Isolation and structure determination of new chymotrypsin inhibitory peptides streptopeptolins B and C

RESEARCH ARTICLE

Isolation and structure determination of new chymotrypsin inhibitory peptides streptopeptolins B and C

Ankit Tiwari[a], Issara Kaweewan[b], Yuto Miyake[a], Hikaru Hemmi[c], and Shinya Kodani[a, b ,d, ∗]

[a]*Graduate School of Integrated Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*;[b]*Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*; [c]*Food Research Institute, National Agriculture and Food Research Organization (NARO), 2–1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan*; [d]*Academic Institute, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*

*To whom correspondence should be addressed: Shinya Kodani, Academic Institute, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan, Tel/Fax; +81(54)238-5008, E-mail; kodani.shinya@shizuoka.ac.jp

Abstract

New chymotrypsin inhibitory peptides named streptopeptolins B and C were isolated from *Streptomyces olivochromogenes*. Structures of streptopeptolins B and C were determined to be cyclic depsipeptides possessing 3-amino-6-hydroxy-2-piperidone unit by interpretation of NMR spectra and ESI-MS. Streptopeptolins B and C showed inhibitory activities to chymotrypsin with IC_{50} of 8.0 and 12.0 μg/mL, respectively.

Keywords: streptopeptolin, peptide, *Streptomyces olivochromogenes*, chymotrypsin inhibitor

1. Introduction

Cyanopeptolin-type peptides are cyclic depsipeptides commonly possessing 3-amino-6-hydroxy-2-piperidone (Ahp) unit and *N*-methylated amino acid in the molecules. Cyanopeptolin-type peptides have been isolated mostly as protease inhibitors from a wide variety of cyanobacteria with different names: cyanopeptolins (Martin et al. 1993; Bister et al. 2004; von Elert et al. 2005), micropeptins (Okino et al. 1993; Ploutno et al. 2002; Yamaki et al. 2005; Kisugi and Okino 2009; Zafrir and Carmeli 2010), microcystilide (Tsukamoto et al. 1993), aeruginopeptins (Harada et al. 2001), nostopeptins (Okino et al. 1997), nostocyclin (Kaya et al. 1996), oscillapeptins (Shin et al. 1995; Sano and Kaya 1996; Itou et al. 1999), jizanpeptins (Gallegos et al. 2018) and somamides(Nogle et al. 2001). Cyanopeptolin-type peptides have been isolated from other bacteria besides cyanobacteria. A cyanopeptolin-type peptide crocapeptin was isolated from the myxobacterium *Chondromyces crocatus* Cm c5 and indicated to be biosynthesized by NRPS (Viehrig et al. 2013; Zaburannyi et al. 2016). Recently a cyanopeptolin-type peptide streptopeptolin (**1** in Fig. 1) was isolated from *Streptomyces olivochromogenes* NBRC 3561 (Kodani et al. 2018). In addition, the biosynthetic gene cluster encoding a NRPS for streptopeptolin was deduced from whole genome data of *S. olivochromogenes* NBRC 3561 (Dohra et al. 2017). In this report, further chemical investigation was performed on the extract of *S. olivochromogenes*, which resulted in isolation of new two analogous peptides streptopeptolins B and C (**2** and **3** in Fig. 1). Here, we describe isolation and structure determination of **2** and **3** from *S. olivochromogenes*.

2. Results and discussion

The new peptide streptopeptolins B (**2**) and C (**3**) were purified from the extract of culture of *Streptomyces olivochromogenes* NBRC 3561. The molecular formula of **2**

was established to be $C_{49}H_{67}N_9O_{14}$ by accurate ESI-MS analysis, as the ion corresponding to $[M - H_2O]^+$ was observed at *m/z* 988.4790 (the calculated *m/z* value, 988.4780). To obtain the chemical structure, the NMR spectra of **2** including ^1H, ^{13}C, DEPT-135, DQF-COSY, TOCSY, ROESY, HMBC, and HSQC were measured using the mix solvent system (0.5 mL, MeCN-d_3/DMSO-d_6; 4:1). By interpretation of TOCSY and DQF-COSY spectra, proton spin systems of seven units of amino acids (Gln1, Thr2, Gln3, Ahp4, Phe5, *N*-Me-Tyr6, and Ala7) were assigned as shown in Fig. S3 and Table S1. The chemical shifts of **2** were very similar to those of previously reported streptopeptolin (Kodani et al. 2018). The assignment of Ahp unit was performed mainly by TOCSY and HMBC spectra (Fig. S3). The proton spin system from amide proton to δ-proton (bold line in Fig. S3) in Ahp unit was constructed by TOCSY and DQF-COSY spectra. The cyclic structure in Ahp unit was constructed by HMBC correlations from α-proton (δH 3.72) and δ-proton (δH 5.14) to carbonyl carbon (δC 170.5). HMBC correlations from α-protons and amide protons to carbonyl carbon in adjacent amino acids (arrow in Fig. S3) indicated the two amino acid sequences (Gln1-Thr2-Gln3-Ahp4-Phe5 and *N*-Me-Tyr6-Ala7). HMBC correlations (H-δ of Ahp4/C-α of Phe5, H-α of Phe5/C-δ of Ahp4, and H-α of Phe5/C=O of Ahp4) indicated the structure of Ahp connecting with tertiary amide of Phe. The connection between Phe and *N*-Me-Tyr was established by HMBC correlations from protons of *N*-Me (δH 2.74) to carbonyl carbon (δC 170.1) of Phe. The connection between Thr2 and Ala7 with lactone structure was indicated by HMBC correlation from β-proton (δH 5.36) in Thr to carbonyl carbon (δC 174.8) in Ala. The presence of 2-methylbut-2-enoic acid (Mba) was indicated by TOCSY correlation between H-3 (δH 6.41) and H-4 (δH 1.72) and HMBC correlations from H-2-Me (δH 1.79) to C-2 (δC 132.7), C-3 (δC 131.6), and carbonyl carbon (δC 170.1).

The connection between Gln1 and Mba was established by HMBC correlation from amide proton (δH 7.50) in Gln1 to carbonyl carbon (δC 170.1) in Mba. The configuration of the double bond in Mba was assigned to be *E* considering the similarity of chemical shifts to tiglic acid, in the same manner with streptopeptolin (Kodani et al. 2018). The proton spin system of 1-amino-2-propanol (Apn) was established by TOCSY spectrum. The characteristic chemical shifts at position 2 (δH 3.66, δC 67.0) indicated the presence of hydroxyl residue. HMBC correlation from amide proton (δH 7.16) of Apn to γ-carbonyl carbon (δC 173.5) of Gln3 indicated that Apn attached to Gln3.

The molecular formula of **3** was established to be $C_{53}H_{74}N_{10}O_{14}$ by accurate ESI-MS analysis, as the ion corresponding to $[M - H_2O]^+$ was observed at *m/z* 1057.5369 (the calculated *m/z* value, 1057.5358). To obtain the chemical structure, the NMR spectra of **3** including ^{1}H, ^{13}C, DEPT-135, DQF-COSY, TOCSY, NOESY, ROESY, HMBC, and HSQC were measured using the mix solvent system (0.5 mL, MeCN-d_3/DMSO-d_6; 4:1). The structure determination of **3** was performed by NMR analysis in the same manner with **2** (Table S1 and Fig. S3). The structure of **3** was confirmed to be a depsipeptide which had the same amino acid sequence with **2** (Fig. 1). In the structure of **3**, *N*-(5-aminopentyl)acetamide (Apa) was attached to Gln3 instead of 1-amino-2-propanol (Apn) in **2**. Briefly, TOCSY and DQF-COSY spectra indicated sequential proton spin system of five sequential methylenes flanked by two amino residues. HMBC correlation from methyl protons (δH 1.78) and amide proton (δH 7.22) to carbonyl carbon (δC 170.5) indicated the presence of acetyl residue. HMBC correlation from amide proton (δH 7.18) to carbonyl carbon (δC 172.8) indicated the attachment of Apa at Gln3.

106 The modified Marfey's analysis (Harada et al., 1996) was performed to determine
107 the absolute stereochemistries of Gln, Thr, Ala, Phe, and *N*-Me-Tyr in **2** and **3**. The
108 hydrolysate of **2** or **3** was analysed by HPLC after derivatization with *N*-α-(5-fluoro-2,
109 4-dinitrophenyl)-L-leucinamide (L-FDLA), to compare with standard amino acid
110 derivatives. For stereochemistry of 1-amino-2-propanol, (*R*)-1-amino-2-propanol and
111 (*S*)-1-amino-2-propanol were respectively derivatized with *N*-α-(5-fluoro-2, 4-
112 dinitrophenyl)-L-leucinamide (L-FDLA) for HPLC comparative analysis. As a result,
113 the stereochemistries of Gln, Thr, Ala, Phe and *N*-Me-Tyr in **2** and **3** were determined
114 to be L and the stereochemistry of Apn was determined to be R. Regarding the
115 relative stereochemistry of Ahp, stereochemistry of Ahp in **2** was determined to be 3*S*,
116 6*R* or 3*R*, 6*S* by ROESY correlations (Fig. S4). The same correlation pattern was
117 observed in ROESY spectrum of **3**, which indicated that Ahp units in **2** and **3** had the
118 same relative stereochemistry. Considering biosynthesis of streptopeptolin by NRPS
119 (Kodani et al. 2018), the stereochemistry of Ahp in **2** and **3** was proposed to be 3*S*, 6*R*
120 as same as that of streptopeptolin.

121 Since the related peptide streptopeptolin was reported to have inhibitory activity
122 against chymotrypsin (Kodani et al. 2018), the inhibitory activities of **2** and **3** was
123 tested against trypsin and chymotrypsin. As a result, compounds **2** and **3** showed
124 inhibitory activities against chymotrypsin with IC_{50} of 8.0 and 12.0 μg/mL,
125 respectively. However, both compounds did not inhibit trypsin at the concentration of
126 50 μg/mL. Previously, we reported that compound **1** showed inhibitory activity
127 against chymotrypin with IC_{50} of 5.0 μg/mL (Kodani et al. 2018). The compounds **2**
128 and **3** showed similar/slightly less inhibitory activities compared with **1**. Considering
129 discrepancy of the structures of **1**-**3** (Fig.1), modification of Gln3 did not have a
130 noticeable effect on the inhibitory activity against chymotrypsin.

In previous report (Kodani et al. 2018), streptopeptolin was proposed to be biosynthesized via NRPS (Accession number: GAX58086). In this paper, we isolated two new analogous peptides that had additional modification on Gln3. We proposed that the compounds **2** and **3** were also biosynthesized by same NRPS. Modification residues including 1-amino-2-propanol and *N*-(5-aminopentyl)acetamide may be biosynthesized from Thr and Lys through decarboxylation for both residues and acetylation for *N*-(5-aminopentyl)acetamide. We found Lys/Orn decarboxylase coding gene (Accession number: GAX58093) and N-acetyltransferase coding gene (Accession number: GAX58092) possibly responsible for biosynthesizing *N*-(5-aminopentyl)acetamide. After biosynthesizing (*R*)-1-amino-2-propanol and *N*-(5-aminopentyl)acetamide, some transferase may function to attach these residues to Gln3 of **1** to afford **2** and **3**. As far as our search on the region close to the biosynthetic gene cluster of streptopeptolin, there is no possible transferase-like protein-coding gene. The biosynthetic system to afford **2** and **3** from **1** is not clear for now.

3. Experimental

3.1. Bacterial strain

Streptomyces olivochromogenes NBRC 3561 was obtained from the NBRC culture collection (Biological Resource Center, National Institute of Technology and Evaluation, Chiba, Japan).

3.2. Isolation of streptopeptolins B and C

S. olivochromogenes NBRC 3561 was cultured on ISP2 agar medium (2 L) at 30 °C for 5 days. Spores and aerial hyphae were harvested by a steel spatula after the cultivation. Double volume of methanol was added to the harvested cell for extraction,

156 followed by filtration using filter paper (Whatman No.1, GE Healthcare Life Sciences,
157 Little Chalfont, UK). The filtrate was evaporated by a rotary evaporator and the
158 concentrated extract was subjected to open column chromatography (styrene-
159 divinylbenzene resin, CHP-20P, Mitsubishi Chemical Corp., Tokyo, Japan) and eluted
160 with 10% MeOH, 60% MeOH and 100% MeOH. The 100% MeOH fraction was
161 concentrated using a rotary evaporator, subjected to HPLC separation using an ODS
162 column (4.6 × 250 mm, Wakopak Handy ODS, WAKO) and an UV detector set at
163 220 nm, and eluted by 22% MeCN containing 0.05% TFA at flow rate 1 mL /min to
164 yield **2** (4.5 mg) and **3** (3.6 mg).

165 ***3.3. NMR experiments***

166 NMR sample was prepared by dissolving **2** or **3** in 500 μl of mix solvent (MeCN-
167 d_3/DMSO-d_6, 4:1). 1D ^{1}H, ^{13}C, DEPT-135, and all 2D NMR spectra were obtained on
168 Bruker Avance800 spectrometer with quadrature detection following the previous
169 report (Kodani et al. 2018).

170 ***3.4. MS experiments***

171 ESI-MS analyses were performed using a JEOL JMS-T100LP mass spectrometer.
172 For accurate MS analysis, reserpine was used as an internal standard.

173 ***3.5. Modified Marfey's method***

174 The modified Marfey's method was applied to **2** or **3**, following previous report
175 (Kodani et al. 2018). The reagents including *N*α-(5-fluoro-2,4-dinitrophenyl)-L-
176 leucinamide (L-FDLA, Tokyo Chemical Industry Co., LTD, Tokyo, Japan) and *N*α-
177 (5-fluoro-2,4-dinitrophenyl)-D-leucinamide (D-FDLA, Tokyo Chemical Industry Co.,
178 LTD, Tokyo, Japan) were used for derivatization. The standard amino acids including
179 L-Glu, L-Thr, L-allo-Thr, L-Ala, L-Phe, and *N*-Me-L-Tyr were purchased from Wako
180 chemical. The HPLC analysis was performed for L-FDLA or D-FDLA derivative of L-

Glu, L-Thr, L-allo-Thr, and L-Ala at a flow rate of 1 mL/min using solvent A (distilled water containing 0.05% TFA) and solvent B (MeCN containing 0.05%TFA) with an isocratic mode from 0 to 30 min at 35 % of solvent B and a linear gradient mode from 30 min to 70 min increasing percentage of solvent B from 35 % to 60%.The retention times (min) of L- or D-FDLA derivatized amino acids in this HPLC condition were following; L-Thr-L-FDLA (13.52 min), L-allo-Thr-L-FDLA (14.29 min), L-Glu-L-FDLA (20.15 min), L-allo-Thr-D-FDLA (20.42 min), L-Glu-D-FDLA (23.81 min), L-Thr-D-FDLA (27.48 min), L-Ala-L-FDLA (28.36 min), L-Ala-D-FDLA (39.48 min). The HPLC analysis was performed for L-FDLA or D-FDLA derivative of L-Phe, and *N*-Me-L-Tyr at a flow rate of 1 mL/min using solvent A (distilled water containing 0.05% TFA) and solvent B (MeCN containing 0.05%TFA) with a linear gradient mode from 0 min to 70 min increasing percentage of solvent B from 25 % to 60%. The retention times (min) of L- or D-FDLA derivatized amino acids in this HPLC condition were following; L-Phe-L-FDLA (48.74 min), L-Phe-D-FDLA (59.09 min), *N*-Me-L-Tyr-L-FDLA (68.68 min), *N*-Me-L-Tyr -D-FDLA (70.98 min).

3.6. Enzyme inhibitory assay

The inhibition assays using trypsin and chymotrypsin were carried out following previous report (Kodani et al. 2018).

Supplementary material

The underlying research materials for this article can be accessed on line.

Acknowledgments

The NMR spectra were recorded on Bruker Avance 600 and Avance III HD 800 spectrometers at the Advanced Analysis Center, NARO.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

Bister B, Keller S, Baumann HI, Nicholson G, Weist S, Jung G, Sussmuth RD, Juttner F. 2004. Cyanopeptolin 963A, a chymotrypsin inhibitor of *Microcystis* PCC 7806. J Nat Prod. 67:1755-1757.

Dohra H, Miyake Y, Kodani S. 2017. Draft genome sequence of *Streptomyces olivochromogenes* NBRC 3561, a bioactive peptide-producing actinobacterium. Genome Announc. 5:e01048-01017.

Gallegos DA, Sauri J, Cohen RD, Wan X, Videau P, Vallota-Eastman AO, Shaala LA, Youssef DTA, Williamson RT, Martin GE et al. 2018. Jizanpeptins, cyanobacterial protease inhibitors from a *Symploca* sp. cyanobacterium collected in the Red Sea. J Nat Prod. 81:1417-1425.

Harada KI, Mayumi T, Shimada T, Fujii K, Kondo F, Park HD, Watanabe MF. 2001. Co-production of microcystins and aeruginopeptins by natural cyanobacterial bloom. Environ Toxicol. 16:298-305.

Itou Y, Ishida K, Shin HJ, Murakami M. 1999. Oscillapeptins A to F, serine protease inhibitors from the three strains of *Oscillatoria agardhii*. Tetrahedron. 55:6871-6882.

231 Kaya K, Sano T, Beattie KA, Codd GA. 1996. Nostocyclin, a novel 3-amino-6-
232 hydroxy-2-piperidone-containing cyclic depsipeptide from the cyanobacterium
233 *Nostoc* sp. Tetrahedron Lett. 37:6725-6728.
234 Kisugi T, Okino T. 2009. Micropeptins from the freshwater cyanobacterium
235 *Microcystis aeruginosa* (NIES-100). J Nat Prod. 72:777-781.
236 Kodani S, Komaki H, Hemmi H, Miyake Y, Kaweewan I, Dohra H. 2018.
237 Streptopeptolin, a cyanopeptolin-type peptide from *Streptomyces*
238 *olivochromogenes*. ACS Omega. 3:8104-8110.
239 Martin C, Oberer L, Ino T, Konig WA, Busch M, Weckesser J. 1993. Cyanopeptolins,
240 new depsipeptides from the cyanobacterium *Microcystis* sp. PCC 7806. J Antibiot
241 (Tokyo). 46:1550-1556.
242 Nogle LM, Williamson RT, Gerwick WH. 2001. Somamides A and B, two new
243 depsipeptide analogues of dolastatin 13 from a Fijian cyanobacterial assemblage
244 of *Lyngbya majuscula* and *Schizothrix* species. J Nat Prod. 64:716-719.
245 Okino T, Murakami M, Haraguchi R, Munekata H, Matsuda H, Yamaguchi K. 1993.
246 Micropeptins A and B, plasmin and trypsin inhibitors from the blue-green alga
247 *Microcystis aeruginosa*. Tetrahedron Lett. 34:8131-8134.
248 Okino T, Qi S, Matsuda H, Murakami M, Yamaguchi K. 1997. Nostopeptins A and B,
249 Elastase Inhibitors from the Cyanobacterium *Nostoc minutum*. J Nat Prod.
250 60:158-161.
251 Ploutno A, Shoshan M, Carmeli S. 2002. Three novel protease inhibitors from a
252 natural bloom of the cyanobacterium Microcystis aeruginosa. J Nat Prod. 65:973-
253 978.
254 Sano T, Kaya K. 1996. Oscillapeptin G, a tyrosinase inhibitor from toxic *Oscillatoria*
255 *agardhii*. J Nat Prod. 59:90-92.

Shin HJ, Murakami M, Matsuda H, Ishida K, Yamaguchi K. 1995. Oscillapeptin, an elastase and chymotrypsin inhibitor from the cyanobacterium *Oscillatoria agardhii* (NIES-204). Tetrahedron Lett. 36:5235-5238.

Tsukamoto S, Painuly P, Young KA, Yang X, Shimizu Y, Cornell L. 1993. Microcystilide A: a novel cell-differentiation-promoting depsipeptide from *Microcystis aeruginosa* NO-15-1840. J Am Chem Soc. 115:11046-11047.

Viehrig K, Surup F, Harmrolfs K, Jansen R, Kunze B, Muller R. 2013. Concerted action of P450 plus helper protein to form the amino-hydroxy-piperidone moiety of the potent protease inhibitor crocapeptin. J Am Chem Soc. 135:16885-16894.

von Elert E, Oberer L, Merkel P, Huhn T, Blom JF. 2005. Cyanopeptolin 954, a chlorine-containing chymotrypsin inhibitor of *Microcystis aeruginosa* NIVA Cya 43. J Nat Prod. 68:1324-1327.

Yamaki H, Sitachitta N, Sano T, Kaya K. 2005. Two new chymotrypsin inhibitors isolated from the Cyanobacterium Microcystis aeruginosa NIES-88. J Nat Prod. 68:14-18.

Zaburannyi N, Bunk B, Maier J, Overmann J, Muller R. 2016. Genome analysis of the fruiting body-forming myxobacterium *Chondromyces crocatus* reveals high potential for natural product biosynthesis. Appl Environ Microbiol. 82:1945-1957.

Zafrir E, Carmeli S. 2010. Micropeptins from an Israeli fishpond water bloom of the cyanobacterium *Microcystis* sp. J Nat Prod. 73:352-358.

279 Figure legends

280 Fig. 1. Chemical structures of streptopeptolins A (**1**), B (**2**) and C (**3**)

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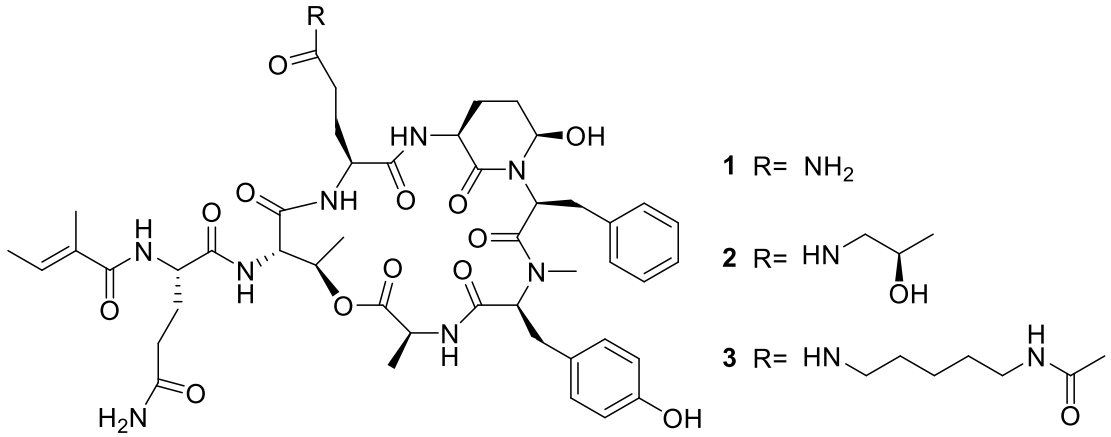
282 Fig. 1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