Supplementary Information

Structural basis for precursor protein-directed ribosomal peptide macrocyclization

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Supplementary Results

Supplementary Table 1. MS profiles of MdnA and MdnA variants

	MS fragments (positive mode)						Experimental	Theoretical
	+3	+4	+5	+6	+7	+8		
MdnA		1430.3	1144.5	953.9	817.8	715.7	5717.5	5718.31
MdnAΔ1		1426.0	1140.8	950.8	815.3		5699.5	
MdnAΔ2		1421.4	1137.3	948.0	812.8	711.3	5681.6	
MdnAΔ3		1416.6	1134.1	944.8	810.2		5663.8	
$MdnA^{1-35}$		978.4	782.9	652.7			3909.8	3910.37
$MdnA^{Ac20-49}$	1189.4	892.3	714.1				3565.3	3563.76
$MdnA^{Ac20-49}\Delta1$	1183.3	887.8	710.5				3547.2	
$MdnA^{11-49}$		1147.0	917.8	765.0			4584.0	4585.07
$MdnA^{11-49}\Delta2$		1138.1	910.7	759.1			4548.5	
$MdnA^{11-49}\Delta3$		1133.6	907.0	756.0			4530.1	
$MdnA^{9-22}$	770.6(+2)						1540.2	1539.84
$MdnA^{36-49}$	913.6(+2)						1825.2	1825.95
$MdnA^{36-49}\Delta3$	886.6(+2)						1771.6	

Supplementary Table 2. ITC-based precursor peptide/cyclase interaction

Protein	Ligand	Site	K_d
MdnC	MdnA	0.95±0.02	112±52 nM
MdnC	$MdnA^{1-35}$	0.90±0.01	146±52 nM
MdnC	$MdnA^{11-49}$	1.04±0.01	308±78 nM
MdnC	$MdnA^{Ac20-49}$	*n.d.**	
MdnC	$MdnA^{9-22}$	1.08±0.02	308±62 nM
MdnC	$MdnA^{36-49}$	*n.d.*	
MdnC	ATP	*n.d.*	
$MdnC^{AA}$	MdnA	*n.d.*	
$MdnC^{KK}$	MdnA	*n.d.*	
$MdnC^{E293A/N295A}$	MdnA	0.84±0.05	575±302 nM
$MdnC^{D281A}$	MdnA	0.71±0.01	568±80 nM
MdnB	MdnA	0.80±0.10	4.85±1.02 μM
MdnB	MdnAΔ2	1.05±0.09	6.45±2.04 μM
MdnB	$MdnA^{1-35}$	1.12±0.02	2.85±0.77 μM
MdnB	$MdnA^{Ac20-49}$	*n.d.*	
MdnB	$MdnA^{9-22}$	1.12±0.01	1.55±0.25 μM
MdnB	$MdnA^{36-49}$	*n.d.*	

* *n.d.* - not detected, with no observable heat change during the titration.

Supplementary Table 3. **Data collection and refinement statistics**

	SeMet Derivative		Native	
	MdnB	MdnC	MdnB	MdnC+MdnA$^{1-35}$
Data collection				
Space group	C121	$P4_1$	C121	$P4_1$
Cell dimensions				
a, *b*, *c* (Å)	128.49, 36.13, 139.90	132.62, 132.62, 196.98	128.62 37.99 139.38	132.56, 132.56, 198.16
α, β, γ (°)	90, 116.91, 90	90, 90, 90	90, 116.55, 90	90, 90, 90
Resolution (Å)	45.2-2.51 (2.57-2.51)*	39.5-2.76 (2.81-2.76)	33.4-2.28 (2.36-2.28)	39.7-2.66 (2.71-2.66)
R_{merge}	0.11 (0.66)	0.18 (0.80)	0.053 (0.41)	0.114 (0.67)
R_{pim}	0.07 (0.46)	0.13 (0.66)	0.034 (0.28)	0.037 (0.22)
CC(1/2)	1 (0.93)	0.99 (0.90)	1 (0.96)	1 (0.93)
$I / \sigma I$	18.7 (1.8)	19.4 (2.1)	17.0 (3.6)	17.1 (3.8)
Completeness (%)	88.3 (69.1)	99.8 (99.7)	99.9 (97.1)	99.9 (92.8)
Redundancy	4.6 (4.5)	12.5 (11.4)	4.9 (4.9)	11.9 (10.8)
Refinement				
Resolution (Å)			33.4-2.28	39.7-2.66
No. reflections			137054	1155055
R_{work} / R_{free}			0.215 / 0.255	0.219 / 0.260
No. atoms			4433	20643
Protein			4376	19872
Precursor peptide			-	771
Water			43	-
B-factors			47.82	55.97
Protein			47.81	55.16
Precursor peptide			-	76.70
Water			48.60	-
R.m.s. deviations				
Bond lengths (Å)			0.009	0.003
Bond angles (°)			1.02	0.64

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

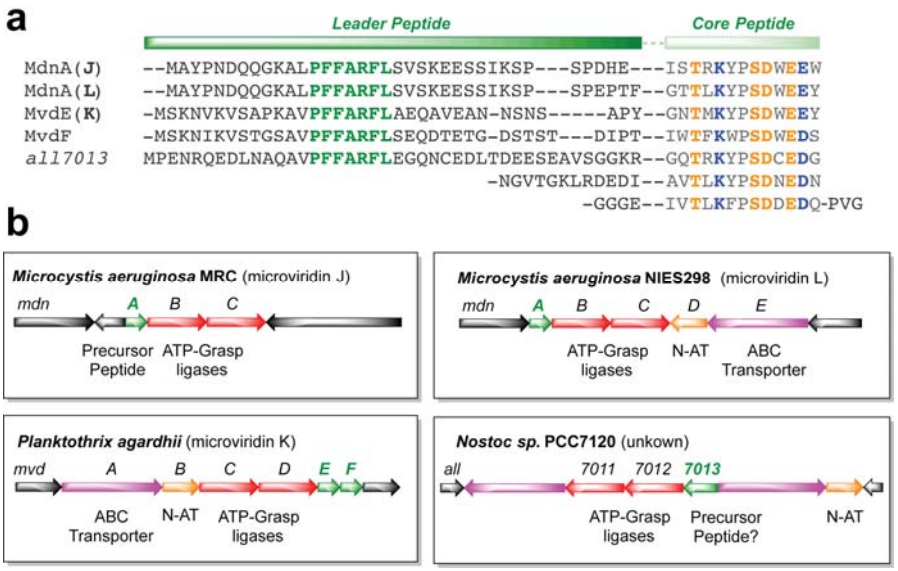
Supplementary Table 4. Key residues in nucleotide interaction

MdnC	MdnB	LysX	RimK	DDL	Proposed interaction
Lys125	Lys131	Lys127	Lys100	Lys97	Binds to β-phosphate of ATP
Lys166	Lys171	Lys87	Lys141	Lys144	Binds to α-phosphate of ATP
Gln207	Gln211	Gln167	Glu178	Glu180	Hydrogen bond to adenosine N6
Glu215	Glu219	Asp176	Asp187	Glu187	Hydrogen bond to ribose O3'
Asp281	Asp284	Asp234	Asp248	Asp257	Metal ion coordination
Glu294	Glu297	Glu250	Glu260	Glu270	Metal ion coordination
Asn296	Asn299	Asn252	Asn262	Asn272	Metal ion coordination

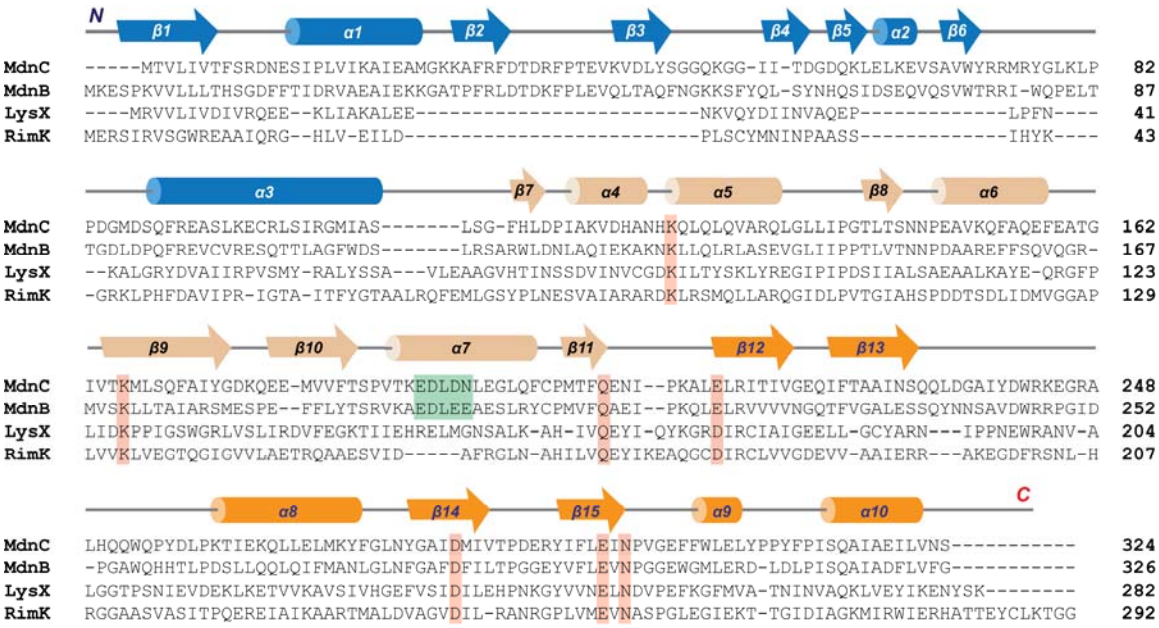
Supplementary Table 5. Oligonucleotides for protein cloning and mutagenesis

Protein		Primers
MdnB	WT	5'-GACCTTCATATGAAAGAATCGCCGAA*
		5'-CTAACTCGAGTCAGTGATGGTGATGGTGA
		TGGGCGGCGGCACCGAACACCAGAAAATC
MdnC	WT	5'-GCCATATGACGGTAGTGATTGTGACG
		5'-GACTCGAGTCAGTGATGATGATGATGATG
		AGCAGCAGCGGAGTTCACCAGAATCTC
	K125A	5'-CGCCAACCACgcaCAACTGCAGC
		5'-TGGTCCACTTTGGCAATC
	K166A	5'-TATTGTCACTgcAATGCTGTCCCAG
		5'-CCGGTCGCTTCAAACTCT
	Q207A	5'-GATGACATTTgcAGAAAACATCCCG
		5'-GGACAAAATTGCAGACCC
	Q215A	5'-AAAGCACTGGcGCTGCGTATTAC
		5'-CGGGATGTTTTCTTGAAATGTC
	D281A	5'-GGCGCCATTGcTATGATCGTG
		5'-ATAGTTCAGGCCGAAATATTTC
	E294A/N296A	5'-tgcTCCGGTTGGTGAGTTCTTC
		5'-atcgCCAGGAAGATATAACGTTCATC
	E191K/D192K	5'-GGTTACAAAAaaaaaaCTGGATAACCTGG
		5'-GGGCTGGTAAAGACGACC
	E191A/D192A	5'-GTTACAAAAGcagcTCTGGATAACCTGGAG
		5'-CGGGCTGGTAAAGACGAC
SeMet	M25K	5'- GCGATCGAAGCAAAAGGCAAAAAGGCC
(MdnC)	M104L	5'- GTATTCGTGGCCTGATTGCCTCACTGTC
	M167L	5'- CGGTATTGTCACTAAACTGCTGTCCCAGTTCG
	M181N	5'-GGGGATAAACAGGAGGAAAACGTCGTCTTTAC
	M282I	5'-CGCCATTGATATCATCGTGACCCCG

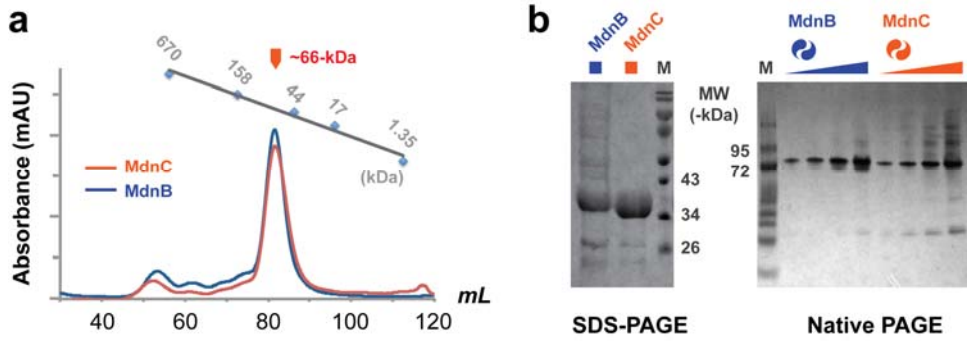
*Restriction sites are underlined.



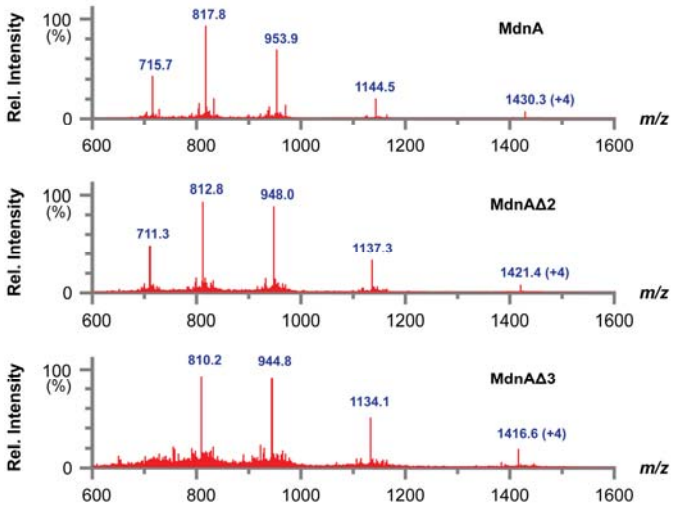
Supplementary Figure 1. Representative precursor peptides and gene clusters in microviridin biosynthetic pathways. (**a**) Aligned precursor peptides have a strictly conserved region (PFFARFL) highlighted in green. Residues involved in the cyclizations are labeled in orange (lactonization) and blue (lactamization). (**b**) The microviridin J biosynthesis gene cluster in *Microcystis aeruginosa MRC* contains the genes of *mdnA-mdnC*, with the notable absence of *mdnD* or *mdnE*-like genes, present in *M. aeruginosa* NIES298. *Planktothrix agardhii* microviridin biosynthetic gene cluster contains two genes (*mvdE* and *mvdF*) encode for two independent precursor peptides. Gene *all7013* in *Nostoc sp.* PCC7120 encodes a putative microviridin precursor peptide with three sequential core peptide region following a leader peptide.



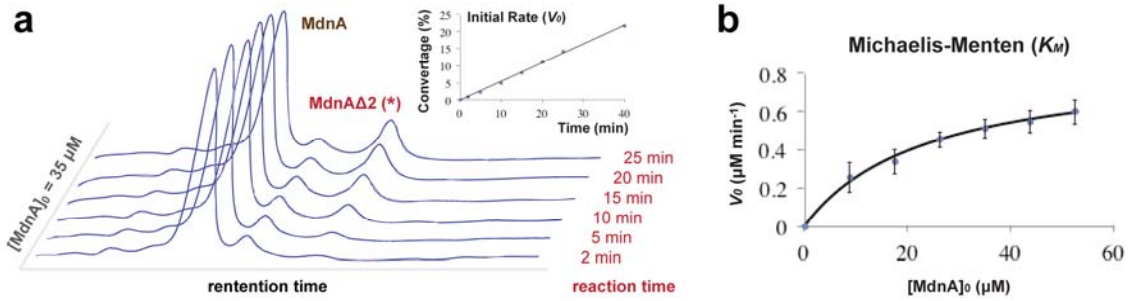
Supplementary Figure 2. **Alignment of MdnC, MdnB and other reported ATP-grasp ligases.** MdnB and MdnC belong to ATP-grasp ligase superfamily consisting of a tri-domain structure. Of deposited structures, LysX is most similar sharing sequence identity of 19.5% and 17.2% with MdnC and MdnB respectively, while MdnB and MdnC share a sequence identity of 40.1%. Key residues corresponding to ATP binding interactions are highlighted in red. The amino acid region highlighted in green corresponds to leader peptide recognition.



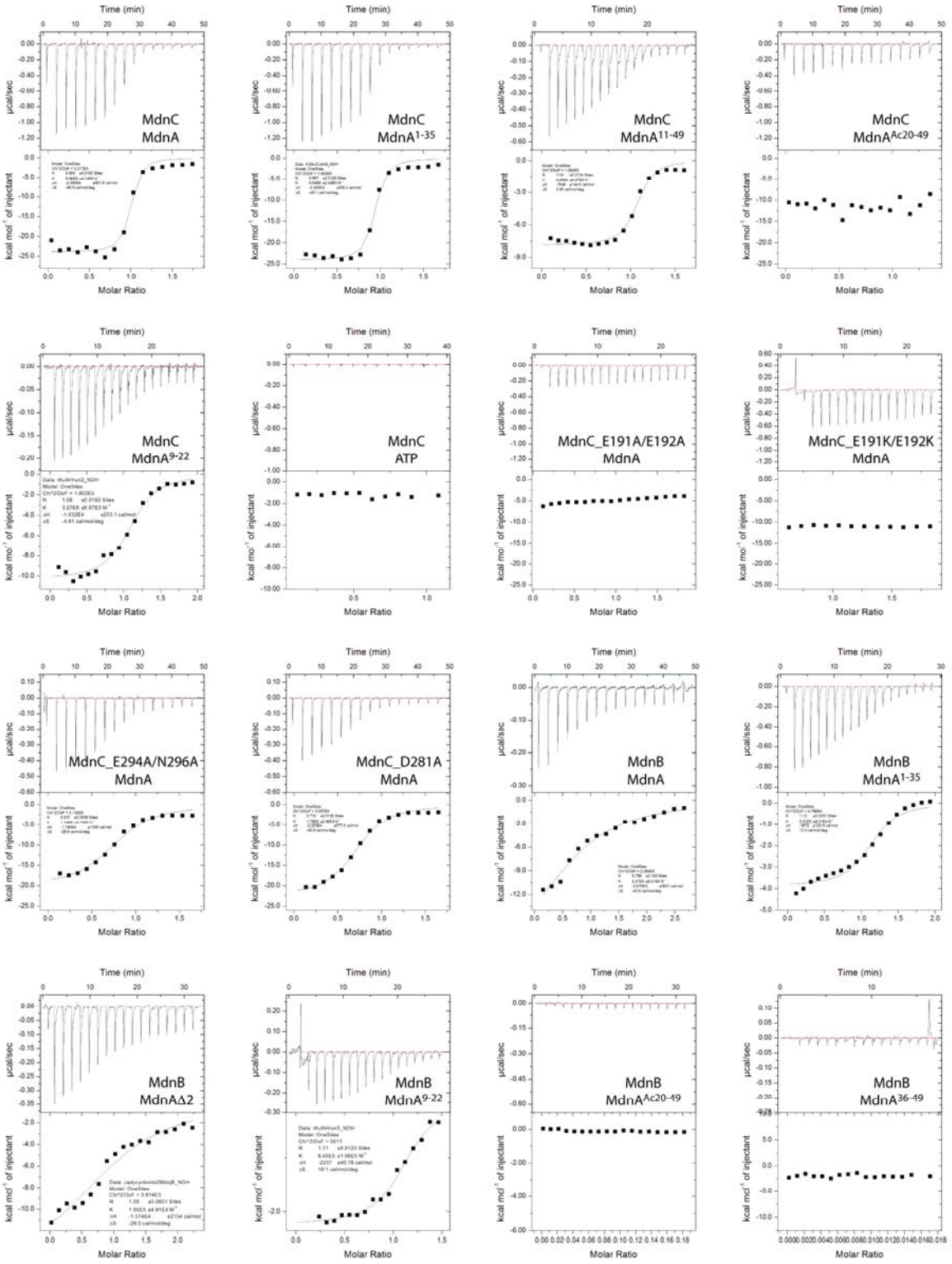
Supplementary Figure 3. Expression and purification of MdnB and MdnC as dimeric proteins. (**a**) MdnC and MdnB shares a similar retention time in size exclusion chromatograph and are predicted to be dimers in solution. Molecular weight standards (BioRad) were plotted as retention volume *vs log* (MW). (**b**) MdnC and MdnB, with a molecular weight of ~35 kDa, run as dimers in native PAGE analysis (~80 kDa).



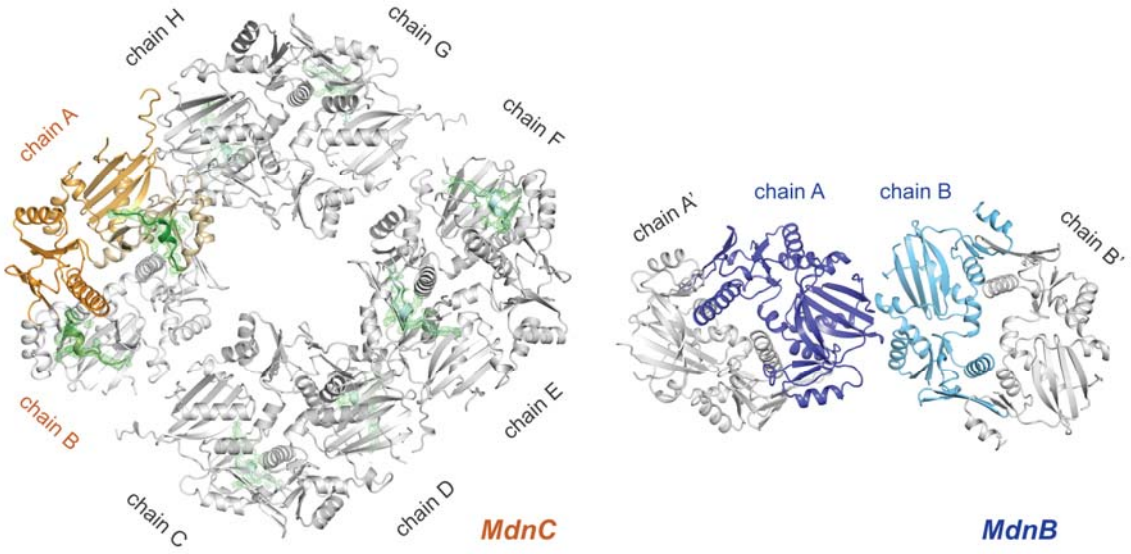
Supplementary Figure 4. Analysis of full-length MdnA cyclizations. MdnA and its cyclic products have been identified in MS analysis. Refer to **Supplementary Table 1** for masses.



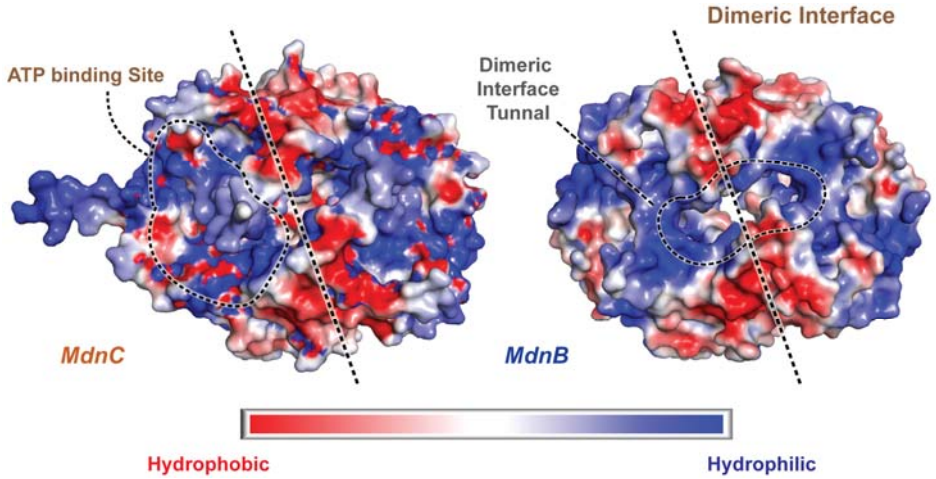
Supplementary Figure 5. Kinetic analysis of the MdnC catalyzed dicyclization. (**a**) Representative HPLC traces for MdnC-catalyzed MdnA dicyclization and initial rate determination. (**b**) Michaelis-Menten plot created with various initial rates at different substrate concentrations. Each initial rate has been determined as triplets.



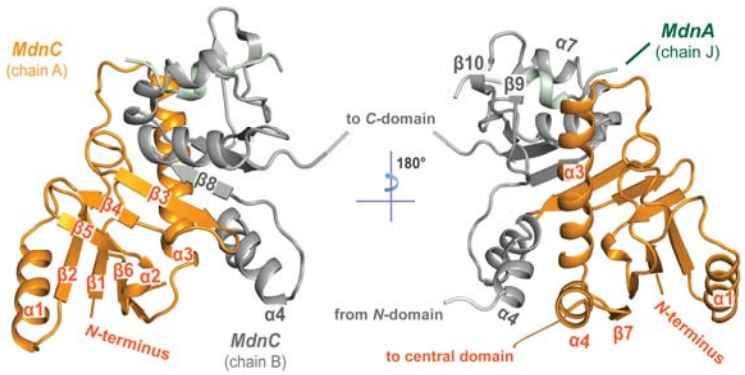
Supplementary Figure 6. ITC profiles of MdnA variants interact with macrocyclases MdnB and MdnC. Detailed fitting information is listed in **Supplementary Table 2**.



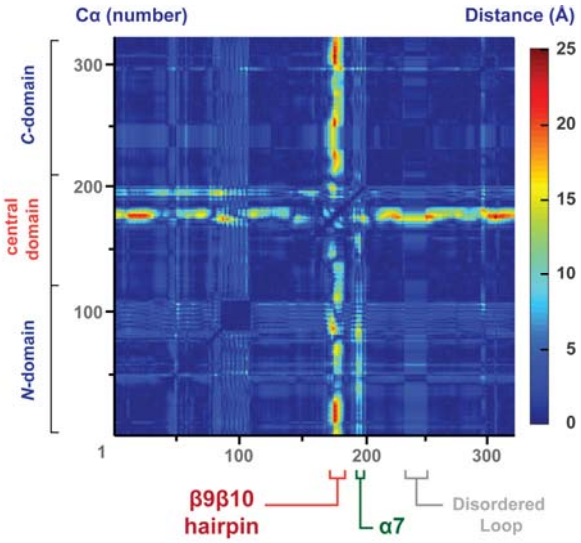
Supplementary Figure 7. Crystallographic packing of MdnC and MdnB. MdnC crystals packs in space group $P4_1$, with four dimers per asymmetric unit. MdnB crystallize in space group C2, consisting of two protomers per asymmetric unit. Observed $MdnA^{1-35}$ bound with MdnC protomers in 1:1 ratio. Calculated *mFo-DFc* difference map corresponds for MdnA fragments is displayed in green at 2.0σ contour level.



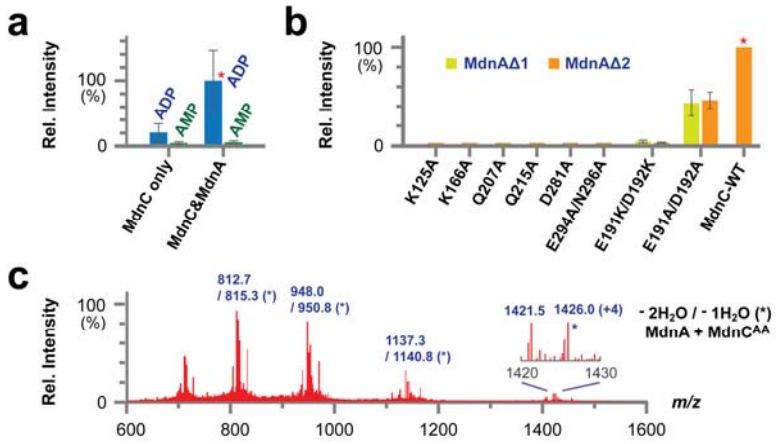
Supplementary Figure 8. Electrostatics map of MdnC and MdnB dimers. Color-coded electrostatic surface map. MdnC and MdnB dimers are the same orientations as shown in **Fig 3**. A tunnel-like feature in MdnB dimeric interface has been observed. Hydrophobic residues are assembled near the dimeric interface, while hydrophilic residues are predominantly near the ATP binding site.



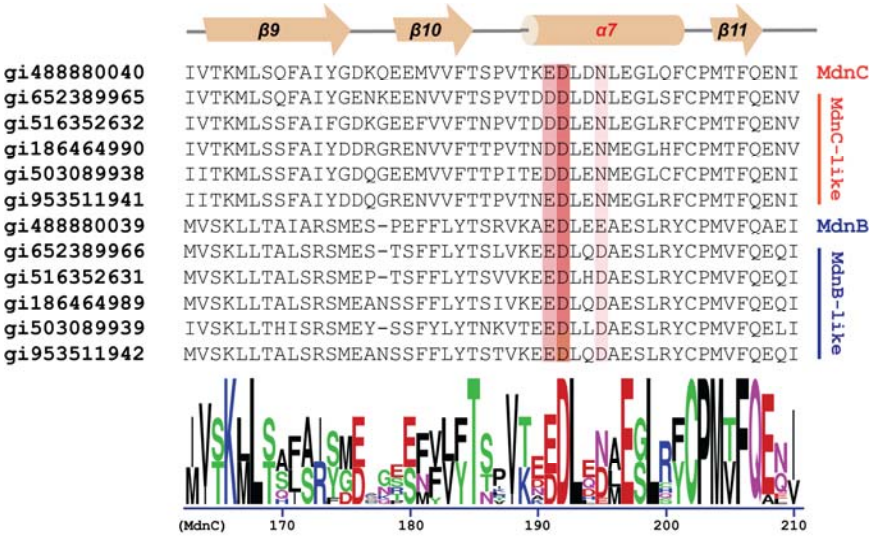
Supplementary Figure 9. MdnC crystallized as a dimer. The *N*-domain of MdnC (chain A) is in orange, while the neighboring central domain (chain B) is in grey. Key interactions between antiparallel β3/β'8 stabilize the dimerization. Other interactions include α3/β'9 and α4/ α'4.



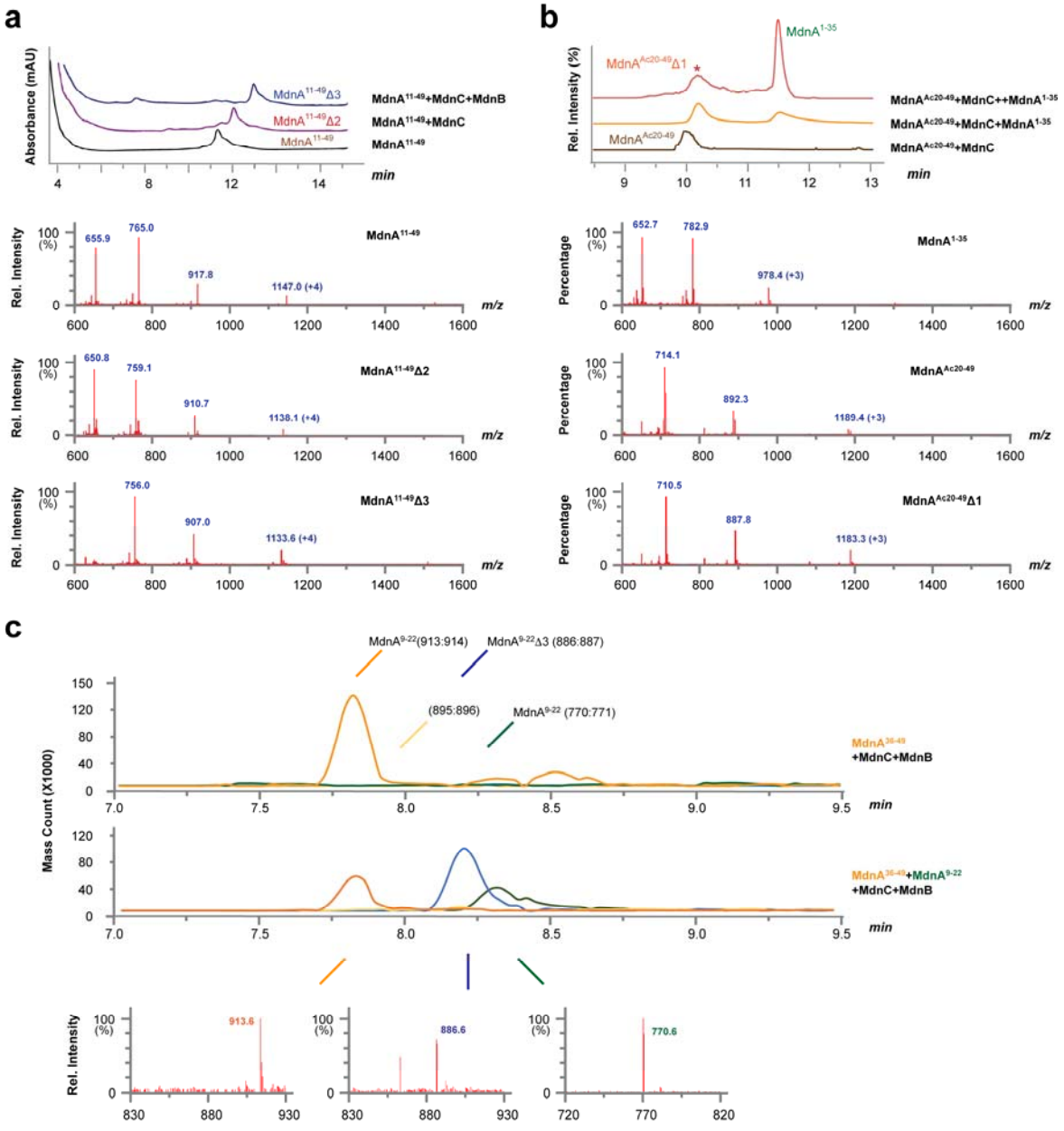
Supplementary Figure 10. C-alpha distance difference plotting between MdnC and MdnB protomers. Plot (X, Y) indicate the absolute difference between the distance of residue X Cα and residue Y Cα in MdnC and MdnB. Amino acid movement between MdnC and MdnB protomers has been observed in β9 β10 hairpin and helix α7 regions.



Supplementary Figure 11. Characterization of determinants for MdnC catalyzed cyclization. (**a**) Extraction of crude reaction mixture followed by mass spec analysis, relative ion intensity corresponding to ADP and AMP are plotted. MdnC produces ADP but not AMP during the catalysis. (**b**) Mass spec analysis of product formation for MdnC and site-directed mutants. Masses (positive mode) are 1137:1138 for MdnAΔ2 and 1140:1141 for MdnAΔ1. Only the MdnC^{AA} mutant produces cyclic product. (**c**) MS for the MdnC^{AA} reaction mixture, indicating the formation of MdnAΔ1.



Supplementary Figure 12. MdnC central domain interacts with the precursor peptide MdnA. Alignment of the β9 β10 hairpin and helix α7 regions among enzymes in microviridin biosynthetic pathways. D192 (dark red) is well conserved in MdnC-like and MdnB-like homologs. E191 is conserved in MdnC, MdnB and MdnB-like homologs, but is substituted as an aspartate in most other MdnC-like macrocyclases.



Supplementary Figure 13. MdnA variants and their macrocyclizations. (**a**) MdnA$^{11\text{-}49}$ can be recognized and processed by both MdnC and MdnB to form the tri-cyclic product, while (**b**) MdnA$^{Ac20\text{-}49}$ alone is not an active substrate for MdnC. MdnC can be constitutively activated in the presence of MdnA$^{1\text{-}35}$, and catalyze the single cyclization of MdnA$^{Ac20\text{-}49}$. However, the single cyclic product, *in trans* reaction cannot be further converged into di (or tri) cyclic product, neither with an extended reaction time, nor with an increased MdnA$^{1\text{-}35}$ concentration. (**c**) Similarly, leader peptide-free MdnA$^{36\text{-}49}$ alone is not an active substrate for MdnC/MdnB, but can be processed *in trans* into the tricyclic product (37°C, 4h reaction time) with inclusion of MdnA$^{9\text{-}22}$. Corresponding mass extraction traces are displayed in colors (M^{+2} ions).