Tetrahedron 64 (2008) 7986–7991

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Seven new prenylated indole diketopiperazine alkaloids from holothurian-derived fungus *Aspergillus fumigatus*

Fazuo Wang, Yuchun Fang, Tianjiao Zhu, Min Zhang, Aiqun Lin, Qianqun Gu *, Weiming Zhu *

Key Laboratory of Marine Drugs, Chinese Ministry of Education, Institute of Marine Drugs and Food, Ocean University of China, Qingdao 266003, PR China

ARTICLE INFO

Article history:
Received 18 February 2008
Received in revised form 11 May 2008
Accepted 3 June 2008
Available online 10 June 2008

Keywords:
Alkaloids
Marine-derived fungi
Aspergillus fumigatus
Structure elucidation
Cytotoxicity

ABSTRACT

Seven new prenylated indole diketopiperazine alkaloids, including compound **1**, 3 spirotryprostatins C–E (**2**–**4**), 2 derivatives of fumitremorgin B (**5** and **6**), and 13-oxoverruculogen (**7**), have been isolated from the holothurian-derived fungus *Aspergillus fumigatus*, along with 12 known ones (**8**–**19**). The structures of the new compounds were determined on the basis of extensive spectroscopic data and amino acid analysis. All new compounds were evaluated for their cytotoxic activities on MOLT-4, A549, HL-60, and BEL-7420 cell lines by the MTT and SRB methods.

1. Introduction

In our ongoing search for antitumor metabolites from marine microorganisms, we found that ethyl acetate extracts of marine-derived fungus *Aspergillus fumigatus* Fres., isolated from holothurian *Stichopus japonicus*, contained different cytotoxic alkaloids constituents that were found from our previous works on the same genera strain by active and chemical screening.[1,2] Therefore, investigations of the active constituents of this fungus led to the isolation of 7 new prenylated indole diketopiperazine alkaloids, including 4 spiro-oxindole diketopiperazines, 2 derivatives of fumitremorgin B, and 1 derivative of verruculogen, along with 13 known ones.

A series of indole diketopiperazine alkaloids, biosynthetically originated from L-tryptophan and L-proline, were discovered with cell cycle inhibitory activity from the broth of *A. fumigatus*.[3–11] Among them, the spirotryprostatins showed the most attention due to their interesting intriguing molecular structures,[12–17] although their biological activity is relatively modest. To the best of our knowledge, only two compounds with this spiro-oxindole skeleton were reported from nature. In this paper we report the isolation, structure elucidation, and cytotoxic activities of these new compounds (**1**–**7**).

2. Results and discussion

A. fumigatus was cultured in liquid medium for nine days and the metabolites were extracted with EtOAc. The crude extract was then subjected to repeated column chromatography and followed by semi-preparative HPLC separation to obtain 7 new indole diketopiperazine alkaloids (**1**–**7**), along with 12 known ones (**8**–**19**). The known compounds **8**–**19** were identified as spirotryprostatin A (**8**),[10] 13-oxofumitremorgin B (**9**),[18–20] fumitremorgin B (**10**)[3,4] verruculogen (**11**),[21] 3-β hydroxy *cyclo*-L-tryptophyl-L-proline (**12**),[22,23] *cyclo*-L-tryptophyl-L-proline (**13**),[24] tryprostatin B (**14**),[7,8] tryprostatin A (**15**),[7,8] *N*-prenyl-*cyclo*-L-tryptophyl-L-proline (**16**),[25] fumitremorgin C (**17**),[8] 12,13-dihydroxyfumitremorgin C (**18**),[8] and cyclotryprostatins A (**19**),[11] respectively. 13-Oxofumitremorgin (**9**) was first reported from the *Aspergillus* group and its stereochemistry was verified by X-ray diffraction analysis.[20]

Compound **1** was isolated as pale yellow amorphous solid. Its molecular formula was determined as $C_{22}H_{25}N_3O_6$ according to HRESIMS (found *m*/*z* 450.1620 $[M+Na]^+$, calcd 450.1641). In the UV spectrum, compound **1** showed characteristic absorption curve suggestive of a 6-*O*-methylindole chromophore with absorption maxima at 220 ($\log \varepsilon$ 4.29), 251 (4.24), 286 (4.09), and 392 (3.53) nm,[7–11] and the IR spectrum of **1** showed absorption bands at 3281 (OH), 1652 (C=O), 1619 (C=C) in the functional group region. Comparisons of the 1D NMR spectra of **1** with those of **8** suggested that **1** showed the similar oxindole diketopiperazine skeleton as **8**,[10] and the differences were presence of two hydroxyl groups [8-OH (δ_H 5.38, br s, δ_C 73.5, C-8) and 9-OH (δ_H 7.42, br s, δ_C 86.3, C-9)]

---

[*] Corresponding authors. Tel.: +86 532 82032065; fax: +86 532 82033054 (Q.G.); tel.: +86 532 82032065; fax: +86 532 82033054 (W.Z.).

E-mail addresses: guqianq@ouc.edu.cn (Q. Gu), weimingzhu@ouc.edu.cn (W. Zhu).

0040-4020/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2008.06.013

Table 1
^{1}H NMR data for compounds **1**–**7** in $CDCl_3$[a]

Position	δ_H (mult., J in Hz)						
	1	**2**	**3**	**4**	**5**	**6**	**7**
4	7.43 (d, 8.7)	6.98 (d, 8.2)	6.98 (d, 8.3)	7.03 (d, 8.2)	7.85 (d, 8.7)	7.85 (d, 8.7)	8.12 (d, 8.7)
5	6.36 (dd, 1.9, 8.7)	6.56 (dd, 2.3, 8.3)	6.57 (dd, 2.3, 8.3)	6.62 (dd, 2.3, 8.2)	6.81 (dd, 2.1, 8.7)	6.80 (dd, 2.3, 8.7)	6.87 (dd, 2.1, 8.7)
7	6.24 (d, 1.9)	6.40 (d, 2.3)	6.42 (d, 2.3)	6.69 (d, 2.3)	6.89 (d, 2.1)	6.72 (d, 2.3)	6.59 (d, 2.1)
8	4.75 (s)	4.82 (s)	4.82 (s)	4.84 (s)	5.73 (d, 2.4)	5.70 (d, 1.9)	
12	4.60 (dd, 7.3, 9.6)	4.62 (dd, 6.7, 9.6)		(4.62, dd, 7.8, 9.2)	4.41 (dd, 7.3, 10.1)	4.40 (dd, 7.3, 10.1)	4.99 (dd, 8.2, 8.3)
13	2.06 (m) 2.37 (m)	2.09 (m) 2.38 (m)	2.28 (m) 2.36 (m)	2.10 (m) 2.38 (m)	2.08 (m) 2.45 (m)	2.07 (m) 2.44 (m)	1.97 (m) 2.43 (m)
14	1.94 (m) 2.06 (m)	1.97 (m) 2.05 (m)	2.03 (m) 2.18 (m)	1.97 (m) 2.05 (m)	1.97 (m) 2.08 (m)	1.95 (m) 2.07 (m)	1.97 (m) 2.23 (m)
15	3.56 (m, 2H)	3.59 (m, 2H)	3.57 (m) 3.77 (m)	3.58 (m, 2H)	3.63 (m, 2H)	3.61 (m, 2H)	3.67 (m, 2H)
18	4.80 (s)	4.86 (d, 8.7)	4.86 (d, 8.9)	4.88 (d, 9.1)	6.00 (d, 10.4)	6.26 (d, 10.4)	6.34 (d, 9.5)
19	4.80 (s)	4.90 (d, 8.7)	4.91 (d, 8.9)	4.90 (d, 9.1)	4.67 (d, 10.4)	4.63 (d, 10.4)	1.87 (d, 13.7) 1.93 (dd, 9.5, 13.7)
21	1.56 (s, 3H)	1.07 (s, 3H)	1.09 (s, 3H)	1.11 (s, 3H)	2.02 (s, 3H)	2.04 (s, 3H)	2.03 (s, 3H)
22	1.76 (s, 3H)	1.61 (s, 3H)	1.62 (s, 3H)	1.62 (s, 3H)	1.63 (s, 3H)	1.62 (s, 3H)	1.01 (s, 3H)
23		4.23 (dd, 15.1, 6.4) 4.40 (dd, 15.1, 6.9)	4.23 (dd, 15.4, 6.4) 4.41 (dd, 15.4, 7.8)	6.62 (d, 15.1)	3.96 (dd, 4.1, 15.1) 4.10 (dd, 8.7, 15.1)	4.05 (dd, 2.3, 15.1) 3.83 (dd, 8.7, 15.1)	6.67 (d, 8.3)
24		5.13 (t, 6.4, 6.9)	5.13 (t)	6.49 (d, 15.1)	4.46 (dd, 4.1, 8.3)	4.25 (br d, 8.7)	5.05 (dd, 7.8, 1.4)
26		1.82 (s, 3H)	1.83 (s, 3H)	1.47 (s, 3H)	4.96 (s) 5.04 (s)	4.98 (s) 5.12 (s)	1.78 (s, 3H)
27		1.73 (s, 3H)	1.73 (s, 3H)	1.47 (s, 3H)	1.79 (s, 3H)	1.86 (s, 3H)	1.71 (s, 3H)
6-OMe	3.80 (s, 3H)	3.81 (s, 3H)	3.82 (s, 3H)	3.83 (s, 3H)	3.86 (s, 3H)	3.86 (s, 3H)	3.82 (s, 3H)
8-OH	5.38 (br s)	2.98 (br s)	2.79 (br s)	3.22 (br s)	4.73 (d, 2.4)	4.76 (d, 1.9)	
9-OH	7.42 (br s)	7.44 (br s)	8.53 (br s)	6.98 (br s)	4.05 (br s)	4.26 (br s)	6.34 (br s)
12-OH			5.13 (s)				

[a] Spectra were recorded at 600 MHz for ^{1}H using TMS as internal standard.

in **1** and the original 2-position amine carbonyl (δ_C 180.5) of oxindole core in **8** being ketone carbonyl (δ_C 200.1) in **1**. It suggested that **1** has a different biosynthetic pathway from that of **8** to form another spiro mode with the spiro carbon (δ_C 75.4, C-2) at the 2-position of the oxindole core (Tables 1 and 2, Fig. 1). These changes of structure were confirmed by the key HMBC correlations from H-8 (δ_H 4.75, s) to C-2 (δ_C 75.4), C-3, and C-17 (δ_C 165.2), from 9-OH (δ_H 7.42, br s) to C-8 (δ_C 73.5), C-9 (δ_C 86.3), and C-17, and from H-4 (δ_H 7.43, d, J=8.7 Hz), H-18 (δ_H 4.80, s), and 8-OH to C-3. All the other locations of protons and carbons were further determined by HMQC, ^{1}H–^{1}H COSY, and HMBC experiments and the key signals of COSY and HMBC correlations are shown in Figure 2.

The relative stereochemistry of **1** was determined on the basis of the NOESY spectrum. NOESY correlations (Fig. 3) between H-8 and H-19, between 8-OH and H-18, H-7, 9-OH, and between 9-OH and H-12, H-18 showed that H-7, H-12, H-18, 8-OH, and 9-OH in **1** were on the same side, while H-8, H-19, and H-4 were on the opposite side. The absolute stereochemistry of C-12 of the proline residue in **1** was determined by chiral capillary electrophoresis analysis of the acidic hydrolyzate of **1** (Fig. 4). The resulting L-configuration of proline was assigned and thus the C-12 was in *S*-configuration. So the absolute stereochemistry of **1** was established as 2*S*,8*S*,9*R*,12*S*,18*S* (Fig. 1).

Compound **2** was isolated as a pale yellow amorphous powder. The molecular formula was determined as $C_{27}H_{33}N_3O_6$ by HRESIMS (found m/z 518.2255 $[M+Na]^+$, calcd 518.2267). Based on the detailed analysis of NMR data (Tables 1 and 2), compound **2** showed the same spiro-oxindole skeleton as that of **8** with the exception of the presence of two hydroxyl groups (8-OH and 9-OH) and an isoprenyl unit (C-23 to C-27) attached to the indole N-1-position in **2**. HMBC correlations from 9-OH (δ_H 7.44, br s) to C-9 (δ_C 87.1) and C-17 (δ_C 165.1) and from H-8 (δ_H 4.82, s) to C-2 (δ_C 178.9), C-3 (δ_C 60.9), and C-17 confirmed the locations of 8-OH (δ_H 2.98, br s) and 9-OH. The isoprenyl group connection to N-1 in **2** was confirmed by HMBC correlations from both protons of H-23a (δ_H 4.23, dd, J=15.1, 6.4 Hz), H-23b (δ_H 4.40, dd, J=15.1, 6.9 Hz) to C-2 and C-7a (δ_C 144.6).

NOESY correlations between H-8 and H-19, H-4, and between 9-OH and H-18, H-12, 8-OH showed that H-8, H-4, and H-19 in **2** were all in the same side, while H-12, H-18, 8-OH, and 9-OH should be oriented in the opposite direction in **2** (Fig. 3). The absolute configuration of C-12 in **2** was determined as *S* configuration by acidic hydrolysis of **2**, as one of the products was identified as L-Pro (Fig. 4). Therefore, the absolute stereochemistry of **2** was

Table 2
^{13}C NMR data for compounds **1**–**7** in $CDCl_3$[a]

Position	δ_C						
	1	**2**	**3**	**4**	**5**	**6**	**7**
2	75.4	178.9	179.0	178.9	131.2	132.6	137.1
3	200.1	60.9	60.8	60.8	105.5	104.7	108.9
3a	112.2	117.5	117.1	117.0	120.5	120.8	118.9
4	126.8	126.9	127.0	127.3	121.4	121.7	123.2
5	110.2	106.5	106.7	107.5	109.6	109.0	111.7
6	169.2	160.6	160.8	160.7	156.4	156.3	157.7
7	94.4	97.2	97.4	97.8	94.2	93.7	95.0
7a	165.2	144.6	144.6	143.7	138.7	137.8	148.0
8	73.5	75.4	75.3	75.6	68.9	69.0	181.9
9	86.3	87.1	86.9	87.1	82.8	82.9	81.9
11	169.0	169.0	167.3	169.0	170.2	170.3	172.7
12	60.5	60.7	90.3	60.7	58.7	58.8	60.2
13	27.6	27.7	34.9	27.7	25.7	25.7	28.4
14	23.2	23.3	21.3	23.3	22.6	22.6	23.3
15	45.1	45.0	45.1	45.1	45.2	45.3	53.5
17	165.2	165.1	166.0	165.1	166.1	166.2	165.1
18	55.2	57.6	57.9	57.8	48.8	49.5	48.0
19	120.0	121.8	121.2	121.4	122.7	122.7	45.5
20	140.9	138.7	139.2	139.2	136.0	135.4	81.3
21	18.5	17.9	17.9	18.1	18.3	18.6	25.6
22	25.8	25.3	25.4	25.4	28.8	28.9	26.5
23		38.6	38.8	120.7	48.7	48.6	86.7
24		117.4	117.1	127.7	74.6	74.1	117.4
25		137.7	138.0	81.6	144.7	144.8	145.3
26		18.2	18.0	24.5	112.9	112.4	18.9
27		25.6	25.7	24.5	18.8	18.9	23.8
6-OMe	55.7	55.6	55.5	55.6	55.8	55.8	55.6

[a] Spectra were recorded at 150 MHz for ^{13}C using TMS as internal standard.

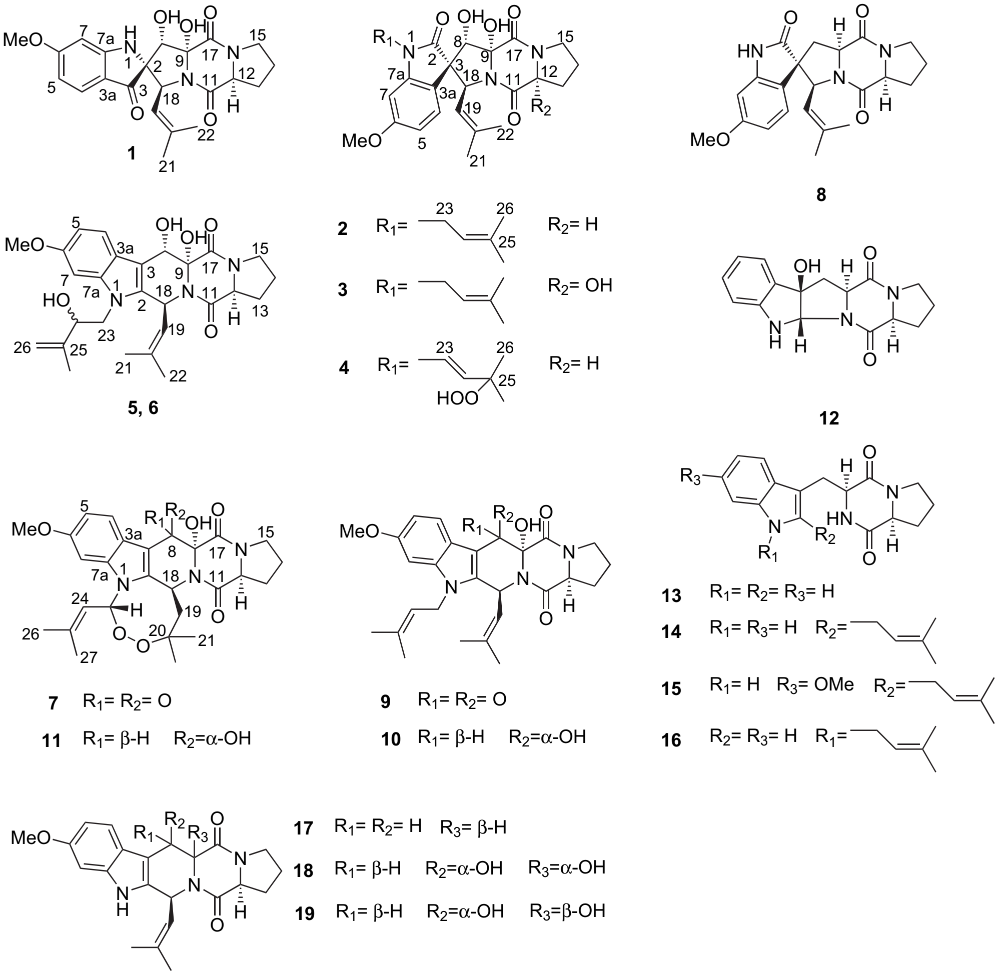

Figure 1. Structures of compounds **1**–**19**.

determined as 3*S*,8*S*,9*R*,12*S*,18*S* in Figure 1, and was given the name spirotryprostatin C.

Compound **3** was obtained as pale yellow amorphous powder. The molecular formula was determined as $C_{27}H_{33}N_3O_7$ by HRESIMS (found *m*/*z* 534.2198 [M+Na]$^+$, calcd 534.2216). Comparison of the ^{1}H and ^{13}C NMR data with those of **2** (Tables 1 and 2) showed that **3** owned the same molecular skeleton as **2**, except for H-12 (δ_H 4.62, t, *J*=6.7, 9.6 Hz) in **2** being replaced by 12-OH in **3**. This difference could be confirmed by the replacement of a methine carbon (δ_C 60.7) in **2** by an oxygenated quaternary carbon (δ_C 90.3) in **3**, which led to the downfield shift of C-13 (δ_C 34.9) and the upfield shift of C-14 (δ_C 21.3) in **3**. The stereochemistry of **3** was identical with **2**, which was determined by NOESY correlations (Fig. 3). Thus, the structure of **3** was determined (Fig. 1), and named spirotryprostatin D.

Compound **4** was isolated as pale yellow amorphous powder. The molecular formula was determined as $C_{27}H_{33}N_3O_8$ by HRESIMS (found *m*/*z* 550.2169 [M+Na]$^+$, calcd 550.2165). Analysis and comparison of 1D NMR data (Tables 1 and 2) suggested that **4** had the same molecular skeleton as **2** with the exception of a hydroperoxy isoprenyl group linked to N-1 in **4**. Detailed analysis of 1D NMR spectra revealed two methyl groups (δ_H 1.47, s, δ_C 24.5, CH_3-26; δ_H 1.47, s, δ_C 24.5, qC, CH_3-27), one bisubstituted double bond (δ_H 6.62, d, *J*=15.1 Hz, H-23; δ_H 6.49, d, *J*=15.1 Hz, H-24, δ_C 120.7, C-23; δ_C 127.7, C-24), and a hydroperoxyl-bearing quaternary carbon (δ_C 81.6, C-25), which indicated that **4** had a hydroperoxy isoprenyl group in agreement with the molecular weight. By further comparisons of NMR data of **4** with those of **2**, the stereochemistry of **4** was established the same as **2**. Thus, the structure of **4** was identified (Fig. 1), and named spirotryprostatin E.

Compounds **5** and **6**, obtained as pale yellow amorphous powder and colorless crystalline solid, had the same molecular formula, $C_{27}H_{33}N_3O_6$, according to HRESIMS (found *m*/*z* 518.2277 and 518.2261 [M+Na]$^+$, calcd 518.2267), respectively. The UV, IR, and NMR spectra all showed the presence of indole diketopiperazine system in **5** and **6**. Comparison of the 1D NMR spectral data of **5** and **6** (Tables 1 and 2) with those of **10** suggested that they had the same molecular framework except for the difference of the isoprenyl side chain attached to N-1. Interpretation of the ^{1}H and ^{13}C NMR spectral data of **5** and **6** revealed that the isopent-2-enyl at N-1 in **10** was substituted by a 2-hydroxyisopent-3-enyl (**5**, δ_H 4.46, dd, *J*=4.1, 8.3 Hz, H-24, δ_C 74.6, d, C-24; **6**, δ_H 4.25, br d, *J*=8.7 Hz, δ_C

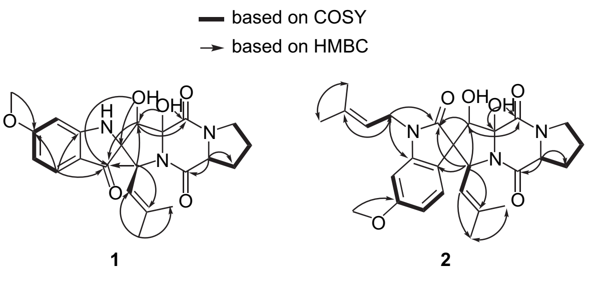

Figure 2. ^{1}H–^{1}H COSY and key HMBC correlations of compounds **1** and **2**.

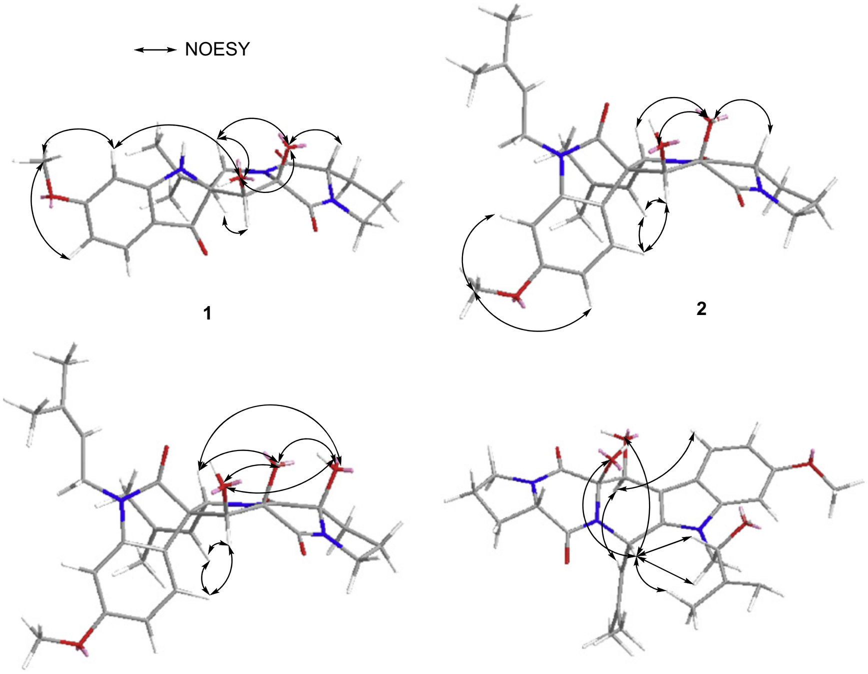

Figure 3. Selected NOESY correlations of compounds **1**–**3** and **5**.

74.1) in **5** and **6** (Tables 1 and 2). Detailed analysis of ^{1}H and ^{13}C NMR spectral data of **5** with those of **6** found that they had the same structure only differing in the stereochemistry of C-24, which also could be confirmed by their optical rotations (**5** $[\alpha]_D^{20}$ −5.7, **6** $[\alpha]_D^{20}$ 15.0). The stereochemistry of the pentacyclic ring skeleton of **5** and **6** was the same as compound **10**, which could be determined by the NOE correlations in **5** between H-8 and H-19, H-4, and between H-18 and 9-OH, 8-OH (Fig. 3). These data suggested that **5** and **6** were the epimers with different chirality only on the side chain.

Compound **7** was isolated as colorless crystalline solid. The HRESIMS (found *m*/*z* 510.2229 $[M+H]^+$, calcd 510.2240), coupled with NMR data, suggested its molecular formula as $C_{27}H_{31}N_3O_7$. The direct comparisons of ^{1}H and ^{13}C NMR spectra of **7** (Tables 1 and 2) with those of **11**, coupled with the structure information from the elemental composition and the UV and IR spectra, led us to consider that **7** was an analogue of **11**. Besides the carbonyl (δ_C 181.9) substitution for the oxygenated methine of C-8 (δ_H 5.70, s, δ_C 69.0) was observed. Accordingly, the compound **7** was determined and named as 13-oxoverruculogen (Fig. 1).

To date, including 7 new compounds (**1**–**7**) and 1 known (**12**) in this paper, there are 25 alkaloids in this family isolated from *A. fumigatus*. Although there are no sufficient evidences, it can be reasonably and safely assumed that these substances are fashioned from L-tryptophan, L-proline, L-methionine (the source of the aromatic methyl ether carbon), and one or more isoprene moieties derived from mevalonate, closely parallels that of the fumitremorgin B (**10**) and verruculogen (**11**).[26–28] The co-existence of these substances in cultures of *A. fumigatus* suggests a biosynthetic relationship between them.[29]

All new compounds, except **1** (**1** only for HL-60 and A549), were evaluated for their cytotoxicities against MOLT-4, HL-60, A549, and BEL-7402 cell lines (IC_{50} values are shown in Table 3). They showed the selected activity to the four cancer cell lines and further analysis of the activity data suggested that compounds **4**–**6** showed better susceptivity to MOLT-4, HL-60, and A549 than those of compounds **1**–**3** and **7**. VP16 was used as positive control and its IC_{50} values to above four cell lines are shown in Table 3.

3. Experimental

3.1. General experimental procedures

Optical rotations were obtained on a JASCO P-1020 digital polarimeter. UV spectra were recorded on Beckman DU 640 spectrophotometer. IR spectra were taken on a Nicolet NEXUS 470 spectrophotometer in KBr disks. ^{1}H, ^{13}C NMR and DEPT spectra, and 2D NMR were recorded on a JEOL JNM-ECP 600 spectrometer. Chemical shifts are given in δ (ppm) scale with TMS as an internal standard. NOESY experiments were carried out using a mixing time of 0.5 s. HRESIMS spectra were measured on a Q-Tof Ultima GLOBAL mass spectrometer. Semi-preparative HPLC was performed using an ODS column [YMC-Pack ODSA, 250×10 mm, 5 μm, 4 mL/min] and monitored by UV detector. Agilent HP^{3D} capillary electrophoresis system (Agilent, USA) was performed

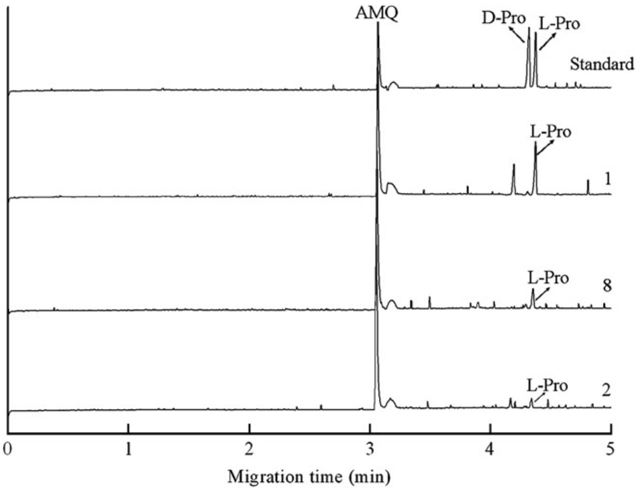

Figure 4. Chiral separation of amino acids derivatives by capillary electrophoresis.

Table 3
Cytotoxicities of compounds **1–7** in four cancer cell lines

Compound	Cytotoxicity (IC_{50}, μM)			
	MTT		SRB	
	MOLT-4 cells	HL-60 cells	A-549 cells	BEL-7402 cells
1	—	125.3	Not active	—
2	25.7	43.5	35.9	68.8
3	25.7	45.0	35.5	17.5
4	3.1	2.3	3.1	98.4
5	11.0	3.4	11.0	7.0
6	11.0	5.4	11.6	10.8
7	25.7	1.9	16.9	25.6
VP16	0.003	0.083	1.400	1.025

using an uncoated fused-silica capillary column [a total length of 55 cm and an effective length of 46.5 cm (Yongnian, Hebei Province, China)].

3.2. Fungal material

The isolated *A. fumigatus* originated from holothurian *S. japonicus*, collected in August 2005 from Lingshan Island, Qingdao, China.

3.3. Fermentation, extraction, and isolation

The fungus was inoculated into 500 mL Erlenmeyer flasks containing 150 mL liquid medium (maltose 20 g, mannitol 20 g, glucose 10 g, monosodium glutamate 10 g, KH_2PO_4 0.5 g, $MgSO_4$ 0.3 g, yeast extract paste 3 g, and maize paste 1 g dissolved in 1 L seawater, pH 6.5), followed by shaking incubation at 165 rpm for nine days at 28 °C. The fermented whole broth (60 L) was filtered through cheesecloth to separate into supernatant and mycelia. The former was concentrated under reduced pressure to about a quarter of the original volume and then extracted three times with ethyl acetate to give an ethyl acetate solution, while the latter was extracted three times with acetone. The acetone solution was concentrated under reduced pressure to afford an aqueous solution. The aqueous solution was extracted three times with ethyl acetate to give another ethyl acetate solution. Both ethyl acetate solutions were combined and concentrated under reduced pressure to give a crude extract (100 g).

The crude extract was subjected to chromatography over silica gel column using a stepwise gradient elution of petroleum ether/$CHCl_3$/MeOH, to yield 10 fractions (1–10). Fraction 3 was subjected to Sephadex LH-20 eluting with $CHCl_3$/MeOH (1:1), followed by chromatographing on a silica gel column eluting with $CHCl_3$/MeOH (60:1), to afford three subfractions, and compounds **2** (110 mg), **3** (2.5 mg), **4** (3.6 mg), **7** (30.1 mg), **5** (7.5 mg), **6** (8.0 mg), **9** (92.5 mg), **10** (28.2 mg), and **11** (3.0 mg) were purified by extensive preparative HPLC using MeOH/H_2O (70:30) from subfraction 3-2. Fraction 5 was subjected to chromatography over silica gel column using a stepwise gradient elution of petroleum ether/acetone to yield subfraction 5-2, and the known compounds **8** (4.1 mg), **18** (69.0 mg), **17** (83.3 mg), **15** (72.1 mg), **14** (16.0 mg), and **16** (9.0 mg) were purified by extensive preparative HPLC using MeOH/H_2O (58:42) from the subfraction 5-2. Fraction 6 was subjected to repeated silica gel column chromatography using a stepwise gradient elution of petroleum ether/$CHCl_3$/MeOH to yield subfraction 6-2-3. The subfraction 6-2-3 was separated by extensive preparative HPLC using MeOH/H_2O (30:70) to yield compounds **12** (2.1 mg), **13** (177.0 mg), **19** (8.7 mg), and **1** (21.6 mg).

3.3.1. Compound **1**

Pale yellow amorphous solid; $[\alpha]_D^{22}$ 147.2 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 202 (4.35), 220 (4.29), 251 (4.24), 286 (4.09), 392 (3.53) nm; IR (KBr) ν_{max} 3281, 2970, 2937, 2877, 1652, 1619, 1414, 1388, 1308, 1209, 1162, 1096, 1063, 1010, 944, 759 cm^{-1}; CD (MeOH) 203 ($\Delta\varepsilon$ +3.7), 221 (−3.2), 233 (−1.2), 255 (−5.2), 269 (−1.6), 277 (−2.0), 307 (+3.9), 331 (+0.4), 387 (+2.3) nm; ESIMS *m/z* 450.2 $[M+Na]^+$, 877.4 $[2M+Na]^+$; HRESIMS *m/z* 450.1620 $[M+Na]^+$ (calcd for $C_{22}H_{25}N_3O_6Na$, 450.1641); ^1H and ^{13}C NMR data are shown in Tables 1 and 2.

3.3.2. Compound **2**

Pale yellow amorphous powder; $[\alpha]_D^{20}$ −76.5 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 204 (5.00), 210 (4.96), 221 (4.75), 257 (3.81), 269 (3.79), 286 (3.79), 294 (3.74) nm; IR (KBr) ν_{max} 3414, 3250, 2932, 1681, 1619, 1491, 1386, 1297, 1161, 1033, 924, 831, 750, 633 cm^{-1}; CD (MeOH) 194 ($\Delta\varepsilon$ +3.4), 208 (−24.8), 221 (−23.9), 222 (−38.4), 240 (+13.7), 253 (−0.8), 265 (+0.3), 292 (−3.5), 327 (−0.2) nm; ESIMS *m/z* 478.1 $[M-H_2O+H]^+$, 518.1 $[M+Na]^+$; HRESIMS *m/z* 518.2255 $[M+Na]^+$ (calcd for $C_{27}H_{33}N_3O_6Na$, 518.2267); ^1H and ^{13}C NMR data are shown in Tables 1 and 2.

3.3.3. Compound **3**

Pale yellow amorphous powder; $[\alpha]_D^{20}$ −73.6 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 204 (4.38), 221 (4.31), 257 (3.53), 268 (3.51), 285 (3.46), 293 (3.40) nm; IR (KBr) ν_{max} 3414, 3148, 2947, 1678, 1616, 1478, 1390, 1297, 1157, 1029, 819, 753 cm^{-1}; CD (MeOH) 196 ($\Delta\varepsilon$ +10.5), 206 (−11.1), 212 (−7.9), 224 (−18.2), 241 (−1.9), 249 (−4.6), 270 (−0.4), 292 (−2.4), 327 (−0.5) nm; ESIMS *m/z* 494.2 $[M-H_2O+H]^+$, 534.2 $[M+Na]^+$; HRESIMS *m/z* 534.2198 $[M+Na]^+$ (calcd for $C_{27}H_{33}N_3O_7Na$, 534.2216); ^1H and ^{13}C NMR data are shown in Tables 1 and 2.

3.3.4. Compound **4**

Pale yellow amorphous powder; $[\alpha]_D^{20}$ −60.9 (*c* 0.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 243 (3.90), 275 (3.41), 284 (3.39), 294 (3.13) nm; IR (KBr) ν_{max} 3313, 3184, 2955, 1678, 1495, 1398, 1262, 1095, 1033, 944, 804, 703, 641 cm^{-1}; ESIMS *m/z* 550.3 $[M+Na]^+$, 1077.6 $[2M+Na]^+$; HRESIMS *m/z* 550.2169 $[M+Na]^+$ (calcd for $C_{27}H_{33}N_3O_8Na$, 550.2165); ^1H and ^{13}C NMR data are shown in Tables 1 and 2.

3.3.5. Compound **5**

Pale yellow amorphous powder; $[\alpha]_D^{20}$ −5.7 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 223 (4.53), 240 (4.29), 266 (3.78), 276 (3.88), 294 (3.88), 303 (3.81) nm; IR (KBr) ν_{max} 3433, 2932, 2858, 1666, 1449, 1371, 1250, 1153, 1084, 955, 819, 753, 617, 555 cm^{-1}; ESIMS *m/z* 496.1 $[M+H]^+$, 518.1 $[M+Na]^+$; HRESIMS *m/z* 518.2277 $[M+Na]^+$ (calcd for $C_{27}H_{33}N_3O_6Na$, 518.2267); ^1H and ^{13}C NMR data are shown in Tables 1 and 2.

3.3.6. Compound **6**

Colorless crystalline solid; $[\alpha]_D^{20}$ 15.0 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 210 (5.17), 221 (4.94), 237 (4.60), 268 (4.05), 276 (4.11), 295 (4.11), 305 (4.02) nm; IR (KBr) ν_{max} 3445, 2932, 1662, 1452, 1375, 1247, 1153, 1080, 955, 816, 750, 617, 540 cm^{-1}; ESIMS *m/z* 496.1 $[M+H]^+$, 518.1 $[M+Na]^+$; HRESIMS *m/z* 518.2261 $[M+Na]^+$ (calcd for $C_{27}H_{33}N_3O_6Na$, 518.2267); ^1H and ^{13}C NMR data are shown in Tables 1 and 2.

3.3.7. Compound **7**

Colorless crystalline solid; $[\alpha]_D^{20}$ 83.8 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 210 (4.45), 220 (4.34), 248 (4.19), 283 (4.02), 320 (3.88) nm; IR (KBr) ν_{max} 3443, 3317, 2971, 2936, 2905, 2882, 2823, 1684, 1648, 1542, 1499, 1452, 1415, 1359, 1242, 1150, 1064, 1041, 932, 816, 664 cm^{-1}; ESIMS *m/z* 510.1 $[M+H]^+$, 532.1 $[M+Na]^+$; HRESIMS *m*/*z* 510.2229 $[M+H]^+$ (calcd for $C_{27}H_{32}N_3O_7$, 510.2240); ^1H and ^{13}C NMR data are shown in Tables 1 and 2.

3.4. Amino acid analysis of 1, 2, and 8

Compounds **1** (1.2 mg), **2** (1.2 mg), and **8** (1.2 mg) were each dissolved in 6 N HCl and heated at 105 °C for 24 h in three sealed tubes. The solutions were evaporated to dryness under reduced pressure. The residue was stirred in 1 mL of deionized water, respectively, and then filtrated with microporous membrane (∅ 50 μm). The amino acid derivatisation was performed according to the specifications of the Waters AccQ-Tag method: 10 μL of sample, 20 μL of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) reagent, and 70 μL of 0.2 M borate buffer (pH 8.8) were mixed in a small tube. This solution was heated for 10 min at 55 °C. Excessive AQC reagent was degraded as 6-aminoquinoline (AMQ), *N*-hydroxysuccinimide (NHS), and carbon dioxide. Standard solutions of amino acids were D-Pro and L-Pro.

Samples were loaded by pressure injection at 50 mbar for 5 s. Analytical conditions: 40 mM Tris–H_3PO_4 (pH 6.50) containing 20 mg/mL HP-β-CD, voltage 30 kV, temperature 25 °C, UV detection wavelength 240 nm. The results showed that the products of acidic hydrolysis of **1**, **2**, and **8** contained L-Pro by comparison with the standard and further confirmed the Pro in the structure of the three compounds were in L-configuration (Fig. 4).

3.5. Biological assays

Active fractions were assayed using the MTT method with P388 cell line. Cytotoxic activities of compounds were evaluated by MTT method[30] using P388 and HL-60 cell lines, and SRB method[31] using A549 and BEL-7402 cell lines.

In MTT assay, the cell suspensions (200 μL) at a density of 5×10^4 cells mL^{-1} were plated in 96-well microtiter plates and incubated for 24 h at 37 °C in a humidified incubator at 5% CO_2. The test compound solution (2 μL in DMSO) at different concentrations was added to each well and further incubated for 72 h in the same condition. Then 20 μL of the MTT solution was added to each well and incubated for 4 h. The old medium (150 μL) containing MTT was then gently replaced by DMSO and pipetted to dissolve any formazan crystals formed. Absorbance was then determined on a Spectra Max Plus plate reader at 540 nm.

In SRB assay, 200 μL of the cell suspensions was placed in 96-cell plates at a density of 2×10^5 cell mL^{-1}. Then 2 μL of the test compound solutions (in MeOH) at different concentrations was added to each well and the culture was further incubated for 24 h at 37 °C in a humidified incubator at 5% CO_2. Following drug exposure, the cells were fixed with 12% trichloroacetic acid and the cell layer was stained with 0.4% SRB. The absorbance of SRB solution was measured at 515 nm. Dose–response curves were generated and the IC_{50} values were defined as the concentration of compound required for inhibiting cell proliferation by 50%.

Acknowledgements

This work was financially supported by the Chinese National Programs for High Technology Research and Development (No. 2007AA09Z447), the Shandong Provincial Natural Science Fund (No. Z2006C13). The *Aspergillus fumigatus* was identified by Professor Hong Kui at the Chinese Academy of Tropical Agricultural Sciences (CATAS) and South China University of Tropical Agriculture (SCUTA). The antitumor assay was performed at the Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

References and notes

1. Zhao, W. Y.; Gu, Q. Q.; Zhu, W. M. *Chem. Res.* **2007**, *18*, 10–13.
2. Zhao, W. Y.; Zhu, Q. S.; Gu, Q. Q. *J. Qingdao Univ. Sci. Technol.* **2007**, *28*, 199–201.
3. Yamazaki, M.; Sasago, K.; Miyaki, K. *J. Chem. Soc., Chem. Commun.* **1974**, 408–409.
4. Yamazaki, M.; Fujimoto, H. *Tetrahedron Lett.* **1975**, 27–28.
5. Cui, C. B.; Kakeya, H.; Okada, G.; Onose, R.; Ubukata, M.; Takahashi, I.; Isono, K.; Osada, H. *J. Antibiot.* **1995**, *48*, 1382–1384.
6. Abaraham, W. R.; Arfmann, H. A. *Phytochemistry* **1990**, *29*, 1025–1026.
7. Cui, C. B.; Kakeya, H.; Okada, G.; Onose, R.; Osada, H. *J. Antibiot.* **1996**, *49*, 527–533.
8. Cui, C. B.; Kakeya, H.; Osada, H. *J. Antibiot.* **1996**, *49*, 534–540.
9. Cui, C. B.; Kakeya, H.; Osada, H. *J. Antibiot.* **1996**, *49*, 832–835.
10. Cui, C. B.; Kakeya, H.; Osada, H. *Tetrahedron* **1996**, *52*, 12651–12666.
11. Cui, C. B.; Kakeya, H.; Osada, H. *Tetrahedron* **1997**, *53*, 59–72.
12. Miyake, F. Y.; Yakushijin, K.; Horne, D. A. *Org. Lett.* **2004**, *6*, 4249–4251.
13. Onishi, T.; Sebahar, P. R.; Williams, R. M. *Tetrahedron* **2004**, *60*, 9503–9515.
14. Edmondson, S.; Danishefsky, S. J.; Sepp-Lorenzino, L.; Rosen, N. *J. Am. Chem. Soc.* **1999**, *121*, 2147–2155.
15. Sebahar, P. R.; Osada, H.; Usui, T.; Williams, R. M. *Tetrahedron* **2002**, *5*, 6311–6322.
16. Bagul, T. D.; Lakshmaiah, G.; Kawabata, T.; Fuji, K. *Org. Lett.* **2002**, *4*, 249–251.
17. Marti, C.; Carreira, E. M. *J. Am. Chem. Soc.* **2005**, *127*, 11505–11515.
18. Nakatsuka, S.; Teranishi, K.; Goto, T. *Tetrahedron Lett.* **1986**, *27*, 6361–6364.
19. Fujimoto, H.; Fujimaki, T.; Okuyama, E.; Yamazaki, M. *Mycotoxins* **2000**, *50*, 93–99.
20. Wang, F. Z.; Zhang, M.; Sun, W.; Gu, Q. Q.; Zhu, W. M. *Acta Crystallogr.* **2007**, *E63*, o1859–o1860.
21. Fayos, J.; Lokensgard, D.; Clardy, J.; Cole, R. J.; Kirksey, J. W. *J. Am. Chem. Soc.* **1974**, *96*, 6785–6787.
22. Maes, C. M.; Potgieter, M.; Steyn, P. S. *J. Chem. Soc., Perkin Trans. 1* **1986**, 861–866.
23. Ley, S. V.; Cleator, E.; Hewitt, P. R. *Org. Biomol. Chem.* **2003**, *1*, 3492–3494.
24. Birch, A. J.; Russell, R. A. *Tetrahedron* **1972**, *28*, 2999–3008.
25. Sanz-Cervera, J. F.; Stocking, E. M.; Usui, T.; Osada, H.; Williams, R. M. *Bioorg. Med. Chem.* **2000**, *8*, 2407–2415.
26. Willingale, J.; Perera, K. P. W. C.; Mantle, P. G. *Biochem. J.* **1983**, *214*, 991–993.
27. Horak, R. M.; Vleggaar, R. *J. Chem. Soc., Chem. Commun.* **1987**, 1568–1570.
28. Day, J. B.; Mantle, P. G. *Appl. Environ. Microbiol.* **1982**, *43*, 514–516.
29. Williams, R. M.; Stocking, E. M.; Sanz-Cervera, J. F. Topics in Current Chemistry; *Biosynthesis: Aromatic Polyketides, Isoprenoids, Alkaloids*; 2000; Vol. 209, pp 97–173.
30. Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55–63.
31. Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107–1112.