The Journal of Antibiotics (2017) 70, 911–914

www.nature.com/ja

NOTE

Cladomarine, a new anti-saprolegniasis compound isolated from the deep-sea fungus, *Penicillium coralligerum* YK-247

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The Journal of Antibiotics (2017) **70,** 911–914; doi:10.1038/ja.2017.58; published online 31 May 2017

The Saprolegniaceae belongs to the Oomycota; among a member of this family, *Saprolegnia parasitica* is a virulent pathogen of fishes and their ova, and causes enormous damage and losses in the freshwater aquaculture,[1,2] such as a salmon hatchery. In fish farms, saprolegniasis has been controlled by malachite green, effectively preventing infection. However, malachite green has been banned in many countries because it was found to be mutagenic. Bronopol is sometimes used as an alternative anti-saprolegniasis agent, but is not as effective as malachite green and is toxic to zooplankton and phytoplankton.[3,4] Therefore, new anti-saprolegniasis compounds are urgently needed.

Fungi are rich sources of useful chemical compounds and mycotoxins, and are often explored to discover new useful substances. The fungi inhabit various environments, from land to sea. Remarkably, diverse fungi are present in deep-sea environments, in spite of the fact that the conditions in deep-sea environments are characterized by high pressure, low temperature and oligotrophy.[5,6] The extreme conditions may affect the production of primary and secondary metabolites in deep-sea fungi, and therefore, there is a high probability that they produce structurally and functionally unique metabolites.[7] Indeed, novel bioactive secondary metabolites from deep-sea fungi are increasingly being reported.[8–10]

On screening 546 cultured broths of 91 deep-sea fungal strains, we discovered that *Penicillium coralligerum* YK-247 could produce compounds with notable anti-*Saprolegnia parasitica* activity. Chromatographic fractionation of the cultured broth led to the isolation of a new compound, named cladomarine (**1**), together with two known compounds, cladosporin (**2**)[11] and 5′-hydroxyasperentin (**3**)[12] (Figure 1a). This study describes the taxonomy of the producing fungal strain, together with the fermentation, isolation, structure elucidation and anti-*Saprolegnia parasitica* activity of **1**, **2** and **3**.

Strain YK-247 was isolated from a sea cucumber collected at São Paulo Plateau, off Brazil (water depth: 3064 m, sampling site: 28°29.9′S and 41°39.3′W), using the human-occupied vehicle *Shinkai 6500* of JAMSTEC under Japan–Brazil cooperative deep-sea investigation named *Iatá-Piúna* cruise. For determination of the morphological characteristics of the strain YK-247, slides were prepared from colonies grown on malt extract agar (MEA). Conidiophores were borne on a basal felt or directly from the agar, simple, smooth, 40–110 μm long, with a thick wall. Penicilli were typically biverticillate, sometimes monoverticillate or terverticillate. Metulae in whorls of 2–4, which were usually relatively appressed, sometimes slightly divergent when forced apart by larger whorls, ~10.5–15.5 × 3.0–4.8 μm near the top, individually more or less cylindrical. Phialides were acerose, 8.0–12.5 × 2.2–3.7 μm. Conidia borne in chains were globose, subglobose to ellipsoidal, slightly roughened, 3.0–4.0 × 2.3–3.7 μm. The internal transcribed spacer (ITS) of ribosomal RNA gene of strain YK-247 was sequenced and its sequence was deposited at the DNA Data Bank of Japan (DDBJ) with the accession number LC214562. The ITS sequence of YK-247 was compared to sequences in the GenBank database by BLASTN 2.5.1 analysis.[13] The ITS sequence of YK-247 was 99.8% similar to that of CBS 123.65 (ex-type of *Penicillium coralligerum*; GenBank accession number NR_111666). Based on the results of morphological characteristics and ITS sequence, the strain YK-247 was identified as a strain of *Penicillium coralligerum*.

A loop of spores from a colony growing on a Miura's medium (LcA) slant was inoculated into 10 ml of GP seed medium (2.0% glucose, 0.2% yeast extract, 0.5% $MgSO_4 \cdot 7H_2O$, 0.5% Polypeptone, 0.1% KH_2PO_4 and 0.1% agar, pH 6.0) in a test tube and incubated at 27 °C for 3 days on a shaker at 300 r.p.m. The seed-culture (10 ml) was inoculated into 500 ml-Erlenmeyer

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Received 23 January 2017; revised 6 April 2017; accepted 20 April 2017; published online 31 May 2017

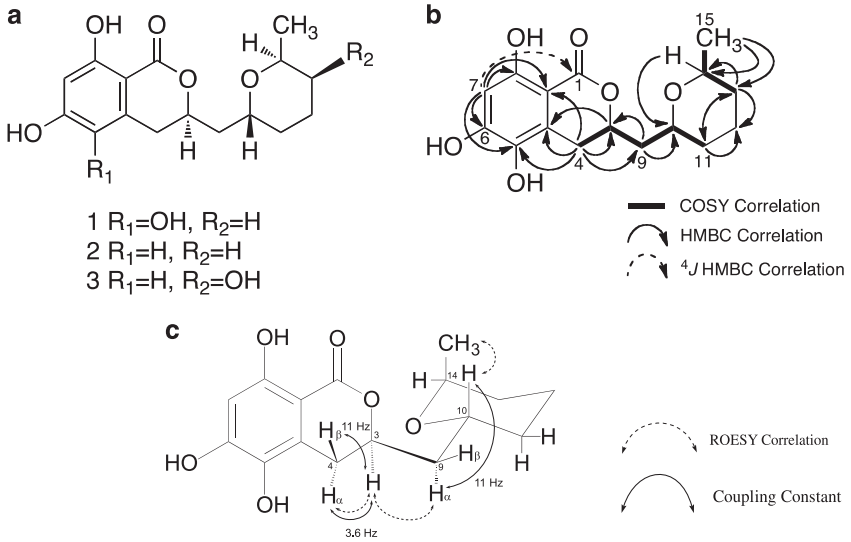

Figure 1 (**a**) Structure of (**1**) cladomarine, (**2**) cladosporin and (**3**) 5′-hydroxyasperentin. (**b**) COSY and HMBC correlation of (**1**) cladomarine. Bold lines show ^{1}H–^{1}H correlation, arrows show ^{1}H–^{13}C correlation and dotted arrows show ^4J HMBC. (**c**) ROESY correlation and coupling constant of (**1**) cladomarine. Arrows show ^{1}H coupling constant and dotted arrows show ROESY correlation.

flasks, each containing 50 g of rice medium was immersed in seawater for 2 h and sterilized (three flasks, total 150 g) and static fermentation occurred for 13 days. The cultured rice medium (150 g) was subsequently added to 300 ml of ethanol and then filtered. The filtrate was concentrated under reduced pressure to remove ethanol and extracted with EtOAc (300 ml) three times. The EtOAc extract (365.3 mg) was applied to an octadecylsilane (ODS) gel column (20φ × 100 mm, YMC Co. Ltd., Kyoto, Japan). The column was eluted stepwise with CH_3OH–H_2O (0, 30, 50, 60, 70, 80 and 100%) and fractionated. The 60% CH_3OH aq. fraction (39.7 mg) was purified by HPLC (Pegasil ODS SP100 (20φ × 250 mm) Senshu Scientific Co. Ltd., Tokyo, Japan) with 40% CH_3OH aq. at a flow rate of 7 ml min^{-1}, using detection by UV 210 nm to obtain **1** (4.8 mg, R_t = 29 min) and **2** (12.1 mg, R_t = 48 min). The 50% CH_3OH aq. fraction (18.0 mg) was separated by silica gel column (1.8φ × 25 mm, Merck KGaA, Darmstadt, Germany) with $CHCl_3$–CH_3OH (100/0, 100/1, 100/3, 100/5, 9/1, 1/1 and 0/100). The 100/5 fraction was evaporated under reduced pressure to yield **3** (3.3 mg).

The molecular formulae of **2** and **3** were elucidated by ESI-MS to be $C_{16}H_{20}O_5$ and $C_{16}H_{20}O_6$, respectively. The analyses of ^{1}H, ^{13}C and 2D NMR spectra data led to the identification of **2** and **3** as cladosporin[11] and 5′-hydroxyasperentin,[12] respectively.

Physico-chemical properties of **1** are shown in Supplementary Table S2. Compound **1** was obtained as dark yellow oil (UV (CH_3OH) λmax nm (ε): 207 (12104), 232 (7515), 272 (5513) and 328 (3819)). The molecular formula of **1** was elucidated as $C_{16}H_{20}O_6$ with seven degrees of unsaturation by a high-resolution electron spray ionization mass spectrometer (*m/z* 307.1185 [M-H]$^-$ (calcd. for $C_{16}H_{19}O_6$, 307.1182)). The IR characteristic absorptions of **1** at 3276, 2923, 2852, 1637, 1199, 1089 and 1016 cm^{-1} suggested the presence of hydroxyl and carbonyl groups. The ^{1}H and ^{13}C NMR spectra of **1** in CD_3OD are summarized in Table 1. Analyses of ^{1}H, ^{13}C NMR and HMQC spectra indicated the presence of one sp^3 methyl group, three sp^3 oxymethine groups, five sp^3 methylene groups and seven sp^2 carbons (including one carbonyl and six aromatic carbons, which suggested the presence of a benzene ring). The ^{1}H–^{1}H COSY analysis revealed two partial structures, from H_2-4 to H_2-11 and from H_2-13 to H_3-15. The HMBC correlations from H_3-15 to C-14 and C-13, from H_2-14 to C-10 and from H_2-11 to C-13 and C-12 suggested the presence of 2-methyltetrahydro-2*H*-pyran ring including two partial structures. Moreover, the HMBC correlations from H_2-4 to C-3, C-4a, C-5, C-8a and C-9 and from H-7 to C-1, C-8a, C-8, C-6 and C-5 revealed that **1** has a 6, 7, 9-trihydroxy-3, 4-dihydroisocoumarin skeleton and should be a new analog of **2** (Figure 1b). The comparison of the ^{13}C chemical shifts at C-6 (δ_C 155.6) and C-8 (δ_C 159.0) with those reported in literature,[14,15] confirmed the presence of this skeleton.

The relative configuration of **1** was established by ROESY correlations and coupling constants (Figure 1c). ROESY correlations of H-10/H_3-15, H-3/H-4α and H-3/H-9α and ^{1}H–^{1}H coupling constant of H-4β/H-3 (10.9 Hz), H-4α/H-3 (3.6 Hz) and H-9α/H-10 (11 Hz) the relative configuration of **1** should be 3 *R**, 10 *R**, 14 *S**, as shown in Figure 1c. Finally, the absolute configuration of **1** was determined to be the same 3 *R*,10 *R*,14 *S* by comparison of the optical rotation of **1** (−12.9) with that of **2** (−18.1), as reported in the literature.[16] The CD spectra of **1** and **2**, with positive Cotton effect (270 nm), also supported this absolute configuration[17] (Supplementary Figures S2-1, S2-2). The results described above confirmed that **1** is a new analog of cladosporin, which was named cladomarine.

Anti-oomycetes assays were carried out by a modification of previously reported methods.[2,3] Anti-oomycetes activity against *Saprolegnia parasitica* kassi1 and another oomycete, *Pythium* sp. sakari1, was evaluated using the paper disc method (8 mm, ADVANTEC, Tokyo, Japan). The two oomycete strains were isolated from Chum salmon hatcheries in Iwate Prefecture, Japan. *Saprolegnia parasitica* was cultured on glucose yeast extract (GY) seed medium (0.25% yeast extract, 0.25% glucose and 0.2% agar) and cultured at 27 °C for 2 days on a shaker at 300 r.p.m. The seed-culture (10%) and GY agar ((0.25% yeast extract, 0.25% glucose and 1.0% agar), 40 °C) were poured into plates and paper disks with samples for testing were placed to each plate. All samples were evaluated at three doses (100, 30 and 10 μg). Plates were incubated at 27 °C for 24 h. The *Pythium* sp. assay was conducted identically. Cultural conditions were as follows; (vegetable juce medium (12% vegetable juce (Kagome, Aichi, Japan) supernatant, 1.0% glucose and 1.0% agar), 3.0% inoculation, 27 °C, 24 h).

Table 1 ^{1}H and ^{13}C NMR data for cladomarine (1) (recorded at 400 MHz ^{1}H NMR and 100 MHz ^{13}C NMR in CD_3OD; δ in p.p.m.)

		1
Position	^{13}C	^{1}H (*J* in Hz)
1	171.8	–
2	–	–
3	77.7	4.61 (dddd, 3.6, 3.8, 9.1, 10.9)
4α	28.5	3.17 (dd, 3.6, 16.9)
4β		2.66 (dd, 10.9, 16.9)
4a	126.1	–
5	135.7	–
6	155.6	–
7	101.8	6.27 (s)
8	159.0	–
8a	100.2	–
9α	39.3	2.15 (ddd, 3.8, 11.0, 14.9)
9β		1.8 (ddd, 3.0, 9.1, 14.9)
10	68.3	4.14 (m)
11	31.4	1.35 (m)
		1.73 (m)
12	19.3	1.70 (m)
		1.70 (m)
13	32.7	1.32 (m)
		1.70 (m)
14	68.3	3.92 (m)
15	20.0	1.18 (d, 6.0)

Measured in CD_3OD.

Antimicrobial activities against two bacteria, a filamentous fungus and a yeast were also measured to reveal any selectivity differences between anti-*S. parasitica* activity and antimicrobial activity. All assays were carried out using the paper disc method described previously.[18] Test organisms (two bacteria and two fungi) used were as follows, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* NBRC 12582, *Aspergillus niger* ATCC 6275 and *Candida albicans* ATCC 64548.

We also determined the MIC of **1**, **2** and **3** against *S. parasitica*. MIC assay was carried out with a modification of previously reported methods.[2,3] *Saprolegnia parasitica* was cultured on corn meal agar (0.2% corn meal and 1.5% agar) at 15 °C for 5 days. 9 ml of GY agar (0.25% yeast extract, 0.25% glucose and 1.0% agar) and 1 ml of sample solution in 50% CH_3OH aq. was poured into a plate, with the final concentrations of samples being (64, 32 and 16 μg ml^{-1}). Subsequently, a corn meal agar seed cultured block of *S. parasitica* was put on each plate; the plates were incubated at 27 °C for 24 h. A solution of malachite green (fin. 25 μg ml^{-1}) was used as a positive control. The growth of *S. parasitica* was checked by observation to determine the MIC values.

Antimicrobial activities of **1**, **2** and **3** are summarized in Table 2. Among them, **2** was the most potent against *S. parasitica* (an MIC value of 2 μg ml^{-1}) along with *Pythium* sp. but was not active against the other organisms tested. Compound **1** was slightly active against the two oomycetes, while **3** did not show any activity. Structurally, because of the lack of a hydroxyl group, **2** is more hydrophobic than **1** and **3**. The difference between **1** and **3** is the position of a hydroxyl group, at C-5 and C-13, respectively. The high hydrophobicity of **2** may be important in affecting anti-oomycete activity. In addition, these compounds are more selective against the two oomycetes tested than malachite green, exhibiting a narrower antimicrobial activity. The results suggest that the cladosporin analog could be a promising lead candidate for the development of a new, less toxic compound to control saprolegniasis.

Table 2 Antimicrobial activity of cladomarine (1), cladosporin (2) and 5′-hydroxyasperentin (3)

	10 μg per disc (MIC: μg ml^{-1})			
Organism	***1***	***2***	***3***	*Malachite green*
Saprolegnia parasitica	13 (>64)	23 (2)	– (>64)	36
Pythium sp.	12	26	–	38
Bacillus subtilis	–	–	–	27
Pseudomonas aeruginosa	–	–	–	10
Aspergillus niger	–	–	–	32
Candida albicans	–	–	–	24

Inhibition zone (mm): no inhibition.

The mode of action on the anti-malarial activity of **2** was proposed to be inhibition of the *Plasmodium falciparum* lysyl-tRNA synthetase.[19] However, *Saccharomyces cerevisiae* lysyl-tRNA synthetase is not inhibited by **2**.[19] This selectivity of **2** against lysyl-tRNA synthetase might be associated with that against oomycetes. Very recently, the biosynthesis of **2** was identified by genome sequencing of *Cladosporium cladosporioides* UAMH 5063, which also produces **2**.[20] However, a gene encording an oxidase such as cytochrome P450 enzyme, which potentially oxidizes **2** at the C-5 position, were not found in the gene cluster of **2**. It seems likely that *P. coralligerum* YK-247, the producer of **1**, has an additional oxidase gene, as well as a similar gene cluster.

In conclusion, we isolated three compounds, **1**, **2** and **3**, from a cultured broth of the deep-sea fungus *P. coralligerum* YK-247. Compounds **1** and **2** showed selective inhibition of the growth of *S. parasitica*, together with a second oomycete, *Pythium* sp. The results demonstrate that cladomarine and cladosporin analogs could be good lead candidates in the development of much-needed novel agents to control saprolegniasis, while also confirming that the deep-sea fungi are indeed promising sources for new drug discovery.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We are grateful to Dr Kenichiro Nagai (School of Pharmacy, Kitasato University) for measurement of MS, to Mr Hiromu Iwasaki (School of Science, Kitasato University) for assistance with fungal cultivation and observation, and to Dr Yukihiro Asami (Kitasato Institute for Life Sciences, Kitasato University) for support concerning this study, and to Dr Hiroshi Kitazato (JAMSTEC) for arranging research cruise of the *Iatá-Piúna* cruise. This work was supported by the research project Tohoku Ecosystem-Associated Marine Sciences from the Ministry of Education, Culture, Sports, Science and Technology.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)