Correlation between Chemical Structures and Inhibitory Activities of Anti-bacterial Substances from Marine Pseudoalteromonas sp. A1-J11

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ABSTRACT—The anti-bacterial substance, AVS-03δ, isolated from the culture of marine Pseudoalteromonas sp. A1-J11 was separated by HPLC with a C18 column. The chemical structure of AVS-03δ was identified as 2-n-pentyl-4-quinolinol (C14H17NO = 215.13) on the basis of mass spectrometry and NMR spectroscopy. AVS-03δ showed strong inhibitory activity against Vibrio harveyi strains as compared with other quinolinol compounds, suggesting that the length of the alkyl side chain of the compound is important for anti-bacterial activity.

Key words: anti-bacterial substance, alkyl-quinolinol, NMR, Pseudoalteromonas

Vibrio harveyi has been associated with diseases in shellfish (Pass et al., 1987), fish (Ishimaru and Muroga, 1998) and crustaceans (Karunasagar et al., 1994; Lavilla-Pitogo and de la Pena, 1998; Vandenberghe et al., 1999). In a study on mass mortalities of the pearl oyster, Pinctada maxima, in Western Australia, Pass et al. (1987) reported that V. harveyi caused losses amounting to 80% of cultured oyster. Bacteria were isolated from the hemolymph of 75% of diseased organisms and of these, 90% were identified as V. harveyi. Ishimaru and Muroga (1997) isolated two pathogenic Vibrio species from milkfish, Chanos chanos from the Philippines and inferred that the Vibrio species infecting the crab might be closely related to V. harveyi. V. harveyi has also been identified as a pathogen of cultured black tiger shrimp, Penaeus monodon. Disease caused by V. harveyi is a major problem in tropical aquaculture that remains, as of yet, not completely controlled.

Several methods have been employed or suggested to combat V. harveyi infection in aquaculture systems. Antibiotics had been used extensively in aquaculture but the emergence of antibiotic resistant strains makes this a dangerous practice. On the other hand, Scholz et al. (1999) demonstrated that some yeasts supplemented in the diets of P. vannamei could increase survival when challenged with V. harveyi. Rengpipat et al. (1998) showed similar results when a Bacillus strain S11 was supplemented to the diet of P. monodon, resulting in 100% survival, as opposed to the 26% survival of the control group.

In a previous paper (del Castillo et al., 2008), the authors reported the isolation of anti-bacterial substances from a marine bacterium, Pseudoalteromonas sp. A1-J11 which was found to be very close to Pseudoalteromonas piscicida by 16S rDNA analysis. In marine environments, microorganisms are thought to develop chemical defense mechanisms to compete with other microorganisms under severe selection pressure (Leisinger and Margraff, 1979; Fenical, 1993; Holstrom and Kjelleberg, 1999). Pseudoalteromonas piscicida is well known to be a producer of antibiotic and cytotoxic compounds including norharman (Zheng et al., 2006).

The highly specific activity of anti-bacterial substances from the strain A1-J11 against V. harveyi as pre-
variably reported (del Castillo et al., 2008) is a bit of an enigma, by virtue of their mode of action being effective in a relatively narrow range against bacteria as compared with those from other pseudoalteromonads. Several microorganisms have been known to inhibit the activity of known fish pathogens and used as probiotics (Balcazar et al., 2006; Sugita et al., 2007). Strain A1-J11 was also suggested to possess the characteristics as a probiotic bacterium to function in aquaculture environments. This paper reports the chemical structure elucidation of anti-bacterial substances produced by a marine bacterium A1-J11, and inhibitory activity of the purified substances and closely related authentic compounds. Once the chemical structure of the substance(s) can be determined, they can be tested for compounds. Once the chemical structure of the purified substances and closely related authentic compounds. Once the chemical structure of the substance(s) can be determined, they can be tested for the study of action mechanisms by comparing with other substances having a similar structure.

Materials and Methods

Bacterial strains and culture media

Pseudoalteromonas A1-J11 was isolated from coastal seawater of Kagoshima Bay, Japan, in July 2001 as described in a previous paper (Phyu Phyu Than et al., 2004). Vibrio harveyi strain ATCC 14126, V. harveyi strain ATCC 35084 and V. alginolyticus strain ATCC 17749 were obtained from the American Type Culture Collection (ATCC), and V. paraaemolotylicus IFO 12711 were obtained from Institute for Fermentation, Osaka (IFO, the present name is NBRC 12711). Vibrio sp. 9M-P5-1 (similar to V. campbellii) was isolated from an aquaculture pond of black tiger shrimp (Penaeus monodon) in the Philippines (de la Cruz et al., 2002), Vibrio fischeri VF-74, Pseudomonas sp. 56a-1 and Pseudomonas sp. 55b-1 were isolated from seawater of Kagoshima Bay.

Bacterial strains were cultured in a modified ZoBell medium (Z-CII) (Sashihara et al., 1975) containing Polypepton (Nippon Seiyaku) 5.0 g/L and yeast extract (Nippon Seiyaku) 1.0 g/L in 3/4 strength of artificial seawater (ASW, Herbst's formula composed of NaCl 30.0 g, KCl 7.0 g, MgCl₂ · 6H₂O 10.8 g, MgSO₄ · 7H₂O 5.4 g, and CaCl₂ · 2H₂O 1.0 g per L). Target strains used for disk diffusion assay were grown at 25°C for 2 days in 10 mL Z-CII broth in a rotating L-shaped test tube.

Chemical reagents

2-Methyl-4-quinolinol (4-hydroxy-2-methyl-quinoline), 4-methyl-2-quinolinol (2-hydroxy-4-methyl-quinoline), and 3-isoquinolinol (3-hydroxysoquinoline) were purchased from Sigma-Aldrich; 1-isoquinolinol (1-hydroxysoquinoline) from Fluka Chemi; 4-quinolinol (4-hydroxyquinoline) and chloroform-d (CDCl₃) with 0.03% tetramethylsilane (TMS) and methanol-d₄ from Wako (Osaka, Japan).

Purification of anti-bacterial substances

The purification process has been slightly modified from the one described in a previous paper (del Castillo et al., 2008). Pseudoalteromonas A1-J11 was grown for 5 days in a shaking culture of 2 L Z-CII broth in 3 L Erlenmeyer flasks. The culture was centrifuged at 10,000 × g and the supernatant was extracted with 500 mL of ethyl acetate. The ethyl acetate extract was concentrated and applied to a silica gel column (Silica Gel 60, 260 mm × 25 mm, Merck, Darmstadt, Germany) and eluted with a mobile phase of chloroform: ethyl acetate: acetone (12:1:1, v:v:v). After a visible yellow substance was eluted, about 200 mL of eluent mixture was collected and concentrated. The silica gel purified fraction dissolved in methanol (MeOH) was applied to high performance liquid chromatograph (HPLC) columns (Mightysil RP-18GP, 250 × 10 mm and 250 × 4.6 mm columns, Kanto Chemical) and eluted with 25% aqueous acetonitrile at a flow rate of 2.5 mL/min or 1.0 mL/min. Active fractions designated as AVS-03a and AVS-03d, which were previously considered as the major anti-bacterial compounds, were monitored by UV absorption at 325 nm, and were collected and used for further experiments.

Chemical structure analyses

The molecular mass was determined using electrospray ionization mass spectroscopy (ESI-MS) on a Finnigan MAT 900XL (Thermo Fisher Scientific). Samples were dissolved in MeOH and applied directly to the instrument. Samples for nucleic magnetic resonance (NMR) spectroscopy were prepared in chloro-

<table>
<thead>
<tr>
<th>Table 1. Comparative physico-chemical characteristics of AVS-03a, AVS-03d, 2-methyl-4quinolinol (2MQ), and 4-methyl-2-quinolinol (4MQ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVS-03a</td>
</tr>
<tr>
<td>---------------------------------</td>
</tr>
<tr>
<td>MW</td>
</tr>
<tr>
<td>Major λmax in UV spectrum (nm)</td>
</tr>
<tr>
<td>HPLC retention time (min)*4</td>
</tr>
<tr>
<td>Powder color</td>
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</table>

*2 2MQ, 2-methyl-4-quinolinol; 4MQ, 4-methyl-2-quinolinol.
** ESI-MS estimate (m/z).
*** The figures in parentheses indicate calculated molecular weight.
*4 HPLC: column (RP-18GP, 250 × 4.6), 25% aqueous acetonitrile, 1.0 mL/min.
form-\(d\) (CDCl\(_3\)) with 0.03% of TMS or methanol-\(d_4\) (CD\(_3\)OD) and applied to a JEOL ECA 600 (JEOL). The chemical shifts were expressed as \(\delta\) values with TMS (\(\delta 0.0\)) or methanol (\(\delta 49.0\)) as the internal standards.

**Assay for inhibitory activity against various bacteria**

Disk diffusion assay was conducted by applying the test substances dissolved in MeOH onto a paper disk (8 mm, in diameter) to a double layer Z-CII agar plate (1.5% agar bottom layer/ 0.5% agar top layer seeded with 10\% v/v of the target strain culture). Inhibitory activity against the target strains was determined by measuring the diameter of the inhibition zone on the double layer agar plate. Inhibitory activity of 10 \(\mu\)g/disk and 50 \(\mu\)g/disk of the synthetic compounds (2-methyl-4-quinolinol, 4-methyl-2-quinolinol, 3-isoquinolinol, 1-isoquinolinol, and 4-quinolinol) and 10 \(\mu\)g/disk of purified AVS-03d was tested by disk diffusion assay against *V. harveyi* ATCC 14126, *V. harveyi* ATCC 35084, *V. alginolyticus* ATCC 17749, *Vibrio* sp. 9M-P5-1, *V. fischeri* VF-74, *V. parahaemolyticus* IFO 12711, *Pseudomonas* sp. 56a-1 and *Pseudomonas* sp. 55b-1, as described in the previous paper (del Castillo *et al.*, 2008).

**Table 2.** \(^1\)H and \(^{13}\)C NMR data and HMBC correlation for AVS-03d in chloroform-\(d_4\)

<table>
<thead>
<tr>
<th>Carbon number</th>
<th>(^{13})C ((\delta))</th>
<th>(^1)H ((\delta))</th>
<th>HMBC correlation (from H to C)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>13.90</td>
<td>0.82 (3H, t)(^{\ast\ast})</td>
<td>2, 3</td>
</tr>
<tr>
<td>2</td>
<td>22.43</td>
<td>1.28 (2H, m)</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>31.31</td>
<td>1.28 (2H, m)</td>
<td>2</td>
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<tr>
<td>4</td>
<td>28.50</td>
<td>1.70 (2H, m)</td>
<td>2, 3, 5, 6</td>
</tr>
<tr>
<td>5</td>
<td>34.47</td>
<td>2.62 (2H, t)</td>
<td>3, 4, 6, 7</td>
</tr>
<tr>
<td>6</td>
<td>154.03</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>108.68</td>
<td>6.20 (1H, s)</td>
<td>5, 6, 9</td>
</tr>
<tr>
<td>8</td>
<td>179.11</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>125.10</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>10</td>
<td>125.77</td>
<td>8.34 (1H, d)</td>
<td>8, 12, 13</td>
</tr>
<tr>
<td>11</td>
<td>123.70</td>
<td>7.31 (1H, d)</td>
<td>9, (12)(^{\ast\ast}), 13</td>
</tr>
<tr>
<td>12</td>
<td>131.93</td>
<td>7.57 (1H, m)</td>
<td>10, (11), 14</td>
</tr>
<tr>
<td>13</td>
<td>117.79</td>
<td>7.54 (1H, d)</td>
<td>(8), 9, 11</td>
</tr>
<tr>
<td>14</td>
<td>140.13</td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

\(\ast\) s, singlet; d, doublet; t, triplet; m, multiplet; br, broad.  
\(\ast\ast\) Parenthesis indicates weak correlation.

Fig. 1. Comparative \(^{13}\)C NMR profiles of AVS-03a (upper), AVS-03d (middle), and 2-methyl-4-quinolinol (lower) in methanol-\(d_4\).
Results

Physico-chemical properties of anti-bacterial substances

As shown in Table 1, UV absorption spectrum of purified active substance AVS-03a, but slightly different from that of authentic compounds 2-methyl-4-quinolinol (2MQ) and 4-methyl-2-quinolinol (4MQ). Retention times in HPLC for 2MQ, 4MQ, AVS-03a and AVS-03d are 4.6, 10.4, 17.6 and 32.2 min, respectively.

Chemical structure elucidation of antibacterial substances

ESI-MS analyses of AVS-03d dissolved in methanol showed major peaks at 216.1 m/z (M + H). $^{13}$C-NMR spectroscopy for AVS-03d in CDCl$_3$ detected 14 carbon signals at $\delta$ 13.90, 22.43, 28.50, 31.31, 34.47, 108.68, 117.79, 123.70, 125.10, 125.77, 131.93, 140.13, 154.03, and 179.11, and $^1$H NMR detected 10 proton signals at $\delta$ 0.82, 1.28, 1.70, 2.62, 6.20, 7.31, 7.54, 7.57, 8.34, and 10.38, as shown in Table 2 and Fig. 1. On the other hand, $^{13}$C-NMR analyses of AVS-03a in methanol- $d_4$ exhibited 13 carbon signals at 12.73, 21.97, 30.99, 33.38, 107.46, 117.77, 123.74, 124.12, 124.61, 132.09, 140.28, 155.84 and 179.31 (Fig. 1), and 9 proton signals at 0.97, 1.43, 1.73, 2.71, 6.22, 7.38, 7.58, 7.67 and 8.19 (Fig. 2). Coupling analyses of NMR (COSY, HMBC and HMQC) for both substances indicated the presence of an aromatic ring, an alkyl side chain, and one hydrogen signal unattached to carbon (Fig. 3).

Comparative studies with authentic 4-methyl-2-quinolinol (4MQ) and 2-methyl-4-quinolinol (2MQ) indicated that NMR signals (Figs. 1 and 2) and UV absorption spectrum (Table 1) of 2MQ include common peaks with those of AVS-03a and AVS-03d. The downfield signals of the $^1$H NMR and $^{13}$C NMR for AVS-03a and AVS-03d in methanol- $d_4$ were similar to those of 2MQ and the upfield signals of both compounds indicated the presence of a butyl (C$_4$) or pentyl (C$_5$) chains in position 2 of 4-hydroxy-quinoline core (Figs. 1 and 2). These results indicated that the chemical structures of AVS-03a and AVS-03d were identical with 2-n-butyl-4-quinolinol ($C_{13}H_{15}NO = 201.27$) and 2-n-pentyl-4-quinolinol ($C_{14}H_{17}NO = 215.13$), for which assignment of

Fig. 2. Comparative $^1$H NMR profiles of AVS-03a (upper), AVS-03d (middle), and 2-methyl-4-quinolinol (lower) in methanol- $d_4$. 
Anti-bacterial substances from marine bacterium

**Fig. 3.** HMBC correlation analysis of AVS-03d in chloroform-d as deduced from $^1$H NMR (horizontal) and $^{13}$C NMR (vertical).

**Fig. 4.** Chemical structures of AVS-03a (A) and AVS-03d (B) identified as 2-$n$-butyl-4-quinolinol and 2-$n$-pentyl-4-quinolinol, respectively. Numbers indicate assignment of carbon positions based on $^{13}$C NMR signals.

Inhibition activity

At 10 µg/disk, AVS-03d was effective against only two strains of *V. harveyi* and one strain of *V. fischeri* among test strains; all other compounds tested were ineffective at this concentration as shown in Table 3. At 50 µg/disk, only 2-methyl-4-quinolinol (2MQ) was effective against two strains of *V. harveyi*, and slightly effective against three strains including *V. fischeri*, *V. parahaemolyticus* and *Vibrio* sp. 9M-P5-1. Other quinolinol and isoquinolinol derivatives were ineffective even at 50 µg/disk concentration.

**Discussion**

The authors isolated and purified three anti-bacterial substances from the culture supernatant of a marine...
bacterium, *Pseudoalteromonas* sp. A1-J11, and showed that the two substances, AVS-03a and AVS-03d inhibited the growth of limited range of vibrios including *V. harveyi, V. fischeri* and *V. anguillarum* strains in the previous paper (del Castillo et al., 2008). In this study, mass spectroscopy and NMR analysis indicated that the chemical structures of AVS-03a and AVS-03d were identical with 2-\textit{n}-butyl-4-quinolinol (BQ, C\textsubscript{13}H\textsubscript{15}NO\textsubscript{x}=201.27) and 2-\textit{n}-pentyl-4-quinolinol (PQ, C\textsubscript{14}H\textsubscript{17}NO\textsubscript{x}=215.13), respectively.

2-\textit{n}-Pentyl-4-quinolinol (PQ) has been first reported by Wratten et al. (1977) from a marine pseudomonad, which has been shown to inhibit *V. anguillarum, V. harveyi* and *Staphylococcus aureus*, and but not *Escherichia coli* strain, and later by Long et al. (2003) from an *Alteromonas* sp. that has been shown to inhibit *Synecheococcus* and phytoplankton growth as well as Gram negative bacteria. The biosynthesis of ‘pyo’ compounds (including 2-alkyl-4-quinolinols) in *Pseudomonas aeruginosa* has been reported by Cornforth and James (1956) and by Leisinger and Margraff (1979). ‘Pyo’ compounds are thought to be condensation products between anthranilic acid and a fatty acid precursor according to the scheme proposed by Conforth and James (1956). Long et al. (2003) inferred that the most sensitive mode of action of PQ was inhibition of respiration through electron transport by cytochromes, with inhibition of DNA and protein synthesis as possible secondary effects. Recovery experiments which they conducted also suggested that PQ was bacteriostatic against test organisms since target bacteria were recovered after the culture with PQ was diluted. They also reported that PQ had no effect on the ectoenzymatic activity of the target bacteria, nor alter the cell surface charge. It could, however, inhibit motility at higher concentrations or longer exposure times.

Wratten et al. (1977) and Long et al. (2003) reported that 2-\textit{n}-pentyl-4-quinolinol (PQ) was the major antibiotic compound from a marine pseudomonad strain, while Taylor et al. described 2-\textit{n}-heptyl-4-quinolinol as the most abundant antibiotic compound produced by *Pseudomonas aeruginosa*. On the other hand, strain A1-J11 produced anti-bacterial substances including AVS-03d (PQ) and AVS-03a, which showed a molecular mass of 201.0 (by ESI-MS spectrometry) and was identified as 2-\textit{n}-butyl-4-quinolinol by NMR analyses. The present paper describes the production of 2-\textit{n}-butyl-4-quinolinol by a marine bacterium for the first time. Since 2-methyl-4-quinolinol did not show anti-bacterial activity at low concentration while AVS-03a and AVS-03d (PQ) did, and AVS-03d showed higher anti-bacterial activity than AVS-03a, it is suggested that the length of the alkyl side chains is critical for anti-bacterial activity of 2-alkyl-4-quinolinol compounds. The ability to produce 2-alkyl-4-quinolinols is well known to be distributed widely among marine pseudomonads and *Pseudoalteromonas* as several papers including this one have reported. Recently, *Pseudomonas aeruginosa* has been found to utilize 4-hydroxy-2-alkylquinolines as a messenger molecule in a cell-to-cell communication pathway (Deziel et al., 2004). Further work is needed to determine the action mechanisms of alkyl-quinolinol compounds against *Vibrio* spp.

Probiotic microorganisms might stimulate immune system of host animals or produce antimicrobial substances to result in antagonistic effect on fish pathogens. Several researchers (Nogami and Maeda, 1992; Vazquez et al., 2005) demonstrated that antibiotic-pro-

### Table 3. Antibacterial activities of AVS-03d (2-\textit{n}-pentyl-4-quinolinol) and synthetic quinolinol compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Vh1</th>
<th>Vh2</th>
<th>Val</th>
<th>Vp</th>
<th>Vl</th>
<th>Vs</th>
<th>Ps1</th>
<th>Ps2</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVS–03d (2-pentyl-4-quinolinol)</td>
<td>10</td>
<td>+</td>
<td>+</td>
<td>50</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2-methyl-4-quinolinol</td>
<td>10</td>
<td>–</td>
<td>–</td>
<td>50</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4-methyl-2-quinolinol</td>
<td>10</td>
<td>–</td>
<td>–</td>
<td>50</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4-quinolinol</td>
<td>10</td>
<td>–</td>
<td>–</td>
<td>50</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1-isoquinolinol</td>
<td>10</td>
<td>–</td>
<td>–</td>
<td>50</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3-isoquinolinol</td>
<td>10</td>
<td>–</td>
<td>–</td>
<td>50</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

\*1 Vh1, V. harveyi ATCC 14126; Vh2, V. harveyi ATCC 35084; Val, V. aiginolyticus ATCC 17749; Vp, V. parahaemolyticus IFO 12711; Vf, V. fischeri VF-74; Vs, *Vibrio* sp. 9M-P5-1; Ps1, *Pseudomonas* 56a-1; Ps2, *Pseudomonas* 55b-1.

\*2 Amount of chemicals (\textmu g/paper disk).

\*3 ++, > 20 mm; +, 10~20 mm; +/-, 8~10 mm; –, 8 mm (disk) of diameter of inhibition zone.
roducing bacteria improved the growth and survival of aquatic animals as biocontrol agents. Strain A1-J11 isolated from coastal sea water should be proved to be useful in protection of aquatic animals against pathogenic microorganisms as potential probiotics in aquaculture systems, particularly for tropical aquaculture in which *V. harveyi* is a major problem for crustaceans and shellfish.

**References**


