ELSEVIER

Steroids 66 (2001) 771–775

Steroids

A novel sterol from Chinese truffles *Tuber indicum*

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Received 19 October 2000; received in revised form 8 January 2001; accepted 19 January 2001

Abstract

From the fruiting bodies of Ascomycetes *Tuber indicum*, a new steroidal glucoside with polyhydroxy ergosterol nucleus, tuberoside (**2**), has been isolated along with additional four known ergosterol derivatives, (22*E*, 24*R*)-ergosta-7, 22-dien-3β, 5α, 6β-triol (**1**), 5α, 8α-epidioxy-(22*E*, 24*R*)-ergosta-6, 22-dien-3β-ol (**3**), (22*E*, 24*R*)-ergosta-5, 22-dien-3β-ol (**4**), and (22*E*, 24*R*)-ergosta-4, 6, 8(14), 22-tetraen-3-one (**5**). The structure of new compound was established as 3-O-β-D-glucopyranosyl-(22*E*, 24*R*)-ergosta-7, 22-dien-5α, 6β-diol (**2**) on the basis of chemical and spectroscopic means (^{1}H NMR, ^{13}C NMR, HMQC, HMBC, MS, and IR). This is the first example of isolation of a polyhydroxylated ergosterol glucoside from higher fungi in nature.

Keywords: Chinese truffles; *Tuber indicum*; Ascomycetes; Tuberoside; Sterol; 3-O-β-D-Glucopyranosyl-(22*E*, 24*R*)-ergosta-7, 22-dien-5α, 6β-diol

1. Introduction

Truffles, also known as the 'black diamonds', are underground mushrooms which grow in symbiosis with certain trees. They are thought to be a 'miracle of nature' and have been, since ancient times, the ultimate in gastronomy. There are more than a hundred different kinds of truffles, but only a few have a gastronomic interest, some are not even edible. The Chinese truffle *Tuber indicum* Cooke et Massee., a generally hypogeous fungus belonging to the family Tuberaceae, is distributed in the provinces of Yunnan and Sichuan of China [1]. This truffle looks a lot like the black winter truffle *Tuber melanosporum* in Europe. Its skin is reddish before becoming brow-black when mature. The flesh is white veins and has an elasticized consistency [1]. Since its significant commercial value and as part of a search for naturally occuring bioactive metabolites of the higher fungi in Yunnan province, the chemical composition of *Tuber indicum* was investigated. From the fruiting bodies of *T. indicum*, a new polyhydroxy sterol glycoside named tuberoside (**2**) was isolated, together with four known ergostane compounds, (22*E*, 24*R*)-ergosta-7, 22-dien-3β, 5α, 6β-triol (**1**), 5α, 8α-epidioxy-(22*E*, 24*R*)-ergosta-6, 22-dien-3β-ol (**3**), (22*E*, 24*R*)-ergosta-5, 22-dien-3β-ol (**4**) (22*E*, 24*R*)-ergosta-4, 6, 8(14), 22-tetraen-3-one (5). Of these five steroids, **4** was found to be a major component of this fungus. In this paper, the isolation and structural elucidation of a new glycoside, 3-O-β-D-glucopyranosyl-(22*E*, 24*R*)-ergosta-7, 22-dien-5α,6β-diol (**2**) is reported.

2. Experimental

2.1. General methods

Melting points were obtained on an XRC-1 apparatus and are uncorrected. Optical rotations were measured on a Horiba SEPA-300 polarimeter. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AM-400 spectrometer, the chemical shifts δ are given in ppm relative to TMS as a internal standard and coupling constants are given in Hz. The multiplicity of ^{13}C NMR was determined as DEPT. Two-dimensional (2D) NMR programs were used for recording the 2D spectra in Bruker DRX-500. High-resolution electron impact mass spectra (HREIMS) and fast-atom bombardment mass spectrometry (FAB-MS) were recorded on a VG AutoSpec 3000 mass spectrometer. Infrared (IR) spectra were obtained with a

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0039-128X/01/$ – see front matter
PII: S0039-128X(01)00105-2

Bio-Rad FTS-135 infrared spectrophotometer with KBr pellets.

Column chromatography was performed over silica gel (200–300 and 230–400 mesh), Merck, LiChroprep RP-8 gel (40–63 μm) and Sephadex LH-20 gel (25–100, Pharmacia). Thin layer chromatography (TLC) was carried out on plates precoated with Merck RP-18 and silical gel F_{254} (Qingdao Marine Chemical Ltd., People's Republic of China).

2.2. Extraction and isolation

The dried fruiting bodies of *T. indicum* were purchased in Yunnan Province in April 2000 and identified by Profs. P.G. Liu, X.H. Wang, Kunming Institute of Botany, the Chinese Academy of Sciences, Kunming, Yunnan, People's Republic of China. A voucher specimen is deposited at the Herbarium of Kunming Institute of Botany.

The dried and powdered fruiting bodies (4.7 kg) were extracted successively three times with $CHCl_3$ and four times with $CHCl_3$/MeOH (1:1) at room temperature, which were concentrated to dryness in vacuo, respectively, to give $CHCl_3$ extracts (154 g) and $CHCl_3$/MeOH extracts (122 g). The latter were chromatographed over silica gel using $CHCl_3$ and increasing concentrations of MeOH in $CHCl_3$ as eluents. The fractions (3.5 g) eluted with $CHCl_3$/MeOH (95:5) were subjected to silica gel column chromatography using $CHCl_3$/MeOH (9:1) to yield a residue (139 mg), which was rechromatographed on silica gel with cyclohexane/acetone (7:3) to furnish pure compound **1** (32 mg). The fractions (3.1 g) eluted with $CHCl_3$/MeOH (9:1) were chromatographed over RP-8 column using MeOH/H_2O (90:10) to produce a residue (93.8 mg), followed by gel permeation through Sephadex LH-20 in MeOH/H_2O (8:2) to afford pure compound **2** (46.7 mg). The $CHCl_3$ extracts were passed through vacuum liquid chromatography (VLC) over silica gel with a petroleum ether/ether mixture containing increasing amount of ether as eluents to yield ten fractions. Fraction 2 afforded compound **5** (9 mg). Fraction 8 gave compound **3** (36 mg). The fractions eluted with petroleum ether /ether (30:70, v/v) were rechromatographed over silica gel with *n*-hexane/ethyl acetate (80:20) followed by crystallization from ethyl acetate to afford compound **4** (2 g).

2.2.1. (22E, 24R)-ergosta-7, 22-dien-3β, 5α, 6β-triol (= cerevisterol) (*1*)

Colorless needles, m.p. 224–227°C (cyclohexane/acetone); $[\alpha]_D^{24} = -22.62°$ (c = 0.21, MeOH); IR (KBr) *v*: 3412, 2951, 2865, 1660, 1456, 1381, 1032, 969 cm^{-1}; EI-MS (70 eV) *m/z* (relative intensities): 430 (M^+, 6.5), 412 (M^+-H_2O, 100), 394 (M^+-2H_2O, 70), 379 (M^+-2H_2O-Me, 65.5), 376 (M^+-3H_2O, 15), 285 [M^+-3H_2O-side chain (C_9H_{17}), 11], 269 (M^+-2H_2O-C_9H_{17}, 34), 251 (M^+-3H_2O-C_9H_{17}, 39), 107(15), 81(28), 69(44); ^{1}H NMR (400 MHz, C_5D_5N) δ: 5.73 (1H, dd, J = 4.8, 2.4 Hz, H-7), 5.24 (1H, dd, J = 15.4, 7.3 Hz, H-23), 5.19 (1H, dd, J = 15.4, 7.8 Hz, H-22), 4.82 (1H, m, H-3), 4.31 (1H, br s, H-6), 0.67 (3H, s, H_3-18), 1.53 (3H, s, H_3-19), 1.07 (3H, d, J = 6.5 Hz, H_3-21), 0.96 (3H, d, J = 6.8 Hz, H_3-28), 0.87 (3H, d, J = 6.6 Hz, H_3-27), 0.86 (3H, d, J = 6.6 Hz, H_3-26); ^{13}C NMR, see Table 1.

Table 1. ^{13}C NMR (DEPT) chemical shifts for **1**, **2** and **2a***

Position	**1** δc	**2** δc	**2a** δc
1	32.59 (CH_2)	33.56 (CH_2)	32.39 (CH_2)
2	33.83 (CH_2)	30.10 (CH_2)	29.69 (CH_2)
3	67.59 (CH)	75.60 (CH)	72.94 (CH)
4	41.90 (CH_2)	38.09 (CH_2)	39.19 (CH_2)
5	76.16 (C)	75.92 (C)	75.18 (C)
6	74.27 (CH)	74.24 (CH)	73.26 (CH)
7	120.4 (CH)	120.3 (CH)	114.0 (CH)
8	141.6 (C)	141.4 (C)	145.8 (C)
9	43.80 (CH)	43.66 (CH)	43.45 (CH)
10	38.09 (C)	38.09 (C)	37.34 (C)
11	22.44 (CH_2)	22.35 (CH_2)	22.07 (CH_2)
12	39.95 (CH_2)	39.88 (CH_2)	39.19 (CH_2)
13	43.80 (C)	43.77 (C)	43.89 (C)
14	55.26 (CH)	55.23 (CH)	54.91 (CH)
15	23.48 (CH_2)	23.46 (CH_2)	22.78 (CH_2)
16	28.42 (CH_2)	28.41 (CH_2)	27.82 (CH_2)
17	56.29 (CH)	56.25 (CH)	56.00 (CH)
18	12.54 (CH_3)	12.50 (CH_3)	12.32 (CH_3)
19	18.76 (CH_3)	18.49 (CH_3)	18.25 (CH_3)
20	40.75 (CH)	40.74 (CH)	40.31 (CH)
21	20.14 (CH_3)	20.12 (CH_3)	19.93 (CH_3)
22	136.2 (CH)	136.2 (CH)	135.3 (CH)
23	132.2 (CH)	132.2 (CH)	132.2 (CH)
24	43.11 (CH)	43.08 (CH)	42.84 (CH)
25	33.38 (CH)	33.35 (CH)	33.06 (CH)
26	20.67 (CH_3)	19.83 (CH_3)	19.63 (CH_3)
27	19.85 (CH_3)	17.81 (CH_3)	19.14 (CH_3)
28	17.85 (CH_3)	17.46 (CH_3)	17.60 (CH_3)
1′		102.6 (CH)	100.6 (CH)
2′		75.36 (CH)	71.50 (CH)
3′		78.62 (CH)	71.77 (CH)
4′		71.77 (CH)	68.55 (CH)
5′		78.13 (CH)	71.64 (CH)
6′		62.87 (CH_2)	62.12 (CH)
5 × $COCH_3$			170.6, 170.2 × 2 169.4 × 2
5 × $COCH_3$			21.26, 21.10, 20.73 20.58 × 2

* ^{13}C NMR spectra at 100 MHz were measured in C_5D_5N except for compound **2a** ($CDCl_3$); assignment on the basis of ^{1}H-^{1}H and ^{13}C-^{1}H correlated 2D spectroscopy (COSY) and DEPT.

2.2.2. 3-O-β-D-glucopyranosyl-(22E, 24R)-ergosta-7, 22-dien-5α, 6β-diol (*2*)

White amorphous powders, m.p. 245–247°C (MeOH); $[\alpha]_D^{26} = -95.52°$ (c = 0.22, pyridine); HR-EIMS *m/z*: 592.3985 (M^+, $C_{34}H_{56}O_8$; requires 592.3975); EI-MS (70 eV) *m/z* (relative intensities): 592 (M^+, 0.1), 574 (M^+-H_2O, 1.2), 412 [M^+-H_2O-hexose ($C_6H_{10}O_5$), 55], 394 (M^+-

$2H_2O$- $C_6H_{10}O_5$, 42.5), 376 (M^+-$3H_2O$-$C_6H_{10}O_5$, 38), 379 (M^+-$2H_2O$-$C_6H_{10}O_5$-Me, 28), 305 [M^+-C_9H_{17}(side chain)-$C_6H_{10}O_5$, 1], 269 (M^+-$2H_2O$-C_9H_{17}-$C_6H_{10}O_5$, 19), 285 (M^+-H_2O-2H-$C_6H_{10}O_5$-C_9H_{17}, 6), 251 (M^+-$3H_2O$-$C_6H_{10}O_5$-C_9H_{17}, 40), 109 (24), 95 (30), 81 (48), 69 (100); negative FABMS *m/z* 591 (M-1). IR (KBr) *v*: 3404 (OH), 2952, 2868, 1635, 1455, 1371, 1158, 1101, 1075, 1037 (C-O), 968 cm^{-1}; ^1H NMR (400 MHz, C_5D_5N) δ 5.73 (1H, dd, J = 4.8, 2.4 Hz, 7-H), 4.93 (1H, m, 3-H), 4.39 (1H, d, J = 4.8 Hz, 6-H), 2.88 (1H, dd, J = 10.2, 9.5 Hz, H_{ax}-4), 0.65 (3H, s, 18-H_3), 1.41 (3H, s, 19-H_3), 0.94 (3H, d, J = 6.8 Hz, 21-H_3), 5.18 (1H, dd, J = 14.9, 7.2 Hz, 22-H_3), 5.22 (1H, dd, J = 14.9, 8.1 Hz, 23-H_3), 0.84 (3H, d, J = 6.7 Hz, 26-H_3), 0.85 (3H, d, J = 6.7 Hz, 27-H_3), 1.04 (3H, d, J = 6.5 Hz, 28-H_3), 4.96 (1H, d, J = 7.6 Hz, 1′-H), 4.05 (1H, t, J = 8.0 Hz, 2′-H), 4.17 (1H, t, J = 8.9 Hz, 3′-H), 4.28 (1H, t, J = 9.1 Hz, 4′-H), 3.73 (1H, m, 5′-H), 4.46 (1H, dd, J = 11.8, 4.8 Hz, 6′-H_a), 4.38 (1H, dd, J = 11.8, 2.1 Hz, 6′-H_b); ^{13}C NMR see Table 1.

2.2.3. Acetylation of compound **2**

To a solution of **2** (6.3 mg) in pyridine (0.2 ml) was added Ac_2O (0.2 ml) and the reaction mixture was left standing overnight at room temperature. After usual work-up, the resulting residue was purified by column chromatography over silica gel with *n*-hexane/ethyl acetate (3:2) as eluent to give the pentacetate (**2a**) of compound **2** (9.3 mg) as a colorless crystal. $[\alpha]_D^{24} = -39.33°$ (c = 1.51, $CHCl_3$); IR(KBr) *v*: 3508, 2864, 1742, 1457, 1375, 1239, 1210, 1046, 976 cm^{-1}; EI-MS *m/z* (relative intensities): 802 (M^+, 0.1), 784 (M^+-H_2O, 3.5), 742 (M^+-H_2O-$COCH_3$+H, 44), 724 (M^+-H_2O-HOAc, 19.8), 453 (M^+-H_2O-331, 1.5), 436 (19.2), 411 (M^+-H_2O-$COCH_3$-glc$(OAc)_4$, 8), 394 (64.8), 376 (86.5), 331 (glc$(OAc)_4^+$, 82), 271 (11.5), 251 (55.5), 229 (8.8), 211 (20), 169 (100), 109 (78.5), 69 (90.8); negative FABMS *m/z*: 801 $(M-1)^-$, 783 [$(M-1)^-$-H_2O, 5], 758 [$(M-1)^-$-$COCH_3$, 8]; 429 [$(M-1)^-$-glc$(OAc)_4$-$COCH_3$+H, 7]; ^1H NMR (400 MHz, $CDCl_3$) δ: 5.22–5.27 (3H, m, 7-H, 22-H, and 23-H), 4.83–5.18 (4H, m, 2′-H, 3′-H, 4′-H, and 5′-H), 4.58 (1H, d, J = 6.4 Hz, 1′-H), 4.26 (1H, dd, J = 9.8 and 3.9 Hz, 6′-H), 4.12 (1H, dd, J = 9.8 and 2.0 Hz, 6′-H), 4.09 (1H, d, J = 5.4 Hz, 6-H), 3.98 (1H, m, 3-H), 0.58 (3H, s, 18-H_3), 1.03 (3H, s, 19-H_3), 0.99 (3H, d, J = 5.4 Hz, 21-H_3), 0.91 (3H, d, J = 5.4 Hz, 26-H_3), 0.85 (3H, d, J = 6.8 Hz, 27-H_3), 0.82 (3H, d, J = 6.4 Hz, 28-H_3), 1.99, 2.02, 2.07 (each 3H, s, 3 × $COCH_3$), 2.03 (6H, s, 2 × $COCH_3$); ^{13}C NMR see Table 1.

2.2.4. Acid hydrolysis of compound **2**

A solution of **2** (6.2 mg) in MeOH (3 ml) was treated with 0.1 mol/l H_2SO_4 (1 ml) at 60°C for 50 min, 2 ml of H_2O was added and the whole was concentrated to 3 ml and then extracted with EtOAc. The organic phase was evaporated in vacuo to give a residue which was purified by silica gel column chromatography with $CHCl_3$/MeOH (19:1-9:1) to afford an aglycone identified as (22*E*, 24*R*)-ergosta-7, 22-dien-3β, 5α, 6β-triol (**1**) by EIMS, ^1H NMR (in pyridine-d_5), and TLC comparison with authentic sample. The aqueous layer was neutralized with saturated $Ba(OH)_2$. The precipitate was filtered off and the filtrate was evaporated to a syrup which was then chromatographed on silica gel using $CHCl_3$/MeOH/H_2O (6:4:0.5) to give methyl-β-D-glucopyranoside which was identified by EIMS *m/z* 194 (M^+), and TLC comparison with authentic sample.

2.2.5. 5α, 8α-Epidioxy-(22E, 24R)-ergosta-6, 22-dien-3β-ol (*3*)

Colorless crystals, m.p. 182–184°C (*n*-hexane); $[\alpha]_D^{23} = -34°$ (c = 0.6, $CHCl_3$); IR (KBr) *v*: 3525, 3309, 2957, 2873, 1653, 1459, 1377, 1046, 1029, 985, 970, 858 cm^{-1}; EI-MS (70 eV) *m/z* (relative intensities): 428 (M^+, 5), 410 (4), 396 (100), 363 (35), 271 (7), 251 (14), 152 (30), 107 (22), 81 (43), 69 (63); ^{13}C NMR (100 MHz, $CDCl_3$) δ 34.7 (C-1), 30.2 (C-2), 66.5 (C-3), 37.0 (C-4), 82.1 (C-5), 135.4 (C-6), 130.8 (C-7), 79.4 (C-8), 51.2 (C-9), 37.0 (C-10), 23.4 (C-11), 39.4 (C-12), 44.6 (C-13), 51.7 (C-14), 20.6 (C-15), 28.6 (C-16), 56.3 (C-17), 12.9 (C-18), 18.2 (C-19), 39.7 (C-20), 20.9 (C-21), 135.2 (C-22), 132.4 (C-23), 42.8 (C-24), 33.1 (C-25), 19.9 (C-26), 19.6 (C-27), 17.6 (C-28); ^1H NMR (400 MHz, $CDCl_3$) δ 3.94 (1H, m, H-3), 6.22 (1H, d, J = 8.5 Hz, H-6), 6.48 (1H, d, J = 8.5 Hz, H-7), 0.86 (3H, s, H_3-18), 1.06 (3H, s, H_3-19), 0.97 (3H, d, J = 6.6 Hz, H_3-21), 5.11 (1H, dd, J = 15.3, 8.0 Hz, H-22), 5.19 (1H, dd, J = 15.1, 7.5 Hz, H-23), 0.83 (3H, d, J = 5.0 Hz, H_3-26), 0.82 (3H, d, J = 5.0 Hz, H_3-27), 0.89 (3H, d, J = 5.3 Hz, H_3-28).

2.2.6. Brassicasterol = (22E, 24R)-Ergosta-5, 22-dien-3β-ol (*4*)

Colorless needles, m.p. 154–156°C (EtOAc); $[\alpha]_D^{24.5} = -65.7°$ (c = 0.37, $CHCl_3$); EI-MS (70 eV) *m/z* (relative intensities): 398 (M^+, 100), 383 (M^+-Me, 13), 380 (M^+-H_2O, 10), 365 (M^+-H_2O-Me, 9.5), 337 (M-C_3H_9O, 18), 300 (61), 271 (M^+-C_9H_{17}-2H, 57.5), 255 (M^+-C_9H_{17}-H_2O, 73.5), 159 (45), 125 ($C_9H_{17}^+$, 30), 105 (50), 91 (54), 81 (70), 69 (96), 55 (100); IR (KBr) *v*: 3427, 2948, 2862, 1665, 1458, 1382, 1048, 968 cm^{-1}; ^{13}C NMR (100 MHz, $CDCl_3$): 37.54 (1-C), 31.95 (2-C), 72.04 (3-C), 42.58 (4-C), 141.0 (5-C), 121.9 (6-C), 32.16 (7-C), 33.34 (8-C), 50.51 (9-C), 36.78 (10-C), 21.32 (11-C), 39.96 (12-C), 42.58 (13-C), 57.12 (14-C), 24.51 (15-C), 28.68 (16-C), 56.36 (17-C), 12.30 (18-C), 20.12 (19-C), 40.26 (20-C), 21.18 (21-C), 136.1 (22-C), 132.0 (23-C), 43.06 (24-C), 33.34 (25-C), 19.84 (26-C), 19.58 (27-C), 17.80 (28-C); ^1H NMR (400 MHz, $CDCl_3$): 3.53 (1H, m, 3-H), 5.35 (1H, d, J = 5.2 Hz, 6-H), 0.69 (3H, s, 18-H_3), 0.83 (3H, s, 19-H_3), 1.00 (3H, d, J = 6.5 Hz, 21-H_3), 5.17–5.20 (2H, m, 22-H and 23-H), 0.84 (3H, d, J = 6.5 Hz, 26-H_3), 0.91 (3H, d, J = 6.8 Hz, 27-H_3), 1.01 (3H, d, J = 6.5 Hz, 28-H_3).

2.2.7. (22E, 24R)-Ergosta-4, 6, 8(14), 22-tetraen-3-one (*5*)

Yellow crystals, m.p. 112–114°C; IR (KBr) *v*: 2982, 1675 (C=O), 1590 (C=C), 1270, 1223, 965 cm^{-1}; EI-MS

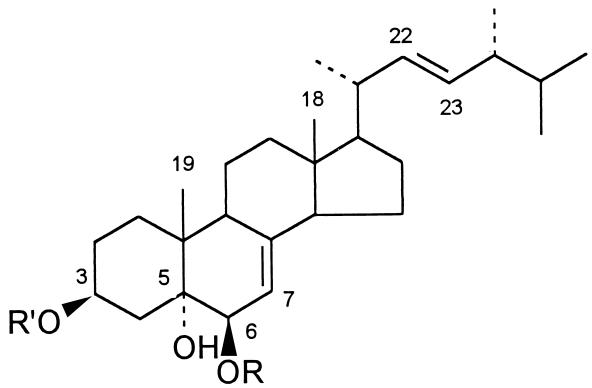
(70 eV) *m/z* (relative intensities): 392 (M^+, 19.5), 377 (M^+-Me, 1.5) 349 (1.5), 268 (42), 253 (6), 240 (3.5), 214 (7.5), 173 (7), 129 (6); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 34.09 (C-1), 18.95 (C-2), 199.2 (C-3), 122.9 (C-4), 164.3 (C-5), 124.4 (C-6), 133.9 (C-7), 124.4 (C-8), 44.28 (C-9), 36.71 (C-10), 25.33 (C-11), 34.09 (C-12), 43.95 (C-13), 156.04) (C-14), 35.55 (C-15), 27.69 (C-16), 55.66 (C-17), 18.95 (C-18), 16.62 (C-19), 39.25 (C-20), 21.19 (C-21), 134.9 (C-22), 132.5 (C-23), 42.83 (C-24), 33.05 (C-25), 19.63 (C-26), 19.96 (C-27), 17.61 (C-28); ^1H NMR (400 MHz, $CDCl_3$) δ: 0.79∼0.83 (2 × 3H, dd, J = 6.7 Hz, 26-H_3 and 27-H_3), 0.9 (3H, d, J = 6.9 Hz, 28-H_3), 0.93 (3H, s, 18-H_3), 0.97 (3H, s, 19-H_3), 1.03 (3H, d, J = 6.6 Hz, 21-H_3), 1.23–2.51 (18H, m, steroid necleus), 5.14–5.26 (2H, m, 22- and 23-H), 5.70 (1H, s, 4-H), 6.00 (1H, d, J = 9.5 Hz, 6-H), 6.57 (1H, d, J = 9.5 Hz, 7-H).

3. Results and discussion

Compound **1**, $C_{28}H_{46}O_3$, showed the presence of two tertiary methyls, four secondary methyls, two methine protons attached to two oxygen bearing carbons and three protons attached to two double bonds in the ^1H NMR spectrum. Its ^{13}C NMR spectrum contained 28 peaks (Table 1). In the mass spectrum of **1**, fragment ion peaks were very similar to those of an ergesterol derivative [2,3]. These facts indicated that compound **1** was (22*E*, 24*R*)-ergosta-7, 22-dien-3β, 5α, 6β-triol, confirmed by direct comparison with literature data [2–5].

Compound **2**, obtained as an amorphous powder, gave a quasi-molecular ion peak at *m/z* 591$[M-1]^-$ in the negative fast-atom bombardment mass spectrometry (FAB-MS). The high-resolution electron impact mass spectra (HREIMS) of **1** indicated a molecular formula $C_{34}H_{56}O_8$ which was derived from the molecular ion peak at *m/z* 592.3895 (calc., 592.3975) and confirmed by the ^{13}C-NMR spectrum. In the IR spectrum, it showed strong absorption of hydroxyl groups at 3400, 1075, and 1037 cm^{-1}. The ^1H-NMR spectrum of **2** showed the presence of two tertiary methyl groups [δ 0.65 (3H, s, H-18), 1.41 (3H, s, H-19)], a 24-methyl-Δ^{22}-sterol side chain [δ 0.84 (3H, d, H-26), 0.85 (3H, d, H-27), 1.04 (3H, d, H-28), 0.94 (3H, d, H-21), 5.18 (1H, H-22), 5.22 (1H, H-23)], two oxygenated methine protons [δ 4.39 (1H, H-6), 4.91 (1H, H-3)], a trisubstituted olefinic proton [δ 5.73 (1H, H-7)] and a glycoside moiety. The ^{13}C NMR (DEPT) spectrum (Table 1) of **2** contained thirty four signals (C × 4, CH × 16, CH_2 × 8, CH_3 × 6) that included a glucose moiety [δ 62.87 (CH_2), 71.77 (CH), 75.36 (CH), 78.13 (CH), 78.62 (CH) and 102.7 (C)]. The remaining signals were very similar to those of compound **1** except the signals at δ 30.1 (CH_2), 75.6 (CH) and 38.1 (CH_2). In addition, from the heteronuclear multiple bond coherence (HMBC) spectrum, the long-range coupling was observed between H-1′ [δ 4.96 (1H, d)] and C-3 [δ 75.60 (CH)]. This suggested that a glucose was attached to the C-3 position of aglycone, and the ^{13}C NMR differences between a downfield shift of the signal at δ 75.6 (C-3, +8.0 ppm) and upfield shifts of signals at δ 30.10 (C-2, −3.7 ppm) and 38.09 (C-4, −3.9 ppm) from those of aglycone **2** were explained by glycosylation shifts on C-3 [6,7]. The electron impact mass spectrum (EIMS) displayed the characteristic fragment ions at m/z 287 (M^+-H_2O-glucose-C_9H_{17}), 269 (M-2H_2O-glucose-C_9H_{17}), and 251 (M-3H_2O-glucose-C_9H_{17}), indicating the presence of a C_9H_{17} side chain containing a double bond, a glucose, and hydroxyl groups located at sterol nucleus in **2**. The correlation between δ 4.39 (1H, H-6) and δ 5.73 (1H, H-7) in ^1H-^1H COSY spectrum of **2** indicated that the olefinic proton is located next to a hydroxyl, the latter being adjacent to a quaternary carbon. The multiplet centered around 4.93 ppm had a complexity normally seen for a 3α-carbinol proton, and unusually high chemical shift suggested the additional tertiary alcohol was located at C-5 [2]. These data were suggestive of a Δ^7-3,5,6-triol structure or, alternatively, a $\Delta^{9(11)}$-3,5,12-triol structure. It was identified as Δ^7-3β,5α,6β-trihydroxysterol on the basis of the H-3, H-4, H-6, H-7, H-18 and H-19 chemical shift value which are typical of this nucleus, in addition, the signals of H-3α, H_{ax}-4 and H-19 showed the typical pyridine-d_5-induced deshielding due to the 1,3-diaxial interaction with C-5 and C-6 hydroxyl groups [4,8]. By examination of HMBC spectrum of **2** the correlation between H-7 and C-5, C-9, C-14 also permits rigorous exclusion of an alternative structure. The stereochemistry of the side chain was determined by comparison of the ^1H and ^{13}C NMR data of **2** with those of (22*E*, 24*R*)-methyl-Δ^{22}-sterol side chain [8,9]. The presence of glucose and (22*E*, 24*R*)-ergosta-7, 22-dien-3β, 5α, 6β-triol was further confirmed by TLC of the acid hydrolysis products of **2**. In the ^1H NMR spectrum, the coupling constant (d, J = 7.6 Hz, H-1′) of the anomeric proton at δ 4.96 suggested the glucose to be a β-anomer. Six distinct ^{13}C NMR signals at δ 62.87 (CH_2), 71.77 (CH), 75.36 (CH), 78.13 (CH), 78.62 (CH), and 102.7 (quaternary carbon) were assigned to glucose, also indicating that the sugar moiety in **1** was in the β-configuration and pyranose form [6,7]. Comparison of the ^{13}C NMR signals attributed to the glucosyl moiety of **1** with published data for model compound [6] confirmed the identity of β-D-glucose.

Compound **2** on acetylation with Ac_2O/pyridine gave a pentacetyl derivative (**2a**) that showed a molecular ion peak at *m/z* 801$(M-1)^-$ in its negative-ion FABMS, consistent with the composition $C_{44}H_{66}O_{13}$ for **2a**. The EIMS of **2a** also gave the diagnostic fragments of the sugar moiety at *m/z* 331, 271, 229, 211, 169 (base peak), and 109, due to an acetylated glucopyranoside. The presence of fragment ion peaks at *m/z* 784 (M^+-H_2O), 742 (M^+-$COCH_3$+H-H_2O), 453 (M^+-331-H_2O), and 411 (M^+-331-H_2O-$COCH_3$). The ^1H NMR spectrum of **2a** showed five acetyl groups resonating at δ 2.07 (3H, s), 2.03 (2 × 3H, s), 2.02 (3H, s), and 1.99 (3H, s) ppm, suggesting one 5α-hydroxy group of six hydroxy groups was unacetylated, due to steric hindrance



1 R=R'=H
2 R=H, R'=glc
2a R=Ac, R'=glc(OAc)$_4$

Fig. 1. Structures of compounds **1, 2** and **2a**.

effect for tertiary hydroxyl. In addition, it displayed absorption in the IR spectrum at 3500 and 1740 cm^{-1}, also indicating the presence of acetyl and hydroxyl groups.

From all of those evidence mentioned above, the structure of **2** was therefore deduced to be 3-O-β-D-glucopyranosyl-(22*E*, 24*R*)-ergosta-7, 22-dien-5α, 6β-diol (**2**) as shown in Fig. 1.

Based upon spectroscopic (MS, IR, ^{1}H- and ^{13}C-NMR) and physical data comparison with literature, the structures of other known compounds were characterized as 5α, 8α-epidioxy-(22*E*, 24*R*)-ergosta-6, 22-dien-3β-ol (**3**) [3,10], (22*E*, 24*R*)-ergosta-5, 22-dien-3β-ol (**4**) [11], and (22*E*, 24*R*)-ergosta-4, 6, 8(14), 22-tetraen-3-one (**5**) [13,14], respectively.

The higher fungi, mainly including both Basidiomycetes and Ascomycetes, produce many ergosterol analogues, but the presence of the glucoside (**2**) of a polyhydroxylated ergosterol derivative is firstly reported from a natural source to the best of our knowledge.

These known ergostane-type compounds were previously obtained from marine organisms [2,4,10,12,13] and macro-fungi [3,5,8,11,14]. Biogenetically, Δ^5-, Δ^6- and Δ^7-ergosteroids seem quite obviously to originate from $\Delta^{5,7}$-ergosterol distributed widely in both fungi and marine organisms [2,4]. The co-occurrence of steroids (**1–5**), of which brassicasterol was the major composition in the Ascomycetes *T. indicum* suggested that they could also derive from ergosterol. Recently, Δ^5-brassicasterol and ergosterol as prominent components have been isolated from the black truffle, *T. melanosporum* [11], which was likely to helpful for throwing light on the biogenetic pathways involved in the formation of these steroids. In general, the occurrence of structurally closely related ergostane derivatives in taxonomically remote species is interesting and may indicate the connection with a common producer, probably symbiotic microorganisms. The above fact suggests that a close correlation between terrestrial fungi and marine organisms appears to exist, which is of evolutionistic significance.

What is more, it is worthwhile to note that this fungus possesses the enzyme system to convert $\Delta^{5,7}$-ergosterol into the glucoside of ergosterol-7,22-dien-3β, 5α, 6β-triol (cerevisterol).

In addition, the biological functions of these sterols and ergosterol glycoside in *T. indicum* remain unknown, they presumably serve as structural constituents of cellular biomembranes and possibly also as precursors of steroid hormones, on the other hand, and as deterrents or phytoalexins. Further investigation will be expected in depth.

Acknowledgments

We wish to acknowledge the financial support from the Natural Science Foundation of Yunnan Province (2000B0067M, 98C086M). We are grateful to Ms. X.H. Wang, Profs. P.G. Liu and D.G. Ji for their help in this project.

References

[1] Mao XL. Economic fungi of China. Science Press. Beijing, 1998:620.
[2] Iorizzi M, Minale L, Riccio R, Lee JS, Yasumoto T. Polar steroids from the marine Scallop *Patinopecten yessoensis*. J Nat Prod 1988; 51:1098–103.
[3] Gao JM, Dong ZJ, Liu JK. Constituents of Basidiomycetes *Russula cyanoxantha*. Acta Botanica Yunnanica 2000;22:85–9.
[4] Madaio A, Piccialli V, Sica D, Corriero G. New polyhydroxysterols from the Dictyoceratid Sponges, *Hippospongia communis*, Spongia officinalis, Ircinia variabilis, and *Spongionella gracilis*. J Nat Prod 2000;52:952–61.
[5] Kawagishi H, Katsumi R, Sazawa T, Mizuno T, Hagiwara T, Nakamura T. Cytotoxic steroids from the mushroom *Agaricus blazei*. Phytochemistry 1988;27:2777–9.
[6] Takaishi Y, Ohashi T, Tomimatsu T. Ergosta-7, 22-dien-3β-ol glycoside from *Tylopilus neofelleus*. Phytochemistry 1989;28:945–7.
[7] Castro Ferreira Gomes DDE, Vilela Alegrio L. Acyl steryl glycosides from *Pithecellobium cauliflorum*. Phytochemistry 1998;49:1365–7.
[8] Ishizuka T, Yaoita Y, Kikuchi M. Sterols from the fruit bodies of *Grifola frondosa*. Chem Pharm Bull 1997;45:1756–60.
[9] Wright JLC, McInnes AG, Shimizu S, Smith DG, Walter JA. Identification of C-24 alkyl epimers of marine sterols by ^{13}C NMR spectroscopy. Can J Chem 1978;56:1898–903.
[10] Gunatilaka AAL, Gopichand Y, Schmitz FJ, Djerassi C. Minor and trace sterols in marine invertebrates. 26. Isolation and structure elucidation of nine new 5α,8α-epidioxy sterols from four marine organisms. J Org Chem 1981;46:3860–4.
[11] Harki E, Klaebe A, Talou T, Dargent R. Identification and quantification of *Tuber melanosporum* Vitt. sterols. Steroids 1996;61:609–12.
[12] Erdman TR, Thomson RH. Sterols from the Sponges *Cliona celata grant* and *Hymeniacidon perleve montagu*. Tetrahedron 1972;28: 5163–73.
[13] Kobayashi M, Krishna MM, Ishida K, Anjaneyulu V. Marine sterols. XXII. Occurrence of 3-oxo-4,6,8(14)-triunsaturated steroids in the sponge *Dysidea herbacea*. Chem Pharm Bull 1992;40:72–4.
[14] Gonzalez AG, Barrera JB, Toledo Marante FJ. The steroids and fatty acids of the Basidiomycete *Scleroderma polyrhizum*. Phytochemistry 1983;22:1049–50.