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

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**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).<sup>1–3</sup> While genome mining studies showed that they are distributed throughout the bacterial domain,<sup>4–8</sup> only lasso peptides of proteo- and actinobacterial origin have been isolated and characterized thus far.<sup>2,3</sup> Their unique defining feature is a 7–9 residue macrolactam ring that is threaded by the C-terminal tail.<sup>1–3,9</sup> The ring is formed between the  $\alpha$ -amino group of the first residue (typically Gly, but some systems were shown to feature Ala, Ser or Cys instead<sup>8,10</sup>) 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 product family. Generally, lasso peptides were shown to exhibit a wide range of diverse biological activities, ranging from antimicrobial<sup>1,3,10–12</sup> to inhibitory<sup>1,3,12</sup> and antagonistic.<sup>1,3,13</sup> However, with the recent increase in lasso peptide isolations by genome mining approaches, a large number with unknown functions have been reported as well.<sup>4–8</sup>

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.<sup>14</sup>

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.<sup>11</sup> Still, recent studies revealed that thermal stability is not intrinsic to all lasso peptides, and since this finding was reported<sup>5</sup> more and more heat sensitive lasso peptides have been identified.<sup>6–8,15,16</sup>

The first lasso peptides that were thoroughly investigated in this regard were the caulosegnins I–III (CsegI–III; Fig. 1).<sup>5</sup> 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.<sup>5</sup> 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



Fig. 1 Schematic representation of the caulosegnin biosynthetic gene cluster from *Caulobacter segnis* ATCC 21756.<sup>5</sup> 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.

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of the lower plug AAs in combination with the size of the macrolactam 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.<sup>15</sup> In another study, it was shown that the heat resistant lasso peptide capistrain, also featuring a Gly1–Asp9 ring, could be converted into a heat sensitive one.<sup>16</sup> This was achieved by exchanging both the original plug (Arg15) and the neighboring Phe16 with Ala. In this R15A/F16A variant, Phe18 of capistrain 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 unthread at high temperatures. Xanthomonin II is another highly stable lasso peptide comprising a ring of only seven AAs (Gly1–Glu7).<sup>9</sup> 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.<sup>9</sup>

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.<sup>5</sup> 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),<sup>17,18</sup> 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 unthreads 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,<sup>17,18</sup> namely P5A, P8A, P13A, P18A, P5A/P8A and P13A/P18A. All were expressed under previously published conditions<sup>5</sup> and production

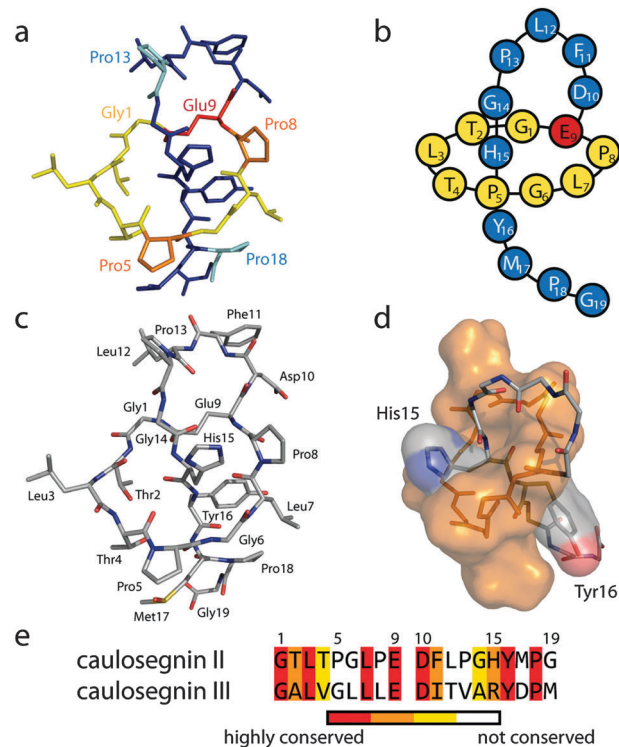


Fig. 2 Crystal structure of CsegII (PDB code 5D9E). (a) Stick representation with the tail shown in blue and the ring in yellow. Further emphasized are Pro5 and Pro8 in orange as well as Pro13 and Pro18 in cyan. The ring forming Glu9 is highlighted in red. (b) Schematic representation. (c) Stick representation, 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.<sup>5</sup> (e) Alignment of CsegII and CsegIII (36.8% identity, 63.2% similarity).

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).<sup>5,6,8,9,15</sup>

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







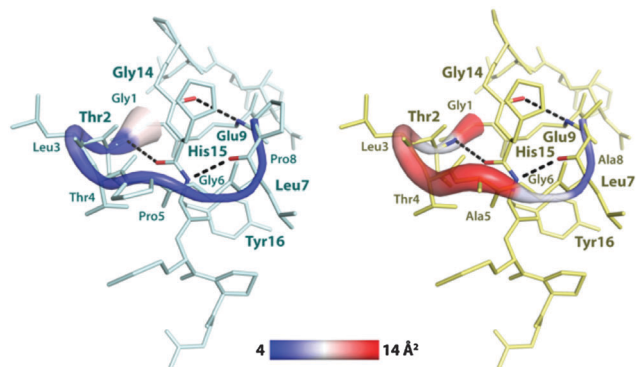


Fig. 5 B-factor mapping of residues in the macrolactam ring of the CsegII WT (left, in cyan) and in the P5A/P8A (right, in yellow) variant. Residues 1 to 8 in both peptides are highlighted as colored cartoons. The B-factor color scale ranges from 4 (blue) to 14 Å<sup>2</sup> (red). Hydrogen bonds are displayed as dashed black lines and the amino acids that form these bonds are labelled in bold.

Interestingly, even though the position of the proline is only shifted by one residue in this variant, the thermal stability was lost nonetheless. This might be explained by the absence of the aforementioned L7–Y16 hydrogen bond due to the L7P replacement and indicates that not only the proline at position 8 alone is essential for thermal stability, but that it might indeed be the concerned rigidity of Pro8 with other factors present in the CsegII scaffold. While this specific way of enabling thermal stability is most likely not present in every other heat stable lasso peptide scaffold, it is interesting to contemplate that these could at least contain similar variations thereof. Hence, it could be feasible that in these lasso peptides incorporation of specific residues at specific positions in the ring could also significantly alter their stability.

## Conclusions

In conclusion, we show in this study for the first time that even though the plug residues are important factors with regard to the maintenance of the lasso fold and its thermal stability, the AAs of the macrolactam ring also contribute to the overall stability of lasso peptides. Based on the crystal structure of CsegII and an alignment of CsegII and CsegIII, we proposed that the large number of prolines in CsegII is the reason for its high resistance against thermally induced unthreading. Surprisingly, our experiments revealed that only Pro8 is essential for a thermally stable lasso fold, while the other prolines can be substituted more or less freely with alanine.

This is especially highlighted by the observation that the thrice substituted P5A/P13A/P18A variant remains mostly heat stable and even though some slight unthreading at 95 °C can be observed, the unthreading process for this lasso peptide is still much slower than for the variant carrying a single P8A point mutation. Our findings were substantiated by MD simulations showing how the ring of CsegII becomes significantly more flexible upon Pro to Ala exchanges. Thus, the results of this study demonstrate the influence of the macrolactam ring's primary structure on the thermal stability of a lasso peptide

and thereby help to better understand the criteria that govern how these natural products behave at elevated temperatures.

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