DOI: 10.1002/cjoc.201100653

ent-Kaurane Diterpenoids from *Isodon eriocalyx* var. *laxiflora*†

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Four new *ent*-kaurane diterpenoids, 6*β*,13*α*,15*β*-trihydroxy-16-ene-3*α*,20-epoxy-*ent*-kaur-1,7-dione (**1**), 6-hydroxy-3*α*,20-epoxy-5(6)-ene-*ent*-kaur-1,7,15-trione (**2**), 6-hydroxy-15*β*-acetoxy-3*α*,20-epoxy-16*β*,17-epoxy-5(6)-ene-*ent*-kaur-1,7-dione (**3**), 3*α*,17-dihydroxy-15(16)-ene-*ent*-kaur-7-one (**4**), along with four known compounds **5**—**8** were isolated from the leaves of *Isodon eriocalyx* var. *laxiflora.* The structures were elucidated by extensive spectroscopic methods (IR, UV, MS and NMR). The cytotoxic activities of these compounds were evaluated by MTT assay. Compound **8** showed moderate inhibitory effects on HL-60, SMMC-7721, MCF-7 and SW-480 cell lines.

Keywords *ent*-Kaurane diterpenoid, *Isodon*, structure elucidation, cytotoxicity

Introduction

The genus *Isodon*, which includes about 150 species, is one of the most widespread members of the *Labiatae* (Lamiaceae) family and has attracted considerable attention as a prolific source of new natural products with diverse structural scaffold and biological diversity.[1] About 600 new diterpenoids with a diversity of highly oxygenated structures have been isolated and characterized, most of which have been recently summarized.[1,2]

Among plants of southwest China, *I. eriocalyx* var. *laxiflora* had been previously investigated phytochemically,[1] which led to the isolation of more than 60 new diterpenoids including 7,20-epoxy-*ent*-kauranoids (laxiflorins H-I),[3] 3,20-epoxy-*ent*-kauranoids (laxiflorins J-M),[4] 6,7-*seco*-*ent*-kauranoids (laxiflorins A—C),[5] 6,7:8,15-*seco*-*ent*-kauranoids (laxiflorins F and G),[6] *ent*-abietanoids (laxiflorin N),[7] and two unprecedented *ent*-kaurane diterpenoids (neolaxiflorins A and B).[8] Our further investigation on this plant led to the isolation of four new *ent*-kauranoids, 6*β*,13*α*,15*β*-trihydroxy-16-ene-3*α*,20-epoxy-*ent*-kaur-1,7-dione (**1**), 6-hydroxy-3*α*,20-epoxy-5(6)-ene-*ent*-kaur-1,7,15-trione (**2**), 6-hydroxy-15*β*-acetoxy-3*α*,20-epoxy-16*β*,17-

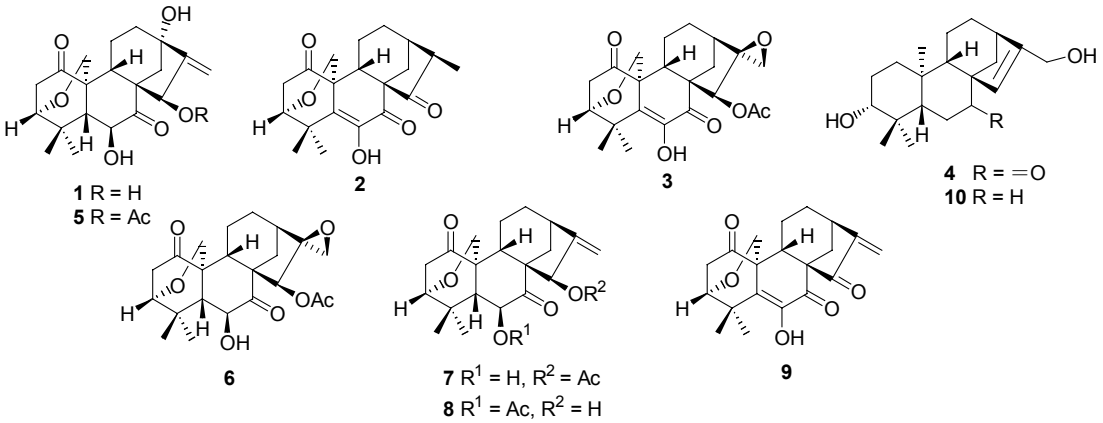

Figure 1 Molecular structures of **1**—**10**.

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Received December 1, 2011; accepted December 28, 2011; published online April 10, 2012.
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cjoc.201100653 or from the author.
† Dedicated to Professor Jun Zhou on the occasion of his 80th birthday.

epoxy-5(6)-ene-*ent*-kaur-1,7-dione (**3**), 3*α*,17-dihydroxy-15(16)-ene-*ent*-kaur-7-one (**4**), and another four known compounds, laxiflorin M (**5**),[9] eriocalyxin C (**6**),[10] maoecrystal A (**7**)[11] and maoecrystal U (**8**).[12] All of the compounds were 3,20-*epoxy*-*ent*-kauranoids except compound **4**, while it has been reported that there were less than 20 *ent*-kauranoids possessing 3,20-epoxy moiety in the thousands of natural *ent*-kaurane diterpenoids. In this paper, we report their isolation, structure elucidation and cytotoxic activities.

Experimental

General

Optical rotations were measured with a JASCO DIP-370 digital polarimeter. UV data were obtained on a Shimadzu UV-2401A spectrophotometer. A BioRad FtS-135 spectrophotometer was used for scanning IR spectroscopy with KBr pellets. 1D and 2D NMR spectra were recorded on DRX-400 spectrometers. Unless otherwise specified, chemical shifts (δ) were expressed with reference to the solvent signals. High-resolution electrospray-ionization mass spectrum (HRESIMS) were performed on a VG Autospec-3000 spectrometer under 70 eV. Column chromatography was performed with silica gel (100—200 mesh; Qingdao Marine Chemical, Inc., Qingdao, People's Republic of China). MPLC was performed on a Buchi C-615 liquid chromatograph with a RP-18, 50 mm×35 cm column. Fractions were monitored by TLC and spots were visualized by heating silica gel plates sprayed with 8% H_2SO_4 in EtOH. All solvents including petroleum ether (60—90 ℃) were distilled prior to use.

Plant material

The leaves of *Isodon eriocalyx* var. *laxiflora* were collected in Yunnan province, People's Republic of China, in September 2009. Voucher specimens (KIB20080028) were deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, and were identified by Prof. Xiwen Li.

Extraction and isolation

The air-dried leaves *Isodon eriocalyx* var. *laxiflora* (10 kg) were extracted with 70% aqueous acetone (40 L ×3, 2 d each) at room temperature. The solvent was evaporated *in vacuo* to afford a crude extract, which was suspended in H_2O, and then extracted successively with EtOAc and *n*-BuOH. The EtOAc-soluble part (600 g) was decolorized on MCI gel with 90% MeOH/H_2O to obtain a yellow gum (427.5 g). The gum was purified by CC (column chromatography on SiO_2 with $CHCl_3$-Me_2CO gradient system 1∶0, 9∶1, 8∶2, 7∶3, 6∶4 and 1∶1) to yield six main fractions, Fr. A—F. Fr. B ($CHCl_3$/acetone 9∶1, 80 g) was submitted to repeated chromatography over silica gel (petroleum ether-acetone, from 30∶1 to 1∶1) to give fractions B1—B4, compounds **7** (2.0 g) and **8** (15 mg) were crystallized from fraction B1 (petroleum ether-acetone, 30∶1). Then, the fraction B1 was purified by ODS column chromatography (50% CH_3CN-H_2O) to afford **2** (3 mg), **3** (5 mg), and **6** (4 mg). Fr. D ($CHCl_3$/acetone 7∶3, 50 g) was eluted with $CHCl_3$/CH_3OH (30∶1, 20∶1 and 10∶1), and yielded subfractions D1—D3. Subfraction D1 (20 g, $CHCl_3$/CH_3OH, 30∶1) was fragmented by repeated CC, first on RP-18 with a gradien elution of MeOH/H_2O (2∶8 to 1∶0) to yield fractions D1/1—D1/8. Subsequently fraction D1/3 (2.27 g) was purified by a silica gel column ($CHCl_3$/CH_3OH 50∶1 to 10∶1) to give subfractions D1/3/1—D1/3/8. Subfraction D1/3/1 (205 mg) was purified by MPLC (15 mL/min, MeOH/H_2O, 40∶60) to yield **5** (12 mg), **4** (5 mg). Subfraction D1/3/2 (180 mg) was purified by MPLC (20 mL/min, MeCN/H_2O 30∶70) to yield **1** (5 mg).

1: White, amorphous powder. $[\alpha]_D^{25}$ −119.2 (*c* 0.14, MeOH); UV (MeOH) λ_{max} (lg ε): 202.6 (3.09), 254.6 (1.98), 285.4 (1.25) nm; ^{1}H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) see Tables 1 and 2; IR (KBr) ν_{max}: 3441, 3000, 2971, 2944, 2933, 2896, 2877, 1743, 1720, 1642, 1636, 1385, 1373, 1087, 1078, 1062, 996, 857, 760 cm^{-1}; positive ESIMS *m/z*: 385 [M + Na]$^+$; positive HRESIMS [M + Na]$^+$ calcd for $C_{20}H_{26}O_6Na$ 385.162, found 7385.1626.

2: White, amorphous powder. $[\alpha]_D^{25}$ −120.0 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (lg ε): 201.5 (6.1), 279.5 (3.4), 313.0 (3.1), 366 (2.9) nm; ^{1}H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) see Tables 1 and 2; IR (KBr) ν_{max}: 3386, 2984, 2969, 2949, 2895, 2847, 1749, 1729, 1657, 1398, 1379, 1363, 1295, 1271, 1242, 1090, 1056, 1007, 987, 788, 752 cm^{-1}; positive ESIMS *m/z*: 367 [M+Na]$^+$; positive HRESIMS [M+Na]$^+$ calcd for $C_{20}H_{24}O_5Na$ 367.1521, found 367.1528.

3: White, amorphous powder. $[\alpha]_D^{25}$ −11.7 (*c* 0.43, MeOH); UV (MeOH) λ_{max} (lg ε): 201.8 (2.9), 278.4 (2.9), 306.8 (2.6), 311.2 (2.6), 360.4 (2.2) nm; ^{1}H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) see Tables 1 and 2; IR (KBr) ν_{max}: 3419, 2929, 2875, 2306, 1737, 1670, 1374, 1279, 1227, 1097, 1076, 985, 789, 775, 754 cm^{-1}; positive ESIMS *m/z*: 425 [M+Na]$^+$; positive HRESIMS [M + Na]$^+$ calcd for $C_{22}H_{30}O_7Na$ 425.1576, found 425.1571.

4: White, amorphous powder. $[\alpha]_D^{25}$ −45.7 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (lg ε): 203.8 (3.0) nm; ^{1}H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) see Tables 1 and 2; IR (KBr) ν_{max}: 3441, 2975, 2927, 2858, 1684, 1389, 1365, 1098, 998, 630 cm^{-1}; positive ESIMS: *m/z* 341 [M + Na]$^+$; positive HRESIMS [M+Na]$^+$ calcd for $C_{20}H_{30}O_3Na$ 341.2092, found 341.2084.

Cellular proliferation assay

Colorimetric assays were performed to evaluate compound activity. The following human tumor cell lines were used: the A549 lung cancer cell line, the

HL-60 human myeloid leukemia cell line, the MCF-7 breast cancer cell line, the SMMC-7721 human hepatocarcinoma cell line, and the SW-480 human pancreatic carcinoma. All cells were cultured in RPMI-1640 or DMEM medium (Hyclone, Logan, UT), supplemented with 10% fetal bovine serum (Hyclone) at 37 ℃ in a humidified atmosphere with 5% CO_2. Cell viability was assessed by conducting colorimetric measurements of the amount of insoluble formazan formed in living cells based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO). Briefly, 100 μL adherent cells were seeded into each well of a 96-well cell culture plate and allowed to adhere for 12 h before drug addition, while suspended cells were seeded just before drug addition, both with initial density of 1×10^5 cells/mL in 100 μL of medium. Each tumor cell line was exposed to the test compound at various concentrations in triplicate for 48 h, with *cis*-Platin (Sigma) as positive control. After the incubation, MTT (100 μg) was added to each well, and the incubation continued for 4 h at 37 ℃. The cells were lysed with 100 μL of 20% SDS-50% DMF after removal of 100 μL of medium. The optical density of the lysate was measured at 595 nm in a 96-well microtiter plate reader (Bio-Rad 680). The IC_{50} value of each compound was calculated by Reed and Muench's method.[13]

Table 1 ^{13}C NMR spectroscopic data of compounds **1**—**4**

No.	δ			
	1	**2**	**3**	**4**
1	209.2 (s)	205.4 (s)	206.2 (s)	38.4 (t)
2	42.2 (t)	41.8 (t)	41.9 (t)	27.9 (t)
3	77.3 (d)	78.0 (d)	77.8 (d)	77.7 (d)
4	38.1 (s)	40.9 (s)	40.8 (s)	39.7 (s)
5	51.9 (d)	133.4 (s)	133.0 (s)	53.0 (d)
6	72.2 (d)	147.1 (s)	146.1 (s)	37.6 (t)
7	211.3 (s)	193.9 (s)	193.7 (s)	213.2 (s)
8	57.0 (s)	60.1 (s)	55.2 (s)	61.9 (s)
9	33.0 (d)	32.5 (d)	27.7 (d)	48.9 (d)
10	52.0 (s)	54.7 (s)	54.2 (s)	38.9 (s)
11	24.3 (t)	19.1 (t)	20.7(t)	18.5 (t)
12	41.2 (t)	23.6 (t)	27.9 (t)	25.1 (t)
13	77.5 (s)	35.2 (d)	40.8 (d)	40.2 (d)
14	44.1 (t)	38.8 (t)	37.9 (t)	42.7 (t)
15	73.1 (d)	217.4 (s)	77.4 (d)	129.4 (d)
16	159.8 (s)	49.3 (d)	68.6 (s)	150.0 (s)
17	105.9 (t)	9.7 (q)	47.6 (t)	60.3 (t)
18	29.5(q)	23.4 (q)	23.5 (q)	28.1 (q)
19	23.2 (q)	22.0 (q)	21.8 (q)	15.6 (q)
20	62.5 (t)	66.7 (t)	67.3 (t)	16.7 (q)
OAc	—	—	169.6 (s), 20.4 (q)	—

Results and Discussion

Structural elucidation

Compound **1**, obtained as a white, amorphous powder, gave the molecular formula of $C_{20}H_{26}O_6$ from its HRESIMS (calcd for $[M+Na]^+$ 385.1627, found 385.1626), indicating eight degrees of unsaturation. IR absorptions at 3441, 1743 and 1720 cm^{-1} implied the presence of hydroxyl and carbonyl groups, respectively. The ^{13}C NMR and DEPT spectra of **1** displayed 20 carbon signals corresponding to two methyls, six methylenes (of which one was oxygenated, one was olefinic carbon), five methines (including three oxygenated), and seven quaternary carbons (including two keto carbonyl groups, one oxygenated, one olefinic carbon) (Table 1).

This was consistent with a skeleton of an *ent*-kaurane diterpenoid.[14] Comparison of its NMR data (Table 1) with those of laxiflorin M (**5**)[9] revealed similarities except for the lack of the signal of an acetoxy group in **5** instead of a hydroxyl group in **1** at C-15, which was supported by the HMBC correlations from H-14a (δ_H 2.26, d, J=12.0 Hz, 1H) and H-14b (δ_H 1.87, d, J=12.0 Hz, 1H) to C-8, C-9, C-12, and C-15, H-15 (δ_H 5.65, s, 1H) to C-7, C-8, C-9, C-13, C-14, C-16, and C-17, and H_2-17 (δ_H 5.65, s, 2H) to C-13, C-15 and C-16. In the ROESY spectrum (Figure 2) of **1**, correlations of H-3 with Me-18, H-5 with Me-18 and H-9 showed that H-3, H-5, H-9, and Me-18 in **1** had *β*-configurations. The ROESY correlations between H-6 and H-15, H-6 and Me-19, Me-19 and H_2-20 showed that H-6, H-15, Me-19, and C-20 had the same *α*-configurations. Therefore, **1** is 6*β*,13*α*,15*β*-trihydroxy-16-ene-3*α*,20-epoxy-*ent*-kaur-1,7-dione.

Compound **2** exhibited the molecular formula $C_{20}H_{24}O_5$ as determined by the positive ion HRESI-MS (calcd for $C_{20}H_{24}O_5Na$ $[M+Na]^+$ 367.1521, found 367.1528). Comparison of its ^1H and ^{13}C NMR data (Tables 1 and 2) with those of maoecrystal P (**9**)[15] indicated that these two compounds are closely related, and the only difference was that the reduction of carbon-carbon double bond at C-16/C-17 in **9** brought a methyl at C-16 in **2**.

The correlations from H_3-17 (δ_H 1.05, d, J=6.8 Hz, 3H) to C-13, C-15 and C-16 and from H-16 (δ_H 2.43, q, J=6.8 Hz, 1H) to C-8, C-12, C-13, C-14, C-15, and C-17 in the HMBC spectrum and the ^1H-^1H COSY correlation between H-16 and H-17 confirmed the reduction of the double bond between C-16 and C-17 in **9**. On the basis of the ROESY correlations of H-13/H-16, the relative configurations of H-16 and Me-17 were deduced to be *α* and *β*-oriented, respectively. The relative configurations of the remaining stereogenic centers in **2** were determined to be the same as **9**, based on detailed analysis of ROESY data. Thus, **2** was elucidated as 6-hydroxy-3*α*,20-epoxy-5(6)-ene-*ent*-kaur-1,7,15-trione.

Table 2 ^{1}H NMR spectroscopic data of compounds **1**—**4**

No.	δ (J in Hz)			
	1	**2**	**3**	**4**
1	—	—	—	1.75—1.77 (m, 1H)
				0.83—0.89 (m, 1H)
2	2.75 (br s, 2H)	2.98 (dd, J=19.4, 3.3, 1H)	3.04 (dd, J=19.5, 3.2, 1H)	1.83—1.90 (m, 2H)
	—	2.85 (brd, J=19.4, 1H)	2.90 (dd, J=19.5, 1.8, 1H)	
3	3.74 (brs, 1H)	3.84 (brs, 1H)	3.86 (t, J=2.6, 1H)	3.39 (dd, J=11.6, 4.9, 1H)
5	1.78 (d, J=11.7, 1H)	—	—	1.21 (dd, J=13.6, 3.0, 1H)
6	5.05 (d, J=11.7, 1H)	—	—	2.55 (dd, J=14.0, 13.6, 1H)
				2.50 (dd, J=14.0, 3.0, 1H)
9	3.59 (br d, J=8.2, 1H)	3.38 (br d, J=7.3, 1H)	3.66 (br d, 7.5, 1H)	1.34 (br d, J=8.4, 1H)
11	2.52—2.56 (m, 1H)	1.47—1.51 (m, 2H)	2.17 (dd, 15.2, 8.0, 1H)	1.61—1.65 (m, 1H)
	1.74—1.78 (m, 1H)		1.60 (dd, 15.2, 5.5, 1H)	1.52—1.55 (m, 1H)
12	1.91—1.95 (m, 1H)	1.49—1.52 (m, 1H)	1.51—1.55 (m, 1H)	1.61—1.63 (m, 1H)
	1.84—1.89 (m, 1H)	1.26 (d, J=3.0, 1H)	1.24—1.28 (m, 1H)	1.52—1.55 (m, 1H)
13	—	2.29 (brs, 1H)	1.93 (brs, 1H)	2.72 (br d, J=3.2, 1H)
14	2.26 (d, J=12.0, 1H)	2.15 (d, J=12.3, 1H)	1.94—1.96 (m, 2H)	2.14 (d, J=10.0, 1H)
	1.87 (d, J=12.0, 1H)	1.93 (brd, J=12.3, 1H)		1.82 (br d, J=10.0, 1H)
15	5.65 (s, 1H)	—	6.60 (s, 1H)	6.21 (s, 1H)
16	—	2.43 (q, J=6.8, 1H)	—	—
17	5.65 (br s, 2H)	1.05 (d, J=6.8, 3H,)	2.99 (d, J=4.7, 1H)	4.48 (s, 2H)
			2.82 (d, J=4.7, 1H)	
18	1.11 (s, 3H)	1.78 (s, 3H)	1.80 (s, 3H)	1.12 (s, 3H)
19	1.67 (s, 3H)	1.30 (s, 3H)	1.30 (s, 3H)	1.01 (s, 3H)
20	4.97 (d, J=9.5, 1H)	4.78 (d, J=9.0, 1H)	4.78 (d, J=10.2, 1H)	1.13 (s, 3H)
	4.29 (d, J=9.5, 1H)	4.31 (d, J=9.0, 1H)	4.37 (d, J=10.2, 1H)	
OAc	—	—	1.92 (s, 3H)	—

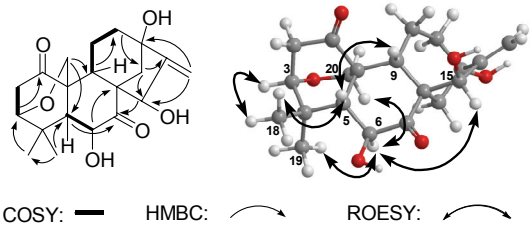

Figure 2 Key COSY, HMBC and ROESY correlations of **1**.

The HRESIMS of compound **3** (calcd for $C_{22}H_{26}O_7Na$ $[M+Na]^+$ 425.1576, found 425.1571) suggested a molecular formula of $C_{22}H_{26}O_7$, with ten degrees of unsaturation. Its ^{1}H and ^{13}C NMR data (Tables 1 and 2) indicated a 3α,20-epoxy-*ent*-kaurane, similar to eriocalyxin C (**6**).[10] The most notable difference was that the single bond (C-5/C-6) in **6** was oxidized to a carbon-carbon double bond in **3**. This was supported by HMBC correlations from H-9 (δ_H 3.66, br d, J=7.5 Hz, 1H), Me-18 (δ_H 1.80, s, 3H), Me-19 (δ_H 1.30, s, 3H), H-20a (δ_H 4.78, 1H, J=10.2 Hz, d), and H-20b (δ_H 4.37, d, J=10.2 Hz, 1H) to C-5. The ROESY experiment indicated that they had the same relative configuration. Consequently, compound **3** was determined to be 6-hydroxy-15β-acetoxy-3α,20-epoxy-16β,17-epoxy-5(6)-ene-*ent*-kaur-1,7-dione.

Compound **4** was found by HRESIMS (calcd for $C_{20}H_{30}O_3Na$ $[M+Na]^+$ 341.2092, found 341.2084) to possess the molecular formula $C_{20}H_{30}O_3$. Detailed analysis of the NMR spectra of **4** and **10**[16] showed that a carbonyl (δ_C 213.2 s) occurred at C-7 in **4**, in good agreement with the observed HMBC correlations from H-5 (δ_H 1.21, dd, J=13.6, 3.0 Hz, 1H), H-6a (δ_H 2.55, dd, J=14.0, 13.6 Hz, 1H), H-6b (δ_H 2.50, dd, J=14.0, 3.0 Hz, 1H), and H-14b (δ_H 1.82, br d, J=10.0 Hz, 1H) to C-7. Analysis of the ROESY data indicated that they had the same relative configuration (Figure 3). Eventually, **4** was established as 3α,17-dihydroxy-15(16)-ene-*ent*-kaur-7-one.

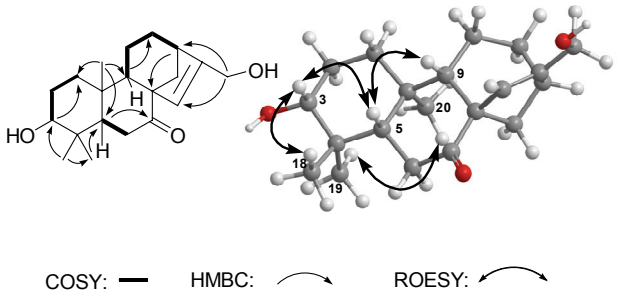

Figure 3 Key COSY, HMBC, and ROESY correlations of **4**.

Cytotoxic bioactivity

Compounds **1**—**8** were tested for the *in vitro* cytotoxicity against A-549, HL-60, MCF-7, SMMC-7721, and SW-480 human cancer cell lines using the MTT method,[13] with *cis*-platin as the postive controls. Only compound **8** exhibited modest cytoxicity activity for HL-60, SMMC-7721, MCF-7 and SW-480 cell lines (Table 3).

Table 3 Cytotoxic activity of compound **8**[a]

Compound	HL-60	SMMC-7721	A-549	MCF-7	SW480
8	4.46	16.20	＞40	8.53	6.41
cis-platin	1.96	16.23	17.50	17.77	12.83

[a] Results are expressed as IC_{50} values in μmol•L^{-1}, and data were obtained from triplicate experiments, and *cis*-platin was used as positive control.

Conclusions

In a summary, eight *ent*-kaurane diterpenoids including four new compounds and seven unusual 3,20-epoxy-*ent*-kauranoids have been isolated from *I. eriocalyx var. laxiflora* collected in the southwest of China. Their structures were determined by analysis of 1D and 2D NMR spectroscopic data. Maoecrystal U (**8**) showed moderate inhibitory effects on several tumor cell lines.

Acknowledgement

This project was supported financially by the NSFC-Joint Foundation of Yunnan Province (No. U0832602 to H.-D. Sun), the National Natural Science Foundation of China (No. 81172939 to J.-X. Pu), the Major State Basic Research Development Program of China (Nos. 2009CB522300 and 2009CB940900), the Science and Technology Program of Yunan Province (Nos. 2008IF010 and 2008CD162), and the Major Direction Projection Foundation of CAS Intellectual Innovation Project (No. 2010KIBA05 to J.-X. Pu).

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