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Insights into the Biosynthesis and Stability of the Lasso Peptide Capistruin

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SUMMARY

Capistruin is a 19-residue ribosomally synthesized lasso peptide encoded by the *capABCD* gene cluster in *Burkholderia thailandensis*. It is composed of an N-terminal 9-residue macrolactam ring, through which the 10-residue C-terminal tail is threaded. Using a heterologous capistruin production system in *Escherichia coli*, we have generated 48 mutants of the precursor protein CapA to gain insights into capistruin biosynthesis. Only 4 residues (Gly1, Arg11, Val12, and Ile13) of the lasso sequence were found to be critical for maturation. Tandem mass spectrometric fragmentation studies of capistruin F16A/F18A proved Arg15 to be responsible for the trapping of the C-terminal tail. Substituting Arg15 and Phe16 by alanine revealed a temperature-sensitive capistruin derivative, which unfolds into a branched cyclic peptide upon heating. In conclusion, our global mutagenic approach revealed a low overall specificity of the biosynthetic machinery and important structure-stability correlations.

INTRODUCTION

Lasso peptides are a structurally unique class of ribosomally synthesized, bioactive natural products of bacterial origin consisting of 16–21 amino acids (Rebuffat et al., 2004; Severinov et al., 2007). They are composed of an 8- or 9-residue N-terminal macrolactam ring generated upon a condensation reaction between the α-NH_2 group of an N-terminal Gly or Cys and the side-chain carboxyl group of an Asp9 or Glu8 (Bayro et al., 2003; Frechet et al., 1994; Iwatsuki et al., 2006; Katahira et al., 1995, 1996; Knappe et al., 2008; Rosengren et al., 2003; Wilson et al., 2003). Their primary structure can therefore be described as a branched cyclic peptide composed of an N-terminal macrolactam ring and a linear C-terminal tail. This tail threads through the macrocycle and is trapped by steric hindrance of bulky side chains within the ring generating the common lariat-protoknot structure of lasso peptides. This rigid and compact lasso fold results in remarkable stability against proteolytic degradation and thermal or chaotrope induced denaturation (Knappe et al., 2008; Rosengren et al., 2004). Lasso peptides are classified depending on the presence (class I) or absence (class II) of four conserved cysteine residues, which are involved in the formation of two intramolecular disulfide bonds (Rebuffat et al., 2004).

By nuclear magnetic resonance (NMR) spectroscopic studies, six bacterial peptides sharing the described primary structure have been proven to adopt the lariat-protoknot structure, namely RES-701-1 (Katahira et al., 1995; Morishita et al., 1994), MS-271 (Katahira et al., 1996; Yano et al., 1996), and RP 71955 (Frechet et al., 1994; Helynck et al., 1993) from different *Streptomyces* species; lariatin A (Iwatsuki et al., 2006) from *Rhodococcus* sp.; microcin J25 (Bayro et al., 2003; Rosengren et al., 2003; Wilson et al., 2003) (MccJ25) from *Escherichia coli*; and capistruin (Knappe et al., 2008) from *Burkholderia thailandensis* E264. Their biological activities range from inhibition of HIV replication (Helynck et al., 1993) (RP 71955) to blockage of Gram-negative bacterial RNA polymerase (Adelman et al., 2004; Mukhopadhyay et al., 2004) (microcin J25). The biosynthetic gene clusters are only known for the best-studied representative MccJ25 (Duquesne et al., 2007a, 2007c; Solbiati et al., 1999) and the recently described capistruin (Knappe et al., 2008).

Capistruin is a 19-amino-acid class II lasso peptide comprising an isopeptide bond between Gly1 and Asp9 resulting in a 9-residue macrolactam ring. NMR spectroscopic studies revealed Arg15 to be the first amino acid within the threading tail located below the macrocyclic ring, and due to the steric hindrance of the arginine side chain it is proposed to be responsible for the trapping of the linear C-terminal tail. Capistruin is the first lasso peptide discovered by a genome mining based approach and displays antibacterial activity against closely related *Burkholderia* and *Pseudomonas* strains. As in the *mcjABCD* gene cluster responsible for MccJ25 production, the capistruin biosynthetic gene cluster consists of four genes encoding (I) the 47-amino-acid capistruin precursor protein CapA, (II) the two processing enzymes CapB and CapC, and (III) the export and immunity protein CapD (Figure 1). It is assumed that the two processing enzymes CapB/CapC convert the ribosomally synthesized precursor protein CapA into the mature lasso peptide capistruin, as it was shown for the maturation of MccJ25 (Duquesne et al., 2007b). CapC is homologous to asparagine synthetase B catalyzing the transfer of ammonia from the amide donor glutamine to the side-chain carboxyl group of aspartic acid, which is activated in an initial reaction as adenylate (Larsen et al., 1999). Therefore, CapC is most likely involved in the side-chain carboxyl group activation of aspartic acid at position 9 generating the electrophile for the condensation reaction. CapB is a putative protease showing weak homology to bacterial transglutaminases (Makarova et al., 1999). It is

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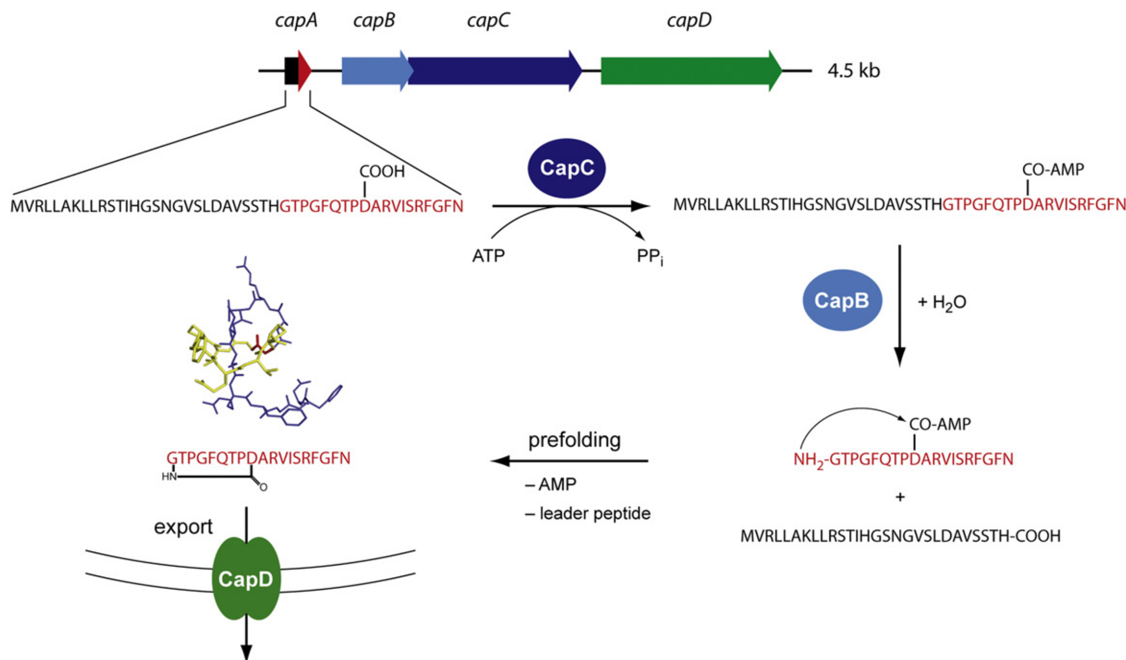

Figure 1. Proposed Biosynthesis of the Lasso Peptide Capistruin

The asn synthetase homolog CapC activates the side-chain carboxyl group of Asp9 by adenylation. The putative protease CapB cleaves the capistruin precursor protein CapA removing the leader peptide (shown in black) and setting free the N-terminal NH_2 group of Gly1. Following prefolding of the lasso sequence (shown in red) this NH_2 group acts as the nucleophile in the subsequent macrocyclization reaction yielding mature capistruin, which is exported out of the producing cell by the ABC transporter homolog CapD. Sequence of the shown reactions can also occur in an inverted manner.

assumed to cleave the precursor protein CapA and to set an N-terminal Gly free, whose α-NH_2 group acts as the nucleophile in the subsequent cyclization reaction. Prior to the macrolactamization, a prefolding of the 19-amino acid peptide into a lasso-like topology is crucial because the sterically demanding residues cannot pass the closed ring. CapD is homologous to ATP-binding cassette (ABC) transporters and may therefore mediate detoxification by exporting capistruin outside of the producing cells, as shown for the homologous MccJ25 export protein McjD (Solbiati et al., 1999, 1996).

A deeper understanding of capistruin biosynthesis has so far been limited by the low solubility of the heterologously expressed processing enzymes, which up to now prevents the purification and hence detailed in vitro studies. Therefore we constructed 48 mutants of the *capA* gene and analyzed these mutants in a previously invented heterologous capistruin production system in *E. coli* (Knappe et al., 2008) to determine (I) critical residues of the protease cleavage site, (II) the specificity of the side-chain carboxyl group activation, and (III) crucial positions within the lasso sequence for capistruin maturation. In addition, based on the results for single amino-acid substitutions, double and triple mutants were constructed to gain further insights into structure-stability relationships of the lasso fold of capistruin. Our mutational studies revealed only four positions within the 19-residue lasso sequence, forming a continuous area on the surface of capistruin, to be critical for processing, folding, and macrocyclization. Furthermore, a conserved threonine residue (Thr27) upstream of the protease cleavage site and the side chain of Asp9, which is involved in isopeptide bond formation, were found to be essential. Mass spectrometry (MS) analysis of capistruin F16A/F18A proved Arg15 to be the plug trapping the tail within the macrocycle. Alanine substitutions of Arg15 and Phe16 generated a labile, temperature-sensitive capistruin derivative, which unfolds into a branched cyclic structure upon heating.

RESULTS

Mutational Analysis of the Protease Cleavage Site

The bottleneck of detailed in vitro studies of capistruin biosynthesis so far has been the very limited solubility of the recombinant expressed processing enzymes CapB and CapC. Because capistruin can be synthesized heterologously in *E. coli* upon cloning of the entire 4.5 kb *capABCD* gene cluster into a pET41a(+) vector (Knappe et al., 2008), a mutational analysis of the *capA* gene was performed to gain first insights into the maturation reactions. In order to simplify the site-directed mutagenesis, the *capA* gene was cloned into a compatible pCDFDuet vector, and capistruin production was restored following co-transformation with pET41a(+)_*capA**BCD* (which contains a T27P mutation within *capA* abolishing capistruin synthesis) into *E. coli*. Thus the pCDFDuet_*capA* vector was utilized as the template for the construction of all mutants described in this study. The proposed maturation of capistruin includes the peptide bond cleavage reaction between His28 and Gly29 of the precursor protein CapA most likely catalyzed by CapB (Figure 1). To determine critical positions of this maturation reaction an alanine scan of the P4 to P1′ position of CapA (Ser25-Gly29) was performed.

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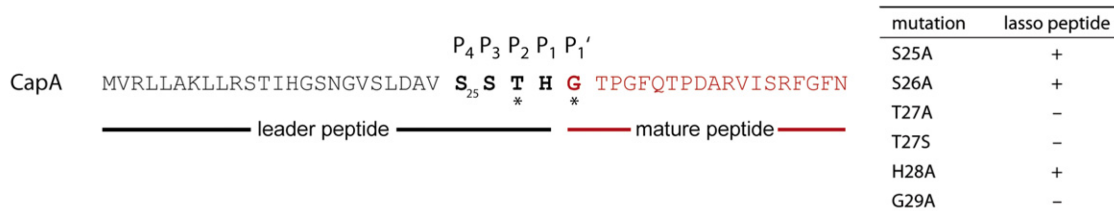

mutation	lasso peptide
S25A	+
S26A	+
T27A	–
T27S	–
H28A	+
G29A	–

Figure 2. Mutational Analysis of the Protease Cleavage Site of CapA

An alanine scan of S25-G29 corresponding to the P4 to P1′ position revealed T27 and G29 to be critical for capistruin production, which could not be restored by a T27S substitution. The two critical positions in the P2 and P1′ position are conserved within the microcin J25 precursor protein McjA (as indicated by an asterisk).

Following fermentation, extracts of the culture supernatants and of the cells were analyzed regarding the synthesis of capistruin or derivatives thereof. If the mutation did not abolish lasso peptide production, capistruin or the corresponding derivative was always detectable by liquid chromatography-mass spectrometry (LC-MS) in the culture supernatant. Substitution of Ser25, Ser26, or His28 corresponding to the P4, P3, and P1 position, respectively, by alanine did not influence the production of capistruin (Figure 2). In contrast, the alanine substitution of Thr27 in the P2 position abolished capistruin production and could not be restored by a conservative T27S replacement. Furthermore, Gly29 in the P1′ position, which is the first residue in the mature lasso peptide (Gly1), could not be substituted by alanine because the capistruin G1A derivative was not produced.

Alanine Scan of the Lasso Peptide Sequence

In addition to the analysis of the protease cleavage site, an alanine scan of the entire lasso peptide sequence was performed. Interestingly, with the exception of Asp9, 14 out of 18 positions could be substituted by alanine without influencing the biosynthesis of the lasso structure (Figure 3). Namely, Thr2-Pro8 corresponding to all residues within the N-terminal macrolactam ring, which are not involved in the cyclization reaction, and Ser14-Asn19 according to the residues in the C-terminal tail located within or below the macrocycle, could be replaced by alanine, yielding lasso structured capistruin derivatives. The production rates of these derivatives were in the range of 0.01 to 0.05 mg l^{-1}, as estimated by peak integration of extracted ion chromatograms (EIC), and therefore significantly lower in comparison to the wild-type peptide (0.1–0.2 mg l^{-1}).

The lasso structure of the capistruin variants was proven by gas-phase fragmentation studies as exemplified by the m/z 1010 doubly protonated species of the capistruin T2A derivative in Figure 3B. The spectrum shows a weak overall fragmentation behavior because the most intense ions observed resulted from the loss of neutral molecules (e.g., water or ammonia), which

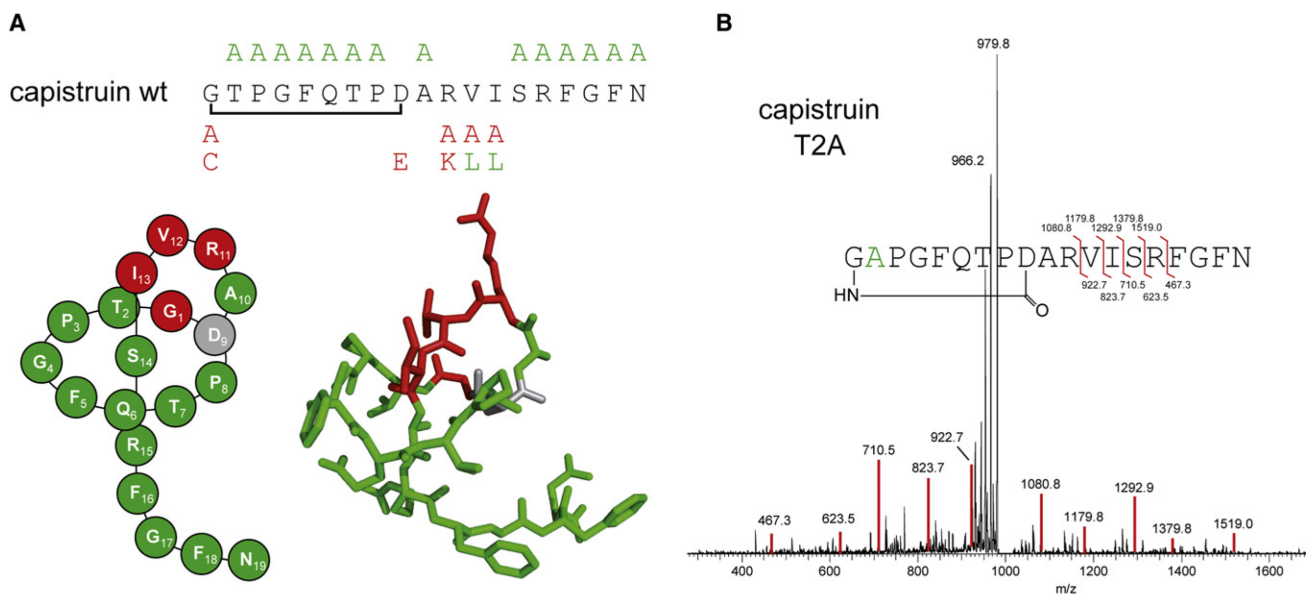

Figure 3. Mutational Analysis of Capistruin

(A) Overview of analyzed single amino acid substitutions within the lasso sequence of capistruin. Influence on the capistruin production is colored according to the traffic light principle (green, substitution yields lasso structured capistruin derivatives; red, no production). The results of the alanine scan are mapped onto a capistruin scheme and the NMR structure in solution shown as sticks using the same color code.

(B) MS^2 spectrum of the m/z 1010.0 doubly charged precursor ion of the capistruin T2A derivative. The main signals (m/z 979.8 and m/z 966.2) correspond to the loss of neutral molecules (e.g., water and/or ammonia) because wide band activation was used. The peaks colored red represent the b and y ions shown schematically.

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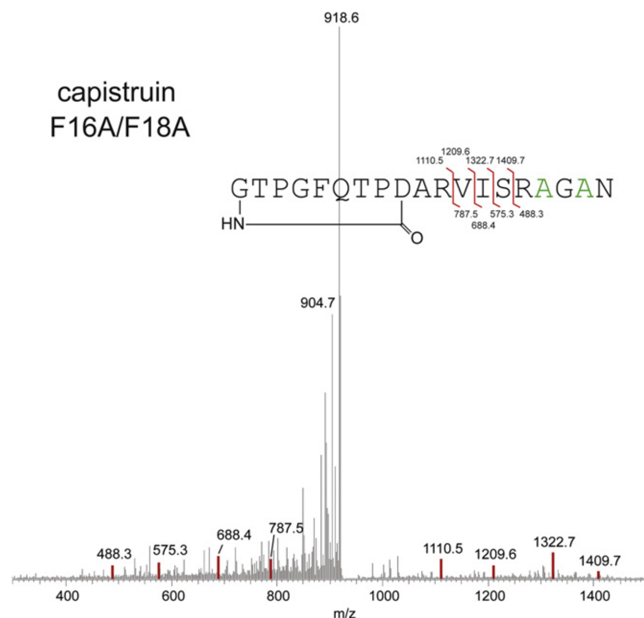

Figure 4. MS^2 Fragmentation Analysis of Capistruin F16A/F18A
MS^2 spectrum of the m/z 949 doubly protonated precursor ion of the capistruin F16A/F18A derivative. The two alanine substitutions are shown in green letters. The main signals correspond to the neutral loss of multiple molecules (e.g., water and/or ammonia) because wide band activation was used. The peaks colored red represent b and y ions shown schematically.

indicates a compact and rigid peptide structure. The remaining weak fragment ions present in the spectra originated from the C-terminal part of the peptide and all broken peptide bonds are located C-terminally to Arg11. All of these findings are consistent with the fragmentation behavior observed for capistruin (Knappe et al., 2008), and therefore prove the folding of the alanine derivatives into the compact lasso structure.

The four positions of the lasso sequence, which could not be replaced by alanine, were glycine at position one and the 3 residues located in the β-turn motif (Arg11-Ile13) of the C-terminal tail. Interestingly, Val12 and Ile13 could be substituted by leucine, generating the capistruin derivatives V12L and I13L, which indicates that conservative substitutions are accepted for these positions. In contrast, the R11K variant is not produced, suggesting an essential role of this position for capistruin maturation.

Because class I lasso peptides contain a cysteine residue at position one, a G1C mutant was generated. However no derivative could be detected. Aspartic acid at position nine plays an important role in the biosynthesis, because its side-chain β-carboxyl group represents the electrophile in the macrolactamization reaction. Therefore, only a substitution by glutamic acid is meaningful because all other proteinogenic amino acids would prevent cyclization and hence adoption of the lasso fold. It was, however, found that the D9E substitution, corresponding to a methylene extension of the macrolactam ring, abolished the biosynthesis of the capistruin derivative.

Lasso Engineering of Capistruin

In order to explore how the capistruin structure can be engineered by mutagenesis, we constructed 13 mutants by C-terminal truncation, extension, ring shortening, or ring extension (see Table S1 available online). Stop codons could be inserted at the positions Asn19, Phe18, and Gly17, yielding capistruin derivatives of 18, 17, or 16 amino acids in length. Further shortening of the lasso peptide was not tolerated by the biosynthetic machinery. One extra alanine could be added to the C terminus, yielding the 20-residue lasso peptide capistruin A20. Addition of further alanines to the C terminus abolished lasso peptide production. Interestingly, neither the ring extension by insertion of alanine or glycine between the positions Gly4-Phe5 or Phe5-Gln6 nor the ring shortening by deletion of Phe5 or Gln6 yielded capistruin derivatives composed of an 8-residue or 10-residue macrolactam ring.

Arginine 15 Traps the Tail within the Macrocyclic Ring of Capistruin

In previous studies we proposed that Arg15, which is the first amino acid positioned below the cycle, is due to its sterically demanding side chain responsible for trapping of the C-terminal tail within the ring (Knappe et al., 2008). Because single alanine substitutions are tolerated inside the C-terminal tail, we constructed all possible double and triple alanine mutants of the three bulky side chains Arg15, Phe16, and Phe18 to explore their influence on capistruin stability (Table S1). Two double mutants (capistruin R15A/F16A and capistruin F16A/F18A) were produced, whereas capistruin R15A/F18A and the triple mutant capistruin R15A/F16A/F18A, which does not contain any bulky side chain acting as a plug to entrap the C-terminal tail, could not be detected. To confirm our previous proposal, we performed gas-phase fragmentation studies of the m/z 949 doubly protonated species of capistruin F16A/F18A. This derivative contains only Arg15 as a bulky and sterically demanding residue within the C-terminal tail that could act as a plug. The MS^2 spectrum of capistruin F16A/F18A (Figure 4) shows a weak overall fragmentation behavior because the most intense ions follow from the loss of neutral molecules. The remaining weak fragment ions arose from the C-terminal tail of the capistruin derivative. Because these findings agree very well with the fragmentation pattern of capistruin (Knappe et al., 2008), we infer that the capistruin F16A/F18A derivative adopts a lasso fold and that Arg15 is responsible for the trapping of the C-terminal tail, because other bulky side chains are not present in this molecule.

Capistruin R15A/F16A Is a Temperature-Sensitive Lasso Peptide

The capistruin R15A/F16A derivative is the second alanine double mutant being heterologously synthesized in *E. coli*. This derivative contains only one sterically demanding side chain out of the three original ones, namely Phe18, and therefore should allow investigation whether phenylalanine in general is able to pass the 9-residue macrolactam ring of capistruin. The EIC of a supernatant extract of capistruin R15A/F16A producing cultures (Figure 5) revealed the presence of two compounds of the expected mass differing in their retention times (22 and 24 min). Because only one compound of the expected mass was observed in the extracts of all other capistruin derivative-producing cultures, this finding suggested the existence of two distinct conformations, most likely of a lasso structure and a branched cyclic structure. To discriminate between these two

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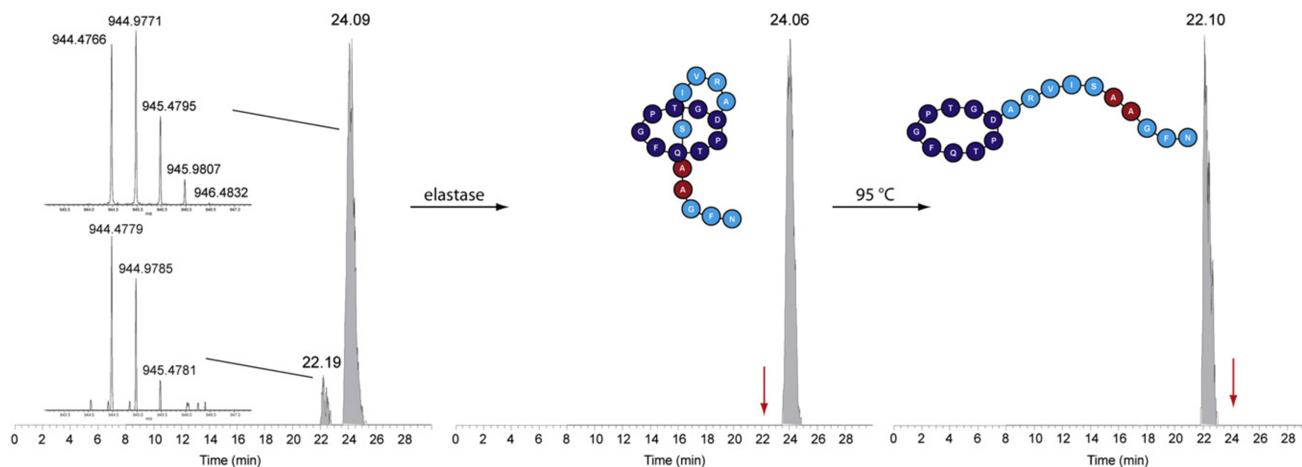

Figure 5. Temperature-Induced Unthreading of the Lasso Structure of Capistruin R15A/F16A

Extracted ion chromatograms (EIC) of an extracted culture supernatant revealed two compounds (RT 22.19 min and RT 24.09 min) corresponding to the calculated mass of the doubly protonated ion of capistruin R15A/F16A ($[M+2H]^{2+}_{calculated}$ = 944.4716). Elastase treatment resulted only in a degradation of the 22 min compound. The remaining 24 min compound could be converted into the one at 22 min upon incubation at 95°C. Further incubation of the heat-converted sample with elastase resulted in a complete digestion, indicating that the 24 min compound corresponds to the lasso-structured conformation and the 22 min compound resembles the branched-cyclic peptide.

conformations, gas-phase fragmentation studies were performed. Interestingly, both compounds were proven to be capistruin R15A/F18A, because the expected peptide fragments of the C-terminal tail were observed in MS^2 studies, but none of the two compounds displayed the prototypical fragmentation pattern of lasso structured capistruin (Figures S1 and S2). Neither the predominating loss of neutral molecules nor low-intensity fragment ions of the tail were found. Instead, both compounds yielded fragment ions of the C-terminal tail in good yields, therefore indicating a branched cyclic structure. A second method to distinguish between a branched cyclic and a lasso structured peptide is the susceptibility to hydrolysis by proteases, because the lasso structure was found to be stable against proteolytic degradation (Knappe et al., 2008), whereas the linear part of branched cyclic peptides composed of proteinogenic amino acids is sensitive to proteolysis. Because two additional alanines were introduced within the C-terminal tail, elastase, which cleaves peptide bonds after small, hydrophobic amino acids (e.g., glycin, alanine, valine), was used to investigate differences in the conformation. Interestingly, elastase treatment of the extract resulted in a complete degradation of the earlier eluting compound (RT 22 min), whereas the compound with a retention time of 24 min was not affected, hence indicating a lasso structure. Heating of the elastase treated extract, which contains only the 24 min compound, for 1 hr at 95°C, yielded an entire conversion into the 22 min compound. Further addition of elastase led to a complete degradation of the heat-converted compound (RT 22 min). Defined peptide fragments could not be detected upon elastase digestion, probably due to the presence of a mixture of cleavage products resulting in a low concentration of each single fragment or due to the presence of the small hydrophilic cleavage products in the flow through during high-performance liquid chromatography analysis.

In conclusion, capistruin R15A/F16A seems to be a temperature-labile lasso peptide, because a small fraction is already unfolded to a branched cyclic peptide during fermentation and extraction. This unfolded conformation has a decreased retention time (22 min) and is susceptible to degradation by elastase. The predominating lasso-structured peptide in the extract (RT 24 min) is not digested by elastase instead, but heat treatment leads to unfolding of the lasso structure into the branched cyclic conformation and converts it into a substrate of elastase.

DISCUSSION

Lasso peptides share the unique lariat-protoknot structure composed of a C-terminal tail entrapped within an 8/9-residue macrolactam ring. Thus they combine a very stable gene-encoded protein scaffold with a diverse spectrum of biological activities. Altogether these features make them ideal candidates for protein engineering efforts. To employ the biosynthetic machinery for such approaches, the maturation of lasso peptides has to be further investigated. In the current study, we performed a systematic mutational analysis of the capistruin precursor protein CapA to determine critical residues for the biosynthetic machinery and to study structure-stability relationships.

The maturation of capistruin from the ribosomally assembled precursor protein CapA involves the cleavage of the peptide bond between His28 and Gly29, a reaction assumed to be catalyzed by CapB. The alanine scan of the P4 to P1′ positions (Ser25-Gly29) revealed Thr27 at P2 and Gly29 at P1′ to be important for capistruin production. Interestingly, these two positions are conserved in the microcin J25 precursor protein McjA and therefore might be generally critical for class II lasso peptide generation (Figure 2). Because Thr27 could not be substituted conservatively by serine, the interaction between the threonine side chain and the S2 substrate binding pocket of the putative CapB protease seems to be important for the processing of CapA. Gly29 is the N-terminal amino acid in the mature peptide and as such involved in macrolactam formation. Therefore, the

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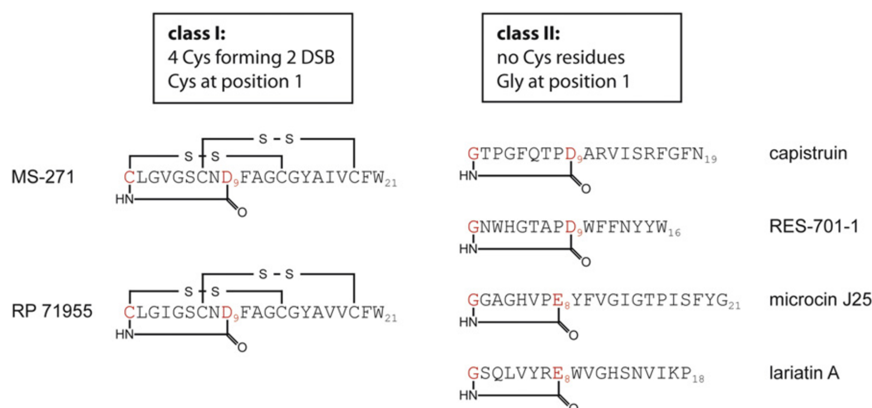

Figure 6. Classification of Lasso Peptides
Overview of the sequences of currently known lasso peptides, whose lariat-protoknot structures have been proven by NMR spectroscopy. Lasso peptides are classified concerning the presence (class I) or absence (class II) of 4 conserved Cys residues. In addition, class I peptides share a Cys at position one involved in the isopeptide bond to Asp9 (shown in red) whereas class II peptides contain a Gly at this position, forming the isopeptide bond to Asp at position 9 or Glu at position 8, respectively.

proteolytic cleavage might be specific for glycine at the P1′ position, but it cannot be ruled out that CapA_G29A is a substrate for the protease and the alanine substitution prevents the subsequent macrocyclization catalyzed by CapC. In addition, a G29C substitution also abolishes lasso peptide production. These findings are consistent with a systematic structure-activity analysis of the MccJ25 coding portion of McjA by Severinov and coworkers, which revealed the N-terminal Gly1 to be also essential for MccJ25 production because no other proteinogenic amino acid was tolerated (Pavlova et al., 2008). Considering that the G1C mutation inhibits the cleavage of the precursor and does not interfere with the cyclization, nature would have developed two different proteases for the biosynthesis of lasso peptides: one being specific for glycine at P1′ present in the gene cluster of class II lasso peptides, and another one cleaving N-terminal of a cysteine involved in the biosynthesis of class I peptides (Figure 6). This varying substrate specificity would limit gene shuffling approaches to synthesize hybrid molecules of both lasso peptide classes.

The second important maturation reaction of capistruin biosynthesis is the activation of the side-chain β-carboxyl group of Asp9 (Figure 1). This activation is most likely achieved by an adenylation reaction, because the in vitro biosynthesis of MccJ25 was shown to be ATP and Mg^{2+} dependent (Duquesne et al., 2007b). It is assumed to be catalyzed by the asparagine synthetase homolog CapC. Interestingly, a D9E substitution inhibits capistruin maturation indicating a high specificity for the length of the carboxyl side chain being adenylated. Taking into account that a E8D substitution within the lasso-coding sequence of McjA abolishes MccJ25 production (Pavlova et al., 2008), the carboxyl group activating enzymes in the capistruin and MccJ25 biosynthesis seem to be specific for aspartic acid and glutamic acid, respectively. To sum up, the enzymatic repertoire of lasso peptide biosynthesis in bacteria seems to contain two proteases, cleaving N-terminal either of glycine or cysteine, and two carboxyl group-activating enzymes specific for aspartic or glutamic acid, respectively.

An alanine scan of the lasso sequence of capistruin identified in addition to the already discussed Gly1, the 3 residues Arg11, Val12, and Ile13, located within the β-turn motif of capistruin, that could not be substituted by alanine. A leucine substitution of Val12 and Ile13, however, yielded lasso-structured capistruin derivatives, whereas Arg11 could not be replaced by lysine, indicating an essential role of Arg11 in the maturation of capistruin. Interestingly, these 4 identified residues form a continuous area on the surface of capistruin (Figure 7). Therefore, we propose, that the biosynthetic machinery most likely recognizes this surface during capistruin maturation. Perhaps it is a discriminating feature of the prefolding reaction. All other positions, including all residues within the ring (Thr2-Pro8), which are not involved in the cyclization reaction, and the residues inside the tail located within or below the macrolactam ring (Ser14-Asn19), tolerate alanine substitutions. These substitutions, however, have an impact on the conversion of the precursor protein into the lasso peptide by the enzymatic machinery, because the production rates of the variants were considerably lower compared with the wild-type peptide. Taking into account the rather weak antibacterial activity of capistruin with MIC values in the range of 12 to 150 μM (Knappe et al., 2008), antibacterial activity studies are currently not feasible. The development of a homologous production system should facilitate increased production levels and thus overcome the current limitations of the heterologous system. However, in conjunction with the mutagenesis results of MccJ25 revealing only three irreplaceable positions (Pavlova et al., 2008), class II lasso peptides, as capistruin or MccJ25, are promising platforms for protein engineering efforts either to improve their intrinsic activities or to introduce new biological activities. Interestingly, all produced capistruin variants were found in the culture supernatant, which is consistent with the results obtained for microcin J25 derivatives (Pavlova et al., 2008), and indicates a low overall specificity of the exporter CapD. An involvement of the unmodified leader peptide in the export process could also be an explanation for this finding.

The capistruin F16A/F18A derivative contains only Arg15 as a sterically demanding residue inside the tail. MS fragmentation studies verified its lasso structure and thus proved Arg15 to act as a plug entrapping the C-terminal tail within the 9-residue ring. This result further explains the fragmentation pattern of capistruin and its derivatives, where only fragments C-terminally of Arg11 were observed (Knappe et al., 2008). Because arginine in general is not capable of passing the ring, a peptide bond breakage N-terminally of Arg11 results in a binary peptide complex comprising a cyclic and a linear peptide, which is trapped by the sterically demanding Arg residues located on opposite sides of the ring. This binary complex cannot be distinguished from native capistruin in MS experiments. The existence

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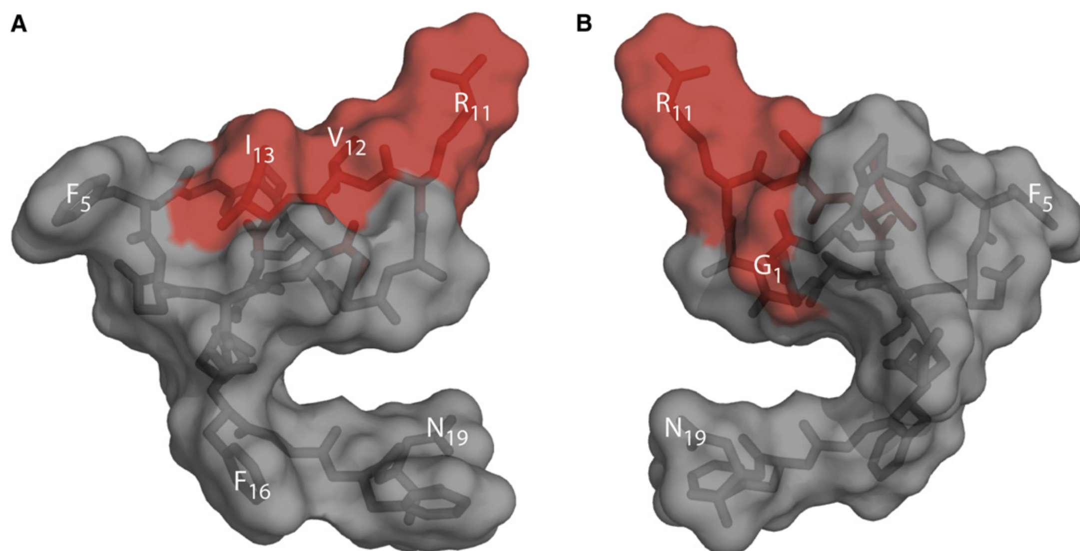

Figure 7. Location of Important Residues for Capistruin Biosynthesis

(A) Capistruin and its solvent-accessible surface are shown. Critical residues for capistruin maturation (Gly1, Arg11, Val12, and Ile13) are highlighted red.

(B) View rotated by 180° along the *y* axis.

of such binary peptide complexes in MS^2 studies has been described for MccJ25 (Rosengren et al., 2003) and a binary complex derived from thermolysin cleavage could even be structurally investigated by NMR spectroscopy (Rosengren et al., 2004). In conclusion, capistruin is the only known example where a basic amino acid is used to trap the linear tail, because in all other lasso peptides aromatic or hydrophobic residues are employed for this purpose.

The capistruin R15A/F16A derivative lacks the bulky side chains of arginine and phenylalanine at position 15 and 16, and therefore only Phe18 can act as a plug. Heating experiments combined with elastase digestion assays proved the R15A/F16A variant to adopt the lasso fold but to be temperature sensitive because it could be converted into an elastase cleavable form upon heating. Therefore, capistruin R15A/F16A is a labile lasso peptide that is resistant to proteolytic cleavage but not stable against temperature-induced unfolding. Interestingly, mass fragmentation studies failed to prove the lariat-protoknot structure of the variant, because no significant difference in the fragmentation pattern of the lasso structured and the branched cyclic peptide was observed (Figures S1 and S2). We propose that the temperature-sensitive lasso peptide already unfolds in the collision cell of the mass spectrometer, and therefore MS^2 studies cannot be applied to distinguish between the two topologies. In summary, Phe18 is able to pass the 9-residue ring in the capistruin R15A/F16A derivative. Surprisingly, in the capistruin R15A variant the two phenylalanines at position 16 and 18 are able to stabilize the lasso structure as indicated by the prototypical fragmentation pattern. Probably the extension of the β-turn in the R15A/F16A variant compared with the R15A assists in the temperature-induced unfolding of the lasso structure. Unfortunately, the R15A/F18A variant, which could support this hypothesis, is not produced.

In conclusion, the mutational analysis provides first insights into the specificity of the processing enzymes involved in capistruin biosynthesis and revealed the determinants for the stabilization of the lariat-protoknot structure. Due to the low specificity of the biosynthetic machinery regarding the overall lasso sequence, combined with the ribosomal coding, extraordinary stability and the ability of fermentative production, capistruin can be regarded as a promising platform for future protein engineering approaches.

SIGNIFICANCE

Lasso peptides are ribosomally synthesized, bioactive peptides of bacterial origin sharing a complex three-dimensional structure. They consist of an N-terminal 8/9-residue macrolactam ring, through which the C-terminal tail is threaded and irreversibly trapped by steric hindrance. The combination of extraordinary stability, various biological activities, and ribosomal synthesis makes lasso peptides ideal platforms for drug design. However, prior to these approaches, the biosynthesis of lasso peptides needs to be further investigated. Capistruin is a 19-residue lasso peptide secreted by *Burkholderia thailandensis* E264 displaying antibacterial activity against closely related *Burkholderia* and *Pseudomonas* species. The 4.5 kb *capABCD* gene cluster located on the chromosome I of *B. thailandensis* is responsible for its biosynthesis. In the present study, we have performed a mutational analysis of the capistruin precursor protein CapA to investigate the specificity of the two processing enzymes CapB and CapC and to study structure-stability correlations. The constructed 48 mutants revealed that only 4 residues of the 19-residue lasso sequence seem to be critical for lasso peptide maturation, indicating a low overall specificity of the biosynthetic machinery. A conserved Thr27 within the leader peptide was essential for CapA processing and the macrocyclization reaction was specific for the β-carboxyl side chain of Asp9

at the branching point. The double alanine mutant capistruin F16A/F18A displayed a prototypical capistruin mass fragmentation pattern, which proved Arg15 to be solely responsible for the trapping of the C-terminal tail within the macrolactam ring. Substitution of Arg15 and Phe16 by alanine generated a temperature-sensitive capistruin variant, which adopts the lasso fold, but unfolds into a branched cyclic, elastase-sensitive peptide upon heating. Our results provide first significant insights into capistruin biosynthesis regarding specificity of the biosynthetic machinery and stability determinants, and should facilitate future protein engineering and gene shuffling approaches to generate lasso peptides of designed stability and biological activity.

EXPERIMENTAL PROCEDURES

Bacterial Strains and General Methods

Escherichia coli NEB 10-beta was purchased from New England Biolabs and used as general host for cloning. *E. coli* BL21(DE3) was purchased from Invitrogen and used for heterologous production of capistruin and derivatives thereof. *Burkholderia thailandensis* E264 was purchased from German Collection of Microorganisms and Cell Cultures (DSMZ). Oligonucleotides were purchased from Eurofins MWG Operon. Pancreatic elastase was purchased from Sigma. DNA dideoxy sequencing confirmed the identity of constructed plasmids.

Cloning and Site-Directed Mutagenesis of pCDF-Duet_*capA*

Phusion High-Fidelity DNA-Polymerase (New England Biolabs) was used for cloning and site directed mutagenesis following the instructions of the manufacturer.

The *capA* gene of the capistruin biosynthetic gene cluster was amplified from genomic DNA of *B. thailandensis* E264 by polymerase chain reaction using the forward primer (*Nde*I) ATATCATATGGTT CGACTTTTGGCGAA GCTGC and the reverse primer (*Xho*I) ATATCTCGAGTTAATTGAAC CCGAA GCGCGAAATGACG. The resulting amplicon was digested with *Nde*I and *Xho*I (New England Biolabs) and cloned into pCDFDuet-1 (Novagen). pCDFDuet_*capA* was used as a template for site-directed mutagenesis. Mutagenesis was performed with Phusion High-Fidelity DNA-Polymerase in HF buffer in the presence of 5% dimethyl sulfoxide. For each targeted codon, a pair of ~35 nt complementary oligonucleotide primers introducing the designated substitutions was used. Mutagenized plasmid DNA was transformed into *E. coli* NEB 10-beta. After overnight incubation on LB agar plates containing 100 μg ml^{-1} spectinomycin, plasmids were prepared from individual transformants and analyzed by sequencing.

Heterologous Production and Extraction of Capistruin and Capistruin Derivatives

For heterologous production of capistruin or derivatives thereof, *E. coli* BL21(DE3) cells were cotransformed with pCDFDuet_*capA* or a pCDFDuet vector containing a *capA* mutant and pET41a(+)_*capA*BCD* (containing a T27P mutation within CapA suppressing capistruin synthesis). Cells were grown in LB medium containing 100 μg ml^{-1} spectinomycin and 50 μg ml^{-1} kanamycin at 37°C overnight. M20 medium supplemented with spectinomycin (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}), thiamine (2 μg ml^{-1}), and biotin (2 μg ml^{-1}) was inoculated with the starter culture to an OD_{600} of 0.01 and cultivated at 37°C. Cells were induced by addition of IPTG to a final concentration of 0.05 mM at an OD_{600} of 0.6 and harvested after further incubation for 12 to 48 hr at 37°C by centrifugation. Cell pellet was extracted twice with 100 ml methanol. The culture supernatant was applied to solid phase extraction using XAD16 resin (Sigma) (~5 g per liter culture supernatant). Upon incubation of the culture supernatant with XAD16 resin, the supernatant was removed by filtration and the resin was washed with water and eluted with methanol. Methanol extracts of the pellet and the supernatant were evaporated to dryness, dissolved in 1.6 ml 20% methanol, and analyzed by LC-MS. Production rates of the various capistruin derivatives were generally lower than the wild-type peptide (0.1–0.2 mg l^{-1}) and were estimated to be in the range of 0.01–0.05 mg l^{-1} based on peak integration of EICs.

Mass Spectrometric Analysis

The mass spectrometric characterization of capistruin or its derivatives was performed with an LTQ-FT instrument (Thermo Fisher Scientific, Bremen, Germany) connected to a microbore 1100 HPLC system (Agilent, Waldbronn, Germany). Separation of extracted capistruin or capistruin derivatives from contaminants was achieved using a 125/2 Nucleodur C18ec column (Macherey-Nagel, Düren, Germany) applying the following gradient of water/0.05% formic acid (solvent A) and acetonitrile/0.045% formic acid (solvent B) at a column temperature of 40°C and a flow rate of 0.2 ml min^{-1}: Linear increase from 10% B to 40% B within 30 min followed by a linear increase to 95% B in 5 min and holding 95% B for additional 2 min.

CID fragmentation studies within the linear ion trap were either done using online LC-MS or utilizing purified capistruin samples. The purified samples were analyzed using a syringe pump at a flow rate of 10 μl min^{-1}. Usually the doubly charged ions were selected for fragmentation in the ion trap, because they were the dominant species in the spectrum. The energy for fragmentation was set to 35 in all cases.

Stability Studies of Capistruin R15A/F16A and R15A

An extract of the supernatant of a 6 l culture was prepared as described above and dissolved in 1.6 ml 20 mM Tris/HCl (pH 8.0). In a 30 μl reaction, 25 μl of the extract was incubated in the presence or absence of 0.025 units elastase (Sigma) for 1 hr at 25°C (20 mM Tris/HCl [pH 8.0] was used to fill up to 30 μl). Afterwards the samples were heated for 1 hr at 95°C, cooled down to 25°C, and incubated again in the presence or absence of additional 0.025 units elastase for 1 hr at 25°C. After each single incubation step, 20 μl of the reaction mixtures was analyzed by LC-MS.

SUPPLEMENTAL DATA

Supplemental Data include two figures and one table and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00400-1.

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