Supporting Information for

Quorum Sensing-Regulated Bactobolin Production by *Burkholderia thailandensis E264*

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General procedures. ^{1}H and 2-D NMR spectra for bactobolins were recorded in the inverse-detection probe of a Varian Inova spectrometer (600 MHz for ^{1}H, 150 MHz for ^{13}C). ^{13}C NMR spectra were recorded on the same instrument with a broad-band probe. Optical rotations were measured on an Autopol IV Automatic Polarimeter (Rudolph Research Analytical). HPLC purifications were carried out on an Agilent 1100 or 1200 Series HPLC system equipped with a photo diode array detector. LC-MS analysis was carried out on an Agilent 1200 Series HPLC system equipped with a diode array detector and a 6130 Series ESI mass spectrometer using an analytical Thermo Scientific Hypercarb column (5 μm, 4.6 × 100 mm) operating at 0.7 mL/min with a gradient of 10 % MeCN in H_2O to 100 % MeCN over 25 min. High resolution mass spectrometry (HR-MS) and tandem MS (MS/MS) were performed at the University of Illinois Urbana-Champaign Mass Spectrometry Facility. Activity assays for activity-guided fractionation were performed using the disc diffusion assay on LB-Agar plates and overnight cultures of *B. subtilis 3610* or *B. subtilis 168* in LB as the test organism.

Cultivation of *B. thailandensis.* *B. thailandensis E264* was obtained from DSMZ. All cultivation was carried out on a shaker/incubator at 30°C and 250 rpm, unless otherwise noted. A small 5 mL culture of *B. thailandensis* in LB was grown to saturation overnight. A 500 mL Erlenmeyer flask containing 125 mL of LB was inoculated with 1 mL of the overnight culture, and subsequently grown to saturation overnight. Each of three 4 L Erlenmeyer flasks containing LB+50 mM Mops, pH 7.0, were inoculated with 15 mL of the overnight culture and grown for three days at 30°C and 225 rpm. The cells were pelleted by centrifugation (8000g, 12 min, 4°C) and bactobolins purified as described below.

Purification of bactobolins. 1.5 L of the cell-free *B. thailandensis* supernatant from above was loaded directly onto an HP-20 column (~120 mL, d=2.5 cm, l=25 cm), which had been equilibrated in water, at ~5 mL/min. Bactobolin eluted in the flow-through, which was loaded directly onto a DEAE column (~80 mL, d=2.5 cm, l=17 cm), which had been equilibrated in

water, at ~5 mL/min. Bactobolin eluted in the flow-through. These initial columns do not bind bactobolin, but provide a crude separation of bactobolin from non-polar and small negatively charged molecules. The flow-through was concentrated 10-fold in vacuo and stored at 4°C overnight. A precipitate that forms after these steps was removed by centrifugation and the supernatant, which contains bactobolin, loaded onto a Hypercarb column (Thermo Scientific, ~25 mL, d=1.25 cm, l=20 cm), which had been equilibrated in 20 % MeCN in water. The column was washed with 5-7 column volumes (CV) of 20 % MeCN, then 10 CV of 50 % MeCN in water, and finally with 8-10 CV of 100 % MeCN; bactobolin eluted in the 50 % MeCN fraction, which was concentrated ~12-fold in vacuo and then loaded with successive 2 mL injections onto an Agilent Eclipse C8 column (5 μm, 9.4 × 250 mm). Bactobolin eluted at 25-30 % MeCN in H_2O. Fractions containing bactobolin were pooled, concentrated and separated to individual congeners on a Supelco Discovery HS C18 column (10 μm, 10 × 250 mm) with an isocratic gradient of 12 % MeCN (+0.1 % formic acid) in H_2O (+0.1 % formic acid) over 25 minutes, followed by a gradient from 12 to 45 % of MeCN (+0.1 % formic acid) over 15 minutes. This was repeated to obtain each analog in >95 % purity (as judged by NMR). Bactobolins A, B, C, and D eluted at 11, 14, 20, and 29 minutes with a yield of 9, 8, 14 and 1.5 mg per 1.5 L, respectively.

NMR spectra of bactobolin A. The 1-D and 2-D NMR spectra used to elucidate the structure of bactobolin A are shown below. These are representative for spectra of other bactobolin analogs characterized herein. NMR data for all bactobolins are summarized in Tables S1-S4. The stereochemistry of bactobolin A can be demonstrated using the data below. The large coupling between H5 and H6 (9.5-9.8 Hz) suggests an axial-axial relationship between these protons. H10 couples to H5 with a similar coupling constant resulting in a triplet H5 signal and indicating an axial-axial interaction between H5-10. H4 appears as a doublet with a 3.3 Hz coupling to H10 indicating an equatorial-axial interaction between H4-H10. H10 is also involved in homoallylic coupling interaction with H7ax with a constant of 2.4 Hz consistent with

the axial position of H10. This orientation is consistent with the ROESY interactions below (Fig. S6). The ROESY signal between H4 and H3b indicates an S orientation at C3. This interaction is highlighted with a black arrow in Fig. S6. There is also a ROESY correlation between H3b and H10 consistent with an axial H10 and an *S* configuration at C3. This interaction is highlighted with a red arrow in Fig. S6. H5 shows a weak ROESY signals to H4' indicated with a blue arrow in Fig. S6. A similar analysis has been applied to other bactobolins with a similar outcome (see Tables S1-S4).

Marfey's Analysis on the Ala residues in bactobolin A-D. To determine the absolute configuration of the Ala residues in bactobolin, 0.1-0.7 mg of bactobolin A-D was dissolved in ~1 mL of 6 N HCl in a 4 mL scintillation vial and heated in an oil bath at 115°C for 1.5 h. The acid was removed in vacuo and the material re-dissolved ~1 mL in water. This was repeated 2× to completely remove the acid. Each sample was then loaded onto a PrepSep C8 cartridge (Fisher Scientific) and Ala was eluted with 10 % MeCN in H_2O. The eluant was dried in vacuo and Marfey's analysis was carried out as previously described.[1,2] Bactobolins B-D only contained L-Ala, bactobolin A contained ~18 % D-Ala which presumably arose from racemization during extended storage of bactobolin A under acidic conditions (0.2 % TFA).

Export of bactobolin. The *btaK* mutant and *btaI1, I2, I3* mutants have been described (see Table S8).[3,4] The *btaT* deletion mutant was generated by a method previously described.[4] Overlap extension PCR was used to generate PCR products with ~1 kb of DNA homologous to the DNA flanking the *btaT* gene. The products were annealed and amplified by PCR. This step introduced primer-encoded XbaI and HindIII sites during amplification. The digested PCR products were ligated to XbaI-HindIII-digested pJRC115 and this suicide vector was introduced into *B. thailandensis* E264 to deliver the *btaT* deletion allele. Correct clones were identified by PCR amplification of the *ΔbtaT* allele. These were further confirmed by sequencing PCR products generated with primers targeting chromosomal DNA flanking the recombination sites.

To examine the export of bactobolin, a 5 mL culture of LB+50 mM Mops, pH 7, was inoculated with Δ*btaK*, Δ*btaT* or wt *B. thailandensis*. After 12 or 24 h, the cells were removed by centrifugation and the supernatant analyzed by LC-MS methods on an analytical Hypercarb column as described above. The volume of supernatant loaded was 30, 100, and 100 μL for wt, *btaK* and *btaT B. thailandensis*, respectively. The mass-ion extracted trace for bactobolin B or bactobolin A was used for quantitation yielding similar results. The results of the 24 h time point analysis are shown in Fig. 2. At the 12 h time point, 18-fold more bactobolin was detected in the wt supernatant vs. that in the *btaT* mutant. No bactobolin was detected in the *btaK* mutant.

MIC Assays. The minimum inhibitory concentrations (MIC) of each bactobolin was determined according to the 2003 guidelines of the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) using the microtiter MIC method. Inocula for each test organism were prepared by suspending a colony from an LB agar plate into Tryptic Soy Broth and growing for 3-5 hours at 35°C with shaking, then adjusting the culture turbidity in PBS to the equivalent of a 1.0 McFarland Standard (3×10^8 CFU/ml). These cell suspensions were used as inocula for microtiter MIC assays. 2.5 μL inoculum, which corresponded to $1x10^6$ cells, was added to a 200 μL well containing each bactobolin diluted in cation-adjusted Mueller-Hinton II broth (actual CFU/ml ranged from 5×10^4 to 1×10^6), and these were incubated without shaking for 24 hours at 35°C. The MIC was defined as the lowest concentration of bactobolin (μg/ml) in which bacterial growth in the well was not measurable by determining the turbidity at 595 nm (A_{595}) on a 96-well plate reader. See Table S8 for strains used in these assays.

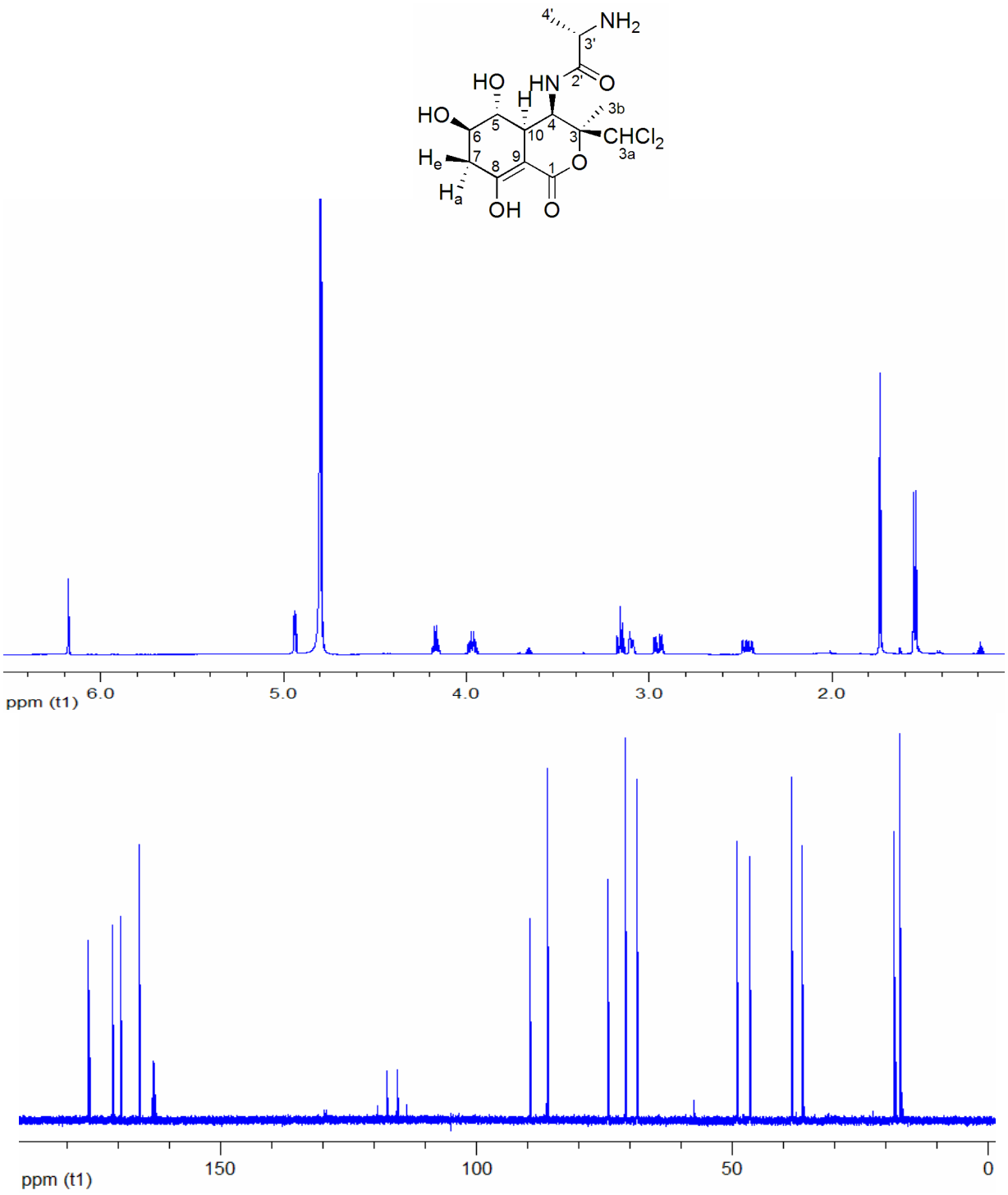

Figure S1. ^{1}H (A) and ^{13}C (B) spectrum of bactobolin A in H_2O (+0.2 % TFA). See Table S1 for assignment of peaks. The structure of bactobolin A is shown at the top.

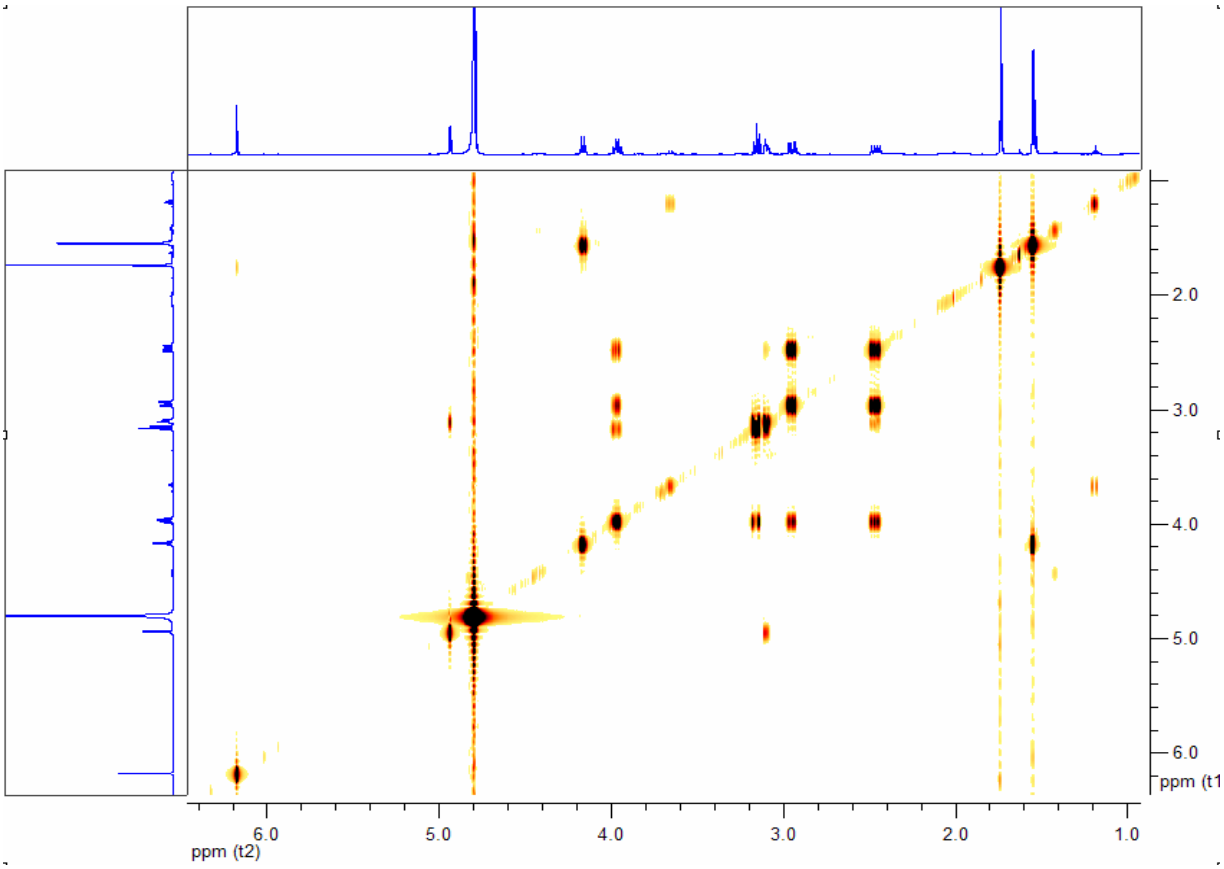

Figure S2. gCOSY spectrum of bactobolin A in H_2O (+0.2 % TFA).

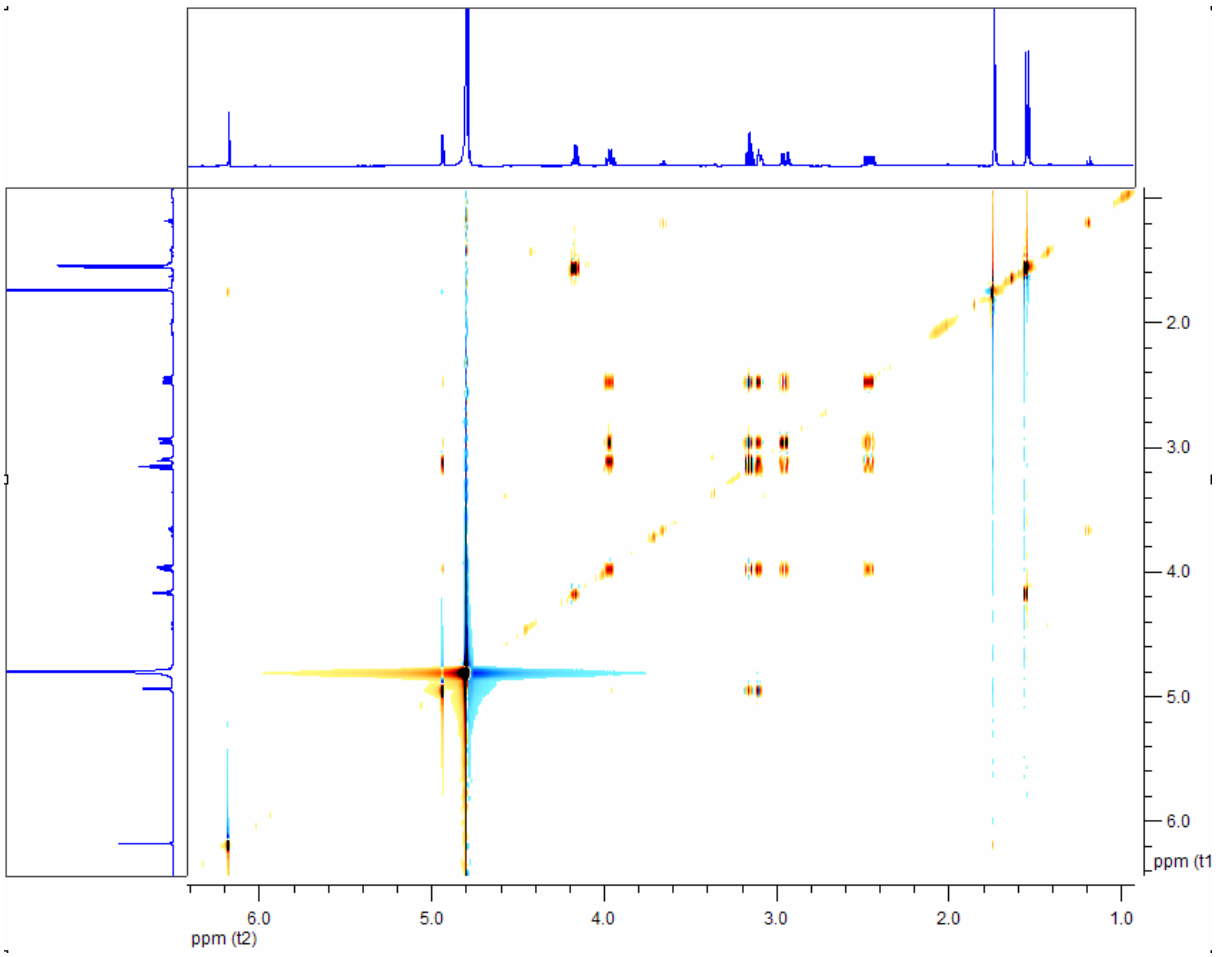

Figure S3. TOCSY spectrum of bactobolin A in H_2O (+0.2 % TFA).

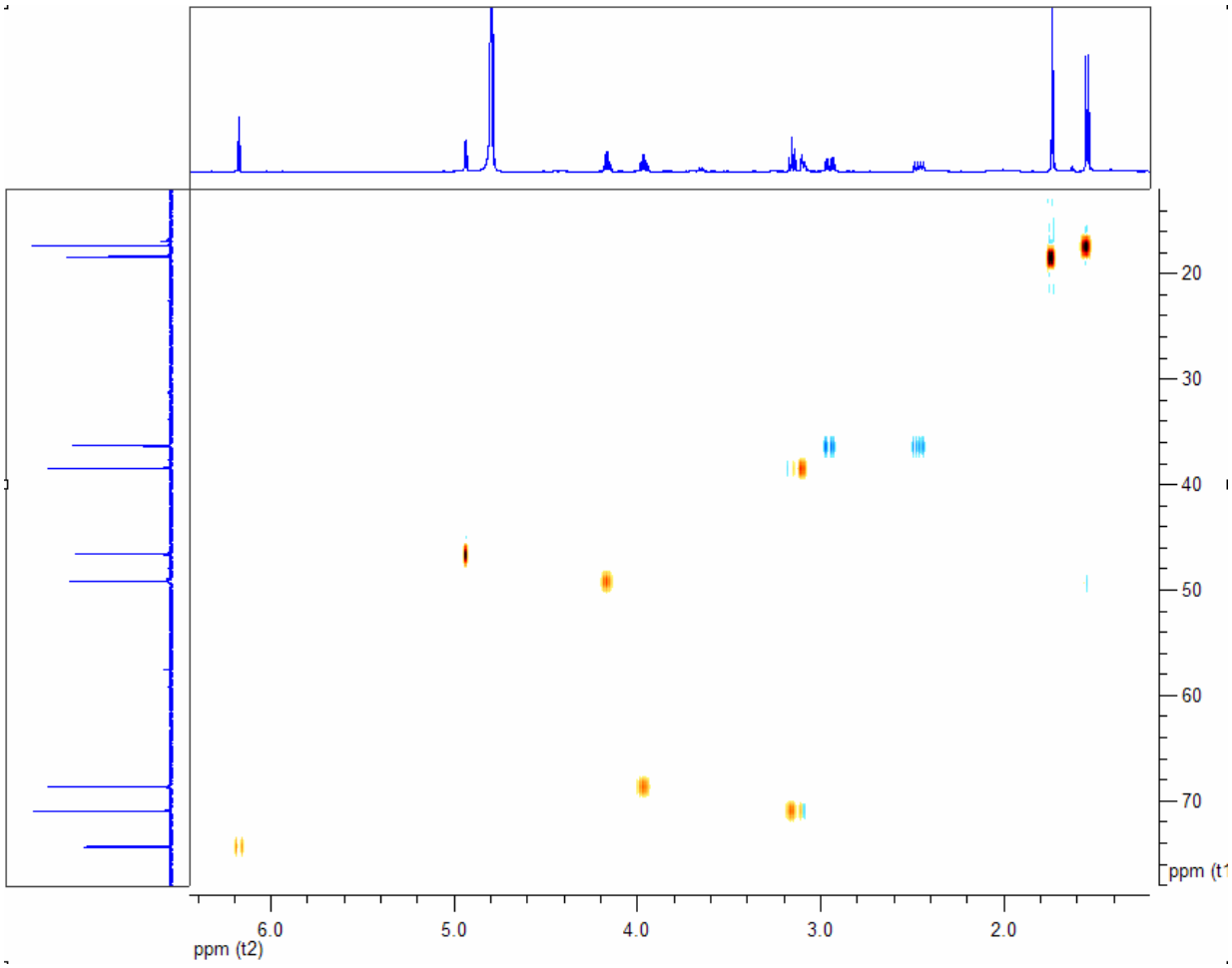

Figure S4. Multiplicity-edited gHSQC spectrum of bactobolin A in H_2O (+0.2 % TFA). Correlations of CH and CH_3 groups are shown in red, those of CH_2 groups are shown in blue.

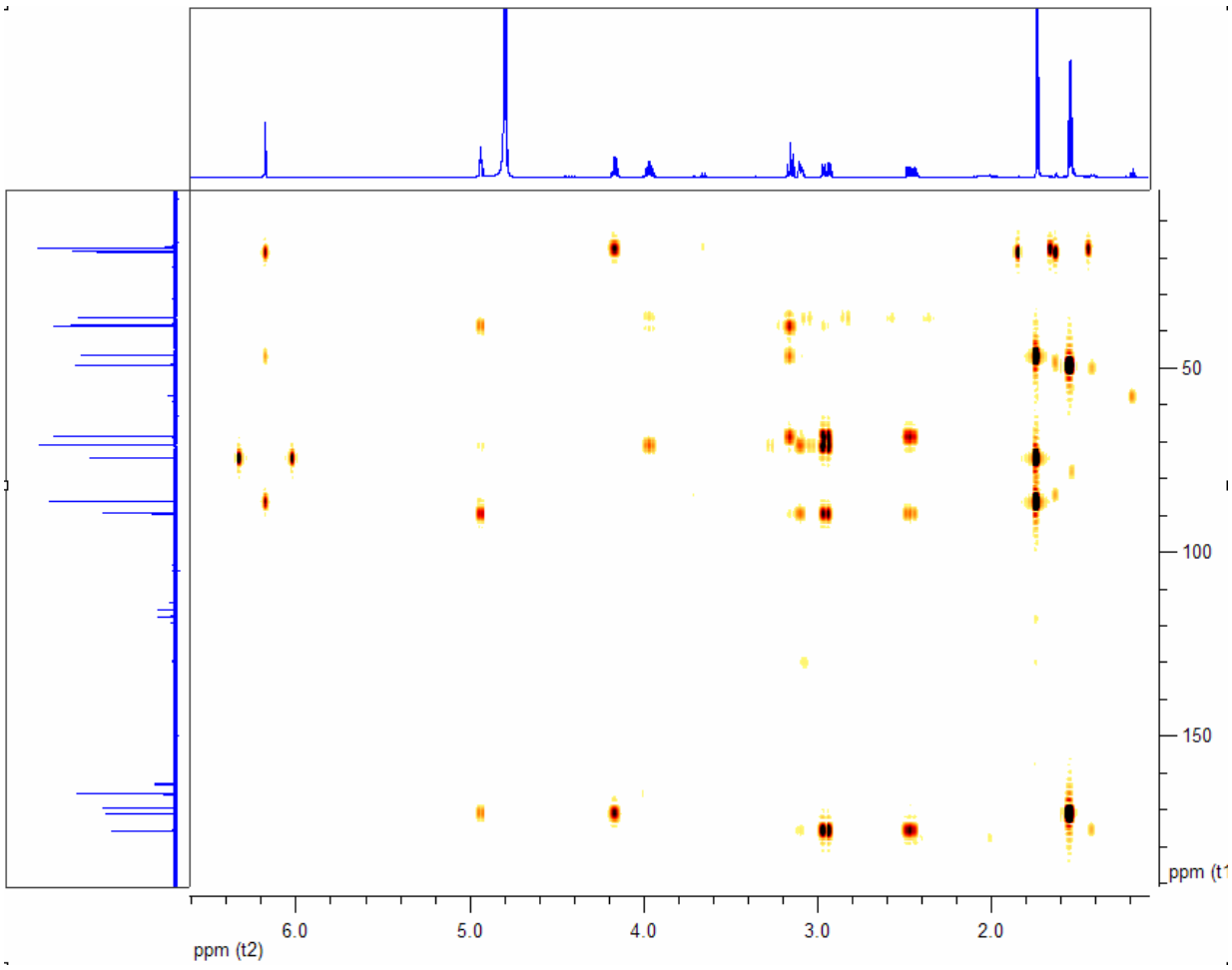

Figure S5. gHMBC spectrum of bactobolin A in H_2O (+0.2 % TFA).

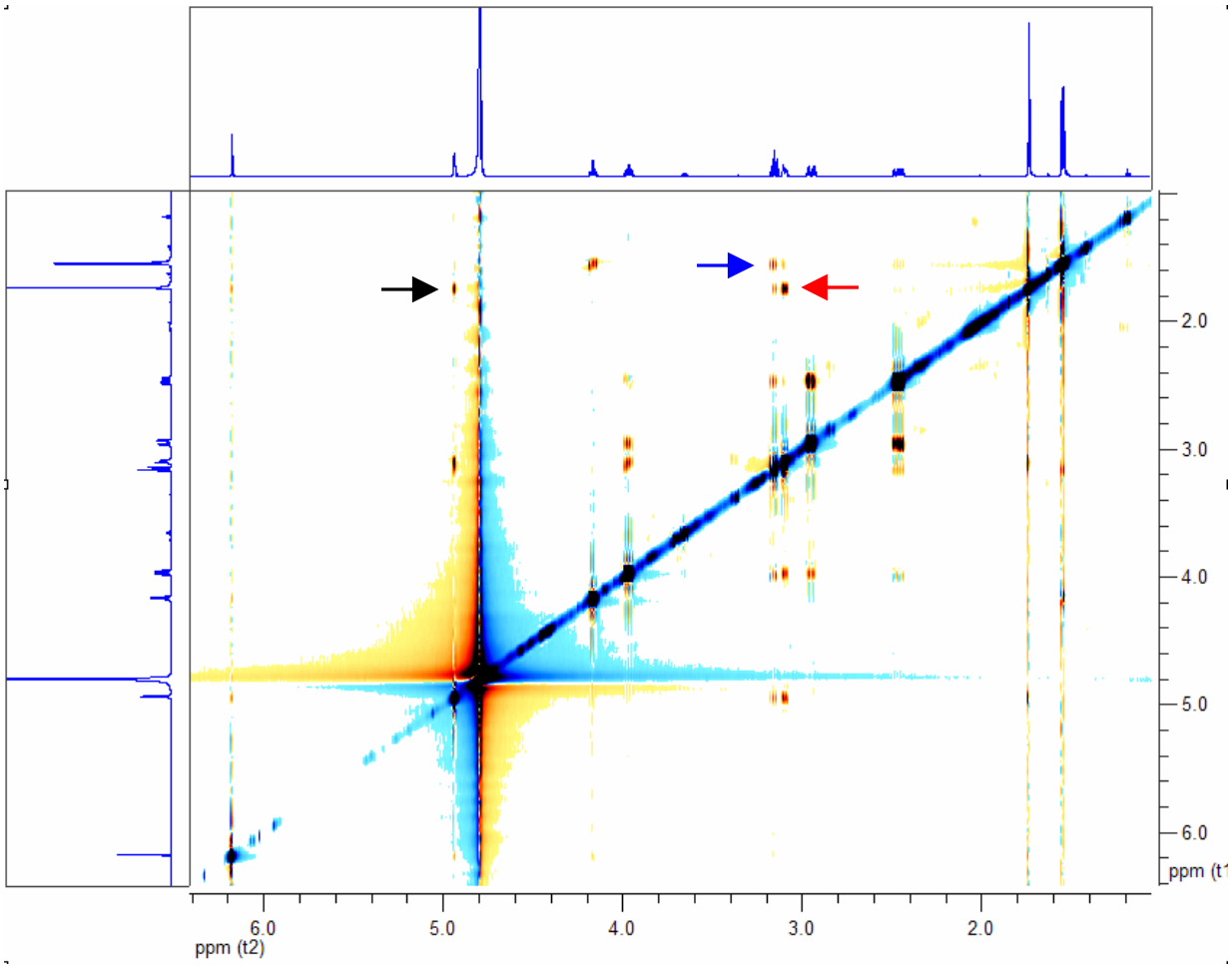

Figure S6. ROESY spectrum of bactobolin A in H_2O (+0.2 % TFA). See text above (p. S3) for details.

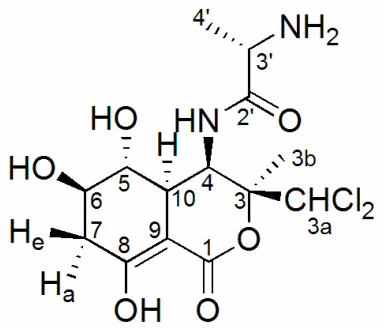

Table S1. NMR spectral data for bactobolin A. The structure of bactobolin A is shown above.

C/H	δ_H	Multiplicity (Hz)	δ_C	HMBC
1			169.4	
3			86.2	
3a	6.18	s	74.4	C3, C3b, C4
3b	1.74	s	18.4	C3, C3a, C4
4	4.94	d (3.4)	46.6	C3, C5, C9, C10
5	3.16	t (9.5)	70.9	C4, C6, C10
6	3.97	dt (6.8, 9.8)	68.7	C5, C7, C10
7 (H_e)	2.95	dd (6.7, 18.8)	36.4	C5, C6, C8, C9
7 (H_a)	2.46	ddd (2.4, 10.0, 18.9)	36.4	C6, C8, C9
8			175.8	
9			89.5	
10	3.10	m	38.4	C5, C9
2'			171	
3'	4.17	q (7.1)	49.2	C2', C4'
4'	1.55	d (7.1)	17.3	C2', C3'

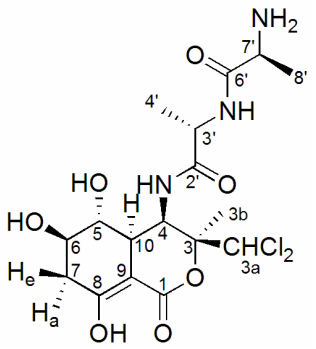

Table S2. NMR spectral data for bactobolin B. The structure of bactobolin B is shown above.

C/H	δ_H	Multiplicity (Hz)	δ_C	HMBC
1			169.7	
3			86.2	
3a	6.19	s	74.5	C3, C3b, C4
3b	1.72	s	18.2	C3, C3a, C4
4	4.88	d (3.3)	46.5	C9, C10, C2'
5	3.20	t (9.5)	70.8	C4, C6, C7, C10
6	3.96	dt (6.9, 9.8)	68.7	C5, C7
7 (H_e)	2.94	dd (6.7, 18.8)	36.3	C5, C6, C8, C9
7 (H_a)	2.46	ddd (2.2, 10.0, 18.9)	36.3	C6, C8, C9
8			175.7	
9			89.5	
10	3.06	m	38.3	C5, C8, C9
2'			174.8	
3'	4.41	q (7.1)	49.9	C2', C4', C6'
4'	1.41	d (7.2)	16.7	C2', C3'
6'			170.6	
7'	4.09	q (7.1)	48.9	C6', C8'
8'	1.53	d (7.1)	16.7	C6', C7'

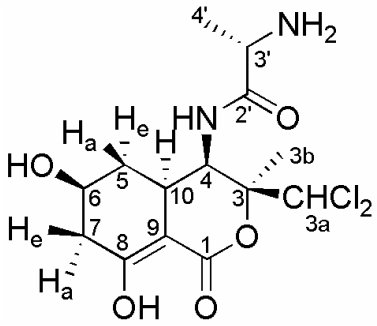

Table S3. NMR spectral data for bactobolin C. The structure of bactobolin C is shown above.

C/H	δ_H	Multiplicity (Hz)	δ_C	HMBC
3			85.3	
3a	6.15	s	74.3	C3, C3b
3b	1.74	s	18.2	C3, C3a, C4
4	4.72	d (3.6)	49.1	C9, C10
5 (H_a)	2.08	m	33.5	C4, C6, C10
5 (H_e)	1.15	q (11.6)	33.5	C4, C6, C7, C10
6	4.17	m	64.4	
7 (H_e)	2.85	dd (6.4, 18.6)	37.5	C5, C6, C8, C9
7 (H_a)	2.35	ddd (2.5, 9.9, 18.6)	37.5	C6, C8, C9
8			176.7	
9			91.4	
10	3.30	m	31.8	
2'			171.1	
3'	4.16	m	49.1	C2', C4'
4'	1.55	d (7.1)	17.2	C2', C3'

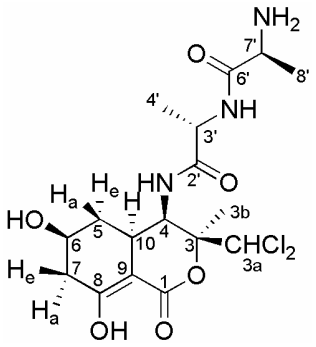

Table S4. NMR spectral data for bactobolin D. The structure of bactobolin D is shown above.

C/H	δ_H	Multiplicity (Hz)	δ_C	HMBC
3			85.8	
3a	6.17	s	74.5	C3, C3b
3b	1.73	s	18.1	C3, C3a, C4
4	4.66	d (3.6)	49.1	C9, C10
5 (H_a)	2.05	m	33.7	C6
5 (H_e)	1.18	m	33.7	C4, C10
6	4.16	m	64.2	
7 (H_e)	2.85	dd (6.3, 18.6)	37.6	C5, C6, C8, C9
7 (H_a)	2.35	ddd (2.4, 9.9, 18.5)	37.6	C6, C8, C9
8			176.2	
9			91.4	
10	3.28	m	31.8	
2'			174.8	
3'	4.41	q (7.2)	49.9	C2', C4', C6'
4'	1.41	d (7.2)	16.7	C2', C3'
6'			170.2	
7'	4.09	q (7.1)	48.9	C6', C8'
8'	1.52	d (7.1)	16.6	C6', C7'

Table S5. Spectral properties of bactobolins.

	Bactobolin A	Bactobolin B	Bactobolin C	Bactobolin D
HR-MS (M+H)obs [a]	383.0777	454.1148	367.0828	438.1212
HR-MS (M+H)calc	383.0769	454.1145	367.0823	438.1199
MS/MS (M+H)obs [b]	312.1	312.1	296.1	296.1
MS/MS (M+H)calc [b]	312.2	312.2	296.2	296.2
$[\alpha]_D$ (c=1, H_2O) [c]	-6.3°	-26.7°	-17.8°	-12.9°
$[\alpha]_{D\ (reported)}$ [d]	-6.3°	-27°	-23.9°	nr [e]
λ_{max} (nm)	264	264	266	266

[a] HR-MS and MS/MS data were acquired under positive ionization mode. [b] MS/MS spectra were acquired under positive ionization mode. The observed (M+H) of the dominant peak is indicated, and corresponds to bactobolin minus the Ala or Ala-Ala fragment. [c] The specific rotation data have been scaled to c=1 for comparison with published values. Optical rotation for bactobolins A, B, C, and D were obtained with c=0.9, c=0.8, c=1 and c=0.15, respectively. [d] The reported $[\alpha]_D$ data are from Ref. 5. [e] nr, not reported.

Table S6. FASTA search results for proteins in the *bta* cluster

Gene	No. of amino acids	Protein homology (Swissport accession no.)	% Amino acid identity [a]
btaA	381	*Burkholderia multivorans* CGD1, 4-hydroxyphenyl-pyruvate dioxygenase (B9B0B6)	79.5
btaB	98	No significant homology [b]	
btaC	307	*Burkholderia pseudomallei* MSHR346, CmaB chlorinase (C4I3I8)	97.1
btaD	81	*Burkholderia pseudomallei*, putative acyl-carrier protein (Q63L26)	91.1
btaE	548	*Burkholderia pseudomallei* 1106b, Linear gramicidin synthetase subunit C – adenylation domain (C5ZLJ3) [c]	89.0
btaF	135	No significant homology	
btaG	410	*Burkholderia pseudomallei*, CPA2 family transporter (C0YG66)	93.4
btaH	285	No significant homology [d]	
btaI2	206	*Burkholderia pseudomallei* S13, autoinducer synthetase (B1H6I9)	91.7
btaJ	693	*Burkholderia pseudomallei* 406e, oligopeptidase A (A8ELY3)	86.9
btaK	1446	*Burkholderia pseudomallei*, putative non-ribosomal peptide synthase (Q63L34) [e]	82.4
btaL	*1524*	*Burkholderia pseudomallei*, type I polyketide synthase (A3NJX3) [f]	86.4
btaM	*1370*	*Burkholderia pseudomallei*, TubD-like polyketide synthase (Q3JMA6) [g]	91.4
btaN	1144	*Burkholeria pseudomallei*, putative non-ribosomal pepdite synthase (A4MH51) [h]	88.1

btaO	779	*Burkholderia pseudomallei* Pakistan 9, type I polyketide synthase (C0YG77) [i]	84.2
btaP	530	*Burkholderia pseudomallei* 668 Metallo-β-lactamase family protein (A3NJW9)	94.5
btaQ	*281*	*Burkholderia pseudomallei* Pakistan 9, GNAT family acetyltransferase (C0YG79)	90.4
btaR2	*234*	*Burkholderia pseudomallei*, N-acyl homoserine lactone-dependent regulatory protein (Q3JMA2)	95.3
btaS	280	*Burkholderia pseudomallei* 1106a, type II thioesterase (A3P5I5)	92.8
btaT	408	*Burkholderia pseudomallei* 1106 a, Major facilitator superfamily/drug resistance protein (C5ZLH5)	90.7
btaU	281	*Burkholderia pseudomallei* MSHR346 TauD/TfdA (C4I3G5)	94.7

[a] Results are from FASTA searches conducted between 7/5/2009 and 9/1/2009. [b] InterProScan searches indicate similarities to an ACP-like domain. [c] The NRPS Predictor software indicates valine specificity for this domain. [d] InterProScan searches indicate similarities to members of the α/β hydrolase superfamily. [e] InterProScan searches indicate a domain architecture of C-A-T-C, corroborated by analysis of the individual domains, where the initial C domain appears to be inactive. NRPS prediction software indicates Gly specificity for the A domain. [f] InterProScan searches suggest a domain architecture of KS-AT-SDR-ACP. No homology was found for a ~300 amino acid stretch preceding the SDR domain. [g] InterProScan searches suggest a domain architecture of KS-KR-ACP, the presence of the AT domain is inferred from results of secondary structure prediction searches using Phyre software.[6] [h] InterProScan and NRPS prediction software searches indicate an A-T-C-T domain architecture and Ala specificity for the A domain. [i] InterProScan searches indicate a KS-AT-ACP architecture.

Table S7. Susceptibility of wt *B. thailandensis*, Δ*btaI1/I2/I3* or Δ*btaT* to bactobolin B or spent medium from wt *B. thailandensis*.

Strain	Bactobolin B MIC (μg/ml) [a]	Susceptible to spent medium of wt *B. thailandensis*?
B. thailandensis E264 (wt)	>25	No
B. thailandensis Δ*btaI1, I2, I3* (JBT112)	>25	No
B. thailandensis Δ*btaT* (BD44)	>25	No

[a] Susceptibility of *B. thailandensis* strains was determined using the CLSI microtiter MIC assay (described above) using purified bactobolin B or filter sterilized wild type culture fluid prepared from wt *B. thailandensis* E264 cultures grown shaking in LB+Mops, pH 7 at 30°C for 24 hours.

Table S8. Bacterial strains used in this study.

Strain	Source
Burkholderia thailandensis E264 wt	DSMZ, Ref. 7
Burkholderia thailandensis Δ*btaK* (BD20)	Ref. 3
Burkholderia thailandensis Δ*btaT* (BD44)	This study
Burkholderia thailandensis Δ*btaI1, I2, I3* (JBT112)	Ref. 4
Staphylococcus aureus COL (methicillin resistant MRSA strain)	Ref. 8
Enterococcus faecalis V583 (vancomycin resistant VRE strain)	Ref. 9
Vibrio parahaemolyticus LM5674	Ref. 10
Bacillus subtilis 3610	Ref. 11
Escherichia coli ATCC25922	ATCC

Supporting Information References

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