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CONCISE ARTICLE

Cytotoxic and antiviral nitrobenzoyl sesquiterpenoids from the marine-derived fungus *Aspergillus ochraceus* Jcma1F17†

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Nitrobenzoyl sesquiterpenoids are rare in nature sources, and fungi *Aspergillus* species are the only sources of them. A new nitrobenzoyl sesquiterpenoid, 6β,9α-dihydroxy-14-*p*-nitrobenzoylcinnamolide (**1**), and a known analogue, insulicolide A (**2**), were isolated from extracts of the culture of marine-derived fungus *Aspergillus ochraceus* Jcma1F17, which was identified by morphological and ITS phylogenetic analyses. The structures were determined by NMR, MS, CD, and optical rotation analysis. Both nitrobenzoyl sesquiterpenoids displayed significant cytotoxicities against 10 cancer cell lines, and the new one (**1**) also showed antiviral activities against H3N2 and EV71. It's the first time to report nitrobenzoyl sesquiterpenoids from *A. ochraceus*.

Introduction

Sesquiterpenoids are mainly found in higher plants, microorganisms, and marine life, and many of them provide encouraging leads for pharmacological agents, especially cytotoxic anticancer agents.[1] Nitrobenzoyl sesquiterpenoids are rare in nature sources. There are only four nitrobenzoyl sesquiterpenoids reported by now, and all of them were obtained from marine-derived *Aspergillus* fungi.[2-4] Insulicolide A displayed significant cytotoxicity against HCT-116 colon carcinoma cells *in vitro* and exhibited moderately selective cytotoxicity toward a panel of renal tumor cell lines.[2] Nitrobenzoyl sesquiterpenoids should capture the attention they deserve because of their good antitumor potential.

In the current study, insulicolide A and another new nitrobenzoyl sesquiterpenoid were obtained from organic extracts of the culture of marine alga-derived *A. ochraceus* Jcma1F17. Both nitrobenzoyl sesquiterpenoids (**1** and **2**, **Figure 1**) displayed significant cytotoxicities against 10 human cancer cell, and the new sesquiterpenoid (**1**) also exhibited inhibitory activities against influenza virus A/Hong Kong/8/68 (H3N2) and human enterovirus 71 (EV71). It is the first time to report nitrobenzoyl sesquiterpenoids obtained from *A. ochraceus*.

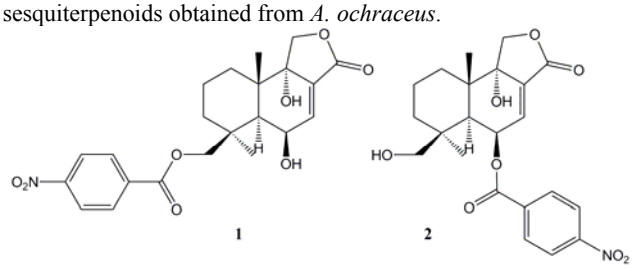

Figure 1. Structures of compound **1** and **2**.

Results

Characterization and identification of isolated strain Jcma1F17

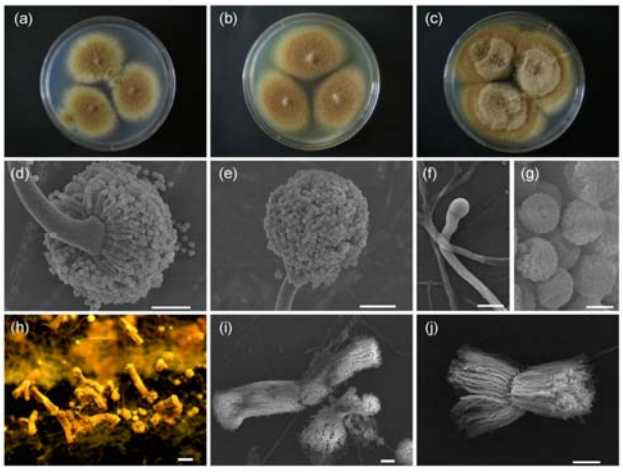

Figure 2. Colony appearance and micromorphology of *A. ochraceus* Jcma1F17.

(**a**–**c**) Colony appearance after 7 days at 25 °C (a, CA; b, SA; c, PDA); (**d**–**e**) Conidiophores after 7 days at 25 °C under SEM; (**f**) Vesicle under SEM; (**g**) Conidia as seen using SEM; (**h**) Conidiophores after 14 days at 25 °C under light microscope; (**i**–**j**) Conidiophores after 14 days at 25 °C under SEM. Bars: 10 μm (d–f), 1 μm (g), 250 μm (h), and 50 μm (i–j).

Fungal strain Jcma1F17, which isolated from a marine alga *Coelarthrum* sp. collected in Paracel Islands, South China Sea, exhibited potential cytotoxic activity in our previous screening tests. After 7 d of growth on Czapek agar (CA) medium at 25 °C,

colonies were 54 mm to 56 mm in diameter, showed good sporulation, were mainly middle yellowish brown at the center, and had a pale orange–yellow periphery with a regular margin, whereas the color of the reverse was the same as that of the surface (**Figure 2a**). After 7 d of growth on Sabouraud agar (SA) medium at 25 °C, colonies were 48 mm to 51 mm in diameter, showed good sporulation, and had a yellow–white center surrounded by a pale orange–yellow edge with a regular margin, whereas the reverse was dominated by a middle yellow color and radioactive wrinkled (**Figure 2b**). After 7 d of growth on potato dextrose agar (PDA) medium at 25 °C, colonies were 64 mm to 68 mm in diameter, displayed very good sporulation, were light yellowish pink at the centre and surrounded by a yellowish brown edge with a regular margin whilst the reverse was dominated by deep yellowish brown (**Figure 2c**).

Scanning electron microscopy (SEM) revealed that the mycelia were composed of branched, septate, smooth-walled hyphae 1.3 µm to 2.7 µm wide (mean = 2.1 µm). Conidial heads radiated, normally splitting into two to four dense columns (up to 500 µm long) with age. Stipes were 69 µm to 242 µm long (mean = 181 µm), were 3.7 µm to 6.0 µm wide (mean = 5.3 µm), had rough walls, and were light orange pigmented. Vesicles were globose, 6.8 µm to 10.9 µm in diameter (mean = 8.9 µm), and biseriate. Metulae were smooth walled and cylindrical, measuring 1.7 to 3.1 (mean = 2.6) × 1.7 to 2.1 (mean = 1.9) µm. Phialides were smooth walled and flask shaped, measuring 4.3 to 5.7 (mean = 4.8) × 1.2 to 1.3 (mean = 1.2) µm, with 0.6 µm to 1.4 µm–long collula. Conidia were middle orange–yellow and globose to subglobose when mature, measuring 1.4 to 1.7 (mean = 1.5) µm (**Figures 2d** to **2j**), and roughened. A teleomorphic state was not observed.

ITS1-5.8S-ITS2 sequence region (550 basepairs (bp), accession number KF793915) of strain Jcma1F17 was amplified by PCR and sequenced. A phylogenetic tree was constructed, using the maximum-likehood method based on similarity of a 536-bp consensus length of ITS1-5.8S-ITS2 sequence (**Figure S1,** Supporting Information). Strain Jcma1F17 was found to belong to a clade related to *A. ochraceopetaliformis* and *A. ochraceus* in the tree, with sequence identities of 99% and 98%, respectively. The properties of culture and morphology of strain Jcma1F17 were consistent with those of *A. ochraceus* as previously. [5] The ITS phylogenetic analyses confirmed that the fungus strain Jcma1F17 belonged to *A. ochraceus*, and was designated as *A. ochraceus* Jcma1F17.

Structural elucidation

After large-scale fermentation and bioassay-guided isolation, a new nitrobenzoyl sesquiterpenoid (6β,9α-dihydroxy-14-*p*-nitrobenzoylcinnamolide, **1**) and a known analogue (insulicolide A, **2**) were obtained and structurally characterized.

Compound **2** was obtained as white amorphous powder; $[\alpha]^{20}_{D}$ −115.3° (*c* 0.3, EtOH); CD (*c* 0.03, EtOH) (*Δ*ε) 212 (−11.698), 262 (−2.42); HRESIMS revealed a molecular ion peak of *m/z* 454.1480 for $C_{22}H_{25}NO_8Na$ $[M + Na]^+$ (Calcd. 454.1472) and suggested 431 as the molecular weight and $C_{22}H_{25}NO_8$ as the molecular formula. Compound **2** was determined to be 9α,14-dihydroxy-6β-*p*-nitrobenzoylcinnamolide (insulicolide A) according to 1D-NMR (^{1}H NMR and ^{13}C NMR, Table 1), HSQC, HMBC, OR, andCD data and by comparison with data reported in literature. [3, 4]

Compound **1** was obtained as white amorphous powder; $[\alpha]^{20}_{D}$ −99.3°(*c* 0.3, EtOH); CD (*c* 0.03, EtOH) (*Δ*ε) 225 (−7.78), 262 (−1.95); HRESIMS revealed a molecular ion peak of *m/z* 454.1480 for $C_{22}H_{25}NO_8Na$ $[M+Na]^+$ (Calcd. 454.1472) (**Figure S2**) and suggested 431 as the molecular weight and $C_{22}H_{25}NO_8$ as the molecular formula, same to compound **2**. The ^{1}H NMR spectrum of **1** (**Figure S3**, **Table 1**) displayed signals attributed to two methyl groups at δ_H 1.20 (3H, s, H-13) and 1.35 (3H, s, H-15); two oxygenated methylenes at δ_H 4.23 (1H, d, *J* = 9.9 Hz, H-11), 4.55 (1H, d, *J* = 9.9 Hz, H-11), 5.02 (1H, d, *J* = 11.4 Hz, H-14), and 5.18 (1H, d, *J* = 11.4 Hz, H-14); an oxygenated methine at δ_H 4.72 (1H, dd, *J* = 4.0, 4.6 Hz, H-6); and five olefinic or aromatic protons at δ_H 6.82 (1H, d, *J* = 4.0 Hz, H-7), 8.26 (2H, br.d, *J* = 8.9 Hz, H-3′, 7′), and 8.38 (2H, br.d, *J* = 8.9 Hz, H-4′, 6′). The signals in the ^{13}C NMR spectrum (**Figure S4**) at δ_C 164.9 (COO, C-1′), 135.9 (C-2′), 130.3 (CH, C-3′, 7′), 123.3 (CH, C-4′, 6′), and 150.7 (-C-NO_2, C-5′), and the HMBC correlations (**Figure. S5**) from H-3′(7′, δ_H 8.26) to C-1′, C-2′, C-5′, respectively, and from H-4′ (6′, δ_H 8.38) to C-5′ indicated a typical *p*-nitrobenzoate moiety (**Figure 3**). Compound **1** was suggested to be an isomer of insulicolide A (**2**), according to 1D-NMR (^{1}H NMR and ^{13}C NMR, **Table 1**) and 2D-NMR (HMBC and HSQC) (**Figures S5** and **S6**) and by comparison with data of compound **2**. The HMBC correlations from H-14 (δ_H 5.02, 5.18, d, *J* = 11.4 Hz) to C-1′ (δ_C 164.9) indicated that the *p*-nitrobenzoate moiety is connected to C-14. The chemical shifts of H-6 and H-14 between compounds **1** and **2** also supported the C-14 position of *p*-nitrobenzoate moiety. Thus, compound **1** was suggested to be 6,9-dihydroxy-14-*p*-nitrobenzoylcinnamolide.

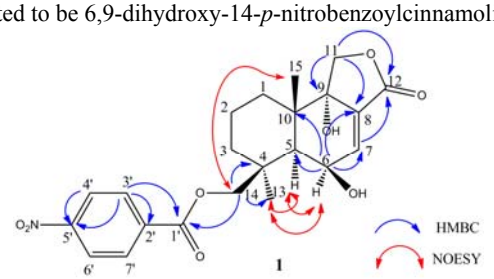

Figure 3. Key HMBC and NOESY correlations of compound **1**.

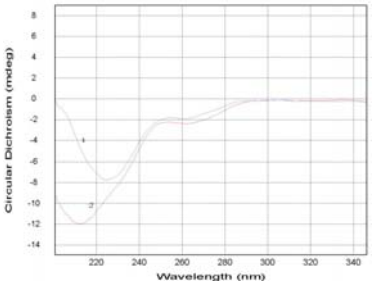

Figure 4. Circular dichroism spectrum of compounds **1** and **2.**

The NOESY correlations (**Figure S7**) from H-14 (δ_H 5.18) to H-15 (δ_H 1.35), H-13 (δ_H 1.20) to H-5 (δ_H 2.20) and H-6 (δ_H 4.72), and H-5 to H-6 revealed the relative configulation of C-4, C-5, C-6, and C-10. The OR data ($[\alpha]^{20}_{D}$ −99.3°, *c* 0.3, EtOH) and CD data {(*c* 0.03, EtOH) (*Δ*ε) 225 (−7.78), 262 (−1.95)} (**Figure 4**) of **1** suggested the same stereochemistry with **2**. Taken together, the complete structure of **1** was resolved as 6β,9α-dihydroxy-14-*p*-nitrobenzoylcinnamolide.

Table 1. NMR Spectroscopic Data (500 MHz, MeOD) for Compounds **1** and **2**.

	1		2	
No.	δ_C, type	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	31.5, CH_2	1.29, m 2.08, m	33.3	1.37, m 2.17, m
2	17.3, CH_2	1.57, m 1.75, m	18.7	1.57, m 1.72, m
3	36.3, CH_2	1.17, m 2.16, m	36.9	1.01, m 2.24, m
4	38.6, C		40.5	
5	47.9, CH	2.20, d (4.6)	48.0	2.49, d (4.6)
6	63.6, CH	4.72, dd (4.6, 4.0)	65.5	6.14, dd (4.6, 4.0)
7	138.9, CH	6.82, d (4.0)	133.3	6.79, d (4.0)
8	129.4, C		134.9	
9	76.6, C		77.8	
10	38.1, C		40.5	
11	75.1, CH_2	4.55, d (9.9) 4.23, d (9.9)	76.4	4.57, d (9.9) 4.27, d (9.9)
12	170.5, C		171.0	
13	26.1, CH_3	1.20, s	27.4	1.12, s
14	68.6, CH_2	5.18, d (11.4) 5.02, d (11.4)	69.0	4.14, d (11.1) 3.53, d (11.1)
15	19.9, CH_3	1.35, s	21.9	1.36, s
1′	164.9, C		165.2	
2′	135.9, C		136.4	
3′ 7′	130.3, CH	8.26, br.d (8.9)	132.1	8.25, br.d (8.9)
4′ 6′	123.3, CH	8.38, br.d (8.9)	125.0	8.39, br.d (8.9)
5′	150.7, C		152.4	

Biological activities

Two sesquiterpenoid nitrobenzoyl esters (**1** and **2**) were evaluated for their cytotoxic, antiviral, and antituberculosis activities. Both displayed significant cytotoxicity against 10 human cancer cell lines (H1975, U937, K562, BGC-823, Molt-4, MCF-7, A549, Hela, HL60, and Huh-7), with IC_{50} values of 1.95 μM to 6.35 μM (**Table 2**). The new compound **1** also exhibited moderate inhibitory activity against two viruses, H3N2 and EV71, with IC_{50} value of 17.0 and 9.4 μM, respectively. Compound **2** revealed no obvious activities against H3N2 and EV71 (**Table 2**). In addition, no obvious antituberculosis activities of both compounds were found against *Mycobacterium tuberculosis* H37Ra in our tests.

Materials and methods

General experimental procedures.

Optical rotations were measured with a PerkineElmer 341 polarimeter. Circular dichroism spectra were measured with a Chirascan circular dichroism spectrometer (Applied Photophysics, Ltd). NMR spectra were obtained on a Bruker AVANCE-500 spectrometer with TMS as internal standard, and chemical shifts were recorded as *δ*-values. ESI-MS and HRESI-MS were performed on a Thremo LCQ-DECA-XP LC-MS spectrometer and a Q-Tof Micro mass spectrometer, respectively. Silica gel (100 to 200 and 200 to 300 mesh), Sephadex LH-20, and ODS (500/400 mesh) for column chromatography were purchased from Qingdao Marine Chemical Group Co. (Qingdao, China), GE Healthcare (Uppsala, Sweden), and YMC (Kyoto, Japan), respectively. All solvents used were of analytical grade (Tianjin Fuyu Chemical and Industry Factory). Semipreparative HPLC was performed using an ODS column (Sunfire, Prep C_{18} OBD, 10 mm × 250 mm, 5 μm).

Table 2 Cytotoxic and Antiviral activities of Compounds **1** and **2.**

Cell lines or viruses	IC_{50} (μM)		
	1	**2**	Positive control[a]
H1975	2.08	4.63	0.09
U937	1.95	3.97	0.05
K562	4.33	4.76	0.16
BGC823	2.32	2.78	0.08
Molt-4	2.39	2.11	0.03
MCF-7	4.25	6.08	0.06
A549	2.41	2.86	0.05
Hela	6.12	6.35	0.10
HL60	2.44	2.34	0.03
Huh-7	3.28	2.35	0.09
H3N2	17	> 50	0.008
EV71	9.4	> 50	0.60

[a] Trichostatin A was used as the positive control in the cytotoxic bioassays against ten human cancer cell lines; the antiviral drug oseltamivir (Tamiflu) was used as the positive control against H3N2 fluvirus; ribavirin was used as the positive control against EV71 virus.

Microbial strains.

The fungal strain Jcma1F17 was isolated from a marine alga *Coelarthrum* sp. collected in Paracel Islands, South China Sea, and grown on MB agar at 25 °C.[6] This strain was stored on MB agar slants at 4 °C and then deposited at the China General Microbiological Culture Collection (Beijing) as CGMCC 8180.

Cultural and morphological properties of strain Jcma1F17.

The cultural properties of strain Jcma1F17 were examined on SA (consisting of 40 g dextrose, 10 g peptone, 2.5 g NaCl, 15 g agar, and 1000 mL distilled water, pH 5.6), CA (consisting of 30 g sucrose, 3 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4$, 2.5 g NaCl, 15 g agar, and 1000 mL distilled water), and PDA (consisting of 200 g potato, 20 g dextrose, 2.5 g NaCl, 15 g agar, and 1000 mL distilled water) after culturing at 25 °C for 7 d. The morphological features of the spores and mycelia in the 7- and 14-d cultures grown on CA were examined. The samples were observed with a Hitachi S-3400N scanning electron microscope and a LEICA MZFLIII light microscope using a previously described cover technique.[7, 8]

ITS region sequence and phylogenetic analysis.

The mycelia of strain Jcma1F17 cultured in SA were sampled and powdered in a mixer mill after liquid nitrogen was added. DNA was isolated through the AxyPrep™ multisource genomic DNA Miniprep Kit according to the manufacturer's protocol. The ITS

region of strain Jcma1F17 was amplified by polymerase chain reaction with the primer pair ITS1–ITS4. The amplified product was purified with a TIANgel mini purification kit (TianGen Biotech Beijing). Pure PCR product was submitted for sequencing together with the primer ITS1 to a commercial service (Invitrogen Biotechnology Co., Ltd.). The derived ITS region sequence was compared against the GenBank database (NCBI) through BLAST-Algorithmus. Similarity analysis was performed using ClustalW program (Thompson et al. 1994).[9] The phylogenetic tree of strain Jcma1F17 was constructed using maximum-likelihood method (ML), as previously described.[10] *Aspergillus versicolor* ATCC9577 was used as an outgroup.

Nucleotide sequence accession number.

The nucleotide sequence of the ITS region reported in this article was assigned the GenBank accession number KF793915.

Fermentation in shaken flasks.

Strain Jcma1F17 stored on MB slants at 4 °C was cultured on MB agar plates and then incubated for 7 d. Seed medium (consisting of 6.25 g maltose, 6.25 g malt extract, 1 g yeast extract, 6.25 g peptone, 1.25 g potassium dihydrogen phosphate, and 1000 mL distilled water, pH 7.0) in 500 mL Erlenmeyer flasks was inoculated with strain Jcma1F17 and then incubated at 25 °C for 3 d on a rotating shaker (120 rpm). Production medium (the same as the seed medium) in 500 mL flasks was inoculated with 10% seed solution. The flasks were incubated at 28 °C statically. After 7 d, broth from 100 flasks (15 L) was harvested to isolate substances.

Fractionation and purification of bioactive compounds.

The broth (15 L) was extracted with 10 L ethyl acetate stiring three times for 30 min. The ethyl acetate was filtrered and then concentrated *in vacuo* to yield a crude extract (9.0 g). The crude extract was subjected to a silica gel column chromatograph and was separated by a linear gradient of petroleum ether (60 °C to 90 °C)/EtOAc (50:0, 50:1, 20:1, 10:1, 5:1, 3:1, 2:1, 1:1, and 0:1) to yield nine fractions (fr.I- fr.IX). fr.VII (petroleum ether/EtOAc, 2:1) was dissolved in methanol and chromatographed over semipreparative HPLC (Sunfire, Prep C_{18} OBD, 10 mm × 250 mm, 5 μm, 7.5 mL/min) with a gradient solvent system from 10% to 50% CH_3CN over 30 min to yield compounds **1** (2.4 mg, t_R 25.17 min) and **2** (20.3 mg, t_R 21.11 min).

6β,9α-Dihydroxy-14-*p*-nitrobenzoylcinnamolide (**1**): white amorphous powder (MeOH); UV (EtOH) λ_{max} (log ε) 254 (4.16) nm; $[\alpha]^{20}_D$ −99.3°(*c* 0.3, EtOH); CD (*c* 0.03, EtOH) (*Δ*ε) 225 (−7.78), 262 (−1.95); ^{1}H NMR and ^{13}C NMR: see **Table 1**; HRESIMS *m/z* 454.1480 (Calcd for $C_{22}H_{25}NO_8Na$ $[M+Na]^+$ 454.1472).

Bioactive assays.

Experimental procedures of the cytotoxic, antiviral against H3N2 and EV71 virus, and antituberculosis bioassays are available in Supporting Information.

Discussion and Conclusion

Insulicolide A is a taxonomic marker for three *Aspergillus* species, *insulicola*, *bridgeri* and *sclerotiorum*, [3, 4] and this acceptance will be amended by our study about insulicolide A and another new nitrobenzoyl sesquiterpenoid obtained from marine-derived *A. ochraceus*. Fungi *Aspergillus* species are the only sources of the existing nitrobenzoyl sesquiterpenoids. Our study, therefore, suggests that marine-derived fungus *A. ochraceus* is one of the promising sources to provide natural cytotoxic nitrobenzoyl sesquiterpenoids.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental procedures of the cytotoxic, antiviral against H3N2 and EV71 virus, and antituberculosis bioassays; Maximum-likehood tree based on ITS1-5.8S-ITS2 sequences of strain Jcma1F17; HRESI-MS, ^{1}H NMR, ^{13}C NMR, HMBC, HSQC, and NOESY spectrum of **1**. See DOI: 10.1039/b000000x/

‡ These authors contributed equally to this paper.

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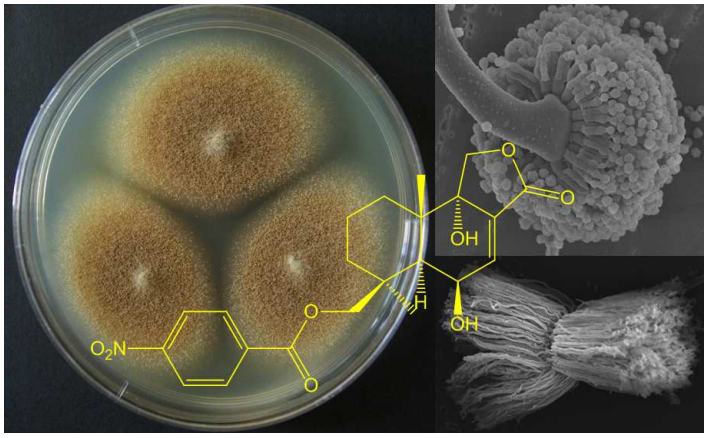

New nitrobenzoyl sesquiterpenoid, with significant cytotoxicities and antiviral activities, was isolated from marine-derived fungus *Aspergillus ochraceus* Jcma1F17.