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Mechanistic Studies on the Substrate-Tolerant Lanthipeptide Synthetase ProcM

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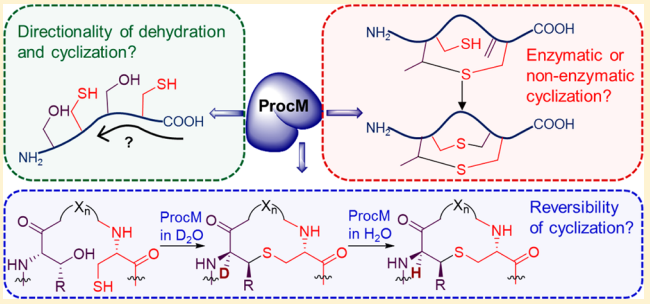
Supporting Information

ABSTRACT: Lanthipeptides are a class of post-translationally modified peptide natural products. They contain lanthionine (Lan) and methyllanthionine (MeLan) residues, which generate cross-links and endow the peptides with various biological activities. The mechanism of a highly substrate-tolerant lanthipeptide synthetase, ProcM, was investigated herein. We report a hybrid ligation strategy to prepare a series of substrate analogues designed to address a number of mechanistic questions regarding catalysis by ProcM. The method utilizes expressed protein ligation to generate a C-terminal thioester of the leader peptide of ProcA, the substrate of ProcM. This thioester was ligated with a cysteine derivative that resulted in an alkyne at the C-terminus of the leader peptide. This alkyne in turn was used to conjugate the leader peptides to a variety of synthetic peptides by copper-catalyzed azide−alkyne cycloaddition. Using deuterium-labeled Ser and Thr in the substrate analogues thus prepared, dehydration by ProcM was established to occur from C-to-N-terminus for two different substrates. Cyclization also occurred with a specific order, which depended on the sequence of the substrate peptides. Furthermore, using orthogonal cysteine side-chain protection in the two semisynthetic peptide substrates, we were able to rule out spontaneous non-enzymatic cyclization events to explain the very high substrate tolerance of ProcM. Finally, the enzyme was capable of exchanging protons at the α-carbon of MeLan, suggesting that ring formation could be reversible. These findings are discussed in the context of the mechanism of the substrate-tolerant ProcM, which may aid future efforts in lanthipeptide engineering.



INTRODUCTION

Lanthipeptides are a class of ribosomally synthesized and post-translationally modified peptides (RiPPs[1]) that contain the characteristic thioether residues lanthionine (Lan) and methyllanthionine (MeLan).[2,3] A subclass of lanthipeptides with antibacterial activity is known as lantibiotics,[4] which are effective against many Gram-positive bacteria including some drug-resistant species.[5] For instance, nisin has been used for over 50 years as a preservative in the food industry to combat food-borne pathogens without significant bacterial resistance.[6] Lanthipeptides that do not display antibacterial activity can exert antiviral,[7] antiallodynic,[8] antinociceptive,[9] and morphogenic[10] activities. Cyclic peptides such as lanthipeptides are also increasingly recognized as promising compounds for disrupting protein−protein interactions.[11−16] Investigation of the synthetases that post-translationally generate lanthipeptides would aid in engineering efforts to produce molecules with desirable properties.[17−19]

During lanthipeptide biosynthesis, the ribosomal machinery first synthesizes linear precursor peptides called LanAs.[3] The LanA peptide comprises an N-terminal leader peptide that is believed to serve several possible roles, including recognition by the synthetase,[20,21] and a C-terminal core peptide that is post-translationally modified. Proteolytic cleavage then removes the leader peptide to produce the final lanthipeptide (Figure 1A).[1] Lan and MeLan formation involves first the dehydration of Ser and Thr residues to generate dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. The dehydrated residues then undergo Michael-type addition by the side-chain thiol of cysteines to generate the thioethers Lan and MeLan, respectively (Figure 1B).[22]

Prochlorosins are a large group of lanthipeptides produced by marine cyanobacteria of the genus *Prochlorococcus*. In *Prochlorococcus* MIT9313, a single enzyme, ProcM, catalyzes the post-translational modification of 29 different substrates (ProcAs), thereby generating many distinct thioether ring topologies within the 29 prochlorosin products (Pcns; Supporting Information Figure S1).[23] As such, the Pcn biosynthetic system is intriguing with respect to the details of thioether ring formation that may explain the remarkable diversity of the products formed by ProcM. A previous study suggested that the enzyme might generate a subset of the thioether rings, which would then preorganize the substrate to facilitate non-enzymatic cyclization of the other rings,[23] as illustrated schematically for a substrate with two thioether rings

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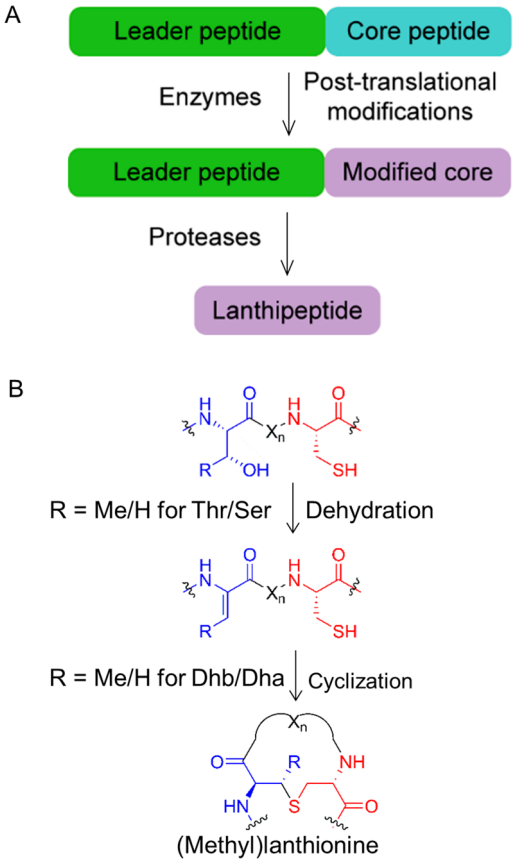

Figure 1. (A) Generic scheme for lanthipeptide biosynthesis. (B) Common post-translational modifications in lanthipeptides.

in Figure S2. Investigation of the stereochemistry of the thioether rings in a subset of Pcns showed that they all had the DL stereochemistry (2*S*,6*R*-Lan and 2*S*,3*S*,6*R*-MeLan),[24] identical to what has been observed for the majority of lanthipeptides analyzed to date.[22] Though this conserved stereochemistry of Michael-type addition suggested enzymatic cyclization, non-enzymatic cyclization of preorganized intermediates with high stereoselectivity could not be ruled out because in previous biomimetic studies of lanthipeptide biosynthesis non-enzymatic cyclization also took place with high selectivity for the DL stereoisomers.[25−27] In this study, we experimentally probed a potential role of non-enzymatic cyclization in prochlorosin maturation.

The substrate-tolerant synthetase ProcM dehydrates core peptides containing a variety of sequences with different residues flanking Ser and Thr and also with different numbers of intervening residues (Figure S1). Currently, it is not known whether ProcM dehydrates its substrates in a directional fashion and whether any observed directionality is general for different substrates. In this study, we expanded upon a previously reported strategy[28] to assign the directionality of dehydration of substrates by ProcM. Additional experiments also revealed the order of ProcM-catalyzed cyclization of these substrates.

In the 29 prochlorosins with diverse ring topologies produced by ProcM, often a single ring structure out of several possible topologies is observed (Figure S1B).[23,24] The observed high site selectivity in prochlorosin cyclization could be the result of either thermodynamic or kinetic control. Thermodynamic control would require the reversible installation of thioether rings, which ultimately results in the ring topology with the lowest free energy. On the other hand, kinetic control would lead to thioether ring formation involving the lowest activation energy barrier. A previous study on the biomimetic cyclization of nisin's B-ring suggested that non-enzymatic thioether ring formation is governed by kinetic control and that the Michael-type addition is irreversible.[29] In the current study, the reversibility of cyclization in the presence of ProcM was investigated.

RESULTS

Choice of Substrates. Two core peptides were chosen for investigation out of the repertoire of 29 possible substrates. The first choice was ProcA2.8, which is transformed into prochlorosin (Pcn) 2.8, a product with two non-overlapping lanthionine rings (Figure 2A). In the majority of the

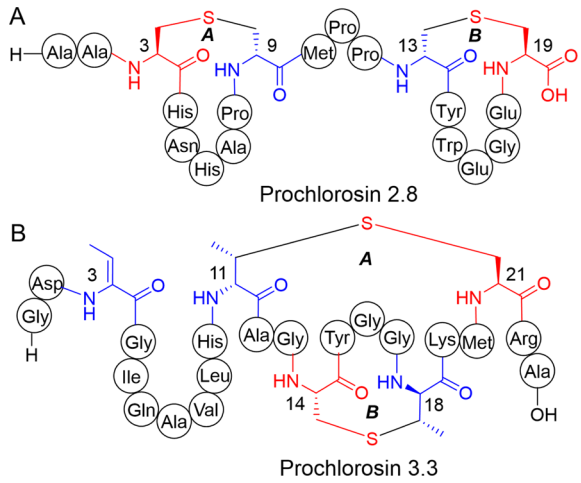

Figure 2. Structures of prochlorosins 2.8 and 3.3. The fragments of the Lan/MeLan residues originating from Cys are in red, and those originating from Ser/Thr residues are in blue.

lanthipeptides discovered to date, the thioether rings are installed by nucleophilic attack of cysteines onto dehydrated residues that are located toward the N-terminus. However, in prochlorosins, the thioether rings are formed by Cys residues located on either side of the dehydrated residues, as illustrated for Pcn2.8 generated from ProcA2.8 (Figure 2; see also Figure S1). Thus, studies with Pcn2.8 could reveal whether perhaps enzymatic cyclization forms rings in one direction and non-enzymatic cyclization forms thioether rings in the opposite direction. In addition, we selected Pcn3.3, a compound containing overlapping thioether rings, to probe the effect of substrate preorganization on non-enzymatic ring formation (Figure 2B). As described below, for both substrates, we determined the order of dehydration and cyclization and investigated the possibility of non-enzymatic cyclization and reversibility as determinants of the ring topology.

ProcM Dehydrates ProcA2.8 Precursor Peptide in C-to-N-Terminal Fashion. Previous studies on LanM lanthipeptide synthetases showed that dehydration is directional, moving from the N-terminus to the C-terminus of the substrate.[30] To investigate whether this would also be the case for the substrate-tolerant ProcM, we used the method developed by Süssmuth and co-workers,[28] in which specific Ser residues are replaced by 2,3,3-deuterium-labeled serine. In such substrates, dehydration of unlabeled Ser involves loss of 18 Da

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($-H_2O$) while dehydration of the labeled serine results in loss of 19 Da ($-HDO$; Figure 3A,B). The lanthipeptide synthetase

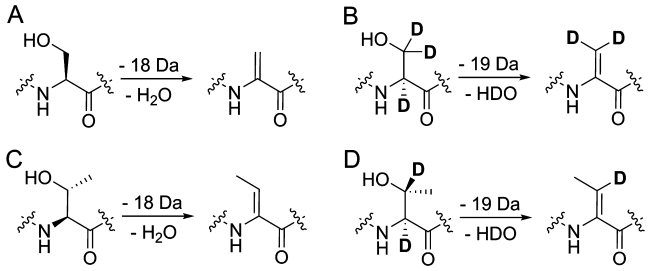

Figure 3. Strategy for determination of directionality of dehydration using [2,3,3-^{2}H]-Ser or [2,3-^{2}H]-Thr residues. (A) Dehydration of Ser incurs a loss of 18 Da. (B) Dehydration of labeled Ser results in a loss of 19 Da. (C) Dehydration of Thr incurs a loss of 18 Da. (D) Dehydration of labeled Thr results in a loss of 19 Da. By tracking which dehydration involves a loss of 19 Da, directionality of dehydration can be established.[28]

ProcM requires an N-terminal leader peptide for maturation of the precursor peptide.[23] The highly conserved leader peptide is ca. 65 amino acid residues long, and the highly variable core peptides comprise between 13 and 32 amino acid residues (Figure S1), resulting in a precursor peptide that is too long to prepare conveniently by linear solid phase peptide synthesis (SPPS). Instead, we used expressed protein ligation (EPL)[31] to generate full-length precursor peptides containing deuterium-labeled serine. The ProcA2.8 core peptide has a cysteine at position 3 that can be used for EPL. Peptide **1** corresponding to the ProcA2.8 leader peptide with two additional Ala residues from the N-terminus of the ProcA2.8 core peptide was generated with a peptide thioester at the C-terminus using intein chemistry (Figure 4). Two ProcA2.8 core peptides **2** and **3** spanning residues 3−19 were synthesized by SPPS with Ser13 or Ser9 replaced with 2,3,3-deuterium-labeled Ser (Figure 4). Native chemical ligation of **2** and **3** with ProcA2.8 leader-AA-thioester **1** afforded the substrates **4** and **5** (Figure 5A,C). These substrates were treated with ProcM, and after various time points, ProcM was removed from a portion of the assay by ultrafiltration. The filtrate was incubated with endoproteinase GluC to remove most of the leader peptide, and the digest was analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and electrospray ionization (ESI) mass spectrometry. With both substrates, the ESI and MALDI-TOF mass spectrometric analyses showed that Ser13 was dehydrated prior to Ser9 (Figure 5B,D and Figures S3 and S4).

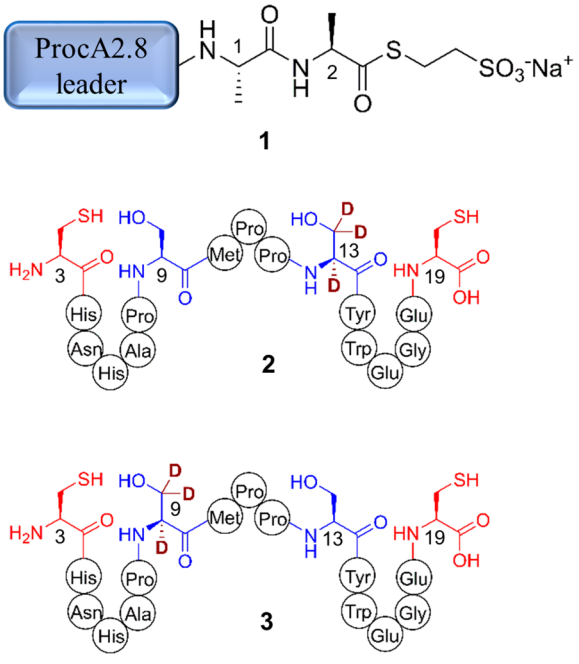

Figure 4. Schematic representation of ProcA2.8 leader-AA-MESNa thioester (**1**), ProcA2.8 core peptide Δ1−2 with Ser13 replaced with [2,3,3-^{2}H]-Ser (**2**), and ProcA2.8 core peptide Δ1−2 with Ser9 replaced with [2,3,3-^{2}H]-Ser (**3**).

Although EPL worked well to determine the directionality of dehydration of ProcA2.8, other substrates do not always have a conveniently located Cys. Hence, we evaluated another hybrid ligation strategy based on both EPL and copper-catalyzed alkyne−azide cycloaddition (CuAAC).[32,33] In previous studies on lacticin 481 synthetase, an alkyne-containing leader peptide was generated by chemical synthesis,[34,35] but in this work, we elected to generate the much longer ProcA leader peptide by heterologous expression in *Escherichia coli* as a fusion protein to an intein and chitin-binding domain.[36] Given the high-sequence similarity of the leader peptides of prochlorosin precursors (Figure S1), the ProcA3.2 leader peptide was arbitrarily chosen for the designed precursor peptides. The C-terminal Gly of the ProcA3.2 leader peptide was mutated to Lys to allow efficient cleavage of the peptide thioester linkage to the chitin resin by 2-mercaptoethanesulfonate sodium salt (MESNa),[37] and to introduce a LysC endoproteinase cleavage site C-terminal to the leader peptide. EPL of the MES-thioester with (*R*)2-amino-*N*-(but-3-yn-1-yl)-3-mercaptopropionamide (**6**, Figure 6) generated the leader peptide with a C-terminal alkyne modification (Figure S5). The cysteine residue incorporated during EPL was protected with iodoacetamide to prevent any potential interference in the enzymatic cyclization reaction (Figure S5).

The core peptides of ProcA2.8 were then synthesized by SPPS, again incorporating deuterium-labeled Ser at positions 9 and 13. In the last step prior to cleaving the peptides from the resin, the CuAAC ligation handles **7** or **8** (Figure 6) were coupled to their N-termini. The building block **7** would enable convenient removal of the leader peptide by photolysis,[38] whereas building block **8** was expected to improve the efficiency of the ligation owing to the copper ligating ability of the pyridine ligand.[39] Two ProcA2.8 precursor peptides **9** and **10** were thus synthesized by CuAAC to probe directionality of dehydration (Figure 7A,C). The substrates were treated with ProcM, and at several time points, ProcM was removed from a portion of the assay by ultrafiltration; the filtrate was incubated with protease LysC and the digest analyzed by MALDI-TOF MS (Figures S6 and S7). With both substrates **9** and **10**, the analysis showed that Ser13 was dehydrated prior to Ser9, indicating C-to-N-terminal dehydration (Figures 7B,D). This observation demonstrated that the presence of a triazole linker in the designed substrate did not affect the directionality of dehydration. Furthermore, GC−MS analysis of the product revealed the presence of Lan rings of the same DL stereochemistry as in the wild-type (wt) product (Figure S8), thus suggesting that ProcM correctly recognizes the substrate with a triazole linker between leader and core peptides. The observation that the substrate analogue generated by CuAAC furnished the same product and with the same directionality as with native substrate enabled us to extend our

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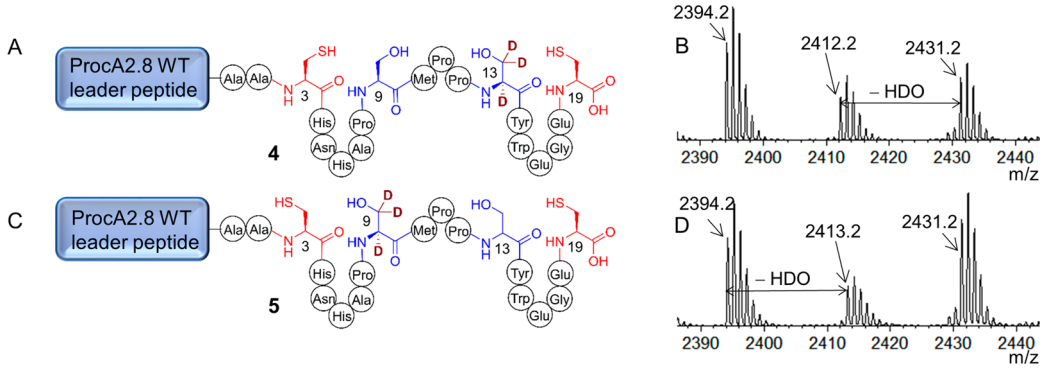

Figure 5. Directionality of dehydration of wt ProcA2.8. (A) Structure of ProcA2.8 precursor peptide **4** assembled using EPL with Ser13 replaced with [2,3,3-^{2}H]-Ser. (B) MALDI-TOF MS of **4** partially dehydrated by ProcM and digested by GluC. (C) Structure of ProcA2.8 precursor peptide **5** assembled using EPL with Ser9 replaced with [2,3,3-^{2}H]-Ser. (D) MALDI-TOF MS of **5** partially dehydrated by ProcM and digested by GluC.

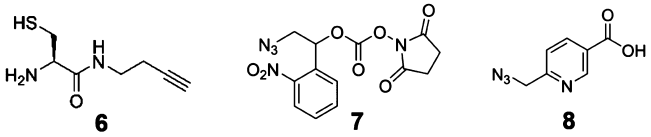

Figure 6. Structure of building block **6** used to attach an alkyne at the C-terminus of the leader peptide by EPL. Building block **7** was used for installation of a photocleavable triazole linker between the leader and the core peptides. The building block **8** was used to facilitate the click reaction via a copper-chelating group.

investigation of directionality of dehydration to other substrates where the sequence of the core peptide does not allow EPL to assemble the native precursor peptide.

Directionality of Dehydration of ProcA3.3 by ProcM. To probe directionality of dehydration in prochlorosins containing threonine residues and overlapping rings, we prepared the ProcA3.3 derivatives **12** and **13** with either Thr18 or Thr11 replaced with [2,3-^{2}H]-Thr, respectively (Figure 8A,C). The peptides were treated with ProcM and after various time points, the enzyme was removed by ultrafiltration. The filtrate was incubated with endoproteinase GluC to remove most of the leader peptide, and the digest was analyzed by MALDI-TOF MS. The data demonstrated that Thr18 was dehydrated first followed by Thr11 and Thr3 (Figure 8B,D and Figures S9 and S10), in agreement with the overall C-to-N-terminal dehydration observed with ProcA2.8.

To confirm that the observed directionality of dehydration of wt ProcA3.3 was the same as that of the analogues **12** and **13**, tandem MS was used to analyze partially dehydrated wild-type ProcA3.3. The order of dehydration of Thr11 and Thr18 cannot be distinguished by this method because of the overlapping rings of prochlorosin 3.3, but the timing of dehydration of Thr3 can be determined. Using ultra-performance liquid chromatography (UPLC) coupled with ESI-MS, peptide ions with predominantly two-fold dehydration were analyzed (Figure S11A). The ion was fragmented, and the observed fragment ions demonstrated that Thr3 escaped dehydration in the two-fold dehydrated ProcA3.3 (Figure S11B), in agreement with the conclusion that we reached from studies on the triazole-containing ProcA3.3 precursor peptide (i.e., Thr3 is dehydrated last).

Directionality of Cyclization by ProcM. ProcM was incubated with expressed and purified His$_6$-ProcA2.8 obtained as reported previously,[23] and the assay was quenched by removing ProcM at various time points. After incubation with endoproteinase LysC to remove most of the leader peptide, the digest was treated with iodoacetamide (IAA). Any noncyclized thiol would react with IAA, and the enzymatic assay conditions were optimized to allow buildup of an incompletely cyclized intermediate that resulted in one IAA adduct (Figure S12A).

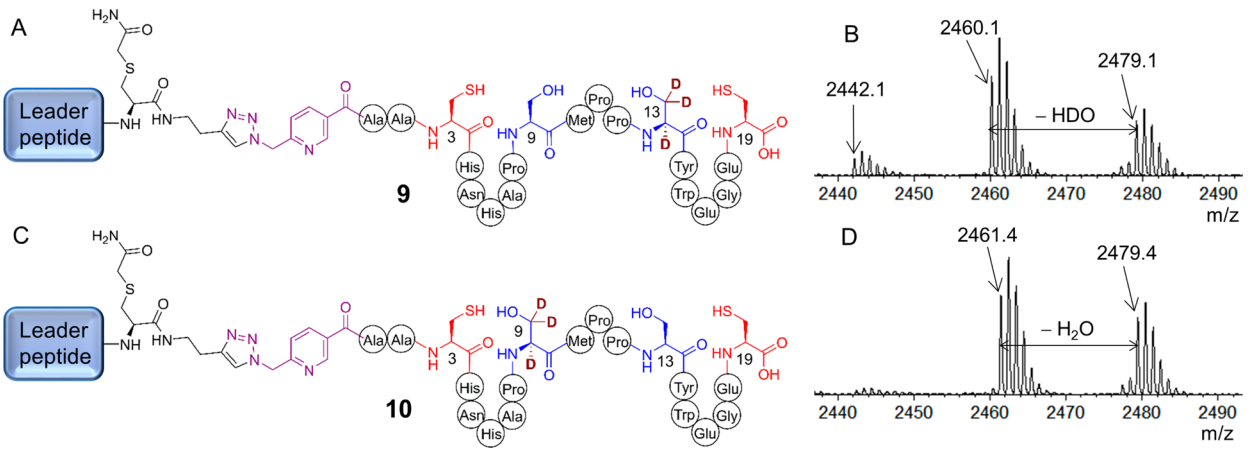

Figure 7. Directionality of dehydration in ProcA2.8 analogues. (A) Structure of ProcA2.8 precursor peptide **9** with Ser13 replaced with [2,3,3-^{2}H]-Ser. (B) MALDI-TOF MS of **9** partially dehydrated by ProcM and after LysC cleavage. (C) Structure of ProcA2.8 precursor peptide **10** with Ser9 replaced with [2,3,3-^{2}H]-Ser. (D) MALDI-TOF MS of **10** partially dehydrated by ProcM and after LysC cleavage.

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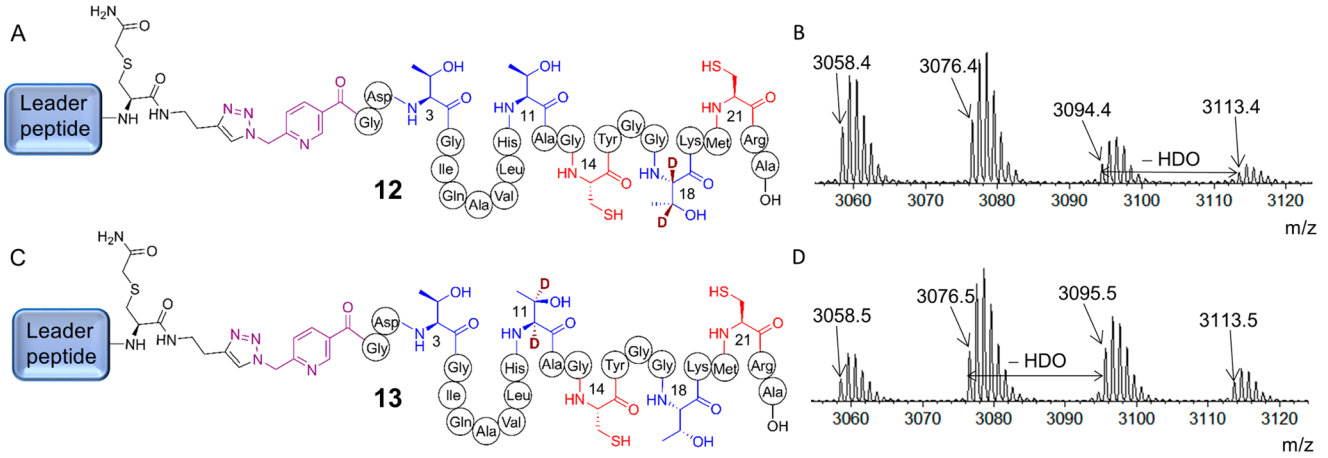

Figure 8. Directionality of dehydration of ProcA3.3 analogues containing a triazole linker. (A) Structure of ProcA3.3 analogue **12** with Thr18 replaced with [2,3-^{2}H]-Thr. (B) MALDI-TOF MS of **12** partially dehydrated by ProcM and digested by GluC. (C) Structure of ProcA3.3 analogue **13** with Thr11 replaced with [2,3-^{2}H]-Thr. (D) MALDI-TOF MS of **13** partially dehydrated by ProcM and digested by GluC.

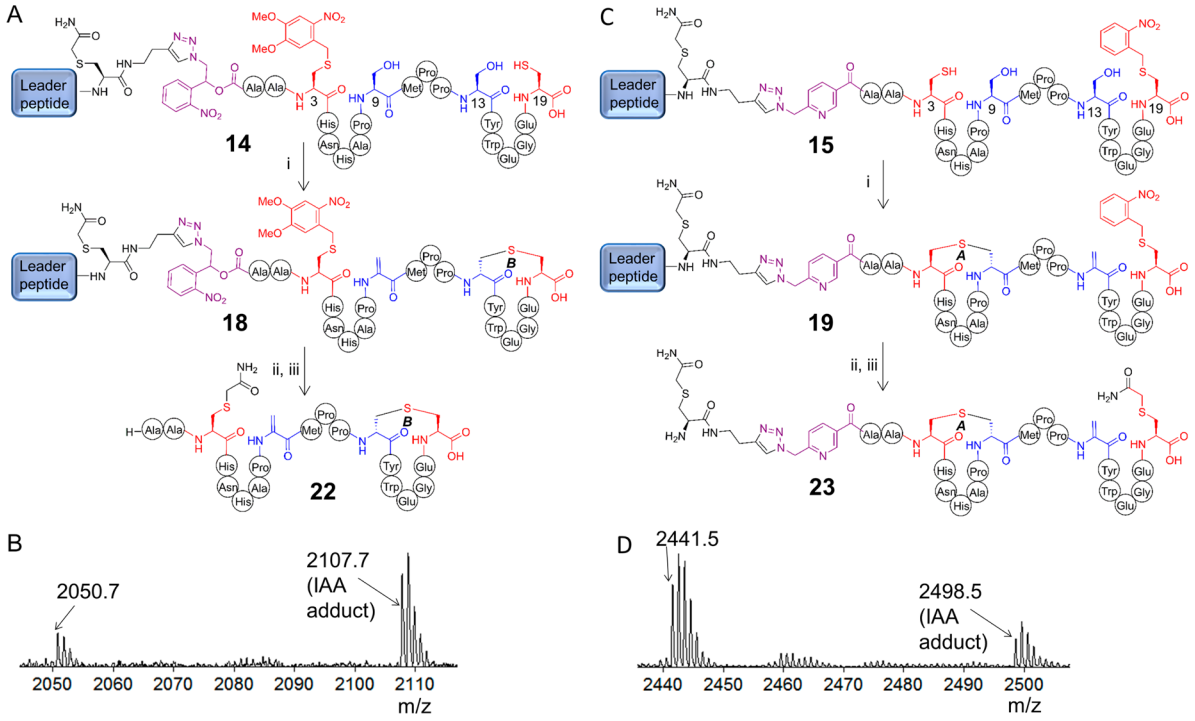

Figure 9. Probing non-enzymatic cyclization in ProcA2.8. (A) Non-enzymatic cyclization of the A-ring in the presence of the enzymatically preinstalled B-ring. Reagents and conditions: (i) HEPES, ATP, TCEP, $MgCl_2$, substrate **14** (50 μM), ProcM (30 μM); (ii) ProcM was removed, the intermediate **18** was desalted and lyophilized, dissolved in 0.1% TFA, and irradiated; (iii) the peptide was dissolved in HEPES buffer (pH 8.0) to allow non-enzymatic cyclization followed by treatment with iodoacetamide. (B) MALDI-TOF MS analysis showed that non-enzymatic cyclization was slow and incomplete, as indicated by the presence of IAA adduct **22**. (C) Non-enzymatic cyclization of the B-ring in ProcA2.8 in the presence of enzymatically preinstalled A-ring. Reagents and conditions: (i) HEPES, ATP, TCEP, $MgCl_2$, ProcM (30 μM), substrate **15** (50 μM); (ii) ProcM was removed, intermediate **19** was irradiated with UV light and lyophilized; (iii) the lyophilized peptide was dissolved in solution containing all components in (i) except ProcM, digested with LysC and treated with excess iodoacetamide. (D) MALDI-TOF MS analysis showed that non-enzymatic cyclization was incomplete, as indicated by the presence of IAA adduct **23**.

ESI-MS/MS was performed on this peptide, and Cys3 was found to be alkylated (Figure S12B). The fragmentation pattern suggested that the B-ring had formed in this intermediate. Hence, cyclization of Cys19 occurred prior to Cys3, suggesting C-to-N-terminal directionality of cyclization.

ProcM was also incubated with His_6-ProcA3.3, and the assay was quenched by removing ProcM at various time points. After endoproteinase LysC digestion, the peptides were treated with IAA. The ProcM assay conditions were again optimized to trap an intermediate that resulted in one IAA adduct (Figure S13A). The observed fragmentation pattern of this peptide by ESI-MS/MS showed that Cys21 was alkylated, suggesting that Cys14 had formed a thioether ring with Dhb18 (Figure S13B). The data suggest that cyclization of ProcA3.3 may not take place with C-to-N-terminal directionality, but that instead the MeLan ring between Cys14 and Dhb18 is formed in the monocyclic intermediate (see Figure 2 for the Pcn3.3 ring topology).

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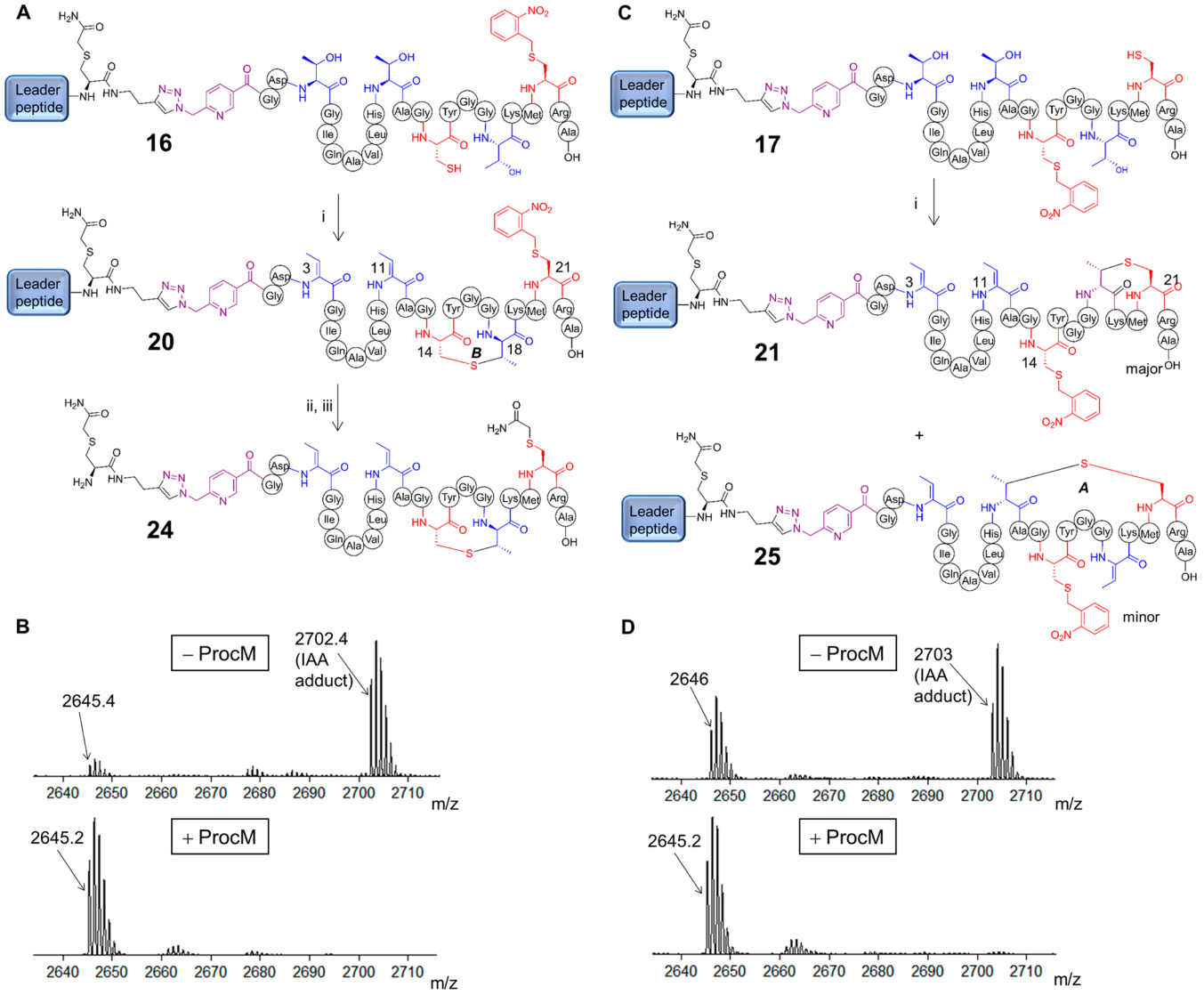

Figure 10. Probing non-enzymatic cyclization in ProcA3.3. (A) Non-enzymatic cyclization of the A-ring in the presence of enzymatically preinstalled B-ring. Reagents and conditions: (i) HEPES, ATP, TCEP, $MgCl_2$, substrate **16** (50 μM), ProcM (20 μM); (ii) ProcM was removed and the intermediate **20** was irradiated with UV light and lyophilized; (iii) the lyophilized peptide was dissolved in assay solution containing all components in (i) with or without ProcM, digested by LysC, and treated with iodoacetamide. (B) MALDI-TOF MS of solution obtained after treatment in (iii). (C) ProcM assay of substrate **17** generated a mixture of intermediates **21** and **25**. (D) ProcM was removed from the mixture of **21** and **25**, and the product mixture was irradiated with UV light and lyophilized. The peptide mixture was dissolved in solution containing all ProcM assay components with or without ProcM. The products were then treated with LysC and IAA. The MALDI-TOF MS spectra of the two assays are presented.

Substrate Design To Probe Non-enzymatic Cyclization in Prochlorosin Maturation. To address whether non-enzymatic cyclization assists ProcM in the maturation of substrates with variable ring topologies, substrates were designed that restrict ProcM installation to selected thioether rings by orthogonal protection of a subset of cysteine thiols in the core peptide. Release of the protecting group would generate an intermediate that would allow investigation of non-enzymatic cyclization in the presence of other preinstalled thioether rings.

A variety of orthogonal protecting groups for the cysteine thiols were tested. *tert*-Butyl disulfide protection proved not suitable because removal by reducing agents like tris(2-carboxyethyl)phosphine (TCEP) and tributylphosphine formed adducts with dehydrated residues. Conversely, acetamidomethyl protection was unsuccessful because its removal required oxidizing agents like iodine, which partially oxidized the thioether rings. However, the *o*-nitrobenzyl and 4,5-dimethoxy-*o*-nitrobenzyl groups were readily introduced into the peptide during SPPS[40] and were cleanly released by UV irradiation (365 nm).

Hence, four precursor peptides were prepared using the previously described hybrid EPL/CuAAC ligation strategy (**14** and **15** in Figure 9 and **16** and **17** in Figure 10), which were treated with ProcM to produce the intermediates **18**–**21** and **25**. Photorelease of *ortho*-nitrobenzyl protecting groups then generated substrates to probe non-enzymatic cyclizations of each of the rings in ProcA2.8 and ProcA3.3 in the presence of other enzymatically installed rings.

Efficient Cyclization of ProcA2.8 Requires ProcM. ProcM correctly processed the ProcA2.8 precursor peptide analogue **14** to generate the B-ring and a dehydroalanine at the ninth position of the core peptide (intermediate **18**) (Figure 9A) as demonstrated by tandem MS (Figure S14). The peptide

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18 was irradiated at 365 nm to release the *o*-nitrobenzyl group on Cys3 along with the leader peptide that was attached via linker **7**. The resulting peptide was incubated in buffered solution at pH 8.0 to probe non-enzymatic cyclization under conditions where enzymatic cyclization is complete. The peptide was then treated with IAA to report on noncyclized Cys residues. MALDI-TOF MS analysis revealed the predominant presence of IAA adduct **22**, which indicated incomplete non-enzymatic cyclization of the A-ring of prochlorosin 2.8 (Figure 9B). Thus, non-enzymatic cyclization of the A-ring in the presence of the B-ring is much slower than enzymatic cyclization. Non-enzymatic cyclization was also conducted at higher pH (pH 8.5) and for a longer time period (12 h), but non-enzymatic cyclization of the A-ring still did not proceed to completion.

ProcM converted the ProcA2.8 analogue **15** (Figure 9C) to an intermediate **19** that contained the A-ring of prochlorosin 2.8 and a dehydroalanine at the 13th position as evidenced by tandem MS (Figure S15). The peptide **19** was irradiated to remove the *o*-nitrobenzyl group from Cys19 and was incubated with ProcM. The peptide was then treated with LysC to remove the leader peptide and with IAA to probe cyclization. Analysis by MALDI-TOF MS showed that, whereas the enzymatic cyclization under these conditions is complete in 1 h, the non-enzymatic cyclization still resulted in a mixture of cyclized product and IAA adduct **23** after 16 h (Figure 9D). Because the observed non-enzymatic cyclization of the B-ring was very slow compared to enzymatic cyclization, these experiments do not provide support for efficient non-enzymatic ring formation facilitated by preorganization upon formation of one of the rings.

Ring Formation in ProcA3.3 Requires ProcM. Treatment of the ProcA3.3 precursor analogue **16** with ProcM resulted in the formation of a product containing a dehydrobutyrine (Dhb) at positions 3 and 11 and a MeLan formed between Cys14 and Dhb18 (Figure 10A), as confirmed by tandem MS after LysC cleavage of **20** (Figure S16). The *o*-nitrobenzyl group was removed from Cys21 by UV irradiation, and the peptide was subjected to both enzymatic and non-enzymatic assay conditions. Following the cyclization assays, the peptides were digested with endoproteinase LysC and treated with IAA. The enzymatic assay did not show IAA adduct formation, whereas the non-enzymatic assay showed predominant formation of the IAA adduct **24** (Figure 10A) as demonstrated by MALDI-TOF MS (Figure 10B). Hence, non-enzymatic cyclization of the A-ring of Pcn3.3 in a peptide that already contained the B-ring is much slower than when this process is catalyzed by ProcM. Thus, the experiments with peptides **18**–**20** show that non-enzymatic cyclization is too slow to be kinetically competent to be a part of the overall process.

The product obtained after ProcM treatment of the ProcA3.3 precursor analogue **17** was not a single peptide but a mixture of peptides **21** and **25** with two different ring topologies (Figure 10C). The major product **21** had a MeLan formed between Cys21 and Dhb18 and the minor product **25** contained a MeLan formed between Cys21 and Dhb11 (as seen in native modified ProcA3.3, Figure 2), as evidenced by tandem ESI-MS on the individual products that were separated on analytical scale by UPLC (Figure S17A,B). These findings can be explained on the basis of the order of cyclization of ProcA3.3 discussed above. In wt ProcA3.3, Cys14 forms a ring with Dhb18 in the observed monocyclic intermediate, and hence only Dhb11 is available to Cys21 for cyclization. However, in peptide **17**, Cys14 is protected, and therefore, both Dhb11 and Dhb18 are available for reaction with Cys21; apparently the enzyme then prefers formation of the smaller ring between Cys21 and Dhb18. Compounds **21** and **25** could not be separated on preparative scale, and therefore, the mixture was subjected to UV irradiation to release the *o*-nitrobenzyl group from Cys14. The mixture of deprotected peptides was subsequently subjected to both enzymatic and non-enzymatic cyclization assay conditions. The peptides were then digested with endoproteinase LysC followed by treatment with IAA. The non-enzymatic assay resulted in the formation of IAA adduct, thus indicating the absence of significant spontaneous non-enzymatic cyclization in the mixture of two peptides, whereas the enzymatic assay resulted in complete cyclization as evidenced by the lack of IAA adduct (Figure 10D). The cyclized product mixture was separated on analytical scale by UPLC and tandem MS analysis revealed the identity of the two products obtained from **21** and **25**. In addition to the overlapping ring topology as observed in Pcn3.3 (Figure S18A), an alternate non-overlapping ring topology was formed (Figure S18B). Hence, manipulation of the order of cyclization by using Cys protecting groups allows access to a ring topology not seen with the native substrate.

Reversibility of Thioether Ring Formation. To test whether thioether ring installation in prochlorosins is reversible, ProcA substrates were modified by ProcM in D_2O, resulting in incorporation of one deuterium at each newly formed α-stereocenter of Lan/MeLan. The modified precursor peptides were then purified and subjected to standard ProcM assay conditions in unlabeled aqueous buffer. If the Michael-type addition is reversible, then the incorporated deuterium would be expected to exchange with a protium in the assay in unlabeled buffer (Figure 11).

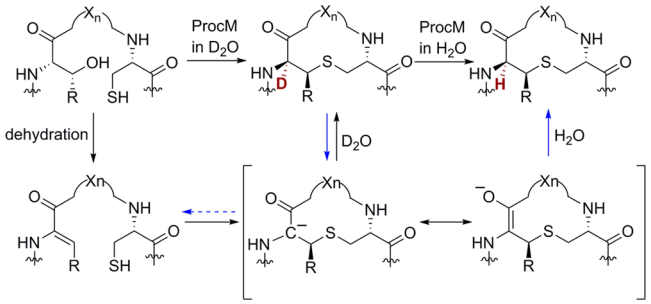

Figure 11. Scheme for the exchange assay. Substrate is modified by ProcM in deuterium-labeled buffer to generate product with one deuterium per Lan/MeLan. Exchange of the installed deuterium was then investigated by treating the modified precursor peptide with ProcM in unlabeled buffer. Exchange can occur by abstraction of deuterium followed by protonation (solid blue arrows) or by retro-Michael-type addition (dashed blue arrow) and recyclization.

His-tagged ProcA2.8 was heterologously expressed in *E. coli* and purified and subjected to ProcM with all assay components dissolved in D_2O. ProcM was removed by ultrafiltration, and the peptide was desalted and lyophilized. A portion of the lyophilized peptide was digested with endoproteinase GluC to generate the modified core peptide with a five amino acid overhang at its N-terminus originating from the leader peptide (Figure 12A). As anticipated, both ESI and MALDI-TOF MS analysis demonstrated the incorporation of two deuterium

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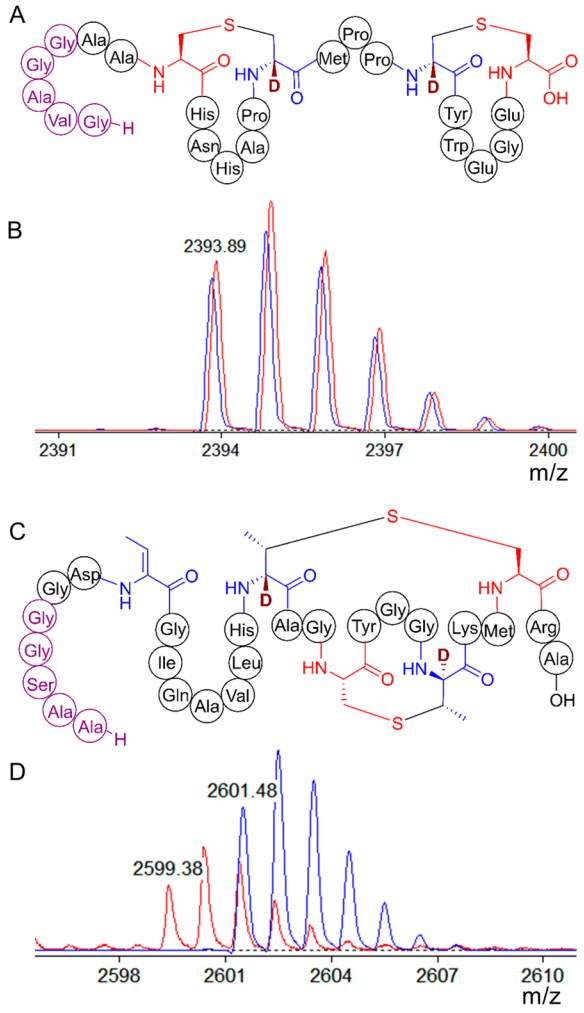

Figure 12. Deuterium incorporation and exchange in ProcA2.8 and ProcA3.3. (A) Structure of GluC cleaved ProcA2.8 core peptide fragment incorporating two deuteriums. Residues in purple originate from the leader peptide. (B) MALDI-TOF MS of GluC cleaved fragment of deuterium-labeled ProcA2.8 (50 μM) after assay without (blue trace) and with ProcM (20 μM) treatment in aqueous buffer (red trace). (C) GluC digested ProcA3.3 core peptide incorporating two deuteriums. Residues in purple originate from the leader peptide. (D) MALDI-TOF MS of GluC digested fragment of modified deuterium-labeled ProcA3.3 (100 μM) without aqueous ProcM treatment (blue trace) and with aqueous ProcM (40 μM) treatment (red trace).

atoms. The full-length peptide was then incubated in unlabeled buffer in the presence or absence of ProcM for 17 h. Exchange of deuterium with protium was not observed (Figure 12B, red). Hence D−H exchange did not occur in ProcA2.8 containing two non-overlapping lanthionine rings, suggesting that such rings are not installed reversibly for this peptide.

ProcM-Cyclized ProcA3.3 Undergoes Enzymatic D−H Exchange. To also explore a substrate containing overlapping rings, ProcA3.3 was heterologously expressed and purified. The peptide was modified by ProcM in D_2O, the enzyme was removed from the modified peptide by ultrafiltration, and the product peptide was desalted and lyophilized. Analysis by MALDI-TOF MS after endoproteinase GluC digestion demonstrated incorporation of two deuterium atoms as expected (Figure 12C). The full-length peptide was then incubated in unlabeled buffer with or without ProcM and digested with GluC before mass spectrometric analysis. The modified ProcA3.3 treated in aqueous buffer without ProcM did not result in any exchange (Figure 12D, blue), but the modified substrate treated in aqueous buffer with ProcM showed exchange of both deuterium atoms with protium (Figure 12D, red). Analysis of the exchange over time at two different concentrations of ProcM revealed that the exchange was time-dependent and dependent on the concentration of ProcM (Figure S19). Also, exchange of one deuterium was significantly faster, with the second deuterium exchange requiring higher enzyme concentration and longer incubation time. Under the conditions of the assay (100 μM modified ProcA3.3 and 5 μM ProcM), the exchange of the first deuterium was already detected after short incubation times (15 min; Figure S19). The dehydration of ProcA3.3 catalyzed by ProcM under the same conditions is complete at this time point, but cyclization is still incomplete. Hence, the observed deuterium exchange appears kinetically competent with the cyclization process.

D−H Exchange Is Not Observed in ProcA2.8 Substrates with Ser Mutated to Thr. Given the observation of D−H exchange in modified ProcA3.3 but not ProcA2.8, it is interesting to note the differences between the two substrates. Pcn3.3 contains overlapping rings whereas Pcn2.8 does not, and the rings in Pcn3.3 are formed by MeLan residues whereas both rings in Pcn2.8 are formed by Lan residues. To investigate if the lack of exchange in cyclized ProcA2.8 was because it contains Lan and not MeLan, two mutants of ProcA2.8 were generated by site-directed mutagenesis—one with Ser9 replaced with Thr (ProcA2.8-S9T) and another with Ser13 replaced with Thr (ProcA2.8-S13T). Both substrates were modified by ProcM in D_2O, incorporating two deuterium atoms in the process. The purified products were subjected to the D−H exchange assay conditions and were digested with GluC. Subsequent analysis by MALDI-TOF MS revealed that D−H exchange had not occurred (Figure S20A−D). Hence, in ProcA2.8 with two non-overlapping rings, even changing a Lan to a MeLan does not lead to D−H exchange, suggesting that ring topology may instead dictate whether ProcM can exchange the α-proton of the cross-linked amino acids.

Exchange in ProcA3.3 Involves the B-Ring. As noted above, the exchange process for cyclized ProcA3.3 involves one deuterium that was exchanged relatively rapidly with a proton from solvent, whereas the second deuterium exchange was much slower. Because cyclization of ProcA3.3 results in a ring within a ring, tandem MS could not be used to determine which of the two MeLan residues is associated with the faster exchange. We therefore mutated Thr11 to Ser such that cyclization would result in one Lan and one MeLan, which in principle can be distinguished by GC−MS after acidic hydrolysis of the product. Thus, ProcA3.3-T11S was first incubated with ProcM under the standard conditions in D_2O. The resulting product was purified and shown to contain two deuterium atoms by mass spectrometry (Figure S21B). The labeled peptide was then treated with ProcM in unlabeled buffer, resulting in relatively rapid exchange of one deuterium (Figure S21C), similar to the observations with wild-type ProcA3.3. The resulting peptide containing one deuterium was then hydrolyzed, and the Lan and MeLan residues were derivatized as previously described.[41,42] Analysis by GC−MS resulted in detection of unlabeled derivatized MeLan and deuterium-labeled derivatized Lan (Figure S22). Hence, the relatively fast exchange occurs in the MeLan in ring B. Both

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Lan and MeLan residues had the correct DL stereochemistry as confirmed by co-injection with synthetic standards (Figure S23).

DISCUSSION

In an effort to understand the remarkable substrate tolerance of ProcM, four aspects of the lanthionine synthetase were investigated. Aided by a hybrid ligation protocol that allowed us to install synthetic core peptides onto a heterologously expressed leader peptide, we investigated the directionality of both dehydration and cyclization, the possibility that a non-enzymatic cyclization step might account for the high diversity of ring topologies of the products, and the possibility of reversibility of ring formation. Use of labeled ProcA substrate unequivocally demonstrated that ProcM dehydrates two very different substrate peptides in a C-to-N-terminal fashion. This same directionality was also reported for the class III lanthipeptide synthetase LabKC[43] and another RiPP synthetase BalhCD,[44] whereas for other class II lanthipeptides, such as LctM and HalM2, the directionality of dehydration was reported to be N-to-C-terminal based on tandem MS.[30] The latter directionality is more readily explained using a proximity effect if the active site for dehydration is close to the leader peptide binding site. Such a model might be expected to lead to an erosion of strict directionality if Ser/Thr residues are close in sequence space, which indeed has been observed.[30] Explanation of a C-to-N-terminal directionality of dehydration as observed here and in LabKC and BalhCD requires a more complex model. Although we cannot completely rule out that the directionality of dehydration by ProcM is simply reflecting the reactivity of each individual site as a result of different flanking residues or secondary structure, the lack of any sequence similarity in the two peptides that are both shown to be dehydrated in C-to-N-terminal direction in this study leads us to favor an explanation that involves a specific juxtaposition of the leader and core peptide binding sites that favors dehydration of C-terminal residues. Structural studies will be required to provide further information.

Interestingly, the cyclization of the two peptides also occurred with a specific order, but this order was not necessarily directional. Whereas for ProcA2.8 the Cys that is located closer to the C-terminus reacted first, in ProcA3.3, it was the Cys that was closer to the N-terminus that appeared to react first. In the latter substrate, this results in the smaller B-ring being formed in the observed intermediate. Preference for formation of a smaller ring was also observed when Cys14 of ProcA3.3 was protected, resulting in Cys21 forming a ring that is not seen in the normal product. Hence, it appears that the order of cyclization catalyzed by ProcM is determined more by the ring size than by directionality of the enzyme. This observation agrees with other recent studies that suggest that the precursor peptides may have an inherent bias for formation of certain rings.[45,46]

The remarkable substrate tolerance of ProcM suggested the possibility that perhaps only a subset of the rings are generated enzymatically and that these enzymatically formed macrocycles preorganize the peptide for subsequent non-enzymatic cyclization. However, our current data show that, for two different substrates, non-enzymatic cyclization of intermediates that contain one ring is too slow to be kinetically competent for the enzymatic process. Another question that has not been previously addressed in lanthipeptide biosynthesis is whether thioether ring formation is reversible or not. Our experiments suggest that enzymatic deprotonation at the α-position of MeLan residues does occur in some rings. Furthermore, although true kinetic studies on the enzyme have thus far been unsuccessful because of the many reactions it catalyzes and the absence of a convenient and quantitative method to measure each step, the general time dependence of exchange and cyclization of ProcA3.3 suggests that deprotonation at the α-carbon of MeLan might be kinetically competent. It is important to note that the D−H exchange assay reports on *the reversibility of the protonation of the enolate* during MeLan formation; it does not necessarily indicate that the cyclization is reversible (i.e., a reversible Michael-type reaction). Attempts to trap a free thiol formed from ring opening involving Lan/MeLan residues were not successful.

The observation that the deuterium in the smaller B-ring exchanges faster than the larger A-ring is somewhat puzzling. If deuterium exchange indeed reports on reversibility of ring formation, then one might have expected that the ring that forms last (A-ring) would open up first. Several explanations may account for the observation that D−H exchange instead occurs in the B-ring. One possibility is that the formation of the two rings are entirely independent, that is, that the kinetics of forming either ring is independent of whether the other ring is already formed (Figure S24). In this scenario, an intermediate with the smaller B-ring installed would indeed be observed in the forward process, and exchange of the same ring could be favored in the reverse process (Figure S24). Although it appears unlikely that ring formation would be entirely independent in a short peptide with overlapping rings such as the ProcA3.3 core peptide, we cannot rule out this possibility. A second possibility is that the intermediate with the B-ring that is observed during the ring formation process is not a productive intermediate en route to the final product. It is possible that formation of the final product requires the A-ring to be formed first (Figure S25). In that scenario, the intermediate with the B-ring formed would be in equilibrium with the starting peptide, and an intermediate with the A-ring installed would not be detected because of very rapid formation of the final product from this intermediate (Figure S25). This model would also readily explain why it is the B-ring that undergoes exchange in the reverse direction. We cannot unambiguously distinguish between both hypotheses, but they do make predictions that can be tested in future studies.

CONCLUSIONS

This study shows that dehydration of ProcA2.8 and ProcA3.3 by ProcM takes place with C-to-N directionality, and that for these two substrates, cyclization is also an ordered process but that the order is determined by the ring topology. Furthermore, non-enzymatic cyclization is shown not to be involved in the formation of two very different ring topologies in prochlorosins 2.8 and 3.3. Whether these observations are general for all ProcA substrates remains to be determined. Finally, for some ring topologies, the protonation of the enolate during the Michael-type addition is reversible (i.e., the enzyme can remove the α-proton from the cyclized product), which possibly may indicate that the enzyme can also catalyze a retro-Michael addition. Future studies will focus on investigating this hypothesis.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures and characterization of compounds and peptides, biochemical assay and molecular biology procedures, and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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