra were then calibrated using known fragment ion masses from this precursor ion. Peak matching criteria for calibration were as follows: S/N ratio, ≥ 10; mass tolerance, ± m/z 0.3; matched peaks, 3; outlier error, ≤ 20 ppm. Data Explorer was used for peak analysis.

LC-MS analyses were performed using an Agilent 6530 Q-TOF mass spectrometer equipped with a JetStream® electrospray ion source. Samples were injected using an Agilent 1260 HPLC system using an Eclipse Plus reversed-phase C_{18} column (30 mm x 2.1 mm, 3.5 µm; Agilent Technologies) at 35°C and a flow rate of 0.4 mL/min. Eluent A was 99.9% H_2O/0.1% NH_4HCO_2. Eluent B was 99.9% MeCN/0.1% HCO_2H. LC analyses were performed with the following gradient: 5% eluent B for 0.5 min, eluent B increased to 100% at 4 min, eluent B held at 100% until 4.5 min, eluent B reduced to 5% at 4.51 min and held until 7 min.

Q-TOF ESI-MS was performed with the following parameters: positive ion mode electrospray ionisation; gas temperature, 300°C; gas flow, 9 L/min; nebulizer, 30 psi; capillary voltage, 3500 V; nozzle voltage, 500 V; fragmentor voltage, 140 V; acquisition rate, 2 spectra/s; mass range, *m/z* 100 – 3200.

Q-TOF ESI-MSMS was performed with the following parameters: max time between MS spectra, 5 s; mass range, *m/z* 100 – 3200; MSMS scan rate, 2 spectra/s; CID collision gas, N2; CID gas pressure, 16 psi; isolation width, 4 amu; collision energies, 50 – 70 eV.

1.3 Purification process

Competition experiments with five co-strains of bacteria (XS 01.96, TKA 04.11, WRG 1.1, WKT 21.8, and TKA 04.12) were cultured to screen for production of tikitericin by whole-cell MALDI-TOF MS. Using pipette tips, pinhead amounts of single bacterial colonies were carefully picked without destroying the surface of the agar and mixed with aliquots of matrix solution (5, 10, and 20 µL, 10 mg/mL 2,5-dihydroxybenzoic acid (DHB) solubilised in 50% 96 MeCN/0.1% trifluoroacetic acid (TFA), v/v in H_2O). Aliquots (1 µL) were spotted onto a stainless steel MALDI MS target plate and dried with hot air. From each competition experiment, scrapings were taken from the centre of the T81 colony, zone of inhibition (if observed), and centre of the co-strain colony for MALDI analysis.

Ultimately, as co-culture experiments did not induce production of tikitericin, Petri dish cultures of strain T81 were grown on solid AOM1 at 50 °C medium for 14 d in batches of 100 plates. The lawns of bacterial growth were scraped by hand (including the first 1 mm of culture medium) and extracted in MeOH (2 x 24 hr). Extracts were filtered and partitioned on

HP-20ss (15 mL) that had been pre-equilibrated with acetone and MeOH. The column was washed with water (45 mL, discarded) and then eluted with 45 mL aliquots of 30%, 60% and 100% $acetone_{(aq.)}$ that were dried *in vacuo*.

Dried 60% acetone fractions from three batches were combined and separated using LH-20 resin (100 g), eluting with 50% $MeOH_{(aq.)}$ at a flow-rate of 0.4 mL/min, collecting for 15 min (6 mL fractions). Fractions were combined based upon LC-MS detection of tikitericin **1**. Final HPLC purification of tikitericin **1** used a Zorbax C_{18} column (100 mm x 4.6 mm i.d., 3.5 µm; Agilent Technologies) with a flow rate of 1 mL/min and a solvent system of eluent A: 0.2 M HCO_2H in 30% MeCN/70% water, and eluent B: 0.2 M HCO_2H in 100% MeCN. The solvent was held at 0% B for 10 min, increased to 15% B over 30 min, increased to 100% B over 1 min and held for 4 min, decreased to 0% B over 2 min, returned to 0% B over 1 min and held for 2 min. The elution of tikitericin **1** was monitored with both ELSD and UV detection (220 nm). Tikitericin **1** (400 µg total combined over four combined batches of 300 plates) eluted at approximately 39% MeCN with a retention time of ~26 min.

Tikitericin **1**: *m/z* 858.1594 $[M+4H]^{4+}$ (calculated, 858.1597 Da; Δ – 0.40 ppm), *m/z* 880.1420 $[M+4Na]^{4+}$ (calculated, 880.1417 Da; Δ 0.33 ppm), *m/z* 1143.8779 $[M+3H]^{3+}$ (calculated, 1143.8772 Da; Δ 0.60 ppm), *m/z* 1166.1927 $[M+3Na]^{3+}$ calculated, 1166.1934 Da; Δ – 0.59 ppm), *m/z* 1715.3153 $[M+2H]^{2+}$ (calculated, 1715.3122 Da; Δ1.82 ppm), *m/z* 1737.3008 $[M+2Na]^{2+}$ (calculated, 1737.2941 Da; Δ 3.86 ppm).

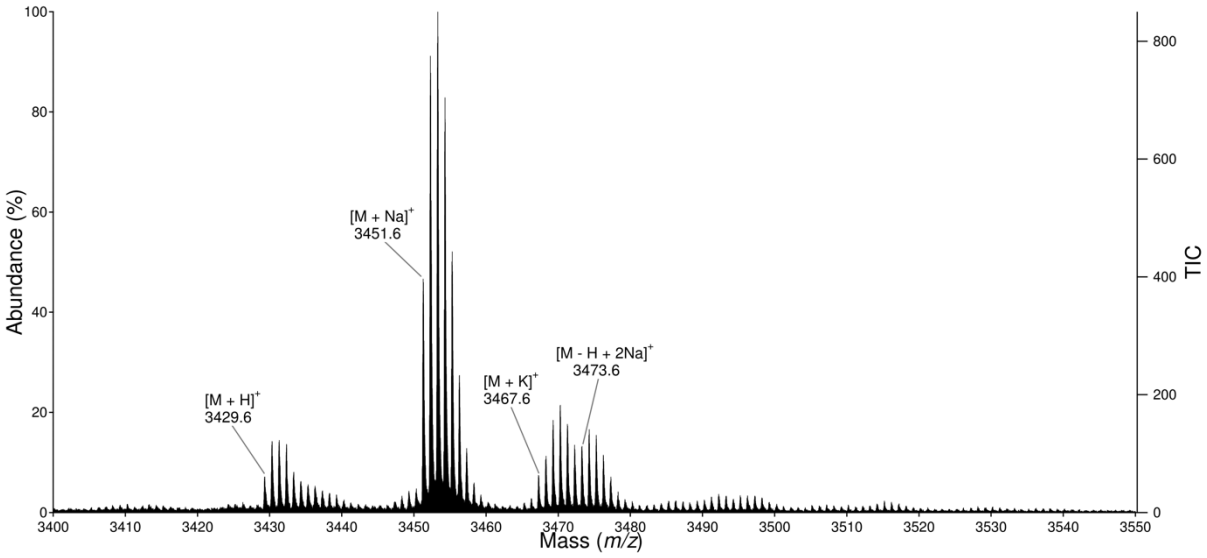
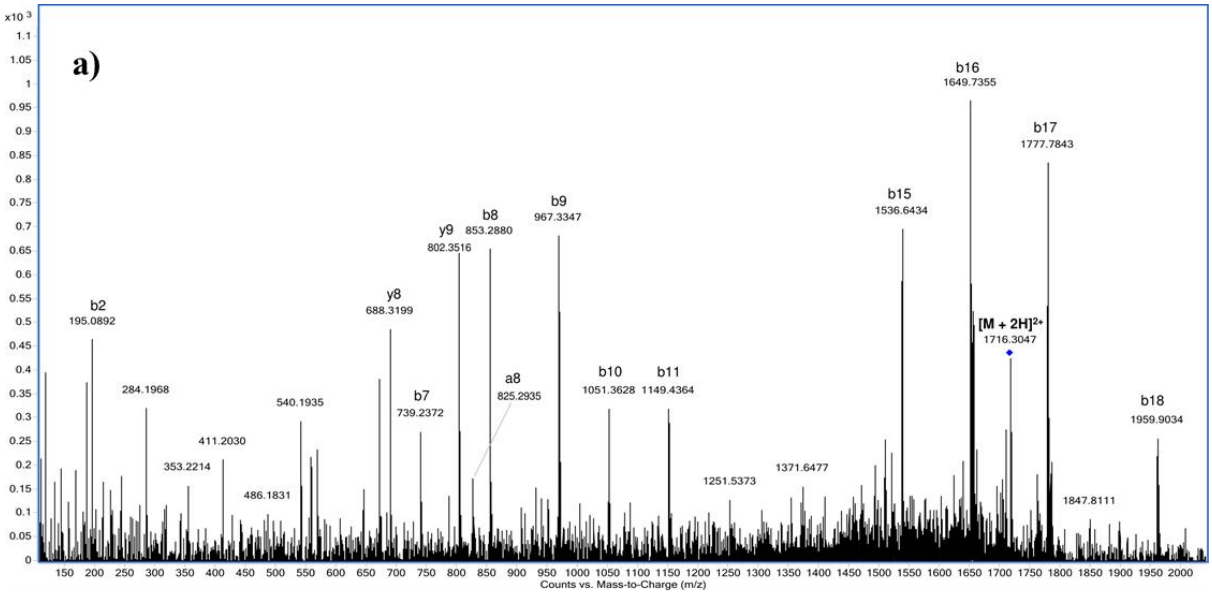
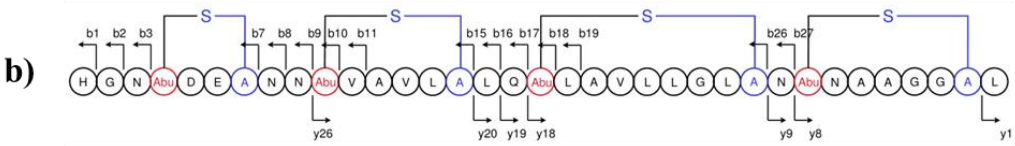


Figure S4. Whole-cell MALDI-TOF MS spectrum of tikitericin **1**. A protonated molecular ion of *m/z* 3429.6 was detected as the protonated $[M+H]^+$ ion of tikitericin **1**. Three additional cationised ions were detected: *m/z* 3451.5 $[M+Na]^+$; *m/z* 3467.6 $[M+K]^+$; *m/z* 3473.6 $[M–H+2Na]^+$.





b ions				y ions			
Pos	Exact (*m/z*)	Observed (*m/z*)	Δ (ppm)	Pos	Exact (*m/z*)	Observed (*m/z*)	Δ (ppm)
1	138.0667	138.0628	– 28.25	34	3292.5587		
2	195.0882	195.0884	1.03	33	3225.5373		
3	309.1311	309.1315	1.29	32	3121.4943		
4	392.1682			31	3038.4572		
5	507.1952			30	2794.3877		
6	636.2378			29	2691.3785		
7	739.2470	739.2469	– 0.14	28	2691.3785		
8	853.2899	853.2898	– 0.12	27	2577.3356		
9	967.3328	967.3331	0.31	26	2463.2927	2463.2780	– 5.83
10	1050.3700	1050.3689	– 1.05	25	2380.2555		
11	1149.4383	1149.4377	– 0.52	24	2281.1871		
12	1220.4745			23	2210.1500		
13	1319.5439			22	2111.0816		
14	1432.6279			21	1997.9975		
15	1535.6371	1535.6386	0.98	20	1894.9883	1894.9920	1.72
16	1648.7212	1648.7233	1.27	19	1781.9043	1781.9060	0.87
17	1776.7798	1776.7806	0.45	18	1653.8457	1653.8340	– 7.35
18	1845.8012	1845.7977	– 1.90	17	1584.8242		
19	1958.8853	1958.8878	1.28	16	1471.7402		
20	2029.9224			15	1400.7031		
21	2128.9908			14	1301.6347		
22	2242.0749			13	1188.5506		
23	2355.1589			12	1075.4665		
24	2412.1804			11	1018.4451		
25	2525.2645			10	905.3610		
26	2628.2737	2628.2564	– 6.58	9	802.3518	802.3516	– 0.19
27	2742.3166	2742.3631	16.96	8	688.3089	688.3077	– 1.67
28	2825.3537			7	605.2718		
29	2939.3966			6	491.2288		
30	3010.4337			5	420.1917		
31	3081.4709			4	349.1546		
32	3138.4923			3	292.1332		
33	3195.5138			2	235.1117		
34	3298.5230			1	132.1025	132.1029	3.41

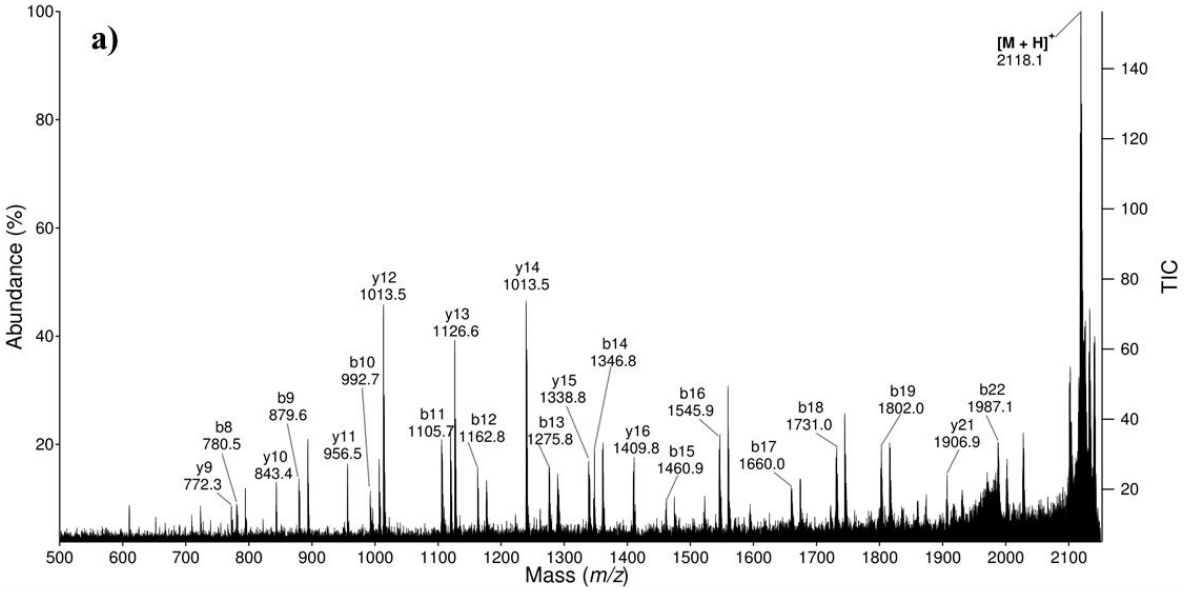
Figure S5. a) HRESI-MSMS spectrum of tikitericin **1**, precursor ion: *m/z* 1716.32 $[M+2H]^{2+}$: a) CID energy = 60 eV; b) HR ESI-MSMS fragmentation pattern of tikitericin **1**, precursor ions: *m/z* 1144.55 $[M+3H]^{3+}$, *m/z* 1716.32 $[M+2H]^{2+}$.

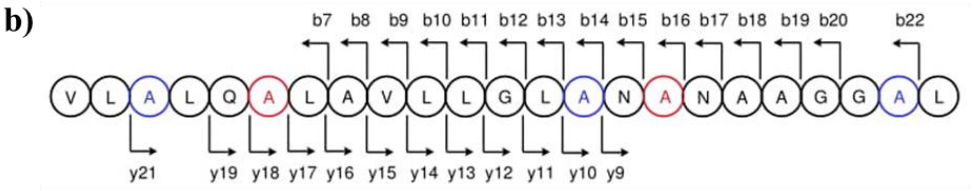
1.4 Raney nickel reduction

LH-20 purified tikitericin **1** (50 µg) was suspended in a 8 M guanidine hydrochloride, 20 mM EDTA and 200 mM Tris-HCl solution (300 _L). After the addition of RaNi (25 mg, active catalyst slurry in H2O), followed by shaking at 55 _C for 15 h and subsequent centrifugation at 5,000 rpm for 2 min, the supernatant was removed and desalted using C18 ZipTips according to the manufacturer's protocol.

RaNi linearised tikitericin Val13-Leu35: *m/z* 706.7598 $[M+3H]^{3+}$ (calculated, 706.7597 Da; 0.14 ppm), *m/z* 1059.6369 $[M+2H]^{2+}$ (calculated, 1059.6359 Da; Δ 1.00 ppm), *m/z* 1081.6209 $[M+2Na]^{2+}$ (calculated, 1081.6178 Da; Δ 2.84 ppm), *m/z* 2118.2645 $[M+H]^{+}$ (calculated, 2118.2645 Da; Δ 0.01 ppm), *m/z* 2140.2574 $[M+Na]^{+}$ (calculated, 2140.2464 Da; Δ 5.11 ppm).

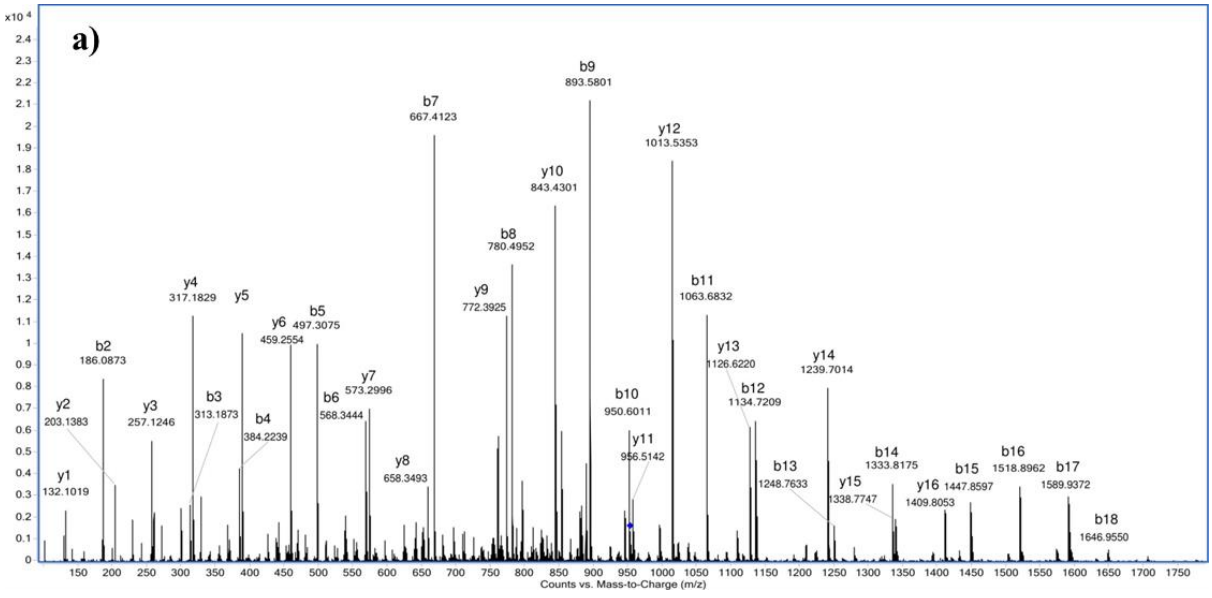
RaNi linearised tikitericin Ala15-Leu35: *m/z* 953.5612 $[M+2H]^{2+}$ (calculated, 953.5596 Da; Δ 1.64 ppm), *m/z* 975.5445 $[M+2Na]^{2+}$ (calculated, 975.5416 Da; Δ 3.01 ppm), 1906.1103 $[M+H]^{+}$ (calculated, 1906.1120 Da; Δ – 0.9 ppm), *m/z* 1928.0924 $[M+H]^{+}$ (calculated, 1928.0940 Da; Δ – 0.8 ppm).

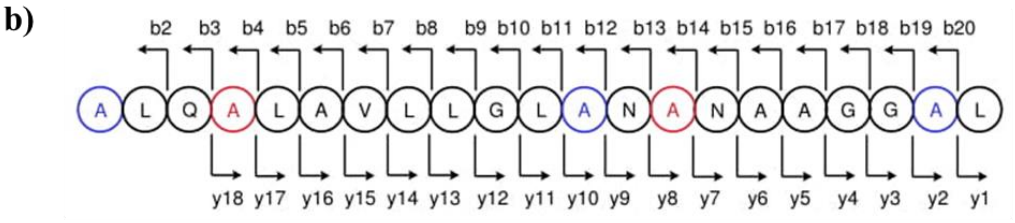




b ions			y ions		
Pos	Exact (*m/z*)	Observed (*m/z*)	Pos	Exact (*m/z*)	Observed (*m/z*)
1	100.08		22	2019.20	
2	213.16		21	1906.11	1905.99
3	284.20		20	1835.08	
4	397.28		19	1721.99	1721.92
5	525.34		18	1593.93	1593.84
6	596.38		17	1522.90	1522.88
7	709.46	709.49	16	1409.81	1409.82
8	780.50	780.49	15	1338.77	1338.77
9	879.57	879.56	14	1239.71	1239.69
10	992.65	992.65	13	1126.62	1126.62
11	1105.73	1105.73	12	1013.54	1013.55
12	1162.76	1162.77	11	956.52	956.52
13	1275.84	1275.85	10	843.43	843.43
14	1346.88	1346.89	9	772.40	772.40
15	1460.92	1460.91	8	658.35	
16	1545.97	1545.97	7	573.30	
17	1660.02	1659.92	6	459.26	
18	1731.05	1731.06	5	388.22	
19	1802.09	1802.10	4	317.18	
20	1859.11	1859.01	3	260.16	
21	1916.13		2	203.14	
22	1987.17	1987.18	1	132.10	

Figure S6. a) MALDI-TOF MSMS spectrum of Raney Nickel linearization product Val13-Leu35, precursor ion *m/z* 2140 $[M+Na]^+$; b) MALDI-TOF MSMS fragmentation pattern of Raney Nickel linearization product Val13-Leu35, precursor ions *m/z* 2118 $[M+H]^+$, *m/z* 2140 $[M+Na]^+$.





b ions				y ions			
Pos	Exact (*m/z*)	Observed (*m/z*)	Δ (ppm)	Pos	Exact (*m/z*)	Observed (*m/z*)	Δ (ppm)
1	72.0449			20	1835.0755		
2	185.1290	185.1284	– 3.24	19	1721.9914		
3	313.1876	313.1873	– 0.96	18	1593.9329	1593.9300	– 1.79
4	384.2247	384.2239	– 2.08	17	1522.8958	1522.8800	– 10.34
5	497.3088	497.3069	– 3.82	16	1409.8117	1409.8053	– 4.50
6	568.3459	568.3444	– 2.64	15	1338.7746	1338.7747	0.11
7	667.4143	667.4138	– 0.75	14	1239.7062	1239.7030	– 2.54
8	780.4943	780.4959	2.05	13	1126.6221	1126.6220	– 0.04
9	893.5824	893.5798	– 2.91	12	1013.5380	1013.5365	– 1.43
10	950.6039	950.6014	– 2.63	11	956.5166	956.5142	– 2.46
11	1063.6879	1063.6863	– 1.50	10	843.4325	843.4320	– 0.53
12	1134.7251	1134.7228	– 2.03	9	772.3954	772.3925	– 3.69
13	1248.7680	1248.7633	– 3.76	8	658.3525	658.3493	– 4.78
14	1333.8207	1333.8181	– 1.95	7	573.2997	573.2993	– 0.61
15	1447.8637	1447.8597	– 2.76	6	459.2608	459.2554	– 11.65
16	1518.9008	1518.8972	– 2.37	5	388.2197	388.2183	– 3.48
17	1589.9379	1589.9372	– 0.44	4	317.1825	317.1815	– 3.00
18	1646.9594	1646.9550	– 2.67	3	260.1611	260.1609	– 0.58
19	1703.9808	1703.9800	– 0.47	2	203.1396	203.1383	– 6.15
b0	1775.0179	1774.9800	– 21.35	1	132.1025	132.1012	– 9.46

Figure S7. a) HR ESI-MSMS spectrum of Raney Nickel linearization product Ala15-Leu35, precursor ion *m/z* 954.06 $[M+2H]^{2+}$,CID energy = 25 eV; b) HR ESI-MSMS fragmentation pattern of Raney Nickel linearization product Ala15-Leu35, precursor ion: *m/z* 954.06 $[M+2H]^{2+}$.

1.5 Base catalysed elimination and thioethanol trapping

LH-20 purified tikitericin **1** (50 µg) was suspended in a 0.24 M NaOH, 0.63 mM 2-thioethanol, 25% EtOH solution (40 µL) and incubated with shaking at 50°C for 1 h. The temperature was raised to 85°C and the reaction incubated for a further 1 h. After the addition of acetic acid (10 µL) the reaction was diluted with H_2O (150 µL) and desalted using C18 ZipTips according to the manufacturer's protocol.

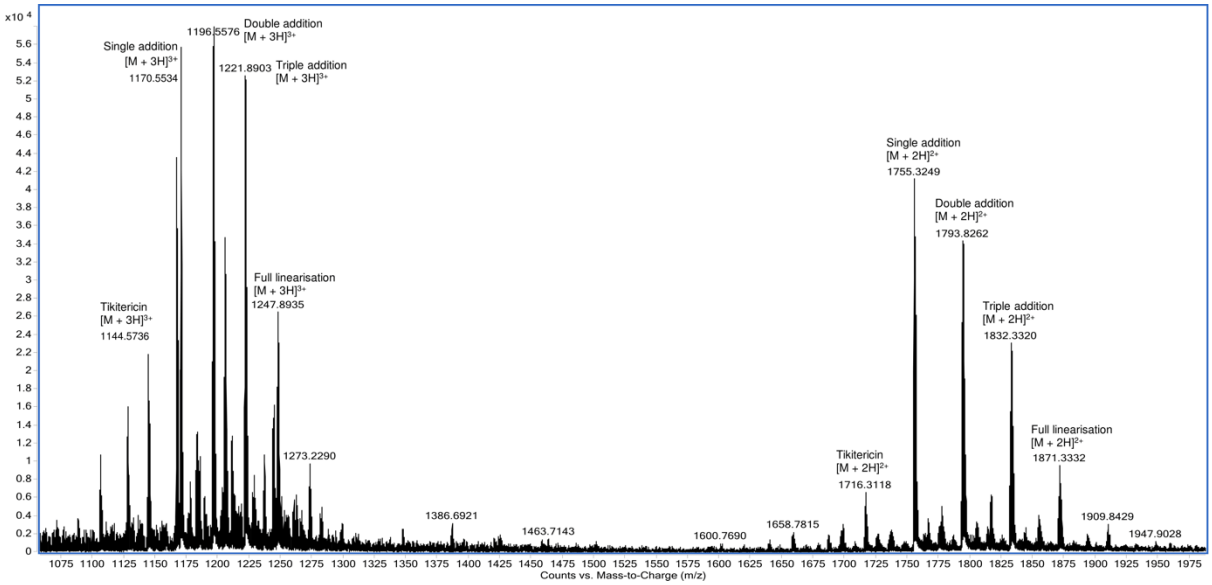
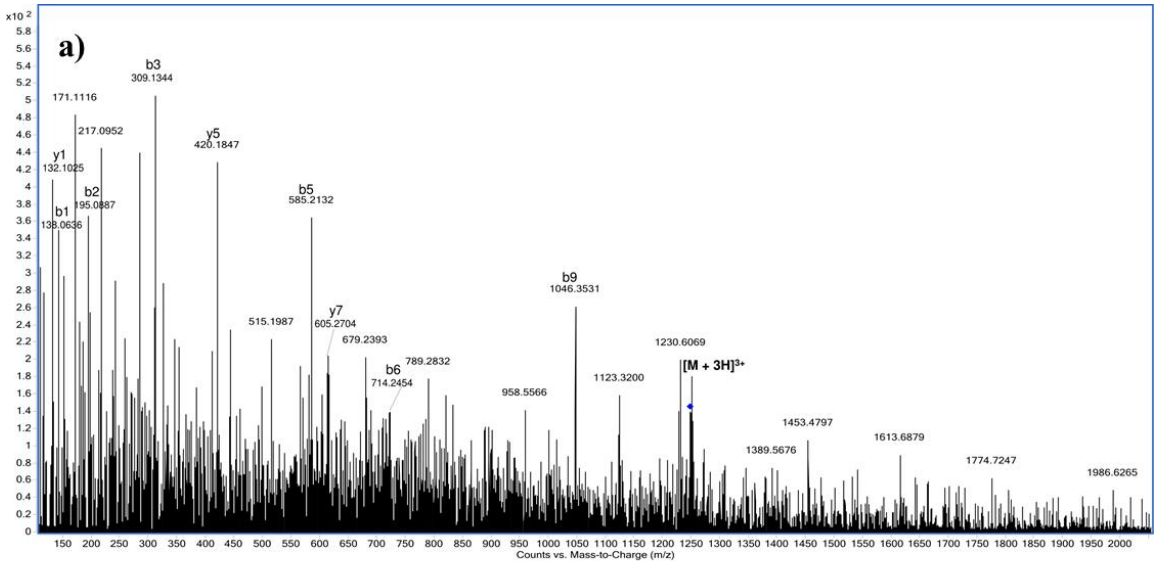
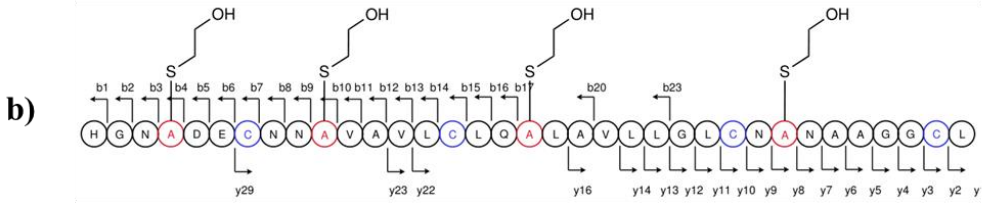


Figure S8. HRESI-MS spectrum of the linearization products of tikitericin **1** following base catalysed elimination and thioethanol trapping: fully linearized tikitericin **1** (*m/z* 1247.89 $[M+3H]^{3+}$, *m/z* 1871.33 $[M+2H]^{2+}$); triple addition (*m/z* 1221.89 $[M+3H]^{3+}$, *m/z* 1832.33 $[M+2H]^{2+}$); double addition (*m/z* 1196.56 $[M+3H]^{3+}$, *m/z* 1793.83 $[M+2H]^{2+}$); single addition (*m/z* 1170.55 $[M+3H]^{3+}$, *m/z* 1755.32 $[M+2H]^{2+}$).





b ions				y ions			
Pos	Exact (*m/z*)	Observed (*m/z*)	Δ (ppm)	Pos	Exact (*m/z*)	Observed (*m/z*)	Δ (ppm)
1	138.0667	138.0636	– 22.45	34	3604.6145		
2	195.0882	195.0887	2.56	33	3547.5930		
3	309.1311	309.1344	10.68	32	3433.5501		
4	470.1822	470.1757	– 13.82	31	3272.4990		
5	585.2091	585.2085	– 1.03	30	3157.4721		
6	714.2517	714.2454	– 8.82	29	3028.4295	3028.3215	– 35.65
7	817.2609	817.2633	2.94	28	2925.4203		
8	931.3038	931.2961	– 8.27	27	2811.3774		
9	1045.3468	1045.3462	– 0.57	26	2697.3345		
10	1206.3978	1206.3864	– 9.45	25	2536.2834		
11	1305.4662	1305.4589	– 5.59	24	2437.2150		
12	1376.5033	1376.5100	4.87	23	2366.1779	2366.1098	– 28.76
13	1475.5717	1475.5808	6.17	22	2267.1095	2267.1243	6.55
14	1588.6558	1588.6620	3.90	21	2154.0254		
15	1691.6650	1691.6530	– 7.09	20	2051.0162		
16	1804.7491	1804.7396	– 5.26	19	1937.9322		
17	1932.8076	1932.8320	12.62	18	1809.8736		
18	2079.8430			17	1662.8382		
19	2192.9271			16	1549.7541	1549.7666	8.10
20	2263.9642	2263.9020	– 27.47	15	1478.7170		
21	2363.0326			14	1379.6486	1379.6282	– 14.75
22	2476.1167			13	1266.5645	1266.6136	38.81
23	2589.2008	2589.0815	– 46.08	12	1153.4805	1153.4870	5.68
24	2646.2222			11	1096.4590	1096.4632	3.88
25	2759.3063			10	983.3749	983.3423	– 33.10
26	2862.3155			9	880.3657	880.3662	0.62
27	2976.3584			8	766.3228	766.2818	– 53.44
28	3137.4094			7	605.2718	605.2704	– 2.23
29	3251.4524			6	491.2288	491.2300	2.54
30	3322.4895			5	420.1917	420.1847	– 16.54
31	3393.5266			4	349.1546	349.1515	– 8.74
32	3430.5481			3	292.1332	292.1311	– 7.02
33	3507.5695			2	235.1117	235.1147	12.97
34	3610.5787			1	132.1025	132.1025	0.38

Figure S9. a) HRESI-MSMS spectrum of fully linearized tikitericin **1**: precursor ion *m/z* 1247.89 $[M+3H]^{3+}$, CID energy = 60 eV; b) HRESI-MSMS fragmentation pattern of linearized tikitericin **1**, precursor ions: *m/z* 1247.89 $[M+3H]^{3+}$, *m/z* 1871.33 $[M+2H]^{2+}$.

1.6 Establishment of (Me)Lan and Lan configurations

HPLC-purified tikitericin **1** (300 µg) was dissolved in 6M HCl (3 mL) and heated in a sealed tube at 110°C for 45 min. After cooling, the solvent was removed *in vacuo.* MeOH (5 mL) was cooled to 0°C, after which acetyl chloride (1.5 mL) was added dropwise. This reagent was added to the hydrolysed tikitericin **1** that was again heated at 110°C for 45 mins. The solvent was removed, DCM (3 mL) and pentafluoropropionic anhydride (1 mL) was added, and the reaction mixture was heated at 110°C for 15 mins. The solution was cooled and dried under a stream of dry $Ar_{(g)}$. The residue was transferred to a 150 µL vial insert in a 2 mL vial, dried under $Ar_{(g)}$, and reconstituted in EtOAc (50 µL) for HR-ESI-MS and GC-MS.

Derivatized Lan: *m/z* 529.0554 $[M+H]^+$ (calculated, 529.0486 Da; Δ 12.92 ppm), *m/z* 546.0754 $[M+NH_4]^+$ (calculated, 546.0751 Da; Δ 0.53 ppm), *m/z* 551.0304 $[M+Na]^+$ (calculated, 551.0305 Da; Δ – 0.15 ppm), *m/z* 567.0043 $[M+K]^+$ (calculated, 567.0044 Da; Δ – 0.22 ppm).

Derivatized MeLan: *m/z* 560.0905 $[M+NH_4]^+$ (calculated, 560.0908 Da; Δ – 0.54 ppm), *m/z* 565.0456 $[M+Na]^+$ (calculated, 565.0462 Da; Δ – 0.97 ppm), *m/z* 581.0161 $[M+K]^+$ (calculated, 581.0201 Da; Δ – 1.76 ppm).

The derivatized MeLan from tikitericin **1** and authentic synthetic MeLan standards were analysed using a Shimadzu QP2010 Plus GCMS equipped an electron impact mass quadrupole spectrometer operating at 70 eV and with an HP-VOC column (60 m x 0.32 mm i.d., 0.18 µm; Agilent Technologies). The samples (1 µL) were introduced to the instrument via splitless injection at an inlet temperature of 270°C and a linear velocity of 40 cm/sec using He carrier gas. The temperature programme was held at 200°C for 1 h, increased to 280°C at 25°C/min, and held at 280°C for 10 min. The MS was operated in SIM mode, monitoring fragment masses of 248 and 379 Da.

The derivatized Lan from tikitericin **1** and authentic Lan synthetic standards were analysed using a Shimadzu QP2010 Plus GCMS equipped an electron impact mass quadrupole spectrometer operating at 70 eV and with an HP-VOC column (60 m x 0.32 mm i.d., 0.18 µm; Agilent Technologies). The samples (1 µL) were introduced to the instrument via a splitless injection at an inlet temperature of 270°C and a linear velocity of 40 cm/sec using He carrier gas. The temperature programme was increased from 150°C to 205°C at a rate of 10°C/min, held at 205°C for 15 min, increased to 250°C at 3°C/min, increased to 280°C at 30°C/min and held at 280°C for 5 min. The MS was operated in SIM mode, monitoring fragment masses of 248 and 365 Da.

The derivatized Lan from tikitericin **1** and authentic synthetic standards were also analysed by HR ESI-MS using a Zorbax C_{18} column (100 mm x 4.6 mm i.d., 3.5 µm; Agilent Technologies). The binary pump was operated with a flow rate of 0.4 mL/min and a solvent system of eluent A: 99.9% H_2O/0.1% NH_4HCO_2, and eluent B: 99.9% MeCN/0.1% HCO_2H. The solvent was held at 40% B for 5 min, increased to 100% B over 30 sec, held at 100% B for 5 min, returned to 40% B over 1.5 min and held for 2 min.

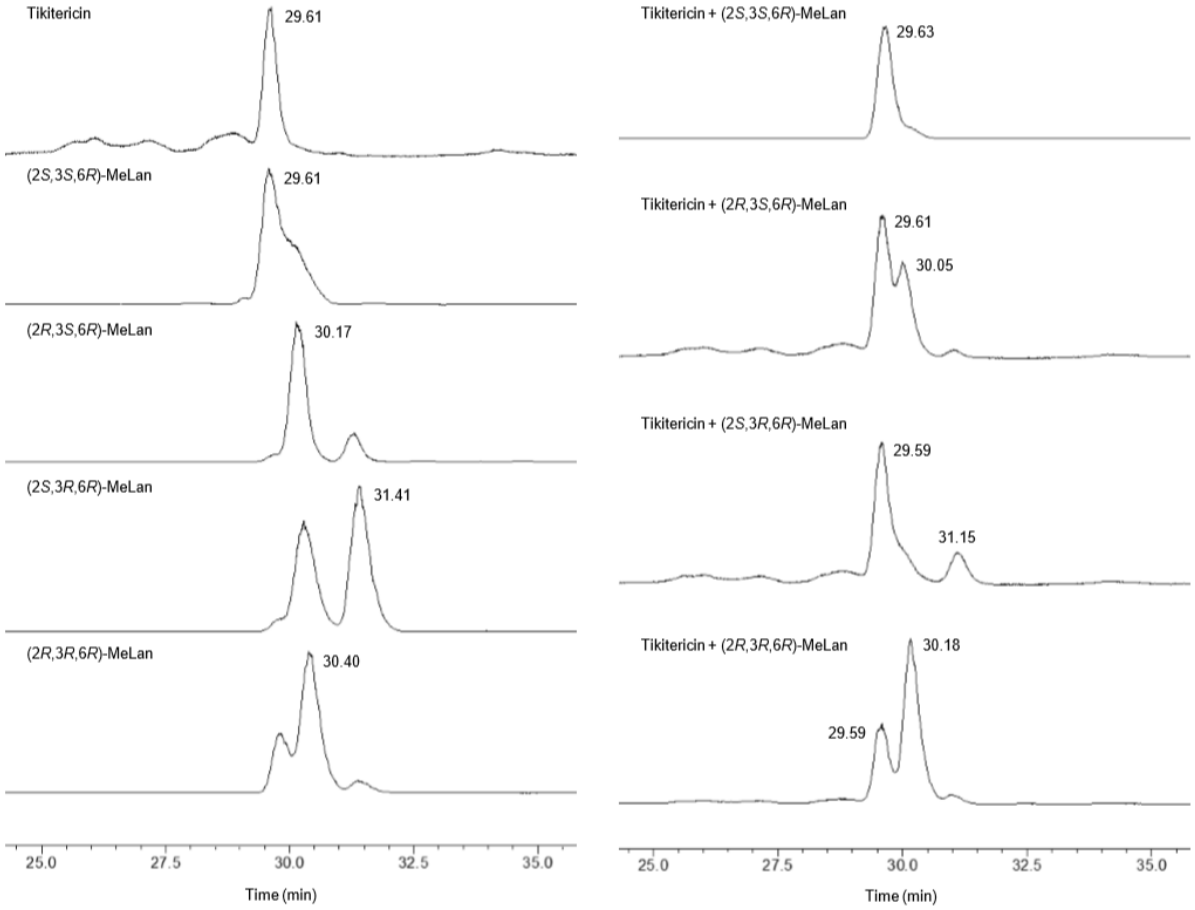


Figure S10. GCMS traces for synthetic, derivatized MeLan standards and hydrolysed and derivatized MeLan residues obtained from tikitericin **1**: a) the hydrolysed tikitericin **1** residue eluted with identical retention time to the (2*S*,3*S*,6*R*) synthetic standard; b) a single peak was observed when the tikitericin **1** residue was co-injected with the (2*S*,3*S*,6*R*) synthetic standard, thus confirming the characterization of the tikitericin **1** MeLan bridges as (2*S*,3*S*,6*R*)-MeLan.

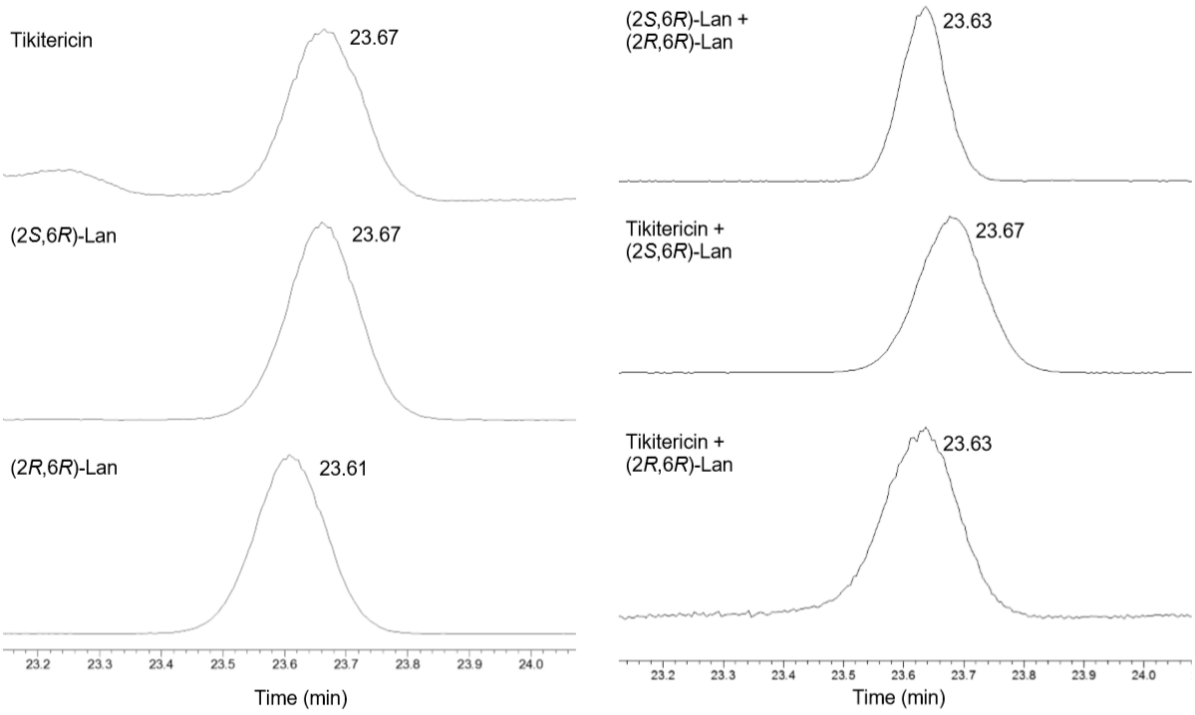
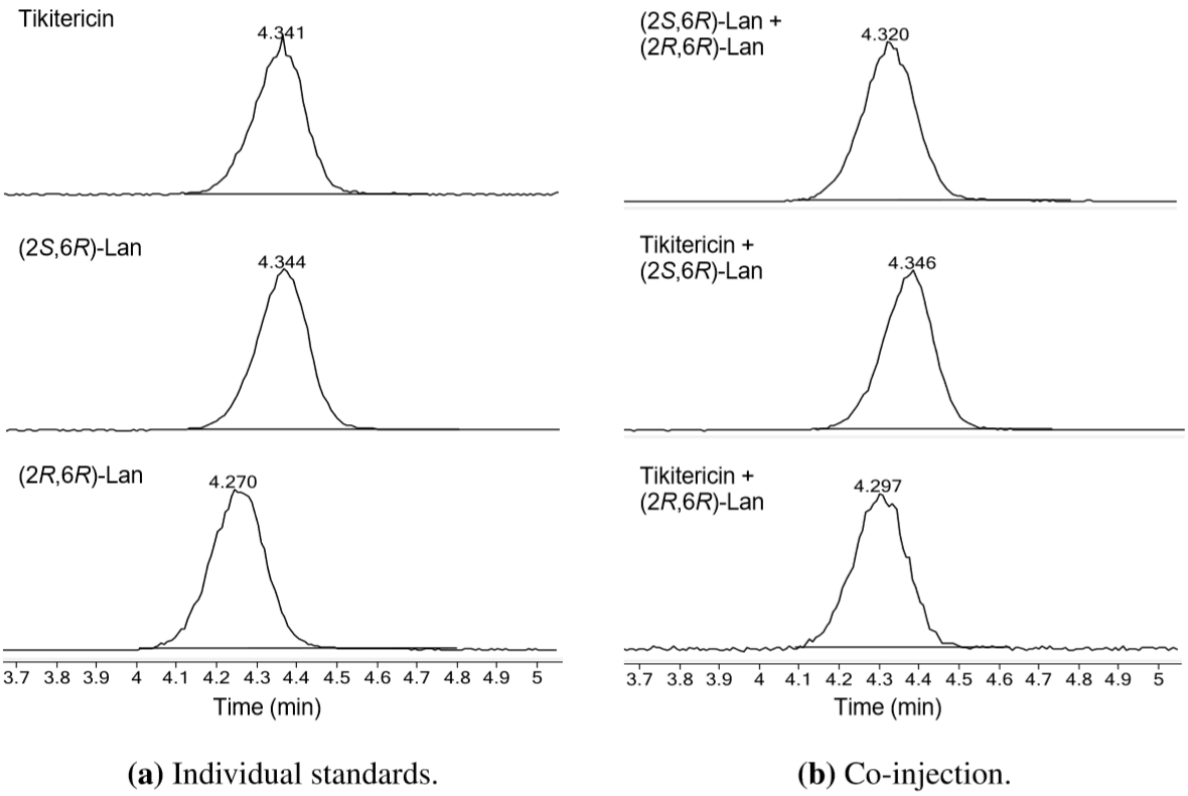


Figure S11. GCMS traces for synthetic, derivatized Lan standards and hydrolysed and derivatized Lan residues obtained from tikitericin **1**: a) when injected individually, residues obtained from tikitericin **1** eluted with the same retention time as the (2*S*,6*R*) standard; b) when the (2*S*,6*R*) and (2*R*,6*R*) standards were co-injected, a retention time between those of the individual standards was observed (23.63 min) and this shift in retention time was similarly observed for a co-injection of tikitericin **1** derived residues with the (2*R*,6*R*)-Lan standard. A co-injection of tikitericin **1** derived residues with the (2*S*,6*R*)-Lan standard did not shift the previously observed retention time of tikitericin **1** derived residues, supporting (2*S*,6*R*) configuration of the tikitericin **1** Lan bridge.



(a) Individual standards. **(b)** Co-injection.

Figure S12. HR ESI-MS traces for synthetic, derivatized Lan standards and hydrolysed and derivatized Lan residues obtained from tikitericin **1**: a) when injected individually, residues obtained from tikitericin **1** eluted with the same retention time as the (2*S*,6*R*) standard; b) when the two standards were co-injected, a retention time between those of the individual standards was observed (4.32 min). A co-injection of tikitericin with the (2*R*,6*R*) standard saw a shift in retention time (4.30 min), while a shift in retention time was not observed for a co-injection of tikitericin with the (2*S*,6*R*) standard, thus supporting (2*S*,6*R*) configuration of the tikitericin **1** Lan bridge.

2. Synthesis of tikitericin 1

2.1 General Information

All the reagents purchased from commercial sources were reagent grade and were used without further purification. Solvents for peptide synthesis and RP-HPLC were purchased as synthesis grade and HPLC grade, respectively. All the required normal Fmoc protected amino acids were purchased from GL Biochem (Shanghai, China). Tentalgel-PHB resin and Polystyrene resin were supplied by Rapp Polymere GmbH (Tuebingen, Germany). Chemmatrix resin was purchased from PCAS BioMatrix Inc. (Quebec, Canada). (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP), 6-chloro-1-hydroxybenzotriazole (6-Cl-HOBt) were obtained from Aapptec (Louisville, USA). *N*,*N*-Diisopropylcarbodiimide (DIC), 1,2-ethanedithiol (EDT), triisopropylsilane (TIPS), phenylsilane, sodium diethyldithiocarbamate, diethylether (Et_2O), piperidine, N,N-diisopropylethylamine (iPr_2NEt), formic acid and morpholine were purchased from Sigma-Aldrich (St. Louis, USA). Tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) were obtained from AK Scientific (Union City, USA). *N*,*N*-Dimethylformamide (DMF, AR grade), acetonitrile (CH_3CN, HPLC grade) and trifluoroacetic acid (TFA) were purchased from Scharlau (Barcelona, Spain). Dichloromethane (CH_2Cl_2) was obtained from ECP Limited (Auckland, New Zealand).

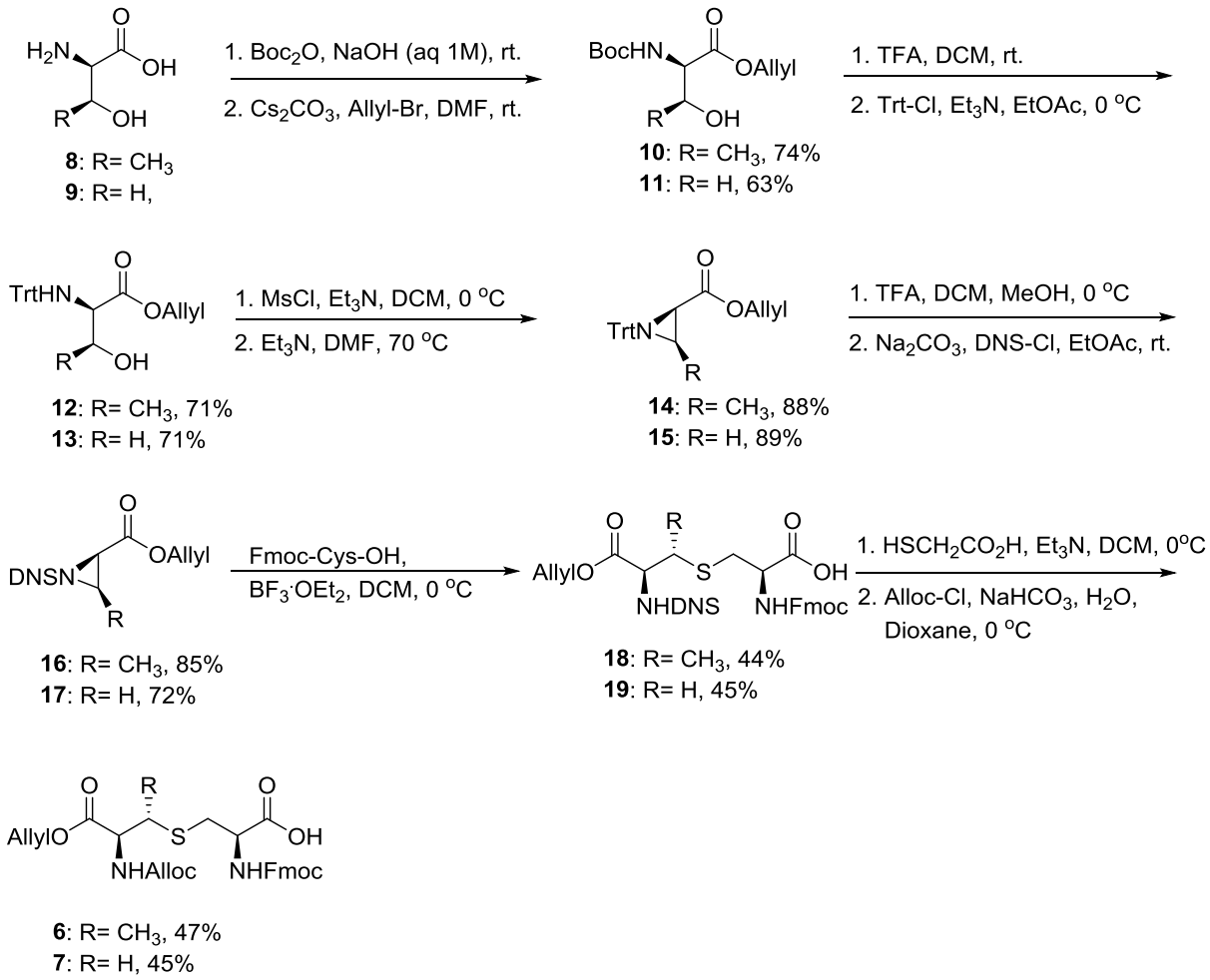
Unless stated otherwise, all reactions were performed under an atmosphere of nitrogen using standard techniques. Analytical thin-layer chromatography (TLC) was carried out using Kieselgel F_{254} 200 µm (Merck) silica plates. The compounds were then visualised by ultraviolet fluorescence or by staining with ninhydrin followed by heating of the plate for a few minutes. Column chromatography was performed using Kieselgel F_{254} S 63-100 µm silica gel with the indicated eluent. Optical rotations were determined at the sodium D line (589 nm) at 25 °C on a Perkin Elmer 341 instrument. Infrared spectra were recorded on a Perkin Elmer (Waltham, MA, USA) Spectrum 100 infrared spectrometer and reported in wavenumbers (ν, cm^{-1}). High resolution mass spectra were recorded on a Bruker micrOTOFQ mass spectrometer.

Nuclear magnetic resonance (NMR) spectra were recorded at room temperature on either a Bruker (Billerica, MA, USA) AVANCE 400 spectrometer (^1H, 400 MHz; ^{13}C, 100 MHz) or a Bruker AVANCE HD 500 spectrometer (^1H, 500 MHz; ^{13}C, 125 MHz). All chemical shifts are reported in parts per million (ppm) from tetramethylsilane ($\delta = 0$) and were measured relative to the solvent in which the sample was analysed ($CDCl_3$: δ 7.26 for ^1H NMR, δ 77.16 for ^{13}C NMR; $(CD_3)_2SO$: δ 2.50 for ^1H NMR, δ 39.52 for ^{13}C NMR; CD_3OD: δ 3.31 for ^1H NMR, δ 49.00 for ^{13}C NMR). The ^1H NMR shift values are reported as chemical shift (δ_H), the corresponding integral, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, td = triplet of doublets, qd = quartet of doublets), coupling constant (*J* in Hz) and assignments. ^{13}C NMR values are reported as the chemical shift (δ_C), the degree of hybridisation and assignment.

Semi-preparative RP-HPLC was performed on a Thermo Scientific (Waltham, MA, USA) Dionex Ultimate 3000 UHPLC equipped with a four channel UV Detector at 210, 225, 254 and 280 nm using either an analytical column (Phenomenex (Torrance, CA, USA) Gemini C_{18}, 110 Å, 250 mm x 4.6 mm, 5 µm) at a flow rate of 1 mL min^{-1} or a semi-preparative column (Phenomenex Gemini C_{18}, 110 Å, 250 mm x 10 mm, 5 µm) at a flow rate of 5 mL min^{-1}.

LC-MS spectra were acquired on either an Agilent Technologies (Santa Clara, CA, USA) 1120 Compact LC equipped with a Hewlett-Packard (Palo Alto, CA, USA) 1100 MSD mass spectrometer or an Agilent Technologies 1260 Infinity LC equipped with an Agilent Technologies 6120 Quadrupole mass spectrometer. An analytical column (Agilent C3, 150 mm x 3.0 mm, 3.5 µm) was used at a flow rate of 0.3 mL min^{-1} using a linear gradient of 5% B to 95% B over 30 min, where solvent A was 0.1% formic acid in H_2O and B was 0.1% formic acid in acetonitrile.

2.2 Synthesis of allyl- and alloc-protected methyllanthionine and lanthionine



8: R= CH_3
9: R= H,

10: R= CH_3, 74%
11: R= H, 63%

12: R= CH_3, 71%
13: R= H, 71%

14: R= CH_3, 88%
15: R= H, 89%

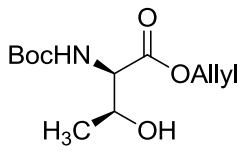
16: R= CH_3, 85%
17: R= H, 72%

18: R= CH_3, 44%
19: R= H, 45%

6: R= CH_3, 47%
7: R= H, 45%

Scheme 1. Optimized synthetic route for the orthogonally protected (Me)Lan building blocks 6 and 7

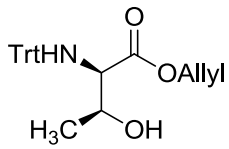
(2R,3S)-Allyl 2-((*tert*-butoxycarbonyl)amino)-3-hydroxybutanoate (10)



To a solution of D-threonine (2.0 g, 16.8 mmol) in 1 N aq. NaOH (40 mL) and dioxane (20 mL) at 0 °C was added di-*tert*-butyl dicarbonate (4.4 g, 20.2 mmol) and the resultant mixture was allowed to warm to r.t. and stirred for 24 h. Dioxane was then removed *in vacuo* and the remaining aqueous solution was washed with Et_2O (60 mL) to remove excess di-*tert*-butyl dicarbonate. EtOAc (100 mL) and 1M aq. HCl solution were then added to the aqueous layer to adjust the pH of the mixture to 2-3. The acidified mixture was then extracted with EtOAc (3 × 100 mL) and the combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo* to afford *N*-Boc-(R)-threonine (3.5 g) as a crude

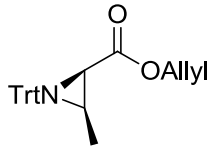
oil without further purification. To a solution of this crude carbamate (3.5 g) in DMF (35 mL) at 0 °C was added cesium carbonate (4.8 g, 14.8 mmol), followed by allyl bromide (1.4 mL, 16.3 mmol). The mixture was allowed to warm to room temperature and stirred overnight, after which the solvent was removed *in vacuo*. The crude residue was then dissolved in EtOAc (100 mL), washed with H_2O (120 mL) and the separated aqueous layer was further extracted with EtOAc (3 × 100 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (Petroleum ether-EtOAc 2:1) to afford *title compound* **10** (2.78 g, 73%) as a colourless oil. $[\alpha]_D^{25}$ +9.5 (*c* 0.83, $CHCl_3$); IR ν_{max} (neat): 3433, 2980, 1692, 1503, 1367, 1159, 735 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 5.90 (m, 1H, C<u>H</u>=CH_2), 5.43 (bs, 1H, Thr-N<u>H</u>), 5.32 (d, 1H, *J* = 17.3 Hz, CH=C<u>H</u>H), 5.23 (d, 1H, *J* = 10.4 Hz, CH=CH<u>H</u>), 4.65 (d, 2H, *J* = 5.4 Hz, OC<u>H</u>$_2$CH=), 4.31-4.24 (m, 2H, Thr-H_α and Thr-H_β), 3.45 (bs, 1H, Thr-OH), 1.43 (s, 9H, ^{t}-Bu), 1.24 (d, 3H, *J* = 6.3 Hz, C<u>H</u>$_3$); ^{13}C NMR (100 MHz, $CDCl_3$): δ171.2, 156.2, 131.5, 118.8, 80.1, 68.1, 66.1, 58.9, 28.3, 19.9. HRMS (ESI) *m/z* calculated for $C_{12}H_{21}NNaO_5$ $(M+Na)^+$ 282.1312, found 282.1322.

(2R,3S)-allyl 3-hydroxy-2-(tritylamino)butanoate (12)



To a solution of compound **10** (8.8 g, 33.9 mmol) in CH_2Cl_2 (90 mL) was added TFA (90 mL) at 0 °C and the resultant mixture was allowed to warm to r.t. and stirred for 2 h. The volatiles was then removed *in vacuo* and the remaining residue was dissolved in EtOAc (100 mL) and cooled to 0 °C. Et_3N (28.4 mL, 203.5 mmol) was added dropwise and stirred for 10 min, followed by the addition of Trt-Cl (17.0 g, 61.1 mmol) in EtOAc (100 mL) *via* an addition funnel over 45 min. The mixture was allowed to warm to r.t. and stirred for 16 h. H_2O (150 mL) was added to the reaction mixture and the separated aqueous layer was further extracted with EtOAc (2 × 100 mL). The combined organic layers were then washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (Petroleum ether-EtOAc 5:1) to afford *title compound* **12** (9.0 g, 66%) as a colourless oil. $[\alpha]_D^{25}$ -9.5 (*c* 0.73, $CHCl_3$); (lit.[3] $[\alpha]_D$ -9.02 (c 2.3, $CHCl_3$)); ^1H NMR (400 MHz, $CDCl_3$): δ 7.47 (d, 6H, *J* = 7.7 Hz, Trt-H), 7.28-7.16 (m, 9H, Trt-H), 5.71-5.61 (m, 1H, C<u>H</u>=CH_2), 5.19-5.13 (m, 2H, CH=C<u>H</u>$_2$), 4.07 (dd, 1H, *J* = 13.1, 6.0 Hz, OC<u>H</u>$_2$CH=), 3.86 (dd, 1H, *J* = 13.1, 6.0 Hz, OC<u>H</u>$_2$CH=), 3.79 (app. dd, 1H, *J* = 6.2 Hz, Thr-CH_β), 3.40 (d, 1H, *J* = 7.4 Hz, Thr-CH_α), 1.21 (d, 3H, *J* = 6.2 Hz, Thr-C<u>H</u>$_3$); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.9, 145.5, 131.5, 128.9, 127.9, 126.7, 118.9, 70.8, 69.8, 65.7, 62.6, 19.0. The NMR data were consistent with those reported in the literature.[3]

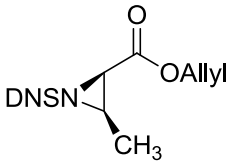
(2R,3R)-allyl 3-methyl-1-tritylaziridine-2-carboxylate (14)



To a solution of compound **12** (2.2 g, 5.5 mmol) in CH_2Cl_2 (25 mL) at 0 °C was added Et_3N (2.3 mL, 16.5 mmol) and after 5 minutes Ms-Cl (0.47 mL, 6.06 mmol) was added dropwise. The resultant mixture was allowed to warm to r.t. and stirred overnight. NH_4Cl (sat.) (25 mL) was then added to quench the reaction and the separated aqueous layer was further extracted with CH_2Cl_2 (3 x 25 mL). The combined organic layers were then washed with brine, dried over anhydrous Na_2SO_4, filtered

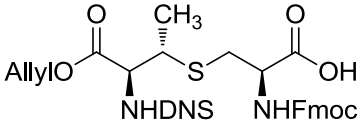
and concentrated *in vacuo* to give an orange oil which was used without further purification. To a solution of this intermediate in DMF (20 mL) was added Et_3N (0.92 mL, 6.61 mmol) and the reaction mixture was heated at 70 °C overnight. After the reaction was cooled to r.t., H_2O (80 mL) and Et_2O (80 mL) were added to the mixture and the separated aqueous layer was further extracted with Et_2O (3 x 80 mL). The combined organic layers were then washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (Petroleum ether-EtOAc 10:1) to afford *title compound* **14** (1.86 g, 88.2%) as a colourless oil. $[\alpha]_D^{25}$ +97.5 (*c* 0.71, $CHCl_3$); (lit.[3] $[\alpha]_D$ +98.4 (c 1.20, $CHCl_3$)); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.52 (d, 6H, *J* = 7.6 Hz, Trt-H), 7.28-7.18 (m, 9H, Trt-H), 5.97-5.88 (m, 1H, C<u>H</u>=CH_2), 5.32 (d, 1H, *J* = 17.4 Hz, CH=C<u>H</u>H), 5.23 (d, 1H, *J* = 10.4 Hz, CH=CH<u>H</u>), 4.66 (m, 2H, OC<u>H</u>$_2$CH=), 1.91 (d, 1H, *J* = 6.5 Hz, Thr-H_α), 1.64 (q, 1H, *J* = 5.5 Hz, Thr-H_β), 1.38 (d, 3H, *J* = 5.5 Hz, Thr-C<u>H</u>$_3$); ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.9, 143.9, 132.2, 129.4, 127.6, 126.9, 118.5, 75.0, 65.4, 36.0, 34.9, 13.3. The NMR data were consistent with those reported in the literature.[3]

(2R,3R)-allyl 1-((2,4-dinitrophenyl)sulfonyl)-3-methylaziridine-2-carboxylate (16)



To a solution of compound **14** (1.9 g, 4.9 mmol) in CH_2Cl_2 (15 mL) and MeOH (5 mL) was added TFA (2.2 mL, 29.1 mmol) dropwise at 0 °C and the resultant mixture was stirred for 2 h at 0 °C. The solvent was then removed *in vacuo* and the residue was partitioned between Et_2O (20 mL) and H_2O (20 mL), and the separated organic layer was further extracted with water (3 x 20 mL). The combined aqueous layers were basified to pH 9 with Na_2CO_3 (sat.) at 0 °C. EtOAc (60 mL) was added to the aqueous solution followed by 2,4-dinitrobenzenesulfonyl chloride (3.0 g, 11.2 mmol) at 0 °C. The biphasic system was warmed to r.t. and stirred vigorously for 24 h. The two layers were then separated and the separated aqueous layer was extracted with EtOAc (3 x 60 mL). The combined organic layers were then washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (Petroleum ether-EtOAc 4:1) to afford *title compound* product **16** (1.36 g, 76 %) as a light yellow oil. $[\alpha]_D^{25}$ +22.7 (*c* 0.78, $CHCl_3$); (lit.[4] $[\alpha]_D^{25}$ +21.3 (c 0.85, $CHCl_3$)); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.65 (d, 1H, *J* = 2.0 Hz, Ar-H), 8.59 (dd, 1H, *J* = 8.6, 2.0 Hz, Ar-H), 8.54 (d, 1H, *J* = 8.6 Hz, Ar-H), 5.97-5.87 (m, 1H, C<u>H</u>=CH_2), 5.36 (d, 1H, *J* = 17.0 Hz, CH=C<u>H</u>H), 5.29 (d, 1H, *J* = 9.0 Hz, CH=CH<u>H</u>), 4.71-4.68 (m, 2H, OC<u>H</u>$_2$CH=), 3.80 (d, 1H, *J* = 7.6 Hz, Thr-H_α), 3.48 (dq, 1H, *J* = 5.6, 7.6 Hz, Thr-H_β), 1.46 (d, 3H, *J* = 5.8 Hz, C<u>H</u>$_3$); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.7, 150.4, 148.6, 137.6, 133.4, 131.1, 127.1, 120.3, 119.5, 66.6, 43.9, 43.3, 12.4. The NMR data were consistent with those reported in the literature.[4]

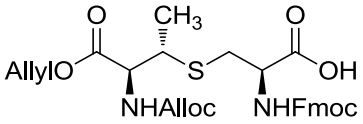
(R)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(((2S,3S)-4-(allyloxy)-3-((2-nitro-4-sulfophenyl)amino)-4-oxobutan-2-yl)thio)propanoic acid (18)



To a solution of compound **16** (3.1 g, 8.2 mmol) in CH_2Cl_2 (150 mL) was added Fmoc-Cys-OH (11.3 g, 32.8 mmol) followed by $BF_3{\cdot}OEt_2$ (8.1 mL, 65.6 mmol) at 0 °C .The resultant mixture was allowed to warm to r.t. and stirred for 48 h. The solvent was then removed *in vacuo* to give a yellow oil which was purified by flash chromatography (Petroleum ether-EtOAc-HOAc 7:3:0.2) to afford *title compound* **18** (2.75 g, 44 %) as a yellow solid. $[\alpha]_D^{25}$ +48.1 (*c* 0.36, $CHCl_3$); (lit.[4] $[\alpha]_D^{25}$ -17.5 (c 0.2,

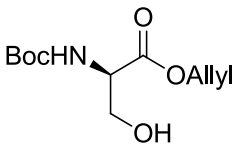
$CHCl_3$)); ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.69 (d, 1H, *J* = 2.1 Hz, DNs-H), 8.47 (dd, 1H, *J* = 8.6, 2.3 Hz, DNs-H), 8.26 (d, 1H, *J* = 8.6 Hz, DNs-H), 7.76 (d, 2H, *J* = 7.6 Hz, Fmoc-ArH), 7.58 (m, 2H, Fmoc-ArH), 7.40 (t, 2H, *J* = 7.4 Hz, Fmoc-ArH), 7.30 (t, 2H, *J* = 7.4 Hz, Fmoc-ArH), 6.65 (d, 1H, *J* = 9.5 Hz, NH), 5.72 (m, 1H, C<u>H</u>=CH_2), 5.63 (d, 1H, *J* = 7.6 Hz, NH), 5.22-5.17 (m, 2H, CH=C<u>H_2</u>), 4.60 (app d, 1H, *J* = 6.1 Hz, H_α), 4.50-4.40 (m, 4H, Fmoc-CH_2 and OC<u>H_2</u>CH=), 4.34 (dd, 1H, *J* = 9.4, 2.8 Hz, H_α), 4.23 (t, 1H, *J* = 5.5 Hz, Fmoc-CH), 3.51 (m, 1H, H_β), 3.07 (dd, 1H, *J* = 13.8, 4.2 Hz, H_β), 2.90 (dd, 1H, *J* = 13.8, 5.7 Hz, H_β), 1.42 (d, 3H, *J* = 7.0 Hz, C<u>H_3</u>); ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.1, 169.2, 156.0, 149.8, 147.8, 143.6, 143.5, 141.3, 139.6, 132.0, 130.6, 127.9, 127.2, 125.0, 120.9, 120.1, 120.0, 67.5, 67.0, 61.7, 53.4, 47.0, 43.4, 33.6, 19.7. The NMR data were consistent with those reported in the literature.[4]

(R)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(((2S,3S)-4-(allyloxy)-3-(((allyloxy)carbonyl)amino)-4-oxobutan-2-yl)thio)propanoic acid (6)



To a solution of compound **18** (0.20 g, 0.28 mmol) in CH_2Cl_2 (5 mL) was added thioglycolic acid (30 µl, 0.42 mmol) and Et_3N (0.16 mL, 1.12 mmol) at 0 °C and the resultant mixture was allowed to warm to r.t. and stirred for 2 h. The reaction was then quenched with Na_2CO_3 (sat.) (5 mL) and the separated aqueous layer was extracted with CH_2Cl_2 (2 x 5 mL). The combined organic layers were then washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to give an orange red oil without further purification. The oil was dissolved in water (5 mL) and dioxane (5 mL) at 0 °C and $NaHCO_3$ (0.09 g, 1.12 mmol) and alloc-Cl (90 µL, 0.84 mmol) were added, and the reaction was allowed to warm to r.t. and stirred overnight. Dioxane was then removed *in vacuo*, and the remaining aqueous solution was diluted with water (5 mL) and extracted with EtOAc (3 x 10 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (Petroleum ether-EtOAc-HOAc 7:3:0.2) to afford *title compound* **6** (75 mg, 47%) as a pale yellow solid. $[\alpha]_D^{25}$ -1.4 (*c* 0.2, CH_2Cl_2); (lit.[4] $[\alpha]_D^{25}$ -0.03 (c 0.4, CH_2Cl_2)); ^{1}H NMR (400 MHz, CD_3OD): δ 7.80 (d, 2H, *J* = 7.5 Hz, Fmoc-ArH), 7.70 (d, 2H, *J* = 7.5 Hz, Fmoc-ArH), 7.41 (t, 2H, *J* = 7.4 Hz, Fmoc-ArH), 7.33 (t, 2H, *J* = 7.4 Hz, Fmoc-ArH), 7.16 (d, 1H, *J* = 9.0 Hz, NH), 6.03-5.91 (m, 2H, C<u>H</u>=CH_2), 5.40-5.31 (m, 2H, CH=C<u>H_2</u>), 5.26-5.19 (dd, 2H, *J* = 17.8, 10.4 Hz, CH=C<u>H_2</u>), 4.66 (d, 2H, *J* = 5.0 Hz, OC<u>H_2</u>CH=), 4.58 (d, 2H, *J* = 5.2 Hz, OC<u>H_2</u>CH=), 4.51-4.33 (m, 4H, Fmoc-CH_2, Fmoc-CH and H_α), 4.26 (t, 1H, *J* = 7.0 Hz, H_α), 3.47 (m, 1H, H_β), 3.11 (dd, 1H, *J* = 13.9, 4.7 Hz, H_β), 2.86 (dd, 1H, *J* = 13.6, 8.6 Hz, H_β), 1.33 (d, 3H, *J* = 7.0 Hz, C<u>H_3</u>); ^{13}C NMR (100 MHz, CD_3OD): δ 173.8, 171.7, 158.6, 158.4, 145.3, 145.2, 142.6, 134.2, 133.1, 128.8, 128.2, 126.3, 120.9, 119.2, 117.8, 68.2, 67.2, 66.9, 60.2, 55.3, 48.4, 43.8, 34.2, 19.8. The NMR data were consistent with those reported in the literature.[5]

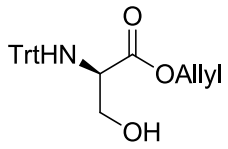
(R)-allyl 2-((tert-butoxycarbonyl)amino)-3-hydroxypropanoate (11)



To a solution of D-serine (1.05 g, 9.5 mmol) in 1 N aq. NaOH (20 mL) and dioxane (10 mL) at 0 °C was added di-*tert*-butyl dicarbonate (2.53 g, 11.4 mmol) and the resultant mixture was allowed to warm to r.t. and stirred for 24 h. Dioxane was then removed *in vacuo* and the remaining aqueous was washed with Et_2O (30 mL) to remove excess di-*tert*-butyl dicarbonate. EtOAc (50 mL) and 1M aq. HCl solution were then added to the aqueous layer to adjust the pH of the mixture to 2-3. The

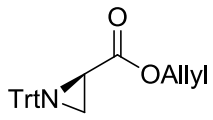
acidified mixture was then extracted with EtOAc (3 × 50 mL) and the combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to give *N*-Boc-(R)-serine (2.0 g) as a colourless oil without further purification. To a solution of this crude intermediate (2.0 g) in DMF (20 mL) was then added cesium carbonate (3.5 g, 10.7 mmol), followed by allyl bromide (1.0 mL, 11.7 mmol) at 0 °C. The mixture was allowed to warm to r.t. and stirred overnight, after which the solvent was removed *in vacuo*. The crude residue was then dissolved in EtOAc (50 mL), and washed with H_2O (60 mL) and the separated aqueous layer was further extracted with EtOAc (2 × 50 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (Petroleum ether-EtOAc 2:1) to afford *title compound* **11** (1.43 g, 60%) as a colourless oil. $[\alpha]_D^{25}$ -0.56 (*c*, 0.89, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.97-5.87 (m, 1H, C<u>H</u>=CH_2), 5.54 (bs, 1H, Ser-N<u>H</u>), 5.35 (app dq, 1H, *J* = 17.1, 1.0 Hz, CH=C<u>H</u>H), 5.26 (app dq, 1H, *J* = 10.4, 0.8 Hz, CH=CH<u>H</u>), 4.68 (d, 2H, *J* = 5.8 Hz, OC<u>H_2</u>CH=), 4.40 (bs, 1H, Ser-H_α), 3.95 (m, 2H, Ser-H_β), 2.76 (bs, 1H, Ser-OH), 1.45 (s, 9H, ^{t}-Bu); ^{13}C NMR (100 MHz, $CDCl_3$): δ170.5, 155.7, 131.4, 118.9, 80.4, 66.2, 63.7, 55.9, 28.3. The NMR data were consistent with those reported in the literature.[6]

(R)-allyl 3-hydroxy-2-(tritylamino)propanoate (13)



To a solution of compound **11** (1.05 g, 4.3 mmol) in CH_2Cl_2 (10 mL) was added TFA (10 mL) at 0 °C and the resultant mixture was allowed to warm to r.t. and stirred for 2 h. The volatiles was then removed *in vacuo* and the remaining residue was dissolved in EtOAc (10 mL) and cooled to 0 °C. Et_3N (3.6 mL, 25.8 mmol) was added dropwise and stirred for 10 min, followed by the addition of Trt-Cl (2.4 g, 8.6 mmol) in EtOAc (10 mL) *via* an addition funnel over 45 min. The reaction was allowed to warm to r.t. and stirred for 16 h. H_2O (20 mL) was added to the reaction mixture and the separated aqueous layer was further extracted with EtOAc (2 × 20 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (Petroleum ether-EtOAc 5:1) to afford *title compound* **13** (1.15 g, 70%) as a colourless oil. $[\alpha]_D^{25}$ -2.8 (*c* 0.83, $CHCl_3$); (lit.[7] $[\alpha]_D^{19}$ -2.0 (c 0.75, $CHCl_3$)); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.49 (d, 6H, *J* = 7.7 Hz, Trt-H), 7.28-7.17 (m, 9H, Trt-H), 5.75-5.65 (m, 1H, C<u>H</u>=CH_2), 5.21-5.14 (m, 2H, CH=C<u>H_2</u>), 4.22 (dd, 1H, *J* = 13.1, 5.8 Hz, OC<u>H_2</u>CH=), 4.09 (dd, 1H, *J* = 13.1, 5.8 Hz, OC<u>H_2</u>CH=), 3.71 (app. dd, 1H, *J* = 6.2 Hz, Ser-CH_α), 3.55 (m, 2H, Ser- CH_β), 3.01 (bs, 1H, NH), 2.31 (bs, 1H, OH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.1, 145.6, 131.6, 128.7, 127.9, 126.7, 118.5, 71.1, 65.7, 64.9, 57.8. The NMR data were consistent with those reported in the literature.[3]

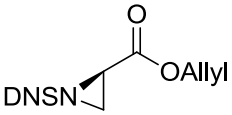
(R)-allyl 1-tritylaziridine-2-carboxylate (15)



To a solution of compound **13** (1.15 g, 3.0 mmol) in CH_2Cl_2 (10 mL) was added Et_3N (1.2 mL, 8.9 mmol) at 0 °C and after 5 minutes Ms-Cl (0.25 mL, 3.27 mmol) was added dropwise. The resultant mixture was allowed to warm to r.t. and stirred overnight. NH_4Cl (sat.) (10 mL) was then added to quench the reaction and the separated aqueous layer was further extracted with CH_2Cl_2 (3 x 10 mL). The combined organic layers were then washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to give as an orange oil without further purification. The oil was dissovled in DMF (10 mL), Et_3N (0.47 mL, 3.38 mmol) was added and the reaction mixture was heated at 70 °C overnight. After the reaction was cooled to

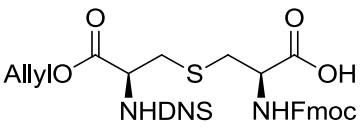
r.t., H_2O (50 mL) and Et_2O (60 mL) were added to the mixture and the separated aqueous layer was further extracted with Et_2O (3 x 60 mL). The combined organic layers were then washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (Petroleum ether-EtOAc 10:1) to afford *title compound* **15** (0.98 g, 89%) as a colourless oil. $[\alpha]_D^{25}$ +77.6 (*c* 0.93, $CHCl_3$); (lit.[3] $[\alpha]_D^{25}$ +73.6 (c 1.00, $CHCl_3$)); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.49 (d, 6H, *J* = 7.4 Hz, Trt-H), 7.31-7.20 (m, 9H, Trt-H), 5.99-5.89 (m, 1H, C<u>H</u>=CH_2), 5.34 (app dq, 1H, *J* = 17.2, 1.4 Hz, CH=C<u>H</u>H), 5.26 (app dq, 1H, *J* = 10.4, 0.9 Hz, CH=CH<u>H</u>), 4.67 (m, 2H, OC<u>H</u>$_2$CH=), 2.27 (dd, 1H, *J* = 2.3, 1.5 Hz, Ser-H_α), 1.91 (dd, 1H, *J* = 6.1, 2.6 Hz, Ser-H_β), 1.41 (dd, 1H, *J* = 6.3, 1.4 Hz, Ser-H_β); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.2, 143.6, 132.0, 129.3, 127.6, 126.9, 118.5, 74.4, 65.5, 31.7, 28.7. The NMR data were consistent with those reported in the literature.[3]

(R)-allyl 1-((2,4-dinitrophenyl)sulfonyl)aziridine-2-carboxylate (17)



To a solution of compound **15** (0.36 g, 0.98 mmol) in CH_2Cl_2 (6 mL) and MeOH (2 mL) was added TFA (0.46 mL, 6.01 mmol) dropwise at 0 °C and the resultant mixture was stirred for 2 h at 0 °C. The solvent was then removed *in vacuo* and the remaining residue was partitioned between Et_2O (5 mL) and H_2O (5 mL), and the separated ether layer was extracted with water (3 x 5 mL). The combined aqueous layers were basified to pH 9 with Na_2CO_3 (sat.) at 0 °C. EtOAc (15 mL) was added to the aqueous solution followed by 2,4-dinitrobenzenesulfonyl chloride (0.60 g, 2.25 mmol) at 0 °C. The biphasic system was warmed to r.t. and stirred vigorously for 24 h. The two layers were then separated and the separated aqueous layer was extracted with EtOAc (3 x 15 mL). The combined organic layers were then washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (Petroleum ether-EtOAc 4:1) to afford *title compound* **17** (0.26 g, 73 %) as a light yellow oil. $[\alpha]_D^{25}$ +37.1 (*c* 1.17, CH_2Cl_2); (lit.[4] $[\alpha]_D^{25}$ +0.2 (c 0.49, CH_2Cl_2)); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.63 (d, 1H, *J* = 2.1 Hz, DNs-H), 8.58 (dd, 1H, *J* = 8.6, 2.1 Hz, DNs-H), 8.50 (d, 1H, *J* = 8.6 Hz, DNs-H), 5.94 (ddt, 1H, *J* = 22.5, 17.4, 6.0 Hz, C<u>H</u>=CH_2), 5.38 (app dq, 1H, *J* = 17.1, 1.3 Hz, CH=C<u>H</u>H), 5.30 (app dq, *J* = 10.5, 1.2 Hz, CH=CH<u>H</u>), 4.71-4.68 (m, 2H, -C<u>H</u>$_2$CH=), 3.71 (dd, 1H, *J* = 7.0, 4.5 Hz, H_α), 3.15 (d, 1H, *J* = 7.1 Hz, H_β), 2.87 (d, 1H, *J* = 4.5 Hz, H_β); ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.8, 150.6, 146.5, 137.0, 133.4,130.9, 126.9, 120.2, 119.6, 66.9, 38.3, 34.7. The NMR data were consistent with those reported in the literature.[4]

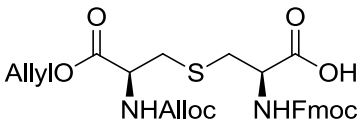
(R)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(((S)-3-(allyloxy)-2-((2-nitro-4-sulfophenyl)amino)-3-oxopropyl)thio)propanoic acid (19)



To a solution of compound **17** (65 mg, 0.18 mmol) in CH_2Cl_2 (5 mL) was added Fmoc-Cys-OH (0.25 g, 0.73 mmol) followed by $BF_3{\cdot}OEt_2$ (0.18 mL, 1.46 mmol) at 0 °C. The resultant mixture was allowed to warm to r.t. and stirred for 48 h. The solvent was then removed *in vacuo* to give a yellow oil which was purified by flash chromatography (Petroleum ether-EtOAc-HOAc 7:3:0.2) to afford *title compound* **19** (58 mg, 45%) as a yellow solid. $[\alpha]_D^{25}$ +48.3 (*c* 1.35, CH_2Cl_2); (lit.[4] $[\alpha]_D^{25}$ +0.11(c 0.3, CH_2Cl_2)); ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.82 (s, 1H, DNs-H), 8.61 (d, 1H, *J* = 7.6 Hz, DNs-H), 8.31 (d, 1H, *J* = 8.7 Hz, DNs-H), 7.90 (d, 2H, *J* = 7.2 Hz, Fmoc-ArH), 7.72 (m, 4H, Fmoc-ArH and 2NH), 7.42 (t, 2H, *J* = 7.1 Hz, Fmoc-ArH), 7.33 (t, 2H, *J* = 7.1 Hz, Fmoc-ArH), 5.70 (m, 1H, C<u>H</u>=CH_2), 5.22 (d, 1H, *J* = 17.3 Hz, CH=C<u>H</u>H), 5.11 (d, 1H, *J* = 10.4 Hz, CH=CH<u>H</u>), 4.44 (d, 2H, *J* = 4.5 Hz, -C<u>H</u>$_2$CH=), 4.27 (m, 4H, Fmoc-CH_2, Fmoc-CH and H_α), 4.02 (m, 1H, H_α), 3.02 (dd, 1H, *J* = 13.8, 4.8 Hz, H_β), 2.93 (dd, 1H, *J* =

13.7, 4.1 Hz, H_β), 2.86 (m, 1H, H_β), 2.74 (dd, 1H, *J* = 12.3, 10.0 Hz, H_β); ^{13}C NMR (100 MHz, DMSO-d_6): δ 172.0, 169.1, 155.9, 149.6, 147.1, 143.7, 140.6, 138.2, 131.7, 127.6,127.1, 125.2, 120.1, 119.9, 118.1, 65.7, 65.5, 56.3, 53.9, 46.5, 33.6, 33.1. The ^{1}H NMR data were consistent with those reported in the literature.[4]

(R)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(((S)-3-(allyloxy)-2-(((allyloxy)carbonyl)amino)-3-oxopropyl)thio)propanoic acid (7)



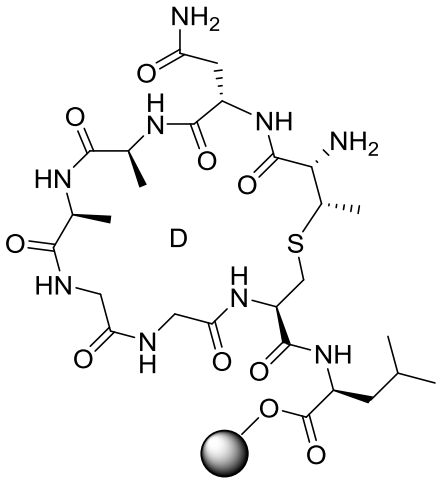
To a solution of compound **19** (182 mg, 0.26 mmol) in CH_2Cl_2 (5 mL) was added thioglycolic acid (30 µl, 0.40 mmol) and Et_3N (0.15 mL, 1.04 mmol) at 0 °C and the resultant mixture was allowed to warm to r.t. and stirred for 2 h. The reaction was then quenched with Na_2CO_3 (sat.) (5 mL) and the separated aqueous layer was extracted with CH_2Cl_2 (2 x 5 mL). The combined organic layers were then washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to give an orange red oil without further purification. The oil was dissolved in water (3 mL) and dioxane (3 mL) at 0 °C and $NaHCO_3$ (88 mg, 1.04 mmol) and alloc-Cl (80 µL, 0.78 mmol) were added, and the reaction was allowed to warm to r.t. and stirred overnight. Dioxane was then removed *in vacuo*, and the remaining aqueous was diluted with water (3 mL) and extracted with EtOAc (3 x 5 mL). The combined organic layers were then washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (Petroleum ether-EtOAc-HOAc 7:3:0.2) to afford *title compound* **7** (67 mg, 46%) as a pale yellow solid. $[\alpha]_D^{25}$ +5.5 (*c* 0.4, $CHCl_3$); (lit.[8] $[\alpha]_D^{26}$ +13.3 (c 3.3, $CHCl_3$)); ^{1}H NMR (400 MHz, CD_3OD): δ 7.81 (d, 2H, *J* = 7.5 Hz, Fmoc-ArH), 7.71 (d, 2H, *J* = 7.4 Hz, Fmoc-ArH), 7.41 (t, 2H, *J* = 7.5 Hz, Fmoc-ArH), 7.33 (t, 2H, *J* = 7.5 Hz, Fmoc-ArH), 5.99-5.89 (m, 2H, C<u>H</u>=CH_2), 5.37-5.30 (m, 2H, CH=C<u>H_2</u>), 5.23-5.17 (m, 2H, CH=C<u>H_2</u>), 4.63 (d, 2H, *J* = 5.5 Hz, OC<u>H_2</u>CH=), 4.56 (d, 2H, *J* = 5.3 Hz, OC<u>H_2</u>CH=), 4.50-4.32 (m, 4H, Fmoc-CH_2, Fmoc-CH and H_α), 4.27 (t, 1H, *J* = 7.0 Hz, H_α), 3.14-3.05 (m, 2H, SC<u>H_2</u>), 3.00-2.93 (m, 2H, SC<u>H_2</u>); ^{13}C NMR (100 MHz, CD_3OD) δ 173.9, 172.0, 158.5, 158.3, 145.3, 145.2, 142.6, 134.2, 133.1, 128.8, 128.2, 126.4, 120.9, 118.9, 117.7, 68.3, 67.1, 66.8, 55.6, 55.4, 48.4, 35.5, 35.2. The NMR data were consistent with those reported in the literature.[8]

2.3 Solid phase peptide synthesis of Tikitericin 1

2.3.1 General procedure for Fmoc Solid Phase Peptide Synthesis (SPPS):

The required Fmoc amino acid (5.0 equiv to resin loading) and 6-Cl-HOBt (5.0 equiv) were dissolved in DMF (5 mL) and to this solution, NMM (10.0 equiv) was added, followed by PyBOP (4.9 equiv). The resulting mixture was pre-activated for 5 min. This activated amino acid solution was then transferred to a pre-swelled resin and reacted for 40 min. The completion of coupling was ascertained by a negative Kaiser test and end capping was performed by exposing the resin to 20% Ac_2O in DMF (*v/v*) for 15 min. Subsequent Fmoc deprotection was achieved using 20% piperidine in DMF (*v/v*) for 2 x 5 mins.

2.3.2 Synthesis of ring D of Tikitericin (2)



Loading of Fmoc-Leu-HMPP (4-(Hydroxymethyl)phenoxypropanoic acid) linker on aminomethyl chemmatrix resin

The initial loading of 0.62 mmol/g of the aminomethyl chemmatrix resin was reduced to 0.10 mmol/g to avoid any interstrand dimerization during the on resin-cyclization step. [4, 5]

To ensure the exact loading, the aminomethyl chemmatrix resin (645 mg, 0.62 mmol/g, 0.4 mmol) was first swelled in CH_2Cl_2 for 30 min, followed by addition of Fmoc-Leu-HMPP linker (85 mg, 0.16 mmol) and DIC (124 uL, 0.80 mmol) that had been pre-activated in CH_2Cl_2 (5 mL) for 5 min. The resulting mixture was shaken for 24 h and filtered. The resin was then washed with DMF (10 mL x 3) and CH_2Cl_2 (10 mL x 3). Any remaining free resin sites were capped with 20 vol % Ac_2O in DMF for 15 min.

Loading test

Adjusted resin loading was calculated as follows:

To each 2 × 10 mm matched Starna Scientific Ltd cuvettes was placed 1.0 mg of dried resin in each cuvette. Fresh 20 vol % piperidine in DMF (*v*/*v*, 3 mL) is placed in a separate 1 x 10 mm Starna Scientific Ltd cuvette. This reference cuvette was placed in a SHIMADZU UV-1280 UV-VIS spectrophotometer and zero at 290 nm. To the 2 × 10 mm cuvettes with 1.0 mg of resin in each was added 20 vol % piperidine in DMF (*v*/*v*, 3 mL) and agitated for 15 min. To the spectrophotometer was placed, in turn, the cuvettes containing the settled resin and the average of the absorbance at 290 nm is recorded at 0.170 absorption. Loading is then calculated based on equation. Loading estimated to be 0.095 mmol/g.

$$\text{Loading (mmol/g)} = (\text{Abs}_{\text{sample}}) / (\text{mg of sample} \times 1.75)^{b}$$

[b] Based on Ɛ = 5253 M^{-1} cm^{-1}

Loading of Fmoc-Alloc/Allyl-MeLan and the required amino acids, deprotection of Allyl, Alloc, Fmoc groups and cyclization to form ring D on solid support

After standard Fmoc deprotection, the orthogonally protected Fmoc-Alloc/Allyl-MeLan (**6**) (2.0 equiv.) was pre-activated with PyBOP (1.9 equiv), 6-Cl-HOBt (2.0 equiv) and NMM (2.0 equiv.) in DMF for 5 min, reacted with the resin-bound peptide for 2 h, and then Fmoc deprotected by the standard protocol. Two Fmoc-Gly-OH were coupled/deprotected by the standard protocol. Two Fmoc-Ala-OH were coupled/deprotected by the standard protocol. Fmoc-Asn(Trt)-OH was coupled by the standard protocol, but the Fmoc was left intact.

A solution of $Pd(PPh_3)_4$ (2 equiv.) and $PhSiH_3$ (10 equiv.) in (1:1) DMF/ CH_2Cl_2 (5 mL) was reacted with the resin bound peptide, protected from light, for 2 h. The solution was drained and the resin was washed in the following sequence: 1) CH_2Cl_2 (2 x 10 mL), 2) 0.5% sodium diethyldithiocarbamate in DMF (3 x 10 mL), 3) DMF (2 x 10 mL). The colour of the resin changed to light yellow after these washings. The Fmoc group was removed with 20% piperidine in DMF (*v/v* 10 mL, 2 x 5 mins). The resin was washed with DMF (3 x10 mL). The cyclization to form ring D was done by adding a solution of PyBOP (4.9 equiv.), 6-Cl-HOBt (5.0 equiv.) and NMM (10.0 equiv.) in DMF (5 mL) to the linear peptide and reacted for 2 h. A small sample (5 mg) of resin was cleaved by shaking with 95/2.5/2.5 (TFA/TIPS/H_2O) for 1 h and then filtered to remove the resin. The filtrate was concentrated *in vacuo* and precipitated with Et_2O to give an off-white solid. ESI-MS: calculated for $C_{27}H_{45}N_9O_{10}S$ $(M+H)^+$ 688.8, found 688.3. No dimer or uncyclized linear precursor peptides were detected by ESI-MS.

The resin (50 mg, 0.005 mmol) was cleaved with (95/2.5/2.5) TFA/TIPS/H_2O (10 mL) for 1 h. The cleavage mixture was filtered to remove the resin and concentrated *in vacuo*, followed by precipitation with cold ethyl ether and centrifugation gave crude ring D of Tikitericin **2** as a light yellow solid (5 mg).

Purification of ring D of Tikitericin 2: The crude peptide was purified by reverse phase HPLC using a Phenomenex Gemini 110 Å / C_{18}, 5 µm 10 x 250 mm column. The crude product was dissolved in 12.5% CH_3CN in H_2O (0.1% TFA) to give a concentration of 1 mg/mL. In the method a 2.0 mL injection was employed and a gradient of water and CH_3CN (0.1% TFA) was used to separate peptides. Gradient: Starting from 5% CH_3CN for 1 min, ramp up to 65% over 60 min, then ramp down to 5% over 2 min, and remaining at this concentration for 5 min (flow rate 5.0 mL/min, detection at 210 and 230 nm). The fraction containing the desired product was collected as a single peak (t_R = 12.5 min), which was lyophilized to give the ring D of Tikitericin **2** as a white solid (1.24 mg, 36.1%, yield based on 0.005 mmol scale).

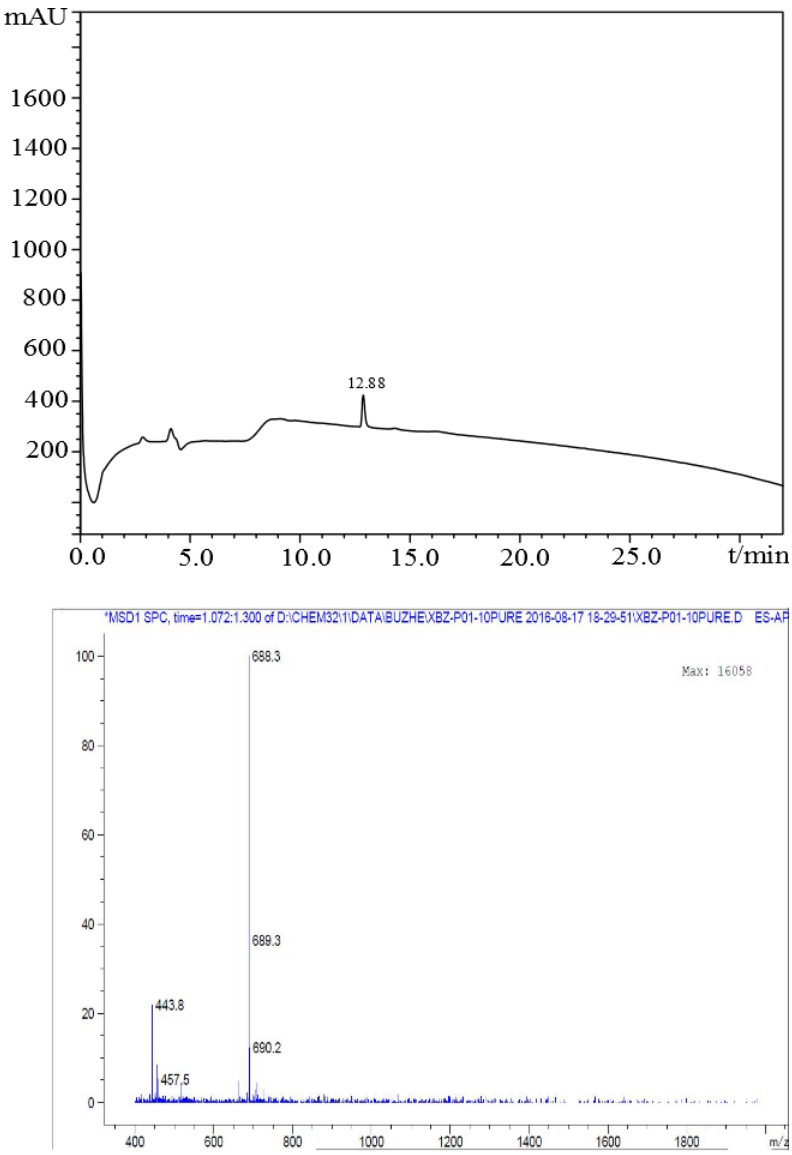
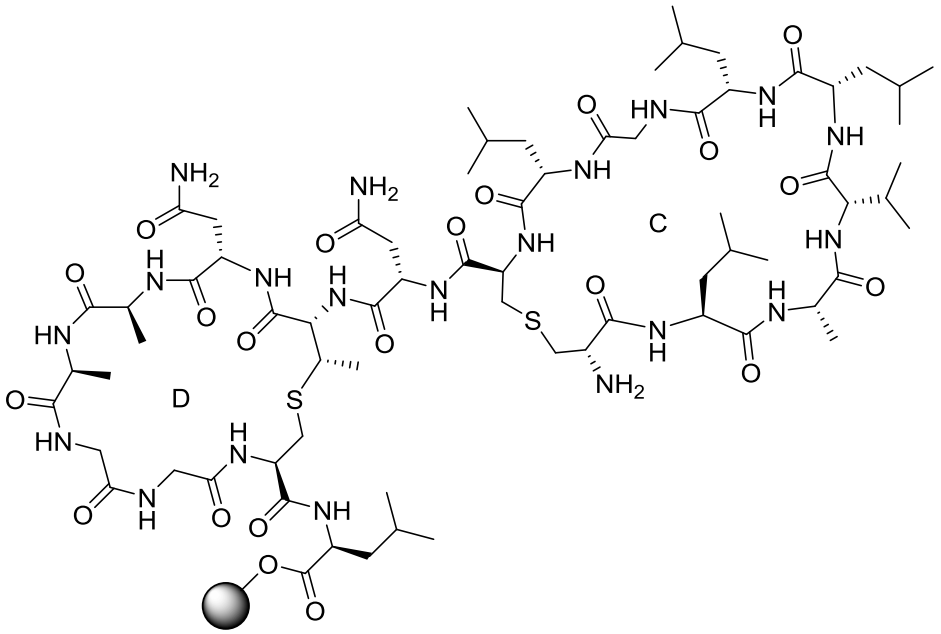


Figure S13. Analytical RP-HPLC of ring D of Tikitericin **2** (*ca*. 98% as judged by peak area of RP-HPLC at 210 nm) were performed on a XTerra® MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 30 min at room temperature, *ca*. 3% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*). ESI-MS (*m/z* $[M+H]^+$ calcd: 688.8; found: 688.3).

2.3.3 Synthesis of ring DC of Tikitericin (3)



Intermediate **2** was elongated using standard Fmoc SPPS with PyBOP to couple protected amino acids in the following order: Fmoc-Asn(Trt)-OH, Fmoc-Alloc/Allyl-Lan (**7**), Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Leu-OH, Fmoc-Val-OH, Fmoc-Ala-OH, Fmoc-Leu-OH. Allyl, Alloc and Fmoc groups were removed according to the procedure described above. Cyclization to form ring C was done with PyBOP similar to ring D. A small portion of resin (5 mg) was cleaved and analyzed by ESI-MS: calculated for $C_{71}H_{120}N_{20}O_{21}S_2$ $(M+2H)^{2+}$ 827.4, found 827.4; calculated for $C_{71}H_{120}N_{20}O_{21}S_2$ $(M+H)^{+}$ 1653.8, found 1653.4.

The resin (55 mg, 0.005 mmol) was cleaved with (95/2.5/2.5) TFA/TIPS/H_2O (10 mL) for 1 h. The cleavage mixture was filtered to remove the resin and concentrated *in vacuo*, followed by precipitation with cold ethyl ether and centrifugation gave crude ring DC of Tikitericin **3** as a light yellow solid (3 mg).

Purification of ring DC of Tikitericin 3: The crude peptide was purified by reverse phase HPLC using a Phenomenex Gemini 110 Å / C_{18}, 5 µm 10 x 250 mm column. The crude product was dissolved in 25% CH_3CN in H_2O (0.1% TFA) to give a concentration of 1 mg/mL. In the method a 2.0 mL injection was employed and a gradient of water and CH_3CN (0.1% TFA) was used to separate peptides. Gradient: Starting from 5% CH_3CN for 1 min, ramp up to 65% over 60 min, then ramp down to 5% over 2 min, and remaining at this concentration for 5 min (flow rate 5.0 mL/min, detection at 210 and 230 nm). The fraction containing the desired product was collected as a single peak (t_R = 40.0 min), which was lyophilized to give the ring DC of Tikitericin **3** as a white solid (0.74 mg, 8.9%, yield based on 0.005 mmol scale).

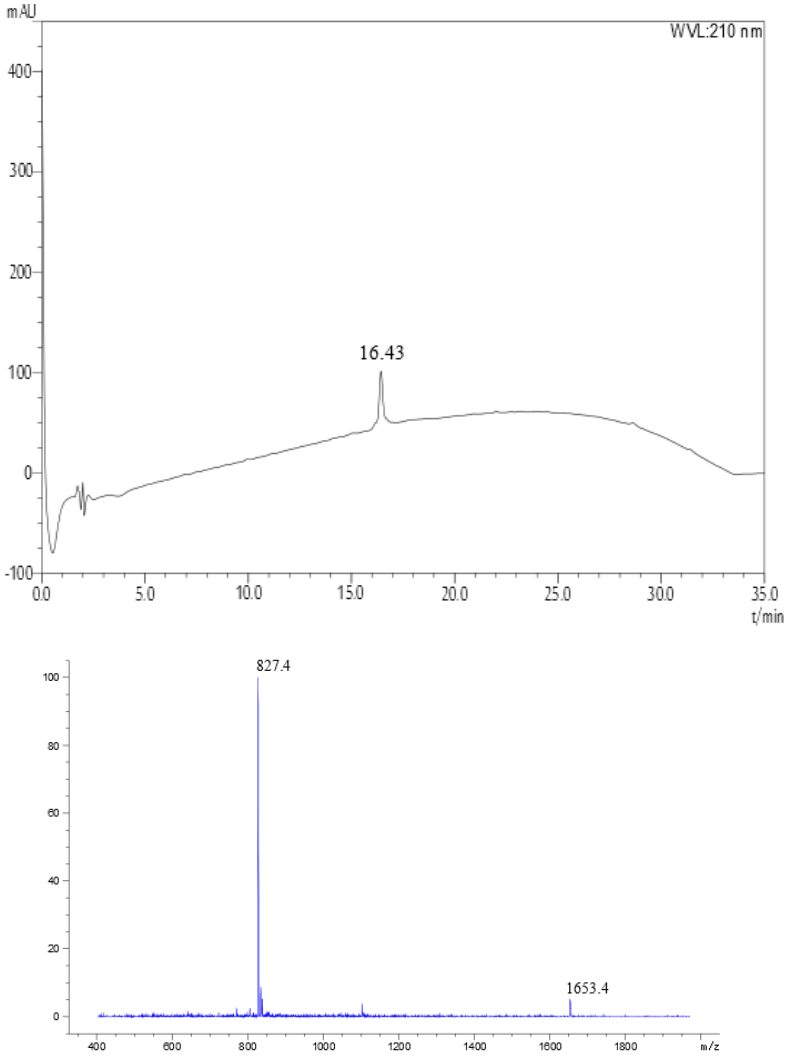
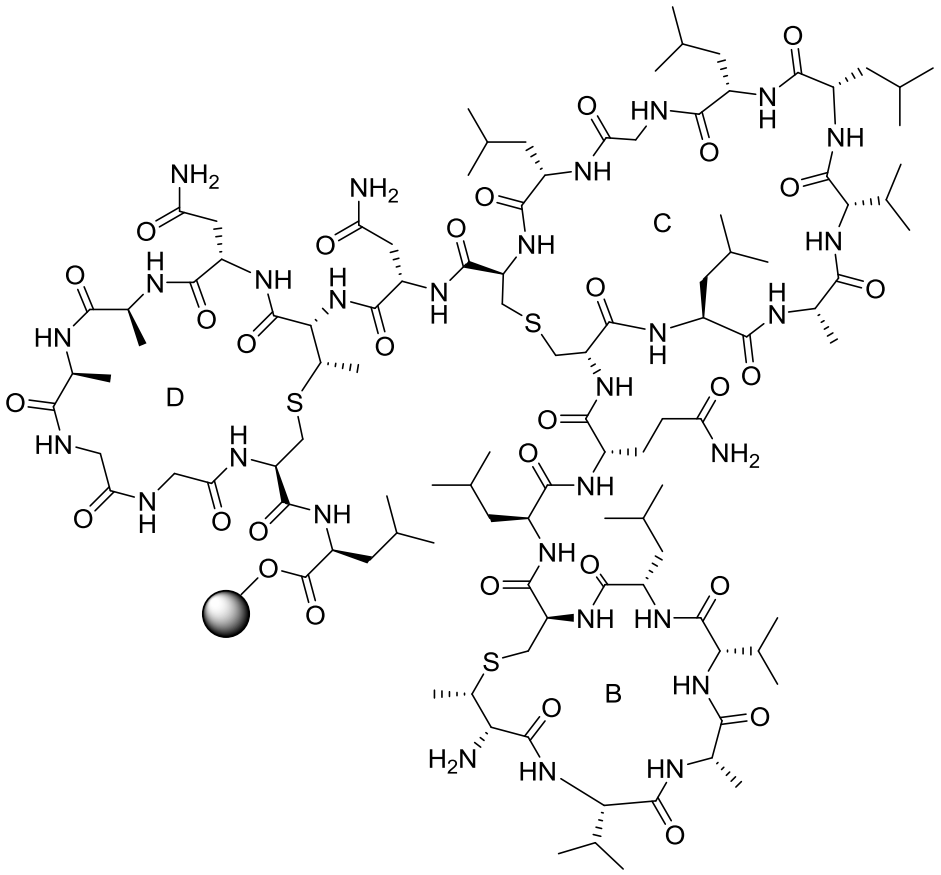


Figure S14. Analytical RP-HPLC of ring DC of Tikitericin **3** (*ca*. 98% as judged by peak area of RP-HPLC at 210 nm) were performed on a XTerra® MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 30 min at room temperature, *ca*. 3% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*). ESI-MS (*m/z* $[M+2H]^{2+}$ calcd: 827.4; found: 827.4; $[M+H]^{+}$ calcd: 1653.8; found: 1653.4).

2.3.4 Synthesis of ring DCB of Tikitericin (4)



Intermediate **3** was elongated using standard Fmoc SPPS with PyBOP to couple protected amino acids in the following order: Fmoc-Gln(Trt)-OH, Lan, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Alloc/Allyl-MeLan (**6**), Fmoc-Leu-OH, Fmoc-Val-OH, Fmoc-Ala-OH, Fmoc-Val-OH. Allyl, Alloc and Fmoc groups were removed according to the procedure described above. Cyclization to form ring B was done with PyBOP similar to ring D. A small portion of resin (5 mg) was cleaved and analyzed by ESI-MS; calculated for $C_{108}H_{183}N_{29}O_{30}S_3$ $(M+2H)^{2+}$ 1232.1, found 1232.4; calculated for $C_{108}H_{183}N_{29}O_{30}S_3$ $(M+3H)^{3+}$ 821.8, found 822.0.

The resin (300 mg, 0.025 mmol) was cleaved with (95/2.5/2.5) TFA/TIPS/H_2O (10 mL) for 1 h. The cleavage mixture was filtered to remove the resin and concentrated *in vacuo*, followed by precipitation with cold ethyl ether and centrifugation gave crude ring DCB of Tikitericin **4** as a light yellow solid (20 mg).

Purification of ring DCB of Tikitericin 4: The crude peptide was purified by reverse phase HPLC using a Phenomenex Gemini 110 Å / C_{18}, 5 µm 10 x 250 mm column. The crude product was dissolved in 50% CH_3CN in H_2O (0.1% TFA) to give a concentration of 1 mg/mL. In the method a 2.0 mL injection was employed and a gradient of water and CH_3CN (0.1% TFA) was used to separate peptides. Gradient: Starting from 5% CH_3CN for 1 min, ramp up to 95% over 30 min, then ramp down to 5% over 2 min, and remaining at this concentration for 5 min (flow rate 5.0 mL/min, detection at 210 and 230 nm). The fraction containing the desired product was collected as a single peak (t_R = 19.8 min), which was lyophilized to give the ring DCB of Tikitericin **4** as a white solid (1.7 mg, 2.8%, yield based on 0.025 mmol scale).

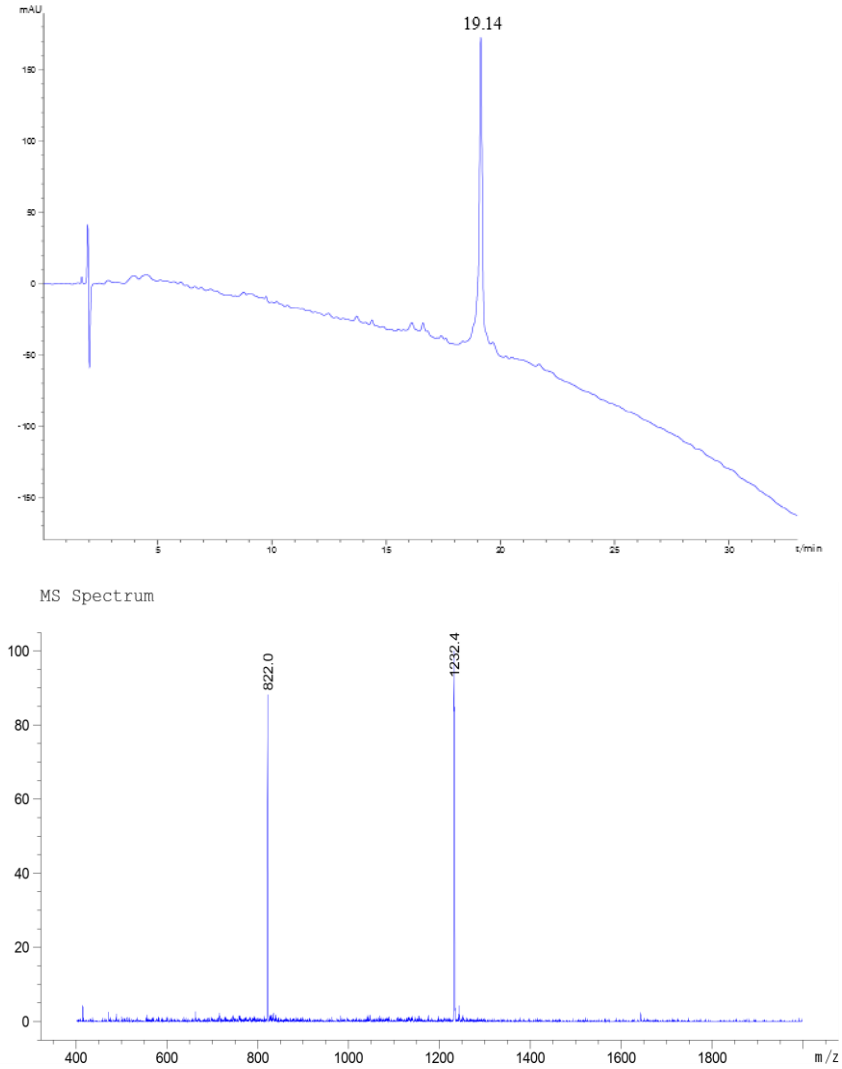
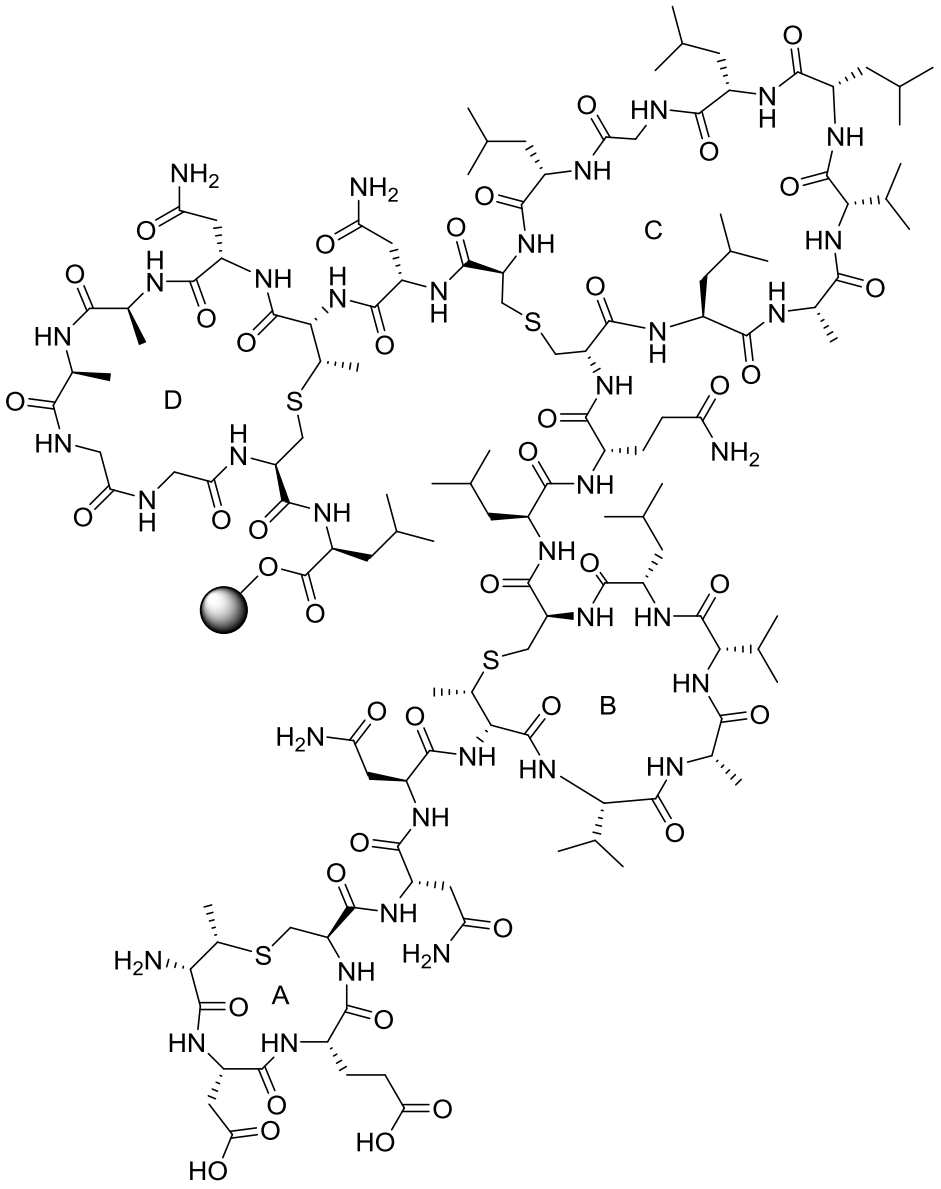


Figure S15. Analytical RP-HPLC of ring DCB of Tikitericin **4** (*ca*. 95% as judged by peak area of RP-HPLC at 210 nm) were performed on a XTerra® MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 30 min at room temperature, *ca*. 3% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*). ESI-MS (*m/z* $[M+2H]^{2+}$ calcd: 1232.1, found: 1232.4; $[M+3H]^{3+}$ calcd: 821.8, found: 822.0).

2.3.5 Synthesis of ring DCBA of Tikitericin (5)



Intermediate **4** was elongated using standard Fmoc SPPS with PyBOP to couple protected amino acids in the following order: Fmoc-Asn(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Alloc/Allyl-MeLan (**7**), Fmoc-Glu(OtBu)-OH, Fmoc-Asp(OtBu)-OH. Allyl, Alloc and Fmoc groups were removed according to the procedure described above. Cyclization to form ring A was done with PyBOP similar to ring D. A small portion of resin (5 mg) was cleaved and analyzed by ESI-MS; calculated for $C_{132}H_{217}N_{37}O_{42}S_4$ $(M+2H)^{2+}$ 1561.2, found 1561.4; calculated for $C_{132}H_{217}N_{37}O_{42}S_4$ $(M+3H)^{3+}$ 1041.2, found 1041.4.

The resin (105 mg, 0.0085 mmol) was cleaved with (95/2.5/2.5) TFA/TIPS/H_2O (10 mL) for 1 h. The cleavage mixture was filtered to remove the resin and concentrated *in vacuo*, followed by precipitation with cold ethyl ether and centrifugation gave crude ring DCBA of Tikitericin **5** as a light yellow solid (8 mg).

Purification of ring DCBA of Tikitericin 5: The crude peptide was purified by reverse phase HPLC using a Phenomenex Gemini 110 Å / C_{18}, 5 µm 10 x 250 mm column. The crude product was dissolved in 50% CH_3CN in H_2O (0.1% TFA) to give a concentration of 1 mg/mL. In the method a 2.0 mL injection was employed and a gradient of water and CH_3CN (0.1% TFA) was used to separate peptides. Gradient: Starting from 5% CH_3CN for 1 min, ramp up to 32% over 2 min, then ramp up to 68% over 48 min, followed by ramping up to 85% over 5 min, and remaining at this concentration for 10 min, then ramp

down to 5% over 2 min, and remaining at this concentration for 5 min (flow rate 4.0 mL/min, detection at 210 and 230 nm). The fraction containing the desired product was collected as a single peak (t_R = 20.5 min), which was lyophilized to give the ring DCBA of Tikitericin **5** as a white solid (0.4 mg, 1.4%, yield based on 0.0085 mmol scale).

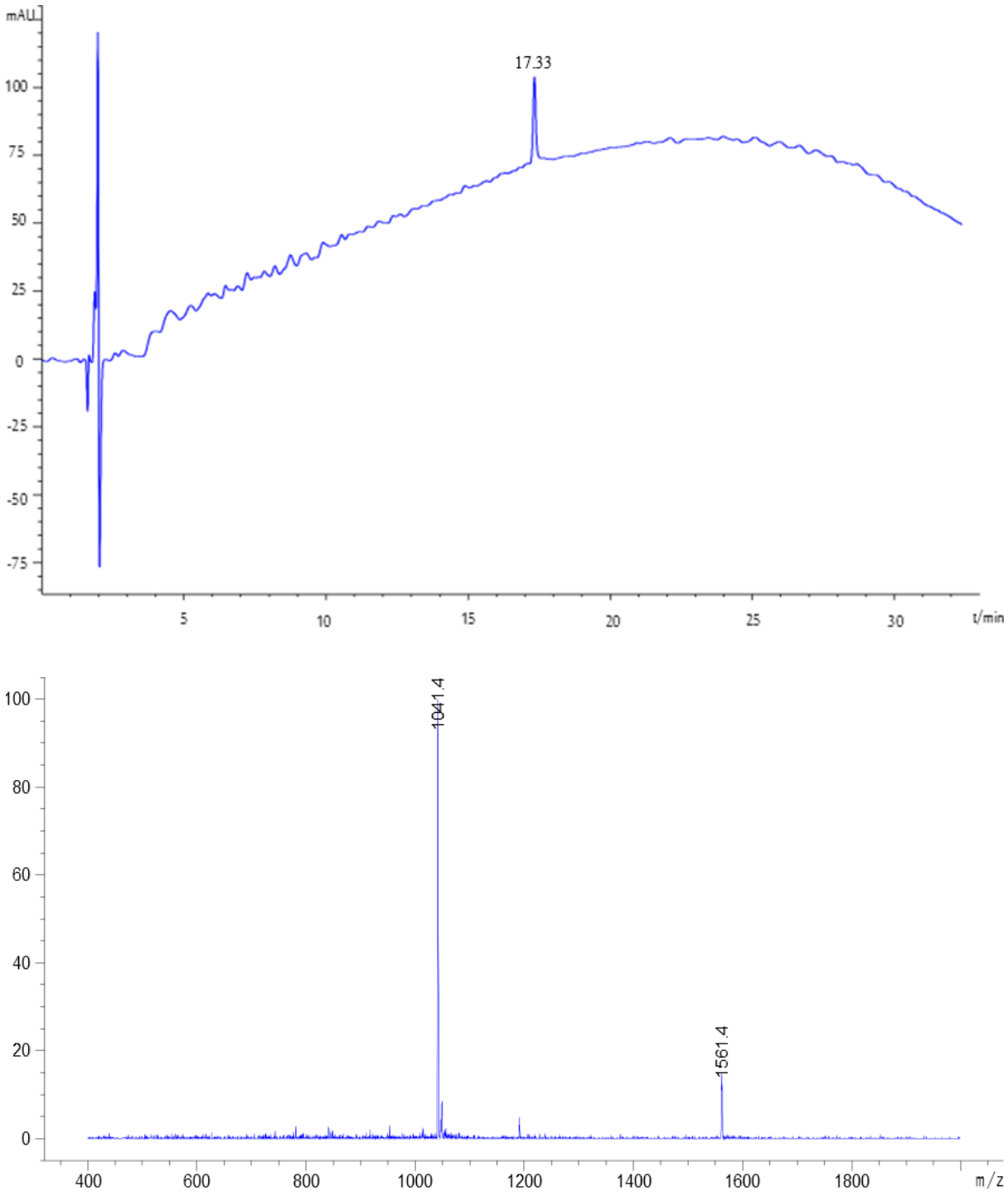
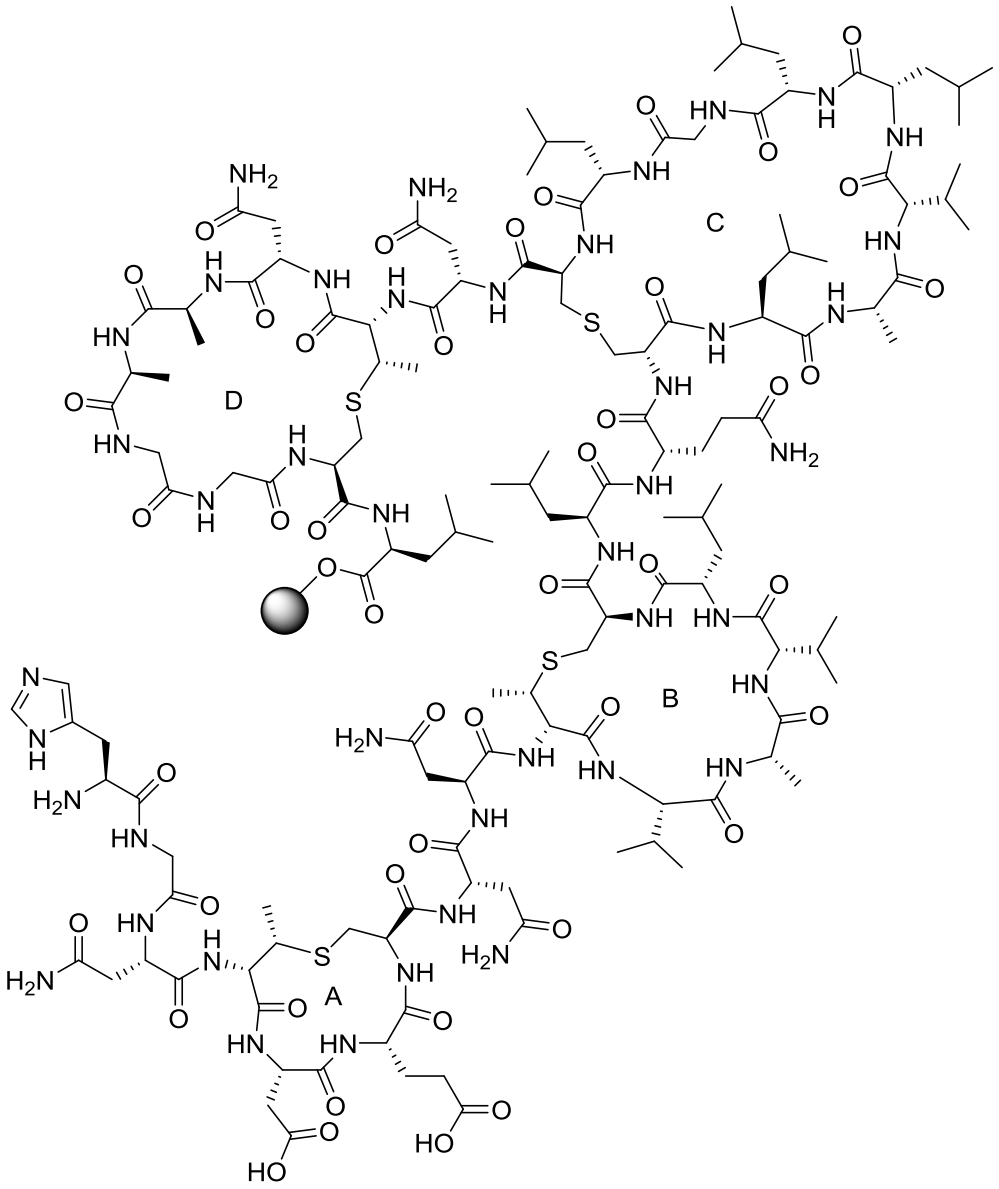


Figure S16. Analytical RP-HPLC of ring DCBA of Tikitericin **5** (*ca*. 98% as judged by peak area of RP-HPLC at 210 nm) were performed on a XTerra® MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 30 min at room temperature, *ca*. 3% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*). ESI-MS (*m/z* $[M+2H]^{2+}$ calcd: 1561.2, found: 1561.4; $[M+3H]^{3+}$ calcd: 1041.2, found: 1041.4).

2.3.6 Synthesis of the full length Tikitericin 1



Intermediate **5** was elongated using standard Fmoc SPPS with PyBOP to couple protected amino acids in the following order: Fmoc-Asn(Trt)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH. The Fmoc group was removed with 20% piperidine in DMF (*v/v* 10 mL, 2 x 5 mins) and the resin was washed with DMF (3 x 10 mL) and CH_2Cl_2 (3 x 10 mL) to remove any traces of piperidine. The resin (430 mg, 0.034 mmol) was cleaved with (94/2.5/2.5/1) TFA/TIPS/H_2O/EDT (10 mL) for 1 h. The cleavage mixture was filtered to remove the resin and concentrated *in vacuo*, followed by precipitation with cold ethyl ether and centrifugation gave crude Tikitericin **1** as a light yellow solid (20 mg).

Purification of Tikitericin 1: the crude peptide was purified by reverse phase HPLC using a Phenomenex Gemini 110 Å / C18, 5 µm 10 x 250 mm column. The crude Tikitericin **1** was dissolved in 50% CH_3CN in H_2O (0.1% TFA) to give a concentration of 1 mg/mL. In the method a 2.0 mL injection was employed and a gradient of water and CH_3CN (0.1% TFA) was used to separate peptides. Gradient: Starting from 5% CH_3CN for 1 min, ramp up to 32% over 2 min, then ramp up to 68% over 48 min, followed by ramping up to 85% over 5 min, and remaining at this concentration for 10 min, then ramp down to 5% over 2 min, and remaining at this concentration for 5 min (flow rate 4.0 mL/min, detection at 210 and 230 nm). The fraction containing the desired product was collected as a broad peak (t_R = 18.0 min), which was lyophilized to give relatively pure peptide. A Second

purification was done using the same conditions. The fractions containing the desired product were lyophilized to give the full peptide of Tikitericin **1** as a white solid (1.0 mg, 0.86%, yield based on 0.034 mmol scale). The calculated MALDI-TOF MS for $C_{144}H_{233}N_{43}O_{46}S_4$ $(M+H)^+$ 3429.6098, found 3429.6189.

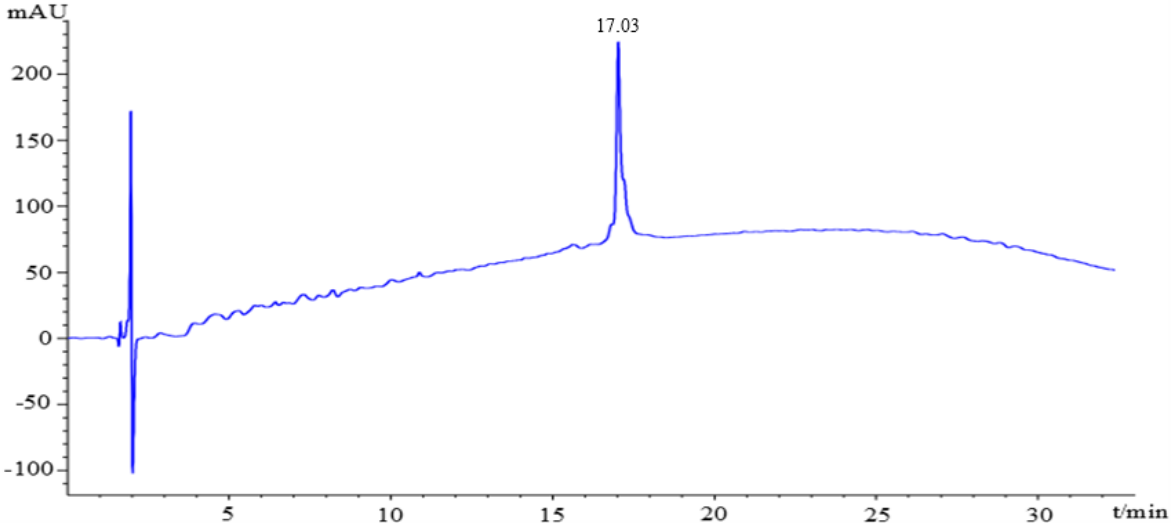


Figure S17. Analytical RP-HPLC of the full peptide of Tikitericin **1** (*ca*. 95% as judged by peak area of RP-HPLC at 210 nm) were performed on a XTerra® MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 30 min at room temperature, *ca*. 3% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

2.4 MALDI-TOF MS and HRESI-MS/MS analysis of synthetic tikitericin 1

High resolution mass spectra (HRMS) experiments were carried out using a Bruker Solarix-XR 7T mass spectrometer (Bruker Daltonics, using FTMSControl v2.1. The peptide was diluted to 0.1 mg/mL in water/acetonitrile (50:50) for nanoelectrospray ionization (Nanospray, Bruker Daltonics) tandem MS experiments. Matrix assisted laser desorption ionization (MALDI) samples were prepared by the dried droplet method with 4-hydroxy-α-cyanocinnamic acid (HCCA) as the matrix for accurate mass determination. Spectra for accurate mass determination were acquired in broadband, positive ion mode from m/z 600–5000. Tandem MS experiments were carried out in broadband, positive ion mode from m/z 150–3000, and performed by isolating a single charge state (5 Da isolation window) followed by collision induced dissociation (collision energy = 20 V). 16 scans were averaged for MS experiments, and 150 for tandem MS.

MS and MS/MS data were processed with DataAnalysis 4.4 (Bruker Daltonics). For MS/MS experiments, monoisotopic masses of the singly charge peptide ($[M+H]^+$) were extra by the DataAnalysis software using the modified THRASH algorithm (SNAP ver 2.0) with a quality factor threshold = 0.5, S/N threshold = 2, and maximum charge state = peptide precursor charge state. Theoretical fragment masses were calculated manually, cross checked with the experimentally identified masses, and manually confirmed to ensure the quality of assignments. A mass accuracy of 5 ppm was used for product ion assignments.

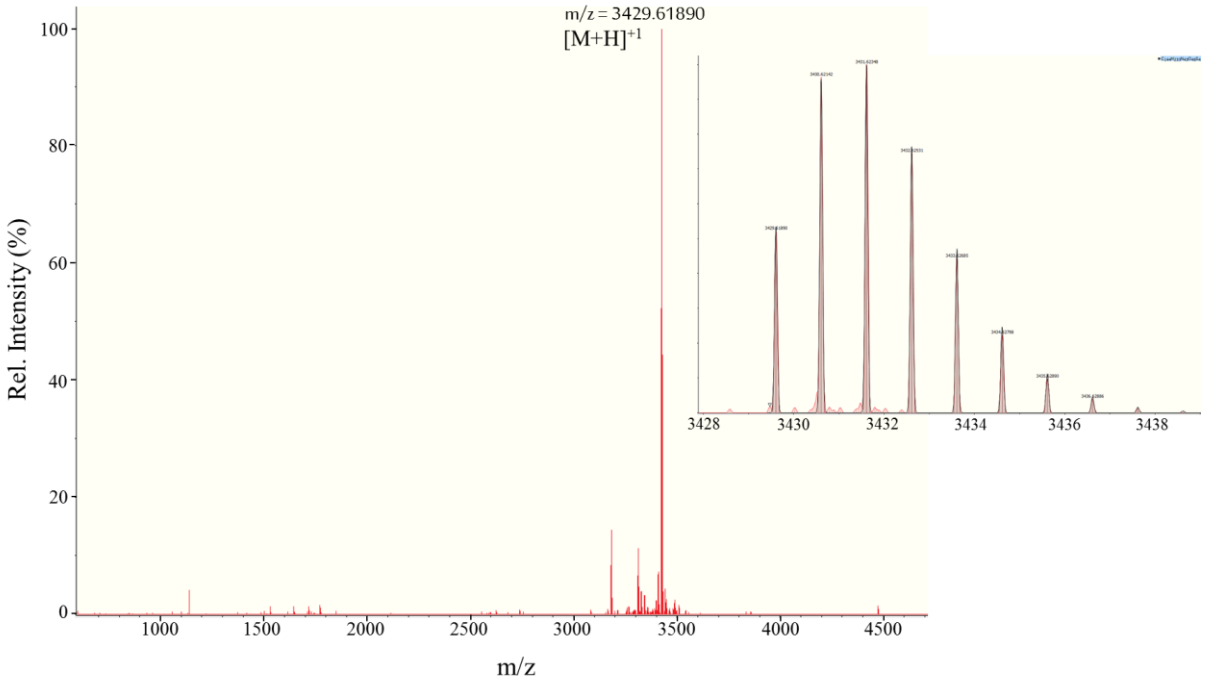


Figure S18. MALDI-TOF MS of the full peptide of Tikitericin **1**

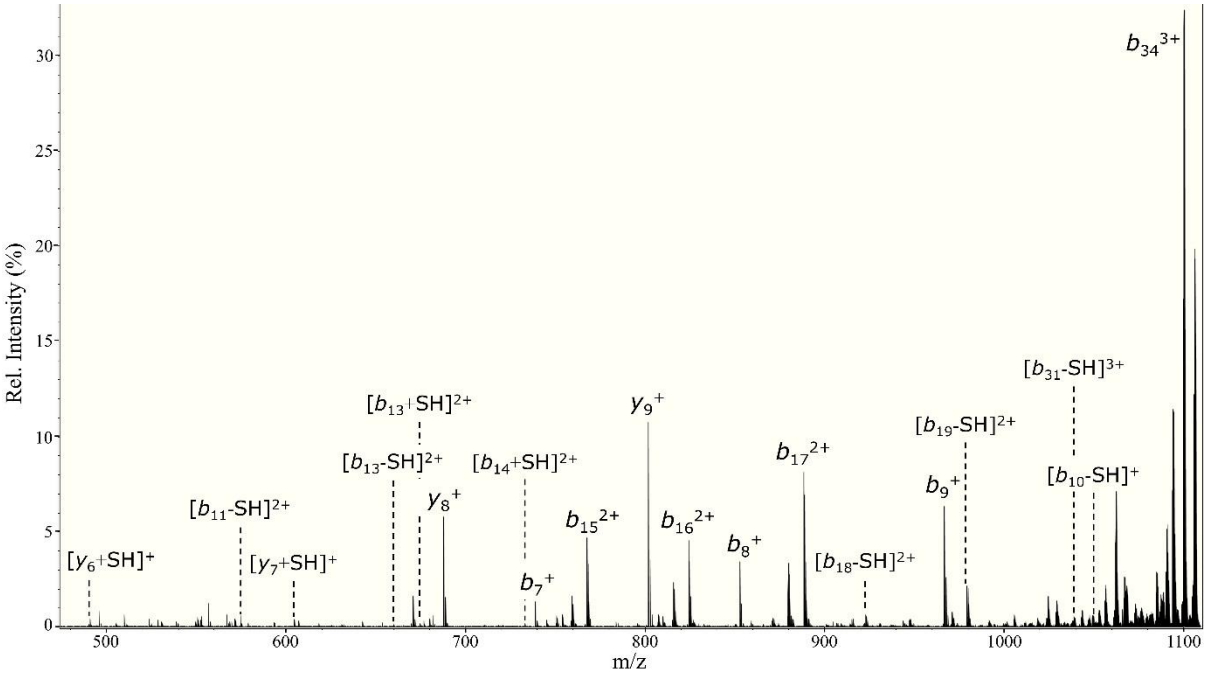


Figure S19. H R ESI-MS/MS spectrum of tikitericin **1** (M = 3428.6098 Da), precursor ion: *m/z* 1143.8756 $[M+3H]^{3+}$, collision energy = 20 V.

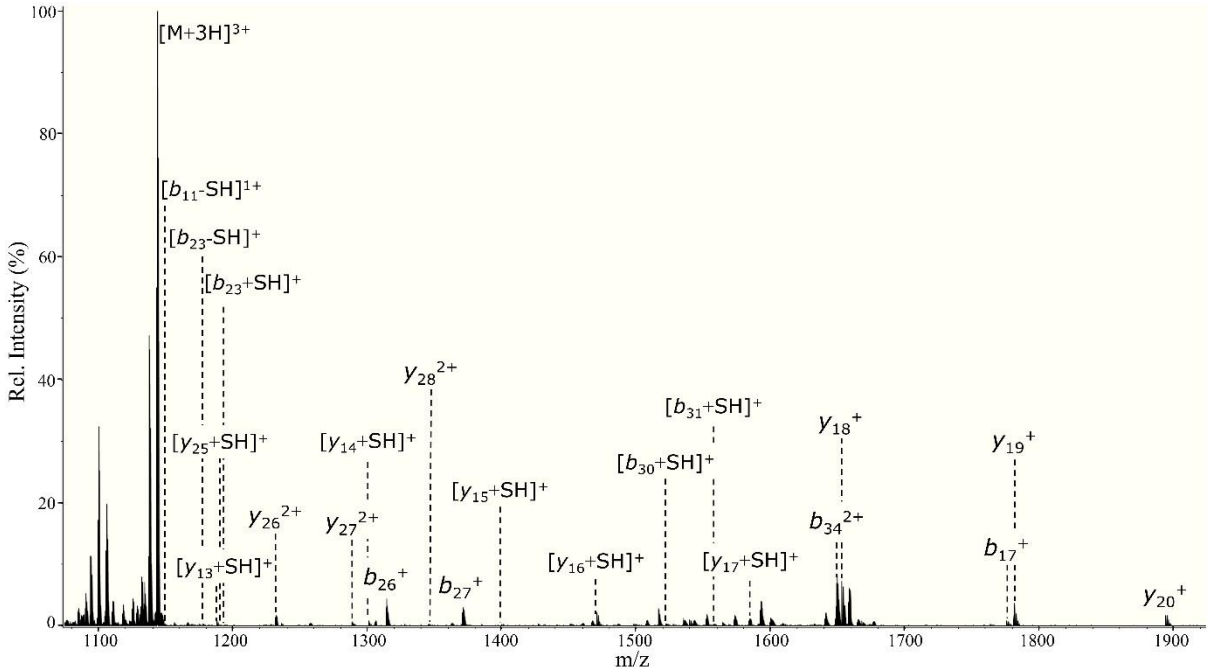
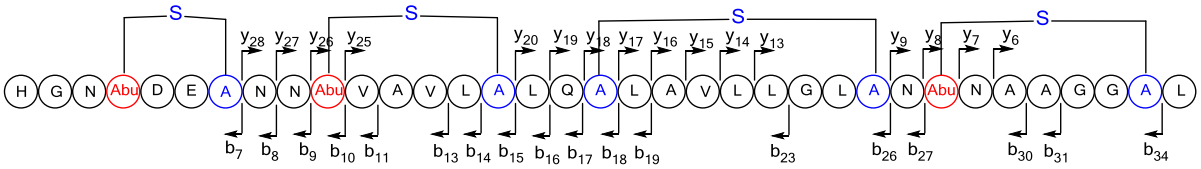


Figure S20. H R ESI-MS/MS spectrum of tikitericin **1** (M = 3428.6098 Da), precursor ion: *m/z* 1143.8756 $[M+3H]^{3+}$, collision energy = 20 V.



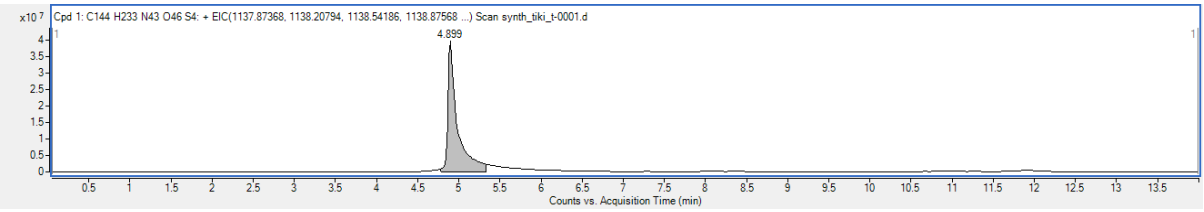
Ion	Theoretical (*m/z*)	Experimental (*m/z*)	Error	Ion	Theoretical (*m/z*)	Experimental (*m/z*)	Error (ppm)
*b*7	739.2470	739.2464	−0.8	[*y*6+SH]	491.2283	491.2280	−0.5
*b*8	853.2899	853.2893	−0.7	[*y*7+SH]	605.2712	605.2715	0.5
*b*9	967.3329	967.3318	−1.1	*y*8	688.3083	688.3083	0.0
[*b*10–SH]	1050.3699	1050.3677	−2.1	*y*9	802.3512	802.3510	−0.3
[*b*11–SH]	1149.4383	1149.4376	−0.6	[*y*13+SH]	1188.5500	1188.5486	−1.2
[*b*13–SH]	1319.5439	1319.5425	−1.1	[*y*14+SH]	1301.6341	1301.6323	−1.4
[*b*13+SH]	1353.5317	1353.5317	0.0	[*y*15+SH]	1400.7025	1400.7025	0.0
[*b*14+SH]	1466.6157	1466.6132	−1.7	[*y*16+SH]	1471.7396	1471.7376	−1.3
*b*15	1535.6372	1535.6364	−0.5	[*y*17+SH]	1584.8237	1584.8183	−3.4
*b*16	1648.7212	1648.7197	−0.9	*y*18	1653.8451	1653.8425	−1.5
*b*17	1776.7798	1776.7778	−1.1	*y*19	1781.9037	1781.9008	−1.6
[*b*18–SH]	1845.8012	1845.7987	−1.3	*y*20	1894.9878	1894.9857	−1.1
[*b*19–SH]	1958.8853	1958.8831	−1.1	[*y*25+SH]	2380.2550	2380.2443	−4.5
[*b*23–SH]	2355.1589	2355.1544	−1.9	*y*26	2463.2921	2463.2899	−0.9
[*b*23+SH]	2389.1467	2389.1441	−1.1	*y*27	2577.3350	2577.3335	−0.6
*b*26	2628.2737	2628.2709	−1.0	*y*28	2691.3779	2691.3724	−2.0
*b*27	2742.3166	2742.3109	−2.1				
[*b*30+SH]	3044.4215	3044.4131	−2.7				
[*b*31+SH]	3115.4586	3115.4589	0.1				
*b*34	3298.5230	3298.5139	−2.7				

Figure S21. HRESI-MS/MS fragmentation pattern of tikitericin **1** (M = 3428.6098 Da), precursor ion: *m/z* 1143.8756 $[M+3H]^{3+}$

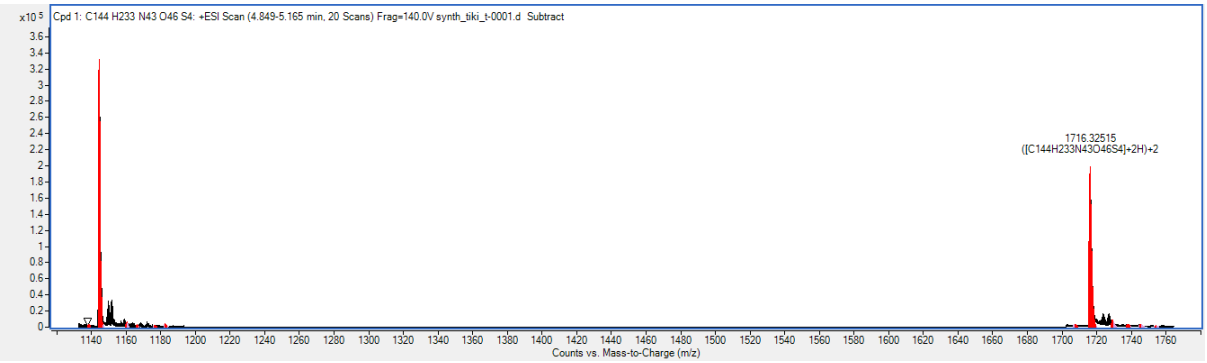
2.5 Comparison of isolated and synthetic tikitericin (All of the MS spectrum experiments below followed section 1.2)

2.5.1 HRESI-MS chromatogram, HRESI-MS and HRESI-MSMS of synthetic intact tikitericin 1

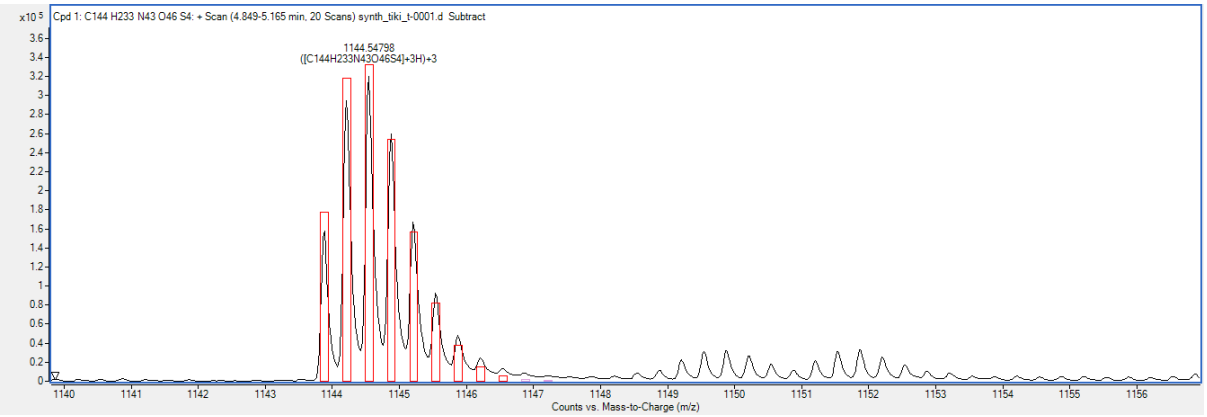
a)



b)



c)



d)

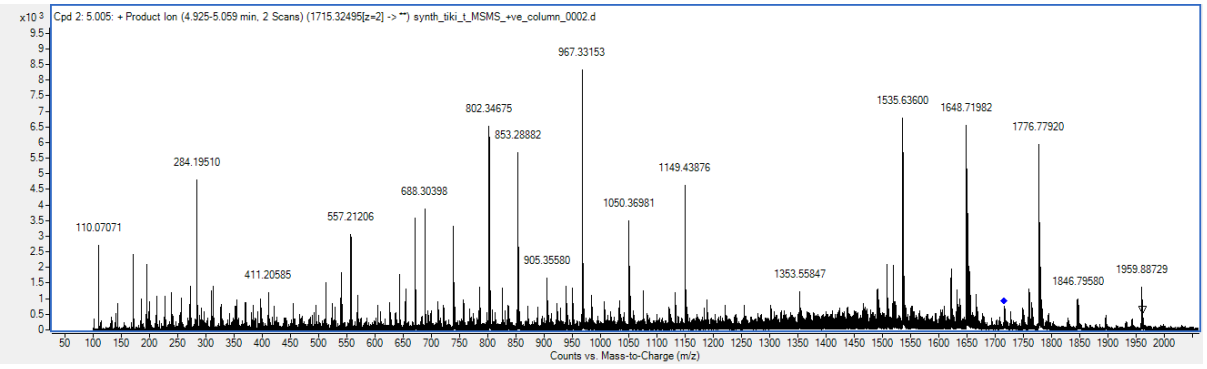
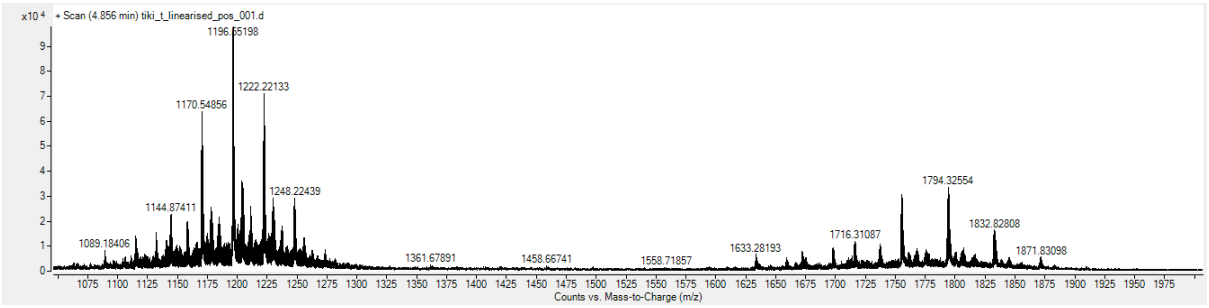


Figure S22. a) HRESI-MS chromatogram of synthetic tikitericin **1,** b) HRESI-MS spectrum of synthetic tikitericin **1,** c) HRESI-MS spectrum of synthetic tikitericin **1** (zoomed), d) HRESI-MSMS spectrum of synthetic tikitericin **1**, precursor ion: *m/z* 1716.32 $[M+2H]^{2+}$, CID energy = 60 eV.

2.5.2 Base catalysed elimination and thioethanol trapping of synthetic tikitericin 1

Synthetic tikitericin **1** (40 µg) was processed in an analogous manner to that indicated above for naturally occurring material, *viz.* it was suspended in a 0.24 M NaOH, 0.63 mM 2-thioethanol, 25% EtOH solution (40 µL) and incubated with shaking at 50°C for 1 h. The temperature was raised to 85°C and the reaction incubated for a further 1 h. After the addition of acetic acid (10 µL) the reaction was diluted with H_2O (150 µL) and desalted using C18 ZipTips according to the manufacturer's protocol.



b)

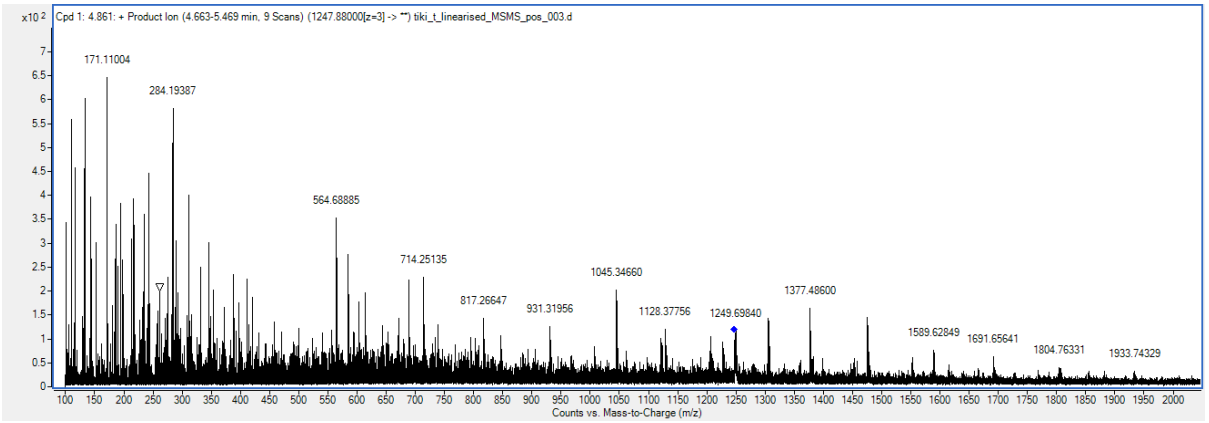
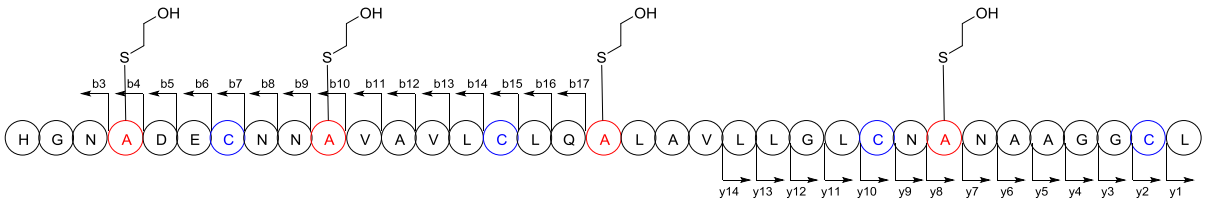


Figure S23. a) HRESI-MS of base eliminated and thioethanol trapped fully linearized synthetic tikitericin **1**. b) HRESI-MSMS spectrum of fully linearized synthetic tikitericin **1**: precursor ion m/z 1247.89 $[M+3H]^{3+}$, CID energy = 70 eV; c) HRESI-MSMS fragmentation pattern of linearized tikitericin **1**, precursor ion: m/z 1247.89 $[M+3H]^{3+}$.



b ions				y ions			
Pos	Exact (*m/z*)	Observed (*m/z*)	Δ (ppm)	Pos	Exact (*m/z*)	Observed (*m/z*)	Δ (ppm)
1	138.0067			34	3604.6145		
2	195.0082			33	3547.5930		
3	309.1311	309.1308	-0.9	32	3433.5501		
4	470.1822	470.1881	12.5	31	3272.4990		
5	585.2091	585.2070	-3.7	30	3157.4721		
6	714.2517	714.2591	10.3	29	3028.4295		
7	817.2609	817.2553	-6.9	28	2925.4203		
8	931.3038	931.3306	28.7	27	2811.3774		
9	1045.3468	1045.3599	12.5	26	2697.3345		
10	1206.3978	1206.3936	-3.5	25	2536.2834		
11	1305.4662	1305.4647	-1.2	24	2437.2150		
12	1376.5033	1376.4944	-6.5	23	2366.1779		
13	1475.5717	1475.5657	-4.1	22	2267.1095		
14	1588.6558	1588.6580	1.4	21	2154.0254		
15	1691.6650	1691.6594	-3.3	20	2051.0162		
16	1804.7491	1804.7439	-2.9	19	1937.9322		
17	1932.8076	1932.8155	4.1	18	1809.8736		
18	2079.8430			17	1662.8382		
19	2192.9271			16	1549.7541		
20	2263.9642			15	1478.7170		
21	2363.0326			14	1379.6486	1379.5288	-86.8
22	2476.1167			13	1266.5645	1266.5579	-5.2
23	2589.2008			12	1153.4805	1153.4682	-10.7
24	2646.2222			11	1096.4590	1096.4517	-6.7
25	2759.3063			10	983.3749	983.3739	-1.0
26	2862.3155			9	880.3657	880.3592	-7.4
27	2976.3584			8	766.3228	766.3150	-10.2
28	3137.4094			7	605.2718	605.2565	-25.3
29	3251.4524			6	491.2288	491.2239	-9.9
30	3322.4895			5	420.1917	420.1890	-6.4
31	3393.5266			4	349.1546	349.1520	-7.5
32	3430.5481			3	292.1332	292.1340	2.7
33	3507.5695			2	235.1117	235.1084	-14.0
34	3610.5787			1	132.1025	132.1016	-6.7

Figure S24. HRESI-MSMS fragmentation pattern of linearized tikitericin **1**, precursor ion: m/z 1247.89 $[M+3H]^{3+}$, CID energy = 70 eV.

2.6 Previous unsuccessful synthesis of tikitericin 1 on Polystyrene and TentaGel-PHB resin

Initial synthesis of the ring D of tikitericin on Polystyrene resin (PS) (0.1 mmol/g), mini-cleavage and analysed by HPLC

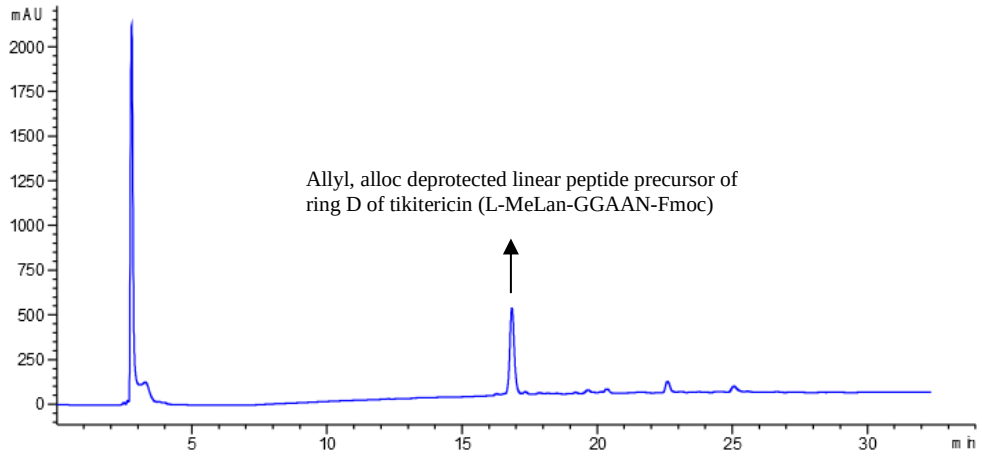


Figure S25. Analytical RP-HPLC of the allyl, alloc deprotected linear peptide precursor of ring D of tikitericin (L-MeLan-GGAAN-Fmoc) mini-cleaved from PS resin were performed on an analytical column (Agilent C3, 150 mm x 3.0 mm, 3.5 µm) and a linear gradient of 5-95% B in 30 min at room temperature, *ca*. 3% B per min at a flow rate of 0.3 mL/min. Buffer A: H_2O containing 0.1% formic acid (*v/v*); Buffer B: acetonitrile containing 0.1% formic acid (*v/v*).

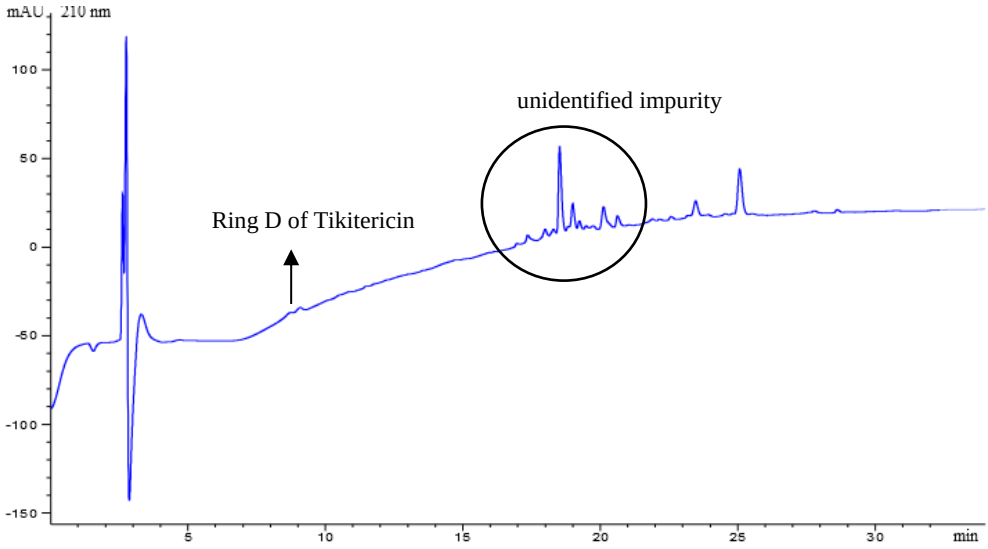


Figure S26. Analytical RP-HPLC of the ring D formation of tikitericin on PS resin were performed on an analytical column (Agilent C3, 150 mm x 3.0 mm, 3.5 µm) and a linear gradient of 5-95% B in 30 min at room temperature, *ca*. 3% B per min at a flow rate of 0.3 mL/min. Buffer A: H_2O containing 0.1% formic acid (*v/v*); Buffer B: acetonitrile containing 0.1% formic acid (*v/v*).

Initial synthesis of the ring DCB of tikitericin on TentaGel-PHB resin (Wang linker, 0.25 mmol/g), mini-cleavage and analysed by HPLC

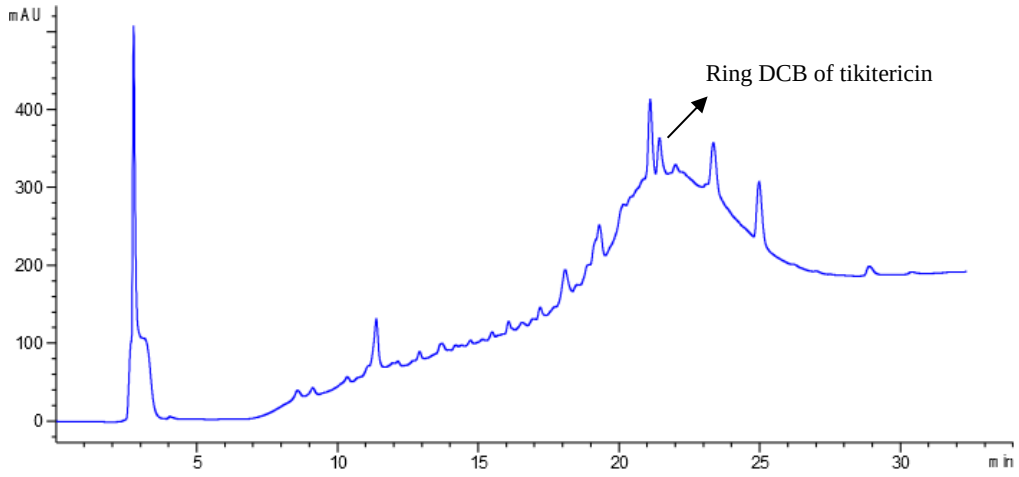


Figure S27. Analytical RP-HPLC of the ring DCB formation of tikitericin on TentaGel-PHB resin were performed on an analytical column (Agilent C3, 150 mm x 3.0 mm, 3.5 µm) and a linear gradient of 5-95% B in 30 min at room temperature, *ca*. 3% B per min at a flow rate of 0.3 mL/min. Buffer A: H_2O containing 0.1% formic acid (*v/v*); Buffer B: acetonitrile containing 0.1% formic acid (*v/v*).

2.7 Liquid culture antimicrobial assays of tikitericin 1 and truncated analogues 2, 3, 4, 5

Antimicrobial susceptibility testing was performed by measuring the minimum inhibitory concentrations (MICs) by broth microdilution. To do so *Staphylococcus aureus* ATCC 6538 was grown overnight (16 hr) at 37°C with shaking at 200 rpm in Tryptic Soy Broth (TSB) (BD™ Difco™). The following day a cell suspension was prepared by diluting the overnight culture to an OD_{600} of 0.1 in TSB. A 96 well polystyrene plate (ThermoFisher Scientific, New Zealand) was then set up to contain a 2-fold dilution series for each test compound (Tikitericin, Ring ABCD, Ring BCD, Ring CD, and Ring D) in 75 µL TSB medium. Once the test compounds were appropriately diluted, the cell suspension (75 µL) was added to the diluted test compounds yielding an OD_{600} (final) of 0.05 in a final volume of 150 µL. Media, compound-free (untreated), and DMSO vehicle controls were included in each microtiter plate in triplicate. The plates were then incubated for 24 hours at 37 °C with shaking at 200 rpm. After incubation, the OD_{600} of wells were determined using a Varioskan Flash plate reader (ThermoFisher Scientific, New Zealand). The MIC was reported as the lowest concentration of the test compound for which no growth occurred.

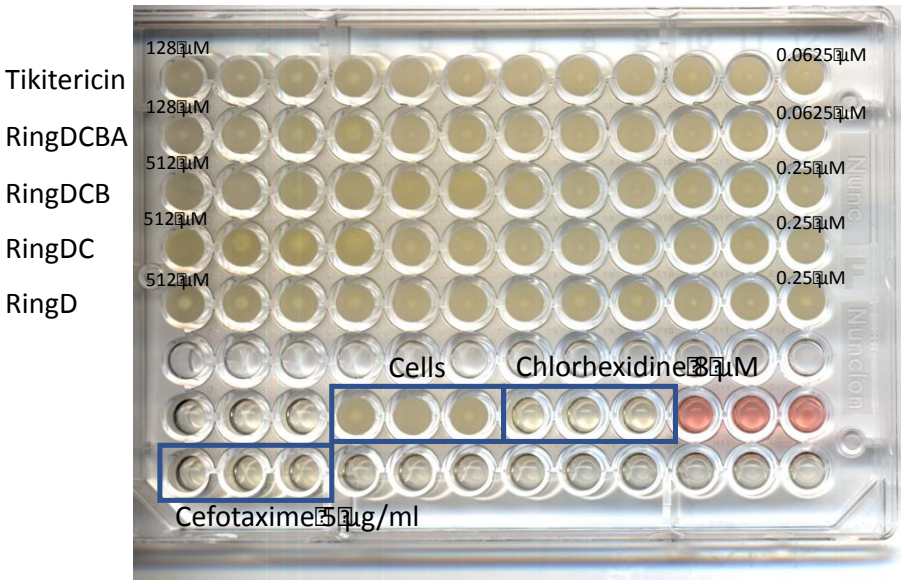


Figure S28. Minimum Inhibitory Concentration (MIC) of Tikitericin samples. Samples tested against *Staphylococcus aureus* ATCC 6538. Samples tested at concentrations indicated, (serial two-fold dilutions). Controls: Untreated cells, Chlorhexidine (8 µM), Cefotaxime (5 µg/ml), and inoculated media.

Table 1. Plate Layout of Tikitericin samples (μM)

Tiki	128	64	32	16	8	4	2	1	0.5	0.25	0.125	0.0625
DCBA	128	64	32	16	8	4	2	1	0.5	0.25	0.125	0.0625
DCB	512	256	128	64	32	16	8	4	2	1	0.5	0.25
DC	512	256	128	64	32	16	8	4	2	1	0.5	0.25
D	512	256	128	64	32	16	8	4	2	1	0.5	0.25
	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty
	Media	Media	Media	Cells	Cells	Cells	Chx	Chx	Chx			
	Cefo	Cefo	Cefo	Media	Media	Media	Media	Media	Media	Media	Media	Media

Table 2. Optical Density (600 nm) measurements of the Minimum Inhibitory Concentration (MIC) of Tikitericin samples.

1.05	1.27	1.42	1.49	1.22	1.45	1.31	1.45	1.22	1.47	1.32	1.45
1.18	1.27	1.45	1.55	1.32	1.47	1.41	1.43	1.32	1.32	1.28	1.23
1.01	1.12	1.44	1.33	1.37	1.46	1.44	1.39	1.3	1.47	1.46	1.38
1.26	1.57	1.51	1.52	1.48	1.49	1.4	1.37	1.34	1.33	1.5	1.34
1.5	1.49	1.48	1.48	1.47	1.46	1.48	1.5	1.5	1.34	1.42	1.51
0.04	0.04	0.04	1.49	1.21	1.5	0.05	0.05	0.05			
0.05	0.07	0.08	0.04	0.04	0.04	0.04	0.04	0.05	0.04	0.04	0.04

S. aurues cells control

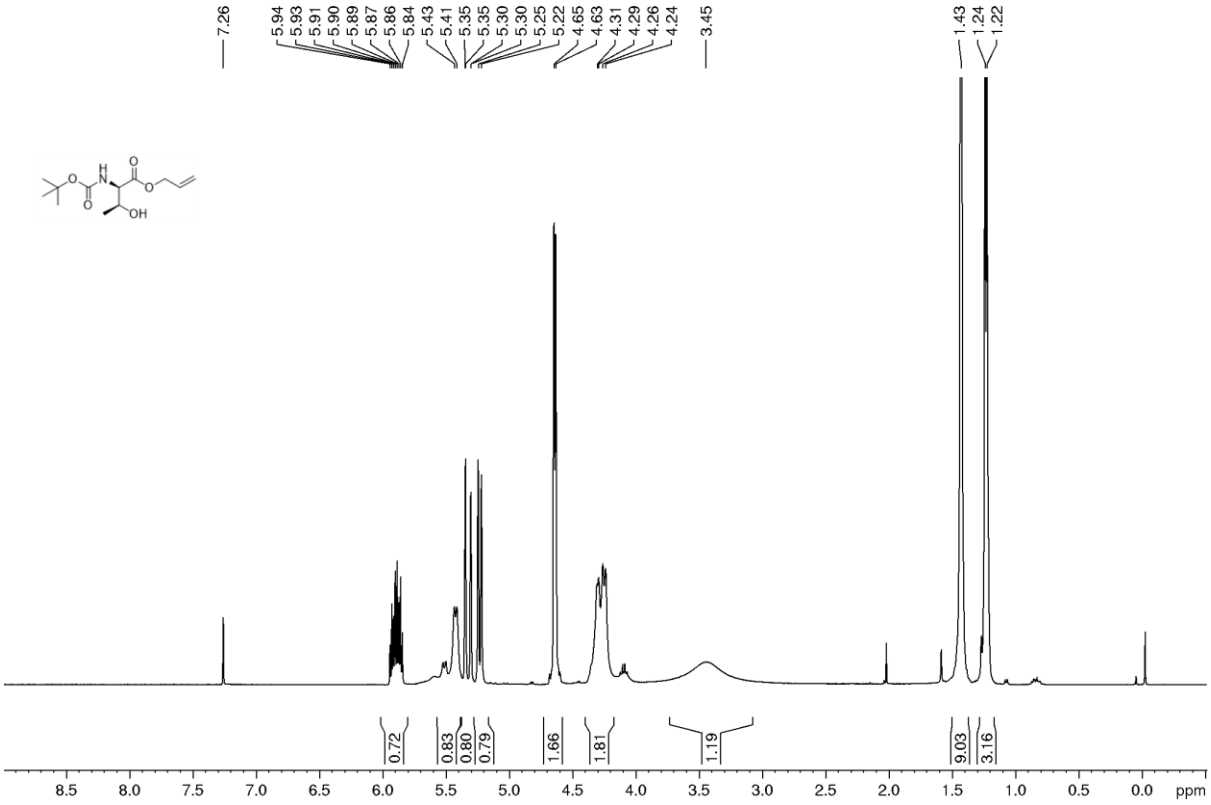
Cefotaxime (5 μg/ml) control

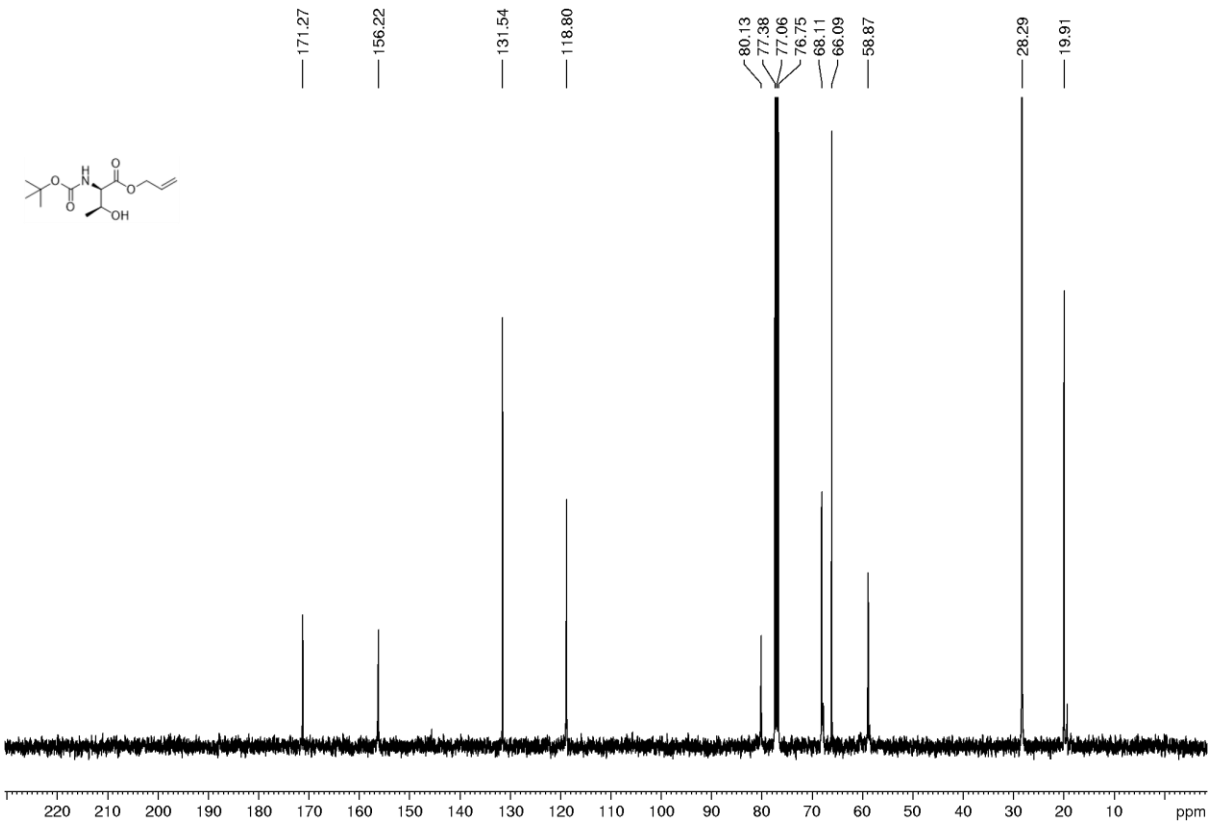
Chlorhexidine (8 μM) control

Sterile Media control

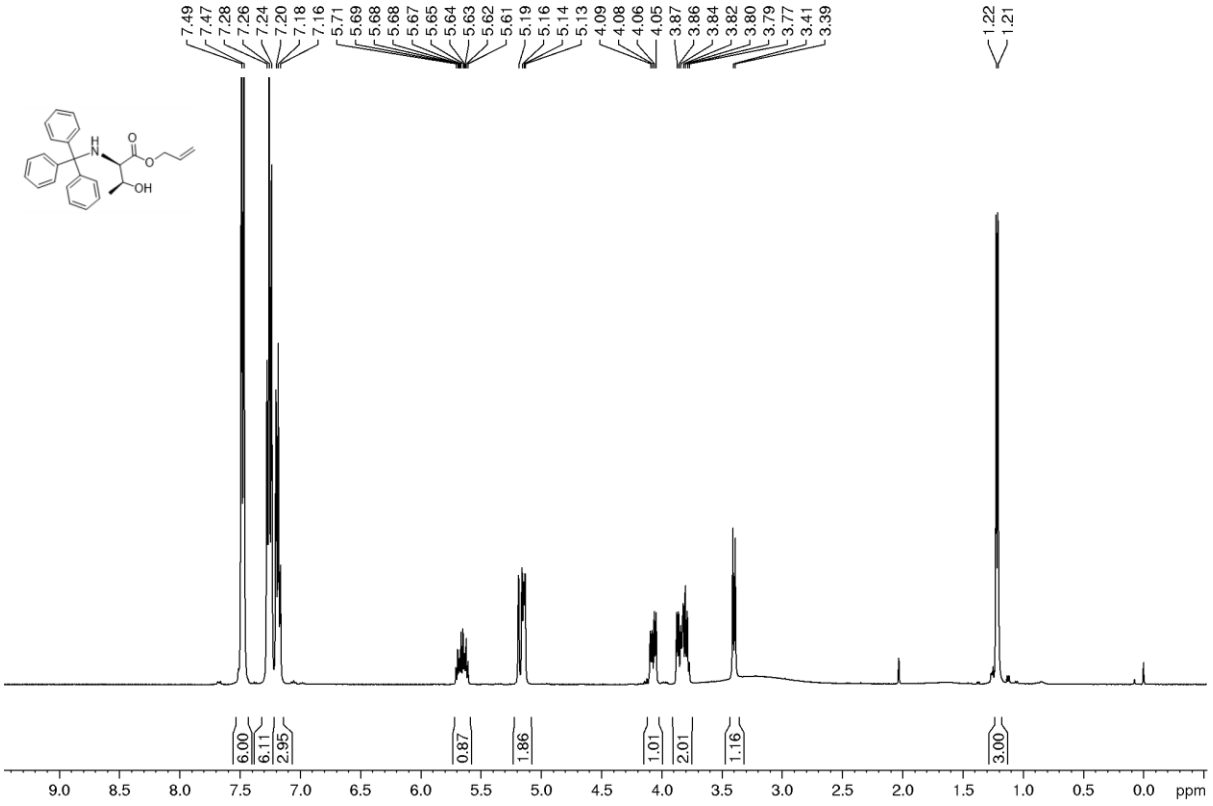
2.8 ^{1}H and ^{13}C NMR Spectra

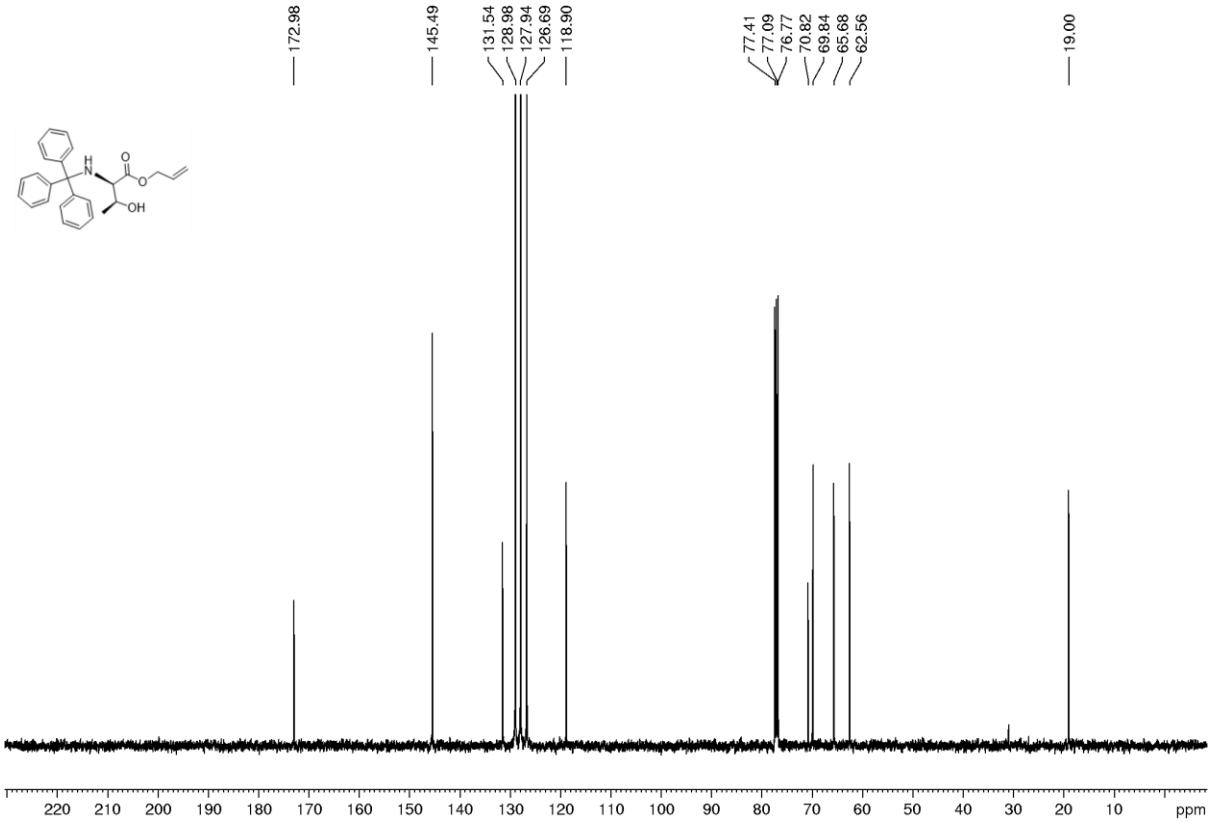
(2R,3S)-allyl 2-((tert-butoxycarbonyl)amino)-3-hydroxybutanoate (10) (^{1}H, $CDCl_3$; ^{13}C, $CDCl_3$)



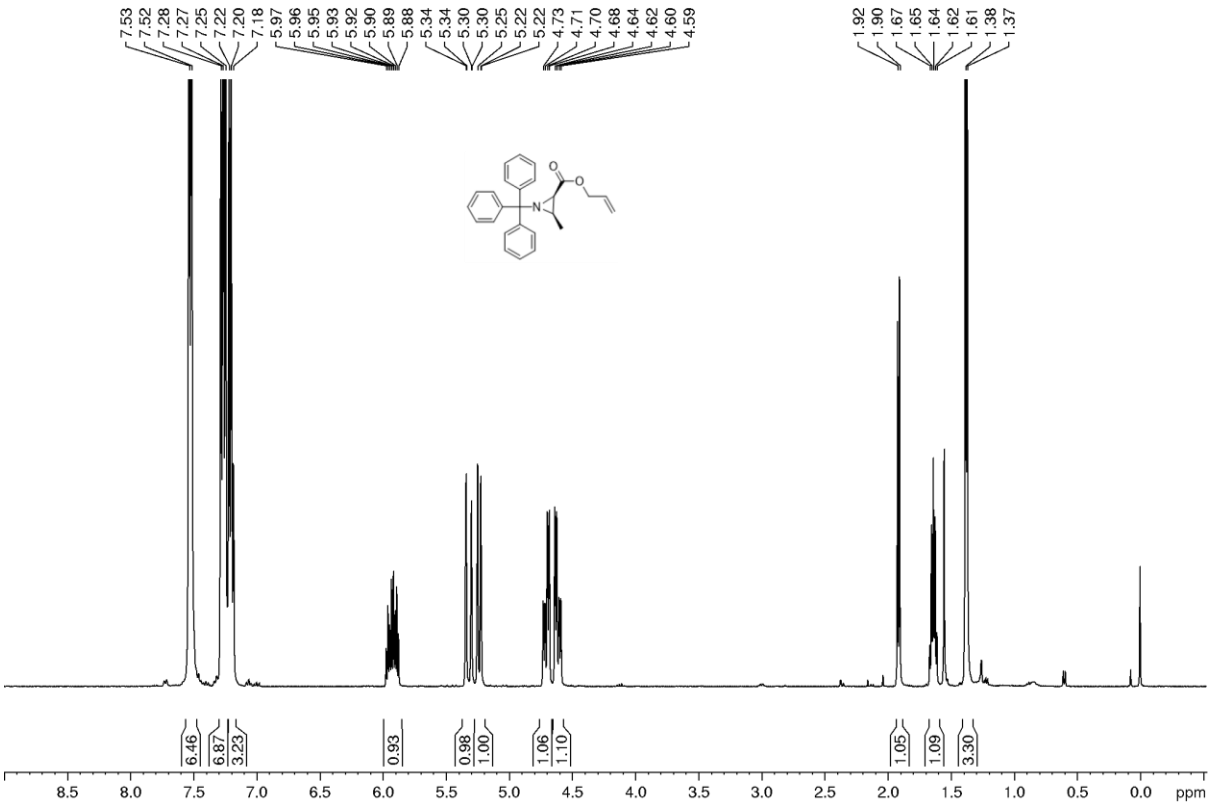


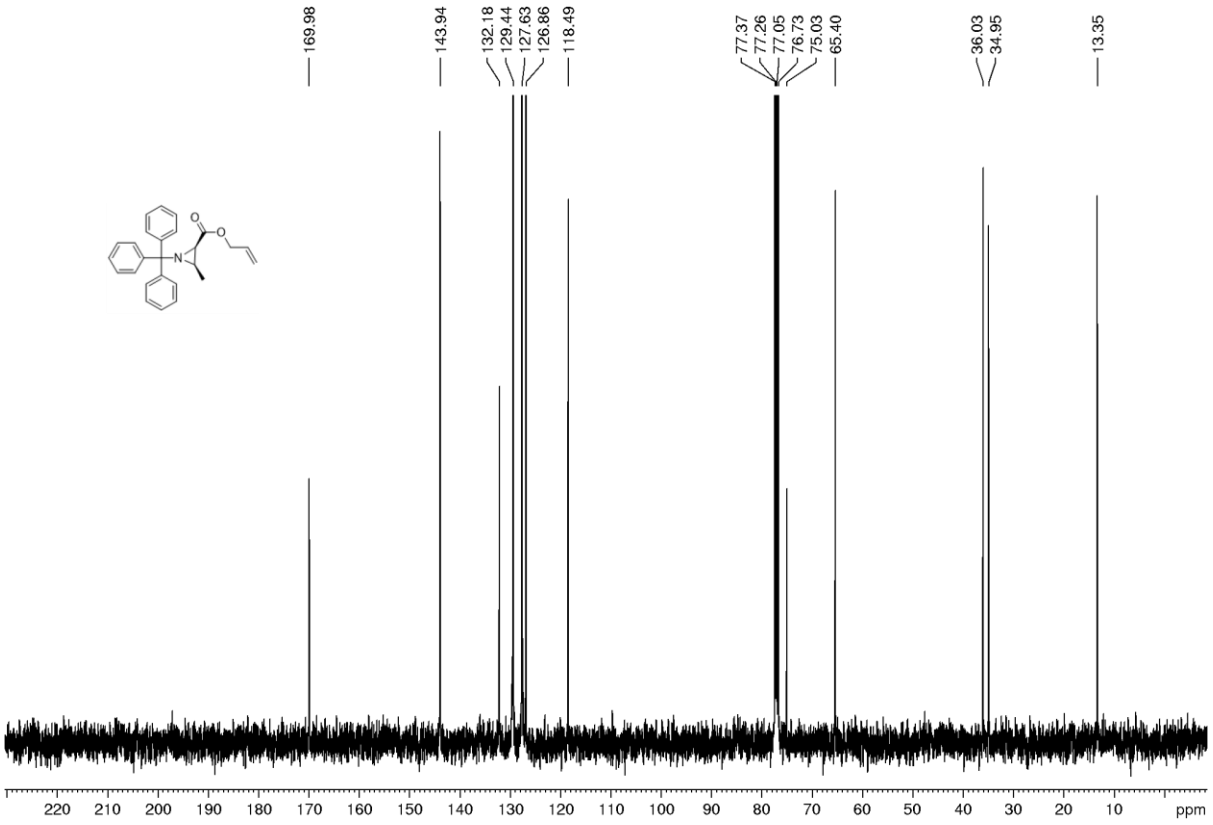
(2R,3S)-allyl 3-hydroxy-2-(tritylamino)butanoate (12) (^{1}H, $CDCl_3$; ^{13}C, $CDCl_3$)



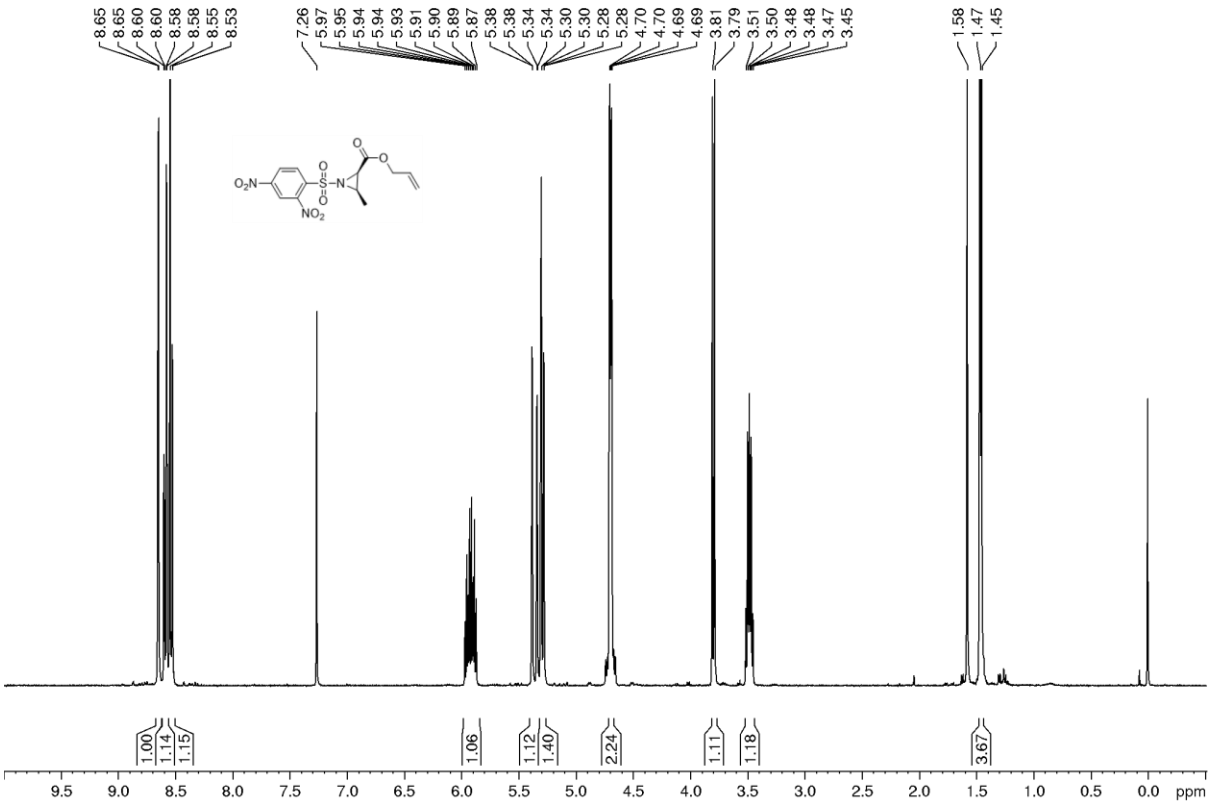


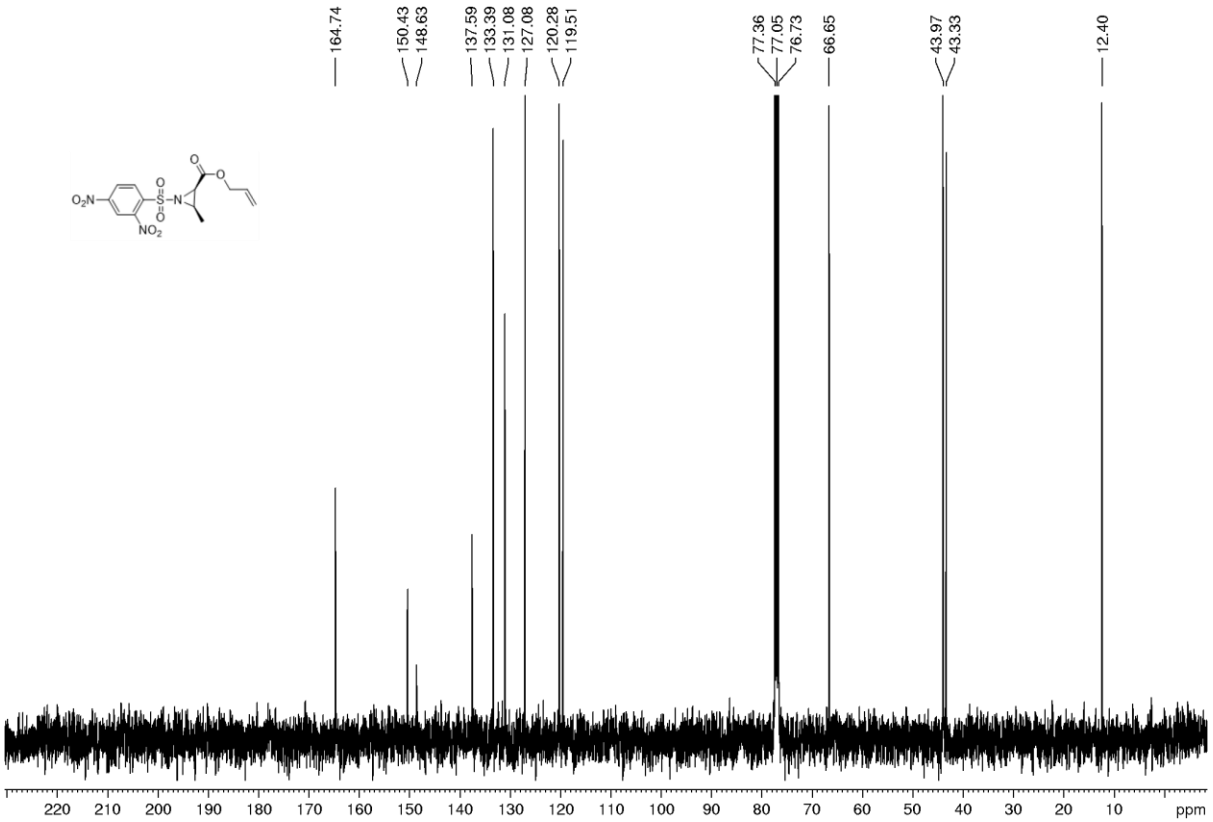
(2R,3R)-allyl 3-methyl-1-tritylaziridine-2-carboxylate (14) (^{1}H, $CDCl_3$; ^{13}C, $CDCl_3$)



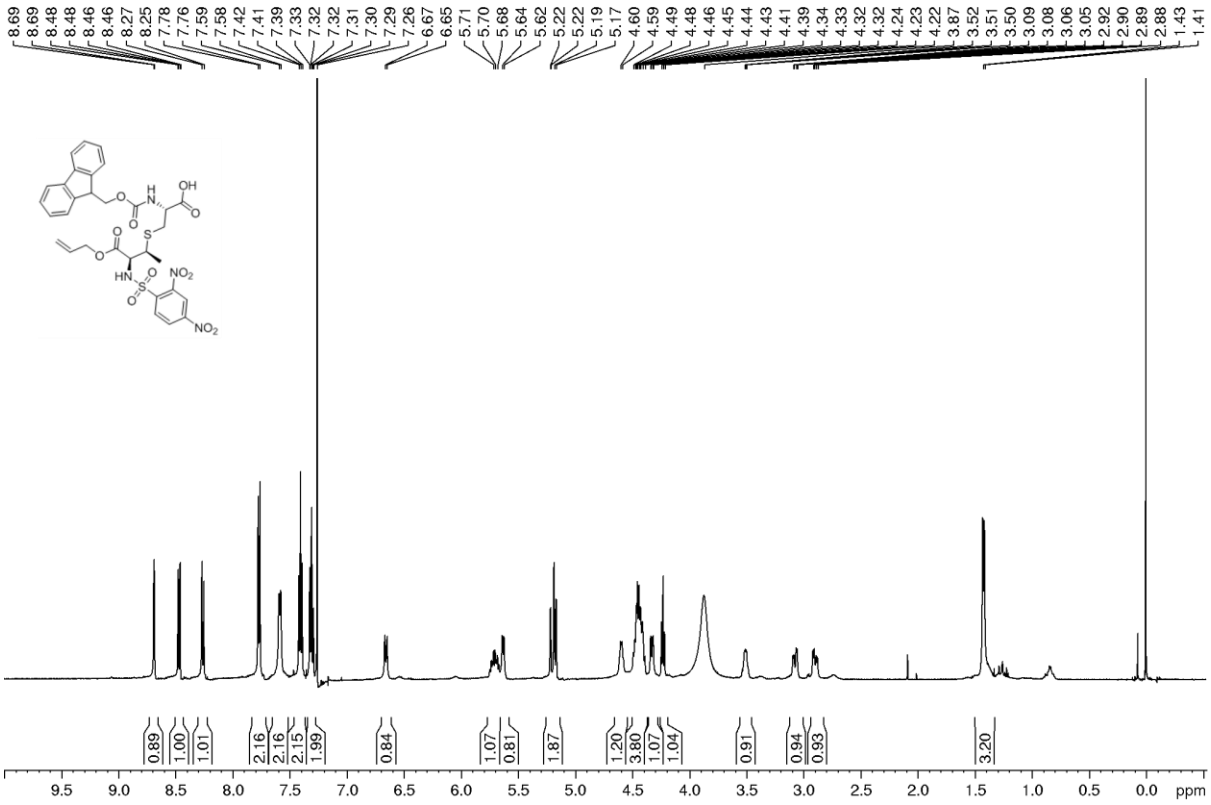


(2R,3R)-allyl 1-((2,4-dinitrophenyl)sulfonyl)-3-methylaziridine-2-carboxylate (16) (^{1}H, $CDCl_3$; ^{13}C, $CDCl_3$)

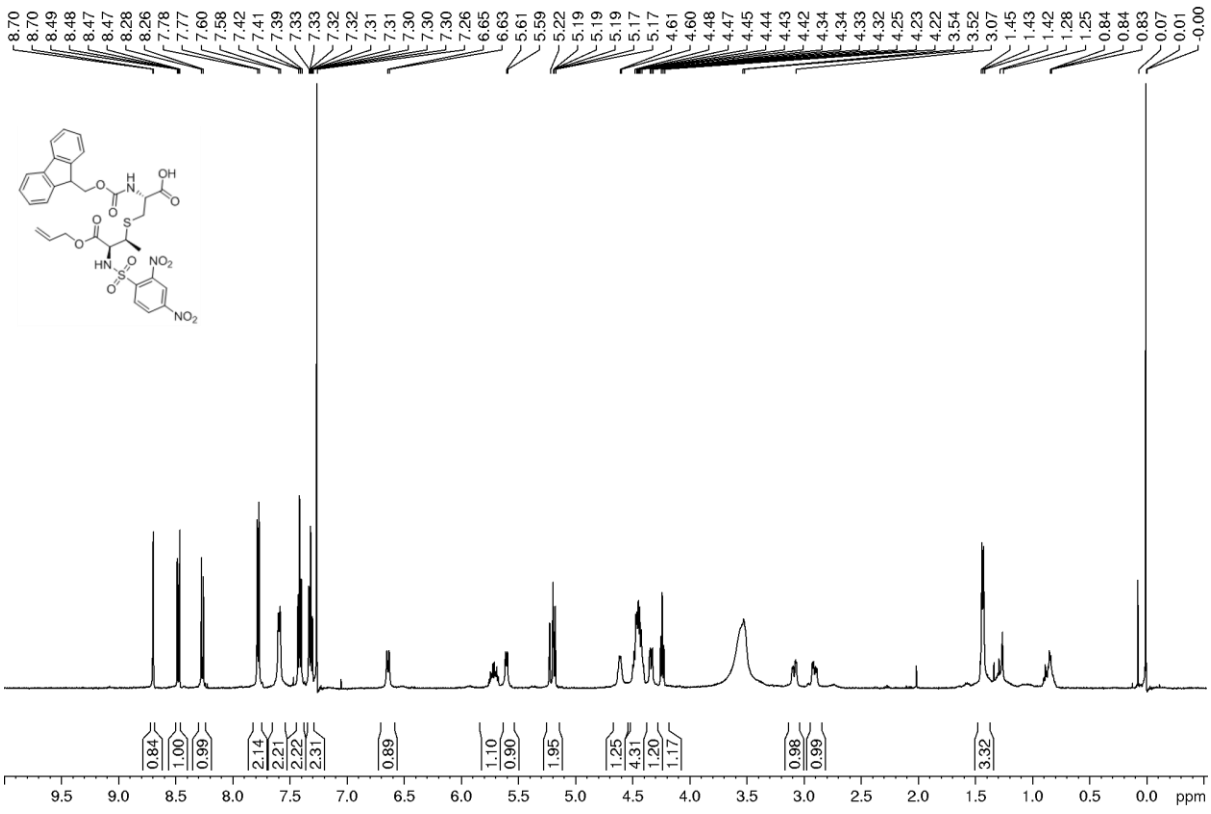


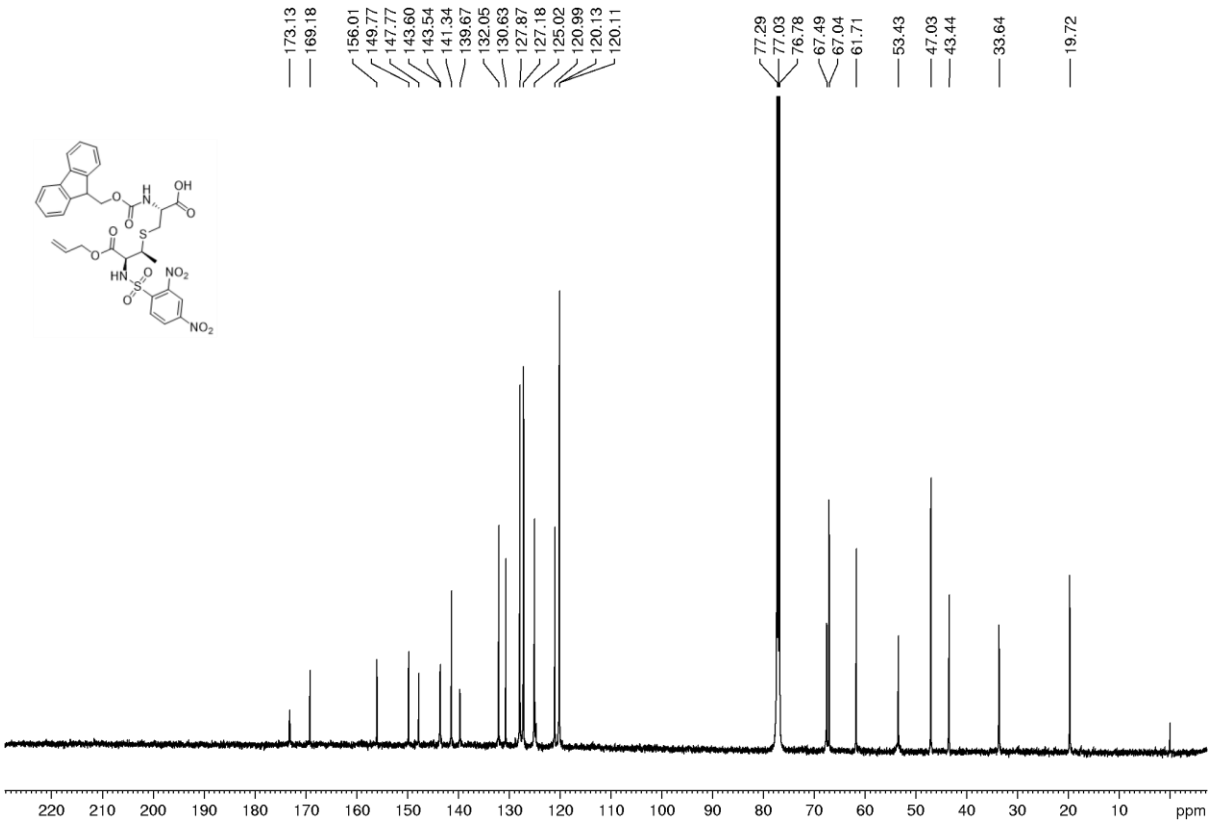


(R)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(((2S,3S)-4-(allyloxy)-3-((2-nitro-4-sulfophenyl)amino)-4-oxobutan-2-yl)thio)propanoic acid (18) (^{1}H, $CDCl_3$; ^{13}C, $CDCl_3$)

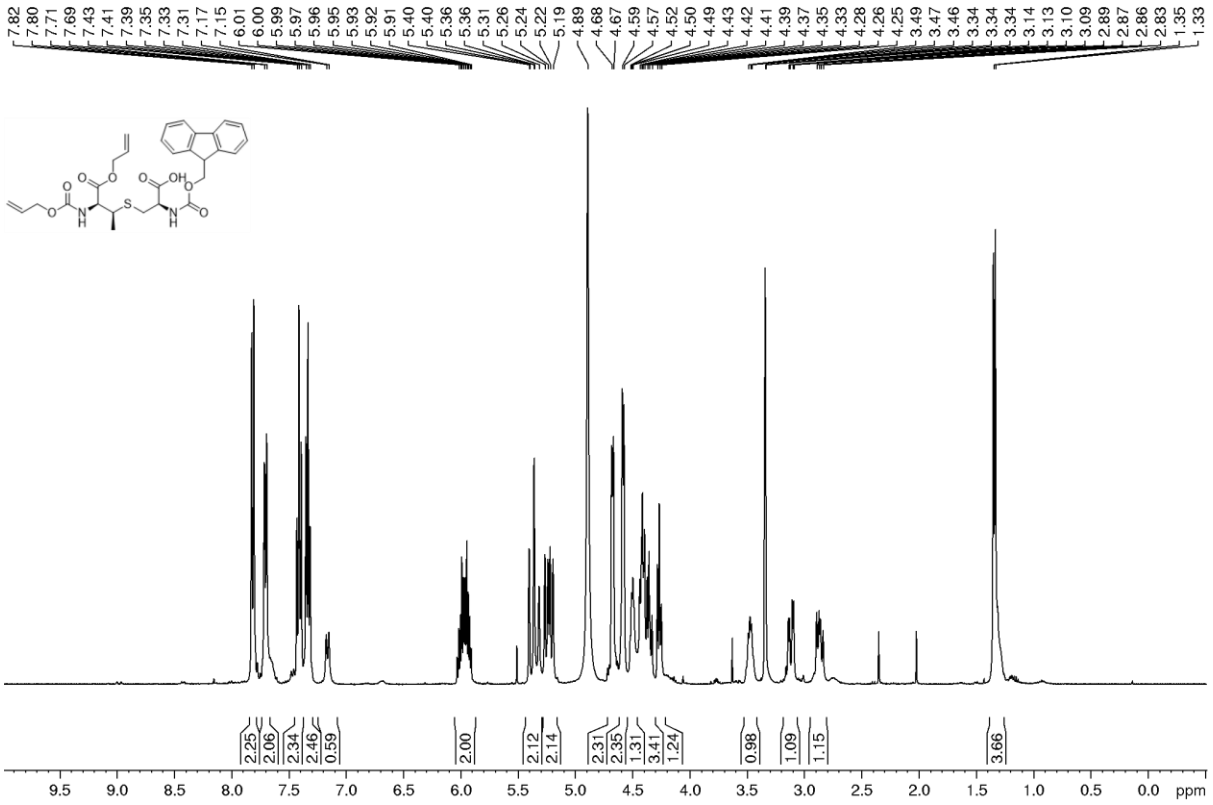


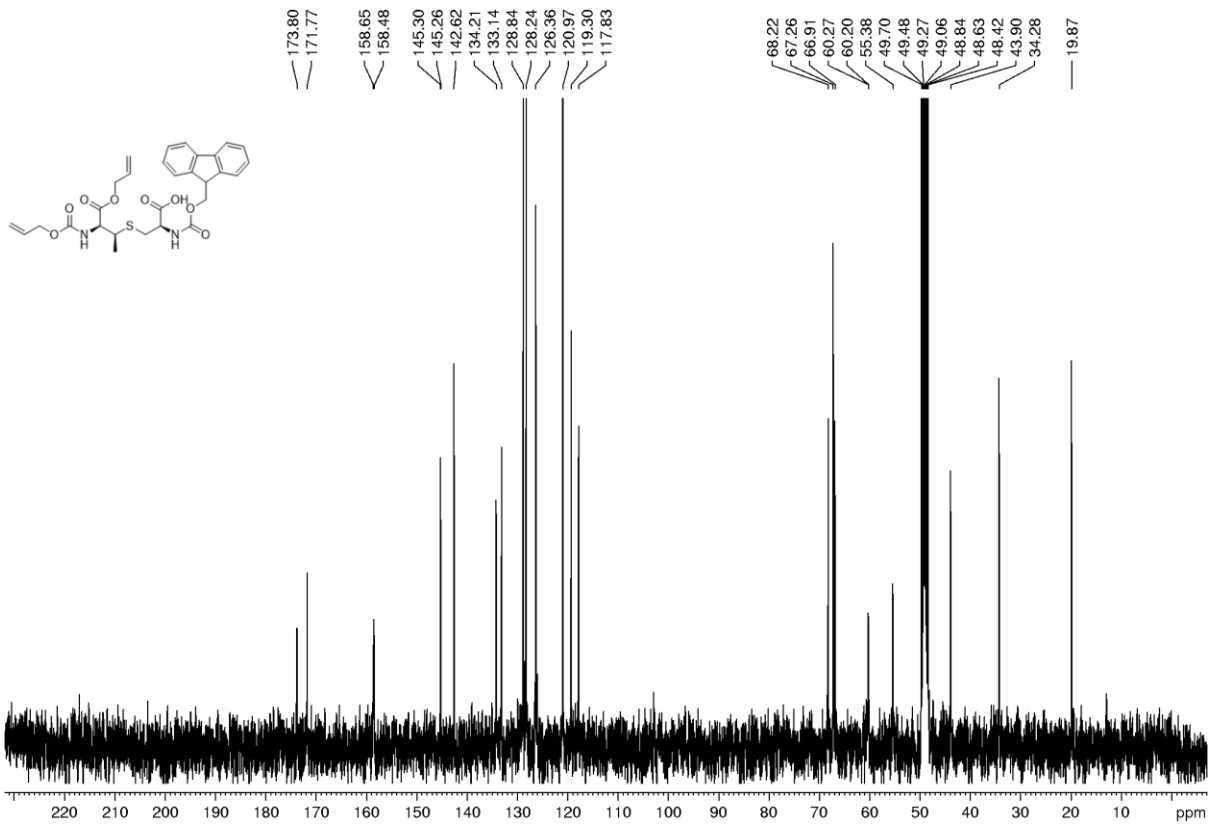
(The peak at 3.87 ppm should be some solvent impurity, it shift to 3.51 ppm when compound's concentration changes.)



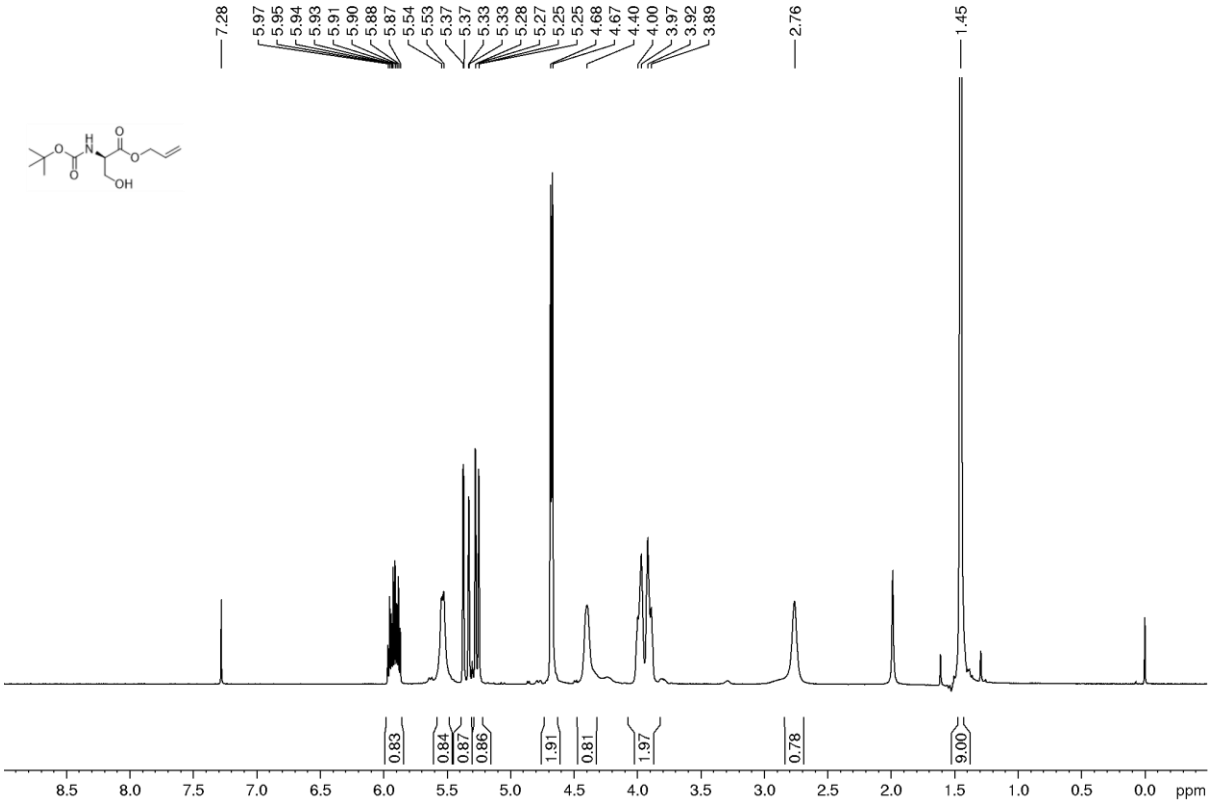

173.13
169.18
156.01
149.77
147.77
143.60
143.54
141.34
139.67
132.05
130.63
127.87
127.18
125.02
120.99
120.13
120.11
77.29
77.03
76.78
67.49
67.04
61.71
53.43
47.03
43.44
33.64
19.72
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

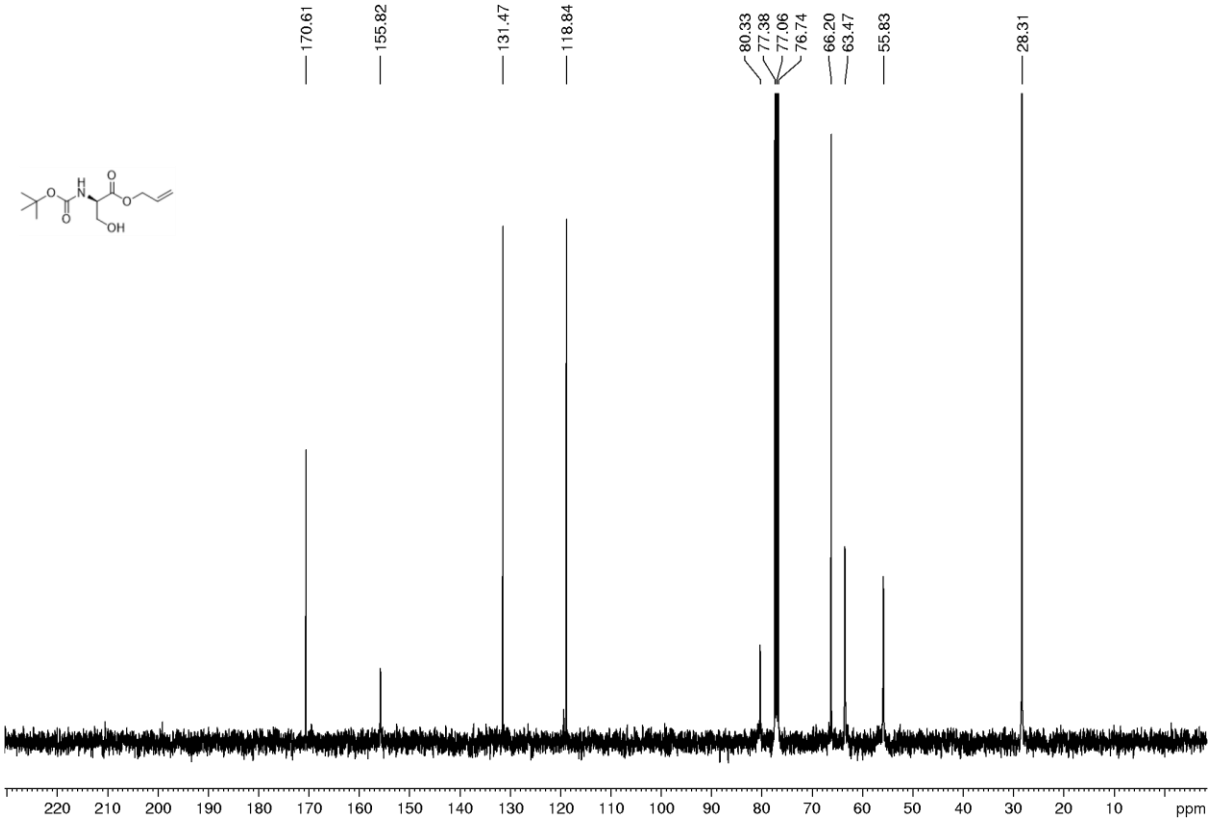
(R)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(((2S,3S)-4-(allyloxy)-3-(((allyloxy)carbonyl)amino)-4-oxobutan-2-yl)thio)propanoic acid (6) (^{1}H, CD_3OD; ^{13}C, CD_3OD)



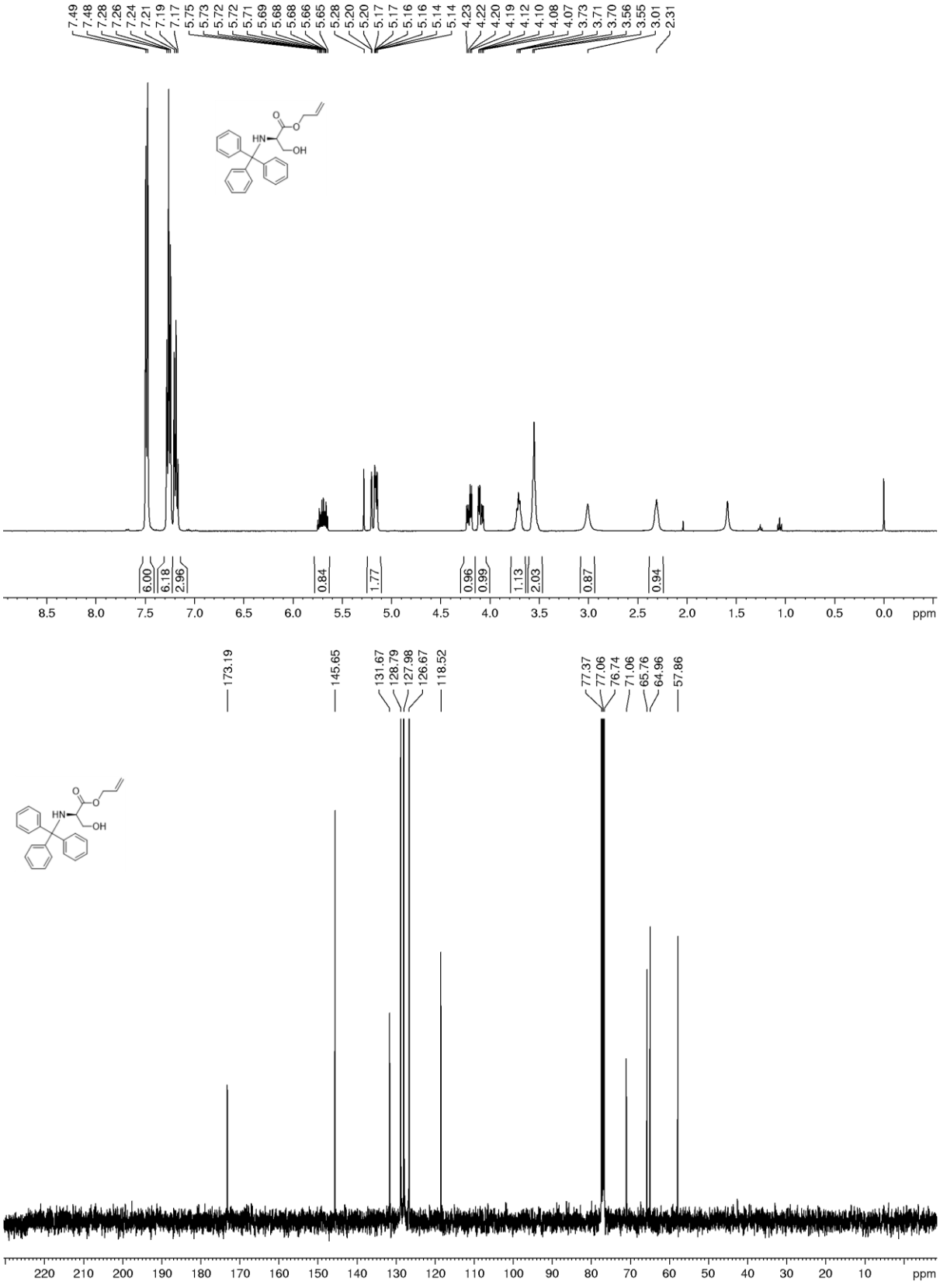


(R)-allyl 2-((tert-butoxycarbonyl)amino)-3-hydroxypropanoate (11) (^{1}H, $CDCl_3$; ^{13}C, $CDCl_3$)

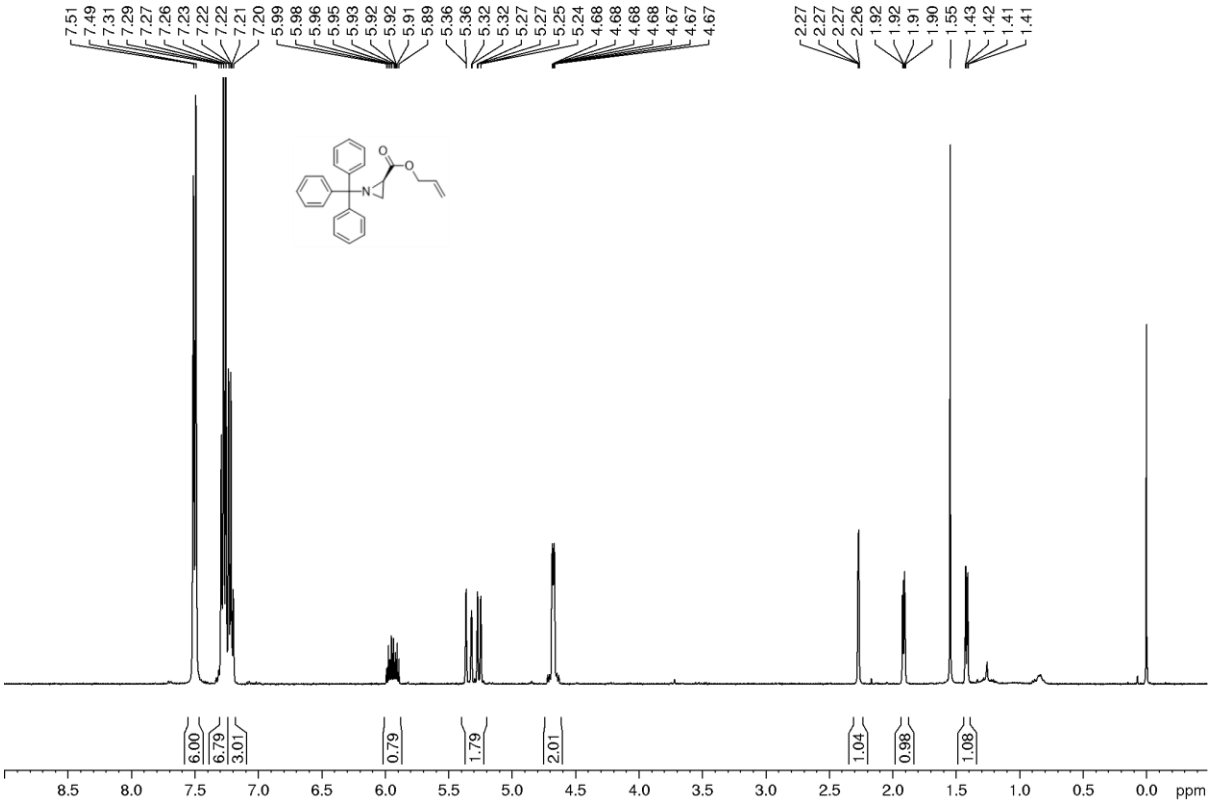


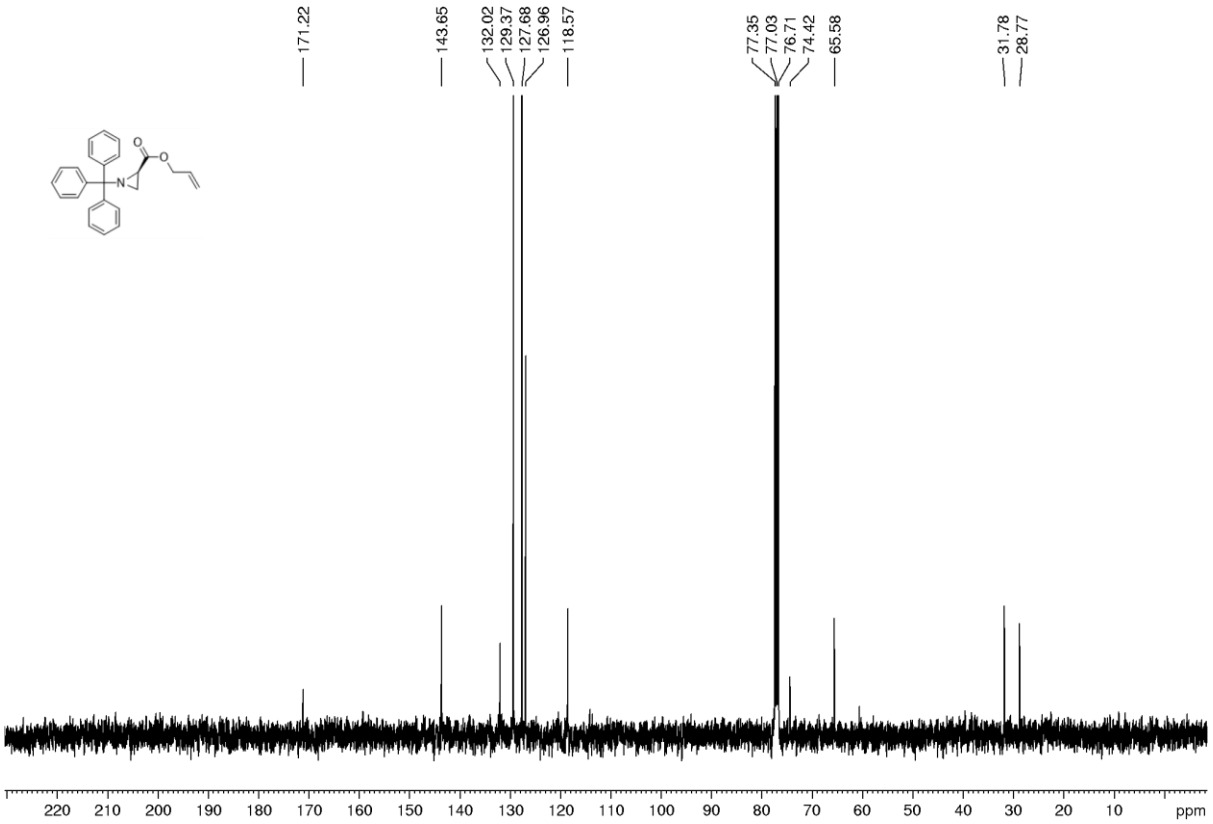


(R)-allyl 3-hydroxy-2-(tritylamino)propanoate (13) (^{1}H, $CDCl_3$; ^{13}C, $CDCl_3$)

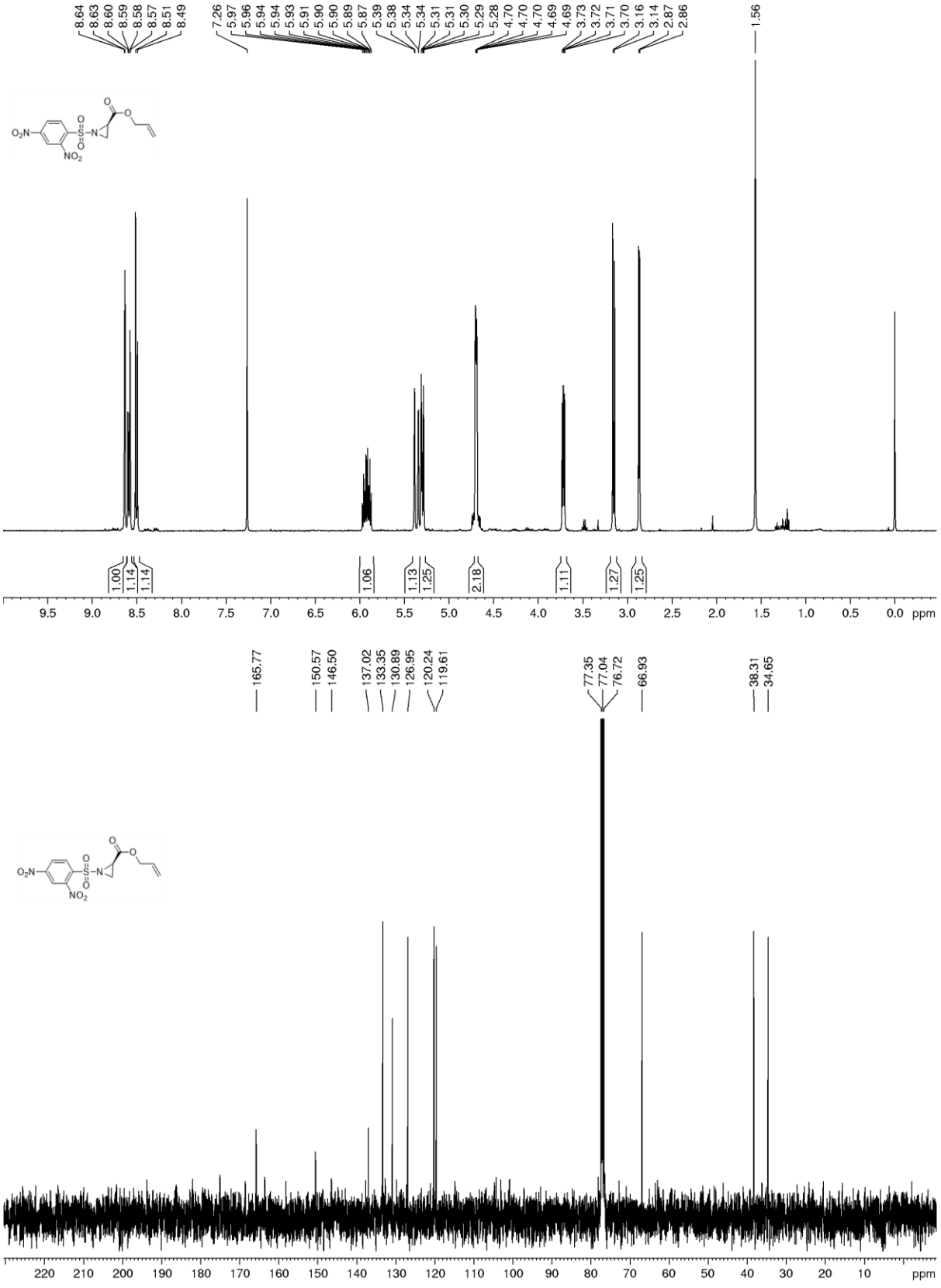


(R)-allyl 1-tritylaziridine-2-carboxylate (15) (^{1}H, $CDCl_3$; ^{13}C, $CDCl_3$)

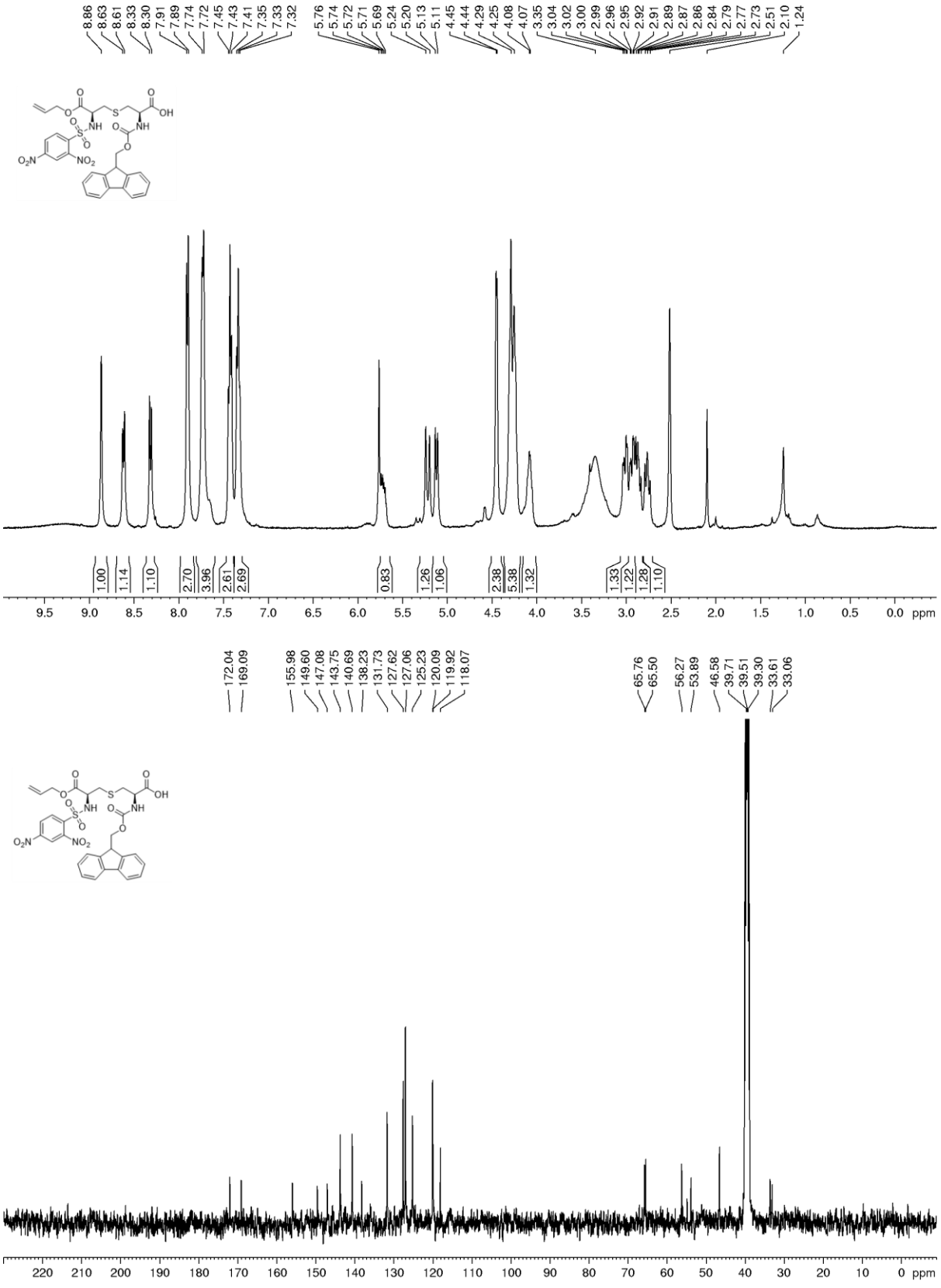




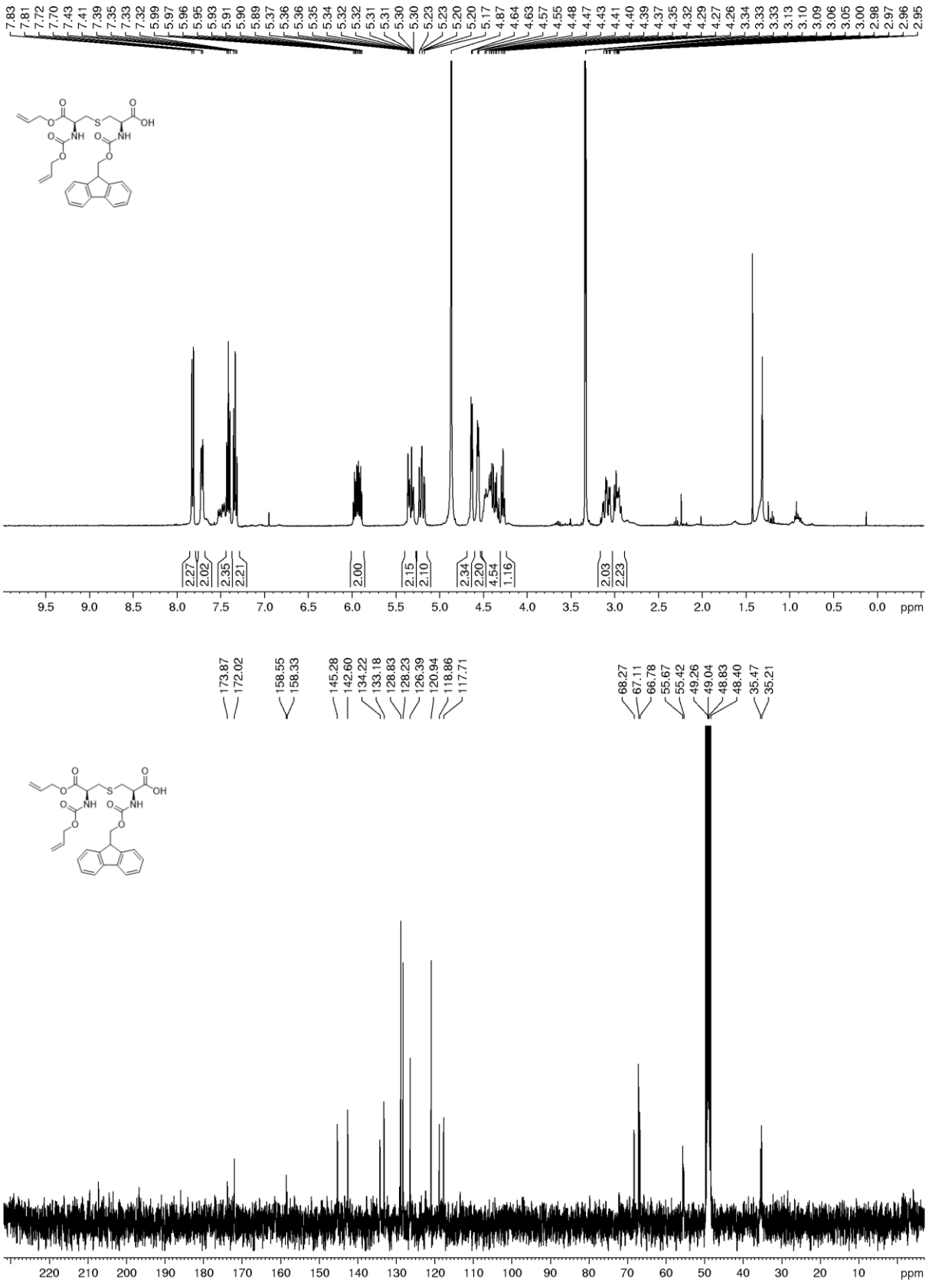
(R)-allyl 1-((2,4-dinitrophenyl)sulfonyl)aziridine-2-carboxylate (17) (^{1}H, $CDCl_3$; ^{13}C, $CDCl_3$)



(R)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(((S)-3-(allyloxy)-2-((2-nitro-4-sulfophenyl)amino)-3-oxopropyl)thio)propanoic acid (19) (^{1}H, DMSO-*d*$_6$; ^{13}C, DMSO-*d*$_6$)



(R)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(((S)-3-(allyloxy)-2-(((allyloxy)carbonyl)amino)-3-oxopropyl)thio)propanoic acid (7) (^{1}H, CD_3OD; ^{13}C, CD_3OD)



3. References

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