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New secondary metabolites from bioactive extracts of the fungus *Armillaria tabescens*

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Ethyl acetate extracts of *Armillaria tabescens* (strain JNB-OZ344) showed significant fungistatic and bacteristatic activities against several major human pathogens including *Candida albicans*, *Cryptococcus neoformans*, *Escherichia coli* and *Mycobacterium intracellulare*. Chemical analysis of these extracts led to the isolation and identification of four new compounds, emestrin-F (**1**), emestrin-G (**2**), 6-O-(4-*O*-methyl-*β*-D-glucopyranosyl)-8-hydroxy-2,7-dimethyl-4*H*-benzopyran-4-one (**3**) and cephalosporolide-J (**4**), along with five other previously known compounds, emestrin (**5**), cephalosporolide-E (**6**), decarestrictine-C_2 (**7**), ergosterol and brassicasterol. Structural elucidation of all compounds was carried out by NMR and MS analyses. Antimicrobial assays revealed that compounds **1** and **5** were responsible for the observed growth inhibitory activities of the fungal extracts against the human pathogens tested.

Keywords: *Armillaria tabescens*; emestrin; chromone; cephalosporolide; antimicrobial activity

1. Introduction

Armillaria tabescens is a major fungal pathogen known to cause root decay and mortality in numerous woody plant species in the southern USA, including many commercially important hardwood tree species within natural forest stands, timberlands, plantations and urban landscapes (Baietto & Wilson, 2010; Wilson, Leininger, Otrosina, Dwinell, & Schiff, 2004). At least 20 species of *Armillaria* are recognised worldwide with virulence varying between species and individual strains of each species (Shaw & Kile, 1991). A series of biologically active protoilludane sesquiterpene aryl esters have been isolated previously from different strains of *A. tabescens* (Donnelly, Konishi, Dunne, & Cremin, 1997).

In our investigations of bioactive secondary metabolites from wood decay fungi, we found that the ethyl acetate extracts of *A. tabescens*, cultured on boiled brown rice grains, showed antimicrobial activity against the fungi *Candida albicans*, *Cryptococcus neoformans* and the bacteria *Escherichia coli* and *Mycobacterium intracellulare*. Chemical analysis of

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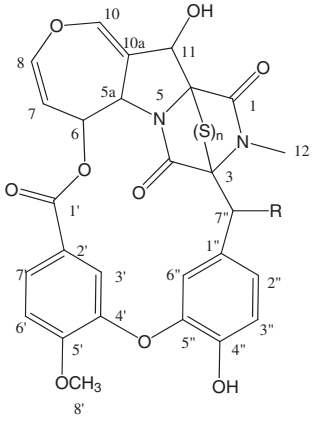
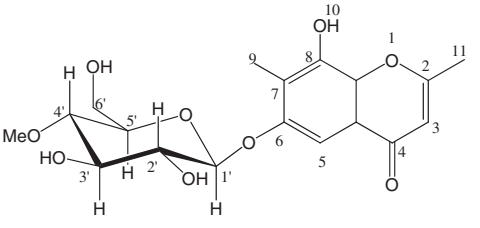
these extracts led to the isolation and identification of emestrin (**5**) (Seya, Nozawa, Nakajima, Kawai, & Udagawa, 1986) and two of its new closely related analogues, emestrin-F (**1**) and emestrin-G (**2**), as the compounds responsible for this activity. In addition to these compounds, we also isolated a new chromone glycoside, [6-*O*-(4-*O*-methyl-*β*-D-glucopyranosyl)-8-hydroxy-2,7-dimethyl-4*H*-benzopyran-4-one] (**3**) and a new diastereomer of the cephalosporolide series, cephalosporolide-J (**4**) along with four other known compounds, cephalosporolide-E (**6**) (Ackland, Hanson, Hitchcock, & Ratcliffe, 1985), decarestrictine C_1 (**7**) (Gohrt et al., 1992), ergosterol and brassicasterol.



(**1**) n=2, R=H, Emestrin-F
(**2**) n=1, R=OH, Emestrin-G
(**5**) n=2, R=OH, Emestrin



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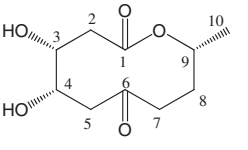

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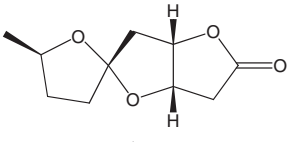

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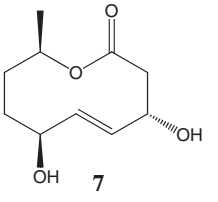

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The identification of the compounds along with physical and spectroscopic data for four new compounds and their biological activities is described.

2. Results and discussion

Emestrin (**5**) (Seya et al., 1986), cephalosporolide-E (**6**) (Ackland et al., 1985), decarestrictine C_1 (**7**) (Gohrt et al., 1992), brassicasterol (Lee, Lee, Cho, Baek, & Lee, 2010) and ergosterol (Kang, Wang, & Chen, 2003) were identified by comparing physical and spectroscopic data with those reported. Ergosterol is the common, predominant sterol structural component in the cell membranes of higher fungi, whereas brassicasterol is a rarer sterol found in the cell membranes of certain fungi.

Compound **1** was isolated as a white solid and its molecular formula was determined as $C_{27}H_{22}N_2O_9S_2$ by high-resolution electrospray ionisation mass spectroscopy (HRESIMS) analysis. Comparison of the molecular formulas of **1** and emestrin (**5**) ($C_{27}H_{22}N_2O_{10}S_2$) indicated that the former has one oxygen atom less than the latter. The only major difference in their ^{1}H-NMR spectra was the replacement of an oxygenated methine signal

in **5** with two geminal protons at the δ3.95 (1H, d, $J = 13.2$ Hz) and 3.45 (1H, d, $J = 13.6$ Hz) in **1**. This was also evident in their ^{13}C-NMR spectra with the appearance of a methylene signal (δ36.9) in **1** in place of an oxygenated methine in **5**. The correlation of the two protons at δ3.95 and 3.45 with the C-3 (81.4), C-4 (163.7), C-1″ (123.5), C-2″ (128.1) and C-6″ (125.7) showed in the HMBC spectra of **1**, suggested that these two protons should be at the C-7″ position. Hence, the structure of **1** was deduced as the 7″-deoxy analogue of emestrin and named emestrin-F. Emestrin-F is rare in having a hydrogen in the C-7″ position instead of a hydroxy group that is typical of most emestrin analogues.

Compound **2** was also isolated as a white solid and HRESIMS analysis indicated the molecular formula $C_{27}H_{22}N_2O_{10}S$ with one less sulphur atom compared to emestrin ($C_{27}H_{22}N_2O_{10}S_2$). The ^{1}H- and ^{13}C-NMR spectroscopic data of both **2** and the emestrin were almost identical. Therefore, the structure of **2** was suggested to be the mono-sulphur derivative of emestrin and it was named emestrin-G.

Compound **3** was isolated as a crystalline solid and the HRESIMS suggested the molecular formula $C_{18}H_{22}O_9$. The ^{1}H-NMR of **3** exhibited singlets due to two aromatic protons (δ6.12 and 7.03), a methoxy group (δ3.87) and two aromatic methyls (δ2.33, δ2.07) and signals typical of a sugar unit. The ^{13}C-NMR spectrum indicated resonances due to 10 low-field carbons, including one each for a carbonyl (δ183.2) and an anomeric carbon (δ101.7), four oxygenated methines, a oxygenated methylene, a methoxy and two aromatic methyls. These data indicated that **3** is a chromone glycoside. In the HMBC spectrum, the aromatic proton at δ6.12 showed a strong correlation with carbons at δ106.1 (C-4a), 167.8 (C-2), 20.3 (C-11) and 183.2 (C-4), while the other aromatic proton at δ7.03 correlated with carbons at δ110.1 (C-7), 162.0 (C-6), 106.1 (C-4a), 156.5 (C-8a) and 183.2 (C-4). Further, the correlation of the methyl protons at δ2.07 (2-CH3) with the carbons at δ167.8 (C-2) and 109.1 (C-3) and the other methyl protons at δ2.33 (7-CH3) with carbons at δ159.6 (C-8), 110.1 (C-7) and 162.0 (C-6) in the HMBC of **3** suggested that the two methyl groups were on C-2 and C-7 of the chromone moiety, respectively. The methoxy group correlated a carbon in the sugar unit suggesting that the aglycone is 6,8-dihydroxy-2,7-dimethylchromone, dulcinone, which was previously isolated from a higher plant (Deachathai et al., 2006). The anomeric proton (δ5.66) of the sugar moiety showed correlation with the C-6 carbon (δ162.0), indicating the attachment of the sugar unit to C-6. The ^{1}H-NMR coupling constants, COSY, HMQC and HMBC spectroscopic data confirmed that the sugar moiety is β-D-glucose and the methoxy group is attached to C-4 of the sugar. The evidence suggested the structure of **3** as 6-*O*-(4-*O*-methyl-β-D-glucopyranosyl)-8-hydroxy-2,7-dimethyl-4*H*-benzopyran-4-one. Even though compounds bearing 4-*O*-methylglucopyranosyl units have not been isolated from higher plants, several of them have previously been isolated from fungi (Hu et al., 2002; Zhang & Xuan, 2007) and a fern (Ye, Fan, Zhang, Yin, & Zhao, 2007).

Compound **4**, a white crystalline solid, was assigned the molecular formula $C_{10}H_{16}O_5$ based on HRESIMS. The NMR spectroscopic data were similar to the previously reported spectroscopic data for cephalosporolide and HMBC correlations indicated that it has the same gross structure as cephalosporolide-C (Ackland et al., 1985). The NOESY spectrum of **4** showed a good correlation between the two protons at δ4.20 (H-3) and 3.38 (H-4), which revealed that both hydroxy groups attached to C-3 and C-4 should be on the same plane of the molecule. However, the carbon and proton signals corresponding to C-2, C-3, C-4, C-5 and C-6 positions of **4** did not match with the values obtained for cephalosporolide-C (Ackland et al., 1985), which have both C-3 and C-4 hydroxy groups at the β orientation. The ^{1}H- and ^{13}C-NMR values which represent the C-7, 8, 9 and 10 positions of both **4** and cephalosporolide-C were almost identical (Ackland et al., 1985). This spectroscopic information suggested that both the C-3 and C-4 hydroxy

Table 1. Antimicrobial activity of compounds **5** and **1** from *A. tabescens*.

	Candida albicans		*Cryptococcus neoformans*		*Mycobacterium intracellulare*		*Escherichia coli*		*Staphylococcus aureus*		Methicillin-resistant *S. aureus*	
Compound	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC
5	3.94	–[a]	0.6	2.5	–	–	2.21	–	4.55	20	2.21	10
1	–	–	3.24	–	1.18	1.25	–	–	–	–	–	–
Amphotericin B[c]	0.42	1.25	0.43	1.25	NT[b]	NT	NT	NT	NT	NT	NT	NT
Ciprofloxacin[c]	NT	NT	NT	NT	0.41	0.5	0.007	0.03	0.12	0.25	0.14	0.50

Notes: IC_{50} and MIC (minimum inhibitory concentration) values are in $\mu g\,mL^{-1}$.
[a]Not active at the highest test concentration of $20\,\mu g\,mL^{-1}$.
[b]Not tested.
[c]Positive control.

groups of **4** have an α-orientation and the compound was named cephalosporolide-J. A diastereomer of **4** named cephalosporolide-G previously has been reported from a fungus (Farooq, Gordon, Hanson, & Takahashi, 1995), whereas its C-4 epimer has been synthesised (Barradas, Urbano, & Carreno, 2009).

None of the compounds showed activity against *Aspergillus fumigatus*, *Candida glabrata* and *Pseudomonas aeruginosa* at the highest test concentration. Emestrin (**5**) exhibited antifungal activity against *C. albicans*, *C. neoformans* and antibacterial activity against both *E. coli* and *Staphylococcus aureus* (Table 1). Compound **1** was only active against *C. neoformans* and *M. intracellulare* whereas compound **2** had no antimicrobial activity. Strong antifungal activity of emestrin (**5**) against *Penicillium expansum* and *Gibberella zeae* has been reported previously (Seya et al., 1986). The mode of action responsible for toxicity against certain eukaryotes (fungi) may be due to the inhibition of ATP synthesis in mitochondria causing an uncoupling of oxidative phosphorylation and depression of respiration (Kawai et al., 1989).

3. Experimental

3.1. *General details*

Melting points were measured with a Uni-melt, Thomas Hoover capillary melting point apparatus. UV and IR spectra were obtained with a Varian-50 Bio UV visible spectrophotometer and a Bruker-Tensor-27 infrared spectrophotometer, respectively. NMR spectra were recorded using a Varian-Mercury-plus-400 or Varian Unity-Inova-600 spectrometer with $CDCl_3$ and methanol-d_4 unless otherwise stated. Mass spectra data were obtained from an Agilent Series 1100 SL equipped with an ESI source (Agilent Technologies, Palo Alto, CA, USA). Column chromatography and preparative TLC were carried out using Merck silica gel 60 (230–400 mesh) and silica gel GF plates ($20 \times 20\,cm^2$, thickness 0.25 mm), respectively.

3.2. *Fungal material*

Armillaria tabescens strain accession number JNB-OZ344 was isolated from contextual hyphae of mushroom basidiomes growing on the surface roots of a live *Quercus velutina* Lam. (black oak) tree with significant root rot in southern Missouri. This strain was identified using a combination of macroscopic and microscopic morphological characters. Cultures were maintained in long-term storage under sterile water in plastic cryotubes,

according to methods described by Burdsall and Dorworth (1994), prior to culture on rice grains for this study.

3.3. *Culture, extraction and purification*

Armillaria tabescens strain JNB-OZ344 was cultured and incubated for 21 days at 26°C followed by 7 days at 10°C on 750 g of previously boiled brown rice grains. The rice substrate was inoculated with 4.5% potato dextrose agar plugs containing mycelium of the fungus. Accumulated mycelial growth on the rice substrate was extracted thrice with 500 mL aliquots of ethyl acetate. The combined organic extract was evaporated to dryness (18.5 g) and thoroughly washed with hexane. The hexane-insoluble portion (1.35 g), later showing antimicrobial activity, was subjected to chromatography on a silica gel column using hexane/ethyl acetate/methanol gradient to yield nine major fractions. The activity was concentrated in fourth and fifth fractions which eluted with hexane/ethyl acetate (80 : 20). Fractions were combined and separated by preparative TLC on silica gel using hexane/ethyl acetate 7 : 3 as the solvent (three developments) to yield compounds **1** (17 mg), **2** (15 mg) and **5** (27 mg)**.** Fraction 6, which eluted with ethyl acetate : hexane (3 : 7), was separated by preparative TLC on silica gel using hexane : ethyl acetate (1 : 1) to yield compounds **4** (11 mg) and **7** (15 mg). Compound **3** (13 mg), which eluted with ethyl acetate, was purified by preparative TLC on silica gel using CH_2Cl_2 : MeOH (98 : 2) as the solvent. Compounds **6** (19 mg), ergosterol (24 mg) and brassicasterol (41 mg) were isolated from fraction 3 by preparative TLC using hexane : ethyl acetate (7 : 3).

Emestrin-F (**1**): ^{1}H-NMR ($CDCl_3$) δ: 7.89 (1H, d, $J = 1.6$ Hz, 6′-H), 7.76 (1H, dd, $J = 8.8$, 2.0 Hz, 7′-H), 7.77 (1H, d, $J = 1.2$ Hz, 3′-H), 7.06 (1H, dd, $J = 8.0$, 2.0 Hz, 2″-H), 7.02 (1H, d, $J = 8.8$ Hz, 6′-H), 6.91 (1H, d, $J = 8.4$ Hz, 3″-H), 6.89 (1H, brs, 10-H), 6.32 (1H, dd, $J = 8.4$, 2.4 Hz, 8-H), 5.62 (1H, dd, $J = 8.4$, 2.4 Hz, 5a-H), 5.55 (1H, brs, OH) 5.29 (1H, s, 11-H), 4.92 (2H, m, 6-H, 7-H), 4.01 (3H, s, 8′-CH_3), 3.95 (1H, d, $J = 13.2$ Hz, 7″-Ha), 3.45 (1H, d, $J = 13.6$ Hz, 7″-Hb) and 3.22 (3H, s, N–CH_3); ^{13}C-NMR (Pyridine-D_5) δ: 166.42 (C-1), 81.4 (C-3), 163.7 (C-4), 61.9 (C-5a), 76.3 (C-6), 108.4 (C-7), 138.2 (C-8), 143.1 (C-10), 114.2 (C-10a), 77.2 (C-11), 81.4 (C-11a), 28.1 (C-12), 165.7 (C-1′), 123.5 (C-2′), 122.9 (C-3′), 146.4 (C-4′), 154.9 (C-5′), 113.1 (C-6′), 125.6 (C-7′), 56.5 (C-8′), 123.5 (C-1″), 128.1 (C-2″), 118.3 (C-3″), 150.3 (C-4″), 145.8 (C-5″), 125.7 (C-6″) and 36.9 (C-7″); HRESIMS $[M-1]^-$ m/z 581.0662 (Calcd for $C_{27}H_{21}N_2O_9S_2$, 581.0688).

Emestrin-G (**2**): ^{1}H-NMR ($CDCl_3$) δ: 8.71 (1H, brs, 6″-H), 8.35 (1H, brs, 3′-H), 7.86 (1H, d, $J = 8.4$ Hz, 7′-H), 7.02 (1H, d, $J = 8.4$ Hz, 6′-H), 6.93 (1H, d, $J = 8.0$ Hz, 2″-H), 6.83 (1H, s, 10-H), 6.80 (1H, d, $J = 8.0$ Hz, 3″-H), 6.36 (1H, d, $J = 8.4$ Hz, 8-H), 5.39 (1H, d, $J = 8.4$ Hz, 5a-H), 5.32 (1H, brd, $J = 8.0$ Hz, 6-H), 5.22 (1H, brd, $J = 12.0$ Hz, 7″-H), 5.00 (1H, dd, $J = 8.0$, 2.0 Hz, 7-H), 4.81 (1H,s, 11-H), 4.43 (1H, brd, $J = 12.4$ Hz, 7″-OH), 4.06 (3H, s, 8′-CH_3) and 3.53 (3H, s, N–CH_3); ^{13}C-NMR ($CDCl_3$) δ: 168.1 (C-1), 77.6 (C-3), 165.5 (C-4), 59.0 (C-5a), 74.8 (C-6), 109.8 (C-7), 139.0 (C-8), 143.0 (C-10), 108.3 (C-10a), 79.8 (C-11), 83.1 (C-11a), 28.8 (C-12), 165.2 (C-1′), 122.6 (C-2′), 123.2 (C-3′), 147.2 (C-4′), 154.5 (C-5′), 112.0 (C-6′), 127.5 (C-7′), 55.5 (C-8′), 127.0 (C-1″), 130.3 (C-2″), 114.4 (C-3″), 149.6 (C-4″), 146.4 (C-5″), 126.8 (C-6″) and 76.6 (C-7″); HRESIMS $[M-1]^-$ m/z 565.0902 (Calcd for $C_{27}H_{21}N_2O_{10}S$, 565.0917).

6-*O*-(4-*O*-methyl-β-D-glucopyranosyl)-8-hydroxy-2,7-dimethyl-4*H*-benzopyran-4-one (**3**): m.p. 132–134°C, ^{1}H-NMR (pyridine-D_6) δ: 13.57 (1H, s, OH), 7.03 (1H, s, 5-H), 6.12 (1H, s, 3-H), 5.66 (1H, dd, $J = 7.6$, 1.6 Hz, 1′-H), 4.34 (3H, m, 2′, 3′, 6′-H), 4.20 (1H, dd, $J = 12.0$, 4.4 Hz, 6′-H), 4.00 (1H, m, 5′-H), 3.89 (1H, overlapped, 4′-H), 3.87 (3H, s, OCH_3), 2.33 (3H, s) and 2.07 (3H, s). ^{13}C-NMR (pyridine-D_6) δ: 183.2 (C-4), 167.8 (C-2), 162.0 (C-6), 159.6 (C-8), 156.6 (C-8a), 110.2 (C-7), 109.1 (C-3), 106.1 (C-4a), 101.7 (C-1′),

93.9 (C-5), 80.5 (C-4′), 78.4 (C-3′), 78.1 (C-5′), 75.1 (C-2′), 62.0 (C-6′), 60.9 (OCH_3), 20.3 (2-CH_3) and 8.2(7-CH_3). HRESIMS $[M+1]^+$ m/z 383.0983 (Calcd for $C_{18}H_{23}O_9$, 383.1342).

Cephalosporolide-J (**4**): ^{1}H-NMR ($CDCl_3$) δ: 5.09 (1H, m, 9-H), 4.20 (2H, m, 3-H, OH), 3.38 (1H, m, 4-H), 2.96 (1H, brs, OH), 2.90 (1H, dd, $J = 17.4$, 3.4 Hz, 2-H_a), 2.76 (1H, dd, $J = 18.4$, 4.8 Hz, 5-H_a), 2.69 (1H, dd, $J = 18.4$, 2.8 Hz, 5-H_b), 2.34 (3H,m, 2-H_b, 7-H_a, 7-H_b), 2.05 (2H, m, 8-H_a, 8-H_b) and 1.37 (3H, s, 10-CH_3); ^{13}C-NMR ($CDCl_3$) δ: 214.4 (C-6), 169.5 (C-1), 74.9 (C-4), 72.1 (C-9), 68.7 (C-3), 43.3 (C-5), 40.6 (C-7), 38.8 (C-2), 33.6 (C-8) and 19.4 (C-10). HRESIMS $[M+1]^+$ m/z 217.1047 (Calcd for $C_{10}H_{17}O_5$, 217.1076).

3.4. *Antimicrobial assay*

Test organisms included the fungi *C. albicans* American Type Culture Collection (ATCC) 90028, *C. glabrata* ATCC 90030, *C. neoformans* ATCC 90113 and *A. fumigatus* ATCC 204305 and the bacteria *S. aureus* ATCC 29213, MRSA ATCC 33591, *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853 and *M. intracellulare* ATCC 23068 obtained from the ATCC (Manassas, VA). Susceptibility testing was performed using a modified version of CLSI methods (National Committee on Clinical Laboratory Standards (NCCLS, 2002a, 2002b, 2003, 2006)), as described by Samoylenko et al. (2009). Two drug controls included in each assay were ciprofloxacin (ICN Biomedicals, OH) for bacteria and amphotericin B (ICN Biomedicals, OH) for fungi. IC_{50}s (concentrations that afford 50% inhibition relative to controls) were calculated using the XLfit 4.2 software (IDBS, Alameda, CA) using fit model 201. The MIC is defined as the lowest test concentration that allows no detectable growth.

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