An Engineered Lantibiotic Synthetase That Does Not Require a Leader Peptide on Its Substrate

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Supporting Information

ABSTRACT: Ribosomally synthesized and post-translationally modified peptides are a rapidly expanding class of natural products. They are typically biosynthesized by modification of a C-terminal segment of the precursor peptide (the core peptide). The precursor peptide also contains an N-terminal leader peptide that is required to guide the biosynthetic enzymes. For bioengineering purposes, the leader peptide is beneficial because it allows promiscuous activity of the biosynthetic enzymes with respect to modification of the core peptide sequence. However, the leader peptide also presents drawbacks as it needs to be present on the core peptide and then removed in a later step. We show that fusing the leader peptide for the lantibiotic lacticin 481 to its biosynthetic enzyme LctM allows the protein to act on core peptides without a leader peptide. We illustrate the use of this methodology for preparation of improved lacticin 481 analogues containing non-proteinogenic amino acids.

Cyclic peptides are attracting increased attention for their potential pharmaceutical applications. They are metabolically more stable than linear peptides and are promising candidates for disruption of protein–protein interactions. Natural product cyclic peptides are generated by both non-ribosomal and ribosomal pathways. The molecules produced by the latter route have rapidly expanded in both number and structural diversity in recent years as a consequence of the explosion in genomic sequence information. These pathways, in which a linear precursor peptide is generated ribosomally and subsequently post-translationally modified, provide many attractive opportunities for bioengineering. First, the amino acid sequence is genetically encoded, allowing site-directed mutagenesis approaches to access analogues. Second, the pathways toward these compounds usually involve a relatively small number of biosynthetic enzymes, which often act iteratively. In turn, such short pathways are more amenable to bioengineering approaches. Third, the biosynthetic enzymes are often highly promiscuous, achieved in part by a biosynthetic strategy in which the enzymes recognize an N-terminal leader peptide that activates them toward catalyzing post-translational modifications of the C-terminal core peptide that will become the natural product (e.g., Figure 1).

Because the biosynthetic enzymes for these ribosomally synthesized cyclic peptides typically recognize the leader peptide, the sequence of the core peptide is often hyper-variable. The post-translational modifications release these natural products from the structural and functional constraints imposed on natural ribosomal peptides, while at the same time restricting conformational flexibility to allow better target recognition and increased metabolic and chemical stability. An example of such a system is shown in Figure 1 for lacticin 481, a member of the lantibiotic group of polycyclic peptides. A single, multifunctional enzyme LctM repeatedly dehydrates Ser and Thr residues in the core peptide to generate the dehydroamino acids dehydroalanine (Dha) and dehydrobutyryl (Dbh), respectively. The enzyme subsequently catalyzes conjugate additions of Cys residues to the αβ-unsaturated structures.

The attractive attributes of ribosomally synthesized cyclic peptides have not gone unnoticed, and a large number of studies have focused on using site-directed mutagenesis to improve their properties. Recently, these studies were extended to substitutions with non-proteinogenic amino acids in Escherichia coli. In addition, an increasing number of these biosynthetic pathways has been reconstituted in vitro, which has enabled the combination of promiscuous biosynthetic enzymes with the power of peptide synthesis to generate molecular diversity. A major current limitation of the latter approach is that the leader peptide needs to be attached to synthetic core peptides, which has been
achieved by various orthogonal ligation strategies.\textsuperscript{31–33} After post-translational modification, the leader needs to be removed, typically with a commercial protease\textsuperscript{34,35,36} or using photochemically labile linkers.\textsuperscript{36} The overall process is inefficient and does not render this strategy readily amenable for use with combinatorial peptide libraries.

In an attempt to overcome the disadvantages of leader peptide attachment and subsequent removal, we explored whether addition of the leader and core peptides in \textit{trans} would result in efficient catalysis. In previous work, this approach was only partially successful for lacticin 481, resulting in incompletely processed core peptides when the leader and core peptides were present in low concentrations.\textsuperscript{37} In this investigation, we increased the concentrations of synthetic leader peptide, which resulted in products that were three- or four-fold dehydrated as determined by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) (Figure 4). After 1 h, significant amounts of an intermediate with three dehydrations and one phosphorylation were still present, but this intermediate is mostly converted to four-fold dehydrated product by 180 min (Figure S4). Furthermore, HPLC analysis, CDAP assays, and bioactivity assays (vide infra) demonstrated that the three thioether rings were formed in the absence of the leader peptide, to predominantly active in its presence (Figure 3A).\textsuperscript{38} This model suggested that attachment of the leader peptide to LctM (Figure 3B) would result in a high effective molarity and more efficient catalysis than providing core and leader peptides in \textit{trans}, thus yielding a constitutively active fusion (ConFusion) enzyme. In addition, the leader peptide would not need to be synthesized independently as it would be obtained during enzyme expression in \textit{E. coli}. A related approach fused the NS3 protease/helicase involved in processing the hepatitis C virus polyprotein to a portion of its activator protein, resulting in a constitutively active protease.\textsuperscript{40}

To test the hypothesis, several heterologous expression constructs were generated that encoded a hexa-His-tagged LctA leader peptide fused to the N-terminus of the LctM synthetase via a (GlySer)\textsubscript{n} linker sequence (\(n = 1, 5, 10, \text{and } 15\); Figure S3; longer linkers decreased protein expression yields) to generate the LctM ConFusion enzyme (LctCE-GS\textsubscript{n}). The hybrid genes were subcloned into a pET28b vector and overexpressed in \textit{E. coli}, and the resulting fusion proteins were purified by immobilized metal affinity chromatography (IMAC). Incubation of the core peptide of LctA, obtained by solid-phase peptide synthesis, with LctCE-GSG, ATP, and Mg\textsuperscript{2+} resulted in dehydrated activity as demonstrated by MALDI-MS. The desired four-fold dehydrated core peptide was observed as a minor product along with intermediates that underwent one, two, and three dehydrations (Figure 4). Reactions utilizing LctCE-GS\textsubscript{5}, LctCE-GS\textsubscript{10} or LctCE-GS\textsubscript{15} resulted in progressively more efficient dehydration such that with LctCE-GS\textsubscript{15}, the four-fold dehydrated core peptide was the major product (Figure 4). After 1 h, significant amounts of an intermediate with three dehydrations and one phosphorylation were still present, but this intermediate is mostly converted to four-fold dehydrated product by 180 min (Figure S4). Furthermore, HPLC analysis, CDAP assays, and bioactivity assays (vide infra) demonstrated that the three thioether rings were formed in the four-fold dehydrated product, demonstrating LctCE-GS\textsubscript{15} to be constitutively active. The protein was obtained in 10 mg/L of \textit{E. coli} cell culture without any optimization of culturing and expression conditions.

Figure 2. Effect of leader peptide concentration on the \textit{in trans} processing of the LctA core peptide by LctM. Shown are MALDI-TOF mass spectra of the LctA core peptide after a 3 h incubation. The final concentrations of the core peptide and enzyme were 20 \(\mu\text{M}\) and 2 \(\mu\text{M}\), respectively. Numbers above the peaks indicate the number of dehydrations; an asterisk indicates a phosphorylated substrate; SM indicates starting material.

Figure 3. (A) The leader peptide is believed to trap an active conformation of the enzyme that is present as a minor fraction in the absence of the leader peptide. (B) Proposed model of a constitutively active lacticin 481 synthetase. The LctA leader peptide (red) is covalently fused to the synthetase via a flexible proteinogenic linker (purple). As with the model in panel A, it was envisioned that the leader peptide would shift the equilibrium population toward the active enzyme species, thereby enabling the modification of LctA core peptide (green).
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Figure 4. Effect of linker length on the processing of the core peptide by a series of lacticin 481 ConFusion enzymes. Shown are MALDI-TOF mass spectra of the LctA core peptide after 1 h of incubation. Indicated to the right of the spectra is the enzyme utilized for the reaction. The final concentrations of substrate and enzyme were 20 μM and 2 μM, respectively. Numbers above the peaks indicate the number of dehydrations. Asterisk indicates phosphorylated substrate; SM = starting material.

To evaluate the efficiency of LctCE-GS15, the modification process of the core peptide catalyzed by LctCE-GS15 and by wild-type LctM with a synthetic leader peptide provided in trans were evaluated using a combination of HPLC and MALDI-MS. The modification assays were carried out at a 10:1 substrate:enzyme ratio (final concentrations 20 μM substrate and 2 μM enzyme). In the assay with His₆-LctM, the LctA leader peptide was supplemented at the same concentration as the synthetase enzyme, thus mimicking the ConFusion enzyme where the leader and synthetase enzyme are present in stoichiometric equivalents. Under conditions in which the leader peptide was provided in trans, the fully modified lacticin 481 core peptide was a minor product in the LctM-catalyzed reaction after 3 h. In contrast, with the ConFusion enzyme LctCE-GS15 the four-fold dehydrated peptide was the major product (Figure S5). Importantly, after purification of this four-fold dehydrated peptide, the antimicrobial activity of the product was comparable to lacticin 481 isolated from the producer organism.

The observation that the LctCE-GS15 enzyme correctly breaks eight chemical bonds and generates six new chemical bonds in the core peptide without the need for a leader peptide on the substrate demonstrates that a covalent bond between the leader and core peptides is not required for ensuring the correct regiochemistry of cyclization. These results also suggest that the enzyme must have binding affinity for the core peptide. A similar conclusion was reached recently by the observation that pull-down of the dehydratase NisB and cyclase NisC from L. lactis has been achieved predominantly by mutagenesis of the precursor peptide to include commercial protease recognition sites. ²⁹,³⁵ Unfortunately, these mutations can have deleterious effects, including reduced heterologous expression levels, diminished or even abolished precursor peptide processing by the biosynthetic enzymes, and non-specific proteolytic cleavage after modification of the core peptide.

To test the generality of the ConFusion enzyme technology, several analogues of lacticin 481 were prepared. Wild-type lacticin 481 demonstrates sub-micromolar inhibitory activity (IC₅₀ = 750 nM) against *L. lactis* HP.³¹ Previous studies have suggested that substitution of Trp19 and/or Phe21 with non-proteinogenic amino acids improves the antimicrobial activity of lacticin 481.³¹ However, the proteolytic step necessary to remove the leader peptide in these studies resulted in lacticin 481 analogues missing the N-terminal lysine, which is important for activity.³¹ Because a proteolytic step is not required to produce lacticin 481 analogues using LctCE, the products would include this important residue. Four mutants of the LctA core peptide were prepared by Fmoc-based solid-phase peptide synthesis containing the following mutations: N15R/F21H, N15R/F21Pal, N15R/W19Nal/F21H, and N15R/W19Nal/F21Pal (Pal = 3-(4′-pyridyl)alanine, Nal = 3- (2-naphthyl)alanine; Figure S6). The four mutant core peptides were treated with purified His₆-LctCE-GS15 in the presence of Mg²⁺ and ATP. Fully modified product was generated in each reaction with the efficiency comparable to that observed with the wild-type core peptide. Partially processed intermediates were readily separated from the desired product via reversed-phase HPLC (Figure S7), and the fully modified material was analyzed for antimicrobial activity against *L. lactis* HP. The IC₅₀ for authentic lacticin 481 was 785 ± 19 nM, agreeing well with a previously reported value (750 nM).³² Interestingly, lacticin 481 N15R/F21Pal and lacticin 481 N15R/F21H displayed greater inhibitory activity compared to authentic lacticin 481 with IC₅₀ values of 213 ± 9 and 428 ± 21 nM, respectively. The triply substituted analogues were not as active as authentic lacticin 481 under the conditions tested (IC₅₀ values of 1370 ± 48 nM for N15R/F21H/W23Nal and 2420 ± 60 nM for N15R/F21Pal/W23Nal). Thus, improved analogues of lacticin 481 can be prepared using this strategy.

The ConFusion approach allows relatively rapid evaluation of synthetic peptides. When hits with improved antimicrobial activity are identified, follow-up studies can focus on generating these analogues in larger amounts by heterologous expression in *E. coli* using stop-codon suppression methodology ⁴² or auxotrophic strains. The successful application of such methodology was recently illustrated for the prochlorosins and lichenicidins, class II lanthionine containing peptides, ²¹,²³ and for the cyanobactins, another group of post-translationally modified peptide natural products. ⁵² Furthermore, in the past year, 11 different lantibiotics have been successfully produced in *E. coli* in several laboratories, ²¹,²³,²⁴−⁴⁵ illustrating the generality of this approach. However, lacticin 481 has not yet been produced in *E. coli*. We therefore constructed a pDUET co-expression vector containing LctM and His₆-LctA to test production of lacticin 481 in *E. coli* BL21 (DE3). After induction of expression with IPTG, harvesting of the cells, lysis, and IMAC purification, His₆-LctA was obtained that had been completely processed (four dehydrations and three cyclizations). Removal of the leader peptide with LysC resulted in the desired compound as shown by HPLC, MS, and bioactivity assays (Figure S8). LysC was used in this final step instead of...
the transmembrane protein LctT that removes the leader peptide in the producer strain. Although the activity of the protease domain of LctT has been reconstituted in vitro,\textsuperscript{46} it possesses very low efficiency.

In summary, this work illustrates a platform that can rapidly transform synthetic peptides into poly cyclic products to be evaluated for biological activity. Because of the modularity of peptide synthesis, this methodology enables SAR studies that can cover a much larger chemical space than conventional site-directed mutagenesis. Once hits are identified, the tools are available to achieve and optimize larger-scale production of the compounds in E. coli.

\section*{ASSOCIATED CONTENT}

\subsection*{Supporting Information}
Description of all molecular biology procedures, protein purifications, solid-phase peptide synthesis, and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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\subsection*{Notes}
The authors declare no competing financial interest.

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\section*{REFERENCES}

(1) Sieber, S. A.; Marahiel, M. A. Chem. Rev. 2005, 105, 715.
(2) Fischbach, M. A.; Walsh, C. T. Chem. Rev. 2006, 106, 3468.
(3) McIntosh, J. A.; Donia, M. S.; Schmidt, E. W. Nat. Prod. Rep. 2009, 26, 537.
(4) Velásquez, J. E.; van der Donk, W. A. Curr. Opin. Chem. Biol. 2011, 15, 11.
(5) Kersten, R. D.; Yang, Y.-L.; Xu, Y.; Cimermancic, P.; Nam, S.-J.; Fenical, W.; Fischbach, M. A.; Moore, B. S.; Dorrestein, P. C. Nat. Chem. Biol. 2011, 7, 794.
(6) Oman, T. J.; van der Donk, W. A. Nat. Chem. Biol. 2010, 6, 9.
(7) Donia, M. S.; Hathaway, B. J.; Sudak, S.; Haygood, M. G.; Rosovitz, M. J.; Ravel, J.; Schmidt, E. W. Nat. Chem. Biol. 2006, 2, 729.
(8) Li, B.; Sher, D.; Kelly, L.; Shi, Y.; Huang, K.; Knerr, P. J.; Joewono, I.; Rutsch, D.; Chisholm, S. W.; van der Donk, W. A. Proc. Natl. Acad. Sci. U.S.A. 2010, 107, 10430.
(9) Haft, D. H.; Basu, M. K.; Mitchell, D. A. BMC Biol. 2010, 8, 70.
(10) Hallen, H. E.; Luo, H.; Scott-Craig, J. S.; Walton, J. D. Proc. Natl. Acad. Sci. U.S.A. 2007, 104, 19097.
(11) Knerr, P. J.; van der Donk, W. A. Annu. Rev. Biochem. 2012, DOI: 10.1146/annurev-biochem-060110-113521.
(12) Xie, L.; Miller, L. M.; Chatterjee, C.; Averin, O.; Kelleher, N. L.; van der Donk, W. A. Science 2004, 303, 679.
(13) Lee, M. V.; Ilknen, L. A.; You, Y. O.; Mcclerren, A. L.; van der Donk, W. A.; Kelleher, N. L. J. Am. Chem. Soc. 2009, 131, 12258.
(14) Lubelski, J.; Khusainov, R.; Kuipers, O. P. J. Biol. Chem. 2009, 284, 25962.