Halogenated Metabolites from the New Okinawan Red Alga *Laurencia yonaguniensis*

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A novel brominated diterpene based on the rare neoirieane skeleton, named neoirietetraol (**1**), has been isolated along with a halogenated C_{15} acetogenin, (3*Z*)-laurenyne (**2**), from a new *Laurencia* species, *L. yonaguniensis* Masuda et Abe, *species inedita*, collected at Yonaguni Island, Okinawa Prefecture, Japan. The structures of these metabolites were elucidated by spectroscopic data (IR, ¹H NMR, ¹³C NMR, 2D NMR, and MS). Neoirietetraol (**1**) was toxic to the brine shrimp (*Altemia salina*; LC$_{50}$, 40.1 μM) and also showed weak antibacterial activities against two marine bacteria, *Alcaligenes aquamarinus* and *Escherichia coli*.

It is well known that species discrimination in the red algal genus *Laurencia* (Rhodomelaceae, Ceramiales) is complicated by a high degree of morphological variation within individual species. As is well documented, species of *Laurencia* are known to produce diverse, unique, halogenated secondary metabolites.[1] Most species of *Laurencia* biosynthesize at least one specific secondary metabolite not found in any others[2,3] or a characteristic set of metabolites.[4] Thus, secondary metabolite chemistry can provide a criterion for taxonomy in *Laurencia*.[4] In our continuing chemotaxonomic studies on Japanese species of *Laurencia* based upon morphological and chemical features as well as genetic affinities,[4−11] we have examined a new species, *Laurencia yonaguniensis* Masuda et Abe, *species inedita* (Masuda, M.; unpublished results), collected at Yonaguni Island, Okinawa Prefecture, Japan, and found this species to contain a novel halogenated diterpene, neoirietetraol (**1**), along with a new halogenated C_{15} acetogenin, (3*Z*)-laurenyne (**2**). We wish to report herein the structural elucidation of these metabolites that seem to be characteristic of this species. The antibacterial activity of these metabolites against marine bacteria and their toxicity against brine shrimp are also described.

L. yonaguniensis collected at Hikawa, Yonaguni Island, Okinawa Prefecture, Japan, was extracted with methanol. A combination of column and thin-layer chromatography of the methanol extract yielded neoirietetraol (**1**) (6.8% w/w of methanol extract) and (3*Z*)-laurenyne (**2**) (14.5% w/w).

Neoirietetraol (**1**) was assigned the molecular formula $C_{20}H_{34}Br_2O_4$ by HRFDMS. Its IR spectrum showed the presence of hydroxyl groups (ν_{max} 3620 and 3400 cm⁻¹), but no carbonyl group absorption. Furthermore, in the IR spectrum, absorptions at ν_{max} 1390 and 1375 cm⁻¹ suggested the presence of a *gem*-dimethyl group,[12] which was further supported by the HMBC spectrum (Table 1). The ¹H NMR spectrum showed five tertiary methyls (δ_H 1.62, 1.49, 1.11, 1.01, and 0.32) and four hydroxyl groups [δ_H 5.78 (1H, s), 4.95 (1H, d, *J* = 2.4 Hz), 0.87 (1H, d, *J* = 3.4

1 **3**

2: 3*Z*
4: 3*E*
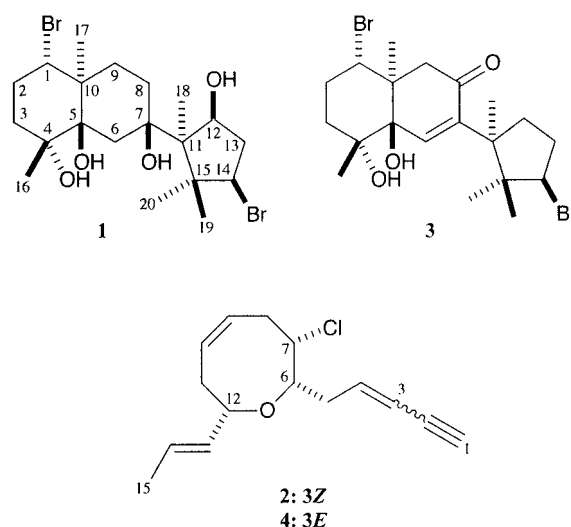
Hz), and 0.44 (1H, br s)], the latter four signals of which were changed by the addition of D₂O.

Detailed analysis of the ¹H and ¹³C NMR, ¹H−¹H COSY, and HETCOR spectra of **1** revealed the presence of partial structural units **1a**−**n**, comprising all elements implied by the molecular formula (Figure 1). In the ¹³C NMR spectrum, the chemical shifts (δ_C 66.3 and 62.9) of the methine carbons in units **1a** and **1b** indicated that the substituents U and V are bromine atoms. Furthermore, the substituents W, X, Y, and Z were verified as hydroxyl groups on the basis of the chemical shifts of the pertinent carbons (δ_C 81.5, 78.8, 75.5, and 83.0, respectively).

Confirmation of the partial structural units and their connectivities was made with the aid of the HMBC spectrum (Table 1). Long-range correlations between the tertiary methyl protons (δ_H 1.49) in **1l** and quaternary carbons (δ_C 78.8 and 43.9) in **1e** and **1h**, a methine carbon (δ_C 66.3) in **1a**, and a methylene carbon (δ_C 32.6) in **1c** established the connection of unit **1h** with units **1a**, **1c**, **1e**, and **1l**, leading to the expanded partial structure **1o**. Long-range correlations between the tertiary methyl protons (δ_H 1.11) in **1m** and quaternary carbons (δ_C 78.8 and 75.5) in **1e** and **1f** and a methylene carbon (δ_C 38.4) in **1a** confirmed the connection of unit **1f** with units **1a**, **1m**, and **1e**, to give the expanded partial structure **1p** with a six-

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Table 1. ^{13}C NMR (100 MHz, DETP), ^{1}H NMR (400 MHz), and HMBC Data for Neoirietetraol (**1**)^a

position^b	δ_C	δ_H	multiplicity, J (Hz)	HMBC correlation
1	66.3	4.98	dd, J = 13.2, 4.4	C-2, C-5, C-9, C-10, C-17
2	31.5	2.52	dddd, J = 13.2, 13.2, 13.2, 4.4; H_{ax}	C-1, C-3, C-10
		2.05	dddd, J = 13.2, 4.4, 4.4, 2.4; H_{eq}	C-3, C-10
3	38.4	2.19	ddd, J = 13.7, 13.2, 4.4; H_{ax}	C-2, C-4, C-16
		0.95	ddd, J = 13.7, 4.4, 2.4; H_{eq}	C-2, C-16
4	75.5			
5	78.8			
6	33.1	2.01	dd, J = 14.2, 2.4; H_{ax}	C-5, C-10, C-11
		1.91	dd, J = 14.2, 2.4; H_{eq}	C-5, C-7, C-8, C-10
7	83.0			
8	30.1	1.84	ddd, J = 13.2, 13.2, 4.4; H_{ax}	C-6, C-9
		1.32	dddd, J = 13.2, 4.4, 2.4, 2.4; H_{eq}	
9	32.6	2.17	ddd, J = 13.2, 13.2, 4.4; H_{ax}	C-8, C-10, C-17
		1.74	ddd, J = 13.2, 4.4, 2.4; H_{eq}	C-7, C-8, C-10, C-17
10	43.9			
11	53.7			
12	81.5	3.19	ddd, J = 7.3, 3.4, 2.9	C-11, C-14, C-18
13	44.9	2.18	ddd, J = 14.7, 9.3, 7.3; H_β	C-11, C-14, C-15
		1.63	ddd, J = 14.7, 9.8, 2.9; H_α	C-14
14	62.9	3.75	dd, J = 9.8, 9.3	
15	48.5			
16	26.8	1.11	s	C-3, C-4, C-5
17	18.9	1.49	s	C-1, C-5, C-9, C-10
18	20.8	0.32	s	C-7, C-11, C-12, C-15
19	25.0	1.62	s	C-11, C-14, C-15, C-20
20	24.8	1.01	s	C-11, C-14, C-15, C-19
OH		5.78	s	
OH		4.95	d, J = 2.4	
OH		0.87	d, J = 3.4	
OH		0.44	br s	

^{a} Measured in benzene-d_6. ^{b} Assignment was made from the HETCOR spectrum.
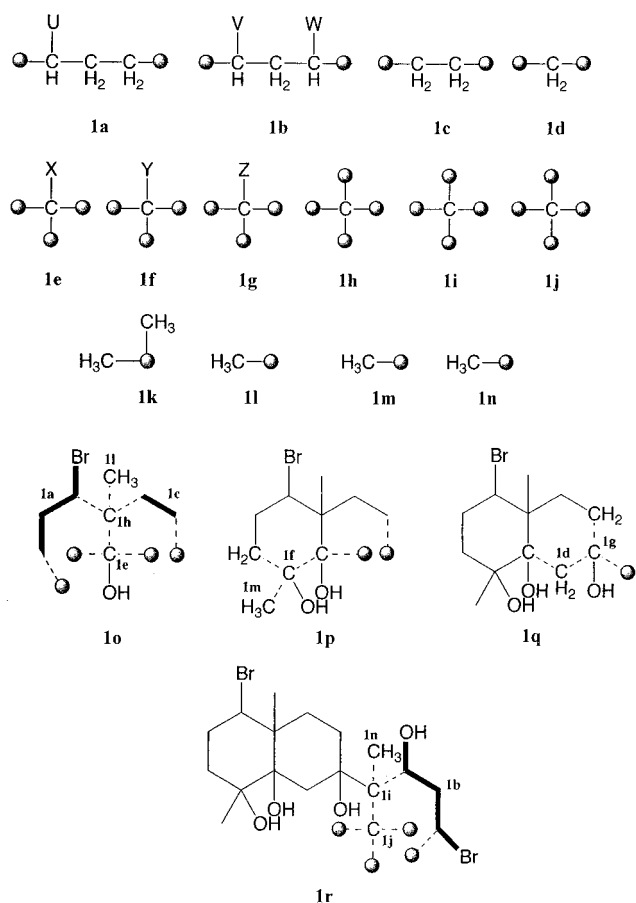
Figure 1. Partial structural units for neoirietetraol (**1**).

membered ring. Furthermore, long-range correlations between one of the protons (δ_H 1.91) of the isolated methylene protons in unit **1d** and one of the methylene carbons (δ_C

30.1) in **1c** and quaternary carbons (δ_C 78.8, 83.0, and 43.9) in **1e**, **1g**, and **1h** revealed the connection of unit **1d** with units **1e** and **1g**. Hence, unit **1p** was expanded to unit **1q** to form a decalin-type skeleton. The connection of unit **1i** to unit **1q** was possible from the HMBC cross-peaks observed between the resonance for one (δ_H 2.01) of the methylene protons in **1d** and that of a quaternary carbon (δ_C 53.7) in **1i**. On the other hand, long-range correlations between the tertiary methyl protons (δ_H 0.32) in **1n** and a hydroxymethine carbon (δ_C 81.5) in **1b** and quaternary carbons (δ_C 83.0, 53.7, and 48.5) in **1g**, **1i**, and **1j** established the connection of unit **1i** with units **1q**, **1n**, **1b**, and **1j**, leading to the proton expanded structure **1r**. Furthermore, the presence of the *gem*-dimethyl group (**1k**) suggested by the IR spectrum was confirmed by mutual long-range correlations between two tertiary methyl groups at δ_H 1.62/δ_C 25.0 and δ_H 1.01/δ_C 24.8. These *gem*-dimethyls showed correlations with quaternary carbons (δ_C 53.7 and 48.5) in **1i** and **1j** and a bromomethine carbon (δ_C 62.9) in **1b**, confirming the connection of unit **1k** with units **1i** and **1b**. Thus, the planar structural formula **1** could be assigned for neoirietetraol, with this compound possessing a neo-irieane skeleton that was first found in neoirieone (**3**) isolated from *Laurencia irieii*.[13]

The relative stereochemistry of **1** was determined by the NOESY spectrum (Figure 2) as well as the coupling constants in the ^{1}H NMR spectrum. The methine proton (H-1) on C-1 showed the coupling constants J = 13.2 and 4.4 Hz, indicating that the H-1 is a typical axial proton on the chair cyclohexane ring, and hence the bromine atom at C-1 is equatorial. The axial configuration of the methyl group (H$_3$-17) at C-10 was shown by NOESY correlations to H$_{ax}$-2 (δ_H 2.52), H$_{ax}$-6 (δ_H 2.01), and H$_{ax}$-8 (δ_H 1.84), which required the decalin ring to be *trans*-fused. Hence, the hydroxyl group at C-5 was shown to be axial. In addition, the hydroxyl group at C-7 was determined as axial on the basis of a NOE correlation between OH-5 (δ_H 5.78) and
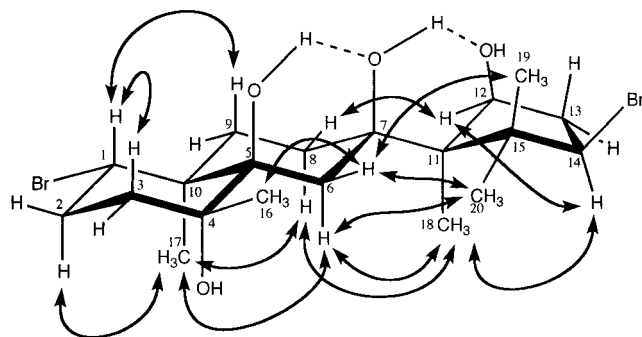
Figure 2. NOEs from NOESY spectrum of neoirietetraol (**1**).

OH-7 (δ_H 4.95). This feature was further supported by the *W*-shaped long-range coupling ($J = 2.4$ Hz) between OH-7 and H_{ax}-6. The equatorial configuration of the methyl group (H_3-16) at C-4 was indicated by a NOE between H_3-16 and H_{eq}-6, which showed *W*-type long-range coupling ($J = 3.4$ Hz) with H_{eq}-8. Furthermore, NOESY correlations between H_{ax}-6/H_3-18, H_{ax}-8/H_3-18, H-12/H_{eq}-8, H_3-20/H_2-6, and H_3-19/H_{eq}-6 indicated that the rotation of the C-7−C-11 bond is restricted probably due to the hydrogen bond between the hydroxyl groups at C-7 and C-12. NOEs between H-12/H_3-18, H-12/H-14, and H-14/H_3-18 revealed that H_3-18, H-12, and H-14 are located on the same face and OH-12 and Br-14 are located on the opposite face of the cyclopentane ring.

Consequently, the structure of neoirietetraol must be represented by formula **1**, which has the relative configurations of $1S^*$, $4R^*$, $5R^*$, $7R^*$, $10S^*$, $11R^*$, $12S^*$, and $14R^*$. An effort to determine the absolute configuration of the secondary hydroxyl group in **1** using the advanced Mosher's method[14] failed due to the inability to transform this compound to (*R*)- and (*S*)-MTPA esters, respectively. We are currently attempting to prepare a single crystal of **1** suitable for X-ray crystallographic analysis to confirm the proposed structure and the absolute configuration for neoirietetraol. Neoirietetraol (**1**) is the second example of a halogenated diterpenoid having a neoirieane skeleton.[1]

The second halogenated metabolite **2** was shown to have the molecular formula $C_{15}H_{19}ClO$ by HREIMS. The IR spectrum showed the presence of a terminal acetylenic (ν_{max} 3310 cm^{-1}) functionality. The presence of a 2-penten-4-ynyl moiety was readily recognized by the ^{1}H NMR spectrum (Table 2) [δ_H 3.11 (1H, d, $J = 2.2$ Hz), 5.54 (1H, m), and 6.07 (1H, dddd, $J = 10.9$, 8.5, 6.4, and 1.0 Hz)]. The coupling constants ($J_{3,4} = 10.9$ Hz) for H-3 and H-4 as well as the chemical shift value (δ_H 3.11) of the acetylenic proton indicated the geometry of the double bond at C-3 to be *Z*.[15] In addition, the presence of a 1-propenyl moiety was revealed by a vinyl methyl signal at δ_H 1.69 (3H, ddd, $J = 6.4$, 1.5, and 1.0 Hz) and the signals at δ_H 5.69 (1H, ddq, $J = 15.2$, 1.0, and 6.4 Hz) and 5.55 (1H, ddq, $J = 15.2$, 5.9, and 1.5 Hz). The *J* value ($J_{13,14} = 15.2$ Hz) for H-13 and H-14 indicated the geometry of the double bond at C-13 to be *E*. Furthermore, an additional double bond was revealed by the signals at δ_H 5.90 (1H, dddd, $J = 10.3$, 8.6, 7.3, and 1.0 Hz) and 5.68 (1H, dddd, $J = 10.3$, 10.0, 8.3, and 1.5 Hz).

Detailed analysis of the ^{1}H and ^{13}C NMR, ^{1}H−^{1}H COSY, and HSQC spectra of **2** revealed the presence of partial structure **2a** (Figure 3). The chemical shift values of the methine carbons at C-6 (δ_C 79.1) and C-12 (δ_C 81.7) indicated that oxygen atoms are attached to these carbons. Moreover, the remaining substituent at C-7 was verified as a chlorine atom on the basis of the chemical shift of the

Table 2. ^{13}C NMR (100 MHz, DETP) and ^{1}H NMR (400 MHz) Data for (3*Z*)-Laurenyne (**2**)^a

position^b	δ_C	δ_H	multiplicity, J (Hz)
1	80.2	3.11	d, $J = 2.2$
2	82.1		
3	110.2	5.54	m
4	141.8	6.07	ddd, $J = 10.9$, 8.5, 6.4, 1.0
5	35.3	2.73	dddd, $J = 14.2$, 8.8, 6.4, 1.5; H_a
		2.55	dddd, $J = 14.2$, 8.5, 4.6, 1.0; H_b
6	79.1	3.89	ddd, $J = 8.8$, 4.6, 2.4
7	65.3	3.99	ddd, $J = 11.5$, 4.9, 2.4
8	34.5	2.98	dddd, $J = 12.7$, 11.5, 10.0, 1.2; H_a
		2.53	m; H_b
9	128.6	5.68	dddd, $J = 10.3$, 10.0, 8.3, 1.5
10	131.1	5.90	dddd, $J = 10.3$, 8.6, 7.3, 1.0
11	34.9	2.47	m; H_a
		2.15	ddd, $J = 14.2$, 8.6, 1.5; H_b
12	81.7	3.76	br dd, $J = 8.8$, 5.9
13	132.1	5.55	ddq, $J = 15.2$, 5.9, 1.5
14	126.2	5.69	ddq, $J = 15.2$, 1.0, 6.4
15	17.8	1.69	ddd, $J = 6.4$, 1.5, 1.0

^{a} Measured in CDCl$_3$. ^{b} Assignment was made from the HSQC spectrum.

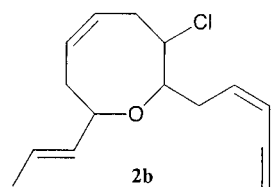
2a: X = Cl or O

2b
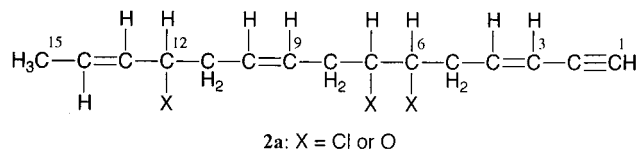
Figure 3. Partial and planar structure for (3*Z*)-laurenyne (**2**).

pertinent carbon at δ_C 65.3. Since the IR spectrum showed no absorption indicative of a hydroxyl group, compound **2**, having five degrees of unsaturation, must contain one oxocene, thus leading to the planar formula **2b** (Figure 3).

The gross structure of compound **2** was the same as laurenyne (**4**), which has previously been found in *Laurencia obtusa* collected at Gökçeada in the Aegean Sea.[16] A detailed comparison of the ^{1}H and ^{13}C NMR data of **2** with **4** revealed that compound **2** is (3*Z*)-laurenyne, a geometrical isomer of laurenyne with respect to the double bond at C-3.

The antibacterial activities of the two new isolated halogenated metabolites of *L. yonaguniensis* were tested using paper disk diffusion assays against six species of marine bacteria. Neoirietetraol (**1**) showed activities against *Alcaligenes aquamarinus* and *Escherichia coli* at 100 μg/disk, while (3*Z*)-laurenyne (**2**) was inactive toward these bacteria at the same doses tested. In addition, neoirietetraol (**1**) and (3*Z*)-laurenyne (**2**) showed toxicity toward brine shrimp with LC$_{50}$ values of 40.1 and 467.0 μM, respectively.

Experimental Section

General Experimental Procedures. Melting points were determined on a Yazawa micro-melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-140 polarimeter. IR spectra were recorded on a JASCO IR-Report-100 spectrophotometer. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were measured in CDCl$_3$ and C$_6$D$_6$ with TMS as an internal standard by using a JEOL JNM-EX-400 spectrometer. EIMS and HREIMS were obtained on a

JEOL JMS-A500 spectrometer, and FDMS, HRFDMS, and HRFABMS were obtained on a JEOL JMS-SX102A spectrometer. Si gel (Merck, Kieselgel 60, 70−230 mesh) was used for column chromatography. Si gel plates (Merck, Kieselgel 60 F_{254S}) were used for preparative TLC.

Plant Material. A sample of *L. yonaguniensis* Masuda et Abe, *species inedita* was collected at Hikawa, Yonaguni Island, Okinawa Prefecture, Japan, in May 2001. This species is similar to *Laurencia intricata* Lamouroux in many respects, but differs from the latter in an important feature, the number of *corps en cerise*:[17] each trichoblast cell includes three to five *corps en cerise*. No such species is present in the genus *Laurencia*. This species will be described as a new species in the near future after examination of sexual reproductive plants, which are not available at present. Voucher specimens are deposited in the Herbarium of the Graduate School of Science, Hokkaido University (SAP 091287-091290).

Extraction and Isolation. The partially dried alga (40 g) was soaked in MeOH for 3 days. The MeOH solution was concentrated in vacuo and partitioned between Et_2O and H_2O. The Et_2O solution was washed with water, dried over anhydrous Na_2SO_4, and evaporated to leave a dark green oil (523 mg). The extract was fractionated by column chromatography on Si gel with a step gradient (hexane and EtOAc). The fraction (144 mg) eluted with hexane−EtOAc (9:1) was further subjected to preparative TLC with toluene to give (3*Z*)-laurenyne (**2**) (75.5 mg, 14.5% based on the weight of the MeOH extract) and a triacylglycerol (12.2 mg, 2.3%). Furthermore, the fraction (107 mg) eluted with hexane−EtOAc (1:1) gave neoirietetraol (**1**) (35.4 mg, 6.8%) by preparative TLC with toluene−EtOAc (4:1).

Neoirietetraol (1): colorless needles; mp 126−128 °C; $[\alpha]^{28}_D$ −43.0° (*c* 0.53, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3620, 3400, 3010, 1470, 1390, 1375, 1220, 1100, 925 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; LRFDMS *m/z* 501, 499, 497 [M + H]$^+$ (17:35:18), 482, 480, 478 [M − H_2O]$^+$ (17:28:16), 446, 444, 442 [M − $3H_2O$]$^+$ (28:44:24), 419, 417 [M − Br]$^+$ (95:100), 364, 362 [M − $3H_2O$ − HBr]$^+$ (24:23), 190, 188 [M − $C_{12}H_{21}O_4Br$]$^+$ (45:47); HRFDMS *m/z* 497.0885 (calcd for $C_{20}H_{35}{}^{79}Br_2O_4$, 497.0902 [M + H]).

(3*Z*)-Laurenyne (2): colorless needles; mp 46−48 °C; $[\alpha]^{28}_D$ +30.4° (*c* 0.63, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3310, 3025, 1450, 1085, 1010, 970 cm^{-1}; ^1H and ^{13}C NMR data, Table 2; LREIMS *m/z* 252, 250 [M]$^+$ (1:3), 237, 235 [M − CH_3]$^+$ (17:50), 215 [M − Cl]$^+$ (11), 187, 185 [M − C_5H_5]$^+$ (5:13), 145 (23), 117 (35), 105 (34), 91 (100), 79 (43), 71 (38), 65 (32); HREIMS *m/z* 250.1096 (calcd for $C_{15}H_{19}{}^{35}ClO$, 250.1124 [M]).

Antibacterial Assays. The antibacterial bioassays for the isolated halogenated compounds were carried out using six species of marine bacteria isolated from an algal bed at Oshoro Bay, Hokkaido, Japan. These bacteria were *Alcaligenes aquamarinus*, *Alteromonas* sp., *Azomonas agilis*, *Erwinia amylovora*, *E. coli*, and *Halococcus* sp. One loopful of each organism was precultured in 5 mL of peptone water (3% NaCl) overnight. The turbidity of the culture was adjusted to an optical density (OD) McFarland 0.5.[18,19] Then, 0.1 mL of the precultured bacterial suspension was used to seed nutrient agar plates (3% NaCl). Paper disks (Whatman, 6 mm) impregnated with 0.1 mg of the respective isolated compound were placed on the seeded agar plates, and the diameters of the inhibitory zones were measured after the plates were incubated at 30 °C for 24 h.

Brine Shrimp Assays. A bioassay of toxicity toward brine shrimp was performed as described in the literature.[20] Briefly, the compounds (0.6 mg) dissolved in DMSO (50 μL) were made up to 1 mg/mL in seawater. Serial dilution was made in the wells of 96-well microplates (Iwaki, Asahi Techno Glass Co., Tokyo, Japan) in triplicate in seawater (100 μL). Brine shrimp eggs obtained locally (Japan Pet Drugs Co., Ltd., Tokyo, Japan) were hatched in filtered seawater and oxygenated with an aquarium pump at 28 °C. After 24 h, a suspension of nauplii containing 10−20 organisms (100 μL) was added to each well and incubated at 24 °C for 24 h, and the numbers of nonmotile and total nauplii in each well were counted in turn.

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