Supplementary Information:

Structural and biochemical studies of an iterative ribosomal peptide macrocyclase

Gengnan Li[1‡], Krishna Patel[1‡], Yi Zhang[2], Jackson Pugmire[1], Yousong Ding[2] and Steven D. Bruner[1*]
[1] Department of Chemistry, University of Florida, Gainesville, FL, 32611, USA.
[2] Department of Medicinal Chemistry, College of Pharmacy, University of Florida, Gainesville, FL, 32610, USA.

* To whom correspondence should be addressed: S.D.B. (bruner@ufl.edu)
‡ Equally contributing authors

Table S1. Observed and accurate masses for peptides released from AMdnA-Δ7 by GluC digestion.

	Peptide Fragment	Calculated	Observed	Error (ppm)
M2- Δ3	y1	148.0605	148.0602	2.0
	y2	205.0819	205.0818	0.7
	y3	262.1034	262.1032	0.8
	y4	319.1249	319.1248	0.2
	y5	433.1678	433.1671	1.6
	b2	229.1183	229.1177	2.7
	b3	300.1554	300.1550	1.5
	b4	399.2239	399.2233	1.4
	y17**$^{2+}$	856.3815	856.3753	7.2
	y16**	1640.7186	1640.6986	12.2
	y15**	1541.6502	1541.6539	2.4
	b16**	1678.7707	1678.7664	2.5
	b15**	1621.7492	1621.7536	2.7
	b14**	1507.7063	1507.6926	9.1
M3-Δ3	y2	175.1078	175.1077	0.3
	y3	272.1605	272.1604	0.5
	y4	400.2191	400.2197	1.5
	b2	213.1598	213.1597	0.5
	y12**	1292.6157	1292.6125	2.5
	y13**	1420.6743	1420.6761	1.3
	b12**$^{2+}$	646.8115	646.8136	3.2
	b13**$^{2+}$	710.8407	710.8410	0.4

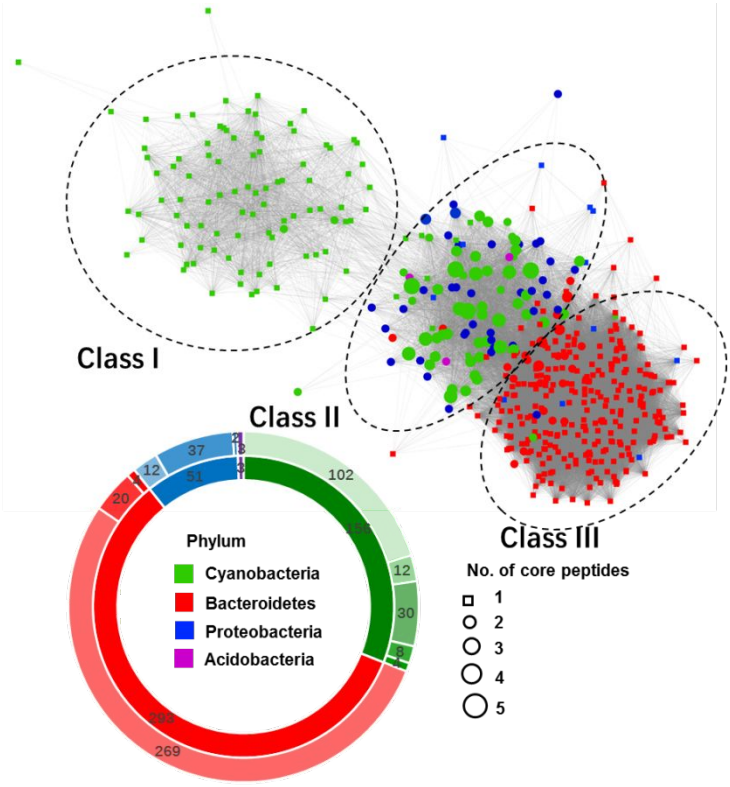

Figure S1. Sequence similarity network of 503 precursor peptides found across various microviridin biosynthetic clusters showing clustering of three classes of precursor peptides corresponding to the number of core peptides and the linker length between the conserved leader peptide and the start of core peptides. The inner circular chart on the left compares the proportion of microviridin precursor peptides identified in the bacterial phyla of cyanobacteria, bacteroidetes, proteobacteria and acidobacteria. The outer circular chart further compares the proportion of microviridin precursor peptides possessing different numbers of core peptides within each phylum. Darker colors correspond to more core peptides the corresponding precursor peptides contain.

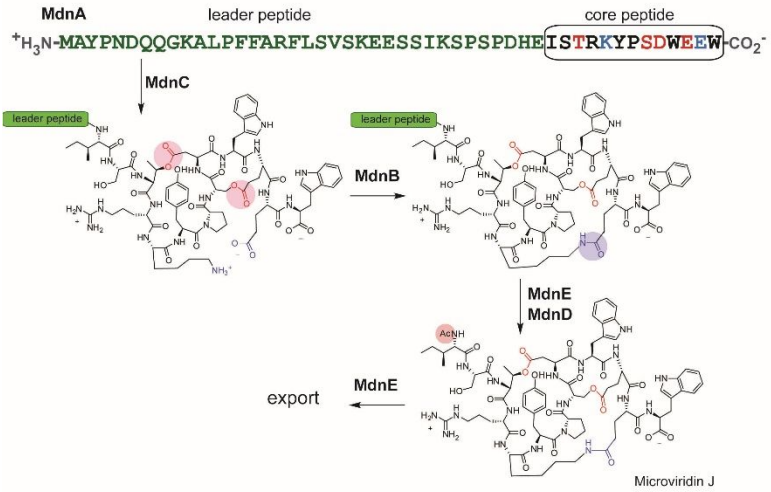

Figure S2. Schematic of the RiPP pathway to microviridins. The five gene product biosynthetic pathway from microviridin J of *Microcystis aeruginosa* UOWOCC MRC is illustrated.

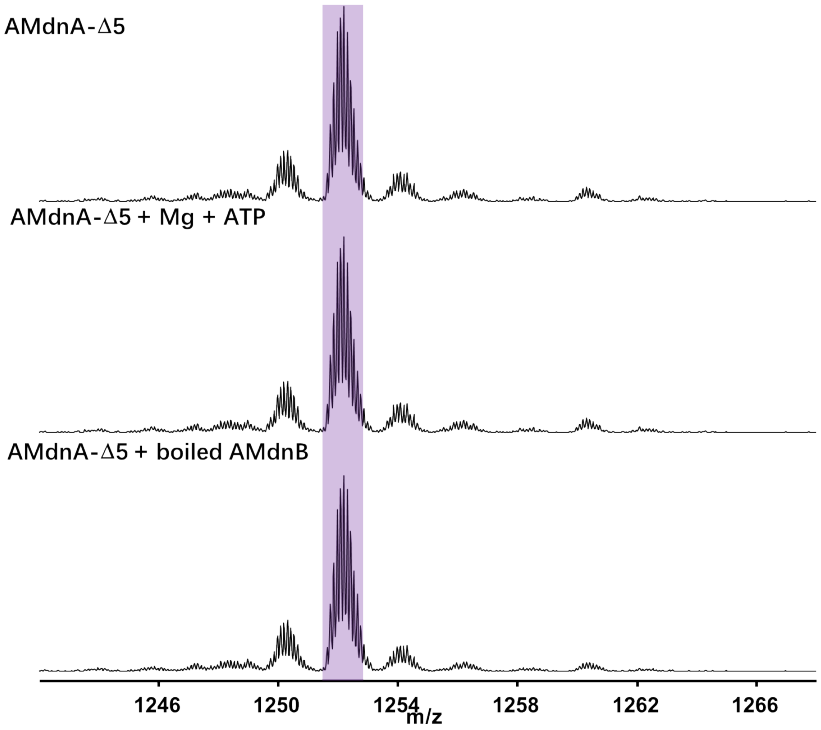

Figure S3. No processing of the substrate AMdnA-Δ5 was observed when using no enzyme control or boiled AMdnB as negative controls.

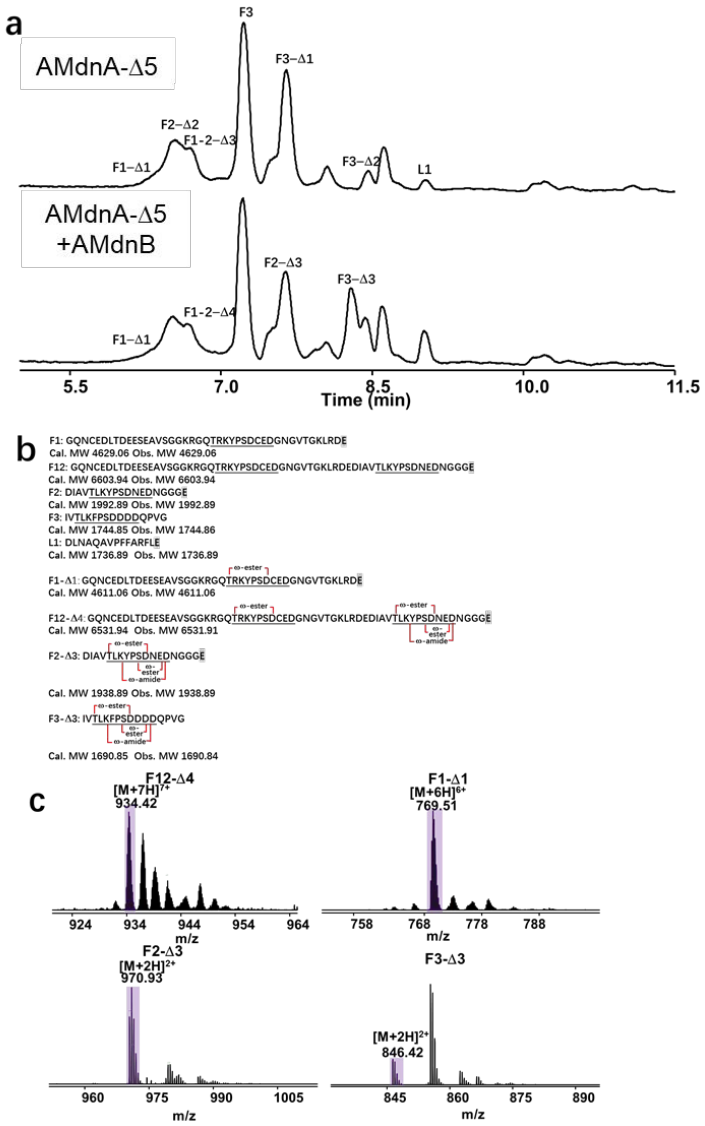

Figure S4. A. HPLC traces of GluC digestion mixture of AMdnA-Δ5 before (top) and after (bottom) AMdnB processing. Key chromatographic peaks were labeled with the names of the corresponding peptides released by GluC. GluC digests target peptides C-terminal to glutamic acid residues and theoretically would release each of the three processed core peptides within AMdnA. Despite the apparent incomplete processing, LC-HR-MS analysis of the digestion mixture of both intact and processed AMdnA-Δ5 identified mass fragments F1-Δ1, F1-2-Δ4, F3-Δ3 and F4-Δ3 carrying the core peptide M1, M1-2, M2 and M3, respectively (Figure 2). F1-Δ1 remains intact during the reaction, while one extra dehydration was observed on F1-2-Δ3 after AMdnB processing, suggesting one extra macrocyclization was installed on M2. Two new major chromatographic peaks with retention times of about 7.6 min and 8.2 min emerged (Figure 2b). HR-MS analysis assigned these two new peaks as F2-Δ3 and F3-Δ3, respectively. Therefore, AMdnB catalyzed two consecutive dehydrations from the M2 and M3 in AMdnA-Δ5. The observed dehydration pattern gave a 1-3-3 ring topology of the major product (Figure 2). We further validated this deduced structure by comparative MS/MS analysis (Figure 2). Expectedly, in tandem MS traces, multiple fragments generated from the F2-Δ2 and F3-Δ2 were shifted after AMdnB processing, corresponding to the tricyclic ring topology on F2-Δ3 and F3-Δ3.
B. The amino acid sequences with calculated and observed monoisotopic masses of each peptide released by GluC digestion. The regions of putative core peptide are underlined and the GluC digestion sites are highlighted in grey. C. Charge numbers and the accurate mass values were labeled for key peaks in the HRMS spectra of the proteolytic peptides.

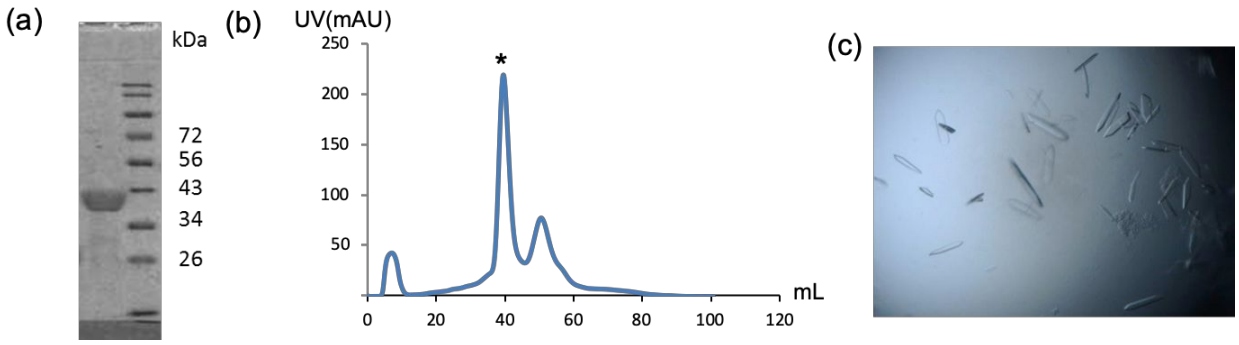

Figure S5. A. SDS-PAGE analysis of AMdnB elution fraction from Ni-affinity chromatography. The size of AMdnB protein was confirmed by polyacrylamide gel electrophoresis. B. Size-exclusion chromatography analysis of AMdnB protein. Protein sample was eluted after 40 minutes of the run. The flow rate of the FPLC purification was 1 mL/min. C. Hexagonal crystals of AMdnB protein in 0.18 M Tri-ammonium citrate, 20% (w/v) PEG 3350 and 0.012 M praseodymium (III) acetate hydrate. Crystals were yielded from the hanging drop vapor diffusion method at room temperature.

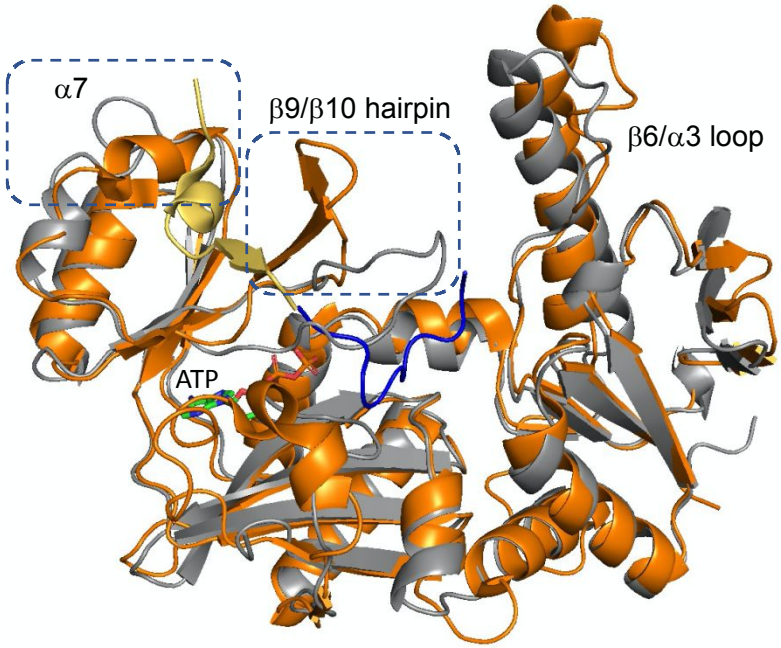

FIGURE S6. Superimposed structures of AMdnB and CdnC (PDB code: 7MGV). A crystallographic AMdnB monomer (grey cartoon representation) is aligned with CdnC complex structure (orange). ATP is shown as a stick model, the precursor peptide (from the CdnC co-complex) is colored in yellow (leader peptide) and blue (core peptide). Major differences between the two structures are boxed.

Figure S7. Sequence alignment of AMdnB with other ATP-grasp ligase superfamily members. Enzymes listed here are top candidates from structure similarity search results. Amino acid residues highlighted in red: bind to β-phosphate of ATP; in orange: bind to α-phosphate of ATP; in purple: hydrogen bond to adenosine N6; in green: hydrogen bond to ribose O3'; in blue: metal ion coordination.

Table S2: Data collection and refinement statistics of AMdnB protein.

Data collection	
Space group	$P12_11$
Cell dimensions	
a, b, c (Å)	61.48, 132.46, 83.01
α, β, γ (°)	90, 91.01, 90
Resolution (Å)	41.5 - 2.493 (2.582 - 2.493)*
R-merge	0.1459 (1.265)
R-pim	0.06129 (0.5103)
CC1/2	0.994 (0.596)
Mean I/sigma(I)	9.29 (1.26)
Completeness (%)	97.68 (93.23)
Redundancy	6.77 (7.00)
Refinement	
Resolution (Å)	41.5 - 2.493
No. reflections	45239
R-work/R-free	0.1986/0.2647
Number of non-hydrogen atoms	9947
Protein	9900
Solvent	47
B-factors	50.23
Protein	50.28
Solvent	40.51
R.m.s deviations	
Bond lengths (Å)	0.012
Bond angles (°)	1.34
Data collection	
Space group	P1211
Cell dimensions	
a, b, c (Å)	61.48, 132.46, 83.01
α, β, γ (°)	90, 91.01, 90

*Single crystal was used for each data set; *Values in parentheses are for highest-resolution shell;

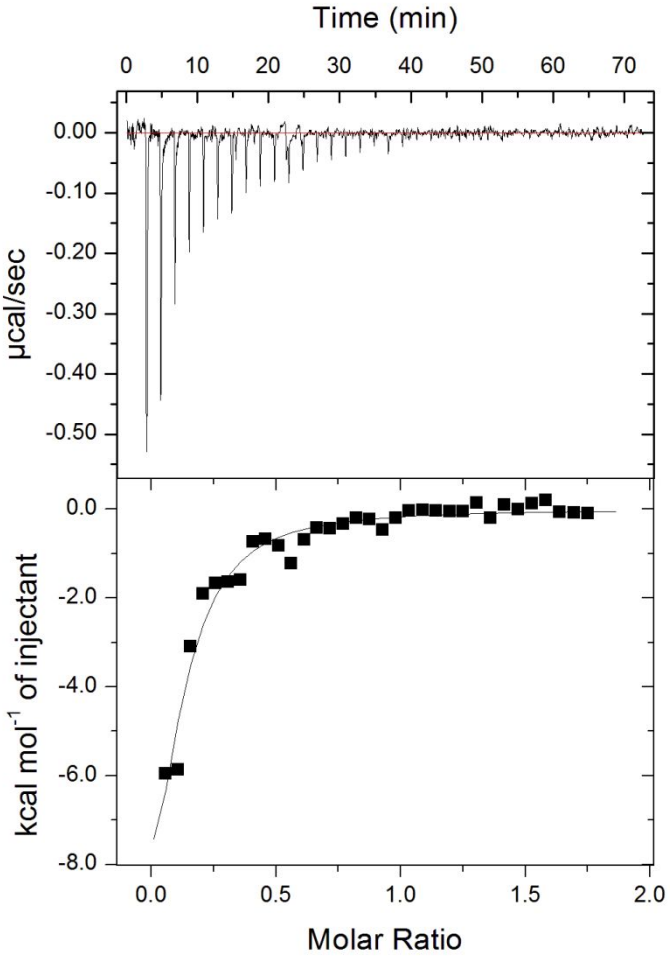

Figure S8. ITC profile of AMdnA$_{1\text{-}24}$ interaction with AMdnB macrocyclase. ITC experiments were performed at 25°C with a total of 33 titrations. Protein concentration was at 0.1 mM and peptide concentration was at 1 mM before starting the titration. The titration was continued for 75 minutes. Data was processed and fitted in Origin.