Identification of *ent*-16*β*,17-Dihydroxykauran-19-oic Acid as an Anti-HIV Principle and Isolation of the New Diterpenoids Annosquamosins A and B from *Annona squamosa*

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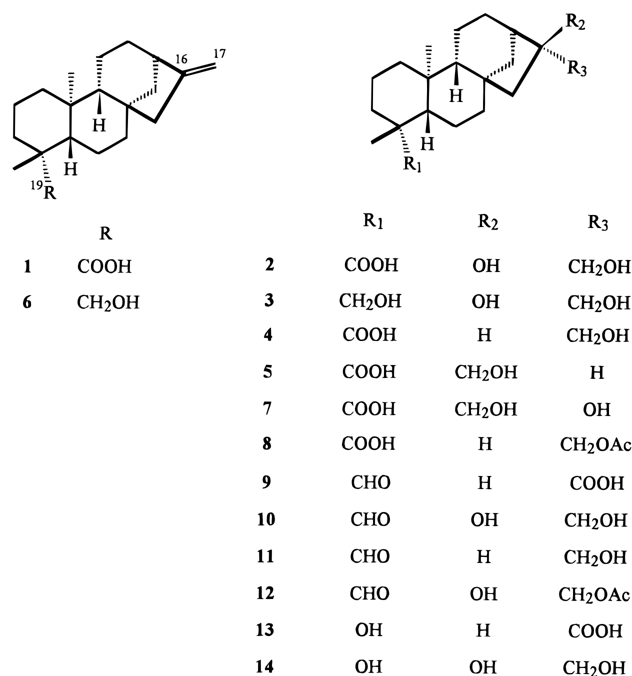
Phytochemical analysis of the fruits of *Annona squamosa* yielded 12 known kaurane derivatives (**1**−**11**, **13**) and two new kaurane diterpenoids, which have been named annosquamosin A (16*β*-hydroxy-17-acetoxy-*ent*-kauran-19-al) (**12**) and annosquamosin B (19-nor-*ent*-kaurane-4α,16*β*,-17-triol) (**14**). The structures of the new compounds were established by spectral analyses and chemical evidence. Among these 14 compounds, 16*β*,17-dihydroxy-*ent*-kauran-19-oic acid (**2**) showed significant activity against HIV replication in H9 lymphocyte cells with an EC_{50} value of 0.8 μg/mL (therapeutic index >5).

As a result of our continuing search for novel bioactive agents from plants, a methanolic extract of the fresh fruits of *Annona squamosa* L. (Annonaceae) was found to show significant inhibition of HIV replication in H9 lymphocytic cells. Bioactivity-guided chromatographic fractionation of the active extract has led to the isolation and characterization of an anti-HIV principle, 16*β*,17-dihydroxy-*ent*-kauran-19-oic acid (**2**),[1,2] and 13 kaurane diterpenoids: *ent*-kaur-16-en-19-oic acid (**1**),[3,4] *ent*-kaurane-16*β*,17,19-triol (**3**),[5] a mixture of 17-hydroxy-16*β*-*ent*-kauran-19-oic acid (**4**) and 17-hydroxy-16α-*ent*-kauran-19-oic acid (**5**),[2] *ent*-kaur-16-en-19-ol (**6**),[6] 16α,17-dihydroxy-*ent*-kauran-19-oic acid (**7**),[2,7] 17-acetoxy-16*β*-*ent*-kauran-19-oic acid (**8**),[8] 19-formyl-*ent*-kauran-17-oic acid (**9**),[9,10] *ent*-16*β*,17-dihydroxykauran-19-al (10),[11] 17-hydroxy-16*β*-*ent*-kauran-19-al (**11**),[12] 16*β*-hydroxy-17-acetoxy-*ent*-kauran-19-al (**12**), 4α-hydroxy-19-nor-*ent*-kauran-17-oic-acid (**13**),[9,10] and 19-nor-*ent*-kaurane-4α,16*β*,17-triol (**14**), which are inactive. Among them, compounds **12** and **14** are new compounds and have been named annosquamosins A and B, respectively. Compounds **2**, **7**, and **9** were isolated previously from the branches of this same plant.[13] Compounds **3**−**5**, **8**, and **10** have been isolated for the first time from this plant. The structural eludication of the isolates was established by spectroscopic and chemical methods.

Annosquamosin A (**12**) was obtained as colorless needles. Its infrared (IR) spectrum showed absorptions due to a free hydroxy group (3505 cm^{-1}) and a pair of strong ester carbonyl bands (1740 and 1725 cm^{-1}). The ^{1}H-NMR spectrum of **12** in $CDCl_3$ exhibited signals for two tertiary methyl groups at δ 0.88 and 0.99 and one aldehyde moiety at δ 9.74, which are typical of the equatorial C-18 and axial C-20 methyl groups of an *ent*-kaurane diterpene with a C-19 axial aldehyde group.



	R
1	COOH
6	CH_2OH

	R_1	R_2	R_3
2	COOH	OH	CH_2OH
3	CH_2OH	OH	CH_2OH
4	COOH	H	CH_2OH
5	COOH	CH_2OH	H
7	COOH	CH_2OH	OH
8	COOH	H	CH_2OAc
9	CHO	H	COOH
10	CHO	OH	CH_2OH
11	CHO	H	CH_2OH
12	CHO	OH	CH_2OAc
13	OH	H	COOH
14	OH	OH	CH_2OH

The other major feature of the ^{1}H-NMR spectrum of **12** was an AB quartet (2H) with doublets centered at δ 3.91 and 4.05 (J = 11 Hz), suggesting the presence of an acetoxy group. The ^{13}C-NMR spectrum (Table 1) and a DEPT experiment on **12** indicated a total of 22 carbons. The carbon types were determined from the DEPT spectrum as three methyls, 11 methylenes (including an acetoxy-bearing carbon at δ 71.2), three methines, four quaternary carbons (including an oxygenated carbon at δ 78.6), and one aldehyde carbon. A comparison of these carbon resonances with those of the related kauranoid diterpene *ent*-16*β*,17-dihydroxykauran-19-al (**10**) (Table 1) suggested that compound **12** possesses the same *ent*-kaurane-type skeleton. The presence of a C-17 acetoxy group in **12** was shown by the carbon resonances at δ 21.0 (Me) and 171.4 (carbonyl). Thus, the structure of **12** was determined as 16*β*-hydroxy-17-acetoxy-*ent*-kauran-19-al.

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Table 1. ^{13}C-NMR Chemical Shift Values for Diterpenes **1**−**14**

carbon no.	compound												
	1	**2**	**3**	**4/5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1	41.8	41.2	40.8	41.6/42.0	40.5	41.1	41.0	41.5	41.8	41.9	41.8	42.1	37.3
2	19.6	19.9	19.3	19.0/19.1	18.3	19.8	18.1	18.3	18.8	19.0	18.7	18.9	18.8
3	38.3	38.8	36.2	37.2	35.7	38.7	37.0	34.2	34.2	39.8	34.3		38.7
4	44.2	44.0	39.3	44.7	39.3	43.9	42.6	48.4	48.4	48.4	48.4	70.5	69.7
5	57.5	57.1	57.8	56.9	56.9	57.0	55.9	56.6	56.5	56.6	56.5	57.5	56.7
6	22.3	22.5	20.7	22.1/22.4	20.5	23.0	21.2	20.0	19.6	20.1	19.7		40.7
7	41.8	42.6	42.8	40.4/40.7	41.7	42.8	42.6	41.4	39.4	40.5	39.8	40.7	42.3
8	44.7	43.9	43.9	43.6/43.7	44.0	45.0	43.4	44.2	43.3	44.0	43.6	43.9	38.8
9	55.6	56.8	57.0	55.3/56.4	56.2	56.3	55.4	55.5	55.4	55.9	55.5	56.8	56.1
10	41.2	40.1	39.6	39.6	38.7	40.1	39.8	39.3	39.7	39.3	39.8	39.5	42.5
11	18.9	19.5	18.8	18.9	18.2	19.0	18.1	18.1	18.3	18.3	18.3	17.7	18.0
12	33.6	27.6	27.5	20.0/31.4	33.2	26.8	25.0	27.1	26.5	25.8	26.5	27.2	26.2
13	44.3	41.7	41.8	36.9/38.1	44.2	45.9	36.3	39.5	40.6	36.9	41.3	39.3	40.4
14	40.2	38.6	38.5	37.8	39.7	37.8	38.6	39.7	38.3	34.2	38.4	39.2	38.7
15	49.4	53.5	53.5	44.2/45.0	49.1	53.9	49.3	40.8	52.3	43.6	52.4	40.3	52.2
16	156.4	79.8	79.6	43.3/43.1	155.9	81.7	38.1	45.3	79.8	43.1	78.6	45.5	78.3
17	103.5	70.5	70.4	64.2/67.4	103.0	66.5	65.2	180.5	69.7	64.1	71.2	177.4	69.1
18	29.5	29.4	28.1	28.9	27.1	29.3	28.0	24.2	24.2	24.2	24.3	22.8	22.1
19	185.0	180.2	64.1	183.7	65.6	180.1	180.7	206.0	206.0	206.0	205.9		
20	16.1	16.0	13.4	15.5	18.1	16.0	14.5	16.3	16.3	16.3	16.3	16.5	16.0
*C*H$_3$CO							20.0				21.0		
CH$_3$*C*O							170.9				171.4		

Annosquamosin B (**14**) was obtained as a white amorphous solid. The major IR absorption bands were characteristic of a hydroxy group (3450−3350 cm^{-1}). The FABMS of **14** gave an intense molecular ion at *m*/*z* 331 [M + Na]$^+$. In the EIMS, a fragment appeared at *m*/*z* 277, indicating the facile loss of CH_2OH; other fragments were found at *m*/*z* 123, 121, 109, and 107. The ^{1}H-NMR spectrum of **14** displayed two methyl singlets at δ 1.05 and 1.30 and a pair of doublets at δ 3.78 and 3.87 (J = 11 Hz), indicating that **14** was probably an *ent*-kaurane diterpene, possessing a tertiary hydroxy group at C-16 and a hydroxymethyl group at C-17. Comparison of the ^{1}H-NMR spectrum of **14** with that of **2** showed that the two methyl groups in **14** had shifted upfield, while the C-17 methylene groups resonated at almost the same positions. The proposed structure of **14** was confirmed by a DEPT experiment and from the ^{13}C-NMR spectrum, which showed an oxygenated methylene chemical shift at δ 69.1, an additional nine methylene carbons, three methine carbons, and four quaternary carbons (including an oxygenated carbon at δ 78.3). Because the ^{13}C-NMR spectrum of compound **14** indicated only 19 carbons, comparison with the ^{13}C-NMR resonances of compound **2** (Table 1) indicated that the C-4 axial carboxylic acid of **2** was replaced by a hydroxy group in **14**. The evidence described above indicated that **14** was a nor-*ent*-kaurane compound and was determined as 19-nor-*ent*-kaurane-4α,16β,17-triol.

ent-16β,17-Dihydroxykauran-19-oic acid (**2**) inhibited HIV replication in H9 lymphocyte cells with an EC_{50} of 0.8 μg/mL (therapeutic index >5). The other 13 compounds (**1**, **3**−**14**) showed no inhibition up to a concentration level of 100 μg/mL. Comparable anti-HIV activity has been reported with other *ent*-kuranes.[14]

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO Model DIP-370 digital polarimeter. The IR spectra were determined on a Hitachi Model 260−30 infrared spectraphotometer. The ^{1}H, ^{13}C, DEPT, and 2D-NMR spectra were taken on a Varian Gemini-200 spectrometer with TMS as an internal standard, and chemical shifts are reported in δ units. MS were obtained on a JEOL TMSD-100 mass spectrometer. Si gel 60 (Merck 70−230, 230−400 mesh) and Sephadex LH-20 were used for preparative TLC and column chromatography, respectively.

Plant Material. Fresh fruits of *A. squamosa* were collected at Chie Shan, Kaohsiung, Taiwan, in June 1991. Voucher specimens are kept in the School of Pharmacy Kaohsiung Medical College, Kaohsiung, Taiwan, Republic of China.

Extraction and Isolation. The fresh fruits (10 kg) were extracted five times with MeOH at room temperature. The combined MeOH extracts were evaporated and partitioned to yield $CHCl_3$ and aqueous extracts. The $CHCl_3$ solution was extracted with 3% HCl to remove alkaloids, and then the neutral $CHCl_3$ solution was dried and evaporated to leave a brownish viscous residue (700 g). The residue was subjected to Si gel chromatography and eluted with gradually more polar $CHCl_3$/MeOH mixtures; the eluants were combined into 31 fractions on the basis of TLC. Fraction 3 eluting with *n*-hexane−$CHCl_3$−MeOH (3:1.5:0.5) was further purified by recrystallization and repeated Si gel column chromatography to give *ent*-kaur-16-en-19-oic acid (**1**) (3 g) and 17-acetoxy-16β-*ent*-kauran-19-oic acid (**8**) (20 mg). Fraction 5 eluting with ethyl acetate−$CHCl_3$−MeOH (9:1:0.5) provided *ent*-kaur-16-en-19-ol (**6**) (3.7 mg). Fraction 8 eluting with *n*-hexane−$CHCl_3$−ethyl acetate (1:2:0.5) and $CHCl_3$−acetone (2:1) was further separated and recrystallized to yield 17-hydroxy-16β-*ent*-kauran-19-al (**11**) (40.9 mg) and annosquamosin A (**12**) (10.3 mg). Fraction 12 eluting with *n*-hexane−ethyl acetate (3:1) was further separated and purified by Sephadex LH-20 and preparative TLC (*n*-hexane−$CHCl_3$−acetone (1:2:0.8)) to afford 19-formyl-*ent*-kauran-17-oic acid (**9**) (37.1 mg), 16β,17-dihydroxy-*ent*-kauran-19-al (**10**) (94.5 mg), and annosquamosin B (**14**) (8.9 mg). Fraction 18 was chromatographed on a Sephadex LH-20 column using $CHCl_3$−acetone (1:1) to yield 16α,17-dihydroxy-*ent*-kauran-19-oic acid (**7**) (160.7 mg). Fractions 14−16 were combined and chromatographed on a Si gel (230−400 mesh) column using *n*-hexane−ethyl acetate (1:1) and ethyl acetate−acetone

(5:1) to yield 16 fractions; the first fraction was further separated and purified by Si gel column chromatography and preparative TLC to give 16*β*-*ent*-kaurane-16,17,19-triol (**3**) (6.2 mg), a ca. 1:2 mixture (15.6 mg) of 17-hydroxy-16*β*-*ent*-kauran-19-oic acid (**4**) and 17-hydroxy-16α-*ent*-kauran-9-oic acid (**5**), and 16*β*,17-dihydroxy-*ent*-kauran-19-oic acid (**2**) (8.9 mg).

The alkaloid layer was chromatographed on a Si gel column using a $CHCl_3$–MeOH mixture of increasing polarity to yield eight alkaloids and 4α-hydroxy-19-nor-*ent*-kauran-17-oic acid (**13**) (20 mg).

***ent*-Kaur-16-en-19-oic acid (1)**: white needles; mp 162–166 °C (lit.[4] mp 167–171 °C); $[\alpha]^{24}_D$ −112° (*c* 0.3, $CHCl_3$) [lit.[4] $[\alpha]^{24}_D$ −110° (*c* 0.2, $CHCl_3$)].

16*β*,17-Dihydroxy-*ent*-kauran-19-oic acid (2): white powder; mp 298–300 °C (lit.[1] mp 296–300 °C); $[\alpha]^{24}_D$ −160° (*c* 0.02, CH_3OH).

16*β*-*ent*-Kaurane-16,17,19-triol (3): white powder; mp 232–234 °C; $[\alpha]^{24}_D$ −184° (*c* 0.02, CH_3OH).

Mixture of 17-hydroxy-16*β*-*ent*-kauran-19-oic acid (4) and 17-hydroxy-16α-*ent*-kauran-19-oic acid (5): white powder; mp 118–120 °C; $[\alpha]^{24}_D$ −79° (*c* 0.1, $CHCl_3$) [lit.[2] $[\alpha]^{24}_D$ −130° (*c* 0.01, $CHCl_3$)].

***ent*-Kaur-16-en-19-ol (6)**: white powder; mp 126–128 °C (lit.[6] mp 140–141 °C); $[\alpha]^{24}_D$ −68° (*c* 0.1, $CHCl_3$) [lit.[6] $[\alpha]^{24}_D$ −82° (*c* 0.42, EtOH)].

16α,17-Dihydroxy-*ent*-kauran-19-oic acid (7) white powder; mp 264–266 °C (lit.[2] mp 264–266 °C); $[\alpha]^{24}_D$ −58° (*c* 0.05, $CHCl_3/CH_3OH$) [lit.[7] $[\alpha]^{24}_D$ −70° (*c* 1.0, C_5H_5N)].

17-Acetoxy-16*β*-*ent*-kauran-19-oic acid (8): white powder; mp 164–166 °C; $[\alpha]^{24}_D$ −97° (*c* 0.07, $CHCl_3/CH_3OH$).

19-Formyl-*ent*-kauran-17-oic acid (9): white powder; mp 178–180 °C (lit.[9] mp 204–205 °C); $[\alpha]^{24}_D$ −21° (*c* 0.03, $CHCl_3$) (lit.[7] $[\alpha]^{24}_D$ −63°).

16*β*,17-Dihydroxy-*ent*-kauran-19-al (10): white needles (CH_3OH); mp 150–152 °C; $[\alpha]^{24}_D$ −58° (*c* 0.2, $CHCl_3$).

17-Hydroxy-16*β*-*ent*-kauran-19-al (11): white needles (CH_3OH); mp 174–176 °C; $[\alpha]^{24}_D$ −49° (*c* 0.6, $CHCl_3$).

Annosquamosin A (16*β*-hydroxy-17-acetoxy-*ent*-kauran-19-al) (12): white needles (CH_3OH); mp 162–164 °C; $[\alpha]^{24}_D$ −64° (*c* 0.2, $CHCl_3$); IR (KBr) ν_{max} 3505, 2950, 1740, 1725, 1480 cm^{-1}, EIMS *m*/*z* 289 (100), 271 (24), 243 (7), 123 (14), 121 (8), 109 (11), 107 (10); FABMS *m*/*z* 363 $[M + H]^+$ (22), 258 (40), 271 (91), 123 (44), 121 (51), 109 (64), 107 (77); ^{1}H-NMR ($CDCl_3$) δ 0.86 (3H, s, H-20), 0.99 (3H, s, H-18), 3.91 and 4.05 (2H, ABq, *J* = 11Hz, H-17), 9.74 (1H, d, H-19), 2.11 (3H, s, OAc); ^{13}C-NMR see Table 1; HREIMS *m*/*z* $[M]^+$ 362.2445 (calcd for $C_{22}H_{34}O_4$, 362.2457).

4α-Hydroxy-19-nor-*ent*-kauran-17-oic acid (13): white powder; mp 280–282 °C (lit.[9] mp 274–275 °C); $[\alpha]^{24}_D$ −55° (*c* 0.2, $CHCl_3$ + CH_3OH).

Annosquamosin B (19-nor-*ent*-kaurane-4α,16*β*,17-triol) (14): white powder; mp 263–266 °C; $[\alpha]^{24}_D$ −47° (*c* 0.2, $CHCl_3$ + CH_3OH); IR (KBr) ν_{max} 3450–3350, 2950, 2860, 1400, 1105, 1050, 1080, 1025 cm^{-1}; EIMS *m*/*z* 277 $[M - CH_2OH]^+$ (83), 259 $[277 - H_2O]^+$ (40), 241 (18), 123 (10), 121 (12), 109 (17), 107 (19); ^{1}H-NMR (C_5D_5N) δ 1.05 (3H, s, H-20), 1.30 (3H, s, H-18), 3.78 and 3.88 (2H, ABq, *J* = 11 Hz, H-17); ^{13}C-NMR see Table 1; HREIMS *m*/*z* $[M]^+$ 308.2343 (calcd for $C_{19}H_{32}O_3$, 308.2351).

HIV Inhibition Assay.[15] The assay employed H9 lymphocytes (3.5 × 10^6 cells/mL) in the presence or absence of HIV-1 (IIIB strain, 0.01–0.1 $TCID_{50}$/cell) for 1 h at 37 °C. Cells were washed thoroughly and resuspended at a final concentration of 2 × 10^5 cells/mL in the presence or absence of compound. After incubation for 3 days at 37 °C, the cell density of uninfected cultures was determined by cell count to assess toxicity of the drug. A p24 antigen capture assay was used to determine the level of virus released into the medium of HIV-infected cultures.

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