Peptide epimerase-dehydratase complex responsible for biosynthesis of the linaridin class ribosomal peptides

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Abstract

Grisemycin, salinipeptin, and cypemycin belong to the linaridin class of ribosomally synthesized and posttranslationally modified peptides that contain multiple dehydrobutyrine and D-amino acid residues. The biosynthetic gene clusters of these linaridins lack obvious candidate genes for the dehydratase and epimerase required to introduce dehydrobutyrine and D-amino acid residues, respectively. However, we previously demonstrated that the grisemycin (*grm*) cluster contained cryptic dehydratase and epimerase genes by heterologous expression of this biosynthetic gene cluster in *Streptomyces lividans* and proposed that two genes (*grmH* and *grmL*) with unknown functions catalyze dehydration and epimerization reactions. In this study, we confirmed that both GrmH and GrmL, which were shown to constitute a protein complex by a pulldown assay, were required to catalyze the dehydration, epimerization, and proteolytic cleavage of a precursor peptide GrmA by *in vivo* experiments.

Keywords: biosynthesis, D-amino acid, epimerase, linaridin, peptide

Introduction

Ribosomally synthesized and posttranslationally modified peptides (RiPPs) are an emerging group of peptide natural products with diverse chemical structures and biological activities (Montalban-Lopez *et al*. 2021). The biosynthesis of RiPPs initiates with translation of a structural gene to synthesize a precursor peptide, which typically contains an N-terminal leader peptide and a C-terminal core peptide. The precursor peptide then undergoes posttranslational modification and subsequent removal of the leader peptide completes the biosynthesis of the mature product. In many cases, the modification enzymes involved in dehydration, methylation, azole/azoline formation, ester/amide bond formation, and macrocyclic formation, are novel enzymes with new functionalities. Therefore, unique structures in RiPPs are expected to be formed by novel enzymes.

Linaridins are a small group of linear RiPPs containing threonine-derived dehydrobutyrines (Dhb). To date, cypemycin (Komiyama *et al*. 1993; minami *et al*. 1994), grisemycin (Claesen and Bibb 2011), salinipeptins (Shang *et al*. 2019), legonaridin (Rateb *et al*. 2015), pegvadins (Georgiou *et al*. 2020), mononaridin (Wang *et al*. 2021), and corynaridin (Pashou *et al*. 2023) have been isolated and some were reported to exhibit narrow-spectrum antimicrobial activities (Ma and Zhang 2020). Among these, salinipeptins and pegvadins were reported to contain multiple D-amino acid residues (Shang *et al*. 2019; Georgiou *et al*. 2020). In addition, we recently demonstrated that cypemycin and grisemycin also contained multiple D-amino acid residues, suggesting that the presence of multiple D-amino acid residues is a common feature of linaridins and that epimerization reactions are involved in their biosynthesis (Figure 1) (Xiao *et al*. 2022). Bioinformatics analysis indicated that all linaridin biosynthetic gene clusters contained genes encoding a precursor peptide LinA (Lin is the generic name for linaridin proteins), a small

protein (~200 aa) with unknown function LinL, and a protein comprising an N-terminal horizontally transferred transmembrane (HTTM) domain and a C-terminal α/β hydrolase (ABHD) domain LinH. In addition, some linaridin clusters such as the salinipeptin (*sin*), grisemycin (*grm*), and cypemycin (*cyp*) gene clusters contained genes encoding a methyltransferase (LinM) and a decarboxylase (LinD) that install an N-terminal *N*,*N*-dimethyl group and a C-terminal aminovinyl cysteine residue, respectively (Ding *et al*. 2018a, 2018b). Although linaridins contain dehydroamino acid, similar to other dehydroamino acid-containing RiPP natural products such as lanthipeptides and thiopeptides, linaridin biosynthetic gene clusters lack the dehydratase-like genes commonly found in lanthipeptide and thiopeptide biosynthetic gene clusters. In addition, linaridin clusters do not contain an obvious candidate epimerase gene required to install D-amino acid residues. Previously, we showed that the grisemycin cluster (*grmA* to *grmP*) contained all of the necessary genes for grisemycin production including a dehydratase and epimerase by heterologous expression of the biosynthetic gene cluster in *Streptomyces lividans* (Xiao *et al*. 2022). Furthermore, we demonstrated that the dehydration and epimerization reactions preceded decarboxylation and methylation, which would be mediated by GrmD and GrmM, respectively, by gene-deletion experiments. Thus, we hypothesized that two proteins with unknown function (GrmH and GrmL) are responsible for the dehydration and epimerization of nascent precursor peptide GrmA and continued our investigation to confirm the plausibility by *in vivo* heterologous expression of *grmA*, *grmH*, and *grmL* genes and in vitro assay with their recombinant enzymes. Very recently, Xue *et al*. showed that heterologous expression of the *cypA*, *cypH*, and *cypL* genes from the cypemycin cluster in *Streptomyces coelicolor* yielded a cypemycin core peptide with D-amino acids and Dhb residues found in cypemycin, suggesting that CypH and CypL catalyzed dehydration, epimerization, and proteolytic cleavage to remove the leader peptide (Xue, Wang,

and Liu 2023). However, the individual functions of CypH and CypL were not investigated. Here, we showed that either GrmH or GrmL alone was able process GrmA and that GrmH and GrmL constituted enzyme complex to catalyze reactions by protein pulldown assay and *in vivo* analysis.

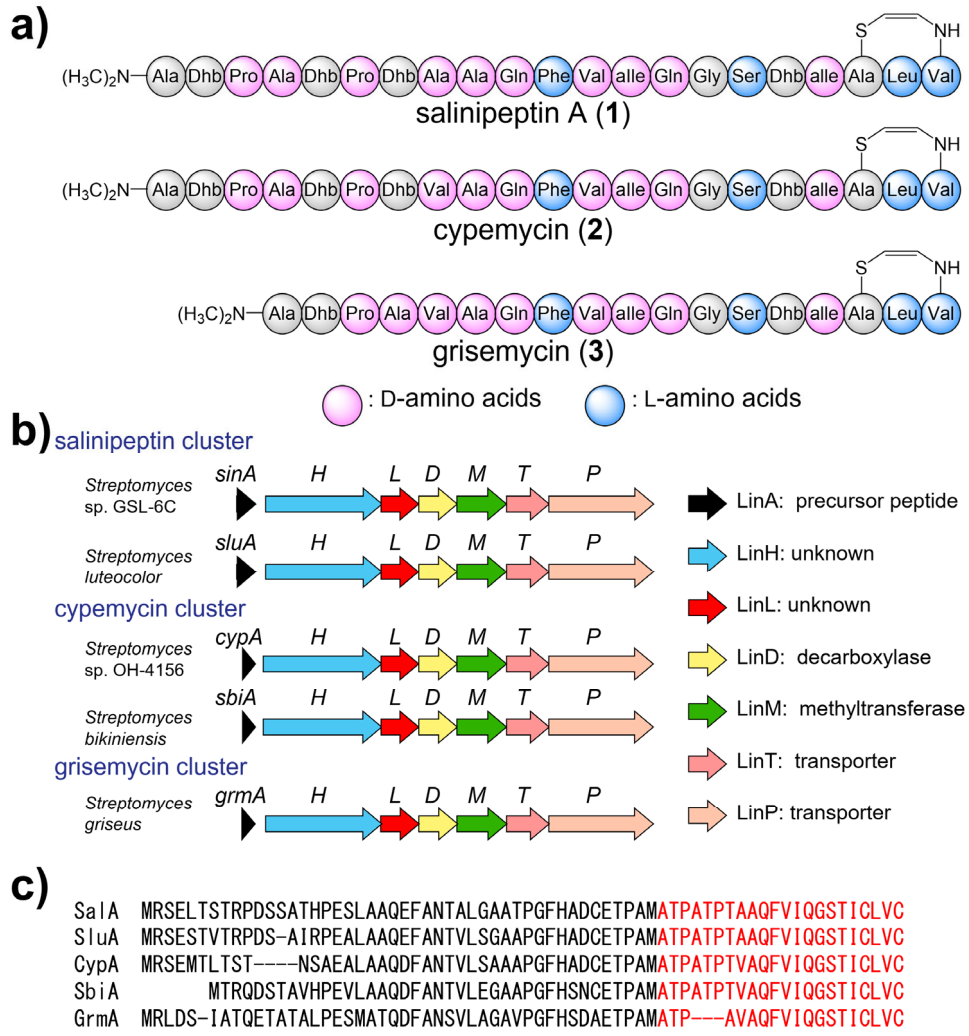

Figure 1. Structure of salinipeptin A, cypemycin, and grisemycin (**a**), the biosynthetic gene clusters (**b**), and deduced amino acid sequences of precursor peptide (**c**). Salinipeptin clusters: *Streptomyces* sp. GSL-6C (*sin*) and *S. luteocolor* NBRC 13826 (*slu*), cypemycin clusters: *Streptomyces* sp. OH-4156 (*cyp*) and *S. bikiniensis* NBRC 14598 (*sbi*), and grisemycin cluster: *S. griseus* NBRC 13350 (*grm*). Core peptide sequences are shown in red in **c**. alle: *allo*-Ile.

Materials and methods

General experimental procedure

All chemicals were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan), Sigma-Aldrich Japan (Tokyo, Japan), or Tokyo Chemical Industry Co. Ltd (Tokyo, Japan) unless specified otherwise. Enzymes and kits for DNA manipulations were purchased from Takara Bio. (Shiga, Japan), Nippon Gene Co. Ltd (Tokyo, Japan), or New England Biolabs Japan Inc. (Tokyo, Japan). Polymerase chain reaction (PCR) assays were carried out using a SimpliAmp thermal cycler (Life Technologies Japan Ltd, Tokyo, Japan) and Tks Gflex DNA polymerase (Takara Bio.). Oligonucleotide synthesis and DNA sequencing were performed in Fasmac (Kanagawa, Japan). General genetic manipulations of *E. coli* and *S. lividans* TK23 were performed according to standard protocols. A vector pSE101N8H was kindly provided by Prof. Tomohisa Kuzuyama at The University of Tokyo.

Construction of plasmids

Oligonucleotides used for PCR in this study are summarized in Table S1. All plasmids were confirmed by DNA sequencing.

pCDFD-His-grmL, pACYCD-His-GrmH, pET21a-SKIK-His-grmA, pET21a-SKIK-His-sluA, and pET21a-SKIK-His-sbiA: A primer pair grmL-F(BamHI)/grmL-R(HindIII) was used to amplify the *grmL* gene and the PCR products, digested with *Bam*HI and *Hin*dIII, were cloned into the same sites of pCDF-Duet1 to obtain pCDFD-His-grmL. A plasmid pACYCD-His-GrmH was constructed with a primer pair, grmH-F(BamHI)/grmH-R(HindIII), and pACYC-Duet1 vector in a similar manner. For the expression of SKIK- and His-tagged GrmA, a DNA fragment containing the *grmA* gene was amplified with the primer pair, grmA-F(NdeI)/grmA-R(HindIII), and cloned

into pET21a vector using *Nde*I and *Hin*dIII restriction enzymes to obtain pET21a-SKIK-His-grmA. Similarly, primer pairs sluA-F(NdeI)/sluA-R(HindIII) and sbiA-F(NdeI)/sbiA-R(HindIII) were used to construct pET21a-SKIK-His-sluA and pET21a-SKIK-His-sbiA, respectively.

pSE101N8H-grmA, pSE101N8H-grmAH, pSE101N8H-grmAL, and pSE101N8H-grmAHL: The DNA fragments containing *grmA* alone, *grmA–grmH*, and *grmA–grmL* were amplified by PCR from the plasmid pWHM3-grm with the primer pairs grmA-F(HindIII)/grmA-R(XbaI), grmA-F(HindIII)/grmH-R(XbaI), and grmA-F(HindIII)/grmL-R(XbaI), respectively. The DNA fragment containing *grmA* and *grmL* was obtained by PCR from the plasmid pWHM3-grm-Δ*grmH* using the primer pair grmA-F(HindIII)/grmL-R(XbaI). Each PCR product was cloned into the *Hin*dIII–*Xba*I site of the pSE101N8H vector so that the N-terminal 8×His-tag and GrmA sequences were expressed in a single open reading frame. The deduced amino acid sequence of the precursor peptide was: MHHHHHHHHKLRLDSIATQETATALPESMATQDFANSVLAGAVPGFHSD-AETPAMATPAVAQFVIQGSTICLVC.

pSE101N8H-sluA-grmHL and pSE101N8H-sbiA-grmHL: To construct pSE101N8H-sluA-grmHL, the DNA fragments containing *sluA* was amplified by PCR with the primer pair sluA-F(HindIII)/sluA-R. Independently, the DNA fragment containing *grmH* and *grmL* was prepared with the primer pair grmH-F/grmL-R(XbaI). Two DNA fragments were then fused with overlap extension PCR with the primer pair sluA-F(HindIII)/grmL-R(XbaI) and the resulting PCR product was cloned into *Hin*dIII–*Xba*I sites of the pSE101N8H vector. Likewise, pSE101N8H-sbiA-grmHL was prepared using primers sbiA-F(HindIII) and sbiA-R. The deduced amino acid sequences of SluA and SbiA were "MHHHHHHHHKLQSESTVTRPDSAIRPEAL-AAQEFANTVLSGAAPGFHADCETPAMATPATPTAAQFVIQGSTICLVC" and "MHHHH-

HHHHKLTRQDSTAVHPEVLAAQDFANTVLEGAAPGFHSNCETPAMATPATPTVAQFVI QGSTICLVC", respectively.

pSE101-grmAH+C-His-grmL: The DNA fragment containing *grmAHL* was amplified with the primer pair grmA-F(HindIII)/grmAHL_C-His-R(XbaI) to introduce a 6×His-tag sequence at the C-terminus of the *grmL* gene. The fragment was then cloned into pSE101 using *Hin*dIII and *Xba*I restriction enzymes.

LC-MS analysis

LC-MS analysis was performed using the Shimadzu Prominence HPLC system equipped with an amaZon SL DB-1 (Bruker, MA, USA) under the following conditions: column, Zorbax 300SB-C8 column (150 × 2.1 mm, 3 μm, Agilent); column temperature, 40 °C; detection, PDA (190–350 nm) and ESI-MS positive mode (*m*/*z* 50–2000); capillary voltage, 4500 V; end plate offset voltage, 500 V; nebulizer gas, 1.5 bar; dry gas flow, 7.0 l min^{-1}; dry temp, 200 °C; mobile phase, A: water with 0.1% trifluoroacetic acid (TFA), B: acetonitrile with 0.1% TFA, 5% solvent B for 0–5 min, a linear gradient to 95% solvent B for 5–30 min, and 95% solvent B for 30–40 min; flow rate, 0.2 ml min^{-1}; injection volume, 10 μL.

Chiral analysis of amino acid residues

The chirality of the amino acid residues in the peptide products was analyzed using a modified Marfey's method. The peptide sample was first hydrolyzed at 110 °C for 6 h with 200 μl of 6 M hydrochloric acid. After evaporation to dryness, the sample was re-dissolved in 100 μl of water. To the 20 μl sample solution, 5 μl of 1 M sodium bicarbonate and 25 μl of 1% 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA) in acetone were added. After incubation for 2 h at 37 °C, the reaction was quenched by the addition of 5 μl of 1 M HCl, and then diluted with 200 μl of

methanol and 250 μl of water. The sample containing L-FDLA derivatives was analyzed by LC-MS using Waters ACQUITY UPLC system equipped with a SQ Detector2 and Acquity PDA detector as described previously (Xiao *et al*. 2022).

Analysis of the interaction between GrmH and GrmL by a pulldown assay

The interaction between GrmH and GrmL was evaluated by a pulldown assay using His-tagged GrmL and untagged GrmH. An *S. lividans* transformant harboring pSE101-grmAH+C-His-grmL was cultivated in YEME medium (300 ml) for 3 days. The cells were collected by centrifugation, lysed by sonication, and His-tagged proteins were purified using Ni-NTA resin as described above. The elution fraction (~300 μl) containing GrmL and the proteins copurified with GrmL were analyzed by SDS-PAGE and LC-MS. LC-MS analysis was performed with an HPLC instrument (Agilent Technologies Inc.) equipped with a Maxis Plus (Bruker). The analytical conditions were as follows: column, Sunshell C8-30HT (2.1 × 150 mm, 3.4 μm, ChromaNik Technologies Inc., Osaka, Japan); flow ratio, 0.3 ml min^{-1}; temperature, 70 °C; mobile phase, (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile; gradient conditions, 30%–60% B (30 min); detection, 280 nm and positive ion mode; injection volume, 10 μl. The mass spectra of multiply charged ions were deconvoluted using the DataAnalysis ver. 4.3 software.

Preparation of recombinant GrmA, SluA, and SbiA

The plasmids pET21a-SKIK-His-grmA, pET21a-SKIK-His-sluA, and pET21a-SKIK-His-sbiA were used to express recombinant SKIK-His-tagged GrmA, SluA, and SbiA, respectively, in *E. coli* BL21(DE3). *E. coli* transformant cells were shaken at 200 rpm at 37 °C, until the OD_{600} reached 0.6–0.8. After induction with isopropyl β-D-1-thiogalactopyranoside (0.5 mM), the cultures were incubated at 16 °C, with shaking at 200 rpm, for 16–20 h. The cells were harvested

by centrifugation (20 000 ×*g*, 10 min, 4 °C) and washed with wash buffer I (50 mM sodium phosphate, 300 mM NaCl, and 20 mM imidazole, pH 8.0). The cell pellets were resuspended with wash buffer I and sonicated. After centrifugation (20 000 ×*g*, 30 min, 4 °C), His-tagged proteins were adsorbed onto Ni-NTA agarose resin (Qiagen), washed with wash buffer II (50 mM sodium phosphate, 300 mM NaCl and 50 mM imidazole, pH 8.0), and eluted with elution buffer (50 mM sodium phosphate, 300 mM NaCl, and 250 mM imidazole, pH 8.0). Purified protein was rebuffered with 50 mM Tris-HCl (pH 8) and concentrated using the Amicon Ultra (MWCO; 3K, Millipore) until the concentration reached 200 μM.

In vitro reactions of the GrmH/GrmL complex with GrmA, SluA, or SbiA

Reaction mixtures (50 μl) containing the precursor peptides (100 μM), the GrmH/GrmL complex (4 μM), and the cell-free extract in reaction buffer (50 mM Tris-HCl, pH 8.0) of *S. lividans* were incubated at 30 °C overnight. The reactions were quenched by the addition of 50 μl of methanol. After centrifugation (16 000 ×*g*, 10 min), the supernatants were analyzed by LC-MS as described above. The cell-free extract of *S. lividans* was prepared as follows: the cell pellet (1 g wet weight) of *S. lividans* TK23 was sonicated in 4 ml of reaction buffer. After centrifugation, the supernatant was passed through an Amicon Ultra (MWCO; 3K) to remove macromolecules.

Results and discussion

To gain insight into the dehydration and epimerization reactions, we first performed *in vivo* experiments by coexpressing *grmA* with *grmH* and/or *grmL* in *Escherichia coli*. To monitor the *in vivo* epimerization reactions, the GrmA product was purified by Ni-NTA affinity chromatography and SDS-PAGE, and the chirality of the amino acid residues in GrmA was analyzed by a modified

Marfey's method after acid hydrolysis. However, no D-amino acids were observed in GrmA even though both GrmL and GrmH were coexpressed, and the same was true when GrmL or GrmH alone was coexpressed with GrmA (Figure S1). Furthermore, LC-MS analysis of purified GrmA revealed that *in vivo* dehydration reactions did not occur (Figure S2). We also carried out coexpression of *sluA* with *sluH* and/or *sluL* from the salinipeptin cluster of *Streptomyces luteocolor*, as well as coexpression of *sbiA* with *sbiH* and/or *sbiL* from the cypemycin cluster of *Streptomyces bikiniensis*. However, the coexpression of recombinant proteins was unsuccessful in most of these combinations and the only successful combinations were SluA with SluH alone and SluA with SluL alone. In addition, no modification of SluA was observed with LC-MS or chiral amino acid analysis in either case. Considering that *grmL* and *grmH* are the only genes of unknown function in the *grm* cluster, GrmL and GrmH may require cofactors absent in *E. coli*.

We therefore used *S. lividans* as the heterologous host. DNA regions containing *grmA*, *grmA–grmH*, and *grmA–grmL* were cloned into pSE101N8H to construct pSE101N8H-grmA, pSE101N8H-grmAH, and pSE101N8H-grmAHL, respectively, resulting in the *grmA* product being fused to the N-terminal 8×His-tag. Similarly, pSE101N8H-grmAL, a plasmid containing His-tagged-*grmA* and *grmL*, was constructed by in-frame deletion of *grmH*. After *S. lividans* TK23 was transformed by the plasmids, the transformants were cultivated in YEME medium for 3 days. The resulting cultures were then centrifuged, and cell extracts prepared with 20% acetone were analyzed by LC-MS. As shown in Figure 2, a specific metabolite **4** was observed in the acetone extract of the cells expressing *grmA*, *grmH*, and *grmL*. The monoisotopic mass of **4** was 925.9988 ($[M+2H]^{2+}$, calcd. for $C_{85}H_{135}O_{23}N_{21}S$: 925.9953), corresponding to the predicted dehydrated product of GrmA core peptide, and importantly, chiral analysis with Marfey's method revealed that **4** contained multiple D-amino acid residues identical to those in grisemycin (Figures

3 and S3). These results clearly indicated that GrmH and GrmL were responsible for the dehydration and epimerization reactions. By contrast, no specific metabolites were observed in the 20% acetone extract of the other transformants containing pSE101N8H-grmA, pSE101N8H-grmAH, or pSE101N8H-grmAL (Figure 2). To further investigate the fate of the precursor peptide in these transformants, we purified GrmA by Ni-NTA affinity chromatography from cell extracts and conducted analysis by SDS-PAGE. The results revealed that GrmA was observed when *grmA* was expressed alone and when *grmA* was coexpressed with either *grmH* or *grmL*, but not when *grmA* was coexpressed with both *grmH* and *grmL* (Figure S4). The observed GrmA was further purified by SDS-PAGE to a single band and the modifications on GrmA were analyzed by LC-MS and Marfey's method (Figures S5 and S6). However, no dehydration or epimerization product was observed. These observations suggested that neither GrmH nor GrmL alone was able to process GrmA. Because both LC-MS and SDS-PAGE analysis of cell extracts indicated that proteolytic cleavage between the leader and core peptides occurred only when *grmA* was coexpressed with both *grmH* and *grmL*, removal of the leader peptide may not be due to endogenous protease in the heterologous host. Taken together, these findings suggest that GrmH and GrmL catalyzed the dehydration, epimerization, and removal of the leader peptide on GrmA to yield **4**.

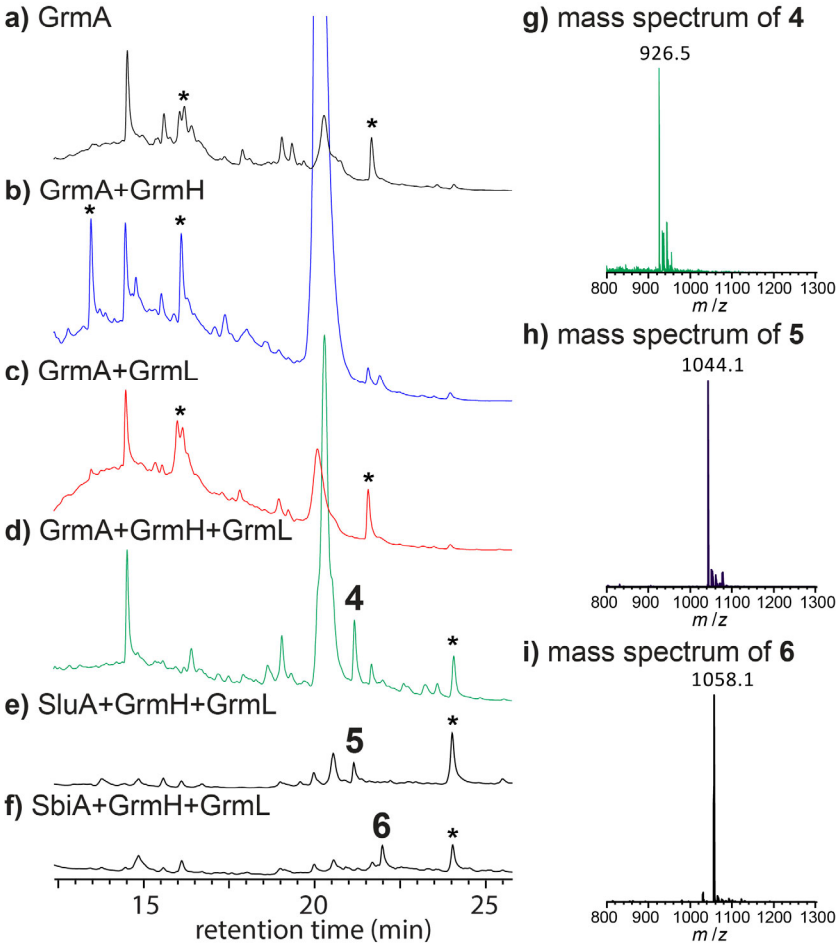

Figure 2. LC-MS analysis of the extract of cells expressing **a)** GrmA, **b)** GrmA + GrmH, **c)** GrmA + GrmL, **d)** GrmA + GrmH + GrmL, **e)** SluA + GrmH + GrmL, and **f)** SbiA + GrmH + GrmL. The chromatograms were monitored by UV absorption at 254 nm.

We next examined substrate tolerance of GrmH and GrmL by replacing *grmA* gene in pSE101N8H-grmAL with *sluA* and *sbiA* which are the precursor peptide genes for salinipeptin and cypemycin, respectively. Although the amino acid sequences of GrmA, SluA, and SbiA are similar to each other, the core peptide in grisemycin is shorter by three amino acids compared to those in salinipeptin and cypemycin (Figure 1). After cultivation of the *S. lividans* expressing *sluA* with *grmH* and *grmL* (pSE101N8H-sluA-grmHL) and *sbiA* with *grmH* and *grmL* (pSE101N8H-sbiA-

grmHL), metabolites were analyzed by LC-MS (Figure 2). Specific metabolites **5** (*m/z* 1043.5439, $[M+2H]^{2+}$, calcd for $C_{96}H_{148}N_{24}O_{26}S$: 1043.5431) and **6** (*m/z* 1057.5584, $[M+2H]^{2+}$, calcd for $C_{98}H_{152}N_{24}O_{26}S$: 1057.5588) were detected in the *S. lividans* transformants harboring pSE101N8H-sluA-grmHL and pSE101N8H-sbiA-grmHL, respectively, and the observed molecular mass was consistent with expected dehydrated products of the core peptides in both cases. Furthermore, chiral analysis revealed that both **5** and **6** contained D-amino acid residues almost identical to those in salinipeptin and cypemycin, respectively (Figure 3), except that **6** contained both D- and L-Glu while all Glu residues in cypemycin have D-configuration. These results indicated that GrmH and GrmL accepted precursor peptides with different lengths. Previously, heterologous expression experiments of cypemycin cluster have revealed that posttranslational modification enzymes in cypemycin biosynthesis were tolerant to single amino acid substitutions on the CypA (Xue, Wang, and Liu 2023; Mo *et al*. 2017). Our results expanded insight into the substrate tolerance of modification enzymes (LinH and LinL) and would be useful to generate unnatural linaridins by precursor peptide bioengineering.

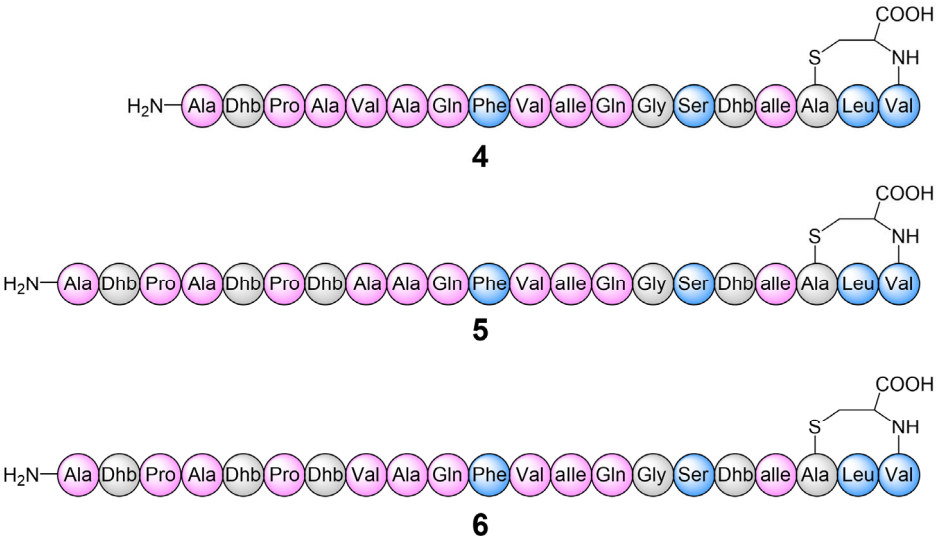

Figure 3. Structure of **4**–**6**. The color coding is the same as that indicated in Figure 1A.

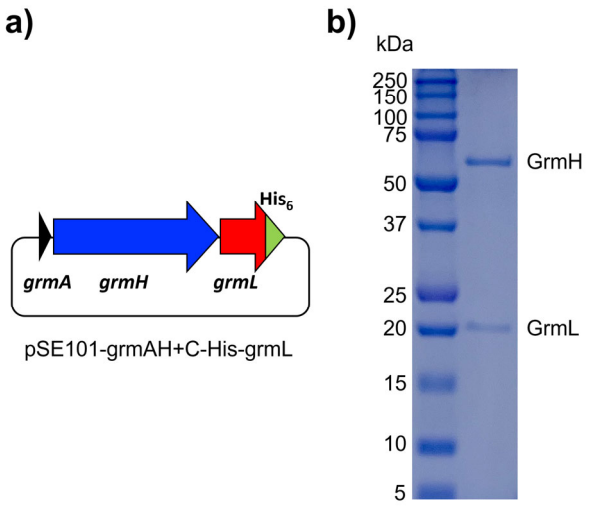

Figure 4. The plasmid to express *grmA*, *grmH*, and *grmL* in *S. lividans* (**a**) and SDS-PAGE analysis of copurified GrmL-GrmH complex (**b**). His-tagged GrmL (21.3 kDa) coeluted with a stoichiometric amount of untagged GrmH (63.2 kDa).

Because AlphaFold 2 analysis using the LocalColabFold program (Mirdita *et al*. 2016) suggested a strong interaction between GrmH and GrmL, we next examined whether GrmH and GrmL form a complex by carrying out a pulldown assay using His-tagged GrmL (or GrmH) and its untagged counterpart (Figure S7). We did not obtain GrmL and GrmH as soluble proteins when we expressed the individual genes in *S. lividans*. However, we succeeded in coexpression of His-tagged GrmL with untagged GrmH as soluble proteins upon additional coexpression of the *grmA* gene. After affinity purification with Ni-NTA resin, we clearly observed two bands (1:1 molar ratio based on the band intensity) with the expected molecular weight of His-tagged GrmL (~21 kDa) and GrmH (~63 kDa) on SDS-PAGE (Figure 4). Furthermore, LC-MS analysis confirmed that the copurified sample contained GrmH (observed: 63242.6 Da and calculated for $C_{2820}H_{4480}N_{830}O_{813}S_7$: 63242.4 Da) and His-tagged GrmL (observed: 21163.3 Da and calculated

for $C_{952}H_{1448}N_{274}O_{269}S_4$: 21162.7 Da) (Figure S8). Although formation of the GrmH/GrmL complex was proposed by AlphaFold2 analysis, this is the first experimental evidence that GrmH and GrmL form a stable complex.

Because we obtained the copurified GrmH/GrmL complex in soluble form, we next performed *in vitro* analysis of the GrmH/GrmL reaction. To prepare full-length GrmA as the substrate in large amounts, we used *E. coli* as the heterologous host. However, at first, no expression of *grmA* was observed on SDS-PAGE. We previously demonstrated that the addition of an N-terminal Ser-Lys-Ile-Lys (SKIK tag) sequence, reported by Ojima-Kato *et al*. (Ojima-Kato, Nagai, and Nakano 2017), immediately after the start codon of the precursor peptide (MslA) of a RiPP natural product MS-271, significantly improved its heterologous expression in *E. coli* (Feng *et al*. 2018; Feng, Ogasawara, and Dairi 2020). Using the same strategy, we successfully expressed and purified GrmA fused with SKIK and His tags at its N-terminus. We also prepared SluA and SbiA in a similar manner, because these precursor peptides have similar amino acid sequences to GrmA and may be accepted by the GrmH/GrmL complex. Because *in vivo* analysis showed that **4** was observed only when *S. lividans* was used as the heterologous host, the GrmH/GrmL reaction may require cofactors present in *S. lividans*. Thus, we incubated GrmA with the GrmH/GrmL complex in the presence of a cell-free extract prepared from *S. lividans*, and the reaction was analyzed by LC-MS. However, the formation of **4** (*m*/*z* 926, $[M+2H]^{2+}$) or another product was not observed in the reaction mixture, despite many different conditions being tried. In addition, incubation of SluA or SbiA with the GrmH/GrmL complex and the cell-free extract did not generate a product corresponding to **5** (*m*/*z* 1043.5, $[M+2H]^{2+}$) for SluA or **6** (*m*/*z* 1057.5, $[M+2H]^{2+}$) for SbiA. Considering that we could detect epimerization activity by the simultaneous coexpression of *grmA*, *grmH*, and *grmL*, simultaneous enzyme expression and the successive

formation of a complex structure of the three enzymes might be necessary for the dehydration and epimerization reactions. Otherwise, other conditions such as an anaerobic environment or the addition of a macromolecule fraction from *S. lividans* may be required for the enzymatic activity of the GrmH/GrmL complex.

Conclusion

In conclusion, we demonstrated that neither GrmH nor GrmL alone could process GrmA but that a GrmH/GrmL complex can catalyze dehydration, epimerization, and proteolytic cleavage. Moreover, we experimentally revealed that GrmH and GrmL constituted a protein complex by a pulldown assay with His-tagged GrmL and untagged GrmH. Although the reactions catalyzed by the GrmH/GrmL complex have yet to be carefully investigated by additional *in vitro* analysis, our results provide a new functionality to catalyze both dehydration and epimerization by the GrmH/GrmL complex involved in the biosynthesis of RiPP natural products.

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Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Author's Contribution

T.D. conceived, designed, and supervised the project. W.X., T.T., C.M., Y.H., and Y.O. conducted experiments and acquired the data. W.X., T.T., and Y.O. analyzed the data. W.X, Y.O, and T.D. mainly wrote the paper. All authors discussed the results and approved the final manuscript.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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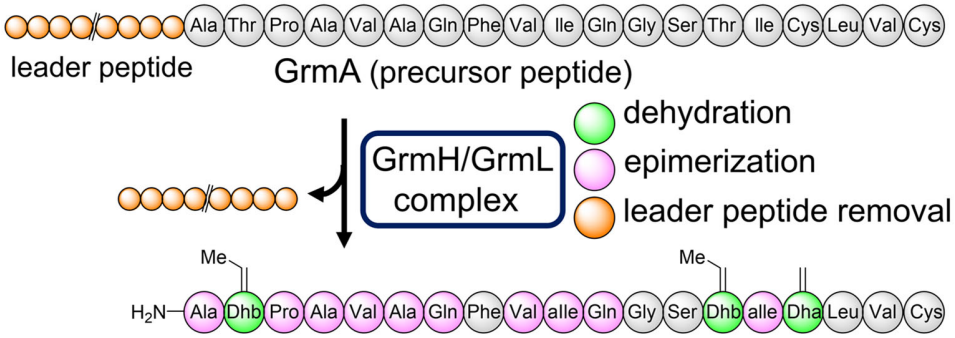

Description of TOC figure: GrmH and GrmL in linaridin natural product biosynthesis constitute an enzyme complex catalyzing dehydration, epimerization, and leader peptide removal.