

ORIGINAL ARTICLE

Antifouling and antibacterial polyketides from marine gorgonian coral-associated fungus *Penicillium* sp. SCSGAF 0023

Jie Bao^{1,2,5}, Yu-Lin Sun^{1,2,5}, Xiao-Yong Zhang¹, Zhuang Han^{1,2}, Hai-Chun Gao³, Fei He¹, Pei-Yuan Qian⁴ and Shu-Hua Qi¹

Two new polyketides, 6,8,5'6'-tetrahydroxy-3'-methylflavone (1) and paecilin C (2), together with six known analogs secalononic acid D (3), secalononic acid B (4) penicillixanthone A (5), emodin (6), citreorosein (7) and isorhodoptilometrin (8) were obtained from a broth of gorgonian coral-associated fungus *Penicillium* sp. SCSGAF 0023. Compounds 1 and 6–8 had significant antifouling activity against *Balanus amphitrite* larvae settlement with EC₅₀ values of 6.7, 6.1, 17.9 and 13.7 $\mu\text{g ml}^{-1}$, respectively, and 3–5 showed medium antibacterial activity against four tested bacterial strains. This was the first report of antibacterial activity of 3–5 against marine bacteria and antifouling activity of 6–8 against marine biofouling organism's larvae. The results indicated that gorgonian coral-associated fungus *Penicillium* sp. SCSGAF 0023 strain could produce antifouling and antibacterial compounds that might aid the host gorgonian coral in protection against marine pathogen bacteria, biofouling organisms and other intruders.

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INTRODUCTION

Gorgonian corals appear to have few predators and survive in the highly competitive and hostile marine environment, mainly relying on a series of secondary metabolites accumulating in their bodies or releasing to their surroundings, which have important roles in protecting the colonies against grazing, feeding and marine biofouling organisms.¹ A variety of studies suggested that coral-associated microbe was a possible first line of chemical defense for corals, possibly through producing bioactive substances.^{2,3} Recent studies showed that gorgonian corals had a large and diverse fungal community, and *Penicillium* was one of the most diverse and common genus in gorgonian corals.^{4,5} However, few attentions were paid to the secondary metabolites of gorgonian coral-associated fungi and their possible chemical defense roles for their hosts.

In our preliminary experiment, we found that the ethyl acetate extract of a culture broth of the marine-derived fungus *Penicillium* sp. SCSGAF 0023 isolated from South China Sea gorgonian coral *Dichotella gemmacea* exhibited significant antibacterial activity against *Escherichia coli* and *Bacillus subtilis* and antifouling activity against *Balanus amphitrite* larvae settlement. Further investigation on the chemical constituents of the extract led to the isolation of eight

polyketides including two new compounds. For these compounds, antifouling and antibacterial activities were evaluated. In this paper, we describe the isolation, structure elucidation and bioactivities of these secondary metabolites from the marine gorgonian coral-associated fungus *Penicillium* sp. SCSGAF 0023 strain.

RESULTS

Isolation and identification of compounds

Totally, eight polyketides were obtained from the ethyl acetate extract of the broth of *Penicillium* sp. SCSGAF 0023 strain. The structures of these compounds were identified by spectroscopic analysis and comparison with published data. They were 6,8,5'6'-tetrahydroxy-3'-methylflavone (1), paecilin C (2), secalononic acid D (3),⁶ secalononic acid B (4),⁷ penicillixanthone A (5),^{8,9} emodin (6),¹⁰ citreorosein (7)¹⁰ and isorhodoptilometrin (8)¹¹ (Figure 1). Among them, 1 and 2 were new compounds, and 3 was the main secondary metabolite of the strain accounting about 2.5% of the crude extract.

Compound 1 was obtained as white power, having the molecular formula C₁₆H₁₂O₆ as deduced from NMR spectra and (+)-ESIMS. The ¹H NMR spectrum showed the signals of one methyl at δ_{H} 2.22 (3H, s), five aromatic protons at δ_{H} 6.35 (1H, d, $J=1.5$ Hz), 6.28

¹Key Laboratory of Marine Bio-Resources Sustainable Utilization/RNAM Center for Marine Microbiology/Guangdong Key Laboratory of Marine Material Medical, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, China; ²Graduate School of the Chinese Academy of Sciences, Beijing, China; ³College of Life Sciences, Zhejiang University, Yuhangtang Road, Hangzhou, China and ⁴Department of Biology, Hong Kong University of Science and Technology, Clearwater Bay, KLN, Hong Kong, China

⁵These authors contributed equally to this work.
Correspondence: Dr S-H Qi, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou 510301, China.

E-mail: shuhuaqi@scsio.ac.cn

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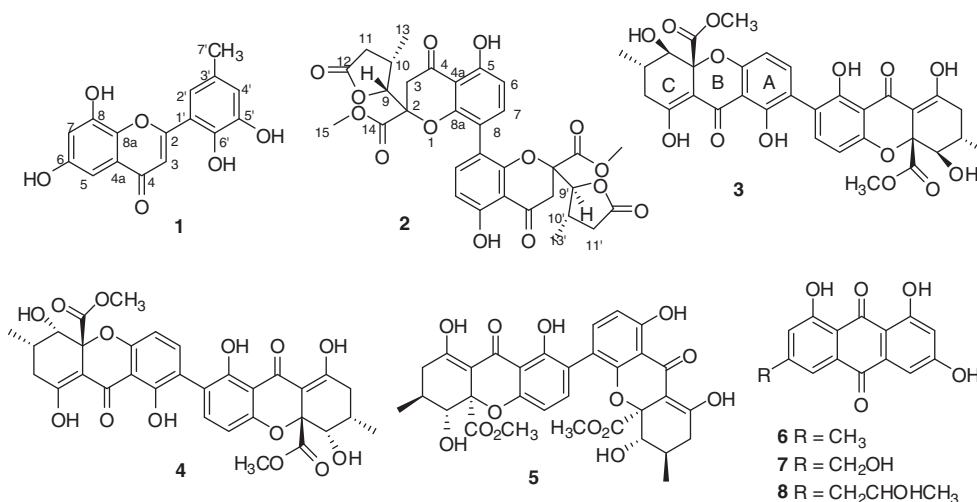


Figure 1 Structures of **1–8** from the gorgonian-associated fungus *Penicillium oxalicum*.

(1H, d, $J=1.5$ Hz), 6.26 (1H, d, $J=1.5$ Hz), 6.24 (1H, s) and 6.23 (1H, d, $J=1.5$ Hz). The ^{13}C and DEPT NMR spectra exhibited 16 carbons including 1 methyl (δ_{C} 20.4), 5 methines (δ_{C} 95.0, 100.2, 101.3, 110.2 and 112.9) and 10 quaternary carbons (δ_{C} 105.4, 113.2, 140.8, 158.4, 160.3, 161.4, 163.3, 166.2, 166.3 and 184.0). These data suggested that **1** should have a flavonoid skeleton.^{12–15} This suggestion was proved by the heteronuclear multiple bond connectivity (HMBC) spectrum (Figure 2). In the HMBC spectrum, HMBC correlations from H-5 (δ_{H} 6.35) to C-4 (δ_{C} 184.0)/C-4a (δ_{C} 105.4)/C-6 (δ_{C} 166.2)/C-7 (δ_{C} 100.2)/C-8a (δ_{C} 160.3), from H-7 to C-5(95.0)/C-6/C-8 (δ_{C} 163.3), suggested the oxygenation of C-6 and C-8. HMBC correlations from H-3 (δ_{H} 6.24) to C-4/C-4a (δ_{C} 105.4)/C-2 (δ_{C} 166.3)/C-1' (δ_{C} 113.2) indicated that **1** was a flavone. Furthermore, HMBC correlations from H-7' (δ_{H} 2.22) to C-2' (δ_{C} 110.2)/C-3' (δ_{C} 140.8)/C-4' (δ_{C} 112.9), from H-2' (δ_{H} 6.28) to C-1'/C-4'/C-6' (δ_{C} 161.4), from H-4' (δ_{H} 6.26) to C-5' (δ_{C} 158.4)/C-6', suggested the oxygenation of C-5'/C-6' and one methyl attached on C-3'. Based on the above data, the structure of **1** was determined as shown in Figure 1.

Compound **2** was obtained as a pale yellow gum, having a molecular formula of $\text{C}_{32}\text{H}_{30}\text{O}_{14}$ as deduced from NMR spectra and (+)-ESIMS. The ^1H NMR spectrum showed the signals of two phenolic hydroxyl protons at δ 11.92 (1H, s), 12.03 (1H, s), two sets of doublets of AB-aromatic system protons at δ 6.64 (1H, d, $J=8.0$ Hz), 7.52 (1H, d, $J=8.5$ Hz), 6.63 (1H, d, $J=8.0$ Hz), 7.54 (1H, d, $J=8.5$ Hz), two oxymethines at δ 4.82 (1H, d, $J=6.5$ Hz), 4.10 (1H, s), two methoxyl groups at δ 3.79 (3H, s), 3.77 (3H, s) and two methyls at δ 1.36 (3H, d, $J=7.0$ Hz), 1.11 (3H, d, $J=6.5$ Hz). The ^{13}C and DEPT NMR spectra exhibited 32 carbons including 2 methyls, 2 methoxyls, 4 methylenes, 2 methines, 2 oxymethines, 2 oxyquaternary carbons, 12 aromatic carbons, 4 carboxyl groups and 2 ketone groups (δ_{C} 195.9, 194.1). The signals of all carbon resonances appeared in pairs, which suggested that **2** should be a dimer. The ^1H and ^{13}C NMR data of **2** showed great similarity to those of paecilin A¹⁶ with the only obvious differences of the chemical shifts of H-9' (from δ_{H} 4.65 (d, $J=7.5$ Hz) to δ_{H} 4.10 (br s)), H-10' (from δ_{H} 2.87 (m) to δ_{H} 2.39 (m)), H-11' (from δ_{H} 2.30 (dd, $J=8.5$, 17 Hz), 1.96 (dd, $J=11$, 17 Hz) to δ_{H} 2.67 (dd, $J=8.0$, 17 Hz), 2.46 (m)), C-9' (from δ_{C} 82.3 to δ_{C} 76.2), C-10' (from δ_{C} 33.8 to δ_{C} 30.7) and C-11' (from δ_{C} 35.2 to δ_{C} 40.2), which indicated that **2** might be an

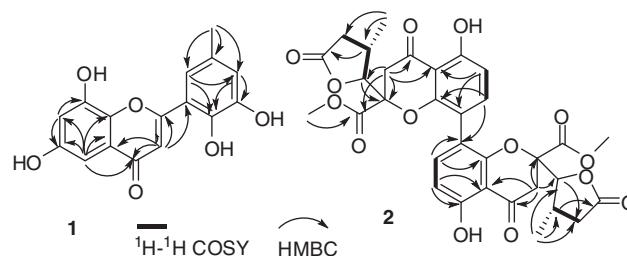


Figure 2 Key HMBC and ^1H - ^1H COSY correlations of **1** and **2**.

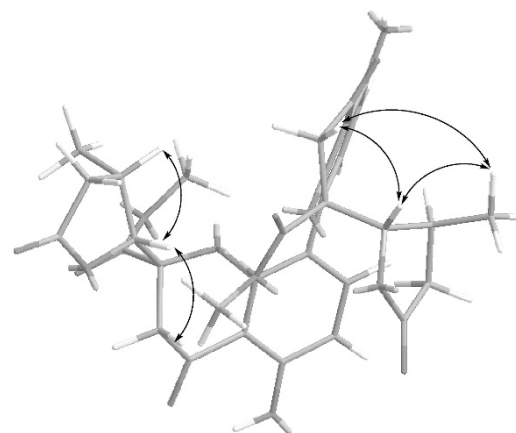


Figure 3 Key NOESY correlations of **2**. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

epimer of paecilin A. The 2D NMR spectra including HSQC, HMBC and ^1H - ^1H COSY spectra (Figure 2) of **2** proved that **2** had the same planar structure as paecilin A. The relative configuration of **2** was further inferred from the NOESY spectrum (Figure 3) and coupling constants of $J_{\text{H-9/H-10}}$ and $J_{\text{H-9'/H-10'}}$. The large coupling constant of $J_{\text{H-9/H-10}}$ (6.5 Hz) and comparison of NMR data of H-9/H-10/H-11, C-9/C-10/C-11 in **2** and paecilin A, combined no NOE correlation between H-9 and Me-13 observed in the NOESY spectrum, suggested the relative configurations of H-9 β , H-10 β and Me-13 α in **2** that were the same as those in paecilin A. In addition, according to the Karplus

curve, the small coupling constant of $J_{H-9'/H-10'}$ (H-9' appeared as singlet) indicated a dihedral angle of $\sim 90^\circ$ between H-9' and H-10'. The presence of NOE correlation between H-9' and Me-13' indicated that H-9' and Me-13' were *cis*- configuration, based on the dihedral angle measurement and small coupling constant of $J_{H-9'/H-10'}$, which suggested the relative configurations of H-9' α , H-10' β and Me-13' α in **2**. Based on the above data, the structure of **2** was inferred as shown in Figure 1. The specific optical rotation of **2** (-71.35°) was opposite to that of paecililic acid ($+61.60^\circ$).

Antibacterial activity

Preliminary antibacterial assay results showed that at concentration of 50 μg per disc compounds **3–5** could inhibit the growth of four tested bacterial strains (*B. subtilis*, *E. coli* JVC1228, *M. luteus* UST950701-006, *P. nigrifaciens* UST010620-005), whereas **1**, **2** and **6–8** had no effect on the growth of any tested bacteria. The MIC values of **3–5** were further tested by standard disc diffusion assay. The results (Table 1) showed that **3–5** had medium antibacterial activity against the four tested bacterial strains, and their antibacterial activities against *M. luteus* UST950701-006 (one marine larval settlement inducing bacterial strain) and *P. nigrifaciens* UST010620-005 (one

marine pathogenic bacterial strain) were stronger than the positive control kanamycin.

As compound **3** showed potent antibacterial activity, the antibacterial activity of **3** against *S. onedensis* MR-1 biofilm was further evaluated using sterile 24-well polystyrene plates. The results (Figure 4) showed that **3** could completely inhibit the growth of *S. onedensis* MR-1 at concentration of 3.125 $\mu\text{g ml}^{-1}$ after 12 h of incubation, whereas as the incubation time increased to 24 h, the pellicle formation recovered well. From the morphological observation, we found that **3** could inhibit the growth of *S. onedensis* MR-1 by interfering cells' regular division, whereas as the culture time increased, cells gradually recovered normal division, and then the bacterium became to grow well.

Antilaval settlement activity

Antifouling activity of **1–8** was evaluated in settlement inhibition assays with *B. amphitrite* larvae. The results (Figure 5) showed that **1** and **6–8** could strongly inhibit the larvae settlement of *B. amphitrite* larvae with EC_{50} values of 6.71, 6.05, 17.9 and 13.67 $\mu\text{g ml}^{-1}$ respectively, whereas **2** and **3–5** didn't have antifouling activity at concentration of 25 $\mu\text{g ml}^{-1}$. No toxicity toward larvae was observed for **1** and **6–8** at the test concentrations of 3.125–100 $\mu\text{g ml}^{-1}$, which indicated that the $\text{LC}_{50}/\text{EC}_{50}$ values of **2** and **6–8** were >14.9 , >16.5 , <15 and <15 , respectively.

Table 1 MIC of **3–5** against four bacterial strains

Compound	MIC ($\mu\text{g ml}^{-1}$)			
	<i>M. luteus</i>	<i>P. nigrifaciens</i>	<i>E. coli</i>	<i>B. subtilis</i>
3	24.4	97.5	24.4	24.4
4	97.5	390.5	97.5	97.5
5	24.4	97.5	24.4	24.4
Kanamycin	6.3×10^3	390.5	6.1	6.1
Penicillin	24.4	6.1	6.1	1.5

DISCUSSION

This study serves as the attempt to find antifouling and antibacterial compounds from gorgonian coral-associated fungus *Penicillium* sp. SC5GAF 0023 and investigates their possible chemical defense for its host. In the study, we obtained one flavone (**1**), four secalonic acid analogs (**2–5**) and three anthraquinones (**6–8**) from the marine fungal species. Previous studies reported that **3** and **4** had strong cytotoxicity,^{17,18} and many attentions have been paid to **3**, however, its application was limited because of its teratogenic effect on murine

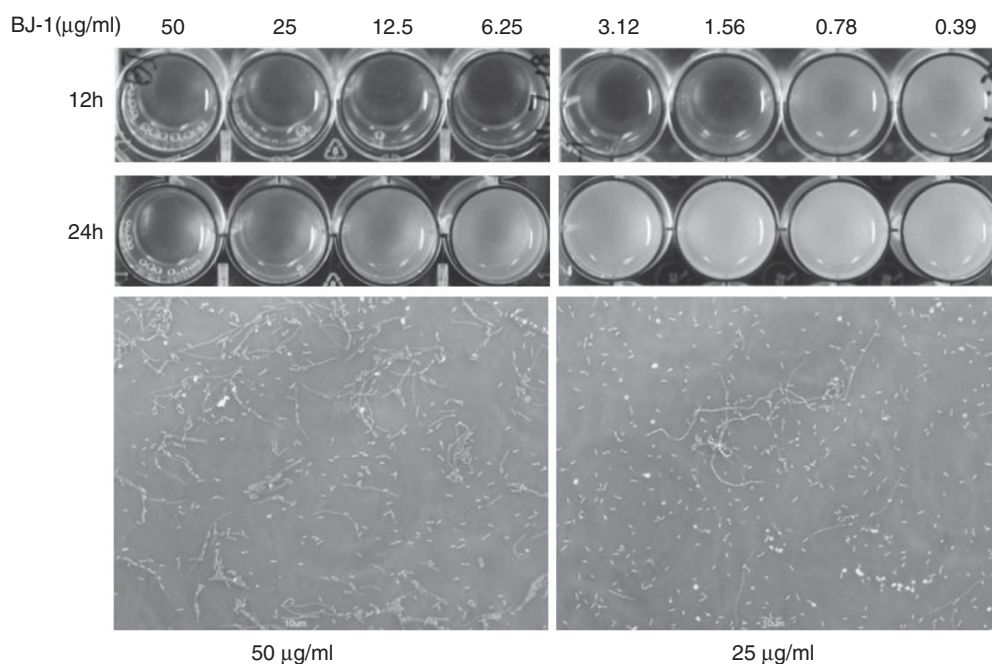


Figure 4 Antibacterial activity of **3** against *Shewanella onedensis* MR-1 biofilm. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

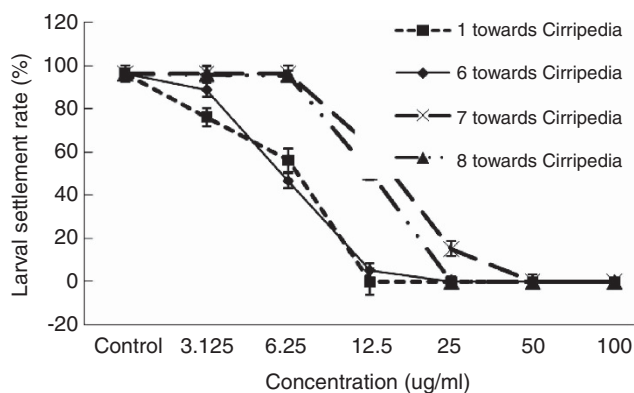


Figure 5 Effect of **1** and **6–8** on larval settlement of *Balanus amphitrite* larvae (Cirripedia).

and chick embryo.^{19,20} Here, we found that **3–5** had medium antibacterial activity against marine larval settlement inducing bacterium *M. luteus*, marine pathogenic bacterium *P. nigrificiens*, and common bacteria *B. subtilis* and *E. coli*, and **3** also showed antibacterial activity against marine bacterium *S. onedensis* MR-1 biofilm. This was the first report of the antibacterial activity of **3–5** against marine bacteria.

In addition, the new compound **1** showed strong antifouling activity toward *B. amphitrite* larvae with EC_{50} value of $6.71 \mu\text{g ml}^{-1}$ and low toxicity with LC_{50}/EC_{50} ratio > 14.9 . Its structure is similar to the flavones that have strong antilarval settlement activity toward *B. amphitrite* larvae.²¹ Anthraquinones **6–8**, especially **6** also exhibited strong antifouling activity against *B. amphitrite* larvae settlement with $EC_{50} < 15 \mu\text{g ml}^{-1}$, and the LC_{50}/EC_{50} ratio of **6** was > 16.5 . Usually, the standard requirement of an efficacy EC_{50} level for natural antifoulant is $25 \mu\text{g ml}^{-1}$ that was established by the US Navy program, and a compound with a LC_{50}/EC_{50} ratio > 15 is often considered as a non-toxic antifouling compound.²² The results indicated that **1** and **6** were potent natural antifoulants. This was the first report of the antifouling activity of **1** and **6–8** against marine biofouling organism's larvae.

In conclusion, gorgonian coral-associated fungus *Penicillium* sp. SCSGAF 0023 strain could produce antifouling and antibacterial secondary metabolites that might aid the host gorgonian coral in protection against marine pathogen bacteria, biofouling organisms and other intruders.

MATERIALS AND METHODS

Fungal strain isolation and identification

The sample of gorgonian coral *Dichotella gemmacea* was collected from the South China Sea, Sanya ($18^{\circ}11'N$, $109^{\circ}25'E$), Hainan Province, China. The sample was rinsed three times in sterile seawater to remove transient and loosely attached microbial organisms, and then cut into pieces measuring ca. 1 cm^3 and thoroughly homogenized in a sterile mortar with two volumes of sterile seawater. A 10-fold dilution was made and 0.1 ml dilutions were plated on glucose yeast extract peptone (GYP) medium (containing 5 g l^{-1} glucose, 10 g l^{-1} yeast extract, 10 g l^{-1} peptone 5, 30 g l^{-1} sea salt and 18 g l^{-1} agar) agar plates. The inoculated plates were incubated at 28°C for 3 weeks. The fungal isolate SCSGAF 0023 recovered on the media was selected on the basis of their morphological differences based on visible examination of growth characteristics, aerial mycelium, substrate mycelium and diffusible pigments.

Total genomic DNA of fungal isolate SCSGAF 0023 was extracted as described by Lai *et al.*²³ From the genomic DNA, the internal transcribed spacer (ITS) gene sequences were amplified by polymerase chain reaction using primers ITS1(5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TC

CTCCGCTTATTGAT ATGC-3'). And the ITS region was sequenced and compared with the GenBank database, which indicated the ITS sequence of fungal isolate SCSGAF 0023 (GenBank access number JN850988) shared a similarity of 99% with *Penicillium oxalicum* strain F3 (JF793525.1).

Fungal strain cultivation and fermentation

The fungal strain was inoculated in potato dextrose agar (PDA) liquid medium (20 potato, 2 glucose and 3% sea salt), and then cultured in potato dextrose agar liquid nutrient medium under shaking. Twelve-liter cultivations were carried out in 500 ml shake flasks with 150 ml broth and incubated on a rotary shaker (150 r.p.m.) at 28° for 6 days.

Extraction and isolation of compounds

The 12 l fermentation broth was filtered through cheesecloth to separate the broth supernatant and mycelia. The broth supernatant was extracted with ethyl acetate, whereas the mycelia was extracted with 80% acetone. The acetone extract was evaporated under reduced pressure to afford an aqueous solution, and then extracted with ethyl acetate. The extracts of broth supernatant and mycelia were combined to give a crude gum (8 g). The crude gum was separated to two parts: methanol soluble and methanol insoluble fractions. The methanol insoluble fraction was repeatedly crystallized from $\text{CHCl}_3/\text{MeOH}$ to yield **3** (200 mg), and its mother liquid was further purified by semi-preparative reversed-phase HPLC (YMC-Pack, ODS S-5 μ $250 \times 10 \text{ mm}$ i.d., 3 ml min^{-1}), eluting with $\text{MeOH}/\text{H}_2\text{O}/\text{CH}_3\text{COOH}$ (v/v/v 90:10:10 $^{-4}$) to yield **5** (10 mg). The methanol soluble fraction was subjected to Rp-18 reverse-phase column chromatography eluting with $\text{H}_2\text{O}/\text{MeOH}$ (v/v from 90:10 to 0:100) to give seven fractions (I–VII). Fraction II was subjected to Sephadex LH-20 column chromatography eluting with MeOH to afford four subfractions. Subfraction II-2 was further purified by reversed-phase-HPLC with $\text{MeOH}/\text{H}_2\text{O}$ (v/v 58:42) to yield **2** (5 mg), and subfraction II-4 was subjected to Sephadex LH-20 column eluting with $\text{CHCl}_3/\text{MeOH}$ (v/v 1:1), then further purified by preparative TLC plate with $\text{CHCl}_3/\text{MeOH}$ (v/v 8:2) to give **1** (4 mg). Fraction V was chromatographed on a Sephadex LH-20 column, eluting with $\text{CHCl}_3/\text{MeOH}$ (v/v 1:1) to obtain eight subfractions. Subfraction V-2 was further chromatographed on a Sephadex LH-20 column eluting with $\text{CHCl}_3/\text{MeOH}$ (v/v 1:1) to give **6** (5 mg). Subfraction V-7 was repeatedly subjected to silical gel column chromatography using a gradient elution of $\text{CHCl}_3/\text{MeOH}$ to yield **4** (2 mg). Fraction IV was applied to a Sephadex LH-20 column chromatography eluting with $\text{CHCl}_3/\text{MeOH}$ (v/v 1:1) to give five subfractions. Subfraction IV-4 was further purified by reversed-phase-HPLC with $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (v/v 45:55) to yield **8** (2 mg), and subfraction IV-5 was purified by preparative TLC plate to give **7** (4 mg).

Physical properties of compounds 1 and 2

The structures of compounds **1–8** were determined by spectroscopic analysis. Optical rotations were measured with a Horiba SEAP-300 spectropolarimeter (Horiba Ltd., Kyoto, Japan). UV spectra were measured with a Shimadzu double-beam 210A spectrophotometer (Shimadzu Corp., Kyoto, Japan) in MeOH solution. ^1H , ^{13}C NMR and 2D NMR spectra were recorded on a Bruker AV-500 MHz NMR spectrometer (Bruker Corp., Karlsruhe, Germany) with TMS as internal standard. MS spectral data were obtained on an LCQDECA XP HPLC/MSn spectrometer (Agilent Technologies Inc., San Jose, CA, USA) for ESIMS.

6,8,5,6'-Tetrahydroxy-3'-methylflavone (1): white powder; UV (MeOH) λ_{max} : 250, 261, 346 nm; IR (KBr): 3358, 1650, 1602, 1496 cm^{-1} ; ^1H NMR (CD_3OD , 500 MHz) δ_{H} : 2.22 (3H, s, H-7'), 6.35 (1H, d, $J = 1.5 \text{ Hz}$, H-5), 6.28 (1H, d, $J = 1.5 \text{ Hz}$, H-2'), 6.26 (1H, d, $J = 1.5 \text{ Hz}$, H-4'), 6.24 (1H, s, H-3), 6.23 (1H, d, $J = 1.5 \text{ Hz}$, H-7); ^{13}C NMR (CD_3OD , 125 MHz) δ_{C} : 20.4 (C-7'), 95.0 (C-5), 100.2 (C-7), 101.3 (C-4'), 105.4 (C-4a), 110.2 (C-2'), 112.9 (C-3), 113.2 (C-1'), 140.8 (C-3'), 158.4 (C-5'), 160.3 (C-8a), 161.4 (C-6'), 163.3 (C-8), 166.2 (C-6), 166.3 (C-2), 184.0 (C-4); (+)-ESIMS m/z 301 $[\text{M} + \text{H}]^+$; (+)-HRESIMS m/z : $[\text{M} + \text{H}]^+$ 301.0706 (calcd. for $\text{C}_{16}\text{H}_{13}\text{O}_6$, 301.0712).

Paecilin C (2): pale yellow gum; $[\alpha]_{\text{D}}^{20}$ -71.35 (c 0.37, CHCl_3); UV (MeOH) λ_{max} : 214, 281, 346 nm; IR (KBr): 3422, 1790, 1738, 1650 cm^{-1} ; ^1H NMR (CDCl_3 , 500 MHz) δ_{H} : 3.29 (2H, d, $J = 17.5 \text{ Hz}$, H-3), 6.64 (1H, d, $J = 8.0 \text{ Hz}$, H-6), 7.52 (1H, d, $J = 8.5 \text{ Hz}$, H-7), 4.82 (1H, d, $J = 6.5 \text{ Hz}$, H-9), 3.00 (1H, m,

H-10), 2.73 (1H, dd, $J=8.0$, 17 Hz, H-11), 2.52 (1H, dd, $J=8.5$, 17.5 Hz, H-11), 1.36 (3H, d, $J=7.0$ Hz, H-13), 3.79 (3H, s, H-15), 11.92 (1H, s, 5-OH), 3.22 (2H, d, $J=17.5$ Hz, H-3'), 6.63 (1H, d, $J=8.0$ Hz, H-6'), 7.54 (1H, d, $J=8.5$ Hz, H-7'), 4.10 (1H, s, H-9'), 2.39 (1H, m, H-10'), 2.67 (1H, dd, $J=8.0$, 17 Hz, H-11'), 2.46 (1H, m, H-11'), 1.11 (3H, d, $J=6.5$ Hz, H-13'), 3.77 (3H, s, H-15'), 12.03 (1H, s, 5'-OH); ^{13}C NMR (CDCl_3 , 125 MHz) δ_{C} : 84.5 (C-2), 39.8 (C-3), 195.9 (C-4), 159.2 (C-5), 107.4 (C-6), 140.9 (C-7), 117.4 (C-8), 82.6 (C-9), 33.5 (C-10), 36.7 (C-11), 174.9 (C-12), 14.9 (C-13), 169.1 (C-14), 53.5 (C-15), 107.5 (C-4a), 158.8 (C-8a), 86.8 (C-2'), 39.8 (C-3'), 194.1 (C-4'), 159.2 (C-5'), 107.3 (C-6'), 141.2 (C-7'), 117.8 (C-8'), 76.2 (C-9'), 30.7 (C-10'), 40.2 (C-11'), 174.9 (C-12'), 13.7 (C-13'), 169.1 (C-14'), 53.7 (C-15'), 107.5 (C-4a'), 158.4 (C-8a'); (+)-ESIMS m/z 639 $[\text{M}+\text{H}]^+$; (+)-HRESIMS m/z $[\text{M}+\text{H}]^+$ 639.1701 (calcd. for $\text{C}_{32}\text{H}_{31}\text{O}_{14}$, 639.1708).

Antibacterial assays

Antibacterial activity of **1–8** was tested against four bacteria (*B. subtilis*, *E. coli* JVC1228, *Micrococcus luteus* UST950701-006, *Pseudoalteromonas nigrifaciens* UST010620-005) using standard disc diffusion assay. Briefly, first, the tested bacteria were cultured on nutrient agar plates at 37 °C for 1–2 days, then scraped to make cell suspension (10^8 CFU ml^{-1}) with sterile seawater and sterile water for marine and common bacterial strains, respectively. Forty microliters cell suspension were then mixed with 20 ml corresponding nutrient agar when the agar temperature was kept in 50–60 °C, and then poured into 100 mm sterile Petri plates. Sterile 6 mm diameter circular discs of filter paper were loaded with 50 μg of tested compound dissolved in methanol, evaporated to dryness and then placed onto the bacterial lawn. An additional set of discs with 50 μg kanamycin or penicillin were used as the positive controls. The agar plates were incubated for 18 h at 37 °C, and then the inhibition zones were measured. The experiment was run in three replicates.

The MICs of active compounds **3**, **4** and **5** were further determined by disc diffusion assay as the above described. Tested samples were dissolved in methanol at a concentration of 50 μg μl^{-1} , which were diluted to 10000, 2500, 625, 156, 39, 9.7, 2.4, 0.6 μg ml^{-1} . Five microliters of each concentration were added into each paper disc. An additional set of discs with gradient-based concentrations of penicillin and kanamycin were used as positive controls. MIC value was calculated as the following equation: $\text{MIC} = \text{C}_n + \text{C}(\text{n}+1)/2$. C_n , the lowest concentration at disc with inhibition zones; $\text{C}(\text{n}+1)$, the highest concentration at disc without inhibition zones.

Antibacterial activity of **3** against *Shewanella oneidensis* MR-1 biofilm was further evaluated using sterile 24-well polystyrene plates.²⁴ *S. oneidensis* MR-1, a facultative Gram-negative anaerobe with a remarkable respiratory versatility, has been extensively studied for its biofilm development. In here, the strain was grown in 3 ml lysogeny broth to an OD_{600} of 0.8–1.0. The culture was then diluted to 1:100 in fresh lysogeny broth at 24-well plate, which containing different concentration of **3**. The stock solution concentration of **3** was 50 μg ml^{-1} . Double dilution method was used to test the effects of **3** on pellicle formation. The culture in 24-well plate was incubated without shaking at room temperature, and after 12 and 24 h of incubation their pellicles were analyzed by visual inspection.

Larval settlement bioassays

Antifouling activity of compounds **1–8** was evaluated in settlement inhibition assays with laboratory-reared *B. amphitrite* (Cirripedia) larvae. Larval settlement bioassays were performed using sterile 24-well polystyrene plates as previously reported.²⁵ Briefly, the stock solution of tested samples in DMSO was diluted with autoclaved filtered sea water to concentrations ranging from 3.125 to 100 μg ml^{-1} . About 20 competent larvae were added to each well in 1 ml of the test solution. Wells containing only filtered sea water with DMSO served as the controls. The plates were incubated at 27 °C for 24 h. The percentage of larval settlement was determined by counting the settled, live individuals under a dissecting microscope and expressing the result as a proportion of the total number of larvae in the well. EC_{50} (inhibits 50% of settlement of *B. amphitrite* larvae in comparison with the control) and LC_{50}

(refers to the concentration that kills 50% of the test organisms in comparison with the control) levels of active compounds were calculated by using the Excel software program.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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