SUPPORTING INFORMATION

Elucidation of the Roles of Conserved Residues in the Biosynthesis of the Lasso Peptide Paeninodin

Julian D. Hegemann,[a,b] Christopher J. Schwalen,[b,c] Douglas A. Mitchell,[b,c,†] and Wilfred A. van der Donk[a,b,†]

[a]Howard Hughes Medical Institute and [b]Department of Chemistry, University of Illinois at Urbana-Champaign, 600 S. Mathews Ave, Urbana, Illinois 61801, United States. [c] Carl R. Woese Institute for Genomic Biology, University of Illinois, Urbana, IL 61801.
†Corresponding authors: vddonk@illinois.edu and douglasm@illinois.edu

Supporting Information Table S1a. Overview of all previous reports about the effects of exchanges of conserved precursor peptide residues in heterologous lasso peptide production systems.

lasso peptide	ring	variant	relative lasso peptide production compared to WT
capistruin[1]	G1-D9	**T-2A**	not detected
		T-2S	not detected
		G1A	not detected
		G1C	not detected
		D9E	not detected
microcin J25[2]	G1-E8	**T-2G**	detected by mass only
		T-2A	detected by mass only
		T-2S	8%
		T-2C	69%
		T-2V	71%
		T-2I	73%
		T-2L	detected by mass only
		T-2D	not detected
		T-2M	detected by mass only
		T-2F	not detected
capistruin[2]	G1-D9	**T-2V**	52%
		T-2A	not detected
		T-2C	74%
		T-2S	84%
		T-2I	39%
		T-2L	not detected
caulosegnin I[3,a]	G1-E8	**T-2A**	<1%
		T-2C	35%
		T-2I	3%
		T-2S	4%
		T-2V	9%
		G1A	detected by mass only
		G1C	<1%
		G1F	not detected
		E8D	not detected

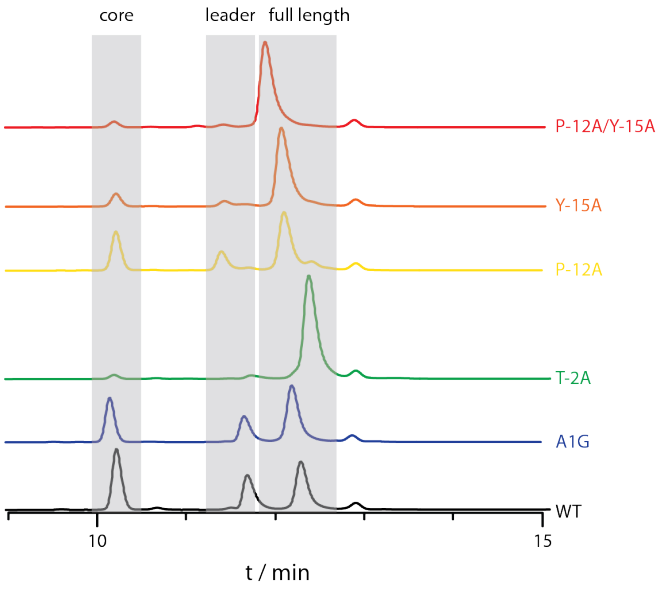
[a]The caulosegnin gene cluster produces lasso peptides with both G1-E8 (caulosegnin I) and G1-E9 macrolactam rings (caulosegnin II-III).

Supporting Information Table S1b. Overview of all previous reports about the effects of exchanges of conserved precursor peptide residues in heterologous lasso peptide production systems.

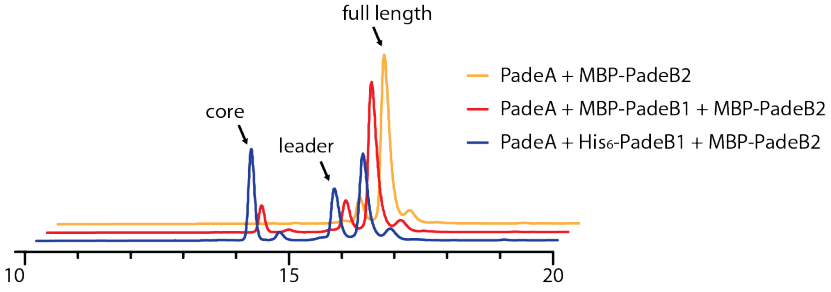
lasso peptide	ring	variant	relative lasso peptide production compared to WT
astexin-1[4]	G1-D9	**T-2A**	detected by mass only
		T-2V	not detected
		T-2S	detected by mass only
		G1C	not detected
		D9E	detected by mass only
xanthomonin II[5]	G1-E7	**T-2A**	<1%
		T-2C	13%
		T-2I	1%
		T-2S	1%
		T-2V	2%
		E7A	not detected
		E7D E8A	not detected
		E7A E8D	not detected
caulonodin V[6,b]	S1-E9	**T-2A**	detected by mass only
		S1G	4%
		S1A	132%
		S1C	detected by mass only
		S1V	detected by mass only
		S1T	detected by mass only
		S1F	detected by mass only
caulonodin VI[6,b]	A1-E9	**A1G**	7%
		A1S	122%
		A1C	detected by mass only
		A1V	detected by mass only
		A1T	1%
		A1F	detected by mass only
paeninodin[7,c]	A1-D9	**T-2A**	40%
		T-2C	63%
		T-2V	44%
		A1C	not detected
		A1G	5%
		A1S	detected by mass only
		D9E	not detected

[b]The gene clusters producing caulonodins IV/V and caulonodins VI/VII both encode precursors with an A1 or S1 residue. The natural occurrence of two different residues at position 1 might explain why S1A/A1S exchanges are readily tolerated, but not exchanges of A1/S1 to other amino acids.

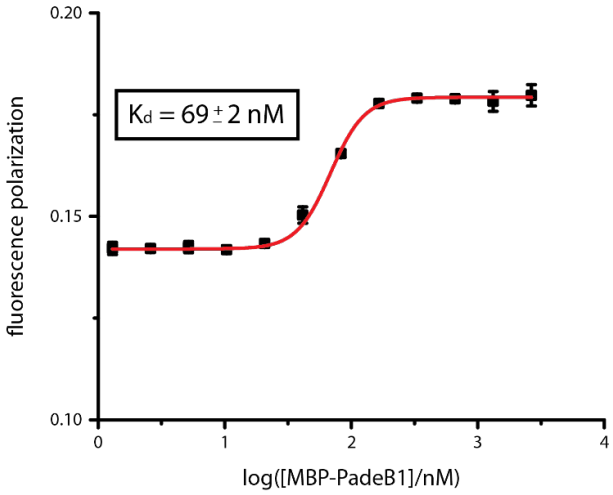
[c]Values were estimated based on bar graphs as no exact numerical values were given.



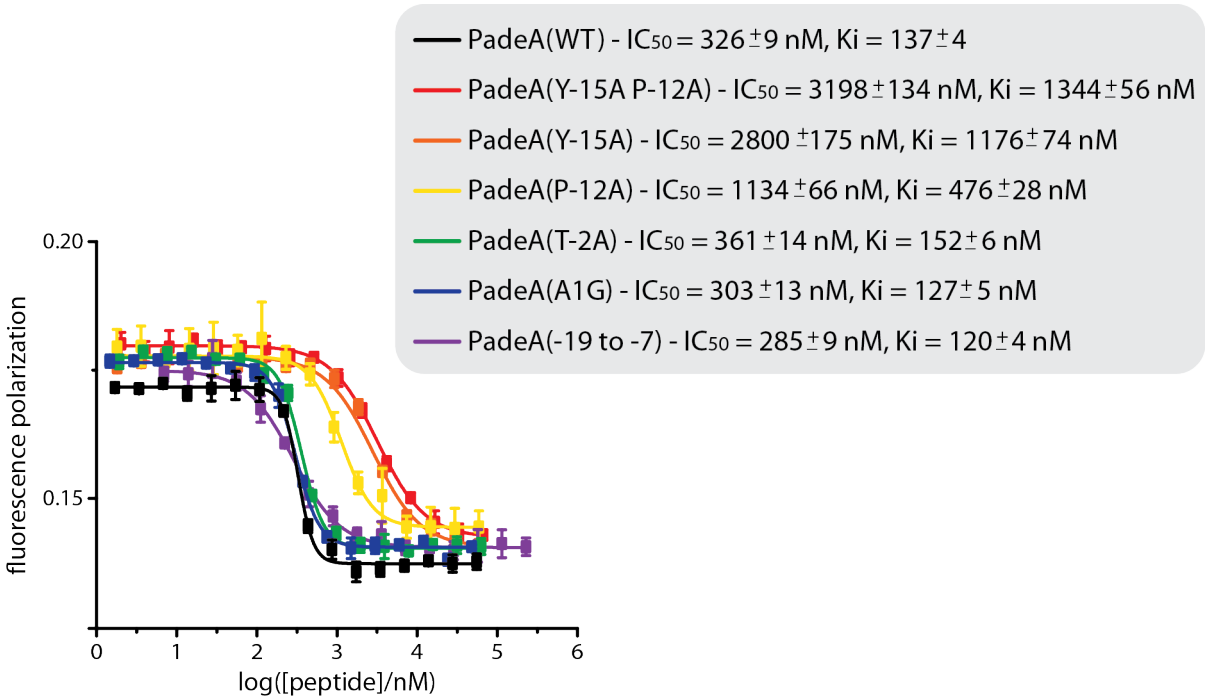
Supporting Information Figure S1. *In vitro* cleavage of 50 μM PadeA (or variants thereof) in the presence of 5 μM of His_6-PadeB1 and 5 μM of His_6-MBP-PadeB2. Assays were incubated overnight (ON) at room temperature (RT). Representative LC traces for each cleavage reaction are shown. The three different peptides observed (core, leader and uncleaved precursor peptide) are highlighted by shading and their identities were confirmed by mass spectrometry. For every peptide tested, the cleavage occurred between Met-1 and the residue at position 1, as expected.



Supporting Information Figure S2. *In vitro* cleavage of 50 μM PadeA in the presence of 5 μM His_6-MBP-PadeB2 and either 5 μM His_6-PadeB1, 5 μM His_6-MBP-PadeB1 or no other protein. Assays were incubated overnight at RT.



Supporting Information Figure S3. FP binding assay using 50 nM Fl-PadeA and various concentrations of His_6-MBP-PadeB1. All experiments were performed in triplicates with errors shown as standard deviation of the mean.



Supporting Information Figure S4. FP competition assays using 50 nM Fl-PadeA, 100 nM His_6-MBP-PadeB1 and various concentrations of the respective competitor peptide. All experiments were performed in triplicates with errors shown as standard deviation of the mean.

Experimental Procedures

Bacterial Strains and Materials. Cloning and mutagenesis was accomplished using *E. coli* DH10b cells, while *E. coli* BL21(DE3) was employed for protein expression. Oligonucleotide primers were obtained from Integrated DNA Technologies, and Phusion DNA polymerase and Gibson Assembly Master mix were purchased from New England Biolabs. Sequencing was performed by ACGT, Inc. to confirm correct sequences of all plasmids generated in this study. Fluorescein-5-maleimide, lysozyme and benzonase were bought from Thermo Fisher Scientific. The paeninodin production plasmid and plasmids for expression of His_6-PadeB1 and His_6-MBP-PadeB2 were kindly provided by Prof. Mohamed A. Marahiel (Philipps-University Marburg, Germany).[7, 8]

Mutagenesis and Cloning. For cloning, recipient vectors were linearized by PCR using the oligonucleotide primers shown in Supporting Information Table S2a. The *padeA* and *padeB1* genes were amplified in a likewise manner with overhangs that allowed subsequent Gibson Assembly. For cloning of *mbp-padeA* pET28a, the ATG start codon of *padeA* was replaced with a Cys codon (TGC) to incorporate a Cys residue that would allow selective labeling of the peptide later on. For mutagenesis, site-directed ligase-independent mutagenesis (SLIM) was employed using the primers listed in Supporting Information Tables S3b-c and standard protocols.[9, 10]

Supporting Information Table S2a. Oligonucleotide primers used for Gibson Assembly cloning. Overhangs for Gibson Assembly are underlined, mutated residues are highlighted in bold.

name	sequence
FP_MBPpET28a_Gibson	GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT C
RP_MBPpET28a_Gibson	GGA TTG GAA GTA CAG GTT CTC AGA TCC ACG CG
FP_Cys-PadeA_Gibson	TCTGAGAACCTGTACTTCCAATCC **TGC** AAA AAG CAG TAC AGC AAG CCG TCC CTT GAA G
RP_PadeA_Gibson	TTGCTGTCCACCAGTCATGCTAGC CTA GCT ATC GTA ATG CAC GTC TTC ATC CGG G
FP_PadeB1-pET28a	GAGAACCTGTACTTCCAATCC ATG AGC AAA CTT CAT TCG ATC ACC CCT GTC GAT AC
RP_PadeB1-pET28a	TTTACCTTCTTCGATTTTCAT ATG GGC GCC GTG ATG GTG ATG GTG ATG TTT C
FP_MBP	ATG AAA ATC GAA GAA GGT AAA CTG GTA ATC TGG
RP_MBP	GGA TTG GAA GTA CAG GTT CTC AGA TCC ACG

Supporting Information Table S2b. Oligonucleotide primers used for SLIM of the paeninodin production system. Overhangs for SLIM are underlined, mutated residues are highlighted in bold.

name	sequence
FP_PadeA_-18to-10	GAA GTG TTG GAT GTT CAT CAG ACC ATG GCT GGC
RP_PadeA_-18to-10	CAT GGT TAC ACC TCC TTT CCT GAG ATT CTT GAC AG
FPTail_PadeA_Y-15A	AAA AAG CAG **GCG** AGC AAG CCG TCC CTT GAA GTG TTG GAT GTT CAT CAG ACC ATG GCT GGC
RPTail_PadeA_Y-15A	AAG GGA CGG CTT GCT **CGC** CTG CTT TTT CAT GGT TAC ACC TCC TTT CCT GAG ATT CTT GAC AG
FPTail_PadeA_P-12A	AAA AAG CAG TAC AGC AAG **GCG** TCC CTT GAA GTG TTG GAT GTT CAT CAG ACC ATG GCT GGC
RPTail_PadeA_P-12A	AAG GGA **CGC** CTT GCT GTA CTG CTT TTT CAT GGT TAC ACC TCC TTT CCT GAG ATT CTT GAC AG
FPTail_PadeA_YPtoAA	AAA AAG CAG **GCG** AGC AAG **GCG** TCC CTT GAA GTG TTG GAT GTT CAT CAG ACC ATG GCT GGC
RPTail_PadeA_YPtoAA	AAG GGA **CGC** CTT GCT **CGC** CTG CTT TTT CAT GGT TAC ACC TCC TTT CCT GAG ATT CTT GAC AG
FP_PadeA_-9to1	GGC CCG GGT ACC TCT ACC CCG GAT GC
RP_PadeA_-9to1	AAG GGA CGG CTT GCT GTA CTG CTT TTT CAT G
FPTail_PadeA_T-2A	GAA GTG TTG GAT GTT CAT CAG **GCG** ATG GCT GGC CCG GGT ACC TCT ACC CCG GAT GC
RPTail_PadeA_T-2A	AGC CAT **CGC** CTG ATG AAC ATC CAA CAC TTC AAG GGA CGG CTT GCT GTA CTG CTT TTT CAT G
FPTail_PadeA_A1G	GAA GTG TTG GAT GTT CAT CAG ACC ATG **GGC** GGC CCG GGT ACC TCT ACC CCG GAT GC
RPTail_PadeA_A1G	**GCC** CAT GGT CTG ATG AAC ATC CAA CAC TTC AAG GGA CGG CTT GCT GTA CTG CTT TTT CAT G

Supporting Information Table S2c. Oligonucleotide primers used for SLIM of *mbp-padeA* pET28a. Overhangs for SLIM are underlined, mutated residues are highlighted in bold. For mutants where FP and FPTail primers are identical to the ones listed in Supporting Information Table S2b, only the RP and RPTail primers are shown.

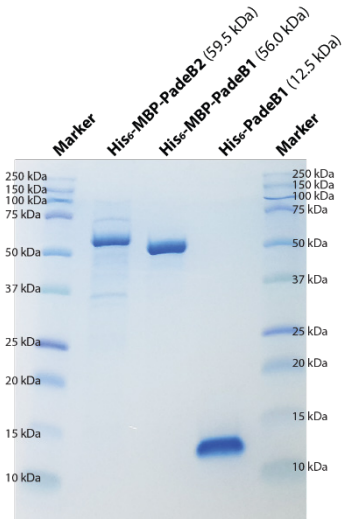
name	sequence
RP_MBP-PadeA_-18to-10	GCA GGA TTG GAA GTA CAG GTT CTC AGA TCC ACG
RPTail_MBP-PadeA_Y-15A	AAG GGA CGG CTT GCT **CGC** CTG CTT TTT GCA GGA TTG GAA GTA CAG GTT CTC AGA TCC ACG
RPTail_MBP-PadeA_P-12A	AAG GGA **CGC** CTT GCT GTA CTG CTT TTT GCA GGA TTG GAA GTA CAG GTT CTC AGA TCC ACG
RPTail_MBP-PadeA_YPtoAA	AAG GGA **CGC** CTT GCT **CGC** CTG CTT TTT GCA GGA TTG GAA GTA CAG GTT CTC AGA TCC ACG
RP_MBP-PadeA_-9to1	AAG GGA CGG CTT GCT GTA CTG CTT TTT GC
RPTail_MBP-PadeA_T-2A	AGC CAT **CGC** CTG ATG AAC ATC CAA CAC TTC AAG GGA CGG CTT GCT GTA CTG CTT TTT GC
RPTail_MBP-PadeA_A1G	**GCC** CAT GGT CTG ATG AAC ATC CAA CAC TTC AAG GGA CGG CTT GCT GTA CTG CTT TTT GC
FP_Pade(-19to-7)-MBP	ACT GGT GGA CAG CAA ATG GGT CGC GGA TC
RP_Pade(-19to-7)-MBP	GGA CGG CTT GCT GTA CTG CTT TTT GCA GG
FPTail_Pade(-19to-7)-MBP	CTTGAAGTGTTGTAGGCTAGCATG ACT GGT GGA CAG CAA ATG GGT CGC GGA TC
RPTail_Pade(-19to-7)-MBP	CATGCTAGCCTACAACACTTCAAG GGA CGG CTT GCT GTA CTG CTT TTT GCA GG

Protein Expression and Purification. For all overnight cultures and single protein expressions, lysogeny broth (LB) medium supplemented with 50 μg/mL kanamycin was used. His_6-PadeB1, His_6-MBP-PadeB1 and His_6-MBP-PadeB2 were all produced under the same conditions: An LB expression culture was inoculated 1:100 with a 37 °C LB overnight culture. Cells were grown at 37 °C until the optical density at 600 nm (OD_{600}) reached 0.2-0.25 and then the temperature was shifted to 18 °C. One hour later, expression (OD_{600} ~0.5-0.7) was induced by addition of 0.2 mL of an isopropyl β-D-1-thiogalactopyranoside (IPTG) stock solution (0.5 M) per 1 L of cell culture (0.1 mM final IPTG concentration). Expression was continued overnight at 18 °C and

then the cells were harvested by centrifugation. For His_6-MBP-PadeA and variants thereof, expression was performed continuously at 37 °C for 3 h after cells were induced by IPTG addition (0.1 mM final concentration) when reaching an OD_{600} of 0.5-0.7.

For protein purification, cell pellets were resuspended in ice-cold lysis buffer (300 mM NaCl, 50 mM HEPES, 10% glycerol, pH 7.5) to a final volume of 10-20 mL per 1 L of original culture volume. Cells were then treated with lysozyme (1 mg/mL final concentration) and benzonase (2.5 μL per 10 mL of cell suspension) for 1 h on ice, before lysis was accomplished by sonication with a Vibra Cell sonicator (Sonics & Materials) at the following instrument settings: 40% amplitude, 5 min total sonication time; alternating between 2 s on/5 s off-pulse. Lysates were cleared by centrifugation and filtration through 0.45 μm syringe filters. For nickel nitrilotriacetic acid (NiNTA) affinity chromatography, a 5 mL NiNTA HisTrap column (GE healthcare) and a peristaltic pump were used. After column equilibration with 25 mL of lysis buffer, the lysate was loaded and then the column was washed with 25 mL of wash buffer buffer (25 mM imidazole, 300 mM NaCl, 50 mM HEPES, 10% glycerol, pH 7.5) and protein was eluted with 15 mL of elution buffer (25 mM imidazole, 300 mM NaCl, 50 mM HEPES, 10% glycerol, pH 7.5).

A second round of purification was performed via size exclusion chromatography (SEC) for His_6-PadeB1, His_6-MBP-PadeB1 and His_6-MBP-PadeB2. NiNTA elution fractions were concentrated in Amicon centrifugal filter units (with suitable MW cut-offs) and applied to an Äkta FPLC (GE Healthcare) system with a Superdex200 column using 300 mM NaCl, 50 mM HEPES, 10% glycerol at pH 7.5 as run buffer at a flow rate of 1 mL/min. After SEC, the purest fractions of each protein were identified by SDS-PAGE, pooled, concentrated and then flash-frozen as aliquots to be stored at -80 °C until later usage (Supporting Information Figure S5).



Supporting Information Figure S5. SDS-PAGE of all proteins used in this study.

HPLC Purification of PadeA and Variants. NiNTA purified His_6-MBP-PadeA was concentrated and buffer exchanged with TEV cleavage buffer (300 mM NaCl, 50 mM HEPES, pH 8.0) in a 30 kDa cut-off Amicon centrifugal filter unit. Then, TEV protease stock (3 mg/mL) was added to accomplish a His_6-MBP-PadeA to TEV protease ratio of ~50:1 and the cleavage reaction was incubated overnight at room temperature (RT). Afterwards, protein denaturation was achieved by incubation for 10 min at 95 °C and the sample was cleared by centrifugation. The clear supernatant was diluted with MeOH and water to obtain ~8 mL at 50% MeOH and tris(2-carboxyethyl)phosphine (TCEP) was added to a final concentration of 1 mM. After overnight incubation at RT, any precipitate was removed by another round of centrifugation. The supernatant was applied to preparative HPLC on a Nexera HPLC system (Shimadzu) using a C18 column (Phenomenex, Luna 10 μm C18(2) 100 Å, 250x10 mm) at RT with a flow rate of 8 mL/min and solvents A (0.1% trifluoroacetic acid in H_2O) and B (0.1% trifluoroacetic acid in MeCN). A linear gradient from 8-80% B was run over 30 min and followed by a linear increase from 80% to 98% over 1 min and finally holding at 98% B for 5 min. The pure peptide fractions were pooled, dried under reduced pressure and then redissolved in water at 10 mg/mL and stored at -20 °C until needed. In this way, pure PadeA with N-terminal Ser-Cys residues was isolated. All variants of PadeA were obtained in a likewise manner in yields usually ranging from 0.5-5 mg of peptide from 1 L of expression culture.

Fluorescein Labeling of PadeA. For fluorescein labeling, pure PadeA was reacted with fluorescein-5-maleimide under the following conditions: 200 μL of a PadeA stock solution (10 mg/mL in water) and 39 μL of a TCEP stock solution (100 mM in water) were mixed with 3475 μL of reaction buffer (100 mM phosphate, pH 7.3). Then, 166 μL of a fluorescein-5-maleimide stock solution (10 mg/mL in DMSO) were added and the reaction was incubated at RT for 1 h while shaking lightly and protecting the sample from light. Afterwards, the reaction was applied to preparative HPLC under the aforementioned conditions, which allowed isolation of pure S-C(fluorescein-5-maleimide)-PadeA (Fl-PadeA).

***In Vivo* Lasso Peptide Production.** *E. coli* production of paeninodin was accomplished under previously established conditions.[7] In short, 1 L aliquots of M9 minimal medium was prepared (6.8 g/L anhydrous Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 1 mL/L $MgSO_4$ solution (2 M), and 0.2 mL/L $CaCl_2$ solution (0.5 M), pH 7.0; after autoclaving, 10 mL/L sterilized glucose solution (40% w/v) and 2 mL/L vitamin mix (see Supporting Information Table S3) were added). The medium was inoculated 1:100 with a 37 °C LB overnight culture and was grown at 37 °C until an OD_{600} of 0.5-0.7 was reached. Then, cells were induced by IPTG addition (0.1 mM final concentration) and cultures were grown overnight at 37 °C. On the next day, cells were harvested by centrifugation and cell pellets were resuspended in 100 mL of MeOH and shaken ON at 4 °C. Afterwards, the samples were centrifuged and the cleared supernatants were collected and dried at reduced pressure. This procedure was performed in triplicate for the WT and all mutant lasso peptide production systems.

Supporting Information Table S3. 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
	add 300 mL ddH_2O*

*After ddH_2O was added to a final volume of 300 mL, 10 M NaOH was slowly added until every component was completely dissolved (at this point, the solution usually has a pH ~12). The cleared solution was then sterile filtered and stored at 4 °C for short-term or -20 °C for long-term.

Dried extracts were then resuspended in 1 mL of 50% MeOH and centrifuged to remove any insoluble components. From the concentrated pellet extracts, 50 μL were used for analytical HPLC employing a 1100 HPLC system (Agilent) with an EC 125/2 Nucleodur 100-5 C18ec column (Macherey-Nagel). The following gradient of solvents A (0.1% trifluoroacetic acid in H_2O) and B (0.1% trifluoroacetic acid in MeCN) was run at RT with a flow rate of 0.2 mL/min: Keeping 2% B for 2 min, followed by a linear increase from 2-30% B in 18 min and then a linear increase from 30-95% B over 15 min and finally holding 95% for 7 min. Fractions absorbing at 220 nm were collected by hand and analyzed by matrix-assisted laser desorption/ionization-time-of-flight MS (MALDI-TOF-MS) on an UltrafleXtreme MALDI TOFTOF instrument (Bruker Daltonics). For sample preparation, 1 μL of each HPLC fraction was mixed with 1 μL of sinapinic acid matrix solution (saturated in 60% MeCN) and crystallized at RT. In this way, the peaks corresponding to the extracted lasso peptides were identified and integrated for quantification (see Supporting Information Table S4).

Supporting Information Table S4. UV integrals observed for the paeninodin lasso peptides during LC analysis of MeOH pellet extracts.

sample	replicate 1	replicate2	replicate3	mean	deviation	relative production
WT	3883	2455	1789	2709	874	100% ± 32%
A1G	222	445	417	361	99	13% ± 4%
T-2A	1493	749	691	978	365	36% ± 13%
P-12A	2205	498	1015	1239	715	46% ± 26%
Y-15A	524	631	846	667	134	25% ± 5%
P-12A/Y-15A	282	915	715	637	264	24% ± 10%

***In Vitro* Cleavage Assays.** The *in vitro* cleavage assays were run under the following conditions: 25 μL of 2x assay buffer (100 mM Tris, 200 mM NaCl, 10 mM $MgCl_2$, pH 8.0) was mixed with stock solutions of His_6-PadeB1 (or His_6-MBP-PadeB1), His_6-MBP-PadeB2 and peptide, and water was added to a final volume of 50 μL (50 μM peptide and 5 μM protein, final concentrations). Cleavage reactions were run overnight at RT and then proteins were precipitated by addition of 5 μL of a 10% TFA solution in H_2O. All reactions were run in triplicates. After centrifugation, 50 μL of the supernatant of each reaction were applied to HPLC employing a 1100 HPLC system (Agilent) with an EC 125/2 Nucleodur 100-5 C18ec column (Macherey-Nagel). The following gradient of solvents A (0.1% trifluoroacetic acid in H_2O) and B (0.1% trifluoroacetic acid in MeCN) was used at RT with a flow rate of 0.2 mL/min:

Linear increase from 10-40% B in 15 min followed by a linear gradient from 40-98% B in 2 min and holding 98% for 5 min. Fractions absorbing at 220 nm were collected by hand and analyzed by MALDI-TOF-MS. For sample preparation, 1 μL of each HPLC fraction was mixed with 1 μL of sinapinic acid matrix solution (saturated in 60% MeCN) and crystallized at RT. In this way, peaks corresponding to core, leader and full length precursor peptide were identified and integrated for quantification (Supporting Information Table S5). As approximation, the sum of the integrals of leader and core peptide was treated to be equivalent to the integral of the same molar amount of full length precursor peptide.

Supporting Information Table S5. UV integrals of core, leader and precursor peptide observed by LC analysis of cleavage assays.

peptide	replicate	core peptide integral	leader peptide integral	precursor peptide integral	sum of all integrals	percentage of core and leader peptide	percentage of full length precursor peptide
WT	1	7580	5026	7806	20412	62%	38%
	2	5749	3931	7705	17385	56%	44%
	3	5577	3845	8780	18202	52%	48%
					mean ± deviation:	**56% ± 4%**	**44% ± 4%**
A1G	1	5570	3706	9658	18934	49%	51%
	2	4314	2892	10443	17649	41%	59%
	3	4163	2822	10262	17247	40%	60%
					mean ± deviation:	**43% ± 4%**	**57% ± 4%**
T-2A	1	434	460	17774	18668	5	95
	2	389	736	16756	17881	6	94
	3	389	703	16129	17221	6	94
					mean ± deviation:	**6% ± 1%**	**94% ± 1%**
P-12A	1	4656	2612	9213	16481	44	56
	2	3533	2315	9506	15354	38	62
	3	3285	1922	9928	15135	34	66
					mean ± deviation:	**39% ± 4%**	**61% ± 4%**
Y-15A	1	1566	751	14192	16509	14	86
	2	1623	962	13033	15618	17	83
	3	1532	734	12368	14634	15	85
					mean ± deviation:	**15% ± 1%**	**85% ± 1%**
P-12A/ Y-15A	1	683	299	15356	16338	6	94
	2	581	668	14577	15826	8	92
	3	546	521	13526	14593	7	93
					mean ± deviation:	**7% ± 1%**	**93% ± 1%**

Fluorescence Polarization Binding Assay. For determination of the K_d of the interaction of His_6-MBP-PadeB1 with Fl-PadeA, a stock solution of 500 nM Fl-PadeA in water and a 1:1 dilution series of His_6-MBP-PadeB1 in buffer (300 mM NaCl, 50 mM HEPES, 1 mM TCEP, 5% glycerol, pH 7.5) were used. In wells of a 384-well solid black polystyrene microplate (Corning), 5 μL of the Fl-PadeA stock solution were mixed with 45 μL of the His_6-MBP-PadeB1 dilutions or plain buffer as control (yielding a final concentration of 50 nM Fl-PadeA in every well). Triplicates were set up for each His_6-MBP-PadeB1 concentration. After mixing, the samples were incubated for 30 min at RT and shielded from light. Then, fluorescence intensities were measured in parallel and perpendicular directions using a Synergy H4 Hybrid Reader (BioTek) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm with a bandwidth of 20 nm (Supporting Information Table S6). Fluorescence polarization (FP) was calculated as the difference of parallel minus perpendicular intensities divided by the sum of both. Under these conditions, the K_d is equal to the turning point between unbound (low FP) and bound (high FP) states of Fl-PadeA when plotting the FP versus the logarithm of the His_6-MBP-PadeB1 concentrations (Supporting Information Figure S3). K_d determination was accomplished by using a non-linear dose-response fit in OriginPro2015 (OriginLab).

Supporting Information Table S6. Data of the fluorescence polarization binding assay using 50 nM Fl-PadeA and varying concentrations of His_6-MBP-PadeB1.

[His6-MBP-PadeB1] / nM	log([His6-MBP-PadeB1] / nM)	replicate 1	replicate 2	replicate 3	mean	standard deviation
2651.27	3.42	0.1795	0.1831	0.1767	0.1797	0.0026
1325.63	3.12	0.1773	0.1816	0.1759	0.1783	0.0025
662.82	2.82	0.1787	0.1796	0.1783	0.1789	0.0005
331.41	2.52	0.1803	0.1789	0.1781	0.1791	0.0009
165.70	2.22	0.1774	0.1774	0.1784	0.1777	0.0005
82.85	1.92	0.1653	0.1667	0.1644	0.1654	0.0010
41.43	1.62	0.1496	0.1530	0.1483	0.1503	0.0020
20.71	1.32	0.1430	0.1444	0.1427	0.1434	0.0007
10.36	1.02	0.1420	0.1421	0.1414	0.1418	0.0003
5.18	0.71	0.1430	0.1438	0.1406	0.1425	0.0014
2.59	0.41	0.1421	0.1426	0.1417	0.1421	0.0003
1.29	0.11	0.1418	0.1405	0.1441	0.1421	0.0015
blank	-	0.1424	0.1404	0.1359	0.1396	0.0027

Fluorescence Polarization Competition Assays. For FP competition assays, a stock solution containing 62.5 nM Fl-PadeA and 125 nM His_6-MBP-PadeB1 in buffer (300 mM NaCl, 50 mM HEPES, 1 mM TCEP, 5% glycerol, pH 7.5) and a 1:1 dilution of each competitor peptide in water

were used. Peptide concentrations were determined for the dilutions at ~125 μg/mL by measuring absorption at 205 nm in triplicate using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific) and employing predicted extinction coefficients at 205 nm.[11] Concentrations of the other dilutions were calculated based on these values. In wells of a 384-well solid black polystyrene microplate (Corning), 10 μL of peptide solution (or water as control) were mixed with 40 μL of the Fl-PadeA/His_6-MBP-PadeB1 stock solution (yielding final concentrations of 50 nM Fl-PadeA and 100 nM His_6-MBP-PadeB1). After mixing, the samples were incubated for 30 min at RT, while being shielded from light. Triplicate measurements were set up for every peptide. Fluorescence intensities were measured and FP values calculated as described above (Supporting Information Tables 8a-8g). For determination of the IC_{50} values, the FP values were plotted against the logarithm of the peptide concentrations and then a non-linear dose-response fit was performed in OriginPro2015 (OriginLab). All fits had an R^2 value >0.99. The K_i was obtained by dividing the IC_{50} value through 1+concentration(Fl-PadeA)/K_d(Fl-PadeA with His_6-MBP-PadeA).

Supporting Information Table S7a. Data of the fluorescence polarization competition assays using 50 nM Fl-PadeA, 100 nM His_6-MBP-PadeB1 and varying concentrations of PadeA WT.

[PadeA] / nM	log([PadeA] / nM)	replicate 1	replicate 2	replicate 3	mean	standard deviation
55430.40	4.74	0.1395	0.1358	0.1378	0.1377	0.0015
27715.20	4.44	0.1386	0.1349	0.1385	0.1373	0.0017
13857.60	4.14	0.1374	0.1370	0.1393	0.1379	0.0010
6928.80	3.84	0.1382	0.1355	0.1370	0.1369	0.0011
3464.40	3.54	0.1375	0.1343	0.1369	0.1362	0.0014
1732.20	3.24	0.1383	0.1337	0.1352	0.1357	0.0019
866.10	2.94	0.1427	0.1388	0.1387	0.1401	0.0019
433.05	2.64	0.1464	0.1439	0.1436	0.1446	0.0013
216.53	2.34	0.1678	0.1656	0.1677	0.1670	0.0010
108.26	2.03	0.1737	0.1681	0.1718	0.1712	0.0023
54.13	1.73	0.1758	0.1696	0.1706	0.1720	0.0027
27.07	1.43	0.1749	0.1702	0.1692	0.1714	0.0025
13.53	1.13	0.1722	0.1690	0.1701	0.1704	0.0013
6.77	0.83	0.1728	0.1716	0.1728	0.1724	0.0006
3.38	0.53	0.1717	0.1703	0.1720	0.1713	0.0008
1.69	0.23	0.1720	0.1711	0.1717	0.1716	0.0004
blank	-	0.1725	0.1690	0.1672	0.1696	0.0022

Supporting Information Table S7b. Data of the fluorescence polarization competition assays using 50 nM Fl-PadeA, 100 nM His_6-MBP-PadeB1 and varying concentrations of Pade(A1G).

[PadeA(A1G)] / nM	log([PadeA(A1G)] / nM)	replicate 1	replicate 2	replicate 3	mean	standard deviation
47763.20	4.68	0.1410	0.1401	0.1406	0.1406	0.0004
23881.60	4.38	0.1374	0.1397	0.1373	0.1381	0.0011
11940.80	4.08	0.1404	0.1423	0.1422	0.1417	0.0009
5970.40	3.78	0.1398	0.1408	0.1423	0.1410	0.0010
2985.20	3.47	0.1393	0.1403	0.1429	0.1408	0.0015
1492.60	3.17	0.1378	0.1427	0.1405	0.1403	0.0020
746.30	2.87	0.1414	0.1439	0.1422	0.1425	0.0010
373.15	2.57	0.1520	0.1539	0.1540	0.1533	0.0009
186.58	2.27	0.1668	0.1714	0.1719	0.1700	0.0023
93.29	1.97	0.1731	0.1738	0.1766	0.1745	0.0015
46.64	1.67	0.1761	0.1744	0.1756	0.1754	0.0007
23.32	1.37	0.1758	0.1775	0.1757	0.1763	0.0008
11.66	1.07	0.1765	0.1767	0.1784	0.1772	0.0009
5.83	0.77	0.1760	0.1778	0.1771	0.1770	0.0008
2.92	0.46	0.1759	0.1764	0.1780	0.1768	0.0009
1.46	0.16	0.1754	0.1766	0.1782	0.1767	0.0012
blank	-	0.1783	0.1787	0.1789	0.1787	0.0003

Supporting Information Table S7c. Data of the fluorescence polarization competition assays using 50 nM Fl-PadeA, 100 nM His_6-MBP-PadeB1 and varying concentrations of Pade(T-2A).

[PadeA(T-2A)] / nM	log([PadeA(T-2A)] / nM)	replicate 1	replicate 2	replicate 3	mean	standard deviation
62675.20	4.80	0.1396	0.1429	0.1396	0.1407	0.0016
31337.60	4.50	0.1387	0.1425	0.1416	0.1409	0.0016
15668.80	4.20	0.1398	0.1416	0.1417	0.1410	0.0008
7834.40	3.89	0.1405	0.1398	0.1392	0.1398	0.0005
3917.20	3.59	0.1396	0.1439	0.1385	0.1407	0.0023
1958.60	3.29	0.1409	0.1426	0.1392	0.1409	0.0014
979.30	2.99	0.1417	0.1447	0.1432	0.1432	0.0012
489.65	2.69	0.1512	0.1505	0.1500	0.1506	0.0005
244.83	2.39	0.1689	0.1702	0.1725	0.1705	0.0015
122.41	2.09	0.1720	0.1747	0.1750	0.1739	0.0014
61.21	1.79	0.1776	0.1769	0.1767	0.1771	0.0004
30.60	1.49	0.1773	0.1766	0.1773	0.1771	0.0003
15.30	1.18	0.1790	0.1778	0.1791	0.1786	0.0006
7.65	0.88	0.1769	0.1778	0.1798	0.1782	0.0012
3.83	0.58	0.1775	0.1788	0.1798	0.1787	0.0009
1.91	0.28	0.1752	0.1784	0.1762	0.1766	0.0013
blank	-	0.1762	0.1786	0.1776	0.1775	0.0010

Supporting Information Table S7d. Data of the fluorescence polarization competition assays using 50 nM Fl-PadeA, 100 nM His_6-MBP-PadeB1 and varying concentrations of Pade(P-12A).

[PadeA(P-12A)] / nM	log([PadeA(P-12A)] / nM)	replicate 1	replicate 2	replicate 3	mean	standard deviation
58876.80	4.77	0.1486	0.1409	0.1438	0.1444	0.0032
29438.40	4.47	0.1491	0.1396	0.1440	0.1442	0.0039
14719.20	4.17	0.1497	0.1406	0.1432	0.1445	0.0038
7359.60	3.87	0.1473	0.1421	0.1439	0.1444	0.0021
3679.80	3.57	0.1579	0.1458	0.1479	0.1505	0.0053
1839.90	3.26	0.1560	0.1511	0.1521	0.1530	0.0021
919.95	2.96	0.1674	0.1603	0.1640	0.1639	0.0029
459.98	2.66	0.1771	0.1726	0.1729	0.1742	0.0021
229.99	2.36	0.1802	0.1748	0.1773	0.1774	0.0022
114.99	2.06	0.1906	0.1735	0.1795	0.1812	0.0071
57.50	1.76	0.1826	0.1754	0.1784	0.1788	0.0030
28.75	1.46	0.1863	0.1744	0.1770	0.1792	0.0051
14.37	1.16	0.1846	0.1750	0.1773	0.1790	0.0041
7.19	0.86	0.1780	0.1752	0.1771	0.1768	0.0011
3.59	0.56	0.1851	0.1760	0.1778	0.1796	0.0040
1.80	0.25	0.1841	0.1759	0.1783	0.1795	0.0034
blank	-	0.1842	0.1733	0.1778	0.1784	0.0044

Supporting Information Table S7e. Data of the fluorescence polarization competition assays using 50 nM Fl-PadeA, 100 nM His_6-MBP-PadeB1 and varying concentrations of Pade(Y-15A).

[PadeA(Y-15A)] / nM	log([PadeA(Y-15A)] / nM)	replicate 1	replicate 2	replicate 3	mean	standard deviation
59209.60	4.77	0.1406	0.1413	0.1411	0.1410	0.0003
29604.80	4.47	0.1412	0.1431	0.1415	0.1420	0.0009
14802.40	4.17	0.1428	0.1464	0.1440	0.1444	0.0015
7401.20	3.87	0.1458	0.1463	0.1479	0.1467	0.0009
3700.60	3.57	0.1549	0.1559	0.1550	0.1553	0.0004
1850.30	3.27	0.1659	0.1681	0.1695	0.1678	0.0015
925.15	2.97	0.1709	0.1746	0.1744	0.1733	0.0017
462.58	2.67	0.1744	0.1774	0.1758	0.1759	0.0012
231.29	2.36	0.1758	0.1751	0.1760	0.1757	0.0004
115.64	2.06	0.1753	0.1774	0.1769	0.1765	0.0009
57.82	1.76	0.1746	0.1757	0.1772	0.1759	0.0011
28.91	1.46	0.1788	0.1792	0.1805	0.1795	0.0007
14.46	1.16	0.1770	0.1790	0.1799	0.1787	0.0012
7.23	0.86	0.1783	0.1777	0.1781	0.1780	0.0002
3.61	0.56	0.1758	0.1760	0.1776	0.1765	0.0008
1.81	0.26	0.1769	0.1744	0.1753	0.1755	0.0010
blank	-	0.1760	0.1772	0.1768	0.1767	0.0005

Supporting Information Table S7f. Data of the fluorescence polarization competition assays using 50 nM Fl-PadeA, 100 nM His_6-MBP-PadeB1 and varying concentrations of Pade(P-12A/Y-15A).

[PadeA(P-12A/Y-15A)] / nM	log([PadeA(P-12A/Y-15A)] / nM)	replicate 1	replicate 2	replicate 3	mean	standard deviation
66390.40	4.82	0.1443	0.1426	0.1418	0.1429	0.0010
33195.20	4.52	0.1459	0.1421	0.1405	0.1428	0.0022
16597.60	4.22	0.1485	0.1438	0.1437	0.1453	0.0022
8298.80	3.92	0.1512	0.1490	0.1502	0.1501	0.0009
4149.40	3.62	0.1584	0.1569	0.1550	0.1568	0.0014
2074.70	3.32	0.1681	0.1681	0.1652	0.1671	0.0013
1037.35	3.02	0.1755	0.1715	0.1734	0.1735	0.0017
518.68	2.71	0.1782	0.1767	0.1776	0.1775	0.0006
259.34	2.41	0.1795	0.1759	0.1768	0.1774	0.0015
129.67	2.11	0.1821	0.1792	0.1763	0.1792	0.0024
64.83	1.81	0.1809	0.1785	0.1789	0.1794	0.0011
32.42	1.51	0.1808	0.1797	0.1772	0.1792	0.0015
16.21	1.21	0.1822	0.1794	0.1810	0.1809	0.0012
8.10	0.91	0.1835	0.1756	0.1794	0.1795	0.0032
4.05	0.61	0.1813	0.1766	0.1778	0.1786	0.0020
2.03	0.31	0.1831	0.1778	0.1794	0.1801	0.0022
blank	-	0.1830	0.1786	0.1781	0.1799	0.0022

Supporting Information Table S7g. Data of the fluorescence polarization competition assays using 50 nM Fl-PadeA, 100 nM His_6-MBP-PadeB1 and varying concentrations of Pade(-19 to -7).

[PadeA(-19 to -7)] / nM	log([PadeA(-19 to -7)] / nM)	replicate 1	replicate 2	replicate 3	mean	standard deviation
228307.20	5.36	0.1430	0.1392	0.1396	0.1406	0.0017
114153.60	5.06	0.1446	0.1380	0.1409	0.1412	0.0027
57076.80	4.76	0.1451	0.1375	0.1398	0.1408	0.0032
28538.40	4.46	0.1422	0.1380	0.1393	0.1398	0.0017
14269.20	4.15	0.1418	0.1404	0.1392	0.1405	0.0011
7134.60	3.85	0.1419	0.1387	0.1405	0.1404	0.0013
3567.30	3.55	0.1465	0.1403	0.1419	0.1429	0.0026
1783.65	3.25	0.1442	0.1409	0.1434	0.1429	0.0014
891.83	2.95	0.1491	0.1459	0.1447	0.1466	0.0018
445.91	2.65	0.1543	0.1500	0.1483	0.1509	0.0025
222.96	2.35	0.1612	0.1610	0.1602	0.1608	0.0004
111.48	2.05	0.1711	0.1652	0.1661	0.1675	0.0026
55.74	1.75	0.1751	0.1711	0.1722	0.1728	0.0017
27.87	1.45	0.1822	0.1747	0.1746	0.1772	0.0036
13.93	1.14	0.1776	0.1719	0.1733	0.1743	0.0024
6.97	0.84	0.1760	0.1739	0.1727	0.1742	0.0014
blank	-	0.1773	0.1726	0.1714	0.1738	0.0025

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