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A New Cytotoxic Sterol Produced by an Endophytic Fungus from *Castaniopsis fissa* at the South China Sea Coast

Hou Jin LI[1], Yong Cheng LIN[1]*, L. L. P. VRIJMOED [2], E. B. G. JONES [2]

[1] Department of Applied Chemistry, Zhongshan University, Guangzhou 510275
[2] Department of Biology and Chemistry, City University of Hong Kong, Hong Kong

Abstract: A new sterol, ergosta-8(9),22-diene-3,5,6,7-tetraol(3β,5α,6β,7α,22*E*)(**A**) together with three known sterols: 3β, 5α,6β-trihydroxyergosta-7,22-diene (**B**), 3β-hydroxy-5α,8α- epidioxyergosta-6,22-diene (**C**) and ergosterol (**D**) were isolated from the mycelia of an unidentified endophytic fungus separated from *Castaniopsis fissa* (chestnut tree). Compound **A** exhibited potent selective cytotoxicity against Bel-7402, NCI4460 and L-02 cell lines with IC_{50} values 8.445, 5.03, 13.621 μg/mL, respectively.

Keywords: Endophytic fungus, sterols, ergosta-8(9), 22-diene-3,5,6,7-tetraol (3β,5α,6β,7α,22*E*), cytotoxicity.

Endophytes are defined as fungi colonizing healthy tissue plant without overt symptoms or no apparent injury to the host. As the structurally novel and biologically active secondary metabolites possessing rich source, the endophytic fungi have gained increased attention in the last decade. In our search for secondary metabolites of endophytic fungi, many cytotoxic and/or novel compounds were isolated[1-4]. In this paper we report the isolation and structural identification. The new sterol (**A**) from the mycelia of the fungus (strain number #2059), which was an unidentified endophytic fungus separated from a leaf of *Castaniopsis fissa* (chestnut tree) in a forest at the South China Sea Coast.

Starter cultures (from Professor Jones E. B. G. and Dr. Vrijmoed L. L. P.) were maintained on potato dextrose agar. Plugs of agar supporting mycelial growth were cut and transferred aseptically to a 250 mL Erlenmeyer flask containing 100 mL liquid medium (1.0% glucose, 0.5% peptone, 0.5% yeast extract, 20% sea water). The flask was incubated at 25°C on a rotary shaker (rpm=120) for 5-7 days. The mycelium was aseptically transferred to 500 mL Erlenmeyer flasks containing 200 mL of the same liquid medium. The flasks were incubated at 25°C on a rotary shaker (rpm=120) for two weeks. 200 L of growth culture were filtered through cheesecloth. The separated mycelia were dried in air. The dried mycelia (550 g) was extracted with 3×2000 mL of methanol for seven days. The combined extracts were concentrated, then chromatographed on silica gel column with a petroleum-EtOAc-MeOH gradient as the eluent. The petroleum-EtOAc (1:3) eluate was further purified using $CHCl_3$-MeOH (7:1) as the eluent to afford **A**

* E-mail: ceslyc@zsu.edu.cn

(66 mg). **B** (55 mg) and **C** (35 mg) were obtained by repeated chromatography on silica gel column. **D** (about 650 mg) were obtained by recrystallization from the solvent.

Ergosta-8(9),22-diene-3,5,6,7-tetraol (3β,5α,6β,7α,22*E*) (**A**), white amorphous solid, m.p.145～150℃, $[\alpha]_D^{20}$ - 54.5 (c=0.22, MeOH). Anal. found: C 75.54; H 10.22. Calcd. for $C_{28}H_{46}O_4$: C 75.34; H 10.31. IR (KBr)cm^{-1}: 3423, 2959, 2872, 1668, 1634, 1461, 1378, 1276, 1158, 1067, 969, 915, 878, 805, 733. UV: λmax 216.00 (ε408.7), 247.00 (ε144.1). APCI-MS: *m/z* 429 $[M-H_2O+H]^+$, 411$[M-2H_2O+H]^+$, 393$[M-3H_2O +H]$, 375$[M-4H_2O+H]$, 267, 251, 187, 171, 131, 105. NMR data were shown in **Table 1**.

The IR spectrum suggested the presence of hydroxy group (3423 cm^{-1}). The fragment ions *m/z* [429$(M-H_2O+H)^+$, 411$(M-2H_2O+H)^+$, 393$(M-3H_2O+H)^+$, 375 $(M-4H_2O+H)^+$] in APCI-MS indicated the molecule possessed four hydroxy group and confirmed by the ^1H NMR spectrum, which was recorded in acetone-d_6 (**Table 1**) and showed four hydroxy proton signals at 2.77 (2H), 3.34 (d, 10.0 Hz), 3.68 (d, 5.0Hz). Two methyl singlets (δ_H 1.13 and 0.64) and four methyl doublets (δ_H 1.05，0.94，0.86 and 0.84) suggested an ergostane skeleton. Its ^{13}C NMR spectrum showed 28 signals consisting of 6 methyls, 7 methylenes, 10 methines and 5 quaternary carbons. Four of these signals were assigned to carbon atoms bearing hydroxyl groups, three secondary carbons at δ68.6，67.5, 63.3 and one tertiary atδ64.9. Four carbons, resonating atδ_C 136.8 (δ_H 5.24, d, 1H, 6.0Hz), 132.6 (δ_H 5.25, d, 1H, 6.0Hz), 128.2 (s), 134.3 (s), indicated the presence of two double bond in the molecule. 2D NMR analysis revealed the position of the two double bonds at $\Delta^{8,9}$ and $\Delta^{22,23}$. The stereochemistry of 3-OH, 5-OH, 6-OH and 7-OH were determined by comparison with (24*S*)-24-ethyl-cholest -8- en-5α,6β,7α-triol, showing only the difference at the side chain. The coupling constant between H-6 and H-7 (J=2.5Hz) indicated 6β,7αconfigurations. The α-orientation of 7-OH group is the lowest energy conformation. The coupling constant is small on account of the dihedral angle H6-C6-C7-H7[5]. The $\Delta^{22,23}$ double bond was assigned in *E* configuration based on the coupling constant between H-22 and H-23 (J=15.0Hz), comparing with the $J_{H-22/H-23}$ of ergosterol (15.0Hz).

Figure 1 The structure of **A** and its HMBC correlations

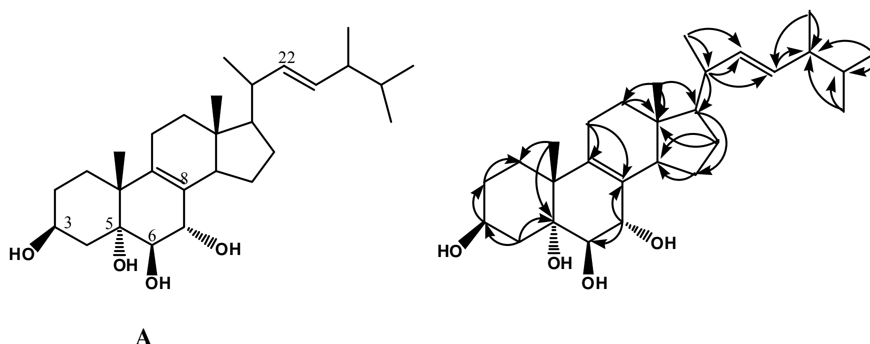

Table 1 The NMR data for **A** (acetone-d6,TMS,δppm,INOVA 500Hz)

position	δ_C	DEPT	δ_H	^1H-^1HCOSY
1	31.1	CH_2	1.54	1.73
			1.73	1.54
2	31.9	CH_2	1.57	1.85, 3.76
			1.85	1.57
3	68.6	CH	3.76	1.57, 2.13
4	40.2	CH_2	1.32	2.13
			2.13	1.32, 3.76
5	64.9	C		
6	67.5	CH	4.20(brd, 10.0Hz)	3.13, 3.34
7	63.3	CH	3.13(d, 2.5Hz)	4.20
8	128.2	C		
9	134.3	C		
10	39.0	C		
11	24.4	CH_2	1.94	1.38, 2.01, 2.02
			2.02	1.38, 1.94, 2.01
12	36.8	CH_2	1.38	
			2.01	
13	42.8	C		
14	50.8	CH	2.22	1.32
15	30.0	CH_2	1.32	2.05, 2.18
			2.05	1.32
16	24.2	CH_2	2.18	1.22, 1.32
17	54.6	CH	1.22	1.32, 2.10, 2.18
18	11.7	CH_3	0.64(s)	
19	22.8	CH_3	1.13(s)	
20	41.3	CH	2.10	1.05, 1.22, 1.32
21	21.4	CH_3	1.05(d, 6.5Hz)	2.10
22	136.8	CH	5.22(dd, 15.0, 6.0Hz)	
23	132.6	CH	5.26(dd, 15.0, 6.0Hz)	
24	43.7	CH	1.88	0.94
25	18.1	CH_3	0.94(d, 7.0Hz)	1.88
26	33.8	CH	1.99	
27	20.3	CH_3	0.86(d, 8.0Hz)	0.84
28	20.0	CH_3	0.84(d, 6.5Hz)	0.86
3-OH		OH	3.68(d, 5.0Hz)	
5-OH		OH	2.77(s)	
6-OH		OH	3.34(d, 10.0Hz)	
7-OH		OH	2.77(s)	

The HMBC and ^1H-^1H COSY spectra of **A** provided the informations of its intact molecular structure. The structure of **A** was determined to be ergosta-8 (9),22-diene-3,5,6,7-tetraol (3β,5α,6β,7α,22*E*).

Although tetrahydroxyergostas had been detected in the wood-rotting fungus *Polyporus versicolor*[6], the fruiting bodies of *Agaricus blazei*[7], the marine spong *Spongia officinalis*[8]. M. Anastasia *et al.* obtained the tetrahydroxyergosta by permanganate oxidation of ergosterol[9]. While the isolation of a tetrahydroxyergosta from endophytic fungi is the first case.

The human cancer cell lines Bel-7402, NCI-4460 and the normal human cell lines L-02 were used to examine the cell growth inhibitory activity of **A**. Compound **A**

exhibited significant cytotoxicity against Bel-7402, NCI-4460 and L-02 with IC_{50} values 8.445, 5.03 and 13.621 μg/mL, respectively.

Other sterols metabolites, such as 3β,5α,6β-trihydroxyergosta-7,22-diene (**B**), 3β-hydroxy-5α,8α-epidioxyergosta-6,22-diene (**C**) and ergosterol (**D**) were also isolated from the mycelia. Their structures were established on the basis of the NMR data by comparison with those reported in the literatures.

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