Lasso Peptides

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Xanthomonins I–III: A New Class of Lasso Peptides with a Seven-Residue Macrolactam Ring**

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Abstract: *Lasso peptides belong to the class of ribosomally synthesized and post-translationally modified peptides. Their common distinguishing feature is an N-terminal macrolactam ring that is threaded by the C-terminal tail. This lasso fold is maintained through steric interactions. The isolation and characterization of xanthomonins I–III, the first lasso peptides featuring macrolactam rings consisting of only seven amino acids, is now presented. The crystal structure of xanthomonin I and the NMR structure of xanthomonin II were also determined. A total of 25 variants of xanthomonin II were generated to probe different aspects of the biosynthesis, stability, and fold maintenance. These mutational studies reveal the limits such a small ring imposes on the threading and show that every plug amino acid larger than serine is able to maintain a heat-stable lasso fold in the xanthomonin II scaffold.*

Lasso peptides are a fascinating group of natural compounds that contain the unique structural motif of a lariat knot. This structural feature consists of a macrolactam ring, formed between an N-terminal α-amino group and the carboxylic acid side chain of an Asp or Glu residue, through which the C-terminal tail is threaded and interlocked through amino acids with sterically demanding side chains. These amino acids, the so-called plugs, are positioned above and below the ring.[1–16] As a consequence of their structure, lasso peptides often exhibit a high stability against proteolytic and chemical degradation, and in some cases also show exceptional thermal stability.[1,3,8,10,14,16–23] They are of general interest not only because of their unprecedented fold and conservation of their noncovalent shape, but also because they are associated with interesting biological properties, which range from antimicrobial[3,10,11,18,20,24,25] to receptor antagonistic[26–28] and inhibitory activities.[1,20,24,25,29–32] As their biosynthetic machinery is highly promiscuous, most residues of their scaffolds can be exchanged, which allowed their successful application for epitope grafting.[32]

Recently, an increasing number of new lasso peptides, particularly of proteobacterial origin, were identified.[2,14,15,23,33] Interestingly, all the lasso peptides known so far feature macrolactam rings consisting of either eight or nine amino acids. These findings gave rise to the notion of lasso peptides being limited to eight- and nine-residue rings, with ten-residue rings being proposed to be too large and seven-residue rings to be too small to maintain the lasso fold.

However, in an extensive genome-mining study on proteobacteria,[23] a small subgroup of putative precursors was found that carried a Glu residue suitable for ring formation at the seventh position of the proposed lasso peptide sequence. This was observed for 7 out of 74 of the suitable precursors identified. To probe if lasso peptides with a seven-residue ring could exist, we chose the putative biosynthetic gene cluster from *Xanthomonas gardneri* for a thorough investigation (Figure 1).

For heterologous expression, the complete cluster was cloned into pET41a. After fermentation in M9 minimal medium at 20°C for 3 days, production of the four amino acid truncated lasso peptide derived from XgaA1 and of the six amino acid truncated lasso peptide derived from XgaA2 could be detected by high-resolution FT mass spectrometry. These compounds were named xanthomonin I and II, respectively. The M9 medium was chosen for expression as it had previously been shown to be the most suitable medium for the heterologous production of lasso peptides.[14,16,23]

As the yield of this initial system was very low, production optimization was carried out as previously described.[14] Compared to the unmodified expression system, the single-precursor constructs (Figure 1c and d) showed a 37- or 11-fold increase in the overall production. By using these constructs, we were able to isolate the four amino acid truncated xanthomonin I in a yield of 12.7 $mg\,L^{-1}$ culture and the six amino acid truncated xanthomonin II in a yield of 4.1 $mg\,L^{-1}$ culture.

Analysis of the primary structure was done by MS^2 and its result was in accordance with the predicted amino acid sequences and the presence of seven-residue rings. To confirm the ring size and lasso topology, both compounds were subjected to crystallization screens, which resulted in the formation of crystals in both cases. While the crystals of the truncated xanthomonin I had a sharp outline and a defined structure, the truncated xanthomonin II formed irregularly shaped crystalline plates. As expected, the latter crystals diffracted poorly, whereas the former crystals yielded high-resolution (0.8 Å) diffraction data that allowed the elucida-

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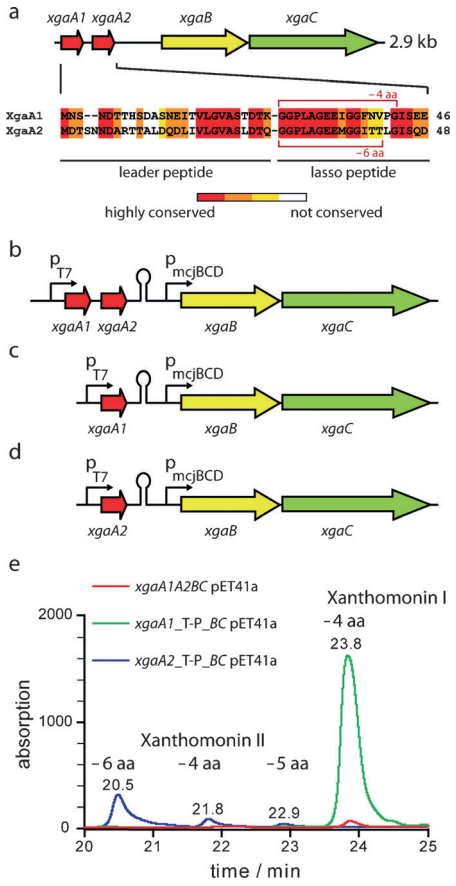

Figure 1. a) Schematic representation of the biosynthetic gene cluster from *X. gardneri* and alignment of the amino acid sequences of the identified precursor peptides. Genes were named according to the established nomenclature. The major product for each precursor is marked. b) Gene cluster after exchange of the intergenic region between *xgaA2* and *xgaB* with the λt_0 terminator (represented as a loop) and *mcjBCD* promoter sequences. c, d) Single-precursor constructs. e) UV traces of the fermentation extracts of the different constructs.

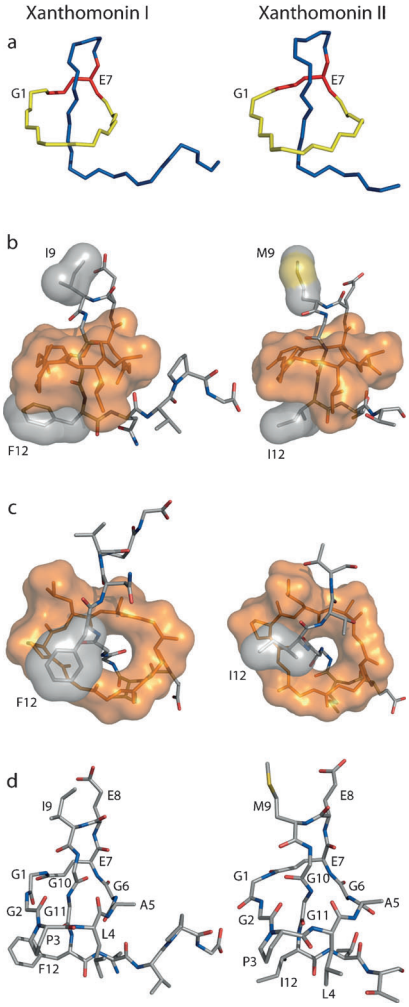

Figure 2. Several representations of the structures of the four amino acid truncated xanthomonin I (left side, PDB code 4NAG) and the six amino acid truncated xanthomonin II (right side, PDB code 2MFV). a) Structures of the lasso peptide backbones. The tail is highlighted in blue, the ring in yellow, and the ring-forming Glu7 residue in red. b, c) The interactions between the C-terminal tail and the macrolactam rings seen from the side (b) and from below (c). d) Structures of the lasso peptides with their side chains colored by elements (nitrogen in blue, oxygen in red, sulfur in yellow, carbon in gray).

tion of the structure of the four amino acid truncated xanthomonin I. This is to date only the second example of a crystal structure of a lasso peptide.[13] The structure of the six amino acid truncated xanthomonin II was solved by using NMR spectroscopic methods. The two structures are shown in Figure 2 (for more details see the Supporting Information). The plug amino acid pairs could be identified as I9/F12 for xanthomonin I and M9/I12 for xanthomonin II.

Several assays were performed to test the physicochemical properties of the xanthomonins (see the Supporting Information). The proteolytic stability was assessed against carboxypeptidase Y, chymotrypsin, and proteinase K. In almost all cases the lasso peptides proved to be completely resistant to proteolytic degradation. The only exception was that carboxypeptidase Y was able to slowly cleave off the two C-terminal amino acids of the already four amino acid truncated xanthomonin I to yield detectable traces of peptides truncated by five and six amino acids. To test the thermal stability both compounds were incubated for up to 8 h at 95 °C. In both cases, no alterations in the retention time occurred, which shows the high thermal stability of the xanthomonins.

These novel lasso peptides were also assessed for their potential antimicrobial activity through spot-on-lawn assays, but showed no activity against any of the tested bacterial strains (see the Supporting Information).

To probe the specificity of the biosynthetic machinery and to further investigate important structural features, a total of 25 xanthomonin II mutants were generated by site-directed ligase-independent mutagenesis.[34,35] Xanthomonin II was chosen for these experiments as the shorter main product facilitates the investigation of the tail region below the ring. An overview of the production of all the variants is shown in Figure 3.

Exchange of the Thr residue at the penultimate position of the leader peptide revealed that the xanthomonin system shows much lower tolerance to substitutions at this position

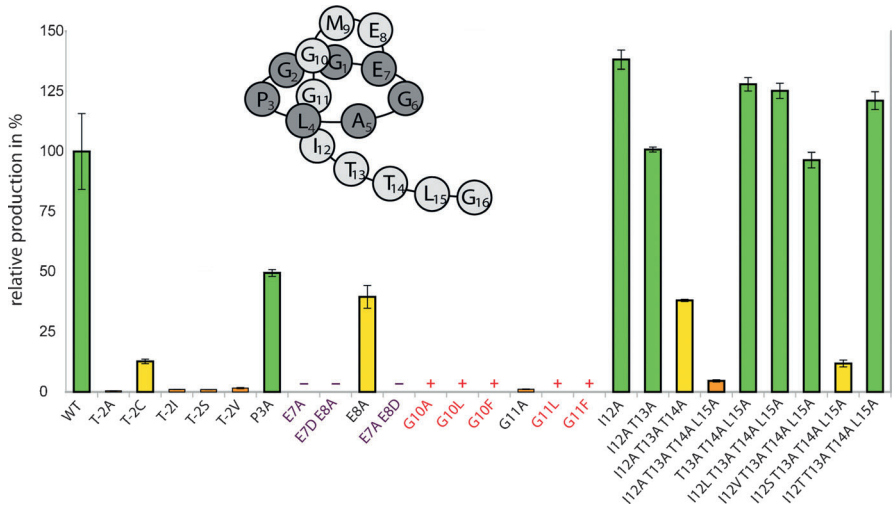

Figure 3. Overview of the overall production of all the tested xanthomonin II variants (see also the Supporting Information). A green bar represents a production of 150–50%, a yellow bar a production of 50–10%, and an orange bar a production of 10–1% compared to the wild type. Variants marked with a red cross were only detectable by mass spectrometry, while variants marked with a violet minus sign were not detectable. A schematic representation of the four amino acid truncated xanthomonin II is shown in the middle.

than other systems. Amino acids structurally similar to Thr are usually tolerated rather well, while others dramatically decrease the overall production of the lasso peptides.[14,16,22,36,37] In the case of the xanthomonin system, not only the T-2A variant, which should serve as a negative control, but also the variants T-2I, T-2S, and T-2V resulted in poor yields. Only the T-2C substitution was tolerated to some extent.

Several mutants were generated to investigate if differently sized macrolactam rings could be generated by either substituting Glu with Asp or replacing Glu7 with Ala. Furthermore, we wanted to assess if Pro3 is crucial for maturation, as the rigidity of Pro3 could be important for the prefolding. While the variants P3A and E8A were produced in good amounts, the mutations E7A, E7D, and E7A/E8D completely abolished production, thus highlighting that the biosynthetic machinery appears to be limited to processing 23-atom macrocycles.

Both amino acids that thread through the ring are glycine residues, which gives rise to the question, if this is the lower limit for a seven-residue ring. To answer this, both G10 and G11 were substituted independently with Ala, Leu, or Phe. The results clearly show that the exchange of either Gly residue with any larger amino acid almost completely abolishes production of the lasso peptide.

Finally, we wanted to investigate how small an amino acid can be to still act as a plug for a seven-residue ring. For this, we first generated a couple of variants to test if and which amino acids of the tail, besides I12, are still capable of maintaining the lasso fold. The variants I12A, I12A/T13A, and I12A/T13A/T14A were produced in good amounts. In contrast, the quadruple Ala substitution (I12A/T13A/T14A/L15A) significantly decreased the production level. Additionally, the main product of this variant was the peptide truncated by 10 amino acids, which corresponds to the macrolactam ring with only three further amino acids as a tail, which is too short for formation of a lariat knot. Based on this data, it is likely that these mutations still allow processing of the precursor, but it also suggests that the maturation is directly followed by unthreading and subsequent proteolytic degradation of the majority of the tail region.

Hence, this variant is the ideal starting point for an investigation of the role of the amino acid plug. A set of I12X/T13A/T14A/L15A variants was thus generated, with X being Ile, Leu, Ser, Thr, or Val. From these, only the variant with Ser was produced in rather poor amounts, as well as having a peptide truncated by 10 amino acids as one of its main products. To assess the topology and heat stability of these variants, a combined carboxypeptidase Y and thermal denaturation assay was performed (see the Supporting Information). While the variants carrying Ile, Leu, Thr, or Val at position 12 could not be shortened by more than six amino acids, which is in accordance with the results of the control reaction, both variants with Ala or Ser at position 12 were degraded until only the macrolactam ring and Glu8 were left.

These results were obtained independently from prior incubation at 95 °C for 4 h, thus showing that no thermally induced changes of the topology occur. Consequently, all compounds that carry Ile, Leu, Thr, or Val at position 12 are heat-stable lasso peptides, while the variants with Ala or Ser at position 12 could only be isolated as branched cyclic peptides. This shows that all amino acids larger than Ser are capable of maintaining the lasso fold of a seven-residue ring, even at high temperatures. This is a remarkable result, as similar studies with caulosegnin I revealed that Leu is barely able to maintain the lasso peptide with an eight-residue ring.[14]

It is also intriguing to see how small the openings in the macrolactam rings of the xanthomonins truly are. The fact that their rings consist of only 23 atoms allows a comparison to the class of chemically accessible [2]rotaxanes, which show some basic structural similarities to lasso peptides. Interestingly, the smallest currently known [2]rotaxanes have macrocycles consisting of only 20 atoms and, thus, are only slightly smaller than the xanthomonin rings.[38–40] Nevertheless, in contrast to a peptide chain, the macrocycles and the groups threading through them consist of only linear chains of atoms in the [2]rotaxanes. Considering this and the fact that these [2]rotaxanes are believed to be the limit of what is possible, or at least to be close to it, it is doubtful that lasso peptides with a six-residue ring could exist. However, a lasso peptide with a seven-residue ring and an Asp instead of a Glu residue as the ring-forming amino acid could still be feasible, even though the xanthomonin system was unable to process the corresponding variant. Unfortunately, there are so far no

known gene clusters with putative precursors that feature an Asp7 residue; therefore, the answer to the question, if such a lasso peptide could be naturally occurring, has to be postponed until further genomic data are available.

To prove that the reported seven-ring lasso peptides were not a special case, we decided to further investigate the gene cluster from *Xanthomonas citri* pv. *mangiferaeindicae* (see the Supporting Information), which was also identified in a previous study.[23] Production optimization was carried out as described before,[14,23] thereby increasing the overall yield fourfold and allowing the isolation of the predicted compound, xanthomonin III, truncated by seven amino acids in a yield of 0.3 $mg\,L^{-1}$ culture. MS^2 confirmed both the predicted amino acid sequence and the seven-residue ring. Combined thermal and carboxypeptidase Y studies resulted in no alteration of the length or retention time of the isolated compound, strongly indicating that it is indeed a true lasso peptide.[14,23] For identification of its plug amino acid, a set of variants was generated that successively replaced all the larger amino acids in the tail from positions 14 to 11 with Ala. As the M11A/S12A/P13A/I14A variant was the only one not produced, it can be concluded that Met11 acts as a plug for xanthomonin III. These experiments show that lasso peptides with seven-residue rings can generally be synthesized and that this class of lasso peptides is not restricted to *X. gardneri*.

In conclusion, we were able to heterologously produce and isolate three novel lasso peptides from two different biosynthetic gene clusters. These lasso peptides are the first representatives of this class of natural compounds to feature a seven-residue macrolactam ring. Thus, the old paradigm that lasso peptides can only be produced with eight- and nine-residue rings could be proven wrong. Moreover, our mutational analysis highlights the limits such a small ring imposes on the lasso fold in terms of the amino acids that can be present in the inside of a seven-residue ring and at the same time reveals the minimum size for a plug amino acid that can still maintain a heat-stable lasso fold. Additionally, we presented the NMR structure of the six amino acid truncated xanthomin II and the crystal structure of the four amino acid truncated xanthomonin I, which was solved at 0.8 Å resolution.

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