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Cite this: DOI: 10.1039/c2sc21190d

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EDGE ARTICLE

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

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Received 6th August 2012, Accepted 5th September 2012
DOI: 10.1039/c2sc21190d

Streptomycete bacteria are a rich source of antibacterial natural products. Increasing antibiotic resistance is a global concern and novel classes of potent antibiotics are rare. Here we report the identification and genetic manipulation of the gene cluster for the cyclic antimicrobial peptide bottromycin in *Streptomyces scabies*. Bottromycin is active towards multi-drug resistant bacteria, such as MRSA and VRE, and contains a biologically unique macrocyclic amidine. The *btm* biosynthetic gene cluster was identified by genome mining and confirmed by genetic experiments. The metabolites of mutant strains were identified using liquid chromatography-mass spectrometry (LCMS), to characterise two radical SAM methyltransferases that are responsible for the β-methylation of three amino acids in bottromycin. A number of genes were also identified that are essential for bottromycin biosynthesis. A biosynthetic pathway has been proposed based on the results of these experiments coupled with bioinformatic analysis of the enzymes encoded in the *btm* cluster.

Introduction

Bottromycin (Fig. 1) is a macrocyclic peptide antibiotic that was first isolated from *Streptomyces bottropensis* in 1957,[1] but its absolute configuration was not determined until a total synthesis in 2009.[2] It possesses potent antibacterial activity towards Gram-positive bacteria and mycoplasma, including life-threatening bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE).[3] The ability of *S. bottropensis* to suppress rice blight has recently been attributed to bottromycin.[4] It functions by blocking aminoacyl-*t*RNA binding to the A site of the 50S ribosome, thus inhibiting bacterial protein synthesis.[5] Bottromycin is a structurally unique natural product, with a macrocyclic amidine architecture and β-methylated amino acids. Novel classes of antimicrobials are rare, so these characteristics make bottromycin an attractive molecule for biosynthetic analysis, especially when increasing antimicrobial resistance is a major concern and enzymes capable of β-methylation or forming a macrocyclic amidine have not previously been identified.

Although its *in vitro* activity is highly promising, bottromycin has not yet entered clinical use, possibly owing to its *in vivo* instability. Its methyl ester is hydrolysed rapidly in blood plasma,[3] which reduces anti-MRSA activity from an MIC of 1.0 μg mL^{-1} to 64 μg mL^{-1}. Recently, bottromycin has been chemically derivatised in an effort to discover more stable bioactive forms of the molecule.[3] A complementary approach to synthetic modifications of a molecule is to engineer its biosynthesis to generate a library of "unnatural" natural products.

However, little is known about the biosynthesis of bottromycin apart from studies by Arigoni and co-workers[6] that determined that the β-methyl groups on 3(*R*)-methyl-proline and 3(*S*)-methyl-phenylalanine and one methyl group of each

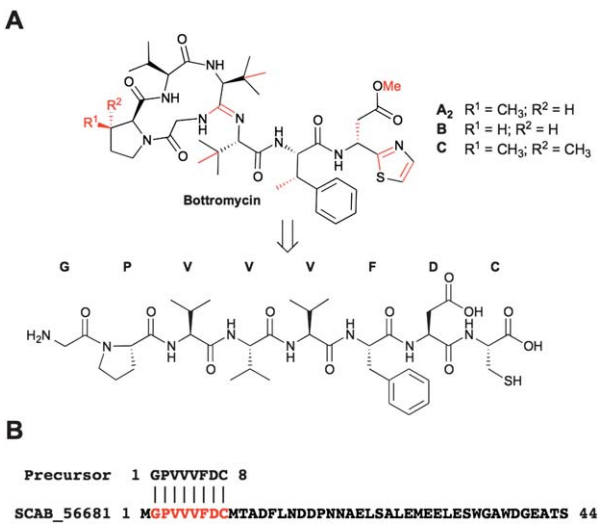

Fig. 1 (A) Structure of bottromycin and its putative unmodified core peptide (post-translational modifications are coloured red). (B) Alignment with SCAB_56681 from *S. scabies*.

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c2sc21190d

tert-leucine originates from *S*-adenosylmethionine (SAM), and are probably transferred from radical SAM methyltransferases (MTases).

Macrocyclic peptides are biosynthesised in two ways:[7] one is by expressing a ribosomal peptide (RP) that can then be post-translationally modified,[8] the other is by using non-ribosomal peptide synthetases (NRPSs), which are multidomain proteins dedicated to the production of peptide secondary metabolites.[9] Bottromycin could feasibly be made by either route, as all of its residues can originate from proteinogenic amino acids and it contains only one D-amino acid (epimerisation is rare but not unprecendented in RPs[10]).

Additionally, it is possible that it could derive from either a single cyclised linear peptide or from the condensation of two peptides to generate the amidine functionality (see ESI†). The ribosomal and non-ribosomal worlds appear to have converged in their ability to generate five-membered heterocycles[6] like the thiazole in bottromycin, and are also adept at producing macrocycles by a variety of routes.[7] However, no macrocyclic amidines have been identified as products from either biosynthetic route.

Here, we demonstrate that bottromycin is biosynthesised from a larger precursor ribosomal peptide. This was proposed following a genome mining analysis of *Streptomyces scabies* and confirmed by a series of gene deletion experiments. This work also identifies *S. scabies* as a previously unknown producer of bottromycin. An almost identical gene cluster was also identified in *S. bottropensis*, an established bottromycin producer.[1] To the best of our knowledge, bottromycin is the first ribosomal peptide natural product that derives from the N-terminus of a larger prepeptide and the first terrestrial peptide to be directly methylated at β-positions.

Results and discussion

To investigate the possibility of a ribosomal pathway to bottromycin, a putative linear core peptide (GPVVVFDC, Fig. 1a) was analysed using a Basic Local Alignment Search Tool (BLAST) homology search. This returned a single hit for a hypothetical protein, SCAB_56681, from *S. scabies* 87.22 (NCBI Reference Sequence: NC_013929.1) an actinomycete[11] that causes common scabs on potatoes.[12] SCAB_56681 is a putative 44 amino acid peptide that has no sequence homology to any known protein, and the putative bottromycin core peptide appears from residues 2–9 of this peptide (Fig. 1b). This is highly unusual for an RP as almost all known precursor peptides for RPs contain N-terminal leader peptide regions,[13] and any C-terminal extensions are very short. A partial exception is the recently discovered YM-216391 system, where YM-216391 derives from the middle of a prepeptide.[14] In conventional ribosomal peptide biosynthesis, the leader peptide functions as a recognition sequence for post-translational modifying enzymes and/or assists with self-resistance and export. However, if post-translational modifications occur prior to peptide cleavage, the bottromycin precursor residues need to be located at the N-terminus of the peptide to allow for the amidine forming cyclisation of the N-terminal glycine onto an internal amide carbonyl.

Analysis of the genes clustered around SCAB_56681 (Table 1) provided additional evidence that this is a cluster for a bottromycin-like molecule (Fig. 2) as it contains genes that encode enzymes that could plausibly participate in its biosynthesis, notably including three radical SAM methyltransferases. The genes in this putative cluster were denoted *btmA-M*, based on likely cluster boundaries. A comparison with other streptomycete genomes shows that the cluster is inserted into the middle of a series of phosphotransferase system proteins. Using this nomenclature, SCAB_56681 was named *btmD*. The gene cluster appears to be arranged into two operons; one for *btmA* and *btmB* and the other for the remaining genes.

Table 1 Putative bottromycin gene cluster

Gene name	Gene cluster	Proposed function
SCAB_56711[a]	*btmA*	Major facilitator superfamily
SCAB_56701[a]	*btmB*	*o*-Methyltransferase
SCAB_56691	*btmC*	Radical SAM MTase
SCAB_56681	*btmD*	Precursor peptide
SCAB_56671	*btmE*	YcaO-domain
SCAB_56661	*btmF*	YcaO-domain
SCAB_56651	*btmG*	Radical SAM MTase
SCAB_56641	*btmH*	α/β Hydrolase
SCAB_56631	*btmI*	Metallo-dependent hydrolase
SCAB_56621	*btmJ*	Cytochrome P450
SCAB_56611	*btmK*	Radical SAM MTase
SCAB_56601	*btmL*	Transcriptional regulator
SCAB_56591	*btmM*	M17 aminopeptidase

[a] Genes transcribed on opposite strand.

S. scabies had not previously been recognised as a producer of bottromycin and it was possible that the cluster is silent or had been incorrectly identified. Therefore, *S. scabies* DSM 41658 was screened for bottromycin production along with *S. bottropensis* DSM 40262 using a bottromycin production medium.[15] After seven days, liquid chromatography/mass spectrometry (LCMS) analysis showed four bottromycins produced by both strains (see Fig. 3A and ESI† for identification of D): A_2 ($[M + Na]^+ = 845.4$), B ($[M + Na]^+ = 831.4$), C ($[M + Na]^+ = 859.4$) and the previously uncharacterised D ($[M + Na]^+ = 817.3$), which lacks two methyl groups compared to bottromycin A_2. Bottromycin A_2 was the most abundant bottromycin, accounting for approximately 70% of all bottromycins in both *S. scabies* and *S. bottropensis*. The compounds produced by these two species eluted with identical HPLC retention times (see ESI†).

Bottromycins A_2 and B from *S. scabies* were analysed by high-resolution mass spectrometry (HRMS): [Bottromycin A_2 + Na]$^+$ HRMS = 845.4360 (calculated $C_{42}H_{62}N_8NaO_7S^+ = 845.4354$); [Bottromycin B + Na]$^+$ HRMS = 831.4203 (calculated $C_{41}H_{60}N_8NaO_7S^+ = 831.4198$). The likely importance of radical SAM methyltransferases to the pathway prompted us to include additional $CoCl_2$ in the production medium, which increased bottromycin production three-fold.

To confirm that the *btm* cluster is associated with bottromycin biosynthesis, the gene encoding the putative precursor peptide, *btmD*, was inactivated by an in-frame deletion using a pYH7-derived plasmid.[16] This deleted a region that encodes for the core bottromycin peptide as well as much of the tail peptide. Bottromycin production was abolished in *S. scabies* Δ*btmD*, thus confirming a connection between this gene and bottromycin biosynthesis (Fig. 3B). Bottromycin production was re-established by two different methods. *Trans*-complementation was achieved by using a pSET152-derived plasmid[17] (Fig. 3B) that contains *btmC* and *btmD* as well as the intergenic region that

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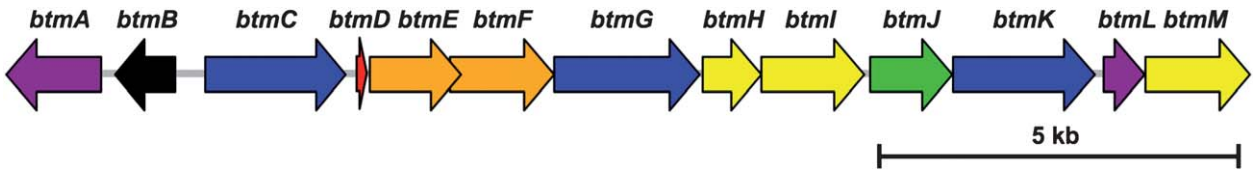

Fig. 2 Bottromycin gene cluster in *S. scabies*. The gene encoding the bottromycin precursor peptide is shown in red, genes encoding radical SAM methyltransferases are blue, the *o*-methyltransferase gene is black, YcaO-domain genes are orange, hydrolase genes are yellow and putative regulatory genes are purple. See ESI† for detailed information about each gene.

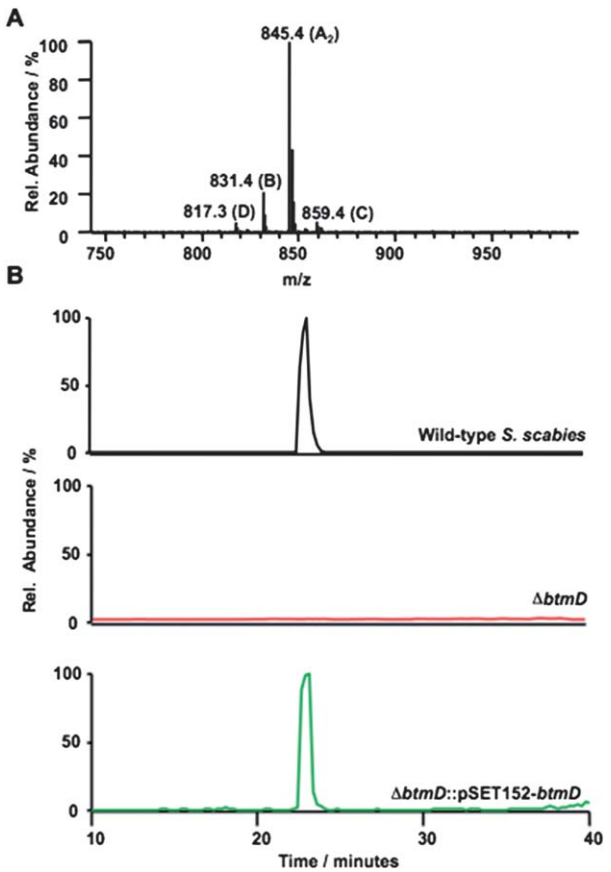

Fig. 3 Bottromycin production in *S. scabies* assessed by LCMS. (A) Mass spectrum showing the distribution of bottromycins A_s, B, C and D. (B) Selective ion monitoring at $m/z = 845.4$ ($[A_2 + Na]^+$) for wild-type *S. scabies* and mutant strains Δ*btmD* and Δ*btmD*::pSET152-*btmD*.

precedes *btmC*, due to its likely role as a transcriptional start site. The *attP* site on this plasmid integrates into an *attB* site in the *S. scabies* genome. *Cis*-complementation was achieved by reintroducing the *btmD* gene into its natural position by a double crossover using a pYH7-derived plasmid. Complementation plasmids based on the established streptomycete promoters[18] P*ermE** and *actII-ORF4*/P*actI* were unable to reconstitute bottromycin biosynthesis (data not shown).

The known bottromycin producer *S. bottropensis* is also associated with potato scab and is closely related to *S. scabies*,[19] although it lacks the genes required for pathogenicity. A PCR-based screen for the *btmD* gene in *S. bottropensis* demonstrated that this gene is present in both organisms. Therefore, the *S. bottropensis* DSM 40262 genome was sequenced to identify the bottromycin gene cluster in this organism. The cluster was assembled from shotgun genome sequence data to reveal an identical arrangement of genes with very few significant differences throughout the cluster region (see ESI†). Overall, the sequences possess 91% identity and their respective *btmD* genes differ at only three nucleotides and encode identical peptides (see ESI†).

β-Methylation is very rare in peptides and the only other structurally characterised example is polytheonamide B, a 48-amino acid peptide produced by a bacterial symbiont associated with the marine sponge *Theonella swinhoei*.[20] NRPs such as daptomycin possess β-methyl groups, but these are not directly installed onto the peptide by a MTase.[21] Bottromycin requires methylation at four non-nucleophilic positions and it is likely that radical SAM methyltransferases are required for these steps. However, only three are encoded by the cluster (BtmC, G and K). One possible explanation is that two valines are methylated by one enzyme. A recent structural analysis of bottromycin showed that these valines exist in a similar environment on the same face of the molecule.[22] Each radical SAM protein has a CxxxxxxxCxxC motif instead of the convential CxxxCxxC motif[23] (Fig. 4A) that is necessary for coordinating a [4Fe-4S] cluster. This four amino acid insertion is not usually seen in radical SAM enzymes, but this motif is conserved throughout a number of uncharacterised radical SAM proteins so it is unlikely that this is a rare inactivating mutation (see ESI†). Additionally, all the radical SAM enzymes contain a vitamin B12-binding domain, which is essential for the generation of a Co(III)–Me intermediate that serves as the methyl donor in class B radical SAM methyltransferases.[23] This motif was identified by an alignment with the characterised cobalamin-binding natural product methyltransferases[24] BcpD (bialaphos), Fom3 (fosfomycin), Fms7 (fortimicin) and CloN6 (clorobiocin). The Motif 2 cobalamin binding site[25] is clearly observed (DxxGx(S/T)x_nGG, Fig. 4A). The Motif 1 consensus sequence of corrinoid proteins is not seen in radical SAM methyltransferases.

btmC, *btmG* and *btmK* were independently inactivated using pYH7-based double crossovers. All deletions were in-frame, scarless and fully deleted the cobalamin and [4Fe-4S] binding sites. Inactivation of *btmC* resulted in abolition of bottromycin production. One possible explanation is that the majority of post-translational modifications are strictly dependent on BtmC methylation as no bottromycin derivative lacking all methyl groups has been identified from the wild-type strain, thus partial methylation is essential for proper bottromycin maturation.

In contrast to the *btmC* deletion, *S. scabies* Δ*btmG* predominantly produced a compound with the same mass as bottromycin D ($m/z = 817.4$, see ESI†), which lacks two methyl groups compared to bottromycin A_2. Total bottromycin yield was also

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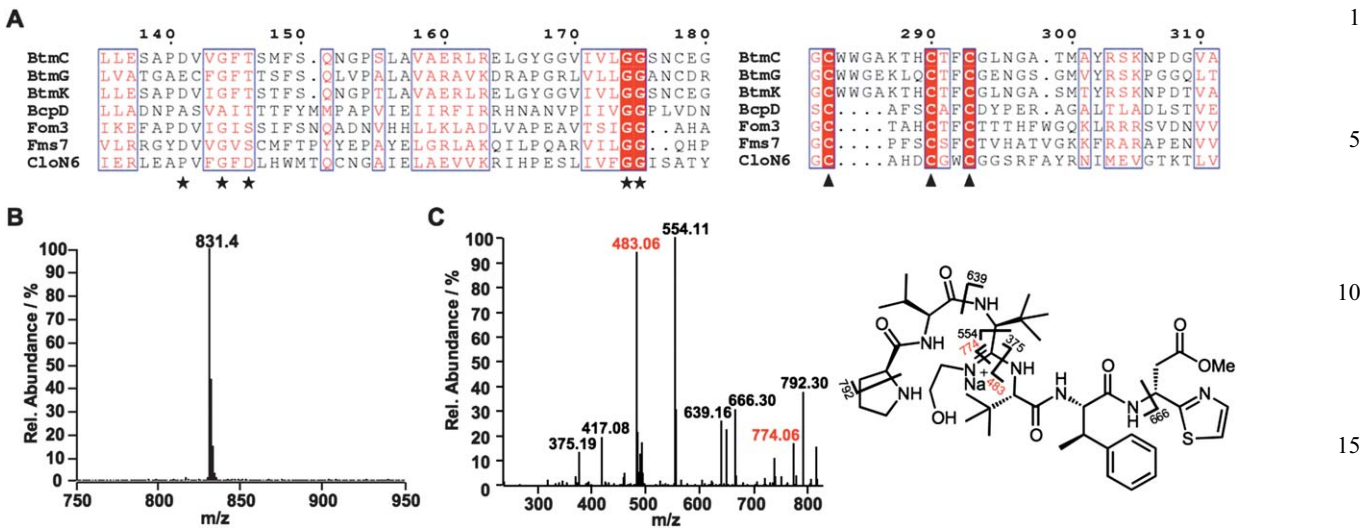

Fig. 4 Analysis of radical SAM methyltransferases BtmC, BtmG and BtmK. (A) Identification of the Motif 2 cobalamin binding site (starred residues) and the motif required for [4Fe-4S] cluster formation (triangles) in comparison to characterised natural product radical SAM MTases. (B) MS analysis of bottromycin production in *S. scabies* Δ*btmK*. (C) ESI-MS^2 analysis of the product of *S. scabies* Δ*btmK* reduced with $NaBH_4$. The red mass refers to a fragment where the sodium is on another part of the molecule.

significantly reduced. The absence of two methyl groups can be explained by either the inability of a later stage methyltransferase to process a demethylated substrate or that BtmG methylates at two positions. The low yield could be explained by the inability of later stage proteins to efficiently process a demethylated substrate. Unfortunately, the low yield prevented a more detailed analysis of the Δ*btmG* metabolite, so the location of methylation in this derivative is currently undetermined.

Inactivation of *btmK* resulted in the production of a compound with a $m/z = 831.4$, and a retention time that was equivalent to bottromycin B (Fig. 4B). This had a yield of about 50% of wild-type levels of bottromycin. The position of methylation on the Δ*btmK* product was investigated by reducing the compound with $NaBH_4$ then analysing the product by tandem mass spectrometry. $NaBH_4$ specifically reduces and cleaves the proline amide bond,[2] thus generating a linear peptide that can be fragmented using MS^n. This analysis (Fig. 4C) showed that the compound with $m/z = 831.4$ had an identical fragmentation pattern to bottromycin B produced by wild type *S. scabies* (see ESI†). This result, along with the absence of bottromycins A_2 and C in the Δ*btmK* strain, indicate that BtmK methylates proline. The high levels of production correlate with the efficient production of bottromycin B in the wild-type strain. These results do not provide detailed information on the precise timing of β-methylation but the differing quantities of bottromycin produced indicate that the order is likely to be BtmC, BtmG and then BtmK. The reduction in yield implies that methylation by BtmC and BtmG are early stage transformations, unless more complex regulatory factors are influencing the expression of *btmD* in these deletion strains.

A plausible biosynthetic pathway can be hypothesised from the proposed functions of the remaining *btm* genes (Fig. 5). Following expression of the bottromycin precursor peptide (BtmD), its N-terminal methionine residue can be cleaved by BtmM, which belongs to the M17 Zn^{2+}-dependent aminopeptidase family.[26] This generates an N-terminal glycine that could then cyclise onto an internal amide carbonyl to generate the amidine moiety. This is an unprecedented biotransformation but shares significant similarities with the formation of 5-membered heterocycles such as thiazoline.[27] The biosynthesis of heterocycles on ribosomal peptides is well-established, and involves a three-component synthetase such as in microcin (McbBCD).[28] In this system, McbB is a cyclodehydratase, McbC is a dehydrogenase and McbD is an ATP-dependent "docking" protein that belongs to the YcaO-domain family of proteins. Recently, Mitchell and co-workers demonstrated that this family of proteins use ATP to activate peptide backbones for cyclisation.[29] McbB and D homologues are sometimes found fused as a single ORF, such as the patellamide heterocyclase PatD.[30]

Surprisingly, there are no McbB homologues in the bottromycin cluster, although BtmE and BtmF both contain YcaO-like domains. A microcin YcaO domain protein has been identified that can slowly catalyse cyclodehydration, but an associated cyclodehydratase is thought to be essential for efficient heterocycle formation.[28] However, it is possible that these proteins contain unidentified domains capable of catalysing cyclodehydration, as BtmE only possesses weak similarity with the YcaO-domain family, whereas BtmF has stronger YcaO-domain similarity but all of its closest homologues are uncharacterised (see ESI†). Thus, one of these proteins could catalyse thiazoline formation and the other could catalyse macrocyclisation. A mechanism for amidine formation can be proposed that involves activation of the peptide bond with ATP followed by nucleophilic attack by the N-terminal amine (see ESI†). A mechanistically similar reaction is the conversion of uridine triphosphate into cytidine triphosphate (CTP), which involves the formation of an amidine from an amide. CTP synthetase[31] catalyses this reaction by first phosphorylating the amide oxygen, which is then displaced by ammonia.

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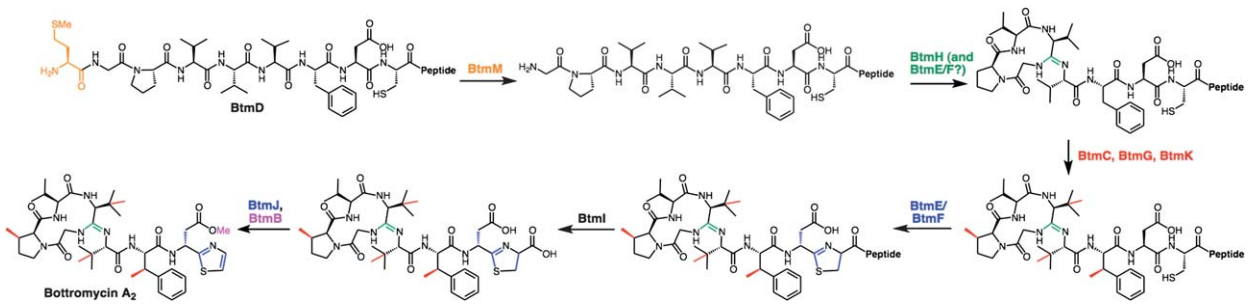

Fig. 5 Proposed biosynthesis of bottromycin A_2. The order and type of transformation proposed are tentative assignments based on biochemical and bioinformatic analyses.

An alternative route to macrocyclisaton would be the use of a putative hydrolytic enzyme, such as BtmH (α/β-hydrolase) or BtmI (Metallo-dependent hydrolase), as either a standalone cyclodehydratase or as a partner to a YcaO-domain protein. An oxidative decarboxylation is also required to transform a thiazoline into a thiazole, assuming that the tail peptide is cleaved prior to oxidation. In other pathways, this oxidation is carried out by a flavin-dependent dehydrogenase (*e.g.* McbC) but one is not present in the cluster. Instead, the cluster encodes BtmJ, a cytochrome P450, which could feasibly catalyse this reaction. Tail peptide hydrolysis could be catalysed by BtmH or BtmI.

Alternatively, in the absence of a convincing cyclodehydratase, a protein from elsewhere in the *S. scabies* genome could catalyse these steps. SCAB_63151 is in a different part of the genome to the bottromycin cluster, but contains the cyclodehydratase and docking domains that are typically necessary for heterocycle formation. Also, it is adjacent to an McbC-like dehydrogenase (SCAB_63141). Cross-talk between bacterial gene clusters is unusual for secondary metabolite biosynthesis but not unprecedented.[32]

btmE, *btmF* and *btmJ* were independently deleted to determine their roles in bottromycin biosynthesis. Bottromycin production was abolished in each of these deletion strains, indicating that these genes are essential for bottromycin biosynthesis. No advanced intermediates could be characterised from any of these mutant strains. This is somewhat surprising given the proposed late stage role of BtmJ. Additonally, no bottromycin was produced by *S. scabies* Δ*btmI*, which reflects either a crucial role in tail peptide hydrolysis or an early stage step in cyclisation.

It is likely that aspartate *o*-methylation is a late-stage step as this modification activates the molecule into an effective antibiotic.[3] The clear candidate for this step is BtmB, which belongs to the COG3315 family of SAM-dependent methyltransferases that methylate carboxylate groups.[33] This methylated aspartate possesses a non-proteinogenic D-stereocentre, and this configuration has been observed before for residues adjacent to a heterocycle in ribosomal peptides, such as in trunkamide A,[10] and may simply be an uncatalysed epimerization caused by difference in diastereoisomer energies. An epimerase that acts on RPs was isolated from platypus venom,[34] but no homologues exist in the *btm* custer. *btmB* is not in the same operon as most other genes described in this cluster but is adjacent to *btmA*, which encodes a trans-membrane protein that belongs to the major facilitator superfamily.[35] This family is responsible for the transport of small molecules across membranes, thus making BtmA a strong candidate as a bottromycin efflux protein. Co-transcription of *btmA* and *btmB* would allow export to be closely coupled to the formation of a potent antibiotic by *o*-methylation.

It is likely that BtmL is a transcriptional regulator of bottromycin biosynthesis. This 20.5 kDa protein contains a DUF2087 domain at its C-terminus. This is proposed to bind nucleic acids,[36] while the remainder of the protein possesses weak sequence homology to the ArsR/SmtB family of transcriptional repressors. De-repression of this family is achieved by metal ion binding, which would be consistent with the requirement of Co^{2+} and Zn^{2+} for at least five proteins in the bottromycin pathway. Deletion of the *btmL* gene did not change the yield of bottromycin produced. It is possible that the presence of divalent metals in the production medium may mean that the BtmL-mediated repression is usually inhibited in wild-type *S. scabies*. A structural prediction using the Phyre2 server[37] confirmed that the first 110 residues are structurally homologous to ArsR-family regulators, although it lacks the conserved residues required for metal binding that are used by its closest structural homologues[38] (see ESI†). Further work will be required to determine the precise role of BtmL in the regulation of bottromycin biosynthesis.

Conclusions

To summarise, we have identified the gene cluster responsible for bottromycin biosynthesis in *S. scabies*, thus identifying a novel producer of this anti-MRSA antibiotic and revealing a ribosomal origin to this highly modified peptide. We also identified a closely related gene cluster in *S. bottropensis*, an established bottromycin producer. Gene deletion experiments have confirmed the roles of two radical SAM methyltransferases (BtmG and BtmK) in the β-methylation of three amino acids and it is very likely that BtmC is responsible for a fourth β-methylation. The essential role of the YcaO-domain proteins BtmE and BtmF in the pathway have been verified. Further investigation is required to elucidate the precise mechanism and timing of some key steps in the pathway, such as the amidine-forming macrocyclisation. To the best of our knowledge, this biosynthetic pathway possesses a number of unique features: it is the first ribosomal peptide that originates from the N-terminus of a precursor peptide, the only terrestrial peptide to be directly β-methylated and bottromycin contains the only known natural macrocyclic amidine. The identification of this pathway should facilitate the generation of a library of bottromycin-like antibiotics.

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Acknowledgements

This work was supported by grants to WJKC from the BBSRC and to AWT from St. John's College, Cambridge. We thank Prof. Peter F. Leadlay for helpful discussions regarding this work.

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EDG ■ C2SC21190D