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Synthetic Biotechnology to Study and Engineer Ribosomal Bottromycin Biosynthesis

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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

