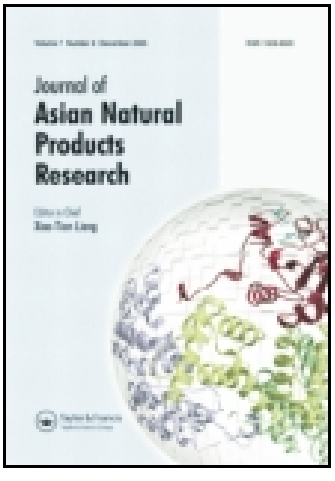

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One new ergostane-type steroid and three new phthalide derivatives from cultures of the basidiomycete Albatrellus confluens

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One new ergostane-type steroid and three new phthalide derivatives from cultures of the basidiomycete *Albatrellus confluens*

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One new ergostane-type steroid, (12β,15β,22*R*,23*S*,24*S*)-22,25-epoxy-12,15,23-trihydroxyergost-4,6,8(14)-trien-3-one (**1**), three new phthalide derivatives, 5-(2′,3′-epoxy-3′,3′-dimethylpropoxy)-7-methoxy-6-methylphthalide (**2**), (2′)-(*Z*)-5-(3′-hydroxymethyl-3′-methylallyloxy)-7-methoxy-6-methylphthalide (**3**), and 5-(3′,3′-dimethylallyloxy)-7-hydroxy-6-methylphthalide (**4**), along with one known phthalide derivative, 5-(3′,3′-dimethylallyloxy)-7-methoxy-6-methylphthalide (**5**), were isolated from cultures of the basidiomycete *Albatrellus confluens*. The structures of the new compounds were established on the basis of extensive spectroscopic data (IR, MS, 1D, and 2D NMR) analyses. All compounds were evaluated for their cytotoxic activities on five tumor cell lines.

Keywords: *Albatrellus confluens*; ergostane-type steroid; phthalide derivatives

1. Introduction

The fungus *Albatrellus confluens* (Alb. & Schwein.) Kotl. & Pouzar, belonging to the Polyporaceae family, is widely distributed all over the world. Pyrazines, aurovertins, cleistanthane-type diterpenes, and isocoumarine have been reported from the fruiting bodies or from the cultures of *A. confluens*, and most of which showed biological activities [1–6]. During our studies on *A. confluens*, albaconol and grifolin were discovered successively, and have been identified to possess immunosuppressive and anti-inflammatory activities and antitumor activity, respectively [7–11]. In the course of our search for more biologically active compounds, we have investigated an enlarged culture on the fungus once more. As a result, one new ergostane-type steroid, 22,25-epoxy-trihydroxyergost-4,6,8(14)-trien-3-one (**1**), three new phthalide derivatives, 5-(2′,3′-epoxy-3′,3′-dimethylpropoxy)-7-methoxy-6-methylphthalide (**2**), (2′)-(*Z*)-5-(3′-hydroxymethyl-3′-methylallyloxy)-7-methoxy-6-methylphthalide (**3**), and 5-(3′,3′-dimethylallyloxy)-7-hydroxy-6-methylphthalide (**4**), along with one known phthalide derivative, 5-(3′,3′-dimethylallyloxy)-7-methoxy-6-methylphthalide (**5**), were obtained (Figure 1). In this paper, we describe the isolation and structure elucidation of the four new compounds.

2. Results and discussion

Compound **1** was isolated as a yellow oil which possessed a molecular formula $C_{28}H_{40}O_5$ as determined by HR-EI-MS at *m/z* 456.2858, indicating nine degrees of unsaturation. The IR spectrum showed absorption bands at 3411 and 1640 cm^{-1} due to hydroxy and unsaturated ketone groups. The latter was also indicated by

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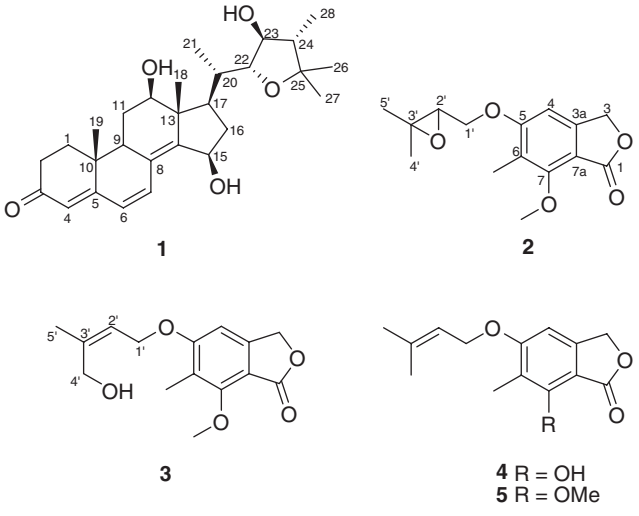

Figure 1. Structures of compounds **1**–**5**.

the ^{1}H NMR signals at δ 5.68 (1H, s, H-4), 6.15 (1H, d, $J = 9.7$ Hz, H-6), and 6.85 (1H, d, $J = 9.7$ Hz, H-7) and the ^{13}C NMR signals at δ_C 198.4 (C-3), 122.9 (C-4), 163.4 (C-5), 125.2 (C-6), 133.6 (C-7), 127.1 (C-8), and 154.9 (C-14). The ^{13}C NMR and DEPT spectra revealed 28 carbon signals including 6 methyls, 4 methylenes, 11 methines (4 oxygenated and 3 olefinic), and 7 quaternary carbons (1 oxygenated, 3 olefinic, and 1 carbonyl). The ^{1}H NMR spectrum showed resonances for four methyl singlets at δ 0.99 (3H, s, H-18), 0.94 (3H, s, H-19), 0.90 (3H, s, H-26), and 1.15 (3H, s, H-27), two methyl doublets at δ 0.92 (3H, d, $J = 6.6$ Hz, H-21) and 0.89 (3H, d, $J = 7.4$ Hz, H-28), and four oxymethine signals at δ 3.33 (1H, m, H-12), 4.53 (1H, dd, $J = 7.7$, 7.4 Hz, H-15), 3.54–3.55 (1H, m, H-22), and 3.37 (1H, dd, $J = 9.0$, 8.9 Hz, H-23) (Table 1). These data revealed that compound **1** should be an ergostane-type steroid with several hydroxy substituents [12]. The ^{1}H–^{1}H COSY correlation of H-1/H-2 and the HMBC correlations from H_3-19 to C-1 (δ 33.6), C-5 (δ 163.4), C-9 (δ 44.6), and C-10 (δ 36.4) suggested the existence of a 4-en-3-one moiety in ring A. Furthermore, the HMBC correlations from the olefinic proton H-7 to C-5 (δ 163.4), C-8 (δ 127.1), C-9 (δ 44.6), and C-14 (δ 154.9), together with the ^{1}H–^{1}H COSY correlation between H-6 and H-7, allowed the locations of the two double bonds at C-6/C-7 and C-8/C-14. These data established a 4,6,8(14)-trien-3-one moiety in the structure of **1**. In addition, a hydroxy group at C-12 was revealed by the ^{1}H–^{1}H COSY correlations of H-9/H-11/H-12 and the HMBC correlations from H_3-18 to C-12 (δ 73.8), C-14 (δ 154.9), and C-17 (δ 52.9). Similarly, the ^{1}H–^{1}H COSY correlations of H-15/H-16/H-17 and the HMBC correlations from H-15 to C-8 (δ 127.1), C-13 (δ 47.6), and C-14 (δ 154.9) indicated a hydroxy substituent at C-15. Further inspection of HMBC and ^{1}H–^{1}H COSY spectra led to the establishment of a side chain from C-20 to C-28. HMBC correlations from the proton H-17 to C-16 (δ 36.3), C-21 (δ 14.4), and C-22 (δ 82.6) and from the methyl protons of Me-28 to C-23 (δ 76.6), C-24 (δ 49.7), and C-25 (δ 79.5) indicated that the oxygenated carbons were C-22, C-23, and C-25, respectively. In addition, the HMBC correlations from OH-23 to C-22 (δ 82.6), C-23 (δ 76.6), and C-24 (δ 49.7), as well as the analysis of MS data, indicated the

Table 1. ^{1}H and ^{13}C NMR spectroscopic data of **1** at 600/150 MHz, respectively, in DMSO-d_6.

Position	δ_H (J, Hz)	δ_C
1	1.89, ddd (13.4, 4.1, 3.0) 1.73, ddd (13.3, 5.0, 4.6)	33.6, CH_2
2	2.48–2.50, m 2.28, br d (16.0)	33.9, CH_2
3		198.4, qC
4	5.68, s	122.9, CH
5		163.4, qC
6	6.15, d (9.7)	125.2, CH
7	6.85, d (9.7)	133.6, CH
8		127.1, qC
9	2.21–2.23, m	44.6, CH
10		36.4, qC
11	1.57–1.61, m 1.52–1.54, m	28.5, CH_2
12	3.31–3.34, m	73.8, CH
13		47.6, qC
14		154.9, qC
15	4.53, dd (7.7, 7.4)	67.5, CH
16	2.20–2.23, m 1.59–1.61, m	36.3, CH_2
17	1.08–1.10, m	52.9, CH
18	0.99, s	14.6, CH_3
19	0.94, s	16.6, CH_3
20	2.11, br s	30.3, CH
21	0.92, d (6.6)	14.4, CH_3
22	3.54–3.55, m	82.6, CH
23	3.37, dd (9.0, 8.9)	76.6, CH
24	1.66–1.68, m	49.7, CH
25		79.5, qC
26	0.90, s	25.1, CH_3
27	1.15, s	29.4, CH_3
28	0.89, d (7.4)	12.3, CH_3
OH-12	4.69, br s	
OH-15	4.95, br s	
OH-23	4.97, br s	

existence of a furan ring formed by C-22, C-23, C-24, and C-25. The $^{1}H-^{1}H$ COSY correlations of H-17/H-20/H-22/H-23/H-24 further supported the connectivity of the side chain from C-20 to C-28 (Figure 2).

The relative configuration of **1** was determined on the basis of an ROESY experiment. ROESY correlations from OH-12 to H_3-19 and OH-15 to H_3-18 indicated that these protons were β-oriented, while the correlations from H-15 to H-9 and H-17 indicated that these protons were α-oriented. The stereochemistry of the side chain was determined by correlations of H-22 to H-24 and OH-23 to H-24 (Figure 2). From the above results, the structure of **1** was established as (12β,15β,22*R*,23*S*,24*S*)-22,25-epoxy-12,15,23-trihydroxyergost-4,6,8(14)-trien-3-one.

Compound **2** was obtained as a yellow oil. Its molecular formula was determined to be $C_{15}H_{18}O_5$ by HR-EI-MS at *m/z* 278.1158 $[M]^+$, corresponding to seven degrees of unsaturation. The ^{13}C NMR and DEPT spectra showed 15 carbon signals that attributed to 4 methyls, 2 methylenes, 2 methines, and 7 quaternary carbons (Table 2). These data were similar to those of 5-(3′,3′-dimethylallyloxy)-7-methoxy-6-methylphthalide (**5**) [13], except that the double bond of the isoprenyl group in **5** was oxidized into an epoxy ring between C-2′ (δ_C 61.1, d) and C-3′ (δ_C 58.3, d) in **2**, as supported by the HMBC correlations from H-2′ at δ_H 3.18 (1H, dd, $J = 6.0$,

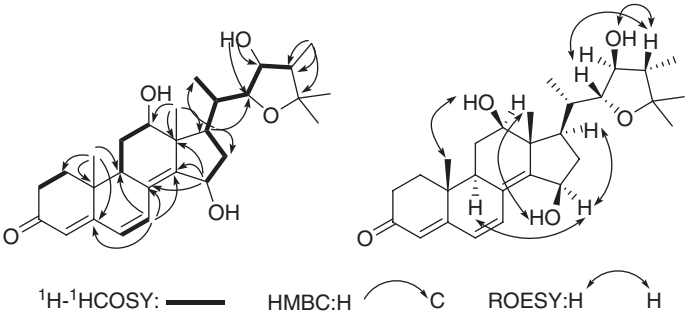

Figure 2. Key $^{1}H-^{1}H$ COSY, HMBC, and ROESY correlations of **1**.

Table 2. ^{1}H and ^{13}C NMR spectroscopic data of **2**–**4** in $CDCl_3$.

	2[a]		**3**[a]		**4**[b]	
Position	δ_H (*J*, Hz)	δ_C	δ_H (*J*, Hz)	δ_C	δ_H (*J*, Hz)	δ_C
1		168.9, qC		168.9, qC		172.9, qC
3	5.19, d (3.2)	68.8, CH_2	5.18, s	68.7, CH_2	5.23, s	70.4, CH_2
3a		148.1, qC		148.1, qC		145.5, qC
4	6.67, s	99.5, CH	6.63, s	99.4, CH	6.46, s	96.7, CH
5		163.0, qC		163.2, qC		164.3, qC
6		120.8, qC		121.9, qC		113.2, qC
7		158.0, qC		157.8, qC		154.7, qC
7a		110.1, qC		109.8, qC		103.8, qC
1′	4.30, dd (11.0, 4.0) 4.10, dd (11.0, 6.0)	68.1, CH_2	4.68, d (6.4)	64.8, CH_2	4.58, d (6.4)	65.8, CH_2
2′	3.18, dd (6.0, 4.0)	61.1, CH	5.65, t (6.3)	121.9, CH	5.47, t (6.0)	119.0, CH
3′		58.3, qC		140.7, qC		138.5, qC
4′	1.38, s	19.1, CH_3	4.24, s	62.0, CH_2	1.74, s	18.3, CH_3
5′	1.42, s	24.6, CH_3	1.90, s	21.4, CH_3	1.80, s	25.8, CH_3
Me-6	2.19, s	8.7, CH_3	2.15, s	8.8, CH_3	2.10, s	7.7, CH_3
OMe-7	4.04, s	62.2, CH_3	4.03, s	62.2, CH_3		

[a] Measured at 600/150 MHz.
[b] Measured at 400/100 MHz.

4.0 Hz) to C-1′ (δ_C 68.1, t) and C-3′, as well as the MS data analysis. Detailed analysis of other 1D and 2D NMR data suggested that the other parts of **2** were the same as those of **5**. However, the stereoconfiguration of this epoxy moiety could not be determined correctly. Therefore, the structure of **2** was determined to be 5-(2′,3′-epoxy-3′,3′-dimethylpropoxy)-7-methoxy-6-methylphthalide.

Compound **3** exhibited the molecular formula $C_{15}H_{18}O_5$, as determined by its HR-EI-MS at *m/z* 278.1159 $[M]^+$, corresponding to seven degrees of unsaturation. Comparison of the spectroscopic data of **3** with those of **5** also indicated similar patterns except for signals of an oxygenated methylene [δ_H 4.24 (2H, s, H-4′); δ_C 62.0 (t, C-4′)] in **3** instead of the methyl in **5**, which was established by the HMBC correlations from the protons of H-4′ to C-3′ (δ 140.7) and C-5′ (δ 21.4). In addition, the ROESY correlation of H-2′ (δ_H 5.65, 1H, t) with H-5′ (δ_H 1.90, 3H, s) suggested that the double bond between C-2′ and C-3′ was cis form. Thus, the structure of **3** was established as (2′)-(*Z*)-5-(3′-hydroxymethyl-3′-methylallyloxy)-7-methoxy-6-methylphthalide, as shown.

The molecular formula of compound **4** was inferred to be $C_{14}H_{16}O_4$ on the basis of its HR-EI-MS. Preliminary analysis of the NMR data also indicated that **4** possessed the same skeleton as that of **5**, except that the methoxy at C-7 was replaced by a hydroxy group, as indicated by the loss of signals for the methyl group and the MS data. Detailed analysis of other spectroscopic data (HSQC, HMBC, ^{1}H–^{1}H COSY, ROESY) established the structure of compound **4** to be 5-(3′,3′-dimethylallyloxy)-7-hydroxy-6-methylphthalide.

All compounds were evaluated for their cytotoxicities against five human cancer cell lines (HL-60, SMMC-7721, A-549, MCF-7, and SW-480) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The results showed that only compound **4** exhibited moderate cytotoxicity against SMMC-7721 and MCF-7, with IC_{50} values of 1.81 and 29.02 μM, respectively.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a Jasco-P-1020 polarimeter (Horiba, Kyoto, Japan). UV spectra were measured on a Shimadzu UV-2401 PC spectrophotometer (Shimadzu, Kyoto, Japan). IR spectra were obtained by using a Bruker Tensor 27 FT-IR spectrometer (Bruker, Karlsruher, Germany) with KBr pellets. NMR spectra were acquired with Avance III 600 or Bruker AV 400 (Bruker) instruments. HR-EI-MS were measured on a Waters Autospec Premier P776 mass spectrometer (Waters, Milford, MA, USA). Preparative HPLC was performed on an Agilent 1100 series with a Zorbax SB-C18 (5 μm, 9.4 mm × 150 mm) column (Agilent Technologies, Santa Clara, CA, USA). Preparative MPLC was performed on a Büchi apparatus equipped with Büchi fraction collector C-660, Büchi pump module C-605, and manager C-615 (Büchi Labortechnik AG, Flawil, Switzerland). Silica gel (200–300 mesh and 80–100 mesh, Qingdao Marine Chemical Inc., Qingdao, China), RP-18 gel (40–75 μm, Fuji Silysia Chemical Ltd, Kasugai, Japan), and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) were used for column chromatography (CC). Fractions were monitored by TLC (Qingdao Marine Chemical Inc.) and spots were visualized by heating silica gel plates immersed in vanillin–H_2SO_4 in EtOH.

3.2 Fungal material and cultivation conditions

The fungus *A. confluens* was collected from Ailao Mountain of Yunnan Province, China, in July 2003, and identified by Prof. Mu Zang, Kunming Institute of Botany. A voucher specimen (HFG0307252) has been deposited at the Herbarium of the Kunming Institute of Botany, CAS. Culture medium: glucose (5%), pork peptone (0.15%), yeast (0.5%), KH_2PO_4 (0.05%), $MgSO_4$ (0.05%). The initial pH was adjusted to 6.0, the fermentation was first carried out on an Erlenmeyer flask for 6 days till the mycelium biomass reached the maximum. Later it was transferred to a fermentation tank (100 l) at 24°C and 250 rpm for 20 days, ventilation was settled to 1.0 vvm (vvm: air volume/culture volume/min).

3.3 Extraction and isolation

The culture broth (70 l) was extracted three times with EtOAc (3 × 10 l). The combined EtOAc extracts were evaporated *in vacuo* to give a residue (50.0 g). The residue was subjected to silica gel CC with a gradient elution system of chloroform–methanol (100:0 → 0:100) to obtain 11 fractions (A–K). Fraction D (4 g) was subjected to preparative MPLC with a reversed-phase C_{18} column (MeOH–H_2O, 0–60%) to obtain subfractions D01–D10. Fraction D03 (800 mg) eluted with petroleum ether (PE)–acetone (6:1) was further separated by preparative HPLC (MeCN–H_2O, 40%, 10 ml/min) to give **1** (5.0 mg) (r.t. = 12 min). Fraction D05 (1.2 g) was eluted with PE–EtOAc (4:1) and then subjected to Sephadex LH-20 CC ($CHCl_3$–MeOH, 1:1) to give **3** (1.0 mg). Fraction E (2.3 g) was chromatographed over a silica gel column using PE–acetone (10:1 → 0:1) to produce fractions E01–E06. Compound **2** (2.0 mg) was obtained by preparative HPLC (MeCN–H_2O, 30%, 10 ml/min, r.t. = 8 min) from fraction E03 (130 mg). Fraction E06 (580 mg) was chromatographed on a RP-18 column (MeOH–H_2O, 50%) and then purified on a silica gel column (PE–acetone, 2:1) to yield **5** (5.0 mg). Compound **4** (8.0 mg) was obtained from fraction F (1.8 g) after Sephadex LH-20 CC ($CHCl_3$–MeOH, 1:1), followed by preparative HPLC (MeCN–H_2O, 10%, 10 ml/min, r.t. = 14 min).

*3.3.1 (12β,15β,22R,23S,24S)-22,25-Epoxy-12,15,23-trihydroxyergost-4,6,8 (14)-trien-3-one (**1**)*

Yellow oil; $[\alpha]_D^{15}$ + 254.9 (c = 0.33, MeOH); UV (MeOH) λ_{max} (log ε) 336 (3.5), 232 (3.0), 203 (3.1) nm; IR (KBr) ν_{max} 3441, 1640 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) and ^{13}C NMR (DMSO-d_6, 150 MHz) spectral data, see Table 1; HR-EI-MS: m/z 456.2858 $[M]^+$ (calcd for $C_{28}H_{40}O_5$, 456.2876).

*3.3.2 5-(2′,3′-Epoxy-3′,3′-dimethylpropoxy)-7-methoxy-6-methylphthalide (**2**)*

Yellow oil; $[\alpha]_D^{15}$ −18.9 (c = 0.06, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 258 (3.1), 214 (3.5) nm; IR (KBr) ν_{max} 3442, 3426, 1753, 1627, 1141, 1096 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) spectral data, see Table 2; HR-EI-MS: m/z 278.1158 $[M]^+$ (calcd for $C_{15}H_{18}O_5$, 278.1154).

*3.3.3 (2′)-(Z)-5-(3′-Hydroxymethyl-3′-methylallyloxy)-7-methoxy-6-methylphthalide (**3**)*

White powder; $[\alpha]_D^{15}$ −20.2 (c = 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 259 (3.4), 215 (3.8) nm; IR (KBr) ν_{max} 3434, 1752, 1608 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) spectral data, see Table 2; HR-EI-MS: m/z 278.1159 $[M]^+$ (calcd for $C_{15}H_{18}O_5$, 278.1154).

*3.3.4 5-(3′,3′-Dimethylallyloxy)-7-hydroxy-6-methylphthalide (**4**)*

White powder; $[\alpha]_D^{15}$ −3.9 (c = 0.40, MeOH); UV (MeOH) λ_{max} (log ε) 332 (2.8), 269 (3.3), 231 (3.7), 197 (3.5) nm; IR (KBr) ν_{max} 3438, 1710, 1638, 1615, 1254, 1142, 1100 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) spectral data, see Table 2; HR-EI-MS: m/z 248.1060 $[M]^+$ (calcd for $C_{14}H_{16}O_4$, 248.1049).

3.4 Cytotoxic assay

All compounds were evaluated for their cytotoxicity against five human cancer cell lines: breast cancer SK-BR-3, hepatocellular carcinoma SMMC-7721, human myeloid leukemia HL-60, pancreatic cancer PANC-1, and lung cancer A-549 cells. Cells were cultured in Roswell Park Memorial Institute-1640 or in Dulbecco's modified eagle medium (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (Hyclone) in 5% CO_2 at 37°C. The cytotoxicity assay was performed according to MTT method in 96-well microplates [14]. Briefly, 100 μl of adherent cells were seeded into each well of 96-well cell culture plates and allowed to adhere for 12 h before addition of test compounds, while suspended cells were seeded just before drug addition with initial density of 1×10^5 cells/ml. Each tumor cell line was exposed to the test compound at concentrations of 0.0625, 0.32, 1.6, 8, and 40 μM in triplicates for 48 h, and all tests were done twice with cisplatin (Sigma, Santa Clara, CA, USA) as a positive control. After compound treatment, cell viability was detected and a cell growth curve was graphed. IC_{50} values were calculated by Reed and Muench's method [15].

Acknowledgments

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