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Phage display and selection of lanthipeptides on the carboxy-terminus of the gene-3 minor coat protein

Johannes H. Urban1, Markus A. Moosmeier1, Tobias Aumüller1, Marcus Thein1, Tjibbe Bosma2, Rick Rink2, Katharina Groth1, Moritz Zulley1, Katja Siegers1, Kathrin Tissot1, Gert N. Moll2 & Josef Prassler1

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are an emerging class of natural products with drug-like properties. To fully exploit the potential of RiPPs as peptide drug candidates, tools for their systematic engineering are required. Here we report the engineering of lanthipeptides, a subclass of RiPPs characterized by multiple thioether cycles that are enzymatically introduced in a regio- and stereospecific manner, by phage display. This was achieved by heterologous co-expression of linear lanthipeptide precursors fused to the widely neglected C-terminus of the bacteriophage M13 minor coat protein pIII, rather than the conventionally used N-terminus, along with the modifying enzymes from distantly related bacteria. We observe that C-terminal precursor peptide fusions to pIII are enzymatically modified in the cytoplasm of the producing cell and subsequently displayed as mature cyclic peptides on the phage surface. Biopanning of large C-terminal display libraries readily identifies artificial lanthipeptide ligands specific to urokinase plasminogen activator (uPA) and streptavidin.
The high degree of chemical and structural diversity found in ribosomally synthesized and post-translationally modified peptides (RiPPs) and other natural products currently sparks the interest in mining and engineering these peptides for drug discovery. Among them, lanthipeptides, a subclass of RiPPs with more than 100 members described to date, are produced in a wide range of bacteria and are characterized by the presence of enzymatically introduced thioether-bridged amino acids, called lanthionines (Lan) and methyllanthionines (MeLan). In a first enzymatic reaction during biosynthesis, a dehydratase recognizes an N-terminal leader sequence within the LanA precursor peptide and catalyzes the dehydration of serines and threonines in the core peptide to 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dbh), respectively. Subsequently, a cyclase supports the addition of cysteine thiols to the unsaturated amino acids Dha and Dbh to form the covalent Lan and MeLan linkages. Whereas dehydration and cyclization of class I lanthipeptides are catalyzed by separate LanB and LanC enzymes, respectively, a single bifunctional dehydratase/cyclase termed LanM is responsible for the orchestrated thioether formation in class II lanthipeptides. The thioether linkages confer thermal and proteolytic stability, frequently increase the affinity to protein targets by reducing the conformational flexibility of the peptide structure, and in contrast to disulfides, are resistant to chemical reduction. Most lanthipeptides characterized so far have antimicrobial activity and are collectively termed lantibiotics. However, screening of microbial strain collections or purified peptides recently identified lanthipeptides with unanticipated antifungal, antiviral, and antiallodynic bioactivities and genome-mining efforts uncovered novel variants that await characterization. The recent advance in lanthipeptide research culminated in the exploitation of heterologous hosts, e.g., Escherichia coli (E. coli) for improved expression, the in vitro reconstitution of several biosynthetic enzymes, and the successful solid-phase synthesis of even complex lanthipeptides with intertwined ring structures. Despite this progress, the systematic engineering of lanthipeptides is still in its infancy. Nonetheless, a proof-of-concept lanthipeptide cell display system based on Lactococcus lactis (L. lactis) was recently described. Unfortunately, the poor transformation efficiency of Gram-positive bacteria, the lack of a robust genetic engineering toolbox, and a tendency toward cell-clumping strictly limits this approach. Another study engineered artificial lanthipeptides by messenger RNA display in a Lan enzyme free system, which is based on chemical thioether bridge introduction via a reactive non-proteinogenic amino acid. In contrast to enzymatic thioether formation, the major drawback of this rather cumbersome method is the lack of stereo- and regioselectivity of cycle formation that requires downstream deconvolution.

We envisioned to use E. coli as heterologous host for the production of large enzymatically modified lanthipeptide libraries to be displayed on the surface of the filamentous bacteriophage M13 and to enable the screening of peptide ligands to targets of choice. Phage display is a robust and widely used in vitro selection technology for the discovery of therapeutic peptides and antibodies. For the successful display of lanthipeptides on phage several requirements must be met: (i) heterologous co-expression of the biosynthetic enzymes along with a genetic fusion comprising the cognate leader peptide, a core peptide library, and a phage coat sequence, (ii) efficient recognition of the leader peptide by the modifying enzymes and modification of the peptide core, both in the context of the fusion protein, (iii) incorporation of the fusion into the inner membrane of E. coli prior to phage assembly, and (iv) assembly and release of infectious particles displaying the cyclic, thioether-bridged peptides. Here, we identified the rarely used carboxy-terminus of the gene-3 minor coat protein (pIII) to be ideally suited for the display and de novo selection of lanthipeptides. C-terminal precursor peptide fusions to pIII ensure prolonged interaction with the heterologous Lan enzymes expressed in the cytoplasm of the producer cell prior to phage assembly and support the display of extensively post-translationally modified peptides on the phage surface. Using this C-terminal display mode on pIII, we were able to select cyclic lanthipeptide ligands to uPA and streptavidin from phage display libraries. We believe that phage display on the C-terminus of pIII is an excellent tool for the mining of novel lanthipeptides and might be suitable for the engineering of other RiPP classes that rely on leader peptide-dependent enzymatic modification.

Results

An ELISA-based reporter assay monitors peptide cyclization. To adapt phage display for the engineering of lanthipeptides, we focused on the class I biosynthetic NisBC system from L. lactis and the class II ProcM system from Prochlorococcus MIT9313, both known to support heterologous expression of lanthipeptides in E. coli. While the well-characterized NisBC two-enzyme system acts on the NisA precursor peptide as sole natural substrate to synthesize the antimicrobial lantibiotic nisin, the bifunctional ProcM turns 29 ProcA precursors with conserved
The figure shows the assessment of the cyclization status of artificial lanthipeptides in cell lysates and displayed on phage. A: Precursor peptides (full sequences in Supplementary Table 1) containing the NisA leader and indicated core sequences (residues involved in thioether formation colored) flanked by affinity tags were expressed with or without NisBC, captured from cell lysates, and subjected to FXa digestion and ELISA detection. The protease resistance relative to untreated (no FXa) samples was calculated and data representing mean ± s.d. of three independent cultures analyzed in duplicate is shown (unpaired, two-tailed t-test). B: As in (a), but core sequences were fused to the ProcA leader (full sequences in Supplementary Table 2) and expressed with or without ProcM enzyme. C: Peptides containing the NisA leader sequence were translationally fused to the N- (left panel) or C-terminus (right panel) of phage pIII and displayed on phage. Herein, a factor Xa (FXa) protease was used to monitor the cyclization status of model peptides in cell lysates or displayed on phage (Fig. 1a). Herein, a factor Xa (FXa) protease cleavage site is flanked by a serine or threonine and a cysteine, the residues involved in enzymatic thioether formation, and two affinity tag sequences which enable ELISA-based capture and detection. Synthetic linear or thioether-bridged peptides of related sequence (Fig. 1b) were tested in a sandwich-ELISA and confirmed that FXa treatment of linear peptides leads to proteolytic cleavage of the core peptide sequence, as judged by FXa cleavage resistance even with NisBC co-expression. The same reporter core peptide sequence fused to the ProcA leader and co-expressed with ProcM enzyme did not result in FXa-resistant cyclization even with NisBC co-expression. The same reporter core peptide sequence fused to the ProcA leader and co-expressed with ProcM enzyme did not result in FXa-resistant cyclization even with NisBC co-expression. The same reporter core peptide sequence fused to the ProcA leader and co-expressed with ProcM enzyme did not result in FXa-resistant cyclization even with NisBC co-expression. The same reporter core peptide sequence fused to the ProcA leader and co-expressed with ProcM enzyme did not result in FXa-resistant cyclization even with NisBC co-expression. The same reporter core peptide sequence fused to the ProcA leader and co-expressed with ProcM enzyme did not result in FXa-resistant cyclization even with NisBC co-expression. The same reporter core peptide sequence fused to the ProcA leader and co-expressed with ProcM enzyme did not result in FXa-resistant cyclization even with NisBC co-expression.

Expression and phage display of artificial lanthipeptides. With this reporter system at hand, a series of FXa site containing peptide variants were fused to the natural NisA and ProcA leader peptides, heterologously expressed in E. coli along with the modifying Lan enzymes, and subsequently cell lysates were screened for peptide cyclization. We identified NisA reporter peptides containing an FXa site flanked by serine/cysteine residues and FLAG and His6-epitopes that are largely resistant to FXa treatment when co-expressed with the modifying NisBC enzymes, but are readily proteolytically cleaved in absence of the Lan enzymes (Fig. 2a). Since enzymatic dehydration of serine to the reactive Dha can result in spontaneous cyclization, a serine to threonine point mutation was introduced, which is more chemically inert after dehydration to Dhb33. Even though the proteolytic resistance was reduced, the threonine-containing mutants still revealed a significant fraction of FXa-resistant peptides when NisBC was co-expressed. Mutating the serine or cysteine residues required for thioether bridge formation to alanine, abrogated FXa cleavage resistance even with NisBC co-expression. The same reporter core peptide sequence fused to the ProcA leader and co-expressed with ProcM enzyme did not result in FXa-resistant peptides. However, when the highly charged FLAG-tag was replaced by a more charge neutral HA-tag enzymatic cyclization of the core peptide sequence, as judged by FXa cleavage resistance, was observed (Fig. 2b). Again, mutation of the serine to threonine reduced the cyclization efficacy, whereas cyclization was abrogated when either serine or cysteine was mutated to alanine. Next we set out to display the identified reporter peptides on phage particles and to assess their cyclization status. A phagemid was constructed that encodes the precursor peptide sequences equipped with an ompA signal sequence for periplasmic transport fused to the N-terminus of the pIII phage coat protein. Phage particles produced with or without co-expression of the cognate Lan enzymes were captured onto ELISA plates using anti-pVIII antibodies and subjected to FXa cleavage assays. Neither in the NisBC nor the ProcM enzymatic
system FXa cleavage-resistant peptides were observed, which suggests that the displayed peptides are largely linear and failed to be efficiently modified (Fig. 2c, d, left panels). In contrast to the widely used N-terminal fusions to phage pIII, an isolated report describes fusions to its C-terminus. The C-terminus of pIII is directed toward the phage core and it was believed that fusions to the C-termini would not be easily accessible in display systems. However, Fuh and Sidhu selected His6-tagged randomized linker libraries fused to the C-terminus of pIII for binding to anti-His antibodies and identified 10 residue linker sequences that enabled display on the pIII C-terminus at similar rates as compared to conventional N-terminal display. We therefore established C-terminal pIII fusions of the same NisA- and ProcA leader containing reporter peptides and assessed their modification status on phage as described above. In this display mode, a significant fraction of FXa cleavage-resistant peptides was observed on phage produced co-expression of both the NisBC (Fig. 2c, right panel) and the ProcM (Fig. 2d, right panel) enzymes. Mutating the serine or cysteine residues required for thioether formation to alanine abrogated FXa resistance. Moreover, when the size of phage-displayed monoycles was gradually increased by insertion of additional residues between the serine and cysteine residues, highly significant peptide modification was observed for all tested constructs (Supplementary Fig. 1). These results demonstrate that C-terminal lanthipeptide fusions to pIII are recognized and modified by the class I and class II biosynthetic machinery, are further incorporated into phage particles, and are displayed in a solvent accessible manner.

**Design and selection of lanthipeptide phage display libraries.** Due to its unprecedented substrate tolerance, the class II ProcM enzymatic system seemed to be the ideal starting point for the generation of lanthipeptide phage libraries displayed on the pIII C-terminus. A comparison of natural prochlorosin peptides with confirmed structure revealed an interesting feature that is present in several of the variants and might serve as a scaffold for library design. Even though unrelated in the primary sequence, ProcA1.1, 2.8, and 2.11 have two larger non-overlapping thioether bridges in common, that are formed from central dehydrated positions to cysteines located further outside in opposite orientation (Fig. 3a). To test whether these natural peptide substrates would be efficiently modified by the ProcM enzyme when expressed in the context of a large fusion protein, we used the maltose-binding protein (MBP) as carrier and surrogate. MBP has approximately the same size as phage pIII (~43 kDa) and comes with the advantage of soluble expression instead of being inserted into the E. coli inner membrane, which facilitates more detailed peptide analytics by electrospray ionization mass spectrometry (ESI-MS). We produced these peptides as C-
terminal His$_6$-tagged fusions to MBP in presence of ProcM, removed MBP by proteolytic cleavage, and analyzed the modification status of purified leader/core peptides by ESI-MS. All three peptides were fully dehydrated by ProcM in the fusion context (Fig. 3b) and the formation of the correct bicyclic core in ProcA2.8 was further confirmed by electron transfer dissociation (ETD) fragmentation (Supplementary Fig. 2; Supplementary Tables 6–8). To further demonstrate that even more complex wild-type thioether cycle topologies are correctly introduced in the context of precursor peptides fused to the C-terminus of MBP, we expressed the lantibiotics nisin and lactacin 481 as MBP fusions and confirmed their antimicrobial activity (Supplementary Fig. 3). Encouraged by these results, three related lantipeptide libraries reminiscent of the ProcA1.1/2.8/2.11 architecture (Fig. 3c) were designed and cloned as C-terminal pIII fusions, which resulted in >1×10$^9$ independent transformants for each library. Phage pools produced with ProcM co-expression were then selected in three iterative rounds of biopanning for binding to uPA (libraries 1, 2, and 3) or streptavidin-coated magnetic beads (libraries 1 and 2). After the third round of selection, the peptide encoding sequences of the enriched phage pools were subcloned as C-terminal fusions to MBP, co-expressed with ProcM in 384 well format, and cell lysates were screened for target binding by ELISA. Screening of the three libraries selected on uPA resulted in 79, 117, and 81 hits, out of 368 tested clones, with ProcM expression were analyzed and tested for streptavidin binding and in a ProcM-dependent manner (Supplementary Fig. 4). More detailed analysis revealed that both, the ProcM-modified PEP332 and PEP334 contained two lanthionines as the dominant species among minor side products, whereas the major product in PEP333 and PEP335 rather contained a single lanthionine (Supplementary Fig. 5; Supplementary Tables 9–12). Even though we did not fully characterize all active species at this stage of the study, our results clearly show that post-translational modification of the selected core peptides by ProcM is a strict requirement to adopt structures that support uPA binding and inhibition.

Screening of the two libraries selected on streptavidin beads resulted in the identification of 49 and 33 hits, which represented two unrelated sequences (Fig. 4d), one of them having acquired an alanine to threonine mutation in the first position of the core peptide. Leader-core peptides produced in absence or presence of ProcM expression were analyzed and tested for streptavidin binding under reducing and non-reducing conditions. While PEP331 was fully dehydrated, one position in PEP330 had largely escaped dehydration (Supplementary Table 9). PEP330 bound streptavidin regardless of ProcM co-expression and reducing conditions had only minor effects, which suggests binding of a linear epitope (Fig. 4e). In contrast, the binding of PEP331 to

**Fig. 4** Phage selection outcome and characterization of identified lantipeptides. a Amino-acid sequences of specific clones selected on uPA. The library in which the clones were found, the number of clones with identical sequences, and a unique clone ID are indicated. Fixed cysteine positions are boxed (gray), putative sites of dehydration (serines and threonines) are underlined, and a conserved three residue motif is highlighted (colored). b Sequence and disulfide pattern of bicyclic and monocyclic uPA-specific peptides described in the literature. c ELISA for uPA binding of purified His$_6$-tagged leader-core peptides produced with (+ProcM) or without (unmodified) ProcM co-expression. Data representing mean ± s.d. of three replicates is shown (curve fit with nonlinear regression). d As in a, but amino-acid sequences of specific clones selected on streptavidin are shown. e ELISA for streptavidin binding of purified His$_6$-tagged leader-core peptide PEP330 produced with or without ProcM co-expression and analyzed under reducing (red) and non-reducing (non-red) conditions. f As in e, but binding curves of PEP331 are shown. Experiments shown in c, e, and f were repeated three times.
streptavidin was strictly dependent on post-translational modifications introduced by ProcM and only a slight reduction in binding was noticed in a reducing environment (Fig. 4f). In-depth analysis confirmed the presence of two efficiently formed lanthionines in both PEP330 and PEP331 that resemble the structural topology found in native ProcA2.8 and which inspired the design of the library scaffold (Supplementary Fig. 5; Supplementary Tables 9, 13–15).

**Discussion**

In summary, we show that the de novo selection of functional lanthipeptides by phage display can be achieved by fusing peptide precursor libraries containing natural leader sequences and a randomized core to the C-terminus of pIII. The co-expression of the lanthipeptide synthetase ProcM along with pIII-peptide precursor fusions leads to the sequential post-translational dehydration and cyclization of the core peptides in the cytoplasm of the producing cells and to their subsequent display on phage particles. We provide two examples in which biopanning of lanthipeptide display libraries resulted in the identification of peptide ligands that depend on ProcM-mediated post-translational modification for recognition of their targets, namely uPA and streptavidin. Interestingly, lanthipeptides selected on uPA share a known sequence motif with mono- and bicyclic disulfide-bridged peptide ligands previously identified by phage display. Moreover, the structures of these disulfide bridged peptides in complex with the catalytic domain of human uPA have recently been solved and their cyclic nature was reported to be indispensable for target binding. The library design in this study was based on the thioether configurations described for some natural prochlorosins and aimed at providing a subtle excess of serines/threonines at flexible positions (to be enzymatically dehydrated) over fixed cysteines within the core peptide to increase the chance of subsequent cyclization. Even though five of the six characterized lanthipeptides identified in this study clearly show the intended lanthionine configuration of two non-overlapping cycles, our analysis revealed that excess of available serines and threonines not necessarily correlates with improved cyclization efficiency, but might rather lead to stable phosphorylated intermediates, which fail to undergo phosphate elimination and conversion to the dehydrated residues.

In addition, some of the peptide preparations contained multiple modification products, which might complicate the identification of the active species (Supplementary Fig. 6, Supplementary Table 9). While site-specific peptide phosphorylation might be of interest for certain applications, this should be taken into account to further improve future library designs and emphasizes that the substrate requirements of promiscuous LanM enzymes are still not fully understood.

Fusions to the C-terminus of pIII are rather unconventional and to our knowledge have not been reported in the literature anymore since the first proof-of-concept study published by Fuh and Sidhu. As emphasized by the authors, C-terminal fusions should be advantageous for the display of cDNA libraries and proteins that require a free C-terminus to support protein–protein interaction. However, as demonstrated in this study, C-terminal fusions to pIII are furthermore ideally suited for the display of peptides that need to undergo post-translational modification in the cytoplasm of the host cell prior to phage assembly. We employed the widely used ompA signal sequence to target pIII-peptide fusions to the secretory (Sec) pathway. Upon translation, pIII is rapidly translocated and spans the bacterial inner membrane with the N-terminus facing the periplasmic space and the C-terminus remaining in the cytoplasm. Hence, the fusion of lanthipeptide precursors to the C-terminus of pIII ensures prolonged exposure to the cognate modifying LanM enzymes located in the cytoplasm prior to phage assembly and further enables the display of cyclic peptides on phage particles (see Fig. 5 for detailed illustration). Besides this spatiotemporal advantage, the translocation of modified lanthipeptides fused to
the C-terminus of pIII is unlikely to be restricted by the pore diameter of the Sec translocon. The SecYEG channel is limited to the transport of proteins <2.9 nm in diameter, which would preclude the translocation of bulky cyclic peptides, such as nisin with estimated dimensions of 2.2 × 2.7 × 4.2 nm, fused to the N-

terminus of pII. This assumption is further supported by the failure to target nisin to the Sec pathway in the natural producer E. coli.35 In C-terminal display, the lanthipeptide fusion bypasses the SecYEG channel and only has to be threaded through the larger phage pII pore (6–8.8 nm) during phage extrusion. However, all of the five phage coat proteins (pII, pVI, pvII, pvIII, and pIVX) have been exploited for phage display34 and we cannot rule out at this point that other display modes might also be suitable for the engineering of lanthipeptides. C-terminal peptide display has further been demonstrated on phage pV35 and pVIII36, even though the reported display rates are well below the levels achieved on the pII C-terminus, which we estimated to be ~0.3 per phage for lanthipeptide precursors. The Sec pathway translocates proteins in their unfolded conformation, whereas the twin-arginine translocation (Tat) pathway catalyzes the translocation of secretory proteins that fold in the cytoplasm.37 It is intriguing to speculate that the Tat pathway might present an alternative to achieve prolonged lanthipeptide precursor interaction with Lan enzymes in the cytoplasm and to further support the efficient display of lanthipeptides on the N-terminus of pIII, but it should be noted that our preliminary attempts were not successful. The bioactivity of several Lan enzymes has been reconstituted in vitro and it seems plausible to modify linear lanthipeptide precursors displayed on phage or on ribosomes by incubation with recombinant Lan enzymes. However, the production of Lan enzymes is laborious, cytostatic in the precursor peptide have to be trapped in a reduced state to allow the enzymatic introduction of the thioether cycles, and the buffers and additives used during the reaction have to be strictly controlled.38 In contrast, the here reported phage display platform essentially follows standard protocols and should be widely accessible to other laboratories.

During the establishment of the phage display procedure, we employed a modular two-plasmid system for the co-expression of Lan enzymes and lanthipeptide precursors that allowed to easily swap and test genetic parts, such as promoters, plasmid replicons, or enzyme- and peptide-encoding genes. We found that expression of ProcM from a low-copy plasmid with only ~3 copies per producer cell and under control of an inducible sigma 70-dependent (housekeeping) promoter is sufficient to display and select post-translationally modified lanthipeptides on phage. This suggests that the system could be further simplified by integration of the procM gene into the E. coli chromosome, thereby eliminating the need for a second plasmid. It was previously known that Lan enzymes recognize and modify lanthipeptide precursors with short N-terminal extensions, e.g., affinity tags, but the finding that also large bulky protein fusions, such as MBP and pIII, are tolerated was not anticipated. Beyond the instrumental impact for successful phage display on the pIII C-terminus, MBP fusions to lanthipeptide precursors warrant further attention. MBP is an excellent carrier protein that confers high level and soluble expression of various fusion partners that would otherwise suffer from poor producibility.39 This applies also to lanthipeptide precursor fusions and enabled not only high-level soluble expression in E. coli, but also affinity screening of lanthipeptides in cell lysates in a 384-well format. We believe that the here described phage display system is widely applicable to generate lanthipeptide ligands to protein targets of choice and can be adapted to other library designs, the use of alternative promiscuous lanthipeptide synthetases, or to incorporate further PTMs, such as glycosylations40 or α-amino acids.41 Moreover, the underexplored C-terminal display of pIII should be suitable for the engineering of other RIPP classes that rely on leader peptide-dependent enzymatic modification and could be a valuable source to identify therapeutic peptide candidates.

**Methods**

**Overview of expression plasmids and phagemids.** The vector maps of the plasmids and phagemids used in this study and their essential features are shown in Supplementary Fig. 7. A detailed description of the plasmids is provided below. A list of oligonucleotide primers used for PCR and cloning is provided in Supplementary Table 16.

**Cloning of Lan enzyme expression plasmids.** All Lan enzyme encoding plasmids for expression in E. coli MC10616 were derived from pZET12MCS (Expressys). The ColE1 origin was swapped for RSFi030 by AvrII/SpiI cloning of a ProcM gene (coding residues 27–361) into the bicistronic operon of phage pII. This resulted in a PPC101 promoter for the expression of RNAP and ProcM. The insertion of ProcM with its expression control into the construction vectors pMB81 and pRSFDuet-1 (Novagen) was performed by HindIII digestion of the product into pII, followed by transformation of E. coli DH5α and isolation of transformants. One colony was purified and sequenced to verify the integration of ProcM into the phage pII. The procM gene was PCR amplified from chromosomal Pro- chocrucococcus MIT9313 DNA (Prosavoli-Guillard National Center for Marine Algae and Microbiota) using primers JUB-007/-009, digested with KpnI/AciI, and ligated into KpnI/MluI digested pII, resulting in a recombinant plasmid pIIRSF_SCS. This pIIRSF_SCS harbors a ColE1 origin and its expression is controlled by the tac promoter.

**Phagemid cloning for lanthipeptide display.** The phagemid pL3_stuffer that was used for display of precursor peptides on the N-terminus of pIII was obtained via cloning intermediate that was designed for peptide display on the major coat protein gp8. In a first step a synthetic DNA fragment (GeneArt) consisting of tandem Xhol/NotI sites followed by a 15 amino acid peptide, a fragment of peptidoglycan associated lipoprotein (PilS) from W. succinogenes and a GGGDSRGGGAAGGGCDSRG sequence as signal peptide, two STOP codons and a myc epitope tag was cloned into HindIII site of pL3C_zeoR-stuffer. The pL3C_zeoR-stuffer plasmid was transformed into E. coli BL21(DE3) and筛选display libraries of the intermediate plasmid pZELSF_MCS. The nic and nisB genes from pRSFDuet-nisC-nisB encode a bicistronic operon with N-terminally myc- or HA-epitope-tagged nic and nisB, respectively. The procM gene was PCR amplified from chromosomal Pro- chocrucococcus MIT9313 DNA (Prosavoli-Guillard National Center for Marine Algae and Microbiota) using primers JUB-136/-137 to obtain plasmid pZELSF_MCS. This pZELSF_MCS harbors a ColE1 origin and its expression is controlled by the tac promoter. For T7-polymerase-driven expression, procM was PCR amplified using primers JUB-011/-012 and cloned into NotI/KpnI digested pIIRSF1_1 resulting in a recombinant plasmid pRSFDuet-1.

**Cloning of reporter lanthipeptide expression.** All expression constructs for reporter lanthipeptides were established from double-stranded synthetic DNA fragments encoding precursor lanthipeptide display for the pII N-terminus were cloned into the BamHI/NotI restriction site of pL3_stuffer. Synthetic DNA fragments encoding precursor lanthipeptides for display on the phage pII were constructed by cloning of a synthetic DNA fragment encoding a SD sequence preceded by an ompA coding sequence, two STOP codons and a linker sequence (YSSAESDTRSAP), a zeocin resistance gene (ble) flanked by BamHI and HindIII restriction sites, via XbaI/HindIII into pL3_stuffer. A SalI linker site was introduced downstream of the ompA coding sequence encoding lanthipeptide display libraries were inserted into the XbaI/Sall site of pL3C_zeoR-stuffer as described below.

**Cloning of reporter lanthipeptide expression for E. coli MC10616.** All expression constructs for reporter lanthipeptides were established from double-stranded synthetic DNA fragments (GeneArt) and cloned into the XbaI/HindIII sites of plasmid pL3_stuffer, which replaced the phage pII gene by the coding sequence of the reporter peptide. The resulting amino acid sequences of precursor peptides are shown in Supplementary Tables 1, 2, 3, 4.

**Cloning of lanthipeptide precursors for production in E. coli BL21(DE3).** The pET21a-MBP_FxZa_zeo-stuffer plasmid was used with T7-polymerase-driven production of lanthipeptides as C-terminal fusions to MBP was constructed by XbaI/ HindIII cloning of a synthetic double-stranded DNA fragment (GeneArt) encoding the murA gene (coding residues 27–391 preceded by an ATG start codon) fused to a linker sequence with integrated FxZa cleavage site (sequence N5TENNN NGTIEGSGPSGSGGAPGS; FxZa site underlined) and a zeocin resistance gene (ble) flanked by BamHI/HindIII sites into pET21a (Novagen). All prepeptide
sequences either derived from synthetic DNA fragments or PCR products obtained on the phagemid libraries using the primer pair JUB-2325/248 were cloned into the pRSET/Codon III sites of pET21a-MBP_Fxx-zeo-stuffer. The amino-acid sequences of the resulting precursor peptide fusions are shown in Supplementary Table 5.

**Library syntheses.** The DNA fragments of the variable regions of the lanthipeptide libraries were synthesized by a modified version of the Slonomics Technology for de novo gene synthesis44, which facilitates both, the consecutive generation of variable positions, as well as the synthesis of a constant sequence by parallel incorporation of complex anchor mixtures during elongation. Herein, every ligation-mediated elongation step is followed by a specific cleavage with a type IIS restriction enzyme generating new overhangs for a further reaction cycle in which a new mixture of anchor molecules (or an individual anchor) is added to the growing DNA fragment through subsequent ligation reactions. Intermediate reaction products were transformed into chemical competent transformed through repeated immobilization of biotinylated DNA fragments on a streptavadin-coated surface during every cycle of the synthesis process. Following the outlined procedure, any variable sequence motif at any position of a given DNA sequence could be introduced, while allowing the precise control of the type, number, and frequency of individual codons. To build the DNA of JPTG for a final concentration of 0.75 mM, and cultures were further incubated for ~20–22 h at 22 °C, 230 rpm. All expressions were performed in 11 centrifuge beakers (Heraeus). Cells were harvested by centrifugation at 6000×g for 30 min at 4 °C (Cryofuge 8505c Heraeus; Rotor 6606/8165). Then the pellet was frozen at ~20 °C for 2 h or until further processing. The bacterial pellets were resuspended in lysis buffer (saturated salting-out solution of 2 M MgCl2, 20 mM β-mercaptoethanol (Roche) and one tablet per 50 ml complete, EDTA-free protease inhibitor cocktail tablets (Roche)) by shaking at 200 rpm for 30 min at room temperature. Cells were disrupted either by chemical lysis (addition of 0.2% (w/v) lysozyme (Roche) to the lysis buffer) or high-pressure homogenization at 1 Mpa using an M110 (Millipore) or enzymes were allowed to proceed for 22 h at 22 °C, shaking at 70 °C, shaking well, and cultures were collected by centrifugation. Phage supernatants were incubated with VCSM13 helper phage at a multiplicity of infection of ~10. Cells were collected by centrifugation, resuspended in 3 ml 2xYT induction media containing 0.5 mM IPTG and appropriate antibiotics, and phage production allowed to proceed for 20 h at 22 °C. Phage supernatants were collected of producer cells by centrifugation and transferr in quadruplicate to 384 well MA2400 plates (Meso Scale Diagnostics) coated with anti-His tag IgG (R&D Systems, MA050; 3 µg ml−1). After affinity capture of the peptides by washing five times with PBS, intact peptides were detected with either biotinylated anti-FLAG (Sigma, F-9291) or anti-HA antibodies (Thermo Scientific, #26183-BTN) and MBD GOLD SULFO-TAG (Meso Scale Diagnostics) conjugated streptavadin. Electrochemiluminescence was measured on a Meso Scale Discovery SECTOR Imager 6000. Signals obtained without FXa treatment were set to 100% (input) and the signal ratio for the corresponding sample after FXa treatment was calculated (signal remaining after FXa treatment (%)). Obtained values therefore reflect the percentage of FXa cleavage-resistant peptide and are a direct measure of thioether bridge formation efficiency. For peptide reporting on phage displayed precursor peptides, triplicate glycerol stocks containing E. coli strains harboring phagemids with or without the cognate peptide. Enzyme and peptide were generated overnight in 96 well plates. For reporter assays on solubly expressed peptide fragments by washing five times with PBS, intact peptides were detected with either biotinylated anti-FLAG (Sigma, F-9291) or anti-HA antibodies (Thermo Scientific, #26183-BTN) and MBD GOLD SULFO-TAG (Meso Scale Diagnostics) conjugated streptavadin. Electrochemiluminescence was measured on a Meso Scale Discovery SECTOR Imager 6000. Signals obtained without FXa treatment were set to 100% (input) and the signal ratio for the corresponding sample after FXa treatment was calculated (signal remaining after FXa treatment (%)). Obtained values therefore reflect the percentage of FXa cleavage-resistant peptide and are a direct measure of thioether bridge formation efficiency.

**Phage selection on uPA and streptavadin-coated magnetic beads.** The number of transducing units (t.u.) present in the established phage libraries was determined by infection titration on susceptible E. coli TG1F+ cells. Phage titers were in the range of 1012 t.u. per ml (>10-fold library size) and no sign of reduced infectivity caused by C-terminally displayed peptides was noted. For the first round of selection, all phage libraries were blocked overnight at 4 °C in 500 µl Chemi-BLOCKER (Millipore) on a rotator. Phage particles were diluted to ~1.5 × 1013 t.u. in 500 µl ChemiBLOCKER containing 0.05% (v/v) Tween 20, incubated for 30 min at room temperature twice with 1 mg streptavadin beads (Dynabeads M-280, ThermoFishier), and once with 2 mg of a randomly biotinylated Fab-fragment immobilized on 2 mg streptavadin beads. Pre-adsorbed phages were transferred to fresh 2 ml tubes, random biotinylated uPA (ProFec, #264-PCR; purified by size exclusion chromatography) added to a final concentration of 100 nM, and samples incubated 1 h at room temperature on a rotator. The samples were then captured on 2 mg streptavadin beads for 20 min at room temperature, washed five times each with PBS containing 0.05% (v/v) Tween 20 or PBS alone, and eluted at the final concentration of 100 nM. Phages were incubated with 2 M Tris pH 8, 22 °C, washing once with 2 M Tris pH 8, 22 °C, washing once with 2 M Tris pH 8, 22 °C, washing once with 2 M Tris pH 8, 22 °C. The infected cells were harvested by centrifugation, plated on large LB/chloramphenicol/ampicillin plates, incubated overnight at 37 °C, and used to produce phage for the next round of selection. Two additional selection rounds were performed with reduced uPA concentration (50 and 25 nM, respectively) and with elongated washing steps. The selection for streptavadin binding was performed essentially as described above using 1.2, 0.6, and 0.3 µg streptavadin-coated beads as target in round one, two, and three, respectively, and the pre-adsorption steps each on 1 µg Protein G Dynabeads (Life Technologies).

**Analysis of MBP-fused prochorosin peptides by mass spectrometry.** Peptide samples were analyzed using an Acquity UPLC System (Waters) coupled to a Synapt G2 Si mass spectrometer with ETD capability (Waters). Peptide identity...
was verified by intact mass measurement following analytical separation on an Agilent UPLC Protein BEH C4 column (2.1 mm × 50 mm, Waters) using a gradient of 3–60% B, and then in water over 20 min at a flow rate of 500 µL min⁻¹. Each eluent was supplemented with 0.1% formic acid. The eluent was passed into the UV detector and the electron spray ionization (ESI) source via a 151 splitter (Waters). The time course of elution was recorded by UV at 214 nm and by MS at a source voltage of 0.8 kV simultaneously. MS data were processed using MaxQuant (Warner et al. 2010 for MW < 10 kDa and MaxEnt1 (Warner et al. 2010) for MS > 10 kDa. The results of all ESI-MS analyses are summarized in Supplementary Table 6. ETD mass spectrometry was used to analyze the structure of leader free ProcA2.8. Following analytical separation on an Agilent UPLC BEH C8 column (2.1 mm × 50 mm, Waters), 3–60% acetonitrile in water over 20 min, flow rate 150 µL min⁻¹), the eluent was passed directly into the ESI source. The 6+ charged ion (m/z 3389.92) of the peptide was selected as precursor and fragmented under ETD conditions using 4-nitrotoluene as an electron donor reagent. The ETD spectrum was analyzed by assigning the ion signals to the c- and z-fragments (c3, c4, c5, z1, z2, z3) expected from the ProcA2.8 sequence. While fragments before, between, and after the N-terminal thioether linker were retained (Supplementary Tables 7, 8), no fragments from the sequence within the proposed thioether bridges could be assigned indicating that the lantionines have been formed in a consecutive arrangement.

Subcloning and affinity screening of selected clones. After the third round of selection plasmid DNA was isolated, the precursor peptide sequences PCR amplified using the 5′-biotinylated primers JUB-225-248, parental plasmid DNA digested with DpnII and the PCR products digested with DpnII and ligated into the BamHI/HindIII and three times sequentially passed over streptavidin-coated 96 well plates (Microcoat) to capture released biotinylated overhangs and undigested DNA. Without further purification the inserts were ligated into the BamHI/HindIII digested vector pET21a (EMBL, the E. coli BL21(DE3) harboring plasmid pRSFD_procM, and plated on large LB/ampicillin/kanamycin plates. 368 transformants from each library output were then picked and plated on large LB/ampicillin/kanamycin plates. 368 transformants from each library output were then picked and plated on large LB/ampicillin/kanamycin plates. 368 transformants from each library output were then picked and plated on large LB/ampicillin/kanamycin plates. 368 transformants from each library output were then picked and plated on large LB/ampicillin/kanamycin plates. 368 transformants from each library output were then picked and plated on large LB/ampicillin/kanamycin plates. 368 transformants from each library output were then picked and plated on large LB/ampicillin/kanamycin plates. 368 transformants from each library output were then picked and plated on large LB/ampicillin/kanamycin plates. 368 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26. Chen, S. et al. Bicyclic peptide ligands pulled out of cysteine-rich peptide libraries. J. Am. Chem. Soc. 135, 6562–6569 (2013).

27. Jiang, L. et al. The binding mechanism of a peptidic cyclic serine protease inhibitor. J. Mol. Biol. 412, 235–250 (2011).

28. Chatterjee, C. et al. Lactacin 481 synthetase phosphorylates its substrate during lantibiotic production. J. Am. Chem. Soc. 127, 15332–15333 (2005).

29. Thibodeaux, G. N. & van der Donk, W. A. An engineered lantibptide synthetase-sequences as a general leader peptide-dependent kinase. Chem. Commun. 48, 10615–10617 (2012).

30. Marvin, D. A., Symmons, M. F. & Straus, S. K. Structure and assembly of filamentous bacteriophages. Prog. Biophys. Mol. Biol. 114, 80–122 (2014).

31. Bonardi, F. et al. Probing the SecYEG translocation pore size with preproteins conjugated with sizable rigid spherical molecules. Proc. Natl Acad. Sci. USA 108, 7775–7780 (2011).

32. Breukink, E. et al. Lipid II is an intrinsic component of the pore induced by nisin in bacterial membranes. J. Biol. Chem. 278, 19898–19903 (2003).

33. Kuipers, A. et al. Sec-mediated transport of posttranslationally dehydrated peptides in Lactococcus lactis. Appl. Environ. Microbiol. 72, 7626–7633 (2006).

34. Velappan, N. et al. A comprehensive analysis of filamentous phage display vectors for cytoplasmic proteins: an analysis with different fluorescent proteins. Nucleic Acids Res. 38, e22 (2010).

35. Jespers, L. S. et al. Surface expression and ligand-based selection of cDNAs fused to filamentous phage gene V1. Biotechnology 13, 378–382 (1995).

36. Held, H. A. & Sidhu, S. S. Comprehensive mutational analysis of the M13 major coat protein: improved scaffolds for C-terminal phage display. J. Mol. Biol. 340, 587–597 (2004).

37. Natale, P., Bruser, T. & Driessen, A. J. M. Sec- and Tat-mediated protein secretion across the bacterial cytoplasmic membrane—distinct translases and mechanisms. Biochim. Biophys. Acta 1778, 1735–1756 (2008).

38. Li, B., Cooper, L. E. & van der Donk, W. A. in Complex Enzymes in Microbial Natural Product Biosynthesis, Part A: Overview Articles and Peptides, 1st edn, Vol. 458 (ed. Hopwood, D.) Ch. 21 (Elsevier, 2009).

39. Capust, R. B. & Waugh, D. S. Escherichia coli maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. Nucleic Acids Res. 38, 1668–1674 (1999).

40. Iorio, M. et al. A glycosylated, labionin-containing lantibiotope with marked antinociceptive activity. ACS Chem. Biol. 9, 398–404 (2014).

41. Huo, L. & van der Donk, W. A. Discovery and characterization of bicereucin, an unusual D-amino acid-containing mixed two-component lantibiotic. J. Am. Chem. Soc. 138, 5254–5257 (2016).

42. Rauchenberger, R. et al. Human combinatorial fab library yielding specific and functional antibodies against the human fibroblast growth factor receptor 3. J. Biol. Chem. 278, 38194–38205 (2003).

43. Prassler, J. et al. HuCAL PLATINUM, a synthetic fab library optimized for sequence diversity and superior performance in mammalian expression systems. J. Mol. Biol. 413, 261–278 (2011).

44. van den Brulle, J. et al. A novel solid phase technology for high-throughput gene synthesis. Biotechniques 45, 340–343 (2008).

45. Zhai, W. et al. Synthetic antibodies designed on natural sequence landscapes. J. Mol. Biol. 412, 55–71 (2011).

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Author contributions
J.H.U. and M.A.M. conceived and designed experiments. T.A. and R.R. performed peptide analytics. K.G. and M.Z. analyzed experiments. M.T. implemented and supervised library syntheses. T.B. provided idea for C-terminal display. K.T., G.N.M., and J.P. provided valuable input. J.H.U. wrote the manuscript with input from all authors.

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Competing interests: J.H.U., M.A.M., J.P., and T.B. are inventors on a patent application related to the ideas described in this manuscript. The remaining authors declare no competing financial interests.

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