The Journal of Antibiotics (2018) 71:741–744
https://doi.org/10.1038/s41429-018-0053-z

JARA Japan Antibiotics Research Association
The Society for Actinomycetes Japan

BRIEF COMMUNICATION

Quellenin, a new anti-*Saprolegnia* compound isolated from the deep-sea fungus, *Aspergillus* sp. YK-76

Konami Takahashi[1,2,3] · Kazunari Sakai[3] · Wataru Fukasawa[4] · Yuriko Nagano[2] · Sakiko Orui Sakaguchi[2] · Andre O. Lima[5] · Vivian H. Pellizari[6] · Masato Iwatsuki[3,4] · Kiyotaka Takishita[2] · Takao Yoshida[2] · Kenichi Nonaka[3,4] · Katsunori Fujikura[2] · Satoshi Ōmura[3]

Received: 25 December 2017 / Revised: 19 March 2018 / Accepted: 27 March 2018 / Published online: 23 April 2018

Abstract

Saprolegnia parasitica, belonging to oomycetes, is one of virulent pathogen of fishes such as salmon and trout, and causes tremendous damage and losses in commercial aquacultures by saprolegniasis. Previously, malachite green, an effective medicine, had been used to control saprolegniasis. However, this drug has been banned around the world due to its mutagenicity. Therefore, novel anti-saprolegniasis compounds are urgently needed. As a new frontier to discover bioactive compounds, we focused on the deep-sea fungi for the isolation of anti-saprolegniasis compounds. In this paper, on the course of anti-saprolegniasis agents from 546 cultured broths of 91 deep-sea fungal strains, we report a new compound, named quellenin (1) together with three known compounds, diorcinol (2), violaceol-I (3) and violaceol-II (4), from deep-sea fungus *Aspergillus* sp. YK-76. This strain was isolated from an *Osedax* sp. annelid, commonly called bone-eating worm, collected at the São Paulo Ridge in off Brazil. Compounds 2, 3 and 4 showed anti-*S. parasitica* activity. Our results suggest that diorcinol and violaceol analogs and could be good lead candidates for the development of novel agents to prevent saprolegniasis.

Saprolegnia parasitica is an oomycete pathogen, a virulent water mold infecting fishes such as salmon and trout, the resultant saprolegniasis causing tremendous damage and losses in commercial aquaculture. Previously, malachite green was used to control saprolegniasis but its use has been widely banned as eating fish contaminated with malachite green poses a significant risk due to its reported carcinogenicity. Therefore, novel anti-saprolegniasis compounds are urgently needed [1, 2]. We focused on deep-sea fungi, which were known as producers of new source of novel useful compounds such as spiromastols A–K [3], emerixanthones A–D [4] and aspiketolactonol [5]. In this paper, we attempt to isolate anti-saprolegniasis compounds from deep-sea fungi.

Our screening for anti-saprolegniasis agents from 546 cultured broths of 91 deep-sea fungal strains has already resulted in the discovery of cladomarine produced by *Penicillium coralligerum* YK-247 [1]. In this paper, we additionally report a new compound, named quellenin (**1**) (Fig. 1a), from the cultured broth of a deep-sea fungus, *Aspergillus* sp. YK-76, which we isolated together with three known compounds, diorcinol

Electronic supplementary material The online version of this article (https://doi.org/10.1038/s41429-018-0053-z) contains supplementary material, which is available to authorized users.

✉ Kenichi Nonaka
ken@lisci.kitasato-u.ac.jp

✉ Satoshi Ōmura
omuras@insti.kitasato-u.ac.jp

1 Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Tokyo, Japan

2 Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Kanagawa, Japan

3 Kitasato Institute for Life Sciences, Kitasato University, Tokyo, Japan

4 Graduate School of Infection Control Sciences, Kitasato University, Tokyo, Japan

5 Department of Biological Sciences, University of Vale do Itajaí, CTTMar, R. Uruguai, 458-CEP, 88302-202 Itajaí, Brazil

6 Institute of Oceanography, University of São Paulo, 191. Praca do Oceanografico, São Paulo 05508120, Brazil

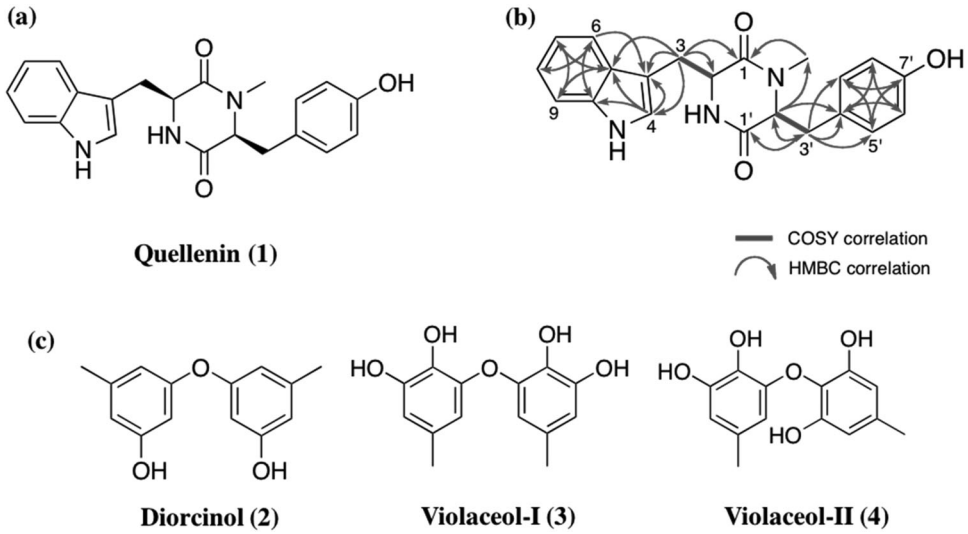

Fig. 1 **a** Structure of quellenin (**1**). **b** COSY and HMBC correlation of quellenin (**1**). Bold lines show ^{1}H–^{1}H correlation, arrows show ^{1}H–^{13}C correlation. **c** Structure of diorcinol (**2**), violaceol-I (**3**) and violaceol-II (**4**)

Table 1 ^{1}H and ^{13}C NMR data for (**1**) (recorded at 400MHz ^{1}H NMR and 100MHz ^{13}C NMR in CD_3OD; ∂ in ppm)

Amino acid	Position	^{13}C	**1** ^{1}H (multiplicity, *J* in Hz)
L-Trp	1	168.5	—
	2	57.2	4.12 (dd, 4.1, 8.3)
	3	31.8	2.16 (dd, 8.3, 14.5)
			2.98 (dd, 4.1, 14.5)
	4	125.5	6.97 (s)
	5	110.1	—
	5a	128.6	—
	6	119.7	7.53 (dd, 1.2, 7.6)
	7	120.1	7.03 (ddd, 1.2, 7.0, 7.6)
	8	122.6	7.12 (ddd, 1.2, 7.0, 8.4)
	9	112.5	7.35 (dd, 1.2, 8.4)
	9a	138.2	—
N-methyl-L-Tyr	1'	169.0	—
	2'	65.1	4.03 (dd, 4.3, 5.7)
	3'	38.4	2.13 (dd, 5.7, 14.3)
			2.77 (dd, 4.3, 14.3)
	4'	128.2	—
	5'	131.9	6.67 (2H, d, 8.8)
	6'	116.5	6.70 (2H, d, 8.8)
	7'	157.9	—
	8'	34.2	2.76 (3H, s)

Measured in CD_3OD

(**2**) [6], violaceol-I (**3**) [7] and violaceol-II (**4**) [7] (Fig. 1c).

Strain YK-76 was isolated from an *Osedax* sp. annelid, commonly called bone-eating worm, collected at the São Paulo Ridge in off Brazil (water depth: 4203 m, sampling site: 28°31.1′S and 41°39.4′W), using the human-occupied vehicle *Shinkai 6500* of the Japan Agency for Marine-Earth Science and Technology (JAMSTEC), as part of a Japan-Brazil cooperative deep-sea investigation named *Iatá-Piúna* cruise [8].

The internal transcribed spacer of ribosomal RNA gene (ITS-rDNA) sequence of YK-76 was compared to sequences in the GenBank database by BLASTN 2.7.1 analysis [9]. The sequence of YK-76 was 99.6% similar to the sequence of NRRL 58600 (ex-type of *Aspergillus jensenii*, GenBank accession number NR_135444). The producing strain YK-76 was assigned to the genus *Aspergillus* based on its morphology and sequence analysis.

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% $MgSO_4$ $7H_2O$, 0.5% Polypeptone, 0.1% KH_2PO_4 and 0.1% agar, pH 6.0) in a test tube (total 20 ml) and incubated at 27 °C for 3 days on a shaker at 300 rpm. The seed-culture (20 ml) was inoculated into 500 g of rice medium in a single culture bag (Ulpak 47, Hokken Co. Ltd., Tochigi, Japan) and static fermentation was carried out at room temperature (about 25 °C) for 13 days. The cultured rice medium (500 g) was subsequently added to 500 ml of ethanol and then filtered. The filtrate was concentrated under reduced pressure to remove ethanol and extracted with EtOAc (500 ml) three times. The EtOAc extract (1.91 g) was applied to an octadecylsilane (ODS) gel column (25φ × 100 mm, YMC Co. Ltd., Kyoto, Japan), eluted stepwise with MeOH-H_2O (0, 30, 50, 60, 70, 80, 90 and 100%) and fractionated. The 50% MeOH aq. fraction (343.5 mg) was separated by MPLC (silica gel column, 25φ × 100 mm, Shoko Scientific Co.,

Table 2 Antimicrobial activity of quellenin (**1**), diorcinol (**2**), violaceol-I (**3**) and violaceol-II (**4**)

organism	**1** 200μg/disc	**2** 30μg/disc	**3** 30μg/disc	**4** 30μg/disc	malachite green 10μg/disc
Saprolegnia parasitica	19.9	17.5	16.5	13.7	36
Pythium sp.	—	13.0	—	—	38
Bacillus subtilis	—	11.8	16.0	14.7	27
Pseudomonas aeruginosa	—	—	—	—	10
Aspergillus niger	—	—	—	—	32
Candida albicans	—	—	—	—	24

Inhibition zone (mm), — No inhibition

Ltd., Kanagawa, Japan) with $CHCl_3$-MeOH (100/0, 100/1, 100/3, 100/5, 9/1, 1/1 and 0/100). Fractions 31-42 eluted with $CHCl_3$-MeOH (100/3-100/5) were combined and purified by HPLC (YMC Actus Triart C18 (20φ × 250 mm) YMC Co. Ltd., Kyoto, Japan) with 25% acetonitrile aq. with a flow rate of 14 ml/min, using detection by UV 210 nm to obtain **1** (4.7 mg, $R_t = 15$ min), **3** (20.4 mg, $R_t = 33$ min) and **4** (27.4 mg, $R_t = 40$ min). The 60% MeOH aq. fraction (321.6 mg) was separated by silica gel column (30φ × 100 mm, Merck KGaA, Darmstadt, Germany) with $CHCl_3$–MeOH (100/0, 100/1, 100/3, 100/5, 9/1, 1/1 and 0/100). The 100/5 fraction (21.2 mg) was purified by HPLC (Develosil C30-UG-5 (20φ × 250 mm) Nomura Chemical Co. Ltd., Aichi, Japan) with 30% acetonitrile aq. with a flow rate of 14 ml/min, using detection by UV 210 nm to obtain **2** (4.7 mg, $R_t = 80$ min).

The molecular formulae of **2**, **3** and **4** were elucidated by HR-ESI-MS to be $C_{14}H_{14}O_3$, $C_{14}H_{14}O_5$ and $C_{14}H_{14}O_5$, respectively. The comparison and analysis of ^1H, ^{13}C and 2D NMR spectra data with the literature led to the identification of **2**, **3** and **4** as diorcinol [6], violaceol-I [7] and violaceol-II [7], respectively.

Physico-Chemical properties of **1** are shown in Table S2. Compound **1** was obtained as a white solid (UV (MeOH) λ_{max} nm (ε): 205 (30,129), 221 (38,550), 280 (6751) and 290 (5,190)). The molecular formula of **1** was elucidated as $C_{21}H_{21}O_3N_3$ by high-resolution electron spray ionization mass spectrometry (m/z 362.1501 $[M–H]^-$ (calcd. for $C_{21}H_{20}O_3N_3$, 362.1505)) with thirteen degrees of unsaturation. The IR characteristic absorptions of **1** at 3432, 1666 and 1639 cm^{-1} suggested the presence of hydroxyl and carbonyl groups. The ^1H and ^{13}C NMR spectra of **1** in CD_3OD are summarized in Table 1. Analyses of ^1H, ^{13}C NMR and HMQC spectra indicated the presence of one nitrogenated methyl group, two sp^3 methylene groups, two nitrogenated sp^3 methine carbons and 14 sp^2 aromatic carbons and two carbonyl carbons. The ^1H-^1H COSY analysis revealed two partial structures, from H-2′ to H_2-3′ and from H-2 to H_2-3 (Fig. 1b). The HMBC correlations from H-9 to C-7 and C-5a, from H-8 to C-6, from H-7 to C-9 and C-5a, from H-6 to C-9a, C-8 and C-5, from H-4 to C-9a, C-5a and C-5 and from H_2-3 to C-5a, C-5, C-2 and C-1 suggested the presence of a tryptophan (Trp) residue, including one partial structure. Moreover, the HMBC correlations from H-6′ to C-4′, from H-5′ to C-7′, C-5′ and C-3′, from H_2-3′ to C-5′, C-4′, C-2′ and C-1′ and H-2′ to C-8 revealed **1** has a *N*-methyl-tyrosine (*N*-methyl-Tyr) moiety. Finally, the HMBC correlations from H_3-8′ to C-1 suggested **1** should be cyclo (Trp-*N*-methyl-Tyr). So far, cyclo (D-Trp-D-*N*-methyl-Tyr) has only been reported as a synthetic compound with possible four stereoisomers [10]. Because ^1H and ^{13}C NMR data in acetonitrle-d_3 for **1** was identical with those of the synthetic one described in the literature, the relative configuration of **1** was the same as the synthetic compound. However, the specific rotation ($[\alpha]_D^{25}$($c = 0.1$, acetonitrle) −99.62) of **1** was opposite to that of the synthetic compound ($[\alpha]_D^{25}$ + 157.1), suggesting that **1** is cyclo (L-Trp-L-*N*-methyl-Tyr), an enantiomer of the synthetic product.

The absolute configuration of **1** was confirmed by the Advanced Marfey's method after acid hydrolysis [11]. Compound **1** was hydrolyzed and derivatized with L-FDLA and analyzed by an UPLC-ESI-MS. As a result of the comparison of retention time with L-FDLA derivatives of standard, Trp was assigned to be the L configuration (Table S3, Fig. S3). The results described above confirmed that **1** is a new analog of diketopiperazine, which we named quellenin.

Antimicrobial activity against bacteria, filamentous fungus and yeast, as well as oomycetes, were measured using the Paper disc method, as described previously [1, 12]. Test organisms were as follows; two oomycetes (*Saprolegnia parasitica* kassi1, *Pythium* sp. sakari1), two bacteria (*Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* NBRC 12582), one filamentous fungus (*Aspergillus niger* ATCC 6275) and one yeast (*Candida albicans* ATCC 64548). Compounds **1**, **2**, **3** and **4** were evaluated at five doses (100, 30, 10, 3 and 1 μg). Compound **1** was also evaluated at a higher dosage of 200 ug.

Compounds **2**, **3** and **4** showed anti-*Saprolegnia* activity, with inhibition zones of 13.7–17.5 mm at 30 µg/disc (Table 2). Although they also showed anti-*Bacillus* activity, they showed anti-*Saprolegnia* activity with better selectivity than malachite green. Compound **1** displayed weaker activity against *S. parasitica*, with an inhibition zone of 19.9 mm at 200 µg/disc, but showed better selectivity against *S. parasitica* (without anti-*Bacillus* activity) compared to **2**, **3** and **4**. We determined the minimum inhibitory concentrations (MICs) of **2**, **3** and **4**, according to a previously reported method [1], finding MICs of 8, 32 and 16, respectively.

Compounds **3** and **4** have been reported to be actin inhibitors, inducing cell shape elongation in fibroblast cells and impairment of mitochondrial function causing uncoupling of oxdative phosphorylation [13, 14]. Therefore, the bioactivity of **3** and **4** may be associated with these functions. Moreover, **3** and **4** have been identified as anti-malarial agents [15], cladosporin, previously reported as an anti-saprolegniasis lead compound by our group [1], also displays anti-malarial properties. Thus, other anti-malarial agents might be a good source for anti-*Saprolegnia* screening. With regards to 1, other compounds containing a diketopiperazine moiety, such as cyclo(L-Pro-L-Leu) and cyclo-(D-Pro-L-Tyr) [16], have been reported to exert many biological activities. The enatiomer of **1** was known as a synthetic intermediate, but hasn't been reported in any bioassays. Further structural modifications of **1** or isolation of other structural analogs may be valuable in the development of new anti-parasitic agents.

In conclusion, three known 2–4 and one new compound **1** were isolated from a cultured broth of deep-sea fungus *Aspergillus* sp. YK-76. Compounds **2**, **3** and **4** showed inhibition of the growth of *S. parasitica*, together with several other microbes. Our results suggest that a diorcinol analog could be a good lead candidate in the development of novel agents to help prevent infection with *Saprolegnia*. Moreover, fungi from deep-sea locations represent a highly-promising new source for the possible discovery of novel bioactive lead compounds.

Acknowledgements We are grateful to Dr. Kenichiro Nagai (School of Pharmacy, Kitasato University) for mass spectra measurements and to Dr. Hiroshi Kitazato (JAMSTEC) for coordination of the research cruise of the *Iatá-Piúna*. This work was supported by a Tohoku Ecosystem-Associated Marine Sciences research project of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Takahashi K, et al. Cladomarine, a new anti-saprolegniasis compound isolated from the deep-sea fungus, *Penicillium coralligerum* YK-247. J Antibiot. 2017;70:911–4.
2. Nakagawa K, Hara C, Tokuyama S, Takada K, Imamura N. Sapromycins A-E, new angucycline antibiotics active against *Saprolegnia parasitica*. J Antibiot. 2012;65:559–607.
3. Niu S, Liu D, Proksch P, Shao Z, Lin W. New polyphenols from a deep-sea *Spiromastix* sp. fungus, and their antibacterial activities. Mar Drugs. 2015;13:2526–40.
4. Fredimoses M, et al. New Prenylxanthones from the deep-sea derived fungus *Emericella* sp. SCSIO 05240. Mar Drugs. 2014;12:3190–202.
5. Chen XW, et al. Nine new and five known polyketides derived from a deep sea-sourced *Aspergillus* sp. 16-02-1. Mar Drugs. 2014;12:3116–37.
6. Itabashi T, Nozawa K, Nakajima S, Kawai K. A new azapholone, falconensin H, from *Emericella falconensis*. Chem Pharm Bull. 1993;41:2040–1.
7. Yamazaki M, Maebayashi Y. Structure determination of violaceol-I and -II, new fungal metabolites from a strain of *Emericella violacea*. Chem Pharm Bull. 1982;30:514–8.
8. Sumida PYG, et al. Deep-sea whale fall fauna from the Atlantic resembles that of the Pacific Ocean. Sci Rep. 2016;6:22139.
9. Altschul SF, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997;25:3389–402.
10. Zhao JC, Yu SM, Qiu HB, Yao ZJ. Total synthesis of ent-(-)-azonazine using a biomimetic direct oxidative cyclization and structural reassignment of natural product. Tetrahedron. 2014;70:3197–210.
11. Harada K, Fujii K, Hayashiand K, Suzuki M. Application of D,L-FDLA derivatization to determination of absolute configuration of constituent amino acids in peptide by advanced Marfey's method. Tetrahedron Lett. 1996;37:3001–4.
12. Iwatsuki M, et al. Guadinomines, Type III secretion system inhibitors produced by *Streptomyces* sp. K01-0509. II. Physicochemical properties and structure elucidation. J Antibiot. 2008;61:230–6.
13. Asami Y, et al. Violaceols function as actin inhibitors inducing cell shape elongation in fibroblast cells. Biosci Biotechnol Biochem. 2012;76:1431–7.
14. Nakamaru T, et al. The effects of toxic metabolites, violaceol-I and -II, from *Emericella violacea* on mitochondrial respiration. Proc Jpn Assoc Mycotoxicol. 1984;19:30–33.
15. Bunyapaiboonsri T, Yoiprommarat S, Intereya K, Kocharin K. New diphenyl ethers from the insect pathogenic fungus *Cordyceps* sp. BCC 1861. Chem Pharm Bull. 2007;55:304–7.
16. Kumar SN, et al. Identification of antimicrobial compound, diketopiperazines, from a *Bacillus* sp. N strain associated with a rhabditid entomopathogenic nematode against major plant pathogenic fungi. J Appl Microbiol. 2012;113:914–24.