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ORIGINAL ARTICLE

The novel anti-*Propionibacterium acnes* compound, Sargafuran, found in the marine brown alga *Sargassum macrocarpum*

Yuto Kamei[1], Miyuki Sueyoshi[1], Ken-ichiro Hayashi[2], Ryuta Terada[3] and Hiroshi Nozaki[2]

We screened extracts of 342 species of marine algae collected from Japanese coastlines for antibacterial activity against *Propionibacterium acnes*, and found a novel antibacterial compound, which we named Sargafuran, from the MeOH extract of the marine brown alga, *Sargassum macrocarpum*. Sargafuran has low cytotoxicity, and the MIC against *P. acnes* was 15 μg ml^{-1}, showing a broad antibacterial activity against Gram-positive bacteria. A time-kill study showed that Sargafuran was bactericidal and completely killed *P. acnes* at 4×MIC by lysing bacterial cells. These results suggest that Sargafuran might be useful as a lead compound to develop new types of anti-*P. acnes* substances and new skin care cosmetics to prevent or improve acne.
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INTRODUCTION

Acne vulgaris is a common skin disease, affecting about 70–80% of adolescents and young adults. It is a multifactorial disease of the pilosebaceous unit.[1] *Propionibacterium acnes*, a common skin organism, is most often recognized in acne vulgaris and produces a number of virulence factors. Clindamycin and erythromycin are most commonly used as topical antibiotics against *P. acnes*.[2]

In our laboratory, we screened the biological activities of marine algae collected from the Japanese coastline and found several bioactive compounds,[3–6] including an antibacterial compound.[7] Other reports show that some distinct seaweeds contain antimicrobial substances against both Gram-positive and -negative bacteria.[8,9] Thus, marine algae are a promising bioresource to find new antibacterial compounds against *P. acnes* and to develop new natural cosmetic products to prevent acne. In this study, we screened a total of 342 species of marine algae, collected from the Japanese coastline, for activity against *P. acnes,* and found a novel anti-*P. acnes* compound from one of these algae.

MATERIALS AND METHODS

Bacterial strains and media

P. acnes (ATCC 11827) was used for the screening of antibacterial activity of marine algal extracts. This test strain was cultured with Reinforced Clostridial Medium (Sigma-Aldrich, Tokyo, Japan). Bacteria used for the antibacterial spectrum assay were methicillin-resistant *Staphylococcus aureus* (ATCC 33591), methicillin-sensitive *S. aureus* (ATCC 25923), *Bacillus subtilis* (IFO 14419), *Escherichia coli* (NBRC 12734), *Enterococcus faecium* (NBRC 3826), *Enterococcus faecalis* (NBRC 3971), *Enterococcus serolicida* (NG 8206), *Streptococcus mutans* (NBRC 13955), *Streptococcus pneumoniae* (GTC 261), *Streptococcus pyogenes* (GTC 262), *Pseudomonas aeruginosa* (IFO 13736) and *Vibrio alginolyticus* (V7) as well as two strains of *P. acnes* (ATCC 1187 and ATCC 25746). These strains were cultured with Tryptic Soy Agar medium (Difco Laboratories, Detroit, MI, USA), except for *P. acnes* and *V. alginolyticus* which was cultured in ZoBell 2216E agar medium.[10]

Marine algae collection and preparation of extracts

During the period from April 1994 to August 2003, marine algae samples were collected at 96 points from north to south along the Japanese coastline and were stored at −20 °C until needed. The marine algae extracts were prepared as described in our earlier paper.[11]

Screening assay for anti-*P. acnes* activity

Sterilized paper disks (φ8 mm; Advantec, Tokyo, Japan) permeated with 50 μl each of phosphate-buffered saline or MeOH algal extract and dried completely were placed on double-layer agar plates inoculated with 5.0×10^{6} CFU ml^{-1} of *P. acnes* strain. These plates were incubated at 37 °C for 2 days under anaerobic condition. After incubation, the zones of inhibition were measured and recorded.

Isolation and purification of Sargafuran

One liter of the MeOH extract from the marine brown alga *Sargassum macrocarpum* (250 g wet weight) was partitioned with a chloroform/water (1:1) mixture. The chloroform/MeOH fraction was concentrated to dryness and redissolved in *n*-hexane/acetone (6:1), and then subjected to a silica gel-60 (Merck, Darmstadt, Germany) column chromatography under continuous elution with *n*-hexane/acetone (6:1, 5:1 and 4:1) and MeOH. The active fractions were pooled and concentrated, and then further chromatographed

[1]Coastal Bioenvironment Center, Saga University, Karatsu, Saga, Japan; [2]Department of Biochemistry, Okayama University of Science, Okayama, Japan and [3]Department of Fisheries Biology and Oceanography, Faculty of Fisheries, Kagoshima University, Shimoarata, Kagoshima, Japan
Correspondence: Dr Y Kamei, Coastal Bioenvironment Center, Saga University, 152-1 Shonan-cho, Karatsu, Saga 847-0021, Japan.
E-mail: kameiy@cc.saga-u.ac.jp
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on a second silica gel column, eluting with *n*-hexane/acetyl ethyl ester (10:1, 6:1 and 3:1) and MeOH, and on a reverse-phase Cosmosil (Nacalai Tesque Inc., Kyoto, Japan) column, eluting with 60–80% MeOH containing 0.1% trifluoroacetic acid (Sigma-Aldrich). The active fractions were finally purified with an HPLC on a reversed-phase column (Mightysil RP-8 GP, ∅ 4.6×250 mm; Kanto Chemical Co. Inc., Tokyo, Japan), eluting with a gradient of acetonitrile and water containing 0.1% trifluoroacetic acid.

Spectrometric analyses of Sargafuran

Optical rotations were measured with an SEPT-200 polarimeter (Horiba, Tokyo, Japan). UV spectra were recorded on a U-3210 spectrophotometer (Hitachi, Tokyo, Japan) and IR spectra on a model 1720 spectrometer (Perkin-Elmer, Waltham, MA, USA). NMR spectra were recorded in $CDCl_3$ on a JEOL lambda 500 NMR spectrometer (JEOL, Tokyo, Japan). Chemical shifts are shown as δ values from TMS as the internal reference. Peak multiplicities are quoted in Hz. Mass spectra were measured on a JMS-700 spectrometer (JEOL).

Antibacterial test

The MICs of Sargafuran were determined by the standard microdilution method described by the National Committee for Clinical Laboratory Standards,[12] using a Tryptic Soy Broth medium (Difco Laboratories), except for *P. acnes* and *V. alginolyticus* which was in ZoBell 2216E broth medium. The final volume of Tryptic Soy Broth, Reinforced Clostridial Medium or ZoBell broth medium containing Sargafuran was 100 μl per well to give a starting inoculum density of 5×10^5 CFU ml^{-1}.

Time-kill curve experiment

The time-kill experiment was conducted by the method described by Aeschlimann and Rybak[13] and Etenza *et al.*[14] The experiments were conducted in test tubes containing 15 ml of fresh Reinforced Clostridial Medium inoculated with an overnight culture of *P. acnes* (ATCC 11827) to give an initial bacterial density of 10^6 cells per ml. The inoculation was carried out immediately after the addition of Sargafuran or Clindamycin (Sigma-Aldrich) at the final concentrations of MIC, 2×MIC and 4×MIC and incubated at 37 °C for 2 days under anaerobic condition.

Bacteriolytic assay

The seed culture of *P. acnes* (ATCC 11827) on an RCA plate was resuspended in Reinforced Clostridial Medium and washed twice with sterile 10 mM Tris-HCl buffer (pH 7.6). The absorbance was adjusted to 0.1 at 660 nm. The bacterial cell suspension (5 ml each) was aliquoted in sterile test tubes and exposed to Sargafuran at various concentrations or to Achromopeptidase (Wako Pure Chemical Industries Ltd, Tokyo, Japan) at 134 μg ml^{-1} as a positive control. Untreated bacterial suspensions were used as negative controls. The concentration of MeOH (the solvent of Sargafuran) in each tube was less than 0.1% (v/v). The test tubes were incubated at 37 °C under anaerobic conditions. The absorbance at 660 nm was measured at 0, 0.15, 0.5, 0.45, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 h after incubation, and the relative absorbance was calculated by dividing each absorbance by that of the negative control. Each treatment was conducted in duplicate.

Cytotoxicity test

The cytotoxic activity of Sargafuran was evaluated as described earlier.[15] Human normal dermal fibroblasts (Morinaga Institute of Biological Science, Yokohama, Japan) were prepared at 5×10^3 cells per 100 μl per well in a 96-well plate. Then Sargafuran, serially diluted twofold, was added to give final concentrations ranging from 3.8 to 480 μg ml^{-1}. The cells were incubated at 37 °C for 3 days. The proliferation of cells was evaluated by an 3-[4,5-dimethylthiazol-2-ly]-2,5-diphenyltetrazolium bromide (MTT) assay[16] and the growth rate relative to the control treatment was calculated.

RESULTS

Marine algae with anti-*P. acnes* activity

We found 13 species of marine algae with anti-*P. acnes* activity, based on disk diffusion assays, using the extracts from a total of 342 species of marine algae collected from the Japanese coastline. The algae are *Laurencia brongniartii, Laurencia okamurae, Osonthalia corymbifera, Rhodomela teres, Dictyopteris divaricata, Dictyopteris undulata, Ishige okamurai, Padina crassa, Sargassum fulvellum, S. macrocarpum, Sargassum siliquastrum, Sargassum yezoense* and *Zonaria diesingiana*, showing approximate MIC ranges of 62.5–1000 μg per disk against *P. acnes* (Table 1). Most of the positive marine algae were brown algae and showed a relatively high anti-*P. acnes* activity at 62.5–250 μg per disk.

Purification of the antibacterial compound, Sargafuran, from *S. macrocarpum*

As described above, several marine algae showed promising anti-*P. acnes* activity. One of those positive algae, *S. macrocarpum,* is widely distributed throughout the Japanese coastline and can be collected in large amounts during any season. Thus, we selected this algal species to proceed with the isolation and purification of the antibacterial compound against *P. acnes* from the MeOH extract. After several purification steps, starting from 250 g wet weight of *S. macrocarpum,* 3.6 mg of the antibacterial compound, which we named Sargafuran, was obtained.

Structure determination of Sargafuran

Sargafuran was isolated as a colorless oil [UV λ_{max} ($CHCl_3$) nm (log ε): 241 (4.27), 263 (3.79); $[\alpha]_D$–1.26°($CHCl_3$, *c* 0.95)]. The molecular formula was determined to be $C_{27}H_{36}O_4$ (found 424.2593, calcd. 424.2614) by high resolution electron impact mass spectrometry (HR-EI-MS). The IR spectrum showed absorption bands at 1686 and 3400 cm^{-1} attributable to a carbonyl and hydroxyl group, respectively. ^1H- and ^{13}C-NMR spectroscopic data indicate that Sargafuran has five singlet methyl groups (C-18, C-19, C-21, C-26 and C-27), seven olefinic bonds, six aliphatic methylenes, a carbonyl group and a quaternary carbon attached to oxygen. Taken together with the presence of seven olefinic bonds and a carbonyl group, 10 units of unsaturation in Sargafuran indicate that the compound has two rings in its structure. ^1H-^1H COSY spectrum revealed the linkages among the methylenes, and olefinic protons (H-6 to H-8, H-10 to H-12 and H-14 to H-16), in addition to two sets of

Table 1 MeOH extracts from marine algae exhibited potent anti-*Propionibacterium acnes* activity

Marine algae	*Anti-P. acnes activity*[a]
Red algae	+++
Laurencia brongniartii	+++
Laurencia okamurae	++
Odonthalia corymbifera	+++
Rhodomela teres	
Brown algae	
Dictyopteris divaricata	++
Dictyopteris undulata	++
Ishige okamurai	++
Padina crassa	+++
Sargassum fulvellum	++
Sargassum macrocarpum	++
Sargassum siliquastrum	++
Sargassum yezoense	++
Zonaria diesingiana	+

[a]+++, ⩾19-mm inhibition zone; ++, 12- to 18-mm inhibition zone; +, ⩽11-mm inhibition zone; anti-*P. acnes* activities of the MeOH extracts were evaluated by the disk diffusion method.

connection between olefinic protons (H-2/H-3 and H-23/H-24). The connections in the triene chain (C-6 to C-21) were established from the HMBC correlations (H-8/C-10, 19, H-19/C-9, 10, H-10/C-9, H-11/C-9, 13, H-12/C-14, 20, H-14/C-13, 20, H-18/C-16, 17 and H-21/C-16, 17) among the olefinic methines (H-8, H-12 and H-16), methyl groups (H-18, -19 and -21) and a carbonyl carbon (C-20). The geometry of olefinic bonds was determined to be *E* (C-8/C-9) and *Z* (C-12/C-13), respectively, by NOESY correlations (H-7/H-19, H-8/H-10 and H-12/H-14). The 2-methyl furan moiety (C-22 to C-26) was revealed by the HMBC correlations (H-23/C-22, 25, H-24/C-22, 25 and H-26/C-24, 25) and the small J_{H-H} coupling value between H-23 and H-24 (J=3 Hz). Additional HMBC correlations (H-2/C-1, -4, H-3/C-1, -4 and H-27/C-1, -2, -5) indicated the presence of 1-methyl-cyclopenta-2,4-dienol moiety (C-1 to C5, and C-27). The remaining carbonyl carbon (C-20) was attributed to a carboxylic acid. The cyclopentadiene moiety was connected with C-6 of the triene chain at C-5 and with C-22 of methylfuran at C-4 on the basis of HMBC correlations (H-7/C-5, H-6/C-1, H-3/C-22 and H-23/C-4), and this was further supported by a strong fragment ion at *m/z* 175 (100%) in the EI-MS spectrum. Thus, the structure of Sargafuran was determined as shown in Figure 1. The assignments of ^{1}H- and ^{13}C-NMR signals are indicated in Table 2.

Minimum inhibitory concentration

Sargafuran showed antibacterial activity against *P. acnes* at 15 μg ml^{-1}. At the same concentration, Sargafuran also showed antibacterial activity against other Gram-positive bacteria, *S. pyogenes* and *S. pneumoniae,* and a Gram-negative bacterium, *V. alginolyticus* (Table 3). However, this compound did not inhibit the bacterial growth of the Gram-negative bacteria, *E. coli* and *P. aeruginosa*, and another Gram-positive bacterium, *S. mutans,* even at a concentration of 120 μg ml^{-1}.

Bactericidal activity of Sargafuran

The time-kill study showed that Sargafuran decreased the bacterial counts of *P. acnes* at 1/2×MIC (7.5 μg ml^{-1}) during a 16-h exposure (Figure 2). At MIC (15 μg ml^{-1}), Sargafuran completely killed the *P. acnes* strain tested within 4 h. However, Clindamycin did not show bactericidal activity against the *P. acnes* strain tested even after 72 h of incubation at 4×MIC (0.1 μg ml^{-1}). This bactericidal property of Sargafuran seems to be very promising for developing new types of antibiotics against the multi-drug-resistant *P. acnes* strains.

Bacteriolytic activity

The reduction in the absorbance of *P. acnes* cell suspensions was not observed in the presence of Sargafuran, at up to 4×MIC (60 μg ml^{-1}), until the end of the incubation period (Figure 3). In contrast, the absorbance of *P. acnes* cell suspensions treated with Achromopeptidase at 134 μg ml^{-1} as the positive control was reduced drastically in the early incubation period. These results indicated that Sargafuran did not lyse *P. acnes* cells.

Cytotoxicity

An MTT assay showed that Sargafuran was not cytotoxic to human normal dermal fibroblast cells (Figure 4). At concentrations up to

Table 2 ^{1}H- and ^{13}C-NMR data of Sargafuran

No.	*^{1}H*	*^{13}C*
1	2.13 (3H, *s*)	15.5 (CH_3)
2		144.9 (C)
3	6.47 (1H, *d*, 3.0)	117.1 (CH)
4	6.32 (1H, *d*, 3.0)	110.3 (CH)
5		148.5 (C)
6		122.9 (C)
7	6.24 (1H, *d*, 10.0)	121.3 (CH)
8	5.57 (1H, *d*, 10.0)	130.7 (CH)
9		77.8 (C)
10		126.4 (C)
11	1.67 (2H, *m*)	40.7 (CH_2)
12	2.11 (2H, *m*)	22.6 (CH_2)
13	5.13 (1H, *dd*, 6.0, 7.3)	124.9 (CH)
14		134.4 (C)
15	2.06 (2H, *t*, 7.5)	39.1 (CH_2)
16	2.58 (2H, *m*)	28.1 (CH_2)
17	5.99 (1H, *dd*, 7.5, 7.0 Hz)	145.7 (CH)
18		130.5 (C)
19	2.26 (2H, *t*, 7.5)	34.5 (CH_2)
20	2.11 (2H, *m*)	27.9 (CH_2)
21	5.09 (1H, *t*, 7.0)	123.0 (CH)
22		132.3 (C)
23	1.67 (3H, *s*)	25.7 (CH_3)
24	1.35 (3H, *s*)	25.9 (CH_3)
25	1.57 (3H, *s*)	15.8 (CH_3)
26		173.3 (C)
27	1.58 (3H, *s*)	17.7 (CH_3)

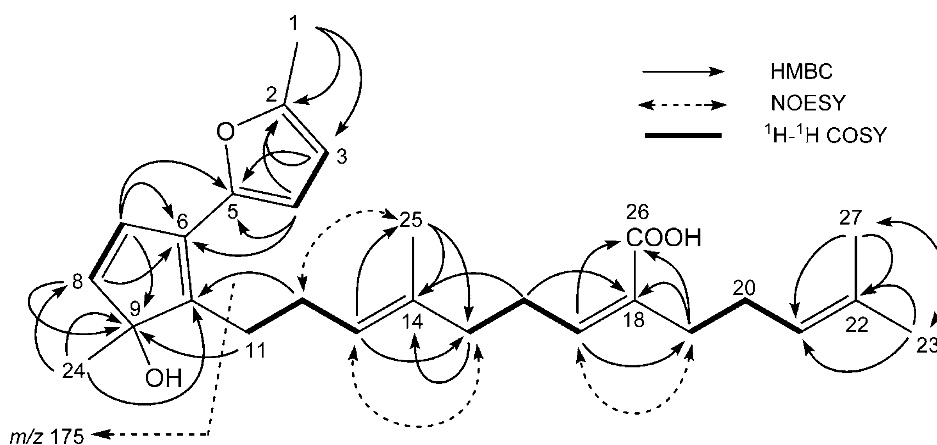

Figure 1 2D NMR correlations and mass fragmentation of Sargafuran.

Table 3 Comparative antibacterial activities of Sargafuran and Clindamycin

	MIC (μg ml^{-1})	
Bacterial strain	Sargafuran	Clindamycin
Propionibacterium acnes (ATCC 11827)	15	0.03
P. acnes (ATCC 25746)	15	0.67
MSSA (ATCC 25923)	30	0.47
MRSA (ATCC 33951)	30	>120
Bacillus subtilis (IFO 14419)	30	1.88
Escherichia coli (NBRC 12734)	>120	>120
Enterococcus faecalis (NBRC 3971)	30	>120
Enterococcus faecium (NBRC 3826)	60	0.90
Enterococcus serolicida (NG 8206)	60	1.88
Streptococcus pyogenes (GTC 262)	15	7.50
Streptococcus pneumoniae (GTC 261)	15	7.50
Streptococcus mutans (NBRC 13955)	>120	3.75
Pseudomonas aeruginosa (IFO 13736)	>120	>120
Vibrio alginolyticus (V-7)	15	30

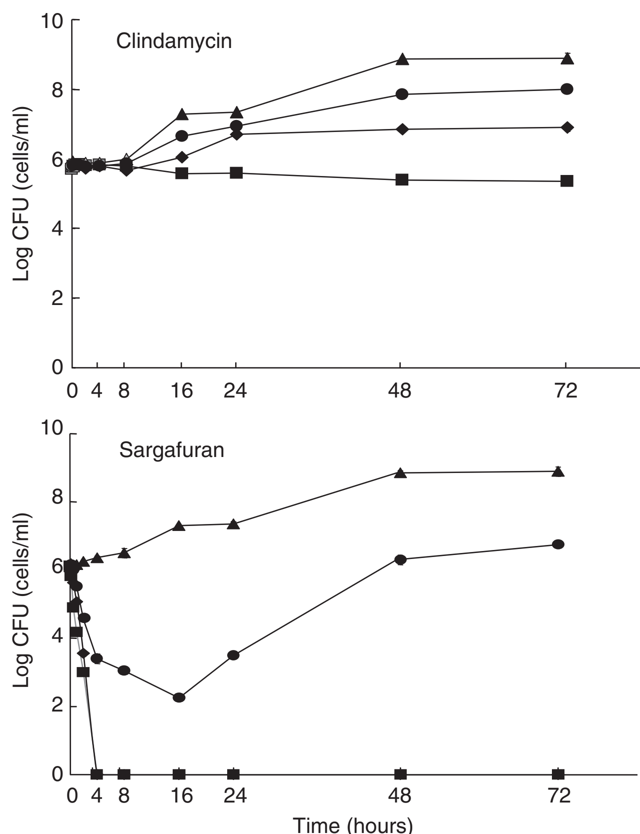

Figure 2 Comparative bactericidal activities of Sargafuran and vancomycin against *P. acnes* (ATCC 11827). ▲, Negative control (antibacterial substance not added); ●, 1/2×MIC; ◆, 1×MIC; ■, 4×MIC. The values with the standard error bars are mean values from duplicate experiments.

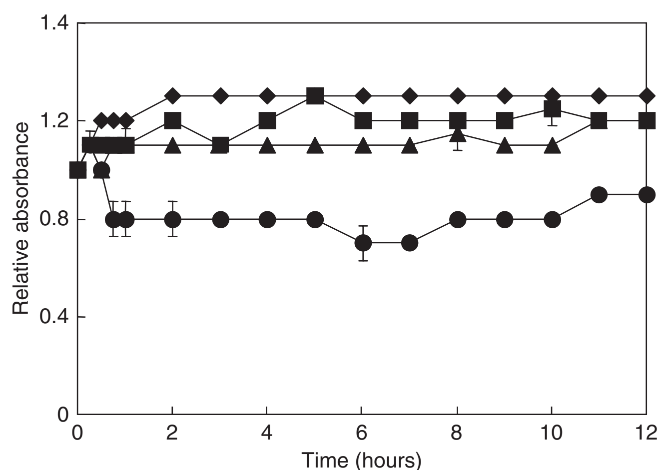

Figure 3 Bacteriolytic activity of Sargafuran against *P. acnes* (ATCC 11827). ●, Positive control (134 μg ml^{-1}) Achromopeptidase; ◆, 1×MIC; ■, 2×MIC; ▲, 4×MIC. Relative absorbance was calculated by dividing the absorbance of the treated tube by that of the negative control tube. The values with the standard error bars are mean values from triplicate experiments.

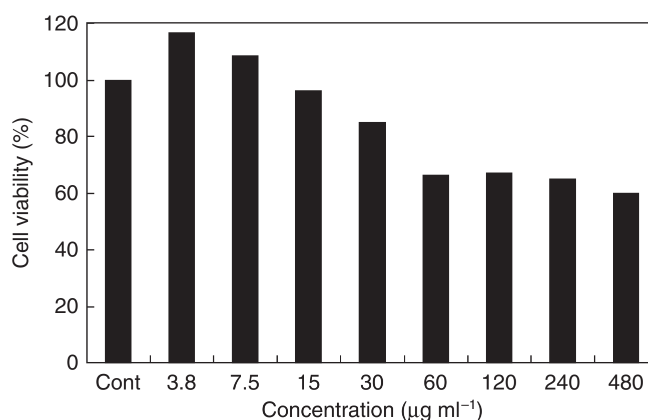

Figure 4 Cytotoxicity of Sargafuran to human normal dermal fibroblast cells.

2× the MIC (30 μg ml^{-1}), it did not significantly affect cell viability and did not reduce cell viability by 50% at any concentrations up to 480 μg ml^{-1}.

DISCUSSION

Acne vulgaris is a multifactorial disease with an unclear etiology and pathogenesis. The factors known to cause acne vulgaris include follicular hyperkeratosis, sebum secretion, *P. acnes* and inflammation.[17] Recently, cosmetics and toiletries containing natural products, such as extracts of herbs, Chinese plant medicine and seaweed, have been prevalent in commercial markets because of consumer concerns over synthetic chemical ingredients.

This study was conducted to screen marine algae collected from the Japan coastline for antibacterial activity against *P. acnes* to develop new types of cosmetics to treat acne. We found a new anti-*P. acnes* compound, Sargafuran, from the marine brown alga, *S. macrocarpum*. We also have reported two neurostimulating substances, sargaquinoic acid and sargachromenol, from this alga[18,19] and determined their structures to be 2-methylquinone-type compounds having side chain (C-20 unit). The 2-methylquinone moiety and C-20 side chain of these compounds would be biosynthesized from sikimic acid (C-7 unit) and geranyl geranyl diphospate (C-20 unit). It is likely that the

biosynthetic pathway of Sargafuran carbon skeleton would share with the pathway of sargaquinoic acid and sargachromenol skeletons, because Sargafuran has a similar side chain and the same number of carbons. Sargafuran was stable against heating up to 60 °C, pH 4–7 and irradiation for 24 h. These properties are suitable for cosmetics or skin care products to prevent acne. Clindamycin is generally used for curing acne as a clinical treatment. However, Clindamycin was shown to be bacteriastatic even at 4×MIC, whereas Sargafuran showed bactericidal activity against *P. acnes*. This is a superior property because bactericidal activity minimizes the chance of development of resistance. Takahashi *et al.*[20] have also reported that eucalyptus leaf extracts and constituent flavonoids showed great antibacterial activity against Gram-positive bacteria, including *P. acnes*. The marine brown alga, *S. macrocarpum,* containing Sargafuran might be a good candidate from which to develop and produce the anti-acne cosmetics or skin care products in the near future.

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