View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Chemistry & Biology
Article

Synthetic Biotechnology to Study and Engineer Ribosomal Bottromycin Biosynthesis

Liujie Huo,[1,4] Shwan Rachid,[1,2,4] Marc Stadler,[3] Silke C. Wenzel,[1,*] and Rolf Müller[1,*]
[1]Department of Microbial Natural Products, Helmholtz Institute for Pharmaceutical Research Saarland, Helmholtz Centre for Infection Research and Pharmaceutical Biotechnology, Saarland University, Saarbrücken, Saarland 66123, Germany
[2]Faculty of Science and Health, University of Koya, Koya, Kurdistan KO50 1001, Iraq
[3]Department of Microbial Drugs, Helmholtz Centre for Infection Research, Braunschweig, Niedersachsen 38124, Germany
[4]These authors contributed equally to this work
*Correspondence: s.wenzel@mx.uni-saarland.de (S.C.W.), rom@helmholtz-hzi.de (R.M.)
http://dx.doi.org/10.1016/j.chembiol.2012.08.013

SUMMARY

Bottromycins represent a promising class of antibiotics binding to the therapeutically unexploited A-site of the bacterial ribosome. By inhibiting translation they are active against clinically important pathogens, such as vancomycin-resistant *Enterococci*. Structurally, bottromycins are heavily modified peptides exhibiting various unusual biosynthetic features. To set the stage for compound modification and yield optimization, we identified the biosynthetic gene cluster, used synthetic biotechnology approaches to establish and improve heterologous production, and generated analogs by pathway genetic engineering. We unambiguously identified three radical SAM methyltransferase-encoding genes required for various methylations at unactivated carbons yielding *tert*-butyl valine, methyl-proline, and β-methyl-phenylalanine residues, plus a gene involved in aspartate methyl-ester formation. Evidence for the formation of the exo-thiazole unit and for a macrocyclodehydration mechanism leading to amidine ring formation is provided.

INTRODUCTION

One of the most prominent global public health threats is caused by antibiotic resistance in conjunction with new and reoccurring infectious diseases. In addition, antimicrobial research in pharmaceutical companies is challenged by a severe disproportion between the degree of investment and the expected profit in the course of drug development. Therefore, access to new hit-and-lead structures addressing novel targets and/or representing new chemical scaffolds exhibiting activity against multidrug-resistant bacteria is of utmost importance (Fischbach and Walsh, 2009; Newman and Cragg, 2012; Li and Vederas, 2009).

Bottromycins were discovered as antibacterial peptides with promising activity against Gram-positive bacteria and mycoplasma from the fermentation broth of *Streptomyces bottropensis* (Waisvisz et al., 1957a, 1957b, 1957c; Waisvisz and van der Hoeven, 1958; Tanaka et al., 1968). Later on it was shown that their antibacterial ability extends to methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE) (Shimamura et al., 2009). The structure elucidation process involved several chemical revisions (Nakamura et al., 1965a, 1965b, 1965c, 1966, 1967; Takahashi et al., 1976; Schipper, 1983) and ultimately led to the assignment of **1** (Figure 1), which was recently confirmed by total synthesis (Shimamura et al., 2009). Mode of action studies revealed the aminoacyl-*t*RNA binding site (A site) on the 50S ribosome as the target of bottromycins, ultimately leading to the inhibition of protein synthesis (Otaka and Kaji, 1976, 1981, 1983). As this site is currently not addressed by clinically used antibiotics, no cross-resistance was observed, and bottromycins are regarded as promising leads to be developed as novel anti-infectives, with renewed interest even in medicinal chemistry (Gouda et al., 2012).

Bottromycins represent octapeptides exhibiting an internal tetrapeptide cycle formed via a unique amidine linkage. The compound harbors an exo-thiazole and several unnatural amino acids, which carry methyl-groups at nonactivated carbons, posing additional challenges for total synthetic approaches aimed toward drug development.

As a valid alternative to total synthesis, biosynthetic engineering can be envisaged to improve structure and yield of any microbial natural product eventually resulting in fermentative production (Fischbach and Voigt, 2010). To achieve this goal the underlying principles of compound production need to be understood, and thus identification of the biosynthetic genes is mandatory. In principle, there are two natural ways for the production of highly modified and bioactive peptide scaffolds: biosynthesis can be achieved in a thiotemplated fashion on large multienzymatic systems termed nonribosomal peptide synthetases (NRPS), which were intensively studied in the past decades (Finking and Marahiel, 2004; Schwarzer et al., 2003). Alternatively, and only recently described as more common than originally anticipated, complex peptides can also be biosynthesized starting from simple, ribosomally made precursor peptides undergoing intriguing modification steps (McIntosh et al., 2009; Nolan and Walsh, 2009; Oman and van der Donk, 2010). Nonribosomally synthesized peptides (NRPs) and the latter compounds of ribosomal origin (RPs) differ mainly in the construction of the core-scaffold as RPs are limited in structural diversity by incorporation of the canonical proteinogenic amino acids only. However, they can be extensively posttranslationally

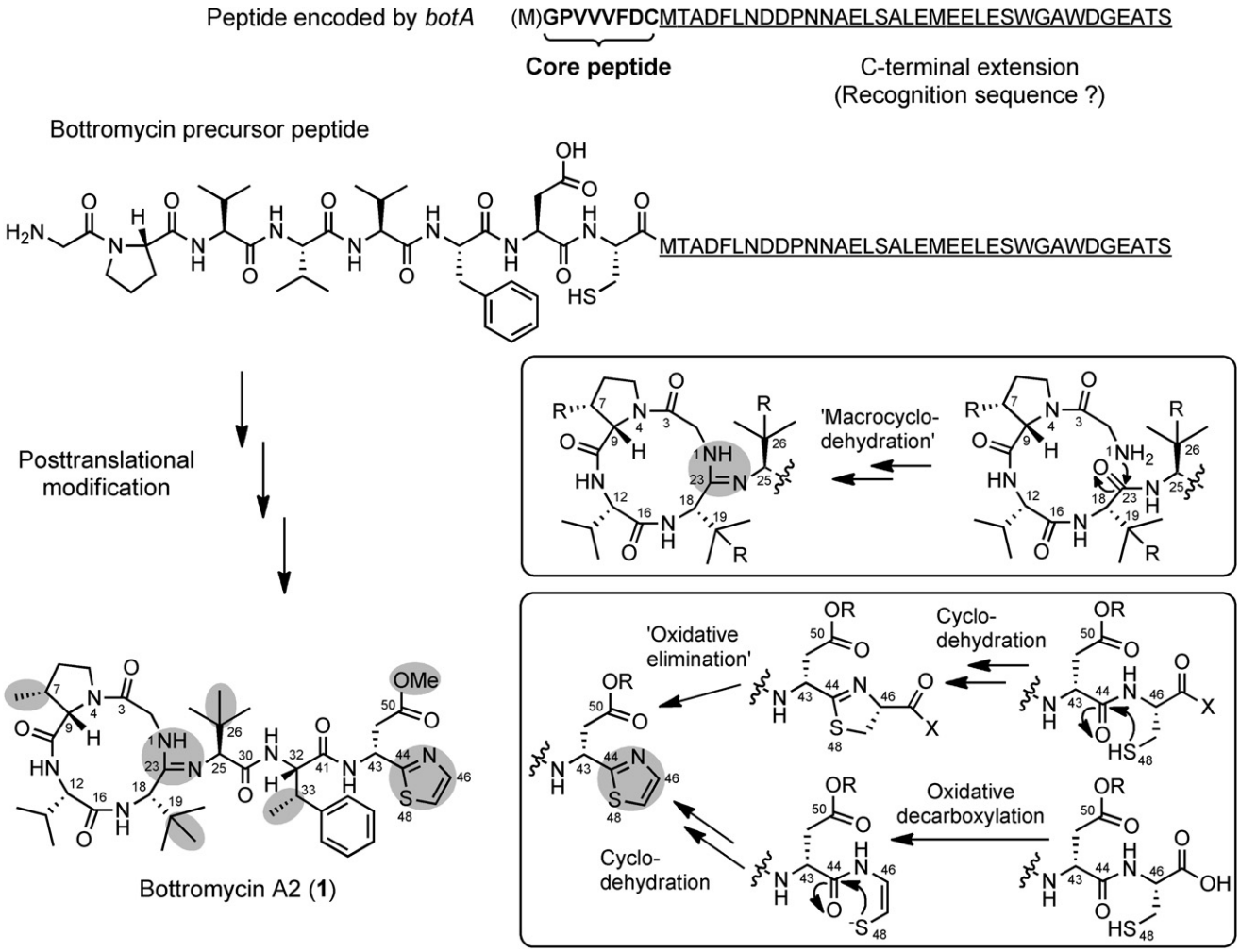

Figure 1. Maturation of the Ribosomal Bottromycin Peptide Scaffold

The bottromycin precursor peptide consists of the core peptide as well as a C-terminal extension (underlined) likely serving as recognition sequence. Maturation of the bottromycin peptide scaffold requires a number of posttranslational modifications (highlighted in gray), the timing of which is currently unknown. These include various methylations and proteolytic digestion as well as "macrocyclodehydration" and thiazole ring formation (proposed mechanisms for the latter two are boxed, for explanations see subsection "In Silico Analysis of the Bottromycin Biosynthetic Pathway"). R, H or Me; X, OH or MTADFLNDDPNNAELSALE MEELESWGAWDGEATS.

modified, which is mostly performed on the precursor peptide state (McIntosh et al., 2009; Oman and van der Donk, 2010). NRPS, in contrast, incorporate during chain assembly numerous "unnatural" amino acids and are thought to undergo less extensive post-NRPS modifications. However and despite their vastly different core biosynthetic routes, RPs and NRPs share many postassembly modifications, which are often required for biological activity. These include heterocyclizations, methylations, and glycosylations, just to name a few (Walsh et al., 2001; McIntosh et al., 2009; Oman and van der Donk, 2010). In NRPS the required enzymatic functionalities are optionally incorporated into the megasynthetases. As discussed above it is difficult to predict from the chemical structure whether modified peptides are based on NRP and RP assembly logic. Therefore, retrobiosynthetic analysis of bottromycin did not yield an assignment of the type of biosynthetic pathway to be expected.

Motivated by the promising activity and the chemical uniqueness of the bottromycin scaffold, we set out to identify the biosynthetic gene locus in the terrestrial actinomycete *Streptomyces* sp. BC16019, which was shown to produce bottromycin A2, B2, and C2 (Lerchen et al., 2006). This work identified a ribosomal locus required for bottromycin biosynthesis and set the stage for synthetic biotechnology approaches toward structure and yield improvement. We heterologously expressed the biosynthetic pathway and optimized initially low bottromycin production titers. Using pathway engineering we were able to assign each of the five unusual methylation reactions to specific genes, which in parallel enabled the production of three, to our knowledge, novel derivatives. In addition, work presented here provides evidence for a unique "macrocyclodehydration" reaction giving rise to the unique amidine linkeage found in the bottromycin scaffold.

Table 1. Proteins Involved in Bottromycin Biosynthesis

Protein	Residues (aa)	Annotation	Proposed Function in Bottromycin Biosynthesis
BotRMT1	641	Radical SAM	*C*-Methylation (of phenylalanine)
BotA	44	Structural gene	Precursor peptide
BotC	434	Cyclodehydratase (YcaO-like family)	Macrocyclodehydration (?)
BotCD	400	Cyclodehydratase (YcaO-like family and docking_ocin superfamily)	Cyclodehydration during thiazole ring formation (?)
BotRMT2	660	Radical SAM	*C*-Methylation (of valines)
BotH	293	Putative α/β hydrolase	Macrocycle or thiazole ring formation (?)
BotAH	465	Putative amidohydrolase	Macrocycle or thiazole ring formation (?)
BotCYP	345	Cytochrome P450 enzyme	Macrocycle or thiazole ring formation (?)
BotRMT3	681	Radical SAM	*C*-Methylation (of proline)
BotR	184	Transcriptional regulator	Pathway regulation
BotOMT	279	*O*-methyl transferase	*O*-Methylation
BotT	441	Multidrug transporter	Efflux pump, self-resistance
BotP	504	Leucyl-aminopeptidase	Proteolytic cleavage

See also Figure S1 and Table S1.

RESULTS AND DISCUSSION

Identification of the Bottromycin Biosynthetic Gene Cluster

Our initial attempts to identify the bottromycin biosynthetic pathway were based on the assumption that the compound is made via a nonribosomal peptide synthetase (NRPS) assembly line, similar to most other cyclic peptide pathways analyzed to date. Those systems usually exhibit a modular architecture, in which each module incorporates one amino acid building block to the growing peptide chain (Finking and Marahiel, 2004; Schwarzer et al., 2003). Retrobiosynthetic analysis revealed that the bottromycin peptide core almost certainly originates from eight amino acids (Gly-Pro-Val-Val-Val-Phe-Asp-Cys), which led us to predict an octamodular NRPS biosynthetic machinery. In accordance with textbook biosynthetic logic, bottromycin thiazole ring formation from a cysteine residue requires the catalytic activity of a heterocyclization (HC) and an oxidation (Ox) domain (Walsh et al., 2001; Schwarzer et al., 2003; Roy et al., 1999; Walsh and Nolan, 2008), which should therefore be included within the putative bottromycin assembly line. As the identification of heterocycle-forming peptide pathways using homologous and heterologous probes for HC and/or Ox domains is well established (Cheng et al., 2002), we considered this strategy to screen for the bottromycin biosynthetic pathway. A cosmid library of the bottromycin producer *Streptomyces* sp. BC16019 was constructed and analyzed for HC/Ox harboring NRPS pathways by hybridization experiments (S.R., L.H., and R.M., unpublished data). However, these efforts did not lead to the identification of a genetic locus possibly involved in bottromycin biosynthesis. To obtain comprehensive information about all putative secondary metabolite pathways of *S.* sp. BC16019 whole-genome shotgun sequence data were generated using the 454 technology. Detailed in silico analysis of the resulting 319 sequence scaffolds revealed no putative NRPS-dependent bottromycin biosynthetic gene cluster, indicating that the compound might rather derive from a ribosomal pathway. In this case, one would expect a small precursor peptide-encoding gene accompanied by a number of "modifying genes" for crafting the complex and bioactive bottromycin scaffold from a rather simple, linear peptide chain assembled from the canonical 20 proteinogenic amino acids. Indeed, we were able to identify a small open reading frame (orf; *botA*) in the sequence data encoding the bottromycin octapeptide sequence, plus some C-terminal extension. In silico analysis of the flanking regions led to the identification of a number of genes encoding enzymes that are most likely involved in the various posttranslational modification (PTM) steps predicted for the maturation of the bottromycin propeptide (see Figure 1 and Table 1). In order to confirm that the identified chromosomal region encodes the bottromycin biosynthetic pathway, we established a genetic modification procedure for the producer strain to perform targeted gene inactivation experiments. Using the suicide vector pKC1132-botOMT-KO, one of the putative-modifying genes, *botOMT*, was inactivated via homologous recombination, resulting in the mutant strain *S.* sp. BC16019::pKC1132-botOMT-KO. High-performance liquid chromatography (HPLC)-mass spectrometry (MS) analysis in comparison to wild-type extracts showed the abolishment of bottromycin production, confirming the involvement of *botOMT* in bottromycin biosynthesis. At this point, polar effects on genes located downstream could not be excluded using the applied insertion strategy. Further experiments (see subsection "Establishment and Optimization of Heterologous Bottromycin Production") clearly established the *bot* locus as the bottromycin biosynthetic gene cluster.

In Silico Analysis of the Bottromycin Biosynthetic Pathway

The gene locus responsible for bottromycin biosynthesis was identified through in silico analysis of genome data coupled with targeted insertion mutagenesis as described above as well as via heterologous expression of the entire pathway (see subsection "Establishment and Optimization of Heterologous Bottromycin Production"). The pathway consists of (at least) 13 genes (*bot* genes) and spans about ~18 kb (see Figure 2). The *botA* gene encodes a 44-amino acid peptide and is flanked

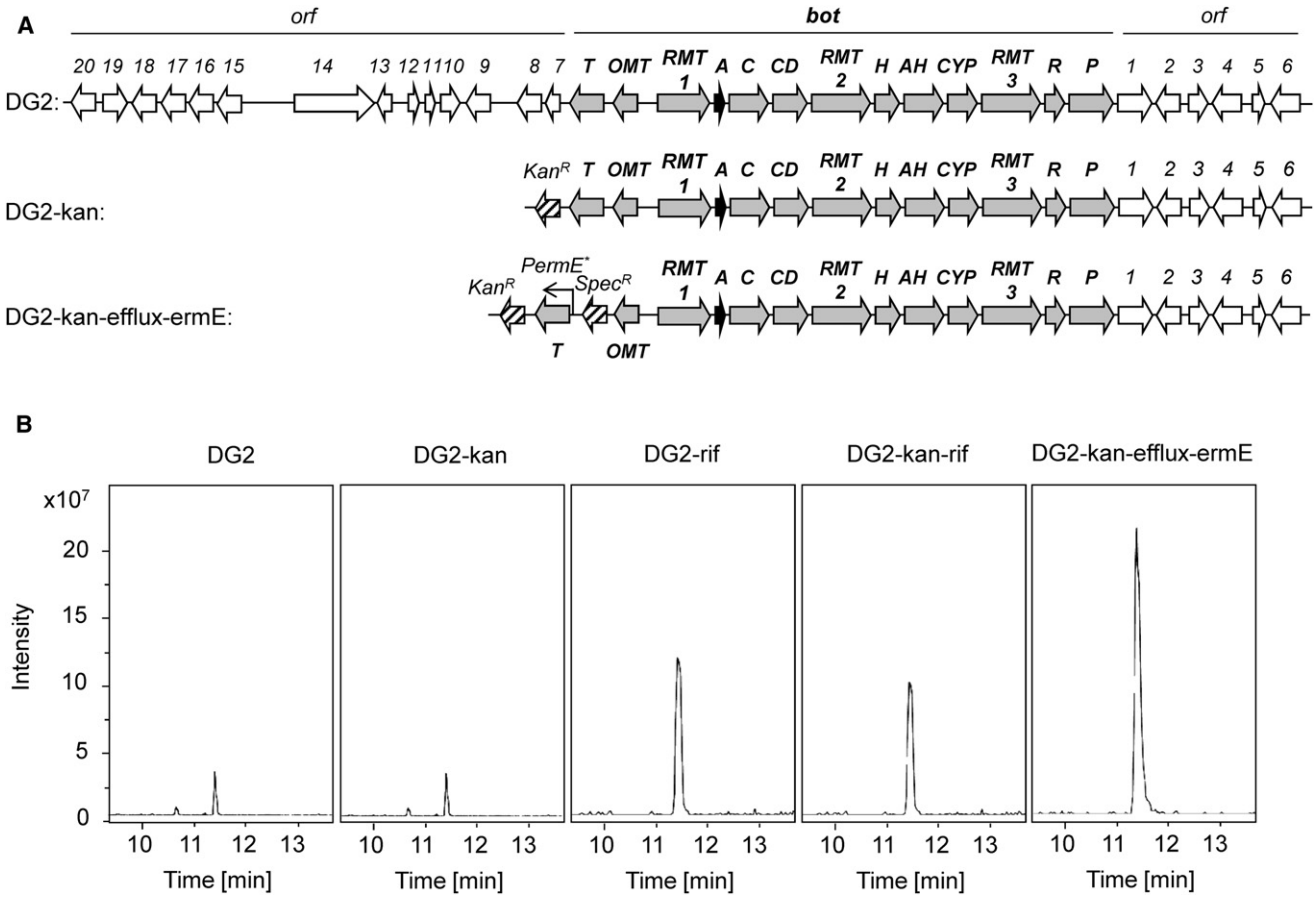

Figure 2. Heterologous Bottromycin Production

(A) Inserts of the three expression constructs (pOJ436 derivatives) used for heterologous bottromycin production. The original cosmid DG2 was modified via Red/ET recombineering to delete the 5′-flanking region (*orf7–orf20*) by insertion of kan^R and, in the next step, to insert the *PermE** promoter upstream of *botT* together with $spec^R$.

(B) Quantification of heterologous bottromycin production by HPLC-MS analysis of the culture extracts from different *S. coelicolor* mutant strains. Sections of extracted ion chromatograms at m/z = 823.45 corresponding to the $[M+H]^+$ ion of bottromycin A2 (**1**) are illustrated as representative readout of productivity. The *S. coelicolor* A3(2) host strains contain one of the three expression constructs shown in (A). With *S. coelicolor*::DG2 and DG2-kan, "rifampicin-induced mutagenesis" was performed ("DG2-rif" and "DG2-kan-rif").

by a set of modifying genes required for maturation to an 8-residue highly modified macrocycle (see Figure 1). The BotA precursor peptide is bipartite: it contains an N-terminal core peptide as well as a C-terminal extension (the N-terminal methionine is most likely cleaved off by a methionine aminopeptidase during translation or by the putative peptidase BotP). This structure is unique among precursor peptides from ribosomal pathways described to date; in other precursors, the core peptides are typically flanked by N-terminal leader sequences, and in some cases additional C-terminal extensions are found (Oman and van der Donk, 2010). It is frequently postulated that the N-terminal leader sequences serve as a recognition motif for the PTM enzymes and that they are removed in the last step of the maturation process (Oman and van der Donk, 2010). The absence of an N-terminal leader sequence in BotA indicates that the C-terminal extension—or parts of it—functions as PTM enzyme recognition sequence. Furthermore, as discussed for the N-terminal leaders, some other functions can be attributed to the C-terminal BotA sequence, for example, serve as secretion signal, act as chaperone (in *cis*) and assist with folding, stabilize the precursor against degradation, and/or keep the peptide inactive during biosynthesis inside the cell (Oman and van der Donk, 2010). (Final) Proteolytic cleavage of the C-terminal extension is most likely catalyzed either by the putative α/β-hydrolase BotH or by BotP, which shows homology to leucyl aminopeptidases (Matsui et al., 2006). For some members of this group, it could be shown that they also prefer methionine as N-terminal amino acid, which correlates well with the predicted cleavage site in BotA (see Figure 1) (Herrera-Camacho et al., 2007). The highly modified bottromycin scaffold results from various PTM reactions, and a number of candidate genes involved in these processes have been identified in the *bot* locus—although most of the encoded proteins exhibit only very low sequence identity to proteins found in the common databases. This finding and the associated difficulties in predicting enzyme function from low similarity scores on the protein level are currently a general challenge for the analysis of ribosomal peptide biosynthesis pathways, which complicates the in silico analysis and

functional gene annotation. However, a striking feature of the bottromycin scaffold is its extensive methylation pattern, and according to our expectations a number of putative *S*-adenosylmethionine (SAM)-dependent methyl transferases (MTs) could be identified (BotRMT1, BotRMT2, BotRMT3, and BotOMT). BotOMT shows homologies to *O*-MTs and was therefore assumed to be involved in *O*-methylation of the aspartate residue. The remaining three MTs (BotRMT1–BotRMT3) belong to the class of radical SAM-dependent MTs (''radical SAMs''; Frey et al., 2008; Atta et al., 2010) and were expected to catalyze the various *C*-methylations at the bottromycin scaffold. According to their didomain protein architecture (N-terminal cobalamin binding domain and C-terminal radical SAM domain), BotRMT1–BotRMT3 belong to class B of the recently classified radical SAM MTs (Zhang et al., 2012), which are indeed hypothesized to contain members methylating unactivated sp^3 carbons. This finding is also in accordance with the proposed methylation sites of BotRMT1–BotRMT3 at proline, phenylalanine, and the two valine residues. However, based on the in silico analysis, it is clearly impossible to assign the exact function of each protein. We therefore carried out further experiments to elucidate the pathway (see subsection ''Functional Studies on the Bottromycin Methylation Pattern: A Suite of Hyperactive Methylases''). The thiazole ring is yet another structural feature of bottromycin, occurring widespread in ribosomally made and nonribosomally assembled peptide scaffolds (Walsh et al., 2001; Schwarzer et al., 2003; Roy et al., 1999; McIntosh et al., 2009; Walsh and Nolan, 2008; Nolan and Walsh, 2009). This heterocycle is typically installed by cyclodehydration starting from a cysteine residue, the thiol of which attacks the carbonyl carbon of the adjacent amino acid to form a thiazoline, which can subsequently undergo oxidation to form the thiazole. For the ribosomal peptide microcin B17, it was shown that a three-protein complex, consisting of a cyclodehydratase, a ''docking protein,'' and a flavin mononucleotide (FMN)-dependent dehydrogenase, is required for thiazole ring formation (Li et al., 1996). Whereas the function of the cyclodehydratase (cyclodehydration reaction) and the dehydrogenase (thiazoline oxidation) was clearly assigned, the precise function of the ''docking protein'' remained elusive until recently, and a direct role in regulating cyclodehydratase activity and assembly of an active complex was debated (Melby et al., 2011). However, a recent study demonstrates that the ''docking protein,'' which belongs to the YcaO-like protein family, itself catalyzes the cyclodehydration under consumption of ATP (Dunbar et al., 2012). This study not only provided first experimental evidence for the requirement of ATP-hydrolysis for cyclodehydration reactions but also showed the dispensability of the subunit formerly annotated as ''cyclodehydratase'' for the reaction. However, it could be shown that this protein can increase the reaction rate by approximately three orders of magnitude. When analyzing the bottromycin biosynthetic pathway for enzyme candidates possibly involved in thiazole ring formation, two putative ''YcaO-like family proteins'' (BotC and BotCD) were identified, whereas genes encoding homologs of the ''cyclodehydratase'' subunit could not be detected (see below). Based on the above findings, we assume that BotC and BotCD autonomously act as cyclodehydratases. Whereas one of these enzymes is most likely involved in thiazoline ring formation, the other candidate might play a role in the unique ''macrocyclodehydration'' reaction as discussed below (see Figure 1). We speculate that BotCD is the enzyme involved in bottromycin thiazole formation, because it shows not only homology to YcaO-like family proteins but also to the ''docking_ocin superfamily'' of proteins (TIGR03604; members of this protein family include enzymes related to SagD; Lee et al., 2008), which includes ''docking proteins'' involved in heterocyclization reactions in other ribosomal pathways. BotC, on the other hand, would thus be a likely candidate for the unique catalyst performing the macrocyclodehydration discussed below. After cyclization conversion of thiazoline moieties into thiazoles usually occurs via dehydrogenation. However, a putative dehydrogenase activity could not be detected in the *bot* locus, suggesting that thiazole ring formation in bottromycin biosynthesis proceeds via a different mechanism. The absence of a dehydrogenase might be explained by the requirements to form an ''exo-thiazole'' moiety, which probably results from an oxidative elimination reaction (see Figure 1). One possible scenario is an oxidative decarboxylation after proteolysis of the C-terminal extension sequence. However, we cannot exclude other ''elimination hypotheses,'' which might rely on further premodifications of the thiazoline ring (e.g., hydroxylation or dehydrogenation). Alternatively, precursor peptide proteolysis and subsequent oxidative decarboxylation of the C-terminal cysteine might occur before cyclodehydration. Similar reactions have been described for the biosynthesis of aminovinyl-cysteine-containing ribosomal peptides, including the generation of reactive ''thio-enol'' intermediates via oxidative decarboxylation of C-terminal cysteine residues (Sit et al., 2011). The enzymes involved were characterized as homo-oligomeric flavin-containing cysteine decarboxylases, but no putative homolog of this enzyme family was found encoded within the bottromycin biosynthetic gene locus. Apparently, the underlying biochemistry for bottromycin ''exo-thiazole'' ring formation cannot be predicted based on the available in silico data and requires further experimental studies.

Another intriguing modification unique for ribosomal peptide biosynthesis is the formation of the bottromycin macrocycle established between the N-terminal glycine and the second valine residue. Similar to textbook cyclodehydration chemistry, we propose a nucleophilic attack of the glycine amino group to the valine carbonyl carbon, followed by dehydration. This transformation may be seen as ''macrocyclodehydration'' reaction, resulting in the formation of an amidine group, which is—to the best of our knowledge—unique among all ribosomal peptides biosynthetically characterized so far and also very rare among other natural products. Coformycin (Nakamura et al., 1974) and ectonine (Inbar and Lapidot, 1988), also described as pyrostatin B (Castellanos et al., 2006), are examples of natural products containing this structural feature. Studies on the genetics and biochemistry of ectoine metabolism revealed that the cyclic amidine moiety is formed via a ''cyclodehydratase''-like reaction similar to the macrocyclization mechanism we postulate for the bottromycin biosynthesis. The enzyme involved is described as ectoine synthase (EctC) and belongs to the family of carbon-oxygen lyases. It is encoded in the ectoine biosynthetic gene locus, which is found widespread in microorganisms (Pastor et al., 2010), and could also be identified in the bottromycin producer as well as in *Streptomyces coelicolor*. However, no homolog of the ectoine synthase family could be identified in

the bottromycin biosynthetic gene cluster, and we assume that one of the putative cyclodehydratases (BotC and BotCD) might be involved in "macrocyclodehydration." BotC is the favored candidate enzyme because of the arguments provided above. However, we cannot exclude the involvement of ectoine biosynthetic pathway enzymes in this reaction, as they are encoded in the chromosomes of both the native and the heterologous bottromycin producers. Additionally, there are some *bot* genes with yet unassigned biosynthetic function, like *botH*, *botAH*, and *botCYP*, encoding a putative α/β hydrolase, an amidohydrolase, and a P450 enzyme, respectively, which might be involved in this unusual "macrocyclodehydration" reaction or in the "exo-thiazole" ring formation discussed above. Besides the discussed enzyme candidates involved in the maturation of the bottromycin precursor peptide, the biosynthetic gene cluster encodes a putative transcriptional regulator (BotR) as well as a putative multidrug transporter (BotT). The latter might play an important role for the self-resistance of the bottromycin producer, which is underpinned by results from genetic engineering studies based on a heterologous expression system (see next subsection).

Establishment and Optimization of Heterologous Bottromycin Production

As genetic manipulation of the native bottromycin producer *S.* sp. BC16019 turned out to be rather difficult and was restricted to the established single-cross-over method with very low efficiency, we aimed to establish a heterologous expression system to further characterize and engineer bottromycin biosynthesis. To mobilize the bottromycin pathway for expression in suitable host strains, the entire ~18 kb biosynthetic gene cluster was required to be subcloned on one physical entity. We were able to identify a cosmid harboring the complete target region plus some flanking sequence when screening the gene library of the bottromycin producer with suitable probes (cosmid DG2, see Figure 2A). As the pOJ436 cosmid backbone already contains genetic elements for conjugation (origin of transfer, *oriT*) and site-specific integration into *Streptomyces* chromosomes (phage ΦC31 integrase gene and attachment site; Bierman et al., 1992), the related host strains *Streptomyces coelicolor* A3(2) and *Streptomyces albus* J1074 were directly transformed with this construct. Transconjugants were verified by PCR and subsequently analyzed for heterologous bottromycin production. Parallel cultivations of host strains with and without the expression construct in comparison to the native bottromycin producer were carried out followed by HPLC-MS analysis of the culture extracts. Successful heterologous bottromycin production could be detected for both of the expression hosts, *S. coelicolor*::DG2 and *S. albus*::DG2, demonstrating that the putative bottromycin pathway was complete and active in the foreign circuits under control of its native regulatory elements. Because the heterologous production yields in both hosts were much lower compared to the native producer (~1 μg/l in *S. albus* and ~4 μg/l in *S. coelicolor*; ~100 times lower than in native host), optimization approaches focused on the *S. coelicolor* expression system were initiated. In a preliminary step, the bulky expression construct was simplified by deletion of a ~16 kb insert fragment at the 5′ end of the *bot* gene cluster. This modification was performed via Red/ET recombineering (Zhang et al., 1998, 2000), resulting in the integration of a kanamycin resistance gene to generate cosmid DG2-kan (see Figure 2A). As expected, subsequent heterologous expression in *S. coelicolor* showed that this deletion does not significantly affect the bottromycin production titer (see Figure 2B), and it also confirmed our assumption that the excised genetic region (*orf7–orf20*) is not required for bottromycin biosynthesis. Aiming at improvement of bottromycin production titers, we next applied an undirected approach based on the observation that certain drug-resistance mutations in the *rpoB* gene (encoding the RNA polymerase β-subunit), or the *rpsL* gene (encoding the ribosomal S12 protein), may effectively enhance secondary metabolite production in Gram-positive bacteria (Hu et al., 2002; Inaoka et al., 2004; Shima et al., 1996; Hu and Ochi, 2001; Tamehiro et al., 2003). Such mutations can be induced, for example, by challenging the bacteria with rifampicin (mutations in *rpoB*) or streptomycin (mutations in *rpsL*). After plating of the expression strains *S. coelicolor*::DG2 and *S. coelicolor*::DG2-Kan on rifampicin-containing medium, spontaneous isolates resistant to the antibiotic were obtained. Quantitative bottromycin production analysis of clones from each "rifampicin mutagenesis" experiment revealed mutant strains (*S. coelicolor*::DG2-rif and *S. coelicolor*::DG2-Kan-rif) with a considerably higher production titer (about ten times higher compared to the starting clones, see Figure 2B). In parallel to this successful undirected approach, we also considered targeted engineering strategies to improve bottromycin production yields; because of the antibacterial activity of bottromycin, we speculated that the self-resistance of the host strain might be a limiting factor for the production titer. Indeed we could show that the growth of *S. coelicolor* is already heavily impaired at bottromycin concentrations of 2 μg/ml. In light of this result, the putative efflux pump (BotT) encoded in the bottromycin biosynthetic gene cluster represented a promising target for directed engineering approaches as we speculated it to play a crucial role for self-resistance. To overexpress the *botT* gene, its native promoter was replaced against the strong *PermE** promoter (Bibb et al., 1994) by insertion of a spectinomycin resistance cassette via Red/ET recombineering (see Figure 2A). *S. coelicolor* A3(2) wild-type was subsequently transformed with the resulting construct DG2-kan-efflux-ermE, and bottromycin production was quantified in comparison to *S. coelicolor*::DG2(-kan) and the corresponding "rif mutants." The HPLC-MS analysis revealed that, once more, bottromycin production could be increased: the generated *S. coelicolor*::DG2-kan-efflux-ermE expression host shows a 20 times higher production titer compared to *S. coelicolor*::DG2(-kan) and an around two times higher production titer compared to *S. coelicolor*::DG2(-kan)-rif. The generated data on the successful enhancement of heterologous bottromycin production for future experiments suggest combining both strategies: the targeted approach (*PermE** promoter insertion) with the undirected approach ("rifampicin mutagenesis"). This could potentially lead to a further increase of bottromycin production yields.

Functional Studies on the Bottromycin Methylation Pattern: A Suite of Hyperactive Methylases

A remarkable characteristic of the bottromycin scaffold is its versatile methylation pattern, which can be attributed to a set

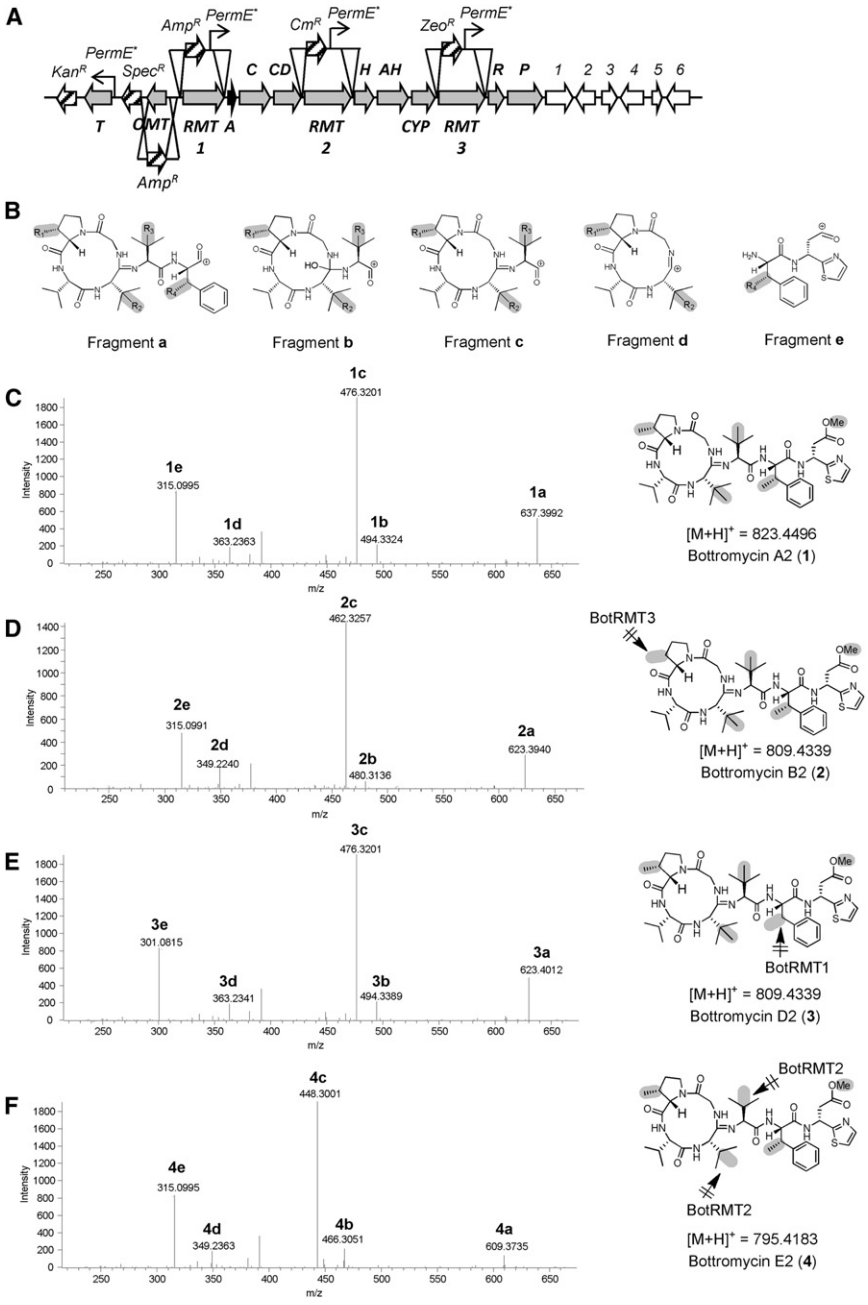

Figure 3. Engineering and Characterization of the Bottromycin "Methylation Machinery"

(A) Modification of the bottromycin biosynthetic gene cluster. The four methyl transferase encoding genes *botRMT1*, *botRMT2*, *botRMT3*, and *botOMT* were deleted from the expression construct DG2-kan-efflux-ermE in four orthogonal Red/ET recombineering steps by insertion of appropriate selection marker cassettes.

(B) Assigned fragment ions used for the comparison of the different bottromycin scaffolds detected in (C)–(F). R_1, R_2, R_3, and R_4 = H or Me.

(C)–(F) High-resolution MS/MS fragmentation analysis of bottromycin A2 reference substance (C) and of bottromycin scaffolds detected in extracts from the *S. coelicolor* A3(2) host strain expressing modified versions of the bottromycin biosynthetic gene cluster: *botRMT3* deletion (D), *botRMT1* deletion (E), and *botRMT2* deletion (F). For the fragment spectrum from the *botOMT* deletion experiment and a detailed comparison of calculated and detected high-resolution masses see Table S2 and Figure S1.

of four putative MTs encoded in the *bot* gene cluster. Based on the in silico analysis, it was quite obvious that BotOMT might catalyze *O*-methylation of the aspartate residue, whereas the action sites of the three radical SAMs (BotRMT1–BotRMT3) could not be assigned (*C*-methylation at C-7, C-19, C-26, and/or C-33, see Figure 1). The established heterologous expression system provided an excellent opportunity to accomplish functional studies on the four MTs by performing targeted gene deletion experiments, followed by the heterologous expression of the modified pathways to elucidate the resulting bottromycin methylation pattern. In order to achieve this goal, four orthogonal gene deletions were performed on the expression construct DG2-kan-efflux-ermE using the Red/ET recombineering technology (Zhang et al., 1998, 2000). The respective MT-encoding genes were replaced by an antibiotic resistance gene (*amp*^{R}, *cm*^{R}, or *zeo*^{R}), and in case of *botRMT1–botRMT3* the *PermE** promoter was simultaneously introduced in front of the downstream genes to ensure proper transcription (see Figure 3A). After *S. coelicolor* A3(2) was transformed with the modified constructs, the heterologous production was studied by intensive HPLC-hrMS/MS analysis on the culture extracts to elucidate the methylation pattern of the produced bottromycin derivatives. By comparing the fragmentation pattern of the detected bottromycin scaffolds, the function of all four MTs could be unambiguously assigned (see Figure 3, Figure S1 [available online], and Table S2). Three bottromycin derivatives can be usually detected in extracts of the native bottromycin producer, which differ in the methylation pattern of the proline residue: the monomethylated main product bottromycin A2 (**1**) plus unmethylated (bottromycin B2 (**2**)) and bimethylated (bottromycin C2) minor components (Nakamura et al., 1967). Deletion of *botRMT3* resulted in the (exclusive) production of a bottromycin scaffold exhibiting a molecular mass as well as a fragmentation pattern identical to authentic bottromycin B2 (**2**) reference substance. Consequently, we can draw the conclusion that *botRMT3* catalyzes radical *C*-methylation of the proline residue. After deleting *botRMT1*, again a bottromycin derivative with a molecular mass identical to bottromycin B2 could be detected, but numerous differences in the fragmentation pattern are obvious. The mass of all ''phenylalanine-containing'' fragments (**3a** and **3e**) is reduced by 14 Dalton (Da) in comparison to the

corresponding fragments of bottromycin A2 and B2 (**1a** and **1e/2e**, **2a** is also reduced by 14 Da as it contains the nonmethylated proline residue). On the other hand, "non-phenylalanine-containing" fragments (**3b–3d**) show the same mass as corresponding fragments from bottromycin A2 (**1b–1d**), strongly indicating that the three other relevant positions (proline and two valine residues) are still methylated. Based on this, BotRMT1 was assigned to catalyze radical *C*-methylation of the phenylalanine residue. The deletion of the third radical SAM-encoding gene (*botRMT2*) resulted in the production of a bottromycin scaffold with a molecular mass 28 Da less than that of bottromycin A2 (**1**) and 14 Da less than that of bottromycin B2 (**2**), indicating the presence of two unmethylated positions. The mass of fragment **4e** verifies the presence of the methyl group at the phenylalanine residue, whereas fragments **4b–4d** indicate the "loss" of methyl groups at two valine sites. This suggests that BotRMT2 is involved in radical *C*-methylation of two of the three valine residues and therefore acts twice during bottromycin biosynthesis. A similar analysis was carried out for the *botOMT* deletion experiment (not shown in Figure 3) and provided experimental evidence for the assigned function: *O*-methylation of the aspartate residue. The produced "free acid" bottromycin scaffold cannot be detected in strains expressing the complete pathway, and the same holds true for compounds **3** and **4** identified after *botRMT1* and *botRMT2* deletion. Thus, in addition to the findings on the functional role of the bottromycin MTs, these experiments also led to the production of three, to our knowledge, novel bottromycin derivatives. It will be very interesting to analyze the bioactivity of the modified scaffolds, which is currently impeded by the very low production yields that also prevented us from characterizing all structures via NMR experiments. The reason(s) for the heavily reduced production yields in the deletion experiments currently remain elusive. Reduced conversion rates of desmethyl precursors by subsequent biosynthetic enzymes or breakdown of the immunity system in the deletion mutants are two of numerous possible explanations. As no cross-complementation reactions could be observed in the mutants analyzed, we conclude that the three radical SAMs act very specifically when crafting the bottromycin scaffold.

SIGNIFICANCE

Bottromycins represent an intriguing family of natural products exhibiting promising activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci*. As protein synthesis inhibitors, they are regarded as attractive lead compounds for the development of novel antibiotics because bottromycins target the unique aminoacyl-*t*RNA binding site of the 50S ribosomal subunit. Here, we discovered the bottromycin biosynthetic pathway from *Streptomyces* sp. BC16019, providing evidence that the heavily modified peptide scaffold is of ribosomal origin. Quite unusually, the bottromycin precursor peptide lacks an N-terminal leader sequence, which is regarded as a common structural feature of ribosomally made natural product peptide precursors. Our data also highlight the bottromycin biosynthetic pathway as an outstanding example for a variety of posttranslational modification reactions, which in general are typical for ribosomal peptide biosyntheses. Maturation of the bottromycin precursor peptide includes macrocyclization via a unique amidine-forming "macrocyclodehydration," a second cyclodehydration most likely coupled to oxidative elimination building a thiazole ring, proteolytic cleavage, and various unusual methylation reactions. By use of synthetic biotechnology approaches, we functionally elucidated the methylation steps involving the action of an *O*-methyl transferase and three radical *S*-adenosylmethionine (SAM)-dependent methyl transferases required for alkylation of nonactivated carbons. The latter enzyme class is currently not well characterized and thus of special interest. These experiments also enabled the production of three bottromycin derivatives based on a heterologous expression system. Initial low yields were addressed by application of targeted and undirected approaches for production optimization. These resulted in significant increases of bottromycin production titers in the heterologous host now providing an important basis for advanced structure and yield improvements. In a broader sense, the discovery and characterization of the ribosomal bottromycin pathway revealed exciting insights into the biosynthesis of ribosomally made peptides—a biotechnologically hardly exploited class of natural products of increasing importance for drug discovery.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions

Streptomyces sp. BC16019, originally obtained from a soil sample collected in Germany (Lerchen et al., 2006), is maintained in the culture collection of InterMed Discovery GmbH, Dortmund, Germany, under liquid nitrogen. For the present study, it was revived and cultivated at 30°C and 180 rpm in liquid yeast and malt extract with glucose (YMG) medium (Stadler et al., 2007). *Streptomyces coelicolor* A3(2) (Bentley et al., 2002) and *Streptomyces albus* J1074 (Chater and Wilde, 1976) were grown at 30°C and 180 rpm in liquid Tryptone soya broth (TSB) medium (Kieser et al., 2000), and their mutants were cultivated in liquid TSB medium containing 60 μg/ml apramycin at 30°C and 180 rpm. *S.* sp. BC16019, *S. coelicolor* A3(2), and *Streptomyces albus* J1074 were grown on solid MS agar plates (Kieser et al., 2000). In addition to *Escherichia coli* HS996 (Invitrogen, Carlsbad, CA, USA) used as general host for molecular biology experiments, *E. coli* GB05-red (Fu et al., 2012) was employed in Red/ET recombineering experiments (Zhang et al., 2000), and *E. coli* ET12567/pUZ8002 (Kieser et al., 2000) was used for conjugation experiments. All *E. coli* strains were grown in Luria Broth (LB) medium at 37°C. If necessary, the medium was supplemented with the following antibiotics for screening appropriate mutants: chloramphenicol 30 μg/ml, spectinomycin 100 μg/ml, ampicillin 100 μg/ml, zeocin 25 μg/ml, kanamycin sulfate 50 μg/ml, and apramycin 60 μg/ml.

Sequencing and In Silico Analysis of the *Streptomyces* sp. BC16019 Genome

Total genomic DNA of *S.* sp. BC16019 was isolated in accordance with the Kirby mix procedure as described previously (Kieser et al., 2000) and sequenced by applying the 454 technology (Rothberg and Leamon, 2008), including 2 kb paired-end reads. The resulting sequence data (2,228 contigs and 319 scaffolds) were subsequently screened for putative open reading frames (*orfs*) encoding peptides that contain the amino acid sequence of the bottromycin scaffold (GPVVVFDC). For this, the EMBOSS "getorf" tool was applied to identify sequences of ORFs (defined as region between two stop codons), in accordance with the standard amino acid code. The output sequence list of all possible, translated *orfs* was subsequently screened for the GPVVVFDC-sequence using standard text editor software. A small *orf* (later named *botA*) was identified, encoding this sequence plus some C-terminal extension (see Figure 1).

Sequence Analysis of the Bottromycin Biosynthetic Gene Cluster

Based on the data from the library screening (see Supplemental Experimental Procedures), cosmid DG2 was chosen for further analysis, including whole cosmid shotgun sequencing as described previously (Silakowski et al., 1999). The obtained sequence of the ~41 kb chromosomal fragment (insert of cosmid DG2) was subsequently analyzed using bioinformatic tools. Prediction of open reading frames was performed with FramePlot 4.0 (http://nocardia.nih.go.jp/fp4/), and functional annotation was based on BlastP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Pfam (http://pfam.sanger.ac.uk/search) searches. The Geneious 5.6.2 software packages (Biomatters, New Zealand) was used for the analysis and annotation of DNA and protein sequences. In addition to the putative ~18 kb bottromycin biosynthetic gene cluster (*bot* genes; see Figure 2 and Table 1) a number of flanking *orfs* (*orf1*– *orf20*; see Figure 2 and Table S1) were identified.

ACCESSION NUMBERS

The GenBank accession number for the bottromycin biosynthetic gene cluster sequence from *Streptomyces* sp. BC16019 reported in this paper is JX235926.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables, one figure, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2012.08.013.

ACKNOWLEDGMENTS

The authors would like to thank Eva Luxenburger and Thomas Hoffmann (Saarland University) for skillful help with various analytical technologies, Andrea Rademacher (InterMed Discovery GmbH) for expert technical assistance, and Jens Bitzer for reference compounds. This work was generously supported by a grant from the Bundesministerium für Bildung und Forschung (FKZ 0315385B) to Saarland University and InterMed Discovery GmbH.

Received: May 25, 2012
Revised: August 3, 2012
Accepted: August 9, 2012
Published online: September 27, 2012

REFERENCES

Atta, M., Mulliez, E., Arragain, S., Forouhar, F., Hunt, J.F., and Fontecave, M. (2010). S-Adenosylmethionine-dependent radical-based modification of biological macromolecules. Curr. Opin. Struct. Biol. *20*, 684–692.

Bentley, S.D., Chater, K.F., Cerdeño-Tárraga, A.M., Challis, G.L., Thomson, N.R., James, K.D., Harris, D.E., Quail, M.A., Kieser, H., Harper, D., et al. (2002). Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature *417*, 141–147.

Bibb, M.J., White, J., Ward, J.M., and Janssen, G.R. (1994). The mRNA for the 23S rRNA methylase encoded by the ermE gene of *Saccharopolyspora erythraea* is translated in the absence of a conventional ribosome-binding site. Mol. Microbiol. *14*, 533–545.

Bierman, M., Logan, R., O'Brien, K., Seno, E.T., Rao, R.N., and Schoner, B.E. (1992). Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. Gene *116*, 43–49.

Castellanos, L., Duque, C., Zea, S., Espada, A., Rodríguez, J., and Jiménez, C. (2006). Isolation and synthesis of (-)-(5S)-2-imino-1-methylpyrrolidine-5-carboxylic acid from *Cliona tenuis*: structure revision of pyrostatins. Org. Lett. *8*, 4967–4970.

Chater, K.F., and Wilde, L.C. (1976). Restriction of a bacteriophage of *Streptomyces albus* G involving *endonuclease SalI*. J. Bacteriol. *128*, 644–650.

Cheng, Y.Q., Tang, G.L., and Shen, B. (2002). Identification and localization of the gene cluster encoding biosynthesis of the antitumor macrolactam leinamycin in *Streptomyces atroolivaceus* S-140. J. Bacteriol. *184*, 7013–7024.

Dunbar, K.L., Melby, J.O., and Mitchell, D.A. (2012). YcaO domains use ATP to activate amide backbones during peptide cyclodehydrations. Nat. Chem. Biol. *8*, 569–575.

Finking, R., and Marahiel, M.A. (2004). Biosynthesis of nonribosomal peptides. Annu. Rev. Microbiol. *58*, 453–488.

Fischbach, M.A., and Walsh, C.T. (2009). Antibiotics for emerging pathogens. Science *325*, 1089–1093.

Fischbach, M., and Voigt, C.A. (2010). Prokaryotic gene clusters: a rich toolbox for synthetic biology. Biotechnol. J. *5*, 1277–1296.

Frey, P.A., Hegeman, A.D., and Ruzicka, F.J. (2008). The Radical SAM Superfamily. Crit. Rev. Biochem. Mol. Biol. *43*, 63–88.

Fu, J., Bian, X., Hu, S., Wang, H., Huang, F., Seibert, P.M., Plaza, A., Xia, L., Müller, R., Stewart, A.F., and Zhang, Y. (2012). Full-length RecE enhances linear-linear homologous recombination and facilitates direct cloning for bioprospecting. Nat. Biotechnol. *30*, 440–446.

Gouda, H., Kobayashi, Y., Yamada, T., Ideguchi, T., Sugawara, A., Hirose, T., Omura, S., Sunazuka, T., and Hirono, S. (2012). Three-dimensional solution structure of bottromycin A2: a potent antibiotic active against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci*. Chem. Pharm. Bull. (Tokyo) *60*, 169–171.

Herrera-Camacho, I., Rosas-Murrieta, N.H., Rojo-Domínguez, A., Millán, L., Reyes-Leyva, J., Santos-López, G., and Suárez-Rendueles, P. (2007). Biochemical characterization and structural prediction of a novel cytosolic leucyl aminopeptidase of the M17 family from *Schizosaccharomyces pombe*. FEBS J. *274*, 6228–6240.

Hu, H., and Ochi, K. (2001). Novel approach for improving the productivity of antibiotic-producing strains by inducing combined resistant mutations. Appl. Environ. Microbiol. *67*, 1885–1892.

Hu, H., Zhang, Q., and Ochi, K. (2002). Activation of antibiotic biosynthesis by specified mutations in the rpoB gene (encoding the RNA polymerase beta subunit) of Streptomyces lividans. J. Bacteriol. *184*, 3984–3991.

Inaoka, T., Takahashi, K., Yada, H., Yoshida, M., and Ochi, K. (2004). RNA polymerase mutation activates the production of a dormant antibiotic 3,3′-neotrehalosadiamine via an autoinduction mechanism in Bacillus subtilis. J. Biol. Chem. *279*, 3885–3892.

Inbar, L., and Lapidot, A. (1988). The structure and biosynthesis of new tetrahydropyrimidine derivatives in actinomycin D producer Streptomyces parvulus. Use of 13C- and 15N-labeled L-glutamate and 13C and 15N NMR spectroscopy. J. Biol. Chem. *263*, 16014–16022.

Kieser, T., Bibb, M., Buttner, M.J., Chater, K.F., and Hopwood, D.A. (2000). Practical Streptomyces Genetics. 613 (Norwich, England: The John Innes Foundation).

Lee, S.W., Mitchell, D.A., Markley, A.L., Hensler, M.E., Gonzalez, D., Wohlrab, A., Dorrestein, P.C., Nizet, V., and Dixon, J.E. (2008). Discovery of a widely distributed toxin biosynthetic gene cluster. Proc. Natl. Acad. Sci. USA *105*, 5879–5884. Published online 2008.

Lerchen, H.-G., Schiffer, G., Brötz-Österheld, H., Mayer-Bartschmid, A., Eckermann, S., Freiberg, C., Endermann, R., Schuhmann, J., Meier, H., Svenstrup, N., et al. (2006). Cyclic iminopeptide derivatives (Germany) [WO 2006/103010].

Li, J.W.H., and Vederas, J.C. (2009). Drug discovery and natural products: end of an era or an endless frontier? Science *325*, 161–165.

Li, Y.M., Milne, J.C., Madison, L.L., Kolter, R., and Walsh, C.T. (1996). From peptide precursors to oxazole and thiazole-containing peptide antibiotics: microcin B17 synthase. Science *274*, 1188–1193.

Matsui, M., Fowler, J.H., and Walling, L.L. (2006). Leucine aminopeptidases: diversity in structure and function. Biol. Chem. *387*, 1535–1544.

McIntosh, J.A., Donia, M.S., and Schmidt, E.W. (2009). Ribosomal peptide natural products: bridging the ribosomal and nonribosomal worlds. Nat. Prod. Rep. *26*, 537–559.

Melby, J.O., Nard, N.J., and Mitchell, D.A. (2011). Thiazole/oxazole-modified microcins: complex natural products from ribosomal templates. Curr. Opin. Chem. Biol. *15*, 369–378.

 ©2012 Elsevier Ltd All rights reserved

Nakamura, H., Koyama, G., Iitaka, Y., Ono, M., and Yagiawa, N. (1974). Structure of coformycin, an unusual nucleoside of microbial origin. J. Am. Chem. Soc. *96*, 4327–4328.

Nakamura, S., Chikaike, T., Yonehara, H., and Umezawa, H. (1965a). Structures of bottromycin A and B. J. Antibiot. (Tokyo) *18*, 60–61.

Nakamura, S., Chikaike, T., Karasawa, K., Tanaka, N., Yonehara, H., and Umezawa, H. (1965b). Isolation and characterisation of bottromycin A and B. J. Antibiot. (Tokyo) *18*, 47–52.

Nakamura, S., Chikaike, T., Yonehara, H., and Umezawa, H. (1965c). Isolation, characterization and structural elucidation of new amino acids from bottromycin A. Chem. Pharm. Bull. (Tokyo) *13*, 599–602.

Nakamura, S., Tanaka, N., and Umezawa, H. (1966). Bottromycin A1, A2 and their structures. J. Antibiot. (Tokyo) *19*, 10–12.

Nakamura, S., Yajima, T., Lin, Y.C., and Umezawa, H. (1967). Isolation and characterization of bottromycins A2, B2, C2. J. Antibiot. (Tokyo) *20*, 1–5.

Newman, D.J., and Cragg, G.M. (2012). Natural products as sources of new drugs over the 30 years from 1981 to 2010. J. Nat. Prod. *75*, 311–335.

Nolan, E.M., and Walsh, C.T. (2009). How nature morphs peptide scaffolds into antibiotics. ChemBioChem *10*, 34–53.

Oman, T.J., and van der Donk, W.A. (2010). Follow the leader: the use of leader peptides to guide natural product biosynthesis. Nat. Chem. Biol. *6*, 9–18.

Otaka, T., and Kaji, A. (1976). Mode of action of bottromycin A2. Release of aminoacyl- or peptidyl-tRNA from ribosomes. J. Biol. Chem. *251*, 2299–2306.

Otaka, T., and Kaji, A. (1981). Mode of action of bottromycin A2: effect on peptide bond formation. FEBS Lett. *123*, 173–176.

Otaka, T., and Kaji, A. (1983). Mode of action of bottromycin A2: effect of bottromycin A2 on polysomes. FEBS Lett. *153*, 53–59.

Pastor, J.M., Salvador, M., Argandoña, M., Bernal, V., Reina-Bueno, M., Csonka, L.N., Iborra, J.L., Vargas, C., Nieto, J.J., and Canovas, M. (2010). Ectoines in cell stress protection: uses and biotechnological production. Biotechnol. Adv. *28*, 782–801.

Rothberg, J.M., and Leamon, J.H. (2008). The development and impact of 454 sequencing. Nat. Biotechnol. *26*, 1117–1124.

Roy, R.S., Gehring, A.M., Milne, J.C., Belshaw, P.J., and Walsh, C.T. (1999). Thiazole and oxazole peptides: biosynthesis and molecular machinery. Nat. Prod. Rep. *16*, 249–263.

Schipper, D. (1983). The revised structure of bottromycin A_2. J. Antibiot. (Tokyo) *36*, 1076–1077.

Schwarzer, D., Finking, R., and Marahiel, M.A. (2003). Nonribosomal peptides: from genes to products. Nat. Prod. Rep. *20*, 275–287.

Shima, J., Hesketh, A., Okamoto, S., Kawamoto, S., and Ochi, K. (1996). Induction of actinorhodin production by rpsL (encoding ribosomal protein S12) mutations that confer streptomycin resistance in *Streptomyces lividans* and *Streptomyces coelicolor* A3(2). J. Bacteriol. *178*, 7276–7284.

Shimamura, H., Gouda, H., Nagai, K., Hirose, T., Ichioka, M., Furuya, Y., Kobayashi, Y., Hirono, S., Sunazuka, T., and Omura, S. (2009). Structure determination and total synthesis of bottromycin A_2: a potent antibiotic against MRSA and VRE. Angew. Chem. Int. Ed. Engl. *48*, 914–917.

Silakowski, B., Schairer, H.U., Ehret, H., Kunze, B., Weinig, S., Nordsiek, G., Brandt, P., Blöcker, H., Höfle, G., Beyer, S., and Müller, R. (1999). New lessons for combinatorial biosynthesis from myxobacteria. The myxothiazol biosynthetic gene cluster of *Stigmatella aurantiaca* DW4/3-1. J. Biol. Chem. *274*, 37391–37399.

Sit, C.S., Yoganathan, S., and Vederas, J.C. (2011). Biosynthesis of aminovinyl-cysteine-containing peptides and its application in the production of potential drug candidates. Acc. Chem. Res. *44*, 261–268.

Stadler, M., Bitzer, J., Mayer-Bartschmid, A., Müller, H., Benet-Buchholz, J., Gantner, F., Tichy, H.V., Reinemer, P., and Bacon, K.B. (2007). Cinnabaramides A-G: analogues of lactacystin and salinosporamide from a terrestrial streptomycete. J. Nat. Prod. *70*, 246–252.

Takahashi, Y., Naganawa, H., Takita, T., Umezawa, H., and Nakamura, S. (1976). The revised structure of bottromycin A_2. J. Antibiot. (Tokyo) *29*, 1120–1123.

Tamehiro, N., Hosaka, T., Xu, J., Hu, H.F., Otake, N., and Ochi, K. (2003). Innovative approach for improvement of an antibiotic-overproducing industrial strain of *Streptomyces albus*. Appl. Environ. Microbiol. *69*, 6412–6417.

Tanaka, N., Nishimura, T., Nakamura, S., and Umezawa, H. (1968). Activity of bottromycin against Mycoplasma gallisepticum. J. Antibiot. (Tokyo) *21*, 75–76.

Waisvisz, J.M., and van der Hoeven, M.G. (1958). The chemistry and partial structure of bottromycin. IV. J. Am. Chem. Soc. *80*, 383–385.

Waisvisz, J.M., van der Hoeven, M.G., and te Nijnhius, B. (1957a). The structure of the sulfur-containing moiety of bottromycin. J. Am. Chem. Soc. *79*, 4524–4527.

Waisvisz, J.M., van der Hoeven, M.G., Hölscher, J.F., and te Nijnhius, B. (1957b). Bottromycin. II. Preliminary degradation studies. J. Am. Chem. Soc. *79*, 4522–4524.

Waisvisz, J.M., van der Hoeven, M.G., van Peppen, J., and Zwennis, W.C.M. (1957c). Bottromycin. I. A new sulfur-containing antibiotic. J. Am. Chem. Soc. *79*, 4520–4521.

Walsh, C.T., and Nolan, E.M. (2008). Morphing peptide backbones into heterocycles. Proc. Natl. Acad. Sci. USA *105*, 5655–5656.

Walsh, C.T., Chen, H.W., Keating, T.A., Hubbard, B.K., Losey, H.C., Luo, L.S., Marshall, C.G., Miller, D.A., and Patel, H.M. (2001). Tailoring enzymes that modify nonribosomal peptides during and after chain elongation on NRPS assembly lines. Curr. Opin. Chem. Biol. *5*, 525–534.

Zhang, Y., Buchholz, F., Muyrers, J.P., and Stewart, A.F. (1998). A new logic for DNA engineering using recombination in *Escherichia coli*. Nat. Genet. *20*, 123–128.

Zhang, Y., Muyrers, J.P.P., Testa, G., and Stewart, A.F. (2000). DNA cloning by homologous recombination in *Escherichia coli*. Nat. Biotechnol. *18*, 1314–1317.

Zhang, Q., van der Donk, W.A., and Liu, W. (2012). Radical-mediated enzymatic methylation: a tale of two SAMS. Acc. Chem. Res. *45*, 555–564.