Halymecins, New Antimicroalgal Substances Produced by Fungi Isolated from Marine Algae

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Novel antimicroalgal substances halymecins A (**1**), B (**2**) and C (**3**) were isolated from the fermentation broth of a *Fusarium* sp. and halymecins D (**4**) and E (**5**) from an *Acremonium* sp. The structures of these halymecins, Fig. 1, were determined based on extensive 2D NMR studies as well as mass spectral data. These chemical structures are conjugates of di- and trihydroxydecanoic acid. Halymecin A showed antimicroalgal activity against *Skeletonema costatum*.

Since marine microorganisms dwell in different circumstances and possess different biological properties compared with terrestrial ones, they can be expected to produce novel chemical substances. Recently marine microorganisms have been used as sources for new substances. Still, very few studies have been reported[1~3].

In the course of searching for new substances, three antimicroalgal substances named halymecins A (**1**), B (**2**) and C (**3**) were isolated from the fungus *Fusarium* sp. FE-71-1 from an algal isolate. We also found other halymecins D (**4**) and E (**5**) from another fungus, *Acremonium* sp. FK-N30, isolated from another algal isolate.

This paper describes the taxonomic studies of the producing organisms, as well as production, isolation, structure determination and some biological activities of the halymecins.

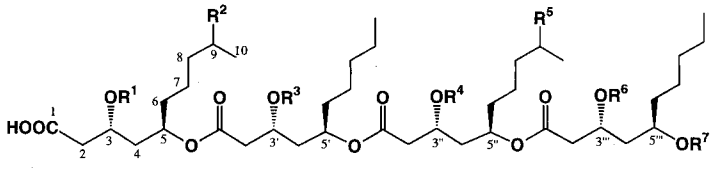
Taxonomic Studies of Producing Strain

The producing fungus of halymecins A, B and C, strain FE-71-1, was isolated from a marine alga (*Halymenia dilatata*) taken from the Palau islands. It was identified as a *Fusarium* sp. through its morphological and physiological properties. Another fungus, producing halymecins D and E, strain FK-N30, was isolated from a marine alga collected from Aburatsubo at Kanagawa prefecture in Japan. It was identified as an *Acremonium* sp. in the same way.

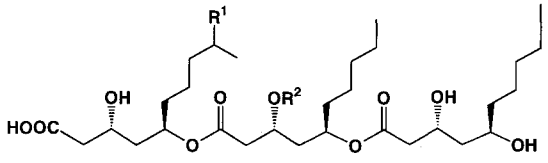
Production of Halymecins by Fermentation

FE-71-1 was cultured in 100-ml Erlenmeyer flasks containing the fermentation medium described in the culture and medium conditions section. A typical time

Fig. 1. Structure of halymecins.



Halymecin A (**1**): $R^1=H$, $R^2=H$, $R^3=Ac$, $R^4=H$, $R^5=H$, $R^6=H$, $R^7=H$
1a: $R^1=Ac$, $R^2=H$, $R^3=Ac$, $R^4=Ac$, $R^5=H$, $R^6=Ac$, $R^7=Ac$
Halymecin B (**2**): $R^1=H$, $R^2=H$, $R^3=Ac$, $R^4=H$, $R^5=H$, $R^6=\beta$-D-mannosyl, $R^7=H$
Halymecin D* (**4**): $R^1=H$, $R^2=OH$, $R^3=H$, $R^4=H$, $R^5=OH$, $R^6=H$, $R^7=H$

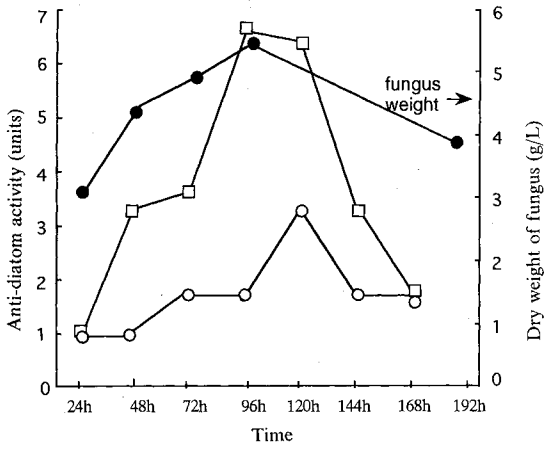


Halymecin C (**3**): $R^1=H$, $R^2=Ac$
Halymecin E* (**5**): $R^1=OH$, $R^2=H$

* Stereochemistries for **4** and **5** have not been confirmed.

Fig. 2. Time course of halymecins fermentation by *Fusarium* sp. FE-71-1.

□ Supernatant, ○ extraction of fungus, ● fungus weight



course of fermentation is shown in Fig. 2, indicating that the highest level of activity was achieved at about 96 hours fermentation time, and the activity in the supernatant medium was three times larger than in the mycelial extract.

Structure of Halymecin A (1)

Halymecin A (**1**) was obtained as a colorless oil. Negative FAB-MS of halymecin A (**1**) gave a pseudomolecular ion at *m/z* 803 $[M-H]^-$. The molecular formula of **1** was determined to be $C_{42}H_{76}O_{14}$ by negative ion high resolution FAB-MS (Found 803.5179, Calcd. 803.5157). The ^1H and ^{13}C NMR spectral data of **1** are summarized in Table 1. The ^1H and ^{13}C NMR showed that the molecule contained one acetyl group, four methyl groups, 24 methylene groups, eight methine groups bound to oxygen and four carbonyl carbons besides that of an acetyl carbon.

Treatment of **1** with acetic anhydride in pyridine afforded an acetyl derivative **1a** in 63% yield. Positive FAB-MS of **1a** gave a pseudomolecular ion at *m/z* 973 $[M+H]^+$, and ^1H NMR showed five acetyl groups, suggesting that **1a** was a tetraacetyl derivative of **1**. The ^1H NMR spectrum was simplified by acetylation indicating that **1a** was the conjugate of equivalent units.

The HMBC spectrum of **1a** indicated that the methylene groups (δ 2.62, H-2) were attached to carbonyl groups. In the COSY experiment, the methylene protons showed coupling to methine protons (δ 5.32, H-3) which were coupled through the H-4 methylene protons to methine protons (δ 4.92, H-5). Furthermore a non-branched pentyl group was found. The chemical shifts of these methine protons at positions 3 and 5 suggested that they were bound to oxygen. In addition, the FAB-MS fragment peaks at 685, 457, 229 produced from the peak at 913 $[M+H-AcOH]^+$ ion by loss of multiples of 228 mu were observed. Therefore the structure of **1a** was elucidated as a tetramer of 3,5-dihydroxydecanoic acid monoacetate.

More detailed NMR analyses of **1** determined the connectivities of each of the 3,5-dihydroxydecanoate units and the position of the acetyl group. The TOCSY spectrum (Fig. 3) of each unit showed the connectivities from the H-2 through the H-6 protons, as well as the assignment of these protons. HMBC spectra showed the correlations between the methine proton (H-5) and the carbonyl carbon (C-1) of adjacent units, revealing that these units were linked by ester linkages through the 5 position. The acetyl carbonyl carbon (δ_C 171.6) was long range coupled to the 3′ proton (δ_H 5.25), so that the acetyl group must be attached to an oxygen bound to 3′ position on the second unit (Fig. 4). Thus the basic structure of **1** was determined.

Stereochemistry of 1

Halymecin A (**1**) showed optical activity. Stereochemistry was determined by chemical degradation. Hydrolysis of **1** with 0.1 N HCl in CH_3CN at 80°C afforded lactone **1b** in 66% yield (Fig. 5). Positive FAB-MS of **1b** gave a pseudomolecular ion at *m/z* 187 $[M+H]^+$, and ^1H NMR suggested a 3-hydroxy-δ-lactone structure. All of the possible four stereoisomers of this lactone have been synthesized and reported by ROMEYKE *et al.*[4]. By the comparison of **1b** with their literature ^1H NMR data and optical rotations[4], **1b** was identified as the 3R, 5R stereoisomer (Fig. 5). Thus the structure of **1** was shown to have the stereochemistry shown in Fig. 1.

Structure of Halymecin B (2)

Halymecin B (**2**) was also obtained as a colorless oil. Negative FAB-MS of halymecin B (**2**) gave a pseudomolecular ion at *m/z* 965 $[M-H]^-$. The molecular formula of **2** was determined to be $C_{48}H_{86}O_{19}$ by high resolution negative ion FAB-MS (Found 965.5701, Calcd. 965.5685) which was greater by $C_6H_{10}O_5$ than **1** and showed quite similar ^1H NMR and ^{13}C NMR spectra compared with those of **1**, except for the existence of a sugar moiety. These results indicate that **2** consists of one sugar moiety attached to **1**. The nonglycosidic moiety was characterized by NMR studies done in the same manner as **1** (Table 1). The HMBC spectrum

Table 1. NMR chemical shifts for **1**, **2** and **3**.

Position	**1**		**2***		**3**	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	178.65		174.94		175.60	
2	43.50	2.31, 2.45	43.15	2.35, 2.45	42.70	2.35, 2.45
3	66.70	3.95	66.20	3.95	66.34	4.01
4	41.78	1.65, 1.82	41.70	1.65, 1.81	41.70	1.65, 1.82
5	73.52	5.05	73.61	5.01	73.46	5.01
6	35.00[a]	1.60	34.87[e]	1.60	34.89[i]	1.60
7	25.60[b]	1.32	25.49[f]	1.32	25.61[j]	1.32
8	32.71[c]	1.32	32.26[g]	1.32	32.69[k]	1.32
9	23.37[d]	1.32	23.17[h]	1.32	23.22[l]	1.32
10	14.40	0.86	14.42	0.86	14.42	0.86
1′	171.19		171.12		171.19	
2′	39.90	2.55, 2.65	39.80	2.55, 2.65	39.85	2.55, 2.65
3′	69.11	5.20	69.00	5.21	69.09	5.20
4′	38.93	1.85, 1.95	38.82	1.85, 1.95	38.93	1.85, 1.95
5′	72.34	4.90	72.24	4.93	72.24	4.93
6′	34.90[a]	1.60	34.78[e]	1.60	34.81[i]	1.60
7′	25.60[b]	1.32	25.49[f]	1.32	25.56[j]	1.32
8′	32.45[c]	1.32	32.36[g]	1.32	32.35[k]	1.32
9′	23.30[d]	1.32	23.19[h]	1.32	23.26[l]	1.32
10′	14.40	0.86	14.42	0.86	14.42	0.86
1″	172.76		172.62		172.68	
2″	43.19	2.45, 2.50	42.62	2.45	43.39	2.56, 2.75
3″	66.61	4.05	66.50	4.07	68.81	4.21
4″	42.00	1.70, 1.80	42.49	1.70, 1.80	43.73	1.56
5″	73.46	5.05	73.39	5.04	71.76	3.75
6″	34.90[a]	1.60	34.78[e]	1.60	38.40	1.41
7″	25.60[b]	1.32	25.49[f]	1.32	25.80	1.41
8″	32.45[c]	1.32	32.36[g]	1.32	32.31[k]	1.32
9″	23.30[d]	1.32	23.19[h]	1.32	23.35	1.32
10″	14.40	0.86	14.42	0.86	14.40	0.86
1‴	173.02		172.91			
2‴	43.50	2.46	41.62	2.56, 2.75		
3‴	69.05	4.20	75.57	4.28		
4‴	43.69	1.56	41.87	1.56		
5‴	71.86	3.75	69.67	3.63		
6‴	38.45	1.41	38.47	1.41		
7‴	25.83	1.41	25.92	1.41		
8‴	32.34[c]	1.32	32.61[g]	1.32		
9‴	23.25[d]	1.32	23.30[h]	1.32		
10‴	14.40	0.86	14.40	0.86		
$-COCH_3$	171.64		171.59		171.64	
$-COCH_3$	21.41	2.01	21.28	2.01	21.28	2.01

NMR are measured in $CDCl_3$ - CD_3OD = 1 : 1.
[a~l] Assignments may be interchanged.
* Assignments for the sugar moiety are listed below.
Position (δ_C, δ_H): 1 (101.15, 4.57), 2 (72.12, 3.87), 3 (74.87, 3.45), 4 (68.26, 3.62), 5 (77.47, 3.45), 6 (62.67, 3.62).

revealed that each unit was linked by ester linkages thorough the 5 position, and an acetyl group was assigned to the C-3′ position. The sugar residue was identified by HPLC analysis of the hydrolysate of **2** which was obtained with 0.5 N hydrochloric acid. The hydrolysate was subjected to HPLC analysis using fluorescent detection after condensation with benzamidine[5]; the sugar peak coincided with mannose. The methanolysis product of **2** was α-methyl mannoside, which was identified as the **D** form by optical rotation[6]. The HMBC spectrum correlated the anomeric proton with the 3‴ methine carbon so that the location of mannose was assigned to position 3‴. A NOESY spectrum showed the correlation of H-1 with H-3 and H-5 positions on the mannoside moiety, so that the linkage of the glycosidic residue was determined to be β (Fig. 6). Thus the structure of **2** was determined as shown in Fig. 1.

Fig. 3. TOCSY data for halymecin A (**1**).

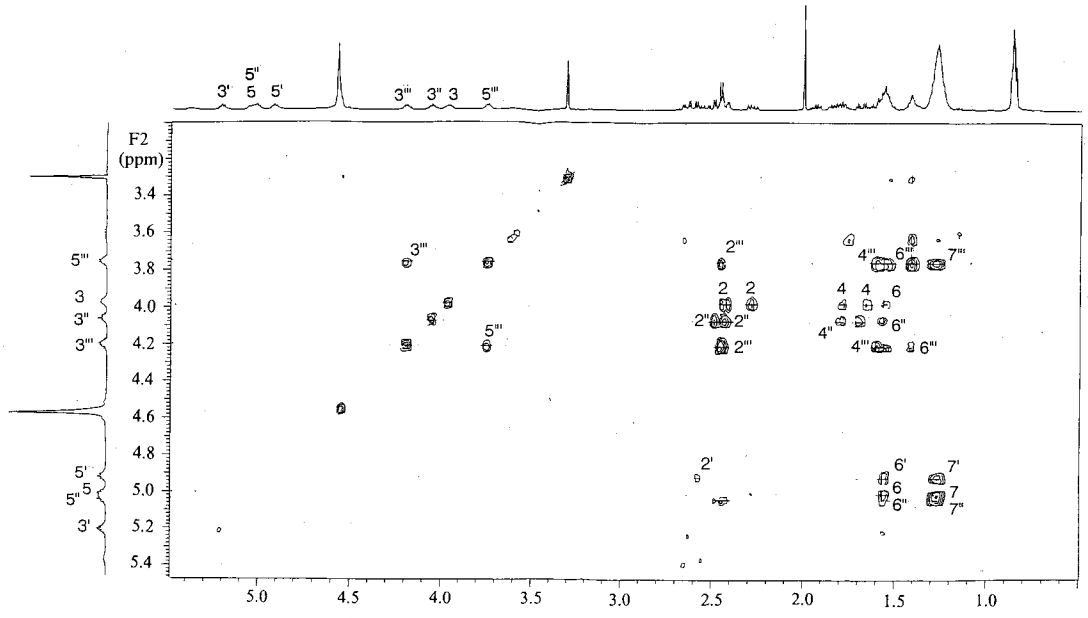


Fig. 4. HMBC data for halymecin A (**1**).

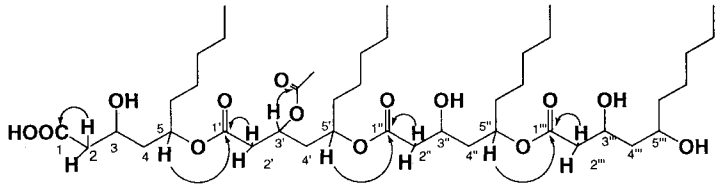


Fig. 5. Acid degradation product of halymecin A (**1**) and ^{1}H NMR chemical shifts (ppm) for the resulting lactone **1b**.

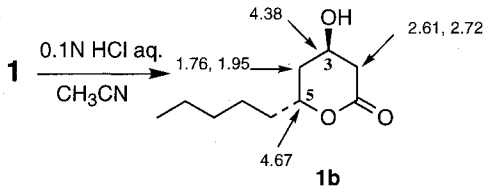
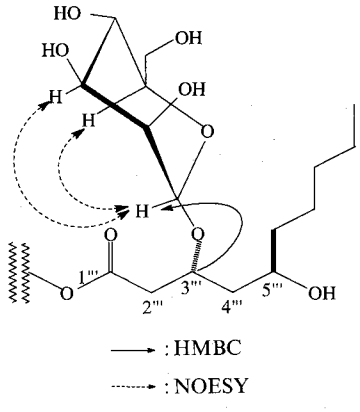


Fig. 6. HMBC and NOESY of halymecin B (**2**).



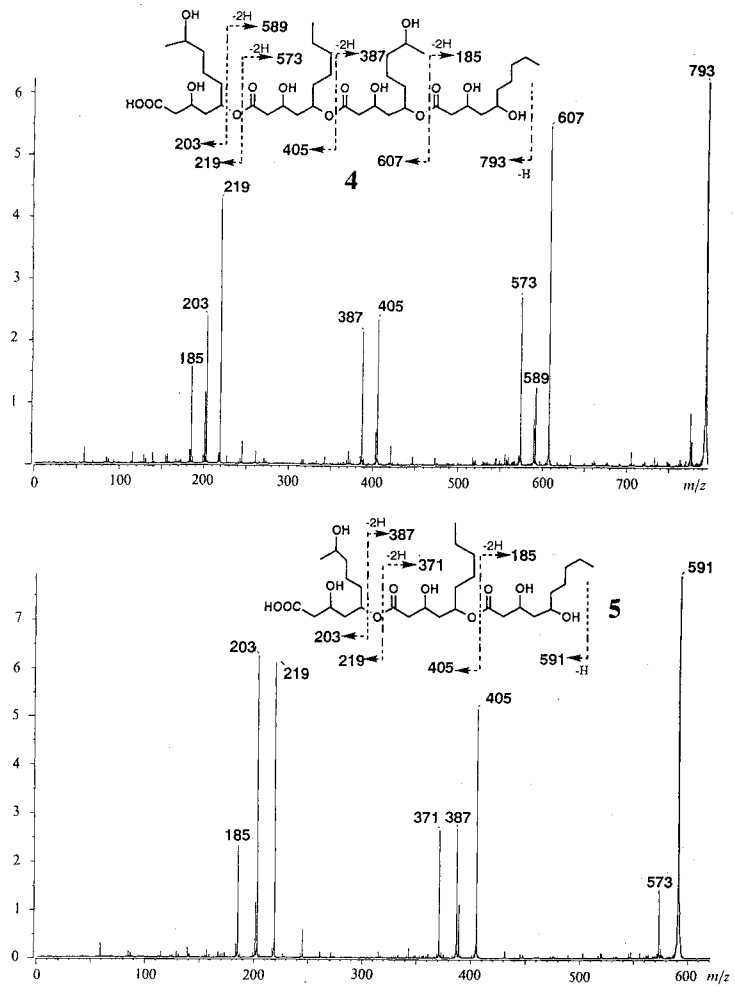
Structure of Halymecin C (**3**)

Halymecin C (**3**) was also obtained as a colorless oil. Negative FAB-MS of halymecin C (**3**) gave a pseudo-molecular ion at m/z 617 $[M-H]^-$. The molecular formula was determined to be $C_{32}H_{58}O_{11}$ by HR negative FAB-MS (Found 617.3901, Calcd. 617.3901). The comparison of this molecular formula with **1** indicated that **3** lacked one 3,5-dihydroxydecanoate moiety, so that **3** was a trimer of 3,5-dihydroxydecanoic acid. The combination of TOCSY, HMBC and HMQC spectra permitted assignment of the chemical shifts of most of the protons, and the position of the acetyl group was determined in the same way as for **1**. Thus the structure of **3** was determined as shown in Fig. 1.

Structure Elucidation of Halymecin D (**4**) and E (**5**)

Compounds from the antimicroalgal fraction purified from strain FK-N30 broth appeared to have similar structures to halymecin A without having an acetyl group by ^{1}H NMR analysis. Since negative FAB-MS with

Fig. 7. MS/MS of halymecin D (**4**) and E (**5**).



nitrobenzyl alcohol gave ions at *m/z* 793 $[M-H]^-$ and 591 $[M-H]^-$, this fraction seemed to contain the mixture of approximately equimolar amounts of the two compounds although they appeared to be a single peak by HPLC. These two compounds were designated as halymecin D and halymecin E. Halymecin D, corresponding to an ion at *m/z* 793 $[M-H]^-$, was determined to have the molecular formula $C_{40}H_{74}O_{15}$ by positive HR-MS ($[M+Na]^+$, Found 817.4895, Calcd. 817.4925). The number of carbons corresponded to four decanoate units, while two extra oxygens were accounted for by assuming that halymecin D contains trihydroxydecanoate units as well as dihydroxydecanoate units. Negative MS/MS on 793 showed fragmented ions derived from the cleavage of ester linkages on all four units, providing us with a structure composed of two dihydroxydecanoates and two trihydroxydecanoates (Fig. 7). The COSY spectrum showed the connectivities from C-2 to C-6 containing C-3 (4.07, 4.20) and C-5 (δ 5.02, 3.76) protons. The methine proton (H-9, δ 3.71) is bound to oxygen and is not involved with this connectivity. It is coupled to terminal methyl groups (δ 1.12, 1.13), so that the hydroxy groups on trihydroxydecanoate were assigned to positions 3, 5 and 9. Thus the structure of **4** was determined as shown in Fig. 1.

Halymecin E (**5**), $[M-H]^-$ *m/z* 591, was determined to have the molecular formula $C_{30}H_{56}O_{11}$ by positive HR-MS ($[M+Na]^+$, Found 615.3726, Calcd. 615.3720), corresponding to two dihydroxydecanoate and one trihydroxydecanoate units. Negative MS/MS on 591 showed fragmentation derived from the cleavage of ester linkages in the same manner as **4** (Fig. 7). Thus the structure of **5** was determined as shown in Fig. 1.

Bioactivity of Halymecin A (**1**)

Antimicroalgal, antibacterial and cytotoxic activities of halymecin A are listed in Table 2. Halymecin A (**1**) showed strong antimicroalgal activity against *Skeletonema costatum* and was also active against *Brachiomonas submarina* and *Prorocentrum micans* and weakly against

Table 2. Bioactivity of halymecin A (**1**).

Antimicrobial activity	MIC (μg/ml)
Staphylococcus aureus	>83
Enterococcus faecium	10
Bacillus subtilis	>83
Klebsiella pneumoniae	10
Escherichia coli	>83
Pseudomonas aeruginosa	>83
Salmonella typhimurium	>83
Proteus vulgaris	10
Shigella sonnei	>83
Candida albicans	>83
Antimicroalgal activity	**MIC (μg/ml)**
Skeletonema costatum	4
Oscillatoria amphibia	500
Brachiomonas submarina	68
Prorocentrum micans	68
Cytotoxicity	**IC_{50} (μg/ml)**
HeLa S3	6
BALB3T3	7

Oscillatoria amphibia. Compound **1** was scarcely active against Gram positive and negative bacteria. The cytotoxicity of **1** was very weak. These characteristics of bioactivity implied the possibility of halymecins acting as antimicroalgal agents.

Experimental

General

Spectral data were recorded on the following instruments: ^1H and ^{13}C NMR, Varian Unity 500; FAB-MS, JEOL JMS-SX102. IR spectra were obtained from a KBr pellet with a JASCO FT-IR 7000 spectrophotometer. Optical rotations were determined on a Horiba SEPA-300 polarimeter at ambient temperature. HPLC separation was performed on a TOSOH CCPM system with a tunable absorbency detector UV 8010 (λ=215 nm). Centrifugal Partition Chromatography (CPC, Model LLB-M, Sanki Laboratories, Inc.) was performed using a TOSOH CCPD system. Merck silica gel 60 (230~400 mesh) was used for column chromatography.

Microorganisms

The fungus producing halymecins A, B, C, strain FE-71-1, was isolated from an algae (*Halymenia dilatata*) from the Palau Islands. Another fungus producing halymecins D, E, strain FK-N30, was isolated from algae (unidentified) at Aburatsubo (Kanagawa, Japan).

Culture and Medium Conditions

Strain FE-71-1

An agar slant of the strain FE-71-1 was inoculated into a 100-ml flask containing 20 ml of fermentation medium consisting of 50% sea water, 1% glucose, 0.5% yeast extract and calcium carbonate (pH 8.0 before sterilization). The flask was incubated at 30°C for 4 days on a reciprocal shaker. The whole portion of this culture was divided and transferred to fifteen 1-liter Erlenmeyer flasks containing 400 ml of the same medium as above. The fermentation was carried out at 30°C for 4 days on a reciprocal shaker.

Strain FK-N30

Fermentation for strain FK-N30 was also performed according to the same procedure for strain FE-71-1 except with a fermentation medium consisting of 50% sea water, 1.0% kelp powder, 1.0% dried bonito powder, 1.0% glucose and 0.5% calcium carbonate (pH 8.0 before sterilization).

Taxonomic Studies

Strain FE-71-1

1) Morphology

Physiological properties were examined with an optical or scanning electron microscope. The culture was prepared with YM broth at 30°C. The hyphae possessed septa, however teleomorphs were not observed. Conidiospores bore a whorl of phialide. Macrocondia, canoe shaped and 4 or 5-celled were formed on sporodochia. Microcondia, ovoid or oblong shaped were formed on sporodochia (phialosphere) which branched from aerial mycelium. Thick-walled chlamydospores were formed by hyphae or macrocondia. Thus this strain possessed anamorphs as explained above. However teleomorphs were not observed.

2) Cultural properties

Aerial mycelia were cotton-like, white and turned to light red with prolonged cultivation. Yellow, reddish yellow, purple or grayish green pigments appeared on different media. The strain could grow in the range of 14 to 33°C and the optimal temperature was 29 to 33°C in YM broth. The strain could grow at the range of pH 3 to 11 in the broth, and the optimal pH was at 4 to 11 with best growth around pH 9. On the basis of these results strain FE-71-1 was identified as *Fusarium* sp. This strain was deposited at the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba-shi, Japan with the accession number FERM P-13910.

Strain FK-N30

1) Morphology

Physiological properties were examined with an optical or scanning transfer electron microscope. The culture was prepared with a YM agar plate at 30°C. Hyphae possessed smooth and well branched septa. Conidia were formed by phialides which were pleurogenetic or acrogenetic on the hyphae. The phialides were borne singly from hyphae, colorless, smooth and the heads

became narrower gradually. The width of phialide is 1~3 μm at largest, while the length is 3~22 μm. The phialides possessed septa at the ends. From the heads of phialides, phialidic and enteroblastoconidium were formed, as well as small myxospore of conidia that were often observed around there. The conidia, single celled, smooth, and ovoid or oblong, was 2.2~9.8 μm in length and 1~3.5 μm wide.

2) Cultural properties

In a YM agar plate at 30°C, the diameter of the colony reached 30 mm after 7 days. The surface of the colony was white and turned to salmon pink. Wrinkles spread radially from the center of the mycelium. The strain could grow in the range of 4 to 38°C and the optimal temperature was 25 to 30°C in YM broth. The strain could grow in the range of pH 3 to 11 in the broth, and the optimal pH was at 3 to 6. On the basis of these results strain FK-N30 was identified as *Acremonium* sp.

Antimicroalgal Activity

Bluegreen algae *Oscillatoria amphibia* (O.a.), Diatom *Skeletonema costatum* (S.c.), Green algae *Brachiomonas submarina* (Br.s.) and Dinoflagellate *Prorocentrum micans* (P.m.) were used for the antimicroalgal activity assay. Microalgae were cultured for one week at 2000 lux (dark and light were alternated every 12 hours), and inoculated with the f/2 medium consisting of 99.9% sea water with 0.1% f/2 metals (shown in Table 3). This medium was sterilized at 121°C for 15 minutes. The inoculation was performed under sterile conditions. Thirty ml of culture for P.m., and 20 ml for the rest of microalgae, were diluted with 10 ml of sterilized F/2 medium, adjusted for the assay culture. Ten μl of the test samples were applied to a 96 well microplate, air-dried and 200 μl of these assay culture were inoculated at 25°C under 2000 lux light (12 hour intervals). Ten μg/ml and 500 μg/ml of dicyclodimethylurea was used for the positive control. The activities against P.m. or Br.s. were examined using a light microscope, while O.a. or S.c. were examined by direct visual observation.

Table 3. Composition of f/2 medium.

$NaNO_3$	75 mg
$NaH_2PO_4 \cdot 2H_2O$	6 mg
Vitamin B_{12}	0.5 mg
Biotin	0.5 mg
Thiamine·HCl	0.1 mg
$Na_2SiO_3 \cdot 9H_2O$	10 mg
f/2 metals*	1 ml
Sea water	999 ml
*f/2 metals	
$Na_2EDTA \cdot 2H_2O$	4.4 g
$FeCl_3 \cdot 6H_2O$	3.2 g
$CoSO_4 \cdot 7H_2O$	12 mg
$ZnSO_4 \cdot 7H_2O$	21 mg
$MnCl_2 \cdot 4H_2O$	180 mg
$CuSO_4 \cdot 5H_2O$	7 mg
$Na_2MoO_4 \cdot 2H_2O$	7 mg
Dist. water	1 liter

Isolation of Halymecins A (1), B (2) and C (3)

The harvested culture (6 liters) was centrifuged at 30°C for 30 minutes at 3,500 rpm. The supernatant fluid was extracted with an equal volume of EtOAc. The cells were extracted with 100 ml of ethyl alcohol, filtered, condensed and extracted with EtOAc. These two EtOAc extracts were combined and evaporated to dryness. The extract was fractionated by using high speed centrifugal countercurrent chromatography (CPC) with a solvent system containing $CHCl_3$ - MeOH - H_2O = 2 : 2 : 1 (upper layer as the stationary phase). The resulting active fractions were further separated using HPLC on a reverse phase Lichrosphere RP-18 column (10 × 250 mm, Merck) with 50% H_2O - MeOH to yield halymecins A (**1**, 80 mg), B (**2**, 11 mg) and C (**3**, 5 mg).

Halymecin A (**1**): Colorless oil; $[\alpha]_D^{26}$ −4.27° (*c* 1.5, CH_2Cl_2); IR (KBr) cm^{-1} 3420, 2932, 2860, 1734, 1655, 1460, 1379, 1170, 1091; ^1H and ^{13}C NMR, see Table 1.

Halymecin B (**2**): Colorless oil; $[\alpha]_D^{26}$ −24.4° (*c* 6.6, CH_2Cl_2); IR (KBr) cm^{-1} 3412, 2932, 2864, 1734, 1576, 1379, 1238, 1172, 1073; ^1H and ^{13}C NMR, see Table 1.

Halymecin C (**3**): Colorless oil; $[\alpha]_D^{26}$ −9.2° (*c* 3.2, CH_2Cl_2); IR (KBr) cm^{-1} 3444, 2934, 2864, 1734, 1377, 1172, 1054; ^1H and ^{13}C NMR, see Table 1.

Preparation of Halymecin A Tetraacetate (1a)

Compound **1** (5 mg) was dissolved in pyridine (1 ml) and 2 ml of acetic anhydride was added. The reaction mixture was kept at room temperature for 30 minutes. and worked up in the usual manner. The residue was purified by Lichrocurt Si (500 mg, Merck) column chromatography (chloroform - MeOH = 15 : 1) to afford 3.8 mg of tetraacetate (**1a**) as a colorless oil: $[\alpha]_D^{26}$ +7.8° (*c* 0.3, CH_2Cl_2); FAB-MS *m/z* 1001 $[M+K]^+$, 995 $[M+Na]^+$, 973 $[M+H]^+$, 913 $[M-AcOH]^+$, 685, 457, 229; IR (KBr) cm^{-1} 3448, 2998, 2858, 1744, 1462, 1379, 1243, 1033; ^1H NMR ($CDCl_3$, δ_H) 0.86 (12H), 1.26 (12H), 1.57 (8H), 2.01 (3H), 2.62 (8H), 4.92 (4H), 5.23 (4H); ^{13}C NMR ($CDCl_3$, δ_C) 14.0, 21.0, 21.1, 21.2, 22.5, 24.8, 31.6, 34.0, 34.1, 37.5, 37.9, 38.0, 39.0, 67.7, 67.9, 68.0, 71.0, 71.4, 71.5, 71.6, 169.6, 170.1, 170.2, 170.3, 170.7.

Acid Hydrolysis of 1

Compound **1** (5 mg) in 3 ml of a 0.1 N HCl - CH_3CN (1 : 1) mixture was heated at 80°C for 1 hour. The reaction mixture was concentrated and extracted with ethyl acetate. The extracts were concentrated and purified Lichrocurt Si (500 mg, Merck) column chromatography (chloroform - MeOH = 15 : 1) to afford 3.2 mg of **1b**: $[\alpha]_D^{26}$ +32.3° (*c* 0.03, CH_2Cl_2); ^1H NMR ($CDCl_3$, δ_H) 1.76, 1.95, 2.10, 2.61, 2.72, 4.67; ^{13}C NMR ($CDCl_3$, δ_C) 36.0, 38.2, 62.3, 75.9.

Acid Hydrolysis of 2

A solution of **2** (5 mg) in 3 ml of a 0.5 N HCl was heated at 90°C for 2 hours. The reaction mixture was

concentrated and extracted with ethyl acetate. The ethyl acetate layer was discarded and the water layer was subjected to the following HPLC analysis:

Column: TSK GEL Amide-80 (4.6 mm × 25 cm).
Solvent: 80% acetonitrile, flow rate 0.8 ml/minute.
Post labeling conditions:
reagent: 100 mM benzamidine, 0.5 N-KOH;
temp.: 110°C; reaction coil 0.4 mm × 10 m;
flow rate: 0.6 ml/minute.
Detection: fluorescence: 287 nm ex, 470 nm em.

Methanolysis of **2**

Compound **2** (10 mg) was treated with 2 ml of 0.1 M HCl-MeOH and heated at 80°C for 1 hour. The reaction mixture was concentrated and extracted with ethyl acetate. The ethyl acetate layer was discarded and water layer was concentrated. The residue was purified on a Lichrocurt RP-18 (100 mg, Merck) column (H_2O-MeOH = 10 : 90) to afford methyl α-D-mannopyranoside: $[\alpha]_D^{26}$ +80.9° (*c* 0.2, H_2O); reported: $[\alpha]_D^{26}$ +79.5°[6]

Isolation of a Fraction Containing Halymecins D (**4**), E (**5**)

The harvested culture (6 liters) was centrifuged at 30°C for 30 minutes at 3,500 rpm. The supernatant fluid was extracted with an equal volume of EtOAc. The extract was fractionated by using high speed CPC with a solvent system containing 1 : 1 hexane - 90% EtOH (lower layer as stationary phase). The active fraction was again fractioned by CPC with $CHCl_3$ - MeOH - H_2O = 2 : 2 : 1 (upper layer as the stationary phase). The resulting active fractions were further separated using HPLC on a reverse phase Lichrosphere RP-18 column (10 × 250 mm, Merck) with 60% H_2O - MeOH to yield a single peak (9 mg), containing two novel compounds, designated as halymecins D (**4**) and E (**5**): ^{1}H NMR ($CDCl_3$ - CD_3OD = 1 : 1): 0.86 (12H), 1.12 (6H), 1.13 (3H), 1.22 ~ 1.84 (64H), 2.24 ~ 2.65 (14H), 3.71 (3H), 3.76 (2H), 4.07 (4H), 4.20 (3H), 5.02 (5H).

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References

1) FAULKNER, D. J.: Marine natural products. Nat. Prod. Rep. 2: 75 ~ 125, 1995
2) IMAMURA, N.; M. NISHIJIMA, K. ADACHI & H. SANO: Novel antimycin antibiotics, Urauchimycins A and B, produced by marine actinomycete. J. Antibiotics 46: 241 ~ 246, 1993
3) IMAMURA, N.; K. ADACHI & H. SANO: Magnesidin A, a component of marine antibiotic magnesidin, produced by *Vivrio gazogenes* ATCC29988. J. Antibiotics 47: 257 ~ 261, 1994
4) ROMEYKE, Y.; M. KELLER, H. KLUGE, S. GRABLEY & P. HAMMANN: Secondary metabolites by chemical screening. 13. Enantioselective synthesis of δ-lactones from streptenol A, a chiral building block from streptomyces. Tetrahedron 47: 3335 ~ 3346, 1991
5) KAI, M.; K. TAMURA, M. YAMAGUCHI & Y. OHKURA: Aromatic amidines as fluorogenic reagents for reducing carbohydrates. Anal. Sci. 1: 59 ~ 63, 1985
6) HANNAFORD, A. J.: Optical rotation of some carbohydrate derivatives in p-dioxane-water mixtures. Carbohydrate Research 3: 295 ~ 299, 1967