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Title	Isolation of a new antibacterial peptide achromosin from <i>Streptomyces achromogenes</i> subsp. <i>achromogenes</i> based on genome mining
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1 Note

2 Isolation of a new antibacterial peptide achromosin from *Streptomyces achromogenes* subsp.

3 *achromogenes* based on genome mining

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15 Running title: Isolation of a new antibacterial peptide achromosin

16 Keywords: *Streptomyces achromogenes* subsp. *achromogenes*; lasso peptide; MS/MS

17 analysis; biosynthetic genes

18

19 Lasso peptides are a class of ribosomally biosynthesized and post-translationally modified  
20 peptides with a common motif of knot structure in the molecule.<sup>1</sup> The amino group of the N-  
21 terminal amino acid forms a peptide bond with  $\gamma$ -carboxyl group of Asp or Glu in the 8th or  
22 the 9th position from the N-terminus, resulting in formation of a macrolactam ring. The  
23 macrolactam ring looks like a loop of a “lasso” with a tail of the C-terminal linear peptide that  
24 normally locates through the ring. Regarding lasso peptides, a wide variety of biological  
25 activities such as anti-HIV,<sup>2</sup> antimycobacterial,<sup>3</sup> endothelin type B receptor antagonist,<sup>4</sup> and  
26 prolyl endopeptidase inhibition<sup>5</sup> were reported. In addition, lasso peptides normally show a  
27 stable property against proteolytic, thermal, and chemical degradation, which makes lasso  
28 peptides attractive in terms of practical application as pharmaceutical reagents.

29 Lasso peptides derived from actinobacteria have been classified into three main classes on  
30 the basis of their *N*-terminal residues and the number of disulfide bridges.<sup>1</sup> The class I lasso  
31 peptides include siamycins I and II,<sup>2</sup> aborycin,<sup>6</sup> and svieceucin,<sup>7</sup> which have an internal peptide  
32 linkage between  $\gamma$ -carboxyl residue of Asp9 (9th amino acid residue from the N-terminus) and  
33 the amino residue of Cys1. These peptides commonly have additional two disulfide bridges  
34 between Cys1 and Cys13, and Cys7 and Cys19. The class II lasso peptides include anantin,<sup>8</sup>  
35 lariatins,<sup>3</sup> propeptin,<sup>5</sup> RES-701-1,<sup>4</sup> SRO15-2005,<sup>9</sup> and sungsanpin.<sup>10</sup> These peptides have an  
36 internal peptide linkage between  $\gamma$ -carboxyl residue of Asp8 or Asp9 and the amino residue of  
37 Gly1 without any disulfide bonds. The class III lasso peptide includes only one peptide named

38 BI-32169.<sup>11</sup> The peptide BI-32169 has an internal peptide linkage between  $\gamma$ -carboxyl residue  
39 of Asp9 and the amino residue of Gly1 with one disulfide bond between Cys6 and Cys19.

40 The lasso peptide microcin J25 was isolated from *Escherichia coli*, which is regarded as the  
41 archetype of lasso peptides.<sup>12</sup> Its biosynthetic gene cluster consists of four genes including a  
42 precursor peptide coding gene: gene A (*mcjA*), two maturation enzymes including gene B (*mcjB*,  
43 cleavage of leader peptide) and gene C (*mcjC*, formation of macrolactam ring) and an ATP-  
44 binding cassette transporter coding gene: gene D (*mcjD*).<sup>13</sup> The protein McjC was reported to  
45 form the macrolactam ring, and the function of the protein McjB was assigned to cleave off the  
46 leader peptide from precursor peptide by in vitro experiments.<sup>14</sup> Normally lasso peptide  
47 biosynthetic genes in proteobacteria have a corresponding set of the genes, although the  
48 transporter gene is optional.<sup>1</sup> In actinobacteria, lasso peptide biosynthetic genes consist of a  
49 similar gene set, except that a maturation enzyme gene B have split-B genes (gene B1 and gene  
50 B2).<sup>1</sup> By genome mining, biosynthetic genes of a lasso peptide sviveucin was found on the  
51 genome of *Streptomyces sviveus*, and the lasso peptide was isolated and structure-determined  
52 by heterologous expression.<sup>7</sup> The lasso peptide SRO15-2005 was identified by matrix-  
53 assisted laser desorption/ionization-time-of-flight tandem mass spectrometry (MALDI-TOF-  
54 MS/MS) from the extract of *Streptomyces roseosporus*, based on genome sequence data.<sup>9</sup>  
55 Based on genome mining, a new lasso peptide chaxapeptin was also isolated as a lung cancer  
56 invasion inhibitor from *Streptomyces leeuwenhoekii*.<sup>15</sup> These results prompted us to find a

57 new lasso peptide from streptomycetes using genome sequence data. By genome search  
58 approach, we found new lasso peptide biosynthetic genes on the genome sequence of  
59 *Streptomyces achromogenes* subsp. *achromogenes*.<sup>16</sup> The new antibacterial peptide was  
60 isolated by chromatographic separation from the culture of *S. achromogenes* subsp.  
61 *achromogenes*. Here we describe isolation and structure determination of a new antibacterial  
62 peptide named achromosin.

63 In the genome sequence of *Streptomyces achromogenes* subsp. *achromogenes*,<sup>16</sup> lasso  
64 peptide modification enzyme coding genes (gene C named *acrC*: WP\_063755122.1, *acrB2*:  
65 WP\_037654156.1, *acrB1*: WP\_037654159.1, shown in Fig 1a) were found by blastp  
66 similarity search. Since the lasso precursor peptide coding gene was not annotated, we  
67 searched for the lasso precursor peptide coding gene in the close region to the modification  
68 enzyme coding genes. Upstream of the gene *acrC* (WP\_063755122.1), a new putative  
69 precursor peptide coding gene for new peptide named achromosin (126 base pairs, 42 amino  
70 acids, Fig. 1b) similar to chaxapeptin<sup>15</sup> was found from position 72827 to 72952 bp in the  
71 genome sequence (GenBank accession number: NZ\_JODT01000002.1). On the upstream of  
72 9 residues of the precursor peptide coding region (72827-72952), Shine-Dalgarno sequence  
73 (AGGAGGA) was present. As shown in Fig. 1b, the expected peptide achromosin was  
74 deduced to have the amino acid sequence of GIGSQTWDTIWLWD (monoisotopic molecular  
75 weight: 1676.7 Da ), after cleaving off the leader peptide at the same position as chaxapeptin

76 (arrow in Fig. 1b). The expected monoisotopic molecular weight of achromosin was  
77 calculated to be 1658.7 Da considering the loss of 18 Da, resulting in macrolactam formation  
78 of lasso peptide biosynthesis. The preliminary chemical investigation of *S. achromogenes*  
79 subsp. *achromogenes* NBRC12735<sup>T</sup> indicated that the expected peptide was present in the  
80 methanol extract of aerial hyphae and spore cells by HPLC and ESI-MS (Data not shown).  
81 Thus, cultivation of *S. achromogenes* subsp. *achromogenes* was performed using 5 L of ISP2  
82 agar media to obtain enough amount of the peptide for structure determination. After 7 days  
83 of cultivation, cells of spore and aerial hyphae were harvested by steel spatula. The cells  
84 were extracted with double volume of methanol (MeOH), followed by centrifugation. After  
85 condensation using rotary evaporation, the extract was subjected to open column  
86 chromatography using hydrophobic resin (CHP-20P), eluted with 10%, 60%, and 100%  
87 MeOH. The expected peptide achromosin was detected in 100% MeOH fraction by HPLC  
88 (Fig. S1) and ESI-MS analysis (Fig. S2). The ESI-MS analysis of the peptide gave an ion  
89 peak at  $m/z$  1659.7 for  $[M+H]^+$ . The 100% MeOH fraction was repeatedly subjected to  
90 HPLC purification to give pure achromosin.

91 The molecular formula of achromosin was established to be  $C_{79}H_{106}N_{18}O_{22}$  by accurate  
92 mass analysis using the ESI-FTICR mass spectrometer ( $[M+2H]^{2+}$  was observed at  $m/z$   
93 830.3941 corresponding to  $C_{79}H_{108}N_{18}O_{22}$  whose calculated value was 830.3937). The  
94 amino acid content analysis was performed on achromosin following the reported method.<sup>17</sup>

95 The amino acid content analysis on achromosin afforded the relative molar ratios of the  
96 constituent amino acids (2 moles each of Asp, Gly, Ile, and Thr, and 1 mole each of Glu, Leu,  
97 and Ser), as shown in Fig. S3. NMR analysis using dimethyl sulfoxide- $d_6$  as a solvent was  
98 not possible due to ambiguous broad peaks in the NMR spectrum. To obtain peptide  
99 sequence, MALDI-TOF MS/MS analysis on achromosin was accomplished. As a result, the  
100 product ions from achromosin at  $m/z$  1659 were of *b*-series peptides, *b*8-*b*13 (Fig. 2a), which  
101 indicated that the sequence of TIWLWD was the C-terminus tail sequence. Macrolactam  
102 ring structure was reported not to give fragment ions,<sup>9</sup> thus we proposed the structure of  
103 achromosin to be shown in Fig. 2a, based on the amino acid sequence of precursor peptide  
104 gene. To confirm the amino acid sequence in the macrolactam ring, C-terminal peptide  
105 bonds of tryptophans were cleaved by BNPS-skatole. After BNPS-skatole reaction, the  
106 cleaved achromosin (BNPS-achromosin) was purified by HPLC separation. ESI-TOF-MS  
107 analysis on BNPS-achromosin gave an ion peak at  $m/z$  1291.5 for  $[M+H]^+$  (Fig. S4). The  
108 molecular formula of BNPS-achromosin was clarified to be  $C_{58}H_{78}N_{14}O_{20}$  by the accurate  
109 mass analysis. That is,  $[M+2H]^{2+}$  was observed at  $m/z$  646.2832 corresponding to  
110  $C_{58}H_{80}N_{14}O_{20}$  whose calculated value was 646.2831. By the reaction of BNPS-skatole the  
111 Trp residue in a peptide is oxidized and transformed to 3-oxindole with a spirolactone, which  
112 increases the molecular weight due to the addition of two oxygens by 32 Da. As shown Fig.  
113 2b, the MALDI-TOF MS/MS of the cleaved achromosin gave the sequence of the peptide

114 with one N-terminus and two C-terminal ends. The product ions of *b1*, *b2*, and *b3* supported  
115 the sequence of DTIW\* and *b4* ion especially indicated that Trp at C-terminus was oxidized  
116 (indicated with asterisk, Fig. 2b). The product ions of *y2* to *y7* supported the sequence of  
117 GIGSQTW\* (Fig. 2b). Above all, the structure of achromosin was proposed to be a peptide  
118 with the sequence of GIGSQTWDTIWLWD having one macrolactam ring which was formed  
119 by peptide bond between amino residue of 1st Gly and  $\gamma$ -carboxyl residue of 8th Asp (Fig.  
120 2a). The structure of achromosin did not include cysteine residues, and it was classified into  
121 class II lasso peptide.

122 The antimicrobial activity of achromosin was tested using a paper disk agar diffusion assay  
123 against microorganisms (Bacterial strains including *Escherichia coli*, *Pseudomonas*  
124 *aeruginosa*, *Serratia marcescens*, *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus*  
125 *luteus*, *Streptomyces antibioticus*; Yeast strains including *Saccharomyces cerevisiae*,  
126 *Schizosaccharomyces pombe*, *Kloeckera apiculata*; fungi strains including *Aspergillus niger*,  
127 *Aspergillus oryzae*, *Mucor hiemalis*). At the dosage of 10  $\mu$ g per disk, achromosin showed  
128 an inhibitory zone of 11 mm diameter against *M. luteus* (Fig. S5). On the other hand,  
129 achromosin did not show any inhibitory activity against the other testing microorganisms at  
130 the same dosage.

131 Biosynthetic genes clusters of lasso peptides of actinobacteria have been identified for lasso  
132 peptides including lariatins,<sup>18</sup> SRO15-2005,<sup>9</sup> lassomycin,<sup>19</sup> svceucin,<sup>7</sup> chaxapeptin,<sup>15</sup> and



133 streptomonicin.<sup>20</sup> The biosynthetic gene cluster of chaxapeptin consisted of 4 genes  
134 including *cptA*, *cptC*, *cptB1*, and *cptB2*.<sup>15</sup> Interestingly, the gene cluster of chaxapeptin  
135 lacked of transporter gene that often exists in the lasso peptide biosynthetic gene cluster.  
136 The gene *cptA* encoded chaxapeptin precursor peptide, and the three genes including *cptC*,  
137 *cptB1*, and *cptB2* were proposed to be involved in macrolactam formation and leader peptide  
138 cleavage. The amino acid sequence of precursor peptide gene *acrA* which was found on the  
139 genome of *S. achromogenes* subsp. *achromogenes*<sup>16</sup> showed high similarity with that of *cptA*  
140 (46% identity, 68% positive matches). By reference to chaxapeptin biosynthetic genes, we  
141 assigned the biosynthetic gene cluster for achromosin, which have 4 genes, *acrA* (annoted in  
142 this study, 42 aa), *acrC* (WP\_063755122.1, 616 aa), *acrB2* (WP\_037654156.1, 150 aa), and  
143 *acrB1* (WP\_037654159.1, 95 aa) in this order with all the same direction (Fig. 1a).  
144 Interestingly, there was no transport protein coding genes near the gene cluster. The lack of  
145 transport gene was also reported in the chaxapeptin gene cluster.<sup>15</sup> Based on the similarity  
146 of each gene, we proposed the functions of the genes as shown in Fig. 1a. The gene *acrA*  
147 encoded the precursor of achromosin and the genes including *acrC*, *acrB1*, and *acrB2* were  
148 proposed to be modification enzymes to give the mature lasso peptide. The gene *acrC*  
149 encoded putative asparagine synthase possibly responsible for formation of the Gly1-Glu8  
150 amide bond, which showed high similarity to *cptC* by using a BLAST homology search (37%  
151 identity, 51% positive matches). The amino acid sequence of *acrB2* showed high similarity

152 to that of *cptB2* by using a BLAST homology search (55% identity, 69% positive matches),  
153 and the amino acid sequence of *acrB1* showed high similarity to that of *cptB1* by using a  
154 BLAST homology search (40% identity, 54% positive matches). Above all, the biosynthetic  
155 genes of achromosin showed the similarity to those of chaxapeptin.

156 So far, no similar peptide has been found by the blastp search, which indicates the novelty  
157 of achromosin. As shown in Fig. 1b, the amino acid sequence of core peptide is different  
158 even from that of chaxapeptin, the closest lasso peptide. The lasso peptide in class II were  
159 reported to have a wide variety of biological activity such as antimycobacterial,<sup>3</sup> endothelin  
160 type B receptor antagonist,<sup>4</sup> and prolyl endopeptidase inhibition<sup>5</sup>. In this paper, the  
161 antimicrobial activity was tested on achromosin, and the further other bioactivity tests may  
162 lead to another activity of achromosin. In addition, the biosynthetic genes of achromosin  
163 was identified from the genome of *S. achromogenes* subsp. *achromogenes*, indicating that the  
164 gene cluster functioned properly enough to produce achromosin in *S. achromogenes* subsp.  
165 *achromogenes*. This information will lead to genetic engineering using the gene cluster to  
166 create mutated lasso peptide based on achromosin by heterologous expression. More potent  
167 antibacterial peptides may be produced based on the discovery of achromosin by the further  
168 genetic engineering experiments.

169

170 **Conflict of interest**

171 The authors declare no conflict of interest.

172 **Acknowledgments**

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176

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246

247 Figure legends

248

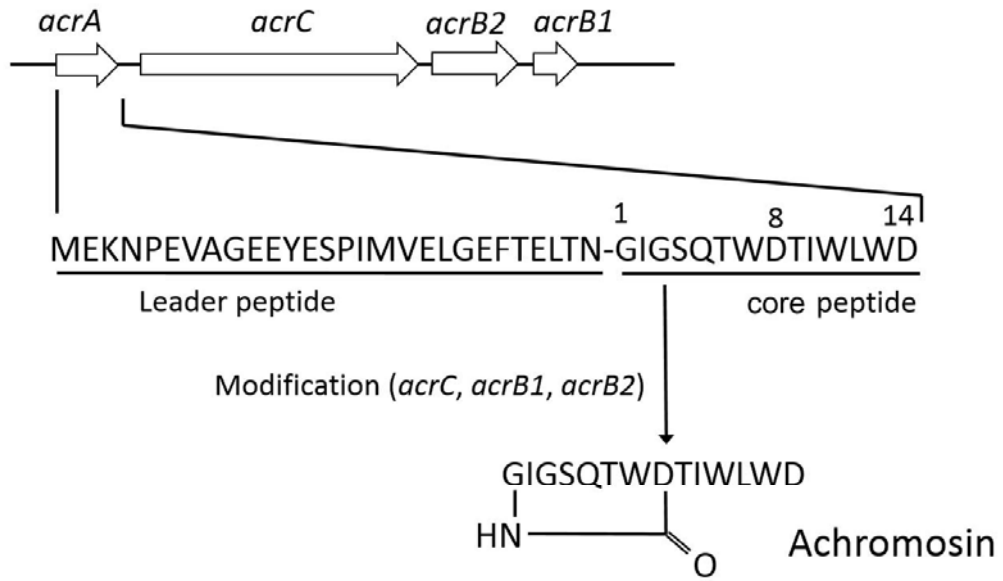
249 Fig. 1. a) Alignment of amino acid sequences of achromosin and chaxapeptin precursor peptide  
250 genes (Letters with underline: leader peptide, bold letter: conserved amino acid, arrow: cleavage  
251 position), b) Gene cluster for biosynthesis of achromosin including 4 genes (*acrA*: structural gene,  
252 and modification genes: *acrC*, *acrB1*, and *acrB2*)

253 Fig. 2. MALDI-TOF-MS/MS analyses of achromosin (a) and BNPS-skatole treated  
254 achromosin (b) (The oxidized Trp residue is marked by an asterisk. ) a) MALDI-TOF-MS/MS  
255 analysis of achromosin, b) MALDI-TOF-MS/MS analysis of BNPS-skatole cleaved  
256 achromosin (oxidization was indicated with asterisk)

257



**a**



**b**

Achromosin precursor    ME-KNPEVAGEEYESPIMVELGEFTEL**TN**GIGSQTWDTIWLWD

Chaxapeptin precursor    MEPQMT**ELOPEAYEAPSLIEVGEFSEDTL**GF<sup>↓</sup>GSKPLDSFGLNFF

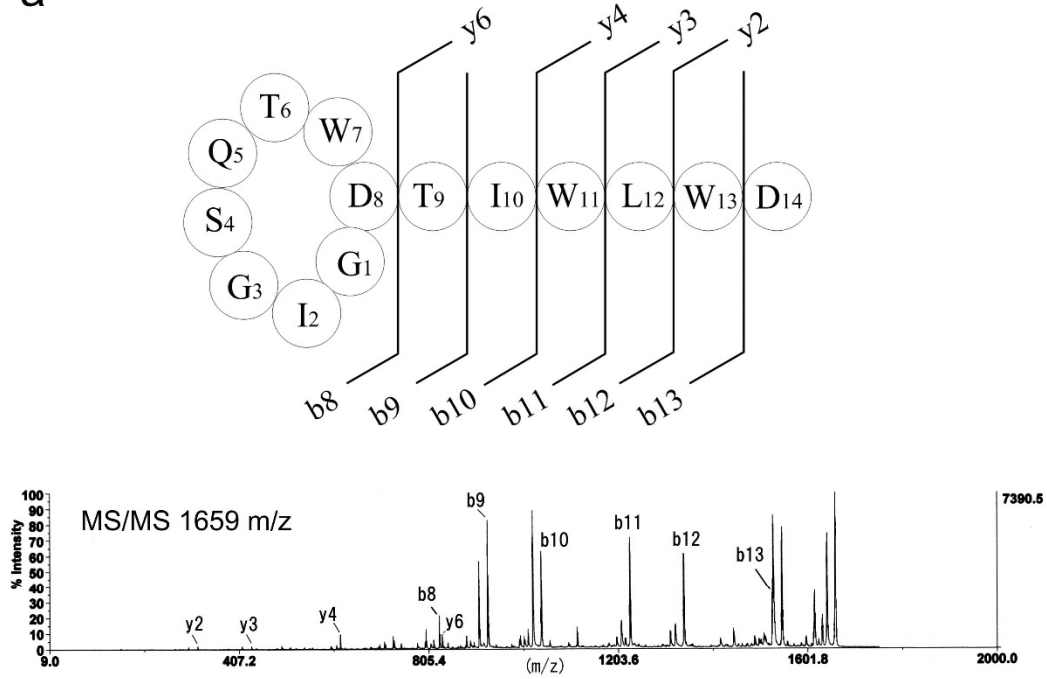
Figure 1. Issara et al.

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260

261

a



b

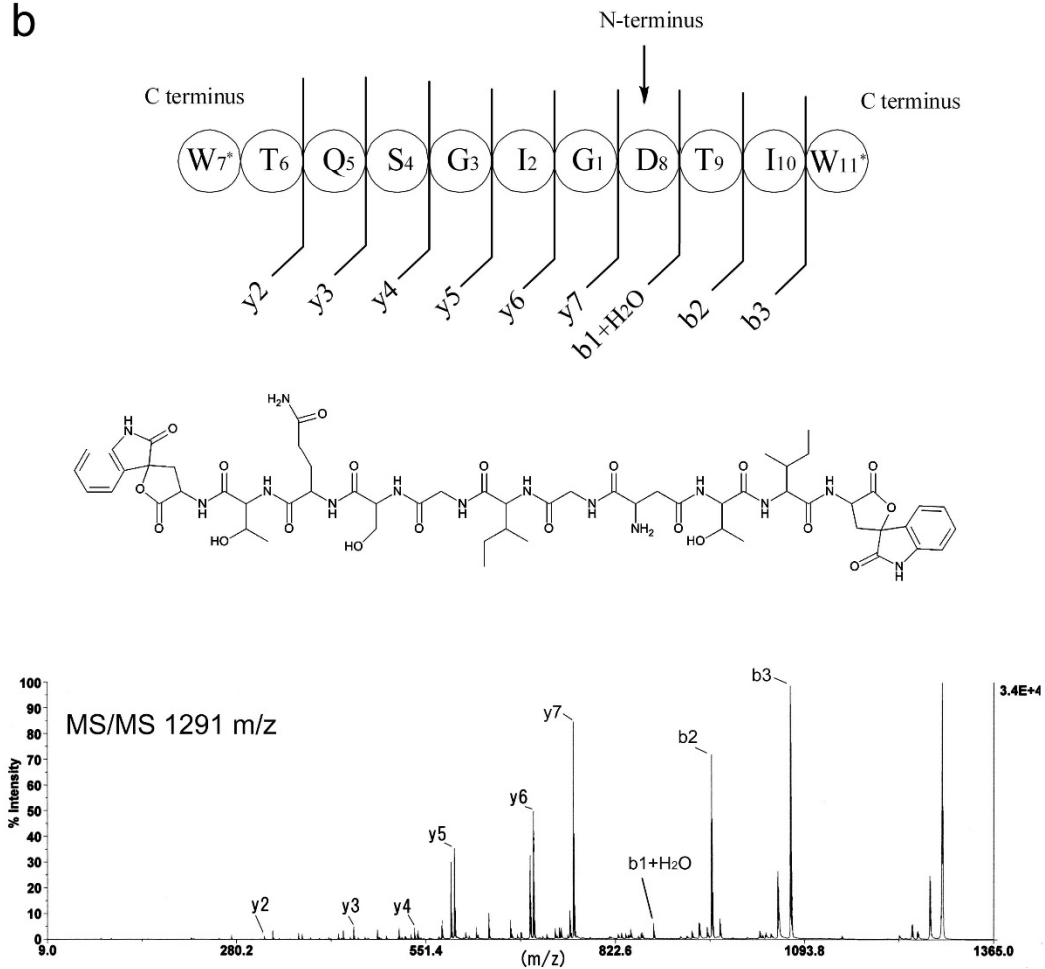


Figure 2. Issara Kaweevan et al.