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Steroids from the leaves of Chinese *Melia azedarach* and their cytotoxic effects on human cancer cell lines

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ABSTRACT

Three new (**1**–**3**) and several known (**4**–**6**) steroids were isolated from the leaves of Chinese *Melia azedarach*. The structures of the new compounds were elucidated by means of spectroscopic methods including 2D NMR techniques and mass spectrometry to be (20*S*)-5,24(28)-ergostadiene-3β,7α,16β,20-tetrol (**1**), (20*S*)-5-ergostene-3β,7α,16β,20-tetrol (**2**), and 2α,3β-dihydro-5-pregnen-16-one (**3**). The cytotoxicities of the isolated compounds against three human cancer cell lines (A549, H460, U251) were evaluated; only compounds **1**, **2**, and (20*S*)-5-stigmastene-3β,7α,20-triol (**4**) were found to show significant cytotoxic effects with IC_{50}s from 12.0 to 30.1 μg/mL.

1. Introduction

The large evergreen tree *Melia azedarach* Linn (Meliaceae), known as Chinaberry or Pride of China, is native of China, India and Iran. It is now cultivated throughout most regions of the world, especially in the tropics [1–3]. Previous phytochemical and pharmacological studies on the root bark of *M. azedarach* collected either in Southern China (Guangdong province) [4,5] or in Eastern China (Jiangsu province) [6,7] revealed that it yielded limonoids and degraded limonoids with antifeedant and cytotoxic effects. During a ongoing project towards the discovery of new anticancer agents from plants [8], two new ergostane-type (**1**, **2**) and one new pregnane-type (**3**) steroids, together with the related known (20*S*)-5-stigmastene-3β,7α,20-triol (**4**) [9], 5-stigmastene-3β,7α-diol (**5**) [9], and 2α,3α,16β-trihydroxy-5α-pregnane 20*R*-methacrylate (azedarachol, **6**) [10] were obtained from the leaves of *M. azedarach* collected from Guangdong, a province of China. In this paper, we report the isolation and structure elucidation of the new steroids (**1**–**3**) and their cytotoxicity against two human lung cancer cell lines (A549, H460) and a human glioma cancer cell line (U251) using the Cell Counting Kit (CCK)-8 assay method.

2. Experimental procedures

2.1. General method

Optical rotations were determined by using a PerkinElmer 341 polarimeter. IR spectra were measured on a Nicolet NEXUS-670 FT-IR spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX-500 spectrometer. Chemical shifts are expressed in δ (ppm). ^{1}H NMR chemical shifts were referenced to the residual solvent signal [δ_H 7.26 ($CDCl_3$) or δ_H 3.30 (CD_3OD)] and ^{13}C NMR to the center peak at δ_C 77.00 ($CDCl_3$) or δ_C 49.00 (CD_3OD). HSQC and HMBC spectra were optimized for $^1J_{CH}$ = 145.0 Hz and $^nJ_{CH}$ (n = 2 and 3) of 7.7 Hz, respectively. High-resolution electrospray ionization mass spectra (HR-ESI-MS) were measured on a Bruker Daltonics micrOTOF mass spectrometer. LR-ESI-MS was carried out on a Bruker Esquire 3000plus instrument. HR-EI-MS (70 eV) was measured on a Finnigan MAT 95 mass spectrometer. Semipreparative HPLC was performed on a Beckman System consisting of a Beckman Coulter System Gold 508 autosampler, Gold 126 gradient HPLC pumps with a Beckman System Gold 166 single wavelength UV detector (254 nm), a Sedex 75 (SEDERE, France) evaporative light-scattering detector (ELSD) and a Beckman Coulter Ultrasphare ODS column (dp 5 μm, 250 mm × 10 mm). Column chromatography (CC) was performed using silica gel (200–300 mesh, Qingdao Ji-Yi-Da Silysia Chemical Ltd., China), MCI gel CHP20P (75–120 μ, Mitsubishi Chemical Industries, Japan), and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Sweden). Silica gel-precoated plates (GF_{254}, 0.25 mm, Yantai Kang-Bi-Nuo Silysia Chemical Ltd., China)

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were used for TLC. Spots were visualized using UV light (254 and/or 366 nm) and 5% H_2SO_4–EtOH.

2.2. Collection, extraction and isolation

The leaves of *M. azedarach* were collected from the Foshan County of Guangdong province, China. The plant was identified by Dr. Hong-Qing Li (Department of Botany, School of Life Science, East China Normal University). A voucher specimen (No. 081220) was deposited at the Herbarium of the Shanghai Key Laboratory of Brain Functional Genomics, East China Normal University.

The dried leaves (4 kg) were extracted three times with 95% ethanol (3 × 10 L) at room temperature. The solvent was removed at reduced pressure to give a brown residue (395 g). The entire crude extract was suspended in H_2O (3 L), and extracted with EtOAc five times (5 × 3 L) to give an EtOAc extract (185 g). This extract was then subjected to column chromatography over silica gel (column: 90 cm × 9 cm) with petroleum ether–acetone gradient (15:1–1:1—acetone neat) to yield twelve fractions (Fr.1–Fr.12). Fr.9 (petroleum ether–acetone 3:1, 1.8 g) was chromatographed on silica gel (column: 70 cm × 4 cm) with a dichloromethane–acetone gradient (4:1–2:1) to furnish compounds **5** (24 mg) and **6** (34 mg). Compounds **3** (17 mg) and **4** (28 mg) were isolated from Fr.10 (petroleum ether–acetone 2:1, 2.5 g) through silica gel CC (column: 70 cm × 4 cm) with petroleum ether–ethyl acetate (1:1) and both were further purified by gel permeation chromatography on Sephadex LH-20 (column: 120 cm × 3 cm) in methanol. Fr.11 was subjected to column chromatography over MCI-gel using a stepwise gradient elution with MeOH/H_2O (from 1:1 to MeOH neat) to yield six subfractions (Fr.11-A to Fr.11-F). Compound **2** (23 mg) was purified from Fr.11-D by using semipreparative HPLC. The method was developed in a way that resulted in a linear gradient of acetonitrile in H_2O from 30 to 50% over 10 min, then followed by an isocratic gradient of 50% acetonitrile for 30 min, and finally followed by 95% acetonitrile for 5 min (flow rate: 3 mL/min; **2**: t_R = 28 min). Compound **1** (12 mg) was obtained from Fr.11-F by using semipreparative HPLC. The method was an isocratic gradient of 30% acetonitrile in H_2O over 8 min, then followed by a linear gradient of acetonitrile from 30 to 50% for 25 min, and eventually followed by 95% acetonitrile for 5 min (Flow rate: 3 mL/min; **1**: t_R = 25 min).

2.3. Spectral data of new compounds

2.3.1. (20S)-5,24(28)-Ergostadiene-3β,7α,16β,20-tetrol (1)

Colorless gum, $[\alpha]_D^{20}$: −63° (*c* 0.23, MeOH), (+) ESI-MS *m/z* 469 $[M+Na]^+$, 915 $[2M+Na]^+$; (−) ESI-MS *m/z* 491 $[M+HCOO^-]^-$, 891 $[2M-H]^-$; HR-ESI-MS *m/z* 469.3271 (calcd for $C_{28}H_{46}O_4Na$, 469.3294); IR (KBr) ν_{max}: 3397 (br), 2923, 2853, 1647, 1466, 1421 and 1384 cm^{-1}; ^1H and ^{13}C NMR data see Tables 1 and 2.

2.3.2. (20S)-5-Ergostene-3β,7α,16β,20-tetrol (2)

Colorless gum, $[\alpha]_D^{20}$: −8.6° (*c* 0.73, MeOH), (+) ESI-MS *m/z* 471 $[M+Na]^+$, 919 $[2M+Na]^+$; (−) ESI-MS *m/z* 493 $[M+HCOO^-]^-$; HR-ESI-MS *m/z* 471.3455 (calcd for $C_{28}H_{48}O_4Na$, 471.3450); IR (KBr) ν_{max}: 3394 (br), 2924, 2853, 1648, 1464, 1420 and 1383 cm^{-1}; ^1H and ^{13}C NMR data see Tables 1 and 2.

2.3.3. 2α,3β-Dihydro-5-pregnen-16-one (3)

Colorless gum, $[\alpha]_D^{20}$: −89° (*c* 0.30, MeOH), (+) ESI-MS *m/z* 355 $[M+Na]^+$; (−) ESI-MS 663 *m/z* $[2M-H]^-$; EI-MS *m/z* 332 $[M]^+$, 314 $[M-H_2O]^+$, 299 $[M-H_2O-Me]^+$, 281 $[M-2H_2O-Me]^+$; HR-EI-MS *m/z* 332.2337 (calcd for $C_{21}H_{32}O_3$, 332.2351); IR (KBr) ν_{max}: 3422 (br), 2922, 2853, 1737, 1647, 1459 and 1383 cm^{-1}; ^1H and ^{13}C NMR data see Tables 1 and 2.

Table 1
^{13}C NMR data of compounds **1**–**4** and **6** ($CDCl_3$, 125 MHz).

No.	1	2[a]	3	4[b]	6
1	37.3	38.0	44.8	37.0	40.9
2	31.3	32.1	72.5	31.4	69.2
3	71.3	72.0	76.2	71.3	72.4
4	42.0	42.9	39.2	42.0	31.8
5	146.5	146.8	139.6	146.3	38.2
6	123.7	124.9	121.8	123.8	27.5
7	65.2	65.8	31.8	65.3	34.2
8	36.5	38.1	30.4	37.4	34.3
9	42.3	43.3	50.0	42.1[c]	54.3
10	37.4	38.5	38.4	36.9	37.0
11	20.4	21.5	20.6	20.6	20.6
12	39.8	41.2	38.5	39.5	39.4
13	42.7	43.6	41.8	42.4[c]	42.3
14	47.7	48.8	50.6	49.6	53.8
15	36.9	37.9	37.9	24.4	37.8
16	74.2	74.5	219.3	22.4[d]	70.3
17	60.1	60.7	65.1	57.0	60.5
18	14.6	15.1	13.4	13.4	14.0
19	18.3	18.6	20.5	18.2	12.4
20	76.7	78.3	17.6	75.4	69.1
21	26.7	26.6	13.3	26.6[d]	20.1
22	42.6	43.2		42.5	166.7
23	29.4	30.2		23.8	136.9
24	156.4	40.7		46.1	125.3
25	33.9	33.4		29.1	18.5
26	21.9	20.7		19.6	
27	22.0	18.5		19.2	
28	106.3	16.0		23.0	
29				12.1	

The literature assignments [9] with the same superscripts (c, d) were revised herein.
[a] Measured in CD_3OD.
[b] Based on its DEPT and 2D NMR experiments and comparison with the literature [9].

2.4. Acetylation of compound 2

A solution of **2** (15.0 mg) in anhydrous pyridine (1 mL) was added to anhydrous acetic anhydride (0.5 mL), and the mixture was stirred at room temperature for 24 h. After evaporation of excess reagent under vacuum, the residue was chromatographed on silica gel with petroleum ether/EtOAc (8:1) to yield (20*S*)-3β,7α,16β-triacetoxy-5-ergostene-20-ol (**2a**) (6.5 mg). ^1H NMR ($CDCl_3$) of **2a**: δ 5.62 (1H, d, *J* = 5.0 Hz, H-6), 5.39 (1H, m, H-16α), 4.88 (1H, dd, *J* = 5.0, 3.8 Hz, H-7β), 4.67 (1H, m, H-3α), 2.36 (2H, m, H-4), 2.28 (1H, m, H-15), 2.09 (3H, s, -OAc), 2.05 (3H, s, -OAc), 2.04 (3H, s, -OAc), 1.70 (1H, m, H-8β), 1.55 (1H, m, H-25), 1.50 (1H, d, *J* = 6.8 Hz, H-17α), 1.40 (1H, m, H-14α), 1.29 (3H, s, Me-21), 1.27 (1H, m, H-9α), 1.20 (1H, m, H-24), 1.19 (1H, m, H-15′), 1.13 (3H, s, Me-18), 1.03 (3H, s, Me-19), 0.86 (3H, d, *J* = 6.5 Hz, Me-26), 0.80 (3H, d, *J* = 6.5 Hz, Me-27), and 0.79 (3H, d, *J* = 6.9 Hz, Me-28).

2.5. Cytotoxicity assay

The human lung cancer cell lines (A549, H460) and the human glioma cancer cell line (U251) were purchased from the cell bank of Shanghai Institute of Cell Biology (Shanghai, China). All cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 units/mL streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were maintained at 37 °C in a humidified 5% CO_2 environment. The cell viability was determined by using the standard Cell Counting Kit (CCK)-8 (Dojindu Laboratory, Japan) assay method [11]. Briefly, the cancer cells were seeded into 96-well plates at an initial density of 5×10^4 cells/well. After 24 h, the cells were treated with compounds at varying concentrations. Fluorouracil (5-FU, Sigma–Aldrich, catalog No. F6627) was used as a positive control. After incubation for 48 h, 10 μL of CCK-8 Kit reagent was added, and incubated for 1 h.

Table 2
^{1}H NMR data of compounds **1**–**3** ($CDCl_3$, 500 MHz, *J* in Hz).

No.	1	2[a]	3
1	1.08, 1H, m 1.85, 1H, m	1.12, 1H, m 1.86, 1H, m	1.09, 1H, overlapped 2.10, 1H, dd, 13.1, 4.5
2	1.50, 1H, m 1.82, 1H, m	1.50, 1H, m 1.84, 1H, m	3.68, 1H, ddd, 12.5, 9.0, 4.5
3	3.57, 1H, m	3.48, 1H, m	3.34, 1H, ddd, 10.4, 9.0, 6.4
4	2.32, 1H, m, overlapped 2.34, 1H, m, overlapped	2.28, 1H, m, overlapped 2.29, 1H, m, overlapped	2.35, 2H, m
6	5.60, 1H, d, 4.2	5.55, 1H, d, 5.0	5.41, 1H, brd, 5.0
7	3.84, 1H, dd, 4.2, 4.0	3.76, 1H, dd, 5.0, 3.8	1.66, 1H, m 2.00, 1H, m
8	1.58, 1H, m	1.52, 1H, m	1.67, 1H, m
9	1.20, 1H, m	1.25, 1H, m, overlapped	1.20, 1H, m
11	1.58, 2H, m	1.58, 2H, m	1.54, 1H, m 1.66, 1H, m, overlapped
12	1.17, 1H, m 2.20, 1H, m	1.16, 1H, m 2.13, 1H, m	1.40, 1H, m 1.93, 1H, m
14	1.30, 1H, m, overlapped	1.29, 1H, m, overlapped	1.45, 1H, dd, 13.1, 8.2
15	1.32, 1H, m 2.42, 1H, m	1.28, 1H, m, overlapped 2.40, 1H, m	1.78, 1H, dd, 18.0, 13.1 2.23, 1H, dd, 18.0, 8.2
16	4.68, 1H, brs	4.60, 1H, brs	
17	1.29, 1H, overlapped	1.30, 1H, d, overlapped	1.66, 1H, overlapped
18	1.16, 3H, s	1.14, 3H, s	0.72, 3H, s
19	1.01, 3H, s	1.02, 3H, s	1.08, 3H, s
20			1.27, 1H, m 1.66, 1H, overlapped
21	1.32, 3H, s	1.26, 3H, s	1.03, 3H, t, 6.8
22	1.78, 1H, m 1.88, 1H, m	1.51, 1H, m 1.90, 1H, m	
23	2.05, 1H, m 2.10, 1H, m	1.06, 1H, m 1.50, 1H, m	
24		1.24, 1H, m, overlapped	
25	2.25, 1H, m,	1.59, 1H, m, overlapped	
26	1.03, 3H, d, 6.9	0.90, 3H, d, 6.5	
27	1.04, 3H, d, 6.9	0.83, 3H, d, 6.5	
28	4.70, 1H, brs 4.74, 1H, brs	0.84, 3H, d, 6.4	

[a] Measured in CD_3OD.

The cell viability was obtained by scanning with a microplate reader at 450 nm. IC_{50} was calculated from the curves generated by plotting the percentage of the viable cells versus the test concentration on a logarithmic scale using SigmaPlot 10.0 software.

3. Results and discussion

The ethanol extract from the leaves of Chinese *M. azedarach* was successively subjected to column chromatography over silica gel, MCI-gel and Sephadex LH-20, and semipreparative HPLC to afford six steroids (**1**–**6**, Fig. 1). Comparing their MS and NMR data, as well as their physical properties, with those reported in the literature, the known steroids were identified as 5-stigmastene-3β,7α,20-triol (**4**) [9], 5-stigmastene-3β,7α-diol (**5**) [9], and 2α,3α,16β-trihydroxy-5α-pregnane 20*R*-methacrylate (azedarachol, **6**) [10]. Compounds **4** and **5** were obtained from this plant for the first time. Compound **4** was previously isolated from the fruits of *Ailanthus altissima* [9]. The *S* configuration at C-20 in **4** was assigned according to the proton chemical shift (*δ* 1.29, recorded in $CDCl_3$) of Me-21 [9]. Previous studies have documented that the proton chemical shift (recorded in $CDCl_3$) of Me-21 is around *δ* 1.10–1.20 when an *R* configuration is assigned to C-20, while this methyl signal is shifted downfield to *δ* 1.25–1.43 when C-20 is in an *S* configuration [12–15]. Therefore, the structure of **4** was updated as (20*S*)-5-stigmastene-3β,7α,20-triol (Fig. 1). During a reinvestigation of the ^{13}C NMR data of **4**, we wished to revise the assignments of the ^{13}C chemical shifts of C-9, C-13, C-16 and C-21 (Table 1) based on the current ^{13}C NMR, DEPT and 2D NMR experiments. Compound **6** was previously isolated from *M. azedarach* var. *japonica* [10]; nevertheless, its ^{13}C NMR data are reported herein for the first time (Table 1).

The molecular weight of compound **1** and its chemical formula of $C_{28}H_{46}O_4$ were determined from the positive mode HR-ESIMS, which resulted in an $[M+Na]^+$ ion peak at *m/z* 469.3271 ($C_{28}H_{46}O_4Na$ requires 469.3294). The IR spectrum of **1** showed strong absorptions (ν_{max}) at 3397 (hydroxyl groups), and 1647 cm^{-1} (double bonds). The ^{13}C NMR and DEPT spectra (Table 1) of compound **1** exhibited the presence of twenty-eight carbons classified as five methyls, one sp^2 (*δ* 106.3) and eight sp^3 methylenes, one sp^2 (*δ* 123.7) and eight sp^3 (three of which were oxygenated at *δ* 74.2, 71.3, 65.2) methines, two sp^2 (*δ* 156.4, 146.5) and three sp^3 (one of which was oxygenated at *δ* 76.7) non-protonated carbons. The ^{1}H NMR spectrum (Table 2) of **1** showed general features similar to those of the known steroid (**4**) [9]. The major differences between compounds **1** and **4** are that: (1) **1** has a terminal methylene with signals at *δ* 4.74 (1H, brs, H-28) and 4.70 (1H, brs, H-28′) instead of an ethyl group bonded to C-24 as in **4** [9]; (2) an additional oxymethine signal at *δ* 4.68 (1H, brs, H-16) was found in the lowfield of the ^{1}H NMR spectrum of **1**. These data together with its molecular formula indicated that **1** is an ergosterol derivative with three secondary hydroxyl groups at C-3 (*δ* 71.3), C-7 (*δ* 65.2), and C-16 (*δ* 74.2), a tertiary hydroxyl group at C-20 (*δ* 76.7), and a double bond between C-24 (*δ* 156.4) and C-28 (*δ* 106.3) in the side chain. These structural features were corroborated by analyzing its ^{1}H–^{1}H COSY, HSQC and HMBC spectra.

Based on biogenetic considerations and by comparison with the known 3β-hydroxylated steroids **4** and **5** [9], an identical stereochemistry at C-3 should be expected. Since H-8 is β-axial, the small coupling constant of H-8 with H-7 ($J_{ax.eq.}$ = 4.0 Hz), as well as the chemical shift of the last hydrogen (*δ* 3.84), indicated the presence of an α alcohol at C-7. In the ^{13}C NMR spectrum of **1**, the highly deshielded non-oxygenated methine of C-17 at *δ* 60.1 enabled us to propose the hydroxyl group at C-16 to be in the β-orientation. Therefore, the *S* configuration at C-16 was established, which is consistent with those found for the previously isolated steroids [12,16–18]. Similar to compound **4**, the *S* configuration at C-20 was determined by the observed proton chemical shift (*δ* 1.32) of Me-21. The predicted configurations at C-16 and C-20 (Fig. 1) implied that a hydrogen bond may be easily formed between the hydroxyl groups at C-16 and C-20, hence explaining the unexpected highly upshifted resonance of C-17 [12,17]. Thus, compound **1** is elucidated to be (20*S*)-5,24(28)-ergostadiene-3β,7α,16β,20-tetrol.

The ^{1}H and ^{13}C NMR data (Tables 1 and 2) of compound **2** were generally similar to those of compound **1**. The apparent difference between **2** and **1** is that **2** has one more secondary methyl group at *δ* 0.84 (3H, d, *J* = 6.4 Hz, Me-28) instead of the terminal methylene group linked at C-24 in the side chain of **1** (Table 2). Therefore, **2** has one degree of unsaturation less than that of **1**, which can be confirmed by its chemical formula of $C_{28}H_{48}O_4$. This was obtained from an $[M+Na]^+$ ion peak at *m/z* 471.3455 in its positive mode HR-ESIMS. Compound **2** was successfully acetylated with acetic anhydride in pyridine to afford a triacetate (**2a**) at the C-3, C-7 and C-16 secondary hydroxyl groups of **2** in good yield. The overlapping of signals between *δ* 1.28 and 1.30 in the highfield of ^{1}H NMR spectrum of **2** (Table 2) prevented clear NOE correlations among H-14, H-16 and H-17 in the NOESY spectrum being acquired. However, the β-orientation of the hydroxyl group at C-16 in **2a** could

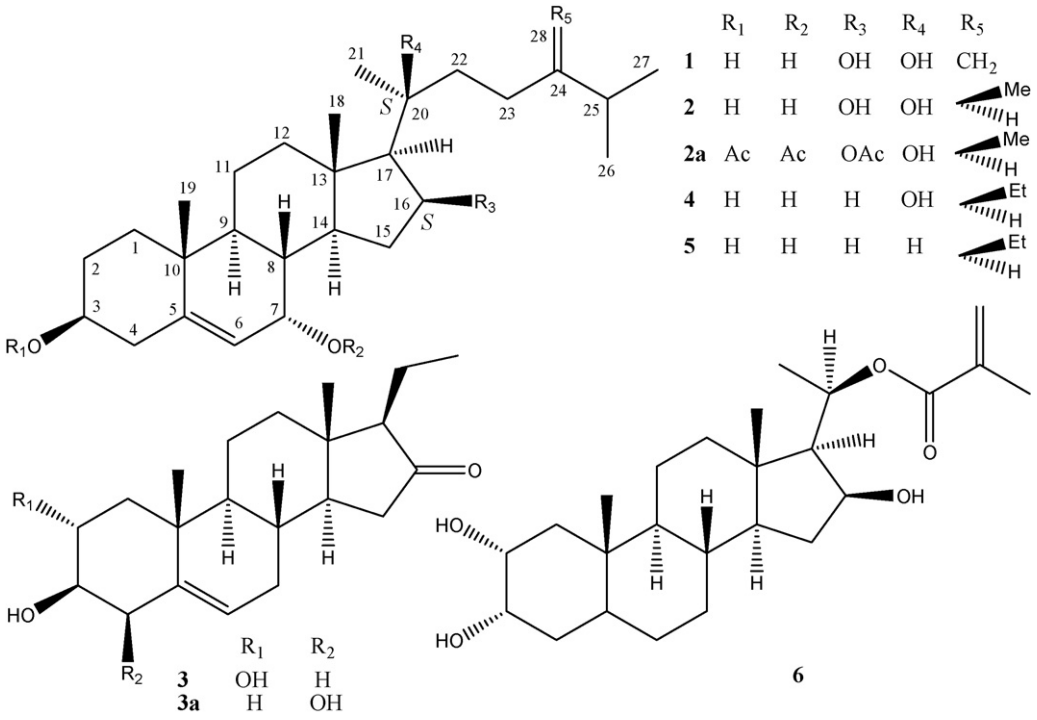

Fig. 1. Chemical structures of compounds **1**–**6**.

be unambiguously established by the observed NOE correlations between H-16α at δ 5.39 and H-14α at δ 1.40, and between H-16α and H-17α at δ 1.50 in the NOESY spectrum of **2a**. Accordingly, compound **2** was deduced to be (20*S*)-5-ergostene-3β,7α,16β, 20-tetrol.

In the HR-EIMS spectrum, compound **3** showed a molecular ion peak $[M]^+$ at *m*/*z* 332.2337 corresponding to its molecular formula $C_{21}H_{32}O_3$. The ^{13}C NMR and DEPT spectra (Table 1) of compound **3** accounted for twenty-one carbons classified as three methyls, seven sp^3 methylenes, one sp^2 (δ 121.8) and six sp^3 (two of which were oxygenated at δ 72.5, 76.2) methines, two sp^3 and two sp^2 (δ 139.6, 219.3) non-protonated carbons. The IR spectrum of **3** displayed absorptions due to the presence of hydroxyl (3422 cm^{-1}), carbonyl (1737 cm^{-1}), and double bond (1647 cm^{-1}) functional groups. In its highfield of ^1H NMR spectrum (Table 2), the signals of two tertiary methyls at δ 0.72 (3H, s, Me-18) and 1.08 (3H, s, Me-19), and one primary methyl at δ 1.03 (3H, t, *J* = 6.8 Hz, Me-21) were detected. Comparison of the above data with that of the known steroids **6** [10] and ekeberin B (**3a**) [19] suggested that compound **3** is a pregnane derivative (Fig. 1).

Detailed 1D and 2D NMR (COSY, HSQC and HMBC) analysis of **3** led to the assignments of two secondary hydroxyl groups each located at C-2 [H-2β: δ 3.68 (1H, ddd, *J* = 12.5, 9.0, 4.5 Hz)] and C-3 [H-3α: 3.34 (1H, ddd, *J* = 10.4, 9.0, 6.4 Hz)], one double bond at C-5 [H-6: δ 5.41 (1H, brd, *J* = 5.0 Hz)], and a ketone at C-16. In the ^1H–^1H COSY spectrums of **3**, a spin system was found between H_2-1 and H-2, between H-2 and H-3, between H-3 and H-4, and between H-4 and H-6 via a long-range allylic coupling. As depicted in Fig. 2, the relative stereochemistry at C-2 and C-3 in ring A was determined by the split patterns and coupling constants observed for both H-2 and H-3, which were in good agreement with those of atratogenin A (**3a**) (Table 2), a 2α,3β-dihydroxyl steroid previously isolated from the root of *Cynanchum atratum* [20]. This inference was further supported by the NOESY spectrum of **3**, in which clear NOE correlations (Fig. 2) between H-2β at δ 3.68 and H-19 at δ 1.08, between H-3α at δ 3.34 and H-1α at δ 2.10, as well as between H-1α and H-9 at δ 1.20 were observed. Similarly to ekeberin B (**3a**) [19], the relative stereochemistry at C-17 was also confirmed by the NOE correlations observed between the singlet methyl signal of H-18 at δ 0.72 and the signals at 1.27/1.66 attributed to the methylene at C-20 (Fig. 2). Therefore, compound **3** is defined as 2α,3β-dihydro-5-pregnen-16-one.

The isolated steroids (**1**–**6**) were tested against a small panel of human cancer cell lines: A549 and H460 (human lung carcinoma), and U251 (human glioma), for their *in vitro* cytotoxicity, by using the CCK-8 assay method. As summarized in Table 3, compounds **1**, **2**

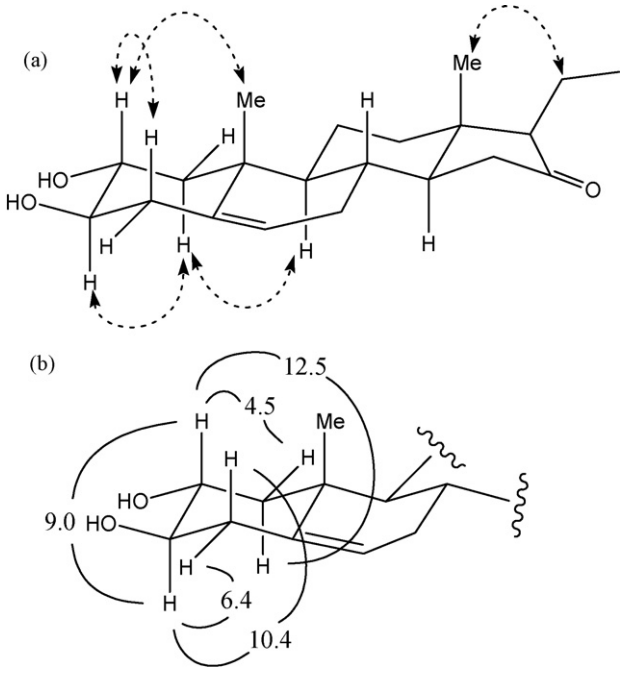

Fig. 2. (a) Key NOESY correlations of **3**; (b) the coupling constants (Hz) of ring A in **3**.

Table 3
The IC_{50}s (μg/mL) of compounds **1**–**6** against human cancer cell lines (A549, H460 and U251).[a].

Compound	A549	H460	U251
1	30.1 ± 1.3	29.5 ± 3.4	12.0 ± 0.8
2	24.5 ± 2.1	13.3 ± 0.6	18.8 ± 3.4
3	NA	NA	NA
4	>80	22.8 ± 2.5	24.0 ± 3.0
5	NA	NA	NA
6	NA	NA	NA
5-FU	6.7 ± 0.7	15.1 ± 1.2	6.7 ± 0.8

[a] The results were expressed as mean ± SEM. NA: Not active.

and **4** showed significant cytotoxic effects, with IC_{50}s ranging from 12.0 to 30.1 μg/mL. It seems that a free hydroxyl group at C-20 is pivotal for their cytotoxic properties. It is worthy to notice that in 2005, J. H. Sheu's group reported that free hydroxyl groups attached at both C-11 and C-20 in several hippuristanol-type steroids could significantly enhance their cytotoxicity against the proliferation of the Hep G2, Hep 3B, MCF-7, and MDA-MB-231 cancer cell lines [21].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2009.04.005.

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