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Dissecting Reactions of Nonlinear Precursor Peptide Processing of the Class III Lanthipeptide Curvopeptin

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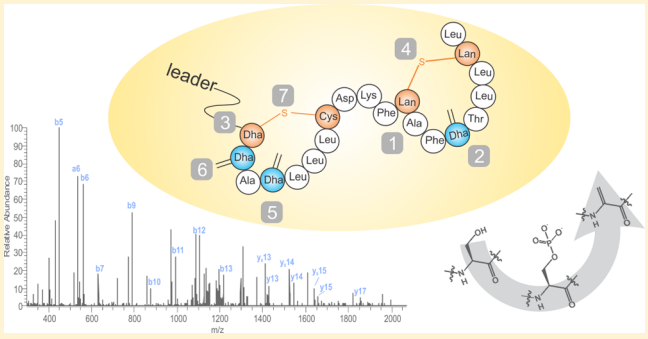
Supporting Information

ABSTRACT: Lanthipeptides are ribosomally synthesized peptides which undergo extensive post-translational modifications. In addition to novel structural features and bioactivities, the *in vitro* study on the biosynthesis of the class III lanthipeptide labyrinthopeptin revealed a unique C- to N-terminal directionality of biosynthetic processing. The recently described class III lanthipeptide curvopeptin allowed investigating the directionality aspect in much greater detail: Structural characterization of nine curvopeptin biosynthesis intermediates by high-resolution mass spectrometry combined with a deuterium-labeling approach enabled for the first time building a comprehensive biosynthesis model featuring all three post-translational modification reactions: phosphorylation, elimination, and cyclization. These results point to a nonlinear processing scheme with a predominant C → N-terminal directionality. Our data give important mechanistic insights into the concerted processing and directionality of the multifunctional class III modifying enzymes. The data are of significance in the light of obtaining a mechanistic understanding of the post-translational biosynthesis machinery of the growing variety of ribosomally synthesized and post-translationally modified peptides.



INTRODUCTION

Lanthipeptides belong to the large family of ribosomally synthesized peptides that acquire their unique structures and bioactivities upon post-translational modifications (PTMs) and processing. These peptides attained great scientific interest due to an exceptional engineering potential, i.e., generation of derivatives by site-directed mutagenesis.[1] Importantly lanthipeptides are one of the most thoroughly investigated groups of ribosomally synthesized and post-translationally modified peptides (RiPPs) in terms of structure, biosynthesis, and bioactivities. The characteristic feature of lanthipeptides is the presence of a lanthionine (Lan) ring.[2] The most common classification scheme divides lanthipeptides into four classes.[3] The recently described labyrinthopeptins, assigned to class III, were shown to contain a novel structural motif named labionin.[4,5] The subsequent characterization of class III peptides, erythreapeptin,[6] flavipeptin,[7] and curvopeptin[8] highlighted the structural heterogeneity within this particular class. In addition, the biological characterization of labyrinthopeptins revealed antiallodynic and antiretroviral activities, which together with high engineering capabilities[9,10] demonstrates the potential of these compounds as lead structures for drug discovery programs.

In order to fully exploit the potential of the lanthipeptide biosynthesis machinery, a deeper understanding of the underlying enzymatic mechanisms and principles is required. During the past decade great progress has been made in elucidating some crucial aspects of lanthipeptide biosynthesis.[2] A gene-encoded precursor peptide composed of two segments, an N-terminal leader and a C-terminal core is subjected to PTMs and upon leader removal maturates to the final peptide product. The PTMs are based on a series of reactions, i.e., phosphorylation, elimination, and a Michael-type cyclization. During the initial biosynthesis steps Ser/Thr residues in the core part of the precursor peptide are phosphorylated in the hydroxyl side-chains providing suitable leaving groups for the subsequent β-elimination reaction.[11,12] Phosphate groups are eliminated from phosphoserines (pSer) or -threonines (pThr) to give 2,3-didehydroalanines (Dha) and 2,3-didehydrobutyrines (Dhb), respectively. Dehydration is followed by a Michael-type addition of a cysteine thiol side chain to the unsaturated double bond of Dha/Dhb establishing the lanthionine structure upon protonation of the resulting enolate.[13,14] Alternatively for class III lanthipeptides subsequent Michael addition of the nucleophilic enolate can result in the formation of labionin (Lab).[5]

In the case of class III lanthipeptides the individual processing steps, i.e., phosphorylation, elimination, and cyclization are assigned to separate domains of a dedicated trimodular lanthipeptide synthetase (LanKC). Due to the lack of structural data on these enzymes the mechanistic details of the precursor peptide processing remain elusive. A particularly interesting aspect is the order in which Ser/Thr/Cys residues

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are modified and cyclized. It was already demonstrated for class II lanthipeptides that dehydrations and lanthionine formation predominantly occur in a directional manner from the N- to C-terminus.[15] Similar findings rendered *in vivo* studies on the class I lanthipeptide nisin.[16]

The only examples for directionality of class III lanthipeptide, namely labyrinthopeptin A2, surprisingly revealed a predominant C- to N-terminal directionality of dehydration events.[17] A similar C- to N-terminal directionality was also reported for a thiazole/oxazole-containing peptide from *Bacillus* sp. Al Hakam.[18] Therefore, it was important to assess if this mode of processing is more common, particularly among class III lanthipeptides. In this study we aimed for a thorough investigation of the directionality of all three reactions: phosphorylation, elimination, and cyclization. In this respect *in vitro* reconstituted biosynthesis of the novel peptide curvopeptin appeared as a well suited system.[8] The herein described characterization of biosynthetic intermediates of CurKC-mediated PTM employs chemical biology tools, i.e., deuterium-labeling, solid phase peptide synthesis, and ESI-MS/MS product ion analysis, rendering a comprehensive picture of the directionality of crucial PTM reactions of a class III lanthipeptide.

RESULTS AND DISCUSSION

Directionality of Phosphorylation and Elimination. The comparably facile synthesis of the CurA precursor peptide together with the expression of the modifying synthetase CurKC of the class III lanthipeptide curvopeptin allowed for studying the complexity of precursor peptide processing, its order, and the timing of consecutive reactions in a comprehensive manner. Curvopeptin is composed of two nonoverlapping lanthionine rings (Figure 1) with five dehydrations of precursor peptide CurA at positions 1, 2, 4, 12, and 15. The biosynthesis was reconstituted *in vitro* with the synthetic precursor peptide CurA, and the reaction was followed by high resolving ESI-mass spectrometry.[8] In order to pinpoint individual sites of PTMs, the reaction mixture was treated with protease GluC before MS/MS analysis, which greatly increased fragmentation in the relevant part of the peptide. Upon incubation of the precursor peptide with CurKC, a heterogeneous mixture of phosphorylated and dehydrated reaction intermediates was detected already after 1−2 min (Figure S1). Despite shortening of the reaction times it was impossible to detect singly and doubly dehydrated intermediates that were not phosphorylated. The least processed product containing solely Dha residues was triply dehydrated peptide (Figure 2, **8**). The subsequent MS/MS analysis located dehydrations at Ser1, Ser12, and Ser15 of the precursor peptide (Figures 2 and S3A). Fragment ion analysis of other curvopeptin biosynthetic intermediates containing 4 and 5 dehydroalanines revealed the two final dehydrations events occurring at Ser4 and Ser2, respectively (Figure S3). These findings already indicated a nondirectional dehydration pattern of CurA and required more detailed studies of this enzymatic reaction.

To further investigate formation of singly and doubly dehydrated curvopeptin intermediates, we performed time dependent *in vitro* assays at different temperatures (18 and 25 °C) and different enzyme-to-substrate ratios (CurKC:CurA: 1:1, 1:15, and 1:50) (Figures S6−9). ATP was used in excess (1 mM) to mimic physiological conditions of the enzymatic reaction.[19,20] However, formation of solely singly and doubly

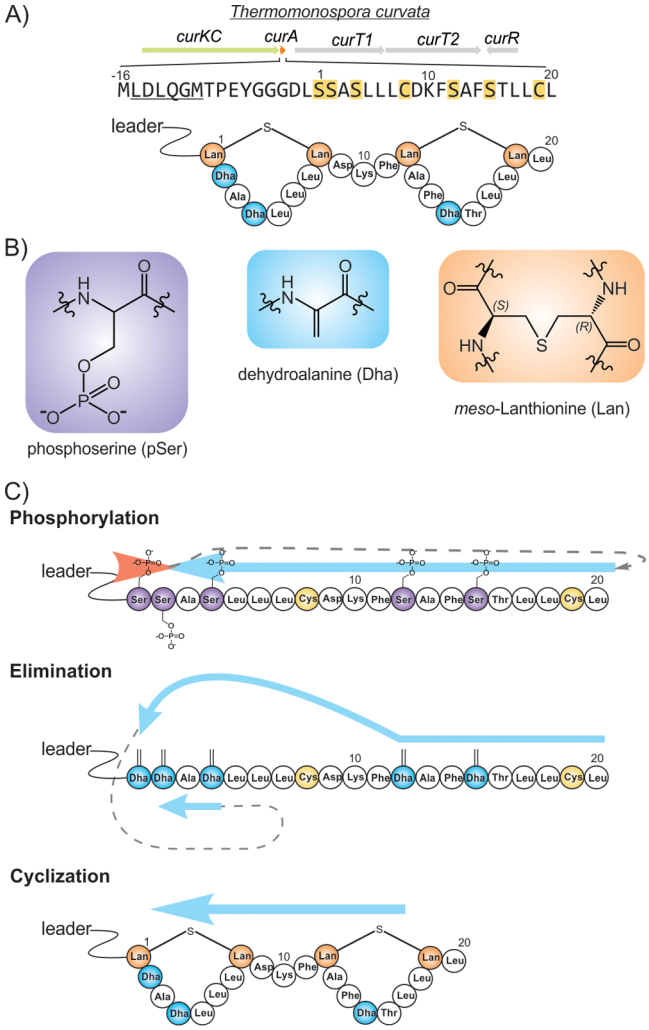

Figure 1. Curvopeptin gene cluster from *Thermomonospora curvata* with *curA* encoding the precursor peptide (modified Ser highlighted) and the structure of mature curvopeptin (A). Structures of important lanthipeptide building blocks: phosphoserine (pSer), dehydroalanine (Dha), and lanthionine (Lan) (B). Directionality of three PTMs performed by CurKC synthetase: phosphorylation, elimination, and cyclization (C).

dehydrated intermediates could never be observed (Figures S6−9). This strongly suggests that a direct conversion of a monophosphorylated CurA peptide into the corresponding singly dehydrated peptide does not occur. Instead the monophosphorylated peptide experiences subsequent phosphorylation and/or dehydration events while maintaining the initial phosphorylation. Together with previous observations it was concluded that the first intermediate devoid of pSer, i.e., a triply dehydrated peptide, is derived from a doubly dehydrated and monophosphorylated peptide. In order to assess the sequence of the two initial dehydration events, deuterium-labeled CurA precursor peptides were employed based on a previously developed method for directionality investigations of RiPPs.[17] Accordingly three CurA variants with deuterium-labeled Ser at positions 15, 12, and 1 were incubated with CurKC (**11**−**13**, respectively) (Figure 3). Subsequent analysis of the series of monophosphorylated intermediates and the triply dehydrated intermediate revealed the first dehydration to occur mainly at Ser15, followed by Ser12 and Ser1 (Figure 3). Intriguingly, deeper analysis and comparison of the observed

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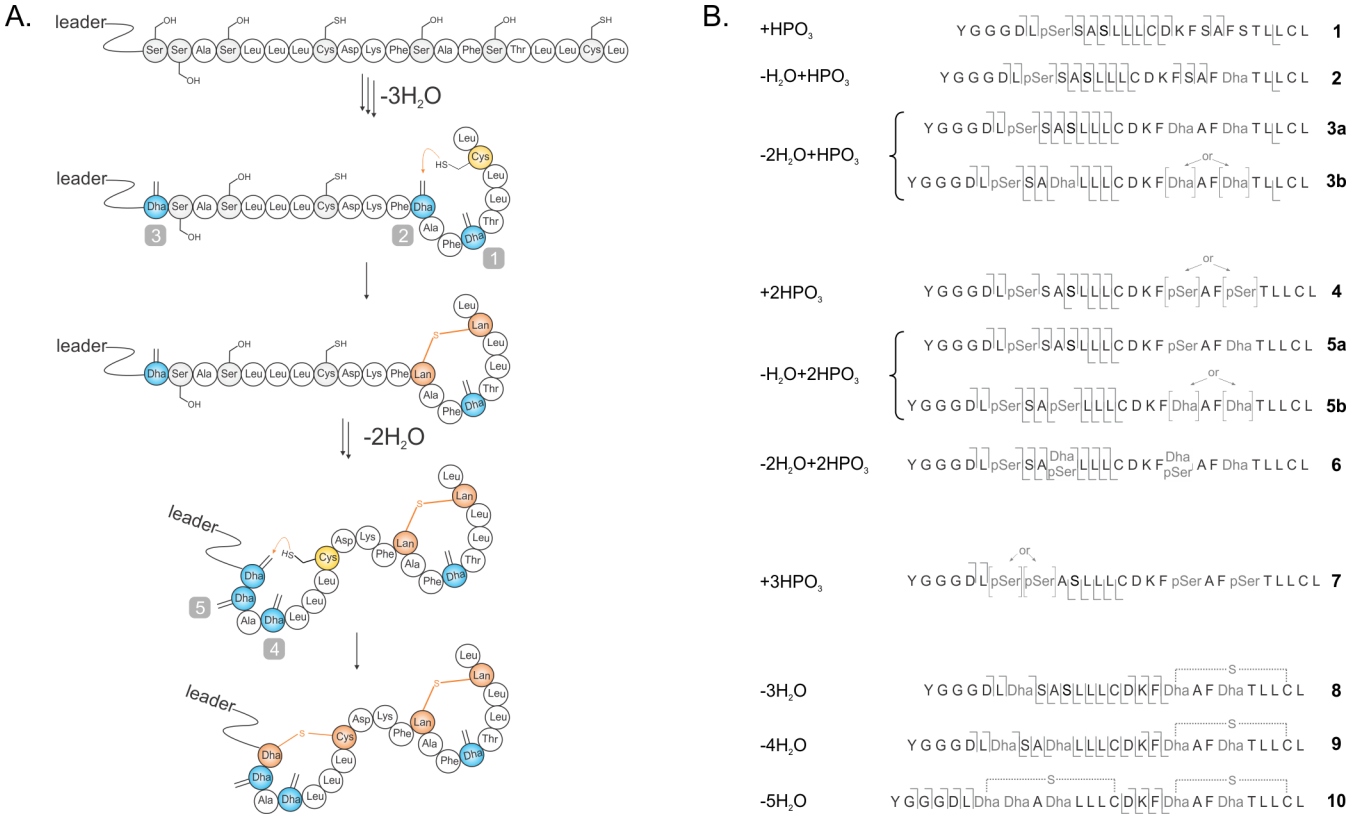

Figure 2. Order of dehydrations and cyclization reactions during the curvopeptin biosynthesis deduced from mass spectrometric data. The order in which particular Dha are installed is indicated by numbers (gray boxes) (A). Analysis of the MS/MS fragmentation pattern of intermediates from *in vitro* reactions with CurKC after partial removal of the leader peptide with protease GluC (B).

and simulated isotopic distribution patterns of different monophosphorylated intermediates revealed also possible alternative processing routes (Figures S18 and S19). As already elucidated with nondeuterated CurA peptides, the ultimate two dehydrations occur at Ser4 and Ser2, respectively. These results allowed deriving the order in which every pSer is eliminated. Accordingly, an intriguing sequence of elimination reactions was observed: within an overall C- to N-terminal directionality of elimination reactions, an exception occurs when Dha1 is formed from pSer1, which is again followed by the C- to N-terminal processing of Ser4 and Ser2.

Due to the presence also of multiple phosphorylated peptides, the directionality of dehydration events alone does not provide a full picture of the precursor peptide processing. Interestingly, CurA peptides carrying up to three phosphate esters were detected (**1**–**7**). To gain further insights into the phosphorylation sequence, the monophosphorylated species were analyzed. A direct assignment of phosphorylation sites by means of ESI-MS/MS using collisionally activated dissociation (CAD) is hampered due to postsource elimination of the phosphate group and formation of Dha. Therefore, a direct distinction between Dha and pSer at a particular position is difficult. However, these modifications could be indirectly assigned, since phosphorylation must precede the elimination reaction. Combining results from MS/MS product ion scans with those of deuterium-labeling gave a concise picture on the catalytic processing of CurKC. Surprisingly, characterization of the first reaction intermediate, i.e., monophosphorylated precursor peptide **1**, revealed the presence of pSer1, indicating that Ser1 is the first residue to be modified by CurKC (Figures 2 and S2A). Further analysis of intermediate **2** bearing a single phosphorylation and single dehydration indicates that Ser1 and Ser15 are modified (Figures 2 and S2B). Analyzing the modification sites of peptide containing one pSer and two Dha (**3a**, **3b**) (Figures 2 and S2C) and aligning all these results with elimination of the deuterium label, we conclude that Ser1 stays phosphorylated, while Ser15 and then Ser12 are subsequently phosphorylated and eliminated rendering Dha. Interestingly, in-depth analysis of the product ion spectrum revealed fragments corresponding to peptides with pSer1, Dha12, and Dha15 (**3a**) and a minor contribution of a peptide with pSer1, Dha4, and Dha12/Dha15 (**3b**) (Figure S2C). Detection of the latter peptide indicates that CurKC, similar to other RiPP modifying enzymes, displays some flexibility in the processing order.[15,17,18] The analysis of two-fold phosphorylated intermediate **4** reveals the modification of Ser1 and either Ser12 or Ser15 (Figures 2 and S4A). As we do not observe singly dehydrated peptide, this doubly phosphorylated intermediate must be a direct precursor of already described monophosphorylated and singly dehydrated peptide **2**. Similarly, the analysis of the CurA derivative **7** bearing three pSer (Figures 2 and S5) showed that these are located in positions 12, 15, and either 1 or 2. The interpretation of the fragmentation pattern from MS/MS spectra of the putative elimination product of triply phosphorylated peptide, namely doubly phosphorylated singly dehydrated peptides **5a** and **5b**, once again shows that CurKC affords two alternative routes (Figures 2 and S4B): MS/MS spectra indicate the presence of derivatives with pSer1, pSer12, and Dha15 (**5a**) as well as pSer1, pSer4, and Dha12/Dha15 (**5b**). Analysis of doubly phosphorylated and doubly dehydrated intermediate **6** reveals pSer1 and Dha15, whereupon for positions 4 and 12 MS/MS

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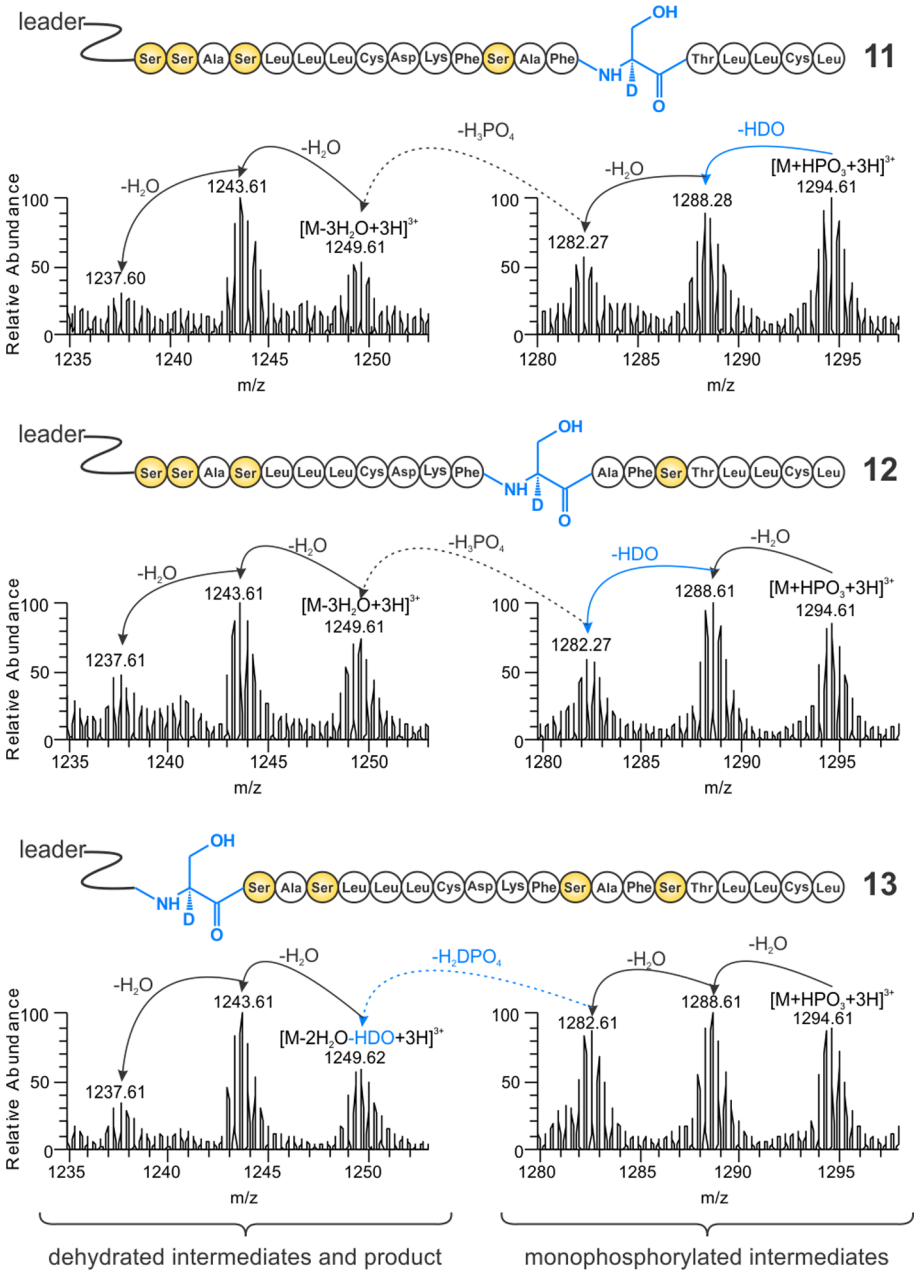

Figure 3. HPLC-ESI-MS spectra of series of monophosphorylated and dehydrated intermediates of deuterium-labeled CurA substrate peptides **11**, **12**, and **13** (α-deuterated Ser15, Ser12, and Ser1, respectively) illustrating that Ser1 remains phosphorylated until the third dehydration event.

spectra indicate either a peptide with Dha4 and pSer12 (**6′**) or a peptide with pSer4 and Dha12 (**6″**) (Figures 2 and S4C). Combining all the results allowed us to come up with a detailed scheme for all phosphorylation and dehydration steps including major and minor pathways (Figure 4).

In conclusion these results suggest a nonlinear curvopeptin processing regarding both PTMs, i.e., phosphorylation and elimination. Both processes show a preferred C → N-terminal directionality with the exception of modification of Ser1. The divergence in Ser1 processing, phosphorylated as first residue and phosphate group elimination occurring as the third residue, highlights the importance of careful studying all partial reactions performed by the enzyme. As outlined in Figure 4, we propose a main biosynthesis route (green arrows) as well as alternative routes (dashed, gray arrows) which may be pursued by CurKC. Similar to herein presented curvopeptin system, parallel processing pathways were reported for phosphorylation of lacticin 481,[15] dehydration of labyrinthopeptin A2,[17] and processing of the RiPP microcin B17,[21] thus highlighting a flexibility of the processing machinery in various biosynthesis systems.

Directionality of the Cyclization. Importantly, the present study concomitantly sheds light on the cyclization reaction. Employing MS/MS experiments, the y^3 product ion could serve as a diagnostic ion indicative of the absence of the C-terminal lanthionine ring. This fragment is observed in all MS/MS product ion spectra of singly phosphorylated intermediates (Figure S2). The first peptide in the processing route lacking the y^3 product ion is the triply dehydrated product **7**. Interestingly, the y^3 ion is present also in the direct upstream intermediate, i.e., phosphorylated and doubly dehydrated peptides **3a**/**3b**. Together with results of

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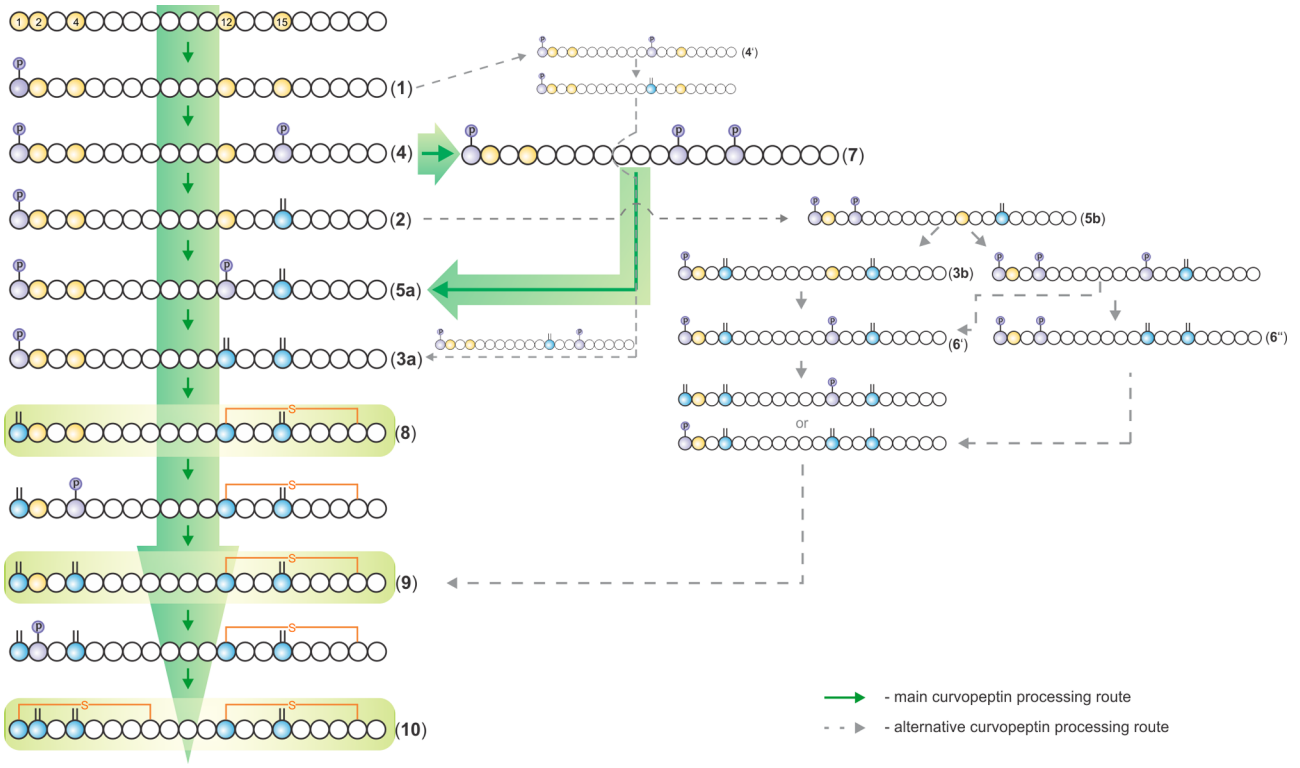

Figure 4. Proposed scheme of PTM events in curvopeptin biosynthesis highlighting different processing routes. The order of modifications and structural characterization of biosynthesis intermediates was based on deuterium-labeling approach and MS/MS product ion analysis; detected and characterized peptides are labeled with numbers referring to Figure 2. Color code of filled spheres: Ser (orange), pSer (purple), and Dha (blue).

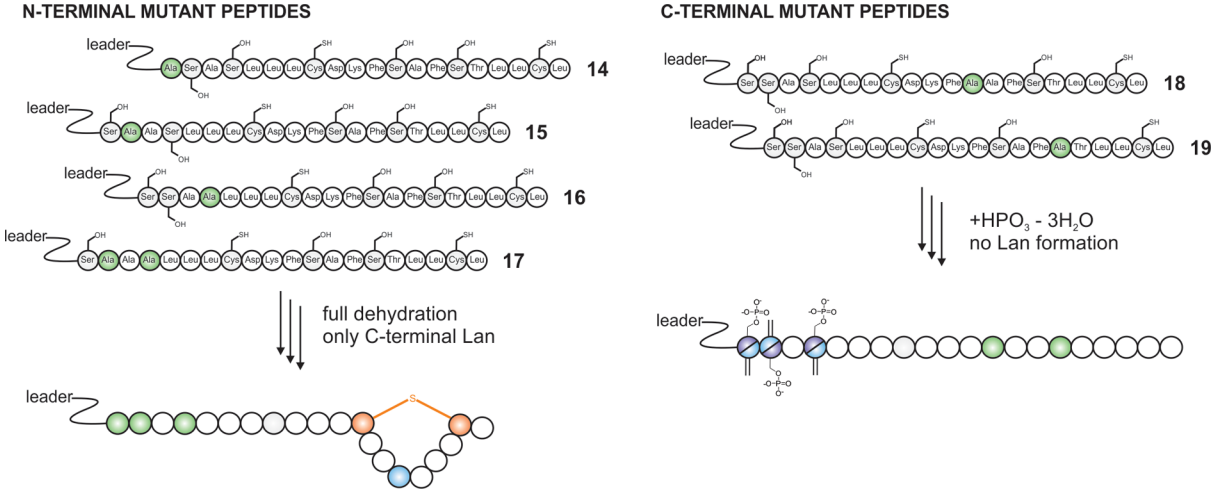

Figure 5. Schematic representation of results from Ser → Ala substitution experiments in CurA peptide, designed to affect the N- and C-terminal Lan formation. Mutation of biosynthetically important Ser within the N-terminal part of the core peptide impedes Lan formation, but has no influence on the formation of C-terminal Lan. Mutations in the C-terminal part fully abolish Lan formation as well as negatively impact dehydration of the remaining Ser.

deuterium-labeled curvopeptins **11**−**13** this is indicative of formation of the C-terminal lanthionine ring only after elimination of the phosphate group from pSer1 and not as expected, directly after dehydration of Ser12. This further suggests that the cyclase domain of CurKC does not process phosphorylated peptides. Equally important is the finding that cyclization in the N-terminal part occurs only after final dehydration of all five Ser residues, despite the fact that Dha1, which is essential for formation of the N-terminal Lan, is present already from earlier dehydration events (peptide **8**). Therefore, it seems that the cyclase domain will not establish the N-terminal Lan until the final dehydration of Ser2. This effect may be based on interference of the hydroxyl side chain of Ser2 or by specific conformational requirements only fulfilled by Dha, enabling a proper positioning of the precursor peptide in the cyclase domain. The C → N-terminal directionality for the cyclization reaction is in line with studies on cyclization directionality of the class III lanthipeptide catenulipeptin.[22] The extensive analytical characterization of CurA cyclization based on both data from MS/MS product ion spectra and deuterium-

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labeling allowed us to assemble the comprehensive scheme of consecutive post-translational steps in curvopeptin biosynthesis (Figure 4). As already mentioned, our studies show that CurKC does not catalyze formation of the N-terminal lanthionine ring immediately after dehydration of Ser1, instead Ser4 and Ser2 must be dehydrated prior to ring formation. To shed more light on the requirements for Lan formation, we employed six synthetic CurA peptides with Ser mutated to Ala: S1A (**14**), S2A (**15**), S4A (**16**), S12A (**18**), and S15A (**19**) as well as double mutations S2A/S4A (**17**). At first we examined the impact of N-terminal mutations: Applying the above enzyme reactions conditions, all N-terminal mutant peptides **14**−**17** were found fully dehydrated by CurKC (Figures 5 and S10−13). Detailed analysis of the MS/MS product ion spectra revealed the presence of the C-terminal Lan ring, whereas formation of the N-terminal Lan ring was impeded. Peptide CurA-S1A (**14**) is unable to undergo the first modification event in the native peptide, i.e., phosphorylation of Ser1. Analysis of fragment spectra of singly phosphorylated curvopeptin S1A now points to Ser2 as the first phosphorylation site (Figure S16 A), followed by the natural modification site at Ser15 (Figure S16 B). Hence in the absence of the first modified residue the modifying enzyme processes the nearest available residues keeping its distinctive processing manner. Subsequently, we investigated the effect of mutations of Ser in the C-terminal part of the peptide. For both peptides S12A (**18**) and S15A (**19**), the main product was singly phosphorylated and triply dehydrated curvopeptin, while no Lan formation was observed. Extended incubation times (>12 h) showed formation of a four-fold dehydrated product. Due to low peak abundance, it was only possible to obtain fragmentation spectra of processed S12A peptide **18** (Figure S17). However, in all cases formation of the N-terminal ring was impaired. These studies show that any Ala substitution of Ser residues in the C-terminal part of curvopeptin is detrimental to both N- and C-terminal Lan formation. This is in agreement with previously reported cyclization directionality and ring formation dependence of another class III lanthipeptide catenulipeptin.[22] The Ser → Ala mutants of curvopeptin further suggest that dehydration of Ser may induce a conformational change important for cyclization reaction. Previous reports on effects of Dha on peptide conformations[23−26] based on X-ray diffraction, NMR spectroscopy, and computational studies suggest steric effects of a roughly planar dehydroalanine on closely located residues and adoption of a constrained conformation which influences secondary structure.

CONCLUSIONS

In summary this study represents the first comprehensive compilation of *in vitro* data of all three modifying reactions of class III lanthipeptide synthetase CurKC. Using synthetic peptides in enzymatic assays followed by MS/MS analytics, the directionality of individual biosynthesis steps, i.e., phosphorylation, elimination, and cyclization, has been assessed. Based on these results we derived a biosynthesis scheme, including all individual steps. Apart from a main biosynthetic route which is mainly nonlinear, alternative processing routes were also identified. The derived biosynthesis scheme has the following features: a preferable C- to N-terminal directionality of phosphorylation with exception of the first phosphorylation event occurring on Ser1. Second, a mainly C- to N-terminal direction of elimination reactions was observed, except for the third elimination event occurring on pSer1. Finally a C- to N-terminal cyclization establishing two Lan rings. Interestingly, it could be shown that the cyclization in the C-terminal part occurs first after dehydration of Ser1. Likewise, the second cyclization in the N-terminal region is catalyzed only after all residues are dehydrated, which indicates auxiliary requirements for N-terminal ring formation. Furthermore, investigation of enzyme reactions of herein presented Ser → Ala mutant peptides for lanthionine formation suggests conformational requirement fulfilled only by Dha. Our data clearly show that careful inspection of all biosynthetic steps is required in order to unequivocally assign the exact sequence of PTMs. The present study constitutes an important step in the understanding of the complexity of the biosynthesis of class III lanthipeptides and underlines the importance of detailed investigations of RiPP biosyntheses for future engineering and drug development purposes.

EXPERIMENTAL METHODS

Expression and Purification of CurKC. Expression and purification of His_6-CurKC were performed as previously described by Krawczyk et al.[8]

***In vitro* Enzymatic Assays with CurKC.** The reaction mixture contained buffer [Tricine (20 mM), pH 8.0], $MgCl_2$ (10 mM), dithiothreitol (1 mM), dATP (1 mM), His_6-CurKC (5 μM), and synthetic curvopeptin precursor peptide (75 μM). The reaction was quenched by the addition of acetonitrile (1:1, v:v) after incubation at 25 °C from 30 s to 2 min for the native curvopeptin precursor peptide and after 1 h for the Ala mutants. Temperature, concentration, and time-dependent experiments were carried out at 18 and 25 °C over 30 s and 1, 3, 6, 10, 30, and 60 min. Enzyme:substrate ratios were 1:1, 1:15, and 1:50 at enzyme concentrations of 5 μM and peptide 5 μM, enzyme 5 μM and peptide 75 μM, enzyme 1 μM and peptide 50 μM, respectively.

For MS/MS analysis the reaction was quenched by addition of EDTA (50 mM). Probes were desalted using a stage tipping protocol.[27] To speed vac-dried processed peptides GluC and GluC reaction buffer (NEB, Frankfurt, Germany) was added and digested for 2 h at 37 °C. For HPLC-ESI-MS/MS analysis probes were once again prepared according to stage tipping protocol.

HPLC-ESI-MS and HPLC-ESI-MS/MS Experiments. All HPLC-ESI-MS and HPLC-ESI-MS/MS analyses were conducted using a LTQ-Orbitrap XL (Thermo Fisher Scientific, Bremen, Germany) coupled to a HPLC system 1200 (Agilent Technologies, Waldbronn, Germany). Chromatographic separations were performed using GromSil 120 ODS-5 ST columns (100 × 2.0 mm or 50 × 2.0 mm, Grace, Deerfield, IL, USA) with a linear mobile phase gradient consisting of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The gradient was 5−100% B over 18 or 10 min, respectively. ESI-MS/MS experiments were recorded in FTMS mode with resolution of 7500. Fragmentation occurred in a high-energy collision dissociation (HCD) cell (normalized collision energy of 25 or 30%). Data were analyzed using the Thermo Xcalibur 2.2 software.

Synthesis of Curvopeptin Precursor Peptides. Detailed procedures are described in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures and mass spectrometric data. 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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