

Antimicrobial ergosteroids and pyrrole derivatives from halotolerant *Aspergillus flocculosus* PT05-1 cultured in a hypersaline medium

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Abstract In order to obtain more structurally novel and bioactive lead compounds for subsequent drug discovery, we have shifted the focus of our study from traditional microbial resources to ‘extremophiles’. In this study, a halotolerant fungus *Aspergillus flocculosus* PT05-1 was isolated from the sediment of Putian saltern of Fujian Province of China in a hypersaline medium. Two new compounds, (22*R*,23*S*)-epoxy-3 β ,11 α ,14 β ,16 β -tetrahydroergosta-5,7-dien-12-one (**1**) and 6-(1*H*-pyrrol-2-yl) hexa-1,3,5-trienyl-4-methoxy-2*H*-pyran-2-one (**5**) (existed as a pair of epimers with the configuration of 1*E*,3*Z*,5*E* and 1*E*,3*E*,5*E* separately), along with nine known compounds were isolated and identified from the fermentation broth of *A. flocculosus* PT05-1 grown at a 10 % saline medium. New ergosteroid **1** together with 7-nor-ergosterolide (**2**) and 3 β -hydroxyergosta-8,24(28)-dien-7-one (**3**) showed cytotoxicity against HL-60 and BEL-7402 cells with IC₅₀ values of 12–18 μ M, and antimicrobial activity against *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, and *Candida albicans* with MIC values of 1.6–15 μ M, respectively. New compound **5** exhibited antibacterial effect on *E. aerogenes* with MIC value of 3.7 μ M. This

study also showed great prospects in developing medicinal resources from extremophiles.

Keywords Halotolerant microorganism · *Aspergillus flocculosus* · Secondary metabolites · Cytotoxicity · Antimicrobial activity

Introduction

As is well known at present, microbes have made a phenomenal contribution to the health and well-being of people throughout the world (Demain and Sanchez 2009). Back in 1928, Alexander Fleming (1929) discovered in a Petri dish seeded with *Staphylococcus aureus* that was killed by a mold. The mold, identified as *Penicillium notatum*, produced an active agent that was named penicillin, which began the microbial drug era. Another example, the well-known immunosuppressant drug ‘cyclosporine’ was initially isolated from the fungus *Tolypocladium inflatum* (*Beauveria nivea*), found in a soil sample obtained in 1969 from Hardangervidda, Norway (Laupacis et al. 1982; Svarstad et al. 2000). There is increasing evidence indicating that microorganisms are capable of producing bioactive natural products for the drug discovery and development, which constitute half of the pharmaceuticals on the market (Cragg et al. 1997).

Nevertheless, unexploited environmental microorganism resources were less and less along with the deepened study (Bredholdt et al. 2007; Jensen et al. 2007). Therefore, many scientists have turned their attention to the extremophiles, e.g., deep-sea microbes (Pettit 2011), thermophiles (Bonch 2012), radiation-resistant extremophiles (Gabani and Singh 2013), acid mine waste extremophiles (Stierle and Stierle 2005) and so on. In order to survive in

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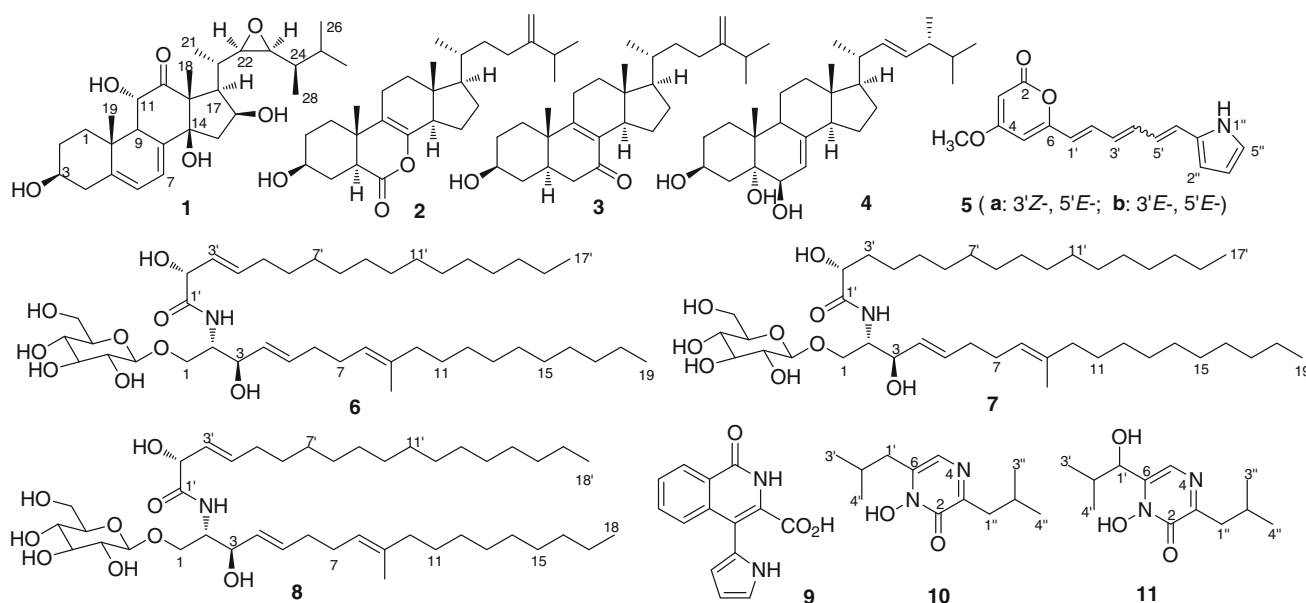


Fig. 1 Chemical structures of the metabolites **1–11** from *A. flocculosus* PT05-1

such extreme environments, microorganisms have developed unique defensive and metabolic systems that have frequently led them to produce novel molecules. Although a large number of reported extremophiles are considered, very few have been screened for production of novel and bioactive secondary metabolites, and this rich source of novel lead compounds for drug remains largely untapped (Wilson and Brimble 2008; Extance 2005).

Among them, halophilic and halotolerant fungi from solar salterns attracted our attention for their rich diversity and abundance (Nina and Jose 2009). We view these microbes grown in specific hypersaline environment as potential assets, and some structurally new and bioactive secondary metabolites have been identified from the fermentation broth of halotolerant fungi (Wang et al. 2007; Zheng et al. 2009; Zhang et al. 2010). As part of our ongoing investigations for secondary metabolites of halotolerant fungi from hypersaline ecological niches, a fungal strain, *Aspergillus flocculosus* PT05-1 was isolated from the sediment of Putian saltern, Fujian Province of China. Generally speaking, marine-derived microbes were grown at the concentration of 3 % sea salt. In this study, we increased the salinity in its culture medium to obtain more chemical and bioactive diversities. Chemical investigation of the EtOAc extract of the fermentation broth of *A. flocculosus* PT05-1 at 10 % salinity resulted in the isolation and identification of a new ergosterol derivative (**1**), a new red pyrrole pigment (**5**) and nine known compounds, 7-norergosterolide (**2**), 3 β -hydroxyergosta-8, 24(28)-dien-7-one (**3**), cerebisterol (**4**), (2*R*,3*E*)-2-hydroxy-*N*-[(2*S*,3*R*,4*E*,8*E*)-1- β -D-glucopyranosyloxy-3-hydroxy-9-methylnonadec-4,8-dien-2-yl]heptadec-3-enamide (**6**), (2*R*)-2-hydroxy-*N*-[(2*S*,

3*R*,4*E*,8*E*)-1- β -D-glucopyranosyloxy-3-hydroxy-9-methylnonadec-4,8-dien-2-yl]hepta decanamide (**7**), cerebroside C (**8**), 4-(1*H*-pyrrol-2-yl)-1-isoquinolone-3-carboxylic acid (**9**), neoaspergillilic acid (**10**) and hydroxyneaspergillilic acid (**11**) (Fig. 1). New ergosteroid **1**, 7-norergosterolide (**2**) and 3 β -hydroxyergosta-8,24(28)-dien-7-one (**3**) showed cytotoxicity against HL-60 and BEL-7402 cells with IC₅₀ values of 12–18 μ M, and antimicrobial activity against *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, and *Candida albicans* with MIC values of 1.6–15 μ M, respectively. New compound **5** exhibited antibacterial effect on *E. aerogenes* with MIC value of 3.7 μ M.

Experimental section

General experimental procedures

Specific rotations were obtained on a JASCO P-1020 digital polarimeter. UV spectra were recorded on a Beckman DU 640 spectrophotometer. IR spectra were obtained on a Nicolet NEXUS 470 spectrophotometer as KBr disks. ¹H NMR, ¹³C NMR, and DEPT spectra and 2D-NMR were recorded on a JEOL JNMECP 600 spectrometer using TMS as internal standard, and chemical shifts were recorded as δ values. ESIMS was measured on a Q-TOF Ultima Global GAA076 LC mass spectrometer. Semipreparative HPLC was performed using an ODS column (YMC-pack ODS-A, 10 \times 250 mm, 5 μ m, 4 mL/min). HPLC was performed using an ODS column (YMC-pack C18, 4.6 \times 250 mm, 5 μ m, 2 mL/min). TLC and column chromatography (CC) were performed on plates precoated

with silica gel GF254 (10–40 μm) and over silica gel (200–300 mesh, Qingdao Marine Chemical Factory, Qingdao, China) and Sephadex LH-20 (Amersham Biosciences, Sweden), respectively.

Fungal material

The halotolerant fungus *A. flocculosus* PT05-1 was isolated from surface of sediment (saline 20 %), Putian saltern of Fujian Province of China. The strain was identified according to its morphological characteristics and internal transcribed spacer (ITS) sequences (GenBank ID: KF305117) by Prof. Kui Hong, Wuhan University. The voucher specimen is deposited in our laboratory at $-80\text{ }^{\circ}\text{C}$. Working stocks were prepared on potato dextrose agar slants stored at $4\text{ }^{\circ}\text{C}$.

Fermentation and extraction

The fungus *A. flocculosus* PT05-1 was cultured on an orbital shaker (165 rpm) at $28\text{ }^{\circ}\text{C}$ for 16 days in two hundred 500-mL conical flasks containing the liquid medium (150 mL/flask, pH 7.0) composed of glucose (10 g/L), maltose (20 g/L) mannitol (20 g/L), monosodium glutamate (10 g/L), yeast extract (3 g/L), corn steep liquor (1 g/L), and 10 % sea salt. The fermentation broth (30 L) was filtered through cheesecloth to separate into supernatant and mycelia. The former was concentrated under reduced pressure to about 5 L and then extracted three times with EtOAc to give an EtOAc 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 EtOAc to give another EtOAc solution. Both EtOAc solutions were combined and concentrated under reduced pressure to give an EtOAc extract (22.0 g). *A. flocculosus* PT05-1 was also incubated under the same conditions except the salinity is 3 % salt. The chemical diversities of the secondary metabolites in EtOAc extract were investigated with HPLC.

Purification and identification

The EtOAc extract (22.0 g) was separated into five fractions (Fractions 1–5) on a silica gel column using a step gradient elution with CHCl_3 -MeOH (1:100, 1:50, 1:30, 1:15, 1:10). Compound **4** (39 mg) was recrystallized from the MeOH solution of Fraction 1. Fraction 3 was separated on Sephadex LH-20 with MeOH- CHCl_3 (1:1) and further purified on semipreparative HPLC (70 % MeOH in water) to give **10** (500 mg, t_R 9 min). Fraction 4 was separated on Sephadex LH-20 with MeOH- CHCl_3 (1:1) and purified on

semipreparative HPLC (65 % MeOH in water) to give **9** (7 mg, t_R 6 min) and **11** (50 mg, t_R 5 min). Fraction 5 was separated on Sephadex LH-20 with MeOH- CHCl_3 (1:1) and the second fraction was separated on silica gel column with CHCl_3 -MeOH (12:1) and then purified on semipreparative HPLC (95 % MeOH in water) to give **6** (100 mg, t_R 14 min), **7** (12 mg, t_R 17 min) and **8** (2 mg, t_R 9 min). Fraction 2 was separated on Sephadex LH-20 with MeOH- CHCl_3 (1:1) to obtain Fractions 2-1 and 2-2. Fraction 2-1 was chromatographed on ODS open column (90 % MeOH in water), and then semipreparative HPLC (95 % MeOH in water) to give **3** (10 mg, t_R 8 min), **2** (12 mg, t_R 7 min) and **1** (15 mg, t_R 4 min). Fraction 2-2 was purified on semipreparative HPLC (70 % MeOH in water) to give **5** (10 mg, t_R 14 min).

(22*R*,23*S*)-epoxy-3 β ,11 α ,14 β ,16 β -tetrahydroxyergosta-5,7-diene-12-one (**1**): colorless needles; $[\alpha]_D^{25} -58.2$ (c 0.3, MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (4.0), 262 (4.2) nm; CD (c 0.1, MeOH) λ_{max} ($\Delta\epsilon$) 277 (-3.5) nm; IR (KBr) ν_{max} 3508, 3441, 2952, 1700, 1646, 1388, 1235, 1076, 838, 606 cm^{-1} ; ^1H NMR data (600 MHz, $\text{DMSO}-d_6$): δ 1.80 (1H, m, H-1a), 1.41 (1H, m, H-1b), 1.68 (1H, m, H-2a), 1.29 (1H, m, H-2b), 3.37 (1H, m, H-3 α), 2.29 (1H, m, H-4a), 2.07 (1H, m, H-4b), 5.61 (1H, dd, $J = 2.3, 5.9$ Hz, H-6), 6.06 (1H, dd, $J = 2.3, 5.5$ Hz, H-7), 2.83 (1H, d, $J = 4.6$ Hz, H-9), 3.90 (1H, d, $J = 4.6$ Hz, H-11), 1.55 (1H, m, H-15a), 1.46 (1H, m, H-15b), 4.23 (1H, m, H-16 α), 1.60 (1H, dd, 2.8, 6.9, H-17 α), 1.16 (3H, s, H-18), 0.77 (3H, s, H-19), 1.63 (1H, m, H-20), 1.06 (3H, d, $J = 5.9$ Hz, H-21), 2.82 (1H, dd, $J = 4.6, 9.2$ Hz, H-22), 2.77 (1H, dd, $J = 4.6, 9.7$ Hz, H-23), 1.30 (1H, m, H-24), 1.72 (1H, m, H-25), 0.88 (3H, d, $J = 6.8$ Hz, H-26), 0.84 (3H, d, $J = 7.3$ Hz, H-27), 0.85 (3H, $J = 6.8$ Hz, H-28), 4.69 (1H, d, $J = 4.1$ Hz, OH-3), 5.19 (1H, d, $J = 4.1$ Hz, OH-11), 4.48 (1H, s, OH-14), 4.20 (1H, brs, OH-16); ^{13}C NMR data (150 MHz, $\text{DMSO}-d_6$): δ 37.7 (t, C-1), 31.1 (t, C-2), 68.5 (d, C-3), 40.2 (t, C-4), 141.4 (s, C-5), 118.7 (d, C-6), 117.9 (d, C-7), 134.1 (s, C-8), 56.9 (d, C-9), 37.6 (s, C-10), 83.9 (d, C-11), 213.0 (s, C-12), 56.7 (s, C-13), 83.4 (s, C-14), 44.6 (t, C-15), 74.8 (d, C-16), 52.9 (d, C-17), 19.5 (q, C-18), 17.9 (q, C-19), 30.0 (d, C-20), 17.2 (q, C-21), 61.1 (d, C-22), 60.0 (d, C-23), 35.6 (d, C-24), 30.5 (d, C-25), 19.9 (q, C-26), 18.0 (q, C-27), 12.4 (q, C-28); HRESI-MS m/z 497.2871 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{28}\text{H}_{42}\text{O}_6\text{Na}$, 497.2879).

A mixture of (1*E*,3*Z*,5*E*)- and (1*E*,3*E*,5*E*)-6-(1*H*-pyrrol-2-yl)hexa-1,3,5-trienyl-4-methoxy-2*H*-pyran-2-one (**5**): red amorphous powder; UV (MeOH) λ_{max} (log ϵ) 205 (3.9), 237 (3.9), 305 (4.2), 418 (4.3) nm; IR (KBr) ν_{max} 3449, 1640, 1534, 453, 1520, 1448, 1323, 1286, 1237, 749 cm^{-1} ; ^1H and ^{13}C NMR data, see Table 1; HRESI-MS m/z 270.1123 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{16}\text{H}_{16}\text{NO}_3$, 270.1130).

Table 1 ^1H (600 MHz) and ^{13}C NMR (150 MHz) data for compounds **5**, **10** and **11** (in DMSO- d_6 , δ ppm)

Position	5a		5b		10		11	
	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)
1								
2	162.8, qC		162.7, qC		152.4, qC		152.3, qC	
3	88.4, CH	5.61, d (2.2)	88.1, CH	5.58, d (2.2)	154.4, qC		154.5, qC	
4	170.8, qC		170.9, qC					
5	100.9, CH	6.25, d (2.2)	100.1, CH	6.19, d (2.2)	121.6, CH	7.15, s	119.9, CH	7.29, s
6	158.6, qC		158.5, qC		137.7, qC		141.2, qC	
1'	122.0, CH	6.31, d (15.4)	120.4, CH	6.26, d (14.8)	36.0, CH ₂	2.51, d (7.3)	69.8, CH	4.67, d (4.1)
2'	130.1, CH	7.51, dd (12.6, 15.3)	135.5, CH	7.05, dd (11.2, 14.8)	26.1, CH	2.04, m	31.6, CH	2.05, m
3'	124.9, CH	6.06, t (11.6)	128.3, CH	6.35, dd (11.5, 14.0)	19.4, CH ₃	0.89, d (4.1)	19.4, CH ₃	0.95, d (6.8)
4'	136.0, CH	6.39, t (11.0)	139.8, CH	6.76, dd (10.4, 14.3)	19.4, CH ₃	0.89, d (4.1)	15.9, CH ₃	0.79, d (6.9)
5'	118.1, CH	7.20, dd (11.6, 15.4)	122.9, CH	6.68, dd (11.0, 15.4)				
6'	127.0, CH	6.60, d (15.4)	126.5, CH	6.60, d (15.4)				
1''		11.46, s		11.23, s	41.7, CH ₂	2.57, d (7.3)	41.6, CH ₂	2.57, d (7.3)
2''	130.3, qC		130.4, qC		26.2, CH	2.14, m	26.3, CH	2.14, m
3''	111.7, CH	6.27, d (3.8)	110.5, CH	6.29, d (3.8)	19.5, CH ₃	0.91, d (4.1)	22.5, CH ₃	0.89, d (6.8)
4''	109.4, CH	6.09, dd (3.3, 5.5)	109.5, CH	6.10, dd (3.3, 5.5)	19.5, CH ₃	0.91, d (4.1)	22.4, CH ₃	0.89, d (6.8)
5''	121.3, CH	6.92, brs	121.0, CH	6.87, brs				
4-OMe	56.3, CH ₃	3.83, s	56.3, CH ₃	3.81, s				

Neospergillilic acid (**10**): yellow needles; UV (MeOH) λ_{max} (log ϵ) 234 (4.0), 329 (4.0) nm; IR (KBr) ν_{max} 3446, 3108, 2950, 2927, 2870, 2504, 2059, 1641, 1586, 1466, 1241, 1157, 1090 cm^{-1} ; ^1H and ^{13}C NMR data, see Table 1; ESI-MS m/z 223.1 $[\text{M} - \text{H}]^-$.

Hydroxyneospargillilic acid (**11**): yellow needles; $[\alpha]_{\text{D}}^{25}$ -49.2 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 240 (4.0), 327 (3.9) nm; IR (KBr) ν_{max} 3382, 3123, 2967, 2873, 2078, 1647, 1569, 1466, 1388, 1337, 1231, 1197, 1152, 1014 cm^{-1} ; ^1H and ^{13}C NMR data, see Table 1; ESI-MS m/z 239.1 $[\text{M} - \text{H}]^-$.

Bioassay

Cytotoxicity against HL-60 and BEL-7402 cancer cell lines was assayed by the MTT method (Mosmann 1983). Cell lines were grown in RPMI-1640 supplemented with 10 % FBS under a humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C. Cell suspensions, 200 μL , at a density of 5×10^4 cell mL^{-1} were plated in 96-well microtiter plates and incubated for 24 h. Then, 2 μL of the test solutions (in MeOH) were added to each well and further incubated for 72 h. The MTT solution (20 μL , 5 mg/mL in RPMI-1640 medium) was then added to each well and incubated for 4 h. Old medium containing MTT (150 μL) 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.

The IC_{50} was calculated as the compound concentration necessary to reduce cell viability by 50 %. Vp-16 (Etoposide) was used as the positive control with IC_{50} values of 0.042 and 0.83 μM , respectively.

Antimicrobial activity against *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* was evaluated using an agar dilution method (Zaika 1988). The tested strains were cultivated in LB agar plates for bacteria and in YPD agar plates for *C. albicans*, at 37 °C. Compounds and positive controls were dissolved in 5 % DMSO- H_2O at different concentrations using continuous 2-fold dilution. The test solutions (5 μL) were absorbed onto paper disks (5 mm diameter) and placed on the assay plates. After 24 h incubation, zones of inhibition (mm in diameter) were recorded. The minimum inhibitory concentrations were defined as the lowest concentration at which an inhibition zone could be observed.

Results and discussion

The selected halotolerant fungus for lead compounds

Eighty-eight strains of halotolerant fungi have been isolated from surface of sediment (saline 20 %), Putian saltern of Fujian Province, China. The halotolerant fungus PT05-1 was selected after a combination of chemical (TLC and HPLC) and biological (antitumor and antimicrobial

activity) screening of their ethyl acetate extracts. The colony picture and micrograph of this strain are shown in Fig. 2a, b, respectively. It was identified as *Aspergillus flocculosus* whose ITS sequences and phylogenetic tree are shown in Supporting Information.

The effects of salt stress on production of secondary metabolites from *A. flocculosus* PT05-1

The halotolerant fungus *A. flocculosus* PT05-1 was isolated from surface of sediment in saltern containing high salinity. There are few references on effect of salt on the secondary metabolites of salt-tolerant fungi. In our continuous

investigation of high salt stress on fungal secondary metabolites (Wang et al. 2011), *A. flocculosus* PT05-1 was cultured in 3 and 10 % saline conditions. The amount of EtOAc extracts of metabolites at 10 % salinity was higher than that at 3 % salinity (70 vs 55 mg). Meanwhile, chemical diversities of the low polar secondary metabolites in 10 % salinity increased (Fig. 3). By comparison of UV absorption (from 200 to 400 nm) and co-elucidation with standards, it was identified that compounds **1–10** were stimulated and produced by *A. flocculosus* PT05-1 in the 10 % salinity medium. The specific metabolites by this extreme microbe in special hypersaline culture environment aroused our interest, and the further structural elucidation and bioactivity test for them were carried out subsequently.

The identification of new metabolites from *A. flocculosus* PT05-1 at 10 % salinity

The fungus *A. flocculosus* PT05-1 was isolated from the sea sediment of Putian saltern of Fujian Province of China. When fermented under 10 % salinity condition, two new compounds, (22*R*,23*S*)-epoxy-3 β ,11 α ,14 β ,16 β -tetrahydroxy-ergosta-5,7-dien-12-one (**1**), a mixture of (1*E*,3*Z*,5*E*)- and (1*E*,3*E*,5*E*)-6-(1*H*-pyrrol-2-yl)hexa-1,3,5-trienyl-4-methoxy-2*H*-pyran-2-one (**5**), together with nine known compounds, 7-nor-ergosterolide (**2**), 3 β -hydroxyergosta-8,24(28)-dien-7-one (**3**) (Cui et al. 2010), cerevisterol (**4**) (Kawagishi et al. 1988), (2*R*,3*E*)-2-hydroxy-*N*-[(2*S*,3*R*,4*E*,8*E*)-1- β -D-glucopyranosyloxy-3-hydroxy-9-methylnonadec-4,8-dien-2-yl]heptadec-3-enamide (**6**) (Jiang et al. 2002), (2*R*)-2-hydroxy-*N*-[(2*S*,3*R*,4*E*,8*E*)-1- β -D-glucopyranosyloxy-3-hydroxy-9-methylnonadec-4,8-dien-2-yl]heptadecanamide (**7**), cerebroside C (**8**) (Shu et al. 2004), 4-(1*H*-pyrrol-2-yl)-1-isquinolone-3-carboxylic acid (**9**) (Zhu and Lin 2006), neoaspergillic acid (**10**) (Yamazaki et al. 1972), hydroxy-neoaspergillic acid (**11**) (Micetich and MacDonald 1964), were isolated and identified from the fermentation broth.

Compound **1** gave an HRESI-MS peak at m/z 497.2871 [$M + Na$] $^+$ (calcd for $C_{28}H_{42}O_6Na$, 497.2879) corresponding to the molecular formula $C_{28}H_{42}O_6$ with seven

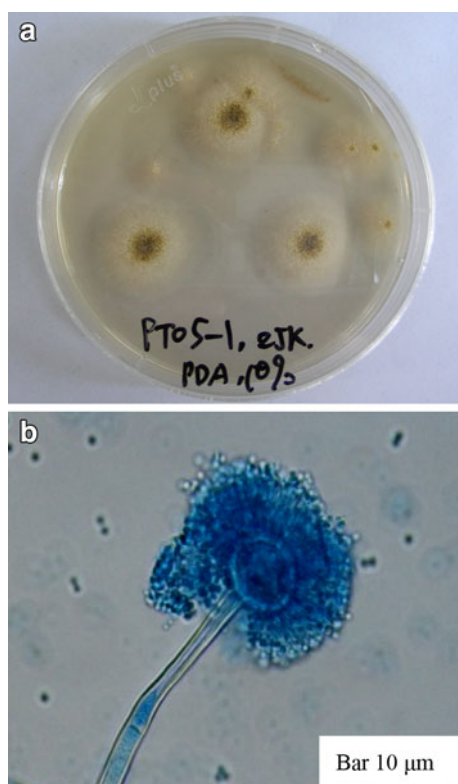
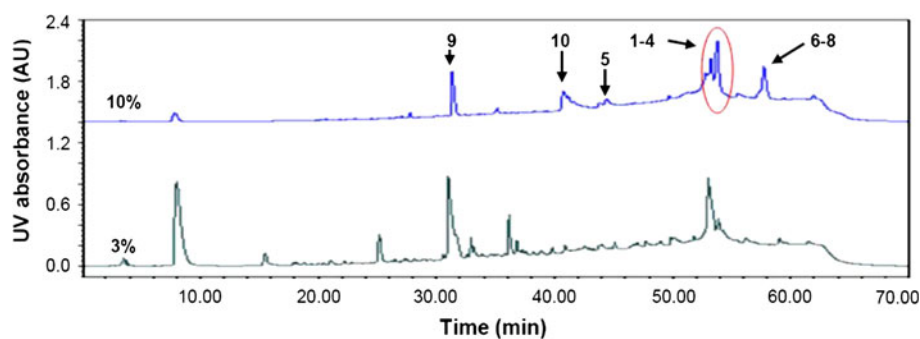


Fig. 2 The colony picture (a) and micrograph (b, staining with cotton blue) of halotolerant *A. flocculosus* PT05-1

Fig. 3 The chromatographic fingerprints of secondary metabolites from *A. flocculosus* PT05-1 grown in condition of 3 and 10 % salt (HPLC gradient elution: 5–65 min, 5–100 % CH_3OH ; UV wavelength: 205 nm; flow rate: 1 mL/min). The specific metabolites produced at high salinity were labeled in numbers



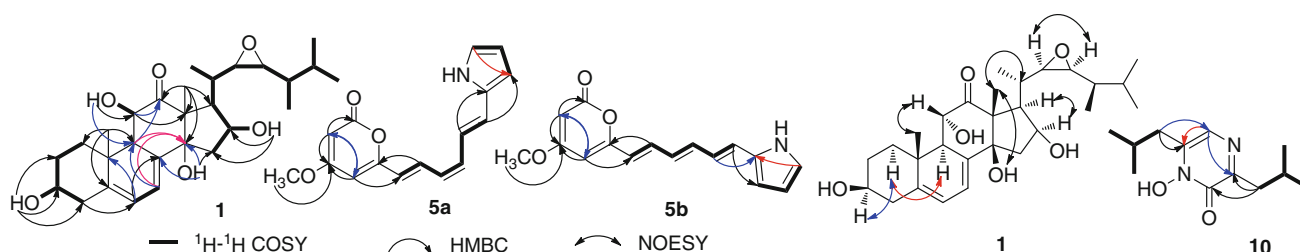


Fig. 4 Selected 2D NMR correlations for compounds **1**, **5** and **10**

degrees of unsaturation. Proton signals of six methyl at δ_{H} 1.16 (s), 0.77 (s), 1.06 (d), 0.89 (d), 0.85 (d) and 0.84 (d) revealed the ergosterol skeleton of **1**. Compared with ergosterol (Zhang et al. 2010), ^{13}C NMR and DEPT spectra of **1** revealed additional one carbonyl (δ_{C} 213.0), a pair of epoxymethines (δ_{C} 60.0 & 61.1), two oxygenated methines (δ_{C} 74.8 & 83.9), and one oxygenated quaternary carbon at δ_{C} 83.4. Correspondingly, two vinyl methines signals disappeared relative to the ^{13}C NMR of ergosterol. ^1H - ^1H COSY from H-20 to H-25 located the epoxy at C-22 and C-23. Two hydroxy groups were positioned at C-3 and C-16 by the ^1H - ^1H COSY of H-1/H-2/H-3/H-4 and OH-3/H-3, and H-15/H-16/H-17 and OH-16/H-16, respectively (Fig. 4). This deduction was further supported by HMBC correlations from OH-3 (δ 4.69) to C-2 (δ 31.1), C-3 (δ 68.5) and C-4 (δ 40.2), and from 16-OH (δ 4.20) to C-15 (δ 44.6) and C-16 (δ 74.8), respectively (Fig. 4). Another two hydroxy groups were confirmed to be located at C-11 and C-14 according to the HMBC correlations between OH-11 (δ 5.19) with C-9 (δ 56.9) and C-11 (δ 83.9), between H-11 (δ 3.90) with C-12 (δ 213.0) and C-13 (δ 56.9); between OH-14 (δ 4.48) with C-8 (δ 134.1), C-14 (δ 83.4) and C-15 (δ 44.6), and between H-7 (δ 6.06) with C-5 (δ 141.4), C-9 and C-14, respectively. And the HMBC correlations from H-9 (δ 2.83), H-11 and H₃-18 (δ 1.16) to C-12 fixed the position of carbonyl. Thus the constitution of compound **1** was determined to be 22,23-epoxy-3,11,14,16-tetrahydroxyergosta-5,7-dien-12-one. NOESY spectrum of **1** showed NOE correlations of H-11 to H₃-19 (δ 0.77), H-3 (δ 3.37) and H-9 to H-1 α (δ 1.41), OH-14 to H₃-18, H₃-18 to H-20 (δ 1.64), and H-16 (δ 4.23) to H-17 (δ 1.60), revealing the configurations of 3-OH, 11-OH, 14-OH and 16-OH to be β -, α -, β -, and β -, respectively. In addition, *cis*-configuration of the epoxy could be deduced from the NOE correlations between H-22 (δ 2.82) and H-23 (δ 2.77), and between H-20 and H-24 (δ 1.30), which was also confirmed by the larger coupling constant ($J_{\text{cis-22,23}}$ 4.6 Hz vs $J_{\text{trans-22,23}}$ 1.8–2.2 Hz) (Tsukamoto et al. 1998, Funel et al. 2004). Thus, compound **1** was elucidated as *cis*-22,23-epoxy-3 β ,11 α ,14 β ,16 β -tetrahydroxyergosta-5,7-dien-12-one. To further assign the relative configuration of the epoxy of **1**, ^{13}C NMR chemical shifts of **1** and its three

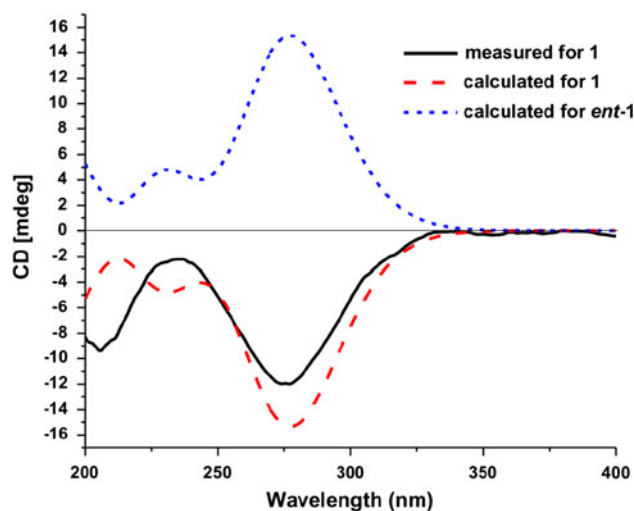


Fig. 5 CD and the calculated ECD spectra of **1**

stereoisomers (22,23-diepi-**1**, 22-epi-**1**, and 23-epi-**1**) were calculated. Conformational search of each were carried out in the HyperChem 7.5 package using an Amber force field. Chemical shifts of ^{13}C NMR were computed at the B3LYP/6-311++G(2d,p)//B3LYP/6-31G(d) level (Zheng et al. 2009). The magnetic shielding values were converted into chemical shifts after the corrections using slope and intercept of the linear-square functions (Zheng et al. 2009), and the relative errors of chemical shifts were computed by subtracting the calculated ^{13}C NMR from the experimental shifts. The result showed that structure **1** displayed the smallest relative errors for C-20 and C-24 (−2.3 and −5.2 ppm, respectively) among the four stereoisomers (Table S1, Supporting Information), indicating the relative configuration of chain for **1** as (20*S**,22*R**,23*S**,24*R**)-. The absolute configuration of **1** was established by CD and the ECD calculation. The ECD of **1** and *ent*-**1** were calculated using the time-dependent density functional theory (TD-DFT) method at the B3LYP/6-31G(d) level in Gaussian 03 (Stephens et al. 2007). The result showed that the CD curve of **1** is good agreement with the calculated ECD spectrum of **1** but opposite to that of *ent*-**1** (Fig. 5), indicating (3*S*,9*R*,10*R*,11*S*,13*R*,14*S*,16*S*,17*R*,20*S*,22*R*,23*S*,24*R*)- configuration of **1**. Thus, compound **1** was elucidated as

Table 2 Antimicrobial activities of compounds **1–3** and **5**

Compound	MIC (μM)			
	<i>Candida albicans</i>	<i>Pseudomonas aeruginosa</i>	<i>Enterobacter aerogenes</i>	<i>Staphylococcus aureus</i>
1	3.3	3.3	1.6	>100
2	1.9	7.5	7.5	>100
3	>100	>100	15	>100
5	>100	>100	3.7	>100
Ketoconazole	0.18	–	–	–
Ciprofloxacin	–	1.9	0.93	3.7

“–” indicating “not measured”

(22*R*,23*S*)-epoxy-3 β ,11 α ,14 β ,16 β -tetrahydroxyergosta-5,7-dien-12-one.

Compound **5** was isolated as a pair of *cis/trans* isomers that gave an HRESI-MS peak at m/z 270.1123 [$M + H$]⁺ (calcd for C₁₆H₁₆NO₃, 270.1130) corresponding to the molecular formula C₁₆H₁₅NO₃. Once this compound was purified, it readily changed into its *cis/trans* isomer with ratio of 2:1 as observed in the HPLC profile. In addition, NMR spectra of **5** showed two set of similar data with the ratio of 2:1 (Table 1 **5a** and **5b**). 1D NMR spectra of **5a** showed signals attributed to eleven sp² methines, three oxygenated quaternary carbons, one methoxy and one NH group. ¹H-¹H COSY correlations of H-1'/H-2'/H-3'/H-4'/H-5'/H-6' and H-3''/H-4''/H-5'', and HMBC correlations of CH₃O- to C-4, H-3 to C-2 and C-4, H-4 to C-3 and C-6, and H-5'' to C-2'' indicated these groups linked into three fragments, 6-substituted 4-methoxy-2*H*-pyran-2-one, 1,6-disubstituted hexa-1,3,5-triene and 2-substituted pyrrole (Fig. 4). The coupling constant values of $J_{1',2'}$ 15.3 Hz, $J_{3',4'}$ 11.0 Hz and $J_{5',6'}$ 15.4 Hz revealed the configuration of $\Delta^{1',3',5'}$ double bonds as *E*, *Z* and *E* in the triene moiety, respectively. Finally, the key HMBC correlations from H-5 to C-1', from H-2' to C-6, and from H-5' to C-2' and from H-6' to C-3' suggested that the triene moiety is connected to the position 6 of pyrone moiety and the position 2'' of pyrrole moiety, respectively. Thus, compound **5a** was identified as (1*E*,3*Z*,5*E*)-6-(1*H*-pyrrol-2-yl)hexa-1,3,5-trienyl-4-methoxy-2*H*-pyran-2-one. Compound **5b** also showed similar 1D NMR data and 2D NMR correlations to those of **5a**. The main difference of NMR data between **5b** and **5a** focused on the triene moiety. The larger coupling constant values of $J_{1',2'}$ 14.8 Hz, $J_{3',4'}$ 14.3 Hz and $J_{5',6'}$ 15.4 Hz clearly indicated all the double bonds in 3-heptenyl moiety as *E*-configuration. Compound **5b** was thus identified as (1*E*,3*E*,5*E*)-6-(1*H*-pyrrol-2-yl)hexa-1,3,5-trienyl-4-methoxy-2*H*-pyran-2-one. The experiment showed that compounds **5a** and **5b** readily interconverted in the light, resulting in the isolation of **5** as a mixture of a pair of geometric isomers.

Being lack of ¹³C NMR data of neoaspergillic acid (**10**) and hydroxyneoaspergillic acid (**11**) in the literatures, the

NMR data of compounds **10** and **11** were reported in this paper. The molecular formula of **10** was determined as C₁₂H₂₀N₂O₂ from the ESI-MS peak at m/z 223.1 [$M - H$][−] and 1D NMR which revealed three sp²-quaternary carbons, one sp²-methine, two sp³-methines, two sp³-methylenes, and four sp³-methyls (Table 1). Two sets of ¹H-¹H COSY correlations from H₂-1' to H₃-3' and H₃-4' through H-2' and from H₂-1'' to H₃-3'' and H₃-4'' through H-2'' indicated two isobutyl moieties in the molecule of **10** (Fig. 4). The remaining moiety, C₄H₂N₂O₂, was deduced to connected into pyrazine skeleton according to the HMBC correlations of H-5 with C-3 and C-6 and the comparison of ¹H NMR with those reported (Yamazaki et al. 1972; Micetich and MacDonald 1964). The key HMBC correlations from H₂-1' to C-5 and C-6, and from H₂-1'' to C-2 and C-3 revealed that the structure of **10** is neoaspergillic acid. And the NMR data of compound **11** were assigned by comparative analysis of NMR data with those of **10**. The main differences of NMR between **11** and **10** are an oxygenated methine signals replacement of a methylene signals and the down field shifts of C-6, C-2' and H-5, indicating **11** to be hydroxyneoaspergillic acid.

The bioactivities of metabolites from *A. flocculosus* PT05-1 at 10 % salinity

Ergosterols **1–3** and the new pigment (**5**) were evaluated for their cytotoxicity against HL-60 and BEL-7402 cell lines by MTT method (Mosmann 1983), and antimicrobial activity against *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* by agar dilution method (Zaika 1988) (Table 2) for the first time. Weak cytotoxicity for **2** against HL-60 and BEL-7402 and **3** against HL-60 cell lines was observed with IC₅₀ values of 12.4 and 17.7 and 13.7 μM , respectively, while compounds **1** and **5** were inactive on the test tumor cell lines (IC₅₀ > 50 μM). Although ergosterols **2** and **3** did not exhibit antimicrobial activities against *S. aureus*, *E. coli*, and *Aspergillus niger*, moderate antimicrobial activities against *C. albicans*, *P. aeruginosa* and *E. aerogenes* for **1** and **2** were observed with MIC values of 3.3/

3.3/1.6 and 1.9/7.5/7.5 μM , respectively. In addition, compound **3** showed weak antibacterial activity against *E. aerogenes* with a MIC value of 15 μM and compound **5** showed moderate antibacterial activity against *E. aerogenes* with a MIC value of 3.7 μM (Table 2). It is worth mentioning that the production of bioactive metabolites **2** and **3** increased approximately 2 and 4 times, respectively under high salt stress. And the active compounds **1** and **5** only produced under high salt stress conditions (10 % salinity).

Conclusions

Microorganisms at extreme environments are important resources of bioactive natural products with new structures. And in our view, halotolerant fungi grown under high salt stress were particularly considered as great treasures for seeking lead compounds in drug discovery. In this study, eleven metabolites including two new compounds were isolated and identified from the fermentation broth of *A. flocculosus* PT05-1 under 10 % salinity conditions. Among them, compounds **1**, **5–8** and **10** were only produced under high salt stress conditions by this halotolerant strain, and the ergosterols **1–3** showed weak cytotoxicity against HL-60 and BEL-7402 cell lines, and moderate antimicrobial activity against the bacteria *E. aerogenes*, *P. aeruginosa* and the fungus *C. albicans*.

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