Cytotoxic Limonoids from *Melia azedarach*

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Abstract

Five new compounds (**1**–**5**), including two limonoids, one triterpenoid, one steroid, and one sesquiterpenoid, along with nine known limonoids (**6**–**14**), were isolated from the bark of *Melia azedarach*. The structures of the new compounds were elucidated by 2D NMR spectroscopy and mass spectrometry. The isolated compounds as well as three acetylated derivatives of **9** were evaluated for their cytotoxicities against five human tumor cell lines (HL-60, SMMC-7721, A-549, MCF-7, and SW480) by an MTT assay. Seven limonoids (**1, 6, 7, 8, 9, 9b**, and **9c**) showed significant inhibitory activities against tested cell lines with IC_{50} values ranging from 0.003 to 0.555 µM, and their preliminary structure-activity relationships are also discussed.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction

Limonoids with highly diverse structures and a broad range of bioactivities have become a hot topic in the fields of natural products and synthetic chemistry [1,2]. The genus *Melia* (Meliaceae) comprises three species in the world and is widely distributed in Asia and the south of tropical Africa [3]. As a traditional Chinese medicine, the bark of *Melia azedarach* Linn. was extensively used as an anthelmintic [3,4]. Previous studies on the genus *Melia* have led to the isolation of a variety of structurally diverse compounds including triterpenoids, steroids, and limonoids. These compounds showed significant biological properties such as antifeedant, insecticidal, antiviral, and cytotoxic activities, which have motivated natural product researchers to search for potential drug leads [4–8]. In our continuing search for the bioactive metabolites from the Meliaceae family [9–11], five new compounds, including two limonoids, mesendanins K (**1**) and L (**2**), one triterpenoid, mesendanin M (**3**), one steroid, 17*β*,20*β*-epoxyergosta-5,24(28)-diene-3*β*,16*β*,22*α*-triol (**4**), and one sesquiterpenoid, 1*α*,4*α*,6*β*-trihydroxyeudesmane (**5**), along with nine known compounds (**6**–**14**), were isolated from the bark of *M. azedarach*. In addition, three acetylated derivatives (**9a**–**9c**) were obtained from compound **9**. All these isolates and the three acetylated derivatives were evaluated for their cytotoxic activities against five human tumor cell lines (HL-60, SMMC-7721, A-549, MCF-7, and SW480) by an MTT assay [12]. In this paper, we present the isolation, structural elucidation, and bioassay results of all the compounds (**Fig. 1**).

Materials and Methods

General experimental procedures

Optical rotations were determined with a JASCO P-1020 polarimeter. IR spectra were measured in a Bio-Rad FTS-135 spectrometer with KBr pellets, whereas UV data were measured using a UV-2401A spectrometer. 1D NMR and 2D NMR were recorded on a Bruker AM-500 spectrometer and a Bruker AM-400 instrument. ESIMS and HRESIMS were measured with a Finnigan MAT 90 instrument and VG Auto Spec-3000 spectrometer, respectively. Semipreparative HPLC was performed on a Merck column (i.d. 10–100 mm; Merck). Column chromatography was performed on silica gel (100–200, 200–300, and 300–400 mesh; Qingdao Marine Chemical, Inc.), MCI gel (CHP 20P, 75–150 µm; Mitsubishi Chemical Industries Ltd.), C_{18} reversed-phase silica gel (25–45 µm; Merck), and Sephadex LH-20 (40–70 µm;

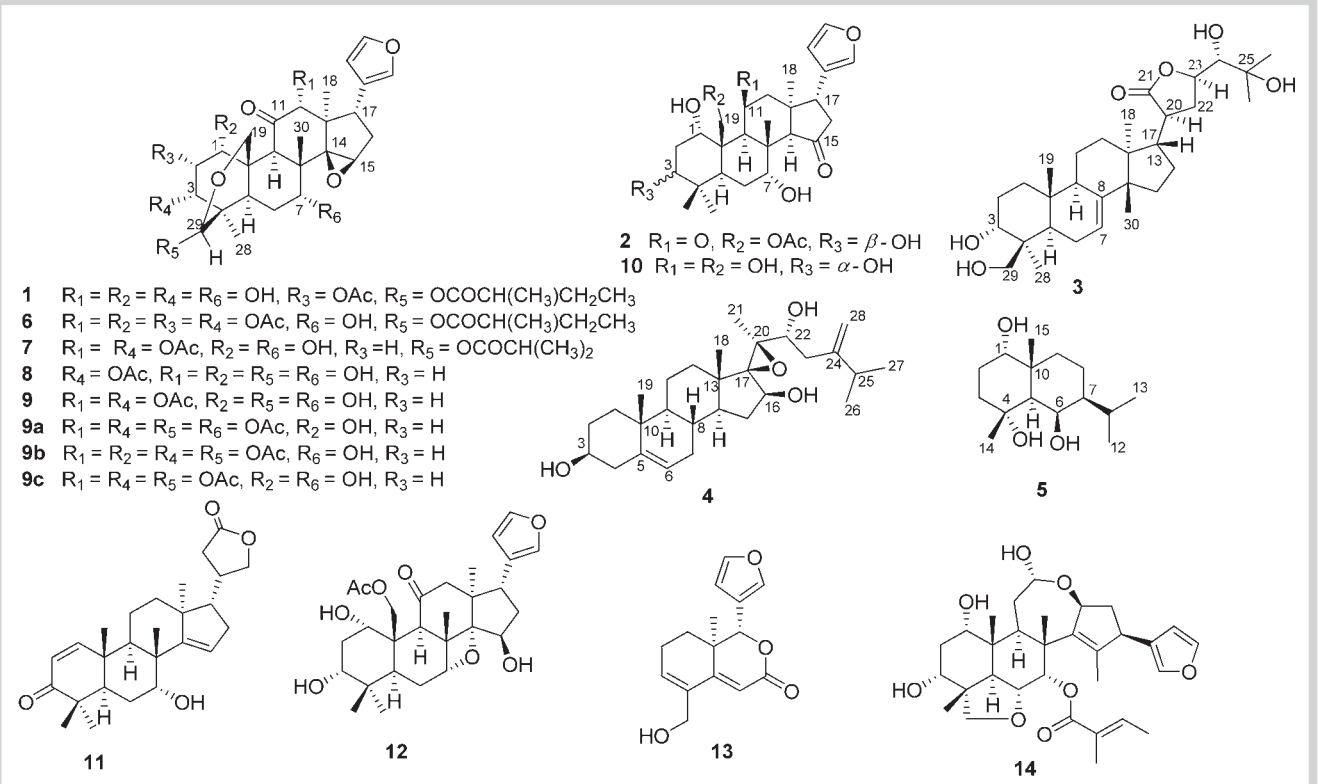

Fig. 1 Chemical structures of compounds **1**–**14**.

Amersham Pharmacia Biotech AB). TLC plates were precoated with silica gel GF_{254} and HF_{254} (Qingdao Haiyang Chemical Plant).

Plant material

The dried bark of *M. azedarach* (8.6 kg) was collected in Shanxi province of China in September 2010 and was identified by one of the authors (G.-H.T.). A voucher specimen (KIB H20101009) was deposited at the Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and isolation

The air-dried powdered bark of *Melia azedarach* (8.6 kg) was extracted with 90% EtOH (20 L × 3) under reflux three times (4, 3, and 3 h, respectively) at 60 °C. The combined EtOH extracts were concentrated under vacuum to give a crude residue (600 g), which was suspended in water. The water layer was successively partitioned with petroleum ether (7 L × 3) and EtOAc (7 L × 4). The EtOAc portion (220 g) was chromatographed on a silica gel column (100–200 mesh, 10 × 100 cm) eluted with petroleum ether/acetone (v/v 9 : 1, 8 : 2, 7 : 3, 6 : 4, and 1 : 1, each 7 L) and then $CHCl_3/CH_3OH$ (v/v 9 : 1, 8 : 2, 7 : 3, and 0 : 1, each 7 L) to yield nine fractions (A–I). Fraction D (15 g) was subjected to an MCI gel column (75–150 µm, 5.5 × 48 cm) eluted with a gradient MeOH–H_2O (v/v 5 : 5, 8 : 2, 9 : 1, and 1 : 0, each 5 L), further purified on Sephadex LH-20 (40–70 µm, 1.5 × 140 cm, eluted with MeOH) and repeated silica gel columns to yield **5** (10 mg), **11** (23 mg), **4** (7 mg), and **10** (4 mg). Fraction E (15 g) was separated over an MCI gel column (75–150 µm, 5.5 × 48 cm) eluted with a gradient MeOH–H_2O (v/v 5 : 5, 8 : 2, 9 : 1, and 1 : 0, each 5 L) to give five fractions (E1–E5). Fraction E2 (8.5 g) was applied to a C_{18} silica gel column (20–45 µm, 4 × 48 cm) eluted with a gradient MeOH–H_2O (v/v 4 : 6, 5 : 5, and 6 : 4, each 2 L) to afford three fractions (E2a1–E2a3). Fraction E2a1 (300 mg) was subjected to a silica gel column (300–400 mesh, 2.5 × 18 cm) eluted with petroleum ether/acetone (v/v 7 : 3) to yield **2** (10 mg) and **14** (10 mg). Fraction E2a2 (500 mg) was separated by Sephadex LH-20 (40–70 µm, 1.5 × 140 cm) eluted with MeOH and then applied to a silica gel column (300–400 mesh, 2.5 × 18 cm) eluted with petroleum ether/EtOAc (v/v 8 : 2) to yield **13** (17 mg). Fraction E3 (400 mg) was chromatographed over a silica gel column (300–400 mesh, 2.5 × 18 cm) eluted with chloroform/acetone (v/v 100 : 2, 100 : 5, 100 : 7, and 100 : 10, each 1 L) to give three parts, each of which was purified by Sephadex LH-20 (40–70 µm, 1.5 × 140 cm, eluted with MeOH) to obtain **8** (10 mg), **6** (8.5 mg), and **7** (5.5 mg). Fraction F (20 g) was first applied to a C_{18} silica gel column (75–150 µm, 4 × 48 cm) eluted with a gradient MeOH/H_2O (v/v 5 : 5, 6 : 4, and 7 : 3, each 7 L) to give five fractions (F1–F8). Fraction F5 (80 mg) was then purified by semipreparative HPLC using MeOH/H_2O (45%) as the mobile phase (3.5 mL/min) to yield **12** (30 mg, t_R 15 min) and **9** (500 mg, t_R 23 min). Fraction G (4.5 g) was subjected to a silica gel column (200–300 mesh, 4.5 × 20 cm) eluted with chloroform/acetone (v/v 9 : 1, 8 : 2, 7 : 3, and 6 : 4, each 3 L) to afford three fractions (G1–G3), and fraction G1 (300 mg) was purified on a silica gel column (300–400 mesh, 2 × 18 cm) eluted with chloroform/acetone (v/v 8 : 2) to yield **3** (21 mg) and **1** (4.8 mg). The purity of compounds **1**–**14** was greater than 95% as determined by TLC and NMR spectra.

*Preparation of **9a, 9b**, and **9c***: Compound **9** (80 mg) was treated with 3 mL $(Ac)_2O$ in 1 mL pyridine for 12 h at room temperature, and then 20 mL water was added to the mixture. The mixture

Table 1 ^{1}H NMR (500 MHz) and ^{13}C NMR (100 MHz) spectroscopic data for **1** and **2**.

No.	1[a]		2[b]	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1	4.06 (d, 2.9)	73.0	4.28 (t, 2.7)	68.6
2*α*	5.71 (t, 2.9)	70.8	1.70 (dt, 15.0, 2.7)	30.5
2*β*			2.14 (dd, 15.0, 7.3)	
3	4.54 (d, 2.9)	72.7	3.24 (dd, 7.3, 2.7)	75.5
4		41.4		37.2
5	2.69 (dd, 14.0, 4.3)	26.3	2.46 (m)	32.9
6*α*	1.68 (m)	25.5	1.49 (m)	23.9
6*β*	1.95 (d, 14.0)		1.83 (t, 14.5)	
7	3.58 (br s)	70.0	4.03 (s)	68.5
8		42.3		43.7
9	4.47 (s)	47.6	2.69 (s)	50.9
10		42.3		48.9
11		213.5		211.1
12*α*	4.01 (s)	78.8	2.10 (d, 14.6)	51.4
12*β*			3.10 (d, 14.6)	
13		46.1		41.1
14		72.7	2.69 (s)	59.8
15	3.69 (s)	59.1		217.4
16*α*	2.26 (dd, 13.2, 6.2)	33.0	2.79 (m)	43.0
16*β*	1.83 (dd, 13.2, 10.8)		2.37 (m)	
17	2.92 (dd, 10.8, 6.2)	38.7	3.30 (m)	42.4
18	1.04 (s, 3H)	14.3	0.73 (s, 3H)	26.3
19a	4.24 (br s)	63.9	4.43 (d, 12.3)	63.1
19b	4.23 (br s)		4.01 (d, 12.3)	
20		123.5		123.1
21	7.14 (s)	140.6	7.57 (s)	140.3
22	6.43 (s)	112.7	6.50 (s)	111.0
23	7.25 (s)	142.2	7.76 (s)	143.3
28	0.91 (s, 3H)	19.4	0.83 (s, 3H)	28.7
29	5.69 (m)	94.1	0.69 (s, 3H)	21.5
30	1.06 (s, 3H)	22.6	1.20 (s, 3H)	19.4
CH_3CO		169.6		170.2
	2.08 (s, 3H)	21.1	1.95 (s, 3H)	20.6
1′		175.4		
2′	2.38 (m)	41.1	1-OH 4.95 (d, 6.0)	
3′a	1.59 (m)	26.5	3-OH 4.80 (d, 7.4)	
3′b	1.46 (m)		7-OH 4.72 (d, 3.7)	
4′	1.10 (d, 7.0, 3H)	16.3		
5′	0.85 (t, 7.4, 3H)	11.4		

[a] Recorded in $CDCl_3$, [b] Recorded in DMSO-d_6

was extracted with EtOAc (20 mL × 3). The EtOAc part (100 mg) was chromatographed on a silica gel column (300–400 mesh, 1.5 × 20 cm) eluted with chloroform/acetone (v/v 200 : 1 and 100 : 1, each 400 mL) to yield **9a** (4.0 mg), **9b** (4.2 mg), and **9c** (15.0 mg).

Mesendanin K (**1**): white amorphous power; $[\alpha]_D^{25}$ − 60.2 (*c* 0.12, CH_3OH); UV (MeOH) λ_{max} (log ε) nm 210 (3.07); IR (KBr) ν_{max} 3448, 2929, 1742, 1716, 1238, 1067 cm^{-1}; NMR data, see **Table 1**; positive ESIMS *m/z* 655 [M + Na]$^+$; HRESIMS *m/z* 655.2732 [M + Na]$^+$ (calcd. for $C_{27}H_{30}O_{10}Na$, 655.2730).

Mesendanin L (**2**): white amorphous power; $[\alpha]_D^{25}$ − 51.8 (*c* 0.23, CH_3OH); UV (MeOH) λ_{max} (log ε) nm 210 (2.94); IR (KBr) ν_{max} 3442, 2957, 2926, 1744, 1679, 1233, 1070 cm^{-1}; NMR data, see **Table 1**; positive ESIMS *m/z* 525 [M + Na]$^+$; HRESIMS *m/z* 525.2460 [M + Na]$^+$ (calcd. for $C_{28}H_{38}O_8Na$, 525.2464).

Mesendanin M (**3**): white amorphous power; $[\alpha]_D^{25}$ − 122.4 (*c* 0.09, CH_3OH); UV (MeOH) λ_{max} (log ε) nm 209 (3.39); IR (KBr) ν_{max} 3431, 2946, 2881 1771, 1377, 1170, 1025 cm^{-1}; NMR data, see **Table 2**; positive ESIMS *m/z* 527 [M + Na]$^+$; HRESIMS *m/z* 527.3355 [M + Na]$^+$ (calcd. for $C_{27}H_{30}O_{10}Na$, 527.3348).

17β,20β-Epoxyergosta-5,24(28)-diene-3β,16β,22α-triol (**4**): white amorphous power; $[\alpha]_D^{25}$ − 33.8 (*c* 0.12, CH_3OH); UV (MeOH) λ_{max} (log ε) nm 206 (2.74); IR (KBr) ν_{max} 3439, 2957, 2930, 1634, 1075, 581 cm^{-1}; NMR data, see **Table 2**; positive ESIMS *m/z* 467 [M + Na]$^+$; HRESIMS *m/z* 467.3128 [M + Na]$^+$ (calcd. for $C_{28}H_{44}O_4Na$, 467.3137).

1α,4α,6β-Trihydroxyeudesmane (**5**): white amorphous power; $[\alpha]_D^{25}$ − 8.4 (*c* 0.13, CH_3OH); UV (MeOH) λ_{max} (log ε) nm 236 (2.33), 201 (2.52); IR (KBr) ν_{max} 3441, 1631 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ_H 4.57 (1H, br s, H-6*α*), 3.21 (1H, br s, H-1*β*), 1.86 (1H, m, H-2b), 1.79 (1H, m, H-3b), 1.67 (1H, m, H-9b), 1.62 (1H, m, H-2a), 1.57 (1H, m, H-8b), 1.47 (1H, m, H-3a), 1.47 (1H, m, H-5*α*), 1.46 (1H, m, H-8a), 1.46 (3H, s, H-14), 1.46 (1H, m, H-11), 1.12 (1H, m, H-9a), 1.11 (3H, s, H-15), 0.90 (3H, d, *J* = 6.6 Hz, H-12), 0.88 (3H, d, *J* = 6.6 Hz, H-13), 0.82 (1H, m, H-7*α*); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 75.0 (CH, C-1), 72.8 (C, C-4), 66.9 (CH, C-6), 50.7 (CH, C-5), 50.5 (CH, C-7), 39.1 (C, C-10), 38.0 (CH_2, C-3), 37.0 (CH_2, C-9), 28.8 (CH, C-11), 27.1 (CH_2, C-2), 25.3 (CH_3, C-14), 21.8 (CH_3, C-15), 21.1 (CH_3, C-13), 20.6 (CH_3, C-12), 20.5 (CH_2, C-

Table 2 ^{1}H NMR (500 MHz) and ^{13}C NMR (100 MHz) spectroscopic data for **3** and **4**.

No.	3[a]		4[b]	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1a	1.38 (m)	32.4	1.04 (m)	37.3
1b	1.56 (m)		1.82 (m)	
2a	1.59 (m)	26.2	1.49 (m)	31.7
2b	1.91 (m)		1.82 (m)	
3	3.86 (br s)	71.1	3.50 (m)	71.9
4a		43.5	2.20 (m)	42.3
4b			2.28 (m)	
5	1.92 (m)	46.7		140.9
6a	1.93 (m)	24.7	5.34 (t, 2.3)	121.3
6b	2.08 (m)			
7a	5.27 (br s)	119.5	1.82 (m)	31.7
7b			2.06 (m)	
8		146.9	1.60 (m)	31.2
9	2.45 (d, 10.5)	50.0	0.95 (m)	49.8
10		35.7		36.7
11a	1.38 (m)	32.4	1.43 (m)	21.1
11b	1.56 (m)		1.58 (m)	
12α	1.76 (m, 2H)	32.3	1.42 (m)	36.5
12b			1.85 (m)	
13		44.8		42.8
14		51.7	1.06 (m)	49.0
15a	1.52 (m)	35.0	1.37 (m, 2H)	34.2
15b	1.60 (m)			
16a	1.59 (m)	24.7	3.73 (t, 7.8)	69.2
16b	1.79 (m)			
17	2.35 (m)	48.6		77.5
18	0.89 (s, 3H)	23.9	0.93 (s, 3H)	15.6
19	0.78 (s, 3H)	14.3	0.99 (s, 3H)	19.5
20	2.82 (dt, 11.2, 5.6)	42.0		70.5
21		181.4	1.51 (s, 3H)	14.7
22	2.27 (m, 2H)	29.3	3.56 (t, 6.7)	71.1
23	4.67 (ddd, 9.6, 6.3, 3.3)	80.3	2.16 (m, 2H)	38.9
24	3.61 (d, 3.3)	78.1		151.6
25		72.8	2.19 (m)	33.5
26	1.23 (s, 3H)	26.8	1.01 (d, 6.7, 3H)	22.1
27	1.21 (s, 3H)	26.4	1.03 (d, 6.7, 3H)	21.9
28a	1.02 (s, 3H)	22.4	4.89 (br s)	110.3
28b			4.83 (br s)	
29a	3.78 (d, 11.3)	65.6		
29b	3.53 (d, 11.3)			
30	1.05 (s, 3H)	27.9		

[a] Recorded in CD_3OD, [b] Recorded in $CDCl_3$

8); positive ESIMS *m/z* 279 $[M + Na]^+$; HRESIMS *m/z* 279.1932 $[M + Na]^+$ (calcd. for $C_{15}H_{28}O_3Na$, 279.1936).

Bioassays

Cytotoxicity bioassays: HL-60 (myeloid leukemia), SMMC-7721 (hepatocellular carcinoma), A-549 (lung cancer), MCF-7 (human breast adenocarcinoma), and SW480 (colorectal cancer) cell lines (Shanghai Cell Bank) were cultured in RPMI 1640 or DMEM medium (Hyclone) supplemented with 10% fetal bovine serum (Hyclone) at 37 °C. The cytotoxicity assay was performed by the MTT method [12] using cisplatin (Sigma, purity ≥ 99.9%) as a positive control. The IC_{50} values were calculated by the Reed and Muench method [12].

Supporting information

The MS, IR, 1D and 2D NMR spectra of compounds **1**–**5**, selected ROESY correlations of **1**–**5**, and HMBC correlations of **1** and **2** are available as Supporting Information.

Results and Discussion

Mesendanin K (**1**) was found to possess the molecular formula $C_{33}H_{44}O_{12}$, as deduced by HRESIMS with 12 degrees of unsaturation. The ^{13}C NMR data along with DEPT experiments showed 33 carbon signals, including six methyls, four methylenes, 14 methines (three olefinic ones), and nine quaternary carbons (one olefinic and three carbonyl ones). The aforementioned information together with the characteristic hemiketal methine signal [δ_H 5.69 (1H, m); δ_C 94.1] indicated that **1** was a trichilin-type limonoid [13]. Extensive analysis of the 1D and 2D NMR data of **1** suggested a high similarity between **1** and 12-deacetyltrichilin I [13], except for the location of an acetyl. The acetoxyl was placed at C-2, and a hydroxyl was located at C-3, which were readily supported by the key HMBC correlation of H-2/OAc-2 as well as the ^{1}H–^{1}H COSY correlations observed between H-2 with H-1 and H-3 (see Supporting Information). Thus, the gross structure of **1** was constructed as depicted.

Compounds	IC_{50} (μM)				
	HL-60	SMMC-7721	A-549	MCF-7	SW480
1	0.020 ± 0.001	0.300 ± 0.021	0.160 ± 0.021	0.010 ± 0.0003	0.050 ± 0.002
6	0.555 ± 0.060	0.364 ± 0.086	0.934 ± 0.197	0.060 ± 0.028	0.043 ± 0.014
7	0.219 ± 0.019	0.233 ± 0.011	0.081 ± 0.011	0.003 ± 0.001	0.005 ± 0.001
8	0.235 ± 0.009	0.302 ± 0.007	0.221 ± 0.034	0.125 ± 0.041	0.107 ± 0.009
9	0.219 ± 0.002	0.292 ± 0.007	0.178 ± 0.012	0.030 ± 0.011	0.034 ± 0.009
9a	10.170 ± 1.650	10.010 ± 0.850	11.810 ± 1.060	10.300 ± 0.640	9.980 ± 0.680
9b	0.225 ± 0.007	0.288 ± 0.022	0.273 ± 0.013	0.208 ± 0.004	0.181 ± 0.005
9c	0.249 ± 0.010	0.303 ± 0.010	0.303 ± 0.010	0.004 ± 0.001	0.026 ± 0.015
2	14.590 ± 0.140	> 40	> 40	> 40	> 40
3	17.800 ± 0.440	> 40	> 40	> 40	> 40
Cisplatin[b]	1.140 ± 0.058	14.480 ± 0.615	12.730 ± 1.175	13.450 ± 1.156	13.630 ± 1.215

Table 3 Cytotoxicity of compounds **1**–**14** against tested cell lines[a].

[a] Compounds **4**–**5** and **10**–**14** were inactive against all cell lines tested (IC_{50} > 40 μM). [b] Positive control

The relative stereochemistry of **1** was assigned by the ROESY spectrum, in which correlations of H-2/H_2-19, H_2-19/H-1, H_2-19/Me-30, Me-30/H-12, H-12/H-17, H-7/Me-30, and H-29/H-6*β* indicated that those groups were cofacial and were arbitrarily assigned as *β*-oriented. The H-3 was inferred to be *β*-oriented on the basis of the small coupling constant ($J_{2,3}$ = 2.9 Hz) between H-2 and H-3, indicating that OH-3 was *α*-oriented. Meanwhile, the *α*-orientations of Me-28, H-5, H-9, Me-18, and H-15 were established by the ROESY cross-peaks between H-5 with H-9 and Me-28, and Me-18 with H-9 and H-15. Therefore, the relative configuration of **1** was elucidated as shown (see Supporting Information).

Mesendanin L (**2**) was obtained as a white powder, and its molecular formula was determined as $C_{28}H_{38}O_8$ in agreement with the $[M + Na]^+$ ion peak at *m/z* 525.2460 in HRESIMS. Further analysis of the 1D NMR data of **2** (◉ **Table 1**) indicated that **2** was similar to mesendanin J [14]. The differences were the presence of a ketone carbonyl at C-15 and a *β*-oriented hydroxyl at C-3, which were assumed by the HMBC correlations of H-17/C-15 (δ_C 217.4) and H-14/C-15, and the crucial ROESY correlation observed from H-3 to H-5 as well as the large coupling constant ($J_{2,3}$ = 7.3, 2.7 Hz) between H-2 and H-3, respectively (see Supporting Information). Therefore, the structure of **2** was established as shown.

Mesendanin M (**3**) gave the molecular formula $C_{30}H_{48}O_6$, as determined by HRESIMS. By comparing the NMR data of **3** (◉ **Table 2**) with those of 21*α*-methylmelianodiol [15], it was evident that they were structural analogues with the differences being in the appearance of an oxygenated methine, a lactone carbonyl, and an oxygenated methylene in the former. The hydroxyl located at C-3 and the lactone carbonyl located at C-21 were assumed by the HMBC correlations from Me-28 and H_2-29 to C-3 (δ_C 71.1), and from H-17 and H_2-22 to C-21 (δ_C 181.4), respectively. In addition, the oxygenated methylene was attached to C-4 by the HMBC correlations of Me-28 and H-5 with C-29. A broad singlet for the H-3 signal at 3.86 indicated the axial OH-3, while the *O*-bearing CH_2 group adopted the *β*-orientation from the ROESY cross-peak of H_2-29/Me-19. The free rotation of C-23/C-24 was fairly fixed due to the stereo-hindrance of the five-membered lactone ring and the side chain at C-23, which was supported by the key ROESY correlations of H_2-22/H-24 and H-23/H-24 along with the small coupling constant ($J_{23,24}$ = 3.3 Hz) between H-23 and H-24. The above data suggested that H-24 took a *β*-orientation (see Supporting Information) [16, 17]. Accordingly, the compound was assigned as shown.

Compound **4** had a quasimolecular ion peak $[M + Na]^+$ at *m/z* 467.3137 in HRESIMS, corresponding to the molecular formula $C_{28}H_{44}O_4$ with seven degrees of unsaturation. The 1D NMR data of **4** resembled those of 3*α*,16*β*,20,22-tetrahydroxyergosta-5,24(28)-diene [18], except for the occurrence of an oxygenated quaternary carbon. The oxygenated quaternary carbon at C-17 was confirmed by the HMBC correlations of Me-18 and Me-21 with C-17. Comparison of ^{13}C NMR data of **4** with those of 17*β*,20*β*-epoxy-23,24-dimethylcholest-5-ene-3*β*,22-diol [19] implied the existence of a 17*β*,20*β*-epoxy ring and a *β*-oriented hydroxyl at C-3. The free rotation of C-20/C-22 was fairly fixed because of the stereo-hindrance of the 17*β*,20*β*-epoxy ring and the side chain at C-20. This was indicated by the key ROESY correlations of H-22/H-16 and H_2-23/Me-21 as well as the large coupling constant ($J_{22,23}$ = 6.7 Hz) between H-22 and H_2-23, suggesting that H-22 was *β*-oriented (see Supporting Information). Thereby, the compound was constructed as 17*β*,20*β*-epoxyergosta-5,24(28)-diene-3*β*,16*β*,22*α*-triol.

The molecular formula of compound **5** was defined as $C_{15}H_{28}O_3$ by means of HRESIMS. The NMR data of **5** showed many similarities to 1*β*,4*β*,6*β*-trihydroxyeudesmane [20]. The difference was the orientation of OH-1 and OH-4. The *β*-orientation of H-1 and Me-14 was deduced from the strong cross-peaks of H-1*β*/Me-15 and Me-15/Me-14 in the ROESY spectrum, then, OH-1 and OH-4 were *α*-oriented. The ROESY correlation of H-5/H-7 assigned the *α*-orientation of H-5 and H-7. In addition, the *α*-oriented H-6 was assumed by the broad singlet of H-6 along with the ROESY correlation between H-5 and H-6 (see Supporting Information). Thus, the stereochemistry of **5** was established as 1*α*,4*α*,6*β*-trihydroxyeudesmane.

The known compounds, 1,12-diacetyltrichilin B (**6**) [13], 29-*iso*-butylsendanin (**7**) [21], 12-hydroxyamoorastin (**8**) [21], 29-deacetylsendanin (**9**) [21], 7-acetylsendanin (**9a**) [21], 1-acetylsendanin (**9b**) [21], sendanin (**9c**) [21], meliatoosenin F (**10**) [22], 6-deacetyloxy-7-deacetylchisocheton (**11**) [23], mesendanin I (**12**) [14], azedarariide (**13**) [24], and nimbolinin B (**14**) [25], were confirmed by comparing their spectroscopic data with the corresponding literature data.

All the compounds were evaluated for their cytotoxicities (◉ **Table 3**) against five human tumor cell lines, HL-60, SMMC-7721, A-549, MCF-7, and SW480, by the MTT method [12]. Seven compounds (**1, 6, 7, 8, 9, 9b**, and **9c**) showed significant inhibitory activities against the five human tumor cell lines, and compound **9a** revealed moderate cytotoxicities against the five cancer cell lines, while compounds **2** and **3** only exhibited cytotoxicity against HL-

60. The structure–activity relationships of limonoids indicated that the presence of a C-19/C-29 lactol bridge and a 17*β*, 20*β*-epoxy group were important for improving their activity. Comparison of the cytotoxicity of **9a** with those of **1, 6–9, 9b**, and **9c** implied that the presence of two acetyl groups located at C-7 and C-29 could be responsible for its lower values. Interestingly, compounds **9** and **9c** showed some relatively stronger inhibitory activities against MCF-7 and SW480 than other human cancer cell lines, whereas compounds **9a** and **9b** did not show this phenomenon. This suggested that only when the hydroxyls of C-3 and C-12 or the hydroxyls of C-3, C-12, and C-29 were acetylated, could the compounds (**9** and **9c**) exhibit stronger cytotoxicities against MCF-7 and SW480. Compound **2** with a ketone at C-11, an acetyl at C-19, and a *β*-oriented hydroxyl at C-3 only exhibited inhibitory activity against HL-60 compared to compound **10**.

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Conflict of Interest

There were no conflicts of interest among all authors in this manuscript.

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