ELECTRONIC SUPPLEMENTARY INFORMATION

Identification and characterisation of the gene cluster for the anti-MRSA antibiotic bottromycin: Expanding the biosynthetic diversity of ribosomal peptides

William J. K. Crone,[a] Finian J. Leeper[a] and Andrew W. Truman[a]

[a] *Department of Chemistry, University of Cambridge, Cambridge, UK CB2 1EW*

Materials

All chemicals were analytical grade and obtained from Sigma Aldrich unless otherwise specified.

Bacterial Strains

Streptomyces scabies DSM 41658 and *Streptomyces bottropensis* DSM 40262 were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and were kept either on agar plates at 4 °C or in 30-40% glycerol stocks at -80 °C. NovaBlue competent cells (Novagen) were used for genetic manipulation and the methylation deficient strain of *E. coli,* ET12657[1], was used for transfer of genetic material to *S. scabies* by conjugation. Cells transformed with plasmids were stored in a 30-40% glycerol stock at -80 °C.

Antibiotics

Antibiotics were used at final concentrations as follows: kanamycin (Melford) was used at 50 μg mL^{-1}, apramycin (Duchefa) at 50 μg mL^{-1}, carbenicillin (Melford) at 30 μg mL^{-1}, chloramphenicol at 25 μg mL^{-1} and nalidixic acid at 25 μg mL^{-1}.

Production of bottromycin

GYM medium (0.4% glucose (Fisher), 0.4% yeast extract, 1.0% malt extract, in Milli-Q (MQ) water[2]) in a 250 mL flask was inoculated with a small piece (approx. 1 cm^2) of bacteria grown on SFM-agar (Soy Flour Medium: 20 g L^{-1} soy flour, 20 g L^{-1} mannitol, 20 g L^{-1} agar, 10 mM $MgCl_2$ in tap water[3]). This was incubated at 250 rpm, 30 °C for 2 days. This starter culture was then used to inoculate production medium[2] (PM: 1% glucose, 1.5% starch, 0.5% yeast extract, 1.0% soy flour, 0.5% NaCl (Breckland), 0.3% $CaCO_3$ (Breckland) in MQ water), at 5% of the PM volume. 1.05 mM of $CoCl_2$ was often included in the PM as it was found to increase production of bottromycin.

LCMS analysis of cell extract

Spectra were obtained using a Hewlett-Packard HPLC 1100 series instrument coupled to a Finnigan MAT LCQ ion trap mass spectrometer fitted with a positive mode ESI source. PM cell culture (100 μL) was diluted with methanol (100 μL) at various time points and centrifuged to pellet cell debris. 40 μL of the supernatant was added to 5 μL of 0.5 mM desvancosaminyl vancomycin (DVV), which was used as a reference compound. Samples were injected onto a Phenomenex Luna C18(2) column (250 mm x 2.0 mm, 5 μm), eluting with a linear gradient of 5 to 95% acetonitrile (Fisher,

HPLC grade) containing 0.1% trifluoroacetic acid (TFA) in water (Rathburn, HPLC grade) + 0.1% TFA over 28 minutes with a flow-rate of 0.3 mL/min.

HPLC-HRMS data were obtained using a Thermo Electron LTQ-Orbitrap. Samples were injected onto a Dionex Acclaim C18 PepMap 100 column (150 mm x 1.0 mm, 3 μm), eluting with a linear gradient of 0 to 100% B in A over 28 min with a flow rate of 50 μL/min (A: 98 % H_2O, 2 % MeCN, 0.1 % formic acid, B: 90 % MeCN, 10 % H_2O, 0.1 % formic acid). The mass spectrometer was run in positive ionization mode.

Purification and reduction of bottromycin

Bottromycins were purified from 50 mL PM cultures after 8-14 days' growth. The culture was centrifuged (5000 rpm, 10 min) to pellet the cell debris. The pH of the supernatant was adjusted to 8.5, and was then extracted with EtOAc (2 x 20 mL). The combined organic phases were dried (Na_2SO_4), and the ethyl acetate was evaporated under reduced pressure, and any residual water was removed by freeze-drying. The residue (typically ~2-5 mg) was dissolved in 500 μL of methanol for LCMS analysis and reduction.

Reduction of bottromycin with $NaBH_4$ selectively reduces the proline amide bond, breaking the macrocycle[4]. This should lead to a much clearer $LCMS^2$ analysis than on non-reduced bottromycin, as the fragmentation of almost any bond will lead to the formation of two fragments that can be identified by their mass/charge ratio. $NaBH_4$ (1 mg) was added to the organic extract dissolved in methanol (40 μL) and the mixture was stirred on ice for 30 min. The reaction was quenched with TFA (2.5 μL), and then diluted with 240 μL of methanol for LCMS and $LCMS^2$ analysis. This was performed as outlined above with additional fragmentation at 849.5 (reduced A_2), 835.5 (reduced B), 863.5 (reduced C) and 821.5 (reduced D), using an isolation width of 3.0 m/z and a normalised collision energy of 35%.

Genomic DNA (gDNA) extraction

gDNA was extracted from cultures grown both in liquid media and on agar plates, using the salting out method[3] or a non-enzymatic method[5].

Construction of deletion plasmids

Δ*btmD* and Δ*btmG* plasmids were constructed using assembly PCR as follows. Regions of approximately 1.5 kbp either side of the target gene were amplified from *S. scabies* gDNA using KOD Hot Start polymerase (Novagen) following manufacturers' instructions. This yielded fragments that overlapped by approximately 25 bp within the target gene. For example, for Δ*btmD* the primers used were *btmD*-KO-Up with *btmD*-KO1 and *btmD*-KO-Down with *btmD*-KO2. These fragments were purified by agarose gel electrophoresis, and 5 μL of each was used as both template and primer in a second PCR reaction for ten cycles to produce a fragment of ~3000 bp with a deletion of 3n nucleotides within the target gene. 0.5 μL of each primer (*btmD*-KO-Up and *btmD*-KO-Down) was added and amplification was repeated for 25 cycles. Gel purification isolated the 3 kbp fragment, which was then digested with NdeI (Fermentas) and ligated (T4 DNA ligase, Fermentas) with pYH7[6] that had been NdeI digested and dephosphorylated with calf alkaline phosphatase (CIP, NEB). pYH7-Δ*btmG* was constructed in an analogous manner.

For all other gene deletion experiments, Gibson's isothermal assembly method[7] was used. Fragments of approximately 2 kbp either side of the target gene were amplified as described above. These fragments overlap by about 25bp with each other as well as with either side of the NdeI restriction site in pYH7. These fragments were purified by agarose gel electrophoresis and were then used in an assembly reaction with NdeI digested pYH7. ~100 ng of NdeI digested pYH7 was used in an isothermal assembly reaction with the two PCR products in a 1:1:1 molar ratio (~20 ng PCR product). Phusion polymerase (Thermo Scientific), Taq Ligase (NEB) and T5 exonuclease (Epicentre) were used at 50 °C for 1 h[7]. The reaction mixture was then transformed into chemically competent NovaBlue cells using standard procedures, selecting for apramycin resistance.

Primers

Primers used are listed in Supplementary Table 2. They were synthesised by Eurofins Genetic Services Ltd or Life Technologies, and specificity was determined using Amplify 3X (Bill Engels, NIH, 2005).

Conjugation

S. scabies was manipulated using the techniques described by Keiser *et al.*[3]. In summary, a conjugation proceeded as follows. 48 h before the conjugation, 25 mL of GYM was inoculated with a ~1 cm^2 patch of *S. scabies* grown on SFM-agar and incubated at 28 ˚C shaking at 220 rpm. Cells from a -80 °C stock of ET12657/pUZ8002 (ET/pUZ) transformed with a gene deletion pYH7 plasmid were used to inoculate 10 mL of LB, containing apramycin, kanamycin and chloramphenicol. Cultures were grown for 16 h, and 1 mL was used to inoculate 15 mL of LB with half the usual antibiotic concentration of the above antibiotics. This was grown to an OD_{600} of 0.4-0.6 (~3 h) and all cultures were centrifuged at 3500 rpm for 8 min at 4 ˚C. The cell pellets were resuspended in 15 mL LB and were then centrifuged again at 3500 rpm for 8 min. ET/pUZ was then resupended in 500 μL LB and the *S. scabies* in 1 mL LB. 50 μL of the *S. scabies* suspension was added to 500 μL ET/pUZ and mixed. 100 μL of this mixture was plated onto SFM-agar plates. The plates were incubated for 16-24 h at 30 °C before being overlayed with water containing apramycin and nalidixic acid to give a final concentration of 50 μg mL^{-1} apramycin and 25 μg mL^{-1} of nalidixic acid.

Plates were incubated for up to 7 days at 30 °C, and exconjugants were patched onto SFM-agar containing apramycin and nalidixic acid. After 48 h, *S. scabies* patches were streaked onto antibiotic free SFM-agar plates, to obtain single colonies and to stimulate the loss of the pYH7 plasmid by homologous recombination. Deletion mutants were screened by streaking single colonies onto paired SFM plates (one + Apra and one -Apra). Bacteria that only grew without apramycin were screened by PCR (DreamTaq polymerase, Fermentas) to confirm gene truncation.

Complementation of *S. scabies* Δ*btmD*

pYH7-*btmD* was generated by using *btmD*-KO-Up and *btmD*-KO-Down to amplify a 3 kbp stretch of DNA containing *btmD*. This was digested with NdeI and ligated into the NdeI site of pYH7. For pSET152-*btmD*, a 2630 bp region including *btmD* along with *btmC* and the region upstream of *btmC* were amplified from *S. scabies* gDNA, using the primers *btmD*-promoter and *btmD*-End. This fragment was digested with XbaI and EcoRI and then ligated into XbaI and EcoRI digested pSET152[8]. ET/pUZ was transformed with these plasmids and they were used to conjugate *S.*

scabies Δ*btmD* as described above. pSET152-*btmD* required a single crossover for genome integration whereas pYH7-*btmD* underwent the same double crossover screening as gene deletion.

S. bottropensis DSM 40262 gene cluster sequencing

Purified genomic DNA from *S. bottropensis* DSM 40262 was sequenced at the DNA sequencing facility at the Department of Biochemistry, University of Cambridge using a Roche 454 Genome Sequencer FLX+. This provided 515,992 reads that assembled into 630 contigs, with an average contig size of 14.9 kb (longest contig = 131 kb). The assembly estimates the genome at 9.1 Mb in size and gives 23x coverage of the consensus. The bottromycin gene cluster was assembled from four contigs by comparison with the sequenced *S. scabies* gene cluster (Supplementary Figure 6).

Table 1 Plasmids used in this study

Name	Source	Resistance Marker	Function
pYH7	[6]	Apramycin	*E. coli-Streptomyces* shuttle vector
pUZ8002	[3]	Chloramphenicol	Facilitates transfer of genetic material by conjugation
pSET152	[8]	Apramycin	Complementation by integration
pYH7-Δ*btmD*	This study	Apramycin	*btmD* deletion
pYH7-Δ*btmE*	This study	Apramycin	*btmE* deletion
pYH7-Δ*btmF*	This study	Apramycin	*btmF* deletion
pYH7-Δ*btmG*	This study	Apramycin	*btmG* deletion
pYH7-Δ*btmH*	This study	Apramycin	*btmH* deletion
pYH7-Δ*btmI*	This study	Apramycin	*btmI* deletion
pYH7-Δ*btmJ*	This study	Apramycin	*btmJ* deletion
pYH7-Δ*btmK*	This study	Apramycin	*btmK* deletion
pYH7-Δ*btmL*	This study	Apramycin	*btmL* deletion
pYH7-*btmD*	This study	Apramycin	*btmD* complementation
pSET152-*btmD*	This study	Apramycin	*btmD* complementation

Table 2 Primers used in this study. Restriction enzyme indicates which restriction site has been introduced with the primer

Primer Name	Primer Sequence (5'→3')	Restriction site
btmD-KO-Up	CCCCTTGCCGCATATGCGGGAACAGAGCG	NdeI
btmD-KO-Down	GTCACCCTCCTCCATATGGTCGCAGCGGG	NdeI
btmD-KO-1	ACCACACCATGGAAGCCACCTCATGAGCGTCGGCCGGTGAGTAGC	
btmD-KO-2	CATGAGGTGGCTTCCATGGTGTGGTCCTTTCGTCTGTCTTCTCGTC	
btmD-End	CGGCGAATTCTCATGAGGTGGCTTC	EcoRI
btmD-Promoter	GACCTCTA GACTCTCTG CGCACAGAAT	XbaI
btmC-KO-Up	GATCAAGGCGAATACTTCATATGGCGCTCGCCATGCTCCGGTGC	NdeI
btmC-KO-Down	CCGCGCGGTCGATCCCCGCATATGCGCCGGGTCGACGAGCATGATCAG	NdeI
btmC-KO-1	ATCCAGGTGGGCGGCACCCGGTTCCGTGATCTCCAG	
btmC-KO-2	GCCGCCGACGACGGCGAGCGAGCGCAGAATACCG	
btmE-KO-Up	GATCAAGGCGAATACTTCATATGCACCAGCTGGCTGAAGGACGTGG	NdeI
btmE-KO-down	CGCGCGGTCGATCCCCGCATATGGTTTCACCTCGATGTTCAGCCAGAACG	NdeI
btmE-KO-1	CGGTGCACCGTGGGCATCCGTGTGTGGGCCGACTC	
btmE-KO-2	ACACGGATGCCCACGGTGCACCGCACGGTCACC	
btmF-KO-Up	GATCAAGGCGAATACTTCATATGGTCGCGCACCGCGAGCACGAGAC	NdeI
btmF-KO-Down	CCGCGCGGTCGATCCCCGCATATGCCATCACGAGTCCGCGCGCTTC	NdeI
btmF-KO-1	GTCGGGGACTGGCACCGTGTGCTGACCCCGCCG	
btmF-KO-2	CAGCACACGGTGCCAGTCCCCGACGGAGGCCAGGG	
btmG-KO-Up	GCGCCGTCCACCATATGGACCCCCTGCGGG	NdeI
btmG-KO-Down	CGGTCCATATGGACGGGGCCGCGAAGGAAG	NdeI
btmG-KO-1	GGTGCGATCCGGCGGAGGGCCGGCGCGGTCTCGTGCTCGCGGT	
btmG-KO-2	GCGCCGGCCCTCCGCCGGATCGCACCCCAGCGCGTCGACGGCG	
btmH-KO-Up	GATCAAGGCGAATACTTCATATGCCCACCGCCGTCATCAGGTTGTCCAG	NdeI
btmH-KO-Down	CCGCGCGGTCGATCCCCGCATATGAACAGCCTGTACCGCCCGTCCATC	NdeI
btmH-KO-1	GAGGTGGTCTCCCGTATCAGGGCGGCCGGACACCT	
btmH-KO-2	CGCCCTGATACGGGAGACCACCTCGAACGGTTCC	
btmI-KO-Up	GATCAAGGCGAATACTTCATATGCAGCGACCGGCCCGCGGAGATG	NdeI
btmI-KO-Down	CCGCGCGGTCGATCCCCGCATATGAAGGCCAATCTGGGGCCGCGTGAG	NdeI
btmI-KO-1	ACCGCTTGGACGCGACCGACGAAGGAGACAGCCCATG	
btmI-KO-2	CTTCGTCGGTCGCGTCCAAGCGGTGACCGTCCCGTC	
btmJ-KO-Up	GATCAAGGCGAATACTTCATATGGAATGCGTGGGCGTGAGCGTGAG	NdeI
btmJ-KO-Down	CCGCGCGGTCGATCCCCGCATATGCTATCTGCACGACCACTTCACCGG	NdeI
btmJ-KO-1	ATCGTGGACGCCCCGCATCCCGGGCAGCGCGCCAC	
btmJ-KO-2	CCCGGGATGCGGGGCGTCCACGATCCCGTCGTACG	
btmK-KO-Up	GATCAAGGCGAATACTTCATATGCGGTGCGAACAGTTCACCCGG	NdeI
btmK-KO-Down	CCGCGCGGTCGATCCCCGCATATGCGCACCTCCGAGCATCTGTACGGC	NdeI
btmK-KO-1	ATCCAGGTGGGCGGCACCCGGTTCCGTGATCTCCAG	
btmK-KO-2	GAACCGGGTGCCGCCCACCTGGATGGACGGACGCCG	
btmL-KO-Up	GATCAAGGCGAATACTTCATATGGGAAGGCCTTCAGGACGTTGCTGTAG	NdeI
btmL-KO-Down	CCGCGCGGTCGATCCCCGCATATGCGTCCATCCAGGTGGGCACGTTG	NdeI
btmL-KO-1	CCGGATTCCCGCGCTCGTGGACGAGGAGCTGCTGC	
btmL-KO-2	TCGTCCACGAGCGCGGGAATCCGGTCATCGGTGGA	

Table 3 Bottromycin gene cluster data for *S. scabies*

Gene name	Gene cluster	Size (AA)	Best Match		Proposed function
SCAB_56711*	*btmA*	436	*Eggerthella lenta* DSM 2243	YP_003181038.1	Major facilitator superfamily
SCAB_56701*	*btmB*	279	*Mycobacterium* sp. JLS	YP_001073721.1	*O*-MTase
SCAB_56691	*btmC*	645	*Salinispora tropica* CNB-440	YP_001159120.1	Radical SAM MTase
SCAB_56681	*btmD*	44	No matches		Precursor peptide
SCAB_56671	*btmE*	418	*Chitinophagapinensis* DSM 2588	YP_003121679.1	YcaO-domain phosphokinase
SCAB_56661	*btmF*	480	*Streptosporangium roseum* DSM 43021	YP_003341786.1	YcaO-domain phosphokinase
SCAB_56651	*btmG*	671	*Salinispora tropica* CNB-440	YP_001159120.1	Radical SAM MTase
SCAB_56641	*btmH*	271	*Micromonospora aurantiaca* ATCC 27029	YP_003838784.1	α/β hydrolase
SCAB_56631	*btmI*	473	*Alkaliphilus oremlandii* OhILAs	YP_001514298.1	Metal-dependent hydrolase
SCAB_56621	*btmJ*	378	*Thermomonospora curvata* DSM 43183	YP_003300966.1	Cytochrome P450
SCAB_56611	*btmK*	652	*Nostoc* sp. PCC 7120	NP_486063.1	Radical SAM MTase
SCAB_56601	*btmL*		*Kribbella flavida* DSM 17836	YP_003381325.1	Transcriptional regulator
SCAB_56591	*btmM*	480	*Aquifex aeolicus* VF5	NP_214437.1	M17 aminopeptidase

* Genes on opposite strand

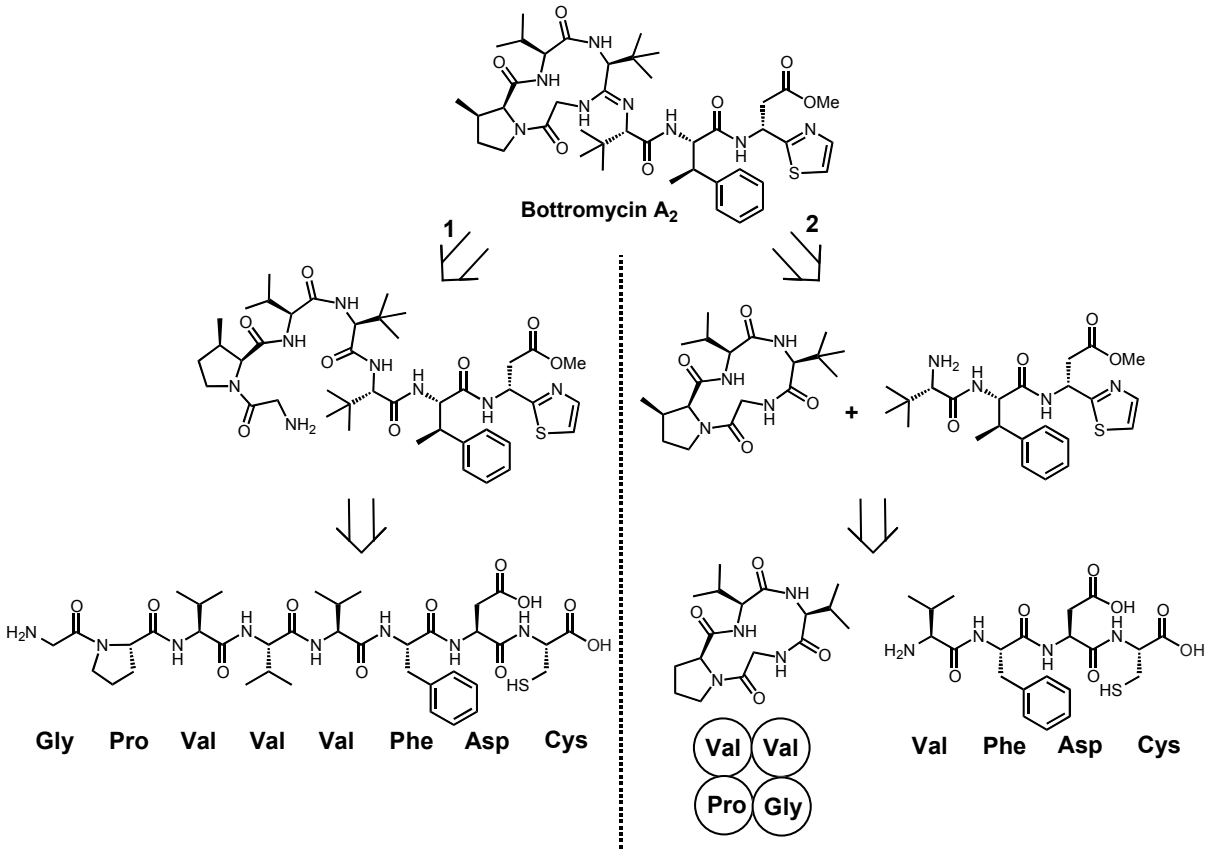


Figure 1 Alternative retrobiosynthetic proposals based on one precursor peptide chain (**1**) and two peptide chains (**2**).

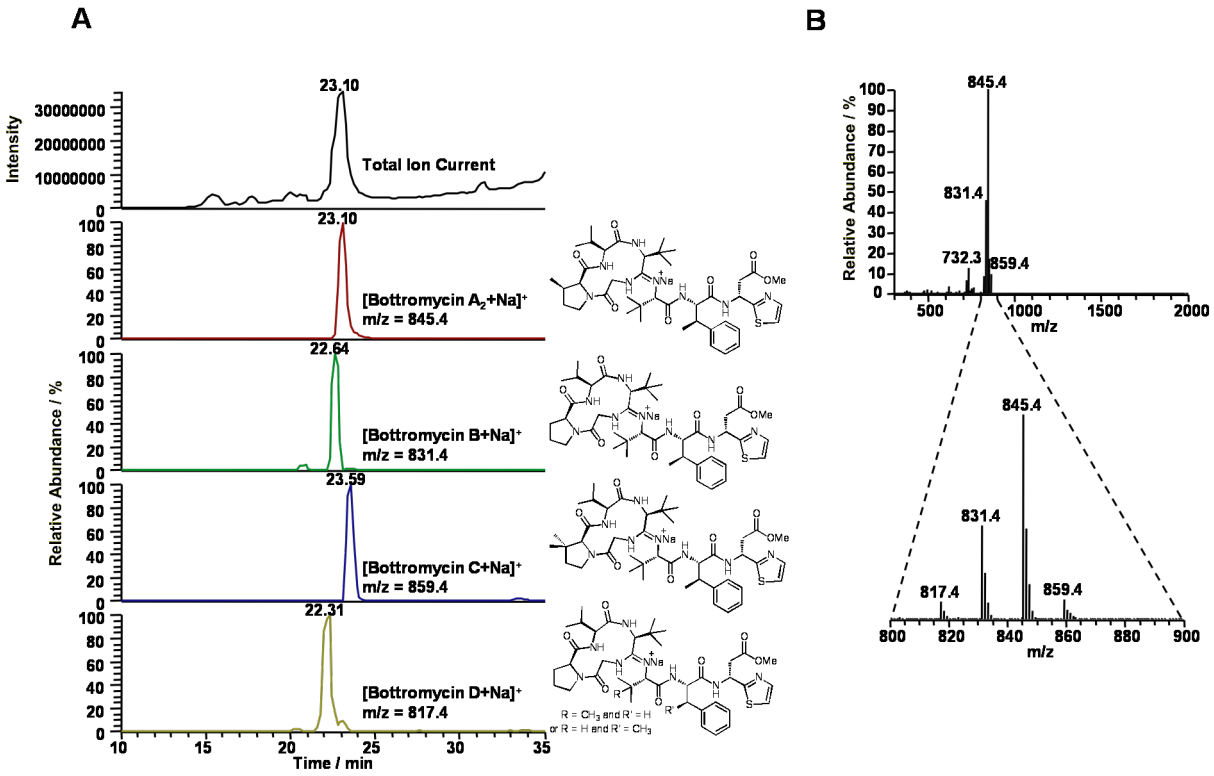


Figure 2 LCMS analysis of bottromycin production by *S. scabies*. **A**. Selective ion monitoring indicating the production of bottromycins A_2, B, C and D in a crude extract of *S. scabies*. Fragmentation analysis was unable to determine the precise location of methylation in bottromycin D (see Supplementary Figure 5). It should be noted that the naming of bottromycin A_2 originates from an early and incorrect structural analysis[9] that reported a bottromycin A_1 compound with a mass consistent with bottromycin B. We assume that A_1 and B are the same molecule. **B**. MS analysis of 22-24 minutes showing the relative proportions of bottromycins produced by *S. scabies*.

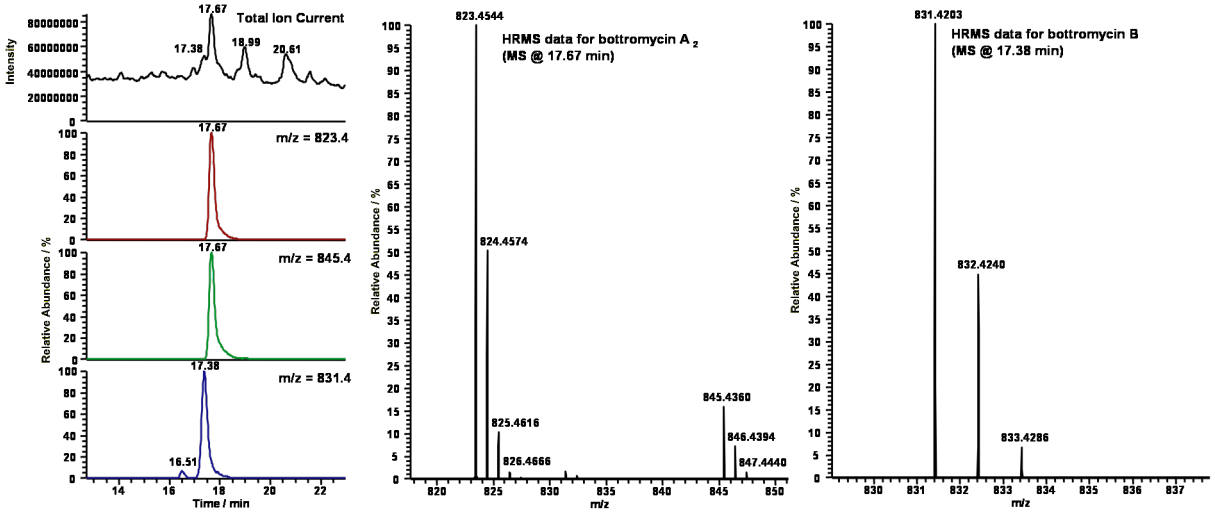


Figure 3 HPLC - high-resolution mass spectrometry (HRMS) analysis of bottromycins A_2 and B produced by *S. scabies* DSM 41658.

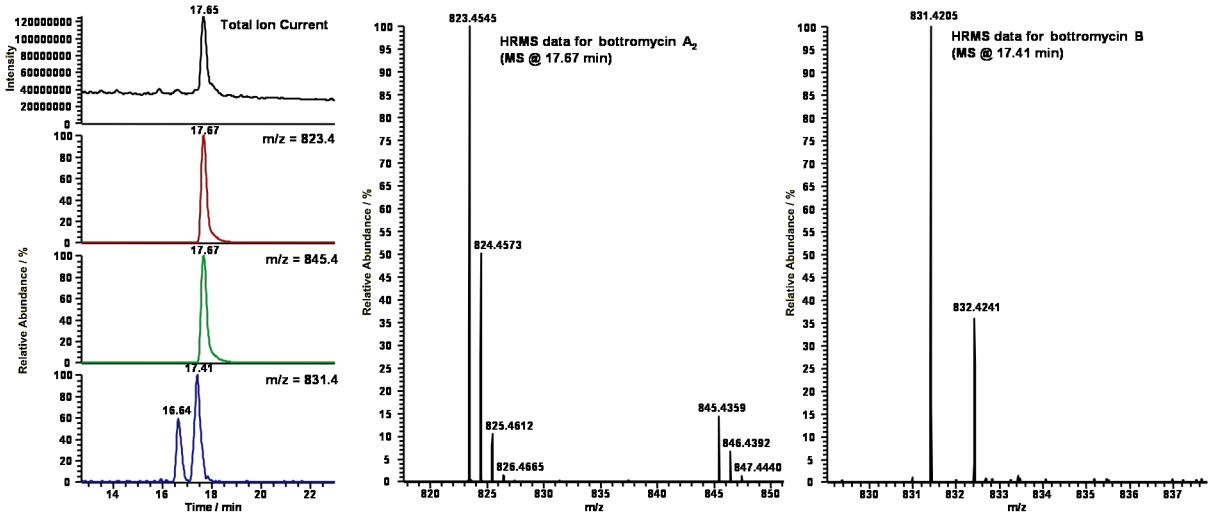


Figure 4 HPLC - high-resolution mass spectrometry (HRMS) analysis of bottromycins A_2 and B produced by *S. bottropensis* DSM 40262. The m/z = 831.4 peak at 16.64 minutes is bottromycin A_2 lacking the methyl ester.

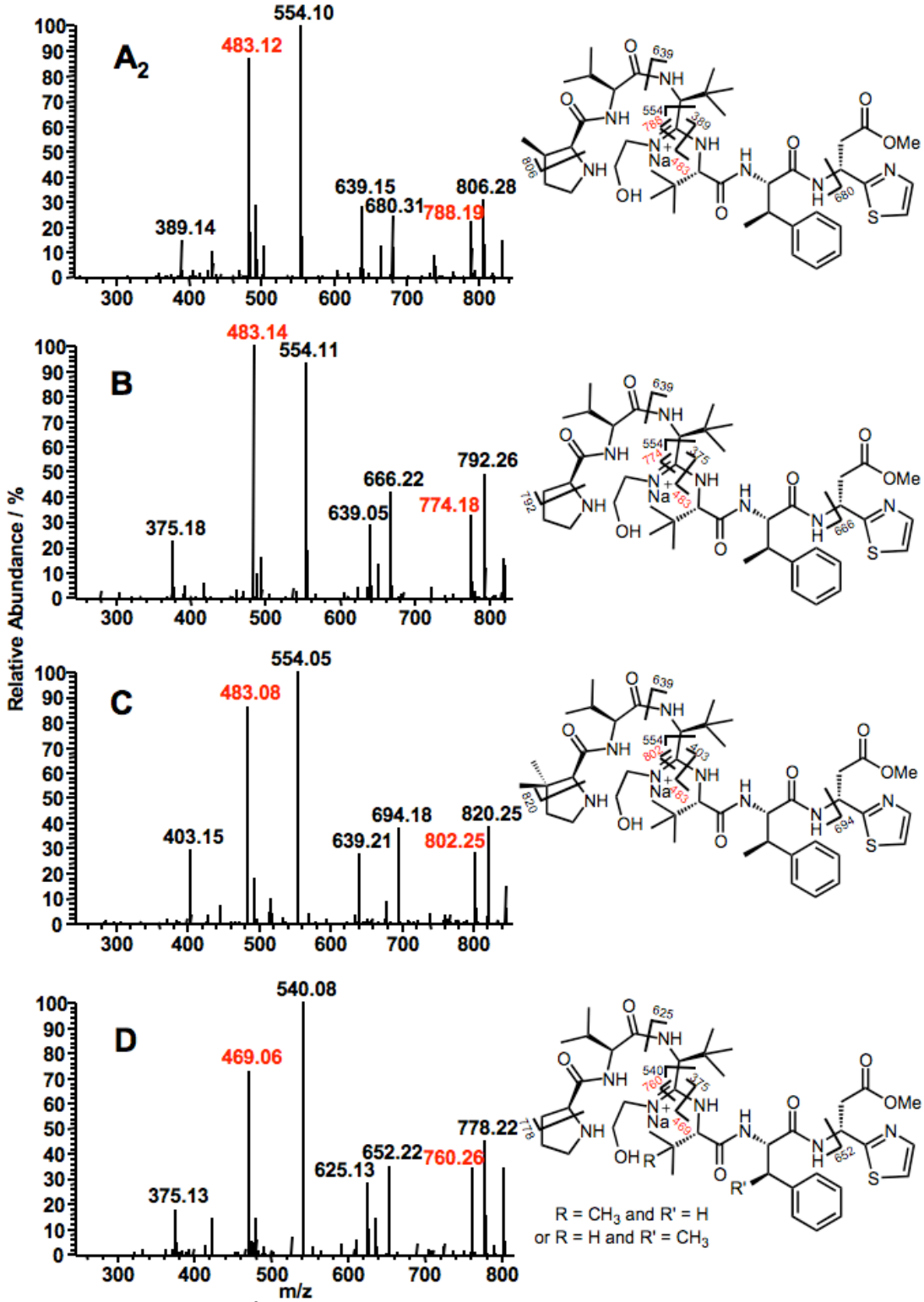
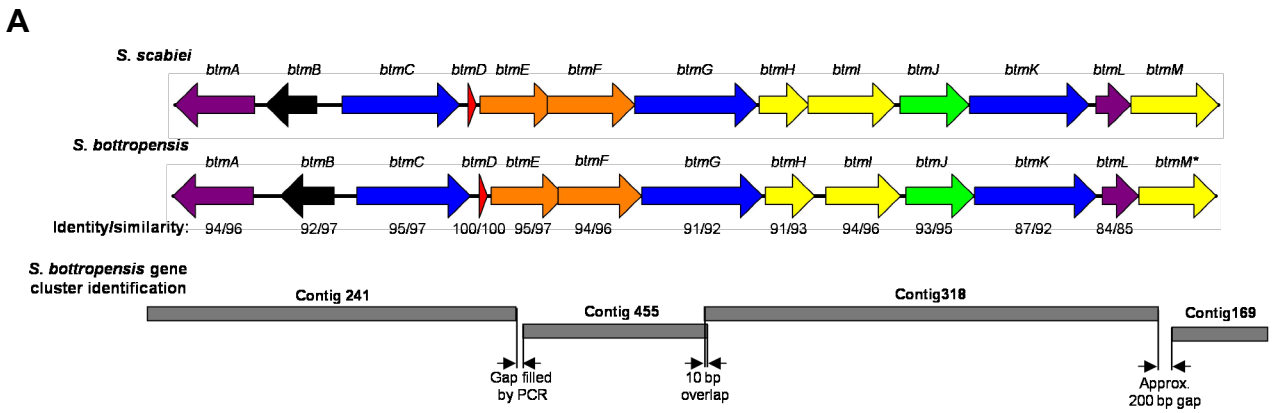


Figure 5 ESI-MS^2 analysis of $NaBH_4$-reduced bottromycin A_2, B, C and D from *S. scabies*. The red text refers to fragments that have the sodium in a different part of the molecule. The unusual fragmentation of the substituted pyrrolidine has previously been observed by ESI-MS/MS[10].



B

```
S. bottropensis      ATGGGACCCGTAGTCGTATTCGACTGCATGACCGCAGACTTCCTCAACGACGACCCCAAC 60
S. scabies           ATGGGACCCGTAGTCGTATTCGACTGCATGACCGCGGACTTCCTCAACGACGACCCCAAC 60
                     ********************************** ************************

S. bottropensis      AACGCGGAACTGAGCGCGCTGGAGATGGAGGAGCTGGAGTCCTGGGGCGCCTGGGACGGC 120
S. scabies           AACGCGGAGCTGAGCGCGCTGGAGATGGAGGAGCTGGAGTCCTGGGGCGCCTGGGACGGT 120
                     ******** ************************************************

S. bottropensis      GAAGCCACCTCATGA 135
S. scabies           GAAGCCACCTCATGA 135
                     ***************
```

Figure 6 Analysis of bottromycin genes in *S. bottropensis*. **A**. Bottromycin gene cluster in *S. bottropensis* compared to the gene cluster identified in *S. scabies* and details of *S. bottropensis* cluster assembly. No similarity score is reported for *btmM* as a 200 bp gap was identified between contigs 318 and 169 that was difficult to sequence. **B**. Alignment between the *btmD* gene in *S. bottropensis* and *S. scabies*.

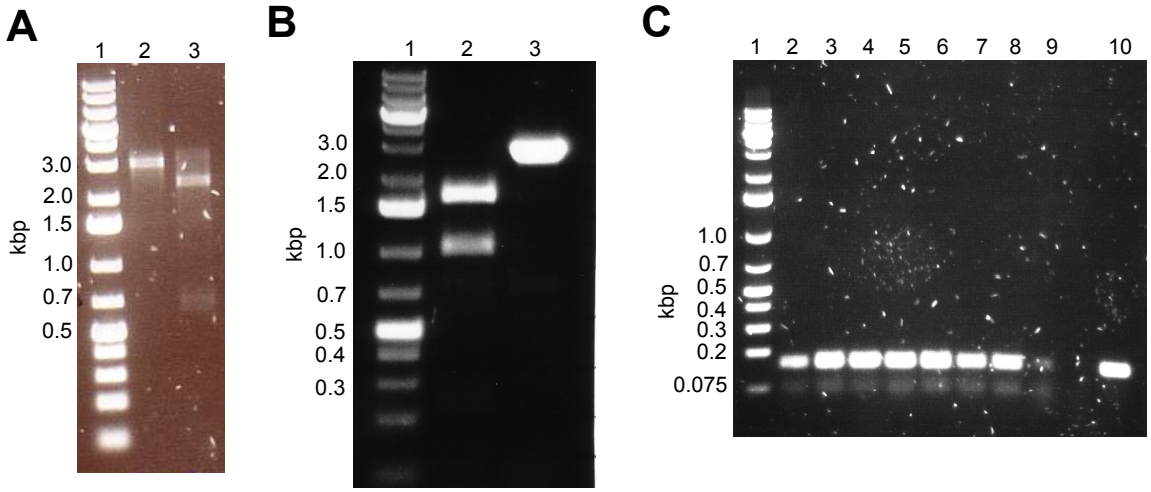


Figure 7 Gel analysis of *btmD* deletion and complementation with pYH7-*btmD* and pSET152-*btmD*. **A**. Agarose gel analysis of *S. scabies* Δ*btmD*. Lane 1: 1 kb+ marker (Fermentas); lane 2: amplification of a 2960 bp region of Δ*btmD* gDNA using the primers *btmD*-KO-up and *btmD*-KO-down; lane 3: digestion of the lane 2 DNA with BlpI. Intact *btmD* contains a BlpI site within the gene, which is removed on deletion. This would therefore digest to give products of 1548, 840 and 689 bp. Δ*btmD* is digested only once, giving products of 2271 and 689 bp, as seen here. **B**. Agarose gel analysis of *trans*-complementation of Δ*btmD* using pSET152-*btmD*. Lane 1: 1 kb+ marker;

lane 2: digestion of the product in lane 3 with NcoI, which gives the expected products of 1528, 956 and 227 bp (faint); lane 3: PCR amplification of Δ*btmD*::pSET152-*btmD* gDNA with primers amplifying the entire (including *btmD* and *btmC*) complementation region. **C**. Agarose gel analysis of a PCR screen for *cis*-complementation of *S. scabies* Δ*btmD* by pYH7-*btmD*. Lane 1: 1kb+ marker; lanes 2-9: colony PCR of successful complementations, using *btmD*-start and *btmD*-end as primers to give a 144 bp product; lane 10: control PCR using wild-type gDNA.

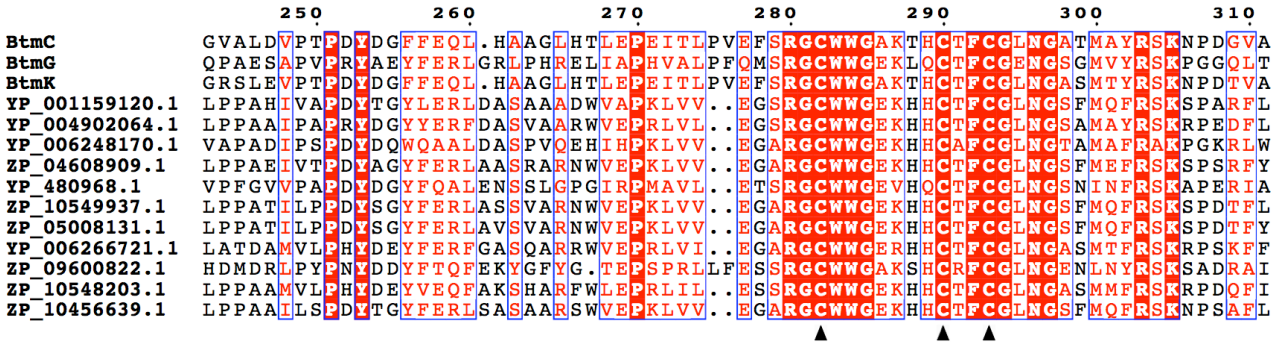


Figure 8 Partial sequence alignment of bottromycin radical SAM methyltransferases with eleven closest homologues. The atypical CxxxxxxxCxxC motif is highlighted with triangles. The sequences were aligned using ClustalW2 (Gonnet Matrix) and visualised using ESPript 2.2[11] where residues that possess at least 60% similarity (Risler matrix) are boxed blue and coloured red and identical residues are displayed as white text on a red background.

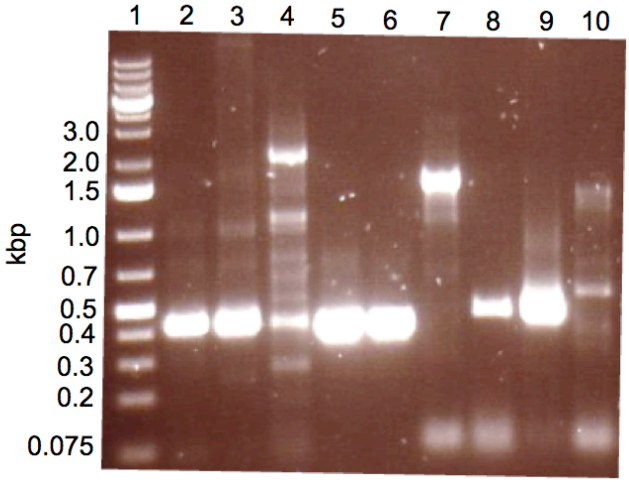


Figure 9 Agarose gel showing deletion of *btmG*, *btmF* and *btmE* using primers to amplify each gene. Lane 1: 1kb+ marker; lane 2: Δ*btmG* gDNA as template; lane 3: pYH7-Δ*btmG* as template; lane 4: WT gDNA; *btmG* is 2070 bp, Δ*btmG* is 420 bp. Lane 5: Δ*btmF* gDNA; lane 6: pYH7-Δ*btmF*; lane 7*:* WT gDNA; *btmF* is 1486 bp, Δ*btmF* is 403 bp. Lane 8: Δ*btmE* gDNA; lane 9: pYH7-Δ*btmE*; lane 10: WT gDNA; *btmE* is 1291 bp, Δ*btmE* is 439 bp.

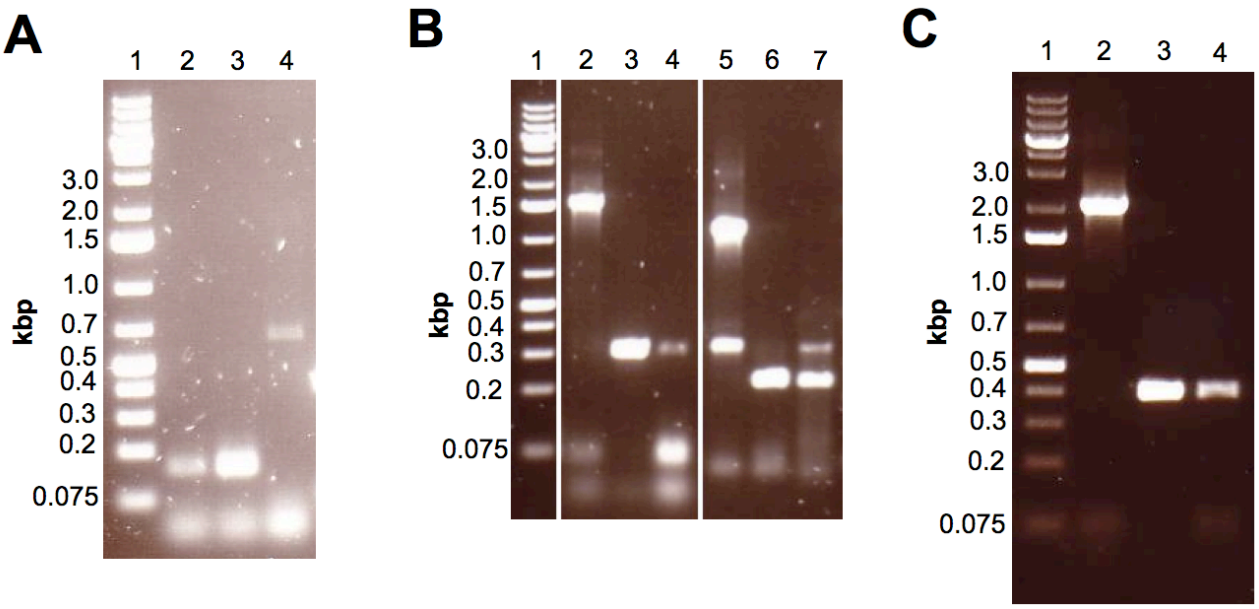


Figure 10 Gel analysis of *S. scabies* Δ*btmL*, Δ*btmI*, Δ*btmJ* and Δ*btmK*. **A**. Agarose gel showing deletion of *btmL* using primers to amplify this gene. Lane 1: 1kb+ marker; lane 2: Δ*btmL* gDNA; lane 3: pYH7-Δ*btmL*; lane 4: WT gDNA; *btmL* is 621 bp, Δ*btmG* is 144 bp. **B**. Agarose gel showing deletions of *btmI* and *btmJ*. The gaps in figure are where lanes containing DNA irrelevant to this figure have been removed; lane 1: 1kb+ marker; lane 2: WT gDNA; lane 3: pYH7-Δ*btmI*; lane 4: Δ*btmI* gDNA; *btmI* is 1483 bp, Δ*btmI* is 330 bp. Lane 5: WT gDNA; lane 6: pYH7-Δ*btmJ*; lane 7: Δ*btmJ* gDNA; *btmJ* is 1168 bp, Δ*btmJ* is 235 bp. **C**. Agarose gel showing deletion of *btmK*. Lane 1: 1kb+ marker; lane 2: WT gDNA; lane 3: pYH7-Δ*btmK*; lane 4: Δ*btmK* gDNA; *btmK* is 2012 bp, Δ*btmK* is 389 bp.

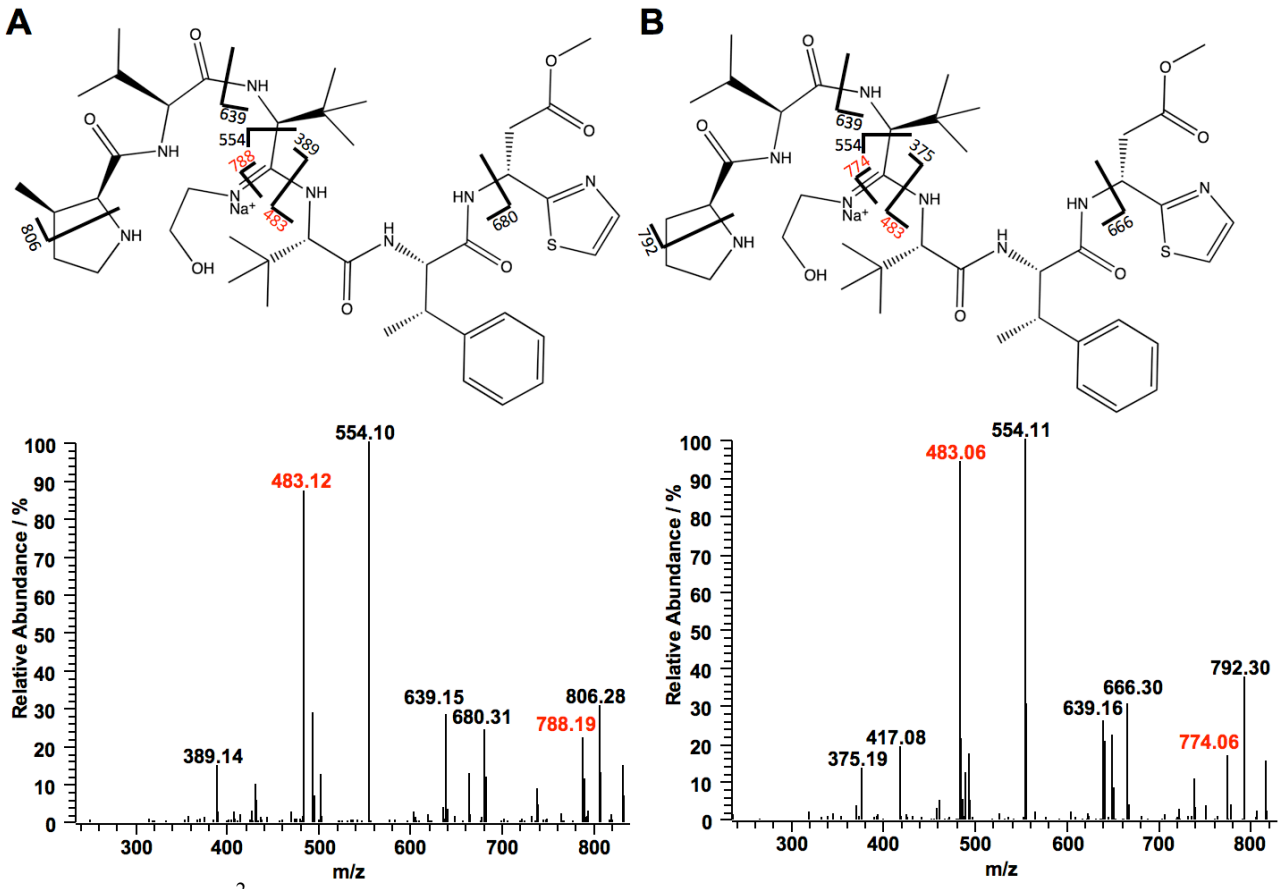


Figure 11 MS^2 analysis of $NaBH_4$-reduced bottromycin A_2 (**A**) from wild-type *S. scabies* and putative bottromycin B (**B**) from *S. scabies* Δ*btmK*. The red text refers to fragments that have the sodium in a different part of the molecule.

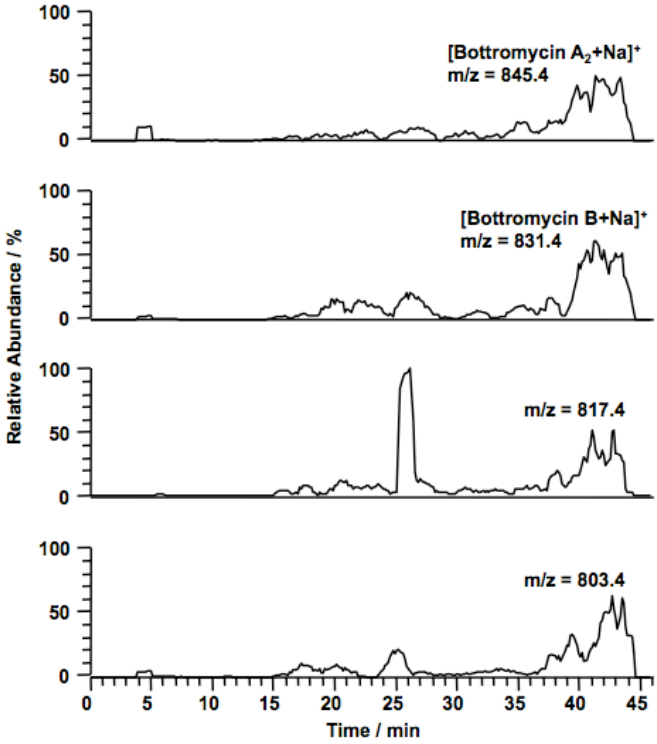


Figure 12 MS analysis of *S. scabies* Δ*btmG*. Selective ion monitoring for bottromycins differing in the number of methyl groups. The retention time of the peak for m/z = 817.4 was identical to that of bottromycin D produced by wild type *S. scabies*.

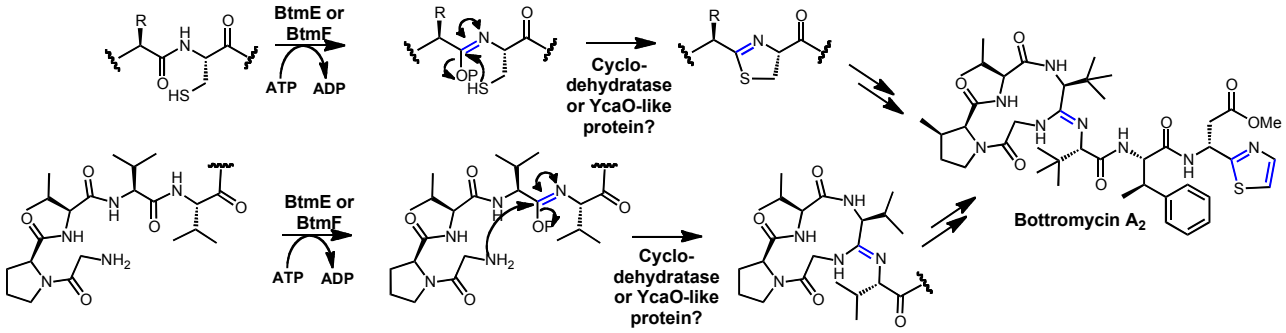


Figure 13 Proposed biosynthesis of heterocycles. BtmE and BtmF both contain YcaO-domains, that have been shown to catalyse the phosphorylation of amide bonds during heterocycle biosynthesis[12]. It is possible that a putative hydrolytic enzyme such as BtmH or BtmI has evolved the ability to catalyse the attack of an intramolecular nucleophile onto an activated amide rather than the attack of water onto an amide.

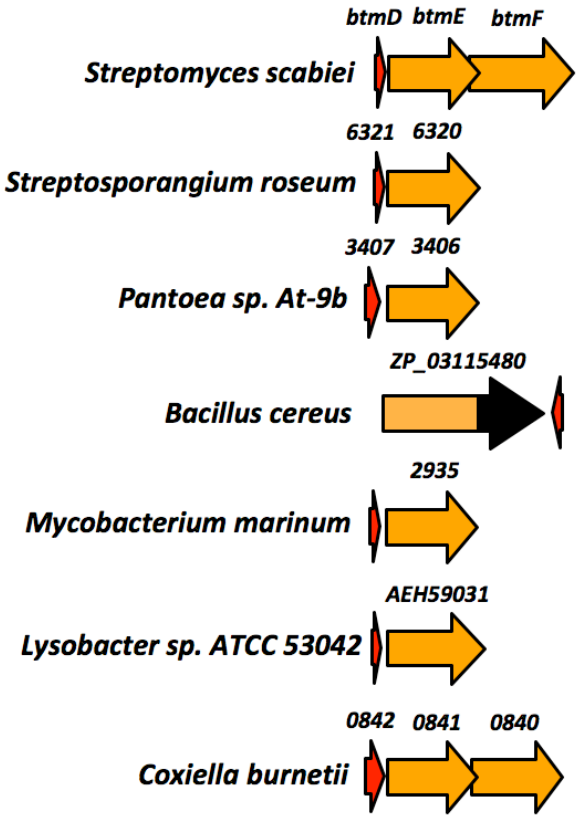
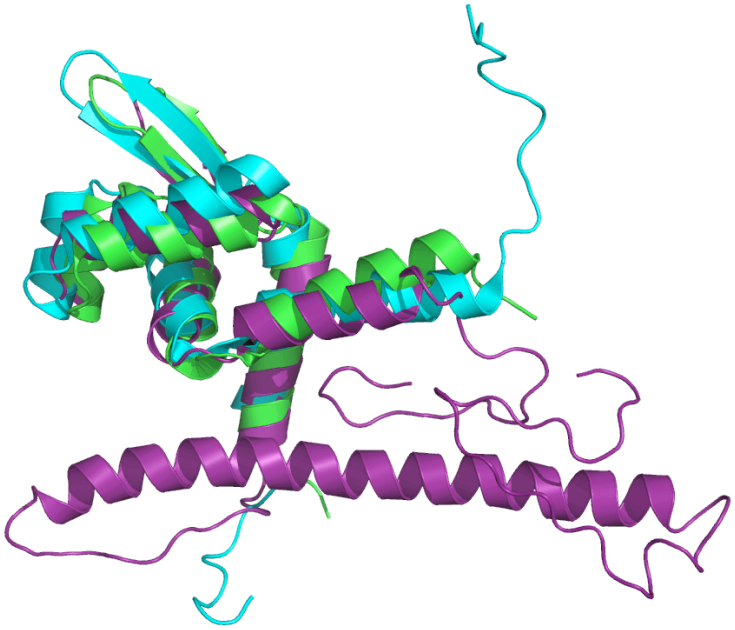


Figure 14 Mining for related pathways using BtmF as a probe. YcaO-like proteins are coloured orange and putative precursor peptides are coloured red. There is no significant homology between the short peptides. The black *C*-terminus of ZP_03115480 contains a methyltransferase-like domain.

A



B

```
                    20        30        40        50        60        70        80
BtmL                .AQDLPPSCRDLIGVLAQTDRRDAFAALALGATSAAEVAERAGLRPAAAAALSKLVDGRIAAY..DADA
YP_006245341.1      .GPDAGERSRQLIGLLAEENRLRAFAAVALGARSTRQVAETAGLTPKDTALALLRLREQGAVT...DGPE
ZP_10090806.1       ..MTAH....ALAGALADDGRRRIFAAIVLGSTSAAEVAQRSGLPTRAVLTGLRRLADAGLVA....GAD
YP_006368441.1      ..MEAG....TIVGLLADPARLRVVAALALGAGTIEEVAEASGLSLKDVALAARRLARAGLVHR..DG..
YP_001432892.1      NSEALSTTVR.YLKVMAHATRLRILGILADGEWSVSELAELLDTTSSTVSRHLAKLQEVDLVRMRMDDAN
YP_003683746.1      .METINQTLLGFFQALADANRLRIVGVLAQGPQTVEQISALLGLGMSTTSHHLRKLAKAGLVEARADGHY
NmtR                DSQAAAQVAS.TLQALATPSRLMILTQLRNGPLPVTDLAEAIGMEQSAVSHQLRVLRNLGLVVGDRAGRS
CzrA                .TDTLERVTE.IFKALGDYNRRIRIMELLSVSEASVGHISHQLNLSQSNVSHQLKLLKSVHLVKAKRQGQS

                    90        100
BtmL                RTYRLIEDTFRLAVQAEVRISGRGG
YP_006245341.1      DGLAVAHDLLRELARPRRTEGSARP
ZP_10090806.1       GALTVDEASLRAAARDGRPADDAEP
YP_006368441.1      HTLALHTERFAEAARTAAAQAPPPP
YP_001432892.1      HVYSLNDETLQRINRDLISPASIVA
YP_003683746.1      SVYSLRTQTLEELAKNLLSRETLPR
NmtR                IVYSLYDTHVAQLLDEAIYHSEHLH
CzrA                MIYSLDDIHVATMLKQAIHHANHPK
```

Figure 15 Bioinformatic analysis of BtmL. A, Structural model of BtmL (purple) generated by the PHYRE2 Protein Fold Recognition Server[13]. It is aligned with its two closest structural homologues, NmtR[14] (cyan, PDB ID = 2LKP) and CzrA[15] (green, PDB ID = 1R1U) using PyMOL (DeLano Scientific, W. L. DeLano). These both belong to the ArsR/SmtB family of metal-dependent transcriptional repressors. B, Sequence alignment of BtmL, NmtR, and CzrA with the five closest full-length BtmL homologues identified by BLAST analysis (only the structurally homologous regions are aligned). The sequences were aligned using ClustalW2 (Gonnet Matrix) and visualised using ESPript 2.2[11] where residues that possess at least 60% similarity (Risler matrix) are boxed blue and coloured red and identical residues are displayed as white text on a red background. The metal binding sites for NmtR (Ni(II)/Co(II)-specific sensor) and CzrA (Zn(II)-specific sensor) are coloured cyan.

References

1. D. J. MacNeil, K. M. Gewain, C. L. Ruby, G. Dezeny, P. H. Gibbons and T. MacNeil, *Gene*, 1992, **111**, 61.
2. *PCT Pat.* WO/2006/103010, 2006.
3. T. Kieser, M. J. Bibb, M. J. Buttner, K. F. Chater and D. A. Hopwood. *Practical Streptomyces Genetics*, John Innes Foundation, Norwich, 2000.
4. H. Shimamura, H. Gouda, K. Nagai, T. Hirose, M. Ichioka, Y. Furuya, Y. Kobayashi, S. Hirono, T. Sunazuka and S. Ōmura, *Angew. Chem. Int. Ed.*, 2009, **48**, 914.
5. A. D. Sharma and J. Singh, *Anal. Biochem.*, 2005, **337**, 354.
6. Y. Sun, H. Hong, M. Samborskyy, T. Mironenko, P. F. Leadlay and S. F. Haydock, *Microbiology*, 2006, **152**, 3507.
7. D. G. Gibson, L. Young, R. Y. Chuang, J. C. Venter, C. A. Hutchison 3rd and H. O. Smith, *Nat. Methods*, 2009, **6**, 343.
8. M. Bierman, R. Logan, K. O' Brien, E. T. Seno, R. N. Rao and B. E. Schoner, *Gene*, 1992, **116**, 43.
9. S. Nakamura, N. Tanaka and H. Umezawa, *J. Antibiot.*, 1966, **19**, 10.
10. T. J. Smyth, V. N. Ramachandran, A. McGuigan, J. Hopps and W. F. Smyth, *Rapid Commun. Mass Spectrom.*, 2007, **21**, 557.
11. P. Gouet, E. Courcelle, D. I. Stuart and F. Metoz, *Bioinformatics*, 1999, **15**, 305.
12. K. L. Dunbar, J. O. Melby and D. A. Mitchell, *Nat. Chem. Biol*., 2012, **8**, 569.
13. L. A. Kelley and M. J. E. Sternberg, *Nat. Protoc.*, 2009, **4**, 363.
14. C. W. Lee, D. K. Chakravorty, F.-M. J. Chang, H. Reyes-Caballero, Y. Ye, K. M. Merz, Jr. and D. P. Giedroc, *Biochemistry*, 2012, **51**, 2619.
15. C. Eicken, M. A. Pennella, X. Chen, K. M. Koshlap, M. L. VanZile, J. C. Sacchettini and D. P. Giedroc, *J. Mol. Biol*., 2003, **333**, 683.