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Isolation and Structural Determination of the Antifouling Diketopiperazines from Marine-Derived *Streptomyces praecox* 291-11

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Marine derived actinomycetes constituting 185 strains were screened for their antifouling activity against the marine seaweed, *Ulva pertusa*, and fouling diatom, *Navicula annexa*. Strain 291-11 isolated from the seaweed, *Undaria pinnatifida*, rhizosphere showed the highest antifouling activity and was identified as *Streptomyces praecox* based on a 16S rDNA sequence analysis. Strain 291-11 was therefore named *S. praecox* 291-11. The antifouling compounds from *S. praecox* 291-11 were isolated, and their structures were analyzed. The chemical constituents representing the antifouling activity were identified as (6*S*,3*S*)-6-benzyl-3-methyl-2,5-diketopiperazine (bmDKP) and (6*S*,3*S*)-6-isobutyl-3-methyl-2,5-diketopiperazine (imDKP) by interpreting the nuclear magnetic resonance and high-resolution mass spectroscopy data. Approximately 4.8 mg of bmDKP and 3.1 mg of imDKP were isolated from 1.2 g of the *S. praecox* 291-11 crude extract. Eight different compositions of culture media were investigated for culture, the TBFeC medium being best for bmDKP and TCGC being the optimum for imDKP production. Two compounds respectively showed a 17.7 and 21 therapeutic ratio (LC_{50}/EC_{50}) to inhibit zoospores, and two compounds respectively showed a 263 and 120.2 therapeutic ratio to inhibit diatoms.

Key words: antifouling; (6*S*,3*S*)-6-benzyl-3-methyl-2,5-diketopiperazine; (6*S*,3*S*)-6-isobutyl-3-methyl-2,5-diketopiperazine; *Streptomyces praecox* 291-11; algal spore

Fouling organisms cause considerable damage to the immersed surfaces of man-made structures such as ships, fishnets, and aquaculture facilities. Most antifouling techniques rely on a coating containing such antifoulants as Irgarol, chlorothalonil, and diuron.[1)] However, these antifoulants are toxic,[2)] and many studies have reported detecting these antifoulants in water and sediment samples in various aquatic environments.[3–5)]

To develop environmentally sustainable antifouling agents, recent research has been focused on the behavior of microorganisms, biofilm formation and metabolite production which can inhibit marine invertebrate larval settlement and the attachment of algal spores. In the case of diatoms, Daume *et al.*[6)] have reported the inhibition of a *Haliotis rubra* settlement by monospecific diatomaceous biofilms. The unsaturated aldehydes isolated from *Thalassiosira rotula* have disrupted egg development and morphologenesis in *Sphaechinus granularis, Crassostrea gigas*, and *Calanus helgolandicus*.[7,8)] Extracts from *Achnantes parvula* and *Nizchia frustulum* have respectively inhibited settling of *Semibalanus balanoides* and the attachment of *B. neritina*.[9)] In the case of bacteria, *Pseudoalteromonas* sp. biofilms have caused significantly lower larval settlement of *Bugula neritina*[9)] which was negatively correlated with the increasing density of *Pseudoalteromonas* sp. A *Pseudoalteromonas* sp. extract has inhibited cyprid settling of the barnacle, *Balamus amphitrite*,[10)] and shown antialgal activity toward *Ulva lactuca*, *Chatonella* sp., *Gymnodium* sp., *Nitzschia paleacea*, and *Navicula* sp.[11)] The ubiquinones isolated from *Alteromonas* sp. KK10304 and 6-bromoindole-3-carbaldehyde isolated from *Acinetobacter* sp. have inhibited cyprid settling of the barnacle, *Balamus amphitrite*.[12)] *Halomonas marina, Vibrio campbelli*,[13)] and *Micrococcus* sp.[14)] have also shown anti-larval settlement activity against the barnacle, *Balamus amphitrite*. However, only a limited number of microorganisms have been screened for their antifouling activity. Moreover, most antifouling metabolites have not been reported from these microorganisms and only a few have been reported as mainly unknown high-molecular-weight carbohydrates.

Marine actinomyces are a significant new chemical resource, and many molecules with interesting bioactivities have been isolated from marine actinomycetes.[15,16)] Actinomycetes possess a tremendous ability to produce structurally diverse secondary compounds[17)] which often serve as leads to develop new pharmaceuticals and industrial chemicals. Despite the success in discovering antibiotic and anticancer agents, marine actinomyces have not been paid much attention for the discovery of antifouling agents. Marine actinomycetes are obligate marine taxa that have been found closely related to terrestrial bacteria by a 16s rDNA sequence analysis.[17)] The genus *Streptomyces* has been focused on due to its ability to produce a variety of biologically active agents,

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Table 1. Composition of Broth Media Used for Actinomycetes Culture in 1 L Seawater (g/L) at pH 8.0

Medium	Tryptone	Casitone	Glucose	Starch	Yeast extract	Peptone	$CaCO_3$	$Fe_2(SO_4)_3$ $4H_2O$	KBr	Reference
TCG	3	5	4							Newton *et al.*, 2008
TCGC	3	5	4				1			In this study
TBFe	3	5	4					0.04	0.1	In this study
TBFeC	3	5	4				1	0.04	0.1	In this study
A1				10	4	2				Moore *et al.*, 1999
A1C				10	4	2	1			Choi *et al.*, 2010
A1BFe				10	4	2		0.04	0.1	Asolkar *et al.*, 2006
A1BFeC				10	4	2	1	0.04	0.1	Bugni *et al.*, 2006

including the cytotoxic actinofuranones and azamerone.[18,19]

We focused this study on marine-derived *Streptomyces* and used its metabolite to develop an antifouling agent. The isolation and identification of the main active antifouling compounds from the most potent *S. praecox* 291-11 are described, and the antifouling compounds are evaluated. The production of these compounds from the strain is also compared by using eight different culture media.

Materials and Methods

Marine actinomycetes culture and extract. We isolated in our previous study the marine actinomycetes from seaweed and sediment collected from a 10-m depth along the coast of Korea (Kangnung, Pohang, Ulsan, Pusan, Tongyoung, and Wando).[20] A total of 185 strains, 87 isolated from seaweed and 98 isolated from marine sediment, were cultured and screened for their antifouling activity. The strains were grown in 1-L flasks containing 500 mL of a TCG fermentation medium (Table 1) for 7 d at 25 °C with 215 rpm shaking. A 20 g/L amount of Amberlite XAD-7 resin (Fluka, Sigma, St. Louis, MO, USA) was then added to the culture, and the slurry was shaken for 6 h. The resin was collected by filtering through cheesecloth, washed with 1-L of deionized water to remove salts, and eluted with acetone to yield the crude extract. Crude extracts of 85–120 mg were obtained from the 1-L cultures, and the antifouling activity was examined. The most active 291-11 strain was selected for additional experiments.

Inhibition assays against zoospore settlement. The fouling macroalga, *Ulva pertusa*, was collected in July 2010 from Anmyoundo, West Sea, Korea. The spore-releasing method followed that of Cho *et al.*,[21] the released spores being used for the settlement assays. Slide glasses (7.5 × 2.5 cm) were cleaned in 10% HCl for 12 h and rinsed in deionized water for 12 h before using for *U. pertusa* spore settlement. Three slide glasses were placed vertically in a 100-mL beaker, and zoospores (7×10^6 cells) were distributed in 75 mL of seawater in the beaker. Each bacterial extract (or compound) was dissolved in DMSO and added to the beaker. The beakers were wrapped and placed in the dark for 6 h at 20 °C to allow the spores to settle, because *U. pertusa* spores attain the stationary phase within 6 h in seawater at 20 °C.[21] The number of spores that settled on each glass (4 × 2.5 cm) was counted, and the relative rate S/C × 100 (where S is the number of settled spores on a slide glass, and C is the number of settled spores in a seawater reference culture) was measured.

Diatom inhibition assays. The biofouling diatom, *Navicula annexa*, was supplied by Korea Marine Microalgae Culture Center at Pukyong National University, South Korea. The diatoms were cultured in an f/2 medium[22] at 20 °C under 70 μmol/m^2/s light intensity in a 12:12 (light:dark) cycle with 50 rpm shaking. The same slide glass method as that just mentioned was used for the inhibition assay. An initial cell density of 2.5×10^4 cells/mL was distributed in 75 mL of the f/2 medium, and the bacterial extract was added. The number of settled diatom cells on each glass (4 × 2.5 cm) was counted after 3 d, and the relative rate (S/C × 100) was measured.

Calculation of the therapeutic ratio. The therapeutic ratio[23] LC_{50}/EC_{50} was used to evaluate the antifouling efficacy of a compound in relation to its toxicity. LC_{50} is the lethal dose of a compound for 50% of the fouling organisms, whereas the EC_{50} is the concentration for 50% inhibition of the fouling organism. The isolated compounds were dissolved in DMSO, and a serial two-fold dilution was made to a concentration with no inhibitory or lethal effect. A concentration–response curve was plotted, and a trend line was constructed for each compound. The commercial antifouling compound, Irgarol, was compared for its antifouling activity.

Identification of the marine bacteria. Chromosomal DNA of strain 291-11 was isolated with a High Pure PCR Template Preparation kit (Roche, Mannheim, Germany) to identify the *Streptomyces* strains producing the antifouling compounds. 16S rDNA was amplified by the polymerase chain reaction (PCR) with 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R universal primers (5′-GGT TAC CTT GTT ACG ACT T-3′), and a PCR premix (Bioneer, Seoul, Korea).[20] The PCR running conditions were as follows: pre-denaturation at 94 °C for 5 min, followed by 30 cycles denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and elongation at 72 °C for 30 s. After agarose gel electrophoresis, the PCR products were purified with a GeneAll Gel SV kit (Generalbiosystem, Seoul, Korea), and the purified PCR products were cloned with a TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA). The plasmid was isolated with a GeneAll Plasmid SV kit (Generalbiosystem, Seoul, Korea) and sequenced with an ABI Prism PE Big Dye Terminator Cycle DNA sequencing kit (Applied Biosystems, Foster City, CA, USA), using an ABI3730XL sequencer (Applied Biosystems, Carlsbad, CA, USA). The 16S ribosomal DNA gene sequence was compared with the NCBI gene database by using the BLASTN program.

Culture media study and scale up culture. Strain 291-11 was cultured in eight different media to maximize the compound production. The media compositions are shown in Table 1. The cultures were conducted for 7 d at 25 °C in 2.8-L Fernbach flasks containing 1-L of the fermentation medium. The bacterial metabolites were extracted from the eight different media and analyzed by liquid chromatography-mass spectrometry (LC-MS) with a 2020 instrument (Shimazu, Kyoto, Japan) equipped with electrospray ionization in the positive mode. The extract was subjected to reversed-phase chromatography in a Luna C_8 column (4.6 mm i.d. × 10 cm, 5 μm; Phenomenex, Torrance, CA, USA) at a flow rate of 0.5 mL/min. The mobile phase consisted of acetonitrile and water solvent systems. Elution was performed with a linear gradient of 10–100% acetonitrile over 30 min. The experiments were conducted at least three times for each independent assay. Differences between the experimental and control treatments were tested for significance by a one-way analysis' of variance ($\alpha = 0.05$). A scaled-up culture was conducted with strain 291-11 in 10 replicate 2.8-L Fernbach flasks containing 1-L of the TBFeC broth medium at 25 °C while shaking at 215 rpm. The cell growth and compound production were determined every 12 h for 9 d. Cell growth was measured by determining the number of colonies per unit (CFU) on a solid TCG medium. The production of the target compound was monitored by LC-MS, using the same method as that already described. Aliquots of 20 mL of the culture medium were withdrawn and extracted with ethyl acetate and then analyzed.

Isolation and identification of the diketopiperazines. The crude strain 291-11 extract was adsorbed onto diatomaceous earth (Celite) and subjected to silica-gel flash column (100 g; 70–230 mesh) chromatography, which was eluted with a step gradient mixture of hexane/ethylacetate and an ethylacetate/methanol mixture to generate 9 fractions. Active fraction obtained was dissolved in methanol for reverse-phased high performance liquid chromatography (HPLC). Separation was achieved on a Luna C_8 column (10 mm i.d. × 25 cm, 10 μm; Phenomenex). The analysis was performed on an Agilent 1200 gradient LC (Agilent Technologies, Santa Clara, CA, USA) system and monitored at 220 nm with a ultraviolet detector. The mobile phase consisted of acetonitrile and water solvent systems. Elution was performed with a isocratic flow of 25% acetonitrile over 100 min at a flow rate of 2 mL/min. Purified compounds were analyzed on a GC-MS-QP5050A (Shimadzu) equipped with a flame ionization detector and compared to spectral data from a database. HR-FABMS data were obtained from a JMS HX 110 tandem mass spectrometer (Jeol, Tokyo, Japan). The nuclear magnetic resonance (NMR) spectra were obtained on a JNM-ECP 600 NMR spectrometer (Jeol, Tokyo, Japan), using DMSO-d_6.

Absolute configuration of the amino acids. The advanced Marfey method[24,25] was applied to determine the absolute configurations of the amino acid constituents of compounds 1 and 2. Each compound (0.5 mg) in 6 N HCl (0.5 mL) was heated at 110 °C for 16 h, and the acid hydrolysate was evaporated to dryness. The residue was soluble in H_2O (200 μL). Half of this (100 μL) was added to 50 μL of a 1% (v/v) solution of 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA) solution (Tokyo Kasei Kogyo, Tokyo, Japan) in acetone and 1 M $NaHCO_3$ (100 μL), and then the mixture was heated to 80 °C for 3 min. After being cooled, 2 N HCl (50 μL) was added, and the solution diluted with acetonitrile (300 μL). Each solution (10 μL) of the FDLA derivatives was analyzed by LC-MS with positive mode electrospray ionization, using a C_8 Luna column (4.6 mm ID × 10 cm, 5 μm) at a flow rate of 0.7 mL/min with 30–50% acetonitrile in water containing 0.01 M TFA for 50 min. Each derivative was identified by its retention time and molecular weight when compared with FDLA standards. The retention times (min) of the L-FDLA-converted standards were L-Ala (25.7 min), L-Phe (31.3 min), and L-Val (26.4 min). The retention times of the D-FDLA-converted standards were L-Ala (32.1 min), L-Phe (39.2 min), and L-Val (40.3 min).

Results

Identification of the antifoulant-producing actinomycetes

Of the total of 185 strains, the crude extracts of 25 strains isolated from marine sediment and 39 strains isolated from seaweed showed an EC50 value of less than 20 against zoospore settlement. Strain 291-11 showing an EC50 value of 15 against zoospore settlement was selected for bacterial identification by its 16S rDNA partial sequence. Strain 291-11, which had been isolated from the seaweed *Undaria pinnatifida* rhizosphere, showed more than a 99% sequence similarity to *S. praecox* (Table 2) based on a NCBI BLAST analysis. This strain was therefore named *S. praecox* 291-11 and was used to isolate the two antifouling diketopiperazines, bmDKP and imDKP.

Antifouling compound production in Streptomyces

Strain 291-11 was cultured in eight different media including the TCG medium, and compound production was measured to identify the most appropriate culture media to produce antifouling compounds. Bacterial metabolites were extracted and analyzed by LC-MS, the weight percentage of the metabolite being estimated from the peak area on the chromatogram. The results show that the production of compounds was significantly higher in TCG-based cultures than that in the A1-based culture. The concentration of bmDKP was highest in the TBFeC medium (0.48 mg/L) and lowest in the A1 medium (0.10 mg/L). The concentration of imDKP was highest in the TCGC medium (0.42 mg/L) and lowest in the A1C medium (0.19 mg/L) (Fig. 1).

Cell growth and production of the compounds

The growth curve for strain 291-11 and the antifouling compounds produced in the TBFeC medium was measured every 12 h (Fig. 2). Strain 291-11 showed the first phase of growth 72–132 h post inoculation. The second phase occurred during 132–168 h, and declined thereafter. This strain produced two target compounds after around 84 h and production increased depending on cell growth. The compounds produced were maximized at the end of the second phase. bmDKP production

Table 2. Results of a BLAST Search Using the 16S rDNA Sequence

Strain	Accession no.	Description	Max identification
291-11	AB184293.1	*Streptomyces praecox*	99%

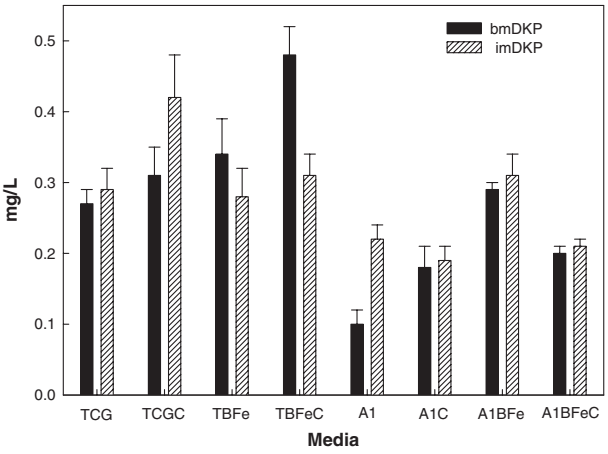

Fig. 1. Antifouling Compound Production of *S. Praecox* 291-11 in Eight Different Media.

The level of production was analyzed after 7 d, all experiments being conducted five times.

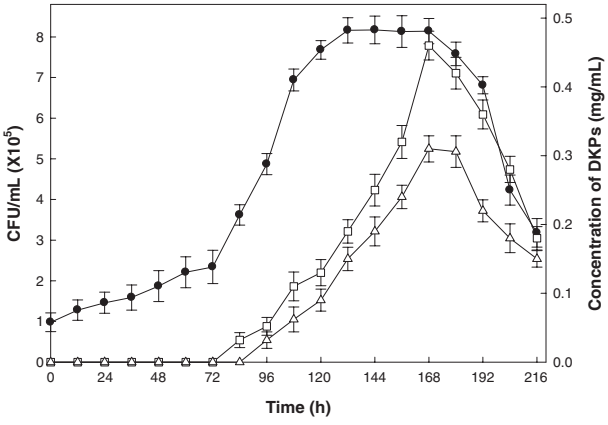

Fig. 2. Cell Growth and Diketopiperazine Production by Strain 291-11.

Cells were cultured in a TBFeC broth medium at 25 °C. Cell growth was measured on a TBFeC solid medium and is expressed as CFU/mL (●). bmDKP (□) and imDKP (△) production were evaluated from their high-performance liquid chromatography peak areas.

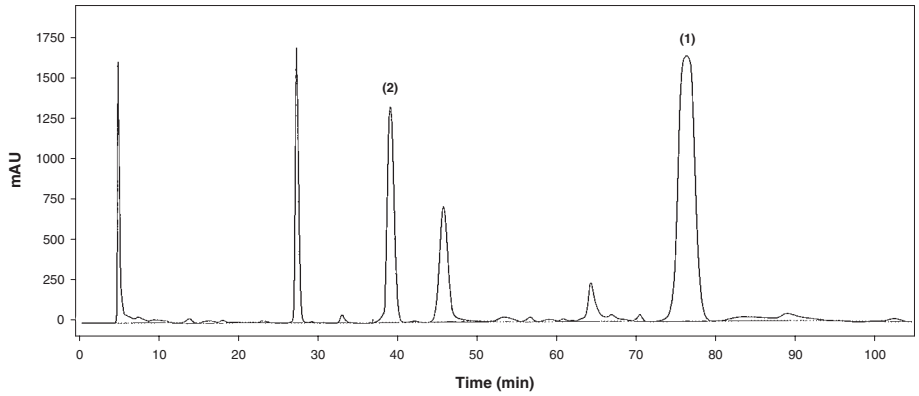

Fig. 3. High-Performance Liquid Chromatographic (HPLC) Profile for Isolating the Antifouling Compounds.
Fraction 6, collected by silica gel chromatography, was subjected to reversed-phase HPLC and eluted with an isocratic flow of 25% acetonitrile over 100 min at a flow rate of 2 mL/min. Peaks in the HPLC profile: 1, (6*S*,3*S*)-6-benzyl-3-methyl-2,5-diketopiperazine; 2, (6*S*,3*S*)-6-isobutyl-3-methly-2,5-diketopiperazine.

increased up to 168 h and was maximal at 0.48 mg/mL, before immediately decreasing. imDKP production was also maximal by 168 h with 0.31 mg/mL, but this lasted for 12 h more, before decreasing after 180 h. Strain 291-11 was therefore cultured in 10 replicates of 2.8-L flasks containing 1-L of the fermentation medium and extracted after 168 h by using the method just described.

Isolation of the antifouling compounds

Following bioassay-guided isolation against zoospore settlement, the extract (1.2 g) was fractionated from a silica gel column, and active fraction 6 (97 mg) was eluted with ethyl acetate:methanol (4:1, v/v) to show antifouling activity with an EC_{50} value of 13. This fraction was further subjected to reverse-phased HPLC. Six peaks from HPLC were respectively generated at 8, 28, 39, 47, 65 and 78 min. The active compounds were eluted at 39 min (compound 2) and 78 min (compound 1) (Fig. 3). Compound 1, bmDKP, weighed 4.8 mg in a yield of 0.4% from the 10-L culture and showed an EC_{50} value of 2.2. Compound 2, imDKP, weighed 3.1 mg in a yield of 0.25% from the 10-L culture and showed an EC_{50} value of 3.1. The peaks eluted at 8 and 28 min showed very weak activity with $EC_{50} > 300$, while the peaks eluted at 47 and 65 min showed respective EC_{50} values of 125 and 167.

Identification of the compounds and their antifouling activity

The antifouling compounds isolated from *S. praecox* 291-11 were identified by interpreting the NMR and MS data. The molecular composition of antifouling compound 1 was $C_{12}H_{14}N_2O_2$ (positive mode, $[M + H]^+$ at m/z 219.14, calculated formula weight of 218.25). The ^{1}H-NMR spectrum revealed the presence of one methyl proton at 1.67 ppm (H-1″), methylene protons from 2.91 to 3.24 ppm (H-1′), two methine protons next to the nitrogen at 4.61 and 4.84 ppm (H-3, H-6), five aromatic protons from 7.17 to 7.31 ppm (H-3′–H-7′), and two amide protons at 6.95 and 7.12 ppm (H-1, H-4). The ^{13}C-NMR spectrum showed two carbonyl carbons (C-2, C-5) at 170.2 ppm, a methyl carbon (C-1″) at 18.1 ppm, a methylene carbon (C-1′) at 38.3 ppm, two methine carbons next to the nitrogen (C-3, C-6) at 52.4 and 55.8 ppm, five aromatic carbons (C-3′–C-7′) from 127.3 to 129.7 ppm, and another quaternary carbon (C-2′) at 140.1 ppm. The spectrum from H-6 to H-7′, amide proton (H-1), and carbonyl carbon (C-5) revealed the presence of a phenylalanine fragment, and the spectrum from H-3 to H-1″, amide proton (H-4), and one more carbonyl carbon (C-2) revealed the presence of an alanine fragment. These two fragment structures established this compound as diketopiperazine composed of the amino acids, phenylalanine and alanine (Fig. 4 and Table 3). Compound 1 was identified as bmDKP from these results. The configuration of the Ala unit was determined by comparing the retention times of the FDLA standards (L, 25.7 min, and D-converted, 32.1 min) with the Ala unit of the compound at 25.5 min. The respective retention times of the L- and D-converted standards as Phe units were 31.3 and 39.2 min. The Phe unit of the compound was eluted at 31.7 min. Based on Marfey's technique, the absolute configuration was determined from the elution order of the L- *versus* D-FDLA derivatives (Table 4). The absolute configuration of compound 1 was therefore L-Ala and L-Phe, and could be assigned as 3*S*, 6*S*. Compound 1 was thus confirmed as (6*S*,3*S*)-6-benzyl-3-methyl-diketopiperazine.

The molecular composition of antifouling compound 2 was $C_9H_{16}N_2O_2$ (positive mode, $[M + H]^+$ at m/z 185.11, calculated formula weight, 184.24). The ^{1}H-NMR spectrum revealed the presence of three methyl protons at 1.16–1.18 (H-3′, 4′, 1″) ppm, methylene protons from 1.84 to 1.98 ppm (H-1′), two methine protons next to the nitrogen at 4.57 and 4.82 ppm (H-6, H-3), one more methine proton at 1.79 ppm (H-2′), and two amide protons at 6.95 and 7.11 ppm (H-1, H-4). The ^{13}C-NMR spectrum showed two carbonyl carbons (C-2, C-5) at 169.8 and 168.2 ppm, three methyl carbons (C-1″, 3′, 4′) at 18.3–23.2 ppm, a methylene carbon (C-1′) at 41.2 ppm, two methine carbons next to the nitrogen (C-6, C-3) at 56.1 and 52.1 ppm, and one more methine carbon (C-2′) at 23.4 ppm. The spectrum from H-6 to H-4′, amide proton (H-1), and carbonyl carbon (C-5) revealed the presence of a valine fragment, and the spectrum from H-3 to H-1″, amide proton (H-4), and one more carbonyl carbon (C-2) revealed the presence of an alanine fragment. These two fragment structures established this compound as diketopiperazine composed of valine and alanine (Fig. 4, Table 3). These results enabled compound 2 to be identified as imDKP. The

Table 3. Nuclear Magnetic Resonance (NMR) Spectral Data for Diketopiperazines

Position	(6*S*,3*S*)-6-benzyl-3-methyl-2,5-diketopiperazine		(6*S*,3*S*)-6-isobutyl-3-methyl-2,5-diketopiperazine	
	δC	δH	δC	δH
1		6.95		6.95
2	170.2		169.8	
3	52.4	4.61	52.1	4.82
4		7.12		7.11
5	170.2		168.2	
6	55.8	4.84	56.1	4.57
1′	38.3	3.24, 2.91	41.2	1.84, 1.98
2′	140.1		23.4	1.79
3′	129.1	7.24	23.2	1.16
4′	129.6	7.31	23.2	1.16
5′	129.7	7.31		
6′	127.3	7.17		
7′	129.4	7.24		
1″	18.1	1.67	18.3	1.18

Table 4. Retention Times (min) of D, L-FDLA Derivatized Amino Acids of Compounds 1 and 2

	$[M + H]^+$ *m/z*	Std FDLA	bmDKP L-FDLA	imDKP L-FDLA
L-Ala	384.2	25.7 (L) 32.1 (D)	25.5	25.1
L-Phe	460.1	31.3 (L) 39.2 (D)	31.7	
L-Val	412.1	26.4 (L) 40.3 (D)		26.7

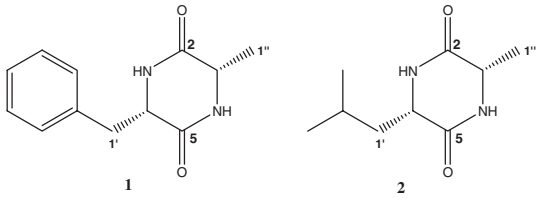

Fig. 4. Structures of the Diketopiperazines Isolated from the Marine *Streptomycetes praecox* 291-11 Culture Extract.
1, (6*S*,3*S*)-6-benzyl-3-methyl-2,5-diketopiperazine; 2, (6*S*,3*S*)-6-isobutyl-3-methyl-2,5-diketopiperazine.

configuration of the Ala unit was the same as that in compound 1. The configuration of the Val unit was determined by comparing the retention times of the FDLA standards (L, 26.4 min, and D-converted, 40.3 min) with the Val unit of the compound at 26.7 min (Table 4). The absolute configuration of compound 2 was therefore L-Ala and L-Val and was assigned as 3*S*, 6*S*. We thus confirmed compound 2 to be (6*S*,3*S*)-6-isobutyl-3-methyl-diketopiperazine.

bmDKP showed a therapeutic ratio for zoospore inhibition (LC_{50}/EC_{50}) of 17.7, while imDKP showed 21, whereas the positive control (Irgarol) was 2 (Table 5). bmDKP showed a therapeutic ratio of 263 for diatom inhibition, imDKP showed 120.2, whereas that of Irgarol was 6 (Table 6).

Discussion

Marine actinomycetes are a rich source of natural products exhibiting such diverse biological properties as cytotoxic, antifungal, and antibiotic activities.[26,27] Although many biosynthetic classes of compounds have been found from marine actinomycetes, only four DKPs have been reported.[28] DKPs are a relatively unexplored class of bioactive peptides that may have great promise in the future.[29] Many recent studies have focused on these compounds because of their significant biological activities, including antimicrobial,[30] antiviral,[31] and antifungal effects.[32] Two DPKs were isolated in the present study from the genus *Streptomyces* and were active against fouling algae.

Table 5. Inhibition of Zoospore Settlement of *Ulva pertusa* by Diketopiperazines

Compound (μg/mL)	LC_{50}	EC_{50}	LC_{50}/EC_{50}
Compound 1	139.1	2.2	17.7
Compound 2	65.3	3.1	21
Irgarol	0.01	0.005	2

LC_{50} represents the lethal dose concentration for 50% of the spores.
EC_{50} represents the minimum inhibiting concentration for 50% of the spores.

Table 6. Inhibition of Diatom Settlement of *Navicula annexa* by Diketopiperazines

Compound (μg/mL)	LC_{50}	EC_{50}	LC_{50}/EC_{50}
Compound 1	210.4	0.8	263
Compound 2	132.3	1.1	120.2
Irgarol	0.012	0.002	6

LC_{50} represents the lethal dose concentration for 50% of the microalgae.
EC_{50} represents the minimum inhibiting concentration for 50% microalgae growth.

Biofouling in the marine environment includes primary colonization of the substrate by microorganisms involving diatoms and macroalgal zoospores.[33] Microbial cells produce extracellular polymers and biofilms,[34] and marine invertebrate larvae utilize these biofilms as a settlement indicator.[35] Inhibiting this primary colonization may be an effective way to eliminate fouling organisms. The two DKPs were therefore assayed against diatoms and zoospores of algae. The antifouling activity of the two compounds in terms of their LC_{50} and EC_{50} values are shown in Tables 3 and 4. The therapeutic ratio (LC_{50}/EC_{50}) > 15 indicates a harmless antifoulant.[36] The two compounds inhibited diatoms and zoospores with a >15 therapeutic ratio, suggesting that they might be successful antifoulants, whereas commercial compound Irgarol showed a therapeutic ratio of <15 (Tables 3 and 4). Since both compounds had a DKP structure, it is possible that this moiety was an important functional group for their antifouling activity. Both the diatom and zoospore inhibition experiments showed bmDKP to be approximately 1.4-fold more potent than imDKP, with EC_{50} values of 0.8–2.2 μg/mL, suggesting that the benzyl moiety may have affected the bioactivity.

Biological metabolites isolated from marine actinomycetes, particularly the genus *Streptomyces*, have a relatively low productivity of <1 mg/L of culture;[18,19,37–40] for example, *Streptomyces* sp. CNB-982 produces 0.1 mg/L of anti-inflammatory cyclomarin,[37] and *Streptomyces* sp. YM14-060 produces approximately 0.35–0.93 mg/mL of antimicrobial piericidins.[38] Wu *et al.*[39] have reported an amorphane productivity of 0.12–0.44 mg/mL isolated from *Streptomyces* sp. M491.

However, the pigment reached 8–44 mg/mL.[41] Different medium compositions often lead to improved rates of production of these compounds.[42] Strain 291-11 in this study produced two DKPs when using the nutritional culture medium recommended for marine actinomycete culture to produce biological metabolites (Table 1). The two DKPs were produced at relatively higher levels in glucose-based cultures than those in starch-based cultures. Adding $CaCO_3$ to the glucose-based culture increased imDKP, and adding two inorganic sources ($Fe_2(SO_4)_3$ $4H_2O$ and KBr) increased the bmDKP level. Despite these observations, the cell growth of strain 291-11 was almost the same under the eight different culture conditions tested (data not shown). $CaCO_3$ added to the TCG medium (TCGC) for the glucose-based culture resulted in an imDKP yield of 1.44-fold that in the TCG medium. Adding $Fe_2(SO_4)_3$ $4H_2O$ and KBr to the culture (TBFeC) resulted in a bmDKP yield 1.77-fold that in the TCG medium, whereas imDKP decreased the yield 1.35-fold compared to that in TCGC. Adding two inorganic sources ($Fe_2(SO_4)_3$ $4H_2O$ and KBr) (A1BFe) to the starch-based culture resulted in a slightly increased yield of the two DKPs, whereas the other three starch-based cultures showed a decreased DKP yield.

The production of these antifouling compounds will be valuable to both marine ecology and to the antifouling paint industry which has been trying to identify a non-toxic paint formula. Although many natural marine compounds with antifouling activity isolated from seaweeds[43–46] and invertebrates[47–50] have been reported, culturable marine microbes can be a good source for discovering antifouling compounds due to their relatively easy supply. Studying the optimal culture conditions will be valuable in future investigations.

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