The ring residue proline 8 is crucial for the thermal stability of the lasso peptide caulosegnin II†

Julian D. Hegemann,a Christopher D. Fage,a Shaozhou Zhu,a Klaus Harms,a Francesco Saverio Di Leva,b Ettore Novellino,b Luciana Marinelli,b and Mohamed A. Marahiel*a

Lasso peptides are fascinating natural products with a unique structural fold that can exhibit tremendous thermal stability. Here, we investigate factors responsible for the thermal stability of caulosegnin II. By employing X-ray crystallography, mutational analysis and molecular dynamics simulations, the ring residue proline 8 was proven to be crucial for thermal stability.

Lasso peptides are intriguing members of the superfamily of ribosomally synthesized and post-translationally modified peptides (RiPPs).1–5 While genome mining studies showed that they are distributed throughout the bacterial domain,4–8 only lasso peptides of proteo- and actinobacterial origin have been isolated and characterized thus far.4,5 Their unique defining feature is a 7–9 residue macrolactam ring that is threaded by the C-terminal tail.1–3,9 The ring is formed between the ω-amino group of the first residue (typically Gly, but some systems were shown to feature Ala, Ser or Cys instead8,10) and the carboxylic acid side chain of an Asp or Glu (Fig. S1, ESI†). This unusual fold is stabilized by bulky side chains of residues above and below the ring, so-called plug amino acids (AAs), which trap the threaded structure by sterical means. The fact that this topology is reminiscent of a lariat knot explains the name of this natural structure by sterical means. The fact that this topology is so far have been reported as well.4–9 Intriguingly, their compact structures confer a high stability against chemical and proteolytic degradation, which makes them useful scaffolds for the grafting of bioactive peptide epitopes.14

Additionally, lasso peptides can exhibit tremendous stability against thermal denaturation. This resistance against thermal denaturation was assumed to be true for all lasso peptides due to their shared topology as early studies on the lasso peptide microcin J25 revealed that it could even withstand autoclaving at 120 °C.11 Still, recent studies revealed that thermal stability is not intrinsic to all lasso peptides, and since this finding was reported more and more heat sensitive lasso peptides have been identified.6–8,15,16

The first lasso peptides that were thoroughly investigated in this regard were the caulosegnins I–III (CsegI–III; Fig. 1).5 These lasso peptides are produced by the same biosynthetic gene cluster and feature either an eight-(CsegI) or nine-residue (CsegII/III) macrolactam ring. Interestingly, while CsegI and CsegIII readily unthread at elevated temperatures, CsegII resists even prolonged incubation at 95 °C. This becomes especially intriguing considering that CsegII and CsegIII share a high level of similarity and utilize both Tyr16 as the lower plug as was previously reported.5 In this study, we explored factors that define the heat resistance of CsegII and thereby were able to identify Pro8 as a key residue responsible for its thermal stability.

So far, the main factors that are considered when discussing the thermal stability of lasso peptides are the nature and location

![Diagram](image-url)

**Fig. 1** Schematic representation of the caulosegnin biosynthetic gene cluster from *Caulobacter segnis* ATCC 21756.5 Below, an alignment of all three precursor peptides is shown. Gly1 of the core peptides, the ring forming Glu8/9 and Thr-2 of the leader regions are highlighted. A red arrow marks the lower plug residues.
of the lower plug AAs in combination with the size of the macro-
lactam rings. For example, the heat sensitive lasso peptide astexin-1
has a Gly1–Asp9 ring and was reported to be converted into a heat
resistant lasso peptide by exchange of its Phe15 plug residue with a
bulkier Trp.15 In another study, it was shown that the heat resistant
lasso peptide capistruin, also featuring a Gly1–Asp9 ring, could be
converted into a heat sensitive one.16 This was achieved by
exchanging both the original plug (Arg15) and the neighboring
Phe16 with Ala. In this R15A/F16A variant, Phe18 of capistruin is
still able to act as lower plug and thereby maintains the lasso fold.
Nevertheless, the double Ala substitution apparently causes such
an increase in the flexibility of the C-terminal tail that it is now able
to unfold at high temperatures. Xanthomonin II is another
highly stable lasso peptide comprising a ring of only seven AAs
(Gly1–Glu7).9 Due to the small diameter of its ring, any AA with a
side chain larger than Ser was found to act as a thermostable plug.9

These studies emphasize the importance of lower plug residues
for the thermal stability of a lasso peptide, but the question remains,
whether other residues can affect the behavior of a lasso peptide
upon exposure to elevated temperatures. This is especially intriguing
for CsegII and CsegIII; two lasso peptides with identical ring sizes,
identical lower plugs (Tyr16) and similar primary structures.5 Unlike
all of the aforementioned compounds, they comprise the largest
macrolactam ring (Gly1–Glu9) known for lasso peptides and employ
Tyr (besides Trp one of the bulkiest side chains possible) as plugs.
Therefore, we proposed that the observed differences in thermal
stability are caused by other residues.

To investigate this hypothesis, we generated a Y16W variant
of the heat sensitive CsegIII by site-directed ligase-independent
mutagenesis (SLIM),17,18 which was isolated and then incubated at
95°C. Even in this variant, Trp was found to be insufficient to confer
thermal stability to the lasso fold, as it still unfolds similarly to the
wild type (WT) CsegIII (see Fig. S2, ESI†). In contrast, the according
E16W substitution in CsegI, which features only an eight-residue
ring, transformed the heat sensitive WT into a heat resistant lasso
peptide, again emphasizing the importance of ring size in this
context (see Fig. S2, ESI†). Nonetheless, the question remained,
which residues other than the plugs may cause the thermal stability
of CsegII.

To allow a better estimate which residues could be important
for the heat resistance of CsegII (or the lack thereof in CsegIII), we
sought to elucidate the 3D structure of at least one of these lasso
peptides. For this, both compounds were applied to crystallization
screens, which yielded rod-shaped crystals of CsegII that diffracted
to a resolution of 0.86 Å and allowed the elucidation of its structure
(Fig. 2; see also Fig. S3 (ESI†) for a comparison with the lasso
peptide macrolactam rings of the compounds discussed above).

A defining feature of this structure is the kinks induced by the
four Pro residues at positions 5, 8, 13 and 18. In comparison to
CsegIII, the abundance of prolines in CsegII (4 out of 19 residues
versus 1 out of 19 in CsegIII) and the rather conserved substitution
of AAs at other positions led us to hypothesize a crucial role of the
prolines for the thermal stability of CsegII.

Therefore, a set of CsegII variants was generated by SLIM;17,18
namely P5A, P8A, P13A, P18A, P5A/P8A and P13A/P18A. All were
expressed under previously published conditions5 and production
was confirmed by analyzing the corresponding pellet extracts via
high resolution LC-MS. We then tested the thermal stability of each
variant by an assay that combines incubation at 95 °C with subsequent
carboxypeptidase Y (cpepY) treatment (Fig. 3).5,6,8,9,15

The results of these assays clearly show that WT, P5A, P13A,
P18A and P13A/P18A are all heat resistant lasso peptides. In contrast,
significant amounts of the P8A and P5A/P8A lasso
peptides were converted to new compounds upon incubation at
95 °C. The resulting compounds were furthermore completely
degraded to −9 AA truncation products by subsequent cpepY
treatment, while the residual lasso peptides were unaffected by this
protease.

As the only variants that showed a loss of thermal stability
were those carrying a P8A (one of the two prolines in the ring)
exchange, we wanted to investigate the lasso peptides featuring
substitutions of the ring prolines in more detail. Therefore, we
isolated the P5A, P8A and P5A/P8A variants and employed the
purified compounds alongside a WT control again for thermal
stability and cpepY assays (Fig. 4).

The results of these experiments were in accordance with the
pellet extract assays described above and clearly showed the

Fig. 2 Crystal structure of CsegII (PDB code 5D9E). (a) Stick representation
with the tail shown in blue and the ring in yellow. Further emphasized are Pro5
and Pro8 in orange as well as Pro13 and Pro18 in cyan. The ring forming Glu9 is
highlighted in red. (b) Schematic representation. (c) Stick representation,
 colored by elements (carbon in gray, nitrogen in blue, oxygen in red and sulfur
in yellow). As can be seen in this depiction, the Met17 thioether was oxidized to
a sulfoxide moiety during crystallization. (d) Surface maps of the macrolactam
ring (orange) and the His15 and Tyr16 side chains. The crystal structure
confirms the mutagenesis based prediction of His15 and Tyr16 as the upper
and lower plugs, respectively, as previously reported.5 (e) Alignment of CsegII
and CsegIII (36.8% identity, 63.2% similarity).
indicating that it is tightly locked in its native (crystallographic)
in the WT the ring shows a very low rmsd value (0.77
Peptide’s conformational flexibility, we computed their
Corresponding to a higher ring flexibility, are instead observed for the
Calculating the rmsd of residues 1–9 (Fig. S4a, ESI†)
Flexibility rate of the macrolactam ring in both peptides by
Heat resistant, though after 2 h at 95
Repetition of the combined thermal stability and cpepY assays
Additional triple substitution variants were generated, namely
Peptides unthread during prolonged incubation at high temperatures and are
Consequently more susceptible to subsequent cpepY mediated degradation.
Therefore, much shorter truncation products (up to the lone macrolactam ring)
Detected after treatment with cpepY.
To investigate in more detail how each ring AA influences the
From simulation trajectories. B-Factor mapping (Fig. 5) shows
that in the WT, residues 1 to 5 are the most flexible ones, while
Residues 6 to 9 appear somehow more rigid. This can be ascribed
to the formation of two tight hydrogen bonds (Fig. 5) between the
E9 and G14 and the L7 and Y16 residues, which are almost
Conserved during the whole simulation (Fig. S4b, ESI†). On the other hand, only one hydrogen bond with the C-terminal tail is
Established by residues 1–5, which is located between T2 and
H15 (Fig. 5 and Fig. S4b, ESI†). As expected, all of the As in the
P5A/P8A variant’s ring show higher B-factors compared to the
WT peptide, particularly those occupying positions 1 to 5. In fact, the
Hydrogen bond between T2 and H15 of the P5A/P8A variant is
Less stable than in the WT, although it is still present during the
MD simulation (Fig. S4b, ESI†). Overall, these results indicate
That the exchange of Pro to Ala, which is devoid of rigid
Constraints on the N–Cζ bond, increases the ring’s flexibility,
Probably allowing this variant to more easily adopt a conformation
Suitable for the unthreading of the C-terminal tail. Furthermore, we
have shown that while the P5A substitution occurs in a flexible
Region of the ring, the P8A exchange can induce fluctuations in a
More rigid ring fragment, explaining why it is crucial for trans-
Forming the heat resistant CsegII to a heat sensitive lasso peptide.
Based on these findings, we were interested if in absence of
Pro8, another Pro residue in the more rigid region of the
Macrolactam ring can fulfill its function and thereby would yield
Again a heat stable lasso peptide variant. For this, we generated
two additional double substitution variants, G6P/P8A and L7P/
P8A. While the G6P/P8A variant was not produced, L7P/P8A could be analyzed for thermal stability and cpepY resistance.
and thereby help to better understand the criteria that govern how these natural products behave at elevated temperatures.

Acknowledgements

The Deutsche Forschungsgemeinschaft (DFG) and the LOEWE Center for Synthetic Microbiology (SYNMIKRO) are gratefully acknowledged for financial support. Furthermore, we would also like to thank Holger Steuber, Julian Koch, Bastian Langer and Shawn Walsh for initial assistance with this project and Pavel Afonine for aid with the PHENIX software.

Notes and references

1 M. O. Maksimov, S. J. Pan and A. James Link, Nat. Prod. Rep., 2012, 29, 996–1006.
2 M. O. Maksimov and A. J. Link, J. Ind. Microbiol. Biotechnol., 2014, 41, 333–344.
3 J. D. Hegemann, M. Zimmermann, X. Xie and M. A. Marahiel, Acc. Chem. Res., 2015, 48, 1909–1919.
4 M. O. Maksimov, I. Pelczer and A. J. Link, Proc. Natl. Acad. Sci. U. S. A., 2012, 109, 15223–15228.
5 J. D. Hegemann, M. Zimmermann, X. Xie and M. A. Marahiel, J. Am. Chem. Soc., 2013, 135, 210–222.
6 J. D. Hegemann, M. Zimmermann, S. Zhu, D. Klug and M. A. Marahiel, Biopolymers, 2013, 100, 527–542.
7 M. O. Maksimov and A. J. Link, J. Am. Chem. Soc., 2013, 135, 12038–12047.
8 M. Zimmermann, J. D. Hegemann, X. Xie and M. A. Marahiel, Chem. Sci., 2014, 5, 4032–4043.
9 J. D. Hegemann, M. Zimmermann, S. Zhu, H. Steuber, K. Harms, X. Xie and M. A. Marahiel, Angew. Chem., Int. Ed., 2014, 53, 2230–2234.
10 M. Metelev, J. I. Tietz, J. O. Melby, P. M. Blair, L. Zhu, I. Livnat, K. Severinov and D. A. Mitchell, Chem. Biol., 2015, 22, 241–250.
11 R. A. Salomon and R. N. Farias, J. Bacteriol., 1992, 174, 7428–7435.
12 E. Gavrish, C. S. Sit, S. Cao, O. Kandror, A. Spoering, A. Peoples, L. Ling, A. Fetterman, D. Hughes, A. Bissell, H. Torrey, T. Akopian, A. Mueller, S. Epstein, A. Goldberg, J. Clardy and K. Lewis, Chem. Biol., 2014, 21, 509–518.
13 O. Potterat, K. Wagner, G. Gemmecker, J. Mack, C. Puder, R. Vettermann and R. Streicher, J. Nat. Prod., 2004, 67, 1528–1531.
14 J. D. Hegemann, M. De Simone, M. Zimmermann, T. A. Knappe, X. Xie, F. S. Di Leva, L. Marinelli, E. Novellino, S. Zahler, H. Kessler and M. A. Marahiel, J. Am. Chem. Soc., 2014, 527–542.
15 M. Zimmermann, J. D. Hegemann, X. Xie and M. A. Marahiel, Chem. Biol., 2013, 20, 558–569.
16 T. A. Knappe, U. Linne, L. Robbel and M. A. Marahiel, Chem. Biol., 2009, 16, 1290–1298.
17 J. Chiu, P. E. March, R. Lee and D. Tillett, Nucleic Acids Res., 2004, 32, e174.
18 J. Chiu, D. Tillett, I. W. Dawes and P. E. March, J. Microbiol. Methods, 2008, 73, 195–198.