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Piericidins, Novel Quorum-Sensing Inhibitors against *Chromobacterium violaceum* CV026, from *Streptomyces* sp. TOHO-Y209 and TOHO-O348

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ABSTRACT

Piericidin A1, 3'-rhamnopiericidin A1, and a novel compound piericidin E, a new quorum-sensing (QS) inhibitor against *Chromobacterium violaceum* CV026, were isolated from the culture broth of *Streptomyces* sp. QS is well known as a microbial signaling system and controls certain types of gene expression resulting in bioluminescence, biofilm formation, swarming motility, antibiotic biosynthesis, and virulence factor production. *C. violaceum* CV026 is commonly used to determine qualitative and quantitative QS activity. The structures of piericidin derivatives were characterized, and their QS activities were determined.

Keywords: Piericidin A1; 3'-Rhamnopiericidin A1; Piericidin E; Quorum-Sensing Inhibitor; *Chromobacterium violaceum* CV026; N-Acylhomoserinlactone

1. Introduction

Quorum sensing (QS) is a process for cell-to-cell communication in bacteria using intermediary substances, which are excreted from bacterial cells into the environment. When the concentration of such an excreted metabolite reaches its threshold level, certain types of gene expression resulting in bioluminescence [1], biofilm formation [2,3], swarming motility [4], antibiotic biosynthesis [5,6] virulence factor production [7,8], etc., are triggered by the metabolite (autoinducer). Disruption of the QS system in pathogenic *Burkholderia cepacia* and *B. pseudomallei* resulted in reduced pathogenicity in murine and hamster infection [9,10], and erythromycin inhibits biofilm formation of *Pseudomonas aeruginosa* below the minimum inhibitory concentration (MIC) [11]. Therefore, compounds that inhibit QS have great potential for use in the treatment of bacterial infectious diseases.

We screened metabolites in the culture broth of about 1000 strains of *Actinomycetes* isolated from soils for their capacity to inhibit the production of violacein in *Chromobacterium violaceum* CV026 [12,13] and found that the culture broth from 103 strains produced QS inhibitors (QSIs). In this study, the structures of piericidin A1 and its derivatives isolated as QSIs were characterized, and the QS inhibitory activity and taxonomical properties of the strains that produced these QSIs were analyzed.

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2. Materials and Methods

2.1. General Experimental Procedures for Screening

Streptomyces sp., isolated from soil, was cultured in a YM medium at 27˚C for 4 - 7 days under vigorous shaking. The culture broth was applied to QSI screening. *C. violaceum* CV026, kindly provided by Dr. Tsukasa Ikeda, Utsunomiya University, was used for the screening. CV026 was inoculated into 5 mL of Luria-Bertani broth (LB) containing 50 $\mu g \cdot mL^{-1}$ of kanamycin (KM) and incubated at 27˚C for about 18 h under shaking (240 rpm). A culture of *C. violaceum* CV026 (200 µL) and 20 µL of 10 mM *N*-hexanoyl-L-homoserine lactone HHL (Santa Cruz Biotechnology Inc.) were added to 20 mL of Luria-Bertani soft agar (LSA), pre-warmed at 45˚C, and mixed

well, and then immediately poured onto an Luria-Bertani agar (LA) plate. The overlaid plate was cooled (10˚C) until the soft agar layer was solidified. Holes were bored in the plate using a sterilized cork borer (4 mm diameter), and then, a small amount of LSA was added to createan agar pocket. To the pocket, 50 μL of a culture broth of *Streptomyces* sp. was added, and the plate was incubated at 27˚C until the purple pigment developed. When a circle (white background) around agar pocket was observed, the culture was considered to contain metabolites with QS inhibitory activity.

2.2. General Experimental Procedures for QS Inhibitory Assay

Serial dilutions of piericidin and its analogs were made with methanol, and the dilutions were added to wells in microtiter plate and air-dried over a clean bench until methanol was completely evaporated. To each well, 200 μL of LSA containing *C. violaceum* CV026 and HHL (the same preparation as QSIs) were added, and then, the microtiter plate was cultured at 27˚C until the purple pigment in the control was well developed. The plate was dried at 60˚C until the water was completely evaporated, and 200 μL of DMSO was added and shaken for 2 h to extract violacein. The OD570 of the DMSO extract was measured.

2.3. Taxonomic Studies

An ISP medium recommended by Shirling and Gottlieb [14] and media recommended by Waksman [15] were used to investigate the cultural and physiological characteristics. The cultures were routinely observed after two weeks of incubation at 27˚C. The color names and hue numbers were determined according to the Color Harmony Manual [16]. The carbon sources were tested by their growth on a Pridham and Gottlieb's medium containing 1.0% carbon at 27˚C [17]. The type of DAP isomers was determined by the method reported by Becker *et al*. [18]. The morphological properties were observed using a scanning electron microscope. 16S rDNA fragments of TOHO-Y209 and TOHO-O348 were amplified by polymerase chain reaction (PCR) using the general bacterial 16S rDNA primers 10F (5'-AGTTTGATCCTGGCTC-3') and 1541R (5'-AAGGAGGTGATCCAGCC-3'). The DNA sequence of the amplified fragments were determined using Genetic Analyzer 3500 (Applied Biosystems, USA) by cycle sequencing with the chain termination technique using dye-labeled dideoxynucleotides.

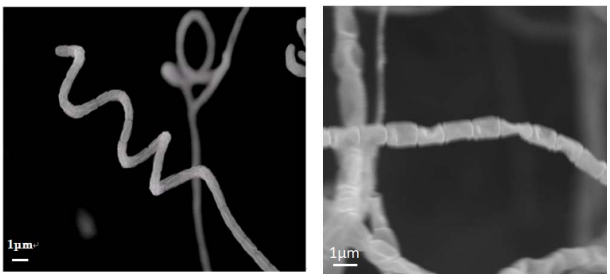
3. Results

3.1. Taxonomical Properties of the Strains That Produced QSIs

The cultural and physiological characteristicsand utilization of carbon source are shown in **Table 1**. The isomer of diaminopimelic acid (DAP) in whole-cell hydrolysates of both the strains was determined as the LL-form. The scanning electron micrograph of these strains showed that the spore chains of TOHO-Y209 were spiral, whereas those of TOHO-O348 were straight (**Figure 1**). The spores of both the strains were cylindrical in shape, 1.0 straight μm in long and had a smooth surface. Whirls, sclerotic granules, sporangia, and flagellate spores were not observed.

Table 1. Comparison of cultural characteristics of strain TOHO-O348 and TOHO-Y209.

	TOHO-Y209	TOHO-O384
Nutrient starch agar		
Substrate mycelium	black	grey
Aerial mycelium	grey	grey
Reverse side colony	cream	cream
Hydrolysis	Negative	Negative
Waksman's starch agar		
Substrate mycelium	black	grey
Aerial mycelium	grey	grey
Reverse side colony	brown	yellow
Utilization of:		
D-Glucose	+	±
D-Xylose	+	−
L-Arabinose	+	±
L-Rhamnose	±	−
D-Fructose	+	±
D-Galactose	+	±
D-Mannitol	+	±
myo-Inositol	+	±
Sucrose	+	−



(a) (b)

Figure 1. Scanning electron micrograph of strains TOHO-Y209 (a) and TOHO-O348 (b).

From the 16S rDNA gene sequence analysis, TOHO-Y209 was found to be closely related to *Streptomyces phaeofaciens* [19] (100.0%), and TOHO-O348 was found to be closely related to *S. aburaviensis* [20] (99.7%). Based on the above results, TOHO-Y209 (AB849954) and TOHO-O348 (AB849955) strains were confirmed to belong to genus *Streptomyces*.

3.2. Fermentation and Isolation

Based on the inhibitory activity in the screening, two strains, *Streptomyces* sp. TOHO-Y209 and TOHO-O348, were selected to identify the metabolites with anti-QS activity.

Strain TOHO-Y209 was cultivated in a yeast malt (YM) medium (10 L) at 27˚C for 14 days. The ethyl acetate extract from the culture broth was concentrated in vacuo. The residue was dissolved in a small amount of chloroform ($CHCl_3$) and subjected to silica gel column chromatography (20 × 107 mm). The column was eluted successively with 100 mL each of $CHCl_3$, $CHCl_3$-1% methanol (MeOH), $CHCl_3$-2% MeOH, and CH_3OH. The QSIs were found in the $CHCl_3$-1% MeOH eluate. This eluate was concentrated, and the brown residue (203.2 mg) obtained was subjected to octadecylsilyl (ODS) column (10 × 175 mm) chromatography and eluted successively with 30%, 60%, and 100% CH_3CN. The active fractions (40 mL each) were concentrated and were subjected to high-performance liquid chromatography (HPLC) (Shim-pack PREP-ODS column, Shimadzu Corporation, 10 × 250 mm; mobile phase, 75% acetonitrile (CH_3CN) containing 0.06% TFA; flow rate, 8.0 mL/min; detection, UV at 210 nm). QSI activity was found in the peaks at 7 (Compound **209-1**) and 13 (Compound **209-2**) min. Finally, 4.3 mg of **209-1** and 7.2 mg of **209-2** were obtained. The incubation period was decided based on the findings that **209-1** was produced after incubation for 5 days and reached a plateau after 16 days of incubation, while **209-2** production reached a plateau after 5 days (**Figure 2**).

TOHO-Y209 was incubated in a YM medium, and 5 mL of culture broth was evacuated at the time designated in the figure and extracted with 5 mL of ethyl acetate. The extract (100 μL) was evaporated and subjected to HPLC to determine the amounts of 209-1 and 209-2.

Strain TOHO-Y348 was cultivated in a YM medium (10 L) at 27˚C for 7 days because the production of both QSIs reached to plateau after 5 days of incubation. The QSI produced by strain TOHO-O348 was extracted with ethyl acetate from 10 L of the culture broth and concentrated. The residue was dissolved in a small amount of $CHCl_3$ and subjected to silica gel column chromatogramphy (25 × 300 mm) and eluted successively with 300 mL each of $CHCl_3$, $CHCl_3$-3% MeOH, $CHCl_3$-5% MeOH, and CH_3OH. The active fractions were combined, concentrated, and subjected to HPLC (Shim-pack PREP-ODS column, Shimadzu Corporation, 10 × 250 mm; mobile phase, 80% CH_3CN; flow rate, 8.0 mL/min; UV detection at 210 nm). The QSI activity was observed in the peaks at 7 min (Compound **348-1**) and 9 min (Compound **348-2**). Finally, 8 mg of **348-1** and 23 mg of **348-2** were obtained.

3.3. Structure Elucidation

The structures of compounds **209-2**, **348-1**, and **348-2** were established on the basis of their UV, ^{1}H-NMR, and ^{13}C-NMR spectra. Compounds **209-2** and **348-2** were found to possess the same structure and were characterized as piericidin A1 (**Figure 3**) [21,22]. Compound **348-1** was found to be the derivative of piericidin A1and was characterized as 3'-rhamnopiericidin A1 (**Figure 4**) [23].

The UV, IR, ^{1}H-NMR, and ^{13}C-NMR spectra of **209-1** resembled those of piericidin A1; however, the chemical shifts of certain C and H atoms were different from those for**209-2**, **348-1**, and **348-2**, as shown in **Table 2**. The molecular formula of **209-1** was determined to be $C_{25}H_{37}NO_5$ by high-resolution mass spectroscopy (HR-MS). By analyzing the^{1}H-NMR, ^{13}C-NMR, and various two-dimensional (2D)-NMR spectra of **209-1**, 35 proton

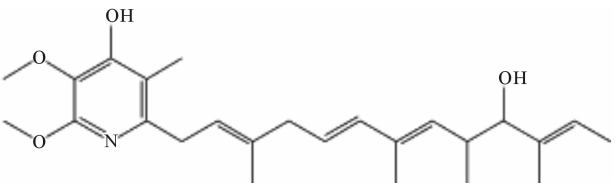


Figure 3. Structure of compounds 209-2 and 348-2.

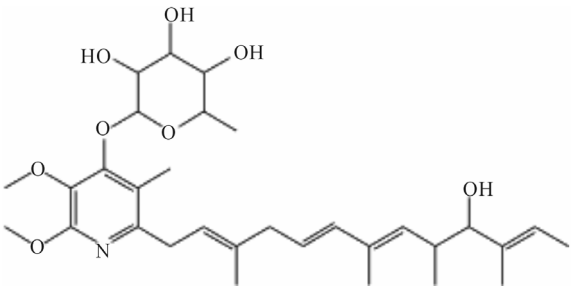


Figure 4. Structure of compound 348-1.

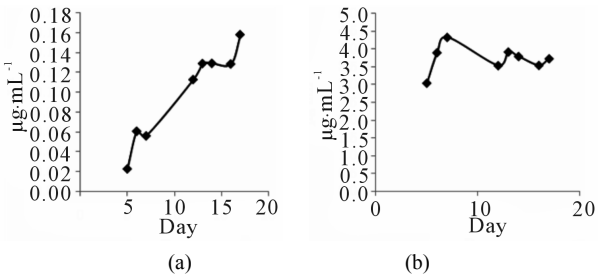


Figure 2. Time course of 209-1 (a) and 209-2 (b) productions.

Table 2. Physicochemical properties of 209-1.

Appearance	Yellow oil
Molecular formula	C25H37NO5
Molecular weight	431.26 (M)+
HR Pos. FAB-MS (m/z)	
Obsd	431.2670
Calcd	431.2672
$[\alpha]_D^{20}$	–98.5
UV λ nm (logε)	269 (3.63)
IR γ cm^{-1}	3423, 2928, 2859, 2361, 1678, 1469, 1463, 1197, 1131

and 25 carbon signals were confirmed, and by analyzing the heteronuclear multiple-quantum correlation (HMQC) spectroscopy, C-H correlations were determined, as shown in **Table 3**.

^{1}H-^{1}H COSY spectral analysis of **209-1** led to the identification of partial structural units (I, II, III, and IV), as shown in **Figure 5**. Next, the connection between these units and the remaining functional groups was determined. The^{1}H-^{13}Cheteronuclear multiple-bond correlation (HMBC) correlations from 3-CH_3 to C-2, C-3, and C-4; and from 4-H to C-2, C-3, and 3-CH_3 established the connection between partial structures I and II. The ^{1}H-^{13}CHMBC correlations from 6-H to C-7 and C-8; from 7-CH_3 to C-6, C-7, and C-8; and from 8-H to C-6 established the connection between partial structures II and III. The ^{1}H-^{13}CHMBC correlations from 10-H to 11-CH_3; from 11-CH_3 to C-10, C-11, and C-12; and from 12-H to C-10 established the connection between partial structures III and IV. Further, HMBC correlations from 1-H to C-2' and C-3'; from 3'-CH_3 to C-2', C-3', and C-4'; from 5'-OCH_3 to C-5'; and from 6'-OCH_3 to C-6' were observed. The ^{1}H-^{13}CHMBC correlations are summarized in **Figure 6**.

Furthermore, the existence of eleven sp^2 carbons suggested the existence of one double bond between the heteroatom and carbon atom, and five carbon-carbon double bonds. Because the index of hydrogen deficiency calculated from molecular formula $C_{25}H_{37}NO_5$ was eight, the partial structure analysis described above indicated the existence of one carbon-carbon double bond and one heteronuclear double bond. The ^{13}C-NMR spectrum confirmed the presence of a pyridine ring. The chemical shifts of C-7 and C-10 suggested that they are connected *via* an ether linkage, indicating the presence of an oxacyclopentane ring. Further, the butylene substituent at C-10 and hydroxyl groups at C-8 and C-4' were characterized. The planner structure of **209-1** was supported by MS fragment analysis (**Figure 7**).

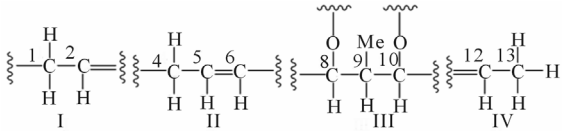


Figure 5. Partial structural units of 209-1.

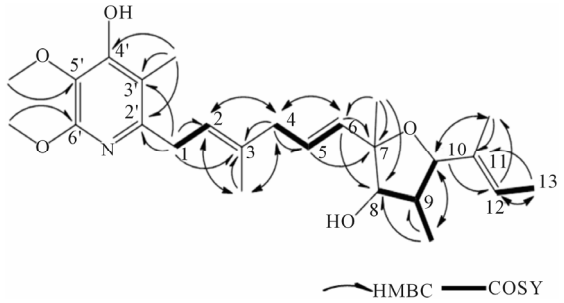


Figure 6. HMBC correlations of 209-1.

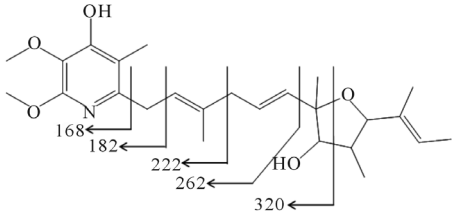


Figure 7. MS fragment analysis of compound 209-1.

The stereochemistry of **209-1** was deduced from nuclear overhauser effect (NOE) and nuclear overhauser effect spectroscopy (NOESY) experiments. The NOE correlations from 4-H to 2-H, from 4-H to 6-H, and from 10-H to 12-H indicated that the double bonds at positions 3, 6, and 11 exist in trans-conformations, and the relative stereochemistry at the oxacyclopentane ring were determined as shown in **Figure 8**. Thus, the structure of **209-1** was determined and designated as piericidin E.

3.4. Effect of Piericidin Derivatives on *C. violaceum* CV026

We investigated the QS inhibitory effects of piericidin A1, 3'-rhamnopiericidin A1, and piericidin E against *C. violaceum* CV026 (**Figure 9**). The three piericidins inhibited the purple pigment (violacein) synthesis controlled by QS, and the inhibitory effect was found to be dose dependent in the range 1to 100 μg·mL^{-1}. Piericidin A1 showed the strongest inhibitory activity, and the half maximal inhibitory concentration (IC50) was 10 μg·mL^{-1}. The antimicrobial activities (MICs) of the metabolites against *C. violaceum* CV026, *Micrococcus luteus* ATCC-9341, *Staphylococcus aureus* ATCC25923, and *P. aeruginosa* ATCC27853 were more than 100 μg·mL^{-1}.

4. Discussion

In this study, certain metabolites isolated from *Actino-*

Table 3. ^{1}H and ^{13}C NMR chemical shifts of compounds.

	piericidin A1 (**209-2** and **348-2**)		3'-rhamnopiericidin A1 (**348-1**)		piericidin E (**209-1**)	
Position	^{13}C	^{1}H (Int, M, *J* = Hz)	^{13}C	^{1}H (Int, M, *J* = Hz)	^{13}C	^{1}H (Int, M, *J* = Hz)
1	34.4	3.45 (2H, d, *J* = 7 Hz)	34.7	3.36 (2H, d, *J* = 7 Hz)	34.4	3.32 (2H, d, *J* = 7 Hz)
2	122.2	5.39 (1H, t, *J* = 7 Hz)	122.0	5.36 (1H, t, *J* = 7 Hz)	122.2	5.34 (1H, t, *J* = 7 Hz)
3	134.8	134.8	135.0	-	134.5	-
3-CH_3	16.6	1.73 (3H, s)	16.7	1.72 (3H, s)	16.5	1.70 (3H, s)
4	43.1	2.77 (2H, d, *J* = 7 Hz)	43.0	2.76 (2H, d, *J* = 7 Hz)	42.5	2.96 (2H, d, *J* = 7 Hz)
5	126.8	5.58 (1H, dt, *J* = 7, 16 Hz)	126.6	5.59 (1H, dt, *J* = 7, 16 Hz)	126.4	5.60 (1H, dt, *J* = 7, 15 Hz)
6	135.7	6.06 (1H, d, *J* = 16 Hz)	135.7	6.05 (1H, d, *J* = 16 Hz)	136.6	5.56(1H, d, *J* = 15 Hz)
7	136.0	-	136.0	-	81.9	-
7-CH_3	13.1	1.78 (3H, s)	13.1	1.78 (3H, s)	21.8	1.23 (3H, s)
8	133.1	5.19 (1H, d, *J* = 9 Hz)	133.1	5.18 (1H, d, *J* = 9 Hz)	84.6	3.63 (1H, m)
9	36.9	2.66 (1H, m)	36.8	2.65 (1H, m)	43.5	1.93 (1H, m)
9-CH_3	17.4	0.78 (3H, d, *J* = 7 Hz)	17.4	0.78 (3H, d, *J* = 7 Hz)	13.9	0.94 (3H, d, *J* = 7 Hz)
10	82.8	3.59 (1H, d, *J* = 9 Hz)	82.9	3.60 (1H, d, *J* = 9 Hz)	87.7	3.66 (1H, d, *J* = 10 Hz)
11	135.5	-	135.5	-	133.6	-
11-CH_3	10.5	1.61 (3H, s)	10.5	1.61 (3H, s)	13.3	1.60 (3H, s)
12	123.5	5.47 (1H, q, *J* = 4 Hz)	123.6	5.46 (1H, q, *J* = 4 Hz)	123.7	5.47 (1H, m)
13	13.2	1.62 (3H, d, *J* = 4 Hz)	13.1	1.60 (3H, d, *J* = 4 Hz)	10.5	1.61 (3H, d, *J* = 5 Hz)
2'	150.8	-	151.1	-	150.8	-
3'	111.9	-	117.3	-	111.8	-
3'-CH_3	10.4	2.07 (3H, s)	11.5	2.05 (3H, s)	10.4	2.06 (3H, s)
4'	153.9	-	154.8	-	153.9	-
4'-OH	-	6.13 (1H)	-	-	-	6.13(1H)
5'	127.8	-	133.3	-	122.7	-
5'-OCH_3	60.6	3.84 (3H, s)	60.5	3.77 (3H, s)	60.6	3.83 (3H, s)
6'	153.5	-	155.8	-	153.5	-
6'-OCH_3	53.0	3.93 (3H, s)	53.3	3.92 (3H, s)	53.0	3.92 (3H, s)
1''	-	-	68.1	4.22 (1H, q, *J* = 7 Hz)	-	-
1''-CH_3	-	-	16.5	1.27 (3H, d, *J* = 7 Hz)	-	-
2''	-	-	72.6	3.76 (1H, brs)	-	-
3''	-	-	66.2	3.99 (1H, brs)	-	-
4''	-	-	70.3	4.11 (1H, brs)	-	-
5''	-	-	102.7	5.61 (1H, brs)	-	-

^{1}H NMR: 500 MHz in $CDCl_3$ (ref. 7.24 ppm, *J* value in Hz); ^{13}C NMR: 125 MHz in $CDCl_3$ (ref. 77.0 ppm).

myces culture broths demonstrated QS inhibiting activity against *C. violaceum* CV026. The major metabolite isolated from *Streptomyces* sp. TOHO-Y209 and TOHO-O348 was piericidin A1. Compound 3'-rhamnopiericidin Awas isolated from TOHO-O348 as a minor component. Moreover, a novel piericidin derivative, designated as

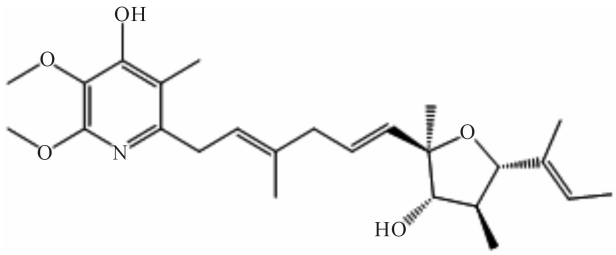


Figure 8. Structure of piericidin E (209-1).

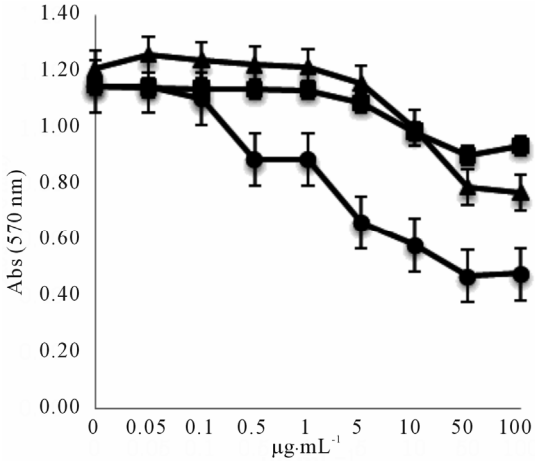


Figure 9. Effect of serial dilutions of piericidin A1 (○), 3'-rhamno piericidin A1(□), and piericidin E (Δ) on violacein production in *C. violaceum* CV026. Violacein was extracted as described in the experimental section and quantified by measuring the optical density at a wavelength of 570 nm (OD570).

piericidin E, was isolated from TOHO-Y209.

Piericidin and its derivatives are known as potent inhibitors of NADH-ubiquinone oxidoreductase, and piericidin A1 inhibits both mitochondrial and bacterial NADH-ubiquinone oxidoreductases [21]. The antimicrobial activities (MICs) of piericidin A1 and 3'-rhamnopiericidin A1 previously reported on certain strains (*Staphylococcus aureus* FDA 209P, *Bacillus subtilis* ATCC 6633, *Escherichia coli* NIHJ, *Klebsiella pneumoniae* ATCC 29655, *P. aeruginosa* IFO 13725, and *Aspergillus oryzae* IFO 4221) [22] were more than 100 $\mu g \cdot mL^{-1}$. Piericidin A1 and its derivatives isolated in this paper showed similar antibacterial activity as mentioned above; therefore, the QS inhibitory activity of the metabolites rather than the antibacterial activity resulted in the inhibition of violacein synthesis of *C. violaceum* CV026.

Until now, the QS inhibitory activity of piericidin derivatives have not been reported. The inhibitory effect was found to be dose dependent. Piericidin A1 showed the strongest QS inhibitory activity among the above mentioned compounds. The QS inhibitory activity of piericidin E was almost the same as that of 3'-rhamnopiericidin A1.

Piericidin and its derivatives were produced by *Streptomyces* sp. [24-27]. Although *S. aburaviensis* and *S. phaeofaciens* are not reported to produce piericidin A1, 3'-rhamnopiericidin A1, or piericidin E, *Streptomyces* sp. TOHO-Y209 was considered to be closely related to *S. phaeofaciens* and *Streptomyces* sp. TOHO-O348 was considered to be closely related to *S. aburaviensis* based on taxonomical studies.

In this study, piericidin A1 and its derivatives including a novel metabolite, piericidin E produced by *Streptomyces* sp. were found to be new QSIs. In this screening project, we found that 103 strains out of 1000 strains isolated from soil showed anti-QS activity in their culture broths; therefore, the strains of *Actinomycetes* are still a good source for screening the QSIs. The QSIs can be evaluated as drugs for antimicrobial chemotherapy.

5. Acknowledgements

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