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# Leader Peptide-Free In Vitro Reconstitution of Microviridin Biosynthesis Enables Design of Synthetic Protease-Targeted Libraries

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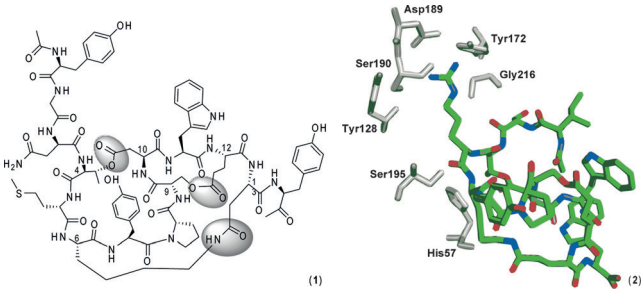
***Abstract:*** *Microviridins are a family of ribosomally synthesized and post-translationally modified peptides with a highly unusual architecture featuring non-canonical lactone as well as lactam rings. Individual variants specifically inhibit different types of serine proteases. Here we have established an efficient in vitro reconstitution approach based on two ATP-grasp ligases that were constitutively activated using covalently attached leader peptides and a GNAT-type N-acetyltransferase. The method facilitates the efficient in vitro one-pot transformation of microviridin core peptides to mature microviridins. The engineering potential of the chemo-enzymatic technology was demonstrated for two synthetic peptide libraries that were used to screen and optimize microviridin variants targeting the serine proteases trypsin and subtilisin. Successive analysis of intermediates revealed distinct structure–activity relationships for respective target proteases.*

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a large group of compounds that combine great chemical complexity at low genetic costs.[1] The increasing knowledge about mechanisms and the scope of peptide-modifying enzymes has provided an avenue for the rational and random design of peptide libraries through synthetic biology methodologies.[2] Microviridins (**1**; see Scheme 1) are one of the most fascinating families of RiPPs with potent activities against various serine proteases.[3] Their unprecedented cage-like architecture results from the activity of two ATP-grasp ligases that introduce non-canonical lactone and lactam rings.[4] Full maturation of microviridins additionally requires the activity of a GNAT-type *N*-acetyltransferase.[4a] In vitro reconstitution studies have revealed a strict order of cyclization reactions and a stringent ring size requirement for microviridin with the large lactone ring being formed first, followed by the smaller lactone ring and the lactam ring.[4c]




***Scheme 1.*** Left: Structure of microviridin K (**1**). The amide bond and the non-canonical ester bonds are highlighted in gray. Right: 3D structure of microviridin J (**2**) derived from the co-crystal structure with trypsin.[3] The interaction of the pivotal position 5 with active site residues of trypsin is indicated.

An increasing number of RiPPs was successfully reconstituted and diversified using chemo-enzymatic approaches that use chemically synthesized precursor peptides.[5] Expansion of the tool-kit for peptide modification has also enabled the design of "unnatural" peptide variants by modularizing different post-translational modification enzymes in vitro.[5] Microviridins feature a unique type of macrocyclization.[1,5] The full in vitro reconstitution of microviridins and thus their exploitation in chemo-enzymatic approaches was so far hampered by the long leader peptide that is essential for peptide maturation and the lack of an appropriate protease for leader peptide removal. Recent studies on lanthipeptide and cyanobactin biosynthetic enzymes have revealed that the functionality of the leader peptide does not necessarily require its direct linkage to the core peptide.[5a,6] Leader peptides were shown to successfully activate the corresponding modifying enzymes when added in *trans* or were alternatively attached to the N-terminus of modifying enzymes.[5a,6] These findings dramatically improve the possibilities for an efficient chemo-enzymatic production of RiPPs as the relatively short core peptides can be synthesized much easier and at reduced costs. Moreover, the fusion of leader peptides and modifying enzymes can potentially simplify further modularization of RiPPs. Koehnke et al. have provided structural insights into the allosteric activation of the heterocyclase LynD by a cyanobactin-type leader peptide.[6b] Common RiPP precursor peptide recognition elements (RREs) with homology to the pyrroloquinoline quinone biosynthesis enzyme PqqD were detected for LynD and a large number of modifying enzymes in diverse RiPP subfamilies.[7] In contrast, both ATP-grasp ligases involved in microviridin maturation do not contain a known RRE motif.[7] Unique features of the microviridin leader peptide

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such as a highly conserved hydrophobic sequence stretch at the N-terminus of the leader peptide further point towards a unique type of interaction between the microviridin leader peptide and its associated modifying enzymes.[8]

Here we report on the successful development of an in vitro reconstitution approach for microviridins that enables an efficient synthesis of tricyclic depsipeptides from short linear core peptides using three enzymes in a one-pot reaction. We have used our efficient chemo-enzymatic synthesis approach for the design of macrocyclic depsipeptide libraries and identified and optimized peptide variants targeted to the bacterial serine proteases subtilisin and trypsin.

The MvdE leader peptide (LP) of *P. agardhii* Cya126 was fused with the N-termini of the two ATP-grasp ligases MvdD and MvdC connected by a 30 amino acid linker (see Figure S1 in the Supporting Information). In a first experiment the resulting engineered enzyme variants LP-MvdD and LP-MvdC were successfully used to transform the linear 14 amino acid microviridin K core peptide (**2**) into a bicyclic variant containing the two characteristic lactone rings (**3**) and a tricyclic variant additionally comprising a lactam ring (**4**; Figures 1 and 2). Full maturation of microviridin K was achieved using the *N*-acetyl transferase MvdB (Figures 1 and 2) in the presence of acetyl-CoA as a co-substrate. The correct

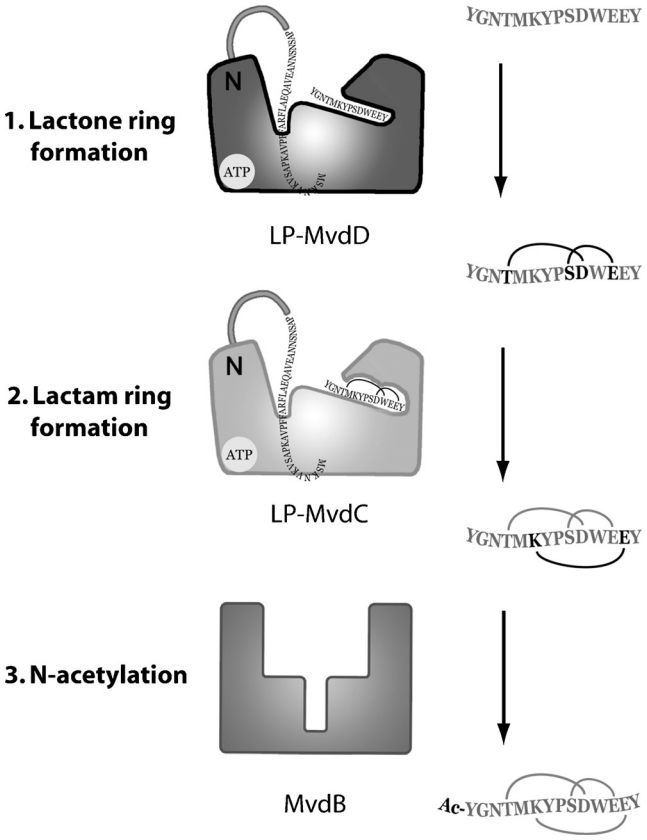


***Figure 1.*** Schematic representation of the in vitro synthesis of microviridin K. First the constitutively active ester ligase LP-MvdD and amide ligase LP-MvdC introduce the two lactone rings and the lactam ring, respectively. Afterwards the acetyltransferase acetylates the tricyclic core peptide at the *N*-terminus, rendering mature microviridin.

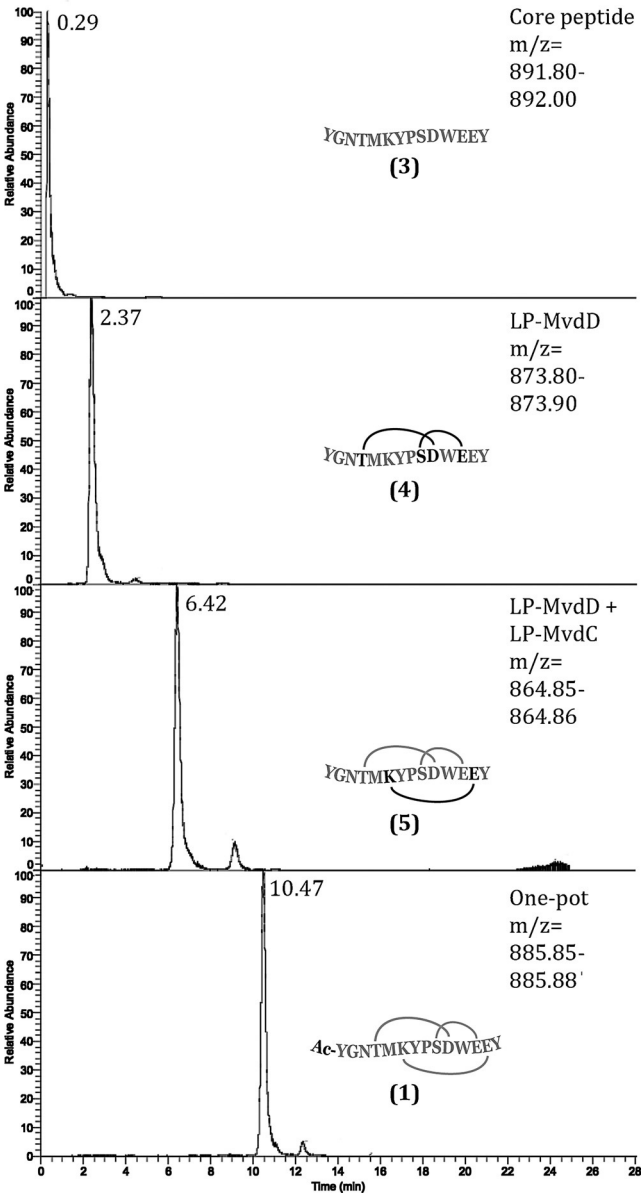


***Figure 2.*** Three-step maturation of the microviridin K core peptide (**3**) using the engineered enzymes LP-MvdD to yield (**4**), LP-MvdD and LP-MvdC to yield (**5**), and a combination of LP-MvdD, LP-MvdC, and MvdB ("one-pot") to yield mature microviridin K (**1**).

cyclizations were confirmed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and electrospray ionization high-resolution tandem mass spectrometry (ESI-HR-MS/MS; Figures S3 and S4). Subsequently, all three biosynthetic enzymes and MvdE core peptides were combined in a one-pot reaction. In our first attempt, acetylated mono- and bicyclic intermediates were detected along with microviridin K. These results clearly demonstrate that the *N*-acetyltransferase can act in variable order. The reaction protocol was improved by combining the enzymes in two steps: an 18 h reaction with the ligases followed by addition of the *N*-acetyltransferase for 6 h. This two-step combination of all three enzymes led to the full reconstitution of microviridin K from the MvdE core peptide (Figure 2). Cyclization

  

of the microviridin core peptide was also achieved when leader peptides were added in *trans* position to the unmodified enzymes MvdD and MvdC (Figure S2). Notably, both the constitutively activated LP-MvdD and LP-MvdC enzymes and unmodified MvdD and MvdC enzymes activated by the leader peptide in *trans* were also able to cyclize the microviridin B core peptide that differs in four N-terminal amino acids from the microviridin K homologue (Figure S4).

Co-crystallization of trypsin with the potent variant microviridin J and rational in vivo engineering of microviridins have recently revealed the immediate interaction of position 5 of microviridins with the catalytic center of serine-type proteases.[3] Hence this position was selected to expand our newly developed in vitro reconstitution concept into a focused library approach.

Two single compound libraries of the core peptide were synthesized by means of solid-phase peptide synthesis (SPPS). At position 5 in either microviridin K or microviridin B (Figure 3) all 20 amino acids were varied rendering $2 \times 20$

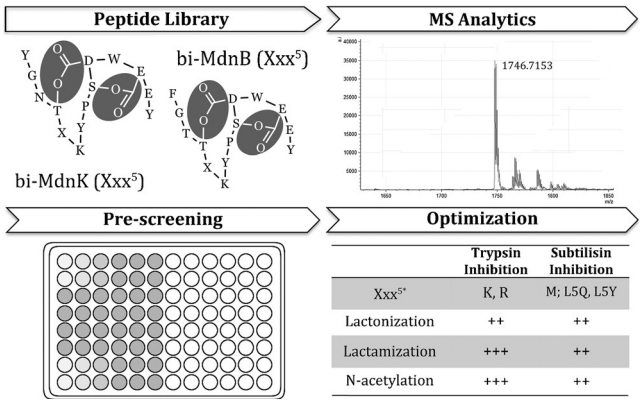


***Figure 3.*** Overview of a microviridin library production and bioassay screening. bi-MdnK ($Xxx^5$) and bi-MdnB ($Xxx^5$) represent two bicyclic depsipeptide libraries generated by SPPS and enzymatic transformation using LP-MvdD. All cyclizations were confirmed using MALDI-MS. Bicyclic variants were pre-screened against trypsin and subtilisin using colorimetric substrates. Selected active bicyclic peptides were further processed and optimized using LP-MvdC and MvdB. The activity of all intermediates was compared to deduce structure activity relationships. + + represents moderate inhibitory activity (low micromolar range); + + + represents strong inhibitory activity (nanomolar range).

peptides. While 17 of these core peptide variants each could be successfully cyclized for both the microviridin K and the microviridin B backbone, Pro, Cys, and Thr at position 5 impeded lactonization for microviridin K types. For microviridin B types, the variant with Cys was poorly cyclized and the variant with Pro was not cyclized at all. In this context it is worth mentioning that the efficiency of the cyclization was not the same for all the variants. In some cases, a monocyclic intermediate could also be detected (Table S4). Peptide bonds to Pro are able to form *cis* and *trans* isomers because both isomers collide sterically with the neighboring substitution. Because the occurring *cis–trans* isomerization is slow, it is able to hinder the progress of peptide folding and this suggests the inability of cyclization in the case of Pro. Restrictions in the cyclization of specific microviridin variants were previously overlooked in our in vivo screening approach. Correct cyclization of each variant was confirmed using MALDI-MS (Figure 3 and Figure S3).

Thus generated bicyclic peptide libraries were subsequently used for a colorimetric screening against the representative serine proteases trypsin and subtilisin (Figure 3). Microviridin B variants harboring either Arg or Lys at position 5 showed inhibition against trypsin in the low micromolar range ($IC_{50}$ 0.76 μM and 0.22 μM, for L5R and L5K, respectively; $IC_{50}$ = half maximal inhibitory concentration), in agreement with the substrate-like interaction of microviridin and trypsin that has been demonstrated in co-crystallization studies with microviridin J.[3] $IC_{50}$ values observed for the corresponding microviridin K types were slightly higher ($IC_{50}$ 1.78 μM and 3.13 μM for M5R and M5K variants). Subtilisin was most efficiently inhibited by variants carrying Met at position 5 ($IC_{50}$ 9.2 μM for L5M microviridin B type and 8.3 μM for M5M microviridin K type, respectively; Figure 3 and Table S5). Additionally, microviridin B variants carrying either Gln or Tyr at position 5 showed moderate inhibitory activities against subtilisin (Table S5). Notably, the corresponding microviridin K variants were inactive. Preselected bicyclic variants were then further cyclized using LP-MvdC and subsequently *N*-acetylated using MvdB. Each of the intermediates and final products were also tested against the respective protease. Interestingly, lactam ring formation and *N*-acetylation had a varying impact on the activity profile. Trypsin inhibition was clearly improved after introduction of the third lactam ring in the L5R microviridin B type ($IC_{50}$ 90 nM) by almost two orders of magnitude. The activity of this variant could be even further improved by N-terminal acetylation ($IC_{50}$ 50 nM) (Figure 3 and Table S5). These results are in agreement with the co-crystal structure of microviridin J and trypsin that has revealed an impact of the N-terminal side chain on target interaction.[3] Similar results were obtained for the corresponding microviridin K variants ($IC_{50}$ 1.78 μM, 0.41 μM, and 0.2 μM for the bicyclic, tricyclic and acetylated tricyclic M5R variants, respectively). However, the third lactam ring as well as the *N*-acetylation turned out to be less important for the activity of other variants, in particular against subtilisin. The activity of the bicyclic, tricyclic and *N*-acetylated L5M microviridin B types towards subtilisin, as an example, remained in a similar low micromolar range ($IC_{50}$: 9.2, 4.3, and 4.8 μM, respectively; Figure 3 and Table S5). The same trend was observed for the corresponding microviridin K types ($IC_{50}$ 8.3 μM, 3.9 μM and 5.2 μM for the bicyclic, tricyclic and *N*-acetylated tricyclic microviridin K variant, respectively).

The stepwise analysis of biosynthetic intermediates in bioassays as performed in the example of the microviridins can thus help to deduce structure–activity relationships in cases where a co-crystal structure with a protease or a general target protein is not available. Notably, highly active bicyclic variants targeted to subtilisin indeed exist in nature. The bicyclic depsipeptide marinostatin from *Alteromonas sp.* in fact represents a biosynthetic intermediate of microviridins.[9] The different processing states of the peptides in nature could

  

thus relate to variations in structure–activity relationships of different target proteases.

The chemo-enzymatic library approach established in the present study is superior to our previously developed in vivo microviridin production platform.[3] Isolation of single microviridin variants from the *E.coli* library required large-scale fermentation and laborious purification steps rendering a few milligram at the most. With our consecutive one-pot in vitro reconstitution approach we now introduce a clean time and cost-effective concept for cyclic depsipeptide production at milligram scale. Whereas variations in cloning, cyclization, and production efficiencies of individual microviridin variants can lead to false-positive or false-negative results during screening of the *E.coli* whole cell library, the novel technique allows following all structural intermediates of the biosynthesis and then to synthesize and test all intermediates of interest individually. The step-by-step procedure enables a tailored design of inhibitors against all serine-type proteases of interest. The cost-effective concept allows for a parallel synthesis and screening of multiple variants. The approach is likely also suitable for modularization with other RiPP enzymes, introduction of non-proteinogenic amino acids, and miniaturization of the relatively large depsipeptides.

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[1] P. G. Arnison, M. J. Bibb, G. Bierbaum, A. A. Bowers, T. S. Bugni, G. Bulaj, J. A. Camarero, D. J. Campopiano, G. L. Challis, J. Clardy, et al., *Nat. Prod. Rep.* **2013**, *30*, 108.
[2] M. A. Ortega, W. A. van der Donk, *Cell Chem. Biol.* **2016**, *23*, 31.
[3] A. R. Weiz, K. Ishida, F. Quitterer, S. Meyer, J. C. Kehr, K. M. Muller, M. Groll, C. Hertweck, E. Dittmann, *Angew. Chem. Int. Ed.* **2014**, *53*, 3735–3738; *Angew. Chem.* **2014**, *126*, 3810–3813.
[4] a) B. Philmus, G. Christiansen, W. Y. Yoshida, T. K. Hemscheidt, *ChemBioChem* **2008**, *9*, 3066; b) N. Ziemert, K. Ishida, A. Liaimer, C. Hertweck, E. Dittmann, *Angew. Chem. Int. Ed.* **2008**, *47*, 7756; *Angew. Chem.* **2008**, *120*, 7870; c) B. Philmus, J. P. Guerrette, T. K. Hemscheidt, *ACS Chem. Biol.* **2009**, *4*, 429.
[5] a) T. J. Oman, P. J. Knerr, N. A. Bindman, J. E. Velasquez, W. A. van der Donk, *J. Am. Chem. Soc.* **2012**, *134*, 6952; b) D. Sardar, Z. Lin, E. W. Schmidt, *Chem. Biol.* **2015**, *22*, 907.
[6] a) Y. Goto, Y. Ito, Y. Kato, S. Tsunoda, H. Suga, *Chem. Biol.* **2014**, *21*, 766; b) J. Koehnke, G. Mann, A. F. Bent, H. Ludewig, S. Shirran, C. Botting, T. Lebl, W. E. Houssen, M. Jaspars, J. H. Naismith, *Nat. Chem. Biol.* **2015**, *11*, 558.
[7] B. J. Burkhart, G. A. Hudson, K. L. Dunbar, D. A. Mitchell, *Nat. Chem. Biol.* **2015**, *11*, 564.
[8] A. R. Weiz, K. Ishida, K. Makower, N. Ziemert, C. Hertweck, E. Dittmann, *Chem. Biol.* **2011**, *18*, 1413.
[9] K. Kanaori, K. Kamei, M. Taniguchi, T. Koyama, T. Yasui, R. Takano, C. Imada, K. Tajima, S. Hara, *Biochemistry* **2005**, *44*, 2462.

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