Minimal lactazole scaffold for in vitro thiopeptide bioengineering

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Lactazole A is a natural product belonging to the thiopeptide group of ribosomally synthesized and post-translationally modified peptides (RiPPs). Thiopeptides are natural products with a lot of therapeutic potential, as they usually possess strong antibiotic activity against Gram-positive bacteria, including methicillin resistant *Staphyrococcus aureus* strains. Lactazole A is biosynthesized from a minimal 9.8 kb biosynthetic gene cluster (BGC), which encodes five enzymes solely responsible for its biosynthesis. The biosynthesis is initiated with ribosomal production of LazA precursor peptide encoded inside laz BGC. The biosynthetic enzymes then utilize the N-terminal 38 residue-long leader peptide of LazA as a recognition sequence to introduce post-translational modifications such as azole and dehydroalanine to the C-terminal core peptide sequence of LazA. Eventually, a pyridine synthase LazC catalyzes formation of a pyridine ring and eliminates the leader peptide, yielding the macrocyclic thiopeptide.

Lactazole is unique in many aspects. It has a 32-membered macrocycle, a low Cys/Ser/Thr content, and it bears an unmodified amino acid in position 2. All of these features are unusual among thiopeptides. Recent bioinformatic studies indicate that the lactazole-like thiopeptides comprise close to half of all predicted thiopeptides (251 out of 508 annotated BGCs) and can be further subdivided into multiple subfamilies, and yet the prototypical laz BGC remains the only characterized member of this family to date. Overall, lactazole-like thiopeptides remain a rather enigmatic family of natural products, as close to nothing is known about their function, structural diversity, and biosynthesis.

Here, we report the construction of a platform for *in vitro* biosynthesis of lactazole A, referred to as the FIT Laz system, via a combination of the flexible in vitro

translation (FIT) system with recombinantly produced lactazole biosynthetic enzymes. Using FIT-Laz as a prototyping tool to study lactazole biosynthesis, we systematically analyzed substrate tolerance of lactazole biosynthetic machinery, and found that the enzymes can accept an unusually broad range of substrates, which stands in stark contrast to thiopeptide BGCs studied to date. These studies led to the development of the “minimal lactazole scaffold”, a construct requiring only 6 post-translational modifications for macrocyclization. We found that the minimal lactazole scaffold can be used to synthesize a number of first-of-its-kind constructs: thiopeptides with the smallest and largest known macrocycles, the largest, 34 amino-long, lactazole variant, as well as pseudo-natural thiopeptides containing up to 4 non-proteinogenic amino acids. Ultimately, we demonstrated that Laz enzymes can accommodate randomization of up to 10 consecutive amino acids inside the primary macrocycle, suggesting that the minimal lactazole scaffold is an excellent candidate for bioengineering, and may be used to discover artificial thiopeptides with de novo designed biological activities for drug lead development efforts. Some preliminary work toward this goal, such as integration of the FIT-Laz system with a powerful in vitro screening platform, mRNA display, is also discussed.