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ISOLATION AND BIOLOGICAL EVALUATION OF SECONDARY METABOLITES OF THE ENDOPHYTIC FUNGUS *Aspergillus fumigatus* FROM *Astragalus membranaceus*

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Endophytic fungi are eukaryotic organisms that live inside plant tissues and are usually specific at the host species level [1, 2]. Endophytic fungi show high potential as sources of novel antimicrobial, antiviral, anticancer, antioxidant, and insecticidal compounds [3].

In the course of our search for biologically active metabolites of endophytic fungi from Chinese medicinal plants, an endophytic fungus, *Aspergillus fumigatus*, was isolated from the root of *Astragalus membranaceus*. The EtOAc extract of the culture showed significant antimicrobial activity. This prompted us to carry out secondary-metabolite studies on the fungus, which resulted in the isolation of eight active compounds, ergosterol (**1**), cerevisterol (**2**), ergosterol peroxide (**3**), fumitremorgin B (**4**), fumitremorgin C (**5**), verruculogen (**6**), cyclotryprostatins B (**7**), and fumiquinazoline J (**8**). Herein we describe the isolation, structural elucidation, and biological test of its secondary metabolites.

The fungal strain AR05 was isolated from the root of *Astragalus membranaceus*, collected in the Hengshan Mountains, Shanxi Province, China, on August 10, 2010. By classical microscopic analysis, the fungus was identified as a member of the genus *Aspergillus*. It was further identified as *Aspergillus fumigatus* according to a molecular biological protocol by DNA amplification and sequencing of the ITS region. The fungal strain has been preserved at the College of Agronomy and Life Science, Shanxi Datong University, Shanxi Province.

Starter cultures were maintained on PDA medium at 28°C for 7 days. Plugs of agar supporting mycelial growth were cut and transferred aseptically to 1000 mL Erlenmeyer flasks containing 400 mL of liquid Czapek medium at 28°C on a rotary shaker set to 120 rmp for 15 days. The fungal culture (40 L) was filtered through cheesecloth. The filtrate was concentrated to 5 L below 60°C and then extracted five times with ethyl acetate (4.5 L). The dried mycelium (55°C, 95 g) was extracted three times with methanol (4 L). All extracts were concentrated at reduced pressure to afford 9.8 g of a dark brown crude extract. The crude extract was subjected to silica gel column chromatography eluting successively with ethyl acetate–methanol gradients (1:0, 50:1, 20:1, 10:1, 5:1, 2:1, 1:1, 0:1) and yielded eight fractions (A–H). Fraction A was purified by column chromatography on silica gel with a gradient of ethyl acetate in petroleum ether to give five subfractions (A1-5). Fraction A1 was separated by silica gel column chromatography with elution with petroleum ether–ethyl acetate (2:1) to give pure compounds **1**–**3**. Fraction B was separated by column chromatography on silica gel with a gradient of ethyl acetate in petroleum ether to give five subfractions (B1-5). Fraction B2 was repeatedly subjected to silica gel column chromatography with a gradient of ethyl acetate in petroleum ether and Sephadex LH-20 with ethyl acetate–methanol (1:1) to yield compounds **4** and **5**. Fraction B3 was repeatedly subjected to silica gel column chromatography with a gradient of ethyl acetate in petroleum ether and Sephadex LH-20 with ethyl acetate–methanol (1:1) to yield compounds **6**–**8**.

The compounds were subjected to characterization using spectroscopic analysis and identified as ergosterol (**1**) [4], cerevisterol (**2**) [5], ergosterol peroxide (**3**) [6, 7], fumitremorgin B (**4**) [8, 9], verruculogen (**5**) [10], fumitremorgin C (**6**) [11, 12], cyclotryprostatins B (**7**) [13], and fumiquinazoline J (**8**) [14, 15] by comparison of their spectral data with the reported data in the literature.

Ergosterol (1), $C_{28}H_{44}O$, colorless needles, mp 155–157°C. ESI-MS *m/z* 397.0 $[M + H]^+$.

Cerevisterol (2), $C_{28}H_{46}O_3$, colorless needles, mp 240–242°C. ESI-MS *m/z* 431.0 $[M + H]^+$.

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TABLE 1. Antimicrobial Activity of Secondary Metabolites of Endophytic Fungus *Aspergillus fumigatus* from *Astragalus membranaceus*, MIC, μg/mL

Strains	Tested samples								Positive control	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	gentamicin	nystatin
Gram Positive Bacteria										
Bacillus subtilis	128	64	128	4	4	2	1	1	0.5	
Staphylococcus aureus	256	32	64	8	8	4	1	1	1	
Gram Negative Bacteria										
Escherichia coli	256	64	64	8	8	2	1	1	0.5	
Salmonella typhimurium	128	128	128	8	4	2	2	1	0.5	
Fungi										
Candida albicans	–	–	–	16	32	8	8	2		4
Penicillium chrysogenum	–	256	–	64	64	16	16	4		4
Fusarium solani	–	–	–	32	16	16	32	2		8

–: not active at concentration up to 256 μg/mL.

Ergosterol peroxide (3), $C_{28}H_{44}O_3$, colorless needles, mp 175–177°C. ESI-MS *m/z* 429.0 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 4.06–3.93 (1H, m, H-3), 6.28 (1H, d, J = 8.5, H-6), 6.53 (1H, d, J = 8.5, H-7), 0.90 (3H, s, H-18), 0.92 (3H, d, J = 6.8, H-19), 1.02 (3H, d, J = 6.6, H-21), 5.15 (1H, dd, J = 15.2, 7.4, H-22), 5.24 (1H, dd, J = 15.2, 7.4, H-23), 0.83 (3H, s, H-26), 0.82 (3H, s, H-27), 0.84 (3H, s, H-28). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 34.65 (C-1), 30.08 (C-2), 66.45 (C-3), 36.88 (C-4), 82.16 (C-5), 135.40 (C-6), 130.73 (C-7), 79.42 (C-8), 51.02 (C-9), 36.93 (C-10), 20.61 (C-11), 39.76 (C-12), 44.54 (C-13), 51.65 (C-14), 23.38 (C-15), 28.66 (C-16), 56.14 (C-17), 12.86 (C-18), 18.17 (C-19), 39.30 (C-20), 20.87 (C-21), 135.19 (C-22), 132.27 (C-23), 42.75 (C-24), 33.04 (C-25), 19.95 (C-26), 19.64 (C-27), 17.55 (C-28).

Fumitremorgin B (4), $C_{27}H_{33}N_3O_5$, white needles, mp 209–211°C. ESI-MS *m/z* 480.0 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 7.85 (1H, d, J = 8.0, H-4), 6.79 (1H, d, J = 8.0, H-5), 6.69 (1H, s, H-7), 5.77 (1H, s, H-8), 4.45 (1H, t, J = 8.0, 8.0, H-12), 2.47 (1H, m, H-13a), 2.08 (1H, m, H-13b), 2.10 (1H, m, H-14a), 1.93 (1H, m, H-14b), 3.63 (2H, d, J = 8.0, H-15), 5.98 (1H, d, J = 12.0, H-18), 4.71 (1H, d, J = 12.0, H-19), 1.99 (3H, s, H-21), 1.63 (3H, s, H-22), 4.53 (2H, s, H-23), 5.03 (1H, s, H-24), 1.85 (3H, s, H-26), 1.70 (3H, s, H-27), 3.84 (3H, s, 6-OMe), 4.74 (1H, s, 8-OH), 4.17 (1H, s, 9-OH). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 131.13 (C-2), 104.42 (C-3), 120.52 (C-3a), 121.34 (C-4), 109.29 (C-5), 156.17 (C-6), 93.82 (C-7), 137.87 (C-7a), 68.95 (C-8), 82.98 (C-9), 170.51 (C-11), 58.76 (C-12), 28.93 (C-13), 22.61 (C-14), 45.26 (C-15), 166.25 (C-17), 49.03 (C-18), 122.97 (C-19), 135.22 (C-20), 18.37 (C-21), 25.71 (C-22), 41.77 (C-23), 120.29 (C-24), 134.61 (C-25), 18.20 (C-26), 25.55 (C-27), 55.72 (6-OMe).

Fumitremorgin C (5), $C_{22}H_{25}N_3O_3$, white needles, mp 124–126°C. ESI-MS *m/z* 380.0 $[M + H]^+$.

Verruculogen (6), $C_{27}H_{33}N_3O_7$, white needle, mp 218–220°C. ESI-MS *m/z* 512.0 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 7.95 (1H, d, J = 8.0, H-4), 6.88 (1H, d, J = 8.0, H-5), 6.58 (1H, s, H-7), 5.66 (1H, s, H-8), 4.45 (1H, t, J = 8.0, 8.0, H-12), 2.45 (1H, m, H-13a), 2.10 (1H, m, H-13b), 2.08 (1H, m, H-14a), 1.94 (1H, m, H-14b), 3.63 (2H, d, J = 8.0, H-15), 6.11 (1H, d, J = 8.0, H-18), 2.00 (1H, m, H-19a), 1.63 (1H, d, J = 8.0, H-19b), 1.99 (3H, s, H-21), 1.02 (3H, s, H-22), 6.73 (1H, s, H-23), 5.03 (1H, s, H-24), 1.77 (3H, s, H-26), 1.70 (3H, s, H-27), 3.84 (3H, s, 6-OMe), 4.74 (1H, s, 8-OH), 4.04 (1H, s, 9-OH). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 131.13 (C-2), 105.50 (C-3), 120.87 (C-3a), 121.64 (C-4), 109.29 (C-5), 156.36 (C-6), 93.82 (C-7), 136.19 (C-7a), 68.96 (C-8), 82.58 (C-9), 166.10 (C-11), 58.76 (C-12), 28.94 (C-13), 22.61 (C-14), 51.11 (C-15), 170.71 (C-17), 49.03 (C-18), 45.29 (C-19), 82.09 (C-20), 25.71 (C-21), 27.00 (C-22), 85.82 (C-23), 118.40 (C-24), 143.11 (C-25), 18.90 (C-26) , 24.09 (C-27), 55.72 (6-OMe).

Cyclotryprostatin B (7), $C_{23}H_{27}N_3O_5$, white needles, mp 130–132°C. ESI-MS *m/z* 426.0 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 8.10 (1H, s, H-1), 7.43 (1H, d, J = 8.0, H-4), 6.81 (1H, d, J = 8.0, H-5), 6.85 (1H, s, H-7), 4.7 (1H, s, H-8), 4.42 (1H, dd, J = 4.0, 4.0, H-12), 2.51 (1H, m, H-13a), 2.05 (1H, m, H-13b), 2.19 (1H, m, H-14a), 1.96 (1H, m, H-14b), 3.75 (1H, m, H-15a), 3.73 (1H, m, H-15b), 6.66 (1H, d, J = 8.0, H-18), 5.62 (1H, d, J = 8.0, H-19), 2.24 (3H, s, H-21), 1.79 (3H, s, H-22), 3.82 (3H, s, 6-OMe), 3.34 (3H, s, 8-OMe). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 133.75 (C-2), 105.22 (C-3), 122.60 (C-3a), 118.68 (C-4), 109.99 (C-5), 156.49 (C-6), 95.23 (C-7), 136.69 (C-7a), 76.83 (C-8), 84.77 (C-9), 167.03 (C-11), 59.93 (C-12), 29.69 (C-13), 22.05 (C-14), 45.82 (C-15), 165.73 (C-17), 49.11 (C-18), 123.54 (C-19), 137.92 (C-20), 18.10 (C-21), 26.03 (C-22), 55.77 (6-OMe), 56.57 (8-OMe).

Fumiquinazoline J (8), $C_{21}H_{16}N_4O_2$, white needles. ESI-MS *m/z* 357.0 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 8.29 (1H, s, H-2), 7.61 (1H, d, J = 8.0, H-7), 7.71 (1H, t, J = 8.0, 4.0, H-8), 7.45 (1H, t, J = 8.0, 4.0, H-9), 8.26 (1H, d, J = 8.0, H-10), 6.07 (1H, s, H-14), 3.37 (1H, dd, J = 4.0, 4.0, H-15a), 3.50 (1H, dd, J = 4.0, 4.0, H-15b), 2.26 (3H, s, H-16), 8.19 (1H, s, H-19), 7.31 (1H, d, J = 4.0, H-21), 7.17 (1H, t, J = 8.0, 8.0, H-22), 7.06 (1H, t, J = 8.0, 4.0, H-23), 7.36 (1H, d, J = 8.0, H-24). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 170.84 (C-1), 54.77 (C-3), 153.29 (C-4), 147.03 (C-6), 127.61 (C-7), 134.60 (C-8), 127.35 (C-9), 126.90 (C-10), 120.68 (C-11), 160.26 (C-12), 54.52 (C-14), 25.90 (C-15), 18.01 (C-16), 107.51 (C-17), 132.42 (C-18), 134.57 (C-20), 111.33 (C-21), 123.45 (C-22), 120.55 (C-23), 118.36 (C-24), 127.82 (C-25).

The isolated compounds **1**–**8** were tested for their antifungal and antibacterial properties towards the tested microorganisms (Table 1). Compounds **4** and **5** showed potent antibacterial and antifungal activities with MICs of 4–64 μg/mL. Moreover, compound **6** and **7** inhibited growth of bacteria and fungi with MICs of 1–32 μg/mL. Compounds **1**, **2** and **3** showed only weak antibacterial activity and no antifungal activity. It is noteworthy that the activity of compound **8** was close to that of gentamicin, a present antibacterial drug, and was stronger than that of nystatin, a present antifungal drug.

The eight compounds were isolated from the culture of the endophytic fungus *Aspergillus fumigatus* from *Astragalus membranaceus* for the first time. Herein, the MICs of the antimicrobial activity were first carried out. The above findings demonstrate that the endophytic fungus *Aspergillus fumigatus* from the root of *Astragalus membranaceus* is a promising source of antimicrobial secondary metabolites and could protect the host by producing bioactive metabolites, which may be toxic or even lethal to phytopathogens.

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