



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

Xinya Xu<sup>a</sup>, Fei He<sup>a</sup>, Xiaoyong Zhang<sup>a</sup>, Jie Bao<sup>a,b</sup>, Shuhua Qi<sup>a,\*</sup>

<sup>a</sup> Key Laboratory of Marine Bio-Resources Sustainable Utilization/Guangdong Key Laboratory of Marine Material Medical, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou 510301, China

<sup>b</sup> University of Chinese Academy of Sciences, Beijing 100049, China

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### ABSTRACT

Nine mycotoxins including six aspergillilic acid group toxins, aluminiumneoaspergillin (1), zirconiumneoaspergillin (2), aspergilliamide (3), ferrineoaspergillin (5), flavacol (6), neoaspergillilic 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<sub>50</sub>) values of 2.59–205.67 μM. This was the first report about zirconium complex obtained from nature and ochratoxins isolated from marine environment.

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### 1. Introduction

Mycotoxins produced by *Aspergillus* species, such as aflatoxins, ochratoxins, and aspergillilic 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 aspergillilic 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, neoaspergillilic acid had toxicity to organisms with lethal dose (LD<sub>50</sub>) 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 aspergillilic 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 Shimadzu 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<sub>3</sub>: δ<sub>H</sub> 7.26 and δ<sub>C</sub> 77.16 ppm; DMSO-*d*<sub>6</sub>: δ<sub>H</sub> 2.50 and δ<sub>C</sub> 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<sub>2</sub>SO<sub>4</sub> 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,

\* Corresponding author. Tel.: +86 20 8902 2112.

E-mail address: [shuhuaqi@scsio.ac.cn](mailto:shuhuaqi@scsio.ac.cn) (S. Qi).

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%,  $\text{KH}_2\text{PO}_4$  0.05%,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.003%, corn steep liquor 0.05%, yeast extract 0.3% and marimum 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/ $\text{H}_2\text{O}$ . 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  $\text{CHCl}_3/\text{MeOH}$  (100:0–70:30, v/v) to give nine fractions. Fr. 2 ( $\text{CHCl}_3/\text{MeOH}$ , 100:0) was separated by Sephadex LH-20 and purified with HPLC using 90:10 MeOH/ $\text{H}_2\text{O}$  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 ( $\text{CHCl}_3/\text{MeOH}$ , 90:10) was subjected to Develosil ODS column eluting with a decreasing polarity of MeOH/ $\text{H}_2\text{O}$  and purified with HPLC at the flow rate of 3 mL/min to obtain **3** (58:42 MeOH/ $\text{H}_2\text{O}$ ,  $t_R$  24.7 min, 4 mg), **2** (96:4 MeOH/ $\text{H}_2\text{O}$ ,  $t_R$  25.3 min, 22 mg), **6** (56:44 MeOH/ $\text{H}_2\text{O}$ ,  $t_R$  31.4 min, 17 mg), and **7** (58:42 MeOH/ $\text{H}_2\text{O}$ ,  $t_R$  36.4 min, 11 mg). Fr. 6 ( $\text{CHCl}_3/\text{MeOH}$ , 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/ $\text{H}_2\text{O}/\text{HCOOH}$ ,  $t_R$  16.5 min, 168 mg), **9** (75:25:0.1, MeOH/ $\text{H}_2\text{O}/\text{HCOOH}$ ,  $t_R$  15.5 min, 24 mg), and **4** (75:25:0.1, MeOH/ $\text{H}_2\text{O}/\text{HCOOH}$ ,  $t_R$  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. Aspergilliac acid group of mycotoxins

Aluminiumneospergillin (**1**). Yellowish solid; UV ( $\text{CHCl}_3$ )  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 316 (3.99);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz) and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 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  $\text{C}_{36}\text{H}_{58}\text{AlN}_6\text{O}_6$ , 697.4228).

Zirconiumneospergillin (**2**). Yellowish solid; UV ( $\text{CHCl}_3$ )  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 319 (4.01);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz) and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 125 MHz) data: see Table 1; ESIMS (+) $m/z$  983 [M+H] $^+$ , 759 [M-C $_{12}\text{H}_{19}\text{N}_2\text{O}_2$ ] $^+$ ; HRESIMS  $m/z$  983.4911 ([M+H] $^+$ , calcd. for  $\text{C}_{48}\text{H}_{77}\text{N}_8\text{O}_8\text{Zr}$ , 983.4906).

Aspergillamide (**3**). Yellowish solid; UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 238 (4.01);  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ , 500 MHz) and  $^{13}\text{C}$  NMR ( $\text{DMSO}-d_6$ , 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  $\text{C}_{11}\text{H}_{19}\text{N}_2\text{O}_3$ , 227.1401).

2-(Hydroxyimino)-4-methylpentanamide (**3a**). Yellowish solid;  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ , 500 MHz)  $\delta$  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, –NH $_2$ ), 11.50 (1H, s, –NOH); ESIMS (+) $m/z$  145 [M+H] $^+$ , 167 [M+Na] $^+$ , 301 [2M+Na] $^+$ .

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

Flavacol (**6**). White solid;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz)  $\delta$  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] $^-$ .

Neospergilliac acid (**7**). Yellowish solid;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz)  $\delta$  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,  $\text{CHCl}_3$ ); UV ( $\text{CHCl}_3$ )  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 334 (3.61), 375 (3.22);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz) and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 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  $\text{C}_{24}\text{H}_{25}\text{ClNO}_6$ , 458.1376).

Ochratoxin A (**8**). Yellowish power;  $[\alpha]_D^{20}$  –61 (c 0.20,  $\text{CHCl}_3$ );  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ , 500 MHz) and  $^{13}\text{C}$  NMR ( $\text{DMSO}-d_6$ , 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,  $\text{CHCl}_3$ );  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ , 500 MHz) and  $^{13}\text{C}$  NMR ( $\text{DMSO}-d_6$ , 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  $\text{NH}_4\text{OH}$  (2 mL) was stirred at room temperature for 1 h. The solution was centrifuged to separate  $\text{Al}(\text{OH})_3$  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  $\text{Zr}(\text{OH})_4$  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  $\text{Fe}(\text{OH})_3$  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% marine salt dissolved in tap water) and incubated at 28 °C for 48 h. A serial concentration of 5  $\mu\text{L}$  test compounds **1–9** and positive control toosendanin dissolved in DMSO were added to 195  $\mu\text{L}$  suspension containing 20–25 organisms in 96-well flat-bottom microplate with final concen-

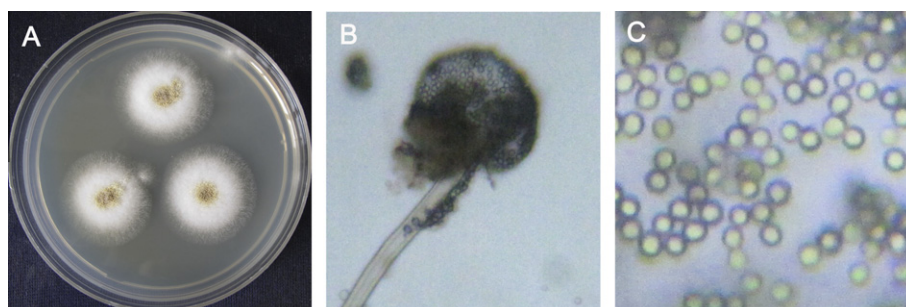


Fig. 1. *Aspergillus* strain SCSGAF0093. (A) Four-day-old cultures on PDA medium. (B) Micrograph photo of conidiophores (400 $\times$ ). (C) Micrograph photo of conidia (400 $\times$ ).

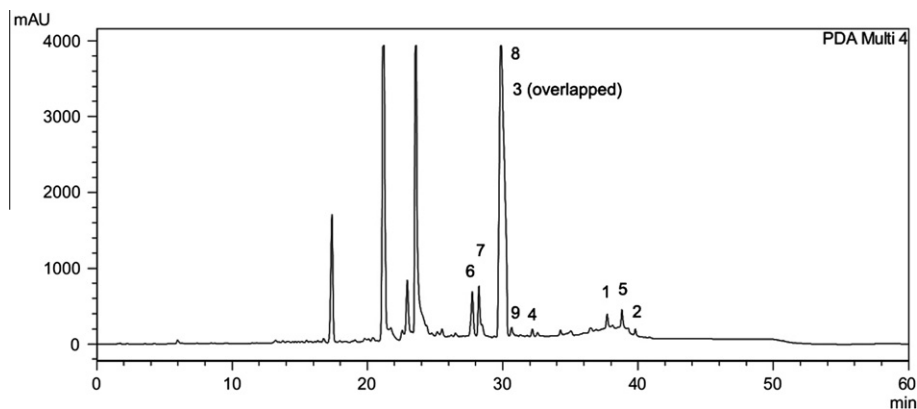


Fig. 2. HPLC finger-ponit spectrum of compounds 1–9 in SCSGA0093 extract.

Table 1  
NMR data for compounds 1, 2 and 3.

	1 <sup>a</sup>		2 <sup>a</sup>		3 <sup>b</sup>	
	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$
2		153.88, s		155.82, s		152.59, s
3		147.93, s		148.55, s		162.68, s
5	7.61–7.62, m	130.37, d	7.48, s	129.68, d	–	–
6		138.33, s		139.00, s		172.42, s
7	2.56–2.84, m	36.36, t	2.63, d, (7.1)	36.37, t	2.49 (overlapped)	45.55, t
8	2.10, m	27.68, d	2.03, m	27.97, d	2.01, m	24.67, d
9	0.81–0.89, m	22.42, q	0.87, m	22.64, q	0.97, d, (6.6)	22.21, q
10						
11	2.56–2.84, m	41.15, t	2.56, d, (7.0)	41.49, t	2.37, d, (7.3)	31.49, t
12	2.10, m	26.84, d	2.03, m	26.72, d	1.91, m	25.85, d
13	0.81–0.89, m	22.42, q	0.87, m	22.64, q	0.85, d, (6.6)	22.57, q
14						
–NOH					9.97, s	

<sup>a</sup> In CDCl<sub>3</sub>.

<sup>b</sup> In DMSO-*d*<sub>6</sub>.

trations of 1, 2.5, 5, 10, 20 and 50  $\mu\text{g}/\text{mL}$  in quadruplicate, respectively. After being incubated at 28 °C for 24 h, the microplates were examined under the microscope to count dead nauplii and total numbers of brine shrimp in each well (Miceli et al., 2011). The corrected mortality rate was calculated according to the following formula:

$$\text{Corrected mortality rate (\%)} = [(T - C)/(1 - C) \times 100]$$

where *T* is the mortality rate of the treatment and *C* is the mortality rate of the negative control. The data was analyzed on SPSS 16.0 (SPSS Inc., USA) to determine LC<sub>50</sub>.

### 3. Result and discussion

#### 3.1. Aspergillic acid group of mycotoxins

Compound **1** was obtained as yellowish solid. Its molecular formula of C<sub>36</sub>H<sub>58</sub>AlN<sub>6</sub>O<sub>6</sub> was determined by HRESIMS (*m/z* 697.4259 ([M+H]<sup>+</sup>, calcd. for, 697.4228) and <sup>1</sup>H and <sup>13</sup>C NMR spectral data. The <sup>1</sup>H NMR spectrum (Table 1) showed signals at  $\delta$  7.61–7.62 (1H, m, H-5), 2.56–2.84 (4H, m, H-7, 11), 2.10 (2H, m, H-8, 12), 0.81–0.89 (12H, m, H-9, 10, 13, 14). The <sup>13</sup>C NMR and DEPT spectral data (Table 1) revealed the presence of 12 carbon signals including four methyls at  $\delta$  22.42 (C-9, 10, 13, 14), two methylenes at  $\delta$  26.84 (C-8), 27.68 (C-12), two methines at  $\delta$  41.15 (C-7), 36.36 (C-11), and four low-field carbons at  $\delta$  153.88 (s, C-2), 147.93 (s, C-3), 138.33 (s, C-6), 130.37 (d, C-5). These NMR data showed great similarity to those of neospergillus acid (**7**) (Assante et al., 1981). Alkali hydrolysis of **1** gave neospergillus acid (**7**) and Al(OH)<sub>3</sub> sediment, which indicated that **1** was an aluminum complex of

neospergillus acid. Combined with the molecular formula, these data displayed that **1** was a complex containing three molecules of neospergillus acid and one aluminum atom and named aluminumneospergillin (Fig. 3).

Compound **2** had the molecular formula of C<sub>48</sub>H<sub>76</sub>N<sub>8</sub>O<sub>8</sub>Zr as inferred from HRESIMS (*m/z* 983.4911 ([M+H]<sup>+</sup>, calcd. for 983.4906) spectrum. The <sup>1</sup>H and <sup>13</sup>C NMR spectral data of **2** (Table 1) showed great similarity to those of **1**. Alkali hydrolysis of **2** gave **7** and Zr(OH)<sub>4</sub> sediment. These data indicated that **2** was a novel complex containing four molecules of neospergillus acid and one zirconium atom and named zirconiumneospergillin. This is the first zirconium complex isolated from nature.

Compound **3** was afforded as yellowish solid. It had the molecular formula of C<sub>11</sub>H<sub>20</sub>N<sub>2</sub>O<sub>3</sub> as inferred from HRESIMS (*m/z* 227.1399 ([M–H]<sup>–</sup>, calcd. for 227.1401) and NMR data (Table 1). The <sup>1</sup>H NMR spectrum of **3** showed similar to those of **1**, **2**, **6** and **7** (Fig. 4). The <sup>1</sup>H NMR data (Table 1) at  $\delta$  2.49 (2H, overlapped, H-7), 2.37 (2H, d, *J* = 7.3 Hz, H-11), 2.01 (1H, m, H-8), 1.91 (1H, m, H-12), 0.97 (6H, d, *J* = 6.6 Hz, H-9, 10), 0.85 (6H, d, *J* = 6.6 Hz, H-13, 14) and <sup>13</sup>C NMR data (Table 1) at  $\delta$  45.55 (t, C-7), 31.49 (t, C-11), 24.67 (d, C-8), 25.85 (d, C-12), 22.21 (q, C-9, 10), 22.57 (q, C-13, 14) suggested that **3** also contained two isobutyl groups. In the HMBC spectrum (Fig. 5), correlations from H-7 to C-6 ( $\delta$  172.42, s)/C-8/C-9/C-10 suggested the existence of 3-methylbutanamide unit. Furthermore, HMBC correlations from H-11 to C-2 ( $\delta$  152.59, s)/C-3 ( $\delta$  162.68, s)/C-12/C-13/C-14, and from OH-4 ( $\delta$  9.97, br s) to C-3, and alkaline hydrolysis of **3** to give 2-(hydroxyimino)-4-methylpentanamide (**3a**) (Fig. 5) suggested the presence

**Table 2**  
NMR data for compounds **4**, **8** and **9**.

	<b>4<sup>a</sup></b>		<b>8<sup>b</sup></b>		<b>9</b>	
	$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}^{\text{a}}$ (J in Hz)	$\delta_{\text{C}}^{\text{b}}$
1		168.04		162.66		162.81
3	4.76, m	75.90	4.73, m	75.33	4.74, m	75.40
4	2.85, dd, (17.4, 11.7) 3.28, dd, (17.4, 3.1)	32.33	2.89, dd, (17.2, 11.7) 3.21, m	31.60	2.84, dd, (17.4, 11.6) 3.27, dd, (17.3, 3.5)	31.55
5		123.18		121.29		121.47
6	8.43, s	139.08	8.07, s	136.97	8.41, s	136.60
7		120.91		120.08		120.03
8		159.11		158.49		158.23
9		110.11		111.32		111.23
10		140.36		141.63		141.67
11		169.78		168.19		168.24
12-NH	8.51, d, (6.2)		8.65, d, (7.2)		8.48, d, (7.1)	
13	5.03, dd, (13.1, 6.4)	54.23	4.83, m	53.92	5.03, dd, (12.8, 6.6)	53.96
14	3.22, m	38.18	3.09, dd, (13.8, 8.0) 3.22, dd, (13.8, 6.8)	36.59	3.17, dd, (13.9, 6.6) 3.25, dd, (13.8, 5.6)	36.54
15		136.14		136.04		135.99
16	7.18, d, (7.1)	129.36	7.19–7.29, m	129.13	7.16, d, (7.0)	129.07
17	7.27, m	128.56		128.19	7.26, m	128.28
18	7.25, m	127.12		126.56	7.24, m	126.69
19	7.27, m	128.56		128.19	7.26, m	128.28
20	7.18, d, (7.1)	129.36		129.13	7.16, d, (7.0)	129.07
21	1.59, d, (6.4)	20.70	1.46, d, (6.3)	20.01	1.58, d, (6.3)	19.98
22		171.43		172.27		171.31
23	4.10, m	65.38			3.73, s	52.01
24	1.59, m	30.56				
25	1.31, m	19.06				
26	0.91, t, (7.4)	13.63				
8-OH	12.70, s		12.85, s		12.72, s	

<sup>a</sup> In CDCl<sub>3</sub>.<sup>b</sup> In DMSO-*d*<sub>6</sub>.

of **3a** unit in **3**. Based on the above data, the structure of **3** was deduced as 2-(hydroxyimino)-4-methyl-*N*-(3-methylbutanoyl)pentanamide and simply named aspergilliamide.

Compounds **5–7** were identified as ferrineoaspergillin (Assante et al., 1981), flavacol (Li et al., 2010), and neoaspergilliac acid (Assante et al., 1981) by comparing spectroscopic data with reported in literatures, respectively.

### 3.2. Ochratoxins

Compound **4** was obtained as light brown powder. The HRESIMS peak of [M–H]<sup>–</sup> at *m/z* 458.1377 and NMR spectra of **4** confirmed its molecular formula as C<sub>24</sub>H<sub>26</sub>ClNO<sub>6</sub>. The <sup>1</sup>H and <sup>13</sup>C NMR data of **4** (Table 2) were similar to those of ochratoxin A (**8**) (VanderMerwe et al., 1965a,b) except for extra signals for an *n*-butyl group at  $\delta_{\text{H}}$  4.10 (2H, m, H-23), 1.59 (2H, m, H-24), 1.31 (2H, m, H-25), 0.91 (3H, t, *J* = 7.4 Hz, H-26) and  $\delta_{\text{C}}$  65.38 (t, C-23), 30.56 (t, C-24), 19.06 (t, C-25), 13.63 (q, C-27). In the HMBC spectrum, correlations from H-23 to C-22/C-24/C-25, and from H-26 to C-24/C-25 suggested the extra *n*-butyl group linked to C-22 of the ochratoxin A skeleton. The optical rotation value of **4** was similar to that of **8**, and *n*-butyl group affected little on optical rotation value, which suggested that **4** should have the same relative configuration as **8**. So, the structure of **4** was determined as shown and named ochratoxin A *n*-butyl ester. During the separation experiment, we did not involve in *n*-butanol, and did not detect the existence of **4** in the mixture of **8** (1 mg) dissolved in *n*-butanol (2 mL) and reacted for two weeks at room temperature, which supported **4** as a natural product.

Compounds **8, 9** were identified as ochratoxin A (VanderMerwe et al., 1965b) and ochratoxin A methyl ester (Steyn and Holzapfel, 1967) by comparing spectroscopic data with reported in literatures.

### 3.3. Brine shrimp bioassay

Brine shrimp lethality activities of compounds **1–9** were evaluated. The results showed that LC<sub>50</sub> values of **1–9** and positive control (toosendanin) were 6.61, 10.76, 71.09, 4.14, 29.62, 205.67, 90.08, 13.74, 2.59, and <1.74  $\mu\text{M}$ , respectively. Among these aspergilliac acid group toxins (compounds **1–3** and **5–7**), **6** exhibited the lowest toxicity, and complexes **1, 2** and **5** showed stronger toxicity than **7**. The result was conformity with the previous report that toxic functionality of aspergilliac acid group compounds were related to hydroxamic acid rather than side-chain substituent at C-3 and C-6 (Cole, 1976). All the ochratoxins (**4, 8, 9**) exhibited significant toxicity against brine shrimp.

Aspergilliac acid group of mycotoxins are a number of closely related pyrazine metabolites. Neoaspergilliac acid (**7**) was first isolated from cultures of *Aspergillus sclerotiorum* Huber (Micetich and MacDonald, 1964). Compound **3** was a novel neoaspergilliac acid analog with medium toxicity. Compounds **1, 2**, and **5** were neoaspergilliac acid complexes, and **2** was a new zirconium complex. Zirconium is one kind of rare metal and mostly spread in sea beach placer as zirconium silicate (zircon). Dissolved zirconium ranges from 12 to 300 pmol/kg in seawater of North Pacific (McKelvey and Orians, 1998) and 185–200 pmol/kg in the Indian and Atlantic Oceans (Boswell and Elderfield, 1988). In here, the zirconium could perhaps have been brought by marimum salt 3%. This was the first report about zirconium complex from nature.

Complexes are common in nature and essential to organisms. For examples, chlorophyll is composed of magnesium ion and porphyrin ligand, and hemoglobin is ferric complex, which provide necessary energy to green plants and higher animals, respectively. Natural copper and ferric complexes were also studied as antibiotics (Itoh et al., 1979; Sun and Wei, 2011). In this study, neoaspergilliac acid complexes **1, 2** and **5** had more potent toxicity to brine shrimp than ligands **6** and **7**. The genus *Aspergillus* was the most

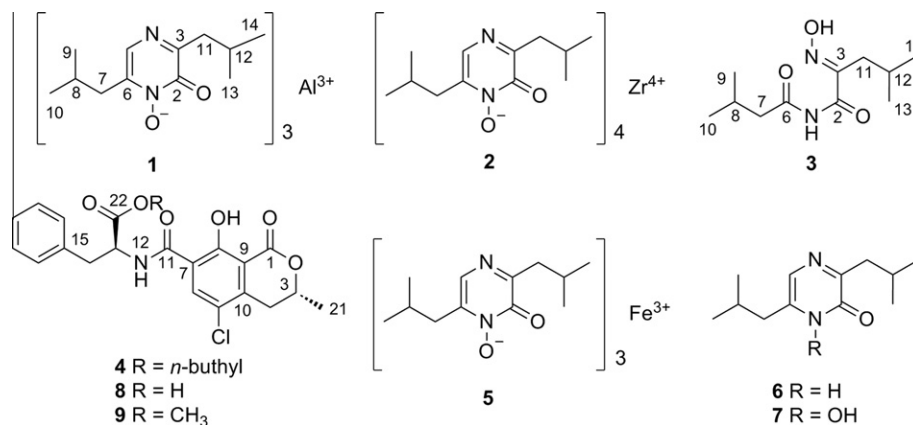


Fig. 3. Structures of 1–9 from gorgonian-derived fungus *Aspergillus* sp. SCSGAF0093.

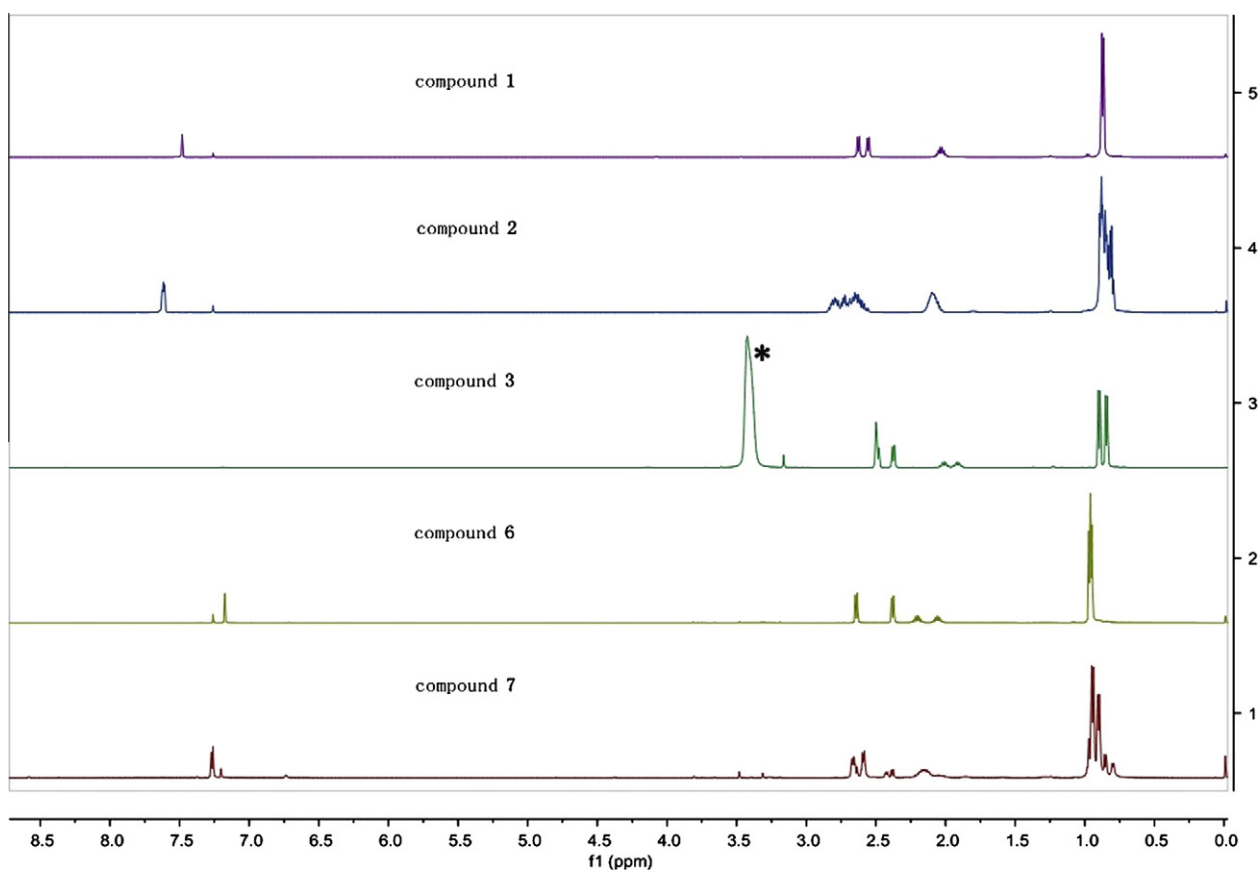


Fig. 4. <sup>1</sup>H NMR spectrum for compounds 1, 2, 3, 6 and 7. \*Solvent residual peak.

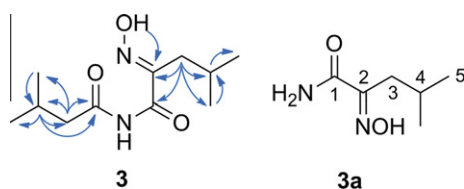


Fig. 5. Key HMBC correlations of 3 and structure of 3a.

allelochemicals to inhibit other microorganism, and played a chemical defense function against gorgonian aspergillosis disease.

Ochratoxin A was first isolated from *Aspergillus ochraceus* (VanderMerwe et al., 1965a,b) and also obtained from *Aspergillus niger*, *Aspergillus carbonarius*, and *Aspergillus flavus* (Anli and Alkis, 2010). *Aspergillus* species producing ochratoxin A can be divided into two distinct groups. One group includes species producing conidia colored golden brown, classified in *Aspergillus* section *Circumdati* (Frisvad et al., 2004). The other group includes species producing black conidia, classified in *Aspergillus* section *Nigri* (Samson et al., 2004). By BLAST search in GenBank, the ITS sequences of marine-derived fungus SCSGAF0093 showed most similarity to those of *Aspergillus ochraceopetaliformis* and *Aspergillus*

commonly distributed in gorgonian species (Zhang et al., 2012; Toledo-hernandez et al., 2007). It was possible that neoaspergillid acid complexes were produced by *Aspergillus* sp. SCSGAF0093 as

*elegans*, so the fungus was identified as *Aspergillus* genus. As this strain can produce ochratoxin A and has golden brown conidia (see Fig. 1), it can be put in the *Aspergillus* section *circumdati*.

*Aspergillus* strains which can produce ochratoxin A, such as *A. ochraceus*, *A. niger*, *A. carbonarius*, and *A. flavus*, are also widespread in marine environment (Zhang et al., 2012; Toledo-Hernandez et al., 2007). Ochratoxin A can be probably transferred into marine organism such as fish through bioaccumulations. Fish and seafood are important parts of human diet. All over the world consumed about 148 million tones fish and seafood in 2010 (FAO Fisheries and Aquaculture Department, 2012). Worldwide per capita supply of fish and seafood was on average 16.3 kg per year in 2002 (Jensen, 2006), while was increased to 18.6 kg per year in 2010 (FAO Fisheries and Aquaculture Department, 2012). Ochratoxin A is a nephrotoxin to all animal species in micro concentration (Bennett and Klich, 2003). It's serious if seafood supply is polluted by ochratoxins. However research and analysis about ochratoxins' contents in seafood and marine products were lacked. Our report will be valuable to develop seafood safety and quality systems.

#### 4. Conclusion

Nine mycotoxins (1–9) including six aspergillilic acid group of toxins and three ochratoxins were isolated from the fermentation broth of marine gorgonian-derived fungus *Aspergillus* sp. SCS-GAF0093. Four of them (1–4) were new mycotoxins. All these compounds exhibited strong or medium brine shrimp toxicity. Neaspergillilic acid complex 2 was the first zirconium complex isolated from nature. Ochratoxins were first isolated from marine-derived fungus. This study reminds us to be alert to mycotoxins from seafood and marine products as from terrestrial products.

#### Conflict of Interest

The authors declare that there are no conflicts of interest.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.fct.2012.11.037>.

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