New Diterpenoids from the Marine Mangrove *Bruguiera gymnorrhiza*

Li Han,[†,‡] Xueshi Huang,[‡] Isabel Sattler,*[,‡] Hans-Martin Dahse,[‡] Hongzheng Fu,[†] Wenhan Lin,[†] and Susanne Grabley[‡]

National Research Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing 100083, People's Republic of China, and Hans-Knoell-Institute for Natural Products Research (HKI), Beutenbergstrasse 11a, D-07745 Jena, Germany

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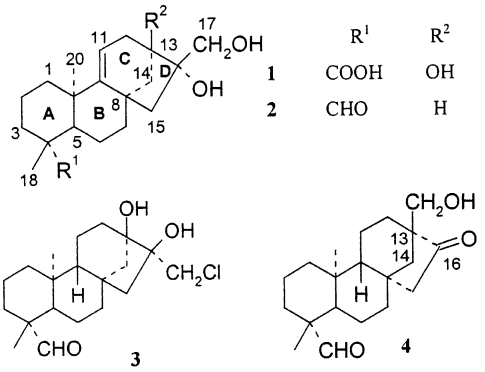
Phytochemical investigation of the stem of *Bruguiera gymnorrhiza* yielded three new *ent*-kaurane diterpenoids (**1**, **2**, and **3**) and one new *ent*-beyerane diterpenoid (**4**) together with nine known *ent*-kaurane diterpenoids. All structures and the relative stereochemistry of the new compounds were determined by NMR spectroscopic studies. The absolute stereochemistry of **4** was determined by CD data. Some of the compounds showed moderate cytotoxic properties.

As part of our investigations on marine sources of bioactive compounds, we have investigated systematically the chemical constituents of the stem of *Bruguiera gymnorrhiza* (L.) Lamk. (Rhizophoraceae). This large evergreen tree was collected from the coast of Xiamen in the south of mainland China. Recent studies on *B. gymnorrhiza* from India have shown the presence of diterpenes, triterpenes, and flavonoids in its leaves and the outer layer of the root bark.[1−4]

In this paper we describe the isolation and structural elucidation of four new diterpenes, named 13,16α,17-trihydroxy-*ent*-9(11)-kaurene-19-oic acid (**1**), 16α,17-dihydroxy-*ent*-9(11)-kaurene-19-al (**2**), 17-chloro-13,16*β*-dihydroxy-*ent*-kauran-19-al (**3**), and (4*R*,5*S*,8*R*,9*R*,10*S*,13*S*)-*ent*-17-hydroxy-16-oxobeyeran-19-al (**4**) together with the known diterpenes methyl-16α,17-dihydroxy-*ent*-kauran-19-oate (**5**),[5] 16α,17-dihydroxy-*ent*-9(11)-kauren-19-oic acid (**6**),[6,7] methyl-16α,17-dihydroxy-*ent*-9(11)-kauren-19-oate (**7**),[7,8] 16α,-17-dihydroxy-*ent*-kauran-19-al (**8**),[9,10] 16α*H*-17-hydroxy-*ent*-kauran-19-oic acid (**9**),[9] 16α*H*-17,19-*ent*-kauranediol (**10**),[9,11,12] 13-hydroxy-16-*ent*-kauren-19-al (**11**),[1] 16-*ent*-kaurene-13,19-diol (**12**),[1] and 16-*ent*-kauren-19-ol (**13**).[13] As attempts to grow single crystals of the new compounds suitable for X-ray crystallography failed, we used detailed NMR spectroscopic analysis, in particular NOESY experiments, to determine the relative stereochemistry of the new compounds. In the case of **4**, analysis of CD data according to the octant rule allowed determination of the absolute stereochemistry. Results of biological testing are presented for compounds **3**−**5** and **7**−**13**.

Compound **1** was obtained as a white amorphous solid and showed a molecular formula of $C_{20}H_{30}O_5$, as determined by HREIMS of the molecular ion at *m*/*z* 350.2095 (calcd 350.2093). The major IR absorption bands indicated a double bond (1652 cm^{-1}), a carboxyl group (1684 cm^{-1}), and hydroxy groups (3440 cm^{-1}). ^1H NMR and ^{13}C NMR spectra of **1** (Table 1) indicated an olefinic proton on a triple-substituted double bond, an oxymethylene group, eight regular methylenes, one methine, and two tertiary methyl groups. The 20 signals of the ^{13}C NMR spectrum and a DEPT experiment of **1** showed the corresponding carbon atoms and in addition a carboxy, two oxygen-bearing quaternary carbons, one sp^2 carbon, and three



	R^1	R^2
1	COOH	OH
2	CHO	H

regular quaternary carbons. The NMR data showed similarities to data reported for methyl-*ent*-9(11)-kauren-13,-17-epoxy-16-hydroxy-19-oate (**14**)[1] except for the missing carbomethoxy group, thus indicating a close similarity in the skeletons of these two compounds. Only one degree less of unsaturation and the chemical shift of C-13 at higher field strength (Δ*δ* −3 ppm) were observed for **1** compared to the known compound. At the same time, **1** carried two more protons and one more oxygen atom without accounting for the additional CH_2 of the methyl ester of **14**. These facts indicated that in **1** the hydroxy groups at C-13 and C-17 have not formed an oxetane and the carboxy group at C-19 is not esterified. Unambiguous and complete assignments of the ^1H and ^{13}C NMR spectra of **1** (Table 1) were achieved with COSY, HMQC, HMBC, and NOESY experiments. The NOESY data (Figure 1) allowed detailed stereochemical analysis of **1**. Several NOE correlations confirmed the relative configuration of the *ent*-kaurane structure, e.g., between H-15α (*δ* 1.50) and H-7*β* (*δ* 1.32) and between H-2α (*δ* 1.80) and Me-20 (*δ* 0.93). Moreover, the NOE cross-peaks observed between H-12*β* (*δ* 2.28) and H-17a (*δ* 3.20) suggested that the hydroxy group at C-16 could be placed on the α face of ring D. Therefore, the structure of **1** was determined to be 13,16α,17-trihydroxy-*ent*-9(11)-kauren-19-oic acid.

Compound **2** was obtained as a white amorphous powder. ESIMS indicated quasi-molecular ion peaks at *m*/*z* 341.2 $[M + Na]^+$, 659.2 $[2M + Na]^+$, and 336.3 $[M + NH_4]^+$. The molecular formula was established as $C_{20}H_{30}O_3$ by HREIMS at *m*/*z* 318.2199 (calcd 318.2195). The ^1H NMR spectrum showed signals for two tertiary methyl groups at *δ* 0.91 and 0.98 and an aldehyde moiety at *δ* 9.96, which represented an equatorial C-18 and an axial C-20 methyl

* To whom correspondence should be addressed. Tel: 0049-3641-656920. Fax: 0049-3641-656679. E-mail: isabel.sattler@hki-jena.de.
† Peking University.
‡ Hans-Knoell-Institute for Natural Products Research.

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Table 1. ^{1}H NMR (300 MHz, δ in ppm, J in Hz) and ^{13}C NMR (75 MHz, δ in ppm) Data for **1**−**4**

	1[a]		**2**[b]		**3**[b]		**4**[b]	
position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	α 1.88 d[c]	40.6 t	α 1.18−2.30[d]	39.9 t	α 1.80 m[d]	39.7 t	α 1.68 d[c]	38.9 t
	β 1.15 td (12.1, 3.0)		β 1.18−2.30[d]		β 0.80 m[d]		β 0.90 t[c]	
2	α 1.80 m[d]	19.8 t	α 1.18−2.30[d]	19.2 t	α 1.63 m[d]	18.2 t	α 1.57 td[c]	18.1 t
	β 1.40 dt[c]		β 1.18−2.30[d]		β 1.45 m[d]		β 1.45 d[c]	
3	α 2.00 d (13.2)	37.9 t	α 1.18−2.30[d]	35.1 t	α 2.12 m[d]	34.2 t	α 2.10 m[d]	34.2 t
	β 0.95 td (13.2, 2.8)		β 1.18−2.30[d]		β 0.98 m[d]		β 1.00 m[d]	
4		43.6 s		48.3 s		48.4 s		48.2 s
5	1.52 t (10.4)	45.5 d	1.18−2.30[d]	45.9 d	1.12 dd (12.6, 2.4)	56.4 d	1.26 br d[c]	56.6 d
6	α 2.48 m[d]	17.5 t	α 1.18−2.30[d]	17.5 t	α 1.65 m[d]	19.9 t	α 1.91 m[d]	19.7 t
	β 1.72 m[d]		β 1.18−2.30[d]		β 1.86 m[d]		β 1.62 m[d]	
7	α 1.82 m[d]	29.9 t	α 1.18−2.30[d]	29.6 t	α 1.67 m[d]	41.7 t	α 1.72 m[d]	41.2 t
	β 1.32 dd[c]		β 1.18−2.30[d]		β 1.42 m[d]		β 1.55 m[d]	
8		39.5 s		42.6 s		41.0 s		39.5 s
9		156.4 s		156.6 s	0.94 br d (8.9)	54.1 d	1.28 br d[c]	54.9 d
10		38.1 s		38.3 s		39.2 s		37.9 s
11	5.25 t (3.0)	114.9 d	5.14 t (3.0)	113.8 d	α 1.77 m[d]	19.9 t	α 1.23 m[d]	19.9 t
					β 1.57 m[d]		β 1.79 m[d]	
12	α 2.19 dd (15.0, 2.8)	37.1 t	α 1.18−2.30[d]	30.1 t	α 1.83 m[d]	32.9 t	α 1.78 m[d]	31.9 t
	β 2.28 dd (15.0, 3.8)		β 1.18−2.30[d]		β 1.75 m[d]		β 1.32 m[d]	
13		76.9 s	1.18−2.30[d]	44.2 d		76.7 s		54.1 s
14	α 1.27 dd (2.0, 10.0)	50.1 t	α 1.18−2.30[d]	42.9 t	α 1.78 m[d]	43.7 t	α 1.31 d[c]	48.9 t
	β 1.80 d (10.0)		β 1.18−2.30[d]		β 1.88 m[d]		β 1.80 br d[c]	
15	α 1.50 d (13.1)	52.4 t	α 1.18−2.30[d]	55.0 t	α 1.56 m[d]	53.0 t	α 2.64 dd (3.9, 18.8)	49.1 t
	β 1.85 d (13.1)		β 1.18−2.30[d]		β 1.70 m[d]		β 1.83 d (18.8)	
16		79.4 s		84.6 s		80.7 s		222.5 s
17	a 3.20 d (13.2)	66.5 t	a 3.62 d (10.8)	68.4 t	a 3.70 d (11.3)	50.5 t	a 3.48 d (11.2)	64.9 t
	b 3.27 d (13.2)		b 3.52 d (10.8)		b 3.75 d (11.3)		b 3.62 d (11.2)	
18	1.09 s	27.9 q	0.98 s	24.2 q	0.97 s	24.2 q	1.00 s	24.3 q
19		178.5 s	9.96 d (1.1)	206.6 d	9.70 d (1.4)	205.5 d	9.69 d (1.3)	205.4 d
20	0.93 s	23.4 q	0.91 s	23.7 q	0.85 s	16.3 q	0.71 s	14.1 q
OH-13					2.37 s			
OH-16					3.12 s			

[a] In DMSO-d_6. [b] In $CDCl_3$. [c] Multiplicity and chemical shift deduced from HMQC spectra. [d] Overlapped signal.

group as well as an axial aldehyde group at C-19.[14] The ^{13}C NMR data of **2** (Table 1) were very similar to those of methyl 16α,17-dihydroxy-*ent*-9(11)-kauren-19-oate (**7**)[7,8] except for the signals of ring A (Table 1). In the ^{13}C NMR spectra of **2**, C-3 was observed at higher ($\Delta\delta$ −5 ppm), C-4 at lower ($\Delta\delta$ +4 ppm), and Me-18 at higher field strength ($\Delta\delta$ −4 ppm) compared to the data of **7**. Together with an additional aldehyde group (δ 206.6) and the missing ester carbonyl and methoxy groups, an axial aldehyde group was proposed at C-19 of **2**. Therefore, the structure of **2** was established as 16α,17-dihydroxy-*ent*-9(11)-kauren-19-al.

Compound **3** was obtained as a white amorphous powder. ESIMS exhibited the quasi-molecular ion at *m*/*z* 377.2 [M + Na]$^+$, 731.0 [2M + Na]$^+$, and 372.2 [M + NH_4]$^+$. Accompanying peaks, e.g., at *m*/*z* 379.0 [(M + 2) + Na]$^+$ with a relative intensity of 1:3 of the peak at 377.2 [M + Na]$^+$, indicated the presence of one chloride atom. The molecular formula of $C_{20}H_{31}NaClO_3$ was determined by HRESIMS at *m*/*z* 377.2276 (calcd 377.1859). The ^{13}C NMR spectrum of **3** (Table 1) was very similar to that of **8**,[9,10] except for differences in chemical shift values of C-12, C-13, C-14, and C-17. It showed an additional oxygenated quaternary carbon signal at δ 76.7, and the methine signal for C-13 of ring D was absent. Thus, like in **1**, a hydroxy group has to be located at C-13. The hydroxy-methylene group of **8** was replaced by a chloromethylene group at C-16, as observed by methylene signals at δ 3.70 (1H, d, J = 11.3 Hz) and 3.75 (1H, d, J = 11.3 Hz) in the ^{1}H NMR spectrum and the carbon signal at δ 50.5, according to a HMQC experiment. The ^{1}H NMR spectrum of **3** (Table 1) exhibited signals for two tertiary methyl groups at δ 0.85 and 0.97 and an aldehyde moiety at δ 9.70, corresponding to the equatorial C-18 and axial C-20 methyl groups of an *ent*-kaurane diterpene with an axial aldehyde group at C-19.[14] HMBC correlations between OH-13 and C-13 (δ 76.7) and between OH-16 and C-13 (δ 76.7), C-15 (δ 53.0), and C-16 (δ 80.7) indicated the assignment of the two hydroxyl protons. NOE effects observed in a NOESY spectrum between the C-16 hydroxy proton (δ 3.12) and the equatorial H-12β (δ 1.75) suggested that OH-16 could be placed on the β face of ring D (Figure 1). This stereochemistry is also consistent with the finding that the protons of C-17 showed NOE effects only with OH-13 and OH-16 and no NOE with any other protons. All other NOE correlations confirmed the assigned structure. On the basis of the above evidence, the structure of **3** was established as 17-chloro-13,16β-dihydroxy-*ent*-kauran-19-al.

Compound **4** was isolated as a white amorphous powder. ESIMS afforded the quasi-molecular ion at *m*/*z* 319.3 [M + H]$^+$, 336.3 [M+NH_4]$^+$, 341.3 [M + Na]$^+$, and 659.2 [2M + Na]$^+$. The molecular formula was established as $C_{20}H_{30}O_3$ by HREIMS at *m*/*z* 318.2205 (calcd 318.2195). The ^{13}C NMR spectrum of compound **4** showed 20 carbon signals, indicating an aldehyde, a carbonyl, an oxymethylene, four quaternary carbons, nine methylenes, two methines, and two tertiary methyl groups (Table 1). Its ^{1}H and ^{13}C NMR spectral data, reminiscent of an *ent*-beyerane diterpenoid, were found to be very close to the data of *ent*-16-oxobeyeran-19-oic acid.[15] The ^{1}H NMR spectrum (Table 1) showed signals for two tertiary methyl groups (δ 0.71 and 1.00) and an aldehyde moiety at δ 9.69, which represented an equatorial C-18 and an axial C-20 methyl group as well as an axial aldehyde group at C-19.[14] The two oxymethylene protons (δ 3.48 d, J = 11.2 Hz and 3.62 d, J = 11.2 Hz) formed an AB system, which suggested that the oxymethylene is connected to a quaternary carbon. HMBC correla-

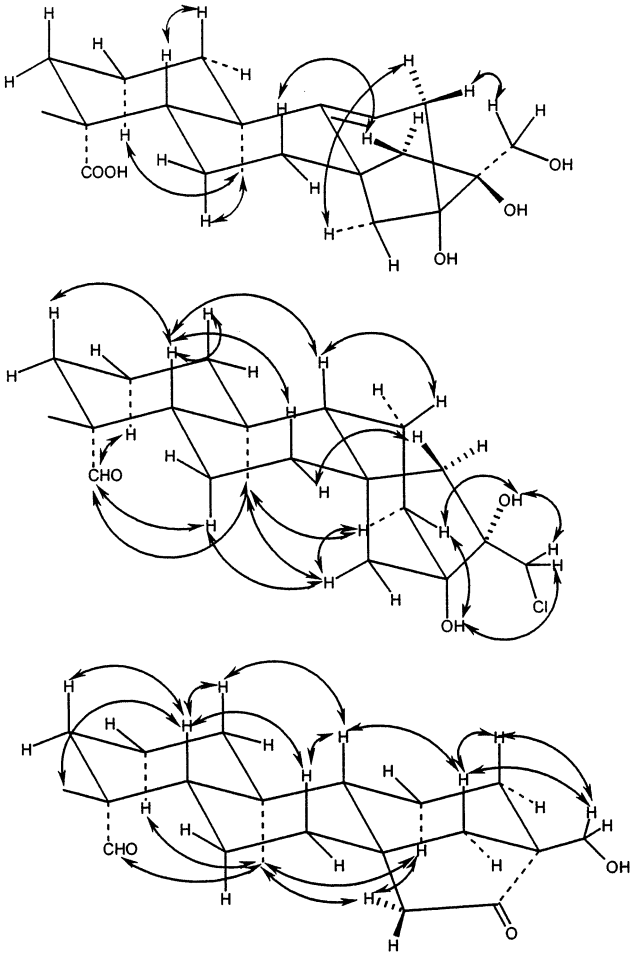

Figure 1. Significant NOE correlations (NOESY data) of **1**, **3**, and **4**.

tions of these protons with carbon signals at δ 31.9 (CH_2), 54.1 (C), 48.9 (CH_2), and 222.5 (CO) helped to locate the oxymethylene group at C-13 and the carbonyl group at C-16. A NOESY experiment allowed the assignment of the relative stereochemistry (Figure 1). Crucial NOEs were observed between H-1β, H-5, and H-9β and between H-15α and Me-20, which were not observed in **3**. These findings confirmed that **4** possesses an *ent*-beyerane skeleton. Like in the kauranes, the A and B rings are *trans* oriented, but the B/C connection is also *trans* configured. In addition, NOE cross-peaks between Me-18 and H-5 confirmed an equatorial position of this methyl group.

The CD spectrum of **4** showed a negative Cotton effect at the $n \rightarrow \pi^*$ transition at 297 nm. Analysis of the geometrical arrangement of the molecule in the eight octants formed by the symmetry planes of the orbitals of the keto group[16] clearly indicated the absolute configuration of **4**. Thus, the structure of **4** is (4*R*,5*S*,8*R*,9*R*,10*S*,-13*S*)-*ent*-17-hydroxy-16-oxobeyeran-19-al.

In the course of our general activity of profiling newly isolated compounds, **3**−**5** and **7**−**13** were tested for cytotoxic activities against L-929 (mouse fibroblasts), K562 (human chronic myeloid leukemia), and HeLa (human cervix carcinoma) cell lines (Table 2). The most promising activity was found for **10**, **11**, and **13** against K562 and L-929 (Table 2), of which **13** showed the greatest selectivity for K562 (IC_{50} 6.8 μg/mL). The potency of our compounds is on the same order of magnitude as that of those with similar skeletons.[17]

Table 2. Antiproliferative (GI_{50}) and Cytotoxic (CC_{50}) Activities of Compounds **3**−**5** and **7**−**13**

compound	L-929 (GI_{50})[a]	K562 (GI_{50})[a]	HeLa (CC_{50})[a]
3	50.0	29.2	38.2
4	45.4	50.0	37.7
5	39.5	27.7	40.5
7	41.9	26.7	38.7
8	50.0	50.0	50.0
9	42.4	32.8	43.0
10	12.8	13.6	35.7
11	11.5	10.5	42.3
12	50.0	50.0	50.0
13	18.2	6.8	32.8
paclitaxel	0.1	0.01	0.01

[a] In μg/mL.

Experimental Section

General Experimental Procedures. Column chromatography: silica gel 60M (230−400 mesh, Macherey-Nagel, Germany), Sephadex LH-20 (Pharmacia Biotech AB, Sweden). TLC: silica gel plates (Sil G/UV_{254}, 0.20 mm, Macherey-Nagel, Germany); spots were detected under a UV lamp and spraying with anisaldehyde/H_2SO_4 (1%/5% in methanol). Optical rotation: Propol digital automatic polarimeter (Dr. Wolfgang Kernchen GMBH, Germany). IR spectra: IFS55 spectrometer (Bruker, Germany). ^1H and ^{13}C NMR spectra: DPX-300, 2DNMR, DPX-500 (Bruker, Germany). ESIMS: triple quadrupole mass spectrometer Quattro (VG Biotech, England). EIMS: 70 eV, direct inlet, high resolution with perfluorokerosine as a standard, MAT 95 XL (Finnigan, Germany). CD spectrum: J-810-150s spectropolarimeter (JASCO, Germany).

Plant Material. The stems of *B. gymnorrhiza* were collected in Xiamen, People's Republic of China, in June 2002 and authenticated by Prof. Peng Lin, Xia Men University, People's Republic of China. A voucher sample of the plant is deposited in the National Research Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing, People's Republic of China (Mangrove XM004). The stem was air-dried and milled.

Extraction and Isolation. The pulverized plant material (6.1 kg) was macerated with methanol (25 L) at room temperature three times for two weeks. The combined methanol extracts were concentrated and yielded 282.6 g of a crude extract. This crude extract was partitioned to yield 23.6 g of a dried EtOAc extract, 39.6 g of a dried *n*-BuOH extract, and an aqueous residue. The EtOAc extract was subjected to silica gel column chromatography (6 × 50 cm, $CHCl_3$/MeOH, 50:1−1:1) The eluents were combined into 25 fractions on the basis of TLC analysis. Fraction 7 was further separated with silica gel column chromatography (3 × 30 cm, $CHCl_3$/MeOH, 100:1), yielding **11** (9.0 mg) and **13** (9.5 mg). Fraction 13 was subjected to Sephadex LH-20 column chromatography (3.5 × 120 cm, $CHCl_3$), then further purified by silica gel column chromatography (1.5 × 30 cm, petroleum ether/EtOAc, 3:1), to give **3** (10.0 mg), **9** (9.0 mg), and **10** (7.0 mg). Fraction 15 was separated by repeated silica gel chromatography to afford **4** (27.8 mg) and **12** (8.6 mg). Fraction 16 was subjected to Sephadex LH-20 column chromatography (3.5 × 120 cm, MeOH) and further purified by silica gel column chromatography (3 × 30 cm, petroleum ether/EtOAc, 2:1) to yield **2** (17.3 mg), **5** (11.6 mg), **7** (28.0 mg), and **8** (7.0 mg). Fractions 18 and 20 were further separated by silica gel column chromatography (1.5 × 30 cm, petroleum ether/EtOAc, 1:1) to yield **1** (5.1 mg) and **6** (4.6 mg), respectively.

13,16α,17-Trihydroxy-*ent*-9(11)-kauren-19-oic acid (1): white amorphous solid; $[\alpha]^{20}_D$ +67.4° (*c* 0.5, $CHCl_3$/MeOH, 1:1); IR (film) ν_{max} 3439, 2927, 2360, 2341, 1684, 1652, 1219 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; ESIMS *m*/*z* 373.3 $[M + Na]^+$, 723.2 $[2M + Na]^+$, 368.3 $[M + NH_4]^+$, 718.4 $[2M + NH_4]^+$, 333.3 $[M + H - H_2O]^+$, 349.3 $[M - H]^-$, 699.1 $[2M - H]^-$; HREIMS *m*/*z* 350.2095 (calcd for $C_{20}H_{30}O_5$, 350.2093).

16α,17-Dihydroxy-*ent*-9(11)-kauren-19-al (2): white amorphous solid; $[\alpha]^{20}_D$ +4.8° (*c* 0.3, $CHCl_3$); ^1H and ^{13}C NMR, see

Table 1; ESIMS *m/z* 341.2 $[M + Na]^+$, 659.2 $[2M + Na]^+$, 336.3 $[M + NH_4]^+$; HREIMS *m/z* 318.2199 (calcd for $C_{20}H_{30}O_3$, 318.2195).

17-Chloro-13,16*β*-dihydroxy-*ent*-kauran-19-al (3): white amorphous solid; $[\alpha]^{20}_D$ −45.0° (*c* 0.3, $CHCl_3$); IR (film) ν_{max} 3440, 2930, 2360, 2341, 1706, 1446, 1364, 1220, 1045, 745, 714 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; ESIMS *m/z* 377.2 $[M + Na]^+$, 379.0 $[M + 2 + Na]^+$, 731.0 $[2M + Na]^+$, 733.3 $[2M + 2 + Na]^+$, 372.2 $[M + NH_4]^+$; HRESIMS *m/z* 377.2276 (calcd for $C_{20}H_{31}NaClO_3$, 377.1859).

(4*R*,5*S*,8*R*,9*R*,10*S*,13*S*)-*ent*-17-Hydroxy-16-oxobeyeran-19-al (4): white amorphous solid; $[\alpha]^{20}_D$ −35.0° (*c* 0.3, $CHCl_3$); CD (*c* 6.37 × 10^{-5} mol/L, MeOH) θ_{251} −725, θ_{297} −6 185, θ_{304} −6 151, θ_{337} −320; IR (film) ν_{max} 3446, 2934, 2360, 2341, 1736, 1704, 1362, 1222 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; ESIMS *m/z* 319.3 $[M + H]^+$, 336.3 $[M + NH_4]^+$, 341.3 $[M + Na]^+$, 659.2 $[2M + Na]^+$; HREIMS *m/z* 318.2205 (calcd for $C_{20}H_{30}O_3$, 318.2195).

Methyl 16α,17-Dihydroxy-*ent*-kauran-19-oate (5),[5] **16α,-17-Dihydroxy-*ent*-9(11)-kauren-19-oic Acid (6),**[6,7] **Methyl 16α,17-Dihydroxy-*ent*-9(11)-kauren-19-oate (7),**[7,8] **16α,17-Dihydroxy-*ent*-kauran-19-al (8),**[9,10] **16α*H*-17-Hydroxy-*ent*-kauran-19-oic acid (9),**[9] **16α*H*-17,19-*ent*-Kauranediol (10),**[9,11,12] **13-Hydroxy-16-*ent*-kauren-19-al (11),**[1] **16-*ent*-Kaurene-13,19-diol (12),**[1] **and 16-*ent*-Kauren-19-ol (13).**[13] The spectral properties were identical with those previously reported.

Biological Testing. Compounds **3**−**5** and **7**−**13** were assayed against L-929 (DSM ACC 2), K562 (DSM ACC 10), and HeLa (DSM ACC 57) cells for their cytotoxic effects as previously described.[18]

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Supporting Information Available: CD spectrum of compound **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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