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Isolation of novel indole diterpenes and dihydrodibenzofuran from the marine fungus *Aspergillus* sp.

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Abstract
Twelve secondary metabolites were earlier isolated from two marine-derived fungal strains, *Aspergillus* sp. AF-119 and *Aspergillus* sp. JQG a-6f. The objectives of this investigation were to isolate and purify natural products produced in these fungi, and identify possible drug candidates. The chemical structures of these compounds were elucidated and characterized by the analysis of 1D and 2D NMR spectra, UV, optical rotation and mass spectroscopy data. Three of the isolated compounds are novel derivatives of indole diterpenes (**1**& **2**), and dihydrodibenzofuran, namely, 9-hydroxy-7-methyl-2, 3-dihydrodibenzo [b, d] furan-1, 4-dione (**3**). The rest of the compounds were also determined from spectroscopy data to be known. These included three indole diterpenes – paspalinine (**4**), paspalicine (**5**) and paspaline (**6**); two ditetopiperazines – fumitremorgin B and lanosulin (**7** & **8**); methyl 2,8-dihydroxy-6-methyl-9-oxo-9H-xanthene-1-carboxylate (**9**); 1,3,8-trihydroxy-6-methylanthracene-9,10-dione (**10**); terphenyl (**11**) and 2-(3-chloro-2-hydroxyphenyl)-5-hydroxy-3,7,8-trimethoxy-4H-chromen-4-one (**12**). Paper disc diffusion assays showed that the novel compounds exhibit antibacterial activity on *S. aureus*, *B. subtilis*, and *E. coli*, but no activity on *C. niger*. MTT assays showed that the three novel compounds have antibacterial activity *in vitro*. These findings further enhanced the concept that natural products synthesized by marine-derived fungi possessed high structural diversity, antimicrobial and antitumor activities. Hence, they are a rich resource for drug discovery.

Keywords: Marine Fungi; Natural product; Indole diterpenes; Cytotoxicity Assay

INTRODUCTION

Microorganisms varied widely in their capacity to synthesize and transform chemicals. They have been a remarkable source of relief when it comes to harnessing potential chemicals. They have proven to be a rich reservoir of structurally novel and biologically active natural products that might be useful leaders in the development of new pharmaceutical agents (Faulkner, 2000). Amongst the endophytic fungi in the marine ecosystem, the genera *Penicillum* and *Aspergillus* are very prolific in synthesizing potent novel secondary metabolites (Strobel, 2004). Natural products from these marine organisms possessed significant relevance in the fight against diseases (Newman *et al*, 2006). The major sources of marine fungi and the beneficial attributes of their natural products in the marine biotope are very important. Natural products synthesized by these organisms have imparted significantly in various ways on the livelihood of humanity (Guo *et al*, 2008; Proksch *et al.*, 2003). A

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number of natural products have been identified and isolated from marine organisms in recent time (Floss, 1997). Secondary metabolites synthesized by marine fungi have ecological roles in the marine environment. They may serve as biochemical protective mechanism through which some microorganisms and other higher forms of life, such as plants and animals, can defend themselves from pathogens; and also serve as volatile attractants and coloring agents in the habitats of the organisms that synthesized them.

The need to identify potential sources of bioactive natural products has increased in contemporary time. This has made the isolation of viable and novel compounds from marine sources important. One of such sources is the vast deposit of endophytic fungi in the marine world.

EXPERIMENTAL

Identification of fungal species. The two strains of marine fungi, AF-119 and JQG a-6f were obtained from the microbial pharmaceutical laboratory at the University of Xiamen; they were pre-screened and PCR was conducted and they were identified as members of the genus *Aspergillus.*

Fermentation, extraction and isolation. The two marine fungi strains (AF-119 and JQG a-6f) were cultured on potato-dextrose-agar (PDA) medium containing 50% sea water, and allowed to ferment for 16 and 20 days respectively. The fermentation products were subjected to extraction for four consecutive days by using ethyl acetate (AF-119) and a mixture of ethyl acetate, MeOH and acetic acid in a composition of 80%, 15%, and 5% v/v/v (JQG a-6f). The filtrates collected were vacuum dried and subsequently dissolved with ethyl acetate and water (1:1) in a separating funnel, thoroughly stirred and separated by partitioning. Again the filtrates were collected and vacuum dried; and the weights of the crude extracts recorded (2.5g for AF-119 & 10.7g for JQG a-6f). The crude extracts were further fractionated into fractions (5 fractions for each strain) using reverse phase C_{18} silica gel chromatography (RP-18 MPLC). Bioautography testing was conducted on each fraction to ascertain whether secondary metabolites synthesized in these marine fungi have bioactivities in vitro in representative microbes (*S. aureus*, *C. albicans*, *E. coli*). The components of each fraction were isolated and purified by using column chromatographic techniques such as Sephadex LH-20, silica gel chromatography, HPLC (Agilent technologies 1200 series), and thin layer chromatography (TLC). The pure compounds obtained were subjected to NMR, UV, and mass spectroscopy, and the 1D and 2D spectral data were used to elucidate their structures. The novel compounds were tested using paper disc diffusion assays and MIC to determine their bioactivity in vitro.

Antimicrobial testing of novel compounds. Antibacterial and antifungal testing was conducted on the three novel compounds (**1**, **2**, & **3**) isolated from these marine fungi. The three novel compounds did not show any activity on the representative fungus used (C. *niger*), indicating that they have no antifungal activity. But they exhibit antibacterial activity on *S. aureus* and *B. subtilis*, and *E. coli*. This showed that they possessed broad-spectrum antibacterial activity. At the concentration of 10µg/µl, the zones of inhibition of **1** were 7mm (*S. aureus*) and 6mm (*B. subtilis*); for **2**, 10mm (*S. aureus* and *E. coli*), and 8mm (*B. subtilis*); for **3,** 12mm (*S. aureus*) and 8mm (*B. subtilis*). At the concentration of 20µg/µl, the zones of inhibition of **1** were 13mm (*S. aureus* and *B. subtilis*) and 12mm (*E. coli*); for **2**, 10mm (*S. aureus* and *E. coli*) and 18mm (*B. subtilis*); for **3**, 15mm (*S. aureus*), 17mm (*B. subtilis*) and 12mm (*E. coli*). The MIC of **1**, **2**, and **3** are 32µg/ml, 16µg/ml and 10µg/ml respectively.

Spectroscopy and other analytical details. NMR spectra were recorded on a Bruker

Avance 500 MHz spectrometer (500 MHz for $_1$H and 125 MHz for $_{13}$C) and chemical shifts were recorded as δ values. Mass spectra were performed on a VG Autospec 3000 mass spectrometer. UV spectra were measured on a Lengguang Gold spectrumlab 54. Optical rotations were measured on an AA-55 polarimeter (Optical Activity Ltd). Silica gel (200–300 mesh), reversed-phase silica gel C18 (40–75 mm, Fuji Silysia Chemical Ltd.) and Sephadex LH-20 (18–110 mm, Merck, Darmstadt, Germany) were used for open column chromatography (CC). High-performance liquid chromatography (HPLC) was done on a Waters 6000A solvent delivery system equipped with UV detection (254nm). All solvents were reagent grade and redistilled from appropriate drying agents before use.

RESULTS AND DISCUSSIONS

In pursuance of identifying and isolating novel molecules from the marine ecosystem, twelve pure secondary metabolites were isolated from two strains of marine fungi belonging to the genus *Aspergillus* (AF-119 & JQG a-6f). Two of the isolated compounds (**1** & **2**) are novel derivatives of indole diterpenoids and one of them is a novel derivative of dihydrodibenzofuran (**3**). The rest of the isolated compounds are known indole diterpenoids (**4-6**), ditetopiperazines (**7-8)** (Nozawa, 1989; Tepaske, 1992); Xanthone (**9**), anthraquinone (**10**), terphenyl (**11**), and chloroflavonin (**12**) (Barsby *et al*, 2006; Chang, 1974). Compounds **1**, **2** and **4-8** were isolated from JQG a-6f; while **3**, and **9-12** were obtained from AF-119.

The chemical structure derived for **2** based on the 1D and 2D NMR spectra, is similar to that of **4** (paspalinine), but there are slight differences. Inducing from the 1D and 2D NMR spectra, and the chemical structure derived is based on the chemical shifts of protons and carbon atoms. It can be seen that **2** is a novel derivative of paspalinine, an indole diterpene. It differs from the other indole diterpenes at C-1, C-2, and C-15a. It contains a short-chain 4, 5 dihydroxyl-5-methyhex-1-en-2-one moiety at C-5 (δ_C 169.2s). ^{1}H and ^{13}C NMR data for **2** are presented in table1. The relative stereochemistry is proposed for **2** by analogy to **4-6** (known indoloditerpenes). Proton spin systems were determined by analysis of ^{1}H-^{1}H COSY data and homonuclear decoupling experiments. Single-bond ^{1}H-^{13}C connectivity was determined by analysis of HMBC data, and quaternary ^{13}C NMR assignments were based upon ^{13}C NMR δ values and selective INEPT results.

The UV and optical activity of **2** are as below: UV: (10µl/ml) 231nm – 0.02248; 280nm – 0.037 Optical activity: 1mg/ml - 0.060 (OROT 589nm 20ºC)

Inspection of the ^{1}H and ^{13}C spectra of **1** revealed eight methine groups at C2 (δC 84.6d; δH 3.20,dd), C6 (δC 44.9d; δH 1.35,m), C9 (δC 35.0d; δH 2.89,m), C13 (δC 127.5d; δH 7.53, dt), C14 (δC 126.9d; δH 7.41, dt), C15 (δC 131.8d; δH 7.55,dt), C16 (δC 127.2d; δH 7.18,br,d), and C23 (δC 85.3d; δH 2.96,m); seven methylene groups at C3 (δC 24.09t; δH 1.61), C4 (δC 37.6t; δH 1.80), C7 (δC 20.3t; δH 1.57), C8 (δC 29.7t; δH 1.57), C10 (δC 48.2t; δH 2.96,m), C21 (δC 33.1t; δH 1.82,m), and C22 (δC 29.7t; δH 1.49); and four methyl groups at C1a (δC 24.14q; δH 1.16, s), C1b (δC 24.14q; δH 1.16, s), C5a (δC 12.3q; δH 0.81), and C20a (δC 17.9q; δH 1.10).

The linkage of these methine, methylene, and methyl groups are confirmed by HSQC and HMBC correlations. The quaternary carbon atoms are connected to their respective methine, methylene, and methyl groups. The 2D HMBC and ^{13}C NMR values support the connectivity of these atoms in the structure (table 2). From the structure elucidated, **1** is novel paspalinine, an indoloditerpenes, whose stereochemistry differed from the others, by an introduction of

an eight-membered ring at C9 (δC 35.0d), C12 (δC 135.4s), C17 (δC 136.8s) and C19 (δC 59.3s), replacing the two five-membered ring (one N-containing ring of the indole ring and one homonuclear ring) of the indoloditerpenes. The relative molecular weight determined by ESI-MS is 453.29; and its molecular formula was confirmed to be $C_{28}H_{39}NO_4$ by electron impact (EI) mass spectrometry and elemental analysis.

The UV and optical activity of 1 were determined as UV: (10μl/ml) 220.9nm – 0.0751; 251.9nm – 0.0159; Optical activity: 3mg/ml -0.100 (OROT 589nm 20ºC)

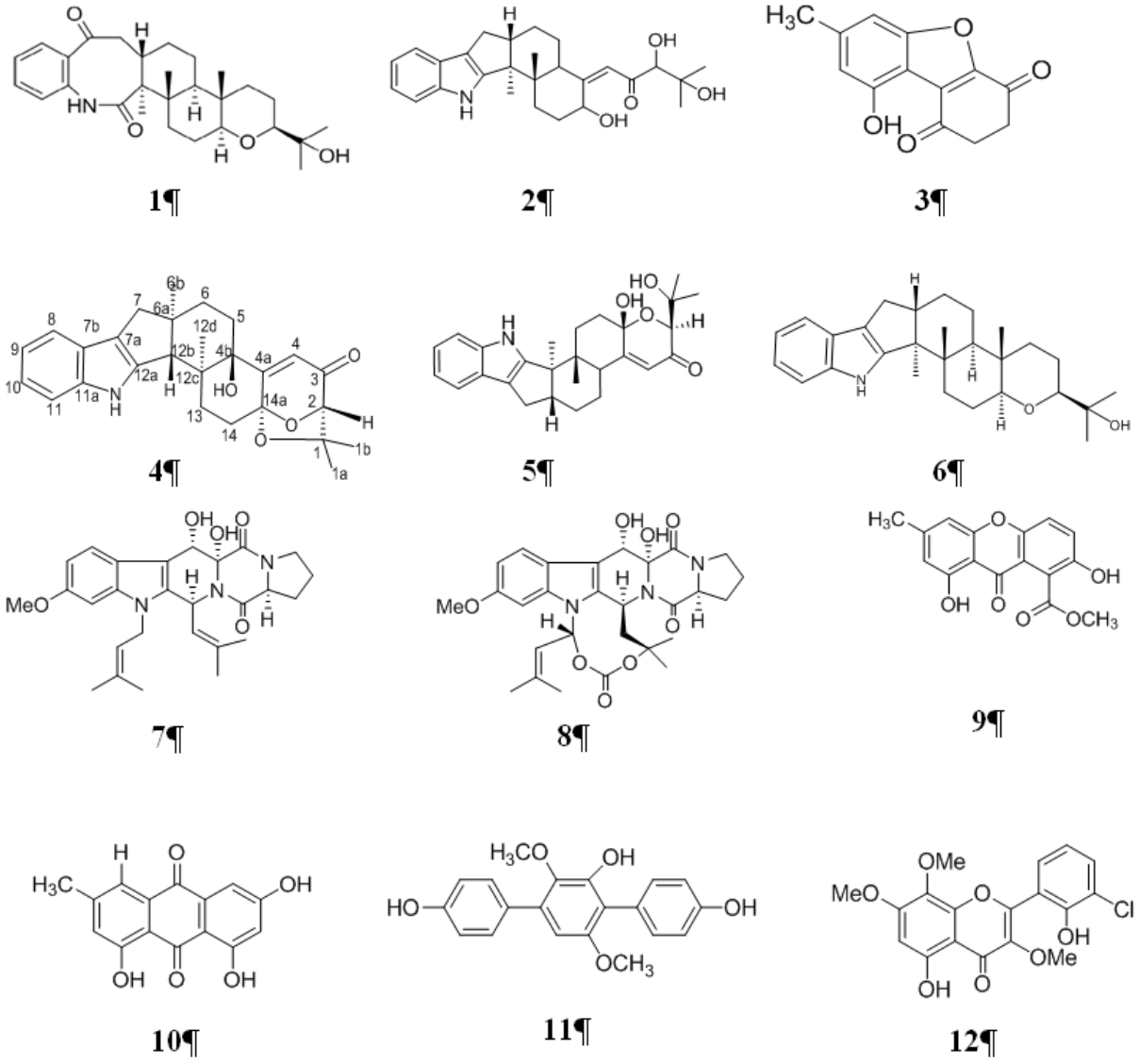


Figure 1: Chemical Structures of Compounds **1-12**

Table 1: 1D and 2D NMR spectral data of **2** (in acetone)

No.	δC (DEPT)	δH	HMBC
1	71.6 (C)		
1a	26.1 (CH_3)	1.90 (m, 3H)	C-1b, 2, 3
1b	24.0 (CH_3)	1.79 (m, 3H)	C-1a, 2, 3

2	82.5 (CH)	3.80 (s, 1H)	C-1, 4
3	187.6 (C)		
4	120.8 (CH)	5.85 (s, 1H)	C-2, 15a
5	169.2 (C)		
5a	84.7 (CH)	3.80 (d, J=11.0, 1H)	C-4, 5, 7
6	21.5 (CH_2)	1.79 (m, 2H)	C-5, 7a, 14
7	32.9 (CH_2)	1.89 (m, 2H)	C-5a, 7b, 8
7a	48.9 (CH)	2.79 (m, 1H)	C-6, 8, 8a
7b	51.5 (C)		
8	27.2 (CH_2)	2.65 (dd, J=6.4, 2H)	C-7, 8a, 9, 13b
8a	111.7 (C)		
9	125.1 (C)		
10	117.8 (CH)	7.30 (t, J=8.5, 1H)	C-8a, 11
11	118.8 (CH)	6.98 (m, 1H)	C-8a, 11
12	119.7 (CH)	6.98 (m, 1H)	C-10, 11
13	111.7 (CH)	7.30 (dd, J=6.8, 1H)	C-9, 11
13a	140.7 (C)		
13b	149.8 (C)		
13c	14.0 (CH_3)	1.10 (m, 3H)	C-7a, 7b, 14a
14	26.5 (CH_2)	1.98 (m, 2H)	C-5a, 13
15	28.3 (CH_2)	1.95 (m, 2H)	C-14, 14a, 15a
15a	74.6 (CH)	4.46 (t, J=9.0, 1H)	C-4, 5, 14
OH-15a		9.94 (s, 1H)	

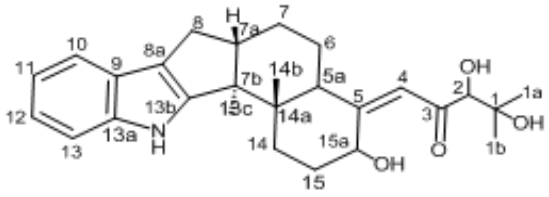


Table 2: 1D and 2D NMR spectral data table of **1** (in methanol)

No.	δC (DEPT)	δH	HMBC
1	71.5 (C)		
1a	24.1 (CH_3)	1.16 (s, 3H)	C-1, 2 3
1b	24.1 (CH_3)	1.16 (s, 3H)	C-1, 2 3
2	84.6 (CH)	3.20 (dd, J=?, 1H)	
3	24.1 (CH_2)	1.61	C-1, 2, 4
4	37.6 (CH_2)	1.80 (, 2H)	C-2, 5, 5a
5	35.8 (C)		
5a	12.3 (CH_3)	0.81	C-4, 5, 6, 23
6	44.9 (CH)	1.35 (m, 1H)	C-18, 19, 20
7	20.3 (CH_2)	1.57 (2H)	C-6
8	29.7 (CH_2)	1.57 (2H)	
9	35.0 (CH)	2.89 (m, 1H)	
10	48.2 (CH_2)	2.96 (m, 2H)	C-8, 9, 11, 19
11	205.0 (C)		
12	135.4 (C)		
13	127.5 (CH)	7.53 (dt, 1H)	C-12, 16
14	126.9 (CH)	7.41 (dt, 1H)	C-12, 16
15	131.8 (CH)	7.55 (dt, 1H)	C-12, 13 or 16
16	127.2 (CH)	7.18 (br, d, 1H)	C-12, 14
17	136.8 (C)		
18	176.7 (C)		
19	59.3 (C)		

20	41.5 (C)		
20a	17.9 (CH_3)	1.10 (s, 3H)	C-6, 20, 19, 21
21	33.1 (CH_2)	1.82 (m, 2H)	C-2, 5
22	29.7 (CH_2)	1.49 (2H)	
23	85.3 (CH)	2.96 (m, 1H)	C-2, 3 5a

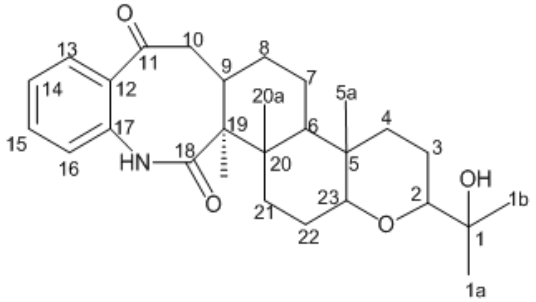
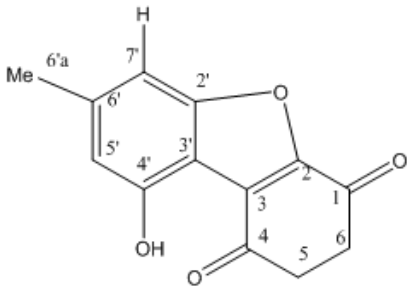


Table 3: 1D and 2D NMR spectral data for **3** (in chloroform)

No.	δC (DEPT)	δH	HMBC
1	189.8 (C)		
2	178.1 (C)		
3	118.1 (C)		
4	197.3 (C)		
5	26.3 (CH_2)	3.11 (dt, J=3.1, 2H)	C-1, 6
6	33.8 (CH_2)	2.75 (dt, J=3.1, 2H)	C-1, 4
2'	156.5 (C)		
3'	108.8 (C)		
4'	162.5 (C)		
5'	114.4 (CH)	6.73 (s, 1H)	C-3', 6'a, 7'
6'	148.2 (C)		
6'a	22.4 (CH_3)	2.44 (s, 3H)	C-5', 6', 7'
7'	108.0 (CH)	6.80 (s, 1H)	C-2',3', 5', 6'a
OH-4'		12.25 (s)	C-3', 4', 5'



4-12 are known secondary metabolites that have already been isolated and characterized from other strains of marine fungi and other microbes (Nozawa, 1989; Floss, 1997).

The structure of **3** deduced from the 1D and 2D NMR spectra showed the presence of one methyl group at C-6'a(δ_C 22.4; δ_H 2.44), two methylene groups at C-5 (δ_C 26.3 & δ_H 3.11) and C6 (δ_C 33.8 & δ_H 2.75), and two methine groups at C-5'(δ_C 114.4 & δ_H 6.73), and C-7'(δ_C 108.0 & δ_H 6.80). Connectivity of the methine, methylene and methyl carbons to the quaternary carbons was established by HMBC spectrum (table 3). Two benzene rings bond to a five-membered oxygen substituted ring with the first benzene ring containing two ketone carbonyl carbon atoms at C-1(δ_C 189.8) and C-4 (δ_C 197.3). One hydroxyl group (δ_H 12.2) is bond to the second benzene at C-4' (δ_C 162.5). The molecular formula determined for **3** is

$C_{13}H_{10}O_4$ and its relative molecular weight obtained from ESI-MS is 230.22.

The UV and optical activity determined for 3 are- UV: (10µg/ml) 245nm- 0.0382; 320nm - 0.0248; Optical activity: 2mg/ml +0.029 (OROT 589 20ºC).

Marine fungi are capable of synthesizing potent novel bioactive natural products that have antimicrobial and anti-tumor activities. Information gathered from other sources indicates that the genera *Penicillium* and *Aspergillus* in the fungal world are very prolific in synthesizing novel biologically active natural products (. And with this findings obtained from this research work thus far, it has rekindled the hope and aspirations of the writer, and other alike, to further delve into more vigorous investigation. Research work on marine fungi will assist in identifying and harnessing the vast repository of novel compounds that are synthesized in these two genera of fungi.

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