Pergamon 0031-9422(94)00740-3 Phytochemistry, Vol. 38, No. 4, pp. 947 950, 1995
Elsevier Science Ltd
Printed in Great Britain
0031-9422/95 $9.50 + 0.00

TWO STEROIDS FROM *CALVATIA CYATHIFORMIS*

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(*Received* 14 *June* 1994)

Key Word Index—*Calvatia cyathiformis*; Lycoperdaceae; steroid; calvasterol A; 14α-hydroxyergosta-4,7,9,22-tetraen-3,6-dione; calvasterol B; 9α,14α-dihydroxyergosta-4,7,22-trien-3,6-dione.

Abstract—Calvasterols A (14α-hydroxyergosta-4,7,9,22-tetraen-3,6-dione) and B (9α,14α-dihydroxyergosta-4,7,22-trien-3,6-dione), two new steroids, have been isolated from *Calvatia cyathiformis*. Their molecular structures have been defined by spectroscopic means and chemical correlations.

INTRODUCTION

In a previous paper [1], we reported the isolation of two new steroids designated as cyathisterone (**3**) and cyathisterol (**4**) from the dichloromethane extract of the fruiting body of *Calvatia cyathiformis* (Bosc.) Morg., a Chinese crude drug ('Ma Bo' in Chinese; 'Mabotsu' in Japanese), used in China as a haemostatic and pharyngodynia [2]. Further investigation of this extract led us to isolate two new steroids designated as calvasterols A (**1**) and B (**2**). The structural elucidation of the above compounds (**1** and **2**) is reported in this paper.

RESULTS AND DISCUSSION

Calvasterol A (**1**), had the molecular formula $C_{28}H_{38}O_3$ as shown by the HR mass spectrum, which had a molecular ion at *m/z* 422 $[M]^+$. The IR absorption at 3450 and 1660 cm^{-1} suggested the presence of a hydroxyl and an α,β-unsaturated carbonyl group. The ^1H NMR spectrum of **1** clearly showed two tertiary methyl signals at δ0.76 and 1.46, four secondary methyl signals at δ0.84, 0.85, 0.93 and 1.04, which suggested an ergostane skeleton [3]. This was supported by the fact that the chemical shift of the signals for the secondary methyl groups together with resonances for a *trans*-disubstituted double bond at δ5.22 and 5.30 were consistent with those of ergosterol. On the other hand, two olefinic proton signals at δ6.17 (1H, *s*) and 6.45 (1H, *s*) in the ^1H NMR spectrum of **1**, together with two tertiary carbon signals at δ121.8 and 127.0, two quaternary carbon signals at δ156.0 and 156.3 and two carbonyl carbon signals at δ188.7 and 199.7 observed in the ^{13}C NMR spectrum of **1** (Table 1) were similar to those of ergosta-4,7,22-triene-3,6-dione (**5**) [1, 4]. Also a negative Cotton effect at 275 nm and a positive Cotton effect at 234 nm, respectively, observed in the CD spectrum of **1** were closely similar to those of **5** (270 nm, negative; 234 nm, positive). All these data indicated that calvasterol A (**1**) was an ergosta-4,7,22-trien-3,6-dione (**5**) derivative including its stereochemistry.

An olefinic proton signal at δ6.22 (1H, *dd*, *J* = 1.8, 6.6 Hz), which was only coupled with the methylene protons at δ2.38 (1H, *dd*, *J* = 6.6, 18.3 Hz) and 2.72 (1H, *dd*, *J* = 1.8, 18.3 Hz), showed three-bond correlations with three quaternary carbon signals at δ38.8 (C-10), 46.3 (C-13) and 156.0 (C-8) in the HMBC spectrum of **1** (Table 2). Thus the olefinic proton and methylene protons were assigned at C-11 and C-12, respectively. Also the assignment of a quaternary carbon signal (δ84.7), which was conjoining a hydroxyl group, was confirmed as C-14. This was supported by the fact that the olefinic proton, which was assigned at C-7, was not coupled with any other protons. Finally, a proton signal at δ2.72, which was assigned at H-12α using a NOE correlation between δ0.76 (H-18) and 2.38 (H-12β) in the NOESY spectrum of **1**, was shifted down-field to δ 3.04 (pyridine-d_5) by the pyridine induced deshielding effect [5] indicating that a hydroxyl group and a proton (OH-14α and H-12α) occupied 1,3-*syn*-periplanar positions. Therefore, the configuration at C-14 has been assigned as *S*. These data led to the conclusion that calvasterol A (**1**) was elucidated as 14α-hydroxyergosta-4,7,9,22-tetraen-3,6-dione.

Calvasterol B (**2**), had the molecular formula $C_{28}H_{40}O_4$ since the high resolution mass spectrum gave a molecular ion at *m/z* 440 $[M]^+$. The IR absorption at 3450 and 1660 cm^{-1} of **2** also suggested the presence of a hydroxyl and α,β-unsaturated carbonyl group. The ^1H NMR spectrum of **2** showed two tertiary methyl signals at δ0.77 and 1.37, four secondary methyl signals at δ0.84, 0.85, 0.93 and 1.05 and a *trans*-disubstituted double bond signal at δ5.20 and 5.29. This information revealed the presence of an ergostane skeleton. The ^1H and ^{13}C NMR spectra of **2** were similar to that of calvasterol

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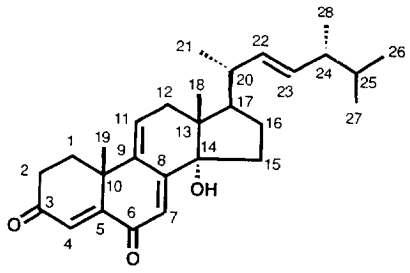

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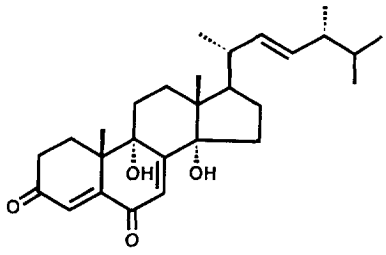

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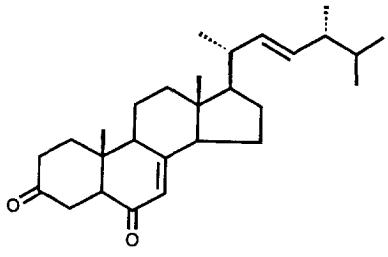

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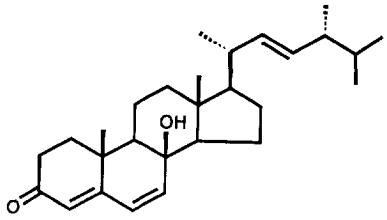

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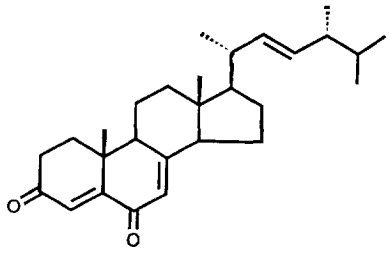

5

A (**1**) except for the disappearance of an olefinic proton signal at δ6.22 (1H, *dd*, J = 1.8, 6.6 Hz) in the ^{1}H NMR spectrum of **1** and the appearance of a secondary carbon signal δ27.6 and a quaternary carbon signal at δ74.4 in the ^{13}C NMR spectrum of **2** instead of a tertiary carbon signal at δ132.9 and a quaternary carbon signal at δ138.5 seen in the spectrum of **1** (Table 1). Thus the structure of **2** was considered to be the dihydroxy derivative of ergosta-4,7,22-trien-3,6-dione (**5**).

Hydroxylation of ergosta-4,7,22-trien-3,6-dione (**5**) was examined to give dihydroxysteroid, which was identical with calvasterol B (2), including the optical rotation. The assignment of two quaternary carbon signals (δ74.4 and 87.0), which were joining a hydroxyl group, were confirmed as C-9 and C-14, respectively, using a HMBC correlation of **2** (Table 2). This was supported by the fact that the olefinic proton, which was assigned at C-7, was not coupled with any other protons. Furthermore, two proton signals at δ2.79 (H-1α) and 2.18 (H-12α) were also shifted downfield to δ3.04 and 2.48 (pyridine-d_5) by the pyridine induced deshielding effect [5] indicating that two hydroxyl groups and two protons (OH-9α and H-1α, OH-14α and H-12α) occupied 1,3-*syn*-periplanar positions. Therefore, the configurations at C-9 and C-14 have been assigned as *S*, respectively. From the above results, the structure of calvasterol **B** (**2**) was established as 9α,14α-dihydroxyergosta-4,7,22-trien-3,6-dione.

A large number of steroids, which have the ergostane skeleton, have been isolated from fungi. However, calvasterols A (**1**) and B (**2**) are the first examples of a naturally occurring hydroxylated ergosta-4,7,22-trien-3,6-dione type of steroid.

EXPERIMENTAL

General. Mps: uncorr. IR spectra were recorded in KBr discs. EI-MS were taken at 70 eV. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) were recorded in $CDCl_3$ with TMS as int. standard. Low pressure LC (LP-LC) was performed on a Nihon Seimitsu NP-FX-20 in a glass column (300 × 10 mm) packed with silica gel CQ-3 (30–50 μ; Wako).

Isolation of metabolites **1** *and* **2**. The fruiting body of *Calvatia cyathiformis* (4 kg) was extracted with CH_2Cl_2, and the organic layer was dried (Na_2SO_4) and evapd *in*

Table 1. ^{13}C NMR data of calvasterols A (**1**) and B (**2**), and related compound **5** (in $CDCl_3$)

C	1	2	5
1	34.5	27.7	35.5
2	34.3	34.3	34.4
3	199.7	200.1	200.1
4	127.0	125.5	124.4
5	156.3	155.4	168.4
6	188.7	188.1	187.7
7	121.8	129.1	126.4
8	156.0	163.4	158.7
9	138.5	74.4	47.3
10	38.8	44.1	39.1
11	132.9	27.6	21.9
12	37.4	27.7	38.6
13	46.3	46.4	44.8
14	84.7	87.0	56.3[a]
15	31.2	31.9	22.6
16	27.2	26.3	27.8
17	50.4	50.2	56.5[a]
18	16.2	16.4	12.9
19	29.5	22.9	19.6
20	40.1	40.0	40.4
21	20.9	21.3	21.2
22	135.4	135.4	135.3
23	133.3	133.4	133.2
24	42.9	43.0	43.0
25	33.2	33.2	33.2
26	20.0	20.0	20.0
27	19.7	19.7	19.7
28	17.6	17.7	17.6

[a]The assignments may be reversed.

Table 2. Three-bond correlations (HMBC experiments) of the steroid ring of calvasterols A (**1**) and B (**2**)

	C	
H	1	2
1	2*, 3, 5, 9, 10*, 19	2*, 3, 9, 10*, 19
2	1*, 3*, 4, 10	1*, 3*, 10
4	2, 5*, 6, 10	2, 5*, 6, 10
7	5, 9, 14	5, 9, 14
11	8, 10, 12*, 13	8, 12*
12	9, 11*, 13*, 14, 18	9, 11*, 13*, 14, 18
15	13, 14*, 16*, 17	14*
16	14, 15*, 20	14, 17*, 20
17	18, 20*	15, 16*, 18, 21
18	112, 13*, 14, 17	12, 13*, 14, 17
19	1, 5, 9, 10*	1, 5, 9, 10*

*Two-bond correlation.

vacuo. The residue (13.5 g) was chromatographed on silica gel with C_6H_6–Me_2CO (10:1) followed by LP-LC using *n*-hexane–EtOAc (5:1) to give a calvasterol B (9α,14α-dihydroxyergosta-4,7,22-triene-3,6-dione) (**2**) (15 mg), and then *n*-hexane–EtOAc (4:1) to give calvasterol A (14α-hydroxyergosta-4,7,9,22-tetraene-3,6-dione) (**1**) (10 mg).

Calvasterol A (**1**). Pale yellow needles (*n*-hexane–$CHCl_3$, 1:1); mp 190–192° $[\alpha]_D^{20}$ + 93° ($CHCl_3$; *c* 0.39). IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1660 (CO). UV λ_{max}^{MeOH} nm (log ε): 250 (4.35), 340 (4.06). EI-MS (probe) 70 eV, *m/z* (rel. int.): 422.2815 $[M]^+$ ($C_{28}H_{38}O_3$ requires 424.2820, 2), 404 $[M - H_2O]^+$ (60), 280 (55), 265 (38). ^1H NMR (600 MHz, $CDCl_3$, TMS as int. std.): δ0.76 (3H, *s*, H-18), 0.84 (3H, *d*, *J* = 6.9 Hz, H-26), 0.85 (3H, *d*, *J* = 6.9 Hz, H-27), 0.93 (3H, *d*, *J* = 6.6 Hz, H-28), 1.04 (3H, *d*, *J* = 5.9 Hz, H-21), 1.44 (1H, *m*, H-16b), 1.46 (3H, *s*, H-19), 1.50 (1H, *m*, H-25), 1.77 (1H, *m*, H-15b), 1.89 (1H, *m*, H-24), 2.00 (2H, *m*, H-15a, H-16a), 2.09 (1H, *m*, H-20), 2.11 (1H, *m*, H-17), 2.28 (1H, *ddd*, *J* = 5.5, 13.6, 14.3 Hz, H-1a), 2.38 (1H, *dd*, *J* = 6.6, 18.3 Hz, H-12b), 2.42 (1H, *ddd*, *J* = 4.5, 5.1, 13.6 Hz, H-1b), 2.59 (1H, *ddd*, *J* = 4.5, 5.5, 19.4 Hz, H-2b), 2.65 (1H, *ddd*, *J* = 5.1, 14.3, 19.4 Hz, H-2a), 2.72 (1H, *dd*, *J* = 1.8, 18.3, H-12a), 5.22 (1H, *dd*, *J* = 7.7, 15.4 Hz), 5.30 (1H, *dd*, *J* = 7.7, 15.4 Hz, H-23), 6.17 (1H, *s*, H-7), 6.22 (1H, *dd*, *J* = 1.8, 6.6 Hz, H-11), 6.45 (1H, *s*, H-4) ^{13}C NMR: Table 1. CD (MeOH; *c* 7.1×10^{-5}): $\Delta\varepsilon_{275}$ − 3.46, $\Delta\varepsilon_{234}$ + 1.57.

Calvasterol B (**2**). Pale yellow needles; mp 173–175°; $[\alpha]_D^{20}$ − 103° ($CHCl_3$; *c* 0.27). IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1660 (CO). UV λ_{max}^{MeOH} nm (log ε): 266 (4.09). EI-MS (probe) 70 eV, *m/z* (rel. int.): 440.2932 $[M]^+$ ($C_{28}H_{40}O_4$ requires 440.2926, 4), 422 $[M - H_2O]^+$ (38), 297 (26), 229 (87). ^1H NMR (600 MHz, $CDCl_3$, TMS): δ0.77 (3H, *s*, H-18), 0.84 (3H, *d*, *J* = 6.9 Hz, H-26), 0.85 (3H, *d*, *J* = 6.9 Hz, H-27), 0.93 (3H, *d*, *J* = 6.9 Hz, H-28), 1.05 (3H, *d*, *J* = 6.6 Hz, H-21), 1.37 (3H, *s*, H-19), 1.46 (1H, *m*, H-16β), 1.49 (1H, *m*, H-25), 1.65 (1H, *m*, H-15β), 1.75 (1H, *br d*, *J* = 13.6 Hz, H-12β), 1.82 (1H, *br d*, *J* = 13.9 Hz, H-1β), 1.83 (1H, *m*, H-11β), 1.88 (1H, *m*, H-24), 1.94 (1H, *m*, H-16α), 1.97 (1H, *m*, H-15α), 2.01 (1H, *m*, H-17), 2.09 (1H, *m*, H-11α), 2.11 (1H, *m*, H-20), 2.18 (1H, *ddd*, *J* = 4.4, 13.2, 13.6 Hz, H-12α), 2.46 (1H, *br s*, OH-9α or 14α), 2.50 (1H, *ddd*, *J* = 5.5, 13.9, 16.8 Hz, H-2β), 2.59 (1H, *ddd*, *J* = 4.0, 5.1, 16.8 Hz, H-2α), 2.79 (1H, *ddd*, *J* = 5.1, 13.9, 13.9 Hz, H-1α), 4.22 (1H, *br s*, OH-9α or 14α), 5.20 (1H, *dd*, *J* = 8.4, 15.0 Hz, H-22), 5.29 (1H, *dd*, *J* = 7.7, 15.0 Hz, H-23), 6.19 (1H, *s*, H-7), 6.60 (1H, *s*, H-4). ^{13}C NMR: Table 1. CD (MeOH; *c* 6.8 $\times 10^{-5}$): $\Delta\varepsilon_{290}$ − 2.94, $\Delta\varepsilon_{248}$ + 4.30.

Hydroxylation of ergosta-4,7,22-trien-3,6-dione (**5**). A 1 M K_2CO_3 soln (0.5 ml) was added to a stirred soln of ergosta-4,7,22-trien-3,6-dione (**5**) (37 mg) in Me_2CO (10 ml) and refluxed for 20 min. The reaction mixture was poured into ice-H_2O and extracted with $CHCl_3$. The evapd residue was purified by LP-LC using *n*-hexane–EtOAc (5:1) to obtain 9α,14α-dihydroxyergosta-4,7,22-trien-3,6-dione (**2**) (2 mg), $[\alpha]_D^{20}$ − 87° ($CHCl_3$; *c* 0.06). This compound was identical with calvasterol B (**2**) on the basis of a comparison of the ^1H NMR and IR spectra and the optical rotation.

Acknowledgements—We are grateful to Dr K. Kawai of the faculty of Phamaceutical Sciences, Hoshi University for mass measurements. This study was performed through Special Coordination Funds for Promoting

Science and Technology (Joint Research Utilizing Scientific and Technological Potential in the Region) of the Science and Technology Agency of the Japanese Government.

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