Bioactive Cyclic Dipeptides from a Marine Sponge-Associated Bacterium, *Psychrobacter* sp.

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**Abstract**  
A bacterial strain with good antibacterial activities against *Staphylococcus aureus* and *Escherichia coli* was isolated from a marine sponge *Stelleta* sp., and it was identified as a *Psychrobacter* sp. by comparative 16S rDNA sequence analysis. In our search for bioactive secondary metabolites from this psychrophillic and halotolerant bacterium, sixteen cyclic dipeptides (1-16) were isolated and their structures were identified on the basis of NMR analysis. In the test of the compounds for the protective effect against *Vibrio vulnificus*-induced cytotoxicity in human intestinal epithelial cells, cyclo-(L-Pro-L-Phe) (5) exhibited significant protective effect. Compounds 2, 6, and 11, which contain D-amino acid, were first isolated from bacteria.

**Keywords:** Sponge-associated, *Psychrobacter* sp., Cyclic dipeptide, D-proline, *Vibrio vulnificus*-induced cytotoxicity

**INTRODUCTION**

Recently, much attention has been given to marine sponge-associated microorganisms, which have emerged to be a rich source of novel and biologically active secondary metabolites (Piel, 2004). As a filter feeder, sponge is able to filter thousands of liters of water per day, which makes sponge harbor a large number of diverse microorganisms in its tissue. Generally, for microbe-abundant sponge, microorganisms can contribute up to 40-70% of the sponge body volume, exceeding microorganisms in seawater by 2-4 orders of magnitude (Hentschel et al., 2006; Li et al., 2007). In almost all cases, development of sponge-derived drug leads is seriously hampered by the environmental and technical problems associated with collecting or cultivating large amounts of animals. The possible existence of a bacterial symbiont, which can produce the relevant bioactive molecules, is therefore especially intriguing, because a sustainable source of sponge-derived drug candidates could be generated by establishing a symbiont culture or by transferring symbiont biosynthetic genes into culturable bacteria (Piel, 2004).

*Psychrobacter* sp., belonging to Gram-negative gamma-proteobacteria, has been isolated from several kinds of marine sponges including *Stelleta tenui*, *Haliclona rugosa*, and *Dysidea avara* (Li et al., 2007). Members of the genus *Psychrobacter*, discovered at both poles, deep sea waters, and other diverse marine environments, are psychrotolerant or psychrophilic and halotolerant (Bowman et al., 1996). In spite of their diversity and abundance, the analysis of the physiological and biochemical adaptations of this kind of bacteria is still fragmented. To the best of our knowledge, this is the first report on the investigation of biologically active metabolites from a bacterium *Psychrobacter* sp.

**MATERIALS AND METHODS**

**Bacterial isolation and culture conditions**  
The sponge *Stelleta* sp. was collected by hand using SCUBA (20 m depth) in 2005 off the coast of Geoje
Island, Korea. Following a rinse with sterile sea water, small pieces (1×1 cm²) of the surface and inner tissue of the sponge were homogenized and then inoculated on Zobell 2216 marine agar (5 g/L of peptone, 1 g/L of yeast extract, 1 mg/L of FePO₄·4H₂O, and 15 g/L of agar in 75% aged sea water; pH was adjusted to 7.4 with 1 M NaOH solution) at 25°C for 3 days. By this process, 12 pure bacterial strains (J05B1-1~J05B1-12) were isolated from the sponge Stelletta sp. Among these 12 bacterial strains, the EtOAc extract of J05B1-11 (20 mg/mL, 10 μL) showed the highest inhibitory effect against *Staphylococcus aureus* 503 (ATCC) (inhibition zone: 16 mm) and *Escherichia coli* TEM (ATCC) (inhibition zone: 16 mm) and *Escherichia coli* (1.3 mL/min). Compounds 15 and 16 were obtained by separation of fraction 5 (inhibition zone: 23 mm against *S. aureus*; 12 mm against *E. coli*) eluting with 50% MeOH (1 mL/min).

**Identification of the bacterium**

Genomic DNA extraction and PCR amplification of the 16S rRNA gene were conducted using the method previously described by Kim et al. (1998). The PCR products were purified using a HiYield™ Gel/PCR DNA extraction kit (Real Biotech Corp.) and sequence analysis was performed according to procedures demonstrated by Kim et al. (1998). The 16S rDNA sequence was aligned with those of representative members of the genus *Psychrobacter* on the basis of similarities in the primary and secondary RNA structures using PHYDIT version 3.1 (http://plaza.snu.ac.kr/~jchun/phydit/).

**Bioactivity-guided isolation of metabolites**

The combined culture broth (80 L) was extracted with EtOAc at room temperature, and the EtOAc extract (4.5 g) was partitioned between 90% MeOH and n-hexane to yield 3.3 g and 1.0 g of residues, respectively. The aqueous MeOH layer, which exhibited good antibacterial properties against *Staphylococcus aureus* 503 (ATCC) (inhibition zone: 15 mm) and *Escherichia coli* TEM (ATCC) (inhibition zone: 5 mm), was subjected to a stepped-gradient MPLC (ODS-A, 120 Å, S-30/50 mesh) eluting with 20-100% MeOH to afford 18 fractions. HPLC was performed on a Gilson 370 pump with a YMC ODS-H80 column (250×10 mm i.d., 4 μm, 80 Å) using RI-101 detector. Compounds 1, 2, 4, 7, 8, 9, and 12-14 were obtained by separation of fraction 2 (inhibition zone: 11 mm against *S. aureus*; 8 mm against *E. coli*) on a reversed-phase HPLC (YMC ODS-H80 column) eluting with 23% aqueous MeOH at the flow rate of 1.3 mL/min. Compounds 3, 5, 6, 10, and 11 were obtained by separation of fraction 4 (inhibition zone: 15 mm against *S. aureus*; 6 mm against *E. coli*) eluting with 40% MeOH (1.3 mL/min). Compounds 15 and 16 were obtained by separation of fraction 5 (inhibition zone: 23 mm against *S. aureus*; 12 mm against *E. coli*) eluting with 50% MeOH (1 mL/min).

**Structure elucidation**

The 1H and 2D NMR (HSQC, HMBC, COSY, NOESY, and TOCSY) spectra were recorded at 500 MHz using Varian INOVA 500 spectrometer, and 13C NMR spectra were recorded on Varian UNITY 400 spectrometer. Chemical shifts were reported with reference to the respective solvent peaks and residual solvent peaks (δ₁H 3.30 and δ₁C 49.0 for CD₃OD; δ₁H 7.28 and δ₁C 77.2 for CDCl₃). Optical rotations were measured using a JASCO P-1020 polarimeter.

Stereochemistry of compound 2 was defined by chemical reaction method reported by Barrow and Sun (1994). Cyclo-(L-Pro-L-Val) (1) (2 mg) was dissolved in NaOH (0.1 N, 1 mL) and the solution was left at room temperature for 4 h. The solution was extracted with EtOAc more than three times and the organic layer was washed with dilute HCl followed by H₂O. The solvent was removed under nitrogen gas and then cyclo-(D-Pro-L-Val) was obtained as a white amorphous solid (1.3 mg). The 1H NMR of the reaction product was compared with that of compound 2. Stereochemistry of other compounds was confirmed by comparing with the previously reported data.

**V. vulnificus** and human intestinal epithelial INT-407 cells

*V. vulnificus* strain MO6:24/O used in this study was isolated from patients. For the infection experiments, the bacterial strain was grown to log phase at 30°C in Luria-Bertani medium supplemented with 2.0% (w/v) NaCl (LBS). Bacterial concentrations were then confirmed via viable cell counts on LBS agar. The human intestinal epithelial cell-line (INT-407) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and maintained at 37°C in 5% CO₂ in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) and antibiotics (10 unit/mL penicillin G and 10 g/mL streptomycin).
Infection of the INT-407 cells

Human intestinal epithelial cells (INT-407) were grown in culture T-75 flasks at 37°C in a 5% CO₂ incubator. Then, the INT-407 cells were seeded onto 96-well plates and cultured for 24 h in antibiotic-free growth medium. Prior to infection, the bacteria were centrifuged for 3 min at 2,500×g, resuspended, and then adjusted to 6×10⁵ CFU/mL in antibiotic and phenol red-free MEM medium. INT-407 cells were incubated with V. vulnificus or cyclic dipeptide (3-5, 8, 9)-mixed V. vulnificus at a multiplicity of infection (MOI) of 10 (number of bacteria/number of epithelial cells = 10:1) in a 5% CO₂ incubator at 37°C.

Cytotoxicity assay (LDH release assay)

The cytotoxicity was determined by measuring the activity of lactate dehydrogenase (LDH) in the supernatant using a cytotoxicity detection kit (Roche, Mannheim, Germany) and expressed using the total LDH activity of the cells that were completely lysed by 1% Triton X-100 as 100% (Lee et al., 2008).

Morphological study

INT-407 (3×10⁵ cells/well) cells were incubated with V. vulnificus or cyclo-(L-Pro-L-Phe) (5) (50 μg/mL)-mixed V. vulnificus in a 12-well plate for 3 h at an MOI of 10. The culture plates were then centrifuged at 3,000×g and washed twice with pre-warmed PBS (pH 7.4), and fixed with 4% para-formaldehyde (Sigma) for 10 min at room temperature. Then, the cells were washed twice with PBS, after which they were stained with Giemsa solution (Molecular Probe) for 1 h at room temperature. To determine the cytotoxic effect of cyclo-(L-Pro-L-Phe) (5) on INT-407 cells, the cells treated with this compound (50 μg/mL) was compared with the blank (INT-407 cells in medium). Images of specimens were then acquired using a microscope (Olympus IX 71, Japan).

Bacterial growth test

Values of the bacterial growth were determined by a broth microdilution assay. Briefly, various dilutions of the cyclo-(L-Pro-L-Phe) (5) were prepared in an appropriate 2% NaCl LB culture medium (100 μL) in sterile 96-well round-bottom polystyrene microtiter plates. The V. vulnificus (6×10⁵ CFU/mL) was diluted in the same medium to 2×10⁵ CFU/100 μL. Each well of the microtiter plate then received 2×10⁵ CFU /100 μL of the inoculated medium, and the plates were incubated at 37°C for up to 13 h. The bacterial growth was determined by microplate reader at 540 nm.

RESULTS

Identification of the bacterium

Comparative analyses of the 16S rDNA sequence (1,390 nt) of strain J05B1-11 with those of representative members of the major lines of descent within the domain *Bacteria* revealed that the isolate was phylogenetically affiliated to the genus *Psychrobacter*. The 16S rDNA gene sequence of strain J05B1-11 showed the highest similarity to that of *P. nivimaris* 882/2-7T (98.7%) (Heuchert et al., 2006); the next highest similarity was observed in *P. proteolyticus* 116T (98.6%) (Denner et al., 2001) and *P. namhaensis* SW-242T (98.5%) (Yoon et al., 2005).

Structure elucidation

The MPLIC fractions 2, 4 and 5 were separated repeatedly by reversed-phase HPLC to give sixteen pure compounds. The structures of compounds 1-16 were elucidated by means of spectroscopic data (1D-NMR, 2D-NMR) analysis, and by comparing with previously reported data. The following cyclic dipeptides were isolated: cyclo-(L-Pro-L-Val) (1) (5.4 mg) (Schmitz et al., 1983; Jayatilake et al., 1996; Li et al., 2006), cyclo-(D-Pro-L-Val) (2) (1.2 mg) (Barrow and Sun, 1994), cyclo-(L-Pro-L-Leu) (3) (11.0 mg) (Jayatilake et al., 1996; Yan et al., 2004; Li et al., 2006), cyclo-(trans-4-hydroxy-L-Pro-L-Leu) (4) (66.0 mg) (Mitova et al., 2004), cyclo-(L-Pro-L-Phe) (5) (17.3 mg) (Jayatilake et al., 1996; Ström et al., 2002; Li et al., 2006), cyclo-(D-Pro-L-Phe) (6) (5.4 mg) (Barrow and Sun, 1994), cyclo-(trans-4-hydroxy-L-Pro-L-Phe) (7) (9.3 mg) (Ström et al., 2002), cyclo-(cis-hydroxy-D-Pro-L-Phe) (8) (8.9 mg) (Shigemori et al., 1998), cyclo-(L-Pro-L-Tyr) (9) (6.2 mg) (Rudi and Kashman, 1994; Jayatilake et al., 1996), cyclo-(L-Pro-L-Trp) (10) (4.2 mg) (Li et al., 2006), cyclo-(D-Pro-L-Trp) (11) (1.7 mg) (Sanz-Cervera et al., 2000), cyclo-(L-Ala-L-Leu) (12) (1.0 mg) (Stark and Hofmann, 2005), cyclo-(L-Ala-L-Ile) (13) (2.7 mg) (Stark and Hofmann, 2005), cyclo-(L-Phe-Gly) (14) (1.1 mg) (Kanoh et al., 1999), cyclo-(L-Phe-L-Val) (15) (1.1 mg) (Stark and Hofmann, 2005), and cyclo-(L-Pro-L-Leu) (16) (0.9 mg) (Tullberg et al., 2006). Among these, compounds 2, 6, 8, and 11 contained D-proline. According to 1D and 2D NMR data, the planar structure of compound 2 was the same with compound 1, however, ¹H chemical shift of compound 2 was different from that of compound 1, which indicated that they were stereoisomers. The stereochemistry of compound 2 was defined by conversion of the common cyclo-(L-Pro-L-Val) to cyclo-(D-Pro-L-Val) by chemical reaction. The ¹H NMR
data of compound 2 and the synthetic cyclo-(D-Pro-L-Val) were identical. Therefore, compound 2 was defined as cyclo-(D-Pro-L-Val). As far as we know, only one literature (Kwon et al., 2001) has reported the 1H and 13C NMR data of cyclo-(D-Pro-L-Val), while it was not match with the data in this study, which seem to correspond more possibly with the L,L- isomer by comparison with previously reported data of cyclo-(L-Pro-L-Val) (Schmitz et al., 1983; Jayatilake et al., 1996; Li et al., 2006).

In the 1H NMR spectra of the cyclic dipeptides except 2, 6, 8, and 11, we observed that H-3 and H-6 were coupled with each other, with coupling constant value smaller than 2 Hz. This transannular long-range proton coupling was clearly observed in CD3OD and could be attributed to homoallylic coupling \( J_{	ext{HH}} \) in the diketopiperazine ring. Lone-pair electrons of nitrogens and oxygens of the peptide bonds will participate in resonance equilibrium, and the pseudo-double bond character of the peptide bond is capable of transmitting the spin information from H-3 to H-6. Davies et al. (1976) mentioned that the observation of \( J_{	ext{HH}} \) could be used to determine the cis and/or trans-conformations of the peptide bond. From this observation, we are able to further confirm the stereochemistry of D-proline containing compounds 2, 6, 8, and 11, which showed no long-range proton coupling because of their trans-conformations. This is the first report for D-amino acid containing compounds 2, 6, and 11 from bacteria.

Cyclo-(L-Pro-L-Val) (1) – colorless, crystalline solid; [\( \alpha \)]\(_D^{28} = -110 \) (c 0.48, MeOH).

Cyclo-(D-Pro-L-Val) (2) – white, amorphous solid; [\( \alpha \)]\(_D^{28} = +25 \) (c 0.12, MeOH). 1H NMR (CDCl\(_3\), 500 MHz) \( \delta \) 6.21 (NH, bs), 4.10 (1H, dd, \( J = 9.5, 6.5 \) Hz, H-6), 3.74 (1H, bs, H-3), 3.68-3.75 (1H, m, H-9a), 3.50-3.55 (1H, m, H-9b), 2.39-2.44 (1H, m, H-7a), 2.21-2.27 (1H, m, H-10), 2.01-2.07 (1H, m, H-8a), 1.85-1.97 (2H, m, H-7b and H-8b), 1.05 (3H, d, \( J = 7.0 \) Hz, H-12), 1.00 (3H, d, \( J = 7.0 \) Hz, H-11); 13C NMR (CDCl\(_3\), 100 MHz) \( \delta \) 169.5 (C-1), 165.4 (C-4), 63.8 (C-3), 58.5 (C-6), 45.8 (C-9), 33.3 (C-10), 29.6 (C-7), 22.1 (C-8), 19.2 (C-12), 17.7 (C-11).

Effects on Vibrio vulnificus-induced cytotoxicity in human intestinal epithelial cells

To determine the protective effect of several cyclic dipeptides (3-5, 8, 9) against V. vulnificus-induced cytotoxicity in human intestinal epithelial cells (INT-407), the levels of lactate dehydrogenase (LDH) release were measured. As shown in Fig. 2A, V. vulnificus highly induced the release of LDH from the INT-407 cells (76.10 ± 2.46 %) when incubated at MOI 10 for 3 h, while in cyclic dipeptide (3-5, 8, 9)-treated cells, LDH release was reduced. Especially, cyclo-(L-Pro-L-Phe) (5) significantly abolished the release of LDH (15.34 ± 2.46 %). The level of LDH release from the cells treated with V. vulnificus and cyclo-(L-Pro-L-Phe) (5) (100 µg/mL) was almost 7-fold less than that of the control (Fig. 2B). As shown in Fig. 2C, the release of LDH in V. vulnificus-infected INT-
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407 cells approached 100% at the incubation time of 4 h, while the cells infected with cyclo-(L-Pro-L-Phe) (5) treated-\textit{V. vulnificus} kept significantly reduced LDH values for as long as 4 h at 50 or 100 µg/mL, which suggested that the protective effect of cyclo-(L-Pro-L-Phe) (5) was not decreased with time dependency. Morphological studies were also carried out using INT-407 cells. As shown in Fig. 2D, many INT-407 cells exhibited marked cellular damage, such as cytoplasmic loss and typical phenotypes of cell death after infection with \textit{V. vulnificus} (D-1). In contrast, fewer damages were observed when incubated with cyclo-(L-Pro-L-Phe) (5) (50 µg/mL) and \textit{V. vulnificus} (D-2). The cells exhibited less-damaged surface and less cytoplasmic loss. And also, cyclo-(L-Pro-L-Phe) (5) (50 µg/mL) itself had no cytotoxic effect in human intestinal epithelial cells (D-4) as compared to blank control (D-3). In the test for the effect of cyclo-(L-Pro-L-Phe) (5) on the growth of \textit{V. vulnificus}, the compound inhibited the growth of \textit{V. vulnificus} for as long as 13 h (Fig. 3A), and the inhibition effect was increased dose-dependently (20-100 µg/mL) (Fig. 3B).

**DISCUSSION**

Despite wide distribution and extreme environmental tolerance, few reports described bioactive secondary
metabolites of the psychrophilic bacterium *Psychrobacter* sp. In this study, sixteen (1-16) cyclic dipeptides were isolated from a *Psychrobacter* sp., derived from a marine sponge *Stelletta* sp. Cyclic dipeptides possess diverse biological activities such as antitumor (Nicholson et al., 2006), antifungal (Houston et al., 2004), antibacterial (Fdhila et al., 2003), and antihyperglycaemic (Song et al., 2003) activities. Due to their chiral, rigid, and functionalized structures, they bind to a large variety of receptors with high affinity, giving a broad range of biological activities (Martins and Carvalho, 2007). Therefore, cyclic dipeptides are attractive structures for the discovery of new lead compounds by combinatorial chemistry, and are considered ideal for the rational development of new therapeutic agents (Martins and Carvalho, 2007). More than 90% of Gram-negative bacteria produce cyclic dipeptides (Mitova et al., 2005), however, their origins, arguably from culture media, have been suspected for dozens of years (Lautru et al., 2002). Recently, it has been proved that sterile media do not contain cyclic dipeptides (Mitova et al., 2004; Li et al., 2005). In addition, gene clusters for the biosynthesis of cyclic dipeptides were identified in some bacterial strains (Lautru et al., 2002; Sioud et al., 2007; Schultz et al., 2008). These evidences proved that cyclic dipeptides are biosynthesized by bacteria under the control of some specific genes, not obtained artificially from the culture media or separation process.

Among the isolated cyclic dipeptides, cyclo-(D-Pro-L-Val) (2), cyclo-(D-Pro-L-Phe) (6), cyclo-(cis-hydroxy-D-Pro-L-Phe) (8), and cyclo-(D-Pro-L-Trp) (11) were composed of D-amino acids. In most cases, proteins or peptides in nature are comprised solely of L-amino acids. D-amino acids, the enantiomer of L-amino acids, are not common in eukaryotic cells. However, in bacteria, there are abundant D-amino acids, originating from cell wall peptidoglycan. D-amino acids have been found to constitute 5 and 15% of peptidoglycan amino acids in a Gram-negative and a Gram-positive bacterium, respectively (Jørgensen et al., 2006). It seems that D-amino acids widely distributed among eubacteria have been eliminated from eukaryotes during the evolution (Nagata et al., 1998). Recently, D-amino acids have also been identified in eukaryotic organisms such as fungi (Lee et al., 1977), algae (Brückner et al., 1994), and mammals (Hamase et al., 2006). And it was assumed that microbial enzymes, in particular racemases and epimerases, are responsible for the formation of D-amino acids (Brückner et al., 1993; Gruenewald et al., 2004). As D-proline was recognized as a sole D-amino acid of the cyclic dipeptides from this bacterium, we presume that an epimerase just responsible for proline epimerization might exist in this *Psychrobacter* sp.

In our search for the compounds exhibiting protective effect against *V. vulnificus*-induced cytotoxicity in human intestinal epithelial cells (INT-407), compounds 3-5, 8, and 9 were used in the test. The pathogenic marine bacterium *V. vulnificus* is a causative agent of food-borne diseases, such as life-threatening septicemia and possibly gastroenteritis, in individuals with underlying predisposing conditions (Strom, et al., 2000; Park, et al., 2006). As a result, we found that cyclo-(L-Pro-L-Phe) (5) (50 µg/mL) significantly reduce *V. vulnificus*-induced cytotoxicity as gauged by the release of LDH from INT-407 cells (Fig. 2AC). In addition, we also clearly observed the protective effect of cyclo-(L-Pro-L-Phe) (5) against *V. vulnificus*-induced cell damage by morphological study (Fig. 2D). In a subsequent test for the effect of cyclo-(L-Pro-L-Phe) (5) on *V. vulnificus* growth, we found that this compound inhibit the growth of *V. vulnificus* in a dose-dependent manner (20-100 µg/mL) (Fig. 3B). Therefore, the protec-
tion of cyclo-(L-Pro-L-Phe) (5) against V. vulnificus-induced cytotoxicity in human intestinal epithelial cells might be due to its antibacterial effect.

As quorum-sensing in bacterial cells is becoming a rising focus of the research for cyclic dipeptides produced by bacteria, we examined the inhibition effect of cyclic dipeptides (3-5, 8, 9) against SmcR, which is an important global regulator for genes associated with the survival and pathogenesis of V. vulnificus (Lee et al., 2007), but none of them exhibited inhibitory activity (data not shown). Considering that cyclic dipeptides exhibit diverse biological and pharmacological activities in higher plants and animals (Martins and Carvalho, 2007), it is quite plausible that cyclic dipeptides do not function as bacteria-to-bacteria signaling molecules, but they might have a role in modulating prokaryotic-eukaryotic interactions (Holden et al., 2000). Understanding the functional roles of cyclic dipeptides in prokaryotic-eukaryotic interactions will greatly assist the research on symbiotic interactions between marine sponges and their bacterial communities.

In summary, we isolated sixteen cyclic peptides (1-16) from a sponge-associated psychrophilic bacterium Psychrobacter sp, among which, compounds 2, 6, and 11 were first obtained from bacteria. And cyclo-(L-Pro-L-Phe) (5) exhibited significant protective effect against V. vulnificus-induced cytotoxicity in human intestinal epithelial cells (INT-407). These cyclic dipeptides, having a variety of pharmaceutical potential, may play important roles in sponge-bacteria interaction to make their co-beneficial symbiotic relationship. Additionally, a better understanding of Psychrobacter's metabolic products may aid in research on potential extraterrestrial cryo-dwelling organisms.

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