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Secondary metabolite steroids isolated from medicinal entomogenous mushroom *Cordyceps jiangxiensis* mycelium

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ABSTRACT

Cordyceps jiangxiensis, a medicinal entomogenous mushroom, showed potent antitumor activity as described previously. In this present study, four steroids including docosanoic acid campesterol-ester (1), hexadecanoic acid ergosterol-ester (2), ergosterol (3), 5α-Ergosta-7,22-dien-3β-ol (4) were isolated from chloroform and ethyl acetate fractions of methanol extract of *Cordyceps jiangxiensis* mycelium by bioassay-guided fractionation, respectively. The chemical structures of four compounds were elucidated on the basis of their physical and spectroscopic properties. Among these compounds, compounds 1 and 2 were two new ergosteryl esters of long-chain alkanes, and compound 4 was obtained from the genus *Cordyceps* for the first time.

KEYWORDS

Cordyceps jiangxiensis;
Steroid;
Antitumor;
Isolation and identification.

INTRODUCTION

Cordyceps, an entomopathogenic macrofungus, has been used for centuries as a rare traditional Chinese medicinal herb. In recent years, the fungi of *Cordyceps* have received an increasing attention around the world as interesting natural drug resources due to their diverse pharmacological actions such as immunomodulatory, antitumor, hypoglycemic and hypocholesterolemic activities, etc.[1,2]. Actually, the large biomedicinal values of *Cordyceps* fungi are connected with their diversiform known and untapped active metabolites or components such as polysaccharides, alkaloids, polypeptides, steroids, and terpenes *etc*.. Among these metabolites, steroids are believed to be one of important components of *Cordyceps* fungi. For example, Bok *et al*. reported that two novel antitumor steroids including 5α,8α-epidioxy-24(*R*)-methylcholesta-6,22-dien-3β-*D*-glucopyranoside and 5α,6α-epoxy-24(*R*)-methylcholesta-7,22-dien-3β-ol, were isolated from the methanol extract of *C. sinensis* mycelium, together with ergosteryl-3-*O*-β-*D*-glucopyranoside and 22-dihydro-ergosteryl-3-*O*-β-*D*-glucopyranoside[3]. 5α, 8α-epidioxy-24(*R*) – methylcholesta -6,22-dien-3β-ol, an ergosterol peroxide, isolated from the methanolic extract of artificial culture of another *Cordyceps* species *Paecilomyces tenuipes*, showed strong cytotoxic effects against various human tumor cells[4]. A recent report indicated that five steroids including ergosterol, ergosterol peroxide,

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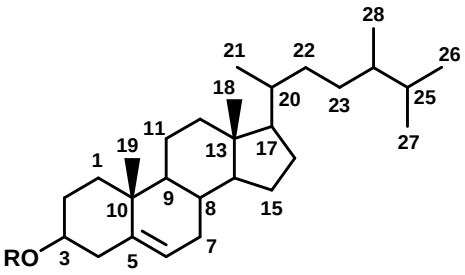
ergosta-4,6,8(14)-tetraen-3-one, eitrostadienol, and ergosta-7,22-dien-3β, 5α,6β-triol were isolated from the cultured *C. militaris*[5], of which ergosterol also indicated significant antitumor activity due to its direct antiangiogenic effect on solid tumors[6]. For this reason, *Cordyceps*-derived steroids may be regarded as valuable compound source for the development of health-promoting functional food and new drug discovery.

C. jiangxiensis, also known as Cao-Mu-Wang in China, is used in Chinese ethnomedicine as a major remedy to cure snakebite for centuries in local hospitals. In 2000, *C. jiangxiensis* was collected and denominated as a new species of the genus *Cordyceps* by Liang's research group[7]. Subsequently further study indicated that the polysaccharide, methanol and chloroform extract of *C. jiangxiensis* mycelium showed potent antitumor activity via both cell cycle arrest and caspase-dependent apoptotic biochemical pathway[8,9], which may possess therapeutic potential in the treatment of cancer diseases. As mentioned above, steroids were major antitumor components in *Cordyceps* fungi, they therefore were isolated as potential target compounds from the extracts of *C. jiangxiensis* mycelium by bioassay-guided fractionations. In this present study, four steroids in Figure 1 including docosanoic acid campesterol-ester **(1)**, hexadecanoic acid ergosterol-ester **(2)**, ergosterol **(3)**, 5α-Ergosta-7,22-dien-3β-ol **(4)** were isolated from both chloroform and ethyl acetate fractions of methanol extract of *C. jiangxiensis* mycelium. Among these compounds obtained, compounds **(1)** and **(2)** were two new ergosteryl esters of long-chain alkanes, and compound **(4)** was isolated from the genus *Cordyceps* for the first time. Here we describe the isolation and structural elucidation of these compounds, and cytotoxicity of fractions of *C. jiangxiensis* mycelium.



(1) R = -OCCH$_2$(CH$_2$)$_{18}$CH$_2$CH$_3$

(2) R1 = -OCCH$_2$(CH$_2$)$_{12}$CH$_2$CH$_3$

(3) R2 = H

(4)

Figure 1 : Structures of compounds

MATERIALS AND METHODS

General experimental procedures

Melting points were detected on an XT-2 micro melting point apparatus (uncorrected). Optical rotations were obtained on an Autopol V polarimeter. IR spectra were taken on a Vector-22 Fourier- transformation infrared spectrometer (KBr disc technique). UV spectra were performed on a HP-1200 UV-VIS spectrometer. EI-MS spectra were recorded on a HP-5973 mass spectrometer and ESI-MS spectra on a HP1100MSD instrument. ^{1}H- NMR and ^{13}C-NMR spectra were recorded on a Varian INOVA-400 spectrometers with TMS as the internal standard. Thin-layer chromatography and/or preparative TLC were carried out on silica gel GF254 (Qingdao Haiyang Chemical Co., Ltd.) and/or RP-18 (Merck) plates, and spots were visualized by spraying with 10% H_2SO_4/EtOH (v/v) reagent followed by heating at 120°C and/or iodine vapor. Column chromatography was performed on silica gel (40-80, 200-300, and 300-400 mesh, Qingdao Haiyang Chemical Co., Ltd.), RP-18 gel (40-63ìm) from Merck, and Sephadex LH-20 from Amersham Biosciences. All chemical reagents used were of analytical grade.

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Fungal materials

Fruiting bodies of *C. jiangxiensis* were collected in Jingangshan Mountain, Jiangxi, China, and identified as a new member of *Cordyceps* family[7]. The voucher specimen has been deposited in Institute of Fungal Resource of Guizhou University, Guiyang, China. Strain JXJP0109 of *C. jiangxiensis* was maintained and cultured as described previously[10]. The *C. jiangxiensis* mycelia were prepared on the basis of previous reports by our research group[11,12].

Extraction and isolation

The dried powder of *C. jiangxiensis* mycelium (3.15 kg) was extracted with hot 90% MeOH/H_2O (8h×3) under refluxing. After the removal of the solvent in *vacuo*, the MeOH extract (about 1.66 kg) was suspended in hot water and then was partitioned with petroleum ether (60°C-90°C), chloroform, and ethyl acetate, respectively.

The chloroform fraction (72g) was fractionated on column chromatography (silica gel 200-300 mesh, 1.45kg, petroleum ether- ethyl acetate 100 : 1→0 : 1) to obtain 120 fractions. On the basis of the results of cytotoxicity, Fr.2 was further separated on a silica gel column chromatography (200-300 mesh), eluting with petroleum ether-ethyl acetate to give 12 subfractions. Subfraction 1 was fractionated by preparative TLC (petroleum ether-ethyl acetate) to afford compounds **(1)** (13mg) and **(2)** (36mg). The ethyl acetate fraction (30g) was fractionated on column chromatography (silica gel 200-300 mesh, 700g, chloroform-MeOH 50 1→0 : 1) to obtain 106 fractions. Subfraction 44 was further fractionated by recrystallization and repeated column chromatographer (silica gel 200-300 mesh, chloroform-MeOH) to afford compounds **(3)** (43mg), and **(4)** (26mg).

Cytotoxic activity assay

The fractions and/or chemical entities from *C. jiangxiensis* mycelium were dissolved in DMSO and diluted with RPMI 1640 medium. The fractions were added to the 96-well cell culture plate from corning Inc. (Acton, MA, USA) at the concentrations of 40, 100 and 200μg/mL as a final concentration, likewise the chemical entities were added to the cell culture plate at the final concentration of 10, 100, 1000nmol/L. The maximum concentration of DMSO in the culture medium was 0.5%. For the cytotoxic activity assay, non-adherent (5,000-40000 cells/well) or adherent (5,000 cells/well) tumor cells were cultured in the 96-well culture plate in the presence or absence of the candidate fractions (total volume 200μl/well). Unless otherwise indicated, drug exposure was continuous for 48 h, and then cell growth inhibition was measured by Sulforhodamine B (SRB) colorimetric method as described previously[13]. Briefly, after 48h of drug exposure, the adherent cell cultures were fixed *in situ* by adding 50 μl of cold 50% (w/v) trichloroacetic acid (final concentration 10% TCA), and incubated for 1 h at 4°C (80% TCA for non-adherent cells, and its final concentration 16% TCA). The supernatant was then discarded and the plates were washed five times with demonized water and air-dried at room temperature. 100 μl of SRB solution (0.4% w/v in 1% acetic acid) was added to each well and incubated for 10 min at room temperature. The unbound SRB was removed by washing five times with 1% acetic acid. Then, the plates were air-dried and 150 μl of 10 mM Tris buffer pH 10.5 were added to each well to solubilize the dye bound to basic amino acids of the cell membrane. The plates were shaken gently for 20 minutes on a plate shaker and the optical density of each well was measured at 490 nm using a microplate reader (SAFIRE, Austria). Cell survival and/or cell growth inhibitory rate was measured as the percentage absorbance compared to that of the control (nontreated cells). Cisplatin (25μg/mL) was used as the positive control in this present study.

Statistical analysis

The data were statistically analyzed using the SPSS 13.0 software, and all data shown were means with the corresponding standard error of the mean. When appropriate, statistical significance was tested using a two-tailed Student's *t*-test, and a *p*-value of <0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

Structures identification

Compound 1

White powder, IR (KBr), 3447cm^{-1}, 2956 cm^{-1}, 2918 cm^{-1}, 2850 cm^{-1}, 1738 cm^{-1}, 1640 cm^{-1}, 1549

cm^{-1}, 1511 cm^{-1}, 1196 cm^{-1}. ^{1}H-NMR($CDCl_3$, C400 MHz) δ_H: 5.37(1H, m, 6-H), 4.61(1H, m, 3-H), 1.02(3H, s, CH_3-19), 0.92(3H, s, J = 6.0 Hz, CH_3-21), 0.92(3H, d, J = 6.8 Hz, CH_3-28), 0.83(3H, d, J = 6.4 Hz, CH_3-27), 0.81(3H, d, J = 7.2 Hz, CH_3-26), 0.68(3H, s, CH_3-18). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ_C: 173.37 (COOR), 139.69(C-5), 122.58(C-6), 73.66(C-3), 56.66(C-17), 55.99(C-14), 49.98 (C-9), 45.78(C-24), 42.28(C-13), 39.69(C-12), 38.13(C-1), 36.97 (C-4), 36.57(C-10), 36.14(C-20), 34.71, 33.90 (C-22), 31.88, 31.83 (C-25), 29.70-29.10, 28.23(C-23), 27.78(C-2), 25.99 (C-7), 25.05, 24.27(C-16), 23.02(C-15), 22.70, 21.00(C-11), 19.81(C-21), 19.31(C-27), 19.31 (C-16), 19.00(C-26), 18.76(C-28), 14.13, 11.84(C-18). These data were similar to the basic physical and spectral properties of the campesterol, only the chemical shift of 3-H for compound **(1)** changed from δ_H 3.52 to δ_H 4.61 by comparison of campesterol as described previously[14], furthermore the ^{13}C-NMR spectrum of compound **(1)** still presented one ester carbonyl group at δ_C 173.37, one terminal methyl group at δ_C 14.13, together with a number of saturated methylenes at δ_C 29.70-29.10, these physical and spectral features outlined above therefore suggested that compound **(1)** was a saturated long-chain fatty acid ester of campesterol. In addition, compound **(1)** was hydrolyzed by 10 % HCl-methanol and analyzed by GC-MS, both campesterol and docosanoic acid were simultaneously observed. On the basis of evidences mentioned above, the structure of compound **(1)** was assigned as docosanoic acid campesterol-ester.

Compound 2

White powder, ^{1}H-NMR($CDCl_3$, 400 MHz) δ_H: 5.57(1H, m, 6-H), 5.38(1H, m, H-7), 5.20(2H, m, H-22,23), 4.72(1H, m, 3-H), 1.04(3H, d, *J* = 6.4 Hz, CH_3-21), 0.96(3H, s, CH_3-19), 0.92(3H, d, *J* = 6.8 Hz, CH_3-28), 0.84(3H, d, *J* = 6.4 Hz, CH_3-27), 0.81(3H, d, J=7.2Hz, CH_3-26), 0.63(3H, s, CH_3-18). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ_C: 173.34(COOR), 141.48 (C-8), 138.62(C-5), 135.56(C-22), 131.94(C-23), 120.12(C-6), 116.29(C-7), 72.45(C-3), 55.67(C-17), 54.50(C-14), 46.01(C-9), 42.79(C-13), 42.79(C-24), 40.45(C-20), 39.00(C-12), 37.91 (C-1), 37.08(C-10), 36.67 (C-4), 34.69, 33.07(C-25), 31.93, 29.70-29.10, 28.29(C-16), 28.13(C-2), 25.05, 22.98(C-15), 22.70, 21.09(C-21), 21.00(C-11), 19.95(C-27), 19.64(C-26), 17.60(C-28), 16.25(C-19), 14.13, 12.07(C-18). The main physical and spectral data of compound **(2)** were similar to that of ergosterol as described previously[5,15], but compound **(2)** had a different chemical shift with δ_H 4.72 at 3-H. As compared with the ^{13}C-NMR spectrum of ergosterol, compound **(2)** still displayed an ester carbonyl carbon at δ_C 173.34, one terminal methyl carbon at δ_C 14.13, together with a number of saturated methylene carbons at δ_C 31.93, 29.70-29.10, 25.05, and 22.70. The above-mentioned evidences and spectral data suggested that compound **(2)** was a saturated long-chain fatty acid ester of ergosterol. Compound **(2)** was further hydrolyzed by 10% HCl-methanol and analyzed by GC-MS, both ergosterol and hexadecanoic acid were simultaneously observed. Based on the mentioned above, the structure of compound **(2)** was identified as hexadecanoic acid ergosterol-ester.

Compound 3

Colorless needles, mp:124~126°C(acetone), EI-MS *m/z*: 396[M]$^+$, 363, 337, 271, 253, 211, 69. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ_H: 5.57(1H, m, H-6), 5.38(1H, m, H-7), 5.20(2H, m, H-22,23), 3.63(1H, m, H-3), 1.03(3H, d, J=6.4Hz, CH_3-21), 0.95(3H, s, CH_3-19), 0.92(3H, d, J=6.8Hz, CH_3-28), 0.84(3H, d, J=6.4Hz, CH_3-27), 0.81(3H, d, J=7.2Hz, CH_3-26), 0.63(3H, s, CH_3-18). ^{13}C-NMR($CDCl_3$, 100 MHz) δ_C: 141.37 (C-8), 139.75(C-5), 135.54(C-22), 131.92(C-20), 119.56(C-6), 116.24(C-7), 70.43(C-3), 55.67(C-17), 54.52(C-14), 46.19 (C-9), 42.79(C-13), 42.79(C-24), 40.74(C-4), 40.44(C-20), 39.03 (C-12), 38.33(C-1), 37.0(C-10), 33.06(C-25), 31.94(C-2), 28.29(C-16), 22.97(C-15), 21.08(C-11), 21.08(C-21), 19.94(C-27), 19.63(C-26), 16.25(C-19), 17.58(C-28), 12.07(C-18). On the basis of the above-mentioned data obtained, compound **(3)** was characterized as (22*E*, 24*R*)-ergosta -5,7,22-trien-3β-ol (ergosterol) by comparison of the physical and spectral data with literatures[5,15].

Compound 4

Colorless needles, mp:137~140°C. ^{1}H-NMR($CDCl_3$, 400 MHz) δ_H: 5.20(2H, m, H-22,23), 4.68(1H, m, H-7), 3.63(1H, m, H-3), 1.03(3H, d,

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J=6.4Hz, CH_3-21), 0.95(3H, s, CH_3-19), 0.92(3H, d, J=6.8Hz, CH_3-28), 0.84(3H, d, J=6.4Hz, CH_3-27), 0.81(3H, d, J=7.2Hz, CH_3-26), 0.63(3H, s, CH_3-18). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ_C: 139.75(C-8), 135.65(C-22), 131.83(C-23), 117.4(C-7), 71.05(C-3), 55.89(C-17), 55.07(C-14), 49.39(C-9), 43.1(C-13), 42.79(C-24), 40.44(C-20), 40.21(C- 5), 39.41(C-12), 37.91(C-4), 37.10(C-1), 34.20(C-10), 33.06(C-25), 31.42(C-2), 29.59(C-6), 28.11(C-16), 22.90(C-15), 21.50(C-11), 21.08(C-21), 19.94(C-27), 19.63(C-26), 17.58(C-28), 13.04(C-19), 12.03(C-18). On the basis of the above-mentioned data obtained, compound **(4)** was identified as 5*α*-Ergosta-7,22-dien-3*β*-ol by comparison of the physical and spectral data with literature[16,17].

Cytotoxic activity

The chloroform fraction from the methanol extract of *C. jiangxiensis* mycelium showed potent antitumor activity[8], therefore it was further separated using the bioassay-guided fractionation as a major active fraction in this present study. As shown in TABLE 1, the results of cytotoxic effects indicated that only six fractions such as Fr.2, Fr.4, Fr.6, Fr.8, Fr.12, and Fr.13 had growth inhibitory activities on human tumor cells, especially Fr.2 at different concentrations ranging from 40μg/mL to 200μg/mL all exhibited the same extent inhibition capability versus first-line antitumor drug cisplatin at the concentration of 25μg/ml. These finding suggested that Fr. 2 was a considerable antitumor fractions for isolating potential new antitumor drug candidate. In addition, compound **(1)** and **(2)** had no cytotoxic effects on human tumor cells tested in this study due to their poor solubility (data not shown), while compound **(3)** show potent cytotoxic activity on tumor cell as reported previously[18].

TABLE 1 : Inhibitory effect of various fractions isolated from *Cordyceps jiangxiensis* mycelium on the growth of human tumor cells

Samples	Inhibitory rate (%)					
	Human SGC-7901 tumor cell			Human A_{549} tumor cell		
	40μg/mL	100μg/mL	200μg/mL	40μg/mL	100μg/mL	200μg/mL
Fr.2	94.11 ± 0.68	99.80 ± 1.90	99.89 ± 2.44	87.74 ± 2.78	97.93 ± 0.40	95.79 ± 3.74
Fr.4	12.59 ± 8.34	39.15 ± 3.90	80.97 ± 3.62	17.42 ± 5.59	35.07 ± 6.53	78.60 ± 2.48
Fr.6	30.85 ± 18.09	1.91 ± 1.51	22.42 ± 8.11	2.25 ± 3.58	2.84 ± 1.91	22.11 ± 1.90
Fr.8	31.29 ± 3.15	77.67 ± 11.02	99.87 ± 3.93	48.38 ± 14.18	73.07 ± 39.7	91.28 ± 2.09
Fr.12	13.29 ± 5.71	27.12 ± 3.02	59.92 ± 1.10	12.77 ± 5.82	27.02 ± 1.42	61.66 ± 1.92
Fr.13	61.74 ± 4.54	30.89 ± 0.70	70.06 ± 3.53	3.75 ± 8.80	25.82 ± 1.85	61.26 ± 6.42
Cisplatin (25μg/mL)	96.95 ± 8.30			90.38 ± 9.62		

CONCLUSIONS

Cordyceps fungi are an unparalleled source for new drug discovery and development due to their diversity of secondary metabolites. In this present study, steroids were isolated as potential target compounds from *C. jiangxiensis* mycelium with antitumor activity by bioassay-guided fractionations. Fr.2 from the chloroform fraction of methanol extract of *C. jiangxiensis* mycelium showed potent cytotoxicity on various human tumor cells at the tested concentration range. Four steroids including docosanoic acid campesterol-ester **(1)**, hexadecanoic acid ergosterol-ester **(2)**, ergosterol **(3)**, 5*α*-Ergosta-7,22-dien-3*β*-ol **(4)** were isolated and identified on the basis of the physical and spectral data. Among these compounds, compounds **(1)** and **(2)** were two new ergosteryl esters of long-chain alkanes and compound **(4)** was obtained from the genus *Cordyceps* for the first time.

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