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Substrate Specificity and Scope of MvdD, a GRASP-like Ligase from the Microviridin Biosynthetic Gene Cluster

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ABSTRACT The cyanobacterial protease inhibitor microviridin K is ribosomally biosynthesized as a prepeptide (MvdE) and subsequently modified posttranslationally by double lactonization followed by lactamization. Two proteins belonging to the GRASP superfamily of ligases catalyze these ring closures. We here show that one of these ligases (MvdD) forms the lactones in a specific order, the larger ring being formed first, and that the ring size requirement for both lactonizations is stringent. However, for the first cyclization MvdD accepts alanine substitution in all C-terminal positions of the microviridin prepeptide that are not directly involved in the cross-linking, whereas the second lactonization is dependent on the presence of specific residues in MvdE. This suggests that MvdD possesses some, albeit limited, substrate tolerance that might be useful for the modification of peptides and proteins not belonging to the microviridin group of metabolites.

In the past 20 years our understanding of nonribosomal peptide biosynthesis has advanced greatly as has been documented in recent reviews (*1, 2*). Progress has also been made with characterization *in vitro* of enzymes responsible for the biosynthesis of peptide natural products of ribosomal origin, particularly the antibacterial peptides of the lactobacilli (*3*) and microcins B17, C7, E492m, and J25 (*4*). An emerging theme in this research is the recruitment of homologues of enzymes from primary metabolism for functions in secondary metabolism (*5, 6*). As more microbial genomes are being sequenced and becoming publicly available, we may expect to encounter new, unexpected biosynthetic mechanisms and biological transformations effected by known classes of enzymes.

We recently reported on the structure and ribosomal origin of microviridin K, a nanomolar inhibitor of elastase, and its biosynthetic gene cluster (*mvd*) from the cyanobacterium *Planktothrix agardhii* CYA 126/8. In the course of that work we had expressed three enzymes of the microviridin pathway that perform posttranslational modifications of the microviridin K prepeptide (MvdE) after its assembly by the ribosome. Specifically, one of these introduces two ester bonds (MvdD) and one an amide bond (MvdC), respectively, into the prepeptide by ligation of ω functional groups. These proteins display homology to members of the GRASP superfamily of N/O/S ligases (*7*). Further maturation of the resulting tricyclic peptide involves cleavage of the leader sequence and capping of the newly exposed N-terminus by acetylation catalyzed by MvdB.

Independently Dittmann *et al.* (*8*) reported results of heterologous expression experiments for microviridin B and J from *Microcystis* sp. In a subsequent commentary on the latter and related work, Moore (*9*) called for an investigation into the mechanism of the cross-linking enzymes to explore their potential for the engineering of new natural products. Additionally, since the posttranslational modifications observed in the microviridins are unprecedented, one could consider the application of the ligases as tools for the introduction of analogous cross-links into substrates unrelated to microviridin, perhaps even proteins. We here report on our findings toward this goal.

In our first communication on the microviridin biosynthetic system, we had been unable to determine through manipulation of incubation conditions the sequence in which the cross-linking ester bonds are introduced into the linear precursor peptide MvdE. At low conversions a monocyclic intermediate could be detected by LCMS in reaction mixtures containing MvdD and ATP, establishing that MvdD is a distributive enzyme, as had been demonstrated for the MccB17 synthetase (*10*), and not a

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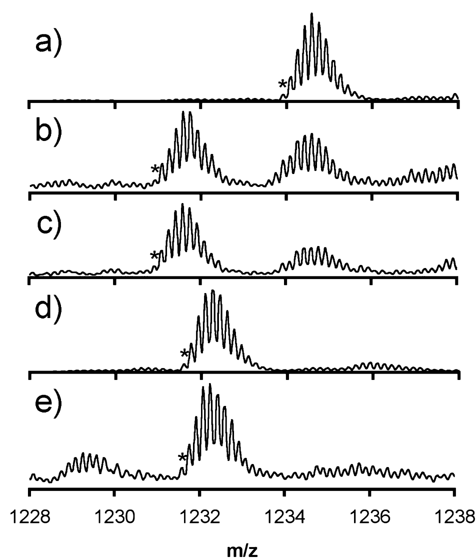

Figure 1. Cross-linking of mutant MvdE by MvdD and MvdC. a) Substrate His$_6$-MvdE-S43A [M + 6H]$^{6+}$ *m*/*z* = 1233.9266; b) His$_6$-MvdE-S43A with MvdD [M + 6H]$^{6+}$ *m*/*z* = 1230.9269; c) His$_6$-MvdE-S43A with MvdD and MvdC [M + 6H]$^{+}$ *m*/*z* = 1230.9258; d) His$_6$-MvdE-T38A [M + 6H]$^{6+}$ *m*/*z* = 1231.6031; e) His$_6$-MvdE-T38A with MvdD and MvdC [M + 6H]$^{6+}$ *m*/*z* = 1231.5883. Asterisk (*) denotes the monoisotopic peak.

processive one, as has been shown for LctM (*11*). Since the monocylic intermediate could not be isolated, we elected to pursue a mutagenesis approach in the C-terminal 14 amino acids of MvdE destined to become microviridin K. We reasoned that replacement of either the serine or the threonine residue, each contributing their β-oxygen to one of the two lactone rings in the natural product, with an amino acid lacking a nucleophilic group in the side chain would result in a pair of mutant substrate molecules, which would differ in their reactivity with MvdD. Thus, if the introduction of the ester bonds follows a specific sequence, one of the two mutant substrates should be unreactive, whereas the other one should undergo one cyclization. If on the other hand the ester bond-forming enzyme MvdD were to introduce these bonds in random order, both mutant substrates should be reactive. In order to establish this experimentally, we cloned MvdE into the pET28b vector, which introduces a N-terminal His$_6$-tag to MvdE during expression in *E. coli* (His$_6$-MvdE) and allows any of the projected mutations to be introduced by site-directed mutagenesis.

In the event, incubation in parallel of the two mutants, His$_6$-MvdE-S43A (Figure 1, panel a) and His$_6$-MvdE-T38A (Figure 1, panel d), with MvdD resulted in the loss of 1 equiv of H_2O from the former (Figure 1, panel b) and no significant reaction of the latter (Figure 1, panel e) as shown by HR-ESI-TOFMS (mass spectrometric data for all experiments are listed in Supplementary Table S-1). This result is compatible with only the model requiring the introduction of the ester bonds in a specific order, namely, formation of the lactone between Asp44 and Thr38 before the formation of the lactone between Glu46 and Ser43 (wild-type numbering).

As we had demonstrated earlier (*6*), the introduction of the two ester bonds precedes that of the amide between the ε-amino group of Lys40 and the δ-carboxyl group of Glu47 (Figure 2, entry a). The production of the monocyclic microviridin K analog His$_6$-MvdE-S43A allowed us to assess whether the monocyclic prepeptide is a substrate for MvdC, the amide-forming enzyme. Addition of the amide-forming ligase MvdC to a reaction mixture containing ATP, His$_6$-MvdE-S43A, and MvdD did not result in lactamization of the monolactone product of MvdD (Figure 1, panel c), even though a parallel incubation with wild-type substrate demonstrated that the MvdC protein used was functional (data not shown).

These results suggest that the cross-linking of the MvdE prepeptide is strictly ordered, in contrast to the multiple pathways of cyclization observed in the biosynthesis of MccB17 (*10*). The presence of both ester bonds may be required before MvdC introduces the amide bond last, although two questions concerning MvdC activity remain open at the present time: First, would the enzyme cyclize a substrate in which the smaller lactone ring, between Glu46 and Ser43, is present while the large lactone between Asp44 and Thr38 is not? Second, is a conformational preorganization of the substrate as a result of the first lactonization a prerequisite for the second cyclization? To answer the former a method must be developed that will allow a suitable substrate to be prepared. The answer to the latter hinges on the expression level of soluble MvdD, which presently is not such that the required amount of a monocyclic MvdE derivative could be obtained in a cell-free system that would allow the structure of this intermediate to be studied by NMR.

Next it was of interest to determine whether MvdD possesses any flexibility with respect to the size of the lactone rings it is capable of forming. For this purpose a triple mutant was constructed on the basis of the reactive mutant substrate His$_6$-MvdE-S43A. The first bore additional N37T and T38A mutations, in effect moving the nucleophilic residue for the lactone formation one amino acid residue toward the N-terminus (see Figure 2, entry d) creating a 27-membered lactone rather than the 23-

	expected	observed	MvdC
a)	LP-Y^35GNTMKY^41PSDWEEY^48	LP-Y^35GNTMKY^41PSDWEEY^48	+
b)	LP-Y^35GNAMKY^41PSDWEEY^48	LP-Y^35GNAMKY^41PSDWEEY^48	+
c)	LP-Y^35GNTMKY^41PADWEEY^48	LP-Y^35GNTMKY^41PADWEEY^48	+
d)	LP-Y^35GTAMKY^41PADWEEY^48	LP-Y^35GTAMKY^41PADWEEY^48	+
e)	LP-Y^35GNATKY^41PADWEEY^48	LP-Y^35GNATKY^41PADWEEY^48	+
f)	LP-Y^35GNTMKY^41SADWEEY^48	LP-Y^35GNTMKY^41SADWEEY^48	-
g)	LP-Y^35GNTMKY^41PSDEAEY^48	LP-Y^35GNTMKY^41PSDEAEY^48	-
h)	LP-A^35AATAKA^41ASDAEEA^48	LP-A^35AATAKA^41ASDAEEA^48	+
i)	LP-A^35AATAKA^41PSDAEEA^48	LP-A^35AATAKA^41PSDAEEA^48	-
j)	LP-A^35AATAKA^41ASDWEEA^48	LP-A^35AATAKA^41ASDWEEA^48	-
k)	LP-A^35AATAKA^41PSDWEEA^48	LP-A^35AATAKA^41PSDWEEA^48	-
l)	LP-A^35AATAKY^41PSDAEEA^48	LP-A^35AATAKY^41PSDAEEA^48	+
m)	LP-A^35AATAKY^41PSDWEEA^48	LP-A^35AATAKY^41PSDWEEA^48	+
n)	LP-A^35AATAKY^41ASDAEEA^48	LP-A^35AATAKY^41ASDAEEA^48	+

LP = GSSHHHHHHSSGLVPRGSH-
MSKNVKVSAPKAVPFFARFLAEQAVEANNSNSAP

Figure 2. Schematic representation of the important substrates tested in this study using one-letter amino acid codes. a) His$_6$-MvdE wild-type; b) His$_6$-MvdE-T38A; c) His$_6$-MvdE-S43A; d) His$_6$-MvdE-N37TT38AS43A; e) His$_6$-MvdE-T38AM39TS43A; f) His$_6$-MvdE-P42SS43A; g) His$_6$-MvdE-W45EE46A; h) His$_6$-MvdE-Ala$_8$; i) His$_6$-MvdE-Ala$_7$PSD; j) His$_6$-MvdE-Ala$_7$SDW; k) His$_6$-MvdE-Ala$_6$PSDW; l) His$_6$-MvdE-Ala$_6$YPSD; m) His$_6$-MvdE-Ala$_5$YPSDW; n) His$_6$-MvdE-Ala$_6$YASDA. (LP = His$_6$-tag from pET28b in green and native MvdE leader peptide sequence in black). Mutated residues are shown in red, putative lactones introduced by MvdD are indicated by a tie bar on top of string, and the lactam created by MvdC is shown below string. Residues are numbered with superscript with respect to position in native MvdE. Inclusion of MvdC in the assay mix is indicated by + sign.

membered lactone characteristic of the wild-type MvdE. However, this modified substrate was completely unreactive. The second triple mutant, His$_6$-MvdE-T38AM39TS43A, which would result in a smaller, 20-membered lactone ring, met with a similar fate: no cyclization was observed when the mutant substrate was incubated with MvdD (Figure 2, entry e). Similarly, the ring size of the smaller, 15-membered lactone ring proved not to be amenable to modification; attempted cyclization with MvdD of His$_6$-MvdE-P42SS43A (Figure 2, entry f) resulted in monocyclization only (Supplementary Figure S-2), no trace of a bicyclic product bearing an 18-membered ring being observed. A His$_6$-MvdE-W45EE46A mutant substrate, which was expected to yield a product with a 12-membered ring, was apparently a poor substrate for MvdD and resulted in a minimal amount of cyclized product (Figure 2, entry g; Supplementary Figure S-3). The use of an alanine scan, the approach chosen for this investigation, must necessarily result in disruption of the motif of two vicinal acidic residues in positions 46 and 47 (MvdE numbering) that is conserved across all microviridins, and this may be the reason for the observed lack of reactivity. Moreover, a W45E mutation will place two acidic residues next to each other (Asp44/Glu45). MvdD forms both lactones sequentially and in the wild-type substrate must distinguish between an isolated acidic residue (Asp44) and a pair of acidic residues (Glu46/Glu47) to form the large and small ring, respectively, with apparent high selectivity. It may therefore not be altogether surprising that a mutant substrate in which the sequence of isolated and vicinal acidic residues is reversed is not a viable substrate for the formation of either ring. An alternative approach was briefly considered: cyclization of a His$_6$-MvdE-W45E mutant substrate, which would place four acidic residues next to each other. MvdD could then perform lactonizations between Ser43 and either Glu45 or Glu46. However, this approach poses formidable analytical challenges as the two products would likely not be separable chromatographically and not be distinguishable by mass spectrometry. This avenue was therefore not pursued.

A His$_6$-MvdE-T38S mutant substrate proved to be a fully viable substrate in assays with combinations of MvdD and MvdC. This mutation does not affect the ring size of the lactone, only the character of the nucleophilic amino acid. Surprisingly, this mutant substrate (see Supplementary Figure S-4, panel a) appeared to react more slowly

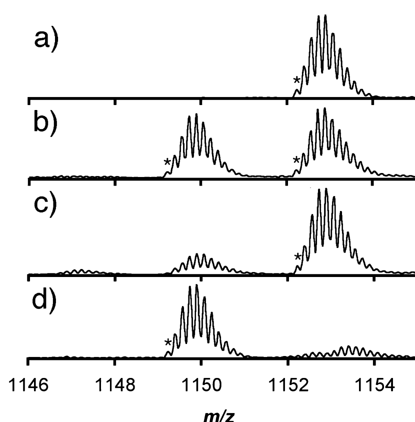

Figure 3. Time-dependent cyclization of His_6-MvdE-Ala_8 by MvdD under modified conditions. a) Substrate alone [His_6-MvdE-Ala_8 + 6H]$^{6+}$ $m/z = 1152.2347$; b) His_6-MvdE-Ala_8 with MvdD (pH 8.0, 37 °C, 12 h) [His_6-MvdE-Ala_8 − H_2O + 6H]$^{6+}$ $m/z = 1149.2313$, [His_6-MvdE-Ala_8 + 6H]$^{6+}$ $m/z = 1152.2380$; c) His_6-MvdE-Ala_8 with MvdD (pH 8.0, 37 °C, 36 h) [His_6-MvdE-Ala_8 + 6H]$^{6+}$ $m/z = 1152.2337$; d) His_6-MvdE-Ala_8 with MvdD 16 h (pH 7.0, 22 °C, 16 h) [His_6-MvdE-Ala_8 -H_2O + 6H]$^{6+}$ $m/z = 1149.2277$. Asterisk (*) denotes the monoisotopic peak.

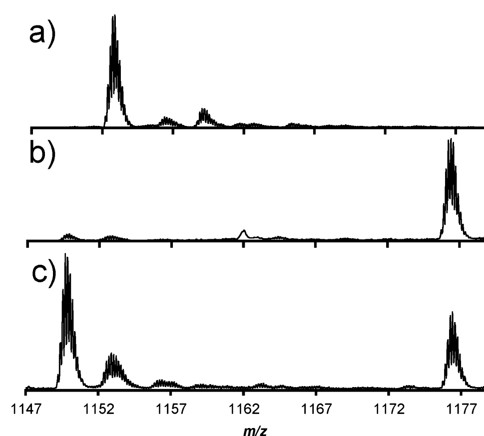

Figure 4. Conversion of a mixture of His_6-MvdE-Ala_8 and His_6-MvdE-Ala_6PSDW by MvdD. a) [M + 6H]$^{6+}$ pseudomolecular ion of His_6-MvdE-Ala_8; b) [M + 6H]$^{6+}$ pseudomolecular ion of His_6-MvdE-Ala_6PSDW; c) [M + 6H]$^{6+}$ pseudomolecular ions of products after incubation with MvdD (pH 7.0, 22 °C, 20 h). Cyclization is indicated by a shift to lower mass (−3 Da).

with MvdD than the wild-type substrate, even though in the latter the nucleophilic group is secondary rather than primary (Supplementary Figure S-4, panels b and c). This observation, in light of later results (*vide infra*), suggested that possibly the cyclized product was not stable and hydrolyzed back to starting material. We therefore conducted cyclization reactions of His_6-MvdE-T38S catalyzed by MvdD and MvdC under modified conditions (pH 7.0, 22 °C), which resulted in clean cyclization to a tricyclic product (Supplementary Figure S-4, panel d).

Another set of experiments was aimed at delineating the substrate specificity of MvdD with respect to the amino acids surrounding the cross-linking sites within MvdE. To this end we performed a global substitution of all eight amino acids in the C-terminal portion of His_6-MvdE not involved in the cross-linking reaction catalyzed by MvdD/MvdC and destined to be part of the mature microviridin K (Figure 2, entry h), while amino acids in the leader peptide were not substituted. This mutant substrate, His_6-MvdE-Ala_8 (Figure 3, panel a), was subject to cyclization by MvdD, resulting in approximately 50% conversion to a monocyclic product after 12 h (Figure 3, panel b). However, the product was not stable under standard incubation conditions (100 mM Tris buffer pH 8.0, 37 °C) and apparently reverted back to starting material over the course of a further 24 h (Figure 3, panel c). Thus, the apparent low conversion at 12 h may reflect some reversion to starting material rather than a low intrinsic reaction rate of the mutant substrate. Under modified incubation conditions of neutral pH and reduced temperature (22 °C), His_6-MvdE-Ala_8 again only undergoes monocyclization. However, the product is apparently stable under these conditions (Figure 3, panel d) as nearly complete conversion is observed after 16 h. Bicyclic product can be detected only at levels close to mass spectrometric limits of detection if at all (data not shown), independent of pH and temperature.

In a next round, mutant substrates were created in which the proline and the tryptophan, located in the conserved PSDW motif found in most microviridins, were reintroduced into the Ala_8 mutant. In three reactions conducted in parallel, the three mutants, His_6-MvdE-Ala_7PSD, His_6-MvdE-Ala_7SDW, and His_6-MvdE-Ala_6PSDW (see Figure 2, entries i−k), were individually subjected to cyclization by MvdD. Only the His_6-MvdE-Ala_7PSD substrate was monocyclized to a similar degree as the His_6-MvdE-Ala_8 substrate (Figure 2, entry i) that was used in a control incubation conducted in parallel. On the other hand, the His_6-MvdE-Ala_7SDW mutant substrate was repeatedly found to be much less reactive (≤5% conversion) than His_6-MvdE-Ala_8, and His_6-MvdE-Ala_6PSDW proved to be completely unreactive (data not shown). In order to prove that this observation is significant, we repeated the incubation, this time using the His_6-MvdE-Ala_8 mutant substrate as an internal standard in the reaction mixture. The His_6-MvdE-Ala_8 substrate (Figure 4, panel a) was cyclized to the monocyclic product (Figure 4, panel c), whereas the His_6-MvdE-Ala_6PSDW substrate (Figure 4, panel b) remained untouched (Figure 4, panel c). A co-incubation of His_6-MvdE-Ala_8 and His_6-MvdE-Ala_7SDW with MvdD resulted in a low (≤5%) but detectable conversion of the latter substrate when compared to

that of the former (see Supplementary Figure S-5). Considering that the His_6-MvdE-Ala_8 mutant undergoes clean cyclization, the lack of reactivity of two mutant substrates that contain the conserved Pro42 and highly conserved Trp45 residues is a confounding result. It is noteworthy that the rate of cyclization of His_6-MvdE-Ala_8 is reduced in the presence of either the His_6-MvdE-Ala_7PSD or the His_6-MvdE-Ala_6PSDW mutant substrate, which suggests that the latter bind to MvdD and that the reason for lack of cyclization must lie elsewhere. We next introduced Tyr41 back into the His_6-MvdE-Ala_7PSD and the His_6-MvdE-Ala_6PSDW mutants, yielding mutant substrates His_6-MvdE-Ala_6YPSD and His_6-MvdE-Ala_5YPSDW, respectively (see Figure 2, entries l and m). Both substrates underwent clean cyclization in the presence of MvdD to yield bicyclic products, which could be cyclized further to fully tricyclic products through the action of MvdC under standard conditions (see Supplementary Figure S-6). Thus, Tyr41 appears to be required for the first cyclization to occur when Trp45 is present in the substrate but not in its absence. These results will require a mechanistic explanation that is beyond the present approach and would probably require conformational analysis of the substrate by spectroscopic techniques. Last we assessed the role of Pro42 in triggering the second lactonization by creation of the variant His_6-MvdE-Ala_6YASD (Figure 2, entry n). Incubation with MvdD and MvdC resulted in the observation of only the monocyclic product (Supplementary Figure S-7). This indicates that the presence of both Tyr41 and Pro42 is required for double lactonization to occur, a result that underscores the need for structural characterization of the cyclization intermediates.

Considered together, these data suggest that for the first lactonization there appears to be some flexibility with respect to the identity of the amino acids surrounding the reactive sites in the C-terminal portion of MvdE involved. However, the stability of the resulting product to hydrolysis may become an issue as in the all-Ala mutant substrate where small alanine residues had substituted mostly large, hydrophobic residues such as tyrosine, tryptophan, and methionine. The alanine residues may not as effectively shield the relatively labile lactone bond from attack by water. The instability to hydrolysis of this Ala_8 mutant peptide also may explain the apparent reduced cyclization rate of the His_6-MvdE-T38S mutant. In this case as well, enzyme-catalyzed cyclization, hydrolysis, and enzyme denaturation may be in competition, giving the appearance of a reduced reaction rate (Supplementary Figure S-4).

In our previous report (*6*) we had proven through reductive quench and subsequent degradation the chemical nature and location of the functional group being formed upon dehydration. Moreover, we showed that the ester bonds were located in the positions one would expect from the NMR structure of microviridin K and not, for instance, in the leader peptide. In the present work we argue by analogy without repeating the chemical proof in every instance. This is justified since the His_6-MvdE-T38A substrate is not dehydrated, whereas the His_6-MvdE-S43A substrate is reactive. If dehydrations were occurring within either the leader peptide or the His_6-tag sequence, both mutants would be reactive. However, given the large change introduced into the His_6-MvdE-Ala_8 mutant by the substitution of eight amino acids by alanine, we chose to apply our reductive quench method to the product of cyclization of His_6-MvdE-Ala_8. Thus, the monocyclic product from the cyclization of His_6-MvdE-Ala_8 after reduction with $LiBH_4$ in MeOH yielded a product that had gained four hydrogen atoms ($2H^- + 2H^+$) as suggested by high resolution mass spectrometry (data not shown). This change in mass is in accord with the reductive ring opening of a monolactone rather than the conjugate reduction of one dehydroalanine/dehydrobutyrate residue (see ref 6 for the full argument). Analysis of the reduced product by LC-MS/MS localized the reduced residue to Asp44 as would be expected if the Thr-Asp bond had been formed, as postulated, and subsequently reduced (Supplementary Figure S-8).

The experiment with His_6-MvdE-Ala_8 also serves as a valuable control for the experiments in which we moved the nucleophilic threonine residue in either the C-terminal or N-terminal direction (Figure 2, entries d and e). The lack of cyclization observed in these experiments could be ascribed to the requirement for specific amino acids to surround the nucleophilic serine or threonine involved in lactone formation, in analogy to sequence requirements surrounding cleavage sites of proteases or to suppression of dehydration in lantibiotic biosynthesis by glycine substitution (*12*). However, MvdD accepts the His_6-MvdE-Ala_8 mutant with an Ala residue to either side of the nucleophilic Thr38 as a substrate for one cyclization. Moreover, for the first lactonization separate mutations to alanine residues are tolerated to either side of the acceptor amino acid, Asp44, (Figure 2, entry f). Thus, a putative sequence requirement on either the donor or the acceptor side cannot be the reason for the observed failure of lactonization of the mutants that were created to study the effect of ring size.

In conclusion, we have shown here that the cross-linking of MvdE and its analogs is strictly ordered with the formation of the large lactone ring between Thr38 and Asp44 occurring first. Second, the presence of the lactone between Ser43 and Glu46 in the substrate is required for MvdC, the lactam-forming enzyme, to be active. Third, we have shown that the ring size of both lactones is not flexible. Last, MvdD appears to be able to accommodate amino acids other than those found in the native substrate in positions surrounding the reacting amino acids involved in the formation of the first lactone.

METHODS

His$_6$-MvdE Expression Construct. See Supporting Information.

Overexpression of Constructs. Plasmids containing the correct sequence were transformed into *E. coli* BL21(DE3) cells and plated on LB agar with kanamycin. A single colony was inoculated into 25 mL of LB media plus kanamycin and grown overnight at 37 °C with shaking at 220 rpm. Overnight culture (5 mL) was inoculated into 500 mL of fresh LB media plus kanamycin and grown at 30 °C until $OD_{600} \approx 0.6-0.8$ (~3.5 h). IPTG was then added to a final concentration of 0.1 mM, and the cultures were grown for an additional 4 h at 30 °C. Cells were collected by centrifugation and stored at −20 °C until use (typically <1 week).

Cells were defrosted on ice and resuspended in 7 mL of lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 5 mM imidazole). Cells were lysed by sonication, and the supernatant was cleared by centrifugation (10,000 rpm, 30 min, 4 °C). The supernatant was transferred to a new tube and was recentrifuged (15,000 rpm, 15 min, 4 °C) to remove residual debris. Cleared supernatant was then incubated with 0.25 mL of Ni-NTA resin, which had been previously washed with 2 mL of 20 mM Tris (pH 7.5) for 1 h at 4 °C with gentle rocking. The resin was then loaded onto a Poly prep column and drained, followed by washing with 2.5 mL of lysis buffer containing 5 mM β-mercaptoethanol and then 2 × 1 mL of buffer 2 (50 mM Tris pH 8.0, 300 mM NaCl, 25 mM imidazole, 5 mM β-mercaptoethanol). The His$_6$-MvdE substrate was eluted with 0.5 mL buffer 3 (50 mM Tris pH 8.0, 300 mM NaCl, 250 mM imidazole, 5 mM β-mercaptoethanol). The MvdE substrates were then heated to 95 °C for 15 min followed by centrifugation (15,000 rpm, 15 min/4 °C), and the cleared supernatant was stored at 4 °C and used without further purification within 2 days of isolation to minimize degradation.

MvdD Activity Assay. Cyclization and isolation of products were performed as previously described (*6*).

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Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

REFERENCES

1. Fischbach, M. A., and Walsh, C. T. (2006) Assembly-line enzymology and non-ribosomal peptide antibiotics: logic, machinery and mechanisms, *Chem. Rev. 106*, 3468–3496.
2. Sattely, E. S., Fischbach, M. A., and Walsh, C. T. (2008) Total biosynthesis: *in vitro* reconstitution of polyketide and nonribosomal peptide pathways, *Nat. Prod. Rep. 25*, 757–793.
3. Chatterjee, C., Paul, M., Xie, L., and van der Donk, W. A. (2005) Biosynthesis and mode of action of lantibiotics, *Chem. Rev. 105*, 633–684.
4. Nolan, E. M., and Walsh, C. T. (2009) How nature morphs peptide scaffolds into antibiotics, *ChemBioChem 10*, 34–53.
5. Fortin, P. D., Walsh, C. T., and Magarvey, N. A. (2007) A transglutaminase homologue as a condensation catalyst in antibiotic assembly lines, *Nature 448*, 824–827.
6. Philmus, B., Christiansen, G., Yoshida, W. Y., and Hemscheidt, T. K. (2008) Posttranslational modification in microviridin biosynthesis, *ChemBioChem 9*, 3066–3073.
7. Galperin, M. Y., and Koonin, E. V. (1997) A diverse family of enzymes with ATP-dependent carboxylate-amine/thiol ligase activity, *Protein Sci. 6*, 2639–2643.
8. Ziemert, N., Ishida, K., Liaimer, A., Hertweck, C., and Dittmann, E. (2008) Ribosomal biosynthesis of tricyclic depsipeptides from bloom-forming cyanobacteria, *Angew. Chem., Int. Ed. 47*, 7756–7759.
9. Moore, B. S. (2008) Extending the biosynthetic repertoire in ribosomal peptide assembly, *Angew. Chem., Int. Ed. 47*, 9386–9388.
10. Kelleher, N. L., Hendrickson, C. L., and Walsh, C. T. (1999) Posttranslational heterocyclization of cysteine and serine residues in the antibiotic Microcin B17: distributivity and directionality, *Biochemistry 38*, 15623–15630.
11. Miller, L. M., Chatterjee, C., van der Donk, W. A., and Kelleher, N. L. (2006) The dehydratase activity of lacticin 481 synthetase is highly processive, *J. Am. Chem. Soc. 128*, 1420–1421.
12. Chatterjee, C., Patton, G. C., Cooper, L., Paul, M., and van der Donk, W. A. (2006) Engineering dehydro amino acids and thioethers into peptides using lacticin 481 synthetase, *Chem. Biol. 13*, 1109–1117.