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The Ring Residue Proline 8 is Crucial for the Thermal Stability of the Lasso Peptide Caulosegnin II

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

SUPPORTING INFORMATION

Materials and Methods

Bacterial Strains and General Methods

E. coli TOP10 was used for the generation of the expression construct mutants, while *E. coli* BL21 (DE3) was used for heterologous lasso peptide production. Successful incorporation of the mutations was validated through dideoxy sequencing performed by GATC Biotech. Phusion polymerase and DpnI were purchased from New England Biolabs, while oligonucleotide primers were obtained from Sigma Aldrich and carboxypeptidase Y was bought from Alfa Aesar.

Generation of the Caulosegnin Variants

All caulosegnin variants were generated in the same manner by employing site-directed ligase-independent mutagenesis (SLIM).[1,2] For this, the corresponding expression plasmids were utilized as templates and mixed overhang PCRs were performed with the primers listed in Supplementary Table S1. Afterward, the PCR products were treated with DpnI to remove residual template DNA, hybridized as described in the literature,[1,2] dialyzed and finally transformed by electroporation. Mutated plasmids were isolated and validated by DNA sequencing.

The template plasmids were optimized for caulosegnin production in a previous study (see also SI Figure S1)[3] and were mostly identical with exception of the genes encoding the corresponding precursor peptides. For caulosegnin I the WT *csegA1* gene was present, while for caulosegnins II and III hybrid genes encoding the caulosegnin I leader and the caulosegnin II/III core sequences were used.

Supplementary Table S1. Oligonucleotide primers used for SLIM PCRs. For every mutation, two PCR reactions were carried out; one featuring the corresponding FP and RP_{Tail}, the other one the corresponding FP_{Tail} and RP primers shown below. The overhang regions of the tail primers are underlined, the mutated positions highlighted in bold.

name	sequence
FP_CsegA1[a]	TGA GGT ACC AGC TTA ATT AGC TGA GCT TGG ACT CCT GTT GAT AG
RP_CsegA1[a]	ATT GAC CGC TTC GGG CTG GCC GAC GAA CGC GCC TTG
FP_{Tail}_E16W_CsegA1[a]	CCG CTC GGC CGC **TGG** ATC CAA GGC TGA GGT ACC AGC TTA ATT AGC TGA GCT TGG ACT CCT GTT GAT AG
RP_{Tail}_E16W_CsegA1[a]	GCC TTG GAT **CCA** GCG GCC GAG CGG ATT GAC CGC TTC GGG CTG GCC GAC GAA CGC GCC TTG
FP_CsegA3[b]	TGA GGT ACC AGC TTA ATT AGC TGA GCT TGG ACT CCT GTT GAT AG
RP_CsegA3[b]	GAT GTC CTC GAG CAG CAG GCC CAC CAG CGC GCC TTG
FP_{Tail}_Y16W_CsegA3[b]	ACC GTG GCC CGC **TGG** GAC CCG ATG TGA GGT ACC AGC TTA ATT AGC TGA GCT TGG ACT CCT GTT GAT AG
RP_{Tail}_Y16W_CsegA3[b]	CAT CGG GTC **CCA** GCG GGC CAC GGT GAT GTC CTC GAG CAG CAG GCC CAC CAG CGC GCC TTG
FP_CsegA2-Ring[c]	GAG GAT TTC CTG CCT GGC CAC TAC ATG CCG GGC TG
RP_CsegA2-Ring[c]	GCC TTG CGT CAG GCG ATG GGC GTC GCC GAC
FP_{Tail}_P5A_CsegA2Ring[c]	ACG CTC ACT **GCG** GGC CTG CCG GAG GAT TTC CTG CCT GGC CAC TAC ATG CCG GGC TG
RP_{Tail}_P5A_CsegA2Ring[c]	CGG CAG GCC **CGC** AGT GAG CGT GCC TTG CGT CAG GCG ATG GGC GTC GCC GAC
FP_{Tail}_P8A_CsegA2Ring[c]	ACG CTC ACT CCG GGC CTG **GCG** GAG GAT TTC CTG CCT GGC CAC TAC ATG CCG GGC TG
RP_{Tail}_P8A_CsegA2Ring[c]	**CGC** CAG GCC CGG AGT GAG CGT GCC TTG CGT CAG GCG ATG GGC GTC GCC GAC
FP_{Tail}_P5A-P8A_CsegA2Ring[c]	ACG CTC ACT **GCG** GGC CTG **GCG** GAG GAT TTC CTG CCT GGC CAC TAC ATG CCG GGC TG
RP_{Tail}_P5A-P8A_CsegA2Ring[c]	**CGC** CAG GCC **CGC** AGT GAG CGT GCC TTG CGT CAG GCG ATG GGC GTC GCC GAC
FP_{Tail}_G6P-P8A_CsegA2Ring[c]	ACG CTC ACT CCG **CCG** CTG **GCG** GAG GAT TTC CTG CCT GGC CAC TAC ATG CCG GGC TG
RP_{Tail}_G6P-P8A_CsegA2Ring[c]	**CGC** CAG **CGG** CGG AGT GAG CGT GCC TTG CGT CAG GCG ATG GGC GTC GCC GAC
FP_{Tail}_L7P-P8A_CsegA2Ring[c]	ACG CTC ACT CCG GGC **CCG GCG** GAG GAT TTC CTG CCT GGC CAC TAC ATG CCG GGC TG
RP_{Tail}_L7P-P8A_CsegA2Ring[c]	**CGC CGG** GCC CGG AGT GAG CGT GCC TTG CGT CAG GCG ATG GGC GTC GCC GAC
FP_CsegA2[c]	GGC TGA GGT ACC AGC TTA ATT AGC TGA GCT TGG ACT CCT G
RP_CsegA2[c]	GAA ATC CTC CGG CAG GCC CGG AGT GAG CGT GC
FP_{Tail}_P13A_CsegA2[c]	CTG **GCG** GGC CAC TAC ATG CCG GGC TGA GGT ACC AGC TTA ATT AGC TGA GCT TGG ACT CCT G
RP_{Tail}_P13A_CsegA2[c]	CGG CAT GTA GTG GCC **CGC** CAG GAA ATC CTC CGG CAG GCC CGG AGT GAG CGT GC
FP_{Tail}_P18A_CsegA2[c]	CTG CCT GGC CAC TAC ATG **GCG** GGC TGA GGT ACC AGC TTA ATT AGC TGA GCT TGG ACT CCT G
RP_{Tail}_P18A_CsegA2[c]	**CGC** CAT GTA GTG GCC AGG CAG GAA ATC CTC CGG CAG GCC CGG AGT GAG CGT GC
FP_{Tail}_P13A-P18A_CsegA2[c]	CTG **GCG** GGC CAC TAC ATG **GCG** GGC TGA GGT ACC AGC TTA ATT AGC TGA GCT TGG ACT CCT G
RP_{Tail}_P13A-P18A_CsegA2[c]	**CGC** CAT GTA GTG GCC **CGC** CAG GAA ATC CTC CGG CAG GCC CGG AGT GAG CGT GC
FP_CsegA2(P13A-P18A)[d]	GAG GAT TTC CTG CGG GGC CAC TAC ATG CGG GGC TG
RP_CsegA2(P13A-P18A)[d]	GCC TTG CGT CAG GCG ATG GGC GTC GCC GAC
FP_{Tail}_P5A_CsegA2(P13A/P18A)[d]	ACG CTC ACT **GCG** GGC CTG CCG GAG GAT TTC CTG CGG GGC CAC TAC ATG CGG GGC TG
RP_{Tail}_P5A_CsegA2(P13A/P18A)[d]	CGG CAG GCC **CGC** AGT GAG CGT GCC TTG CGT CAG GCG ATG GGC GTC GCC GAC
FP_{Tail}_P8A_CsegA2(P13A/P18A)[d]	ACG CTC ACT CCG GGC CTG **GCG** GAG GAT TTC CTG CGG GGC CAC TAC ATG CGG GGC TG
RP_{Tail}_P8A_CsegA2(P13A/P18A)[d]	**CGC** CAG GCC CGG AGT GAG CGT GCC TTG CGT CAG GCG ATG GGC GTC GCC GAC

[a] *csegA1_T-P-BC* pET41a was used as PCR template[3]

[b] *csegA1-QG-A3_T-P-BC* pET41a was used as PCR template[3]

[c] *csegA1-QG-A2_T-P-BC* pET41a was used as PCR template[3]

[d] *csegA1-QG-A2(P13A/P18A)_T-P-BC* pET41a was used as PCR template

Cultivation and Lasso Peptide Extraction

The respective plasmids were expressed in *E. coli* BL21 (DE3). For this, 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 (see Supplementary Table S2) were added) containing 50 µg/mL kanamycin was inoculated with an LB overnight culture and grown at 37 °C until an OD_{600} of ~0.4. Then, the temperature was reduced and after reaching 20 °C, the expression was induced by addition of 100 µL/L IPTG stock solution (0.5 M) at an OD of ~0.6. Subsequently, the cells were fermented for 3 days at 20 °C and then harvested by centrifugation.

Supplementary Table S2. M9 vitamin mix.

component	amount
choline chloride	1.0 g
folic acid	1.0 g
pantothenic acid	1.0 g
nicotinamide	1.0 g
myo-inositol	2.0 g
pyridoxal hydrochloride	1.0 g
thiamine	1.0 g
riboflavin	0.1 g
disodium adenosine 5'-triphosphate	0.3 g
biotin	0.2 g
	ad 300 mL ddH2O[a]

[a]10 M NaOH was slowly added until all components were dissolved at a pH of ~12. Afterward, the clear, orange solution was sterile filtered and stored at 4 °C for short term use or at -20 °C for longer storage durations.

The cell pellets were resuspended and extracted with 50 mL of pure methanol per liter of culture by shaking overnight at 4 °C. Afterward, the extracts were cleared by centrifugation and filtration and the solvent was evaporated at 40 °C and reduced pressure.

For LC-MS analyses of the extracts, the constructs were expressed in a total of 600 mL M9 medium contained within a 2 L baffled culture flask and in the end, the dried extracts were resuspended in 1 mL of 50% methanol. The resuspended extracts were then prepared for LC-MS analysis by centrifugation (30 min, 13000 rpm) and filtration through a 0.20 µm sterile filter. For lasso peptide isolation, larger cultures (a total volume of 3 L, which was also aliquoted in portions of 600 mL medium in 2 L baffled culture flasks) were fermented and their dried extracts were resuspended in 9 mL of 50% methanol and prepared in a likewise manner for preparative HPLC.

MS Analysis

For analysis of cell extracts, high resolution LC-FT-MS was performed, employing a microbore 1100 HPLC system (Agilent) that was connected to a LTQ-FT ultra instrument (Thermo Fisher Scientific). For the measurements, an EC 125/2 Nucleodur 300-5 C18ec column (Macherey-Nagel) was used with a flow rate of 0.2 mL/min and separation of the compounds was achieved by 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: Holding 2% B for 2 min, then a linear increase from 2% to 30% B in 18 min, followed by an additional linear increase from 30% to 95% B in 15 min and finally holding 95% B for another 2 min.

The resulting data was analyzed for the presence of the predicted masses for full length lasso peptides and all corresponding truncation products down to -10 aa (which corresponds to the lone macrolactam ring). For all peptide sequences containing a methionine, also signals were checked that were increased by the mass of one or two oxygen atoms, to take into account the possibility of methionine oxidation. In these cases, often a once-oxidized compound could be detected in significant amounts, while doubly-oxidized compounds were at most observed in negligible traces.

For a semi-quantitative determination of the amounts of the analyzed compounds, all peaks in the corresponding extracted ion chromatograms (EICs) that showed the exact predicted masses (of unoxidized and, if possible and present, oxidized species) were integrated and their values were added together to serve as a rough indicator of the total production. The integral of each observed truncated species (plus the integral of their oxidized version, if present) was correlated to the total integral, to yield a relative estimate of the distribution of the different truncation products in a sample.

For analysis of the assays with purified lasso peptides, another microbore 1100 HPLC system (Agilent) coupled with a 1100 series MSD (Hewlett-Packard) was utilized. In these cases, sample separation was achieved by using an EC 125/2 Nucleodur 100-3 C18ec column (Macherey-Nagel) at room temperature and a flow rate of 0.3 mL/min. The same solvent system as described before was used with the following gradient for caulosegnin II, III and variants thereof: Linear increase from 35 to 55% B in 15 min, followed by another linear increase up to 95% B in 2 min and subsequently holding 95% B for another 5 min. For caulosegnin I and its E16W variant another gradient was applied: Linear increase from 20 to 35% B in 15 min, then an increase up to 95% B in another 2 min and finally holding 95% B for additional 5 min.

Isolation and Purification of Lasso Peptides

For isolation and purification of selected lasso peptides, a preparative HPLC system (microbore 1100 HPLC System (Agilent) with a VP 250/21 Nucloedur C18 Htec 5 mm column

(Macherey-Nagel); 18 mL/min flow rate at room temperature) was used. The WT lasso peptides were isolated as described previously with similar yields.[3]

In every other case, the lasso peptides were purified by two rounds of HPLC with different gradients of the aforementioned solvent systems. For the first round, 9 mL of cleared pellet extracts from each production culture (see *Cultivation and Lasso Peptide Extraction* for more details) were applied separately to the following gradient: A linear increase from 10 to 60% B in 30 min, followed by a subsequent linear increase to 95% B in another 3 min. For the second round, a less steep gradient was employed: Linear increase from 20 to 40% B in 30 min, followed by another linear increase to 95% B in additional 2 min and holding 95% B for 3 minutes. The yields of the thereby purified variants are shown in Supplementary Table S3.

Supplementary Table S3. Yields of the purified lasso peptide variants in mg per liter of production culture.

variant	yield
caulosegnin I (E16W)	0.31 mg/L
caulosegnin II (P5A)	0.33 mg/L
caulosegnin II (P8A)	0.19 mg/L
caulosegnin II (P5A/P8A)	0.11 mg/L
caulosegnin III (Y16W)*	0.09 mg/L

*only the species with a singly oxidized Met19 could be isolated from the large scale culture

Crystallization and Structure Elucidation

Caulosegnin II and III stock solutions were prepared in 30% MeCN with a final concentration of 20 mg/mL of the corresponding lasso peptide. These stock solutions were applied to sparse-matrix screens purchased from Qiagen, where they were mixed in an 1:1 ratio with the respective crystallization conditions. Rod-shaped crystals of caulosegnin II were obtained at 293 K by mixing with a reservoir solution consisting of 0.2 M LiCl and 20% (w/V) PEG 3350. No crystals were obtained for caulosegnin III.

Caulosegnin II crystals were cryoprotected with 10% (V/V) glycerol in the aforementioned reservoir solution and then flash-frozen in liquid nitrogen. Data were collected at 100 K at Bessy II (Helmholtz-Zentrum Berlin, Germany), Beamline 14.1 using a Dectris PILATUS detector.[4] To obtain high-resolution data and to improve the signal-to-noise ratio, a wavelength and oscillation range of 0.800 Å and 0.1°, respectively, were utilized.

Data were processed with the program XDS,[5] prepared for phasing in XPREP (Bruker AXS GmbH, Karlsruhe, Germany), and then solved by dual-space direct methods in SHELX.[6] For refinement, the hkl reflection file output by XDS was converted to mtz format in Combat, and then scaled (with free-R flags added to 5% of reflections) in Aimless,[7] both programs of the CCP4software suite.[8] Despite the completeness of the highest-resolution bin (51.6%), data were not truncated to lower

resolution due to reasonable ‹*I*/σ(*I*)› and *CC*1/2 values (2.8 and 81%, respectively).[9,10] The coordinate file output by SHELX was refined to the electron density map of the scaled mtz using Coot[11] and phenix.refine of the PHENIX software suite.[12] In the latter, B-factors of all atoms except for hydrogens and water oxygens were refined anisotropically; additionally, a "riding" hydrogen model was applied, X-ray/stereochemistry and X-ray/ADP weights were optimized, and the isopeptide bond between Gly1N and Glu9CD was restrained (ideal distance = 1.4, sigma = 0.03). These procedures yielded the complete electron density for all 19 residues of the single monomer in the asymmetric unit of a $P2_12_12_1$ unit cell and coordinates of the final model of caulosegnin II were submitted to the Protein Data Bank (PDB) with accession code 5D9E.

Combined Thermal Stability and Carboxypeptidase Y Assays

For the extract assays, 55 µL of the corresponding extracts were mixed with 55 µL ddH_2O. For thermal treatment, such a solution was then incubated for 2 h at 95 °C. Treated and untreated samples were then dried in a Concentrator 5301 (Eppendorf) at 30 °C under reduced pressure.

For subsequent carboxypeptidase Y treatment, the dried samples were dissolved in 35 µL MES buffer (50 mM MES, 1 mM $CaCl_2$, pH 6.75) and then 20 µL of carboxypeptidase Y solution (1 mg/mL in MES buffer) was added. After mixing, the samples were incubated for 4 h at 25 °C. For the control reactions without carboxypeptidase Y, 20 µL of plain MES buffer were added instead of the carboxypeptidase Y solution and then these samples were also incubated for 4 h at 25 °C. Subsequently, all samples were quenched with 55 µL of pure methanol and stored at -20 °C until analyzed via high-resolution FT-LC-MS. For analysis, 100 µL of each sample was injected. In this way, for each variant four different conditions were analyzed; namely untreated, only heated for 2 h at 95 °C, only treated with carboxypeptidase Y, and both heated for 2 h at 95 °C and subsequently treated with carboxypeptidase Y. Results for these assays are shown in Supplementary Tables S4-S13.

Interestingly, the lasso peptide variants containing a P18A exchange showed an accumulation of -1 to -3 aa truncation products during incubation with carboxypeptidase Y that was not observed for variants that still contained a proline at this position. Closer inspection also revealed that for the P13A/P18A double and the P8A/P13A/P18A triple substitutions, the -2 aa truncation is already the main product that is isolated from the cell pellet. Nonetheless, as this behavior was independent from prior incubation at 95 °C and as all three truncation products still contain the Tyr16 plug residue, it can be assumed that the lasso fold is not affected by this. Rather, it is likely that this partial proteolysis can be explained by Pro18 normally slowing down the proteolytic degradation by carboxypeptidase Y. This hypothesis is further substantiated by the observation that WT caulosegnin II and its variants with Pro18 also show a very slight production of these truncation products under

the reaction conditions. Thus, the last residues below the ring appear to be at least slightly accessible for carboxypeptidase Y, which can therefore slowly cleave off these amino acids. This degradation is slowed down even further by the presence of a proline. Still, as long as the lasso fold is present, the Tyr16 plug is completely shielded by the macrolactam ring, which explains why no truncations below -3 aa are observed in significant amounts for these lasso peptides.

For the assays with pure lasso peptides, 11 µL of the respective lasso peptide solutions (1 mg/mL in ddH_2O) were mixed with 24 µL of MES buffer and for thermal treatment were incubated for either 1, 2 or 4 h at 95 °C. For carboxypeptidase Y treatment, samples that were either not heated or incubated for 4 h at 95 °C were mixed with 20 µL of carboxypeptidase Y solution and subsequently incubated for 4 h at 25 °C. For the other samples that should not be treated with carboxypeptidase Y, only 20 µL of pure MES buffer were added. The samples were then mixed with 55 µL pure methanol and stored at -20 °C until measured by LC-MS. For each sample, 100 µL was injected for analysis. In this way, six conditions were analyzed for each lasso peptide. These are untreated, only incubated for 1, 2 or 4 h at 95 °C, only treated with carboxypeptidase Y, and first heated for 4 h at 95 °C and then treated with carboxypeptidase Y.

Supplementary Table S4. Results of the combined thermal stability and carboxypeptidase Y assays for WT caulosegnin II. The numbers shown are the total integrals of the corresponding peaks in the EICs on the left, **relative amounts** in the middle and retention times on the right. For peptides containing a methionine, only the retention times of the unoxidized species are shown.

truncation	untreated	2 h at 95 °C	treated with carboxypeptidase Y	2 h at 95 °C, then treated with carboxypeptidase Y
full length	1.59E+09 / **99.75%** / 30.1 min	1.49E+09 / **99.70%** / 30.1 min	1.48E+09 / **92.09%** / 30.0 min	1.86E+09 / **92.66%** / 29.7 min
-1	9.04E+05 / **0.06%** / 30.7 min	7.72E+05 / **0.05%** / 30.6 min	1.18E+08 / **7.36%** / 30.6 min	8.32E+07 / **4.15%** / 30.5 min
-2	- / - / -	- / - / -	4.43E+06 / **0.28%** / 30.9 min	1.14E+07 / **0.57%** / 30.9 min
-3	- / - / -	- / - / -	- / - / -	2.58E+05 / **0.01%** / 28.3 min
-4	- / - / -	- / - / -	- / - / -	1.11E+06 / **0.06%** / 27.8 min
-5	- / - / -	- / - / -	- / - / -	- / - / -
-6	- / - / -	- / - / -	- / - / -	- / - / -
-7	- / - / -	- / - / -	- / - / -	- / - / -
-8	- / - / -	- / - / -	- / - / -	3.15E+06 / **0.16%** / 26.5 min
-9	3.12E+06 / **0.20%** / 21.2 min	3.78E+06 / **0.25%** / 21.0 min	4.34E+06 / **0.27%** / 21.3 min	4.80E+07 / **2.39%** / 21.4 min
-10	- / - / -	- / - / -	- / - / -	- / - / -
total	**1.59E+09**	**1.50E+09**	**1.60E+09**	**2.01E+09**

Supplementary Table S5. Results of the combined thermal stability and carboxypeptidase Y assays for caulosegnin II (P5A). The numbers shown are the total integrals of the corresponding peaks in the EICs on the left, **relative amounts** in the middle and retention times on the right. For peptides containing a methionine, only the retention times of the unoxidized species are shown.

truncation	untreated	2 h at 95 °C	treated with carboxypeptidase Y	2 h at 95 °C, then treated with carboxypeptidase Y
full length	7.37E+08 / **99.63%** / 28.7 min	1.22E+09 / **99.73%** / 28.8 min	6.96E+08 / **98.91%** / 28.7 min	1.16E+09 / **96.09%** / 28.6 min
-1	2.82E+05 / **0.04%** / 29.4 min	1.64E+05 / **0.01%** / 29.3 min	4.41E+06 / **0.63%** / 29.3 min	3.27E+06 / **0.27%** / 29.6 min
-2	- / - / -	- / - / -	5.72E+05 / **0.08%** / 29.6 min	2.01E+06 / **0.17%** / 29.8 min
-3	- / - / -	- / - / -	- / - / -	- / - / -
-4	- / - / -	- / - / -	- / - / -	- / - / -
-5	- / - / -	- / - / -	- / - / -	- / - / -
-6	- / - / -	- / - / -	- / - / -	- / - / -
-7	- / - / -	- / - / -	- / - / -	- / - / -
-8	- / - / -	- / - / -	- / - / -	2.91E+06 / **0.24%** / 26.1 min
-9	1.19E+06 / **0.16%** / 20.6 min	1.57E+06 / **0.13%** / 20.7 min	2.62E+06 / **0.37%** / 20.8 min	3.87E+07 / **3.21%** / 20.8 min
-10	1.27E+06 / **0.17%** / 21.5 min	1.59E+06 / **0.13%** / 21.5 min	7.47E+04 / **0.01%** / 22.0 min	2.02E+05 / **0.02%** / 22.0 min
total	**7.39E+08**	**1.22E+09**	**7.04+08**	**1.21E+09**

Supplementary Table S6. Results of the combined thermal stability and carboxypeptidase Y assays for caulosegnin II (P8A). The numbers shown are the total integrals of the corresponding peaks in the EICs on the left, **relative amounts** in the middle and retention times on the right. For peptides containing a methionine, only the retention times of the unoxidized species are shown.

truncation	untreated	2 h at 95 °C	treated with carboxypeptidase Y	2 h at 95 °C, then treated with carboxypeptidase Y
full length	5.35E+08 / **89.27%** / 29.7 min	1.62E+08 / **27.07%** / 29.7 min	4.80E+08 / **80.55%** / 29.6 min	1.84E+08 / **33.18%** / 29.6 min
full length (unthreaded)	- / - / -	3.72E+08 / **62.16%** / 29.1 min	- / - / -	- / - / -
-1	6.16E+04 / **0.01%** / 30.2 min	- / - / -	2.78E+07 / **4.85%** / 30.1 min	6.64E+06 / **1.20%** / 30.1 min
-2	- / - / -	- / - / -	1.66E+07 / **2.79%** / 30.5 min	3.16E+06 / **0.57%** / 30.5 min
-3	- / - / -	- / - / -	1.76E+05 / **0.03%** / 28.7 min	2.35E+06 / **0.43%** / 28.9 min
-4	- / - / -	- / - / -	5.18E+05 / **0.09%** / 28.2 min	1.18E+06 / **0.21%** / 28.2 min
-5	5.80E+05 / **0.10%** / 28.3 min	3.71E+05 / **0.06%** / 28.2 min	7.54E+04 / **0.01%** / 28.3 min	2.39E+05 / **0.04%** / 28.3 min
-6	- / - / -	- / - / -	- / - / -	- / - / -
-7	- / - / -	- / - / -	- / - / -	- / - / -
-8	- / - / -	- / - / -	- / - / -	2.48E+07 / **4.49%** / 25.4 min
-9	4.60E+07 / **7.68%** / 20.2 min	4.64E+06 / **7.75%** / 20.3 min	6.96E+07 / **11.68%** / 20.3 min	3.31E+08 / **59.88%** / 20.4 min
-10	1.76E+07 / **2.94%** / 20.6 min	1.77E+07 / **2.96%** / 20.7 min	- / - / -	- / - / -
total	**5.99E+08**	**5.98E+08**	**5.96E+08**	**5.53E+08**

Supplementary Table S7. Results of the combined thermal stability and carboxypeptidase Y assays for caulosegnin II (P5A/P8A). The numbers shown are the total integrals of the corresponding peaks in the EICs on the left, **relative amounts** in the middle and retention times on the right. For peptides containing a methionine, only the retention times of the unoxidized species are shown.

truncation	untreated	2 h at 95 °C	treated with carboxypeptidase Y	2 h at 95 °C, then treated with carboxypeptidase Y
full length	1.97E+08 / **96.44%** / 28.3 min	1.40E+08 / **53.41%** / 28.2 min	1.11E+08 / **99.59%** / 28.2 min	1.38E+08 / **59.68%** / 28.2 min
full length (unthreaded)	- / - / -	1.14E+08 / **43.61%** / 29.7 min	- / - / -	- / - / -
-1	- / - / -	- / - / -	7.66E+04 / **0.04%** / 28.8 min	- / - / -
-2	- / - / -	- / - / -	- / - / -	- / - / -
-3	- / - / -	- / - / -	- / - / -	6.02E+05 / **0.26%** / 29.4 min
-4	- / - / -	- / - / -	- / - / -	1.10E+06 / **0.47%** / 28.8 min
-5	- / - / -	- / - / -	- / - / -	- / - / -
-6	- / - / -	- / - / -	- / - / -	- / - / -
-7	- / - / -	- / - / -	- / - / -	- / - / -
-8	- / - / -	- / - / -	- / - / -	4.46E+06 / **1.93%** / 27.1 min
-9	4.85E+06 / **2.38%** / 22.4 min	5.24E+06 / **1.99%** / 22.4 min	7.46E+05 / **0.37%** / 22.4 min	8.69E+07 / **37.65%** / 22.4 min
-10	2.40E+06 / **1.18%** / 23.0 min	2.60E+06 / **0.99%** / 23.1 min	- / - / -	- / - / -
total	**2.04E+08**	**2.63E+08**	**2.00E+08**	**2.31E+08**

Supplementary Table S8. Results of the combined thermal stability and carboxypeptidase Y assays for caulosegnin II (P13A). The numbers shown are the total integrals of the corresponding peaks in the EICs on the left, **relative amounts** in the middle and retention times on the right. For peptides containing a methionine, only the retention times of the unoxidized species are shown.

truncation	untreated	2 h at 95 °C	treated with carboxypeptidase Y	2 h at 95 °C, then treated with carboxypeptidase Y
full length	1.29E+08 / **100%** / 30.1 min	1.30E+08 / **100%** / 30.1 min	1.22E+08 / **86.35%** / 30.0 min	1.16E+08 / **85.84%** / 29.7 min
-1	- / - / -	- / - / -	1.72E+06 / **1.22%** / 29.9 min	1.65E+06 / **1.22%** / 29.9 min
-2	- / - / -	- / - / -	1.73E+07 / **12.30%** / 30.1 min	1.65E+07 / **12.19%** / 30.1 min
-3	- / - / -	- / - / -	6.39E+04 / **0.05%** / 28.9 min	7.58E+04 / **0.06%** / 28.9 min
-4	- / - / -	- / - / -	- / - / -	- / - / -
-5	- / - / -	- / - / -	- / - / -	- / - / -
-6	- / - / -	- / - / -	- / - / -	- / - / -
-7	- / - / -	- / - / -	- / - / -	- / - / -
-8	- / - / -	- / - / -	- / - / -	1.42E+05 / **0.11%** / 26.5 min
-9	- / - / -	- / - / -	1.18E+05 / **0.08%** / 22.0 min	7.98E+05 / **0.59%** / 21.6 min
-10	- / - / -	- / - / -	- / - / -	- / - / -
total	**1.29E+08**	**1.30E+08**	**1.41E+08**	**1.35E+08**

Supplementary Table S9. Results of the combined thermal stability and carboxypeptidase Y assays for caulosegnin II (P18A). The numbers shown are the total integrals of the corresponding peaks in the EICs on the left, **relative amounts** in the middle and retention times on the right. For peptides containing a methionine, only the retention times of the unoxidized species are shown.

truncation	untreated	2 h at 95 °C	treated with carboxypeptidase Y	2 h at 95 °C, then treated with carboxypeptidase Y
full length	8.46E+08 / **88.61%** / 29.9 min	5.25E+08 / **85.14%** / 29.9 min	2.62E+08 / **18.73%** / 29.1 min	2.90E+08 / **20.54%** / 29.1 min
-1	9.72E+07 / **10.18%** / 30.3 min	1.03E+08 / **13.36%** / 30.4 min	8.33E+08 / **59.65%** / 30.2 min	8.23E+08 / **58.24%** / 30.2 min
-2	4.03E+06 / **0.42%** / 30.7 min	3.97E+06 / **0.51%** / 30.8 min	1.93E+08 / **20.97%** / 30.7 min	2.74E+08 / **19.39%** / 30.7 min
-3	- / - / -	- / - / -	6.11E+04 / **0.01%** / 28.6 min	3.03E+05 / **0.02%** / 28.5 min
-4	- / - / -	- / - / -	- / - / -	- / - / -
-5	- / - / -	- / - / -	- / - / -	- / - / -
-6	- / - / -	- / - / -	- / - / -	- / - / -
-7	- / - / -	- / - / -	- / - / -	- / - / -
-8	- / - / -	- / - / -	- / - / -	1.12E+06 / **0.08%** / 26.6 min
-9	7.49E+06 / **0.78%** / 21.7 min	7.57E+06 / **0.98%** / 21.9 min	8.97E+06 / **0.64%** / 21.7 min	2.44E+07 / **1.72%** / 21.7 min
-10	- / - / -	- / - / -	- / - / -	- / - / -
total	**9.55E+08**	**7.73E+08**	**1.40E+09**	**1.41E+09**

Supplementary Table S10. Results of the combined thermal stability and carboxypeptidase Y assays for caulosegnin II (P13A/P18A). The numbers shown are the total integrals of the corresponding peaks in the EICs on the left, **relative amounts** in the middle and retention times on the right. For peptides containing a methionine, only the retention times of the unoxidized species are shown.

truncation	untreated	2 h at 95 °C	treated with carboxypeptidase Y	2 h at 95 °C, then treated with carboxypeptidase Y
full length	1.57E+07 / **12.64%** / 29.3 min	1.62E+07 / **13.13%** / 29.2 min	2.02E+06 / **1.64%** / - min[a]	1.88E+06 / **1.55%** / - min[a]
-1	4.40E+07 / **35.36%** / 29.5 min	4.32E+07 / **35.00%** / 29.5 min	3.99E+07 / **32.48%** / 29.5 min	4.12E+07 / **34.06%** / 29.6 min
-2	6.47E+07 / **52.00%** / 30.1 min	6.40E+07 / **51.87%** / 30.1 min	8.03E+07 / **65.41%** / 30.0 min	7.52E+07 / **62.26%** / 30.1 min
-3	- / - / -	- / - / -	1.19E+05 / **0.10%** / 28.9 min	1.05E+05 / **0.09%** / 28.9 min
-4	- / - / -	- / - / -	- / - / -	- / - / -
-5	- / - / -	- / - / -	- / - / -	- / - / -
-6	- / - / -	- / - / -	- / - / -	- / - / -
-7	- / - / -	- / - / -	- / - / -	- / - / -
-8	- / - / -	- / - / -	- / - / -	- / - / -
-9	- / - / -	- / - / -	4.52E+05 / **0.37%** / 21.9 min	2.46E+06 / **2.04%** / 21.7 min
-10	- / - / -	- / - / -	- / - / -	- / - / -
total	**1.24E+08**	**1.23E+08**	**1.23E+08**	**1.21E+08**

[a]only the singly oxidized species could be detected, while the unoxidized one was missing. The observed retention times of the singly oxidized full length lasso peptides were 28.4 min (untreated), 28.4 min (2 h at 95 °C), 28.3 min (treated with carboxypeptidase Y) and 28.4 min (2 h at 95 °C, then treated with carboxypeptidase Y).

Supplementary Table S11. Results of the combined thermal stability and carboxypeptidase Y assays for caulosegnin II (P5A/P13A/P18A). The numbers shown are the total integrals of the corresponding peaks in the EICs on the left, **relative amounts** in the middle and retention times on the right. For peptides containing a methionine, only the retention times of the unoxidized species are shown.

truncation	untreated	2 h at 95 °C	treated with carboxypeptidase Y	2 h at 95 °C, then treated with carboxypeptidase Y
full length	1.48E+08 / **71.24%** / 26.7 min	1.40E+08 / **63.59%** / 26.8 min	3.05E+07 / **14.21%** / 26.8 min	2.94E+07 / **13.77%** / 26.7 min
full length (unthreaded)	- / - / -	1.56E+07 / **7.08%** / 28.9 min	- / - / -	- / - / -
-1	3.80E+07 / **18.30%** / 27.8 min	3.77E+07 / **17.16%** / 27.8 min	6.99E+07 / **32.58%** / 27.6 min	7.54E+07 / **35.38%** / 27.7 min
-1 (unthreaded)	- / - / -	3.08E+06 / **1.40%** / 29.2 min	- / - / -	- / - / -
-2	2.10E+07 / **10.11%** / 28.5 min	2.02E+07 / **9.20%** / 28.4 min	1.14E+08 / **53.17%** / 28.4 min	1.08E+08 / **50.50%** / 28.4 min
-2 (unthreaded)	- / - / -	2.66E+06 / **1.21%** / 29.3 min	- / - / -	- / - / -
-3	- / - / -	- / - / -	- / - / -	- / - / -
-4	- / - / -	- / - / -	- / - / -	- / - / -
-5	- / - / -	- / - / -	- / - / -	- / - / -
-6	- / - / -	- / - / -	- / - / -	- / - / -
-7	- / - / -	- / - / -	- / - / -	- / - / -
-8	- / - / -	- / - / -	- / - / -	6.58E+05 / **0.31%** / 26.2 min
-9	- / - / -	- / - / -	- / - / -	- / - / -
-10	7.10E+05 / **0.34%** / 22.1 min	7.83E+05 / **0.36%** / 22.0 min	9.24E+04 / **0.04%** / 22.0 min	7.40E+04 / **0.03%** / 22.0 min
total	**2.08E+08**	**2.19E+08**	**2.15E+08**	**2.01E+09**

Supplementary Table S12. Results of the combined thermal stability and carboxypeptidase Y assays for caulosegnin II (P8A/P13A/P18A). The numbers shown are the total integrals of the corresponding peaks in the EICs on the left, **relative amounts** in the middle and retention times on the right. For peptides containing a methionine, only the retention times of the unoxidized species are shown.

truncation	untreated	2 h at 95 °C	treated with carboxypeptidase Y	2 h at 95 °C, then treated with carboxypeptidase Y
full length	- / - / -	- / - / -	- / - / -	- / - / -
-1	9.82E+04 / **0.97%** / 29.6 min	9.79E+04 / **0.97%**[a] / 29.7 min	- / - / -	- / - / -
-2	8.70E+06 / **86.20%** / 30.0 min	8.32E+06 / **82.35%**[a] / 29.9 min	4.14E+06 / **41.01%**[a] / 29.9 min	2.18E+05 / **2.16%**[a] / 29.9 min
-3	- / - / -	- / - / -	5.79E+04 / **0.57%**[a] /29.4 min	1.51E+05 / **1.49%**[a] / 29.4 min
-4	- / - / -	- / - / -	- / - / -	- / - / -
-5	- / - / -	- / - / -	- / - / -	- / - / -
-6	- / - / -	- / - / -	- / - / -	- / - / -
-7	- / - / -	- / - / -	- / - / -	- / - / -
-8	- / - / -	- / - / -	- / - / -	- / - / -
-9	1.29E+06 / **12.83%** / 14.1 min	1.21E+06 / **11.93%**[a] / 14.1 min	- / - / -	- / - / -
-10	- / - / -	- / - / -	- / - / -	- / - / -
total	**1.01E+07**	**9.62E+06**	**4.20E+06**	**3.69E+05**

[a]as carboxypeptidase Y degrades this compound apparently very efficiently (especially after thermal treatment) and as the corresponding degradation products could not be identified, the relative amounts were all calculated on basis of the total integral of the untreated sample, to put the results into perspective.

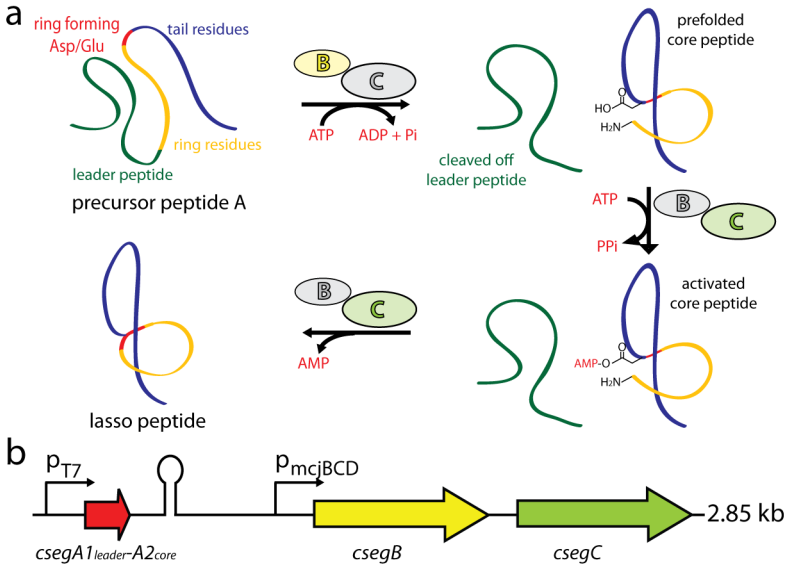
Supplementary Table S13. Results of the combined thermal stability and carboxypeptidase Y assays for caulosegnin II (L7P/P8A). The numbers shown are the total integrals of the corresponding peaks in the EICs on the left, **relative amounts** in the middle and retention times on the right. For peptides containing a methionine, only the retention times of the unoxidized species are shown.

truncation	untreated	2 h at 95 °C	treated with carboxypeptidase Y	2 h at 95 °C, then treated with carboxypeptidase Y
full length	3.57E+06 / **35.73%** / 28.9 min	2.36E+06 / **20.89%** / 28.9 min	3.39E+06 / **38.47%** / 28.9 min	1.82E+06 / **22.06%** / 28.8 min
full length (unthreaded)	- / - / -	1.53E+06 / **13.56%** / 28.0 min	- / - / -	- / - / -
-1	- / - / -	- / - / -	1.17E+05 / **1.33%** / 31.0 min	2.04E+05 / **2.47%** / 31.1 min
-1 (unthreaded)	- / - / -	- / - / -	- / - / -	- / - / -
-2	- / - / -	- / - / -	- / - / -	- / - / -
-2 (unthreaded)	- / - / -	- / - / -	- / - / -	- / - / -
-3	- / - / -	- / - / -	- / - / -	- / - / -
-4	- / - / -	- / - / -	- / - / -	- / - / -
-5	- / - / -	- / - / -	- / - / -	- / - / -
-6	- / - / -	- / - / -	- / - / -	- / - / -
-7	- / - / -	- / - / -	- / - / -	- / - / -
-8	- / - / -	- / - / -	- / - / -	- / - / -
-9	4.93E+06 / **49.41%** / 14.8 min	5.42E+06 / **47.99%** / 14.7 min	4.93E+06 / **49.41%** / 14.8 min	4.99E+06 / **60.44%** / 14.9 min
-10	1.48E+06 / **14.86%** / 15.0 min	1.98E+06 / **17.55%** / 15.1 min	1.48E+06 / **14.86%** / 15.0 min	1.24E+06 / **15.03%** / 15.0 min
total	**9.98E+06**	**1.13E+07**	**8.80E+06**	**8.25E+06**

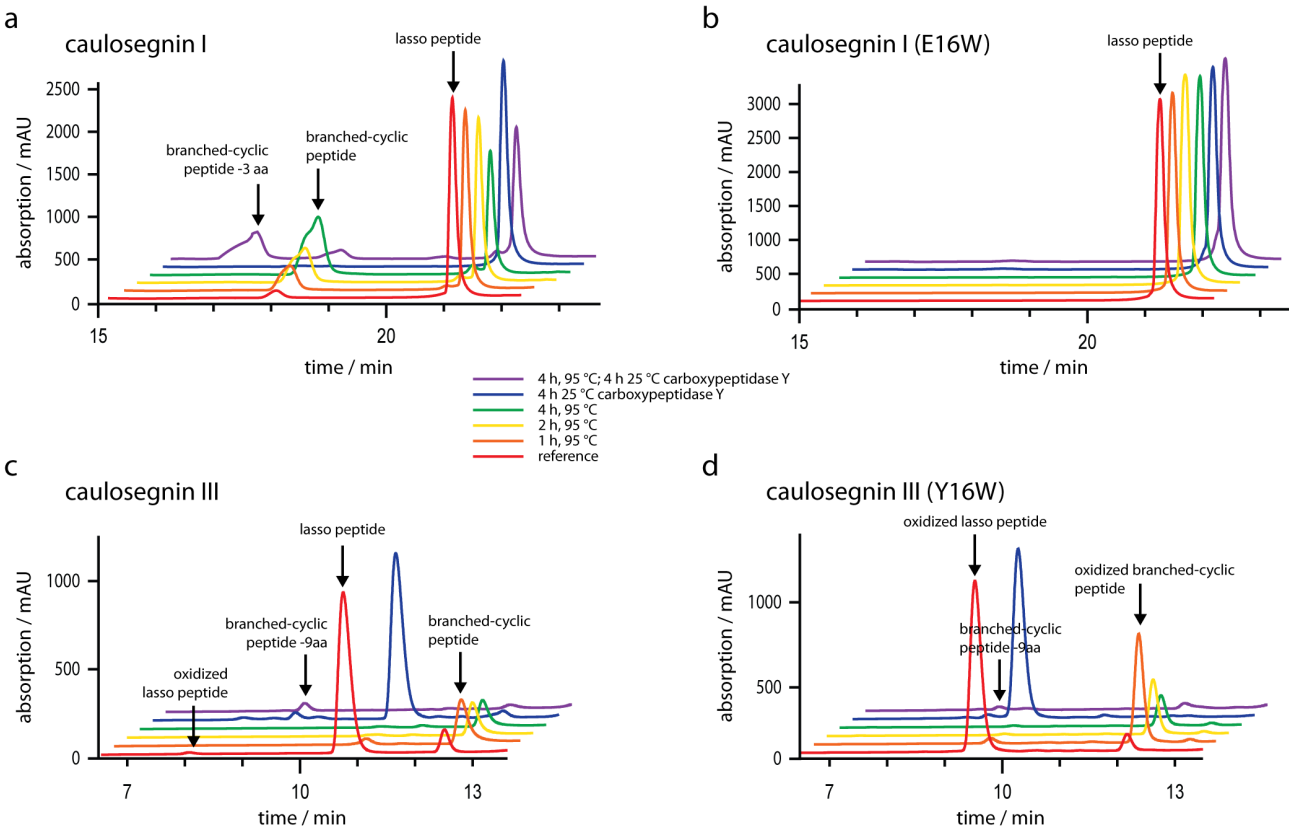
MD simulations

The three-dimensional structure of the caulosegnin II P5A/P8A variant was obtained by manually modifying the crystal structure of the WT peptide. Both lasso peptides were parametrized using the ff14SB force field[14] together with the parameters for the uncommon head-to-side chain amide bond between Gly1 and Glu9, which were developed in a previous work.[3] Each peptide was then solvated in a 10 Å layer cubic box using the TIP3P water model parameters.[15] In each system, neutrality was ensured by addition of two Na^+ ions. Simulations were performed using the NAMD 2.9 MD code.[16] Lennard-Jones potential was used to compute atom-pair interactions, using a 10.0 Å cutoff. The long-range electrostatic interactions were computed by means of the particle mesh Ewald (PME) method using a 1.0 Å grid spacing in periodic boundary conditions. The RATTLE algorithm was applied to constrain bonds involving hydrogen atoms, thus allowing for the use of a 2 fs time step interval. Each system was minimized and heated up to 368 K while keeping harmonic constraints on the peptide, which were gradually released along the thermalization process. Production runs were then performed under NPT conditions at 1 atm and 368 K, so as to reproduce the experimental conditions.

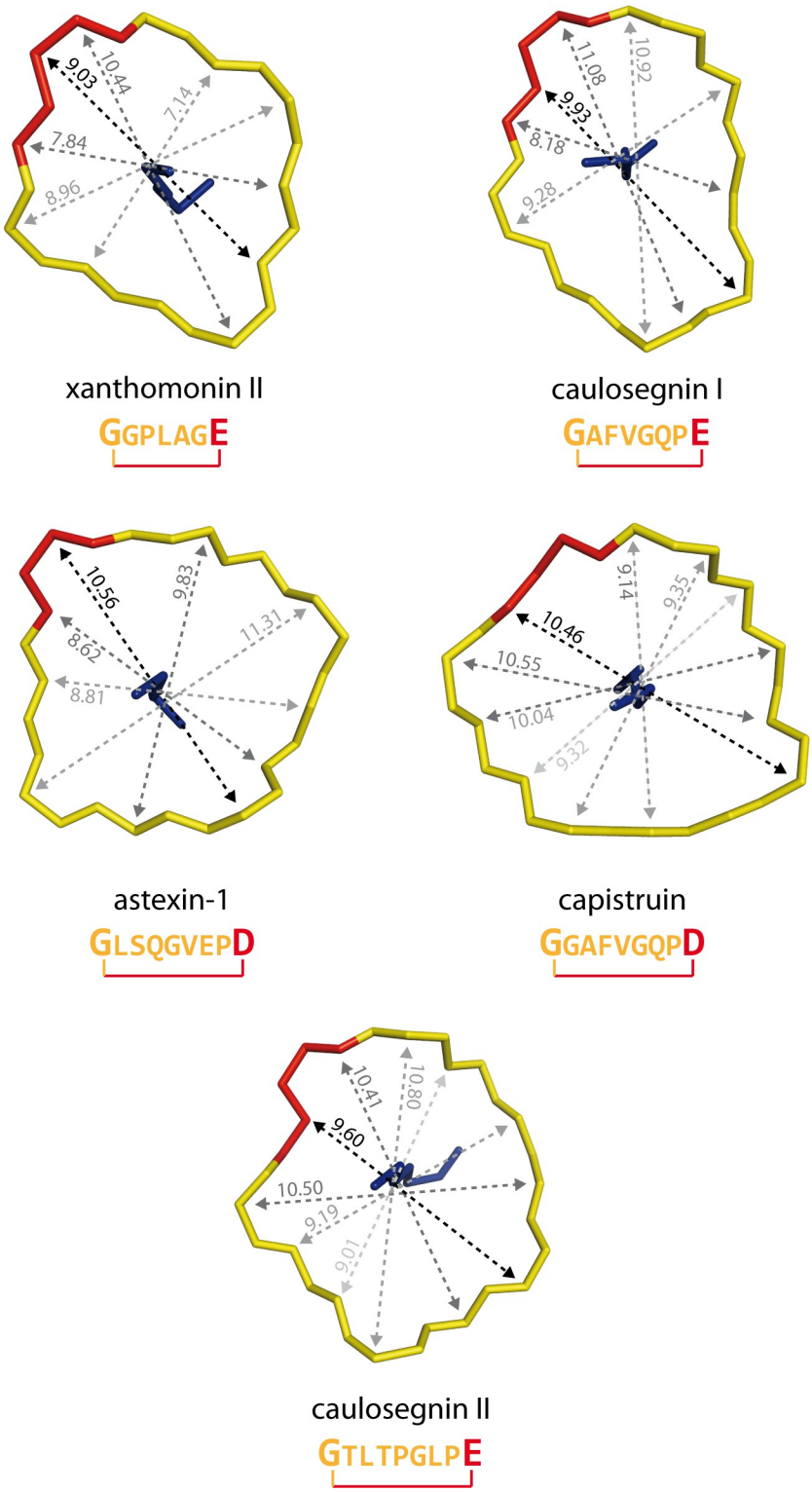
R.m.s.d. and B-factor analyses were performed using GROMACS 4.6.7.[17] Figures S3a and 5 were rendered with Grace (http://plasma-gate.weizmann.ac.il/Grace/) and Pymol (www.pymol.org), respectively.



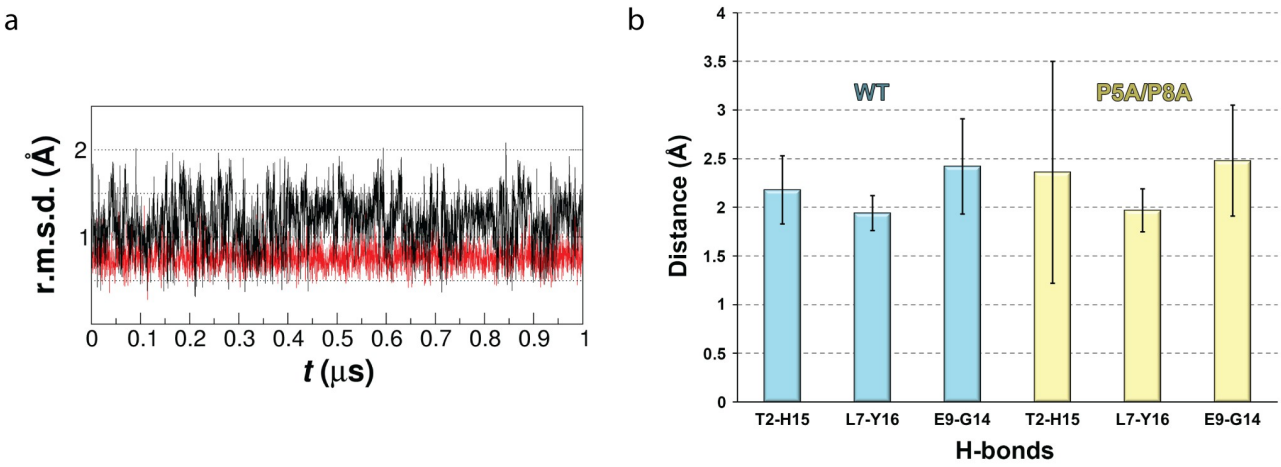
Supplementary Figure S1. (a) Proposed mechanism of lasso peptide biosynthesis.[13] First, the leader peptide gets cleaved off by the B protein, a cysteine protease that might also mediate the prefolding of the core peptide under ATP consumption. Subsequently, the C protein, an ATP-dependent lactam synthetase, activates the Asp/Glu side chain and catalyzes the ring formation, yielding the mature lasso peptide.[13] (b) The optimized heterologous expression system that is present in a pET41a vector is shown.[3] Expression of the $CsegA1_{leader}$-$A2_{core}$ hybrid precursor peptide is governed by a T7 promoter and the gene is followed by a terminator region, while the PmcjBCD promoter from the microcin J25 system controls expression of the processing enzymes.



Supplementary Figure S2. Combined thermal stability and carboxypeptidase Y assays for pure (a) WT caulosegnin I, (b) caulosegnin I (E16W), (c) WT caulosegnin III and (d) caulosegnin III (Y16W), with a singly-oxidized Met19 residue. From these four compounds, only caulosegnin I (E16W) is not affected by both incubation at 95 °C and carboxypeptidase Y treatment. In all other instances, the lasso peptides are resistant against carboxypeptidase Y digestion if directly treated. Incubation at 95 °C on the other hands leads to the conversion to branched-cyclic peptides, which is proven by the observation that these compounds are readily degraded upon exposure to carboxypeptidase Y.



Supplementary Figure S3. A comparative overview of the macrolactam rings of the lasso peptides discussed in this work. The distances shown are given in Å. As before, the backbones of the ring residues are shown in yellow, the parts of the isopeptide bond-forming Asp or Glu residues in the rings are depicted in red and in the middle the backbones of threading portions of the C-terminal tails are highlighted in blue. It has to be considered that these ring conformations are only static averages and therefore only allow a general comparison, while neglecting the inherent dynamics of such structures in solution. Xanthomonin II (PDB code 2MFV) features a seven-residue macrolactam ring formed between Gly1 and Glu7; caulosegnin I (PDB code 2LX6) an eight-residue macrolactam ring formed between Gly1 and Glu8; both astexin-1 (PDB code 2M37) and capistruin (BMRB accession number 20014) nine-residue macrolactam rings formed between Gly1 and Asp9; and caulosegnin II (PDB code 5D9E) a nine-residue macrolactam ring formed between Gly1 and Glu9.



Supplementary Figure S4. (a) R.m.s.d. of the Cα carbons of residues from position 1 to 9 in the WT caulosegnin II (red plot) and in the P5A/P8A variant (black plot). (b) Histogram of representative backbone-backbone hydrogen bond distances (mean ± S.D.) in WT caulosegnin II (cyan) and in the P5A/P8A (yellow) variant.

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