JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Article

pubs.acs.org/JACS

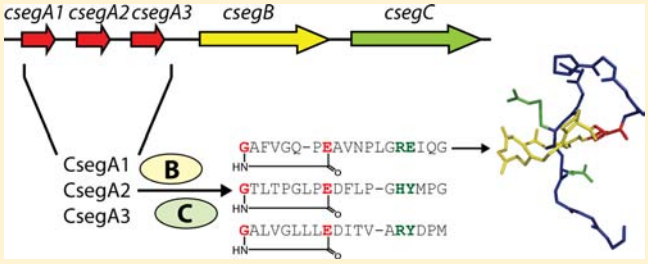
Caulosegnins I–III: A Highly Diverse Group of Lasso Peptides Derived from a Single Biosynthetic Gene Cluster

Julian D. Hegemann, Marcel Zimmermann, Xiulan Xie, and Mohamed A. Marahiel*

Department of Chemistry, Biochemistry, Philipps-University Marburg, Hans-Meerwein-Strasse 4 and LOEWE-Centre for Synthetic Microbiology, D-35032, Marburg, Germany

Supporting Information

ABSTRACT: Lasso peptides are natural products of ribosomal origin with a unique knotted structural fold. Even though only a few of them are known, recent reports of newly isolated lasso peptides were scarce. In this work, we report the identification of a novel lasso peptide gene cluster from *Caulobacter segnis*, that produces three new lasso peptides (caulosegnins I, II, and III) using a single biosynthetic machinery. These lasso peptides possess different ring sizes and amino acid sequences. In this study, we have developed a system for enhanced lasso peptide production to allow isolation of these compounds through heterologous expression in *Escherichia coli*. We were able to elucidate the structure of the most abundant lasso peptide caulosegnin I via NMR spectroscopic analysis and performed a thorough mutational analysis that gave insight into their biosynthesis and revealed important factors affecting the stabilization of the lasso fold in general. The caulosegnins also show a diverse behavior when subjected to thermal denaturation, which is exceptional as all lasso peptides were believed to have an intrinsic high thermal stability.



INTRODUCTION

Lasso peptides are ribosomally assembled post-translationally modified peptides that show an inimitable knotted fold. They feature a macrolactam ring formed between the N-terminus of a glycine or cysteine residue at position 1 and the side chain of a glutamate or aspartate residue at position 8 or 9.[1−13] Additionally, they have a C-terminal tail that is threaded through the macrolactam ring and sterically trapped by bulky amino acids.[3,6,11,12,14−20] This structural motif is the main reason why lasso peptides often exhibit an extraordinary stability against heat, chemicals and proteolytic degradation[2,3,6,8,11,21−25] and it is worth mentioning that up to date it is still not possible to generate such structures through synthetic means.[26,27] Known lasso peptides can be divided into three classes. Class I lasso peptides feature a cysteine residue at position 1 and have a total of four cysteine residues forming two disulfide bonds. Class II lasso peptides feature a glycine residue at position 1 and do not contain any cysteines. Class III lasso peptides combine features of the first two classes, containing a glycine at position 1 and two cysteines forming one disulfide bond.[14]

In addition to their unique structural fold, lasso peptides often possess interesting biological activities such as inhibition of the HIV-protease,[6,7] the glucagon receptor,[10] the atrial natriuretic factor[1] and other medicinally relevant targets,[4,5,8,9] as well as antimicrobial activities.[2,3,7−9,11,12,28−31] While the antimicrobial activities, which are often directed against closely related species or bacteria living in the same habitat, are assumed to give the producer an advantage against competitors when nutrients are scarce, the anti-enzymatic activities may not reflect the natural functions of the lasso peptides, as they were observed in systematic screens for compounds with such activities.

Lasso peptides are interesting not only for their intrinsic bioactivities, but also for their genetic accessibility and their highly promiscuous processing enzymes, allowing their use for epitope grafting. It was recently shown that microcin J25 (MccJ25), the best studied lasso peptide, could be utilized as a scaffold to carry the RGD peptide epitope, enabling a nanomolar inhibition of the $\alpha_v\beta_3$ integrin receptor that plays major roles in angiogenesis and tumor growth.[32]

Even though lasso peptides feature such interesting characteristics and activities, the biosynthetic gene clusters of only four lasso peptides (as well as two putative clusters from lasso peptides only observed via mass spectrometric analysis) are currently known.[12,13,33−35]

The gene cluster of MccJ25 contains four genes. The *mcjA* gene encodes the precursor peptide, while *mcjB* encodes an ATP-dependent cysteine protease with homology to transglutaminases, which was shown to cleave off the leader peptide and is assumed to have some chaperone activity because of its ATP-dependency.[36−38] An asparagine synthetase homologue is encoded by *mcjC*, which catalyzes the ring formation in an ATP-dependent manner,[36−38] whereas *mcjD* encodes an ABC-transporter, that confers immunity to its host.[33] The capistruin

Received: August 22, 2012
Published: December 8, 2012

   dx.doi.org/10.1021/ja308173b | *J. Am. Chem. Soc.* 2013, 135, 210−222

biosynthetic gene cluster is arranged in a similar manner,[12] while that of lariatin contains an additional gene coding for an 84 amino acid small protein of unknown function that was shown to be essential for lasso peptide maturation.[34] The gene cluster of the recently reported astexin-1 contains homologues to *mcjB* and *mcjC*, but unlike all clusters mentioned above lacks an ABC-transporter.[35] As the ATP-dependent cysteine proteases have no known homologues outside of lasso peptide biosynthetic gene clusters, they are useful targets for genome mining approaches.[12]

Through use of a genome mining strategy combined with an optimized heterologous expression system, we were able to identify a new lasso peptide biosynthetic gene cluster from *Caulobacter segnis* and could show that this single enzymatic system is able to produce three class II lasso peptides, designated caulosegnins I–III, with different primary structures, ring sizes and stability profiles. Furthermore, we provide the NMR structure of caulosegnin I and a detailed mutagenesis study of this novel system.

EXPERIMENTAL PROCEDURES

Bacterial Strains and General Methods. *C. segnis* (DSM no. 7131) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). *Escherichia coli* TOP10 was used for cloning and *E. coli* BL21 (DE3) was used for heterologous expression. Both strains were purchased from Invitrogen. DNA dideoxy sequencing confirmed the identity of constructed plasmids and mutants thereof. Oligonucleotides, proteinase K and carboxypeptidase Y were purchased from Sigma Aldrich. Elastase, trypsin and chymotrypsin were purchased from Promega. Restriction enzymes, Phusion polymerase and T4 DNA ligase were purchased from New England Biolabs.

Fermentation of *C. segnis*. For homologous fermentation of *C. segnis*, different culture media were used, namely, LB medium (10 g/L bactotryptone, 5 g/L yeast extract, 5 g/L NaCl, pH = 7.0), M9 minimal medium (17.1 g/L $Na_2HPO_4 \cdot 12\ H_2O$, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 1 mL/L $MgSO_4$ solution (2 M), 0.2 mL/L $CaCl_2$ solution (0.5 M), pH = 7.0, after autoclaving, 10 mL/L glucose solution (40% w/v) and 0.2 mL/L vitamin mix (Supporting Information Table S1) were added), M20 minimal medium (20 g/L L-glutamic acid, 0.2 g/L L-alanine, 1.0 g/L sodium citrate, 20 g/L $Na_2HPO_4 \cdot 12\ H_2O$, 0.6 g/L KCl, 0.6 g/L Na_2SO_4, 0.02 g/L $FeSO_4 \cdot 7\ H_2O$, 2 mL/L $MgCl_2$ solution (0.5 M), 103 μL/L $CaCl_2$ solution (0.5 M), 346 μL/L $MnSO_4$ solution (0.1 M), pH = 7.0, after autoclaving, 0.2 mL/L biotin solution (10 mg/L) and 0.2 mL/L thiamine solution (10 mg/mL) were added), M63 minimal medium (13.6 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 1 g/L bactopeptone, 1 mL/L $MgSO_4$ solution (2 M), pH = 7.0, after autoclaving, 5 mL/L glucose solution (40% w/v), 0.2 mL/L biotin solution (10 mg/L) and 0.2 mL/L thiamine solution (10 mg/mL) were added) and PYEGR medium (2 g/L bactopeptone, 1 g/L yeast extract, 0.8 mL/L $MgSO_4$ solution (2 M), pH = 7.0, after autoclaving, 2.5 mL/L glucose solution (40% w/v) and 0.2 mL/L riboflavin (2 mg/mL) were added).

A total of 300 mL of each medium was inoculated with 3 mL of *C. segnis* PYEGR overnight cultures and cultivated for 7 days at 20 or 30 °C. Cultures were harvested by centrifugation. Cell pellets were extracted with 50 mL of MeOH. Culture supernatants were applied to solid phase extractions using XAD16 resin (Sigma Aldrich, ~7 g/L culture supernatant). After 1 h of stirring the XAD16 containing supernatant at room temperature, the supernatant was removed by filtration. The resin was then washed with water and subsequently eluted with a total of 50 mL of MeOH. Solvent was removed via evaporation at 40 °C and reduced pressure. Dry pellet and supernatant extracts each were resuspended in a total of 900 μL of 50% MeOH, cleared by centrifugation and analyzed via LCMS.

Mass Spectrometric Analysis. Mass spectrometric analysis of extracts was performed with a LTQ-FT ultra instrument (Thermo Fisher Scientific) connected to a microbore 1100 HPLC system (Agilent) using 100 μL extract for each measurement. For detection, the UV absorption at 215 nm was recorded. Separation was achieved using a CC 125/2 Nucleosil 300–8 C18 column (Macherey-Nagel) applying the following gradient of water/0.1% trifluoroacetic acid (solvent A) and MeCN/0.1% trifluoroacetic acid (solvent B) at a column temperature of 40 °C and a flow rate of 0.2 mL/min: holding 2% B for 2 min, followed by a linear increase from 2% to 30% B in 18 min, a subsequent linear increase from 30% to 95% B in 15 min and holding 95% B for additional 2 min.

Mass spectrometric analysis of the thermal stability and protease assays of the purified lasso peptides was performed with a 1100 series MSD (Hewlett-Packard) coupled with a microbore 1100 HPLC system (Agilent). Separation was achieved using a EC 125/2 Nucleodur 100-3 C18ec column and specific gradients. For caulosegnin I the following gradient was used at 50 °C column temperature at a flow rate of 0.2 mL/min: Linear increase from 25% to 35% B in 15 min, followed by a linear increase from 35% to 95% B in 2 min and holding 95% B for additional 5 min. For caulosegnin II and III, another gradient at 25 °C column temperature and a flow rate of 0.3 mL/min was used: Linear increase from 30% to 50% B in 15 min, followed by a linear increase from 35% to 95% B in 2 min and holding 95% B for additional 5 min.

Collision-induced dissociation fragmentation studies within the linear ion trap were done using online LCMS. In all cases, the doubly charged ions were selected for fragmentation as they were the dominant species in the spectrum. The energy for fragmentation was set to 35% for every measurement performed.

For quantification UV-peak areas were integrated and relative production was determined by comparison to the UV-peak area of the corresponding wild type.

Cloning, Mutagenesis, and Modification of the Caulosegnin Gene Cluster. For cloning, the *csegAAABC* gene cluster was amplified from genomic DNA of *C. segnis* by PCR using appropriate oligonucleotide primers (Supporting Information Table S2), Phusion polymerase, GC-buffer and 10% DMSO. Annealing temperature was set to 65 °C and extension was performed for 4.5 min at 72 °C. After purification, the resulting ~3.2 kb amplicon was digested with *NdeI* and *HindIII* and cloned into the likewise digested pET41a vector.

Mutagenesis was performed via PCR using the above-mentioned conditions and 5′-phosphorylated oligonucleotide primers (Supporting Information Table S3–S5) containing the mutated sequence. The linear DNA amplicon (carrying the desired mutation) was purified, subjected to *DpnI* digestion to remove residual template DNA and a blunt end ligation at 16 °C overnight was performed. For ligation, 150–500 ng of purified DNA amplicon was used. Deletions and sequence substitutions were achieved in a likewise manner with appropriate 5′-phosphorylated oligonucleotide primers (Supporting Information Table S6).

Ligated DNA was transformed into *E. coli* TOP10. After overnight incubation on LB agar plates containing 50 μg/mL kanamycin, plasmids were prepared from indiviual transformants and isolated plasmids were analyzed by DNA sequencing. For heterologous expression, plasmids bearing the correct sequences were retransformed into *E. coli* BL21(DE3).

Heterologous Synthesis of Caulosegnins I–III in *E. coli*. LB medium was supplemented with 50 μg/mL kanamycin, inoculated with *E. coli* BL21 cells carrying the respective plasmids and grown overnight at 37 °C. With such overnight cultures, the fermentation media were inoculated to an OD_{600} of 0.01.

Initial screens were done in M9, M20 and M63 minimal medium and in LB medium and fermented for either 1 day at 37 °C or 3 days at 20 °C. Expression was induced by the addition of IPTG to a final concentration of 0.05 mM at an OD_{600} of ~0.6. For fermentation at 20 °C, cells were at first grown to an OD_{600} of ~0.4 at 37 °C, then slowly cooled to 20 °C in the course of 1 h, induced when reaching the target OD_{600} of ~0.6 and furthermore cultivated at 20 °C until harvested.

For optimization screenings, 1200 mL of M9 minimal medium were used for each construct. For variants (and respective wild type controls) 600 mL M9 minimal medium were used for each variant. In

dx.doi.org/10.1021/ja308173b | J. Am. Chem. Soc. 2013, 135, 210–222

both cases, the cultures were fermented as described for 3 days at 20 °C. At the end of each fermentation of a variant, the OD_{600} was measured and compared to the OD_{600} of the corresponding wild type fermentation to rule out that a possible toxicity of the produced compound hindered the cell growth and thus caused a low yield. Cells were harvested by centrifugation and cell pellets were extracted with 50 mL of MeOH. Solvent was removed at 40 °C and reduced pressure. Dried extracts were resuspended in a total of 800 μL of 50% MeOH, cleared by centrifugation and analyzed via LCMS.

For isolation of the lasso peptides, 6 L of M9 minimal medium was fermented as described for 3 days at 20 °C and cells were harvested by centrifugation. The cell pellet of each large-scale fermentation was extracted with 400 mL of MeOH and the solvent was evaporated at 40 °C and reduced pressure. Dried extracts were resuspended in 10 mL of 50% MeOH, cleared by centrifugation and filtration and were then subjected to preparative HPLC.

Purification of Caulosegnins I–III. Lasso peptides were purified by preparative HPLC using a microbore 1100 HPLC system (Agilent) with a VP 250/21 Nucleodur C18 Htec 5 μm column (Macherey-Nagel). For detection, the UV absorption at 215 nm was recorded.

Crude pellet extracts were subjected to the following gradient of water/0.045% formic acid (solvent A) and MeOH/0.05% formic acid (solvent B) at room temperature and a flow rate of 18 mL/min: Linear increase from 40% to 85% B in 30 min, followed by a linear increase from 85% to 95% B in 5 min and holding 95% B for additional 3 min. Retention time of caulosegnin I was 17.5 min, retention time of caulosegnin II was 18.0 min and retention time of caulosegnin III was 23.5 min. Lasso peptide containing fractions were pooled and solvent was evaporated at 40 °C and reduced pressure.

For the second round of purification, 1–3 mg of semipure lasso peptide was dissolved in 10 mL of 20% MeCN and subjected to a gradient of water/0.1% trifluoroacetic acid (solvent C) and MeOH/0.05% trifluoroacetic acid (solvent D) at room temperature and a flow rate of 18 mL/min. For purification of caulosegnin I, the following gradient was used: Linear increase from 30% to 45% D in 30 min, followed by a linear increase from 45% to 95% D in 2 min and holding 95% B for additional 3 min. Retention time of caulosegnin I was 12.5 min. For purification of caulosegnin II and III, another gradient was used: Linear increase from 20% to 60% D in 60 min, followed by a linear increase from 60% to 95% D in 5 min and holding 95% B for additional 3 min. Retention time of caulosegnin II was 27.5 and 29.0 min for caulosegnin III. After the second purification, lasso peptide containing fractions were pooled and solvent was evaporated via lyophillization to yield pure product.

NMR Spectroscopy. Samples for NMR measurements contained 4.97 mg of caulosegnin I in 250 μL of methanol-d_3 or the same amount of caulosegnin I in the same volume of methanol-d_4 in Wilmad 3 mm tubes (Rototec Spintec). Spectra were recorded on a Bruker Avance 600 MHz spectrometer equipped with an inverse triple resonance $^{1}H-^{13}C-^{15}N$ probe with z-gradient. Temperature effect on the structure was surveyed by recording ^{1}H spectra at variable temperatures. Thus, all 2D spectra were recorded at 296 and 288 K while structure determination was done on the basis of spectra at 288 K. For sequential assignment, DQF-COSY,[39] TOCSY,[40] NOESY,[41] ROESY,[42] and E.COSY[43] experiments were performed in phase-sensitive mode using States-TPPI.[44] TOCSY spectra were recorded with mixing times of 50 and 80 ms. NOESY spectra were taken at 100, 200, and 300 ms mixing times, while ROESY spectra were observed at 120 ms. Water suppression was fulfilled by using excitation sculpting[45] with gradients for DQF-COSY, TOCSY, NOESY, and ROESY experiments, while presaturation was used for E.COSY. 1D spectra were acquired with 65 536 data points, while 2D spectra were collected using 4096 points in the F_2 dimension and 512 increments in the F_1 dimension. For 2D spectra, 32 transients were used, with an exception of 48 transients for E.COSY. Relaxation delay was 3.0 s. Chemical shifts of ^{1}H were referenced to the rest signal of methanol-d_3/d_4 (δ_{1H} = 3.31 ppm). To study the proton/deuterium exchange, ^{1}H and TOCSY spectra were recorded with the sample in methanol-d_4 at 288 K sequentially 30 min, 1 d, 7 d, 14 d, 21 d and 28 d after sample preparation. All spectra were processed with Bruker TOPSPIN 2.1. NOE cross-peaks were analyzed within the program Sparky.[46]

Structure Calculations. Standard procedures were used.[47] In brief, on the basis of the vicinal coupling constants $^{3}J_{HH\alpha}$ and $^{3}J_{\alpha\beta}$, torsion angles ϕ and χ_1 were determined. NOESY cross-peaks with mixing time 100 ms at 288 K were used to create distance constraints. These constraints were used in the simulated annealing protocol and the structure calculations were done with the program CYANA 2.1.[48] The coordinates of the 15 lowest energy structures to present the solution structure of caulosegnin I have been submitted to the Protein Data Bank (PDB) and assigned the accession number 2LX6.

Thermostability Assays. For testing the thermal stability of the lasso peptides, a solution of 10 μg of purified lasso peptide was incubated at 95 °C for either 1, 2, 4, or 8 h, or for 4 h at either 20, 35, 50, 65, 80, or 95 °C. Samples were cooled to 4 °C and were subsequently analyzed via LCMS using appropriate gradients.

For testing the heat stability of the variants, 50 μL of the respective extracts was incubated at 95 °C for 1 h. The samples were cooled and were analyzed with the LTQ-FT ultra instrument under the above-mentioned conditions. As reference, additional 50 μL of the respective untreated extract was measured under the same conditions.

Protease Assays. To assess the proteolytic stability of caulosegnins I–III, assays were performed using 10 μg of purified lasso peptide and different proteases. Additionally, these assays were performed for the branched cyclic analogues of caulosegnins I–III, which were obtained by heating 10 μg purified lasso peptide to 95 °C for 1 h and were then incubated under the same conditions.

For trypsin, the assays were performed with 0.5 μg of trypsin in a buffer containing 50 mM Tris-HCl and 1 mM $CaCl_2$ at pH 7.6 for 4 h at 25 °C. For chymotrypsin, the assays were performed with 0.5 μg of chymotrypsin in a buffer containing 100 mM Tris-HCl and 10 mM $CaCl_2$ at pH 8.0 for 4 h at 25 °C. For elastase, the assays were performed with 0.5 μg of elastase in a buffer containing 50 mM Tris-HCl at pH 8.5 for 4 h at 37 °C. For proteinase K, the assays were performed with 1 U proteinase K in a buffer containing 50 mM Tris-HCl at pH 7.5 for 4 h at 37 °C. For carboxypeptidase Y, the assays were performed with 0.5 U carboxypeptidase Y in a buffer containing 50 mM MES and 1 mM $CaCl_2$ at pH 6.75 for 4 or 16 h at 25 °C.

Differentiation between Lasso and Branched Cyclic topology. To investigate the topology of a produced compound, several experiments were carried out. The first hint for a lasso peptide topology was derivable from thermal treatment and subsequent LCMS analysis of samples of the putative lasso peptides, where the time and temperature dependent conversion into another topology can be observed by the generation of a new compound with the exact same mass but a different retention time. The putative lasso peptides and their derivatives were then incubated with carboxypeptidase Y to further prove them having either a lasso or a branched cyclic topology. Furthermore, tandem mass spectrometry was applied to gain characteristic fragmentation patterns for both lasso peptides and branched cyclic peptides. For all produced variants discussed in this study, thermal treatment and LCMS analysis combined with tandem mass spectrometry were applied to assess if the isolated variants were of lasso or branched cyclic topology.

Antibacterial Assays. The antibacterial activity of caulosegnin I–III was assayed by spot-on-lawn assays against *Asticaccaulis excentricus*, *Bacillus subtilis*, *Burkholderia thailendensis*, *Burkholderia rhizoxinica*, *Caulobacter crescentus*, *Caulobacter* sp. K31, *C. segnis*, *Micrococcus flavus*, *Sphingobium japonicum*, *Sphingopyxis alaskensis* and *Xanthomonas gardneri*. For this a bacteria, soft agar overlay was prepared by inoculation of 10 mL of an appropriate soft agar medium (6 g/L agar) to an OD of ~0.01 with a culture of the respective bacteria in the same medium during its exponential growth phase. The bacterial suspension was deposited on a 20 mL agar (15 g/L agar) layer of the same medium in a Petri dish. After solidification, droplets containing 0, 5, 10, and 20 nmol of the tested lasso peptide were placed on the agar overlay on marked spots. After 1 and 2 days of incubation at 30 °C, plates were analyzed for the presence of inhibition halos.

LB medium was used for every organism except *C. crescentus* and *C.* sp. K31, which do not grow on LB medium. For these bacteria, the assays were performed with PYEGR medium.

RESULTS AND DISCUSSION

Genome Mining Reveals the Biosynthetic Gene Cluster of the Lasso Peptides Caulosegnins I–III. Through an initial BLAST homology search of the B protein from the capistruin biosynthetic gene cluster, a 648 bp homologue was identified in the genome of *C. segnis* (GenBank accession no. NC_014100) and was designated as *csegB*, according to the general nomenclature for lasso peptide biosynthetic gene clusters.[12,33] Closer inspection of the flanking regions of this gene revealed an 1851 bp gene encoding the asparagine synthetase homologue (*csegC*) as well as three short ORFs with sizes of 111 to 126 bp each. These small peptides were analyzed for the presence of conserved features of lasso peptide precursors, namely a glycine or cysteine residue in the N-terminal region of the peptide, which will be position 1 of the mature lasso peptide, an aspartate or glutamate residue at position 8 or 9 and a threonine residue at the penultimate position of the leader peptide (position −2), which is important for recognition by the processing enzymes.[49] All three peptides met these criteria and their respective genes were named *csegA1*, *csegA2* and *csegA3*. Interestingly CsegA1 features a glutamate capable of macrolactam ring formation at position 8, while CsegA2 and CsegA3 possess suitable glutamate residues at position 9.

Unlike other known lasso peptide biosynthetic gene clusters,[12,13,33,34] that of *C. segnis* does not only feature more than one precursor peptide, but also does not contain a gene encoding an ABC-transporter, which in the cases of MccJ25, capistruin and lariatin were linked with conferring an immunity against the produced compounds.[12,33,34] The latter was also observed for the just recently identified biosynthetic gene cluster of astexin-1,[35] making both systems the first examples of lasso peptide biosynthetic gene clusters arranged in this manner. Interestingly these two systems also share similarities in the genomic regions flanking their gene clusters, as upstream to both clusters a gene encoding a putative GntR family regulator is situated, while downstream a reverse facing gene cluster is positioned, comprising genes proposed to encode a transcriptional regulator, an anti-FecI sigma factor FecR, a TonB-dependent receptor and a peptidase. This is also true for the second putative lasso peptide biosynthetic gene cluster found in the genome of *A. excentricus*, which was predicted by Link and co-workers.[35] Furthermore, it should be noted that even though the gene cluster from *C. segnis* was also identified by the precursor-centric genome mining approach from Link and co-workers, their prediction lacked the precursor peptide from caulosegnin I, showing that a manual quality check for precursor presence and suitability should always be considered.

A scheme of the complete cluster from *C. segnis*, including the precursor sequences, is shown in Figure 1. A comparison of all newly identified genes with their homologues from the MccJ25, astexin-1 and capistruin biosynthetic gene clusters is shown in Table 1.

Fermentation of *C. segnis*. *C. segnis* is a nonpathogenic aquatic alpha proteobacterium of the *Caulobacteraceae* family. This bacterial family is known for their interesting asymmetrical cell division, which is extensively studied using *C. crescentus* as a model organism.[50] Analysis of the *C. segnis* genome with antiSMASH[51] revealed, that it lacks NRPS and PKS gene clusters and thus seems to be confined to the production of secondary metabolites of ribosomal origin like lasso peptides.

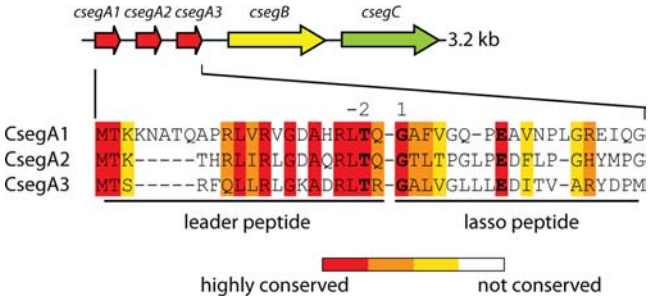


Figure 1. Schematic representation of the caulosegnin biosynthetic gene cluster and alignment of the three precursor peptides.

After identification of the putative lasso peptide gene cluster of *C. segnis* via genome mining, the strain was cultivated in LB medium, PYEGR medium and several minimal media (M9, M20 and M63) at 20 and 30 °C for 7 days. Best growth was observed in LB and PYEGR medium, while growth in M9 minimal medium was moderate and growth in M20 and M63 minimal media was very poor. Supernatant and pellet extracts were screened for the molecular masses of the predicted lasso peptides by LCMS using a high-resolution Fourier transform mass spectrometer. In the LB, M20 and M63 extracts, no detectable amounts of any lasso peptide were present. In M9 medium, traces of caulosegnin I were found at both 20 and 30 °C in the pellet and supernatant extracts, while PYEGR medium contained traces of caulosegnin I and II in both pellet and supernatant extracts of the 20 °C fermentation and traces of only caulosegnin I in the pellet and supernatant extracts of the 30 °C fermentation (data not shown). But even though it was possible to observe the production of two out of the three predicted lasso peptides, the produced amounts were far too low for isolation and made an heterologous approach necessary.

Heterologous Expression in *E. coli* and Optimization of Caulosegnin Production. For our first efforts to heterologously produce caulosegnins in *E. coli*, the complete 3.2 kb gene cluster was cloned into the pET41a expression vector (Figure 2a).

This construct was expressed in *E. coli* BL21 in LB medium and M9, M20 and M63 minimal media and each fermentation was carried out for either 1 day at 37 °C and 3 days at 20 °C (data not shown). We observed that expression for 3 days at 20 °C in M9 medium was best suited for the production of caulosegnins, while LB was the only medium tested where no lasso peptides could be detected. With this initial system, we were able to detect all three lasso peptides via LCMS, with caulosegnin I showing the best production. Still the production was too low for isolation, which is why single precursor constructs were generated and tested under the same conditions (Figure 2b). Again caulosegnin I showed the best production, which was ∼15-fold higher compared to the production in the initial system, while caulosegnin II and III were produced at ∼5% of the amount of caulosegnin I. Since the caulosegnin I precursor peptide has a much longer leader sequence compared to the other caulosegnins (Figure 1), we reasoned that this could cause the higher production of caulosegnin I. As consequence hybrid precursor peptides, which carried the leader sequence of CsegA1 and the lasso sequences of CsegA2 and CsegA3, respectively (named CsegA1-QG-A2 and CsegA1-QG-A3), were generated to investigate the effects

dx.doi.org/10.1021/ja308173b | J. Am. Chem. Soc. 2013, 135, 210–222

Table 1. Coding Sequences from the Caulosegnin Biosynthetic Gene Cluster

protein	amino acids	proposed function	identity/similartiy to MccJ25 cluster homologues[a]	identity/similartiy to capistruin cluster homologues[a]	identity/similartiy to astexin-1 cluster homologues[a]	GenBank accession no.
CsegA1[b]	42	precursor peptide	-	-	-	YP_003593640.1
CsegA2[b]	37	precursor peptide	-	-	-	YP_003593639.1
CsegA3[b]	37	precursor peptide	-	-	-	YP_003593638.1
CsegB	216	protease	20%/35%	24%/34%	28%/43%	YP_003593637.1
CsegC	617	adenylation, cyclization	15%/30%	26%/40%	26%/40%	YP_003593636.1

[a]Determined using the BLOSUM62 algorithm. [b]As precursor peptides are small and as their amino acid sequences are highly variable, identity and similarity was not determined.

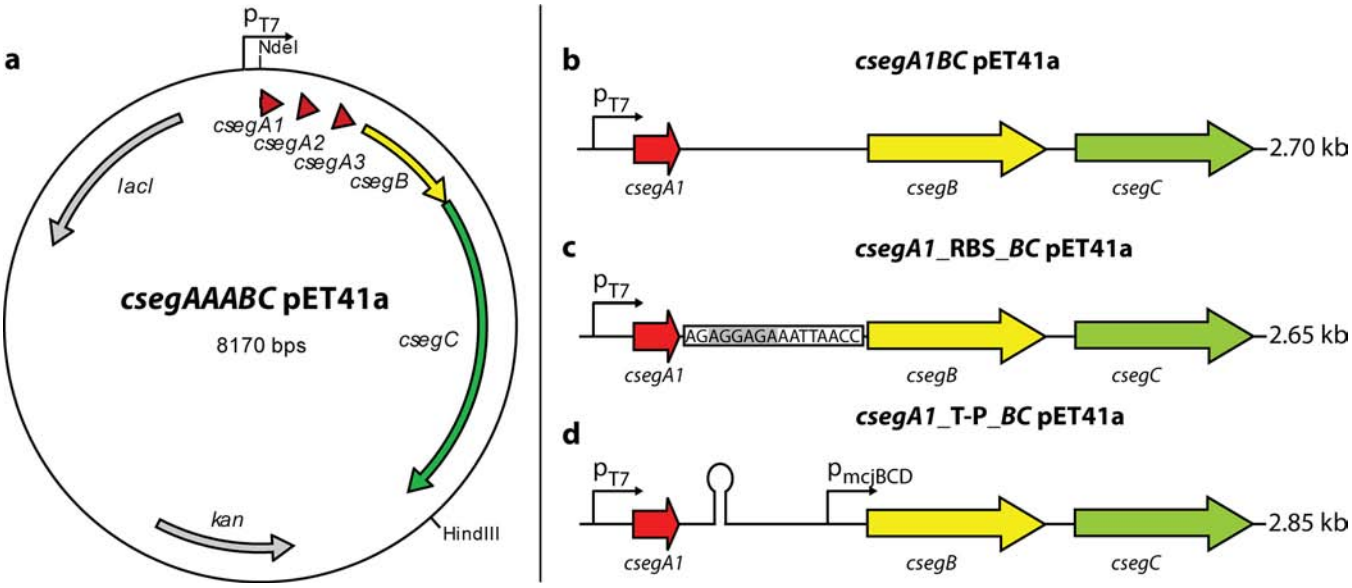


Figure 2. Schematic representation of the different constructs developed for optimized lasso peptide production. (a) Complete construct consisting of the pET41a vector backbone with the inserted *csegAAABC* gene cluster. (b) Gene cluster after deletion of *csegA2* and *csegA3*, which was used as basis for further optimization. (c) Gene cluster after the intergenic region was replaced by an *E. coli* optimized ribosomal binding site (RBS) marked in gray. (d) Gene cluster after the intergenic region was replaced by a terminator sequence (symbolized by a loop) and the subsequent *mcjBCD* promoter.

of the leader peptide on lasso peptide production. This modification led to an improved yield of the lasso peptides, with caulosegnin II now being produced at ~40% and caulosegnin III at ~10% of the amount of caulosegnin I. As caulosegnin I still showed the highest production of all three lasso peptides, it was chosen for additional optimization approaches. In recent publications by Link et al., the production of capistruin,[49] astexin-1[35] and MccJ25[52] was improved through different means. In case of capistruin and astexin-1, production was optimized through exchange of the intergenic region between their respective precursor and processing enzyme genes with an *E. coli* optimized ribosomal binding site. For MccJ25, an improved expression system was generated, where the precursor was under control of the strong inducible T5 promoter, while the expression of the processing enzymes was put under control of their native promoter system.

On the basis of this, one construct (*csegA1*_RBS_*BC* pET41a, Figure 2c) was generated, where the intergenic region between *csegA1* and *csegB* was replaced with the same ribosomal binding site (RBS) and another construct was generated (*csegA1*_T-P_*BC* pET41a, Figure 2d), where this region was replaced with a terminator sequence, followed by the mcjBCD promoter, causing *csegA1* expression to be under control of the strong inducible T7 promoter, while *csegBC* expression levels were now regulated by the mcjBCD promoter system.

The yields with these constructs were vastly improved, being ~11-fold higher for the *csegA1*_RBS_*BC* pET41a and ~16-fold higher for the *csegA1*_T-P_*BC* pET41a construct compared to the *csegA1BC* pET41a construct. As the terminator-promoter (T-P) variant showed the best production, it was also introduced in the *csegA1-QG-A2* and the *csegA1-QG-A3* hybrid systems. The production improvements for caulosegnin II and III were even more dramatic with a ~24-fold increase for caulosegnin II and a ~50-fold increase for caulosegnin III. A comparison of the production of all constructs is shown in Figure 3.

Isolation of Caulosegnins I–III. With the optimized heterologous production of caulosegnins I–III, we performed large-scale fermentations to isolate these lasso peptides. The observed production was ~0.30 mg/L for caulosegnin I, ~0.15 mg/L for caulosegnin II and ~0.10 mg/L for caulosegnin III after two HPLC purification runs (Supporting Information Figure S1–S3) and in accordance with the ratios obtained in the test fermentations. As caulosegnin II and III contain one

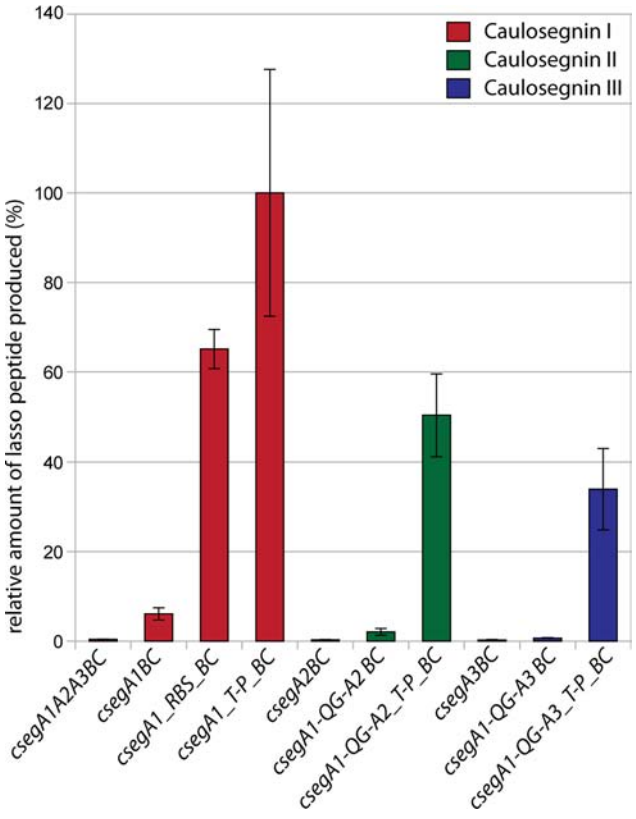


Figure 3. Comparison of relative lasso peptide production for the different pET41a constructs.

methionine residue each, oxidation of these lasso peptides occurs to a small extent when removing the solvent after HPLC purification at 40 °C at reduced pressure. Even though caulosegnin I does not contain any methionine residues, solvent evaporation at 40 °C and reduced pressure led to the formation of small amounts of another compound with exactly the same mass, but a different retention time in the LCMS analysis. This can be explained by the fact that caulosegnin I tends to unthread at higher temperatures.

To prevent the formation of oxidated and unthreaded lasso peptide derivatives, the solvent was removed by lyophillization directly after the final HPLC purification step. This way, pure samples of each lasso peptide could be obtained.

NMR Studies and Structure Elucidation of Caulosegnin I. To gain a better understanding of the structural factors contributing to the unique lasso fold, the structure of the most abundant lasso peptide, caulosegnin I, was elucidated via NMR.[53] Assignments of the ^{1}H signals were obtained by standard procedures.[47] A combination of DQF-COSY and NOESY produced sequential assignments (i.e., all αH and NH and their sequence in the backbone), and a combination of DQF-COSY and TOCSY resulted in assignments of the side chains. One pure conformation was observed. Full assignments of ^{1}H signals were thus obtained (Supporting Information Figure S4−S7, Tables S9−S10). Strong NOE contacts between the NH of Gly1 and the γH of Glu8, showing an internal linkage between the two residues, were observed. Strong sequential αH−δH NOESY cross peaks were detected, which showed the trans-conformation of Pro7 and Pro12. Inspection of ^{1}H spectra between 278 and 298 K (Supporting Information Figure S8) revealed almost no temperature dependence of the NHs of Ala2, Val4, Gln6, Glu8 (positioned in the macrolactam ring), Arg15 and Glu16 (positioned in the tail region and involved in the tail threading/trapping). Variable delay ^{1}H spectra in methanol-d_4 (Supporting Information Figure S9) were consistent with a very slow deuterium exchange of the amide protons of these residues. Furthermore, a large number of long-range NOE contacts were observed. These are the $d_{NN}(i,j)$ connectivities between Ala2−Arg15 and Gln6−Glu16; the $d_{\alpha N}(i,j)$ connectivities between Phe3−Arg15, Pro7−Glu16, and Arg15−Glu8; the $d_{\beta N}(i,j)$ connectivities between Arg15−Val4, Glu16−Gly1, and Glu16−Ala2; and the $d_{\alpha\beta}(i,j)$ connectivity between Val4−Arg15. In addition, contacts between the NH of Gly5 and the δH of Arg15 and between the βH of Phe3 and the εH of Arg15 were also observed. As summarized in Figure 4, all these short distances revealed in the NOESY spectrum are in favor of the lasso structure of caulosegnin I.

Structure calculations were performed with the program CYANA 2.1.[48] The internal linkage was realized by setting the distance constraints between N of Gly1 and Cδ of Glu8 to be 1.33 Å. NOE cross-peaks observed in the 100 ms mixing time NOESY experiment were converted into distance constraints manually. In this way, 148 unambiguous distance constraints were obtained, 43 for the backbone, 28 for long-range interactions, and 77 for the side-chains. Thus, there were an average of 7.8 distance constraints per residue. In addition, constraints of torsion angles ϕ and χ^1 were determined by analyzing the vicinal coupling constants $^3J_{HN\alpha}$ and $^3J_{\alpha\beta}$ (see Supporting Information).

The above-mentioned constraints were used in the simulated annealing protocol for the calculation in the CYANA 2.1 program. The calculation initiated with 50 random conformers and the resultant structures were engineered by the program package Sybyl 7.3[54] to include the covalent linkage between the nitrogen of Gly1 and Cδ of Glu8, followed by energy minimization under NMR constraints using TRIPOS force field within Sybyl. Thus, on the basis of low energies and minimal violations of the experimental data, a family of 15 structures was chosen. These 15 energy-minimized conformers show an average root-mean-square deviation (rmsd) of 0.02 Å and are kept to represent the solution structure of caulosegnin I.

The structure is in accordance with the notion of caulosegnin I being a lasso peptide with an eight amino acid macrolactam ring. The superimposition of the 15 lowest energy structures (Figure 5) shows the backbone adopting a very rigid fold, as the structures show very little deviation from each other, with the last three amino acids below the ring being the most flexible part of the whole lasso peptide.

In regard to the tail passing the ring, Arg15 is located above and Glu16 is the first residue below the ring, suggesting that these amino acids are sterically locking the tail in position (Figure 6). Interestingly Arg15 and Glu16 as well as the C-terminal Gly19 are the only charged residues in caulosegnin I.

It is worth mentioning that the bend caused by the Pro12 residue is the major turning point of the loop and thus Pro12 could play an important role in the prefolding of the precursor peptide and the stabilization of the lasso fold.

Antibacterial Activity Assays. As it is known, for other lasso peptides to exhibit antibacterial activity against related species (e.g., MccJ25[2,28−30] and capistruin[12,31] are able to inhibit the RNA polymerase of some Gram-negative bacteria, but not those of Gram-positive bacteria), caulosegnins I−III were tested primarily for possible antibacterial activity by spot-

dx.doi.org/10.1021/ja308173b | J. Am. Chem. Soc. 2013, 135, 210−222

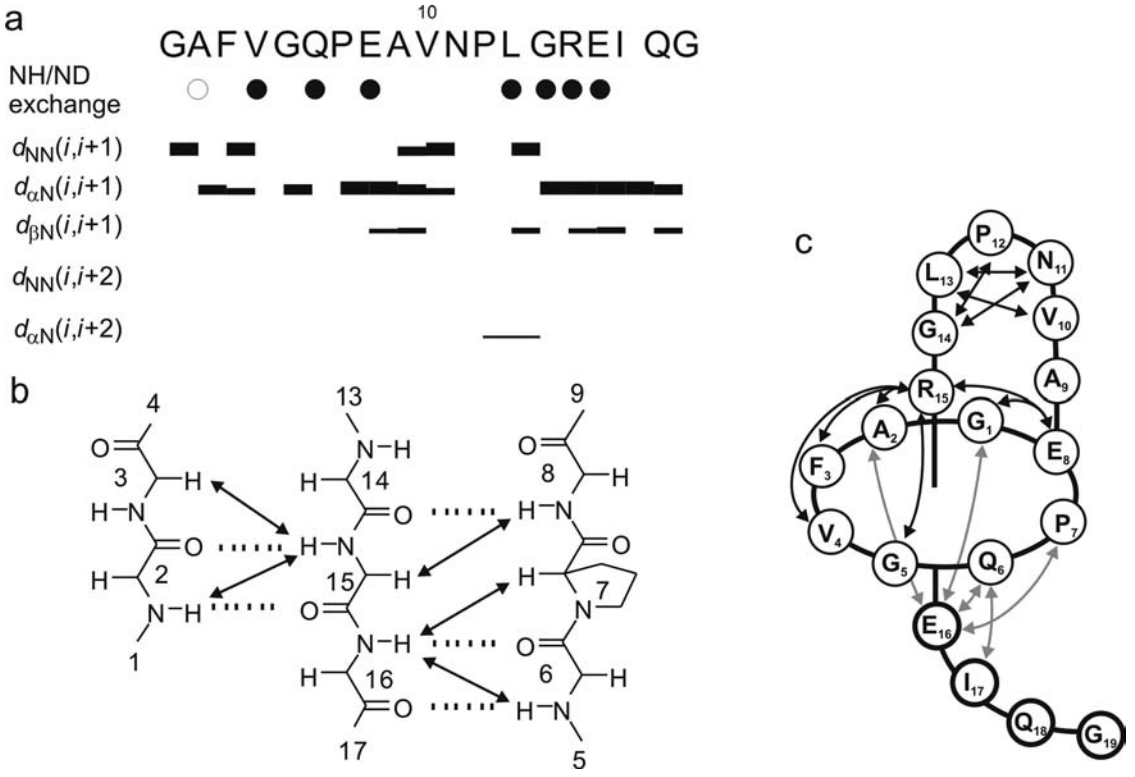


Figure 4. Summary of the NMR data that define the lasso structure of caulosegnin I. (a) The slow (observable in a TOCSY spectrum after 2 days in methanol-d_4 at 288 K) and very slow (observable in a TOCSY spectrum after 4 weeks in methanol-d_4 at 288 K) exchanging amide protons are portrayed with open and filled circles respectively and the sequential and medium range NOE connectivities (observed in a NOESY spectrum of 100 ms mixing time at 288 K in methanol-d_3) are depicted with bars. The thickness of the bars corresponds to the NOE strength. (b) Key backbone−backbone NOE connectivities (100 ms 288 K in methanol-d_3) shown through arrows and hydrogen bond interactions deduced from the amide proton/deuterium exchange experiments and structure calculations shown as broken lines. (c) Schematic view of the caulosegnin I structure showing the long-range NOE connectivities (100 ms 288 K in methanol-d_3) defining the lasso threading. Long-range NOE connectivities in the loop region are shown as black straight arrows and those above and below the ring are shown as black and gray curved arrows respectively.

on-lawn assays against other proteobacteria, namely, *A. excentricus*, *B. thailendensis*, *B. rhizoxinica*, *C. crescentus*, *Caulobacter* sp. K31, *C. segnis*, *S. japonicum*, *S. alaskensis* and *X. gardneri*. Additionally, antibacterial activity was assessed against the Gram-positive bacteria *B. subtilis* and *M. flavus*. In these assays, caulosegnins I−III showed no antibacterial activity against any of the tested strains. An explanation for this could be the lack of an ABC-transporter in the caulosegnin biosynthetic gene cluster, which suggests that these lasso peptides could serve other purposes than being antibacterial agents.

Heat Stability Assays Illustrate the Unique Behavior of Each Caulosegnin. Lasso peptides are often reported to possess an extraordinary stability against thermal degradation.[2,12,22] To investigate if this is also true for the caulosegnins, they were incubated for different time intervals at 95 °C and at different temperatures for 4 h (Supporting Information Figure S11). Interestingly each caulosegnin showed a different stability profile.

While caulosegnin II showed only minimal unthreading even after 8 h at 95 °C, caulosegnin I was completely unthreaded after 4 h at 95 °C. Additionally, at temperatures above 35 and below 95 °C, caulosegnin I shows a strong tendency to form a deamidated derivative, where the carboxamide function of either Gln6, Asn11 or Gln18 is hydrolyzed to a carboxylic acid, resulting in a weight loss of 1 Da and a slight shift of retention time.[55] Caulosegnin III, on the other hand, does not only unthread when exposed to increased temperatures, but also readily decomposes, resulting in small detectable amounts of a derivative missing the last two amino acids and a degradation product which consists of only the nine amino acid macrolactam ring and one additional amino acid. It is worth mentioning that both degradation products feature a C-terminal aspartate, which is in accordance with the known fact that an aspartate residue is able to catalyze the self-hydrolysis of a peptide via formation of a cyclic imide intermediate.[56] As a full-length branched cyclic peptide analogue of caulosegnin III can also be detected, degradation does not seem to be required before the unthreading of caulosegnin III can occur.

For caulosegnin II and III, these assays were performed with samples containing small amounts of oxidized lasso peptide (~15%) to see if the oxidized forms would behave differently. For both caulosegnins, the oxidized forms showed a similar behavior compared to the unmodified lasso peptides.

Different protease assays were performed to compare the stability of the lasso peptides and their branched cyclic analogues. The results of these assays were in agreement with previous observations[22] that lasso peptides are far less susceptible to proteolytic digestion than branched cyclic peptides with the same amino acid sequence (Supporting Information). In this regard, the results from the carboxypeptidase Y digests were most notable, as they allowed a clear distinction between the lasso peptides and their branched cyclic analogues. While the latter were readily digested, the lasso peptides were still intact after protease treatment. Subsequent

dx.doi.org/10.1021/ja308173b | J. Am. Chem. Soc. 2013, 135, 210−222

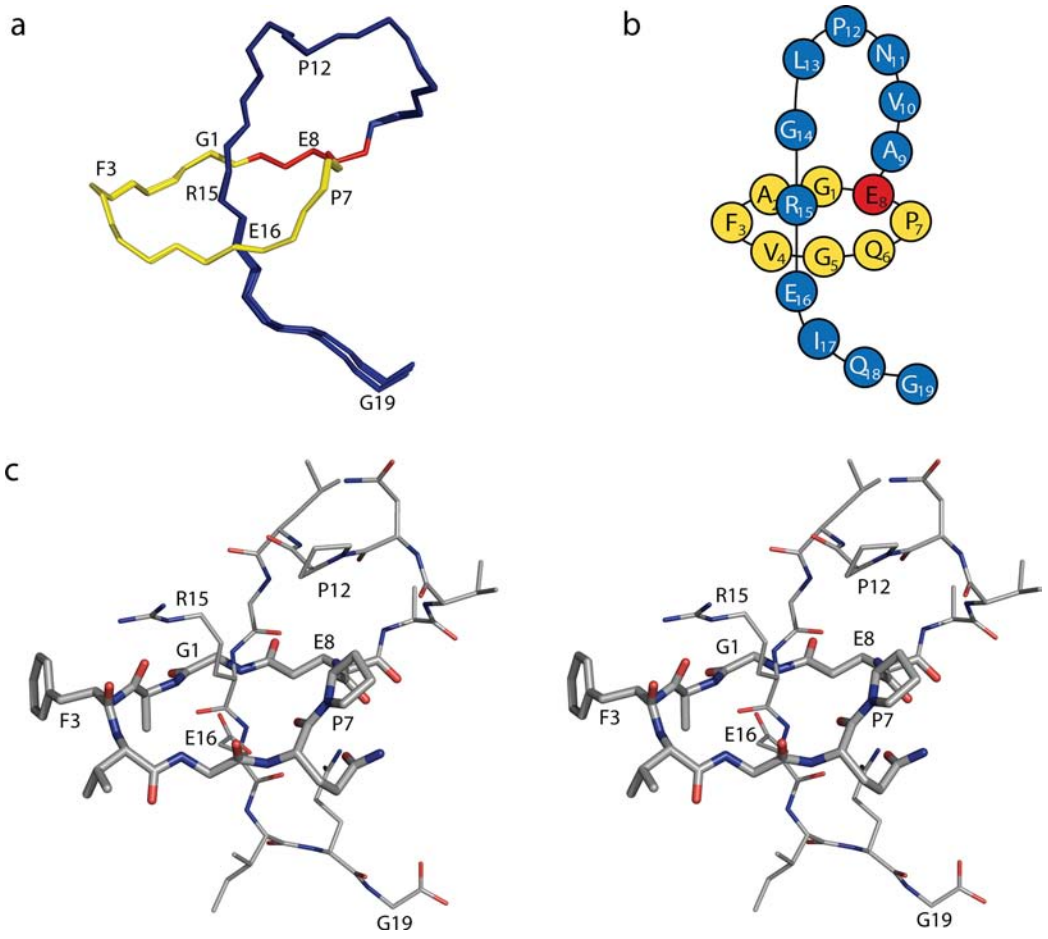


Figure 5. Structure of caulosegnin I. (a) Superimposition of the 15 lowest energy structures of caulosegnin I. Structures are superimposed over all backbone atoms. The isopeptide bond forming Glu8 is colored in red, while the remaining ring amino acids are colored in yellow and the tail amino acids are colored in blue. (b) Schematic representation of the structure of caulosegnin I. (c) Representative average structure of caulosegnin I colored by elements (nitrogen in blue, oxygen in red and carbon in gray) shown in stereoview.

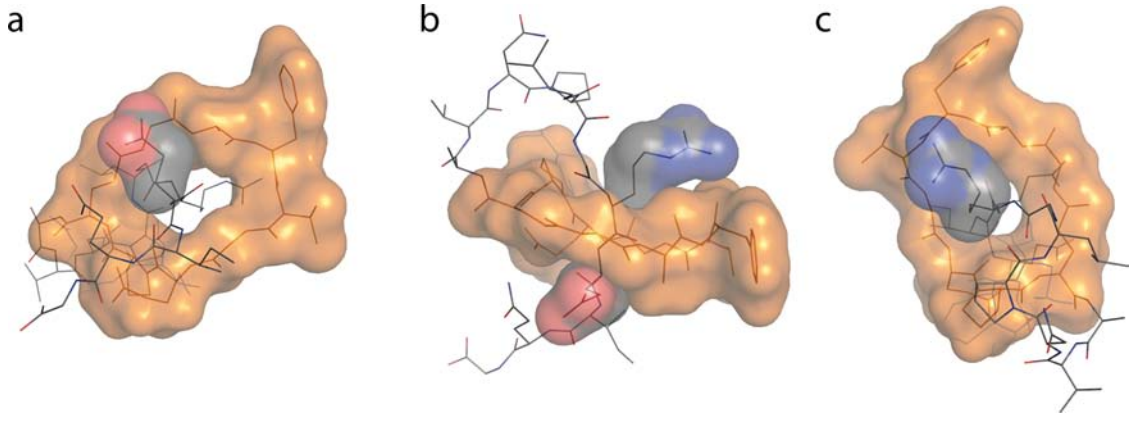


Figure 6. Interactions between the C-terminal tail and the macrocyclic ring of caulosegnin I. The surface of the ring is colored in orange, the surface of the side chains from Arg15 and Glu16 are colored by elements. Steric hindrance caused by the depicted side chains traps the tail within the ring. (a) Shown from below, (b) from the side and (c) from above the ring. A version of this figure in stereoview can be found in the Supporting Information (Figure S10).

MS^2 analysis of the lasso peptides and their branched cyclic analogues revealed distinct fragmentation patterns for the different topologies, where all three lasso peptides differed in similar ways from their branched cyclic analogues (Supporting Information pages S3–S4, Figures S15–S17). As the lasso topology of caulosegnin I was confirmed by NMR structure elucidation, the common features of all three proposed lasso peptides and their branched cyclic analogues are a very strong indication that both caulosegnin II and III are true lasso peptides, although their 3D structures were not yet determined.

Mutational Analysis of Caulosegnin I. In the case of the caulosegnins, the presence of three lasso peptides with different

dx.doi.org/10.1021/ja308173b | J. Am. Chem. Soc. 2013, 135, 210–222

primary structures is an indication for the high promiscuity of the processing enzymes. This observation is not uncommon for lasso peptide processing enzymes as mutational studies of MccJ25 and capistruin have also shown a very relaxed specificity toward amino acid substitutions in their respective lasso peptide sequences.[22,49,57−59] To further examine which residues are important for efficient processing of the caulosegnin I precursor, a total of 23 mutants was generated (Table 2). To assess if toxicity of a new variant was the reason

Table 2. Production of the Generated Variants of Caulosegnin I[a]

	relative production	topology	thermal stability
T-2A	*	lasso	no
T-2C	***	lasso	no
T-2I	**	lasso	no
T-2S	**	lasso	no
T-2V	**	lasso	no
G1A	*	lasso	no
G1C	*	lasso	no
G1F	-	-	-
E8D	-	-	-
P12A	**	lasso	no
ΔL13	**	lasso	no
R15A	***	lasso	no
E16A	**	branched cyclic	-
E16D	****	lasso	no
E16K	***	lasso	no
E16L	**	lasso	no
E16N	**	lasso	no
E16Q	***	lasso	no
E16A/I17A	**	branched cyclic	-
I17A	****	lasso	no
I17STOP	*	lasso	no
Q18STOP	***	lasso	no
G19STOP	****	lasso	no

[a]Produced amounts were determined via integration of the corresponding UV signals and comparing them to the wild type lasso peptide. The variants are classified according to their relative production with ***** for >120%, **** for 120−70%, *** for 70−15%, ** for 15−1% and * for <1% of the amount of wild type lasso peptide being produced. Variants that were not produced are marked with "-". For the truncation variants I17STOP, Q18STOP and G19STOP the corresponding amino acid codon was replaced with the TAA stop codon.

for a low production, OD_{600} was measured at the end of each fermentation and compared to the OD_{600} of a wild type fermentation. As all OD_{600} values obtained (data not shown) were in the same range, it was shown that the production of caulosegnin I and all its variants had no toxic effects on the cells.

The mutational analysis shows that the processing enzymes of the caulosegnin biosynthetic gene cluster tolerate substitutions of the threonine residue at the penultimate position of the leader peptide, but to a lesser extent than the processing enzymes of capistruin and MccJ25.[49] In agreement with the studies for capistruin and MccJ25, the T-2A variant is produced only in trace amounts, while T-2I, T-2S and T-2V are produced by an order of magnitude less than wild type. The best tolerated substitution was T-2C, showing a production that was at least in the same order of magnitude as the wild type.

In accordance with other mutational studies of capistruin[22] and MccJ25,[57] the substitution of Gly1 proved to massively hinder the maturation of caulosegnin I, but unlike capistruin and MccJ25 the G1A and G1C mutations did not completely abolish production as trace amounts of the predicted lasso peptides were still detectable, while the G1F variant did not show any production at all. This is interesting considering that all class I lasso peptides contain a cysteine at position 1, as it again illustrates the different specificity of class I and class II processing enzymes concerning this residue. The E8D variant was also not produced, suggesting that not only the charge of the side chain, but also its length is important for enzymatic recognition. This is consistent with results obtained by the capistruin[22] and MccJ25[57] studies.

The truncation variants I17STOP, Q18STOP and G19STOP provided further information about the enzymatic recognition, as they show that parts of the tail appear to be necessary for a proper maturation of the precursor peptide. While G19STOP is still produced in amounts comparable to wild type, Q18STOP already shows a slight impairment of the production, while the I17STOP variant reduces lasso peptide production to a minimal level.

As expected from the NMR structure, Pro12 seems to play an important role in lasso peptide production. We suggest this is due to the fact that Pro12 induces a bend in the loop region, which could support the prefolding and could facilitate the maintenance of the correct fold during the enzymatic maturation of caulosegnin I.

The R15A mutation was tolerated surprisingly well, indicating that neither a plug above the ring is entirely necessary for the stability of the lasso fold of caulosegnin I, nor that the charge of Arg15 plays a crucial role in the enzymatic recognition. The production of the E16A variant shows, that the charge of the glutamate side chain is also not essential for the enzymatic recognition, but at the same time illustrates that Glu16 is indeed needed to sterically maintain the lasso fold, which apparently can not be sustained by Ile17 or Gln18 as the E16A and E16A/I17A variants could be shown to be branched cyclic peptides. Identification of E16A and E16A/I17A as branched cyclic peptides was possible due to their retention time being much closer to unthreaded than to correctly folded caulosegnin I and due to the fact that retention time was not altered by exposing these variants to increased temperatures (Supporting Information Figure S14), proving that they were already unthreaded.

Furthermore, the systematic exchange of Glu16 with several amino acids revealed that the lasso fold can be maintained by amino acids as small as leucine and asparagine, when they are introduced at position 16 of the lasso peptide. Still the asparagine of the E16N variant is barely able to trap the tail inside of the macrolactam ring, as this lasso peptide is already mostly unthreaded after its isolation. Additionally, the data indicates that an amino acid with a charged side chain at position 16, regardless of being positively or negatively charged, leads to an improved lasso peptide production when compared to amino acids with uncharged side chains.

It is interesting to see that the E16A/I17A mutation results in a branched cyclic peptide, even though the tail features a glutamine residue at position 18, as the E16Q variant was shown to be a lasso peptide. This observation is in accordance with previous reports of capistruin R15A being a heat stable lasso peptide, whose fold is sustained by Phe16, while a R15A/F16A mutation results in a heat labile lasso peptide, whose fold

is still maintained by a phenylalanine, but now at position 18 instead of position 16.[22] In case of both capistruin R15A/F16A and caulosegnin I E16A/I17A the increased flexibility of the tail, caused by the plug being further away from the position where it crosses the macrolactam ring, could explain their altered stability properties even though a suitable plug amino acid is generally present.

Identification of the Plug Amino Acid of Caulosegnin II and III via Mutagenesis. When comparing the amino acid sequences of caulosegnin I with caulosegnin II and III (Figure 7), it is noticeable that each peptide contains a positively

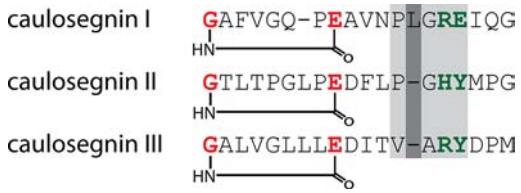


Figure 7. Comparison of the amino acid sequences of caulosegnin I–III. Amino acids involved in the macrolactam ring formation are presented in red. The plug amino acids of caulosegnin I and the proposed plug amino acids of caulosegnin II and III are presented in dark green. Highlighted in light gray are positions which are suggested to have similar importance for the lasso fold. Highlighted in dark gray is the position of Leu13, which in caulosegnin I is inserted in this area.

charged residue in the same distance to the C-terminus, namely Arg15 for caulosegnins I and III, and His15 for caulosegnin II. In analogy to the Glu16 of caulosegnin I, caulosegnin II and III possess a tyrosine residue at position 16, which would be the most suitable plug for the nine amino acid sized ring being present in both of these lasso peptides. Additionally all caulosegnins carry a small amino acid, either glycine or alanine, directly in front of their respective positively charged residue and caulosegnins I and II feature a proline residue four amino acids after their respective macrolactam ring forming glutamate, while caulosegnin III possesses a valine residue at this position.

To identify the plug of caulosegnins II and III, an alanine scan of the last five amino acids of caulosegnin II and III was performed, with exception of the Gly19 residue of caulosegnin II. The results of these mutational studies are shown in Table 3. To assess a possible toxicity of any new lasso peptide variant, OD_{600} was measured and compared to the OD_{600} of the wild type fermentation as described for the mutational analysis of caulosegnin I. Again toxic effects caused by lasso peptide expression were not observed.

As expected, the mutational analysis shows that the Tyr16 residue is important for both caulosegnin II and III. In case of caulosegnin III, the Y16A mutation completely abolishes production, while for caulosegnin II, the Y16A mutation reduces the production to a minimal level of a peptide with a branched cyclic topology. This is in agreement with mutational studies of both capistruin[22] and MccJ25,[57,58] where variants with their lower plug amino acids being replaced with alanine where not produced, while the MccJ25 Y20I variant could only be isolated as a branched-cyclic peptide. The notion of Tyr16 being the plug of both caulosegnin II and III is also supported by results of MS^2 fragmentation studies of the caulosegnins (Supporting Information).

The lack of production of the R15A variant of caulosegnin III highlights that this residue also seems to be very important for either processing, prefolding or stabilization of this lasso

Table 3. Production of the Generated Variants of Caulosegnin II and III[a]

	relative production	topology	thermal stability
caulosegnin II			
P13A	***	lasso	yes
H15A	***	lasso	yes
Y16A	*	branched cyclic	-
M17A	***	lasso	yes
P18A	*****	lasso	yes
caulosegnin III			
V13P	*****	lasso	no
R15A	-	-	-
Y16A	-	-	-
D17A	**	lasso	no
P18A	***	lasso	no
M19A	*****	lasso	no

[a]Produced amounts were determined via integration of the corresponding UV signals and comparing them to the respective wild type lasso peptides. The variants are classified according to their relative production with ***** for >120%, **** for 120–70% *** for 70–15%, ** for 15–1% and * for <1% of the amount of wild type lasso peptide being produced. Variants that were not produced are marked with "-".

peptide. As caulosegnin I R15A and caulosegnin II H15A are produced in rather good amounts, it is most likely that Arg15 is needed to maintain the lasso fold of caulosegnin III, rather than being an important factor for enzymatic recognition and thus for processing of the lasso peptide precursor. This seems plausible since it was already shown that wild type caulosegnin III easily unthreads and it can be assumed that the increased flexibility of the tail through loss of the upper plug would facilitate the unthreading of the lasso peptide even more.

Tyr16 being the plug for both caulosegnin II and III would mean that these lasso peptides must have a loop shortened by one amino acid compared to caulosegnin I, as they feature an one amino acid bigger macrolactam ring. To investigate if this is generally possible, a caulosegnin I ΔL13 variant was generated (Table 2). This variant was successfully processed into a mature lasso peptide, but the strongly reduced production shows that, although this structure is indeed possible for caulosegnin I, a less constraint loop region seems to be preferable for a caulosegnin with an eight amino acid sized macrolactam ring. If this is due to problems during the prefolding step or with the enzymatic recognition remains unclear at this point.

Interestingly, the production of caulosegnin III M19A is improved compared to the wild type. As caulosegnin I and II already feature a small amino acid at the last position of the tail (glycine and alanine respectively) and as the truncation variant of caulosegnin I lacking this last amino acid is produced in amounts comparable to wild type, it could be suggested that a bigger amino acid at this position could lead to a steric repulsion in the enzymatic binding pocket during the lasso peptide maturation and thus decreases the produced amounts of lasso peptide.

As both caulosegnin I and caulosegnin II feature a proline in the same distance to their respective macrolactam ring forming glutamate, a P13A variant of caulosegnin II and a V13P variant of caulosegnin III were generated. These variants further highlight the importance of the proline residue at this position, as the caulosegnin III V13P variant exhibits a strongly increased production, while the produced amounts of caulosegnin II

dx.doi.org/10.1021/ja308173b | J. Am. Chem. Soc. 2013, 135, 210–222

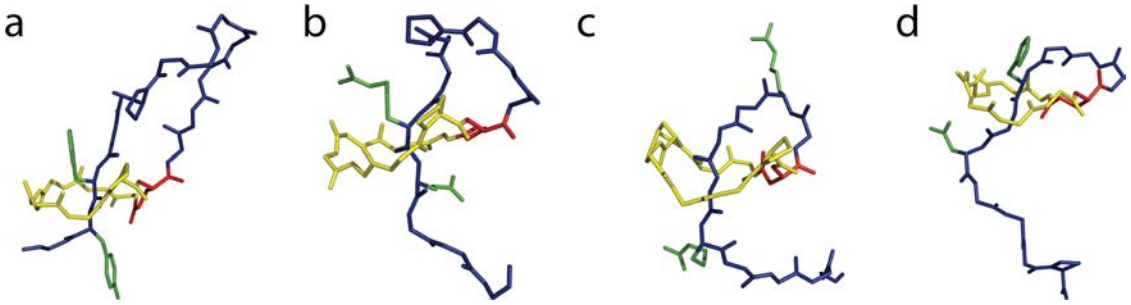


Figure 8. Comparison of class II lasso peptide structures. The ring forming aspartate/glutamate is shown in red, the remaining amino acids of the macrolactam ring are shown in yellow, the tail amino acids are shown in blue and the side chains of the amino acids which were proposed to sterically trap the tail inside the ring are highlighted in green. The shown lasso peptides are McJ25[18] (a), caulosegnin I (b), capistruin[12] (c) and lariatin A[11] (d).

Table 4. Comparison of Structural Features of Astexin-1, Capistruin, Caulosegnin I, Lariatin A, McJ25 and RES-701-1

	total length	ring size	tail length	loop length	no. of residues below the ring	ring forming amino acid	upper plug	lower plug
astexin-1	23 aa	9 aa/28 atoms	14 aa	8 aa	6 aa	Asp9	Glu16	Arg18
capistruin	19 aa	9 aa/28 atoms	10 aa	5 aa	5 aa	Asp9	Arg11	Arg15
RES-701-1	16 aa	9 aa/28 atoms	7 aa	4 aa	3 aa	Asp9	Phe12	Tyr14
McJ25	21 aa	8 aa/26 atoms	13 aa	11 aa	2 aa	Glu8	Phe19	Tyr20
caulosegnin I	19 aa	8 aa/26 atoms	11 aa	7 aa	4 aa	Glu8	Arg15	Glu16
lariatin A	18 aa	8 aa/26 atoms	10 aa	4 aa	6 aa	Glu8	His12	Asn14

P13A decreased compared to its wild type, even though not as drastic as for caulosegnin I P12A. Still it can be assumed that a proline induced bend in the loop could facilitate the prefolding and processing of a lasso peptide precursor and thus could have a positive influence on the produced amounts of lasso peptide.

Comparison of Caulosegnin I to Other Class II Lasso Peptides. There are five other examples of class II lasso peptides with known structures besides caulosegnin I. These are astexin-1,[35] capistruin,[12] lariatin,[11] McJ25[17−19] and RES-701-1.[20] A comparison of caulosegnin I with capistruin, lariatin and McJ25 is shown in Figure 8 and Figure S18.

Even though all six lasso peptides belong to the same class, their structures have little in common besides having a lasso fold. All peptides possess differently sized loops and tail sections below the ring that differ in length, with McJ25 having both the longest loop and shortest tail section below the ring and lariatin A having both the shortest loop and longest tail section below the ring. In regard to the size of the macrolactam ring, astexin-1, capistruin and RES-701-1 are the only ones of these six peptides with a nine membered ring instead of an eight membered one (Table 4). It is noticeable that all these lasso peptides use an aspartate residue for ring formation, while both caulosegnins II and III use a glutamate residue and thus are having a slightly larger macrolactam ring. Analysis of the predicted precursor sequences by Link et al.[35] reveals that the use of an aspartate residue as ring forming amino acid at position 9 appears to be much more common than the use of a glutamate residue at this position. It could be reasoned that through the slightly longer side chain of glutamate, a stabilization of a nine amino acid macrolactam ring could be more difficult, making the use of an aspartate residue favorable as it could lead to a more stable lasso fold.

Interestingly, the amino acids used as plugs are rather diverse, ranging from tyrosine to a comparably small amino acid like asparagine. In general, it appears more and more likely that the size of the amino acid itself is not the only important factor in regard to lasso fold maintenance. In contrast, the size of the loop and the resulting level of strain of its conformation, as well as the distance of the plugs to the part of the tail that passes the ring, could play a major role in stabilizing the lasso fold. This assumption would explain why tyrosine is not capable of stabilizing a 9 aa ring variant of McJ25,[58] while it was shown that it most likely stabilizes both caulosegnins II and III. The fact that a R15A variant of capistruin is a heat stable lasso peptide, whose fold is sustained by its Phe16 residue, while a R15A/F16A mutation results in a heat labile variant, even though the stabilizing amino acid is still a phenylalanine residue (but now at position 18 instead of 16), further backs this notion. In this case, the elongated tail area that fits through the ring and the corresponding increase of flexibility apparently allow that even a bulky amino acid like phenylalanine may pass through the macrolactam ring.[22]

Taking all this information into consideration, it seems necessary to update the former assumption that only bulky amino acids are capable of maintaining the lasso fold and that the scope of feasible structures of class II lasso peptides could be much more diverse than previously anticipated. As factors like loop length, conformational constraints and orientation of side chains of ring amino acids can hardly be predicted solely based on the amino acid sequence, future genome mining approaches should be realized with an open mind in regard to amino acids capable of being a plug, as even smaller amino acids could be important for the lasso fold.

■ CONCLUSION

In this work, we demonstrate the practical application of the combination of our genome mining approach with an optimized system for heterologous expression in *E. coli* for the isolation of novel lasso peptides. With this approach, we were able to identify a lasso peptide biosynthetic gene cluster within the genome of *C. segnis* and were able to isolate three lasso peptides (caulosegnins I−III). This is the first report since the isolation of capistruin[12] and astexin-1[35] that a new lasso peptide biosynthetic gene cluster was systematically identified

dx.doi.org/10.1021/ja308173b | J. Am. Chem. Soc. 2013, 135, 210−222

and proven to produce previously unknown lasso peptides. At the same time, this lasso peptide biosynthetic gene cluster is the first instance, together with the cluster from *A. excentricus*, where no immunity conferring ABC-transporter is present.

It is interesting to see that the enzymes of this gene cluster are capable of processing lasso peptides with different ring sizes. Although recent studies showed that it is possible to expand the ring size of MccJ25 through insertion of an alanine residue between the fifth and sixth amino acid of the ring, the result was a mere branched cyclic peptide with a nine membered ring,[58] showing that either a flawed prefolding occurred or that the bulky side chains were no longer able to trap the tail inside the expanded ring. Still the caulosegnin biosynthetic gene cluster is the first known native system to feature not only more than one lasso peptide, but is also the first system being able to process lasso peptides with differently sized rings. Additionally, the production of a lasso peptide featuring a nine amino acid macrolactam ring using as ring forming amino acid a glutamate instead of an aspartate is something not shown before.

The caulosegnins were exposed to temperatures up to 95 °C to test their thermal stability, showing caulosegnin I to unthread, caulosegnin III to unthread and decompose and caulosegnin II to be very stable at increased temperatures. The thermal lability of both caulosegnin I and III is in contrast to the former assumption that lasso peptides have an intrinsic heat stability and raises new questions about factors affecting lasso peptide stabilization. Subsequent assessment of the proteolytic stability of these lasso peptides once more revealed the high resistance of the lasso fold against proteolytic degradation, while the thermally unthreaded analogues of caulosegnin I and III were readily digested by the tested enzymes. Furthermore the structure of caulosegnin I was determined via NMR spectroscopic methods, which showed that a surprisingly small amino acid, Glu16, was maintaining the lasso fold through sterical means.

Via mutational analysis it was shown that the plugs for both caulosegin II and III are most likely their respective Tyr16 residues. To confirm or disprove this assumption, structure elucidation of both lasso peptides is essential and will be possible after their production is further improved to allow isolation of sufficient amounts for NMR spectroscopic analysis. Until then, it will also remain unclear why these peptides show such a different behavior toward increased temperatures, as both feature a nine membered macrolactam ring and possibly use the same amino acid below the ring as plug.

In regard to the biosynthesis of the caulosegnins, our mutational studies revealed that enzymatic recognition most likely does not occur via ionic interactions and that a tail section below the ring of at least three amino acids is needed for efficient processing, while it was also shown that the loop size can be varied between 6 and 7 amino acids.

Our work also shows that the lasso fold of caulosegnin I can be maintained by an amino acid as small as asparagine or leucine, questioning the former notion that only amino acids with big, bulky side chains are capable of fulfilling this function and shifting the paradigm to loop length, structural rigidity and the orientation of the side chains of other amino acids in spacial proximity as additional important factors for lasso peptide stability. The shown importance of a proline residue in the loop region of the caulosegnins further supports this idea, as does the fact that an E16Q variant of caulosegnin I results in a lasso peptide, while an E16A/I17A variant exhibits a branched cyclic topology, even though a glutamine residue is present at position 18 of the tail.

Additionally, our mutational analysis confirmed the importance of the threonine residue at the penultimate position of the leader peptide and the glycine at position 1 of the lasso peptide, which further highlights that these criteria appear to be universal for the maturation of class II lasso peptides. Still we could also show for the first time that the substitution of the glycine at position 1 is not completely unfeasible.

As our antibacterial assays all turned out negative, the native function of these lasso peptides remains elusive. If an antibacterial activity is found in future works, it will further solidify the belief of lasso peptides generally being antibacterial agents giving a selective advantage to its producer under certain conditions. Still, the lack of an immunity conferring ABC-transporter gives rise to the idea of the caulosegnins serving a different function, suggesting that lasso peptides could have a far more diverse scope of biological functions than previously expected.

All these questions illustrate the importance of the systematic discovery, isolation and characterization of new lasso peptides and will lead to a better understanding of their role in nature and the physicochemical factors stabilizing these fascinating molecules.

■ ASSOCIATED CONTENT

Supporting Information

Protease stability tests of the caulosegnins and their branched cyclic analogues, MS^2 studies of the caulosegnins and their branched cyclic analogues, chromatograms of the purifications and of the thermostability assays, DQF-COSY-, TOCSY-, NOESY- and $^{1}H/^{15}N$-HSQC-spectra, ^{1}H spectra at variable temperatures, ^{1}H variable delay spectra, tables showing all used primers, relative production of the different generated constructs and variants, structural statistics of the 15 structures selected to represent the solution structure of caulosegnin I, assignment of ^{1}H and ^{15}N signals and stereoplots of Figures 5 and 7. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

marahiel@staff.uni-marburg.de

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Financial support from the Deutsche Forschungsgemeinschaft and the LOEWE program of the State of Hesse is gratefully acknowledged. We would like to thank Prof. A. James Link from the University of Princeton for providing us the pJP3 plasmid.

■ REFERENCES

(1) Weber, W.; Fischli, W.; Hochuli, E.; Kupfer, E.; Weibel, E. K. *J. Antibiot.* **1991**, *44*, 164−171.
(2) Salomón, R. A.; Farías, R. N. *J. Bacteriol.* **1992**, *174*, 7428−7435.
(3) Potterat, O.; Stephan, H.; Metzger, J. W.; Gnau, V.; Zähner, H.; Jung, G. *Liebigs Ann. Chem.* **1994**, 741−743.
(4) Morishita, Y.; Chiba, S.; Tsukuda, E.; Tanaka, T.; Ogawa, T.; Yamasaki, M.; Yoshida, M.; Kawamoto, I.; Matsuda, Y. *J. Antibiot.* **1994**, *47*, 269−275.

(5) Ogawa, T.; Ochiai, K.; Tanaka, T.; Tsukuda, E.; Chiba, S.; Yano, K.; Yamasaki, M.; Yoshida, M.; Matsuda, Y. *J. Antibiot.* **1995**, *48*, 1213−1220.
(6) Fréchet, D.; Guitton, J. D.; Herman, F.; Faucher, D.; Helynck, G.; Monegier du Sorbier, B.; Ridoux, J. P.; James-Surcouf, E.; Vuilhorgne, M. *Biochemistry* **1994**, *33*, 42−50.
(7) Tsunakawa, M.; Hu, S. L.; Hoshino, Y.; Detlefson, D. J.; Hill, S. E.; Furumai, T.; White, R. J.; Nishio, M.; Kawano, K.; Yamamoto, S. *J. Antibiot.* **1995**, *45*, 433−434.
(8) Yano, K.; Toki, S.; Nakanishi, S.; Ochiai, K.; Ando, K.; Yoshida, M.; Matsuda, Y.; Yamasaki, M. *Bioorg. Med. Chem.* **1996**, *4*, 115−120.
(9) Kimura, K.; Kanou, F.; Takahashi, H.; Esumi, Y.; Uramoto, M.; Yoshihama, M. *J. Antibiot.* **1997**, *50*, 373−378.
(10) Potterat, O.; Wagner, K.; Gemmecker, G.; Mack, J.; Puder, C.; Vettermann, R.; Streicher, R. *J. Nat. Prod.* **2004**, *67*, 1528−1531.
(11) Iwatsuki, M.; Tomoda, H.; Uchida, R.; Gouda, H.; Hirono, S.; Omura, S. *J. Am. Chem. Soc.* **2006**, *128*, 7486−7491.
(12) Knappe, T. A.; Linne, U.; Zirah, S.; Rebuffat, S.; Xie, X.; Marahiel, M. A. *J. Am. Chem. Soc.* **2008**, *130*, 11446−11454.
(13) Kersten, R. D.; Yang, Y. L.; Xu, Y.; Cimermancic, P.; Nam, S. J.; Fenical, W.; Fischbach, M. A.; Moore, B. S.; Dorrestein, P. C. *Nat. Chem. Biol.* **2011**, *7*, 794−802.
(14) Knappe, T. A.; Linne, U.; Xie, X.; Marahiel, M. A. *FEBS Lett.* **2010**, *584*, 785−789.
(15) Nar, H.; Schmid, A.; Puder, C.; Potterat, O. *ChemMedChem* **2010**, *5*, 1689−1692.
(16) Constantine, K. L.; Friedrichs, M. S.; Detlefsen, D.; Nishio, M.; Tsunakawa, M.; Furumai, T.; Ohkuma, H.; Oki, T.; Hill, S.; Bruccoleri, R. E.; Lin, P. F.; Mueller, L. *J. Biomol. NMR* **1995**, *5*, 271−286.
(17) Bayro, M. J.; Mukhopadhyay, J.; Swapna, G. V. T.; Huang, J. Y.; Ma, L.-C.; Sineva, E.; Dawson, P. E.; Montelione, G. T.; Ebright, R. H. *J. Am. Chem. Soc.* **2003**, *125*, 12382−12383.
(18) Rosengren, K. J.; Clark, R. J.; Daly, N. L.; Göransson, U.; Jones, A.; Craik, D. J. *J. Am. Chem. Soc.* **2003**, *125*, 12464−12474.
(19) Wilson, K. A.; Kalkum, M.; Ottesen, J.; Yuzenkova, J.; Chait, B. T.; Landick, R.; Muir, T.; Severinov, K.; Darst, S. A. *J. Am. Chem. Soc.* **2003**, *125*, 12475−12783.
(20) Katahira, R.; Shibata, K.; Yamasaki, M.; Matsuda, Y.; Yoshida, M. *Bioorg. Med. Chem.* **1995**, *3*, 1273−1280.
(21) Wyss, D. F.; Lahm, H. W.; Manneberg, M.; Labhart, A. M. *J. Antibiot.* **1991**, *44*, 172−180.
(22) Knappe, T. A.; Linne, U.; Robbel, L.; Marahiel, M. A. *Chem. Biol.* **2009**, *16*, 1290−1298.
(23) Yamasaki, M.; Yano, K.; Yoshida, M.; Matsuda, Y.; Yamaguchi, K. *J. Antibiot.* **1994**, *43* (3), 276−280.
(24) Blond, A.; Cheminant, M.; Destoumieux-Garzón, D.; Ségalas-Milazzo, I.; Goulard, C.; Rebuffat, S. *Eur. J. Biochem.* **2002**, *269*, 6212−6222.
(25) Rosengren, K. J.; Blond, A.; Afonso, C.; Tabet, J. C.; Rebuffat, S.; D., J. *Biochemistry* **2004**, *43*, 4696−4702.
(26) Soudy, R.; Wang, L.; Kaur, K. *Bioorg. Med. Chem.* **2012**, *20*, 1794−1800.
(27) Katahira, R.; Shibata, K.; Yamasaki, M.; Matsuda, Y.; Yoshida, M. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1595−1600.
(28) Yuzenkova, J.; Delgado, M.; Nechaev, S.; Savalia, D.; Epshtein, V.; Artsimovitch, I.; Mooney, R. A.; Landick, R.; Farias, R. N.; Salomon, R.; Severinov, K. *J. Biol. Chem.* **2002**, *277*, 50867−50875.
(29) Adelman, K.; Yuzenkova, J.; La Porta, A.; Zenkin, N.; Lee, J.; Lis, J. T.; Borukhov, S.; Wang, M. D.; Severinov, K. *Mol. Cell* **2004**, *14*, 753−762.
(30) Semenova, E.; Yuzenkova, Y.; Peduzzi, J.; Rebuffat, S.; Severinov, K. *J. Bacteriol.* **2005**, *187*, 3859−3863.
(31) Kuznedelov, K.; Semenova, E.; Knappe, T. A.; Mukhamedyarov, D.; Srivastava, A.; Chatterjee, S.; Ebright, R. H.; Marahiel, M. A.; Severinov, K. *J. Mol. Biol.* **2011**, *412*, 842−848.
(32) Knappe, T. A.; Manzenrieder, F.; Mas-Moruno, C.; Linne, U.; Sasse, F.; Kessler, H.; Xie, X.; Marahiel, M. A. *Angew. Chem.* **2011**, *50*, 8714−8717.
(33) Solbiati, J. O.; Ciaccio, M.; Farías, R. N.; González-Pastor, J. E.; Moreno, F.; Salomón, R. A. *J. Bacteriol.* **1999**, *181*, 2659−2662.
(34) Inokoshi, J.; Matsuhama, M.; Miyake, M.; Ikeda, H.; Tomoda, H. *Appl. Microbiol. Biotechnol.* **2012**, *95*, 451−460.
(35) Maksimov, M. O.; Pelczer, I.; Link, A. J. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 15223−15228.
(36) Yan, K. P.; Zirah, S.; Goulard, C.; Knappe, T. A.; Marahiel, M. A.; Rebuffat, S. *ChemBioChem* **2012**, *13*, 1046−1052.
(37) Duquesne, S.; Destoumieux-Garzón, D.; Zirah, S.; Goulard, C.; Peduzzi, J.; Rebuffat, S. *Chem. Biol.* **2007**, *14*, 793−803.
(38) Pan, S. J.; Rajniak, J.; Cheung, W. L.; Link, A. J. *ChemBioChem* **2012**, *13*, 367−37.
(39) Rance, M.; Sørensen, O. W.; Bodenhausen, G.; Wagner, G.; Ernst, R. R.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *117*, 479−485.
(40) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *65*, 355−360.
(41) Jeener, J.; Meier, B. H.; Bachmann, P.; Ernst, R. R. *J. Chem. Phys.* **1979**, *71*, 4546−4553.
(42) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *63*, 207−213.
(43) Griesinger, C.; Sørensen, O. W.; Ernst, R. R. *J. Magn. Reson.* **1987**, *75*, 474−492.
(44) Marion, D.; Ikura, M.; Tschudin, R.; Bax, A. *J. Magn. Reson.* **1989**, *85*, 393−399.
(45) Hwang, T.-L.; Shaka, A. J. *J. Magn. Reson., Ser. A* **1995**, *112*, 275−279.
(46) Goddard, T. D.; Kneller, D. J. *Sparky 3*; University of California: San Francisco.
(47) Wüthrich, K. *NMR of Protein and Nucleic Acids*; Wiley: New York, 1986.
(48) Herrmann, T.; Güntert, P.; Wüthrich, K. *J. Mol. Biol.* **2002**, *319*, 209−227.
(49) Pan, S. J.; Rajniak, J.; Maksimov, M. O.; Link, A. J. *Chem. Commun.* **2012**, *48*, 1880−1882.
(50) Curtis, P. D.; Brun, Y. V. *Microbiol. Mol. Biol. Rev.* **2010**, *74*, 13−41.
(51) Medema, M. H.; Blin, K.; Cimermancic, P.; de Jager, V.; Zakrzewski, P.; Fischbach, M. A.; Weber, T.; Takano, E.; Breitling, R. *Nucleic Acids Res.* **2011**, *39*, W339−3W46.
(52) Pan, S. J.; Cheung, W. L.; Link, A. J. *Protein Expression Purif.* **2010**, *71*, 200−206.
(53) Xie, X.; Marahiel, M. A. *ChemBioChem* **2012**, *13*, 621−625.
(54) *Sybyl 7.3*; Tripos, Inc.: St. Louis, MO.
(55) Radkiewicz, J. L.; Zipse, H.; Clarke, S.; Houk, K. N. *J. Am. Chem. Soc.* **2001**, *123*, 3499−3506.
(56) Skribanek, Z.; Mezo, G.; Mák, M.; Hudecz, F. *J. Pept. Sci.* **2002**, *8*, 398−406.
(57) Pavlova, O.; Mukhopadhyay, J.; Sineva, E.; Ebright, R. H.; Severinov, K. *J. Biol. Chem.* **2008**, *283*, 25589−25595.
(58) Ducasse, R.; Yan, K. P.; Goulard, C.; Blond, A.; Li, Y.; Lescop, E.; Guittet, E.; Rebuffat, S.; Zirah, S. *ChemBioChem* **2012**, *13*, 371−380.
(59) Pan, S. J.; Link, A. J. *J. Am. Chem. Soc.* **2011**, *133*, 5016−5023.