Bioactive Kaurane Diterpenoids from *Annona glabra*

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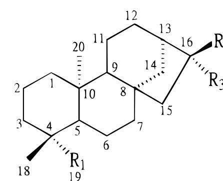
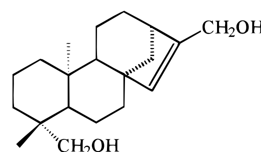
Phytochemical analysis of the fruits of *Annona glabra* yielded two new kaurane diterpenoids, annoglabasin A (methyl-16β-acetoxy-19-al-*ent*-kauran-17-oate)(**1**) and annoglabasin B (16α-hydro-19-acetoxy-*ent*-kauran-17-oic acid)(**2**), along with 11 known kaurane derivatives (**3**−**13**). The structures of the new compounds were established by spectral and chemical evidence. Among these, methyl-16α-hydro-19-al-*ent*-kauran-17-oate (**11**) exhibited mild activity against HIV replication in H9 lymphocyte cells, and 16α-17-dihydroxy-*ent*-kauran-19-oic acid (**4**) showed significant inhibition of HIV-reverse transcriptase.

In a continuing search for novel bioactive agents from plants, a methanolic extract of the fresh fruits of *Annona glabra* 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 two new kaurane diterpenoids, annoglabasin A (methyl-16β-acetoxy-19-al-*ent*-kauran-17-oate) (**1**) and annoglabasin B (16α-hydro-19-acetoxy-*ent*-kauran-17-oic acid) (**2**). Eleven known compounds, *ent*-kaur-16-en-19-oic acid (**3**),[1−3] 16α, 17-dihydroxy-*ent*-kauran-19-oic acid (**4**),[1,4,5] 16β-hydroxy-17-acetoxy-*ent*-kauran-19-oic acid (**5**),[5] 16β-hydro-*ent*-kauran-17-oic acid (**6**),[6,7] 16α-hydro-*ent*-kauran-17-oic acid (**7**),[8] *ent*-kaur-16-en-19-ol (**8**), *ent*-kaur-15-ene-17,19-diol (**9**),[9,10] 16α-hydro-19-al-*ent*-kauran-17-oic acid (**10**),[1,11,12] methyl-16α-hydro-19-al-*ent*-kauran-17-oate (**11**),[12] 16β-hydroxyl-17-acetoxy-*ent*-kauran-19-al (**12**),[1] and 19-nor-*ent*-kauran-4α-ol-17-oic acid (**13**),[1,11,12] were also isolated. Only compound **3** was isolated previously from this plant.[13] Structure elucidation of the compounds was established using spectroscopic and chemical methods.



	R_1	R_2	R_3
1	CHO	OAc	$COOCH_3$
2	CH_2OAc	COOH	H
3	COOH	$=CH_2$	
4	COOH	CH_2OH	OH
5	COOH	OH	CH_2OAc
6	CH_3	H	COOH
7	CH_3	COOH	H
8	CH_2OH	$=CH_2$	
10	CHO	COOH	H
11	CHO	$COOCH_3$	H
12	CHO	OH	CH_2OAc
13	OH	COOH	H



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Results and Discussion

Annoglabasin A (**1**) was obtained as a colorless, amorphous powder. Its IR spectrum showed absorption bands at 1730 and 1715 cm^{-1} due to ester carbonyl and aldehyde functions. The ^1H NMR spectrum of **1** ($CDCl_3$) exhibited signals for two tertiary methyl groups at δ 0.85, 0.99 and an aldehyde moiety at δ 9.74, which are typical for equatorial C-18 and axial C-20 methyl groups of an *ent*-kaurane diterpenoid with a C-19 axial aldehyde group. The other major features of the ^1H NMR spectrum of **1** were a methine signal at δ 2.39, an acetoxy signal at δ 2.04, and a methoxy−carbonyl signal at δ 3.71. The ^{13}C NMR spectrum (Table 1) and a DEPT experiment indicated that **1** had a total of 23 carbons. The carbon types were determined from the DEPT spectrum as four methyls at δ 24.2 (C-18), 16.4 (C-20), 21.1 (acetoxyl methyl group), and 52.2 (methyl carbon of methoxy−carbonyl); nine methylenes between δ 16.9 and 51.2; four methines at δ 56.3(C-5), 54.8 (C-9), 46.1(C-13), and 205.8 (C-19, aldehyde carbon); and six quaternary carbons at δ 39.5 (C-10), 44.69 (C-8), 48.4 (C-4), 89.1 (C-16, acetoxy-bearing carbon), 170.5 (C-17,

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Table 1. ^{13}C NMR Chemical Shift Values for Diterpenes **1** and **2**

carbon no.	compound **1** (mult)	**2** (mult)
1	41.6 (t)	41.6 (t)
2	18.3 (t)	18.1 (t)
3	34.3 (t)	36.2 (t)
4	48.4 (s)	39.1 (s)
5	56.3 (d)	57.1 (d)
6	20.0 (t)	20.6 (t)
7	39.3 (t)	41.8 (t)
8	44.7 (s)	44.3 (s)
9	54.8 (d)	56.6 (d)
10	39.5 (s)	39.1 (s)
11	16.9 (t)	18.0 (t)
12	26.2 (t)	27.2 (t)
13	46.1 (d)	39.7 (d)
14	38.0 (t)	40.2 (t)
15	51.2 (t)	40.5 (t)
16	89.1 (s)	45.3 (d)
17	170.5 (s)	180.1 (s)
18	24.2 (q)	27.5 (q)
19	205.8 (d)	67.2 (t)
20	16.36 (q)	18.0 (q)
O*C*OCH$_3$	170.9 (s)	171.5 (s)
OCO*C*H$_3$	21.1 (q)	21.0 (q)
COO*C*H$_3$	52.2 (q)	

ester carbonyl carbon), and 170.9 (acetoxy carbonyl carbon). Comparison of these ^{13}C NMR chemical shifts with those of the related kauranoid diterpenes **11** and **12**, suggested that **1** possessed the same *ent*-kaurane-type skeleton with an aldehyde located at C-19. The missing signals for H-16 and the highfield proton shift of H-13 (−0.19 ppm) suggested the presence of an acetyl group at C-16 and a carbonyl−methoxy at C-17. Thus, the structure of **1** was determined to be methyl-16β-acetoxy-19-al-*ent*-kauran-17-oate.

Annoglabasin B (**2**) was obtained as colorless needles (MeOH), and the major IR absorption bands were characteristic of carbonyls. The FABMS of **2** gave a molecular ion at m/z 363 [(M + 1)$^+$, 9%]. In the EIMS, a base peak appeared at m/z 289, indicating the facile loss of CH_2OCOCH_3; other fragments were found at m/z 271 [289 − H_2O], 243[271 − CO], 192, 123, 109, and 107. The ^1H NMR spectrum of **2** displayed methyl singlets at δ 0.94 and 1.01, a pair of doublets at δ 4.21 and 3.87, an acetyl group at δ 2.04, and two methine protons at δ 2.57 and 2.93, indicating that **2** was probably an *ent*-kaurane diterpene possessing a carboxylic acid at C-17 and an acetoxy group at C-19 [by comparison with the data of related diterpenes 16α-hydro-*ent*-kauran-17-oic acid (**7**) and an acetyl derivative of *ent*-kaur-16-en-19-ol (**8**)]. The proposed structure of **2** was confirmed by ^{13}C NMR and DEPT spectra, which showed a total of 22 carbons and indicated an *ent*-kaurane diterpene skeleton and an acetyl group. The carbons of *ent*-kaurane diterpene were assigned, from DEPT and HETCOR experiments, as two methyls at δ 18.01 (C-20) and 27.45 (C-18); 10 methylenes [including an acetoxy-bearing methylene at δ 67.16(C-19)]; four methines at δ 39.71 (C-13), 45.32 (C-16), 56.64 (C-9), and 57.11 (C-5); and four quaternary carbons at δ 36.25 (C-4), 39.06 (C-10), 44.26 (C-8); and a carboxylic acid carbon at δ 180.22 (C-17), along with acetoxyl carbons at δ 171.5 (O*C*OCH$_3$) and 20.95 (OCO*C*H$_3$). The stereochemical relationship of H-16 was α in **2** as determined by the NOESY spectrum. Thus, the evidence described above indicated that **2** is 16α-hydro-19-acetoxyl-*ent*-kauran-17-oic acid, a new *ent*-kaurane diterpene.

Table 2. Inhibition of HIV Replication of Compounds **1**, **2**, **5**−**7**, and **11** in H9 Lymphocyte Cells

compound	IC_{50} (μg/mL)	EC_{50} (μg/mL)	therapeutic index
1	15	10	1.5
2	40	no suppression	
5	65	85	0.8
6	32	24	1.3
7	40	no suppression	
11	20	5	4
AZT	500	0.01	50 000

Table 3. The Inhibitory Effect of Compounds **3**, **4**, **8**, and **10** on HIV Reverse Transcriptase

compound	% inhibitory effect at 33 μg/mL (%)
3	1
4	46
8	7
10	14

To our knowledge, there is no clear nomenclature to clarify 16-hydro kaurane diterpenes (e.g., compounds **2**, **6**, **7**, **10**, and **11**) given in previous reports. We defined the orientation of H-16 by using the words "16α-hydro" or "16β-hydro" to indicate the stereochemistry.

Methyl-16α-hydro-19-al-*ent*-kauran-17-oate (**11**) inhibited HIV replication in H9 lymphocyte cells,[1] with an EC_{50} of 5 μg/mL (therapeutic index = 4) (Table 2). 16α-17-Hydroxyl-*ent*-kauran-19-oic acid (**4**) gave 46% of inhibition against HIV reverse transcriptase at a concentration 33 μg/mL (Table 3).[14]

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. Melting points were determined using a Yanagimoto micro-melting point apparatus and were uncorrected. The IR spectra were measured on a Hitachi 260-30 spectrophotometer. ^1H NMR spectra were recorded with Varian NMR spectrometers at 400 and 200 MHz, and ^{13}C NMR spectra were recorded with Varian NMR spectrometers at 100 and 50 MHz, in $CDCl_3$ using TMS as internal standard. LREIMS and LRFABMS spectra were obtained with a JOEL JMS−SX/SX 102A mass spectrometer or a Quattro GC/MS spectrometer having a direct inlet system. HREIMS were measured on a Jeol JMS−HX 110 mass spectrometer. Si gel 60 (Macherey−Nagel, 230−400 mesh) was used for column chromatography, precoated Si gel plates (Macherey−Nagel, SIL G-25 UV_{254}, 0.25 mm) were used for analytical TLC, and precoated Si gel plates (Macherey−Nagel, SIL G/UV_{254}, 0.25 mm) were used for preparative TLC. The spots were detected by spraying with Dragendorff's reagent or 50% H_2SO_4 and then heating on a hot plate.

Plant Material. Fresh fruits of *A. glabra* L. were collected from Chia-Yi-Hsien, Taiwan, in July 1994. Voucher specimens are deposited in the Graduate Institute of Natural Products, Kaohsiung Medical College, Kaohsiung, Taiwan, Republic of China.

Extraction and Isolation. The fresh fruits (6 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 (165 g). The residue was subjected to Si gel column chromatography and eluted with gradually more polar $CHCl_3$−MeOH mixtures; the eluents were combined into 38 fractions on the basis of TLC. Fraction 2, eluting with *n*-hexane−$CHCl_3$−EtOAc (1:1:0.3), was further purified by recrystallization and repeated Si gel column chromatography to give **1** (13 mg), **3** (481.8 mg), **6** (15.8 mg), **8** (16.2 mg), and **11** (41.3 mg). Fraction 3, eluting with *n*-hexane−$CHCl_3$−EtOAc (1:2:1), provided **2** (72.2 mg). Fraction 4, eluting with *n*-hexane−$CHCl_3$−EtOAc (1:2:0.5) and $CHCl_3$−MeOH (5:1), was further separated and recrystallized to yield **10** (32.6 mg) and **13** (54.6 mg). Fraction 5, eluting with *n*-hexane−$CHCl_3$−EtOAc (1:2:1), was further separated and purified to afford **5** (10.2 mg) and **9** (16.9 mg). Fraction 6 was chromatographed on a Sephadex LH-20 column using $CHCl_3$−EtOAc (1:1) to yield **7** (158.3 mg). Fraction 8, eluting with *n*-hexane−$CHCl_3$−EtOAc (1:2:1), was recrystallized and purified by repeated Si gel column chromatography to obtain **12** (23.6 mg). Fraction 11 was chromatographed on a Si gel column using *n*-hexane−$CHCl_3$−Me_2CO (2:5:2) to give **4** (23.8 mg).

Annoglabasin A (methyl-16*β*-acetoxy-19-al-*ent*-kauran-17-oate)(1): white powder; mp 138−140 °C; $[\alpha]^{24}_D$ −74.8 (*c* 0.22, $CHCl_3$); IR (KBr) ν_{max} 2900, 1730, 1715, 1250 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 9.74 (1H, d, $J = 1.2$ Hz, H-19), 3.71 (3H, s, $COOCH_3$), 2.39 (1H, br s, H-13), 2.04 (3H, s, $OCOCH_3$), 0.99 (3H, s H-18), 0.85 (3H, s, H-20); ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 1; EIMS(70 eV) *m*/*z* 330 (11), 301 (8), 271 (4), 233 (14), 123 (19), 109 (22), 91 (34); HRFABMS *m*/*z* $[M + 1]^+$ 391.2489 (calcd for $C_{23}H_{34}O_5$, 391.2484).

Annoglabasin B (16α-hydro-19-acetoxy-*ent*-kauran-17-oic acid)(2): white needles (MeOH); mp 106−108 °C; $[\alpha]^{24}_D$ −41.3 (*c* 0.6, $CHCl_3$); IR (KBr) ν_{max} 2900, 1735, 1710, 1230 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 4.21 (1H, d, $J = 11.2$ Hz, H-19a), 3.88 (1H, d, $J = 11.2$ Hz, H-19b), 2.93 (1H, dt, $J = 12.0$, 6.0 Hz, H-16), 2.57 (1H, br s, H-13), 2.05 (3H, s, $OCOCH_3$), 1.01 (3H, s H-18), 0.94 (3H, s, H-20); ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 1; EIMS (70 eV) *m*/*z* 330 (11), 344 (4), 289 (57), 271 (4), 243 (4), 192 (17), 123 (90), 109 (57), 107 (30), 91 (36); HRFABMS *m*/*z* $[M + 1]^+$ 363.2529 (calcd for $C_{22}H_{35}O_4$, 363.2535).

***ent*-Kaur-16-en-19-oic acid (3):**[1] white needles (MeOH); mp 166−168 °C (lit.[1] mp 162−166 °C); $[\alpha]^{24}_D$ −110 (*c* 0.6, $CHCl_3$){lit.[1] $[\alpha]^{24}_D$ −112 (*c* 0.3, $CHCl_3$)}.

16α, 17-Dihydroxy-*ent*-kauran-19-oic acid (4):[1] white powder; mp 264−266 °C (lit.[1] mp 264−266 °C); $[\alpha]^{24}_D$ −65 (*c* 0.1, $CHCl_3$){lit.[1] $[\alpha]^{24}_D$ −58 (*c* 0.05, $CHCl_3$−MeOH)}.

16*β*-Hydroxy-17-acetoxy-*ent*-kauran-19-oic acid (5):[5] white powder; mp 162−164 °C; $[\alpha]^{24}_D$ −43 (*c* 0.2, $CHCl_3$).

16*β*-Hydro-*ent*-kauran-17-oic acid (6):[6,7] white needles (MeOH); mp 204−206 °C (lit.[6,7] mp 208−209 °C); $[\alpha]^{24}_D$ −34 (*c* 0.3, $CHCl_3$){lit.[6,7] $[\alpha]^{24}_D$ −67.5 (*c* 0.7, $CHCl_3$)}.

16α-Hydro-*ent*-kauran-17-oic acid (7):[8] white needles (ethyl acetate); mp 190−192 °C (lit.[7,8] mp 189−190 °C); $[\alpha]^{24}_D$ −66 (*c* 0.36, $CHCl_3$){lit. [7,8] $[\alpha]^{24}_D$ −50 (*c* 0.8, $CHCl_3$)}.

***ent*-Kaur-16-en-19-ol (8):**[1] white powder; mp 137−138 °C (lit.[1] mp 126−128 °C); $[\alpha]^{24}_D$ −80 (*c* 0.4, $CHCl_3$)-{lit.[1] $[\alpha]^{24}_D$ −82 (*c* 0.4, $CHCl_3$)}.

***ent*-Kaur-15-ene-17,19-diol (9):**[9,10] white powder; mp 185−187 °C (lit.[9] mp 193−195 °C); $[\alpha]^{24}_D$ −32 (*c* 0.32, $CHCl_3$−MeOH){lit.[9] $[\alpha]^{24}_D$ −37 (*c* 0.3, $CHCl_3$)}.

16α-Hydro-19-al-*ent*-kauran-17-oic acid (10):[1,11,12] white powder; mp 178−182 °C (lit.[1] mp 178−180 °C); $[\alpha]^{24}_D$ −58 (*c* 0.2, $CHCl_3$){lit.[1] $[\alpha]^{24}_D$ −21 (*c* 0.03, $CHCl_3$)}.

Methyl-16α-hydro-19-al-*ent*-kauran-17-oate (11):[12] white needles (MeOH); mp 174−176; $[\alpha]^{24}_D$ −58 (*c* 0.3, $CHCl_3$).

16*β*-Hydroxyl-17-acetoxy-*ent*-kauran-19-al (12):[1] white needles (MeOH); mp 160−163 °C (lit. [1] mp 162−164 °C); $[\alpha]^{24}_D$ −58 (*c* 0.2, $CHCl_3$){lit.[1] $[\alpha]^{24}_D$ −64 (*c* 0.2, $CHCl_3$)}.

19-Nor-*ent*-kauran-4α-ol-17-oic acid (13):[1] white powder; mp 277−279 °C (lit.[1] mp 280−282 °C); $[\alpha]^{24}_D$ −65 (*c* 0.2, $CHCl_3$){lit.[1] $[\alpha]^{24}_D$ −55 (*c* 0.2, $CHCl_3$)}.

HIV Inhibition Assay. The anti-HIV activity assays were carried out according to procedures described in the literature.[1,14]

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