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New mycotoxins from marine-derived fungus *Aspergillus* sp. SCSGAF0093

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ABSTRACT

Nine mycotoxins including six aspergillic acid group toxins, aluminiumneoaspergillin (**1**), zirconiumneoaspergillin (**2**), aspergilliamide (**3**), ferrineoaspergillin (**5**), flavacol (**6**), neoaspergillic acid (**7**), and three ochratoxins, ochratoxin A *n*-butyl ester (**4**), ochratoxin A (**8**), ochratoxin A methyl ester (**9**), were isolated from the fermentation broth of marine gorgonian derived fungus *Aspergillus* sp. SCSGAF0093. Four of them (**1–4**) were new mycotoxins, and their structures were elucidated on the basis of spectroscopic analysis and chemical evidence. The bio-toxicity of compounds **1–9** were determined by brine shrimp lethality bioassay with median lethal concentration (LC_{50}) values of 2.59–205.67 μM. This was the first report about zirconium complex obtained from nature and ochratoxins isolated from marine environment.

1. Introduction

Mycotoxins produced by *Aspergillus* species, such as aflatoxins, ochratoxins, and aspergillic acid group are capable of causing disease and death in humans and other animals (Cole, 1976). Ochratoxins, including ochratoxins A, B, C and their analogs are often found in terrestrial feed and food materials such as cereal, coffee, wine (Bennett and Klich, 2003). They are nephrotoxin, liver toxin, immune suppressant, potent teratogen and carcinogen to all animals (Bennett and Klich, 2003). The Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) expert committee established a provisional tolerable weekly intake (PTWI) of 100 ng/kg body weight on the basis of deterioration of renal function in pigs (WHO, 2007), and the European Commission amended the maximum level of contaminant ochratoxin A in unprocessed cereal from 5 to 3 μg/kg in Commission Regulation (EU) No 594/2012 (The European Commission, 2012). There was still no report about ochratoxins isolated from marine fungus. The aspergillic acid group of mycotoxins were a number of closely related pyrazine metabolites, and most of them had antibiotic properties (Dunn et al., 1949; MacDonald et al., 1964), however, most of them also had strong toxicity, for example, neoaspergillic acid had toxicity to organisms with lethal dose (LD_{50}) in mice 125 mg/kg (MacDonald, 1973).

As in terrestrial environment, mycotoxins were also metabolized by marine-derived *Aspergillus* species (Tepsic et al., 1997; Grovel et al., 2003) and had potential danger for marine organism and their consumers even human through bioaccumulations (Sallenave-Namont et al., 2000; Kalaitzis et al., 2010). During the course of our investigation on bioactive natural products from marine organisms, we found the extract of marine fungus SCSGAF0093, which was isolated from South China Sea gorgonian *Melitodes squamata* and identified as *Aspergillus* sp., exhibited definite brine shrimp cytotoxicity. Bioassay-guided isolation led to the obtainment of nine mycotoxins including six aspergillic acid group toxins and three ochratoxins. Herein we described the fermentation, isolation, identification and their brine shrimp toxicity of **1–9**.

2. Materials and methods

2.1. General experimental procedures

Optical rotations were measured on a Perkin-Elmer 343 polarimeter (Perkin-Elmer, Inc., Waltham, MA). Ultraviolet (UV) spectra were obtained with a Shimazu UV-1750 spectrophotometer (Shimadzu Corp. Kyoto, Japan). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV-500 NMR spectrometer using solvent residual peaks ($CDCl_3$: δ_H 7.26 and δ_C 77.16 ppm; DMSO-d_6: δ_H 2.50 and δ_C 39.52 ppm) as references. High-resolution electrospray ionization mass spectrometry (HRESIMS) and mass spectrometry (MS) were obtained on Bruker maXis mass spectrometer and Bruker amaZon SL mass spectrometer (Bruker Daltonics, Inc., Bremen, Germany), respectively. Thin-layer chromatography (TLC) was conducted on precoated silica gel HSGF-254 plates (Yantai Jiangyou Silica Gel Development Co., Ltd., Yantai, China), and spot detection was performed under fluorescent light (λ = 254 and 365 nm) and then spraying 10% H_2SO_4 in EtOH, followed by heating. Column chromatography was performed over silica gel (200–300 mesh, Qingdao Haiyang Chemical Co., Ltd., Qingdao, China), Develosil ODS (S-75 μm, Nomura Chemical Co., Ltd., Seto, Japan), macroporous resin Diaion Amberlite XAD-16 (Rohm and Haas Co., Philadelphia, USA), and Sephadex LH-20 (Amersham Bio-sciences,

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Uppsala, Sweden). High-performance liquid chromatography (HPLC) was carried out on a Shimadzu LC-20AT liquid chromatography equipped with a Shimadzu SPD-M20A refractive index detector (Shimadzu Corp., Kyoto, Japan). Inertsil ODS-SP column (150 × 4.6 mm inner diameter, GL Sciences Inc., Tokyo, Japan) and YMC-Pack ODS-A column (150 × 10 mm inner diameter, YMC Co., Ltd., Kyoto, Japan) were used for analytical and semi-preparative purposes, respectively.

2.2. Fungal material

The fungus SCSGAF0093 strain was isolated from a healthy tissue of *Melitodes squamata* collected from the South China Sea, near Sanya City (18°11′N, 109°25′E), Hainan Province, China. The strain was analyzed following the internal transcribed spacers (ITS) sequences (accession number JN851021) and identified as *Aspergillus* sp. (see Fig. 1).

2.3. Fermentation and extraction

Spores were inoculated into 500 mL Erlenmeyer flasks each containing 120 mL liquid medium (glucose 1%, maltose 2%, monosodium glutamate 1%, KH_2PO_4 0.05%, $MgSO_4{\cdot}7H_2O$ 0.003%, corn steep liquor 0.05%, yeast extract 0.3% and marinum salt 3%, dissolved in tap water, pH 6.5) (Lu et al., 2008). After 35 days of stationary cultivation at 28 °C, the whole broths (25 L) were filtered through cheesecloth. Sterilized XAD-16 resin (20 g/L) was added to the liquor and shaken at 200 rpm for 30 min to absorb the organic products. The resin was washed with distilled water to remove medium residue and saccharides then eluted with methanol. The methanol solvent was removed under vacuum to give a brown residue (40.7 g). The mycelium portion was smashed and extracted twice with 80% acetone/H_2O. The acetone soluble fraction was dried *in vacuo* to yield 14.6 g residue. The residues of liquor and mycelium extracts were combined together by thin layer chromatography (TLC) detecting.

2.4. Isolation and identification of metabolites

The combined extract (50 g) was subjected to silica gel column (840 g) and eluted with $CHCl_3$/MeOH (100:0–70:30, v/v) to give nine fractions. Fr. 2 ($CHCl_3$/MeOH, 100:0) was separated by Sephadex LH-20 and purified with HPLC using 90:10 MeOH/H_2O as the mobile phase at the flow rate of 3 mL/min to yield compounds **1** (t_R 28.4 min, 160 mg) and **5** (t_R 35.0 min, 140 mg). Fr. 3 ($CHCl_3$/MeOH, 90:10) was subjected to Develosil ODS column eluting with a decreasing polarity of MeOH/H_2O and purified with HPLC at the flow rate of 3 mL/min to obtain **3** (58:42 MeOH/H_2O, t_R 24.7 min, 4 mg), **2** (96:4 MeOH/H_2O, t_R 25.3 min, 22 mg), **6** (56:44 MeOH/H_2O, t_R 31.4 min, 17 mg), and **7** (58:42 MeOH/H_2O, t_R 36.4 min, 11 mg). Fr. 6 ($CHCl_3$/MeOH, 80:20) was separated over Develosil ODS column and purified with HPLC at the flow rate of 3 mL/min to afford **8** (66:34:0.1, MeOH/H_2O/HCOOH, t_R 16.5 min, 168 mg), **9** (75:25:0.1, MeOH/H_2O/HCOOH, t_R 15.5 min, 24 mg), and **4** (75:25:0.1, MeOH/H_2O/HCOOH, t_R 17.4 min, 4 mg). The crude extract was analyzed by HPLC with DAD-detector and compounds **1**–**9** were marked in the spectrum (see Fig. 2).

2.5. Aspergillic acid group of mycotoxins

Aluminiumneoaspergillin (**1**). Yellowish solid; UV ($CHCl_3$) λ_{max} (log ε) 316 (3.99); ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data: see Table 1; ESIMS (+)*m*/*z* 697 $[M+H]^+$, 719 $[M+Na]^+$, 735 $[M+K]^+$; HRESIMS *m*/*z* 697.4259 ($[M+H]^+$, calcd. for $C_{36}H_{58}AlN_6O_6$, 697.4228).

Zirconiumneoaspergillin (**2**). Yellowish solid; UV ($CHCl_3$) λ_{max} (log ε) 319 (4.01); ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data: see Table 1; ESIMS (+)*m*/*z* 983 $[M+H]^+$, 759 $[M-C_{12}H_{19}N_2O_2]^+$; HRESIMS *m*/*z* 983.4911 ($[M+H]^+$, calcd. for $C_{48}H_{77}N_8O_8Zr$, 983.4906).

Aspergilliamide (**3**). Yellowish solid; UV (MeOH) λ_{max} (log ε) 238 (4.01); ^{1}H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz) data: see Table 1; ESIMS (+)*m*/*z* 251 $[M+Na]^+$, 479 $[2M+Na]^+$; (−)*m*/*z* 227 $[M-H]^-$; HRESIMS (−)*m*/*z* 227.1399 ($[M-H]_-$, calcd. for $C_{11}H_{19}N_2O_3$, 227.1401).

2-(Hydroxyimino)-4-methylpentanamide (**3a**). Yellowish solid; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 2.35 (2H, d, *J* = 7.5 Hz, H-3), 1.92 (1H, m, H-4), 0.84 (6H, d, *J* = 6.5 Hz, H-5, 6), 7.08–7.19 (2H, s, –N*H*$_2$), 11.50 (1H, s, –NO*H*); ESIMS (+)*m*/*z* 145 $[M+H]^+$, 167 $[M+Na]^+$, 301 $[2M+Na]^+$.

Ferrineoaspergillin (**5**). Red pigment; The NMR spectrum couldn't be measured. ESIMS (+)*m*/*z* 726 $[M+H]^+$, 748 $[M+Na]^+$.

Flavacol (**6**). White solid; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.17 (1H, s, H-5), 2.64 (2H, d, *J* = 7.1 Hz, H-7), 2.20 (1H, m, H-8), 0.96 (6H, d, *J* = 6.7 Hz, H-9, 10), 2.38 (2H, d, *J* = 7.3 Hz, H-11), 2.05 (1H, m, H-12), 0.95 (6H, d, *J* = 6.7 Hz, H-13, 14); ESIMS (+)*m*/*z* 231 $[M+Na]^+$; (−)*m*/*z* 207 $[M-H]^-$.

Neoaspergillic acid (**7**). Yellowish solid; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.32 (1H, s, H-5), 2.72 (2H, d, *J* = 7.0 Hz, H-7), 2.20 (2H, m, H-8, 12), 1.00 (6H, d, *J* = 6.6 Hz, H-9, 10), 2.65 (2H, d, *J* = 7.0 Hz, H-11), 0.96 (6H, d, *J* = 6.5 Hz, H-13, 14); ESIMS (−)*m*/*z* 223 $[M-H]_-$.

2.5.1. Ochratoxins

Ochratoxin A *n*-butyl ester (**4**). Light brown power; $[\alpha]_D^{20}$ −139 (*c* 0.20, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 334 (3.61), 375 (3.22); ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data: see Table 2; ESIMS (+)*m*/*z* 460 $[M+H]^+$, 482 $[M+Na]^+$; (−)*m*/*z* 458 $[M-H]^-$; HRESIMS *m*/*z* 458.1377 ($[M-H]^-$, calcd. for $C_{24}H_{25}ClNO_6$, 458.1376).

Ochratoxin A (**8**). Yellowish power; $[\alpha]_D^{20}$ −61 (*c* 0.20, $CHCl_3$); ^{1}H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz) data: see Table 2; ESIMS (+)*m*/*z* 404 $[M+H]^+$, 426 $[M+Na]^+$, 442 $[M+K]^+$; (−)*m*/*z* 402 $[M-H]^-$.

Ochratoxin A methyl ester (**9**). Light brown power; $[\alpha]_D^{20}$ −43 (*c* 0.39, $CHCl_3$); ^{1}H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz) data: see Table 2; ESIMS (+)*m*/*z* 418 $[M+H]^+$, 440 $[M+Na]^+$; (−)*m*/*z* 416 $[M-H]^-$.

2.6. Alkaline hydrolysis of **1** and **2**

A mixture of **1** (2 mg) in MeOH (0.2 mL) and 3 M NH_4OH (2 mL) was stirred at room temperature for 1 h. The solution was centrifuged to separate $Al(OH)_3$ that could re-solve in excess KOH and the supernatant was extracted with ether to obtain compound **7** (1.5 mg). Through similar procedures, compound **7** (1.4 mg) and $Zr(OH)_4$ sediment were yielded from hydrolysis of **2** (2 mg).

2.7. Alkaline hydrolysis of **3**

Compound **3** (0.5 mg) was solved in 4% NaOH (2 mL) and stirred at room temperature for 4 h. Then the mixture was neutralized with dilute hydrochloric acid and separated with HPLC to obtained compound **3a** (0.3 mg).

2.8. Alkaline hydrolysis of **5**

A solution of compound **5** (5 mg) in MeOH (0.5 mL) was stirred with 2 M KOH (2 mL) at room temperature for 1 h. The mixture was centrifuged to separate $Fe(OH)_3$ and the supernatant was extracted with ether (Assante et al., 1981). The solvent was removed under vacuum to give white solid (4 mg) that was identified as compound **7** by comparison of HPLC retention time and NMR spectral data.

2.9. Brine shrimp bioassay

Brine shrimp (*Artemia salina*) eggs (Aquatic Lifeline, Inc., Utah, USA) were hatched in artificial seawater (3% marinum salt dissolved in tap water) and incubated at 28 °C for 48 h. A serial concentration of 5 μL test compounds **1**–**9** and positive control toosendanin dissolved in DMSO were added to 195 μL suspension containing 20–25 organisms in 96-well flat-bottom microplate with final concen-

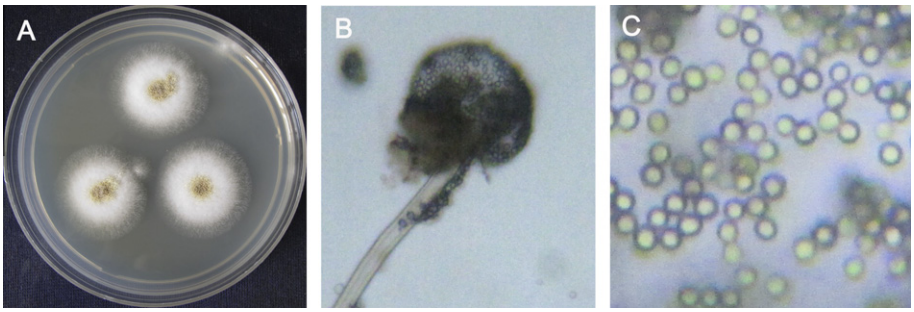

Fig. 1. *Aspergillus* strain SCSGAF0093. (A) Four-day-old cultures on PDA medium. (B) Micrograph photo of conidiophores (400×). (C) Micrograph photo of conidia (400×).