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A cytotoxic 4α-methyl steroid from the aerial parts of *Cimicifuga foetida* L

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ABSTRACT

A new 4α-methyl sterol, cimisterol A (**1**), together with five known compounds (**2–6**), were isolated from the aerial parts of *Cimicifuga foetida* L. The new compound's structure was determined with the help of extensive 1D and 2D NMR spectroscopy. Compound **1** exhibited broad-spectrum and potent cytotoxic activities against human HL-60, Jurkat, K562, U937, HepG-2, and SGC-7091 cell lines, with IC_{50} values of 7.23, 2.89, 6.88, 3.38, 4.21, and 4.89 μM, respectively. Compound **3** showed moderate to weak activities to all cell lines, except for SGC-7091, having IC_{50} values ranging from 13.37 to 17.72 μM. This is the first time a cytotoxic 4α-methyl sterol constituent was discovered from *Cimicifuga* spp.

1. Introduction

4α-Methyl sterols are a small group of important natural products, especially for the elucidation of sterols' biogenetic implications, which represented the most direct evidence for the theory that squalene or a squalenoid precursor is concerned with sterol biogenesis in plants and as the unambiguous biomarkers for organic matter derived from dinoflagellates in sediments and crude oils [1,2]. Although many 4-methyl sterols have been identified from the marine dinoflagellates [3] and soft corals [4], the presence of 4α-methyl sterols is rare in plants [1,2,5].

The genus *Cimicifuga* (now *Actaea* in Europe and USA) consists of 28 species and the roots have been used in traditional medicine worldwide [6–8]. In Europe and the United States, *C. racemosa*, commonly called black cohosh, is a well-known dietary supplement for women's health in alleviating menstrual pain and menopausal disorders [9,10]. In China, the roots of *C. foetida* are an important traditional Chinese medicine and have been officially listed in the Chinese Pharmacopoeia as a cooling and detoxifying remedy [11]. Chemical and pharmacological studies shown that its main bioactive constituents were 9,19-cyclolanostane triterpenoids, chromones, and caffeic acid derivatives [12–19]. As a part of our work to explore potential antitumor constituents from Chinese Traditional Medicine, we have been studying on the bioactivity compounds of *C. foetida* since 2003 and successively reported a series of cycloartane triterpenoids from the roots of this plant, which exhibited cytotoxicities against various tumor cell lines [20–22].

The aerial parts of *C. foetida*, however, were discarded as a waste byproduct in China. In an attempt to fully explore chemical and bioactive properties of this medicinal plant, our research groups paid attention to the aerial parts of *C. foetida* and a series of cytotoxic 9,19-cycloartane triterpene glycosides were reported recently [23]. In our continuing work on the aerial parts of *C. foetida*, an unusual 4α-methyl sterol, cimisterol A (**1**), for the first time, was isolated from the genus *Cimicifuga*. Besides, five known compounds, 12β-*O*-acetylcimiracemonol (**2**) [24], cimicifoetisides A (**3**) [20], 3β,6β-dihydroxyolean-12-en-27-oic acid (**4**) [25], aceriphyllic A (**5**) [26], and ferulic acid (**6**) [27] were also obtained, of which **4** and **5** were isolated from *Cimicifuga* spp for the first time too. Described herein are the isolation, structure elucidation, and biological activities of the isolated compounds Scheme 1.

2. Experimental

2.1. General

Optical rotations were obtained with a Horiba SEAP-300 polarimeter. ^{1}H and ^{13}C NMR spectra were measured on

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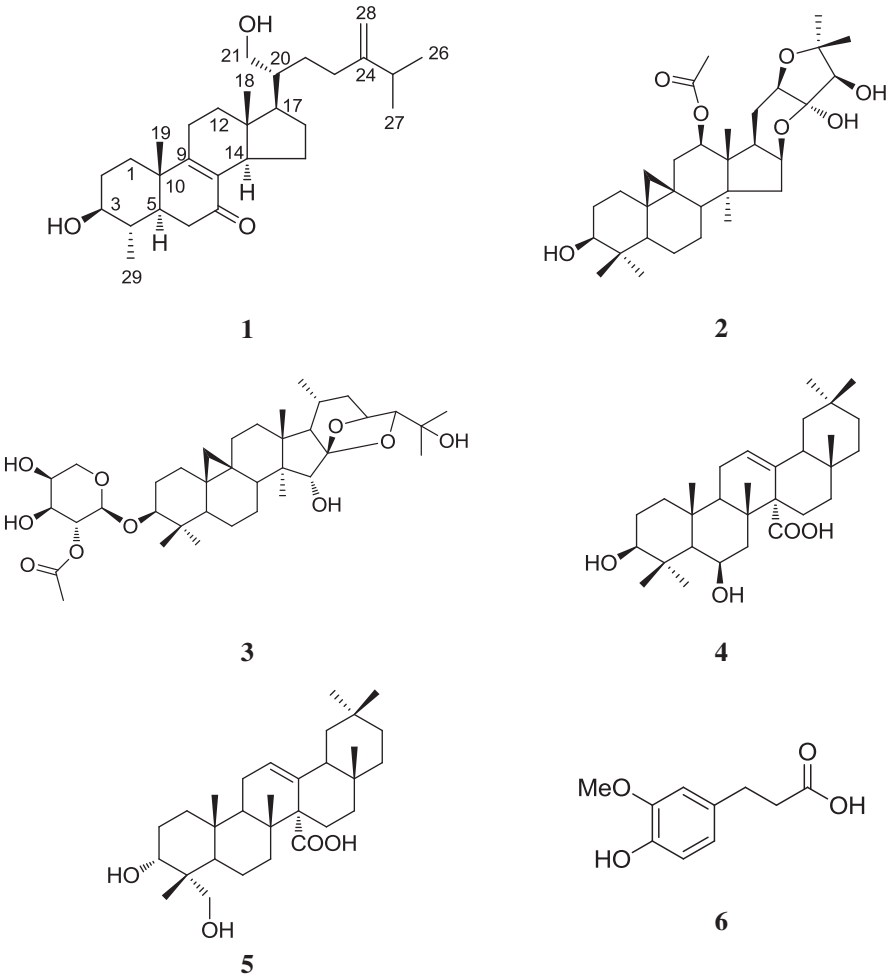

Scheme 1. Structures of compounds isolated from the aerial parts of *C. foetida.*

Bruker DRX-500 and Avance III-600 instruments (Bruker, Zűrich, Switzerland) using TMS as internal standard for chemical shifts. Chemical shifts (δ) were expressed in ppm with reference to the TMS resonance. FABMS and HRTOF-ESIMS data were recorded on a VG Autospec-3000 spectrometer. Infrared spectra were recorded on a Shimadzu IR-450 instrument by using KBr pellets. TLC was performed on precoated TLC plates (200–250 μm thickness, F254 Si gel 60 and F_{254} RP-18 Si gel 60, Qingdao Marine Chemical, Inc.) with compounds visualized by spraying the dried plates with 10% aqueous H_2SO_4 followed by heating until dryness. Silica gel (200–300 mesh, Qingdao Marine Chemical, Inc.), Lichroprep RP-18 (40–63 μm, Merk), and Sephadex LH-20 (20–150 μm, Pharmacia) were used for column chromatography (cc).

The human Leukemia cell lines HL-60, Jurkat, K562, U937, human hepatoma cell line HepG2 and human gastric carcinoma cell line SGC-7901were from the American Type Culture Collection (ATCC). All cell lines were grown in RPMI-1640 medium (GIBCO) supplemented with 10% heat-inactivated bovine serum, 2 nM L-glutamine, 10^5 IU/L penicillin, 100 mg/L streptomycin and 10 mM HEPES pH 7.4. Cells were kept at 37 °C in a humidified 5% CO_2 incubator.

2.2. Plant material

Aerial parts of *Cimicifuga foetida* L. (2 kg) were collected from Shangrila County, Yunnan Province, China, in September 2008 and identified by Prof. Shengji Pei, Kunming Institute of Botany, Chinese Academy of Science. A voucher specimen (KUN No. 200808024) has been deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, PR China.

2.3. Extraction and isolation

The dried and milled aerial parts of *C. foetida* (2 kg) were extracted with MeOH (5 L × 24 h × 3) at room temperature to give a residue (205 g) after evaporating in vacuum at 50°. This residue was suspended in water and then extracted successively with PE, AcOEt and n-BuOH. The AcOEt extract (55 g) was chromatographed on silica gel (CH_3Cl/MeOH = 1:0 → 0:1) to afford Fractions 1–4. Fraction 3 (8 g) was re-subjected to repeated CC (SiO_2; CH_3Cl/Me_2CO = 20:1 → 10:1; then RP-18, MeOH/H_2O = 60:40 → 100:0) to afford Fractions 2.1–2.5. Then Fraction 2.3 (1.5 g) was subjected to CC (SiO_2; CH_3Cl/Me_2CO = 20:1) to

give crud compounds, and then purified on Sephadex LH-20 (MeOH) to afford **1** (3 mg), **2** (10 mg), and **6** (5 mg). Compounds **3** (16 mg), **4** (5 mg), and **5** (4 mg) were purified from Fraction 2.2 (1.9 g) by conducting silica gel cc, eluting with $CHCl_3$–Me_2CO (10:1), followed by Sephadex LH-20, eluted with MeOH.

3β,21-dihydroxy-4α-methyl-5α,14α-ergosta-8,24(28)-dien-7-one: white powder. M.p. 175−178°; [α] 22 D −14.0 (c 0.13, MeOH); UV λ_{max} (MeOH) nm (log ε) 255 (4.88); IR ν_{max} (KBr) cm^{-1}: 3421, 2941, 1655, 1640, 887; ^1H and ^{13}C NMR: see Table 1.

Positive HRTOF-ESIMS *m/z*: 443.3499 $[M+H]^+$, molecular formula $C_{29}H_{46}O_3$ calc for 443.3525.

2.4. Cytotoxicity assay

Cytotoxicity evaluations were performed using the MTT method for the human cancer cell lines HL-60, Jurkat, K562, U937, HepG2 and SGC-7901 and cisplatin was used as a positive control [28]. Briefly, 2×10^4/mL cells were added to each well (100 μL/well), and incubated with various concentrations of drugs (100, 30, 10, 3, 1, 0.3 μg/mL) in three replicates for 48 h at 37 °C in a humidified atmosphere of 5% CO_2. After 48 h, 20 μL of methyl thiazol tetrazalium (MTT) solution (5 mg/mL) were added to each well, which were incubated for another 4 h. Then 10% SDS–5% isobutanol–0.012 M HCl was added to each well (100 μL/well). After 12 h at room temperature, the OD value of each well was recorded on Model680 (BIO-RAD) reader at 595 nm.

3. Results and discussion

Cimisterol A (**1**), was obtained as white powder, which gave an $[M+H]^+$ ion at *m/z* 443 in positive-ion FAB-MS. A molecular formula of $C_{29}H_{46}O_3$ for the compound was deduced by HRTOF-ESIMS (found 443.3499 $[M+H]^+$, calcd 443.3525). The UV and IR spectra showed absorption bands for hydroxyl groups (ν_{max} 3421 cm^{-1}), a conjugated enone (λ_{max} 255 nm; ν_{max} 1655 cm^{-1}), and a terminal methylene group (ν_{max} 1640, 887 cm^{-1}). The assignment of ^1H and ^{13}C NMR spectroscopic data of **1** (Table 1) was based on HSQC, HMBC, and ^1H–^1H COSY spectroscopic data (Fig. 1). In the ^1H spectrum (Table 1), the signals due to two tertiary methyl groups at δ_H 0.72 and 1.12; one secondary methyl groups at δ_H 1.13 (brd, $J=6.0$ Hz); protons of a terminal olefinic methylene at δ_H 4.88 (1H, brs) and 4.85 (1H, brs), and an isopropyl group [δ_H 1.02, 1.04 (each 3H, brd, $J=4.5$ Hz, 2.28 (1H, m)] were observed. The ^{13}C and DEPT NMR spectra of **1** (Table 1) showed signals ascribable to a hydroxymethine group at δ_C 74.6 (C-3), a hydroxymethylene group at δ_C 62.1 (C-21), a terminal methylene group at δ_C 106.6 (C-28), and 156.9 (C-28), and an α, β-unsaturated ketone moiety at δ_C 132.9 (C-8), 165.4 (C-9), and 198.4 (C-7).

All of the aforementioned evidence suggests **1** is not a 9,19-cycloartane triterpene, which is commonly isolated from *C. foetida*. The UV, IR and NMR spectroscopic data (Tables 1 and 2) of **1** resembled those of ergostane-type steroids, especially 3β-dihydroxy-4α,14α-dimethyl-5α-ergosta-8,24(28)-dien-7-one (**7**) [29,30], except for the main differences below. There are twenty nine C-atoms of **1**, which is one carbon less than that of **7**. In the ^{13}C and DEPT NMR spectra of **1**, two tertiary methyl signals and six methine signals were observed. While, in **7**, there were three signals for tertiary methyl groups and five for methine groups. In addition, for **1**, signals for three secondary methyl groups and a hydroxymethylene group were observed. Whereas, there were four signals for secondary methyl groups and no hydroxymethylene signal in **7**. Based on the

Table 1
^1H and ^{13}C NMR spectroscopic data of cimisterol A in pyridine-d_5.

Position	Cimisterol A		Position	Cimisterol A	
	δ_H mult.	δ_C mult.		δ_H mult.	δ_C mult.
1	1.70 m 1.26 m	34.7 t	16	1.88 m 1.80[a]	28.9 t
2	2.05 m 1.50 m	29.1 t	17	1.79 brt (11.5)	48.7 d
3	3.25 m	74.6 d	18	0.72 s	11.9 q
4	1.62 m	39.9 d	19	1.12 s	18.2 q
5	1.48 m	47.2 d	20	1.48 m	43.7 d
6	2.64 dd (4.5, 15.5) 2.30 m	39.6 t	21	4.08 brd (11.5) 3.95 dd (5.0, 12.3)	62.1 t
7		198.4 s	22	2.05 m 1.76[a]	31.7 t
8		132.9 s	23	2.22 brd (12.0) 1.62 m	35.7 t
9		165.4 s	24		156.9 s
10		39.0 s	25	2.28 m	34.1 d
11	2.35 m 2.24 m	25.6 t	26	1.02 brd (4.5)	22.0 q
12	2.35 m 2.18 m	31.8 t	27	1.04 brd (4.5)	22.1 q
13		42.7 s	28	4.88 brs 4.85 brs	106.6 t
14	2.37 m	48.9 d	29	1.13 brd (5.5)	15.0 q
15	2.88 m 1.68 m	25.6 t			

Chemical shifts are in δ scale with *J* values in parentheses. [a]Signals overlapped.

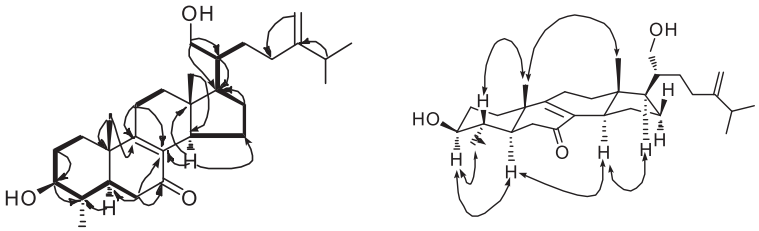

Fig. 1. Major HMBC (→), Key ROESY (↔) and $^{1}H–^{1}H$ COSY (—) Correlations of cimisterol A.

foregoing evidence, we deduced that compared to **7**, a tertiary methyl group was eliminated and a hydroxyl group substituted at a secondary methyl group in **1**. In the HMBC spectrum (Fig. 1), significant HMBC correlations between proton signal at δ_H 2.37 and the quaternary carbons at δ_C 42.7 (C-13), the methylene group at δ_C 25.6 (C-15), and olefinic carbon at δ_C 132.9 (C-8) were observed, located the removed methyl group at C-14 position. In $^{1}H–^{1}H$ COSY spectrum (Fig. 1), the correlation between H-14 signal at δ_H 2.37 and H-15 signal at 1.68 and 2.88 further confirmed the conclusion. The hydroxyl group was located at C-21 position based on the HMBC and $^{1}H–^{1}H$ COSY correlations of the hydroxymethylene signals at δ_H 4.08 brd (11.5) and 3.95 dd (5.0, 12.3) with C-20 and H-20 at δ_c 43.7 and δ_H 1.48 (m), respectively.

In the ROESY spectrum (Fig. 1), H-3 and Me-29 showed correlations with H-5 (biogenetically α oriented), while H-14 showed a correlation with H-5 and H-17. Based on these observations, H-3, H-14, H-17 and Me-29 were assigned in an α-orientation. Hence, **1** was elucidated as 3β,21-dihydroxy- 4α-methyl-5α,14α-ergosta-8,24(28)-dien-7-one.

The cytotoxicities of **1**–**6** were evaluated against human HL-60, Jurkat, K562, U937, HepG-2, and SGC-7091 cell lines. Among them, compound **1** exhibited more potent inhibitory activities than the positive control Cisplatin to all testing cell lines, except for HL-60, with IC_{50} values of 7.23, 2.89, 6.88, 3.38, 4.21, and 4.89 μM, respectively. Compound **3** showed moderate to weak activities to all cell lines, except for SGC-7091, having IC_{50} values ranging from 13.37 to 17.72 μM. Other compounds showed no cytotoxic activities up to a highest concentration of 40.00 μM in any of the cell lines tested (Table 2).

As we reported before, 9,19-cyclolanostane triterpenoids were responsible for the cytotoxicities of *C. foetida*. In the present paper, a cytotoxic 4α-methyl sterol was obtained from the aerial parts of this medicinal plant. This find offers us a new perspective about the cytotoxic constituents of *C. foetida*, and gives us a new clue to explore potential antitumor constituents of this plant. Moreover, because of economic factors, Asian *Cimicifuga* species, such as *C. simplex*, *C. foetida*, and *C. dahurica* were adulterated with black cohosh extracts. Most of the published chemical methods focused on the identification of triterpene glycosides from the roots of these plants [31–37], but no report related to the aerial parts of *Cimicifuga*. In this study, cimisterol A, together with two olean-type triterpenes (**4** and **5**) were isolated from the aerial parts of *C. foetida* for the first time. Thus, these compounds may be the biomarkers for distinguishing the aerial parts of *C. foetida* and black cohosh.

Although 4α-methyl sterols are important for the theory of sterol biogenesis and are the unambiguous biomarkers for organic matter derived from dinoflagellates in sediments and crude oils, their bioactivities, specially, cytotoxicities were rarely reported. In the present paper, a 4α-methyl sterol with noticeable cytotoxicities was obtained. This find will attract more research on this subject.

Table 2
Cytotoxicity of compounds isolated from the aerial parts of *C. foetida* (IC_{50} values; μM).

Compound	HL-60	Jurkat	K562	U937	HepG-2	SGC-7901
1	7.23	2.89	6.88	3.38	4.21	4.89
2	>40	>40	>40	>40	>40	>40
3	13.37	14.80	15.65	17.35	17.72	>40
4	>40	>40	>40	>40	>40	>40
5	>40	>40	>40	>40	>40	>40
6	>40	>40	>40	>40	>40	>40
Cisplatin	1.92	3.66	17.36	5.48	4.58	5.79

Acknowledgment

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.fitote.2011.11.001.

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