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Three new sterigmatocystin analogues from marine-derived fungus *Aspergillus versicolor* MF359

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Abstract During the systematic screening of active compounds from marine-derived fungi, the extract of a strain of *Aspergillus versicolor* MF359 isolated from a marine sponge of *Hymeniacidon perleve* was identified for detailed chemical investigation. Three new secondary metabolites, named hemiacetal sterigmatocystin (**1**), acyl-hemiacetal sterigmatocystin (**2**), and 5-methoxydihydrosterigmatocystin (**3**), together with a known compound, aversin (**4**), were characterized. **1** represents a first structure of sterigmatocystin hemiacetal from nature. The antibacterial activities of these identified compounds were evaluated against *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*. Compound **3** showed activity against *S. aureus* and *B. subtilis* with MIC values of 12.5 and 3.125 μg/mL, respectively.

Keywords Marine-derived fungus · *Aspergillus versicolor* · Sterigmatocystin · *Staphylococcus aureus* · *Bacillus subtilis*

Introduction

The microorganism is the most important resource during the drug discovery program. Although the first antibiotic from marine microbe is cephalosporin C from a marine-derived fungus in the 1940s, scientists gave considerable attention to marine natural products 50 years later. Marine-derived fungi possess a great ability to produce structurally unique and biologically active natural products, and a growing number of novel bioactive secondary metabolites have been reported from marine-derived fungi (Blunt et al. 2006; Bugni and Ireland 2004; Saleem et al. 2007; Rateb and Ebel 2011).

The genus *Aspergillus* was a major contributor to the active compounds of marine fungal origin. Many classes of compounds have been isolated from *Aspergillus*, such as cytotoxic (Belofsky et al. 1998) and antibacterial (Liu et al. 2012) sesqueterpenoids, cytotoxic cyclopentapeptides (Fremlin et al. 2009), antibacterial diketopiperazines (Song et al. 2012a), antibacterial anthraquinone (Zhang et al. 2012), and cytotoxic indole alkaloids (He et al. 2012) from *Aspergillus versicolor*, Ca^{2+}-ATPase and *Micrococcus luteus* inhibitory pentacyclic oxindole alkaloid from *Aspergillus tamari* (Suda et al. 2003), antioxidant sesquiterpenes and xanthone derivatives (Trisuwan et al. 2011), from *Aspergillus sydowii*, cytotoxic tripeptides (Toske et al. 1998), antibacterial sesquiterpenoids (Wei et al. 2010), and cytotoxic-prenylated indole alkaloids from *Aspergillus* sp. (Kato et al. 2007). To investigate the great potential of marine-derived fungal sources and screening novel bioactive metabolites against pathogenic bacteria, we assessed a library of marine-derived bacteria (4,024) and fungi (533) (Ashforth et al. 2010; Chen et al. 2012, 2013; Song et al. 2010, 2012a, 2012b; Wang et al. 2013) for growth-inhibitory activity against *Staphylococcus aureus*. The extract of a strain of *A. versicolor* (MF359) isolated from a marine sponge sample of *Hymeniacidon perleve* collected from the Bohai Sea showed moderate activity with an IC_{50} value of 25 μg/mL. This strain was prioritized for further fermentation and chemical analysis. MF359 was cultured in 10 different media and the extract of solid rice medium gave good chemical diversity. So, a large-scale

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culture of *A. versicolor* (MF359) in rice medium was subjected to chemical investigation, which resulted in three new secondary metabolites, named hemiacetal sterigmatocystin (**1**), acyl-hemiacetal sterigmatocystin (**2**), and 5-methoxydihydrosterigmatocystin (**3**), together with a known compound, aversin (**4**) (Fig. 1). Their structures were elucidated by extensive spectroscopic analysis as well as comparison with previously reported data. The bioactivities of these four compounds were evaluated as well.

Materials and methods

General experimental procedures

IR spectra were recorded on a Nicolet 5700 Fourier transform infrared spectroscopy (FT-IR) Microscope spectrometer (FT-IR Microscope Transmission). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 600 MHz spectrometer at 600 MHz for ^{1}H and 150 MHz for ^{13}C. High-resolution electrospray ionization mass spectrometry (HRESIMS) data were obtained using a Bruker APEX III 7.0 T spectrometer. Optical rotations were acquired with a PerkinElmer 343 polarimeter. High-performance liquid chromatography (HPLC) was performed using an Agilent Eclipse XDB-C18 (5 μm) semi-preparative column (9.4×250 mm). Absorbance values were read by Envision 2103 multilabel reader (PerkinElmer, USA).

Microbial strains

The strain MF359 was isolated from a marine sponge sample of *H. perleve* collected from the Bohai Sea, China. The strain was grown on a marine yeast extract peptone dextrose agar medium slant consisting of yeast extract 1 %, peptone 2 %, dextrose 2 %, NaCl 3.5 %, and agar 2 % at 28 °C. The *A. versicolor* MF359 was identified by analysis of the internal transcribed spacer (ITS) regions (GenBank accession number HQ000003) and morphology, and assigned the accession number CGMCC No. 3.15366 at the China General Microbiological Culture Collection Center (CGMCC). Generally, the primer pair ITS1 (5″-TCCGTAGGTGAACC TGCGG-3″) and ITS4 (5″-TCCTCCGCTTATT GATATGC-3″) were employed to amplify the ITS region of MF359. PCR amplification (25-μL final volume: 0.4 μL each primer, 2.5 μl 10×buffer, 2.5 μL 2.5-nM dNTP, 0.4-μL rTap polymerase, and 1-μL DNA template) of the ITS sequence was performed on ABI PCR Thermal Cycler with the initial denaturation at 94 °C for 5 min, 25 cycles of denaturation (94 °C 1 min), annealing (55 °C 1 min), and elongation (72 °C 45 s), and a final elongation at 72 °C for 10 min.

Bacillus subtilis (ATCC 6633), *S. aureus* (ATCC 6538), methicillin-resistant *S. aureus* (MRSA), and *Pseudomonas aeruginosa* (ATCC 15692) were used as testing strains for antibacterial activities of compounds. The MRSA strain used here was a clinical isolate from Beijing Chaoyang Hospital (Beijing, People's Republic of China) in November 2007.

Fermentation and isolation

A stock culture of the strain MF359 was grown and maintained on an marine yeast extract peptone dextrose agar medium slant consisting of yeast extract 1 %, peptone 2 %, dextrose 2 %, NaCl 3.5 %, and agar 2 % at 28 °C. The stock culture was inoculated into 250-mL Erlenmeyer flasks containing 50-mL seed medium consisting above components without agar. The culture was incubated on a rotary shaker (140 rpm) at 28 °C for 96 h. Five milliliters of the seed culture was transferred to 1,000-mL Erlenmeyer flasks containing 130 g of the producing medium which contained 100 g of rice, 3.25 g of soybean powder, and 30 mL of artificial seawater (3.4 % Pro-Reef sea salt (Tropic Marin, Wartenberg, Germany, in distilled water). The fermentation flasks were incubated at 28 °C for 20 days without shaking.

The cultivation products were extracted exhaustively with EtOAc/MeOH (80:20). The combined extracts were reduced to dryness in vacuo and the residue was partitioned between EtOAc and H_2O. The EtOAc layer (8.1 g after drying in vacuo) was fractionated by silica gel chromatography (50× 80 mm column, TLC H grade silica) using a stepwise gradient

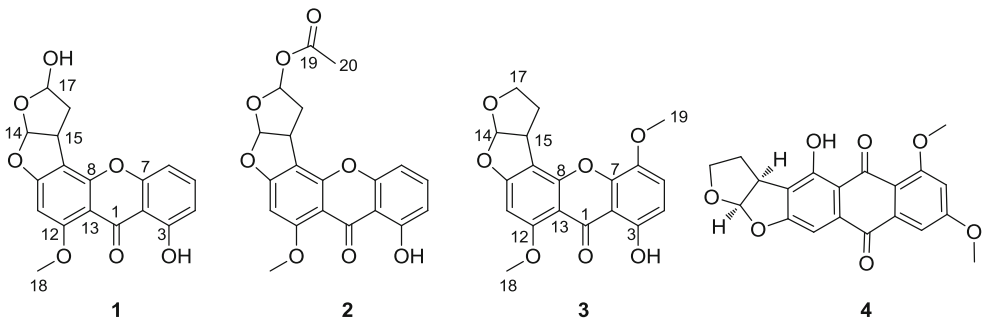

Fig. 1 Structures of compounds **1**–**4**

of 50–100 % light petroleum/CH_2Cl_2 and then 0–100 % MeOH/CH_2Cl_2 to afford 17 fractions (A–Q; 500 mL each). Fraction E fractionated on a Sephadex LH-20 column (800× 30 mm) using an isocratic elution of light petroleum/CH_2Cl_2/MeOH (5:5:1), to give eight subfractions (E1-E8). Subfraction E5 was further fractionated by HPLC (Agilent Zorbax SB-C3 250×9.4 mm, 5-μm column, 1.25 mL/min, isocratic elution 53 % MeCN/H_2O) to yield **1**, **2**, and **3**. Fraction G was fractionated on a Sephadex LH-20 column (800×30 mm) using an isocratic elution of CH_2Cl_2/MeOH (2:1), to give six subfractions (G1-G6). Subfraction G2 was further fractionated by HPLC (Agilent Zorbax SB-C3 250×9.4 mm, 5-μm column, 3.5 mL/min, isocratic elution 60 % MeCN/H_2O) to yield **4**.

Antibacterial assays

Antimicrobial assays were performed according to the Antimicrobial Susceptibility Testing Standards outlined by the Clinical and Laboratory Standards Institute using the bacteria *S. aureus* (ATCC 6538), methicillin-resistant *S. aureus* (clinical strain from Chaoyang Hospital, Beijing, China), *B. subtilis* (ATCC 6633), and *P. aeruginosa* (ATCC 15692). For each organism, a loopful of glycerol stock was streaked on an LB-agar plate, which was incubated overnight at 37 °C. A single bacterial colony was picked and suspended in Mueller-Hinton Broth to approximately 1×10^4 cfu/mL. A twofold serial dilution of each compound to be tested (4,000 to 31.3 μg/mL in dimethyl sulfoxide (DMSO)) was prepared and an aliquot of each dilution (2 μL) was added to a 96-well flat-bottom microtiter plate (Greiner). Vancomycin and ciprofloxacin were used as positive controls and DMSO as the negative control. An aliquot (78 μL) of bacterial suspension was then added to each well (to give the final compound concentrations of 80 to 0.80 μg/mL in 2.0 % DMSO) and the plate was incubated at 37 °C aerobically for 16 h. Finally, the optical density of each well at 600 nm was measured with an EnVision 2103 multilabel plate reader (PerkinElmer Life Sciences). Minimum inhibitory concentration (MIC) values were defined as the minimum concentration of compound that inhibited visible bacterial growth. All the experiments were performed in triplicate.

Results

Characterization and identification of isolated strain MF359

The marine-derived fungus *A. versicolor* MF359 was isolated from a marine sponge sample of *H. perleve* collected from the Bohai Sea of China and identified by morphology and sequence analysis of its ITS region and 5.8 S rDNA (GenBank accession number HQ000003) using conventional primer pair ITS1(5″-TCCGTAGGTGAACCTGCGG-3″) and ITS 4(5″-TCCTCCGCTTATTGATATGC-3″). The total genomic DNA of marine-derived fungus MF359 was extracted using the E.Z.N.A. kit (Omega). The polymerase chain reaction product is 534 bp. The purified PCR products were sequenced (Huada Bio., Beijing, China). Multiple alignments with sequences of most closely related fungi and calculations of levels of sequence similarity were carried out using CLUSTAL W (Thompson et al. 1994). The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987) and using MEGA 4.0 (Tamura et al. 2007), as shown in Fig. 2. The topology of phylogenetic tree was evaluated by bootstrap resampling method with 1,000 replicates (Felsenstein. 1985). The fungus has been assigned the accession number MF359 in the culture collection at the Institute of Microbiology, Chinese Academy of Sciences, Beijing.

Structure elucidation

1 was obtained as a light yellow amorphous powder; $[\alpha]_D^{25}$ (*c* 0.13, −78.7, MeOH/$CHCl_3$ 5:3). Its HRESIMS revealed a molecular ion peak of *m/z* 343.0818 for $[M + H]^+$ (Calcd. 343.0818) and suggested a molecular formula of $C_{18}H_{14}O_7$. UV spectrum showed the maximal absorbance at 250 and 324 nm. The ^{13}C NMR spectrum showed 18 carbon signals. The ^1H NMR, together with ^{13}C NMR (Table 1) and HSQC NMR data of **1** revealed the presence of one methoxyl group at δ_H 3.90 (δ_C 56.8, C-18); one sp^3 hybridized methene at δ_H 2.58 (m) and 2.39 (d, 13.8 Hz) (δ_C 36.1, C-16); one sp^3 hybridized methine at δ_H 4.34 (dd, 9.0 and 6.0 Hz) (δ_C 41.7, C-15); two acetal or hemiacetal groups at δ_H 6.65 (d, 5.4 Hz) (δ_C 113.8, C-14) and δ_H 6.35 (d, 4.8 Hz) (δ_C 98.5, C-17); one aromatic proton signal at δ_H 6.69 (δ_C 90.8, C-11); and one ABX system at δ_H 6.75 (d, 8.4 Hz), 7.62 (t, 8.4 Hz) and 6.97 (d, 8.4 Hz) (δ_C 110.8, C-4; 136.2, C-5; 106.3, C-6); and one hydroxyl signal at δ_H 13.36 (s, 3-OH). Analysis of ^1H–^1H COSY confirmed the bold moieties in Fig. 3. Besides the above assigned carbon signals, there were nine sp^2 hybridized quaternary carbons, including δ_C 180.7 (conjugated carbonyl carbon, C-1), δ_C 108.3 (C-2), 107.1 (C-9), 104.9 (C-13), five oxygenated aromatic quaternary carbons (δ_C>140) at δ_C 161.5 (C-3), 154.4 (C-7), 153.4 (C-8), 164.8 (C-10), and 163.2 (C-12). These NMR data suggested that compound **1** is a xanthone derivative (Asai et al. 1995). In the heteronuclear multiple-bond correlation spectroscopy (HMBC) spectrum (Fig. 3), the cross peaks from H-3-OH to C-2, C-3, and C-4 and from H-6 to C-2 and C-4 identified the chemical shift of C-2 (δ_C 108.3). C-7 was characterized by the HMBC correlations from H-5 to C-3 and C-7. The HMBC signals from H_3-18 to C-12 and from H-11 to C-9, C-10, C-12 and C-13 demonstrated that the methoxyl group was attached to C-12. The cross peaks in HMBC spectrum from H-14 to C-17 and

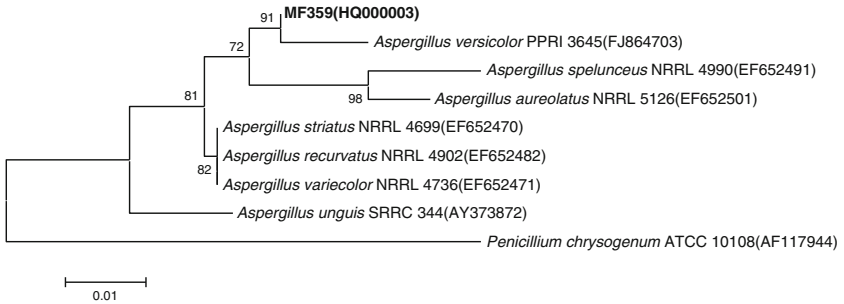

Fig. 2 Neighbor-joining phylogenetic tree of strain MF359. *Numbers at nodes* indicate levels of bootstrap support (%) based on a neighbor-joining analysis of 1,000 resampled datasets; only values >50 % are given. NCBI accession numbers are given in *parentheses. Bar* 0.01 nucleotide substitutions per site

from H-17 to C-14 revealed the connection from C-14 through one oxygen atom to C-17. The HMBC correlations from H-14 to C-10 and from H-15 and H-16b to C-9 suggested that the connection from C-15 to C-9 and from C-14 through one oxygen atom to C-10. With the help of HRESIMS data of **1**, there should be a hydroxyl group at C-17. Thus, the structure of **1** was assigned as in Fig. 1 which was hemiacetal of sterigmatocystin. This was the first report of sterigmatocystin hemiacetal from nature. Chen described the semisynthesis of **1** from reduction of sterigmatocystin (Chen et al. 1977).

2 was obtained as a light yellow amorphous powder; $[\alpha]_D^{25}$ (*c* 0.15, −20, MeOH/$CHCl_3$ 5:3). HRESIMS revealed a molecular ion peak of *m*/*z* 385.0910 for $[M + H]^+$ (Calcd. 385.0918) and suggested 384 as the molecular weight and $C_{20}H_{16}O_8$ as the molecular formula. The molecular weight difference of 43 between **2** and **1** suggested that **2** should be

Table 1 NMR data of compounds **1**, **2**, and **3** in DMSO-d_6

	1		**2**		**3**	
Position	δ_H, mult (*J* in Hz)	δ_C	δ_H, mult (*J* in Hz)	δ_C	δ_H, mult (*J* in Hz)	δ_C
1		180.7		181.1		180.3
2		108.3		108.9		108.8
3		161.5		162.0		154.1
4	6.75, d (8.4)	110.8	6.73, d (8.4)	111.3	6.66, d (9.0)	109.0
5	7.62, t (8.4)	136.2	7.62, t (8.4)	136.8	7.40, d (9.0)	120.8
6	6.97, d (8.4)	106.3	6.95, d (8.4)	106.9		139.1
7		154.4		155.1		144.0
8		153.4		154.5		153.8
9		107.1		107.2		105.5
10		164.8		165.1		165.9
11	6.69, s	90.8	6.69, s	91.7	6.62, s	90.3
12		163.2		163.8		163.0
13		104.9		105.9		105.0
14	6.65, d (5.4)	113.8	6.60, d (6.0)	113.1	3.99, s	56.7
15	4.34, dd (9.0, 6.0)	41.7	4.36, q (6.0)	42.3	6.54, d (6.0)	113.5
16a	2.58, m	36.1	2.54, m	36.4	4.25, t (7.2)	43.5
16b	2.39, d (13.8)		2.49, m			
					2.24, m	30.5
17	6.35, d (4.8)	98.5	6.25, t (4.8)	98.6	4.10, m	67.4
18	3.90, s	56.8	3.89, s	57.4	3.53, m	
19				170.0	3.98, s	57.5
20			2.05, s	21.5		
3-OH	13.36, s		13.30, s			

Measured at 150 MHz for ^{13}C, 600 MHz for ^{1}H

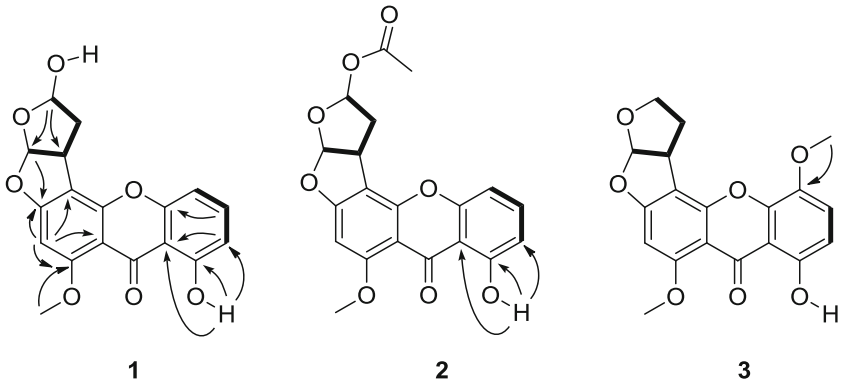

Fig. 3 The key ^{1}H–^{1}H COSY (*bold line*) and HMBC (H → C) correlations of compounds **1**–**3**

the acetylated product of **1**. UV spectrum showed the maximal absorbance at 248 and 322 nm. In the ^{1}H and ^{13}C NMR spectra of **2**, except for the similar signals of **1**, there is a group signals for one acetyl group at δ_C 170.0 (C-19) and 21.5 (C-20; δ_H 2.05, s) (Table 1). Since the hydroxyl proton at C-1 was observed in proton spectra and HMBC correlations from H-OH to C-2, C-3 and C-4 were observed (Fig. 3), the acetyl group was attached to C-17. Therefore, the structure of **2** was assigned.

3 was obtained as a light yellow amorphous powder; $[\alpha]_D^{25}$ (*c* 0.10, –168.7, MeOH/$CHCl_3$ 5:3). HRESIMS revealed a molecular ion peak of *m/z* 357.0976 for $[M + H]^+$ (Calcd. 357.0969) and suggested 356 as the molecular weight and $C_{19}H_{16}O_7$ as the molecular formula. UV spectrum showed the maximal absorbance at 252 and 328 nm. Comparison with the ^{1}H the ^{13}C NMR spectra of **1**, in the NMR data of **3**, one hemiacetal moiety was replaced by one oxygenated methane, and one aromatic proton was substituted by methoxyl group. In the ^{1}H-^{1}H-COSY spectra of **3**, the correlation from H-14 to H-15, to H_2-16, then to H_2-17 revealed presence of oxygenated methane at δ_C 67.4 (C-17; δ_H 4.10 and 3.53, m) (Table 1). Therefore, the structure of **3** was assigned as 5-methoxydihydrosterigmatocystin. Mori reported the semisynthesis of **3** from its unsaturated compound 5-methoxysterigmatocystin. (Mori et al. 1986)

4 was isolated as a light yellow solid. It was identified as aversin by comparing the NMR data with reported data by Shao (Shao et al. 2007). Biological activities of compounds **1**–**4** were evaluated for activities against *B. subtilis*, *S. aureus*, *MRSA*, and *P. aeruginosa*. The results were shown in Table 2. Compounds **3** exhibited potent activities against *B. subtilis* and *S. aureus* with MIC values of 3.125 and 12.5 μg/mL, respectively (Table 2).

Discussion

Sterigmatocystins and its analogues aflatoxins are poisonous fungal metabolites produced by certain strains of the molds *A. flavus*, *Aspergillus parasiticus*, and *Aspergillus* sp. (Cole and Cox 1981). Exposure to it can result into acute and chronic toxic responses and cancer. Ashley reported that trifluoroacetic acid-catalyzed addition of water across the vinyl ether double bond yielded α- and β-hemiacetal configurations (Ashley et al. 1987). Chen also reported this reduction of sterigmatocystin to its hemiacetal derivative under reflux in acetone containing 10 % H_2SO_4 (Chen et al. 1977). Sterigmatocystin exerted a marked influence on gastric mucus and gland cells, showing dominant gastritis, erosion events, polyps, and intestinal metaplasia in these animals (Kusunoki et al. 2011). Sterigmatocystin also showed mosquito larvicidal activities with LC_{50} and LC_{90} values of 13.3 and 73.5 ppm (Matasyoh et al. 2011).

Our study firstly reported the isolate of these sterigmatocystin analogues and sterigmatocystin hemiacetal from marine-derived fungus. Compound **3** showed potential activities against *S. aureus* and *B. subtilis*. Our results suggested that marine-derived fungus can generate more

Table 2 Antimicrobial activities of compounds **1**–**4**

Microorganism (strain)	Minimum inhibitory concentration (μg/mL)				
	1	2	3	4	Positive control
Staphyloccocus aureus (ATCC 6538)	>100	>100	12.5	>100	1[a]
MRSA[b] (clinical isolate 309)	>100	>100	>100	>100	1[a]
Pseudomonas aeruginosa (ATCC 15692)	>100	>100	>100	>100	1[c]
Bacillus subtilis (ATCC 6633)	>100	>100	3.125	>100	0.5[a]

[a] Vancomycin

[b] Methicillin-resistant *Staphylococcus aureus*

[c] Ciprofloxacin

sterigmatocystic analogues and will be a potential resource for producing these compounds for biology study.

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