Heterologous expression of a cryptic gene cluster from Marinomonas fungiae affords a novel tricyclic peptide marinomonasin

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	作成者: Kaweewan, Issara, Nakagawa, Hiroyuki, Kodani, Shinya
	メールアドレス:
	所属:
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1 Title: Heterologous expression of a cryptic gene cluster from *Marinomonas fungiae*

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4 Authors: Issara Kaweewan[1], Hiroyuki Nakagawa[2,3], and Shinya Kodani[1,4,5*]

5 Affiliations: [1]Faculty of Agriculture, Shizuoka University, Shizuoka, Japan; [2]Food

6 Research Institute, National Agriculture and Food Research Organization (NARO),

7 Ibaraki, Japan; [3]Advanced Analysis Center, National Agriculture and Food Research

8 Organization (NARO), Ibaraki, Japan; [4]Shizuoka Institute for the Study of Marine

9 Biology and Chemistry, Shizuoka University, Shizuoka, Japan; [5]College of Agriculture,

10 Academic Institute, Shizuoka University, Shizuoka, Japan

11 *To whom correspondence should be addressed: Shinya Kodani, College of

12 Agriculture, Academic Institute, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka

13 422-8529 Japan, Tel/Fax; +81(54)238-5008, E-mail; kodani.shinya@shizuoka.ac.jp,

14 ORCID:0000-0002-6792-1184

15

16   **Abstract**

17   The ω-ester-containing peptides are a group of ribosomally synthesized and

18   posttranslationally modified peptides. The biosynthetic gene clusters of ω-ester-

19   containing peptides commonly include ATP-grasp ligase coding genes and are

20   distributed over the genomes of a wide variety of bacteria. A new biosynthetic gene

21   cluster of ω-ester-containing peptides was found in the genome sequence of the marine

22   proteobacterium *Marinomonas fungiae.* Heterologous production of a new tricyclic

23   peptide named marinomonasin was accomplished using the biosynthetic gene cluster in

24   *Escherichia coli* expression host strain BL21 (DE3). By ESI-MS and NMR

25   experiments, the structure of marinomonasin was determined to be a tricyclic peptide 18

26   amino acids in length with one ester and two isopeptide bonds in the molecule. The

27   bridging patterns of the three intramolecular bonds were determined by the

28   interpretation of HMBC and NOESY data. The bridging pattern of marinomonasin was

29   unprecedented in the ω-ester-containing peptide group. The results indicated that the

30   ATP-grasp ligase for the production of marinomonasin was a novel enzyme possessing

31   bifunctional activity to form one ester and two isopeptide bonds.

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36   Key points

37   New tricyclic peptide marinomonasin was heterologously produced in *Escherichia coli.*

38   Marinomonasin contained one ester and two isopeptide bonds in the molecule.

39   The bridging pattern of intramolecular bonds was novel.

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Introduction

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43   Ribosomally synthesized and posttranslationally modified peptides (RiPPs) include

44   more than twenty subclasses (Budisa 2013). By genome mining, the biosynthetic gene

45   clusters (BGCs) of a new class of RiPPs, ω-ester-containing peptides (OEPs), were

46   found to be distributed over the genomes of bacteria (Lee et al. 2020b). This class of

47   RiPPs was renamed graspeptides because the biosynthetic system included ATP-grasp

48   ligase (Montalban-Lopez et al. 2021). The minimum set of BGCs of OEPs

49   (graspeptides) was indicated to consist of two genes (precursor and ATP-grasp ligase

50   coding genes) that are essential for the production of mature cyclic peptides. BGCs with

51   at least two essential genes were found by searching the genome data of bacteria in the

52   database. A total of ~1500 candidate OEPs (graspeptides) were indicated based on the

53   similarity of the amino acid sequences of precursor peptides, and the OEPs

54   (graspeptides) were classified into 12 groups (Lee et al. 2020b).

55   Among the 12 groups, group 1 contained cyanobacterial peptides called microviridins

56   (Dehm et al. 2019; Fujii et al. 2000; Ishitsuka et al. 1990; Murakami et al. 1997; Okino

57   et al. 1995; Reshef and Carmeli 2006; Rohrlack et al. 2003; Shin et al. 1996). The

58   structure of microviridins commonly contains two ester and one isopeptide bond

59   biosynthesized by two ATP-grasp ligases (Philmus et al. 2008; Ziemert et al. 2008).

60   Attempts have been reported to generate new cyclic peptides by genetic engineering

61   utilizing the intramolecular macrocyclization mechanism of the core peptide in

62   microviridins (Ahmed et al. 2017; Gatte-Picchi et al. 2014; Reyna-Gonzalez et al. 2016;

63   Weiz et al. 2011; Weiz et al. 2014; Zhang et al. 2018; Ziemert et al. 2010). In the

64   genomes of bacteria belonging to Proteobacteria, Bacteroidetes, and Cyanobacteria,

65   BGCs of microviridin-type peptides were found to be widely distributed (Ahmed et al.

66   2017). Utilizing the BGC of the marine proteobacterium *Grimontia marina*,

67   heterologous production of a new microviridin-type peptide named grimoviridin was

68   reported (Unno et al. 2020). Recently, chryseoviridin, a new type of multicore RiPP,

69   was produced by *in vitro* synthesis using the precursor peptide CdnA3 and the ATP-

70   grasp ligase CdnC of *Chryseobacterium gregarium* (Zhao et al. 2021). Interestingly, the

71   ATP-grasp ligase CdnC was found to install single and bicyclic ω-ester rings on

72   multiple domains of the precursor peptide CdnA3(Zhao et al. 2021). Regarding the

73   peptides in group 2, heterologous production of the peptide plesiocin was reported using

74   the BGC of the marine myxobacterium *Plesiocystis pacifica* (Lee et al. 2020a; Lee et al.

75   2017). Plesiocin contained four repeats of a distinct hairpin-like bicyclic structure

76   containing two ester bonds each and showed potent inhibitory activity against proteases.

77   A peptide named thuringinin (group 3) was produced using BGC derived from *Bacillus*

78   *thuringiensis serovar huazhongensis* in the same manner as plesiocin (Roh et al. 2019).

79   Similar to plesiocin, thuringinin had three tandemly repeating hairpin-like structures

80   with two ester bonds each. The bridging pattern of ester bonds was different from that

81   of plesiocin. In addition, new OEPs (OEP4-1, OEP5-1, OEP6-1) were produced using

82   the BGCs of groups 4, 5, and 6, respectively (Lee et al. 2020b). Recently, we reported

83   the heterologous production of a new peptide named prunipeptin belonging to group 11

84   (Unno and Kodani 2021). Prunipeptin was determined to be a bicyclic peptide

85   possessing one ester and one isopeptide bond. Interestingly, OEPs (graspeptides) had

86   diverse intramolecular bond formation patterns in the seven groups (groups 1-6 and 11).

87   These data indicated that the specificity of the ATP-grasp enzyme of each group

88   determines the bridge formation pattern of intramolecular bonds. Further investigation

89   on OEPs (graspeptides) in the rest of the groups is needed to clarify the characteristics

90   of the ATP-grasp enzyme and generate new cyclic peptides.

91   Based on this background, we accomplished the heterologous production of a new

92   tricyclic peptide named marinomonasin using BGC of the marine proteobacterium

93   *Marinomonas fungiae*. By analyses of MS and NMR data, the structure of

94   marinomonasin was determined. Here, we describe the heterologous production,

95   isolation and structure determination of marinomonasin.

Materials and methods

Bacterial strain

Marinomonas fungiae strain JCM 18476^T was obtained from Japan Collection of

Microorganisms (JCM), RIKEN BioResource Research Center, Ibaraki, Japan.

Construction of the expression vector pET41a-18476ABCD

Genomic DNA was extracted from *M. fungiae* strain JCM 18476^T using DNeasy Blood

& Tissue Kit (Qiagen, Venlo, Netherlands). The diluted DNA was used as a template

for PCR amplification. The gene fragment of marinomonasin containing four genes in

Fig. 1A (2179 bp) was amplified by PCR with a primer pair (18476-ATP-F-XbaI and

18476-ATP-R-KpnI, Table S1 and Figure S1) using EmeraldAmp PCR Master Mix

(Takara Bio Inc., Shiga, Japan), following the manufacturer's instructions. Amplified

PCR product was purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel,

Germany). 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), following the

manufacturer's instructions. To obtain the vector pET41a-18476ABCD, the digested

insert DNA fragment and the digested vector pET-41a (+) were ligated using Ligation-

Convenience Kit (Nippon Gene Co., Ltd., Tokyo, Japan), according to manufacturer's

113   instructions. The 5 μL of ligation mixture was transformed into *Escherichia coli* DH5α

114   cells, and the transformants were spread on LB agar plates containing kanamycin (30

115   μg/mL, final concentration). The vector pET41a-18476ABCD was extracted and

116   purified using FastGene Plasmid Mini Kit (Nippon Genetics Co., Ltd., Tokyo, Japan),

117   following the manufacturer's instructions. For heterologous expression of

118   marinomonasin, the vector pET41a-18476ABCD was transformed into the expression

119   host *E. coli* BL21(DE3) by chemical competence method.

Construction of the expression vector pET41a-18476ABC

121   To construct the expression vector pET41a-18476ABC containing *marA*, *marB* and the

122   uncharacterized protein coding gene (accession number: CUB04734.1), the vector

123   pET41a-18476ABCD was used as a DNA template for PCR amplification with a primer

124   pair (18476-ATP-Del-KpnI-F and 18476-ATP-Del-ABC-KpnI-R, in Table S1 and

125   Figure S2), using EmeraldAmp PCR Master Mix (Takara Bio Inc., Shiga, Japan),

126   following the manufacturer's instructions. Amplified PCR product was purified using

127   NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Germany) and was digested with

128   *Kpn*I-HF (New England Biolabs, Ipswich, MA, USA), following the manufacturer's

129   instructions. To obtain the vector pET41a-18476ABC, self-ligation was performed

130   using Ligation-Convenience Kit (Nippon Gene Co., Ltd., Tokyo, Japan), according to

131   manufacturer's instructions. The 5 μL of ligation mixture was transformed into *E. coli*

132   DH5α cells, and the transformants were spread on LB agar plates containing kanamycin

133   (30 μg/mL, final concentration). The vector pET41a-18476ABC was extracted and

134   purified using FastGene Plasmid Mini Kit (Nippon Genetics Co., Ltd., Tokyo, Japan),

135   following the manufacturer's instructions. Finally, the vector pET41a-18476ABC was

136   transformed into the expression host *E. coli* BL21(DE3) by chemical competence

137   method.

138   **Construction of the expression vector pET41a-18476AB**

139   To construct the expression vector pET41a-18476AB containing *marA* and *marB*, the

140   vector pET41a-18476ABCD was used as a DNA template for PCR amplification with

141   the primer pair (18476-ATP-Del-KpnI-F and 18476-ATP-Del-AB-KpnI-R, in Table S1

142   and Figure S3), using EmeraldAmp PCR Master Mix (Takara Bio Inc., Shiga, Japan),

143   following the manufacturer's instructions. Amplified PCR product was purified using

144   NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Germany) and was digested with

145   *Kpn*I-HF (New England Biolabs, Ipswich, MA, USA), following the manufacturer's

146   instructions. To obtain the vector pET41a-18476AB, self-ligation was performed using

147   Ligation-Convenience Kit (Nippon Gene Co., Ltd., Tokyo, Japan), according to

148   manufacturer's instructions. The 5 μL of ligation mixture was transformed into *E. coli*

149 DH5α cells, and the transformants were spread on LB agar plates containing kanamycin

150 (30 μg/mL, final concentration). The vector pET41a-18476AB was extracted and

151 purified using FastGene Plasmid Mini Kit (Nippon Genetics Co., Ltd., Tokyo, Japan),

152 following the manufacturer's instructions. Finally, the vector pET41a-18476AB was

153 transformed into the expression host *E. coli* BL21(DE3) by chemical competence

154 method.

155 **Heterologous expression of marinomonasin**

156 *E. coli* BL21(DE3) harboring the vector pET41a-18476ABCD, pET41a-18476ABC or

157 pET41a-18476AB was cultured using modified basal agar medium (MBM) containing

158 kanamycin (30 μg/mL, final concentration) and isopropyl-β-D-thiogalactopyranoside

159 (IPTG) (0.1 mM, final concentration) at 30°c for 24 h. The modified basal agar medium

160 was prepared by adding inorganic compounds (K_2SO_4, 2 g; K_2HPO_4, 3 g; NaCl, 1 g;

161 NH_4Cl, 5 g; $MgSO_4·7H_2O$, 80 mg; $CuCl_2$, 0.5 mg; $MnSO_4·H_2O$, 0.35 mg; $FeCl_3$, 0.5

162 mg; $CaCl_2·2H_2O$, 0.5 mg) and 15 g agar in 1 L of distilled water, followed by adjusting

163 pH to 7.3. After autoclaving, the medium was supplemented with separately sterilized

164 glucose and yeast extracts at final concentrations of 0.25 and 0.4%, respectively.

165 Bacterial cells on agar medium surface were harvested using laboratory spatula and

166 were extracted with double volume of MeOH. After centrifugation, the extract was

167   analyzed by high-performance liquid chromatography (HPLC) using Wakopak Handy

168   ODS column (4.6 × 250 mm, Fujifilm Wako Pure Chemical Co., Japan) with gradient

169   mode using solvent A (MeCN containing 0.05% TFA) and solvent B (H_2O containing

170   0.05% TFA). The gradient mode was performed by increasing percentage of solvent A

171   from 5% to 25% during 20 min. The UV detector was set at 220 nm and the flow rate

172   was set at 1 mL/min.

173   **Isolation of marinomonasin**

174   *E. coli* BL21(DE3) harboring the vector pET41a-18476AB was cultured using total 4 L

175   of the modified basal agar medium (MBM) containing kanamycin (30 μg/mL, final

176   concentration) and IPTG (0.1 mM, final concentration) at 30 °C for 24 h. Bacterial cells

177   on agar medium surface were harvested using laboratory spatula and were extracted

178   with double volume of MeOH. After centrifugation (4000 rpm, 10 min), the supernatant

179   was removed from insoluble material and concentrated by rotary evaporation. In order

180   to obtain pure compound, the concentrated extract was repeatedly subjected to HPLC

181   purification (Figure S4 and S6). Marinomonasin was isolated by HPLC using ODS

182   column (Wakopak Handy ODS, 4.6 × 250 mm, Fujifilm Wako Pure Chemical Co.,

183   Japan) with isocratic mode; 18% MeCN/water containing 0.05% TFA (Retention time;

184   14.80 min, Fig. S6b). The UV detector was set at 220 nm and the flow rate was set at 1

185   mL/min. The pure compound obtained from HPLC was lyophilized by freeze-dryer.

186   Marinomonasin (11.5 mg) was obtained as white powder after lyophilization.

187   **Mass spectrometry experiments**

188   The accurate mass measurement was conducted using an ESI Orbitrap mass 158

189   spectrometer (Orbitrap Velos ETD, Thermo Fisher Scientific, Waltham, MA, USA)

190   following the previous report (Unno et al. 2020).

191   **NMR experiments**

192   NMR sample was prepared by dissolving 7.9 mg of marinomonasin in 500 μL of

193   DMSO-d_6. 1D and 2D NMR spectra were obtained from Bruker Avance800

194   spectrometer with quadrature detection following the previous report (Kodani et al.

195   2018).

196   **Protease inhibition assay**

197   Protease inhibition assay was performed by modified method of the previous report

198   (Unno et al. 2020). Enzyme solution (trypsin, chymotrypsin and elastase) was prepared

199   by dissolving enzyme in 50 mM Tris-HCl buffer. Trypsin from Porcine Pancreas

200   (35544-94, Nacalai Tesque, Inc., Kyoto, Japan) and chymotrypsin from Bovine

201   Pancreas (09041-84, Nacalai Tesque, Inc.) were dissolved in 50 mM Tris-HCl buffer

202   (pH 7.6) to obtain 150 U/mL and 15 U/mL enzyme solutions, respectively. Elastase

203   from Porcine Pancreas (E1250, Sigma-Aldrich, Inc., St. Louis, MO, USA) was

204   dissolved in 50 mM Tris-HCl buffer (pH 8.6) to obtain 0.6 U/mL enzyme solution.

205   Substrate solution for trypsin was prepared by dissolving 43.3 mg of $N\alpha$-Benzoyl-DL-

206   arginine 4-nitroanilide hydrochloride (B4875, Sigma-Aldrich, Inc.) in 1 mL of dimethyl

207   sulfoxide (DMSO), followed by addition of 99 mL of 50 mM Tris-HCl buffer (pH 7.6).

208   Substrate solution for chymotrypsin was prepared by dissolving N-Succinyl-L-

209   phenylalanine-p-nitroanilide (S2628, Sigma-Aldrich, Inc.) in 50 mM Tris-HCl buffer

210   (pH 7.6) (final concentration of 1 mg/mL). Substrate solution for elastase was prepared

211   by dissolving N-Succinyl-Ala-Ala-Ala-p-nitroanilide (S4760, Sigma-Aldrich, Inc.) in

212   50 mM Tris-HCl buffer (pH 8.6) (final concentration of 1 mg/mL). For enzyme

213   inhibition assay, 100 μL of enzyme solution and 60 μL of 0.4 M Tris-HCl buffer (pH

214   7.6 for trypsin and chymotrypsin, pH 8.6 for elastase) were prepared in

215   spectrophotometer cuvettes. The 40 μL of marinomonasin (100 μg/mL) was added into

216   the mixture and the reaction mixture was pre-incubated at 37 °C for 5 min. After

217   addition of 200 μL of substrate solution, the absorbance at 405 nm was immediately

218   measured (A_{405} start). The reaction mixture was incubated at 37 °C for 30 min and then

219   the absorbance at 405 nm was measured (A_{405} end) to clarify enzyme inhibitory activity

220   of marinomanasin.

221

Results

Assignment of the biosynthetic gene cluster

In group 7 OEPs (graspeptides), 113 BGCs were found to be distributed mostly

among Proteobacteria (Lee et al. 2020b). The BGCs of group 7 commonly contain a

minimum of two genes, a precursor peptide coding gene and an ATP-grasp ligase

coding gene. We found typical BGCs in the genome of the marine proteobacterium

Marinomonas fungiae. The BGC contained two genes coding for a precursor peptide

(*marA,* accession number: CUB04731.1) and an ATP-grasp ligase (*marB,* accession

number: CUB04732.1), as shown in Fig. 1A. Two other genes, including an

uncharacterized protein-coding gene (accession number: CUB04734.1) and a quinol

monooxygenase-coding gene (accession number: CUB04736.1), existed close to the

BGC (Fig. 1A), although it was unclear whether the additional two genes were involved

in the biosynthesis of the OEP (graspeptide). To find analogous peptide-coding genes, a

similarity search was performed by BLASTP search using the amino acid sequence of

the precursor peptide gene (*marA*). Many analogous genes were found in the genomes

of Proteobacteria, such as *Vibrio,* and the alignment of the genes is shown in Fig. 1B.

Apparently, the leader peptide region at the *N*-terminus does not have similarity except

for the short sequences H-(V/I/L)-L and G-X-W shown by bold letters in Figure 1B. On

240   the other hand, the core sequence at the *C*-terminus has a conserved sequence: (T/S)-K-

241   K-X-D-X-E-T-G-E-D-X-K-G-(E/Q). We checked the production of the expected OEP

242   (graspeptide) in *M. fungiae.* Briefly, *M. fungiae* was cultured using marine agar 2216

243   medium. The cells of *M. fungiae* were extracted by MeOH, and the extract was analysed

244   by HPLC and ESI-MS (data not shown). As a result, the expected peptide was not

245   detected in the MeOH extract. Thus, we planned to perform heterologous expression of

246   the BGC of *M. fungiae* to obtain a new OEP (graspeptide).

247   Heterologous production of marinomonasin

248   First, we performed molecular cloning of the region including the four genes shown

249   in Fig. 1A, considering the possibility that the two additional genes, an uncharacterized

250   protein coding gene (CUB04734.1) and a quinol monooxygenase coding gene

251   (CUB04736.1), might also participate in the biosynthesis. To obtain crude genomic

252   DNA as a template for PCR, *Marinomonas fungiae* JCM18476 cells were extracted

253   using a DNA extraction kit. The gene fragment including the four genes was amplified

254   by PCR using template genomic DNA and primers (Table S1). The amplified DNA

255   fragment was cut by restriction enzymes followed by ligation with the cut plasmid of

256   pET41a (+) to give the expression vector pET41a-18476ABCD (Figure S1). The

257   bacterium *E. coli* BL21(DE3) possessing the vector pET41a-18476ABCD was cultured

258   on modified basal agar medium containing kanamycin and isopropyl-β-D-

259   thiogalactopyranoside (IPTG) at 30 °C for 24 h. The cells were harvested with a

260   laboratory spatula and extracted with a double volume of MeOH. The MeOH extract

261   was analysed by HPLC and ESI-MS. The resulting transformant produced several

262   peptides, including marinomonasin. To determine the essential gene set for the

263   production of marinomonasin, two expression vectors (pET41a-18476ABC containing

264   *marA*, *marB*, and the uncharacterized protein coding gene CUB04734.1 in Figure S2,

265   pET41a-18476AB containing *marA* and *marB* in Figure S3) were constructed. To

266   construct the two expression vectors (pET41a-18476ABC and pET41a-18476AB), PCR

267   was applied using pET41a-18476ABCD as template DNA to obtain two DNA

268   fragments (Figure S2 and S3). After the treatment of each DNA fragment with the

269   restriction enzyme *Kpn*I, ligation was performed to obtain pET41a-18476ABC or

270   pET41a-18476AB (Figure S2 and S3), followed by transformation into *Escherichia coli*

271   DH5α. After cloning of each plasmid in *E. coli* DH5α, each plasmid was transformed

272   into *E. coli* BL21(DE3). The production of peptides was compared by HPLC among

273   three different transformants of *E. coli* BL21(DE3) containing one of the vectors

274   (pET41a-18476ABCD, pET41a-18476ABC, or pET41a-18746AB). The HPLC

275   chromatographs of the MeOH extracts of all three transformants indicated exactly the

276   same profile (Figure S5). This result indicated that the essential gene set to produce

277   marinomonasin was *marA* (precursor peptide coding gene) and *marB* (ATP-grasp ligase

278   coding gene). The highest production yield was observed in the transformant containing

279   pET41a-18476AB (Figure S5). Thus, the production of marinomonasin was

280   accomplished using the transformant containing pET41a-18476AB.

281   **Isolation and structure determination of marinomonasin**

282   The transformant *E. coli* BL21(DE3) harbouring the vector pET41a-18476AB was

283   cultured using modified basal agar medium. The bacterial cells were directly harvested

284   by laboratory spatula from the agar medium and extracted with MeOH. The MeOH

285   extract was repeatedly subjected to HPLC purification to obtain marinomonasin (Fig.

286   S4 and S6). To determine the chemical structure, including the bridging pattern, NMR

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

288   marinomonasin dissolved in 0.5 mL of DMSO-d_6 were analysed (Figures S7-S39). All

289   18 amino acids in marinomonasin were assigned using spin system identification from

290   the 2D NMR spectral data (Figure 2 and Table S2). The peptide sequence of 18 amino

291   acids was determined by the NOESY correlations between the α-proton and amide

292   proton of adjacent amino acids (double ended arrow in Figure 2). The bridge between

293   Thr4 and Asp14 was determined by HMBC correlations. HMBC correlations to the β-

294   carbonyl carbon (δC 171.3) from β-protons (δH 2.88, δH 3.29) of Asp14 and β-proton

295   (δH 5.34) of Thr4 were observed (Figure S39), which indicated the presence of an ester

296   bond between the side chains of Thr4 and Asp14. The NOESY correlation between the

297   ε-NH of Lys5 and the γ-protons of Glu13 was observed, which indicated an isopeptide

298   bond between the side chains of Lys5 and Glu13 (Figure S31). In the same manner, the

299   NOESY correlation between the ε-NH of Lys6 and the γ-protons of Glu10 was observed

300   (Figure S31), which indicated an isopeptide bond between the side chains of Lys6 and

301   Glu10. Above all, the structure of marinomonasin was determined to be a tricyclic

302   peptide with three intramolecular bonds, including one ester bond (Thr4/Asp14) and

303   two isopeptide bonds (Lys5/Glu13 and Lys6/Glu10), as shown in Figure 2. To

304   determine the molecular formula, accurate MS analysis of marinomonasin was

305   accomplished in positive ion mode (Figure S40). Marinomonasin gave an ion peak of

306   decarboxylated molecule [M - COOH + Na + H] $^{2+}$ *m/z* 944.9419 (the calculated m/z

307   value was 944.9453: $C_{78}H_{128}O_{28}N_{23}SNa$). In the NMR spectrum, all amino acids were

308   assigned, and no decarboxylated residue was observed. Therefore, we concluded that

309   decarboxylation occurred as fragmentation of the ionization process of ESI-MS.

310   **Protease inhibition assay**

311   As several OEPs (graspeptides), including plesiocin (Lee et al. 2020a; Lee et al.

312   2017) and microviridins (Dehm et al. 2019; Fujii et al. 2000; Ishitsuka et al. 1990;

313   Murakami et al. 1997; Okino et al. 1995; Reshef and Carmeli 2006; Rohrlack et al.

314   2003; Shin et al. 1996), were reported to possess inhibitory activity against proteases,

315   we performed protease inhibitory assays using trypsin, chymotrypsin, and elastase.

316   Marinomonasin did not show inhibitory activity against all tested proteases at a

317   concentration of 100 μg/mL (Table S3).

318

Discussion

Several OEPs (graspeptides), including microviridins, plesiocin, and chryseoviridin,

were characterized as protease inhibitors. Among the inhibitors, the inhibitory

mechanism of microviridin J was clarified at the molecular level by X-ray

crystallographic analysis of cocrystals of microviridin J and trypsin (Weiz et al. 2014).

Microviridin J binds to the hydrophobic pocket located on the surface of trypsin by

forming a substrate-like trypsin binding motif. The methyl group of the Thr4 side chain

of microviridin J points towards Leu99 of the S2 pocket, while the Arg5 side chain

interacts with the carboxyl group of Asp189 at the bottom of the S1 pocket (Weiz et al.

2014). In this report, we tested marinomonasin against serine proteases, including

trypsin, chymotrypsin, and elastase. However, no protease inhibitory activity was

observed in the marinomonasin assay. The amino acid sequence and bridging pattern of

marinomonasin were different from those of the inhibitory peptides (microviridins,

plesiocin and chryseoviridin). Marinomonasin may not have such a strong binding

affinity to active pockets of proteases as other protease inhibitory OEPs (graspeptides).

The topology of bridging patterns of OEPs (graspeptides) that were determined thus

far is summarized in Figure 3B. In groups 1, 5, and 11, OEPs (graspeptides) possess

intramolecular isopeptide bond in addition to ester bonds. In group 1, the two ATP-

337   grasp ligases (MdnB and MdnC in the case of microviridin B) were indicated to form

338   two ester bonds and one isopeptide bond (Hemscheidt 2012; Ziemert et al. 2008). The

339   two ligases have different functions: the ligase MdnC catalyses the formation of two

340   ester bonds, and the other ligase MdnB catalyses the formation of isopeptide bond (Li et

341   al. 2016). Recently, chryseoviridin, a peptide with single and bicyclic ω-ester rings, was

342   produced by *in vitro* synthesis using the ATP-grasp ligase CdnC of *Chryseobacterium*

343   *gregarium* (Zhao et al. 2021). In a previous report (Lee et al. 2020b), the BGC of *C.*

344   *gregarium* was classified into group 1 based on the similarity of the amino acid

345   sequence of the precursor peptide. However, the ester-forming pattern of chryseoviridin

346   was novel (Figure 3B), and it is proposed to be classified into a subclass of group 1

347   (chryseoviridin). Interestingly, the ATP-grasp ligase of group 5 was indicated to possess

348   bifunctional activity to catalyse two ester bonds and one isopeptide bond to give the

349   mature peptide OEP5-1 (Lee et al. 2020b). Recently, an OEP (graspeptide) named

350   prunipeptin was produced by heterologous expression of the BGC of *Streptomyces*

351   *prunicolor* (Unno and Kodani 2021). The prunipeptin molecule contained one ester

352   bond and one isopeptide bond. The ATP-grasp ligase PruB was bifunctional, similar to

353   the ATP-grasp ligase in group 5. The topology of the intramolecular bridges in

354   prunipeptin was completely different from that of OEP5-1. As shown in Figure 3A, the

355　precursor peptide MarA is modified by the ATP-grasp enzyme MarB to form one ester

356　bond and two isopeptide bonds. In the biosynthesis of RiPPs, specific proteases often

357　cleave the leader peptide region from the precursor peptide. The BGC of

358　marinomonasin apparently lacks a protease-encoding gene. In the heterologous

359　expression system, an endogenous protease of *Escherichia coli* seems to cleave the *N*-

360　terminus region of the precursor to give mature marinomonasin (Figure 3A). The

361　bridging pattern of marinomonasin is a new combination of intramolecular bonds (two

362　isopeptide bonds and one ester bond), and the topology of bridges is novel among

363　groups of OEPs (graspeptides), as shown in Figure 3B. This indicates that the ATP-

364　grasp enzyme MarB is a novel bifunctional enzyme that forms two isopeptide bonds and

365　one ester bond in the molecule. In fact, the amino acid sequence of MarB had low

366　similarity (less than 35% identity) to other ATP-grasp enzymes of other groups (Table

367　S4).

368　　X-ray crystallography analysis of the ATP-grasp ligase MdnC (group 1) was

369　accomplished to clarify the reaction mechanism (Li et al. 2016). According to a

370　previous report (Li et al. 2016), the leader sequence is recognized by the specific

371　pocket, followed by recruitment of the core peptide region into the active pocket near

372　the ATP-binding site. In the core peptide, the side chain carboxyl residue of Asp/Glu is

373 phosphorylated using ATP to form the mixed carboxylate-phosphate anhydride, and the

374 hydroxyl group of Thr or Ser reacts with it to form the ester bond. In the case of other

375 OEPs (graspeptides), a similar mechanism was proposed (Lee et al. 2020b). The ATP

376 grasp ligase CdnC was indicated to have dual functions in installing single and bicyclic

377 ω-ester rings to afford chryseoviridin (Zhao et al. 2021). X-ray crystallographic analysis

378 of a quaternary complex including ATP-grasp ligase (CdnC) bound to ADP, the

379 conserved leader peptide and the peptide substrate illustrated that macrocyclization

380 occurs in the direction of the *N*- to *C*-terminus of the core peptide. The amino acid

381 residue Arg 217 in CdnC recognizes the participating Asp residue to lead the substrate

382 into the active site of the enzyme for phosphorylation (Zhao et al. 2021). In this study,

383 marinomonasin (OEP/graspeptide in group 7) possessed a novel bridging pattern of

384 ester and isopeptide bonds, which indicated that the ATP-grasp ligase MarB had a novel

385 reaction specificity of ligation. The reaction mechanism of MarB is not clear at present,

386 and further X-ray crystallography experiments are needed to clarify the mechanism.

387

388

389 **Author Contributions**

390 I.K. designed and performed experiments. H.N. performed MS and NMR analyses. S.K.

391   conceived and supervised the project. I.K. and S.K. wrote the manuscript.

399   **Compliance with ethical standards**

400   **Conflict of interest**

401   The authors declare that they have no conflict of interest.

402   **Ethical approval**

403   This article does not contain any studies with human participants or animals performed

404   by any of the authors.

405   **Data Availability Statement**

406   All data generated or analysed during this study are included in this published article

407   and its supplementary information file.

408

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517

518   Figure legends

519   Figure 1. A) biosynthetic gene cluster for marinomonasin (marA and marB) and two

520   closely located genes including uncharacterized protein coding gene (CUB04734.1) and

521   quinol monooxygenase coding gene (CUB04736.1), B) alignment of precursor peptides

522   in group 7 of OEPs (graspeptides), accession numbers are following: [1]CUB04731.1,

523   [2]WP_088701948.1, [3]WP_194089323.1, [4]WP_039979867.1, [5]WP_133320119.1,

524   [6]WP_033377333.1, [7]WP_094397536.1, [8]WP_100996147.1,[9]NOT87337.1,

525   [10]WP_128751671.1, [11]WP_128821517.1, [12]MBB5053603.1, [13]WP_103684573.1,

526   [14]WP_142901796.1, [15]WP_104805249.1, [16]WP_008817734.1, [17]WP_151170560.1,

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528   Figure 2. Key 2D NMR correlations of marinomonasin

529   Figure 3. A) biosynthetic pathway of marinomonasin, B) topology of intramolecular

530   bridging patterns in OEPs (graspeptides), ester bond: blue, isopeptide bond: red

531

532

Figure 1

A

100bp

marA   *marB*   CUB04734.1   CUB04736.1

B

```
Marinomonas fungiae¹                      MSNHVLIALAKRKPFVVTPEMPEGCSYDYVRGLWMKANDV-LVSHGSEFGVQATKKCDVETGEDQKGE
Halomonas campaniensis²                   MSDHVLITLAKRKPYEVVPNMPEGCSYDYDRGLWTKSNEV-LVSHSSEFGVQVTKKCDVETGEDQKGE
Vibrio hibernica³                         MSQHILIELAKRKPYSISPEMPENAIFDSAKGYWLSGSEP-LVSPGSKYGALVSKKCDQETGEDQKGE
Vibrio sagamiensis⁴                       MRQHILITLATKKPFSEQPEMPEGASYDFSRGYWFKSDE-ILVSYHSEFGTMVSKKCDIETGEDLKGE
Vibrio cholerae⁵                          MQQHILIKLATKKPFSEQPEMPEGTFYDSSRGYWFKSDE-ILVSYHSEFGTMVSKKCDIETGEDQKGE
Desulfurispora thermophila⁶               MSNHLLIQKAYKYPLPKQAPNMDGCYYDHKKGYWISKTNNLPVILDKNFIKPRTKKADRETGEDQKGE
Thermoanaerobacterium thermosaccharolyticum⁷ MSYHLLIKKAYKYPLPKQVPNMDGCYYDHEKGYWISKTNNLPVILDKNFIKPRTKKEDRETGEDQKGE
Lacinutrix sp. Bg11-31⁸                    -MKHILFQKALVYKRKEITPPKNYEYDYILGAWKDKLSN-LLINSTDFKAQATKKLDIETGEDNKGQ
Lysobacter sp.⁹                            --MHILEMLAVSSPARDSTPMPIGCQYDFAAGIWK-SEHG-AILALDPAFEQQTKKMDLETGEDQKGQ
Tissierellia sp. JN-28¹⁰                   MLKHLLIEKAYEYPLPHKEPTMQGCKYDNVKGYWTYENNNKPVILDKNFIKPRTKKADRETGEDQKGE
Aeromonas allosaccharophila¹¹             MSQHVLIKLATKKPYLDKPEMPEGSFYDSVKGYWVKAGES-LVSYSSEFGVMATKKCDIETGEDQKGE
Afipia massiliensis¹²                      MTDHVLIQLAVRRPHVNAPTLPEGATYDTISGCWQTRGE-------GGLTPMSTKKNDIETGEDMKGE
Marortus luteolus¹³                        MQNHLLIKFAKRKPFQQKPEMPKGSVFDHKRGIWMYENK-PLVSHTSKFGTQATKKCDVETGEDQKGE
Klebsiella pneumoniae¹⁴                    MIDHILLQRSVRRPHSDVPCLGSEVEYRAEEGLWYASSE-----VSKYFRPPMTKKFDIESGEDMKGE
Blautia marasmi¹⁵                          MKEHILLDKAYIYENIDRDLSPKDCFYDRMCGLWRVSSTGEVMMVSSSVQRAETKKSDIETGEDMKGE
Clostridium innocuum¹⁶                     MKQHILLEKAYCYPEPGEAQIPENCTFIQKNGYWRNNSTGEIMMLSNDPRRPQTKKADIETGEDQKGE
Photobacterium damselae¹⁷                 MQQHVLIKLATKKPFSEQPEMPEDAFYDNARGYWVKAGE-SLVSYNSEYGTMVTKKLDIETGEDNKGQ
Chlamydia trachomatis¹⁸                   MREHVLIEKAFMFDNVKEPTKPKGCYYDRFVGLWRVDGTGEVMMLSDSFEKPATKKCDIETGEDQKGE
Pseudomonas lini¹⁹                         --MHVLQRKAKTTECSKIDAPEDATYDFEA-GVWRNETG---LVAYDPRHAQSTKKNDIETGEDQKGQ
```

Fig. 1. Kaweewan et al.

Figure 2

Fig. 2. Kaweewan et al.

550   Figure 3

551   A

552   marA   marB

554   MSNHVLIALAKRKPFVVTPEMPEGCSYDYVRGLWMKANDVLVSHGSEFGVQATKKCDVETGEDQKGE

Ester and isopeptide bonds formation (MarB)
Cleavage by endogenous protease of *Escherichia coli*

556   VQATKKCDVETGEDQKGE

557   marinomonasin

558   B

Group 1 (microviridin-type)

559   TXKXPSDX(D/E)(D/E)

Group 1 subgroup (chryseoviridin)

TXXXDXXTXXXXDXXTXXXXDXXTXXXXDXXXTKXPSDXDE

Group 2 (plesiocin)

TTXXXXEE

Group 3 (thuringinin)

TXXTXXXEXXDXD

Group 4 (OEP4-1)

TXXTXTXEXXDXXE

Group 11 (prunipeptin)

SXXXKXXXDD

Group 5 (OEP5-1)

TKTXXXXEXDD

Group 6 (OEP6-1)

TXTXTXXXXEXXDXD

Group 7 (marinomonasin, this study)

TKKXXXEXXED

Fig. 3. Kaweewan et al.