Ent–kauren-19-oic acid derivatives from the stem bark of *Croton pseudopulchellus* Pax

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Abstract

Two new *ent*-kauren-19-oic acid derivatives, *ent*-14*S**-hydroxykaur-16-en-19-oic acid and *ent*-14*S**,17–dihydroxykaur-15-en-19-oic acid together with eleven known compounds *ent*-kaur-16-en-19-oic acid, *ent*-kaur-16-en-19-al, *ent*-12β-hydroxykaur-16-en-19-oic acid, *ent*-12β-acetoxykaur-16-en-19-oic acid, 8*R*,13*R*-epoxylabd-14-ene, eudesm-4(15)-ene-1β,6α-diol, (–)-7-epivaleran-4-one, germacra-4(15),5*E*, 10(14)-trien-9β-ol, acetyl aleuritolic acid, β-amyrin, and stigmasterol were isolated from the stem bark of *Croton pseudopulchellus* (Euphorbiaceae). Structures were determined using spectroscopic techniques. *Ent*-14*S**-hydroxykaur-16-en-19-oic acid, *ent*-kaur-16-en-19-oic acid, *ent*-12β-hydroxykaur-16-en-19-oic acid, *ent*-12β-acetoxykaur-16-en-19-oic acid and 8*R*,13*R*-epoxylabd-14-ene were tested for their effects on Semliki Forest virus replication and for cytotoxicity against human liver tumour cells (Huh-7 strain) but were found to be inactive. *Ent*-kaur-16-en-19-oic acid, the

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major constituent, showed weak activity against the *Plasmodium falciparum* (CQS) D10 strain.

Keywords

Euphorbiaceae, *Croton pseudopulchellus*, *ent*-14*S**-hydroxykaur-16-en-19-oic acid, *ent*-14*S**,17–dihydroxykaur-15-en-19-oic acid, *ent*-kaur-16-en-19-oic acid, *ent*-kaur-16-en-19-al, *ent*-12β-hydroxykaur-16-en-19-oic acid, *ent*-12β-acetoxykaur-16-en-19-oic acid, 8*R*,13*R*-epoxylabd-14-ene, eudesm-4(15)-ene-1β,6α-diol, (–)-7-epivaleran-4-one, germacra-4(15), 5*E*,10(14)-trien-9β-ol, acetyl aleuritolic acid, β-amyrin, Semliki Forest virus, Huh-7 liver tumour cells, *Plasmodium falciparum* (CQS) D10 strain.

1. Introduction

Croton pseudopulchellus Pax is a shrub that grows to about 4 m tall and is commonly called the Small Lavender Croton. It is widespread in drier woodlands of the warmer regions of East, South-central and parts of West Africa (Burkill, 1994; Schmidt et al., 2002). In the coastal area of Kenya *C. pseudopulchellus* is employed as a condiment when material is burnt and the smoke used to flavour fresh milk (Pakia and Cooke, 2003). Poles from this species are durable and termite-proof, and accordingly used for hut building in Tanzania (Arbonnier, 2004). A decoction from the roots is used to treat asthma (Githens, 1948; Watt and Breyer-Brandwijk, 1962) and the powdered root taken as a snuff for headaches (Burkill, 1994). Leaves are applied by Tanzanians to their chest, for chest ailments (Burkill, 1994). Such treatment of tussive conditions, likely associated with viral infections, has prompted the current investigation of anti-viral activity of compounds isolated from *C. pseudopulchellus.*

Githens (1948) reported that leaves and roots of *C. pseudopulchellus* contain the toxalbumin crotin. The chloroform extract of the stem bark of *C. pseudopulchellus* has been found to give a 82% inhibition against PfUP1 at 50 µg/ml, a chloroquine resistant strain of the malarial parasite *Plasmodium falciparum* and in cytotoxicity tests showed an ID_{50} of 64µg/ml against vervet monkey kidney cells (Prozesky et al., 2001).

We report the isolation of a number of *ent*-kauren-19-oic acid derivatives from the stem bark of *C. pseudopulchellus*, their effects on Semliki Forest virus replication using baby hamster kidney (BHK) cells as the host, cytotocity assays against BHK and human liver tumour cells (Huh-7 strain) and the activity of *ent*-kaur-16-en-19-oic acid against the *Plasmodium falciparum* (CQS) D10 strain.

2. Results and discussion

Two new *ent*-kauren-19-oic acid derivatives, *ent*-14*S**-hydroxykaur-16-en-19-oic acid (**1**), and *ent*-14*S**,17–dihydroxykaur-16-en-19-oic acid (**2**) together with eleven known compounds *ent*-kaur-16-en-19-oic acid (Vieira et al., 2002), *ent*-kaur-16-en-19-al (Piozzi et al., 1971), *ent*-12β-hydroxykaur-16-en-19-oic acid (Ortega et al., 1985), *ent*-12β-acetoxykaur-16-en-19-oic acid (Bohlmann et al., 1980), 8*R*,13*R*-epoxylabd-14-ene (Zhou et al., 1995), eudesm-4(15)-ene-1β,6α-diol (Sun et al., 2004), (-)-7-epivaleran-4-one (Srikrishan et al., 2004), germacra-4(15), 5*E*, 10(14)-trien-9β-ol (Brown et al., 2003), β-amyrin, acetyl aleuritolic acid and stigmasterol were isolated from the hexane and methylene chloride extracts of the stem bark of *Croton pseudopulchellus*. *Ent*-kaur-16-en-19-oic acid was isolated from this plant in large quantities (7 % of combined hexane and dichloromethane extracts).

Ent-14*S**-hydroxykaur-16-en-19-oic acid (**1**) was isolated as a white solid. HR-ESI-MS indicated a molecular formula of $C_{20}H_{30}O_3$, indicating six degrees of unsaturation. The IR spectrum showed absorption bands at 3340 and 1691 cm^{-1} due to hydroxyl and carbonyl stretches respectively. Similarities between the NMR spectra and those for the co-isolated e*nt*-kaur-16-en-19-oic acid suggested the presence of a hydroxylated derivative (Vieira et al., 2002). The ^{13}C NMR spectra showed twenty carbon resonances, including alkene carbon resonances at δ 107.5 (CH_2, C-17) and 149.2 (C-16), a carbonyl carbon resonance at δ 183.7 (C-19) and an oxymethine carbon resonance at δ 69.4 (C-14). The ^1H NMR spectrum of compound **1** showed the presence of two methyl group proton resonances at δ 0.99 (s, 3H-20) and δ 1.24 (s, 3H-18), an oxymethine proton resonance (δ 4.00, dt, J = 9.4, 2.0 Hz, H-14) and exocyclic methylene proton resonances (δ 4.84, d, J = 1.9 Hz; δ 4.68, d, J = 1.9 Hz, 2H-17).

The placement of the additional hydroxyl group at C-14 was confirmed by coupling seen in the COSY spectrum between the H-14 resonance at δ 4.00 (d, *J* = 9.4 Hz) and the typical H-13 (δ 2.25, dd, *J* = 3.3, 2.0 Hz) resonance (Vieira et al., 2002) and HMBC correlations seen between the H-14 resonance and resonances assigned to C-8 (δ 38.7), C-15 (δ 46.9), C-16 (δ 149.2), C-12 (δ 39.0) and C-13 (δ 44.9). Placement of the hydroxyl group at C-12 was ruled out as both *ent*-12α-hydroxykaur-16-en-19-oic acid and *ent*-12β-hydroxykaur-16-en-19-oic acids have been reported and their ^{1}H NMR spectroscopic data differ (Ortega et al., 1985) from those of compound **1.**

The optical rotation of -160 confirmed that compound **1** belonged to the *ent* series, with H-13 on the α face of the molecule (Vieira et al., 2002). When the hydroxyl group at C-14 is placed in the β-position, a model indicates that the H-13,14 dihedral angle is around 90° (Spartan08 calculation indicates 104.7°) resulting in the small coupling constant of 2.0 Hz observed (Takeda et al., 1989; Takeda et al., 1987) whereas the observed coupling constant of 9.4 Hz is attributable to long-range coupling with the H-12α resonance. NOE effects were observed between the H-14α proton resonance and resonances ascribed to H-11α (δ 1.42), H-12α (δ 1.45) and H-13, (δ 2.25) further confirming the assignment. Therefore the structure of compound **1** was determined to be *ent*-14*S**-hydroxykaur-16-en-19-oic acid.

Jones oxidation of compound **1** yielded *ent*-14-oxokaur-16-en-19-oic acid, **1a**, previously synthesized from stevioside (Yun-Xing et al., 1995; Bowden et al., 1946). Assignment of the NMR spectra was undertaken as literature data for **1a** is incomplete [**Table 1**]. The HMBC spectrum of **1a** showed correlations between the C-14 resonance (δ 213.1) and the H-13 (δ 2.94, s) and the two H-15 (2.69 dd *J* = 2.4, 17.0 and 1.78 m) resonances.

Compound **2,** *ent*-14*S**,17–dihydroxykaur-15-en-19-oic acid, was isolated as its diacetate derivative after acetylation of an inseparable mixture. Compound **2** was found to be the 14α-hydroxy derivative of the kaurene diterpenoid, *ent*-17–hydroxykaur-15-en-19-oic acid, reported previously from *Baccharis illinita* (Verdi et al., 2004). HR-ESI-MS analysis of compound **2a** indicated a molecular formula of $C_{24}H_{34}O_6$. The IR spectrum of **2a** showed a hydroxyl band at 3423 cm^{-1} and two carbonyl bands at 1736 cm^{-1} and 1692

cm^{-1} attributable to acetate carbonyl and carboxylic acid carbonyl group stretches respectively.

The ^{13}C NMR spectrum of **2a** showed twenty-four carbon atoms including a carboxylic acid carbonyl carbon resonance (δ 183.0, C-19), two acetyl carbonyl carbon resonances (δ 171.1, 170.6), two alkene carbon resonances (δ 142.1, C-15; 140.2, C-16), an oxymethylene carbon resonance (δ 62.4, C-17) and an oxymethine carbon resonance (δ 69.3, C-14). The ^{1}H NMR spectrum showed an alkene proton singlet resonance at δ 5.47 (H-15), an oxymethine proton resonance at δ 4.97 (dt, J = 7.2, 3.5 Hz, H-14), and an oxymethylene proton resonance at δ 4.66 (s, 2H-17). The characteristic ent-kaurenoic acid H-13 resonance at δ 2.63 (t, J = 3.5 Hz) was observed (Ortega et al., 1985). The placement of the hydroxyl group at C-14 was confirmed by a correlation seen in the COSY spectrum between the H-13 and H-14 resonances and correlations seen in the HMBC spectrum between the H-14 resonance and resonances ascribable to C-8 (48.2), C-13 (δ 46.2) and C-15 (142.1).

The negative optical rotation of – 55.9 indicated that compound **2a** belonged to the *ent* series of kaurenoic acid-type diterpenoids (Garcia et al., 2007), as for all the diterpenoids isolated from this extract. The H-14 proton resonance displayed a correlation in the NOESY spectrum with the H-7α (1.58, m), H-11α (2.01, m), H-12α (1.32, dd, J = 3.5, 11.0 Hz) and H-13α resonances, thus the C-14 hydroxy group was assigned the β – configuration. This was again supported by the small $J_{13,14}$ coupling constant (Takeda et al., 1989; Takeda et al., 1987).

Ent-14*S**-hydroxykaur-16-en-19-oic acid (**1**), *ent*-kaur-16-en-19-oic acid, *ent*-12β-hydroxykaur-16-en-19-oic acid, *ent*-12β-acetoxykaur-16-en-19-oic acid and 8*R*,13*R*-epoxylabd-14-ene were tested for their capacity to inhibit Semliki Forest virus (SFV) replication using baby hamster kidney (BHK) cells as the host for the viral infection. SFV is a positive-strand RNA virus that is widely studied as a prototype of *Alphavirus* genus. However, all the compounds were found to be inactive against the virus at a concentration of 50μM (**Table 2**). Cytotoxicity assays against BHK and human liver tumour cells (Huh-7 strain) in a single dose of 50 μM were also performed on the six compounds to check for preliminary toxicity against animal cells and they displayed low activity (**Table 2**). In addition, the compounds were not able to inhibit the enzyme β-

lactamase, a potential target for antimicrobial combination therapy. *Ent*-kaur-16-en-19-oic acid, the major component of the extract, showed a moderate *in vitro* anti-plasmodial activity against the chloroquine sensitive strain of *P. falciparum* with an IC_{50} value of 31.77 μg/ml (compared to chloroquine IC_{50} 14.26 ng/ml).

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a JASCO P-1020 polarimeter. FTIR spectra were recorded using a Perkin-Elmer (2000) spectrometer. 1D and 2D NMR spectra were recorded in $CDCl_3$ on a 500 MHz Bruker AVANCE NMR instrument at room temperature. Chemical shifts (δ) are expressed in ppm and were referenced against the solvent resonances at 7.26 and 77.23 ppm for ^{1}H and ^{13}C NMR respectively. ESI mass spectra were recorded on a Bruker MicroToF mass spectrometer using an Agilent 1100 HPLC to introduce samples (University of Oxford).

3.2 Plant material

The stem bark of cultivated material of *C. pseudopulchellus* was collected in August 2005 in Durban, of KwaZulu-Natal, South Africa, and a voucher retained at the KwaZulu-Natal herbarium for verification purposes (*Crouch 1050*, NH). .

3.3 Extraction, isolation and characterization of compounds

The ground stem bark (2.6kg) was successively extracted with hexane, methylene chloride and methanol. TLC analysis of the hexane (22.4g) and methylene chloride extracts (11.4g showed they were similar, so they were combined and separated using column chromatography over silica gel (Merck 9385) using a hexane/ methylene chloride/methanol step gradient collecting fractions of 75 mL. NMR analysis of the methanol extract showed that it contained only sugars and was not analysed further. Fractions 10 – 19 (20% DCM: 80% Hex) gave *ent*-kaur-16-en-19-al (27.0 mg), 8*R*, 13*R* -epoxylabd-14-ene (11.6 mg) and (–)-7-epivaleran-4-one (13.2 mg). Fractions 50 – 120 (50% DCM: 50% Hex) gave *ent*-kaur-16-en-19-oic acid (2.2 g), acetyl aleuritolic acid (36.9 mg) and germacra-4(15), 5*E*, 10(14)-trien-9β-ol (10.1 mg); fractions 110 – 130

(60% DCM: 40% Hex) gave β-amyrin (4.9 mg) and stigmasterol (16.0 mg), fractions 125 – 138 (70% DCM: 30% Hex) gave eudesm-4(15)-ene-1β,6α-diol (6.8 mg), fractions 150 – 153 (100% DCM) yielded *ent*-12α-hydroxykaur-16-en-19-oic acid (25.0 mg), fractions 155 – 158 (100% DCM) gave *ent*-12β-acetoxykaur-16-en-19-oic acid (25.6 mg), fractions 160 – 170 (100% DCM) gave *ent*-14*S**-hydroxykaur-16-en-19-oic acid (38.0 mg), fractions 200 – 212 (2% MeOH: 98% DCM) gave impure *ent*-14*S**,17–dihydroxykaur-15-en-19-oic acid which was purified using column chromatography (100% DCM) after acetylation to yield *ent*-14*S**,17–diacetoxykaur-15-en-19-oic acid (6.0 mg). *Ent*-14*S**-hydroxykaur-16-en-19-oic acid (**1**) was oxidised using Jones reagent (Bowden et al., 1946) to yield compound **1a**. A mixture containing *ent*-14*S**,17–dihydroxykaur-15-en-19-oic acid (15 mg) was acetylated using pyridine (1 ml) and acetic anhydride (1 ml) and the reaction left to stand for 24 hours. **2a** was isolated using column chromatography.

3.3.1 Ent-14S-hydroxykaur-16-en-19-oic acid* (**1**)

White solid; mp 126–129° C; $[\alpha]_D^{24.7}$ = - 160 (0.01, $CHCl_3$), IR 3340, 3078, 2929, 2851, 1691; ^1H and ^{13}C NMR see Table **1**; HRESIMS *m/z* $[M - H]^+$ 317.2124 (calcd for $C_{20}H_{29}O_3$, 317.2122)

3.3.2 Ent-14-oxokaur-16-en-19-oic acid (**1a**)

Yellow oil; $[\alpha]_D^{24}$ = - 101 (0.01, $CHCl_3$), IR 3422, 3070, 2926, 2845, 1722 and 1692 cm^{-1}; ^1H and ^{13}C NMR see Table **1**; EIMS (70eV) *m/z* 316 ($C_{20}H_{28}O_3$), 281, 249, 203, 189, 135, 109, 95 (100), 55.

3.3.3 Ent-14S,17–dihydroxykaur-15-en-19-oic acid* (**2**)

Colourless oil: IR 3412, 1696 cm^{-1}; ^1H and ^{13}C NMR see Table **1**; GC-EI-MS (70eV) *m/z* 334 ($C_{20}H_{30}O_4$)

3.3.4 Ent-14S,17–diacetoxykaur-15-en-19-oic acid* (**2a**)

Colourless oil: $[\alpha]_D^{24}$ = - 55.9 (0.005, $CHCl_3$); IR 3423, 1736, 1692 cm^{-1}; ^1H and ^{13}C NMR see Table **1**; HRMS *m/z* 441.2251 (calcd for $C_{24}H_{34}O_6Na$, 441.2253).

3.4 Biological assays

The SFV assay was done as previously described (Pohjala et al., 2008) to measure the effect of test compounds on Semliki Forest Virus replication using baby hamster kidney (BHK) cells as host cells. The preliminary toxicity on animal cell viability was studied with two cell lines, baby hamster kidney (BHK) cells and Huh-7 hepatocytes (from human hepatocellular carcinoma), as previously described (Pohjala et al., 2007). BHK and Huh-7 cells were exposed to test compounds for 24h and 48h, respectively. Cell viability after exposure was assessed by measuring the intracellular ATP levels with a CellTiter-Glo assay kit (Promega). Inhibition of the β-lactamase enzyme was tested according to Payne et al. (1994). Inhibitors of this enzyme have possible applications in antimicrobial therapy.

Ent-kaur-16-en-19-oic acid was tested in duplicate against the chloroquine sensitive (CQS) strain of *Plasmodium falciparum* (D10). Continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum* were maintained using a modified method of Trager and Jensen (Trager and Jensen, 1976). Quantitative assessment of anti-plasmodial activity *in vitro* was determined *via* the parasite lactate dehydrogenase assay using a modified method described by Makler et al. (1993). *Ent*-kaur-16-en-19-oic acid was also dissolved and diluted to a 2 mg/ml stock solution in 10% methanol and sonicated to enhance solubility and stock solution stored at – 20 $^{\circ}$C. Further dilutions were prepared on the day of the experiment in complete medium. Chloroquine (CQ) was used as the reference drug. A full dose-response was performed for the compound to determine the concentration inhibiting 50 % of parasite growth (IC_{50} – value). The Test sample was tested at a starting concentration of 100 μg/ml, which was serially diluted 2-fold in complete medium to give 10 concentrations, with the lowest concentration being 0.2 μg/ml. CQ was tested at a starting concentration of 100 ng/ml. The highest concentration of solvent to which the parasites were exposed had no measurable effect on the parasite viability (data not shown). The IC_{50} - values were obtained using a non-linear dose-response curve fitting analysis *via* graph pad prism v.4.0 software.

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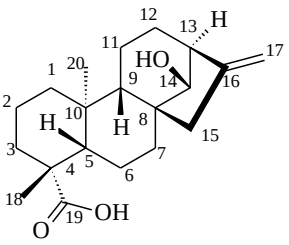
Table 1

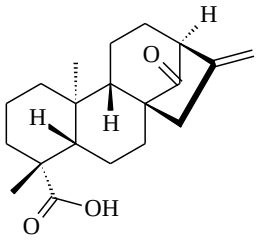
NMR Data (500 MHz) of 1, 1a, 2 and 2a (multiplicities, *J* in Hz)

No.	1	1	1a	1a	2	2	2a	2a
1α	39.9 CH_2	1.66 m	39.6 CH_2	1.55, d (12.9)	40.7 CH_2	1.80 m	40.6 CH_2	1.71 m
1β		0.86 m		0.88 m		0.80 m		0.79 m
2α	18.8 CH_2	1.40 *	18.7 CH_2	1.41, d (14.0)	20.7 CH_2	1.84 m*	19.1 CH_2	1.42, d (12.0)
2β		1.92 *		1.84*		1.84 m*		1.84 m
3α	38.1 CH_2	2.13, d (13.4)	37.9 CH_2	2.16 m	38.1 CH_2	2.11 m	38.0 CH_2	2.17 m
3β		1.01 m		1.03, td (4.0, 9.6)		1.00 m		1.01*
4	43.7 C	-	43.8 C	-	44.0 C	-	44.0 C	-
5	57.0 CH	1.04 m	56.7 CH	1.14, t (7.6)	56.7 CH	1.08 m	56.7 CH	1.09, d (12.0)
6α	20.5 CH_2	1.76 m*	20.2 CH_2	1.84*	19.2 CH_2	1.95 m	20.6 CH_2	1.81 m*
6β		1.76 m*		1.84*		1.38 m		1.81 m*
7α	39.9 CH_2	1.15 m	38.3 CH_2	1.30*	38.5 CH_2	1.30 m*	38.9 CH_2	1.59 m
7β		1.52 m		1.66 m		1.30 m*		1.68 m
8	38.7 C	-	38.5 C	-	48.3 C	-	48.2 C	-
9	51.7 CH	1.09 m	51.2 CH	1.32*	48.5 CH	1.15, d (9.5)	47.7 CH	1.19, d (10.0)
10	34.6 C	-	37.2 C	-	39.1 C	-	38.7 C	-
11α	21.1 CH_2	1.42 m*	27.4 CH_2	1.69 m	28.7 CH_2	1.60 m*	25.2 CH_2	1.56 m
11β		1.90 m*		1.92, t (11.5)		1.60 m*		2.01 m
12α	39.0 CH_2	1.45 m	46.7 CH_2	2.13 m	36.9 CH_2	1.24 m	37.7 CH_2	1.32, dd (3.5, 11.0)
12β		1.84 m		2.20 m		2.43, d (10.5)		2.40, d (11.0)
13	44.9 CH	2.25, dd (2.0, 3.3)	55.5 CH	2.94 s	47.9 CH	2.61 s	46.2 CH	2.63 t (3.5)
14	69.4 CH	4.00, dt (9.4, 2.0)	213.1 C	-	66.7 CH	4.07 br s ($W_{1/2}$ = 14.0)	69.3 CH	4.97 dd (3.5, 7.2)
15	46.9 CH_2	1.80 m	46.0 CH_2	1.78 m	138.6 CH	5.47 s	142.1 CH	5.47 s
		1.96 m		2.69, dd (2.4, 17.0)		-	-	-
16	149.2 C	-	143.0 C	-	145.7 C		140.2 C	-
17	107.5 CH_2	4.84, d (1.9)	110.8 CH_2	4.93 s	61.4 CH_2	4.21, d (4.5)*	62.4 CH_2	4.66 s*
		4.68, d (1.9)		4.80 s		4.21, d (4.5)		4.66 s*
18	29.0 CH_3	1.24 s	29.1 CH_3	1.25 s	28.6 CH_3	1.21 s	29.2 CH_3	1.26 s
19	183.7 C	-	183.5 C	-	182.7 C	-	183.0 C	-
20	13.6 CH_3	0.99 s	12.6 CH_3	0.75 s	14.7 CH_3	1.08 s	13.7 CH_3	1.02 s*
17-**Ac**			-		-	-	170.6 C	-
17-**Ac**	-	-	-	-	-	-	21.2 CH_3	2.08 s
14-**Ac**	-	-	-	-	-	-	171.1 C	-
14-**Ac**	-	-	-	-	-	-	21.7 CH_3	2.03 s
OH	-	-	-	11.1 br s	-	-	-	-

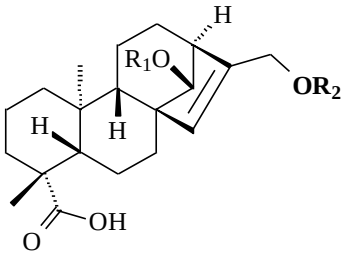
*Refer to overlapped/superimposed proton resonances



1



1a



2: $R_1 = R_2 = H$
2a: $R_1 = R_2 = Ac$

Table 2: Summary of bioassay results

Compound	SFV replication[a]	BHK cell viability[b]	Huh-7 cell viability[c]	β-lactamase[d]
Ent-kaur-16-en-19-oic acid	72.7	87.4	105.7	120.1
Ent-12β-hydroxykaur-16-en-19-oic acid	76.5	93.0	96.9	122.8
Ent-12β-acetoxykaur-16-en-19-oic acid	86.0	85.6	101.5	129.6
Ent-14*S**-hydroxykaur-16-en-19-oic acid	115.8	86.4	99.9	118.1
8*R*,13*R*-epoxylabd-4-ene	56.7	85.0	87.6	89.8
Positive control	17.4	21.3	19.8	13.4

Results represent the surviving fraction (%) of virus / cells / enzyme activity compared to vehicle-treated control in each assay. All experiments were performed at 50 µM concentration in triplicate. Positive controls were 3'-amino-3'-deoxyadenosine[a] (20 µM), polymyxin B sulphate[b,c] (7500 IU/ml), and clavulanic acid[d](50 µM). SFV, Semliki Forest virus; BHK, baby hamster kidney; Huh-7, human hepatocyte cell line.