Supplementary Information

Cloacaenodin, an Antimicrobial Lasso Peptide with Activity against *Enterobacter*

Drew V. Carson[1], Monica Patiño[1], Hader E. Elashal[1], Alexis Jaramillo Cartagena[2], Yi Zhang[1], Megan E. Whitley[1], Larry So[1], Angelo K. Kayser-Browne[3], Ashlee M. Earl[2], Roby P. Bhattacharyya[2,4], A. James Link[1,3,5,*]

[1]Department of Chemical and Biological Engineering, Princeton University, Princeton, New Jersey 08544, United States

[2]Infectious Disease and Microbiome Program, Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, United States

[3]Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544, United States

[4]Infectious Diseases Division, Department of Medicine, Massachusetts General Hospital, Boston, Massachusetts 02114, United States

[5]Department of Chemistry, Princeton University, Princeton, New Jersey 08544, United States

*To whom correspondence should be addressed: ajlink@princeton.edu

Table of Contents

Detailed methods: Pages S2-S7

30 supplementary figures: Pages S8-S34

11 supplementary tables: Pages S35-S46

Methods

Materials

For cloning, *E. coli* XL-1 blue cells were used, while for peptide expression, *E. coli* BL-21 cells were used. Q5 DNA polymerase from New England Biolabs (NEB) was used for PCR, and all restriction enzymes and T4 DNA ligase used were purchased from NEB. Plasmids were purified using mini-prep spin columns from QIAGEN. DNA fragments for molecular cloning were gel extracted using Zymoclean Gel DNA recovery kits from Zymo. Commercial strains for testing cloacaenodin activity were purchased from Leibniz Institute DSMZ or American Type Culture Collection, and are listed in Table S4A. Primers and gBlocks were ordered from Integrated DNA Technologies and cloned plasmids were sequence confirmed with Genewiz (now Azenta) before expression. All primer sequences are listed in Table S5 and resulting plasmids are listed in Table S6. After expression, cultures were spun down with an Avanti J26S XP centrifuge from Beckman Coulter. For supernatant purification, we used HyperSep C8 columns from Thermo Fisher with a 6 mL column volume. Methanol extracts were rotovapped with a Buchi Rotovapor R-210. LC-MS analysis and LC-MS/MS analysis were done using an Agilent 6530 QTOF connected to an Agilent 1260 LC, with all analysis done using electrospray ionization with the instrument in positive ion mode. LC-MS data were visualized using Agilent MassHunter software, and MS/MS data were visualized using mMass software. The column used for LC-MS analysis was an Agilent Zorbax C18 column, with size 2.1 mm by 50 mm and 3.5 µm particle size. For HPLC analysis, an Agilent 1200 series HPLC was used, with extracts purified using an Agilent Zorbax C18 column, with size 9.4 mm by 250 mm and 5 µm particle size. HPLC-grade solvents were used for LC-MS and HPLC analysis, with acetonitrile purchased from Sigma-Aldrich. Collected HPLC fractions were lyophilized using a Labconco FreeZone 4.5.

Identification of Gene Cluster and Bioinformatic Search of Cloacaenodin-like Gene Clusters

We used our precursor-centric genome mining algorithm as described previously[1], but specifically looked for precursors with the Tyr after the expected ring and penultimate Tyr. Upon identification of the cloacaenodin BGC in *Enterobacter hormaechei* strain LB3, a BlastP search was conducted on the amino acid sequence for CloA using the following default parameters: standard database search set, non-redundant protein sequences database, blastp algorithm, 100 maximum target sequences, parameters automatically adjusted for short input sequences, expected threshold of 0.05, word size of 6, 0 max matches in a query range, BLOSUM 62 matrix, gap costs of existence: 11 and extension: 1, conditional compositional score matrix adjustment, low complexity regions filtered. We then manually searched nearby the identified *A* genes to confirm the presence of the *B*, *C*, and *D* genes. To search for the total number of assembled *Enterobacter cloacae and Enterobacter hormaechei* genomes, an assembly search was conducted on NCBI with "*Enterobacter cloacae*" or "*Enterobacter hormaechei*" as the search query on August 25th, 2022, and the total number of "Latest GenBank" assemblies was recorded. To compare the amino acid sequences of the CloA, CloB, CloC, and CloD-like proteins, the multiple sequence alignment tool was used through Clustal Omega on the web server.[2]

Cloning and Plasmid Construction

The BGC of cloacaenodin (consisting of the *ABCD* architecture) was codon-optimized for *E. coli* using DNAWorks.[3] The codon-optimized sequence was used for the gene refactoring into pQE-80. This refactored gene cluster contains the *A* precursor under the control of the isopropyl-ß-D-thiogalactopyranoside (IPTG)-inducible T5 promoter in pQE-80, with the other genes of the BGC (*B*, *C*, and *D*) placed under the natural constitutive *mcjBCD* promoter of the microcin J25 gene cluster. Briefly, the *cloA* gene was cloned following the T5 promoter and ribosome binding site (RBS) of pQE-80 using *Eco*RI and *Hin*dIII restriction sites. This was assembled with primers listed in Table S10. gBlocks encoding the codon-optimized *cloBCD* genes were amplified via overlap PCR with a preceding p_{mcjBCD} promoter. These gBlock sequences are listed in Table S11. The resulting purified PCR product was then cloned into the plasmid containing *cloA* using the *Nhe*I and *Nco*I restriction sites. This resulted in formation of the pAK2 plasmid (p_{T5}-*cloA* p_{mcjBCD}-*cloBCD*), which was verified by sequencing from Genewiz (now Azenta).

Cloacaenodin variants were constructed using site-directed mutagenesis. Mutant precursor genes were amplified from the wild-type precursor in pAK2 using mutagenic primers. Following PCR amplification of the mutated precursor gene, the purified PCR product was digested and ligated into pAK2 with the *Eco*RI and *Hin*dIII restriction sites. For the pMP3 plasmid for deletion of the *cloD* gene, the *D* gene was first disrupted by digestion of pAK2 with *Bam*HI and *Nco*I, which removed the entire *cloD* gene and the C-terminal portion of the *cloC* gene. The digested plasmid was then ligated with an insert that restored the C-terminal portion of the *cloC* gene. All mutants were sequence confirmed by Genewiz (now Azenta) before use.

Expression and Purification of Cloacaenodin and Variants

pAK2 was transformed via electroporation into *E. coli* BL-21 cells before plating on an LB agar plate supplemented with 100 µg/mL of ampicillin. Following overnight incubation of the plate at 37 °C, a single colony was then used to inoculate 5 mL of LB broth supplemented with 100 µg/mL of ampicillin. This culture was then grown at 37 °C with 250 rpm shaking overnight. The following day, the OD_{600} of the overnight culture was measured, and this was diluted to an OD_{600} of 0.02 in 500 mL M9 minimal media in a 2L flask. The M9 minimal media consisted of M9 salts, 0.2% glucose, 1 mM $MgSO_4$, 0.00005 wt% thiamine, and the 20 amino acids each at a concentration of 40 mg/L. 100 µg/mL of ampicillin was also added to the culture for plasmid selection. Following inoculation with the overnight culture, the 500 mL cultures were allowed to grow at 37 °C with shaking at 250 rpm. Once the OD_{600} of these cultures reached approximately 0.2 (~3-4 hours), 1 mM of IPTG was added to the cultures to induce expression of cloacaenodin. The culture was allowed to grow overnight at room temperature, with shaking at 250 rpm.

After expression, the cells and supernatant were separated by centrifugation at 4000 x *g* for 15 minutes at 4 °C. To purify the supernatant, the supernatant was extracted through a C8 column through the use of a vacuum chamber. The column was activated with 6 mL of 100% methanol before being washed with 12 mL of DI water. The supernatant was then added to the column and allowed to flow through. After the supernatant was flowed through, the column was washed with 12 mL of DI water, and

then 6 mL of 100% methanol was added to elute the extract. The methanol was then dried with a rotovap, and following this, 1 mL of DI water per liter of expression was used to resuspend the dried extract. The extract was then spun down further on a tabletop centrifuge before injection on an LC-MS instrument.

The LC-MS instrument was operated at 0.5 mL/min of a water/acetonitrile gradient with the addition of 0.1% formic acid. From 0-1 min, 90% water/10% acetonitrile flowed through the column, followed by a linear gradient from 90% water/10% acetonitrile to 50% water/50% acetonitrile from 1-20 minutes, followed by a linear gradient from 50% water/50% acetonitrile to 10% water/90% acetonitrile from 20-25 minutes.

Via LC-MS analysis, cloacaenodin was detected in the supernatant extract. Thus, the supernatant extract was used for RP-HPLC purification of cloacaenodin. 20-60 µL of the supernatant extract was injected onto a C18 semi-preparative column. The HPLC instrument was operated at 4 mL/min of a water/acetonitrile gradient with the addition of 0.1% trifluoroacetic acid. From 0-1 min, 90% water/10% acetonitrile flowed through the column, followed by a linear gradient from 90% water/10% acetonitrile to 50% water/50% acetonitrile from 1-20 min, followed by a linear gradient from 50% water/50% acetonitrile to 10% water/90% acetonitrile from 25-29 minutes. Multiple peaks on the chromatogram were collected and checked via LC-MS for cloacaenodin's expected mass. The prominent peak with a retention time of 15.0 minutes matched the expected mass of cloacaenodin. This peak was then collected from the HPLC instrument and frozen at -80 °C within minutes of collection. This was done to minimize unthreading of cloacaenodin in solution in the HPLC collection vial. After freezing fully, the frozen sample was then lyophilized and re-suspended in pure water. To calculate concentration of purified cloacaenodin, a NanoDrop spectrophotometer was used to measure the absorbance at 280 nm. From the amino acid sequence of cloacaenodin, an extinction coefficient of 2560 cm^{-1} M^{-1} was calculated and used for the NanoDrop measurements.[4]

Cloacaenodin varants were expressed in the same way as wild-type and purified from the supernatant. The production levels of each variant were judged via HPLC relative to the wild-type. Variants with identifiable peaks via HPLC analysis and appreciable production levels were purified for further assays.

For the Y10A variant, a second round of HPLC was required to further purify the peptide with a flatter gradient. For the second run, the HPLC instrument was operated at 4 mL/min of a water/acetonitrile gradient with the addition of 0.1% trifluoroacetic acid. From 0-1 min, 90% water/10% acetonitrile flowed through the column, followed by a linear gradient from 90% water/10% acetonitrile to 75% water/25% acetonitrile from 1-3 minutes, followed by a linear gradient from 75% water/25% acetonitrile to 70% water/30% acetonitrile from 3-30 minutes. The prominent peak was collected at ~14.7 minutes using this gradient and confirmed with LC-MS analysis to match the peptide's expected mass.

Cloacaenodin Stability

A 200 µL sample of purified ~18 µM cloacaenodin in water was incubated at 37 °C for 72 hours. 30 µL of the sample was analyzed via LC-MS at 24-hour increments.

NMR Data Collection

NMR studies were performed at the Princeton University Department of Chemistry NMR Facilities using a Bruker Avance III HD 800 MHz NMR spectrometer. Purified cloacaenodin was prepared at a concentration of 6.7 mg/mL (2.6 mM) in 95:5 H_2O:D_2O. The NMR spectra were acquired at 4 °C to minimize any cloacaenodin unthreading. A ^{1}H-^{1}H TOCSY spectrum at a mixing time of 80 ms was acquired, as well as ^{1}H-^{1}H NOESY spectra at 150 ms and 300 ms. Water suppression was used in the collection of all NMR data. To verify that the lasso peptide was not undergoing unthreading, 1D ^{1}H NMR was collected in between each 2D NMR acquisition and analyzed to see that the 1D spectra stayed consistent. As further confirmation that the lasso peptide had not undergone any conformational change during the NMR acquisition and that no degradation or contamination of the sample had occurred, an aliquot of the NMR sample was analyzed via LC-MS following NMR data collection.

Determination of Structure through NMR Analysis

NMR spectra were processed and analyzed with the use of MNova (Mestrelab). Residues were manually assigned from an overlay of the 80 ms TOCSY and the 300 ms NOESY. After residue assignment, the 150 ms NOESY was used for through-space distance measurements, where cross-peaks were manually chosen and integrated. These peaks were inputted to CYANA 2.1 to be used as distance constraints. Further explicit distance constraints were inputted regarding the amino acids involved in the isopeptide bond (Gly1 and Glu9) and are listed in Table S7. These distances were calculated from the crystal structure of the similarly 9-member-ringed lasso peptide rubrivinodin[5] (PDB 5OQZ).

We used CYANA for seven cycles of initial structural calculations, with 100 initial structures, resulting in 20 final structures. These 20 structures were then energy minimized using Avogadro, with force field MMFF94 and the steepest descent algorithm used.

Cloacaenodin Antimicrobial Activity

Cloacaenodin was evaluated against several common laboratory strains as well as commercially acquired strains using a previously described spot-on-lawn assay.[6] Strains were streaked out onto LB agar plates and incubated overnight at their recommended temperatures. A single colony was then used to inoculate an overnight culture in 5 mL of LB broth. After shaking at 250 rpm at the recommended temperature for each strain, 50 µL of the overnight culture was used to inoculate 5 mL of LB broth (volume ratio 1:100). These cultures were grown for a few hours until they reached exponential phase (OD_{600} ~0.4-0.6) before being added to 10 mL of soft M63 agar at a final cell density of 10^7 CFUs/mL, or 10^8 CFUs total in 10 mL. The M63 soft agar was composed of 2 g/L of $(NH_4)_2SO_4$ (EMD MilliporeSigma), 13.6 g/L of KH_2PO_4 (Fisher), 40 mg/L of each of the 20 common amino acids, 0.2% glucose (Sigma), 0.00005% w/v thiamine hydrochloride (Sigma), and 0.65% w/v bacteriological-grade agar (Apex Bioresearch). The inoculated agar was then poured on top of a 10 mL M63 hard agar plate (contains same components of M63 soft agar but is instead 1.5% w/v agar and does not contain amino acids) and allowed to cool. Upon solidification, 10 µL of purified

cloacaenodin in water at two-fold dilutions were then spotted on the agar and allowed to dry. The plates were incubated overnight at the strains' recommended temperatures and analyzed the next morning for activity. The MIC is defined as the last dilution where a spot was visible.

12 clinical isolates were tested at the Broad Institute of MIT and Harvard, with a subset of strains from BioProject PRJNA292902, BioProject PRJNA271899, BioProject PRJNA201976, and BioProject 219285. The strains were tested following the same spot-on-lawn assay protocol described above, but with BD Bacto Agar used instead. All cloacaenodin-treated isolates were incubated at 37 °C overnight, and the assay was repeated at least three times (on biological replicates) for each of the 12 strains. We defined a strain to be susceptible if it was reliably susceptible in at least three biological replicates.

For liquid inhibition assays, 5 mL LB broth was inoculated with 40-50 µL of an overnight culture of *E. cloacae* or *E. amnigenus*. Once the culture reached exponential phase, the culture was diluted to an OD_{600} of 0.0005 in a 96-well plate in M63 media (same components as M63 soft agar but lacks agar) with varying concentrations of cloacaenodin. The plate was shaken at 30 °C at 250 rpm for *E. cloacae*, and 37 °C at 250 rpm for *E. amnigenus*. The OD_{600} was measured at 8-hour and 16-hour increments. The MIC is defined as the lowest concentration of cloacaenodin for which growth (as assessed by the OD_{600}) was inhibited.

Microscopy

After a 96-well plate of *E. cloacae* grew for 16 hours at 30 °C with varying concentrations of cloacaenodin in M63 media, samples were imaged using a Zeiss Observer Z1 automated inverted microscope with a cage incubator kept at 37 °C. The microscope was used with a 100X Zeiss chroma objective with oil immersion and 35 ms exposure transmitted light, and controlled using SlideBook software. Images were viewed using the software ImageJ.

Carboxypeptidase Digestion

Carboxypeptidase assays were done in 50 µL of 50 mM sodium acetate, pH 6.0 with 1 unit each of carboxypeptidase B and carboxypeptidase Y. Reactions were incubated overnight at 20 °C for 16 hours and then quenched with 1% formic acid. An aliquot of the reaction was then analyzed via LC-MS to compare with an untreated control.

Protease Digestion

Sequencing grade trypsin (Promega) was added to 50 µM peptide samples at a 1:100 trypsin:peptide weight ratio in a buffer of 50 mM ammonium bicarbonate. The reaction was allowed to proceed at room temperature for 30 minutes to 1 hour and then quenched by 1% formic acid. An aliquot was then analyzed via LC-MS.

α-chymotrypsin from bovine pancreas (Sigma-Aldrich) was first resuspended in 1mM HCl, 2 mM $CaCl_2$. The enzyme was added to 50 µM peptide samples at a final enzyme concentration of about 0.04 mg/mL in a buffer of 100 mM Tris, 10 mM $CaCl_2$, pH

8. The reactions were then allowed to incubate at 25 °C for about 1 hour before quenching with 1% formic acid, and an aliquot was analyzed via LC-MS.

Elastase (Promega) was resuspended in 50 mM Tris pH 9.0. The enzyme was added to 50 µM peptide samples at a final enzyme concentration of about 0.04 mg/mL in a buffer of 50 mM Tris, pH 9. The reactions were then allowed to incubate at 25 °C for about 1 hour before quenching with 1% formic acid. An aliquot was analyzed via LC-MS

Thermolysin from *Geobacillus stearothermophilus* (Sigma-Aldrich) was first resuspended in 50 mM Tris, 0.5 mM $CaCl_2$. The enzyme was then added to 50 µM peptide samples at a final concentration of about 0.04 mg/mL in a buffer of 50 mM Tris, 0.5 mM $CaCl_2$, pH 8. The reactions were then allowed to incubate at 30 °C for about 1 hour before quenching with 1% formic acid. An aliquot was then analyzed via LC-MS.

Data Deposition

The structure of cloacaenodin with its atomic coordinates has been deposited to the Protein Data Bank under PDB code 8DYN and to the Biological Magnetic Resonance Data Bank under BMRB entry 31037.

Supplementary References

1. Maksimov, M. O.; Pelczer, I.; Link, A. J. Precursor-Centric Genome-Mining Approach for Lasso Peptide Discovery. *Proc Natl Acad Sci USA* **2012**, *109* (38), 15223–15228. https://doi.org/10.1073/pnas.1208978109.
2. Madeira, F.; Pearce, M.; Tivey, A. R. N.; Basutkar, P.; Lee, J.; Edbali, O.; Madhusoodanan, N.; Kolesnikov, A.; Lopez, R. Search and Sequence Analysis Tools Services from EMBL-EBI in 2022. *Nucleic Acids Res* **2022**, *50* (W1), W276–W279. https://doi.org/10.1093/nar/gkac240.
3. Hoover, D. M.; Lubkowski, J. DNAWorks: An Automated Method for Designing Oligonucleotides for PCR-Based Gene Synthesis. *Nucleic Acids Res* **2002**, *30* (10), e43–e43. https://doi.org/10.1093/nar/30.10.e43.
4. Gill, S. C.; von Hippel, P. H. Calculation of Protein Extinction Coefficients from Amino Acid Sequence Data. *Anal Biochem* **1989**, *182* (2), 319–326. https://doi.org/10.1016/0003-2697(89)90602-7.
5. Jeanne Dit Fouque, K.; Lavanant, H.; Zirah, S.; Hegemann, J. D.; Fage, C. D.; Marahiel, M. A.; Rebuffat, S.; Afonso, C. General Rules of Fragmentation Evidencing Lasso Structures in CID and ETD. *Analyst* **2018**, *143* (5), 1157–1170. https://doi.org/10.1039/c7an02052j.
6. Pan, S. J.; Cheung, W. L.; Link, A. J. Engineered Gene Clusters for the Production of the Antimicrobial Peptide Microcin J25. *Protein Expres Purif* **2010**, *71* (2), 200–206. https://doi.org/10.1016/j.pep.2009.12.010.

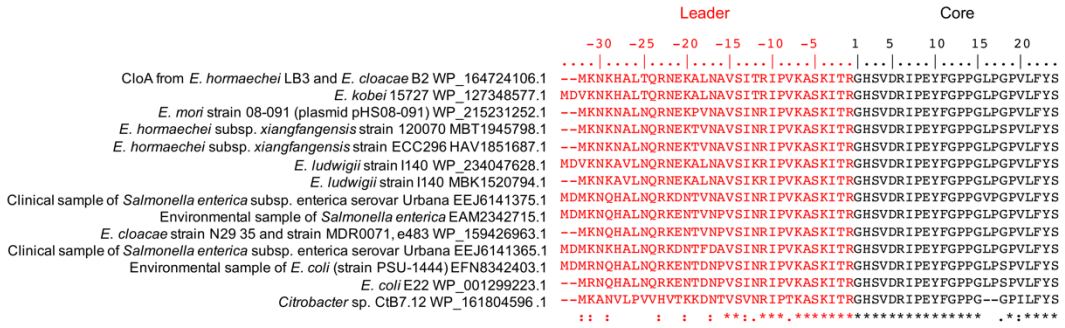


Figure S1. An alignment of the A proteins from cloacaenodin-like BGCs, found from a BlastP search on the CloA sequence. The region with the least degree of similarity is the N-terminal portion of the leader, which is consistent with our understanding that most of the recognition by the B protein lies at the C-terminal end of the leader and the beginning of the core peptide. All of the leader peptides consist of 32-34 amino acids with a core peptide of 24 amino acids (with the exception of the *Citrobacter* core at 22 amino acids).

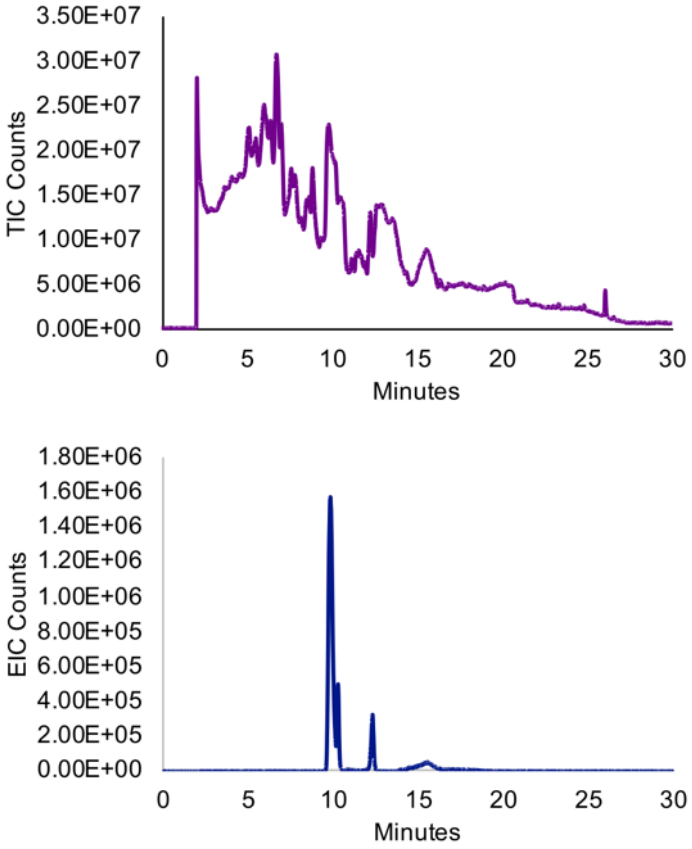
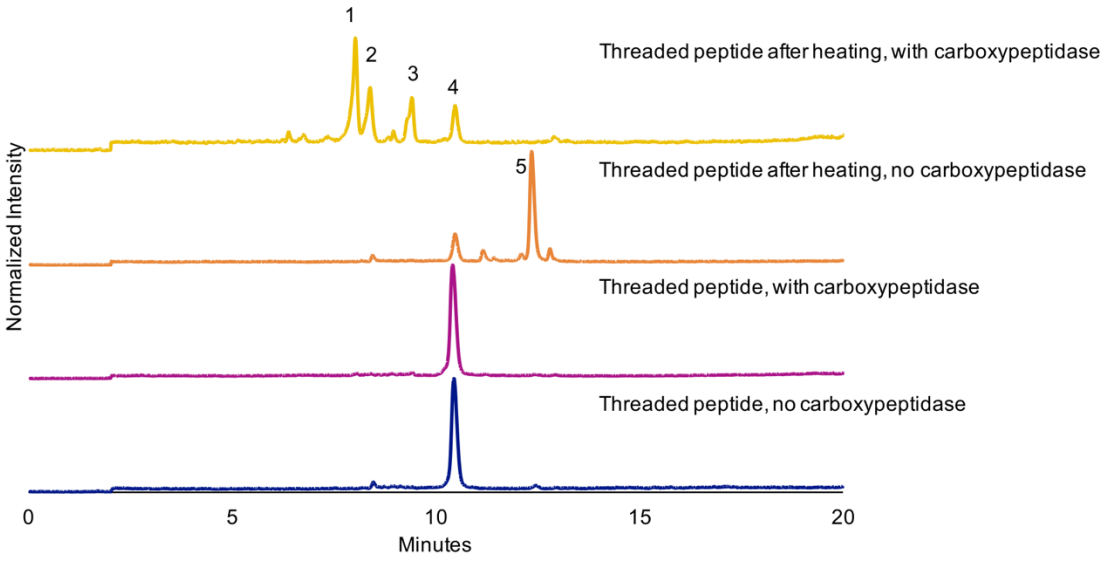


Figure S2. LC-MS trace of supernatant extract for expression of cloacaenodin. Top: Total ion current (TIC) chromatogram of supernatant extract. Bottom: Extracted ion current (EIC) chromatogram of supernatant extracted for expected +3 and +2 mass-charge states. We extracted for the predicted monoisotopic charge-states of a +3 m/z of 861.7725 and a +2 m/z of 1292.1552.



Peak	Observed Monoisotopic Mass (Da)	Expected Monoisotopic Mass (Da)	Sequence
1	537.26 (+3)	537.26 (+3)	GHSVDRIPEYFGPPG
2	518.26 (+3)	518.26 (+3)	GHSVDRIPEYFGPP
3	626.32 (+3)	626.32 (+3)	GHSVDRIPEYFGPPGLPG
4	861.78 (+3)	861.77 (+3)	GHSVDRIPEYFGPPGLPGPVLFYS (threaded)
5	861.78 (+3)	861.77 (+3)	GHSVDRIPEYFGPPGLPGPVLFYS (unthreaded)

Figure S3. The threaded cloacaenodin stays intact when treated with carboxypeptidase, while the unthreaded cloacaenodin, which can be generated by heating at 95 °C, is susceptible to proteolysis by carboxypeptidase. C-terminally truncated fragments of cloacaenodin can be detected only when the unthreaded peptide is treated. Top: TIC chromatograms of cloacaenodin under the conditions indicated. Bottom: Table of observed masses and proposed sequence. For each value, the charge state is shown in parentheses.

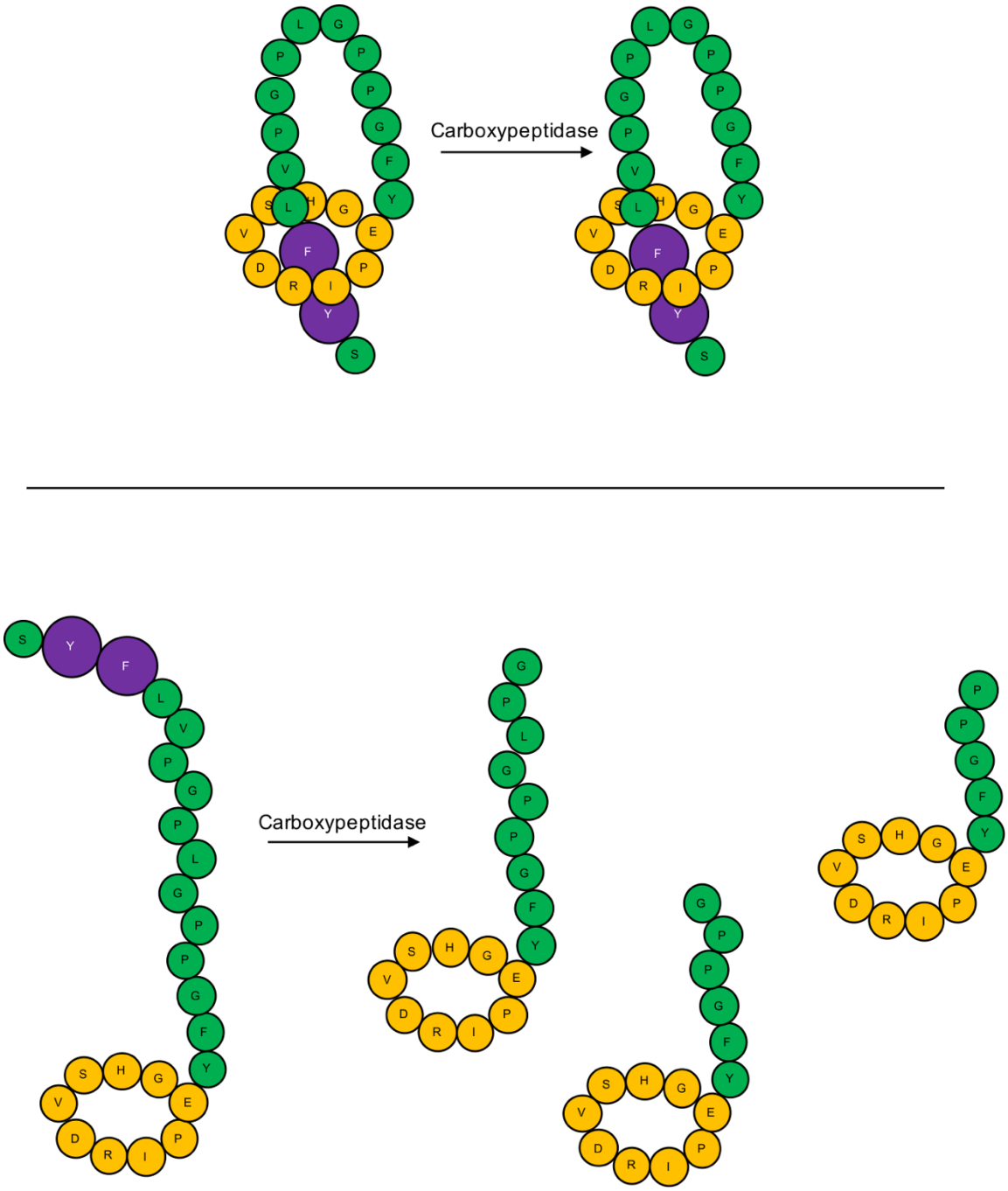


Figure S4. Cartoon depicting the major products of threaded and unthreaded cloacaenodin upon treatment with carboxypeptidase. The threaded peptide is not proteolyzed by carboxypeptidase as its C-terminus is protected by the ring. The unthreaded cloacaenodin can be truncated at the C-terminus by 6, 9, or 10 amino acids. Loop/tail residues are shown in green and ring residues are shown in orange. Steric lock residues are shown in purple.

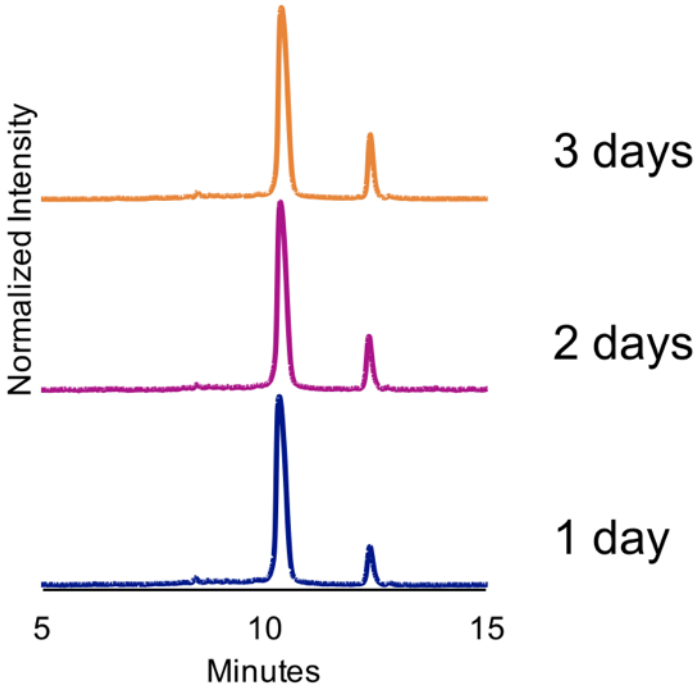
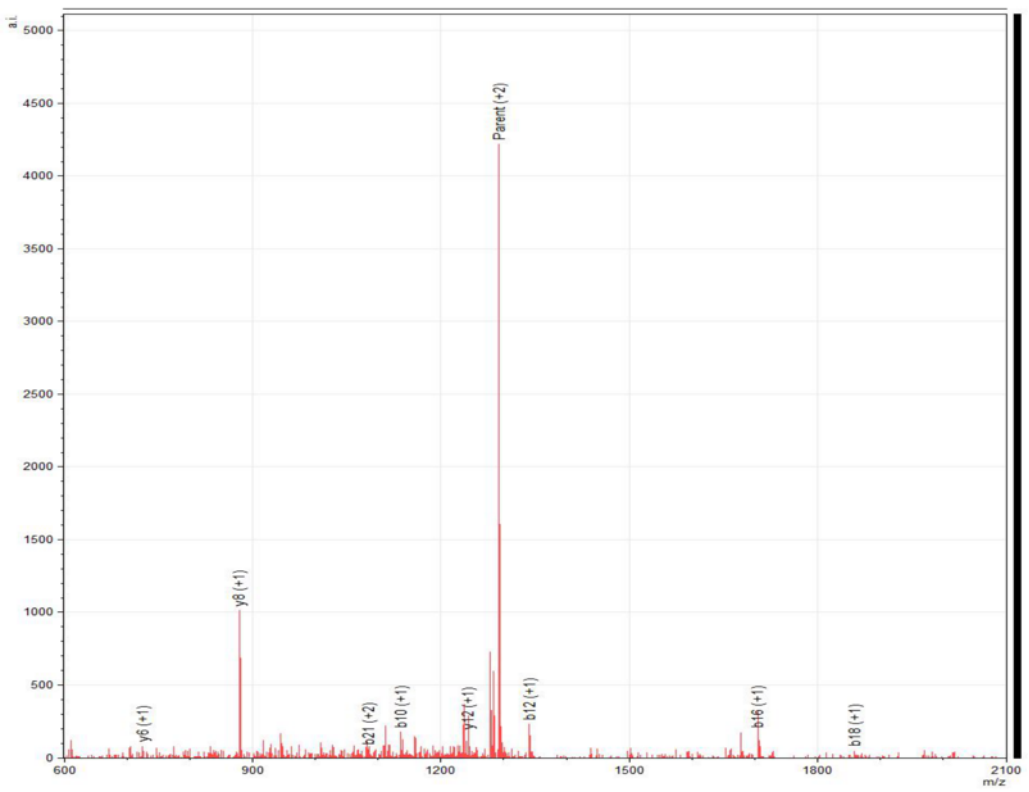
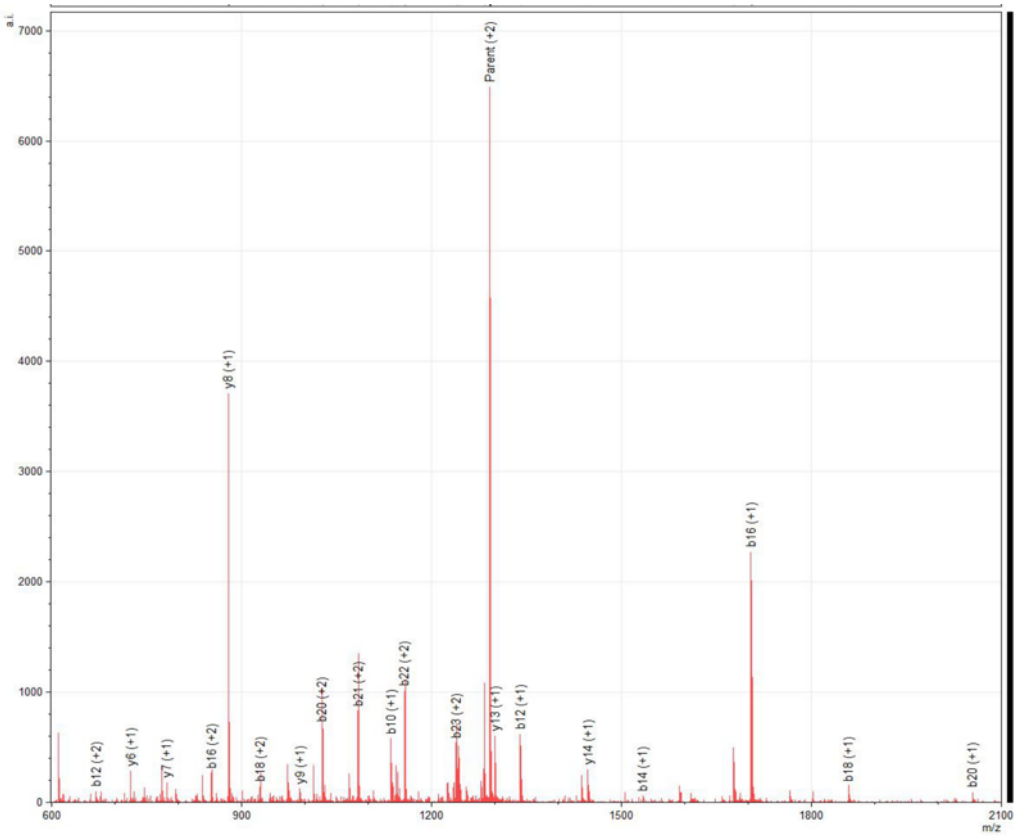


Figure S5. An 18 μM sample of cloacaenodin stays >80% threaded after incubating at 37°C over a period of 3 days, showing that the peptide's bioactivity would not be completely inactivated by unthreading at this timescale.

A)


a.i.
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
600
900
1200
1500
1800
2100
m/z
Parent (+2)
y8 (+1)
y6 (+1)
b21 (+2)
b10 (+1)
y12 (+1)
b12 (+1)
b16 (+1)
b18 (+1)

B)


a.i.
7000
6000
5000
4000
3000
2000
1000
0
600
900
1200
1500
1800
2100
m/z
Parent (+2)
y8 (+1)
b16 (+1)
b12 (+2)
y6 (+1)
y7 (+1)
b16 (+2)
b18 (+2)
y9 (+1)
b20 (+2)
b21 (+2)
b10 (+1)
b22 (+2)
b23 (+2)
y13 (+1)
b12 (+1)
y14 (+1)
b14 (+1)
b18 (+1)
b20 (+1)

C)

Ion	Sequence	Predicted Mass (Da)	Observed Mass in Unthreaded (Da)	Observed Mass in Threaded (Da)
y_6	PVLFYS	725.3869 (+1)	725.3898 (+1)	725.4243 (+1)
y_7	GPVLFYS	782.4083 (+1)	782.4057 (+1)	N/A
y_8	PGPVLFYS	879.4611 (+1)	879.4663 (+1)	879.4665 (+1)
y_9	LPGPVLFYS	992.5451 (+1)	992.5549 (+1)	N/A
y_{12}	PPGLPGPVLFYS	1243.6721 (+1)	1243.6839 (+1)	1243.6666 (+1)
y_{13}	GPPGLPGPVLFYS	1300.6936 (+1)	1300.6952 (+1)	N/A
y_{14}	FGPPGLPGPVLFYS	1447.7620 (+1)	1447.7818 (+1)	N/A
b_{10}	GHSVDRIPEY	1136.5483 (+1)	1136.5572 (+1)	1136.5586 (+1)
b_{12}	GHSVDRIPEYFG	670.8227 (+2) and 1340.6382 (+1)	670.8178 (+2) and 1340.6514 (+1)	1340.6426 (+1)
b_{13}	GHSVDRIPEYFGP	1437.6910 (+1)	1437.6991 (+1)	N/A
b_{14}	GHSVDRIPEYFGPP	1534.7437 (+1)	1534.7512 (+1)	N/A
b_{16}	GHSVDRIPEYFGPPGL	852.9283 (+2) and 1704.8493 (+1)	852.9415 (+2) and 1704.8575 (+1)	1704.8680 (+1)
b_{18}	GHSVDRIPEYFGPPGLPG	929.9654 (+2) and 1858.9235 (+1)	929.9614 (+2) and 1858.9415 (+1)	1858.9205 (+1)
b_{20}	GHSVDRIPEYFGPPGLPGPV	1028.0260 (+2) and 2055.0447 (+1)	1028.0271 (+2) and 2055.0554 (+1)	N/A
b_{21}	GHSVDRIPEYFGPPGLPGPVL	1084.5680 (+2)	1084.5737 (+2)	1084.5698 (+2)
b_{22}	GHSVDRIPEYFGPPGLPGPVLF	1158.0980 (+2)	1158.1022 (+2)	1158.1157 (+2)
b_{23}	GHSVDRIPEYFGPPGLPGPVLFY	1239.6339 (+2)	1239.6530 (+2)	N/A

Figure S6. Both threaded and unthreaded cloacaenodin yield similar MS/MS fragmentation patterns, but the threaded cloacaenodin is more resistant to fragmentation due to its interlocked structure. Various peaks were only observable for the unthreaded species. A) MS/MS of threaded cloacaenodin. B) MS/MS of unthreaded cloacaenodin. C) Fragment ions observed. For each value, the charge state is shown in parentheses.

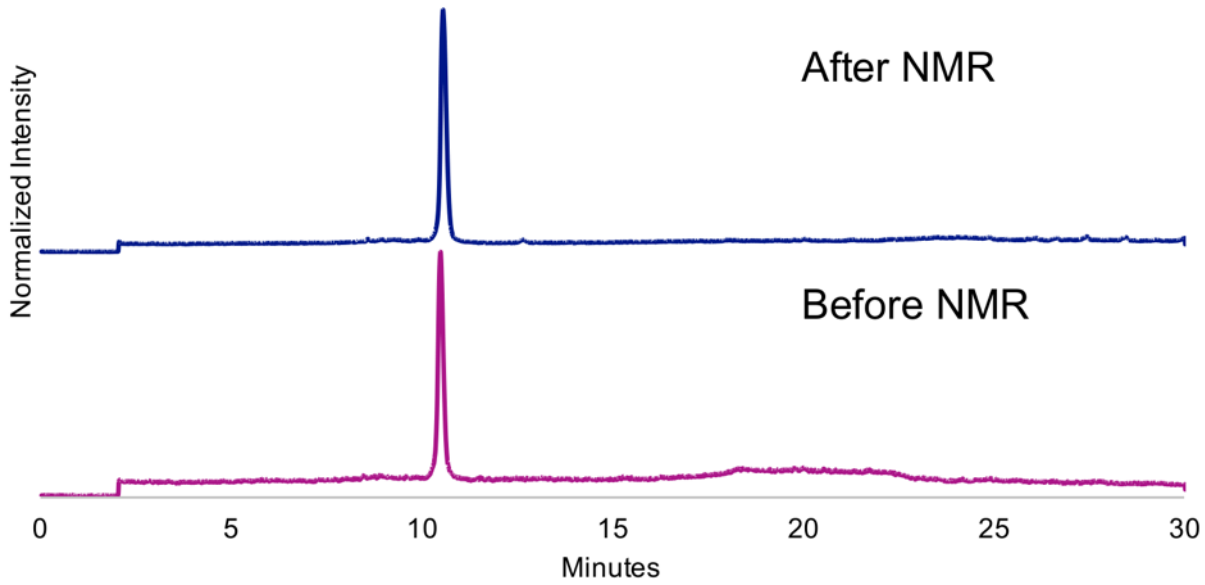


Figure S7. Cloacaenodin remained threaded throughout NMR acquisition by keeping it at 4°C, as shown by the single peak on LC-MS before and after acquisition.

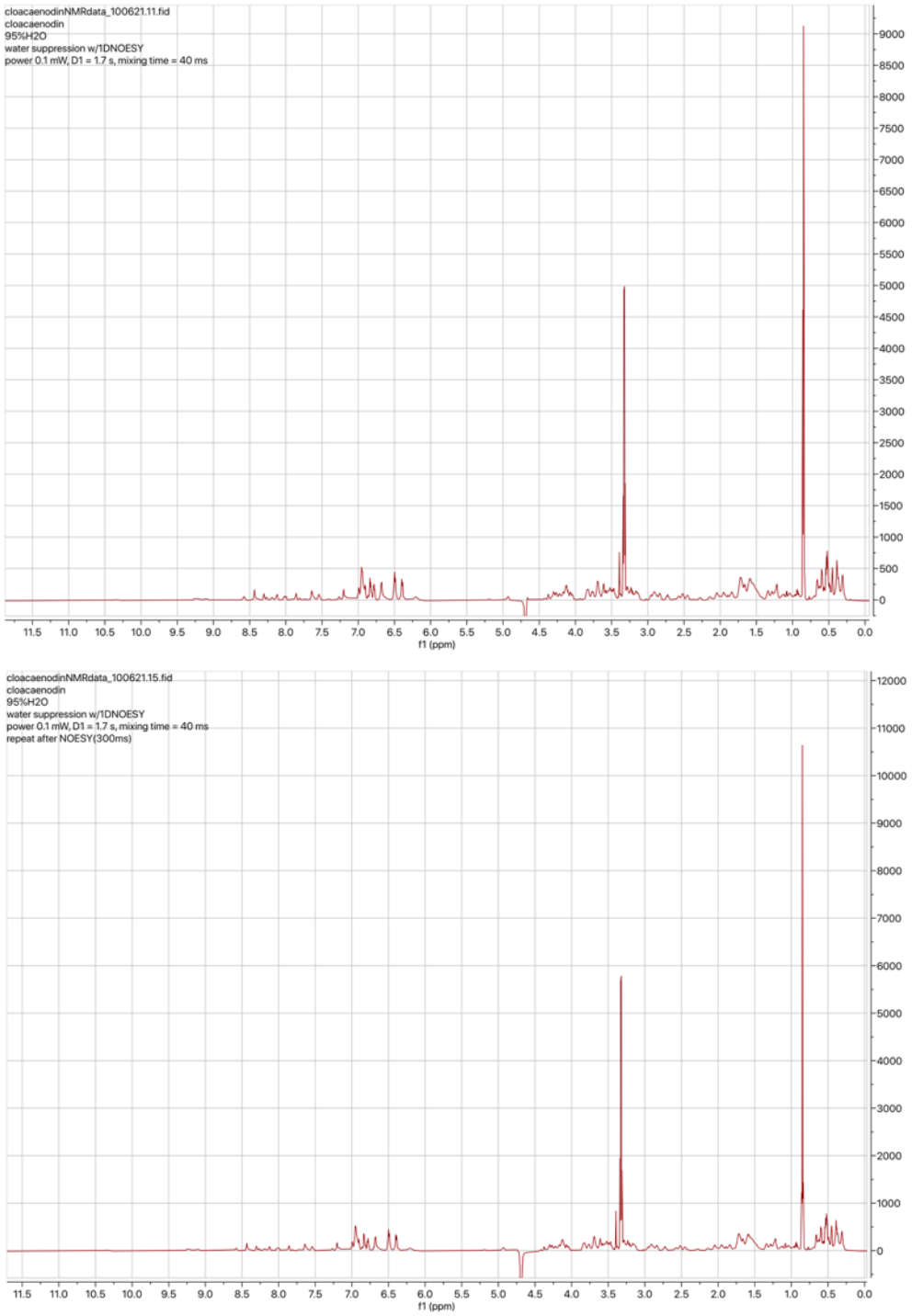


Figure S8. 1D NMR spectrum taken before (top) and after (bottom) 2D NMR acquisition ensured the sample remained stable. The 1D NMR spectrum stayed consistent.

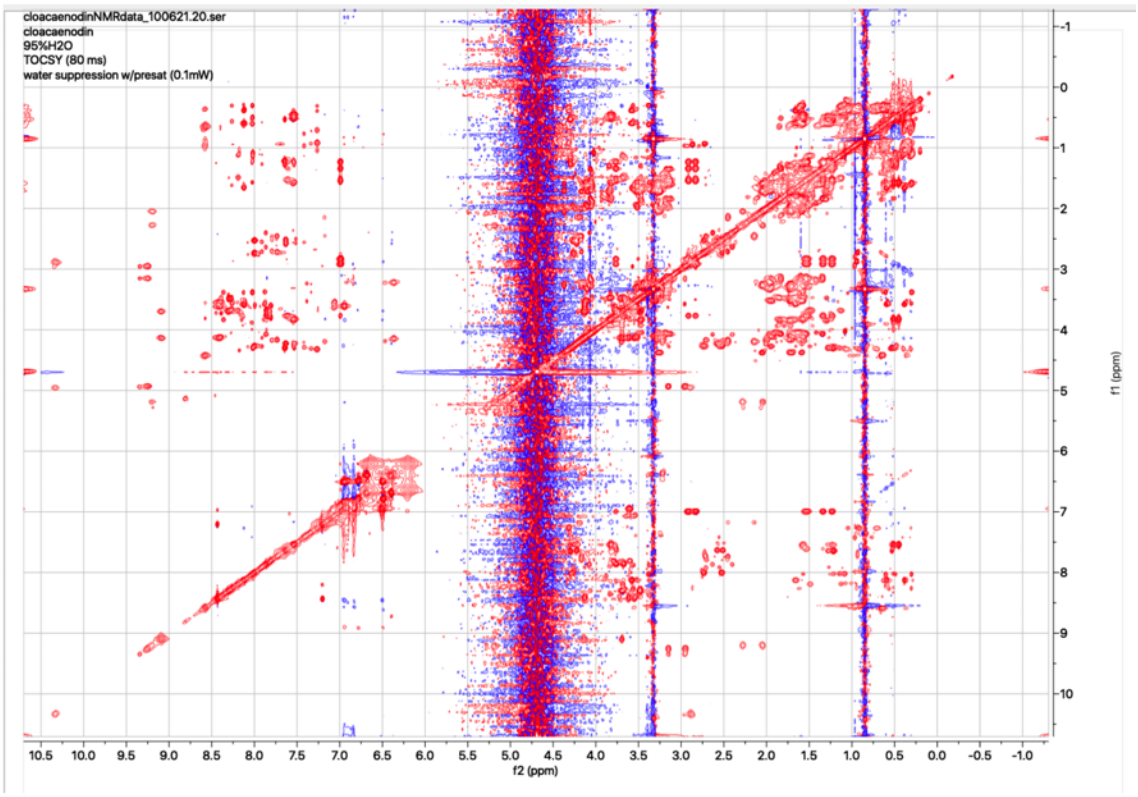


Figure S9. TOCSY of cloacaenodin at a mixing time of 80 ms.

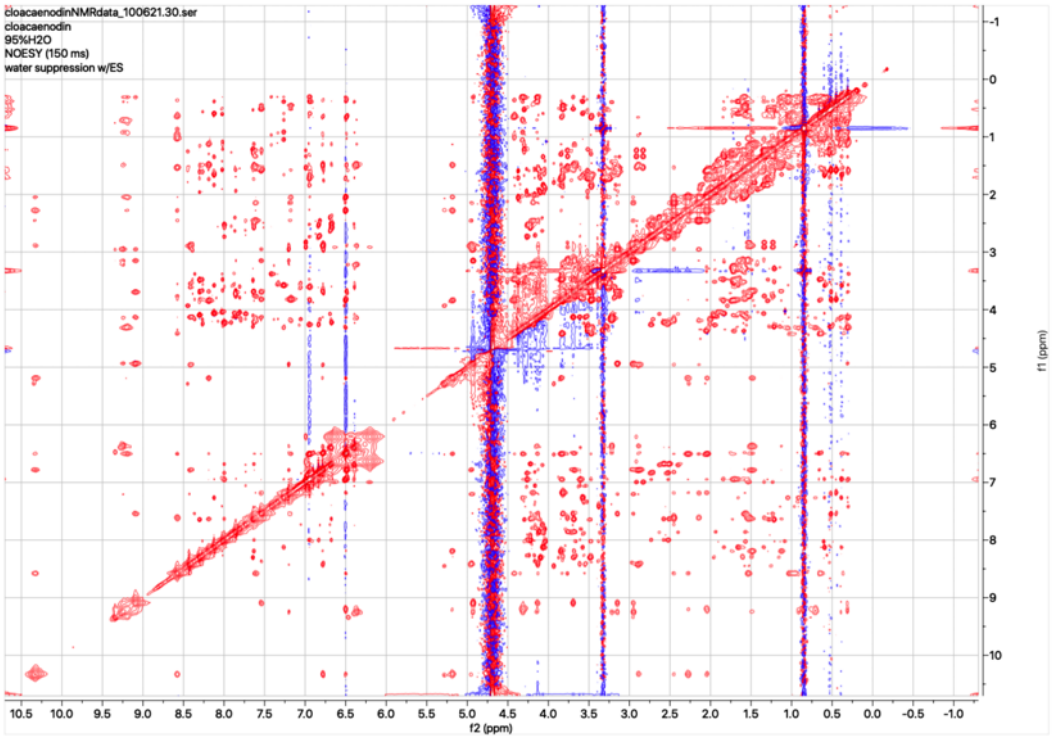


Figure S10. NOESY of cloacaenodin at a mixing time of 150 ms.

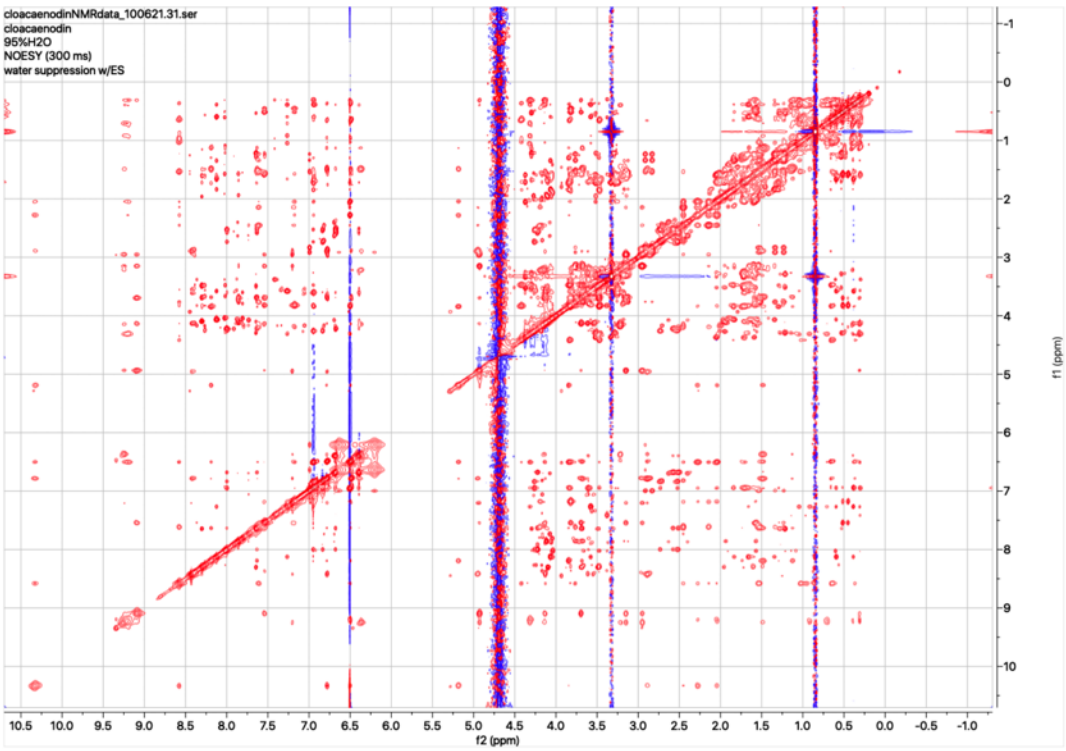


Figure S11. NOESY of cloacaenodin at a mixing time of 300 ms.

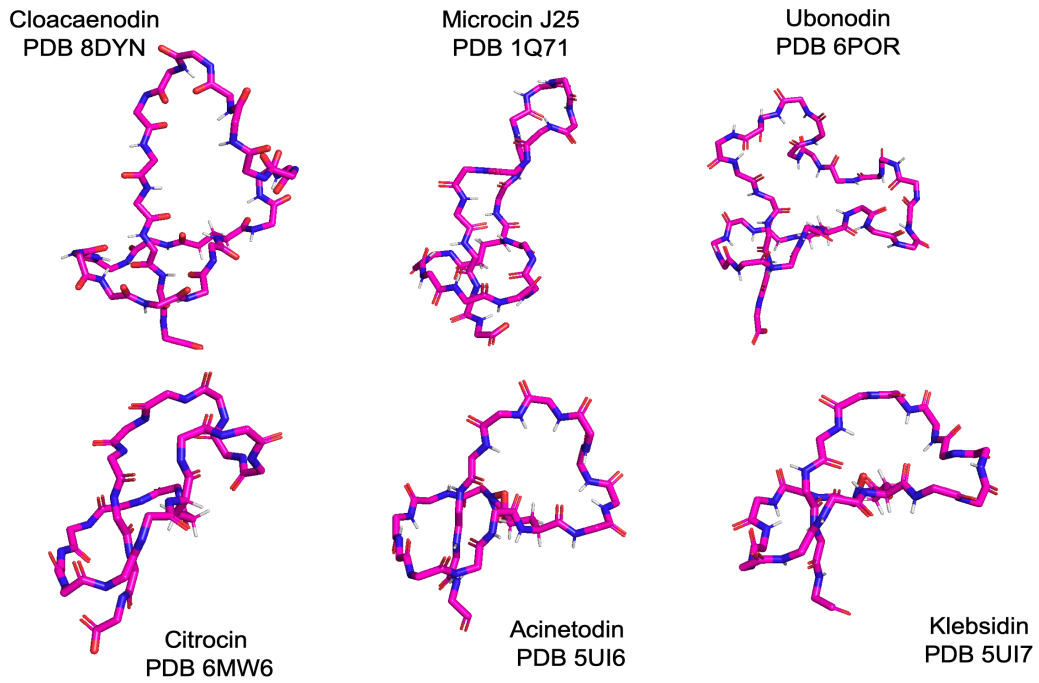
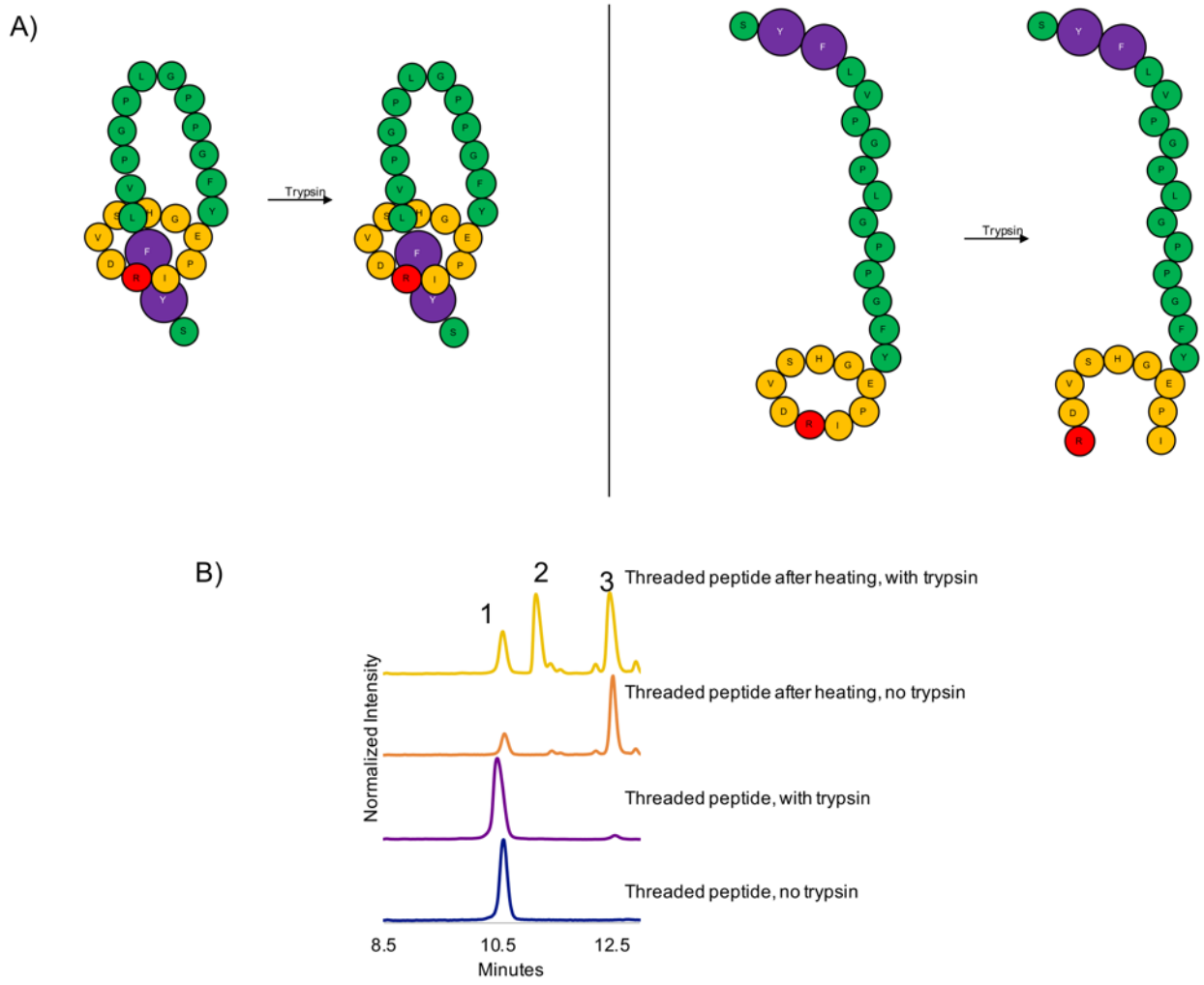
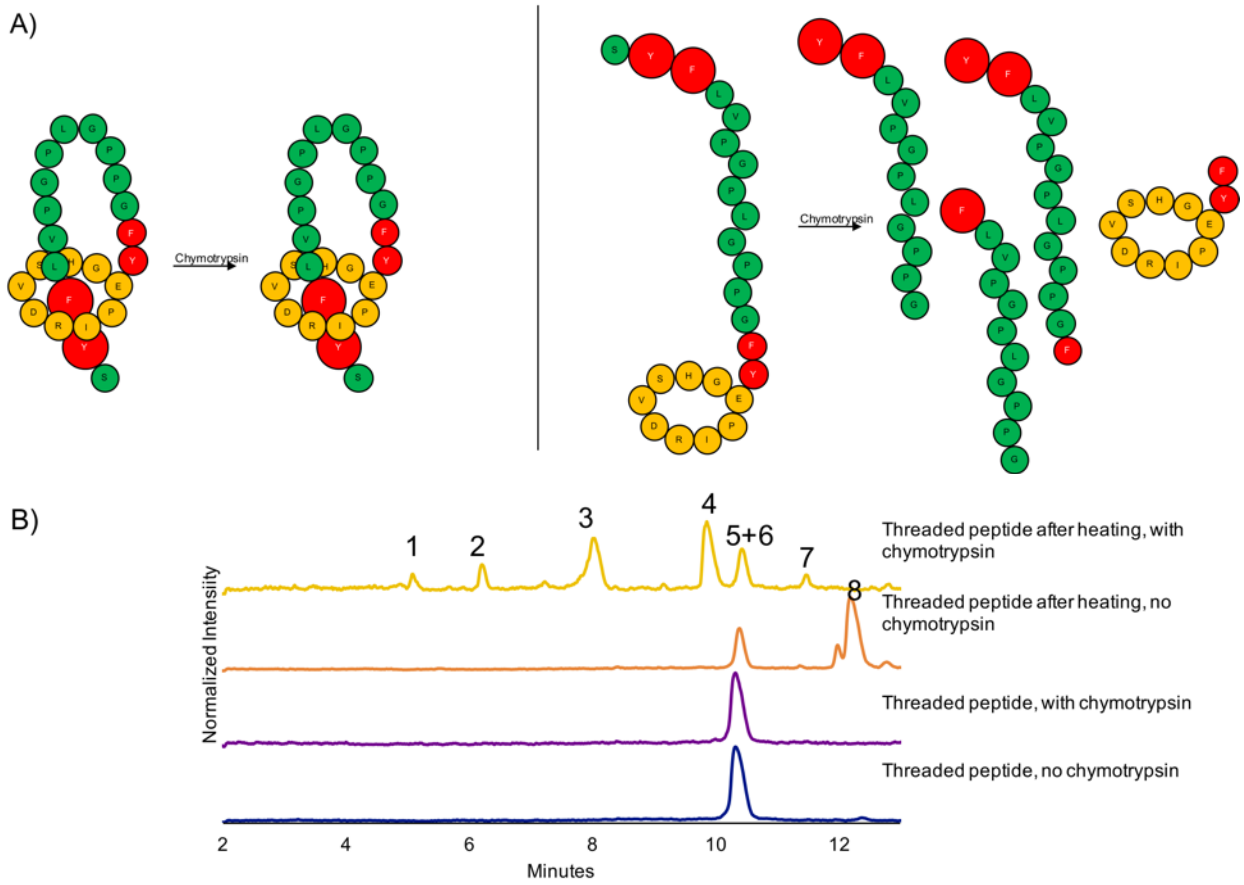


Figure S12. NMR structures of cloacaenodin and other Gram negative-targeting lasso peptides. The lasso peptides share a large loop, short tail structure.



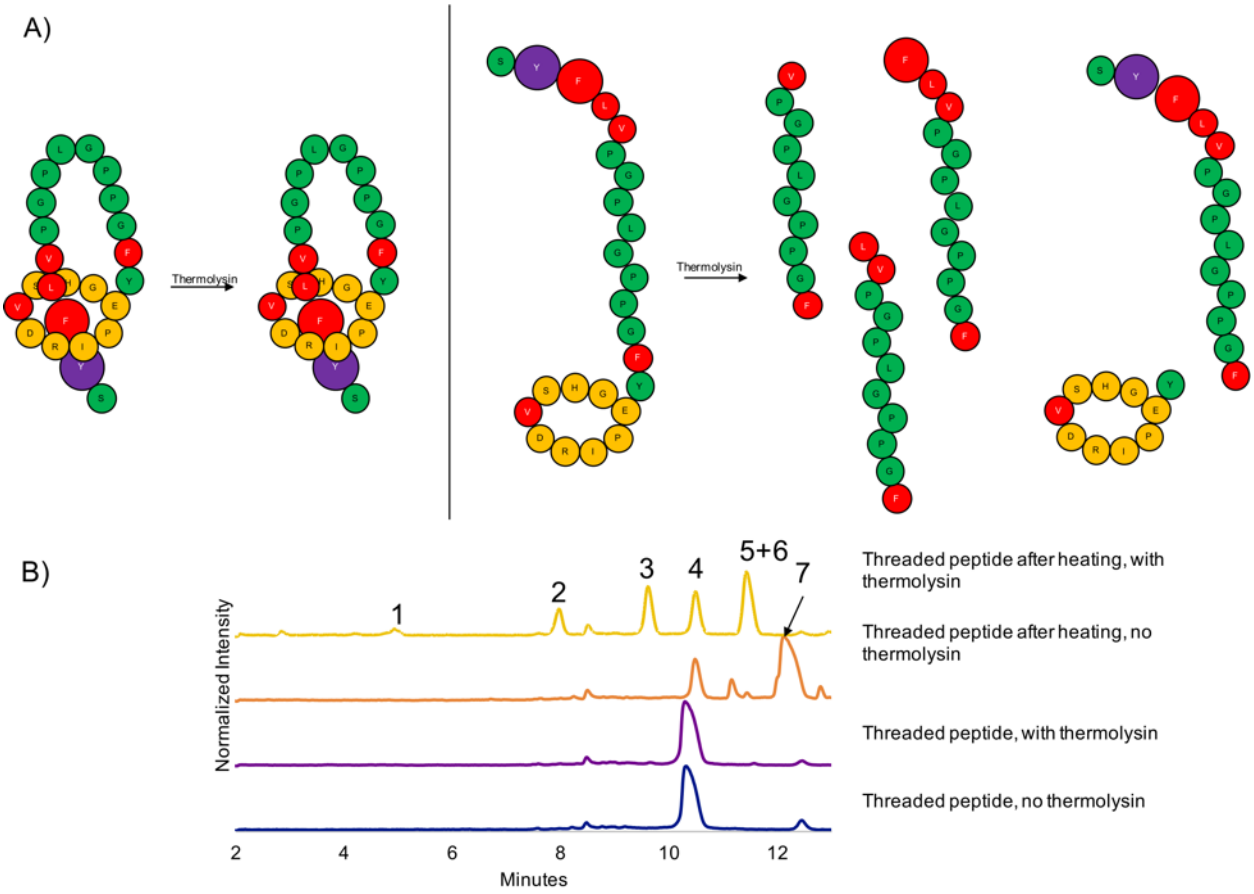
Peak	Predicted Monoisotopic Mass (Da)	Observed Monoisotopic Mass (Da)	Sequence
1	861.77 (+3)	861.75 (+3)	GHSVDRIPEYFGPPGLPGPVLFYS (threaded)
2	867.77 (+3)	867.75 (+3)	GHSVDR-IPEYFGPPGLPGPVLFYS - = cleavage site
3	861.77 (+3)	861.75 (+3)	GHSVDRIPEYFGPPGLPGPVLFYS (unthreaded)

Figure S13. Threaded cloacaenodin is resistant to trypsin digestion while unthreaded cloacaenodin can be cleaved by trypsin. A) Cartoon depicting major products of threaded and unthreaded cloacaenodin upon trypsin treatment. Ring residues are orange, loop/tail residues are green, and steric lock residues are purple. Residues that are preferentially susceptible to trypsin digestion are in red. B) LC-MS data of trypsin proteolysis experiment on threaded and unthreaded cloacaenodin, with a table of observed masses and proposed sequence. The charge state for each value is shown in parentheses.



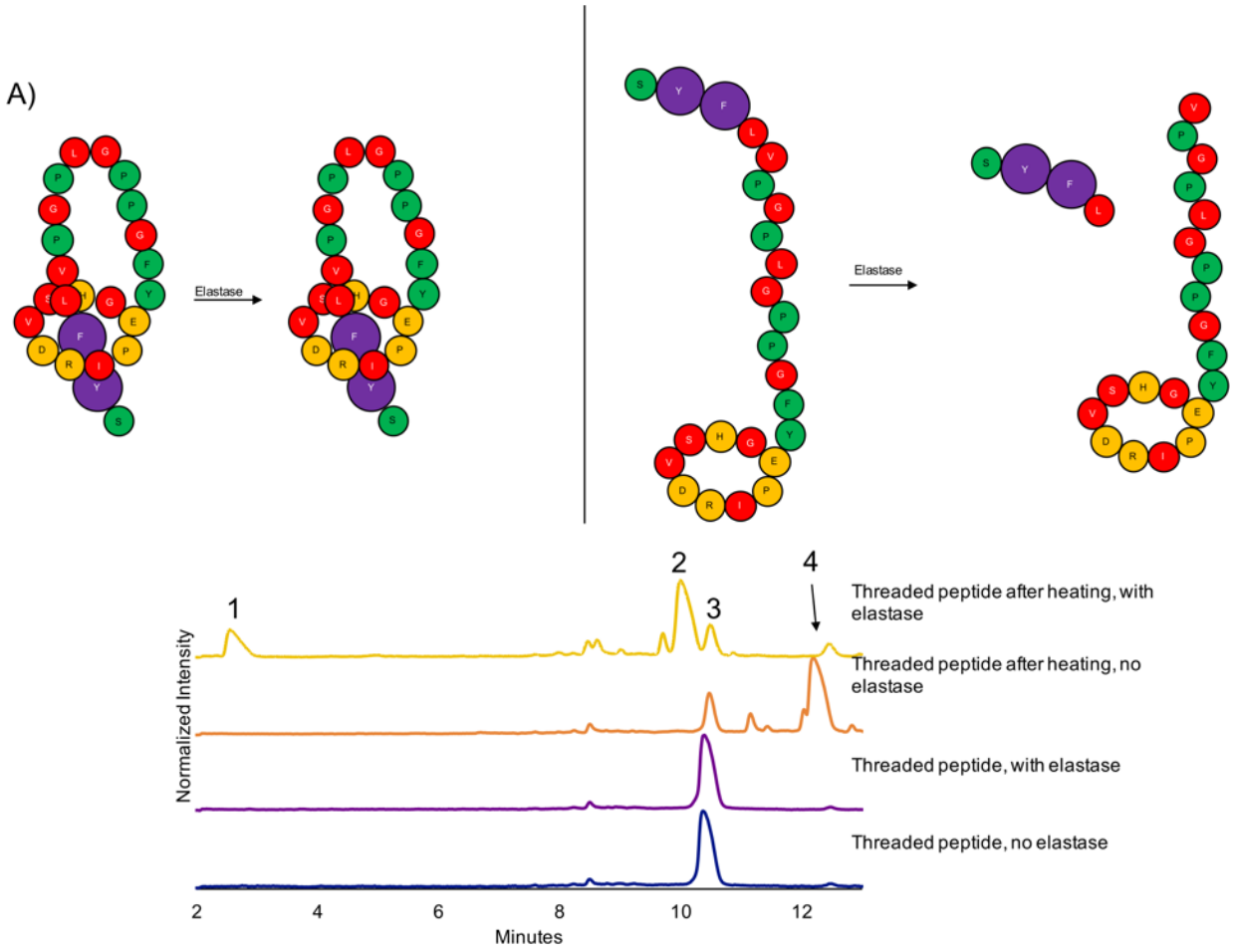
Peak	Predicted Monoisotopic Mass (Da)	Observed Monoisotopic Mass (Da)	Sequence
1	660.32 (+3)	660.29 (+3)	GHSVDRIPEYF + 18 Da
2	660.32 (+3)	660.29 (+3)	GHSVDRIPEYF + 18 Da
3	651.32 (+3)	651.29 (+3)	GHSVDRIPEYF
4	525.80 (+3)	525.78 (+3)	GPPGLPGPVLF
5	1292.15 (+2)	1292.08 (+2)	GHSVDRIPEYFGPPGLPGPVLFYS (threaded)
6	607.33 (+3)	607.30 (+3)	GPPGLPGPVLFY
7	599.34 (+3)	599.31 (+3)	FGPPGLPGPVLF
8	1292.15 (+2)	1292.11 (+2)	GHSVDRIPEYFGPPGLPGPVLFYS (unthreaded)

Figure S14. Threaded cloacaenodin is resistant to chymotrypsin digestion while unthreaded cloacaenodin can be cleaved by chymotrypsin. A) Cartoon depicting major products of threaded and unthreaded cloacaenodin upon chymotrypsin treatment. Ring residues are orange and loop/tail residues are green. Residues that are preferentially susceptible to chymotrypsin digestion are in red. B) LC-MS data of chymotrypsin proteolysis experiment on threaded and unthreaded cloacaenodin, with a table of observed masses and proposed sequence. For each value, the charge state is shown in parentheses.



Peak	Predicted Monoisotopic Mass (Da)	Observed Monoisotopic Mass (Da)	Sequence
1	577.78 (+2)	577.79 (+2)	GHSVDRIPEY
2	937.51 (+1)	937.52 (+1)	FGPPGLPGPV
3	1050.60 (+1)	1050.61 (+1)	FGPPGLPGPVL
4	861.77 (+3)	861.78 (+3)	GHSVDRIPEYFGPPGLPGPVLFYS (threaded)
5	1197.67 (+1)	1197.68 (+1)	FGPPGLPGPVLF
6	1447.76 (+1)	1447.77 (+1)	FGPPGLPGPVLFYS
7	861.78 (+3)	861.78 (+3)	GHSVDRIPEYFGPPGLPGPVLFYS (unthreaded)

Figure S15. Threaded cloacaenodin is resistant to thermolysin digestion while unthreaded cloacaenodin can be cleaved by thermolysin. A) Cartoon depicting major products of threaded and unthreaded cloacaenodin upon thermolysin treatment. Ring residues are orange, loop/tail residues are green, and steric lock residues are purple. Residues that are preferentially susceptible to thermolysin digestion are in red. B) LC-MS data of thermolysin proteolysis experiment on threaded and unthreaded cloacaenodin, with a table of observed masses and proposed sequence. For each value, the charge state is shown in parentheses.



Peak	Predicted Monoisotopic Mass (Da)	Observed Monoisotopic Mass (Da)	Sequence
1	529.27 (+1)	529.27 (+1)	LFYS
2	1037.03 (+2)	1037.04 (+2)	GHSVDRIPEYFGPPGLPGPV
3	861.77 (+3)	861.77 (+3)	GHSVDRIPEYFGPPGLPGPVLFYS (threaded)
4	861.77 (+3)	861.77 (+3)	GHSVDRIPEYFGPPGLPGPVLFYS (unthreaded)

Figure S16. Threaded cloacaenodin is resistant to elastase digestion while unthreaded cloacaenodin can be cleaved by elastase. A) Cartoon depicting major products of threaded and unthreaded cloacaenodin upon elastase treatment. Cartoon depicting major products of threaded and unthreaded cloacaenodin upon elastase treatment. Ring residues are orange, loop/tail residues are green, and steric lock residues are purple. Residues that are preferentially susceptible to elastase digestion are in red. B) LC-MS data of elastase proteolysis experiment on threaded and unthreaded cloacaenodin, with a table of observed masses and proposed sequence. For each value, the charge state is shown in parentheses.

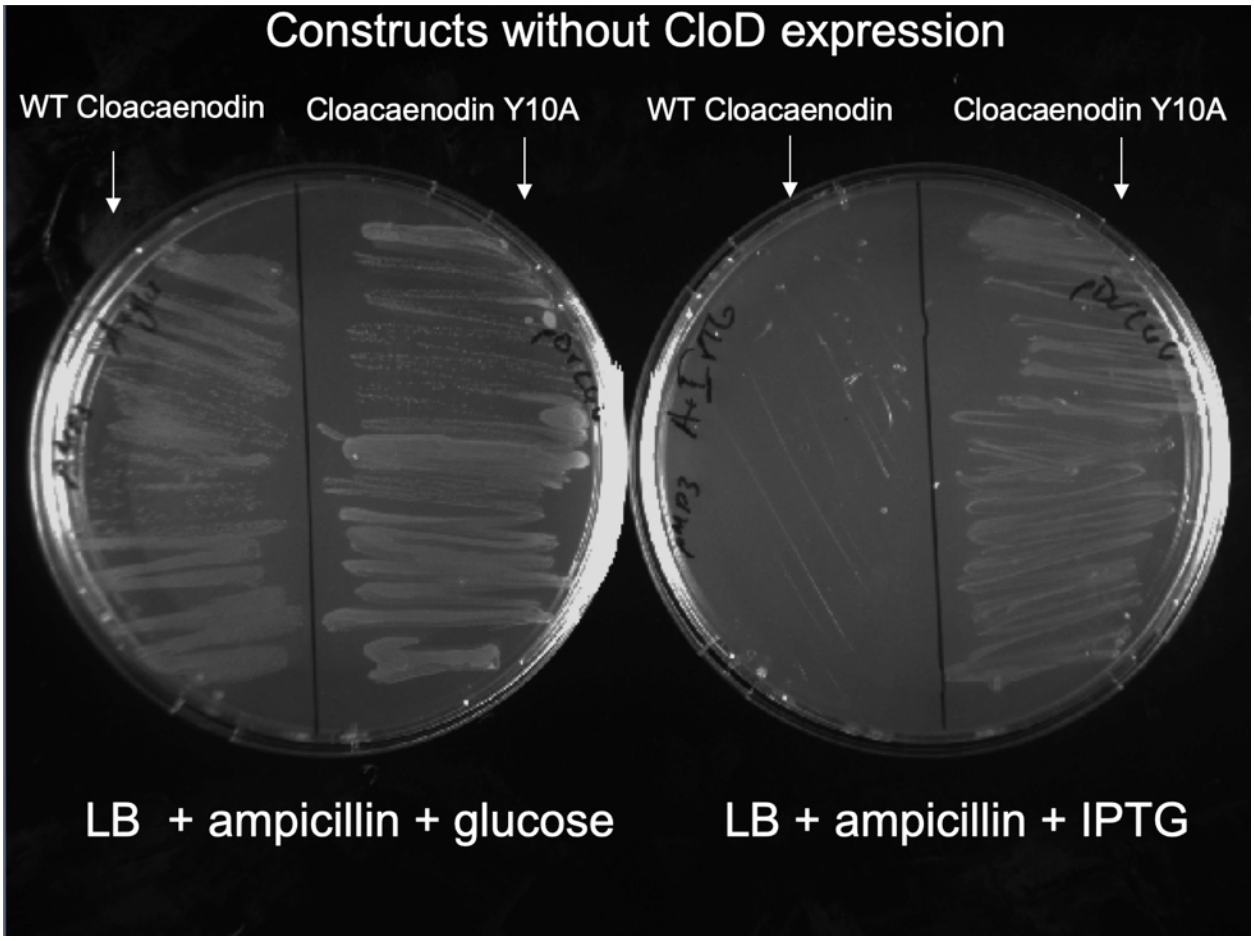


Figure S17. When the *cloD* gene is deleted from the cloacaenodin expression plasmid for *E. coli*, *E. coli* cells can no longer grow in media that induces expression of cloacaenodin. Cloacaenodin Y10A is not toxic intracellularly to *E. coli*.
On left plate: Grown on media with glucose to repress expression of pQE-80 derived plasmid, *E. coli* cells containing expression plasmid for wild-type cloacaenodin without CloD (left) and *E. coli* cells containing expression plasmid cloacaenodin Y10A without CloD (right).
On right plate: Grown on media with IPTG to induce expression of pQE-80 derived plasmid, *E. coli* cells containing expression plasmid for wild-type cloacaenodin without CloD (left) and *E. coli* cells containing expression plasmid cloacaenodin Y10A without CloD (right).

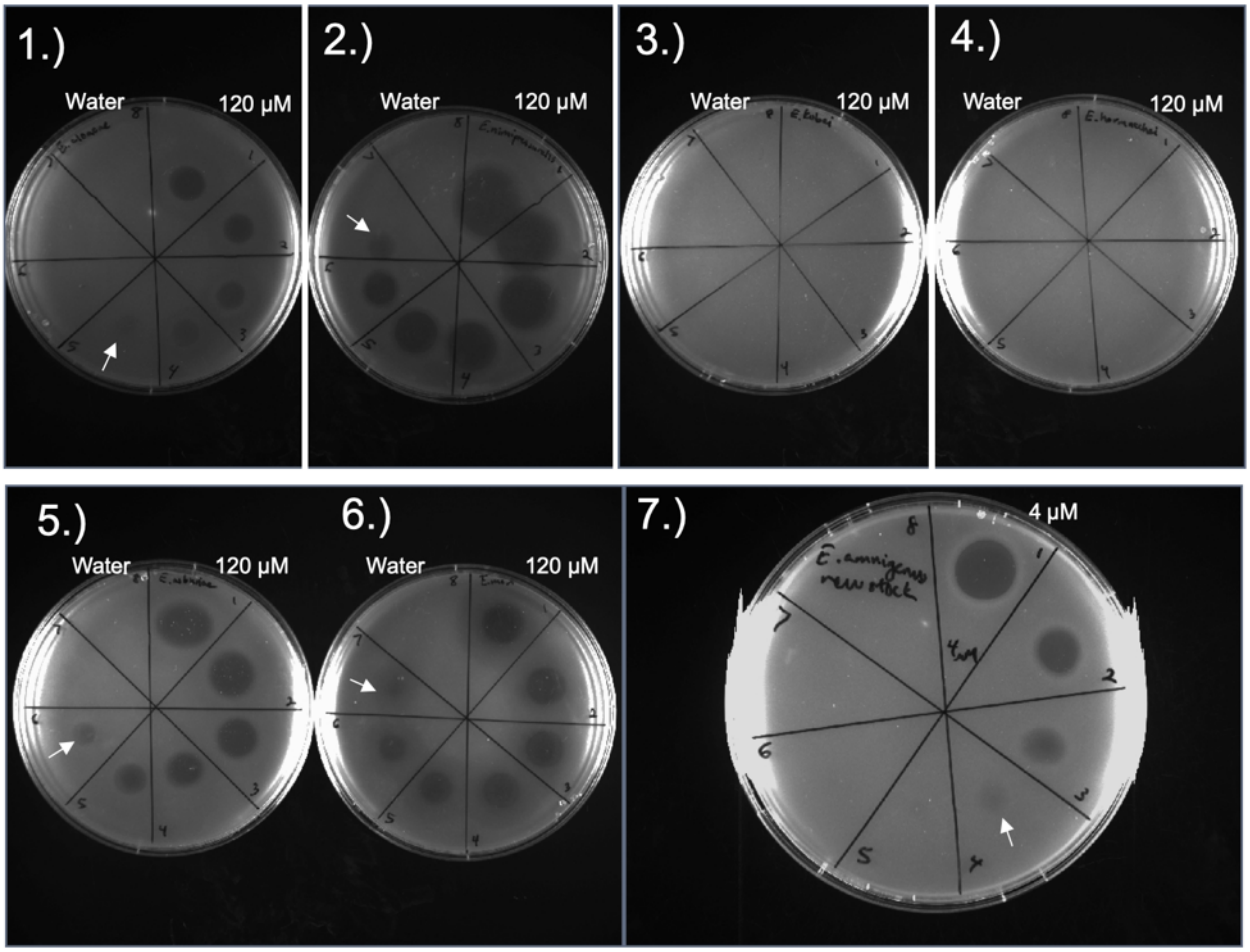


Figure S18A. Spot-on-lawn assays in M63 agar of cloacaenodin against commercially acquired *Enterobacter* species. 1.) *Enterobacter cloacae* ATCC 13047 2.) *Enterobacter nimipressuralis* DSM 18955 3.) *Enterobacter kobei* BAA-260 4.) *Enterobacter hormaechei* ATCC 700323 5.) *Enterobacter asburiae* DSM 17506 6.) *Enterobacter mori* DSM 26271 7.) *Enterobacter amnigenus* ATCC 33072.

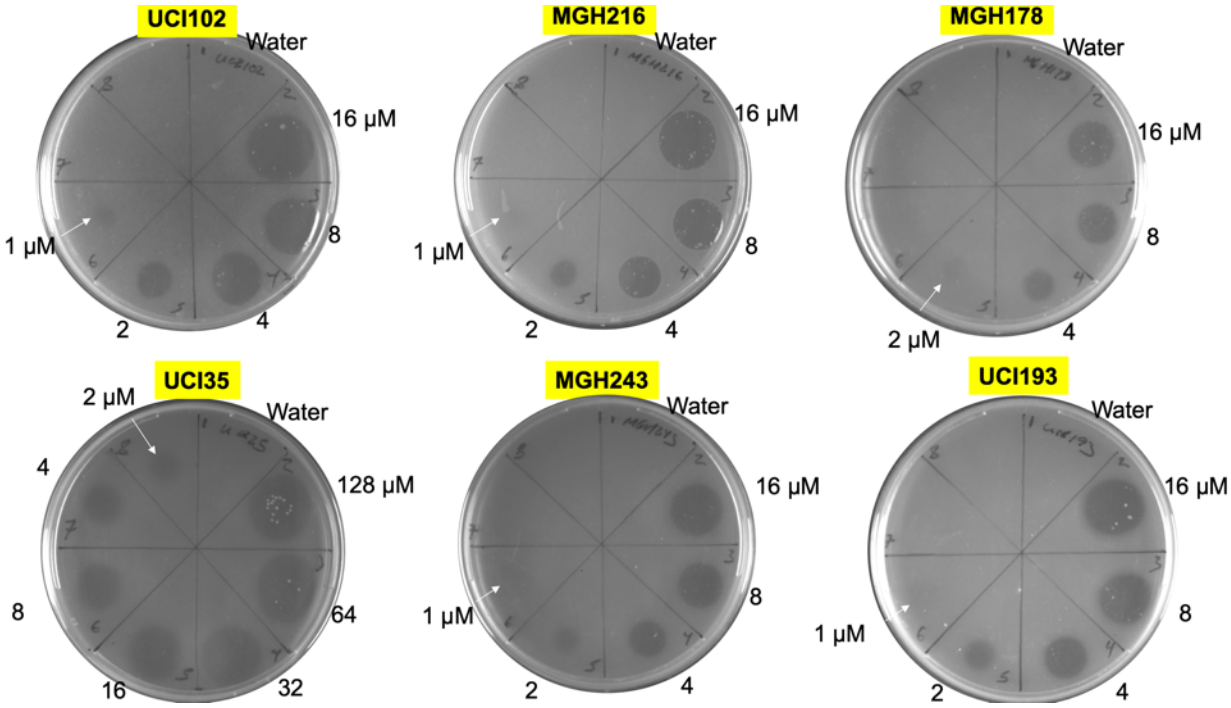


Figure S18B. Selected spot-on-lawn assays in M63 agar of cloacaenodin against clinical isolates of *Enterobacter* species (full list of tested strains in Table S4B). These strains consistently showed low micromolar MICs in the assay. UCI35, MGH243, and UCI193 are classified as carbapenem resistant.

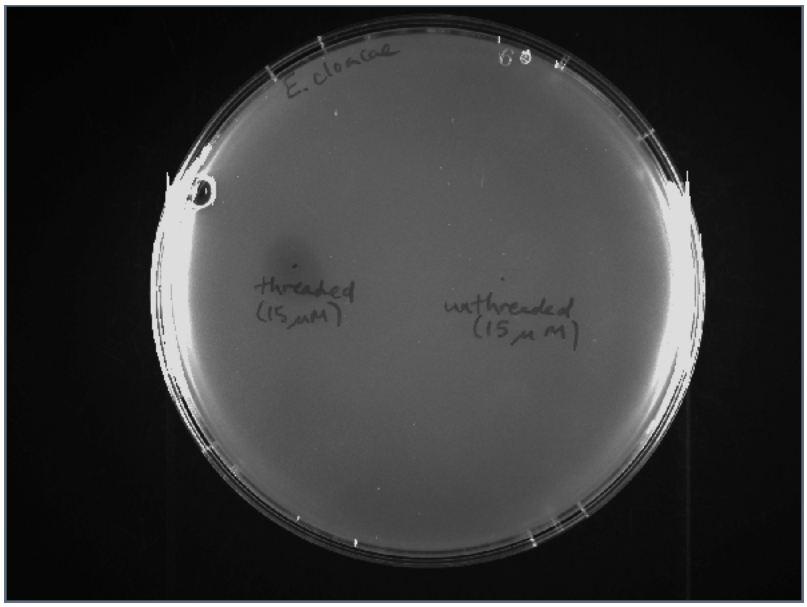


Figure S19. At a concentration of 15 μM, only threaded cloacaenodin has activity against *E. cloacae.* Unthreaded cloacaenodin has no activity at this concentration against *E. cloacae*.

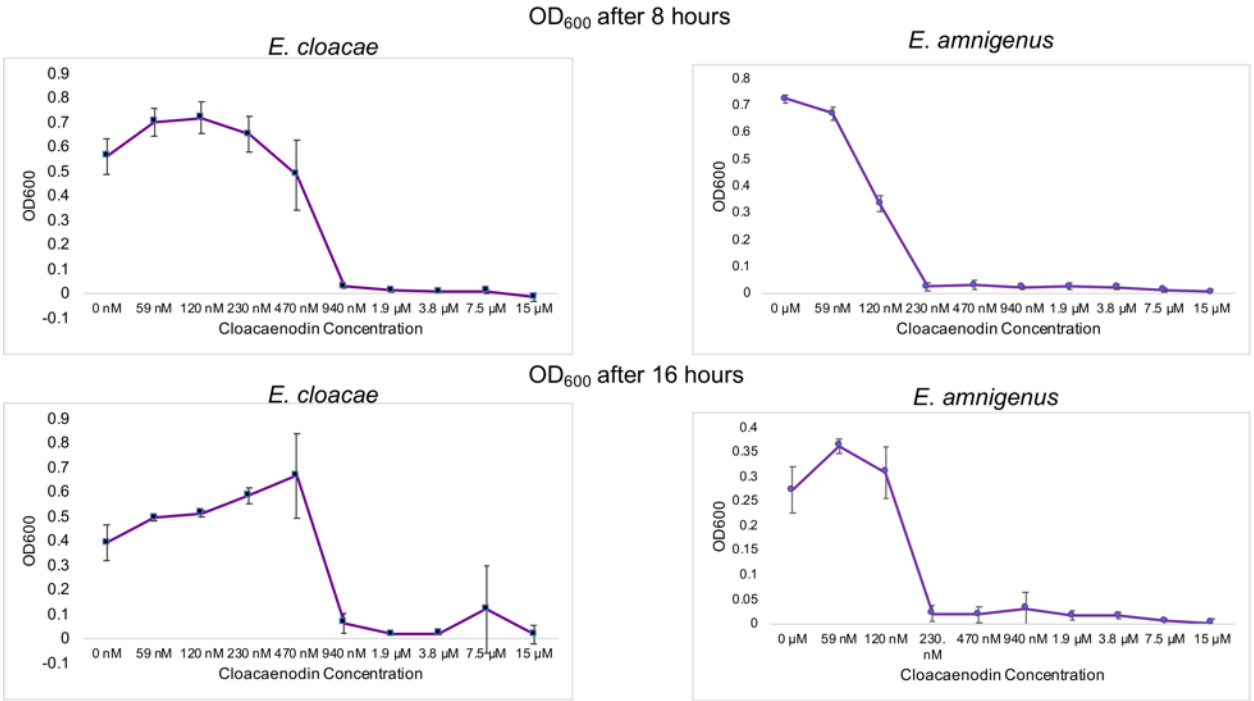


Figure S20. Liquid inhibition assays in M63 media of cloacaenodin activity against *E. cloacae* and *E. amnigenus.*

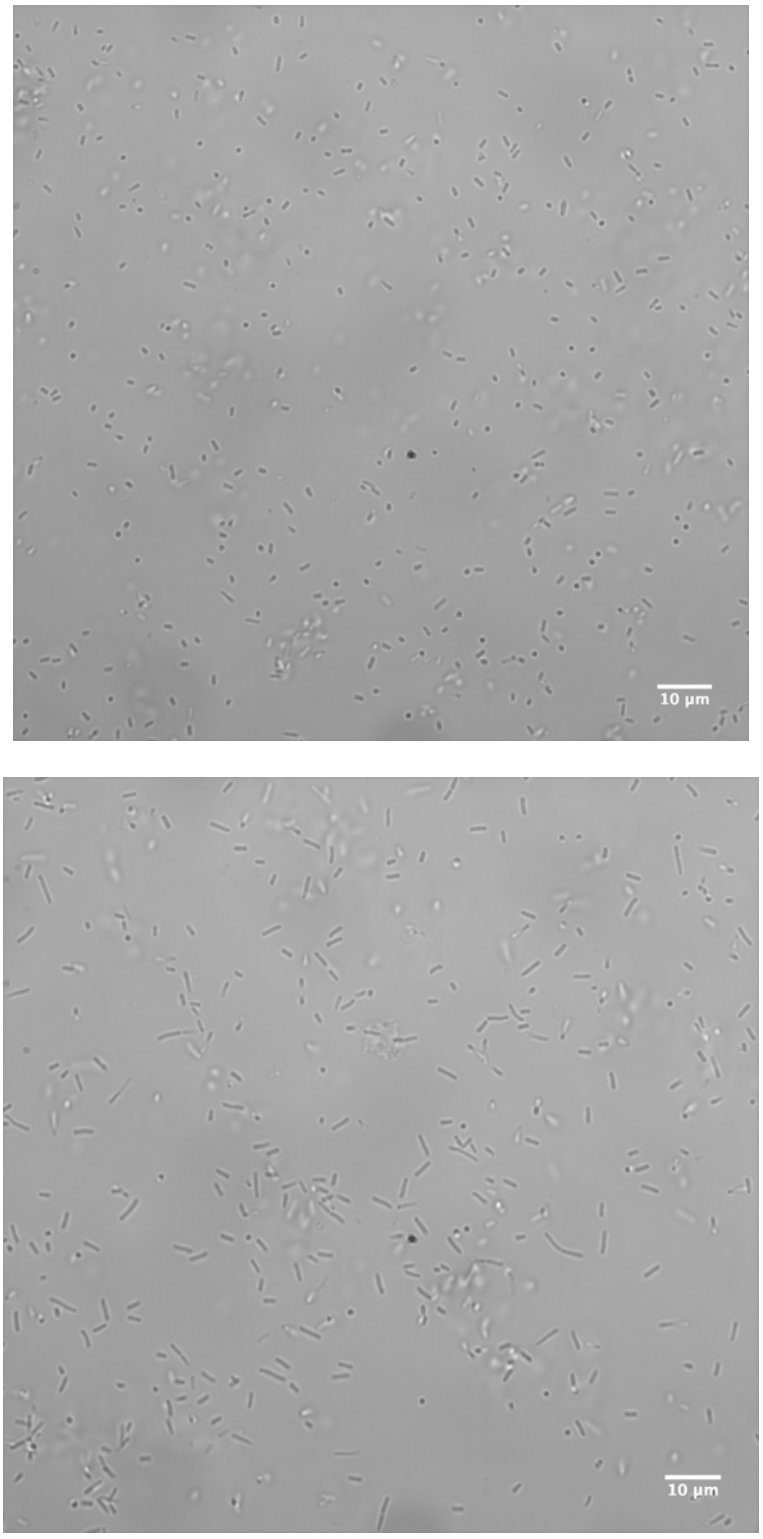


Figure S21. When treated with sub-MIC values of cloacaenodin, *E. cloacae* exhibits a filamentation phenotype. Top: Untreated *E. cloacae*, grown for 16 hours in M63 media. Bottom: *E. cloacae* treated with 230 nM of cloacaenodin and grown for 16 hours in M63 media.

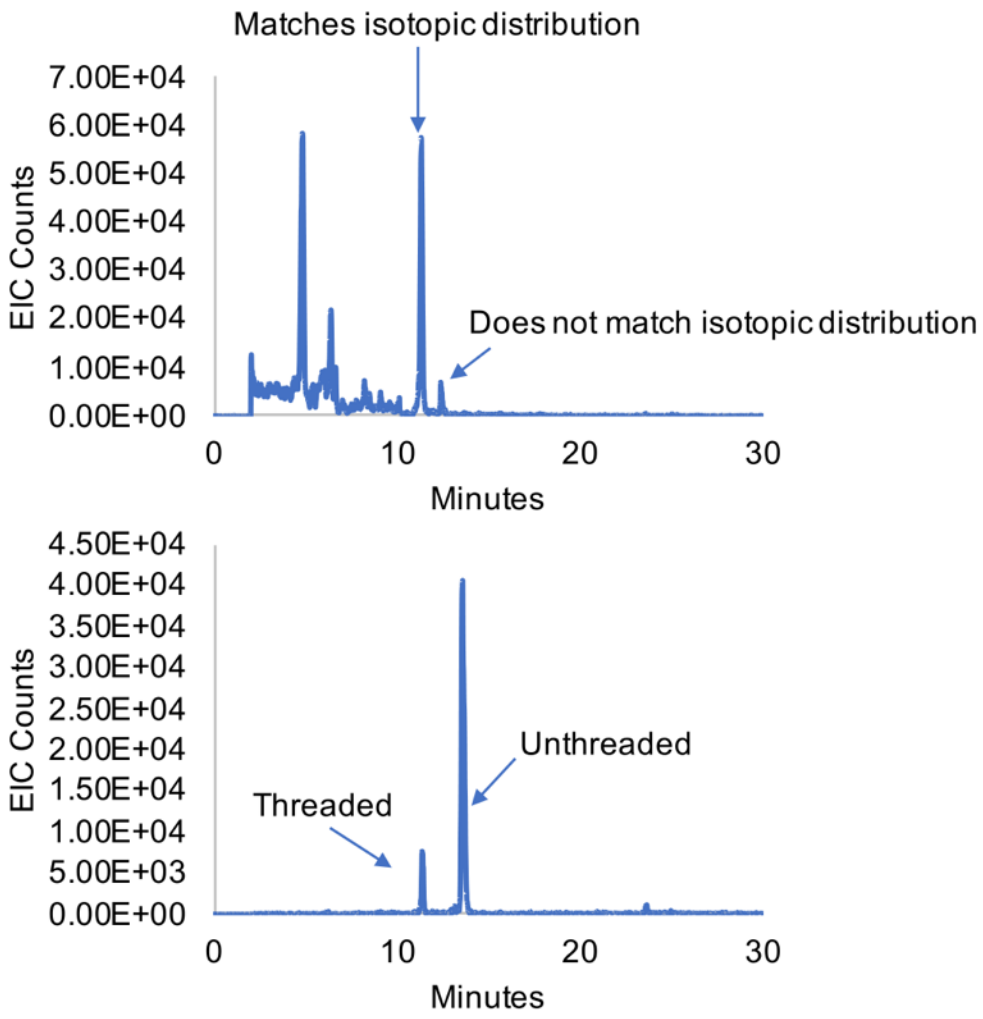
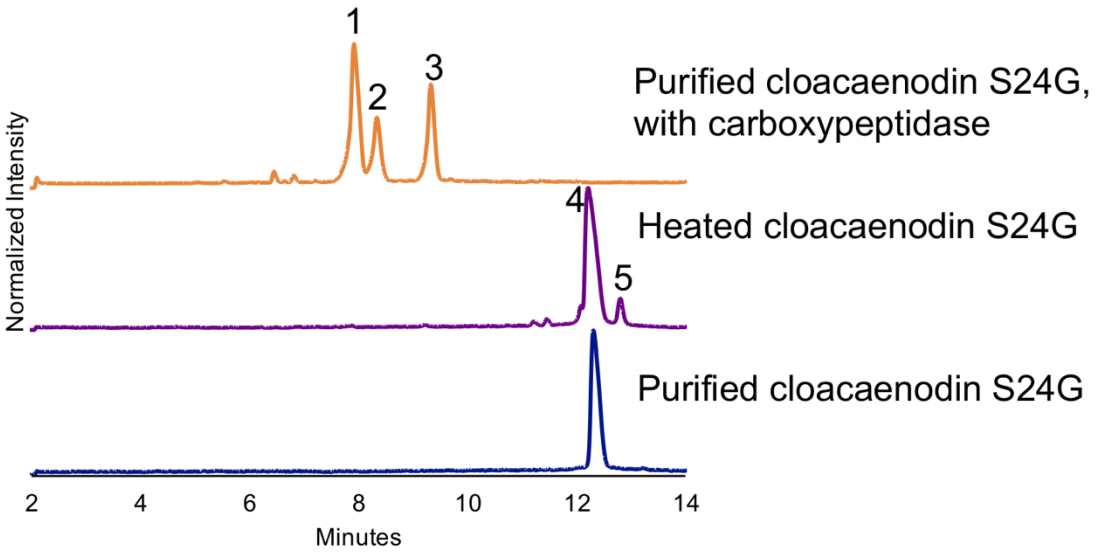


Figure S22. Changing of the lower steric lock (Tyr23) to a bulkier Trp residue leads to only threaded cloacaenodin Y23W observed in the supernatant extract. Top: EIC of supernatant extract of cloacaenodin Y23W, extracted for the expected monoisotopic +2 and +3 charge states (1303.6632 Da and 869.4445 Da, respectively). Bottom: EIC of cloacaenodin Y23W in an HPLC fraction and heated at 95 °C. This was done to verify that the peak at around 11.5 minutes in the supernatant extract (top trace) was threaded. The peak representing unthreaded cloacaenodin Y23W (bottom trace) was not observed In the supernatant extract (top trace).



Peak	Observed Monoisotopic Mass (Da)	Expected Monoisotopic Mass (Da)	Sequence
1	537.27 (+3)	537.26 (+3)	GHSVDRIPEYFGPPG
2	518.26 (+3)	518.26 (+3)	GHSVDRIPEYFGPP
3	626.32 (+3)	626.32 (+3)	GHSVDRIPEYFGPPGPGLPG
4	851.76 (+3)	851.76 (+3)	GHSVDRIPEYFGPPGLPGPVLFYG (unthreaded)
5	845.76 (+3)	845.76 (+3)	GHSVDRIPEYFGPPGLPGPVLFYG (unthreaded) – 18 Da

Figure S23. After expression and purification of the peptide, the major cloacaenodin S24G product was unthreaded. This is supported by the later retention time compared to wild-type cloacaenodin, a lack of shift in retention time upon heating, and susceptibility to carboxypeptidase. The dehydrated product **5** is likely due to Asp dehydration in the ring.

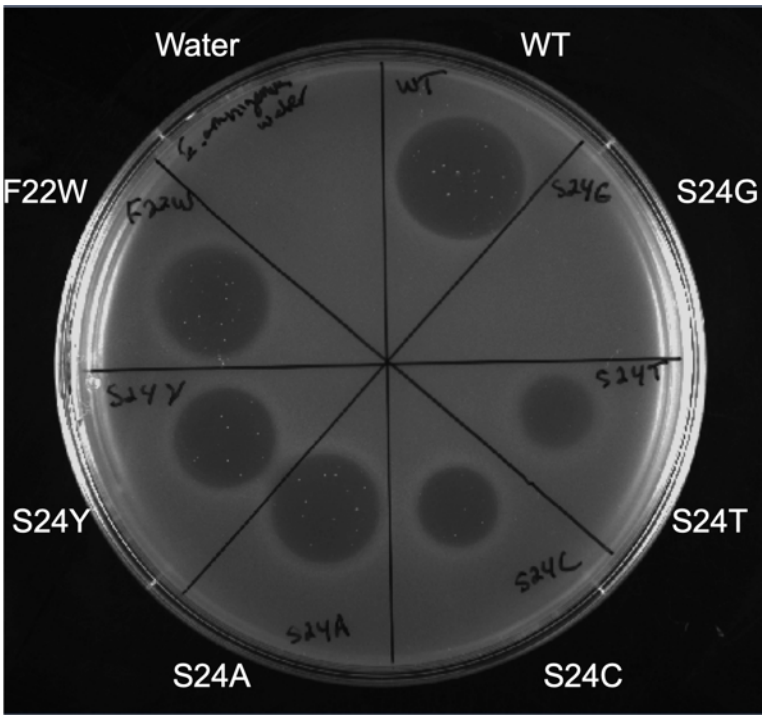


Figure S24. Bioactivity of cloacaenodin variants against *E. amnigenus.* Peptides were tested at a concentration of 10 μM. Based on inhibition diameter, all variants appear to have similar or reduced activity compared to wild-type cloacaenodin.

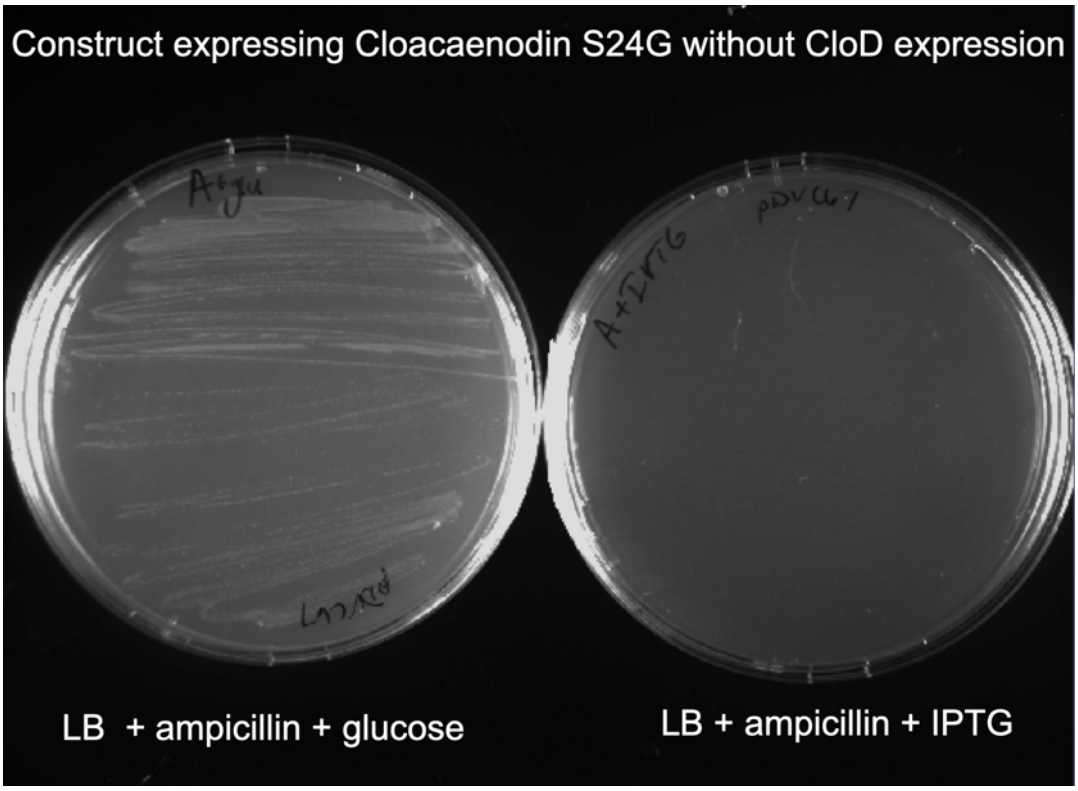


Figure S25. Despite being mostly unthreaded after purification, cloacaenodin S24G exerts intracellular toxicity in *E. coli* XL-1 blue cells when expression is induced with IPTG in a construct lacking *cloD*. This result indicates that at least some threaded peptide exists intracellularly to exert antimicrobial activity.

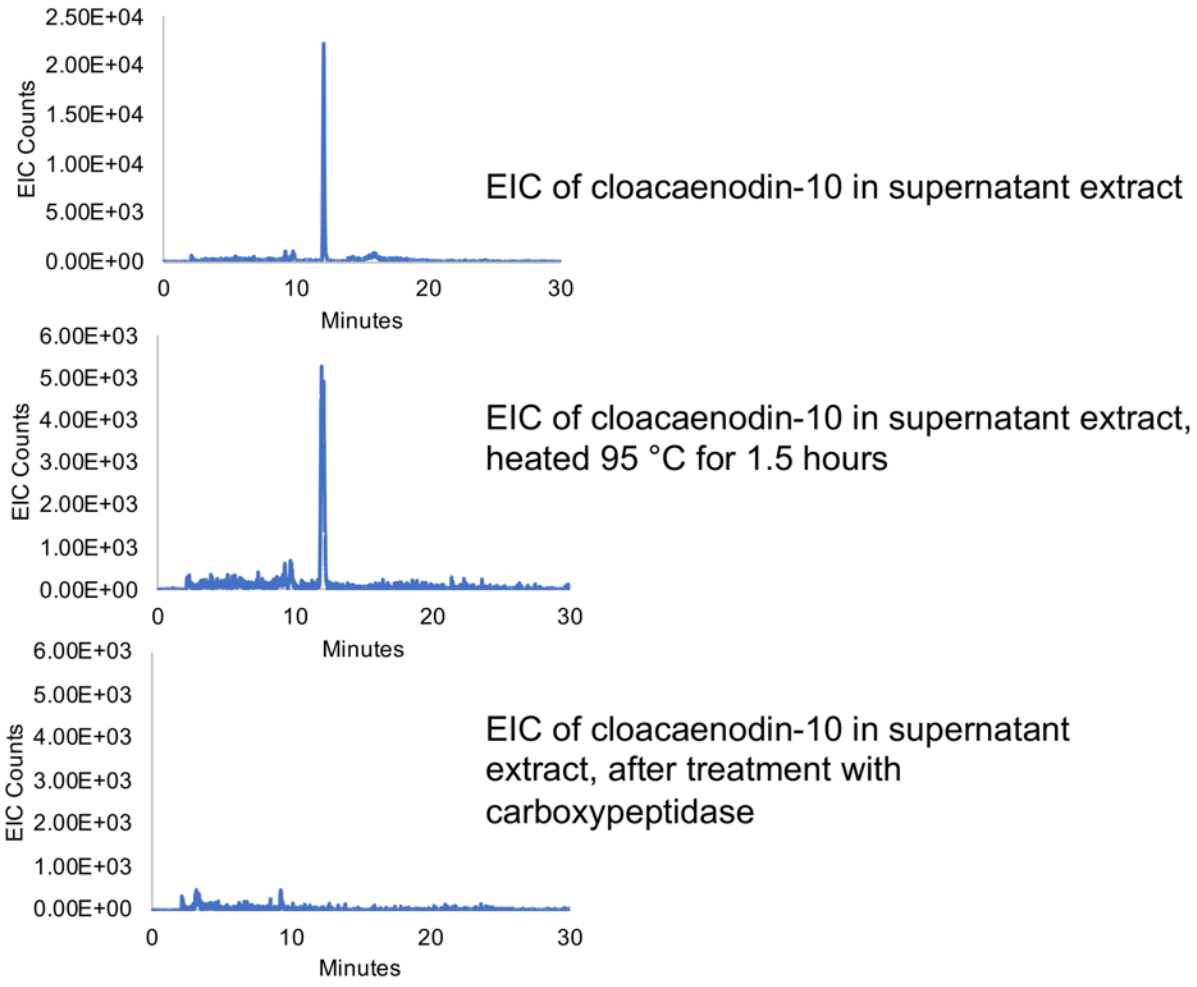


Figure S26. A variant of cloacaenodin with a 10-membered ring can be biosynthesized and is detected in the supernatant. Top: EIC of cloacaenodin-10 in the supernatant extract. Middle: EIC of cloacaenodin-10 in supernatant extract after heating. The retention time does not shift. Bottom: EIC of cloacaenodin-10 in supernatant extract after treatment with carboxypeptidase. The peak detected at 12.1 minutes is no longer detected, suggesting that it was susceptible to degradation by carboxypeptidase. We extracted for the predicted monoisotopic charge states of a +3 m/z of 885.4516 and a +2 m/z of 1327.6737.

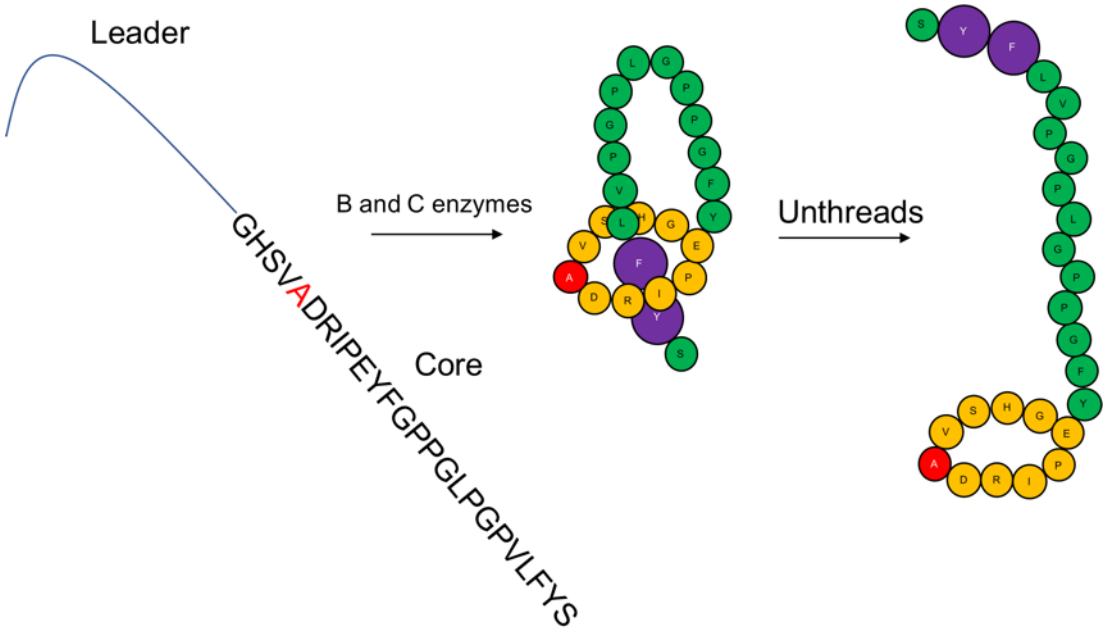


Figure S27. Cartoon depicting proposed biosynthesis of cloacaenodin with a 10-membered ring. We hypothesize that the lasso peptide variant is made threaded and then unthreads upon secretion into the supernatant and during purification.

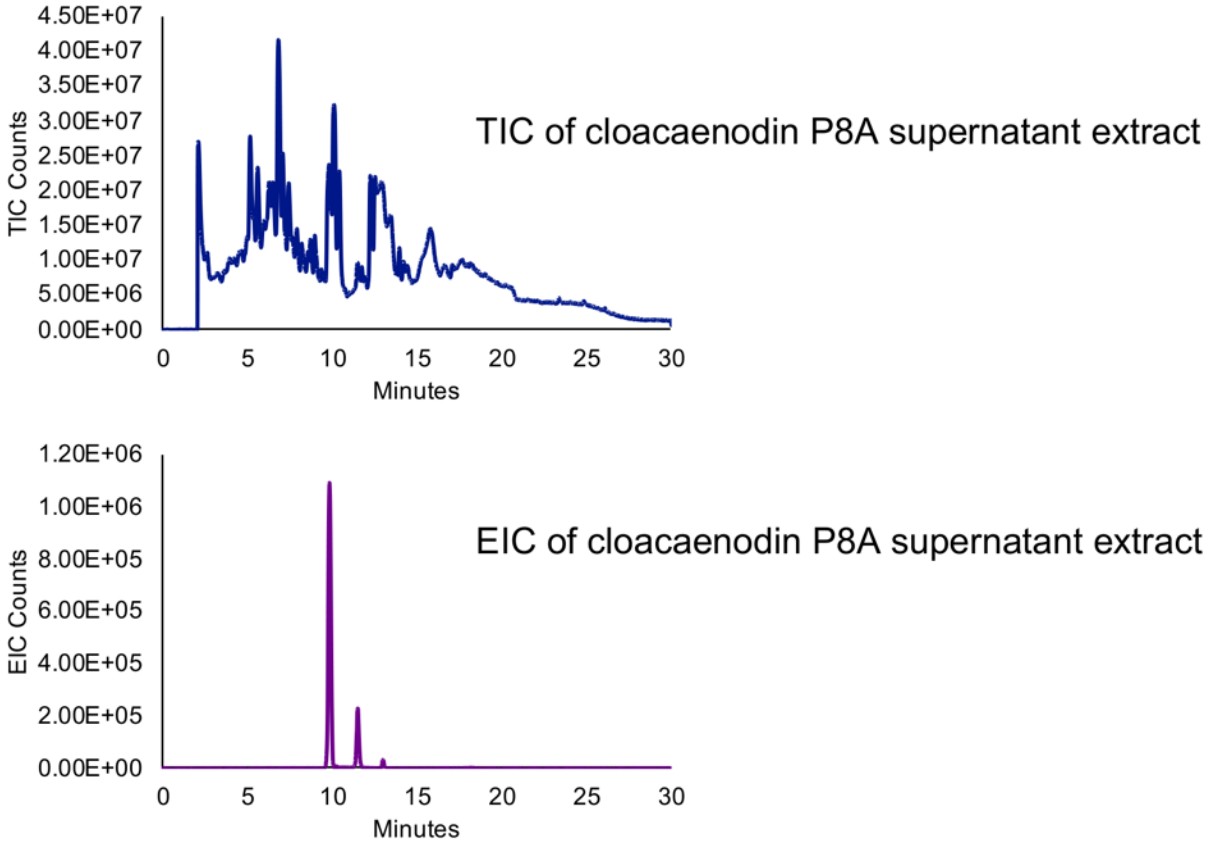


Figure S28. TIC (top trace) and EIC (bottom trace) chromatograms of a cloacaenodin P8A variant from supernatant extract. We extracted for the predicted monoisotopic charge states of a +3 m/z of 853.1007 and a +2 m/z of 1279.1473.

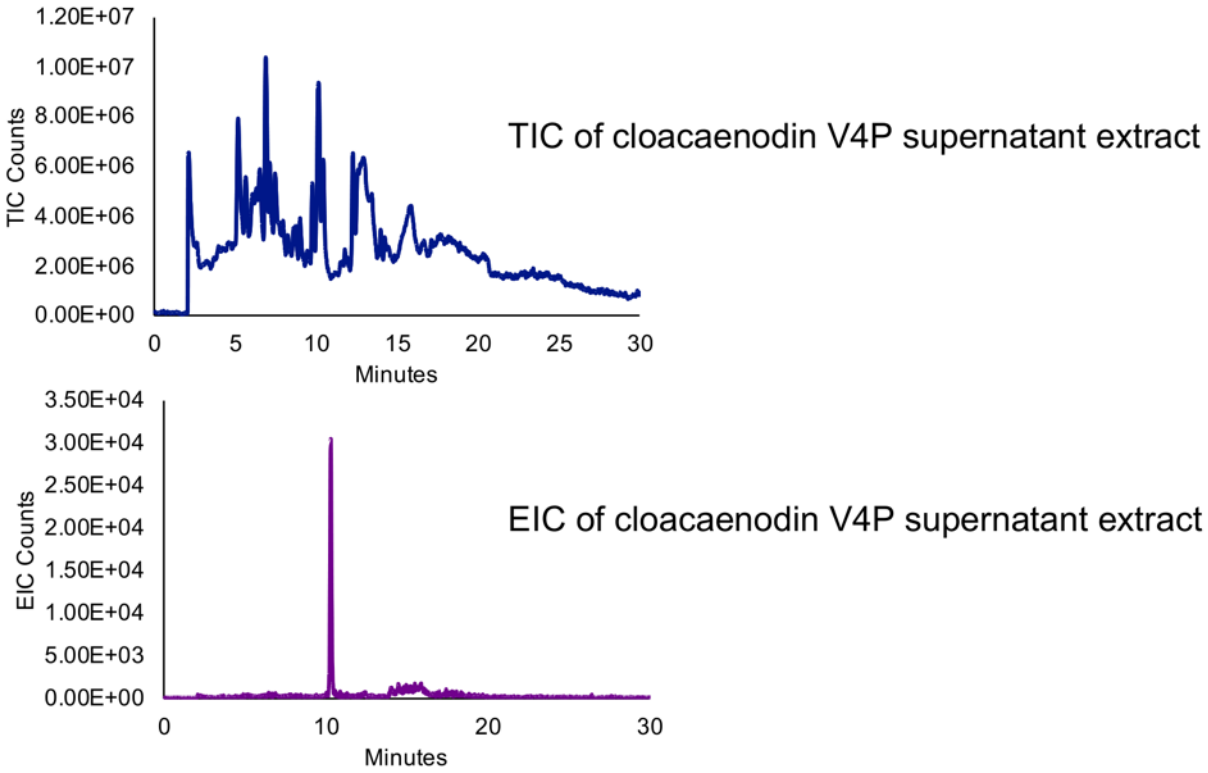


Figure S29. TIC (top trace) and EIC (bottom trace) chromatograms of a cloacaenodin V4P variant from supernatant extract. We extracted for the predicted monoisotopic charge states of a +3 m/z of 861.1007 and a +2 m/z of 1291.1473.

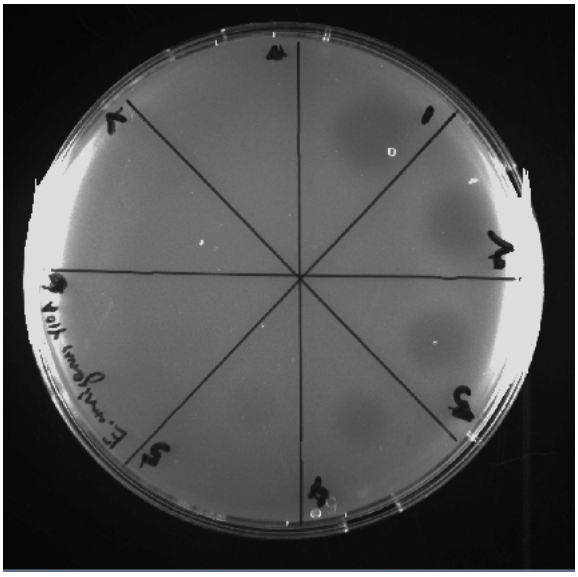


Figure S30. Purified cloacaenodin Y10A has drastically reduced bioactivity against *E. amnigenus*. Spot 1 is 120 μM, and two-fold serial dilutions were spotted clockwise. Similar data for the wild-type peptide is shown in Figure S18A.

Table S1. B proteins found near cloacaenodin-like *A* genes

Species	Accession	Percent Identity
E. hormaechei LB3, *E. cloacae* B2, *E. kobei* 15727	WP_046889164.1	100
Enterobacter mori strain 08-091 (plasmid pHS08-091)	WP_215231253.1	91.63
Enterobacter hormaechei subsp. *xiangfiangensis* strain 120070	MBT1945799.1	91.63
E. hormaechei subsp. *xiangfangensis* strain ECC296	HAV1851688.1	91.63
Enterobacter ludwigii strain I140	WP_104440573.1 and MBK1520793.1	99.53
Clinical sample of *Salmonella enterica* subsp. *enterica* serovar Urbana	EEJ6141376.1	79.53
Environmental sample of *Salmonella enterica*	EAM2342716.1	80.00
E. cloacae strain N29 35 and strain MDR0071, e483	WP_194517084.1 (strain N29 35) and WP_063955191.1 (strain MDR0071, e483)	79.07 (strain N29 35) and 78.60 (strain MDR0071, e483)
Clinical sample of *Salmonella enterica* subsp. *enterica* serovar Urbana	EEJ6141366.1	77.21
Environmental sample of *E. coli* (strain PSU-1444)	*B* disrupted	*B* disrupted
E. coli E22	*B* disrupted	*B* disrupted
Citrobacter sp. CtB7.12	WP_054177363.1	53.95

Table S2. C proteins found near cloacaenodin-like *A* genes

Species	Accession	Percent Identity
E. hormaechei LB3, *E. cloacae* B2, *E. kobei* 15727	WP_046889163.1	100
Enterobacter mori strain 08-091 (plasmid pHS08-091)	WP_215231254.1	86.19
Enterobacter hormaechei subsp. *xiangfiangensis* strain 120070	MBT1945800.1	86.19
E. hormaechei subsp. *xiangfangensis* strain ECC296	HAV1851689.1	86.19
Enterobacter ludwigii strain I140	WP_185791751.1 and MBK1520792.1	99.25
Clinical sample of *Salmonella enterica* subsp. *enterica* serovar Urbana	EEJ6141377.1	72.05
Environmental sample of *Salmonella enterica*	EAM2342717.1	72.42
E. cloacae strain N29 35 and strain MDR0071, e483	WP_194517083.1 (strain N29 35) and WP_063955193.1 (strain MDR0071, e483)	72.98 (strain N29 35) and 73.17 (strain MDR0071, e483)
Clinical sample of *Salmonella enterica* subsp. *enterica* serovar Urbana	EEJ6141367.1	72.04
Environmental sample of *E. coli* (strain PSU-1444)	*C* disrupted	*C* disrupted
E. coli E22	*C* disrupted	*C* disrupted
Citrobacter sp. CtB7.12	WP_054177364.1	52.91

Table S3. D proteins found near cloacaenodin-like *A* genes

Species	Accession	Percent Identity
E. hormaechei LB3, *E. cloacae* B2, *E. kobei* 15727	WP_072057270.1	100
Enterobacter mori strain 08-091 (plasmid pHS08-091)	WP_215231255.1	94.64
Enterobacter hormaechei subsp. *xiangfiangensis* strain 120070	MBT1945801.1	94.29
E. hormaechei subsp. *xiangfangensis* strain ECC296	HAV1851690.1	94.29
Enterobacter ludwigii strain I140	WP_185791750.1 and MBK1520791.1	99.83
Clinical sample of *Salmonella enterica* subsp. *enterica* serovar Urbana	EEJ6141378.1	85.84
Environmental sample of *Salmonella enterica*	EAM2342718.1	85.15
E. cloacae strain N29 35 and strain MDR0071, e483	WP_194517082.1 (strain N29 35) (sequence cut short) and WP_080471846.1 (strain MDR0071, e483)	85.49 (strain MDR0071, e483)
Clinical sample of *Salmonella enterica* subsp. *enterica* serovar Urbana	EEJ6141368.1	85.15
Environmental sample of *E. coli* (strain PSU-1444)	EFN8342409.1	84.80
E. coli E22	WP_001299236.1	84.80
Citrobacter sp. CtB7.12	WP_054177365.1	70.93

Table S4A. Commercial strains or laboratory strains tested for cloacaenodin activity. An asterisk indicates that the strain was susceptible to cloacaenodin.

Strain
* *Enterobacter cloacae* subsp. *cloacae* ATCC 13047
Klebsiella aerogenes ATCC 13048
Enterobacter hormaechei ATCC 700323
* *Enterobacter asburiae* DSM 17506
* *Enterobacter mori* DSM 26271
Enterobacter kobei BAA-260
* *Lelliottia nimipressuralis* DSM 18955
* *Enterobacter amnigenus* ATCC 33072
Escherichia coli MG1655
Bacillus subtilis 168
Salmonella enterica sp. *enterica* serovar Newport

Table S4B. Clinical strains tested for cloacaenodin activity. Strains designated with BIDMC were isolated at Beth Israel Deaconess Medical Center in Boston, MA. Strains designated with BWH were isolated at Brigham and Women's Hospital in Boston, MA. Strains designated with UCI were isolated at University of California in Irvine, CA. Strains designated with MGH were isolated at Massachusetts General Hospital in Boston, MA. The information displayed can also be obtained from Reference 67 in the main text. AR0154 was obtained from the Antibiotic Resistance Bank from the U.S. Centers for Disease Control. An asterisk indicates that the strain was susceptible to cloacaenodin.

Strain ID	Species	Isolation source	Meropenem MIC (μg/mL)	Classified as Carbapenem Resistant?
AR0154	*Enterobacter cloacae*	N/A	N/A	Yes
BIDMC120	*Enterobacter cloacae*	Foot ulcer	4	Yes
BIDMC87	*Enterobacter hormaechei*	Blood culture	16	Yes
BWH39	*Enterobacter hormaechei*	Urine	2	Yes
MGH3	*Enterobacter cloacae*	Respiratory	≥16	Yes
* UCI35	*Enterobacter hormaechei*	Surgical Procedure Specimen	4	Yes
* MGH178	*Enterobacter kobei*	Wound	≤0.25	No
* MGH216	*Enterobacter ludwigii*	Urine	≤0.25	No
MGH239	*Enterobacter roggenkampii*	Urine	1	Intermediate
* MGH243	*Enterobacter asburiae*	Tissue Wound	16	Yes
* UCI102	*Enterobacter cloacae*	Urine	0.063	No
* UCI193	*Enterobacter asburiae*	Sputum	23	Yes

Table S5. Primer sequences used in this study. The sequences shown are from the 5' end to 3' end.

Primer Description	Sequence
Cloacae forward	CGATCGTGAATTCATTAAAGAGGAGAAATTAACTATGGATG
Cloacae Y23W reverse	CGATGCAAGCTTTTAGCTCCAAAACAGGACTG
Cloacaa F22W reverse	CGATGCAAGCTTTTAGCTATACCACAGGACTGG
Cloacae S24G reverse	TGACGAAGCTTTTAGCCATAAAACAGGACT
Cloacae S24T reverse	ACAGAAAAGCTTTTAGGTATAAAACAGGACTGG
Cloacae S24C reverse	AAGCAAAAGCTTTTAGCAATAAAACAGGACTG
Cloacae S24A reverse	AAGCAAAAGCTTTTAGGCATAAAACAGGACTGGA
Cloacae S24Y reverse	ACAGAAAAGCTTTTAATAATAAAACAGGACTGGAC
Cloacae S24W reverse	ACAGAAAAGCTTTTACCAATAAAACAGGACTGG
Cloacae S24F reverse	ACAGAAAAGCTTTTAAAAATAAAACAGGACTGG
Cloacae reverse	GCTAATTAAGCTTTTAGCTATAAAACAGGAC
Cloacae BGC D removal forward	ACGATGGATCCGTCGTGAGG
Cloacae BGC D removal reverse	GGCATACCATGGTTAAACAAACTCAAAAAATTCATGCCTTG
Cloacae G1A internal reverse	ACGCTATGGGCACGTGTAATTTTGCTCGCTTTC
Cloacae G1A internal forward	CGTGCCCATAGCGTGGATCGTATTC
Cloacae E9D internal reverse	GTAGTCCGGAATACGATCCACGC
Cloacae E9D internal forward	GGATCGTATTCCGGACTACTTTGGCCCTCCGG
Cloacae Y10A internal reverse	GAGGGCCAAAGGCCTCCGGAATACGATCCAC
Cloacae Y10A internal forward	GGAGGCCTTTGGCCCTCCGG
Cloacae P8A internal reverse	CCGCAATACGATCCACGCTATGG
Cloacae P8A internal forward	CGTGGATCGTATTGCGGAGTACTTTGGCCCTC
Cloacae V4P internal reverse	CGATCCGGGCTATGGCCACGTGTAATTTTG
Cloacae V4P internal forward	CCATAGCCCGGATCGTATTCCGGAGTAC
Cloacae 10-member ring internal reverse	AATACGATCCGCCACGCTATGGCCACG
Cloacae 10-member ring internal forward	CGTGGCGGATCGTATTCCGGAGTACT

Table S6. Plasmids used in this study.

Plasmid Name	Description	Host vector
pAK2	Refactored cloacaenodin BGC	pQE-80L
pMP1	Cloacaenodin Y23W	pAK2
pMP2	Cloacaenodin F22W	pAK2
pMP3	Cloacaenodin BGC without *D* gene	pAK2
pMP4	Cloacaenodin S24G	pAK2
pMP5	Cloacaenodin S24T	pAK2
pMP6	Cloacaenodin S24C	pAK2
pMP7	Cloacaenodin S24A	pAK2
pMP8	Cloacaenodin S24Y	pAK2
pMP9	Cloacaenodin S24W	pAK2
pMP10	Cloacaenodin S24F	pAK2
pMP11	Cloacaenodin G1A	pAK2
pMP12	Cloacaenodin E9D	pAK2
pMP14	Cloacaenodin Y10A	pAK2
pDVC060	Cloacaenodin P8A	pAK2
pDVC061	Cloacaenodin V4P	pAK2
pDVC062	Cloacaenodin 10-membered ring	pAK2
pDVC066	Cloacaenodin Y10A in cloacaenodin BGC without *D* gene	pMP3
pDVC067	Cloacaenodin S24G in cloacaenodin BGC without *D* gene	pMP3

Table S7. Explicit distance constraints used for cloacaenodin model building in CYANA.

Residue Number	Residue	Atom	Residue Number	Residue	Atom	Distance (Angstroms)
1	Gly	N	9	Glu	CD	1.33
1	Gly	N	9	Glu	OE1	2.26
1	Gly	N	9	Glu	CG	2.42
1	Gly	H	9	Glu	CD	2.06
1	Gly	CA	9	Glu	CD	2.47
1	Gly	H	1	Gly	CA	2.02
1	Gly	CA	9	Glu	OE1	2.82
1	Gly	H	9	Glu	OE1	3.15

Table S8. Chemical shift assignments for NMR in water. Both shift assignments are listed for Pro17 and Gly18 but due to ambiguity these were left out in model building. Other shifts are listed for protons that could be unambiguously assigned for input into CYANA.

Residue	Hydrogen Atom	δ (ppm)
Glycine 1	H	6.364
	A1	4.141
	A2	3.222
Histidine 2	H	9.243
	A	4.932
	B2	3.148
	B3	2.960
	D2	7.203
	E1	8.421
Serine 3	H	9.094
	A	4.134
	B	3.697
Valine 4	H	7.542
	A	3.823
	B	1.576
	QG1	0.520
	QG2	0.449
Aspartic Acid 5	H	7.175
	A	4.191
	B2	2.444
	B3	2.139
Arginine 6	H	7.612
	A	3.758
	B2	1.342
	B3	1.231
	D2	2.912
	D3	2.834
	QG	1.529
	E	6.992
Isoleucine 7	H	8.578
	A	4.417
	B	1.023
	G12	0.966
	G13	0.875
	QD	0.872
	QG	0.646
Proline 8	A	3.835
	B2	1.847
	B3	1.718
	G2	
	G3	1.296
	QD	3.477
Glutamic Acid 9	H	8.189
	A	3.688
	QB	1.130
	QG	1.482
Tyrosine 10	H	7.631
	A	4.217
	B2	2.570

	B3	2.513
	QD	6.678
	QE	6.387
Phenylalanine 11	H	7.996
	A	4.250
	B2	2.726
	B3	2.516
	QD	6.504
	QE	6.945
	HZ	
Glycine 12	H	6.945
	QA	3.606
Proline 13	A	4.373
	B2	2.050
	B3	1.717
	G2	
	G3	1.599
	QD	3.265
Proline 14	A	4.171
	B2	2.001
	B3	1.717
	G2	
	G3	1.552
	QD	3.312
Glycine 15	H	8.267
	A2	3.664
	A3	3.477
Leucine 16	H	8.018
	A	4.293
	B2	1.215
	B3	1.145
	QQD	0.504
Proline 17	A	4.115
	B2	1.937/1.834
	B3	1.695/1.595
	QG	1.553/1.346
	QD	3.323/3.324
Glycine 18	H	7.856/8.303
	A2	3.758/3.688
	A3	3.688/3.487
Proline 19	A	4.067
	B2	1.834
	B3	1.595
	G2	
	G3	1.346
	QD	3.172
Valine 20	H	8.123
	A	3.579
	B	1.660
	QG1	0.594
	QG2	0.381
Leucine 21	H	7.264
	A	4.315
	B2	1.052
	B3	0.919

	G QD1 QD2	0.708 0.379 0.309
Phenylalanine 22	H A B2 B3 QD QE HZ	9.210 5.190 2.271 2.045
Tyrosine 23	H A QB QD QE	10.320 4.955 2.890 6.780 6.485
Serine 24	H A B2 B3	8.420 4.134 3.613 3.547

Table S9. NOEs observed between lock and ring residues.

Position	Residue	Hydrogen	Position	Residue	Hydrogen
23	Tyr	H	5	Asp	HB2
23	Tyr	H	5	Asp	HB3
23	Tyr	H	7	Ile	HG12
23	Tyr	H	9	Glu	QG
23	Tyr	H	8	Pro	HA
23	Tyr	QD	9	Glu	H
23	Tyr	HA	2	His	HD2
23	Tyr	QD	2	His	HD2
23	Tyr	QB	2	His	HD2
23	Tyr	QD	9	Glu	QG
23	Tyr	QD	9	Glu	QB
23	Tyr	QD	7	Ile	HG12
23	Tyr	QD	7	Ile	QG2
23	Tyr	QD	9	Glu	HA
23	Tyr	QE	7	Ile	QG2
23	Tyr	QE	9	Glu	HA
23	Tyr	QE	9	Glu	QG
23	Tyr	QB	1	Gly	H
23	Tyr	HA	5	Asp	HB2
23	Tyr	HA	5	Asp	HB3
23	Tyr	QB	1	Gly	HA2
22	Phe	HB2	7	Ile	H
22	Phe	HB3	7	Ile	H
22	Phe	HA	9	Glu	H
22	Phe	HB2	6	Arg	H
22	Phe	HB2	5	Asp	H
22	Phe	HA	8	Pro	HA
22	Phe	HA	9	Glu	QB
22	Phe	HA	9	Glu	QG
22	Phe	HB2	8	Pro	HA
22	Phe	HB3	8	Pro	HA
22	Phe	HB2	5	Asp	HB3
22	Phe	HB3	5	Asp	HB3
22	Phe	HB2	5	Asp	HB2

Table S10. Primers used to assemble *cloA* gene in pAK2. The sequences shown are from the 5' to 3' end.

Primer	Sequence
1F	CACACAGAATTCATTAAAGAGGAGAAATTAACTATGGATGTGATGA
2R	GGTCAGCGCGTGTTTATTCTTCATCACATCCATAGTTAATTTCTCCT
3F	AGAATAAACACGCGCTGACCCAGCGTAATGAAAAAGCGCTGAATGCG
4R	TTCACCGGTATACGGGTGATGCTCACCGCATTCAGCGCTTTTTCATT
5F	CATCACCCGTATACCGGTGAAAGCGAGCAAAATTACACGTGGCCATA
6R	AAGTACTCCGGAATACGATCCACGCTATGGCCACGTGTAATTTTGCT
7F	GTGGATCGTATTCCGGAGTACTTTGGCCCTCCGGGCCTGCCGGGTCC
8R	CTAATTAAGCTTTTAGCTATAAAACAGGACTGGACCCGGCAGGCCC

Table S11. gBlock sequences used to assemble pAK2. The sequences shown are from the 5’ to 3’ end.

gBlock	Sequence
1	GCGTTTTTTATTGGTGAGAATCCAAGCTAGCCATCAATTAAGAAAAAAATTTAGCTT GTAGATAAATTCAGAAGTTTTATTATTCCAATTGAGTGTAAAGGCATAACTACAGGA GGGAGTGTGCAAAATGCTGATTACGCCGGATACCCTGAAAATTACCTGCTTTAACA AGGATCTGGTGGTGCTTGACTTACTGAAAGATGAATATACCGTGCTGGAAAATACC AGCGATAGCTTCCTGACCTTTCAGATTCCGAACCCTGACGTAGAGGCGTTTCTGAA TGGCGAAAAAAACTGCCGTTATCTGCAGGCGGATAGCCACAATGATCCCGAATCTT TTCTGGAAGTGCGTTGGATGAAACCGCAGGTGCGTATTAAACATTCTAGCGCCCTG GCTCGTATCTCGGCGCTGCGTGCGATGCTGTATTGTCGGCATGGCCTGAATCATG GCGGCTTGATTGCGCTGAAACGTCAGTTTTGCGAAAGCCAGACCATTGGCTGCGC GACCAAAATCACTCCGGAAGTGGACATTCGTATCAAGAAAGAAATTGCGGCGTTGA ATAGCATTTTTGATCTGATTGATCACGATAATCCGTGCCTGGTCTATTCGTGCGCG CTTAAACAGCGCCTGATTAGCCGTAAAATTGGCGCAACCCTGGTGATTGGCGTGC GTACCCGTCCGTTTTATAGCCATGCGTGGGTGGAGGTGGATAGCACCGTGGTGAG CGATGATCCGGACCTGAGAAAGAAAACGAGCGTCATACTGGAGTGCTGACCATGC TGATTGTGCCAGTGATGCTGTCCGAAAGCGTGGCAGTGCCGGCTCATTTTACCGC TGTGCGTACCGGGATGTACCTGATTTATCTGGACGCGGGCTGGAGCTATCGGGAA GATGACGGAAAACTGTGCATTTTTAGCGGCTGGGCGATTACCGGCAATGGCGAAG TGTTATCGTTGAGCGAGACCGCACCTGATGTGCTGGAGAAAGAGCTTAAAAGCGC CGATGGCTATTTTTGCTTTATTGTTATAGAAAATGATGTGATACAAGTAATGAAGAG CCTGTATCGTTATTGCGATGTGTACTACGGCAAGACTGCGTTTGGCGCTATTATTG GCACCGGCTTGAAAGATGTGGCGCGGATTAGCGGGACCAAAACCATTACTCGGGA CTTTATTCGTAGCTTCATACGGGATAGCTTTCAGGCGGCGTTTCTGAGCCCGCTGC ATAATACCGGAAAGGCGTACTCCGGCTGCTTGACCCGGATTGACCGTGAAAATATC CATACCGAAGCGTTTGTGAATCTGACCCCTCTGAAGAAAAACACCCTGGACGTGAT TATTACGATTGTGAAAAAACTGCCAGCCGATCTGCCGCTGGCGTTATATCTGTCCG GCGGATTGGATAGCAGCATCGTGTTCTTCTGCCTGCTGGCTGCGGATAAACCGTTT A
2	TGCTGGCTGCGGATAAACCGTTTACCGTGTTTCATTGCTTACCGTTCAGCTTTGAA ACCGACGCAGAACGGGCGGACGCGCATCGTCTGTGCCGTAAGTATAACATTACCC TGACCGAGCTGAAACCTGGCTATACCACCGATCGCCAAATTAGCAATCGTGTAAAT CATCCGAGCGATTGTCCGGTGGTGAATACCTTTTGGCTGAATGCGAGTGACGGCC TGACACAAGATATGAGCGGGTGGTTCTGCTTGGACGGTCATGGCGGCGACAGCGT GTTTATTCAGAGCCCGAGCGTGCGCATCATTCGTGACTTAATTCGCGTGGGCAAAC CGATTCAAGCGTTGCGGACCGCATTCCGTCTTGCAGCGTTGAAAAGCCGCTCTTTA CACAGCGTACTGGCGAGCGCATACAAAACCGAACCGGCGAAACGTACCCATGTTC ATCCCCTTCTGGTGGGCCATATTCCAGGCACCGCATGGTATGAATACTTGGAAGAA CTTATCCGTATTAGCGAAAGCACAGTGTCAATTTTTCCGGATGGACCGGCGGTGAA ATTTAGTCCTCTTATTAGCCTGAGCGTGATTCGTTCACGTCTGGCGGTGCGTTACG AGGATAACATACGTGCTGGGCATGATCGTGCGGGGATCCGTCGTGAGGCGCTGA CCCGTTTTAAAGATCCGGTGTTCATAAAGAAAACTAAGCGTTCCAGCAGCCAATTG ATTTTTACAATTATTCGGCAGTATGAGGACGAACTGATTGCGTTCGTGGTCCGTTAT GGCACCTATATTCTGGACAACCCTGAAGCGATGATTGAGGAAATTCGTTATAATGC TCATGTGGAATTAAACGGCAGCCTGCCGGAAATTTTGTCCCTGATTAAACTGATGG TCTTTAGCCAGAAGATGGAAGCTGATTTTTTCGACGCGGAGTCAAGGCATGAATTT TTTGAGTTTGTTTAAAACCGTGAGCCGTGCGACCGGCACCCGTCATGTGGGCAAA ATTACGTTCATTATCCTGCTGAGCGTGTTAAGCAGCGTGCTGATCATAATTTCTCCG GTCCTGATTAGTCGTACAACCGGCTACCTGATGAGCGATACCGCGGATAGCTTTC GTGTGATATTTACCTTGGGTGCGGTGTATGTGTTTGCCATTTCAGCGCAAAAGATA ATTGCTTTTTGGGCGACCTACCTGCAGAGCGCCCTGCGCGTGGAATGTATTGCGA GCATAAGCGAGTTTTTCCTTAAGAAACTGTATTATAGCGGTAGCCAGAAGGACAAC GCTGGTGGAATTAGCCAGCAACTTAATCAAGCCACCAATGACATTTACATAATCATC

	CGTGAACTGACCTTCGGCCTGTTACCACCGCTGATTCAGCTGTTTATTGCCCTGTG GACCATTTTAGCGAGCGATGATGTGATTGTGGCGTGCAT
3	TGATGTGATTGTGGCGTGCATGTTTCTGATGTTTGCGATTAGCTTTGTGTTTACCAA TTATGTGTATACCCATAAAATCGTGGCGGCTCGTCAGGCGCTGATGGACAGCAGC CGTAAAACCTACACCTTATTATCCGATTCGGTCCGTAATATTCCGGTGGTGCGGGT GTTTAACGGCTTTGGCGTGTTTATGAAGCGTTTCAACGGCCAGCTGACAGAGGATG CGCGTACCCAGCGTAGCTTTTGGAAAGTTAGCTTCCATGCGCAGCTGGTGTCGGC GTTACTGCAGATAATCTTTTTTGGCTCCGCGTTCTTTTATACTCTGTATGGCGCGAG CACCGGTCAGGTGACCATTGAGCATTTCATTCTGGTGAGCAGCTACATTCTGATTT TGAGTGCCCCCTTGGAAAATCTGGGCCAGTCCTATATCAATTTTCGTCAGAGTGCT CATGCGTTTAGCGCATTTAGCGGCGGTTGGCTGGAAGAACCGGTGGATCGTGATG GCAATCTGCGCCGTCCCCAAAACGGGGATATTACTCTGAGCGGCGTGTCATTTCG TTACCCGGAAAGCCCGAGCTGCACACTGCATGACGTGAATTTACACTTCCGCACG GGCGAGTTCATAACCATTACCGGCAAGTCCGGTTCAGGCAAAAGCACCCTGTTGA AAATCCTGGCACGTCAGCTTCCGCTGACCATAGGGAACTATACACTGGGAGGCTG CCCTAATGAAGAACTGCCTGAGCAAGTGTTCTACGAAAACATTGCATATGTGAGCC AAGAAGAATATGTCTTTATGGATAGCGTGGCGTATAACTTACAGATCGCGAATCCG GAAGCGACTCGTGAAGAGATGATCTCGGCCCTGGCGAAAAGCGGACTGCAAATTG GACATCTGCAAGGCGAAGCGCTGCTGGATTTGAACCTGGCCAATGAAGGCACCAA CATTTCCGGAGGTCAACGCCAACGGCTGAGCTTGGCGCGTTTGTTTTTGCGTAAC CCGGCAGTGATACTGTTGGACGAGATTACCTCTAGCCTGGATCTTATATCTGAAAC CCACGCAATTAACGCGATTTTGTCAGACTTTCCGACCGCGACCATTATTTCGATCT CGCATCGTATAAGCACCTTTAACTATAGCGATCGTATTATTGTGATGCATAACGGTC GGGTTAGCGATGCAGGCCCCCTGAACGAACTGCTGCAACGTAACAACTTCATAGC TCAACTGCACCGTAGCGAAGAAAGTCCACCTGTGAAAGCGCCATGGGCAAATATTA TACGCAAGGCGAC

It was found after assembling the gBlocks that a stop codon was missing on the cloned *cloD* gene; we corrected this with the following primer sequence:
5'-GCGTATAATATTTGCCCATGGTTACGCTTTCACAGGTGGACTTTCTTCGC-3'