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Heterologous expression of a cryptic gene cluster from Grimontia marina affords a novel tricyclic peptide grimoviridin

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Title: Heterologous expression of a cryptic gene cluster from *Grimontia marina* affords a novel tricyclic peptide grimoviridin

Running headline: Heterologous production of grimoviridin

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Abstract

Microviridins are a class of ribosomally synthesized and posttranslationally modified peptides (RiPPs) that have been isolated from a wide variety of cyanobacterial strains. There are similar gene clusters of RiPPs distributed in the genomes of bacteria belonging to the phyla Proteobacteria and Bacteroidetes. A cryptic gene cluster for the production of microviridin-type peptides was found in the genome of the marine γ-Proteobacterium *Grimontia marina.* Heterologous production of new microviridin-type peptide named grimoviridin was accomplished in *Escherichia coli* using the biosynthetic gene cluster of *G. marina.* The structure of grimoviridin was determined by analysis of MS and NMR data. Grimoviridin contained one isopeptide and two ester bonds, which had exactly the same bridging pattern as other microviridin-type peptides. The absolute stereochemistries of constituent amino acids were determined to be all L-forms by modified Marfey's analysis. Grimoviridin showed potent inhibitory activity against trypsin with an IC_{50} value of 238 nM. This is the first report of heterologous production of microviridin-type peptide using a biosynthetic gene cluster from a Proteobacterium.

Keywords: heterologous production, biosynthesis, peptide, protease inhibitor, microviridin, *Grimontia marina*

Key Points

- Heterologous production afforded new microviridin-type peptide named grimoviridin.
- This is the first report of microviridin-type peptide from proteobacterial origin.
- Grimoviridin showed potent inhibitory activity against trypsin.

43 **Introduction**

44 Ribosomally synthesized and posttranslationally modified peptides (RiPPs) are a
45 class of naturally occurring peptides that includes more than twenty subclasses such as
46 lantibiotics (Budisa 2013; Letzel et al. 2014; Li et al. 2016; Link 2015; Sardar and
47 Schmidt 2016; Zhang et al. 2018a). Among the RiPPs, a group of cyanobacterial
48 peptides called microviridins commonly possess a tricyclic structure with two ester
49 bonds between the carboxyl group of Asp/Glu side chain and the hydroxyl group of
50 Thr/Ser and an amide bond between the carboxyl group of Asp/Glu side chain and an
51 amine of Lys side chain (Fujii et al. 2000; Ishitsuka et al. 1990; Murakami et al. 1997;
52 Okino et al. 1995; Reshef and Carmeli 2006; Rohrlack et al. 2003; Shin et al. 1996).
53 Microviridins showed potent inhibitory activities against various serine proteases:
54 microviridin B against elastase with an IC_{50} value of 26 nM, (Okino et al. 1995);
55 microviridin 1777 against chymotrypsin with an IC_{50} value of 100 nM, (Sieber et al.
56 2020); and microviridin J against trypsin with an IC_{50} value of 20-90 nM (Rohrlack et
57 al. 2003). Dysregulation of serine proteases may lead to many diseases, such as
58 cancers, type 2 diabetes, and Alzheimer's disease (Schilling and Findeisen 2014); thus,
59 understanding the biosynthesis of protease inhibitors, such as microviridins, is very
60 important to produce new selective protease inhibitors by genetic engineering.

In 2008, the biosynthetic gene clusters (BGCs) of microviridin B and microviridin K were identified in the genomes of the Cyanobacteria *Microcystis aeruginosa* NIES-298 (Ziemert et al. 2008) and *Planktothrix agardhii* NIVA-CYA 126/8 (Philmus et al. 2008), respectively. The biosynthetic gene cluster of microviridin B contained five genes, including the precursor peptide coding gene *mdnA*, two ligase coding genes *mdnB* and *mdnC*, the acetyl transferase coding gene *mdnD*, and the ABC-transporter coding gene *mdnE* (Ziemert et al. 2008). The two ligase enzymes, MdnB and MdnC, possessed an ATP-grasp domain and were modification enzymes for the formation of two ester bonds and one isopeptide bond in the molecule (Hemscheidt 2012). The function of MdnC was determined to be the formation of two ester bonds, and the other modification enzyme, MdnB, was shown to function as a ligase for isopeptide bond (Li et al. 2016). The ABC transporter MdnE was shown to be essential for the cyclization and processing of microviridins for stability of the biosynthesis complex (Weiz et al. 2011). The intramolecular macrocyclization of the core peptide with both ester and isopeptide bonds indicated the importance of a unique macrocyclization strategy for RiPP biosynthesis in terms of the application of this system for the generation of new bioactive peptides (Ahmed et al. 2017; Reyna-Gonzalez et al. 2016; Weiz et al. 2011; Zhang et al. 2018b; Ziemert et al. 2010). The potent trypsin inhibitor microviridin J

was cocrystallized with trypsin and the mode of inhibition was described as competitive inhibition (Weiz et al. 2014). A metagenomic approach was applied to generate new microviridins using the fosmid library system (Gatte-Picchi et al. 2014). There are many analogous BGCs of microviridin-type peptides distributed over Cyanobacteria (Micallef et al. 2015), although they are mostly not explored. To upregulate the biosynthetic genes, a culture control experiment of *Nostoc punctiforme* using RNA-seq and fluorescence reporter analysis revealed new microviridins along with other cyanobacterial peptides (Dehm et al. 2019).

The distribution of BGCs of microviridin-type peptides was revealed in the genome sequences of bacteria belonging to the phyla Proteobacteria, Bacteroidetes, and Cyanobacteria (Ahmed et al. 2017). Microviridin-type peptides have been isolated from a wide variety of Cyanobacteria, and marinostatins (microviridin analogous bicyclic peptides) were isolated from the marine Proteobacterium *Algicola sagamiensis* (Imada et al. 1985; Kanaori et al. 2005; Miyamoto et al. 1998). To date, no tricyclic microviridin-type peptide has been reported from Proteobacteria or Bacteroidetes. Based on the genome mining report (Ahmed et al. 2017), we focused on the BGC of microviridin-type peptides in the genome of the marine Proteobacterium *Grimontia marina* (Choi et al. 2012). We performed heterologous production of a new peptide

named grimoviridin using the BGC of *G. marina*. To the best of our knowledge, this is the first report of tricyclic microviridin-type peptide production using biosynthetic genes of Proteobacteria. Here, we describe the production and structure determination of the new peptide grimoviridin (Fig. 1A).

Materials and Methods

Bacterial strain

The bacterium *Grimontia marina* NBRC 105794^{T} was obtained from the NBRC culture collection (NITE Biological Resource Center, Chiba, Japan).

Construction of the expression vector pET-41a-105794G

The gene cluster of grimoviridin was integrated into the expression vector pET-41a(+) by performing amplification and integration of the partial sequences twice. For the template of PCR amplification, the crude genome DNA was extracted from the cells of bacterium *G. marina* NBRC 105794^{T} using DNeasy Blood & Tissue (Qiagen, Venlo, Netherlands). The partial sequence (2476 bp, *grmA-C*) of the gene cluster was amplified by PCR with the primer pair (105794-F1-*Xba*I: 5'-CCGTCTAGAGGCTAATGTTCAAAAAATAACC -3' and 105794-R1-*Kpn*I: 5'-TGCGGTACCACAATAAACTTTTGGTTAGC -3') using EmeraldAmp PCR Master Mix (Takara Bio Inc., Shiga, Japan) following the manufacturer's instructions. The insert DNA fragment and the pET-41a(+) vector were double-digested with *Xba*I and *Kpn*I-HF (New England Biolabs, Ipswich, MA, USA), according to the manufacturer's instructions. The DNA products were ligated using Ligation Convenience Kit (Nippon gene Co., Ltd., Tokyo, Japan) to afford the vector pET-41a-105794S. *Escherichia coli*

120 DH5α cells were transformed with 10 μL of the ligation mixture by chemical
121 competence transformation, and the cells were plated on LB agar plates containing
122 kanamycin (final concentration of 30 μg/mL). The vector pET-41a-105794S was
123 purified using FastGene Plasmid Mini Kit (Nippon Genetics Co., Ltd., Tokyo, Japan)
124 following the manufacturer's instructions. The remaining partial sequence (3387 bp,
125 *grmT1* and *grmT2*) of the gene cluster was amplified by PCR with the primer pair
126 (105794-F2-KpnI: 5'- AATTAGGTACCATTGGAGATCACTAATGAGCCAGC -3' and
127 105794-R2-SalI: 5'- AATGAGTCGACTTGGTTTATAGATCCGCTAAGTTC -3')
128 using EmeraldAmp PCR Master Mix (Takara Bio Inc.). The insert DNA fragment and
129 the pET-41a-105794S were double-digested with *Kpn*I-HF and *Sal*I-HF (NEB). The
130 DNA products were ligated using Ligation Convenience Kit (Nippon gene Co. Ltd.) to
131 afford the vector pET-41a-105794G, which contained the whole gene cluster of
132 grimoviridin. *E. coli* DH5α cells were transformed with 10 μL of the ligation mixture
133 by chemical competence transformation, and the cells were plated on LB agar plates
134 containing kanamycin (final concentration of 30 μg/mL). The vector pET-41a-
135 105794G was purified using FastGene Plasmid Mini Kit (Nippon Genetics Co., Ltd.).
136 Finally, the vector pET-41a-105794G was transformed into the expression host *E. coli*

137 BL21(DE3) by chemical competence method, for the heterologous expression of

138 grimoviridin.

139 **Isolation of grimoviridin**

140 The bacterium *E. coli* BL21(DE3) harboring pET-41a-105794G was cultured using

141 2.0 L of modified basal agar medium containing kanamycin (30 μg/mL, final

142 concentration) and isopropyl-β-D-thiogalactopyranoside (0.1 mM, final concentration)

143 at 30 °C for 24 h. The modified basal agar medium was prepared by adding the

144 inorganic compounds (K_2SO_4, 2 g; K_2HPO_4, 3 g; NaCl, 1 g; NH_4Cl, 5 g; $MgSO_4 \cdot 7H_2O$,

145 80 mg; $CuCl_2$, 0.5 mg; $MnSO_4 \cdot H_2O$, 0.35 mg; $FeCl_3$, 0.5 mg; $CaCl_2 \cdot 2H_2O$, 0.5 mg) and

146 15 g agar in 1 L of distilled water with adjusting pH 7.3. After autoclaving, the

147 medium was supplemented with separately sterilized glucose and yeast extract solutions

148 at final concentrations of 0.25% and 0.4%, respectively. The bacterial cells were

149 harvested with a laboratory spatula and extracted with twice volume of MeOH. After

150 centrifugation, the MeOH extract was subjected to preparative HPLC using Wakopak

151 Navi C30-5 column (4.6 × 250 mm, FUJIFILM Wako Pure Chemical Co., Osaka,

152 Japan) with isocratic mode (flow rate: 1 mL/min, solvent: 17% MeCN containing

153 0.05% TFA). HPLC purification was performed repeatedly with the UV detector set at

154 220 nm to obtain grimoviridin (retention time: 16.0 min). The yield was 7.6 mg from
155 2.0 L culture.

156 **Mass spectrometry experiments**

157 The accurate mass measurement was conducted using an ESI Orbitrap mass
158 spectrometer (Orbitrap Velos ETD, Thermo Fisher Scientific, Waltham, MA, USA).
159 The peptide was appropriately diluted with 50% MeOH containing 0.1% formic acid,
160 and was supplied to ESI Orbitrap mass spectrometer by direct infusion with
161 electrospray ionization (ESI) in the positive polarity. MALDI-TOF MS and MS/MS
162 analysis was conducted using a MALDI-TOF/TOF mass spectrometer (4800 Plus
163 TOF/TOF analyzer, Sciex, Redwood City, CA, USA). Peptide was mixed with equal
164 volume of α-Cyano-4-hydroxycinnamic acid (4-CHCA) (Shimadzu GLC Ltd., Tokyo,
165 Japan) matrix solution (prepared as 10 mg/mL in 50% MeCN containing 0.1% TFA),
166 and aliquot of the mixture (0.5 μL) was spotted onto a standard stainless plate. After
167 dried up, MS and MS/MS spectra were measured in the positive-ion mode. The mass
168 spectrometer was tuned and calibrated using calibration standards of polytyrosine
169 (Thermo Fisher Scientific Pierce Biotechnology, Rockford, IL, USA) and the peptide
170 mixture (Peptide Calibration Standard II, Bruker Daltonics), respectively, prior to the
171 measurements.

172 **NMR experiments**

173 NMR sample was prepared by dissolving 3.0 mg of grimoviridin in 500 μL of DMSO-

174 d_6. 1D ^{1}H, ^{13}C, DEPT-135, and all 2D NMR spectra were obtained on Bruker Avance800

175 spectrometer with quadrature detection following the previous report (Kodani et al. 2018).

176 **Modified Marfey's method**

177 Grimoviridin (1.5 mg) was subjected to acid hydrolysis with 6 N HCl at 110 °C for

178 16 h for detection of amino acids. The hydrolysates were completely evaporated using

179 rotary evaporator, followed by adding 200 μL of water. 10 μL of *N*α-(5-Fluoro-2,4-

180 dinitrophenyl)-L-leucinamide (L-FDLA, Tokyo Chemical

181 Industry Co., Ltd., Tokyo, Japan) in acetone (10 μg/μL) and 100 μL of 1 M $NaHCO_3$

182 solution were added to the hydrolysate and the mixture was incubated at 80 °C for

183 3 min. The reaction mixture was cooled down at room temperature, followed by

184 neutralization with 50 μL of 2 N HCl and dilution with 1 mL of 50% MeCN. For

185 standard amino acids, each amino acid was derivatized with L-FDLA and D-FDLA in

186 the same method. Approximately 30 μL of each FDLA derivatives was subjected to

187 HPLC analysis with Wakopak Handy ODS column (4.6 × 250 mm, FUJIFILM Wako

188 Pure Chemical Co.). The DAD detector (MD-2018, JASCO Corporation, Tokyo,

189 Japan) was used for detection of the amino acid derivatives accumulating the data of the

190 absorbance from 200 nm to 420 nm. The HPLC analysis for all amino acids was
191 performed at a flow rate of 1 mL/min using solvent A (distilled water containing 0.05%
192 TFA) and solvent B (MeCN containing 0.05% TFA) with a linear gradient mode from 0
193 min to 70 min, increasing percentage of solvent B from 25% to 60%. The retention
194 times (min) of L- or D-FDLA derivatized amino acids were as follows; L-Lys-D-FDLA
195 (17.75 min), L-Arg-D-FDLA (18.53 min), L-Lys-L-FDLA (22.06 min), L-Arg-L-FDLA
196 (22.60 min), L-Thr-L-FDLA (25.12 min), L-Ser-L-FDLA (27.09 min), L-allo-Thr-L-
197 FDLA (27.15 min), L-Asp-L-FDLA (27.19 min), L-Asp-D-FDLA (27.52 min), L-Ser-D-
198 FDLA (28.27 min), L-allo-Thr-D-FDLA (30.14 min), L-Glu-L-FDLA (30.44 min), L-
199 Glu-D-FDLA (32.84 min), L-Pro-L-FDLA (33.68 min), L-Ala-L-FDLA (34.07 min), L-
200 Thr-D-FDLA (34.40 min), L-Pro-D-FDLA (39.62 min), L-Ala-D-FDLA (41.07 min), L-
201 allo-Ile-L-FDLA (45.23 min), L-Ile-L-FDLA (45.58 min), L-Phe-L-FDLA (48.96 min),
202 L-Phe-D-FDLA (59.54 min), L-Ile-D-FDLA (61.37 min), L-allo-Ile-D-FDLA (61.71
203 min).

204 **Protease inhibition assay**

205 Serine protease inhibitory assay (trypsin, chymotrypsin, and elastase) was carried out
206 by the modified method of previous report (Shin et al. 1996). Each assay mixture
207 containing 120 μL of 0.4 M Tris-HCl buffer (pH 7.6 for trypsin and chymotrypsin, pH

208 8.6 for elastase), 200 μL of enzyme solution and 80 μL of solution of grimoviridin
209 (prepared in distilled water) were pre-incubated at 37 °C for 5 min. Each reaction was
210 started with addition of 400 μL of substrate solution and the absorbance at 405 nm was
211 immediately measured. The absorbance at 405 nm was measured after 30 min
212 incubation at 37 °C. The inhibition rate was calculated based on data of absorbance.
213 Trypsin from Porcine Pancreas (35544-94, Nacalai Tesque, Inc., Kyoto, Japan) and
214 chymotrypsin from Bovine Pancreas (09041-84, Nacalai Tesque, Inc.) were dissolved in
215 50 mM Tris-HCl buffer (pH 7.6) to prepare 150 U/mL and 15 U/mL enzyme solutions,
216 respectively. Elastase from Porcine Pancreas (E1250, Sigma-Aldrich, Inc., St. Louis,
217 MO, USA) was dissolved in 50 mM Tris-HCl buffer (pH 8.6) to prepare 0.6 U/mL
218 enzyme solution. Regarding substrate solution for trypsin, 43.3 mg of *N*α-Benzoyl-
219 DL-arginine 4-nitroanilide hydrochloride (B4875, Sigma-Aldrich, Inc.) was dissolved in
220 1 mL of dimethyl sulfoxide (DMSO), followed by addition of 99 mL of 50 mM Tris-
221 HCl buffer (pH 7.6). Regarding substrate solution for chymotrypsin, *N*-Succinyl-L-
222 phenylalanine-*p*-nitroanilide (S2628, Sigma-Aldrich, Inc.) was dissolved in 50 mM
223 Tris-HCl buffer (pH 7.6) to adjust to the final concentration of 1 mg/mL. Regarding
224 substrate solution for elastase, *N*-Succinyl-Ala-Ala-Ala-*p*-nitroanilide (S4760, Sigma-
225 Aldrich, Inc.) was dissolved in 50 mM Tris-HCl buffer (pH 8.6) to adjust to the final

concentration of 1 mg/mL. The subtilisin inhibitory assay was performed according to the methods described previously (Weiz et al. 2014). Subtilisin from *Bacillus licheniformis* (P5380, Sigma-Aldrich, Inc.) was used for the subtilisin inhibitory assay.

Results

Assignment of the biosynthetic gene cluster of grimoviridin

The BGCs of microviridin groups were previously described to distribute broadly over genomes of bacteria belonging to the phyla Proteobacteria, Bacteroidetes, and Cyanobacteria (Ahmed et al. 2017). The precursor peptide coding genes were classified into three classes according to structural characteristics (Ahmed et al. 2017). Class I precursor peptides contained *N*-terminal leader peptides with a single core peptide. Class II precursor peptides contained a single leader peptide with up to five consecutive core peptides. Class III precursor peptides contained a single core peptide with leader peptide and multiple precursors existed in the gene cluster. Among these classes, we focused on the typical BGCs of microviridin-type peptides in the genome of marine bacteria including *Grimontia marina* (Choi et al. 2012), *G. celer* (Pujalte et al. 2016), *Vibrio penaeicida*, (Kawato et al. 2018) and *V. caribbeanicus* (Hoffmann et al. 2012). The BGCs of *G. marina, G. celer*, and *V. caribbeanicus* had one precursor

244 peptide gene (*grmA*, *grcA*, and *vcaA*, respectively) in each BGC. On the other hand,
245 the BGC of *V. penaeicida* contained two precursor peptide genes (*vpeA1* and *vpeA2*).
246 The sequences of precursor peptides of marine bacteria were aligned with those of other
247 cyanobacterial precursor peptides (MdnA, MdnA2 and MdnA3, Fig. 2). At the leader
248 sequence at the *N*-terminus, the motif sequence K-X-X-P-F-F-X-X-F (bold letters in
249 Fig. 2) was conserved in all precursor peptides. This motif sequence forms an α-helix,
250 which is recognized by the modification enzyme MdnC (Li et al. 2016). As indicated
251 by the underline in Fig. 2, the precursor peptides possessed the microviridin peptide
252 motif -T-X1-K-X2-P-S-D-X3-(D or E)-(D or E)- in the core peptides. This region has
253 a tricyclic structure with two ester bonds and one isopeptide bond. The precursor
254 peptide genes of *V. penaeicida* contained two microviridin peptide motifs in each
255 sequence. The sequences of the peptides of marine Proteobacteria possessed a GG
256 motif before the core peptide, which is possibly exposed to bacteriocin processing
257 peptidases. As shown in Fig.3, the BGC in *G. marina* consisted of five genes
258 including genes coding for precursor (*grmA*, accession number CZF84009.1), two
259 modification enzymes (*grmB*, CZF84007.1, *grmC*, CZF84004.1), HlyD family secretion
260 protein (*grmT1*, CZF84002.1) and ABC-transporter (*grmT2*, CZF83999.1). The two
261 modification enzymes, GrmB and GrmC, encoded ATP-grasp domain-containing

262 proteins. The amino acid sequences of GrmB and GrmC showed high similarity to
263 those of MdnB (CAQ16117.1) (56% similarity, 38% identity) and MdnC
264 (CAQ16118.1) (65% similarity, 45% identity), respectively. The amino acid sequence
265 of GrmT1 showed similarity to the colicin V secretion protein Cva of *E. coli*
266 (GDA71566.1) (48% similarity and 27% identity). Regarding ABC transporters, the
267 amino acid sequence of GrmT2 showed similarity to that of MdnE (CAQ16120.1) (45%
268 similarity, 27% identity). The proteins GrmT1 and GrmT2 may function cooperatively
269 to export mature peptides to the outside of the cell. Interestingly, the biosynthetic gene
270 cluster in *G. marina* lacks an acetyl transferase coding gene corresponding to *mdnD*.
271 Overall, we expected that an "unacetylated" microviridin-type peptide would be derived
272 from the precursor GrmA by modification of GrmB and GrmC. We checked the
273 production of the expected microviridin-type peptide in *G. marina*. Briefly, *G. marina*
274 was cultured using 2216 marine liquid medium. The culture of *G. marina* was
275 extracted by MeOH, and the extract was analyzed by HPLC and ESI-MS (data not
276 shown). As a result, the expected peptide was not detected in the MeOH extract of *G.*
277 *marina*. Thus, we accomplished heterologous production of a new microviridin-type
278 peptide named grimoviridin in *Escherichia coli*.

279 **Heterologous production**

To perform heterologous production of grimoviridin, the full gene cluster was cloned. The gene cluster was amplified by PCR and integrated into the expression vector pET-41a(+) to afford pET-41a-105794G (containing the *grmA-T2* genes, Fig. S1). The plasmid was cloned into *E. coli* DH5α, and then transformed into *E. coli* BL21(DE3). The bacterium *E. coli* BL21(DE3) possessing the plasmid pET-41a-105794G was cultured on modified basal agar medium containing kanamycin and isopropyl-β-D-thiogalactopyranoside (IPTG) at 30 °C for 24 h. The bacterial cells were harvested from solid agar medium efficiently to completely separate cells. Compared with culture using liquid medium, agar medium culture had the advantages of avoiding contamination of nutrient substances such as glucose at the following extraction step. The peptide production yield using modified basal medium was higher than the yield with commonly used LB medium (data not shown). The cells were harvested with a laboratory spatula and extracted with double volume of MeOH; the resulting MeOH extract was analyzed by HPLC and ESI-MS. The transformant produced a new peptide named grimoviridin (Fig. S2). To obtain enough grimoviridin for structure determination and biological tests, the transformant (*E. coli* BL21(DE3) possessing pET41a-105794G) was cultured using modified basal agar medium. After HPLC

297 purification (Fig. S3), approximately 7.6 mg of grimoviridin was obtained from cultured

298 cells using 2 L of media.

299 **Structure determination**

300 The molecular formula of grimoviridin was determined to be $C_{67}H_{99}N_{19}O_{24}$ by

301 accurate MS (Fig. S4) since the ion corresponding to $[M+2H]^{2+}$ was observed at *m/z*

302 777.8625 (the calculated *m/z* value was 777.8628). Compared with the molecular

303 formula of the core peptide, dehydration seemed to occur with the removal of three

304 units of H_2O from the core peptide. To confirm the amino acid sequence, an MS/MS

305 experiment was performed on grimoviridin to obtain the amino acid sequence. As a

306 result, limited fragmentation ion peaks (*y13*, *y14*, *b12*, *b13*, *b14*) were observed,

307 indicating the *N*-terminal sequence (Ile1-Ala2) and C-terminal sequence (Gly13-Phe14-

308 Ser15), since the cyclic structure in grimoviridin hampered fragmentation in the MS/MS

309 experiment (Table S1). To further determine the chemical structure, the analyses of

310 NMR spectra (^{1}H, ^{13}C, DEPT-135, DQF-COSY, TOCSY, NOESY, HMBC, and HSQC)

311 were performed on grimoviridin dissolved in 0.5 mL of DMSO-d_6 (Fig. S5 - S30). In

312 the ^{1}H NMR spectrum, α-protons of amino acid residues at 3.60 - 4.73 ppm and amine

313 protons at 6.84 - 9.11 ppm were observed. The assignments of the constituent 15

314 amino acids in grimoviridin were completed using spin-system identification (Fig. 1B

315 and Table S2). The sequential analysis of amino acids was performed by NOESY
316 correlations between amino protons and α-protons in adjacent amino acids (double-end
317 arrows in Fig. 1B). The two sequences (Ile1-Ala2-Thr3-Arg4-Lys5-Ala6 and Pro7-
318 Ser8-Asp9-Asp10-Asp11-Glu12-Gly13-Phe14-Ser15) were obtained by sequential
319 NOESY walk. The connection between Ala6 and Pro7 was established by HMBC
320 correlation from the α-proton of Pro7 (δH 4.17) to the carbonyl of Ala6 (δC 171.7).
321 The isopeptide bond between Lys5 and Glu12 was determined by the HMBC
322 correlation from the ε-amine proton of Lys5 (δH 7.21) to the γ-carbonyl of Glu12 (δC
323 171.9). The two ester bonds were elucidated by HMBC correlations from the β-proton
324 of Thr3 (δH 5.46) to the β-carbonyl of Asp9 (δC 170.0) and from the β-proton of Ser8
325 (δH 4.10) to the β-carbonyl of Asp11 (δC 169.9). The bridging pattern of grimoviridin
326 (one isopeptide and two ester bonds) had the same topology as those of other
327 microviridins. The stereochemistries of constituent amino acids were determined by
328 modified Marfey's method. After hydrolysis of grimoviridin, the hydrolysate was
329 derivatized by *N*α-(5-Fluoro-2,4-dinitrophenyl)-L-leucinamide (L-FDLA).
330 Comparative HPLC analysis was performed with standard amino acids with L-FDLA or
331 D-FDLA (Fig. S31 - S43). As a result, all constituent amino acids in grimoviridin

332 were determined to be L-form. Thus, the chemical structure including absolute

333 chemistry was determined as shown in Fig. 1A.

334 **Protease inhibition assay**

335 To determine the inhibitory activities of grimoviridin against proteases, including

336 trypsin, chymotrypsin, elastase, and subtilisin, inhibition tests were performed

337 according to previous reports. As a result, grimoviridin inhibited chymotrypsin,

338 trypsin, and subtilisin with IC_{50} values of 5.2 μM, 238 nM, and 55 μM, respectively

339 (Table 1). However, grimoviridin did not show inhibitory activity against elastase at a

340 concentration of 64 μM.

341

342 **Discussion**

343 **Biosynthetic gene cluster of grimoviridin**

344 As shown in Fig. 3, the BGC of grimoviridin comprised five genes (*grmA-T2*). The

345 precursor peptide GrmA contains a leader peptide (dotted underline in Fig. 3), double

346 Gly sequence (no underline in Fig. 3), and core peptide (underline in Fig. 3). The

347 precursor peptide is modified by the modification enzymes GrmB and GrmC, giving

348 one isopeptide and two ester bonds in the molecule. The protein GrmT1 is analogous

349 to protein the colicin V excretion protein CvaA (Gilson et al. 1987). CvaA was

350 described as a membrane fusion protein that possibly forms a bridge that connects the
351 inner and outer membranes via interaction with CvaB, an ABC transporter, and TolC, an
352 outer membrane channel protein (Hwang et al. 1997). The protein GrmT1 may
353 interact with the ABC transporter GrmT2, possibly functioning to coordinate the export
354 of mature peptide to the outside of the cell. The *N*-terminal sequence of GrmT2
355 contains a double-glycine peptidase domain that possibly cleaves off the leader peptide
356 at the double Gly position in the precursor peptide GrmA. Although the host
357 bacterium *E. coli* BL21(DE3) lacks proteases (Lan and OmpT), other endogenous
358 proteases of *E. coli* may truncate C-terminal and *N*-terminal peptide sequences from the
359 core peptide to afford grimoviridin (Fig. 3). The structural peptide of 15 amino acids
360 in length has intermolecular bonds that give the molecule resistance to endogenous
361 proteases of *E. coli*.

362 **Structure-activity relationship in the microviridin-type peptides**

363 Previously, the structure-activity relationship of the microviridin-type peptides was
364 summarized (Ziemert et al. 2010). The consensus sequence (T-X-K-Y-S-P-D-X-E-
365 D/E) in microviridins was reported by comparison of microviridin variants (Table. 1).
366 We added the sequence and protease inhibitiory activities of grimoviridin to the
367 sequences of microviridin variants (Table. 1). As a result, the consensus sequence T-

368 X1-K-X2-P-S-D-X3-E/D-E/D was observed in grimoviridin (Fig. 1C and Table 1).
369 Tyr at the X2 position was conserved in all microviridin variants; on the other hand,
370 grimoviridin uniquely possessed Ala at the X2 position. Philmus et al. indicated that
371 ester and amide bonds formation was caused by MvdD (MdnC) and MvdC (MdnB) by
372 in vitro experiments. They reported that the ester formation activity of MvdD (MdnC)
373 was lost by substitution of Tyr to Ala at the X2 position of the core peptide (Philmus et
374 al. 2009). The analogous enzyme GrmC seemed to be different from MdnC in terms
375 of substrate specificity. Position X3 should have aromatic amino acids in all
376 microviridin variants. On the other hand, grimoviridin possessed the hydrophilic
377 amino acid Asp at the X3 position. Microviridin molecules normally contain two ester
378 bonds and one isopeptide bond. However, some microviridin variants such as
379 microviridins C-F, seem to be incomplete products that have one ester bond or no ester
380 bond with the isopeptide bond in the molecule. In this study, such incomplete peptides
381 were not detected by HPLC and ESI-MS analysis (data not shown). The inhibitory
382 mechanism was clarified by X-ray crystallographic analysis on cocrystals of trypsin and
383 microviridin J (Weiz et al. 2014). In the cocrystal, the methyl group of the Thr side
384 chain in microviridin J points towards Leu99 in the S2 pocket of trypsin, whereas the
385 Arg side chain in microviridin J is coordinated by the carboxyl group of Asp189 at the

bottom of the S1 pocket, resulting in rigid complex formation (Weiz et al. 2014). The motif of grimoviridin contains -Thr-Arg- similar to microviridin J, so it is reasonable that grimoviridin has potent inhibitory activity against trypsin.

In the genomes of Proteobacteria and Bacteroidetes, there are many BGCs of microviridin-type peptides (Ahmed et al. 2017). We revealed that heterologous production using *E. coli* was applicable to obtain microviridin-type peptides. The combination of genome mining and heterologous production will lead to the discovery of new microviridin-type peptides, such as grimoviridin, by exploring other unexploited BGCs.

Author contribution

SK designed the research and wrote the manuscript. KU and IK conducted experiments of cloning and structure determination. HN conducted MS experiments. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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539

Figure legends

Fig. 1 A) Chemical structure of grimoviridin, B) Key 2D NMR correlations on grimoviridin (HMBC, TOCSY, and NOESY), C) Amino acid sequence of tricyclic region in microviridin analogues

Fig. 2 Alignment of amino acid sequences of precursor peptides of grimoviridin related peptides; GrmA from BGC of grimoviridin in *Grimontia marina* CECT 8713 (accession number: CZF84009.1); GrcA from *G. celer* CECT 9029 (WP_062661690.1) ; VpeA1 and VpeA2 from *Vibrio penaeicida* TUMSAT-NU1 (WP_104398650.1 and WP_104398651.1); VcaA from *V. caribbeanicus* ATCC BAA-2122 (WP_081454958.1); MdnA from BGC of microviridin B in *Microcystis aeruginosa* NIES-298 (CAQ16116.1); MdnA1 from *M. aeruginosa* NIES-87 (GBE73820.1); MdnA2 from *Planktothrix agardhii* NIES-204 (BBD53454.1)

Fig. 3 Proposed biosynthetic pathway of grimoviridin in *Escherichia coli*

Figure 1

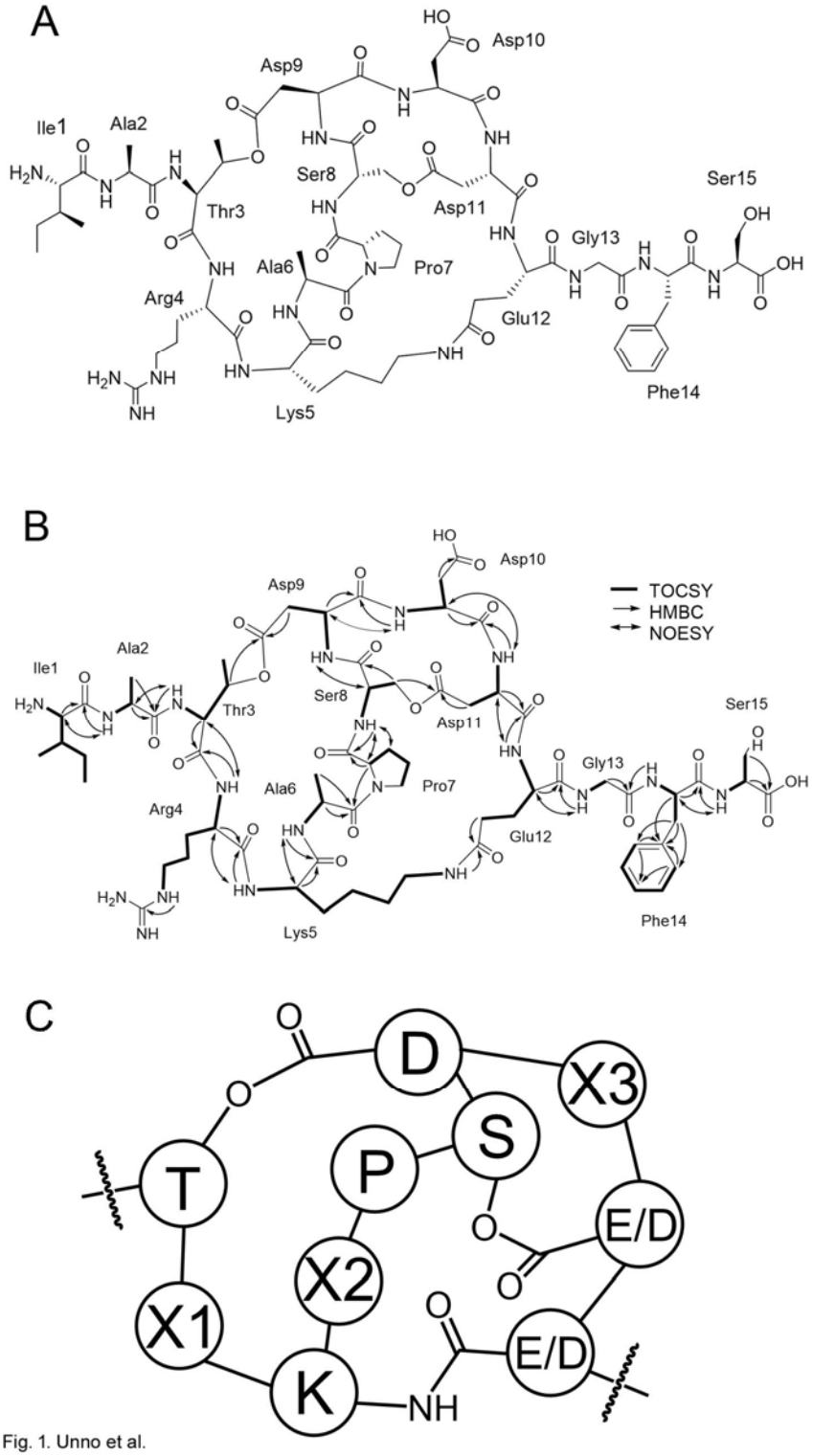

Fig. 1. Unno et al.

563 Figure 2

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GrmA   MD--------KNTPFFSNFIEADTITKEEANQIS---------------GGWNFPNEIATRKAPSDDDEGFSSSAIVFPTDELPALPELS
GrcA   MS--------KNTPFFSNFMEAQTLTKEETKQATGGLDFVKPGDLP-GGFDPSKEPIYVTMKHPSDDDEGFDAPAFLR
VpeA1  MN--------KNTPFFSNFIESQIEELSAQETKQCA-------------GGIVGDFDVVTMKAPSDDDEGLEFVLKPGDLVTMKHPSDSDEGNDFQSF
VpeA2  MS--------KNTPFFNNFIEADTLTEEEANQISGGTINFGGISTRKAPSDDDEGFAIATRKAPSDDDEGFEIATRKAPSDDDEGTAIVFPTW
VcaA   MN--------KNTPFFNNFIEAETLTEEEANQIA---------GGCANFSTSSFSASDLTRKYPSDDDEGNSSISGVNFPGSASGVNFPGSSSKVNFPS
MdnA   MAYPNDQQG-KALPFFARFLSVSKEE-------------------SSIKSPSPEPTFGTTLKYPSDWEEY
MdnA2  MSKNVKVSAPKAVPFFARFLAEQA---------------------VEANNSNSAPYGNTMKYPSDWEEY
MdnA3  MSENNN----KALPFFARFLEEQQSEPEE---------------------DAPKPFPFTYKFPSDWEDR
```

Fig. 2 Unno et al.

565 Figure 3

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568

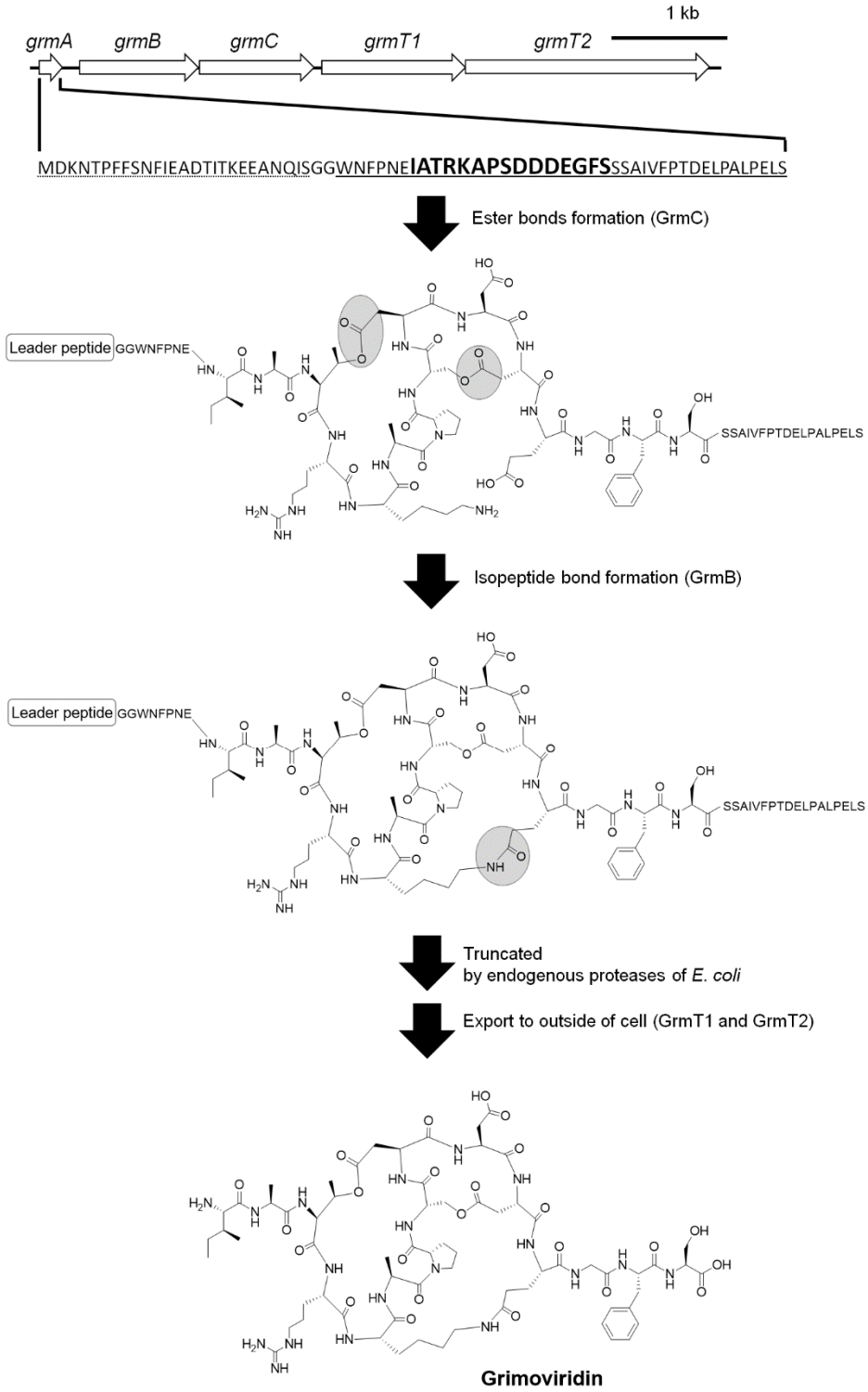

Fig. 3 Unno et al.