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Pregnane, coumarin and lupane derivatives and cytotoxic constituents from *Helicteres angustifolia*

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Abstract

2α,7β,20α-Trihydroxy-3β,21-dimethoxy-5-pregnene (**1**), 6,7,9α-trihydroxy-3,8,11α-trimethylcyclohexo-[d,e]-coumarin (**2**), 3β-hydroxy-27-benzoyloxylup-20(29)-en-28-oic acid (**3**), and 3β-hydroxy-27-benzoyloxylup-20(29)-en-28-oic acid methyl ester (**4**), along with 24 known compounds were isolated and structurally characterized from roots and aerial parts of *Helicteres angustifolia* (Sterculiaceae). In a preliminary bioassay, the two cucurbitacin derivatives, cucurbitacin D and J exhibited significant inhibitory activities against the growth of both hepatocellular carcinoma BEL-7402 cells and malignant melanoma SK-MEL-28 cells in vitro.

Keywords: *Helicteres angustifolia*; Sterculiaceae; Pregnane; Coumarin; Triterpenoid; Cytotoxicity

1. Introduction

The dried roots and stems of *Helicteres angustifolia* (L.) (Sterculiaceae) are used in folk medicine as analgesic, anti-inflammatory and anti-bacterial agents (Jiangsu New Medical College, 1986) in the south of China under the name *Shan-Zhi-Ma*; tumor growth inhibitory activity has also been reported for this plant (Chiu and Chang, 1995). Previous phytochemical investigations of *Helicteres* plants mainly led to identification of quinones derived from oxidation of a cadinane-type sesquiterpenoid (Chen et al., 1990) as well as triterpenoids with lupane and oleanane-type skeletons (Liu and Wang, 1985; Chang et al., 2001). In a continuation of our efforts to find new anti-tumor natural products from Chinese herb medicine, the constituents of *Helicteres angustifolia* (L.) were investigated systematically. As a result, 28 compounds were isolated and elucidated, of which four were new based on extensive NMR analysis and chemical methods. In a preliminary bioassay, the two cucurbitacin derivatives, cucurbitacin D and J were found to exhibit significant inhibitory activities against the growth of both hepatocellular carcinoma BEL-7402 cells and malignant melanoma SK-MEL-28 cells.

2. Results and discussion

Compound **1** was obtained as white amorphous powder with an elemental formula $C_{23}H_{38}O_5$ as deduced by HR-EIMS and NMR analyses. Its IR spectrum indicated the existence of hydroxy (3425 cm^{-1}) and olefinic functional (1630 cm^{-1}) groups in its structure. The ^1H NMR spectrum of **1** exhibited one singlet olefinic proton signal at δ_H 5.40, two methoxyl resonances at δ_H 3.46 and 3.43, and two singlet methyl signals at δ_H 1.12 and 0.77. Its ^{13}C NMR

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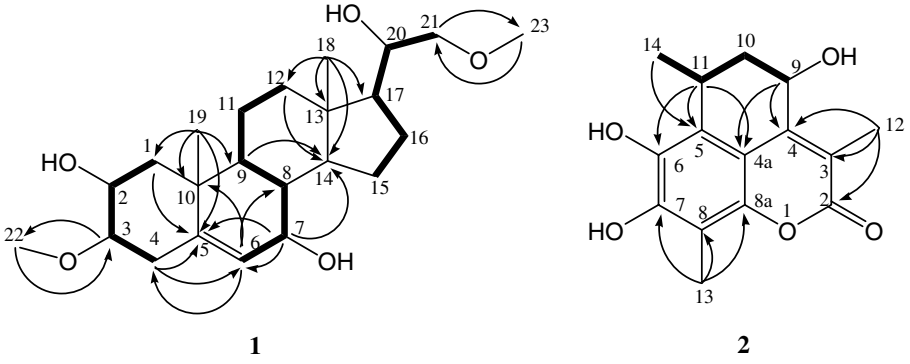
spectrum displayed 23 resonances, which through a DEPT experiment were subclassified into four methyls (two for methoxyls), seven methylenes (one oxygenated methylene), nine methines (one for a sp^2 methine and four for oxygenated methines), and three quaternary carbons (one for a sp^2 carbon). Analysis of the ^1H–^1H COSY spectrum of **1** revealed the following three fragments –C-1–C-2–C-3–C-4–, –C-6–C-7–C-8–C-9–C-11–C-12–, and –C-14–C-15–C-16–C-17–C-20–C-21– in its structure. The structure of **1** was established on the basis of analysis of the HMBC spectrum, in which ^1H–^{13}C long-range correlation signals were observed as follows between: H-19 and C-1, C-5, C-9; H-18 and C-12, C-14, C-17; H-22 and C-3; H-23 and C-21; C-5 and H-1, H-7; H-6 and C-4, C-8, C-10, and C-14 and H-7, H-9, H-12 (Fig. 1). In the NOESY spectrum of **1**, NOE correlations were observed at H-19/H-1β, H-19/H-2, H-19/H-4β, H-3/H-4α, H-7/H-9, H-7/H-14, and H-18/H-20 (Fig. 2). The relative configuration of **1** was thus characterized completely, except for C-20. The relative configuration at C-20 was subsequently deduced as follows: since only one of the protons attached to C-21 showed a NOE correlation signal to H-17, the analysis of the 3D-structure of **1** generated from the molecular modeling (CS Chem3D Ultra 9.0) using a MM2 force field calculation for energy minimization revealed that H_a-21 was closer to H-17 than H_b-21, no matter whether the 20-OH group was in an α or β orientation. Therefore, H_a-21 gave a NOE correlation with H-17 (Fig. 2). The dihedral angles between H-20 and H_a-21 were close to 180° and 45°, when the 20-OH group was in an α or β orientation, respectively. Therefore, the coupling constants between H-20 and H_a-21, and between H-20 and H_b-21 could be used in the determination of the relative configuration of 20-C of **1**. The relatively large coupling constant between H-20 and H_a-21 (8.5 Hz), and the small coupling constant between H-20 and H_b-21 (2.9 Hz) indicated that the 20-OH group was in an α orientation (Fig. 3). According to the literature, the configuration of the 20-OH group of pregnane derivative could also be determined by the magnitude of the upfield shift of the 18-CH_3 group of the 20-OH acylated product. The principles used were the van der Waals effect of the 20-OH group and the diamagnetic effect of the acyl group to 18-CH_3, which was stronger in the 20β-OH series than in the 20α-OH series (Lee and Wolff, 1967). To confirm the configuration of C-20, **1** was peracetylated to give **1a**, and little variance was observed between the chemical shifts of CH_3-18 of **1** and **1a** ($\Delta\delta_H = 0.01$ ppm) thereby suggesting an α-configuration of the 20-OH group in **1** (Nakatani et al., 1985). Therefore, the structure of **1** was determined to be 2α,7β,20α-trihydroxy-3β,21-dimethoxy-



1 **2**

Fig. 1. ^1H–^1H COSY (▬) and key ^1H–^{13}C long-range correlation signals (⌒) in the HMBC spectra of **1** and **2**.

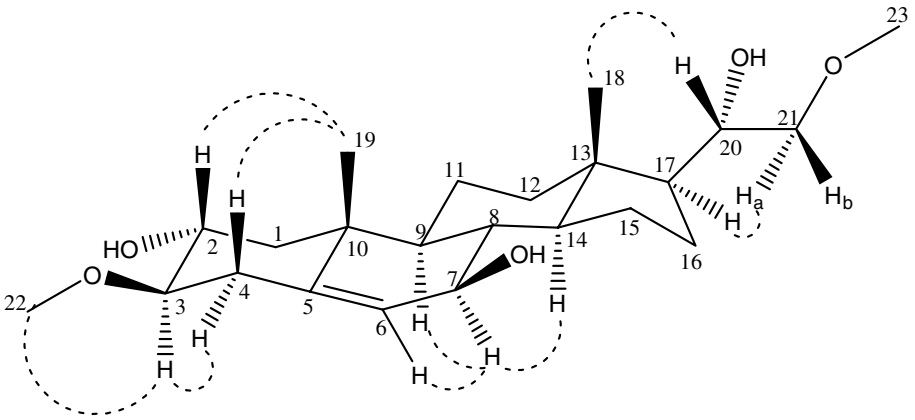
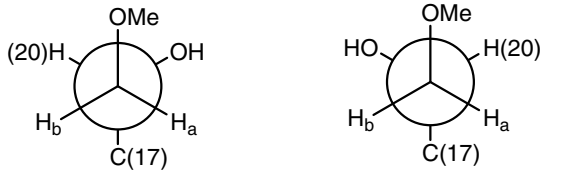


Fig. 2. Main NOE signals (⋰⋱) observed in the NOESY spectrum of **1**.



1
J (H-20, H_a-21) = 8.5 Hz
J (H-20, H_b-21) = 2.9 Hz

20-epimer of **1**
J (H-20, H_a-21) = 2.9 Hz
J (H-20, H_b-21) = 8.5 Hz

Fig. 3. Newman projection diagrams of C(20)–C(21) in **1** (20α-OH) and its epimer (20β-OH).

5-pregnene. To the best of our knowledge, compound **1** is a new compound.

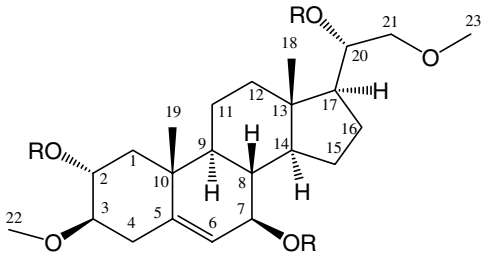
Compound **2** was obtained as brown amorphous powder with an elemental formula $C_{15}H_{16}O_5$ as also deduced by HR-EIMS and NMR analyses. The maxima absorption at 320 nm in the UV spectrum revealed a conjugated system in its structure, and the IR spectrum indicated the existence of hydroxy (3398 cm^{-1}), conjugated carbonyl (1664 cm^{-1}), and olefinic (1614 cm^{-1}) functionalities. In the ^{13}C NMR spectrum, the 15 carbon signals consisted of three methyls, one methylene, two methines, and nine quaternary carbons (one for an ester carbonyl and the other eight for sp^2 carbons). The ^1H NMR spectrum of **2** exhibited two singlet methyl signals at δ_H 2.18 and 2.10, and one doublet methyl resonance at δ_H 1.36 (3H, *d*, $J = 7.3$ Hz). A structural fragment –C-9–C-10–C-11–C-14– could be deduced from the ^1H–^1H COSY spectrum of **2**. The structure of **2** was further established on the basis of analysis of its HMBC spectrum (Fig. 1), in which the ^1H–^{13}C long-range correlation signals were observed between: H-12 and C-2, C-3, C-4; H-13 and C-7, C-8, C-8a; H-9 and C-4, C-4a; H-11 and C-4a, C-5, C-6, and H-14 and C-5, respectively. The relative configurations of C-9 and C-11 were determined according to NOE correlations between H-9 and H-11, between H-11 and H-10β, and between H-14 and H-10α in its NOESY spectrum. The structure of **2** was therefore established as 6,7,9α-trihydroxy-3,8,11α-trimethylcyclohexo-[d,e]-coumarin. It was a highly oxidized derivative possessing cadinane-type skeleton (Wang et al., 1988), and to the best of our knowledge, it is a new compound.

Compound **3** was obtained as a white amorphous powder with a molecular formula $C_{37}H_{52}O_5$ as also deduced by the HR-EIMS and NMR analyses. Its ^1H NMR spectrum revealed the existence of one mono-substituted phenyl, one terminal methylene, one oxygenated methylene, one oxygenated methine, and five single methyl groups (Table 2). The ^{13}C NMR spectrum of **3** displayed 37 signals which following a DEPT analysis were separated into five methyl, twelve methylene (one for a sp^2 methylene and one for an oxygenated methylene), eleven methine (one for an oxygenated methine and five for sp^2 methines), and nine quaternary carbon (two for ester/carboxylic carbonyl, and two for sp^2 carbons) functionalities, respectively. The ^{13}C NMR spectrum of **3** was similar to that of the known compound helicteric acid (**5**) isolated from the same plant (Chang et al., 2001), except for differences in chemical shifts at C-2 (+3.8 ppm), C-3 (−1.9 ppm), and C-4 (+1.0 ppm) (see Table 2), i.e. due to the absence of the acetyl group at 3-OH. Acetylation of **3** with acetic anhydride in pyridine yielded **5** as the sole product. Therefore, compound **3** was characterized to be 3β-hydroxy-27-benzoyloxylup-20(29)-en-28-oic acid.

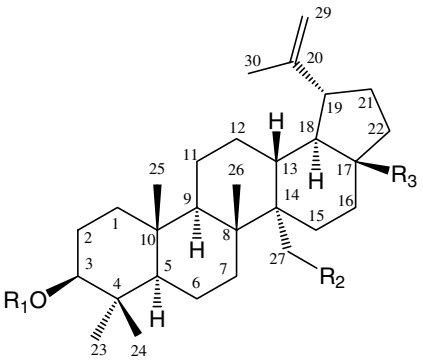
Compound **4** was obtained as white amorphous powder, and its molecular formula was deduced as $C_{38}H_{54}O_5$ by HR-EIMS and NMR spectroscopic analyses. Its NMR spectra were superimposed with those of **3** except for the up-field shift of C-28 (−3.9 ppm), and additional resonances for one methoxyl group, suggesting that **4** was the methyl ester of **3**. Therefore, the structure of **4** was determined to be 3β-hydroxy-27-benzoyloxylup-20(29)-en-28-oic acid methyl ester. To the best of our knowledge, **3** and **4** are new compounds.

Besides the four new natural products, 24 other known compounds were also isolated from *Helicteres angustifolia*, and their structures were characterized by comparison of the physical and NMR data with those reported in literature, or co-TLC with authentic samples as helicteric acid (**5**) (Chang et al., 2001), methyl helicterate (**6**) (Chang et al., 2001), 3β-acetoxyl-27-(*p*-hydroxylbenzoyloxy)-lup-20(29)-en-28-oic acid methyl ester (**7**) (Chang et al., 2001), 3β-acetoxylup-20(29)-en-28-ol (**8**) (Hiroya et al., 2002), 3β-hydroxylup-20(29)-en-28-oic acid 3-caffeate (**9**) (Pan et al., 1994), betulinic acid (**10**), cucurbitacin D (**11**) (Velde and Lavie, 1983), cucurbitacin J (**12**) (Kanchanapoom et al., 2002), oleanolic acid (**13**), β-sitosterol (**14**), mansonone E (**15**) (Chen et al., 1990), mansonone F (**16**) (Chen et al., 1990), mansonone H (**17**) (Chen et al., 1990), mansonone H methyl ester (**18**) (Chen et al., 1990), lariciresinol (**19**) (Fonseca et al., 1978), dihydrodehydrodiconiferyl alcohol (**20**) (Fukuyama et al., 1996), (+)-pinoresinol (**21**) (Ludwig et al., 1964), lirioresinol-B (**22**) (Tatematsu et al., 1984), rosmarinic acid (**23**) (Satake et al., 1999), 8-*O*-β-D-glucuronyl-hypolaetin 4′-methyl ether (**24**) (Billeter et al., 1991), coniferyl alcohol (**25**) (Steeves et al., 2001), 3-(3,4-dimethoxyphenyl)-2-propenal (**26**) (Ito et al., 2001), kaempferol 3-*O*-β-D-glucopyranoside (**27**) (Beninger and Hosfield, 1998), 5,8-dihydroxy-7,4′-dimethoxyflavone (**28**) (Ramesh and Yuvarajan, 1995). Among them, **8**, **9**, **11**, **12**, **19-22**, and **25-27** were reported for the first time from the genus *Helicteres*.

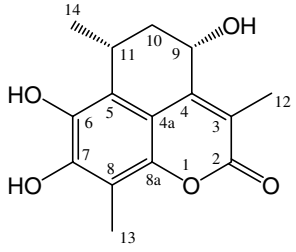
Helicteres angustifolia was reported to exhibit tumor growth inhibitory activity (Chiu and Chang, 1995). According to the literature, cucurbitacins possessed significant anti-tumor activity (Gitter et al., 1961; Gallily et al., 1962; Duncan et al., 1996), and betulinic acid was found to be a selective inhibitor of human melanoma functioned by induction of apoptosis (Pisha et al., 1995). In order to identify the anti-tumor constituents of *H. angustifolia*, compounds **3**–**12** and **15**–**18** were evaluated against the growth of human tumor cell lines, including hepatocellular



1 R = H
1a R = acetyl



3 R_1 = H, R_2 = benzoyloxy, R_3 = COOH
4 R_1 = H, R_2 = benzoyloxy, R_3 = $COOCH_3$
5 R_1 = acetyl, R_2 = benzoyloxy, R_3 = COOH
6 R_1 = acetyl, R_2 = benzoyloxy, R_3 = $COOCH_3$
7 R_1 = acetyl, R_2 = 4-hydoxylbenzoyloxy, R_3 = $COOCH_3$
8 R_1 = acetyl, R_2 = H, R_3 = CH_2OH
9 R_1 = cafeoyl, R_2 = H, R_3 = COOH
10 R_1 = R_2 = H, R_3 = COOH



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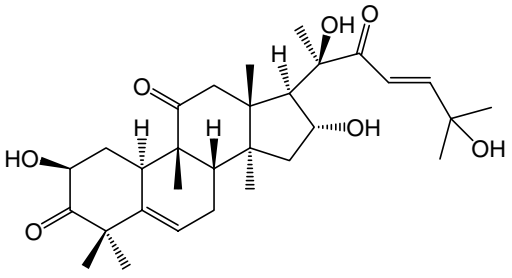

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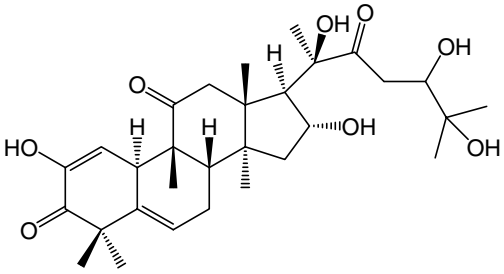

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carcinoma BEL-7402 cell line and malignant melanoma SK-MEL-28 cell line. It was found that the two cucurbitacin derivatives, **11** and **12**, significantly inhibited the proliferation of BEL-7402 cells (with IC_{50}s of 1.41 and 1.37 μM, respectively) and SK-MEL-28 cells (with IC_{50}s of 1.22 and 1.28 μM, respectively). The three betulinic acid derivatives **3**, **5** and **9** possessed only mild inhibitory activity against SK-MEL-28 cells with IC_{50}s of 20.5, 81.7 and 32.0 μM, respectively. These cytotoxic components found from *H. angustifolia* may contribute in part to the tumor growth inhibitory activity of this medicinal plant.

3. Experimental

3.1. General

Optical rotations were measured with Perkin–Elmer 241MC polarimeter or Perkin–Elmer 341 polarimeter. UV spectra were recorded with a Beckman DU-7 spectrometer. IR spectra were recorded using a Perkin–Elmer 577 spectrometer. LR-ESIMS was measured using a Finnigan LCQ-DECA instrument, LR-EIMS were obtained on a MAT-95 spectrometer, and HR-EIMS were obtained on Kratos 1H spectrometer. NMR spectra were acquired on a Bruker AM 400 spectrometer with TMS as internal standard. CC graphic separations were carried out using a LiChroprep RP-18 Lobar column (40–63 μm, Merck), silica gel H60 (300–400 mesh, Qingdao Haiyang Chemical Group Corporation, Qingdao, People's public of China), MCI Gel CHP20P (75–150 μm; Mitsubishi Chemical Industries) and Sephadex LH-20 (Pharmcia Biotech AB, Uppsala, Sweden), respectively. HSGF254 silica gel TLC plates (Yantai Chemical Industrial Institute, Yantai, People's Republic of China) and RP-18 WF_{254s} TLC plates (Merck) were used for analytical TLC.

3.2. Plant material

Whole plants of *Helicteres angustifolia* (L.) were collected in the suburb of Guangzhou, Guangdong Province, P.R. China, in May 2003, and identified by Professor Zexian Li of the South China Institute of Botany, Chinese Academy of Sciences. A voucher specimen (No.

SIMMS0305) is deposited in the herbarium of Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

3.3. Extract and isolation

Powdered air-dried roots of *Helicteres angustifolia* (L.) (500 g) were extracted with boiling methanol thrice (1.5 L × 3, each time for 2 hours). After evaporation of the methanol in vacuo, the residue was suspended into 1.0 L water and then extracted with $CHCl_3$ and *n*BuOH (both 500 mL × 3) successively, to afford $CHCl_3$ (8 g) and *n*-BuOH fraction (40 g) solubles. The chloroform solubles were subjected to silica gel CC, eluted with a gradient of petroleum ether–EtOAc (10:1–0:1) to give Fr.C1 (902 mg), Fr.C2 (1.34 g), Fr.C3 (1.91 g), Fr.C4 (943 mg) and Fr.C5 (2.53 g). Fr.C1 was purified by silica gel CC eluted with petroleum ether–acetone (40:1) to give **6** (87 mg); Fr.C2 was subjected to repeated column chromatography over silica gel using petroleum ether–acetone (25:1) as eluent to yield **5** (400 mg), **8** (22 mg), and **14** (68 mg); Fr.C3 was also subjected to silica gel CC eluted with petroleum ether–acetone (20:1) afforded **3** (10 mg), **4** (19 mg), **7** (19 mg), **10** (12 mg), and **13** (10 mg); **9** (25 mg) was obtained from Fr.C4 by silica gel CC, eluted with petroleum ether–acetone (10:1); Fr.C5 was also subjected to repeated silica gel CC using petroleum ether–acetone (8:1) as eluent to give **15** (20 mg), **16** (40 mg), **17** (10 mg), and **18** (5 mg).

Powdered air-dried aerial parts of *Helicteres angustifolia* (L.) (3.0 kg) were percolated at room temperature with EtOH–H_2O (95:5, 5.0 L × 3). The filtrate was concentrated to dryness in vacuo and then suspended in EtOH–H_2O (1:4) overnight. After filtration of the precipitated chlorophyll and evaporation of the EtOH in the filtrate, the aqueous residue (1.0 L) was extracted with $CHCl_3$ and *n*-BuOH (both 500 mL × 3), successively, to yield $CHCl_3$ (10 g) and *n*-BuOH (25 g) solubles. The chloroform solubles were subjected to silica gel CC, eluted with a gradient of petroleum ether–EtOAc (10:1–0:1) to give Fr.C′1 (3.11 g), Fr.C′2 (1.7 g) and Fr.C′3 (4.6 g). Fr.C′1 was subjected to repeated silica gel CC, using petroleum ether–EtOAc (5:1) as eluent, to afford **26** (3 mg), **19** (28 mg), **21** (28 mg) and **25** (10 mg); Fr.C′2 was separated by a silica gel CC, eluted with petroleum ether–EtOAc (2:1), and then subjected to preparative TLC developed with Et_2O to afford **1** (67 mg), **11** (34 mg), and **12** (5 mg); Fr.C′3 was applied to silica gel column which was eluted with a gradient of $CHCl_3$–acetone (20:1–1:1) to give Fr.C′3 A (300 mg), Fr.C′3B (310 mg) and Fr.C′3 C (3.6 g). **22** (36 mg) was obtained from Fr.C′3A by Sephadex LH-20 CC eluted with EtOH–H_2O (95:5), while **20** (43 mg) was obtained from Fr.C′3B by a RP-18 column eluted with MeOH–H_2O (35:65). The *n*-BuOH fraction was separated by a MCI column successively, eluted with H_2O, MeOH–H_2O (1:1) and MeOH–H_2O (9:1), to give Fr.B1 (20 g), Fr.B2 (2.0 g) and Fr.B3 (300 mg), respectively. Fr.B2 was subjected to silica gel CC eluted with a gradient of $CHCl_3$–MeOH (10:1–1:1) to give Fr.B2A (800 mg), Fr.B2B (648 mg) and Fr.B2C (351 mg), respectively. Fr.B2A was separated by preparative TLC developed with $CHCl_3$–EtOAc (5:1), to afford **28** (3 mg). Fr.B2B was successively subjected to Sephadex LH-20 CC eluted with EtOH–H_2O (95:5), and silica gel CC eluted with $CHCl_3$–MeOH–H_2O (4:1:0.1), to yield **24** (80 mg) and **27** (4 mg). Fr.B2C was separated by preparative TLC developed with $CHCl_3$–MeOH–H_2O (4:1:0.1), to give **23** (5 mg); Fr.B3 was purified by a RP-18 column eluted with MeOH–H_2O (1:1) to afford **2** (40 mg).

3.4. 2α,7β,20α-Trihydroxy-3β,21-dimethoxy-5-pregnene (*1*)

White amorphous powder, $[\alpha]_D^{20}$: +6.1 (*c* 0.43, $CHCl_3$); IR (KBr): ν_{max} cm^{-1}: 3425, 2937, 1689, 1630, 1458, 1385, 1194, 1099, 1022, 731; EIMS (probe) 70 eV, *m*/*z* (rel. int.) 394 $[M]^+$ (9), 376 (44) 362 (100), 149 (54), 86 (54); HR-EIMS, *m*/*z*: 394.2711 $[M]^+$ (Calcd for $C_{23}H_{38}O_5$, 394.2719); for ^1H and ^{13}C NMR spectroscopic data, see Table 1.

3.5. Acetylation of compound *1*

Compound **1** (10 mg) and Ac_2O (0.2 mL) were added to pyridine (2 mL), with the resulting mixture stirred at 80 °C

Table 1
^1H (400 MHz) and ^{13}C (100 MHz) NMR spectroscopic data of **1** ($CDCl_3$) and **2** (CD_3OD) (*J* in Hz)

No.	**1**		**2**	
	^{13}C NMR	^1H NMR	^{13}C NMR	^1H NMR
1	44.0 *t*	1.11, *m* (α); 2.14, *dd*, 4.8, 12.7 (β)		
2	70.5 *d*	3.75, *m*	166.5 *s*	
3	85.0 *d*	2.97, *m*	119.2 *s*	
4	34.8 *t*	2.20, *dd*, 2.2, 11.5 (α); 2.56, *dd*, 4.9, 11.5 (β)	149.5 *s*	
4a			109.2 *s*	
5	142.0 *s*		126.2 *s*	
6	126.3 *d*	5.40, *br s*	140.8 *s*	
7	73.1 *d*	3.92, *br d*, 7.8	147.7 *s*	
8	40.1 *d*	1.44, *m*	111.0 *s*	
8a			149.3 *s*	
9	48.4 *d*	1.17, *m*	65.2 *d*	4.98, *br s*
10	37.9 *s*		37.1 *t*	1.90, *m*; 2.11, *m*
11	26.4 *t*	1.95, *m*	27.4 *d*	3.32, *m*
12	38.3 *t*	1.17, *m* (α); 1.82, *m* (β)	12.5 *q*	2.10, *s*
13	52.2 *s*		8.9 *q*	2.18, *s*
14	55.4 *d*	1.25, *m*	22.6 *q*	1.36, *d*, 7.3
15	20.8 *t*	1.63, *m*		
16	24.8 *t*	1.95, *m*		
17	42.2 *d*	1.44, *m*		
18	12.8 *q*	0.77, *s*		
19	20.2 *q*	1.12, *s*		
20	71.1 *d*	3.80, *m*		
21	76.5 *t*	3.28, *dd*, 8.5, 9.3; 3.54, *dd*, 2.9, 9.3		
22	56.6 *q*	3.46, *s*		
23	58.9 *q*	3.43, *s*		

for 5 h. Further cooling to room temperatures, as evaporation of the solvent to dryness in vacuo, the residue was subjected to preparative TLC developed with petroleum ether–$CHCl_3$ (1:6) to afford **1a** (7 mg). 2α,7β,20α-triacetoxy-3β,20-dimethoxy-5-pregnene (**1a**): white amorphous powder, $[\alpha]_D^{20}$: −25.6 (*c* 0.348, $CHCl_3$); ESI-MS, *m/z*: 543 $[M+Na]^+$, 1063 $[2M+Na]^+$; ^1H NMR (400 MHz, in $CDCl_3$): δ 5.32 (1H, *br s*, H-6), 5.12 (1H, *m*, H-20), 5.06 (1H, *br d*, 7.8, H-7), 5.02 (1H, *m*, H-2), 3.50 (1H, *dd*, 8.5, 9.3, H-21), 3.44 (1H, *dd*, 2.9, 9.3, H-21), 3.41 (3H, *s*, 21-OCH_3), 3.37 (3H, *s*, 3-OCH_3), 3.17 (1H, *m*, H-3), 2.10, 2.08 and 2.05 (each 3H, *s*, 3 × -$COCH_3$), 1.17 (3H, *s*, 19-CH_3), 0.76 (3H, *s*, 18-CH_3).

3.6. 6,7,9α-Trihydroxy-3,8,11α-trimethylcyclohexo-[d,e]-coumarin (2)

Brown amorphous powder, $[\alpha]_D^{20}$: +30 (*c* 1.17, MeOH); UV (MeOH) λ_{max} nm (log ε): 320 (3.6), 209 (4.3); IR (KBr): ν_{max} cm^{-1}: 3398, 2929, 1664, 1614, 1574, 1387, 1244, 1192, 1132, 1016, 970, 775; EIMS, *m/z* (rel. int.) 276 $[M]^+$ (100), 261 (28), 243 (60), 231 (23), 215 (27); HR-EIMS, *m/z*: 276.0989 $[M]^+$ (Calcd. for $C_{15}H_{16}O_5$, 276.0998); for ^1H and ^{13}C NMR spectroscopic data, see Table 1.

3.7. 3β-Hydroxy-27-benzoyloxylup-20(29)-en-28-oic acid (3)

White amorphous powder, $[\alpha]_D^{20}$: −2.2 (*c* 0.31, $CHCl_3$); UV (MeOH) λ_{max} nm (log ε): 230 (4.2), 275 (w); IR (KBr): ν_{max} cm^{-1}: 3429, 3070, 2945, 2872, 1716, 1641, 1603, 1452, 1377, 1315, 1273, 1176, 1117, 1070, 1026, 984, 885, 712; EIMS, *m/z* (rel. int.): 576 $[M]^+$ (4), 558 $[M-H_2O]^+$ (7), 454 (74), 436 (55), 246 (38), 234 (26), 207 (95), 189 (80), 175 (26), 135 (41), 105 (100); HR-EIMS, *m/z*: 576.3823 $[M]^+$ (Calcd. for $C_{37}H_{52}O_5$, 576.3815); for ^1H and ^{13}C NMR spectroscopic data, see Table 2.

3.8. 3β-Hydroxy-27-benzoyloxylup-20(29)-en-28-oic acid methyl ester (4)

White amorphous powder, $[\alpha]_D^{20}$: −5.8 (*c* 0.38, $CHCl_3$); UV (MeOH) λ_{max} nm (log ε): 230 (4.2), 275 (w); IR (KBr): ν_{max} cm^{-1}: 3535, 3070, 2949, 2872, 1718, 1643, 1603, 1452, 1377, 1315, 1271, 1165, 1117, 1070, 1026, 974, 885, 712; EIMS, *m/z* (rel. int.): 590 $[M]^+$ (2), 572 $[M-H_2O]^+$ (4), 468 (95), 260 (38), 207 (80), 187 (72), 105 (100); HR-EIMS, *m/z*: 590.3981 $[M]^+$ (Calcd. for $C_{38}H_{54}O_5$, 590.3971); for ^1H and ^{13}C NMR spectroscopic data, see Table 2.

3.9. Acetylation of compound 3

Acetylation of **3** (1 mg) was carried out as above. The product was identified to be identical with **5** by co-TLC analysis (developed with petroleum ether–acetone 6:1).

Table 2
^1H (400 MHz) and ^{13}C (100 MHz) NMR spectroscopic data of **3** and **4** ($CDCl_3$) (*J* in Hz)

No.	3		4	
	^{13}C NMR	^1H NMR	^{13}C NMR	^1H NMR
1	38.9 *t*		38.9 *t*	
2	27.4 *t*		27.4 *t*	
3	78.8 *d*	3.20, *dd*, 5.2, 10.8	78.7 *d*	3.15, *dd*, 5.3, 11.1
4	38.8 *s*		38.8 *s*	
5	55.5 *d*		55.5 *d*	
6	18.3 *t*		18.3 *t*	
7	35.4 *t*		35.4 *t*	
8	41.6 *s*		41.5 *s*	
9	52.1 *d*		52.1 *d*	
10	37.6 *s*		37.5 *s*	
11	21.1 *t*		21.1 *t*	
12	25.4 *t*		25.4 *t*	
13	39.3 *d*		39.2 *d*	
14	45.8 *s*		45.7 *s*	
15	24.4 *t*		24.3 *t*	
16	32.7 *t*		32.6 *t*	
17	56.2 *s*		56.4 *s*	
18	49.7 *d*		49.9 *d*	
19	46.9 *d*	3.06, *m*	46.9 *d*	3.02, *m*
20	150.1 *s*		150.1 *s*	
21	30.5 *t*		30.5 *t*	
22	36.7 *t*		36.6 *t*	
23	27.9 *q*	0.74, *s*	27.9 *q*	0.73, *s*
24	16.7 *q*	1.03, *s*	16.7 *q*	0.99, *s*
25	16.5 *q*	0.91, *s*	16.4 *q*	0.89, *s*
26	15.4 *q*	0.87, *s*	15.4 *q*	0.85, *s*
27	63.9 *t*	4.54, *d*, 12.9; 4.78, *d*, 12.9	63.9 *t*	4.50, *d*, 12.9; 4.67, *d*, 12.9
28	180.5 *s*		176.6 *s*	
29	110.0 *t*	4.63, *s*; 4.75, *s*	109.9 *t*	4.60, *d*, 1.8; 4.67, *d*, 1.8
30	19.5 *q*	1.72, *s*	19.5 *q*	1.68, *s*
28-$COOCH_3$			51.4 *q*	3.66, *s*
Benzoyl moiety				
1′	130.6 *s*		130.6 *s*	
2′, 6′	129.5 *d*	8.03, *dd*, 1.6, 7.2	129.5 *d*	8.00, *dd*, 1.6, 7.2
3′, 5′	128.5 *d*	7.47, *m*	128.5 *d*	7.45, *m*
4′	133.0 *d*	7.58, *m*	133.0 *d*	7.56, *m*
7′	166.8 *s*		166.7 *s*	

3.10. Determination of cell proliferation by MTT assay

Cells (5×10^4 cells/ml) were incubated with different concentrations of compounds **3**–**12** and **15**–**18** for 72 h. Cell proliferation was determined by MTT assay (Vistica et al., 1991). The assays were carried out by triplicate in at least three independent experiments.

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References

Beninger, C.W., Hosfield, G.L., 1998. Flavonol glycosides from the seed coat of a new manteca-type dry bean (*Phaseolus vulgaris* L.). Journal of Agricultural and Food Chemistry 46, 2906–2910.

Billeter, M., Meier, B., Sticher, O., 1991. 8-Hydroxyflavonoid glucuronides from *Malva sylvestris*. Phytochemistry 30, 987–990.

Chang, Y.S., Ku, Y.R., Lin, J.H., Lu, K.L., Ho, L.K., 2001. Analysis of three lupane type triterpenoids in *Helicteres angustifolia* by high-performance liquid chromatography. Journal of Pharmaceutical and Biomedical Analysis 26, 849–855.

Chen, C.M., Chen, Z.T., Hong, Y.L., 1990. A mansonone from *Helicteres angustifolia*. Phytochemistry 29, 980–982.

Chiu, N.Y., Chang, K.S., 1995. The Illustrated Medicinal Plants of Taiwan (I). Southern Materials Center, Inc, Taipei, p. 104.

Duncan, K.L., Duncan, M.D., Alley, M.C., Sausville, E.A., 1996. Cucurbitacin E-induced disruption of the action and vimentin cytoskeleton in prostate carcinoma cells. Biochemical Pharmacology 52, 1553–1560.

Fonseca, S.F., Campello, J., Barata, L., Ruveda, E.A., 1978. ^{13}C NMR spectral analysis of lignans from *Araucaria angustifolia*. Phytochemistry 17, 499–502.

Fukuyama, Y., Nakahara, M., Minami, H., Kodama, M., 1996. Two new benzofuran-type lignans from the wood of *Viburnum awabuki*. Chemical and Pharmaceutical Bulletin 44, 1418–1420.

Gallily, R., Shohat, B., Kalish, J., Gitter, S., Lavie, D., 1962. Further studies on the antitumor effect of cucurbitacins. Cancer Research 22, 1038–1045.

Gitter, S., Gallily, R., Shohat, B., Lavie, D., 1961. Studies on the antitumor effect of cucurbitacins. Cancer Research 21, 516–521.

Hiroya, K., Takahashi, T., Miura, N., Naganuma, A., Sakamoto, T., 2002. Synthesis of betulin derivatives and their protective effects against the cytotoxicity of cadmium. Bioorganic & Medicinal Chemistry 10, 3229–3236.

Ito, J., Chang, F.R., Wang, H.K., Park, Y.K., Ikegaki, M., Kilgore, N., Lee, K.H., 2001. Anti-AIDS agents. 48. Anti-HIV activity of moronic acid derivatives and the new melliferone-related triterpenoid isolated from Brazilian propolis. Journal of Natural Products 64, 1278–1281.

Jiangsu New Medical College, 1986. Dictionary of Chinese Herb medicines. Shanghai Scientific and Technologic Press, Shanghai, pp. 178–179.

Kanchanapoom, T., Kasai, R., Yamasaki, K., 2002. Cucurbitane, hexanorcucurbitane and octanorcucurbitane glycosides from fruits of *Trichosanthes tricuspidata*. Phytochemistry 59, 215–228.

Lee, H., Wolff, M.E., 1967. C-18 functional steroids and D-homo steroids. The Journal of Organic Chemistry 32, 192–196.

Liu, W.G., Wang, M.S., 1985. Three new triterpenoids from *Helicteres angustifolia*. Acta Pharmaceutica Sinica 20, 842–851.

Ludwig, C.H., Nist, B.J., Mccarthy, J.L., 1964. Lignin. XII. The high resolution nuclear magnetic resonance spectroscopy of protons in compounds related to lignin. Journal of the American Chemical Society 86, 1186–1196.

Nakatani, M., Takao, H., Miura, I., Hase, T., 1985. Azedarachol, a steroid ester antifeedant from *Melia azedarach* var. *japonica*. Phytochemistry 24, 1945–1948.

Pan, H., Lundgren, L.N., Andersson, R., 1994. Triterpene caffeates from bark of *Betula pubescens*. Phytochemistry 37, 795–799.

Pisha, E., Chai, H., Lee, I.S., Chagwedera, T.E., Farnsworth, N.R., Cordell, G.A., Beecher, C.W., Fong, H.H., Kinghorn, A.D., Brown, D.M., Wani, M.C., Wall, M.E., Hieken, T.J., Gupta, T.K., Pezzuto, J.M., 1995. Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. Nature Medicine 1, 1046–1051.

Ramesh, P., Yuvarajan, C.R., 1995. A new flavone methyl ether from *Helicteres isora*. Journal of Natural Products 58, 1242–1243.

Satake, T., Kamiya, K., Saiki, Y., Hama, T., Fujimoto, Y., Kitanaka, S., Kimura, Y., Uzawa, J., Endang, H., Umar, M., 1999. Studies on the constituents of fruits of *Helicteres isora* L. Chemical and Pharmaceutical Bulletin 47, 1444–1447.

Steeves, V., Forster, H., Pommer, U., Savidge, R., 2001. Coniferyl alcohol metabolism in conifers-I. Glucosidic turnover of cinnamyl aldehyde by UDPG: coniferyl alcohol glycosyltransferase from pine cambium. Phytochemistry 57, 1085–1093.

Tatematsu, H., Kurokawa, M., Niwa, M., Hirata, Y., 1984. Piscicidal constituents of *Stellera chamaejasme* L II. Chemical and Pharmaceutical Bulletin 32, 1612–1613.

Velde, V.V., Lavie, D., 1983. ^{13}C NMR spectroscopy of cucurbitacins. Tetrahedron 39, 317–321.

Vistica, D.T., Skehan, P., Scudiero, D., Monks, A., Pittman, A., Boyd, M.R., 1991. Tetrazolium-based assays for cellular viability: a critical examination of selected parameters affecting formazan production. Cancer Research 51, 2515–2520.

Wang, M.S., Liu, W.G., Li, J.R., Shen, F.L., Lin, X.Y., Zheng, Q.T., 1988. Structure elucidation of heliclactone. Acta Chimica Sinica 46, 768–771.