Two New Cycloartane Triterpenoids from *Kleinhovia hospita*

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Two new cycloartane triterpenoids, (23*R*)-21,23:23,27-diepoxycycloarta-1,24-diene-3,27-dione (**1**) and (3α)-(α-L-arabinopyranosyloxy)-1α-hydroxy-23-oxocycloartan-28-oic acid (**2**), together with six known pentacyclic triterpenoids, **3**–**8**, and five known C_{29} steroids, **9**–**13**, were isolated from *Kleinhovia hospita*. The structures of these compounds were determined by analysis of their spectroscopic data. Moreover, the absolute configuration of **1** was confirmed by quantum-chemical TDDFT calculation of its ECD spectrum. All compounds were evaluated for their cytotoxic activities against human colon carcinoma (HCT116) and gastric carcinoma (SGC7901) cell lines, and compounds **6**, **7**, **8**, **11**, and **12** exhibited antiproliferative activities with IC_{50} values ranging from 23.0 to 91.8 μM.

Introduction. – Before our first discovery of cycloartane triterpenoids with spiro side chains from *Kleinhovia hospita* (Annonaceae) in 2009, only a few fatty acids and flavonoids had been reported from this medicinal plant [1]. Later, kaempferol 3-*O*-β-D-glucoside and eleutherol were isolated from *K. hospita* as antioxidant agents [2]. Moreover, our re-investigation of this medicinal plant in 2012 resulted in the isolation of unprecedented cycloartane-triterpenoid alkaloids possessing spiro α,β-unsaturated γ-lactamlactone side chains and 9α,10α-cyclopropyl rings [3]. Besides all the compounds reported previously, the re-investigation also provided 13 compounds, including two new cycloartane triterpenoids, (23*R*)-21,23:23,27-diepoxycycloarta-1,24-diene-3,27-dione (**1**) and (3α)-(α-L-arabinopyranosyloxy)-1α-hydroxy-23-oxocycloartan-28-oic acid (**2**), six known pentacyclic triterpenoids, **3**–**8**, and five known C_{29} steroids, **9**–**13** (*Fig. 1*). We report herein the isolation and structure elucidation of these compounds, as well as their cytotoxic activities against human colon carcinoma (HCT116) and gastric carcinoma (SGC7901) cell lines.

Results and Discussion. – Compound **1** was obtained as white amorphous powder. Its molecular formula was determined as $C_{30}H_{40}O_4$ by a HR-ESI-MS (*m*/*z* 465.3000 ($[M+H]^+$; calc. 465.2999)). Based on our previous study of the spiro-cycloartane triterpenoids [1], the IR spectrum of **1** showed two characteristic absorption peaks for the α,β-unsaturated ketone of ring *A* (1664 cm^{-1}) and the α,β-unsaturated γ-lactone group of ring *F* (1764 cm^{-1}). Similarly, the UV spectrum showed two characteristic maximum absorption peaks at 266 and 224 nm. The ^{1}H-NMR spectrum of **1** (*Table*)

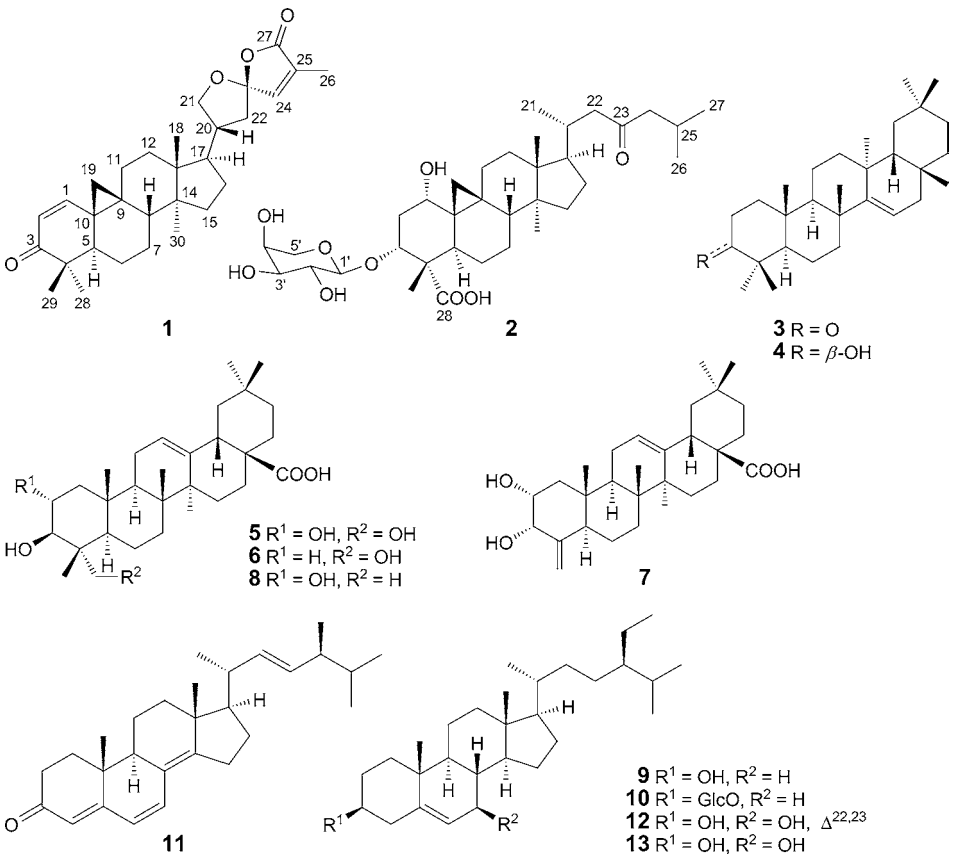

Fig. 1. *Structures of compounds* **1**–**13**

displayed two intercoupling olefinic *doublet*s assignable to the α,β-unsaturated ketone of ring *A* at δ(H) 6.76 and 5.96 (2*d*, $J = 10.1$). An aliphatic *doublet* at δ(H) 0.72 (*d*, $J =$ 4.7, 1 H) indicated the presence of the cyclopropane ring. A olefinic *quadruplet* at δ(H) 6.68 (*q*, $J = 1.6$, 1 H) and a Me *doublet* at δ(H) 1.92 (*d*, $J = 1.6$) were assigned to H–C(24) and Me(26), respectively. Signals of two intercoupling O-bearing H-atoms at δ(H) 3.59 (*dd*, $J = 8.1, 9.8$) and 4.30 (*dd*, $J = 8.1, 8.1$) suggested the presence of a CH_2O group, probably in the side chain. In the ^{13}C-NMR spectrum, the α,β-unsaturated ketone of ring *A* can be confirmed by three C-atom signals at δ(C) 205.3, 153.4, and 127.1. The C-atom signals of the α,β-unsaturated γ-lactone group of ring *F* appeared at δ(C) 171.4, 144.6, and 133.6. Compared to the ^{13}C-NMR spectrum of the known spiro-cycloartane triterpenoids [1], the C-atom signal around δ(C) 110 for the acetal C(23)-atom remains unchanged, and another acetal signal was replaced by that of an O-bearing CH_2 group at δ(C) 74.2. Therefore, analysis of these data allowed the primary assignment of the structure of **1** as a *C(21)*-saturated derivative of the known diacetal spiro-cycloartane triterpenoids. The above elucidation was confirmed by 2D-NMR spectra (HSQC, HMBC, NOESY; *Fig. 2*). In the HMBC spectra, besides ordinary

Table. *^{1}H- and ^{13}C-NMR* (500 and 125 MHz, resp.) *Data of Compounds* **1** *and* **2**. δ in ppm, J in Hz.

Position	**1**[a])		**2**[b])	
	δ(H)	δ(C)	δ(H)	δ(C)
1	6.76 (*d*, *J* = 10.1)	153.4	3.35 (overlapped)	70.7
2	5.96 (*d*, *J* = 10.1)	127.1	1.90, 1.63 (both overlapped)	36.1
3		205.3	4.40 (*dd*, *J* = 12.2, 4.5)	78.9
4		46.0		53.2
5	2.13 (*dd*, *J* = 12.8, 3.9)	44.1	2.44 (*dd*, *J* = 12.6, 4.5)	36.8
6	1.58, 1.27 (both overlapped)	23.0	1.11, 0.87 (both overlapped)	22.1
7	1.56, 1.12 (both overlapped)	19.3	1.03, 1.19 (both overlapped)	25.1
8	2.06 (*dd*, *J* = 8.1, 8.0)	42.7	1.46 (*dd*, *J* = 11.8, 4.5)	47.5
9		25.0		20.1
10		30.2		29.0
11	1.84 (overlapped), 1.65 – 1.72 (*m*)	27.7	2.29, 1.19 (both overlapped)	25.1
12	1.55, 1.27 (both overlapped)	31.0	1.56 (overlapped)	32.5
13		45.8		48.6
14		48.9		45.0
15	1.33, 1.41 (both overlapped)	34.6	1.22 (overlapped)	35.3
16	1.87, 1.43 (both overlapped)	27.5	1.81, 1.17 (both overlapped)	27.9
17	1.81 (overlapped)	50.9	1.59 (overlapped)	51.8
18	0.99 (*s*)	17.8	0.94 (*s*)	18.0
19	1.34 (overlapped), 0.72 (*d*, *J* = 4.7)	28.3	0.57 (*d*, *J* = 4.0), 0.39 (*d*, *J* = 4.0)	28.9
20	2.67 – 2.76 (*m*)	40.9	1.86 (overlapped)	32.3
21	3.59 (*dd*, *J* = 8.1, 9.8), 4.30 (*dd*, *J* = 8.1, 8.1)	74.2	0.79 (*d*, *J* = 6.3)	19.3
22	1.81 (overlapped), 2.18 (*dd*, *J* = 12.5, 6.2)	42.6	2.40 (*dd*, *J* = 16.2, 2.2), 2.13 (*dd*, *J* = 16.2, 9.9)	50.3
23		112.1		210.5
24	6.68 (*q*, *J* = 1.6)	144.6	1.97 – 2.03 (*m*), 2.27 (overlapped)	24.1
25		133.6	2.27 (overlapped)	51.7
26	1.92 (*d*, *J* = 1.6)	10.7	0.83 (*d*, *J* = 7.1)	22.6
27		171.4	0.85 (*d*, *J* = 7.1)	22.5
28	1.09 (*s*)	18.5		178.0
29	0.96 (*s*)	21.5	0.89 (*s*)	19.1
30	0.88 (*s*)	19.3	0.99 (*s*)	9.5
1′			4.20 (*d*, *J* = 5.5)	103.6
2′			3.29 (*dd*, *J* = 5.5, 8.3)	70.9
3′			3.31 (overlapped)	72.3
4′			3.61 (overlapped)	66.9
5′			3.64, 3.30 (both overlapped)	64.1

[a]) Recorded in $CDCl_3$. [b]) Recorded in (D_6)DMSO.

signals for the known skeleton, the key changes at CH_2(21) were indicated by correlations from CH_2(21) to C(23), C(22), and C(17). The NOESY correlations H_β–C(19)/Me(29), H–C(8), and Me(18); from Me(18)/H–C(8), H–C(20), H_β–C(21), and H_β–C(16); and H–C(20)/H_β–C(21) and H_β–C(16) revealed the β-orientation of these H-atoms. The α-facial H-atoms and the configuration at C(23) were deduced on the basis of correlations from H–C(17) to H_α–C(21) and Me(30), from H–C(5) to Me(30) and Me(28), and from H_α–C(22) to H–C(24) and H_α–C(16) (*Fig. 2*).

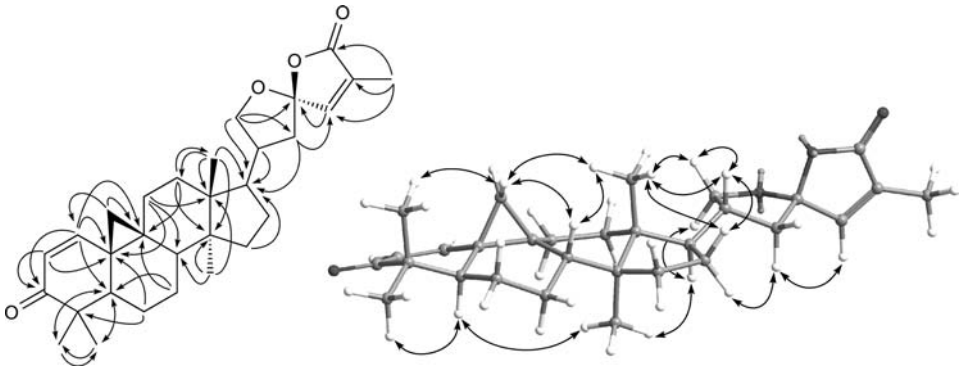

Fig. 2. *Selected HMBCs and NOESY correlations of compound* **1**

The absolute configuration of **1** was confirmed by quantum-chemical TDDFT calculation of the electronic circular dichroism (ECD) spectra. In the 190–425-nm region (*Fig. 3*), both of the experimental and theoretical ECD spectra showed a first negative *Cotton* effect at *ca.* 350 nm and a positive one at *ca.* 235 nm, which allowed the determination of the absolute configuration of **1**. The key molecular orbital involved in the characteristic first negative *Cotton* effects at *ca.* 350 nm (contributed by excited state 1) indicated that this effect is determined by configurations of ring *A* and the cyclopropane ring, which is in consistent with our previous interpretation [3]. The

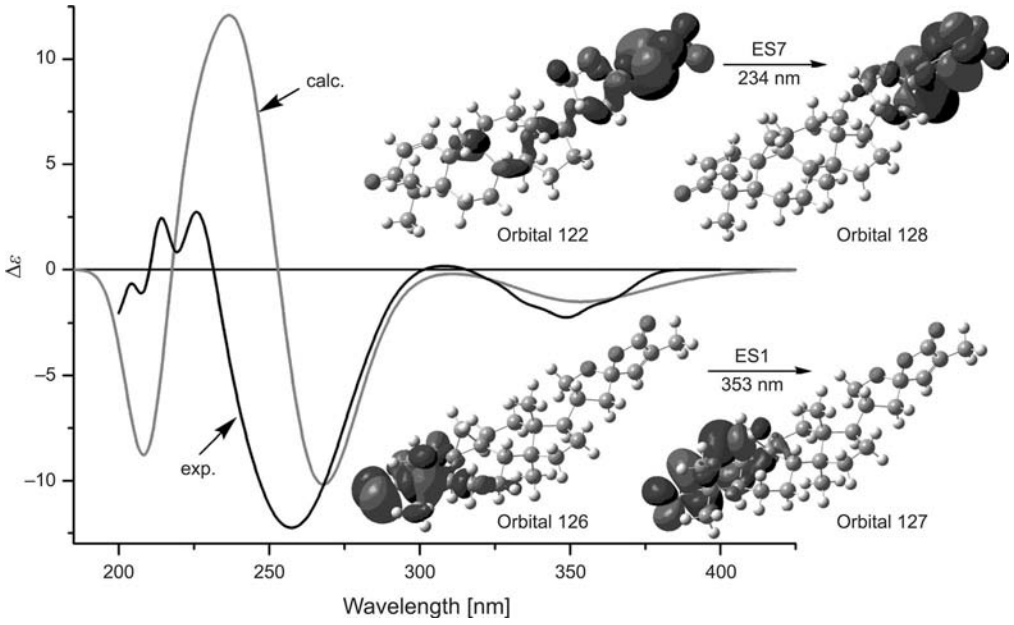

Fig. 3. *Experimental and B3LYP/6-311++G(2d,2p)//B3LYP/6-31+G(d)-calculated ECD spectra and the key molecular orbitals involved in the characteristic* Cotton *effects of* **1**

Cotton effects at *ca.* 235 nm was mainly contributed by electronic excitations from molecular orbitals 120–124 to 128 that surrounding the stereogenic C-atom C(23). Therefore, compound **1** was identified as (23*R*)-21,23:23,27-diepoxycycloarta-1,24-diene-3,27-dione (**1**).

Compound **2** was isolated as white amorphous powder, and its molecular formula was determined as $C_{35}H_{56}O_9$ by HR-ESI-MS. The ^{1}H-NMR spectrum showed typical signals for CH_2(19) of the cyclopropane ring at δ(H) 0.57 (*d*, *J* = 4.0) and 0.39 (*d*, *J* = 4.0), as well as six Me signals, including three *singlet*s at δ(H) 0.99 (*s*), 0.89 (*s*), and 0.94 (*s*), two intercoupling *doublet*s at δ(H) 0.85 (*d*, *J* = 7.1) and 0.83 (*d*, *J* = 7.1) for an ^{i}Pr terminal, and one *doublet* at δ(H) 0.79 (*d*, *J* = 6.3) assignable to Me(21). The MS data and the ^{1}H-NMR signals from 3.0 to 5.0 ppm indicated the presence of a pentopyranose moiety. In the ^{13}C-NMR spectrum, two signals at δ(C) 210.5 and 178.0 indicated C=O and COOH groups, respectively. One signal at δ(C) 103.6 could be assigned to the anomeric C-atom of the pentopyranose moiety. The presence of six C-atom signals from 60 to 80 ppm indicated four O-bearing C-atoms for the pentopyranose and two C-atoms of the aglycone. These data pointed to a cycloartane-type triterpenoid-saponin structure of for **2**. The C-skeleton, and the positions of the C=O, OH, and pentopyranose groups were determined by 2D-NMR techniques, including HSQC, HMBC, and NOESY experiments. The main C-skeleton of **2** was confirmed by HMBC features depicted in *Fig. 4*. The C=O group was located at C(23) as deduced from the HMBCs CH_2(22)/C(23) and CH_2(24)/C(23). The COOH group was assigned to C(28) by HMBC Me(29)/C(28). The key correlations CH_2(19)/H–C(1) and Me(29)/H–C(3) disclosed the positions of the two O-bearing C-atoms in ring *A*. Full assignment of the five C-atom signals at δ(C) 103.6, 72.3, 70.9, 66.9, and 64.1 to the pentopyranose based on a literature survey of characteristic C-atom signals for pentopyranose moiety of triterpenoid saponins [4][5], as well as the HMBC between H–C(1′) and H–C(3) suggested an α-L-arabinose moiety at C(3). In the NOESY spectrum, correlations from H–C(1) and H–C(3) to the β-facial H-atoms, H_a–C(19) and Me(29), respectively, evidenced their β-orientation (*Fig. 4*). Therefore, the structure of **2** was determined as (3α)-(α-L-arabinopyranosyloxy)-1α-hydroxy-23-oxocycloartan-28-oic acid.

By comparison of their spectroscopic data with those reported in the literature, the structures of the known compounds were identified as taraxerone (**3**) [6], taraxerol (**4**) [7], 2α,3β,23-trihydroxyolean-12-en-28-oic acid (**5**) [8], 3β,23-dihydroxy-12-oleanan-28-oic acid (**6**) [9], 2α,3α-dihydroxy-24-noroleana-4(23),12-dien-28-oic acid (**7**) [10], maslinic acid (**8**) [11], β-sitosterol (**9**) [12], daucosterol (**10**) [13], (22*E*,24*S*)-ergosta-4,6,8(14),22-tetraen-3-one (**11**) [14], stigmasta-5,22-diene-3β,7β-diol (**12**) [15], and 7β-hydroxysitosterol (**13**) [16]. Except **9** and **10**, all of the other known compounds have been isolated from *K. hospita* for the first time.

All compounds were evaluated against human colon carcinoma (HCT116) and gastric carcinoma (SGC7901) cell lines for their cytotoxic activities. Compounds **6**–**8** exhibited cytotoxic effects against the HCT116 cell line with IC_{50} values of 91.8, 23.0, and 54.2 μM, respectively. Compounds **6**, **7**, **11**, and **12** showed cytotoxic effects against the SGC7901 cell line with IC_{50} values of 80.7, 32.9, 57.4, and 49.8 μM, respectively. Taxol (IC_{50} 0.0059 μM for HCT116, 0.0013 μM for SGC7901) was used as positive control.

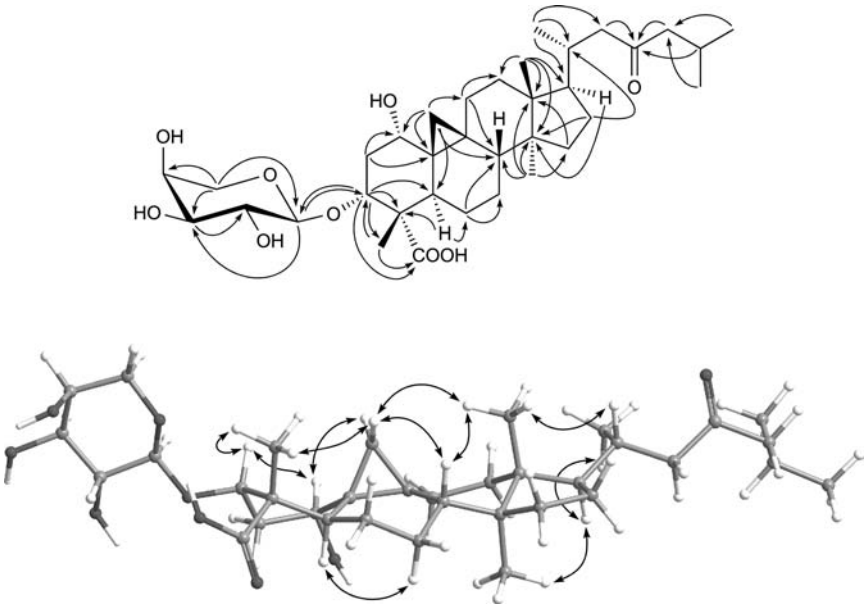

Fig. 4. *Selected HMBCs and NOESY correlations of compound* **2**

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Experimental Part

General. TLC: Precoated silica-gel plates (SiO_2; *HSGF*$_{254}$, *Yantai Jiangyou Silica Gel Development Co., Ltd.*). Column chromatography (CC): commercial SiO_2 for TLC (*Qingdao Haiyang Chemical Group Co., Ltd.*), C_{18} column (*Phenomenex 00G-4324-N0*; 10 μm, 10 mm (i.d.) × 250 mm), and *MCI* gel (*Mitsubishi*, Japan). HPLC: *Agilent1100* system (Englewood, U.S.). Optical rotations: *JASCO-P-1010* polarimeter. UV Spectra: *Beckman-DU-600* spectrometer; λ_{max} (log ε) in nm. IR Spectra: *JASCO-FT/IR-4100* spectrophotometer; KBr pellets; $\tilde{\nu}$ in cm^{-1}. NMR Spectra: *Bruker-AVIII-500M* spectrometer (*Bruker Co.*, Switzerland); δ in ppm rel. to Me_4Si as internal standard; *J* in Hz. ESI-MS: *Micromass-Quattro* triple-quadrupole mass spectrometer equipped with an ESI source (*Micromass*, Manchester, UK); in *m*/*z* (rel. %). HR-ESI-MS: *Agilent Q-TOF 1290 LC/6224* MS; in *m*/*z*.

Plant Material. Leaves and twigs of *K. hospita* (8 kg) were collected in July 2012 from Hainan Island, P. R. China, and authenticated by Prof. *Shi-Man Huang*, Department of Biology, Hainan University, P. R. China. A voucher specimen has been deposited with the Institute of Modern Chinese Medicine, Zhejiang University (accession No. KH-2012-I).

Extraction and Isolation. The dried plant material of *K. hospita* was ground and extracted at r.t. with 95% EtOH (3 × 50 l; for 5 d each time). The solvent was evaporated under reduced pressure to give a crude extract (163 g). The extract was suspended in 1.5 l of H_2O and partitioned successively with pertroleum ether (PE), AcOEt, and BuOH (3 × 2.0 l for each). The AcOEt fraction (55 g) was then subjected to CC (*MCI* gel; aq. EtOH 40, 50, 60, 70, 80, and 90% (*v*/*v*), each 2 l): *Frs. A – G. Fr. C* (1.8 g) was purified by CC (SiO_2; CH_2Cl_2/MeOH 90 : 1): **5** (55 mg). *Fr. D* (4.0 g) was separated by CC (SiO_2; PE/AcOEt 4 : 1): *Frs. D1 – D5*. *Fr. D3* (552 mg) was further purified by CC (SiO_2; PE/acetone 5 : 1; and C_{18}; 60% aq. EtOH): **6** (23 mg) and **7** (27 mg). Solvent precipitation of *Fr. D5* (220 mg) afforded **2** (70 mg). *Fr. E* (4.1 g) was separated on CC (SiO_2; cyclohexane/AcOEt 12 : 1): **8** (700 mg). *Fr. F* (1.7 g) was

subjected to CC (SiO_2; CH_2Cl_2/MeOH/HCOOH 200:20:1): **1** (32 mg). The PE fraction (65 g) was separated by CC (SiO_2; PE/CH_2Cl_2/MeOH 1:0:0 to 0:1:0.1): *Frs. pA–pK*. Compounds **3** (20 mg), **4** (176 mg), and **9** (439 mg) were obtained by recrystallization of *Frs. pA*, *pD*, and *pG*, resp. *Fr. pH* (5.6 g) was subjected to CC (*Sephadex LH-20*; CH_2Cl_2/MeOH 1:1; and C_{18}; 80% aq. EtOH): **11** (22 mg). *Fr. pI* (8.7 g) was submitted to CC (SiO_2; cyclohexane/AcOEt 10:1): *Frs. pI1–pI9*. *Fr. pI9* (42 mg) was separated by prep. HPLC (C_{18}; 100% MeOH): **12** (3 mg) and **13** (8 mg). *Fr. pK* (2 g) was subjected to CC (SiO_2; CH_2Cl_2/MeOH 30:1): **10** (400 mg).

*(23*R*)-21,23:23,27-Diepoxycycloarta-1,24-diene-3,27-dione* (=*(5α,9β,17β)-4,4,14-Trimethyl-17-[(3*R*,5*R*)-8-methyl-7-oxo-1,6-dioxaspiro[4.4]non-8-en-3-yl]-9,19-cycloandrost-1-en-3-one*; **1**): White amorphous powder. $[\alpha]_D^{25} = -66.4$ ($c = 0.75$, $CHCl_3$). UV (MeOH): 266 (3.86), 224 (4.35). IR (KBr): 2956, 2926, 2869, 1764, 1664, 1603, 1462, 1377, 1303, 1099, 1073, 1051, 939, 870, 764. ^{1}H- and ^{13}C-NMR: see the *Table*. ESI-MS: 465 ($[M+H]^+$), 951 ($[2M+Na]^+$). HR-ESI-MS: 465.3000 ($[M+H]^+$, $C_{30}H_{41}O_4^+$; calc. 465.2999).

*3α-(α-*L*-Arabinopyranosyloxy)-1α-hydroxycycloarta-23-one-28-oic Acid* (=*(1α,3α,9β)-3-(α-*L*-Arabinopyranosyloxy)-1-hydroxy-23-oxo-9,19-cyclolanostan-28-oic Acid*; **2**): White amorphous powder. $[\alpha]_D^{25} = +73.5$ ($c = 0.70$, MeOH). IR (KBr): 2956, 2925, 2855, 1711, 1463, 1375, 1248, 1171, 1087, 1071, 995. ^{1}H- and ^{13}C-NMR: see the *Table*. ESI-MS: 643 ($[M+Na]^+$). HR-ESI-MS: 643.3939 ($[M+Na]^+$, $C_{35}H_{56}NaO_9^+$, calc. 643.3817).

Quantum-Chemical TD DFT Calculations of ECD Spectra. Conformational search was first carried out *via* Monte Carlo using molecular mechanism with MMFF 94 force field in Spartan 08 software package [17]. Only one dominant conformer with relative energy within 2 kcal/mol was identified for compound **1**. Subsequently, the resulting conformer were re-optimized using DFT at the B3LYP/6-31 + G(d) level in the GAUSSIAN 09 program [18]. The B3LYP/6-31 + G(d) harmonic vibrational frequencies were further calculated to confirm their stability. The energies, oscillator strengths, and rotational strengths of the first 40 electronic excitations of the conformer were calculated by the TDDFT methodology at the B3LYP/6-311 + + G(2d,2p) level, and the ECD spectrum was then simulated by the overlapping Gaussian function [19] in which the velocity rotatory strengths of the first 15 electronic excitations were adopted (σ 0.3 eV).

Assay of Cytotoxic Activities. Suspended human colon carcinoma HCT116 and gastric carcinoma SGC7901 cells were cultured in *Dulbecco*'s Modified *Eagle*'s (DME) medium (*Gibco*, Grand Island, N.Y.), and supplemented with 10% fetal bovine serum (FBS; *Hangzhou Sijiqing*, P. R. China), L-glutamine (2 nM), penicillin (100 IU/ml), and streptomycin (100 μg/ml) at 37° in a humidified atmosphere with 5% CO_2. The logarithmic phase cells (100 μl) were seeded onto 96-well plates at the concentration of $5 \cdot 10^3$ cells-well. After 24 h, different concentrations of the sample, dissolved in DMSO, were added at 10 μl/well, and three parallel wells for each concentration were tested. Control cells were treated with DMSO alone and positive controls with taxol. The cells were cultivated for 72 h and then fixed with 10% CCl_3COOH for 1 h and washed by dist. H_2O. Sulforhodamine B (SRB) was dissolved at 4 mg/ml in phosphate-buffered saline (PBS). To each well, 100 μl of this soln. were added, and the cells were stained for 20 min. The supernatant was then removed, and 100 μl of *Tris* buffer (10 mM) were added into each well. The absorbance (*A* value) at 515 nm was measured with a microplate reader (*Thermo*). The inhibition rates were calculated by using *OD* mean values from inhibition rate = $(OD_{control} - OD_{sample})/OD_{control}$. The IC_{50} value was determined by the *Bliss* method.

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