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Three New Chlorine Containing Antibiotics from a Marine-derived Fungus *Aspergillus ostianus* Collected in Pohnpei

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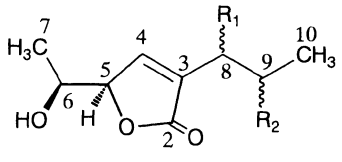
A marine bacterium *Ruegeria atlantica* (designated as strain TUF-D) was isolated from a glass plate submerged in the coastal water. Three new chlorine containing compounds (**1**~**3**) together with penicillic acid (**4**) were obtained from a marine-derived fungus *Aspergillus ostianus* strain TUF 01F313 isolated from a marine sponge at Pohnpei as antibacterial components against *R. atlantica*. The structures of three new antibiotics were determined based on their spectral data as 8-chloro-9-hydroxy-8,9-deoxyasperlactone (**1**), 9-chloro-8-hydroxy-8,9-deoxyasperlactone (**2**), and 9-chloro-8-hydroxy-8,9-deoxyaspyrone (**3**). Compound **1** inhibited the growth of *R. atlantica* at 5 μg/disc (inhibition zone: 12.7 mm), while **2** and **3** were active at 25 μg/disc (10.1 and 10.5 mm, respectively).

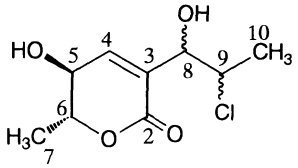
Filamentous fungi isolated from marine environments are now recognized as an important resource of biologically active secondary metabolites[1~7]. The term marine fungi may be restrictedly used for the fungus, whose habitat is elucidated to be obligate marine, while facultative or unidentified fungi obtained from marine environments are called marine-derived fungi[1,8]. Marine bacteria are also interesting sources for biologically active metabolites[3~7,9]. Some marine bacteria are recognized as the first organism attached to natural and artificial constructions in the sea after adhesion of organic matter leading to the sequential biofauling by zoo planktons and macroorganisms such as barnacles, hydrozoa, and shellfish[10].

As a part of our studies on biologically active metabolites from the sea[11], we isolated a marine bacterium *Ruegeria atlantica* (designated as strain TUF-D) attached to a glass plate submerged in the coastal water and screened antibacterial substances against this bacterium from the culture broths of marine-derived fungi isolated from marine sponges at Pohnpei. Three new compounds (**1**~**3**, Fig. 1)

Fig. 1. Structures of 8-chloro-9-hydroxy-8,9-deoxyasperlactone (**1**), 9-chloro-8-hydroxy-8,9-deoxyasperlactone (**2**), 9-chloro-8-hydroxy-8,9-deoxyaspyrone (**3**), and penicillic acid (**4**) isolated from a marine-derived fungus *Aspergillus ostianus* strain TUF 01F313 collected in Pohnpei.



1: R_1=Cl, R_2=OH
2: R_1=OH, R_2=Cl



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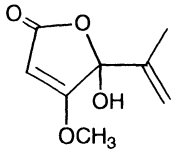

penicillic acid (**4**)

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have been isolated from *Aspergillus ostianus* strain TUF 01F313 together with penicillic acid (**4**) as antibacterial components to *R. atlantica*. We report here the identification of the producing fungus and isolation and structure assignment of three new chlorine containing antibiotics (**1**~**3**).

Materials and Methods

Spectral Analysis

NMR spectra were measured on either a JEOL JNM A-500 NMR spectrometer or a Bruker DMX-500 NMR spectrometer. ^{1}H and ^{13}C signals were assigned by ^{1}H-^{1}H COSY, HSQC, and HMBC spectra. Mass spectra were obtained by either a JEOL HX-110 mass spectrometer (FAB mode, *m*-nitrobenzylalcohol as matrix) or a Finnigan TSQ 700 triple quadrupole mass spectrometer (ESI mode). UV and IR spectra were recorded on a Hitachi U-3000 and on a JASCO A-102, respectively. Optical rotations were taken on a JASCO DIP-1000 polarimeter.

Marine Bacterium *Ruegeria atlantica* Strain TUF-D

Five clean slide glasses were submerged in the coastal water for a day at Hayama, Kanagawa Prefecture, Japan. Each glass was washed with sterilized seawater (25 ml) by shaking in a sterile plastic bottle. Two glass plates were each placed on a Marine Agar (Difco) plate for several minutes and then removed. The other two glass plates were shaken vigorously in each 30 ml of sterile seawater in a plastic bottle. A 200 μl aliquot of the seawater was inoculated on Marine Agar. The biofilm of the fifth glass plate was scraped from the surface into sterilized seawater by a sterile platinum loop, and the suspension (200 μl) was inoculated on Marine Agar. The agar plates were incubated at 20°C for 5 days. Six different bacteria were isolated from these agar plates. *R. atlantica* strain TUF-D, isolated from the fifth glass plate, was selected for the antibacterial bioassay and identified by the 16S rDNA sequence[12].

Antibacterial Assay

R. atlantica strain TUF-D was cultured in PY-SW medium (0.5% peptone and 0.1% yeast extract in seawater) at 25°C for 5 days. A water solution of glycerol (20%) was added to the same volume of cultured broth (final concentration of 10%), and the suspension was divided into small plastic tubes (each 100 μl) and frozen at −80°C. The frozen stock was thawed, inoculated into Marine Broth (Difco, 2 ml), and cultured for 5 days at 25°C. This was mixed with autoclaved Marine Agar (100 ml) to make assay plates. Samples were dissolved in methanol or ethanol and 40 μl of each solution was absorbed on a disc (8 mm in diameter). After placing the discs, the assay plates were incubated at 25°C for a day. Diameters of the inhibition zones were measured.

Three new compounds **1**~**3** were also tested for antimicrobial activity against *Escherichia coli* IAM 12119T, *Staphylococcus aureus* IAM 12544T, *Saccharomyces cerevisiae* IAM 14383T, and *Mucor hiemalis* IAM 6088.

Isolation and Identification of *Aspergillus ostianus* Strain TUF 01F313

The fungus, designated as strain TUF 01F313, was isolated from an unidentified marine sponge collected at Pohnpei in 2001. Marine sponges were collected by SCUBA diving and sealed in a sterile plastic bag in the water. Small pieces of each sponge were placed in a sterile mortar with 1~2 ml of sterile seawater and homogenized with a pestle. Two hundred microliters of the liquid portion was inoculated on an agar plate (0.02% yeast extract, 0.1% soluble starch, 2% agar, and 200 ppm chloramphenicol in 90% natural seawater). The organism remaining in the mortar was pressed by the pestle to remove liquid, and three pieces were applied on an agar plate. The strain TUF 01F313, grown from the sponge, was inoculated on a slant in a culture tube.

Identification of the strain TUF 01F313 was conducted according to the methods of KLICH & PITT[13] and KLICH[14]. The color names used in this study were taken from KORNERUP & WANSCHER[15].

Isolation of Antibacterial Components **1**~**4**

A. ostianus strain TUF 01F313 was cultured in four 500 ml flasks each containing 150 ml of 1/2 PD medium (hot water (500 ml) extract of potato (200 g), 10 g dextrose, 500 ml natural seawater) for three weeks at 20°C. The broth was filtered, and the filtrate was extracted three times with EtOAc (each 500 ml). The EtOAc extract was evaporated to yield 265 mg of material that was chromatographed on a silica gel column (30 g) with $CHCl_3$-MeOH (gradient elution) into 10 fractions. Fractions 5 (44.7 mg), 6 (24.7 mg), and 7 (65.1 mg) showed antibacterial activity against *R. atlantica* strain TUF-D and were subjected to HPLC separation using an ODS column (Mightysil RP-18, 10 mm×25 cm; flow rate, 2 ml/minute). Fraction 6 (15 mg) was separated with 60% MeOH-H_2O into four fractions, and the second fraction (11.0 mg) was further separated by HPLC with 20% MeOH-H_2O (0.1% AcOH) to give **1** (7.5 mg) and **2** (2.2 mg). Fraction 7 (24 mg) afforded **2**

(4.4 mg) and **3** (5.4 mg) with 40% MeOH-H_2O (0.1% AcOH). Fraction 5 (38 mg) gave penicillic acid (**4**, 25.0 mg) with 50% MeOH-H_2O.

Compound **1**: $[\alpha]_D^{24}$ +22.5° (*c* 0.17, $CHCl_3$). HRFABMS *m/z* 221.0596; calcd for $C_9H_{14}O_4Cl$ $[M+H]^+$, 221.0581. UV $\lambda_{max}^{MeOH\text{-}chloroform\ (9:1)}$ nm (ε) 230 (2,500). IR ν_{max}^{neat} (cm^{-1}) 3356, 2980, 2934, 1748, 1379, 1197, 1069, 941. ^1H ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data are listed in Table 1.

Compound **2**: $[\alpha]_D^{26}$ +76° (*c* 0.015, $CHCl_3$). HRFABMS *m/z* 221.0590; calcd for $C_9H_{14}O_4Cl$ $[M+H]^+$, 221.0581. UV $\lambda_{max}^{MeOH\text{-}chloroform\ (9:1)}$ nm (ε) 230 (2,300). IR ν_{max}^{neat} (cm^{-1}) 3364, 2980, 2933, 1746, 1380, 1200, 1065, 981, 934. ^1H ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data are listed in Table 2.

Compound **3**: $[\alpha]_D^{25}$ +17.2° (*c* 0.17, $CHCl_3$). HRFABMS *m/z* 221.0586; calcd for $C_9H_{14}O_4Cl$ $[M+H]^+$, 221.0581. UV $\lambda_{max}^{MeOH\text{-}chloroform\ (9:1)}$ nm (ε) 230 (1,600). IR ν_{max}^{neat} (cm^{-1}) 3393, 2983, 2935, 1705, 1649, 1449, 1384, 1218, 1149, 1122, 1052, 982, 951, 916. ^1H (500 MHz) and ^{13}C NMR

Table 1. ^1H (500 MHz) and ^{13}C NMR (125 MHz) data for **1** in $CDCl_3$.

C#	^{13}C	^1H (*J* in Hz)	COSY	HMBC
2	171.3 s	-----		
3	133.4 s	-----		
4	150.4 d	7.54 br s	5, 8	2, 3, 5, 8
5	85.2 d	4.87 ddd (4.5, 1.2, 1.2)	4, 6	2, 3, 4, 6, 7
6	67.6 d	4.03 dq (6.5, 4.5)	5, 7	4, 5, 7
7	18.9 q	1.26 d (6.5)	6	5, 6
8	58.0 d	4.64 br d (4.5)	4, 9	2, 3, 4, 9, 10
9	70.0 d	4.19 dq (6.5, 4.5)	8, 10	3, 10
10	19.1 q	1.18 d (6.5)	9	8, 9

Table 2. ^1H (500 MHz) and ^{13}C NMR (125 MHz) data for **2** in $CDCl_3$.

C#	^{13}C	^1H (*J* in Hz)	COSY	HMBC
2	171.8 s	-----		
3	133.7 s	-----		
4	148.7 d	7.49 dd (1.4, 1.4)	5, 8	2, 3, 5, 8
5	85.3 d	4.94 ddd (4.8, 1.4, 1.4)	4, 6	2, 3, 4, 6, 7
6	67.9 d	4.07 dq (6.5, 4.8)	5, 7	4, 5, 7
7	18.9 q	1.34 d (6.5)	6	5, 6
8	71.4 d	4.66 br s	4, 9	2, 3, 4, 9, 10
9	59.0 d	4.47 dq (6.7, 4.8)	8, 10	3, 10
10	19.0 q	1.47 d (6.7)	9	8, 9

Table 3. ^1H (500 MHz) and ^{13}C NMR (125 MHz) data for **3** in $CDCl_3$ - CD_3OD (85 : 15).

C#	^{13}C	^1H (*J* in Hz)	COSY	HMBC
2	163.8 s	-----		
3	129.6 s	-----		
4	146.1 d	6.82 d (2.5)	5, 8	2, 6, 8
5	68.1 d	4.10 dd (8.5, 2.5)	4, 6	3, 4, 6, 7
6	79.0 d	4.23 dq (8.5, 7.0)	5, 7	4, 5
7	17.9 q	1.35 d (7.0)	6	5, 6
8	75.8 d	4.37 d (5.5)	4, 9	2, 3, 4, 9, 10
9	58.6 d	4.25 dq (7.0, 5.5)	8, 10	3, 8, 10
10	19.9 q	1.36 d (7.0)	9	8, 9

Table 4. ^1H (500 MHz) and ^{13}C NMR (125 MHz) data for **3** in C_6D_6.

C#	^{13}C	^1H (*J* in Hz)	COSY	HMBC
2	163.5 s	-----		
3	129.8 s	-----		
4	146.0 d	6.25 br s	5	2, 6, 8
5	67.7 d	3.32 dd (9.0, 7.5)	4, 6, 5-OH	3
6	78.5 d	3.74 dq (9.0, 6.5)	5, 7	4, 5
7	17.6 q	0.96 d (6.5)	6	5, 6
8	75.9 d	4.20 dd (12.5, 7.5)	9, 8-OH	2, 3, 4, 10
9	59.2 d	4.35 dq (12.5, 6.5)	8, 10	3, 8, 10
10	19.7 q	1.30 d (6.5)	9	8, 9
5-OH		0.90 d (7.5)	5	5, 6
8-OH		3.02 d (7.5)	8	3, 8

(125 MHz) data obtained in $CDCl_3$ - CD_3OD (85 : 15) and in C_6D_6 are listed in Tables 3 and 4, respectively.

Results and Discussion

Marine Bacterium *Ruegeria atlantica* Strain TUF-D

Six marine bacteria were isolated from glass plates submerged in the coastal water for a day. The bacteria were examined as potential bioassay organisms. Three bacteria grew well in Marine Broth and were observed under an electron microscope. Two bacilli, which required NaCl for growth, and a coccus were detected. One of two bacilli (strain TUF-D) was selected because it was easier to culture than the other one. The sequence analysis of the 16S rDNA revealed that this strain is classified as *R. atlantica* (100% identity)[12].

R. atlantica strain TUF-D was cultured in PY-SW medium to make frozen stocks, and Marine Broth and Marine Agar were used for pre-culture and bioassay, respectively. The culture broths of 239 marine-derived fungi collected at Pohnpei in 2001 were screened for antibacterial activity against *R. atlantica*, and the strain designated as TUF 01F313 showed the strongest inhibitory activity among them.

Taxonomy of the Fungus Strain TUF 01F313

Colonies on Czapek yeast extract agar reached 33~38 mm in diameter after 7 days at 25°C, and were velutinus and wrinkled. Conidia were grayish yellow (4B4). Mycelia were white. Exudates and white sclerotia were produced. No soluble pigments were observed. On the reverse side, the colonies were dull yellow (3B4) or pale red (8A3). Colonies on Malt extract agar reached 24~25 mm in diameter after 7 days at 25°C. Conidia were light yellow (4A4). Mycelia were inconspicuous and white. On the reverse side, the colonies were light yellow (4A4). Colonies on Czapek yeast extract agar with 20% sucrose reached 51~53 mm in diameter after 7 days at 25°C, and were wrinkled. Conidia were olive (3D4)~grayish yellow (3C3). On the reverse side, the colonies were olive (3E8)~grayish yellow (3B5). No growth was observed at 37°C. Conidial heads were radiate. Stipes were 550~1100×5~10 μm, with rough and thick walls. Vesicles were 20~42 μm in diameter, and nearly globose. Aspergilla were biseriate. Metulae were 8~10×3.5~4 μm, covering the entire surface of the vesicle. Phialides were 8~10×2.5~3 μm. Conidia were 3.5~4.5 μm, subglobose to ovoid, and with smooth to finely roughened walls.

Based on the above characteristics, strain 01F313 was identified as *Aspergillus ostianus* Wehmer.

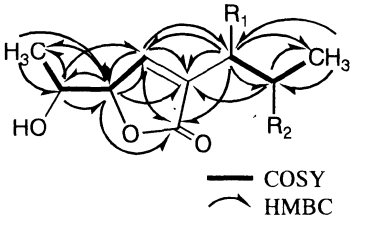
Isolation and Structures of Antibacterial Components

The marine fungus *A. ostianus* strain TUF 01F313 was cultured in 1/2 PD medium (50% seawater), and the broth was filtered. The filtrate was extracted with EtOAc. Bioassay-guided separation of the EtOAc extract by silica gel column chromatography followed by HPLC yielded four antibacterial compounds (**1**~**4**, Fig. 1). Three chlorine containing compounds (**1**~**3**) were revealed to be new, and the forth component (**4**) was identified as penicillic acid by the comparison of ^1H and ^{13}C NMR data for **4** with those of the reported values for penicillic acid[8].

Compound **1** showed molecular ions at *m/z* 221 and 223 with the ratio of 3 : 1 in the FAB and ESI mass spectra, which suggested the presence of chlorine. The molecular formula of **1** was determined from HRFABMS and NMR data as $C_9H_{13}O_4Cl$. The ^{13}C NMR spectrum of **1** revealed signals due to a carboxyl, two olefinic (singlet and doublet), three oxygenated methine, a methine, and two methyl carbons (Table 1). The olefinic proton and its ^{13}C signals were observed at lower field (δ_H 7.54 and δ_C 150.4, respectively), which indicated that this carbon was ascribable to the β-position of an α,β-unsaturated carboxyl moiety. The presence of an α,β-unsaturated γ-lactone was suggested by the IR (1748 cm^{-1}) and NMR data for **1**. The ^1H-^1H COSY spectrum of **1** showed the connectivity of carbons at 4-5-6-7 and 8-9-10 (Fig. 2). The skeletal structure of **1** was assigned by the analysis of HMBC data as shown in Fig. 2.

Compound **2** showed the same molecular ions at *m/z* 221 and 223 (3 : 1) in the FAB and ESI mass spectra as those detected in those of **1**. The molecular formula ($C_9H_{13}O_4Cl$) was deduced from the HRFABMS and NMR data (Table 2). The ^1H and ^{13}C NMR spectra of **2** resembled those of

Fig. 2. ^1H-^1H COSY and HMBC correlations for compounds **1** (R_1=Cl, R_2=OH) and **2** (R_1=OH, R_2=Cl).

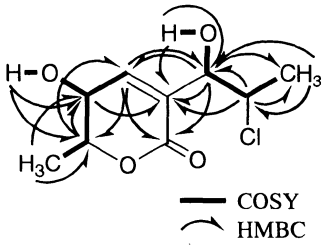


1. The presence of an α,β-unsaturated γ-lactone was suggested by the IR (1746 cm^{-1}) and NMR data (δ_H 7.49; δ_C 171.8, 133.7, and 150.4). These data revealed that **2** was an isomer of **1**. ^1H-^1H COSY data for **2** showed that the signal of a methine proton (δ 4.47, H-9) attached to the chlorinated carbon coupled with a methyl doublet (H_3-10) at δ 1.47 and an oxygenated methine signal (H-8) at δ 4.66 (Fig. 2). The connectivity of carbons at 4-5-6-7 was also determined by the ^1H-^1H COSY spectrum of **2** (Fig. 2). The HMBC spectrum of **2** was very similar to that of **1**, and the data (Table 2) revealed all connections of carbons. Thus, the skeletal structure of **2** was elucidated as shown in Fig. 2.

Compound **3** showed the molecular ions at *m/z* 221 and 223 with the 3 : 1 ratio in the FAB and ESI mass spectra, the same as those of **1** and **2**. The molecular formula ($C_9H_{13}O_4Cl$) was determined from the HRFABMS and NMR data (Tables 3 and 4). The ^1H NMR spectrum of **3** measured in $CDCl_3$ showed complex multiplets between δ 4.20 and 4.35 due to four protons. ^1H signals were separated when measured in a mixture of $CDCl_3$ and CD_3OD (85 : 15) (Table 3). A better ^1H NMR spectrum was obtained in C_6D_6 solution (Table 4). The ^1H NMR spectra of **3** in $CDCl_3$-CD_3OD and in C_6D_6 were similar to those of **1** and **2**. The presence of an α,β-unsaturated lactone (or ester) was suggested by the IR (1705 cm^{-1}) and NMR data (Tables 3 and 4). The ^1H-^1H COSY spectrum of **3** in C_6D_6 revealed the connectivity of carbons at 4-5-6-7 and 8-9-10, and the couplings between H-5 (δ 3.32) and 5-OH (δ 0.90), and between H-8 (δ 4.20) and 8-OH (δ 3.02) were also detected (Fig. 3). The analysis of HMBC data for **3** (Tables 3 and 4) connected all carbons assigning the skeletal structure of **3** as shown in Fig. 3.

Compounds **1** and **2** are chlorinated derivatives of asperlactone (8,9-epoxide)[17,18], and **3** is a derivative of aspyrone (8,9-epoxide)[19~21]. Asperlactone and aspyrone have been isolated from the terrestrial fungi *Aspergillus ochraceus* and *Aspergillus melleus*. The absolute stereochemistries of asperlactone and aspyrone were determined by X-ray crystallography and chemical transformations as (5*R*,6*S*,8*S*,9*S*) and (5*S*,6*R*,8*S*,9*S*) configurations, respectively[18]. ^{13}C NMR data for **1** and **2** at C-2~C-7 were very similar to those for asperlactone[22]. The ^{13}C signals due to C-3 [**1**: $\Delta\delta$ 0.9, **2**: $\Delta\delta$ 1.2] and C-4 [**1**: $\Delta\delta$ 2.6, **2**: $\Delta\delta$ 0.9] were observed at lower field than those of the reported values for asperlactone. ^1H NMR data for **1** and **2** at H-4~H-7 were also very similar to those for asperlactone, which revealed the lower field shifts of H-4 [**1**: $\Delta\delta$ 0.20~0.26, **2**: $\Delta\delta$ 0.15~0.21] and H-5 [**2**: $\Delta\delta$ 0.06~0.12][18,23]. These shifts would be ascribable to the effect of different functional groups at C-8. The relative configurations at C-5 and C-6 in **1** and **2** are, therefore, considered to be the same as asperlactone (Fig. 1).

Fig. 3. ^1H-^1H COSY and HMBC correlations for compound **3** measured in C_6D_6.



Comparison of the ^{13}C NMR data for **3** in $CDCl_3$-CD_3OD (85 : 15) and aspyrone in $CDCl_3$ at C-2~C-7 showed resembled chemical shifts except the lower field shift [$\Delta\delta$ 4.4] of C-4 of **3**[21]. The chemical shifts of H-5~H-7 of **3** in $CDCl_3$ were detected at very similar fields to those of aspyrone, and the lower field shift [$\Delta\delta$ 0.16~0.17] of H-4 of **3** was also observed[18,21]. Moreover, the coupling constants between H-4 and H-5, H-5 and H-6, and H-6 and H-7 of **3** in $CDCl_3$ - CD_3OD (85 : 15) were identical to those of aspyrone. These data suggested that the relative configurations at C-5 and C-6 of **3** are the same as those of aspyrone (Fig. 1).

Asperlactone and aspyrone are biogenetically synthesized *via* the same intermediate[17,20,22,24~29]. If the compounds **1**~**3** are biosynthesized similar to or *via* asperlactone and aspyrone, **1** may have a (8*R**,9*S**) configuration and **2** and **3** a (8*S**,9*R**) configuration. Studies on the stereochemistries and biosynthesis of **1**, **2**, and **3** are now under investigation.

Antimicrobial Activity

Three new chlorinated compounds (**1**~**3**) showed antibacterial activity against *R. atlantica* strain TUF-D (Table 5). The growth of *E. coli* and *S. aureus* were also inhibited by these compounds. *R. atlantica* was more sensitive than *E. coli* and *S. aureus* (Table 5). Compounds **1**~**3** did not inhibit the growth of *S. cerevisiae* and *M. hiemalis* even at 100 μg/disc. Penicillic acid (**4**) showed an inhibition zone of 17.3 mm at 10 μg/disc against *R. atlantica*. Compound **1** was the most potent among the three new components. Therefore, the position of Cl affects the activity of these compounds.

Table 5. Antimicrobial activity of compounds **1**~**3**.

	Ruegeria atlantica					*Escherichia coli*			*Staphylococcus aureus*			
	100[a]	50	25	10	5	100	50	25	100	50	25	10
1	33.1[b]	29.2	24.9	17.0	12.7	17.6	11.6	---[c]	18.6	13.2	10.2	---
2	19.6	14.1	10.1	---	---	10.8	---	---	13.1	9.9	---	---
3	23.5	17.8	10.5	---	---	10.3	---	---	11.4	9.7	---	---

[a]Concentration (μg/disc). [b]Diameter of inhibition zone (mm). [c]Not active.

Antimicrobial activities of asperlactone and aspyrone were reported[30]. These compounds inhibited the growth of several filamentous fungi at 20 μg/ml and yeast at 100~200 μg/disc. Asperlactone showed weaker activity than aspyrone. *S. cerevisiae* was not affected by these compounds, which is the same as compounds **1**~**3**. The weak growth inhibition of some bacteria by asperlactone and aspyrone (25~200 μg/disc) were reported[30]. The activities to *E. coli* and *S. aureus* of these compounds were almost the same as those of **2** and **3**. Therefore, **1** has stronger activity against bacteria than asperlactone and aspyrone. Asperlactone and aspyrone have nematocidal, insecticidal, and ovicidal activities besides their antimicrobial properties[21,23]. Therefore, bioactivities of **1**~**3** to microorganisms other than five species tested in this study and to invertebrates are interesting future studies.

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